

Characterization of *Fusarium semitectum* from Isolates Vegetable Fruits (Pencirian Pencilan *Fusarium semitectum* daripada Buah Sayuran)

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ABSTRACT

Fusarium semitectum is a widespread species occurring in various types of substrate. Fifteen *F. semitectum* isolates were recovered from several types of vegetable fruits showing fruit rot symptoms, namely long bean (*Vigna sesquipedalis*), okra (*Abelmoschus esculentus*), loofa (*Luffa acutangula*), bitter melon (*Momordica charantia*), cucumber (*Cucumis sativus*) and green chilli (*Capsicum annuum*). The identification of the *F. semitectum* isolates was based on morphological characteristics of macroconidia and microconidia, presence of mesoconidia and colony pigmentation. The isolates were then characterized using vegetative compatibility group (VCG) and sequencing of translation elongation factor-1 α gene (*TEF-1 α*). From VCG analysis, 11 isolates were assigned to six VCGs and another four isolates were self-incompatible. The results of the phylogenetic analysis using *TEF-1 α* sequences showed that the isolates were phylogenetically distinct although the morphological characteristics were very similar and generally the grouping of the isolates was not according to the host. Both VCG and phylogenetic analyses indicated that *F. semitectum* isolates from vegetable fruits were highly variable which suggested that the isolates belong to a species complex.

Keywords: *Fusarium semitectum*; *TEF-1 α* ; VCG; vegetable fruits

ABSTRAK

Fusarium semitectum merupakan spesies yang tersebar dengan meluas, menduduki pelbagai jenis substrat. Lima belas pencilan *F. semitectum* telah diperolehi daripada beberapa jenis buah sayuran yang menunjukkan gejala reput buah iaitu kacang panjang (*Vigna sesquipedalis*), okra (*Abelmoschus esculentus*), petola (*Luffa acutangula*), peria (*Momordica charantia*), timun (*Cucumis sativus*) dan cili hijau (*Capsicum annuum*). Pencilan *F. semitectum* telah dikenal pasti berdasarkan ciri-ciri morfologi makrokonidia, mikrokonidia, kehadiran mesokonidia dan pigmentasi koloni. Pencilan tersebut kemudiannya dicirikan menggunakan kumpulan keserasian vegetatif (VCG) dan penjujukan gen faktor pemanjangan translasi (*TEF-1 α*). Daripada analisis VCG, 11 pencilan diletakkan ke dalam enam kumpulan VCG dan empat pencilan yang selebihnya menunjukkan ketidakserasian sendiri. Keputusan analisis filogenetik menggunakan jujukan *TEF-1 α* menunjukkan pencilan tersebut berbeza secara filogenetik walaupun ciri-ciri morfologinya adalah serupa dan secara amnya pencilan tersebut tidak dikumpulkan mengikut perumah. Kedua-dua analisis VCG dan filogenetik menunjukkan pencilan *F. semitectum* daripada buah sayuran mempunyai variasi yang tinggi yang dicadangkan pencilan tersebut tergolong dalam satu kompleks spesies.

Kata kunci: Buah sayuran; *Fusarium semitectum*; *TEF-1 α* ; VCG

INTRODUCTION

Fusarium semitectum isolates are commonly isolated from soils (Latiffah et al. 2007, 2009). They are widely distributed as saprophytes in soils and most probably exist as soil inhabitants (Burgess et al. 1988; Leslie 1990). The species is also commonly isolated from aerial plant parts in subtropical and tropical regions but is not regarded as an important plant pathogen (Leslie & Summerell 2006) although *F. semitectum* has been implicated in causing several diseases such as canker of walnut (Seta et al. 2004), pod rot, seed rot and root rot of beans (Dhingra & Muchovej 1979), corky dry rot of cantaloupe (Carter 1979), storage rot of banana (Griffiee 1976; Griffiee & Burden 1976) and wilting of alfalfa (Zaccardelli et al. 2006). These studies showed that *F. semitectum* is widespread and occurred in various substrates. During a survey on

Fusarium species associated with fruit rot of vegetable fruits, several *F. semitectum* isolates were recovered. As current knowledge on the occurrence and diversity of *F. semitectum* in Malaysia is still limited, the present study was conducted to characterize the *F. semitectum* isolates from vegetable fruits using vegetative compatibility group (VCG) and sequencing of the *TEF-1 α* gene.

