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Morphological and molecular identification of *Fusarium* spp. and *Colletotrichum* spp. isolated from infected vanilla orchid

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ABSTRACT

Aims: This study aimed to isolate and identify fungi involved in causing diseases to *Vanilla planifolia* as well as to study their pathogenicity level in causing disease.
Methodology and results: The diseased parts of vanilla plants were collected from vanilla farms located in Pahang and Sabah, Malaysia from May 2015 to May 2016. Diseases tissue transplantation was adopted to isolate the fungi for morphology identification prior to the polymerase chain reaction (PCR) amplification of internal transcribed spacer (ITS) regions using universal primers for fungi, ITS1 and ITS4. After being isolated, the fungi pathogenicity was tested on detached fresh and mature vanilla leaves. A total of 22 fungal isolates were identified, *Fusarium fujikuroi* and *F. oxysporum* were the two most recovered species, followed by *Colletotrichum gloeosporioides*, *Fusarium* sp., *F. proliferatum* and *F. solani*. Pathogenicity test revealed a significantly high pathogenicity of *F. oxysporum* and *C. gloeosporiodes* (p<0.01) on detached vanilla leaf, with high level of damage.
Conclusion, significance and impact of study: This study provides valuable information on fungi-associated diseases on vanilla plants grown in Malaysia and can be used for future development in disease management.

Keywords: Colletotrichum, fungal, *Fusarium*, disease, pathogenicity

INTRODUCTION

Vanilla planifolia is an economically important herbaceous perennial vine grown in warm and humid climates (Bhai *et al.*, 2009). The crop especially the beans contain sweet natural flavour known as vanillin, has contributed a pleasant flavour in the culinary world besides being coveted for medicinal purposes over the years (Greule *et al.*, 2015; Bartoňková and Dvořák, 2018). Not only that, vanilla is used in household products, dairy products, pharmaceutical products, oral care, perfumes and toy industry (Havkin-Frenkel *et al.*, 2011). Vanilla is placed as the fourth of the fastest-growing products from 2000 to 2013, following Bambara beans, walnut and pistachios (Food and Agriculture Organization, 2015). Despite the high demand for this commodity, its production is limited by fungal diseases, thus affecting the world production (Adame-García *et al.*, 2015). Some diseases cause huge losses in vanilla production since they affect the whole plant. The common diseases affecting vanilla plants are stem rot, leaf rot, fruit rot, root rot, stem blight, brown spots and anthracnose, due to some phytopathogenic fungi (Pinaria *et al.*, 2010). In the Asia-pacific region, stem rot in vanilla plants is caused by several pathogens such as *Fusarium* sp., *Colletotrichum* sp., and *Phytophthora* sp. (Pinaria *et al.*, 2010). In Malaysia, vanilla is a newly emerged crop, so the studies on diseases associated with this plant are still limited. Most studies focus on vanilla tissue culture (Tan *et al*., 2011; Izzati *et al*., 2013).

In fact, the identification and characterisation of pathogen are based on morphological and cultural characteristics (Zakaria and Bailey, 2000; Than *et al.*, 2008). The macroscopic and microscopic features of pure colony on the media plate are observed through the eye, aided by microscope (Than *et al.*, 2008; Zainudin *et al.*, 2017). Although these criteria are useful in morphological identification, the outcomes sometimes overlap between species (Than *et al.*, 2008). Cultural characteristic is one of the crucial elements in differentiating the fungal species although similar species can express extensive variation in culture. This is because the culture condition such as the media and the age of culture, environmental conditions such as the temperature can affect the cultural characteristic of the cultured fungi (Zakaria and Bailey, 2000). To address the limitation in morphological analysis, molecular identification is used to confirm the

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identification via morphological approach (Singha *et al.*, 2016). At present, nucleotide sequence information from the conserved regions is utilised to identify eukaryotic organisms using the polymerase chain reaction (PCR) amplification. The PCR technique is often utilised for early diagnosis, identification and characterisation of microbes and pathogens (Singha *et al.*, 2016). The conserved regions that are valuable for species identification include the internal transcribed spacer (ITS) region from the conserved ribosomal RNA genes (Schoch *et al.*, 2012; Singha *et al.*, 2016). The ITS region is used as the fungi barcode marker since it has the highest probability to identify a wide range of fungi (Than *et al.*, 2008; Schoch *et al.*, 2012). The current study investigates the *Fusarium* spp. and *Colletotrichum* spp. associated with wilt, rot and anthracnose disease from different parts of vanilla obtained from three vanilla orchards in Malaysia. The identification of the disease associated species from vanilla plant samples were conducted by using micromorphological characterizations, cultural characterizations and molecular technique of ITS region's amplifications.

