PCR and Sequencing Base Detection of Gummosis Disease on *Citrus aurantifolia* Caused by *Lasiodiplodia theobromae* and Evaluation of Its Antagonisms

Md. Faruk Hasan¹, Md. Asadul Islam¹ and Biswanath Sikdar¹*

¹Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi, 6205, Bangladesh.

Authors’ contributions

This work was carried out in collaboration among all authors. Author MFH designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author BS managed the analyses of the study. Author MAI managed the literature searches. All authors read and approved the final manuscript.

ABSTRACT

Diplodia gummosis disease caused by *Lasiodiplodia theobromae* is an economically important postharvest fruit decay that occurs on all types of citrus grown in Bangladesh. The present investigation was conducted to isolate and identify the pathogenic fungus responsible for postharvest diplodia gummosis disease (DGD) of citrus as well as the evaluation of its biological control through microbial antagonists. DGD causing pathogen was identified by physiological, morphological and molecular methods. The pathogenic fungus was isolated from the surface of postharvest lime fruits. The optimum growth of the fungi was observed in maltose, 2% NaCl, 15% sugar and pH 7 containing PDA medium at 35 °C. PCR products of the internal transcribed spacer (ITS) region of the fungus showed approximately 650 bp size clear band in gel electrophoresis. The
prognosis is considered to be an important all over the world. Consequently, diplodia quality of fruits not only in Bangladesh but also can limit the extension of storage, shelf different pathogenic diseases. Postharvest decay numerous health benefits, they are being fruits are valued for its nutritional qualities and both men and women. Though hepatic used for the treatment of cardiovascular antiparasitic inflammation described as antibacterial und. Ancylostoma dubum. The traditional uses of lime viz. essential oil and pectin of fruit peel are used in the cosmetic and products of lime viz. es other important constituents carotenoids, coumarins, essential oils, flavonoids, phenolic acids, triterpenoids and secondary metabolites like, alkaloids, carotenoids, coumarins, essential oils, flavonoids, phenolic acids, triterpenoids and other important constituents [4]. Different products of lime viz. essential oil and pectin of fruit peel are used in the cosmetic and pharmaceutical industries [5]. The traditional uses or phytochemical properties of C. aurantifolia from several literature reviews are described as antibacterial [6], antidiabetic, antifungal [7], antihypertensive [8], antiinflammation [9], antilipidemia [10], antioxidant, antiparasitic [11] and antiplatelet activities. It is used for the treatment of cardiovascular [12], hepatic [13], osteoporosis [14] and urolithiasis diseases and acts as a fertility promoter [15] for both men and women. Though C. aurantifolia fruits are valued for its nutritional qualities and numerous health benefits, they are being experienced in different marketing problems after harvesting and during storage [16], because of different pathogenic diseases. Postharvest decay can limit the extension of storage, shelf-life and quality of fruits not only in Bangladesh but also all over the world. Consequently, diplodia gummosis is considered to be an important problem confronting the citrus fruits industry and marketing, causes by a pathogenic fungus [17]. Amplified region of the fungus showed 99.62% similarities with the sequences of L. theobromae. Artificially inoculation of the fungus in malta, musumbi, sweet orange, lime, and guava fruits showed similar size clear band and typical diplodia gummosis disease symptom. Methanol extracts of *Datura metal* displayed the highest inhibition (75.25%) against the isolated fungus. Non-pathogenic fungi *Trichoderma viride* showed the highest antagonistic efficiency followed by *Neofusicoccum mangifera* against the isolated fungus. The tested soil bacteria did not show significant antagonistic activity against the isolated fungus. Therefore, the DGD of citrus control system should be integrated into the overall citrus postharvest decay control system to reduce all citrus postharvest diseases and to protect fresh citrus fruit values.

