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ISOLATION AND IDENTIFICATION OF FUNGI CAUSING POST-HARVEST SPOILED MANGO FRUITS VENDED IN HANOI, VIETNAM

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ABSTRACT

Mango (*Mangifera indica* L.) is one of the most popular and nutritious fruits cultivated widely in Vietnam. However, under increasingly harsh climate conditions, mangoes are easily susceptible to fungal invasion and spoiled, thereby reducing mango yield. Therefore, this study was carried out to determine the pathogenic fungal strains of mango to provide useful information for finding effective measures to prevent the diseases. Rotten mango fruits were collected from different markets in Hanoi, Vietnam. Three fungal strains (M1, M2 and M3) were isolated from the studied mangoes samples. All strains were demonstrated as fungal agents associated with mango rot through pathogenicity tests. Microscopic observation showed that the mycelium of these fungal strains was branched and septate. M1 strain formed dark-brown conidiophores and conidia produced on conidiophores. M2 strain produced α- and β-conidia, as well as sub-ovoid and brown sclerotia. Whereas the M3 strain could not produce spores. Additionally, this study determined that all three fungal isolates showed the fastest growth on PDA at 30^{o}C. The optimum growth of the M1 and M3 strains was observed at pH 5.0 while the M2 strain grew actively at pH 7.0 and 8.0. All selected strains showed the ability to produce extracellular enzymes, in which the M1 strain synthesized both cellulase and pectinase while the M2 and M3 strain secreted only pectinase. Finally, by molecular identification method, the isolates (M1, M2 and M3) were identified as *Aspergillus niger* isolate M1, *Phomopsis* sp. M2, *Lasiodiplodia theobromae* M3, respectively.

Keywords: *Aspergillus niger*, *Lasiodiplodia theobromae*, mango spoilage, *Phomopsis sp.* pathogenic fungal strains

INTRODUCTION

Mango (*Mangifera indica* L.) is one of the important fruits cultivated widely in tropical and subtropical regions of the world countries such as India, Myanmar, Malaysia, Thailand, and Vietnam (Le *et al.*, 2020). Additionally, mango cultivation has been widespread in some other countries, including Africa, Americas, and the Caribbean region (Ahmed and Mohammed, 2014). According to statistics, mangoes have been grown in more than 100 countries, of which India ranked first in global mango production with 24 million tons in 2020 (FAO, 2023), followed by Thailand and

China (Mitra, 2014). In Vietnam, the mango is the second-most popular fruit (after bananas) and is popularly grown in the South (Le *et al.*, 2020). Mango fruits provide many vitamins and minerals necessary for proper body metabolism, as well as some highly biologically active chemicals boosting the human immune system (Le *et al.*, 2020; Spina *et al.*, 2024). Therefore, mango consumption has been increasing in many countries (Spina *et al.*, 2024). However, mangoes are rapidly perishable fruits, especially post-harvest mangoes, which are highly susceptible to microbial invasion. Microorganisms, especially fungi, are known as a major agent causing mango spoilage (Rajmane and Korekar, 2016). Fungal pathogens can infect the fruits either on the trees or during storage (Ahmed and Mohammed, 2014; Diedhiou *et al.*, 2010). In 2016, Rajmane and Korekar isolated and identified 8 fungal isolates as *Alternaria Alternata, Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Botryodiplodia theobromae, Collectotracum gloeosporioides, Penicillum crysogenum* and *Rhizopus stolonifer* from different varieties of spoiled mango in India (Rajmane and Korekar, 2016). Similarly, in Saudi Arabia, Al-Najada also carried out his study and determined fungi from rotten mangoes such as *Aspergillus niger, Aspergillus flavus, Alternaria* spp., *Rhizopus* spp., *Penicillium* spp., *Botryodiplodia* and *Phomopsis* spp (Al-Najada, 2019). In Vietnam, Nguyen Vu Mai Linh also isolated the fungal strain XB1 from stem-end rot mango fruits and then identifed the strain as *Lasiodiplodia theobromae* (Nguyen *et al.*, 2021). Currently, synthetic fungicides have been mainly used to control most crop pathogens, however, the application of the fungicides for a long time may increase concerns to human and environmental health (Wu *et al.*, 2023). Moreover, until now, the information on mango diseases has been limited (Rahi *et al.*, 2017). Therefore, this study was conducted to isolate, determine the morphological characteristics as well as identify molecularly the fungi causing post-harvest spoilage on mango to find out an effective environmentally friendly biological measure to manage the diseases on mango and other fruits.

