BIOTROPIA No. 12,1999 .31-41

VEGETATIVE COMPATIBILITY GROUPS OF *FUSARIUM OXYSPORUM*, THE CAUSAL ORGANISM OF VASCULAR WILT ON ROSELLE IN MALAYSIA

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ABSTRACT

Forty strains of *Fusarium oxysporvm* isolated from roselle *(Hibiscus sabdariffa* var. *sabdariffa)* showing vascular wilt symptoms in three states (Terengganu, Penang and Ipoh) in the northern Malaysian Peninsula were used to investigate the vegetative compatibility. Nitrate-nonutilizing *(nil)* mutants were recovered from all the strains tested and subsequently used to study vegetative compatibility groups (VCG) within the population by *nit* mutants pairings on minimal medium. Thirteen VCGs were found and none were vegetatively compatible with those of other formae speciales (f. spp.) such as *asparagi* and *cubense*, and non-pathogenic strains from paddy and oil palm. The results indicate that there is substantial genetic diversity in *F. oxysporum* that causes vascular wilt disease on roselle as reflected by multiple VCGs, but the distribution of strains into the VCGs is not even as there are 26 representatives in VCG-1001M, two in VCG-1003M and VCG-1013M and only one in the other VCGs. This study may provide new insight into the establishment of a new forma specialis off. *oxysporum.*

Key words: Vegetative Compatibility Groups/M/ mutants/fitsarium oxysporwm/Roselle/Vascular wilt/ Malaysia.

INTRODUCTION

Fusarium oxysporum Schlechtend.: Fr. is an asexual, soil-borne fungus found in agricultural soils throughout the world. This species includes many pathogenic strains, known as formae speciales (f. spp.). Each forma specialis (f. sp.) is characterized by its ability to cause vascular wilt on a limited taxonomic range of host plants (Booth 1971).

Individual strains of the fungus show a high degree of host specificity which led Snyder and Hansen (1940) to classify strains of the fungus into f. spp. on the basis of the host plants attacked. For example, *F. oxysporum* f. sp. *melonis* attacks muskmelon *(Cucumis melo* L.), whereas *F. oxysporum* f. sp. *vasinfectum* is a pathogen of cotton *(Gossypium* spp.). Strains of the fungus, whether from the same or different f. spp. are usually morphologically indistinguishable (Puhalla 1985).

Currently, pathogenicity tests are the primary means to distinguish different pathogenic *Fusarium* strains. However, such tests do not indicate whether various strains of a given f. sp. or a physiologic race are genetically related. Puhalla (1985) used nitrate-nonutilizing *(nit)* mutants to show that strains in different f. spp. of *F. oxysporum* were in distinct vegetative compatibility groups (VCG), based on the ability of complementary *nit* mutants to anastomose and form wild-type

heterokaryons. These *nit* mutants could be recovered without mutagen treatment from selective media containing potassium chlorate ($KC1O_3$) (Puhalla 1985). Heterokaryon formation between complementary mutants was indicated by the development of dense aerial growth where mycelia of two thin *nit* mutant colonies touched and anastomosed when cultured side-by-side on minimal medium with nitrate as a sole nitrogen source (Klittich & Leslie 1988; Puhalla & Spieth 1985). Mycelia outside the anastomosed region remained thin. Diversity within pathogenic strains of F. *oxysporum* has been evaluated by characterizing strains in terms of vegetative compatibility. Strains capable of forming a successful vegetative heterokaryon are referred to as vegetatively compatible. Strains that are vegetatively compatible with one another are frequently described as members of the same vegetative compatibility group, or VCG (Leslie 1993). Strains from different groups would not form heterokaryons with each other. In asexually reproducing fungi such as *Fusarium*, vegetatively compatible strains are much more likely to be genetically similar than vegetatively incompatible strains (Correll *et al.* 1987).

Puhalla (1985) used *nit* mutants to test for vegetative compatibility among 21 strains of F. *oxysporum.* He found a correlation between VCG and f. sp. i.e. members of the same VCG belong to the same f. sp. Vegetative compatibility tests, therefore, have been shown to be a powerful tool for studying genetic diversity in F. *oxysporum* (Correll *et al.* 1987).

Most of the studies revealed a limited number of distinct VCGs within a f. sp. (Correll *et al.* 1986a; Jacobson & Gordon 1988; and Larkin *et al.* 1988). In contrast, 97 strains of F. *o.* f. sp. *asparagi* were found to include a minimum of 42 VCGs (Elmer & Stephens 1989), and 110 strains of F. *oxysporum* isolated from celery roots but nonpathogenic on celery included a minimum of 14 VCGs (Correll *et al.* 1986b).

