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Molecular detection of *Fusarium oxysporum* f. sp. *niveum* and *Mycosphaerella melonis* in infected plant tissues and soil

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Abstract

We developed two species-specific PCR assays for rapid and accurate detection of the pathogenic fungi *Fusarium oxysporum* f. sp. *niveum* and *Mycosphaerella melonis* in diseased plant tissues and soil. Based on differences in internal transcribed spacer (ITS) sequences of *Fusarium* spp. and *Mycosphaerella* spp., two pairs of species-specific primers, Fn-1/Fn-2 and Mn-1/Mn-2, were synthesized. After screening 24 isolates of *F. oxysporum* f. sp. *niveum*, 22 isolates of *M. melonis*, and 72 isolates from the Ascomycota, Basidiomycota, Deuteromycota, and Oomycota, the Fn-1/Fn-2 primers amplified only a single PCR band of approximately 320 bp from *F. oxysporum* f. sp.*niveum*, and the Mn-1/Mn-2 primers yielded a PCR product of approximately 420 bp from *M. melonis*. The detection sensitivity with primers Fn-1/Fn-2 and Mn-1/Mn-2 was 1 fg of genomic DNA. Using ITS1/ITS4 as the first-round primers, combined with either Fn-1/Fn-2 and or Mn-1/Mn-2, two nested PCR procedures were developed, and the detection sensitivity increased 1000-fold to 1 ag. The detection sensitivity for the soil pathogens was 100-microconidia/g soil. A duplex PCR method, combining primers Fn-1/Fn-2 and Mn-1/Mn-2, was used to detect *F. oxysporum* f. sp. *niveum* and *M. melonis* in plant tissues infected by the pathogens. Real-time fluorescent quantitative PCR assays were developed to detect and monitor the pathogens directly in soil samples. The PCR-based methods developed here could simplify both plant disease diagnosis and pathogen monitoring as well as guide plant disease management.

Keywords: Molecular detection; *Fusarium oxysporum* f. sp. *niveum*; *Mycosphaerella melonis*; Real-time PCR

1. Introduction

Watermelon *Fusarium* wilt and watermelon or melon gummy stem blight are the most destructive diseases to melon crops and are caused by two soil-borne pathogens, *Fusarium oxysporum* f. sp. *niveum* and *Mycosphaerella melonis*. These diseases are a yield-limiting factor in watermelon and melon production worldwide. Watermelon *Fusarium* wilt can occur at all growing stages of the crop throughout all watermelon-growing regions. Symptoms include the wilting of stems and leaves early on and vascular discoloration of roots and stems later [1]. Because the fungus persists in the soil for many years, watermelons should not be replanted in infected soils for at least five years. Gummy stem blight is a mid- to late-season disease. Symptoms include early stem and leaf wilt and black spots on stems and leaves later [2]. These two diseases have similar symptoms in the early stages, beginning with plant wilt and waterlogged stems.

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Few effective, economical, and environmentally safe management options are available for *Fusarium* wilt and gummy stem blight. A major reason for this lack is the inability to accurately detect the presence and identity of the two fungal pathogens, especially in plant tissues and soil. Also, plants affected by sudden wilt are generally infected by a number of different fungal species, including *Pythium*, *Phytophthora*, *Fusarium*, *Rhizoctonia* and others. Traditional methods to detect or isolate these pathogens involve plating infected plant parts or soil on selective medium and conducting a pathogenicity assay. However, this method is limited by its lack of sensitivity and specificity, since *Fusarium oxysporum* f. sp. *niveum* and *M. melonis* share similar morphology with certain other fungal pathogens when growing on medium. Microscopic methods are used to identify the pathogens, but it is often difficult to detect specific fungi in roots and soil that contain morphologically similar pathogens [3]. In addition, these laborious, time-consuming methods preclude processing large numbers of samples and require extensive knowledge of fungal taxonomy.

A rapid detection method for pathogens and a diagnostic assay for disease would facilitate pathogen identification and lead to more effective management practices, such as directing the application of the proper fungicide to prevent serious diseases. Polymerase chain reaction (PCR) techniques offer advantages over traditional methods of detection and diagnosis, because the fungi do not need to be cultured prior to detection by PCR, and the technique is rapid and sensitive [4–11]. In this study, two pairs of species-specific primers were developed to detect pathogens.

Quantitative real-time PCR technology can accurately quantify the extent of pathogen biomass and, with multiplex formats, can simultaneously detect different organisms. Real-time PCR is being used increasingly in plant pathology for the accurate detection and quantification of plant pathogens, even at very low levels of infection [12–16]. However, its application in the detection and quantification of fungi from soil samples remains limited [17–20]. Here, we describe the use of real-time PCR conjugated with fluorescent SYBR Green I dye to quantify *F. oxysporum* f. sp. *niveum* and *M. melonis* directly from soil.

Internal transcribed spacer (ITS) regions of the ribosomal RNA gene possess characteristics that allow the identification of pathogens at the species level. In this paper, we describe the development of PCR primers derived from ITS sequences for the specific detection of *F. oxysporum* f. sp. *niveum* and *M. melonis*. The specificity and sensitivity of the reaction were tested on a range of wild *Fusarium* and *Mycosphaerella* species and representatives of other fungal divisions, as well as of the host plants. The sensitivity of the PCR assay was determined, and the PCR protocols were tested for their ability to detect *F. oxysporum* f. sp. *niveum* and *M. melonis* in diseased plant tissues and soil samples collected in the field. We also describe the use of real-time PCR in conjunction with fluorescent SYBR Green I dye to quantify *F. oxysporum* f. sp. *niveum* and *M. melonis* directly from soil samples.

