Secondary metabolite profiles and mating populations of *Fusarium* species in section Liseola associated with bakanae disease of rice

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**ABSTRACT**
A total of 25 strains of Fusarium species that belong to *F. fujikuroi* (a pathogen of bakanae disease), *F. proliferatum*, *F. sacchari*, *F. subglutinans* and *F. verticillioides* were isolated from rice plants showing typical bakanae symptoms in Malaysia and Indonesia and screened for their secondary metabolites. The objectives of the studies were to determine the physiological variability based on production of moniliformin (MON), fumonisin (FB₁) and gibberellic acid (GA₃) and fusaric acid (FA) as well as to ascertain the mating populations (MPs) within the Gibberella fujikuroi species complex based on their ability to produce perithecia and viable ascospores. Production of GA₃ could be used to separate *F. fujikuroi* that belongs to MP-C from other species. In crosses with seven standard testers of MPs, 76% of strains could be assigned to at least one of the *G. fujikuroi* species complex namely MP-A (*G. moniliformis*), MP-B (*G. sacchari*), MP-C (*G. fujikuroi*) and MP-D (*G. intermedia*). Single strain (M3237P) that was assigned as MP-C, and has also been identified morphologically as *F. fujikuroi* was also crossed-fertile with MP-D tester. The secondary metabolite profiles and the presence of MP-A, MP-B, MP-C and MP-D strains on samples of bakanae-infected rice plants are new records in Malaysia.

**Keywords:** Secondary metabolite, mating population, bakanae, Fusarium, Gibberella.

**INTRODUCTION**
Bakanae is caused by *Fusarium moniliforme* Sheldon and the pathogen was later identified as *F. fujikuroi* Nirenberg the anamorph stage of Gibberella fujikuroi Sawada (Nirenberg, 1976). In Malaysia, the disease was seriously observed in 1985 during the second rice planting season in Kedah, Kelantan and Perak (Saad, 1986).

In the beginning, identification of *Fusarium* spp. was usually based on the differences in anamorphic morphological characteristics (Wollenweber and Reinking, 1935; Snyder and Hansen, 1945; Nelson et al., 1983; Nirenberg, 1989). However, identification of *Fusarium* species based on this limited characteristic is insufficient to resolve all the physiological and biologically meaningful entities, especially in identifying the closely related *Fusarium* species in section Liseola. For example, *F. fujikuroi* and *F. proliferatum* is similar based on morphological characteristics. Both species commonly is distinguished by mating types, chemotaxonomic criteria and molecular marker (Leslie and Summerell, 2006). Secondary metabolites, particularly mycotoxins and mating population (MP) are characters in identification of *Fusarium* to species and sometimes to sub-species levels (Leslie and Summerell, 2006).

Many efforts were undertaken to generate a comprehensive classification system for *Fusarium* species especially those in section Liseola. During the last 20 years, studies on chemotaxonomic criteria have become evident that each *Fusarium* species has a specific profile of secondary metabolite (Thrane, 2001). Thus, physiological studies, especially by using chemotaxonomic criteria, may serve as supplements to morphological characteristics in delimitation of *Fusarium* species (Nelson et al., 1993; Thrane and Hansen, 1995).

Normally, different species of *Fusarium* produced different profiles of secondary metabolites e.g. most members of *F. verticillioides* produce fumonisin B₁ (FB₁) and little or no moniliformin (MON), whereas most members of *F. fujikuroi* produce both FB₁ and MON (Marasas et al., 1986; Leslie et al., 1992).

Another possible way to solve these taxonomic difficulties is to use MP based on the sexual stage to distinguish the species. Mating is an essential step in the life cycle of sexually reproducing organisms. Mating pattern within each of these MPs are heterothallic and governed by two alleles at a single mating-type locus.
Members of the same MP are sexually fertile with one another but not with members of different MPs. Later, nine different MPs (designated by letter A to I) have been distinguished (Leslie, 1991; Klittich and Leslie, 1992; Kerényi et al., 1997; Steenkamp et al., 2000; Zeller et al., 2003).

The objectives of this study were: i) determine the physiological variability based on production of secondary metabolites i.e. MON, FB1, gibberellic acid (GA3) and fusaric acid (FA); ii) to determine nature of the compatibility and MPs of Fusarium strains isolated from rice showing bakanae symptoms in Malaysia and Indonesia.

