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Quantitative real-time PCR assay for rapid detection of plant and human pathogenic *Macrophomina phaseolina* from field and environmental samples

Bandamaravuri Kishore Babu[1]
Sukumar Mesapogu
Anu Sharma
Saida Reddy Somasani
Dilip K. Arora

National Bureau of Agriculturally Important Microorganisms (NBAIM), Post Box 06, Maunath Bhanjan, Uttar Pradesh-275 101, India

Abstract: A real-time qPCR assay was developed to detect and quantify *Macrophomina phaseolina* abundance in rhizosphere soil and plant tissue. Both TaqMan and SYBR green techniques were targeted on ~ 1 kb sequence characterized amplified region (SCAR) of *M. phaseolina* and two sets of specific primers were designed for SYBR green (MpSyK) and TaqMan (MpTqK) assays. No cross-hybridization and no fluorescent signal exceeding the baseline threshold was observed in TaqMan and SYBR green assays, respectively. The minimum detection limit or sensitivity of TaqMan assay was 30 fg/μL of *M. phaseolina* DNA and limit of quantification of *M. phaseolina* viable population was estimated as 0.66×10^5 CFU/g soil^{-1} equivalent to 10 pg/μL of target DNA. This is the first report which demonstrated real-time qPCR assays with greater specificity and sensitivity to detect *M. phaseolina* population in soil and plant materials.

Key words: rhizosphere, SCAR, SYBR green assay, TaqMan probe

INTRODUCTION

Macrophomina phaseolina (Tassi) Goid. is a soilborne plant pathogen infecting about 500 crop plants worldwide (Mihail et al. 1995). Reports have indicated that *M. phaseolina* can be an opportunistic fungal pathogen causing infections to immunosuppressive patients (Tan et al. 2008, Srinivasan et al. 2009). The conventional methods to identify the pathogen most often have relied on symptoms, isolation and culturing of pathogen followed by morphological observations and biochemical tests (Byadgi and Hedge 1985, Cloud and Rupe 1991, Pearson et al. 1987, Tan et al. 2008). Attempts to develop molecular identification and detection of *M. phaseolina* with specific primers and probes have been targeted on the internal transcribed spacer (ITS) region (Babu et al. 2007). Even though primers showed high specificity, dot-blot hybridization assays with oligonucleotide probes were not sufficiently sensitive. Furthermore the dot-blot technique is time consuming and subject to cross reactivity under field trials.

Quantitative real-time PCR (qRT-PCR) based assays have advantages of speed, accuracy and sensitivity over other detection techniques (Gachon et al. 2004, Schaad and Frederick 2002, Schena et al. 2004, Wong et al. 2005). In addition the qRT-PCR method has been employed to a wide range of fungal pathogens. In such studies various target sequences were used to distinguish the target microbe from the nearest neighbors under laboratory and field conditions (Bonants et al. 2004, Boonham et al. 2004, Ippolito et al. 2004, Weller et al. 2000, Hayden et al. 2006, Hughes et al. 2006, Tooley et al. 2006, Tomlinson et al. 2007). However in *M. phaseolina* no such specific approach has been described to date.

The sequence characterized amplified regions (SCAR) approach also has been used to develop specific primers to monitor and detect fungal strains released into the field (Ye et al. 2006, Hagn et al. 2007, Savazzini et al. 2008, Kim et al. 2008). Although such an approach requires more time and expertise relative to conserved and housekeeping genes, it provides specific target sequences to detect target organisms in complex environments.

In plant infections by *M. phaseolina* the sclerotia were considered to be a major source of inoculum, the disease occurrence and severity has been directly related to the population of viable sclerotia in soil (Khan 2007). In human infections *M. phaseolina* hyphae have been detected in skin lesions and purulent discharge of immunosuppressive patients (Tan et al. 2008, Srinivasan et al. 2009), and thus *M. phaseolina* might be considered significant to both plant and human health. Moreover the development of a rapid method to detect and quantify the viable population of *M. phaseolina* mycelia as well as sclerotia in clinical, agricultural and environmental samples would have potential applications. Therefore the aim of this study was to detect specific SCARs in *M. phaseolina* genome; to develop species-specific primers and probe for real-time PCR and to test the assay for enumeration and quantification of *M. phaseolina* vegetative population under laboratory and field conditions.