MATERIALS AND METHODS

Sample collection and fungal isolation

The plant parts that are affected with wilt, rot and anthracnose symptoms such as roots, stems, leaves and beans were collected from two vanilla orchards in Ulu Cheka, Pahang (2015) and Papar, Sabah (2016). Five samples for each infected plant parts were collected for each disease. The fungi were isolated using tissue transplantation and with the standard procedures (Torres-Calzada *et al.*, 2012; Heng *et al.*, 2013). The infected vanilla parts were surface-sterilised with 10% Clorox for 1 min, washed with sterilised water for 3 times, submerged into 75% ethanol for 30 sec and left to dry on the sterilised filter paper. The specimens then were cut into pieces with the advanced margin of a lesion, placed onto the fresh potato dextrose agar (PDA) media amended with 5% streptomycin and incubated at 25 °C. The emerging fungi were transferred aseptically to the new PDA media to obtain pure colonies. The pure colonies were established by transferring tips of new emerging fungal hyphae from the cultured plant tissue using sterile loop to new PDA plate (Torres-Calzada *et al.*, 2012; Adame-García *et al.*, 2015). Then the isolates were then growing as single colony on fresh PDA plate for 7 days at 25 ± 2 °C. Each isolate was purified by single-spore transfer to PDA and maintained on PDA slants at 4 °C for species identification.

Fungal identification through morphological approach

The morphological identification of pure isolate was performed by aseptically punched three 5 mm plugs of 5-days-old actively growing cultures at the edge of the colony (Leslie and Summerell, 2008; Torres-Calzada *et al.*, 2012). Each plug was placed onto new PDA plates and incubated at room temperature for 7 days. The colony size and colour were recorded. Colony diameter was recorded daily for 7 days to calculate the growth rate (mm/day). The growth rate was calculated as the average of 7 days of mycelia growth (Torres-Calzada *et al.*, 2012). The morphology and microscopic features such as macroconidia, microconidia, hyphae, chlamydospores and setae were observed on the culture slide using microscope under 40× and 100× magnification (Leica DM750). The experiments were repeated twice using the starter culture to avoid degeneration of the culture.

DNA extraction, DNA amplification and sequence analysis

The fungal DNA was extracted using the protocol described by Than *et al.* (2008). The DNA concentration of each isolate was measured with Nanodrop Spectrophotometer (DeNovix DS-11, USA) before being diluted into working solution of 50 ng/µL.

The PCR reaction volume (50 µL) contained 1 µL 10 mM dNTP mixture, 4 µL 25 mM $MgCl_2$, 5 µL 10× Taq buffer (1st BASE Pte Ltd, Singapore), 5 µL DNA, and 1 unit of Taq DNA polymerase (1st BASE Pte Ltd, Singapore), 0.2 µL 100 mM of each primer; ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT ATT GAT ATG C), provided by Vivantis Technologies Sdn Bhd (Heng *et al*., 2013). PCR was performed with Mastercycler (Eppendorf, Germany), using the parameters; 95 °C for 3 min followed by 30 cycles of 95 °C for 1 min, 52 °C for 50 sec, 72 °C for 1 min, completed with 72 °C for 10 min. The PCR products were then purified and sequenced by MyTACG Bioscience Enterprise (Kuala Lumpur, Malaysia).

All the sequence data received were used for multiple alignment purposes, utilising the Bio-Edit Sequence Alignment Editor software (http://www.bioeditor.sdsc.edu/download.shtml). A consensus sequence known as contigs of the forward and reverse sequences was generated for each sample. The sequences were adjusted to accommodate gaps. The large unaligned regions at the end of the sequences were 'trimmed'. The nucleotide sequences of all 22 samples were compared to those in the NCBI (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov) public domain database using BLASTN (Basic Local Alignment Search Tool for Nucleotide Sequences). The alignment of the ITS DNA sequences was done using ClustalW programme, implementing the maximum likelihood method in *MEGA* version 7.2 (Kumar *et al*., 2016). The 1000 replications of bootstrap with support values of ≥ 70% were used for phylogeny test. The nucleotide sequences of *Fusarium oxysporum* f. sp. *vanilla* (MG905424.1) was included into the phylogenetic analysis. *Trichoderma harzianum* T32 (KX632729.1) was used as outgroup. Their sequences were obtained from NCBI.

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Pathogenicity test on leaves

The pathogenicity test of each fungal species was conducted on the fresh detached vanilla leaves. The leaves were surface-sterilised with 10% Clorox for 1 min, washed with sterilised water for 3 times, submerged into 75% ethanol for 30 sec and left to dry on the sterilised filter paper. Three leaves were used for each isolate and the experiment was repeated twice. The leaves were needle-pricked and a 10 mm diameter fragment of each 7-day fungi-isolate grown on PDA was placed onto the pricked leaves (Adame-García *et al.*, 2015). The control was set by inoculating the pricked leaf with cleaned PDA fragment. The inoculated leaves were incubated at room temperature (29-32 °C) and kept in humid conditions for daily observation until the symptoms appear. The diameter of lesion developed on the leaves (mm) was recorded. The level of damage was determined using a descriptive key as follow: (1) leaves with no symptom, (2) leaves with chlorosis, (3) leaves with rot, and (4) leaves showing necrosis or dead leaves (Adame-García *et al.*, 2015). The data on pathogenicity were subjected to one-way ANOVA (SPSS Inc., Chicago, IL) and the means were compared using the Tukey's studentized range test (HSD) at $p<0.05$.

