DIVERSITY OF *FUSARIUM* SPECIES ASSOCIATED WITH BAKANAE DISEASE OF RICE IN MALAYSIA AND INDONESIA

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by

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LIST OF ABBREVIATIONS

µg	Microgram (10^{-3} gram)
µl	Microliter (10^{-3} ml)
µm	Micrometer (10^{-3} mm)
µM	Micromolar
AFLP	Amplified restriction fragment polymorphisms
$AlCl_3$	Aluminium chloride
ANOVA	Analysis of variance
bp	Base pair
BS	Biological species
C	Cytosine
CA	Carrot agar
CH_3CN	Acetonitrile
CH_3COOH	Acetic acid
$CHCl_3$	Chloroform
CLA	Carnation leaf-piece agar
ClO_3	Chlorate
cm	Centimeter
CM	Complete medium
CRD	Complete randomized design
CRSs	Chlorate resistant sectors
dai	Day after inoculation
ddH_2O	Dionized distilled water
DMRT	Duncan's multiple range test
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
DON	Deoxynivalenol
DSI	Disease severity index
EtBr	Ethidium bromide
EtOAc	Ethyl acetate
EtOH	Ethanol
FA	Fusaric acid
FB_1	Fumonisin B_1
$FeSO_4\,7H_2O$	Ferum sulphate dehydrated
G	Guanine
g	Gram
GA_3	Gibberellic acid
h	Hour
H_2O	Water
H_2SO_4	Sulphuric acid
ha	Hectare
HCl	Hidrochloride acid
het	Heterokaryon incompatibility
HSC	Heterokaryon self-compatible
HSI	Heterokaryon self-incompatibility
HX^-	Hypoxanthine
K	Kalium

K_2HPO_4	Dikalium hydrogen phosphate
KCl	Potassium chloride
kg	Kilogram
KH_2PO_4	Kalium dihydrogen phosphate
L	Liter
MeOH	Methanol
MG	Mating group
mg	Milligram
$MgCl_2$	Magnesium chloride
$MgSO_4\,7H_2O$	Magnesium sulfate dehydrated
min	Minute
ml	Milliliter
MM	Minimal medium
mm	Millimeter
MMC	Minimal medium agar amended with chlorate
MON	Moniliformin
MP	Mating population
MS	Mass spectroscopy
N	Nitrogen
NaOCl	Sodium hypochlorite
ng	Nanogram
NH_4^-	Ammonium
nit	Nitrate non-utilizing
nm	Nanometer
NMR	Nuclear magnetic resonance
NO_2^-	Nitrite
NO_3^-	Nitrate
NTSYS	Numerical taxonomy and multivariate analysis system
°C	Degree centigrade
OPA	Operon technologies primer series A
OPT	Operon technologies primer series T
OPU	Operon technologies primer series U
OPV	Operon technologies primer series V
P	Phosforus
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDC	Potato dextrose agar amended with chlorate
PPA	Peptone pentachloronitrobenzene agar
psi	Per square inch
RAMs	Random amplified microsatellites
RAPD	Random amplified polymorphic DNA
R_f	Retention factor
RFLP	Restriction fragment length polymorphism
rpm	Revolution per min
SEA	Soil extract agar
SMC	Simple matching coefficients
SNA	Spezieller-Nahrstoffarmer agar
spp.	Species
SPSS	Statistical Package for social science
TE	Tris-EDTA

TLC	Thin layer chromatography
UPGMA	Underweight pair-group method with arithmetic
UV	Ultraviolet light
v/v	Volume/volume
VC	Vegetative compatibility
VCG	Vegetative compatibility group
vic	Vegetative incompatibility
W	Watt
WA	Water agar
ZEN	Zearalenone

DIVERSITY OF *Fusarium* SPECIES ASSOCIATED WITH BAKANAE DISEASE OF RICE IN MALAYSIA AND INDONESIA

ABSTRACT

Many diseases were reported to be associated with rice; one of the diseases is bakanae that caused by *Fusarium* spp.. The main objective of these studies was to characterize *Fusarium* spp. in Section Liseola isolated from bakanae-infected rice in Malaysia and Indonesia by using several approaches in order to determine the similarities and variabilities between the species. Results during sampling bkanae disease were widespread in Peninsular Malaysia and three provinces in Indonesia (East Java, Padang and Samarinda) except in Yan, Kedah. The typical symptoms of bakanae disease were abnormal elongation, yellowish leaves, produced a few tillers and wiry adventitious roots. Some infected plants were stunted and those survived appeared normal until maturity but produced empty and discoloured grains. A total of 212 strains of *Fusarium* were isolated from bakanae-infected rice plants and they were initially identified using morphological characteristics for species delimitation. The highest number (127) of *Fusarium* strains were classified into five species in Section Liseola i.e. *F. fujikuroi* (most frequently, 37.3%), *F. verticillioides, F. proliferatum, F. sacchari* and *F. subglutinans*. Other species were also isolated such as *F. semitectum, F. oxysporum, F. solani*, *F. longipes*, *F. chlamydosporum* and *F. equiseti*. Pathogenicity test showed *F. fujikuroi* was confirmed as the pathogen of bakanae disease on rice in Malaysia and Indonesia with varying levels of virulence. The strains of *F. fujikuroi, F. proliferatum, F. verticillioides, F. sacchari* and *F. subglutinans* were grouped into

26, 8, 8, 7 and 4 vegetative compatibility groups (VCGs), respectively. In crosses with seven standard testers of mating populations (MPs), 69.3% of strains could be assigned to at least one of the *Gibberella fujikuroi* species complex (MP-A to MP-E) based on their ability to produce perithecia and viable ascospores. However, five fertile strains that were assigned as MP-C and have been identified morphologically as *F. fujikuroi* were also crossed-fertile with MP-D tester. Furthermore, 25 strains of *Fusarium* species in Section Liseola were used in detection of some secondary metabolites e.g. moniliformin (MON), fumonisin B_1 (FB_1), fusaric acid (FA) and gibberellic acid (GA_3). Only *F. fujikuroi* strains examined were able to produce GA_3. This phenomenon could be used as a main physiological character in separating *F. fujikuroi* from the other four *Fusarium* species in Section Liseola. RAPD banding patterns of the five *Fusarium* species in Section Liseola were different. The results thus showed that the five species of *Fusarium* are clearly separated into different clusters. It is therefore concluded that the pathogen of rice bakanae is identified as *F. fujikuroi* by using morphological characteristics, pathogenicity test, VCGs, MPs, secondary metabolite profiles and RAPD analysis.

