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Quantitative assessment of phytopathogenic fungi in various substrates using a DNA macroarray

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Summary

Detection, identification and quantification of plant pathogens are the cornerstones of preventive plant disease management. To detect multiple pathogens in a single assay, DNA array technology currently is the most suitable technique. However, for sensitive detection, polymerase chain reaction (PCR) amplification before array hybridization is required. To evaluate whether DNA array technology can be used to simultaneously detect and quantify multiple pathogens, a DNA macroarray was designed and optimized for accurate quantification over at least three orders of magnitude of the economically important vascular wilt pathogens *Verticillium albo-atrum* and *Verticillium dahliae*. A strong correlation was observed between hybridization signals and pathogen concentrations for standard DNA added to DNA from different origins and for infested samples. While accounting for specific criteria like amount of immobilized detector oligonucleotide and controls for PCR kinetics, accurate quantification of pathogens was achieved in concentration ranges typically encountered in horticultural practice. Subsequently, quantitative assessment of other tomato pathogens (*Fusarium oxysporum*, *Fusarium solani*, *Pythium ultimum* and *Rhizoctonia solani*) in environmental samples was performed using DNA array technology and correlated to measurements obtained using real-time PCR. As both methods of quantification showed a very high degree of correlation, the reliability and robustness of the DNA array technology is shown.

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Introduction

For many processes in which microorganisms are involved, it is important to know not only which microbes are present but also the size of the respective populations. In plant pathology, the imposed strategy of disease management is not simply to combat a pathogen whether or not it is present, but to apply corrective measures only when its presence is confirmed and its magnitude is expected to result in disease development, increasing distribution and inoculum potential, and/or economic loss. Therefore, detection and quantification of pathogens is increasingly gaining interest.

Until recently, detection of pathogenic microorganisms has been performed by plating plant parts, soil or soil extracts onto selective media, or by biochemical, chemical and immunological analyses (Singleton *et al*., 1992). However, these methods are often time-consuming, laborious, and require extensive knowledge of classical taxonomy (McCartney *et al*., 2003). Furthermore, quantification, based on these culture-plating techniques, is considered relatively inaccurate and unreliable (Tsao and Guy, 1977; Jeffers and Martin, 1986; Thorn *et al*., 1996; Termorshuizen *et al*., 1998; Goud and Termorshuizen, 2003).

Advances in molecular biology, in particular the advent of polymerase chain reaction (PCR), have provided new tools for detecting and quantifying microbial DNA without resorting to culturing. Additionally, non-culturable microorganisms can also be detected and quantified, an aspect that is considerably important as possibly less than 1% of the microorganisms in an environmental sample may be cultured (Amann *et al*., 1995). In addition, diagnosis time can be reduced from weeks, typically experienced with culture plating, to a few days.

Real-time PCR has been a powerful development, especially with regard to quantification purposes (Heid *et al*., 1996). This technology is more sensitive, more accurate and less time-consuming than conventional,

end-point quantitative PCR because it monitors PCR products as they accumulate during the reaction. This allows template quantification during the exponential phase of the reaction, before reaction components become limiting. Increasingly, real-time PCR is being used for accurate detection and quantification of specific plant pathogens as well as for monitoring pathogen infection (Schaad and Frederick, 2002; Brouwer *et al*., 2003). Although not yet applied to plant pathogens, multiplex formats enable simultaneous detection and quantification of different organisms (Boivin *et al*., 2004; Candotti *et al*., 2004). However, the total amount of PCR reactions in a single tube is severely limited by the number of different fluorescent dyes available and the energizing light source used in real-time PCR instruments (Mackay *et al*., 2002). As a result, detection of more than a handful of different pathogens at the same time is currently impossible.

In contrast, DNA array technology can, in principle, be used to detect an unlimited number of different pathogens in a single assay (Martin *et al*., 2000; Lévesque, 2001; Wilson *et al*., 2002; Lievens *et al*., 2003). The advantage of this technology is that it combines nucleic acid amplification with the unlimited screening capability of DNA arrays, resulting in high degrees of sensitivity, specificity and throughput capacity. With this technology, taxon-specific oligonucleotide detectors are immobilized on a solid support, such as a nylon membrane. In general, the target DNA is amplified with universal primers and labelled simultaneously, and subsequently hybridized to the array under stringent conditions. This technology was originally developed as a technique to screen for human genetic disorders (Saiki *et al*., 1989), but has also been successfully applied to detect and identify human and animal pathogens (Fiss *et al*., 1992; Anthony *et al*., 2000; González *et al*., 2004). In plant pathology, DNA array technology has been successfully applied to identify oomycete, nematode and bacterial pathogen DNA from pure cultures (Lévesque *et al*., 1998; Uehara *et al*., 1999; Fessehaie *et al*., 2003). Recently, we showed the utility of DNA arrays for multiplex detection and identification of plant pathogens from complex environmental samples including those derived from soils and plants (Lievens *et al*., 2003). DNA arrays may be used for the detection and identification of microorganisms that are important for agricultural and horticultural practice. A DNA array for simultaneous detection of over 40 different plant pathogenic soilborne fungi and 10 bacteria that frequently occur in greenhouse crops has been developed. This array, called DNA Multiscan® (http://www.DNAmultiscan.com), is routinely used worldwide by companies that offer disease diagnostic services and advice to commercial growers. A major limitation of the current technology, however, is that reliable quantification of pathogen presence is not yet available. Hence, only qualitative detection can be conducted. To fully exploit the potential use of DNA arrays in plant pathology, and more specifically, to determine minimal threshold densities of the pathogen to start application of appropriate soil or crop treatments, quantitative ability is a prerequisite. In this article we describe, as a proof-of-principle, a format of the previously designed DNA macroarray (Lievens *et al*., 2003), which has been further developed for accurate quantitative assessment of the economically important vascular wilt pathogens *Verticillium albo-atrum* and *Verticillium dahliae* for concentration ranges typically encountered in horticultural practice. In addition, in order to generalize the data obtained and to address the robustness of the technology, quantitative assessment of other fungi in artificially inoculated and naturally infested samples from diverse origin is demonstrated.

Results

Optimizing PCR conditions to permit end-point quantification

For sensitive pathogen detection using DNA arrays, PCR amplification is required. There are, however, limitations to the use of PCR in a quantitative approach, as bias in template-to-product ratios may be introduced due to typical PCR amplification kinetics (Suzuki and Giovannoni, 1996). As a result, the dynamic range of the targets to be detected may not always be reflected by the outcome of the assay. This bias in template-to-product ratio can be caused by two technical artefacts, namely: (i) differential PCR efficiency between samples or (ii) analysis of samples, which are no longer in the exponential phase of the reaction. To monitor the first potential problem, for each sample 1 ng of exogenous control DNA derived from *Saccharomyces cerevisiae* was added to each sample and amplified in a separate PCR reaction. In Table 1, PCR

Table 1. Comparison of PCR efficiencies[a] between different soil samples.

