ความสามารถก่อโรคและการตรวจหาโมเลกุลของเชื้อ *Fusarium solani* ฟอร์ม A และ B จากโรครากเน่าของหม่อน

Pathogenicity and Molecular Detection of *Fusarium solani* Form A and B on Root Rot of Mulberry

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บทคัดย่อ

เชื้อ *Fusarium solani* (Mart.) Appel & Wollenw. ที่แยกได้จากโรครากเน่าหม่อนมี 2 ฟอร์ม ให้ชื่อว่า ฟอร์ม A และฟอร์ม B เชื้อฟอร์ม A สร้างโคโลนีสีชมพูและเชื้อฟอร์ม B สร้างโคโลนีสีขาวบนอาหารเลี้ยงเชื้อพีดีเอ เมื่อนำเชื้อฟอร์ม A และฟอร์ม B มาทดสอบความสามารถก่อโรคกับรากและบนกิ่งพันธุ์ของหม่อนพบว่าก่อโรคได้ในระดับความรุนแรง 33.8-85 % และ 30-100% ตามลำดับ หลังจากปลูกเชื้อแล้วสามารถแยกเชื้อกลับมาจากรากฝอย รากแขนงและโคนต้นได้ 76-100, 60-90 และ 40-62 % ตามลำดับ ไพรเมอร์ Fsol-F1 และ Fsol-R1 มีความจำเพาะในระดับสปีชีส์ สามารถใช้ตรวจหาดีเอ็นเอที่ขนาด 300 คู่เบส ของเชื้อทั้งฟอร์ม A และฟอร์ม B จากเส้นใยและจากรากหม่อนที่ติดเชื้อแล้ว

Abstract

Two morphological forms of *Fusarium solani* (Mart.) Appel & Wollenw., designated as form A (FS-A) and form B (FS-B) were isolated from mulberry roots with symptoms of root rot disease. All of FS-A and FS-B forms were pink and white colonies, respectively. The pathogenicity test of both forms on mulberry roots by detached root technique and on mulberry cutting showed infection capability of root rot at 33.8 - 85% disease severity and 30-100% disease incidence, respectively. All isolates can be recovered from fibrous roots, lateral roots and basal of the cutting trees at 76 - 100, 60 - 90, and 40 - 62%, respectively. The primer Fsol-F1 and Fsol-R1 showed good specificity for *F. solani*, at the specific level, the 300 bp PCR product was amplified exclusively from both mycelium and infected mulberry roots.

คำสำคัญ: *Fusarium solani* ฟอร์ม รากเน่าหม่อน ไพรเมอร์จำเพาะ
Keywords: *Fusarium solani*, Forms, Mulberry root rot, Specific primer

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Introduction

Root rot disease is considered to be the most destructive disease of mulberry (*Morus alba* L.) in the Northeast of Thailand and Asian Countries. The disease can be found wherever the plants are grown and crop losses due to the disease can reach 100% within 3 years (Sanoamuang and Saksirirat, 1984). The disease was first reported in 1954, since then its etiology has been extensively studied (Kerewan and Chantrasrikul, 1967; Sittipongse et al., 1969; Aoki, 1971; Keupracone et al., 1983; Sanoamuang et al., 1987; Yamakawa et al., 1991; Kaosiri, 1998). For more than 50 years, plant pathologists have failed to confirm the causal agents of mulberry root rot. Several plant pathogenic fungi, bacteria and nematodes were frequently isolated from diseased tissues, such as *Botryodiplodia theobromae* (Keupracone et al., 1983; Sanoamuang and Saksirirat, 1984; Sanoamuang et al., 1987; Kaosiri 1998), *Fusarium solani* (Sanoamuang and Saksirirat, 1984, 1986; Philip et al., 1995; Kaosiri 1998), *Fusarium* spp. (Kerewan and Chantrasrikul, 1967; Sittipongse et al., 1969; Aoki, 1971; Keupracone et al. 1983; Sanoamuang et al., 1987 and Kaosiri 1998), *Phytophthora* spp. (Boonnab, 1981; Sanoamuang and Saksirirat, 1984), *Pythium* sp., *Macrophomina* sp., *Neocosmospora* sp., *Rhizopus* sp., *Sphaeropsis* sp., *Sclerotium rolfsii*, *Erwinia carotovora, Pseudomonas fluorescens, Aphelenchoides* sp., *Criconemella* sp., *Helicotylenchus* sp., *Hoplolaimus* sp., *Meloidogyne* sp., *Pratylenchus* sp. and *Rotylenchulus* sp. (Sutthisa, 2010). Charoenstaporn et al. (1999) concluded that the disease was caused by complex agents. The more microorganisms got involved with the complex root rot disease of mulberry, the more confusion to study on its' etiology and control of the disease will be obtained. Sanoamuang et al. (1987) reexamined the associated organisms again and reported that *F. solani* was isolated consistently, this fungus always found associated with the mulberry roots in both healthy and infected plants collected from the Sericulture Experiment Stations in the northeast region of Thailand. In addition, Philip et al. (1995), Herald (2005), and Mostafa and Kamran (2006) reported, in India, that severe root rot of mulberry trees were associated with *F. oxysporum* and *F. solani*. Obviously from previous reports (Sanoamuang et al. 1987), *Fusarium* spp. especially *F. solani* seemed to be the most frequently involved with the disease. In 2010, Sutthisa et al. intensively studied on the *Fusarium* spp. isolated from both healthy and diseased mulberry roots and proved that *F. solani* is the causal agent of mulberry root rot.