**Keywords:** *Citrus aurantifolia; PCR; sequencing; Lasiodiplodia theobromae; antagonisms.*

### 1. INTRODUCTION

Lime, *Citrus aurantifolia* (Christm.) Swingle, is one of the most popular fruit of the citrus species belonging to the family of Rutaceae, which reproduces asexually [1], commonly known as 'lebu' in Bangla. It is originated in Southern China but now grown commercially worldwide in tropical, subtropical, and some warm temperate regions [2]. Lime is rich of vitamin C that potentially provides advantage and good for health [3]. The fruits are very widely used in Indian subcontinent for preparation of lemon juice, used in salad, making pickles and beverages for flavouring jams, jellies, lemonade and marmalades. The fruits contain different secondary metabolites like, alkaloids, carotenoids, coumarins, essential oils, flavonoids, phenolic acids, triterpenoids and other important constituents [4]. Different products of lime viz. essential oil and pectin of fruit peel are used in the cosmetic and pharmaceutical industries [5]. The traditional uses or phytochemical properties of *C. aurantifolia* from several literature reviews are described as antibacterial [6], antidiabetic, antifungal [7], antihypertensive [8], antiinflammation [9], antilipidemia [10], antioxidant, antiparasitic [11] and antiplatelet activities. It is used for the treatment of cardiovascular [12], hepatic [13], osteoporosis [14] and urolithiasis diseases and acts as a fertility promoter [15] for both men and women. Though *C. aurantifolia* fruits are valued for its nutritional qualities and numerous health benefits, they are being experienced in different marketing problems after harvesting and during storage [16], because of different pathogenic diseases. Postharvest decay can limit the extension of storage, shelf-life and quality of fruits not only in Bangladesh but also all over the world. Consequently, diplodia gummosis is considered to be an important problem confronting the citrus fruits industry and marketing, causes by a pathogenic fungus [17]. Major factors responsible for postharvest loss of fresh fruits are mechanical damage, spoilage by fungi, bacteria, insects and other microorganisms. All the citrus fruits contain high levels of sugars, nutrient elements, and low pH values make them specially targeted to fungal decayed [18]. Citrus is one of the most cultivated fruits around the world, and fungal diseases are badly serious threats to its production and postharvest quality in Bangladesh. Postharvest DGD caused by *Lasiodiplodia theobromae* has become an enormous economically important problem in almost all over the world. It has a wide host range estimated to be more than 280 plant species [19,20] with varied pathological effects on its hosts and environmental conditions. Twumasi et al. [21] reported that this fungus was isolated from the four different crops, namely, cocoa, mango, banana and yam in Ghana. The fungal stain, *L. theobromae* is a causal agent of *Prunus persica* (L.) tree’s gummosis, a serious disease affecting peach cultivation and production [22]. Saeed et al. [17] showed that the fungus, *L. theobromae* is responsible for the mango dieback disease in the United Arab Emirates. Zhang et al. [23] reported the transcriptional response of grapevine to infection with the fungal pathogen *Lasiodiplodia theobromae*. Saeed et al. [17] reported fungicide treatments against *L. theobromae* as an effective and reliable approach to reduce the economic losses associated with mango disease. They also showed that the implementation of integrated disease management programs which combine cultural, chemical and biological approaches are highly recommended to control mango dieback, reduce cost and improve production efficiency. Despite its negative impact on the environment and human health, the use of chemicals continues to be the major strategy to lessen the menace of crop diseases in different countries. Surprisingly, there is very limited research and report about the pathogenicity capability of this devastating pathogenic fungus
affecting food and citrus fruits in Bangladesh. Moreover, there is no suitable report for detection of the causal agent for postharvest DGD of lime and evaluation of its ecofriendly biological control measures. Hence, the present study was aimed to detect the causal agent of citrus gummosis disease by morpho-physiological and molecular approaches as well as find out the effectiveness of the selected antagonistic agents to control this economically devastating and pathogenic fungus in Bangladesh.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Diseased samples of *Citrus aurantifolia* fruits were collected from different markets in Rajshahi, Bangladesh which were used to pathogen isolation.

2.2 Collection of Antagonistic Agents

For the present research, five non-pathogenic soil bacterial strains (*Bacillus subtilis*, *Brevibacillus brevis*, *Pseudomonas* spp., *Rhizobium phaseoli* and *Rhizobium leguminosarum*) and five non-pathogenic fungal strains (*Colletotrichum gloeosporioides*, *Neofusicoccum mangifera*, *Pestalotiopsis* spp., *Phomopsis* spp. and *Trichoderma viride*) were collected from Microbiology Lab., Dept. of Genetic Engineering and Biotechnology, University of Rajshahi which were identified previously. Microbes were storage at 4°C refrigerador for further experiments.

2.3 Isolation of the Pathogen Responsible for the Disease

At the laboratory, the infected fruit tissues were excised with a sterilized scalpel at the point of progression of disease symptom; then surface sterilized by 70% ethanol solution for 2 minutes. The tissues were then washed with sterilized distilled water three times and were placed on sterile paper towels for drying [24], followed by incubation on PDA (Hi-Media, India) medium at 35°C in the dark room for 3 days. The medium was supplemented with 0.5 gL⁻¹ of streptomycin sulphate (Sigma-Aldrich, USA) and 1 mL⁻¹ of lactic acid. The mycelium emerged from diseased sample were re-isolated using a single spore technique and transferred onto the petri dish of fresh PDA medium. The petri dishes were kept in dark room at 35°C for 7 days. The experiments were performed at different times for pure culture. Isolated fungus was sub-cultured on PDA medium to grow. The sub-cultured plates were preserved in a refrigerator at 4°C for further experiments.

