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Quantification in Multiplex Format as a Challenging Goal for Plant Pathogen Molecular Diagnostic Assays

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ABSTRACT

Accurate detection and identification of plant pathogens is fundamental to plant disease management. The lack of rapid, accurate and reliable means by which plant pathogens can be detected and identified has been one of the main limitations in plant disease management, and has prompted the development of alternative diagnostic technologies. Increasingly, molecular methods that are based on the detection of nucleic acids are implemented for routine plant pathogen diagnosis. Now that a number of molecular assays have been established, the latest challenge is to be able to quantify pathogen biomass, preferentially in multiplex format, with these assays. However, the development of technology for accurate quantification bears a number of pitfalls that will be highlighted in this review. The ability to perform pathogen quantification will result in a novel challenge; namely to be able to predict disease development based on pathogen densities in a specific environment. Issues that are of relevance to these topics are discussed in this review.

Keywords: DNA array, population density, pitfall, real-time PCR, disease risk
Abbreviations: cDNA, complementary DNA; **CFU**, colony forming units; **EMA**, ethidium monoazide; **IC-PCR**, immunocapture PCR; **MDA**, multiple displacement amplification; **PCR**, polymerase chain reaction; **PMA**, propidium monoazide; **rDNA**, ribosomal DNA; **RT-PCR**, reverse transcriptase PCR

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INTRODUCTION

The lack of rapid, accurate and reliable means by which different (types of) plant pathogens can be detected and identified is still one of the main limitations in plant disease management. This limitation has pushed the development of novel diagnostic technologies, of which those based on detection of nucleic acids are the most predominant (McCartney *et al*. 2003; Lievens *et al*. 2005b). This trend is enhanced by the growing availability of sequence data in public databases and the increased availability of microbial whole-genome sequences. In general, nucleic acid-based methods are much faster, more specific, more sensitive and more accurate than traditional methods based on (selective) culturing of pathogenic microorganisms and can be performed and interpreted by personnel with no specialized taxonomical expertise. However, perhaps even more important, these technologies allow the detection of microorganisms that cannot be cultured *in vitro* (McCartney *et al*. 2003; Lievens *et al*. 2005b). The number of pathogens that can be diagnosed by these methods has now grown to the extent that only few pathogens cannot be accurately identified. The remaining pathogens mainly concern species that contain pathogenic as well as non-pathogenic strains, such as the *formae speciales* of the fungal species *Fusarium oxysporum*. In such cases, the differences between these strains

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can not be resolved by targeting the conserved genes that are generally used, and thus the development of molecular markers becomes less straightforward because novel diagnostic regions need to be identified (Recorbet *et al*. 2003). The breadth of nucleic acid-based techniques for plant pathogen diagnosis is exemplified in **Table 1**, in which the studies that are discussed in this review are further dissected.

Although nucleic acid-based methods are routinely used in the diagnosis of human diseases (Sebire *et al*. 1998), and an online PCR primer database for plant pathogenic fungi and oomycetes is available (Ghignone and Migheli 2005), nucleic acid-based methods are not yet widely implemented for routine plant pathogen diagnosis. Several reasons, mainly of technical and economical nature, may explain this slow uptake. These include the lack of quantitative nature and multiplexing capabilities (i.e. the ability to detect many pathogens at the same time) of most available assays that should allow pathogen quantification and the detection of large numbers of pathogens in a single assay, respectively (Lievens and Thomma 2005). While quantification serves as the basis for the determination of population thresholds at which disease may occur and action thresholds when control measures should be taken to limit or prevent losses, simultaneous detection of multiple pathogens enables cost-effective comprehensive screening of complex samples. Ultimately, multiplexing capability should lead to comprehensive diagnostic kits that can detect all possible pathogens of a given crop in a single assay.

Recently developed novel molecular technologies can meet such demands (**Table 2**). While currently real-time PCR is the most accurate technique to quantify pathogen presence, DNA array technology is the most appropriate to simultaneously detect large numbers of microorganisms (Lievens *et al*. 2005b). With respect to sensitivity, both technologies are equally suited to detect microbial pathogens in concentration ranges in which they typically occur in agricultural and horticultural practice (Lievens *et al*. 2005a, 2007). However, since both technologies differ considerably in nature, obviously quantification in a multiplex format bears a number of technical difficulties and limitations. Furthermore, once accurate pathogen quantification is established, novel challenges will emerge. The ability to predict disease development based on pathogen densities in a certain environment is still in its infancy. Several aspects that are of relevance to novel developments in molecular plant pathogen diagnosis are discussed in this review.

REAL-TIME PCR

In order to quantify the presence of a certain pathogen by DNA-based techniques, the amount of its DNA should be directly correlated to the amount of its biomass. If PCR is used for target amplification in order to allow sensitive detection, several potential pitfalls that may skew accurate quantification are introduced. The non-linear nature of PCR amplification makes it challenging to relate the final amount of amplicon obtained to the amount of target DNA that was initially present in the sample. Although several studies have shown that upon extensive optimization of PCR conditions quantification in endpoint analysis-based PCR assays can be performed (e.g. Hu *et al*. 1993), the introduction of real-time PCR technology that is characterrized by on-line measurement of amplicons as they accumulate with each PCR cycle (Heid *et al*. 1996) has improved and simplified methods for accurate template quantification. Furthermore, since post-reaction processing has become useless, real-time PCR analysis is faster than conventional PCR analysis and the risk of post-PCR carry-over contamination of amplicons is eliminated. As extensively discussed in other reviews (Schaad and Frederick 2002; McCartney *et al*. 2003; Schena *et al*. 2004; Lievens *et al*. 2005b), accumulating amplicons can be detected with several detection chemistries that make use of either fluorescent DNA-intercalating dyes or sequence-specific fluorescent probes. While use in practice has shown that most of these chemistries are highly comparable in terms of overall performance, with respect to other traits (such as cost issues) they all have their own advantages and disadvantages.