Symptom of mulberry root rot disease is similar to soybean sudden death syndrome (SDS) which the causal agent of SDS is the fungus *F. solani* (Mart.) Appel. & Wollenw. emend. Snyd. & Hans. (Roy et al. 1989; Rupe, 1989), but the disease is often associated with soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe. The presence of the nematode has been reported to hasten the appearance and increase the severity of SDS (Roy et al., 1989; Rupe et al. 1989), but SDS also occurs in fields not infested with SCN (Rupe et al., 1989). Later, this same

fungus then called strain B and apparently different strain of *F. solani*, then called strain A, was found associated with roots of soybean plants symptomatic for SDS. Roy (1997a) reported that the two fungi are different and distinguishable on the basis of host specialization and the types of symptoms they incite on soybeans. *F. solani* form A, the causal agent of SDS, was designated *F. solani* (Mart.) Sacc. f.sp. glycines. Cultural and morphological characteristics of *F. solani* form B, the cause of seedling disease and root rot of soybean. PCR-based assays have been applied to microbial ecology and environmental sciences to detect and monitor microorganisms in rhizosphere/ rhizoplane and soil (Steffan and Atlas, 1991), and to plant disease diagnosis.

Abd-Elsalam et al. (2003) developed two taxon-selective primers for quick identification of *Fusarium* genus. These primers ITS-Fu-f and ITS-Fu-r were designed by comparing the aligned sequences of internal transcribed spacer regions (ITS) of a range of *Fusarium species*. The primers showed good specificity for the genus *Fusarium*, and the approximately 389 bp product was amplified exclusively. Li and Hartman (2003) developed simple and effective methods to extract *F. solani* f.sp. *glycines* DNA directly from field-grown roots and soils. Detection of *F. solani* f.sp. *glycines* in field soil samples using a single round PCR was not consistent, probably because of the low concentration of the target DNA. By contrast, they used nested PCR assay using primers Fsg1/Fsg2 and FsgEF1/FsgEF2, the size of the PCR product amplified by Fsg1/Fsg2 (438 bp) was greater than the product amplified by FsgEF1/FsgEF2 (237 bp), which allowed it to be detected more easily. Our previous study (Sutthisa et al. 2009) designed a pair of primers from the internal transcribed spacer (ITS) region, Fsol-F1 and Fsol-R1 the primer showed good specificity for *F. solani* and produced PCR product of 300 bp. The purpose of this study was to prove and compare pathogenicity of *F. solani* form A and B for root rot of mulberry and to use the methodology developed for DNA extraction and PCR assay on the specific detection of *F. solani.*

Materials & Methods

Study site

This study was carried out at the Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kaen University as part of a PhD thesis funded by AG-Bio/Perdo-CHE. Isolates of *Fusarium solani* were from mulberry plantations in Mahasarakam and Roi-et provinces, the areas where the root rot disease of mulberry occurred dramatically, in the year 2008.

