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A robust identification and detection assay to discriminate the cucumber pathogens *Fusarium oxysporum* f. sp. *cucumerinum* and f. sp. *radicis-cucumerinum*

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Summary

The fungal species *Fusarium oxysporum* is a ubiquitous inhabitant of soils worldwide that includes pathogenic as well as non-pathogenic or even beneficial strains. Pathogenic strains are characterized by a high degree of host specificity and strains that infect the same host range are organized in so-called formae speciales. Strains for which no host plant has been identified are believed to be non-pathogenic strains. Therefore, identification below the species level is highly desired. However, the genetic basis of host specificity and virulence in *F. oxysporum* is so far unknown. In this study, a robust random-amplified polymorphic DNA (RAPD) marker-based assay was developed to specifically detect and identify the economically important cucumber pathogens *F. oxysporum* f. sp. *cucumerinum* and *F. oxysporum* f. sp. *radicis-cucumerinum*. While the *F. oxysporum radicis-cucumerinum* strains were found to cluster in a separate clade based on elongation factor-1α phylogeny, strains belonging to *F. oxysporum* f. sp. *cucumerinum* were found to be genetically more diverse. This is reflected in the observation that specificity testing of the identified markers using a broad collection of *F. oxysporum* strains with all known vegetative compatibility groups of the target formae speciales, as well as representative strains belonging to other formae speciales, resulted in two cross-reactions for the *F. oxysporum* f. sp. *cucumerimum* marker. However, no cross-reactions were observed for the *F. oxysporum* f. sp. *radicis-cucumerimum* marker. This *F. oxysporum* f. sp. *radicis-cucumerimum* marker shows homology to *Folyt1*, a transposable element identified in the tomato pathogen *F. oxysporum* f. sp. *lycopersici* and may possibly play a role in host-range specificity in the target forma specialis. The markers were implemented in a DNA array that enabled parallel and sensitive detection and identification of the pathogens in complex samples from diverse origins.

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Introduction

Fusarium oxysporum Schlechtend. Fr. is an anamorphic ubiquitous soil-inhabiting fungal species that includes plant pathogenic as well as non-pathogenic strains for which no host plants have been identified. The pathogenic *F. oxysporum* strains cause vascular wilt or cortical rot diseases in a broad range of horticultural and agricultural crops. Historically, pathogenic strains have been grouped in formae speciales based on specificity to host species, and furthermore in races based on cultivar specificity (Armstrong and Armstrong, 1981; Di Pietro *et al.*, 2003). In addition, based on the ability to form heterokaryons, *F. oxysporum* strains have been grouped into vegetative compatibility groups (VCGs; Puhalla, 1985), and different formae speciales and races may contain multiple VCGs (Katan, 1999; Katan and Di Primo, 1999). As the species *F. oxysporum* also contains non-pathogenic or even beneficial strains, identification of the pathogenic strains is highly desirable. Currently, identification of pathogenic *F. oxysporum* isolates is mainly based on bioassays that are time-consuming and laborious (Recorbet *et al.*, 2003). Increasingly, attempts are made to replace these methods by culture-independent molecular identification techniques. Ideally, molecular identification of pathogenic strains is based on the detection of targets that are directly linked to pathogenicity (Lievens and Thomma, 2005). However, so far the genetic basis of host specificity

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and virulence in *F. oxysporum* is unknown (Di Pietro *et al*., 2003). Furthermore, molecular discrimination of *F. oxysporum* isolates is complicated by the observation that different isolates that are classified into a single forma specialis may have independent evolutionary (polyphyletic) origins (O'Donnell *et al*., 1998; Baayen *et al*., 2000; Skovgaard *et al*., 2001; Cramer *et al*., 2003), and that isolates that belong to different formae speciales may share a common ancestor (monophyletic origin; Kistler, 1997).

Generally, molecular identification of plant pathogenic fungi is based on the detection of polymorphisms in ubiquitously conserved genes, such as ribosomal RNA, β-tubuline or translation elongation factor (EF)-1α genes (McCartney *et al*., 2003; Lievens *et al*., 2005a). The use of universal primers that anneal to conserved sequences flanking variable domains within these genes offers the possibility of simultaneous detection and identification of multiple plant pathogens in a single assay, for instance by DNA array hybridization (Lievens *et al*., 2003; Seifert and Lévesque, 2004; Tambong *et al*., 2006). This strategy has proven to be successful for species identification, even when different species can only be discriminated by a single nucleotide polymorphism (Lievens *et al*., 2006a). However, housekeeping genes do not generally reflect sufficient sequence variation for the discrimination below the species level, such as for formae speciales (Lievens *et al*., 2003). Therefore, additional strategies have been exploited to identify selective target sequences. This can be achieved using sequence-unbiased approaches, such as random-amplified polymorphic DNA (RAPD) (Williams *et al*., 1990) or amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995) technology. Markers identified with these approaches can be used to design specific sequence-characterized amplified region (SCAR) primers (Paran and Michelmore, 1993) that specifically amplify the selected markers resulting in a robust identification assay (McDermott *et al*., 1994; Larsen *et al*., 2002). This approach has proven to be effective for the identification of some formae speciales and races of *F. oxysporum* (García-Pedrajas *et al*., 1999; De Haan *et al*., 2000; Chiocchetti *et al*., 2001; Alves-Santos *et al*., 2002; Jiménez-Gasco and Jiménez-Díaz, 2003).

Fusarium oxysporum f. sp. *cucumerinum* J. H. Owen and *F. oxysporum* f. sp. *radicis-cucumerinum* D. J. Vakalounakis are two formae speciales that cause severe losses in the worldwide production of cucumber (*Cucumis sativus* L.; Vakalounakis and Fragkiadakis, 1999). Both formae speciales cause distinct diseases. While *F. oxysporum* f. sp. *cucumerinum* causes vascular wilt disease of cucumber as its unique host (Ahn *et al*., 1998), *F. oxysporum* f. sp. *radicis-cucumerinum* is the causal agent of root and stem rot on multiple hosts (Vakalounakis, 1996). For *F. oxysporum* f. sp. *cucumerinum*, 11 VCGs and three races were identified (Armstrong *et al*., 1978; Vakalounakis and Fragkiadakis, 1999; Vakalounakis *et al*., 2004), while for *F. oxysporum* f. sp. *radicis-cucumerinum* only two distinct VCGs, one bridging VCG interconnecting members of both VCGs, and no races have been described (Vakalounakis, 1996; Vakalounakis and Fragkiadakis, 1999; Vakalounakis *et al*., 2004). Finally, while *F. oxysporum* f. sp. *cucumerinum* is thought to have a polyphyletic origin, *F. oxysporum* f. sp. *radicis-cucumerinum* is suggested to be monophyletic (Vakalounakis and Fragkiadakis, 1999; Vakalounakis *et al*., 2004). At present, only time-consuming bioassays can be used to identify these cucumber pathogens (Vakalounakis and Fragkiadakis, 1999).

