Article

Mangrove Dieback and Leaf Disease in *Sonneratia apetala* and *Sonneratia caseolaris* in Vietnam

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Abstract: Even though survival rates for mangrove restoration in Vietnam have often been low, there is no information on fungal pathogens associated with mangrove decline in Vietnam. Therefore, this research was undertaken to assess the overall health of mangrove afforestation in Thanh Hoa Province and fungal pathogens associated with tree decline. From a survey of 4800 *Sonneratia* trees, the incidence of disorders was in the order of pink leaf spot > shoot dieback > black leaf spot for *S. caseolaris* and black leaf spot > shoot dieback > pink leaf spot for *S. apetala*. Approximately 12% of *S. caseolaris* trees had both pink leaf spot and shoot dieback, while only 2% of *S. apetala* trees had black leaf spot and shoot dieback. Stem and leaf samples were taken from symptomatic trees and fungi were cultured in vitro. From ITS4 and ITS5 analysis, four main fungal genera causing leaf spots and shoot dieback on the two *Sonneratia* species were identified. The most frequently isolated fungal taxa were *Curvularia* aff. *tsudae* (from black leaf spot), *Neopestalotiopsis* sp.1 (from stem dieback), *Pestalotiopsis* sp.1 (from pink leaf spot), and *Pestalotiopsis* sp.4a (from black leaf spot). The pathogenicity of the four isolates was assessed by under-bark inoculation of *S. apetala* and *S. caseolaris* seedlings in a nursery in Thai Binh Province. All isolates caused stem lesions, and *Neopestalotiopsis* sp.1 was the most pathogenic. Thus, investigation of fungal pathogens and their impact on mangrove health should be extended to other afforestation projects in the region, and options for disease management need to be developed for mangrove nurseries.

Keywords: afforestation; *Curvularia*; leaf spot; *Neopestalotiopsis*; pathogenicity; *Pestalotiopsis*

1. Introduction

Mangroves provide crucial environmental services including habitat for birds, fish, and invertebrates. They are responsible for coastal protection from hurricanes, floods, sea-level rise, wave action, and erosion [1,2]. Mangrove systems are among the most threatened ecosystems globally [3,4] due to the fact of increasing urban and industrial development [5–7]. In addition, abiotic factors, such as hurricanes, lightning strikes, salinity, and flooding, are reported to be affecting the health of mangroves [2,8]. Extreme weather, such as drought, hot water, hot air, and the temporary drop in sea level, have contributed to mangrove dieback [9]. For example, approximately 7400 ha of mangroves in northern Australia died in the summer of 2015–2016 [10]. As well as primary pathogens and insects, abiotic stress can expose trees to a variety of opportunistic pests that can cause tree declines [11,12]. In addition, primary pathogens and insects can cause tree

degradation. Unlike in terrestrial ecosystems, there are fewer reports of pathogenic fungi in mangroves [13–15], and these have mostly been descriptive and focus on the morphology and taxonomy of mangrove fungi [16–20]. A range of fungal pathogens have been associated with disease in mangroves (Table 1). Overall, given the pantropical distribution of mangroves, the number of investigations into mangrove health remain limited [21].

Table 1. Fungi associated with diseases in mangroves.

Fungus	Disease	Host	Location	Reference
Acanthus ilicifolius	leaf spot	*Corynespora cassiicola*	China	[22]
Anthostomella rhizomorphae	leaf spot	*Rhizophora mangle*	Puerto Rico	[23]
Botrosphaeria ribis	death of twigs and branches	*Heritiera fomes*	Bangladesh	[24]
Celoporthe guangdongensis	canker	*Sonneratia apetala*	China	[25]
Cercospora	leaf spot	*Rhizophora mangle*	Florida	[26]
Cercospora	leaf disease	*Rhizophora mangle*	Florida	[27]
Colletotrichum sp.	leaf spot	*Rhizophora* sp.	Australia	[13]
Cyphellophora sp.	stem canker, branch dieback	*Avicennia marina*	South Africa	[28,29]
Cytospora rhizophorae	dieback	*Rhizophora mangle*	Puerto Rico	[30]
Cytospora rhizophorae	canker	*Sonneratia apetala*	China	[25]
Cytospora sp.	dieback	*Rhizophora mangle*	Hawaii	[31]
Eutypella sp.	stem/branch canker	*Avicennia marina*	South Africa	[28,29]
Fulvifomes	heart/butt infections	*Xylocarpus granatum*	Thailand	[32]
Fulvifomes sp.	decay	*Xylocarpus granatum*	Thailand	[33]
Lasiodiplodia theobromae	branch dieback, branch/stem canker	*Avicennia marina*	South Africa	[28,29]
Oomycete	mortality	*Avicennia marina*	Australia	[34]
Pestalotiopsis sp.	leaf spot	*Rhizophora mucronata*	China	[35]
Phyllosticta hibiscina	necrosis and death	*Avicennia germinans*	Florida	[36]
Polystigma sonneratiae	leaf spot	*Rhizophora* sp.	Australia	[13]
Pseudocercospora avicenniae	leaf spot	*Avicennia marina*	Australia	[37]
Pseudocercospora mapelanensis	disease on fruit, flowers and leaves	*Barringtonia racemosa*	South Africa	[28]
Pseudocercosporella	leaf spot	*Ceriops decandra*, *Sonneratia caseolaris*, *Rhizophora stylosa* and *Osbornia octodonta*	Philippines	[38]

It is estimated that the total area of mangrove forests in Vietnam declined from 408,500 ha in 1943 [39] to 235,569 ha in 2019 [40]. According to recent studies, although many afforestation projects have been undertaken in Vietnam, the survival of out-planted nursery stock has often been unsatisfactory [41,42]. The few studies on mangrove damage or death in the country did not consider pathogens but focused on environmental processes and human activities such as industrial development, over-harvesting, and land conversion to agriculture. These included dieback of 20 year old *Rhizophora apiculata* in Ben Tre Province [43], where the disease was likely influenced by increased anthropogenic disturbances [44]. Other reports included mortality of mangroves in Quang Ngai [45] and in Ha Tinh [46] provinces. Large gaps remain in the documentation of mangrove diseases for most parts of northern Vietnam. As the average rate of success of mangrove restoration in northern Vietnam is approximately 30% [47], understanding whether mangrove diseases are a contributing factor could help improve the management of mangroves in future projects. Therefore, this study was undertaken to document the extent and impact of disease problems on afforested mangroves in Thanh Hoa Province. The objectives were to (i) assess the health of mangrove restoration and (ii) to identify likely causes of mangrove decline.

2. Materials and Methods

2.1. Study Area and Canopy Assessment

Thanh Hoa Province in north–central Vietnam was chosen because afforestation trials had been established in 2015, and remote sensing imagery showed that many plantings

had been unsuccessful [47]. Thanh Hoa city (20.0° N, 105.5° E) has a tropical climate with intermittent rainfall concentrated in the summer (1447 mm/year), and the average annual temperature is 27.3 °C. Preliminary health surveys were randomly conducted in mangroves in two geographical areas (Table 2) to assess canopy conditions in 2018. This revealed shoot dieback and leaf spots as the main tree health problems. An afforestation plantation planted in April 2018 in Hoang Phu District was then chosen for a detailed study of these problems. The afforestation trial was established using *Sonneratia caseolaris* and *Sonneratia apetala* with single and mixed plantings at a spacing of 2 × 3 m. A rating system (Table 3) was used to quantify the condition of 4800 trees, 16 months after out-planting, in August 2019. Shoot dieback and foliar symptoms of pink and black spots are illustrated (Figure 1).

2.2. Sample Collection

During preliminary sampling in December 2018, 30 leaf and stem samples were collected at random from symptomatic trees in Hoang Phu and Sam Son districts (Table 1), and transported to the Plant Clinic, Forest Protection Research Center (Vietnamese Academy of Forest Sciences, Hanoi) where fungal cultures were obtained. In August 2019, more intensive sampling of two leaf spots (pink, black) and branch dieback was undertaken from the 2018 afforestation trials in the Hoang Phu District. Samples were collected from the two *Sonneratia* species from single and mixed plantings. A total of 105 samples were collected from each species comprising 70 leaf samples and 35 branch samples.