Cultural and morphological characteristics of forms of *F. solani*

Eleven isolates of *F. solani* obtained from mulberry roots were grown on Potato dextrose agar (PDA) in 9 cm diameter Petri dishes and after 7 days spore suspension was prepared. Each spore suspension, 100 µl aliquot was spread over the surface of water agar (WA) in 9 cm diameter Petri dishes, after 24 hours single spore isolation of each isolates was performed. There were 10 plates per isolate. Typical colony characteristics of the FS-A and FS-B were shown in Figure 1.

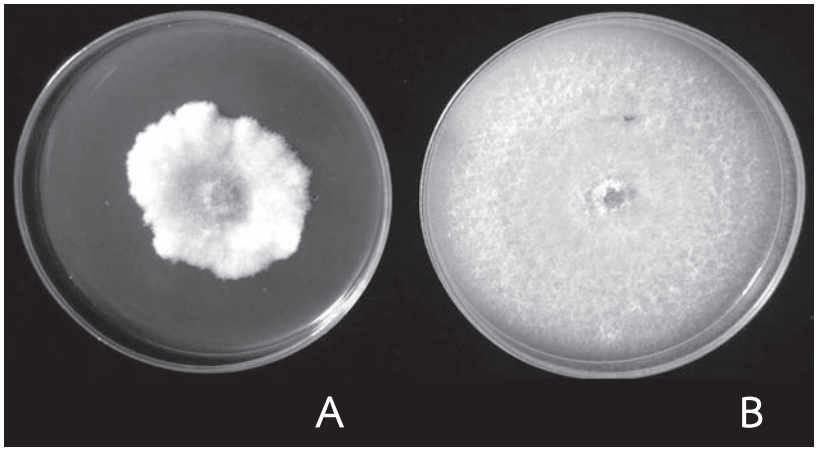

Figure1. Colonies of *Fusarium solani*, A. form A (FS-A) and B. form B (FS-B) growing on PDA plates at 28°C for 7 days

Pathogenicity tests

by detached roots technique

To obtain inoculums, FS-A and FS-B were grown on PDA and incubated at 28°C for 7 days. Plugs of mycelium at the edge of colonies were cut with a 0.8 cm diameter cork borer.

Mulberry roots were washed in running tab water, and cut into pieces approximately 5.2 cm in length. They were surface disinfested in 0.6% sodium hypochlorite for 5 minutes, rinsed twice in sterile distilled water, and blotted dry on sterile filter paper. Agar disks of the fungus or without fungus as control treatment were placed onto the root tissue on the top and placed in plastic moist chamber. Treatments (isolates) were arranged in a randomized complete block design with five replications. Disease severity was measured daily. Percentage of disease severity was rated as root rot areas compared with normal areas. Data from this experiment were subjected to analysis of variance (ANOVA). Treatment means were compared by Duncan's test of MSTAT.

On mulberry cuttings

Pathogenicity tests were conducted using Koch's Postulates to confirm the FS-A and FS-B as the causal agent of root rot of mulberry. The plants used were 4 months old grown from cuttings put in a nursery block filled with moist rice hull ash and showed no disease symptoms.

To obtain inoculums, FS-A and FS-B were grown on pieces of mulberry twigs that were cut into 0.5 mm and autoclaved twice. The plugs of FS-A and FS-B were added into the pieces and incubated at 28°C for 14 days.

Mulberry cutting Noi cultivar were washed and surface sterilized before grown in 10 cm diameter plastic glasses using sterile peat moss and FS-A and FS-B inoculums were placed on the bottom before planting. The experiment was conducted in a randomized completely block design (RCBD) with ten replications for each FS-A, FS-B and control. Data collections were conducted 30, 60 and 75 days after inoculation, by

counting disease incidence. Disease incidence of plants was based on the percentage of plant with foliar symptoms typical of root rot. Symptoms of the disease appear as sudden leaf withering starting from the bottom of the branch upwards, followed by defoliation (Figure 2 & 3). Data were subjected to analysis of variance (ANOVA), comparing mean values statistically using Duncan's test of MSTAT.

Fungal DNA extraction

Pure cultures of the isolates were maintained on PDA plates and incubated at 28°C for 7 days and one plug (8 mm in diameter) of fungal mycelium was aseptically transferred to 50 ml aliquots of liquid potato dextrose broth in 250 ml Erlenmeyer flasks. Cultures were grown at 28°C with shaking 120 rpm for 1 week. The mycelium was filtered from liquid medium through filter paper (Whatman No. 1). Total DNA was extracted according to the protocol of Zang et al. (2005).