In this article, we describe the development of a RAPD marker-based assay to specifically identify and discriminate the two cucumber pathogens *F. oxysporum* f. sp. *cucumerinum* and *F. oxysporum radicis-cucumerinum* from each other, and from other strains that are not pathogenic on cucumber. Based on the RAPD markers that were identified, robust SCAR markers were developed that allow specific detection of these pathogens in environmental samples, encompassing plant tissue, recirculating water and potting mix samples. The markers were implemented in a DNA macro-array that has been developed to detect multiple plant pathogens including those belonging to the genus *Fusarium* and the species *F. oxysporum* (Lievens *et al*., 2003). This study is the first to describe robust markers for genetically different and economically important formae speciales of the fungal species *F. oxysporum* that can be used for pathogen detection in diverse environmental samples.

Results

Genetic organization of F. oxysporum *f. sp.* cucumerinum *and* F. oxysporum *f. sp.* radicis-cucumerinum *within the* F. oxysporum *species complex*

To examine the genealogies of *F. oxysporum* f. sp. *cucumerinum* and *F. oxysporum* f. sp. *radicis-cucumerinum* within the *F. oxysporum* complex, EF-1α sequences from 20 *F. oxysporum* f. sp. *cucumerinum* and 20 *F. oxysporum* f. sp. *radicis-cucumerinum* isolates, representing all known VCGs, were compared with EF-1α sequences from 98 isolates belonging to other formae speciales. Based on the nucleotide sequence alignment a cladogram was constructed (Fig. 1) that perfectly displays the different clades within the *F. oxysporum* species complex as proposed by O'Donnell and colleagues (1998). Irrespective of their geographic origin or VCG, nearly all *F. oxysporum* f. sp. *radicis-cucumerinum* strains grouped together in a single cluster within clade 3 (Fig. 1).

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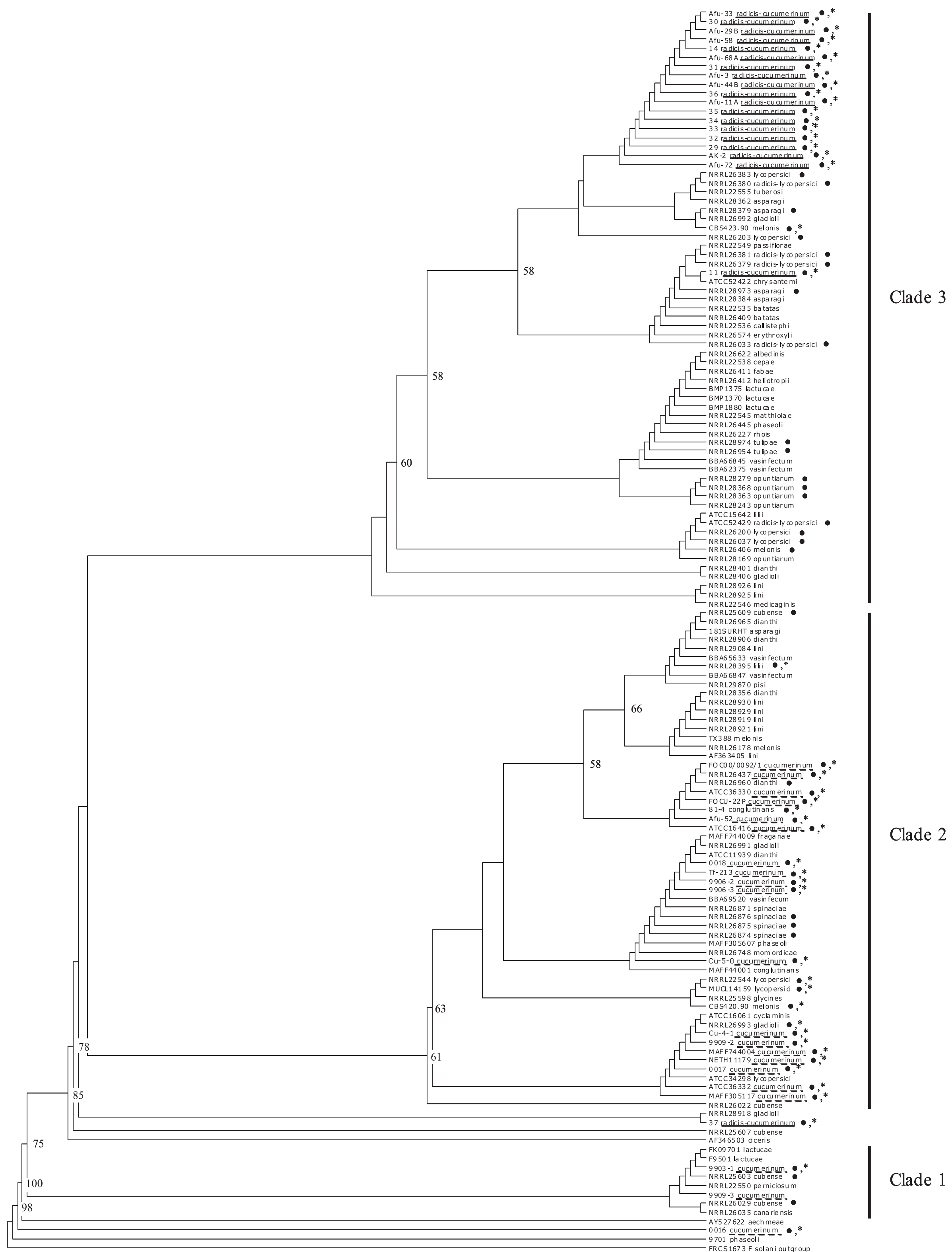

Fig. 1. Neighbour-joining cladogram showing genetic relationships within the *Fusarium oxysporum* complex based on elongation factor (EF)-1α sequences. The tree is based on 47 *Fusarium oxysporum* strains (denoted by asterisks; GenBank accession numbers in Table 1) and 92 sequences retrieved from GenBank. Clades 1, 2 and 3 were previously defined (O'Donnell *et al.*, 1998). Bootstrap percentages > 50% based on 1000 replications are shown at the major nodes. Strains denoted by a dot were used to test the specificity of the diagnostic assay and are listed in Table 1. The *F. solani* FRCS 1673 EF-1α sequence was used as outgroup.