Sample ID	PCR efficiency[a]
03-111	0.85 ± 0.13
03-115	0.76 ± 0.11
03-142	0.85 ± 0.10
03-176	1.01 ± 0.12
03-193	0.91 ± 0.06
03-224	0.94 ± 0.15
03-226	0.78 ± 0.12
03-307	1.02 ± 0.17
03-337	0.95 ± 0.09
04-200	0.84 ± 0.16

a. Reported as the ratio between the hybridization signals for the detector oligonucleotides to target the exogenous control DNA derived from *S. cerevisiae* (Sce1) and the digoxigenin-labelled reference control (Dig1). Values are means ± standard errors ($n = 4$ from two independent analyses).

Table 2. Yield[a] of PCR product (ng μl^{-1}) after a specific number of PCR cycles.

Sample	Sample ID	Template DNA (ng)	Yield of PCR product after PCR cycle number			
			25	30	35	40
V. albo-atrum		5	81.7	96.3	112.1	118.3
		0.5	21.2	59.7	97.9	99.6
		0.05	3.7	24.3	40.3	43.5
		0.005	0.0	0.9	1.2	2.3
		0.0005	0.0	0.0	0.0	0.0
V. dahliae		25	69.8	377.7	442.9	441.7
		2.5	14.8	82.0	218.3	254.7
		0.25	0.0	19.4	34.0	34.7
		0.025	0.0	8.4	12.2	32.9
		0.0025	0.0	0.0	0.0	7.9
Soil	03-311	2.75	12.5	47.3	105.7	166.4
	03-324	12.75	20.9	71.6	67.6	45.4
Plant	03-312	1.5	19.1	48.9	127.3	178.6
	P58	8.75	21.9	86.3	216.6	176.9

a. Polymerase chain reaction (PCR) products were quantified after gel electrophoresis by comparison to a DNA ladder.

efficiencies are shown for DNA extracted from several soil samples that were obtained from commercial vegetable growers. Polymerase chain reaction efficiencies appeared stable for all conditions tested. For all other experiments, PCR efficiencies between analysed samples were highly comparable. In addition to spiking DNA samples with exogenous control DNA, PCR reaction parameters were adjusted to ensure hybridization with amplicons arising from the exponential phase of the PCR reaction. To this end, a 10-fold dilution series of genomic DNA from *V. albo-atrum* (5 ng to 0.5 pg) and *V. dahliae* (25 ng to 2.5 pg) was amplified using either 25, 30, 35 or 40 cycles. Polymerase chain reaction products were quantified by comparison to standard DNA, showing that up to at least 30 cycles, most PCR reactions remained in the exponential phase (Table 2). In addition, genomic DNA isolated from naturally infested soils and infected plants was amplified under the same conditions, essentially showing similar results (Table 2). A comparable experiment was performed for other fungal pathogens, including the tomato pathogens *Fusarium oxysporum* f. sp. *lycopersici* and *Fusarium solani*. In all cases, PCR reactions were found to be in the exponential phase up to at least 30 cycles (data not shown). Therefore, all following PCRs were performed using 30 cycles to ensure detection at the exponential phase of the PCR reaction in combination with high sensitivity.

Quantification of DNA dilutions using a DNA macroarray

One potential problem that can hamper quantification using DNA arrays is interspot variability caused by printing errors or spatial effects. To test this, 8.0 fmol of detector oligonucleotide Vda1 (Table 3) was spotted in duplicate at six different locations on a membrane and labelled *V. dahliae* amplicon (10 ng ml^{-1}) was hybridized

Table 3. Detector oligonucleotides used for DNA array analysis.

Code	Specificity	Sequence (5′–3′)	Origin
Fox2[a]	*F. oxysporum*	GTTGGGACTCGCGTTAATTCG	ITS II
Fso1[b]	*F. solani*	ATCAACCCTGTGAACATACCTAA	ITS I
Pap1[b]	*P. aphanidermatum*	TTGGAGTATAGATCAGTATTAGGTAAA	ITS I
Pul1[b]	*P. ultimum*	TGCTGACTCCCGTTCCAGTG	ITS I
Rso1[b]	*R. solani*	GCCTGTTTGAGTATCATGAAAT	ITS II
Val2[a]	*V. albo-atrum*	CATCAGTCTCTTTATTCATACCAA	ITS I
Vda1[a]	*V. dahliae*	AACAGAGAGACTGATGGACCG	ITS I
Fun1[b,c]	Fungi	GCTGCGTTCTTCATCGATGC	5.8S rDNA
Sce1[d]	*S. cerevisiae*	GTGTTTTGGATGGTGGTAAGAA	*ERG11* gene
Uni1[a]	Eukaryotes	TCCTCCGCTTATTGATATGC	28S rDNA
Dig1[a,e]	None	GTCCAGACAGGATCAGGATTG	–
Con1[a]	None	GTCCAGACAGGATCAGGATTG	–

a. Lievens *et al.* (2003).
b. This study.
c. ITS2 primer (White *et al.*, 1990).
d. Reverse complement of detector oligonucleotide pSce (Posteraro *et al.*, 2000).
e. 3′-end digoxygenin labelled.

to the membrane. The signals obtained showed an average relative optical density of 112.4 with a standard error of 4.6, demonstrating that the interspot variability is limited.

To investigate the quantitative properties of DNA arrays, accurate quantification of a 10-fold dilution series of *V. dahliae* genomic DNA after PCR amplification was pursued. The fungal template ranged from 2.5 ng to 0.25 pg, and 30 cycles of PCR amplification were performed. On the DNA array, different amounts of the same detector oligonucleotide (Vda1) were spotted. In principal, those detectors that show a perfect correlation between the signal intensity and the amount of template before PCR amplification will permit accurate template quantification. Hybridization results revealed that signals increased by increasing the amount of printed detector oligonucleotide, especially when spotted at amounts lower than 2.0 fmol (Fig. 1A). For all spotted amounts, a linear logarithmic relationship between the integrated spot intensity and template DNA concentration could be obtained for a specific concentration range. When considering the complete concentration range, the correlation was almost linear ($R^2 = 0.99$) when 0.5 fmol of oligonucleotide was immobilized per spot. In other cases, however, the curves deviate from linearity for the lower or higher DNA concentrations used in this dilution range. The latter was particularly observed when signals were strong and at the saturation level of the immobilized oligonucleotide. When 2.0 or 8.0 fmol was spotted, hybridization signals were saturated at 25 pg of template DNA or more. Additions of 2.5 ng of DNA extracted from sandy greenhouse soil or from healthy tomato leaf material to the samples of the dilution series before DNA amplification did not influence the hybridization results (data not shown). A similar experiment was conducted for *V. albo-atrum* (Fig. 1B) as well as for other fungal pathogens, including the tomato pathogens *F. oxysporum* f. sp. *lycopersici* and *F. solani* (data not shown), confirming the outcome of this experiment.