DNA extraction from mulberry roots

Roots from inoculated pots were collected. The roots were cut into pieces approximately 1 to 3 mm in length. They were surface disinfested in 5% sodium hypochlorite for 5 min, rinsed twice in sterile distilled water, and blotted dry on sterile filters. Total DNA was extracted according to the protocol of Doyle and Doyle (1987).

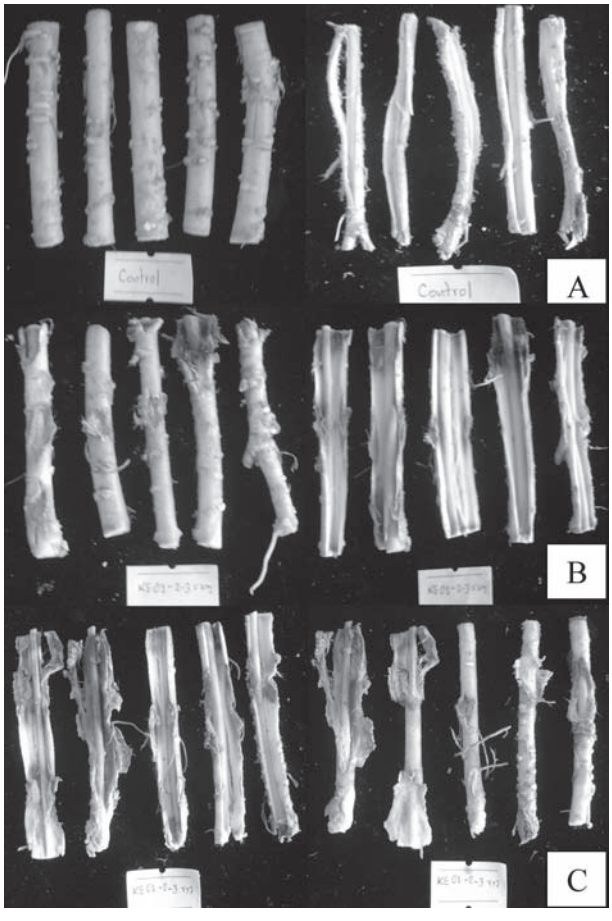

Figure2. Disease severity of mulberry root inoculation with plugs of mycelium (3 weeks after inoculation) A: Control, B: FS-A, C: FS-B

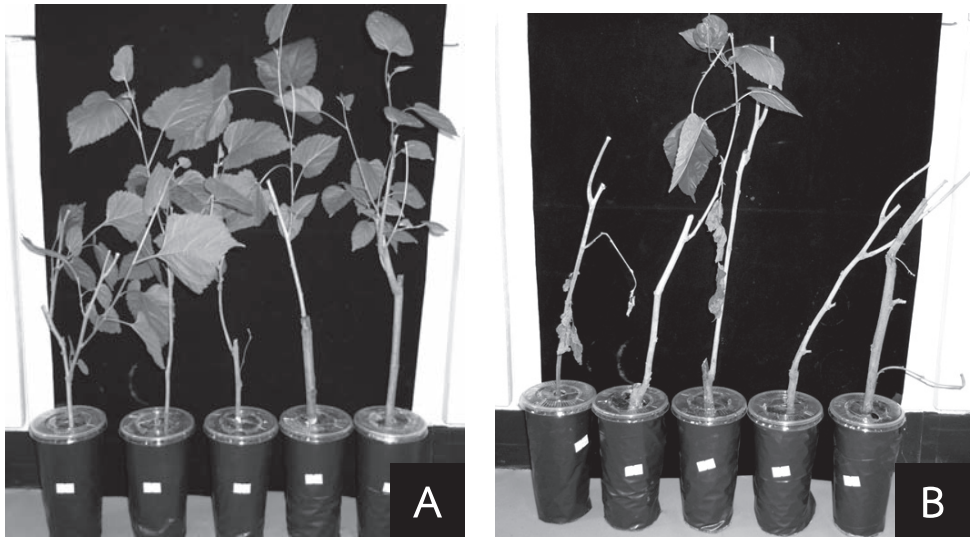

Figure3. Mulberry root rot symptoms, 75 days after inoculation; A: Control, B: Inoculated with *F. solani*

