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Real-time PCR for detection and quantification of fungal and oomycete tomato pathogens in plant and soil samples

Bart Lievens [a,b], Margreet Brouwer [a], Alfons C.R.C. Vanachter [b], Bruno P.A. Cammue [a], Bart P.H.J. Thomma [c,*]

[a] *Centre of Microbial and Plant Genetics (CMPG), Katholieke Universiteit Leuven, Kasteelpark Arenberg 20, B-3001 Heverlee-Leuven, Belgium*
[b] *Scientia Terrae Research Institute, Fortsesteenweg 30A, B-2860 Sint-Katelijne-Waver, Belgium*
[c] *Laboratory of Phytopathology, Wageningen University, Binnenhaven 5, 6709 PD Wageningen, The Netherlands*

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Abstract

Although new, rapid detection and identification technologies are becoming available more and more for various plant pathogens, pathogen quantification remains one of the main challenges in the disease management of many crops. Currently, real-time polymerase chain reaction (PCR) is the most straightforward technique to quantify pathogen presence. This manuscript describes the use of real-time PCR to quantitatively assess the presence of a number of economically important fungal and oomycete tomato pathogens in biological samples. We demonstrate that pathogen DNA can be accurately quantified over at least four orders of magnitude. Additionally, we demonstrate the feasibility of the technique to quantify pathogen biomass in complex biological samples.

Keywords: *Fusarium solani*; Internal transcribed spacer (ITS); *Pythium ultimum*; *Rhizoctonia solani*; SYBR Green I; *Verticillium*

1. Introduction

Soilborne fungi and oomycetes are the causal agents of many diseases that severely impact the agronomic performance of a large number of crops. Also for tomato (*Lycopersicon esculentum*), diseases caused by soilborne pathogens lead to economic losses worldwide. For this economically important plant species major diseases caused by soilborne fungi and oomycetes include root rot caused by *Fusarium solani*, *Fusarium* and *Verticillium* wilt and damping-off and tissue rot caused by *Rhizoctonia solani*, and root rot and damping-off caused by several *Pythium* species, respectively [1].

Early, accurate detection and identification of plant pathogens are essential for effective plant disease management. Until recently, conventional methods to detect and identify fungal and oomycete pathogens have often relied on plating onto selective media, or on biochemical, chemical and immunological analyses [2]. The major drawbacks of these methods, although fundamental to plant pathogen diagnostics, are the reliance on the ability of the organism to be cultured, the time consuming and labor intensive nature, and the requirement for skilled taxonomical expertise [3,4]. Molecular techniques can overcome many of the shortcomings of the conventional assays, especially if they make use of the polymerase chain reaction (PCR) [3,4]. In general, these methods are more sensitive, more accurate, more specific, and much faster than conventional techniques. In addition, generally no culturing step of the pathogen is required, making these techniques also applicable for non-culturable microorganisms [3,4].

In addition to detection and identification, pathogen quantification is an important aspect with respect to plant disease management, since it provides the information required for determining the necessity, and the extent, of appropriate control strategies. While quantification based on culturing techniques is considered relatively inaccurate and in some cases even unreliable [5–8], the development of real-time PCR has been a powerful development with regard to pathogen quantification [9]. Real-time PCR differs from

* Corresponding author. Tel.: +31 317 484536; fax: +31 317 483412.
E-mail address: bart.thomma@wur.nl (Bart P.H.J. Thomma).

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classical end-point PCR by the measurement of the amplified PCR product at each PCR cycle. Since the development of the exponential phase of the reaction is monitored, real-time PCR allows accurate template quantification [10]. Increasingly, real-time PCR is being used for plant pathogen diagnosis, including detection and quantification of different plant pathogenic fungi, oomycetes, bacteria, nematodes and viruses as well as biocontrol agents [3,11–13]. However, with the exception of a few studies [12–14] the interaction between a single plant species and only one or two related pathogens has been reported to date.

In this manuscript, we describe the use of real-time PCR to quantitatively assess the presence of a number of economically important fungal and oomycete pathogens of tomato in environmental samples, including those derived from cultivated soils and plants. The target pathogens comprised the fungi *F. solani*, *R. solani*, and *Verticillium* species that cause tomato wilt, and the oomycete *Pythium ultimum*. In addition to tomato, these pathogens are able to attack a broad range of other plant species. In order to address the robustness of the developed assays, quantitative assessment of these pathogens in naturally infested samples from multiple origins is demonstrated.

2. Materials and methods

2.1. Fungal and oomycete isolates, cultivation and DNA extraction

Fungal and oomycete isolates used in this study are listed in Table 1. All isolates were grown on potato dextrose agar in the dark at 22 °C. Genomic DNA from a patch of mycelium (approximately 2 cm^2) of 5- to 10-day-old cultures was isolated as described previously [15]. For DNA extraction from soil and plant samples, bulk DNA was extracted from 0.75 g (fresh weight) starting material using the UltraClean Soil DNA Isolation Kit and the UltraClean Plant DNA Isolation Kit according to the manufacturer's instructions (Mo Bio Laboratories Inc., Solana Beach, CA, USA) and subsequently diluted 10-fold. DNA yield and purity were determined spectrophotometrically.

2.2. Real-time PCR

PCR primers were designed based on the internal transcribed spacer (ITS) regions I and II. To this end, the region between the small and large subunit of the rRNA gene of

Table 1
Fungal and oomycete isolates used in this study to evaluate primer specificity in real-time PCR assays

Phylum	Order	Species[a]	Isolate[b]	Origin	Host or substrate	Specificity[c] obtained with primer pairs			
						ITS1F/ AFP346	AFP276/ ITS4	ST-RS1/ ITS4	ITS1F/ ST-VE1
Ascomycota	Dothideales	*Didymella lycopersici*	CBS 378.67	The Netherlands	*Lycopersicon esculentum*	–	–	–	–
	Helotiales	*Botrytis cinerea*	MUCL 28919	Belgium	*L. esculentum*	–	–	–	–
		Sclerotinia minor	CBS 339.39	Italy	*Lactuva sativa*	–	–	–	–
		S. sclerotiorum	DSM 1946	Unknown	*Medicago sativa*	–	–	–	–
	Hypocreales	*Cylindrocladium spathiphylli*[*]	MUCL 40062	Unknown	Unknown	–	–	–	–
		Fusarium graminearum[*]	HCK PH1	Unknown	Unknown	–	–	–	–
		F. oxysporum f. sp. *lycopersici*	CBS 645.78	Morocco	*L. esculentum*	–	–	–	–
		F. oxysporum f. sp. *radicis-lycopersici*	CBS 101587	Unknown	*L. esculentum*	–	–	–	–
		F. solani	CBS 165.87	Denmark	*S. tuberosum*	+	–	–	–
		F. solani	CABI 17960	Brazil	*S. tuberosum*	+	–	–	–
		F. solani	HCK S-66	Unknown	Unknown	+	–	–	–
		Nectria haematococca	MUCL 20259	Belgium	Soil	+	–	–	–
		Trichoderma asperellum[*]	MUCL 41923	Unknown	Unknown	–	–	–	–
		T. harzianum[*]	MUCL 19412	Unknown	Unknown	–	–	–	–
	Microascales	*Thielaviopsis basicola*	MUCL 8363	The Netherlands	*Primula* sp.	–	–	–	–
	Phyllachorales	*Colletotrichum coccodes*	DSM 2492	Unknown	*L. esculentum*	–	–	–	–
		C. gloeosporioides	CBS 503.97	USA	*Aeschynomene virginica*	–	–	–	–
		Verticillium albo-atrum	CBS 451.88	Belgium	Unknown	–	–	–	+

