Malaysian Journal of Microbiology, Vol 17(2) 2021, pp. 165-177
DOI: http://dx.doi.org/10.21161/mjm.200938

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Morphological and molecular characterization of *Fusarium* spp. associated with *Fusarium* wilt disease of *Piper nigrum* L. in Northwestern region of Sarawak

Wan Joo Tan[1], Tu Anh Vu Thanh[1], Elissa Stella Rafael[1], Yi Shang Chen[2] and Freddy Kuok San Yeo[1*]

[1]Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia.
[2]Malaysia Pepper Board, Lot 1115, Jalan Utama, Tanah Putih, P.O. Box 1653, 93916 Kuching, Sarawak, Malaysia.
Email: yksfreddy@unimas.my

Received 14 July 2020; Received in revised form 21 August 2020; Accepted 16 November 2020

ABSTRACT

Aims: *Piper nigrum* L. (black pepper) is an economically important commodity plant in Malaysia, which generated RM 200.95 million from pepper export in the year of 2018. However, the increase in pepper production is restricted by diseases. *Fusarium* wilt is one of the major diseases of *P. nigrum* L. The objectives for this study were to isolate *Fusarium* spp. associated with *Fusarium* wilt of *P. nigrum* L. from selected pepper farms in the northwestern region of Sarawak and to characterize the *Fusarium* spp. isolated morphologically and molecularly.
Methodology and results: *Fusarium* spp. were isolated from diseased root samples. The pathogen was grown on potato dextrose agar (PDA) under dark condition at circa (ca.) 25 °C for morphological characterisation. Molecular characterisation was done by using internal transcribed spacer (ITS). Phylogenetic tree was constructed to study the genetic relationship of the isolates. *Fusarium solani*, *F. oxysporum, F. proliferatum* were the three *Fusarium* species identified. There were variations in morphological characters observed between and among the species, including the colony form, margin, elevation, surface appearance and pigmentation. No distinctive morphological characteristic was specific to a location. In addition, growth rate, macroconidia sporulation rate, and microconidia sporulation rate of the isolates were not correlated. In molecular phylogeny, the three *Fusarium* species were separated into three distinct clades representing the three identified species. The genetic relatedness between isolates within each species was depicted in the tree.
Conclusion, significance and impact of study: Variations were observed among isolates in this study based on morphological and molecular characterization. This study would contribute information on the variations of *Fusarium* spp. associated with *Fusarium* wilt of *P. nigrum* L. from the northwestern region of Sarawak.

Keywords: Fusarium, *Fusarium* wilt, internal transcribed spacer (ITS), *Piper nigrum* L., black pepper

INTRODUCTION

Black pepper (*Piper nigrum* L.) is a perennial woody vine which produces berries that are commonly known as peppercorns. As this spice is being widely used in traditional medicine and culinary purposes, *P. nigrum* L. is the most economically important species among the *Piperaceae* family. The plants are commercially cultivated in countries such as Malaysia, Brazil, Vietnam, India and Indonesia (Tiing *et al.*, 2012). According to Malaysian Pepper Board (2018), the pepper production of the nation reached 31,073 tonnes with the total export of 11,714 tonnes that worth RM 200.95 million in 2018. Among the total coverage area of 17,437 hectares of pepper plantation, 98% of the cultivation is located in Sarawak and operated intensively by the local smallholders with an average 2000 vines per hectare (Adama *et al.*, 2018).

Crop loss due to diseases is one of the primary constraints in increasing the yield of pepper production in Malaysia which results in diminishing of 2% of the pepper cultivation area annually (Krishnamurthy *et al.*, 2010). One of the major diseases is *Fusarium* wilt, which is also known as slow wilt or yellowing disease (Gordo *et al.*, 2012; Tiing *et al.*, 2012). The disease may cause as high as 32% of annual loss in Indonesia (Mustika, 1990). Duarte and Albuquerque (1991) also reported that the pepper plantation economic lifespan will drop from 20 years to 6-8 years due to *Fusarium* infestation.

The most typical symptom of the disease is the brown to black lesion in the xylem tissue of the plant root (Eng, 2011). The infected plant will start yellowing and drooping of leaves occurs in whole canopy, then the plant appeared to be flaccid, foliar and foot rot due to the malfunction of the roots (Eng, 2011; da Luz *et al.*, 2017). Later, the

***Corresponding author**

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malaysian Journal of Microbiology, Vol 17(2) 2021, pp. 165-177
DOI: http://dx.doi.org/10.21161/mjm.200938

severely infected plant will dieback due to the loss of its feeder roots. Most of the studies reported that *Fusarium* wilt disease is caused by *Fusarium. solani* (de Souza *et al.*, 2011; Shahnazi *et al.*, 2012). However, some studies claimed that *Fusarium. oxysporum* is responsible for the disease (Duarte *et al.*, 2001; Wiratno *et al.*, 2019)

Correct identification of the pathogen and adequate knowledge on the pathogen diversity are crucial in developing disease early detection protocols of the pathogen and thus effective disease management measures. However, the study of *Fusarium* spp. associated with *Fusarium* wilt and their relatedness are still poor in Sarawak. Hence, the objectives of this study were: (1) to isolate *Fusarium* spp. associated with *Fusarium* wilt of *P. nigrum* L.; (2) to characterize the *Fusarium* isolates morphologically and molecularly.

MATERIALS AND METHODS

Sample collection

Samples of diseased roots of black pepper were collected from 15 pepper farms located in Kuching, Serian, Sri Aman and Betong division of Sarawak (Table 1). Three diseased root samples were collected from each farm.

Fungal isolation

Isolation method based on Shahnazi *et al.* (2012) was used. Briefly, the root samples were cleaned under running tap water and surface sterilised using sodium hypochlorite (1% v/v) for 5 min. The samples were then rinsed three times with sterile distilled water. Cross section of the diseased root was thinly sliced off and three slices for each sample were placed on surface of water agar (WA) plate. The plates were then incubated at room temperature (ca. 25 °C) in dark condition and observed daily for the growth of fungal mycelia. Plugs of mycelia from the edge of predominantly growing fungal colony was transferred onto fresh potato dextrose agar (PDA) plates separately, for pure culture establishment in the next step.

Establishment of pure culture

Hyphal fragment isolation method was used based on Leyronas *et al.* (2012). Under a dissecting microscope, a tiny block of well separated hypha grown from a colony was transferred onto new PDA plate for each isolate. The plates were incubated at room temperature (ca. 25 °C) in dark condition and observed daily. The steps were repeated until pure culture of each isolate was established.

