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Vegetative Compatibility Groups within *Fusarium* Species Isolates from Tomato in Selangor, Malaysia

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ABSTRACT

Vegetative compatibility provides valuable information on genetic diversity of certain fungal population including *Fusarium* species. *Fusarium* species are capable of causing mass spoilage of perishable vegetable fruits such as tomato either in the field or in storage. A total of 81 *Fusarium* isolates comprising *F. oxysporum* (54 isolates), *F. semitectum* (22 isolates) and *F. subglutinans* (5 isolates) were examined for vegetative compatibility groups (VCGs). *Nit* mutants were generated from minimal medium with chlorate (MMC) and potato dextrose agar with chlorate (PDC) media under varying degrees of chlorate ($KClO_3$) concentrations from 4.5 - 6.0%. Four phenotyping media containing different nitrogen sources (NO_2, NO_3, NH_4 and HX) were used to phenotype the *nit* mutants into different classes: *nit1*, *nit3* and NitM. All heterokaryon self-compatible (HSC) *nit* mutants of *Fusarium* species were paired in all pairwise possible combinations on MM to classify them into VCG. Based on the index and distribution of the VCGs, isolates of *F. oxysporum* demonstrated high genetic diversity where 11 VCGs were recovered. Meanwhile, only three VCGs were recovered in *F. semitectum* isolates. *Fusarium subglutinans* isolates had the least number of VCGs where only two groups were recovered.

Keywords: Chlorate resistant sectors (CRSs), *Fusarium*, *Nit* mutants, vegetative compatibility groups (VCGs)

ITRODUCTION

Fusarium species were reported to cause fruit rot of tomato and have been isolated from infected samples in Malaysia including *F. semitectum, F. oxysporum, F. equiseti, F. subglutinans* and *F. solani* (Abu Bakar, Nur Ain Izzati, & Umi Kalsom, 2013). Realizing

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that the information on genetic diversity of *Fusarium* species is limited in a tropical area, this study examined the genetic diversity of *Fusarium* species isolated from *Fusarium* fruit rot of tomato based on vegetative compatibility (VC).

Vegetative Compatibility Groups (VCGs) provide a crude marker for population genetic studies in numerous underlying genes as well as producing a promising result when two strains are compared. VCGs are employed to provide a means of characterizing a variation based on genetics of the fungus, the origin and relatedness among *Fusarium* strains (Deacon, 2006; Desjardins, Plattner, & Gordon, 2000; Korolev, Katan, & Katan, 2000; Leslie & Summerell, 2006; Pasquali, Dematheis, Gilardi, Gullino, & Garibaldi, 2005). The phenomenon by which two hyphae can anastomose and fuse to form a stable heterokaryon is referred to as VC (Carvalho, & Mendes-Costa, 2011; Somrith, Singburaudom, & Piasai, 2011; Wang, Brubaker, Summerell, Thrall, & Burdon, 2010).

In *Fusarium* and *Neurospora*, VC is defined as the ability of auxotrophic strains to form a prototrophic heterokaryon also known as heterokaryon self-compatible (HSC). Strains for which no prototrophic heterokaryon is formed may be due to vegetative incompatibility or the physical inability of one (or both) of the strains to form heterokaryons with any other strains. Strains that lack of ability to form heterokaryons between mutants derived from the same strain are termed heterokaryon self-incompatible (HSI). Asexually reproducing fungi can only exchange the genetic material through parasexual recombination; therefore, VC becomes a prerequisite for sharing genetic materials between fungi. Heterokaryosis, barrages, and complementation have been recognized in *Fusarium* and other fungal population (Pasquali et al., 2005). The objective of this study was to classify *Fusarium* isolates from post-harvest fruit rot of tomato into respective vegetative compatibility groups (VCG).

