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MOLECULAR DETECTION OF *FUSARIUM OXYSPORUM* IN THE INFECTED CUCUMBER PLANTS AND SOIL

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Abstract

In this study, a one-step PCR protocol was developed for rapid and accurate detection of the pathogen *Fusarium oxysporum* in infected cucumber plants and soil. The primers Fc-1 and Fc-2 were designed according to *F. oxysporum* internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA). A specific 315-bp PCR product was amplified from all the tested *F. oxysporum* isolates , infected cucumber plants and soil under the optimized PCR conditions using primers Fc-1 and Fc-2. While no PCR product was obtained from other fungi, bacteria, healthy cucumber plants and non-infected soil. For the detection sensitivity, the minimal quantity of genomic DNA of purified *F. oxysporum* was 100fg and that of soil pathogens was 1000 spores per gram of soil. Furthermore, the PCR protocol enabled detection of *F. oxysporum* in symptomless cucumber root 6 days after inoculation with the pathogen. Therefore, this PCR-based method can be used to detect *F. oxysporum* rapidly, sensitively with reliability in infected cucumber plants and soil. Our detection protocol also allowed for early monitoring and diagnosis of *F. oxysporum* to facilitate disease management.

Introduction

Fusarium species are worldwide pathogenic fungi of many crop plants. *Fusarium oxysporum* is one of the most important phytopathogens causing *Fusarium* wilt disease in more than a hundred species of plants (Liu *et al.*, 2004). Cucumber *Fusarium* wilt disease is one of the most serious fungal diseases in cucumber production in the world (Jenkins & Wehner 1983; Martinez *et al.*, 2003; Yang *et al*., 2007). In China, an epidemic cucumber *Fusarium* wilt disease often occurred and led to a major yield loss in cucumber production. Generally, it caused cucumber yield losses of ~10% to 30% and poor quality products resulting in severe economic losses (Li *et al.*, 2009).

Cucumber *Fusarium* wilt disease may occur at all growth periods of the cucumber plant (Celetti 2007). The pathogens can survive as durable spores for many years with or without plant debris in soil, and it retains the ability to infect cucumber plants causing pre- or post-emergence damping-off, vascular discoloration of roots and stems, and eventually the entire plant wilts or dies. The disease management of *Fusarium* wilt usually consists of soil fumigation, seed treatment, use of disease resistant varieties and biocontrol bacteria to reduce infection and disease severity (Celetti 2007; Rose & Parker 2003; Zhang *et al.*, 2008). However, these disease management tools do not keep the plant healthy during all the growth stages. Therefore, to effectively prevent cucumber wilt disease, the pathogen in soil and cucumber seedling must be detected prior to transplantation.

In China, cucumber Fusarium wilt was caused by several *Fusarium* species, including *F. oxysporum*, *F. equiseti, F. solani, F. moniliforme* and *F. proliferatum*. Among them, *F. oxysporum* is the major pathogen (Huang 1990; Huang & Yang 1990; Chen *et al.*, 2010). However, the wilt symptom caused by all the pathogens is very similar. Using conventional methods to distinguish them are time consuming due to relying on the biochemical and morphological identification, isolation and culture of the pathogen *in vitro* as well as characterization based on pathogenicity (Yang *et al.*, 2007). In addition, other diagnostic methods, such as microscopy and serological detection methods are labor intensive and require pathogen-specific antibodies (Tsunehiro *et al.*, 1989; Wang *et al.*, 2008). Therefore, a rapid, effective, sensitive and specific diagnostic method is needed.

Compared to conventional detection methods, polymerase chain reaction (PCR) techniques are suitable for routine analysis and have been applied extensively to the detection of fungal pathogens in plants and soil due to its many advantages such as specificity, sensitivity and rapidity, etc. PCR can also be performed and interpreted by personnel without taxonomical expertise. The key step for the development of a PCR method for pathogen detection is to design specific oligonucleotide primers. Internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) has been useful to design specific PCR primers because of the high variability in ITS regions among different species, enabling identification of fungal pathogens at the species level (Epaminondas 2006). A molecular diagnostic method for detecting *F. oxysporum* by PCR-RFLP and nested PCR has been reported (Chen *et al.*, 2008). Both procedures can be used to detect *F. oxysporum* in cucumber tissues without visible disease symptoms. *F. oxysporum* can be detected in inoculated cucumber tissues on the third day after inoculation using PCR-RFLP method, and the pathogen can be detected five days after inoculation using a nested-PCR method. However, a nested PCR detection assay requires 2 sets of primers and a two-step PCR. A one-step PCR assay that uses one set of primers in a single tube for specific detection of *F. oxysporum* in soil and plant tissues has not been reported. A one-step PCR protocol would reduce both time and the risk of contamination during sample manipulation. In this study, a one-step and non-nested PCR method was developed for specific detection *F. oxysporum* in plants and soil in the

early stage. Early diagnoses can be helpful to control cucumber *Fusarium* wilt.