MATERIALS AND METHODS

FUNGAL ISOLATES

Isolates of *F. semitectum* used in this study were isolated from several vegetable fruits showing fruit rot symptoms (Table 1). The isolates were grown on potato sucrose agar (PSA) and purified using the single spore isolation method.

Based on pathogenicity test, only isolate KPJ N1 recovered from long bean (*Vigna sesquipedalis*) was pathogenic to the host. Other isolates from okra (*Abelmoschus esculentus*), loofa (*Luffa acutangula*), cucumber (*Cucumis sativus*) and green chilli (*Capsicum annuum*) were non-pathogenic to their host (unpublished data).

For identification, the isolates were cultured on carnation leaf agar (CLA) for observation of primary characteristics namely, shape of macroconidia and microconidia, presence and absence of chlamydo-spores. Secondary characteristics such as the mycelial growth rates and pigmentation were observed on PDA. Species descriptions in The *Fusarium* Laboratory Manual (Leslie & Summerell 2006) were used to identify the *Fusarium* isolates.

VEGETATIVE COMPATIBILITY GROUP ANALYSIS

Fifteen isolates of *F. semitectum* were used in VCG analysis (Table 1). Grouping of VCGs were determined using complementation of nitrate non-utilizing (nit) mutants. The nit mutants, nit1, nit3 and nitM were generated for each isolate on minimal media (Puhalla & Spieth 1985) amended with KClO₃ from 1.5% (w/v) to 3.0% (w/v). Complementation or pairing tests of the nit mutants were also conducted on minimal media incubated at 20°C for 7–14 days. The nit mutants were paired in all possible combinations.

TABLE 1. *Fusarium semitectum* isolates used in this study

Code	Host
BND J2	Okra (<i>Abelmoschus esculentus</i>)
BND J3	Okra (<i>Abelmoschus esculentus</i>)
BND J5	Okra (<i>Abelmoschus esculentus</i>)
BND J6	Okra (<i>Abelmoschus esculentus</i>)
BND R3	Okra (<i>Abelmoschus esculentus</i>)
BND R4	Okra (<i>Abelmoschus esculentus</i>)
BND T1	Okra (<i>Abelmoschus esculentus</i>)
BND T3	Okra (<i>Abelmoschus esculentus</i>)
BND T5	Okra (<i>Abelmoschus esculentus</i>)
LJH N1	Green chili (<i>Capsicum annuum</i>)
KPJ N1	Long bean (<i>Vigna sesquipedalis</i>)
PRI S2	Bitter gourd (<i>Momordica charantia</i>)
PTL N2	Loofa (<i>Luffa acutangula</i>)
PTL S4	Loofa (<i>Luffa acutangula</i>)
TMN S2	Cucumber (<i>Cucumis sativus</i>)

Vegetatively compatible isolates were recognized by growth of dense aerial mycelial at the interface of two nit mutants, which indicated the formation of heterokaryons. Paired isolates that were compatible were assigned to the same VCG and those that did not produce heterokaryons were considered incompatible.

DNA EXTRACTION

For DNA extraction, the isolates were cultured on PSA, overlaid with dialysis membrane and incubated for 7 days

at 25°C. The harvested mycelial were ground in a mortar and pestle using liquid nitrogen until it becomes a fine powder. The genomic DNA was extracted using the DNeasy plant mini kit (Qiagen) according to the instructions given by the manufacturer.

PCR AMPLIFICATION OF TEF-1 α GENE

The translation elongation factor-1 α gene was amplified using primer pair EF1 (5'-ATG-GGT-AAG-GAG-GAC-AAG-AC-3') and EF2 (5'-GGA-AGT-ACC-AGT-GAT-CAT-GTT-3') according to the reaction conditions described by O'Donnell et al. (1998). PCR amplification was carried out in a 50 μ L reaction mixture containing 1 \times PCR buffer, 1 mM MgCl₂, 1 mM of each primer, 200 μ M of dNTP mix, 1 U of *Taq* polymerase (Promega) and 3 ng genomic DNA. PCR amplification was performed in a Peltier thermal cycler (PTC-100, MJ Research) starting with an initial denaturation at 94°C for 85 s, followed by 35 cycles of denaturation at 95°C for 35 s, annealing at 46°C for 55 s, extension at 72°C for 90 s and final extension at 72°C for 10 min. The PCR products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced from both ends.

