INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
21–0135/2021/26–5–607–616
DOI: 10.17957/IJAB/15.1873
http://www.fspublishers.org

Full Length Article

Identification and Characterization of Pathogen Responsible for Causing Southern Corn Leaf Blight (SCLB) Disease in Malaysia

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Received 02 February 2021; Accepted 14 May 2021; Published 15 November 2021

Abstract

Corn is considered an important cereal crop world over. This work aimed to characterize the causative agent of southern corn leaf blight disease. Diseased samples with fusiform, elliptical and elongated lesions on the leaves were obtained from affected farms of four different areas within Malaysia. The morphological characters of the 10 isolates were observed. The conidia were curved and elongated. The results of morphological characteristics showed that potato sucrose agar was suitable for the rapid growth of pathogen with a mean of 10.19 mm day^{-1}, followed by corn meal agar (8.56 mm day^{-1}) and potato dextrose agar (5.46 mm day^{-1}). The temperature of 30°C was found the most suitable for pathogen growth with a mean of 7.30 mm day^{-1}. These isolates were classified into 4 groups in terms of colony color: dark gray, light gray, gray to green and gray. The conidial length ranged from 44.12 μm to 81.61 μm for isolate CH006 and CH004, respectively. Likewise, the number of septa ranged from 4–6 to 8–10 for isolates CH006 and CH004, respectively. The pathogenicity test on corn variety Thai Super Sweet (TSS) showed that the isolates CH001 and CH009 were the most aggressive while the isolate CH010 was the least aggressive. Results from molecular and morphology studies confirmed that all the 10 isolates were identified as *C. heterostrophus*. We suggest that as a result of the race diversity of *C. heterostrophus*, further investigations should be carried out on virulence determination and race detection of this pathogen. © 2021 Friends Science Publishers

Keywords: Aggressiveness; *Cochliobolus heterostrophus*; Corn; Identification; Southern corn leaf blight; Virulence

Introduction

Corn (*Zea mays* L.) belongs to family Poaceae and it is presently one of the most widely grown cereal crops worldwide (Kang *et al.* 2018; Sun *et al.* 2020). Corn is produced on a small scale in Malaysia due to several diseases that are affecting this crop and the planting of susceptible cultivars (Bashir *et al.* 2017b). *Bipolaris* is the anamorph of the ascomycetous genus *Cochliobolus*, the genus has over 100 species and it serves as an important genus of different pathogens of plants (Bengyella *et al.* 2018). Southern corn leaf blight (SCLB) incited by *Cochliobolus heterostrophus*is considered as an important disease of corn world over (Manamgoda *et al.* 2014; Bengyella *et al.* 2018). In Malaysia southern corn leaf blight, rust and leaf spots remain the main foliar diseases of corn in relatively most areas where corn is grown. *C. heterostrophus* (teleomorph) (Nisikado 1929) or *Bipolaris maydis* (anamorph) (Shoemaker 2011) is a necrotrophic pathogen and the causative agent of SCLB disease worldwide. This disease is usually found in hot period and humid corn production areas (Balint-Kurti *et al.* 2007). The disease cycle of this pathogen is polycyclic and can be sexual ascospores or asexual spores to infect the seedlings of the corn. The asexual cycle is of essential concern and known to occur in nature. Upon warm and favorable moist conditions, the conidia are discharged from the infected corn lesions and conveyed to the adjacent plants by means of rain splashing or wind (Soumya and Ramachandr 2019). When the conidia reach the leaf sheath of a healthy seedling, *C. heterostrophus* will grow on the leaf tissue by the method of polar germ tubes. The germ tubes either penetrate through the leaf or enter through a characteristic opening, for example, the hydathode or stomata.

There are four physiological races of *C.*

To cite this paper: Kutawa AB, K Ahmad, A Ali, SAM Zobir, MAA Wahab (2021). Identification and characterization of pathogen responsible for causing southern corn leaf blight (SCLB) disease in Malaysia. *Intl J Agric Biol* 26:607–616

heterostrophus that cause SCLB disease namely race O, S (Sun *et al*. 2020), C and T. Race T was found to be pervasive in the US Corn Belt in the year 1970 (Turgeon and Baker 2007). This race was exceedingly pathogenic on the cytoplasm of Texas male-sterile (cms-T), bringing about serious epidemic from 1970–1971. The pestilence of SCLB disease in the U.S brought about a huge loss in yield. A similar severe disease epidemic was reported in Hubei Province, China in 1968 and it brought about more than 400 million kg of loss in yield (Ye *et al*. 2012). Likewise, there were reports of SCLB in countries like Denmark, Nepal, Bahamas, Australia, Jamaica, Nicaragua, New Zealand, Egypt, Nigeria, Bolivia, Malawi, Brunei Darussalam, Brazil, Bhutan, Gambia, India, Malaysia and Ghana (Balint-Kurti *et al*. 2007). Manamgoda *et al*. (2014) reported that SCLB represents 20–30% or more significant yield losses to the corn.

