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Full Length Article

Characterization of *Fusarium proliferatum* through Species Specific Primers and its Virulence on Rice Seeds

Shireen A. Jahan Quazi[1], Sariah Meon*[2], Hawa Jaafar[3] and Zainal Abidin B.M. Ahmad[2]
[1]*Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[2]*Department of Plant Protection, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[3]*Department of Crop Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
*For Correspondence: sariahm@putra.upm.edu.my; shireenbrri@yahoo.com

Abstract

Bakanae is one of the important diseases of rice (*Oryzae sativa* L.). To evaluate the pathogen(s) responsible for bakanae disease of rice in Malaysia, 12 isolates of *Fusarium spp*. were obtained from infected rice plants samples from rice growing in Tanjung Karang and Sekinchan areas of Selangor. All isolates were identified as *F. proliferatum* based on morphological characteristics and confirmed by amplification of DNA with species specific primer pairs Pro 1/2 at 554 bp. The rDNA-ITS primer sequences showed 99% homology with *F. proliferatum* isolâtes AJ810449.1, X94171.1, GU363955.1, EU151488.1, HQ380789.1, HQ332533.1, GU594758.1 and EU03930366.1. Pathogenicity testing on susceptible rice variety MR 211 proved all isolates to be pathogenic based on increase in plant height (%), decrease in main root length (%) and decrease in lateral roots number (%) of inoculated plants compared to control plants.

Keywords: *Fusarium proliferatum*; Bakanae disease, Pathogenicity, *Oryza sativa* L.; rDNA-ITS

Introduction

Bakanae, is one of the major fungal diseases of rice including blast, sheath blight, brown spot and sheath rot (Sharma and Thind, 2007). Among the main rice diseases, bakanae is common in Asia. It is difficult to develop bakanae resistant rice varieties due to the high genetic variation of the causal pathogens (Serafica and Cruz, 2009). Although both blast and bakanae are becoming a threat for sustainable rice production due to high genetic variation of the causal pathogens, but bakanae is more alarming compared to blast as the disease is not influenced solely by environment and mainly infection increases through "passive transmission" from the infected seeds (Cother, 2002). Bakanae is therefore a major concern in the affected rice growing areas in Asia and is also becoming a threat for sustainable rice production in other parts of the rice growing world.

Bakanae disease is economically important in the Asian rice growing areas due to the significantly large amount of losses estimated at approximately 20% in epidemic areas (Cumagun *et al*., 2011). According to a survey conducted by the Philippine Rice Research Institute in 2006, the incidence of bakanae was 46, 39 and 54% in Ilocos, Norte, and Agusan varieties, respectively (Serafica and Cruz, 2009). In Nepal, both local and improved varieties of rice have been identified as susceptible to bakanae (Desjardins *et al*., 2000). In recent years bakanae disease has been spreading and has been reported from newer parts of Asia where bakanae was not previously recorded. For instance, in Pakistan bakanae has become a major disease over the last five years (Bhalli *et al*., 2001). In Bangladesh 21% yield loss was reported in 2006 (Angeles, 2006). After that, Mainul *et al.* (2011) reported that bakanae has been increasing in Bangladesh in recent years at an alarming rate. In Malaysia, the disease was reported to have a significant effect on Malaysian rice varieties in the major rice growing areas of Kedah, Kelantan and Perak (Zainudin *et al*., 2008a).

A total of five *Fusarium* spp. belonging to "*Gibberella fujikuroi* species complex" under section *Liseola* (i.e. *F. fujikuroi, F. proliferatum, F. sacchari*, *F. subglutinans* and *F. verticillioides*) have been isolated and found to be associated with bakanae disease in Malaysian rice varieties (Zainudin *et al.*, 2008b; Nur Izzati and Salleh, 2009). *F. fujikuroi*, has been identified and reported as a pathogen of bakanae disease (Zainudin *et al*., 2008b; Amatulli *et al*., 2010). However, the role of the other *Fusarium* spp. association in relation to disease initiation and/or disease development is still unclear. *F. fujikuroi* species complex produces a complex of disease symptoms including seedling blight, root and crown rot, stunting and the typical symptoms of etiolation and abnormal elongation (Desjardins *et al*., 2000). The most visible symptom is highly elongated tillers both in the seedbed and in the field. The complex disease symptoms produced by the pathogen can be easily observed in pathogenicity tests.