Table 1 (*Continued*)

Phylum	Order	Species[a]	Isolate[b]	Origin	Host or substrate	Specificity[c] obtained with primer pairs			
						ITS1F/ AFP346	AFP276/ ITS4	ST-RS1/ ITS4	ITS1F/ ST-VE1
		V. albo-atrum	CBS 321.91	The Netherlands	*L. esculentum*	–	–	–	+
		V. albo-atrum	CBS 385.91	The Netherlands	*L. esculentum*	–	–	–	+
		V. dahliae	CBS 386.49	The Netherlands	*Solanum melongena*	–	–	–	+
		V. dahliae	CBS 179.66	The Netherlands	*L. esculentum*	–	–	–	+
		V. dahliae	CBS 381.66	Canada	*L. esculentum*	–	–	–	+
		V. tricorpus	MUCL 9792	UK	*L. esculentum*	–	–	–	+
	Pleosporales	*Alternaria alternata*	CBS 105.24	Unknown	*Solanum tuberosum*	–	–	–	–
		Pyrenochaeta lycopersici	DSM 62931	Germany	*L. esculentum*	–	–	–	–
Mitosporic ascomycota		*Phoma destructiva*	CBS 133.93	Guadeloupe	*L. esculentum*	–	–	–	–
Basidiomycota	Aphyllophorales	*Athelia rolfsii*	MUCL19443	Belgium	Soil	–	–	–	–
	Ceratobasidiales	*Rhizoctonia oryzae*[*]	CBS 273.38	USA	*Oryza sativa*	–	–	–	–
		R. solani AG 1-1B	CBS 101761	The Netherlands	*L. sativa*	–	–	+	–
		R. solani AG 3	CBS 101590	Unknown	*L. esculentum*	–	–	+	–
		R. solani AG 1	CBS 323.84	The Netherlands	*L. sativa*	–	–	+	–
		R. solani	MUCL 9418	Unknown	*L. esculentum*	–	–	+	–
		R. solani	ST 36.01	Belgium	*Beta vulgaris*	–	–	+	–
		R. solani	ST 44.02	Belgium	*Cichorium endivia*	–	–	+	–
		R. solani	ST 50.03	Belgium	*L. sativa*	–	–	+	–
Oomycota	Peronosporales	*Phytophthora cactorum*[*]	CBS 112275	Unknown	*Fragaria ananassa*	–	–	–	–
		P. capsici	CBS 554.88	Argentina	*L. esculentum*	–	–	–	–
		P. cinnamomi[*]	MUCL 43491	Australia	Soil	–	–	–	–
		P. cryprogea	CBS 113.19	Ireland	*L. esculentum*	–	–	–	–
		P. drechsleri	DSM 62679	Iran	*B. vulgaris*	–	–	–	–
		P. infestans	MUCL 43257	Unknown	*S. tuberosum*	–	–	–	–
		P. nicotianae	MUCL 40633	Zimbabwe	*Nicotiana tabacum*	–	–	–	–
		Pythium aphanidermatum	CABI 15272	Unknown	*L. sativa*	–	–	–	–
		P. arrhenomanes	CBS 324.62	USA	*Zea mays*	–	–	–	–
		P. dissotocum[*]	CBS 166.68	USA	*Triticum aestivum*	–	–	–	–
		P. irregulare[*]	CBS 461.48	Australia	Unknown	–	–	–	–
		P. myriotylum	CBS 254.70	Israel	*Arachis hypogaea*	–	–	–	–
		P. polymastum[*]	CBS 810.70	The Netherlands	*L. sativa*	–	–	–	–
		P. sylvaticum[*]	CBS 225.68	The Netherlands	Soil	–	–	–	–
		P. ultimum	CBS 101588	Unknown	*Cucumis sativus*	–	+	–	–
		P. ultimum	CBS 805.95	Canada	*N. tabacum*	–	+	–	–
		P. ultimum var. *ultimum*	CBS 656.68	The Netherlands	*L. esculentum*	–	+	–	–
		P. ultimum var. *ultimum*	MUCL 16164	UK	*Pisum sativum*	–	+	–	–
		P. ultimum	HAJH P211	USA	*Euphorbia pulcherrima*	–	+	–	–

[a] Unless marked with an asterisk, fungal and oomycete species are reported as tomato pathogens [1].

[b] CABI: Centre for Agriculture and Bioscience International, Surrey, UK; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; HAJH: from collection of HAJ Hoitink, Ohio State University, Wooster, OH, USA; HCK: from collection of HC Kistler, University of Minnesota, St. Paul, MN, USA; MUCL: Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; ST: from collection of Scientia Terrae Research Institute, Belgium.

[c] +: significant amplification; –: no significant amplification. A sample was considered positive only if it exhibited an exponential phase of amplification and fluorescence exceeded the baseline threshold. The experiment was repeated twice with similar results.

Table 2
Real-time PCR primers used in this study

Code[a]	Organism	Sequence (5′–3′)	Target	Amplicon size (bp)	T_{ann}[b]	t_{el}[c]	T_m[d]
AFP346[e,f] (R)	*Fusarium solani*	GGTATGTTCACAGGGTTGATG	ITS I	104	60	6	82.5
AFP276[e,g] (F)	*Pythium ultimum*	TGTATGGAGACGCTGCATT	ITS II	150	58	8	81.0
ST-RS1[e,g] (F)	*Rhizoctonia solani*	AGTGTTATGCTTGGTTCCACT	ITS II	187	60	8	83.0
ST-VE1[f] (R)	*Verticillium* spp.	AAAGTTTTAATGGTTCGCTAAGA	ITS I	200	60	9	85.7
ITS1-F[h] (F)	Fungi	CTTGGTCATTTAGAGGAAGTAA	18S rDNA	x[i]	x	x	x
ITS4[j] (R)	Universal	TCCTCCGCTTATTGATATGC	28S rDNA	x	x	x	x
$P450_1$[k] (F)	*Saccharomyces cerevisiae*	ATGACTGATCAAGAAATCGCTAA	Cytochrome P450	343	50	14	83.5
$P450_2$[k] (R)	*S. cerevisiae*	TGTAACCTGGAGAAACCAAAAC	Cytochrome P450				

