Saudi Journal of Biological Sciences (2011) **18**, 341–351

King Saud University

**Saudi Journal of Biological Sciences**

www.ksu.edu.sa
www.sciencedirect.com

ORIGINAL ARTICLE

# Molecular characterization of pathogenic *Fusarium* species in cucurbit plants from Kermanshah province, Iran

**K. Chehri [a,*], B. Salleh [a], T. Yli-Mattila [b], K.R.N. Reddy [a], S. Abbasi [c]**

[a] *School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia*
[b] *Department of Biochemistry and Food Chemistry, University of Turku, Finland*
[c] *Department of Plant Pathology, Faculty of Agriculture, Razi University, Kermanshah, Iran*

Received 18 August 2010; accepted 30 January 2011
Available online 4 February 2011

**KEYWORDS**

*Fusarium*;
Pathogenicity;
PCR;
ITS-RFLP;
Cucurbits;
Iran

**Abstract** *Fusarium* is one of the important phytopathogenic genera of microfungi causing serious losses on cucurbit plants in Kermanshah province, the largest area of cucurbits plantation in Iran. Therefore, the objectives in this study were to isolate and identify disease-causing *Fusarium* spp. from infected cucurbit plants, to ascertain their pathogenicity, and to determine their phylogenetic relationships. A total of 100 *Fusarium* isolates were obtained from diseased cucurbit plants collected from fields in different geographic regions in Kermanshah province, Iran. According to morphological characters, all isolates were identified as *Fusarium oxysporum*, *Fusarium proliferatum*, *Fusarium equiseti*, *Fusarium semitectum* and *Fusarium solani*. All isolates of the five *Fusarium* spp. were evaluated for their pathogenicity on healthy cucumber (*Cucumis sativus*) and honeydew melon (*Cucumis melo*) seedlings in the glasshouse. *F. oxysporum* caused damping-off in 20–35 days on both cucurbit seedlings tested. Typical stem rot symptoms were observed within 15 days after inoculation with *F. solani* on both seedlings. Based on the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) restriction fragment length polymorphism (RFLP) analysis, the five *Fusarium* species were divided into two major groups. In particular, isolates belonging to the *F. solani* species complex (FSSC) were separated into two RFLP types. Grouping among *Fusarium* strains derived from restriction analysis was in agreement with criteria used in morphological classification. Therefore, the PCR-ITS-RFLP method provides a simple and rapid procedure for the differentiation of

* Corresponding author. Tel.: +60 4 6534001; fax: +60 4 6565125.
E-mail address: khchehri@gmail.com (K. Chehri).



Peer review under responsibility of King Saud University.
doi:10.1016/j.sjbs.2011.01.007

Production and hosting by Elsevier

*Fusarium* strains at species level. This is the first report on identification and pathogenicity of major plant pathogenic *Fusarium* spp. causing root and stem rot on cucurbits in Iran.



## 1. Introduction

Cucurbit plants (*Cucurbitaceae*) are the main agricultural crops, particularly in the Kermanshah province in Iran. Annually it is estimated that over 3000 ha of agricultural land in the province are under cucurbits. Root and stem rots of cucurbits have significantly increased in incidence and severity in the past 20 years and they are a yield-limiting factor in many intensive cucurbit production, especially in cucumber (*Cucumis sativus*), watermelon (*Citrullus lunatus*) and honeydew melon (*Cucumis melo*), resulting sudden death and complete destruction of these economic plants (Alymanesh et al., 2009). Diseased plants were characterized by yellowing of the leaves, stem necrotic lesions, phloem discolorations, and collapse. There are many pathogens capable of producing these vine decline symptoms in cucurbits (Boughalleb et al., 2005).

The most important pathogens that cause root and stem rots in cucurbit plants are *Fusarium* spp., which are responsible for vascular wilts, such as those on melons (cantaloupe and muskmelon) caused by *Fusarium oxysporum* f. sp. *melonis*. *Fusarium proliferatum* and *Fusarium solani* f. sp. *cucurbitae* cause crown and foot rots of summer squash, melon, pumpkin, and a fruit rot of pumpkin (Pivonia et al., 1997; Namiki et al., 1994). There are two ''races'' of *F. solani* f. sp. *cucurbitae* causing fruit, crown and foot rots in cucurbit plants. *F. solani* f. sp. *cucurbitae* race 1 (Fsc1) causes crown, fruit, and root rots of cucurbits whereas *F. solani* f. sp. *cucurbitae* race 2 (Fsc2) causes only a fruit rot (Hawthorne et al., 1992). Fsc1 and Fsc2 are not easily distinguished morphologically, but identification requires mating tests, pathogenicity tests and molecular assays (Mehl and Epstein, 2007; Hawthorne et al., 1994; Boughalleb et al., 2005). *F. solani* f. sp. *cucurbitae* race 1 has been reported as the causal agent of melon root and foot rot from Khorasan province in the eastern part of Iran (Alymanesh et al., 2009).

Characterization of the population structure of fungal pathogens is important for understanding the biology of the organism and for development of disease-control strategies (Malvick and Percich, 1998), and for molecular studies among individuals, which is one of the components of population structure (Leung et al., 1993). Universally, *F. solani* species complex (FSSC) has an extensive host range and very high levels of diversity in pathogenicity and morphology (Brasileiro et al., 2004). However, the classification system based only on morphology has not provided an accurate tool for the identification of FSSC, neither has morphological classification system resolved the relationship of isolates within FSCC. So, a molecular approach is promising in establishing the objective (O'Donnell and Gray, 1995; Zhang et al., 2006; O'Donnell et al., 2008). Among the methods which researchers have used to analyze the phylogenetics of *F. solani* species are rDNA-IGS, rDNA-ITS regions, large submit RNA gene and translation elongation factor-alpha (tef) (Zhang et al., 2006). Internal transcribed spacer (ITS) region is probably the most widely sequenced region of DNA in fungi. rDNA-ITS and rDNA-IGS (intergenic spacer) regions show a higher degree of diversity than other ribosomal regions such as small subunits (SSU) and large subunits (LSU) (O'Donnell and Gray, 1995; Depriest and Been, 1992; O'Donnell, 2000; Brasileiro et al., 2004). Therefore, the objectives of this study were: (i) to isolate and identify disease causing *Fusarium* spp. from infected cucurbit plants in Kermanshah province; (ii) to determine their pathogenicity; and (iii) to determine phylogenetic relationships and usefulness of the PCR-ITS-RFLP as a genetic marker within the *Fusarium* spp.

## 2. Materials and methods

### 2.1. Sample collection

Infected cucurbit plants were collected from different regions of Kermanshah province, Iran (Table 1). Each sample were stored in a paper envelope and kept in a cool box with dry ice. In the laboratory, roots and stems of diseased samples were washed in running tap water and cut into small blocks (1.5 cm) for isolation.

### 2.2. Isolation and identification

For isolation of *Fusarium* spp., the blocks were surface-sterilized with 1% sodium hypochlorite for 3 min and rinsed with several changes of sterile water. The sterilized samples were placed onto a general medium (water agar) (Burgess et al., 1994) and a semi-selective medium for *Fusarium*, i.e., peptone-pentachloronitrobenzene agar (PPA) plates (Nash and Snyder, 1962), and incubated under a standard growth condition (Salleh and Sulaiman, 1984). The resulting *Fusarium* colonies were single-spored and transferred onto potato dextrose agar (PDA), carnation leaf agar (CLA) (Fisher et al., 1982), spezieller nahrstoffarmer agar (SNA) (Nirenberg, 1976), and potassium chloride agar (KClA) plates (Fisher et al., 1983) for morphological identification (Leslie and Summerell, 2006).