MATERIALS AND METHODS

Generation of Nitrate Non-utilizing (*nit*) Mutants

Eighty-one isolates of *Fusarium* species used in this study were obtained from Laboratory of Mycology, Department of Biology, Faculty of Science, UPM according to fungal stock availability. All isolates were previously isolated from infected-post-harvest of tomato at storage areas throughout Selangor, Malaysia. The isolates were identified based on morphological characteristics and translation elongation factor (*tef*) 1-α gene sequence analysis by Abu Bakar et al. (2013) and Murad, Kusai and Zainudin (2016). *Nit* mutants were generated on minimal medium with chlorate (MMC) and potato dextrose with chlorate (PDC) media under varying degrees of chlorate ($KClO_3$) concentrations (4.5 - 6.0%). Four fragments of mycelia (2 mm^2) were placed on MMC and PDC plates incubated at standard incubation condition for 14 days in darkness. Spontaneous chlorate-resistant

sectors (CRSs) usually appeared like fan-shaped with thin transparent mycelium at the edges from the parent colonies were transferred and sub-cultured on minimal medium (MM). Meanwhile, colonies that grew with thin transparent aerial mycelia were identified as *nit* mutants and were used for phenotyping. On the other hand, those germinated with dense non-transparent aerial mycelia (regarded as *crn* mutants) were discarded, as they were representatives of the wild-type or reverted cultures.

Phenotyping of *nit* Mutants

Four phenotyping media containing four different nitrogen source media: MM ($NaNO_3$ medium), nitrite ($NaNO_2$ medium), ammonium (NH_4^+ medium) and hypoxanthine (HX^- medium) were prepared and used to phenotype the *nit* mutants and classify them into different classes (*nit*1, *nit*3 and NitM). A complementation test was conducted on *nit* mutants of the same strain paired on MM in all possible combinations (*nit1* × NitM, *nit3* × NitM and *nit1* × *nit3*) to classify them into HSC and HSI *nit* mutants. Colonies with robust growth of mycelia at the line of intersection were evaluated as HSC strains, whereas those with thin growth of mycelia evenly across the area of the plate were analyzed as HSI strains.

Complementation Test

All HSC *nit* mutants were further paired in all the pairwise possible combinations (*nit1* X NitM, *nit3* x NitM and *nit1* x *nit3*) on MM to classify them into VCGs. Those strains in the same VCGs often depicted a dense aerial mycelium at the line where colonies intersected, whereas strains in the different VCGs showed a thin growth of aerial mycelium. All inter-pairings of the complementation test were repeated at least three times. All HSC *nit* mutants were then labeled with VCGs.

RESULTS

Generation of *nit* Mutants

Spontaneous CRSs were successfully generated from all three *Fusarium* species (*F. oxysporum, F. semitectum* and *F. subglutinans*). The CRSs were produced on MMC and PDC with chlorate concentrations of 4.5 - 6.0%. Single, thin, and transparent CRSs were obtained at the edge of the growing colonies on MMC and PDC plates for most of the isolates. The CRSs kept growing radially like fan-shaped from the center of the colonies with aging. Figure 1 displays the generation of spontaneous CRSs on MMC for isolate of *F. semitectum* (B605T) recovered from Sri Serdang, Selangor. Hence, more than 1000 *crn* mutants were obtained and discarded constituting 75% of *nit* mutants from *F. semitectum* and *F. subglutinans*. A total of 676 *nit* mutants were generated and produced from all *Fusarium* species tested. *Fusarium oxysporum* produced the highest frequency number of *nit* mutants (63%) followed by *F. semitectum* (23%), while the least number was recorded by *F. subglutinans* (14%).

