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AN OUTBREAK OF *NEOPESTALOTIOPSIS* SP. CAUSING RED LEAF SPOT OF SAPOTA IN BANGLADESH

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Abstract

Sapota (*Manilkara zapota* is an economically important fruit crop in Bangladesh that was affected by the different types of red colored leaf spot symptoms from 2019 to 2021. The study aimed to characterize red colored leaf spot symptoms of sapota by morphological and molecular analysis. Infected sapota were collected from three southern coastal districts of Bangladesh. Causal organism of the disease was isolated for morphological and molecular characterization. After 24 months of the plantation, about 70% disease prevalence in the experimental areas was recorded. The present investigation suggested that symptoms were caused by *Neopestalotiopsis* sp. Phylogenetic analysis using the internal transcribed spacer (ITS) region of ribosomal DNA additionally confirmed *Neopestalotiopsis* sp. in red leaf spot symptoms of sapota. For further validation, a pathogenicity test was carried out using three isolates on six months old saplings under Net-house conditions, and same symptom was developed in inoculated leaves after 14 days inoculation. The present investigation confirmed the outbreak of sapota red leaf spot disease, caused by *Neopestalotiopsis* sp. in Bangladesh.

Introduction

Manilkara zapota L. commonly known as the sapota, Sofeda in Bangladesh, is a long–lived evergreen tree belonging to the Sapotaceae family. It is originated in tropi-cal America and now widely cultivated in the tropics, including India, Pakistan, Bang-ladesh, Mexico, Vietnam, Guatemala, and Venezuela (Roy *et al.*,1997; Rahim *et al.*, 2011). Sapota grows throughout Bangladesh, however, extensively grown in coastal areas like Barisal, Khulna, Jashore, Chittagong, and Chittagong Hill Tract districts (Rahim *et al.*, 2009; Hossain *et al.*, 2015) Sapota has high calories, 83 calories per 100 grams, with a good source of dietary fiber; and its pulp functions as an ex-cellent laxative (Singh *et al.*, 2021). It is loaded with a rich array of vitamins A, C, niacin, folate, pan-tothenic acid, minerals iron, potassium, and copper (Singh *et al.*, 2021). Since this crop appear in Bangladesh, a few phytopathological studies have been conducted. Several fungal taxa affecting sapota with different symptoms have been reported (Bagheri *et al.*, 2017). Based on morpho-logical data and phylogenetic analysis of internal transcribed spacer

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(ITS), *Pestalotiopsis* Steyaert was separated into three genera namely *Neopestalotiopsis*, Pestalotiopsis, and *Pseudopestalotiopsis* (Senanayake *et al.*, 2020; Maharachchikumbura *et al.*, 2014) The genus *Neopestalotiopsis* Maharachch was recently segregated from Pestalotiopsis Steyaert. The morphology of the *Neopestalotiopsis*-like taxa varies on the isolating environment and the host. Therefore, the separation of species by phenotypic characteristics is difficult (Maharachchikumbura, 2016). Genomic analysis of the internal transcribed spacer (ITS) region is the best way to validate the *Neopestalotiopsis* sp. (Martin and Rygiewicz, 2005).

In 2019–2021, red leaf spot symptoms was noticed in commercial sapota orchards in the southern regions of Bangladesh but there were not any disease incidences. The outbreak of *Neopestalotiopsis* sp, was suspected therefore, in the present study the red leaf spot in sapota was undertaken to (i) determine the disease prevalence and incidence, and (ii) identify its fungal taxa in Bangladesh. We characterized sapota red leaf spot symptoms morphologically and molecular phylogenetic analyses.

Materials and Methods

Sample collection

Ten sapota saplings were planted at ten different farmer's plots in three southern districts of Bangladesh (Jashore, Khulna, and Satkhira). We coded ten farmer's plot as F1, F2, F3, F4, F5, F6, F7, F8, F9, and F10. We noticed different types of red spots in sa-pota leaves in the study area. All types of red spot diseased sapota leaf samples were collected.

