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Detection of *Phytophthora nicotianae* in Soil with Real-time Quantitative PCR

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Abstract

Phytophthora nicotianae is one of the most important soil-borne plant pathogens. A rapid, specific and sensitive real-time polymerase chain reaction (PCR) detection method for *P. nicotianae* was established, which used primers targeting the internal transcribed spacer (ITS) regions of rDNA genes of *Phytophthora* spp. Based on the nucleotide sequences of ITS2 of 15 different species of *Phytophthora*, the primers and probe were designed specifically to amplify DNA from *P. nicotianae*. With a series of 10-fold DNA dilutions extracted from *P. nicotianae* pure cultures, the detection limit was 10 pg/μl in conventional PCR, whereas in SYBR Green I PCR the detection limit was 0.12 fg/μl and in TaqMan PCR 1.2 fg/μl, and real-time PCR was 10^4–10^5 times more sensitive than conventional PCR. The simple and rapid procedures maximized the yield and quality of recovered DNA from soil and allowed the processing of many samples in a short time. The direct DNA extractions from soil were utilized to yield DNA suitable for PCR. By combining this protocol with the real-time PCR procedure it has been possible to specifically detect *P. nicotianae* in soil, and the degree of sensitivity was 1.0 pg/μl. The system was applied to survey soil samples from tobacco field sites in China for the presence of *P. nicotianae* and the analyses of naturally infested soil showed the reliability of the real-time PCR method.

Introduction

Phytophthora nicotianae (*P. nicotianae*) Breda de Haan, a major genus of plant pathogen within the oomycetes, has a host range of more than 72 genera of plants (Hickmann, 1958). Soil-borne diseases caused by *P. nicotianae* are often intractable due to the release into soil of resistant perennating structures, oospores and/or chlamydospores, formed by the pathogen. Healthy nursery materials, fungicides and crop rotations are important elements in disease control, but they require early diagnosis and detection of the pathogen either in the plant, soil or water. Rapid and accurate identification and detection of *P. nicotianae* would improve diagnosis and prophylaxis, especially where several pathogens have the same host range, but are not equally severe in the disease that they cause. Moreover, measurements of inoculum in soil could be used to predict the scale of possible losses and for timing fungicide applications.

In the case of *P. nicotianae*, detection traditionally requires the isolation and culture of the fungus from soil or diseased plant material with identification based on morphological or physiological characters. This is time- and labour-consuming and requires considerable knowledge of the fungus (Tsao, 1983). Recently, new techniques have been used to lessen or eliminate some of these difficulties. Serology has been used to detect *Phytophthora* in a number of instances (Jones and Shew, 1988; Miller et al., 1989; McDonald et al., 1990). Specific DNA probes have also been used to detect *P. nicotianae* sensitively (Goodwin et al., 1990). However, the use of radioactivity prohibits a routine use of this method.

Alternatively, the polymerase chain reaction (PCR) can exponentially amplify specific DNA sequences of the fungus by *in-vitro* DNA synthesis. PCR for detection of a single pathogen or many members of a group of related pathogens can be done more quickly and at a lower cost than specific DNA probes or antibodies and has therefore considerable potential in a multipurpose detection method. Conventional PCR-based techniques have contributed to the alleviation of some of the issues associated with the detection, identification, control and spread of *P. nicotianae* (Lacourt and Duncan, 1997; Grote et al., 2002; Ippolito et al., 2002; Kong et al., 2003; Schena et al., 2004) and other plant pathogens (Bonants et al., 1997; Bridge and Spooner, 2001; Schena et al., 2002b). Nevertheless, technical limitations related to postamplification procedures (gel electrophoresis and ethidium bromide staining) and

