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Characterization of *Lasiodiplodia pseudotheobromae* associated with citrus stem-end rot disease in Bangladesh

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Abstract

Stem-end rot is the most common postharvest citrus fruit diseases in Bangladesh and worldwide. The aim of the present study was to identify the causal pathogen and assays to *in vitro* control of the pathogen against different plant extracts. The pathogen was isolated from infected fruit on PDA media forming white to grey colonies. The maximum mycelial growth of isolate was recorded at temperature 25°C and 30°C with radial growth 88.00 mm and 79.00 mm respectively on PDA media. Different pH, carbohydrates, and salts were also used to lead the growth profiling which also revealed the morphology of isolated pathogen. The isolated fungus was identified based on a combination of morphological characteristics and sequencing of the internal transcribed spacers (ITS) region and the gene encoding lp1 small subunit ribosomal RNA gene. The nucleotide sequence comparison and phylogenetic analyses indicate approximately 99% identity with the fungal pathogen *Lasiodiplodia pseudotheobromae*. Based on the morphological characteristics, molecular and phylogenetic approaches, the causal fungus was identified as *L. pseudotheobromae*. Different plants extract were used to evaluate *in vitro* control of the citrus fruit end-rot disease pathogen. The methanolic extract of *Azadirachta indica* and *Psidium guajava* significantly inhibited the mycelial growth (93.78% and 90.90% respectively) of *L. pseudotheobromae in vitro*.

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Introduction

Citrus fruits are one of the most edible fruits in the worldwide belonging to the family of Rutaceae (Spiegel and Goldschmidt, 1996). It leads a second most planting and production fruit plant around the world. Citrus fruits like lemons and limes reflect various therapeutic qualities such as skin problems, scabies, healing blow, increased sperm quality, preventing hair loss etc (Ram and Singh, 2006). Bangladesh increase to reach a level of 155,936 tonnes in 2016, it went through a trough reaching a low of 17,971 tonnes in 1978 (Sarker *et al.,* 2017). Every year citrus is affected by numerous bacterial, fungal and viral diseases like citrus canker, bacterial spot, bacterial blast, black pit, *Alternaria* rot, Anthracnose, Brown rot, stem-end rot, and *Trichoderma* rot, yellow mosaic virus, CCDV virus, leaf curl etc and cause severe loss of citrus fruit production in Bangladesh (Fulton *et al.,* 1986; Droby *et al.,* 1998; Ismail and Zhang, 2004; Kader and Rolle, 2004; Islam *et al.,* 2017). Fungi causing postharvest diseases may possess a long inactive (quiescent) stage after infection before causing disease symptoms. Stem-end rot disease of citrus is the most considerable disease in the citrus plant because it reduces 60-80% of productivity.

This disease is caused by *L. pseudotheobromae* is an important opportunistic pathogen (Sathya *et al.,* 2017). This pathogen is a member of *Botryosphaeriaceae,* is a cosmopolitan fungus strain occurring in different tropical and subtropical regions causing several types of disease in many plant species (Punnithaligam, 1980; Droby *et al.,* 1998). *L. pseudotheobromae* is an economically important fungus known to cause a major loss of citrus, mango, banana etc (Rieger and Mehrota, 1995). The symptoms of stem-end rot disease appeared on fruits, which has a buff to brown colored, leathery area encircling the stem end of the fruit. The disease starts as water soaked large patches on the basal portions of the stem near the ground level. Bark in such parts dries, shrinks and cracks and shreds in lengthwise vertical strips. Later profuse exudation of gum from the bark of the trunk occurs. A considerable amount of gum formation in sweet oranges may be observed (Coutinho *et al.* 2017). The knowledge on specific characterization in host-pathogen identification is crucial for proper disease management.

The present work focuses on isolation and identification of *L. pseudotheobromae* fungal pathogen through the morphological and molecular approach and also resembles its antifungal activity against isolated fungal strain by using different plant extracts.