Materials and Methods

Source of isolates: All isolates used in the study are listed in Table 1. Four pathogenic *F. oxysporum* were collected from diseased cucumber plants growing near Harbin city of China in 2007. Other ten pathogenic *F. oxysporum*, 33 other fungal and 3 bacterial isolates were obtained from different places or CGMCC (China General Microbiological Culture Collection Center) (Table 1). Fungi were stored on potato dextrose agar at 4°C. Bacteria were stored on LB agar at 4°C. The isolates were maintained in the Institute of Microbiology, Heilongjiang Academy of Sciences, China.

Table 1. Fungal and bacterial species used to screen for specificity of primers Fc-1 and Fc-2.

Species	Origin	Number of isolates
F. oxysporum	Heilongjiang, China	4
	CGMCC, China	4
	Shandong, China	1
	Hebei, China	3
	Liaoning, China	2
F. avenaceum 3.3628	CGMCC, China	1
F. graminearum 04-3	Russia	1
F. moniliforme 3.2835	CGMCC, China	1
F.proliferatum 3.3635	CGMCC, China	1
F. solani	Heilongjiang, China	2
F. equiseti 04-02	Heilongjiang, China	1
F. graminearum 05-03	Canada	1
F. nivale 04-05	Russia	1
F. nivale 06-02	Heilongjiang, China	1
F. poae 06-03	Heilongjiang, China	1
F. vasinfectum 3.4305	CGMCC, China	1
F. oxysporum f. sp. *soybean*	Heilongjiang, China	1
Fusarium sp. 06-05	Heilongjiang, China	1
Botrytis cinerea HWS-03	Heilongjiang, China	2
Alternaria dauci HWS-14	Heilongjiang, China	1
Colletortichum liademuthianum HWS-15	Heilongjiang, China	1
Sclerotinia sclerotiorum HWS-15	Heilongjiang, China	1
Alternaria solani 06-08	Heilongjiang, China	1
Pythium sp. 06-09	Heilongjiang, China	1
Rhizoctonia solani 03-04	Canada	1
Colletotrichum lagenarium 05-12	Heilongjiang, China	1
Phytophthora melonis 05-09	Heilongjiang, China	1
Alternaria cucumerina 05-07	Heilongjiang, China	1
Rhizoctonia solani 05-04	Heilongjiang, China	1
Cladosporium fulvum 2	Heilongjiang, China	1
Verticillium dahliae 04-06	Russia	1
Pyricularia oryzae HWS-21	Heilongjiang, China	1
Sphacelotheca reiliana HWS-16	Heilongjiang, China	1
Ascochyta citrallina HWS-18	Heilongjiang, China	1
Pythium aphanidermatum HWS-19	Heilongjiang, China	1
Septoria lycopersici HWS-23	Heilongjiang, China	1
Bacillus subtilus B29	Heilongjiang, China	1
E.coli JM109	Heilongjiang, China	1
Bacillus amyloliquefaciens TF28	Heilongjiang, China	1

CGMCC: China General Microbiological Culture Collection Center.

Collection of cucumber plants and soil samples: Five infected and 5 healthy cucumber roots and stems were collected from Chenxi, Jianguo and Xuejia areas, Harbin, in 2009. Five naturally infected soil samples per area were collected from the rhizosphere of infected cucumber. All samples were brought to the laboratory using sterilized polythene bags and stored at -70°C for DNA extraction (Moller *et al*., 1992).

Mycelia and conidia preparation: All fungal species were cultured on potato dextrose broth (PDB) at 28°C for 7 days. After incubation, the mycelial pads were harvested by filtration and stored at -70°C for genomic DNA extraction. Conidia of *F. oxysporum* were prepared from plate cultures on PDA at 28°C for 9 days. Conidia were collected by adding 5ml sterile distilled water into the plate and rubbing the surface mycelium gently with a sample collection swab. The spores were filtered by 4 layers of cheesecloth, and adjusted to the desired concentration using a hemocytometer for DNA extraction and soil inoculation according to the below method.

Bacterial species were cultured in LB at 30°C for 1 day, the cultures were stored at 4°C for genomic DNA extraction.

