Identification and Characterization of Macrophomina phaseolina Causing Leaf Blight on White Spider Lilies (Crinum asiaticum and Hymenocallis littoralis) in Malaysia

Abd Rahim Huda-Shakirah, Yee Jia Kee, Abu Bakar Mohd Hafifi, Nurul Nadiah Mohamad Azni, Latifah Zakaria and Masratul Hawa Mohd

School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia

ABSTRACT

Crinum asiaticum and Hymenocallis littoralis, commonly known as spider lilies are bulbous perennial and herbaceous plants that widely planted in Malaysia as ornamental. During 2015-2016, symptom of leaf blight was noticed on the hosts from several locations in Penang. The symptom appeared as irregular brown to reddish lesions surrounded by yellow halos. As the disease progressed, the infected leaves became blighted, dried, and fell off with the presence of black microsclerotia and pycnidia on the lesions parts. The present study was conducted to investigate the causal pathogen of leaf blight on C. asiaticum and H. littoralis. Based on morphological characteristics and DNA sequences of internal transcribed spacer (ITS) region and translation elongation factor 1-alpha (TEF1-a) gene, the causal pathogen was identified as Macrophomina phaseolina. Phylogenetic analysis of combined dataset of ITS and TEF1-a grouped the isolates studied with other isolates of M. phaseolina from GenBank. The grouping of the isolates was supported by 96% bootstrap value. Pathogenicity test proved the role of the fungus in causing leaf blight on both hosts.

1. Introduction

Crinum asiaticum and Hymenocallis littoralis are evergreen and bulbous species which produce narrow and strap-like leaves with white flowers. C. asiaticum and H. littoralis are locally known as bunga tembaga suasa and melong kecil, respectively [1,2] in which both are belong to family Amaryllidaceae and Order Asparagales. The spider lilies are usually planted in Malaysia as ornamental and landscape purposes. They have been claimed to have multiple ethnobotanical uses [3,4]. For instance, C. asiaticum capable of treating joint inflammation and sprain, induced vomiting, treating hemorrhoids, contusion, fracture, and earache [3,5] while H. littoralis was used to treat freckles and blemishes [4,6,7].

Like many other ornamental plants, C. asiaticum and H. littoralis were susceptible to a number of fungal diseases. C. asiaticum was reported to be associated with anthracnose caused by Colletotrichum truncatum, C. boninense, C. fructicola and C. siamense [8–10], ring spot caused by Drechslera avenacea [11], and leaf blight caused by Drechslera sp. [12]. Meanwhile, H. littoralis was reported to be infected with leaf blight caused by Neoscytalidium dimidiatum [13], leaf spot caused by Phyllosticta hostae and Fusarium oxysporum [14,15], brown leaf caused by Phyllosticta hymenocallidicola [16] and leaf tip blight caused by Curvularia eragrostidis [17].

Preliminary observation in Permatang Pauh and Universiti Sains Malaysia showed outdoor plantings of C. asiaticum and H. littoralis have been infected with leaf blight. The symptom was irregular brown to reddish lesions surrounded by yellow halos formed on the leaves. The presence of black microsclerotia and pycnidia was also observed on the infected leaves (Figure 1(A)). Severe infection causing the blighted leaves dried and fell off. The purpose of this study was to investigate the causal agent of leaf blight on C. asiaticum and H. littoralis based on morphological characteristics, DNA sequences, and phylogenetic analysis.

2. Materials and methods

2.1. Isolation and morphological identification

Blighted leaves of C. asiaticum and H. littoralis were collected from Permatang Pauh and Universiti Sains Malaysia, Penang. To isolate the suspected pathogen,
the symptomatic samples were cut into small pieces for surface sterilization in 70% ethanol (C₂H₅OH) and 1% sodium hypochlorite (NaOCl) for 3 min each. Then, the samples were rinsed in three changes of sterile distilled water for 1 min each and left to dry on sterile filter papers before plating on potato dextrose agar (PDA) and incubated at 25 ± 2°C for 2–3 days. Single spore isolation was performed to obtain a pure culture of the fungal colony by transferring the hyphal tip onto a new PDA plate and incubated as above. A mycelial disc of 6 mm diameter was placed onto the center of PDA plate and incubated as above for 7 days. Water agar (WA) overlaid with toothpicks [18] was used to induce the formation of microsclerotia and pycnidia and incubated as above for 7 days. The size of 50 randomly selected conidia and the diameter of 50 microsclerotia and pycnidia were measured.

