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Characterisation and Pathogenicity of *Fusarium* Species Associated with Yellowing Disease in Black Pepper (*Piper nigrum* L.)

(Pencirian dan Kepatogenan Spesies *Fusarium* Berkaitan dengan Penyakit Kuning pada Lada Hitam (*Piper nigrum* L.))

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ABSTRACT

Yellowing disease has become one of the main threats to black pepper (*Piper nigrum* L.) production. This study was conducted to isolate and characterize *Fusarium* species associated with yellowing disease based on morphological and molecular characteristics, and to evaluate their pathogenicity on black pepper. Fungal isolates were recovered from symptomatic black pepper plants located in major growing areas in Sarawak. Based on morphological characteristics, a total of 73 *Fusarium* isolates were identified. Genomic DNA sequence analysis of nuclear ribosomal DNA internal transcribed spacer (ITS) and translation elongation factor-1 alpha (EF-1α) had confirmed the identity of the *Fusarium* isolates. The four identified *Fusarium* species were *F. solani* (90.4%), *F. oxysporum* (5.5%), *F. concentricum* (2.7%), and *F. mangiferae* (1.4%). Based on morphological characterisation, *F. solani* isolates were grouped into two distinct morphotypes, morphotype I and II. Phylogenetic analysis of *F. solani* has shown a high level of intraspecific variation among the isolates. The two major clades formed were found to correspond to the morphotype grouping. Pathogenicity test showed that all four *Fusarium* species are pathogenic to black pepper based on disease incidence and severity. This study provides new insights into the *Fusarium* species associated with yellowing disease in black pepper, and highlights the high genetic variability among the *Fusarium* isolates. This result calls for a critical revision of the current strategies in disease management, and resistance breeding programs in black pepper.

Keywords: *Fusarium solani* species complex; phylogeography; population genetic; root rot disease; slow wilt

ABSTRAK

Penyakit kuning telah menjadi salah satu ancaman utama kepada pengeluaran lada hitam (*Piper nigrum* L.). Kajian ini dijalankan untuk memencil dan mencirikan spesies *Fusarium* yang dikaitkan dengan penyakit kuning berdasarkan ciri morfologi dan molekul serta menilai sifat kepatogenannya ke atas lada hitam. Pencilan kulat diperoleh daripada pokok lada hitam bergejala yang terdapat di kawasan penanaman utama Sarawak. Berdasarkan ciri morfologi, sebanyak 73 pencilan *Fusarium* telah dikenal pasti. Analisis jujukan DNA genom bagi penjarak dalaman yang ditranskripsikan (ITS) DNA ribosom nuklear dan faktor pemanjangan translasi-1 alfa (EF-1α) telah mengesahkan identiti pencilan *Fusarium*. Sebanyak empat spesies *Fusarium* yang dikenal pasti iaitu *F. solani* (90.4%), *F. oxysporum* (5.5%), *F. concentricum* (2.7%) dan *F. mangiferae* (1.4%). Berdasarkan pencirian morfologi, pencilan *F. solani* telah dikelaskan kepada dua morfotip yang berbeza iaitu morfotip I dan II. Analisis filogenetik *F. solani* telah menunjukkan tahap variasi intrakhusus yang tinggi dalam kalangan pencilan. Dua klad utama yang terbentuk didapati sepadan dengan kumpulan morfotip. Kajian kepatogenan mendedahkan bahawa kesemua empat spesies *Fusarium* adalah patogen kepada lada hitam berdasarkan kejadian dan keterukan penyakit. Kajian ini memberikan pandangan baharu tentang spesies *Fusarium* yang dikaitkan dengan penyakit kuning pada lada hitam dan menyerlahkan kebolehubahan genetik yang tinggi dalam kalangan pencilan *Fusarium*. Hasil kajian ini mencadangkan semakan kritikal dibuat terhadap strategi semasa dalam pengurusan penyakit dan program pembiakan kerintangan bagi lada hitam.

Kata kunci: Filogeografi; genetik populasi; kelayuan perlahan; penyakit akar reput; spesies kompleks *Fusarium solani*

Introduction

Black pepper (*Piper nigrum* L.; Piperaceae) is a perennial flowering vine primarily cultivated for its peppercorns. It presently has a wide application in the food, cosmetics, pharmaceutical industries and home remedies. Today, black pepper is rightfully known as the king of spices by dominating 20% of the global spice market. Black pepper is mainly exported in whole peppercorns (95%), and the remaining are in the form of ground pepper, pepper extracts and pepper oils. Global production of black pepper was 591,946 metric tons amounted to an export value of USD1.29 billion in 2019 (Perwara & Munggaran 2020). Malaysia contributed 24,000 metric tons of black pepper in 2019, which was equivalent to 4.1% of global black pepper production. Sarawak accounted for 98% of Malaysia's black pepper production, with a total cultivation area of 17,477 ha generating RM145.6 million of export revenue in 2019 (DOA 2020).

The major challenges faced by the Malaysian black pepper industry are shortage of labour and planting materials, low productivity, and high pest and disease outbreaks (Chen, Dayod & Tawan 2018; Kho & Chen 2017). Yellowing disease is one of the most dreaded diseases in black pepper, leading to yield reduction and plant death. The disease was reported to cause a 30% yield reduction and gradually killing the plants in 3 to 4 years (Ramana & Eapen 2000; Shahnazi et al. 2012). This disease was reported in other major black pepper-producing countries such as Brazil, India, Costa Rica, Thailand and Vietnam. The infected black pepper may appear asymptomatic at the early infection stage but progressively decline in vigour. The initial disease symptoms are discoloration and yellowing of leaves that start from the bottom. Flaccidity, gradual defoliation and dieback are common symptoms of the yellowing disease at the advanced stage. The underground symptoms are necrotic lesions on the primary and secondary roots, and loss of feeder roots leading to plant death (Wong 2010). A higher mortality rate was reported when the plants were predisposed to soil-borne root-knot nematodes (*Meloidogyne incognita*) and burrowing nematodes (*Radopholus similis*) (Ramana & Eapen 2000; Thuy et al. 2012).

Fusarium solani and *F. oxysporum* were reported as the causal agents of this disease (Biju et al. 2019). These cosmopolitan *Fusarium* fungi are among the most notable plant pathogens, causing vascular wilt and root rot diseases on many crops (O'Donnell et al. 2018). The genus *Fusarium* belongs to the Nectriaceae family. The occurrence of species complex has been reported in these two species. Thus, the identification of *Fusarium* species must be based on a polyphasic approach using a combination of morphological and molecular characteristics (Summerell 2019). Based on host specificity, *Fusarium* species can be further classified into formae specialis (f. sp.) and races (Sisic et al. 2018). This is due to the presence of genetic variability among individuals of the same species. The cause of genetic variability in *Fusarium* fungi arises from the rapid fungal adaptation to environmental changes, agronomic practices and crop genetics (Klix, Beyer & Verreet 2008).

Yellowing disease can spread through splash dispersal, vegetative propagation and chlamydospores as survival structures. Fungicide treatment, soil solarisation, microbial antagonists and good agricultural practices were implemented to control yellowing disease in black pepper (Biju et al. 2019). Nevertheless, yellowing disease management can be improved by breeding the resistant black pepper cultivars. The use of resistant cultivars is an economical and sustainable method of disease prevention. However, the development of resistance host plants requires a thorough understanding of the pathogen population genetics. Therefore, this study was conducted to isolate and characterise *Fusarium* species associated with the yellowing disease through morphological and molecular characteristics, and to evaluate their pathogenicity on black pepper.