Identification and differentiation of *F. fujikuroi* and *F.*

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proliferatum as well as *F. subglutinans* and *F. sacchari* based on morphological characteristics alone are "still incomplete and inconclusive" (Leslie and Summerell, 2006). Therefore, there is need to integrate "molecular characterization" as additional criteria with morphological characteristics for species characterization and identification of *Fusarium* spp. associated with bakanae disease. PCR-based techniques have been identified as a powerful tool for pathogen identification (Maheshwar and Janardhana, 2010; Amatulli *et al*., 2012). The limited research carried out using species specific primers for causal pathogen characterization has been done sporadically and not extensively over the different rice growing areas. Gene sequencing analysis of the causal pathogen has not been carried out before. Therefore, the aim of the present study was to (1) isolate the bakanae causing pathogen(s) from infected plant samples, (2) characterize the pathogen by using species specific primers followed by gene sequencing analysis, and 3) observe pathological effects of the causal pathogen on susceptible rice variety MR 211.

Materials and Methods

Fungal Isolation

Bakanae infected rice plants showing symptoms of internode elongation, pale green to yellowing leaves and formation of adventitious roots on the upper 3rd to 4th internodes were collected randomly from the major rice growing areas of Tanjung Karang, Sabak Bernam and Sekinchan in Selangor, Malaysia in August, 2010.

Infected plant samples were washed with water to remove adhering soil. Tissue sections, approximately 1-2 cm long, were cut from the node region, where adventitious roots were formed; surface sterilized with 70% ethanol, washed with sterile water and allowed to dry on sterilized filter paper. The sterilized sections were placed on Potato Dextrose Agar (Oxoid, PDA) and incubated at 25ºC day and 20ºC night temperatures with a 12 h photoperiod for 5 days (Burgess *et al*., 1994). White or pinkish *Fusarium* colonies growing from the tissue sections were sub cultured on fresh PDA and incubated for 14 days for sporulation.

Pure cultures obtained from a single spore of each isolate were grown on PDA to study colony morphology and pigmentation. Single-spored cultures grown on Spezieller Nährstoffarmar agar (SNA) were used to study the shape, size and type of macroconidia and microconidia, conidiogenous cells and chlamydospores (Pérez-Sierra *et al*., 2007). PDA plates were incubated at 28^0C in darkness and SNA plates were incubated at 28^0C with a 12-h photoperiod. All isolates were examined after 7-14 days using light microscopy and scanning electron microscopy (SEM). Samples were prepared for SEM by the modified technique of Al-Awadhi *et al*. (2002). Three sets of samples of each treatment were examined under SEM and 20 conidia of each type (macro and micro) were selected randomly for shape and size measurement. The isolates were identified according to the morphological characteristics described by Leslie and Summerell (2006).

Molecular Characterization

DNA extraction: Three mycelia plugs from 7-days old cultures of each fungal isolate was inoculated aseptically into 100 mL of potato dextrose broth (PDB) separately and incubated for 5 days at 28°C. The mycelia of each isolate was filtered through a double layer of sterile muslin cloth, washed with sterile distilled water, dried on filter paper and ground in liquid nitrogen. The genomic DNA of all *Fusarium* isolates was extracted from 200 mg of ground fresh mycelia according to the method of Maheshwar and Janardhana (2010) with slight modifications. The ground mycelium was taken in 1.5 mL microfuge tubes, vortexed and centrifuged at 12000 rpm for 10 min and the supernatant was discarded. The mycelium was re-suspended in cell lysis buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, and 100 mM Tris-HCl, pH 8.0, pre-heated at 65°C) and heated at 65°C for 20 min in a water bath. An equal volume of phenol: chloroform (1:1) was added to each tube, vortexed and centrifuged at 12000 rpm for 5 min. The supernatants were transferred to new microfuge tubes and an equal volume of isopropyl alcohol was added. The microfuge tubes were incubated at –20°C for 2 h and then centrifuged at 13000 rpm for 10 min to precipitate the DNA. The DNA pellet was air-dried and re-suspended in 100 µL of nuclease free water and used directly for PCR analysis. Quality and concentration of each of the genomic DNA was assessed using a Nano Drop ND-1000 Spectrophotometer (LMS Co., Ltd., Tokyo Japan). The UV absorbance at 260 and 280 nm were measured and the 260/280 absorbance ratio was computed.