cross contamination still limit large-scale applications of PCR for plant pathogen diagnosis. These difficulties may be overcome to a certain extent by real-time PCR, which combines the sensitivity of conventional PCR with the generation of a specific real-time fluorescent signal throughout the reaction. Real-time PCR enables automation of the technique and is suitable for large-scale sample processing and has the potential for an accurate quantification of target DNA (Schmittgen, 2001). Quantitative analyses are of basic importance for development of predictive diagnostic test to identify high-risk fields where pathogens inoculum is above threshold values (Cullen et al., 2001). Among the PCR-based real-time techniques, four main methods are currently used for the application of this technique in plant pathology, which can be grouped into amplicon sequence non-specific (SYBR Green I) and sequence-specific (TaqMan, Molecular beacons and Scorpion-PCR) methods. For the amplicon sequence non-specific method, if a single peak representing the specific products is observed, SYBR Green I is a simple and reliable low-cost method for monitoring PCR amplicons and for quantifying template DNA. For the amplicon sequence-specific detection methods, Taq Man seems much more promising and has been widely applied to identify different fungal and oomycete plant pathogens (Haugland et al., 2002; Vandemark et al., 2002; Mayer et al., 2003; Van de Graaf et al., 2003; Bonants et al., 2004).

In this study, we have assessed the primers and probe designed from internal transcribed space regions (ITS) (Cooke and Duncan, 1997) for their specificity towards *P. nicotianae* and their ability to detect the fungus. The effective detection of this pathogen in artificially inoculated soil as well as in naturally infested soil samples was reported. These molecular approaches to the sensitive identification and detection of *P. nicotianae* open up new pathways for monitoring the epidemiology of this economically important plant pathogen.

Materials and Methods

Fungal isolates and DNA extraction

All the fungal strains used in this study are listed in Table 1. *Phytophthora nicotianae* strains were isolated from diseased tobacco and tomato plants in Chongqing in 2006; other *Phytophthora* strains were available in our laboratory or from Southwest University. All the fungal strains were stored on potato dextrose agar slants at 20°C.

To extract total DNA, fungi were incubated in malt extract agar covered with sterile cellophane sheets before inoculation, to facilitate collection of the mycelium. Genomic DNA was extracted from fungal mycelium by using a hexadecyltrimethylammonium bromide procedure described by Talbot et al. (1993). Between 25 and 50 mg of mycelia, collected after 5–10 d of incubation at 24°C, were grounded in liquid nitrogen and suspended in 400 μl of extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 100 mM EDTA). Each sample was added into 50 μl of SDS (20%) before bathed for 1 h at 37°C and then was added into 75 μl of NaCl (5 M) and mixed gently. Sixty-five microlitre of CTAB/NaCl (10% CTAB, 0.7 M NaCl) was added before incubated at 65°C for 20 min. The mixture was extracted with an equal volume of chloroform/isoamyl alcohol (24 : 1) and mixed sufficiently and centrifuged for 10 min at 13 000 × ***g***. The aqueous phase was collected and precipitated with 0.6 volumes of 100% cold (−20°C) isopropyl alcohol. The precipitated DNA was washed with 70% cold ethanol and finally dissolved in 50 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6), quantified using a spectrophotometer and diluted to 50 ng/μl.

Soil DNA extraction

To evaluate DNA recovery and purity after direct extraction procedures, soil samples were inoculated with 5 g of mycelia of *P. nicotianae* per 50 g of soil, and uninoculated soil samples were included as negative control. Each inoculated sample was vortex-mixed for several minutes sufficiently and then incubated at

Table 1
Strains used in this study

Strains	Number of isolates	Host or Source	Conventional PCR[a]	Real-time PCR[b,c]
Phytophthora nicotianae	18	Tobacco, tomato	+	+ + +
Phytophthora boehmeriae	1	Cotton boll	−	−
Phytophthora cactorum	2	Apple	+	+
Phytophthora capsici	3	Unknown	−	−
Phytophthora cinnamomi	1	Avocado	−	−
Phytophthora citrophthora	2	Citrus	−	−
Phytophthora cryptogea	1	Atractylodes	−	−
Phytophthora drechsler	1	Cucumber	−	−
Phytophthora megasperma	2	Soybean	−	−
Phytophthora palmivora	1	Palm	−	−
Phytophthora infestans	1	Tomato	−	−
Phytophthora spp.	5	Unknown	−	−
Domain fungi isolates in from tobacco plant rhizosphere	4	−	−	−