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[1] Corresponding author. E-mail: kishore_bandam@yahoo.co.in

TABLE I. *M. phaseolina* isolates collected from hosts and locations in this study

Isolate number/ NBAIM accession number	Biological and geographical origin	GenBank accession No.
mpk1 F-302	Sorghum, Jorhat, Assam	GU081134
mpk2 F-1277	Sorghum, Gulberga	
mpk3 F-1291	Sorghum, Delhi	GU081135
mpk4 F-1276	Sorghum, Solapur, Maharastra	GU081142
mpk5 F-1275	Sorghum, Andhra Pradesh	
mpk6 F-1287	Sorghum, Ballia, Uttar Pradesh	GU081136
mpk7 F-1292	Sorghum, Varanasi, Uttar Pradesh	
mpk8 F-1262	Soil, Kansas, USA	
mpk9 CABI-277878[a]	NA[b]	
mpk10 CABI-263176	NA	
mpk11 F-1265	Chickpea, Varanasi, Uttar Pradesh	GU081137
mpk12 F-300	Chickpea, Jorhat, Assam	
mpk13 F-1296	Chickpea, Mau, Uttar Pradesh	
mpk14 F-1272	Chickpea, Varanasi, Uttar Pradesh	
mpk15 F-1281	Chickpea, Karnataka	GU081138
mpk16 F-1296	Chickpea, Mau, Uttar Pradesh	
mpk17 F-1271	Chickpea, Delhi	GU081139
mpk18 F-1295	Chickpea, Ballia, Uttar Pradesh	
mpk19 F-1274	Soybean, Jhansi, Uttar Pradesh	GU081140
mpk20 F-1273	Soybean, Karnataka	
mpk21 F-1269	Soybean, Delhi	GU081141
mpk22 F-1268	Soybean, Madhya Pradesh	
mpk23 F-1284	Soybean, Andhra Pradesh	
mpk24 F-1268	Soybean, Madhya Pradesh	
mpk25 F-1262	Soybean, Varanasi, Uttar Pradesh	

[a] CABI = Centre for Agriculture and Biosciences International, United Kingdom.
[b] NA = Data not available.

MATERIALS AND METHODS

Fungal cultures and extraction of genomic DNA.—In this study 25 isolates of *M. phaseolina* from different geographical and biological origins were obtained from the National Bureau of Agriculturally Important Microorganisms (NBAIM), India (TABLE I). The fungal DNA was extracted as described by Babu et al. (2007) while DNA from actinomycetes and bacteria were extracted with the aid of a Wizard Genomic DNA Purification Kit (Promega, USA).

DNA extraction from soil and infected plant materials.—Host plants (chickpea, soybean and pigeon pea) infected with *M. phaseolina* and rhizosphere soil samples were collected from experimental fields of Indian Council of Agricultural Research (ICAR) and farm fields nearby. The soil samples and infected plant materials (roots and stems) were used for in vitro culture of the pathogen. About 1 g rhizosphere soil and infected plant materials was subjected to total DNA extraction with a Power Soil DNA Isolation Kit (MoBio Laboratories, California) and samples were stored at 4 C for further use.

URP-PCR for SCAR fingerprinting.—Preliminary screening was done to identify SCAR markers that produce distinct banding patterns among the *M. phaseolina* isolates. Out of eight universal rice primers screened, the URP-9F (5′-ATGTGTGCGATCAGTTGCTG-3′) (Kang et al. 2002) was selected for random amplification of *M. phaseolina* genome. PCR was carried out in a total volume of 25 μL containing 2.5 μL 10× PCR buffer (100 mM, Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 250 mM KCl), 1 U *Taq* DNA polymerase (Bangalore Genei, India), 160 μM dNTP mixture, 50 pmol primer and 50 ng genomic DNA in sterile distilled water(SDW). The PCR program was initiated by denaturation at 95 C (7 min), followed by 30 cycles at 94 C (1 min), 54 C (1 min), and 65 C (8 min) the reaction was terminated with an extension step of 72 C (10 min).

PCR-amplified products were resolved by gel electrophoresis (4 Vcm^{-1}) on 1.4% agarose in 1× TAE buffer containing 0.5 mg mL^{-1} ethidium bromide (EtBr) and viewed under UV transilluminator.