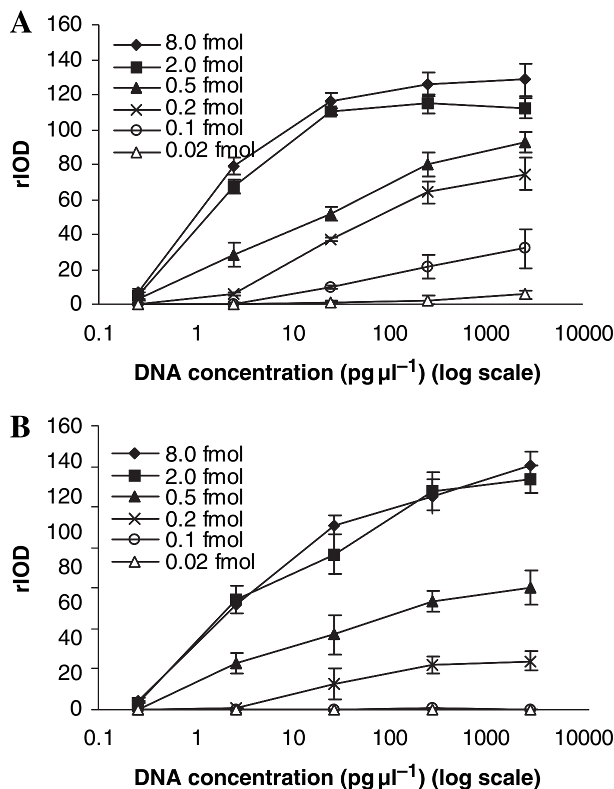

Fig. 1. Quantification of a dilution series of *V. dahliae* (A) and *V. albo-atrum* (B) genomic DNA after PCR using different amounts of detector oligonucleotides on a DNA macroarray. Detector oligonucleotides were spotted at several amounts ranging from 0.02 to 8.0 fmol. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labelled reference control (rIOD) and plotted versus the logarithmic DNA concentration. Data represent the average of two independent analyses of hybridization signals ($n = 4$) using detector sequences Vda1 and Val2 respectively. Error bars indicate standard errors. The experiment was repeated twice with similar results.

Direct quantification of fungal DNA in soil samples using real-time PCR

To find the most appropriate amount of immobilized detector oligonucleotide for quantification of pathogen presence in environmental samples, the range of relevant DNA concentrations was defined for a number of different plant pathogens. Initially, 10 soil samples obtained from commercial vegetable growers at different periods during the growing season were assessed for pathogen occurrence using the DNA Multiscan®. Subsequently, for all pathogens detected, the amount of genomic DNA was quantified using real-time PCR (Table 4). In addition to *V. dahliae*, pathogens detected by DNA Multiscan® included *F. solani*, *Pythium sylvaticum*, *Pythium ultimum* and *Rhizoctonia solani*. Besides these pathogens, the species *F. oxysporum*, which encompasses both pathogenic and non-pathogenic strains, was detected. The tested DNA extracts contained on average 5 ng µl^{-1} genomic DNA of which between 1 and 300 pg µl^{-1} was of fungal or oomycete origin. Up to 25% of this DNA turned out to be from a single pathogen. The average concentration of DNA from a single pathogen was established at 3 pg µl^{-1} and the maximum found in this assay was 12 pg µl^{-1}. Based on these findings, a detector oligonucleotide amount of 8.0 fmol per spot was selected for further experiments. At this amount, detection was most sensitive and a linear logarithmic relationship was obtained for concentrations up to 25 pg µl^{-1}, which represents a realistic range of plant pathogen DNA concentrations that are relevant for naturally infested greenhouse soils (Table 4).

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Table 4. Quantification of fungal genomic DNA (pg μl^{-1}) in soil samples using real-time PCR.

Sample ID	Sampling date	Template DNA[a] (ng μl^{-1})	Fungi	Oomycetes	*F. oxysporum*	*F. solani*	*P. sylvaticum*	*P. ultimum*	*R. solani*	*V. dahliae*
03-111	01/16/03	2.75	8.02	0.88	0.19	0.02	x	x	0.18	x
03-115	01/22/03	6.25	6.69	4.58	x	x	x	x	x	x
03-142	02/14/03	4.00	299.80	11.42	x	x	x	x	x	x
03-176	03/18/03	5.25	29.03	7.20	1.19	x	x	x	x	x
03-193	04/03/03	0.50	13.07	5.70	0.20	x	x	x	x	x
03-224	04/29/03	12.75	10.49	304.50	0.41	x	1.90	12.18	1.50	x
03-226	05/02/03	2.00	7.93	3.99	x	x	x	x	x	x
03-307	07/30/03	10.25	317.80	50.93	4.13	0.34	3.68	x	x	x
03-337	09/02/03	3.00	22.80	2.71	6.30	x	x	x	x	x
04-200	03/30/04	7.00	6.20	1.40	0.50	x	x	x	x	0.19
Maximum		12.75	317.80	304.50	6.30	0.34	3.68	12.18	1.50	0.19
Minimum		0.50	6.20	0.88	0.19	0.02	1.90	12.18	0.18	0.19
Mean		5.38	72.18	39.33	1.85	0.18	2.79	12.18	0.84	0.19

a. Determined spectrophotometrically at 260 nm.
x, absent according to DNA Multiscan® analysis.

Influence of non-target DNA on target quantification using a DNA macroarray

Because the ultimate goal of this work was to quantify pathogen presence in DNA extracts from complex biological samples, the possible interference of non-target DNA of different origins with accurate detection and quantification was tested. A 10-fold dilution series of genomic DNA from *V. albo-atrum* and *V. dahliae* ranging from 0.25 pg (reflecting a light or early infestation) to 25 pg (resembling a strong infestation) was amplified in the presence of a specific amount of non-target DNA. Either 25 pg, 250 pg or 2.5 ng of non-target DNA was added to the PCR mixture, which resulted in testing pathogen:non-target DNA ratios of 1:100, 1:1000 and 1:10 000 respectively. DNA templates isolated from bacterial (*Rhizobium vitis*), oomycete (*P. ultimum*) and fungal (*F. solani*) cultures, and from healthy tomato plant and sandy soil were used. After PCR amplification, amplicons were hybridized to the array and analysed. Figure 2 represents a typical example of signals after hybridization, showing similar hybridization strengths irrespective the presence of non-target DNA. Apart from this, the high sensitivity of the technique is demonstrated by this figure. Regardless the presence of 250 pg of non-target DNA, in all cases as little as 0.25 pg of target DNA can clearly be detected (Fig. 2). Non-target fungal DNA affected hybridization results when present at certain ratios (Table 5), whereas signal intensities were not influenced by bacterial-, oomycete-, plant- or soil-derived DNA and as little as 0.25 pg of target DNA could be detected. In general, non-target fungal DNA did not interfere with detection and quantification up to a target:non-target ratio of 1:1000. For the lowest concentration of target DNA (0.25 pg), the highest concentration of fungal non-target (2.5 ng; target:non-target ratio 1:10 000) resulted in an inability to detect the target. In that case, increasing the number of PCR cycles to 40, however, made detection of target DNA possible. Similar experiments were also performed for *F. oxysporum* f. sp. *lycopersici*, which essentially provided similar results (data not shown). It can thus be concluded that this high amount of fungal non-target DNA, which is, however, not likely to occur in practice (Table 4), results in an underestimation of target DNA. A non-discriminative fungal detector oligonucleotide (Fun1; Table 3), based on 5.8S rDNA sequences, was added to the array in order to measure the total pool of fungal DNA in the sample and thus address possible underestimation of the target. In general, if signals were obtained when this detector was spotted at an amount of 0.02 fmol, the target was determined to be underestimated (Table 5).