### 2.3. Pathogenicity test

All isolates of the *Fusarium* species were tested for their pathogenicity on apparently healthy and uniform 20 days-old seedlings of cucumber (*C. sativus*) and honeydew melon (*C. melo*) in the glasshouse. Roots and stems of the cucumber and honeydew melon seedlings were washed in running tap water before inoculation. Conidial suspension of each individual isolate was prepared by pouring sterile distilled water and gently scraping the conidia of 7 days-old cultures on PDA plates grown under the standard growth condition (Salleh and Sulaiman, 1984). The concentration of the pooled suspension was adjusted to $2 \times 10^6$ conidia/ml by using a haemocytometer. The roots of the seedlings were soaked in 20 ml conidial suspension for 20 min for root inoculation technique. For stem inoculation technique, 20 ml of the conidial suspension of each *Fusarium* species was sprayed on the stems. The control plants were inoculated by booth techniques with 20 ml of sterile distilled water. Three replicates were performed for each isolate and the experiment was repeated twice. All the

**Table 1** *Fusarium* species isolated from different hosts in different sampling locations in Kermanshah province.

| Place of sample collection | Host | Source | *Fusarium* species |
|---|---|---|---|
| Dorood Faraman-Maoqufeh | Watermelon | Root | *F. ox*, *F. eq*, *F. so* |
| Road Kamyaran-Varmele | Honeydew melon | Crown and Stem | *F. ox*, *F. eq*, *F. so* |
| Miandarband-JaFar Abad | Watermelon | Root | *F. ox*, *F. pr*, *F. so* |
| Qazanchi-Ahmad Abad | Melon | Crown and Stem | *F. ox*, *F. eq*, *F. se* |
| Qazanchi-Ahmad Abad | Honeydew melon | Root | *F. ox*, *F. eq*, *F. so* |
| Road Allah-yari | Honeydew melon | Root | *F. ox*, *F. so* |
| Road Ravansar-Kamyaran | Honeydew melon | Root | *F. ox*, *F. eq*, *F. se* |
| Kangavar-Pol Shekasteh | Honeydew melon | Root | *F. ox*, *F. eq*, *F. so* |
| Kangavar-Rahmat Abad | Honeydew melon | Root | *F. ox*, *F. pr*, *F. so* |
| Kangavar-Gaodin | Cucumber | Crown | *F. ox*, *F. eq*, *F. so* |
| Gaodin | Honeydew melon | Root and Crown | *F. ox*, *F. eq*, *F. pr* |
| Sonqor | Honeydew melon | Root | *F. ox*, *F. se*, *F. so* |
| Sonqor | Honeydew melon | Root | *F. ox*, *F. eq*, *F. so* |
| Road Sonqor- Asad Abad | Honeydew melon | Root | *F. ox*, *F. eq*, *F. so* |
| Harsin | Honeydew melon | Root | *F. ox*, *F. se*, *F. pr* |
| Dinavar-Shirkhan | Honeydew melon | Root and Crown | *F. ox*, *F. eq*, *F. so* |
| Bisotun-Barnaj | Honeydew melon | Root | *F. ox*, *F. pr*, *F. so* |
| Bisotun-Hosein Abad | Honeydew melon | Root | *F. ox*, *F. eq*, *F. so* |
| Bisotun-Chehr | Pumpkin | Root | *F. ox*, *F. so* |
| Bisotun | Pumpkin | Root | *F. ox*, *F. eq*, *F. so* |
| Road Kermanshah Sarab NilooFar | Pumpkin | Root and Crown | *F. ox*, *F. eq*, *F. so*, *F. se* |
| Road Kermanshah- Sarab NilooFar | Honeydew melon | Root | *F. ox*, *F. pr*, *F. so* |
| Sarab NilooFar | Honeydew melon | Stem | *F. eq*, *F. so*, *F. se* |
| Road Koozaran-Boor Boor | Honeydew melon | Crown and Stem | *F. se*, *F. eq*, *F. so* |
| Road Koozaran-Chehar Zabar | Honeydew melon | Stem | *F. pr*, *F. eq*, *F. so* |
| Gilan Garb | Cucumber | Root and Crown | *F. ox*, *F. eq*, *F. so* |
| Qasr Shirin | Cucumber | Stem | *F. so*, *F. se* |
| Road Paveh-Ravansar' | Honeydew melon | Stem | *F. so* |
| Paveh- Shemshir | Cucumber | Crown and Stem | *F. pr*, *F. eq*, *F. so* |
| Sarpol Zohab | Watermelon | Stem | *F. so* |

*F. ox* = *F. oxysporum*, *F. eq* = *F. equiseti*, *F. so* = *F. solani*, *F. pr* = *F. proliferatum*, *F. se* = *F. semitectum*.

inoculated and controls seedlings were placed in the glasshouse with day and night temperatures of 30–35 °C and 23–30 °C, respectively. Development of symptoms on inoculated and control seedlings were observed every 2 days for 45 days. The inoculated fungi were re-isolated from the infected plants to prove the Koch's postulates.

### 2.4. Growth condition and DNA extraction

All *Fusarium* isolates were grown in 250 ml of potato dextrose broth (PDB) (Difco) in a rotary shaker at 180 rpm for 48 h at 28 °C. After vacuum filtration, the mycelium of each isolate was dried, ground with sterile sea sand in a mortal and pestle, and stored at −20 °C for further studies. Genomic DNA was extracted using a modified method of Kim et al. (1992). Approximately 0.5 g of the ground mycelium was suspended in CTAB extraction buffer (0.7 M NaCl, 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 1% 2-mercaptoethanol, 1% CTAB), and extracted with phenol/chloroform/isoamylalcohol (25:24:1) and chloroform/isoamylalcohol (24:1). RNA was degraded by treatment with RNase (Qiagen) (50 mg/ml) for 30 min at 37 °C. DNA was then precipitated by adding 2.5 volumes of absolute ethanol and pelleted by centrifugation for 15 min at 12,000 rpm. The pellet was washed with 70% ethanol, air-dried and re-suspended in 1 mM TE buffer {10 mM Tris–HCl, 1 mM EDTA (pH 8.0)}. DNA concentration and purity was measured using a spectrophotometer (Shimadzu UV-120) at 260 and 280 nm.

### 2.5. PCR condition

The ITS region of *Fusarium* spp. was amplified with primers ITS1 (5′-TCCGTTGGTGAACCAGCG G-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). Amplification was performed in 100 µl of reaction mixture containing 50 pmol of primers, 2.5 units of *Taq* DNA polymerase (Takara), 200 mM of each dNTP, 10 ml of 10× PCR buffer and 0.2 mg of template DNA. The mixture was subjected to PCR in a Thermo cycler. The PCR cycles began with an initial denaturation for 3 min at 95 °C, followed by 30 cycles of annealing for 40 s at 58 °C, extension for 40 s at 72 °C and denaturation for 40 s at 94 °C before a final extension for 5 min at 72 °C. The PCR product obtained was run on 1.4% agarose gels, stained with ethidium bromide (EtBr) and visualized under a UV transilluminator (Brand and company).

