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Morphological and RAPD Analysis of *Fusarium* Species Associated with Root and Stem Rot of *Dendrobium* Orchid in Northern Peninsula Malaysia

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A total of 29 *Fusarium* isolates were isolated from *Dendrobium* orchid showing symptoms of root and stem rots. Based on morphological characteristics, three species were identified namely, *F. oxysporum, F. solani,* and *F. proliferatum* which were recovered from root and stem rots of *Dendrobium*. Cluster analysis of RAPD bands clearly separated *F. oxysporum, F. proliferatum,* and *F. solani* into distinct clusters. The present studies showed that three *Fusarium* species were isolated from root and stem rot of *Dendrobium* and cluster analysis of RAPD bands was in agreement with morphological characterization of the *Fusarium* species from root and stem rot of *Dendrobium.*

Key words: orchid, *Fusarium*, root rot, stem rot, RAPD

INTRODUCTION

Fusarium species are one of the most common soil borne plant pathogens, causing serious diseases in many popular ornamental plants such as carnation, chrysanthemum, lily, and orchid. For orchidaceous plant, several *Fusarium* species have been reported to be the causal agent of root rot, dry rot, and leaf spot diseases (Benyon *et al*. 1996; Chang *et al*. 1998; Ichikawa & Aoki 2000; Lee *et al*. 2002).

During a disease survey, root and stem rots of *Dendrobium* orchid were encountered in nurseries in Taiping, Perak and Penang Island, Pulau Pinang, Peninsula Malaysia. Typical symptoms on the root were dark discolorations which indicated rotting of the tissues. Infected stems showed yellowish discolorations with water soaked appearance and were very friable. In preliminary studies, *Fusarium* species were consistently isolated from the infected root and stem rot of the orchid plant.

From the results pathogenicity tests on *Dendrobium* orchid, the symptoms observed corresponded with symptoms on diseased plant in the nurseries. *Fusarium* species were reisolated from inoculated plants which indicated that Koch's postulate has been fulfilled and *Fusarium* species were identified as the causal agent of root and stem rots of *Dendrobium* orchid (NurHayati 2007).

As the information on *Fusarium* species associated with orchid plants in Malaysia is limited and not well documented, the study was conducted to identify and characterize the *Fusarium* species from root and stem rot of *Dendrobium* orchids using morphological characteristics and Random Amplified Polymorphic DNA (RAPD) analysis.

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MATERIALS AND METHODS

Isolation of *Fusarium* Isolates. *Fusarium* isolates were isolated from *Dendrobium* orchids showing symptoms of root and stem rots, from three nurseries in Taiping, Perak and Penang Island, Pulau Pinang. The root and stem samples (about 1.5-2.0 cm) were surface sterilized with 1% sodium hypochlorite and rinsed in sterile distilled water. The samples were then plated onto peptone pentachloronitro benzene agar (PPA) and incubated at 27 $\pm$ 1 ^{o}C for 7 days or until visible sign of mycelial growth from the samples. The mycelium was then sub-cultured onto potato sucrose agar (PSA).

Morphological Characterization. Each recovered isolate was single spored and the single colonies were transferred to carnation leaf agar (CLA) and potato dextrose agar (PDA) for species identification. PDA media was used to study pigmentation and CLA media to examine microscopic characteristics such as microconidia, macroconidia, conidiogenous cells and formation of chlamydospores (Nelson *et al*. 1983). Determination of species was according to Nelson *et al.* (1983) and descriptions in The *Fusarium* Laboratory Manual by Leslie and Summerell (2006).

RAPD Analysis. A total of 40 *Fusarium* isolates from root and stem rots of orchid were used in RAPD analysis (Table 1). Twenty nine isolates were recovered from root and stem rot of *Dendrobium* and eight isolates namely, *F. proliferatum* (1325, 1374, 1377, 1378, 1380, 1381), *F. solani* (1257) and *F. oxysporum* (1493) were obtained from *Fusarium* Culture Collection, School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia. These cultures were isolated from stem rot of orchid in a disease survey conducted in the late 1999. Another three isolates comprising *F. oxysporum* (AP7 and AP9) and *F. solani* (AP8) were from root rot of *Oncidium* orchid obtained in a nursery in Penang Island.