Materials and methods

Source and collection of infected citrus samples

The present investigation was done during the early winter season of 2017-2018 at Professor Joarder DNA and Chromosome Research Laboratory in the Department of Genetic Engineering and Biotechnology, University of Rajshahi, Bangladesh. Stem-end rot symptoms infected citrus fruits were collected from the Rajshahi University botanical garden, Bangladesh. The infected samples were washed with distilled water, and finally kept into plastic bags and placed in a refrigerator at 4°C (Figure. 1A).

Pure culture of L. pseudotheobromae

For obtaining a pure culture of infected pathogens, the fruit samples were thoroughly washed with the distilled water and sterilized in 1% sodium hypochlorite solution (Sigma-Aldrich Cheniere) by dipping for 1 minutes and rinsed three times with sterilized distilled water (SDW). After that, citrus tissue was excised (2-4 cm) from the diseased portion with the help of surgical blade and placed on Petri plates containing PDA (potato dextrose agar) medium amended with 85% lactic acid to prevent bacterial growth (Rangaswami, 1958). Then the plates were incubated at 28°C for seven to ten days (Figure. 1B).

After fungal mycelium growth, mycelium again transferred to a new PDA Petri plates (Munirah *et al.,* 2017). The pure cultures were maintained and preserved at -4°C using a modified filter paper method (Fong, 2000).

Morphological and microscopic studies

In this study, two morphological (macro- and micromorphological) characteristics were figured out. For macro-morphological characteristics, the colony appearance and pigmentations cultures were observed after seven days of incubation on PDA medium. Isolated fungus strain growth was measured in triplicate after 72 hours of incubation. Micro-morphological characteristics of conidia (shape, size, color, texture, reverse color, dry weight) were observed after two weeks of incubation. For this purpose, lactophenol cotton blue reagents were used and subjected to analysis of colony morphology under the 40 x light microscope (LABOMED, LX400, USA) (Woo *et al.,* 2008).

Growth profiling of L. pseudotheobromae

Growth profiling at different temperature

For analyzing the optimum growth in different temperature of isolated fungal strain, three Petri plates with around 25 ml PDA media were used. 5 mm mycelial discs of the isolated strain from pure culture were placed at the center of the Petri dishes. Then the three Petri plates were kept in 25ºC, 30ºC, and 37ºC temperature respectively and incubated for 7 days (Felix *et al.,* 2016).

Growth profiling in different pH

For optimum growth in different pH of isolated fungi, three Petri-plates were used. Then the pH of the medium was adjusted at 5, 7, and 9 respectively with HCl and NaOH. After prior to sterilization, 5mm diameter mycelial discs were cut from a pure culture on PDA media and inoculated in the culture newly sterilized Petri plates. Then the plates were incubated at room temperature for 7 days. After 7 days, the dry weight is measured using electrical balance by collecting the fungal mycelium (Dhandhukia and Thakkar, 2007).

Growth profiling in different carbohydrates

In this study, different carbohydrates were used at the same concentration (20gm/L) such as fructose, lactose, and maltose. After the solidification of the media, mycelia discs were transferred at the center of the top of the media plates and incubated at room temperature for 7 days with the help of cork borer and needles. After 7 days, the dry weight were calculated and fungal mycelial characteristics were studied (Dhandhukia and Thakkar, 2007).

Growth profiling in different salts

The three different salts were used here such as $MgCl_2$, NaCl, and KCl. After the solidification of the media, mycelia discs were transferred at the center of the media and incubated at room temperature for 7 days. After 7 days the characterization study and the dry weight study were done (Kour *et al.,* 2008).

The cellulolytic activity of L. pseudotheobromae

For this analysis, a cellulose strip of 5cm/1cm was used. This strip was placed in liquid PDA culture bottle before fungus incubation. Then mycelia was picked from the PDA plate and placed on newly sterilized PDA bottle. After that, the bottle was kept on 25ºC. The cellulolytic activity was observed after 7 days of incubation (Lee *et al.,* 1985).