[a] F: forward primer; R: reverse primer.
[b] Annealing temperature (°C).
[c] Elongation time (s).
[d] Melting temperature (°C) at which a specific dissociation peak of increased fluorescence is generated in the melting curve analysis.
[e] Ref. [25].
[f] In combination with ITS1-F.
[g] In combination with ITS4.
[h] Ref. [17].
[i] x, depends on second primer used.
[j] Ref. [18].
[k] Ref. [19].

all *F. solani*, *P. ultimum*, *R. solani*, and *Verticillium* isolates listed in Table 1 was amplified and sequenced as previously described [15]. Following sequence alignment using the ClustalW algorithm with related ITS sequences found in Genbank [16] species-specific primers were designed and checked for lack of significant homology with other DNA sequences using BLAST analysis. For each target pathogen, single real-time PCR primers were designed (Table 2) and used for amplification in combination with either the fungal-specific forward primer ITS1-F [17] or the universal reverse primer ITS4 [18]. Real-time PCR amplifications were performed in glass capillaries in a total volume of 20 μl using the intercalating dye SYBR® Green I on a Lightcycler® instrument (Roche Diagnostics Corp., Indianapolis, IN, USA). To perform several parallel reactions a master mix was prepared, which was then aliquoted into separate capillaries. Each reaction contained 2 μl of the target DNA extract, 4 μl of the Lightcycler FastStart DNA Master^{PLUS} SYBR® Green I kit (Roche Diagnostics Corp., Indianapolis, IN, USA), 1 μl of each primer (10 μM), and 12 μl sterile distilled water. Thermal cycling conditions consisted of 10 min at 95 °C followed by 45 amplification cycles of 10 s at 95 °C, 5 s at the annealing temperature indicated in Table 2, and elongation at 72 °C for the time period indicated in Table 2. Fluorescence (520 nm) was detected at the end of the elongation phase for each cycle. To evaluate amplification specificity, melt curve analysis was performed at the end of each PCR run. A melt curve profile was obtained by heating the mixture to 95 °C, cooling to 65 °C (15 s), and slowly heating to 95 °C at 0.1 °C s^{-1} with continuous measurement of fluorescence at 520 nm.

2.3. Exogenous control

To monitor different PCR kinetics between separate samples, 150 pg μl^{-1} of exogenous control DNA from *Saccharomyces cerevisiae* MUCL 28426 was added to each sample and subsequently amplified and quantified in separate real-time PCR reactions. To minimize variability between samples *S. cerevisiae* DNA was added to the PCR master mix. A quantitative real-time PCR assay was developed for *S. cerevisiae* using primers $P450_1$ and $P450_2$ [19]. PCR amplification conditions were those as described above. Comparing the efficiency of the amplification of *S. cerevisiae* control DNA allowed comparing PCR efficiencies between samples.

2.4. Quantification of pathogen DNA

The developed PCR assays were validated in several steps. Initially, for each target pathogen, the performance of the selected primer pair was further evaluated. Experiments were performed using isolates *F. solani* CBS 165.87, *P. ultimum* CBS 101588, *R. solani* CBS 323.84, and *V. dahliae* CBS 381.66. For each target pathogen, 100 and 1 pg genomic DNA μl^{-1} DNA extract, reflecting a heavy and an early or light infestation, respectively, was amplified in the presence of 20 ng μl^{-1} genomic DNA extracted from a healthy tomato plant and sandy soil (Sint-Katelijne-Waver, Belgium). In addition, specific amounts of target DNA (either 100, 50, 10, or 1 pg μl^{-1}) were added to samples containing 10 pg μl^{-1} DNA isolated from nine other pathogens. In addition to the remaining three target pathogens, these encompassed six tomato pathogens including *Athelia rolfsii* MUCL 19443, *Botrytis cinerea* MUCL 28919, *Fusarium oxysporum* f. sp. *lycopersici* CBS 101587, *Phytophthora nicotianae* MUCL 40633, *Pythium dissotocum* CBS 166.68, and *Sclerotinia sclerotiorum* DSM 1946. For all samples, two replicates were analyzed.

Subsequently, to evaluate the correlation between inoculum density and quantified DNA, artificially infested soil samples (0.75 g fresh weight; sandy soil, Sint-Katelijne-Waver, Belgium) were produced with *P. ultimum* CBS 101588, *R. solani* CBS 323.84, *V. albo-atrum* CBS 451.88, and *V. dahliae* CBS 381.66. Following an incubation period of 14 days at 22 °C fresh inoculum prepared in sterilized chopped potato soil [20]

was serially diluted with non-infested soil resulting in a 5-, 10-, 50-, 100-, and 1000-fold dilution of the starting inoculum. As a check for thorough inoculum colonization, 100 soil aggregates (Ø1–2 mm) from the starting inoculum were plated on semi-selective medium [6,20,21] and incubated at 22 °C in darkness. In addition, a specific number of microsclerotia (5, 10, 40, and 100) from *V. dahliae* CBS 381.66 produced according to the method described by Hawke and Lazarovits [22] were added to 0.75 g (fresh weight) sandy soil (Sint-Katelijne-Waver, Belgium). For each experiment, non-infested soil served as negative control. Two samples were used for DNA extraction and independently analyzed using real-time PCR (two replicates). Inoculum density and molecular analyses were subjected to first degree regression analysis.

Finally, the assays were validated using naturally infested samples. Since a wide range of economically important crops can be infected by all pathogens selected [23], sampling was not limited to tomato crops or tomato fields. Various environmental samples, including ten soil and five plant samples from diverse origins, were gathered from commercial vegetable and ornamental growers. Soil samples were collected using a core borer (Ø3 cm) to a depth of 20–30 cm from at least 10 locations per field. Plant samples were taken from infected plant parts, and more in particular at the range of diseased and healthy tissue. Samples were homogenized and subsamples were used for DNA extraction (0.75 g fresh weight) and molecular detection and quantification as well as for classical disease diagnosis. Initially, DNA samples were assessed for pathogen occurrence using an extended version of the DNA array designed previously [15]. Subsequently, for all samples in which any of the studied pathogens was detected, the amount of genomic DNA was determined using real-time PCR and the specificity of the assay was confirmed by sequencing the generated amplicons. In addition, a parallel set of samples was analyzed using classical diagnostic methods. For each soil sample, a series of three 10-fold dilutions was prepared from 10 g (fresh weight) of soil and 100 μl aliquots of each dilution were plated in triplicate on several semi-selective media [6,20]. Plates were incubated at 22 °C in darkness. For the soil samples 04-200A and 04-200B which were taken from two fields of which the cultivated crops displayed *Verticillium* wilt, the number of viable *V. dahliae* microsclerotia was determined by the wet sieving technique using 12.5 g air-dried soil [24,25]. With regard to the plant samples, infected plant parts were plated in triplicate on semi-selective medium [6,20] following surface sterilization. After incubation at 22 °C in darkness the identity of the pathogen was confirmed using classical taxonomy techniques.

