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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 (FB_1), gibberellic acid (GA_3) 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 GA_3 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 metabolites 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 B_1 (FB_1) and little or no moniliformin (MON), whereas most members of *F. fujikuroi* produce both FB_1 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.

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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, FB_1, gibberellic acid (GA_3) 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 filtrated by using a sterile double-layered muslin cloth to remove the mycelial debris and the conidia concentration was adjusted to 1×10^5 conidia/mL by using a heamocytometer.

For the detection of MON and FB_1, 30 g of cornmeal grits in a 300-mL Erlenmeryer flask was added with 15 mL of distilled water and autoclaved two times for 30 min at 15 psi, 121 °C. Sterile cornmeal grits was inoculated with 1 mL of conidial suspension in three replicates and shaken once or twice daily for 3 days to aid in even distribution of the inoculum. The cultures were incubated in the dark at the standard growth conditions (Salleh and Sulaiman, 1984) for 28 days.

GA_3 production was stimulated by inoculating the conidial suspension of individual strains in 50 mL of sterile Richard's Solution (Johnston and Booth, 1983) in three replicates and incubated in static as above for 10 days. For the production of FA, conidial suspension of individual strains were inoculated in 150 mL of sterile Czapek-Dox medium in three replicates and shaken at 150 rpm by using an orbital shaker (Model Certomat®) for 28 days Burmeister *et al.* (1985). 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 = $CHCl_3$:MeOH (97:3), v/v; C = EtOAc:CH_3COOH:H_2O (6:3:1), v/v/v; D = $CHCl_3$:MeOH:CH_3COOH (6:3:1), v/v/v; E = isopropanol:ammonia:H_2O (21:1:1), v/v/v; F = isopropanol:ammonia:H_2O (10:1:1), v/v/v; G = $CHCl_3$:EtOAc:formic acid (5:4:1), v/v/v; H = toluene:acetone:MeOH (5:3:2), v/v/v, and I = isopropanol:EtOAc:H_2O:CH_3COOH (4.0:3.8:2.0:0.2), v/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 cm^2, 0.25 mm thick silica gel 60 F_{254}) 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).

FB_1: 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 FB_1 (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). FB_1 was visualized following Tseng *et al.* (1995) and Fadl Allah (1998).

GA_3: The cultured media were filtered and pH of the 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 GA_3 (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). GA_3 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 (R_f 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 R_f value. The R_f 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

Fusarium **species**	**Strains**	**Location**	**MP**	**Secondary metabolites**			
				MON	**FB_1**	**GA_3**	**FA**
F. fujikuroi	B3102P	Sekinchan, Kuala Selangor, Selangor	C	+	+	+	+
	B3110P	Sekinchan, Kuala Selangor, Selangor	C	+	+	+	+
	B3120P	Sekinchan, Kuala Selangor, Selangor	C	+	+	+	-
	B3127P	Sungai Leman, Sungai Besar, Selangor	C	+	+	+	+
	B3140P	Sungai Leman, Sungai Besar, Selangor	C	+	+	+	+
	B3143P	Sungai Leman, Sungai Besar, Selangor	C	+	+	+	+
	I3208P	Padang, Sumatra, Indonesia	C	+	+	+	-
	K3219P	Pendang, Kedah	C	+	+	+	-
	M3237P	Merlimau, Melaka	C, D	+	+	+	-
	T3068P	Kg Apal, Jabi, Terengganu	C	+	+	+	+
F. verticillioides	A3053P	FELCRA, Seberang Perak, Perak	Sterile	+	+	-	-
	A3055P	FELCRA, Seberang Perak, Perak	A	+	+	-	-
	A3057P	FELCRA, Seberang Perak, Perak	A	+	+	-	-
	A3063P	FELCRA, Seberang Perak, Perak	Sterile	-	+	-	-
	D3070P	Padang Pak Amat, Pasir Puteh, Kelantan	Sterile	-	-	-	-
	I3410P	Tulung Agung, East Java, Indonesia	A	-	+	-	+
F. proliferatum	B3095P	Sekinchan, Kuala Selangor, Selangor	D	+	+	-	+
	A3054P	FELCRA, Seberang Perak, Perak	D	+	-	-	+
	C3083P	LKPP, Padang Sg. Laka, Rompin, Pahang	D	+	+	-	+
	C3089P	LKPP, Padang Sg. Laka, Rompin, Pahang	D	+	+	-	+
	D3074P	Paklekbang, Tumpat, Kelantan	D	+	+	-	+
F. sacchari	C3080P	LKPP, Padang Sg. Laka, Rompin, Pahang	Sterile	-	-	-	-
	K3222P	Pendang, Kedah	B	-	-	-	
F. subglutinans	B3124P	Sawah Sempadan, Kuala Selangor, Selangor	Sterile	-	-	-	-
	D3077P	Ladang Ana Fasa 2, Tumpat, Kelantan	Sterile	-	-	-	-
Control			-	-	-	-	-