DNA extraction and PCR parameters

Total genomic DNA was extracted from 8-10 days old cultures using OMEGA Bio-Tek fungal DNA isolation kit followed by a dry specimen protocol. The internal transcribed spacers (ITS) region of 18s rRNA sequence was amplified using the primers NSI (5′-GTAGTCATATGCTTGTCTC-3′) (Chen *et al.,* 2016) and NSIV (5′-CTTCCGTCAATTCCTTTAAG-3′) (Alves *et al.,* 2008). For PCR amplification, 50 µl of the reaction mixture was prepared which contained 25 µl Taq blue PCR master mix (2X), 1 µl of forward and 1 µl of reverse primer, 1 µl of genomic DNA and rest of the PCR water. PCR conditions (Q-cycler 96, Hain Life Science) were as follows: initial denaturation at 95ºC for 2 min, followed by 35 cycles of denaturation at 94ºC for 1 min, primer annealing 52ºC for 30 seconds, primer extension 72ºC for 1 min each with a final elongation at 72ºC for 5 min. The PCR product was amplified at 1% agarose gel in 0.5% Tris/Borate/EDTA buffer and the amplified bands

were detected against 1 kb ladder (DL5000 DNA Marker, TSINGKE).

Sequencing and phylogenetic studies

The final PCR products were purified by Thermo Fisher PCR purification kit. After getting the sequencing result, the analysis was done through the BlastN search of GenBank analysis software and database. CLUSTAL W and MEGA6 software were used for multiple alignments and comparisons with identifying strain for each of the genes (Tamura *et al.,* 2013). The fungal isolate nucleotide sequences were deposited in the Gen Bank database.

Antifungal activity of plant extracts against L. pseudotheobromae

Source of plant materials

The extracts of six different plant have been evaluated for their antifungal activity against the isolated fungal strain. Table 1 shows a list of the plants along their source, family, local name, usable parts and traditional uses (Chakraborty *et al.,* 1991; Rastogi and Mehrota, 1995). These plant materials were collected from Rajshahi University Botanical garden area.

Preparation of plant extracts and fungal growth inhibition

Fresh plant materials were washed thoroughly with SDW and allowed to dry at 25°C. The dried plant samples were crushed with the help of sterilized mortar and pestle to a fine powder (total 3gm). Each plant powder was added in methanol (0.5 g/ml).

The extracts were filtered through sterilized filter paper and then centrifuged at 10000 rpm x g for 10 min. 1 ml of each methanol plant extracts were added to 25 ml of PDA media. After that, the media plates were allowed to solidify and fungal isolates were inoculated (5 mm disk) from seven-day old culture and grown at 28°C for seven to ten days (Sha *et al.,* 2005; Ullah *et al.,* 2017).

Bio-efficacy of plant extracts against L. pseudotheobromae

The antifungal activity of plant extracts was observed on PDA plate by using the following formula (Vincent 1947).

Percentage of inhibition: [% = C-T/C x 100]

C= Growth in absence of plant extracts

T= Growth of fungi in the presence of plant extracts

Statistical analysis tools

Above the experiment of the present study was conducted in triplicate for consistency of exact results and statistical analysis. All the data were expressed as a mean and standard error (M±SE) using Microsoft Excel software 2013. The actual values with $P<0.05$ were calculated statistically significant.

Results

Isolation of fungi

The fungal isolate was obtained from infected citrus fruit (Figure 1A). After seven days of incubation, the fungal colony was appeared due to mycelial growth. At the first appearance, the colony looked white cottony to fluffy (Figure 1B). In later stage, it undergoes to grey color.

Table 1. List of six medicinal plants for antifungal activity.