DNA extraction: Genomic DNA was extracted from pure fungal mycelia and purified spores using a Biospin Fungus Genomic DNA Extraction Kit (Bioer Technology C Ltd. Japan) according to the manufacturer`s manual.

Total DNA was extracted from infected or healthy cucumber tissues (root, stem) according to Moller *et al*., (1992). Approximately 20mg of cucumber roots or stems frozen at -70°C were ground in a sterile mortar and pestle, suspended in 500µl of TES buffer (100 mM Tris, pH8.0, 10 mM EDTA, 2% SDS) with 100µg proteinase K and incubated at 60°C for 30 min to 60 min with occasional gentle shaking. 140µl of 5M NaCl and 65µl of 10% CTAB were added into the tube, and then incubated at 65°C for 10 min. The mixture was incubated for 30 min at 0°C and centrifuged at 4°C for 10 min after addition of 700µl of SEVAG solution (chloroform: isoamylalcohol 24:1). The supernatant was transferred into a fresh tube with 510µl of isopropanol and placed on ice for 30 min to precipitate DNA. The DNA was pelleted by centrifugation at 12,000×g for 10 min. The precipitated DNA was washed with 70% cold ethanol, dissolved in 50µl distilled deionized H_2O. The amount of DNA and purity were determined by measuring the absorbance value at 260 and 280 nm. DNA samples were stored at -20°C (Moller *et al*., 1992).

The DNA was extracted from soil samples directly according to Zhou *et al*., (1996). 0.5 gram of soil sample frozen at -70°C was ground in a sterile mortar and pestle, transferred into 50ml Erlenmeyer flasks. One milliliter of DNA extraction buffer, containing 100 mM Tris HCl (pH 8.0), 100mM EDTA (pH 8.0), 100mM phosphate buffer (pH 8.0), 1.5 M NaCl, 1% CTAB, and 100µl proteinase K (100 $mg \cdot ml^{-1}$), was added to the flask. After shaking at 37°C for 30 min at the speed of 225 rpm, 300µl of 10% SDS was added into the flask. After incubation at 65°C for 2h with occasional gentle mixing, the mixture was transferred into a centrifuge tube and centrifuged at 12,000 g for 10 min to remove soil and debris. The supernatant was transferred to a new centrifuge tube and then extracted twice with equal volume of chloroform-isoamylalcohol (24:1). The mixture was centrifuged at 12,000 g for 15 min. The aqueous phase was collected and precipitated with 0.6 volume of isopropanol. The DNA pellet was washed with 70% cold ethanol twice and dissolved in 50µl distilled deionized H_2O (Zhou *et al*., 1996). Bacteria genomic DNA was extracted using alkaline lysis method according to Sambrook *et al*., (1989).

Primer design and PCR amplification conditions: One pair of specific primers, Fc-1 (5'CATACCACTTGTTGCCTC 3') and Fc-2 (5'ATTAACGCGAGTCCCACC3'), were designed using software Gene Runner 3.05 by alignment ITS sequences of *F. oxysporum* and 20 different *Fusarium* sp. in GenBank. The sequence alignment revealed that Fc-1 and Fc-2 are specific for *F. oxysporum*.

The GenBank accession numbers and species are HM 179530.1(*F. oxysporum* f. sp. *cucumerinum*), GU301778.1(*F. oxysporum* f. sp. *cucumerinum*), DQ 452450.1(*F. oxysporum* f. sp. *cucumerinum*), FJ040179 (*Fusarium proliferatum*), FJ154074 (*Fusarium verticillioides*), FJ154076 (*Fusarium oxysporum* f. sp. *melonis*), FJ156282 (*Fusarium oxysporum* f. sp. *niveum*), AF133843 (*F. lichenicola*), AF310981 (*F. lateritium*), AF414968 (*F. poae*), AF430129 (*F. mangiferae*), AY147368 (*F. equiseti*), HM635739.1 (*F. tricinctum*), FN597588.1 (*F. incarnatum*), FN547457.1 (*F. lateritium*), EF408543 (*F. virguliforme*), EF408532 (*F. tucumaniae*), EF408521 (*F. phaseoli*), EF 408519.1 (*F. cuneirostrum*), EF408516.1 (*F. brasiliense*), AF178397 (*F. ambrosium*), AF129105 (*F. solani*), AF414969 (*F. pulverosum*), AF414971 (*F. kyushuense*), AY043478 (*F. solani*), AY210330 (*F. oxysporum*) and AJ246148 (*F. cerealis*) respectively.