RESULTS

***Fusarium* and *Colletotrichum* species isolation and identification**

A total of 22 isolates were obtained in this study. The isolates and their identification using ITS are summarised in Table 1. The species were isolated from the plant parts affected by the diseases, either roots, stems, leaves or beans (Figure 1). The microscopic characteristics and morphological observations are summarized in Table 2 and illustrated in Figure 2. The cultural characteristics (Figure 2A), microscopic characteristics and molecular identification show that the 22 isolates are from six species; Group 1 (*Fusarium oxysporum*), Group 2 (*Fusarium* sp.), Group 3 (*Fusarium proliferatum*), Group 4 (*Fusarium solani*), Group 5 (*Fusarium fujikuroi*) and Group 6 (*Colletotrichum gloeosporioides*). Conidia of *Fusarium* species have either canoe or fusiform shapes, whereas *C. gloeosporioides* has oblong conidia (Figure 2B). Chlamydospores (resting spores) was observed in *F. oxysporum* and appressoria in *C. gloeosporioides* (Figure 2B). The PCR products of the amplified ITS regions of all 22 PCR isolates show ~550 bp amplicon size for *Fusarium* spp. and *Colletotrichum* spp., which is consistent with the study of *Fusarium* sp. associated with rot in vanilla plants (Casillas-Isiordia *et al*., 2017). Based on the BLASTn search, the 22 sequences show homology of being 100% identical to the species deposited in GenBank (Table 1).

Analysis of the ITS sequence demonstrates the isolates are categorised into four major clades (Figure 3). Clade A consists of *Fusarium* sp., *F. proliferatum* and *F. fujikuroi*. Clade B consist of all six *F. oxysporum* isolates

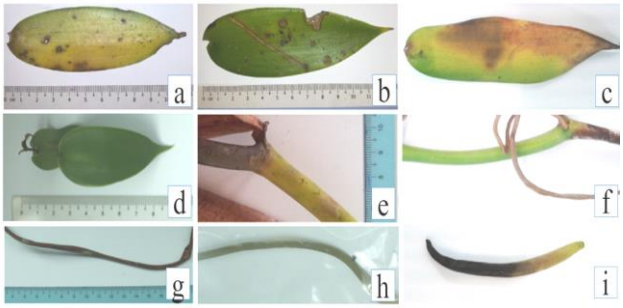

Figure 1: The diseased samples collected from different parts of vanilla plant. Leaves; leaf showed discolouration and black spot on the leaf (a), inconsistent colour and black spot on the leaf (b), discolouration and heat stress (c) and healthy leaves (d). Stems; discolouration and rot (e, f), unhealthy roots (g, h, i); Bean rot collected from vanilla farms in Pahang 1 (a, e, g), Pahang 2 (b, d, h), and Sabah (c, f, i).

and one strain retrieved from Genbank, *Fusarium oxysporum* f. sp. *vanillae* (*Forv*) (MG905424.1). Clade C consists only *F. solani* with low clade reliability support (69%) whereas clade D consists of *C. gloeosporiodes* strains. Nucleotide sequence from the Genbank database, *Trichoderma harzianum* T32 was used as the outgroup for phylogenetic analysis, which is located at cluster B. The phylogenetic analysis based on ITS gene has grouped the genus and species into separate clades except for the *Fusarium* species complex, namely *Fusarium* sp., *F. fujikuroi* and *F. proliferatum*. The grouping of these three *Fusarium* species into the same clade indicates that these species are closely related.

Pathogenicity of fungal isolates

The symptoms on leaves for *F. oxysporum* and *C. gloeosporioides* isolates were observed ten days after inoculation (Figure 4). Based on the disease severity index, the results revealed significant differences ($p<0.01$) in pathogenicity of the isolates. Severity index and lesion diameter demonstrate both *F. oxysporum* and *C. gloeosporioides* are highly pathogenic to vanilla, causing high level of damage (3 = rot and 4 = tissue death), $p<0.01$ (Figure 5). The lesion diameter of these two species shows significant differences from the control ($p<0.05$) (Figure 6). On the other hand, *Fusarium* sp., *F. proliferatum*, *F. solani* and *F. fujikuroi* do not produce lesion, there was only yellow to black discolouration, which is not significantly different from the control ($p>0.05$) (Figure 6).

DISCUSSION

The fungal-associated diseases in vanilla plants have gained attention in the world vanilla producer countries such as Madagascar, India and Indonesia. The effect of the diseases is tremendous, which may ruin the farm production due to the plant's deteriorating health, thus affecting the quality and quantity of the vanilla bean.