Scientific name	Local name	Family	Usable parts	Traditional uses
Ocimum sanctum	Tulsi	Lamiaceae	Leaves	Fresh leaves bruised and applied externally for curing ringworm and other skin diseases
Ficus carica	Dumur	Moraceae	Leaves	Leaves used in a wide range of ailments related to digestive, endocrine, reproductive, and respiratory systems.
Allium sativum	Roshun	Amaryllidaceae	Bulb	Juice used as a rubefacient in skin diseases, used in atonic dyspepsia, flatulence, and colic
Azadirachta indica	Neem	Meliaceae	Leaves	Applied to boils as a poultice, decoction antiseptic, used in ulcers and eczema
Moringa olifera	Sajina	Moringaceae	Leaves	Used as purgative and for a cough
Psidium guajava	Peyara	Myrtaceae	Bulb	Used as a gargle for mouthwash in swollen gum, locally applied to the ulcer

Morphological and microscopic identification

In microscopic observation, the isolated fungal strains showed septate conidia which are very long (Figure. 1C), irregular hyphae (Figure. 1D), and enlarged mycelium (Figure. 1E). The conidia were initially hyaline, thin-walled and aseptate, cylindrical to subovoid in shape. The isolates produced anamorph structures on the pine needles on PDA media within 2 weeks. The aerial mycelium was initially white, turning whitish–grey color after 10-112 days at 25°C in the dark. Table 2 showed the overall morphological characterization of *L. pseudotheobromae* on PDA media.

Table 2. Morphological characterization of *L. pseudotheobromae* on PDA media.

No	Morphology	Characteristics	Growth time
1	Colony color	Fluffy white to grey	Initial
2	Form	Circular	8 days
3	Margin	Entire	10 days
4	Surface	Rough	8 days
5	Back color	Dark black	11 days
6	Diameter (mm)	89±2	12 days
7	Dry weight (mg)	47±0.5	12 days

Growth analysis of L. pseudotheobromae

Growth analysis or profiling tests were done to resemble which conditions are appropriate for fungus growth. Different temperature, pH, carbohydrates, and salts tests were optimized in this study. Temperature is a vital factor which plays a significant role in the different enzymatic studies. *L. pseudotheobromae* fungi were grown in three different temperature to check the effect of temperature on the fungus growth such as 25°C, 30°C, and 37°C respectively. So the colony radial growth was obtained 88mm, 79mm, and 8mm with 48±0.5gm, 36±0.5gm, and 5±0.5gm dry weight respectively (Figure. 2A).