MATERIALS AND METHODS

Fusarium strains
A total of 25 strains of five Fusarium species i.e. F. fujikuroi, F. verticillioides, F. proliferatum, F. sacchari and F. subglutinans were isolated and identified initially by using morphological characteristics (Table 1) following Leslie and Summerell (2006).

Secondary metabolites profiles

Preparation of inoculum and inoculation
The strains were cultured on potatoes dextrose agar (PDA) for 7 days. Each culture plate was flooded with 10 ml sterile distilled water and the conidia gently dislodged by using a sterile “hockey stick” glass rod. The suspension was filtered by using a sterile double-layered muslin cloth to remove the mycelial debris and the suspension was filtrated by using a sterile “hockey stick” glass rod. The cultures were incubated in the dark at the standard growth conditions (Salleh and Sulaiman, 1984) for 28 days. The suspension was filtered after 28 days of incubation and the pH was adjusted to 3.5 – 4.0 with 5N HCl and resuspended in 95% EtOH. The control flasks were produced in the same manner but were inoculated with sterile distilled water.

Extraction and chemical analysis
The solvent systems were used for chemical analysis i.e. solvent systems A = toluene:acetone:MeOH (5:3:2), v/v/v; B = CHCl3:MeOH (97:3), v/v; C = EtOAc:CH3COOH:H2O (6:3:1), v/v/v; D = CHCl3:MeOH:CH3COOH (6:3:1), v/v/v; E = isopropanol:ammonia:H2O (21:1:1), v/v/v; F = isopropanol:ammonia:H2O (10:1:1), v/v/v; G = CHCl3:EtOAc:formic acid (5:4:1), v/v/v; H = toluene:acetone:MeOH (5:3:2), v/v/v, and I = isopropanol:EtOAc:CH3COOH (4:0:3.8:2:0.2), v/v/v.

MON: The cultures were assayed for MON following Kamimura et al. (1981) with slight modifications. The residues were dissolved in MeOH, and 10 µL of the suspended residues was spotted onto a silica gel TLC plate (Merck, Darmstadt, Germany) (20 cm², 0.25 mm thick silica gel 60 F254) along with a standard marker (Sigma) of MON. The plates were dried with a heat gun and developed in solvent systems A and B as described by Burmeister et al. (1979) and Kamimura et al. (1981). MON was visualized according to Kamimura et al. (1981).

FB1: The extracts were filtered and the supernatants were evaporated to dryness following Scott et al. (1999). The residues were dissolved in acetone. About 10 µL of the suspended residues was spotted on a silica gel TLC plate along with a standard of FB1 (Sigma) and developed in specific solvent systems C and D as described by Ross et al. (1991), Tseng et al. (1995) and Fadl Allah (1998). FB1 was visualized following Tseng et al. (1995) and Fadl Allah (1998).

GA3: The cultured media were filtered and pH of filtered product was adjusted to 2.5 by using 1N HCl (Hasan, 2002). Then, the filtered product was extracted following Hasan (2002) with slight modifications. The suspended residue was spotted on a silica gel TLC plate along with standard GA3 (Sigma) and developed in vertical direction by using specific solvent systems E, F and G as described by Hasan (2002) and Chang and Jacobs (1973). GA3 was visualized according to Hasan (2002).

FA: The cultures were filtered after 28 days of incubation and the pH was adjusted to 3.5 – 4.0 with 5N HCl and resuspended in 95% EtOH. The suspended residue was spotted on a silica gel TLC plate along with FA standard (Sigma) and developed in specific solvent systems H and I as described by Burmeister et al. (1985). The TLC plates were observed under longwave UV light (365 nm), following Burmeister et al. (1985).
Retention factor value (Rf value)

The distance that the spot of a particular mycotoxin and the standards moved up on the TLC plate relative to the distance moved by the solvent front is called the retention factor or Rf value. The Rf values of individual secondary metabolites were calculated by following Fessenden et al. (2001);

\[ R_f = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent}} \]

Table 1: Secondary metabolite profiles of strains of Fusarium spp. in Section Liseola isolated from bakanae disease of rice

