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FUNGAL DISEASES

Colletotrichum and *Diaporthe* species associated with soybean stem diseases in Myanmar

Myo Zaw[1] · Seint San Aye[2] · Masaru Matsumoto[3]

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Abstract

In 2017, premature abscission of leaves and dry rot with discoloration on the epidermis of the stem were observed in the field of reproductive-stage soybean in southern Shan State and the Nay Pyi Taw region in Myanmar. On the basis of morphological characteristics, two fungal genera, *Colletotrichum* and *Diaporthe*, were identified. Multilocus phylogenetic analyses based on the ITS, *TUB* and *EF1-α* genes for *Diaporthe* and the ITS, *ACT*, *GAPDH*, and *CHS-1* genes for *Colletotrichum* were used to identify the species as *Colletotrichum plurivorum*, *C. truncatum*, *Diaporthe endophytica* and *D. melonis*. The pathogenicity of the collected isolates was confirmed by inoculation of soybean cultivar (Yezin-10), and all species except for *C. plurivorum* were virulent. The isolates of *C. plurivorum* were less aggressive than the other isolates. Koch's postulate was fulfilled by reisolation of the original inoculated fungal isolates from the symptomatic tissue. In two locations in Myanmar, *C. plurivorum*, *D. endophytica* and *D. melonis* occurred in southern Shan State, whereas only one *C. truncatum* was found in the Nay Pyi Taw region. This is the first report of *Colletotrichum* and *Diaporthe* species associated with soybean stem diseases in Myanmar.

Keywords *Colletotrichum plurivorum* · *C. truncatum* · *Diaporthe endophytica* · *D. melonis* · Fungal diseases

Introduction

Soybean (*Glycine max*) is a major crop in Myanmar, where it is cultivated on 0.15 million hectares and yield averaged 1.07 tons per ha in 2016 (CSO 2017); however, the yield is approximately one-third of the world average yield (USDA 2018) because of low agricultural inputs and problems with pests and diseases. The yield and quality of the product are critical to soybean farmers. Unfortunately, plant diseases are a significant constraint on soybean production and caused losses of approximately 59.9 million metric tons in the top eight producing countries in 2006 (Wrather et al. 2010). Commonly in Myanmar, soybean farmers do not adopt any control measures against plant diseases, so they face losses when weather conditions favour disease development.

Fungal pathogens were the causal agents of the majority of the 24 reported soybean diseases in the top 10 soybean-producing countries in 1994 (Wrather et al. 1997). Among these diseases, stem blight, pod rot and seed decay are caused by *Diaporthe* spp. (and their *Phomopsis* anamorphs) and result in yield and quality losses (Santos et al. 2011). Moreover, a *Diaporthe* species complex (including *D. endophytica*, *D. longicolla*, *D. phaseoli*, *D. phaseolorum* and *D. sojae*) is associated with soybean (Gomes et al. 2013; Udayanga et al. 2015; Zhang et al. 1998).

Soybean anthracnose is mainly caused by *Colletotrichum truncatum* (Schwein.) Andrus and W. D. Moore and is an economically important disease in soybean production. The estimated yield reductions caused by anthracnose in China and India in 2006 were 1.66 million tons and 0.18 million tons, respectively (Wrather et al. 2010). Other species such as *C. gloeosporioides* (Penz) Penz & Sacc. (teleomorph *Glomerella cingulata*), *C. coccodes* (Wallr.) Hughes, and *C. destructivum* O'Gara (teleomorph *G. glycine* F.Lehm. and F.A.Wolf) are also related to anthracnose diseases in soybean (Chen et al. 2006; Manandhar 1986; Riccioni et al.

✉ Masaru Matsumoto
mmatsu@agr.kyushu-u.ac.jp

[1] Graduate School of Integrated Sciences for Global Society, Kyushu University, Fukuoka, Japan

[2] Yezin Agricultural University, Nay Pyi Taw, Myanmar

[3] Institute of Tropical Agriculture, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0359, Japan

1998). However, some of the strains that were previously identified as *C. gloeosporioides* and *C. truncatum* could in fact be any of the newly described species in the *Colletotrichum orchidacearum* species complex (Damm et al. 2019). One species that belongs to this species complex, *C. plurivorum* (formerly *C. cliviae*), was recently reported in Brazil as a causal agent of soybean disease (Barbieri et al. 2017).

As described above, soybean pod and stem blight are caused by several *Diaporthe* spp., and soybean anthracnose is caused by the *Colletotrichum orchidacearum* species complex. Identification of causal pathogens is important for disease management. Traditional techniques of identification based on morphological characteristics present limitations because of phenotypic variation in the different geographical locations and environmental conditions in which *Colletotrichum* species occur (Bailey and Jeger 1992). Moreover, the identification of *Diaporthe* species is complicated due to inter- and intraspecific variability and a wide host range (Mostert et al. 2001; Rehner and Uecker 1994). Therefore, identification at the species level based on morphological characteristics alone is impossible for these species. Currently, species delimitation via molecular identification based on internal transcribed spacer (ITS) sequences of ribosomal DNA (rDNA) and other loci such as actin (*ACT*), beta-tubulin (*TUB*), chitin synthase (*CHS-1*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and translation elongation factor 1-alpha (*EF1-α*) has become the standard (Santos et al. 2011; Udayanga et al. 2015; Weir et al. 2012).