2. Materials and methods

2.1. Source of isolates

Table 1 lists the 24 *F. oxysporum* f. sp. *niveum* isolates, 22 *M. melonis* isolates, and other isolates used in this study, including ascomycetes, basidiomycetes, deuteromycetes, and oomycetes obtained from several sources. All isolates were stored either on lima bean agar (LBA) slants at 10 °C (*Phytophthora* spp. and *Pythium* spp.) or on potato dextrose agar (PDA) at 4 °C (other fungi). The isolates were maintained in a collection at the Department of Plant Pathology, Nanjing Agricultural University, PR China.

2.2. Mycelium and microconidia preparation

For genomic DNA extraction, 20 pieces of agar culture (ca. $1 \times 1 \times 2$ mm) obtained from the advancing margin of 3-day-old colonies growing on plates of LBA (*Phytophthora* and *Pythium*) or PDA (other fungi) were placed in 100 ml of Plich's liquid medium [21] in a 250-ml flask. After incubation in the dark at 25 °C on a shaker for 6 days, the mycelia were collected on filter paper and stored at −70 °C until use.

Microconidia of the fungus *F. oxysporum* f. sp. *niveum* were prepared by growing plate cultures on PDA at 25 °C for 10 days in darkness to induce sporulation. Microconidia were harvested from the plates by rubbing the surface mycelium gently with a rubber swab and collecting the spores in distilled water. Hyphal debris was removed from the spores by centrifuging the crude spore preparation through a 40% sucrose pad, with the spores settling to the bottom of the tube and the rest of the cellular debris remaining on the surface of the sucrose pad. Spores were adjusted to the desired concentration (10^4 spores ml^{-1}) by counting them in a hemocytometer for DNA extraction or soil inoculation.

2.3. Soil preparation and inoculation

To detect fungi in soil-based samples, 2-ml titers of the *F. oxysporum* f. sp. *niveum* spore suspension were inoculated onto 1 g of twice-autoclaved soil substrate in 15-ml conical tubes. To compare the detection threshold of DNA extracted from inoculated soils to that extracted from pure spore suspensions, 1 ml each of the serially diluted spore concentration of the fungus was

Table 1
Fungal isolates used to screen the specificity of the primers

Species	Host	Origin	Number of isolates	Amplification with primers	
				Fn-1/Fn-2	Mn-1/Mn-2
Fusarium oxysporum f. sp.*niveum*	*Citrullus lanatus*	Shanghai	7	+	–
	Citrullus lanatus	Lingxia	3	+	–
	Citrullus lanatus	Jiangsu	2	+	–
	Citrullus lanatus	Fujian	2	+	–
	Citrullus lanatus	Shanghai	3	+	–
	Citrullus lanatus	Jiangsu	2	+	–
	Citrullus lanatus	Heibei	5	+	–
Mycosphaerella melonis	*Citrullus lanatus*	Shanghai	2	–	+
	Cucumis melo	Shanghai	19	–	+
	Benincasa hispida	Jiangsu	1	–	+
Tilletia indica	*Triticum aestivum*	USA	2	–	–
T. walkeri	*Triticum aestivum*	USA	1	–	–
T. controversa	*Triticum aestivum*	USA	2	–	–
T. caries	*Triticum aestivum*	China	1	–	–
Ustilago nuda	*Triticum aestivum*	Jiangsu	1	–	–
U. nuda	*Triticum aestivum*	Heilongjiang	2	–	–
U. maydis	*Zea mays*	Jiangsu	1	–	–
Alternaria solani	*Lycopersicum esculentum*	Jiangsu	1	–	–
Alternaria sp.	Unknown	Jiangsu	1	–	–
A. longipes	*Nicotiana tabacum*	Fujian	1	–	–
Ascochyta fabae	*Vicia faba*	Fujian	1	–	–
Botrytis cinerea	*Lycopersicon esculentum*	Jiangsu	1	–	–
B. cinerea	*Lactuca scariola*	Fujian	1	–	–
B. cinerea	*Vitis vinifera*	Fujian	1	–	–
Colletotrichum orbiculare	*Cucumis sativus*	Jiangsu	1	–	–
C. orbiculare	*Citrullus lanatus*	Jiangsu	1	–	–
C. higgisianum		Jiangsu	1	–	–
C. gloeosporioides	*Diospyros kaki*	Fujian	1	–	–
C. truncatum	*Glycin max*	Fujian	1	–	–
C. capsici	*Capsicum annuum*	Jiangsu	1	–	–
F. equiseti	Unknown	CGMCC*	1	–	–
F. avenaceum	Unknown	CGMCC	1	–	–
F. nivale	Unknown	CGMCC	1	–	–
F. sambucinum	Unknown	CGMCC	1	–	–
F. culmorum	Unknown	CGMCC	1	–	–
F. oxysporum f. sp. *vasinfectum*	*Gossyphum*	Jiangsu	1	–	–
F. oxysporum f. sp. *cucumerinum*	*Cucumis sativus*	Jiangsu	1	–	–
	Cucumis sativus	Fujian	1	–	–
F. oxysporum f. sp. *cubense*	*Musa sapientum*	Fujian	1	–	–
F. graminearum	*Triticum aestivum*	Jiangsu	2	–	–
F. moniliforme	*Oryza*	Jiangsu	1	–	–
F. moniliforme	*Gossyphum*	Jiangsu	1	–	–
F. solani	Unknow	Jiangsu	2	–	–
Fusarium sp.	*Alternanthera philoxeroides*	Jiangsu	1	–	–
Fusarium sp.	Field soil	Jiangsu	20	–	–
Macrophoma kawatsukai	*Malus pumila*	Jiangsu	1	–	–
Rhizoctonia	*Gossyphum*	Jiangsu	1	–	–
R. solani	*Cucumis melo*	Fujian	1	–	–
Verticillium albo-atrum	*Gossyphum*	Jiangsu	1	–	–
V. dahliae	*Gossyphum*	Jiangsu	1	–	–
Phytophthora boehmeriae	*Gossyphum*	Jiangsu	2	–	–
P. colocasiae	*Colocasiu esculenta*	Jiangsu	1	–	–
P drechsleri	*Lycopersicum eslulentum*	Jiangsu	1	–	–
P. medicaginis	*Medicago*	Ho HH	1	–	–
P. sojae	*Glycine max*	Ho HH	1	–	–
P. palmivora	*Ficus carica*	Ko WH	1	–	–
Pythium aphanidermatum	Unknown	Jiangsu	1	–	–