KEPELBAGAIAN *Fusarium* SPESIES YANG BERASSOSIASI DENGAN PENYAKIT BAKANAE PADA PADI DI MALAYSIA DAN INDONESIA

ABSTRAK

Banyak penyakit telah dilaporkan berasosiasi dengan padi; salah satunya ialah penyakit bakanae yang disebabkan oleh *Fusarium* spp.. Objektif utama kajian ini adalah untuk mengenalpasti *Fusarium* spp. dalam Seksyen Liseola yang dipencilkan daripada padi yang menunjukkan gejala penyakit bakanae di Malaysia dan Indonesia menggunakan beberapa teknik yang bertujuan untuk melihat kesamaan dan kevariabelannya di antara spesies. Semasa tinjauan dijalankan, penyakit bakanae telah menular secara meluas di Semenanjung Malaysia dan tiga provinsi di Indonesia (Jawa Timur, Padang dan Samarinda) kecuali di Yan, Kedah. Gejala khas penyakit bakanae adalah pemanjangan yang abnormal, daun kekuningan, menghasilkan sedikit anak dan juga akar adventitus yang keras seperti wayar. Sesetengah tumbuhan terjangkit menjadi terencat dan yang berjaya hidup kelihatan normal sehingga matang tetapi menghasilkan biji benih yang kosong dan kotor. Sebanyak 212 strain *Fusarium* berjaya dipencilkan daripada padi yang dijangkiti dan dikenalpasti menggunakan ciri morfologi hingga ke tahap spesies. Bilangan tertinggi (127) strain *Fusarium* dikategorikan kepada lima spesies dalam Seksyen Liseola iaitu *F. fujikuroi* (paling kerap dipencilkan, 37.3%), *F. verticillioides, F. proliferatum, F. sacchari* dan *F. subglutinans*. Spesies lain yang dipencilkan ialah *F. semitectum, F. oxysporum, F. solani*, *F. longipes*, *F. chlamydosporum* dan *F. equiseti*. Ujian kepatogenan menunjukkan *F. fujikuroi* adalah patogen penyakit bakanae pada padi di Malaysia dan Indonesia dengan pelbagai peringkat

kevirulenan. Strain *F. fujikuroi, F. proliferatum, F. verticillioides, F. sacchari* dan *F. subglutinans* masing-masing dikategorikan ke dalam 26, 8, 8, 7 dan 4 kumpulan keserasian vegetatif (VCGs). Keputusan kajian persilangan dengan tujuh populasi mengawan (MPs), 69.3% strain dikelompokkan ke dalam sekurang-kurangnya satu kompleks spesies *Gibberella fujikuroi* (MP-A hingga MP-E) berdasarkan kepada keupayaannya menghasilkan peritesia dan askospora yang vaibel. Walau bagaimanapun, lima strain yang dikelaskan sebagai MP-C dan telah dikenalpasti secara morfologinya sebagai *F. fujikuroi* juga mengawan dan subur dengan pencilan penguji MP-D. Sebagai tambahan, 25 strain *Fusarium* spesies dalam Seksyen Liseola digunakan dalam mengesan penghasilan metabolit sekunder iaitu moniliformin (MON), fumonisin B_1 (FB_1), asid fusarik (FA) dan asid gibberellik (GA_3). Hanya strain *F. fujikuroi* yang diperiksa berupaya menghasilkan GA_3. Fenomena ini boleh digunakan sebagai ciri fisiologi utama dalam mengasingkan *F. fujikuroi* daripada empat spesies *Fusarium* yang lain dalam Seksyen Liseola. Corak jalur RAPD bagi spesies *Fusarium* dalam Seksyen Liseola adalah berbeza-beza. Oleh itu, keputusan ini menunjukkan bahawa spesies *Fusarium* tersebut telah diasingkan dengan jelas ke dalam kluster yang berbeza-beza. Dengan ini dapat disimpulkan bahawa patogen tersebut dicirikan sebagai *F. fujikuroi* mengunakan ciri morfologi, pengujian kepatogenan, VCGs, MPs, profil metabolit sekunder dan analisis RAPD.

CHAPTER 1

GENERAL INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important staple food that supply more than 50% of all calories consumed (MacLean *et al.,* 2002), and provides major sources of energy for a large percentage of the Asian populations (Chin and Supaad, 1986; Webster and Gunnell, 1992). Rice, like most crops, is host to a correspondingly diverse spectrum of microorganisms, and very vulnerable to several pests and diseases. Chin and Supaad (1986) reported that in Malaysia, at least 50 different diseases such as blast, tungro, sheath blight, bacterial blight and bakanae have been recorded on rice.

Bakanae was one of the first diseases of rice described scientifically and recognized in 1828 in Japan, and formally described in 1898 (Webster and Gunnell, 1992). The disease was widely distributed in Asia and all rice growing areas of the world (Ou, 1985; Webster and Gunnell, 1992). Holiday (1980) reported yield losses as high as 70% in Australia and 15 - 20% in Asia due to bakanae disease. Then, Webster and Gunnell (1992) also reported in Asia that bakanae was responsible for yield losses of up to 50%. In Malaysia, however, the disease was seriously observed in 1985 during the second rice planting season in Kedah, Kelantan, and Perak (Saad, 1986). However, in Indonesia, the disease was first observed in 1938 (Semangun, 1991). If the disease becomes an outbreak due to lack of prevention through early detection, there will therefore be problems to worldwide food resources, especially for the majority of Asian.