<table>
<thead>
<tr>
<th>Fusarium species</th>
<th>Strains</th>
<th>Location</th>
<th>MP</th>
<th>Secondary metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MON</td>
<td>FB1</td>
</tr>
<tr>
<td><em>F. fujikuroi</em></td>
<td>B3102P</td>
<td>Sekinchan, Kuala Selangor, Selangor</td>
<td>C</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>B3110P</td>
<td>Sekinchan, Kuala Selangor, Selangor</td>
<td>C</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>B3120P</td>
<td>Sekinchan, Kuala Selangor, Selangor</td>
<td>C</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>B3127P</td>
<td>Sungai Leman, Sungai Besar, Selangor</td>
<td>C</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>B3140P</td>
<td>Sungai Leman, Sungai Besar, Selangor</td>
<td>C</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>B3143P</td>
<td>Sungai Leman, Sungai Besar, Selangor</td>
<td>C</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>I3208P</td>
<td>Padang, Sumatra, Indonesia</td>
<td>C</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>K3219P</td>
<td>Pendang, Kedah</td>
<td>C</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>M3237P</td>
<td>Merlimau, Melaka</td>
<td>C, D</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>T3068P</td>
<td>Kg Apal, Jabi, Terengganu</td>
<td>C</td>
<td>+</td>
</tr>
<tr>
<td><em>F. verticillioides</em></td>
<td>A3053P</td>
<td>FELCRA, Seberang Perak, Perak</td>
<td>Sterile</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>A3055P</td>
<td>FELCRA, Seberang Perak, Perak</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>A3057P</td>
<td>FELCRA, Seberang Perak, Perak</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>A3063P</td>
<td>FELCRA, Seberang Perak, Perak</td>
<td>Sterile</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D3070P</td>
<td>Padang Pak Amat, Pasir Puteh, Kelantan</td>
<td>Sterile</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>I3410P</td>
<td>Tulung Agung, East Java, Indonesia</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td><em>F. proliferatum</em></td>
<td>B3095P</td>
<td>Sekinchan, Kuala Selangor, Selangor</td>
<td>D</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>A3054P</td>
<td>FELCRA, Seberang Perak, Perak</td>
<td>D</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>C3083P</td>
<td>LKPP, Padang Sg. Laka, Rompin, Pahang</td>
<td>D</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>C3089P</td>
<td>LKPP, Padang Sg. Laka, Rompin, Pahang</td>
<td>D</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>D3074P</td>
<td>Paklekbang, Tumpat, Kelantan</td>
<td>D</td>
<td>+</td>
</tr>
<tr>
<td><em>F. sacchari</em></td>
<td>C3080P</td>
<td>LKPP, Padang Sg. Laka, Rompin, Pahang</td>
<td>Sterile</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>K3222P</td>
<td>Pendang, Kedah</td>
<td>B</td>
<td>-</td>
</tr>
<tr>
<td><em>F. subglutinans</em></td>
<td>B3124P</td>
<td>Sawah Sempadan, Kuala Selangor, Selangor</td>
<td>Sterile</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D3077P</td>
<td>Ladang Ana Fasa 2, Tumpat, Kelantan</td>
<td>Sterile</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MP = Mating population; MON = Moniliformin; FB1 = Fumonisn B1; GA3 = Gibberellic acid; FA = Fusaric acid
+ = Detected; - = Not detected
Control = Non-inoculated corn grit or media for strains growth
Mating population

Crosses were made on carrot agar (CA) following protocol of Klittich and Leslie (1992). The standard mating-type testers strains (MPA to MP-G) were obtained from the Department of Plant Pathology, Kansas State University, Kansas, U.S.A. Strains expected as female parents were inoculated on CA and strains serving as the male were inoculated on complete medium agar slant. Crosses were scored as fertile if the mature perithecia were observed, that contained eight ascospores in the asci. Perithecia were observed in situ by using a compound microscope (FESEM Leo Supra Model 50VP Carl-Zeiss SMT). Asci and ascospores were observed through mounted slides by using a light microscope (Olympus model BX-50F4). Both in situ and slide observations were photographed by using a camera (JVC model FY-F55BE) with a image analyzer-SIS programme.

RESULTS AND DISCUSSION

TLC analysis is a qualitative analysis for determination of multiple mycotoxins as well as other secondary metabolites (Burmeister et al., 1979; Steyn, 1981; Ross et al., 1991; Fadl Allah, 1998). Metabolite profiles of 25 strains of five Fusarium spp. isolated from rice plants showing typical symptoms of bakanae disease are shown in Table 1. No detectable spots of any of the four secondary metabolites were observed on the plates spotted with extracts obtained from the control (non-inoculated) media. For the highest degree of confidence, direct comparison with standards of the secondary metabolites purchased from Sigma® were also applied throughout the studies.

