NITRATE NON-UTILIZING MUTANTS AND VEGETATIVE COMPATIBILITY GROUPS OF *FUSARIUM PROLIFERATUM* AND *F. SACCHARI* ISOLATED FROM RICE IN THE PENINSULAR MALAYSIA AND KALIMANTAN, INDONESIA

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Abstract: A total of 26 isolates of *Fusarium proliferatum* and *F. sacchari* were isolated from rice in the Peninsular Malaysia and Kalimantan, Indonesia. Spontaneous chlorate-resistant sectors (CRSs) were recovered from all wild type of both *Fusarium* species when cultured on two chlorate media. The non-utilizing (*nit*) mutants were generated as *crn* (chlorate resistant, nitrate utilizing), *nit1, nit3* and nitM based on phenotyping growth-types on diagnostic media with different sources of nitrogen. The *nit* mutants were paired on minimal medium (MM) for examining the vegetative compatibility. The majority of *nit* mutants (32.3–46.5%) recovered were *nit1.* Eight and seven vegetative compatibility groups (VCGs) of *F. proliferatum* and *F. sacchari* were identified, respectively. The isolates of *F. proliferatum* and *F. sacchari* were genetically diverse as shown by the number and distribution of the VCGs. No strong correlation was observed between VCGs of both species and location.

Key words: *Fusarium proliferatum, F. sacchari,* rice, vegetative compatibility

INTRODUCTION

Fusarium proliferatum and *F. sacchari* are belong to the section Liseola and are widely distributed as saprophytes on rice (Leslie and Summerell 2006). Although *F. proliferatum* and *F. sacchari* have teleomorphs *Gibberella intermedia* and *G. sacchari,* respectively, perithecia of both species are rarely found in nature. Perithecia can be produced in the laboratory by crossing the field isolates with female tester isolates of the "D" and "E" mating populations (MP) of *G. fujikuroi* species complex (Leslie and Summerell 2006). Since asexually reproducing fungi can only exchange the genetic material through parasexual recombination, therefore vegetative compatibility (VC) becomes a prerequisite for sharing genetic material between fungi. Vegetatively compatible isolates from the same vegetative compatibility group (VCG) usually share more traits than isolates in different VCGs (Leslie 1993).

VC refers to the ability of undergoing mutual hyphal anastomosis between two fungal isolates, which results in viable heterokaryons (Deacon 2006; Leslie and Summerell 2006). A stable and functional heterokaryon would develop only if the nuclei of different isolates proliferate in the apical cells (Deacon 2006). The hyphal anastomosis and heterokaryosis are the only means of exchanging genetic information among different isolates for an anamorphic fungus with unknown sexual stage (Korolev *et al*. 2000). So, the newly formed hyphae contain both types of nuclei. It is a genetic trait controlled by many *vic* loci and *Fusarium* species were usually estimated to possess 10–15 *vic* loci that control VC (Klittich and Leslie 1988). VC is considered homogenic, that is two fungal isolates are vegetatively compatible only if the alleles at each of their corresponding *vic* loci are identical. If the alleles at one (or more) of the *vic* loci are different, then the hyphae are unable to fuse and form heterokaryon, therefore the hyphae between these isolates are considered vegetative incompatible (VIC) (Correll and Klittich 1987). Isolates in the same VCG are generally more genetically similar than those in different VCGs (Puhalla 1979; Leslie 1993). Therefore, VCG identification is useful for studing genetic diversity of fungal population (Baymen and Cotty 1991).

The objectives of this study were to generate the *nit* mutants and to investigate genetic diversity of *F. proliferatum* and *F. sacchari* isolates associated with rice by classifying the isolates into VCGs.

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MATERIALS AND METHODS

Media

Minimal medium (MM) was prepared by adding 2.0 g sodium nitrate ($NaNO_3$) to 1 L of basal medium (BM) following Correll *et al*. (1987). Chlorate resistant sectors (CRSs) were generated on two media i.e. MM and PDA containing 2.5% chlorate ($KClO_3$), represented as MMC and PDC, respectively. The chlorate served as a toxic analogue of nitrite (Corell *et al.* 1987). Four phenotyping media (Correll *et al.* 1987) were used i.e., i) sodium nitrate ($NaNO_3$); ii) sodium nitrite ($NaNO_2$), 0.5 g of $NaNO_2$ was added to 1l BM; iii) hypoxanthine (HX^-), 0.2 g of HX^- was added to 1l BM; iv) ammonium tartrate (NH_4^+), 1.0 g NH_4^+ was added to 1l BM. MM was also used in pairing of the complementary *nit* mutants.