Materials and Methods

Sampling and fungal isolation

Black pepper farms located in seven major black pepper growing districts (Belaga, Betong, Bintangor, Bintulu, Julau, Miri and Mukah) in Sarawak were surveyed. Infected plants showing clear symptoms of leaf chlorosis and rot at the lower stem were sampled. Root samples were washed under running tap water to remove soil particles and cut into small pieces (5 × 5 mm). These small pieces were surface sterilised using 1% (v/v) sodium hypochlorite for 1 min, rinsed twice with sterile distilled water, and dried on sterilised filter paper. The surface-sterilised pieces were plated onto potato dextrose agar (PDA) (Oxoid, UK) amended with 100 mg/L PCNB, 250 mg/L, and 100 mg/L rifampicin. The plates were incubated at 26 ± 2 °C for 3 days. The actively growing mycelia were transferred onto new PDA plates and incubated for 7 days.

MORPHOLOGICAL IDENTIFICATION AND CHARACTERIZATION

The isolated fungi were tentatively identified as *Fusarium* species based on the morphological characteristics as described by Leslie and Summerell (2006). The identified cultures were single-spored onto PDA using the serial dilution method to obtain pure fungal cultures. Mycelial discs of 5 mm were obtained from 7 day-old pure fungal cultures and transferred onto new PDA plates. Mycelial growth rate (mm/day) was determined by measuring the two perpendicular colony diameters on the 6th day of incubation. The isolates were grouped according to growth category namely slow (<10 mm/day), medium (10-12 mm/day) and fast (>12 mm/day). Colony macromorphology such as mycelial form, colony colour and pigmentation was observed on PDA on the 6th day. Colony colour and pigmentation were determined by using the Munsell colour chart. Microscopic characteristics such as conidial shapes, length and branching of conidiogenous cells, and the presence of chlamydospore were observed on carnation leaf agar (CLA) incubated at 26 ± 2 °C for 4 weeks. Fifty conidia were observed randomly, the number of septa, length and width were measured. Analysis of variance (ANOVA) in SAS version 9.4 program was used to analyse the mycelial growth rate, size of conidia and chlamydospores. Means were compared using the Tukey test at $P \leq 0.05$.

PCR AMPLIFICATION

Genomic DNA was extracted from 7 day-old cultures grown in potato dextrose broth (PDB) at 26 ± 2 °C. Genomic DNA was extracted by adopting the cetyltrimethylammonium bromide (CTAB) method according to the protocol outlined by Doyle and Doyle (1990) with slight modification. Fungal mycelia (100 mg) was homogenised using BioMasher-II (Optima, Japan). The concentration and purity of the extracted DNA were quantified using Pico200 Microliter UV/Vis spectrophotometer (Quantica, USA). The nuclear rDNA ITS1-ITS4 (ITS) sequences were amplified using ITS1 (5'-TCCGTAGGTGAACCTTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers, and the partial translation elongation factor-1 alpha (EF-1α) sequences were amplified using primers, EF1-728F (5'-CATCGAGAAGTTCGAGAAGG-3') and EF2 (5'-GGAGTACCAGTATCA-3'). PCR amplification was performed in a 50 µL reaction mixture containing 1X power Taq MasterMix (BioTeke Corporation, China), 0.4 µM of each primer, and 100 ng of DNA template in MiniOpticon Real-Time PCR System (BioRad, USA). PCR program consisted of initial denaturation at 95 °C for 2 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 50 (ITS) and 55 °C (EF-1α), respectively, for 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. PCR amplicons were electrophoresed on 1% agarose gel run parallel to a 100 bp DNA ladder (BioTeke Corporation, China). The size of the ITS (~600 bp) and EF-1α (~500 bp) amplicons were visualized in a UV transilluminator.

MOLECULAR IDENTIFICATION AND PHYLOGENETIC ANALYSIS

PCR amplicons were purified using Gel Extraction and PCR Purification Combo Kit (BioTeke Corporation, China), according to the manufacturer's instructions. The sequence was compared to sequences in three validated databases namely the GenBank database at National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov), *Fusarium* Isolate database (http://isolate.fusariumdb.org/blast.php) and *Fusarium* Multilocus Sequence Typing (MLST) database (http://fusarium.mycobank.org/). The sequences obtained in this study were submitted to GenBank (accession number listed in Supplementary Table 1). ITS and EF-1α sequences from other *Fusarium* species were obtained from the NCBI GenBank database as reference sequences for phylogenetic analysis (Supplementary Table 2). DNA sequences were aligned with the MUSCLE algorithm in SeaView software version 4 (Gouy, Guindon & Gascuel 2010). Phylogenetic trees were generated for individual and concatenated sequences using the Maximum Likelihood method under the general time-reversible (GTR) model of sequence evolution with 1000 bootstrap replicates. Bootstrap values of <50% were considered insignificant support and collapsed.

PATHOGENICITY TEST

One isolate from each *Fusarium* species was selected for the pathogenicity test. Spore inoculum was harvested from 7 day-old cultures grown in PDB at 26 ± 2 °C and 100 rpm agitation speed. The cultures were filtered with two layers of sterile cheesecloth, and a spore suspension of 2×10^6 spores/mL was prepared. Roots of the 1 month-old black pepper (cv. Kuching) cuttings were washed under running tap water and wounded with a sterile scalpel. The injured roots were immersed and swirled several times in 500 mL spore suspension for

5 min. The cuttings were transplanted into polybags containing 3 kg of sterile soil mixture and drenched with 200 mL of spore suspension around the collar regions. Control plants were wounded and treated with sterile distilled water. Fungal infection was repeated 2 weeks after inoculation by drenching the collar regions with fresh spore suspensions. This was to ensure continuous fungal infection at the early stage of the pathogenicity test. Cuttings were arranged in a completely randomized design (CRD) with 6 replications per treatment. The cuttings were maintained in the glasshouse, watered daily, and NPK fertilisers were applied every 2 weeks. Each cutting was rated at monthly intervals according to a slight modification of the disease scale described by Fang et al. (2012) for up to 9 months, where 0 = plant without visible symptoms, 1 = plant slightly stunted, 2 = plant stunted and yellowing, 3 = plant severely stunted and yellowing, and 4 = plant wilted or dead. The disease severity index (DSI) was calculated using the formula:

$$\frac{\Sigma(\text{Number of infected plants x Disease score})}{[(\text{Total number of plants}) \times (\text{Maximum disease score})]} \times 100$$

The virulent classification was based on DSI values, namely weakly virulent (<40), moderately virulent (40 - 60), and highly virulent (>60) (Zhu et al. 2014). Black pepper plants were harvested at 9 months after inoculation. Plant height, internode length, total number of leaves, fresh and dry weight of shoot and root were determined. ANOVA was used to compare means by Tukey test at $P \leq 0.05$. Colonisation assays were carried out to determine the percentage of roots that *Fusarium* fungi have colonized. The harvested root samples were washed under running tap water, surface sterilised with 1% (v/v) sodium hypochlorite for 1 min, rinsed twice with sterile distilled water, randomly segmented into 5 mm in length, and plated on PDA. The cultures were incubated for 5 days at 26 ± 2 °C. Fungal isolates recovered from the roots were morphologically compared to the original isolates. Percentage of root colonisation was calculated based on the number of infected root segments divided by the total number of root segments plated for each plant.