Disease prevalence, incidence, and morphological characterization

The disease prevalence (DP) (Spronk *et al.*, 2019) and disease incidence (DI) (Chiang *et al.*, 2017) were calculated by the following formula:

$$\text{DP (\%)} = \frac{\text{Number of field with disease infection in the surveyed area}}{\text{Total number of field surveyed}} \times 100$$

$$\text{DI (\%)} = \frac{\text{Infected area of the sample plant}}{\text{Total area of sample plants}} \times 100$$

Collected diseased leaves were transported to the Microbiology and Bio-control laboratory, Bangladesh Agricultural University, Mymensingh, Bangladesh for cultur-ing the associated pathogen. Diseased leaf lesions were cut into small pieces (4–5 mm), sterilized with 10% sodium hypochlorite (NaOCl) for 2 minutes followed by 70% eth-anol for 30 seconds, and washed three times with sterile distilled water. Sterilized leaves were cultured on potato dextrose agar (PDA) plates at 28 °C for isolation of the pathogen. Three fungal isolates were re-cultured by transferring hyphal-tip and purified from the selected three types of symptoms. Characteristics of nine isolates of fungi on PDA plates were recorded at 24 hours intervals up to 10 days at room temperature. Isolated fungal pathogens were identified through morphological characteristics. The compactness, texture, color, and size of the conidia were considered to characterize pathogens on PDA culture plates. The experiment was set using a complete random-ized design with three replications. The radial mycelial growth of each isolate at five different temperatures (15, 20, 25, 30, and 35°C) for five to ten days was recorded. Mean radial mycelial growth was calculated using the following formula (Sultana *et al.*, 2022).

Mean radial mycelial growth = (Length + Width)/2

Data were statistically analyzed by one-way ANOVA using Duncan's multiple range test ($p<0.01$) by Statistix10.

DNA extraction, amplification, and sequencing

Fungal mycelia were grown on PDA media at 28°C for genomic DNA extraction. Total genomic DNA of 4 days of fungal mycelia was extracted following the liquid Nitrogen method (Serna-Domínguezusing *et al.*, 2018). Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). The genomic DNA was treated with RNase A to get RNA-free genomic DNA. The DNA extraction was confirmed in 1% agarose gel.

Fragments of the rDNA internal transcribed spacer (ITS) region were amplified for each isolates using the primer pairs ITS4 and ITS5 (Martin *et al.*, 2005). The 25 µl PCR reaction volume was prepared using 1 µl of forward primer, 1 µl of reverse primer, 12.5 µl of GoTaq Green Master Mix (Promega, Wisconsin, USA), 9.5 µl of nuclease-free water, and 1 µl of ge-nomic DNA. PCR reaction volume was subjected to a thermal cycler at 95°C for 5 min followed by 35 cycles of 95°C for 30 sec, 54 °C for 30 sec, and 72°C for 45 sec to am-plify the genomic DNA (Maharachchikumbura *et al.*, 2012). The PCR amplifications were electrophoresed at 1% aga-rose gel and visualized by staining. After the successful amplification, sequencing was performed by Macrogen Inc. (Seoul, Korea). Sequences were deposited in The National Center for Biotechnology Information (NCBI) under accession numbers OL454511 to OL454513 for the ITS sequences.

Phylogenetic analysis

The sequences of our study were compared with sequences retrieved from Gen-Bank. ITS gene sequence data were assembled using the alignment program BioEdit 7.2.5 (Hall *et al.*, 1999) and aligned by the online alignment program MAFFT version 7 (https://maf.cbrc.jp/alignment/server/41). The maximum likelihood (ML) tree was an-alyzed for each alignment. ML tree and bootstrapping analyses were conducted using MEGA11 (Tamura *et al.*, 2021). The ML analysis was performed using the Maximum composite likeli-hood model with 1000 bootstrap iterations (Darapanit *et al.*, 2021). Support values (ML bootstrap) were calculated for all analyses.

Pathogenicity test

Pathogenicity of three fungal isolates (identified by molecular analysis) was tested in their original hosts, six months old saplings of sapota, at Net-house. The cross-inoculation experiment was conducted on the host plant. Three fresh leaves from each sapling were dusted with carborundum powder and inoculated with conidial suspension (105 conidia per ml sterilized distilled water). All the inoculated and con-trol plants were covered with polybags for two days. After the appearance of symptoms, selected leaves were cut and taken to the laboratory, cleaned with tap water, and sterilized by dipping them into 70% ethanol for 3 mins. Isolations of fungi procedures were repeated as previously described. Each of the reisolated fungus was placed in a PDA medium plate and incubated at 25±2 °C. All inoculated leaves were visually assessed daily and species comparison was performed to confirm the causal pathogen.