Species identification

All *Fusarium* spp. isolated from diseased root samples of *P. nigrum* L. were pre-identified and grouped based on the morphological characteristics of colony, such as texture, margin, colony elevation and pigmentation. Then, a total of 24 isolates representing different groups, different morphological variations, sampling locations and species (Table 2) were randomly selected for molecular identification using internal transcribed spacer (ITS) sequence and morphological characterization. Fungal genomic DNA was extracted using Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1987) with modification. The mycelia of four days old culture were scraped and transferred into a microcentrifuge tube. The mycelia were ground together with 500 µL of CTAB solution, which was then incubated in 65 °C water bath for an hour. After incubation, the sample was spun down, and 500 µL of chloroform isoamyl alcohol (CIA) were added and mixed well. The mixture was spun at 13,500 rpm for 15 min before the supernatant was transferred into a new microcentrifuge tube. Then, proper amount of isopropyl alcohol was added, mixed well and stored overnight at 4 °C. The obtained DNA pellet was then washed with 1 mL of 70% ethanol and centrifuged for another 15 min at 13,500 rpm. After that, the ethanol was discarded, and the DNA pellet was air-dried. After the DNA pellet was completely dried, 50 µL of sterile distilled water was added. The dissolved DNA was stored in -20 °C before use. The quality and concentration

Table 1: Location of pepper farms from which diseased roots were collected for pathogen isolation.

Farm code	Location	Division	GPS coordinate
JHF	Stass	Kuching	N01°23’11.259”, E109°58’27.6384”
SMG	Semenggok	Kuching	N01°23’35.2356”, E110°19’35.637”
JMF	Paon Gahat	Serian	N00°56’58.1352”, E110°39’7.8408”
JDF	Linsat	Serian	N01°03’41.4642”, E110°43’21.594”
CXF	Linsat	Serian	N01°03’15.6558”, E110°43’37.0842”
RNF	Linsat	Serian	N01°02’48.015”, E110°43’13.4214”
BLF	Jaong	Sri Aman	N01°04’44.1228”, E110°59’23.6652”
JG1	Jaong	Sri Aman	N01°05’38.9256”, E110°59’16.3422”
JG2	Jaong	Sri Aman	N01°05’47.6628”, E110°59’24.1758”
PTU	Pantu	Sri Aman	N01°05’9.7476”, E111°06’40.9098”
GMF	Gua Ming	Sri Aman	N01°04’30.5358”, E111°17’10.1796”
SDM	San Demam	Sri Aman	N01°07’13.9794”, E111°35’25.875”
JLK	Jelukong	Sri Aman	N01°10’4.6704”, E111°39’32.3784”
BTG	Saka	Betong	N01°23’15.8208”, E111°33’0.0426”
JNF	Saka	Betong	N01°22’49.1334”, E111°33’53.3232”

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malaysian Journal of Microbiology, Vol 17(2) 2021, pp. 165-177
DOI: http://dx.doi.org/10.21161/mjm.200938

of extracted genomic DNA were determined using spectrophotometer (IMPLEN NanoPhotometer® P-Class).

DNA samples were used for ITS amplification. Universal primer pair ITS-1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS-4: 5'-TCCTCCGCTTATTGATATGC-3' was used (Shahnazi *et al.*, 2012; Hussin *et al.*, 2020). The polymerase chain reaction (PCR) was performed in a total volume of 25 µL reaction mixture, containing 1× PCR buffer, 0.2 mM dNTP mixture, 1.5 mM $MgCl_2$, 1 U/µL *Taq* DNA polymerase (First Base), 0.1 pmol of forward and reverse primers and template DNA in a Sensoquest labcycler (Sensoquest, Gottingen, Germany) with the following conditions: pre-denaturation at 95 °C for 2 min; 35 cycles of denaturation at 95 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 1 min; and final extension at 72 °C for 10 min.

The PCR products were then analysed using electrophoresis together with 100 bp DNA ladder on a 1% agarose gel with 1× TAE buffer. Ethidium bromide was used for gel staining before visualizing the PCR products under UV-transilluminator. QIAquick® Gel Extraction Kit (QIAGEN) was used to purify the PCR products, which were then sent for direct sequencing (Apical Scientific Sdn. Bhd.). ITS sequences of the isolates were BLASTN against sequences in NCBI (National Centre for Biotechnology Information) database. Percentage of identity was used to verify the species (Table 2).

Morphological characterisation

Morphological identification of the *Fusarium* isolates was based on the description by Nelson *et al.* (1983) and Leslie and Summerell (2007). Three replicates were done for each isolate. Colony characteristics such as texture, margin, colony elevation and pigmentation were recorded for three replicates of each isolates. The shape of macroconidia and microconidia was observed under a compound microscope (Nikon Eclipse E100) and recorded.

Colony growth rate from the three replicate plates for each isolate were observed daily and colony radius were measured using ruler. A twelve-day old colony was washed using 5 mL of distilled water. Serial dilution was carried out until the concentration of 10^{-2}. Then, the diluted suspension was used to study the sporulation rate with the aid of haemocytometer (LW Scientific). Quantity of both macroconidia and microconidia were calculated and recorded. There were three replicates for each isolate. The data collected for both growth rate and sporulation rate were analysed using one-way ANOVA followed by Tukey HSD post hoc test (p<0.05) using IBM SPSS Statistics 25 software.

Phylogenetic analysis

A phylogenetic dendrogram was constructed based on ITS sequences of the 24 *Fusarium* spp. viz. ten isolates of *F. solani*; three of *F. oxysporum* and 11 of *F. proliferatum* from this study together with ten isolates of *F. solani* and *F. proliferatum,* respectively from previous study by Shahnazi *et al.* (2012). ITS sequences were aligned using Clustal W (MEGA X). Neighbour joining (NJ) phylogenetic tree generated using MEGA X. *Fusarium staphyleae* strain NRRL 22316 was taken as outgroup species. Phylogenetic dendrogram with the bootstrap value which illustrated 1,000 replicate heuristic search was generated.

RESULTS

***Fusarium* spp. isolation**

A total of 93 *Fusarium* spp. isolates were isolated and pre-identified from 43 diseased root samples of *P. nigrum* L. Based on the colony morphology characteristics, such as form, margin, colony elevation, surface texture and pigmentation, the isolates were grouped into nine groups (data not shown).

A total of 24 isolates representing different groups, different morphological variations and sampling locations (Table 2) were randomly selected for species identification and replicated for morphological characterization.

Species identification

The ITS sequence length of the 24 selected isolates, representing different groups, different morphological variations, sampling locations and species (Table 2), ranged from 517-525 bp. The sequenced ITS and 5.8S rDNA (ribosomal DNA) regions had a high percentage of identity (99.2-100%) in comparison to the *Fusarium* sequences in NCBI database (Table 2). Three species, viz. *F. solani*, *F. oxysporum* and *F. proliferatum* were identified. There were ten isolates of *F. solani*; three of *F. oxysporum* and eleven of *F. proliferatum* (Table 2).

Morphology characterisation

Six colony morphological characteristics i.e. form, margin, elevation, colony surface, and pigmentation on both upper view and reverse view were studied. Generally, *F. solani* isolates displayed the most variation compared to *F. oxysporum* and *F. proliferatum*, with 21, 11 and 20 types variations in total, respectively on the six characteristics studied (Table 3).

There were two categories of variation on the form, margin, and elevation, respectively and four variations of colony surface among the *F. solani* isolates. Variation of pigment among *F. solani* were the most, with six variation of pigment ranging from grey-white to brown-grey on upper view and five variation of pigment from greyish-pink to brown white on reverse view.