+, 327-bp and 422-bp products amplified by primers Fn-1/Fn-2 and Mn-1/Mn-2, respectively.
– No amplified products.
CGMCC is China General Microbiological Culture Collection Center.

placed in sterile 2.0-ml screw-capped Eppendorf tubes. The tubes were vortexed at maximum speed for 1 min, freeze-dried for 23 days, ground in liquid nitrogen to produce a fine powder, and stored at −70 °C prior to DNA extraction.

2.4. DNA extraction

DNA was prepared by a modified method of Panabieres et al. [22]. Approximately 10 mg of freeze-dried mycelium or conidia were ground in liquid nitrogen. The resulting powder was suspended in 0.5 ml of NIB buffer (100 mM NaCl, 30 mM Tris–HCl, pH 8.0, 10 mM EDTA, and 10 mM β-mercaptoethanol), 0.5% (v/v) NP-40 and centrifuged at 12,000*g* for 1 min. The pellet was treated once as above, then resuspended in 0.8 ml of homogenization buffer (0.1 M NaCl, 0.2 M sucrose, 10 mM EDTA) before adding 0.2 ml of lysis buffer (0.25 M EDTA, 0.5 M Tris, pH 9.2, 2.5% sodium dodecyl sulfate). The homogenate was incubated for 30 min at 55 °C, then extracted twice with one volume of phenol–chloroform–isoamyl alcohol (25:24:1). This mixture was vortexed at 3000 rpm for 10 min and centrifuged for 15 min at 12,000*g*. The aqueous phase was collected, extracted with an equal volume of chloroform/isoamyl alcohol (24:1) and precipitated with two volumes of 100% cold (−20 °C) ethanol. The precipitated DNA was washed with 70% cold ethanol, dissolved in 50 μl of TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). The $A_{260\ nm}/A_{280\ nm}$ ratio of these DNA preparations was between 1.7 and 2.0, and their $A_{260\ nm}/A_{230\ nm}$ ratio was between 1.6 and 2.0, suggesting that they were essentially free of protein and carbohydrate. All DNA preparations were kept at −70 °C.

DNA was extracted from infected plant tissues according to the methods of Tooley et al. [23]. A 10-mg sample of diseased tissue (stem or leave) was cut from each plant with a sterile scalpel, placed into 10 μl of freshly prepared 0.5 M NaOH in a 1.5-ml microcentrifuge tube, and macerated with a plastic pestle. After the tubes were centrifuged at 12,000*g* for 5 min to pellet the tissue, 5 μl of supernatant were removed and immediately diluted with 195 μl 100 mM Tris (pH 8.0). The samples were then either used immediately for PCR (1 μl per 25 μl reaction mixture) or frozen at −20 °C for later use.

DNA was extracted from soil samples directly by modifying the method of Li and Hartman [10]. Soil samples (0.5 g per sample) were first lyophilized with liquid nitrogen. A TENS buffer, containing 50 mM Tris (pH 8.0), 20 mM EDTA, 100 mM NaCl, 1% (w/v) sodium dodecyl sulfate, plus 10 μg ml^{-1} proteinase K, used as a DNA extraction buffer, was added to the lyophilized soil suspended in 0.5 mL 0.4% dry milk powder solution. After vortexing, samples were incubated at 55 °C for 1–3 h. After incubation, samples were centrifuged at 12,000*g* for 10 min to remove soil and debris. The supernatant was transferred to a new centrifuge tube. DNA was precipitated with one volume of 7.5 M ammonium acetate and two volumes of 100% ethanol at −20 °C for 2 h or overnight. The DNA was pelleted, rinsed with 70% ethanol, dried, dissolved in sterile 0.1 TE buffer (10 mM Tris–HCl pH 8.0 and 1 mM EDTA, pH 8.0) and used as a template for PCR amplification or stored at −20 °C until use.