Currently, in molecular plant pathology, real-time PCR is the most reliable technique to accurately quantify the amount of DNA from specific pathogens, including fungi (Brouwer *et al*. 2003; Filion *et al*. 2003; Gao *et al*. 2004; Lievens *et al*. 2006a), oomycetes (Brouwer *et al*. 2003; Schroeder *et al*. 2006), bacteria (Brouwer *et al*. 2003; Salm and Geider 2004), viruses (Roberts *et al*. 2000), viroids (Boonham *et al*. 2004), phytoplasmas (Bianco *et al*. 2004), and virus vectors (Walsh *et al*. 2005). However, the total amount of real-time PCR reactions in a single tube presently is severely limited. While simultaneous detection and quantification of multiple target organisms is not possible when using non-specific detection chemistries (DNA-intercalating dyes such as SYBR Green®), 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. Similarly, Bertolini *et al*. (2003) developed an assay for simultaneous detection of five microbial pathogens, encompassing 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 dyes available and the nature of the energizing light source that can be used in real-time PCR instruments (Mackay *et al*. 2002). As a consequence, current real-time PCR instrumentation needs to be technologically improved to allow simultaneous quantification of more targets. Therefore, one of the present challenges in molecular disease diagnosis is the development of quantitative multiplex pathogen assays that can effectively screen for large numbers of targets present in a given sample (Lievens and Thomma 2005).

DNA ARRAYS

Detection of multiple pathogens in a single assay has been a continuous technological challenge in plant pathology. For highly sensitive detection of pathogens, target amplification is generally required. However, because of technical limitations the number of targets that can be simultaneously amplified with PCR is limited to a few. DNA arrays, originally developed for gene expression profiling (Schena *et al*. 1996; Lashkari *et al*. 1997), can be used to detect, in theory, an unlimited number of different organisms in a single assay (Martin *et al*. 2000; Lievens and Thomma 2005). With this technology, specific detector oligonucleotides are immobilized on a solid support, essentially allowing reverse dot-blot hybridization. Depending on the size and spacing of the dots, low-density macroarrays (e.g. on a nylon membrane) or high-density microarrays (e.g. on a glass slide) can be generated. For signal amplification, in general the target DNA of interest is amplified using universal PCR primers that anneal to conserved sequences flanking diagnostic domains, labeled, and subsequently hybridized to the array. Like this, numerous targets can be amplified with a single primer pair, while target discrimination occurs afterwards on the array. This strategy has proven to be successful for the detection and identification of various microorganisms including fungi (Lievens *et al*. 2003; Nicolaisen *et al*. 2005), oomycetes (Lévesque *et al*. 1998; Tambong *et al*. 2006), bacteria (Fessehaie *et al*. 2003), nematodes (Uehara *et al*. 1999), and viruses (Boonham *et al*. 2003). Especially for viral pathogens for which no appropriate universal primers are available, the combination of sequence-nonspecific amplification techniques and DNA arrays may be employed in order to increase the amount of pathogens that can be simultaneously detected. In a recently published study, the distinct RNA viruses *Cucumber mosaic virus*, *Potato virus Y*, and *Potato leaf roll virus* were detected simultaneously using a macroarray with several 70-mer oligonucleotide detector oligonucleotides per virus (Agindotan and Perry

Table 1 Recent studies that are referred to in the text using PCR-based techniques for plant pathogen identification, detection and quantification.