### 2.6. Enzyme digestion

Aliquots of 12 µl of PCR products were digested with 10 units of restriction enzymes *Eco*RI, *Sph*I, *Pst*I, *Hae*III, *Hin*fI, *Msp*I and *Sma*I according to the manufacturer's instructions (Fermentas). The restriction fragments were separated on 2.5% agarose gel, run for 140 min at 80 V, 400 mA and stained with EtBr. The restriction fragments were visualized under the UV visualizer and 100 bp DNA ladder (GeneRulers™, Fermentas) was used to estimate the size of the restriction fragments. The restriction analysis was repeated twice.

**Table 2** Morphological characters of *Fusarium* spp. isolated from cucurbit plants.

| *Fusarium* Species | Chla. shape | Pigmentation on PDA | Number of septa in macroconidia | Microconidia shape | Types of conidiogenous cells | | General morphology | | Macroconidia size (μm) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Poly | Mono | Apical cell | Basal cell | |
| *F. equiseti* | ro | Brown | 5–7 | – | – | + | Tapered, elongated | Fs | 44–78 × 3.3–5.6 |
| *F. oxysporum* | sm | Violet | 3 | ov to el | – | + | Curved | Fs | 32–56 × 3.1–5.7 |
| *F. proliferatum* | – | Violet | 3–5 | cl, na | + | + | Curved | Pdfs | 25–58 × 3.0–5.0 |
| *F. semitectum* | ro | Brown | 3–5 | – | + | + | Curved and tapered | Fs | 37–58 × 3.0–5.0 |
| *F. solani* (morphotype II) | sm | Red | 5 | el to tr, cl | – | + | Tapered | Fs | 32–68 × 3.6–6.0 |
| *F. solani* (morphotype I) | ro | White | 3 | re | – | + | Rounded and curved | Nfs | 30–50 × 3.5–5.7 |

+ = presence, – = absence, Poly = polyphialidic, Mono = monophialidic, Pdfs = poorly developed foot shape, Nfs = Notch or foot shape, Fs = foot shape, Lfs = long foot shape, Chla = chlamydospore, ro = rough, sm = smooth, el = ellipsoid, tr = truncate, cl = clavate, re = reniform, ov = oval.

### 2.7. Data analysis

The molecular size of each fragment was estimated using a standard curve of migration versus the log of the molecular size of 100 bp ladder. Each fragment was scored on the basis of the presence (1) or absence (0) of particular fragments. A data matrix was constructed based on the presence or absence of the fragments and converted to a similarity matrix. The similarity matrix was then subjected to the unweighted pair group method with arithmetical mean (UPGMA) cluster analysis based on simple matching coefficient (SMC) (Romesburg, 1994). The data analysis was performed by using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc, version 2.1) (Rohlf, 2000) to analyze the relationship among all isolates of *Fusarium* species.

## 3. Results

### 3.1. Occurrence and pathogenicity

In this study, a total of 100 *Fusarium* isolates were obtained from diseased cucurbit plants in 30 different locations in the Kermanshah province (Table 1), and based on their morphological characteristics, these isolates were identified as *F. oxysporum*, *F. proliferatum, Fusarium equiseti, Fusarium semitectum*, and *F. solani* species complex (FSSC) (Table 2, Figs. 1 and 2). On the basis of mean lesion sizes on stems, the pathogenicity of each *Fusarium* isolates was classified into three groups: virulent (2.5–1.80 $cm^2$), moderately virulent (0.8–0.5 $cm^2$) and nonvirulent (0.0–0.0 $cm^2$) (Table 3).

The results of the pathogenicity test revealed that 25 isolates of *F. oxysporum* were the major causal agent of cucumber and honeydew melon root rot. The symptoms were observed within 20–35 days after inoculation as necrosis and brown discoloration of the root phloem and yellowing of the canopy plants. Also, 14 isolates of *F. solani*, 3 isolates of *F. proliferatum* and 2 isolates of *F. equiseti* caused discoloration and necrosis and then brown rot of the stems. The plants were maintained in the glasshouse for 45 days for symptom development. Their initial symptoms were observed on the 15th day after inoculation as water-soaked lesions on the stems. The inoculated fungi were consistently isolated from the diseased plants but not from negative control plants and nonvirulent isolates (Table 3); thus fulfilled the Koch's postulates.

### 3.2. Molecular analysis

A PCR product from each isolate of the three *Fusarium* species was amplified by using primer pairs ITS1 and ITS4. *F. oxysporum*, *F. equiseti* and *F. semitectum* produced approximately 550 bp band, *F. solani* species complex about 570 bp and *F. proliferatum* approximately 560 bp bands (Fig. 3). Table 4 and Fig. 3 shows estimated sizes of the restriction bands produced after digestion of the ITS + 5.8S using *Eco*RI, *Sph*I, *Pst*I, *Hae*III, *Hin*fI, *Msp*I and *Sma*I for *F. oxysporum*, *F. equiseti*, *F. semitectum*, *F. solani* species complex and *F. proliferatum*. Generally, the restriction patterns by the restriction enzymes could differentiate the five *Fusarium* species (Fig. 3).

Banding patterns of all *Fusarium* spp. after digestion analysis were presented in Table 4. Digestion of the PCR products with *Eco*RI generated two banding patterns in all *Fusarium* isolates except for isolate FSQA, which indicated that there is no restriction site for the restriction enzyme *Eco*RI within it. Digestion with *Eco*RI produced the same patterns for the *F. oxysporum*, *F. semitectum* and *F. equiseti* isolates. It also showed one common and one different band for *F. proliferatum* and *F. solani* isolates except for isolate FSQA. Digestion of the ITS + 5.8S region with *Sph*I showed two banding patterns in all isolates. The fragment of 320 bp was present in all isolates. The fragment of 250 bp was present in all *F. proliferatum* and *F. solani* isolates and the fragment of 230 bp was found in all *F. oxysporum*, *F. semitectum* and *F. equiseti* isolates. Digestion with enzyme *Pst*I mostly indicated that there is no recognition site for the restriction except for *F. solani* isolate FSQA and *F. proliferatum* isolates with two fragments of 150 and 420 bp. Digestion with *Hae*III produced identical patterns of four fragments for *F. solani* isolates, three identical fragments for the *F. oxysporum*, *F. semitectum* and *F. equiseti* isolates and three identical fragments for *F. proliferatum* isolates. Digestion with *Hin*fI exhibited three banding patterns producing three fragments in the *F. oxysporum*, *F. semitectum* and *F. equiseti* isolates and two fragments in the *F. solani* and *F. proliferatum* isolates. The fragment of 270 was found in all