Cloning and sequencing of SCAR product.—The ~ 1 kb monomorphic band was excised from the gel (FIG. 1) with a spin gel extraction kit (Bangalore Genie, India) and quantified. The pure ~ 1 kb product was ligated to pGEM®-T vector and transformed into competent *E. coli* JM107 (Promega, UK). Plasmid DNA was isolated and purified with QIAGEN mini-prep kit (QIAGEN, UK), and ~ 1 kb insert was used for sequencing on an ABI automated DNA sequencer with ABI Big Dye termination cycle sequencing ready reaction kit following the protocol of the manufacturer. The resulting sequences were analyzed for homology with sequences in the GenBank database at NCBI.

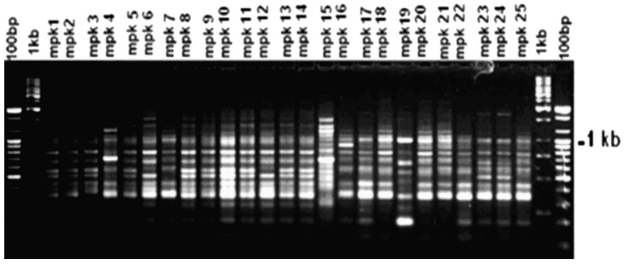

FIG. 1. PCR fingerprinting pattern: Genomic DNA of *M. phaseolina* isolates obtained from different biological origin were amplified by URP-9F primer. −1 kb represents specific monomorphic band (present in all the isolates) used for SCAR development. 100 bp and 1 kb are molecular markers.

Design of SYBR green, TaqMan primer and probe.—The real-time PCR assays were targeted on conserved regions (SCAR) of *M. phaseolina*. Sequences from the monomorphic fragments were analyzed by BLAST queries against non-redundant (nr) database at GenBank to aid the design and testing of the assays for species specificity. The SCAR-derived sequence initially was targeted to design the candidate primers with the aid of the online software Primer3Plus (Steve and Helen 2000), and four pairs of candidate primers were designed in such a way that PCR products were shorter than 200 bp. Other important parameters, such as melting temperature (55–65 C), primer length and absence of secondary structure, also were optimized. For the qPCR assay a normal minor grove binding (MGB) TaqMan probe was designed for the standard two-step qPCR amplification protocol with Primer Express 5.1 software (Applied Biosystems, USA). And 5′ extension of the forward primer (MpTqK F) was adjusted to locate appropriate 5′ hydrolysis of the probe. Theoretical matching and specificity of all the oligonucleotides for the species-specific qPCR assays was verified against the universal and fungal-specific GenBank database (http://blast.ncbi.nlm.nih.gov/) with nucleotide Mega BLAST (Altschul et al. 1997). The primer and probe sequences (TABLE II) were synthesized by the Applied Biosystems oligonucleotide factory.

Real-time PCR assay.—Real-time PCR was performed with StepOne™ real-time PCR system (Applied Biosystems, USA). Fluorescent molecules (SYBR green, dual-labeled TaqMan probe) were included in 20 µL PCR master mix. The reaction conditions specific to each detection technology were set up as follows: In SYBR green assay all reactions performed in 0.5 mL thin-walled, optical-grade PCR tubes and PCR assay with 0.4 µM of each primer (MpSyK F and R, TABLE II), and 1× SYBR® Green PCR Master Mix (Applied Biosystems, USA) with 5 µL template DNA. PCR cycling was set at 95 C (15 min), 40 cycles at 94 C (15 s), 60 C (30 s), and 72 C (30 s), and fluorescence read at 72 C.

For the TaqMan assay each reaction mixture contained 5 µL DNA template, 1× TaqMan® Universal Master Mix, (Applied Biosystems, USA) and the concentration optimized were 18 µM and 5 µM for primers (MpTqK F and R) and probe (MpTqK P) respectively (TABLE II). The standard two-step qPCR thermal cycling conditions consisted of initial soak (2 min) at 50 C and 10 min at 95 C, followed by 40 cycles of 15 s (95 C) and 1 min (60 C) and fluorescence for extension during 65–68 C recorded. Determination of cycle threshold (C_T) and data analysis were carried out with the help of Detection 1.2 software (Applied Biosystems, USA).