***Fusarium* isolates**

Twenty-six isolates originating from single spores of both *Fusarium* species were previously collected from the granary areas in Peninsular Malaysia (Pahang, Kelantan, Kedah and Selangor) and Kalimantan, Indonesia. All isolates were identified as *F. proliferatum* (13 isolates) and *F. sacchari* (13 isolates) based on morphological identification and mating population (MP) following Burgess *et al.* (1994), Klittich and Leslie (1992) and Leslie and Summerell (2006). This experiment was conducted at *Fusarium* Collection Laboratory, School of Biological Sciences, Universiti Sains Malaysia.

Generation of *nit* mutants

Four fragments of mycelium (2 mm^2) for each isolate were transferred from complete medium (CM) to potato dextrose agar (PDA) and minimal medium (MM) containing of 2.5% potassium chlorate ($KClO_3$), presented as PDC and MMC, respectively. The plates were incubated for 14 days and afterwards the CRSs were transferred to MM. Those that grew as transparent colonies with no thick aerial mycelium were considered as *nit* mutants and isolates that reverted to a dense aerial mycelium on MM were considered as chlorate-resistant nitrate-utilizing (*crn*) mutants were discarded.

Phenotyping of *nit* mutants

A mycelial fragment (2 mm^2) of the *nit* mutants was subcultured on each of the four media containing different nitrogen sources: NO_3^-, NO_2^-, NH_4^+ and HX^-. Six mutants were transferred to each phenotyping medium and were incubated in room temperature for 7 days before colony morphology was scored. Colony with abundant aerial mycelium (fluffy) was considered as having a "+" growth; colony with sparse, transparent mycelium was considered as having a "–" growth. The *nit* mutants were classified into four phenotypic classes (*nit*1, *nit*3, NitM and *crn*) according to Correll *et al*. (1987).

Complementation tests

Nit mutants from the same isolates were paired on each MM plate in all possible combinations (NitM x *nit*1, *nit*1 x *nit*3, *nit*3 x Nit M) to verify the heterokaryon self-compatible (HSC) isolate. The pairings were incubated at room temperature in a completely dark chamber and complementation was scored after 7 to 14 days of culturing. HSC mutants of each isolate of *F. proliferatum* and *F. sacchari* were paired for assigning them into VCGs. The isolates in the same VCG showed a dense mycelial growth at the line where colonies merged that is known for heterokaryon. However, for those isolates in different VCGs, the *nit* mutants generated from the same isolate only paired by themselves (HSC). All complementation tests were repeated and for the pairings that scored as negative were repeated at least three times.

RESULTS

Generation of *nit* mutant

The CRSs were unable to utilize nitrate as a sole of nitrogen source, therefore the colonies were thin and transparent with no aerial mycelium on MM and were designated as *nit* mutants. *Nit* mutants emerged from restricted growth on chlorate media after 5–11 days. Eight to 42 *nit* mutants were obtained from the spontaneous CRSs for each isolate of *F. proliferatum* and *F. sacchari*. A total of 539 *nit* mutants were obtained from the 26 isolates when cultured and incubated for 14 days on MMC and PDC with 2.5% $KClO_3$ (table 1). In several isolates, the sectors were recovered but had wild-type colony morphology on MM and were classified as *crn* mutants and discarded.

***Nit* mutant phenotype**

All *nit* mutant phenotypes were identified after growing on media that contained one of four different nitrogen sources (NO_3, NO_2^2, NH_4^+ and HX) as shown in figure 1. Three phenotypic *nit* mutant classes i.e. *nit*1, *nit*3 and NitM were identified. For both species (*F. proliferatum* and *F. sacchari*), most of the *nit* mutants recovered from MMC and PDC were *nit*1 (table 2). However, the frequency of the *nit*1 mutants recovered was considerably higher on MMC (42.9–46.5%) than those on PDC (32.3–34.4%). Based on the mean percentage of the *nit* mutants, MMC (4.9–10.4%) was higher in generating the NitM than on PDC (2.5–7.3%), followed by the *nit*3 on MMC (4.4–6.7%) was higher than on PDC (1.6–6.1%).