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Table 1: Sources of the isolates identified in this study and GenBank accession numbers of *Fusarium* spp. and *Colletotrichum* spp. isolated from the diseased vanilla samples.

Group	Isolate	Part	Species identified	Location	Accession number	Homology identical (%)
1	G1P1	Root	*F. oxysporum*	Pahang2	KY798175.1	100
1	G1P2	Leaf	*F. oxysporum*	Pahang	MG727665.1	100
1	G1P3	Leaf	*F. oxysporum*	Pahang	LC317608.1	100
1	G1P4	Root	*F. oxysporum*	Pahang2	KY798175.1	100
1	G1S3A	Stem	*F. oxysporum*	Sabah	MG727665.1	100
1	G1S52	Bean	*F. oxysporum*	Sabah	MG727665.1	100
2	G2P5	Leaf	*Fusarium* sp.	Pahang2	MG519516.1	100
2	G2P6	Leaf	*Fusarium* sp.	Pahang	JQ621875.1	100
3	G3P7	Leaf	*F. proliferatum*	Pahang	LC101936.1	100
3	G3P8	Leaf	*F. proliferatum*	Pahang	EU835478.1	100
4	G4P9	Root	*F. solani*	Pahang	KY785016.1	100
2	G5P10	Stem	*F. fujikuroi*	Pahang2	MG798789.1	100
5	G5P11	Stem	*F. fujikuroi*	Pahang	MF510829.1	100
5	G5P12	Stem	*F. fujikuroi*	Pahang	MF510829.1	100
5	G5P13	Leaf	*F. fujikuroi*	Pahang	MF510829.1	100
5	G5P14	Leaf	*F. fujikuroi*	Pahang	MF510829.1	100
5	G5P15	Stem	*F. fujikuroi*	Pahang	MF510829.1	100
5	G5P16	Stem	*F. fujikuroi*	Pahang	MF510829.1	100
6	G6S4A	Leaf	*C. gloeosporioides*	Sabah	KP900236.1	100
6	G6S7A	Leaf	*C. gloeosporioides*	Sabah	MG832471.1	100
6	G6S9C	Leaf	*C. gloeosporioides*	Sabah	JX869041.1	100
6	G6S14	Leaf	*C. gloeosporioides*	Sabah	MG832471.1	100

Cultural and conidial characteristic**s** are one of the elements in identifying fungi, even when same species can express variations in culture (Zakaria and Bailey, 2000). Due to the limitation in morphological identification, genomic sequencing utilising the internal transcribed spacer (ITS) regions of the species ribosomal DNA was carried out to gain higher accuracy in species identification. The morphological grouping according to the cultural and microscopic characteristic is in line with the phylogeny derived from molecular data in this study. However, there were some overlapping, especially in the conidial size among the *Fusarium* species**.** All 22 isolates match the morphological study, in which they are 100% homological identical when blasting with the NCBI database. The frequency of the *Fusarium* species isolated in this study is high, indicating that the species is widely spread in vanilla farm in Malaysia, both for pathogenic and non-pathogenic strains. The *Fusarium* species isolated in new farms, Pahang 2 (less than two years old), is less diverse compared to the old farm, Pahang (more than six years). Thus, proper farm management need to be applied to avoid favorable environment for the disease to outbreak.

The results indicate that *F. oxysporum* and *C. gloeosporiodes* are the two disease-causing pathogens in vanilla plants grown in Malaysia. *Fusarium oxysporum* which causes rots and lesions in pathogenic assay is shown to be under the same clade as *F. oxysporum* f. sp. *vanillae* (*Forv*), a pathogenic fungus known to cause rot in vanilla worldwide. *C. gloeosporiodes* was only isolated from vanilla plants in Sabah but not in peninsular Malaysia, suggesting the need to broaden the sampling area in Peninsular and also increase the sampling frequency (seasons sampling). This is because, temperature, humidity and light are the favorable environmental factors that can speed up the disease infection (Zakaria and Bailey, 2000). Additionally, the media for isolating the fungus also need to be varied since some species may not be able to grow on PDA due to its high carbohydrates contents (Leslie and Summerell, 2008). *Colletotrichum* species can cause anthracnose disease in many crops worldwide (De Silva *et al*., 2017). The *Colletotrichum* species also are associated with post-harvest disease on fruits and vegetables, in fact, they also causes diseases in leaves, stems, fruits (Than *et al.*, 2008; Sangdee *et al.*, 2011), tubers (Green and Simons,

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Table 2: Summary of cultural and microscopic characteristics of the isolated fungal species grown on the PDA media.