2.4 Morpho-physiological Characterization of Isolated Fungus

Different morphological characteristics of the fungal colonies such as form, margin, elevation, surface, opacity, front colour, back colour and dry weight were observed. Cotton blue staining of the isolated fungus was performed by Sathya et al. [25] method. For microscopic observations, microscopic mounts were made from PDA medium growth colonies. The lacto-phenol cotton blue (LPCB) wet mount preparation is the most widely used method of staining and observing fungi. A wet mount of the fungi was prepared by suspending a little bit of fungal culture collected using a spatula and a needle in a few drops of lacto-phenol cotton blue solution on a microscope slide and then cover with a cover slip and view under a light microscope (LABOMED LX400, USA) at 40X magnification. Different media contain different nutrient supplements, which have an important effect on fungus growth and development. In the present study, actively growing isolates were placed at the center of the petri dish, containing different semi-solid media in each. The cultural characters of the isolates were studied on three different semi-solid media, like PDA, Sabouraud Dextrose Agar (SDA) and NA media. The isolated fungus was cultured in different nutrients containing media and the culture dishes were incubated at 35°C for 7 days. The resulting growth of fungus mycelium was harvested and filtered through pre-weighed Whatman No.1 filter paper and washed thoroughly with distilled water. The isolated mycelium was dried at 40°C in a hot air oven and the dry weights were recorded using an electric balance (LABOMED, USA). Colonies on each medium were compared for their overall dry weight. In the present study, isolated fungus was cultured on PDA medium and incubated in dark condition at different temperatures i.e., 5°C, 15°C, 25°C, 35°C, and 45°C to standardize the best condition for proper growth and development. The isolated fungus was cultured in PDA medium and incubated in a dark room at 5°C, 15°C, 25°C, 35°C, and 45°C respectively for proper growth. After 7 days of incubation, fungal mycelia were harvested from culture, dry and weighted using a digital electric balance (LABOMED, USA). The optimum pH for mycelial growth was determined on PDA, where pH was...
adjusted from 3.0 to 9.0 at 2.0 intervals. After sterilization, the culture bottles were kept in laminar flow hood for cooling down. After cooling down, five days old isolate culture growing on PDA plates were separately punched aseptically with a 7 mm diameter sterile cork borer. The fungal discs were then put on the centre of all culture vessels containing PD broth. After seven days of inoculation, colony morphology, growth characteristics, colony diameter and mycelial dry weight was recorded using a previously described procedure [26]. Values were means of three replications and the data were analyzed statistically. To study about the effect of salts on the mycelia of the strain, 5% of different salts namely, MgCl$_2$, CaCl$_2$, KCl and NaCl were included in the PDA medium. The isolated fungus was cultured in PDA medium containing different salts and the culture vessels were incubated at 35°C in a dark room for 7 days. After proper growth, the dry weight of the fungus was taken using an electric balance for statistical analysis comparisons to other salts. The effects of NaCl on the isolated fungus depend on different concentrations of NaCl. To detect the effects of NaCl, different amounts of NaCl, such as 1%, 2% and 4% was added in the PDA medium. The isolated fungus was cultured in PDA medium containing different amounts of NaCl and the cultures were incubated at 35°C for 7 days. After proper growth, fungal mycelia were harvested, dried and dry weight was measured using an electric balance. To standardize the effects of carbohydrates on the growth of fungus, 5% different carbohydrates such as lactose, fructose, maltose, starch and dextrose were used as sole carbon source in modified PDA media to check the effect of carbohydrates on the growth of fungi. PDA medium was prepared in four culture vessels then mixed 5% lactose, fructose, maltose, starch and dextrose of each of the vessels. The isolate was inoculated in different carbohydrates treated culture vessels and were incubated in a dark room at 35°C for 7 days. After fungus has grown properly, the mycelia were isolated, dried and weighted in an electric balance separately for statistical analysis.

To observe the best concentration of sugar, different percentages of sugar was added in the PDA medium. In the present study, for the growth profiling of the isolated fungus different concentrations of sugar, like 5%, 10%, 15% and 20% of maltose were added in PDA medium instead of 2% dextrose. The isolated fungus was cultured in different concentration of maltose treated culture vessels and incubated in a dark place at 35°C for 7 days. The fungal mycelia were isolated after proper grown as well as dried in a hot air oven and weighted in an electric balance for comparison to other concentrations.