MATERIALS AND METHODS

Sample collection

Post-harvest spoiled mangoes are purchased from five major markets in Hanoi, Vietnam They were stored in sterile paper envelopes separately, were labeled appropriately, and were transported to the laboratory of the Faculty of Biotechnology, Vietnam National University of Agriculture (Hasan and Zanuddin, 2018).

Isolation of fungi

Fungal isolation technique was carried out according to Wanjiku *et al.* (2020), rotten mango fruits were washed with tap water, sterilized surface with 2% sodium hypochlorite for one minute, washed twice with sterilized distilled water and then allowed to dry. Small pieces of symptomatic flesh were cut off from fruits, plated on Potato Dextrose Agar (PDA) medium by a sterile sharp knife and incubated at 30°C for 5-7 days. The fungi grown from the infected pieces were re-inoculated on PDA medium several times to obtain pure cultures (Wanjiku *et al.*, 2020).

Pathogenicity test

Isolated fungi were screened for their potential to cause diseases on mango fruits

by an artificial infection technique. For this, fungal mycelium was grown on PDA media at 30^{o}C for 7 days. Healthy mango fruits were surface sterilized with 75% alcohol, washed twice with sterilized distilled water and then allowed to dry. A sterile cork borer was used to make a wound on each fruit and then placed the fungal mycelia of the isolates on the artificial wound. The inoculated wound was sealed with muslin cloth. Control samples were prepared in the same way; however, PDA pieces were placed on the artificial wound without fungal mycelia. The inoculated fruits and the controls were incubated at 30°C. Observed and compared disease symptoms day by day with isolated samples to select the fungal strains capable of causing disease through artificial infection (Wanjiku *et al.*, 2020).

Morphological characterization of the isolates

The selected isolates were studied on morphological characteristics (Tafinta *et al.*, 2013). Fungal strains were cultured on PDA, and cover slips were placed gently at an angle of 45° on the media. After every 12 hours, a cover slip was gently removed and placed under a light microscope (40x, 100x objective) to observe fungal features such as hyphae, conidiophores, conidia and other special structures.

Optimal growth conditions of fungi

Mycelial growth of the selected isolates was evaluated on five media, such as Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA), International Streptomyces Project 2 (ISP2), Malt Extract Agar (MEA) and Czapeck Dox Agar (CDA). The fungi were taken from 7 days old culture and transferred to the center of all media. Then the media were incubated for 7 days at 30^{o}C. Radial growth of each fungus was measured daily. The colony morphology also was noted (Kim *et al.*, 2005).

The effect of temperature and pH on mycelial growth was also evaluated by inoculating the fungi on the selected medium at different temperatures (20°C, 30°C, 37°C, 40°C, 50°C) and different pHs (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0). Radial growth of each fungus was measured daily. The colony morphology also was noted (Kim *et al.*, 2005).

Screening for extracellular enzyme activities

Fungal isolates were screened to produce cellulase and pectinase. The media included phosphate buffer, agar, and a substrate suitable for a given enzymatic reaction (CMC and pectin, respectively). All evaluated isolates were cultured in PDB, and the suspension was centrifuged at 10.000 rpm for 10 min at 4^{o}C to receive crude enzymes. Then, 0.1 mL of the supernatant was added to each well of the plates. The plates were placed in a refrigerator at 4°C for 2 hours and incubated at 30°C for 24 hours. After incubation, the plates were flooded with Lugol solution and observed for zone of clearance (Ogórek, 2016).