For detection and identification of SCLB pathogen, molecular methods based on nuclear rDNA sequence of internal transcribed spacer (ITS) regions and *β*-tubulin gene, have supplemented the traditional method of classification and help for fast and precise identification of species from different hosts (Begoude *et al*. 2010; Manamgoda *et al*. 2014; Marin-Felix *et al*. 2017).

For many years SCLB disease remained the most serious disease affecting different corn farmland in Malaysia (Bashir *et al.* 2017b). The disease caused yield loss of about 20–30% if appropriate control methods were not used. So far, not many investigations have been carried out to study SCLB disease in Malaysia; therefore, it might be a quite challenging task to know the background as well as the status of SCLB disease in Malaysia because of the inadequacy of published works. To our knowledge, the molecular identification and characterization of SCLB pathogen have not been studied in Malaysia. There is a need to investigate more about SCLB pathogen with regards to pathogenic variability of the isolates, morphological and molecular characteristics for effective and accurate identification and understanding of the nature of the pathogen. Therefore, this work was carried out to investigate the cultural and morphological features of the pathogen as well as the molecular characterization using ITS and *β*-tubulin genes.

Materials and Methods

Sampling, isolation and identification of the pathogen

Four different areas were selected for collection of samples and a total of 15 infected leaf samples were obtained from each farm in four different states: Selangor (Batu Arang), Perak (Titi Gantong and Sungai Siput), Pahang (Lembah Bertam) and Johor (Kluang). The samples were labelled, transferred into the cold box and brought to the Mycology laboratory (Faculty of Agriculture, Universiti Putra Malaysia) for further examinations.

The isolation of the pathogen from infected leaves samples was conducted according to the method described by (Bashir *et al.* 2017a). A small portion of the diseased tissue with an adjacent healthy tissue of about 0.5 cm x 0.5 cm in diameter was cut up using a knife. The excised cut pieces were then surfaced sterilized in 10% of alcohol for 4–5 min in order to reduce the contaminants on the leaf. The leaf parts were transferred into sterilized distilled water and later onto a sterilized filter paper for moisture absorbance. Finally, the parts were plated on PDA media and the Petri-plates were then sealed using parafilm, incubation was followed at 26℃ for 10 days. A small portion of the mycelium from the matured colony was transferred to a fresh media (PDA) for obtaining a pure culture of the fungus.

Morphological and cultural characterization

Seven-day old culture in the Petri-dishes were aseptically opened under a laminar flow, a sterilized slide was carefully placed on the colony surfaces, the plates were resealed and further incubated to induce spores. The ten isolates were examined for cultural and morphological studies when the culture was 10–14 days old. The texture and colony color were observed and recorded. Conidial length and width were measured, while the number of septa and color of 50 spores per each isolate were studied. The conidial length and width were measured using eyepiece micrometer and compound light microscope (Bashir *et al.* 2017a; Hossain *et al*. 2021; Kutawa *et al.* 2021; Rashed *et al.* 2021).

Effect of media on pathogen colony growth

Cultural conditions of three different media were studied on *Cochliobolus heterostrophus* colony growth. The three different types of media including corn meal agar (CMA) a conventional medium prepared by using fresh corn leaf and synthetic agar, potato sucrose agar (PSA) a conventional medium prepared by using fresh potato, synthetic sucrose and agar, as well as potato dextrose agar (PDA) a commercial medium purchased from Sigma Aldrich company (U.S.A) were used to study the growth rate of all the isolates incubated at the same temperature (25 ± 1℃) for a period of two weeks. A plug of (0.5 cm) in diameter of mycelia from each of the isolates was cut up from an active growing part of the culture (4–5 days old). These plugs were sub-cultured and placed at the center of the media mentioned above to study the texture, appearance and growth rate of the mycelium. Colony growth was measured daily in two different perpendicular directions, D1 & D2 when the plates were completely sealed, until the time when the mycelium had fully grown and covered the Petri plates, a total of three replicates were used for each medium. Each of the isolates was having three replicates and it was incubated at 26 ± 1℃. Completely randomized design (CRD) design was used and the data obtained were analyzed to determine the disparity of the isolates statistically.

The growth rate data (mm day^{-1}) for all the media were subjected to analysis of variances (ANOVA). Means of the treatments were separated based on Duncan multiple range test (DMRT) at ($P \leq 0.05$) using SAS software, version 9.4.

Effect of temperature on pathogen colony growth

For the effect of different temperatures on pathogen growth was studied on PSA medium. A total of four temperatures 35℃, 30℃, 25℃ and 20℃ were used in this study. All isolates were in three replicates and incubated at different temperatures stated above. The growth rate of each isolate was determined on the 3rd, 5th and 7th day of incubation. The data obtained were analyzed to determine the difference in the treatments.