Group	Colony colour		Growth rate (mm/day)	Macroconidia		Microconidia		Chlamydo-spores	Phialides
	Front	Back		Size (µm) length × width (average)	Shape	Size (µm) length × width	Shape		
1	White to pale pink	Pale pink	7.97	30.2 × 4.2	Canoe	5.0 × 1.8	Fusiform to Ellipsoidal	Present	Mono
2	Dark purple	Pale purple	7.80	28.3 × 4.0	Fusiform	6.3 × 2.8	Fusiform	Absent	Mono
3	Maroon	White	7.43	28.5 × 4.0	Canoe	4.9 × 2.1	Fusiform	Absent	Mono and poly
4	Beige to brown	Beige	10.70	33.0 × 4.6	Canoe	6.3 × 1.7	Fusiform	Absent	Mono and poly
5	Pale purple	Pale purple	8.60	28.8 × 3.8	Slight curved	6.5 ×1.9	Fusiform	Absent	Mono and poly
6	Beige to brown	Pale brown	11.00	10.5 × 3.0	Oblong	10.5 × 3.0	Oblong	Present	Mono

1994), and seedlings (Ogbebor *et al*., 2007; Bhai and Kumar, 2008). The environmental factors such as temperature, humidity and light have a significant effect on the development and spread of diseases caused by *C. gloeosporioides* (Zakaria and Bailey, 2000). The symptoms of *Colletotrichum* infection in this study was observed a bit late (>10 days after inoculation) as compared to the previous study (4 days) (Casillas-Isiordia *et al.*, 2017). The lesions observed in this study suggested that the *F. oxysporum* and *C. gloeosporioides* are pathogenic to vanilla. This is fulfill the requirement with 12 days is needed to determine the pathogenicity of *Fusarium* species on vanilla plants (Adame-García *et al.*, 2011). However, the symptoms observed during sample collection (Figure 4a) were more obvious than laboratory test (Figure 4A). It is also suggested that the mixture of several isolates contributes to higher level of damage (Adame-García *et al*., 2015). However, culture degeneration is one of the factors causing the pathogenicity of the fungal (Leslie and Summerell, 2008). Cultural degeneration is a significant problem in the studies of Fusarium. The occurrence is promoted by frequent subculturing on carbohydrate-rich media such as PDA and via the mass transfer of mycelium. However, this limitation can be lessened by using natural substrate or low nutrient media such as a carnation leaf agar (CLA) or Spezieller Nährstoffarmer agar (SNA) and using a single germinated conidium or a hyphal tip to initiate subcultures (Leslie and Summerell, 2008).

The results also demonstrated that *F. proliferatum*, *F.* solani, *Fusarium* sp., and *F. fujikuroi* do not produce lesion but they cause yellow to black discolouration, which is the same as the control (p>0.5). This supports the previous study on vanilla, where *F. proliferatum* is regarded to be non-pathogenic in Mexico (Adame-García *et al*., 2015), it is in fact a saprophyte or endophytic coloniser in Indonesia (Pinaria *et al*., 2010). Although there was no symptom observed on the detached leaf inoculated with *F. proliferatum*, mycotoxin contamination may occur. This is suggested based on the previous study, where *F. proliferatum* infection on crops such as rice cause no physiological changes but the grain was detected with mycotoxin contamination (Kushiro *et al*., 2012). However, mycotoxin level was not tested in this study. Furthermore, *F. proliferatum* was reported to infect multiple plants from different climatic zones (Stępień *et al.*, 2011) such as corn (Zainudin *et al.*, 2017), alfalfa (Cong *et al.*, 2016) and rice (Kushiro *et al*., 2012).

There is only one *F. solani* isolate found in this study, illustrating that the species frequency in vanilla farms is low, in line with the literature (Gordon *et al*., 1989). Although it is present in relatively low frequency, *F. solani* was found to cause diseases in potato, chickpea, wheat, rice, melon and olive (Zaccardelli *et al.*, 2008), but not in vanilla (Pinaria *et al*., 2010). This study also showed *F. solani* has no symptoms associated with diseases in vanilla, in line with no discolouration observed on vanilla leaves in Indonesia