Ref.	Target pathogen(s)	Sample(s) tested	Technique	Target gene(s)	Sensitivity	Specificity	N° strains *
a	*Cucumber mosaic virus* (CMV), *Potato virus Y* (PVY) and *Potato leaf roll virus* (PLRV)	Plant samples	Anchor primer-based PCR amplification and DNA macroarray hybridization	Multiple genome sequences	10^{-2} dilution of a virus-infected plant RNA extract	No cross-reactions observed	8
b	CMV, *Cherry leaf roll virus* (CLRV), *Strawberry latent ringspot virus* (SLRSV), *Arabis mosaic virus* (ArMV) and *Pseudomonas savastanoi* pv. *savastanoi*	Plant samples	Multiplex nested reverse transcriptase (RT-) PCR	Coat protein gene (CMV, ArMV), 3' untranslated region (3' UTR) (CLRV, SLRSV) and IAA-lysine synthetase (iaaL) gene (*P. savastanoi* pv. *savastonoi*)	10^{-4} – 10^{-5} dilution of virus-infected tissue and 1 cell ml^{-1}	Not tested	-
c	Phytoplasmas associated with grapevine flavescence dorée (subgroups 16SrV-C and 16SrV-D)	Plant samples	Real-time PCR using TaqMan chemistry	16S rRNA gene	Not tested	No cross-reactions observed for primer annealing at 64°C	19
d	*Potato virus Y, X, A,* and *S* (PVY, PVX, PVA and PVS)	Plant samples	Species specific primer-based PCR amplification and DNA microarray hybridization	Polyprotein (PVY) and coat protein gene (PVX, PVA and PVS)	1/1,600 dilution of virus-infected tissue	No discrimination between strains with 80-90% sequence identity	5
e	Potato spindle tuber viroid (PSTVd)	Plant samples	Real-time RT-PCR using TaqMan chemistry	Viroid RNA	10^{-6} dilution of a viroid-infected plant RNA extract	Cross-reaction with the closely related pospiviroid Tomato chlorotic dwarf viroid	17
f	*Alternaria brassicicola, Botrytis cinerea, Peronospora parasitica*, *Erwinia carotovora* and *Pseudomonas syringae*	*In vitro* cultures and plant samples	Real-time PCR using SYBR Green chemistry	5.8S rRNA and cutinase gene (*A. brassicicola*), tubulin and actine gene (*B. cinerea*), 16S rRNA gene and a species-specific sequence (*E. carotovora*), 18S rRNA gene and a species-specific sequence (*P. parasitica*), and the outer-membrane proteine (oprF) gene (*P. syringae*)	Not tested	Not tested	-
g	*Leptosphaeria maculans* and *Pyrenopeziza brassicae*	Spore suspensions and spore trap samples	Conventional (*L. maculans*) and nested (*P. brassicae*) PCR	Long interspersed repetitive sequence	10 spores	Not tested	-
h	*Clavibacter michiganensis* ssp. *sepedonicus, E. carotovora* ssp. *atroseptica* and *carotovora*, *E. chrysantemi* and *Ralstonia solanacearum*	*In vitro* cultures	Universal primer-based PCR amplification and DNA macroarray hybridization	16S-23S intergenic spacer (IGS)	Not tested	Cross-reactions were rare and occurred only for closely related (sub)species	64
i	*Fusarium solani* f. sp. *phaseoli* and *Glomus intraradices*	*In vitro* cultures and soil samples	Real-time PCR using SYBR Green chemistry	Elongation factor 1 alpha gene (*F. solani*) and 18S rRNA gene (*G. intraradices*)	5 pg DNA	No cross-reactions observed	35
j	*F. solani* f. sp. *glycines*	*In vitro* cultures and root samples	Real-time PCR using TaqMan chemistry	Mitochondrial small-subunit rRNA gene	90 fg DNA	No cross-reactions observed	11
k	*Alternaria brassicae*	*In vitro* cultures and cruciferous seeds	Real-time PCR using SYBR Green chemistry	A nonribosomal peptide synthase (NRPS) and an ATP-binding cassette (ABC) transporter gene	500 fg DNA	No cross-reactions observed	28
l	*Verticillium albo-atrum* and *V. dahliae*	*In vitro* cultures and plant samples	PCR	Internal transcribed spacers (ITS) I and II	Not tested	Not tested	-
m	*Phytophthora ramorum*	*In vitro* cultures and plant samples	Real-time PCR using TaqMan chemistry	ITS I	10 pg DNA	Slight cross-reaction with the closely related species *Phytophthora lateralis*	57

Ref. = Reference: a = Agindotan and Perry (2007); b = Bertolini *et al.* (2003); c = Bianco *et al.* (2004); d = Boonham *et al.* (2003); e = Boonham *et al.* (2004); f = Brouwer *et al.* (2003); g = Calderon *et al.* (2002); h = Fessehaie *et al.* (2003); i = Filion *et al.* (2003); j = Gao *et al.* (2004); k = Guillemette *et al.* (2004); l = Hu *et al.* (1993); m = Hughes *et al*. (2006)

* N° of strains used to test specificity

Table 1 (cont.)

Ref.	Target pathogen(s)	Sample(s) tested	Technique	Target gene(s)	Sensitivity	Specificity	N° strains *
n	*Tomato mosaic tobamovirus* (ToMV), and *Tobacco mosaic tobamovirus* (TMV)	Plant samples	Singleplex and multiplex immunocapture RT-PCR	Coat protein and viral movement protein gene	10-100 fg (singleplex assay) and 1-10 pg virus ml^{-1} plant extract (multiplex assay)	No cross-reactions observed	33
o	*Cylindrocarpon destructans* f. sp. *panacis*	*In vitro* cultures and soil samples	Real-time PCR using SYBR Green chemistry	Intergenic spacer (IGS)	100 fg DNA	No cross-reactions observed	26
p	*Phytopthora* spp., *P. cinnamomi*, *Pythium acanthicum*, *P. aphanidermatum* and *P. ultimum*	*In vitro* cultures	Universal primer-based PCR amplification and DNA macroarray hybridization	ITS I and II	Not tested	Cross-reactions were rare and occurred only for closely related species	166
q	*Fusarium* spp., *F. oxysporum*, *Verticillium* spp., *V. albo-atrum* and *V. dahliae*	*In vitro* cultures, soil, plant and water samples	Universal primer-based PCR amplification and DNA macroarray hybridization	ITS I and II	500 fg DNA	Cross-reactions were rare and occurred only for closely related species	72
r	*F. oxysporum*, *F. solani, Pythium aphanidermatum*, *P. ultimum*, *Rhizoctonia solani*, *V. albo-atrum* and *V. dahliae*	*In vitro* cultures, soil and plant samples	Universal primer-based PCR amplification and DNA macroarray hybridization	ITS I and II	500 fg DNA	No cross-reactions observed	175
s	*F. solani*, *Rhizoctonia solani*, *P. ultimum*, and *Verticillium* spp.	*In vitro* cultures, soil and plant samples	Real-time PCR using SYBR Green chemistry	ITS I and II	500 fg DNA	No cross-reactions observed	55
t	*R. solani*, *Trichoderma* spp., *T. hamatum* isolate 382 (T_{382})	*In vitro* cultures, potting mix and plant samples	Universal primer-based PCR amplification and DNA macroarray hybridization	ITS II (*R. solani* and *Trichoderma* spp.) and an unknown T_{382}-specific DNA sequence	500 fg DNA	Cross-reactions for strains closely related to T_{382}	225
u	*Monilinia fructicola* and *Botryoshaeria dothidea*	*In vitro* cultures, plant and spore trap samples	Nested PCR	Microsatellite DNA sequences	1 fg DNA/2 conidia	No cross-reactions observed	122
v	*Tilletia* spp.	*In vitro* cultures and wheat seeds	Real-time PCR using TaqMan chemistry	28S rRNA gene	50 pg DNA	No cross-reactions observed	36
w	*Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV)	Plant samples	Singleplex and multiplex real-time PCR using TaqMan chemistry	Coat protein (BaYMV) and 3' UTR (BaMMV)	10^{-3} (BaYMV) and 10^{-4} (BaMMV) dilution of a virus-infected plant RNA extract	No cross-reactions observed	79 field samples
x	*Fusarium graminearum/F. culmorum*, *F. pseudograminearum*, *F. poae*, *F. sporotrichioides*, *F. equiseti*, *F. langsethiae*, and *F. tricinctum/F. avenaceum*	*In vitro* cultures and wheat seeds	Universal primer-based PCR amplification and DNA microarray hybridization	ITS II	Not tested	Cross-reactions were rare and occurred only for closely related species	55
y	*Tomato spotted wilt virus* (TSWV)	Plant samples	Real-time RT-PCR using TaqMan chemistry	N gene	500 fg of total plant RNA sample	Not tested	-
z	*Erwinia amylovora*	*In vitro* cultures and plant samples	Real-time PCR using SYBR Green and TaqMan chemistry	Plasmid pEA29	50 cells	No cross-reactions observed	19
aa	*Pseudomonas syringae* pv. *phaseolicola*	*In vitro* cultures and bean seed extracts	BIO-PCR using two consecutive PCR rounds (nested PCR)	Phaseolotoxin (tox) gene	0.1 pg DNA/1-2 cells	Not tested	-