In the case of Myanmar, scientific information on soybean fungal diseases is scant, and it is very difficult to precisely diagnosis the species, which is essential for disease management. During a field survey in the rainy season of 2017, we found that soybean plants in farmers' fields were affected by fungal diseases causing premature leaf fall and unfilled pods. Therefore, the objective of this study was to characterize the causal fungal species community in soybeans from two major soybean production areas to develop disease control strategies in Myanmar.

Materials and methods

Sample collection and isolation

Samples were collected in September 2017 in a survey of two major crop production locations in Myanmar. The first location, in central Myanmar in the Nay Pyi Taw area (19.8°N, 96.20°E), has a tropical climate with intermittent rainfall, and the second location, Lawksawk (21.78°N, 96.92°E), is a mountainous area and has a mild climate with high humidity in the growing season. During field collections, premature leaf fall, dry rot, discoloration on the epidermis of the stem and pods with shrivelled seeds were observed (Fig. 1). These typical disease symptoms were widespread and easily observed throughout the fields of both locations during the field survey. Twenty diseased stem samples with black specks and blotching were collected from each location. The epidermis of the collected stem samples was cut into 2–3 mm pieces and surface-sterilized with 2% (v/v) NaOCl solution, then incubated on water agar at 25 ± 1 °C for 24 h. The emerging mycelial tips were transferred to potato dextrose agar (PDA) plates, and single-spore cultures and single mycelial tips from the cultures for non-spore-forming isolates were prepared to obtain pure cultures for further studies.

Morphological characterization

Agar discs (5 mm diameter) of each fungal isolate were placed on PDA and incubated at 25 ± 1 °C with four replicates. Colony diameter was measured at 24-h intervals for 7 days, and colony morphology was examined. The isolates

Fig. 1 Field symptoms of soybean stem diseases in Lawksawk (**a-1** = infected plants and **a-2** = magnified view of symptoms) and Nay Pyi Taw (**b-1** = infected plants and **b-2** = magnified view of symptoms)

were placed on sterilized soybean stems on water agar to observe the morphology of the fruiting bodies. Images were obtained using a light microscope mounted with an Olympus DP70 camera (Olympus, Tokyo, Japan). Only *Colletotrichum* and *Diaporthe* species were isolated from the diseased stem samples, and representative isolates were selected from each morphologically similar group for the experiment.

Pathogenicity on soybean

The pathogenicity of the collected isolates was examined using a stem cutting inoculation technique (Li et al. 2010) with slight modifications. Soybean (Yezin-10 cultivar) seeds were surface-sterilized with a 2% (v/v) NaOCl solution and grown in autoclaved potted soil in a phytotron (Biotron Application Center, Kyushu University) at 25 ± 2 °C. Two-week-old seedling stems were cut into equal lengths at 15 cm above the soil line but below the first trifoliate leaf. Then, small mycelial discs were collected from the margins of 10-day-old cultures on PDA plates using the large end of 200 µl micropipette tips and immediately placed onto fresh-cut stems. Inoculation with PDA-only discs served as the control. A completely randomized design (CRD) was used with five replications, and each replicate included three plants. The micropipette tips were removed 2 days after inoculation, and stem and diseased lesion lengths were measured at 10 days after inoculation. Lesion length was measured and calculated as a percentage of total stem length using the following formula: Lesion length (as % of total stem length) = (Lesion length/Stem length) × 100 (Li et al. 2010). Analysis of variance with CRD was performed, and the mean values from each treatment were compared on the basis of the least significant difference at $P \leq 0.05$ with Statistix version 8.0 (Analytical Software, Tallahassee, FL, USA). The inoculated fungal isolates were reisolated from the symptoms that developed after inoculation, and the morphological characteristics were checked to confirm their pathogenicity and whether Koch's postulates were fulfilled.

DNA extraction, PCR amplification and sequencing analysis

Genomic DNA from 15 randomly selected isolates was extracted using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). After the extraction of template DNA, the samples were stored at − 20 °C until use.

For the identification of *Diaporthe* spp., the ITS, beta-tubulin (*TUB*) and translation elongation factor 1-α (*EF1-α*) genes were amplified using the ITS1 and ITS4, Bt-2a and Bt-2b, and EF1-728F and EF1-986R primers, respectively. For *Colletotrichum* spp., ITS, actin (*ACT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and chitin synthase (*CHS-1*) were amplified by using primer pairs ITS1 and ITS4, ACT-512F and ACT-783R, and GDF and GDR, and CHS-79F and CHS-345R, respectively (Table 1).

PCR amplification was carried out in a 25 µl reaction volume containing 2.5 µl of 10 × reaction buffer, 2 µl of dNTPs, 1.0 µl of each primer, 0.25 µl of *Taq* polymerase (2.5 U/µl) (Toyobo, Osaka, Japan), 2 µl of template DNA and 16.25 µl of MilliQ water. PCR was performed in a thermal cycler (TProfessional Basic Gradient Thermocycler, Biometra, Göttingen, Germany). The PCR conditions for ITS were 95 °C for 2 min, 39 cycles 95 °C for 30 s, 55 °C for 50 s, and 72 °C for 1 min, and a final step at 72 °C for 5 min; the annealing temperature was different for other genes: 58 °C for *TUB* and *EF1-α* (Udayanga et al. 2014), 58 °C for *ACT* and *CHS-1*, and 60 °C for *GAPDH* (Weir et al. 2012).