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Only one strain (11) localized in a different cluster within clade 3 (58% bootstrap support). Among all *F. oxysporum* f. sp. *radicis-cucumerinum* strains tested, strain 11 was the only strain of which the EF-1α region contained 649 bp, whereas all other *F. oxysporum* f. sp. *radicis-cucumerinum* strains amplicons were 650 bp in length. In addition, this strain differed in at least six nucleotides from the other *F. oxysporum* f. sp. *radicis-cucumerinum* strains. Remarkably, strain 37 did not cluster in clade 3 at all, but landed rather isolated in a different part of the tree. As with strain 11, at least six single nucleotide differences were observed for this strain compared with the other *F. oxysporum* f. sp. *radicis-cucumerinum* strains. While all except one *F. oxysporum* f. sp. *radicis-cucumerinum* strains clustered within clade 3 of the *F. oxysporum* complex, most of the strains belonging to *F. oxysporum* f. sp. *cucumerinum* resolved within clades 1 and 2 (Fig. 1). Within clade 2, two subgroups of *F. oxysporum* f. sp. *cucumerinum* were formed (63% bootstrap support) that correspond well to the division of these strains in different VCGs. While all test isolates belonging to VCG 0180, 0183 and 0184 landed in one subgroup, the strains belonging to VCG 0181 and 0182 clustered in a second subgroup. Remarkably, strains Tf-213 and 9909-2, both belonging to the same VCG (0185) landed each in one of the two different subgroups, suggesting a polyphyletic nature of this VCG. Nevertheless, because this observation goes against earlier observations that demonstrate the clonal nature of *F. oxysporum* VCGs (Kistler, 1997), this observation should be verified by using other sequences such as for example the mitochondrial small subunit ribosomal RNA gene (O'Donnell *et al*., 1998; Skovgaard *et al*., 2001) to confirm the degree of relatedness and also by confirmation of the VCG grouping of the two isolates. Furthermore, only the strains belonging to VCG 0186 were grouped in clade 1. However, while strain 0016 that belongs to VCG 187 clearly resolved as a separate group, isolate 0020 that belongs to the same VCG clustered within clade 2.

Identification of RAPD markers for the F. oxysporum *cucumber pathogens*

In order to identify genetic markers that can be used to develop a diagnostic assay for the two selected *F. oxysporum* formae speciales, RAPD analyses were performed using a strategy of several elimination rounds, starting with 115 decamer oligonucleotides on genomic DNA of a random selection of *F. oxysporum* isolates and subsequent screening of the most discriminative oligonucleotides on a larger number of strains. Eventually, the remaining discriminative oligonucleotides (two for *F. oxysporum* f. sp. *cucumerinum* and five for *F. oxysporum* f. sp. *radicis-cucumerinum*) were tested on all isolates listed in Table 1. These isolates represent a wide collection of *F. oxysporum* strains, encompassing all known VCGs of the target formae speciales as well as representative strains of other *F. oxysporum* formae speciales. Of the initially tested 115 primers, oligonucleotide OPB-07 produced a clear diagnostic band of 277 bp for most of the tested *F. oxysporum* f. sp. *radicis-cucumerinum* strains, while oligonucleotide OPZ-12 was found to produce a diagnostic band of 865 bp for all *F. oxysporum* f. sp. *cucumerinum* strains. Both markers appeared as bright bands after gel electrophoresis and were consistently amplified in at least three polymerase chain reactions (PCR). Remarkably, the 277 bp diagnostic band was not obtained for the *F. oxysporum* f. sp. *radicis-cucumerinum* strains 11 and 37, which were found not to belong to the main *F. oxysporum* f. sp. *radicis-cucumerinum* clade based on EF-1α sequences (Fig. 1). Similar results were obtained for a recently obtained isolate (isolate 10) (data not shown). To check for misclassification, a pathogenicity test (Vakalounakis and Fragkiadakis, 1999) was carried out on cucumber revealing that strain 37 was not able to cause the typical *F. oxysporum* f. sp. *radicis-cucumerinum* symptoms. Rather, strain 37 induced cucumber wilting, which is typical for *F. oxysporum* f. sp. *cucumerinum* (data not shown). Moreover, RAPD analysis with oligonucleotide OPZ-12 resulted in the production of the diagnostic band for *F. oxysporum* f. sp. *cucumerinum*. These results show that strain 37 was originally misidentified as *F. oxysporum* f. sp. *radicis-cucumerinum* and should be classified as *F. oxysporum* f. sp. *cucumerinum*. In our bioassays, strains 10 and 11 were not able to infect cucumber at all (data not shown). These strains were originally isolated from soil and identified solely on morphological characters. Therefore, these are most likely saprophytic *F. oxysporum* strains.

While the selective band for *F. oxysporum* f. sp. *radicis-cucumerinum* was only obtained for strains belonging to this forma specialis, the diagnostic band for *F. oxysporum* f. sp. *cucumerinum* was also obtained for some isolates that are classified in other formae speciales. In addition to strain 37 that was originally classified as *F. oxysporum* f. sp. *radicis-cucumerinum*, these encompassed *F. oxysporum* f. sp. *conglutinans* 81–4, *F. oxysporum* f. sp. *dianthi* NRRL 26960, *F. oxysporum* f. sp. *gladioli* NRRL 26993, *F. oxysporum* f. sp. *lilii* NRRL 26955 and the *F. oxysporum* f. sp. *lycopersici* strains MUCL 14159 and NRRL 22544. For these strains, amplicon sequences did not display significant differences ($\geq$ 98% homology) with the selected RAPD marker. Apart from strain 37, pathogenicity testing (Vakalounakis and Fragkiadakis, 1999) revealed that none of these isolates was able to cause cucumber wilt, and thus do not belong to *F. oxysporum* f. sp. *cucumerinum*.

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Table 1. Strains of the *Fusarium oxysporum* complex and related strains used in this study.