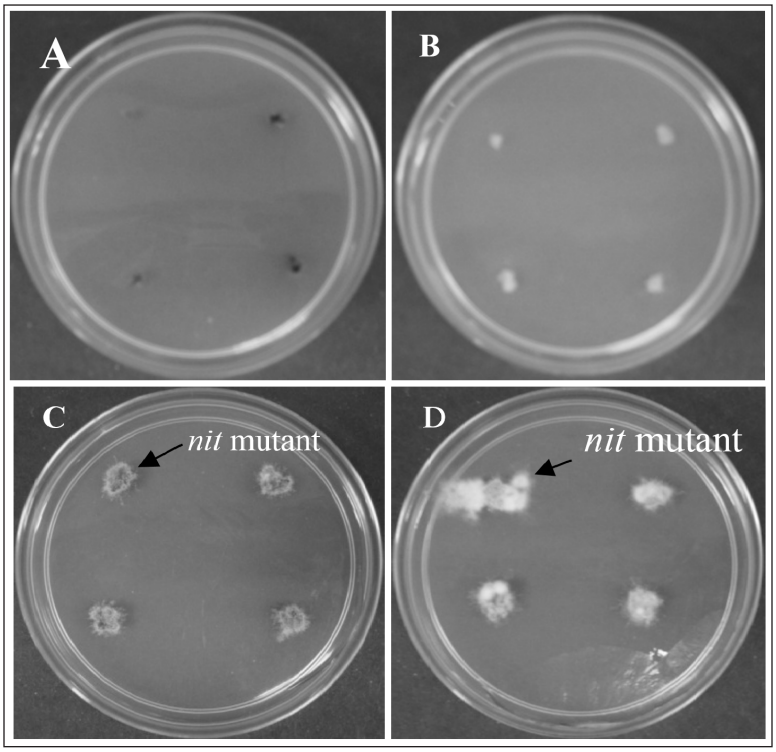

Figure 1. Generation of spontaneous CRSs using different concentrations of chlorate ($KClO_3$) in MMC for *F. semitectum* (Isolate B605T): (A) 4.5% (B) 5.0% (C) 5.5% (D) 6.0%

Nit Mutant Phenotype

The *nit* mutant phenotype of 81 isolates of the *Fusarium* was successfully conducted. *Nit1* mutants produced very thinly mycelia on nitrate medium, but vigorous growth was formed on the nitrite, ammonium, and hypoxanthine media. *Nit3* mutants did not grow well on nitrate and nitrite media while the thick growth of mycelium was formed on the ammonium and hypoxanthine media. NitM mutants did not grow well on nitrate and hypoxanthine media, whereas vigorous growth of mycelium occurred on the nitrite and ammonium media. From the findings, all the *nit* mutants showed different patterns of growth on these media since they possessed different nitrate metabolism pathways. *Nit1* was formed because of mutation in a nitrate reductase holoenzyme while *nit3* was formed due to the mutation at the nitrate-pathway specific regulatory protein. NitM mutants occurred because of mutation of a molybdenum-containing co-factor in the nitrate reductase enzyme. Essentially, these results of phenotyping of *nit* mutants provided a route in which heterokaryosis would be established for all *Fusarium* species. Once the HSC was identified on the isolates, complementation test was then conducted to assign them into VCGs.

The frequencies of *nit* mutants isolated from all three species of *Fusarium* recovered from tomato fruits were obtained. Eighty-five percent (85%) of the total isolates produced *nit* mutants, whereas 15% failed. All classes of the *nit* mutants (*nit1*, *nit3* and NitM) were generated in this study. Table 1 provides a summary of the *nit* mutants recovered from MMC and PDC for all three species. *Fusarium oxysporum, F. semitectum* and *F. subglutinans* generated 427, 152 and 97 *nit* mutants, respectively. Mean of *nit* mutants on both MMC and PDC was considerably higher on *nit1* followed by *nit3* and NitM for all three species of *Fusarium*.