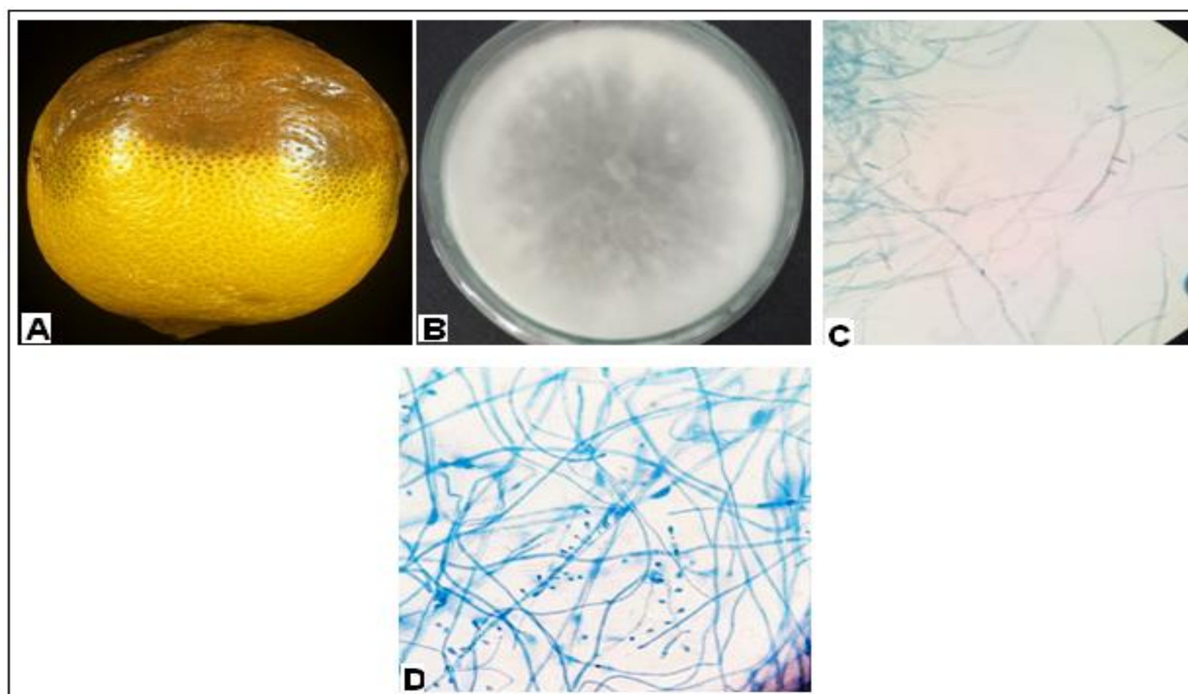

Fig. 1. Macroscopic and microscopic observations of *L. pseudotheobromae* . The stem-end rot infected citrus fruit A; *L. pseudotheobromae* colony growth on PDA media after seven days of incubation B; Morphology of conidia of *L. pseudotheobromae* C; irregular hyphae of *L. pseudotheobromae* D; enlarged mycelium of *L. pseudotheobromae* E; Magnification under light microscope 40x.

To check the effects of pH, the *L. pseudotheobromae* fungus was grown at three different pH ranges. The colony radial growth was obtained in the pH 5, 7, and 9 as 76mm, 65mm, and 70mm with 44±0.5gm, 36±0.5gm, and 28±0.5gm dry weight respectively (Figure. 2B). In carbohydrate growth profiling test, isolated fungi influenced by different carbohydrates.