Isolate[a]	VCG[b]	Host/substrate	Origin (source)[c]	Year of isolation	EF-1α GenBank accession number[d]	Specificity[e] obtained with SCAR primers		Hybridization signal strength[f] obtained with detector oligonucleotides					
						FocF1/ FocR2	ForcF1/ ForcR2	Fgn2	Fox2	Foc1	Foc2	Forc1	Forc2
F. oxysporum f. sp. *cucumerinum*													
707E	0180	*Cucumis sativus*	Israel (A)	1993		+	–	■	■	■	■		
Afu-50(B)	0180	*C. sativus*	Crete (B)	1994		+	–	■	■	■	■		
Afu-52	0180	*C. sativus*	Crete (B)	1994	EF056744	+	–	■	■	■	■		
Afu-57(B)	0180	*C. sativus*	Crete (B)	1995		+	–	■	■	■	■		
ATCC 16416	0180	*C. sativus*	USA, Florida	1956	EF056783	+	–	■	■	■	■		
ATCC 36330	0180	*C. sativus*	Israel	1970	EF056745	+	–	■	■	■	■		
Cu:4–1	0181	*C. sativus*	Japan (B)	Unknown	EF056747	+	–	■	■	■	■		
FOCU-16F	0180	*C. sativus*	Israel (A)	1994		+	–	■	■	■	■		
FOCU-17W	0180	*C. sativus*	Israel (A)	1994		+	–	■	■	■	■		
FOCU-22P	0180	*C. sativus*	Israel (A)	1995	EF056746	+	–	■	■	■	■		
FOCU-26E	0180	*C. sativus*	Israel (A)	1995		+	–	■	■	■	■		
FOCU-33N	0180	*C. sativus*	Israel (A)	1995		+	–	■	■	■	■		
FOCU-39E	0180	*C. sativus*	Israel (A)	1995		+	–	■	■	■	■		
FOCU-45K	0180	*C. sativus*	Israel (A)	1995		+	–	■	■	■	■		
FOCU-48F	0180	*C. sativus*	Israel (A)	1995		+	–	■	■	■	■		
FOCU-CM1C	0180	*C. sativus*	Israel (A)	1994		+	–	■	■	■	■		
NETH 10782(B)	0181	*C. sativus*	the Netherlands (B)	Unknown		+	–	■	■	■	■		
NETH 11179	0181	*C. sativus*	the Netherlands (B)	1979	EF056748	+	–	■	■	■	■		
ATCC 36332	0182	*C. sativus*	Japan (B)	1973	EF056750	+	–	■	■	■	■		
0018	0183	*C. sativus*	China (B)	2000	EF056756	+	–	■	■	■	■		
Cu:5–0	0183	*C. sativus*	Japan (B)	Unknown	EF056751	+	–	■	■	■	■		
9906–2	0184	*C. sativus*	China (B)	1999	EF056757	+	–	■	■	■	■		
9906–3	0184	*C. sativus*	China (B)	1999	EF056755	+	–	■	■	■	■		
9909–2	0185	*C. sativus*	China (B)	1999	EF056752	+	–	■	■	■	■		
Tf-213	0185	*C. sativus*	Japan (B)	1985	EF056753	+	–	■	■	■	■		
9901-2	0186	*C. sativus*	China (B)	1999		+	–	■	■	■	■		
9903-1	0186	*C. sativus*	China (B)	1999	EF056758	+	–	■	■	■	■		
9903-2	0186	*C. sativus*	China (B)	1999		+	–	■	■	■	■		
9904-1	0186	*C. sativus*	China (B)	1999		+	–	■	■	■	■		
9909-3	0186	*C. sativus*	China (B)	1999	EF056759	+	–	■	■	■	■		
0016	0187	*C. sativus*	China (B)	2000	EF056760	+	–	■	■	■	■		
0017	0187	*C. sativus*	China (B)	2000	EF056754	+	–	■	■	■	■		
0020	0187	*C. sativus*	China (B)	2000		+	–	■	■	■	■		
10196	Unknown	*C. sativus*	Unknown (C)	Unknown		+	–	■	■	■	■		
ATCC 42352	Unknown	*C. sativus*	Japan	Unknown		+	–	■	■	■	■		
ATCC 42357	Unknown	*C. sativus*	Japan	Unknown		+	–	■	■	■	■		
D1-RL	Unknown	*C. sativus*	Taiwan (D)	Unknown		+	–	■	■	■	■		
D4-33	Unknown	*C. sativus*	Taiwan (D)	Unknown		+	–	■	■	■	■		
FOC 00/0092/1	Unknown	*C. sativus*	Unknown (E)	Unknown	EF056749	+	–	■	■	■	■		
MAFF 103054	Unknown	*C. sativus*	Japan	Unknown		+	–	■	■	■	■		
MAFF 305116	Unknown	*C. sativus*	Japan	Unknown		+	–	■	■	■	■		
MAFF 305117	Unknown	*C. sativus*	Japan	Unknown	EF056761	+	–	■	■	■	■		
MAFF 727508	Unknown	*C. sativus*	Japan	Unknown		+	–	■	■	■	■		

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Table 1. *cont.*

Isolate[a]	VCG[b]	Host/substrate	Origin (source)[c]	Year of isolation	EF-1α GenBank accession number[d]	Specificity[e] obtained with SCAR primers		Hybridization signal strength[f] obtained with detector oligonucleotides					
						FocF1/ FocR2	ForcF1/ ForcR2	Fgn2	Fox2	Foc1	Foc2	Forc1	Forc2
MAFF 744004	Unknown	*C. sativus*	Japan	Unknown	EF056762	+	–	■	■	■	■		
MAFF 744005	Unknown	*C. sativus*	Japan	Unknown		+	–	■	■	■	■		
NRRL 26437	Unknown	*C. sativus*	USA, South Carolina	Unknown		+	–	■	■	■	■		
F. oxysporum f. sp. *radicis-cucumerinum*													
Afu-11(A)	0260	*C. sativus*	Crete (B)	1992	EF056763	–	+	■	■			■	■
Afu-29(B)	0260	*C. sativus*	Crete (B)	1993	EF056764	–	+	■	■			■	■
Afu-3	0260	*C. sativus*	Crete (B)	1992	EF056765	–	+	■	■			■	■
Afu-33	0260	*C. sativus*	Crete (B)	1993		–	+	■	■			■	■
Afu-44(B)	0260	*C. sativus*	Crete (B)	1993	EF056766	–	+	■	■			■	■
Afu-58	0260	*C. sativus*	Crete (B)	1996	EF056767	–	+	■	■			■	■
Afu-68(A)	0260	*C. sativus*	Crete (B)	1997	EF056768	–	+	■	■			■	■
Afu-72	0260	*C. sativus*	Crete (B)	1997	EF056769	–	+	■	■			■	■
Afu-4(A)	0261	*C. sativus*	Crete (B)	1992		–	+	■	■			■	■
AK-2	0261	*C. sativus*	Crete (B)	1990	EF056770	–	+	■	■			■	■
8	Unknown	*C. sativus*	Canada (F)	1997		–	+	■	■			■	■
10	Unknown	soil	Canada (F)	1998		–	–	■	■				
11	Unknown	soil	Canada (F)	1998	EF056778	–	–	■	■				
14	Unknown	*C. sativus*	Canada (F)	1997	EF056779	–	+	■	■			■	■
16	Unknown	*C. sativus*	Canada (F)	1997		–	+	■	■			■	■
20	Unknown	*C. sativus*	Canada (F)	1998		–	+	■	■			■	■
21A	Unknown	*C. sativus*	Canada (F)	1998		–	+	■	■			■	■
22	Unknown	*C. sativus*	Canada (F)	1998		–	+	■	■			■	■
24	Unknown	*C. sativus*	Canada (F)	1998		–	+	■	■			■	■
28	Unknown	*C. sativus*	Canada (F)	1998	EF056780	–	+	■	■			■	■
29	Unknown	*C. sativus*	Canada (F)	1998	EF056771	–	+	■	■			■	■
30	Unknown	*C. sativus*	Canada (F)	1996	EF056781	–	+	■	■			■	■
31	Unknown	*C. sativus*	Canada (F)	2000	EF056772	–	+	■	■			■	■
32	Unknown	*C. sativus*	Canada (F)	2001	EF056773	–	+	■	■			■	■
33	Unknown	*C. sativus*	Canada (F)	2000	EF056774	–	+	■	■			■	■
34	Unknown	*C. sativus*	Canada (F)	2000	EF056782	–	+	■	■			■	■
35	Unknown	*C. sativus*	Canada (F)	2000	EF056775	–	+	■	■			■	■
36	Unknown	*C. sativus*	Canada (F)	2001	EF056776	–	+	■	■			■	■
37	Unknown	*C. sativus*	Canada (F)	2001	EF056777	+	–	■	■	■	■		
38	Unknown	*C. sativus*	France (F)	2001		–	+	■	■			■	■
FORC 00/0092/2	Unknown	*C. sativus*	Unknown (E)	Unknown		–	+	■	■			■	■
Avirulent isolates of *F. oxysporum* from cucumber													
13	Unknown	*C. sativus*	Canada (F)	1996/1997		–	–	■	■				
Afu-39(B)	Unknown	*C. sativus*	Crete (B)	1993		–	–	■	■				
Afu-65(A)	Unknown	*C. sativus*	Epirus (B)	1993		–	–	■	■				
F. oxysporum f. sp. *asparagi*													
NRRL 28973	1001	*Asparagus officinalis*	USA	Unknown		–	–	■	■				
NRRL 28362	1002	*A. officinalis*	USA	Unknown		–	–	■	■				
NRRL 28379	1008	*A. officinalis*	USA	Unknown		–	–	■	■				