Results and Discussion

Study of the red leaf spot symptom of the sapota plants in the experimental areas

Different types of sapota leaf spots were observed in the field. The spots were round to irregular in shape and red in color (Fig. 1). Size increased from 2 to 4 mm in diameter to whole leaves within time passes. Spots appeared both at the middle and margin of the leaves. The disease was observed first in the immature leaves. Sometimes narrow brown to black margins appeared at the older spots.

Red leaf spot disease prevalence and disease incidence of sapota in the experimental areas

The disease first appeared after 4 months on the plantation at Jashore, Khulna, and Satkhira. Disease prevalences were 10, 40, 50, 50 and 70% at Jashore at 4, 8, 12, 16, 20, and 24 months after planting (MAP), respectively (Fig. 2). At Khulna, 20% 40, 50, 60 and 70% disease prevalence were found respectively at 4, 8, 12, 16, 20, and 24 MAP (Fig. 2). At Satkhira, 30, 40, 50, 60 and 70% were rec-orded at 4, 8, 12, 16, 20, and 24 MAP (Fig. 2). Our data suggested that sapota red leaf spots showed about 70% disease prevalence at the 2 years of planting.

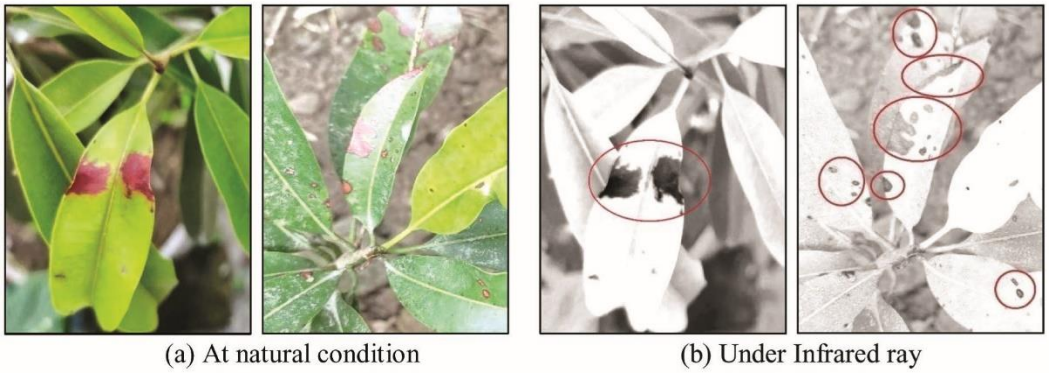

Fig. 1. Different red spot symptoms in Sapota leaves (a) and observed symptoms under infrared ray (b).

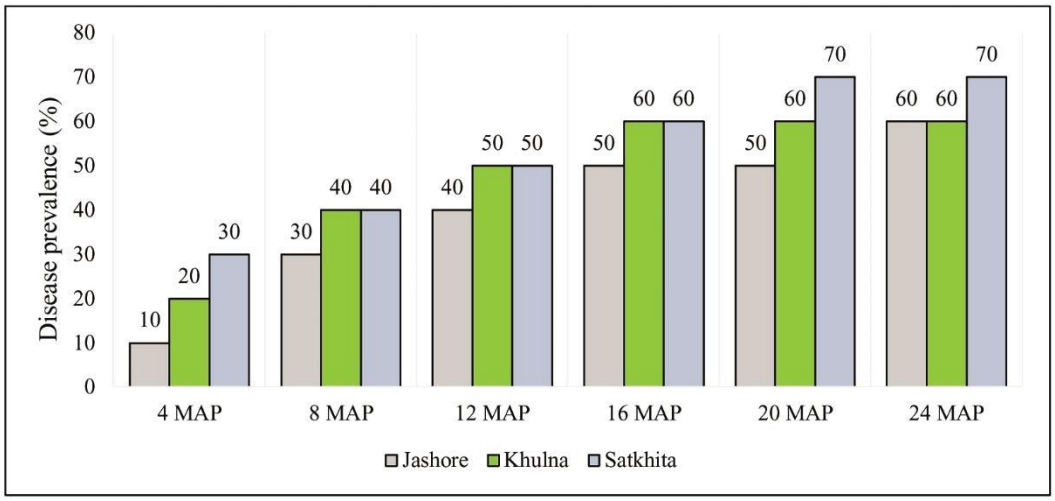

Fig. 2. Disease prevalence (%) of the red leaf spot disease of sapota at different locations of Bangladesh.