Bakanae is a Japanese word that means 'foolish seedling' and refers to an abnormal elongation frequently seen in the infected plants (Webster and Gunnell, 1992). The same disease is called 'white stalk' in China, 'palay lalake' (man rice) in the Philippine, 'penyakit Fusarium' in Indonesia and also 'man rice' in Guyana (Ou, 1985). In the field, infected seedlings are taller than normal plants, with yellowish leaves, produced few tillers and may die before transplanting (Ou, 1985). Those that survived in the field are also elongated and produced a few tillers either dries up before maturity or produced early but sterile panicles, and usually the infected seeds are discolored (Ou, 1985). In the late stages of infection, a layer of white to pink fungal mass that represents sporodochia of the conidia may formed on the sheath and stiff adventitious roots may be produced on upper nodes (Ou, 1985; Chin and Supaad, 1986; Schnitzler, 1989; Webster and Gunnell, 1992). On certain conditions such as water shortage, plants are stunted (Ou, 1985). In many cases, gibberellin hormones produced by the pathogen cause plant abnormal elongation and fusaric acid causes stunting (Booth, 1971; Roncero *et al.,* 2003).

Bakanae is a seedborne disease caused by *F. moniliforme* Sheldon (Booth, 1971) and the pathogen was later identified as *F. fujikuroi* Nirenberg (Nirenberg, 1976), the anamorph stage of *Gibberella fujikuroi* Sawada. Some earlier phytopathologists, who used morphological characters in their species delimitation, considered that *F. moniliforme* was the only species involved in the bakanae disease complex (Snyder and Hansen, 1945; Nirenberg, 1976; Nelson *et al.,* 1983; Semangun, 1991). However, this taxon comprises a number of distinct species now collectively termed *G. fujikuroi* species complex, and divided into three mating populations (MPs), that also known as mating groups

(MG) or biological species (BS) i.e. 'MP-A', 'MP-B' and 'MP-C' (Hsieh *et al.,* 1977). Later, the number of MPs was increased to six ('MP-A' to 'MP-F') (Klittich and Leslie, 1992). Induction of *Gibberella* sexual stages could be used to distinguish MP within this group (Hsieh *et al.,* 1977; Kuhlman, 1982; Leslie, 1991; Leslie, 1995). 'MP-C' (the anamorph *F. fujikuroi*) was first defined in 1977 among strains from rice in Taiwan (Hsieh *et al.,* 1977) and usually recognized as the sole cause of this disease since its member produced large quantities of gibberellins in culture and induced bakanae symptoms in artificially inoculated rice (Sun and Snyder, 1981; Ellis, 1989). However, recent work confused these issues and suggested that another MPs such as 'MP-D' (the anamorph *F. proliferatum*) and 'MP-A' (the anamorph *F. verticilloides* [synonym *F. moniliforme*]) were involved. The strains of 'MP-D' were isolated from rice in Asia and 'MP-A' were isolated from rice in Africa, Australia and the United State (Amoah *et al.,* 1995; 1996; Desjardins *et al.,* 1997). Thus, more than one species of *Fusarium* in the *G. fujikuroi* species complex may be able to infect rice (Desjardins *et al.,* 2000), and cause symptoms of bakanae disease. Regardless of its peculiarly importance, bakanae is therefore one of the important and interesting diseases of rice worldwide.

The major issues faced in classification and identification of *Fusarium* strains associated with rice bakanae disease and other plants diseases are that too many characteristics are influence by different environmental factors. As we know, *Fusarium* spp. in Section Liseola are filamentous fungi, widespread cosmopolitan and one of the most economically important genera of pathogenic species on a wide range of hosts and most frequently isolated by phytopathologists (Nelson *et al.,* 1983; Burgess *et al.,* 1994; Roncero *et al.,*

2003). For examples, *Fusarium* species in the *G. fujikuroi* species complex have wide host range which is also infecting several graminaceous crops such as maize (*Zea mays*), sorghum (*Sorghum bicolor*) and sugarcane (*Saccharum officinarum*) (Booth, 1971; Chin and Supaad, 1986; Roncero *et al.,* 2003). In Indonesia, Semangun (1991) reported that the species complex could infect maize and others graminaceous plants such as sugarcane (Pohkkah boeng disease).

Fusarium species are also involved in diseases of animals and human. Some caused major storage rots on food and feeds, and at the same time contaminating the substrates with harmful substances known as mycotoxins (Booth, 1971; Marasas *et al.,* 1986; 1988; Desjardins *et al.,* 2000; Desjardins and Proctor, 2001). Nevertheless, many species are common soil saprophytes where they are also act as a primarily soil inhabitant (Nelson *et al.,* 1983; Leslie and Summerell, 2006). Therefore, they are abundantly endowed with means of survival, one of the mechanisms of which is the capacity for rapid changes, often morphologically as well as physiologically, to a new environment. Thus, they can survive on a wide range of substrates. Because of their capacity to change rapidly, species identification presents certain problems (Booth, 1971).

Essentially, taxonomy of the genus *Fusarium* has been studied since early 1800s, but it is still poorly defined. In the beginning, the identification of *Fusarium* spp. was usually based on the differences in anamorphic morphological characters (Wollenweber & Reinking, 1935; Snyder and Hansen, 1945; Nelson *et al.,* 1983; Nirenberg, 1989). Within the teleomorphic *G. fujikuroi* species complex, however, the available classical morphological characters are insufficient to resolve all the biologically meaningful entities. At present, the

different biological species in this species complex can be distinguished by modest differences in secondary characters such as growth rate, pigmentation, presence of sporodochia and microconidial chain length (Klittich and Leslie, 1992), virulence test (Bosland and Williams, 1987), vegetative compatibility groups (VCGs) (Leslie, 1993), sexual cross-fertility (Hsieh *et al.,* 1977; Kuhlman, 1982; Leslie, 1991; Leslie, 1995; Leslie and Summerell, 2006), ability to synthesize secondary metabolites (Leslie *et al.,* 1992a; 1992b; 1996), sensitivity to antifungal agents (Yan *et al.,* 1993), DNA-DNA thermal renaturation profiles (Ellis, 1988; 1989), and phylogenetic species concepts (Leslie *et al.,* 2001; Summerell *et al.*, 2003).

Morphological species concepts are based on the similarity of observable morphological characters e.g. growth rate, pigmentation, production of microconidia, macroconidia and chlamydospores. On the other hand, variability mechanisms can also be used for identification of *Fusarium* spp. by looking at the genetic characteristics e.g. by using vegetative compatibility groups (VCGs), to determine whether or not the strains are in the same VCGs. If the strains are grouped in the same VCGs, its means the strains are in the same species. Frequently, the strains in the same VCGs have similarity in genetic characteristics (Leslie, 1993).