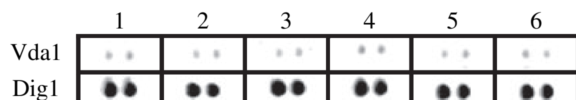

Fig. 2. Influence of non-target DNA on hybridization signals. Signals after hybridization of amplicons resulting from amplification of 0.25 pg of *V. dahliae* and 250 pg of non-target DNA to the oligonucleotide detectors Vda1, to detect *V. dahliae*, and Dig1, for calibration, spotted in duplicate. Non-target DNA isolated from a bacterial (*R. vitis*; 1), oomycete (*P. ultimum*; 2) or fungal (*F. solani*; 3) culture or from tomato plant (4) or sandy soil (5) were used to test possible interference. In panel 6, no non-target DNA was added.

Quantitative assessment of pathogen presence in artificially inoculated and naturally infested samples using a DNA macroarray

To quantify pathogen biomass in complex biological samples, soil was infested with specific amounts of conidia from either *V. albo-atrum* or *V. dahliae*, or microsclerotia from *V. dahliae*. The relationships of hybridization strength to the logarithmic number of *V. dahliae* spores and microsclerotia are presented in Fig. 3A and B, respectively, demonstrating that quantitative detection of the pathogen

Table 5. Influence of non-target fungal (*F. solani*) DNA on target DNA quantification.

	Hybridization signals for different target DNA amounts of								
	0.25 pg *V. albo-atrum*			2.5 pg *V. albo-atrum*			25 pg *V. albo-atrum*		
F. solani template[a]	Ratio[b]	Val2[c]	Fun1[d]	Ratio	Val2	Fun1	Ratio	Val2	Fun1
Con	1:0	3.9 ± 2.2[e]	0.0 ± 0.0	1:0	51.7 ± 3.8	0.0 ± 0.0	1:0	90.6 ± 5.0	0.0 ± 0.0
25 pg	1:100	3.6 ± 0.4	0.0 ± 0.0	1:10	61.2 ± 6.9	0.0 ± 0.0	1:1	88.9 ± 17.2	0.0 ± 0.0
250 pg	1:1000	1.8 ± 0.6	0.0 ± 0.0	1:100	58.9 ± 6.3	0.2 ± 0.1	1:10	93.7 ± 15.8	1.4 ± 0.4
2.5 ng	1:10 000	0.0 ± 0.0	4.2 ± 1.2	1:1000	28.9 ± 12.6	6.6 ± 1.2	1:100	81.7 ± 15.3	8.6 ± 2.9
	Hybridization signals for different target DNA amounts of								
	0.25 pg *V. dahliae*			2.5 pg *V. dahliae*			25 pg *V. dahliae*		
F. solani template[a]	Ratio	Vda1[c]	Fun1	Ratio	Vda1	Fun1	Ratio	Vda1	Fun1
Con	1:0	6.7 ± 2.3	0.0 ± 0.0	1:0	78.8 ± 4.7	0.0 ± 0.0	1:0	116.5 ± 4.5	0.0 ± 0.0
25 pg	1:100	10.9 ± 4.4	0.0 ± 0.0	1:10	90.9 ± 10.6	0.2 ± 0.1	1:1	121.7 ± 3.2	0.1 ± 0.1
250 pg	1:1000	11.5 ± 0.9	0.0 ± 0.0	1:100	53.9 ± 10.5	0.0 ± 0.0	1:10	127.0 ± 4.5	0.7 ± 0.3
2.5 ng	1:10 000	0.0 ± 0.0	2.9 ± 0.2	1:1000	36.1 ± 7.4	10.3 ± 2.3	1:100	134.7 ± 3.3	6.1 ± 1.1

a. Amount of *F. solani* DNA template in the DNA mixture.
b. Target:non-target ratio.
c. Hybridization to the detector oligonucleotides Val2 and Vda1 (8.0 fmol) to detect *V. albo-atrum* and *V. dahliae* respectively.
d. Hybridization to the non-discriminative fungal detector Fun1 spotted at an amount of 0.02 fmol.
e. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin labelled reference control (Dig1). Values are means ± standard errors ($n = 4$ from two independent analyses).

was successful in artificially infested mixes. A linear correlation was obtained with a coefficient of determination of 0.96 and 0.99 between 10^3 and 10^6 spores or 5 and 40 microsclerotia per 0.75 g of soil sample, respectively, each representing realistic ranges by which these pathogens occur under natural conditions (Xiao and Subbarao, 1998). In addition, we evaluated whether the DNA array could also be used for estimating fungal biomass in naturally infested soils. Based on the results shown in Fig. 3B, the relation between the hybridization strength and the number of *V. dahliae* microsclerotia is described by the regression equation $y = 12.3x - 4.73$, with y being the relative integrated optical density and x representing the logarithmic number of microsclerotia. This formula was used to estimate the number of microsclerotia present in the soil of two fields exhibiting wilt symptoms. Using the DNA array, the number of microsclerotia in 0.75 g of air-dried soil was estimated at 10 and 7, corresponding to 13 and 9 microsclerotia per gram of soil respectively. With the classical wet sieving technique in both soils the number of recovered microsclerotia was established at seven microsclerotia per gram of soil. However, as a portion of the microsclerotia generally gets lost by sieving (Goud and Termorshuizen, 2003), it was expected to find more microsclerotia using the DNA array than by using the classical method.

In order to generalize data obtained in this study, we finally used our findings to pursue the development of quantitative detector oligonucleotides for other pathogens as well. In a first experiment the oomycete *Pythium aphanidermatum* was chosen as the target organism and a detector oligonucleotide (Pap1; Table 3) was spotted at 8.0 fmol. Tomato seedlings grown for 10 days in nutrient solution containing specific concentrations (10^2–10^4 zoospores ml^{-1}) of *P. aphanidermatum* zoospores were rated for foot and root rot severity (Fig. 4B). At that time symptoms of reduced plant growth were well developed. In addition, DNA was extracted from the nutrient solution for hybridization to the array (Fig. 4A). The results of this experiment showed a strong correlation between the hybridization signal intensity, the initial amount of zoospores and disease severity (Fig. 4), demonstrating the feasibility of the technique to quantitatively monitor plant health and to quantitatively detect a different pathogen.

