Katholieke Universiteit Leuven
Faculteit Bio-ingenieurswetenschappen
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DISSERTATIONES DE AGRICULTURA

Doctoraatsproefschrift nr. 712 aan de faculteit Bio-ingenieurswetenschappen
van de K.U.Leuven

Development of a DNA Array for Multiplex Detection and Quantification of Plant Pathogens

Proefschrift voorgedragen tot
het behalen van de graad van
Doctor in de
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door

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Development of a DNA Array for Multiplex Detection and Quantification of Plant Pathogens

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Samenvatting

Accurate detectie en identificatie van plantpathogenen zijn onontbeerlijk voor de diagnose van plantenziekten dat de basis vormt van een veilige en duurzame gewasbescherming. De specifieke tekortkomingen van de klassieke kweek- en morfologie-gebaseerde identificatiemethoden hebben geleid tot de ontwikkeling van moleculaire benaderingen die geen kweek van de te identificeren micro-organismen vereisen. In de laatste decennia zijn verschillende serologische en nucleïnezuurgebaseerde technieken ontwikkeld voor het opsporen en identificeren van plantpathogenen (Hoofdstuk 1). Bepaalde van deze technieken laten bovendien een betrouwbare kwantificatie van de doelwitpathogeen toe en verschaffen aldus de vereiste informatie voor het inschatten van de risico's wat betreft ziekteontwikkeling, inoculumverspreiding en economische verliezen. De belangrijkste uitdaging bij de aanvang van het onderzoek beschreven in deze thesis bestond erin een multiplex test te ontwikkelen die geschikt is voor het gelijktijdig opsporen en kwantificeren van een breed gamma aan plantpathogenen.

In deze thesis wordt de ontwikkeling van een DNA "macroarray" beschreven die aan deze vereisten voldoet. In eerste instantie werden de algemene voorwaarden bepaald voor het ontwikkelen van selectieve detectoroligonucleotiden (Hoofdstuk 2). De bruikbaarheid van DNA "arrays" om "single nucleotide polymorphisms" te detecteren is aangetoond door specifieke criteria zoals de positie van de "mismatch", de sequentie van het oligonucleotide en de lengte en hoeveelheid van de gemerkte amplicons in acht te nemen. Op basis van deze criteria werd vervolgens een DNA "macroarray" ontwikkeld die getest is voor een snelle en efficiënte detectie en identificatie van een beperkte set schimmelpathogenen in biologisch complexe stalen zoals plant- en grondstalen (Hoofdstuk 3). Als "proof-of-principle" werd de "macroarray" ontwikkeld voor een aantal belangrijke ziekteverwekkers van tomaat dat wereldwijd één van de economisch belangrijkste vruchtgewassen is. Meer bepaald werd gekozen voor de vaatbundelpathogenen *Fusarium oxysporum* f. sp. *lycopersici*, *Verticillium albo-atrum* en *V. dahliae*. In Hoofdstuk 5 werd deze "array" verder geoptimaliseerd om een accurate kwantificatie van de pathogenen te bewerkstelligen over tenminste drie grootte-ordes die praktijkrelevant zijn. Een sterke correlatie werd vastgesteld tussen de hybridisatiesignalen en de pathogeenconcentraties, zowel voor standaard DNA (al dan niet

in aanwezigheid van niet-doelwit DNA) als voor besmette stalen. Wanneer specifieke criteria zoals de hoeveelheid gebonden oligonucleotiden en bepaalde controles voor het amplificatieproces in acht werden genomen, kon een accurate kwantificatie bewerkstelligd worden voor praktijkrelevante pathogeenconcentraties. Gezien kwantificatie op basis van kweekmethoden als relatief onnauwkeurig of zelfs onbetrouwbaar wordt beschouwd, werd real-time PCR, een reeds gevestigde techniek om DNA te kwantificeren (Hoofdstuk 4), als referentietechniek gebruikt ter validatie van de kwantificering met behulp van de DNA "array". Het feit dat beide kwantificatiemethoden sterk gecorreleerd waren illustreert de betrouwbaarheid en robuustheid van het kwantitatieve karakter van DNA "macroarrays". In het kader van een geïntegreerde gewasbeschermingstrategie werd tenslotte een kwantitatieve "macroarray" ontwikkeld om simultaan zowel pathogenen als biocontrole agentia te detecteren, alsook om hun interacties te bestuderen en hun aanwezigheid te koppelen aan ziekteontwikkeling en symptoomexpressie. Doordat momenteel geen gestandaardiseerde biotoets beschreven is voor tomaat, werd de reeds uitvoerig bestudeerde interactie tussen het biocontrole agens *Trichoderma hamatum* isolaat 382 en de pathogeen *Rhizoctonia solani* aangewend in een standaard biotoets van *R. solani*, de veroorzaker van omvalziekte, op radijs (Hoofdstuk 6). Uit deze studie kan geconcludeerd worden dat DNA "macroarrays" met succes kunnen gebruikt worden voor het gelijktijdig detecteren en kwantificeren van verschillende plantpathogenen in biologisch complexe stalen. Naast zijn toepassingsmogelijkheden voor het routinematig detecteren van plantpathogenen, heeft deze techniek bovendien het potentieel om ingezet te worden in diverse ecologische en epidemiologische studies.

Abstract

Accurate detection and identification of plant pathogens are fundamental to plant pathogen diagnostics and thus plant disease management. The specific limitations of culture-based morphological techniques to adequately identify plant pathogens have led to the development of culture-independent molecular approaches. In the last two decades, many different serological and nucleic acid-based techniques have been developed for the detection and identification of plant pathogens (discussed in Chapter 1). Some of these techniques also permit reliable quantification of the target pathogen, and supply the information that is required to estimate risks with respect to disease development, spread of the inoculum, and economic losses. The major challenge at the start of the research described in this thesis was the development of a multiplex assay that allows accurate detection and quantification of multiple pathogens in a single assay.

In this thesis, the development of a DNA macroarray is described to meet these requirements. First, the overall conditions were determined for the design of highly discriminative detector oligonucleotides (Chapter 2). The utility of DNA array technology is shown to distinguish single base pair differences while accounting for specific criteria such as the position of the mismatch, the sequence of the oligonucleotide, and the length and amount of labeled amplicons that are hybridized. Based on these results, a DNA macroarray was designed for rapid and efficient detection and identification of a comprehensive set of fungal pathogens in complex samples, including artificially and naturally infested plant and soil samples (Chapter 3). As a proof-of-principle, the array was developed for a number of economically important fungal pathogens of tomato which is one of the most important vegetable crops worldwide. The pathogens selected for this study comprised the vascular wilt pathogens *Fusarium oxysporum* f. sp. *lycopersici*, *Verticillium albo-atrum*, and *V. dahliae*. In Chapter 5, this array has been further optimized for accurate pathogen quantification over at least three orders of magnitude. A strong correlation was observed between hybridization signals and pathogen concentrations for standard DNA, in the absence of or added to non-target DNA from different origins, and for infested samples. While accounting for specific criteria like amount of immobilized detector oligonucleotide and specific controls for PCR kinetics, accurate quantification of pathogens was achieved in

concentration ranges typically encountered in horticultural practice. Since quantification based on culturing techniques is considered relatively inaccurate, real-time PCR, a well-established and reliable technique to quantify DNA levels (Chapter 4), was used as a reference technique to validate DNA array-based quantification. As both methods of quantification showed a very high degree of correlation, the reliability and robustness of the DNA array technology is shown. Finally, in the frame of an integrated pest management (IPM) based disease management strategy, a quantitative DNA macroarray was developed to simultaneously monitor populations of pathogens and biocontrol agents, as well as to investigate their interactions, and relate their presence to disease development. Since currently no standard biocontrol assay was available for the model crop tomato, the well established interaction between the biocontrol agent *Trichoderma hamatum* isolate 382 and the pathogen *Rhizoctonia solani* was used in a standard damping-off of radish bioassay (Chapter 6). Altogether, it is shown that DNA macroarrays can be successfully used to simultaneously detect and quantify multiple plant pathogens in samples from various biological sources including those gathered from horticultural practice. Apart from its applicability in routine plant pathogen diagnosis, this technique has the potential to become a reliable tool for diverse ecological and epidemiological studies.

List of abbreviations

3SR	self-sustained sequence replication
AFLP	amplified fragment length polymorphism
AG	anastomosis group
ANOVA	analysis of variance
BLAST	basic local alignment search tool
bp	base pairs
CFU	colony forming units
CPB	composted pine bark amended potting mix
cv.	cultivar
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
DSM	disease suppressive microorganism
DSP	dark Sphagnum peat based potting mix
ELISA	enzyme-linked immunosorbent assay
EMA	ethidium monoazide
f. sp.	*forma specialis*
FRET	fluorescent resonance energy transfer
IC-PCR	immunocapture PCR
IPM	integrated pest management
ITS	internal transcribed spacer
LCR	ligase chain reaction
LSD	least significant difference test
mRNA	messenger RNA
NASBA	nucleic acid sequence based amplification
PCR	polymerase chain reaction
PDA	potato dextrose agar
RAPD	random amplified polymorphic DNA
RCA	rolling circle amplification
rDNA	ribosomal DNA – ribosomal RNA gene
rIOD	relative integrated optical density
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase PCR
SCAR	sequence characterized amplified region
SDS	sodium dodecyl sulfate
SNP	single-nucleotide polymorphism
sp.	species
SSC	sodium chloride sodium citrate
T_{382}	*Trichoderma hamatum* isolate 382
T_{ann}	annealing temperature
t_{el}	elongation time
TMA	transcription mediated amplification
var.	variety
vol	volume
wt	weight

1 Recent developments in diagnostics of plant pathogens*

1.1 Introduction

Diseases caused by plant pathogens, including fungi, oomycetes, bacteria, nematodes and viruses, can cause serious economic losses to both agricultural and horticultural crops. In general, synthetic pesticides have been used intensively to prevent or control diseases. However, largely driven by concerns about the detrimental effects of the use of these chemicals on the environment and on public health, integrated pest management (IPM) has become a mainstream strategy for managing plant diseases over the last few decades (Jarvis, 1992; Shea *et al*., 2000). IPM relies preferentially on non-chemical means and involves the integration of different control strategies of biological, chemical, and cultural nature to reduce pathogen and pest populations below an economical threshold (Apple and Smith, 1976). However, IPM has been severely limited by the lack of fast, accurate, and reliable means by which plant pathogens can be timely detected (preferably before symptoms occur), identified, and accurately quantified. Quantification is particularly important since it serves as the basis for establishing population thresholds whereby a pathogen causes disease, and at which point measures may be employed to effectively limit or prevent losses.

Conventional methods to detect plant pathogens have often relied on interpretation of symptoms, biochemical or morphological identification, usually following isolation and culturing of the organism *in vitro* and, sometimes, on further characterization based on pathogenicity tests (Singleton *et al.*, 1992). Although these methods are fundamental to diagnostics, the accuracy and reliability of these methods largely depend on skilled taxonomical expertise. In addition, diagnosis requiring a culturing step is time consuming and labor intensive. Furthermore, quantification based on these culturing 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). Finally, these techniques rely on the ability of the organism to be cultured *in vitro*. This latter aspect is a considerable limitation since possibly less than 1% of the microorganisms

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in an environmental sample may be cultured *in vitro* (Amann *et al.*, 1995; Rapp and Giovannoni, 2003).

In contrast, more recently developed methods that are based on molecular approaches are increasingly being used to detect and identify plant pathogens. These include immunological (or serological) and nucleic acid-based techniques. Compared to conventional assays, these techniques are more suitable for routine analyses since they are generally faster, more specific, more sensitive and more accurate, and can be performed and interpreted by personnel with no taxonomical expertise. In addition, since no culturing step is required, these techniques are equally suitable for the detection of culturable as well as non-culturable microorganisms.

Many different molecular assays have been described for the detection and identification of pathogens, each requiring its own protocol, equipment, and expertise. In this chapter, some recent advances in molecular plant pathogen diagnostics with an emphasis on molecular diagnostics for fungal and oomycete plant pathogens are outlined.

1.2 Serological techniques

A first development towards techniques for molecular pathogen detection was the advent of serological or antibody-based detection methods almost 30 years ago. These techniques were originally developed to detect viruses, as those can not be cultured *in vitro*. Serological techniques are based on the binding between diagnostic antibodies and specific antigenic determinants of the target pathogen. Several serological plant pathogen detection methods have been described (Lopez *et al.*, 2003; Ward *et al.*, 2004) of which the enzyme-linked immunosorbent assay (ELISA; Clark and Adams, 1977) is by far the most widespread technique. Although different types of ELISA have been developed, all involve an enzyme-mediated color change reaction to detect and often also quantify antibody binding as a measure for pathogen presence. Since its introduction in the late 1970s ELISA assays have been routinely used for virus and bacteria detection because of their high-throughput capacity, the rapid, relatively cheap and simple nature, and the possibility to quantify the amount of target pathogen (Hampton *et al.*, 1990; Schaad *et al.*, 2001).

A major limitation for the development of serological methods is the labor-intensive procedure to obtain reliable assays, often due to the difficulty to generate selective antibodies. Although polyclonal antibodies, which recognize multiple epitopes of the pathogen, have been used successfully for detecting many viruses, they do not always

display the desired degree of target specificity and, importantly, the specificity may vary with each newly produced batch. The accuracy of detection is often improved by using either monoclonal or recombinant antibodies. Both of these allow the selection of specific target epitopes to avoid “false positives”. However, developing antibodies with the required degree of specificity is difficult for relatively complex organisms such as bacteria, fungi and oomycetes. For this kind of organisms, it is often hard to find reliable species-specific epitopes that are ubiquitously shared within a species but not with other species. Therefore, most antibody-based assays currently available are for the detection of relatively unsophisticated organisms such as plant viruses (Sward and Eagling, 1995; Torrance, 1995) while those available for the detection of fungi, oomycetes and bacteria are less common (Dewey and Thornton, 1995; Spire, 1995). On the other hand, immunological techniques can not be applied to poorly sophisticated organisms such as viroids because viroids are infectious naked RNAs that lack detectable proteins (Hadidi *et al*., 2003).

1.3 Nucleic acid-based techniques

Before the possibility to amplify nucleic acid sequences existed, the sensitivity of detection based on those sequences totally relied on the method to translate their presence into a detectable signal, e.g. using radioactive DNA-DNA hybridization (Cheung *et al.*, 1980; Horn *et al.*, 1986; Yao *et al.*, 1991). Since the introduction of amplification methods for nucleic acids, in particular the polymerase chain reaction (PCR; Mullis and Faloona, 1987), nucleic acid-based methods are increasingly developed for the detection and identification of plant pathogens. This trend is enhanced by the growing availability of sequence data in public databases like GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) and COGEME (http://www.cogeme.man.ac.uk/) (Soanes *et al.*, 2002; Benson *et al.*, 2004) and also by the increased availability of microbial full genome sequences (http://www.broad.mit.edu/annotation/fgi/ and http://www.sanger.ac.uk/Projects/Microbes/).

A crucial step in the development of nucleic acid-based diagnostic assays is the selection of sequences that can be employed for pathogen identification. In general, there are two approaches to select target sequences. The first, and most widespread, strategy involves the use of ubiquitously conserved genes, carrying target specific sequences. Currently, the primary target in the development of molecular diagnostics for bacterial as well as fungal or oomycete plant pathogens is the nuclear ribosomal DNA (rDNA), which has been extensively used in molecular phylogenetic studies and is therefore well

characterized (White *et al.*, 1990). In addition, a large amount of rDNA sequence data is available in public databases, which aids the design of diagnostic assays. These extensive sequence data allow comparison of sequences which permits, in turn, determining diagnostic regions that can be used to design selective primers or probes. This is facilitated even more by the structural nature of this type of gene since it contains alternating regions with high and low degrees of conservation. This allows to design primers on sequences that are conserved between species which span variable domains that can be used for species identification (White *et al*., 1990). Apart from the discriminatory potential, the high copy number of rDNA genes in any genome permits a highly sensitive detection.

Fungal as well as oomycete rDNA occurs as a repeated, structured unit consisting of three, relatively conserved, ribosomal RNA (rRNA) subunit genes which are separated by internal transcribed spacers (ITS). This ITS region is an area of particular importance to fungal diagnostics since it contains areas of relatively high variability, which allows classification over a wide range of taxonomic levels (White *et al.*, 1990), sometimes even below the species level (Atkins *et al.*, 2003). However, ribosomal sequences do not always reflect sufficient sequence variation to discriminate between particular species (Tooley *et al.*, 1996). Therefore, but also to corroborate discrimination based on ITS sequences, other housekeeping genes are becoming more intensively studied, including beta-tubulin (Fraaije *et al.*, 1999; Hirsch *et al.*, 2000), actin (Weiland and Sundsbak, 2000), elongation factor 1-alpha (O'Donnell *et al.*, 1998; Jimenez-Gasco *et al.*, 2002), and mating type genes (Wallace and Covert, 2000; Foster *et al.*, 2002).

The second strategy to select target sequences for detection of plant pathogens involves the screening of random parts of the genome to find diagnostic sequences. This can be achieved by several techniques, including random amplified polymorphic DNA (RAPD; Williams *et al.*, 1990) and amplified fragment length polymorphism (AFLP; Vos *et al.*, 1995) technology. Diagnostic markers identified with these approaches can be sequenced and used to design specific sequence characterized amplified region (SCAR) primers (Paran and Michelmore, 1993; Radisek *et al*., 2004). Nevertheless, as these sequences can be derived from anywhere in the genome, there often is few sequence data available for comparison to multiple other organisms. Therefore, extensive experimental screening is required to ensure specificity of the marker.

Nucleic acid-based techniques can be divided into DNA- and RNA-based technologies which are separately addressed below.

1.3.1 DNA-based techniques

Compared to RNA, DNA is a more attractive target for the detection of plant pathogens in biological samples because it is easier to handle and more resistant to degradation. In addition, with improved extraction methods (McCartney *et al.*, 2003) and commercially available extraction kits (Faggian *et al.*, 2003; Lievens *et al*., 2005a) highly purified DNA can rather easily be obtained from complex environmental samples.

Some typical features of the most important DNA-based techniques for detection of plant pathogens, including PCR, real-time PCR, and ligase chain reaction (LCR), are discussed below. In addition, attention is given to another amplification strategy, rolling circle amplification (RCA), since this technology holds promise to result in highly sensitive pathogen detection.

1.3.1.1 Polymerase Chain Reaction (PCR)

Using PCR, millions of copies of specific DNA sequences may be rapidly synthesized in a thermocyclic process that consists of repetitive cycles of DNA denaturation, primer annealing, and extension using a thermostable DNA polymerase (Mullis and Faloona, 1987). If a DNA sequence unique to a particular organism is determined, specific PCR primers can be designed that enable determination of the presence or absence of that sequence, and thus of the specific organism. The presence of amplified DNA is traditionally detected by gel electrophoresis, but alternative detection formats including colorimetric and fluorimetric assays do exist (Mutasa *et al.*, 1996; Fraaije *et al.*, 1999). PCR-based detection methods are very sensitive and can detect minute quantities of pathogen DNA, even the amount derived from a single fungal spore (Lee and Taylor, 1990). To improve specificity and sensitivity, PCR products may also be detected using a probe (Mutasa *et al.*, 1995), or alternatively the use of immunocapture PCR (IC-PCR) or nested PCR can be included. IC-PCR utilizes antibodies to isolate the pathogen from a sample prior to PCR amplification and has mainly been used to detect plant pathogenic viruses (Jacobi *et al.*, 1998). Nested PCR involves two consecutive PCR reactions, the second one using primers that share a sequence within the target DNA fragment that is amplified in the first reaction (Lacourt *et al.*, 1997). As a result, aspecific reaction products that are generated in the first PCR reaction should not be amplified in the second reaction.

Many reports describe specific applications of PCR technology in plant pathology (Haas *et al.*, 1995; Hamelin *et al.*, 1996; Zijlstra *et al.*, 1997; Judelson and Tooley, 2000;

Amiri *et al.*, 2002; Nie and Singh, 2003). In addition, companies providing diagnostic services are increasingly using PCR to routinely detect and identify plant pathogens, often for quarantine testing or to ensure the identity of a pathogen (and to complement classical diagnostic tools).

Quantification of the amount of pathogen DNA, supplying the information required for disease management decisions, and for monitoring the effects of these decisions, has also been pursued using PCR-based methods. Although it is relatively easy to quantify the amount of amplicon generated, it is more difficult to relate this quantity to the initial amount of target DNA present in a sample. This is caused by the typical non-linear kinetics of template amplification. Nevertheless, in theory, the exponential nature of PCR allows the initial amount of DNA to be calculated from the amount of product at any time point in the reaction. In practice, however, as the reaction proceeds reagents become limiting and a plateau level is reached where the amount of product is no longer proportional to the original amount of template. However, target DNA can be quantified using competitive PCR, which is based on the co-amplification of target DNA and competitor DNA, both with the same primer pair (Siebert and Larrick, 1992). The amount of target DNA is subsequently determined on agarose gel by comparing the relative amounts of target and competitor PCR product. This method has been used to successfully quantify, for instance, Verticillium wilt pathogens (Hu *et al.*, 1993).

1.3.1.2 Real-time PCR

Especially with respect to quantification purposes real-time PCR is a powerful development (Heid *et al.*, 1996). This technology differs from conventional PCR by monitoring PCR products on-line while they accumulate at each reaction cycle in a closed tube format, without the need of post-reaction processing such as gel electrophoresis. As a consequence, real-time PCR is faster than conventional PCR, enabling high throughput analyses. In addition, the risk of post-PCR carry-over contamination of amplicons is eliminated. Real-time PCR allows accurate template quantification during the exponential phase of the reaction, before reaction components become limiting. Typically, DNA amplification is monitored each cycle based on the emission of fluorescence (Heid *et al*., 1996; Mackay *et al.*, 2002). In general, the initial amount of target DNA is related to a threshold cycle, defined as the cycle number at which fluorescence increases above the background level.

Target DNA is quantified using a calibration curve that relates threshold cycles to a specific amount of template DNA.

Amplicons can be detected using several chemistries, which can be divided into either amplicon non-specific (Morrison *et al.*, 1998) and amplicon specific (Holland *et al.*, 1991; Livak *et al.*, 1995; Tyagi and Kramer, 1996; Wittwer *et al.*, 1997; Livak, 1999; Thelwell *et al.*, 2000; Mhlanga and Malmberg, 2001) methods, using DNA-binding dyes and sequence-specific probes, respectively (Fig. 1-1). The use of DNA-intercalating dyes such as SYBR® Green is a more straightforward and less expensive approach compared to using probes, but it is also less specific since the dye binds to all double stranded DNA (dsDNA) present in the sample (Fig. 1-1). In addition, the interpretation of results can be disturbed by formation of primer-dimers or aspecific reaction products. It is therefore crucial to use specific primers and to determine optimal reaction conditions (Mackay *et al.*, 2002; Papp *et al.*, 2003). In addition, melt curve analysis at the end of the PCR reaction allows evaluating the accuracy of the amplification reaction.

In contrast to amplicon non-specific chemistries, probe-based assays often offer the advantages of increased specificity, certainly in combination with specific primers, and reducing signals due to mispriming or primer-dimer formation (Livak *et al.*, 1995). Most applications to date have used TaqMan® probes (Livak *et al.*, 1995; Livak, 1999). These probes are single stranded, short oligonucleotides which are labeled with a fluorophore and a fluorogenic quencher (Fig. 1-1). Because of the close proximity of both groups, the fluorescent signal is quenched. During the annealing phase of each PCR cycle the probe hybridizes to a specific region within the target amplified fragment. The probe is degraded by 5' exonuclease activity when the DNA polymerase extends the primer. Consequently the fluorophore and the quencher are released independently, resulting in a fluorescent signal (Fig. 1-1). Variants of this quenching chemistry include hairpin shaped Molecular Beacons® (Fig. 1-1; Tyagi and Kramer, 1996; Mhlanga and Malmberg, 2001) and Scorpion® primers (Fig. 1-1; Thelwell *et al.*, 2000). Whereas the loop portion of these molecules contains the probe sequence, the stem, which is formed by complementary sequences added to both ends of the probe, holds a fluorophore and a quencher in close proximity. In addition, Scorpion® primers couple the stem-loop based probe to a PCR primer. Specific binding of the probe to its target opens the structure, producing a fluorescent signal (Fig. 1-1). A completely different detection chemistry comprises the use of fluorescent resonance energy transfer (FRET) probes (Fig. 1-1; Wittwer *et al.*, 1997). With this technology, two oligonucleotide probes are designed such that they hybridize in very close proximity to the amplified

fragment. Whereas one of the probes contains a donor fluorophore at its 3' end, the other probe is labeled at its 5' end with an acceptor fluorophore. When both probes properly hybridize to the target fragment, the energy excited by the donor is transferred to the acceptor resulting in a fluorescent signal (Fig. 1-1).

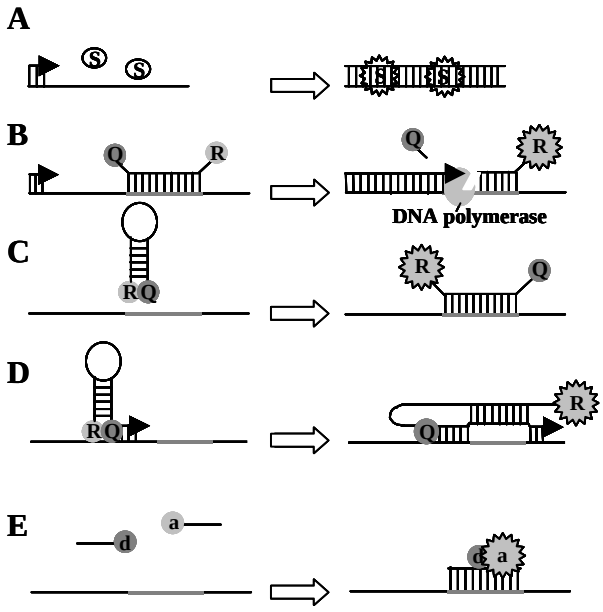

Fig. 1-1. Main chemistries for amplicon detection in real-time PCR applications. **A,** As a DNA-intercalating dye such as SYBR® Green (S) binds to double stranded DNA, fluorescence is recorded. **B,** Taqman® probes, **C,** Molecular Beacons® as well as **D,** Scorpion® primers use a strategy to extinguish fluorescence at certain conditions using a reporter fluorophore (R) and a fluorogenic quencher (Q). Upon physical separation of both molecules fluorescence is emitted. **E,** The use of FRET probes involves the hybridization of two labeled oligonucleotides in close proximity. When both probes bind to the target fragment, energy is transferred from the donor (d) to the acceptor (a) molecule resulting in fluorescence.

Closely related microbial species often only differ in a single or a few bases of ubiquitously conserved genes such as the rDNA. The high degree of specificity of real-time PCR technology allows, independent of the detection chemistry, the detection of single-nucleotide polymorphisms (SNPs), meaning that specificity is determined by a single base pair (Livak, 1999; Thelwell *et al.*, 2000; Mhlanga and Malmberg, 2001; Papp *et al.*, 2003). Therefore, this technology offers many opportunities in plant pathogen diagnostics. In recent years, real-time PCR assays have been developed for accurate detection and/or quantification of specific plant pathogens (Bohm *et al.*, 2001; Boonham *et al.*, 2002; Winton *et al.*, 2002; Mercado-Blanco *et al.*, 2004) as well as for monitoring pathogen infections

(Brouwer *et al.*, 2003). Although not yet used routinely in phytodiagnostics, real-time PCR has a large potential for future applications.

1.3.1.3 Ligase Chain Reaction (LCR)

LCR uses two complementary pairs of oligonucleotides that hybridize in close proximity on the target fragment (Fig. 1-2). Only when the oligonucleotides correctly hybridize to the target sequence, the remaining nick between the oligonucleotides is ligated by a DNA ligase and a fragment equating to the total sequence of both oligonucleotides is generated. Similar as in a PCR reaction, the products of one reaction serve as templates for subsequent cycles, resulting in an exponential amplification of the desired fragment (Fig. 1-2). To further enhance sensitivity and sometimes also specificity, LCR can also be used following a PCR preamplification (Wiedmann *et al.*, 1993; Tooley *et al.*, 2002). Detection of LCR products can be performed by polyacrylamide gel electrophoresis. With this technology, SNPs can easily be differentiated (Barany, 1991). Although LRC is regularly applied in human disease detection (Barany, 1991; Wiedmann *et al.*, 1993; Andrews *et al.*, 1997), it has rarely been reported for detection of plant pathogens (Wilson *et al.*, 1994; Tooley *et al.*, 2002).

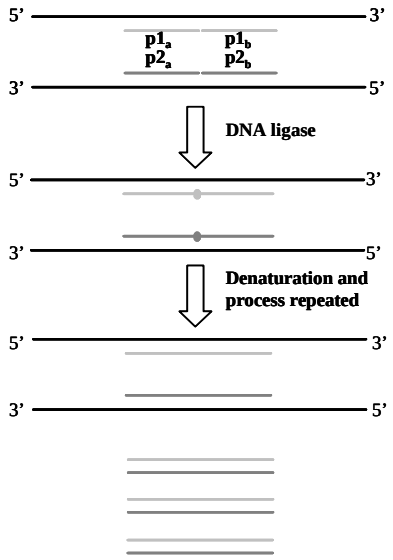

Fig. 1-2. General principle of the Ligase Chain Reaction (LCR). Two complementary pairs of adjacent oligonucleotides ($p1_a$ and $p1_b$; $p2_a$ and $p2_b$) bind to the target sequence. Only if the oligonucleotides bind in close proximity DNA ligase seals the nicks and the cycle can be repeated.

1.3.1.4 Rolling Circle Amplification (RCA)

Originally, padlock probes (Nilsson *et al.*, 1994) were developed as a new approach for molecular analysis of DNA samples, including analysis of alleles and point mutations in the human genome (Nilsson *et al.*, 1997). A padlock probe consists of a single stranded linear oligonucleotide of about 70-100 nucleotides in length with a target-complementary region at both ends and a linker segment in between. The 5' and 3' end regions are designed to hybridize next to each other on a target strand. When properly hybridized to the target sequence, the molecule can be circularized upon ligation. Because of the need for precise base pairing at the junction where ligation should take place and the simultaneous hybridization of two different fragments, padlock probes ensure high specificity (Nilsson *et al.*, 1997).

For sensitive pathogen detection, however, signal amplification is a prerequisite. One approach for the amplification of padlock probes is a PCR reaction using primers that hybridize to sequences within the spacer region of the probe (Thomas *et al.*, 1999). Another method to amplify padlock probes is rolling circle amplification (RCA), analogous to replication mechanisms of several viruses with circular genomes (Fire and Xu, 1995; Baner *et al.*, 1998; Thomas *et al.*, 1999; Nilsson *et al.*, 2002). Two types of RCA have been described: linear and hyperbranched RCA. In the first procedure, a primer hybridized at some point on the circular DNA is extended continuously using a DNA polymerase that lacks exonuclease activity. As a result, a long linear fragment composed of many tandem repeats of the complement to the circularized molecule is generated. In addition, hyperbranched (or cascade) RCA (Fig. 1-3) uses a second primer that binds to each generated RCA repeat. During elongation, the exoncuclease deficient DNA polymerase displaces the polymerized strand in front of it. Next, the displaced strands which are tandem repeats with identical sequences to the original padlock probe, serve again as template for the first primer, resulting in a cascade of DNA amplification (Fig. 1-3).

As for conventional PCR, detection of amplified products can be achieved using gel electrophoresis (Baner *et al.*, 1998; Lizardi *et al.*, 1998) or labeled probes (Nilsson *et al.*, 2002) enabling real-time monitoring of the amplification process. However, although RCA is considered to be one of the most sensitive amplification methods, the procedure is fairly complicated (Andras *et al.*, 2001) and relatively expensive.

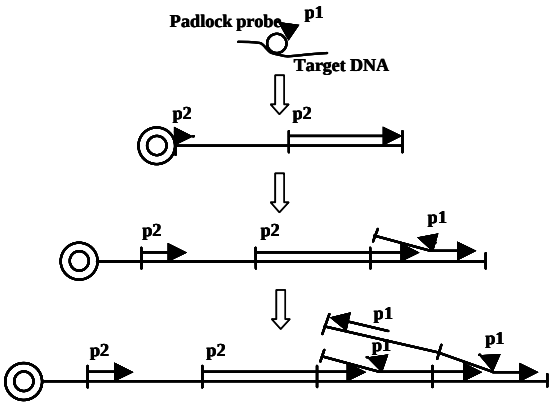

Fig. 1-3. General principle of hyperbranched Rolling Circle Amplification (RCA). The 5' and 3' ends of a linear padlock probe are designed to hybridize next to each other on a target strand. When properly hybridized, the molecule is circularized by ligation. Synthesis of the complementary strand of the circularized padlock probe is initiated by primer p1. As a strand of linear tandem repeats is generated, a second primer (p2) hybridizes to each newly generated repeat. During elongation, the exonuclease-deficient DNA polymerase displaces the polymerized strand in front of it which, in turn, serves as template for the first primer.