The form of *F. solani* isolates were mostly circular (Figure 1A-1D) except for isolate Fs-6RN which was filamentous in shape. The margin of *F. solani* isolates were curled except that of isolate Fs-9T1 which had piliform margin. Isolate Fs-6RN and Fs-9T1 were also the only two isolates with raised colony elevation while the other *F. solani* isolates had flat colony (Table 3).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malaysian Journal of Microbiology, Vol 17(2) 2021, pp. 165-177
DOI: http://dx.doi.org/10.21161/mjm.200938

Table 2: Identification of *Fusarium* spp. based on ITS region sequencing.

Fusarium spp.	Isolate code	Sampling farm code	Location	Accession number	Sequence with best match	Max identity (%)	E - value
F. solani	Fs-7SMG	SMG	Semonggok, Kuching	MT730093	LC055798	99.60	0.0
	Fs-9T1	SMG	Semonggok, Kuching	MT730096	JQ922211	99.22	0.0
	Fs-6RN	RNF	Linsat, Serian	MT730091	LC055798	99.60	0.0
	Fs-8JD	JDF	Linsat, Serian	MT730094	HQ696788	99.23	0.0
	Fs-8JM	JMF	Paon Gahat, Serian	MT730095	KY776027	99.61	0.0
	Fs-5JG	JG1	Jaong field 1, Sri Aman	MT730090	MH517359	99.61	0.0
	Fs-JOG1	JG1	Jaong field 1, Sri Aman	MT730097	MH517359	99.81	0.0
	Fs-JOG2	JG1	Jaong field 1, Sri Aman	MT730098	MH517359	99.61	0.0
	Fs-JOG3	JG1	Jaong field 1, Sri Aman	MT730099	MH517359	100	8e - 167
	Fs-6SDM	SDM	San Demam, Sri Aman	MT730092	MF579583	99.81	0.0
F. oxysporum	Fo-4JH	JHF	Stass, Kuching	MT730112	JN859834	100	0.0
	Fo-4RN	RNF	Linsat, Serian	MT730113	JN859834	100	0.0
	Fo-2BL	JG2	Jaong field 2, Sri Aman	MT730111	JN859834	100	0.0
F. proliferatum	Fp-1JH	JHF	Stass, Kuching	MT730101	MH715405	100	0.0
	Fp-3JH	JHF	Stass, Kuching	MT730105	MH715405	100	0.0
	Fp-9T2	SMG	Semonggok, Kuching	MT730110	MG543732	100	0.0
	Fp-2RN	RNF	Linsat, Serian	MT730102	MH715405	100	0.0
	Fp-3CX	CXF	Linsat, Serian	MT730104	MH715405	100	0.0
	Fp-3RN	RNF	Linsat, Serian	MT730108	MH715405	100	0.0
	Fp-3JM	JMF	Paon Gahat, Serian	MT730106	MH715405	100	0.0
	Fp-5JM	JMF	Paon Gahat, Serian	MT7301019	MH715405	100	0.0
	Fp-1GM	GMF	Gua Ming, Sri Aman	MT730100	MG543732	100	0.0
	Fp-3PTU	PTU	Pantu, Sri Aman	MT730107	MH715405	100	0.0
	Fp-3BTG	BTG	Saka, Betong	MT730103	MH715405	100	0.0

All the *F. solani* colonies were zonate (formation of ring) and the colony surface was covered by aerial mycelia varying from fine and sparse to dense and woolly texture (Figure 1A-1D). Uniquely, aerial mycelia of isolate Fs-6RN formed feather-like pattern radiating from centre of the colony, while isolate Fs-9T1 have wool-like surface (data not shown). Most isolate formed creamy and slimy sporodochia on the surface after day 14. Clusters of perithecia were found on upper view of the colony for both isolates Fs-6SDM and Fs-8JD, which were molecularly identified as *Nectria haematococca*, a sexual form of *F. solani* (data not shown).

Generally, the pigment of colony of *F. solani* varied from brown to greyish colour on upper view, some isolates such as Fs-5JG and Fs-JOG1 have brown patches found on the edge of the colony (Figure 1A). On the contrary, isolate Fs-6RN had violet hue instead of brown (Table 3). *N. haematococca* isolates (Fs-6SDM and Fs-8JD) had orange ring near the edge of the colony (Figure 1C). On the reverse view, most of the isolates had brown to creamy white pigment, except isolate Fs-6RN which had violet hue pigment. Brown patches were observed for the isolates which had brown patches on upper view (Figure 1A). Yellow background, with tiny orange dots that looks like chia seed was uniquely found on the reverse colony of isolate Fs-6SDM and Fs-8JD (Figure 1D).

The shape of macroconidia of *F. solani* were generally curved with pointed apical cell. Three to five septa, but mostly five, were found in *F. solani* macroconidia (Figure 1E). The microconidia were mostly oval, ellipsoidal or reniform in shape. Microconidia with nil to one septum were observed (Figure 1F).

Two variations were observed on the form of *F. oxysporum* isolates; three variations on margin, and no variation were observed on the elevation of the isolates. The characteristic of colony surface was separated into two types among the *F. oxysporum* isolates. There was no variation of upper view pigment observed among *F. oxysporum,* while two types of pigments were observed on the reverse view of the colony, which were violet white and pink white.

For *F. oxysporum* isolates, the form of the colony was divided into filamentous and circular. Most of the *F. oxysporum* colonies were found to have entire margin except for isolate Fo-2BL with undulate margin (Figure 1G), and isolate Fo-4JH with curled margin. The colony elevation of all the isolates were raised (Table 3).

The surface of *F. oxysporum* colonies were covered with abundant aerial mycelia ranged from sparse to fluffy texture. Distinctively, colony of isolate Fo-2BL and Fo-4JH was zonate with aerial mycelia grown and formed feather-like pattern on the colony surface, while isolate Fo-4RN expanded randomly in a vein-like pattern from the culture plug (Figure 1G).

All the *F. oxysporum* isolates had greyish white upper view. However, the colour of the colonies' reverse view varied. The reverse view of isolate Fo-2BL and Fo-4JH showed violet colour from the centre and gradually turn to white at the edge of the colony (Figure 1H), whereas the

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malaysian Journal of Microbiology, Vol 17(2) 2021, pp. 165-177
DOI: http://dx.doi.org/10.21161/mjm.200938

Table 3: Summary of colony morphological characteristics of 24 *Fusarium* spp. isolates.