Recently, many taxonomists and phytopathologists used molecular tools in the identification of *Fusarium* species based on PCR-based techniques i.e. Random Amplified Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphism (RFLP). Lately, Amplified Restriction Fragment Polymorphisms (AFLP) was established in generation of characters to form phylogenies and those that are part of the same monophyletic group (Summerell *et al.,* 2003).

Physiological and chemotaxonomic criteria may also serve as supplements to morphological, genetic and molecular characteristics. During the last 20 years it has become evident that each *Fusarium* species has a specific profile of secondary metabolites (Thrane, 2001).

Because of the above reasons, these studies were focused on vegetative compatibility groups (VCGs), biological species (BS) based on mating population (MP) and molecular techniques by using RAPD analysis as well as physiological studies based on secondary metabolites profiles in assisting morphological identification of *Fusarium* spp. in Section Liseola isolated from rice showing typical bakanae disease symptoms.

Generally, the studies were carried out to characterize *Fusarium* spp. in Section Liseola isolated from rice showing typical bakanae symptoms in Malaysia and Indonesia by using several approaches in order to determine the similarity and variability between species. The detail explanation and justification of these objectives are highlighted below:

A. To determine the distribution, symptoms development, incidence and severity of bakanae disease in Peninsular Malaysia.

   Survey of disease incidence was done in granary areas in 6 states of Peninsular Malaysia (Perak, Kedah, Selangor, Pahang, Terengganu and Kelantan) based on the IRRI Standards Evaluation System for rice (Anon., 1996).

B. To identify *Fusarium* strains from rice plants showing typical bakanae symptoms, to determine the diversity of species and to examine the morphological characteristics of *Fusarium* species strains in Section Liseola from Malaysia and Indonesia.

   All strains were identified by using morphological characteristics which emphasized on growth rates, colony features, mode of production of microconidia and presence or absence of monophialides, polyphialides and chlamydospores for species delimitation and to develop a key for quick identification.

C. To determine whether or not the *Fusarium* species isolated from bakanae infected rice are pathogenic.

   Pathogenicity test was carried out by using monoconidial strains of *Fusarium* spp. in Section Liseola isolated from rice plants showing bakanae symptoms, in order to fulfill the Koch's postulate.

D. To investigate the genetic variability of five species in Section Liseola and to classify the strains of those *Fusarium* species into VCGs.

   VCG is one of the tools to observe into the genetic characteristics of *Fusarium* species. Formation of heterokaryon in two *nit* mutants by mycelial fusion showed the strains are in the same VCG and of the same species. However, if no fusion takes place the strains are classified in different VCGs.

E. To determine the nature of the compatibility and mating population (MP) of *Fusarium* species in Section Liseola

Members of the same MP are sexually fertile with one another but do not mate with different mating populations (MPs). The indicator is being able to produce perithecia of teleomorph stage of *Fusarium* species (*Gibberella* sp.). These different MPs are associated with different anamorphic (*Fusarium*) species.

F. To distinguish the secondary metabolite profiles i.e. fumonisins, moniliformin, gibberellic acid and fusaric acid produced by the *Fusarium* strains.

Thin layer chromatography (TLC) analysis was used for detection of these four secondary metabolites. Different species of *Fusarium* spp. produced different profile of secondary metabolites.

G. To study variability of the *Fusarium* strains by molecular techniques based on Random Amplified Polymorphisms DNA (RAPD).

RAPD was used to determine their genetic relatedness between the species. The underweight pair-group method with arithmetic averaging (UPGMA) analysis was used to designate the strains into clusters from different biological species.

CHAPTER 2

LITERATURE REVIEW

2.1 Rice in Malaysia and Indonesia

Rice (*Oryza sativa*) is cultivated in a wide range of geographical regions from the tropics to the warm temperate regions (Chin and Supaad, 1986). Generally, *O. sativa* has three races but in tropical areas, Indica and Japonica races are the two most important races (Webster and Gunnell, 1992). Most countries in the Asian region depend so exclusively on a single staple food as does in Malaysia (Arriffin and Nik Fuad, 2003) and Indonesia (MacLean *et al.,* 2002). Rice provides 21% of human per capita energy, 15% of per capita protein, minerals, vitamins, and fiber, although all constituents except carbohydrates are reduced by milling (MacLean *et al.,* 2002). On average, Malaysian consumes about 1.8 million metric tonne of rice every year with per capita consumption is estimated to be around 81 kg per annum (Ou, 1985) and was increased to 88.4 kg per capita per annum in 1999 (MacLean *et al.,* 2002). However, Indonesian rice consumption in 1999 is 154 kg milled rice per capita. In contrast with Malaysia, Indonesia is one of the top 10 rice-producing countries which covered more than 13,000 islands, including five of the world's largest islands: Sumatra, Kalimantan (Indonesian part of Borneo), Irian Jaya (western New Guinea), Sulawesi (Celabes) and Java. Therefore, Indonesia has 11,015,000 ha of rice planting areas (MacLean *et al.,* 2002).

Rice in Malaysia has a long history and very significant in our daily life. Hill (1977) claimed that rice was cultivated in Terengganu in early 14th century. Then, in early 16th century, several rice growing areas were identified in Kedah,

Perak, Melaka, Johor, and Pahang (Chin and Supaad, 1986). In these states, cultivation of rice was initially encouraged by the colonial economies to provide food for their settlement and soldiers, and later in support of the plantation industry. However, rice only began to obtain priority attention from the Government of Malaysia (then Malaya) following independence in 1957. Rural development and poverty eradication became the major prongs of the Government policy, and rice cultivation received a substantial increased in the form of subsidies, improvements in infrastructure, and extension (Chin and Supaad, 1986).

In 2000, Malaysia produced a total of 2.04 million tones of rough rice (merely 0.3% of world rice production) from the physical land areas of about 692,389 ha gazetted for rice with an average of 2.94 t/ha yield (MacLean *et al.,* 2002). The major granary areas are located in nine irrigation schemes i.e. West Coast of Selangor, KADA (including Southern Kelantan), Besut, Krian and Sg. Manik, MADA, Seberang Perak, Kemasin and Semerak (Chin and Supaad, 1986). Like any other crops, rice is also facing with multiple pests and diseases attack from time to time. One of the diseases knows amongst scientific community as bakanae disease.