1.3.2 RNA-based techniques

Whereas DNA-based detection techniques are increasingly being used to detect and identify pathogenic fungi, oomycetes, bacteria as well as nematodes, RNA-based techniques are mainly used to detect plant viruses since most of them have RNA genomes. However, since messenger RNA (mRNA) may reflect metabolically active pathogen material more accurately than DNA, these RNA-based techniques are highly attractive to selectively detect viable pathogen propagules (Baeumner *et al.*, 2001; Bentsink *et al.*, 2002; Marois *et al.*, 2002; Van Beckhoven *et al.*, 2002; Bleve *et al.*, 2003; Morin *et al.*, 2004). In addition, since RNA is less stable than DNA, the risk of accidental contamination via aerosols is lower using RNA-based techniques than using DNA-based techniques. However, because of its extreme sensitivity to degradation, specific precautions should be taken to extract RNA from environmental samples.

The main RNA-based detection techniques used in plant pathology are discussed below, including reverse transcriptase PCR (RT-PCR) and nucleic acid sequence based amplification (NASBA), also known as transcription mediated amplification (TMA) or self-sustained sequence replication (3SR).

1.3.2.1 Reverse Transcriptase PCR (RT-PCR)

Since PCR can only amplify double stranded templates such as DNA, RNA should be converted to DNA (called complementary DNA or cDNA) prior to use in a PCR-based assay. Typically, such RT-PCR consists of an annealing step for one primer and an extension step to synthesize the complementary or second strand, followed by a (real-time) PCR reaction (Raineri *et al.*, 1991; Tan and Weis, 1992). In plant pathology, RT-PCR is a common strategy to detect plant viruses (Waterhouse and Chu, 1995).

1.3.2.2 Nucleic Acid Sequence Based Amplification (NASBA), Transcription Mediated Amplification (TMA), or Self-Sustained Sequence Replication (3SR)

NASBA, also known as TMA or 3SR, has been used for the direct amplification of RNA (Compton, 1991). In contrast to conventional PCR, amplification is carried out in an isothermal process (avoiding the need for a thermocycler) using three different enzymes, including a reverse transcriptase, RNase H, and T7 RNA polymerase (Fig. 1-4). Initially, a primer containing an RNA polymerase promoter sequence at its 5' end and a target-specific sequence at its 3' end is extended by reverse transcription to produce a cDNA strand. The resulting hybrid is a substrate for RNase H, which degrades the original RNA strand. Subsequently, a second DNA strand is produced from a primer designed to bind to the 3' end of the cDNA, resulting in a dsDNA molecule that contains the sequence information of the original RNA and the promoter sequence of the T7 RNA polymerase. In a next step, T7 RNA polymerase initiates DNA transcription leading to the production of a large number of antisense RNA molecules. Each antisense RNA molecule is used to generate new dsDNA molecules based on the same principle, and initiates a new round of replication (Fig. 1-4).

The amplification products can be visualized using a specific labeled probe which hybridizes to the RNA amplicons (Oehlenschlager *et al.*, 1996; Lanciotti and Kerst, 2001). In addition, amplicons can be monitored in real-time using a specific detection probe such as a Molecular Beacon®. This procedure is referred to as AmpliDet RNA and combines the advantages of both NASBA and real-time PCR (Klerks *et al.*, 2001; Van Beckhoven *et al.*, 2002).

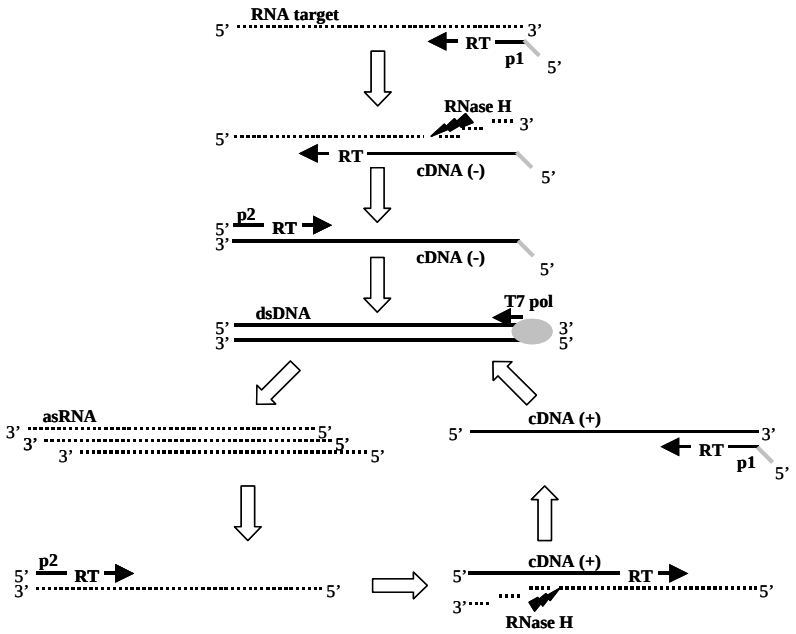

Fig. 1-4. General principle of Nucleic Acid Sequence Based Amplification (NASBA). Upon binding of primer p1 that is tailed with a T7 RNA polymerase promoter, reverse transcriptase (RT) generates a cDNA strand. The resulting hybrid is a substrate for RNase H, which degrades the original RNA strand. Subsequently, reverse transcriptase generates a complementary strand to the first cDNA strand (cDNA(-)) using a second primer (p2), resulting in double stranded DNA (dsDNA) with a T7 RNA polymerase promoter. This is a template for T7 RNA polymerase (T7 pol) that transcribes a large number of antisense RNA molecules (asRNA) which, in turn, are converted into dsDNA for a next amplification cycle.

1.4 Multiplex detection

One of the limitations of most detection procedures, whether serological, DNA- or RNA-based, is that only one or a very few pathogens is detected per assay. However, as most crops can be infected by a multitude of pathogens, detecting multiple pathogens in a single assay is desirable with respect to efficiency, cost, time, and labor. In addition, plant disease symptoms often result from infection by multiple pathogens rather than by a single pathogen, complicating classical diagnosis. In addition to pathogen detection, quantification of its presence is of high importance, since it can be used to estimate potential risks regarding disease development, spread of the inoculum, and economic losses. Therefore, multiplex detection and quantification, enabling to detect and quantify a large number of pathogens in a single assay, is a major challenge in plant disease diagnostics and disease management.

Multiplex PCR assays, using several primers in the same reaction, have been developed for the simultaneous detection of several microorganisms (Wilton and Cousins, 1992). However, the development of efficient and accurate multiplex formats is often difficult and typically requires extensive optimization of reaction conditions in order to properly discriminate at least a few amplicons per reaction. Besides designing selective primer pairs that can be used under the same conditions, the generated PCR products need to have different sizes to ensure clear discrimination of amplicons on agarose gels (Henegariu *et al.*, 1997). This latter limitation does not apply for real-time PCR applications based on amplicon-specific detection probes since different fluorophores can be used for the labeling of different probes. Nevertheless, for this technology the total amount of PCR reactions in a single tube is limited by the availability of dyes emitting fluorescence at different wavelengths on one hand, and the monochromatic character of the energizing light source in real-time PCR instruments on the other hand (Mackay *et al.*, 2002). As a result, detection of more than a few pathogens per assay is currently not possible using these strategies.

In contrast, array hybridization technology offers the possibility to add a multiplex aspect to PCR-based detection. In theory, DNA arrays, originally designed to study gene expression or to generate SNP profiles (Schena *et al*., 1996; Lashkari *et al*., 1997), can be used to detect an unlimited amount of different organisms in parallel. The virtually unlimited screening capability of DNA arrays, coupled with PCR amplification, results in high levels of sensitivity, specificity, and throughput capacity (Martin *et al.*, 2000; Lévesque, 2001; Lievens and Thomma, 2005; Lievens *et al.*, 2005b). With this technology, detector oligonucleotides, each specific for a DNA or RNA sequence of a respective target organism, are immobilized on a solid support, to create, depending on the size of the dots, a macro- (e.g. on a nylon membrane) or microarray (e.g. on a glass slide). For signal amplification, in general the target DNA to be tested (including genomic DNA, cDNA or even padlock probes harboring a specific random sequence in the spacer region (Szemes *et al*., 2005)), is amplified using universal PCR primers, labeled, and subsequently hybridized to the array under stringent conditions. In this way, it may thus be possible to differentiate a large number of organisms using a single PCR, provided that sufficient discriminatory potential exists within the region that is used.

This technology was originally developed as a technique to screen for human genetic disorders (Saiki *et al.*, 1989; Kawasaki and Chehab, 1994), but has also been successfully applied to detect and identify human and animal pathogens of diverse nature (Fiss *et al.*,

1992; Anthony *et al.*, 2000; Gonzales *et al.*, 2004). In plant pathology, this approach was applied for identifying oomycete (Fig. 1-5), nematode, bacterial and fungal DNA from pure cultures (Lévesque *et al.*, 1998; Uehara *et al.*, 1999; Fessehaie *et al.*, 2003; Lievens *et al.*, 2003) as well as for the identification of a number of viruses (Boonham *et al.*, 2003).

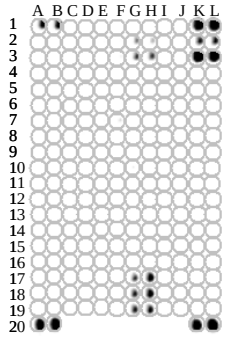

Fig. 1-5. Example of the identification of an oomycete (*Pythium ultimum*) culture using a DNA macroarray. Each detector oligonucleotide is spotted in duplicate on nylon membrane. Specificity of the analysis is enhanced by using multiple oligonucleotides for each target species. In addition to the immobilized target-specific oligonucleotides, the DNA array contains control oligonucleotides for the hybridization (1A & B, 1K & L, 20A & B, 20K & L) and a reference for detection and calibration (2K & L, 3K & L). PCR-labeled amplicons hybridize to genus-specific oligonucleotides for *Pythium* (2G & H, 3G & H) and species-specific oligonucleotides for *P. ultimum* (17G & H, 18G & H, 19G & H). Based on the location of the signals, identification is performed.

Despite these studies, for application in practice, identification of pathogens from pure cultures is not very relevant as, eventually, pathogens should be preferably assessed directly from plant and soil samples. In addition, quantification should be pursued using DNA arrays in order to fully exploit the potential use of DNA arrays in plant pathology, and more in particularly to determine threshold densities when a certain treatment has to be applied to prevent losses. Ultimately, such multiplex approach should lead to a comprehensive diagnostic kit that can detect and quantify all relevant pathogens of a specific crop. Undoubtedly, the availability of such multiplex assays will contribute to IPM programs. After all, with timely and regular qualitative as well as quantitative diagnoses, preventive treatments can be properly prescribed and performed and, in case actual infections are monitored, the afflicted plants can be cured or removed to avoid spread of the disease. Until now, preventive treatments were applied frequently, based on the theoretically calculated risk for disease incidence. Preventive treatments based on actual monitoring of pathogen populations will probably reduce the number of treatments and thus result in reduced environmental impacts.

1.5 Objectives and outline of this thesis

The overall objective of the research described in this thesis was to develop and optimize a DNA macroarray, that allows to simultaneously detect and quantify multiple plant pathogens, to be used for routine plant pathogen diagnosis. In order to achieve this goal, several experiments have been performed. First, the discriminating power of arrayed oligonucleotides was assessed, aiming at the discrimination of single base pair differences (Chapter 2). In this chapter the conditions were determined to obtain the desired specificity. Based on these results, an ITS-based DNA macroarray was designed for the detection and identification of a comprehensive set of fungal pathogens in complex samples, including plant and soil samples (Chapter 3). As a proof-of-principle, the array was developed for a number of economically important fungal pathogens of tomato which is one of the most important vegetable crops worldwide. These pathogens comprised the vascular wilt pathogens *F. oxysporum* f. sp. *lycopersici*, *V. albo-atrum*, and *V. dahliae*. In Chapter 5, this array has been further optimized for accurate pathogen quantification of concentration ranges typically encountered in horticultural practice. Since quantification based on culturing techniques is considered relatively inaccurate, real-time PCR, a well-established technique to quantify DNA levels, was used as a reference technique to validate DNA array-based quantification. In Chapter 4, the feasibility and robustness of real-time PCR for quantification of a number of tomato pathogens in biologically complex samples was evaluated. Finally, in the frame of an IPM-based disease management strategy, a quantitative DNA macroarray was developed to simultaneously monitor populations of pathogens and biocontrol agents, as well as to investigate their interactions, and relate their presence to disease development. Since currently no standard biocontrol assay was available for the previously used model crop tomato, the well established interaction between the biocontrol agent *Trichoderma hamatum* isolate 382 and the pathogen *Rhizoctonia solani* was used in a standard damping-off of radish bioassay (Chapter 6). Altogether, it is shown that DNA macroarrays can be successfully used to simultaneously detect and quantify multiple plant pathogens in samples from various biological sources including those gathered from horticultural practice.

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2 Detecting single nucleotide polymorphisms using DNA macroarrays for plant pathogen diagnosis*

2.1 Introduction

As more extensively described in the previous chapter the lack of rapid and reliable means for pathogen identification has been one of the main limitations in plant disease management and has pushed the development of highly specific molecular approaches (McCartney *et al*., 2003, Lievens *et al*., 2005b). Most of these approaches are designed for the identification of one or small numbers of pathogens at once. In contrast, DNA array technology is the most suitable technique for identification of several isolates in a single assay (Lévesque *et al*., 1998; Uehara *et al.* 1999; Martin *et al.* 2000; Lévesque, 2001; Fessehaie *et al.*, 2003; Lievens *et al.*, 2003; 2005b; Lievens and Thomma, 2005;). With this technology, specific detector oligonucleotides are immobilized on a solid support and used for target microorganism identification. Generally, target DNA is PCR-amplified and labeled using universal primers spanning a genomic region harboring microorganism-specific sequences. Subsequently, labeled amplicons are hybridized to the array.

Generally, ubiquitously conserved genes are targeted for molecular diagnostics, of which the rRNA gene with its ITS regions is most commonly targeted (Chapter 1; McCartney *et al.*, 2003; Lievens and Thomma, 2005; Lievens *et al.*, 2005b). Closely related pathogens, that may have completely different host ranges or pathogenicity, often differ in a single to a few base pairs for such conserved genes (Nazar *et al.*, 1991; Cooke *et al.*, 2000). As a consequence, discrimination of SNPs should be pursued when developing molecular diagnostic assays. With regard to immobilized detector oligonucleotides, factors such as the type of the mismatch, as well as its position and number, are believed to play an important role in hybridization kinetics and thus in the outcome of the assay (Bodrossy *et al.*, 2003). In

* Results described in this chapter have been published in "Detecting single nucleotide polymorphisms using DNA arrays for plant pathogen diagnosis"; Lievens, B., Claes, L., Vanachter, A. C. R. C., Cammue, B. P. A., and Thomma. B. P. H. J.; FEMS Microbiology Letters 255:129-139 (2006).

addition, it is generally accepted that center mismatches are the most destabilizing (Kawasaki and Chehab, 1994; Bodrossy *et al.*, 2003). However, it has never been explored at which number or at which position mismatches significantly influence the outcome of the diagnostic assay. In general, relatively high amplicon concentrations are used to generate strong, unambiguous hybridization signals (Lévesque *et al.*, 1998; Uehara *et al.* 1999; Fessehaie *et al.*, 2003). However, the lack of sufficient oligonucleotide specificity combined with the hybridization of an excess of amplicons potentially increases the risk of "false positives".

In this chapter, the discriminating power of immobilized oligonucleotides is assessed, aiming at the discrimination of SNPs. Multiple oligonucleotides were mutated at one or more positions and used for hybridization assays using different concentrations of labeled PCR products. In addition, as ultimately the use of DNA arrays for direct detection of pathogens in environmental samples is pursued, we investigated whether cross hybridizations to mismatch oligonucleotides are relevant when analyzing environmental samples.

2.2 Materials and methods

2.2.1 Fungal and oomycete isolates used in this study

The fungal isolates *Fusarium oxysporum* f. sp. *lycopersici* CBS 645.78 and *Verticillium dahliae* CBS 381.66 (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) and the oomycete isolates *Phytophthora nicotianae* MUCL 28775 and *Pythium ultimum* MUCL 16164 (Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium) were cultured on potato dextrose agar (PDA) in darkness at 24°C.

2.2.2 DNA extraction

Genomic DNA was extracted from 5- to 10-day old cultures. A patch of mycelium (approximately 2 cm^2) was scraped from the margin of a colony and suspended in 300 µl lysis buffer (2.5 M LiCl, 50 mM Tris, 62.5 mM EDTA, and 4.0% Triton X-100, pH 8.0) together with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v) and approximately 75 µl of glass beads (212-300 µm). Cells were mechanically disrupted in a Fast Prep system (Thermo Savant, Holbrook, NY, USA) by reciprocal shaking the samples for 30 s at maximum speed. The supernatant was collected after centrifugation (9300 x g)

and the DNA was precipitated upon addition of two volumes of absolute ethanol followed by incubation for 15 min at – 20°C and subsequent centrifugation (5 min at 9300 x g). The DNA pellet was washed with 70% ethanol, air-dried, and resuspended in 50 µl 10 mM Tris (pH 8.0).

For DNA isolation from soil and plant samples, genomic DNA was extracted from 0.75 g (fresh weight) sample material using the UltraClean Soil DNA Isolation Kit and the UltraClean Plant DNA Isolation Kit, respectively, according to the manufacturer's protocol (Mo Bio Laboratories, Solana Beach, CA, USA). Subsequently, DNA samples were diluted 10-fold to avoid inhibitory concentrations of potential PCR inhibitors. For water samples, DNA was isolated from 200 ml using the UltraClean Water DNA Isolation Kit as described by the manufacturer (Mo Bio Laboratories, Solana Beach, CA, USA).

DNA yield was determined spectrophotometrically at 260 nm. All DNA extracts were stored at – 20°C until further analysis.

2.2.3 Selection of oligonucleotides

In order to test the discriminatory potential of arrayed detector oligonucleotides, specific oligonucleotides were selected from ITS sequences and mutated at various positions (Table 2-1). Perfect match oligonucleotides were selected from either ITS I or ITS II sequences from four unrelated species: *F. oxysporum*, *P. nicotianae*, *P. ultimum*, and *V. dahliae* (Fig. 2-1). ITS sequences from the target species as well as from the closest relatives were derived from Genbank and the sequence database of Scientia Terrae Research Institute and aligned using the Clustal W algorithm in order to identify diagnostic oligonucleotides. The length of these oligonucleotides was adjusted to obtain detector sequences with a melting temperature of 55°C $\pm$ 5°C as calculated using the nearest neighbor method. The ability to form dimers and hairpin structures was checked using Vector NTI software and the sequences with the lowest tendency to form such structures were chosen.

For each species, initially several perfect match detector oligonucleotides were designed of which the oligonucleotides that provided the most consistent hybridization signals were selected for this study. These encompassed Fox1 and Fox2, Pni1 and Pni2, Pul1 and Pul2, and Vda1, to detect *F. oxysporum*, *P. nicotianae*, *P. ultimum*, and *V. dahliae*, respectively. Although these oligonucleotides differ in length, GC content, and origin, these oligonucleotides all provide uniform and strong hybridization signals upon hybridization with 10 ng labeled target amplicons per ml hybridization buffer (data not shown), and were

therefore selected. To explore the discriminatory power of immobilized oligonucleotides, nucleotides were substituted (Table 2-1). In addition to these oligonucleotides, a digoxigenin-labeled control oligonucleotide (Dig1) with no homology to a known sequence was designed and used as a reference for detection and calibration. The same oligonucleotide was also synthesized without labeling as a negative control. All oligonucleotides were synthesized with a 5'-C6-amino linker for covalent binding to a nylon membrane.

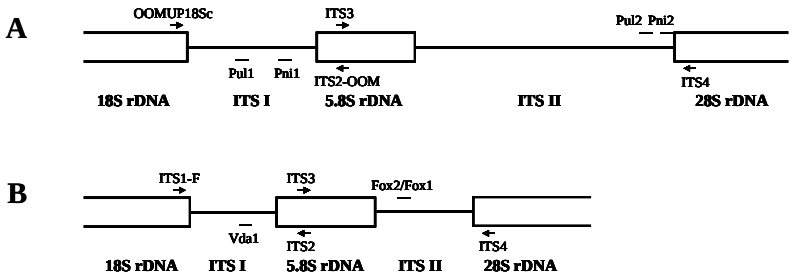

Fig. 2-1. Schematic representation of an **A,** oomycete and **B,** fungal ribosomal cistron showing the location of the PCR primers (→) and the detector oligonucleotides (—) used in this study. Sense sequences are indicated at the top, antisense sequences at the bottom of the cistron.

Table 2-1. Sequences of match[a] and mismatch[b] detector oligonucleotides used in this study[c]

Array[c]	Code[a]	Sequence[b] (5'-3')	Substitution[d]	Length	T_m[e] (°C)	GC (%)	Target[f]
1	Fox1*	TTGGGACTCGCGTTAATTCG		20	55.4	50.0	ITS II
	Fox1-1	**A**TGGGACTCGCGTTAATTCG	T1A	20	55.2	50.0	
	Fox1-2	**C**TGGGACTCGCGTTAATTCG	T1C	20	55.9	55.0	
	Fox1-3	**G**TGGGACTCGCGTTAATTCG	T1G	20	56.2	55.0	
	Fox1-4	TTGG**C**ACTCGCGTTAATTCG	G5C	20	56.1	50.0	
	Fox1-5	TTGG**A**ACTCGCGTTAATTCG	G5A	20	53.3	45.0	
	Fox1-6	TTGG**T**ACTCGCGTTAATTCG	G5T	20	52.8	45.0	
	Fox1-7	TTGGGACTC**C**CGTTAATTCG	G10C	20	54.7	50.0	
	Fox1-8	TTGGGACTC**A**CGTTAATTCG	G10A	20	52.6	45.0	
	Fox1-9	TTGGGACTC**T**CGTTAATTCG	G10T	20	52.1	45.0	
	Fox1-10	TTGGGACTCGCGTT**T**ATTCG	A15T	20	55.4	50.0	
	Fox1-11	TTGGGACTCGCGTT**C**ATTCG	A15C	20	57.9	55.0	
	Fox1-12	TTGGGACTCGCGTT**G**ATTCG	A15G	20	57.9	55.0	
	Fox1-13	TTGGGACTCGCGTTAATTC**C**	G20C	20	55.2	50.0	
	Fox1-14	TTGGGACTCGCGTTAATTC**A**	G20A	20	54.4	45.0	
	Fox1-15	TTGGGACTCGCGTTAATTC**T**	G20T	20	54.1	45.0	
	Fox1-16	**AA**GGGACTCGCGTTAATTCG	T1A; T2A	20	55.2	50.0	
	Fox1-17	TTGG**CT**CTCGCGTTAATTCG	G5C; A6T	20	55.6	50.0	
	Fox1-18	TTGGGACTC**CG**GTTAATTCG	G10C; C11G	20	54.7	50.0	
	Fox1-19	TTGGGACTCGCGTT**TT**TTCG	A15T; A16T	20	56.3	50.0	
	Fox1-20	TTGGGACTCGCGTTAATT**GC**	C19G; G20C	20	56.0	50.0	
	Fox1-21	**A**TGG**C**ACTCGCGTTAATTCG	T1A; G5C	20	56.0	50.0	
	Fox1-22	**A**TGGGACTC**C**CGTTAATTCG	T1A. G10C	20	54.6	50.0	
	Fox1-23	**A**TGGGACTCGCGTT**T**ATTCG	T1A; A15T	20	55.2	50.0	
	Fox1-24	**A**TGGGACTCGCGTTAATTC**C**	T1A; G20C	20	55.1	50.0	
	Fox1-25	TTGG**C**ACTC**C**CGTTAATTCG	G5C; G10C	20	55.5	50.0	
	Fox1-26	TTGG**C**ACTCGCGTTA**T**TTCG	G5C; A15T	20	56.1	50.0	
	Fox1-27	TTGG**C**ACTCGCGTTAATTC**C**	G5C; G20C	20	56.0	50.0	
	Fox1-28	TTGGGACTC**C**CGTTA**T**TTCG	G10C; G20C	20	54.7	50.0	
	Fox1-29	TTGGGACTC**C**CGTTAATTC**C**	G10C; G20C	20	54.5	50.0	
	Fox1-30	TTGGGACTCGCGTT**T**ATTC**C**	A15T;G20C	20	55.2	50.0	
	Fox1-31	**AA**GGGACTCGCGTTAATTC**C**	T1A; T2A; G20C	20	55.0	50.0	
	Fox1-32	**A**TGGGACTCGCGTTAATT**GC**	T1A; C19G; G20C	20	55.8	50.0	
	Fox1-33	**AA**GGGACTCGCGTTAATT**GC**	T1A; T2A; C19G; G20C	20	55.7	50.0	
2	Fox2*	GTTGGGACTCGCGTTAATTCG		21	56.4	52.4	ITS II
	Fox2-1	GTTG**C**GACTCGCGTTAATTCG	G5C	21	56.9	52.4	
	Fox2-2	GTTGGGACT**G**GCGTTAATTCG	C10G	21	56.5	52.4	
	Fox2-3	GTTGGGACTCGCGT**A**AATTCG	T15A	21	56.4	52.4	

Table 2-1 (continued).

Array[c]	Code[a]	Sequence[b] (5'-3')	Substitution[d]	Length	T_m[e] (°C)	GC (%)	Target[f]
3	Pni1[*]	AAAAAAGACTACTAAATCAGGCC		23	51.0	34.8	ITS I
	Pni1-1	AAAA**T**AGACTACTAAATCAGGCC	A5T	23	50.2	34.8	
	Pni1-2	AAAAAAGAC**A**ACTAAATCAGGCC	T10A	23	51.9	34.8	
	Pni1-3	AAAAAAGACTACTA**T**ATCAGGCC	A15T	23	50.2	34.8	
	Pni1-4	AAAAAAGACTACTAAATCA**C**GCC	G20C	23	51.4	34.8	
4	Pni2[*]	TTTGGGAACTTAATGTGTACTTC		23	51.0	34.8	ITS II
	Pni2-1	TTTG**C**GAACTTAATGTGTACTTC	G5C	23	51.6	34.8	
	Pni2-2	TTTGGGAAC**A**TAATGTGTACTTC	T10A	23	51.0	34.8	
	Pni2-3	TTTGGGAACTTAAT**C**TGTACTTC	G15C	23	50.5	34.8	
	Pni2-4	TTTGGGAACTTAATGTGTA**G**TTC	C20G	23	51.0	34.8	
5	Pul1[g,*]	TGCTGACTCCCGTTCCAGTG		20	59.6	60.0	ITS I
	Pul1-1	**A**GCTGACTCCCGTTCCAGTG	T1A	20	59.3	60.0	
	Pul1-2	**C**GCTGACTCCCGTTCCAGTG	T1C	20	60.5	65.0	
	Pul1-3	**G**GCTGACTCCCGTTCCAGTG	T1G	20	60.4	65.0	
	Pul1-4	TGCT**C**ACTCCCGTTCCAGTG	G5C	20	59.6	60.0	
	Pul1-5	TGCT**A**ACTCCCGTTCCAGTG	G5A	20	57.0	55.0	
	Pul1-6	TGCT**T**ACTCCCGTTCCAGTG	G5T	20	57.0	55.0	
	Pul1-7	TGCTGACTC**G**CGTTCCAGTG	C10G	20	60.1	60.0	
	Pul1-8	TGCTGACTC**A**CGTTCCAGTG	C10A	20	57.3	55.0	
	Pul1-9	TGCTGACTC**T**CGTTCCAGTG	C10T	20	56.9	55.0	
	Pul1-10	TGCTGACTCCCCGTT**G**CAGTG	C15G	20	60.3	60.0	
	Pul1-11	TGCTGACTCCCCGTT**A**CAGTG	C15A	20	56.9	55.0	
	Pul1-12	TGCTGACTCCCCGTT**T**CAGTG	C15T	20	57.4	55.0	
	Pul1-13	TGCTGACTCCCGTTCCAGT**C**	G20C	20	59.3	60.0	
	Pul1-14	TGCTGACTCCCGTTCCAGT**A**	G20A	20	58.0	55.0	
	Pul1-15	TGCTGACTCCCGTTCCAGT**T**	G20T	20	58.8	55.0	
	Pul1-16	**AC**CTGACTCCCGTTCCAGTG	T1A; G2C	20	59.1	60.0	
	Pul1-17	TGCT**CT**CTCCCGTTCCAGTG	G5C; A6T	20	59.1	60.0	
	Pul1-18	TGCTGACTC**GG**GTTCCAGTG	G10C; C11G	20	59.6	60.0	
	Pul1-19	TGCTGACTCCCCGTT**GG**AGTG	C15G; C16G	20	59.6	60.0	
	Pul1-20	TGCTGACTCCCGTTCCAG**AC**	T19A; G20C	20	59.3	60.0	
	Pul1-21	**A**GCT**C**ACTCCCGTTCCAGTG	T1A; G5C	20	59.3	60.0	
	Pul1-22	**A**GCTGACTC**G**CGTTCCAGTG	T1A; C10G	20	59.9	60.0	
	Pul1-23	**A**GCTGACTCCCGTT**G**CAGTG	T1A; C15G	20	60.1	60.0	
	Pul1-24	**A**GCTGACTCCCGTTCCAGT**C**	T1A; G20C	20	59.1	60.0	
	Pul1-25	TGCT**C**ACTC**G**CGTTCCAGTG	G5C; C10G	20	60.1	60.0	
	Pul1-26	TGCT**C**ACTCCCGTT**G**CAGTG	G5C; C15G	20	60.3	60.0	
	Pul1-27	TGCT**C**ACTCCCGTTCCAGT**C**	G5C; G20C	20	59.3	60.0	
	Pul1-28	TGCTGACTC**G**CGTT**G**CAGTG	C10G; C15G	20	60.8	60.0	

Table 2-1 (continued).

Array[c]	Code[a]	Sequence[b] (5'-3')	Substitution[d]	Length	T_m[e] (°C)	GC (%)	Target[f]
	Pul1-29	TGCTGACTC**G**CGTTCCAGT**C**	C10G; G20C	20	59.9	60.0	
	Pul1-30	TGCTGACTCCCGTT**G**CAGT**C**	C15G;G20C	20	60.0	60.0	
	Pul1-31	**ACG**TGACTCCCGTTCCAGTG	T1A; G2C; C3G	20	59.4	60.0	
	Pul1-32	TGCT**CTG**TCCCGTTCCAGTG	G5C; A6T; C7G	20	59.6	60.0	
	Pul1-33	TGCTGACTC**GGC**TTCCAGTG	C10G; C11G, G12C	20	59.8	60.0	
	Pul1-34	TGCTGACTCCCGTT**GGT**GTG	C15G; C16G; A17T	20	60.0	60.0	
	Pul1-35	TGCTGACTCCCGTTCCA**CAC**	G18C; T19A; G20C	20	59.8	60.0	
	Pul1-36	**A**GCT**C**ACTC**G**CGTTCCAGTG	T1A; G5C; C10G	20	59.9	60.0	
	Pul1-37	TGCT**C**ACTC**G**CGTT**G**CAGTG	G5C; C10G; C15G	20	60.8	60.0	
	Pul1-38	TGCTGACTC**G**CGTT**G**CAGT**C**	C10G; C15G; G20C	20	60.6	60.0	
	Pul1-39	**AC**CTGACTCCCGTTCCAGA**C**	T1A; G2C; G20C	20	58.8	60.0	
	Pul1-40	**A**GCTGACTCCCGTTCCAG**AC**	T1A; T19A; G20C	20	59.1	60.0	
	Pul1-41	**AC**CTGACTCCCGTTCCAG**TC**	T1A; G2C; A19T; G20C	20	58.8	60.0	
	Pul1-42	**ACG**TGACTCCCGTTCCA**CAC**	T1A; G2C; C3G; G18C; T19A; G20C	20	59.7	60.0	
	Pul1-43	**AGC**T**C**ACTC**G**CGTTC**G**AGT**C**	T1A; C2G; G3C; G5C; C10G; C15G; G20C	20	59.5	60.0	
6	Pul2*	TGTATGGAGACGCTGCATTT		20	54.5	45.0	ITS II
	Pul2-1	TGTA**A**GGAGACGCTGCATTT	T5A	20	54.4	45.0	
	Pul2-2	TGTATGGAG**T**CGCTGCATTT	A10T	20	54.5	45.0	
	Pul2-3	TGTATGGAGACGCT**C**CATTT	G15C	20	53.7	45.0	
7	Vda1*	AACAGAGAGACTGATGGACCG		21	56.2	52.4	ITS I
	Vda1-1	AACA**C**AGAGACTGATGGACCG	G5C	21	56.7	52.4	
	Vda1-2	AACAGAGAG**T**CTGATGGACCG	A10T	21	56.2	52.4	
	Vda1-3	AACAGAGAGACTGA**A**GGACCG	T15A	21	56.1	52.4	

[a] 100 % match oligonucleotides are indicated with an asterisk.

[b] Nucleotide substitutions are in bold and underlined.

[c] In total seven DNA arrays were designed. Specificity of the oligonucleotides was tested with labeled amplicons from *Fusarium oxysporum* (arrays 1 and 2), *Phytophthora nicotianae* (arrays 3 and 4), *Pythium ultimum* (arrays 5 and 6), and *Verticillium dahliae* (array 7).

[d] Notation is as follows: the first character indicates the original and substituted nucleotide at the position indicated by the second character. The third character indicates the nucleotide by which the original one is substituted.

[e] Melting temperature calculated using the nearest neighbor method.

[f] Target of the 100% match oligonucleotide.

[g] Lévesque *et al.* (1998).