2.5 Molecular Identification of the Isolate

Total genomic DNA of isolated fungus was extracted using the Maxwell® 16 LEV Plant DNA Kit, an automated DNA extractor and purifier of genomic DNA (gDNA) from microbe samples by following the manufacturer protocols (PCR purification kits, Promega, USA). Isolated gDNA was quantified using NanoDrop Spectrophotometer (ND2000, Thermo Scientific, USA) by following the manufacturer protocols (PCR purification kits, Promega, USA). Gene fragments specific for the highly variable 'Internal Transcribed Spacer' (ITS) region of the fungal rDNA was amplified by PCR method. The ITS region of the isolated fungus was amplified using the universal primers of ITS5F (5' GGAAGTAAAAGTCGTAACAAGG 3') and ITS4R (5' TCCTCCGCTATTGATATGC 3') [27]. PCR amplification (Gene Atlas, Astec, Japan) was performed in a 25 µl volume of reaction mixture comprising 9.5 µl deionized distilled water, 12.5 µl hot start green master mix, 1 µl of each forward and reverse primers (Promega, USA) and 1 µl cDNA (con. 25-65 ng/µl). PCR amplification was performed according to the kits instructions. Amplicons were separated by agarose gel electrophoresis (1%) in 0.5X Tris-EDTA buffer. A 1 kb DNA marker and DNA binding dye (Promega, USA) was used for the visualization, identification and measuring the size of PCR products by electrophoretic spectrophotometre (Thermo Scientific, USA). PCR products were purified using the Wizard SV Gel and PCR Clean Up System, according to the manufacturer protocol (PCR purification kits, Promega, USA). The ITS5F/ITS4R amplification products obtained starting from the DNA of the isolated fungus. The ITS region were obtained from the GenBank database at the National Centre for Biotechnology Information (NCBI) and aligned using the Pileup program available as part of the GCG package of rRNA gene. Sequence analysis was performed using a BlastN search of GenBank of NCBI analysis software and database from their website (www.ncbi.nlm.nih.gov). All the sequences were also aligned with ClustalW program [28] for constructing a phylogenetic tree. The phylogenetic tree was constructed with the neighbor joining method. The numbers on the nodes correspond to the percentages, with which
a cluster appears in a bootstrap test based on 54661 genes. The bars denote the relative branch length. The ITS region was identified from the GenBank database (www.ncbi.nlm.nih.gov). A phylogenetic tree was constructed using Mega 6 software.

2.6 Pathogenicity Test

The pathogenicity test of the isolated fungus was performed by modified wound method [17]. The pathogenicity of isolated fungus was evaluated against some postharvest citrus fruits, like malta, musumbi, sweet orange, lime and guava fruits. Healthy and fresh fruits of citrus were surface-sterilized with 70% ethanol followed by washing with distilled water. Fruits were wounded using sterile glass rod and wound was inoculated with mycelia from a 7 days old culture of isolated fungus. The uncultured pure PDA medium was used as control. Inoculated fruits were covered with poly bags, and incubated at 35°C for 7 days. After 7 days of incubation, fungal mycelia were collected from infected fruit samples, cultured in PDA medium and harvested the newly grown fungus. DNA was isolated, and PCR were performed, respectively. PCR products were purified and sequenced consequentially.

2.7 Biological Control Assessment

2.7.1 Screening of antifungal activities

For evaluating antifungal activity of plants extracts, a slightly modified poisoned food technique [29] was used. Selected plant specimens namely, Centella asiatica, Cassia alata, Datura metel, Coccinia grandis, Psidium guajava and Azadirachta indica were collected, dried under shade, milled and extracted with methanol using previously reported method [30]. Here, 20 ml PDA medium was plated in each petri dish. When the plated medium was cooled, it was inoculated with isolated fungus where 50 μg plant extract were added at 20 ml of poured the mixture into each petri dish. After solidification, six mm of mycelial disc was cut from the actively growing colonies of the fungal isolate by using sterile cork borer and placed it in the centre of petri dish containing the extract amended medium. The medium with the inoculum disc but without any extract served as negative control. Finally, the fungal plates were incubated at 35°C for 7 days in a dark room. After 7 days of incubation, fungal mycelia were harvested, dried and weighted by an electric balance for each of the plates. Subsequently, the inhibition of the growth was calculated by Arora and Dwivedi method [31].

2.7.2 Antagonistic assay

Antagonistic activity of the tested microorganisms was evaluated by disc diffusion method [32]. Previously made filter paper discs (6mm) were taken into the screw capped tube and sterilized in an autoclave at 121°C for 20 minutes to ensure sterilization. To evaluate the antagonistic efficiency of five non-pathogenic soil bacterial strains (B. subtilis, B. brevis, Pseudomonas spp., R. phaseoli and R. leguminosarum) and five non-pathogenic fungal strains (C. gloeosporioides, N. mangifera, Pestalotiopsis spp., Phomopsis spp. and T. viride) were co-culture with the isolated fungus. Previously cultured antagonistic agents were taken with help of micropipette or wire loop and applied on paper discs one by one and the culture dishes were incubated at 35°C for 7 days. After 7 days of incubation, the antagonistic activities of the test samples were determined by measuring the diameter of growth of soil bacteria and non-pathogenic fungi against the isolated fungus with a transparent scale in mm.