The quality of the PCR products was checked by electrophoresis in 1.5% (w/v) agarose stained with ethidium bromide, and the products were purified with a QIAquick PCR kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Then the purified PCR products were sent to Fasmac Co. (Kanagawa, Japan) for sequencing.

Table 1 List of primers for phylogenetic analysis

Gene	Primer	Sequence (5´–3´)	References
ACT	ACT-512F ACT-783R	ATGTGCAAGGCCGGTTTCGC TACGAGTCCTTCTGGCCCAT	Carbone and Kohn (1999)
CHS-1	CHS-79F CHS-345R	TGGGGCAAGGATGCTTGGAAGAAG TGGAAGAACCATCTGTGAGAGTTG	Carbone and Kohn (1999)
EF1-α	EF1-728F EF1-986R	CATCGAGAAGTTCGAGAAGG TACTTGAAGGAACCCTTACC	Carbone and Kohn (1999)
GAPDH	GDF GDR	GCCGTCAACGACCCCTTCATTGA GGGTGGAGTCGTACTTGAGCATGT	Templeton et al. (1992)
ITS	ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	White et al. (1990)
TUB	Bt-2a Bt-2b	GGTAACCAAATCGGTGCTGCTTTC ACCCTCAGTGTAGTGACCCTTGGC	Glass and Donaldson (1995)

Phylogenetic analysis

The accession numbers of all sequences that were newly generated in this study are listed in Table 2. The sequences of the ITS, *TUB* and *EF1-α* genes of *Diaporthe* species were separately aligned with verified sequences published by Udayanga et al. (2015). The reference sequences of the ITS, *ACT*, *GAPDH* and *CHS-1* genes of *Colletotrichum* spp. were obtained from Damm et al. (2019) and Weir et al. (2012). The nucleotides of the DNA sequences were aligned via the ClustalW method (Thompson et al. 1994) using MEGA software version 7 (Kumar et al. 2016), and alignment gaps were treated as gap data. Multilocus phylogenetic analyses of the *Colletotrichum* (based on ITS, *ACT*, *GAPDH*, and *CHS-1* genes) and *Diaporthe* (based on ITS, *EF1-α* and *TUB* genes) genera were performed separately by the maximum likelihood (ML) method with a distance matrix based on Kimura's two-parameter correlation for multiple hits (Kimura 1980) using MEGA 7. The confidence estimate for tree topologies was determined by bootstrap analysis with 1000 replicates. Additionally, phylogenetic trees for the two genera with Bayesian probabilities were also constructed using the Markov chain Monte Carlo (MCMC) algorithm with MrBayes (version 3.1.2) (Ronquist et al. 2012). Nucleotide substitution models for each gene were selected by using PartitionFinder (version 1.1.1) (Lanfear et al. 2012). The analysis of MCMC chains was run for 1,000,000 generations, and trees were sampled every 1000th generation. The first 25% of the trees were discarded, and the posterior probabilities were calculated using the remaining trees. The resulting tree was viewed by using MEGA 7.

Results

Morphological characteristics of the collected fungal isolates

From the diseased soybean samples from the two locations in Myanmar, 12 representative fungal isolates were obtained from the Lawksawk and three from Nay Pyi Taw areas (Table 2). The observed morphological characteristics indicated that the isolates were species of *Colletotrichum* and *Diaporthe*. Species identification was completed based on the molecular phylogenetic analysis. The morphological characteristics of the four pathogenic fungal species identified in this experiment were as follows.

Diaporthe melonis: Colony colour of the isolates was whitish to grey, and no colour staining was observed on PDA. The growth rate of the isolates was 6.1 ± 0.2 mm/day ($n = 12$) in the dark at 25 °C. Pycnidia on sterilized soybean stems were globose and 189.9 ± 28.2 µm ($n = 50$) in diameter containing abundant α-conidia. The alpha conidia were hyaline, smooth, ovoid to ellipsoid, biguttulate, and 6.9 ± 0.5 µm × 2.4 ± 0.2 µm ($n = 50$) in size. Beta conidia were not observed (Fig. 2a-1, a-4). The morphology of *D. melonis* in this experiment was the same as the taxonomic description of Udayanga et al. (2015).

Diaporthe endophytica: The colony morphology of the fungus was similar to that of *D. melonis.* The growth rate was 5.6 ± 0.3 mm/day on PDA in the dark at 25 °C. There was no sporulation on PDA. However, pycnidia and alpha conidia were observed on lesions of the inoculated soybean plant stems. Pycnidia were round to ovoid with ostioles,

Table 2 GenBank accessions for fungal isolates from soybean in two locations of Myanmar