3. Results

The target tomato pathogens that were chosen in our study comprise the fungi *F. solani*, *R. solani* and the tomato wilt causing *Verticillium* complex encompassing *V. albo-atrum*, *V. dahliae* and *V. tricorpus* and the oomycete *P. ultimum* [1,26,27]. For each target, several real-time PCR primers were designed based on ITS sequences and combined either with ITS1-F [17] or ITS4 [18]. Of the initially tested primers one set of primers for each species was selected that provided the most consistent DNA amplification of a single amplicon following optimization of the PCR reaction (Table 2). The primer sets selected for further experiments were ITS1-F combined with AFP346 or ST-VE1 to detect *F. solani* or the target *Verticillium* species, respectively, and ITS4 in combination with ST-RS1 or AFP276 to detect *R. solani* or *P. ultimum*, respectively (Table 2). The resulting amplicons varied in size from 104 to 200 bp (Table 2). As an example for the in silico predictability of primer specificity, a sequence alignment for the target pathogen and the most related fungi or oomycetes from Table 1 is shown in Fig. 1. In addition, specificity of the selected primer sets was tested using genomic DNA extracted from all isolates listed in Table 1. These isolates, representing 17 genera and 38 species, were selected to represent a wide range of ascomycetous, basidiomycetous, and oomycetous pathogens commonly found on tomato [1]. In all cases, no amplification could be detected using template DNA from any of the non-target species tested (Table 1). As a check for DNA quality, all extracts were successfully subjected to PCR analysis using the universal ribosomal primers ITS 5 and ITS 4 [18] (data not shown). Therefore, our results show that, despite the use of only one species-specific primer, PCR assays were selective for the target organisms (Table 1). For all positive runs, melt curve analysis of the PCR products revealed a single dissociation peak of increased fluorescence at the melting temperature indicated in Table 2, demonstrating the amplification specificity.

To quantify unknown concentrations of pathogen DNA in biological complex samples, for each target pathogen, a standard curve (Fig. 2) was generated by the amplification of a 10-fold dilution series of target DNA in the presence of plant- or soil-derived DNA at an average concentration when assaying environmental DNA samples [25]. For each pathogen, the correlation between the C_T-value and the target DNA concentration was high ($R^2 > 0.992$), irrespective of the presence of non-target DNA (Fig. 2). All standard curves obtained demonstrated that the selected primer sets were highly accurate over a linear range of at least four orders of magnitude. With regard to the selected *Verticillium* primer pair ITS1-F and ST-VE1, standard curves were identical when using genomic DNA from each of the three target *Verticillium* species.

The eventual goal of this study was to quantify pathogen DNA in biological samples. Therefore, the possible interference of non-target DNA of diverse origins with accurate detection and quantification of target pathogen DNA was further investigated. Initially, 100 and 1 pg μl^{-1} pathogen DNA, representing DNA concentrations that are relevant in horticultural practice were added to 20 ng μl^{-1} genomic DNA from a healthy tomato plant or from sandy soil. The results presented in Fig. 3 shows that neither plant DNA nor soil DNA significantly interfered with target DNA quantification. In a next analysis, specific amounts of target pathogen DNA (approximately 100, 50, 10, or 1 pg μl^{-1}, respectively) were added to a DNA mixture containing 10 pg μl^{-1} genomic DNA of nine other fungal or oomycete tomato pathogens. The results show that irrespective of the presence of non-target fungal or oomycete DNA, in all cases the amount of template DNA was