2.3. Fungal Isolation

Branches and leaves were surface sterilized with 70% ethanol for 1 min, rinsed with sterile water. Then 2 × 2 mm pieces containing leaf spots were excised, 1–2 cm lengths were cut from the junction of healthy and dead wood of branches, and the pieces were placed on sterile moist paper in Petri dishes. The Petri dishes were sealed with cling wrap and incubated at 25 °C in the dark. After 2–3 days, aerial mycelia of putative pathogens were transferred aseptically to potato dextrose agar (Gibco, ThermoFisher Scientific, Abingdon, UK) (PDA) supplemented with 0.75% streptomycin sulfate (strep) and incubated at 25 °C in the dark. When fungal cultures had grown several cm in diameter, hyphal tips were sub-cultured onto new PDA + strep Petri dishes. Isolates cultured on PDA at 25 °C were used to describe colony development and for DNA extraction. The morphology and size of spores were obtained using an OLYMPUS BX50 (Tokyo, Japan) compound microscope. A total of 227 fungal isolates were cultured from the 135 samples and grouped into 6 morphotypes. Of these, 9 isolates were selected for sequencing. All fungal cultures are lodged in the Vietnam National University of Agriculture.

Table 2. Details of the mangrove health study undertaken in north–central Vietnam.

Location	*Sonneratia* Species	Year Planted	Preliminary Study	Main Study
Sam Son town, Thanh Hoa Province	*S. apetala*	2015, 2016	December 2018	
	S. caseolaris	2015, 2016	December 2018	
Hoang Phu District, Thanh Hoa Province	*S. caseolaris*	2018	December 2018	August 2019
	S. apetala	2018	December 2018	August 2019

Table 3. Dieback and leaf spot rating of mangroves based on the severity of symptoms.

| Rating	Dieback Criteria	Leaf Spot Criteria
1	No dead branches visible	Dense, healthy foliage; no leaf spots
2	Few dead twigs present; may have an occasional large branch stub on an upper bole	Dense foliage; spots (pink/black) present at low density on leaves
3	Many dead twigs present; an occasional large dead branch; may have large branch stubs on the upper bole	Foliage density appears subnormal; numerous pink or black spots present
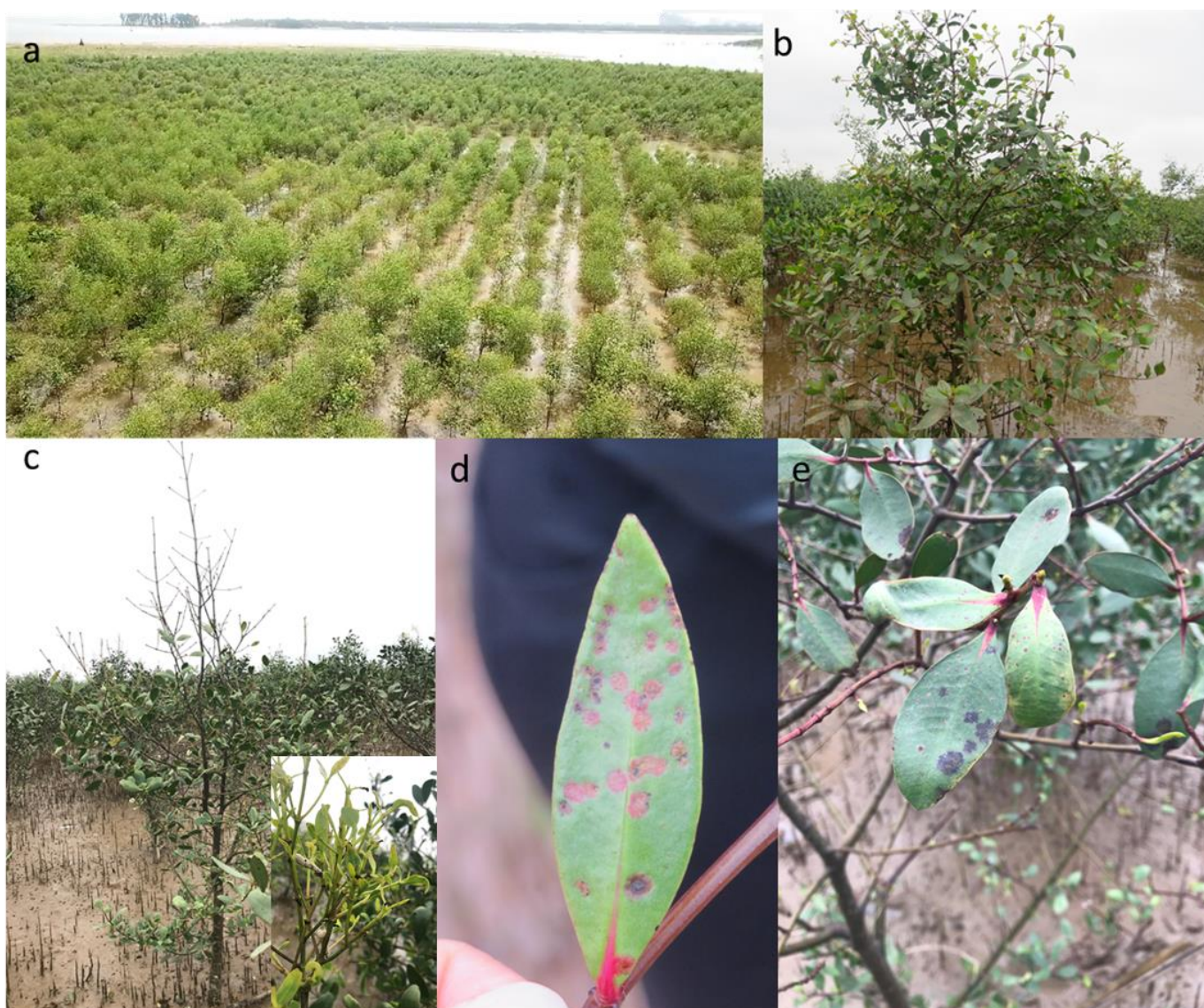
Figure 1. Mangrove afforestation project in Thanh Hoa Province, showing trees planted in a completely randomized design (**a**); healthy tree (**b**); unhealthy tree with shoot dieback (**c**) and a shoot with leaves turning yellow before dieback occurs (insert); symptoms of pink leaf spots (**d**); symptoms of black leaf spots (**e**).

2.4. Fungal Identification

- DNA extraction, PCR amplification, and sequencing analysis

Fungal cultures obtained from symptomatic plant materials were grouped based on colony morphology, color, fruiting bodies, and spore characteristic and used for DNA extraction. DNA was extracted using a CTAB (cetyltrimethyl ammonium bromide, Novo Nordisk Pharmatech, Køge, Denmark) method [48] with minor modification. For each isolate, approximately 100 mg of fresh mycelia mat was transferred into a 1.5 mL Eppendorf tube, washed with distilled water, and homogenized with 500 µL extraction buffer (2 M NaCl, 25 mM EDTA, 100 mM Tris-HCl, 2% PVP, and 2% CTAB) using a plastic pestle. The genomic DNA was extracted with chloroform–isoamyl alcohol (24:1) two times and precipitated with cold propanol. DNA pellets were washed two times with 70% ethanol, air-dried for 30 min, resuspended in 50 µL of TE, and stored at −20 °C.

The primers ITS4 (forward primer) and ITS5 (reverse primer) [49,50] were used to amplify the ITS gene region. PCRs were performed in a final reaction volume of 25 µL containing 2.5 µL DreamTaq PCR X 10 buffer (Fermantas, Waltham, MA, USA), 0.5 µL

dNTPS (10 mM each, Roche, Capetown, South Africa), 0.5 µL of each primer (20 µM), 0.5 µL DreamTaq polymerase (5U/µL, Fermentas), and 0.5 µL DNA. The reactions were performed with the following steps: preliminarily denaturation step at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 40 s, annealing at 52 °C for 45 s, extension at 72 °C for 1 min, and then a final extension step for 5 min at 72 °C. The PCR products were purified from agarose gel using the PureLink Quick Gel Extraction Kit (Invitrogen, Waltham, MA, USA), estimated for the DNA concentration using agarose gel electrophoresis. The mixture of purified PCR product and primers was sent to 1st BASE in Singapore (https://order.base-asia.com/, accessed on 10 July 2021) for sequencing and reading.