DNA Extraction. Mycelium was grown on potato sucrose agar (PSA) and incubated for 6 days. DNA was extracted using DNeasy Plant Mini Kit (Qiagen) according to the manufacturers' instructions. The extracted DNA was stored at -20 °C until used.

Table 1. *Fusarium* isolates used in RAPD analysis

Isolate	Host and plant part	Location
F. oxysporum		
AP1	root (*Dendrobium*)	Relau, Penang
AP2	root (*Dendrobium*)	Relau, Penang
AP3	root (*Dendrobium*)	Relau, Penang
AP5	root (*Dendrobium*)	Relau, Penang
AT14	root (*Dendrobium*)	Taiping, Perak
AT15	root (*Dendrobium*)	Taiping, Perak
AP7	root (*Oncidum*)	Relau, Penang
AP9	root (*Oncidium*)	Relau, Penang
BP1	stem (*Dendrobium*)	Relau, Penang
BP2	stem (*Dendrobium*)	Relau, Penang
BP3	stem (*Dendrobium*)	Relau, Penang
BP4	stem (*Dendrobium*)	Relau, Penang
BT6	stem (*Dendrobium*)	Taiping, Perak
BT8	stem (*Dendrobium*)	Taiping, Perak
BT9	stem (*Dendrobium*)	Taiping, Perak
1493	stem (stock culture)	Kuala Lumpur
F. solani		
AP4	root (*Dendrobium*)	Relau, Penang
AT13	root (*Dendrobium*)	Taiping, Perak
AT16	root (*Dendrobium*)	Taiping, Perak
AT17	root (*Dendrobium*)	Taiping, Perak
AP8	root (*Oncidium*)	Relau, Penang
BP5	stem (*Dendrobium*)	Relau, Penang
BT7	stem (*Dendrobium*)	Taiping, Perak
BT13	stem (*Dendrobium*)	Taiping, Perak
1257	stem (stock culture)	Kuala Lumpur
F. proliferatum		
AP6	root (*Dendrobium*)	Relau, Penang
AP10	root (*Dendrobium*)	Relau, Penang
AP11	root (*Dendrobium*)	Relau, Penang
AP12	root (*Dendrobium*)	Taiping, Perak
BT10	stem (*Dendrobium*)	Taiping, Perak
BT11	stem (*Dendrobium*)	Taiping, Perak
BT12	stem (*Dendrobium*)	Taiping, Perak
BT14	stem (*Dendrobium*)	Taiping, Perak
BT15	stem (*Dendrobium*)	Taiping, Perak
1325	stem (stock culture)	Kuala Lumpur
1374	stem (stock culture)	Kuala Lumpur
1377	stem (stock culture)	Kuala Lumpur
1378	stem (stock culture)	Kuala Lumpur
1380	stem (stock culture)	Kuala Lumpur
1381	stem (stock culture)	Kuala Lumpur

PCR Amplification. After primer screening, three random primers, OPA2 (5'TGCCGAGCTG) , OPA4 (5'AATCGGGCTG), and OPA10 (5'GTGATCGCAG) were used in the analysis based on consistency of the bands generated. Amplification reactions were done in a 25 µl reaction mixture containing 2.5 mM $MgCl_2$, 200 µM dNTP, 0.6 µM for each primer, 5 ng DNA template and one unit of *Taq* polymerase (Promega, Madison, WI). Each reaction was overlaid with 25 µl of mineral oil. PCR amplification was performed in a programmable thermalcycler (PCR DNA Engine Peltier Thermal Cycler Model PTC-100) using an initial denaturation of 95 °C for 2 min, followed by 39 cycles of denaturation at 94 °C for 30s; annealing at 37 °C (for OPA4 and OPA10) and 40 °C (for OPA2) for 1 min; extension at 72 °C for 30s and a final extension at 72 °C for 10 min.