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F. oxysporum f. sp. *conglutinans*											
81–4	Unknown	Unknown	Unknown (G)	Unknown	EF056786	+	–	■	■	■	■
F. oxysporum f. sp. *cubense*											
NRRL 25603	0120	*Musa acuminata*	Australia	Unknown		–	–	■	■		
NRRL 26029	01210	*Musa* sp.	USA	Unknown		–	–	■	■		
NRRL 25609	01214	*Musa* sp.	Malawi	Unknown		–	–	■	■		
F. oxysporum f. sp. *dianthi*											
NRRL 26147	0020	*Dianthus caryophyllus*	USA	Unknown		–	–	■	■		
NRRL 26960	0025	*D. caryophyllus*	the Netherlands	Unknown		–	–	■	■		
F. oxysporum f. sp. *gladioli*											
NRRL 28914	0340	*Crocus* sp.	the Netherlands	Unknown		–	–	■	■		
NRRL 26993	0343	*Gladiolus* × *grandiflorus*	Italy	Unknown	EF056787	+	–	■	■	■	■
NRRL 26990	0345	*Freesia* sp.	the Netherlands	Unknown		–	–	■	■		
F. oxysporum f. sp. *lilii*											
NRRL 26955	0190	*Lilium* sp.	the Netherlands	Unknown		–	–	■	■		
NRRL 28395	0190	*Lilium* sp.	Italy	Unknown	EF056788	–	–	■	■		
F. oxysporum f. sp. *lycopersici*											
FOL-295A	0030	*Lycopersicon esculentum*	Israel (A)	Unknown		–	–	■	■		
BFOL-51	0031	*L. esculentum*	USA, Los Angeles (A)	Unknown		–	–	■	■		
FOL-MM59	0032	*L. esculentum*	USA, Arkansas (A)	Unknown		–	–	■	■		
FOL-MM 10	0033	*L. esculentum*	USA, Arkansas (A)	Unknown		–	–	■	■		
MUCL 14159	Unknown	*L. esculentum*	Unknown	Unknown	EF056784	–	–	■	■		
NRRL 26037	Unknown	*L. esculentum*	USA, Florida	Unknown		–	–	■	■		
NRRL 26200	Unknown	*L. esculentum*	Unknown	Unknown		–	–	■	■		
NRRL 26203	Unknown	*L. esculentum*	Unknown	Unknown		–	–	■	■		
NRRL 26383	Unknown	*L. esculentum*	Unknown	Unknown		–	–	■	■		
NRRL 22544	Unknown	*L. esculentum*	Unknown	1930	EF056785	–	–	■	■		
F. oxysporum f. sp. *luffae*											
Fol-114	Unknown	*Luffa cylindrica*	Taiwan (D)	Unknown		–	–	■	■		
Fol-167	Unknown	*L. cylindrica*	Taiwan (D)	Unknown		–	–	■	■		
F. oxysporum f. sp. *melonis*											
NRRL 26406	0136	*Cucumis melo*	USA	Unknown		–	–	■	■		
CBS 420.90	Unknown	*C. melo*	Israel	1986	EF056790	–	–	■	■		
CBS 423.90	Unknown	*C. melo*	Israel	1988	EF056789	–	–	■	■		
NRRL 26046	Unknown	*C. melo*	USA, California	Unknown		–	–	■	■		
F. oxysporum f. sp. *niveum*											
CBS 187.60	Unknown	Unknown	Germany	Unknown		–	–	■	■		
CBS 418.90	Unknown	*Citrullus lanatus*	Israel	1986		–	–	■	■		
CBS 419.90	Unknown	*C. lanatus*	Israel	1986		–	–	■	■		

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Table 1. *cont.*

Isolate[a]	VCG[b]	Host/substrate	Origin (source)[c]	Year of isolation	EF-1α GenBank accession number[d]	Specificity[e] obtained with SCAR primers		Hybridization signal strength[f] obtained with detector oligonucleotides					
						FocF1/FocR2	ForcF1/ForcR2	Fgn2	Fox2	Foc1	Foc2	Forc1	Forc2
F. oxysporum f. sp. *opuntiarum*													
NRRL 28363	0450	*Rhipsalidopsis* sp.	the Netherlands	Unknown		–	–	■	■				
NRRL 28368	0451	*Disco placentiformis*	the Netherlands	Unknown		–	–	■	■				
NRRL 28279	0454	*Ferocactus* sp.	Germany	Unknown		–	–	■	■				
F. oxysporum f. sp. *radicis-lycopersici*													
ATCC 52429	0090 I	*L. esculentum*	Canada	Unknown		–	–	■	■				
FORL-C58M	0091 I	*L. esculentum*	Israel (A)	Unknown		–	–	■	■				
FORL-C815A	0092	*L. esculentum*	Israel (A)	Unknown		–	–	■	■				
FORL-C202	0093	*L. esculentum*	Israel (A)	Unknown		–	–	■	■				
01150	0094 I	*L. esculentum*	Belgium (A)	Unknown		–	–	■	■				
FORL-C622A	0096	*L. esculentum*	Israel (A)	Unknown		–	–	■	■				
NRRL 26033	Unknown	*L. esculentum*	Unknown	Unknown		–	–	■	■				
NRRL 26379	Unknown	*L. esculentum*	Unknown	Unknown		–	–	■	■				
NRRL 26380	Unknown	*L. esculentum*	Unknown	Unknown		–	–	■	■				
NRRL 26381	Unknown	*L. esculentum*	Unknown	Unknown		–	–	■	■				
F. oxysporum f. sp. *spinaciae*													
NRRL 26874	0330	*Spinacea oleracea*	USA	Unknown		–	+[g]	■	■				
NRRL 26875	0331	*Rhipsalidopsis* sp.	USA	Unknown		–	+[g]	■	■				
NRRL 26876	0332	*Rhipsalidopsis* sp.	USA	Unknown		–	+[g]	■	■				
F. oxysporum f. sp. *tulipae*													
NRRL 22556	0230	*Tulipa* × *gesneriana*	Germany	Unknown		–	–	■	■				
NRRL 26954	0230	*Tulipa* × *gesneriana*	the Netherlands	Unknown		–	–	■	■				
NRRL 28974	0230	*Tulipa* × *gesneriana*	the Netherlands	Unknown		–	–	■	■				
F. javanicum													
CBS 410.62	–	*Cucurbita viciifolia*	the Netherlands	Unknown		–	–	■					
CBS 616.66	–	*C. viciifolia*	the Netherlands	Unknown		–	–	■					
F. solani													
CABI 17960	–	*Solanum tuberosum*	Brazil	Unknown		–	–	■					
CBS 165.87	–	*S. tuberosum*	Denmark	1986		–	–	■					