Pathogenicity test

In this study, susceptible seedlings of corn variety called Thai Super Sweet (TSS) was used, the corn plants were grown in pots (25 cm in diameter) that contain sterilized soil. About 55 plastic pots were arranged in five replicates for this study, and a total of 50 plastic pots which contain corn plants were inoculated with the fungus when the seedlings were at 3–5 leaf stages using 10^5 spores/mL of pathogen conidial suspension, five corn seedlings were treated with only distilled water which served as the control. These pots were kept in a glasshouse for the purpose of maintaining the 25–30 ± 2ºC incubation temperature. The corn seedlings were inspected daily for initial symptom development. The pathogen was then re-isolated from symptomatic leaf portions on PSA media and the pathogen was subjected to a pathogenic variability test to fulfill Koch's postulates. Corn plants were monitored for the presence of symptoms on weekly basis, for a period of four weeks after inoculation (WAI). The indices of disease studied were disease severity index (DSI) and disease incidence (DI) using a rating scale by Bashir *et al.* (2017b). The pathogenic differences of the ten isolates were classified using five different virulent scales, highly virulent (> 50%), virulent (31–50%), moderate (21–30%), mild (11–20%), weak (DSI=1–10%) (Bashir *et al.* 2017b).

Molecular characterization

Identification of *C. heterostrophus* was done using the molecular method, the DNA extraction of *C. heterostrophus* was performed using the method described in DNEasy plant mini kit (QIAGEN Biotechnology Malaysia Sdn. Bhd). The polymerase chain reaction was conducted to amplify the regions of DNA based onITS and β-tubulin genes. ITS amplification by ITS1 as forward (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 as reverse (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.* 1990), while β-tubulin gene amplification by TUBUF2 as forward (5'-CGGTAACAACTGGGCCAAGG-3') and TUBUR1 as reverse (5'-CCTGGTACTGCTGGTACTCAG-3') (Kroon *et al.* 2004). Fragments of DNA were amplified by using an automated thermocycling machine (VITAR SEGATEC, Bio-Rad, USA). The total volume used for amplification of DNA was 30 μL reaction which comprised of 15 μL of mastermix (Taq DNA polymerase-BIOMAX Company), 10 μL of nuclease-free water, 2 μL of DNA template and 1.5 μL of each primer (ITS1 and ITS4). Thermo cycling procedure includes initial denaturation (95°C) for 4 min; this was followed by 30 different cycles with denaturation (95°C for 30 s), annealing 956.6°C), extension (72°C 1 min) and final extension (72°C 5 min.). The gel was prepared by dissolving agarose (powder, 1%) in TAE buffer which contained 20 m*M* acetic acid,1 m*M* EDTA and 40 m*M* Tris, it was ran at (70 volt) for 60 min under room temperature (15–25ºC). Flourosafe stain was used to stain the gel and molecular marker (DNA Ladder Mix, 1 kb) was used to determine the band size. The products of PCR were photographed using gel documentation system and viewed using UV light. Lastly, after PCR the products were sent to MyTACG company (Bioscience Enterprise) for sequencing. The sequences were aligned using BioEdit software (version 7.2). Both ITS and sequences of *β*-tubulin were compared with the deposited sequences in the GenBank (http://www.ncbi.nlm.nih.gov) based on BLASTn search (Altschul *et al.* 1997). Accession numbers of the ten isolates for ITS and *β*-tubulin sequences were generated after sending the sequences (consensus) to the GenBank. The phylogenetic tree was inferred using neighbor-joining tree analysis by using MEGA6 software (version 4.0).

Results

Morphological and cultural characterization

Based on the morphological study, the results showed that most of the conidia were elongated and curved in shapes. In *C. heterostrophus* the conidia, other than being multi-celled they are also pigmented, usually in brown or black shades, and these characters are unique and only found in *C. heterostrophus* spore (Fig. 1A–B). The mycelial color was brown and found to grow faster when compared to some species of fungi as presented in Fig. 2. The cultural characteristics showed that differences existed among the isolates in terms of colony growth and color. Based on the color of the colony, the fungal isolates were classified into four groups: light grey (CH002, CH003, CH008, CH009, and CH010), dark gray (CH004), grey to green (CH001 and CH005) and gray (CH006 and CH007). In terms of growth, the isolates were classified into 3 categories namely, moderate growth, poor growth and professed growth. Results in term of length of the conidial were 44.12 μm and 81.61 μm for the isolates CH006 and CH004, respectively. While the width of the conidia was 11.34 μm and 17.43 μm

Table 1: Growth and conidial measurements of *Cochliobolus heterostrophus* for all isolates obtained from infected corn