Complementation tests of *nit* mutants

Complementation between different *nit* mutants was indicated by the development of dense aerial growth when the mycelia of the *nit* mutant colonies merged and anastomosed to form a heterokaryon. The growth of heterokaryon was more rapid and robust in pairings of NitM with *nit*1 than those in pairings of *nit*1 with *nit*3. Usually when *nit*1 and *nit*3 were paired, the complementation was often slow and taken about 3 weeks for formation of heterokaryon. In some isolates, NitM and NitM combination will pair but not for the reciprocal. However, this possible outcome was quite rare. The isolates from different VCGs were attempted for complementation by pairing with other mutants from different VCGs in the same *Fusarium* species. However, the isolates did not fuse even after repeated attempts.

Table 1. Frequency, phenotype of the *nit* mutants and VCGs of *F. proliferatum* and *F. sacchari* isolated from rice

Fusarium species	VCGs	[a]Isolates	Location	Number of *nit* mutants	[b]*nit* mutant classes ([c]MMC) (%)			[b]*nit* mutant classes ([c]PDC) (%)		
					nit1	*nit3*	NitM	*nit1*	*nit3*	NitM
F. proliferatum (13 isolates)	C01	B3095P	Sekinchan, Kuala Selangor, Selangor	25	32.0	24.0	0	40.0	4.0	0
		B3096P	Sekinchan, Kuala Selangor, Selangor	32	59.4	0	3.1	28.1	9.4	0
	C02	B3125P	Sungai Nibong, Sungai Besar, Selangor	13	46.2	0	7.7	46.1	0	0
		B3126P	Sungai Nibong, Sungai Besar, Selangor	30	63.3	0	10.0	26.7	0	0
	C03	D3072P	Paklekbang, Tumpat, Kelantan	13	38.5	0	23.1	15.4	0	23.1
		D3073P	Paklekbang, Tumpat, Kelantan	14	57.1	0	21.4	21.4	0	0
	C04	D3074P	Paklekbang, Tumpat, Kelantan	11	45.5	27.3	0	27.3	0	0
		D3075P	Paklekbang, Tumpat, Kelantan	9	33.3	0	33.3	33.3	0	0
	C05	D0679P	Rantau Panjang, Kelantan	14	42.9	0	7.1	42.9	7.1	0
		K0664P	Jitra, Kedah	18	55.6	11.1	0	33.3	0	0
	C06	A3054P	FELCRA, Seberang Perak, Perak	36	55.6	19.4	11.1	11.1	0	2.8
	C07	C3083P	LKPP, Sungai Laka, Rompin, Pahang	32	37.5	6.3	6.3	43.8	0	6.3
	C08	C3089P	LKPP, Sungai Laka, Rompin, Pahang	8	37.5	0	12.5	50.0	0	0
F. sacchari (13 isolates)	D01	K3222P	Pendang, Kedah	28	39.3	3.6	3.6	50.0	3.6	0
		K3223P	Pendang, Kedah	14	28.6	0	0	50.0	0	21.4
		K3224P	Pendang, Kedah	18	44.4	5.6	11.1	33.3	5.6	0
	D02	C3078P	LKPP, Sungai Laka, Rompin, Pahang	22	63.6	0	0	18.2	13.6	4.5
		C3081P	LKPP, Sungai Laka, Rompin, Pahang	22	54.5	9.1	0	27.3	4.5	4.5
	D03	C3080P	LKPP, Sungai Laka, Rompin, Pahang	24	29.2	8.3	0	50.0	0	12.5
		C3087P	LKPP, Sungai Laka, Rompin, Pahang	24	54.2	4.2	4.2	25.0	12.5	0
	D04	C3082P	LKPP, Sungai Laka, Rompin, Pahang	11	27.3	0	0	45.5	27.3	0
		C3084P	LKPP, Sungai Laka, Rompin, Pahang	26	65.4	3.8	11.5	15.4	0	3.8
	D05	I3420P	Samarinda, Kalimantan, Indonesia	34	44.1	2.9	11.8	35.3	0	5.9
		I3421P	Samarinda, Kalimantan, Indonesia	42	52.4	2.4	9.5	31.0	0	4.8
	D06	C3079P	LKPP, Sungai Laka, Rompin, Pahang	8	12.0	0	12.0	52.0	12.0	12.0
	D07	C3088P	LKPP, Sungai Laka, Rompin, Pahang	11	42.8	17.1	0	14.3	0	25.7