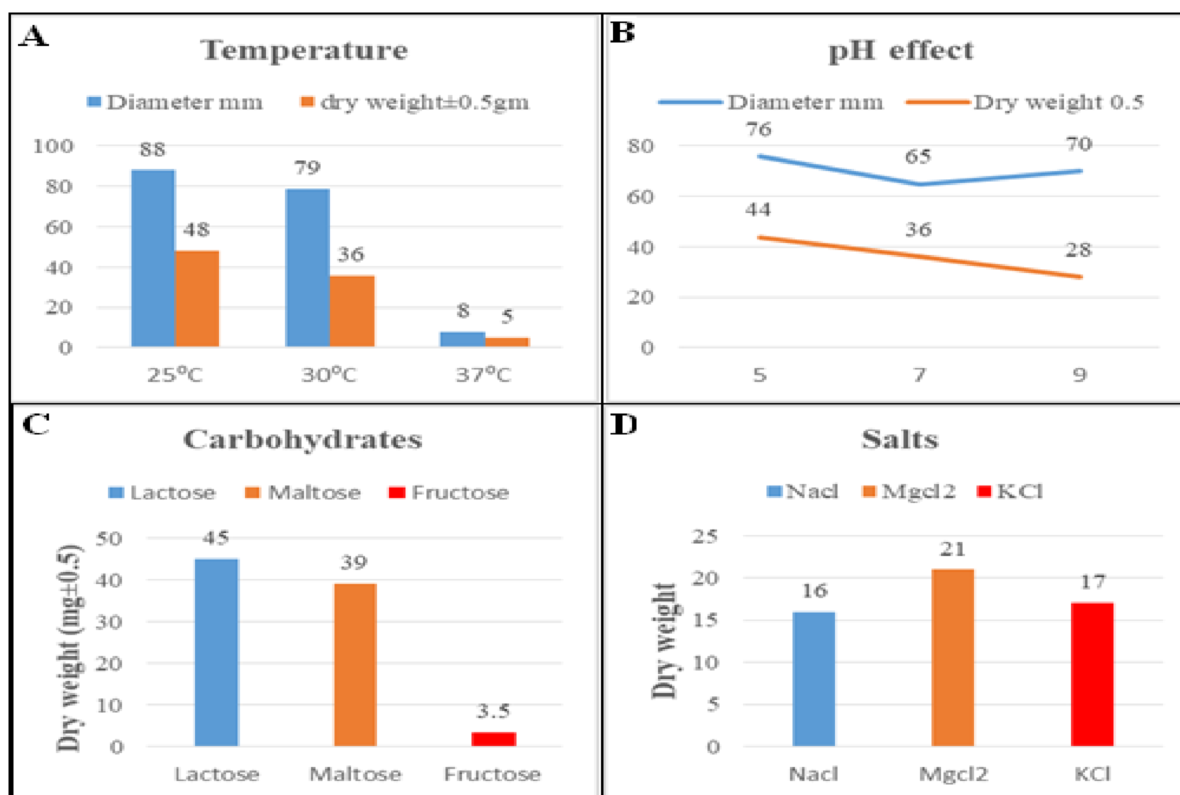

Fig. 2. Growth profiling test of isolated fungi. Different temperature A; pH B; carbohydrates C; salts D.

Three different carbohydrates have been used for this experiments. The dry weight of mycelium obtained on lactose, maltose, and fructose was 45±0.5gm, 39±0.5gm, and 32±0.5 respectively (Figure. 2C). Finally, three different salts have been used for evaluating the growth of isolated fungus strain. The dry weight of mycelium measured on NaCl, $Mgcl_2$, and KCl was 16±0.5gm, 21±0.5gm, and 17±0.5gm respectively (Figure. 2D).

Molecular characterization of the isolated pathogen

The sequence of internal transcribed regions (ITS) of *L. pseudotheobromae* was successfully amplified by using the primers of nuclear sequence NSI and NSIV respectively. The amplified target band were observed with the appropriate size ranging from 1300 bp. The 527 bp sequenced nucleotide was performed for a BlastN search in the NCBI database (https://www.ncbi.nlm.nih.gov/) to find out the best homologs of the sequence and the phylogenetic relationship of the isolated fungi. The sequence comparison and phylogenetic relationship revealed that *L. pseudotheobromae* strains have approximately 99% identity with several other *L. pseudotheobromae* spp. The obtained sequence was deposited in the Gen Bank and got the strain accession no. MH244216 (Figure. 3A, 3B).

Effect of plant extracts on radial growth of L. pseudotheobromae

Six medicinal plant extracts have been used for the antifungal activity test. Among these plant extracts *Azadirachta indica, Psidium guajava* extracts showed significant radial growth with 93.78% and 90.90% inhibition. While *Ficus carica, Moringa olifera, Ocimum sanctum* showed moderate radial growth as 31.18%, 35.81%, and 25% inhibition against *L. pseudotheobromae. Allium sativum* showed the

lowest inhibition (22.22%) results against the *L. pseudotheobromae* (Figure. 4).

Discussion

The stem-end rot infected citrus fruit was collected from Rajshahi University campus and similar symptoms were described by Zhang and Swingle *et al.* (2005), *Zhang et al.,* (2007), Sathya *et al.* (2017). *L. pseudotheobromae* isolates were morphologically characterized on PDA medium. The maximum growth was observed at 25˚C temperature and pH 5.