Ref. = Reference: n = Jacobi *et al.* (1998); o = Kernaghan *et al.* (2006); p = Lévesque *et al.* (1998); q = Lievens *et al.* (2003); r = Lievens *et al.* (2005a); s = Lievens *et al.* (2006a); t = Lievens *et al.* (2007) ; u = Ma *et al.* (2003); v = McNeil *et al.* (2004); w = Mumford *et al.* (2004); x = Nicolaisen *et al.* (2005); y = Roberts *et al.* (2000); z = Roberts *et al.* (2000); aa = Schaad *et al.* (1995)

* N° of strains used to test specificity

Table 1 (cont.)

Ref.	Target pathogen(s)	Sample(s) tested	Technique	Target gene(s)	Sensitivity	Specificity	N° strains *
bb	*Pythium abappressorium*, *P. attrantheridium*, *P. heterothallicum*, *P. irregulare* group I and IV, *P. paroecandrum*, *P. rostratifingens*, *P. sylvaticum* and *P. ultimum*	*In vitro* cultures and soil samples	Real-time PCR using SYBR Green	ITS I and II	10 fg DNA	No cross-reactions observed	77
cc	Fusarium circinatum	*In vitro* cultures, spores and spore trap samples	Real-time PCR using SYBR Green	IGS	10 pg DNA or 100 spores/100 µl	No cross-reactions observed	31
dd	*B. cinerea*, *Penicillium expansum*, *Podosphaera leucotricha*, *Venturia inaequalis* and *E. amylovora*	*In vitro* cultures, plant and spore trap samples	Universal primer-based PCR amplification and DNA macroarray hybridization	ITS I and II	20 or 30 conidia per leaf disk or I-rod, respectively	Cross-reactions were rare and occurred only for closely related species	57
ee	*Acidovorax avenae* ssp. *avenae*	*In vitro* cultures and rice seeds	BIO-PCR using two consecutive PCR rounds (nested PCR)	16S-23S IGS	1-2 cells	No cross-reactions observed	161
ff	All *Pythium* species	*In vitro* cultures and soil samples	Universal primer-based PCR amplification and DNA macroarray hybridization	ITS I and II	Not tested	Cross-reactions were rare and occurred only for closely related species	103
gg	*P. ramorum*	*In vitro* cultures and plant samples	Multiplex real-time PCR using TaqMan chemistry	ITS I	100 fg DNA	Slight cross-reaction with the closely related species *Phytophthora lateralis*	59
hh	*P. ramorum* and *P. pseudosyringae*	*In vitro* cultures and plant samples	Multiplex real-time PCR using TaqMan chemistry	Spacer sequences between the cytochrome oxidase (COX) I and II genes	1 fg DNA	No cross-reactions observed	85
ii	*Pratylenchus brachyurus*, *P. coffeae*, *P. crenatus*, *P. loosi*, *P. penetrans*, *P. vulnus* and *P. zeae*	Pure nematode cultures	Universal primer-based PCR amplification and DNA macroarray hybridization	ITS I	Not tested	No cross-reactions observed	29
jj	*R. solanacearum* and *R. solanacearum* biovar 2A	*In vitro* cultures, water and plant samples	Multiplex real-time PCR using TaqMan chemistry	16S rRNA gene (*R. solanacearum*) and an unknown biovar 2A-specific DNA sequence (*R. solanacearum* biovar 2A)	100 cells ml^{-1}	Few cross-reactions for the *R. solanacearum* assay; no cross-reactions for the *R. solanacearum* biovar 2A assay	42

Ref. = Reference: bb = Schroeder *et al.* (2006); cc = Schweigkofler *et al.* (2004); dd = Sholberg *et al.* (2005); ee = Song *et al.* (2004); ff = Song *et al.* (2004); gg = Tomlinson *et al.* (2005); hh = Tooley *et al.* (2006); ii = Uehara *et al.* (1999); jj = Weller *et al.* (2000)
* N° of strains used to test specificity

2007). To this end, upon RNA extraction of plant material, complementary DNA (cDNA) and second-strand syntheses were performed using random pentamers with an anchor primer sequence at the 5′ end. PCR amplification using the anchor primers resulted in unbiased amplification of plant and viral RNAs that were used as a target in hybridization analyses on the array.