2.2.4 DNA array production

DNA macroarrays were produced as follows. The selected oligonucleotides were diluted in sodium bicarbonate buffer (0.5 M, pH 8.4) containing 0.004% bromophenol blue and kept in a microtiter plate according to a predesigned array template (Table 2-1). Oligonucleotides were spotted in duplicate on Immunodyne ABC membrane strips (PALL Europe Limited, Portsmouth, UK) using a 384-pin replicator (V & P Scientific, San Diego, CA, USA) at an amount of 8.0 fmol per spot. For the reference oligonucleotide Dig1, 2.0 fmol was printed. The distance between two spots, having a surface of nearly 1 mm^2, was approximately 3 mm. Membranes were air dried, blocked for 30 min at room temperature, again air dried, and stored at room temperature until use.

2.2.5 PCR amplification and labeling

Target ITS regions were amplified and simultaneously labeled with alkaline-labile digoxigenin (Roche Diagnostics GmbH, Mannheim, Germany). The region between the small and the large subunit of the rRNA gene was amplified using the primers ITS1-F and ITS4 (Gardes and Bruns, 1993) or OOMUP18Sc and ITS4 (Lievens *et al.*, 2004), for fungi or oomycetes, respectively. Depending on whether the target is a fungus or an oomycete, ITS I sequences were amplified using the fungus-specific primer set ITS1-F and ITS2 (White *et al.*, 1990) or the oomycete-specific primer set OOMUP18Sc and ITS2-OOM (5'-GCAGCGTTCTTCATCGATGT-3'). In order to amplify the ITS II region, ITS3 was combined with ITS4 (White *et al.*, 1990). Samples were amplified in 20 µl, containing 5 ng genomic DNA from a pure microbial culture or 1 µl DNA from an environmental sample. Amplification was performed using 0.15 mM digoxigenin-11-d-UTP mix (Roche Diagnostics GmbH, Mannheim, Germany), 0.5 µM of each primer, and 1 unit Titanium Taq DNA polymerase (Clontech Laboratories, Inc., Palo Alto, CA, USA). Before amplification, DNA samples were denatured at 94°C for 2 min. Next, 35 cycles were run consisting of 45 s at 94°C, 45 s at 59°C, and 45 s at 72°C, with a final extension at 72°C for 10 min. After gel electrophoresis, the resulting Dig-dUTP-labeled amplicons were quantified by comparison to a DNA ladder (Smartladder SF, Eurogentec, Seraing, Belgium) using Labworks 4.0 Image Acquisition and Analysis Software (UVP, Upland, CA, USA).

2.2.6 DNA array hybridization

Prior to hybridization, membranes were prehybridized for at least 1.5 h at 54°C in hybridization buffer (6x sodium chloride sodium citrate (SSC), 0.1% sarcosine, and 0.02% sodium dodecyl sulfate (SDS)) amended with 1% casein. Labeled amplicons were denatured by boiling in hybridization buffer for 10 min and subsequently hybridized overnight at 54°C in 6 ml of hybridization buffer. Hybridization was followed by two washing steps in stringency buffer (6x SSC and 0.1% SDS) at hybridization temperature, and three final washing steps in washing solution (0.1 M maleic acid and 0.15 M sodium chloride; pH 7.5) at room temperature. Detection of digoxigenin was performed using anti-digoxigenin alkaline phosphatase conjugate and CDP-Star substrate (both from Roche Diagnostics GmbH, Mannheim, Germany). Chemiluminescence was detected cumulatively during 45 min at intervals of 30 s using a highly sensitive digital CCD camera (BioChemi System; UVP, Upland, CA, USA). Hybridization signals were quantified and analyzed using Labworks 4.0 Image Acquisition and Analysis Software. Hybridization strength was reported relative to the average integrated optical density of the digoxigenin-labeled reference control (Dig1). All hybridizations were carried out at least twice.

2.3 Results and discussion

2.3.1 Hybridization of amplicons derived from pure cultures

Despite the generally accepted high potential of DNA array technology as an identification tool (Lévesque *et al*., 1998; Uehara *et al.* 1999; Martin *et al.* 2000; Lévesque, 2001; Fessehaie *et al.*, 2003; Lievens *et al.*, 2003; 2005b; Lievens and Thomma, 2005), not much is known about the discriminatory potential of detector oligonucleotides, especially under the high amplicon concentrations used to ensure sufficient sensitivity. In this study, ITS I- or ITS II-specific oligonucleotides were mutated (mismatch oligonucleotides; Table 2-1) and tested for hybridization. Initially, one or more nucleotides were substituted in the *P. ultimum* ITS I oligonucleotide Pul1 (Lévesque *et al.*, 1998) (Table 2-1). In addition to Pul1, 43 mismatch oligonucleotides were arrayed. For hybridization, *P. ultimum* ITS I amplicons from different PCR reactions were pooled and used at 1 ng, 10 ng, 100 ng, or 200 ng ml^{-1} of hybridization buffer.

For the mismatch oligonucleotides hybridization signals as well as cross hybridization increased with increasing amounts of amplicon (Fig. 2-2). When only a single nucleotide

was substituted, mismatches at the fifth nucleotide were the most selective (Pul1-4, Pul1-5, and Pul1-6; Fig. 2-2), allowing SNP discrimination irrespective of the amplicon amount or the nucleotide used for the substitution. In contrast, with mismatches at the extreme 5' or 3' end (Pul1-1, Pul1-2, Pul1-3 and Pul1-13, Pul1-14, Pul1-15, respectively), oligonucleotides were the least discriminatory. With two adjacent substitutions at any location, amplicons did not cross hybridize to the mismatch oligonucleotides, except when they were positioned at the extreme 5' (Pul1-16) or 3' end (Pul1-20). Similar observations were made with multiple mismatches that were spread throughout the oligonucleotide (Pul1-22, Pul1-23, Pul1-24, Pul1-31, Pul1-40, Pul1-41; Fig. 2-2). However, no cross hybridization occurred in combination with a mismatch at the fifth nucleotide (Pul1-21), again demonstrating the high selectivity of this nucleotide (Fig. 2-2).

To test whether amplicon length affects specificity, amplicons were generated from the ITS I-5.8S rDNA-ITS II region (approximately 900 bp, compared to 300 bp for the ITS I amplicons). In all cases, when longer amplicons were hybridized signal intensities slightly increased due to the larger number of labeled nucleotides incorporated per amplicon, but oligonucleotides also provided lower specificity (Fig. 2-2). Specificity is enhanced by hybridizing a lower amplicon amount. However, although highly specific at an amplicon concentration of 1 ng ml^{-1}, hybridization signals produced by olignucleotide Pul1 were rather weak (Fig. 2-2).

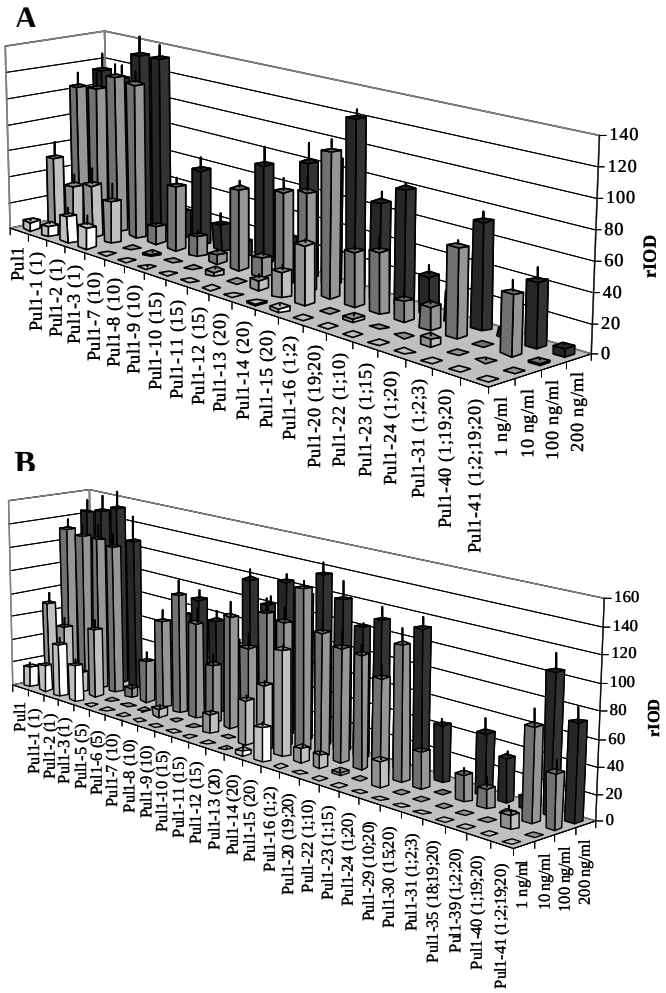

Fig. 2-2. Discriminatory potential of Pul1-derived mismatch detector oligonucleotides upon hybridization with different amounts of **A,** ITS I and **B,** ITS I-5.8S rDNA-ITS II amplicons from *Pythium ultimum*. Results are only shown for those oligonucleotides that resulted in detectable hybridization signals. Mismatch positions are indicated following the code of the oligonucleotide. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD). Data represent means from three hybridization runs (n = 6). Error bars indicate standard errors. Before hybridization, amplicons from different PCR reactions were pooled to minimize variability due to differences in DNA amplification.

A similar experiment was performed for a set of oligonucleotides derived from a *F. oxysporum* ITS II detector sequence, Fox1. As shown in Fig. 2-3, similar results as for Pul1 substitutions were obtained for mutations of Fox1. However, whereas the fifth position was the most selective for the Pul1-derived oligonucleotide, for Fox1 the highest specificity was obtained with a center mismatch oligonucleotide (Fox1-7, Fox1-8, and Fox1-9; Fig. 2-3). This illustrates that the selectivity of a specific SNP oligonucleotide also depends on its sequence.

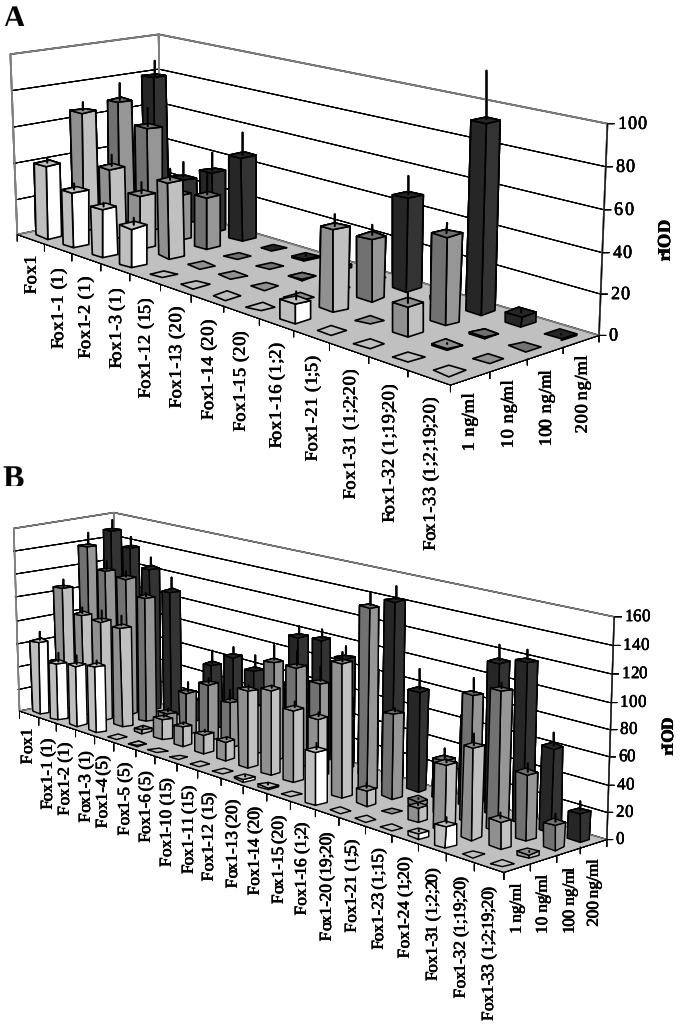

Fig 2-3. Discriminatory potential of Fox1-derived mismatch detector oligonucleotides upon hybridization with different amounts of **A,** ITS II and **B,** ITS I-5.8S rDNA-ITS II amplicons from *Fusarium oxysporum*. Results are only shown for those oligonucleotides that resulted in detectable hybridization signals. Mismatch positions are indicated following the code of the oligonucleotide. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD). Data represent means from three hybridization runs (n = 6). Error bars indicate standard errors. Before hybridization, amplicons from different PCR reactions were pooled to minimize variability due to differences in DNA amplification.

These observations were confirmed by experiments with other SNP oligonucleotides designed on ITS sequences from *P. nicotianae* (Pni1 and Pni2) and *V. dahliae* (Vda1) and additional oligonucleotides for *F. oxysporum* (Fox2) and *P. ultimum* (Pul2) (Table 2-2). For Pni2 and Pul2 the 15th nucleotide was found to be the most selective (Pni2-3 and Pul2-3) and for Pni1 the 20th nucleotide (Pni1-4). For Fox2 and Vda1 mutations, specificity could not be obtained when hybridizing complete ITS I-5.8S rDNA-ITS II amplicons. However, when ITS I amplicons were hybridized, specificity was obtained for all mismatch oligonucleotides under all conditions (Table 2-2).

Table 2-2. Discriminatory potential of different single nucleotide mismatch detector oligonucleotides upon hybridization with different amounts of amplicons generated from several fungal and oomycete isolates

Oligonucleotide[a]	Target organism	Hybridization signal[b]							
		ITS I or ITS II amplicon[c,d]				ITS I-5.8S rDNA-ITS II amplicon[c]			
		200 ng ml^{-1}	100 ng ml^{-1}	10 ng ml^{-1}	1 ng ml^{-1}	200 ng ml^{-1}	100 ng ml^{-1}	10 ng ml^{-1}	1 ng ml^{-1}
Fox1	*F. oxysporum*	79.1 ± 8.3	68.7 ± 9.2	66.0± 4.3	40.4 ± 2.6	148.8 ± 7.2	138.2 ± 9.7	105.7 ± 6.4	62.5 ± 6.2
Fox1-4 (5)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	18.3 ± 5.6	11.7 ± 2.3	2.8 ± 0.8	0.0 ± 0.0
Fox1-7 (10)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Fox1-10 (15)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	58.6 ± 10.0	37.2 ± 8.5	15.0 ± 2.2	0.0 ± 0.0
Fox2		67.9 ± 2.3	75.5 ± 5.0	78.5 ± 4.2	57.2 ± 2.8	115.9 ± 1.7	106.1 ± 4.0	81.3 ± 10.8	85.6 ± 5.6
Fox2-1 (5)		0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	84.8 ± 4.2	78.3 ± 3.7	36.4 ± 11.1	2.8 ± 0.9
Fox2-2 (10)		0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	68.8 ± 6.3	60.7 ± 8.6	30.1 ± 9.2	1.1 ± 0.5
Fox2-3 (15)		4.0 ± 0.9	0.8 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	58.6 ± 15.6	66.2 ± 11.8	61.5 ± 5.3	7.5 ± 2.8
Pni	*P. nicotianae*	102.2 ± 2.3	99.6 ± 6.7	89.4 ± 7.3	81.9 ± 8.9	135.4 ± 2.0	119.7 ± 1.1	63.6 ± 8.3	68.4 ± 2.7
Pni1-1 (5)		30.5 ± 6.0	33.6 ± 5.8	15.1 ± 2.2	1.6 ± 0.4	98.3 ± 4.5	90.6 ± 6.6	9.6 ± 3.3	0.4 ± 0.1
Pni1-2 (10)		10.8 ± 3.8	14.0 ± 2.2	3.8 ± 0.7	0.4 ± 0.3	79.6 ± 8.8	76.8 ± 5.1	7.6 ± 1.5	0.0 ± 0.0
Pni1-3 (15)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	38.9 ± 9.5	24.8 ± 5.4	0.0 ± 0.0	0.0 ± 0.0
Pni1-4 (20)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 0.9	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.6
Pni2		88.9 ± 5.1	94.5 ± 2.7	76.9 ± 6.0	40.3 ± 7.7	99.3 ± 7.7	101.6 ± 6.6	93.8 ± 4.1	32.1 ± 5.7
Pni2-1 (5)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	29.0 ± 6.4	13.0 ± 5.5	0.8 ± 0.4	0.0 ± 0.0
Pni2-2 (10)		1.1 ± 0.2	1.2 ± 0.3	0.0 ± 0.0	0.1 ± 0.1	43.0 ± 3.4	14.5 ± 4.1	2.1 ± 0.6	0.0 ± 0.0
Pni2-3 (15)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Pni2-4 (20)		1.4 ± 0.5	1.8 ± 0.9	0.0 ± 0.0	0.0 ± 0.0	55.7 ± 5.6	29.8 ± 4.5	6.9 ± 2.1	0.0 ± 0.0
Pul1	*P. ultimum*	114.3 ± 10.2	104.3 ± 9.0	52.1 ± 10.2	6.2 ± 1.1	143.9 ± 6.2	131.8 ± 6.2	69.3 ± 8.0	16.7 ± 2.0
Pul1-4 (5)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Pul1-7 (10)		20.4 ± 2.7	13.2 ± 1.4	0.0 ± 0.0	0.0 ± 0.0	75.2 ± 7.5	72.6 ± 7.5	0.4 ± 0.2	0.0 ± 0.0
Pul1-10 (15)		11.3 ± 4.0	6.3 ± 1.6	0.0 ± 0.0	0.0 ± 0.0	64.9 ± 8.3	48.0 ± 8.3	0.0 ± 0.0	0.0 ± 0.0
Pul2		87.3 ± 5.1	108.1 ± 2.8	94.8 ± 0.9	59.8 ± 5.9	119.3 ± 4.2	104.1 ± 4.8	99.8 ± 5.8	75.7 ± 0.9
Pul2-1 (5)		2.0 ± 0.7	9.7 ± 1.1	7.9 ± 2.5	0.2 ± 0.1	73.6 ± 10.3	51.7 ± 5.1	35.2 ± 6.1	0.6 ± 0.2
Pul2-2 (10)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	35.9 ± 8.4	20.5 ± 5.3	10.5 ± 3.0	0.0 ± 0.0
Pul2-3 (15)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Table 2-2 (continued).

Oligonucleotide[a]	Target organism	Hybridization signal[b]							
		ITS I or ITS II amplicon[c,d]				ITS I-5.8S rDNA-ITS II amplicon[c]			
		200 ng ml^{-1}	100 ng ml^{-1}	10 ng ml^{-1}	1 ng ml^{-1}	200 ng ml^{-1}	100 ng ml^{-1}	10 ng ml^{-1}	1 ng ml^{-1}
Vda1	*V. dahliae*	85.4 ± 2.7	73.7 ± 4.7	79.8 ± 3.9	63.5 ± 4.3	134.6 ± 5.5	134.1 ± 8.1	88.6 ± 12.2	72.1 ± 13.7
Vda1-1 (5)		0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	105.6 ± 6.0	106.4 ± 4.4	41.8 ± 15.2	2.8 ± 0.9
Vda1-2 (10)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	85.5 ± 5.2	92.2 ± 4.8	29.7 ± 14.1	0.0 ± 0.0
Vda1-3 (15)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	84.7 ± 5.1	89.9 ± 5.2	45.5 ± 12.4	0.1 ± 0.1

[a] Mismatch positions are indicated between brackets.

[b] Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (Dig1). Values are means ± standard errors (n = 6 from three hybridization runs).

[c] Before hybridization, amplicons from different PCR reactions were pooled to minimize variability due to differences in DNA amplification.

[d] Pul1-, Pni1-, and Vda1-derived detector oligonucleotides were hybridized with ITS I amplicons from the respective oomycete and fungal isolates whereas Fox1-, Fox2-, Pul2-, and Pni2-derived detector oligonucleotides were hybridized with ITS II amplicons.

2.3.2 Hybridization of amplicons derived from naturally infested biological samples

Identification from pure cultures is not very relevant for use in practice as isolating and culturing is time-consuming. In addition, since only a small part of the microorganisms in an environmental sample can be cultured *in vitro* (Amann *et al.* 1995; Rapp and Giovannoni, 2003), direct pathogen detection and identification from environmental samples should be pursued. To investigate whether cross hybridizations to mismatch oligonucleotides are relevant when analyzing environmental samples by DNA array technology, practical conditions were mimicked by DNA dilutions reflecting those typically encountered in horticultural practice (Lievens *et al.*, 2005a). A ten-fold dilution series of genomic DNA from *F. oxysporum* f. sp. *lycopersici* and *P. ultimum*, ranging from 1 ng to 0.1 pg, was amplified and 10 µl of labeled amplicons were hybridized. When disregarding mismatches at the extreme ends, cross hybridization to mismatch oligonucleotides was rare, especially with template amounts equal or less than 100 pg (Fig. 2-4). When *P. ultimum* amplicons were hybridized, cross hybridization to single mismatch oligonucleotides was only observed for Pul1-8 and Pul1-11, both carrying a C to A mutation, at the tenth and fifteenth position, respectively (Fig. 2-4). When *F. oxysporum* amplicons were hybridized, again no cross hybridization was observed for the center mismatch oligonucleotides Fox1-7, Fox1-8 and Fox1-9. Whereas weak signals were obtained for PCR amplification of 1 ng or 100 pg DNA with the oligonucleotides mutated at position 5 or 15, no cross hybridization was observed when amplifying 10 pg DNA or less. Moreover, in these cases, cross hybridization was strictly limited to the oligonucleotides with a 5’ end mismatch (Fig. 2-4).

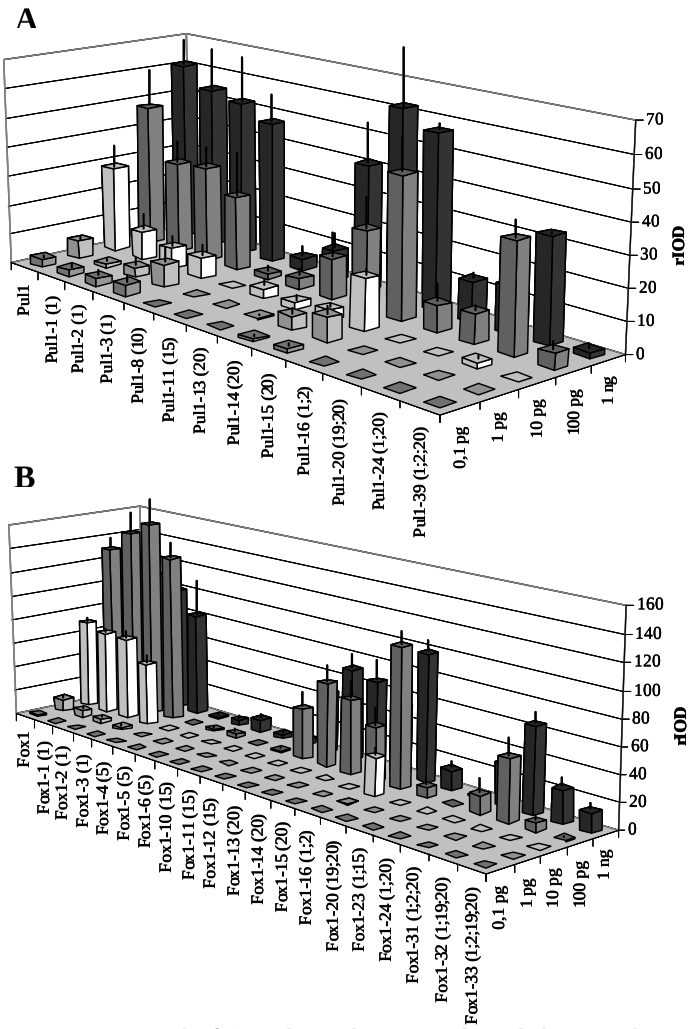

Fig 2-4. Discriminatory potential of **A,** Pul1- and **B,** Fox1-derived detector oligonucleotides after PCR amplification of DNA dilutions. Results are only shown for those oligonucleotides that resulted in detectable hybridization signals. Mismatch positions are indicated following the code of the oligonucleotide. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD). Data represent means from two hybridization runs (n = 4). Error bars indicate standard errors.

Finally, the mismatch *F. oxysporum* and *P. ultimum* oligonucleotides were tested for hybridization with amplicons from naturally infested plant, soil and water samples (Table 2-3). Ten microlitres of labeled amplicons were hybridized in 6 ml of hybridization buffer. As shown in Table 2-3, cross hybridization was strictly limited to the oligonucleotides with mismatches at one or both extreme ends, except for the analysis of plant sample 04-376B which was heavily infected with *P. ultimum*. For this sample, amplicons additionally

hybridized at low levels to Pul1-8 and Pul1-11. Nevertheless, the overall results of these experiments suggest that cross hybridization to oligonucleotides with a mismatch that is not located at one or both extreme ends is not of major concern when analyzing environmental samples. When amplicons corresponding to one of the two ITS regions were hybridized, signals were very weak, even for the signals produced by the perfect match oligonucleotides. In addition, in some cases even no signals were observed (data not shown) demonstrating that, under the labeling conditions used, relatively short amplicons are not suitable for assessing pathogen presence in environmental samples.

In conclusion, the results presented in this chapter illustrate the high specificity that can be obtained with DNA arrays, even allowing discrimination of single base pair differences. As a consequence, when using appropriate oligonucleotide sequences, closely related microbial species can be differentiated. Furthermore, we demonstrated that center mismatches do not always provide the highest degree of specificity, and that the discriminatory potential of a single mismatch oligonucleotide depends on the sequence of the oligonucleotide used. As a consequence, in order to differentiate SNPs, multiple oligonucleotides harboring the unique polymorphism at different positions should be screened for specificity when developing an oligonucleotide array. Nevertheless, based on our results, hybridization may generally be prevented when the mismatch occurs in the 3' half of the immobilized oligonucleotide. In addition, we showed that hybridization of 10 ng amplicons ml^{-1} hybridization buffer should be an appropriate concentration when pure cultures need to be identified. When disregarding mismatches at the extreme ends, cross hybridization signals are generally weak at this amplicon concentration and do not interfere with recognition of specific signals.

Table 2-3. Discriminatory potential of Pul1- or Fox1-derived detector oligonucleotides[a] upon hybridization of ITS I-5.8S rDNA-ITS II amplicons generated from environmental samples naturally infested with *Pythium ultimum* or *Fusarium oxysporum*, respectively

Oligonucleotide[b]	Sample ID								
	Soil			Plant				Water	
	04-285	04-336C	04-495B	04-349	04-376B	04-398	04-446	04-495E	
Pul1	31.9 ± 6.3[c]	27.3 ± 5.3	16.1 ± 3.3	21.6 ± 5.8	58.0 ± 10.2	7.3 ± 3.8	57.0 ± 5.4	8.4 ± 1.5	
Pul1-1 (1)	40.5 ± 7.2	26.5 ± 5.1	18.3 ± 5.0	15.0 ± 3.3	72.0 ± 13.6	7.6 ± 4.1	66.7 ± 4.2	10.6 ± 5.2	
Pul1-2 (1)	32.1 ± 5.2	37.2 ± 10.1	17.3 ± 2.0	25.0 ± 8.0	75.0 ± 16.1	7.0 ± 4.2	61.4 ± 8.8	12.6 ± 7.5	
Pul1-3 (1)	23.1 ± 6.2	30.9 ± 10.1	14.8 ± 3.3	27.0 ± 6.6	63.7 ± 17.0	8.8 ± 4.6	41.4 ± 10.9	9.6 ± 5.9	
Pul1-8 (10)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3	4.9 ± 2.9	0.2 ± 0.2	0.8 ± 0.7	0.0 ± 0.0	
Pul1-11 (15)	0.0 ± 0.0	0.5 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	8.3 ± 2.9	0.0 ± 0.0	0.8 ± 0.7	0.0 ± 0.0	
Pul1-13 (20)	2.6 ± 0.9	0.4 ± 0.4	0.3 ± 0.3	0.1 ± 0.1	28.5 ± 7.1	0.1 ± 0.1	6.2 ± 2.4	0.0 ± 0.0	
Pul1-14 (20)	5.9 ± 1.5	4.5 ± 1.8	1.2 ± 1.1	0.9 ± 0.6	34.4 ± 6.9	0.4 ± 0.4	11.3 ± 4.0	1.4 ± 1.3	
Pul1-15 (20)	20.3 ± 4.4	11.9 ± 3.1	3.4 ± 1.7	6.9 ± 1.6	55.6 ± 7.9	0.5 ± 0.3	51.5 ± 3.6	4.1 ± 0.8	
Pul1-16 (1;2)	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	11.1 ± 2.5	0.0 ± 0.0	10.8 ± 0.7	0.0 ± 0.0	
Pul1-20 (19;20)	0.2 ± 0.1	0.0 ± 0.0	1.1 ± 1.1	0.0 ± 0.0	6.9 ± 3.7	0.0 ± 0.0	5.2 ± 3.0	0.0 ± 0.0	
Pul1-24 (1;20)	0.8 ± 0.3	0.9 ± 0.6	0.0 ± 0.0	0.2 ± 0.1	30.7 ± 5.2	0.0 ± 0.0	16.1 ± 6.0	0.5 ± 0.5	
Pul1-39 (1;2;20)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.0 ± 2.2	0.0 ± 0.0	5.8 ± 3.0	0.0 ± 0.0	
	04-285			04-348	04-411	04-507		04-494	04-569C
Fox1	11.5 ± 2.2[c]			33.4 ± 8.1	74.1 ± 12.9	3.3 ± 1.3		36.0 ± 5.2	17.9 ± 8.0
Fox1-1 (1)	8.1 ± 0.7			12.6 ± 2.8	58.9 ± 14.6	1.2 ± 0.7		19.6 ± 3.7	11.0 ± 6.4
Fox1-2 (1)	3.4 ± 0.7			33.8 ± 7.8	79.6 ± 9.7	1.0 ± 0.8		11.6 ± 5.0	3.9 ± 2.3
Fox1-3 (1)	2.3 ± 0.9			49.9 ± 11.2	87.3 ± 8.7	0.7 ± 0.6		2.2 ± 1.3	2.5 ± 0.8
Fox1-13 (20)	0.0 ± 0.0			5.7 ± 3.1	0.1 ± 0.0	0.0 ± 0.0		0.0 ± 0.0	0.0 ± 0.0
Fox1-14 (20)	0.0 ± 0.0			5.3 ± 2.4	0.1 ± 0.1	0.0 ± 0.0		0.0 ± 0.0	0.0 ± 0.0
Fox1-15 (20)	0.0 ± 0.0			7.5 ± 2.7	0.3 ± 0.2	0.0 ± 0.0		0.0 ± 0.0	0.0 ± 0.0
Fox1-16 (1;2)	0.0 ± 0.0			35.1 ± 16.4	59.3 ± 21.5	0.0 ± 0.0		18.4 ± 10.2	8.1 ± 3.0
Fox1-24 (1;20)	0.0 ± 0.0			19.1 ± 9.5	10.6 ± 1.4	0.0 ± 0.0		0.0 ± 0.0	0.0 ± 0.0

[a] Results are only shown for those oligonucleotides that displayed hybridization signals.

[b] Mismatch positions are indicated between brackets.

[c] Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (Dig1). Values are means ± standard errors (n = 4 from two hybridization runs).

3 Design and development of a DNA macroarray for rapid detection and identification of multiple tomato vascular wilt pathogens*

3.1 Introduction

Fusarium and Verticillium wilt are known as devastating diseases of tomato (*Lycopersicon esculentum* Mill.) worldwide. They are caused by the soilborne fungal pathogens *Fusarium oxysporum* f. sp. *lycopersici* and the *Verticillium* species *V. albo-atrum* and *V. dahliae*. *F. oxysporum* f. sp. *lycopersici* is host-specific, but may also exist in symptomless alternate hosts representing many species (Katan, 1971). In contrast, both *Verticillium* species are able to infect a broad range of plant species, including cultivated crops and weeds (Pegg, 1981). These three tomato wilt fungi are related since they all invade susceptible plants through the roots and plug the water conducting vessels, causing wilt symptoms (Hutson and Smith, 1983; Bishop and Cooper, 1993). For both diseases, brown vascular discoloration can be observed in stem tissue cross sections near the soil line, even though these stems remain firm and green on the outside.

Currently, there are no effective curative treatments for plants afflicted with these wilts. Thus, effective preventative measures must be applied before these pathogens infect crop plants. Control of these fungi in infested soils can be established by reducing their presence through long crop rotations or soil fumigation. Furthermore, resistant cultivars to each of these diseases are available (Kawchuck *et al.*, 2001; Sela-Buurlage *et al.*, 2001). However, fungal strains that are not restricted by these resistant host varieties do appear (Paternotte and Vankesteren, 1993; O'Neill, 2002). Alternatively, attempts are made to develop biocontrol strategies against these vascular wilt diseases (ElAbyad *et al.*, 1993; Nagtzaam *et al.*, 1998; Duijff *et al.*, 1999; Solaraska *et al.*, 2000). However, so far, these

* Results described in this chapter have been published in "Design and development of a DNA array for rapid detection and identification of multiple tomato vascular wilt pathogens"; Lievens, B., Brouwer, M., Vanachter, A. C. R. C., Lévesque, C. A., Cammue, B. P. A., and Thomma. B. P. H. J.; FEMS Microbiology Letters 223:113-122 (2003).

have not yet been applied in practice, mainly because of poor efficacy and poor consistency under variable environmental conditions.

In order to perform appropriate preventative treatments, early detection of the pathogens' presence is required. In principle, a multitude of organisms can be simultaneously differentiated using DNA array technology, even if they differ in only a single to a few bases in the target gene (Chapter 2; Lievens *et al*., 2006). So far, this technology has not been used to detect and identify plant pathogens from complex extracts isolated from artificially or naturally infested samples.