Results

Fungal isolation and morphological characterisation

Black pepper plant samples exhibiting yellowing disease symptoms were collected from 21 fields across the seven selected districts. These fields were separated by at least 500 m apart. Yellowing disease symptoms can be confused with other root diseases caused by *Phytophthora capsici* and *Rigidoporus lignosus*. Hence, further examination is required to identify the causal agents. A total of 73 isolates were identified as *Fusarium* fungi based on morphological characteristics such as moderately fast-growing with abundant aerial mycelia, sickle-shaped macroconidia, oval to ellipsoid-shaped microconidia, and the presence of conidiogenous cells, following the description by Leslie and Summerell (2006). Based on morphological characterisation, the four identified *Fusarium* species were *F. solani* (66 isolates), *F. oxysporum* (4 isolates), *F. concentricum* (2 isolates) and *F. mangiferae* (1 isolate). Each species were morphologically indistinguishable.

Morphological characterisation of *Fusarium* species

F. solani species complex (FSSC)

Sixty-six isolates of *F. solani* were grouped into two different morphotypes based on morphological characteristics. Morphotypes I and II were comprised of 15 and 51 isolates, respectively (Table 1).

Morphotypes I

Culture Characteristics

On PDA, the mycelial growth rate of morphotype I was moderately fast-growing at 11.9 ± 0.9 mm/day. Colonies were sparse to floccose, mycelia were off-white, and pigments were dark red to brownish orange (Figure 1). No odour was detected.

Microscopic Characteristics

On CLA, cream colour and occasionally green colour sporodochia were formed abundantly on leaf pieces. Macroconidia were abundantly found in sporodochia. Macroconidia were thin-walled, 3 to 5 septate but typically 3, straight to slightly curved, curved or pointed at the apical and foot-shaped basal cell (Figure 1). Macroconidia measured at 41.0 ± 5.2 µm (length) × 4.7 ± 0.3 µm (width). Microconidia were abundant in aerial mycelia, oval or ellipsoid, none to 1 but occasionally 2 septa, and measured at 12.2 ± 1.1 µm (length) × 3.5 ± 0.3 µm (width). Conidiogenous cells are monophialidic and short. Chlamydospores were abundant, singly or in pairs but occasionally in short chains on the terminal or intercalary but occasionally sessile hyphae, globose to oval, smooth or rough walled, and diameter measured at 8.0 ± 0.3 µm.

Morphotypes II

Culture Characteristics

On PDA, the mycelial growth rate of morphotype II reached 11.7 ± 0.8 mm/day. The colonies of morphotypes II formed delicate, sparse to floccose aerial mycelia, and zonation patterns were observed in specific isolates. The colonies produced pale yellow to yellow pigments. No odour was detected.

Microscopic Characteristics

On CLA, macroconidia were found abundantly in cream colour and occasionally green colour sporodochia. Macroconidia were thin-walled, 3 to 5 septate, straight to slightly curved, curved or pointed at the apical cell, foot-shaped basal cell, and measured at 42.6 ± 4.1 µm (length) × 4.6 ± 0.3 µm (width). Microconidia were abundant in aerial mycelia, produced in false heads on short and long monophialides, thin-walled, oval or ellipsoid, none to 1 but occasionally 2 septa, and measured at 12.1 ± 1.1 µm (length) × 3.5 ± 0.3 µm (width). Chlamydospores were abundant, singly or in pairs but occasionally in short chains on the terminal or intercalary but occasionally on sessile hyphae, globose to oval, smooth or rough walled, and diameter measured at 8.0 ± 0.3 µm.

F. oxysporum

Cultural Characteristics

On PDA, the mycelial growth rate was the fastest (12.4 ± 1.2 mm/day) among the four species. Aerial mycelia were sparse to floccose, and no zonation pattern was observed. Colonies were off-white, and purple pigments were observed on agar. No odour was detected.

Microscopic Characteristics

On CLA, macroconidia were abundantly found in cream colour sporodochia on leaf pieces. Macroconidia were thin-walled, 3 to 5 septate but typically 3, straight to slightly curved, curved or pointed at the apical and foot-shaped basal cell. Macroconidia measured at 38.5 ± 6.1 µm (length) × 4.2 ± 0.6 µm (width). Microconidia were abundant in aerial mycelia, oval or ellipsoid, none but occasionally 1 to 2 septa and measured at 12.5 ± 3.1 µm (length) × 3.4 ± 0.8 µm (width). Conidiogenous cells are monophialidic and short. Chlamydospores were abundant, usually singly or in pairs on the terminal or intercalary hyphae, globose in shape, smooth or rough walled, and diameter measured at 8.0 ± 0.8 µm.

F. concentricum

Cultural Characteristics

Colonies on PDA were floccose, and alternating concentric rings of red and orange were observed. The mycelial growth rate was the slowest (9.5 ± 0.3 mm/day) among the four species. No odour was detected.

Microscopic Characteristics

Macroconidia were abundant on cream colour sporodochia formed on leaf pieces. Macroconidia were thin-walled, 3 to 5 septate, relatively slender, straight to slightly curved, hooked or beaked apical cell, foot-shaped basal cell, and measured at 43.9 ± 6.0 µm (length) × 2.6 ± 0.3 µm (width). Microconidia were abundant in aerial mycelia, oval, slender, and straight to slightly curved, none to 1 but occasionally 2 septa, and measured 13.1 ± 5.8 µm (length) × 2.4 ± 0.4 µm. Long and short monophialidic and polyphialidic conidiogenous cells were observed. Chlamydospore is absent.

F. mangiferae

Cultural Characteristics

The morphological features of *F. mangiferae* were floccose with purplish-pink pigments on PDA. The mycelial growth rate was characterised as slow with 9.7 ± 0.3 mm/day. No odour was detected.

Microscopic Characteristics

Thin-walled macroconidia were abundant on cream colour sporodochia, relatively slender, straight to slightly curved, hooked or beaked apical cell, barely notched basal cell, 3 to 5 septate, and measured at 38.2 ± 4.5 µm (length) × 2.8 ± 0.3 µm (width). Microconidia were abundant in aerial mycelia, oval or ellipsoid, typically none or less frequently 1 septate, and measured at 10.1 ± 4.1 µm (length) × 2.3 ± 0.4 µm (width). Conidiogenous cells were short monophialidic and polyphialidic. Chlamydospore is absent.

MOLECULAR IDENTIFICATION

PCR amplification of all 73 morphologically identified *Fusarium* isolates has produced a clear single-band amplification corresponding to the amplicon sizes of ITS and EF-1α. ITS and EF-1α sequences have been deposited in the NCBI GenBank database. Based on the closest match of the BLAST analysis, similar analysis percentages ranged from 98% to 100% to known *Fusarium* species in all three databases. Molecular identification was consistent with morphological identification of *Fusarium* species.