The discriminative power of DNA array technology has proven to be very high since even microbes whose target sequences only differs by a single nucleotide polymorphism can be discriminated if the appropriate hybridization conditions are met (Lievens *et al*. 2006b). Currently, one of the most comprehensive arrays described allows the identification and detection of more than 100 species that all belong to the *Pythium* genus (Tambong *et al*. 2006). Similarly, a DNA array has been developed to detect a wide range of fungal and bacterial pathogens that occur in horticultural crops and turf grasses (Lievens and Thomma 2005; www.DNAmultiscan.com).

Recently, it has been demonstrated that array technology can not only be used for multiplex pathogen detection, but also for the quantitative determination of microbial population densities as under certain conditions the hybridization signals on the array are proportional to the quantity of target DNA present in the sample (Lievens *et al*. 2005a; Sholberg *et al*. 2005). As a result, multiple pathogens can be simultaneously detected and quantified in a single assay. Taking into account the unlimited expanding possibilities of DNA arrays, this technique has the potential to become the new benchmark in plant pathogen diagnosis.

PITFALLS AND LIMITATIONS OF PATHOGEN QUANTIFICATION

Despite the advantages of using molecular methods for plant pathogen diagnosis, significant limitations that can hamper accurate detection and identification remain to be solved. However, many of these limitations also apply to the classical detection techniques and are not inherent to the use of molecular methods. For pathogen quantification, additional difficulties arise that can hamper accurate analysis. These will be discussed in this section.

Table 2 Comparison of technical and economical features of the most currently used techniques for the detection and quantification of plant pathogens.

	ELISA	PCR	Real-time PCR	DNA macroarray	DNA microarray
Ease of development	+	+++	++	++	++
Ease of sample preparation	++	++	++	++	++
Specificity	++	+++	+++	+++	+++
Sensitivity	+	++	+++	++	++
Quantification	++	+	+++	+++	++
Multiplexing capabilities	+	+	+	+++	+++
Speed	+++	+++	+++	+	+
Potential portability	+	-	+++	-	-
Cost effectiveness per assay	+++	+++	++	++	+
Cost effectiveness per detected organism	+	+	+	+++	+++

'+' and '-' signs indicate the capacity of the technique to satisfy the respective requirement: - means not possible; + below average; ++ average; +++ good.

Quality and purity of the extracted nucleic acids

Obtaining nucleic acids of high quality used to be a major bottleneck in nucleic acid-based detection procedures (Wilson 1997). Therefore, first of all, the quality and purity of the extracted nucleic acids is crucial for successful quantification. Several approaches have been described to extract genomic DNA or RNA from complex samples. The presence of humic substances, heavy metals, polysaccharides and phenolic secondary metabolites that are co-extracted from soil and plant samples can greatly affect PCR efficiencies, even to the level that PCR amplification is completely inhibited. In many cases these problems may be circumvented by improved extraction methodologies (McCartney *et al*. 2003), for instance through the use of commercially available extraction kits (Lievens *et al*. 2005a). To obtain high yields, the majority of these methods involve mechanical disruption of the sample to release nucleic acids. Although DNA quality is a crucial factor for diagnostic analysis, also the amount of genomic DNA obtained may be limiting, even for PCR-based methods. Some samples, such as those containing rigid and harsh materials, are relatively resilient to physical disruption and may still be difficult to extract sufficient DNA of high quality from. A relatively new technique that overcomes this limitation and that generates sufficient DNA for PCR analysis is multiple displacement amplification (MDA) by which whole genomes can be amplified efficiently (Dean *et al*. 2002; Foster and Monahan 2005). In contrast to conventional PCR, with this method amplification is carried out in an isothermal process using phosphorothioate-modified random hexamer oligonucleotides that act as primers and bacteriophage Phi29 DNA polymerase. A typical reaction is performed at 30°C for 8-16 h. During elongation, the exonuclease-deficient DNA polymerase displaces the polymerized DNA strand in front. Next, the displaced strands serve again as templates for other hexamers, resulting in a cascade of DNA amplification (Foster and Monahan 2005). MDA has the potential to become an important technology in plant pathogen diagnosis, especially to process samples that contain low levels of DNA or poor quality DNA, such as herbarium samples or samples exhibiting low pathogen densities. Whether this technology is also suitable as a basis for reliable quantification still remains to be determined.

The quality of extracts can be monitored through the use of several controls. In many plant-based assays, controls have been designed for the detection of endogenous plant genes (Weller *et al*. 2000; Mumford *et al*. 2004). Alternatively, the extract can be spiked with exogenous control DNA that can be amplified in the same (Cubero *et al*. 2002) or in a separate reaction (Lievens *et al*. 2006a). A different approach is to spike the sample with an additional target containing the same primer annealing sites as the target pathogen, but with a different internal sequence that can be detected by a different fluorescently labeled probe (Kox *et al*. 2005) or sequence-specific detector oligonucleotide. However, while this approach has advantages over other controls, it may have a negative effect on the sensitivity of the assay and furthermore requires considerable optimization efforts.

Sample collection

In addition to the quality and purity of the extracted nucleic acids, also the way in which samples are composed requires attention. Current molecular detection techniques often start from sample amounts of less than a gram of biological material. This is in particular enhanced by the generally high sensitivity of these technologies. As a result, appropriate sampling strategies that account for possible spatial variability in pathogen populations and that ensure a statistically representative sample will become even more challenging than it already used to be. Pooling multiple small subsamples into one sample, or processing subsamples from a homogenized bulk sample is a desired sampling method (Schroth and Kolbe 1994). Furthermore, a standard sample size can minimize variation between different analyses, and facilitate to relate DNA amounts to pathogen densities. Expressing the amount of soil sample in units of dry weight will decrease variation and allow fair comparison between different samples. For accurate pathogen quantification in plant samples, reliability of the assay may be enhanced by calibrating against the amount of plant DNA in the sample (Gao *et al*. 2004). However in other cases, for instance when plant tissue is heavily necrotized due to pathogen infection, an equal amount of (leaf) surface area may be a more appropriate calibration measure (Brouwer *et al*. 2003).