[a]PCR amplification signal with the primers P.nic1 and P.nic2. + and −, positive and negative respectively.
[b]Real-time PCR: SYBR Green I PCR and TaqMan PCR.
[c]+ + +, + and −, very strong fluorescence, weak fluorescence and no fluorescence respectively.

room temperature for 3 d to allow the binding of cells to soil particles before DNA extraction. There were three replicates of each soil sample.

Five different methods were tested to evaluate DNA extraction from soil in terms of yield and purity. They are Soil DNA Kit (Taibaike Biotechnology Co. LTD, Beijing, China), TENS lysis (Kuske et al., 1997), PBS lysis (Edgcomb et al., 1999), Freeze-thaw lysis (Sun et al., 2006) and modified freeze-thaw lysis (based on Sun et al., 2006). Briefly, soil samples (0.5 g dry weight) were thoroughly mixed with 0.2 g of water-insoluble polyvinylpolypyrrolidone and then suspended in 3 ml of DNA extraction buffer [100 mM Tris-HCl (pH8.0), 100 mM EDTA (pH8.0), 100 mM Na_3PO_4 (pH8.0), 1.5 M NaCl, 10 g/l CTAB] and vortexed for 1 min before being frozen in liquid nitrogen for 2 min and then thawed in a 65°C water bath for 3 min. After three successive freeze-thaw cycles, 20 μl of proteinase K (10 mg/ml) and 500 μl of lysozyme (50 mg/ml) were added into each sample, shaken in 37°C for 30 min before addition of 600 μl of 10% SDS and bathed in 65°C for 20 min. The supernatant was collected and 0.05 g of water-soluble polyvinylpyrrolidone was added and mixed sufficiently before they were centrifuged at 13 000 × ***g*** for 10 min. Other manipulations were the same as those described above.

Primers design, specificity and sensitivity

Design of primers and TaqMan probe

Primers design was performed according to Ippolito et al. (2002) with modifications. Briefly, sequenced ITS regions from *P. nicotianae* were recovered using the Nucleotide Sequence Search Program provided by the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/Entrez). Conserved regions, internal to the DNA sequence flanked by universal primers ITS4 and ITS6 (Cooke and Duncan, 1997), were analysed using the similarity search tool Advanced BLAST 2.0 (http://www.ncbi.nlm.nih.gov/blast/blast.cgi?CMD=Web&PAGE_TYPE=BlastHome) (Altschul et al., 1997) to find significant nucleotide homologies in the molecular data retrieved from NCBI's integrated databases GenBank, EMBL and DDBJ. The primers and probe internal to the universal primers ITS4 and ITS6 (Cooke and Duncan, 1997) were designed using the PRIMER EXPRESS 1.0 (PE Biosystems, Foster City, CA, USA).

P.nic1: 5′-GAACAATGCAACTTATTGGACGTTT-3′

P.nic2: 5′-AACCGAAGCTGCCACCCTAC-3′

Pn-Pro: 5′-TTCACCAGTCCATCACGCCACAGC-3′

The probe was labelled with FAM (6-carboxy-fluorescein) and contained 6-carboxy-tetramethyl rhodamine as quencher. The specificity of the primers was tested by analyses of PCR products on gel and the specificity of the fluorescent probes was determined in real-time PCR.