TABLE 1. Morphological characteristics of *Fusarium* spp. isolated from symptomatic black pepper plants

	F. solani (Morphotype I)	*F. solani* (Morphotype II)	*F. oxysporum*	*F. concentricum*	*F. mangiferae*
Pigmentation on PDA	Dark red to brownish orange	Pale yellow to yellow	Purple	Alternating red and orange concentric rings	Purplish-pink
Growth rate	11.9 ± 0.9[a]	11.7 ± 0.[8a]b	12.4 ± 1.2[a]	9.5 ± 0.3[c]	9.7 ± 0.3[bc]
Macroconidia					
Sporodochia	Present, cream, and less frequently green colour	Present, cream, and less frequently green colour	Present, cream colour	Present, cream colour	Present, cream colour
Abundance	Abundant in sporodochia on leaf pieces	Abundant in sporodochia on leaf pieces	Abundant in sporodochia on leaf pieces	Abundant in sporodochia on leaf pieces	Abundant in sporodochia on leaf pieces
Number of septa	3 - 5	3 - 5	3 to 5 (usually 3)	3 - 5	3 - 5
Apical cell	Curved or pointed	Curved or pointed	Curved or pointed	Hooked or beaked	Hooked or beaked
Basal cell	Foot-shaped	Foot-shaped	Foot-shaped	Foot-shaped	Barely notched
Shape	Straight to slightly curved	Straight to slightly curved	Straight to slightly curved	Relatively slender and straight to slightly curved	Relatively slender and straight to slightly curved
Length (µm) *	41.0 ± 5.2[a]	42.6 ± 4.1[a]	38.5 ± 6.1[a]	43.9 ± 6.0[a]	38.2 ± 4.5[a]
Width (µm) *	4.7 ± 0.3[a]	4.6 ± 0.3[a]	4.2 ± 0.6[a]	2.6 ± 0.3[b]	2.8 ± 0.3[b]
Microconidia					
Abundance	Abundant in aerial mycelia	Abundant in aerial mycelia	Abundant in aerial mycelia	Abundant in aerial mycelia	Abundant in aerial mycelia
Conidiogenous cells	Short and long monophialides	Short and long monophialides	Short monophialides	Short and long monophialides, polyphialides	Short monophialides and polyphialides
Number of septa	0 - 1, occasionally 2	0 - 1, occasionally 2	0, occasionally 1 - 2	0 - 1, occasionally 2	0, occasionally 1
Shape	Oval or ellipsoid	Oval or ellipsoid	Oval or ellipsoid	Oval, slender, and straight to slightly curved	Oval or ellipsoid
Length (µm) *	11.5 ± 1.3[ab]	12.2 ± 1.1[ab]	12.5 ± 3.1[ab]	13.1 ± 5.8[a]	10.1 ± 4.1[b]
Width (µm) *	3.4 ± 0.4[a]	3.5 ± 0.3[a]	3.4 ± 0.8[a]	2.4 ± 0.4[b]	2.3 ± 0.4[b]
Chlamydospores					
Presence	Present	Present	Present	Absent	Absent
Abundance	Abundant on mycelia	Abundant on mycelia	Abundant on mycelia		
Location	Usually singly or in pairs (occasionally in short chains) on the terminal or intercalary (occasionally sessile) hyphae	Usually singly or in pairs (occasionally in short chains) on the terminal or intercalary (occasionally sessile) hyphae	Usually singly or in pairs on the terminal or intercalary hyphae		
Appearance	Globose to oval and smooth or rough walled	Globose to oval and smooth or rough walled	Round and smooth or rough walled		
Diameter (µm) *	8.3 ± 0.3[a]	8.0 ± 0.3[a]	8.0 ± 0.8[a]		

*Mean ± standard deviation of 50 randomly picked conidia and chlamydospores. Values followed by the same letter in the same row do not differ significantly (P = 0.05) in Tukey's range test

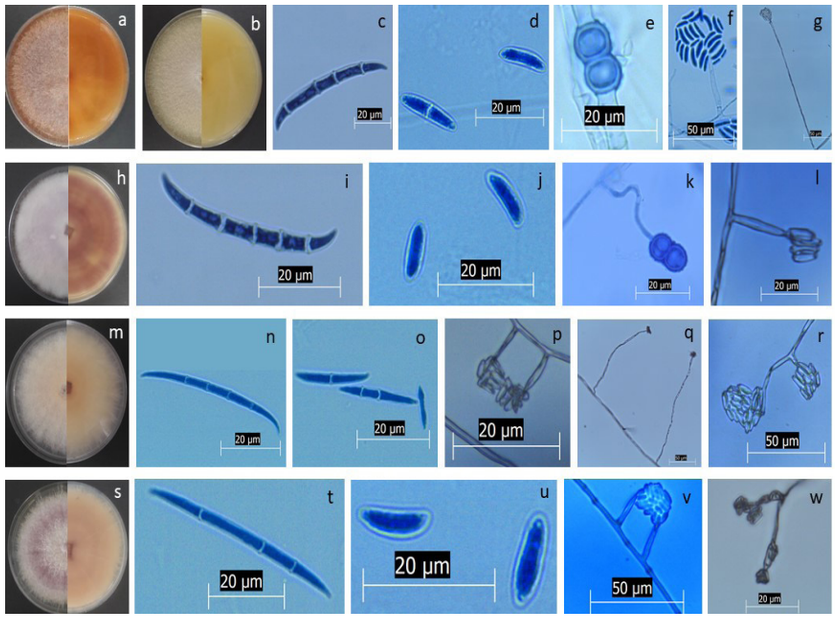
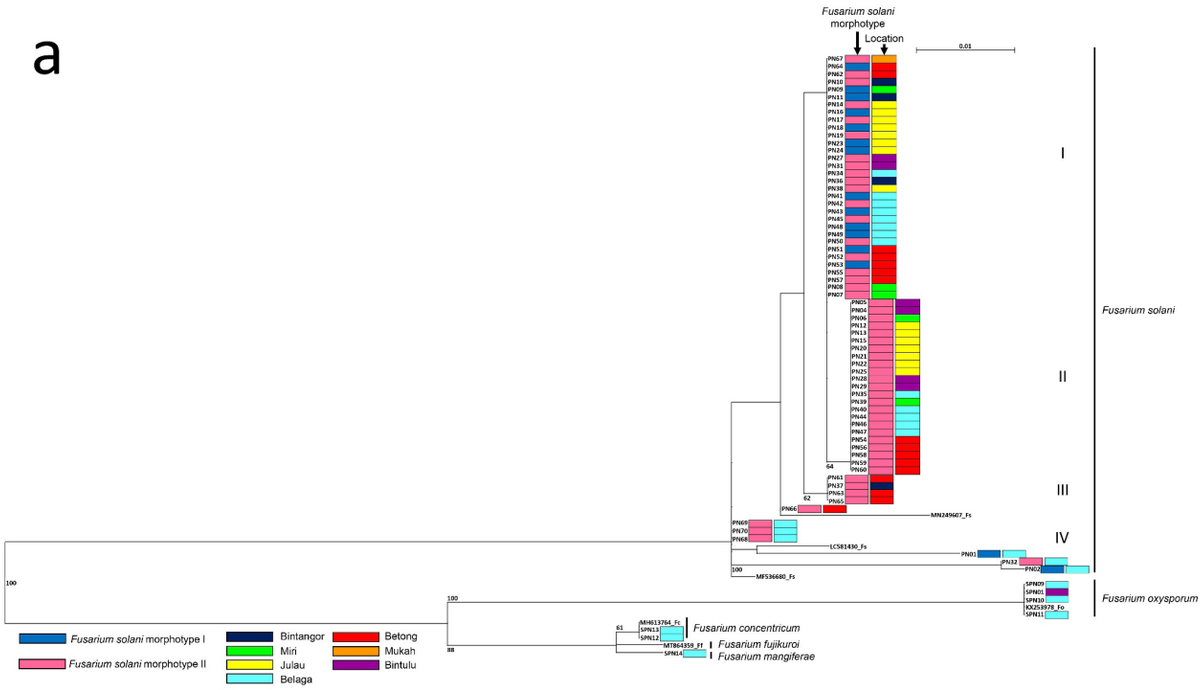
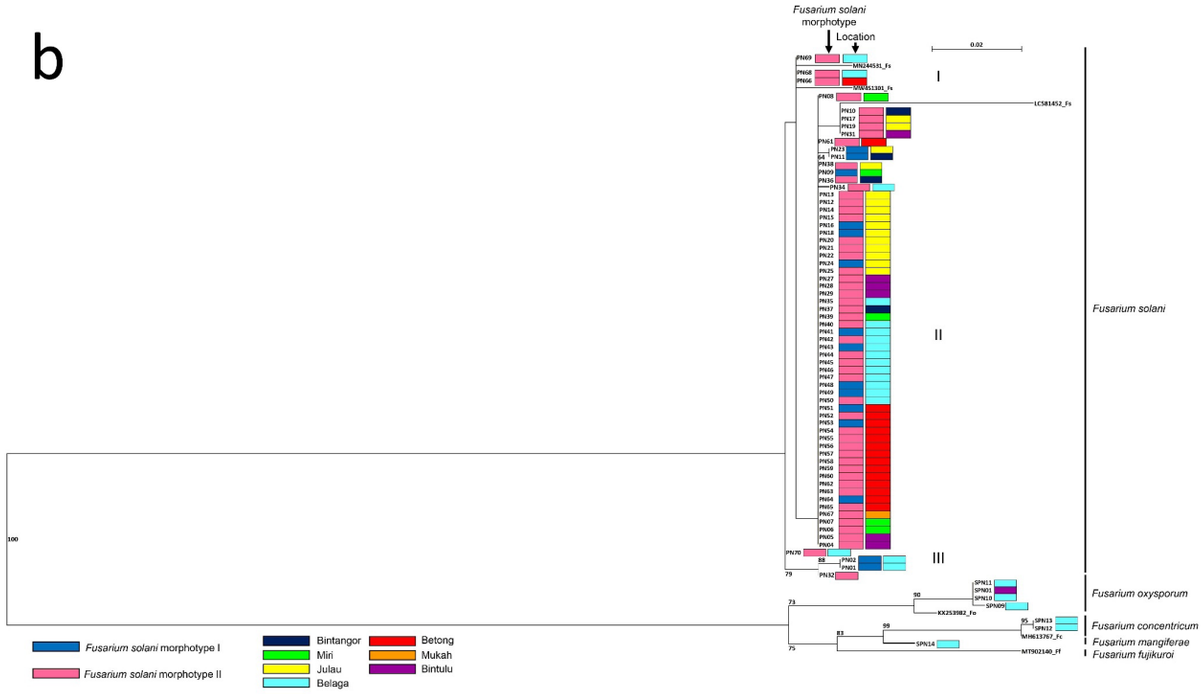