[a] a total of 26 isolates of *F. proliferatum* and *F. sacchari* were used
[b] *Nit* mutant phenotypes were determined according to growth on basal medium amended with different nitrogen sources. *nit1* = mutation in a nitrate reductase structural locus, *nit3* = mutation in a nitrate-assimilation pathway-specific regulatory locus, NitM = mutation in one of five loci that affect the assembly of a molybdenum-containing co-factor necessary for nitrate reductase activity
[c] Chlorate medium contained 2.5% of $KClO_3$ concentration on MM (=MMC) and PDA (=PDC)

Table 2. Frequency of *nit* mutants recovered on MMC and PDC

Fusarium Species	*Nit* mutants		Mean percentage of *nit* mutants [%]					
			MMC			PDC		
	Total	[a]Mean	*nit1*	*nit3*	NitM	*nit1*	*nit3*	NitM
F. proliferatum	255.0	19.6	46.5	6.7	10.4	32.3	1.6	2.5
F. sacchari	284.0	21.9	42.9	4.4	4.9	34.4	6.1	7.3

[a] Mean for each isolate

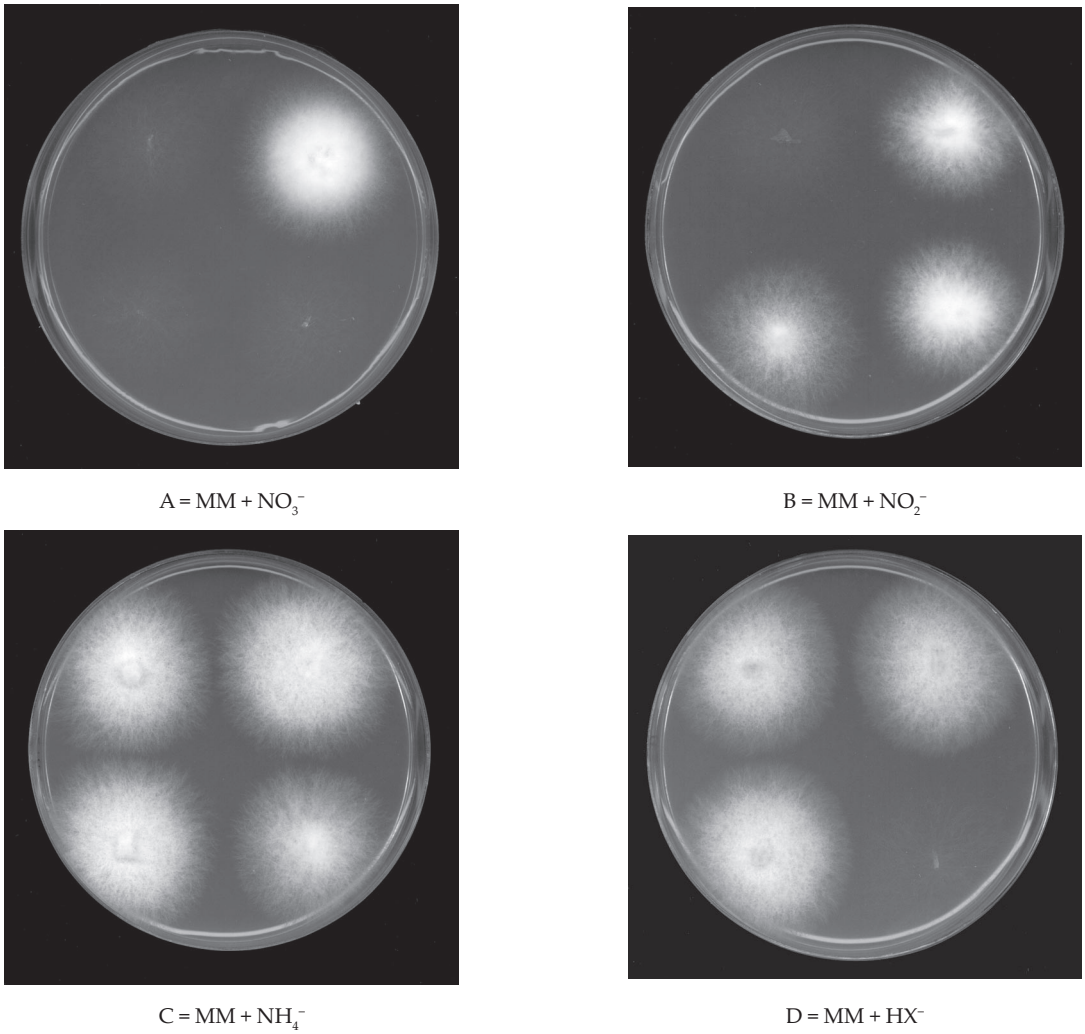

Fig. 1. Growth of wild-type parental *Fusarium* strain and three nitrate non-utilizing (*nit*) mutant phenotypes on media with one of four different nitrogen sources; upper right, *crn*; upper left, *nit3*; lower right, NitM; and lower left, *nit1*. A, nitrate medium, thin growth of three *nit* mutants; B, nitrite medium, thin growth of *nit3*; C, ammonium medium, all dense growth; D, hypoxanthine medium, thin growth of NitM

VCGs of *F. proliferatum*