PCR protocol

The conventional PCR was performed as follows: the reaction mixture (25 μl) consisted of 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.2 μM of each P.nic1 and P.nic2, 0.2 mM dNTPs, 1 units of Taq DNA polymerase (Promega, Mannheim, Germany) and 50 ng of DNA template. The amplification was carried out in a Perkin Elmer 9600 cycler (PE Applied Biosystems, Weiterstadt, Germany). After a denaturation step (94°C for 4 min), 30 cycles of amplification (94°C for 40 s, 55°C for 40 s and 72°C for 1 min) and 10 min at 72°C were performed. Ten microlitres of PCR products were analysed by electrophoresis in 1.5% agarose gels, stained by ethidium bromide and visualized under UV light.

SYBR Green I PCR reactions performed in a total volume of 25 μl containing 1× Hotstart Buffer, 1.5 mM $MgCl_2$, 0.3 μM of each P.nic1 and P.nic2, 0.2 mM dNTPs, 1 units of Hotstart Taq DNA polymerase (TIANGEN BIOTECH CO. LTD, Beijing, China) and 1 μl of DNA template, 1× SYBR Green I, 6.0% (v/v) dimethyl sulfoxide (DMSO) and 0.2 μg/ml of bovine serum albumin (BSA). Amplification was conducted in sealed tubes in a 96-well microtiter plate (Bio-Rad, Hercules, CA, USA) and consisted of an initial denaturing step at 95°C for 5 min followed by 42 cycles, each consisting of 15 s at 95°C and 45 s at 55°C. Fluorescence was monitored at 55°C using a spectrofluorometric thermal cycler (iCycler Thermal Cycler, Bio-Rad, Hercules, CA, USA) for real-time data collection during annealing-extension. In the TaqMan PCR, reactions were performed in a 25 μl mixture containing 1× Hotstart Buffer, 3 mM of $MgCl_2$, 0.2 μM of each P.nic1 and P.nic2, 0.1 μM TaqMan probe (FAM-labelled), 0.2 mM dNTPs, 1 units of Hotstart Taq DNA polymerase (TIANGEN BIOTECH CO. LTD) and 1 μl of DNA template, 6.0% (v/v) DMSO and 0.2 μg/ml of BSA. Amplification conditions and fluorescence monitoring were the same as those described above for SYBR green I.

To compare the sensitivity between SYBR Green I and TaqMan PCR reactions, the total DNA extracted from of *P. nicotianae* was serially diluted in the PCR reaction mixture. Water was used as a negative control to replace template. All reactions were conducted as described above. PCR line subtracted CF RFU was plotted and utilized to calculate the average background fluorescence emission in the initial PCR cycles before fluorescence increased. Threshold fluorescence intensity was determined at a level 10-fold higher than the standard deviation in the initial PCR cycles and any sample that reached a fluorescence value exceeding the fluorescence threshold value was considered positive. The PCR cycle at which fluorescence exceeded the threshold was defined as the cycle threshold (Ct). Therefore, data from molecular analyses of soil samples were recorded as Ct.

Soil samples from naturally infested soils

Tests were carried out with soils collected in May 2007 from the fields where diseased tobacco plants were observed. Forty-six soil samples were collected from

18 villages and towns of Pengshui (seven samples from three villages), Qianjiang (12 samples from five villages), Youyang (14 samples from five villages), Wulong (13 samples from five villages) in Chongqing. Three healthy soils (YW1, YW2 and YW3) collected from wheat fields in Youyang were used as negative controls. Soil samples were collected with a drill at a depth of 15–20 cm and kept at 4°C until processed. Each naturally infested soil sample was accurately crumbled, mixed and sieved before analyses. Three soil sample replicates of 0.5 g were collected from each infested soil and processed to extract DNA for molecular analyses according to the procedure described above. All soils were analysed by SYBR Green I PCR and TaqMan PCR, respectively, with primers P.nic1 and P.nic2 and probe Pn-pro as described before, and the Ct value is the average of three replicates.