Sensitivity

A potential factor that may hamper sensitive pathogen detection and quantification is the presence of large excess of non-target DNA. Nevertheless, recent studies have shown that target quantification by real-time PCR or DNA arrays is not influenced by large amounts of non-target DNA that typically reflect agricultural and horticultural practice (Brouwer *et al*. 2003; Lievens *et al*. 2005a; Hughes *et al*. 2006; Schroeder *et al*. 2006; Lievens *et al*. 2007). However, in order to increase sensitivity, for certain pathogens concentrating pathogen inoculum or pathogen-derived nucleic acids may be appropriate, especially for those with limited distribution capabilities, or when low pathogen densities may have considerable disease-causing capabilities. Immunocapture PCR (IC-PCR), which uses antibodies to selectively isolate and enrich the target prior to PCR amplification, is a powerful method to tackle this problem. This approach has been successfully used to detect a number of plant pathogenic viruses (Jacobi *et al*. 1998), but may be applied to other pathogens as well. For example, motile zoospores may be trapped by immobilized antibodies (Cahill and Hardham 1994) and compose a sample that can be used in subsequent detection assays.

Distinction between viable and non-viable organisms

The rate of DNA degradation from dead cells is relatively high in complex matrices such as soils or growing media due to the high microbial activity (Herdina *et al*. 2004), sug-

gesting that interference by DNA derived from non-viable cells may be trivial. Nevertheless, another potential bottleneck for pathogen quantification is the possibility to detect DNA from dead or non-active organisms, which may lead to over-quantification of pathogen presence. To exclude detection of dead organisms, a culturing step prior to PCR amplification can be included (BIO-PCR; Schaad *et al*. 1995). However, disadvantages of this approach are the labor-intensive and time-consuming nature, implications for quantification since the initial amount of target is amplified in an uncontrolled manner, and the inability to detect non-culturable organisms. As an alternative, attempts have been made to use chemicals such as ethidium monoazide (EMA; Rudi *et al*. 2005) or propidium monoazide (PMA; Nocker *et al*. 2006) to differentiate between viable and non-viable organisms. Both EMA and PMA can only penetrate compromised membranes (that generally occur in dead cells) after which it intercalates into DNA upon photoactivation of the azide group. Cross-linking of these chemicals to DNA renders the DNA insoluble, resulting in its removal during subsequent genomic DNA extraction (Nocker *et al*. 2006). These chemicals cannot cross the intact membranes of living cells, of which the DNA is isolated during normal extraction procedures. Another alternative to prevent detection of dead organisms is the use of RNA as a target, instead of DNA, in combination with reverse transcriptase PCR (RT-PCR; Tann and Weis 1992). Since RNA is less stable than DNA, RNA will be degraded even more quickly in dead organisms. In addition, messenger RNA (mRNA) is only produced by metabolically active cells, making mRNA suitable to specifically detect viable microorganisms (Bleve *et al*. 2003; Morin *et al*. 2004). However, working with RNA is often considered to be technically challenging because of its fragile nature.

Occurrence of different pathogen propagules

Especially with respect to fungal pathogens, reliable quantification may be complicated since most fungi can occur as different propagules including spores, resting structures such as (micro)sclerotia, and mycelium, which are likely to be co-extracted during DNA extraction (Dickie *et al*. 2002). However, so far, most experiments have been conducted with artificially infested samples, inoculated with either individual spores or mycelia (Filion *et al*. 2003), whereas in general the developed assays have not yet been validated under field conditions. At present it is still unclear how the proportion of different propagules varies under practical conditions, and also the relationships between densities of these different structures and disease development are largely unknown. Nevertheless, 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).

Target gene copy number

Another factor that may complicate accurate quantification is the multicopy nature of many of the genomic regions targeted in molecular plant pathogen diagnosis. Currently, ribosomal DNA (rDNA) sequences are the primary target for the development of molecular diagnostics for bacterial, fungal or oomycete plant pathogens as well as for nematodes for several reasons (McCartney *et al*. 2003; Lievens *et al*. 2005b). First, this gene is ubiquitously present in all organisms and, because of its high discriminatory potential, has been used extensively in phylogenetic studies. As a consequence, many rDNA sequences are available in public databases, aiding the development of diagnostic assays. Furthermore, in general rRNA genes are present in high copy numbers, which facilitates sensitive detection. However, this latter feature may also complicate quantification, as accurate quantification may be biased if the copy number varies significantly between genomes from different strains of the same species. So far, such variation has been described for a few bacterial species where little variation in rRNA copy number was observed for different strains belonging to the same species (Patra *et al*. 2002; Tourova 2003). Nevertheless, varying rRNA copy numbers between different strains of the same species have not yet been reported for fungal, oomycete or nematode species. Furthermore, small variation that does not exceed a significant order of magnitude is often not considered to have significant conesquences for the prediction of disease development.