SEQUENCE ANALYSIS OF TEF-1 α

The TEF-1 α sequences were aligned using the molecular evolution and genetic analysis software (MEGA5) (Tamura et al. 2011). The alignments were corrected manually where needed. All the sequences were compared with sequences of *Fusarium* species available in the FUSARIUM-ID database (Geiser et al. 2004; <http://fusarium.cbio.psu.edu>). *Fusarium equiseti* (DQ85454) from GenBank was used as an outgroup.

Multiple sequence alignment was performed using ClustalW 1.6 and phylogenetic analysis was performed using neighbour joining method in MEGA5. For neighbour joining analysis, substitution model of Jukes-Cantor was applied and all positions containing gaps were eliminated. To evaluate the stability of each branch, bootstrap analysis with 1000 bootstrap replicates was performed.

RESULTS AND DISCUSSION

Colonies of *F. semitectum* isolates recovered from different types of vegetable fruits showing fruit rot symptoms were similar with light brown pigmentation and the mycelia were floccose. Conidiophores had branched monophialides and polyphialides, mesoconidia which looked like 'rabbit ears' were abundant on the aerial mycelia. Macroconidia had 3–5 septa and chlamydo-spores occurred singly and in pairs. The morphological and cultural characteristics observed conform to the description in The *Fusarium* Laboratory Manual (Leslie & Summerell 2006).

The vegetative compatibility provides a method for characterizing variations based on genetics of the isolates rather than based on host-pathogen interaction (Leslie 1993). The overall VCG diversity was 40.0% which was

calculated by dividing the number of individual VCG groups by the total number of isolates (Smith-White et al. 2001). The results showed that based on VCG analysis, considerable genetic variation exists among the isolates. From complementation test, 11 isolates were assigned to six VCGs consisting of 1 – 4 isolates (Table 2). VCG1 and VCG3 consisted of isolates from okra. VCG2 comprised isolates from two different vegetable fruits, long bean and loofa. VCGs 4, 5 and 6 contain only one isolate each. Four isolates, BND J3, BND J6, BND R4 and PTL N2 were determined to be self-incompatible. According to Leslie (1993), generally isolates within a VCG tend to be more genetically similar than isolates in different VCG. From the present study, only isolates in VCG1 and VCG3 were isolated from the same host. Although VCG is useful for demonstrating genetic variations or genetic similarities, the inability of some isolates to form nit mutants on chlorate medium demonstrate the restriction of VCG analysis. To exclude self-incompatible isolates from genetic variation analysis would result in a negative implication on the overall variations (Smith-White et al. 2001). Therefore, DNA sequencing was conducted to provide more information on the genetic variation of *F. semitectum* isolates from vegetable fruits.

From PCR amplification of TEF-1 α , a single band of 750 bp was amplified from all the *F. semitectum* isolates. BLAST search for similarities using the Fusarium-ID database showed the isolates were similar to *F. incarnatum-equiseti* species complex with similarity ranging from 96% - 99%. *Fusarium semitectum* is synonymous with *F. incarnatum* and *F. pallidoroseum*, and as suggested by Leslie and Summerell (2006), until more work is done on the taxonomic and nomenclature problems of this species, the name *F. semitectum* is preferred as it is widely known. The phylogenetic trees constructed showed that the isolates of *F. semitectum* from vegetable fruits were grouped into two main groups (I and II) and the grouping for most of the isolates was not correlated to either VCG or host except for five isolates from okra (BND J5, BND R4, BND J3, BND J6 and BND J5) which were grouped in the same group (Figure 1). The results of the phylogenetic analysis showed that *F. semitectum* isolates from vegetable fruits

are phylogenetically distinct although the isolates are morphologically similar.

Fusarium semitectum is common in tropical and subtropical countries and often recognized as weak or wound parasite or secondary invader of plant tissues. The species has been reported as causing storage rot of groundnut, banana, citrus, tomato and cucurbits (Booth 1971). In the present study, only isolate KPJ N1 recovered from long bean was pathogenic causing rot to the host. The rest of the isolates were non-pathogenic which could indicate that *F. semitectum* isolates from other vegetable fruits are either endophytes or saprophytes. According to McDonald (1964), *F. semitectum* is often associated with a disease complex but is seldom pathogenic based on the observation on sesame disease complex.