2.8 Statistical Analysis

Data presented as mean ± SD (Standard Deviation) and graphs were prepared by using Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA). For pathogenicity tests on fruits green, healthy fruits were used. Analysis of variance (ANOVA) and Duncan’s multiple range test were performed to determine the statistical significance at p < 0.05. All experiments were independently repeated three times with similar results.

3. RESULTS

3.1 Isolation of the Causal Agent

The fungus Lasiodiplodia theobromae were isolated from citrus fruits showing symptoms of diplodia gummosis disease (Fig. 1A). The isolated fungus took 3-5 days invariably to cover the 90 mm petri plates (data not shown) on the PDA medium at 35°C. Colour of the isolate varied from grey, greyish white, white, greyish black, grayish brown, blackish grey and blackish white in early growth stages (Fig. 1B). The isolate produced irregular or smooth margin. The
The lowest dry weight of mycelia was observed in the medium followed by 0.65±0.04 gm at 2% NaCl containing the PDA medium at 35°C. The lowest dry weight of mycelia was found to be 0.77±0.03 gm at 2% NaCl containing the PDA medium followed by 0.65±0.04 gm mycelia at 4% NaCl having the PDA medium at 35°C. The lowest dry weight of mycelia was observed 0.56 ± 0.04 gm at 1.0% concentration of NaCl containing the PDA medium at 35°C. Different concentrations of NaCl having the PDA medium showed no color variation but the size and weight were different. The highest dry weight of mycelia was observed to be 0.67±0.04 gm/dish in case of fructose followed by 0.620.01± gm in case of glucose containing PDA medium. On the left hand, the lowest growth of fungal mycelia were found to be 0.49 ± 0.07 gm/dish in case of maltose having the PDA medium. Moreover, in case of fructose and dextrose, the dry weight of mycelia was found to be 0.58 ± 0.06 gm and 0.59±0.01 gm/dish, respectively. Dextrose containing the PDA medium showed pinkish-white color fungal mycelia but other carbohydrates showed greenish-white color fungus. The highest dry weight of mycelia were found to be 1.27 ± 0.10 gm at 15% of sugar having the PDA medium followed by 1.18±0.19 gm mycelia at 20% sugar having PD broth medium. The lowest dry weight of mycelia was observed 0.55 ± 0.03 gm at 5.0% of sugar containing the PDA medium at 35°C. No remarkable morphological and color changes were found in the PDA medium having different percentages of sugar. The effect of different parameters for mycelia growth and development on isolated fungus are given in Fig. 2A-F.

3.2 Microscopic Observation

In cotton blue staining, copious broad, septate melanized fungal hyphae were demonstrated under the electronic microscope. Pycnidia lined internally with a dense layer of conidiophores and paraphyses. Conidiophores hyaline, cylindrical or tapering, short, arising directly from the pseudoparenchymatous inner wall of the pycnidia. Conidiogenous cells holoblastic, cylindrical or with an ampulliform swelling near the apex (Fig. 1D). Culture yielded an initially nonsporulating, dark pigmented mycelium ultimately identified as Lasiodiplodia theobromae.