After PCR, the RAPD products were analyzed by electrophoresis using 1.7% agarose gel in Tris borate EDTA (TBE) buffer for 2h 40 min at $5Vcm^{-1}$. 1 kb DNA marker (GeneRuler, Fermentas, Lithuania) and 100 bp DNA ladder (Promega, Madison, WI) were used to estimate the size of the RAPD bands. The gel was stained with 0.5 µg/ml ethidium bromide and visualized under the UV light and photographed.

RAPD bands were scored based on presence and absence of a particular band with reference to the standard markers. A binary matrix was compiled and subjected to cluster analysis. A genetic similarity was constructed using Simple Matching Coefficient. The Numerical Taxonomy System of Multivariate Program (NT-SYS) software package Version 2.0 (Rohlf 2000) was used to analyze the data. A dendrogram was constructed using unweighted pair-group method with arithmetic averages (UPGMA) cluster analysis to infer the relationships within and between the *Fusarium* isolates from root and stem rots of orchid.

RESULTS

Morphological Characteristics. A total of 29 *Fusarium* isolates were isolated from root and stem rots of *Dendrobium* comprising 14 isolates from *Dendrobium* roots and 15 isolates from *Dendrobium* stems. Based on morphological characteristics described by Nelson *et al*. (1983) and description in The *Fusarium* Laboratory Manual (Leslie & Summerell 2006), three species were identified namely, *F. oxysporum*, *F. solani*, and *F. proliferatum* (Table 2). The

Table 2. Description of morphological characteristics of *Fusarium* species isolated from root and stem rot of *Dendrobium*

Characteristic	Species		
	F. oxysporum	*F. solani*	*F. proliferatum*
Microconidia	Abundant in aerial mycelia, single-celled, produced in false head, oval to kidney-shaped (reniform), 0 septa	Abundant in aerial mycelia, single-celled, thick-walled, oval to kidney-shaped (reniform), 0-1 septa	Abundant, single-celled, club-shaped with flattened base, form in short chains and in false head, 0 septa
Macroconidia	Abundant, slender and relatively straight, thin-walled, short to medium length, sickle-shaped apical cell, foot-shaped basal cell, 3-4 septa	Abundant, relatively straight, stout, thick- walled, blunt apical cell, foot-shaped basal cell, 5-7 septa	Abundant, relatively straight, thin-walled, slightly sickle-shaped, curved apical cell, 3-5 septa
Conidiogenous cells	Short monophialides	Long monophialides	Monophialides and polyphialides
Chlamydospore	Present in pairs and singly	Present in pairs and singly	Absent
Pigmentation	Purple to dark purple	Cream to brown	Dark purple

species identified from root rot of *Dendrobium* were *F. oxysporum* (seven isolates) and four isolates each for *F. solani* and *F. proliferatum*. From stem rot of *Dendrobium*, seven *F. oxysporum*, three *F. solani* and five *F. proliferatum* were recovered.

Fusarium oxysporum isolates were identified based on the production of microconidia in false heads borne on short monophialides. The microconidia were oval to kidney shaped and macroconidia, straight to slightly curved. There was abundance of chlamydospores.

For *F. solani* isolates, the monophialides were long and bears microconidia. The microconidia were wider and oval shaped, and macroconidia were straight and stout. Chlamydospores were also abundant.

Fusarium proliferatum microconidia were formed in short chains with V-shaped polyphialides and in false head. The conidiogeneous cells were both polyphialides and monophialides. Macroconidia were almost straight and slightly sickle-shaped. Chlamydospores were absent.

RAPD Analysis. The overall RAPD banding patterns generated by random primers OPA2, OPA4, and OPA10 showed variable patterns within the same species.