Vascular wilt caused by F. *oxysporum* is the most important soil-borne disease on roselle in Malaysia (Ooi *et al.* 1998). Roselle *(Hibiscus sabdariffa* var. *sabdariffa)* has recently been domesticated and is being planted in a large scale for growing juice, jam and confectionary industries in Malaysia (Mat Isa *et al.* 1985; Tan & Said 1994). The seeds contain 17% oil similar in properties to cotton seed oil. Roselle is suitable to be planted on mineral or bris soils (Chin 1986). However, the cultivation of this newly domesticated crop in Malaysia has been disrupted due to various diseases mainly vascular wilt caused by a soil borne fungus, F. *oxysporum.* The pathogenicity of F. *oxysporum* isolated from the roots and stems of roselle showing vascular wilt symptoms in the field was proven in the greenhouse (Ooi *et al.* 1998).

In the present study, forty strains of F. *oxysporum* isolated from roselle showing vascular wilt symptoms in the northern Malaysian Peninsula were used to investigate the vegetative compatibility among the fungal strains.

MATERIALS AND METHODS

Strains

Forty strains of *F. oxysporum* isolated from roselle in three states of the Malaysian Peninsula were examined (Table 1). The strains were each grown on peptone pentachloronitrobenzene (peptone-PCNB) agar (Nash & Snyder 1962) for 5-7 days. Conidia from these strains were transferred to 3% water agar (WA), and individual uninucleate microconidia were isolated using a micromanipulator and transferred to potato sucrose agar (PSA). Colonies from these single-spore cultures were produced on PSA under standard incubation conditions and the resultant colonies were stored in liquid nitrogen (Salleh & Sulaiman 1984; Salleh & Strange 1988). These parent cultures were used as the starting inoculum for all subsequent tests.

Table 1, Complementation reactions between nitrate-nonutilizing *(nit)* mutants *ofF. oxysporum*[1]

	nitl	*nit3*	NitM
nit I	-or±	±or-	+
nit3	±or-	-	+
NitM	+	+	+

' + = Complementation occurs readily, - = no complementation occurs, ± = weak and/or slow complementation occurs.

Media

The basal medium (BM) was described by Correll *et al.* (1987). Minimal medium (MM) was made by adding 2.0 g of sodium nitrate ($NaNO_3$) to 1 L of the basal medium. *Nit* mutants were generated on minimal agar medium with chlorate (MMC) as described by Correll *et al.* (1987).

PSA were made as described by Booth (1971). Peptone-PCNB medium was prepared by adding the following to 1 L of distilled water: Difco peptone, 15.0 g; KH_2PCv 1.0 g; $MgSO_4$, 0.5 g; pentachloronitrobenzene (PCNB 75 WP), 1.0 g; agar, 20.0 g; and streptomycin, 300 ppm (Nash & Snyder 1962).

Recovery of nitrate-nonutilizing mutants *(nit* mutants)

Nit mutants were generated on MMC at 1.5 % concentration of chlorate. Mycelial transfers (approximately 2-mm^3 PSA blocks) of the fungus were placed on three equidistant places of three plates of MMC and incubated under standard incubation conditions (Salleh & Sulaiman 1984) and examined periodically (3, 5 and

7 days after incubation) for the appearance of fast-growing fan-shaped sectors from the initially restricted colony. All sectors were transferred to MM and those that grew as thin expansive colonies with no aerial mycelium were considered *nit* mutants. All *nit* mutants were resistant to chlorate and showed wild-type growth on PSA.

Nit mutant phenotypes

The physiological phenotypes of *nit* mutants recovered were distinguished by their growth on MM amended with different compounds as the sole nitrogen source (Correll *et al.* 1987; Klittich & Leslie 1988). The plates were incubated as described above, and colony morphology was scored relative to the wild-type parent after four days.

Complementation tests

Vegetative compatibility was determined by observing heterokaryon formation between complementing *nit* mutant on MM. Pairings were made by placing mycelia from each *nit* mutant 1-3 cm apart on MM. Pairings were incubated as described above for 7-10 days and then scored for complementation. Phenotypically distinct *nit* mutants, a *nitl* and a NitM, were obtained from 10 strains. These *nit* mutants were paired in all possible combinations to establish which strains were vegetatively compatible. NitM from one isolate in each of the VCGs thus identified was selected to serve as a tester for each group. Two *nitls* were obtained from each of the remaining 30 strains and paired with a NitM from each of the established VCGs. From within the group of strains that did not pair with any of the testers, a second group of testers was obtained and the procedure repeated. This process was continued until all strains were assigned to a VCG. Each pairing was repeated at least once (Gordon & Okamoto 1991). All of the *nit* mutants recovered from the same parent were paired with at least one *nitl,* one *nit3,* and one NitM mutant from that parent to test for self-compatibility.