Species	Isolate	Location	GenBank Accession no					
			ITS	*EF1-α*	*TUB*	*ACT*	*GAPDH*	*CHS-1*
D. melonis	SoyYS1701	Lawksawk	LC360096	_	LC377201	_	_	_
D. melonis	SoyYS1703	Lawksawk	LC360097	LC377208	LC377202	_	_	_
D. melonis	SoyYS1704	Lawksawk	LC360098	LC377209	LC377203	_	_	_
D. melonis	SoyYS1706	Lawksawk	LC360099	LC377210	LC377204	_	_	_
D. melonis	SoyYS1711	Lawksawk	LC360100	LC377211	LC377205	_	_	_
D. melonis	SoyYS1712	Lawksawk	LC360101	LC377213	LC377206	_	_	_
D. melonis	SoyYS1713	Lawksawk	LC360102	LC377212	LC377207			
D. endophytica	SoyYS1732	Lawksawk	LC381426	LC381703	LC381705	_	_	_
D. endophytica	SoyYS1733	Lawksawk	LC381427	LC381704	LC381706	_	_	_
C. truncatum	SoyYZ1715	Nay Pyi Taw	LC360104	_	_	LC440990	LC441002	LC440996
C. truncatum	SoyYZ1716	Nay Pyi Taw	LC360105	_	_	LC440991	LC441003	LC440997
C. truncatum	SoyYZ1725	Nay Pyi Taw	LC360108	_	_	LC440992	LC441004	LC440998
C. plurivorum	SoyYS1707	Lawksawk	LC383779	_	_	LC440987	LC440999	LC440993
C. plurivorum	SoyYS1709	Lawksawk	LC383780	_	_	LC440988	LC441000	LC440994
C. plurivorum	SoyYS1726	Lawksawk	LC383781	_	_	LC440989	LC441001	LC440995

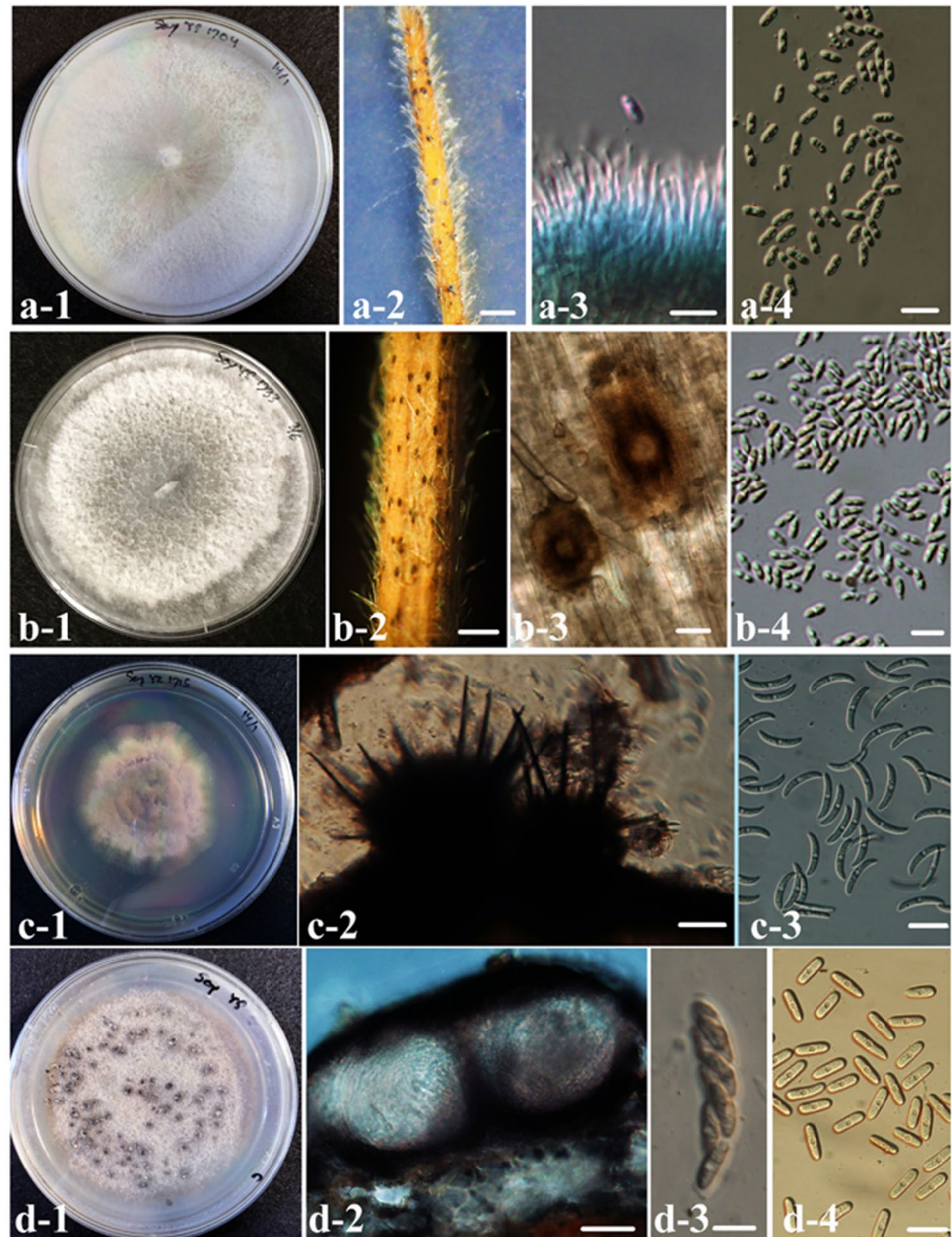


Fig. 2 Morphological characteristics of fungal pathogens isolated in this study. **a-1** to **a-4** *Diaporthe melonis*. **a-1** Colony on PDA after 10 days, **a-2** pycnidia on inoculated sterilized soybean stems on water agar, **a-3** conidiophores, **a-4** alpha conidia (bars: a-2 = 3 mm, a-3 and a-4 = 10 µm). **b-1** to **b-4** *Diaporthe endophytica*. **b-1** Colony on PDA after 10 days, **b-2** lesions after inoculation of soybean stem, **b-3** pycnidia on epidermis of inoculated soybean stem, **b-4** alpha conidia (bar: b-2 = 2 mm, b-3 = 50 µm, and b-4 = 10 µm). **c-1** to **c-3** *Colletotrichum truncatum*. **c-1** Colony on PDA after 7 days, **c-2** acervulus on sterilized soybean stem, **c-3** conidia (bar: c-2 = 50 µm and c-3 = 20 µm). **d-1** to **d-4** *Colletotrichum plurivorum*. **d-1** Colony on PDA after 10 days, **d-2** ascocarp on inoculated soybean stem, **d-3** ascus and ascospores, and **d-4** conidia (bar: d-2 = 50 µm, d-3 = 10 µm, d-4 = 20 µm)