The complementation test identified 8 VCGs among 13 isolates with a genetic diversity (number of VCGs/number of isolates) of 0.62. Three VCGs belonged to a single-member isolate; while five VCGs contained two members (fig. 2, table 1). The isolate within VCG failed to fuse after pairing with isolates from other VCG. Only VCG C05 contained isolates from two different locations, while VCGs C01, C02, C03, C04, C06, C07 and C08 were limited to isolates from a single location. There was no correlation between VCG grouping of *F. proliferatum* with the location except for VCG C05.

VCGs of *F. sacchari*

The complementation test enabled to identify 7 VCGs among 13 isolates of *F. sacchari*. The genetic diversity of *F. sacchari* based on number of the VCGs per number of the isolates is 0.54. Two VCGs contained a single member isolate and the other 5 VCGs consisted of more than one isolate member (fig. 2, table 1). VCGs D01 to VCG D07 were limited to isolates from a single location. Therefore, we can conclude there was no correlation between VCGs of *F. sacchari* with the location.

DISCUSSIONS

Vegetative compatibility (VC) or heterokaryon compatible (HC) test is widely used in many fungal populations such as *F. oxysporum* (Puhalla 1984; Correll *et al*. 1987), *Verticillium albo-atrum* (Gordon *et al*. 1986), *V. dahliae* (Chen 1994), *Neurospora crassa* (Marzluf 1981) and *Aspergillus flavus* (Papa 1986). Previous studies on VCGs of these fungal by naturally occurring genetic markers were used to differentiate the isolates involved. The *nit* mutants were essential for determination of VC of isolates by forcing it to generate heterokaryon. This genetic complementation of mutants can prove the potential for anamorphic forms to exchange genetic information by examining the heterokaryon.