- Phylogenetic analyses

Consensus sequences of the ITS gene region were compared to data of previously published species obtained from GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 10 July 2021). The obtained ITS consensus sequences were used to perform BLAST searches in GenBank and to identify isolates at the genus and species level, where possible. The highly similar sequences "megablast" option was selected. Sequences of closely related sister taxa to those obtained from mangroves were selected as outgroups in the individual sequence data sets. This information was used to generate data sets for further phylogenetic analyses. The basic sequence statistics, including nucleotide frequencies, transition/transversion (ns/nv) ratio, and variability in different regions of sequences, were computed by Molecular Evolutionary Genetics Analysis (MEGA X) [51], which is offline software that performs optimum alignment of sequences. The sequence data were analyzed by the neighbor-joining method using the NEIGBOR program unweighted pair group mean average (UPGMA) methods with MEGA X. Bootstrap values were equal to or greater than 50% derived from 1000 iterations. Aligned sequences were manually edited using BioEdit (Biological sequence alignment editor).

2.5. Pathogenicity Tests

Four sequenced isolates, P8 (MZ127510, *Curvularia* aff. *tsudae*), T28 (MZ127517, *Neopestalotiopsis* sp.1), P4 (MZ127507, *Pestalotiopsis* sp.1), and Pesta23 (MZ127514, *Pestalotiopsis* sp.4a) were chosen for pathogenicity trials on *Sonneratia apetala* and *S. caseolaris* in a mangrove nursery in Dong Hoang commune, Tien Hai District, Thai Binh Province. The fungi selected were representative of the most frequently isolated putative fungal pathogens. The preliminary health survey showed that the number of trees having pink or black leaf spot disorders was higher than trees with shoot dieback. Thus, one isolate T28 (MZ127517, *Neopestalotiopsis* sp.1, host source *S. apetala*) from dieback, two isolates from black spot symptoms (P8: MZ127510, *Curvularia* aff. *tsudae*, host source *S. caseolaris*; Pesta23: MZ127514, *Pestalotiopsis* sp.4a, host source *S. apetala*), and one isolate from pink spot symptoms (P4: MZ127507, *Pestalotiopsis* sp.1, host source *S. caseolaris*) were selected for the pathogenicity trial.

Pathogenicity was assessed on three-month-old seedlings with a stem diameter of 0.5–0.8 cm and a height of 50–70 cm. Ten seedlings of each host species was used for each fungus and the experiments were replicated four times. The inoculation method of Osorio et al. [28] was used. As inoculation on leaves in the field nursery was difficult to achieve, pathogenicity tests were conducted on the stem. Briefly, midway up each stem, a sterile knife was used to expose the cambium, and a 6 mm diameter plug of 6 day old colonized agar grown on PDA at 25 °C was placed, mycelial surface face down, on the cambium. Controls were inoculated with sterile agar plugs. The inoculated wounds were sealed with Parafilm® to reduce contamination and to prevent desiccation of the inoculum.

Lesion lengths (L) in the bark and cambium were measured 6 weeks after inoculation with five lesion classes as follows: L = 0 cm (nil), L ≤ 10 cm (weak), 10 < L ≤ 20 cm (average), 20 < L ≤ 30 cm (strong), and L > 30 cm (very strong). Five sections of each inoculated stem were retained for fungal re-isolation and re-sequencing to confirm Koch's Postulates. Disease index (DI) was calculated as follows: $DI = \frac{\sum_{0}^{4} n_i v_i}{N}$ in which: v_i = class

of disease scoring (lesion length); ni = number of seedlings observed in this class; N = total seedlings observed. Based on DI, pathogenicity was evaluated as: DI = 0 (no pathogenicity); 0 < DI ≤ 1 (weak); 1 < DI ≤ 2 (average); 2 < DI ≤ 3 (strong); 3 < DI ≤ 4 (very strong).

2.6. Statistical Analysis

Descriptive statistics for the preliminary health survey were analyzed using Excel. Other data were analyzed by non-parametric tests using the software SPSS v22 [52]. Non-parametric Kruskal–Wallis analyses of variance were used to determine whether the four isolates induced larger bark and cambium lesions than the controls at a significance threshold of $p < 0.05$.

3. Results

3.1. Tree Health Survey

During the preliminary assessment in December 2018, the three most commonly observed mangrove disorders were shoot dieback and pink and black leaf spots (Table S1, Figure 1). Therefore, these three disorders were then explored in detail.

From the canopy assessment (Table 3) of 4800 trees, the occurrence and extent of leaf spots and dieback were quantified (Table 4). In single stands, for dieback symptoms, on average, there was approximately 12.2% of trees with a dieback rating of 3 on *S. caseolaris*, and the values for rating 2 and rating 1 were 7.5% and 80.3%, respectively. The number of *S. apetala* trees with dieback symptoms with a rating of 3 was much lower at 1.4%. For mixed stands of the two species, the percentage of dieback symptoms with a rating of 3 was 10.1% for *S. caseolaris* and 1.5% for *S. apetala*, which were similar to the single species stands, being 13.6% and 1.3%, respectively. The number of *S. caseolaris* trees with pink leaf spot accounted for 33.3% (mixed stands) and 30.7% (single stands), while the values for *S. apetala* were 0.9% in both mixed and single stands. The incidence for black leaf spot was different from pink leaf spot. Approximately 35.2% of *S. apetala* trees had black leaf spot with a rating of 2, but for *S. caseolaris* it was only 1.1%. In the single stands, approximately 35% of *S. apetala* trees had black leaf spot, while the number for *S. caseolaris* was only 1.1%. Very few trees had all three disorders: 0.2% of *S. caseolaris* in mixed stands and 0.5% in single species stands, and 0.2% of *S. apetala* in single stands. However, the percentage of *S. caseolaris* with two symptoms (dieback and pink leaf spot) made up 11.6% (mixed stands) and 12.8% (single stands), while the numbers for *S. apetala* were <1% in both single and mixed stands. The joint incidence of dieback and leaf black spot was low but slightly higher in *S. apetala* (2.8%) than in *S. caseolaris* (0.5%).

Table 4. Number of *Sonneratia apetala* and *S. caseolaris* trees (percent of the total in brackets) in the afforestation trial with symptoms of dieback and leaf spots in three damage classes.

	Trees with Dieback (Damage Class)			Trees with Pink Spot (Damage Class)			Trees with Black Spot (Damage Class)		
	1	2	3	1	2	3	1	2	3
Mixed stands									
Sonneratia apetala	1354 (94.8)	53 (3.7)	22 (1.5)	1415 (99)	12 (0.8)	2 (0.1)	926 (64.8)	487 (34.1)	16 (1.1)
Sonneratia caseolaris	1129 (81)	107 (7.7)	157 (11.3)	929 (66.7)	438 (31.4)	26 (1.9)	1377 (98.9)	14 (1)	2 (0.1)
Single stands									
Sonneratia apetala	885 (94.8)	37 (4)	12 (1.3)	925 (99)	7 (0.7)	2 (0.2)	663 (71)	253 (27.1)	18 (1.9)
Sonneratia caseolaris	746 (79.1)	69 (7.3)	128 (13.6)	654 (69.4)	261 (27.7)	28 (3)	938 (99.5)	3 (0.3)	2 (0.2)
Total									
Sonneratia apetala	2239 (94.8)	90 (3.8)	34 (1.4)	2340 (99)	19 (0.8)	4 (0.2)	1589 (67.2)	740 (31.3)	34 (1.4)
Sonneratia caseolaris	1875 (80.3)	176 (7.5)	285 (12.2)	1583 (67.8)	699 (29.9)	54 (2.3)	2315 (99.1)	17 (0.7)	4 (0.2)

3.2. Symptoms of Disease on Sonneratia Species

Typical symptoms of shoot dieback include yellowing, stunted shoots with leaves often cupped and with dead margins (Figure 1c). The bark and wood around the lesions were darker than usual. In trees with dieback, the canopies turned yellow, followed by wilting, leaf drying, leaf fall, and branch death. In some trees, new shoots proliferated from the trunk, but in others the trunks also died.