In addition, various environmental samples, including plant and soil samples, were assessed for pathogen quantification using DNA array analysis and quantitative real-time PCR. Analyses were carried out for four different pathogens previously detected using the DNA Multiscan®, including *F. oxysporum*, *F. solani*, *P. ultimum* and *R. solani*. In addition to the control oligonucleotides Uni1, Con1, Sce1 (8.0 fmol per spot) and Dig1 (2.0 fmol per spot), the respective detector oligonucleotides Fox2, Fso1, Pul1 and Rso1 (Table 3) were spotted at 8.0 fmol on a single membrane. For each target organism, a linear logarithmic correlation ($R^2 > 0.91$) was obtained between DNA array hybridization signal strength and the calculated DNA concentration obtained by real-time PCR analysis (Fig. 5), thus demonstrating the robustness and

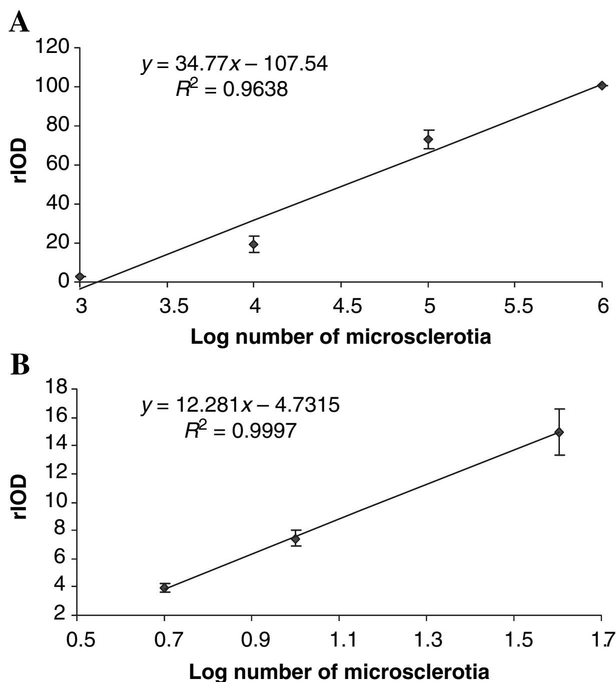

Fig. 3. Quantitative assessment of *V. dahliae* presence in artificially infested soil samples.
A. Regression line for DNA array analysis of a 10-fold dilution series of *V. dahliae* conidia added to 0.75 g of soil.
B. Regression line for DNA array analysis of a series of 40, 10 and 5 microsclerotia from *V. dahliae* added to 0.75 g of soil.
Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labelled reference control (rIOD) and plotted versus the logarithmic number of pathogen propagules. Data represent averages of hybridization signals generated by Vda1 from two independent analyses ($n = 4$). Error bars indicate standard errors.

breadth of the developed quantitative DNA array-based assay.

Discussion

DNA array technology has the potential to detect multiple microorganisms in a single assay from diverse environments (Lévesque, 2001; Lievens *et al.*, 2003). One major shortcoming until now, however, has been its lack of quantitative character allowing the evaluation of the severity of an infestation. This has implications for interpretation of pathogen assessment surveys and for decision making as to whether or not disease control strategies should be undertaken based on the presence of certain signals. In this article we describe a new format of the previously designed DNA macroarray (Lievens *et al.*, 2003), which has been further developed for accurate pathogen quantification for concentration ranges typically encountered in horticultural practice. To our knowledge, this is the first report describing the use of DNA array technology to quantify microbial communities.

This study shows that by including several controls, hybridization results can be standardized and accurately quantified allowing a quantitative estimate of pathogen biomass. To use DNA arrays for diagnostic purposes in plant pathology, PCR amplification is required to obtain the desired sensitivity. However, end-point quantification after PCR is often accompanied by bias in template-to-product ratio (Suzuki and Giovannoni, 1996). This ratio may be skewed by two major technical artefacts, namely variability in PCR efficiency and template saturation. Variability in PCR efficiency is generally caused by compounds in sample materials that reduce or inhibit amplification efficiency (including phenolic compounds, humic acids, fulvic acids, heavy metals and excessive non-target DNA). In this study, 1 ng of exogenous control DNA (derived from *S. cerevisiae*, generally not a common soil inhabitant) was added to each sample and amplified in a separate PCR reaction. Using the immobilized detector Sce1, PCR efficiency between samples could be monitored. In this study, PCR efficiency between all samples analysed was highly comparable, regardless of the sample matrix from which DNA was isolated. Obviously, the quality of the DNA to be amplified is critical (López *et al.*, 2003; McCartney *et al.*, 2003). Therefore, these results also suggest that high-quality purified DNA was obtained

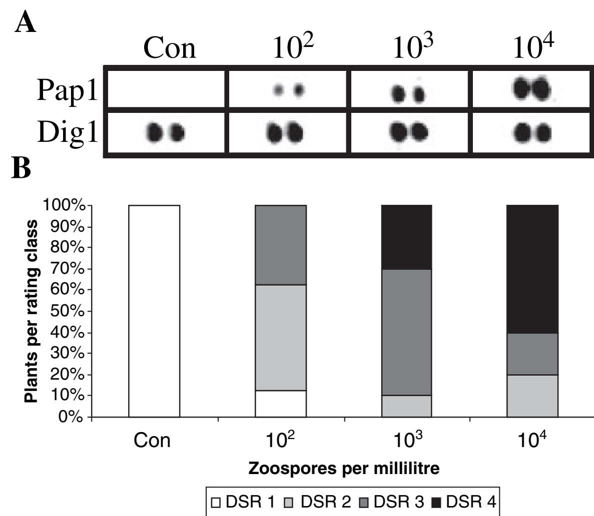

Fig. 4. Quantitative assessment of *P. aphanidermatum* in artificially infested water-based samples.
A. DNA array analysis 10 days after inoculation of nutrient solution with 0 (Con), 10^2, 10^3 or 10^4 *P. aphanidermatum* zoospores per millilitre. Signals after hybridization to the detector oligonucleotides to detect *P. aphanidermatum* (Pap1) and the digoxigenin-labelled reference (Dig1), spotted in duplicate, are shown.
B. Disease severity rating (DSR) for root and foot rot expressed as the percentage of plants per treatment ($n = 10$). Plants were rated 10 days after treatment for disease severity on a 1–5 scale:
1 = symptomless, 2 = light browning and/or superficial lesions present, 3 = dark browning and/or sunken lesions present,
4 = development of coalescing lesions and necrosis, 5 = plant death.
The experiment was repeated twice with similar results.