FIGURE 1. Morphological characteristics of *Fusarium* species recovered from black pepper. *F. solani* species complex (FSCC) (a-g), *F. oxysporum* (h-l), *F. concentricum* (m-r), and *F. mangiferae* (s-w). Colony morphology of *Fusarium* species on PDA 6 days after inoculation (a, b, h, m, s). The plate in each pair is the upper surface (right) and under surface (left). FSSC morphotype I produced brown to reddish-brown pigment (a), and morphotype II produced cream colour pigment on PDA (b). Macroconidia (c, i, n, t); microconidia with 0 to 2 septa (d, j, o, u); intercalary (e) and terminal (k) chlamydospores; false heads of microconidia on short (f, l, p, v) and long monophialides (g, q), and polyphialides (r, w)

PHYLOGENETIC ANALYSIS

DNA analysis showed sequence variations among the *F. solani* isolates where there were single-nucleotide (G/A) substitution, nucleotide insertion and deletion. Phylogenetic trees of individual and concatenated gene sequences were constructed to study the taxonomic placement of the *Fusarium* isolates (Figure 2). No strongly conflicting clades were detected in the individual and concatenated ITS and EF-1α phylogenies. This indicated that ITS and EF-1α sequences can be concatenated for phylogenetic analysis. The concatenated sequence is in congruence with the single-gene analysis but with increased phylogenetic resolution. Phylogenetic analysis based on the concatenated sequences proposed three major clades among the *Fusarium* isolates (Figure 2(c)). Isolates of the same species were clustered in the same clade. The separated clades showed that the species are not closely related. Phylogenetic analysis has clustered the *F. solani* isolates into five sub-clades, I-V. The clustering of sub-clades has corresponded with the grouping of morphotypes I and II. Most of the morphotype I have clustered in sub-clade I, and isolates of morphotype II were distributed across sub-clades II-V. However, there was no correlation between the clustering of *F. solani* isolates and their geographical origins except for sub-clade V. Clade V contained *F. solani* isolates originated from the Belaga district. Sub-clades II-V were heterogeneous with isolates from different geographical origins. *F. concentricum* was morphologically distinct from *F. mangiferae,* with pigments forming alternating red and orange concentric rings. Interestingly, phylogenetic analysis has clustered *F. concentricum* and *F. mangiferae* as closely related species. Genetic variation was not observed in *F. concentricum* isolates. However, this might be due to the small sample size used in this analysis. More isolates are required to validate the findings of this study.


a
Fusarium solani
morphotype
Location
0.01
I
II
III
IV
Fusarium solani
Fusarium oxysporum
Fusarium concentricum
Fusarium fujikuroi
Fusarium mangiferae
Fusarium solani morphotype I
Fusarium solani morphotype II
Bintangor
Miri
Julau
Belaga
Betong
Mukah
Bintulu


b
Fusarium solani
morphotype
Location
0.02
I
II
III
Fusarium solani
Fusarium oxysporum
Fusarium concentricum
Fusarium mangiferae
Fusarium fujikuroi
Fusarium solani morphotype I
Fusarium solani morphotype II
Bintangor
Miri
Julau
Belaga
Betong
Mukah
Bintulu