Table 1
Nit mutants recovered on MMC and PDC

Fusarium species	*Nit* mutants		Mean of *nit* mutants					
			MMC			PDC		
	Total	°Mean	*nit1*	*nit3*	NitM	*nit1*	*nit3*	NitM
F. oxysporum	427	8.1	1.6	0.4	0	3.9	1.3	0.9
F. semitectum	152	6.9	0.9	0.3	0	3.1	1.3	1.3
F. subglutinans	97	19.4	4.6	2.4	0.4	8.4	2.4	1.2

º Mean for each isolate

Complementation Test

The complementation test was carried out to classify the isolates into VCGs. *Nit* mutants from the same isolates were firstly paired on MM and if the dense growths of mycelia were formed at the line of intersection of the colonies, then they were confirmed as heterokaryon self-compatible (HSC) *nit* mutants. The isolates that did not produce heterokaryons were considered as heterokaryon self-incompatible (HSI). Interpairings between different HSC isolates were then conducted to assign them into VCGs. The heterokaryon in most of the complementation test is often formed rapidly between *nit1* and NitM or *nit3* and NitM. The pairings between *nit1* and *nit3* in most situations produced weak heterokaryons. A typical complementation test of *F. oxysporum* is illustrated in Figure 2 shows isolates B712T (plate A) and B762T (plate B) formed heterokaryons (HSC). The pairing between isolates B622T and B640T indicated that they belonged to the same VCGs (plate C) while complementation between the isolates B622T and B707T suggested that they belonged to the different VCGs (plate D). Therefore, based on the complementation tests, 10 VCGs were established for 53 HSC isolates of *F. oxysporum* (Table 2). The genetic diversity (number of VCGs/number of isolates) of *F. oxysporum* isolates was 0.187.

Based on the complementation test, three VCGs were established in 22 *F. semitectum* isolates. The ratio of VCGs was 0.136 as depicted in Table 2. Figure 2 shows the complementation test of *F. semitectum* HSC (isolates B745T and B765T). The pairing between B601T and B605T showed

that they belonged to the same VCGs (Figure 2; Plate G), whereas, the pairing between B601T and B767T depicted that they belonged to the different VCGs (Figure 2; Plate H).

Five isolates of *F. subglutinans* were categorized into two VCGs (Table 2). The typical complementation test of HSC on MM for *F. subglutinans* (isolates B658T and B681T) are presented in Figure 2 (Plates I and J). The pairings in plates L and M showed no complementation between vegetatively incompatible isolates, B658T and B679T, as well as B658T and B681T. The genetic diversity of *F. subglutinans* isolates was 0.4.