Fusarium spp.	Division	Isolate	Form	Margin	Elevation	Surface appearance	Pigmentation	
							Upper view	Reverse view
F. solani	Kuching	Fs-7SMG	Circular	Curled	Flat	Fine mycelia	Maroon-grey to white	Brown to cream white
		Fs-9T1	Circular	Piliform	Raised	Fine, woolly texture	Grey to white	Greyish pink to white
	Serian	Fs-6RN	Filamentous	Curled	Raised	Fine mycelia with feather-like pattern radiate from centre	Greyish violet	Violet to white
		Fs-8JD	Circular	Curled	Flat	Fine mycelia with clusters of perithecia formed	Yellow-grey to white. Orange outer ring formed	Yellow with tiny orange dots
		Fs-8JM	Circular	Curled	Flat	Fine mycelia	Brown to greyish white	Pale brown to cream white
	Sri Aman	Fs-5JG	Circular	Curled	Flat	Fine mycelia	Brown to greyish white	Pale brown with brown patches
		Fs-6SDM	Circular	Curled	Flat	Fine mycelia with clusters of perithecia formed	Yellow-grey to white. Orange outer ring formed	Yellow with tiny orange dots
		Fs-JOG1	Circular	Curled	Flat	Fine mycelia	Greyish white with brown patches	Pale brown with brown patches
		Fs-JOG2	Circular	Curled	Flat	Fine mycelia	Brown to greyish white	Pale brown to cream white
		Fs-JOG3	Circular	Curled	Flat	Fine mycelia	Brown to greyish white	Pale brown to cream white
F. oxysporum	Kuching	Fo-4JH	Filamentous	Curled	Raised	Fine mycelia with feather-like pattern radiate from centre	Greyish white	Violet to white
	Serian	Fo-4RN	Circular	Entire	Raised	Fine mycelia with vein-like pattern radiate from centre	Greyish white	Pinkish white
	Sri Aman	Fo-2BL	Filamentous	Undulate	Raised	Fine mycelia with feather-like pattern radiate from centre	Greyish white	Violet to white
	Kuching	Fp-1JH	Circular	Entire	Raised	Fine, woolly texture with vein-like	Greyish white	Pinkish white

(Continued)

ISSN (print): 1823-8262, ISSN (online): 2231-7538

						pattern radiate from centre		
		Fp-3JH	Circular	Entire	Raised	Fine, woolly texture with vein-like pattern radiate from centre	Greyish white	Pinkish white
		Fp-9T2	Circular	Curled	Flat	Fine mycelia	Brownish white	Brown to cream white
F. proliferatum	Serian	Fp-2RN	Irregular	Piliform	Raised	Fine, woolly texture	White	Violet to white
		Fp-3CX	Circular	Entire	Raised	Fine, woolly texture with vein-like pattern radiate from centre	White	Colourless
		Fp-3JM	Circular	Entire	Raised	Fine, woolly texture with vein-like pattern radiate from centre	Greyish white	Pinkish white
		Fp-3RN	Filamentous	Entire	Raised	Fine, woolly texture with vein-like pattern radiate from centre	Greyish white	Pinkish white
		Fp-5JM	Circular	Entire	Raised	Fine, woolly texture with vein-like pattern radiate from centre	Greyish white	Pinkish white
	Sri Aman	Fp-1GM	Filamentous	Undulate	Raised	Fine mycelia with feather-like pattern radiate from centre	White	Violet to white
		Fp-3PTU	Circular	Entire	Raised	Fine, woolly texture with vein-like pattern radiate from centre	Greyish white	Pinkish white
		Fp-3BTG	Circular	Entire	Raised	Fine, woolly texture with vein-like pattern radiate from centre	Greyish white	Pinkish white

reverse view of isolate Fo-4RN was found to be pinkish white in colour.

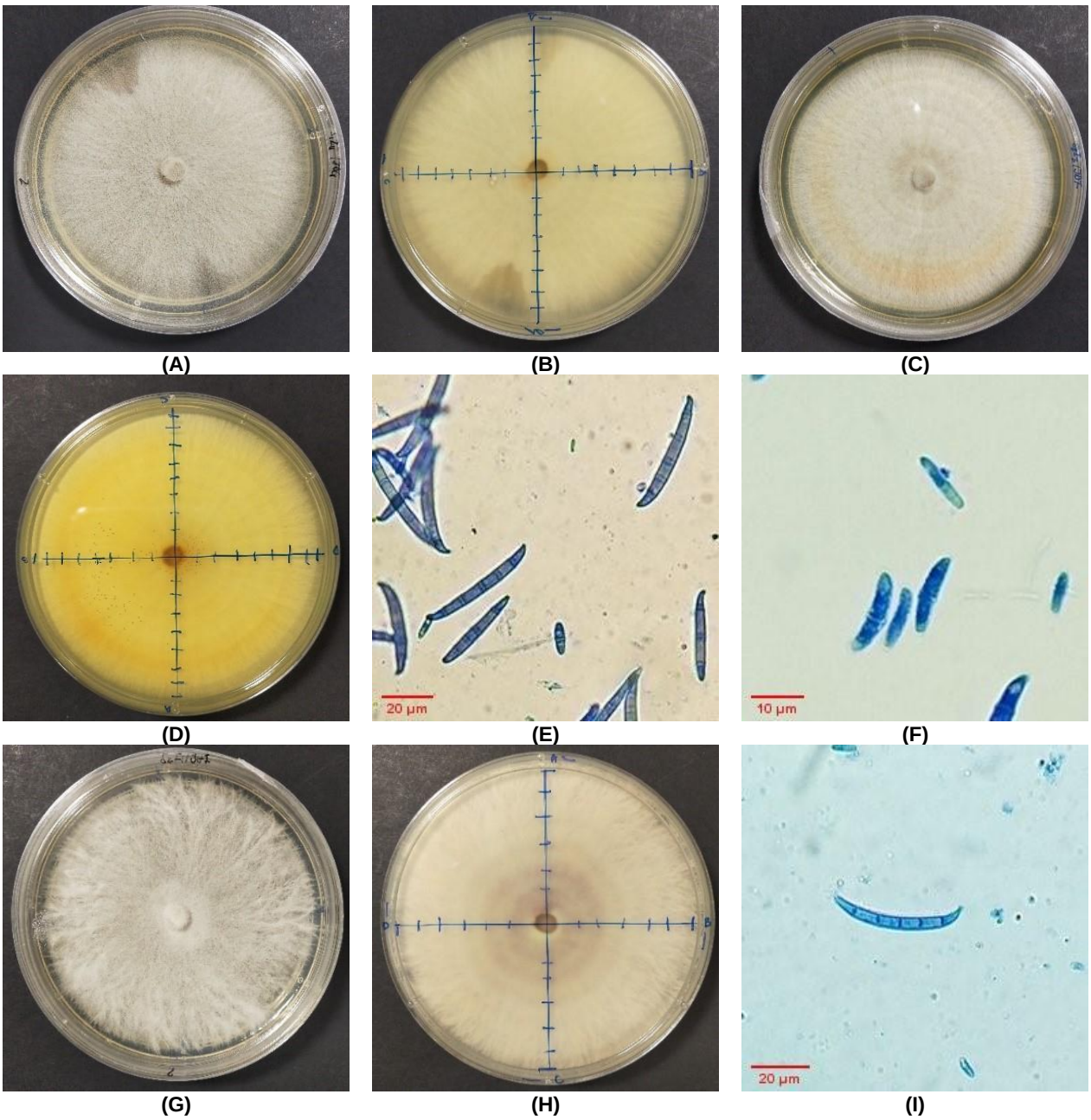
The macroconidia of *F. oxysporum* were curved and slender. Hook shape was observed on the apical cell of *F. oxysporum* macroconidia (Figure 1I). Typically, three to five septa were observed. Microconidia of *F. oxysporum* were quite similar that of *F. solani* which they were oval, ellipsoidal or reniform in shape with nil to one septum (Figure 1J).