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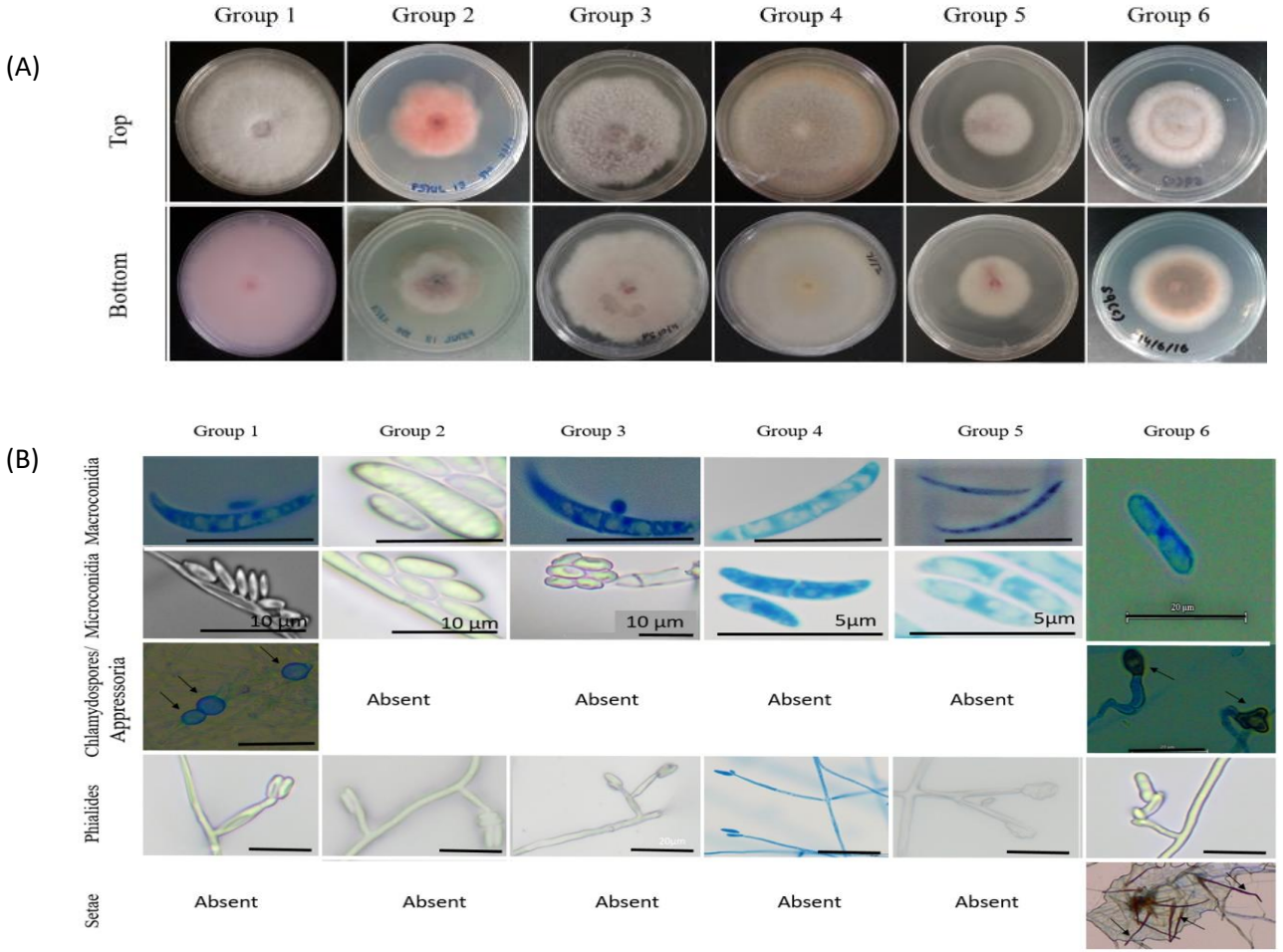

Figure 2: (A) The cultural observation of Group 1 (*F. oxysporum*), Group 2 (*Fusarium* sp.), Group 3 (*F. proliferatum*), Group 4 (*F. solani*), Group 5 (*F. fujikuroi*) and Group 6 (*C. gloeosporioides*). (B) The microscopic features of the isolates. Chlamysospores were observed in *F. oxysporum* isolates whereas appressoria were observed in *C. gloeosporioides* isolates. Setae were only observed in *C. gloeosporioides.* The scale: 20 µm or as labelled under magnification 40× (stained) and 100× magnification.

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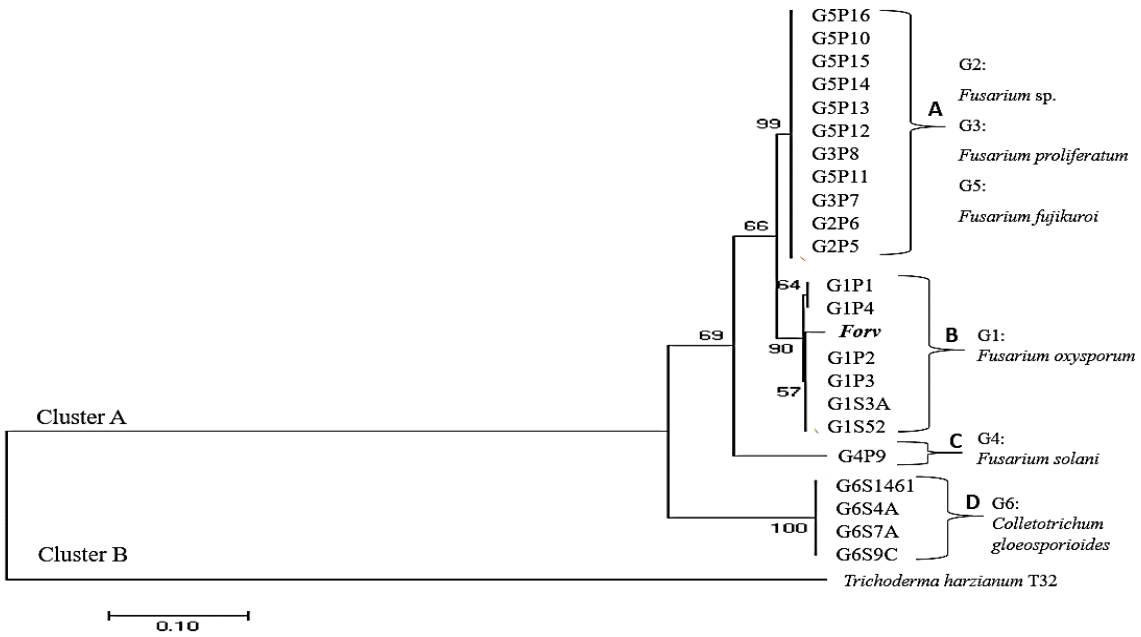