Assessment of damage and action thresholds

Finally, pathogen quantification results in the need to relate DNA amounts via pathogen densities to damage thresholds and to action thresholds at which appropriate control measures should be employed to effectively limit economical loss. Traditionally, the assessment of disease risks has been based on the number of colonies formed (colony forming units; CFU) by dilution plating on selective media (Davet and Rouxel 2000). Alternatively, for certain soil-borne diseases such analyses have been carried out using bioassays in which susceptible hosts are grown in aliquots of infested field soil. In the case where nucleic acid-based techniques are used to quantify the occurrence of a pathogen, DNA concentrations should be linked to such population and inoculum measurements (Ippolito *et al*. 2004; Schroeder *et al*. 2006) which, in turn, can be related to disease development. However, to reliably predict the risk of disease, a direct correlation between DNA concentrations and the actual disease occurrence should be realized. In this respect, recent studies have shown that real-time PCR and DNA array-based quantification methods are similar in reliability, or even more reliable, to assess fungal populations than bioassays or CFU data (Kernaghan *et al*. 2006; Lievens *et al*. 2007). However, predicting disease severity solely based on pathogen densities is not obvious as disease is also significantly influenced by the environment in which the interaction between pathogen and host takes place. The presence of other pathogens may enhance disease symptoms, and pathogens causing similar symptoms may complicate the interpretation of disease severity data. Furthermore, soil characteristics may significantly impact disease expression, and the same pathogen densities in different soils may lead to a completely different outcome with respect to disease development. Therefore, disease severities obtained in greenhouse assays with a given pathogen inoculum density may not be representative for other environments and conditions.

EXAMPLES OF APPLICATIONS

Some of the technologies described so far in this review are currently being implemented in a wide variety of plant pathogen diagnostic applications. Some examples of applications in which rapid pathogen detection and accurate quantification are equally important criteria are described in this section.

Monitoring seed health

Detection and quantification of seed-borne pathogens is important to guarantee high quality seed, more in particular for seed certification and for deciding whether a disinfestation treatment should be performed or not. As low inoculum levels in seeds may still result in disease later on, highly sensitive detection methods are required. Whereas in some cases no seed infestation is tolerated at all (e.g. for quarantine organisms), in other cases infestation below a certain level may be tolerated as long as this will not result in significant losses. In such latter cases, seed health assays need to be quantitative in nature. Traditionally, seed health testing involves an assay in which samples of a seed lot are grown and inspected for symptoms. Alternatively, seeds can be plated on semi-selective media enabling growth of specific pathogens. In addition, seed washings can be performed of which the washing fluids are subsequently inoculated on in-

dicator plants. All these methods are generally slow, labor-intensive and require skilled personnel, and they are not suited for rapid, high-throughput screening. The advent of rapid, sensitive and quantitative diagnostic techniques may open alternative ways for seed quality testing (Reeves 1998). For example, Song *et al*. (2004) developed a BIO-PCR assay, combining a preculturing step with DNA amplification, for specific and sensitive detection of *Acidovorax avenae* ssp. *avenae* in rice seeds. Alternatively, Guillemette *et al*. (2004) used real-time PCR technology for the detection of *Alternaria brassicae* in cruciferous seed. With regard to quantifying the level of seed contamination, a real-time PCR assay has been developed to quantify the level of *Tilletia* spp. in wheat-seed (McNeil *et al*. 2004).

Monitoring airborne inoculum

Airborne inoculum plays an important role in the spread of certain plant diseases. Consequently, the ability to accurately detect and quantify pathogen inoculum directly from air samples provides a reliable method to assess disease risks (Gilles *et al*. 2000). In the past, spore traps have been developed to collect spores and other pathogen propagules from the air. In general, spores are impacted on adhesive coated plastic tape followed by microscopic identification and enumeration. With the advent of molecular methods to detect and quantify pathogen DNA, various types of these samplers are now being used to collect airborne spores prior to PCR-based identification (Calderon *et al*. 2002; Ma *et al*. 2003; Schweigkofler *et al*. 2004; Sholberg *et al*. 2005). A major advantage of this combination is that difficulties with microscopic identification are avoided, resulting in accurate monitoring of potential pathogens before the actual disease takes place. As a result, this should lead to proper preventive measures to control potential diseases.

Monitoring plant and substrate health based on pathogen population densities

The disease-causing potential of soils, artificial substrates, or recirculating water from greenhouses is generally evaluated using labor-intensive bioassays that depend on scoring of symptoms, which is often subjective. As a result, other methods than just visually observing plant symptoms are desired to measure plant or substrate health (Lievens *et al*. 2007). Furthermore, to be able to take preventive measures, forecasting of potential disease outbreaks is warranted. With the advent of molecular techniques, especially those allowing quantification in a multiplex format, preventive treatments can be properly prescribed and performed based on the population densities measured in the test sample. Whereas previously preventive treatments were frequently applied without the knowledge of actual pathogen populations, this approach should result in well-founded control measures with minimal environmental impact. Currently, several companies start to implement DNA arrays as multi-pathogen monitoring tools to prevent diseases in hydroponic growing systems by regularly assaying recirculating water samples. Nevertheless, profiling the presence of potential pathogens in recirculating water, growing media and diseased plants still requires considerable expertise with respect to data interpretation. Making the discrimination between the primary pathogens that actually caused the disease, and the secondary pathogens that subsequently caused opportunistic infections may be difficult. Furthermore, establishing action levels presently still requires expertise. Further optimization of plant pathogen detection technologies should focus on these issues.