Each PCR was carried out in 25 µl volume that included 1U of EasyTag DNA polymerase, 1 × PCR buffer (20 mM Tris-HCl pH 8.4, 20 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 m M $MgSO_4$), 0.2 mM of dNTPs (Beijing TransGen Biotech Co., Ltd.), 0.4 µM Fc-1 and Fc-2 primer, and 10 ng of gemonic DNA. A negative control contained all reaction compounds except template DNA, and a positive control contained all reaction compounds with 10ng genomic DNA from *F. oxysporum* in each PCR reaction. All PCR amplifications were performed in a GeneAmp PCR System 9700 Thermocycler (PE Applied Biosystems). Denaturation was conducted at 94°C for 5 min followed by 40 cycles of 30 s at 94°C, 1 min at 60°C, and 1 min at 72°C, and a final extension for 10 min at 72°C. All PCR were repeated three times. Five microlitres of the PCR volume were analyzed on GelRed-stained 1% agarose gels in 1 × TAE buffer (40mM Tris acetate, 1mM EDTA, pH 8.0) and were visualized under UV light using UVP BioImaging Systems (UVP Co.)

ITS gene cloning and sequencing: After electrophoresis on agarose gels, 4 ITS fragments of 4 *F. oxysporum* isolates (HWS-11, HWS-12, 10-01 and 10-04) were cut from the gels and recovered using a DNA Extraction Kit

(HaiGene Biotechnology Co., Ltd., Harbin), and cloned into a pMD19-T vector (TaKaRa Biotechnology Co., Ltd., Dalian) according to the manufacturer's manual. Positive recombinants were selected by PCR using primers Fc-1/Fc-2. Eight positive recombinants were sequenced (TaKaRa Biotechnology Co., Ltd., Dalian). The ITS sequence of 8 positive recombinants is identical, which corresponds to ITS1 and ITS2 partial sequence and complete 5.8s ribosomal RNA sequence. The sequence from *F. oxysporum* HWS-12 was deposited in GenBank (accession number GQ287398).

Primer specificity and sensitivity: A primer specificity assay was tested using 10ng of genomic DNA of all tested isolates as templates according to the PCR protocol described as above. The sensitivity assay was performed using purified *F. oxysporum* genomic DNA from mycelia and spores respectively, serially diluted from 10ng to 1fg, as the template for PCR. In addition, total DNA was extracted from inoculated soil with spores from 1×10^5 to 10 spores per gram of soil as the templates for PCR respectively.

Pathogen detection at different stages of disease development: To check the feasibility of PCR amplification for detecting *F. oxysporum* infection prior to visible symptoms, 15-day-old cucumber seedlings were inoculated by dipping the roots into a spore suspension (1×10^7 spores/ml). Control plants were dipped in sterile water. The seedlings were planted in plastic pots (one seedling per pot) and maintained in a greenhouse. Ten Seedlings were harvested at 1, 3, 5, 7, 9, 15, 21, 30 days after inoculation. The roots were washed with water, and the symptoms were examined. All the root samples were stored at -70°C for DNA extraction. Total DNA was extracted from the frozen roots. 10ng of DNA was used as a template for PCR amplification.

Results and Discussions

Specificity of PCR amplification: The specificity of primers was analyzed using DNA template from 14 *F. oxysporum* isolates, in addition to 33 other fungal and 3 bacterial species. All *F. oxysporum* isolates had positive PCR results using the primer pair Fc-1/ Fc-2. A unique DNA fragment of the expected 315-bp size was amplified in all *F. oxysporum* isolates while no PCR products were visualized in 33 other fungal and 3 bacterial species (Fig. 1).

Sensitivity of PCR amplification: Using the method described as above, a strong PCR amplicon of 315-bp size was obtained with the specific primers when using 10ng to 100fg of *F. oxysporum* genomic DNA as the template, but no PCR product was observed at 10fg and 1fg of DNA templates. Therefore, the sensitivity of PCR amplification with the primer pair Fc-1/ Fc-2 was 100fg genomic DNA purified from *F. oxysporum* (Fig. 2a) for 25µl PCR reaction. A PCR product was consistently amplified using the specific primers from 1000 spores per gram of soil (Fig. 2b).

Detection in infected cucumber plants and soil: A single–step PCR assay was performed to detect *F. oxysporum* in naturally infected cucumber tissues and in infected soil from three different areas. The expected specific DNA band of 315-bp size was detected from all diseased plant tissues (stems and roots) and infected soil. No visible PCR product was detected from healthy cucumber tissues (stems and roots) and non-infected soils (Fig. 3a & 3b).