MON was extracted from inoculated cornmeal grits by using MeOH before TLC analysis. MON was visually discernible at Rf value near 0.37 when developed in solvent system A (Table 2). The results were similar with those of Burmeister et al. (1979) although the sources of the fungal strains were different, and the strains were not isolated from bakanae-infected rice. For comparison, solvent system B was also used, however, the Rf value was too low (Table 2), hence difficult to be estimated, near 0.02. The solvent system is therefore categorized as a weak solvent and not practically applicable for MON. The three species of Malaysian strains identified as F. fujikuroi, F. verticillioides and F. proliferatum were therefore able to produce MON in the cultures. These results were in agreement with Marasas et al. (1986), who published the first report on MON production by Fusarium cultures from rice with bakanae disease. Other researchers also reported that F. proliferatum and F. fujikuroi produced MON in varying levels (Abdalla et al., 2000; Desjardins et al., 2000). However, the results indicated that strains of F. subglutinans, F. sacchari and a few strains of F. verticillioides did not produce the compound. There were contrasting reports regarding the production of MON by cultures of F. subglutinans. Marasas et al. (1986) for instance, reported that F. subglutinans has the ability to produce high levels of MON. It could be because of the fungi was isolated from the different hosts and locations.

Twelve strains of Fusarium spp. in section Liseola produced FB1 with Rf values in ranging from 0.360 - 0.474 and 0.537 - 0.620 in the solvent systems C and D, respectively (Table 2). On TLC plates, FB1 migrated at Rf 0.56 in developing solvent system D. In contrast, Tseng et al. (1995) and Fadl Allah (1998) reported FB1 migrated to Rf values of 0.44 and 0.17, respectively in the same solvent system (Table 2). The variation in Rf values showed that many factors probably were involved during the TLC analysis. The same phenomenon was also observed in the solvent system C with Rf value of 0.42. The result was also dissimilar with that of Ross et al. (1991), who reported that the Rf value of FB1 was 0.25. The differences in Rf values of FB1 by different markers could be due to deterioration of FB1 products and/or external factors such as time and temperature, long-term storage of solvent systems as well as the hosts and geographical areas of the Fusarium strains, could influence the Rf values during running of TLC plates. The results in this experiment indicated that 19 strains of F. fujikuroi, F. proliferatum and F. verticillioides were able to produce FB1 in cultures. Production of FB1 by the three species and F. sacchari has been reported by several researchers (Ross et al., 1990; Leslie et al., 1992; Tseng et al., 1995; Fadl Allah et al., 1998). F. sacchari strains in this study does not produce FB1 probably due to long period between isolation and detection of FB1, that will changed the ability of strains to produce the metabolite. The production of FB1 by F. proliferatum, F. fujikuroi and F. verticillioides poses a huge potential in contaminating our most important grains such as rice, sorghum, millet and corn. FB1 have been reported to cause a serious and lethal toxicity known as equine leukoencephalomalacia in horses (Ross et al., 1991; Moss, 1996; Gelderblom et al., 1988) and also caused a variety of negative effects in animal epidemiological evidences, as well as esophageal cancer in humans (Richard et al., 1996).

In detection of GA3, the Rf values of GA3 in the solvent systems E, F and G were 0.438 - 0.584, 0.322 - 0.509 and 0.529 - 0.680, respectively (Table 2). GA3 is a plant growth hormone that was first isolated from G. fujikuroi. Some researchers reported only F. fujikuroi has the ability to produce GA3 (Marasas et al., 1984; Desjardins et al., 2000). Therefore, data from our experiments showed the only F. fujikuroi strains were able to produce GA3 and the species was determined as the pathogen of bakanae disease of rice. Strains of F. proliferatum, F. sacchari, F. subglutinans and F. verticillioides were not able to produce GA3. This characteristic could be used as a main physiological character in separating F. fujikuroi that belongs to MP-C from the other four species of Fusarium isolated from
Table 2: Rf values and colours of standard markers of four secondary metabolites developed in different solvent systems

<table>
<thead>
<tr>
<th>Secondary metabolites</th>
<th>Moniliformin (MON)</th>
<th>Fumonisins B1 (FB1)</th>
<th>Giberellic acid (GA3)</th>
<th>Fusaric acid (FA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent systems</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>Rf value (Range)</td>
<td>0.24 – 0.37</td>
<td>0.01 – 0.02</td>
<td>0.36 – 0.62</td>
<td>0.36 – 0.47</td>
</tr>
<tr>
<td>Rf value (Mean)</td>
<td>0.31</td>
<td>0.02</td>
<td>0.56</td>
<td>0.50</td>
</tr>
<tr>
<td>Colour (under UV light 366nm)</td>
<td>yellow greenish florescence</td>
<td>red purple florescence</td>
<td>greenish florescence</td>
<td>yellow greenish florescence</td>
</tr>
</tbody>
</table>


Bakanae-infected rice. This is the only species of *Fusarium* that produced this plant growth hormone that caused abnormal elongation when presence in higher levels in infected plants.