2.2 Bakanae Disease of Rice

2.2.1 Occurrence

The disease is caused by a fungus and also known as foot rot or elongation disease (Schnitzler, 1989). The same disease has been called white stalk in China, and 'palay lalake' (man rice) in the Philippines (Ou, 1985).

Countries facing the bakanae disease incidence include Australia, Guyana, Cameroon, Sri Lanka (Ceylon), China, the Former French Equatorial Africa, India, Italy, Ivory Coast, Kenya, Nigeria, Surinam, Tanzania, Thailand, Trinidad, Uganda, U.S.A, Venezuela and Vietnam (Ou, 1985). Sun and Snyder (1981) reported that the disease was one of the earliest serious diseases of rice in the Asian region, but, nowadays, the disease is widely distributed in all rice growing areas especially in the tropics (Ou, 1985; Webster and Gunnell, 1992). Due to its mode of transmission i.e. seedborne, the disease could easily be distributed from one location to another through infected seeds (Ou, 1985; Schnitzler, 1989).

No one knows exactly when and how the first bakanae disease appeared in Malaysia and Indonesia. The disease became serious in Malaysia (Kedah, Perlis, Kelantan and Perak) during the second rice growing season in 1985. A susceptible variety is Seberang, also known as MR 77 was widely planted during that season (Saad, 1986; Rosmayati, 1988). These signified that the disease is widely distributed in Malaysia. However, in Indonesia the disease was observed since 1938 (Semangun, 1991). Therefore, the disease is being taken into consideration in the rice breeding programme as a selection criterion for varietal screening for resistance.

2.2.2 The causal organism

The disease was first described by Hori in 1898 and considered to be caused by the infection of *F. heterosporum* Nees. Later, the perithecial stage *Lisea fujikuroi* Sawada was found in 1917 (cited from Ou, 1985; Schnitzler, 1989). Wollenweber (1931) expressed the view that the fungus was identical

with *F. moniliforme* Sheldon and removed *L. fujikuroi* to the genus *Gibberella* as *G. fujikuroi* Sawada (Padwick, 1950; Ou, 1985; Schnitzler, 1989). *F. moniliforme* Sheldon was therefore recognized as the fungus responsible in causing bakanae (Padwick, 1950; Ou, 1985; Schnitzler, 1989). In contrast, Gerlach and Nirenberg (1982) and O'Donnell *et al.* (1998) used *F. fujikuroi* Nirenberg, widely acceptable by most plant pathologists and mycologists.

F. fujikuroi is the anamorph stage of *G. fujikuroi* and also known as mating population C ('MP-C') that usually produces large quantities of gibberellin in culture and induces bakanae symptoms in the field and on artificially inoculated rice (Sun and Snyder, 1981; Gerlach and Nirenberg, 1982; Ellis, 1989). At the moment, it is believed that bakanae disease may be caused by one or more *Fusarium* species and was classified into a complex of disease symptoms. Although bakanae disease was first described more than 100 years ago, it is still unclear which species of *Fusarium* are responsible in causing the variable symptoms of the disease (Sun and Snyder, 1981; Ou, 1985; Webster and Gunnell, 1992).

2.2.3 Disease symptoms

The most conspicuous and typical symptoms shown by naturally infected plants in the field are the typical bakanae symptoms i.e. abnormal or excessive elongation of the infected plants. Infected seedlings are up to several inches taller and thinner than normal plants, yellowish-green, and subsequently drying out (Sun and Snyder, 1981; Ou, 1985; Chin and Supaad, 1986; Schnitzler, 1989). Severely diseased seedlings die before transplanting, and those that survived may die after transplanting. However, not all infected seedlings show

the above-mentioned typical bakanae symptoms. In certain conditions, infected seedlings are stunted or appeared normal (Sun and Snyder, 1981; Ou, 1985; Chin and Supaad, 1986; Schnitzler, 1989).

For mature crop, the infected plants are taller, and the thin tillers bear pale yellowish-green leaves which are conspicuous above the general level of healthy crop. Infected plants usually have fewer tillers compared to normal plants, and the leaves dry-up in a few weeks. Sometimes, infected plants survive until maturity but produced empty panicles with discolored seeds. While infected plants are dying, the fungal pathogen produced a white or pink growth on lower parts of the plant, comprising mycelium and sporodochia that consist a large number of conidia, and extend upwards after the plants are dead (Sun and Snyder, 1981; Ou, 1985; Chin and Supaad, 1986; Schnitzler, 1989). Another obvious symptom associated with infected plants is the development of stiff (wiry) adventitious roots from the first, second, and sometimes the third nodes from the ground level (Chin and Supaad, 1986; Ou, 1985; Sun and Snyder, 1981). Overall, the development of bakanae symptoms depends on strains of the causal organism (Ou, 1985; Webster and Gunnell, 1992).

2.3 Pathogenicity Test

Some diseases caused by fungi can be recognized from typical symptoms alone especially when characteristics appearance of the pathogen, or known as aethiology is so obvious e.g. mildew, rust, and smut (Waller, 2001). Although some of the fungi are not pathogenic but they are obviously associated with unhealthy plants. In many cases, plant pathologists have made many mistakes by assuming the cause of a disease because of they are

frequently isolated from disease parts such as necrotic root, crowns and stems, where many *Fusarium* species have the ability to live as saprophytes. Majority of *Fusarium* species are fast growing in culture media but most pathogenic species are relatively slow-growing (Nelson *et al.,* 1983). Thus, Koch's postulate must first be fulfilled to ensure the pathogenic status of the fungus after isolation completed. The pathogenicity test should be assessed by using a technique that enables us to reproduce the typical symptoms of the disease in the glasshouse or field situation. At the same time, the hosts must be at susceptible stages and the conditions must be favorable for the development of the disease. As a seedborne disease, the effective technique of inoculation is through direct application on seed surface with spore suspension of the fungus suspected as a pathogen (Waller, 2001).