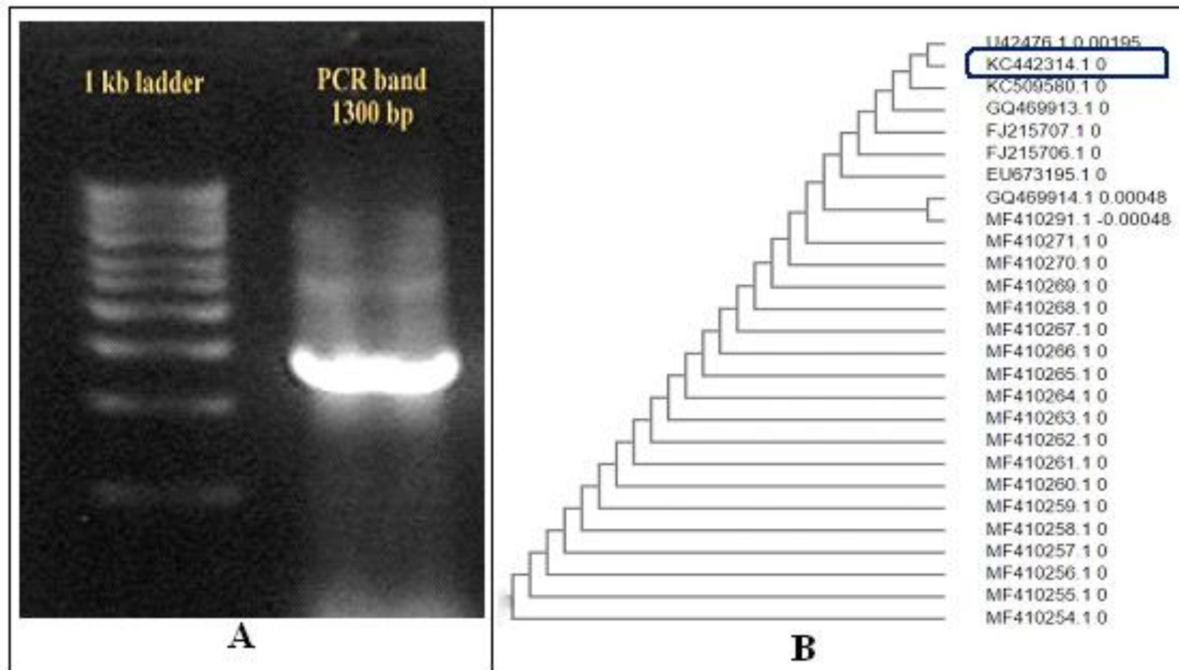

Fig. 3. PCR amplification and phylogeny.1300bp DNA fragment was amplified with NSI and NSIV primer A; the Evolutionary relationship was obtained Neighbor-Joining method B. The analysis involved 25 nucleotide sequences and it was conducted in CLUSTAL W and MEGA 6 software.

These findings were similar to Jacobs & Rehner (1998) where they indicating the observed temperatures ranging from 25 - 30°C are the most favorable for the majority of *Lasiodiplodia* sp. Similar results consisting of limited morphological differences in isolates of *L. pseudotheobromae* have already been reported by Al-Adawi *et al.* (2003) and also supported by Punithalingam (1980) who reported that size of conidia.

The growth profiling of isolated fungal strain in different carbohydrates showed, lactose carbohydrate in 20 gm/L concentration are suitable for growth of isolated fungal strain in PDA media, which is confirmed by Brouwer *et al.* (2014). Additionally, growth profiling of isolated fungal strain in different salts in 20 gm/L showed, $MgCl_2$ salts are optimum for the isolated fungal strain growth. This result showed similarity with the result Brouwer *et al.* (2014).