2.5. Primer design and PCR amplification

Two specific primers for *F. oxysporum* f. sp. *niveum* (Fn1/Fn2) and *M. melonis* (Mn-1/Mn-2) were designed by the comparison of the ITS of 55 different *Fusarium* sequences[2] and 50 *Mycosphaerella* sequences[3] in GenBank. The Fn pair of specific primers, Fn-1 (5′-TAC-

[2] The Genbank accession numbers and species are: AF006356(*Fusarium. venenatum*); AF111059(*F. chlamydosporum*); AF111066(*F. tricinctum*); AF129105(*F. solani* f.sp.*mori*); AF133843(*F. lichenicola*); AF178397(*F. ambrosium*); AF178408 (*F. solani* f.sp.*batatas*); AF178422(*F. solani* f.sp.*piperis*); AF29106(*F. proliferatum*); AF310981(*F. lateritium*); AF414968(*F. poae*); AF414969(*F. pulverosum*); AF414971(*F. kyushuense*); AF414972(*F. sporotrichioides*); AF414973(*F. sporotrichioides* var.*minor*); AF430129(*F. mangiferae*); AF430130(*F. sterilihyphosum*); AJ246148(*F. cerealis*); AY043478(*F. solani*); AY147334(*F. culmorum*); AY147368(*F. equiseti*); AY210330(*F. oxysporum*); AY264267(*F. oxysporum* f.sp.*vasinfectum*); FAU34573(*F. acutatum*); FAU61670(*F. annulatum*); FAU61671(*F. anthophilum*); FBEORRNA(*F. beomiforme*); FBU34576(*F. bactridioides*); FBU61673(*F. begoniae*); FBU61675(*F. brevicatenulatum*); FBU61676(*F. bulbicola*); FCU61678(*F. concentricum*); FCU61679(*F. concolor*); FDLARRNA(*F. dlaminii*); FDU61680(*F. denticulatum*); FGU34562(*F. guttiforme*); FIU34577(*F. inflexum*); FLU61681(*F. lactis*); FLU85537(*F. lunulosporum*); FNAPRRNA(*F. napiforme*); FNYGRRNA(*F. nygamai*); FPOLRRNA(*F. polyphialidicum*); FPU34563(*F. pseudonygamai*); FPU34574(*F. phyllophilum*); FPU61683(*F. pseudoanthophilum*); FREDRRNA(*F. redolens*); FRU61684(*F. ramigenum*); FRU85539(*F. robustum*); FSACRRNA(*F. sacchari*); FSU34561(*F. succisae*); FSU34569(*F. pseudocircinatum*); FSU61685(*F. nisikadoi*); FSU61687(*F. globosum*); FSUBRRNA(*F. subglutinans*); FTU85542(*F. tumidum*); FUU34575(*F. udum*).

[3] The Genbank accession numbers and species are: AF013227(*Mycosphaerella. pini*); AF181704(*M. citri*); AF181705 (*M. fijiensis*); AF243392 (*M. populorum*); AF309590(*M. parkii*); AF309591 (*M. marasasii*); AF309601 (*M. keniensis*); AF309602(*M. africana*); AF309603 (*M. flexuosa*); AF309611(*M. crystalline*); AF309612 (*M. colombiensis*); AF362049 (*M. macrosporai*); AF362052 (*M. brassicicola*); AF362056 (*M. bixae*); AF362058(*M. confusa*); AF362059 (*M. recutita*); AF362060 (*M. rubella*); AF362062 (*M. berberidis*); AF362063 (*M. arbuticola*); AF362067 (*M. latebrosa*); AF362070 (*M. dearnessii*); AF449099 (*M. nubilosa*); AF449101 (*M. molleriana*); AF452517 (*M. heimii*); AF468868 (*M. fori*); AF468873 (*M. marksii*); AF468875 (*M. ellipsoidea*); AF468878(*M. irregulariramosa*); AF468880 (*M. juvenis*); AF468883 (*M. lateralis*); AF509735 (*M. musicola*); AF708(*Phaeosphaerianodorum*); AY045498 (*M. cryptica*); AY045500 (*M. vespa*); AY045502 (*M. walker* i); AY045504 (*M. suberosa*); AY045515 (*M. tasmaniensis*); AY045516 (*M. grandis*); AY045518 (*M. intermedia*); AY045519 (*M. suttoniae*); AY150331 (*M. aurantia*); AY150675 (*M. ambiphylla*); AY257484 (*M. musae*); AY266147 (*M. berkeleyi*); AY266152 (*M. fijiensis*); AY266153 (*M. cruenta*); MGU77363 (*M. graminicola*); MTA238469 (*M. tassiana*); AF222831 (*Mycovellosiella bellynckii*); AF222832 (*M. vaginae*); AF309617 (*M. eucalypti*).

CACTTGTTGCCTCGGC-3′) and Fn-2 (5′-TTGAGGAACGCGAATTAAC-3′), was designed to amplify PCR products of 327 bp of *F. oxysporum* f. sp. *niveum*. The Mn pair of specific primers, Mn-1 (5′-GGATCATTACCTAGAGTTG-3′) and Mn-2 (5′-ACGTCGTCGTTGTGAGTG-3′), was designed to amplify PCR products of 442 bp of *M. melonis*.