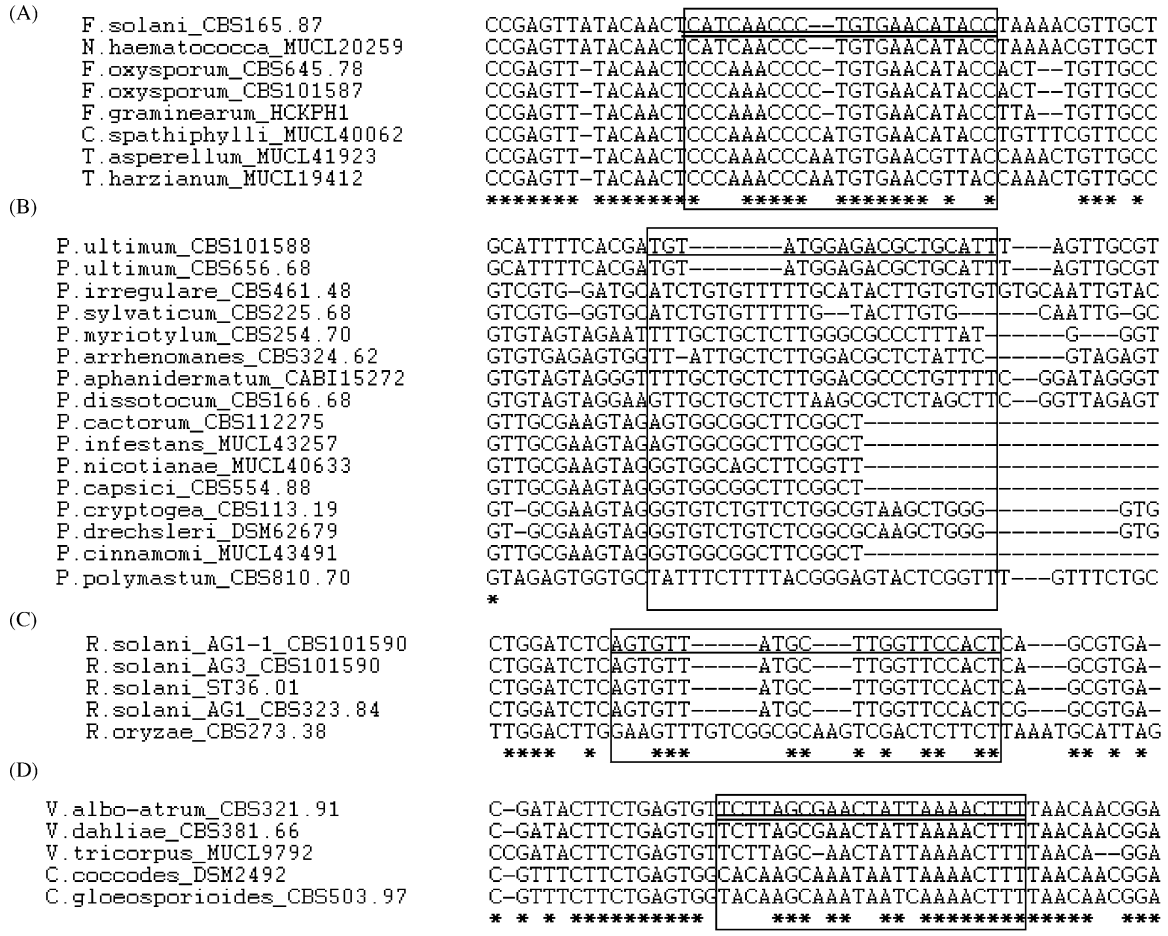

Fig. 1. Alignments of ITS sequences in the regions used for primer design (boxed area) for (A) *Fusarium solani*, (B) *Pythium ultimum*, (C) *Rhizoctonia solani*, and (D) *Verticillium* species that cause tomato wilt. To illustrate primer specificity, sequences of the target pathogen are aligned with sequences of the most related fungi or oomycetes shown in Table 1. Identical nucleotides are marked with an asterisk and gaps are indicated by dashes. Selected forward primers that are combined with the universal reverse primer ITS4 [18] are underlined with a single line. Selected reverse primers (reverse complement sequence) that are combined with the fungal-specific forward primer ITS1-F [17] are double underlined.

accurately quantified (Fig. 4). For each curve generated, a slope of approximately 1 and a low intercept was obtained. In all cases, melt curve analysis revealed a single dissociation peak at the melting temperature indicated in Table 2, demonstrating the specificity of the amplification process. Therefore, these experiments show that the desired amplicons can be generated, detected and quantified in complex DNA mixtures.

To quantitatively assess pathogen presence in biological samples, artificially infested soil samples were produced for *P. ultimum*, *R. solani*, *V. albo-atrum*, and *V. dahliae*. Initially, inoculum was serially diluted with non-infested soil resulting in soil mixtures containing progressively lower pathogen concentrations. The logarithmic relationships between the calculated DNA concentrations using real-time PCR and the inoculum density are shown in Fig. 5(A–D). For each pathogen, a linear correlation was obtained with a coefficient of determination higher than 0.91. In addition, soil was infested with specific amounts of microsclerotia from *V. dahliae*. As shown in Fig. 5E, again a linear correlation was obtained ($R^2 = 0.98$), demonstrating the feasibility of the technique to quantify pathogen biomass in biological samples.

In our final assays, we evaluated whether the developed PCR assays could also be used for the assessment of pathogen biomass in naturally infested samples obtained from commercial growers. Soil samples were taken from six infested fields of which crops showed different kinds of disease symptoms, and four soils on which crops were asymptomatic. In addition, samples were collected from infected plants with clear disease symptoms. Initially, samples were assessed for pathogen occurrence using a DNA macroarray designed for pathogen detection [15]. Subsequently, for all samples in which any of the studied pathogens was detected, the amount of genomic DNA was determined using real-time PCR (Table 3). For all samples, pathogens detected using the DNA macroarray were also detected in the real-time PCR analyses. Using the DNA array all 10 soil samples were diagnosed with multiple microorganisms (data not shown). In five samples, *P. ultimum* was found in DNA concentrations ranging from 0.31 to 8.32 ng DNA per gram of soil. For the crops growing on three of the corresponding soils, no disease symptoms were observed, even if DNA levels of *P. ultimum* were relatively high (e.g. 04-285). For the soils carrying the highest (03-224) and the lowest

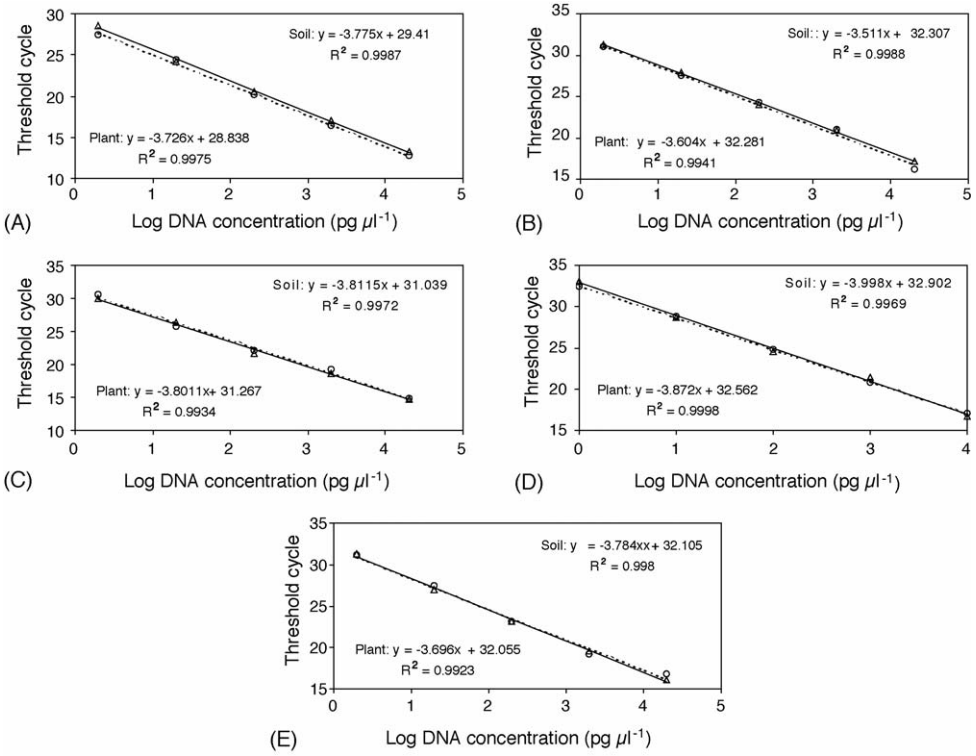

Fig. 2. Standard curves used for the quantification of target DNA in biological complex samples using real-time PCR for (A) *F. solani* CBS 165.87, (B) *P. ultimum* CBS 101588, (C) *R. solani* CBS 323.84, (D) *Verticillium dahliae* CBS 381.66, and (E) *Saccharomyces cerevisiae* MUCL 28426. Standard curves were obtained with amplification of a 10-fold dilution series of target DNA in the presence of 15 ng DNA extracted from a healthy tomato plant (○) or a sandy soil (△). Data represent means of three replicates (error bars, representing standard errors, are too small to be displayed graphically).