All isolates of *F. proliferatum* and *F. sacchari* which emerged as sectors that appeared like a fan-shaped and fast growing were unable to utilize chlorate or nitrate and were resistant to chlorate. This could be caused by transposons (transposable elements) that encode enzymes for their own replication, reverse transcriptase which synthesizes new copies of DNA that subsequently insert at various points in the same or other chromosome, leading to alteration in gene expression (Deacon 2006). The high fre

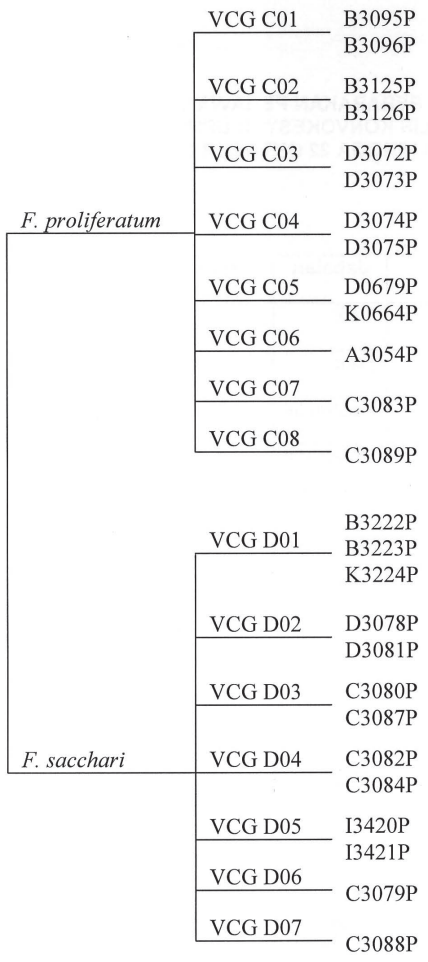

Fig. 2. Classification of *F. proliferatum* and *F. sacchari* based on vegetative compatibility groups (VCGs)

quency of spontaneous mutation among isolates reflects the different transposing movement when fungi were grown on chlorate medium (Klittich and Leslie 1988; Klittich *et al*. 1988). Most of these sectors may also produce mutants that contain genetic lesions in either structural or regulatory loci in nitrate metabolism or related pathways and sectors produced in were colonies proven to differ in morphological, virulence, and other characteristics from the wild-type parents (Garrett and Amy 1978; Marzluf 1981; Klittich and Leslie 1988).

The sectors were then transferred to MM which contained $NaNO_3$ as sole nitrogen source. Those that grew as transparent colonies with no thick aerial mycelium were considered as *nit* mutants that were unable to utilize nitrate because they could not synthesize the nitrate reductase enzyme (Puhalla 1985). There were some isolates that reverted to a dense aerial mycelium on MM and were considered as *crn*. In many *Fusarium* species, the phialides can produce multinucleate spores if a single nucleus enters the developing spore and then divide to produce several nuclei (Deacon 2006). These spores will be either homokaryotic or heterokaryotic depending on cells that produce them, and *crn* may occur when sectors from homokaryotic or heterokaryotic were transferred to MM (Correll *et al.* 1987). The homokaryotic sectors had wild-type morphology on MM and presumably mutants that were both chlorate-resistant and able to utilize nitrate (*crn* mutants) whereas the heterokaryotic sectors might have contained a mixture of *nit* mutant conidia, wild-type conidia and/or *crn* mutant conidia (Correll *et al.* 1987).

A total of 539 *nit* mutants were generated from 26 isolates of *F. proliferatum* and *F. sacchari* and about 8 to 42 *nit* mutants were generated from each isolate. The *nit* mutants could be divided into three distinct phenotypic classes that are associated with nitrate assimilation pathway. The majority of *nit* mutants recovered on both MMC and PDC were *nit*1 mutants. However, the frequency of *nit*1 recovered was considerably higher on PDC than on MMC. The difference between loci in susceptibility to mutation could be related to the physical size of the gene, with larger genes representing a larger target (Klittich and Leslie 1988). In MM, *nit*1 were assumed to be able to use nitrate, ammonium and hypoxanthine, NitM was unable to utilize nitrate and hypoxanthine whereas *nit*3 were unable to utilize nitrate and nitrite. Based on nitrate metabolism pathway, these classes (*nit*1, *nit*3 and NitM) may represent *nit*1 as a mutation in a nitrate reductase structural locus, whereas NitM represent a mutation in one of several loci coding for the assembly of a molybdenum–containing co-factor necessary for the activity of nitrate reductase (Cove 1976; Marzluf 1981; Correll *et al*. 1987). For mutants in the *nit*3 class, the mutation may occur at the nitrate-catabolism pathway-specific regulatory locus (Correll *et al.* 1987).