135 ± 28.2 µm in diameter, and the morphology of the alpha conidia was similar to those of *D. melonis,* with a size of 6.8 ± 0.5 µm × 2.6 ± 0.3 µm (*n* = 50). Beta conidia were not observed (Fig. 2b-1, b-4). However, sporulation of *D. endophytica* was not reported on either media or sterilized host plant tissue by Gomes et al. (2013). In our experiment, the morphology of *D. endophytica* was almost the same as that of *D. melonis.*

Colletotrichum truncatum: The colony on PDA was grey to dark grey with white to grey aerial mycelia, and the PDA was stained black. The colony was thick, and the growth rate was 4.6 ± 0.7 mm/day in the dark at 25 °C. Acervuli formed on the inoculated stems, and black setae were dominant, with a width of 208.8 ± 62.1 µm. The conidia were falcate and tapered at each end, 26.2 ± 1.5 × 4.5 ± 0.4 µm (*n* = 50) (Fig. 2c-1, c-3). The conidial dimensions were similar to those of *C. truncatum* isolate CBS112998 (Damm et al. 2009).

Colletotrichum plurivorum: The colony was whitish grey initially and later became darker grey; the aerial mycelia were sparse, and the ascomata (sexual morph) formed clusters at 2 weeks after incubation on PDA at 25 °C. Conidia (asexual morph) were rarely found on PDA and were mainly observed on inoculated soybean stems; they were smooth-walled, one-celled, hyaline, and cylindrical to oblong with rounded ends,

15.5 ± 0.9 µm × 5.1 ± 0.3 µm (n = 50). The ascostroma were dark and globose to subglobose, and they contained asci with thin walls; in addition, they were unitunicate and clavated, with dimensions of 45.2 ± 9.8 µm × 8.9 ± 2.1 µm (n = 50). Eight ascospores were arranged in each ascus and slightly curved, and they were narrow at each rounded end, with dimensions of 16.5 ± 3.2 µm × 5.5 ± 0.9 µm (n = 50) (Fig. 2d-1, d-4). The morphological characteristics of the isolates (SoyYS1707, SoyYS1709, SoyYS1726, SoyYS1730, and SoyYS1731) matched those of *C. plurivorum* (CBS 125,474) (Damm et al. 2019).

Pathogenicity test

Twelve representative isolates consisting of four isolates of *D. melonis*, two isolates of *D. endophytica*, three isolates of *C. truncatum* and three isolates of *C. plurivorum* were used for the pathogenicity tests. Pathogenicity was determined by measuring the percentage lesion length on the inoculated soybean stems. The inoculated symptoms of the representative fungal isolates of *Colletotrichum* spp. and *Diaporthe* spp. are shown in Fig. 3. Lesion lengths were 19.05–53.62% of the respective stem length, and there were significant differences in percentage lesion length among the species (Table 3). *D. melonis* had the longest lesions at 45.16%, significantly larger than *C. plurivorum*, *C. truncatum* and *D. endophytica*. Lesion lengths of *C. truncatum* and *D. endophytica* did not differ significantly. However, *C. plurivorum* caused the shortest lesions (19.48% of the stem length).

Table 3 Percentage lesion lengths based on the stem length of soybean (cultivar Yezin-10) at 10 days after inoculation with different fungal species

No.	Species	No. of isolates	Lesion length %[a]	
			Mean	Standard error of mean
1	*D. melonis*	4	45.16 a	1.77
2	*D. endophytica*	2	38.40 b	2.17
3	*C. truncatum*	3	34.12 b	1.20
4	*C. plurivorum*	3	19.48 c	1.16
Mean			34.85	
CV			18.00	

[a]Lesion length % = (Lesion length/Stem length) × 100; data are mean values for isolates from each species in the greenhouse and include four replications for each isolate. Means followed by the same letter are not significantly different according to the least significant difference (LSD) test at $P \leq 0.05$

Phylogenetic analysis

Phylogenetic analysis of *Diaporthe* spp. based on ITS, *EF1-α* and *TUB* sequences of 64 strains, including closely related reference *Diaporthe* spp. strains. Gene boundaries were as follows: ITS, 1–492; *EF1-α*, 493–871; *TUB*, 872–1318. *D. vaccinia* (DP5032) was used as an outgroup. For the Bayesian analysis, the substitution models used were as follows: GTR + I + G for the ITS and HKY + G for the *EF1-α* and *TUB* genes. The topologies of the phylogenetic trees obtained via the maximum likelihood method using MEGA 7 software and Bayesian analysis were similar, and parsimony bootstrap (≥ 70%) and Bayesian posterior probability