Molecular identification of fungal species

Fungal isolates were cultured in PDB at 30°C for 3 days and the fungal biomass was collected by centrifugation for 5 min at 10.000 rpm. Genomic DNA was extracted from the fungal isolates following the CTAB method (Zhang *et al.*, 2010). The extracted DNA was used for PCR amplification by primers ITS1 (F-

TCCGTAGGTGAACCTGCGG) and ITS4 (R-TCCTCCGCTTATTGATATGC) (Gonzalez *et al.*, 2008). The PCR products were then analyzed on 1% agarose gel. Successfully amplified DNA samples were sent to Singapore 1st Base for determining ITS regions of each fungal isolate. The obtained sequences were used to compare with other related sequences using BLAST tool (http:/www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic relationships were determined using MEGA 6.0 for the selected fungi related species retrieved from GenBank.

RESULTS AND DISCUSSION

Isolation of fungi

In this study, three fungal trains (M1, M2 and M3) were isolated from the rotten of the collected spoiled mango fruits purchased from different markets in Hanoi, Vietnam. The isolates had different cultural features in terms of color, surface characteristics, reverse and edge. The results were shown in Figure 1 and Table 1.

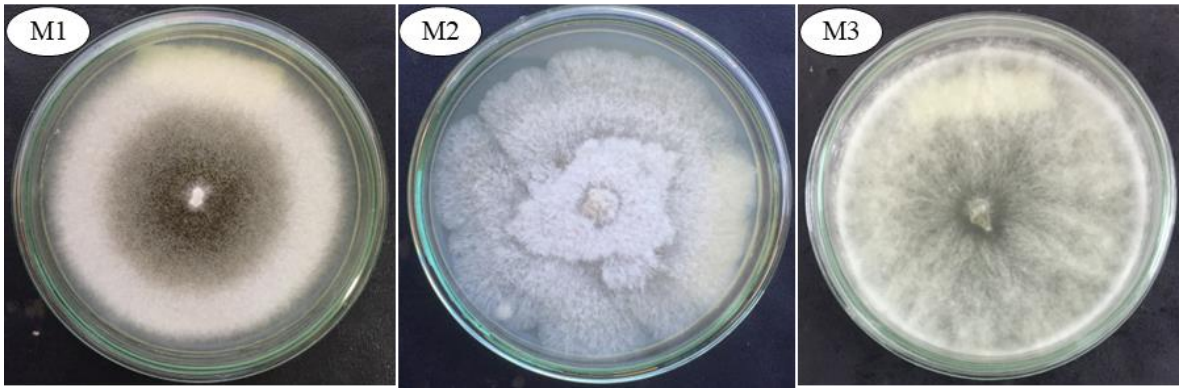


Figure 1. Colonies of fungal strains after 5 culture days on PDA medium

Table 1. Cultural characteristics of three isolated fungal strains

Characteristics	M1 strain	M2 strain	M3 strain
Form	Filamentous	Filamentous	Filamentous
Margin	Filiform	Filiform	Filiform
Elevation	Umbonate	Umbonate	Umbonate
Surface	Rough with black conidia	Rough with wavy growth	Rough
Front color	White with brown center	Yellowish white	White with grey center
Back color	White with brown center	Yellowish white	White with grey center

The study was conducted during the mango harvest season (May to July) to easily collect rotten mango samples and accurately isolate the fungi causing mango rot. Similarly,

Ahmed *et al.* also selected mango spoilage during that time for their research and they determined that *A. niger*, *A. flavus* and *Penicillium* sp. were the commonest fungi causing spoilage on mangoes, especially in developing countries where postharvest handling techniques are limited along with poor infrastructures (Ahmed and Mohammed, 2014). Therefore, collecting spoiled mango samples at that time as well as isolating fungal strains from the samples play an important role in this study.