HSC in each *F. proliferatum* and *F. sacchari* isolate were paired in all possible combinations (NitM x *nit*1, *nit*1 x *nit*3, *nit*3 x NitM) where *nit* mutant works as genetic marker that allow isolates from the same or different species to be tested for their ability to form heterokaryon as a result of complementation tests (Brooker *et al.* 1991). Heterokaryon with dense aerial mycelium can be seen after 2–3 weeks incubation in the dark. The complementation of NitM with either *nit*1 or *nit*3 usually form a dense and visible heterokaryon between isolates whereas combination of *nit*1 and *nit*3 gives a weak appearance of heterokaryon. NitM mutants are the most reliable in VC tests because the mutants help to reduce the occurrence of false-negative complementation reaction between VC isolates (Correll *et al.* 1987). Therefore, in each isolate the NitM should be recovered as it produces better heterokaryon when paired.

Isolates in the same VCG form heterokaryons which means that *nit* mutants anastomosed and the cytoplasm may mix, mitotic recombination may occur, and finally a process in exchanging genetics information for asexual fungal were completed (Puhalla 1985; Correll *et al*. 1986; Correll and Klittich 1987; Jacobson and Gordon 1988; Ploetz and Correll 1988). The isolates from different VCGs that failed to fuse after pairing with each other *nit* mutants were considered VIC. This phenomenon occurs when isolates from different VCGs fuse to form heterokaryotic cells but with different degrees of cytoplasmic incompatibility, which then causes the produced heterokaryon to be unstable, and the fused cell of cytoplasm dies (Leslie and Summerell 2006; Deacon 2006). Isolates in the same VCG are more similar in molecular traits and are different from isolates in other VCGs of the same species (Gordon and

Okamoto 1992; Tantaoui *et al.* 1996). VC is controled by several gene loci and this may results in the genetic isolation of isolates within a population (Perkins *et al*. 1982; Puhalla and Speith 1983). The complementation results indicated that there was the ability to anastomose where genetic traits exchange occurs between isolates in the species. The genetic diversity varied in the two *Fusarium* species; it was the highest *F. proliferatum* (0.62) followed by *F. sacchari* (0.54).

Swift *et al.* (2000) also reported the isolates in the same VCG frequently share the geographical origins as well as pathogenicity and physiological characteristics. In the present study, most of the *Fusarium* isolates that were grouped in the same VCG is originated from the same geographical areas, thus no correlation between isolates and location was observed.

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POLISH SUMMARY

MUTANTY NIE ZUŻYWAJĄCE AZOTU ORAZ GRUPY ZGODNOŚĆ WEGETATYWNEJ *FUSARIUM PROLIFERATUM* I *F. SACCHARI* WYIZOLOWANE Z RYŻU NA PÓŁWYSPIE MALEZYJSKIM I KALIMANTAN W INDONEZJI

Na Półwyspie Malezyjskim i w Kalimantan (Indonezja) wyizolowano ogółem 26 izolaty *Fusarium proliferatum* i *F. sacchari* z ryżu. W hodowli na dwóch pożywkach chloranowych uzyskano spontanicznie powstające sektory ze wszystkich dzikich izolatów obydwu gatunków *Fusarium*. Otrzymano mutanty nie zużywające azotanu (*nit 1*, *nit 3* i nit M), a także odporne na chloran mutanty zużywające azotan, w oparciu o cechy typu wzrostu, na pożywkach diagnostycznych z różnymi źródłami

azotu. Mutanty *nit* łączono parami na pożywce mineralnej (MM) w celu sprawdzenia zgodności wegetatywnej. Większość mutantów nit (32,3–46,5%) określono jako *nit 1*. Zidentyfikowano 8 i 7 grup zgodności wegetatywnej odpowiednio dla *F. proliferatum* i *F. sacchari*. Izobaty *F. proliferatum* i *F. sacchari* były różne pod względem genetycznym, co wykazano na podstawie liczby i rozmieszczenia grup zgodności wegetatywnej. Nie stwierdzono wysokiej korelacji pomiędzy grupami zgodności wegetatywnej w ramach tych gatunków oraz miejscowości.