a. ATCC, American Type Culture Collection, Manassas, VA, USA; CABI, Centre for Agriculture and Bioscience International, Surrey, UK; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; MAFF, Genetic Resources Management Section, Genebank, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan; MUCL, Mycotèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; NRRL, Agriculture Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA.
b. VCG, vegetative compatibility group.
c. A = T. Katan, Hebrew University of Jerusalem, Jerusalem, Israel; B = D.J. Vakalounakis, National Agricultural Research Foundation (N.AG.RE.F), Plant Protection Institute, Heraklion, Crete, Greece; C = A.C.M. Clerckx, Plant Research International, Wageningen, the Netherlands; D = Y.-T. Wang, Soochow University, Taipei, Taiwan; E = F. Vey, Laboratoire National de la Protection des Végétaux, Fleury-les-Aubrais Cédex, France; and F = Z.K. Punja, Simon Fraser University, Burnaby, B.C., Canada; G = H.C. Kistler, University of Minnesota, St. Paul, MN, USA.
d. GenBank accession number of elongation factor-1α sequences (EF056744–EF056790) determined in this study.
e. +, amplicon detected; –, no amplicon detected. A band of 865 bp and 277 bp is expected for *F. oxysporum* f. sp. *cucumerinum* (primers FocF1 and FocR2) and *F. oxysporum* f. sp. *radicis-cucumerinum* (primers ForcF1 and ForcR2) respectively.
f. Blank = no hybridization signal; ■ = strong hybridization signal.
g. Amplicon of 256 bp.

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OPB07_277      GGTGACGCAGCAGTCTAGAAAGGGCTATTATGATTAAGGTCAGCTAATTCCAGAGTCTGC 60
AF057141       ---------------TAGAGATGGAT-------TTAATGCTGCATCATTACATAAAATGC 38
                              **** * ** *       **** *     * *** ** *   ***

OPB07_277      CTGTGATTGGTGCTTTGCCTCGTCACAATGATTTCAGCATGGGTGGCACGGATCACCATG 120
AF057141       ----GCTTTTTGCATTAAATATTTAAAGCAA----GACGCGCTTTGTATGCATTATTTCC 90
                   * **  *** **   *  * * *   *      *  *  * * * * ** *

OPB07_277      TGTCGCGCTGAGCGTGCTTAAAGGGTTAAAGTGAATGTGATGAGCAGCAGCAAAAATGTG 180
AF057141       TGTCGCACTGAGCGTGCTTAAAGGGTTAGAGTGAATATGATGAGCAGCAGCAAA--TGTG 148
               ****** ********************* ******* *****************  ****

OPB07_277      TTGATTGATGTCTTAAGTTATCTCTTGTGCGGGGTATTCCATCCCGCAGAGTTACTTAAA 240
AF057141       TTGATTGATGTCTTAAGTTTTCTCTTGTGCGGGGTATTCCATCCCGCACAGTTACTTAAA 208
               ******************* **************************** ***********

OPB07_277      GCATATAGCTAGATTACTAATGCCTACCCTGCGTCAC 277
AF057141       GCATATAGCTAGATTACTAATGCCTACCCTGCGACAT 245
               ********************************* **
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Fig. 2. Alignment of the complete *Fusarium oxysporum* f. sp. *radicis-cucumerinum* RAPD marker sequence OPB-07$_{277}$ with the corresponding part of the *F. oxysporum* f. sp. *lycopersici Folyt1* DNA sequence (GenBank Accession number AF057141) (Gómez-Gómez *et al.*, 1999). Identical nucleotides are marked with asterisks and gaps are indicated by dashes.

For each target forma specialis, the respective amplicons were selected as most discriminative RAPD markers, named OPZ-12$_{865}$ and OPB-07$_{277}$, and sequenced (GenBank Accession numbers EF056791 and EF056791 respectively). Subsequently, sequence variation was assessed in eight different strains of the respective forma specialis for each marker, showing a high degree of conservation (≥ 99% homology). While OPZ-12$_{865}$ was found not to have significant homology to any sequence present in public databases, OPB-07$_{277}$ showed strong similarity (74%) with *Folyt1*, a transposable element identified in the tomato pathogen *F. oxysporum* f. sp. *lycopersici* (Gómez-Gómez *et al.*, 1999). Whereas the 3′-part of OPB-07$_{277}$ was found to be almost identical to the 3′-part of the *Folyt1* element, a rather high nucleotide diversity was observed in the 5′-part of the marker (Fig. 2).

Real-time PCR (Livak and Schmittgen, 2001; Li *et al.*, 2004) was used to investigate whether the selected markers represent single or multiple copy DNA sequences in the genome. To this end, for each marker primers were designed to result in amplicons of similar length (Table 2). Part of the single copy mating type gene *MAT1-1* (Arie *et al.*, 2000) was used as a reference sequence in this approach. Amplification efficiencies were similar among the different reactions (Fig. 3), allowing accurate determination of the relative copy number of the markers. In order to study the prevalence of the markers, the difference in C_T values between the target and the normalizer (ΔC_T) was calculated for 10 strains. In all cases, ΔC_T values were very close to zero (Table 3), indicating that both markers represent single copy genomic DNA sequences.

Development of SCAR markers

In order to develop a diagnostic assay and overcome interlaboratory reproducibility problems that are often associated with RAPD assays (Jones *et al.*, 1997), the selected RAPD markers were used to develop robust and reliable SCAR primers. To this end, the original RAPD primers were elongated to oligonucleotide sequences of approximately 20 bp that consistently generated a single PCR product of the same size and sequence as the respective RAPD markers. Polymerase chain reaction amplification using SCAR primers ForcF1 and ForcR2 resulted in a highly specific assay for the identification of *F. oxysporum* f. sp. *radicis-cucumerinum* isolates (Table 1). Nevertheless, while for all target strains a 277-bp amplicon was generated, a slightly smaller fragment (256 bp) was produced for the three *F. oxysporum* f. sp. *spinaciae* isolates. For the misclassified isolates lacking the RAPD marker (strains 10, 11 and 37), as expected, no PCR product was generated (Table 1). With regard to the identification of *F. oxysporum* f. sp. *cucumerinum*, amplicons of the expected size were generated for all target isolates using SCAR primers FocF1 and FocR2 (Table 1). In addition, the number of cross-reacting isolates that was obtained with the original RAPD primer (OPZ-12) significantly decreased when the SCAR

Table 2. Real-time PCR primers used for copy number determination.