Pathogenicity test

The fungal isolates were used for artificial infection of healthy ripe and unripe fruits to access the effect of spoilage disease on ripening stages of mangoes. The results showed that all three isolated strains were capable of causing spoilage on both ripe and unripe mango fruits, especially a significant increase in disease severity was observed when fruit ripening increased. After two days of re-infection with all three strains M1, M2, and M3, the symptoms of spoilage began to appear. However, on the 3rd day of re-infection, the symptoms of the disease were clearly observed on ripe mangoes and spread more widely than on green mangoes in the following days (Figure 2 and Table 2). Moreover, the disease symptoms obtained were like those on the selected spoiled mangoes. Rajmane and Korekar also reported similar results that fungi isolated from rotten mango samples were determined to be pathogenic on mango fruits at different ripening stages and disease severity on ripen mangoes was increased more than on green mangoes (Rajmane and Korekar, 2016). Previously, in the study of Palejwala *et al*. (2016), it was confirmed that ripe mangoes are more susceptible to spoilage than green mangoes. Specifically, citric and malic acids in unripe mangoes can partially inhibit fungal growth. However, when mangoes ripen, the amount of sugar in the mango increases and this stimulates the growth of fungi (Palejwala *et al.*, 1984). Thus, the fungal isolates isolated from the selected mango samples (M1, M2 and M3 strains) were used for subsequent experiments.

Table 2. Disease symptoms caused by the isolated fungal strains on mango fruits

Fungi	Symptoms
M1 strain	The symptoms were first observed as small, light yellow color suppressed lesions. The lesions increasing in size resulted in depressed mesocarp and a soft rot condition. The center of the lesion became sunken and was covered with brownish black spores.
M2 strain	The symptoms started as a dark brown to black rot at the fruit. The rot progresses into decay, turning brown, softening the tissues with the emission of a foul smell
M3 strain	The symptoms were a soft brown to black lesion that enlarges very rapidly

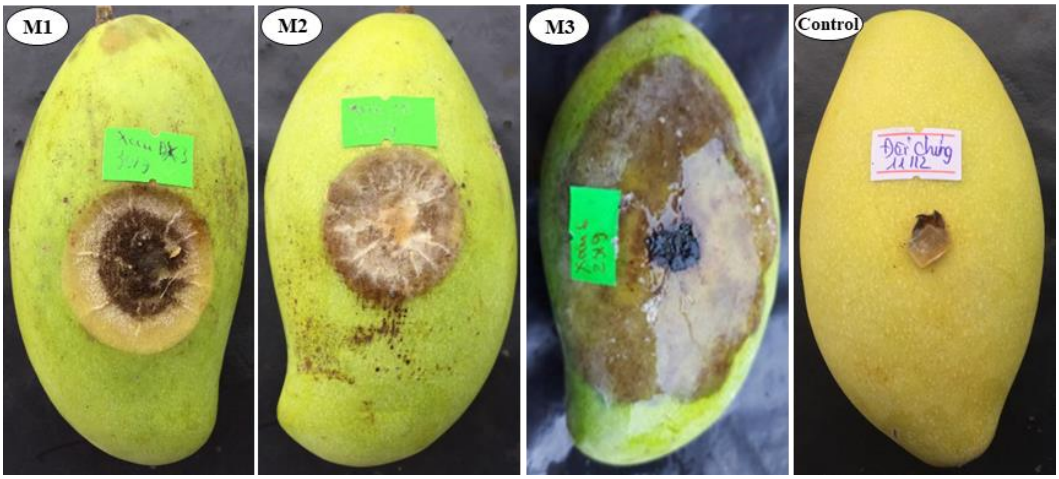


Figure 2. Re-infection of isolated fungal strain (M1, M2, M3) on mango fruits and control sample on the 4th day