Rosmayati (1988) established the pathogenicity test of bakanae disease successfully by using conidial suspension (2×10^6 spores/ml) of *F. moniliforme* applied on the seed. The result showed that *F. moniliforme* was pathogenic with varying levels of virulence while control plants remained uninfected and healthy. Before that, Ahmed *et al.* (1986) established that 1.25×10^5 spore/ml was the optimum spore concentration in screening rice varieties for resistance to bakanae. This concentration showed maximum elongation with moderate root growth but the shoot elongation was significantly higher with 3.1×10^4 spores/ml.

Rosmayati (1988) also reported that the most susceptible rice variety from Malaysia was Seberang (MR 77) while the most resistant was MR-97. Significant differences between treatments (strains) were also detected. All infected plants showed typical bakanae symptoms i.e. abnormal elongation, thin

and yellowish leaves with stiff (wiry) adventitious roots. The symptoms were rated based on Rosmayati (1988); (1) healthy and uninfected plants; (3) normal growth and yellowish-green leaves; (5) abnormal growth, elongated, thin and yellowish-green leaves. Seedlings infected also shorter or longer than normal seedlings; (7) abnormal growth, elongated, chlorotic, thin and brownish leaves, and (9) heavily infected and died.

2.4 History of *Fusarium* Taxonomy

Taxonomy is the science of systematic classification of living and fossilized organisms (Booth, 1975). The taxonomy of the genus *Fusarium* was a subject of controversy for too many years. In the beginning, there was a confusion and disorder in *Fusarium* taxonomy, with more than 1000 species, varieties, and forms were used on the basis of superficial observation (Toussoun and Nelson, 1975). The genus *Fusarium* was divided into Sections, and a Section is used for one or more genera with a large number of species. The original idea was to group *Fusarium* species according to similar morphological characteristics (Nelson *et al.,* 1994). All *Fusarium* species have one typical taxonomic feature i.e. production of distinctly shaped macroconidia, usually with a foot-shaped basal cell, when produced in sporodochia. This main feature can be used as a basis for the classical approach to *Fusarium* taxonomy when combine with primary and secondary criteria (Nelson *et al.,* 1983).

The taxonomy of *Fusarium* began with the description of the genus by Link in 1809, based on the presence of fusiform non-septate conidia borne on a stroma (cited from Toussoun and Nelson, 1975). However, in 1935, the monograph *Die Fusarien* by Wollenweber and Reinking become the foundation

of the present system of classification, where the genus *Fusarium* was reduced to 143 species, varieties, and forms that were grouped in 16 Sections. Since then, knowledge on variation in fungi was increased, and then, further advances in the taxonomy of *Fusarium* have been achieved.

Snyder and Hansen (1945) classified *Fusarium* into 9 species with no varieties or forms. However, 44 species and 7 varieties were grouped in 2 Sections according to Booth (1971). Joffe (1974) classified about 26 species and 29 varieties in 9 Sections. Nelson *et al.* (1983) published an excellent monograph and widely used as practical guide to assist in the identification of *Fusarium* species. The most comprehensive treatment of *Fusarium* species is culminated in the *Fusarium* Laboratory Manual by Leslie and Summerell (2006).

In the beginning, many *Fusarium* taxonomists used morphological species concepts in their identification approaches. At the moment, basically there are three different species concepts being employed in identification approaches i.e. morphological, biological and phylogenetics (Summerell *et al.*, 2003).

2.5 Section Liseola

Wollenweber and Reinking (1935) included 3 species and 3 varieties in Section Liseola. However, Snyder and Hansen (1945) reduced the number to a single species i.e. *F. moniliforme* Sheldon emended Snyder and Hansen. Booth (1971) recognized only one species i.e. *F. moniliforme* with one variety *subglutinans* but Nelson *et al.* (1983) documented 6 species. Gerlach and Nirenberg (1982) increased the number to 9 species and 5 varieties. Numbers of Fusarium species in Section Liseola are varied according to different

taxonomic systems (Table 2.1). Nirenberg and O'Donnell (1998) developed a key to describe *Fusarium* species of the *G. fujikuroi* complex and related species for identification of *Fusarium* spp. or associated with Section Liseola (Appendix 1). At the moment, 27 *Fusarium* species are classified into or possibly allied to Section Liseola (Table 2.2). However, the number is still increasing, especially when chlamydospore-producing species; which is not the characteristics of *Fusarium* species in Section Liseola, are also included.

Table 2.1: Species of *Fusarium* in Section Liseola by different taxonomic systems

Wollenweber and Reinking (1935)	Snyder and Hansen (1945)	Booth (1971)	Gerlach and Nirenberg (1982)	Nelson *et al.* (1983)
F. lactis *F. moniliforme* *F. moniliforme* var. *anthophilum* *F. moniliforme* var. *minus* *F. moniliforme* var. *subglutinans* *F. neoceras*	*F. moniliforme*	*F. moniliforme* *F. moniliforme* var. *subglutinans*	*F. annulatum* *F. anthophilum* *F. fujikuroi* *F. lactis* *F. neoceras* *F. proliferatum* var. *minus* *F. proliferatum* var. *proliferatum* *F. sacchari* var. *elongatum* *F. sacchari* var. *sacchari* *F. sacchari* var. *subglutinans* *F. succisae* *F. verticilloides*	*F. annulatum* *F. anthophilum* *F. moniliforme* *F. proliferatum* *F. subglutinans* *F. succisae*

Table 2.2: *Gibberella fujikuroi* species complex and it's related species

Species	References
F. acutatum	Nirenberg and O'Donnell, 1998
F. andiyazi	Marasas *et al.*, 2001
F. anthophilum	Nirenberg,1976
F. begoniae	Nirenberg and O'Donnell, 1998
F. beomiforme	Nelson *et al.*, 1987
F. brevicatenulatum	Nirenberg *et al.*, 1998
F. bulbicola	Nirenberg and O'Donnell, 1998
F. circinatum	Nirenberg and O'Donnell, 1998
F. concentricum	Nirenberg and O'Donnell, 1998
F. denticulatum	Nirenberg and O'Donnell, 1998
F. dlamini	Marasas *et al.*, 1985
F. fujikuroi	Nirenberg,1976
F. globosum	Marasas *et al.*, 1987
F. guttiforme	Nirenberg and O'Donnell, 1998
F. konzum	Zeller *et al.*, 2003
F. lactis	Nirenberg and O'Donnell, 1998
F. mangifera	Britz *et al.*, 2002
F. miscanthi	Gams *et al.*, 1999
F. napiforme	Marasas *et al.*, 1987
F. nygamai	Burgess and Trimboli, 1986
F. nisikadoi	Nirenberg and Aoki, 1997
F. phyllophilum	Nirenberg and O'Donnell, 1998
F. proliferatum	Nirenberg,1976
F. pseudoanthophillum	Nirenberg *et al.*, 1998
F. pseudocircinatum	Nirenberg and O'Donnell, 1998
F. pseudonygamai	Nirenberg and O'Donnell, 1998
F. ramigenum	Nirenberg and O'Donnell, 1998
F. sacchari	Gerlach and Nirenberg, 1982
F. subglutinans	Nelson *et al.* 1983
F. thapsinum	Klittich *et al.*, 1997
F. verticillioides	Nirenberg,1976