In this chapter, we describe, as a proof-of-principle, the design and development of an ITS-based DNA macroarray to specifically detect and identify the economically important tomato vascular wilt pathogens *F. oxysporum* f. sp. *lycopersici*, *V. albo-atrum*, and *V. dahliae* to the species level. The array was validated using both artificially and naturally infested soil and plant samples, demonstrating the opportunities for utilization of DNA arrays in practice.

3.2 Materials and methods

3.2.1 Fungal and oomycete isolates and DNA extraction

A collection of over 175 fungal and oomycete isolates of plant pathogens that frequently occur in greenhouse crops was used. The most relevant isolates of this collection, including isolates belonging to the target species as well as to closely related species, are listed in Table 3-1. All isolates were cultured on PDA and incubated in darkness at 22°C. Genomic DNA was extracted from 5- to 10-day old cultures as described in Chapter 2.

Table 3-1. *Fusarium* and *Verticillium* isolates used in this study

Species	Isolate[a]	Origin	Host or substrate
Fusarium graminearum (lineage 7)	HCK PH1	unknown	unknown
F. oxysporum f. sp. *conglutinans*	HCK 81-4	unknown	unknown
F. oxysporum f. sp. *cucumerinum*	ATCC 201950	Florida	*Cucumis sativus*
F. oxysporum f. sp. *cucumerinum*	ATCC 36330	New Guinea	*C. sativus*
F. oxysporum f. sp. *lycopersici* (race 1)	CBS 645.78	Morocco	*Lycopersicon esculentum*
F. oxysporum f. sp. *lycopersici* (race 1)	CBS 412.90	Israel	*L. esculentum*
F. oxysporum f. sp. *lycopersici* (race 2)	CBS 646.78	Morocco	*L. esculentum*
F. oxysporum f. sp. *lycopersici* (race 2)	CBS 413.90	Israel	*L. esculentum*
F. oxysporum f. sp. *lycopersici* (race 1)	HCK FOL1	unknown	unknown
F. oxysporum f. sp. *lycopersici* (race 2)	HCK FOL2	unknown	unknown
F. oxysporum f. sp. *radicis-cucumerinum*	Afu-68(A)	Crete, Greece	*C. sativus*
F. oxysporum f. sp. *radicis-cucumerinum*	Afu-58	Crete, Greece	*C. sativus*
F. oxysporum f. sp. *radicis-lycopersici*	MUCL 39789	Belgium	*L. esculentum*
F. oxysporum f. sp. *radicis-lycopersici*	CBS 873.95	Israel	*L. esculentum*
F. oxysporum f. sp. *radicis-lycopersici*	CBS 101587	unknown	*L. esculentum*
F. oxysporum f. sp. *radicis-lycopersici*	HCK 0-1090/B	unknown	unknown
F. solani	CBS 165.87	Denmark	*Solanum tuberosum*
F. solani	CABI 17960	Brazil	*S. tuberosum*
F. solani	HCK S-66	unknown	unknown
*Gibberella fujikuroi (*anamorph: *F. verticillioides)*	MUCL 43506	Nepal	*Zea mays*
*Nectria haematococca (*anamorph: *F. solani)*	MUCL 20259	Belgium	Soil
Verticillium albo-atrum	CBS 451.88	Belgium	unknown
V. albo-atrum	CBS 321.91	Netherlands	*L. esculentum*
V. albo-atrum	CBS 385.91	Netherlands	*L. esculentum*
V. dahliae	CBS 386.49	Netherlands	*S. melongena*
V. dahliae	CBS 179.66	Netherlands	*L. esculentum*
V. dahliae	CBS 381.66	Canada	*L. esculentum*
V. dahliae	RCR V44	USA	*Gossypium* sp.
V. dahliae	RCR PH	USA	*Pistacia* sp.
V. dahliae	RCR 115	Syria	*Gossypium* sp.
V. dahliae	RCR 70-21	USA	*Capsicum* sp.
V. dahliae	RCR BB	USA	*S. tuberosum*
V. dahliae	RCR S39	USA	Soil
V. nubilum	MUCL 8266	Germany	Soil
V. tricorpus	MUCL 9792	UK	*L. esculentum*

[a] ATCC: American Type Culture Collection, Manassas, VA, USA; Afu: collection of D. J. Vakalounakis, N. AG. RE. F., Plant Protection Institute, Heraklio, Crete, Greece; CBS: Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; HCK: collection of H. C. Kistler, University of Minnesota, St. Paul, MN, USA; MUCL: Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; RCR: collection of R. C. Rowe, Ohio State University, Wooster, OH, USA.

3.2.2 Selection of oligonucleotides and DNA array production

Target-specific detector olignucleotides were designed based on ITS sequences. To this end, the region between the small and the large subunit of the rRNA gene of all *F. oxysporum, V. albo-atrum*, and *V. dahliae* isolates listed in Table 3-1 was amplified and sequenced using the universal primers ITS5 and ITS4 (White *et al.*, 1990). Amplification was carried out in 25 µl containing 5 ng genomic DNA using Platinum *Pfx* DNA polymerase (Invitrogen Corporation, San Diego, CA, USA) according to the manufacturer's protocol. The following

thermal profile was used: 94°C for 2 min followed by 35 cycles of 45 s at 94°C, 45 s at 58°C, and 45 s at 68°C with a final 10-min extension step at 68°C. Subsequently, the nucleotide sequence of all PCR products was determined (Eurogentec, Seraing, Belgium).

Genbank was searched to find matching and closely related ITS sequences. Subsequently, relevant ITS sequences were aligned using the ClustalW algorithm and multiple oligonucleotides were selected as described in Chapter 2. In addition to the previously used control oligonucleotides Dig1, as a reference for the detection and calibration, and Con1, as a negative control, a universal oligonucleotide (Uni1) supposed to detect the presence of any eukaryotic DNA was designed as a control for the amplification and hybridization. All oligonucleotides were synthesized with a 5'-C6-amino linker for covalent binding to nylon membrane and DNA arrays were produced as described in Chapter 2.

3.2.3 PCR amplification, labeling, and hybridization

The target ITS region of all isolates used in this study was amplified and simultaneously labeled with alkaline-labile digoxigenin using the universal primers ITS5 and ITS4 (White *et al.*, 1990). The target samples (25 µl) containing 5 ng genomic DNA were amplified using 1.25 units Platinum *Taq* DNA polymerase (Invitrogen Corporation, San Diego, CA, USA), 0.15 mM Dig-dUTP mix (Roche Diagnostics GmbH, Mannheim, Germany) and 0.5 µM of each primer, according to the same thermal profile as described above, with an elongation temperature of 72°C. After gel electrophoresis, the resulting Dig-dUTP-labeled amplicons were quantified by comparison to a DNA ladder (Smartladder SF, Eurogentec, Seraing, Belgium) using Labworks 4.0 Image Acquisition and Analysis Software (UVP, Upland, CA, USA). Approximately 10 ng of labeled amplicons per ml of hybridization buffer were hybridized to the array in a total volume of 6 ml as described in Chapter 2. All hybridizations were performed at least twice.

3.2.4 Validation of the DNA array

The DNA array was validated using complex samples from different biological origins. First, artificially infested samples were produced attempting to mimic samples obtained under current horticultural practices. Potting mix (DCM potting mix for House and Garden, DCM, Grobbendonk, Belgium) was heated for 5 days at 60°C and subsequently inoculated with either *V. dahliae* or *F. oxysporum* f. sp. *lycopersici*, or mock-inoculated as a negative

control. Per gram fresh weight of potting mix, 50 *V. dahliae* (CBS 179.66) microsclerotia were added (Hawke and Lazarovits, 1994). For *F. oxysporum* f. sp. *lycopersici* (CBS 645.78), soil inoculum was prepared in chopped potato soil as described by Ko and Hora (1971). For inoculation, air-dried inoculum was ground with a mortar and pestle followed by sequential sieving through 2.0 mm and 1.0 mm sieves. Particles remaining on the 1.0 mm sieve were used to infest potting mix at 0.5 g inoculum per pot (800 ml). Directly after inoculation, ten-day old tomato seedlings (*L. esculentum* Mill. cv. Saint-Pierre) were transferred to both inoculated and mock-inoculated potting mixes. Plants were grown in a growth chamber with a 16 h photoperiod (225 µE m^{-2} s^{-1}) at 22 °C and an 8 h period of darkness at 18°C. Stems, roots, and potting mix samples were collected for DNA array analysis at 7 and 10 weeks after transplantation, at which time the plants had not yet developed symptoms. Genomic DNA was extracted from 0.75 g (fresh weight) homogenized sample material using the UltraClean Plant DNA Isolation Kit (for stems and roots) and the UltraClean Soil DNA Isolation Kit (for potting mix) as described by the manufacturer (Mo Bio Laboratories, Inc., Solana Beach, CA, USA). DNA extracts were diluted 10-fold and amplified using the primers ITS1-F and ITS4 (Gardes and Bruns, 1993) as described in Chapter 2. Ten µl of labeled amplicons were hybridized in 6 ml of hybridization buffer. A parallel set of samples was retained for traditional plating on semi-selective medium (Nadakavukaren and Horner, 1959; Komada, 1975).

In addition, the DNA array was validated using naturally infested samples gathered from commercial tomato growers. Soil samples were collected using a core borer (∅ 3 cm) to a depth of 20-30 cm from several locations per field. Plant samples were taken from infected plant parts. Samples were homogenized and subsamples were assessed using the DNA array as well as by classical disease diagnostic methods. Genomic DNA was isolated from 0.75 g (fresh weight) sample, amplified and hybridized as described above. All hybridizations were performed at least twice. In addition, to confirm the identity of the pathogens isolated, ITS amplicons derived from purified isolates were sequenced as described above.

3.3 Results and discussion

3.3.1 Development of the DNA array

Based on the ITS sequences of *F. oxysporum*, *V. albo-atrum*, and *V. dahliae*, 10 taxon-specific oligonucleotide detectors with comparable theoretical hybridization kinetics were designed (Table 3-2). *Fusarium* is an anamorphic genus for which clear species boundaries are lacking (Kistler, 1997). Two genus-specific oligonucleotides were developed for *Fusarium* (Fgn1 and Fgn2), both covering a wide and largely overlapping range of *Fusarium* species. In addition, two species-specific oligonucleotides were designed for the detection of *F. oxysporum* (Fox1 and Fox2). Specific oligonucleotides for the subspecies *F. oxysporum* f. sp. *lycopersici* could not be designed because of the lack of ITS sequence variation between different *formae speciales*.

Also the genus *Verticillium* contains anamorphic species that are genetically very diverse. This diversity is also reflected at the nucleotide level for the ITS region. Since a genus-specific oligonucleotide of which the sequence is shared by a wide range of *Verticillium* species was not identified, two oligonucleotide sequences (Vgn1 and Vgn2) that are common for a subgroup of homologous *Verticillium* species including *V. albo-atrum*, *V. dahliae*, *V. longisporum*, and *V. tricorpus* were selected. Furthermore, species-specific oligonucleotides were designed for the detection of *V. albo-atrum* (Val1 and Val2) and for *V. dahliae* (Vda1 and Vda2).

Table 3-2. Sequences of detector oligonucleotides used for the DNA array

Code	Specificity	Sequence (5'-3')	Target
Fgn1	*Fusarium* sp.	CACGTCGAGCTTCCATAGC	ITS II
Fgn2	*Fusarium* sp.	CCAACTTCTGAATGTTGACC	ITS II
Fox1	*F. oxysporum*	TTGGGACTCGCGTTAATTCG	ITS II
Fox2	*F. oxysporum*	GTTGGGACTCGCGTTAATTCG	ITS II
Vgn1	*Verticillium* sp.	GCCGAAGCAACAATATGGTT	ITS I
Vgn2	*Verticillium* sp.	GTTGTTAAAAGTTTTAATAGTTCG	ITS I
Val1	*V. albo-atrum*	GCCGGTACATCAGTCTCTTTATTCA	ITS I
Val2	*V. albo-atrum*	CATCAGTCTCTTTATTCATACCAA	ITS I
Vda1	*V. dahliae*	AACAGAGAGACTGATGGACCG	ITS I
Vda2	*V. dahliae*	GTCCATCAGTCTCTCTGTTTAT	ITS I
Con1	None	GTCCAGACAGGATCAGGATTG	-
Uni1[a]	Universal	TCCTCCGCTTATTGATATGC	28S rDNA
Dig1[b]	None	GTCCAGACAGGATCAGGATTG	-

[a] ITS4 primer (White *et al.*, 1990).

[b] 3'-end digoxygenin-labeled.

3.3.2 Evaluation of the specificity and sensitivity of the DNA array

Specificity of the detector oligonucleotides was tested in hybridization reactions with labeled amplicons from the target pathogens as well as from a large collection of related and non-related species. Amplicons of the tested *F. oxysporum*, *V. albo-atrum* or *V. dahliae* isolates hybridized strongly to their respective detector oligonucleotides as shown in Table 3-3. Amplicons generated from oomycete or fungal species other than from the genus *Fusarium* or *Verticillium* did not show hybridization (data not shown). Differential hybridization strengths were observed for the two different oligonucleotides that were used to detect the genus *Verticillium* and the species *V. dahliae*. In general, hybridization to the oligonucleotides Vgn2 and Vda1 produced stronger signals than hybridization to the oligonucleotides Vgn1 and Vda2, respectively. For detection of *F. oxysporum* and *V. albo-atrum*, both oligonucleotides displayed similar hybridization strengths. Amplicons generated from *V. nubilum* did not hybridize to any of the *Verticillium* oligonucleotides, while amplicons of all *F. solani* (*Nectria haematococca*, teleomorph) isolates tested only hybridized to Fgn2. This was anticipated because the designed genus-specific oligonucleotides do not cover all species within the genus due to diversity at the nucleotide level.

Only for Val1, an oligonucleotide designed for the detection of *V. albo-atrum*, cross hybridization with a non-target species was observed demonstrating the high specificity of the oligonucleotides on the membrane. In addition to hybridization with *V. albo-atrum* amplicons, this oligonucleotide cross hybridized with amplicons from *V. tricorpus*. This latter fungus, together with *V. dahliae* and *V. albo-atrum*, is one of the three *Verticillium* pathogens of potato, but only occasionally causes Verticillium wilt of tomato (Huisman, 1988). The oligonucleotide Val2 did not display this cross hybridization. This difference in specificity can be explained by the position of the two adjacent nucleotides in the oligonucleotide that do not match with the *V. tricorpus* amplicon. While for Val2 the mismatches occur in the central region of the oligonucleotide, they are at the extreme 3' end for Val1, which is, as also shown in Chapter 2, the most destabilizing position in an immobilized oligonucleotide. Based on these findings the oligonucleotides Fgn2, Fox2, Vgn2, Val2, and Vda1 were selected for further experiments.

Table 3-3. Hybridization results of digoxigenin-labeled PCR amplicons from selected fungal cultures to the DNA array[a]

	Detector oligonucleotides												
Fungal isolate	Fgn1	Fgn2	Fox1	Fox2	Vgn1	Vgn2	Val1	Val2	Vda1	Vda2	Con1	Uni	Dig1
Fusarium graminearum HCK PH1	■	■										■	■
F. oxysporum f. sp. *conglutinans* HCK 81-4	■	■	■	■								■	■
F. oxysporum f. sp. *cucumerinum* ATCC 201950	■	■	■	■								■	■
F. oxysporum f. sp. *cucumerinum* ATCC 36330	■	■	■	■								■	■
F. oxysporum f. sp. *lycopersici* CBS 645.78	■	■	■	■								■	■
F. oxysporum f. sp. *lycopersici* CBS 412.90	■	■	■	■								■	■
F. oxysporum f. sp. *lycopersici* CBS 646.78	■	■	■	■								■	■
F. oxysporum f. sp. *lycopersici* CBS 413.90	■	■	■	■								■	■
F. oxysporum f. sp. *lycopersici* HCK FOL1	■	■	■	■								■	■
F. oxysporum f. sp. *lycopersici* HCK FOL2	■	■	■	■								■	■
F. oxysporum f. sp. *radicis-cucumerinum* Afu 68(A)	■	■	■	■								■	■
F. oxysporum f. sp. *radicis-cucumerinum* Afu 58	■	■	■	■								■	■
F. oxysporum f. sp. *radicis-lycopersici* MUCL 39789	■	■	■	■								■	■
F. oxysporum f. sp. *radicis-lycopersici* CBS 873.95	■	■	■	■								■	■
F. oxysporum f. sp. *radicis-lycopersici* CBS 101587	■	■	○	■								■	■
F. oxysporum f. sp. *radicis-lycopersici* HCK 0-1090/B	■	■	■	■								■	■
F. solani CBS 165.87		■										■	■
F. solani CABI 17960		■										■	■
F. solani HCK S-66		■										■	■
Gibberella fujikuroi MUCL 43506	■	■										■	■
Nectria haematococca MUCL 20259		■										■	■
Verticillium albo-atrum CBS 451.88					○	■	■	■				■	■
V. albo-atrum CBS 321.91					■	■	■	■				■	■
V. albo-atrum CBS 385.91					○	○	○	○				■	■
V. dahliae CBS 386.49					■	■			■	○		■	■
V. dahliae CBS 179.66					○	■			■	■		■	■
V. dahliae CBS 381.66					○	■			■	■		■	■
V. dahliae RCR V44					○	■			■	■		■	■
V. dahliae RCR PH					○	■			■	■		■	■
V. dahliae RCR 115					■	■			■	■		■	■
V. dahliae RCR 70-21					○	■			■	■		■	■
V. dahliae RCR BB					○	■			■	○		■	■
V. dahliae RCR S39					○	■			■	■		■	■
V. nubilum MUCL 8266												■	■
V. tricorpus MUCL9792					○	■	■					■	■

[a] Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD) and classified into three categories: blank = no signal (rIOD $\leq$ 2); ○ = weak signal (rIOD > 2 and $\leq$ 70); ■ = strong signal (rIOD > 70).

In order to determine the detection limit of the DNA array, a dilution series of DNA from *V. albo-atrum* CBS 451.88, *V. dahliae* CBS 386.49, and *F. oxysporum* f. sp. *lycopersici* CBS 645.78 was made prior to PCR amplification. The amount of undiluted DNA varied between 250 and 500 ng and was subsequently diluted in six ten-fold dilution steps. After PCR, samples were hybridized (10 µl per 6 ml) to the membrane and analyzed. For *V. dahliae*, 2.5 pg of DNA could still be detected (Fig. 3-1). Moreover, for *V. albo-atrum* and *F. oxysporum* a signal could easily be detected in the last dilution, representing as little as 0.35 pg DNA for *V. albo-atrum* and 0.50 pg DNA for *F. oxysporum* (Fig. 3-1). This reveals the high sensitivity of this technique, which is comparable with the sensitivity of other molecular techniques (Mercado-Blanco *et al*., 2003; Suarez *et al*., 2005; Szemes *et al*., 2005) and should allow detection of these pathogens at densities in which they are likely to occur in the field.

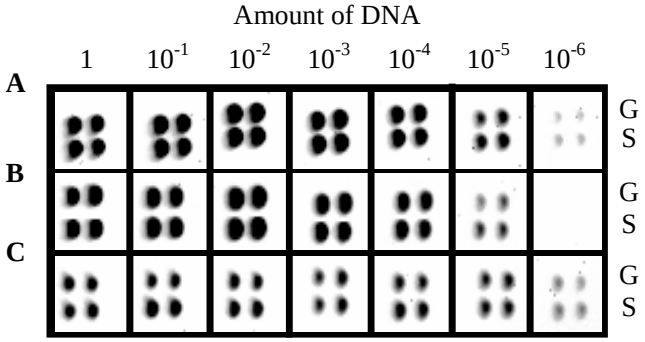

Fig. 3-1. Sensitivity of the DNA array. Blots are shown for detection of **A,** *Verticillium albo-atrum*, **B,** *V. dahliae*, and **C,** *Fusarium oxysporum* f. sp. *lycopersici* after PCR amplification of a ten-fold serial dilution of fungal DNA. Ten µl of labeled amplicons were hybridized in a total volume of 6 ml. Figure panels represent a genus-specific oligonucleotide detector (upper signal (G), Vgn2 and Fgn2 for *Verticillium* and *Fusarium*, respectively) and a species-specific oligonucleotide detector (lower signal (S), Val2, Vda1 and Fox2 for *V. albo-atrum*, *V. dahliae* and *F. oxysporum* f. sp. *lycopersici*, respectively) horizontally spotted in duplicate. The undiluted amount (1) of DNA is 350 ng for *V. albo-atrum*, 250 ng for *V. dahliae*, and 500 ng for *F. oxysporum* f. sp. *lycopersici*.

To test the discriminatory potential of the oligonucleotide array, genomic DNA of *F. oxysporum* f. sp. *lycopersici* CBS 645.78, *V. albo-atrum* CBS 451.88, and *V. dahliae* CBS 386.49 were mixed in different combinations (5 ng for each isolate) prior to PCR amplification of the ITS region. As a control, *F. solani* CBS 165.87 DNA was also incorporated in some of these mixes. In all cases, the expected hybridization pattern for each mixture was obtained, demonstrating that the desired amplicons were generated and detected from a fungal DNA mix using the DNA array (Table 3-4).

Table 3-4. Hybridization results of digoxigenin-labeled PCR amplicons from DNA mixtures from selected fungal cultures to the DNA array[a]

	Detector oligonucleotides							
Fungal isolate mixture[b]	Fgn2	Fox2	Vgn2	Val2	Vda1	Con1	Uni1	Dig1
Fol + Vaa + Vd	■	■	■	■	■		■	■
Fs + Vaa + Vd	■		■	■	■		■	■
Fol + Vaa	■	■	■	■			■	■
Fol + Vd	■	■	■		■		■	■
Vaa +Vd			■	■	■		■	■

[a] Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD) and classified into three categories: blank = no signal (rIOD $\leq$ 2); ○ = weak signal (rIOD > 2 and $\leq$ 70; not observed in this experiment); ■ = strong signal (rIOD > 70).

[b] Fol: *Fusarium oxysporum* f. sp. *lycopersici* CBS 645.78; Fs: *F. solani* CBS 165.87; Vaa: *Verticillium albo-atrum* CBS 451.88; Vd: *V. dahliae* CBS 386.49.

3.3.3 Validation of the DNA array using biological samples

To validate the DNA array using biologically complex samples, initially artificially infested samples were produced. Tomato seedlings were transferred to potting mix that was preheated and inoculated with *V. dahliae* or *F. oxysporum* f. sp. *lycopersici*. Seven and ten weeks after plant transfer, plant material and potting mix was sampled and evaluated for the presence of the pathogen. At this stage, plants did not show any wilting symptoms. For some plants, however, slight vascular discoloration was observed in stem cuttings. For PCR amplification of the ITS region, the forward primer ITS1-F (Gardes and Bruns, 1993) was used in combination with ITS4 (White *et al.*, 1990). In contrast to primer ITS5 which hybridizes to an rDNA sequence shared by all eukaryotes, ITS1-F hybridizes specifically to a fungal-specific rDNA sequence, thus enhancing fungal detection in complex biological samples. Furthermore, based on an experiment with different DNA polymerases, we observed that the yield of the PCR product was consistently higher when using Titanium *Taq* DNA polymerase (Fig. 3-2). Therefore, this DNA polymerase was used for PCR amplification of DNA extracted from environmental samples.

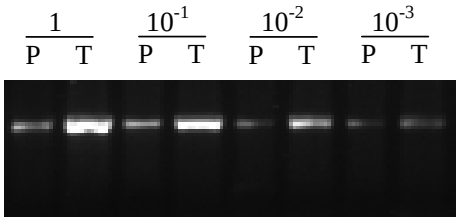

Fig. 3-2. Comparison of DNA polymerase enzymes. Comparison of Titanium *Taq* DNA polymerase (T) versus Platinum *Taq* DNA polymerase (P) for product yield in a PCR reaction with primers ITS5 and ITS4 in a ten-fold serial dilution of *Fusarium oxysporum* f. sp. *lycopersici* (CBS 645.78) DNA. Sample 1 is undiluted (350 ng).

Both pathogens could be detected in roots and stems from plants that were grown in inoculated potting mixes when using the DNA array, demonstrating that detection of the pathogen was successful in plants even at the pre-symptomatic stage of infection (Table 3-5). Furthermore, both pathogens could also be detected in the potting mix. The pathogen was not detected in plant or potting mix samples of non-inoculated control treatments. The signal obtained with the universal detector oligonucleotide (Uni1) in the roots and potting mix for mock-inoculated potting mix indicates the presence of fungi that associate with the roots after recolonization of the potting mix. All of the results above were confirmed by (selective) plating methods (data not shown).

Table 3-5. Hybridization results of digoxigenin-labeled PCR amplicons from biologically complex samples to the DNA array[a]

		Detector oligonucleotides							
Sample		Fgn2	Fox2	Vgn2	Val2	Vda1	Con1	Uni1	Dig1
Fusarium oxysporum f. sp.	stem	■	■					■	■
lycopersici CBS 645.78	roots	■	■					■	■
	potting mix	■	■					■	■
Verticillium dahliae CBS	stem			■		■		■	■
179.66	roots			■		■		■	■
	potting mix			■		■		■	■
Mock-inoculated	stem								■
	roots							■	■
	potting mix							○	■

[a] Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD) and classified into three categories: blank = no signal (rIOD $\leq$ 2); ○ = weak signal (rIOD > 2 and $\leq$ 70); ■ = strong signal (rIOD > 70).

Finally, we evaluated whether the DNA array could also be used for diagnosis of biological samples gathered from commercial tomato growers. Out of fifteen plant samples analyzed, one was diagnosed with *V. dahliae* and one with *F. oxysporum*, while four carried *V. dahliae* as well as a *Fusarium* species, though not *F. oxysporum*. From eight soil samples

tested, all contained *Fusarium* (in three samples *F. oxysporum*) and two contained *V. dahliae* (See Fig. 3-3 for representative examples).

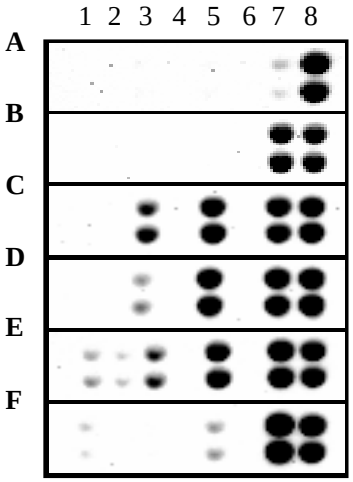

Fig. 3-3. Diagnosis of greenhouse samples. DNA was isolated from greenhouse samples gathered from commercial tomato growers, amplified and hybridized to the DNA array. Detector oligonucleotides are vertically spotted in duplicate: Fgn2 (1), Fox2 (2), Vgn2 (3), Val2 (4), Vda1 (5), Con1 (6), Uni1 (7), and Dig1 (8). Samples are diagnosed as follows: **A,** control (water); **B,** *Pythium* infected tomato plant; **C** and **D,** *Verticillium dahliae* infected tomato plant; **E,** *Fusarium oxysporum* and *V. dahliae* infested soil; and **F,** soil with slight *V. dahliae* and *Fusarium* infestation.

For all samples, results from the DNA array were corroborated by classical plating and taxonomy techniques (data not shown). In addition, the pathogen identity was confirmed by sequencing the ITS region of purified isolates, demonstrating that both the classical and the DNA array approaches are equally reliable. However, the major advantage of the DNA array is that the diagnosis is completed much faster, generally within 36 hours, whereas plating for these organisms takes at least one to several weeks. In addition, this array could be enlarged to include other tomato pathogens of fungal, oomycete, bacterial, and viral origin as well as parasitic nematodes. Ultimately, this may lead to a complete pathogen assessment for a specific crop in a single assay. Alternatively, this array could be enlarged to include vascular wilt pathogens of other crops in order to obtain a universal vascular wilt detection array.

The results of all these tests illustrate the power of DNA arrays for routine analysis of samples from different biological sources. In this chapter, we demonstrated that this technology can easily be implemented for pathogen assessment *in planta*, even at the

presymptomatic stage of infection. Although *F. oxysporum* could not be resolved to the subspecies level by targeting ITS sequences, detection at the *forma specialis* level is not crucial when analyzing stem samples since Fusarium tomato wilt can only be caused by *F. oxysporum* f. sp. *lycopersici*. However, successful implementation of this technology for soil diagnosis will require additional effort, especially with regard to complex species such as *F. oxysporum*. Within this species, over 120 *formae speciales* have been defined, each consisting of strains with the ability to cause disease on a specific host (Hawksworth *et al*., 1995). Morphologically, all *F. oxysporum* strains are identical and discrimination based on the commonly used housekeeping genes, including the rRNA, beta-tubulin or elongation factor-1 alpha genes, is not possible (data not shown). However, recently, genes have been identified in *F. oxysporum* f. sp. *lycopersici* that are directly linked to pathogenicity (Rep *et al*., 2004). Currently, the array is being adapted to include these markers for accurate detection and identification of this *forma specialis* (data not shown). The next challenge will be to implement a quantification aspect to the DNA array. This is essential to quantify the amount of pathogen infestation, to monitor disease development, and to judge whether and when control measures should be taken.

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

4.1 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* Mill.), 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 (Jones *et al*., 1997).

Timely, accurate detection and identification of plant pathogens are essential for effective plant disease management. In addition, pathogen quantification is an important aspect 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 (Tsao and Guy, 1977; Jeffers and Martin, 1986; Thorn *et al*., 1996; Termorshuizen *et al*., 1998; Goud and Termorshuizen, 2003), the development of real-time PCR (Heid *et al*., 1996) has been a great step forward with regard to quantification. Increasingly, real-time PCR is being used for plant pathogen diagnosis (McCartney *et al*., 2003; Schaad *et al*., 2003; Gachon *et al*., 2004; Schena *et al*., 2004; Lievens *et al*., 2005b) as well as for monitoring pathogen infection (Brouwer *et al*., 2003).

In this chapter, 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

* Results described in this chapter have been published in "Real-time PCR for detection and quantification of fungal and oomycete tomato pathogens in plant and soil samples"; Lievens, B., Brouwer, M., Vanachter, A. C. R. C., Cammue, B. P. A., and Thomma. B. P. H. J.; Plant Science 171:155-165 (2006).

target pathogens comprised the fungi *F. solani*, *R. solani* and *Verticillium dahliae*, and the oomycete *P. 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.

4.2 Materials and methods

4.2.1 Fungal and oomycete isolates and DNA extraction

Fungal and oomycete isolates used in this study are listed in Table 4-1. All isolates were grown on PDA in the dark at 22°C. Genomic DNA was isolated from 5- to 10-day-old cultures as described in Chapter 2. 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.

Table 4-1. 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			
						ITS1-F/ AFP346	AFP276/ ITS4	ST-RS1/ ITS4	ITS1-F/ 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	*Solanum 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	-	-	-	+
		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	*S. 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	*S. 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*	-	-	+	-

Table 4-1 (continued).

Phylum	Order	Species[a]	Isolate[b]	Origin	Host or substrate	Specificity[c] obtained with primer pairs ITS1-F/ AFP346	AFP276/ ITS4	ST-RS1/ ITS4	ITS1-F/ ST-VE1
		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*	-	+	-	-

[a] Unless marked with an asterisk, fungal and oomycete species are reported as tomato pathogens (Jones *et al*., 1997).

[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: collection of H. A. J. Hoitink, Ohio State University, Wooster, OH, USA; HCK: collection of H. C. Kistler, University of Minnesota, St. Paul, MN, USA; MUCL: Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; ST: 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.

4.2.2 Real-time PCR

PCR primers were designed based on ITS sequences. To this end, the region between the small and large subunit of the rRNA gene of all *F. solani (Nectria haematococca*, teleomorph*), P. ultimum*, *R. solani*, and *V. dahliae* isolates listed in Table 4-1 was amplified and sequenced as described in Chapter 3. Following sequence alignment using the ClustalW algorithm with related ITS sequences found in Genbank (Benson *et al*., 2004) species-specific primers were designed and checked for lack of significant homology with other DNA sequences using the Basic Local Alignment Search Tool (BLAST). For each target pathogen, single real-time PCR primers were designed (Table 4-2) and used for amplification in combination with either the fungus-specific forward primer ITS1-F (Gardes and Bruns, 1993) or the universal reverse primer ITS4 (White *et al.*, 1990).

Table 4-2. Real-time PCR primers used in this study

Code	Specificity	Sequence (5'-3')	Target	Amplicon size (bp)	T_{ann} [a]	t_{el} [b]	T_m [c]
AFP346[d,e]	*Fusarium solani*	GGTATGTTCACAGGGTTGATG	ITS I	104	60	6	82.5
AFP276[f,g]	*Pythium ultimum*	TGTATGGAGACGCTGCATT	ITS II	150	58	8	81.0
ST-RS1[f,g]	*Rhizoctonia solani*	AGTGTTATGCTTGGTTCCACT	ITS II	187	60	8	83.0
ST-VE1[d,e]	*Verticillium dahliae*[h]	AAAGTTTTAATGGTTCGCTAAGA	ITS I	200	60	9	85.7
ITS1-F[f,i]	Fungi	CTTGGTCATTTAGAGGAAGTAA	18S rDNA	x[j]	x	x	x
ITS4[d,k]	Universal	TCCTCCGCTTATTGATATGC	28S rDNA	x	x	x	x
$P450_1$[f,l]	*Saccharomyces cerevisiae*	ATGACTGATCAAGAAATCGCTAA	*erg11* gene	343	50	14	83.5
$P450_2$[d,l]	*S. cerevisiae*	TGTAACCTGGAGAAACCAAAAC	*erg11* gene				

[a] Annealing temperature (°C).