PCR amplification of ribosomal DNA (rDNA) and partial calmodulin gene region: All isolates were tested with the universal primer ITS 1/4 for amplifying and sequencing (representative isolate) the fungal rDNA-ITS region and species-specific primer pairs PRO1/2, VERT1/2 were used to amplify the calmodulin-encoding regions (partial) (Mule *et al*., 2004). The nucleotide sequence of ITS1/4 (F: 5c`-GAAGTAAAAGTCGTAACAAG-3c`, R: 5c`-CCTCCGCTTATTGATATGC-3c`) for identifying *Fusarium* spp; and species-specific primer pairs PRO1/2 (F: 5c`-CTTTCCGCCAAGTTTCTTC-3c`, R: 5c`-TGTCAGTAACTCGACGTTGTTG-3c`) and VERT1/2 (F: 5c`-CTTCCTGCGATGTTTCTCC-3c`, R: 5c`-AATTGGCCATTGGTATTATATATCTA-3 c`) for identifying *F. proliferatum* and *F. verticillioides* respectively, were supplied by First Base Laboratories Sdn. Bhd., Selangor, Malaysia. The reaction mixture contained 1.0 µL of each primer (20 pmol), 0.2 µL of Taq DNA polymerase (5 U/µL), 1.5 µL of 10x PCR buffer, 1.8 µL of $MgCl_2$ (25 mM) and 0.2 µL of (25 mM) dNTPs. The final volume was brought up to 15 µL with nuclease free water.

Thermal cycling reaction for ITS 1/4 was: denaturation at 95ºC for 5 min, 35 cycles of denaturation at 95ºC for 1 min, annealing at 60ºC for 1 min, extension at 72°C for 2 min, final extension at 72°C for 10 min, followed by cooling at 4°C until recovery of the samples. Thermal cycling reactions of Pro 1/2 and VERT1/2 were: denaturation at 94ºC for 2 min, 35 cycles of denaturation at 94ºC for 1 min, annealing at 60ºC for 30 sec, extension at 72°C for 1 min, final extension at 72°C for 5 min, followed by cooling at 4°C until recovery of the samples. A 100 bp ladder (New England Biolabs, Ipswich, MS, USA) was used as a molecular size standard marker. The PCR products were separated by electrophoresis (at 80V for 40 min) on 2% (w/v) agarose gels with 1 × TBE buffer. The gels were then stained with ethidium bromide to visualize products under UV light using gel documentation system (Bio-Rad, Philadelphia, PA, USA).

DNA purification and sequencing: PCR product of a representative isolate (Iso-8) was purified using the QIAquick PCR Purification Kit (QIAgen, USA) following the manufacturer's instructions and sequenced commercially (NHK BIOSCIENCE SOLUTIONS, Kuala Lumpur, Malaysia) using the Bioneer 3730. The gene sequence was compared with sequences of *F. proliferatum* using the BLAST network services for similarities present in the National Centre for Biotechnology Information (NCBI) database (Altschul *et al.*, 1990). Multiple sequence alignment was performed using Clustal W (version 1.8) (Thompson *et al*., 1994).

Pathogenicity Test

All the 12 *Fusarium* isolates identified as *F. proliferatum* were used for pathogenicity testing on susceptible rice variety MR 211. Two inoculation methods involving seed inoculation and soil inoculation were evaluated. Data collected included increase in plant height (%) compared to control, plant infection (%) in different Disease Severity scale (DSS), Disease Severity Index (DSI), decrease in main root length (%), and decrease in number of lateral roots (%) compared to control. The Disease Severity Scale used was slightly modified from that described by Zainudin *et al*. (2008b) and Amatuli *et al*. (2010); where, 0 = Healthy and symptomless plant, 1 = Stunted plant with yellowing leaves, 2 = Thin plant with narrow, pale yellow leaves, 3= Abnormal elongated internodes with chlorotic or brownish leaves, 4 = Root formation from the upper 3rd- 4th internodes and necrosis symptoms on the lower internodes, 5 = Fungal mass seen on the infected plant or dead plant. The scores obtained were standardized on a 0–100 scale and the DSI was calculated according to Zainudin *et al.* (2008b).

Seed inoculation: Fourteen day old isolates grown on PDA were flooded with sterile water (100 mL) and scraped with a sterile spatula separately. The resulting suspensions were pooled and filtered through two layers of sterile muslin cloth and the final concentration was adjusted to 1 × 10^6 spores/mL by adding sterile distilled water and used for inoculation. Seeds of variety MR 211 were surface sterilized with 70% ethanol, washed with sterilized distilled water and then soaked in sterilized distilled water overnight. The water was drained out and seeds were further soaked in spore suspension of the respective isolates (10^6 conidia/mL) for 48 h. The seeds were then planted in sterilized soil (40% sand, 30% clay, 30% peat) in trays (2 kg soil/tray) measuring 38 × 28 × 11 cm arranged in a completely randomized design with 3 replications per isolate (20 seeds/replication). Seeds for the control treatment were soaked in sterile distilled water for 72 h before sowing. All trays were maintained in a glasshouse with day and night temperatures of 30.0–35.0^{o}C and 23.0–30.0^{o}C, respectively and watered daily. Fertilizer comprising of N:P:K (15:15:15) were applied at 4 g/tray, twice at 15 day intervals. Development of disease symptoms were recorded weekly beginning 7 days after inoculation.