2.2. Molecular identification and phylogenetic analysis

Identification of the fungal isolates was further confirmed by DNA sequences. All the isolates were grown in potato dextrose broth (PDB) and incubated as above for 7 days. The fungal mycelium was harvested and ground in liquid nitrogen to a fine powder. A total of 60 mg of the fine powdered mycelium were extracted by using Invisorb Spin Plant Mini Kit (Stratec Biomedical AG, Birkenfeld, Germany) following the manufacturer’s instructions.

PCR amplification of internal transcribed spacer (ITS) and translation elongation factor 1-alpha (TEF1-α) was performed using the primer pairs of ITS1/ITS4 [19] and EF1-728F [20]/EF2 [21], respectively. The amplification was carried out in a total volume of 50 µl PCR mixture containing 8 µl Green buffer (Promega, Madison, WI), 8 µl MgCl₂, 1 µl deoxynucleotide triphosphate polymerase (dNTP), 8 µl of each primer (Promega, USA), 0.3 µl Taq polymerase, and 1 µl genomic DNA. The reaction was performed in a MyCycler™ Thermal Cycler (Bio-Rad, Hercules, CA) with the following condition: an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 5 min. The amplified PCR products were separated in 1% agarose gel, run at 80 V and 400 mA for 90 min. The size of fragment was estimated based on comparison with 100 bp DNA ladder (Generulers™; Fermentas, Waltham, MA).

The PCR products were sent to a service provider (First BASE Laboratories Sdn Bhd, Seri Kembangan, Malaysia) for DNA purification and sequencing. The DNA sequences obtained were deposited in GenBank. The generated consensus sequences were compared with other sequences in GenBank database (http://www.ncbi.nlm.nih.gov) by using BLAST to determine the identity of the fungal isolates.

Multiple sequence alignment was performed and used to construct maximum likelihood (ML) tree using Molecular Evolutionary Genetic Analysis (MEGA7) [22] based on substitution model by Kimura-2-parameter [23]. The robustness of each grouping and branch was analyzed by 1000 bootstrap replications [24]. The fungal sequences obtained in this study were compared with several isolates of M. phaseolina and Macrophomina spp.
The formation of microsclerotia was observed on the abundant of microsclerotia embedded (Figure 1(C)). Cultures showed black and dark pigmentation with other reverses side of the plates, the olive colonies which turned darker as the culture age used as an outgroup (Table 1).

Table 1. Isolates used for the phylogenetic analysis in this study.

| Species                        | Isolate\(^\text{a}\) | Host                  | Locality       | ITS GenBank accession No. | TEF1-\(\alpha\) GenBank accession No. |
|--------------------------------|---------------------|-----------------------|----------------|---------------------------|-------------------------------------|
| Macrophomina phaseolina        | CMM3650\(^\text{b}\) | Jatropha curcas       | Brazil: Espirito Santo | KF234552                   | KF226710                            |
| M. phaseolina                  | PD112\(^\text{c}\)   | Prunus dulcis        | USA            | GU251105                   | GU251237                            |
| M. phaseolina                  | CMM3615\(^\text{d}\) | J. curcas            | Brazil: Minas Gerais | KF234547                   | KF226693                            |
| M. phaseolina                  | MUC531\(^\text{e}\)   | Sesbania formosa     | Western Australia: Kununurra | EF585505                   | EF585560                            |
| M. phaseolina                  | CBS205.47\(^\text{f}\) | Phaeosclerus vulgaris | Italy          | KF51622                    | KF51997                             |
| M. phaseolina                  | PPCA213              | Cinnamum asiaticum   | Malaysia: Penang | MK408582                   | MK408571                            |
| M. phaseolina                  | PPCA30               | C. asiaticum         | Malaysia: Penang | MK408583                   | MK408572                            |
| M. phaseolina                  | PPCA29               | C. asiaticum         | Malaysia: Penang | MK408584                   | MK408573                            |
| M. phaseolina                  | PPHL26               | Hymenocallis littoralis | Malaysia: Penang | MK408585                   | MK408574                            |
| M. phaseolina                  | PPHL25               | H. littoralis        | Malaysia: Penang | MK408586                   | MK408575                            |
| M. phaseolina                  | PPHL23               | H. littoralis        | Malaysia: Penang | MK408587                   | MK408576                            |
| Macrophomina pseudophasoelina  | CMM3633\(^\text{g}\) | J. curcas            | Brazil: Minas Gerais | KF369262                   | KF533906                            |
| M. pseudophasoelina            | CMM4231\(^\text{h}\) | Arachis hypogaea     | Brazil: Rio Grande do Norte | KU058951                   | KU058921                            |
| M. pseudophasoelina            | CPC21460\(^\text{i}\) | A. hypogaea          | Senegal: Louga   | KF51788                    | KF521520                            |
| M. pseudophasoelina            | CPC21502\(^\text{j}\) | Hibiscus sabdariffa  | Senegal: Saint Louis | KF51797                    | KF521529                            |
| M. pseudophasoelina            | CPC21417\(^\text{k}\) | Arachis hypogaea     | Senegal: Louga   | KF51791                   | KF521533                            |
| Macrophomina ephiricocola      | CM4045\(^\text{m}\)   | Jatropha gossypifolia | Brazil: Paraiba  | KU058928                   | KU058989                            |
| M. ephiricocola                | CM4134\(^\text{n}\)   | Ricinus communis     | Brazil: Bahia    | KU058936                   | KU058906                            |
| M. ephiricocola                | CM4145\(^\text{o}\)   | Ricinus communis     | Brazil: Bahia    | KU058937                   | KU058907                            |
| Macrophomina vaccinii           | CGMCC3.19508\(^\text{p}\) | Vaccinium sp.        | China: Fujian    | MK687455                   | MK687431                            |
| M. vaccinii                    | CGMCC3.19509\(^\text{q}\) | Vaccinium sp.        | China: Fujian    | MK687456                   | MK687432                            |
| M. vaccinii                    | CGMCC3.19510\(^\text{r}\) | Vaccinium sp.        | China: Fujian    | MK687457                   | MK687433                            |
| Botryosphaeria dothidea         | CBS110302\(^\text{s}\) | Vitex vinifera       | Portugal        | AT259092                   | AT575218                            |