Code	Sequence (5′–3′)	Target organism	Origin	Amplicon size (bp)	T_m[a]
FocF3 (F)	AAACGAGCCCGCTATTTGAG	*F. oxysporum* f. sp. *cucumerinum*	OPZ-12$_{865}$	244	80.3
FocR7 (R)	TATTTCCTCCACATTGCCATG				
ForcF5 (F)	TCGTCACAATGATTTCAGCAT	*F. oxysporum* f. sp. *radicis-cucumerinum*	OPB-07$_{277}$	198	81.4
ForcR2 (R)	GTGACGCAGGGTAGGCAT				
Falpha1 (F)	CGGTCAYGAGTATCTTCCTG	*F. oxysporum*	*MAT1-1*[b]	222	83.0
FalphaR5 (R)	AGAGCTGGGTCAGGAAACC				

a. Melting temperature (°C) at which a specific dissociation peak of increased fluorescence is generated in the melting curve analysis.
b. Primer sequences based on GenBank Accession number AB0011379 (Arie *et al.*, 2000).
F, forward primer; R, reverse primer.

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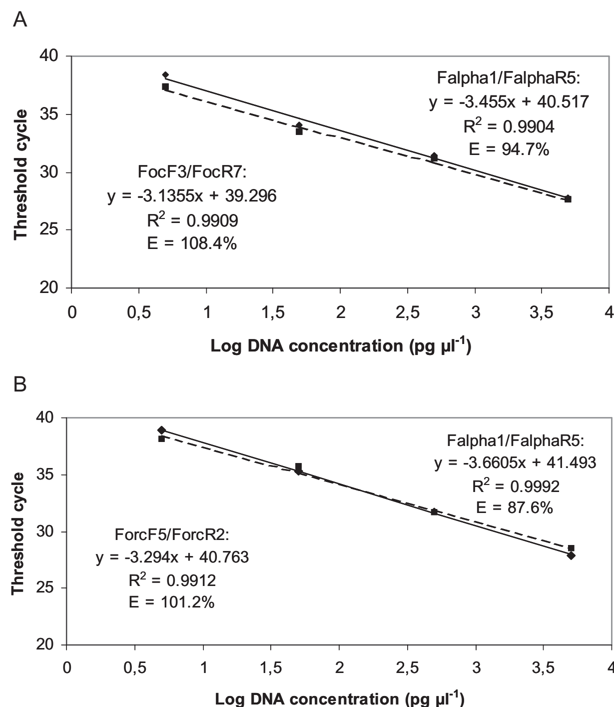

Fig. 3. Determination of the copy number of the discriminative markers using real-time PCR. Standard curves generated using real-time PCR to assess the copy number of (A) the *Fusarium oxysporum* f. sp. *cucumerinum* (OPZ-12_{865}) and (B) *F. oxysporum* f. sp. *radicis-cucumerinum* (OPB-07_{277}) RAPD markers, relative to the single copy mating type gene *MAT1-1*. Curves were generated using primers FocF3 and FocR7 (OPZ-12_{865}; –■–), ForcF5 and ForcR2 (OPB-07_{277}; –■–) and Falpha1 and FalphaR5 (*MAT1-1*; –◆–), for a 10-fold dilution series of genomic DNA. Data represent means of two replicates. The efficiency of each reaction was calculated using the formula $E = 10^{-1/\text{slope}} - 1$.

primers were used. In this case, cross-reactions were limited to the isolates *F. oxysporum* f. sp. *conglutinans* 81–4 and *F. oxysporum* f. sp. *gladioli* NRRL 26993. As expected, the misclassified *F. oxysporum* f. sp. *radicis-cucumerinum* 37 which, in this study, is determined to be *F. oxysporum* f. sp. *cucumerinum* also produced a band (Table 1).

Design and validation of a DNA array for detection and identification of F. oxysporum *pathogens from cucumber*

In order to increase the specificity and sensitivity of the developed assay, as well as to implement the developed SCAR markers in a previously designed DNA array enabling parallel detection of multiple pathogens and targets in a single assay (Lievens *et al.*, 2003), multiple oligonucleotide detectors were designed based on the sequence of the selected markers. Of the initially tested detector oligonucleotides, two oligonucleotides per target forma specialis were selected and implemented in the DNA array (Table 4). The performance of the array containing oligonucleotides to detect and identify the genus *Fusarium*, the species *F. oxysporum* and the formae speciales *cucumerinum* and *radicis-cucumerinum* was tested. All isolates listed in Table 1 were correctly identified to the species level using the DNA array (Table 1). In addition, the previously obtained results using the SCAR-based PCR assays were corroborated by the hybridization results (Table 1). However, amplicons from the *F. oxysporum* f. sp. *spinaciae* strains did not hybridize to the *F. oxysporum* f. sp. *radicis-cucumerinum* oligonucleotides (Table 1), demonstrating the increased specificity of the assay.

To test the sensitivity and discriminatory range of the newly designed assay, a dilution series of genomic DNA ranging from 5 ng to 0.5 pg was used. The experiment was performed with DNA from *F. oxysporum* f. sp. *cucumerinum* ATCC 16416 and *F. oxysporum* f. sp. *radicis-cucumerinum* Afu-68(A). In the absence of non-target DNA, both forma specialis-specific oligonucleotides successfully detected their targets to a DNA amount of 5 pg (Table 5; results are shown for *F. oxysporum* f. sp. *cucumerinum*). The same detection level was found in the presence of excess non-target DNA, either from a non-related fungus (*Rhizoctonia solani*), a related fungus (the other *F. oxysporum* forma specialis) (Table 5; results are shown for *F. oxysporum* f. sp. *cucumerinum*) or from a healthy cucumber plant (data not shown).

Finally, we evaluated whether the developed procedure is suitable for the detection and identification of the *F. oxysporum* cucumber pathogens in environmental samples. To this end, artificially inoculated cucumber plants as well as their corresponding potting mixes were analysed at 7 days (no clear disease symptoms) and 20 days (clear disease symptoms) after inoculation. At both stages, the cucumber pathogens could be detected and identified in the plant tissue and potting mix samples using the DNA array. In addition, water, root or foot samples were collected from different hydroponic systems at commercial cucumber production greenhouse settings that had cucumber plants displaying typical *F. oxysporum* symptoms. Subsequently, these samples were assessed using classical plating techniques and the DNA array. Using the DNA array, out of five plant samples, three were diagnosed with *F. oxysporum* f. sp. *radicis-cucumerinum* and two with *F. oxysporum* f. sp. *cucumerinum*. From five water samples tested, one sample contained *F. oxysporum* f. sp. *cucumerinum*, three samples contained *F. oxysporum* f. sp. *radicis-cucumerinum* and in one sample both pathogens were detected. In all cases, plating on selective medium and morphological characteristics confirmed *F. oxysporum* as the pathogen causing the disease. As a control, water samples were also taken from commercial cucumber growing greenhouse settings that had no cucumber plants with *Fusarium* symptoms. Remarkably, out of five

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Table 3. Real-time PCR assay[a] to determine whether the selected markers for *Fusarium oxysporum* f. sp. *cucumerinum* (OPZ-12_{865}) and *F. oxysporum* f. sp. *radicis-cucumerinum* (OPB-07_{277}) represent single or multiple copy DNA sequences.