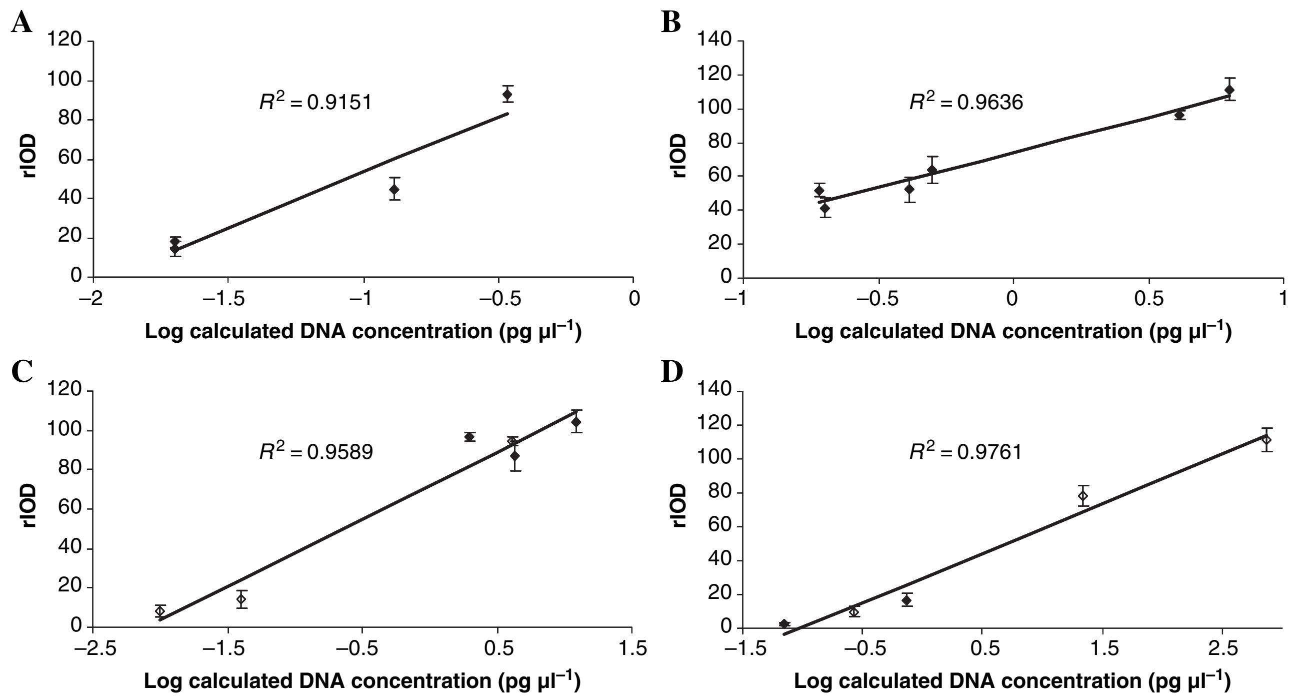

Fig. 5. Quantitative assessment of microbial presence in naturally contaminated environmental samples for (A) *F. solani*, (B) *F. oxysporum*, (C) *P. ultimum* and (D) *R. solani*. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labelled reference control (rIOD) and plotted versus the logarithmic calculated DNA concentration using real-time PCR. Data represent the average of two independent analyses of hybridization signals ($n = 4$) using detector sequences Fso1, Fox2, Pul1 and Rso1 to detect and quantify *F. solani*, *F. oxysporum*, *P. ultimum* and *R. solani* respectively. Error bars indicate standard errors. ◆, soil sample; ◇, plant sample.

in this work by using the commercially available Mo Bio Ultra Clean DNA extraction kits. In addition, the high quality of the DNA extracted was confirmed by standard spectrophotometric readings at 260 and 280 nm.

Based on the data obtained in this study, it can be stated that an immobilized oligonucleotide amount of 0.5 fmol allows quantification of template DNA over a wide concentration range. In contrast, a detector amount of 2.0 fmol or more allows a more sensitive detection at the lower concentrations accompanied by a loss of resolution at the higher concentrations. Thus, the choice of detector oligonucleotide amount should depend on the range of concentrations that need to be measured, which is determined by the range of concentrations by which these pathogens are found in horticultural practice. By considering pathogen biomass and, hence, their corresponding DNA concentrations that typically occur in cultivated horticultural soils, our results revealed that accurate DNA quantification is optimal when 8.0 fmol detector oligonucleotide, which is equivalent to 4.82×10^{12} molecules, was immobilized per spot (approximately 1 mm^2), irrespective the presence or absence of non-target DNA. With this amount of detector oligonucleotide, the assay was determined to be the most sensitive and quantitative over a range covering at least three orders of magnitude that are relevant to horticultural conditions. Increasing the amount of detector oligonucleotide did not enhance detection sensitivity (data not shown), probably due to steric hindrance caused by the high packing of the oligonucleotides in the spot. Additional experiments have revealed that certain oligonucleotides are easily saturated, even at low DNA concentrations. As a consequence, the choice of detector oligonucleotide amount depends on the detector sequence and should therefore be determined for each oligonucleotide individually.

In a previous study (Lievens *et al.*, 2003), we have shown that the detection limit of the DNA array technique largely depends on the detector sequences used. Generally, less than a picogram of DNA from a single target organism could easily be detected, if the appropriate detector oligonucleotide sequence is used. However, based on the data obtained in this study, it can be concluded that the detection limit of a detector oligonucleotide is, in addition, determined by the total population of microorganisms whose DNA is amplified by the same primer pair that amplifies the DNA of the target organisms. In this study, the lowest amount of fungal target DNA tested (0.25 pg) could not be detected when the amount of fungal non-target DNA exceeded the target DNA over 1000-fold. Experiments with other fungal detector oligonucleotides show that the interference caused by fungal non-target DNA is a general phenomenon. To check for pos-

sible underestimation of target presence, a non-discriminative fungal detector oligonucleotide (Fun1) was added to the membrane. In general, if signals were obtained when this detector was printed at an amount of 0.02 fmol, the target was underestimated.

Based on the results obtained in this study, the power of the technology for quantitative assessment of the presence of multiple pathogens in various biological matrices is shown (Figs 3–5). However, as all DNA-based detection methods might detect DNA from dead or non-active organisms as well, detection of non-viable propagules cannot be ignored. However, DNA derived from dead cells in soils will be degraded fairly rapid due to the generally high microbial activity, suggesting that interference by DNA from dead cells might be of less importance (England *et al.*, 1998; Schena and Ippolito, 2003).

Currently, the rDNA operon is the main genomic region targeted in fungal molecular diagnostics (McCartney *et al.*, 2003), mainly because it provides a powerful means for analysing phylogenetic relationships over a wide range of taxonomic levels, including genus, species (White *et al.*, 1990), and even below (Atkins *et al.*, 2003). However, for polytypic species such as *F. oxysporum*, *F. solani* and *R. solani* that can harbour several genetically distinct strains, the rDNA target locus may not always resolve taxa at the species level. Therefore, multiple detector oligonucleotides may be required to consistently detect all strains within a given species. In addition, with regard to complex species that include both pathogenic and non-pathogenic strains, additional efforts are required for accurate disease diagnosis. Whereas the oligonucleotides Fox2, Fso1 and Rso1 detect and quantify the presence of the respective species *F. oxysporum*, *F. solani* and *R. solani* present in a sample, they provide no information about their pathogenicity. Currently, no molecular markers are known for these species that allow to discriminate between pathogenic and non-pathogenic strains. As soon as specific sequences are identified allowing this type of discrimination, the array can be adapted for accurate detection and quantification of pathogenic strains. Until then, pathogenicity tests with host differentials will have to be performed to determine whether or not a specific isolate is a pathogen of a specific crop.