3.3 Morpho-physiological Characterization of Isolated Fungus

In growth profiling of isolated fungus, the PDA medium showed the highest 0.96 ± 0.04 gm dry weight of mycelia per 90 mm diameter petri dish followed by 0.19 ± 0.0 gm dry weight of mycelia in the SDA medium per 90 mm diameter petri dish at 35°C. On the other hand, the NA medium showed the lowest 0.05 ± 0.01 gm dry weight of mycelia in a 90 mm diameter dish at the same conditions. In different media, fungus mycelia were different in color and size. The highest dry weight of mycelia was observed to be 0.75 ± 0.03 gm/dish at 35°C followed by 0.46 ± 0.03 gm/dish at 25°C in the PDA medium. On the left hands, no fungus growth and development was observed at 5°C. Moreover, at 15°C and 45°C the dry weight of mycelia was found to be 0.1 ± 0.00 gm and 0.44 ± 0.02 gm/dish respectively. The isolated fungus showed different color, size and growth concentration. In fungal growth and development, pH plays an important role. The highest dry weight of mycelia was found to be 0.55 ± 0.03 gm/dish at pH 7 followed by 0.38 ± 0.02 gm/dish at pH 5 in the PDA medium at 35°C. On the other hand, the lowest dry weight of mycelia were found to be 0.18 ± 0.05 gm/dish at pH 3 at same condition. In low pH levels, fungus was found to be pinkish-white and in high pH fungus was greenish-white. The highest dry weight of mycelia was found to be 0.72 gm ± 0.3/dish at 0.05 g m NaCl containing the PDA medium followed by 0.65 ± 0.03 gm/dish mycelia at 0.05 gm KCl containing PDA medium at 35°C. The lowest dry weight of mycelia was observed 0.56 ± 0.0 gm/dish at 0.05 gm CaCl2 containing the PDA medium at 35°C. NaCl having the PDA medium showed pinkish-white in color and KCl containing PDA medium showed greenish-white color fungus morphology. The highest dry weight of mycelia was found to be 0.77±0.03 gm at 2% NaCl containing the PDA medium followed by 0.65± 0.04 gm mycelia at 4% NaCl having the PDA medium at 35°C. The lowest dry weight of mycelia was observed 0.56 ± 0.04 gm at 1.0% concentration of NaCl containing the PDA medium at 35°C. Different concentrations of NaCl having the PDA medium showed no color variation but the size and weight were different. The highest dry weight of mycelia was observed to be 0.67± 0.04 gm/dish in case of fructose followed by 0.620.01± gm in case of glucose containing PDA medium. On the left hand, the lowest growth of fungal mycelia were found to be 0.49 ± 0.07 gm/dish in case of maltose having the PDA medium. Moreover, in case of fructose and dextrose, the dry weight of mycelia was found to be 0.58 ± 0.06 gm and 0.59±0.01 gm/dish, respectively. Dextrose containing the PDA medium showed pinkish-white color fungal mycelia but other carbohydrates showed greenish-white color fungus. The highest dry weight of mycelia were found to be 1.27 ± 0.10 gm at 15% of sugar having the PDA medium followed by 1.18±0.19 gm mycelia at 20% sugar having PD broth medium. The lowest dry weight of mycelia was observed 0.55 ± 0.03 gm at 5.0% of sugar containing the PDA medium at 35°C. No remarkable morphological and color changes were found in the PDA medium having different percentages of sugar. The effect of different parameters for mycelia growth and development on isolated fungus are given in Fig. 2A-F.

3.4 Molecular Identification of the Isolates

The primers ITS4 and ITS5 pair gave rise to amplification of fungal DNA products of different intensity (Fig. 3A). The PCR products showed a strong and clear band of 650 bp size approximately, comparison of 1 kb DNA ladder marker. On the other hand, negative control did not show any band.

The universal primers ITS4 and ITS5 were used to confirm the identity of the fungal isolate and draw phylogenetic relationship. The ‘ITS’ region of ribosomal DNA was amplified and sequenced. The 550 bp sequenced nucleotide sequence was performed for a BlastN search in GenBank (https://www.ncbi.nlm.nih.gov). It was revealed that, the strains had approximately 99.62%
similarity with *L. theobromae* isolate BPPCA265 small subunit 5.8S ribosomal RNA gene. The data was aligned and phylogenetic tree that showed the other similar species of *L. theobromae*. The phylogenetic tree of ITS region with other various yeasts is shown in Fig. 3B.

### 3.5 Pathogenicity Test of the Isolated Fungus

Some selected citrus fruit namely, malta, musumbi, sweet orange, lime, and guava fruits were used for pathogenicity determination. Infected fruits show typical gummosis disease symptoms after incubation at 35°C for 7 days. Color of the isolate varied from grey, greyish white, white, greyish black, grayish brown, blackish grey and blackish white in early growth stages. The control fruit did not show any symptoms of gummosis disease. The isolated fungal pathogen from infected fruits were isolated, cultured and DNA was extracted from the isolates subsequently. In PCR products, the isolates B, D, E and F showed clear band of 650 bp length size in gel electrophoresis. The isolate C showed an unexpected 700 bp size band. Negative control didn’t show any band. Sequencing was performed using the PCR.

![Fig. 1. Naturally infected postharvest citrus fruit showing symptoms of DGD and morphological phenotypes of *Lasiodiplodia theobromae* conidia. (A) infected citrus fruit; (B) fungus culture (blackish-white) at day 7, (C) septate filaments of *L. theobromae* and (D) *L. theobromae* hyaline, aseptate, thick-walled mature conidia from a 7 days old PDA culture under the microscope](image)

![Fig. 2. Growth profiling of isolated fungus, measuring the dry weight of mycelia after 7 days of incubation. (A) different media, (B) different temperatures, (C) pH levels, (D) different concentrations of NaCl, (E) different carbohydrates at 5% level and (F) percentages of sugar](image)
products and they showed similar result (Data not given) to isolate. Infested results of fruits are given in Fig. 4 and PCR results of pathogenicity are presented in Fig. 5.