Isolate Codes	Location	Type of pathogen growth	Conidia measurement		No. of septa
			Length (μm)	Width (μm)	
CH001	Batu Arang (Selangor)	Poor growth	59.91cd ± 0.58	13.01f ± 0.53	5-7
CH002	Batu Arang (Selangor)	Profused growth	64.43cbd ± 0.63	13.21e ± 0.57	4-7
CH003	Batu Arang (Selangor)	Profused growth	66.37b ± 0.67	14.67d ± 0.61	5-7
CH004	Batu Arang (Selangor)	Profused growth	81.61a ± 0.82	17.43a ± 0.65	8-10
CH005	Batu Arang (Selangor)	Poor growth	58.45ed ± 0.56	12.65f ± 0.45	5-8
CH006	Daerah Kluang (Johor)	Profused growth	44.12cb ± 0.47	13.25h ± 0.58	4-6
CH007	Daerah Kluang (Johor)	Profused growth	48.32ed ± 0.49	12.68g ± 0.46	6-8
CH008	Titi Gantong (Perak)	Poor growth	72.64a ± 0.76	16.3b4 ± 0.63	5-8
CH009	Sungai Siput (Perak)	Moderate growth	68.13e ± 0.63	11.34c ± 0.33	6-8
CH010	LembahBertam (Pahang)	Moderate growth	80.89a ± 0.81	17.34a ± 0.64	7-9

Values (mean ± standard deviation) within rows followed by the same letter are not significantly different at $P \leq 0.05$ by Duncan multiple range test. μm= micrometer

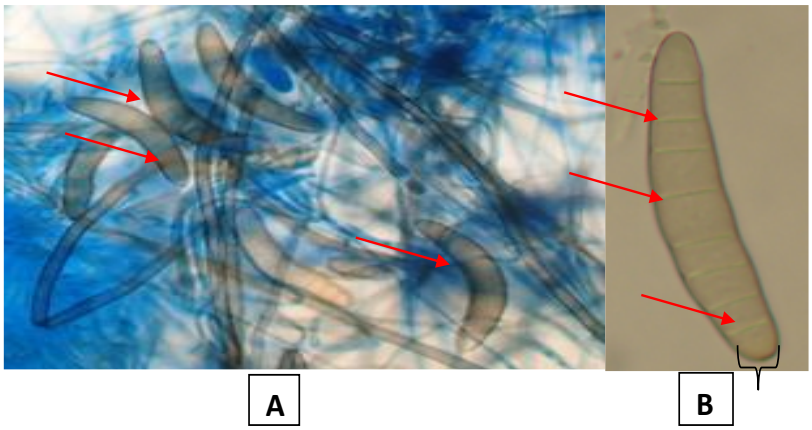

Fig. 1: (**A**) The elongated and curved shaped *C. heterostrophus* conidia and (**B**) The elongated conidia with a conspicuous simple septae arranged in a linear order viewed under light microscope at 40x magnification

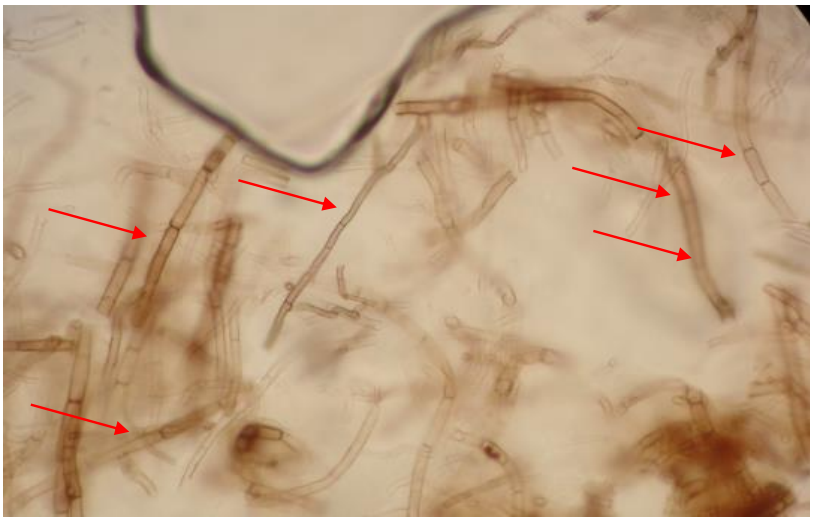

Fig. 2: A brownish color mycelia of *C. heterostrophus* grown on PDA after incubation for 14 days viewed under light microscope at 40x magnification

for isolates CH009 and CH004, respectively (Table 1). Similarly, the septa number of the isolates ranged from 4–6 to 8–10 for isolate CH006 and CH004 respectively. Based on the morphological characteristics, the ten isolates were identified as *C. heterostrophus.*

Effect of media on pathogen colony growth

The colony growth rates of 10 fungal isolates on PDA, CMA and PSA showed a significant difference in growth, with PSA showing the highest growth rate of 10.19 mm

Table 2: Effect of media on *C. heterostrophus* colony growth rate at incubation period of 14 days

Isolate	PSA (mm day^{-1})	PDA (mm day^{-1})	CMA (mm day^{-1})
CH001	9.60a	4.10d	7.70a
CH002	11.00a	8.60ab	7.70a
CH003	9.60a	5.90a	7.30a
CH004	10.70a	4.90bcd	7.70a
CH005	10.70a	4.50cd	7.70a
CH006	7.70a	6.40a	11.00a
CH007	11.00a	4.90bcd	9.60a
CH008	11.00a	3.90d	9.60a
CH009	11.00a	5.90ab	9.60a
CH010	9.60a	5.50abc	7.70a
Mean	10.19a	5.46c	8.56b
Number of days to cover plates	6-10	9-14	7-10