RAPD analysis of *F. oxysporum* isolates produced 28 banding patterns and the size of the bands ranged from 250-3,000 bp with similarity values of 73-92.7%. Figure 1 shows diagrammatical banding patterns of some isolates of *F. oxysporum* using OPA2. There were intraspecific variations between the isolates from *Dendrobium* root and stem rot, *Oncidium* root rot (AP7 and AP9) and the stock culture (1493).

Amplification of *F. solani* isolates produced 21 banding patterns with molecular sizes ranging from 250-3,000 bp. *F. solani* isolates from root and stem rots of *Dendrobium*, stock culture (1257) and *Oncidium* root rot (AP8) showed similar patterns. Figure 2 shows diagrammatical banding patterns of some isolates of *F. solani* generated using OPA4. The RAPD banding patterns were also variable with similarity values ranging from 70.7-97.6%.

Fusarium proliferatum isolates produced 19 banding patterns with sizes of the amplified bands ranging from 250-3,000 bp. The similarity values ranged from 78.1-97.6%. Like *F. oxysporum* and *F. solani*, the RAPD banding patterns were also variable among all the isolates from root and stem rots of

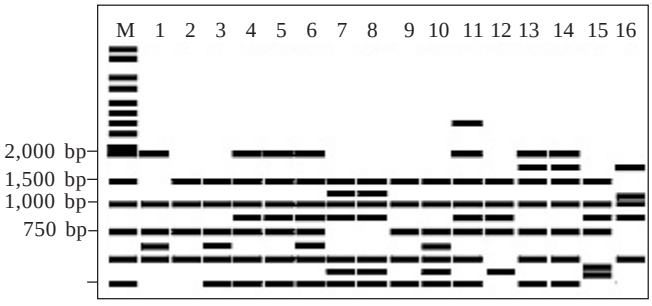

Figure 1. Diagrammatical RAPD banding patterns of *F. oxysporum* isolates using OPA2 primer. Lanes 1-8: root isolates (1: AP1, 2: AP2, 3: AP3, 4: AP5, 5: AP7, 6: AP9, 7: AT14, 8: AT15). Lanes 9-15: stem isolates (9: BP1, 10: BP2, 11: BP3, 12: BP4, 13: BT6, 14: BT8, 15: BT9). Lane 16: stock culture (1493), M = marker.

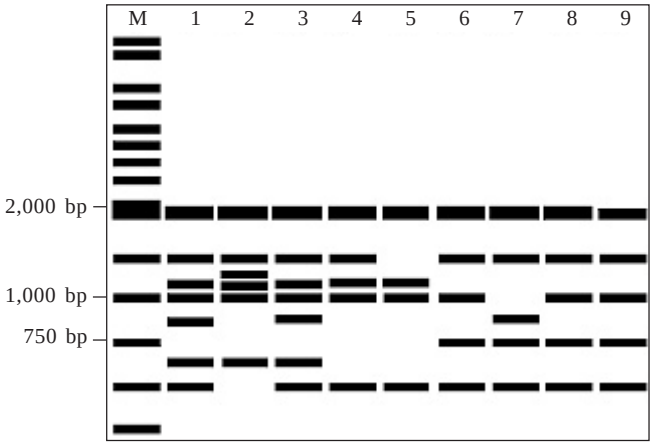

Figure 2. Diagrammatical RAPD banding patterns of *F. solani* isolates using OPA4 primer. Lanes 1-5: root isolates (1: AP4, 2: AP8, 3: AT13, 4: AT16, 5: AT17). Lanes 6-8: stem isolates (6: BP5, 7: BT7, 8: BT13). Lane 9: 1257 (stock culture). M = marker.