Vegetative compatibility tests with testers of other f. spp. and non-pathogenic strains of *F. oxysporum*

Two *nit* mutants (one *nitl* and one NitM) of at least one isolate from each of the established VCGs were paired on MM with four *nit* testers of the following two f. spp.: *asparagi* (two testers) and *cubense* (two testers) ; and four *nit* testers of non-pathogenic strains of *F. oxysporum* from paddy *(Oryza saliva)* (one tester) and oil palm *(Elaeis guineensis)* (three testers).

RESULTS

Nit mutant isolation

Spontaneous chlorate resistant sectors were readily recovered from all 40 strains of *F. oxysporum* when cultured on MMC. The majority of the chlorate resistant sectors recovered were unable to utilize nitrate as a sole nitrogen source and consequently grew as thin expansive colonies with no aerial mycelium on MM. These sectors were designated *nit* mutants. The rate of radial expansion of these *nit* mutants is compatible to that of the wild types. Successive subcultures of the *nit* mutants onto MM continue to show this thin growth habit. Most of the *nit* mutants were stable, but a few (2-4%) of them developed small patches of heavy growth after prolonged incubation on MM.

Nil mutants phenotype identification

Recovered *nit* mutants could be divided into three distinct phenotypic classes by their growth and colony morphology on media containing one of five different nitrogen sources. The classes presumably reflect mutations at a nitrate reductase structural locus *(nitl),* a nitrate assimilation pathway-specific regulatory locus *(nit3)* and loci (at least five) that affect the assembly of a molybdenum-containing cofactor necessary for nitrate reductase activity (NitM). The majority of *nit* mutants recovered on MMC were *nitl.*

Several sectors recovered from some of the strains were resistant to chlorate but had wild-type colony morphology on MM. Correll *et al.* (1987) have observed this type of chlorate resistant nitrate utilizing sectors in *F. oxysporum.* They examined in more detail such sectors to determine if these sectors resulted from a mutation in a single locus or from the heterokaryotic growth of complementary mutations in two or more different nuclei. The analysis of microconidia from these sectors indicated that the chlorate-resistant nitrate utilizing sectors could be heterokaryotic or homokaryotic. Microconidia from homokaryotic sectors had a wild-type morphology on MM and presumably were mutants that were both chlorate-resistant and able to utilize nitrate *(cm* mutants). Microconidia recovered from heterokaryotic sectors were often a mixture of *nit* mutants conidia, wild-type conidia, and/or *crn* mutant conidia.

Complementation tests

Complementation occurred between *nit* mutants with different phenotypes. Complementation occurred more rapidly and growth of the resulting heterokaryon was more robust in pairings of NitM with *nitl* or *nit3* mutants than in pairings *of nitl* with *nit3* mutants (Table 1). When *nitl* and *nit3* mutants were paired,

complementation reaction was weak (very little aerial mycelium). To confirm that weak reactions could not result from cross feeding without anastomosis, 10 pairs of weakly reacting strains were tested with sterilized cellophane separating the two *nit* mutants following a method devised by Puhalla (1985). Where cellophane prevented hyphal contact, there was no visible reaction. Outside the cellophane barrier, where hyphae of the two *nit* mutants came into direct contact, a weak reaction was observed. This result was consistent with a requirement for hyphal anastomosis to produce weak reactions.

In our delineation of compatibility groupings, an isolate was placed in a VCG only if it reacted strongly and developed dense aerial growth where the mycelia of the *nit* mutant colonies came in contact and anastomosed to form a heterokaryon, with at least one other isolate in that group. On this basis, 40 strains were assigned to a total of 13 VCGs (Table 2). The largest of these included 26 strains, whereas 10 VCGs were represented by only a single strain while two VCGs were represented by 2 strains. In no case was a strain vegetatively compatible with strains from two different VCGs.