There were three variations observed on the form of *F. proliferatum* isolates; four variations on margin, and two variations were observed on the elevation of the isolates among the *F. proliferatum* isolates. Four variations were observed on the colony surface of the *F. proliferatum* isolates. Variation of pigment among *F. proliferatum* were less compared to *F. solani* isolates but more compared to *F. oxysporum* isolates, with three variations of pigment, white, grey-white and brown-white on upper view and four variations of pigment from colourless, pink white, violet white to brown white on reverse view.

For *F. proliferatum*, all isolates were found to have colony with circular form (Figure 1K-1L), except for isolate

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malaysian Journal of Microbiology, Vol 17(2) 2021, pp. 165-177
DOI: http://dx.doi.org/10.21161/mjm.200938



(A) (B) (C)

(D) (E) (F)

(G) (H) (I)

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malaysian Journal of Microbiology, Vol 17(2) 2021, pp. 165-177
DOI: http://dx.doi.org/10.21161/mjm.200938

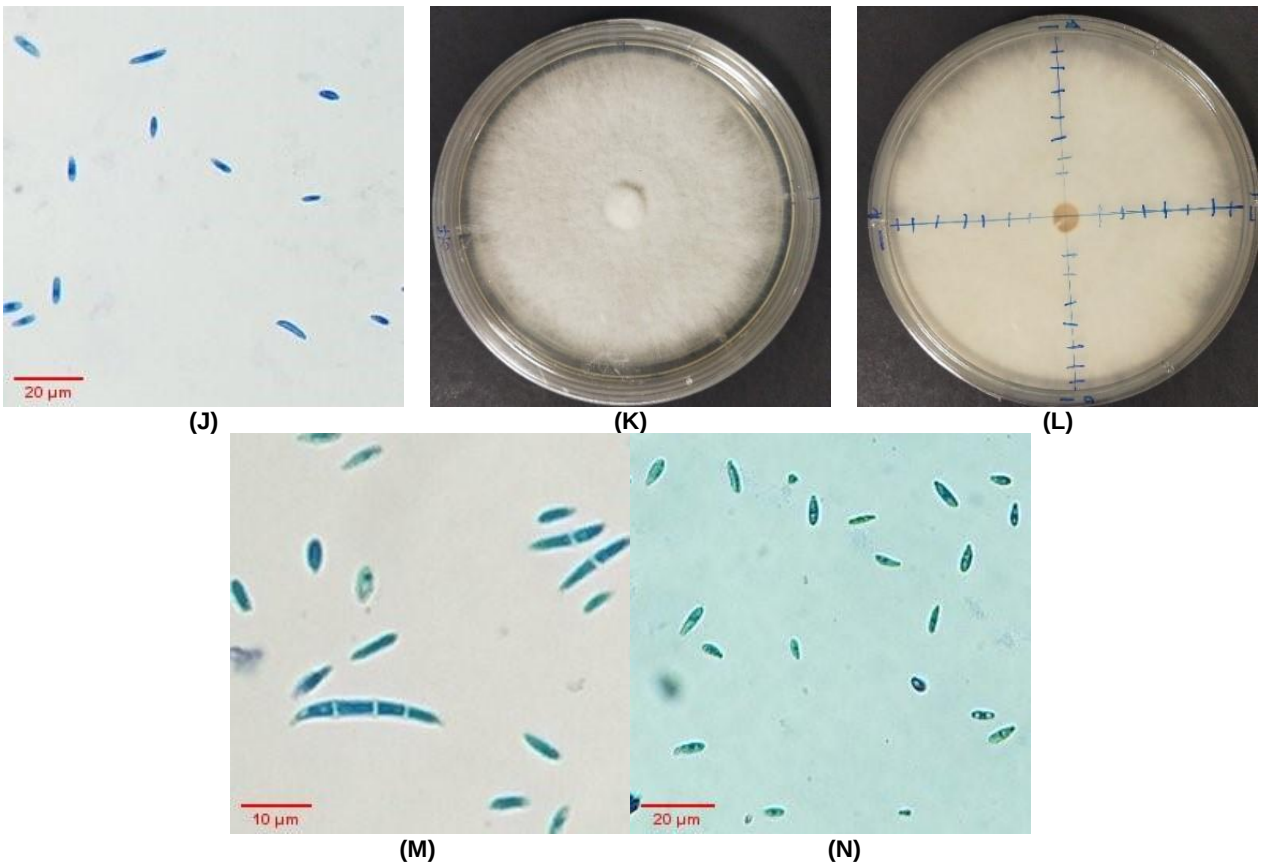


Figure 1: Macromorphology and micromorphology under microscope at 40× magnification of *Fusarium* isolates. A, upper view of *F. solani* isolates with brown patch. B, reverse view of *F. solani* isolates with brown patch. C, upper view of *N. haematococca*. D, reverse view of *N. haematococca*. E, macroconidia of *F. solani* (bar = 20 µm). F, microconidia of *F. solani* (bar = 10 µm). G, upper view of *F. oxysporum*. H, reverse view of *F. oxysporum*. I, macroconidia of *F. oxysporum* (bar = 20 µm). J, microconidia of *F. oxysporum* (bar = 20 µm). K, upper view of *F. proliferatum*. L, reverse view of *F. proliferatum*. M, macroconidia of *F. proliferatum* (bar = 10 µm). N, microconidia of *F. proliferatum* (bar = 20 µm).

Fp-1GM and Fp-3RN which had filamentous form and isolate Fp-2RN with irregular form. Four types of colony margin were found among the *F. proliferatum* isolates. Eight isolates had entire margin; one isolate with curled margin; one with piliform and one with undulate margin. All *F. proliferatum* isolates had colony with raised elevation except for isolate Fp-9T2 which had flat colony (Table 3).

The colony of *F. proliferatum* was generally zonate and appeared to have woolly texture (Figure 1K). The aerial mycelia grew out randomly in vein-like pattern in most of the *F. proliferatum* isolates. Isolates with distinctive surface features were observed. Isolate Fp-2RN had colony covered with fluffy aerial mycelia without vein-like pattern. On the other hand, isolate Fp-1GM was found to be zonate, and aerial mycelia grew into feather-like pattern that resembled *F. oxysporum* isolates, whereas isolate Fp-9T2 resembled majority of *F. solani* isolates with sparse to dense aerial mycelia.

The upper view colony pigment of *F. proliferatum* isolates varied from greyish white to white hue except for isolate Fp-9T2 which was brownish white in colour. On the reverse side, most of the isolates were found to be pinkish white in colour (Figure 1L). Two isolates (Fp-1GM and Fp-2RN) had violet colour at the centre on the reverse view and gradually become white at the edge. For isolate Fp-9T2, the reverse view had brownish white in colour. Isolate Fp-3CX had no pigmentation on the reverse side.

The macroconidia of *F. proliferatum* were curved with pointed apical cell. Generally, three to four septa were found in the macroconidia (Figure 1M). The microconidia were pyriform and club-shaped with flattened base. In general, nil to one septum was found on microconidia for some isolates (Figure 1N).

From the observations, the colony morphology characteristics of the isolates were not much related to their origins. There was no unique feature of colony morphology found to be specific to certain locations.