The traditional PCR was performed in reaction volumes of 25 μl. Each reaction consisted of 1 μl genomic DNA, 0.5 μM primers, a 0.5-μl mixture that contained 50 μM of each dNTP, 2.5 μl 10 × PCR buffer, 2 mM Mg^{2+}, 2.5 μl 1% BSA, 0.25 μl Tw-20, and 1.25 U*Taq* DNA polymerase (Promega). Mineral oil was overlaid on the mix. Amplification was performed with a PE2400 PCR System DNA thermal cycler (Perkin–Elmer Applied Biosystems, Foster City, CA, USA) programmed for one cycle at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 54 °C (for primers Fn-1/Fn-2) or 58 °C (for primers Mn-1/Mn-2) for 30 s, and 72 °C for 30 s. A 7-min extension at 72 °C was conducted after 35 cycles. The nested PCR included two rounds of amplification using the universal primers ITS1/ITS4 for the first round and the internal *F. oxysporum* f. sp. *niveum*-specific primers Fn-1/Fn-2 or *M. melonis*-specific primers Mn-1/Mn-2 for the second round. Duplex PCR was performed in 50 μl of PCR mixture, including 1 μl DNA from diseased plant samples prepared as described; 0.5 μM primers Fn-1/Fn-2 and 0.5 μM Mn-1/Mn-2; a 2.5-μl mixture containing 12.5 μM of each dNTP; 5 μl 1% BSA; 0.5 μl Tw-20; and 1.25 U *Taq* DNA polymerase (Promega). The PCR program was 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, and extension at 72 °C for 7 min. Real-time fluorescent quantitative PCR reactions were carried out in a 50-μl volume that consisted of 0.5 μM primer; a 2.5-μl mixture containing 12.5 μM of each dNTP; 5 μl 10 × PCR buffer; 2.5 mM Mg^{2+}; 1.25 U *Taq* DNA polymerase (Promega); and 2.5 μl 20 × SYBR Green I (OPE). Each sample was subjected to an initial denaturation of 12 min at 94 °C, followed by 40 amplification cycles of denaturation at 95 °C for 30 s and 30 s of annealing at 54 °C (for primers Fn-1/Fn-2) or 58 °C (for primers Mn-1/Mn-2), then extension at 72 °C for 30 s. Quantitative PCR experiments were performed using an ABI Prism 7000 sequence detection system.

3. Results

3.1. Specificity and sensitivity of the PCR amplification

The specificity of the primers was assessed against 24 *F. oxysporum* f. sp. *niveum* isolates, 22 *M. melonis* isolates, and 72 isolates of other fungi and oomycetes. The primer pair Fn-1/Fn-2 was able to amplify a unique DNA fragment of approximately 320 bp (Fig. 1) of all *F. oxysporum* f. sp. *niveum* isolates from different provinces in China. However, isolates of *M. melonis* and other fungi tested yielded no amplification product. Similarly, the primer pair Mn-1/Mn-2 amplified a unique DNA fragment of approximately 420 bp from all *M. melonis* isolates (Fig. 2), but did not amplify 24 *F. oxysporum* f. sp. *niveum* and 72 other fungal isolates tested. All fungal and oomycete isolates tested had a positive PCR reaction using the ITS universal primers ITS1/ITS4 (data not shown).

The sensitivity of the primer sets Fn-1/Fn-2 and Mn-1/Mn-2 was 1 fg/25 μl reaction (Fig. 3(a)) from the pure template of *F. oxysporum* f. sp. *niveum* and *M. melonis* total genomic DNA (Fig. 4(a)). Nested PCR increased the sensitivity of the primers at least 1000-fold to 1 ag/25 μl reaction using primers ITS1/ITS4 for the first round and Fn-1/Fn-2 and Mn-1/Mn-2 for the second round (Figs. 3(b) and 4(b)). The sensitivity of the primer pair Fn-1/Fn-2 varied from 10 pure microconidia in the 25-μl reaction to 100 microconidia per gram soil (Fig. 5).

3.2. Detection in plant tissues

Duplex PCR assays combining the two pairs of species-specific primers detected *F. oxysporum* f. sp. *niveum*

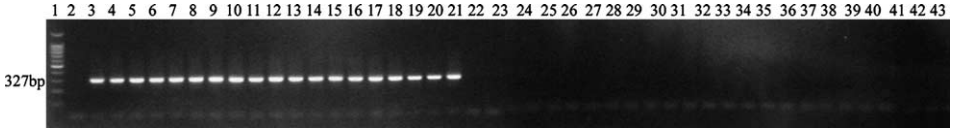

Fig. 1. Agarose gel electrophoresis of PCR-amplified products using the specific primers Fn-1/Fn-2. Lane 1, 100-bp DNA ladder marker; Lane 2, ck; Lanes 3–21, *F. oxysporum* f. sp. *niveum* isolates; Lanes 22–43, *M. melonis* isolates. The same results were obtained in 4 replicates.

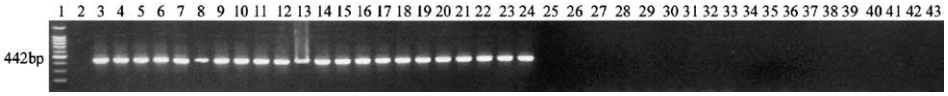

Fig. 2. Agarose gel electrophoresis of PCR-amplified products using the specific primers Mn-1/Mn-2. Lane 1, 100-bp DNA ladder marker; Lane 2, ck; Lanes 3–24, *M. melonis* isolates; Lanes 25–43, *F. oxysporum* f. sp. *niveum* isolates. The same results were obtained in 4 replicates.

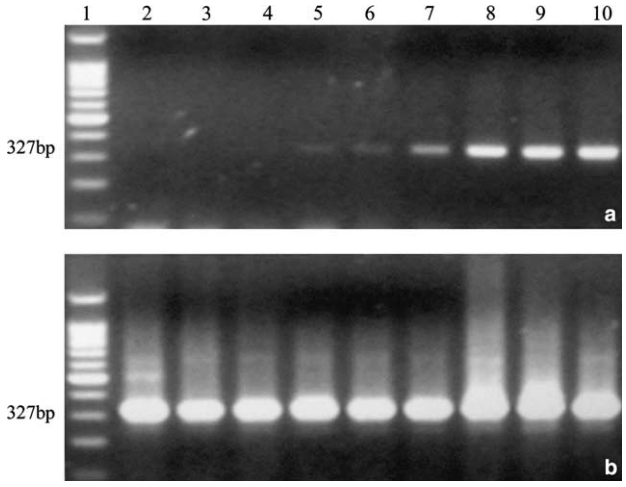

Fig. 3. Sensitivity of single and nested PCR for the detection of *F. oxysporum* f. sp. *niveum*. (a) Sensitivity of PCR with primers Fn-1/Fn-2 using different concentrations of DNA. (b) Nested PCR using primers ITS1/ITS4 for the first round of amplification and primers Fn-1/Fn-2 for the second round of amplification. Lane 1, 100-bp DNA ladder marker; Lanes 2–10, Amplified products using DNA at concentrations of 1 ag, 10 ag, 100 ag, 1 fg, 10 fg, 100 fg, 1 pg, 10 pg, and 100 pg in a 25-μl PCR reaction. The same results were obtained in 3 replicates.