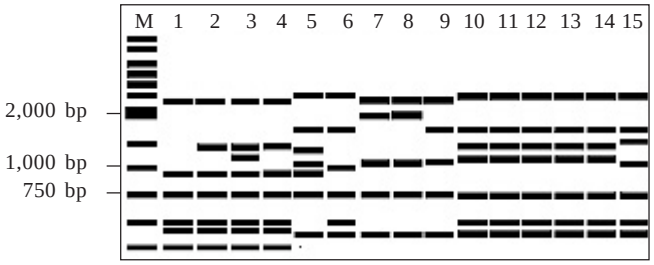

Figure 3. Diagrammatical RAPD banding patterns of *F. proliferatum* isolates using OPA2 primer. Lanes 1-4: root isolates (1: AP6, 2: AP10, 3: AP11, 4: AT12). Lanes 5-9: stem isolates (5: BT15, 6: BT14, 7: BT12, 8: BT11, 9: BT10). Lanes 10-15: stock cultures (10: 1325, 11: 1377, 12: 1378, 13: 1380, 14: 1391, 15: 1374), M = Marker.

Dendrobium as well as the isolates dfrom the stock cultures derived from the stem. Figure 3 shows diagrammatical banding patterns of some isolates of *F. proliferatum* using OPA2.

As RAPD bands of the three morphologically identified *Fusarium* species showed variable banding patterns, cluster analysis was perfomed to estimate the variability and to group the isolates. The dendrogram based on UPGMA cluster analysis of RAPD bands showed two main clusters, I and II, and several sub-clusters (Figure 4). The cluster analysis clearly discriminated *F. proliferatum, F. solani,* and *F. oxysporum* into separate clusters. *Fusarium proliferatum* isolates were clustered in main cluster I, *F. solani* and *F. oxysporum* in main cluster II i.e. in sub-cluster C and D, respectively.

DISCUSSION

Fusarium oxysporum, *F. solani*, and *F. proliferatum* recovered from root and stem rots of *Dendrobium* in the present study have also been reported to be associated with several diseases of orchid plants. The three *Fusarium* species were pathogenic, causing root rot of *Cymbidium* orchid (Benyon *et al*. 1996), dry rot of *Cymbidium* (Lee *et al*. 2002) and root rot of moth orchid (*Phalaenopsis* spp.) (Kim *et al.* 2002). *Fusarium proliferatum* has also been reported to cause