There were variations in the growth rate among the species. In general, the growth rate ranged from 4.00 to 4.90 mm/day for *F. solani*, 4.90 to 5.30 mm/day for *F. oxysporum* and 4.10 to 5.60 mm/day for *F. proliferatum*.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malaysian Journal of Microbiology, Vol 17(2) 2021, pp. 165-177
DOI: http://dx.doi.org/10.21161/mjm.200938

Table 4: Growth rate and spore concentration of *Fusarium* spp. isolates.

Fusarium spp.	Division	Isolate	Growth rate (mm/day)	Macroconidia sporulation rate	Microconidia sporulation rate
F. solani	Kuching	Fs-7SMG	4.90 ^{c}	$4.4 \times 10^{6} \pm 4.0 \times 10^{5}$ ^{a}	$4.0 \times 10^{7} \pm 2.6 \times 10^{5}$ ^{a}
		Fs-9T1	4.30 ^{abc}	$2.4 \times 10^{7} \pm 1.4 \times 10^{6}$ ^{c}	$4.2 \times 10^{8} \pm 2.5 \times 10^{7}$ ^{cd}
	Serian	Fs-6RN	4.90 ^{bc}	$2.5 \times 10^{6} \pm 8.8 \times 10^{4}$ ^{a}	$8.6 \times 10^{7} \pm 1.7 \times 10^{6}$ ^{ab}
		Fs-8JD	4.00 ^{a}	$3.1 \times 10^{7} \pm 3.1 \times 10^{6}$ ^{d}	$3.0 \times 10^{8} \pm 2.3 \times 10^{7}$ ^{bcd}
		Fs-8JM	4.90 ^{c}	$1.0 \times 10^{7} \pm 1.1 \times 10^{6}$ ^{b}	$4.4 \times 10^{8} \pm 1.4 \times 10^{8}$ ^{d}
	Sri Aman	Fs-5JG	4.40 ^{abc}	$3.3 \times 10^{5} \pm 1.3 \times 10^{5}$ ^{a}	$2.0 \times 10^{8} \pm 7.3 \times 10^{6}$ ^{abc}
		Fs-6SDM	4.00 ^{a}	$3.0 \times 10^{6} \pm 2.3 \times 10^{5}$ ^{a}	$2.5 \times 10^{8} \pm 7.5 \times 10^{6}$ ^{abcd}
		Fs-JOG1	4.30 ^{ab}	$1.2 \times 10^{6} \pm 2.7 \times 10^{5}$ ^{a}	$1.4 \times 10^{8} \pm 1.6 \times 10^{7}$ ^{ab}
		Fs-JOG2	4.80 ^{bc}	$2.3 \times 10^{6} \pm 4.9 \times 10^{5}$ ^{a}	$1.5 \times 10^{8} \pm 1.1 \times 10^{7}$ ^{ab}
		Fs-JOG3	4.90 ^{c}	$1.9 \times 10^{6} \pm 4.3 \times 10^{5}$ ^{a}	$3.0 \times 10^{7} \pm 5.2 \times 10^{6}$ ^{a}
F. oxysporum	Kuching	Fo-4JH	4.90 ^{a}	$9.3 \times 10^{5} \pm 8.8 \times 10^{4}$ ^{ab}	$7.1 \times 10^{7} \pm 2.5 \times 10^{6}$ ^{a}
	Serian	Fo-4RN	5.10 ^{a}	$1.4 \times 10^{6} \pm 2.9 \times 10^{5}$ ^{b}	$4.2 \times 10^{8} \pm 4.4 \times 10^{7}$ ^{c}
	Sri Aman	Fo-2BL	5.30 ^{a}	$1.6 \times 10^{6} \pm 1.9 \times 10^{5}$ ^{b}	$6.6 \times 10^{7} \pm 7.7 \times 10^{6}$ ^{a}
F. proliferatum	Kuching	Fp-1JH	5.40 ^{c}	$4.2 \times 10^{6} \pm 3.8 \times 10^{5}$ ^{b}	$4.6 \times 10^{8} \pm 5.7 \times 10^{6}$ ^{f}
		Fp-3JH	5.10 ^{bc}	$1.1 \times 10^{6} \pm 1.7 \times 10^{5}$ ^{a}	$4.7 \times 10^{8} \pm 2.4 \times 10^{7}$ ^{f}
		Fp-9T2	4.40 ^{ab}	$9.8 \times 10^{6} \pm 3.3 \times 10^{4}$ ^{c}	$2.3 \times 10^{8} \pm 2.4 \times 10^{7}$ ^{c}
	Serian	Fp-2RN	4.10 ^{a}	$1.4 \times 10^{6} \pm 3.5 \times 10^{5}$ ^{a}	$7.0 \times 10^{7} \pm 2.8 \times 10^{6}$ ^{a}
		Fp-3RN	5.10 ^{bc}	$5.3 \times 10^{6} \pm 2.0 \times 10^{5}$ ^{b}	$2.4 \times 10^{8} \pm 5.9 \times 10^{6}$ ^{c}
		Fp-3JM	5.60 ^{c}	$5.4 \times 10^{6} \pm 7.5 \times 10^{5}$ ^{b}	$2.0 \times 10^{8} \pm 9.4 \times 10^{6}$ ^{bc}
		Fp-3CX	4.30 ^{a}	$2.9 \times 10^{7} \pm 5.5 \times 10^{5}$ ^{d}	$1.6 \times 10^{8} \pm 7.8 \times 10^{6}$ ^{b}
		Fp-5JM	4.90^{abc}	$2.0 \times 10^{6} \pm 1.5 \times 10^{5}$ ^{a}	$3.3 \times 10^{8} \pm 1.9 \times 10^{7}$ ^{d}
	Sri Aman	Fp-1GM	4.90 ^{abc}	$2.0 \times 10^{6} \pm 1.5 \times 10^{5}$ ^{a}	$3.9 \times 10^{8} \pm 4.8 \times 10^{6}$ ^{de}
		Fp-3PTU	4.80 ^{abc}	$7.3 \times 10^{5} \pm 2.6 \times 10^{5}$ ^{a}	$1.7 \times 10^{8} \pm 9.6 \times 10^{6}$ ^{b}
	Betong	Fp-3BTG	4.90 ^{abc}	$4.0 \times 10^{6} \pm 1.7 \times 10^{5}$ ^{b}	$4.5 \times 10^{8} \pm 2.4 \times 10^{6}$ ^{ef}

Difference between isolates in terms of growth rate, macroconidia sporulation rate and microconidia sporulation rate were compared using variance analysis (ANOVA) followed by Tukey HSD post hoc test (p<0.05). The different alphabet indicates significant difference.

Significant difference in the growth rate was observed for isolates of *F. solani* and *F. proliferatum* but not for those of *F. oxysporum*. Isolate Fs-7SMG, Fs-8JM and Fs-JOG3 of *F. solani* grew significantly faster than isolates Fs-8JD and Fs-6SDM (Table 4), with the difference of approximately 2 days. The former covered the entire surface of the Petri dish within 9 days while the latter reached full plate around the 11th day (data not shown). For *F. proliferatum* isolate Fp-1JH and Fp-3JM grew significantly faster than Fp-2RN and Fp-3CX, 8 days versus 12 days to fully cover the surface of PDA plates (data not shown).