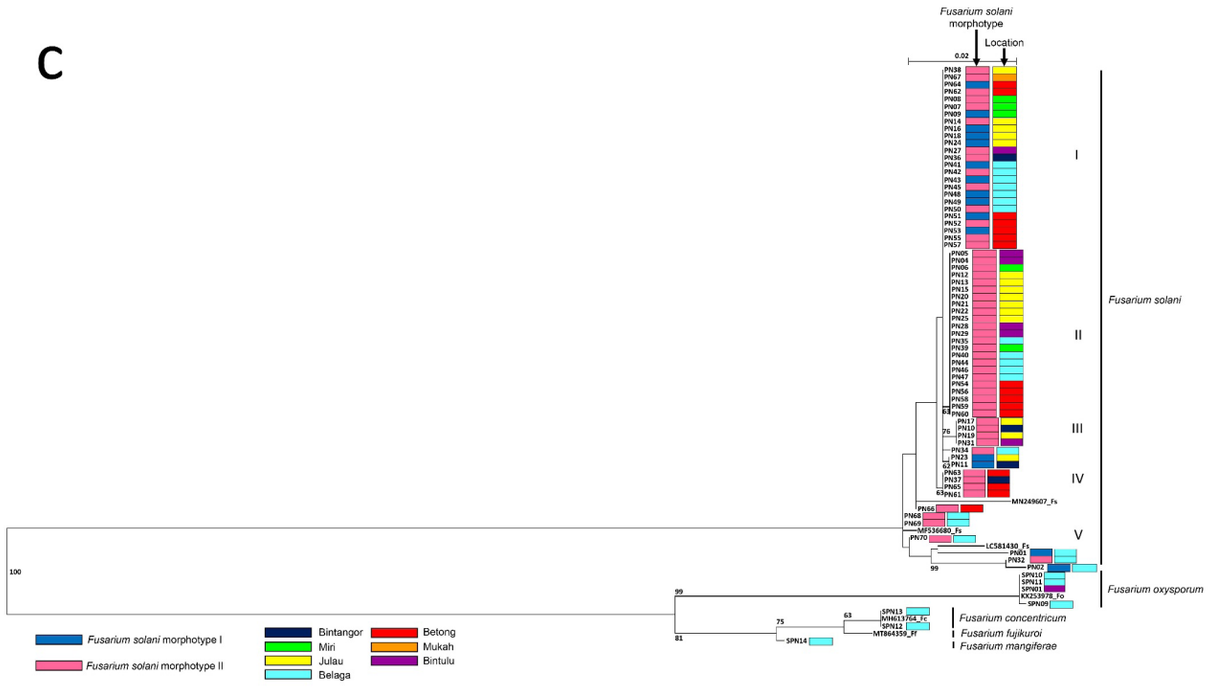


FIGURE 2. Phylogenetic analysis of 73 *Fusarium* isolates from black pepper based on ITS (A), EF-1α (B), and concatenated ITS and EF-1α (C) sequences. Phylogenetic tree was generated from the Maximum Likelihood method using SeaView version 4. Values above the branching nodes represent the bootstrap percentage calculated from 1000 replicates. Branches with bootstrap values <50% were collapsed. The branch length is drawn to scale and in the same unit as evolutionary distance

PATHOGENICITY TEST

Disease symptoms such as stunted growth and leaf yellowing were observed as early as 3 months after inoculation (MAI) in black pepper plants infected with *F. solani* and *F. oxysporum* (Figure 3). The first appearance of disease symptoms was observed at five MAI in *F. concentricum* and *F. mangiferae*-infected plants. No disease symptom was observed in control plants. Since the first observation of leaf yellowing, more leaves turned yellow in the subsequent months. The initial leaf yellowing symptom started from the lower leaves and progressively moved upwards. Disease progression was followed by the formation of smaller and wrinkled leaves.

All *Fusarium*-infected cuttings showed significantly stunted growth compared to the control based on growth parameters such as plant height, internode length, total number of leaves and plant biomass (Table 2). However, the growth reduction rate was significantly higher in *F. solani* and *F. oxysporum*-infected plants than in the other two *Fusarium* species. In addition, disease incidence (DI) and disease severity index (DSI) results also showed that *F. solani* and *F. oxysporum* were the most aggressive among the *Fusarium* species. At nine MAI, 100% DI and 46 - 50% DSI were noted in *F. solani* and *F. oxysporum*-infected plants. Results of DI and DSI of the tested *Fusarium* species were summarised in Table 2. The highest percentage of root colonisation was detected in *F. solani* and *F. oxysporum*-infected plants with 37.1% and 34.3%, respectively. Control plants did not show positive results in the root colonisation assay. *Fusarium* fungi were successfully reisolated from the inoculated cuttings to support Koch's postulate, and the control plants did not yield any *Fusarium* fungus. The isolated fungi were morphologically identical to the characteristics described earlier.

DISCUSSION

Field study has showed the prevalence of the yellowing disease in major black pepper growing areas in Sarawak. The disease was observed in all seven surveyed districts. Based on field observation, Sarawak's popular

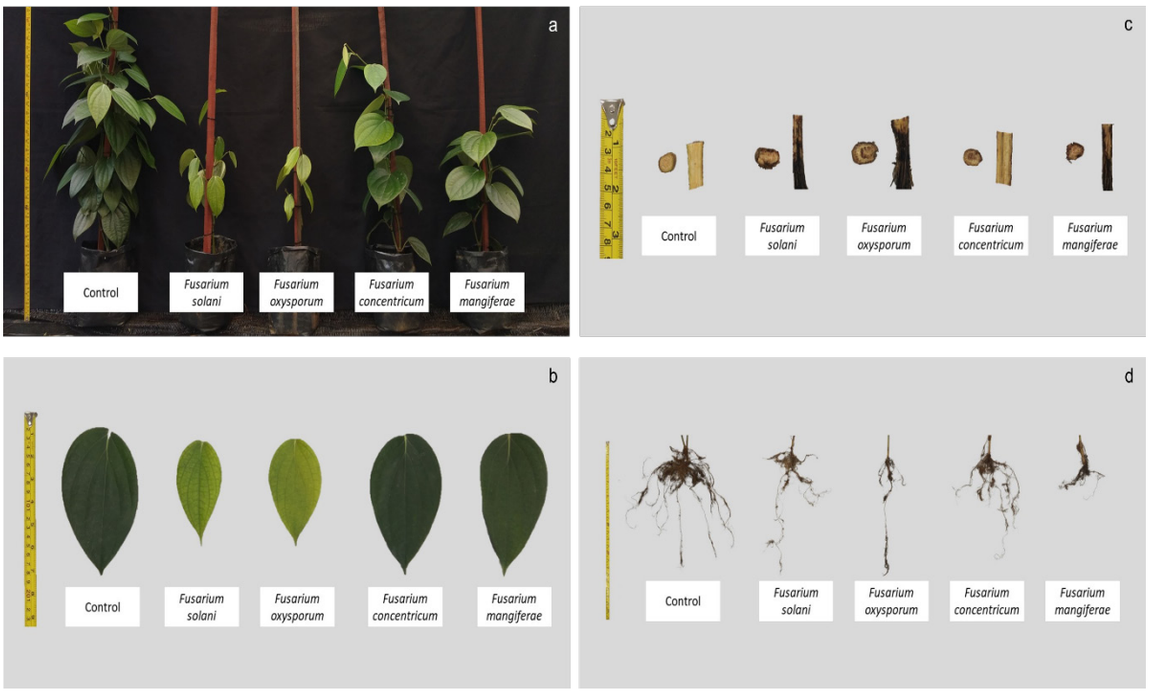


FIGURE 3. Comparison between healthy and *Fusarium*-infected black pepper plants at nine MAI. *Fusarium*-infected plants showed disease symptoms such as stunted growth (a), leaf yellowing (b), discoloration of vascular tissue (c), and reduced root mass and secondary growth (d)

TABLE 2. Plant growth parameters, disease incidence, disease severity index, and percentage of root colonisation at nine MAI