Table 3
Real-time PCR quantification of fungal and oomycete genomic DNA in different environmental samples

Sample	Cultivated crop		Sample ID	Observed field symptoms	Calculated DNA concentration (ng g^{-1} soil or plant sample) for			
	Latin name	Common name			*Fusarium solani*	*Pythium ultimum*	*Rhizoctonia solani*	*Verticillium* sp.
Sandy soil	*Rosa* sp.	Rose	03-111	Foot and stem rot	0.01	x[a]	0.12	x
Sandy soil	*Fragaria ananassa*	Strawberry	03-224	Root rot; reduced growth	0.01	8.32	1.00	x
Sandy soil	*Lycopersicon esculentum*	Tomato	03-307	None	0.23	x	x	x
Sandy soil	*Apium graveolens*	Celery	04-188	None	x	0.57	x	x
Sandy soil	*Phalaenopsis* sp.	Orchid	04-191B[b]	Root rot; reduced growth	x	x	1.31	x
Sandy soil	*Brassica oleracea*	Cauliflower	04-200A	Wilting	x	x	x	0.13
Sandy soil	*B. oleracea*	Cauliflower	04-200B	Wilting	x	x	x	0.08
Sandy soil	*Lactuca sativa*	Lettuce	04-285	None	x	2.84	x	x
Sandy soil	*Apium graveolens*	Celery	04-329B[c]	Crater rot	0.09	0.31	0.49	x
Sandy soil	*L. sativa*	Lettuce	04-336C	None	x	1.32	x	x
Plant	*L. esculentum*	Tomato	03-182Aw[d]	Root rot; wilting; nettle-like head	x	0.03	0.18	x
Plant	*Carpinus* sp.	Hornbeam	04-118	Damping-off	x	2.72	x	x
Plant	*Phalaenopsis* sp.	Orchid	04-191B2[b]	Root rot; reduced growth	x	x	14.67	x
Plant	*L. sativa*	Lettuce	04-178A	Vein rot; browning of leaves	x	x	x	x
Plant	*A. graveolens*	Celery	04-329C[c]	Crater rot	x	x	494.400	x

[a] x, absent according to a DNA macroarray analysis, by which over 40 different plant pathogenic fungi and oomycetes can be detected [15].
[b] Corresponding samples.
[c] Corresponding samples.
[d] Pepino mosaic virus (PepMV) infected plant as confirmed by ELISA (enzyme linked immunosorbent assay; Agdia, Elkhart, IN, USA) testing.

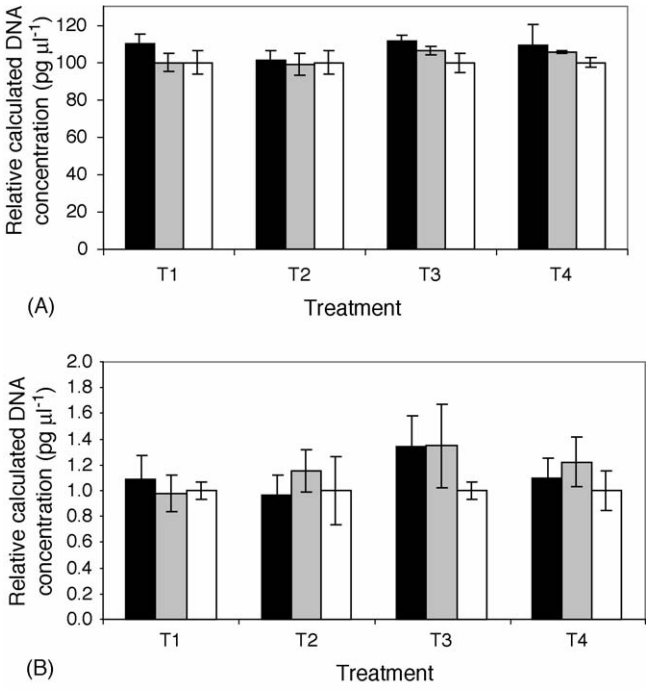

Fig. 3. Influence of non-target DNA on target DNA quantification using real-time PCR. Samples containing (A) 100 pg μl^{-1} or (B) 1 pg μl^{-1} pathogen DNA were amplified in the presence of 20 ng μl^{-1} genomic DNA extracted from a healthy tomato plant (■) or a sandy soil (▒). As a control no non-target DNA was added (□). Target DNA was isolated from *F. solani* CBS 165.87 (T1), *P. ultimum* CBS 101588 (T2), *R. solani* CBS 323.84 (T3), and *V. dahliae* CBS 381.66 (T4). Calculated DNA concentration is reported relative to the calculated DNA concentration for the control treatment. Data represent means of two replicates. Errors bars indicate standard errors.

(04-329B) concentration of pathogen DNA, plants displayed disease symptoms (Table 3). For sample 03-224, *P. ultimum* was identified as the main biological cause of the disease. For 04-329B, other pathogens including *F. solani* and *R. solani* were detected as well (Table 3) of which, based on the observed symptoms, *R. solani* was determined to be the cause of disease. One reason for these differences in *P. ultimum* levels is host preference. Whereas *P. ultimum* is a well known pathogen of strawberry [28], this species has, to our knowledge, not been reported as a pathogen of celery and is not a primary pathogen of harvestable lettuce [29]. *R. solani* was identified in four of the analyzed soil samples and in all cases the corresponding crops displayed typical *Rhizoctonia* symptoms. DNA concentrations were found between 0.12 and 1.31 ng DNA per gram of soil. In all cases, sequencing of the amplicons and BLAST analysis of the ITS sequences confirmed the identity of the pathogens, illustrating the specificity and robustness of the developed assays. In addition, parallel sets of all soil samples were plated on semi-selective media to validate detection and quantification. However, using these poorly discriminative techniques it was impossible to accurately filter out, and thus quantify, the target pathogens in these environmental samples.

Based on the results shown in Fig. 5E, showing the relation between the calculated DNA concentration using real-time PCR analysis and the number of *V. dahliae* microsclerotia, the number of microsclerotia in samples (04-200A and 04-200B) of two fields of which crops exhibited *Verticillium* wilt was estimated at 8 and 13 microsclerotia per gram of soil, respectively. By the classical wet sieving technique, in both soils the number of microsclerotia was estimated at 7/gm of