\(^a\)Reference isolate used as comparison in phylogenetic analysis.

from various hosts and Botryosphaeria dothidea was used as an outgroup (Table 1).

2.3. Pathogenicity test

A total of six healthy seedlings of *C. asiaticum* and *H. littoralis* were used for pathogenicity test. Mycelial plug was applied as an inoculum, preparing from 7-day-old PDA culture using a sterile cork borer (6 mm diameter). The leaves of *C. asiaticum* and *H. littoralis* were surface sterilized with 70% ethanol and wounded with a sterile toothpick or cork borer [32]. The mycelial plug was inoculated on the wounded area as a treatment while the PDA plug without mycelia was inoculated as a control. The plugs were wrapped with wet cotton to maintain moisture content. Each fungal isolate was done in triplicate and the test was repeated twice. The inoculated plants of *C. asiaticum* and *H. littoralis* were placed in a plant house of School of Biological Sciences, Universiti Sains Malaysia for 2 weeks of incubation. Disease signs and symptoms were checked daily. 

3. Results

3.1. Morphological identification

A total of six fungal isolates were obtained from the infected leaves of *C. asiaticum* (PPCA29, PPCA30, and PPCA213) and *H. littoralis* (PPHL23, PPHL25, and PPHL26). All the fungal isolates produced dark olive colonies which turned darker as the culture age (Figure 1(B)) and for the reverse side of the plates, the cultures showed black and dark pigmentation with abundant of microsclerotia embedded (Figure 1(C)). The formation of microsclerotia was observed on the PDA plates and also on the toothpicks (Figure 1(D)) after 7 days of incubation. The microsclerotia were black, smooth, round to oblong, uniformly reticulate, formed from hyphal aggregates, 40.1 ± 5.8 \(\mu m\) in diameter (Figure 1(E)). None of the isolates produced pycnidia from PDA culture and WA overlaid with toothpicks. Pycnidia were present on the infected leaves of *C. asiaticum* and *H. littoralis* collected from the fields (Figure 1(F)). The pycnidia were dark to grayish, globose, membranous, 96.8 ± 12.9 \(\mu m\) in diameter with a truncate ostiole. The structure of conidiogenous cell and conidia can be seen from the crushed pycnidia. The conidiogenous cell was hyaline, short obpyriform to subcylindrical with immature conidia near the apex (Figure 1(G)). The conidia were a single cell, hyaline, aseptate, ellipsoid to obovoid and 22.3 ± 2.0 × 7.2 ± 0.5 \(\mu m\) in size (Figure 1(H)).

3.2. Molecular identification and phylogenetic analysis

The accession numbers for all fungal isolates in the present study were MK408582 to MK408587 for ITS and MK408571 to MK408576 for TEF1-\(\alpha\). Based on BLAST search, all the isolates showed 99.81% and 98.89% identities to KF234552 (ITS) and KF226710 (TEF1-\(\alpha\)) of *Macrophomina phaseolina*, respectively.