The most obvious symptoms of pink spot were circular lesions 3–5 mm in diameter on leaves which then turn dark grey (Figure 1d). Pink leaf spots can be single, but they often coalesce to cover the whole leaf. Leaf spots occur on both surfaces of the leaf and sometimes extend through to the opposite side of leaves. Black leaf spots began as small spots, stay separate, and then enlarged in size to 3–5 mm in diameter (Figure 1e). Black spots were present on the upper and/or lower surface.

3.3. Taxonomy

Phylograms were built using MEGA X 10.2.5 software (Molecular Evolutionary Genetics Analysis) across computing platforms [51] (Figures 2–4). Bootstrap values were equal to or greater than 50% derived from 1000 iterations. The results show that isolate P8 groups with *Curvularia studae* with a 90% bootstrap value using *Alternaria alternariacida* as the outgroup. Isolate T28 lies within the *Neopestalotiopsis mesopotamica* branch with a 95% bootstrap value using *Pseudopestalotiopsis theae* as the outgroup. Isolates Pesta23 and Y6 lie in the branch with *Pestalotiopsis australasiae* and *P. telopeae* with a 61% bootstrap value; isolate Y61 lies within a sub-branch with a 74% bootstrap value from *Pestalotiopsis lushanensis*. Isolates P20, T27, P4, and P2 lie within the *P. neolitseae* and *P. humus* branch with P20 closest to *P. neolitseae* and P2 closest to *P. humus*. The findings are summarized (Table 5).
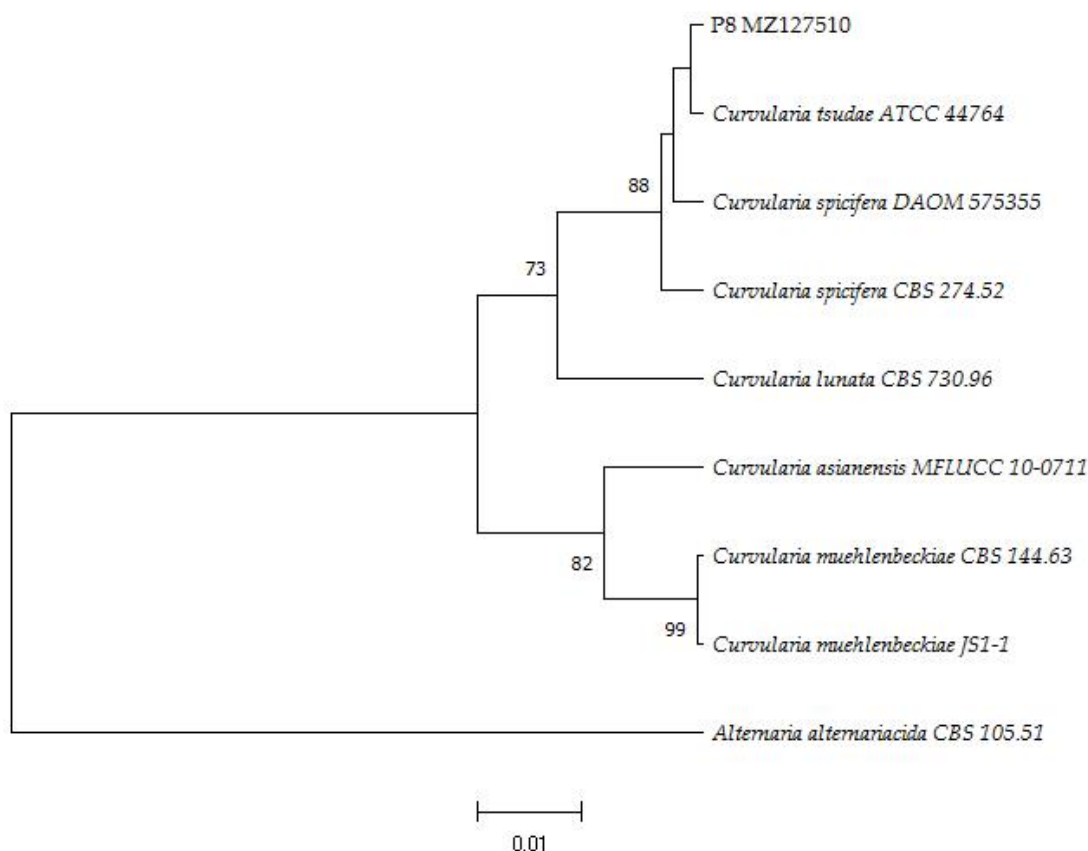