3.6 Biological Control Assessment

Methanol extract of leaf of *Datura metel* showed highest 75.25% of growth inhibition of mycelia followed by 69.31% of growth inhibition of mycelia by methanol extract of *Cassia alata* leaf in the PDA medium having 50µg extract/disc. On the other hand, the lowest growth inhibition of mycelia was found to be 35.65% by methanol extract of *Azadirachta indica* leaf at the same concentration. Moreover, *Centella asiatica*, *Coccinia grandis* and *Psidium guajava* leaf extracts showed 54.55%, 65.35% and 47.53% inhibition of mycelial growth, respectively. Effects of different plants extract against the isolated fungus are presented in Graph 6 and in Fig. 6.

To evaluate the antagonistic efficiency, all the tested soil bacteria reduced the growth capability of the isolated fungus non-significantly. On the other hands, non-pathogenic fungi was significantly effective for inhibit the growth of isolated fungus. *Trichoderma viride* fungi showed highest inhibition zone followed by *Neofusicoccum mangifera* fungus against the isolated fungus. Others fungi like, *Colletotrichum gloeosporioides*, *Pestalotiopsis spp.* and *Phomopsis spp.* was also effective antagonistic agents against the isolated fungus. Effects of different antagonistic agents against the isolated fungus are presented in Fig. 7.

![Fig. 3. Molecular identification of L. theobromae by PCR amplification of the ITS region in infected citrus fruit using the pairs of primers ITS5 and ITS4; (A) M: 1 kb DNA ladder (Marker), I: isolated fungus and N: negative control, (B) dendrogram showing phylogenetic relationships of the fungal sequence of the specimen](image)

![Fig. 4. The pathogenicity assays of L. theobromae on citrus fruits; A: negative control (sweet orange), B: malta, C: musumbi, D: sweet orange, E: lime and F: guava fruits](image)
Fig. 5. Molecular identification of *L. theobromae* by PCR amplification of the ITS region in artificially inoculated citrus fruit using the pairs of primers ITSS and ITS4; M: Marker, A: positive control (isolated fungus), B: malta fruit fungus, C: musumbi fruit fungus, D: sweet orange fruit fungus, E: lime fruit fungus, F: guava fruit fungus, and N: negative control

Fig. 6. Inhibition percentages of mycelia by different plant extract after 7 days of incubation. Effect of different plants extracts against the isolated fungus (A) Positive control (B) *Cassia alata* (C) *Datura metel* (D) *Coccinia grandis* (E) *Psidium guajava* and (F) *Azadirachta indica*

4. DISCUSSION

*Citrus aurantifolia* is one of the most popular juice fruit crops in Bangladesh as well as all over the world. Diplodia gummosis disease caused by *Lasiodiplodia theobromae* (synonyms: *Botryodiplodia theobromae* and *Diplodia natalensis*) is an economically important postharvest fruits decay that occurs on all types of citrus grown in Bangladesh. Bangladesh has been motivated to widely culture lemon fruits for salad and to fulfill the necessity of vitamin c and others in recent years. *L. theobromae* is a geographically widespread species of *Botryosphaeriaceae* [33], causing different diseases in different trees and fruits [17,34,35, 21], growing areas in the world. This fungal pathogen could be found alone or in combination with other fungal pathogens to occur gummosis of citrus disease.