Using DNA array technology, in principle, an unlimited number of microorganisms can be detected in a single assay. The most advanced DNA array platforms are high-density DNA microarrays for which oligonucleotides are either synthesized *in situ* or spotted onto an impermeable rigid support such as a glass slide (Cunningham, 2000). There are a number of disadvantages to the use of microarrays compared with macroarrays for disease diagnostic use in plant pathology. Microarray technology is generally less sensitive than conventional membrane hybridization due to the limitations in the amount of detector oligonucleotide that can be spotted on the solid support and the corresponding limit in loading capacity (Voordouw *et al.*, 1993; Cho and Tiedje, 2002). In addition, quantification still remains problematic due to difficulties in spot detection and inter- and intraspot variability (Cuzin, 2001; Cho and Tiedje, 2002). In contrast, we observed that interspot variability for our macroarray is limited. Finally, highly specialized instruments are needed for microarray fabrication, as well as for hybridization and reading of the hybridization results, making implementation of this technology for plant disease diagnosis expensive. In contrast, a macroarray-based detection procedure for plant pathogens has been proven to be relatively cost-effective. Ultimately, this approach may lead to a complete pathogen assessment method to detect and quantify all known pathogens (including fungi, bacteria, nematodes, as well as viruses) of specific crops. In addition, this technology may also be used for the description of soil microbial communities (Mazzola, 2004). Currently, other techniques such as DGGE (denaturing gradient gel electrophoresis) or T-RFLP (terminal restriction fragment length polymorphism) are most frequently used for this purpose. However, the considerably higher resolution that can be obtained using DNA arrays is a major advantage (Mazzola, 2004). The ability to perform accurate quantification makes the use of DNA arrays even more appealing for diverse applications.

Experimental procedures

Microorganisms and cultivation

The fungal isolates *F. oxysporum* f. sp. *lycopersici* CBS 646.78, *F. solani* CBS 165.87, *V. albo-atrum* CBS 451.88, *V. dahliae* CBS 381.66, *P. ultimum* CBS 656.68 (Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands) and *P. aphanidermatum* ST 59.04 (Scientia Terrae Research Insititute, Sint-Katelijne-Waver, Belgium) were cultured on potato dextrose agar. *Saccharomyces cerevisiae* MUCL 28426 (Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium) and the bacterial strain *R. vitis* LMG 258 (Laboratory of Microbiology, Ghent University, Gent, Belgium) were cultured on malt extract agar supplemented with yeast extract (2%) and nutrient agar respectively. All cultures were incubated in darkness at 24°C.

Production of artificially inoculated samples

To quantify pathogen occurrence using DNA arrays, different samples were produced containing biologically relevant pathogen concentrations. Initially, microsclerotia from *V. dahliae*, or conidia from either *V. albo-atrum* or *V. dahliae* were added to 0.75 g of sandy field soil. These samples were subsequently used for DNA extraction and DNA array analysis. Microsclerotia were produced according to the method described by Hawke and Lazarovits (1994). The microsclerotia were suspended in sterile distilled water, vortexed briefly

to disrupt microsclerotia aggregates, washed through a 125 μm sieve with sterile distilled water and collected on a 32 μm sieve. Conidia were obtained by gently washing a culture plate with sterile distilled water. Conidial cells were counted by direct light microscopy using a haemocytometer, serially diluted and adjusted to the desired concentration. In addition, water-based samples were collected to examine whether or not a relationship could be established between hybridization signals obtained for the tomato pathogen *P. aphanidermatum* and disease development. Ten 14-day-old tomato (*Lycopersicon esculentum* Mill. cv. Clotilde) seedlings were transferred to test tubes filled with 6 ml of nutrient solution (Cooper, 1979) containing 10^2, 10^3 or 10^4 zoospores per millilitre. Zoospores were produced in 20 ml of sterile mineral salt solution (0.68 mM Ca^{2+}, 0.05 mM Mg^{2+}, 0.73 mM K^+ and 0.06 Fe^{3+}) inoculated with 10 agar plugs (diameter 5 mm) of *P. aphanidermatum* grown on V8 agar. After 1 day of incubation at 24°C under continuous illumination, zoospores were harvested and counted as described above. Plants were incubated in a growth chamber with a 16 h photoperiod (225 μE m^{-2} s^{-1}) at 22°C. After 10 days, disease severity rating for plant root and foot rot was scored on a 1–5 scale: 1 = symptomless, 2 = light browning and/or superficial lesions present, 3 = dark browning and/or sunken lesions present, 4 = development of coalescing lesions and necrosis, 5 = plant death. At the same time, the remaining nutrient solution was collected and used for DNA extraction and DNA array analysis.

Collection of environmental samples

To define the range of relevant DNA concentrations by which soilborne pathogens occur in their natural habitats, several soil samples were collected from commercial greenhouses at various times during the growing season. Subsamples were retained for DNA extraction. Separately, soil samples were collected from two fields that were naturally infested with *V. dahliae*. After a 2-week period of air-drying the number of viable *V. dahliae* microsclerotia was determined using the wet sieving technique (Harris *et al.*, 1993). Briefly, 12.5 g of air-dried soil was wet sieved, followed by suspending the 20–100 μm fraction in 0.08% agar. Subsequently, 0.8 ml of this suspension was spread on modified soil extract agar medium (Harris *et al.*, 1993). Plates were incubated in darkness at 24°C. After 4 weeks, soil particles were removed from the plates and clusters of *Verticillium* microsclerotia were counted. Subsamples of the air-dried soil were retained for DNA extraction. In addition, several samples from various matrices, including infected plants and infested soils, were gathered from commercial growers to address the robustness of the technology for quantitative assessment of pathogen presence.

DNA extraction

Genomic DNA from all microorganisms was isolated as previously described (Lievens *et al.*, 2003). For DNA isolation from soil and plant samples, genomic DNA was extracted from 0.75 g starting material using the UltraClean Soil DNA Isolation Kit and the UltraClean Plant DNA Isolation Kit as described by the manufacturer (Mo Bio Laboratories, Solana Beach, CA, USA), and subsequently diluted 10-fold. For water-based samples, DNA was extracted using the UltraClean Water DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA, USA) as described by the manufacturer's protocol. DNA yield and purity were determined spectrophotometrically.