Characterization of isolated fungal strains

In general, all three fungal isolates (M1, M2 and M3) grew well on PDA at 30°C (Figure 3). Microscopically, the strains showed branched and septate hyphae. M1 strain had big, globose, dark-brown conidial heads and conidiophore stipes were hyaline or turning dark towards the conidial heads. M2 strain was able to form sub-ovoid, light-yellow sclerotia, which eventually turned pale brown as the strain matured. Moreover, M2 strain also produced α-conidia and β-conidia. The α-conidia were hyaline, aseptate, ellipsoidal to cylindrical, rounded at both ends. The β-conidia were hyaline filiform, and aseptate. Whereas the conidia of M3 strain could not produce spores.

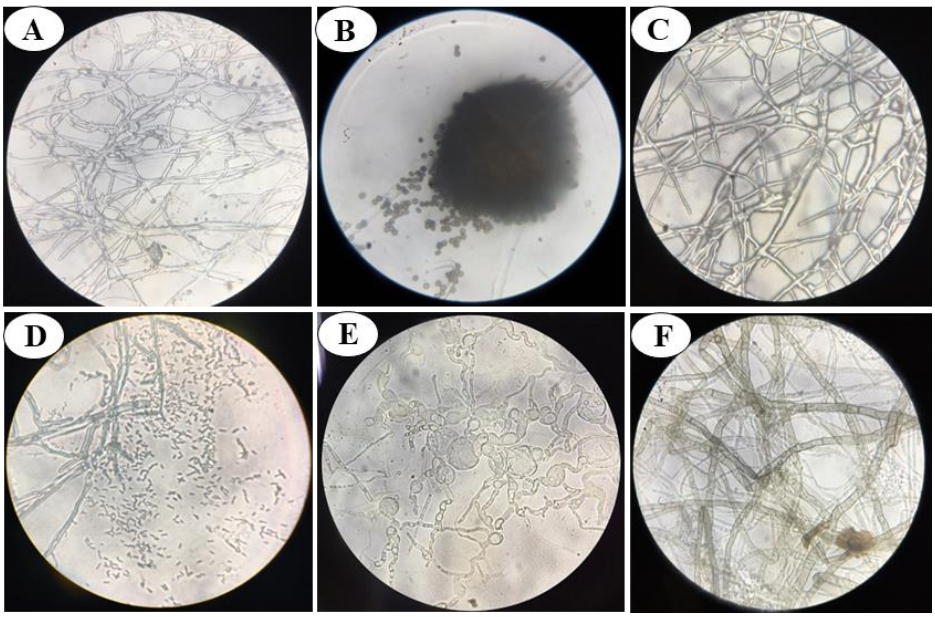


Figure 3. Microscopic observation of the isolated fungi

M1 strain - Septae hyphae (A) and conidia (B); M2 strain - Septae hyphae (C), α, β-conidia (D), and chlamydospore (E); M3 strain – Septae hyphae (F)

Optimal growth conditions of fungi

The radial mycelial growth rates of M1, M2 and M3 strains were significantly affected by culture media. In general, PDA was the most favorable medium for the fast radial growth of the mycelium of all three tested isolates. Apart from that, the M1 strain also grew well on SDA, while the M2 and M3 strains showed the ability to grow fast on MEA. All isolated strains grew slowly on CDA. Thus, PDA was the suitable medium used for other experiments to determine the influence of pH and temperature on colony growth. The results showed that the isolates grew differently at different pH values. The optimum growth of the M1 and M3 was observed at pH 5.0 while the M2 strain grew actively at pH 7.0 and 8.0. The isolates showed reduction in mycelial growth beyond this value. Similarly, the fungal isolates also showed different responses to temperature of incubation. However, the maximum mycelial growth of all three strains was at 30^{o}C. As the temperature went below and above this range, the isolates showed a decrease in growth diameter and mycelial density. Rahi *et al.* also carried out the study of evaluating the effect of different media, pH and temperature on fungal growth. As a result, they reported that the fungal strains grew the most rapidly on CDA media with pH 9.0 (Rahi *et al.*, 2017). Although the mycelial growth of the fungi increased at the same range temperature (25-30^{o}C) of our study, 25^{o}C was the optimal temperature of the fungi according to Rahi *et al*. (2017).