During the plantation of sapota, there was 0% disease incidence in all the experi-mental areas. F2, F3, F6, and F8 from Jashore showed 0% disease incidence from planta-tion time to 24 MAP. F1 and F9 farmer's plots of Jashore showed the highest 30% disease incidence after 24 MAP. Most of the farmers from Jashore showed 5 to 20% disease in-cidence. F3, F5, and F9 from Khulna showed 0% disease incidence from plantation time to 24 MAP. F1, F4, F6, F8, and F10 farmers' plots of Khulna showed the highest 30% disease incidence after 24 MAP. Most of the

farmers from Khulna showed 10 to 30% disease incidence. After 24 MAP, F3 and F9 from Satkhira showed 0% disease incidence from plantation time to 24 MAP. F1, F2, F4, F8, and F10 farmers' plots of Satkhira showed the highest 30% disease incidence (%). Most of the farmers from Satkhira showed 5 to 30% disease incidence (Fig. 3).

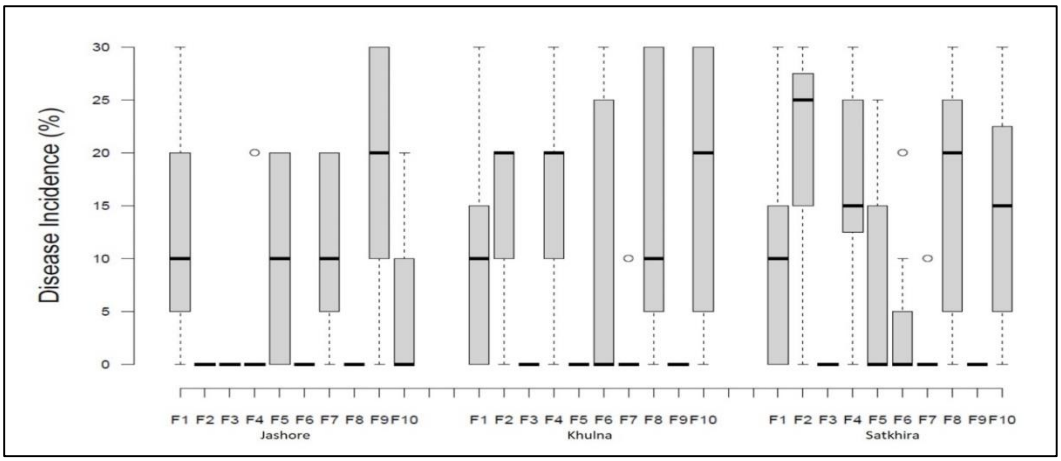

Fig. 3. Disease incidence (%) of the red leaf spot disease of sapota at different locations in Bangladesh. Here, F1; farmer plot 1, F2; farmer plot 2, F3; farmer plot 3, F4; farmer plot 4, F5; farmer plot 5, farmer plot, F6; farmer plot 6, F7; farmer plot 7, F8; farmer plot 8, F9; farmer plot 9, and F10; farmer plot 10.

Isolation of fungal pathogens and morphological characterization of the isolates

In total, nine fungal isolates were collected from different sapota leaves with red spot disease symptoms from Jashore, Khulna, and Satkhira. All isolates were morphologically studied. Different isolates were similar in their cultural and morphological properties viz. mycelial growth, colony compactness, colony shape, colony texture, and colony color (Fig. 4, Table 1). All the isolates were compact, colony shapes were round, cottony textured, and white in color (Fig. 4, Table 1).

Fig. 4. Selected fungal isolates of red leaf spot of sapota.

Radial mycelial growth of each isolate at different temperatures

Different temperatures viz. 15, 20, 25, 30, and 35°C were imposed to study the effect of temperature on radial mycelia growth of isolates of leaf spot of Burmese grape for 5 days. Radial mycelia growth of the disease was increased with the increase of time and temperatures change (Table 2, Table 3, Table 4). The highest radial mycelial growth (13.67 mm) was observed at I4 and I7 after 1 day at 30°C followed by I1 and I6 (13.33 mm) at 30°C (Table 2). After 3 days the highest growth (32.67 mm) was rec-orded at I9 and I6 at 25 °C followed by I1 and I7 (32.33 mm) at the same temperature (Table 3). The highest growth (58.00 mm) after 5 days was observed at I1 at 25°C fol-lowed by I4 (56.83 mm) at the same temperature (Table 4).