Results

Primers specificity and sensitivity

The protocol for extracting DNA from pure cultures gave 0.5–2.0 $\mu g/\mu l$ of DNA. The specificity of primers and probe was determined against *P. nicotianae* and other *Phytophthora* isolates (Table 1). Primers P.nic1and P.nic2 amplified a unique DNA fragment of approximately 120 bp from *P. nicotianae* and *P. cactorum*, and no amplification was achieved with the other species (Table 1). In real-time PCR, a significant increase in fluorescence was observed for all *P. nicotianae* isolates after amplification by primers P.nic1 and P.nic2 and probe Pn-pro; however, for *P. cactorum* isolates, delayed fluorescence increases (higher Ct values) (data not shown) and lower fluorescence were achieved (Table 1). The sensitivity of the primers was assessed using serial dilutions of total DNA extracted from *P. nicotianae* and the detection limit in conventional PCR was 10 $pg/\mu l$ of DNA (Fig. 1).

SYBR Green I PCR, TaqMan PCR and sensitivity for DNA extracted from *Phytophthora nicotianae*

In the 10-fold dilutions, series of *P. nicotianae* DNA extracted from pure cultures, the detection limit was 0.12 $fg/\mu l$ in SYBR Green I PCR (Fig. 2a) and 1.2 $fg/\mu l$ in TaqMan PCR (Fig. 3a) respectively. Standard curves showed a linear correlation between input DNA and Ct after both SYBR Green I PCR (Fig. 2c) and TaqMan PCR (Fig. 3b). The correlation coefficients (r^2) of the standard curve were 0.999 and 0.996 for SYBR Green I PCR and TaqMan PCR respectively.

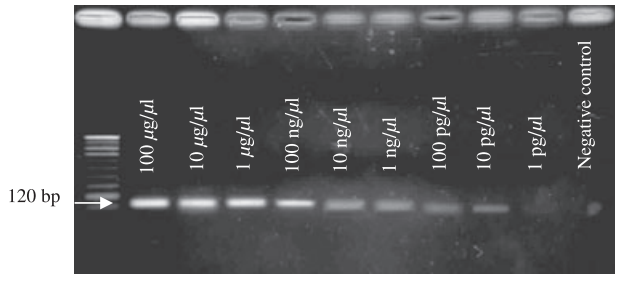

Fig. 1 Sensitivity of polymerase chain reaction (PCR) using a series of 10-fold dilutions of *Phytophthora nicotianae* DNA. The detection limit was 10 $pg/\mu l$ for *P. nicotianae* after PCR with primers P.nic1 and P.nic2. Negative control: containing no DNA. The arrow marks the position of the expected PCR product of approximately 120 bp. Marker (100-bp DNA ladder (TAKARA BIO INC, China) was loaded in the first lane on the left

Influence of soil DNA purity on successful amplification

Five different methods were tested to evaluate total genomic DNA extraction from soil in terms of yield and purity and the results indicated that no significant difference in DNA yield, but DNA absorbance A_{260}/A_{280} is very different between different preparations (Table 2). The frequent presence of humic substances in DNA with low purity greatly reduced the detection limit.

The method which we have optimized is inexpensive and with a small number of efficient lysis and purification steps to maximize the yield and quality of recovered DNA and allow the processing of many samples in a short time. Total processing time for DNA extraction from soil by the modified free-thaw lysis was at most 2 h.

Detection of *Phytophthora nicotianae* in artificially inoculated soils

The applicability of SYBR Green I PCR and TaqMan PCR to soil DNA was assessed. In the 10-fold dilutions series of soil DNA isolated by modified freeze-thaw lysis, the detection limit was 100 $fg/\mu l$ in SYBR Green I PCR and 1.0 $pg/\mu l$ in TaqMan PCR respectively. Standard curves showed a linear correlation between input DNA and Ct after both SYBR Green I PCR and TaqMan PCR. The correlation coefficients (r^2) of the standard curve were 0.996 and 0.940 for SYBR Green I PCR and TaqMan PCR respectively.