Comparison of sequences showed that the six isolates of *M. phaseolina* recovered from *C. asiaticum* and *H. littoralis* were grouped into the same clade with reference isolates of *M. phaseolina* from GenBank including CMM3650, PD112, CMM3615, and MUCC531 based on ITS and TEF1-\(\alpha\). The grouping of the isolates was supported by 96% bootstrap value (Figure 2).
3.3. Pathogenicity test

All the inoculated leaves of *C. asiaticum* and *H. littoralis* showing the typical symptom of leaf blight as observed in the fields except for the control which remained asymptomatic. Initially, the symptom appeared as irregular brown to reddish lesion surrounded by yellow halo. Then, the lesion expanded and turned darker (Figure 3(A–C)). As the disease progressed, the production of small black microsclerotia and pycnidia was scattered on the infected area (Figure 3(D)). The infected leaf tissue was taken and examined under the dissecting microscope. Hyphae produced from germinated microsclerotium were observed and used to penetrate into the host cell (Figure 3(E)). Reisolation and reidentification of the fungal isolates were conducted and fulfilled the Koch’s postulates.

4. Discussion and conclusion

The present study highlighted the causal pathogen of leaf blight on spider lilies (*C. asiaticum* and *H. littoralis*) in Malaysia was proved as *M. phaseolina*. *M. phaseolina* is a well-known plant pathogen causing several important diseases such as charcoal rot [33,34], crown rot [35], Ashy stem blight [36], wilt [37], leaf blight [38], and stem, collar, and root rot [39–41]. This seed-borne or soil-borne pathogen was responsible for causing diseases on numerous hosts namely sunflower, strawberry, soybean, watermelon, guava, and mungbean [42]. Result of pathogenicity test showed the presence of microsclerotia and pycnidia on the inoculated leaves. The germinated microsclerotia will produce infection hyphae which then penetrate through the epidermal cells and colonize intercellularly permitting the fungus to survive prolonged in the soil [42,43].

Besides spider lilies, the other plant from order Asparagales in India, *Chlorophytum borivilianum* was reported to be infected with *M. phaseolina* causing leaf spot disease [44]. This seed-borne or soil-borne pathogen was responsible for causing diseases on numerous hosts namely sunflower, strawberry, soybean, watermelon, guava, and mungbean [42]. Result of pathogenicity test showed the presence of microsclerotia and pycnidia on the inoculated leaves. The germinated microsclerotia will produce infection hyphae which then penetrate through the epidermal cells and colonize intercellularly permitting the fungus to survive prolonged in the soil [42,43].
only conidia and microsclerotia were observed. Pycnidia are rarely produced on the culture and their formation depends on the host and the specific nature of the fungal isolates [42,46]. Identification of *M. phaseolina* solely based on morphology is difficult and challenge as the fungus has two asexual subphases namely saprophytic phase and pathogenic phase. Saprophytic phase (*Rhizoctonia bataticola*) forms microsclerotia and mycelia, while pathogenic phase (*M. phaseolina*) presents in host tissues and forms microsclerotia, mycelia, and pycnidia [42]. Both subphases were observed from the fungal isolates in the present study and to support their morphological identification, molecular analysis was carried out.

Molecular identification of *M. phaseolina* in the present study was verified using ITS region and TEF1-α as adopted by Sousa et al. [47]. Most of the previous studies relied on ITS to recognize *M. phaseolina* [48–50] and the others used species-specific primers [51–53].

In conclusion, to our knowledge, this is the first report of *M. phaseolina* causing leaf blight on white spider lilies (*C. asiaticum* and *H. littoralis*). Findings in the present study will be beneficial in disease monitoring, quarantine, and management purposes of the host.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This research was supported by Fundamental Research Grant Scheme (FRGS) [203.PBIOLOGI.6711575] and Research University Grant (RUI) [1001/PBIOLOGI/8011061].

**ORCID**

Abd Rahim Huda-Shakirah [http://orcid.org/0000-0002-2665-0525](http://orcid.org/0000-0002-2665-0525)

Yee Jia Kee [http://orcid.org/0000-0002-4273-8332](http://orcid.org/0000-0002-4273-8332)

Abu Bakar Mohd Hafifi [http://orcid.org/0000-0001-9299-3932](http://orcid.org/0000-0001-9299-3932)

Latiffah Zakaria [http://orcid.org/0000-0002-2388-1424](http://orcid.org/0000-0002-2388-1424)

Masratul Hawa Mohd [http://orcid.org/0000-0003-2250-2821](http://orcid.org/0000-0003-2250-2821)

**References**

[1] Asmawi MZ, Arafat OM, Amirin S, et al. In vivo antinociceptive activity of leaf extract of *Crinum asiaticum* and phytochemical analysis of the bioactive fractions. Int J Pharmacol. 2011;7(1):125–129.