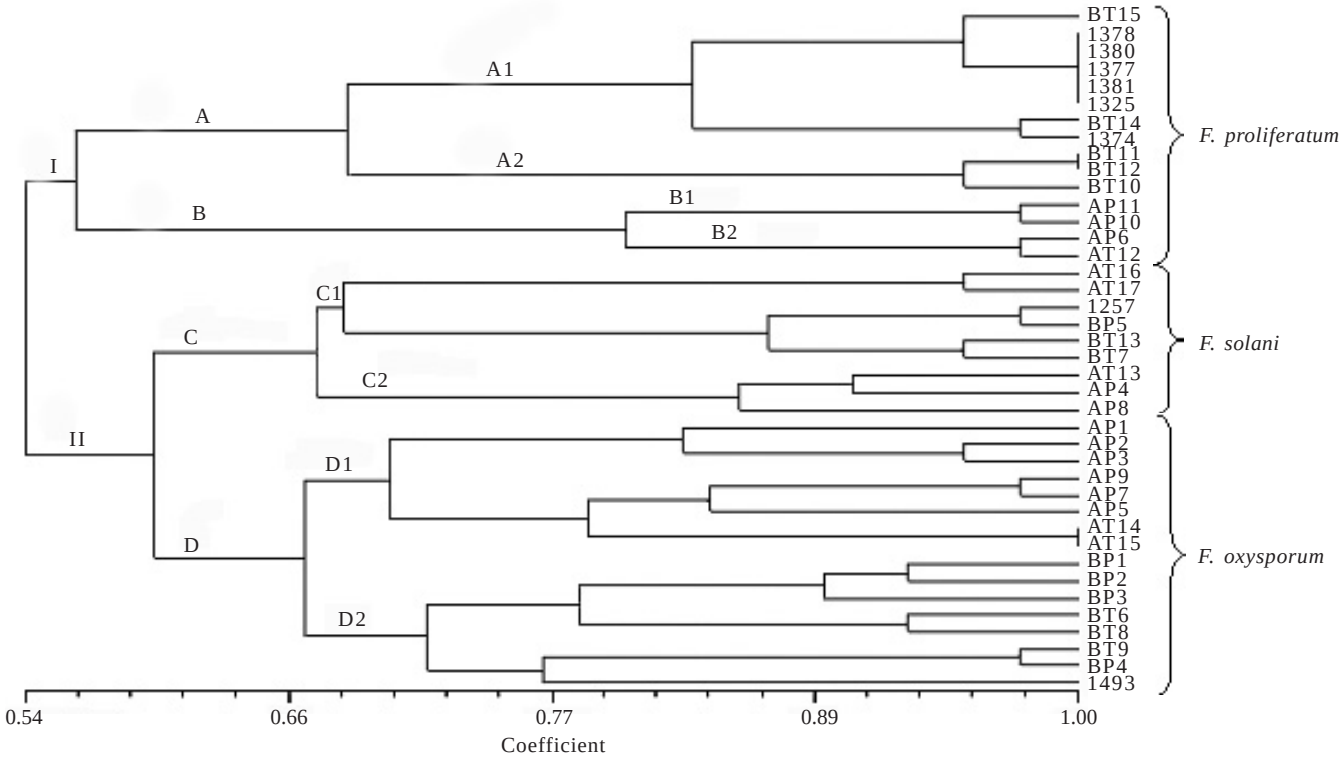

Figure 4. Dendrogram from UPGMA cluster analysis using simple matching coefficient based on RAPD bands of *F. oxysporum, F. proliferatum,* and *F. solani* from root and stem rot sof orchid.

yellow spot disease in *Cymbidium* by Ichikawa and Aoki (2000) and leaf spot of *Cymbidium hybrida* (Chang *et al.* 1998). Although *F. subglutinans* was isolated and associated with root rot and yellow spot disease of orchidaceous plants (Benyon *et al*. 1996; Ichikawa & Aoki 2000), however in this study, *F. subglutinans* was not recovered from any of the plant parts.

Variability within *F. oxysporum* isolates from orchid plants agrees with several studies which showed that pathogenic and non-pathogenic *F. oxysporum* are genetically diverse species complex that may comprised several varieties and races (Armstrong & Armstrong 1981; Waalwijk *et al.* 1996; Nelson *et al*. 1997; O'Donnell & Cigelnik 1997; Edel *et al.* 2001). Variability within *F. oxysporum* isolates was also observed in PCR-RFLP studies by Donaldson *et al.* (1995) and Llorens *et al.* (2006).

Fusarium solani isolates from orchid plants were also very variable. Several studies have indicated that *F. solani* is a species complex comprising several species and varieties (Booth 1971; Gerlach 1981; Nirenberg 1995). Sequence analysis of 28S rDNA also indicated that *F. solani* isolates might comprised a group of species (Guadet *et al*. 1989).

Like *F. oxysporum* and *F. solani*, *F. proliferatum* isolates from root and stem rots of orchid were also variable. Although *F. proliferatum* is not considered as a species complex, considerable variations within the species have been observed. Donaldson *et al*. (1995) reported some polymorphism of restriction patterns in the internal transcribed spacer region of *F. proliferatum* isolates from conifers. In another study, genotypic diversity of *F. proliferatum* isolates from root zone of *Liviatona mariae* palms was observed using Amplified Fragment Length Polymorphism (AFLP).

In conclusion, three *Fusarium* species namely, *F. oxysporum, F. solani,* and *F. proliferatum* were recovered from root and stem rots of *Dendrobium* orchid. UPGMA cluster analysis of RAPD bands were in agreement with morphological characteristics in which the three *Fusarium* species were clustered into different clusters.

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REFERENCES

Armstrong GM, Armstrong JK. 1981. Formae speciales and races of *Fusarium oxysporum* causing wilt diseases. In: Nelson PE, Toussoun TA, Cook R (eds). *Diseases, Biology and Taxonomy*. University Park: The Pennsylvania State Univ Pr. p 391-399.