Detection of *Phytophthora nicotianae* in naturally infested soils

The SYBR Green I PCR and TaqMan PCR were applied to detect *P. nicotianae* in a survey of 46 soil samples from 18 villages and towns in Chongqing, China. Tobacco black shank caused by *P. nicotianae* was reported in 2007 in these field sites. The PCR amplifiability of all DNA samples directly extracted from soil was confirmed by the amplification of 16S rDNA fragments (data not shown). Positive signals were found in soil DNA of 44 samples in SYBR Green I PCR and similar results were achieved in TaqMan PCR. Ct value ranges from 20.1 to 24.6 in SYBR Green I PCR and from 24.6 to 27.0 in TaqMan PCR, which indicated that the latter is less sensitive than the former. Vestiges of *P. nicotianae* could not be detected in two soil samples in both systems, indicating that *P. nicotianae* was either not present or was present under the concentrations of 1.0 pg per reaction. Negative control (without DNA) and soil samples from

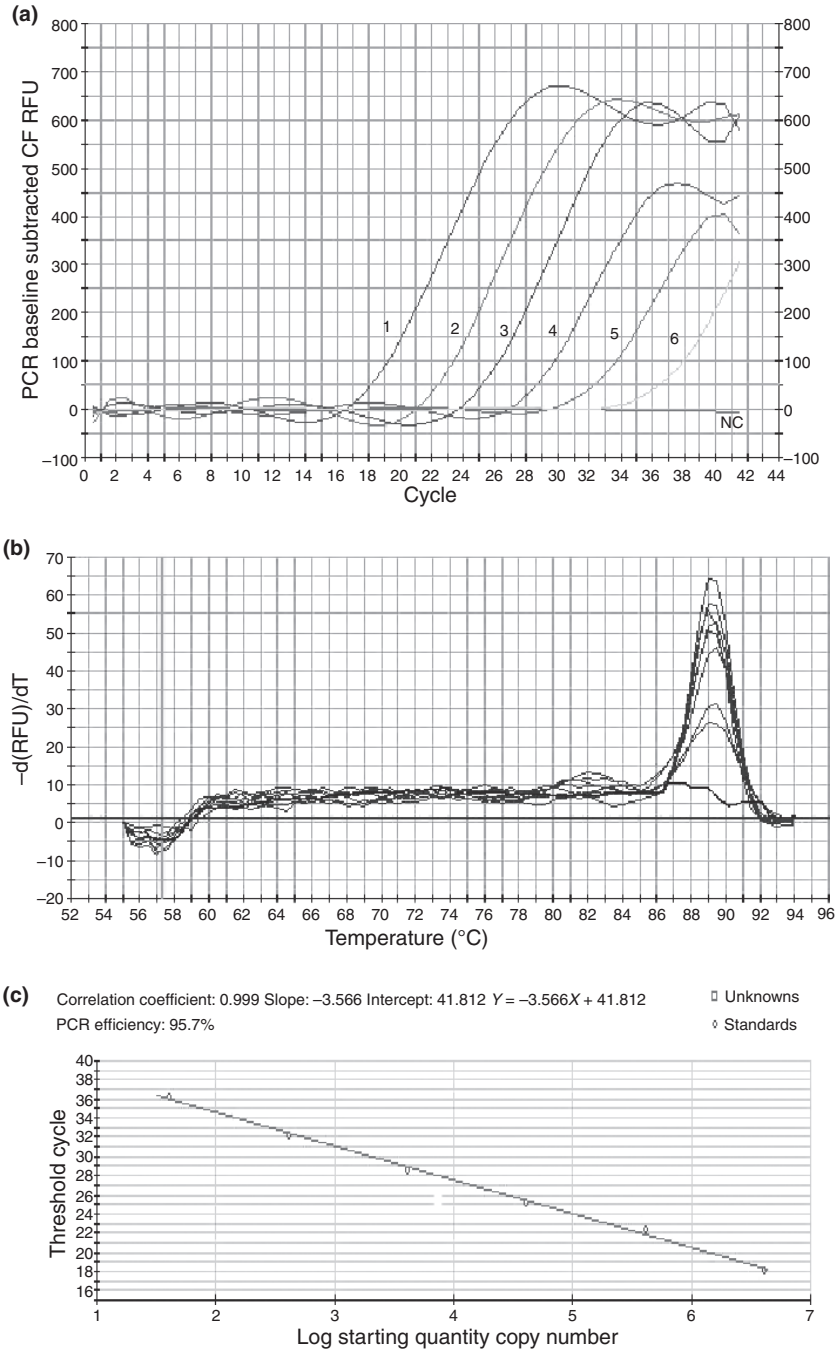

Fig. 2 Sensitivity of SYBR Green I PCR assessed by 10-fold serial dilutions of *Phytophthora nicotiana*e DNA extracted from pure cultures. (a) Real-time amplification curve of different concentration of DNA. Line 1–6: 10-fold dilution of *P. nicotiana*e DNA (12 pg/μl–0.12 fg/μl), NC: PCR-negative control containing no DNA. (b) Melting curve analysis. (c) Standard curve analysis. The relationship between copy number and corresponding Ct value was reported. Linear regression equation of Ct (x) on Log (starting quantity) (y) was: $Y = -3.566X + 41.812$

wheat fields were always negative and no fluorescence was observed.

Discussion

The main goal of this study was to develop a sensitive and effective method suitable for large-scale analysis of *P. nicotianae* in soil. The primers P.nic1 and P.nic2 were less specific for *P. nicotianae*, with amplification from the pathogen as well as from *P.cactorum*, but there is a difference in the fluorescence increase in real-time PCR. For all *P. nicotianae* isolates, a significant fluorescence increase was observed after amplification by primers P.nic1 and P.nic2; however, for *P. cactorum* isolates, delayed fluorescence increases (higher Ct values) and lower fluorescence were achieved, which was consistent with previous results (Ippolito et al., 2004). So, the real-time PCR system was capable of detection of *P. nicotianae* in soil with high sensitivity and specificity. To verify the specificity of the system, four different domain fungi strains isolated from the tobacco plant rhizosphere were used as templates to amplify and no PCR product was obtained.

Special emphasis was placed on the applicability real-time PCR to soil DNA. One of the major obstacles to the use of PCR on soil samples is the frequent presence of organic matter, such as humic substances, which are strong inhibitors of PCR, possibly by interacting with the DNA polymerase or interfering the