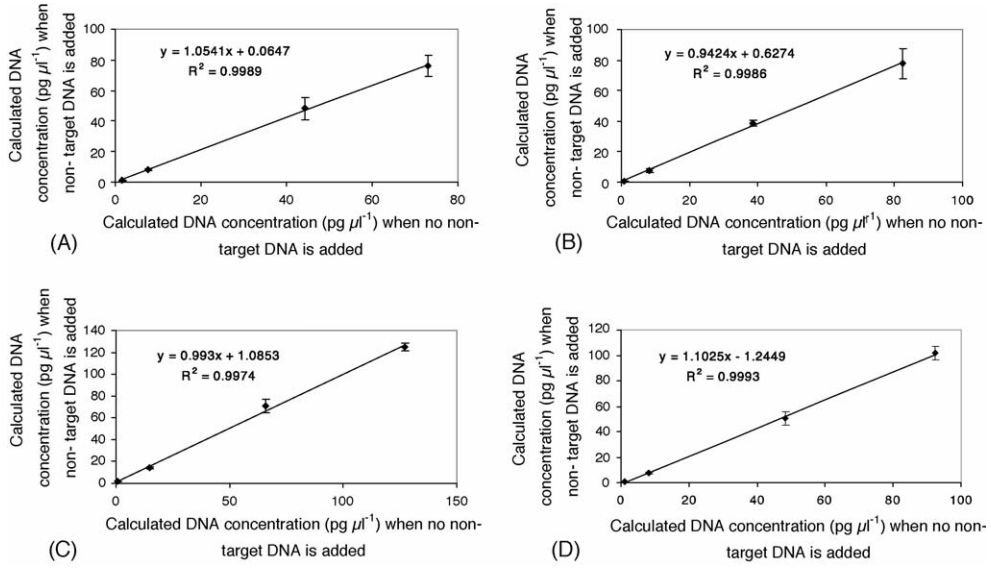

Fig. 4. Influence of non-target fungal and oomycete DNA on target DNA quantification using real-time PCR. Curves for (A) *F. solani* CBS 165.87, (B) *P. ultimum* CBS 101588, (C) *R. solani* CBS 323.84, and (D) *V. dahliae* CBS 381.66 were obtained by plotting the calculated DNA concentration (pg μl^{-1}) when non-target DNA was added against the calculated concentration (pg μl^{-1}) when no non-target DNA was added to the target sequences. Non-target DNA represented a mixture of genomic DNA of nine other fungal and oomycete tomato pathogens (10 pg μl^{-1} per pathogen). The experiment was performed using genomic DNA extracted from *Athelia rolfsii* MUCL 19443, *Botrytis cinerea* MUCL 28919, *Fusarium oxysporum* f. sp. *lycopersici* CBS 101587, *F. solani* CBS 165.87, *Phytophthora nicotianae* MUCL 40633, *Pythium dissotocum* CBS 166.68, *P. ultimum* CBS 101588, *R. solani* CBS 323.84, *Sclerotinia sclerotiorum* DSM 1946, and *V. dahliae* CBS 381.66. Data represent means of two replicates. Errors bars indicate standard errors.

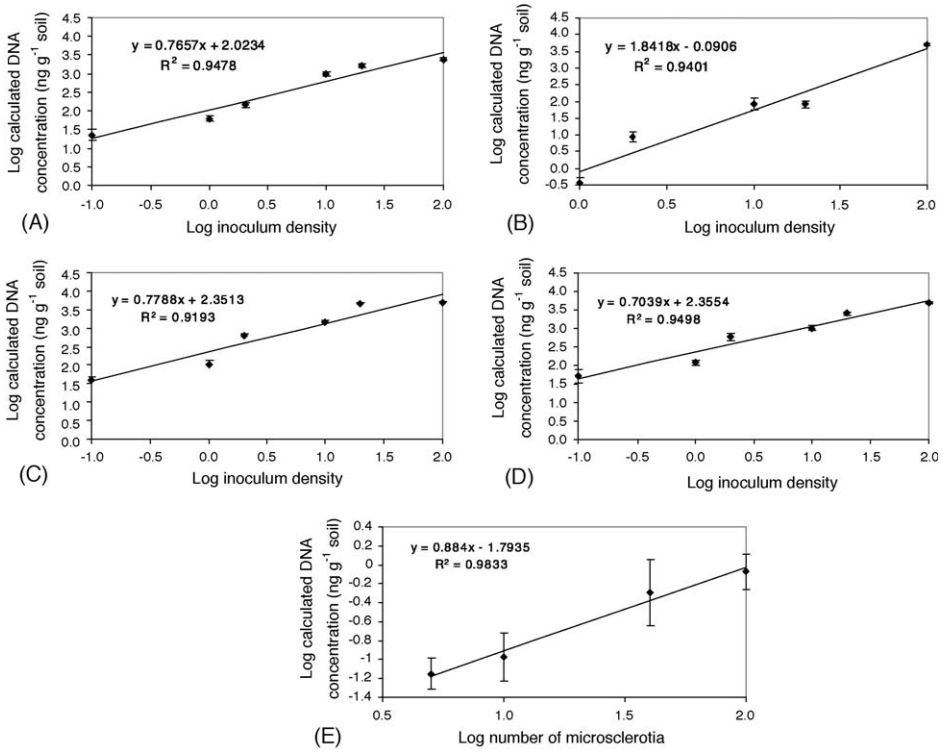

Fig. 5. Quantitative assessment of pathogen presence in artificially infested soil samples. (A–D) Regression lines for real-time PCR analysis of a dilution series of pathogen inoculum from (A) *P. ultimum* CBS 101588, (B) *R. solani* CBS 323.84, (C) *Verticillium albo-atrum* CBS 451.88, and (D) *V. dahliae* CBS 381.66. Inoculum density is expressed as the percentage of fungal growth out of 100 inoculum soil aggregates. (E) Regression line for real-time PCR analysis of a series of 100, 40, 10, and 5 microsclerotia from *V. dahliae* CBS 381.66 added to 0.75 g (fresh weight) soil. Data represent means of four measurements: two samples were used for DNA extraction and analyzed in duplo. Error bars indicate standard errors.

soil. However, as microsclerotia often get lost by sieving [8], it was anticipated that real-time PCR might detect more microsclerotia. On the other hand, since DNA-based detection techniques cannot differentiate between DNA from hyphae and sclerotia of *Verticillium*, sclerotial numbers may be over-estimated using real-time PCR as well. In addition to the soil samples, five plant samples were analyzed, four of which (03-182Aw, 04-118, 04-191B2, and 04-178A) contained more than one pathogen (data not shown). In plant samples 04-191B2 and 04-329C, which displayed typical *Rhizoctonia* symptoms, *R. solani* DNA concentrations were established at 14.67 and 494.40 ng/gm of plant sample, respectively. The corresponding soil samples, 04-191B and 04-329B, contained less but detectable levels of DNA of this pathogen. In sample 04-118, the amount of genomic DNA from *P. ultimum* was calculated to be 2.72 ng g^{-1} plant tissue. In addition to this pathogen, *Pythium sylvaticum* was detected with the DNA macroarray. Either one or both of these *Pythium* species can explain the typical damping-off symptoms observed. For each plant sample, the accurateness of the detection was confirmed by classical plating and standard taxonomy techniques.