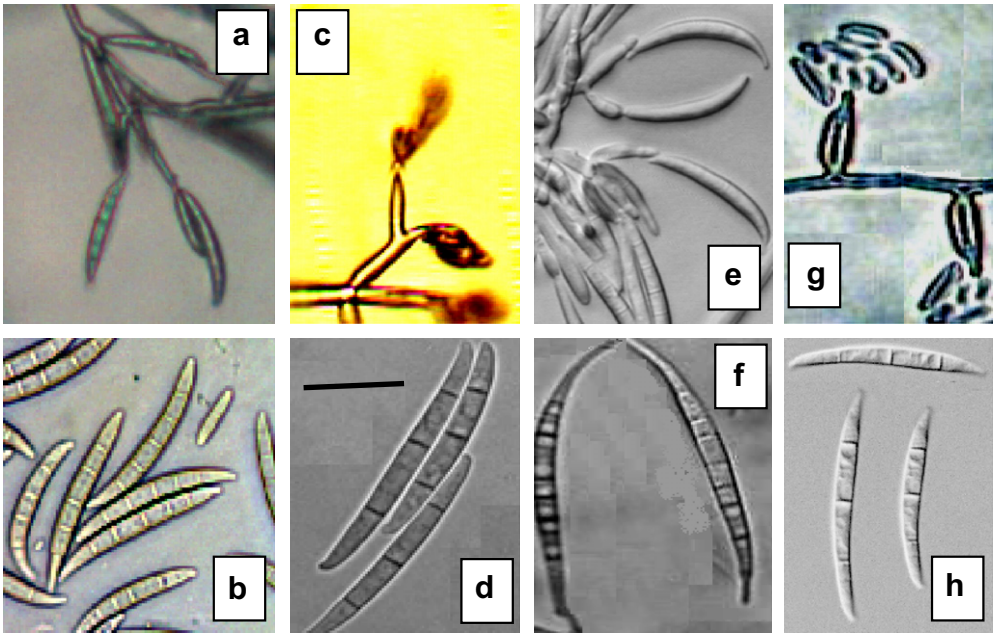


**Figure 1** Conidiophores and macroconidial characters of *Fusarium* spp. a and b = *F. semitectum*, c and d = *F. proliferatum*, e and f = *F. equiseti*, g and h = *F. oxysporum* (scale bar = 25 μm).

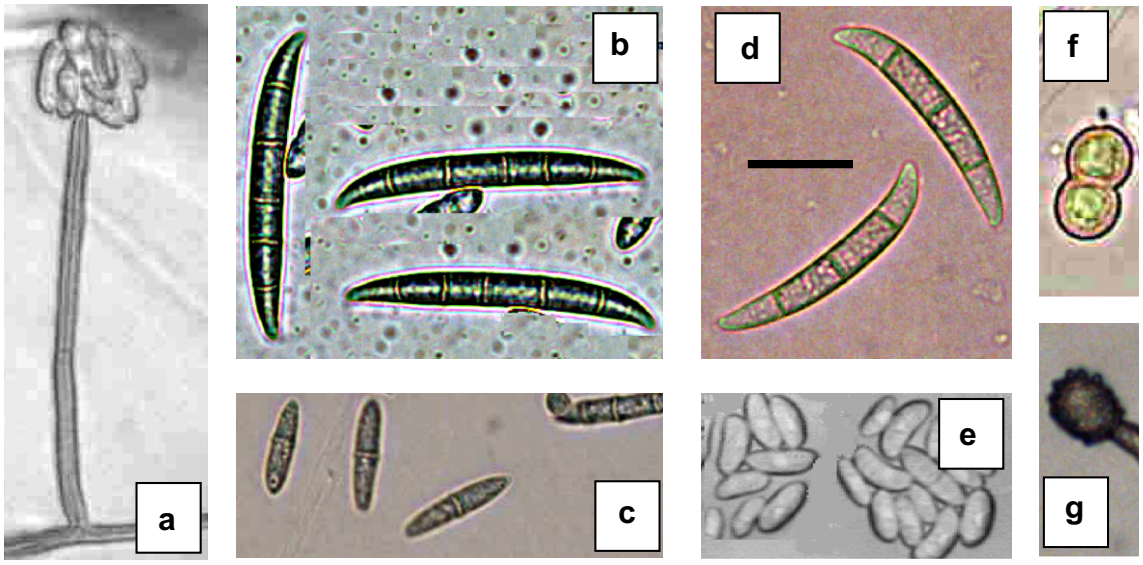


**Figure 2** Morphological characters of *Fusarium solani*. a = Conidiophores; b, c and f = macroconidia, microconidia and chlamydospores of FSQA isolate (morphotype II); d, e and g = macroconidia, microconidia and chlamydospores of FSMV isolate (morphotype I) (scale bar = 25 μm).

*F. solani* isolates except for isolate FSQA that produced the fragment of 290 bp. Digestion with *Msp*I indicated that there is no restriction site for *F. semitectum* and *F. equiseti* isolates whereas there are three banding patterns in the *F. proliferatum* isolates and two banding patterns in *F. oxysporum* and *F. solani* isolates. Digestion with *Sma*I exhibited two identical fragments (230 and 350 bp) in the *F. solani* and *F. proliferatum* isolates but there was no restriction site for *F. oxysporum*, *F. semitectum* and *F. equiseti* isolates.

The cluster analysis clearly discriminate the five *Fusarium* species into five separate clusters. *F. solani* isolates were clustered in subcluster A and *F. proliferatum* isolates in subcluster B in major cluster I and *F. oxysporum* isolates were clustered in subcluster C, *F. equiseti* isolates in subcluster D and *F. semitectum* isolates in subcluster E in major cluster II (Fig. 4).

## 4. Discussion

*Fusarium* species are ubiquitous in roots and stalks of most plants, including cucurbits, and may exist as saprophytes in plant tissues or as opportunistic pathogens awaiting pre-