Detection of the pathogen at different stages of disease development: A PCR product was amplified using the specific primers from cucumber root at 7 days after inoculation with *F. oxysporum.* Meanwhile, the same PCR products were also observed with the specific primers on the 9th, 15th, 21st and 30th day after inoculation (Fig. 4). However, Fusarium wilt symptoms were not visible until the 15th day after inoculation when five roots became brown. On the 21st day, two seedlings began to show wilt symptoms. On the 30th day, six seedlings began to show wilt symptoms.

In this study, we developed an accurate and useful molecular assay method using species-specific PCR to detect and identify the soil-borne pathogens *F. oxysporum* in cucumber root, stem and field soil. The developed single-step PCR assay using the specific primers Fc-1/ Fc-2 enabled reliable detection of *F. oxysporum* in cucumber plant and soil samples. A specific PCR product of 315-bp size was consistently amplified from fourteen isolates of *F. oxysporum* from infected cucumber root, stem and soil, while no PCR product was observed for other negative control samples such as fungi, bacteria, healthy cucumber tissues and non-infected soil. The detection sensitivity for genomic DNA of purified *F. oxysporum* and contaminating soil sample were 100fg and 1000 spores g^{-1} dry soil respectively. Furthermore, *F. oxysporum* in symptomless cucumber root 6 days after inoculation with the pathogen suspensions could be detected using the PCR protocol, whereas wilt symptoms were not visible until 15 days. Molecular detection of *F. oxysporum* was reported using RFLP-PCR and a nested PCR method that could detect *F. oxysporum* 3 and 5 days after inoculation, but the assay was not used for naturally infected cucumber tissues and soil (Chen *et al.*, 2008). Although the sensitivity of the PCR assay described here for inoculated cucumber roots is lower than Chen's report, the one-step PCR assay also could detect the *F. oxysporum* in symptomless cucumber root. Moreover, The PCR protocol is faster than the two-step protocol (RFLP-PCR and nest PCR), and reduces the risk of contamination during sample preparation. The one-step PCR method for detecting *F. oxysporum* has potential value for commercial application in the future.

Plant fungal diseases represent a major problem in the agricultural industry worldwide. Thus, the rapid and accurate diagnostic techniques of plant fungal pathogens are very important for disease prevention and management. PCR-based assays have been applied to detect and monitor plant pathogens in the plant roots and in soil (Cao *et al.*, 2007). Microbial diversity living in plant roots and rhizospheric soil makes it labor intensive and time consuming to isolate *F. oxysporum* from plant roots and soil. Agar plating and morphological identification require considerable expertise to

differentiate *Fusarium* species due to the overlap of morphological properties among species. At least one month is required to detect *F. oxysporum* from plant roots and soil by traditional methods, which may delay disease management. In China, cucumber Fusarium wilt was caused by several Fusarium genus, including *F. oxysporum F. equiseti, F. solani, F. moniliforme* and *F. proliferatum*. *F. oxysporum* is the major pathogen (Huang, 1990; Huang & Yang, 1990; Chen *et al.,* 2010). It is time consuming to distinguish them using conventional methods. However, the PCR detection method developed here provided a definitive diagnosis of *F. oxysporum* within a few hours. Moreover, the method is easily operated.