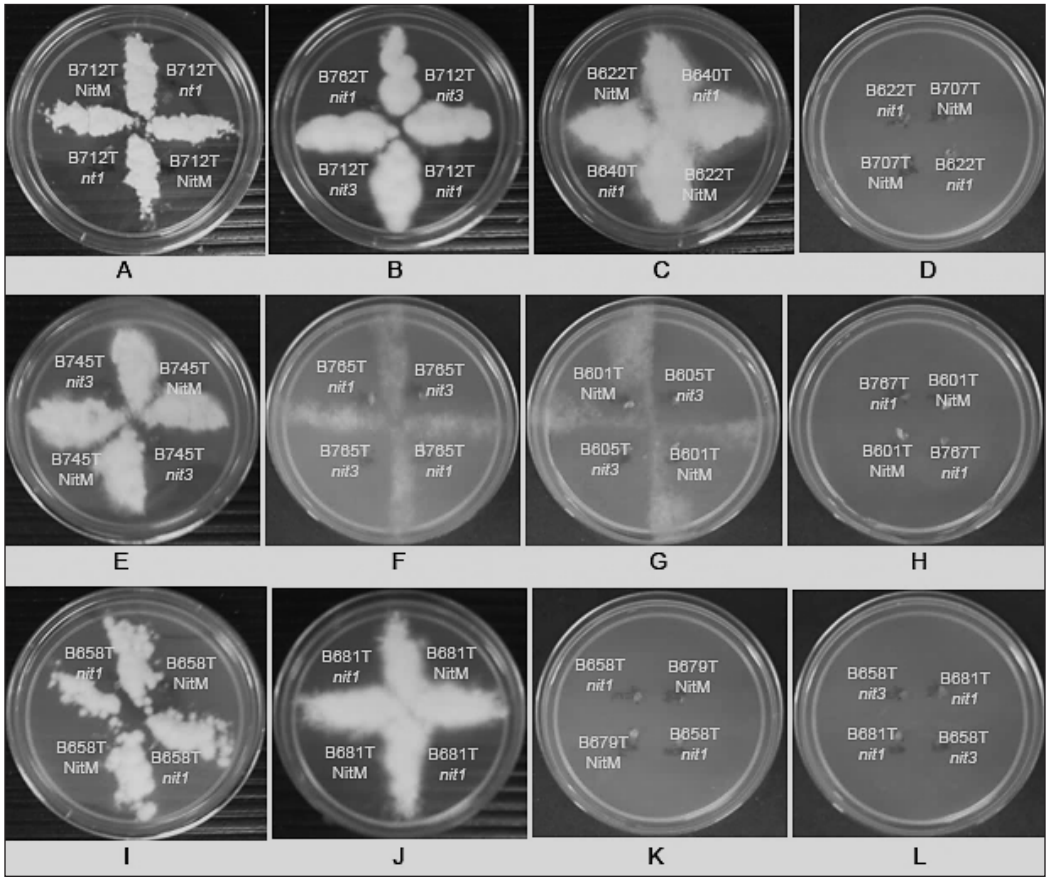

Figure 2. HSC strain of *F. oxysporum* on MM: (A) Isolate B712T (B) Isolate B762T. Complementation test of *F. oxysporum* strains on MMC: (C) Heterokaryon formed between vegetatively compatible isolates B622T and B640T. (D) No heterokaryon occurred between vegetatively incompatible isolates B622T and B707T. HSC strain of *F. semitectum* on MM: (E) Isolate B745T; (F) Isolate B765T. Complementation test of *F. semitectum* on MM: (G) Heterokaryon formed between vegetatively compatible isolates B601T and B605T (H) No complementation occurred between vegetatively incompatible isolates B601T and B767T. HSC strain of *F. subglutinans*: (I) Isolate B658T (J) Isolate B681T. Complementation test of *F. subglutinans* on MM: No complementation was formed between vegetatively incompatible isolates (K) B658T and B679T; and, (L) B658T and B681T

Table 2
Vegetative compatibility groups (VCGs) of Fusarium species isolated from post-harvest fruit rot of tomato in Selangor, Malaysia

VCGs	Strain	Species identification based on VCGs	Location
A01	B622T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B623T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B624T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B625T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B632T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B633T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B635T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B637T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B640T	*F. oxysporum* f.sp. *lycopersici*	Serdang
A02	B757T	*F. oxysporum*	Seri Kembangan
	B758T	*F. oxysporum*	Seri Kembangan
	B759T	*F. oxysporum*	Seri Kembangan
	B760T	*F. oxysporum*	Seri Kembangan
	B761T	*F. oxysporum*	Seri Kembangan
	B762T	*F. oxysporum*	Seri Kembangan
	B763T	*F. oxysporum*	Seri Kembangan
	B764T	*F. oxysporum*	Seri Kembangan
A03	B688T	*F. oxysporum*	Kajang
	B689T	*F. oxysporum*	Kajang
	B690T	*F. oxysporum*	Kajang
	B691T	*F. oxysporum*	Kajang
	B692T	*F. oxysporum*	Kajang
	B693T	*F. oxysporum*	Kajang
A04	B711T	*F. oxysporum* f.sp. *lycopersici*	Kajang
	B712T	*F. oxysporum* f.sp. *lycopersici*	Kajang
	B713T	*F. oxysporum* f.sp. *lycopersici*	Kajang
	B714T	*F. oxysporum* f.sp. *lycopersici*	Kajang
	B715T	*F. oxysporum* f.sp. *lycopersici*	Kajang
	B716T	*F. oxysporum* f.sp. *lycopersici*	Kajang
A05	B645T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B646T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B654T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B655T	*F. oxysporum* f.sp. *lycopersici*	Serdang
A06	B695T	*F. oxysporum*	Kajang
	B696T	*F. oxysporum*	Kajang
	B697T	*F. oxysporum*	Kajang
	B699T	*F. oxysporum*	Kajang