2.6 Traditional Taxonomic (Morphological) Characteristics

Species delimitation by using traditional taxonomic characters are the basis in *Fusarium* identification (Burgess *et al.*, 1994; Leslie and Summerell, 2006). The primary characters used to separate species in *Fusarium* taxonomy are based on the morphology of the macroconidia, microconidia, microconidiophores, and chlamydospores (Nelson *et al.*, 1994). The morphology of macroconidia is the main characteristic for identification not to the species but also the genus levels. The shape of the macroconidia formed in

sporodochia is stable when the fungus is grown on low medium or natural substrates under standard growth conditions (Nelson *et al.,* 1983; Burgess *et al.,* 1994). Microconida of *Fusarium* species have various shapes and sizes. The presence and absence of microconidia is a primary character in *Fusarium* taxonomy. The shape of microconidia (fusiform, oval, obovoid, obovoid with a truncate base, allantoid, napiform, pyriform, and turbinate) and conidiogenesis (mode of conidial formation; whether singly, in false heads only or in false heads and in chains) are observed when microconidia are present (Nelson *et al.,* 1983; Burgess *et al.,* 1994; Nelson *et al.,* 1994; Leslie and Summerell, 2006). These characters are best observed *in situ* on CLA cultures (Fisher *et al.*, 1982). When chlamydospores are present, they may be formed singly, in pairs, clumps or chains, with either rough or smooth walls (Nelson *et al.,* 1983; Burgess *et al.,* 1994; Nelson *et al.,* 1994; Leslie and Summerell, 2006).

In addition, secondary characters are also useful to identify the species when the cultures are grown under standard growth conditions (light, temperature, and substrate). The morphology and pigmentation of the colony, the length and width of the macroconidia may be useful to differentiate the species (Nelson *et al.,* 1983; Burgess *et al.,* 1994; Leslie and Summerell, 2006).

Generally, morphological appearance of *Fusarium* spp. in Section Liseola are light pigmentations (i.e. colorless to typically purple, sometimes yellow, never red) with thin to dense aerial mycelium on PDA; microconidia borne in chains (short, medium or long), and/or false heads with varying shapes such as oval to fusiform, clavate, globose or napiform. Microconidiophores are monophialides and/or polyphialides. Macroconidia is pedicellate, thin-walled,

and almost straight. Chlamydospores are absent (Nelson *et al.,* 1983; Windles, 1991; Leslie and Summerell, 2006).

2.7 Genetic Characteristics (Vegetative Compatibility Groups, VCGs)

2.7.1 History and general properties of vegetative compatibility

Vegetative compatibility (VC), also known as heterokaryon compatibility, reveals the genetic relatedness between two strains of fungi. Basically, VC is controlled by the action of a set of vegetative incompatibility (*vic*) or heterokaryon incompatibility (*het*) loci in Ascomycetes (Glass and Kuldau, 1992). VC means that anastomosis could take place between two hyphae and fuse, and forms a stable heterokaryon (Leslie, 1993). When the heterokaryon is stable the strains are said to be vegetatively compatible with one another and are frequently described as members of the same vegetative compatibility group (VCG) (Leslie, 1993; 1996; Leslie *et al.*, 2001). Incompatibility reaction may be homogenic or allelic, in which a stable heterokaryon is formed only when the two interacting strains carry the same alleles at all *vic* loci, or heterogenic, in which the alleles at one locus interact with alleles of other loci (Puhalla, 1981).

An experiment on fungal, VC was first conducted by Garnjobst (1953) on *Neurospora crassa*. The study included classical genetics, in which five *vic* or *het* loci were identified. However, the numbers of loci involved were increased to 10 *vic* loci after Mylyk (1975) used a chromosome re-arrangement technique.

Although a progress in basic genetic studies of *vic* loci have been made in *Fusarium*, the observation of hyphal interactions were merged with genetic

theory developed for model genera such as *Aspergillus* and *Neurospora*. Besides, the merger has been used primarily in population studies of *F. oxysporum* in an attempt to develop new diagnostic techniques (Puhalla and Spieth, 1983; Puhalla 1985; Puhalla and Spieth, 1985). A few basic studies was completed, however, and there is an evidence for genetic segregation of *vic* loci in both the heterothallic *F. moniliforme* (perfect stage *G. fujikuroi*) (Puhalla and Spieth, 1983) and the homothallic *F. graminearum* (prefect stage *G. zeae*) (Bowden and Leslie, 1992). In *F. moniliforme,* one *vic* locus (*vic1*) has been mapped and there are strains that are known to differ at only this locus, and based on the segregation of different VCG types from a cross, at least ten *vic* loci are expected in *F. moniliforme* (Puhalla and Spieth, 1983). To conclude two strains in the same VCG, the both strains must be identical of each other at of least 10 different *vic* or *het* loci (Leslie *et al.,* 1992a).

Leslie (1996) suggested that VC can be used to subdivide fungal populations into different VCGs and that these subdivisions were correlated with pathogenicity. The laboratory analysis of VCG with complementary nitrate non-utilizing (*nit*) mutants is technically simple, and requires little more than basic microbiological materials (Correll *et al.,* 1987a). VCG is a direct multi-genic assessment of a trait of adaptive importance within fungal populations, and well-suited for measuring genotypic diversity e.g. the frequency of different genotypes within a population and for determining if two strains are identical to one another. However, the VCG technique is not a panacea for population analyses of pathogenic fungi. VCG analyses are not an appropriate for determining strains that belong to different biological species or for assessing

differences that occur above the species level. The VCG technique is therefore useful for measuring genotypic diversity (Leslie, 1996).