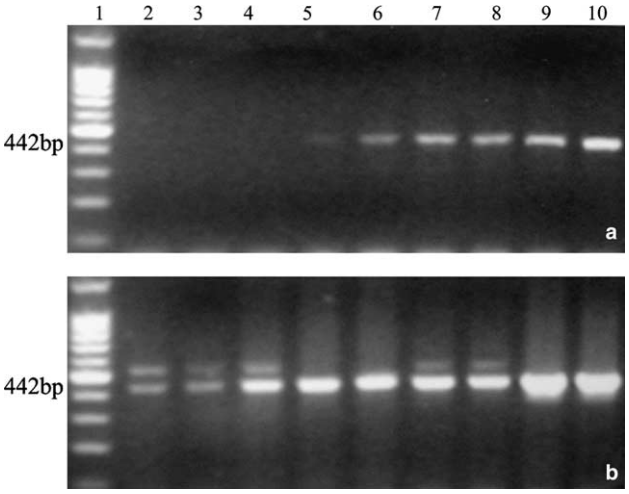

Fig. 4. Sensitivity of single and nested PCR for the detection of *M. melonis*. (a) Sensitivity of PCR with primers Mn-1/Mn-2 using different concentrations of DNA and (b) nested PCR using primers ITS1/ITS4 for the first round of amplification primers Mn-1/Mn-2 for the second round of amplification. Lane 1, 100-bp DNA ladder marker; Lanes 2–10, Amplified products using DNA at concentrations of 1 ag, 10 ag, 100 ag, 1 fg, 10 fg, 100 fg, 1 pg, 10 pg, 100 pg in a 25-μl PCR reaction The same results were obtained in 3 replicates.

and *M. melonis* in naturally infected plant tissues from Shanghai, Jiangsu, and Anhui. The expected specific products showed up in agarose gel electrophoresis (Fig. 5). This duplex PCR method succeeded in amplifying specific products using DNA samples extracted from all diseased plant parts, including both wilted and waterlogged sections. In addition, five colonies from random

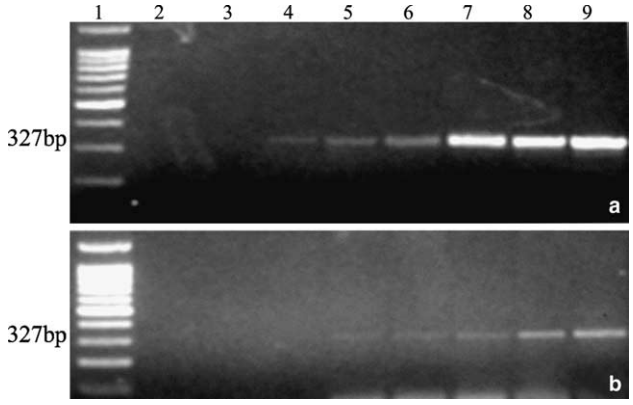

Fig. 5. Sensitivity of PCR using primers Fn-1/Fn-2 with *F. oxysporum* f. sp. *niveum* DNA from microconidia Lane 1, 100-bp DNA ladder marker; Lanes 2–10, Lane 2, -ck; Lanes 3–9, numbers of microconidia were 10^0, 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6, respectively. (b) Amplified product with the microconidia DNA extracted from soil samples Three independent replicates gave the same results.

plating of field-diseased plant samples appeared similar in cultural morphology to purified isolates of *F. oxysporum* f. sp. *niveum* and *M. melonis*. This indicates that the DNA from diseased plants that was amplified in PCR assays by primer sets Fn-1/Fn-2 and Mn-1/Mn-2 was derived from *F. oxysporum* f. sp. *niveum* and *M.melonis* isolates, respectively (Fig. 6).

3.3. Detection in field soil

A real-time PCR method was developed to quantitatively detect the two pathogenic fungi in soil. We first established a standard curve by plotting the log of a known concentration (ten-fold dilution series from 100 fg to 10 ng in reaction volumes of 50 μl) of DNA from *F. oxysporum* f. sp. *niveum* or *M. melonis* against the Ct values and melting curve (Fig. 7). Ct values resulting from assays of unknown samples were plotted onto this curve, and the inferred concentration of *F. oxysporum* f. sp. *niveum* or *M. melonis* was calculated. DNA extracted from three field soil samples collected in a diseased watermelon field and from three others collected in a diseased melon field in Shanghai was subjected to real-time fluorescent quantitative PCR using primers Fn-1/Fn-2 or Mn-1/Mn-2. The DNA samples extracted from 1 g of soil were suspended in 10 μl of ultrapure water, and 1 μl was used for PCR amplification. The amount of DNA per gram of soil was obtained from the standard curve (Fig. 7 and Table 2).