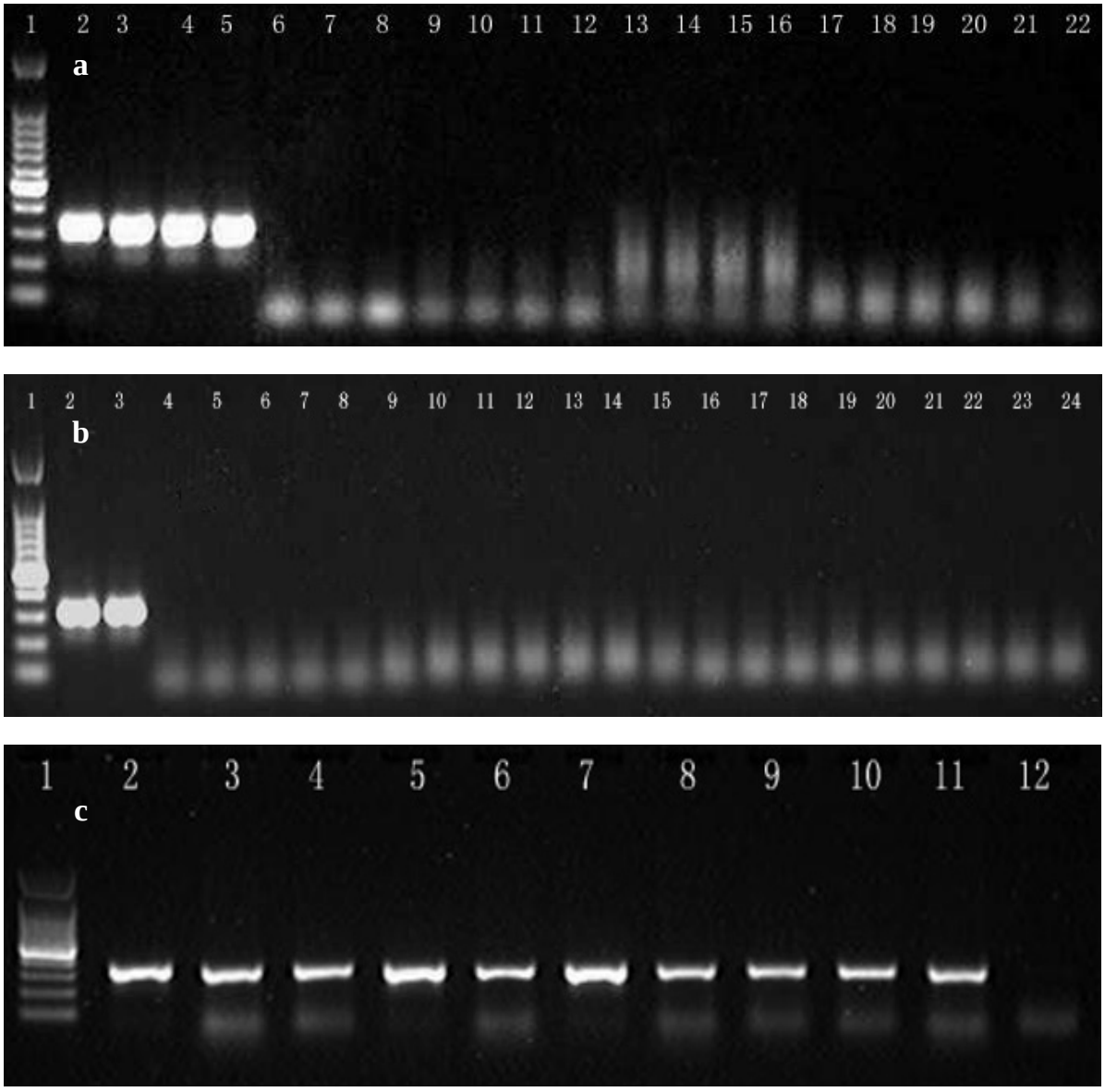

Fig. 1. PCR-amplified products using the specific primers Fc-1/ Fc-2 from genomic DNA of fungal and bacterial species.

a, Lane 1: 100-bp DNA ladder marker, lanes 2-5: *F. oxysporum* (HWS-11,HWS-12, HWS-18 and HWS-23) , lane 6: *F. avenaceum*, lane 7: *F. proliferatum*, lane 8: *F. solani*, lane 9: *F. poae*, lane 10: *Botrytis cinerea*, lane 11: *Alternaria solani*, lane 12: *Ascochyta citrallina*, lane 13: *Septoria lycopersici*, lane 14: *Pythium aphanidermatum*, lane 15: *Fusarium spp*., lanes 16-17: *Rhizoctonia solani*, lane 18: *Cladosporium fulvum*, lane 19: *Sclerotinia sclerotiorum*, lane 20: *F. graminearum*, lane 21: *Verticillium dahliae*, lane 22: negative control, b, Lane 1: 100-bp DNA ladder marker, lanes 2-3: *F. oxysporum* (HWS-11 and HWS-12) , lane 4: *F. moniliforme*, lane 5: *F. solani*, lane 6: *F. equiseti*, lane 7: *F. vasinfectum*, lane 8: *F. oxysporum* f. sp. *soybean*, lane 9: *Phytophthora melonis*, lane 10: *Bacillus subtilus* B29, lane 11: *E.coli* JM109, lane 12: *Bacillus amyloliquefaciens* TF28, lanes 13-14: *F. nivale*, lane 15: *Colletortichum liademuthianum*, lane 16: *Sphacelotheca reiliana*, lane 17: *Pyricularia oryzae*, lane 18: *Botrytis cinerea*, lane 19: *Alternaria cucumerina*, lane 20: *Alternaria dauci*, lane 21: *Colletotrichum lagenarium*, lanes 22-23: *Pythium spp*., lane 24: negative

control. C, Lane 1: 100-bp DNA ladder marker, lanes 2-11: *F. oxysporum* (10-01, 02, 03, 04, 05, 06, 07, 08, 09, 10) lane 12: negative control. The same results were obtained in 3 replicates.