Specificity of PCR amplification

Specificity of the PCR amplification for FS-A and FS-B using primers Fol-F1 (5'GAGGACCCCCTAACTCT 3') and Fsol-R1 (5' GGGACCGCCACTGTATT 3') was tested. Amplification was performed in 25 µl of reaction mixture containing 100 ng genomic DNA (extraction from mycelium and mulberry roots), 2.0 µM each primers, 0.25 unit *Taq* polymerase, 2.5 mM $MgCl_2$, 0.2 mM each dNTP (dATP, dCTP, dGTP and dTTP), 10 µl of 5X PCR buffer, sterile distilled water to a final volume of 25 µl. Amplifications were performed in a Gradient DNA Thermal Cycler programmed for the following parameter: 95°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min, and a final incubation at 72°C for 5 min. Amplification products (5 µl of a 50 µl reaction) were electrophoresed in 2% agarose gels with 1X TBE running buffer, stained with ethidium bromide and either scanned into a computer imaging file or photographed (Sambrook et al., 1989).

Results

Cultural and morphological characteristics and forms of *F. solani*

After 3-5 days in Petri dishes, two forms of colonies were distinguished by color, pink and white colonies (Figure. 1). There were designated forms *F. solani* form A (FS-A) and *F. solani* form B (FS-B) by color, respectively. Colonies of FS-A were pink and FS-B were white, and growth of FS-A isolates was less than FS-B.

Pathogenicity test by detached root technique

Inoculation of mulberry roots with FS-A and FS-B resulted in development of root rot symptoms that could be measured 3 weeks after inoculation. Conversely, non-inoculated control roots remained healthy. In this study, inoculation of mulberry roots with FS-A showed no significant differences in disease severity in comparison to the roots inoculated with FS-B (Table 1, Figure. 2).

Table 1. Pathogenicity of *Fusarium solani* form A (FS-A) and B (FS-B) on mulberry roots by detached root technique in moist plastic boxes

Isolates	Disease severity (%)[1]
KE 01-2-3 (FS-A)	45.4 ^{bcde}
KE 01-2-3 (FS-B)	70.8 ^{abc}
KE 02-1-1 (FS-A)	68.5 ^{abcd}
KE 02-1-1 (FS-B)	85.0 ^{a}
KE 02-1-2 (FS-B)	37.7 ^{de}
KE 03-2-1 (FS-B)	46.9 ^{bcde}
KE 05-1-3 (FS-A)	46.5 ^{bcde}
KE 05-1-3 (FS-B)	59.2 ^{abcde}
KE 05-1-6 (FS-A)	34.6 ^{e}
KE 05-1-6 (FS-B)	45.8 ^{bcde}
Roi-et 03-1-2 (FS-A)	45.4 ^{bcde}
Roi-et 03-1-2 (FS-B)	33.8 ^{e}
Roi-et 03-1-3 (FS-B)	41.5 ^{bcde}
Roi-et 03-1-5 (FS-A)	35.4 ^{de}
Roi-et 03-1-5 (FS-B)	60.0 ^{abcde}
Roi-et 04-1-1 (FS-B)	74.6 ^{ab}
SIC 01-1-1 (FS-A)	36.5 ^{de}
SIC 01-1-1 (FS-B)	54.8 ^{abcde}
Control	0^{f}

[1] Percentage of disease severity determined 3 weeks after inoculation. Means (n=5) followed by the same letter are not significantly different (P = 0.05) based on a protected test of least significant difference.

On mulberry cutting

After inoculation, thirty days, sixty days and seventy-five days disease incidence were observed. The results showed that FS-B isolates Roi-et03-1-5 was the highest pathogenic, causing root rot at 100% disease incidence. There were no significant differences of disease incidence among *F. solani*, FS-A and FS-B. All isolates studies except FS-A isolates Roi-et03-1-5 were pathogenic on mulberry cutting, with disease incidence 50 – 100% (Table 2, Figure. 3).