Extracellular enzyme activity

Mango peel mainly consists of cellulose, and pectin (Poonam *et al.*, 2017). Thus, the three fungal species inhabiting mango fruits were assessed for detecting activity of extracellular enzymes that are involved in spoilage of mango. For this assessment, two kinds of enzymes were examined. The results showed that the M1 had abilities to secrete both cellulase and pectinase, while the M2 and M3 strains produce only pectinase (Figure 4). Therefore, based on the ability to secrete cellulase and pectinase that degrade some components on the cell wall of mango, the fungi used in the study can penetrate and rot the mango fruit.

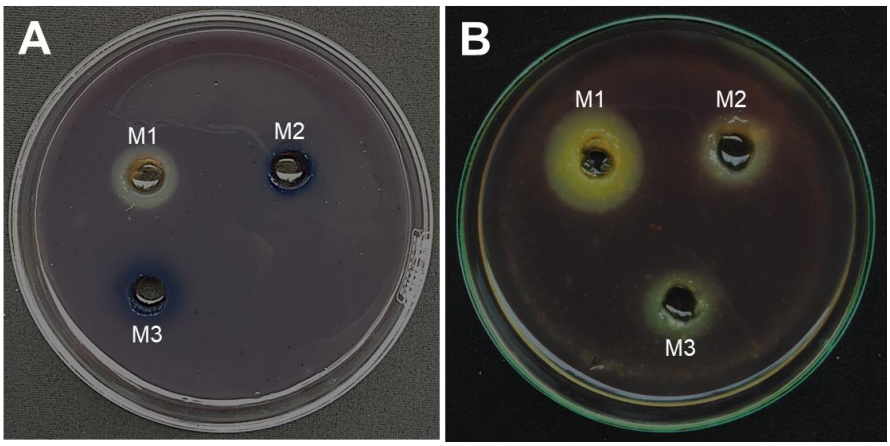
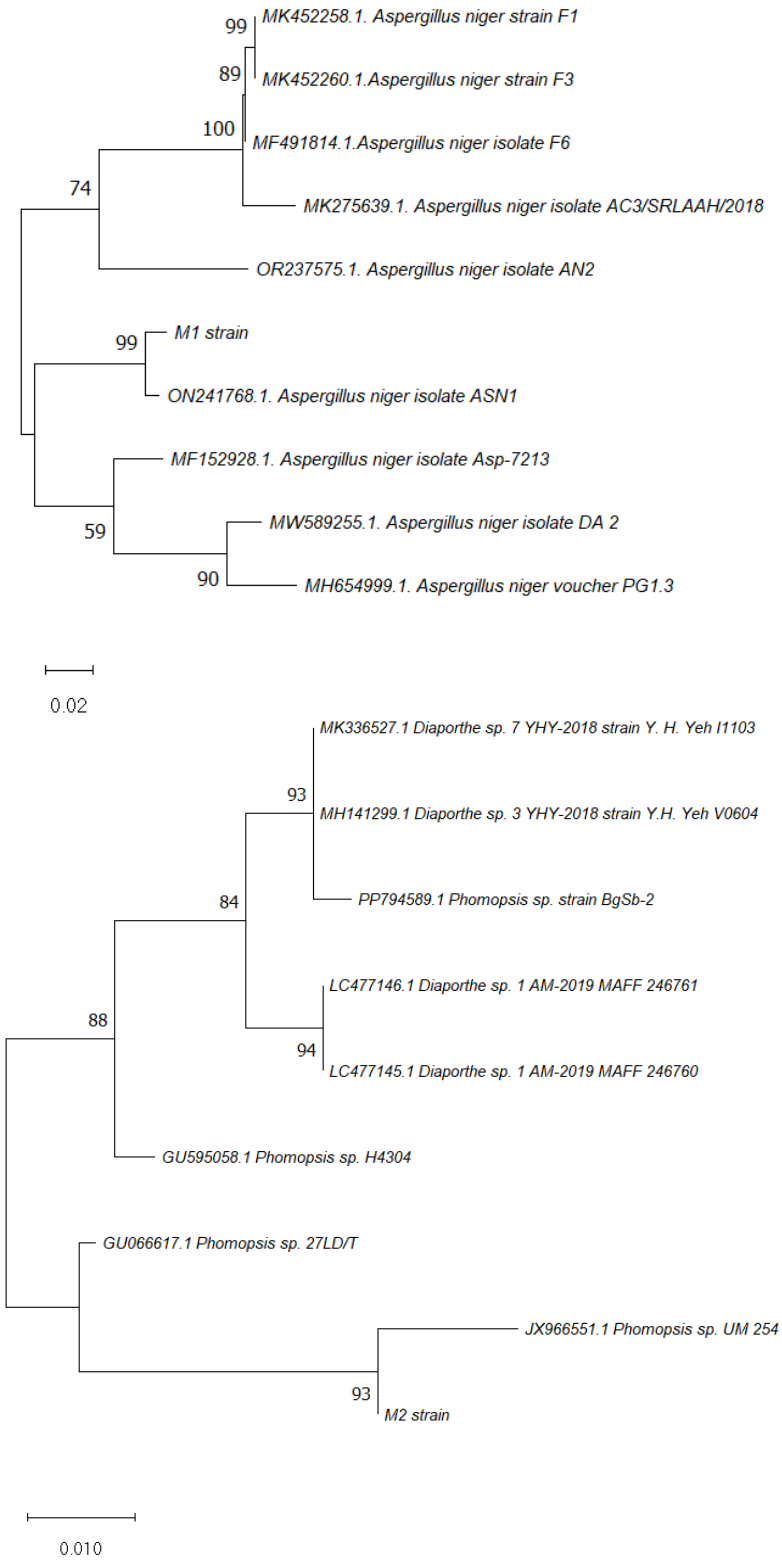