Figure 2. Phylogram obtained from MEGA X software analyses of the ITS data set. Isolate P8 groups close to *Curvularia tsudae*. Numbers on the branches are bootstrap values (>70%/posterior probabilities). The insertion bar corresponds to 1% of the corresponding sequence difference. The tree was rooted to *Alternaria alternariacida* (CBS 105.51).
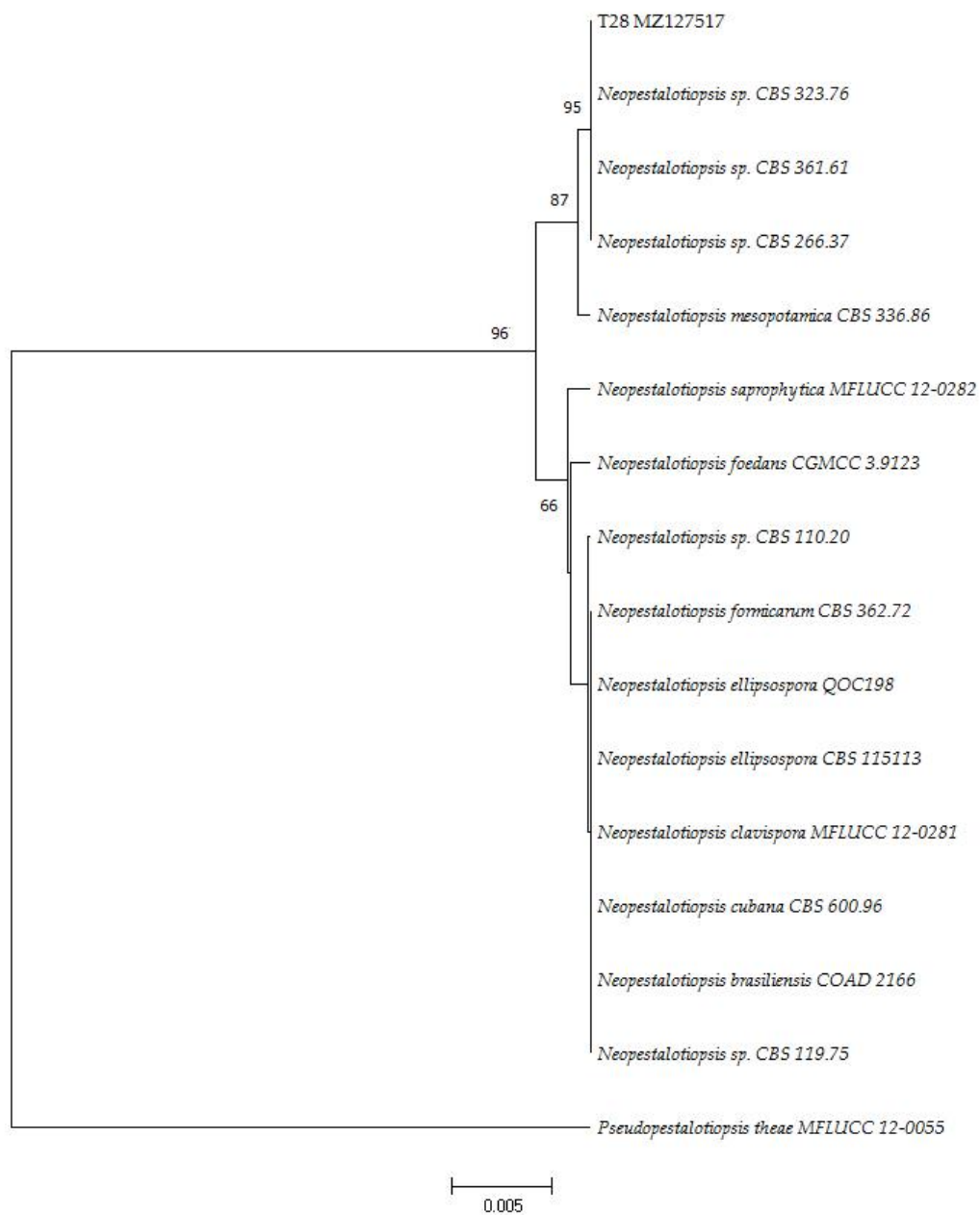
Figure 3. Phylogram obtained from MEGAX software analyses of the ITS data set. Isolate T28 groups with a branch containing *Neopestalotiopsis mesopotamica*. Numbers on the branches are bootstrap value (>60%/posterior probabilities). The insertion bar corresponds to 0.5% of the corresponding sequence difference. The tree was rooted to *Pseudopestalotiopsis theae* (MFLUCC 12-0055).
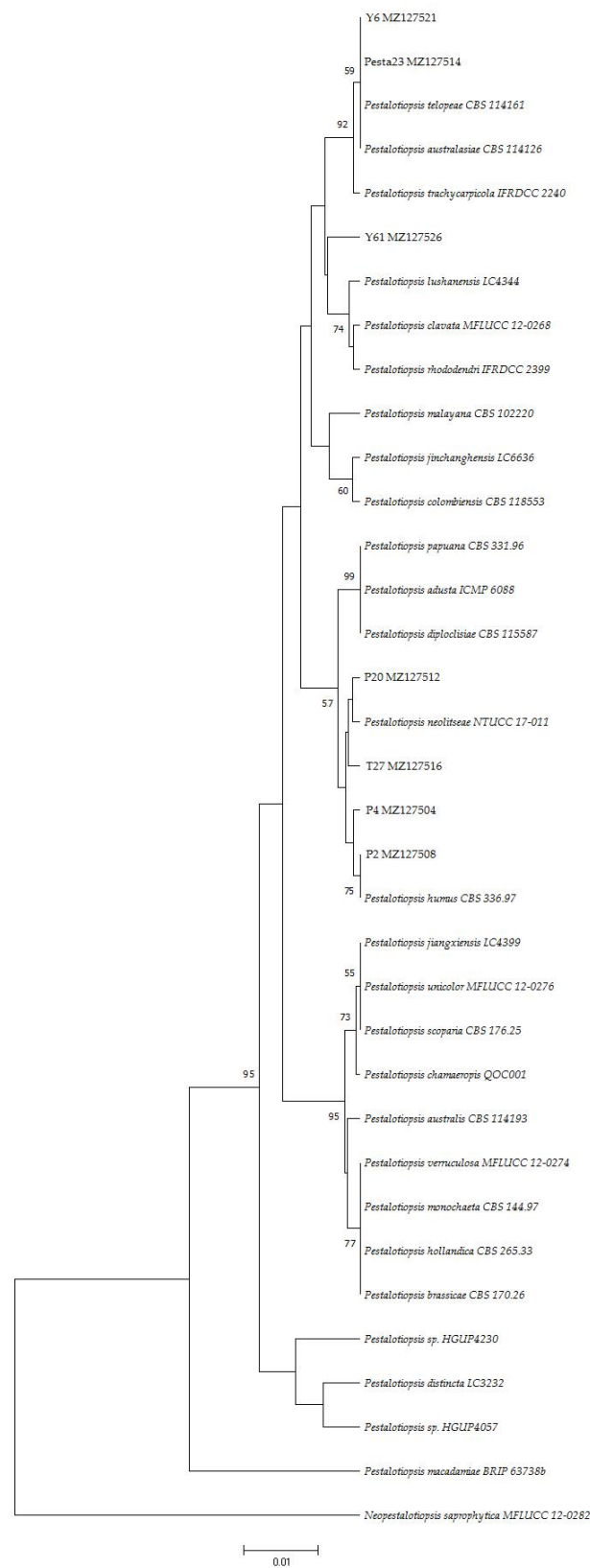
Figure 4. Phylogram obtained from MEGAX software analyses of the ITS data set. Isolates Pesta23, Y6, Y61, P20, T27, P4, and P2 group among *Pestalotiopsis* species. Numbers on the branches are bootstrap value (>55%/posterior probabilities). The insertion bar corresponds to 1% of the corresponding sequence difference. The tree was rooted to *Neopestalotiopsis saprophytica* (MFLUCC 12-0282).

Table 5. Representative fungal isolates obtained from diseased mangrove trees in Thanh Hoa Province that were used for molecular taxonomy. Isolates in bold were used in nursery pathogenicity experiments and are described in the text.

Isolate Sequenced	Number of Isolates Obtained	*Sonneratia* Host	Associated Disease	GenBank Accession Number (ITS)	Fungal Taxa
P8	34	*S. apetala*	Black leaf spot	**MZ127510**	*Curvularia* **aff.** *tsudae*
T28	39	*S. apetala*	Stem dieback	**MZ127517**	*Neopestalotiopsis* **sp.1**
P2	11	*S. caseolaris*	Pink leaf spot	MZ127508	*Pestalotiopsis* aff. *humus*
P4	45	*S. caseolaris*	Pink leaf spot	**MZ127507**	*Pestalotiopsis* **sp.1**
Y61	19	*S. apetala*	Black leaf spot	MZ127526	*Pestalotiopsis* sp.2
P20	16	*S. caseolaris*	Pink leaf spot	MZ127512	*Pestalotiopsis* aff. *neolitseae*
T27	17	*S. caseolaris*	Stem dieback	MZ127516	*Pestalotiopsis* sp.3
Y6	23	*S. apetala*	Black leaf spot	MZ127521	*Pestalotiopsis* sp.4b
Pesta23	23	*S. caseolaris*	Black leaf spot	**MZ127514**	*Pestalotiopsis* **sp.4a**

3.4. Morphology of the Main Taxa

Taxa that had a high frequency of isolation from field samples were selected for descriptions and for testing pathogenicity as follows:

- *Curvularia* aff. *tsudae*

Associated with black leaf spots of *S. apetala* and *S. caseolaris*. Small black rufous spots appeared initially and then gradually enlarged, changing to tawny circular ring spots with a dark brown or black border and smooth edge (Figure 5a). Colonies on PDA reached 5 cm in diameter after 6 days at 25 °C. Young and old mycelium of isolate P8 (*Curvularia* aff. *tsudae*) (Figure 5) on PDA was dark grey, fluffy, cottony, reverse dark brown. Conidia and conidiophores were observed on the PDA plates after 14 days post-inoculation. Spores were 13.5–27 μm in length and 8.7–12.5 μm in width, brown, straight or slightly curved, septate, and unbranched.

- *Neopestalotiopsis* sp.1

Associated with shoot dieback of *S. apetala* and *S. caseolaris*. Symptoms included yellowing of foliage, loss of leaves, canopy thinning, and dead branches (Figures 1c and 6a). Dieback occurred on terminal shoots, lateral branches as well as the main stem. Colonies on PDA reached 6 cm in diameter after 5 days at 25 °C with light aerial mycelium on the surface (Figure 6c). Young and old mycelium of isolate T28 (*Neopestalotiopsis* sp.1) on PDA was milky white. Black and globular pycnidial conidiomata appeared after 14 days at 25 °C in the light. Conidia (Figure 6b) were 14.1–22.7 μm in length and 5.3–6.5 μm in width, fusiform, straight to slightly curved, four-celled, and periclinal walls were darker than the rest of the cell. The basal cell was conical and hyaline. Three median cells were dark brown, septa, and the periclinal walls darker than the rest of the cell. The apical cell was hyaline and filiform, with 2–4 apical appendages, 15.3–17.4 μm long.

- *Pestalotiopsis* sp.1

Associated with pink leaf spot of *S. apetala* and *S. caseolaris*. Symptoms were small irregular-shaped spots, slightly sunken on the adaxial leaf surface, expanding over time (Figure 7a). Colonies on PDA reached 4–6 cm in diameter after four days at 25 °C in the light. Colonies were filamentous to circular, medium in density, aerial mycelium on the surface were flat or raised, with a filiform margin. Young and old mycelium of isolate P4 on PDA were milky white above and brown below (Figure 7c,d). The spores (Figure 7b) ranged from 13.5 to 22.5 μm in length and 5.4 to 6.3 μm in width, fusiform to clavate, straight to slightly curved, with three septa.