[b] Elongation time (s).

[c] Melting temperature (°C) at which a specific dissociation peak of increased fluorescence is generated in the melting curve analysis.

[d] Reverse primer.

[e] In combination with ITS1-F.

[f] Forward primer.

[g] In combination with ITS4.

[h] Experiments showed no species specificity but specificity to the three *Verticillium* species capable of causing tomato wilt, including *V. albo-atrum*, *V. dahliae* and *V. tricorpus*.

[i] Gardes and Bruns (1993).

[j] x, depends on second primer used.

[k] White *et al*. (1990).

[l] Morace *et al*. (1997).

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 separated 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), 1µl 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 (T_{ann}) indicated in Table 4-2, and elongation at 72°C for the time period (t_{el}) indicated in Table 4-2. Fluorescence 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 slowly heating the mixture from 65°C to 95°C at 0.1°C s^{-1} with continuous measurement of fluorescence at 520 nm.

4.2.3 Exogenous control

To monitor potential 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 $P450_2$ (Morace *et al*., 1997). 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.

4.2.4 Standard curves

For all target pathogens as well as for the exogenous control *S. cerevisiae*, standard curves were generated by plotting the threshold cycle (C_T) for a 10-fold dilution series of pure genomic DNA (three replicates) against the logarithm of the DNA concentration (Brouwer *et al*., 2003). This threshold cycle is defined as the cycle number when the amplification is in the exponential phase and the fluorescence exceeds the background level. In addition, in order to evaluate possible alteration in amplification efficiency when environmental samples would be assessed, all samples of the dilution series were spiked with plant- or soil-derived

DNA at an average concentration when assaying environmental DNA samples (15 ng; Lievens *et al.*, 2005a).

4.2.5 Quantification of pathogen DNA

The eventual goal of this study was to quantify pathogen DNA in complex biological samples using real-time PCR. Therefore, 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 or sandy soil (Sint-Katelijne-Waver, Belgium) as described above. 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, and *V. dahliae* CBS 381.66. Following an incubation period of 14 days at 22°C fresh inoculum prepared in sterilized chopped potato soil (Ko and Hora, 1971) 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 (∅ 1-2 mm) from the starting inoculum were plated on semi-selective medium (Nadakavukaren and Horner, 1959; Ko and Hora, 1971; Jeffers and Martin, 1986) and incubated at 22°C in darkness. Plates were checked daily for fungal growth. 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 (1994) 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 in duplo using real-time PCR. 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 (Jones *et al.*, 1997; Agrios, 2005), 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 (∅ 3 cm) to a depth of 20-30 cm from at least ten 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 (Chapter 3; Lievens *et al*., 2003), by which over 40 different fungal and oomycete plant pathogens can be detected in a single assay. 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 (Nadakavukaren and Horner, 1959; Ko and Hora, 1971; Jeffers and Martin, 1986). Plates were incubated at 22°C in darkness and checked daily for fungal growth. 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 (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. With regard to the plant samples, infected plant parts were plated in triplicate on semi-selective medium (Nadakavukaren and Horner, 1959; Ko and Hora, 1971; Jeffers and Martin, 1986) following surface sterilization. After incubation at 22°C in darkness the identity of the pathogen was confirmed using classical taxonomy techniques.

4.3 Results

4.3.1 Primer selection and specificity

For each selected target pathogen, several real-time PCR primers were designed based on ITS sequences and amplified either with ITS1-F (Gardes and Bruns, 1993) or ITS4 (White *et al*., 1990). 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 4-2). The primer sets selected for further experiments were ITS1-F combined with AFP346, or ST-VE1 to detect *F. solani*, or *V. dahliae*, respectively, and ITS4 in combination with ST-RS1 or AFP276 to detect *R. solani* or *P. ultimum*, respectively (Table 4-2). Specificity of the selected primer sets was tested using genomic DNA extracted from all isolates listed in Table 4-1. 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 (Jones *et al*., 1997). As a check for DNA quality, all DNA extracts were successfully subjected to PCR analysis using the universal ribosomal primers ITS5 and ITS4 (White *et al*., 1990) (data not shown). Except for the *V. dahliae* primers, all primer pairs displayed the desired species specificity (Table 4-1), showing that, despite the use of only one species-specific primer, PCR assays were selective for the target organisms. With regard to the primers ITS1-F and ST-VE1, developed to specifically detect and quantify *V. dahliae*, amplification was also observed for *V. albo-atrum* and *V. tricorpus*, both of which are closely related to *V. dahliae* (Table 4-1). 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 4-2, demonstrating the amplification specificity.

4.3.2 Quantification of pathogen DNA in complex biological samples

To quantify unknown concentrations of pathogen DNA, for each target pathogen, a standard curve (Fig. 4-1) was generated by the amplification of a 10-fold dilution series of target DNA. All standard curves obtained demonstrate that pathogen DNA can be accurately quantified over at least four orders of magnitude. For each pathogen, the correlation between the C_T-value and the logarithmic target DNA concentration was very high (R^2 > 0.996). For each species, slopes were very similar, resulting in amplification efficiencies (calculated using the formula $E = 10^{(-1/slope)}-1)*100$) ranging from 80% (for *R. solani* and *V.*

dahliae) to 93% (for *P. ultimum)*. In addition, when plant or soil-derived DNA was added at an average concentration when assaying environmental samples (Lievens *et al*., 2005a), template quantification was not affected (Fig. 4-1), demonstrating that amplification efficiency is not influenced by non-target DNA concentrations that are likely to occur in horticultural practice. Based on these findings, the standard curves obtained for PCR amplification in pure water were used throughout the rest of the study to calculate concentrations of pathogen template.

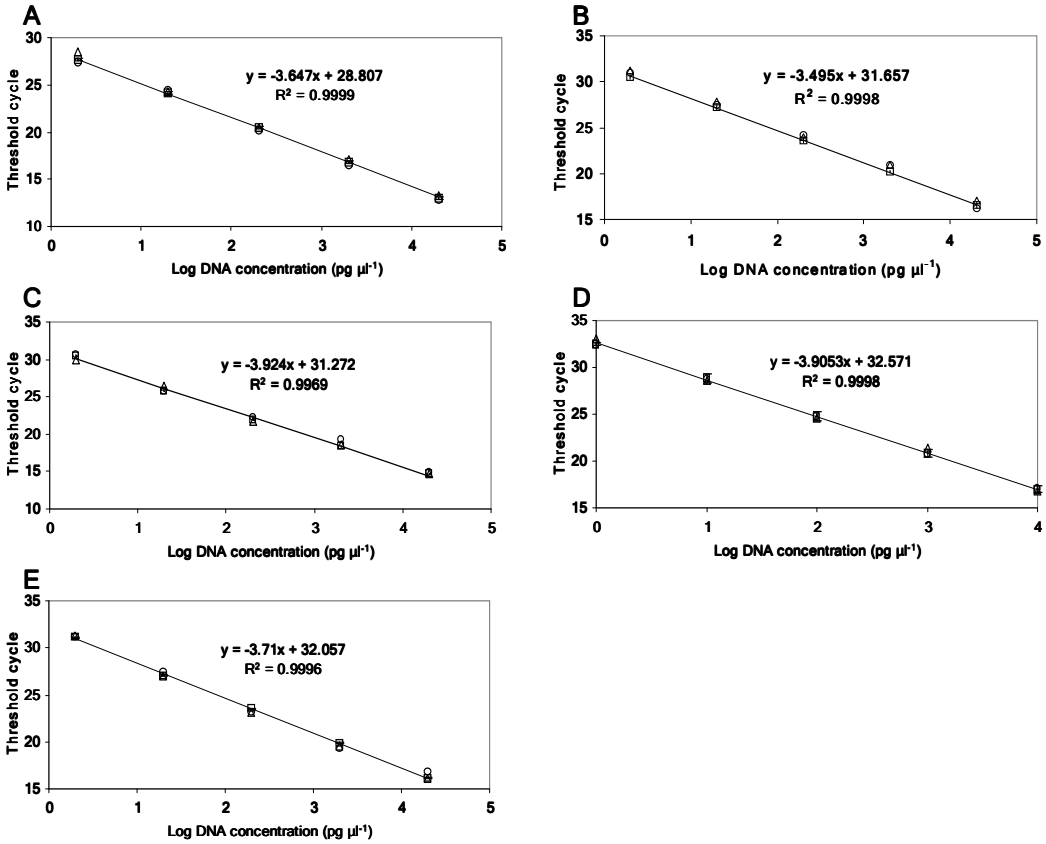

Fig. 4-1. Standard curves used for the quantification of target DNA using real-time PCR for **A,** *Fusarium solani* CBS 165.87; **B,** *Pythium ultimum* CBS 101588; **C,** *Rhizoctonia solani* CBS 323.84; **D,** *Verticillium dahliae* CBS 381.66; and **E,** *Saccharomyces cerevisiae* MUCL 28426. Curves were obtained using a 10-fold dilution series of target DNA amplified in water (□). Data represent means of three replicates (n = 3). Error bars, representing standard errors, not visible are too small to be displayed graphically. In addition, the mean threshold cycles obtained with amplification in the presence of 15 ng DNA extracted from a healthy tomato plant (◇) or a sandy soil (△) are indicated on the plots.

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 were added to 20 ng μl^{-1} genomic DNA from a healthy tomato plant or from the total DNA present in a sandy soil. As was also observed in Fig. 4-1, the results presented in Fig. 4-2 show that neither plant DNA nor soil DNA significantly interfered with target DNA quantification.

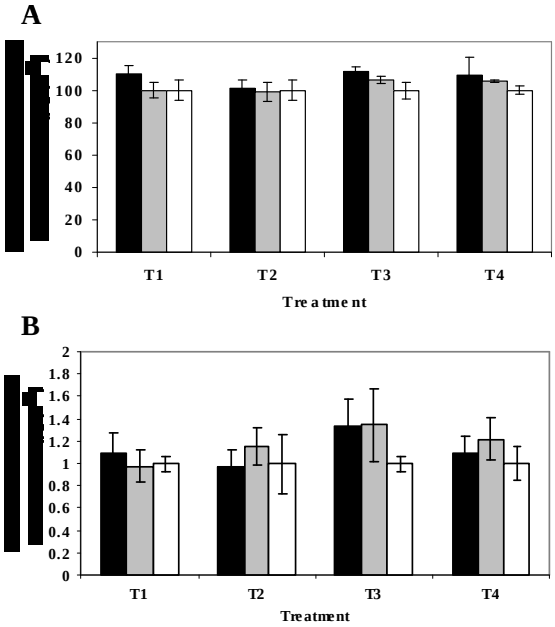

Fig. 4-2. Influence of non-target DNA on target DNA quantification using real-time PCR. Samples containing **A,** 100 pg μl^{-1} or **B,** 1 pg μl^{-1} pathogen DNA were amplified in the presence of 20 ng μl^{-1} genomic DNA extracted from a healthy tomato plant (■) or a sandy soil (▩). As a control no non-target DNA was added (□). Target DNA was isolated from *Fusarium solani* CBS 165.87 (T1), *Pythium ultimum* CBS 101588 (T2), *Rhizoctonia solani* CBS 323.84 (T3), and *Verticillium 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 (n = 2). Errors bars indicate standard errors.

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 9 other fungal or oomycete tomato pathogens. The results show that irrespective the presence of non-target fungal or oomycete DNA, in all cases the amount of template DNA was accurately quantified (Fig. 4-3). 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 4-2, demonstrating the specificity of the amplification process (data not shown). Therefore, these experiments show that the desired amplicons can be generated, detected and quantified in complex DNA mixtures.

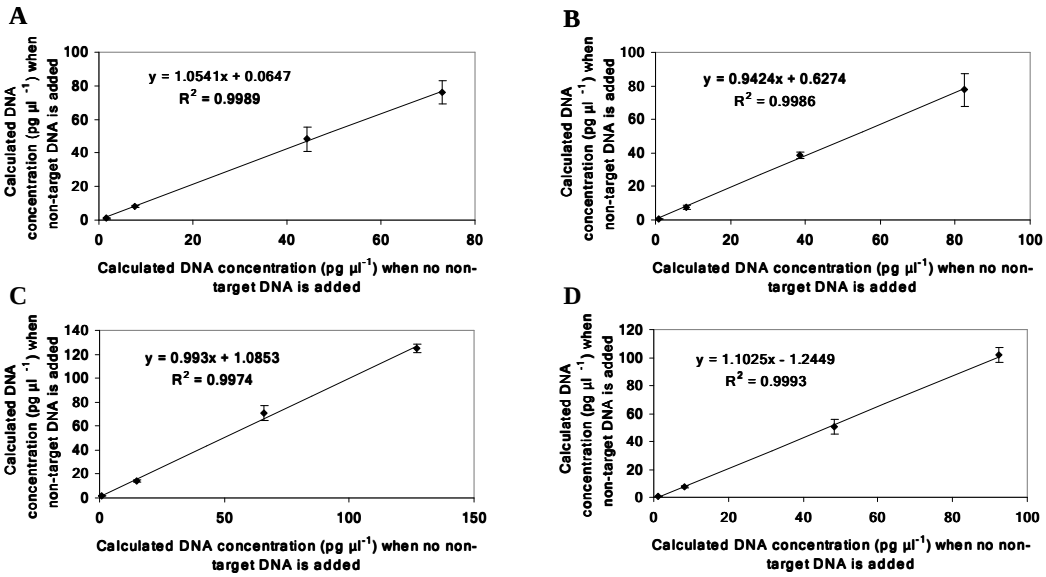

Fig. 4-3. Influence of non-target fungal and oomycete DNA on target DNA quantification using real-time PCR. Curves for **A,** *Fusarium solani* CBS 165.87; **B,** *Pythium ultimum* CBS 101588; **C,** *Rhizoctonia solani* CBS 323.84; and **D,** *Verticillium dahliae* CBS 381.66 were obtained by plotting 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 (n = 2). Errors bars indicate standard errors.

To quantitatively assess pathogen presence in biological samples, artificially infested soil samples were produced for *P. ultimum*, *R. solani*, 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. 4-4 (A-C). For each pathogen, a linear correlation was obtained with a coefficient of determination higher than 0.94. In addition, soil was infested with specific amounts of microsclerotia from *V. dahliae*. As shown in Fig. 4D, again a linear correlation was obtained (R^2 = 0.98), demonstrating the feasibility of the technique to quantify pathogen biomass in biological samples.

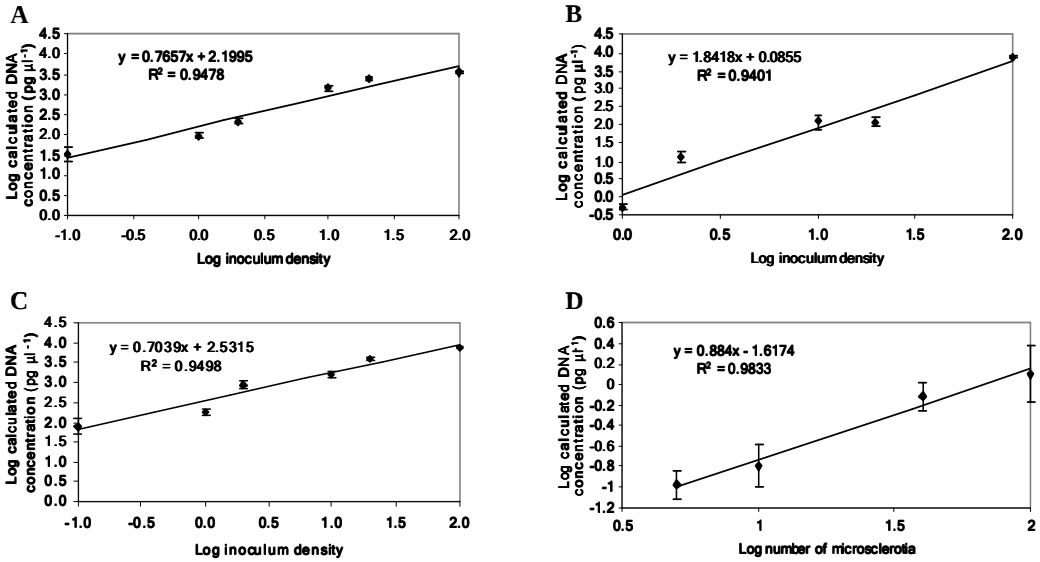

Fig. 4-4. Quantitative assessment of pathogen presence in artificially infested soil samples. **A-C,** Regression lines for real-time PCR analysis of a dilution series of pathogen inoculum from *Pythium ultimum* CBS 101588 **(A)**, *Rhizoctonia solani* CBS 323.84 **(B)**, and *Verticillium dahliae* CBS 381.66 **(C)**. Inoculum density is expressed as the percentage of fungal growth out of 100 inoculum soil aggregates. **D,** 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 (n = 4): two samples were used for DNA extraction and analyzed in duplo. Error bars indicate standard errors.

In our final assays, it was 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 an extended version of the DNA macroarray designed in the previous chapter (Lievens *et al.*, 2003). 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 4-3).

Table 4-3. Real-time PCR quantification of fungal and oomycete genomic DNA in different environmental samples

Sample	Cultivated crop		Sample ID	Observed symptoms	Calculated DNA concentration[a] (pg µl^{-1}) for			
	Latin name	Common name			*Fusarium solani*	*Pythium ultimum*	*Rhizoctonia solani*	*Verticillium* sp.
Sandy soil	*Rosa* sp.	Rose	03-111	Foot and stem rot	0.02	x[b]	0.18	x
Sandy soil	*Fragaria ananassa*	Strawberry	03-224	Root rot; reduced growth	0.02	12.18	1.50	x
Sandy soil	*Lycopersicon esculentum*	Tomato	03-307	None	0.34	x	x	x
Sandy soil	*Apium graveolens*	Celery	04-188	None	x	0.86	x	x
Sandy soil	*Phalaenopsis* sp.	Orchid	04-191B[c]	Root rot; reduced growth	x	x	1.97	x
Sandy soil	*Brassica oleracea*	Cauliflower	04-200A	Wilting	x	x	x	0.19
Sandy soil	*B. oleracea*	Cauliflower	04-200B	Wilting	x	x	x	0.12
Sandy soil	*Lactuca sativa*	Lettuce	04-285	None	x	4.26	x	x
Sandy soil	*Apium graveolens*	Celery	04-329B[d]	Crater rot	0.13	0.47	0.74	x
Sandy soil	*L. sativa*	Lettuce	04-336C	None	x	1.98	x	x
Plant	*L. esculentum*	Tomato	03-182Aw[e]	Root rot; wilting; nettle-like head	x	0.04	0.27	x
Plant	*Carpinus* sp.	Hornbeam	04-118	Damping-off	x	4.08	x	x
Plant	*Phalaenopsis* sp.	Orchid	04-191B2[c]	Root rot; reduced growth	x	x	22.01	x
Plant	*L. sativa*	Lettuce	04-178A	Vein rot; browning of leaves	x	x	x	x
Plant	*A. graveolens*	Celery	04-329C[d]	Crater rot	x	x	741.6	x

[a] Prior to PCR amplification, DNA was diluted 10-fold to avoid inhibitory concentrations of potential PCR inhibitors. Calculated DNA concentrations are those in the diluted DNA samples.

[b] x, absent according to a DNA array analysis (Lievens *et al.*, 2003) by which over 40 different plant pathogenic fungi and oomycetes can be detected.

[c,d] Corresponding samples.

[e] Pepino mosaic virus (PepMV) infected plant as confirmed by ELISA (Agdia, Elkhart, IN, USA) testing.

For all samples, pathogens detected using the DNA array were also detected in the real-time PCR analyses. Using the array, all ten soil samples were diagnosed with multiple microorganisms (data not shown). In five of these samples, *P. ultimum* was found in DNA concentrations ranging from 0.47 to 12.18 pg μl^{-1}. 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 (04-329B) concentration of pathogen DNA, plants displayed disease symptoms (Table 4-3). 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 4-3) 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 (Maas, 1998), this species has, to our knowledge, not been reported as a pathogen of celery and is not a primary pathogen of harvestable lettuce (Davis *et al*., 1997). *R. solani* was identified in three other soil samples and in all cases the corresponding crops displayed typical Rhizoctonia symptoms. DNA concentrations were found between 0.18 and 1.97 pg μl^{-1}. In all cases, BLAST analysis of the sequenced amplicons 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 multiple 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 (data not shown).

Based on the results shown in Fig. 4D, showing the relation between the calculated DNA concentration using real-time PCR analysis and the logarithmic number of *V. dahliae* microsclerotia, the number of microsclerotia in two samples (04-200A and 04-200B) of fields exhibiting 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 per gram of soil. However, as microsclerotia often get lost by sieving (Goud and Termorshuizen, 2003), it was anticipated that real-time PCR might detect more microsclerotia.

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 22.01 and 741.6 pg μl^{-1} 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 4.08 pg μl^{-1} (Table 4-3). In addition to this pathogen, *Pythium sylvaticum* was detected with the DNA array. 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 (data not shown).

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 4-4. 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) 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.

Table 4-4. Example of the amplification efficiency[a] of several DNA extracts containing genomic DNA from a specific pathogen and 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 ($n = 2$) ± standard errors.

4.4 Discussion

The results described in this chapter illustrate that pathogen DNA can be accurately quantified over a large concentration range using real-time PCR, irrespective of the presence of non-target DNA. 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.

In our as well as in other studies (Wang *et al*., 2003a; 2003b), specific PCR assays could be obtained by the use of a single species-specific primer combined with an overall fungus or universal primer (in this chapter demonstrated for *F. solani*, *P. ultimum* and *R. solani*). 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. In contrast, the primer set developed for *V. dahliae* did not display the desired specificity and cross-amplified DNA from its closest relatives, including *V. albo-atrum* and *V. tricorpus*. This lack of specificity can be explained by the high homology at the nucleotide level for these species. However, while for primer ST-VE1 a single mismatch occurs in the middle of the primer, greater specificity might be obtained when the mismatch is at the extreme 3' end of the primer. These three *Verticillium* species are related in that they all cause Verticillium wilt in tomato. Whereas both *V. albo-atrum* and *V. dahliae* are the well known causal agents of tomato vascular wilt (Pegg *et al*., 1981; Jones *et al*., 1997), *V. tricorpus* occasionally causes Verticillium wilt of tomato (Huisman, 1988). In addition, ITS1-F and ST-VE1 generated identical standard curves for these three species (data not shown). Therefore, this primer set can be used to detect and quantify the presence of all three *Verticillium* species capable of causing Verticillium wilt in tomato.

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 (Agrios, 2005). So far, 14 AGs have been described, of which AGs 3 and 4 are associated with tomato diseases (Sneh *et al.*, 1994). 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 (Agrios, 2005). 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* (12.18 pg μl^{-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.

In this chapter we described the use of real-time PCR to quantitatively assess the presence of different tomato pathogens in complex biological samples. Nevertheless, for broad applicability, the availability of a multiplex format for all major pathogens of a single crop is desirable. However, as discussed in Chapter 1, detection and quantification of more than a handful of different pathogens is not possible using this technology. In contrast, as shown in the previous chapter, DNA array technology can be used to detect an, in theory, unlimited number of pathogens in a single assay (Martin *et al*., 2000; Lévesque, 2001; Lievens *et al*., 2003; 2005b). Therefore, qualitative detection using a DNA array followed by accurate quantification using real-time PCR for the detected pathogens could be a solid basis for disease management decisions. Nevertheless, combining techniques makes routine analysis of samples laborious and inefficient. Therefore, the major challenge currently is the implementation of a reliable quantitative aspect to DNA arrays, making this technology even more appealing for plant pathogen diagnosis.

5 Quantitative assessment of phytopathogenic fungi and oomycetes in various substrates using a DNA macroarray*

5.1 Introduction

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, in addition to pathogen detection and identification, quantification of its presence is increasingly gaining interest.

As shown in the previous chapter, real-time PCR (Heid *et al.*, 1996) has been a powerful development, especially with regard to accurate detection and quantification of specific plant pathogens (Schaad and Frederick, 2002; McCartney *et al*., 2003; Schaad *et al*., 2003; Lievens *et al*., 2005b) as well as for monitoring pathogen infection (Brouwer *et al.*, 2003). However, currently, the total amount of PCR reactions in a single tube is severely limited, independent of the detection chemistry used. Simultaneous detection and quantification of multiple target organisms is not possible when using non-specific detection chemistries such as SYBR Green®. On the other hand, multiplex formats do exist for probe-derived detection chemistries. For example, Tooley and coworkers (2006) were able to simultaneously detect and quantify two *Phytophthora* species using real-time PCR technology. In an analogous way, Bertolini *et al.* (2003) developed an assay for the simultaneous detection of five microbial pathogens, including four RNA viruses and one bacterium. Nevertheless, detection of more than a handful of different pathogens at the same time is currently impossible because of the limited number of different fluorescent

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dyes available and the energizing light source used in real-time PCR instruments (Mackay *et al.*, 2002).

In Chapter 3, 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). However, a major limitation of the current technology is that reliable quantification of pathogen presence was not yet established. Hence, only qualitative detection can be conducted. To fully exploit the potential use of DNA arrays in plant pathology, implementation of a quantification aspect has to be pursued.

In this chapter, we describe, as a proof-of-principle, a new format of the previously designed DNA macroarray (Chapter 3; Lievens *et al.*, 2003), which has been further developed and optimized for accurate quantitative assessment of the economically important vascular wilt pathogens *Verticillium albo-atrum* and *V. 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 as well as oomycetes in artificially inoculated and naturally infested samples from diverse origin is demonstrated. In order to validate the quantitative results obtained using the DNA array, real-time PCR is used as a reference technique.

5.2 Materials and methods

5.2.1 Microorganisms and cultivation

The fungal isolates *Fusarium oxysporum* f. sp. *lycopersici* CBS 646.78, *F. solani* CBS 165.87, *V. albo-atrum* CBS 451.88 and *V. dahliae* CBS 381.66 and the oomycete isolates *Pythium ultimum* CBS 656.68 (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) and *P. aphanidermatum* ST 59.04 (Scientia Terrae Research Institute, Sint-Katelijne-Waver, Belgium) were cultured on PDA. *Saccharomyces cerevisiae* MUCL 28426 (Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium) and the bacterial strain *Rhizobium 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 22°C.

5.2.2 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 (fresh weight) of sandy field soil (Sint-Katelijne Waver, Belgium). 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 the 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 ml^{-1}. Zoospores were produced in 20 ml 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 (Ø 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 to 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; and 5 = plant death. At the same time, the remaining nutrient solution was collected and used for DNA extraction and DNA array analysis.

5.2.3 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. Samples were collected as described in the previous chapter. Subsamples were retained for DNA extraction. Separately, soil samples

were collected from two fields that were naturally infested with *V. dahliae*. The number of viable *V. dahliae* microsclerotia was determined using the wet sieving technique as described in Chapter 4 (Harris *et al.*, 1993). Subsamples of the soil were retained for DNA extraction and DNA array analysis. 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.

5.2.4 DNA extraction

Genomic DNA from all microorganisms was extracted as described in Chapter 2. For DNA isolation from soil and plant samples, genomic 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 as described by the manufacturer (Mo Bio Laboratories, Inc., 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, Inc., Solana Beach, CA, USA). DNA yield and purity were determined spectrophotometrically.

5.2.5 Selection of oligonucleotides and DNA array production

Part of the set of detector oligonucleotides used in this study (Table 5-1) was previously selected (Chapter 3; Lévesque *et al.*, 1998; Lievens *et al.*, 2003). In addition, oligonucleotides were developed to detect *F. solani* (Fso1), *P. aphanidermatum* (Pap1), 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 fungal and oomycete isolates (data not shown). In addition to the previously used control oligonucleotides (Uni1, Con1, and Dig1) oligonucleotides were designed to target exogenous control DNA from *S. cerevisiae* (Sce1) and the total amount of fungal DNA (Fun1). All oligonucleotides were synthesized with a 5'-C6-amino linker for covalent binding to nylon membrane and DNA arrays were produced as described in Chapter 2. For this study, the detector oligonucleotides Fun1, Fox2, Fso1, Val2, and Vda1 were initially spotted 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, and Sce1) or 2.0 (Dig1) fmol was printed.

Table 5-1. Detector oligonucleotides used for DNA array analysis

Code	Specificity	Sequence (5'-3')	Target
Fox2[a]	*Fusarium oxysporum*	GTTGGGACTCGCGTTAATTCG	ITS II
Fso1	*F. solani*	ATCAACCCTGTGAACATACCTAA	ITS I
Pap1	*Pythium aphanidermatum*	TTGGAGTATAGATCAGTATTAGGTAAA	ITS I
Pul1[b]	*P. ultimum*	TGCTGACTCCCGTTCCAGTG	ITS I
Rso1	*Rhizoctonia solani*	GCCTGTTTGAGTATCATGAAAT	ITS II
Val2[a]	*Verticillium albo-atrum*	CATCAGTCTCTTTATTCATACCAA	ITS I
Vda1[a]	*V. dahliae*	AACAGAGAGACTGATGGACCG	ITS I
Fun1[c]	Fungi	GCTGCGTTCTTCATCGATGC	5.8S rDNA
Sce1[d]	*Saccharomyces cerevisiae*	GTGTTTTGGATGGTGGTAAGAA	*erg11* gene
Uni1[a]	Eukaryotes	TCCTCCGCTTATTGATATGC	28S rDNA
Dig1[a,e,f]	None	GTCCAGACAGGATCAGGATTG	-
Con1[a,e]	None	GTCCAGACAGGATCAGGATTG	-

[a] Chapter 3; Lievens *et al.* (2003).

[b] Lévesque *et al*. (1998).

[c] ITS2 primer (White *et al.*, 1990).

[d] Reverse complement of detector oligonucleotide pSce (Posteraro *et al.*, 2000).

[e] Chapter 2.

[f] 3'-end digoxigenin-labeled.

5.2.6 PCR amplification, labeling and hybridization

In order to determine the optimal number of PCR cycles that permits end-point quantification, the target 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, Inc., Palo Alto, CA, USA), 0.15 mM dNTP mix, 0.5 µM of each primer, and 1 µl genomic DNA. Prior to amplification, samples were preheated 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, Inc., Upland, CA, USA).

For DNA array analysis, the target ITS region was amplified and simultaneously labeled with alkaline-labile digoxigenin (Roche Diagnostics GmbH, Mannheim, Germany) using the primers ITS1-F and ITS4 (Gardes and Bruns, 1993) or OOMUP18Sc and ITS4 (Lievens *et al.*, 2004), for fungi or oomycetes, respectively, according to the same PCR protocol described above. As a control for PCR efficiency between different samples, 100 pg exogenous control DNA from *S. cerevisiae* was added to each sample, amplified and simultaneously labeled in separate PCR reactions using primers $P450_1$ and $P450_2$ (Morace *et al.*, 1997). Ten µl of both target and control DNA amplification reactions were combined

and simultaneously hybridized in 6 ml of hybridization buffer as described in Chapter 2. All hybridizations were performed at least twice.

5.2.7 Real-time PCR

To define the range of relevant DNA concentrations by which fungal or oomycete pathogens occur in naturally infested greenhouse soils and to verify the robustness and accuracy of the quantitative DNA array-based assay, the amount of pathogen 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 that were detected in these samples using an extended version of the DNA array designed previously (Chapter 3; Lievens *et al*., 2003), by which over 40 different fungal and oomycete plant pathogens can be simultaneously detected. Real-time PCR amplification reactions were conducted using SYBR® Green I technology on a Lightcycler™ instrument (Roche Diagnostics Corp., Indianapolis, IN, USA) as described in Chapter 4. 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 5-2, to generate amplicons smaller than 300 bp. The design and robustness of some of these assays have been shown in the previous chapter. 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 5-2), 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 (T_{ann}) indicated in Table 5-2, and elongation at 72°C for the time period (t_{el}) indicated in Table 5-2. Fluorescence was detected at the end of the elongation phase for each cycle. To confirm amplification specificity, a melting curve temperature profile was obtained as described previously (Chapter 4). For each pathogen, standard curves were generated by plotting the threshold cycle (C_T) of a 10-fold dilution series of genomic DNA against the logarithm of the DNA concentration. The regression line was used to calculate the respective pathogen DNA concentration in the studied sample via its C_T- value (Chapter 4; Brouwer *et al*., 2003).

Table 5-2. Primers used for real-time PCR

Code	Specificity	Sequence (5'-3')	Target	T_m [a]	t_{el} [b]
AFP308[c]	*Fusarium oxysporum*	CGAATTAACGCGAGTCCCAAC	ITS II	60	9
AFP346[c,d]	*F. solani*	GGTATGTTCACAGGGTTGATG	ITS I	60	6
AFP356[c]	*Pythium sylvaticum*	CAATGCAAAGTCAGCAGTGC	ITS I	60	9
AFP276[d,e]	*P. ultimum*	TGTATGGAGACGCTGCATT	ITS II	58	8
ST-RS1[d,e]	*Rhizoctonia solani*	AGTGTTATGCTTGGTTCCACT	ITS II	60	8
AFP307[c]	*Verticillium dahliae*	CAGAGAGACTGATGGACCG	ITS I	60	9
ITS2-O[c]	Oomycetes	GCAGCGTTCTTCATCGATGT	5.8S rDNA	60	12
ITS1-F[e,f]	Fungi	CTTGGTCATTTAGAGGAAGTAA	18S rDNA	x[g]	x
OOMUP18Sc[e,h]	Oomycetes	TGCGGAAGGATCATTACCACAC	18S rDNA	x	x
ITS4[c,i]	Universal	TCCTCCGCTTATTGATATGC	28S rDNA	x	x

[a] Annealing temperature (°C).