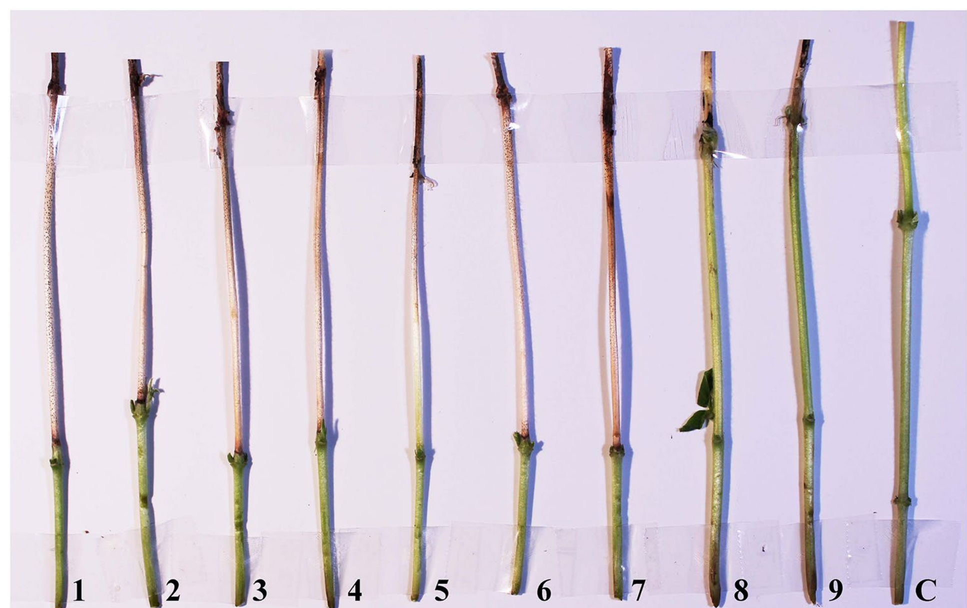
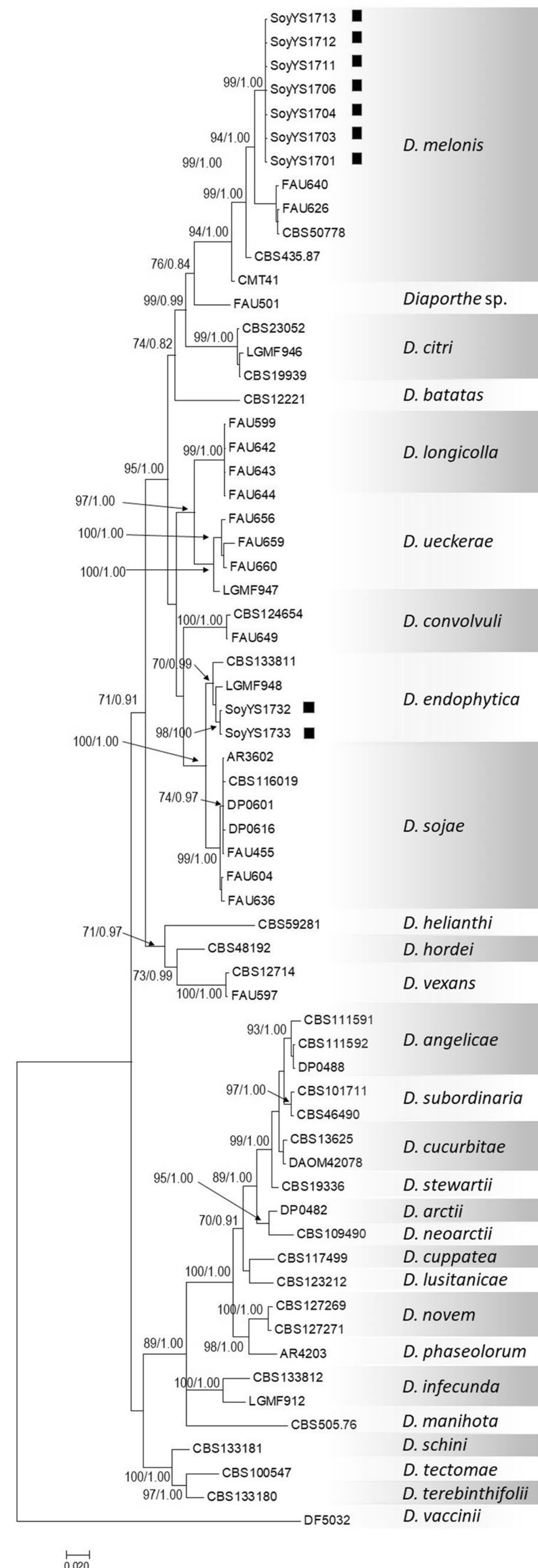


Fig. 3 Symptoms on soybean cv. Yezin 10 after stem cutting inoculation with representative fungal isolates (1–3 = *D. melonis,* 4 and 5 = *D. endophytica*, 6 and 7 = *C. truncatum*, 8 and 9 = *C. plurivorum* and C = control check)

Fig. 4 Phylogenetic trees inferred from the ITS, *EF1-α*, and *TUB* ▸ genes of *Diaporthe* species using the maximum likelihood method. Bootstrap support values (≥70%)/Bayesian posterior probabilities (≥0.90) are displayed at each branch, and black squares (filled rectangle) indicate isolates from the present study



(≥0.90) values are shown on the branches in Fig. 4. The isolates obtained in the present study (SoyYS1701, SoyYS1703, SoyYS1704, SoyYS1706, SoyYS1711, SoyYS1712 and SoyYS1713) formed a single clade and were very closely related to *D. melonis* (CBS435.87). Two isolates (SoyYS1732 and SoyYS1733) fell into a single clade with the ex-type isolates of *D. endophytica* (LGMF948 and CBS133811).