Similarly, macroconidia sporulation rate varied among the isolates, ranging from 3.3×10^{5} to 3.1×10^{7} for *F. solani*; 9.3×10^{5} to 1.6×10^{6} for *F. oxysporum* and 7.3×10^{5} to 2.9×10^{7} for *F. proliferatum* isolates. Both *F. solani* and *F. proliferatum* isolates showed significant difference in macroconidia sporulation rate among isolates but not between isolates of *F. oxysporum*. Isolate Fs-8JD significantly produced more macroconidia compared to other *F. solani* isolates at 3.1×10^{7}, followed by isolate Fs-9T1 at the rate of 2.4×10^{7}. Conversely, isolate Fp-3CX yielded significantly more macroconidia compared to other 10 isolates of *F. proliferatum,* at the rate of 2.9×10^{7}.

The microconidia sporulation rate for *F. solani* ranged from 3.0×10^{7} to 4.4×10^{8}, 6.6×10^{7} to 4.2×10^{8} for *F. oxysporum* and 7.0×10^{7} to 4.7×10^{8} for *F. proliferatum*. The microconidia sporulation rate of *F. solani* and *F. proliferatum* isolates was found to have significant difference between isolates but not for *F. oxysporum*.

Based on the results, the growth rates, macroconidia sporulation rate and microconidia sporulation rate of the isolated were not correlated.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malaysian Journal of Microbiology, Vol 17(2) 2021, pp. 165-177
DOI: http://dx.doi.org/10.21161/mjm.200938

Phylogenetic study

A phylogenetic dendrogram was constructed based on ITS sequences of the 24 *Fusarium* spp. (viz. ten isolates of *F. solani*; three isolates of *F. oxysporum* and eleven isolates of *F. proliferatum*), together with 10 isolates of *F. solani* and *F. proliferatum*, respectively from Shahnazi *et al.* (2012). All *Fusarium* spp. isolates were clearly separated into three distinct clades representing the three identified species (Figure 2). Within their respective clade, the isolates with similar morphology were grouped together under the same lineage regardless of their origins.

All the *F. proliferatum* isolates were in one clade with bootstrap value of 93%. All the 11 *F. proliferatum* isolates from this study which portrayed minor morphology variation among them were grouped together. There is, however, a tendency of grouping for isolates from Sibu and Sarikei.

Sister relationship between *F. proliferatum* and *F. oxysporum* was shown in molecular phylogeny as they

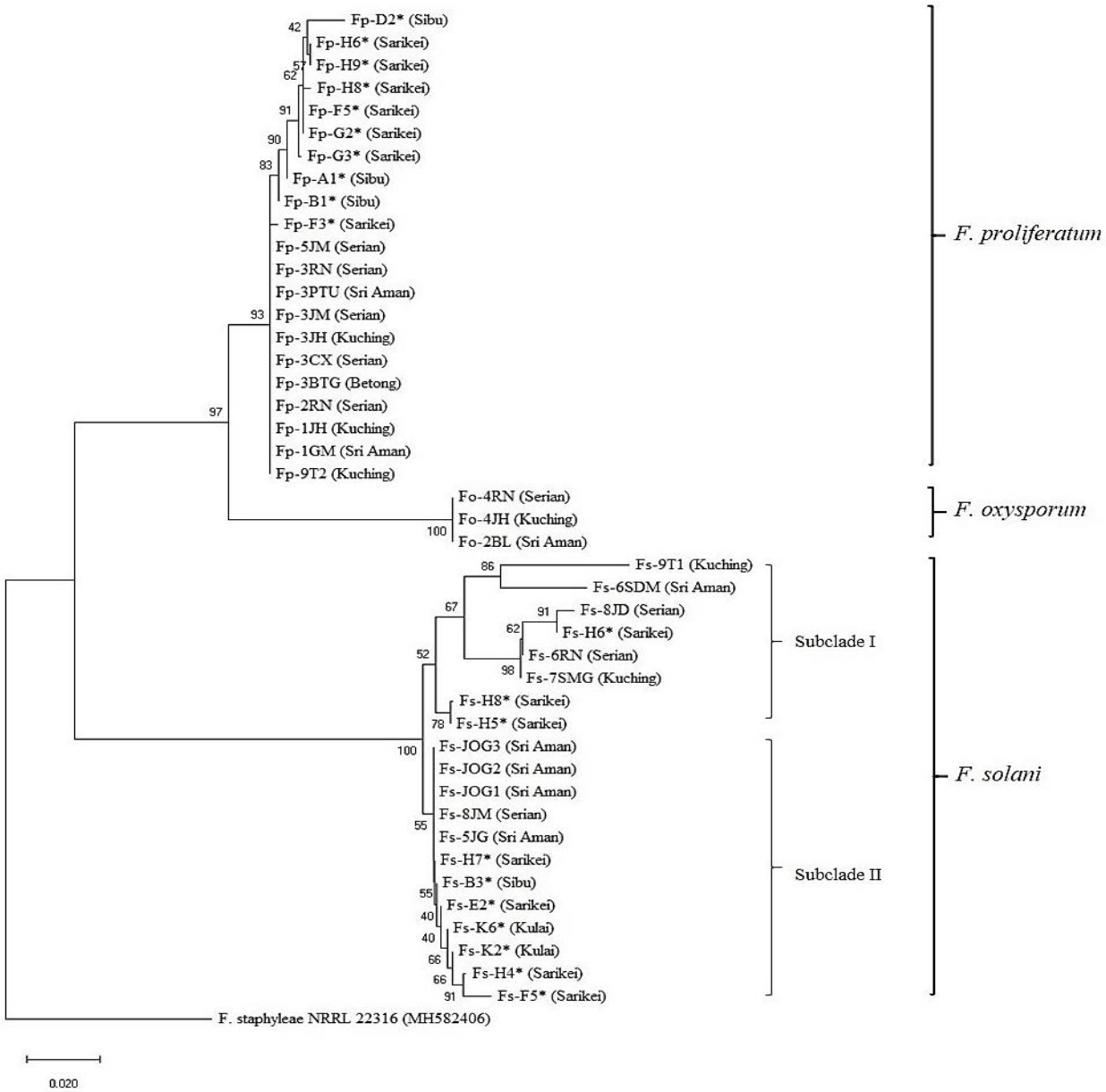


Figure 2: Neighbour joining (NJ) phylogenetic tree of *Fusarium* isolates from Malaysia using maximum likelihood method supported by bootstrap test 1,000 replicates, with 0.02 unit of base substitution per site. Isolates from Shahnazi *et al.* (2012) study were marked with *.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

shared more recent common ancestor. *Fusarium oxysporum* isolates showed 100% homology among the isolates although their origins covered three different divisions in Sarawak (Kuching, Serian and Sri Aman). The clade was supported with 91% of bootstrap value.

Lastly, *F. solani* isolates portrayed diverse phylogenetic affinities among the members. The isolates were split into two subclades. The isolates in Subclade I were very similar to each other, whereas higher variation was observed in Subclade II.