[b] Elongation time (s).

[c] Reverse primer.

[d] Chapter 4.

[e] Forward primer.

[f] Gardes and Bruns (1993).

[g] x, depends on second primer used.

[h] Lievens *et al.* (2004).

[i] White *et al.* (1990).

5.3 Results

5.3.1 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 in the outcome of the assay. This bias in template-to-product ratio can be caused by two technical artifacts, 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 100 pg of exogenous control DNA derived from *S. cerevisiae* was added to each sample and amplified in a separate PCR reaction. In Table 5-3, PCR efficiencies are shown for DNA extracted from several soil samples that were obtained from commercial vegetable growers. PCR efficiencies appeared fairly stable for all conditions tested. For all other experiments, PCR efficiencies between analyzed samples were highly comparable.

Table 5-3. Comparison of PCR efficiencies[a] between different soil samples

Sample ID	PCR efficiency[a]
03-111	0.85 ± 0.13
03-142	0.85 ± 0.10
03-176	1.01 ± 0.12
03-193	0.91 ± 0.06
03-224	0.94 ± 0.15
03-307	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 *Saccharomyces cerevisiae* (Sce1) and the digoxigenin-labeled reference control (Dig1). Values are means ± standard errors (n = 4 from two independent analyses).

In addition to spiking DNA samples with exogenous control DNA, PCR reaction parameters were adjusted to ensure hybridization with amplicons 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 25, 30, 35, or 40 cycles. PCR products were quantified following gel electrophoresis by comparison to standard DNA, showing that up to at least 30 cycles, most PCR reactions remained in the exponential phase (Table 5-4). In addition, genomic DNA isolated from naturally infested soils and infected plants was amplified under the same conditions, essentially showing similar results (Table 5-4).

Table 5-4. 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
Verticillium 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[b]	03-311	0.275	12.5	47.3	105.7	166.4
	03-324	1.275	20.9	71.6	67.6	45.4
Plant[b]	03-312	0.15	19.1	48.9	127.3	178.6
	P58	0.875	21.9	86.3	216.6	176.9

[a] PCR products were quantified after gel electrophoresis by comparison to a DNA ladder.

[b] Prior to PCR amplification, DNA was diluted 10-fold to avoid inhibitory concentrations of potential PCR inhibitors. Template DNA amounts are those of the diluted samples.

A comparable experiment was performed for other fungal pathogens, including the tomato pathogens *F. oxysporum* f. sp. *lycopersici* and *F. 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.

5.3.2 Quantification of DNA dilutions using a DNA array

One potential problem that can hamper quantification using DNA arrays is inter-spot variability caused by printing errors or spatial effects. To test this, 8.0 fmol of detector oligonucleotide Vda1 (Table 5-1) was spotted in duplicate at 6 different locations on a membrane and labeled *V. dahliae* amplicon (10 ng ml^{-1} hybridization buffer) was hybridized to the membrane. The signals obtained showed an average relative integrated optical density (rIOD) of 112.4 with a standard error of 4.6, demonstrating that the inter-spot 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 theory, 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. 5-1A). For all spotted amounts, a linear logarithmic relationship between the rIOD 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 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 prior to DNA amplification did not influence the hybridization results (data not shown). A similar experiment was conducted

for *V. albo-atrum* (Fig. 5-1B) 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.

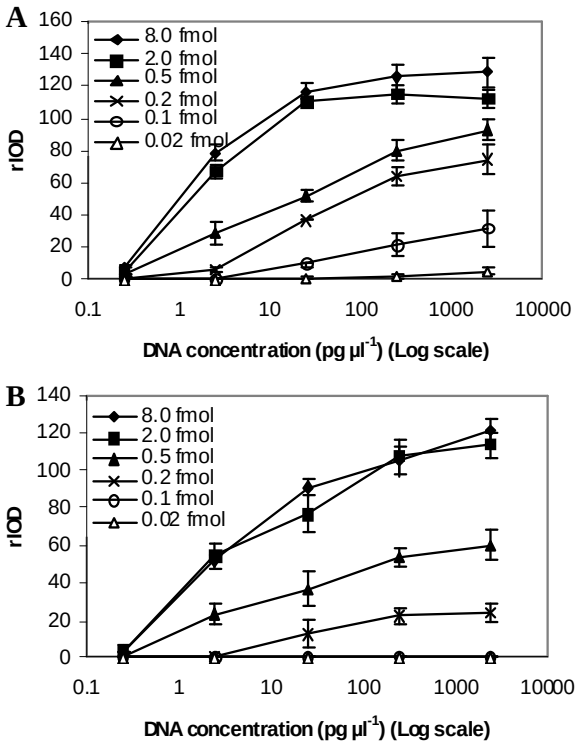


Fig. 5-1. Quantification of a dilution series of **A,** *Verticillium dahliae* and **B,** *V. albo-atrum* genomic DNA after PCR using different amounts of detector oligonucleotides on a DNA macroarray. Detector oligonucleotides were spotted at several amounts ranging from 0.02 to 8.0 fmol. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD) and plotted against the logarithmic DNA concentration. Data represent means 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.

5.3.3 Direct quantification of fungal and oomycete 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, ten soil samples obtained from commercial vegetable growers at different periods during the growing season were assessed for pathogen occurrence using a DNA array by which more

than 40 different plant pathogenic fungi and oomycetes can be detected. Subsequently, for all pathogens detected, the amount of genomic DNA was quantified using real-time PCR (Table 5-5). In addition to *V. dahliae*, pathogens detected by the DNA array included *F. solani*, *P. sylvaticum*, *P. ultimum*, and *R. 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 500 pg μl^{-1} genomic DNA after 10-fold dilution 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 5-5).

Table 5-5. Quantification of fungal and oomycete genomic DNA[a] (pg μl^{-1}) in soil samples using real-time PCR

Sample ID	Sampling date (dd/mm/yy)	Template DNA[b] (ng μl^{-1})	Fungi	Oomycetes	*Fusarium oxysporum*	*F. solani*	*Pythium sylvaticum*	*P. ultimum*	*Rhizoctonia solani*	*Verticillium dahliae*
03-111	16/01/03	2.75	8.02	0.88	0.19	0.02	x[c]	x	0.18	x
03-115	22/01/03	6.25	6.69	4.58	x	x	x	x	x	x
03-142	14/02/03	4.00	299.80	11.42	x	x	x	x	x	x
03-176	18/03/03	5.25	29.03	7.20	1.19	x	x	x	x	x
03-193	03/04/03	0.50	13.07	5.70	0.20	x	x	x	x	x
03-224	29/04/03	12.75	10.49	304.50	0.41	0.02	1.90	12.18	1.50	x
03-226	02/05/03	2.00	7.93	3.99	x	x	x	x	x	x
03-307	30/07/03	10.25	317.80	50.93	4.13	0.34	3.68	x	x	x
03-337	02/09/03	3.00	22.80	2.71	6.30	x	x	x	x	x
04-200	30/03/04	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] Prior to PCR amplification, DNA was diluted 10-fold to avoid inhibitory concentrations of potential PCR inhibitors. Calculated DNA concentrations are those in the diluted DNA samples.

[b] DNA concentration in the undiluted DNA extract; DNA concentration was determined spectrophotometrically at 260 nm.

[c] x, absent according to a DNA macroarray analysis (Lievens *et al*., 2003), by which over 40 different plant pathogenic fungi and oomycetes can be detected.

5.3.4 Influence of non-target DNA on target quantification using a DNA array

Because the ultimate goal of this work was to quantify pathogen presence in DNA extracts from complex biological samples using a DNA array, 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 (*R. 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 analyzed. Fig. 5-2 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 non-target DNA, in all cases as little as 0.25 pg target DNA can clearly be detected (Fig. 5-2).

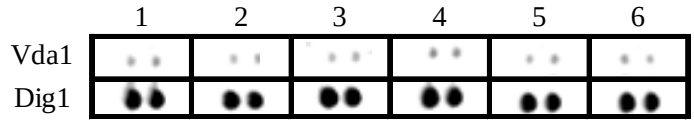


Fig. 5-2. Influence of non-target DNA on hybridization signals. Signals after hybridization of amplicons resulting from amplification of 0.25 pg *Verticillium dahliae* and 250 pg non-target DNA to the oligonucleotide detectors Vda1, to detect *V. dahliae*, and Dig1, for calibration, spotted in duplicate. Non-target DNA isolated from a bacterial (*Rhizobium vitis*; 1), oomycete (*Pythium ultimum*; 2), or fungal (*Fusarium 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.

Non-target fungal DNA affected hybridization results when present at certain ratios (Table 5-6), whereas signal intensities were not influenced by bacterial-, oomycete-, plant- or soil-derived DNA and as little as 0.25 pg 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 5-5), results in an underestimation of target DNA. A non-discriminative fungal detector oligonucleotide (Fun1; Table 5-1), 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 5-6).

Table 5-6. Influence of non-target fungal (*Fusarium solani*) DNA on target DNA quantification

	Hybridization signals for different target DNA amounts of								
Fusarium solani	0.25 pg *Verticillium albo-atrum*			2.5 pg *V. albo-atrum*			25 pg *V. albo-atrum*		
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 *Fusarium 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 *Verticillium 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-labeled reference control (Dig1). Values are means ± standard errors (n = 4 from two independent analyses).

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

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 Figs. 5-3A and B, respectively, demonstrating that quantitative detection of the pathogen 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. 5-3B, 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 was estimated at 13 and 9 microsclerotia per gram soil. This corresponds very well to the real-time PCR analysis performed on these samples (Chapter 4) by which the number of microsclerotia was estimated at 13 and 8 per gram soil, respectively. With the classical wet sieving technique in both soils the number of recovered microsclerotia was established at 7 microsclerotia per gram soil. However, since often a portion of the microsclerotia gets lost by sieving (Goud and Termorshuizen, 2003), one can expect to find more microsclerotia using the DNA array than by using the classical method.

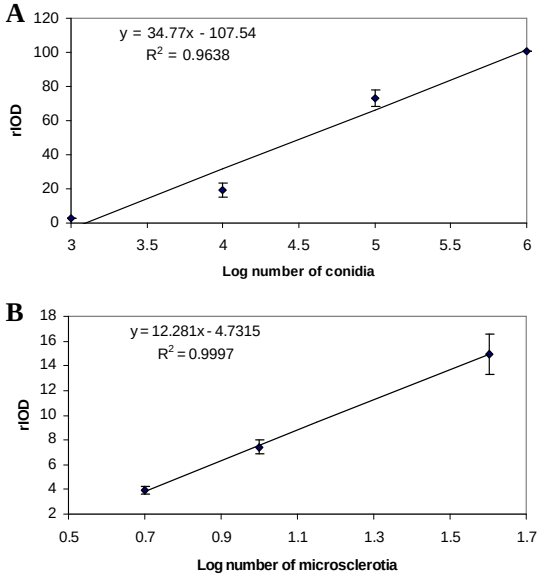


Fig. 5-3. Quantitative assessment of *Verticillium dahliae* presence in artificially infested soil samples. **A,** Regression line for DNA array analysis of a 10-fold dilution series of *V. dahliae* conidia added to 0.75 g (fresh weight) soil. **B,** Regression line for DNA array analysis of a series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

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 *P. aphanidermatum* was chosen as the target organism and a detector oligonucleotide (Pap1; Table 5-1) was spotted at 8.0 fmol. Tomato seedlings grown for 10 days in nutrient solution containing specific concentrations (10^2 to 10^4 zoospores ml^{-1}) of *P. aphanidermatum* zoospores were rated for foot and root rot severity (Fig. 5-4B). 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. 5-4A). The results of this experiment showed a strong correlation between the hybridization signal intensity, the initial amount of zoospores, and disease severity (Fig. 5-4), demonstrating the feasibility of the technique to monitor plant health based on pathogen densities and to quantitatively detect a different pathogen.

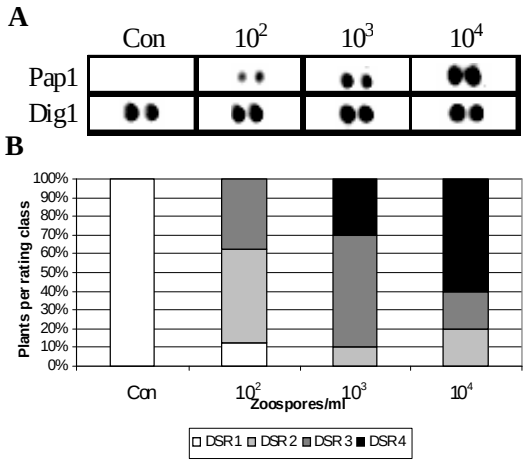


Fig. 5-4. Quantitative assessment of *Pythium aphanidermatum* in artificially infested water-based samples. **A,** DNA array analysis 10 days after inoculation of nutrient solution with 0 (Con), 10^2, 10^3 or 10^4 *P. aphanidermatum* zoospores ml^{-1}. Signals after hybridization to the detector oligonucleotides to detect *P. aphanidermatum* (Pap1) and the digoxigenin-labeled 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 to 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; and 5 = plant death. The experiment was repeated twice with similar results.

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 done for four different pathogens previously detected in these samples, 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 5-1) 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. 5-5), thus demonstrating the robustness and breadth of the developed quantitative DNA array-based assay.

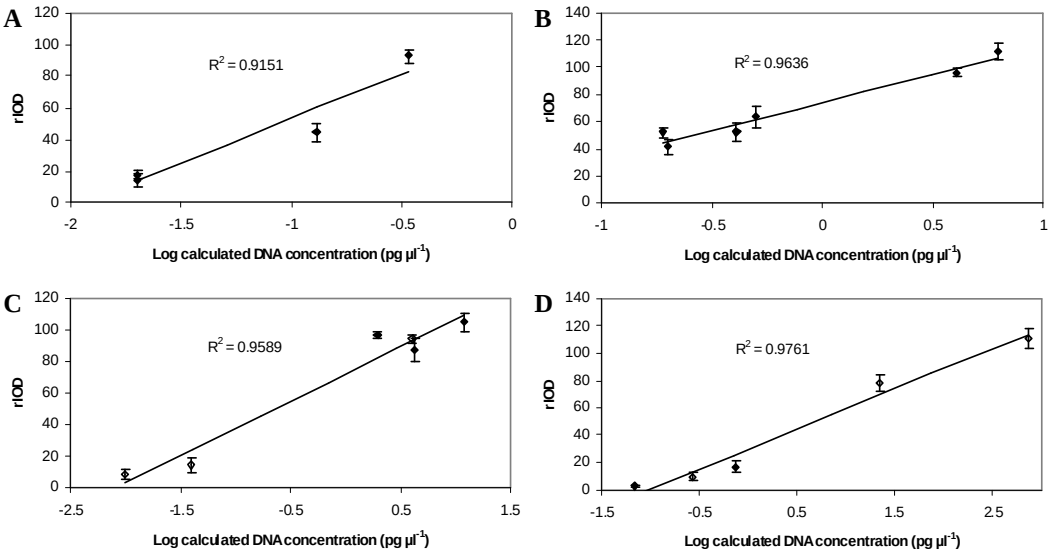


Fig. 5-5. Quantitative assessment of microbial presence in naturally contaminated environmental samples for **A,** *Fusarium solani*, **B,** *F. oxysporum*, **C,** *Pythium ultimum*, and **D,** *Rhizoctonia solani*. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD) and plotted against 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. Symbols: ♦ = soil sample; ◊ = plant sample.

5.4 Discussion

DNA array technology has the potential to detect multiple microorganisms in a single assay from diverse environments (Chapter 3; Martin *et al*., 2000; Lévesque, 2001; Lievens *et al*., 2003; 2005b; Lievens and Thomma, 2005). 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 chapter we show 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 artifacts, 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). Analogous to the previous chapter, 100 pg of exogenous control DNA (derived from *S. cerevisiae*), 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 analyzed was highly comparable, regardless of the sample matrix from which DNA was isolated. Obviously, the quality of the DNA to be amplified is critical (Lopez *et al.*, 2003; McCartney *et al.*, 2003). Therefore, these results also suggest that high-quality purified DNA was obtained 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.

Based on the results presented in this chapter, 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 x 10^{12} molecules, was immobilized per spot, 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 3 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 Chapter 3, we have shown that the detection limit of the DNA array 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 be detected in the presence of a large excess of fungal non-target DNA, with a dynamic range of 1000. When the amount of fungal non-target DNA exceeded the target DNA around 1000-fold, the amount of target DNA was underestimated. Experiments with other fungal or oomycete detector oligonucleotides show that the interference caused by fungal or oomycete non-target DNA, respectively, is a general phenomenon. To check for possible 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. Obviously, the sensitivity and the dynamic range of the developed method is, among other factors, strongly dependent on the PCR step, the amount of immobilized oligonucleotide and the detection system used. In this chapter, the high sensitivity of the technique as well as the broad dynamic range that can be obtained is demonstrated, which compare favorably with those of other multiplex diagnostic systems (Bodrossy *et al*., 2003; Denef *et al*., 2003; Castiglioni *et al*., 2004; Szemes *et al*., 2005).

In conclusion, based on the results described in this chapter, the power of DNA array technology for quantitative assessment of the presence of multiple pathogens in various biological matrices is shown. While accounting for specific criteria, pathogen DNA could be accurately quantified in concentration ranges typically encountered in horticultural practice by using a single PCR and DNA array hybridization. These criteria include optimizing of PCR conditions, inclusion of the proper controls, and spotting the appropriate amounts of detector oligonucleotides. Whereas the first two are used to monitor potential bias in

template-to-product ratio, the latter is important when it comes to accurate quantification of the hybridization signals. However, to fully aid plant disease management, additional effort is necessary in order to correctly interpret the obtained hybridization signals. Therefore, the next challenge will be to correlate hybridization patterns and hybridization strength to disease threshold levels and disease development.

6 Assessing populations of a disease suppressive microorganism and plant pathogen using a DNA macroarray*

6.1 Introduction

Largely driven by concerns about the detrimental effects of the use of synthetic chemicals on the environment and on public health, IPM has become the imposed strategy for managing plant diseases over the last few decades (Jarvis, 1992; Shea *et al*., 2000). However, IPM has been severely limited by the lack of fast, accurate, and reliable means by which plant pathogens can be timely detected, identified, and accurately quantified. Accurate quantification is particularly important since it serves as the basis for establishing population thresholds whereby a pathogen causes disease and at which point measures may be employed to effectively limit or prevent losses.

Beneficial microorganisms are used in IPM programs to increase plant health and yield by limiting or minimizing disease severity and incidence. These disease suppressive microorganisms (DSMs) may be indigenous to the soil or, alternatively, introduced into the rhizosphere. In general, DSM-mediated disease suppression can only be achieved when the pathogen is present under a certain threshold level, and the DSM is active and above a certain threshold level (Paulitz, 2000). Therefore, as with plant pathogens, accurate assessment (including both detection and quantification) of beneficial microorganisms is important when DSMs are being considered in IPM programs.

Currently, DNA array technology is the most suitable technique to detect several target organisms simultaneously (Chapter 3; Martin *et al*., 2000; Lévesque, 2001; Lievens *et al*.,

* Results described in this chapter will be published in "Assessing populations of a disease suppressive microorganism and plant pathogen using DNA arrays"; Lievens, B., Claes, L., Vanachter, A. C. R. C., Krause, M. S., Cammue, B. P. A., and Thomma. B. P. H. J. Plant Disease. In press.

2003; 2005b; Lievens and Thomma, 2005). This can be conducted in such a manner that reliable detection and quantification of multiple microorganisms in one assay is possible (Chapter 5; Lievens *et al*., 2005a). This technology has been successfully applied for diagnostics of human, animal, and plant pathogens (Lievens and Thomma, 2005). However, the possibilities of using DNA arrays for simultaneously assessing the populations of both pathogens and specific DSMs and relating such data to disease severity and disease incidence have not been reported to date.

In this chapter, we describe the use of DNA macroarrays to simultaneously measure population densities of a specific DSM and a fungal pathogen and relate their presence to disease development. Overall, the work described in this chapter describes a technical advance with potential applications for studying population dynamics and ecology of target populations in complex media such as potting mixes or soils. Since no biocontrol assay was available for the previously used model crop (tomato), the well established interaction between the biocontrol agent *Trichoderma hamatum* isolate 382 and the pathogen *Rhizoctonia solani* in a standard damping-off of radish bioassay (Kwok *et al*., 1987) was selected as a model for this study. As in the previous chapter, real-time PCR was used as a reference technique to validate the quantitative results obtained using the DNA array.

6.2 Materials and methods

6.2.1 Selection of oligonucleotides and DNA array production

A number of oligonucleotides used in this study (Table 6-1) was selected in the previous chapters, including the *R. solani* detector (Rso1) and the control oligonucleotides (Fun1, Sce1, Dig1, and Con1) (Lievens *et al*., 2003; 2005a). In addition, oligonucleotides to detect the genus *Trichoderma* (Tgn1) and the isolate T_{382} (Tha382) were designed as described in Chapter 3 (Lievens *et al*., 2003). Whereas the first oligonucleotide is based on an ITS sequence, the latter is derived from the RAPD marker SCE16 (Abbasi *et al*., 1999).

Specificity of the oligonucleotides selected was checked by BLAST analysis and cross-hybridization testing with over 225 related and non-related fungal and oomycete isolates of which the most relevant are listed in Table 6-2. The quantitative character of the oligonucleotides selected was verified as described previously (Chapter 5; Lievens *et al*.,

2005a). To conduct these tests, DNA extraction from reference cultures, PCR amplification, labeling, and hybridization were performed as described in Chapters 2 and 5. In addition, PCR reaction parameters were adjusted to ensure hybridization with amplicons from the exponential phase of the PCR reaction.

Table 6-1. Detector oligonucleotides used for DNA array analysis

Code	Specificity	Sequence (5'-3')	Target
Rsol[a]	*Rhizoctonia solani*	GCCTGTTTGAGTATCATGAAAT	ITS II
Tgn1	*Trichoderma* sp.	GTCATTTCAACCCTCGAACCC	ITS II
Tha382	*T. hamatum* isolate 382	ATTCACGACATATGATCTAATC	SCE16[b]
Fun1[a]	Fungi	GCTGCGTTCTTCATCGATGC	5.8S rDNA
Sce1[a]	*Saccharomyces cerevisiae*	GTGTTTTGGATGGTGGTAAGAA	*erg11* gene
Dig1[a,c,d]	None	GTCCAGACAGGATCAGGATTG	-
Con1[a,c]	None	GTCCAGACAGGATCAGGATTG	-

[a] Chapter 5; Lievens *et al*. (2005a).

[b] Abbasi *et al.* (1999).

[c] Chapters 2 and 3; Lievens *et al*. (2003).

[d] 3'-end digoxigenin-labeled.

Table 6-2. *Rhizoctonia* and *Trichoderma* isolates used in this study

Species	Isolate[a]
Rhizoctonia oryzae	CBS 273.38, CBS 474.82
R. oryzae-sativae	CBS 235.91
R. solani	19 (AG-4), CBS 101590 (AG-4), CBS 101761, CBS 323.84, MUCL 9418, ST 44.02, ST 50.03
Trichoderma aggressivum f. *aggressivum*	CPK 361, CPK 365
T. aggressivum f. *europeum*	CPK 366, CPK 375
T. asperellum	CPK 247,CPK 358, CPK 654, CPK 655, MUCL 41923, MUCL 41924, MUCL 41925, MUCL 41926, MUCL 41927, MUCL 41928
T. atroviride	CPK 369
T. hamatum	T_{382}, CPK 253, CPK 301, CPK 308, CPK 309, CPK 310, CPK 311, CPK 313, CPK 314, CPK 316, CPK 328, CPK 357
T. harzianum	CPK 51, CPK 206, CPK 211, CPK 217, CPK 221, CPK 261, CPK 271, CPK 274, MUCL 19412, MUCL 28446
T. inhamatum	CPK 239
T longibrachiatum	CPK 41D, CPK 47D, CPK 57D, CPK 59D
T. pubescens	CPK 489
T. virens	CPK 389, CPK 396, CPK 400, CPK 432, CPK 521
T. viride	CPK 421, CPK 525

[a] CBS: Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CPK: collection of C. P. Kubicek, Vienna University of Technology, Vienna, Austria; MUCL: Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; ST: collection of Scientia Terrae Research Institute, Sint-Katelijne-Waver, Belgium; AG, anastomosis group.

Oligonucleotides were synthesized with a 5' NH_2 group and a C6 linker for covalent membrane binding. DNA macroarrays were produced as described in Chapter 2. For all oligonucleotides, except the control oligonucleotides Dig1 and Fun1, 8.0 fmol was spotted on the membrane. The oligonucleotides Dig1 and Fun1 were spotted at 2.0 fmol and at different amounts including 8.0, 0.2, 0.1, and 0.02 fmol, respectively.

6.2.2 Potting mixes

Two types of potting mixes that differ in disease suppressive potential were used. The first type, "dark Sphagnum peat" (DSP) mix, is a substrate that, because of its highly decomposed organic matter and subsequent low microbial carrying capacity, typically provides little or no support to DSMs that induce suppression to damping-off diseases (Boehm and Hoitink, 1992; Boehm *et al*., 1993; Boehm *et al*., 1997; Krause *et al*., 2001). This potting mix was formulated by blending dark Sphagnum peat (H_3–H_4 on the von Post peat decomposition scale (Puustjärvi and Robertson, 1975); Bas van Buuren, Maasland, The Netherlands) with medium horticultural grade perlite (7:3, vol/vol), and 1.1 g superphosphate and 1.0 g K_2SO_4 added per liter of mix. The second type of potting mix used was a "composted pine bark" (CPB) mix that, due to its low but stable decomposition level and consequent higher microbial carrying capacity, is able to support the disease suppressive activities of DSMs such as T_{382} against Rhizoctonia damping-off diseases (Krause *et al*., 2001). This mix was prepared with the same batch of dark peat used in the previous mix, composted pine bark (DCM Corp., Grobbendonk, Belgium), medium horticultural grade vermiculite, and medium horticultural grade perlite at ratios of 45:30:15:10 (vol/vol). Agricultural-grade $CaCO_3$ and $Ca(NO_3)_2$ (<100 mesh) were added at a ratio of 4:1 (wt/wt) to adjust potting mixes to pH 5.8–6.0. In addition, tap water was added to potting mixes to adjust moisture content to 35–40% of the water-holding capacity of each mix.

Portions of each potting mix were subjected to either a 25°C or a 60°C ("heated") initial incubation treatment for 5 days to promote the colonization by or diminish levels of mesophilic microflora in the mixes, respectively (Kwok *et al*. 1987). T_{382}-fortified potting mix treatments were prepared by thoroughly blending a dry microgranular preparation of T_{382} (Sylvan Bioproducts, Inc., Cabot, PA, USA) with these potting mixes to achieve an initial density of 2 x 10^7 CFU per liter potting mix, which is a recommended rate to achieve disease suppression (Krause *et al*., 2001). Portions of each potting mix not blended with T_{382} served as non-fortified

("natural") control treatments. Thereafter, all potting mixes were incubated at 25°C for 7 days to allow T_{382} to colonize fortified mixes. Immediately before planting, slow release fertilizer (8–5–7, 1:1 Ecomix 1-Ecomix 4 blend, DCM Corp., Grobbendonk, Belgium) was incorporated into all mixes at a rate of 2.5 g per liter (fresh material).

6.2.3 Rhizoctonia damping-off of radish bioassay

Experiments were conducted using the Rhizoctonia damping-off of radish (*Raphanus sativus* L. cv. 'Early Scarlet Globe') bioassay developed by Kwok *et al*. (1987) to (i) identify the relationships between hybridization signal intensity and Rhizoctonia damping-off severity as well as incidence of severe disease, and (ii) evaluate the application of the DNA array for studying the interactions between a DSM and a pathogen in a disease suppressive system.

Potato soil inoculum of *R. solani* isolate 19, belonging to anastomosis group 4 (AG-4) and originally isolated from poinsettia (The Ohio State University, OH, USA), was prepared as described by Ko and Hora (1971). Air-dried inoculum was ground using a mortar and pestle and was sieved to retain 1–2-mm-sized particles (Nelson and Hoitink, 1982; Kwok *et al*., 1987; Krause *et al*., 2001). In the first experiment, this inoculum was mixed at fertilizer incorporation at rates of 0.125, 0.25, 0.375, 0.5, and 0.625 g per liter potting mix. In the second experiment, potting mixes were inoculated with 0.5 g of this inoculum per liter mix. In both experiments, non-infested control mixes did not received *R. solani* inoculum. Using a vacuum seeder, 32 radish seeds (85% germination, Shaffer Seeds, Akron, OH, USA) were deposited evenly across the surface of 10-cm-diameter polystyrene foam pots containing approximately 400 ml of potting mix. Seeds were covered with approximately 1 cm of potting mix and pots were irrigated initially until mix saturation. As a fungicide control treatment, tolclofos-methyl (0.03 mg a.i. ml^{-1} water) was applied as a drench to pathogen-infested non-fortified potting mixes (125 ml of solution per pot). Pots were incubated in a growth chamber at 24°C under continuous illumination (225 µE m^{-2} s^{-1}) and were irrigated as needed. All bioassays were configured according to a randomized complete block design with five pots (replicates) per treatment and were conducted twice with similar results.

Damping-off severity was determined 7 days after incubation based on a damping-off severity rating scale in which: 1 = symptomless; 2 = small root or stem lesion; 3 = large root or stem lesion; 4 = post-emergence damping-off; and 5 = pre-emergence damping-off. Incidence

of severely diseased seedlings (i.e. proportion of plants with a disease severity rating >2) was also evaluated using this data as described previously (Krause *et al*., 2001). Immediately after rating, for each of the five replicate pots in each treatment, five samples of mix were taken by inserting a 1.5-cm-diameter cork borer completely through the depth of the pot. Subsequently, samples were combined, homogenized and used for DNA extraction and plating on semi-selective medium. Additionally, the causal agent of the disease was verified by recovery of the pathogen from surface disinfected seedlings on a *Rhizoctonia* semi-selective medium (1.5 % water agar amended with 250 ppm chloramphenicol and 250 ppm metalaxyl) followed by microscopic examination.

6.2.4 DNA extraction, amplification, labeling and hybridization

Genomic DNA was extracted from 0.5 g (fresh weight) potting mix using the UltraClean Soil DNA Isolation Kit according to the manufacturer's specifications (Mo Bio Laboratories, Inc., Solana Beach, CA, USA) and subsequently diluted 10-fold. Fungal rDNA spanning the target ITS region was amplified using the primer set ITS1-F and ITS4 (Gardes and Bruns, 1993) and was simultaneously labeled with alkaline-labile digoxigenin (Roche Diagnostics GmbH, Mannheim, Germany). To specifically detect T_{382}, amplification and labeling was performed using primers OPE16-F and OPE16-R (Abbasi *et al*., 1999). PCR amplification and labeling was performed as described in Chapter 5, using 30 PCR cycles to ensure accurate end-point quantification (Chapter 5; Lievens *et al*., 2005a). In order to check for differences in PCR efficiency, a separate PCR reaction was run for each sample in which 100 pg of exogenous control DNA from *Saccharomyces cerevisiae* was added to 1 µl target DNA (Chapter 5; Lievens *et al*., 2005a). Amplification and simultaneous labeling was done using primers $P450_1$ and $P450_2$ (Morace *et al*., 1997). Labeled amplicons were subsequently combined and used for DNA array hybridization as previously described (Chapters 2 and 5). All assays were conducted at least twice.

6.2.5 Plating and real-time PCR as reference techniques

In order to validate DNA array analysis, both culture-dependent classical plating, and culture-independent real-time PCR methods were used. For classical enumeration of *Trichoderma*

propagules, a series of 10-fold dilutions of 10 g (fresh weight) potting mix was made, followed by spreading 100 µl aliquots of each dilution in triplicate on a *Trichoderma* selective medium (Chung and Hoitink, 1990). *Trichoderma* colonies were counted after five days of incubation at 25°C in darkness. Since dilution plating cannot be used to adequately recover and quantify discrete propagules of *R. solani*, 50 randomly picked clumps of the potting mix were directly plated on *Rhizoctonia* selective medium (Ko and Hora, 1971; Henis *et al*., 1978). Plates were subsequently incubated at 25°C in darkness and were checked daily for *R. solani* growth.

In parallel, real-time PCR amplifications were performed in a total volume of 25 µl using the intercalating dye SYBR® Green I on a SmartCyclerII® instrument (Cepheid, Sunnyvale, CA, USA). Each reaction mixture contained 2 µl of the target DNA extract, 12.5 µl of the QuantiTect™ SYBR® Green PCR Master Mix (Qiagen, Inc., Valencia, CA, USA), 0.625 µl of each primer (20 µM), and 9.25 µl sterile distilled water. The forward primers ST-RS1 (Chapters 4 and 5; Lievens *et al*., 2005a) and ST-Tgn1 (5'-TTCAACCCTCGAACCCCTC) were combined with the universal reverse primer ITS4 (White *et al*., 1990) to detect and quantify rDNA from *R. solani* and *Trichoderma* species, respectively. Thermal cycling conditions consisted of 10 min at 95°C followed by 45 amplification cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C with a final 2-min elongation step at 72°C. Fluorescence was detected at the end of the elongation phase of each cycle. To evaluate amplification specificity, melt curve analysis was performed at the end of the PCR run as described in Chapter 4. Standard curves were generated by plotting the threshold cycle (C_T) of a 10-fold dilution series of standard DNA against the logarithm of the concentration. The regression line was used to calculate the DNA concentration of *R. solani* and *Trichoderma* in the analyzed samples via their C_T-values (Chapter 4; Brouwer *et al*., 2003).