Figure 4. Cellulase (A) and Pectinase (B) activity of the isolated fungi

Molecular identification of fungi

Three fungal isolates were identified by ITS region amplification using ITS1 and ITS4 primers. The PCR products were then subjected to sequencing and the sequences were aligned with reference sequences in GenBank using the BLAST tool to determine the relationships and similarities of the isolated fungi with the identified strains in previous studies. The results showed that isolates M1, M2, M3 shared 98-100% similarity and a high boostrap value (92-100%) with *A. niger* isolate ASN1, *Phomopsis* sp. UM254, and *L. theobromae* ARM28, respectively (Figure 5). Therefore, based on the morphological and molecular characteristics, three strains, M1, M2 and M3, were identified as *A. niger* isolate M1, *Phomopsis* sp. isolate M2 and *L. theobromae* isolate M3, respectively. Many studies reported that *A. niger* was the most common species affecting different kinds of fruits. Some studies also indicated that *A. niger* isolated from infected mangoes was considered as a main causative agent of post-harvest diseases of mango (Ahmed and Mohammed, 2014; Fatima *et al.*, 2019; Mireille *et al.*, 2015). In 2012, Abreu *et al*. found that fungi of the genus *Phomopsis* (Teleomorph Diaporthe) caused mango diseases (Abreu *et al.*, 2012). In addition, *Phomopsis fukushiii* was recorded to cause decay of mango fruits (Choi *et al.*, 2017). Similarily, Mascarenhas *et al.* also discovered that *Lasiodiplodia theobromae (Botryodiplodia theobromae)* was able to cause spoilage of mango (Mascarenhas *et al.*, 1996). According to Dukare *et al.* (2019), *Botryodiplodia theobromae* was an etiological agent of stem end rot of mango (Dukare *et al.*, 2019).