2.7.2 Nitrate non-utilizing (*nit*) mutants

Chlorate, a nitrate analogue, has been used for studying heterokaryosis in *Fusarium* spp.. In general, the growth of chlorate-sensitive strains is restricted by the internal reduction of chlorate to toxic chlorite by nitrate reductase. Chlorate-resistant strains do not take up chlorate or are unable to reduce chlorate to chlorite. *Nit* mutants are usually unable to reduce chlorate to chlorite because of a lesion at one or more of the loci that control nitrate reductase, thus rendering them chlorate-resistant (Liu and Sundheim, 1996).

Most fungi can utilize nitrate as nitrogen sources by reducing it to ammonia (Figure 2.1) via nitrate reductase and nitrite reductase pathways (Garraway and Evans, 1984). *Nit* mutants were readily recovered from *F. moniliforme* (Klittich *et al.*, 1986; Klittich and Leslie, 1988). In the current investigation, complementary *nit* mutants recovered from each strain were categorized into one of the several phenotypic classes by their relative growth on phenotyping media containing different nitrogen sources. These classes presumably represent a mutation at a nitrate reductase structural locus (*nit1*), a nitrate-assimilation pathway-specific regulatory locus (*nit3*), and one of the several loci (*nit2, nit4, nit5, nit6, nit7*) that affected the assembly of the molybdenum-containing cofactor necessary for nitrate reductase activity, commonly called NitM (Correll *et al.*, 1987a; Klittich and Leslie, 1988).

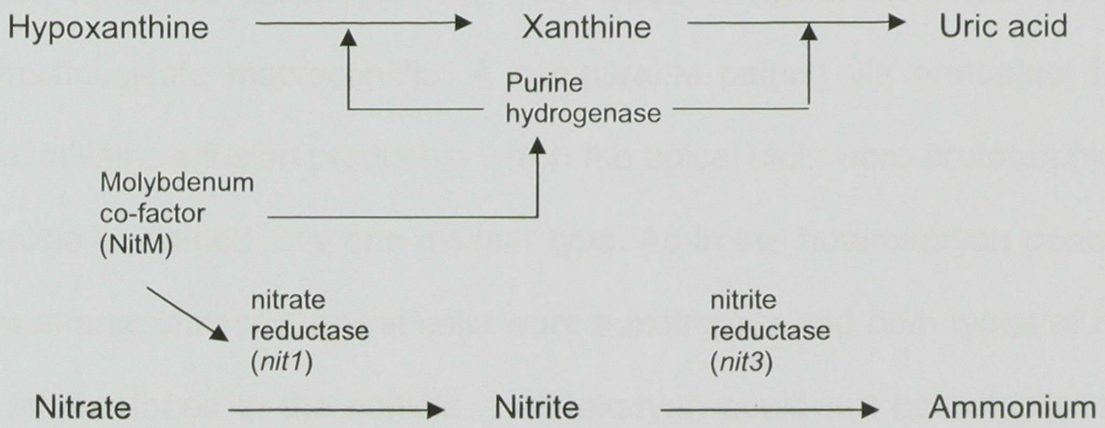

Figure 2.1: NO_3 metabolism pathway as it relates to the generation and classification of *nit* mutants for VCG testing (Leslie and Summerell, 2006)

2.7.3 Heterokaryon formation

Heterokaryon formation was established by pairing mutants that were unable to reduce nitrate and indicated by dense growth where the two mutant colonies merged (Puhalla, 1985). VC systems generally act to restrict the transfer of nuclear and cytoplasmic elements during growth. In each of these fungi, hyphal fusion usually occurs normally, even between strains that carry *vic* nuclei. The incompatibility systems in fungi are all allelic in the nature and field populations usually are rather polymorphic. Conceptually, the simplest systems are those in which strains that are identical at particular set of loci are capable of forming stable heterokaryons, while those that differ at any of these loci are incapable of forming a vegetatively stable heterokaryon (Leslie, 1993; 1996; Leslie *et al.*, 2001).

Adams *et al.* (1987a) distinguished the heterokaryosis in *G. zeae*. It varies depending upon the compatibility of the fused strains and the manner in which the strain are fused. Analysis of nutrionally complementing auxotrophic markers in pairings via hyphal anastomoses revealed a nuclear distribution in which heterokaryosis was restricted to anastamoses cells. Apical cells,

however, remained auxotrophic but both types of nuclei could be recovered from multinucleate macroconidia. A comparable pairing via protoplast fusion yielded, initially, a fusion product in which the apical cells were prototrophic and the conidia contained only one nuclear type. As in the heterokaryon generated by hyphal anastomoses, apical cells were auxotrophic and both types of nuclei could not be found in the conidia. Heterokaryon could not be established by pairing *vic* hyphal cells but the fusion of protoplasts from incompatible cells yielded a slow-growing, prototrophic colony in which conidia resolved only one nuclear type. This nuclear type was different from either of the parental type, and all conidia were capable of growth on MM (Adams *et al.,* 1987a).

2.7.4 Heterokaryon self-incompatibility (HSI)

Mutants with a novel phenotype i.e. inability in forming heterokayon due to improper fusion and the *vic* reaction occurs among themselves, have been described and termed as heterokaryon self-incompatibility (HSI) (Jacobson and Gordon, 1988; Correll *et al.,* 1989). Strains carrying mutations that prevent them from fusing to form heterokaryon, even with themselves, have been identified in field populations of *F. oxysporum* (Bosland and Williams, 1987; Jacobson and Gordon, 1988), *F. moniliforme* (Klittich and Leslie, 1988; Correll *et al.,* 1989; Campbell *et al.,* 1992), and *F. subglutinans* (Correll *et al.,* 1992).

In *F. moniliforme* (Correll *et al.,* 1989), naturally HSI mutant strains cultured under laboratory condition form 2 - 16% of the hyphal fusions formed by heterokaryon self-compatible (HSC) strain, which can form heterokaryons. Some of HSI strains can form weak heterokaryons with HSC strains (Jacobson and Gordon, 1991; Campbell *et al.,* 1992), however, the HSI strains appear to