6.2.6 Statistical analyses

Analysis of variance (ANOVA) was used to determine the effects of potting mix treatment on damping-off severity and on the hybridization signals obtained. Two aspects of damping-off severity were analyzed as response variables, including mean disease severity and mean incidence of severe disease (Krause *et al*., 2001). The first response variable, disease severity rating for each replication (R), was transformed to $R^* = (R^{1.5}-1)/1.5$ to obtain a linear scale and an approximately normal distribution with constant variance. The second response variable,

defined as the proportion of seedlings in each replication with damping-off severity rating >2 (y), was transformed to $y^* = \arcsin(\sqrt{y})$ to obtain a constant variance. To determine the effects of potting mix treatment on the hybridization signals obtained, data were not transformed. Least significant differences (LSD) at $P = 0.05$ were calculated to compare means. Minitab (Release 13, Minitab Inc., State College, PA, USA) was used to conduct ANOVA analyses.

6.3 Results

6.3.1 Quantification of DNA dilutions using a DNA array

Prerequisite to any study related to assessing microbial populations and monitoring their dynamics are accurate detection, identification, and reliable quantification of the microorganisms of interest. For detection of *R. solani*, the previously selected ITS-based oligonucleotide Rso1 (Chapter 5; Lievens *et al*., 2005a) was used. *Trichoderma* species are present in virtually all soils and may act as indigenous buffers against a wide range of plant diseases. Therefore, Tgn1, a genus-specific oligonucleotide, was developed for this genus, covering many species that have been reported to suppress various plant diseases. However, since disease suppression ability may differ among communities, species and isolates of *Trichoderma*, specific detection of T_{382}, a well-known and documented DSM (Trillas-Gay *et al*., 1986; Kwok *et al*., 1987; Zhang *et al*., 1996; Krause *et al*., 2001; 2003; Ryckeboer, 2001; Khan *et al*., 2004; Horst *et al*., 2005), was pursued. Discrimination of T_{382} from other *T. hamatum* isolates was not possible based on ITS sequences. Therefore, another genomic region, namely the RAPD marker SCE16 (Abbasi *et al*., 1999), was utilized to develop an isolate-specific oligonucleotide (Tha382). To investigate the quantitative properties of these detector oligonucleotides, a 10-fold dilution series of genomic DNA from *R. solani* isolate 19 and T_{382} was quantified after 30 cycles of PCR amplification. The amount of template ranged from 2.5 ng to 0.25 pg. Hybridization signals revealed that a linear logarithmic relationship between the signal intensity and template DNA concentration occurred from 0.25 to 25 pg for the oligonucleotides Tgn1 and Tha382 (Fig. 6-1). For Rso1, the relation was nearly linear over the complete concentration range tested ($R^2 = 0.99$). As was also observed in the previous chapter, adding 2.5 ng of DNA extracted from plant or potting mixes to all samples of the dilution series did not influence template quantification, demonstrating that amplification and hybridization

efficiency is not affected by non-target DNA (data not shown). PCR efficiencies evaluated by addition of 100 pg exogenous *S. cerevisiae* control DNA to each sample followed by PCR amplification and hybridization were highly comparable among samples and were consistent with other experiments, as was also shown in the previous chapter.

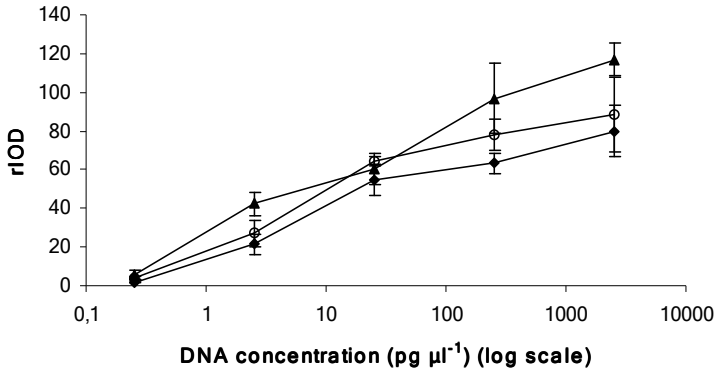


Fig. 6-1. Quantification of a dilution series of *Rhizoctonia solani* isolate 19 and *Trichoderma hamatum* isolate 382 (T_{382}) genomic DNA after PCR using a DNA array containing detector sequences Tgn1 (—♦—), Tha382(—○—), and Rso1 (—▲—) to detect the genus *Trichoderma*, T_{382} and the pathogen *R. solani*, respectively. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD) and plotted against the logarithmic DNA concentration. Data represent means from two hybridization runs using amplicons from a single PCR reaction (n = 4). Error bars indicate standard errors. The experiment was repeated twice with similar results.

6.3.2 Quantification of fungal biomass of T_{382} using a DNA array

Since the population density of T_{382} is a crucial factor for effective suppression of Rhizoctonia damping-off and crown and root rot diseases (Kwok *et al*., 1987; Chung and Hoitink, 1990; Krause *et al*., 2001), accurate quantification of T_{382} using the DNA macroarray was pursued. At the end of a bioassay, a DSP mix containing 1.17 x 10^6 CFU T_{382} per gram fresh weight (determined by dilution plate enumeration) was serially diluted with non-fortified potting mix. In Fig. 6-2, the logarithmic relationship between the hybridization signals obtained with detector Tha382 and the number of CFU g^{-1} fresh weight is presented, demonstrating that accurate quantification of the biocontrol agent is possible between 10^3 and 10^6 CFU g^{-1} fresh weight (R^2 = 0.98).

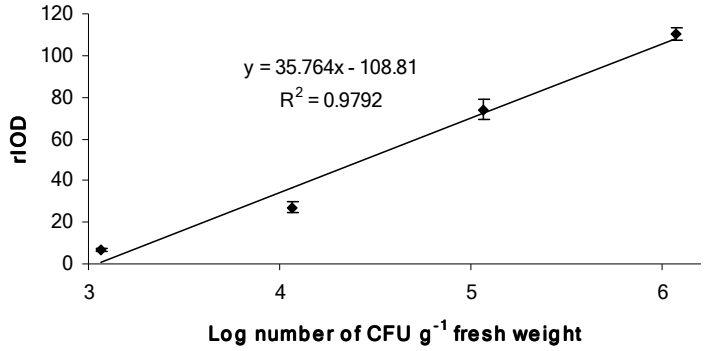


Fig. 6-2. Quantitative assessment of *Trichoderma hamatum* isolate 382 (T_{382}) using a DNA array. Regression line for a serially diluted dark Sphagnum peat mix containing 1.17 x 10^6 T_{382} CFU g^{-1} fresh weight. Dilutions were made using non-fortified potting mix. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD) and plotted against the amount of T_{382} present in the sample (log CFU g^{-1} fresh weight). Data represent means of hybridization signals generated by Tha382 from two hybridization runs using amplicons from a single PCR reaction (n = 4). Error bars indicate standard errors. The experiment was repeated twice with similar results.

Based on these results, the relationship between hybridization strength and the number of T_{382} propagules per gram potting mix is described by the regression equation $y = 35.76x - 108.81$, with y representing the relative integrated optical density (rIOD) and x the logarithmic number of T_{382} CFU g^{-1} fresh weight. This formula was used to estimate the density of T_{382} in multiple fortified potting mixes, encompassing four DSP and four CPB mixes. The inoculum densities of T_{382} calculated using the regression equation were highly comparable to the inoculum densities determined by the classical plating technique as well as to the inoculum density values calculated by real-time PCR analysis (Table 6-3), demonstrating that T_{382} can be accurately quantified in potting mixes based on this formula.

Table 6-3. Determination of *Trichoderma hamatum* isolate 382 (T_{382}) amount in different potting mixes using classical dilution plate enumeration, DNA array[a] analysis, and real-time PCR[b] analysis

	Log CFU g^{-1} fresh weight							
Calculation method	Dark Sphagnum peat mix				Composted pine bark mix			
Dilution plate enumeration	3.76	6.87	3.52	6.07	3.00	5.73	4.00	6.39
DNA array analysis	4.04	6.61	3.50	6.13	3.55	5.47	4.28	5.85
Real-time PCR analysis	3.66	7.53	4.11	5.54	4.13	6.09	3.51	5.90

[a] Based on hybridization strength, the amount of T_{382} was estimated using the regression equation $y = 35.76x - 108.81$, with y representing the relative integrated optical density (rIOD) for detector Tha382 and x the logarithmic number of *Trichoderma* CFU g^{-1} fresh weight.

[b] Based on the calculated DNA concentration, the amount of *Trichoderma* was estimated using the regression equation $y = 0.95x - 2.91$, with y representing the logarithmic calculated DNA concentration using primers ST-Tgn1 and ITS4 and x the logarithmic number of *Trichoderma* CFU g^{-1} fresh weight.

Regression equations were obtained by analyzing a 10-fold dilution series of dark Sphagnum peat mix containing 1.17×10^6 CFU of T_{382} g^{-1} fresh weight.

6.3.3 Correlation between hybridization signal intensity and disease severity and incidence

Establishing a relationship between the pathogen inoculum density in the substrate and any resulting severity or incidence of the disease is essential for understanding and predicting potential outcomes of the disease as well as for taking appropriate control measures. While the density of pathogen propagules is generally related to disease development, it is, in case of DNA array hybridization, however, more efficient to directly link hybridization signal strength to these disease characteristics. Radish plants grown in potting mixes infested with different densities of *R. solani* inoculum were rated for damping-off severity after 7 days of incubation, at which point each of the different damping-off severity ratings were observed. In addition, DNA was extracted from the potting mixes for DNA array hybridization (Fig. 6-3). Rso1 hybridization signal intensity correlated positively with both mean damping-off severity (R^2 = 0.78; Fig. 6-3A) and mean incidence of severely diseased seedlings (R^2 = 0.76; Fig. 6-3B), demonstrating the feasibility of the technique to monitor substrate and plant health.

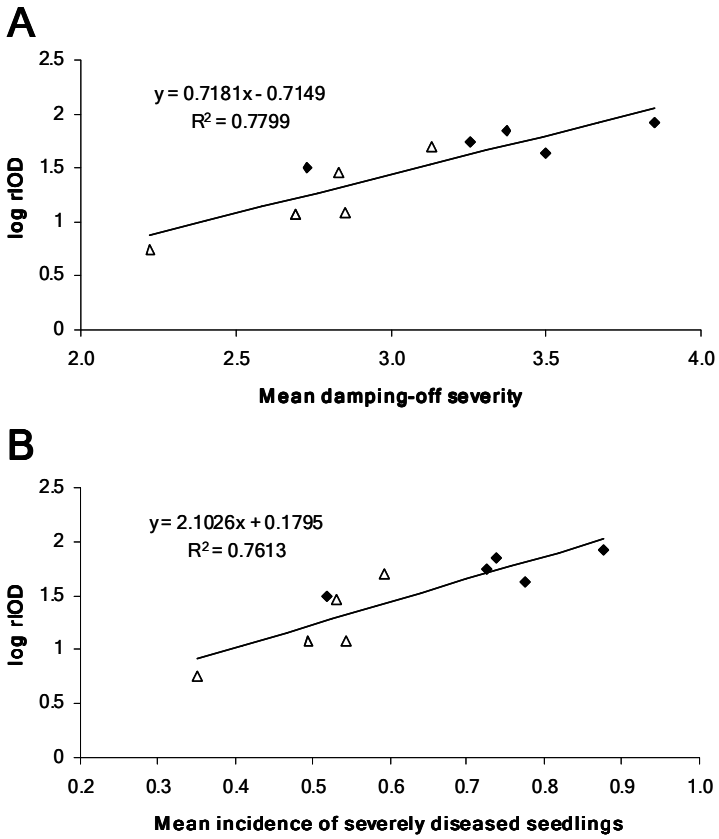


Fig. 6-3. Quantitative assessment of *Rhizoctonia solani* in artificially infested potting mixes. Regression lines for DNA array analysis refer to **A,** mean Rhizoctonia damping-off severity and **B,** mean incidence of severely diseased plants (proportion of plants with a disease severity rating > 2). Damping-off severity and incidence of severely diseased seedlings was determined seven days after planting. Rating was based on five pots of 32 plants each and a damping-off severity scale in which 1 = symptomless; 2 = small root or stem lesion; 3 = large root or stem lesion but not damped-off; 4 = post-emergence damping-off; and 5 = pre-emergence damping-off. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD). Data represent means of hybridization signals generated by Rso1 from two hybridization runs using amplicons from a single PCR reaction (n = 4). ◆, dark Sphagnum peat mix; △, composted pine bark mix. The experiment was repeated twice with similar results.

6.3.4 Assessing populations of T_{382} and *R. solani* in potting mixes with a DNA array

The effects of the different potting mix treatments on the severity of Rhizoctonia damping-off, the incidence of severely diseased seedlings, and the hybridization signal intensities obtained

using the array are summarized in Table 6-4. Transformed mean damping-off severity as well as transformed mean incidence of severely diseased seedlings in the infested natural non-heated treatment was significantly ($P \leq 0.05$) lower with the CPB mix than with the DSP mix. These observations were corroborated by DNA array data that displayed significantly ($P \leq 0.05$) weaker hybridization signals for *R. solani* in the CPB mix than in the corresponding DSP treatment. Heating these mixes eliminated or decreased this suppressive effect as both the transformed mean disease severity and incidence of severely diseased seedlings increased strongly (Table 6-4). In parallel, for both mixes hybridization signal strength more than doubled, reaching a rIOD of 110.51 and 76.76 for the natural infested DSP and CPB mix, respectively. Heating the mixes followed by fortification with T_{382} significantly decreased transformed mean damping-off severity and incidence of severe symptoms again. Likewise, the hybridization signals for *R. solani* decreased from a rIOD of 110.51 in the natural heated DSP mix to a rIOD of 68.02 in the T_{382}-fortified heated DSP mix. However, this decrease was not observed among the same treatments of the CPB mix. For both fortified mixes similar, strong hybridization signals were observed for the *Trichoderma* and T_{382} oligonucleotides (Table 6-4). When these mixes were not preheated, addition of T_{382} did not significantly affect transformed mean disease severity and incidence of heavily diseased seedlings, nor for the DSP mix, nor for the CPB mix (Table 6-4).

In general, hybridization signal strength for *R. solani* corresponded well to disease severity and incidence of severe disease, which is consistent with the data presented in Fig. 6-3. However, this was not observed when the fungicide was applied into the infested potting mixes. In the case of the fungicide-drenched heated treatment, plants were not diseased yet hybridization intensities for the pathogen were as strong as with treatments for which significant disease was observed, suggesting that DNA from the killed pathogen was detected. A non-discriminative detector oligonucleotide (Fun1; Table 6-1) was added at different amounts to the array in order to measure the total amount of fungal DNA. No signals were obtained for Fun1 when mixes were heated, non-fortified, and non-infested when this oligonucleotide was spotted at 0.1 fmol, thus confirming the pasteurizing effect of heating potting mixes. In contrast, clear signals were obtained for the non-heated, non-fortified, and non-infested mixes for this oligonucleotide, indicating a high endogenous fungal growth in these mixes. When mixes were heated, inoculated with the pathogen, and drenched with the fungicide, hybridization signals for Fun1 spotted at 0.1 fmol were just detectable (DSP mix) or not detectable at all (CPB mix).

Table 6-4. Effects of different potting mixes on suppression of Rhizoctonia damping-off of radish and on the development of *Trichoderma hamatum* isolate 382 (T_{382}) and *Rhizoctonia solani* measured by a DNA array

				Rhizoctonia damping-off[e]				Hybridization signal strength[f]				
Potting mix[a]	T_{inc}[b]	*R. solani* control method[c]	*R. solani*[d]	Mean disease severity (R)[g]	Transformed mean disease severity (R^*)[h]	Mean incidence of severe disease (y)[i]	Transformed mean incidence of severe disease (y^*)[j]	Tgn1 (8.0 fmol)	Tha382 (8.0 fmol)	Rso1 (8.0 fmol)	Fun1 (0.1 fmol)	Sce1 (8.0 fmol)
DSP	25	-	-	1.28 ± 0.08	0.30 ± 0.09	0.07 ± 0.02	0.24 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	12.32 ± 3.36	90.85 ± 4.71
	60	-	-	1.18 ± 0.09	0.20 ± 0.10	0.04 ± 0.02	0.18 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	91.02 ± 2.71
	25	T_{382}	-	1.20 ± 0.06	0.21 ± 0.07	0.05 ± 0.02	0.20 ± 0.05	25.08 ±8.69	35.84 ± 4.06	0.00 ± 0.00	17.06 ± 6.94	94.83 ± 3.41
	60	T_{382}	-	1.23 ± 0.08	0.24 ± 0.09	0.06 ± 0.02	0.21 ± 0.06	86.07 ± 6.73	127.61 ± 4.17	0.00 ± 0.00	25.12 ± 10.67	95.15 ± 3.40
	25	-	+	3.19 ± 0.20	3.15 ± 0.36	0.67 ± 0.05	0.96 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	46.21 ± 9.78	26.69 ± 8.49	93.88 ± 2.12
	60	-	+	4.34 ± 0.16	5.37 ± 0.33	0.90 ± 0.03	1.27 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	110.51 ± 8.25	8.79 ± 4.25	97.87 ± 3.91
	25	T_{382}	+	3.66 ± 0.15	4.02 ± 0.29	0.80 ± 0.04	1.11 ± 0.04	35.28 ± 15.66	16.46 ± 8.64	63.27 ± 19.65	29.42 ± 11.75	91.85 ± 2.43
	60	T_{382}	+	3.38 ± 0.15	3.49 ± 0.28	0.71 ± 0.04	1.00 ± 0.04	61.90 ± 4.95	110.39 ± 2.77	68.02 ± 21.81	5.00 ± 3.80	94.98 ± 3.56
	25	Fungicide	+	1.65 ± 0.20	0.78 ± 0.26	0.16 ± 0.05	0.4 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	1.22 ± 0.75	17.70 ± 2.04	89.69 ± 2.72
	60	Fungicide	+	1.09 ± 0.02	0.09 ± 0.02	0.03 ± 0.01	0.14 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	70.79 ± 8.09	1.75 ± 1.09	93.41 ± 2.69
CPB	25	-	-	1.23 ± 0.06	0.24 ± 0.07	0.06 ± 0.02	0.23 ±0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	13.44 ± 1.18	97.55 ± 4.75
	60	-	-	1.33 ± 0.13	0.36 ± 0.15	0.08 ± 0.03	0.25 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	94.08 ± 3.53
	25	T_{382}	-	1.31 ± 0.05	0.33 ± 0.06	0.08 ± 0.01	0.27 ± 0.03	17.05 ± 5.29	18.12 ± 8.24	0.00 ± 0.00	26.34 ± 4.86	90.45 ± 2.71
	60	T_{382}	-	1.23 ± 0.14	0.26 ± 0.16	0.06 ± 0.03	0.18 ± 0.08	82.06 ± 4.74	86.75 ± 4.15	0.00 ± 0.00	30.01 ± 3.32	96.20 ± 4.46
	25	-	+	2.57 ± 0.08	2.08 ± 0.13	0.48 ± 0.03	0.76 ± 0.03	0.00 ±0.00	0.00 ± 0.00	21.25 ± 7.35	33.18± 10.72	90.11 ± 2.12
	60	-	+	4.19 ± 0.11	5.06 ± 0.22	0.94 ± 0.03	1.38 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	76.76 ± 6.36	0.96 ± 0.43	92.81 ± 3.42
	25	T_{382}	+	2.24 ± 0.22	1.60 ± 0.31	0.38 ± 0.06	0.65 ± 0.06	18.44 ± 5.53	44.35 ± 6.12	35.83 ± 8.88	47.08 ± 3.91	96.40 ± 3.78
	60	T_{382}	+	3.29 ± 0.14	3.32 ± 0.25	0.68 ± 0.04	0.97 ± 0.04	71.07 ± 15.31	100.32 ± 2.83	87.03 ± 9.37	12.13 ± 4.65	90.22 ± 3.20
	25	Fungicide	+	1.25 ± 0.08	0.27 ± 0.08	0.06 ± 0.02	0.22 ± 0.06	7.80 ± 3.70	0.00 ± 0.00	0.00 ± 0.00	34.68 ± 8.78	94.83 ± 4.97
	60	Fungicide	+	1.30 ± 0.06	0.33 ± 0.07	0.08 ± 0.02	0.27 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	37.75 ± 12.19	0.00 ± 0.00	90.12 ± 3.88
$LSD_{0.05}$[k]				-	0.56	-	0.16	16.80	9.83	24.25	16.39	9.86

[a] DSP = dark Sphagnum peat mix; CPB = composted pine bark mix.

[b] Incubation temperature (°C). Potting mixes were incubated at 25°C or heated at 60°C for five days prior to fortification or incubation.

[c] Potting mixes were fortified with T_{382} to achieve an initial density of 2 x 10^7 CFU per liter potting mix (T_{382}), not fortified (-), or drenched with tolclofos-methyl (fungicide; 0.03 mg a.i. ml^{-1} water; 125 ml of solution per pot).

[d] + = potting mixes infested with 0.5 g of *R. solani* isolate 19 inoculum per liter of mix; - = not infested.

[e] Damping-off severity and incidence of severely diseased seedlings was determined seven days after planting. Rating was based on five pots of 32 plants each and a damping-off severity scale in which 1 = symptomless; 2 = small root or stem lesion; 3 = large root or stem lesion but not damped-off; 4 = post-emergence damping-off; and 5 = pre-emergence damping-off. Values are means $\pm$ standard errors (n = 5).

[f] Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD). Values are means $\pm$ standard errors (n = 4 from two hybridization runs using amplicons from a single PCR reaction). All detector oligonucleotides with the exception of Fun1 for which hybridization strength is shown were spotted at 8.0 fmol. Fun1 was spotted at 0.1 fmol.

[g] Mean damping-off severity (R).

[h] Disease rating for each replication (R) was transformed to $R^* = (R^{1.5}-1)/1.5$ to obtain a linear scale and an approximately normally distributed variable with constant variance.

[i] Mean proportion of plants in each replication with a disease severity rating > 2 (y).

[j] Mean proportion of plants in each replication with a disease severity rating > 2 (y) was transformed to $y^* = \text{arcsine}(\sqrt{y})$ to obtain a constant variance.

[k] Differences in R^*, y^*, and rIOD larger than the LSD calculated are significantly different (P = 0.05).

In addition to the hybridization assays, real-time PCR analyses were performed and parallel sets of the samples were plated on semi-selective medium to validate detection and quantification. The population of *Trichoderma* propagules in the fortified mixes consisted of approximately 10^4 and 10^6 CFU g^{-1} fresh weight in the DSP mix and in the CPB mix, respectively. Once again, these values corresponded very well to those calculated with the formula derived from the regression equation in Fig. 6-2 (data not shown), as was also presented in the results in Table 6-3. Because of the lack of a good discriminative medium for *R. solani*, it was impossible to accurately distinguish and thus quantify the pathogen in the samples. However, in all instances where hybridization signals were obtained for *R. solani*, except for fungicide treatments, the presence of the pathogen was confirmed by plating (data not shown). Nevertheless, a very high degree of correlation (R^2 = 0.90) was found between hybridization strength and the calculated DNA concentration obtained by real-time PCR analysis (Fig. 6-4.), demonstrating the reliability of the quantitative results obtained with the DNA macroarray.

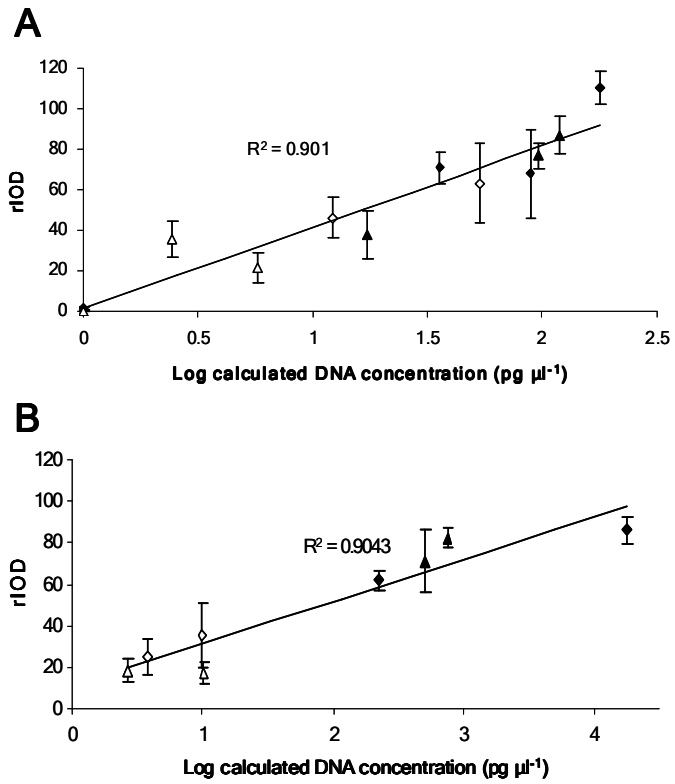


Fig. 6-4. Quantitative assessment of **A,** *Rhizoctonia solani* and **B,** *Trichoderma hamatum* isolate 382 (T_{382}) in all artificially infested and T_{382}-fortified potting mixes from a single bioassay, respectively. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD) and plotted against the logarithmic calculated DNA concentration using real-time PCR. Data represent means of hybridization signals generated with Rso1 and Tgn1 from two hybridization runs using amplicons from a single PCR reaction (n = 4). Error bars indicate standard errors. ◇, ◆,dark Sphagnum peat mix incubated at 25°C or heated for five days at 60°C prior to inoculation or planting, respectively; △, ▲, composted pine bark mix incubated at 25°C or heated for five days at 60°C prior to inoculation or planting, respectively.

6.4 Discussion

Soil and plant health status has become the main focus in integrated crop management. Soils, soil-less growing media, and other substrates may naturally contain a multitude of potential plant pathogens and beneficial microorganisms, including those that naturally suppress plant diseases. Alternatively, controlled inoculation with specific DSMs frequently helps to assure that the proper microorganisms are in place to suppress various diseases. *T. hamatum* isolate

382, one such DSM, has been shown to suppress a broad spectrum of plant diseases caused by different soilborne (Trillas-Gay *et al*., 1986; Kwok *et al*., 1987; Krause *et al*., 2001; Ryckeboer, 2001; Khan *et al*., 2004) and foliar pathogens (Zhang *et al*., 1996; Krause *et al*., 2003; Horst *et al*., 2005) when it is incorporated into suitable growing media. However, effective use of T_{382} and other DSMs for disease suppression is based on numerous factors, including physical, chemical and organic matter qualities of the substrate or niche, pathogen infestation level, and DSM density (Paulitz, 2000).

Microbial detection and identification methods continue to evolve, each with its own strengths and limitations. In recent years there has been a shift towards molecular techniques that provide powerful tools by which microorganisms can be precisely measured (McCartney *et al*., 2003; Lievens *et al*., 2005b). Although most of these techniques have targeted individual microorganisms, DNA array technology has been used to qualitatively and quantitatively detect several microorganisms using a single assay (Chapters 3 and 5; Martin *et al*., 2000; Lievens *et al*., 2003; 2005a; 2005b; Lievens and Thomma, 2005), making them highly attractive for analyzing the dynamics of microbial populations in a specific environment as a basis for further study of their potential interactions. In this chapter, we demonstrated the usefulness of this technique for simultaneously assessing populations of a DSM and a pathogen, T_{382} and *R. solani*, respectively. In addition, the technology was successfully used to measure plant health and predict disease severity based on pathogen population densities in the substrate. We demonstrated that DNA array-based quantification of a pathogen could be effectively correlated with two different types of disease analysis, namely disease severity and disease incidence.

In this study, ITS-based detector oligonucleotides were selected to detect and identify several members of the *R. solani* species complex (Rso1) and the genus *Trichoderma* (Tgn1), encompassing several pathogenic groups and disease suppressive species, respectively. Current classification of *R. solani* is largely based on grouping isolates into anastomosis groups (AG), defined on the basis of hyphal fusion reactions (Agrios, 2005). So far, 14 AGs have been described (Sneh *et al*., 1994). Based on BLAST analysis, a wide range of *R. solani* isolates should be detectable with oligonucleotide Rso1.

Nevertheless, ribosomal sequences do not always provide the desired degree of selectivity, especially below species level (Chapter 3; Tooley *et al*., 1996; Bao *et al*., 2002;

Lievens *et al*., 2003). As a result, other diagnostic regions of the genome should be assessed, preferably directly linked to virulence in case of pathogens, or disease suppressive activity in case of DSMs. This can be achieved by several techniques, including RAPD analysis to generate randomly amplified fragments from the genome (Williams *et al*., 1990). Using this strategy, Abbasi *et al*. (1999) developed a series of PCR primers to specifically detect T_{382}. However, under this protocol that was developed to ensure reliable detection, three individual PCR reactions are required to rule out the presence of other isolates (Abbasi *et al*., 1999), rendering the assay unattractive to conduct and unsuitable for direct quantification. In this study, a detector sequence derived from one of these markers (Tha382) was added to the DNA array containing the ITS-based oligonucleotides. Whereas the primers OPE16-F and OPE16-R generated amplicons of the expected length for more than the half of the *Trichoderma* isolates tested, cross-hybridization to Tha382 was limited to three of the *Trichoderma* isolates tested, including *T. harzianum* CPK 51, *T. inhamatum* CPK 239, and *T. longibrachiatum* 57D (data not shown). It is currently unknown whether these isolates manifest any levels of suppressiveness to diseases caused by *R. solani*.

The power of DNA arrays to simultaneously measure different microbial populations as a basis for further study of their potential interactions is shown in this chapter. Any bias resulting from potential PCR inhibitors was excluded as equal signals were obtained between different samples for the exogenous control DNA (Table 6-4), demonstrating the accuracy of the assay. An alternative for detecting, and, in particular, quantifying microorganisms, is real-time PCR technology (Chapter 4; Schaad and Frederick, 2002; Brouwer *et al*., 2003; McCartney *et al*., 2003; Schaad *et al*., 2003; Lievens *et al*., 2005b). In this study, all results obtained using the DNA array were confirmed by real-time PCR (Fig. 6-4; Table 6-3), as was also shown in Chapter 5. However, the detection capabilities of this technology are limited to a few organisms in a single assay (Mackay *et al*., 2002). The higher resolution that can be obtained using DNA arrays represents their major advantage. Nevertheless, while all DNA-based techniques require isolation of DNA, non-destructive technologies also exist for monitoring particular microorganisms, including measurement of GUS (β-glucuronidase) or GFP (green fluorescent protein) activity in microorganisms transformed with the bacterial *uidA* or *gfp* gene, respectively (Bae and Knudsen, 2000). However, interference of plant factors with the measured parameter constitutes a major drawback for these methods (Thomma *et al*., 1999). In

addition, DSMs transformed to contain these traits are transgenic and will likely suffer the stigmas associated with genetically modified crops. In contrast, the use of DNA arrays may prove very useful for tagging a particular isolate of interest in a complex system without genetic transformation of the organism and without major artifacts caused by external factors.

In this chapter, the power of DNA arrays to predict disease severity by analyzing the growing medium (Fig. 6-3) is shown, which corroborate the results obtained with a preliminar experiment in the previous chapter (Lievens *et al*., 2005a). A reasonable correlation ($R^2 \geq 0.76$) was found between Rso1 hybridization signal and disease severity as well as incidence of severe disease. The lack of a stronger correlation may be explained by the biological variation that impacts symptom development but also by the way the severity of disease is scored.

We further demonstrated the utility of the DNA macroarray approach to measure different microbial populations and the interactions between them (Table 6-4). Sustained biological suppression of *R. solani* requires the presence of specific DSMs that eradicate pathogen propagules by predation or suppress pathogen growth by production of biostatic agents. The most important microorganisms that are suppressive to diseases caused by *R. solani* are members of the genus *Trichoderma* (Elad *et al*., 1980; Kuter *et al*., 1983; Harman and Björkman, 1998; Lewis *et al*., 1998) and *Penicillium* (Hadar and Gorodecki, 1991). These organisms often interact with a number of bacterial species that enhance their suppressiveness (Kwok *et al*., 1987; Tuitert *et al*., 1998). When monitoring a mycopredatory interaction, one would expect a decline in the pathogen level concomitant with an increase in the predator population. However, in this study, we only observed a significant decline in the hybridization signals for *R. solani* in the heated DSP mix (Table 6-4). This suggests that also other factors than eradication by T_{382} contributed to suppression of Rhizoctonia damping-off, most probably factors inherent to the mixes themselves, including the undetected presence of naturally occurring DSMs. The microbial carrying capacity of the potting medium, which is determined by the decomposition level of the organic matter fraction (Boehm *et al*., 1997), is an important factor in suppression of Rhizoctonia damping-off (Krause *et al*., 2001). Assessment of the microbial activity by the rate of hydrolysis of fluorescein diacetate (FDA; Schnürer and Rosswall, 1982) revealed higher microbial activity in the CPB mixes than in the DSP mixes (data not shown). Though it is rare for potting mixes to be naturally suppressive to Rhizoctonia damping-off without allowing several months for these materials to be colonized by naturally

occurring DSMs (Krause *et al*., 2001; Kuter *et al*., 1983), it may be possible that the source of composted pine bark used in our experiments already contained such suppressive microflora and/or more in general antimicrobial compounds. Hence, the higher microbial carrying capacity of the CPB mixes coupled with the natural presence of suppressive microorganisms in the compost may possibly explain why natural suppression of Rhizoctonia damping-off was observed in the infested natural CPB mix and why fortifying this mix with T_{382} did not further reduce disease severity and incidence of severe disease.