To confirm that all samples were amplified with the same efficiency, each sample analyzed was spiked with 150 pg μl^{-1} *S. cerevisiae* DNA (generally not a soil inhabitant) and amplified in a separate real-time PCR reaction. Throughout our experiments, amplification of exogenous control DNA never altered significantly. A typical example of efficiency measurement is given in Table 4. PCR efficiencies, reported as the calculated DNA concentrations for the control DNA, are shown for a mixture containing 100 pg μl^{-1} *F. solani* (CBS 165.87)

Table 4
Amplification efficiency[a] of several DNA extracts containing genomic DNA from a specific pathogen as well as from a healthy tomato plant or a sandy soil

DNA mixture[b]	PCR efficiencies at plant- or soil-derived DNA amounts of			
	0 pg μl^{-1}	200 pg μl^{-1}	2 ng μl^{-1}	20 ng μl^{-1}
Fusarium solani; *Saccharomyces cerevisiae;* tomato	149.6 ± 9.0[c]	164.6 ± 12.1	158.3 ± 11.6	154.5 ± 10.7
F. solani; *S. cerevisiae*; sandy soil	150.0 ± 13.0	145.1 ± 8.5	160.1 ± 14.0	155.8 ± 5.5

[a] Reported as the calculated concentration of *Saccharomyces cerevisiae* DNA (pg μl^{-1}) in the sample using a real-time PCR assay for *S. cerevisiae*.
[b] Each sample, containing a mixture of 100 pg μl^{-1} DNA from *Fusarium solani* CBS 165.87 and a specific amount of non-target DNA isolated from a healthy tomato plant or sandy soil, was spiked with 150 pg μl^{-1} exogenous DNA derived from *S. cerevisiae* MUCL 28426.
[c] Values are the means of two replicates ± standard errors.

DNA and a specific amount of DNA isolated from a healthy tomato plant or a sandy soil. In all cases the expected amount of *S. cerevisae* DNA (150 pg μl^{-1}) was recovered and no significant differences ($P \leq 0.05$) were established between the calculated DNA concentrations, demonstrating that PCR efficiencies between the analyzed samples were highly comparable.

4. Discussion

In this study we have demonstrated for a number of common tomato pathogens that pathogen DNA can be accurately quantified over a large concentration range using real-time PCR. Only for *F. solani*, similar assays have been described to date [30,31]. In addition, we have demonstrated the feasibility of the technique to quantify pathogen biomass in biological samples, using artificially and naturally infested samples from diverse origins. Obviously, soils contain many different organisms. For most naturally infested soil samples studied, it was impossible to accurately distinguish the target pathogens from non-target microorganisms using poorly discriminative techniques like plating on semi-selective medium. Therefore, for these complex samples DNA concentrations were related to the symptoms displayed by the cultivated crops. However, for all artificially infested soil samples that were sterilized before infestation a high degree of correlation was observed between inoculum density and the calculated template DNA concentration, demonstrating the potential of the technique to accurately quantify pathogen occurrence in environmental samples.

V. albo-atrum, *V. dahliae* and *V. tricorpus* are related in that they all cause *Verticillium* wilt in tomato [1,26]. Whereas the first two species are the well known causal agents of tomato vascular wilt, the latter fungus occasionally causes *Verticillium* wilt of tomato [27]. Currently, there are no effective treatments available to cure infected plants. Therefore, preventative measures must be applied in order to avoid that these pathogens infect susceptible crops. Since all three species are able to cause tomato wilt and since there is no difference in managing these pathogens, differentiation to the species level is not required. Therefore, in this study, a single PCR assay using primers ITS1-F and ST-VE1 was developed to detect and quantify the presence of all three *Verticillium* species capable of causing tomato wilt.

For the *R. solani* complex, current classification of isolates that are pathogenic on different hosts is largely based on grouping into anastomosis groups (AG), defined on the basis of hyphal fusion reactions [23]. So far, 14 AGs have been described, of which AGs 3 and 4 are associated with tomato diseases [32]. However, it is not unlikely that other AGs also harbor tomato pathogens. Therefore, in this study, a primer pair (ST-RS1 and ITS4) was chosen to detect a wide range of *R. solani* strains. Whereas this primer pair can easily be used for the in planta detection and quantification of *R. solani* strains, its implementation for soil diagnosis is not that straightforward. Once *R. solani* is detected in a soil sample, pathogenicity tests need to be performed to determine the pathogenic capacity of the isolate.

Pythium species are present in virtually all cultivated soils and depending on the crop regarded as primary or weak, secondary pathogens, implicating that its presence not necessarily results in disease. However, when attacking stressed plants damage is likely to occur [23]. This is endorsed by the data obtained in the present study. Out of ten soil samples collected from fields where diverse crops were cultivated, five were diagnosed with *P. ultimum*. For only two of them, plants displayed disease symptoms. In one soil sample, containing the highest level of *P. ultimum* (8.32 ng g^{-1}), this pathogen was identified as the main cause of the disease (based on DNA macroarray analysis and the observation of root necrosis). For the other sample, plants displayed typical *Rhizoctonia* symptoms. Similar conclusions could be made for the prevalent soilborne complex species *F. solani*. Although this species was found in four of the analyzed samples, in none of the cases its presence could be related with the observed symptoms. Based on these findings, it may be concluded that although real-time PCR is a powerful tool that offers several advantages over the traditional methods of pathogen identification, epidemiological expert interpretation is still required.

In our as well as in other studies [33,34], specific PCR assays were obtained by the use of a single target-specific primer combined with an overall fungus or universal primer. However, cross hybridization of the developed primers to DNA from closely related species cannot be ruled out. In our study, specificity of the developed primers was confirmed based on a representative collection of tomato pathogens. Nevertheless, sequencing of amplicons generated from diverse naturally infested soil samples confirmed the identity of all species, suggesting lack of specificity should not be of major concern. Currently, the ribosomal DNA operon is the main genomic region targeted for PCR primer development in molecular diagnostics, partially because this region provides a powerful means for analyzing phylogenetic relationships over a wide range of taxonomic levels [18,35]. When these ribosomal sequences are not suitable for species identification or discrimination other ubiquitously conserved regions of the genome can be exploited. In addition, efforts aimed to develop diagnostic tools based on genes involved in virulence are undertaken [35–37]. This is especially valuable for species that harbor pathogenic as well as non-pathogenic strains, like *F. solani* and *R. solani* [35].

Rapid real-time PCR diagnosis can result in appropriate control measures and/or eradication procedures more rapidly and accurately than the conventional methods of pathogen isolation and quantification. Nevertheless, additional efforts are required to allow successful implementation for quantitative soil diagnosis [35]. In consideration that PCR can also amplify DNA from dead or non-active organisms, detection of non-viable propagules may not be ruled out. However, DNA from dead cells in soils should be degraded fairly rapid due to the high microbial activity, suggesting that amplification of DNA from dead propagules might be of less importance [38–40]. In addition, sampling methods require special attention when using this highly sensitive technology. Finally, also a link between pathogen DNA concentrations and threshold levels needs to be established [35].

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