Specificity and accuracy of the assay.—The specificity of each TaqMan and SYBR green assays was verified in separate experiments with an array of *M. phaseolina* isolates obtained from different agro-climatic zones (TABLE I), including the cultures used in Babu et al. (2007), along with other tester microbes (TABLE III). DNA extractions were prepared as described above. A total of 5–60 ng template DNA added to the reaction mixture, *M. phaseolina* genomic DNA used in positive control and DNA from other test microbes was used in unknown targets. All these assays were analyzed in duplicate reactions.

Sensitivity, amplification efficiency and standard curve.—Genomic DNA of *M. phaseolina* (mpk4) diluted in a series of 1:10 (initial concentration 100 ng/µL) was used to estimate the amplification efficiency of real-time PCR assays. The quantity of the target molecules determined with the C_T comparative cycle threshold method (Haugland et al. 1999, Roe et al. 2001). The data were analyzed in terms of log of template concentration vs. C_T values. PCR efficiency was calculated with the formula $E = (10^{(-1/\text{slope})} - 1) \times 100$, where E is the amplification efficiency (relative increase in the product per cycle) and the slope derived from the log of template concentrations vs. Cycle threshold (C_T). A slope of 3.32 represents 100% amplification efficiency. The sensitivity or minimum detection limit of the assay was estimated as minimum quantity of target DNA detected when the cycle threshold was being attained up to 40 cycles.

Enumeration and quantification of M. phaseolina.—About 50 g sterile sandy soil was mixed with 10 mL *M. phaseolina*

TABLE II. Species-specific oligonucleotides developed for identification and quantification of *M. phaseolina*

Sl. No.	Name	Sequence (5′–3′)	Chemistry
1	MpSyK F	ATCCTGTCGGACTGTTCCAG	SYBR green
	MpSyK R	CTGTCGGAGAAACCGAAGAC	
2	MpTqK F	GCCTTACAAGGGTCTCGTCAT	TaqMan
	MpTqK R	CCCTTGGCGATGCCGATA	
	MpTqK P	6-FAM-CAGGCCACAGGATCTT-MGBNFQ	

FAM = 6-carboxy-fluoroscein, MGBNFQ = minor groove binder non-fluorescent quencher.

TABLE III. Microbial cultures used in this study

Microorganism	NBAIM accession No.
Fusarium udum	F-138
Fusarium oxysporum f. sp. *ciceri*	F-2791
Neurospora crassa	F-1425
Aspergillus niger	F-583
Alternaria alternate	F-143
Alternaria brassicicola	F-076
Trichoderma viride	F-1316
Chaetomium globosum	F-444
Trametes lactinea	F-1852
Pencillium brevicompactum	F-1496
Verticillium lecanii	F-2102
Phytophthora cambivora	F-1648
Rhizopus oryzae	F-1747
Beauveria bassiana	F-298
Sclerotium rolfsii	F-1766
Rhizoctonia solani	F-1723
Metarhizium anisopliae	F-1311
Phialophora calciformis	NA[a]
Psuedomonas putida	B-231
Bacillus megaterium	B-67
Streptomyces	B-475
Sinorhizobium meliloti	B-471
Serrotia marcescens	B-459
Mesorhizobium ciceri	B-313
Burkholderia cepacia	B-273
Lactobacillus acidophilus	B-304
Klebsiella pneumoniae	B-302
E. coli.	B-283

[a] NA = Data not available.

sclerotial suspension and 1 g of the above mixture resuspended in 10 mL SDW with 0.01% Tween 80. Colony-forming units (CFU) were determined from serial 10-fold dilutions after 5 min shaking, 1 min settling. A total of 1 mL each dilution was spread on Petri dishes containing semiselective media (PDA supplemented with 100 μg g^{-1} rose Bengal). Total CFUs were counted after 3–5 d incubation at 28 C. From each dilution treatment the same amount of suspension (1 mL) was used for DNA extraction. The TaqMan qRT-PCR assay was performed in replicate reactions with the template DNA extracted from sclerotial suspensions. Standards were prepared with *M. phaseolina* DNA and were amplified under same conditions as described above. Comparisons between estimated CFU (No. of CFU × dil. factor/g $soil^{-1}$) and expected sclerotial DNA concentrations (ng/μL) were made to account for accuracy of the approach.