FA production was shown to be highly variable within strains of the same *Fusarium* species screened. FA was successfully detected as a yellow greenish fluorescent spotted on TLC plates and the compound migrated to the Rf values 0.80 and 0.57 in developing solvent systems H and I, respectively (Table 2). *G. fujikuroi* species complex (including *F. fujikuroi*, *F. verticillioides*, *F. subglutinans* and *F. proliferatum*) have previously been reported to produce FA (Burmeister et al., 1985; Leslie, 1991; Miller et al., 1995; Bacon et al., 1996). However, at least one strain in each species of *F. sacchari* and *F. subglutinans* is capable for producing high levels of FA (Bacon et al., 1996). This report is in agreement with data from our experiment that showed only a single strain of *F. sacchari* has produced FA. Production of FA could be used to separate *F. verticillioides* and *F. subglutinans* from other *Fusarium* species isolated from rice with bakanae symptoms. FA is one of the most widely distributed secondary metabolites produced by *Fusarium*. Indeed, FA may well serve as a presumptive indicator of *Fusarium* contamination in food and feed grains (Bacon et al., 1996).

Some earlier phytopathologists who used morphological characteristics in their species delimitation, considered that *F. moniliforme* (reidentified as *F. fujikuroi*) was the only species involved in the bakanae disease complex (Snyder and Hansen, 1945; Nelson et al., 1983; Nirenberg, 1976). However, recent work confused these issues and suggested that another MPs such as MP-D (the anamorph *F. proliferatum*) was involved; the strains were isolated from rice in Asia and MP-A (the anamorph *F. verticillioides* [synonym *F. moniliforme*) has been isolated from rice in Africa, Australia and the United State (Amoah et al., 1995; Amoah et al., 1996; Desjardins et al., 1997).

Figure 1: Ascospores of *G. moniliformis* (MP-A)

Figure 2: Ascospores of *G. sacchari* (MP-B)
MP techniques were found to be useful in assisting the secondary metabolite profiles for the correct identification of closely related *Fusarium* species particularly species in section Liseola. Out of 25 strains, 19 strains of *Fusarium* were crossed-fertile with standard mating population tester's strains (Table 1). MP that existed in the strains of the five *Fusarium* species collected were therefore designated as MP-A (*G. moniliformis*), MP-B (*G. sacchari*), MP-C (*G. fujikuroi*) and MP-D (*G. intermedia*). The mature perithecia of the four teleomorphs stage which produced eight ascospores in asci as shown in Figures 1, 2, 3 and 4. However, a single strain of *F. fujikuroi* could interbreed with MP-C and MP-D were completed the meiosis process and produced viable progenies. Leslie *et al.* (2004) have also reported that some strains of MP-C and MP-D (minority) are crossed-fertile (interbred) and produced viable ascospores. The strains were assigned to these species and MPs generally differ in the ability to produce at least some secondary metabolites. For example, *G. fujikuroi* produced large amounts of GA₃, while strains of *G. intermedia* generally produced high level of FB₁, but did not produce GA₃ (Tudzynski, 1999; Rheeder *et al.*, 2002).

The six sterile strains were carried non-functional alleles, which often sterile and could blocked meiosis process in the perithecia development. These strains could be the sterile members of one of the seven MPs. It's also possible the strains are belonged to one of the *Fusarium* species within the section Liseola could be associated with other perithecial stages such as *F. anthophilum*, *F. beomiforme*, *F. diamini* and *F. succisae*. However, from morphological characteristics, all the sterile strains were identified as either to *F. sacchari*, *F. subglutinans* or *F. verticilloides*. Therefore, the strains were assigned as the sterile strains.

**CONCLUSIONS**

This is the first study carried out on the secondary metabolites profiles such as productions of MON, FB₁, GA₃ and FA by *Fusarium* species in section Liseola isolated from rice in Malaysia and Indonesia. The information obtained is useful for assessing the risk of mycotoxin contamination in rice as well as for assisting in identification process of the closely related species such as *F. fujikuroi* and *F. proliferatum*.

**REFERENCES**