Table 1. Morphological characteristics of different fungal isolates of red leaf spot of Sapota.

Sl. No.	Isolates	Compactness	Colony shape	Texture	Color
I1	BD_MBC_S_1	Compact	Round	Cottony	White
I2	BD_MBC_S_2	Compact	Round	Cottony	White
I3	BD_MBC_S_3	Compact	Round	Cottony	White
I4	BD_MBC_S_4	Compact	Round	Cottony	White
I5	BD_MBC_S_5	Compact	Round	Cottony	White
I6	BD_MBC_S_6	Compact	Round	Cottony	White
I7	BD_MBC_S_7	Compact	Round	Cottony	White
I8	BD_MBC_S_8	Compact	Round	Cottony	White
I9	BD_MBC_S_9	Compact	Round	Cottony	White

Table 2. Radial mycelial growth of each isolate at different temperature at one day after inoculation.

Sl. No.	Temperature									
	15°C		20°C		25°C		30°C		35°C	
I1	10.33	a	13.00	a	10.67	a	13.33	a	10.00	
I2	10.67	a	12.33	ab	10.83	a	12.67	ab	10.00	
I3	10.50	a	11.67	b	11.17	a	11.83	b	10.00	
I4	10.33	a	11.67	b	10.83	a	13.67	a	10.00	
I5	10.50	a	12.33	ab	10.83	a	12.67	ab	10.00	
I6	10.67	a	11.33	ab	11.17	a	13.33	a	10.00	
I7	10.50	a	13.00	a	10.67	a	13.67	a	10.00	
I8	10.67	a	12.33	ab	10.83	a	11.83	b	10.00	
I9	10.33	a	12.33	ab	10.67	a	12.67	ab	10.00	
CV%	2.24		3.02		2.65		2.29			

Isolates those sharing similar letters are statistically identical at 1% level of significance.

The average radial mycelial growth of the isolates was found highest (12.78 mm) at 30°C followed by 20°C (12.22 mm) after 1 day. After 3 days, the highest mycelial growth (29.19 mm) was observed at 25°C followed by at 30°C (24.96 mm). The aver-age radial mycelial growth of

the isolates was found highest (44 mm) at 25°C followed by 20°C (37.85 mm) after 5 days (Fig. 5). Considering all the temperatures, higher radial mycelial growth was recorded at 25°C followed by 20°C after 5 days. There was significant growth found at 15, 20, 25, and 30°C after 5 days. Mycelial growth was also found at 35°C.

Table 3. Radial mycelial growth of each isolate at different temperature at 3 days after inoculation.

Sl. No.	Temperature								
	15°C		20°C		25°C		30°C		35°C
I1	21.33	ab	28.33	a	32.33	a	24.17	a	10.00
I2	19.17	b	18.50	c	23.00	b	25.33	a	10.00
I3	23.50	a	20.67	b	31.67	a	25.33	a	10.00
I4	21.33	ab	20.33	b	31.00	a	25.00	a	10.00
I5	19.17	b	18.50	c	23.00	b	24.67	a	10.00
I6	21.33	ab	20.00	b	32.67	a	25.33	a	10.00
I7	21.33	ab	21.33	b	32.33	a	24.33	a	10.00
I8	19.17	b	19.00	c	24.00	b	24.83	a	10.00
I9	21.67	ab	19.33	c	32.67	a	25.67	a	10.00
CV%	3.49		2.77		1.29		2.00		

Isolates those sharing similar letters are statistically identical at 1% level of significance.

Table 4. Radial mycelial growth of each isolate at different temperature at 5 days after inoculation.

Isolates	Temperature								
	15°C		20°C		25°C		30°C		35°C
I1	33.33	a	45.33	a	58.00	a	38.67	a	10.00
I2	28.33	b	33.50	b	24.00	c	37.67	a	10.00
I3	28.67	b	35.33	b	50.33	b	36.90	a	10.00
I4	29.00	a	42.00	a	56.83	a	37.00	a	10.00
I5	28.33	b	33.50	b	49.33	b	37.67	a	10.00
I6	28.67	b	36.33	b	26.83	c	35.83	a	10.00
I7	33.33	a	34.33	b	52.33	ab	38.67	a	10.00
I8	27.67	b	44.67	a	28.00	c	35.33	a	10.00
I9	28.67	b	35.67	b	50.33	b	36.90	a	10.00
CV%	1.92		1.81		0.76		1.81		

Isolates those sharing similar letters are statistically identical at 1% level of significance.