99
MK452258.1. Aspergillus niger strain F1
89
MK452260.1.Aspergillus niger strain F3
100
MF491814.1.Aspergillus niger isolate F6
74
MK275639.1. Aspergillus niger isolate AC3/SRLAAH/2018
OR237575.1. Aspergillus niger isolate AN2
99
M1 strain
ON241768.1. Aspergillus niger isolate ASN1
MF152928.1. Aspergillus niger isolate Asp-7213
59
MW589255.1. Aspergillus niger isolate DA 2
90
MH654999.1. Aspergillus niger voucher PG1.3
0.02
MK336527.1 Diaporthe sp. 7 YHY-2018 strain Y. H. Yeh I1103
93
MH141299.1 Diaporthe sp. 3 YHY-2018 strain Y.H. Yeh V0604
84
PP794589.1 Phomopsis sp. strain BgSb-2
LC477146.1 Diaporthe sp. 1 AM-2019 MAFF 246761
88
94
LC477145.1 Diaporthe sp. 1 AM-2019 MAFF 246760
GU595058.1 Phomopsis sp. H4304
GU066617.1 Phomopsis sp. 27LD/T
JX966551.1 Phomopsis sp. UM 254
93
M2 strain
0.010

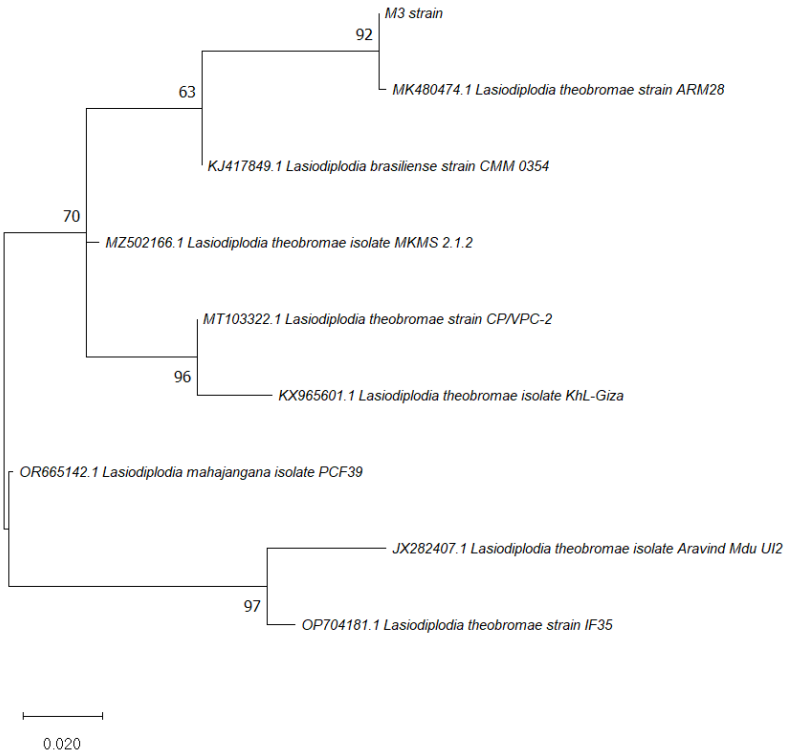


Figure 5. Phylogenetic tree of the M1, M2 and M3 strains

CONCLUSION

Mangoes are rapidly perishable fruits and highly susceptible to fungal invasion. Our findings revealed the characteristics of spoilage fungi capable of causing pathogencity of mangoes purchased in Hanoi, Vietnam. In addition, these fungal strains were identified as *Aspergillus niger* isolate M1, *Phomopsis* sp. isolate M2, and *Lasiodiplodia theobromae* isolate M3. Therefore, this can be useful scientific information to find disease control measures on mangoes, especially during the post-harvest period.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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