- *Pestalotiopsis* sp.4a

Associated with black leaf spots of *S. apetala* and *S. caseolaris* (Figure 8a). Small black rufous spots appeared initially and then gradually enlarged, changing to tawny circular ring spots with a black border and smooth edge. Young and old mycelium of isolate Pesta23

on PDA were dark grey (Figure 8c,d). The length of conidia ranged from 13.2 to 21.4 µm in length and 5.0 to 6.1 µm in width, fusiform to clavate, straight to slightly curved, with four septa (Figure 8b). A separate group of 23 isolates was similar to Pesta23, except the mycelium was creamy white above and creamy brown below (not illustrated). Pesta23 and Y6 lie within the same arm of the phylogram in Figure 4, for convenience the mycelial types are referred to as *Pestalotiopsis* sp.2a for the dark mycelial isolates and *Pestalotiopsis* sp.2b for the light mycelial isolates.
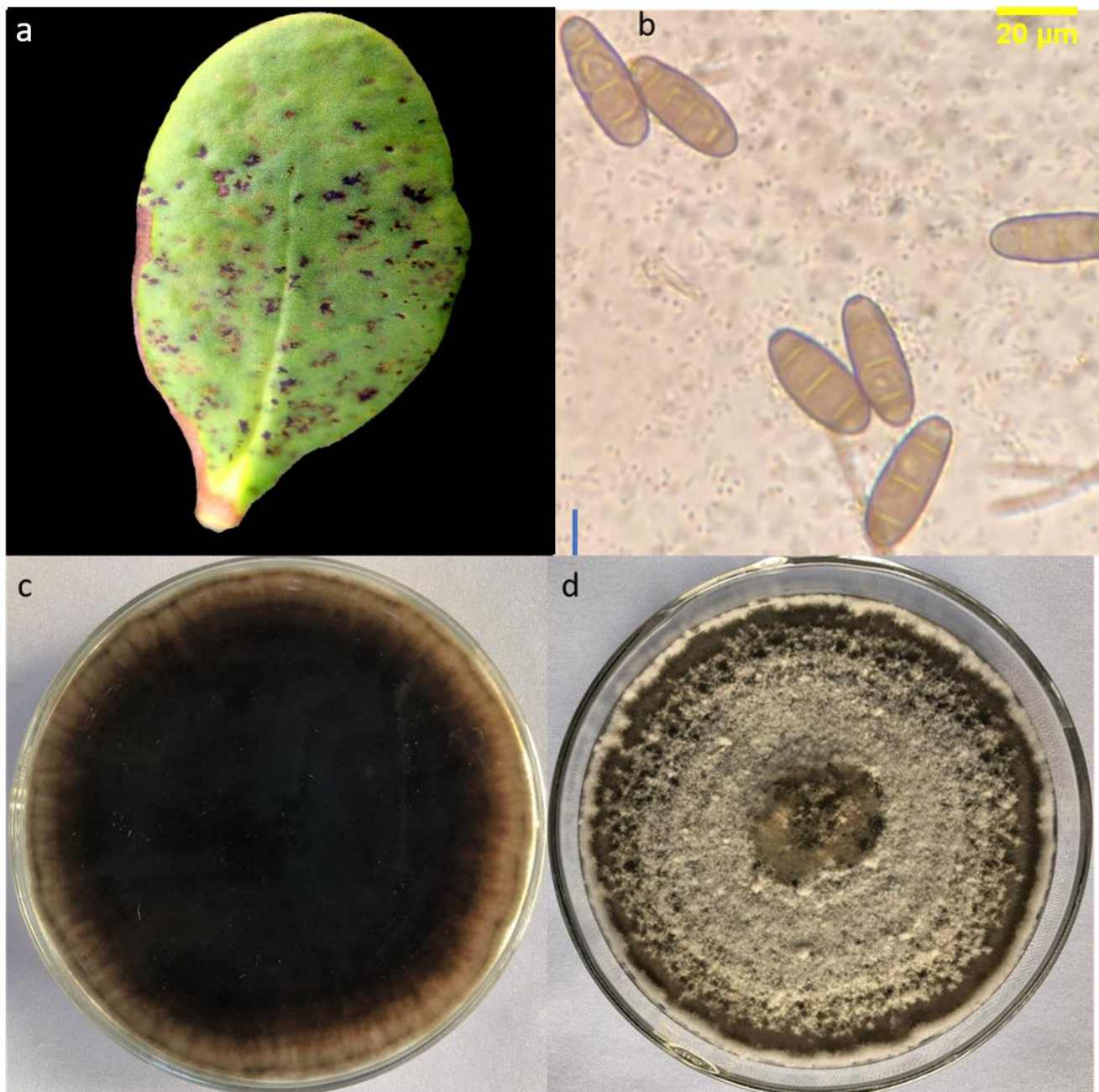