Parameters	Control	*Fusarium solani*	*Fusarium oxysporum*	*Fusarium concentricum*	*Fusarium mangiferae*
Plant height (cm)	114.3 ± 14.8^{a}	64.1 ± 11.7^{bc}	62.3 ± 15.9^{c}	83.9 ± 10.6^{b}	79.9 ± 10.6^{bc}
Leaves number	53.0 ± 8.4^{a}	24.0 ± 8.4^{b}	23.0 ± 9.75^{b}	32.4 ± 9.6^{b}	26.7 ± 9.4^{b}
Internode length (cm)	230.0 ± 42.2^{a}	129.7 ± 26.4^{c}	122.2 ± 28.1^{c}	189.0 ± 38.5^{ab}	152.2 ± 32.4^{bc}
Shoot fresh weight (g)	138.8 ± 21.6^{a}	56.7 ± 15.3^{c}	56.4 ± 15.7^{c}	90.7 ± 15.0^{b}	73.8 ± 14.1^{bc}
Root fresh weight (g)	7.7 ± 1.7^{a}	4.0 ± 1.1^{cb}	4.1 ± 1.0^{cb}	5.9 ± 1.2^{ab}	3.1 ± 1.0^{c}
Shoot dry weight (g)	33.6 ± 6.3^{a}	16.2 ± 3.3^{c}	16.5 ± 3.3^{c}	23.9 ± 4.0^{b}	21.0 ± 3.43^{bc}
Root dry weight (g)	2.8 ± 0.7^{a}	1.6 ± 0.4^{b}	1.6 ± 0.4^{b}	2.1 ± 0.5^{ab}	1.5 ± 0.4^{b}
Disease incidence (%)	0.0^{b}	100.0^{a}	100.0^{a}	57.1^{a}	71.4^{a}
Disease severity index (%)	0.0^{b}	46.4^{a}	50.0^{a}	25.0^{ab}	25.0^{ab}
Root colonisation (%)	0.0^{b}	37.1^{a}	34.3^{a}	7.1^{b}	8.6^{b}

*Mean ± standard deviation. Values followed by the same alphabet in the same row did not differ significantly with 0.05 alpha value in Tukey's range test

black pepper cultivars (Kuching, Semongok Emas, Semongok Perak, and Semongok Aman) were susceptible to the disease. The first attempt to identify *Fusarium* species associated with black pepper in Malaysia was conducted by Shahnazi et al. (2012). They have identified two species of *Fusarium* associated with black pepper in Sarawak, which are *F. solani* (64%) and *F. proliferatum* (36%). Based on pathogenicity assay, *F. solani* was identified as the causal agent, and *F. proliferatum* was regarded as saprophytic despite its relatively high occurrence in black pepper. Nevertheless, *F. proliferatum* is a serious fruit rot pathogen on chili pepper, but no previous study has reported it as a causal agent of yellowing disease in black pepper (Rampersad & Teelucksingh 2011). Interestingly, *F. proliferatum* was not found in this study since the occurrence of *Fusarium* species may vary in different sampling areas. This study has identified the two previously reported agents (*F. solani* and *F. oxysporum*) in black pepper and two unreported *Fusarium* species, *F. concentricum* and *F. mangiferae* (Biju et al. 2019). Based on the fungal isolation frequency, *F. solani* is the primary causal agent of black pepper yellowing disease in Sarawak. The isolation frequency of *F. oxysporum*, *F. concentricum,* and *F. mangiferae* was comparatively low.

Cultural and morphological characteristics are useful in *Fusarium* species identification. Colony pigmentation, macroconidia shape and size, presence of chlamydospore, and types of conidiogenous cells have provided the highest resolution for species identification. Yet, morphological identification can be subtle due to ambiguity caused by environmental factors such as culture medium (salt concentrations), temperature, pH, light, and humidity (Leslie & Summerell 2006). These factors directly affect fungal biomass, bioactive compounds, and enzymatic activities (Hamedi, Ghanati & Vahidi 2012). Thus, standardisation in culture conditions is important for fungal morphological characterization. This also highlights the need for morphologically identified species to be reassessed with molecular data. Colony pigmentation was notably varied among species and within species. Interestingly, *F. solani* isolates produced different shades of pigments on PDA. Pigmentation was reported as a virulence factor in *Fusarium* species linked to fungal pathogenesis (Malz et al. 2005). Pathogenicity test has demonstrated that the wine-red pigmented *F. solani* isolates were more virulent than the colourless isolates (Shahnazi et al. 2012). Toxicity test conducted using culture filtrate of red pigments producing-*F. solani* has shown the most intense vein discoloration in black pepper (Duarte & Archer 2003). Thus, the red pigments of *F. solani* could be associated with toxigenic activity during infection. A study on red pigment has demonstrated that it has antioxidant properties to reduce oxidative stress (Menezes et al. 2020). Hence, the bioactive pigments produced by *F. solani* could protect against oxidative stress and tissue damage induced by plant defense mechanisms. The high degree of morphological variation exhibited by the *F. solani* isolated from black pepper suggests that they may vary in virulence.

Phylogenetic analysis was in accordance with the highly variable morphological characteristics detected among the isolates. The *F. solani* isolates from the Belaga district were found to have a higher degree of genetic variation at the species level than in other districts. *F. solani* population from Belaga district has formed a monophyletic group showing a positive correlation with geographical region. The variation could be due to the rapid adaptation of fungi to the local-scale environmental conditions, agronomic practices, and different black pepper varieties (Hafizi et al. 2013; Klix, Beyer & Verreet 2008). The influence of crop genetics on pathogen population structure was demonstrated in vineyards planted with more distinctive grape varieties. A more differentiated *Saccharomyces cerevisiae* population was found in these vineyards (Schuller et al. 2012). In addition, our finding agreed with the classification of *F. solani* isolates from black pepper in Malaysia into two significant clusters based on inter simple sequence repeat (ISSR) analysis (Shahnazi et al. 2014). This could pose a risk for the cross-district distribution of infected black pepper cuttings for propagation purposes. The rapid evolution of the pathogen's genetics also demands effective resistance breeding programs in black pepper.
Based on the pathogenicity assay, *F. solani* and *F. oxysporum* were the most aggressive among the *Fusarium* species. *Fusarium*-infected black pepper plants will gradually decline in vigor and appear similar to nitrogen deficiency. The infected plants are not likely to cause sudden death, but the productivity per plant will be reduced. Vegetative cuttings for black pepper propagation have often resulted in the fungal spread. Local dispersal of *Fusarium* inoculum can be directed by rain splash and irrigation system. The infection usually involves invasive fungal penetration through the root tips and progressively advancing to the xylem vessels. Understanding *Fusarium* epidemiology in black pepper is crucial in developing an effective disease management strategy.

Conclusions

This study has identified four *Fusarium* species associated with black pepper by morphological and molecular characteristics. Based on the pathogenicity assay, all four species were identified as pathogenic to black pepper. Field surveys have confirmed that *F. solani* is the most prevalent causal agent of yellowing disease. Morphological characterisation and phylogenetic analysis have suggested a high level of genetic diversity in *F. solani*. Hence, future research could be extended to investigate the population genetics of *F. solani* in black pepper paired with a comparative pathogenicity study. The information obtained could help to formulate effective disease management strategies and breed resistant black pepper varieties.