Figure 3: Molecular phylogenetic analysis via the maximum likelihood of 18 isolates of *Fusarium* species and four isolates of *Colletotrichum* species inferred from the ITS regions. The tree was generated from the Tamura-Nei model (Tamura and Nei, 1993), using *MEGA* version 7 (Kumar *et al.*, 2016).

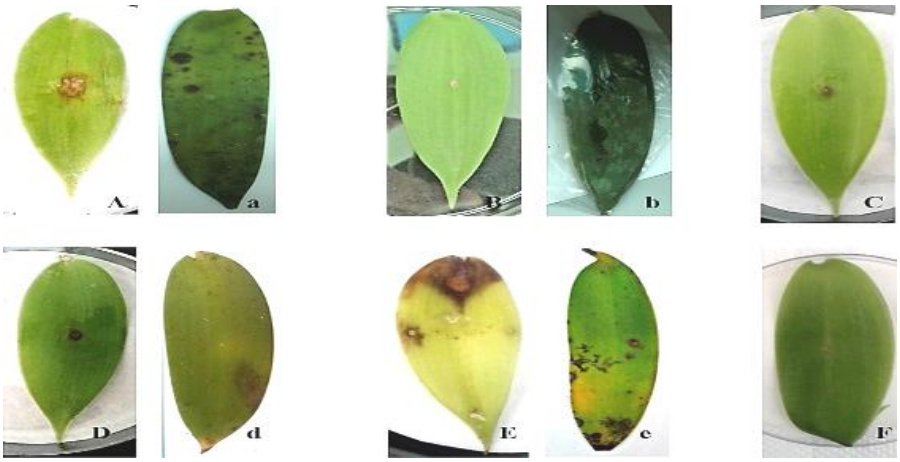

Figure 4: Pathogenicity test conducted on the detached leaves of vanilla orchids at 14 days post inoculation, A) *F. oxysporum* produces brownish lesion and discoloration to the leaf, B) *F. proliferatum* produces no lesion, C) *F. solani* produces no lesion, D) *F. fujikuroi* produces no lesion, E) *C. gloeosporioides* produces significant lesion on the leaf base and margin and cause discolouration to the leaf, F) control treatment with scar due to pricking. The capital letter denote donates the laboratory test while the lowercase letter denote the field sample. *F. solani* (C) do not have field sample as the species was isolated from the root. The black dot on C and D is not lesion but the dried PDA fragment.

(Pinaria *et al.*, 2010). Similar situation was found in *F. fujikuroi*, where the small discolouration produced by this species causes *F. fujikuroi* to be deemed as endophytic coloniser of vanilla grown in Indonesia (Pinaria *et al*., 2010). Nevertheless, *F. fujikuroi* has causes diseases in other crops in Malaysia, such as fusariosis in pineapple (*Ananas comosus*) (Ibrahim *et al.*, 2016) and stem rot in red-fleshed dragon fruit (*Hylocereus polyrhizus*) (Masratul-Hawa *et al.*, 2017).

The phylogenetic analysis has shown that *Fusarium* sp., *F. proliferatum* and *F. fujikuroi* are grouped in the same cluster although the morphological description of these three colony are quite different. The culturing media or repetitive sub culturing might result in the inconsistency

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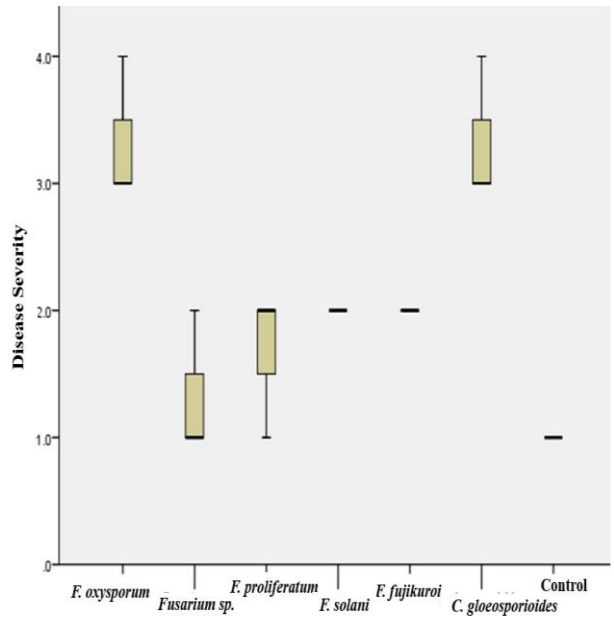

Figure 5: Variations in the pathogenicity of fungal isolates on leaves 14 days after inoculation. *F. oxysporum* and *C. gloeosporioides* demonstrated scores of 3 (leaves with rot) and 4 (leaves showing necrosis or dead leaves). Meanwhile, other species scored 1 (leaves with no symptom) and 2 (leaves with chlorosis). The scores are based on the literature (Adame-García *et al.*, 2015).