Figure 5. Symptoms of leaf black spot on a Sonneratia apetala leaf caused by *Curvularia* aff. *tsudae* (**a**); isolate P8 (MZ127510) conidia (**b**); mycelia ((**c**) from below; (**d**) from above) that were obtained from the leaf illustrated in (**a**).
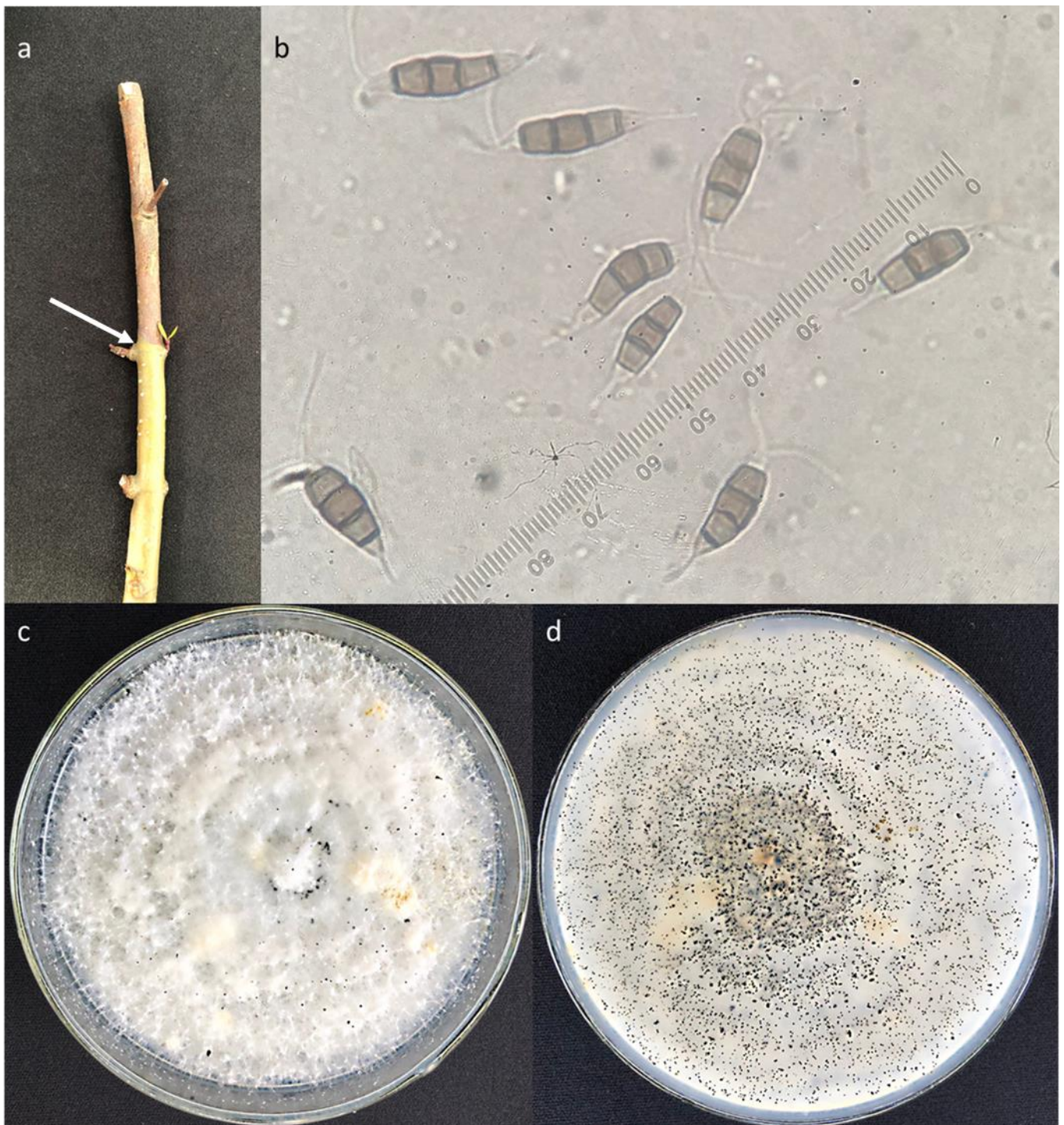
Figure 6. *Neopestalotiopsis* sp.1 isolate T28 (MZ127517) was obtained from a Sonneratia caseolaris stem at the junction of dead and live wood (arrow) (**a**); conidia (**b**); mycelia ((**c**) from above; (**d**) from below) that were obtained from the lesion illustrated in (**a**).
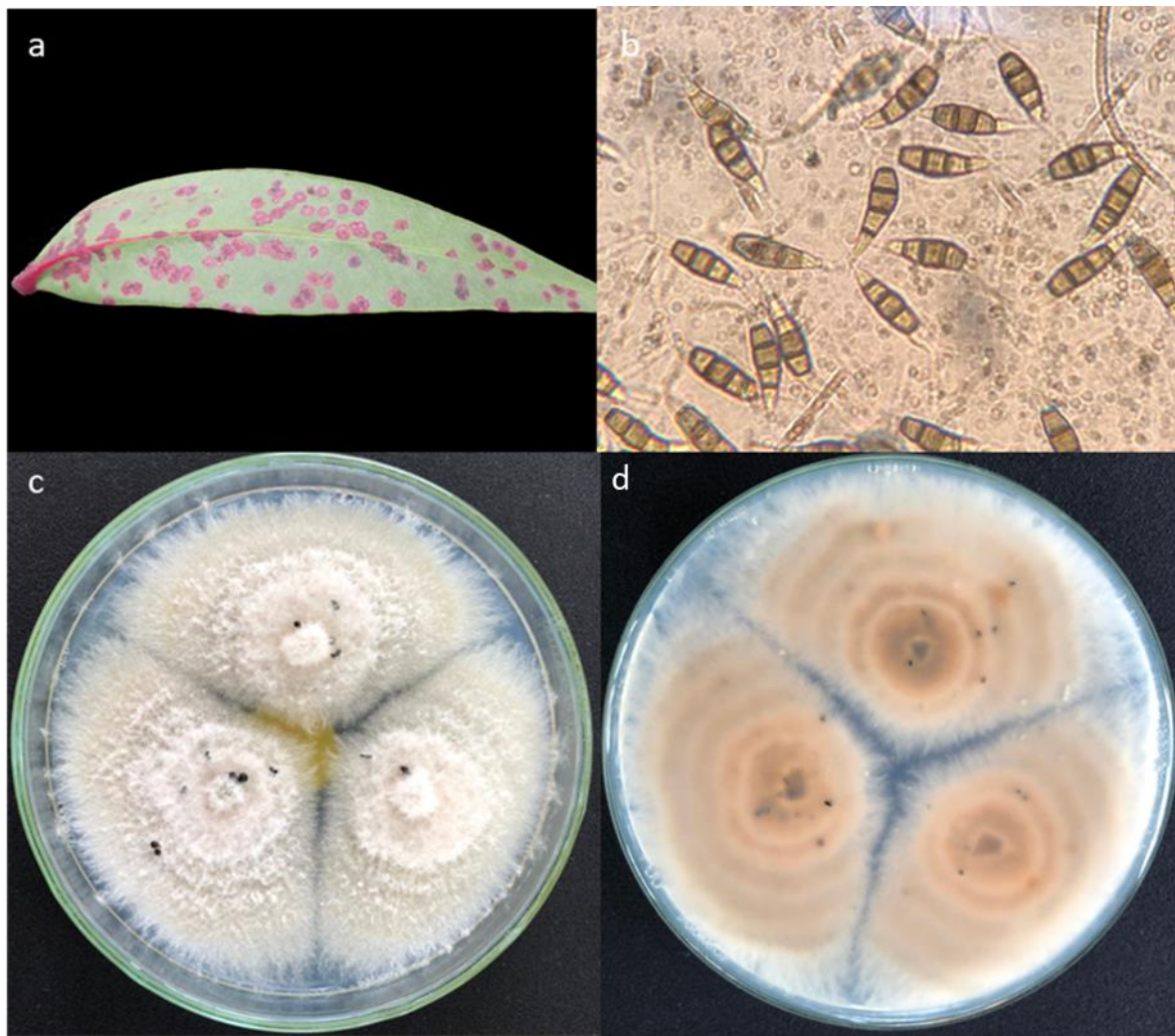
Figure 7. *Pestalotiopsis* sp.1 (Isolate P4-MZ127507) associated with pink leaf spot disorder on Sonneratia caseolaris. Disease symptom (**a**); spores (**b**); mycelia (upper view—(**c**), lower view—(**d**)) that were obtained from the leaf illustrated in (**a**).