In this study, the isolated fungal was negative to cellulolytic activity because cellulose strip didn't degrade. Molecular identification of isolated fungal strain with NSI forward and NSIV reverse was obtained and their sizes (base pairs) were characterized of the species. These Two distinct primers showed a distinct band during agarose gel electrophoresis in genomic and PCR band. BLAST analysis of obtained DNA sequences of isolated strain showed 99% similarity with the corresponding region of the *L. pseudotheobromae* strain. The obtained sequence accession no. provided by the Gen Bank was MH244216. Sequence analysis of isolated fungus showed that the identity of the isolates to be same as that inferred from NS patterns. Meskza *et al.* (2016) did a similar finding in *L. pseudotheobromae.*

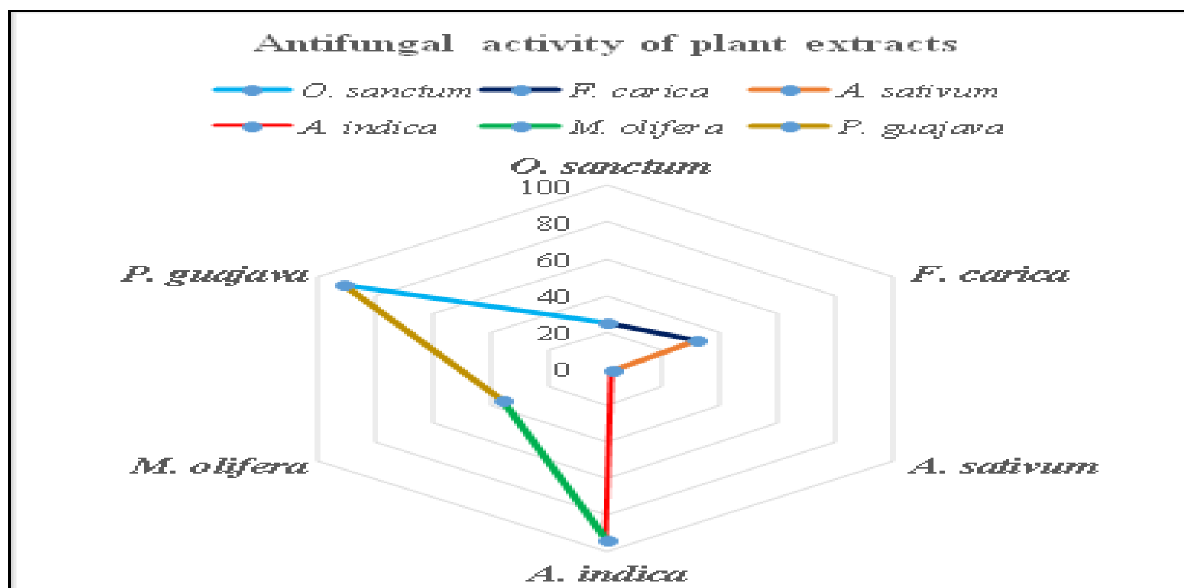

Fig. 4. The radial chart indicates the reduction of radial growth by using the different medicinal plant extract against *L. pseudotheobromae* isolates.

The present study tested the antifungal activity of six different plant extracts against the isolated fungal strain. Except for *Allium sativum* rest of all showed antifungal efficiency at varying degree in concentrations. Significant results were obtained in the previous study conducted by Sharma *et al.* (2013) in which, *A. indica* leaf extract showed greater fungicidal activity against *L. pseudotheobromae*. *P. guajava* showed 40-50% reduction in fungal radial growth; these findings were in excellent agreement with work of Bashir and Tahira (2012) and explained that the leaf extract of *P. guajava* was the most efficient antifungal agent against *Fusarium sp.*

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Disclosure statement

No potential conflict of interest regarding the publication.

Conclusion

In the present study, we have identified and characterized the fungal pathogen *Lasiodiplodia pseudotheobromae* associated with stem-end rot disease of citrus fruit and evaluate *in vitro* control of this pathogen against different plant extracts.

The correct identification of a plant pathogen is utmost important for quarantine and control of specific plant disease. Our findings provide some inside into the proper identification and *in vitro* bio-control assays of *L. pseudotheobromae* isolate in response to selective plant extracts. Future study on the distribution and epidemiology are important and could aid in finding effective management strategies of this pathogen that represent a threat to the citrus fruit in Bangladesh.

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