Means within rows followed by the same letter are not significantly different at $P \leq 0.05$ by Duncan multiple range test. mm day^{-1} = millimeter per day

Table 3: Effect of temperature on the growth of *C. heterostrophus* after incubation for a period of one week

Isolate	20°C mm day^{-1})	25°C (mm day^{-1})	30°C (mm day^{-1})	35°C (mm day^{-1})
CH001	3.30ed	5.70d	7.50a	1.40a
CH002	4.20bcd	6.25c	7.70a	1.42a
CH003	4.00ecd	6.80b	7.50a	1.47a
CH004	5.70a	6.70b	7.50a	1.80a
CH005	3.10e	7.00b	7.50a	2.00a
CH006	6.10a	7.70a	4.60b	3.167a
CH007	5.10ba	7.70a	7.70a	1.65a
CH008	4.65bc	6.30c	7.70a	1.48a
CH009	3.00e	3.68e	5.90ab	1.18a
CH010	3.10e	7.10b	7.70a	1.60a
Mean	4.23c	6.49b	7.30a	1.72d

Means within rows followed by the same letter are not significantly different at $P \leq 0.05$ by Duncan multiple range test. mm day^{-1} = millimeter per day

Table 4: Disease severity index (%) of *C. heterostrophus* isolates obtained from infected corn plants tested on TSS corn seedlings

Isolate	1WAI (%)	2WAI (%)	3WAI (%)	4WAI (%)	AUDPC (units^2)
CH001	36.00	48.00	76.00	80.00	182.00
CH002	32.00	48.00	68.00	72.00	168.00
CH003	40.00	44.00	76.00	76.00	178.00
CH004	34.00	36.00	64.00	72.00	153.00
CH005	28.00	52.00	56.00	60.00	152.00
CH006	26.00	48.00	48.00	64.00	141.00
CH007	28.00	36.00	36.00	52.00	112.00
CH008	40.00	52.00	52.00	68.00	158.00
CH009	36.00	52.00	60.00	80.00	170.00
CH010	18.00	22.00	24.00	28.00	69.00
Control	-	-	-	-	0.00

WAI= week after inoculation. AUDPC = area under disease progressive curve

day^{-1} and the mycelia covered the Petri-dish between 6–10 days. It was then followed by CMA with 8.56 mm day^{-1} and the mycelia covered the plate in 7–10 days, while PDA media having 5.46 mm day^{-1} and the mycelia covered the Petri-dish between 9–14 days, respectively (Table 2 and Fig. 3).

Effect of temperature on pathogen colony growth

Temperature 30°C was the most suitable among all, for growing the pathogen by having a mean of 7.30 mm day^{-1}, followed by 25°C with 6.49 mm day^{-1}. While at 20°C, the mean of the growth was 4.23 mm day^{-1}. And 35°C was found to be the least temperature for growing southern corn leaf blight pathogen with a growth rate mean of 1.72 mm day^{-1} (Table 3). In term of growth on the individual isolates, isolates CH007 was found to grow faster than the other isolates with 5.10, 7.70, 7.70 and 1.65-mm day^{-1} for temperature 35, 30, 25 and 20°C, respectively. Isolate CH009 was found to be the least with 3.00, 3.68, 5.90 and 1.18 mm day^{-1} for temperature 35, 30, 25 and 20°C respectively (Table 3).

Pathogenicity test

The symptom of SCLB disease first appeared as brown-red spots on the leaf surface. The lesions developed and coalesced to turn into zonate, of 2–4 cm long, elliptic in the beginning and thereafter prolonged longitudinally to become rectangular when spots are confined by veins. The symptoms progressed to form single, fusiform, elongated, elliptical and long lesions or blighted zones (Fig. 4A–B).

Fig. 3: Colonies of isolate CH010 of *C. heterostrophus* grown on PDA, CMA and PSA media incubated for a period of two weeks

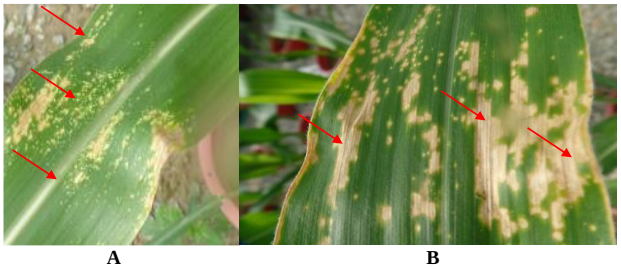

Fig. 4: (**A**) The symptom of SCLB first appeared as elliptical brownish red spots on the surface of the leaf, (**B**) Over time, the symptoms progressed to form necrotic, long lesion or blighted zones