Table 2. Pathogenicity test of *Fusarium solani* form A (FS-A) and B (FS-B) on mulberry cuttings

Isolates	Disease incidence (%)[1/]			Recovery (%)[1/]		
	After inoculation (day)			Fibrous root	Lateral root	Basal of tree
	30 days	60 days	75 days			
KE01-2-3 (FS-A)	40 ^{ab}	60 ^{ab}	90 ^{ab}	94 ^{ab}	84 ^{abc}	62 ^{a}
KE01-2-3 (FS-B)	20 ^{b}	30 ^{bc}	50 ^{bc}	86 ^{abc}	62 ^{c}	48 ^{ab}
KE02-1-2 (FS-A)	20 ^{b}	50 ^{ab}	50 ^{bc}	94 ^{ab}	78 ^{abc}	56 ^{a}
KE05-1-2 (FS-B)	40 ^{ab}	60 ^{ab}	80 ^{ab}	84 ^{bc}	72 ^{abc}	56 ^{a}
KE05-1-3 (FS-A)	40 ^{ab}	40 ^{abc}	50 ^{bc}	84 ^{bc}	76 ^{abc}	52 ^{ab}
KE05-1-3 (FS-B)	0 ^{b}	0 ^{c}	60 ^{bc}	78 ^{c}	66 ^{bc}	50 ^{ab}
Roi-et03-1-5 (FS-A)	80 ^{a}	80 ^{a}	100 ^{a}	76 ^{d}	60 ^{c}	40 ^{b}
Roi-et03-1-5 (FS-B)	20 ^{b}	20 ^{bc}	30 ^{cd}	100 ^{a}	90 ^{a}	62 ^{a}
F. solani (wild-type)	40 ^{ab}	60 ^{ab}	80 ^{ab}	88 ^{abc}	76 ^{abc}	54 ^{ab}
Control	0 ^{b}	0 ^{c}	0 ^{d}	0 ^{d}	0 ^{d}	0 ^{c}

[1] Means (n=10) followed by the same letter are not significantly different (P = 0.05) based on a protected test of least significant difference.

Specificity of PCR amplification

All isolates studies gave strong amplification of single PCR product about 300 bp using primer Fsol-F1 and Fsol-R1 (Figure. 4). Detection of *F. solani*, FS-A and FS-B in mulberry roots, a 300 bp amplification product was obtained using primer Fsol-F1 and Fsol-R1 (Figure. 5).

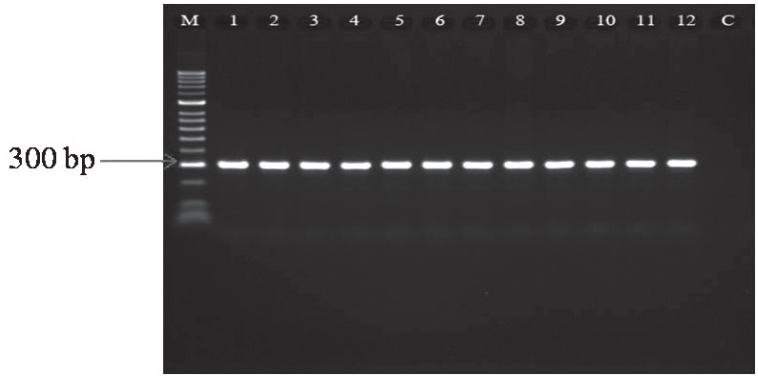

Figure4. Specificity of the PCR assay (Fsol-F1 and Fsol-R1 primer pair) with genomic DNA from *F. solani*, FS-A and FS-B (extracted from mycelium). M: molecular weight markers (Hyper ladder II); lanes 1-4: DNA amplified from *F. solani*; lanes 5, 7, 9 and 11: FS-A; lanes 6, 8, 10 and 12: FS-B; C: negative control (H_2O)