DNA macroarray production

Part of the set of detector oligonucleotides used in this study (Table 3) was previously selected (Lievens *et al.*, 2003). In addition, oligonucleotides were developed to detect *F. solani* (Fso1), *P. aphanidermatum* (Pap1), *P. ultimum* (Pul1) and *R. solani* (Rso1). All oligonucleotides were found to be specific upon BLAST analysis and cross-hybridization testing with over 175 related and non-related strains (data not shown). In addition to the previously used control oligonucleotides Uni1 and Con1, as a control for the hybridization, and Dig1, as a reference for the detection and calibration (Lievens *et al.*, 2003), an oligonucleotide was designed to target exogenous control DNA derived from bakers yeast (Sce1). All oligonucleotides were synthesized with a 5′-C6-amino linker for covalent binding to nylon membrane and were diluted in sodium bicarbonate buffer (0.5 M, pH 8.4) in a microtitre plate and spotted in duplicate on Immunodyne ABC membranes (PALL Europe, Portsmouth, UK) using a multiblot replicator (V and P Scientific, San Diego, CA, USA). The distance between two spots, having a surface of nearly 1 mm^2, was approximately 3 mm. For this study, the detector oligonucleotides Fun1, Fox2, Fso1, Val2 and Vda1 were initially spotted on the membrane at different quantities, namely 8.0, 2.0, 0.5, 0.2, 0.1 and 0.02 fmol. Detectors Pap1, Pul1 and Rso1 were spotted at 8.0 fmol per spot. For the control oligonucleotides, 8.0 fmol (Uni1, Con1, Sce1) or 2.0 fmol (Dig1) was printed on the membrane. After a 10 min drying step, blots were transferred into blocking solution [2× standard saline citrate (SSC), 0.5% casein, and 0.05% Tween 20] and agitated for 30 min at room temperature. Membranes were stored in 2× SSC at 4°C until use.

Polymerase chain reaction amplification and labelling

In order to determine the optimal number of PCR cycles that permits end-point quantification, the target internal transcribed spacer (ITS) region of fungal rDNA was amplified using the primers ITS1-F and ITS4 (Gardes and Bruns, 1993). Amplification was carried out in 20 μl reaction volume using 1 unit Titanium *Taq* DNA polymerase (Clontech Laboratories, Palo Alto, CA, USA) according to the manufacturer's protocol. Before amplification, samples were pre-heated to 94°C for 2 min. Next, 25, 30, 35 or 40 cycles of a PCR reaction protocol consisting of 45 s at 94°C, 45 s at 59°C and 45 s at 72°C, with a final 10 min extension step at 72°C, were run. After gel electrophoresis, PCR products were quantified by comparison to a DNA ladder (Smartladder SF, Eurogentec, Seraing, Belgium) using Labworks Image Acquisition and Analysis Software (version 4.0; UVP, Upland, CA, USA).

For DNA array analysis, the target ITS region was amplified and simultaneously labelled with alkaline-labile digoxigenin

(Roche Diagnostics GmbH, Mannheim, Germany) using the fungal primer set ITS1-F and ITS4 (Gardes and Bruns, 1993) or the oomycete primer set OOMUP18Sc and ITS4 (Lievens *et al*., 2004). As a control for PCR efficiency between different samples, 1 ng of exogenous control DNA from *S. cerevisiae* was added to each sample, amplified and simultaneously labelled in separate PCR reactions using primers $P450_1$ and $P450_2$ (Morace *et al*., 1997). The samples were amplified for 30 cycles in 20 μl using 1 unit Titanium *Taq* DNA polymerase in the presence of 7.5 μM digoxigenin-11-d-UTP (Dig-dUTP; Roche Diagnostics GmbH, Mannheim, Germany) according to the same thermal profile as described above. The resulting Dig-dUTP-labelled amplicons were subsequently used as targets in hybridization reactions to the array.

DNA hybridization of PCR amplicons

Hybridization was conducted as described previously (Lievens *et al*., 2003). Ten microlitres of labelled amplicons of both target and control DNA amplification reactions were combined and simultaneously hybridized to the array in 6 ml of hybridization buffer. Chemiluminescence was detected cumulatively at 30 s intervals over 45 min using a highly sensitive digital CCD video imaging system (BioChemi System; UVP, Upland, CA, USA). Quantification of hybridization signals was carried out using Labworks Image Acquisition and Analysis Software (version 4.0; UVP, Upland, CA, USA). Hybridization signal strength was reported relative to the average integrated optical density of the digoxigenin-labelled reference control. All hybridization assays were conducted at least twice.

Real-time quantitative PCR

To define the range of relevant DNA concentrations by which fungal pathogens occur in naturally infested greenhouse soils and to verify the robustness and accuracy of the quantitative DNA array-based assay, the amount of fungal DNA was quantified in a set of representative soil samples and various environmental samples, respectively, using real-time PCR. This was done for a number of different pathogens detected in these samples using the DNA Multiscan® (De Ceuster, Sint-Katelijne-Waver, Belgium). Real-time PCR amplification reactions were conducted using SYBR® Green I technology on a Lightcycler™ instrument (Roche Diagnostics, Indianapolis, IN, USA). Each reaction mixture contained 2 μl of DNA extract, 4 μl of the Lightcycler FastStart DNA Master^{PLUS} SYBR® Green I kit (Roche Diagnostics, Indianapolis, IN, USA), 1 μl of each primer (10 μM) and 12 μl of sterile distilled water. For each pathogen, the forward primer ITS1-F (Gardes and Bruns, 1993) or OOMUP18Sc (Lievens *et al*., 2004), which hybridizes to a fungal- or oomycete-specific rDNA sequence, respectively, or the universal reverse primer ITS4 (White *et al*., 1990) was combined with the appropriate reverse or forward species-specific primer as presented in Table 6, to generate amplicons smaller than 300 bp. The amount of total fungal and oomycete DNA was quantified using the primer pair ITS1-F and ITS2 (White *et al*., 1990), and OOMUP18Sc and ITS2-O (Table 6) respectively. Samples were preheated to 95°C for 10 min and were then subjected to PCR amplification reactions consisting of 45 amplification cycles of 10 s at 95°C, 5 s at the annealing temperature indicated in Table 6, and elongation at 72°C for the time period indicated in Table 6. Fluorescence (520 nm) was detected at the end of the elongation phase for each cycle. After the final amplification cycle, a melting curve temperature 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. For each pathogen, standard curves were generated by plotting the threshold cycle (C_t) of a 10-fold dilution series of genomic DNA versus the logarithm of the concentration. The regression line was used to calculate the respective pathogen DNA concentration in the studied sample via its C_t-value.

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Table 6. Primers used for real-time PCR.

Code	Organism	Sequence (5′–3′)	Origin	T_m	t_{el}
ITS2-O (R)	Oomycetes	GCAGCGTTCTTCATCGATGT	5.8S rDNA	60	12
AFP308 (R)	*F. oxysporum*	CGAATTAACGCGAGTCCCAAC	ITS II	60	9
AFP346 (R)	*F. solani*	GGTATGTTCACAGGGTTGATG	ITS I	60	6
AFP356 (R)	*P. sylvaticum*	CAATGCAAAGTCAGCAGTGC	ITS I	60	9
AFP276 (F)	*P. ultimum*	TGTATGGAGACGCTGCATT	ITS II	58	8
ST-RS1 (F)	*R. solani*	AGTGTTATGCTTGGTTCCACT	ITS II	60	8
AFP307 (R)	*V. dahliae*	CAGAGAGACTGATGGACCG	ITS I	60	9

F, forward primer; R, reverse primer; T_m, annealing temperature (°C); t_{el}, elongation time (s).

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