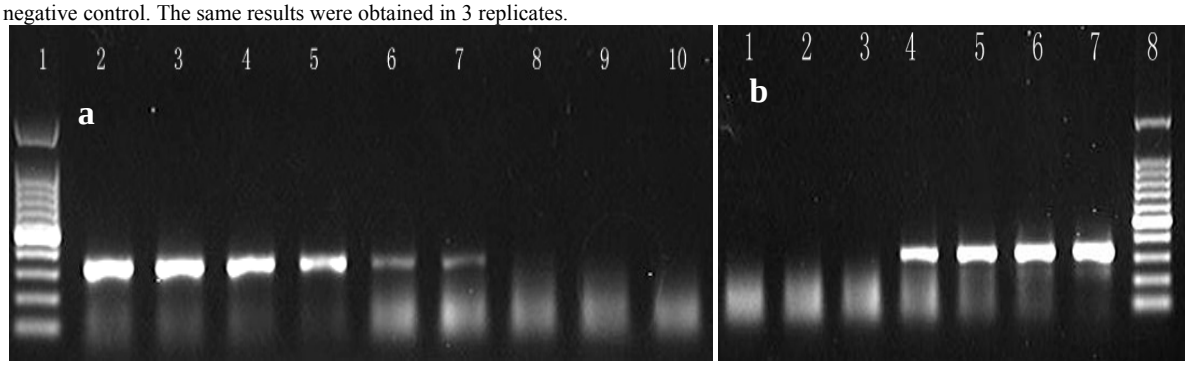

Fig. 2. Sensitivity of PCR for detection of *F. oxysporum* using the specific primers Fc-1/ Fc-2.
a: Sensitivity of PCR using different concentrations of DNA from purified *F. oxysporum* HWS-12 genomic DNA. b: Sensitivity of PCR using spore DNA in infected soils. a, Lane 1: 100-bp DNA ladder marker, lanes 2-9: PCR products using DNA at concentrations of 10 ng, 1 ng, 100pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, lane 10: negative control.b, Lane 1: negative control, lanes 2-6: numbers of spores were 10, 10^2, 10^3, 10^4 and 10^5, respectively, lane 7: positive control, lane 8: 100-bp DNA ladder marker. The same results were obtained in 3 replicates.