Soil inoculation

Soil and spore suspensions for inoculation were prepared in the same manner as for seed inoculation. Surface sterilized MR 211 seeds were pre-soaked in water (24 h) and sown in sterilized soil, followed by spraying with the respective spore suspension (200 mL/tray @10^6 conidia/mL) of *F. proliferatum* isolates. The control treatments received the same volume of distilled water instead of spore suspension. Evaluations were determined based on DSI and other parameters described above. Re-isolation of pathogen from infected plants was carried out by random plating of inoculated tissues on PDA. Morphological characterization of *Fusarium* colonies derived from the infected tissues were compared with the identified *F. proliferatum*.

Results

Identification and Morphological Characteristics of *F. proliferatum*

A total of 12 isolates of *Fusarium spp*. were obtained from the bakanae infected rice plant samples collected from Tanjung Karang (eight isolates) and Sekinchan (four isolates) areas. All the isolates from the two sites were classified as *F. proliferatum* based on morphological characteristics. No *Fusarium spp.*, presumed to be associated with bakanae disease as reported by earlier researchers, was isolated from the samples collected from Sabak Bernam area.

Isolates of *F. proliferatum* produced white to light pinkish floccose aerial mycelium on PDA with an average growth rate of 0.8 cm/day. Pigments produced on PDA varied from white, light yellowish- brown to reddish-brown, light pink, light to deep purple brown with or without concentric rings and light violet or deep violet with concentric rings (Table 1). Sporodochia were orange coloured and black sclerotia were produced from four isolates (Iso: 3, 5, 6 and 8).

Macroconidia were hyaline, delicate, slightly sickle-

Table 1: Growth rate and pigmentation of *F. proliferatum* isolates isolated from bakanae infected rice plants

Isolates (*F. proliferatum*)	Colony diameter (cm) (after 7 days)	Pigmentation on Potato Dextrose Agar (PDA) medium
Iso*-1	6.3	Light pink
Iso-2	6.2	Light yellow
Iso-3	5.8	Deep purple brown
Iso-4	5.5	Light pink
Iso-5	5.6	Light pink with concentric rings
Iso-6	6.0	Light purple-brown with concentric ring
Iso-7	6.5	Light yellowish
Isp-8	5.6	Deep purple with concentric rings
Iso-9	5.6	Light yellowish brown
Iso-10	5.5	Light reddish-brown
Iso-11	5.7	White
Iso-12	5.7	Light brown
Average	5.9	

*Iso-Isolate

**pigmentation on PDA (Potato Dextrose Agar) medium was observed after 14 days of incubation

Table 2: Pathogenicity testing of *F. proliferatum* isolates on rice seeds using seed and soil inoculation methods

