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

Nurdiana Ab Kadir¹, Laila Naher¹,²*, Fatimah Kays¹,², Noorhashira Sidek¹, Norhazifah Md. Zain¹,² and Tengku Halimatun Sa’adiah T. Abu Bakar¹,²

¹Faculty of Agro-Based Industry, Universiti Malaysia Kelantan, 17600, Jeli, Kelantan, Malaysia.
²Institute of Food Security and Sustainable Agriculture, Universiti Malaysia Kelantan, 17600, Jeli, Kelantan, Malaysia.

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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. gloeosporioides (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 vanilla, 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 Bahama 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-Garcia 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

*Corresponding author
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 fungal barcode marker since it has the highest probability to identify a wide range of fungi (Than et al., 2012; 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 fungal hyphae 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-Álvaro 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 MgCl₂, 5 µL 10X 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 GCT TAT CAA TAT CGA AGG T) and ITS4 (TCC GCT TAT CAA TAT CGA AGG T), 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 BioEdit Sequence Alignment Editor software (http://www.bioedit.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. vanillae (MG905424.1) was included into the phylogenetic analysis. Trichoderma harzianum T32 (KX632723.1) was used as outgroup. Their sequences were obtained from NCBI.
**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 fungus-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 isolates were obtained from the plant parts affected by the diseases, either roots, stems, leaves or beans (Figure 1). The microscopic characteristics and morphological observations are summarised 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 lance or fusiform shapes, whereas *Colletotrichum* species 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).

![Image](image.png)

Figure 1: The diseased samples collected from different parts of vanilla plant. Leaves, leaf showed discoloration and black spot on the leaf (a), inconsistent colour and black spot on the leaf (b), discoloration and heat stress (c) and healthy leaves (d). Stoms; discoloration 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).

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 consists of all six *F. oxysporum* isolates and one strain retrieved from GenBank, *Fusarium oxysporum* f. sp. *vanillae* (Fov) (MG905424.1). Clade C consists only *F. solani* with low clade reliability support (69%) whereas clade D consists of *C. gloeosporioides* 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 discoloration, 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.
Cultural and conidial characteristics 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% homologous 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. gloeosporioides* are the two disease-causing pathogens in vanilla plants grown in Malaysia. *F. 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. gloeosporioides* 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; Sangedee et al., 2011), tubers (Green and Simons, 1994), and seedlings (Ogbebor et al., 2007; Bhai and Kumar, 2008). The

### 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         | JG621875.1       | 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                    |
The results also demonstrated that *F. proliferatum*, *F. solani*, *Fusarium oxysporum* sp., and *F. fujikuroi* do not produce lemon 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-Garcia *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, chickepea, 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 discoloration observed on vanilla leaves in Indonesia.
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. Chlamyospores 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.
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).

![Phylogenetic tree](image)

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 discoloration to the leaf, F) control treatment with scar due to pricking. The capital letter denote 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 discoloration 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...
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 citron. 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.

**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-Garcia 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).
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