**Table 3** Relative virulence of *Fusarium* spp. based on the pathogenicity test on cucurbit seedlings.

| Name of species | Number isolate | Pathogenicity test | | |
|---|---|---|---|---|
| | | Virulent | Moderately virulent | Non virulent |
| *F. equiseti* | FEQ101 | | | + |
| *F. equiseti* | FEQ21 | | | + |
| *F. equiseti* | FEQ51 | | | + |
| *F. equiseti* | FEQUI | + | | |
| *F. equiseti* | FEQ22 | | + | |
| *F. equiseti* | FEQUII | + | | |
| *F. equiseti* | FEQ10 | | | + |
| *F. equiseti* | FEQ2 | | | + |
| *F. equiseti* | FEQ5 | | | + |
| *F. equiseti* | FEQ104 | | | + |
| *F. equiseti* | FEQ27 | | | + |
| *F. oxysporum* | FOX142 | + | | |
| *F. oxysporum* | FOAY | + | | |
| *F. oxysporum* | FOX247 | | + | |
| *F. oxysporum* | FOX142 | + | | |
| *F. oxysporum* | FOX148 | + | | |
| *F. oxysporum* | FOX27 | | + | |
| *F. oxysporum* | FOX112 | + | | |
| *F. oxysporum* | FOX148 | + | | |
| *F. oxysporum* | FOX247 | | + | |
| *F. oxysporum* | FOX112 | + | | |
| *F. oxysporum* | FOX118 | + | | |
| *F. oxysporum* | FOX271 | | + | |
| *F. oxysporum* | FOX121 | + | | |
| *F. oxysporum* | FOX188 | + | | |
| *F. oxysporum* | FOX278 | | + | |
| *F. oxysporum* | FORK | + | | |
| *F. oxysporum* | FOX158 | + | | |
| *F. oxysporum* | FOX257 | | + | |
| *F. oxysporum* | FOX182 | + | | |
| *F. oxysporum* | FOX127 | + | | |
| *F. oxysporum* | FOX186 | + | | |
| *F. oxysporum* | FOX273 | | + | |
| *F. oxysporum* | FOX122 | + | | |
| *F. oxysporum* | FOX158 | + | | |
| *F. oxysporum* | FOX287 | | + | |
| *F. oxysporum* | FOX152 | + | | |
| *F. oxysporum* | FOX1228 | + | | |
| *F. oxysporum* | FOX1258 | + | | |
| *F. oxysporum* | FOX277 | | + | |
| *F. oxysporum* | FOX127 | + | | |
| *F. oxysporum* | FOX187 | + | | |
| *F. oxysporum* | FOX278 | | + | |
| *F. oxysporum* | FOX127 | + | | |
| *F. oxysporum* | FOX188 | + | | |
| *F. oxysporum* | FOX278 | | + | |
| *F. oxysporum* | FOX189 | + | | |
| *F. oxysporum* | FOX278 | | + | |
| *F. proliferatum* | FPRFI | + | | |
| *F. proliferatum* | FPR78 | | | |
| *F. proliferatum* | FPRFII | + | | |
| *F. proliferatum* | FPR78 | | | |
| *F. proliferatum* | FPR811 | | | |
| *F. proliferatum* | FPR785 | + | | |
| *F. proliferatum* | FPR88 | | | |
| *F. proliferatum* | FPR711 | | | |
| *F. proliferatum* | FPR885 | | | + |
| *F. proliferatum* | FPR88 | | | + |
| *F. proliferatum* | FPR811 | | | + |
| *F. semitectum* | FSEM | | | + |
| *F. semitectum* | FSE186 | | | + |
| *F. semitectum* | FSE287 | | | + |
| *F. semitectum* | FSE168 | | | + |

**Table 3** (*continued*)

| Name of species | Number isolate | Pathogenicity test | | |
|---|---|---|---|---|
| | | Virulent | Moderately virulent | Non virulent |
| *F. semitectum* | FSE16 | | | + |
| *F. semitectum* | FSE76 | | | + |
| *F. semitectum* | FSE287 | | | + |
| *F. semitectum* | FSE16 | | | + |
| *F. solani* | FSQA | | | + |
| *F. solani* | FSO89 | | | + |
| *F. solani* | FSO22 | | | + |
| *F. solani* | FSO694 | + | | |
| *F. solani* | FSO639 | | | |
| *F. solani* | FSO193 | + | | |
| *F. solani* | FSO1 | + | | |
| *F. solani* | FSO298 | + | | |
| *F. solani* | FSO23 | + | | + |
| *F. solani* | FSO559 | | | + |
| *F. solani* | FSO55 | | | + |
| *F. solani* | FSO18 | | | |
| *F. solani* | FSO288 | | | |
| *F. solani* | FSO273 | + | | |
| *F. solani* | FSO585 | + | | |
| *F. solani* | FSO17 | + | | |
| *F. solani* | FSMV | | | + |
| *F. solani* | FSM555 | | | + |
| *F. solani* | FSO57 | | | + |
| *F. solani* | FSO288 | + | | |
| *F. solani* | FSO237 | + | | |
| *F. solani* | FSM555 | + | | |
| *F. solani* | FSO58 | | | |
| *F. solani* | FSO253 | | | |
| *F. solani* | FSRS | + | | |
| *F. solani* | FSPS | + | | |
| *F. solani* | FSO237 | + | | |
| *F. solani* | FSO555 | | + | |
| *F. solani* | FSO55 | | + | |
| *F. solani* | FSO287 | | | |
| *F. solani* | FSO239 | | | + |
| *F. solani* | FSO558 | | | + |
| *F. solani* | FSO98 | | | + |

+ = virulent (mean of lesion size = 2.5–1.80 $cm^2$), moderately virulent (mean of lesion size = 0.8–0.5 $cm^2$), non virulent (mean of lesion size = 0.0–0.00 $cm^2$).

disposed conditions such as stress in their hosts. Many species of *Fusarium* are viewed as opportunistic or weak pathogens that are capable of attacking only plants that were weakened previously by some other stress factors. Certainly, factors such as those induced by drought, wind and insects are known to affect the amount and disease incidence (Palmer and Kommedahl, 1960). Our observations in rainfed cucurbit fields in Kermanshah province showed that the severity of the disease may be increased under certain environmental conditions in the middle of the summer. There are also observations showing that in irrigated fields the severity of the disease may increased under flooding, and irregular furrow irrigation. Thus, the time and type of irrigation in the cucurbit fields in Kermanshah province may be considered as one of the feasible cultural practices for better disease control.

*Fusarium* species have been recorded from several parts of the world and they are known to be pathogenic to many plants, especially to cucurbits. The results of this study are in agreement with those of the previous literatures, e.g., Boughalleb et al. (2005), Mehl and Epstein (2007). The cluster analysis based on restriction bands formed two major groups, I and II. The group including *F. solani* and *F. proliferatum* isolates was supported at the similarity level of ca. 56%. The two subgroups of *F. solani* species complex (FSSC) were supported at the similarity level of 69%. The second group, that included *F. oxysporum*, *F. semitectum* and *F. equiseti*, was supported at the similarity level of ca. 80% similarity. Within this group, the group of *F. semitectum* and *F. equiseti* was supported at the similarity level of 90%. PCR–RFLP of ITS + 5.8S have been used by Suga et al. (2000) in distinguishing formae speciales (f. spp.) of *F. solani* and Lee et al. (2000) for comparing genetic relationships between 12 *Fusarium* species from different sections. Digestion of *F. solani* PCRs product with *Eco*RI, *Pst*I, *Hin*fI and *Msp*I produced variable restriction patterns. Digestion with *Hae*III, *Sph*I and *Sma*I generated the same restriction patterns for all *F. solani* isolates obtained from