MP = Mating population; MON = Moniliformin; FB_1 = Fumonisin B_1; GA_3 = 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 R_f 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 R_f 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 FB_1 with R_f 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, FB_1 migrated at R_f 0.56 in developing solvent system D. In contrast, Tseng *et al.* (1995) and Fadl Allah (1998) reported FB_1 migrated to R_f values of 0.44 and 0.17, respectively in the same solvent system (Table 2). The variation in R_f values showed that many factors probably were involved during the TLC analysis. The same phenomenon was also observed in the solvent system C with R_f value of 0.42. The result was also dissimilar with that of Ross *et al*. (1991), who reported that the R_f value of FB_1 was 0.25. The differences in R_f values of FB_1 by different markers could be due to deterioration of FB_1 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 R_f 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 FB_1 in cultures. Production of FB_1 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 FB_1 probably due to long period between isolation and detection of FB_1, that will changed the ability of strains to produce the metabolite. The production of FB_1 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. FB_1 have be reported to cause a serious and lethal toxicosis 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 GA_3, the R_f values of GA_3 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). GA_3 is a plant growth hormone that was first isolated from *G. fujikuroi.* Some researchers reported only *F. fujikuroi* has the ability to produce GA_3 (Marasas *et al.,* 1984; Desjardins *et al.,* 2000). Therefore, data from our experiments showed the only *F. fujikuroi* strains were able to produce GA_3 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 GA_3. 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: R_f values and colours of standard markers of four secondary metabolites developed in different solvent systems

Secondary metabolites	Moniliformin (MON)		Fumonisin B_1 (FB_1)		Giberellic acid (GA_3)			Fusaric acid (FA)	
Solvent systems	A	B	C	D	E	F	G	H	I
R_f value (Range)	0.24 – 0.37	0.01 – 0.02	0.54 – 0.62	0.36 – 0.47	0.44 – 0.58	0.322 – 0.509	0.53 – 0.68	0.75 – 0.84	0.51 – 0.64
R_f value (Mean)	0.31	0.02	0.56	0.42	0.50	0.40	0.62	0.80	0.57
Colour (under UV light 366nm)	yellow greenish florescence		red purple florescence		greenish florescence			yellow greenish florescence	

Solvent systems: A = toluene:acetone:MeOH (5:3:2), v/v/v; B = $CHCl_3$:MeOH (97:3), v/v; C = EtOAc:CH_3COOH:H_2O (6:3:1), v/v/v; D = $CHCl_3$:MeOH:CH_3COOH (6:3:1), v/v/v; E = isopropanol:ammonia:H_2O (21:1:1), v/v/v; F = isopropanol:ammonia:H_2O (10:1:1), v/v/v; G = $CHCl_3$:EtOAc:formic acid (5:4:1), v/v/v, H = toluene:acetone:MeOH (5:3:2), v/v/v; I = isopropanol:EtOAc:H_2O:CH_3COOH (4.0:3.8:2.0:0.2), v/v/v/v

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 R_f 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. verticilllioides* [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).

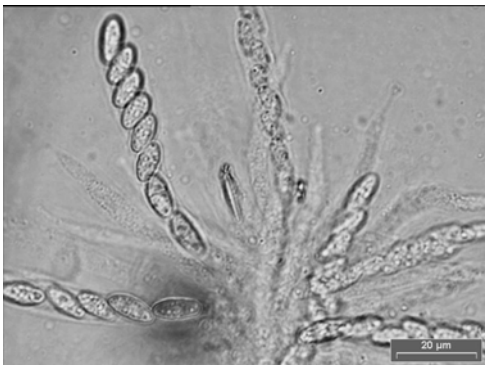

Figure 1: Ascospores of *G. moniliformis* (MP-A)

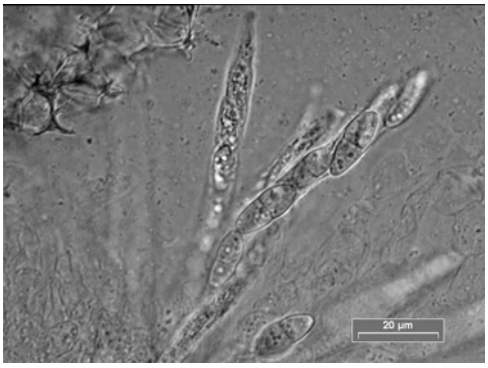

Figure 2: Ascospores of *G. sacchari* (MP-B)

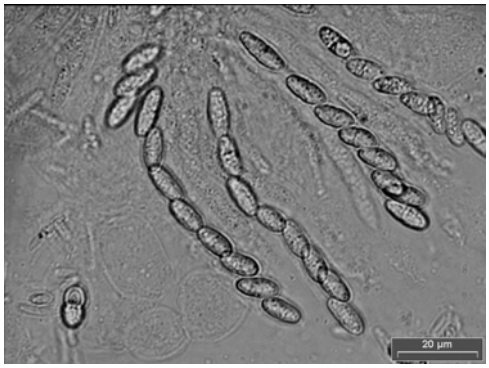

Figure 3: Ascospores of *G. fujikuroi* (MP-C)

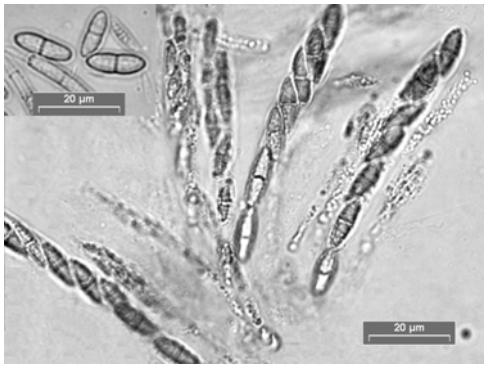

Figure 4: Ascospores of *G. intermedia* (MP-D)

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 (interbreed) 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 GA_3, while strains of *G. intermedia* generally produced high level of FB_1 but did not produce GA_3 (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. dlamini* and *F. succisae*. However, from morphological characteristics, all the sterile strains were identified as either to *F. sacchari, F. subglutinans* or *F. verticillioide*s. 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, FB_1, GA_3 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.*

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