Isolates	plant height (cm) elongation (%) increase in infected plants over control plants		Disease Severity Scale (DSS)		Disease Severity Index (DSI) after 21 days of inoculation	
	Seed inoculation	Soil inoculation	Seed Inoculation scale: (infected plants)	Soil inoculation scale: (infected plants)	Seed inoculation	Soil inoculation
Iso-1*	63.23a**	51.51a	0: (3%), 1: (5%), 2 - 3: (84%), 4: (3%) and 5: (5%).	0: (4%), 1: (3%), 2 - 3: (91%), 5: (2%).	2.1	2.05
Iso-2	35.76f	12.43g	0: (4%), 1: (2%), 2 - 3: (90%), 5: (4%).	0: (5%). 1: (2%), 2 - 3: (92%), 5: (1%).	1.9	1.60
Iso-3	48.48d	46.32bc	0: (3%), 1: (3%), 2 - 3: (86%), 5: (8%).	0: (4%), 1: (3%), 2 - 3: (90%), 5: (3%).	2.3	1.95
Iso-4	43.36e	26.38e	0: (3%), 1: (2%), 2 - 3: (90%), 5: (5%).	0: (8%), 1: (1%), 2 - 3: (88%), 5: (3%).	1.8	1.65
Iso-5	52.55c	26.38e	0: (2%), 1: (3%), 2 - 3: (91%), 5: (4%).	0: (4%), 1: 2%), 2 - 3: (91%), 5: (3%).	1.6	1.55
Iso-6	43.27e	37.34d	0: (2%), 1: (3%), 2 - 3: (93%), 5: (2%).	0: (5%), 1: (3%), 2 - 3: (90%), 5: (2%).	1.7	1.7
Iso-7	47.18d	10.53g	0: (29%,) 1: (3%), 2 - 3: (68%), 5: (0%).	0: (33%), 1: (2%), 2 - 3: (65%), 5: (0%).	0.7	0.9
Iso-8	63.56a	54.88a	0: (0%), 1: (5%), 2 -3: (81%), 4: (5%), 5: (9%).	0: (7%), 1: (5%), 2 - 3: (80%), 5: (8%)	3.0	2.40
Iso-9	43.27e	39.13d	0: (6%), 1: (4%), 2 - 3: (88%), 5: (2%).	0: (10%), 1: (2%), 2 - 3: (87%), 5: (1%).	1.7	1.55
Iso-10	42.47e	19.15f	0: (4%), 1: (3%), 2 - 3: (91%), 5: (2%).	0: (6%), 1: (4%), 2 - 3: (87%), 5: (1%).	1.3	1.30
Iso-11	9.25g	3.00h	0: (5%), 1: (1%), 2: (91%), 5 :(3%).	0: (4%), 1: (3%), 2 - 3: (90%), 5: (3%).	2.4	2.10
Iso-12	57.05b	43.45cd	0: (5%), 1: (4%), 2 - 3: (82%), 4 :(1%), 5: (9%).	0: (7%), 1: (5%), 2 - 3: (87%), 5: (1%)	1.9	1.65
Lsd at 5%	3.644	6.598				

*Iso-Isolate

**Means tagged with different letters within column are significantly different using lsd at p≤0.05

shaped or almost straight, 3-5 septate (Fig. 1A) and produced in sporodochia (Fig. 1B). Distinct foot-shaped cell was observed at the base. The size of macroconidia averaged 17.39–38.1 × 1.9–3.1 µm. Chlamydospores were absent. Microconidia were hyaline, obovoid with distinct bases, 1–2 celled, fusiform to oval (Fig. 1C). The microconidia were agglutinated in short to long chains and or in false heads (Fig. 1D, E and F). Microconidia were produced mostly from polyphialides (Fig. 1G). Most of the microconidia were found to be produced from polyphialides in "V" shaped chains. The size of microconidia averaged 2.4–11.9 × 1.2–3.8 µm.

Amplification of ITS and Calmodulin Region

All isolates were used for molecular identification on the basis of morphological differentiation and pigmentation on PDA. PCR products of all isolates were DNA amplified with ITS1 and ITS4 primers resulting in approximately 550 bp fragments in ITS region (Fig. 2A). Further, the PCR products of the isolates were DNA amplified in the calmodulin region with species specific primer pairs Pro1 and Pro 2. Single bands were obtained with species-specific primers from all strains belonging to *F. proliferatum* and fragment size obtained was approximately 550 bp (Fig. 2B). No isolate was identified as *F. verticillioides* when used