Detection of M. phaseolina in field samples.—Naturally infected plants and their rhizosphere soil samples were collected, and the presence of *M. phaseolina* was confirmed by standard in vitro culture. Direct DNA isolation from the samples was performed as described above. Detection and quantification of *M. phaseolina* were performed in the TaqMan qRT-PCR assay with DNA extracted from each field sample used as unknown target. Genomic DNA of *M. phaseolina* was used as a positive control, and healthy, uninfected (chickpea) plant DNA was used as a negative control. The PCR assay for standard graph was performed under similar conditions described above.

RESULTS

Genomic DNA from 25 isolates of *M. phaseolina* amplified with RAPD and URP primers failed to produce distinct banding pattern (data not shown). The fingerprinting pattern by URP-9F produced bands 200 bp to 1.5 kb (FIG. 1). One monomorphic band of ~ 1 kb was observed from all the isolates of *M. phaseolina* (FIG. 1). This unique and prominent ~ 1 kb fragment from *M. phaseolina* (mpk4) was gel extracted, cloned and sequenced.

Analysis of SCAR sequences.—The DNA sequence GU018142 (888 bp) derived from mpk4 exhibited no significant similarity (> 80%) with the sequences in GenBank. Candidate primers initially were generated corresponding to the SCAR fragment and tested against different isolates of *M. phaseolina* and other microbes in normal PCR. The primer set targeted for ~ 150 bp region has shown specific amplified product from *M. phaseolina* but not with other microbes tested (TABLE III). The ~ 150 bp sequences from eight *M. phaseolina* isolates were aligned completely and BLAST queries against GenBank found no significant matches. This primarily confirmed that the specific (SCAR) sequence existed only in *M. phaseolina* species.

Real-time PCR assays.—SYBR green assay. Species-specific primers (MpSyK F and R) designed for SYBR green assay first were demonstrated with serially diluted *M. phaseolina* (mpk4) genomic DNA. The standard fluorescent amplifications representing exponential growth of PCR products was observed at the end of each cycle, and the mean threshold cycle (C_T) value of 26.65 was obtained with an amplification efficiency of 79.02%. The melting curve for PCR products at the end of the cycling reactions revealed single dissociation peak at 82–83 C, which indicates the specific binding of the primers (FIG. 2).

TaqMan assay. Two primers (MpTqK F and R) and one MGB probe (MpTqK P) had lower C_T values when assay conditions (e.g. PCR components, cycling conditions and extension temperature/time) were optimized. The standard curve between log of DNA concentration vs. C_T value generated a linear fit with a slope of −3.041 and linear regression coefficient (R^2) 0.999. The PCR amplification efficiency was 113.336% over at least seven orders of magnitude. The minimum detection limit of TaqMan assay (30 fg/μL) was

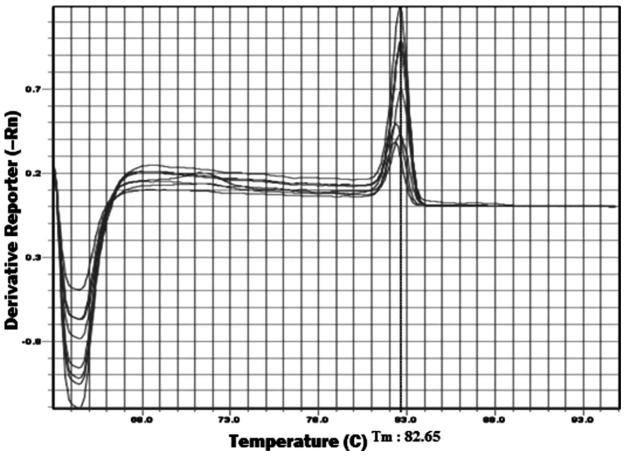

FIG. 2. Melting curve of amplified products obtained by real-time PCR. The single peak at 82.65 C with *M. phaseolina* DNA as template indicates the specificity of the MpSyK F and R primers.