Table 2. Strains of *F. oxysporum* classified by vegetative compatibility and their source

Strain[1]	Source (naturally wilted roselle) [1]	Location
VCG 1001M		
72558%	Root	Mengabang Bakung, 7erengganu
T2560%	root	Mengabang Bakung, 7erengganu
T2561%	root	Kuala Berang, 7erengganu
T2563%	root	Rhu 7apai, 7erengganu
T2564%	root	Rhu 7apai, 7erengganu
T2566%	root	Rhu 7apai, 7erengganu
T2568%	root	Rhu 7apai, Terengganu
T2577%	root	Kuala Berang, 7erengganu
T2605%	nonsterile seed	Kuala 7erengganu, Terengganu
12607%	rotten fruit	Mengabang Bakung, 7erengganu
72608%	rotten stem	Mengabang Bakung, 7erengganu
72609%	rotten leaf	Kampung Pasir Nering, 7erengganu
72610%	rotten leaf	Kampung Pasir Nering, 7erengganu
72623%	stem	Mengabang Bakung, Terengganu
72625%	root	Kuala Berang, 7erengganu
72633%	rotten stem	Mengabang Bakung, Terengganu
P2883%	stem	USM, Penang
P2884%	stem	USM, Penang
P2885%	root	USM, Penang
P2886%	root	USM, Penang
P2887%	root	USM, Penang
P2888%	stem	USM, Penang
P2889%	rotten root	USM, Penang
P2890%	rotten root	USM, Penang
P2893%	stem	USM, Penang
P2894%	stem	USM, Penang

Table 2. Continued

Strain'	Source (naturally wilted roselle)[2]	Location
VCG 1002M		
T2567%	root	Rhu 7apai, 7erengganu
VCG 1003M		
12573%	root	Marang, 7erengganu
12618%	stem	Kampung Pasir Nering, 7erengganu
VCG 1004M		
T2575%	root	Marang, 7erengganu
VCG 1005M		
72620%	leaf	Kampung Pasir Nering, 7erengganu
VCG 1006M		
T2621%	leaf	Kampung Pasir Nering, 7erengganu
VCG 1007M		
72622%	Stem	Mengabang Bakung, 7erengganu
VCG 1008M		
72628%	Root	Mengabang Bakung, 7erengganu
VCG 1009M		
72629%	Root	Rhu 7apai, 7erengganu
VCG 1010M		
72637%	healthy root	Rhu 7apai, 7erengganu
VCG 1011M		
A2891%	fruit	Ipoh, Perak
VCG 1012M		
A2882%	fruit	Ipoh, Perak
VCG 1013M		
A2892%	fruit	Ipoh, Perak
A2898%	healthy root	Ipoh, Perak

'T= Strains from Terengganu A= Strains from Perak P= Strains form Penang

'%= Strains from roselle

[!]Parts of roselle where the strains were isolated

Heterokaryon self-incompatibility

No complementation occurred between any *nit* mutants of eight strains tested, even after repeated attempts. Furthermore, no complementation was observed when the *nitl* or NitM mutants were paired among themselves. The lack of complementation between phenotypically distinct *nit* mutants recovered from these strains lead us to designate these strains as heterokaryon self-incompatible (Table 3).

Vegetative compatibility tests with testers of other f. spp. and non-pathogenic strains of F. *oxysporum*

Two *nit* mutants (one *nitl* and one NitM) from each of the established VCGs were paired with four *nit* testers from two f. spp.: *asparagi* and *cubense'*, and four *nit*

Table 3. Source of self-incompatible strains of *F. oxysporum*

Strain[1]	Source[2]	Location
12566%	root	Rhu Tapai, Terengganu
T2575%	root	Marang, Terengganu
T2605%	nonsterile seed	Kuala Terengganu, Terengganu
T2608%	rotten stem	Mengabang Bakung, Terengganu
T2609%	rotten leaf	Kampung Pasir Nering, Terengganu
T2637%	healthy root	Rhu Tapai, Terengganu
A2882%	fruit	Ipoh, Perak
P2883%	stem	USM, Penang

T = Strains from Terengganu A= Strains from Perak P= Strains from Penang

'%= Strains from roselle
[2]Parts of roselle where the strains were isolated

testers from non-pathogenic strains. No heterokaryon formed (no complementation) between the testers from each VCG with any of the other f. spp. and the non-pathogenic strains.

DISCUSSION

Fusaria are often considered to be genetically unstable because they frequently produce sectors in culture which differ from the original colony in morphology, virulence, or other characteristics (Bumett 1984; Puhalla 1981). A moderate degree of genetic instability could have a selective advantage for a plant-pathogenic fungus such as *Fusarium,* allowing rapid adaptation to environmental stress such as fungicides or to the introduction of resistant genes into the hosts (Sapumohotti & Salleh 1992). A source of genetic variability is essential particularly for organisms such as *F. oxysporum,* that depend entirely on asexual reproduction. Genetic instability could generate variants that are fungicide resistant, able to overcome host resistance, or tolerant to toxic wastes in the soil. A high mutation rate could be an important means of generating variability if sexual or parasexual recombination is rare (Klittich & Leslie 1988).