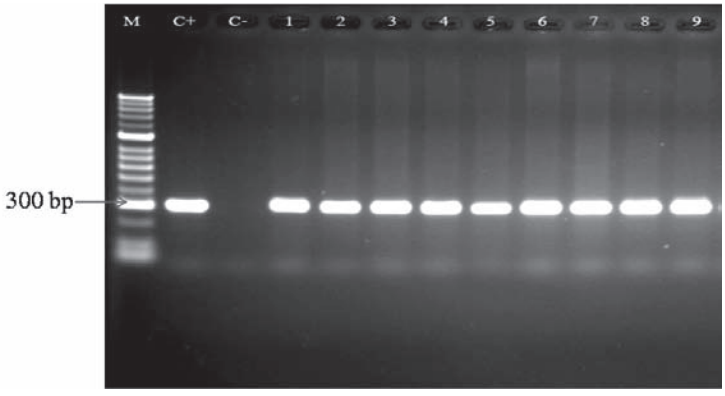

Figure 5. Specificity of the PCR assay (Fsol-F1 and Fsol-R1 primer pair) with genomic DNA from *F. solani*, FS-A and FS-B (extracted from infected root). M: molecular weight markers (Hyper ladder II); C+: positive control (*F. solani*); C-: negative control (H2O); lane 1: DNA amplified from KEO5-1-3 (FS-A); lane 2: KEO5-1-3 (FS-B); lane 3: Roi-et03-1-5 (FS-A); lane 4: Roi-et03-1-5 (FS-B); lane 5: KE01-2-3 (FS-A); lane 6: KE01-2-3 (FS-B); lane 7: KE05-1-2 (FS-A); lane 8: KE05-1-2 (FS-B); lane 9: *F. solani*

Discussion

Pathogenicity of *F. solani*, FS-A and FS-B on root rot of mulberry was not significantly different. Consistently, Sakurai and Matuo (1959) reported that *Hypomyces solani* Reinke et Berthold [*Fusarium solani* (Mart.) Snyd. et Hans.] was another causal fungus of *Fusarium* blight of mulberry trees and the strains of this fungus isolated from mulberry stems were divided into two groups (α and β). The α group of the fungus was pathogenic on mulberry stems. The β group of the fungus was pathogenic not only on mulberry stems (less virulent than α group) but also to pea seedlings and sweet potato sprouts and most vigorously to potato tubers. Roy (1997a) reported that two morphological distinct forms of *F. solani* pathogenic on soybean, forms A and B. *F. solani* form A caused the symptoms characteristic of sudden death syndrome but *F. solani* form B caused seedling disease and root rot of soybean (Roy et al., 1989). Recently, FSA was designated *F. solani* (Mart.) Sacc. f.sp. *glycines* form nov. on the basic of physiological specialization, its distinction from *F. solani* form B and other strains and formae speciales of *F. solani*, including *F. solani* f.sp. *phaseoli* (Roy, 1997b).

PCR based assays have been applied to microbial ecology and environmental science to detect and monitor microorganisms in rhizosphere, rhizoplane and soils (Steffan and Atlas, 1991) and to plant disease diagnosis (Henson and French, 1993). Field-grown plant roots and field soils are microbially complex, making it difficult to develop convenient and reliable procedures for extracting PCR amplifiable DNA (Lovic et al., 1995). Li and Hartman (2003) reported that detection of *F. solani* f.sp. *glycines* in field soil

samples using a single round of PCR was not consistent, probably because of the low concentration of the target DNA, but in our study we can detected *F. solani*, *F. solan*i form A and B from inoculated mulberry roots using primer Fsol-F1 and Fsol-R1, we gave strong amplification of a single PCR product about 300 bp. Li and Hartman (2003) used the methodology developed for DNA extraction and PCR assay on the specific detection of *F. solani* f.sp. *glycines* in plants and soil samples. Detection of *F. solani* f.sp. *glycines* using the described PCR assay was positive for all SDS diseased plant expressing symptoms, infected but symptomless young seedlings and infested field soil. Abd-Elsalam et al. (2006) reported that a PCR assay based on a pair of oligonucleotide primers targeting the 16S and 2S rRNA genes was used to detect *F.oxysporum* f.sp. *vasinfectum* (Fov), a fungus causing Fusarium wilt of cotton seedlings. By using the primer pair Fov1-Eg-f and Fov1-Eg-r, a 438 bp DNA fragments were consistently amplified Egyptian isolates of Fov (race 3), while no amplification was obtained from template genomic DNAs obtained from other fungal pathogens. Fov DNA was detected in infected cotton roots, while no amplification was obtained from other plant structures, such as stem and leaf.

The PCR assay has potential as a diagnostic tool for detecting *F. solani* in field-grown mulberry much earlier before the appearance of the root rot symptom.

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