Leslie and Summerell (2006) indicated that *F. semitectum* is probably a species complex. Based on VCG and phylogenetic analyses, the results suggested that *F. semitectum* isolates from vegetable fruits are variable as the isolates were divided into a number of VCGs and were grouped in several sub-groups in the phylogenetic tree. Fungi that reproduce only asexually, generally have a large number of VCGs which implies that variation exists between individuals (Correll 1991; Leslie 1993). Similar observations were also noted in *F. poae* and *F. avenaceum* which showed a high degree of VCG and molecular diversity and both species have no known sexual stage (Kerenyi et al. 1997; Satyaprasad et al. 2000). A variety of hosts or substrates could contribute to genetic variation in *F. semitectum* isolates as the species is a common secondary invader of many plants. Genetic variation may enhance the adaptation to different environments or host and the fungus may show differences in toxigenicity as well as pathogenic ability.

Although the number of isolates in this study were small, the data provides information regarding the occurrence and diversity of *F. semitectum* in Malaysia. The species has been recovered from soils (Latiffah et al. 2007), healthy roots of *Musa acuminata* (Latiffah & Nur Hidayah 2011) and dragon fruits (Masratul Hawa et al. 2010) which indicated that *F. semitectum* is widespread in Malaysia and occupy diverse ecological substrates.

TABLE 2. VCG of *F. semitectum* isolates from vegetable fruits

VCG	Isolate	Host
1	BND R3	Okra
	BND T1	Okra
	BND T3	Okra
	BND T5	Okra
	KPJ N1	Long bean
2	PTL S4	Loofa
3	BND J2	Okra
	BND J5	Okra
4	LJH N1	Green chili
5	PRI S2	Bitter gourd
6	TMN S2	Cucumber

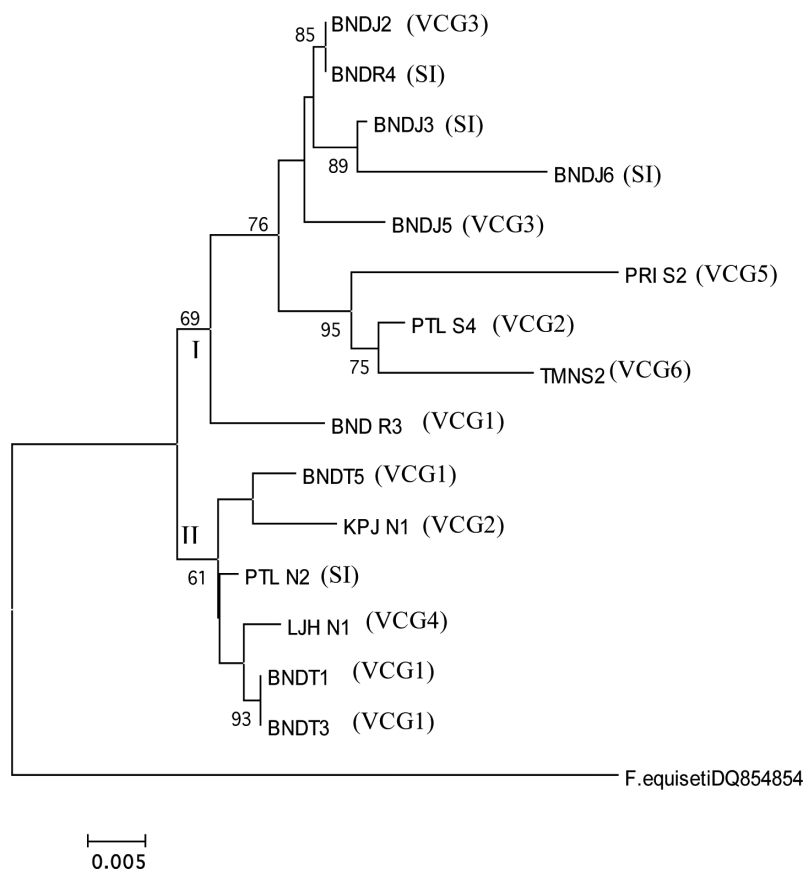


FIGURE 1. Phylogenetic tree of *F. semitectum* isolates from vegetable fruits inferred from neighbor-joining method and the VCG of the isolates. Isolates with SI are 'self-incompatible'. *F. equiseti* (DQ854844) is the outgroup

CONCLUSION

In conclusion, based on VCG analysis and sequencing of the TEF-1 α gene, isolates of *F. semitectum* from different types of vegetable fruits were highly variable which suggested that the isolates could be a species complex.

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