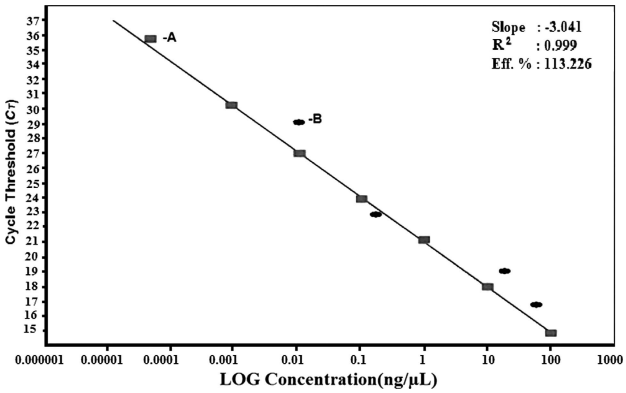

FIG. 3. qRT-PCR assay: Standard curve established between Log of DNA concentration vs. cycle threshold (C_T) obtained in TaqMan assay. ■ Represents serially diluted (1 : 10) *M. phaseolina* genomic DNA (initial concentration 100 ng/µL). ⬬ Indicates concentrations of DNA obtained from diluted sclerotial suspension. A. Represents minimum detection limit/sensitivity of the assay (30 fg/µL). B. Limit of quantification of viable/vegetative population of *M. phaseolina* estimated through sclerotial CFUs (10 pg/µL or equivalent to 0.66×10^5 CFU/g soil^{-1}).

obtained by extrapolation of the regression line to a C_T value of 40 (FIG. 3).

Detection and quantification of M. phaseolina in soil and environmental samples.—In preliminary experiments specificity tests were applied with real-time PCR primers in both chemistries (SYBR green and TaqMan) and verified with genomic DNA from pure cultures of microbes (TABLE III). No cross hybridization and no fluorescent signal exceeding the baseline threshold was observed respectively in TaqMan and SYBR green assays.

Further validation of quantitative real-time PCR experiments was performed in TaqMan assays. The CFU counts for four diluted sclerotial suspensions were 6.5×10^2–0.66×10^5 g soil^{-1}, and the corresponding qPCR results revealed the DNA concentrations range of 75 ng to 10 pg/µL (TABLE IV). In further dilutions (i.e. $> 10^6$) of sclerotia either CFU counts or *M. phaseolina* DNA was detected. The lowest detection limit of *M. phaseolina* vegetative population was estimated at 10 pg/µL or equivalent to 0.66×10^5 CFU/g soil^{-1} (TABLE IV). These tests demonstrated the accuracy and sensitivity of the qRT-PCR method at the five tested sclerotial concentrations.

For field validation experiments the qRT-PCR assay was optimized to detect the pathogen in the DNA samples directly extracted from soil and plant materials. The plate culture of plant materials (roots and stem) showed in planta colonization of *M. phaseolina* and was confirmed by providing positive signals in qRT-PCR assay. In field soil samples CFUs of pathogen were counted in plate assay and corresponding target DNA concentrations were estimated in qRT-PCR (TABLE IV). The lowest target DNA concentration of *M. phaseolina* (50 fg/µL) was detected from field soil sample 4; however no CFUs were obtained in corresponding plate assay.

DISCUSSION

Fungal diagnostics has dramatically increased with the inception of molecular tools in general and real-time PCR technology in particular. Development of specific sequences enables detection of fungal pathogens in samples without the need for culturing. The technology has multifaceted applications in plant pathology, especially in rapid screening of quarantine and suspected samples for detection of fungal pathogens. The main objective of the present work was to develop improved methods for detection and quantification of *M. phaseolina* more specifically suited for field applications. Although we did not include any clinical samples in this study, the real-time detection assay was tested with DNA obtained from different source, such as mycelia, sclerotia, soil and plant samples, and so has the potential for clinical applications. Moreover to represent the assay as a universal tool for detection of *M. phaseolina* an array of isolates obtained from different agro-climatic zones (TABLE I) along with other *M. phaseolina* isolates reported in Babu et al. (2007) were tested in assays and positive signals were obtained.