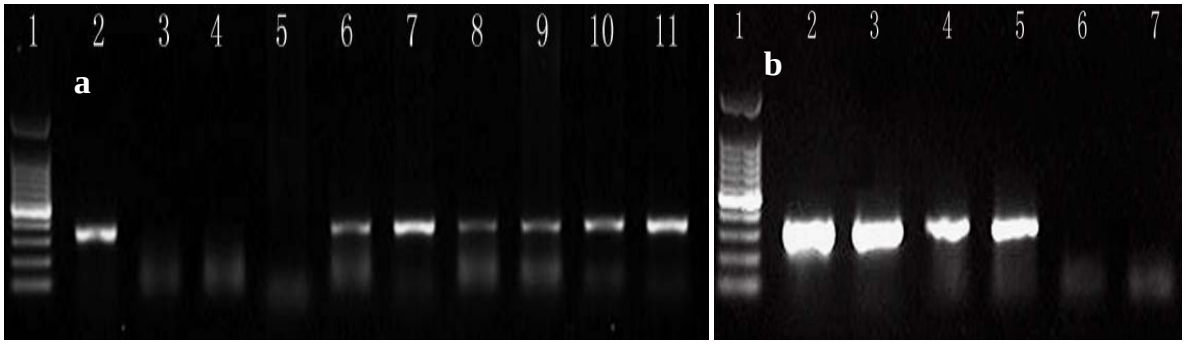

Fig. 3. PCR-amplified products using the specific primers Fc-1/ Fc-2 from DNA extracted from naturally infected and healthy cucumber tissues and rhizosphere soil.
a: PCR-amplified products using the specific primers Fc-1/ Fc-2 from DNA extracted from naturally infected and healthy cucumber tissues (Chengxi, Xuejia and Jianguo areaa). Lane 1: 100-bp DNA ladder marker, lane 2: positive control, lane 3: negative control, lane 4: healthy cucumber root, lane 5: healthy cucumber stem, lane 6: infected cucumber stem (Chengxi area, China), lane 7: infected cucumber root (Chengxi area, China), lane 8: infected cucumber stem (Xuejia area, China), lane 9: infected cucumber root (Xuejia area, China), lane 10: infected cucumber stem (Jianguo area, China), lane 11: infected cucumber root (Jianguo area, China).
b: PCR-amplified products using the specific primers Fc-1/ Fc-2 from DNA extracted from rhizosphere soil (Chengxi, Xuejia and Jianguo areaa). Lane 1: 100-bp DNA ladder marker, lane 2: positive control, lane 3: rhizosphere soil of infected cucumber (Chengxi area, China), lane 4: rhizosphere soil of infected cucumber (Xuejia area, China), lane 5: rhizosphere soil of infected cucumber (Jianguo area, China), lane 6: rhizosphere soil of healthy cucumber (Xuejia area, China), lane 7: negative control. The same results were obtained in 3 replicates.

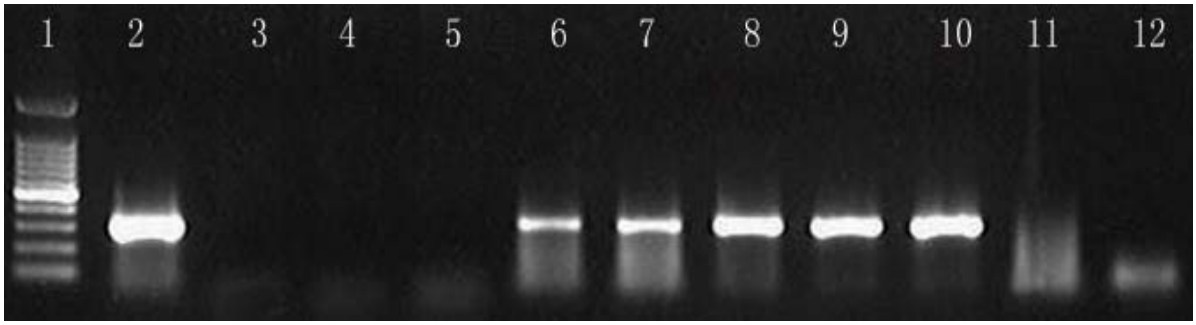

Fig. 4. PCR-amplified products using the specific primers Fc-1/ Fc-2 from DNA extracted from healthy and infected cucumber root at different times after inoculation with *F. oxysporum*.

Lane 1: 100-bp DNA ladder marker, lane 2: positive control, lane 3: root 1 days after inoculation, lane 4: root 3 days after inoculation, lane 5: root 5 days after inoculation, lane 6: root 7 days after inoculation, lane 7: root 9 days after inoculation, lane 8: root 15 days after inoculation, lane 9: root 21 days after inoculation, lane 10: root 30 days after inoculation, lane 11: healthy root, lane 12: negative control. The same results were obtained in 3 replicates.

F. oxysporum caused vascular wilt diseases in a wide variety of economically important crops. Plant pathogenic forms of *F. oxysporum* are divided into formae speciales based on the host they attacked (Armstrong & Armstrong, 1981). They are characterized by a high degree of host specificity. So far, several formae speciales of *Fusarium oxysporum*, including *F. oxysporum* f. sp. *vasinfectum* of cotton (Elsalam *et al.,* 2006), *F. oxysporum*. f. sp. *niveum* of watermelon (Zhang *et al.,* 2005), *F. oxysporum* f. sp. *cubense* of banana (Lin *et al.,* 2009), etc., have been identified through the PCR assay. To our knowledge, no report was found that using one-step PCR method detected cucumber formae specials of *Fusarium oxysporum*. Although the PCR detection method developed here only distinguish *Fusarium oxysporum* from other Fusarium genus, not distinguish formae speciales of *Fusarium oxysporum*, it was still useful due to the high degree of host specificity of different formae speciales. Therefore, the one-step PCR detection would be valuable in early monitoring and diagnosis of *F. oxysporum*.

Briefly, the one-step PCR based protocol developed in this study is useful to detect *F. oxysporum* rapidly, sensitively and reliably in infected cucumber plants and soil. Our detection protocol also allowed for early monitoring and diagnosis of *F. oxysporum* to facilitate disease management. The minimal quantity of genomic DNA of purified *F. oxysporum* was 100 fg and that of soil pathogens was 1000 spores per gram of soil.

Acknowledgments

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