For phylogenetic analysis of *Colletotrichum* spp., the ITS, *GAPDH*, *CHS-1* and *ACT* sequences of the three representative isolates (SoyYZ1715, SoyYZ1716 and SoyYZ1725) from Nay Pyi Taw and three representative isolates (SoyYS1707, SoyYS1709, and SoyYS1726) from Lawksawk were aligned with closely related verified sequences for phylogenetic analysis. The alignment contained 37 taxa, and *Monilochaetes infuscans* (CBS 869.96) served as the outgroup. The gene boundaries, including gaps, were as follows: ITS, 1–519; *GAPDH*, 520–826; *CHS-1*, 827–1116; and *ACT*, 1117–1364. The substitution models selected for Bayesian analysis were GTR + G for ITS, HKY + I for *GAPDH* and HKY + G for the *CHS-1* and *ACT* genes. The results showed that the SoyYZ1715 and SoyYZ1716 isolates were identical to the CBS195.32 and CMES 1036 sequences, and the three isolates obtained in this study were also closely related to the *C. truncatum* type strain (CBS151.35). The other three isolates (Soy YS1707, SoyYS1709 and SoyYS1726) were in the same clade as *C. plurivorum* (CBS125474 and LFN0008) (Fig. 5).

Discussion

The symptoms of soybean stem diseases caused by the *Diaporthe* species complex are described as black blotching with or without pycnidia on the stem (Kmetz et al. 1978). Moreover, groups of *Colletotrichum* species are also associated with the stem and cause anthracnose on soybeans (Yang et al. 2014), and the morphological characteristics among the *Colletotrichum* species were also similar. Consequently, we differentiated the collected isolates only to the genus level as *Colletotrichum* spp. and *Diaporthe* spp. based on morphology.

The pathogenicity test using the stem cutting inoculation method reproduced necrotic lesions with fruiting bodies on the cut stems of soybean. This method is mainly used for pathogenicity testing and varietal resistance screening experiments for *Phomopsis* diseases, *Sclerotinia* stem rot,