This study demonstrated two species-specific primer sets designed for the same target sequence (SCAR)

TABLE IV. Evaluation of TaqMan qPCR assay

Sample number	Sample	Number of CFU × dilution. factor/g soil^{-1}	TaqMan qPCR assay log of DNA concentration(ng/μL)
Assay for soil samples mixed with sclerotial suspension			
		6.5×10^2	75
		3.2×10^3	20
		1×10^4	0.200
		0.66×10^5	0.10
Field assay for plant and rhizosphere soil samples			
1	Chickpea	+	3
	Soil	2×10^4	1
2	Pigeon pea	+	25
	Soil	11×10^4	70
3	Soybean	+	10
	Soil	6×10^4	20
4	Soil	−	50 fg/μL

M. phaseolina presence (+) and absence (−) in plate culture assay.

for two different RT-PCR chemistries (TaqMan and SYBR green). Specific primers developed based on the SCAR approach have been deployed for detection and quantification of *Trichoderma* spp. in soils with real-time PCR (Hagn et al. 2007, Savazzini et al. 2008). However the assays targeted on specific SCARs and/or gene sequences apparently have greater sensitivity and specificity over the ITS-based quantification assays in field applications (Tomlinson et al. 2007). On comparative grounds the TaqMan assay seems to be more sensitive than the SYBR green assay. Similarly several researchers developed real-time PCR based detection techniques (TaqMan, SYBR green and molecular beacons) for detection of *Phytophthora ramorum* and have conducted comparative studies concerning the degree of sensitivity of the assays to provide optimum methodology for field application (Bilodeau et al. 2007, Hayden et al. 2006, Tomlinson et al. 2007). From our results, although both chemistries showed high specificity to *M. phaseolina*, the TaqMan assay is recommended because of its high amplification efficiency and quicker detection. Therefore validation and quantification experiments were carried out with TaqMan assay and discussed further.

With regard to validation tests the standard curves were established with known concentrations of serially diluted genomic DNA extracted from *M. phaseolina* and the sensitivity or minimum detection limit of TaqMan assay was 30 fg/μL (FIG. 3). We demonstrated the correlation between quantification and estimated CFUs in serially diluted sclerotial suspensions to achieve the absolute concentration or limit of quantification (LOQ) of viable population of *M. phaseolina* was 10 pg/μL or equivalent to 0.66×10^5 CFU/g soil^{-1}. In soil sample 4 the concentration of target molecules turned out to be 50 fg/μL although no CFUs were detected in a way demonstrating the probability that the dead propagule or partially degraded target DNA failed to develop into CFU (TABLE IV). It was clearly indicated in the present study that the detection of ≥ 10 pg/μL *M. phaseolina* DNA in the sample indicates the presence of at least 1(≥ 1) CFU/vegetative hyphae/sclerotium. Because the TaqMan assay was derived from SCARs (not based on any gene) we have not correlated the CFUs with gene copy number. However such correlations have been determined for *Trichoderma atroviride* SC1 targeted on endochitinase 42 (*ech42*) gene (Savazzini et al. 2008). *Alternaria brassicae* real-time assay was designed to detect pathogenicity and toxin-producing genes (Guillemette et al. 2004). Similarly Mogens et al. (2009) also correlated the mycotoxin data with quantification of *Fusarium* sp. with RT-PCR assay. However in such a case mycotoxin biosynthesis genes can be influenced by several environmental factors. Furthermore finding a correlation between the PCR amplification signal and the concentration of the target mycotoxin(s) seems unlikely until assays are developed based on gene expression. In *M. phaseolina* to date no such mycotoxin or pathogenesis genes were characterized, which help in detection and quantification of this pathogen. Therefore SCAR-derived qPCR assay reported in this article has become first description of a real-time PCR-based quantification method for *M. phaseolina*.

In conclusion the present study led to the development and application of a species-specific real-time quantitative PCR method, with optimal specificity, sensitivity and rapidity. The assay was useful for the evaluation of a plant pathogen population in the soil, and it seems possible to estimate the vegetative population of *M. phaseolina*

following direct extraction of soil DNA without culturing. The developed method therefore could provide a useful tool for rapid and reliable detection of *M. phaseolina* in diseased plants as well as in field samples, thus reflecting the feasibility of monitoring of seedborne outbreaks of pathogens in the environment. Moreover this technique can be applied directly in clinical diagnosis of *M. phaseolina* infections in humans, which could help in administration of antifungal therapy.

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