Identification of the isolates by the spores of different isolates

The conidia were septate (usually 3–4 septa), brown colored, smooth-walled, straight to slightly curved, and sub-cylindrical. The range of average conidial size was 24.00 to 30.60 µm where the average size range of the apical cell was 6.54–7.95 × 4.54–6.32 µm, the median cell was 6.7-9 × 6.7–7.03 µm and the basal cell was 6.73–7.55 × 5.02–7.02 µm. Apical cells had two to

three appendages of 10.48–47.56 µm and the basal cells bear a single appendage of 3.82–4.58 µm in length (Fig. 6).

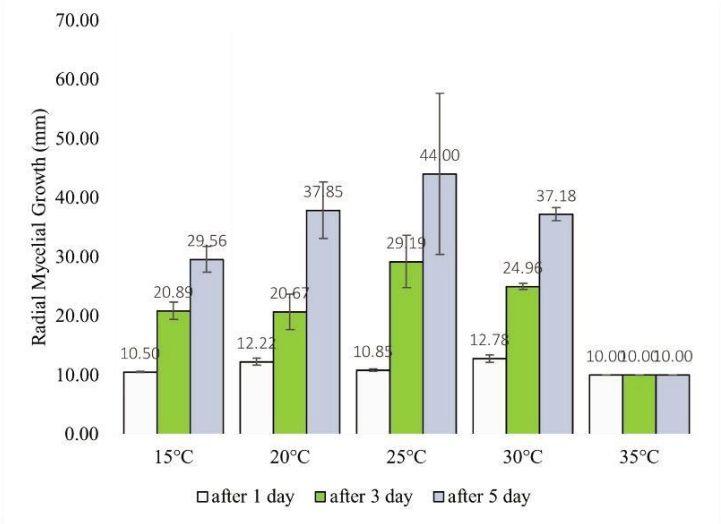

Fig. 5. Average radial mycelial growth of each isolate at different temperature.

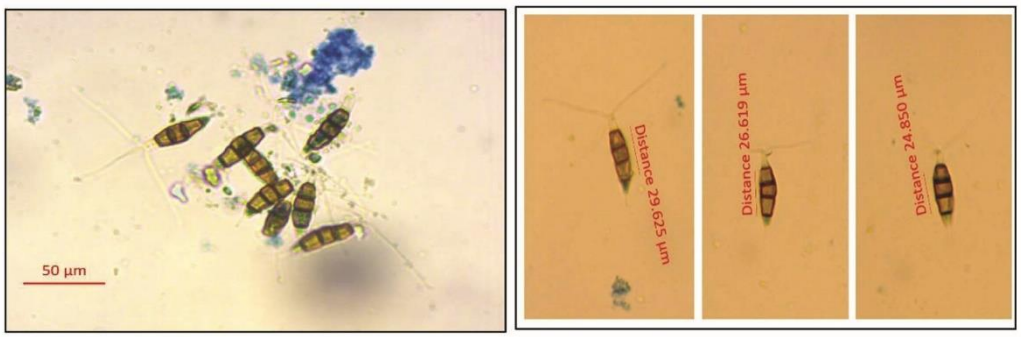

Fig. 6. Pictorial view of the spores of the isolates

Identification of the isolates through molecular studies

Three (I1, I4, and I7 named BD_MBC_S_1, BD_MBC_S_4, and BD_MBC_S_7, re-spectively) isolates were selected for molecular characterization based on their myce-lial growth morphological and physiological characters (Fig. 4). Selected three iso-lates were characterized at the molecular level based on PCR amplification of the in-ternal transcribed spacer (ITS4 and ITS5).

ITS sequences separated the different isolates into different clusters (Fig. 7). ITS regional analysis suggested that all three isolates (BD_MBC_S_1, BD_MBC_S_4, and BD_MBC_S_7) belonged to *Neopestalotiopsis* sp. that were supported by a bootstrap value ranging from 98% to 100% (Fig. 7). ITS sequences of. BD_MBC_S_1 (OL454511) and BD_MBC_S_4 (OL454512) showed >99% similarity with the sequence from *Neopestalotiopsis* sp. strain LC427171,

and >98% similarity with *Neopestalotiopsis* sp. strain LC427189 in GenBank. Sequence identity for ITS of BD_MBC_S_7 (OL454513) was 100% similar to *Neopestalotiopsis* sp. strain MW775515.