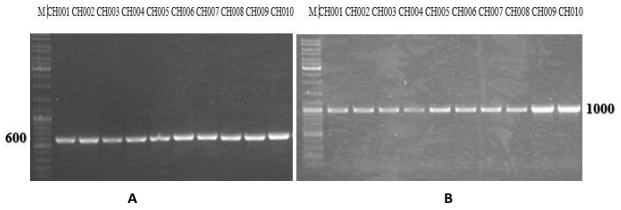

Fig. 5: (**A**) Bands of PCR products from ITS region and the fragments of the amplification were approximately 600 bp. (**B**) Gel electrophoresis showing bands of PCR product (*β*-tubulin gene) and the fragments of the amplification were approximately 1000 bp

The disease severity index (DSI) was calculated based on the data collected ata weekly interval. The findings of this study showed that CH001, CH009, CH003, CH002 and CH004 were the most aggressive isolates by having 80, 80, 76, 72 and 72%, with the area under disease progressive curve (AUDPC) value of 182, 170, 178, 168 and 153 unit^2, respectively at 4 weeks after inoculation (WAI). On the other hand, isolate CH010, was the least aggressive among the isolates tested with 28% and AUDPC value of 69 unit^2 as presented in Table 4. The seedlings (control) did not show any SCLB disease symptoms.

Molecular characterization

The results of molecular identification re-affirmed that, the tenisolates were identified as *C. heterostrophus*. After amplification of DNA, all the ten representative isolates showed bands of around 600 base pairs (bp) for ITS region as indicated in Fig. 5A. Similarly, for the *β*-tubulin gene, all the isolates also showed a band size of around 1000 bp or 1 kb as presented in Fig. 5B.

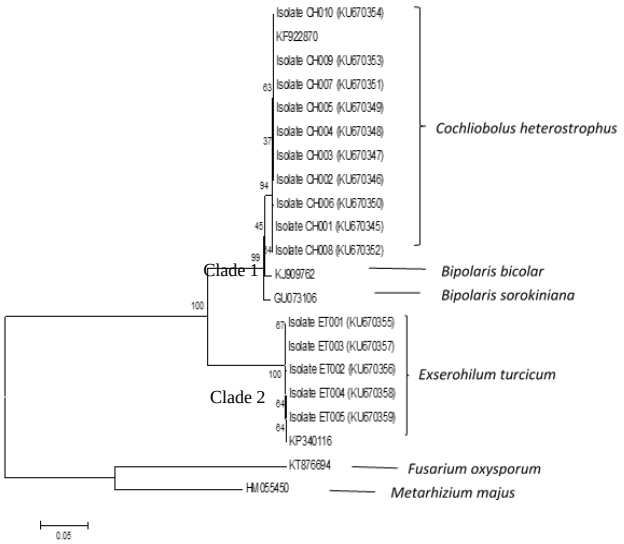

Fig. 6: Showing the phylogenetic relationship (ITS region) of *C. heterostrophus* isolates that were compared with accession numbers of other fungal species. The phylogenetic tree was inferred by neighbor joining tree analysis in term of rDNA sequences. The numbers below the branches indicate the percentage for each of the branch in 1000 bootstrap replications

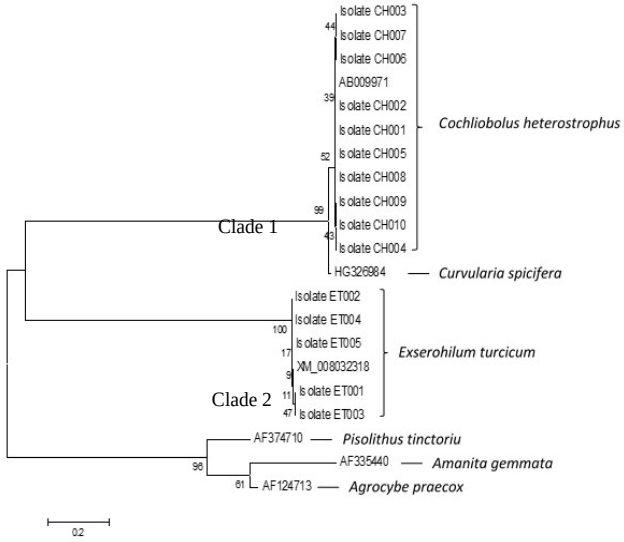

Fig. 7: Showing the phylogenetic relationship (*β*-tubulin gene) of *C. heterostrophus* isolates that were compared with accession numbers of other fungal species. The phylogenetic tree was inferred by neighbor joining tree analysis in term of rDNA sequences

DNA sequencing and phylogenetic analysis

Based on the results of ITS region and *β*-tubulin genes, the

Table 5: Blast results of ten isolates of *C. heterostrophus* obtained from infected corn (ITS Region)

Isolate code	Accession No. of isolate	Accession No. equivalent	Maximum score	Total score	Query coverage (%)	Maximum identity (%)
CH001	KU670345	KT363892	913	913	100	100
CH002	KU670346	HF934924	1037	1037	100	100
CH003	KU670347	HF934924	1035	1035	100	100
CH004	KU670348	HF934924	1033	1033	100	100
CH005	KU670349	KC005707	1042	1042	100	100
CH006	KU670350	HF934924	1038	1038	100	100
CH007	KU670351	HF934924	1033	1033	100	100
CH008	KU670352	KC005707	1048	1048	100	100
CH009	KU670353	HF934924	1033	1033	100	100
CH010	KU670354	HF934924	1033	1033	100	100