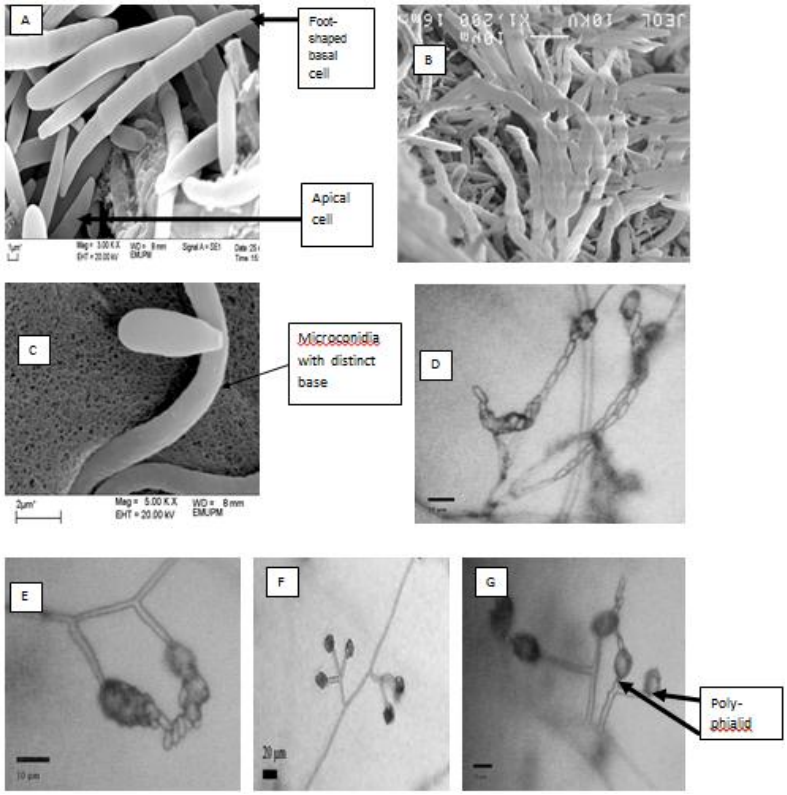

Fig. 1: Scanning Electron Micrographs of *F. proliferatum*: (A) Macroconidia, (B) Sporodochia and (C) Microconidium. Microconidia of *F. proliferatum* produced in (D) long chain, (E) short chain, F) false heads, and (G) from poly phialid.

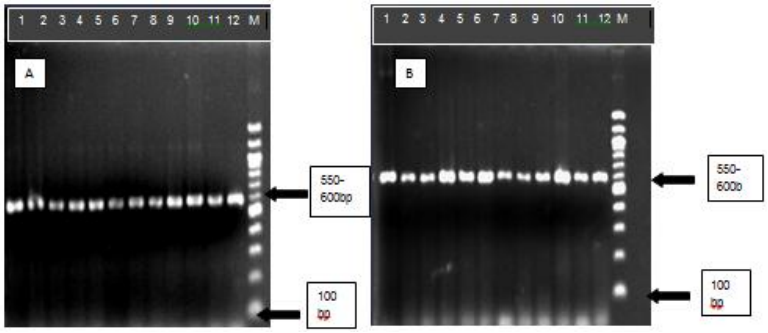

Fig. 2: PCR amplification of *F. proliferatum* isolates [A. Amplification with *Fusarium species* specific primer pairs ITS 1 and ITS 4; B. Amplification with specific species primer pairs for *F. proliferatum* PRO1/PRO2. Lane: 1-12 isolated species from bakanae infected rice plant. Lane M: DNA size marker (100 bp)]

with VERT1 and VERT2 primer pairs.

The amplified and purified DNA fragment of *F. proliferatum* isolate (Iso-8) was sequenced in both directions using the ITS region for further confirmation. The sequence obtained from the isolate was deposited in the GeneBank (NCBI Acc. JQ 807850). The ITS Primer sequences obtained from both directions showed 99% homology with the *F. proliferatum* isolates AJ810449.1, X94171.1, GU363955.1, EU151488.1, HQ380789.1, HQ332533.1, GU594758.1, EU03930366.1.

Pathogenicity Test

The development of symptoms of *F. proliferatum* infection in the glasshouse was observed to be similar in both seed and soil inoculation methods. Initial symptoms were observed as pale green to yellowing of foliage and thinner plants. These plants progressively turned brownish, became dry and died. Infected plants that survived this stage produced adventitious roots on the upper 2-3 internodes above the soil. At later stages of infection, white fungal