3.5. Pathogenicity Trial

The pathogenicity trials showed that all four fungi produced larger lesions in the stems than the wounded control. Ten weeks after inoculation, five samples of each treatment were collected for re-isolation and identification to confirm Koch's postulates. All four species were successfully re-isolated from stem lesions (data not shown).

Pathogenicity of *Curvularia* aff. *tsudae* (P8, MZ127510), *Neopestalotiopsis* sp.1 (T28, MZ127517), *Pestalotiopsis* sp.1 (P4, MZ127507), and *Pestalotiopsis* sp.4a (Pesta23, MZ127514) were significantly different from the control (PDA) ($p < 0.001$) when inoculated on seedlings of *S. apetala* and *S. caseolaris*. The pathogenicity of the isolates and the control fell into three groups: strong (one isolate), average (three isolates), and nil (control) (Table 6).
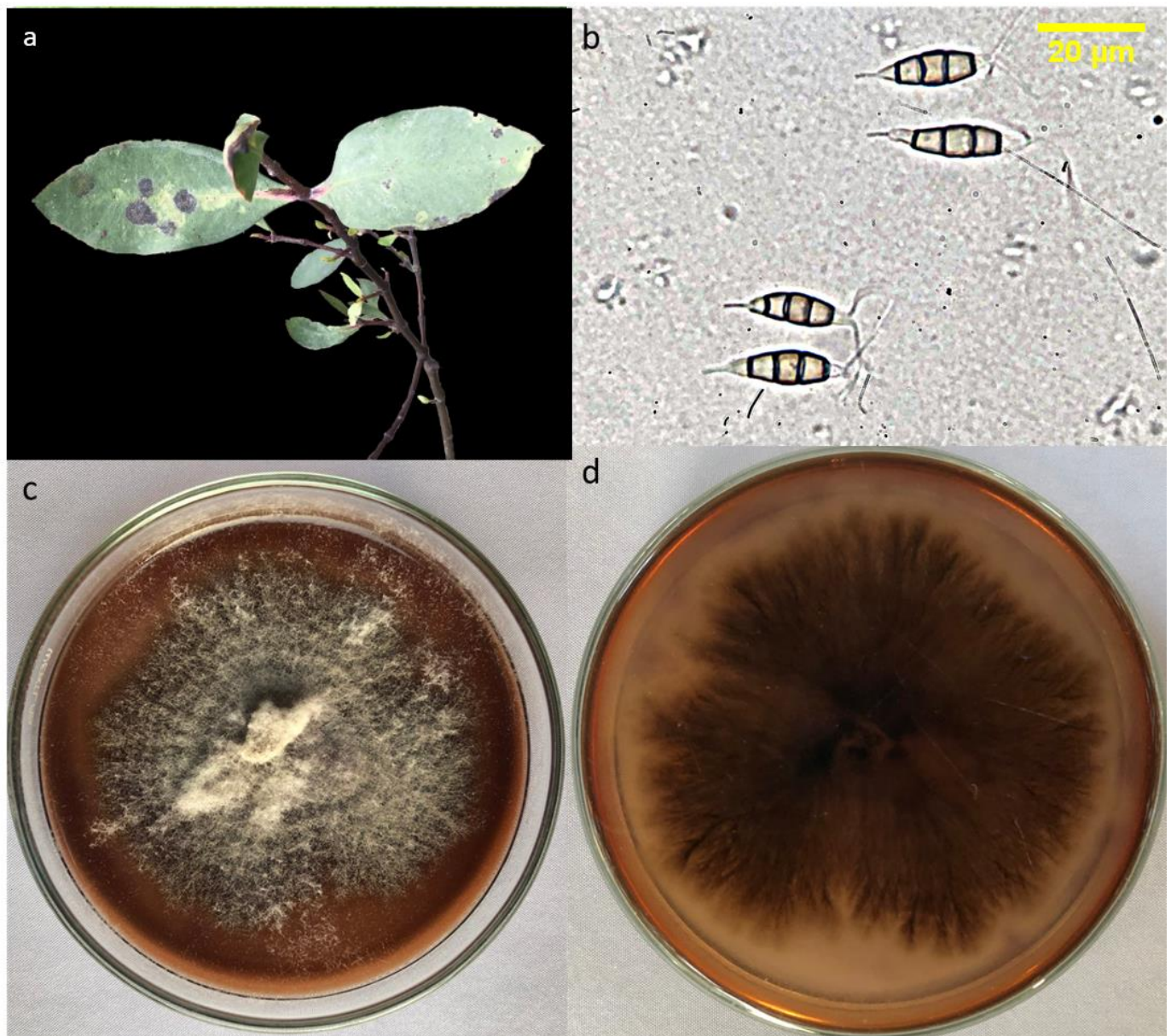
Figure 8. Black leaf spot on Sonneratia caseolaris caused by *Pestalotiopsis* sp.4a (isolate Pesta23-MZ127514) (**a**); spores (**b**); mycelia (upper view—(**c**), lower view—(**d**)) that were obtained from the leaf illustrated in (**a**).

Table 6. Disease index and pathogenicity of the *Pestalotiopsis, Neopestalotiopsis*, and *Curvularia* isolates inoculated onto stems of 3 month old seedlings of *Sonneratia apetala* and *S. caseolaris* in a nursery in Thai Binh Province.

Host Species	Isolate	Fungal Taxa	Disease Index	Pathogenicity
Sonneratia apetala	P8	*Curvularia* aff. *tsudae*	1.90	average
	T28	*Neopestalotiopsis* sp.1	1.82	average
	P4	*Pestalotiopsis* sp.1	1.76	average
	Pesta23	*Pestalotiopsis* sp.4a	1.55	average
	Control (PDA)		0.00	nil
	Chi-Square	48.9		
	P8	*Curvularia* aff. *tsudae*	1.93	average
	T28	*Neopestalotiopsis* sp.1	2.36	strong
	P4	*Pestalotiopsis* sp.1	1.93	average
Sonneratia caseolaris	Pesta23	*Pestalotiopsis* sp.4a	1.89	average
	Control (PDA)		0.00	nil
	Chi-Square	53.6		
	p-Value	0.000		

4. Discussion

This is the first study to explore whether fungal pathogens might be contributing to mangrove decline in Vietnam. The incidence of shoot dieback and leaf spots observed in young afforestation stands of two *Sonneratia* species suggests that diseases may be contributing to the poor survival rates in some mangrove restoration projects, particularly in central and north Vietnam where *Sonneratia* species have been widely planted. In the present study, 227 fungal isolates that were obtained from diseased tissue fell into nine morphotypes, and results from BLAST searches in GenBank for the ITS region indicated the presence of at least eight taxa within the plant pathogen genera *Curvularia*, *Neopestalotiopsis*, and *Pestalotiopsis*. Further collections are required before the unidentified new taxa can be described using more isolates to compare morphology and sequences.

Unlike for Vietnam, a range of mangrove disorders have been previously reported in other countries. Black leaf spot on *Sonneratia apetala* and small black leaf spot on *S. caseolaris* were associated with *Olletotrichum lindemuthianum*, *Pestalotiopsis guepinii*, and *Phoma betae* in Bangladesh [53]; Abbas et al. [54] described a black leaf spot on *Conocarpus erectus* caused by *Alternaria alternata* in Pakistan. Black leaf spot disease also occurs on *Ceriops decandra*, *Osbornia octodonta*, *Rhizophora stylosa*, and *S. caseolaris* associated with *Pseudocercosporella* in the Philippines [38]. Dark brown leaf spots caused by *Orynespora cassiicola* were found on *Acanthus ilicifolius* in China [22]. Regarding mangrove dieback, the high frequency of dieback and mortality were found in *R. mangle* in coastal mangrove forests in Puerto Rico [30,55]. Canker on the trunks, branches or twigs of *S. apetala* were observed in China [25], and top dieback was reported in *Heritiera fomes* in Bangladesh [24]. Symptoms of pink leaf spot disorders have so far not been recorded on mangroves.

More than 200 fungal species have been isolated and identified from mangroves [21,56]. Disease reports include branch/stem cankers, leaf spots, defoliation, dieback, and stem rot of mangroves [36,57–71]. For example, *Diaporthe* is known as an endophytic fungus on mangrove trees [69], and *Pestalotiopsis* is predominant in mangrove trees in China [72] and causes leaf spots on *Rhizophora* [35]. *Pestalotiopsis microspora* has been reported as an endophyte in *Rhizophora mucronata* [73] and *Nigrospora oryzae* was isolated from *R. mucronata* in Malaysia [71].

According to Shamsi et al. [53], fungi associated with black spot of *S. apetala* were *Aspergillus fumigatus*, *Colletotrichum lindemuthianum*, *Pestalotiopsis guepinii*, and *Phoma betae*. Fungi associated with small leaf spot of *S. caseolaris* were *Curvularia fallax*, *Fusarium* sp., *Penicillium digitatum*, and *Phoma betae* [53]. *Alternaria alternata* was considered to cause leaf spot on *Conocarpus erectus* in Pakistan [54].

In Vietnam, *Curvularia* species such as *C. gloeosporioides* are known to cause anthracnose disease in coffee [74], and *C. lunata* causes postharvest problems in rice [75] and dragon fruit [76]. *Pestalotiopsis virgatula* and *P. clavispora* were recorded as causal agents of postharvest rot diseases on rambutan [77], and an unidentified *Pestalotiopsis* was associated with crown rot disease of strawberry [78].

The source of the fungi causing leaf spots and shoot dieback of *Sonneratia* in afforestation in Vietnam is unknown. One possibility is that fungal spores are spread by wind from other mangrove stands in the area or infested fruits are carried by the tide to afforested sites. We observed some natural recruitment on site in the first year after planting *Sonneratia*. Thus, research on fungal pathogens should also be undertaken in old stands in the future. Another possibility is that disease might be carried from nurseries to the field. Two nurseries, one in Thai Binh Province and another in Thanh Hoa Province, provide seedlings for most mangrove afforestation projects in the north of Vietnam. This means that there is a risk of widespread infection if disease management in nurseries is poor.

5. Conclusions

This is the first report of diseases affecting mangrove afforestation in Vietnam. *Neopestalotiopsis* sp.1 and *Pestalotiopsis* sp.3 were associated with shoot dieback of *Sonneratia apetala* and *S. caseolaris*; *Pestalotiopsis* aff. *humus*, *Pestalotiopsis* aff. *Neolitseae*, and *Pestalotiopsis* sp.1 were

associated with pink leaf spots; *Curvularia* aff. *tsudae*, *Pestalotiopsis* sp.2, and *Pestalotiop-sis* sp.4 were associated with black leaf spots. Further work is required to characterize the fungi at the species level and to develop options for disease management.

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