*Accession number equivalent obtained from GenBank database (http://www.ncbi.nlm.nih.gov/Blast)

Table 6: Blast results of ten isolates of *C. heterostrophus* obtained from infected corn (*β*-tubulin gene)

Isolate code	Accession No. of isolate	Accession No. equivalent	Maximum score	Total score	Query coverage (%)	Maximum identity (%)
CH001	KU670330	XM_014226937	1748	1748	99	99
CH002	KU670331	XM_014226937	1694	1694	100	99
CH003	KU670332	AY749035	1578	1578	94	99
CH004	KU670333	XM_014226937	1696	1696	100	99
CH005	KU670334	XM_014226937	1687	1687	100	99
CH006	KU670335	XM_014226937	1724	1724	99	99
CH007	KU670336	XM_014226937	1757	1757	99	100
CH008	KU670337	XM_014226937	1772	1772	99	99
CH009	KU670338	XM_014226937	1768	1768	99	99
CH010	KU670339	XM_014226937	1748	1748	100	99

*Accession number equivalent obtained from GenBank database (http://www.ncbi.nlm.nih.gov/Blast)

nucleotide sequences of the isolates with their accession numbers were deposited in the NCBI (GenBank) database. BLAST search from the NCBI database using ITS and β-tubulin gene sequences of the ten isolates confirmed them as species of genus *Helmenthosporoids* (*Cochliobolus heterostrophus*). Based on ITS sequences, isolates CH001, CH002, CH003, CH004, CH005, CH006, CH007, CH008, CH009 and CH010 were highly homologous to *C. heterostrophus* (100% similarity) as presented in Table 5. The isolates showed 99% nucleotide sequence similarities with the *β*-tubulin gene except for isolate CH007 (100%) as indicated in Table 6.

Phylogenetic relationship of the ten isolates (ITS region), in this study, all the isolates that were obtained from infected corn were clustered together in the same clade (clade 1). These isolates were found to show high nucleotide similarity with the reference isolate of KF922870 (*C. heterostrophus*). *Bipolaris bicolor* and *B. sorokiniana* were also clustered in the first clade (clade 1) but in different sub-clade. On the other hand, six accession numbers (KU670355, KU670356, KU670357, KU670358, KU670359 and KP340116) of *E. turcicum* obtained from the GenBank were clustered together in a separate clade (clade 2) because they belong to the same genus with the 10 isolates. Other species of *Fusarium oxysporum* and *Metarhizium majus* were grouped outside the main clade and they served as an outgroup, since they were clearly separated from *E. turcicum* and *C. heterostrophus* (Fig. 6).

Fig. 7 showed the phylogenetic relationship of all the isolates (*β*-tubulin gene). All the 10 isolates that were isolated from infected corn were clustered together in the same clade (clade 1). These isolates were found to have high similarity with the reference isolate AB009971 (*C. heterostrophus*). *Curvularia spicifera* (HG326984) was placed under the same clade 1 but in a different sub-clade. On the other hand, six accession numbers (KU670340, KU670341, KU670342, KU670343, KU670344 and XM_008032318) of *E. turcicum* obtained from the GenBank were clustered together in a separate clade (clade 2). While other species of *Pisolithus tinctoriu (*AF374710), *Amanita gemmata* (AF335440) and *Agrocybe praecox* (AF124713) were clearly grouped outside the main clade and they served as an outgroup.

Discussion

Based on the morphological characteristics, the ten isolates were identified as *Cochliobolus heterostrophus.* The isolates of *C. heterostrophus* are similar to *Helminthosporium* and are described morphologically based on multi-celled conidia, the cells of the conidia are arranged in a linear organization and not in irregular as in the case of *Alternaria* (a member of the genus that is related). The findings of this work are in conformity with the work of Sivanesan (1987) and Sun *et al.* (2020) who stated that conidial length and width of *C. heterostrophus* was 93.5 and 13.9 *µ*m, respectively. More so, the color of the colony was dark grey to black and grey to greyish black while the shape of the multi-celled conidia was curved. In another work by Degani (2014) reported that the rate at which *C. heterostrophus* pathogen grow fast and sporulate was after four days of incubation on PDA media.

In term of the effect of different media on the growth of *C. heterostrophus*, PSA media was found the highest and then followed by CMA and PDA, respectively. However, these findings are not in line with the work of Sun *et al.* (2020) who stated that SCLB pathogen grow well and faster on PDA for a period of seven days. Moreover, Didvania *et al*. (2012) studied the growth of *Drechslera bicolor* on different media and reported that PDA was the best for sporulation and mycelial growth (82.1 mm). In addition, excellent mycelial sporulation and growth of sub-genera *Drechslera bicolor* (*Helminthosporium*) genus was found on PDA, then next was malt extract and Richard's media according to Didvania *et al*. (2012). The findings of this work are not consistent with the findings of Naz *et al*. (2012) who reported that Richards agar was the best for supporting the growth of *C. heterostrophus*. Based on these findings, it can be recommended that PSA is the optimum media to be used for *in vitro* culturing of *C. heterostrophus*. Different types of media significantly affected the pattern and growth rate of various isolates.