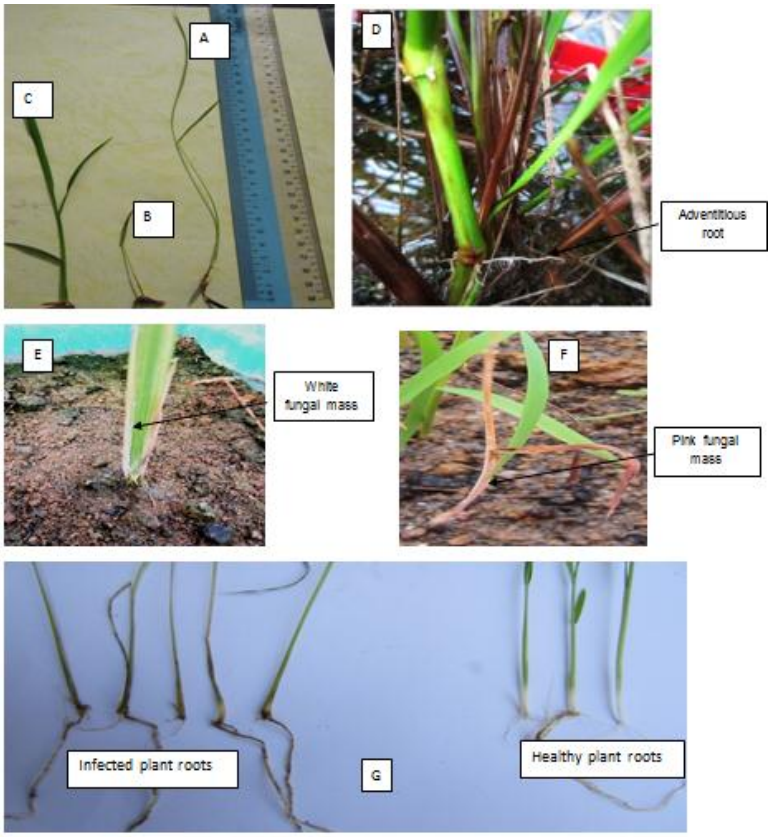

Fig. 3: Different types of symptoms observed in bakanae infected rice plants
A. Thin, pale green, elongated plant. B. Stunted plant. C. control plant. D. Aadventitious root formation on the upper nodes of infected plant. E. White mycelial mass produced on heavily infected plant. F. Fungal mass turning to pink color with time on infected dead plant. G. Crown rot and root necrosis occurred in bakanae infected plants compared to healthy (control) plants

mycelial mass was observed on infected plants at water level, which turned pinkish in color with time. Root necrosis was apparent. All symptoms due to bakanae infection are illustrated in Fig. 3.

All isolates were found to be pathogenic, but there was variation in the degree of infection observed among the isolates. All isolates in both methods of inoculation showed a low percentage (1-5%) of plants with a score of 1 within 7 days of inoculation, Symptoms with a score of 2 (68-90%) occurred between 5-10 days, and all plants with a score of 2 showed an increase to a score of 3, starting after 10 days of inoculation. In seed inoculation method, approximately 1-5% of plants showed symptoms with a score of 4 for Isolate 1, Isolate 8 and Isolate 12. In both methods of inoculation plants with symptom scores of 1 to 4 finally resulted in a score of 5 over time starting from 18 days after inoculation. None of the control plants showed any type of bakanae disease symptoms.

Based on increase in plant height (%), DSI, decrease in main root length (%) and decrease in number of lateral roots (%), seed inoculation was found to be the most suitable method for assessing bakanae disease development compared to soil inoculation in all the 12 isolates tested (Table 2, Fig. 4 and 5).

Discussion

Identification of the causal pathogen of bakanae disease was necessary due to the complex nature of the pathogen(s) associated with the disease development. As different *Fusarium* spp. belonging to the section *Liseola*, associated with bakanae disease have been isolated and reported, therefore the causal pathogen could be different and could depend on geographic as well as on climatic variations. Two causal pathogens, *F. pseudograminearum* and *F. graminearum* (*Gibberella zeae*) also have been identified and reported to causing "head blight of wheat" and are responsible in different geographical and as well as in different climatic regions (Burgess *et al*., 1987; Aoki and O'Donnell, 1999). This however, is the first report of *F. proliferatum* as the causal agent of bakanae disease in Malaysia.

Morphological features including growth characteristics and pigmentation on PDA of isolated

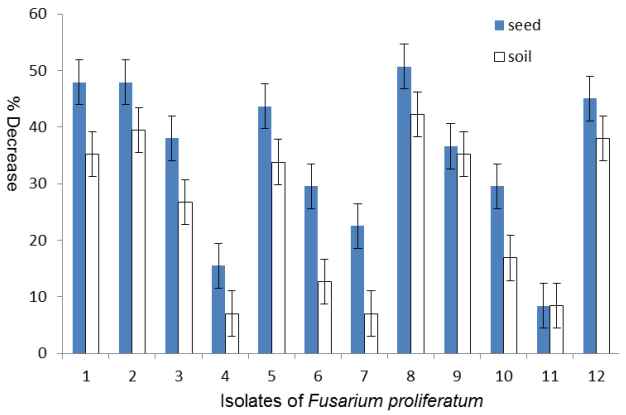

Fig. 4: Decrease (%) of main root length of infected plants over main root length of control plants using seed and soil inoculation methods, 14 days after inoculation