Table 2 *(continue)*

VCGs	Strain	Species identification based on VCGs	Location
A07	B703T	*F. oxysporum*	Kajang
	B704T	*F. oxysporum*	Kajang
	B705T	*F. oxysporum*	Kajang
	B706T	*F. oxysporum*	Kajang
A08	B707T	*F. oxysporum*	Kajang
	B708T	*F. oxysporum*	Kajang
	B709T	*F. oxysporum*	Kajang
	B710T	*F. oxysporum*	Kajang
A09	B717T	*F. oxysporum* f.sp. *lycopersici*	Semenyih
	B718T	*F. oxysporum* f.sp. *lycopersici*	Semenyih
	B719T	*F. oxysporum* f.sp. *lycopersici*	Semenyih
	B720T	*F. oxysporum* f.sp. *lycopersici*	Semenyih
A10	B725T	*F. oxysporum*	Puchong
	B726T	*F. oxysporum*	Puchong
	B727T	*F. oxysporum*	Puchong
	B728T	*F. oxysporum*	Puchong
A11	B1358T	*F. oxysporum*	Selayang
B01	B741T	*F. semitectum*	Shah Alam
	B742T	*F. semitectum*	Shah Alam
	B743T	*F. semitectum*	Shah Alam
	B744T	*F. semitectum*	Shah Alam
	B745T	*F. semitectum*	Shah Alam
	B746T	*F. semitectum*	Shah Alam
	B747T	*F. semitectum*	Shah Alam
	B748T	*F. semitectum*	Shah Alam
B02	B765T	*F. semitectum*	Ampang
	B766T	*F. semitectum*	Ampang
	B767T	*F. semitectum*	Ampang
	B768T	*F. semitectum*	Ampang
	B769T	*F. semitectum*	Ampang
	B770T	*F. semitectum*	Ampang
	B771T	*F. semitectum*	Ampang
	B772T	*F. semitectum*	Ampang
B03	B601T	*F. semitectum*	Sri Serdang
	B602T	*F. semitectum*	Sri Serdang
	B603T	*F. semitectum*	Sri Serdang
	B604T	*F. semitectum*	Sri Serdang
	B605T	*F. semitectum*	Sri Serdang
	B606T	*F. semitectum*	Sri Serdang

Table 2 *(continue)*

VCGs	Strain	Species identification based on VCGs	Location
C01	B678T	*F. subglutinans*	Selayang
	B679T	*F. subglutinans*	Selayang
	B680T	*F. subglutinans*	Selayang
	B681T	*F. subglutinans*	Selayang
C02	B658T	*F. subglutinans*	Sri Serdang

DISCUSSION

In this study, chlorate resistant sectors (CRSs) were successfully generated from all three species (*F. oxysporum*, *F. semitectum* and *F. subglutinans*) and using 4.5 - 6.0% $KClO_3$ on both MMC and PDC. A very few CRSs were obtained when 2.5 – 4.0% $KClO_3$ were used. All generated isolates of *F. oxysporum, F. semitectum* and *F. subglutinans* produced thin transparent mycelia on the minimal media (MM) made up of sodium nitrate ($NaNO_3$) as a sole nitrogen source that was required by the nitrate non-utilizing chlorate resistant sectors and the *nit* mutants. More than 1000 *crn* mutants (wild types) were generated in this study with *F. oxysporum* accounted for 50% of the total mutants, followed by *F. semitectum* (30%) and *F. subglutinans* (20%). All these mutants were then discarded.