Heterokaryosis and other types of mycelial interactions have been recognized in the genus *Fusarium* for at least 100 years (Page 1961; Stover 1959). A heterokaryon is a multinucleate cell containing genetically distinct nuclei in a common cytoplasm. Such cells are often created by hyphal fusions between vegetatively compatible strains. Heterokaryosis provides an opportunity for genetic recombination via the parasexual cycle in sterile, homothallic, or imperfect fungi such as *Fusarium* (Adams *etal.* 1987).

In this experiment, spontaneous *nit* mutants were readily recovered without mutagenic treatment. Because these mutations probably affect at least seven loci with three distinct phenotypes, the *nit* mutants can be used as forcing markers in the

formation of heterokaryon to test fungal strains for vegetative compatibility (Correll *et al.* 1986a, 1987; Gordon *et al.* 1986; Sidhu 1986; Jacobson & Gordon 1988; Elmer 1991).

Studies of fungal populations using VCGs as a means to measure diversity have become widespread in recent years. VCGs serve as a natural means to subdivide fungal populations. The *vie* loci and alleles that define VCGs are presumed to be selectively neutral with respect "to traits such as pathogenicity and vegetative viability. Different strains are vegetatively compatible *(i.e.,* capable of forming a heterokaryon) only if alleles at all vegetative compatibility loci are identical. Vegetative compatibility groups (VCGs) are ideal markers for population studies because they occur naturally and are easy to score using spontaneous *nit* mutants.

Strains which belong to the same VCG can form heterokaryons in which cytoplasms may mix, mitotic recombination may occur, and deleterious cytoplasmic agents, such as mycoviruses, can be exchanged. VCGs, which are analogous to anastomosis groups, have been correlated with pathogenicity in many fungal species including *Fusarium* spp. (Bosland & Williams 1987; Correll *et al.* 1986a; Correll *et al.* 1987; Jacobson & Gordon 1988; Ploetz & Correll 1988; Puhalla 1985).

Results obtained from this study suggest that 26 (65%) of the 40 strains of *F. oxysporum* tested belonged to a dominant VCG designated as 1001M, but only 2 strains (5%) were grouped in VCG 1003M and 1013M, respectively. VCG 1002M and 1004M-1012M each consist of only one strain (0.025%). This result suggests that anastomosis occurs infrequently among strains in this population and implies that the wilt pathogen of roselle in Malaysia is genetically diversified. But the distribution of different genotypes into VCGs among the strains is not even. There are some closely related strains and grouped into one VCG, while some others which showed no relatedness (vegetatively incompatible) with other strains were grouped into several VCGs separately as isolated groups.

At least one strain from each of the 13 VCGs was tested and the results showed that they were vegetatively incompatible with the wilt pathogens, F. *oxysporum* f. sp. *cubense* and f. sp. *asparagi;* and also incompatible with the non-pathogenic strains from paddy and oil palm. Thus, the wilt pathogen of roselle in Malaysia likely appears to constitute a distinct genetic population within the F. *oxysporum* complex.

Generally, mutants deficient in the molybdenum-containing cofactor (NitM) are infrequently isolated but readily form complementing heterokaryons, making them the most suitable choices for vegetative compatibility tests. On the other hand, *nit I* and *nit3* mutants may form weak, slow-growing heterokaryons, giving ambiguous results. We also found that the strength of a heterokaryon varied depending on the combination of the *nit* mutants used. The ideal combinations were mY7-NitM anc NitM-NitM which form more stable heterokaryons.

Our study demonstrates the importance of characterizing heterokaryosis in F *oxysporum.* Once the heteroploids are better characterized, they should be useful ir studying the effects of genetic interactions on the expression of economical!}

important traits such as virulence and the production of secondary metabolites such as mycotoxins. This study may also provide new insight into the establishment of a new forma specialis of F. *oxysporum*, causing wilt of roselle.

ACKNOWLEDGMENTS

This research is supported by the Government of Malaysia (1RPA Grant 01-02-05-6003). We thank our research assistants Mr. Kamarudin Mohd. Maidin and Miss Wan Faridah Maydin for their capable assistance, the Department of Agriculture (State of Terengganu) and the owners of the roselle sampling plots throughout the northern Malaysian Peninsula.

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