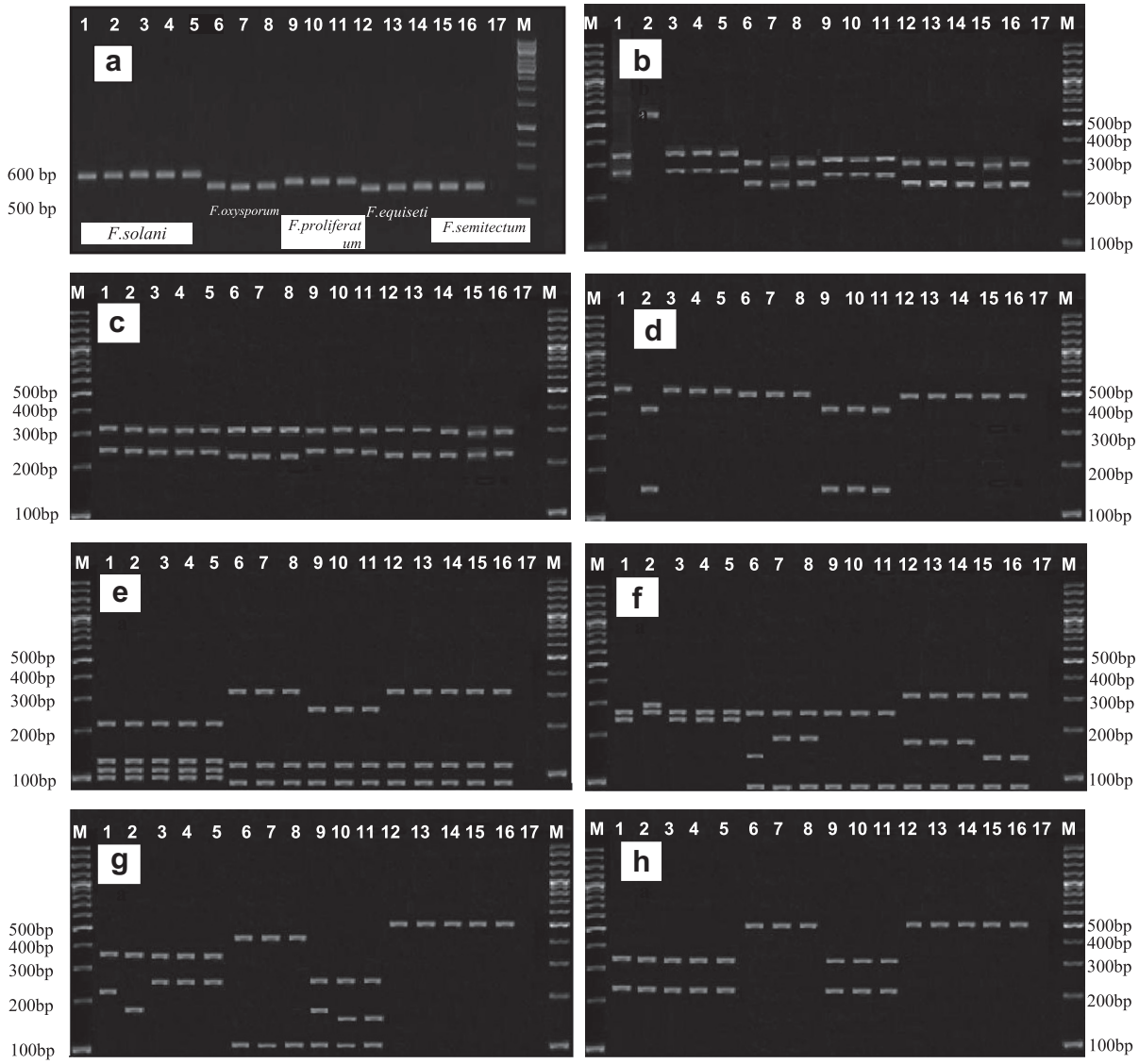


**Figure 3** Agarose gels showing: a = amplification of the ITS region (ITS1, ITS2 and 5.8S) and restriction patterns of PCR-amplified rDNA digested with *Eco*RI (b), *Sph*I (c), *Pst*I (d), *Hae*III (e), *Hinf*I (f), *Msp*I (g) and *Sma*I (h). M. DNA size marker of 100 bp ladder, (1) USM5708 (*F. solani*), (2) FSQA (*F. solani*), (3) FSMV (*F. solani*), (4) FSRS (*F. solani*), (5) FSPS (*F. solani*), (6) USM4484 (*F. oxysporum*), (7) FOAY (*F. oxysporum*), (8) FORK (*F. oxysporum*), (9) USM11558 (*F. proliferatum*), (10) FPRFI (*F. proliferatum*), (11) FPRFII (*F. proliferatum*), (12) USM14999 (*F. equiseti*), (13) FEQUI (*F. equiseti*), (14) FEQUII (*F. equiseti*), (15) USM11548 (*F. semitectum*), (16) FSEM (*F. semitectum*), (17) negative control.

cucurbits showing root and stem rot symptoms in the field. According to the RFLP results, *F. solani* isolate FSQA was different from other isolates having a *Pst*I, *Hinf*I and *Msp*I restriction site and the absence of the *Eco*RI restriction site. This result correlated with the report that *F. solani* f. sp. *phaseoli* is a distinct species within *F. solani* complex (O'Donnell and Gray, 1995). This result is in agreement with that of O'Donnell and Gray (1995), who reported that isolates *F. solani* f. sp. *phaseoli* from soybeans, could be easily distinguished from the FSSC shown by the presence of a unique *Pst*I restriction site within the ITS2 region.

Brasileiro et al. (2004) have used two restriction enzymes, *Eco*RI and *Hae*III for studying the diversity of the ITS region of the *F. solani*. Also, four restriction enzymes have been used to compare formae speciales (f. spp.) of *F. solani* by Suga et al. (2000). In the present work, the PCR products of *F. oxysporum* isolates were undigested after digestion with *Pst*I and *Sma*I and a band of 550 bp was observed which indicated that there was no restriction site for the restriction enzyme within the ITS + 5.8S of the isolates. *F. proliferatum* isolates from cucurbits stem and root rot as well as from the stock culture showed similar restriction patterns when digested with *Eco*RI, *Sph*I, *Pst*I, *Hae*III, *Hinf*I and *Sma*I. Only *Msp*I produced variable restriction patterns. There was no restriction site for the restriction enzymes *Msp*I, *Pst*I and *Sma*I within the ITS + 5.8S of the *F. semitectum* and *F. equiseti* isolates. Also,

**Table 4** Restriction fragment size (in bp) of *Fusarium* ITS region digested with *Eco*RI, *Sph*I, *Pst*I, *Hae*III, *Hin*fI, *Msp*I and *Sma*I.

| Isolates | Isolates number | ITS total size | *Eco*RI | *Sph*I | *Pst*I | *Hae*III | *Hin*fI | *Msp*I | *Sma*I |
|---|---|---|---|---|---|---|---|---|---|
| *F. solani* | USM5708 | 570 | 280, 320 | 250, 320 | 570 | 100, 110, 130, 230 | 270, 280 | 210, 360 | 230, 350 |
| *F. solani* | FSQA | 570 | 570 | 250, 320 | 150, 420 | 100, 110, 130, 230 | 280, 290 | 180, 360 | 230, 350 |
| *F. solani* | FSMV | 570 | 280, 320 | 250, 320 | 570 | 100, 110, 130, 230 | 270, 280 | 240, 360 | 230, 350 |
| *F. solani* | FSRS | 570 | 280, 320 | 250, 320 | 570 | 100, 110, 130, 230 | 270, 280 | 240, 360 | 230, 350 |
| *F. solani* | FSPS | 570 | 280, 320 | 250, 320 | 570 | 100, 110, 130, 230 | 270, 280 | 240, 360 | 230, 350 |
| *F. oxysporum* | USM4484 | 550 | 250, 300 | 230, 320 | 550 | 90, 120, 340 | 100, 150, 280 | 100, 450 | 550 |
| *F. oxysporum* | FOAY | 550 | 250, 300 | 230, 320 | 550 | 90, 120, 340 | 100, 200, 280 | 100, 450 | 550 |
| *F. oxysporum* | FORK | 550 | 250, 300 | 230, 320 | 550 | 90, 120, 340 | 100, 200, 280 | 100, 450 | 550 |
| *F. proliferatum* | USM11558 | 570 | 280, 310 | 250, 320 | 150, 420 | 90, 120, 280 | 100, 280 | 100, 180, 250 | 230, 350 |
| *F. proliferatum* | FPRFI | 570 | 280, 310 | 250, 320 | 150, 420 | 90, 120, 280 | 100, 280 | 100, 150, 250 | 230, 350 |
| *F. proliferatum* | FPRFII | 570 | 280, 310 | 250, 320 | 150, 420 | 90, 120, 280 | 100, 280 | 100, 150, 250 | 230, 350 |
| *F. equiseti* | USM14999 | 550 | 250, 300 | 230, 320 | 550 | 90, 120, 340 | 100, 200, 320 | 550 | 550 |
| *F. equiseti* | FEQUI | 550 | 250, 300 | 230, 320 | 550 | 90, 120, 340 | 100, 200, 320 | 550 | 550 |
| *F. equiseti* | FEQUII | 550 | 250, 300 | 230, 320 | 550 | 90, 120, 340 | 100, 200, 320 | 550 | 550 |
| *F. semitectum* | USM11548 | 550 | 250, 300 | 230, 320 | 550 | 90, 120, 340 | 100, 150, 320 | 550 | 550 |
| *F. semitectum* | FSEM | 550 | 250, 300 | 230, 320 | 550 | 90, 120, 340 | 100, 150, 320 | 550 | 550 |