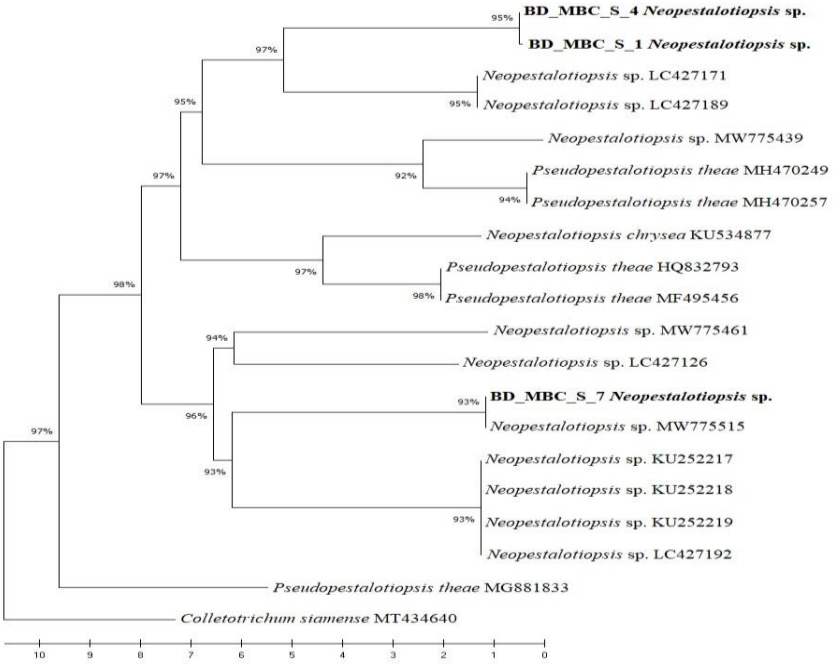

Fig. 7. Phylogenetic relationships among the selected isolates by analysis of ITS sequences.

Pathogenicity test of the selected isolates on the growing plants

Three identified isolates (from molecular analysis) were selected for pathogenicity test in six months old saplings of sapota in Net-house. Three fresh leaves from each sapling were dusted with carborundum powder and inoculated with conidial suspen-sion (105 conidia per ml sterilized distilled water, DIW). All the inoculated and control plants were covered with a polybag for two days. Within 14 days typical symptoms were developed in all inoculated leaves (Fig. 8) and control plants were symptom-less. *Neopestalotiopsis* sp. was successfully re-isolated from all the inoculated plants.

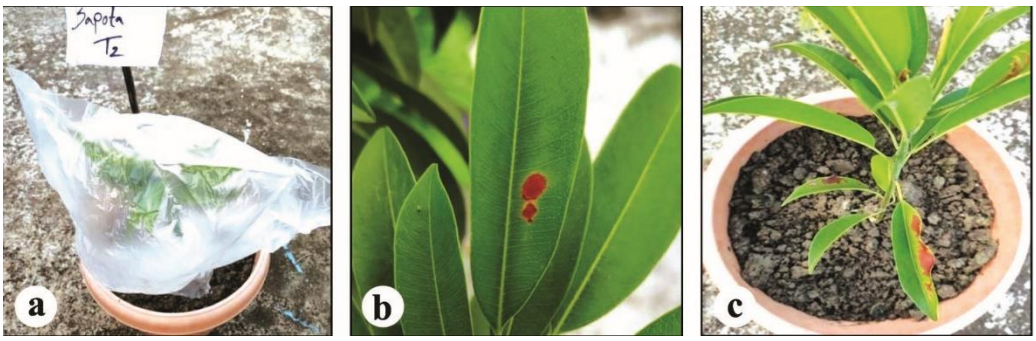

Fig. 8. Pathogenicity test of *Neopestalotiopsis* sp. on sapota at net house (a) Sapota plant covered with polythene bag, (b) visible red leaf spot symptom after 14 days and (c) red leaf spot symptom after 1 month.