4. Discussion

The rapid and reliable detection of pathogens is important in formulating strategies for disease management in plant nurseries. Our objective was to develop a

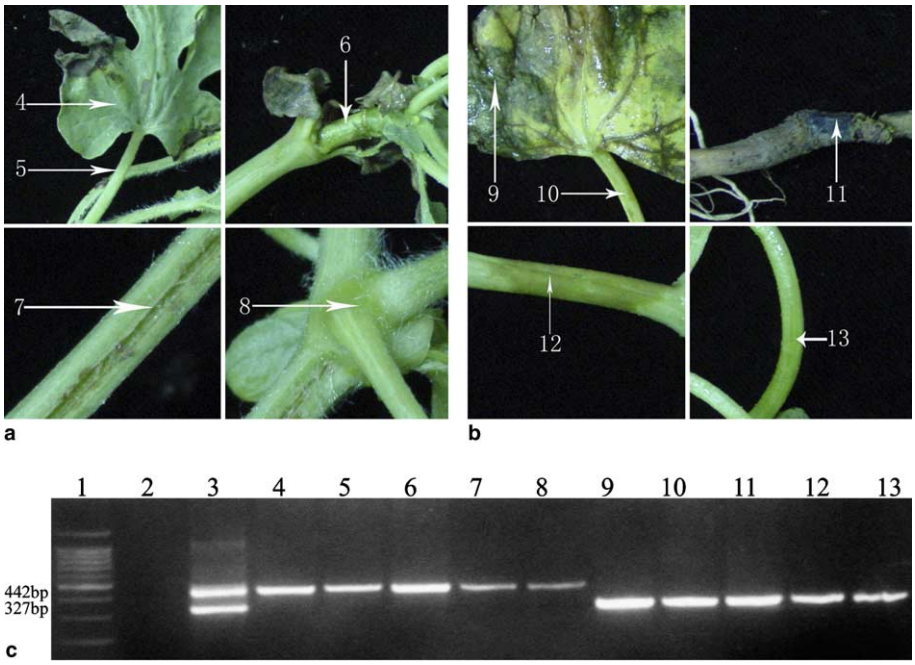

Fig. 6. (a) Diseased plant infected by *F. oxysporum* f. sp. *niveum*. (b) Diseased plant infected by *M. melonis*. (c) Duplex PCR amplification of DNA extracted from diseased plants (a and b): Wilting is indicated by arrows 4 and 9 (leaves); 5 and 10 (stalks); and 6 and 11 (stems). Water-logged stems are indicated by arrows 7, 8, 12, and 13. Lane 1, 100-bp DNA ladder marker; Lane 2, healthy plant; Lane 3, +ck; Lanes 4–13, amplified result with DNA templates extracted from diseased plants as shown in (a) and (b) Three independent replicates gave the same results. The results are shown as the means ± SD of five independent experiments.

rapid, sensitive, and effective molecular method using species-specific PCR to identify and detect the soil-borne pathogens *F. oxysporum* f. sp. *niveum* and *M. melonis*, which cause watermelon *Fusarium* wilt and watermelon or melon gummy stem blight.

PCR-based assays have been applied to microbial ecology and environmental sciences to detect and monitor microorganisms in the rhizosphere and in soils [12,17–19,24,25], as well as to diagnose plant diseases [9,18,23,26]. Field-grown plant roots and field soils harbor microbial complexes, making it tedious and time-consuming to isolate the soil-borne pathogens *F. oxysporum* f. sp. *niveum* and *M. melonis* from soil by traditional agar plating. Plating requires considerable expertise to differentiate *Fusarium* species based on morphology due to the overlap of morphological characteristics among species. Moreover, identifying *forma specialis* of *F. oxysporum* requires many pathogenicity tests. Identification of *M. melonis* depends on perfect stage characters. At least one month is required to detect *F. oxysporum* f. sp. *niveum* and *M. melonis* from soil by traditional isolation methods, which can delay disease-management decisions. However, the PCR detection method reported here can provide a definitive diagnosis of the two pathogens in soils and plants within hours, and can be used to more accurately survey the occurrence and distribution of the two pathogens in soil. Moreover, this method is very easy to use and requires minimal training.

PCR specificity is adequate for the detection of the pathogens *F. oxysporum* f. sp. *niveum* and *M. melonis* in soil. The primer sets Fn-1/Fn-2 and Mn-1/Mn-2 could not only distinguish *F. oxysporum* f. sp. *niveum* and *M. melonis*, respectively, from other pathogens but could also accurately diagnose and survey the two pathogens from diverse areas of China. This suggests that our methods may detect the two pathogens from broader origins. Moreover, the two primer sets had no cross-reactions from among a considerable number of different fungi. Specificity is essential for detecting *F. oxysporum* f. sp. *niveum* and *M. melonis* in soil. Agricultural field soil is a complex ecosystem with a diverse microbial community [27]. For example, more than hundreds of different species of *Phytophthora*, *Pythium*, *Fusarium*, *Verticillium*, *Rhizoctonia*, in addition to various bacteria and nematodes, have been found in field soil.

The sensitivity of PCR assays is an important concern in the molecular detection of plant pathogens in field soil. In a 25-μl reaction assaying *F. oxysporum* f. sp. *niveum* and *M. melonis*, conventional PCR was able to detect 1 fg of pure genomic DNA, 10 pure microconidia, or 100 microcondia in 1 g of artificially inoculated soil. In contrast, in a 100-μl reaction using conventional PCR with the primer set Fsg1/Fsg2, the detection level for *F. solani* f. sp. *glycines* was 10 pg from a pure template of total genomic DNA of the pathogen. For another primer set, the detection level was 1 ng and ranged as low as 10^3 macroconidia g^{-1} soil [10].