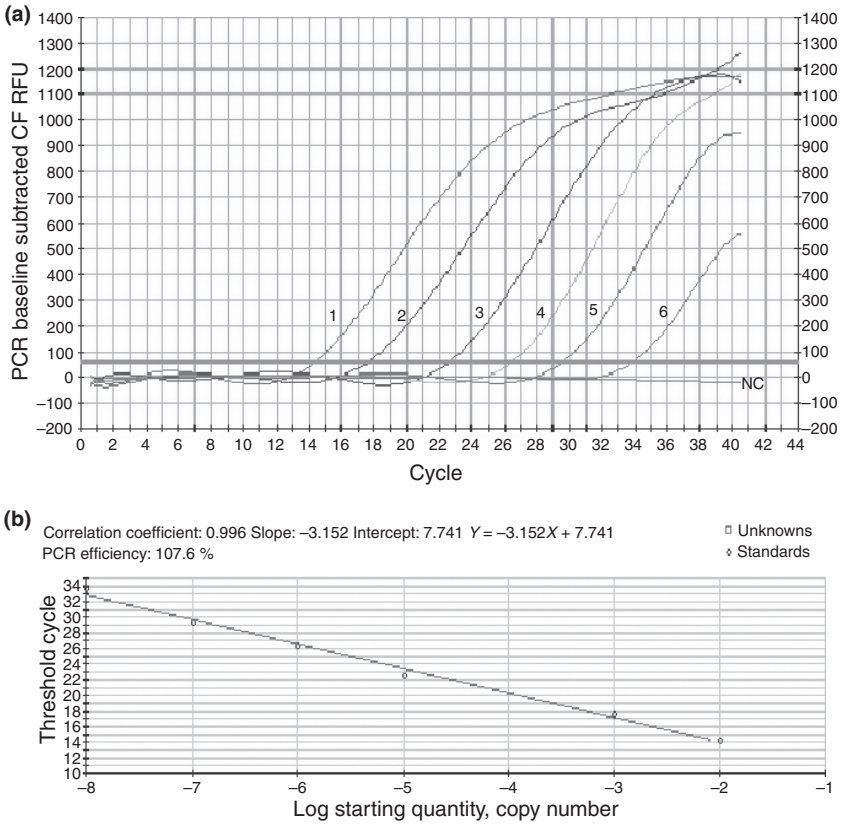

Fig. 3 Sensitivity of TaqMan PCR assessed by 10-fold serial dilutions of *Phytophthora nicotianae* DNA extracted from pure culture. (a) Real time amplification curve of different concentration of DNA; 1–6: 10 fold dilution of *P. nicotianae* DNA (120 pg/μl–1.2 fg/μl), NC: PCR-negative control containing no DNA. (b) Standard curve analysis. The relationship between copy number and corresponding Ct value was reported. Linear regression equation of Ct (x) on Log (starting quantity) (y) was: $Y = -3.152X + 7.741$

Table 2
DNA yield and purity from different preparations

Extraction methods	DNA yield (μg DNA per g dry soil)[a]	Absorbance (A_{260}/A_{280})[b]
Modified freeze-thaw lysis	15.67	1.72
Freeze-thaw lysis (Sun et al., 2006)	15.60	1.60
TENS lysis (Kuske et al., 1997)	17.12	1.41
PBS lysis (Edgcomb et al., 1999)	17.89	1.49
Soil DNA Kit (Taibaike Biotechnology Co. LTD, Beijing, China)	15.38	1.78

[a,b]The values were the average of three replicates.

binding of the primers to the template, and thereby greatly reducing the detection limit (Tsai and Olson, 1992). In this study, the optimized freeze-thaw lysis was generally more efficient than other DNA extraction methods reported previously (Kuske et al., 1997; Edgcomb et al., 1999; Sun et al., 2006) and important to increase the efficiency of detection. The high sensitivity of the real-time PCR combined with the DNA extraction from soil by modification freeze-thaw lysis enabled the detection of *P. nicotianae* in artificially inoculated soils. On the basis of these results, it proved that the system was capable of detection of *P. nicotianae* in naturally infected soils.

In conclusion, the real-time PCR combined with a fast protocol to extract DNA from soil led to the development of a specific, sensitive and rapid detection method for *P. nicotianae* in soil. The procedure is robust, rapid, automated and quantitative, with high sample throughput potential, permitting analysis of up to 96 samples in 4–5 h. Avoidance of laborious post-PCR gel electrophoresis and greatly reduced opportunity for contamination of reaction mixtures with target DNA further increase the suitability of this assay for routine diagnostic testing. The availability of such a method has great practical importance, as checking the soil for the presence of the pathogen before planting is essential to prevent disease developing. Another promising application of the method is in monitoring the fate of the pathogen over time. Shifts in the *P. nicotianae* population size can be detected by quantifying the PCR product, for example, by real-time PCR. Thus, the main advantage of the approach is the possibility to follow the fate of the pathogen without the necessity of introducing a special marker and the detection results can direct the management of the pathogen. Moreover, this system could be used for large-scale testing of the pathogen for the production of disease-free plantlets.

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