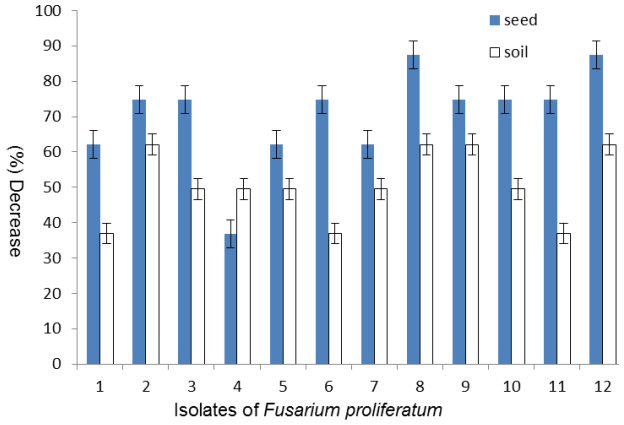

Fig. 5: Decrease (%) of lateral root numbers of infected plants over lateral root numbers of control plants using seed and soil inoculation methods, 14 days after inoculation

Fusarium species were found to be similar to *F. proliferatum* as reported by Burgess *et al.* (1994). The size of macro and micro conidia were also found to be similar to that reported by Gohari *et al.* (2007) and Zhan *et al.* (2010). All the isolated species were identified as *F. proliferatum* based on molecular identification using species specific primer pairs and confirmed with sequencing analysis for *F. proliferatum* (Mule *et al*., 2004; Leslie and Summerell, 2006; Rahjoo *et al.,* 2008). Earlier researchers had also isolated *F. proliferatum* from bakanae infected rice plants and reported that *F. proliferatum* would be one of the main *Fusarium* species causing for bakanae disease (Desjardins *et al*., 2000; Hsuan *et al*., 2011) but those authors did not carry out pathogenicity test.

F. fujikuroi, the most virulent species causing bakanae disease, produced excessive gibberelin hormones responsible for internode elongation of bakanae infected plants, which was low or absent in *F. proliferatum* (Desjardins *et al*., 2000; Malonek *et al.*, 2005). In the present pathogenicity tests, increase in plant height (%) was found to be significantly higher in plants inoculated with *F. proliferatum* isolate compared to the control. The explanation for increase in plant height might be due to the ability of the inoculated species to genetically or under mutation to produce gibberellin. Leslie (2004) had also identified one strain of *F. prolifeartum* that caused bakanae disease. Proctor (2010) reported five strains of *F. proliferatum* that could produce gibberellins. The authors however assumed that the strains might be a hybrid (mutant) between *F. fujikuroi* and *F. proliferatum* and the gene for gibberellic acid biosynthesis might be transferred to the hybrid strain.

This assumption is further supported by Cumagun *et al*. (2011) who suggested that "mixed reproduction" and "gene flow" might be responsible for formation of new genotype. The other explanation for production of symptoms of bakanae infection and excessive increase in plant height might be due to production of metabolites other than gibberellins. Desjardins *et al*. (2000) also suspected that the bakanae infection due to *F. proliferatum* might be due to production of metabolites, although the role of metabolites was not clear in bakanae disease development. Metabolite production by *F. proliferatum* might induce production of gibberellins in infected plants, or increase in plant height might be due to hormonal imbalance. Phylogenetically *F. proliferatum* and *F. fujikuroi* are closely related, whereas biologically both represent "variants of the same species" (Glenn, 2007).

Earlier researchers assumed *F. proliferatum* as non-pathogenic for causing bakanae infection (Zainudin *et al.*, 2008b; Amatulli *et al*., 2010). The results presented here provide evidence through pathogenicity testing, that this organism is pathogenic and causes bakanae disease. Both inoculation methods tested were found to be suitable for bakanae disease development. The symptoms observed and recorded in bakanae infected plants were similar to those recorded by other authors (PRRI, 2006; Graves, 2009). Approximately, 30% of plants were dead within 3 weeks of inoculation in both methods of seed treatment. Similar observation was also observed by Agarwal *et al*. (1989) where highly infected plants died between 14-42 days after inoculation. Although both methods were found to be suitable for assessing the pathogenic potential of *F. proliferatum* isolates in rice, however seed inoculation was considered better based on the higher values of DSI, increase in plant height (%), decrease in main root length (%) as well as the lateral root numbers (%). Moreover, symptom expression was observed 2-3 days earlier in the seed inoculation method compared to the soil inoculation method. Zainudin *et al.* (2008b) and Amatulli *et al*. (2010) had also reported good bakanae infection through seed inoculation.

In conclusion, *F. proliferatum* is also an important pathogen responsible for causing bakanae infection and disease development besides *F. fujikuroi*. Seed inoculation was found to be a more suitable method for disease development.

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