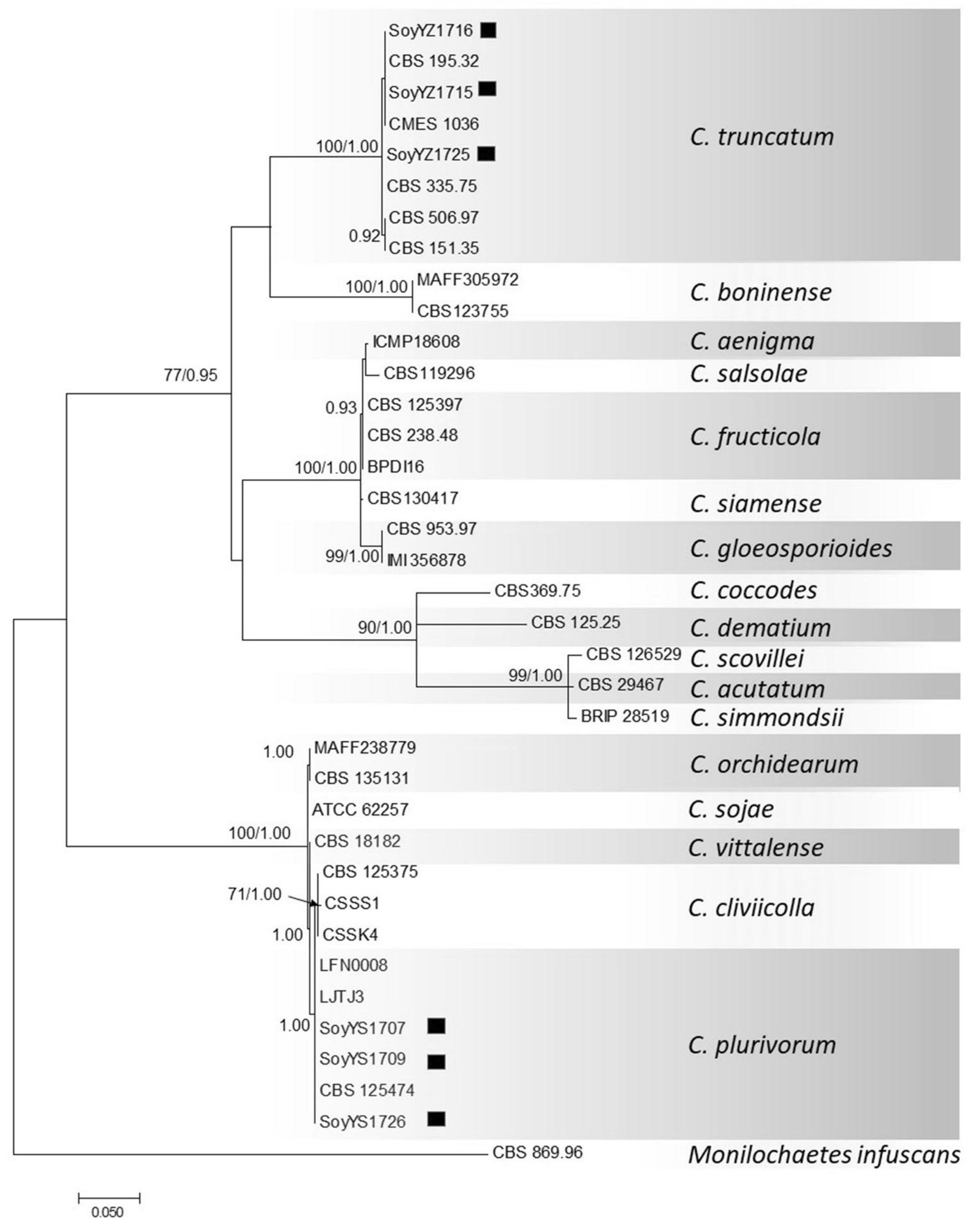


Fig. 5 Phylogenetic trees inferred from the ITS, *ACT*, *GAPDH* and *CHS-1* genes of *Colletotrichum* species using the maximum likelihood method. Bootstrap support values (≥ 70%)/Bayesian posterior probabilities (≥ 0.90) are displayed at each branch, including detailed descriptions of the symbol as per Fig. 4

and charcoal rot disease in soybean (Kull et al. 2007; Li et al. 2001; Shan et al. 2013; Twizeyimana et al. 2012). However, the inoculation test with the isolates of *Colletotrichum* spp. also reproduced necrotic lesions on cut stems, and we assumed that these species were fungal pathogens of soybean. Therefore, isolates of these two genera were identified separately by molecular phylogenetic analysis.

Currently, molecular tools such as the sequencing of different genes or intergenic regions have been shown to be applicable and more accurate for the identification of fungal species (Ash et al. 2010; Gomes et al. 2013; Yang et al. 2014). In strategies involving the use of genetic barcodes of different genes for species delimitation, the ITS region of r-DNA has been widely applied (Gardes and Bruns 1993; Schoch et al. 2012; Shishido et al. 2006). However, fungal species identification on the basis of ITS alone has limitations (Nilsson et al. 2008), and the ITS, *HIS* or *TUB* genes should be analysed for the species description of *Diaporthe* spp. (Gomes et al. 2013). Similarly, many *Colletotrichum* species cannot be identified using only the ITS (Weir et al. 2012). Therefore, other fungal barcoding genes were aligned with other sequences of verified reference isolates, and a phylogenetic study was carried out. According to the multilocus phylogenetic analysis, the isolates of *Diaporthe* from the Lawksawk area were similar to *D. endophytica* and *D. melonis*. The *Colletotrichum* isolates from Lawksawk and Nay Pyi Taw were identified as *C. plurivorum* and *C. truncatum*, respectively.

Known hosts of *Diaporthe melonis* include *Annona squamosa*, *Carapa guianensis*, *Cucumis melo* and *Glycine max* (Dissanayake et al. 2017), and this species is phylogenetically closely related to *D. longicolla* and *D. sojae* (Udayanga et al. 2015). *D. endophytica* has been isolated from seeds of *Glycine max*, as an endophyte on the leaves of *Schinus terebinthifolius* and on the petioles of *Maytenus ilicifolia* in Brazil (Gomes et al. 2013). In our pathogenicity tests, *D. endophytica* and *D. melonis* were pathogenic on soybean plants. On the other hand, *C. truncatum* is a common cause of anthracnose disease in major soybean-growing countries (Wrather et al. 1997, 2010), including Myanmar (CAB-International 2001). However, *C. plurivorum* has been reported on soybean in Brazil and Japan (Barbieri et al. 2017; Damm et al. 2019) and belongs to the *C. orchidearum* species complex (Damm et al. 2019). This species was recently reported as a pathogen that is associated with anthracnose disease on chili in China and the Andaman and Nicobar Islands (Liu et al. 2016; Sakthivel et al. 2018), on papaya in Taiwan (Sun et al. 2019), and on *Pyrus* spp. in China (Fu et al. 2018). In the present study, we revealed that *C. plurivorum* was weakly virulent, whereas *C. truncatum* was strongly virulent on soybean.

We identified isolates of *C. plurivorum*, *D. endophytica* and *D. melonis* in Lawksawk Township, southern Shan State, but only one species, *C. truncatum*, was isolated from the diseased samples from the Nay Pyi Taw area. More diverse fungal plant pathogen species were found in southern Shan State, which seems to be facilitated by mild temperatures (not very hot or cold) and humid weather. TeKrony et al. (1983) reported that disease incidence caused by *Diaporthe* species was significantly related to relative humidity and air temperature and that seed infection by these fungal pathogens is more dependent on moisture. The maximum disease incidence of soybean anthracnose caused by *C. truncatum* occurred at 28.4 °C and 76% relative humidity in September 1995 in India (Singh et al. 2001).

In Myanmar, 15 other species of *Diaporthe* and 16 species of *Colletotrichum* have been reported on different host plants (Thaung 2008). Additionally, infection of soybean by *C. plurivorum*, *C. truncatum*, *D. endophytica* and *D. melonis* in Myanmar was never checked before this study. During the field survey of two major soybean-growing areas, fungal diseases were prominent and severely affected the yield and income of the farmers. To our knowledge, this is the first report of the characterization of fungal pathogens on soybean in Myanmar, and effective control measures are urgently needed by farmers. We hope that the results of this investigation will support further experiments on the development of disease control and management strategies for soybean production.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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