[2] Subramaniam S, Sundarasekar J, Sahgal G, et al. Comparative analysis of lycorine in wild plant and callus culture samples of *Hymenocallis littoralis* by HPLC-UV method. Scientific World J. 2014:408306.

[3] Singh KA, Kumar R, Rao GRK, et al. Crinumin, a chymotrypsin-like but glycosylated serine protease from *Crinum asiaticum*: purification and physicochemical characterisation. Food Chem. 2010;119(4):1352–1358.

[4] Noormi R, Murugaiyah V, Subramaniam S. Optimization of callus induction medium for *Hymenocallis littoralis* (Melong kecil) using root and bulb explants. J Med Plants Res. 2012;6(12):2309–2316.

[5] Ilavenil S, Kaleeswaran B, Ravikumar S. Evaluation of antibacterial activity and phytochemical analysis of *Crinum asiaticum*. Int J Curr Res. 2010;1:35–40.

[6] Ocampo R, Balick MJ, Plants of Semillas Sagradas: an ethnomedicinal garden in Costa Rica. San José: Finca Luna Nueva Extractos de Costa Rica; 2009.

[7] Sundarasekar J, Sahgal G, Subramaniam S. Anticandida activity by *Hymenocallis littoralis* extracts for opportunistic oral and genital infection
Kamalakannan A, Mohan L, Valluvaparidasan V, et al. First report of Macrophomina root rot (Macrophomina phaseolina) on medicinal coleus (Coleus forskohlii) in India. Plant Pathol. 2006; 55(2):302.

Kaur S, Dhillon GS, Brar SK, et al. Emerging phytopathogen Macrophomina phaseolina: biology, economic importance and current diagnostic trends. Crit Rev Microbiol. 2012;38(2):136–151.

Baird RE, Watson CE, Scruggs M. Relative longevity of Macrophomina phaseolina and associated mycobiota on residual soybean roots in soil. Plant Dis. 2003;87(5):563–566.

Dadwal VS, Bhartiya S. New report of a leaf spot disease of Chlorophytum borivillianum caused by Macrophomina phaseolina from India. J Mycol Plant Pathol. 2012;42(3):397–398.

Crous PW, Slippers B, Wingfield MJ, et al. Phylogenetic lineages in the Botryosphaeriaceae. Stud Mycol. 2006;55:235–253.

Ahmed N, Ahmed QA. Physiologic specialisation in Macrophomina phaseoli (Maubl.) Ashby., causing stem rot of jute, Corchorus species. Mycopathol Mycol Appl. 1969;39(2):129–138.

Sousa ES, Melo MP, Pires LL, et al. First report of Macrophomina phaseolina causing charcoal rot in lima bean (Phaseolus lunatus) in Brazil. Plant Dis. 2017;101(8):1551.

Chakraborty BN, Chakraborty U, Dey PL, et al. rDNA Sequence and phylogenetic analysis of Macrophomina phaseolina, root rot pathogen of Citrus reticulata (Blanco). Glob J Mol Sci. 2011; 6(2):26–34.

Zhang JQ, Zhu ZD, Duan CX, et al. First report of charcoal rot caused by Macrophomina phaseolina on mungbean in China. Plant Dis. 2011;95(7):872.

Leyva-Mir SG, Velázquez-Martinez GC, Tlapal-Bolaños B, et al. First report of charcoal rot of sugarcane caused by Macrophomina phaseolina in Mexico. Plant Dis. 2015;99(4):553.

Babu BK, Saxena AK, Srivastava A, et al. Identification and detection of Macrophomina phaseolina by using species-specific oligonucleotide primers and probe. Mycologia. 2007;99(6):797–803.

Sánchez S, Gambardella M, Henríquez JL, et al. First report of crown rot of strawberry caused by Macrophomina phaseolina in Chile. Plant Dis. 2013; 97(7):996.

Hajlaoui MR, Mnari-Hattab M, Sayeh M, et al. First report of Macrophomina phaseolina causing charcoal rot of strawberry in Tunisia. New Dis Rep. 2015;32:14.