*F. semitectum* and *F. equiseti* isolates showed similar restriction patterns when digested by using *Eco*RI, *Sph*I and *Hae*III. Only *Hin*fI restriction patterns produced variable patterns within isolates of these two species. *Hin*fI revealed the highest variation in both fragment sizes and restriction sites within all species. Intraspecies variations could be due to minor changes in nucleotide composition within the ITS + 5.8S, which might lead to different restriction patterns. Similar results were obtained by Konstantinova and Yli-Mattila (2004), in their study using PCR–RFLP of ribosomal intergenic spacer region to analyse *Fusarium* species in section *Sporotrichiella*. Further studies, such as rDNA RFLP analysis with more restriction enzymes and different genes (e.g., IGS region) have demonstrated convincingly the morphological and molecular classifications of several *Fusarium* species including *F. oxysporum*, *F. semitectum* and *F. proliferatum* isolates (Paavanen-Huhtala et al., 1999; Hawa et al., 2010; Lee et al., 2000).

In this study, biological characterization, including culture morphology, pathogenicity, and molecular study were used to compare isolates of *F. solani* associated with cucurbit plants. These isolates caused typical disease symptoms on stems of the tested cucurbit seedlings. Molecular approach by PCR-ITS-RFLP analyses strongly supported the existence of two distinct clades among *F. solani* isolates in the present study. Based on morphological characters, the isolates USM5708, FSRS, FSMV, and FSPS are classifies as morphotype I while FSQA as morphotype II and this observation is in agreement with previous molecular studies, whereas based on morphological characters, there is no difference between isolates USM5708, FSRS, FSMV, FSPS. The results of the present taxonomical studies using molecular method and grouping among *Fusarium* strains derived from restriction analysis were in agreement with previous molecular and morphological classification criteria. Therefore, the PCR-ITS-RFLP method described in this paper provides a simple and rapid procedure for the differentiation of *Fusarium* strains at the species level. Further studies using ITS + 5.8S sequence analysis and other sequence analyses, such as IGS sequences, would be necessary to compare the genetic variations observed in *Fusarium* isolates from root and stem rot of cucurbit plants.

## 5. Conclusion

Five *Fusarium* species were isolated from root and stem rot of naturally diseased cucurbit plants grown in Kermanshah province, Iran. Based on the morphological characteristics, the 100 isolates were identified as *F. oxysporum*, *F. proliferatum, F. equiseti, F. semitectum* and two morphotypes of *Fusarium solani*. From the pathogenicity test, *F. oxysporum* and other *Fusarium* species were the major causal agents of root and stem rot of cucurbit plants in the province. PCR–RFLP of ITS + 5.8S analysis used in this study, offers

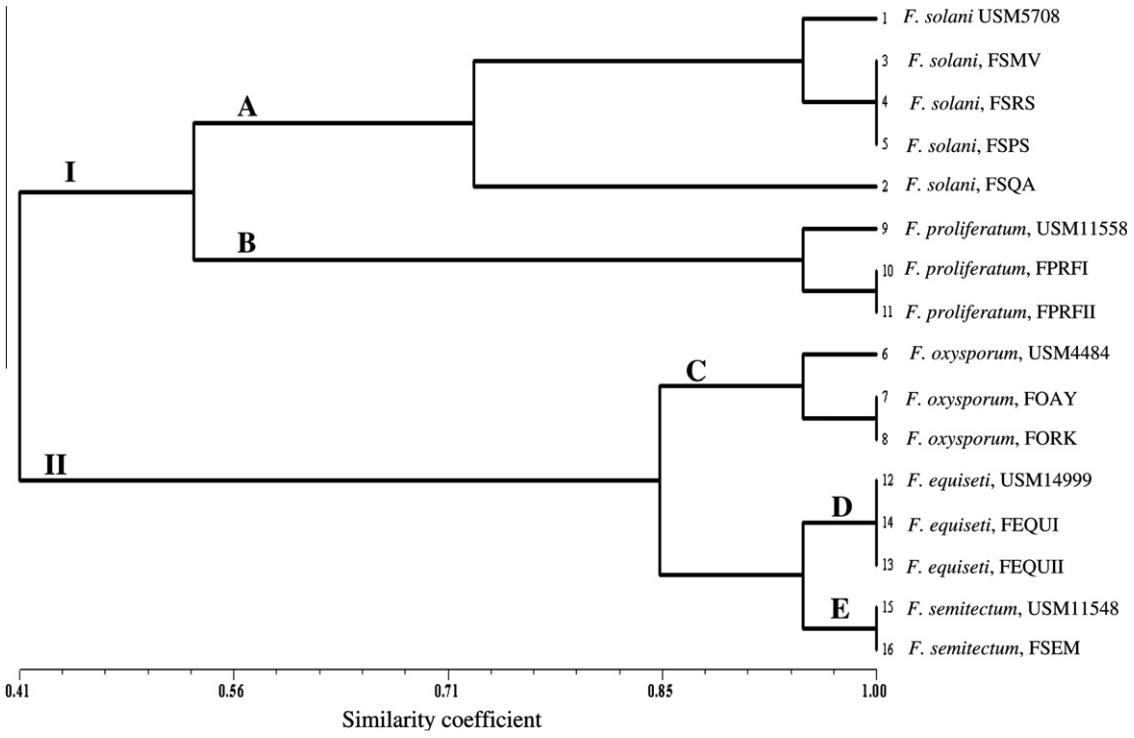


**Figure 4** UPGMA dendrogram showing relationships among the 12 isolates of *Fusarium* based on restriction site data.

a convenient tool for characterization and analyzing variations of *Fusarium* species associated with root and stem rot of cucurbit plants.


## Acknowledgements

Khosrow Chehri acknowledges the Razi University, Iran and Universiti Sains Malaysia (USM), Penang, Malaysia for providing necessary facilities. We are grateful for the Research Grant 300/PBIOLOGI/811009 provided by USM.


## References


Alymanesh, M.R., Falahatirastegar, M., Jafarpour, B., Mahdikhanimoghadam, E., 2009. Genetic diversity in the fungus *Fusarium solani* f. sp. cucurbitae race 1, the casual agent of root and crown rot of cucurbits in Iran, using molecular markers. Pak. J. Biol. Sci. 12, 836–843.