CONCLUSIONS AND FUTURE PERSPECTIVES

Recent developments in molecular plant pathogen diagnosis have spurred the increased use of nucleic acid-based techniques in practice. Increasingly, laboratories that provide diagnostic services are implementing nucleic acid-based methods for swift routine detection and identification of plant pathogens. Now that a number of molecular assays have been established, pathogen quantification is becoming more and more important, especially with regard to estimating the risks on disease, spread of inoculum and economic losses. In addition, pathogen quantification enables monitoring the effects of control measures applied to combat a pathogen. Recently, new technologies have been developed that can be used to estimate pathogen amounts, either in singleplex or in multiplex format (McCartney *et al*. 2003; Lievens and Thomma 2005; Lievens *et al*. 2005b). With increased efforts to circumvent the potential limitations associated with molecular quantification techniques, the technologies discussed in this review will undoubtedly be at the frontline of reliable pathogen quantification and become a valuable tool in plant disease management. Nevertheless, at present molecular diagnostics are still relatively expensive in terms of capital investment and facilities and, as a result, are only pertinent to well equipped laboratories. Therefore, the next challenge is to bring molecular diagnostics into the field allowing on-site pathogen testing and quantification (Lievens and Thomma 2005; Mumford *et al*. 2006). Antibody-based lateral flow devices, originally developed for pregnancy testing, have successfully been used for on-site diagnosis of human diseases (Smits *et al*. 2001), but also for detection of plant viruses and even fungi (Danks and Barker 2000; Thornton *et al*. 2004). Such lateral-flow assays generally consist of a nitrocellulose detection strip encompassing a sample application pad, a reagent pad that contains dried labeled antibodies, a test pad, and an absorption pad. A sample extract is applied on the application pad and transported through the reagent pad by capillary forces. At the test line, other immobilized antibodies bind the antigens to produce a visual signal. A control line antibody confirms the test ran successfully, meaning that the sample flowed through the complete length of the test strip. These tests are relatively inexpensive, simple, can be performed in a few minutes, and may even allow (semi-) quantitative detection (Chan *et al*. 2003; Thornton *et al*. 2004). However, although producing antibodies with high specificity is relatively easy for viruses it is more difficult for bacterial plant pathogens or more complex organisms such as fungi and oomycetes (Ward *et al*. 2004). Furthermore, antibody-based techniques can not be applied to poorly sophisticated organisms such as viroids that lack detectable proteins (Hadidi *et al*. 2003). These limitations do not occur for nucleic acid-based diagnostic assays. Recently, real-time PCR platforms have become available for the detection and quantification of micro-organisms in the field (Schaad *et al*. 2003). The development of such instruments has been driven by clinical and veterinary medicine as well as by increased attention for biosecurity and biological warfare, but they now have found their way towards applications in plant pathogen diagnosis as well (Schaad and Frederick 2002; Tomlinson *et al*. 2005). One of the first portable real-time PCR platforms is the SmartCycler (Cepheid, Sunnyvale, CA, USA) that allows up to 16 samples to be tested simultaneously, each with independently controlled thermal cycling and fluorescence monitoring. Other real-time PCR instruments for the detection of microorganisms in the field involve the R.A.P.I.D. (Ruggedized Advanced Pathogen Identification Device), the RAZOR instrument developed by Idaho Technologies (Salt Lake City, UT, USA), and the Bioseeq developed by Smiths Industries (Edgewood, MD, USA). Apart from their use in plant disease management, they could have merit for monitoring imported plants and plant products at borders and other points of inspection. Obviously, these new developments pose new challenges for sample processing as several limitations inherent to field-testing need to be overcome, such as PCR reagents that are stable at ambient temperature (Klatser *et al*. 1998; Tomlinson *et al*. 2005).

Several diagnostic laboratories have implemented DNA array technology for routine plant pathogen detection, as well as for monitoring plant and substrate health. However, although DNA macroarrays provide efficient, rapid and

cost-effective analyses, a major drawback is the still relatively labor-intensive nature of these analyses and the need of a well-equipped laboratory. Therefore, the challenge is to develop more automated systems that can screen for multiple targets. Considerable progress can be expected from PCR arrays, combining the advantages of real-time PCR and DNA array technology, resulting in high throughput capacity and accurate quantification (Belgrader *et al*. 1998). Essentially, PCR arrays provide a high-throughput platform on which spatially separated simplex PCR reactions are performed. This technique is exemplified by developments from BioTrove (Woburn, MA, USA) utilizing OpenArray™ technology in which over 3000 separate real-time PCR assays can be performed simultaneously in 33 nl holes on a single microscope slide-sized plate. These arrays contain 48 subarrays of 64 reactions, allowing parallel testing of 64 pathogens in up to 48 samples. However, compared to conventional DNA arrays or real-time PCR platforms, sensitivity and accuracy of quantification may suffer from the ultra low-reaction volumes used. Another interesting development is the lab-on-a-chip instrument, which has been developed for the medical field and which integrates several steps from DNA extraction to DNA analysis within a single, portable, and fully automated instrument (Anderson *et al*. 2000; Wang 2000). Which technologies will eventually be implemented in plant pathogen diagnostics is unclear, but obviously only those technologies that are cost-effective may get introduced. After all, compared to clinical diagnostics, the willingness to spend money on expensive plant disease diagnosis is limited due to the low profit margins in agriculture and horticulture, and the low emotional value of a crop.

AUTHOR-RECOMMENDED WEBSITES

BioTrove OpenArray technology: http://www.biotrove.com
Cepheid SmartCycler System: http://www.cepheid.com
Consortium for the functional genomics of microbial eukaryotes: http://www.cogeme.man.ac.uk
DNA arrays for plant pathogen diagnosis: http://www.DNAmultiscan.com
Fungal genome initiative: http://www.broad.mit.edu/annotation/fgi/
Fungal tree of life: http://www.aftol.org
Genomes database: http://ergo.integratedgenomics.com
Genomes database: http://www.sanger.ac.uk/Projects/Microbes/
Idaho Technologies R.A.P.I.D. and RAZOR real-time PCR instrument: http://www.idahotech.com
Joint Genome Initiative: http://genome.jgi-psf.org/euk_cur1.html
Lateral flow devices: http://www.pocketdiagnostic.com
National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov
Pathogen-Host Interactions database (PHI-base): http://www.phi-base.org/
PCR primer database for phytopathogenic fungi: http://www.sppadbase.com
Smiths Industries Bioseeq RT-PCR instrument: http://trace.smithsdetection.com

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