Acknowledgements

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SUPPLEMENTARY TABLE 1. Location of sampling sites and species of *Fusarium* isolates from black pepper in Sarawak

No.	Isolate	*Fusarium* Species	Location	Source	ITS[a]	EF-1α [b]
1	PN01	*F. solani*	Belaga	Berry	MK903034	MN393191
2	PN02	*F. solani*	Belaga	Berry	MK903035	MN393192
3	PN04	*F. solani*	Bintulu	Leaf	MK909936	MN393193
4	PN05	*F. solani*	Bintulu	Leaf	MK909937	MN393194
5	PN06	*F. solani*	Miri	Root	MK909938	MN393195
6	PN07	*F. solani*	Miri	Root	MK909939	MN393196
7	PN08	*F. solani*	Miri	Root	MK909940	MN393197
8	PN09	*F. solani*	Miri	Root	MK909941	MN393198
9	PN10	*F. solani*	Bintangor	Root	MK909942	MN393199
10	PN11	*F. solani*	Bintangor	Root	MK909943	MN393200
11	PN12	*F. solani*	Julau	Basal stem	MK909944	MN393201
12	PN13	*F. solani*	Julau	Basal stem	MK909945	MN393202
13	PN14	*F. solani*	Julau	Basal stem	MK909946	MN393203
14	PN15	*F. solani*	Julau	Basal stem	MK909947	MN393204
15	PN16	*F. solani*	Julau	Root	MK909948	MN393205
16	PN17	*F. solani*	Julau	Root	MK909949	MN393206
17	PN18	*F. solani*	Julau	Basal stem	MK909950	MN393207
18	PN19	*F. solani*	Julau	Root	MK909951	MN393208
19	PN20	*F. solani*	Julau	Root	MK909952	MN393209
20	PN21	*F. solani*	Julau	Basal stem	MK909953	MN393210
21	PN22	*F. solani*	Julau	Root	MK909954	MN393211
22	PN23	*F. solani*	Julau	Root	MK909955	MN393212
23	PN24	*F. solani*	Julau	Root	MK909956	MN393213
24	PN25	*F. solani*	Julau	Root	MK909957	MN393214
25	PN27	*F. solani*	Bintulu	Root	MK909959	MN393216
26	PN28	*F. solani*	Bintulu	Root	MK909960	MN393217
27	PN29	*F. solani*	Bintulu	Root	MK909961	MN393218
28	PN31	*F. solani*	Bintulu	Root	MK909963	MN393220

29	PN32	*F. solani*	Belaga	Basal stem	MT328730	MZ226962
30	PN34	*F. solani*	Belaga	Berry	MZ045755	MZ226963
31	PN35	*F. solani*	Belaga	Berry	MZ045756	MZ226964
32	PN36	*F. solani*	Bintangor	Root	MZ045757	MZ226965
33	PN37	*F. solani*	Bintangor	Berry	MZ045758	MZ226966
34	PN38	*F. solani*	Julau	Leaf	MZ045759	MZ226967
35	PN39	*F. solani*	Miri	Root	MZ045760	MZ226968
36	PN40	*F. solani*	Belaga	Leaf	MZ045761	MZ226969
37	PN41	*F. solani*	Belaga	Basal stem	MZ045762	MZ226970
38	PN42	*F. solani*	Belaga	Basal stem	MZ045763	MZ226971
39	PN43	*F. solani*	Belaga	Root	MZ045764	MZ226972
40	PN44	*F. solani*	Belaga	Leaf	MZ045765	MZ226973
41	PN45	*F. solani*	Belaga	Leaf	MZ045766	MZ226974
42	PN46	*F. solani*	Belaga	Leaf	MZ045767	MZ226975
43	PN47	*F. solani*	Belaga	Basal stem	MZ045768	MZ226976
44	PN48	*F. solani*	Belaga	Root	MZ045769	MZ226977
45	PN49	*F. solani*	Belaga	Root	MZ045770	MZ226978
46	PN50	*F. solani*	Belaga	Root	MZ045771	MZ226979
47	PN51	*F. solani*	Betong	Root collar	MZ045772	MZ226980
48	PN52	*F. solani*	Betong	Root collar	MZ045773	MZ226981
49	PN53	*F. solani*	Betong	Root collar	MZ045774	MZ226982
50	PN54	*F. solani*	Betong	Root collar	MZ045775	MZ226983
51	PN55	*F. solani*	Betong	Root collar	MZ045776	MZ226984
52	PN56	*F. solani*	Betong	Root collar	MZ045777	MZ226985
53	PN57	*F. solani*	Betong	Root collar	MZ045778	MZ226986
54	PN58	*F. solani*	Betong	Root collar	MZ045779	MZ226987
55	PN59	*F. solani*	Betong	Root collar	MZ045780	MZ226988
56	PN60	*F. solani*	Betong	Root collar	MZ045781	MZ226989
57	PN61	*F. solani*	Betong	Root collar	MZ045782	MZ226990
58	PN62	*F. solani*	Betong	Root collar	MZ045783	MZ226991
59	PN63	*F. solani*	Betong	Root collar	MZ045784	MZ226992
60	PN64	*F. solani*	Betong	Root collar	MZ045785	MZ226993
61	PN65	*F. solani*	Betong	Root collar	MZ045786	MZ226994
62	PN66	*F. solani*	Betong	Root collar	MZ045787	MZ226995
63	PN67	*F. solani*	Mukah	Root	MZ045788	MZ226996
64	PN68	*F. solani*	Belaga	Root	MZ045789	MZ226997
65	PN69	*F. solani*	Belaga	Root	MZ045790	MZ226998
66	PN70	*F. solani*	Belaga	Leaf	MZ045791	MZ226999
67	SPN01	*F. oxysporum*	Bintulu	Root	MN133945	MN393221
68	SPN09	*F. oxysporum*	Belaga	Basal stem	MT328734	MZ293789
69	SPN10	*F. oxysporum*	Belaga	Root	MT328735	MZ293790
70	SPN11	*F. oxysporum*	Belaga	Leaf	MZ045792	MZ227000
71	SPN12	*F. concentricum*	Belaga	Leaf	MZ045793	MZ227001
72	SPN13	*F. concentricum*	Belaga	Leaf	MZ045794	MZ227002
73	SPN14	*F. mangiferae*	Belaga	Leaf	MZ045795	MZ227003

[a]GenBank accession number nuclear rDNA ITS1-ITS4 (ITS) sequences

[b]GenBank accession number for translation elongation factor-1 alpha (EF-1α) partial sequences

SUPPLEMENTARY TABLE 2. *Fusarium* species ITS and EF-1α sequences retrieved from the NCBI GenBank database

No.	*Fusarium* Species	Isolate	Country	Host	ITS	EF-1α
1	*F. solani*	NQH-6	China	Qian Hu	MN249607	MN244531
2	*F. solani*	MRR-035	India	Mulberry	MF536680	MW451301
3	*F. solani*	DPCT0101-1	Thailand	Mango	LC581430	LC581452
4	*F. oxysporum*	GXR-1	China	Giant Bamburanta	KX253978	KX253982
5	*F. concentricum*	FJAT-31669	China	Himalayan Paris	MH613764	MH613767
6	*F. fujikuroi*	HJYB-4	China	Winged Prickly Ash	MT864359	MT902140