A typical round to irregular shaped, 2 to 4 mm in diameter, red colored leaf spot symptom was noticed in 1 to 2 years of newly established sapota garden in Jashore, Khulna, and Satkhira coastal area. Primarily occurrence of *Neopestalotiopsis* sp. caus-ing grapevine leaf spots was characterized by a roughly circular to irregular with 2–4 mm in diameter that was typically described by narrow brown to black margin leaf le-sions on the leaf surface (Jayawardena *et al.*, 2016). Lesions of sapota red leaf spots in our study were initially reddish-brown which is similar to grapevine leaf spots identified by Jayawardena *et al.* (2016). Disease symptoms appeared at every experimental district after 4 months of plantation. The maximum 70% sapota red leaf spot disease prevalence was recorded at Satkhira at 24 MAP while 60% was at Jashore and Khulna; and at the same time, 30%, 5-20%, and 5-30% disease incidence appeared at Khulna, Jashore, and Satkhira, respectively. It suggests that all three coastal districts are vulnerable to red leaf spot disease in sapota.

It was found that white colored cottony mycelia in this study. Median cells of *Neopestalotiopsis clavispora* were brown or darker, and the apical and basal cells were hya-line and white, one of which had 2–4 appendages (Shi *et al.*, 2022). On the 5th day, it was recorded that the radial mycelial growth of all isolates of sapota red leaf spots at 15, 20, 25, 30, and 35°C temperature where mycelial growth was highest at 25 °C. Our findings fit with the ranges of optimum temperature, 22–27°C, for the mycelial growth of *Neopestalotiopsis* sp associated sapota red leaf spot disease (Gerardo *et al.*, 2020). On the other hand, our results are not under– or over–estimated with the radial mycelial growth conditions (temperature) of *Neopestalotiopsis* sp. causing macadamia nut flower disease in Australia (Prasannath *et al.*, 2021). The radi-al mycelia growth of all isolates increased with the increase of time while it started to decrease their growth after crossing temperature of 25°C suggesting the lower rate of infestation in high temperatures. However, mycelial development at 35°C means the possibility of infection even at 35°C. Bangladesh has some popular sapota cultivars namely BARI Safeda-1, BARI Safeda-2, BARI Safeda-3, FTIP-BAU Sopheda-1, FTIP-BAU Sopheda-2, and FTIP-BAU Sopheda-3; and their planting time is June–September. After four months, sapota plants started to show the red colored leaf spot symptoms at 4 MAP in our study while the average temperature ranges from 15–25°C (October–January). It suggests that saplings of all popular sapota cultivars in Bangla-desh are vulnerable to sapota red leaf spot disease. The mycelia survived with a high temperature of 35°C and low temperature of 15°C suggesting the possibility of the severity in the next season in the Bangladeshi environment.

The conidia were hyaline, light brown, smooth walled, septate, and straight to slightly curved and subcylindrical. The conidial characteristics of *Neopestalotiopsis* sp. isolated from Rhapisexcelsa, Rhododendron simsii, Rhododendron championiae, and Erythropalum scandens were also hyaline, rarely light brown, smooth-walled, and obclavate (Yang *et al.*, 2021). The range of average conidial size was 24.00–30.60 µm whereas the av-erage size range of the median cell was 6.7-9 × 6.7–7.03 µm. The minimum and maxi-mum values for the length of apical appendages of *Neopestalotiopsis* and Pestalotiopsis spp. were 11.54 and 34.8 µm, respectively; and the minimum and maximum values for the length of basal append-ages were 3.00 and 6.75 µm, respectively (Solarte, *et al.*, 2018). In our study, apical cells had two to three appendages of 10.48–47.56 µm and the basal cells bear a single appendage of 3.82–4.58 µm in length. Conidial size from our study also reconfirms the Neopestalo-tiopsis sp.

The Internal transcribed spacer (ITS) region genes of each isolate were amplified using the primers ITS4/ITS5 ITS sequences of all three isolates, viz. BD_MBC_S_1 (OL454511), BD_MBC_S_4 (OL454512), and BD_MBC_S_7 (OL454513) showed >98% to 100% similarity with the sequence from *Neopestalotiopsis* sp. strain LC427171 and MW775515 in GenBank. The

result support Ismail *et al.,* (2017) and Wu *et al.*, (2021) who studied ITS4 and ITS5 regions, and found *Neopestalotiopsis* sp. similar to other strains of GenBank from the phylogenetic study.

Our study confirmed that the fungal properties of the sapota red leaf spot disease occurred in the presence of *Neopestalotiopsis* sp. *Neopestalotiopsis* sp was not noticed previously in Bangladesh suggesting a new outbreak of this disease. After the confir-mation of the genus, we are now investigating the identification of the species level of the pathogen and antifungal properties that can be analyzed for developing fungicides to control the disease.

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