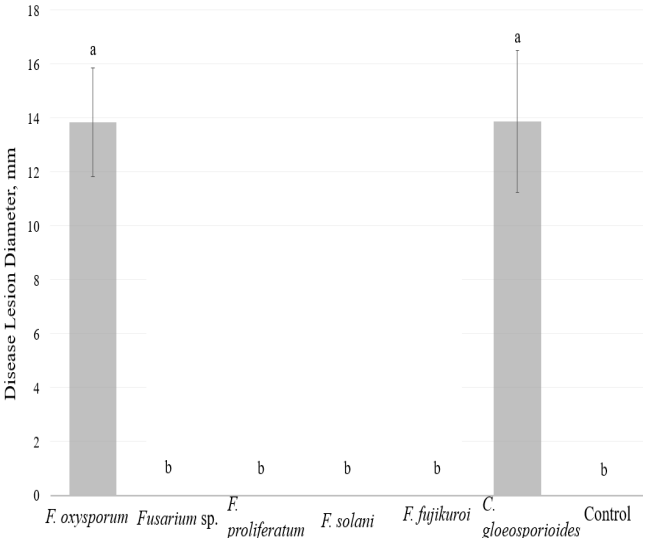

Figure 6: The diameter (mm) of lesions produced 14 days after inoculation, of six fungal species; *F. oxysporum, Fusarium* sp., *F. proliferatum*, *F. solani*, *F. fujikuroi* and *C. gloeosporioides* on the detached vanilla leaves. The control was set by inoculating the incised leaf with cleaned PDA fragment. The error bars show standard error measurement (SEM). Similar letter denotes no significant difference (p>0.05).

in the morphological observation (Leslie and Summerell, 2008). Colony morphology, pigmentation and growth rates of cultures of most *Fusarium* species on PDA are reasonably consistent if prepared in a consistent and standard conditions although the conidia formed on PDA are not consistent in either size or shape as those formed on CLA or SNA media (Leslie and Summerell, 2008). Although PDA is not recommended to isolate *Fusarium* species, since it can harbor many saprophytic fungi and bacteria, it can be used for fungi recovery from plant material by decreasing the concentration of PDA and the use of antibiotics, such as streptomycin (Leslie and Summerell, 2008). Additionally, the use of ITS regions works well in most cases, and the most useful regions for fungal species-level identification. This is because ITS regions is the fastest evolving portion of the rRNA cistron. Moreover, ITS is an official barcode for fungi by a consortium of mycologist since the regions is easy to amplify, widespread use and have appropriately large barcode gap (Schoch *et al.*, 2012). However, it is recommended to use a combination of micromorphological, cultural and molecular characters for fungi identification. Furthermore, the use of entire ITS alone or in combination with the first two domains of LSU (large subunit) and one or more protein coding genes should be compared with authenticated and published sequences (Raja *et al*., 2017). In contrast, some study suggested that the morphological analysis and amplification of ITS regions were not suitable for species elucidation of identity (Casillas-Isiordia *et al.*, 2017).

CONCLUSION

Severity index and lesion diameter demonstrates both *F. oxysporum* and *C. gloeosporioides* showed high pathogenic to vanilla. The study findings provide valuable information on the diseases in vanilla grown in Malaysia. The potential of the non-pathogenic *Fusarium* species and other endophytic fungi in disease management is worthy to be further studied for the future of agricultural industry. The plant's physiological conditions, host genotypes and environmental conditions sometimes switch the nature of endophytic fungus, from mutualistic to pathogenic since the plant-fungus interaction is influenced by abiotic and biotic factors (De Silva *et al.*, 2017; Hardoim *et al.*, 2015). The recently recognised rot-causing pathogen in vanilla worldwide, *Fusarium oxysporum* f. sp. *vanillae* was not isolated in this study, illustrating that the pathogen is not found in Malaysian farms. This urges strict quarantine procedures in the import and export of the vines, so that the pathogen does not spread in this country and harm the cultivars. The data of *Fusarium* species associated with vanilla plants, either pathogenic or non-pathogenic will contribute to the future development in disease management. In addition, the non-pathogenic species be significant in disease suppression. An integrated disease management need to be scrutinised to ensure the strategies can overcome the disease pathogenicity level to combat the spread of the disease.

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