Boughalleb, N., Armengol, J., El Mahjoub, M., 2005. Detection of races 1 and 2 of *Fusarium solani* f. sp. cucurbitae and their distribution in watermelon fields in Tunisia. J. Phytopathol. 153, 162–168.

Brasileiro, T.R.V.B., Coimbra, M.R.M., Antonio de Morais Jr., M., Tinti de Oliveria, N., 2004. Genetic variability within *Fusarium solani* species as revealed by PCR-fingerprinting based on PCR markers. Braz. J. Microbiol. 35, 205–210.

Burgess, L.W., Summerell, B.A., Bullock, P., Backhouse, D., 1994. Laboratory Manual for Fusarium Research, third ed., Department of crop science, University of Sydney, Sydney, Australia, p. 133.

Depriest, P.T., Been, M.D., 1992. Numerous group I introns with variable distributions in the ribosomal DNA of a lichen fungus. J. Mol. Biol. 228, 315–321.

Fisher, N.L., Burgess, L.W., Toussoun, T.A., Nelson, P.E., 1982. Carnation leaves as a substrate and for preserving *Fusarium* species. Phytopathology 72, 151–153.

Fisher, N.L., Marasas, W.F.O., Toussoun, T.A., 1983. Taxonomic importance of microconidial chains in *Fusarium* section *Liseola* and effects of water potential on their formation. Mycologia 75, 693–698.

Hawa, M.M., Salleh, B., Latiffah, Z., 2010. Characterization and intraspecific variation of *Fusarium semitectum* (Berkeley and Ravenel) associated with red-fleshed dragon fruit (*Hylocereus polyrhizus* [Weber] Britton and Rose) in Malaysia. Afr. J. Biotechnol. 9, 273–284.

Hawthorne, B.T., Rees-George, J., Broadhurst, P.G., 1992. Mating behavior and pathogenicity of New Zealand isolates of *Nectria haematococca* (*Fusarium solani*). NZ J. Crop Hortic. Sci. 20, 51–57.

Hawthorne, B.T., Ball, R.D., Rees-George, J., 1994. Genetic analysis of variation of pathogenicity in *Nectria haematococca* (*Fusarium solani*) on Cucurbita sp. Mycol. Res. 98, 1183–1191.

Kim, D.H., Martyn, R.D., Magill, C.W., 1992. Restriction fragment length polymorphism groups and physical map of mitochondrial DNA from *Fusarium oxysporum* f. sp. niveum. Phytopathology 82, 346–353.

Konstantinova, P., Yli-Mattila, T., 2004. IGS-RFLP analysis and development of molecular markers for identification of identification of *Fusarium poae*, *Fusarium langsethiae*, *Fusarium sporotrichioides* and *Fusarium kyushuense*. Int. J. Food Microbiol. 95, 321–331.

Lee, Y.M., Choi, L.Y., Min, B.R., 2000. PCR–RFLP and sequence analysis of rDNA its region in the *Fusarium* spp. J. Microbiol. 38, 66–73.

Leslie, J.F., Summerell, B.A., 2006. The *Fusarium* Laboratory Manual. Blackwell Publish Ltd., UK, p. 388.

Leung, H., Nelson, R.J., Leach, J.E., 1993. Population structure of plant pathogenic fungi and bacteria. In: Andrews, J.H., Tommerup, I.C. (Eds.), Advances in Plant Pathology. Academic Press, London, pp. 157–204.

Malvick, D.K., Percich, J.A., 1998. Genotypic and pathogenic diversity among pea-infecting isolates of *Aphanomyces euteiches* from the central and western United States. Phytopathology 88, 915–921.

Mehl, H.L., Epstein, L., 2007. Identification of *Fusarium solani* f. sp. cucurbitae race 1 and race 2 with PCR and production of disease-free pumpkin seeds. Plant Dis. 91, 1288–1292.

Namiki, F., Shiomi, T., Kayamura, T., Tsuge, T., 1994. Characterization of the formae speciales of *Fusarium oxysporum* causing wilts of cucurbits by DNA fingerprinting with nuclear repetitive DNA sequences. Appl. Environ. Microbiol. 60, 2684–2691.

Nash, S.M., Snyder, W.C., 1962. Quantitative and estimations by plat counts of propagules of the bean rot *Fusarium* in field soils. Phytopathology 73, 458–462.

Nirenberg, H.L., 1976. Untersuchungen uber die morphologische und biologische differenzierung in der Fusarium section Liseola. Mitteilungen aus der biologischen bundesanstalt fur land-und forstwirtschaft (Berlin-Dahlem) 169, 1–117.

O'Donnell, K., 2000. Molecular phylogeny of the *Nectria haematococca–Fusarium solani* species complex. Mycologia 92, 919–938.

O'Donnell, K., Gray, L.E., 1995. Phylogenetic relationships of the soybean sudden death syndrome pathogen *Fusarium solani* f. sp. *phaseoli* inferred from rDNA sequence data and PCR primers for its identification. Mol. Plant Microbe Interact. 8, 709–716.

O'Donnell, K., Sutton, D.A., Fothergill, A., McCarthy, D., Rinaldi, M.G., Brandt, M.E., Zhang, N., Geiser, D.M., 2008. Molecular phylogenetic diversity, multilocus haplotype nomenclature, and in vitro antifungal resistance within the *Fusarium solani* species complex. J. Clin. Microbiol. 46, 2477–2490.

Paavanen-Huhtala, S., Hyvönen, J., Bulat, S.A., Yli-Mattila, T., 1999. RAPD–PCR, isozyme, rDNA RFLP and rDNA sequence analyses in identification of Finnish *Fusarium oxysporum* isolates. Mycol. Res. 103, 625–634.

Palmer, L.T., Kommedahl, T., 1960. Root-infecting *Fusarium* species in relation to rootworm infestations in corn. Phytopathology 59, 1613–1617.

Pivonia, S., Cohen, R., Kafkafi, U., Ben Ze'ev, I.S., Katan, J., 1997. Sudden wilt of melons in southern Israel: fungal agents and relationship with plant development. Plant Dis. 81, 1264–1268.

Rohlf, F.J., 2000. NTSYS-pc Numerical Taxonomy and Multivariate Analysis System, version 2.1. Exeter Publishing Ltd., Setauket, New York, USA.

Romesburg, H.C., 1994. Cluster Analysis for Researchers. Lifetime Learning Publications Belmont, California.

Salleh, B., Sulaiman, B., 1984. *Fusarium* associated with naturally diseases plants in Penang. J. Plant Prot. Tropics 1, 47–53.

Suga, H., Hasegawa, T., Mitsui, H., Kageyama, K., Hyakumachi, M., 2000. Phylogenetic analysis of the phytopathogenic fungus *Fusarium solani* based on the rDNA-ITS region. Mycol. Res. 104, 1175–1183.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, pp. 315–322.

Zhang, N., O'Donnell, K., Sutton, D.A., Nalim, F.A., Summerbell, R.C., Padhye, A.A., Geiser, D.M., 2006. Members of the *Fusarium solani* species complex that cause infections in both humans and plants are common in the environment. J. Clin. Microbiol. 44, 2186–2190.