In this research, we aimed to determine the causal agent of gummosis in lime fruits, and to find an effective biological agent for the potential threat associated with this disease in Bangladesh. The pathogen was isolated and identified morphologically and phylogenetically from the postharvest lime fruits peels.
Microscopy demonstrated that the pathogen is a prolific producer of immature and mature conidia on PDA medium. We observed that with age, mature conidia became two-celled, dark brown, with longitudinal striated appearance. Consistent with Saeed et al. [17] immature conidia were initially hyaline, unicellular, ellipsoid to oblong, thick walled with granular contents. In the present investigation, optimum growth of the fungi was observed in maltose, 2% NaCl, 15% sugar and pH 7 containing PDA medium at 35°C. Baloch et al. [36] reported that the highest growth and development of Botryodiplodia theobromae fungus was observed at pH 7-8 containing PDA medium at 30-40°C. This results support our present findings. Hasan et al. [26] reported the similar condition for growth profiling of the causal agent of Cercospora leaf spot disease of okra. Gao et al. [22] reported that L. theobromae JMB-122 was cultured on PDA medium at 28°C for growth and development which is contradictory to our present findings. For rapid identification of L. theobromae fungus, molecular study is needed, and reference may be built to several well documented sequences available on GenBank [37]. The primers ITS4/ITS5 pair gave rise to amplification of fungal DNA products of different intensity. PCR products of the ITS region of the fungus showed approximately 650 bp length clear band. The amplified region of the fungus showed 99.62% similarities with the sequences of L. theobromae BPPCA265 (GenBank). The universal primers ITS4 and ITS5 were used to confirm the identity of the fungal isolate and draw phylogenetic relationship, the ‘ITS’ region of ribosomal DNA was amplified and sequenced. Chowdhury et al. [38] reported 100% similarity with ITS sequence of Fusarium oxysporum using ITS primers. We also investigate a phylogenetic data analysis of the isolated pathogen from lime fruit. PCR amplification of ITS of the rDNA gene from mycelium of infected tissues was carried. Our results confirming that L. theobromae is frequently associated with all gummosis disease symptoms on postharvest lime fruits in Bangladesh. We compared the identified strain with those available in GenBank based on a phylogeny tree. For that purpose, the ITS rDNA gene was used as a single gene set. The concatenated gene set was sequenced and deposited in GenBank. In the present investigation Lasiodiplodia species, revealed that the isolated pathogen is placed adjacent to L. theobromae BPPCA265, distinguishing the obtained isolate from those belonging to other species of L. Diplodia, or Phyllosticta. Our phylogenetic analysis supports that the species L. theobromae dominates in Bangladesh causing gummosis disease on citrus fruits. To prove the microbial capability of the disease by verifying the existence of the pathogen and its progression in fruits skin of lime were inoculated artificially in marla, musumbi, sweet orange, lime and guava fruits. The results of inoculation on fruits tissues were similar to the disease symptoms of the pathogen isolated from the inoculated plants. The previous pathogenicity test reports of different fruits [21,35,39,40,41] were supported the present data. But, it would be virtually impossible to distinguish between Lasiodiplodia species based on their morphological traits only. To confirm the isolated pathogens, DNA extraction and PCR amplification were performed. The PCR products showed 650 bp length size which was similar to the first isolated pathogen PCR results. Jahan et al. [42] reported similar results which support our

Fig. 7. Antagonistic test of soil borne bacteria and fungi against the isolated fungus (A) Bacillus subtilis (B) Brevibacillus brevis (C) Pseudomonas spp. (D) Rhizobium phaseoli (E) Rhizobium leguminosarum (F) Colletotrichum gloeosporioides (G) Neofusicoccum mangifera (H) Pestalotiopsis spp. (I) Phomopsis spp. and (J) Trichoderma viride
present findings. In antifungal activity, methanol extracts of *Datura metal* displayed highest diameter of zone of inhibition at the concentration of 200 µgm/disc against the isolated fungus. The antifungal activity of some plant extracts have been reported by some researches [43,44,45]. Ehiobu and Ogu, [46] revealed that *Manihot esculenta* leaf extract was significant at 75 g/L, with 68.2% inhibition against *B. theobromae* fungus and leaf extracts of *Discoria rotundata*, and *Colocasia exculenta* also possess significant in vitro antifungal activities against *B. theobromae* at the same concentration. Chowdhury et al. [38] reported that methanolic extracts of *Ficus racemosa*, *Moringa oleifera*, *Azadirachta indica*, *Cassia alata*, and *Senegalia catechu* have been significant antifungal activities against some pathogenic fungi. Findings of the present confirmed that plant extracts can be used as natural fungicides. In this study, *Trichoderma viride* showed highest antagonistic efficacy while soil bacteria did not showed significant antagonistic effect against the isolated fungus. Previous report showed that the antagonism of *Trichoderma spp.* (*T. harzianum* and *T. viride*) or *Aspergillus niger*, can be effective against *L. theobromae* [47]. Sultana and Ghaffar [48] reported that under laboratory and field conditions to protect bottle gourd (*Lagenaria siceraria*) against seedling and root rot diseases, plants treated with *Bacillus subtilis*, *T. harzianum* or *T. viride* reduce the pathogenic effect of *L. theobromae* by more than 90%. Our present study provided insights into the mechanisms of lime gummosis caused by *L. theobromae* fungus. However, the role of gum in lime fruit response to pathogen attacks requires further investigation.

5. CONCLUSION

In the present study, isolate of fungus from postharvest *citrus aurantifolia* fruit was investigated by morphological, physiological and molecular identification. Here, fungus was isolated from infected citrus fruits and characterized by morpho-physiological characteristics. Moreover, PCR amplification and gene sequencing results confirmed the detection of *L. theobromae* fungal stain as causal agent of postharvest diplodia gummosis disease of citrus fruits. Different biological agents were evaluated for in vitro controlling the pathogenic fungus responsible for diplodia gummosis of citrus. So, it can be concluded that the present findings with detailed data will be helpful to manage this devastating disease.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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