The substitution rate of *F. proliferatum* was the lowest among the three clades. *Fusarium proliferatum* and *F. oxysporum* isolates were genetically closer to each other compared to *F. solani* isolates as the clades of both *F. proliferatum* and *F. oxysporum* formed monophyletic group, supported with 97% bootstrap value. On the other hand, *F. solani* isolates mostly showed lower substitution rate compared to *F. oxysporum*, except isolates Fs-8JD, Fs-H6, Fs-6RN, Fs-7SMG, Fs-9T1 and Fs-6SDM (Figure 2).

DISCUSSION

This study generated information on the diversity of the *Fusarium* spp. associated with *Fusarium* wilt disease of *P. nigrum* L. Three species of *Fusarium* were recovered from the diseased samples collected from four divisions in Sarawak, which indicates their association with *Fusarium* wilt. However, which species was the causal agent of *Fusarium* wilt disease among all of the three were still yet to find out. Previous studies reported that the disease was caused by *F. solani* (Hamada *et al.*, 1988; Da Silva *et al.*, 2014; da Luz *et al.*, 2017). On the other hand, Duarte *et al.* (2001) and Edward *et al.* (2013) claimed that *F. oxysporum* was also an important pathogen for *Fusarium* wilt in *P. nigrum* L. Meanwhile, *F. proliferatum* was reported not pathogenic to *P. nigrum* L. plants (Shahnazi *et al.*, 2012).

There were variations in colony morphological characteristics observed among the isolates and the findings matched with previous studies whereby the isolates of the same species displayed different level of morphological variations (Shahnazi *et al.*, 2012; Haapalainen *et al.*, 2016; Berruezo *et al.*, 2018). Most of the colony morphological characteristics of the 24 isolates in this study matched the description by Nelson *et al.* (1983) and Leslie and Summerell, (2007). For example, macroconidia with mostly five septa were found in *F. solani* isolates; macroconidia with hook-shaped apical cell were observed in *F. oxysporum* isolates; and woolly textured aerial mycelia were observed in *F. proliferatum* isolates. Clusters of perithecia found on upper view of the colony for both Fs-6SDM and Fs-8JD also matched with the description for *N. haematococca,* sexual form of *F. solani*, by Wu *et al.* (2011) and Vaz *et al.* (2012).

No distinct characteristic was found to be specific to isolates from one division. Similarity in morphology of the isolates among the same species from different origin observed in this study indicates that there was no correlation between the isolate morphology and their origin. The finding was similar to the previous studies on *F. proliferatum*, *F. oxysporum* and *F. solani* (Shahnazi *et al.*, 2012; Haapalainen *et al.*, 2016; Robles-Carrión *et al.*, 2016; Berruezo *et al.*, 2018).

Variations observed in growth rate and sporulation rate of both macroconidia and microconidia of the isolates may be due to the effect of nutrient intake by the isolates (Robles-Carrión *et al.*, 2016). For instance, fast growing isolates such as Fs-8JM and Fp-3JM might be able to absorb more nutrient as compared to slow growing isolates such as Fs-6SDM and Fp-3CX. However, this assumption yet to be confirmed. No correlation observed between the growth rates, macroconidia sporulation rate, and microconidia sporulation rate, in agreement with previous study by Robles-Carrión *et al.* (2016) on *F. oxysporum* in Ecuador and also other fungal pathogens such as *Pyricularia oryzae* of Sarawak (Hussin *et al.*, 2020). Information on both the growth rate and sporulation rate of the pathogen may help in designing efficient and sustainable strategies to control the disease (Robles-Carrión *et al.*, 2016).

Diverse pigmentation among the isolates were observed ranging from white to violet to brown may suggest differences in the virulence of the isolates in this study towards *P. nigrum* L. Duarte and Archer (2003) suggested that different pigmentation of the pathogen may have different toxigenic activity. Similarly, Saviola (2014) also reported pigment production in pathogen associated with increment in their virulence towards the host. Shahnazi *et al.* (2012) reported that *F. solani* isolated from *P. nigrum* L. with dark red pigmentation showed stronger pathogenicity.

In molecular phylogeny, all the *Fusarium* spp. isolates were separated into three distinct clades, representing the three identified species. It was found that *F. proliferatum* and *F. oxysporum* shared recent common ancestor which in accordance to Haapalainen *et al.* (2016) and Kim *et al.* (2016). *F. proliferatum and F. oxysporum* isolates showed simpler clade compared to *F. solani* clade as they were grouped within one main branch (Figure 2). Haapalainen *et al.* (2016), also had the same observation. This result indicates that they were genetically close to each other as their timing of evolution were near to each other. Among the *F. proliferatum* isolates, Fp-D2 showed the longest branch length (Figure 2), which indicates that the isolate evolved at the lowest substitution rate compared to other *F. proliferatum* isolates. For *F. oxysporum*, there is no branching for the clade observed. This is probably due to low number of *F. oxysporum* isolates isolated in this study, which in turn not enough to display the variations.

On the other hand, *F. solani* isolates which does not share recent common ancestor with the other two *Fusarium* species were split into two main lineages within the clade in agreement with previous studies (Shahnazi *et al.*, 2012). *F. solani* isolates had more lineages within the subclade as compared to the other clades. The finding matched with both Shahnazi *et al.* (2012) and Hafizi *et al.* (2013) study where the clade of *F. solani* consisted more lineages. Isolate Fs-9T1 showed longest branch length among all the *Fusarium* isolates which indicates that the

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

isolate evolved at different timing compared to other isolates with the lowest substitution rate among all the *Fusarium* spp. isolated. Although *F. solani* portrayed variation in terms of molecular phylogeny, no correlation was observed between the origin of the isolates and their genetic relatedness. Therefore, it can be deduced that the genetics of the isolates were closely related to each other despite their origin. This was in agreement with previous study by Shahnazi *et al.* (2012) and Hafizi *et al.* (2013).

CONCLUSION

Three species of *Fusarium*, viz. ten isolates of *F. solani*; three isolates of *F. oxysporum* and eleven isolates of *F. proliferatum* were successfully isolated from diseased root samples of *P. nigrum* L. and identified. Different isolates collected in the northwestern region of Sarawak in this study showed that there were variations observed based on morphological and molecular characterization. Although there were morphological variations observed, no distinctive characteristic shown to be unique to specific location. Variations in growth rate, macroconidia sporulation rate, and microconidia sporulation rate were observed but they were not correlated. In molecular phylogeny, the three *Fusarium* species were separated into three distinct clades representing the three identified species. The genetic relatedness between the isolates within each species is depicted in the tree. This study would contribute information on the variations of *Fusarium* spp. associated with *Fusarium* wilt of *P. nigrum* L. from the northwestern region of Sarawak.

ACKNOWLEDGEMENTS

This project is supported by Research Acculturation Grant Scheme from Malaysia Ministry of Education, Grant No: RAGS/WAB01(1)/1319/2015(13). The authors would like to acknowledge Faculty of Resource Science and Technology, UNIMAS for the facilities provided.

CONFLICT OF INTEREST

The authors declare no conflict of interest in preparing this article.

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