A temperature of 30°C was more suitable in growing the pathogen; it was followed by 25°C, 20°C, and 35°C, respectively. A similar study conducted by Naz *et al*. (2012) proved that the best temperature for growing *C. heterostrophus* was 30°C with a maximum growth of colony size (80 mm) and the pathogen grow poor at a temperature of 35°C with a colony size of 35 mm. Didvania *et al.* (2012) studied seven different temperatures (40, 35, 30, 25, 20, 15 and 10°C) and stated that the ideal temperature for *C. heterostrophus* growth was 25°C with (90 mm) colony size and this goes contrary to the finding of this study.

Based on pathogenicity, the findings of this study showed that isolate CH001, CH009, CH003, CH002 and CH004 were the most aggressive among the isolate tested. Previous works have shown that the virulence of *C. heterostrophus* isolates was diversified and the species of the fungus have many pathotypes (Zhang *et al*. 2013; Lu *et al*. 2014; Wang *et al.* 2017; Gan *et al*. 2018). Our pathogenicity tests showed that virulence variability existed among different isolates of *C. heterostrophus* based on their locations, which was found to be consistent with the findings of Sun *et al*. (2020).

These findings were in conformity with the previous works (Guo *et al*. 2016, 2017; Dai *et al.* 2017), who reported that fusiform, elliptical and elongated lesions were the typical symptoms of SCLB disease. Degani (2014) critically tested virulence levels of *C. heterostrophus* pathogen and reported that detached leaves are significantly more vulnerable to infection of SCLB disease than the intact leaves. Bashir *et al*. (2017b) assessed virulence level of *C. heterostrophus* strains and found to have a different level of aggressiveness. Moreover, the findings are also consistent with the work of Soumya and Ramachandr (2019) who tested the pathogenic variability of 11 isolates and their DSI means were found to range from 10.31–61.73%. Durrishahwar *et al.* (2008) screened different lines of corn and found some lines that are resistant to SCLB disease. We suggest that in the future study as a result of the race diversity of *C. heterostrophus* and the disease symptoms on corn are very complicated, thus, further studies should be carried out on virulence determination and race detection of *C. heterostrophus* and to the explanation of the mechanisms of pathogen variability.

The findings of molecular characterization showed that the isolates tend to split based on species not in term of geographical origins. These findings were consistent with the work of Manamgoda *et al.* (2012) who studied the relationship of different fungal species that belong to the genus *Helminthosporium* (*Bipolaris*, *Cochliobolus* and *Curvularia*). Some previous works have used only ITS locus to describe and identify *Cochliobolus* species (Ahmadpour *et al*. 2012; Cunha *et al*. 2012). The ITS alignment studied by Kang *et al*. (2018) helped in differentiating *Cochliobolus heterostrophus* and *Cochliobolus carbonum* from 13 other *Cochliobolus* species. The use of universal primers (ITS and *β*-tubulin) have resulted in high similarities. Both *β*-tubulinand ITS sequences helped us to examine and analyze the phylogenetic relationship of *C. heterostrophus* and other fungal species that are related closely.

Conclusion

Cochliobolus heterostrophus pathogen responsible for causing SCLB disease in Malaysia was investigated using morphological and molecular methods and the pathogen was identified as *C. heterostrophus*. The pathogen was found to grow in different areas specifically at low land areas in Malaysia. Based on the pathogenicity test, the aggressive levels of the 10 isolates showed that the isolates were virulent and pathogenic; the aggressiveness was based on their geographical locations. The findings of this study contribute immensely to the understanding of the pathogen responsible for causing SCLB disease. This research work would in the future serve as the background for *in vivo* and *in vitro* studies of SCLB pathogen of corn. Further studies should be conducted on race detection of *C. heterostrophus* as well as the determination of mechanisms of pathogen variability.

Acknowledgements

The authors would like to thank Assoc. Prof. Dr. Kamaruzaman Sijam for his immense support, advice and contributions from beginning to the end of this work. Moreover, the authors thank the entire staff of the Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia for numerous technical assistance.

Author Contributions

All authors in this research manuscript have read and agree to the published version of the manuscript. Writing original

draft preparation, ABK; writing, proofreading, and editing KA, AA, MZH and MAA; supervision, KA, AA, MZH and M AA; funding acquisition, KA. All the authors in this manuscript have agreed to publish this manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Data Availability

The data presented in this study will be available on a request to the corresponding author.

Ethics Approval

Not applicable in this research work.

Funding Source

The authors of this manuscript are grateful to the Malaysian Government through its Malaysian Agricultural Research and Development Institute (MARDI) and Green World Genetics (GWG) for the financial support provided during this research work.

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