Based on the phenotyping of the *nit* mutants on each of the four media containing different nitrogen sources, 676 *nit* mutants were generated from 81 *Fusarium* isolates. All *Fusarium* species were able to produce three different classes of *nit* mutants: *nit1, nit3* and NitM. *Fusarium oxysporum* produced 63% of the total *nit* mutants followed by *F. semitectum* (23%) and *F. subglutinans* (14%). Generally, *nit1* mutants were the most abundantly recovered (66%) on both MMC and PDC from all the *Fusarium* species, followed by *nit3* (21%) and NitM (13%). The frequency of *nit1* mutants was significantly higher on PDC than MMC as observed in the previous studies by Carvalho and Mendes-Costa (2011), Desjardins et al. (2000), Wang et al. (2010) and Zainudin et al. (2009). However, a different pattern was observed by Masratul Hawa (2008) of which the highest recovered mutant was *nit3*, contributing approximately 40% of the total mutants. Therefore, this result was inconsistent with the report of the present study and those from previous studies in which *nit1* mutants were obtained at greater frequencies in *F. oxysporum* (66%) (Desjardins et al., 2000; Somrith et al., 2011; Wang et al., 2010) and *F. poe* (63%) (Liu & Sunheim, 1996). Heterokaryons often with dense aerial mycelia were formed after 1 – 2 weeks incubation in the dark. The complementation of NitM between both *nit1* and *nit3* often formed a dense heterokaryon, whereas the pairing of *nit1* and *nit3* formed a weak heterokaryon and required a longer incubation period of 1 – 3 weeks (Carvalho & Mendes-Costa, 2011; Desjardins et al., 2000; Masratul Hawa, 2008; Pasquali et al., 2005).

Based on the results of the present study, all *Fusarium* species were vegetatively compatible but a greater magnitude of heterokaryosis occurred in 11 VCGs of *F. oxysporum* followed by 3 VCGs of *F. semitectum* and 2 VCGs of *F. subglutinans*. Previous studies indicated that heterokaryosis has been recognized in numerous species of *Fusarium* including *F. culmorum* (Balali & Iranpoor, 2006; El-Fadly, El-Kazzaz, Hassan, & El-Kot, 2008), *F. dimerum*, *F. chlamydosporum*, *F. avenaceum*, *F. scirpi*, *F. acuminatum*, *F. equiseti, F. graminearum, F. sambucinum, F. sulfureum, F. lateritium*, *F. graminearum* (El-Fadly et al., 2008), *F. fujikuroi* (Zakaria, Hsuan, & Salleh, 2011)*, F. proliferatum, F. verticillioides* (Zainudin et al., 2009), *F. oxysporum* (Gunn & Summerall, 2002; Leong, Latiffah, & Baharuddin, 2010; Swift, Wickliffe, & Schwartz, 2002), *F. sacchari* (Athman, 2006; Zakaria et al., 2011), *F. solani* (Balali & Iranpoor, 2006; Wang, Brubaker, & Burdon, 2004), *F. semitectum* (Abd-Elsalam, Schniederl, Asran-Amal, Khalil, & Verrett, 2003; Hawa, Salleh, & Latiffah, 2010), and *F. subglutinans* (Desjardins et al., 2000; Zheng & Ploetz, 2002). Based on the results of this study, all species isolates belonged to the same locations were grouped into the same VCGs. Similar observation was noted by Carvalho and Mendes-Costa (2011), Pasquali et al. (2005), Somrith et al. (2011) and Swift et al. (2002).

CONCLUSION

In conclusion, the complementation results indicated that *Fusarium* isolates would fuse to form a stable heterokaryon due to the genetic exchange of their cellular constituents. In the present study, it was evident that *Fusarium* isolates originated from the same geographical areas were clustered into the same VCGs. The results obtained essentially proved that VC is a useful tool for studying the diversity among the isolates of *Fusarium* species, *F. oxysporum, F. semitectum* and *F. subglutinans*. To the best of our knowledge, this was the first report on the classification of *F. oxysporum, F. semitectum* and *F. subglutinans* into VCGs isolated from post-harvest *Fusarium* fruit rot of tomato in Selangor and in the Peninsular Malaysia. Therefore, the findings of the study would possibly draw the attention of the concerned authority to formulate suitable measures to investigate the growth of the pathogens of this important cash crop.

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