Benyon F, Summerell BA, Burgess LW. 1996. Associations of *Fusarium* species with root rot of *Cymbidium* orchids. *Aust Pl Pathol* 25:226-228.

Booth C. 1971. The Genus *Fusarium*. Kew, Surrey, England: Commonwealth Mycological Institute.

Chang M, Hyun IK, Lee YH, Lee DH. 1998. Leaf spot of *Cymbidium hybrida* caused by *Fusarium proliferatum*. *Korean J Pl Pathol* 14: 664-667.

Donaldson GC, Alexandra BL, Axelwood PE, Glass NL. 1995. Primer sets developed to amplify conserved genes from filamentous Ascomycetes are useful in differentiating *Fusarium* species associated with conifers. *Appl Environ Microbiol* 62:1331-1340.

Edel V, Steinberg C, Gautheron N, Recorbet G, Alabouvette C. 2001. Genetic diversity of *Fusarium oxysporum* populations isolated from different soils in France. *FEMS Microbiol Ecol* 36:61-71.

Gaudet J, Julien J, Lafay JF, Brygoo Y. 1989. Phylogeny of some *Fusarium* species as determined by large-subunit rRNA sequences comparison. *Mol Biol Evol* 6:227-24.

Gerlach W. 1981. The present concept of *Fusarium* classification. In: Nelson PE, Toussoun TA, Cook R (eds). *Diseases, Biology and Taxonomy.* University Park: The Pennsylvania State Univ Pr. p 413-426.

Ichikawa K, Aoki T. 2000. New leaf spot disease of *Cymbidium* species caused by *Fusarium subglutinans* and *Fusarium proliferatum*. *J Gen Pl Pathol* 66:213-218.

Kim WG, Lee BD, Kim WS, Cho WD. 2002. Root rot of moth orchid caused by *Fusarium* species. *Plant Pathol J* 18:225-227.

Lee BD, Kim WG, Cho WD, Sung JM. 2002. Occurrence of dry rot on *Cymbidium* orchids caused by *Fusarium* spp. in Korea. *Plant Pathol J* 18:156-160.

Leslie JF, Summerell BA. 2006. The *Fusarium* Laboratory Manual. USA: Blackwell Publ.

Llorens A *et al*. 2006. Characterization of *Fusarium* spp. isolates by PCR-RFLP analysis of the intergenic spacer region of rRNA (rDNA). *Int J Food Microbiol* 106:297-306.

Nelson AJ, Elias KS, Arevalo GE, Darlington LC, Bailey BA. 1997. Genetic characterization of RAPD analysis of isolates of *Fusarium oxysporum* f. sp. *erythroxyli* associated with an emerging epidemic in Peru. *Phytopathology* 87:1220-1225.

Nelson PE, Toussoun TA, Marasas WFO. 1983. *Fusarium species: An Illustrated Manual for Identification*. University Park: Pennsylvania State Univ Pr.

Nirenberg HI. 1995. Morphological and phytopathological differentiation of *Fusarium* strains within Martiella section. In: *International Seminar on Fusarium Mycotoxins, Taxonomy and Pathogenicity*. Italy: Martina Franca. p 147.

NurHayati MZ. 2007. Characterisation of *Fusarium* species from orchid using morphological, molecular and pathogenicity test [Thesis]. Penang, Malaysia: Universiti Sains Malaysia.

O'Donnell K, Cigelnik E. 1997. Two divergent intragenomic rDNA ITS2 type within monophyletic lineage of the fungus, *Fusarium* are nonorthologous. *Mol Phylogenet Evol* 7:103-116.

Rohlf FJ. 2000. NTSYS-pc Numerical Taxonomy and Multivariate Analysis System, Version 2.0. Exeter Publishing Ltd. Setauket, New York, USA.

Waalwijk CJ, de Koning RA, Baayen RP, Gams W. 1996. Discordant groupings of *Fusarium* spp. from section Elegans, Liseola and Dlaminia based on ITS1 and ITS2 sequences. *Mycologia* 88:316-328.