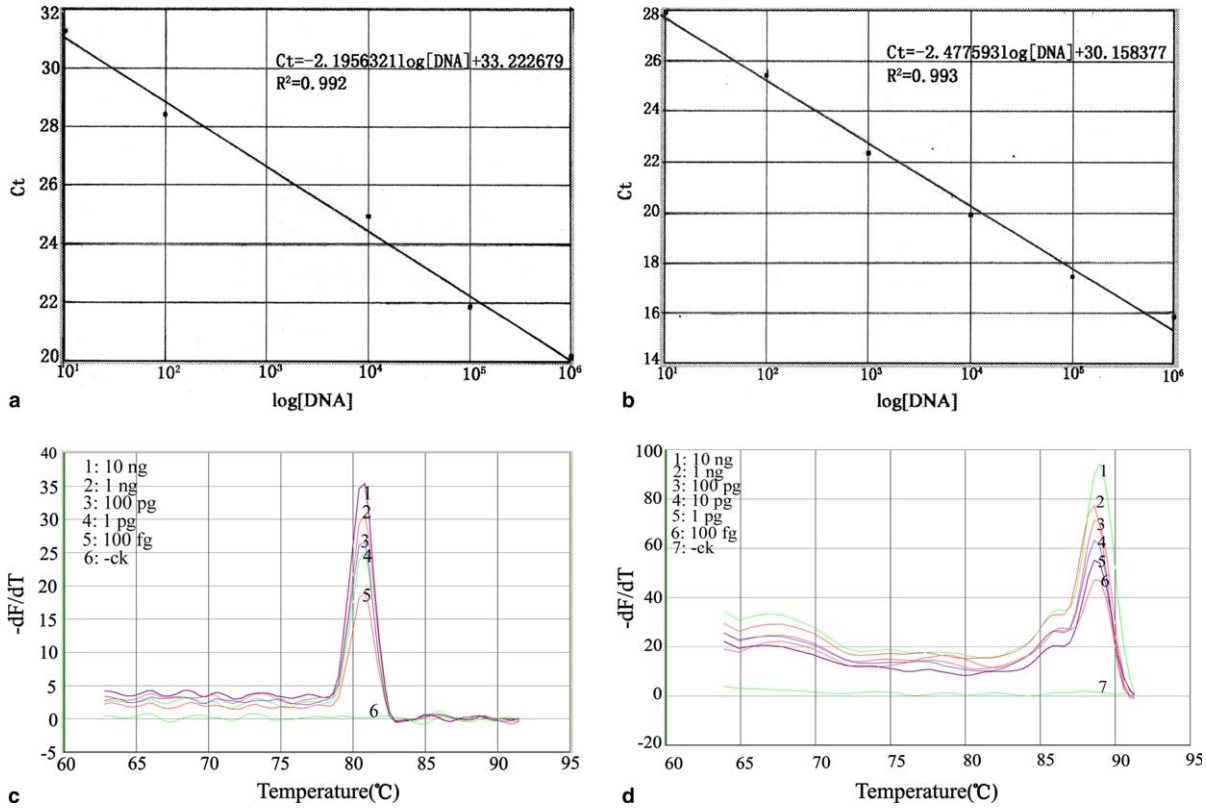

Fig. 7. Standard curve (a) and (b) obtained by plotting the log of a known DNA concentration of *F. oxysporum* f. sp. *niveum* and *M. melonis* (100 fg–10 ng) against the Ct values obtained from real-time quantitative PCR assays. (c) Melting curve profile for real-time PCR amplification of *F. oxysporum* f. sp. *niveum* pure genomic DNA. (d) Melting curve profile for real-time PCR amplification of *M. melonis* pure genomic DNA. The negative derivative of fluorescence with respect to temperature is plotted as dF/dT versus temperature to obtained a graphical representation of the melting peaks.

Table 2
Results of quantitative PCR assay

	Water melon *Fusarium* wilt	Melon gummy stem blight
	DNA g^{-1} soil	DNA g^{-1} soil
Soil sample collected from diseased plant root No. 1	29.7 ± 3.4 pg	10.2 ± 1.5 ng
Soil sample collected from diseased plant root No. 2	365 ± 20.3 fg	20.2 ± 2.8 ng
Field soil sample	3.3 ± 8.6 fg	–

We also report the development of two approaches to increasing the sensitivity of molecular assays. The first method is nested PCR. This includes two rounds of amplification, first using universal primers (ITS1/ITS4) to increase the target DNA templates, and then using internal specific primers for the second round. The nested PCR method in this study provided consistent and reproducible results. Nested PCR has been reported to increase detection sensitivity by factors of 10–1000 [10,28–30]. In our study, the sensitivity of nested PCR assays using Fn-1/Fn-2 (or Mn-1/Mn-2) and the universal primers (ITS1/ITS4) was 1000 times higher than conventional PCR. The nested PCR assay therefore has potential as a diagnostic tool for detecting and surveying pathogens in diseased plants and soil.

In this study, we also describe the use of real-time PCR to quantify *F. oxysporum* f. sp. *niveum* and *M. melonis* directly from soil. To our knowledge, this is the first reported study on the detection and quantification of *F. oxysporum* f. sp. *niveum* and *M. melonis*, two soil-borne pathogenic fungi, using quantitative real-time PCR. Since both pathogens are soil-inhabiting fungi, their quantitative detection will benefit disease management.

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