As further discussed in more detail in Chapter 7, a potential limitation of DNA-based techniques is the possibility of detecting DNA from inactive or dead organisms. Indeed, in this study, *R. solani* was still detected in heated mixes that were drenched with a fungicide specifically labeled for protection against *R. solani* diseases (Table 6-4). Based on the hybridization signals generated by Fun1, the non-discriminative fungal detector oligonucleotide, we concluded that the presence and, consequently, the activity of fungi was rather low in these mixes. As DNA degradation is dependent on microbial activity (England *et al*., 1998; Herdina *et al*., 2004), this may potentially explain why *R. solani* was still detected in these heated, fungicide-drenched mixes using the DNA array. When these mixes were reanalyzed 3 weeks after planting, general fungal presence was higher and the pathogen was no longer detectable (data not shown), thus confirming this hypothesis.

In conclusion, the results shown in this chapter illustrate, apart from the diagnostic power of DNA arrays (e.g. shown in Chapters 3 and 5), their feasibility to simultaneously assess populations of specific DSMs and pathogens, offering perspectives for studying population dynamics and ecology of target populations in certain environments. Furthermore, we showed that DNA arrays can be used to measure plant health and estimate disease severity as well as incidence of severe disease based on population densities in the growing medium. Taking into account the unlimited extension possibilities of DNA arrays to include detectors for other and more microorganisms, this technique has the potential to become a valuable tool for diagnostic, ecological as well as epidemiological studies. Ultimately, this will allow the development of novel methods for integrated measurements of soil health.

7 General discussion*

The failure to adequately identify plant pathogens from culture-based morphological techniques has led to the development of molecular approaches, of which PCR-amplification of pathogen-specific nucleic acid targets is the most predominant. In general, these methods are much faster, more specific, more sensitive and more accurate, and can be performed and interpreted by personnel with no specialized taxonomical expertise. Perhaps even more important, these techniques allow detection of non-culturable microorganisms (Chapter 1; McCartney *et al.*, 2003; Lievens *et al.*, 2005b). However, although these methods are routinely used in the diagnosis of human diseases (Sebire *et al.*, 1998) and an online PCR primer database for phytopathogenic fungi and oomycetes is available (Ghignone and Migheli, 2005; www.sppadbase.com), they are not yet widely used for routine plant pathogen detection. One of the reasons is that, although generally most of these assays are reliable, they target only a single pathogen, making comprehensive screening of complex samples unprofitable. Therefore, the main objective of this thesis was the development of a multiplex pathogen detection assay that, in addition, allows quantification and is suitable for implementation in practice.

In this chapter we discuss, with a special attention to molecular diagnosis of fungal and oomycete plant pathogens, the different criteria that have to be fulfilled for the development of robust detection procedures that can be routinely used by diagnostic laboratories in relation to the results presented in this thesis. In addition, potential limitations of molecular detection and identification techniques as well as some future perspectives that are likely to impact future plant disease controlling and preventing strategies are discussed.

* This chapter is a modified version of the manuscript "Recent developments in pathogen detection arrays: implications for fungal plant pathogens and use in practice"; Lievens, B., and Thomma. B. P. H. J.; Phytopathology 95:1374-1380 (2005).

7.1 Requirements for technology implementation in practice

Since its introduction in the mid 1980's (Mullis and Faloona, 1987), PCR has become a fundamental aspect of molecular diagnostics, and several technologies based on PCR have been developed since then (McCartney *et al.*, 2003; Lievens *et al.*, 2005b). However, although extensively used as a tool in (academic) research, the use of PCR-based technologies in horticultural and agricultural practice is still limited (McCartney *et al.*, 2003; Schaad *et al.*, 2003). Nevertheless, several areas would benefit from the commercial availability of such detection assays. With the opening of the borders of many countries and increased free-trade agreements, rapid testing for possible contamination with quarantine organisms is in high question. In addition, in order to be able to take timely control measures, the question from commercial growers for rapid, affordable pathogen detection assays is increasing. However, different requirements have to be met before new detection methods are implemented in practice. These requirements can be separated into technical and economical demands. Whereas the technical demands are absolutely required for the development of any successful diagnostic method, the economical criteria are important guidelines for the development of a commercially attractive assay.

7.1.1 Technical demands

When developing a tool for plant pathogen diagnostic purposes, several technical aspects related to plant disease management on one hand, and regulatory issues on the other hand, are to be considered. These aspects mainly concern specificity, sensitivity, and robustness. In addition, multiplexing and quantification are increasingly becoming required features for a diagnostic assay.

7.1.1.1 Specificity

The ability to specifically detect the target pathogens is vital for all diagnostic applications. One of the most important advantages that molecular-based detection techniques have over conventional diagnostic methods is the power to, in principle, distinguish closely related organisms. Obviously, the specificity of nucleic acid-based techniques is determined by the sequences that are targeted. Common approaches to select target sequences are in detail

discussed in Chapter 1. For molecular diagnostics, ubiquitously conserved genes are frequently used as target genes. Closely related microbial species often differ in a single to a few bases in such genes. However, the high degree of specificity of nucleic acid-based detection techniques, achieved through the use of PCR primers (Papp *et al.*, 2003), hybridization probes (Livak, 1999), or arrayed detector oligonucleotides (Chapter 2; Consolandi *et al.*, 2001; Lievens *et al.*, 2006) allows detecting such SNPs. Since closely related pathogens might have a different host range or display a completely different pathogenicity, this is an extremely important trait. However, ultimately, to enhance specificity of a diagnostic assay, a combination of multiple unique diagnostic regions can be exploited.

7.1.1.2 Sensitivity

Early detection of pathogens, e.g. before crops are infected or symptoms have developed, is essential to prevent diseases, spread of the inoculum, and economic losses. Therefore, diagnostic procedures should be highly sensitive. Prior to the introduction of nucleic acid amplification methods, in particular PCR, nucleic acid-based diagnostics mainly involved the use of specific probes to report the presence of a certain organism (Yao *et al.*, 1991). However, these methods often led to "false negatives" because of too low sensitivity. Because of this, PCR has been introduced in most molecular diagnostic assays in recent years, allowing detection of minute quantities of pathogen DNA. However, high sensitivity also causes one of the potential pitfalls of PCR technology: the slightest carry-over contamination can give rise to "false positive" results. Therefore, stringent conditions and controls are necessary such as guarding the reagents and samples for accidental DNA contamination via aerosols, running negative controls simultaneously with the test samples, and having separate dedicated areas for pre- and post-PCR handling (Kwok and Higushi, 1989).

Recently, attempts are being made to assess the potential use of relatively novel highly sensitive amplification techniques such as rolling circle amplification (Chapter 1; Baner *et al.*, 1998) for pathogen detection. However, compared to PCR this procedure is fairly complicated (Andras *et al.*, 2001) and relatively expensive. Therefore, it is important to realize what level of sensitivity is required when selecting an appropriate method for plant pathogen detection. Techniques more sensitive than those based on conventional PCR amplification will probably not be required when assessing whether measures have to be taken to prevent yield losses, as

the threshold level that has to be crossed can easily be detected using PCR. In contrast, sensitivity is very important for quarantine organisms for which a zero-tolerance is wanted.

7.1.1.3 Multiplexing

Most current molecular diagnostic assays used in plant pathology target one specific pathogen. However, as crops can be infected by numerous pathogens which are, in addition, often present in plants as complexes, it is desirable to develop assays that can detect multiple pathogens simultaneously. The first multiplex PCR-based strategies involved the use of multiple primer sets in the same reaction. Nevertheless, the development of a reliable multiplex PCR, in order to resolve at least a few amplicons by gel electrophoresis, is a significant technical challenge (Elnifro *et al.*, 2000). For real-time PCR (Heid *et al.*, 1996) the amplification process is monitored on-line, meaning that the size difference of amplicons to discriminate them on gels is not necessary. Nevertheless, in this case multiplexing is limited by the availability of dyes emitting fluorescence at different wavelengths on one hand, and the monochromatic character of the energizing light source in real-time PCR instruments on the other hand (Mackay *et al.*, 2002). As a result, detection of more than a few pathogens per assay is currently not possible using these strategies.

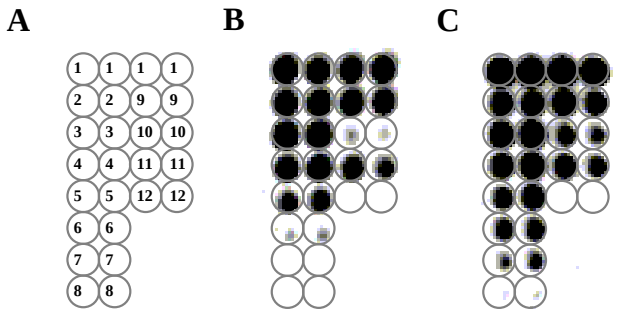
In contrast, array hybridization technology offers the possibility to add a multiplex aspect to PCR-based detection. In theory, DNA arrays, originally designed to study gene expression or to generate SNP profiles, can be used to detect an unlimited amount of different organisms in parallel (Martin *et al.*, 2000; Lévesque, 2001; Lievens *et al.*, 2005b). The virtually unlimited screening capability of DNA arrays, coupled with PCR amplification, results in high levels of sensitivity, specificity, and throughput capacity. In plant pathology, this approach was applied for identifying DNA from pure cultures (Lévesque *et al.*, 1998; Uehara *et al.*, 1999; Fessehaie *et al.*, 2003). Despite these studies, for application in practice, identification of pathogens from pure cultures is not very relevant as, in the end, pathogens should be preferably assessed directly from plant and soil samples. In this work, the utility of this technology for the diagnosis of multiple pathogens in such environmental samples was shown (Chapter 3; Lievens *et al.*, 2003; 2004). Ultimately, such multiplex approach should lead to a comprehensive diagnostic kit that can detect all relevant pathogens of a specific crop. In an analogous manner, recently the first cross-pathogen group DNA array to detect human pathogens has been

developed for high confidence identification of 11 bacterial species, five viruses and two eukaryotic pathogens (Wilson *et al.*, 2002).

7.1.1.4 Quantification

With respect to plant disease management, especially quantification of a pathogen upon its detection and identification is an important aspect as it can be used to estimate potential risks regarding disease development, spread of the inoculum, and economic losses. Apart from this potential, it provides the information required to take appropriate management decisions. However, the non-linear nature of PCR amplification makes it challenging to relate the amount of amplicon produced in the reaction to the amount of target DNA initially present in the sample. Nevertheless, several studies have shown that by extensive optimization of PCR conditions quantification in endpoint analysis-based PCR assays can be performed (Hu *et al.*, 1993). More recently, the introduction of real-time PCR technology (Heid *et al.*, 1996), which is characterized by on-line measurement of amplicons as they accumulate during each cycle has improved and simplified methods for PCR-based quantification. Currently, in plant pathology, real-time PCR is the most reliable culture-independent technique to quantify the presence of specific pathogens (Schaad and Frederick, 2002; McCartney *et al*., 2003; Gachon *et al.*, 2004; Lievens *et al*., 2005b) as well as for the quantification of disease progress (Brouwer *et al*., 2003). The power of real-time PCR for plant pathogen diagnosis is illustrated in Chapter 4 as the feasibility of the technique to specifically quantify pathogen biomass in biological samples was demonstrated for a number of tomato pathogens. However, to quantify more than a handful of plant pathogens in a single assay, real-time PCR instrumentation needs to be adapted or other techniques should be pursued (Mackay *et al*., 2002). As concluded previously, DNA array technology offers the most suitable technology for multiplex detection of plant pathogens. Therefore, implementation of a quantitative aspect to this technology would be highly desirable. In Chapter 5 (Lievens *et al.*, 2005a), it is shown that while accounting for specific criteria like optimizing of PCR conditions, the amount of immobilized oligonucleotides and controls for PCR kinetics, pathogen DNA could be accurately quantified in concentration ranges typically encountered in horticultural practice by using a single PCR and macroarray hybridization (Fig. 7-1). In addition, a high degree of correlation was found between hybridization signal intensity and real-time PCR quantification, demonstrating the

accuracy of the technique (Chapters 5 and 6; Lievens *et al.*, 2005a).



D

105.6	**105.6**
100.0	**98.9**
107.2	**8.7**
95.5	**69.6**
68.5	**0.0**
8.4	
0.0	
0.0	

E

122.1	**123.9**
100.0	**100.8**
121.2	**69.1**
116.0	**92.8**
97.4	**0.0**
78.6	
42.6	
1.6	

Fig. 7-1. Detection and quantification of fungal DNA utilizing a DNA macroarray. **A,** Scheme for the localization of the oligonucleotide detectors on the macroarray. **B,** Signals obtained upon hybridization of amplicons resulting from co-amplification of 50 pg *Fusarium solani* genomic DNA and 5 ng *Verticillium albo-atrum* genomic DNA. **C**, Signals obtained upon hybridization of amplicons resulting from co-amplification of 5 ng *Fusarium solani* genomic DNA and 5 ng *Verticillium albo-atrum* genomic DNA. **D** and **E,** Quantification of hybridization signals obtained in **B** and **C**, respectively. Hybridization signal strength is reported relative to the average integrated optical density of a labeled reference control, marked 2 in panel A. Values are means of two replicates. The target oligonucleotides (spotted at 8.0 fmol per spot) to detect *F. solani* and *V. albo-atrum* are marked 10 and 11, respectively. In addition, several control oligonucleotides were spotted on the membrane, including a positive control for the hybridization (1), an oligonucleotide to target exogenously added control DNA (9), a dilution series of a universal fungal detector oligonucleotide (absolute quantities are 8.0 (3), 2.0 (4), 0.5 (5), 0.2 (6), 0.1 (7), and 0.02 fmol (8)), The labeled reference oligonucleotide for detection and calibration (2) is also synthesized without label and spotted as a negative control (12).

7.1.1.5 Robustness

Obviously, each diagnostic assay developed should be robust, meaning that the methodology is highly reproducible. Therefore, the assay must be optimized and thoroughly tested against multiple targets and taxonomically related organisms, preferably isolated from various hosts or cultivars and from different geographical areas. There are, however, specific obstacles that can affect the robustness and reliability of PCR-based diagnostic assays. PCR efficiency can be

drastically reduced or even inhibited due to a variety of naturally occurring compounds that are co-extracted with the nucleic acids, suchs as phenolic compounds, humic acids, fulvic acids, and heavy metals. However, in many cases these specific problems have been circumvented by improved extraction methods (McCartney *et al.*, 2003) or by the use of optimized extraction kits (Chapter 5; Lievens *et al.*, 2005a) by which highly purified DNA can be obtained from complex environmental samples. To improve reliability, PCR efficiency can be monitored by spiking the DNA extract with a certain amount of exogenous control DNA that can be amplified in the same reaction (Cubero *et al.*, 2002) or in parallel (Lievens *et al.*, 2005a) as demonstrated in Chapters 4, 5 and 6.

7.1.1.6 Validation

In many cases, detection procedures are developed to be used in specific research areas. As a consequence, these tools are generally only evaluated to work under the experimental conditions used. The steps required to evaluate new detection techniques for their direct use in practice are, however, rarely taken. New diagnostic procedures should be validated and standardized using worldwide ring tests before entering the market. Factors involved in validation include (i) specificity, (ii) sensitivity, (iii) reproducibility, (iv) accuracy of results, and (v) consistency and reliability of detection. The reliability of the test must be demonstrated unequivocally in blind tests in several different laboratories and results should be interpreted without any ambiguity. Preferably, the evaluation process must be monitored by an internationally recognized organization that ensures suitable expertise for the crop(s) and pathogen(s) involved in evaluating the test. As soon as new methods and reagents are validated, they can be officially recognized and recommended for plant pathogen detection and eventually replace more conventional gold standards in specific control directives (Stead, 1999; Martin *et al.*, 2000).

7.1.2 Economical demands

Apart from the technical criteria several economical aspects have to be considered in the development of reliable detection methods that can be used by diagnostic laboratories. These demands include short diagnosis time and high-throughput capability. In addition, it should be possible to perform the test with a minimum of taxonomical expertise and at a minimum of

cost. With regard to quarantine and export legislation speed is the most important factor. When it comes to routine diagnosis requested by growers especially cost is of high importance.

7.1.2.1 Speed

The speed at which results can be obtained is a very important issue for any commercial diagnostic tool, especially for the detection of pathogens of high-risk potential or when it comes to take timely disease management decisions. Whereas culture-based traditional techniques are often laborious and time-consuming, and typically take days to weeks to complete, molecular detection techniques can generate accurate results much faster. In general, most molecular analyses can be accurately performed within one or two days, which is a considerable gain of time compared to the more conventional analyses.

7.1.2.2 High-throughput sample analysis

Another requirement for commercial applicability is the possibility to screen a large number of samples in a short period of time. Nowadays, when using molecular techniques, comprehensive screening of samples is made possible because of recent developments in automated high-throughput DNA extraction systems and because of the introduction of 96- to 384-well plate PCR systems. In addition, the development of DNA arrays for plant pathogen diagnosis has enabled screening of multiple pathogens in a single assay, eliminating the need of performing several singleplex assays. However, when many samples need to be processed using such multiplex assays in a short time, there is still a lack of high sample throughput capacity. Nevertheless, it can be expected that this will be achieved in the near future by the use of low-density arrays in a multi-well configuration (Szemes *et al*., 2005), similar to the array systems that are currently being used for the pharmaceutical industry (Eggers, 2000).

7.1.2.3 Expertise

Traditionally, the predominant techniques used to identify pathogens have relied upon morphological criteria and require highly specialized taxonomical expertise, which may take extensive education plus years of work in practice to acquire. However, since many pathogens are difficult to identify using morphological criteria, these techniques often lead to incomplete or even wrong diagnoses. Therefore, companies that provide diagnostic services are intensively

searching for generic diagnostic tools that can be executed relatively easily and interpreted for standard analyses by technicians with a general education in molecular biology.

7.1.2.4 Cost issues

Cost is perhaps the most important consideration for routine pathogen testing since, relative to human clinical diagnostics, the willingness to spend money on expensive plant disease diagnosis is limited. This is caused by the fact that profit margins in agriculture and horticulture are often low as is the emotional value of a crop. On the other hand, when it comes to regulatory issues and risk management of exotic pathogens, other criteria like specificity, sensitivity, and speed are more important than cost.

While nucleic acid-based assays provide an excellent opportunity for rapid and precise detection, currently their success largely depends on well-equipped laboratory facilities. Therefore, first of all, companies that provide diagnostic services should compare the many advantages afforded by nucleic acid-based diagnostic assays to the costs of establishing and maintaining a suitable laboratory environment.

When large numbers of samples need to be assessed for the presence of one specific pathogen (as in some quarantine testing programs), diagnostic assays that can detect only single pathogens may be the most cost-effective. In contrast, when one has no idea about the causal agent of a disease or when several pathogens need to be targeted simultaneously, detection of multiple pathogens increases efficiency, reduces costs, and saves time. With regard to a grower, it is the price that he has to pay that counts. In general, a multiplex assay, like a DNA array-based test, is the most cost effective per sample as the use of singleplex assays often requires multiple consecutive analyses to determine and confirm the cause of a disease, hence increasing the price per sample analyzed. Nevertheless, the price of such DNA array-based analysis is largely determined by the instruments used. In general, there are two types of DNA arrays, including membrane-based macroarrays and high density microarrays using a glass slide (Schena *et al.* 1996) or beads (Yang *et al.*, 1998). Whereas a macroarray-based detection generally does not require specialized equipment, highly specialized instruments are needed for microarray fabrication and reading. As a consequence, implementation of microarray technology for disease diagnostic use in plant pathology is currently relatively expensive and therefore commercially unattractive. In addition,

macroarrays are generally more sensitive than microarrays since spots on a macroarray often contain higher amounts of detector oligonucleotides (Cho and Tiedje, 2002), favoring the use of macroarrays. Besides, high density is probably not a necessity for host-based diagnostic assays since the number of major pathogens of a given crop is usually not more than one hundred.

7.2 Potential pitfalls and limitations

Molecular methods have become increasingly important to specifically detect pathogens and, as indicated in the first chapter, different regions of the genome can be targeted to obtain the desired specificity. In recent years multiplexing, as well as quantification, are being implemented as traits to several of these technologies. However, despite all their advantages there remain limitations to molecular technologies that can hamper accurate pathogen detection and quantification. Nevertheless, most of these are inherent to the classical detection methods as well.

First, misclassification of strains is a regularly occurring phenomenon in microbial taxonomy. Historically, closely related microorganimisms have been grouped into a single species and subsequently to a certain genus largely based on similarities in morphological and biological features (Taylor *et al.*, 2000). However, very poorly defined genera and genera containing asexual fungal species such as *Fusarium*, *Rhizoctonia*, and *Verticillium* are known to often contain unrelated species (Roberts, 1999; Leslie *et al.*, 2001). As a result, relationships based on these morphological and biological traits are not always reflected by the phylogenies that are revealed using nucleic acid-based characterization techniques (Taylor *et al.*, 2000). Consequently, finding DNA sequences that are shared by all members of a given species or genus may be challenging. Therefore, efforts to solve misclassifications should be closely monitored. Presently, there is a trend to reconstruct phylogenies based on orthologous DNA sequences, known as “DNA barcodes” (Herbert *et al.*, 2003). However, controversy exists over the value of DNA barcoding, largely because species determinations based solely on the amount of genetic divergence in a single gene could result in incorrect species recognition (Will and Rubinoff, 2004).

Another difficulty for molecular detection of certain plant pathogens is the existence of species that contain pathogenic as well as non-pathogenic or even beneficial strains. This is a known phenomenon for complex species such as *Fusarium oxysporum, F. solani,* and *Rhizoctonia solani* (Recorbet *et al.,* 2003). Since these differences can very often not be resolved by targeting the known and generally used conserved genes, target sequences should preferably be derived from genes that are directly linked to pathogenicity (Johnson *et al.,* 2000; Recorbet *et al.,* 2003; Rep *et al*., 2004). As long as no molecular markers are available for these species complexes that allow for discrimination between pathogenic and non-pathogenic strains, pathogenicity tests with different hosts or cultivars need to be performed to determine whether or not a specific isolate is a pathogen of a specific crop.

In addition, the lack of adequate sequence information can hamper the development of a reliable molecular diagnostic assay. However, sequence data in public databases is continuously increasing and also allows validating current phylogenetic classifications. As a result, integration of more organisms into detection systems should become possible and identification of emerging pathogens is likely to become an easier task. In this respect, the increasing availability of full-genome sequences of plant pathogens is a desirable development.

Another potential limitation of DNA-based techniques is the possibility to detect DNA from dead or non-active organisms, as was also observed in Chapter 6. As a result, detection of non-viable propagules, and thus the risk of overestimation of viable cells or “false positives”, should be taken into account. This is particularly relevant for pathogens subjected to elimination treatments such as the application of chemicals or antibiotics. Nevertheless, the rate of DNA degradation from dead cells in soils should be considered fairly high due to the high microbial activity, suggesting that interference by DNA derived from non-viable cells might be of less importance (Herdina *et al.,* 2004). The rate of DNA breakdown depends on soil type (Romanowski *et al.,* 1992) and moisture content (Brim *et al.,* 1994). As DNA degradation occurs more slowly in dehydrated soils (Brim *et al.,* 1994), reliable diagnosis especially of samples from dry fields may be perverted by detection of non-viable organisms. However, since persisting soil desiccation generally does not occur in horticultural or agricultural practice, this should not be of major concern. To exclude detection of non-viable organisms, PCR-based diagnostics may be combined with a preculturing step (Schaad *et al.,* 1995). Because only the viable propagules will grow, selection of living organisms is guaranteed. In

addition, by this culturing step the detection limit is increased and potential PCR inhibiting compounds from the original sample are eliminated (Penyalver *et al.*, 2000; Schaad *et al.*, 1995). However, disadvantages to this approach are the labor intensive and time-consuming nature, implications for quantification since the initial amount of target is influenced in an uncontrolled manner, and the inability to detect organisms that are either slow or difficult to grow or non-culturable. A perhaps more attractive alternative is the use of DNA-binding dyes such as ethidium monoazide (EMA) to distinguish viable from non-viable organisms (Rudi *et al.*, 2005). Since dead cells have compromised plasma membranes, EMA is able to selectively penetrate dead cells where it intercalates into DNA upon photoactivation. Once this takes place, EMA-bound DNA inhibits PCR amplification and thus allows the selective amplification of targets from living organisms. Another alternative is the use of RNA as a target instead of DNA, in combination with RT-PCR. Since RNA is less stable than DNA, RNA will be degraded more quickly in dead organisms. In addition, mRNA is only produced by metabolically active cells, making mRNA suitable to selectively detect living microorganisms. However, because of the extreme sensitivity to degradation, specific precautions should be taken to isolate RNA from environmental samples.

As mentioned in Chapter 1, rDNA sequences are currently the primary target for diagnostic development. Whereas the high copy number of this gene allows sensitive detection, this may, however, complicate pathogen quantification in environmental samples. Accurate quantification would be biased if it should appear that the copy number of the rDNA repeats significantly varies between different isolates of the same species. Nevertheless, to our knowledge, this has not been reported to date. Another factor that can hamper reliable quantification is the potential presence of both spores and mycelium, which are co-extracted during DNA extraction (Dickie *et al.*, 2002). However, at present it is still unclear how this proportion varies under horticultural conditions. In addition, as the majority of spores are likely to be found in the upper layers of a soil, they are less likely to be of concern when deeper soil samples are taken (Dickie *et al.*, 2002).

Other potential bottlenecks are sampling procedures and sampling size. As the amount of material necessary for analysis reduces with the development of more sensitive technologies, developing appropriate sampling strategies that account for possible spatial variability is becoming even more challenging than previously. The sampling plan should be performed in a

manner that ensures a statistically representative sample. Nevertheless, Ranjard and coworkers (Ranjard *et al.*, 2003) found that DNA extracted from multiple sampling aliquots of soil ≥1g had no effect on the assessment of fungal diversity, whereas variations were observed between replicates of smaller samples. Therefore, to minimize variation between different molecular analyses a standard sample size should be used. In addition, expressing the amount of soil sample per unit of dry weight will decrease potential variation and allow fair comparison between different samples. Currently, pooling multiple small samples taken from a plant into one extraction or using subsamples taken from a homogenized soil sample may be the preferred sampling method. However, concentrating pathogen inoculum or DNA may be appropriate for certain pathogens, especially for those with limited distribution capabilities. With regard to accurate pathogen quantification in plant samples, the accuracy of the assay may be enhanced further by calibrating against the amount of plant DNA.

Finally, and very importantly with respect to plant disease management, pathogen densities need to be coupled to thresholds at which damage may occur, and translated in accurate advice to growers. In the previous chapter, it is demonstrated for the model pathosystem radish seedling-*Rhizoctonia solani* that DNA array-based quantification of the pathogen in the growing substrate could be effectively correlated with disease development. However, in order to take the proper disease management decisions, such relationships need also to be established under practical conditions, as well as for other pathosystems for which the array is meant to be used. Therefore, extensive ecological and epidemiological studies still need to be conducted, studying the behavior of a pathogen in relation to both biotic and abiotic factors of its environment. In addition, although molecular assays can be performed routinely without any skilled taxonomical expertise, experts will still be necessary to interpret DNA array hybridization patterns, which may be fairly complex for matrices which contain a multitude of organisms such as soils or other growing media, and translate these patterns into an appropriate advice. After all, it is likely that more and other microorganisms will be detected using such sensitive, multiplex assays, than those that have been detected in the past using conventional techniques. For example, in Lievens *et al*. (2004), we reported the first case of root and foot rot of tomato caused by *Phytophthora infestans*, a pathogen which was, until then, not known to cause this disease. Without any doubt, the establishment of a database holding information on several disease related parameters, both of biotic and abiotic nature, will contribute to assessing the risk of a disease and losses as well as to taking the appropriate management decisions.

7.3 Conclusions and future perspectives

Increasingly, diagnostic laboratories and inspection agencies are searching for fast routine methods that provide reliable identification, sensitive detection, and accurate quantification of potentially plant pathogenic organisms. In addition, multiplex detection is an important aspect, taking into account efficiency, cost, time, and labor. Currently, DNA array technology is the most suitable technique to detect multiple plant pathogens in a single assay, even if they differ in only a single to a few bases in the gene that is targeted (Chapter 2; Lievens *et al*., 2006). As shown in Chapter 5, a quantitative aspect was added to a macroarray-based assay (Lievens *et al.*, 2005a), making this technology highly attractive for its use in practice. Currently, several diagnostic companies are using an extended version of the DNA array developed in our work, by which in its current format over 50 different plant pathogens, including fungi, oomycetes and bacteria, can be detected and quantified (www.DNAMultiscan.com). For instance, Microbiometrix (Belgium), Relab Den Haan (the Netherlands), and the Plant Diagnostic Clinic of the University of Guelph (Canada) are using this tool, called DNAMultiscan®, for routine plant pathogen diagnosis. In addition to diagnosis, the same approach is used by Blgg (Spain) as a pathogen monitoring tool (Riscover®) in hydroponics to prevent diseases by regularly assaying water samples. With timely and regular analyses, preventive treatments can be properly prescribed and performed, and in case infections are monitored, the afflicted plants can be cured or removed. Whereas previously preventive treatments were frequently applied without the knowledge of actual pathogen populations, this approach should result in well-founded control measures. Ultimately, this concept should reduce the number of treatments and thus result in minimal environmental impacts.

Obviously, the future will bring new technologies for detecting plant pathogens, largely because of the current efforts in genomics and molecular biosystematics and because of new platforms that have been developed primarily in the field of clinical medicine or even in the field of biological warfare. Whenever appropriate they generally find their way somewhat later to plant pathogen diagnostics as well. This can be illustrated by DNA array hybridization, essentially a reverse dot blot technique, which was originally developed to detect mutations related to different human genetic disorders (Saiki *et al.*, 1989) and was subsequently successfully applied to plant pathology (Lévesque *et al.*, 1998; Uehara *et al.*, 1999; Fessehaie

et al., 2003; Lievens *et al.*, 2003; Nicolaisen *et al.*, 2005). Another example includes the development of affordable, portable real-time PCR instruments such as the SmartCycler (Cepheid, Inc., Sunnyvale, CA) that was originally designed for military personnel to rapidly detect biological threats in the field. Nowadays, it enables sensitive on-site diagnosis of specific pathogens (Schaad *et al.*, 2003).

Most progress can be expected from the development of simple and rapid devices for on-site pathogen detection. Recently, new formats using antibody-based detection for very rapid presumptive on-site diagnosis have become available. These do not require specialized equipment or knowledge. Most of them use a membrane-based lateral flow assay, in which capillary forces generate a migration of the sample extract over specific antibodies (Fig. 7.2; Smits *et al.*, 2001; www.pocketdiagnostics.com). In case the antibodies recognize specific antigenic determinants a visual signal is generated immediately. One of the drawbacks, however, is the relatively low sensitivity, impeding widespread use. Nevertheless, because these assays are relatively inexpensive and require little labor and knowledge, there is a gaining interest to use these tests for in-field plant pathogen diagnostics (Danks and Barker, 2000).

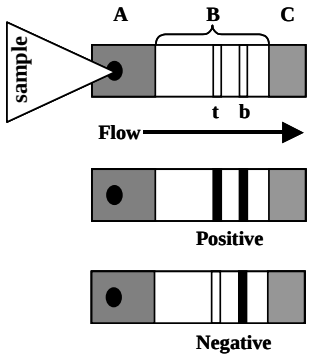


Fig 7-2. Membrane-based lateral flow assay. Capillary forces establish the migration of a sample extract from the sample pad (A) to the absorbent pad (C). The sample pad contains antibody-coated latex beads that capture target antigens if present in the sample. The mixture migrates along the detector strips (B) containing target-specific antibodies (t) and bead-specific antibodies (b). Beads containing antigenic determinants are trapped on the test line, whereas superfluous beads are trapped on the control strip as a control for the assay. Binding of beads to the detector strips immediately results in a visible line.

In clinical diagnostics biosensors that use DNA-based methods are currently developed (Wang, 2000). These devices contain a biological recognition element (e.g. specific DNA sequences) coupled with a physical transducer that translates recognition (e.g. hybridization)

into a measurable electronic signal such as light, current or frequency. Whenever applied to the diagnosis of plant pathogens this should lead to the development of simple, rapid, on-site detection systems. Another interesting development in medical diagnostics is the lab-on-a-chip instrument which integrates several processes (from DNA extraction to DNA analysis) within a single, portable, and fully automated instrument (Anderson *et al.*, 2000; Wang, 2000). However, it is unlikely that many of these devices will meet the desired requirements mentioned before in the near future. It is likely that those technologies that are cost-effective will only be used in routine plant pathogen diagnostics. In addition, only when new technologies become integrated with conventional tools and human expertise they will lead to a better understanding and, ultimately, prevention of diseases.

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