Isolate	VCG[b]	OPZ-12_{865} C_T	*MAT1-1* C_T	ΔC_T[c]
F. oxysporum f. sp. *cucumerinum*				
ATCC16416	0180	27.60; 27.74	27.84; 27.72	–0.11
Afu-50(B)	0180	31.87; 33.33	32.70; 33.12	–0.31
Cu:4–1	0181	29.80; 28.91	28.67; 29.35	0.35
ATCC36332	0182	28.88; 30.23	30.11; 30.30	–0.65
9906–2	0184	28.92; 29.40	29.59; 30.19	–0.73
9909–2	0185	30.31; 31.04	30.56; 30.67	0.06
9901–2	0186	30.87; 30.12	28.57; 28.92	1.75
0016	0187	36.72; 35.95	36.00; 35.95	0.36
00/0092/1	Unknown	32.17; 31.90	32.71; 32.59	–0.61
NRRL26437	Unknown	33.22; 33.59	33.32; 32.66	0.42
		OPB-07_{277} C_T	*MAT1-1* C_T	ΔC_T
F. oxysporum f. sp. *radicis-cucumerinum*				
Afu-68(A)	0260	28.55; 28.52	28.29; 27.38	0.70
Afu-3	0260	28.46; 28.58	28.37; 30.35	–0.84
Afu-33	0260	29.54; 29.89	29.62; 30.51	–0.35
Afu-44(B)	0260	29.24; 29.78	29.57; 30.73	–0.64
Afu-58	0260	34.15; 33.40	33.92; 34.94	–0.65
AK-2	0261	29.08; 29.17	30.27; 30.91	–1.46
00/0092/2	Unknown	31.34; 31.30	31.82; 33.45	–1.32
8	Unknown	28.42; 29.36	29.53; 31.30	–1.53
14	Unknown	35.07; 34.24	34.33; 35.05	–0.03
29	Unknown	30.54; 30.39	30.12; 31.37	–0.28

a. Data represent threshold cycle (C_T) numbers at which fluorescence exceeded the baseline threshold upon amplification of 500 pg μl^{-1} genomic DNA. Values are presented for two separate real-time PCR assays. The primers used to perform the assays are listed in Table 2.
b. VCG, vegetative compatibility group.
c. ΔC_T = mean C_T value for the target – mean C_T value for the normalizer (*MAT1-1*).

samples, three contained *F. oxysporum* f. sp. *radicis-cucumerinum*. However, inquiries at the end of the growing season revealed that the respective growers observed Fusarium root and stem rot at later stages in their crops. A selection of representative DNA array analyses from environmental samples is shown in Fig. 4. In all cases, sequencing of the amplicons generated by DNA amplification corresponded to the developed SCAR markers, confirming the identifications.

Discussion

Because the species *F. oxysporum* harbours pathogenic as well as non-pathogenic or even beneficial strains, identification below the species level is highly desired. In this study, a robust SCAR marker-based assay was developed for the detection and identification of the economically important cucumber pathogens *F. oxysporum* f. sp. *cucumerinum* and *F. oxysporum* f. sp. *radicis-cucumerinum*. Specificity of the assay was tested using a wide collection of *F. oxysporum* strains, encompassing all known VCGs of the target formae speciales as well as representative strains of other formae speciales (Table 1). In addition, the specificity of the markers was tested against another set of *F. oxysporum* strains, including 72 strains of *F. oxysporum* f. sp. *lycopersici* and 65 *F. oxysporum* f. sp. *radicis-lycopersici* strains (data not shown). Within these large collections, no cross-reactions were

Table 4. Detector oligonucleotides used for DNA array analysis.

Code	Sequence (5′–3′)	Specificity	Origin
Uni1[a]	TCCTCCGCTTATTGATATGC	*Universal*	28S rDNA
Fgn2[a]	CCAACTTCTGAATGTTGACC	*Fusarium sp.*	ITS II
Fox2[a]	GTTGGGACTCGCGTTAATTCG	*F. oxysporum*	ITS II
Foc1	ATGCAGTGACAGTTCCATGGC	*F. oxysporum* f. sp. *cucumerinum*	OPZ-12_{865}
Foc2	CTGGTTCCACAAGCGACGG	*F. oxysporum* f. sp. *cucumerinum*	OPZ-12_{865}
Forc1	GTGCTTTGCCTCGTCACAAT	*F. oxysporum* f. sp. *radicis-cucumerinum*	OPB-07_{277}
Forc2	GATGTCTTAAGTTATCTCTTG	*F. oxysporum* f. sp. *radicis-cucumerinum*	OPB-07_{277}
Dig1[a,b]	GTCCAGACAGGATCAGGATTG	None	–

a. Lievens and colleagues (2003).
b. 3′-end digoxigenin-labelled.

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Table 5. Hybridization signals[a] after PCR amplification of different amounts of genomic DNA of *Fusarium oxysporum* f. sp. *cucumerinum* ATCC 16416 in the absence or presence of non-target DNA.

Detector oligonucleotide	Control Target DNA (pg)					Non-target DNA (5 ng): *F. oxysporum* f. sp. *radicis-cucumerinum* Target DNA (pg)					Non-target DNA (5 ng): *Rhizoctonia solani* Target DNA (pg)				
	5000	500	50	5	0.5	5000	500	50	5	0.5	5000	500	50	5	0.5
Dig1	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Uni1	■	■	■	■	○	■	■	■	■	■	■	■	■	■	■
Fgn2	■	■	■	■	○	■	■	■	■	■	■	■	■	○	
Fox2	■	■	■	■	○	■	■	■	■	■	■	■	■	○	
Foc1	■	■	■	○		■	■	■	○		■	■	■	○	
Foc2	■	■	■	○		■	■	■	○		■	■	■	○	

a. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin labelled reference control (Dig1) and classified into three categories: blank = no signal; ○ = weak signal; ■ = strong signal.

observed for the *F. oxysporum* f. sp. *radicis-cucumerinum* marker (Table 1). Despite the higher genetic diversity within *F. oxysporum* f. sp. *cucumerinum,* all target strains contained the forma specialis-specific marker. Only two cross-reactions were observed for this marker, namely with *F. oxysporum* f. sp. *conglutinans* 81–4 and *F. oxysporum* f. sp. *gladioli* NRRL 26993 (Table 1). Pathogenicity testing showed that these two strains are unable to infect cucumber, but both isolates are genetically closely related to *F. oxysporum* f. sp. *cucumerinum* based on EF-1α sequences (Fig. 1). Nevertheless, in order to fully eliminate potential misidentification of these isolates, their belonging to their respective forma specialis should be confirmed by pathogenicity tests as well. Furthermore, with our assay we were able to identify three misclassifications within the collection which was confirmed by pathogenicity testing. Whereas two strains (strains 10 and 11) were found to be most likely saprophytic, strain 37 was found to belong to *F. oxysporum* f. sp. *cucumerinum* based on EF-1α sequences, the markers developed in this study, and pathogenicity testing.

Previously, SCAR markers have been identified for other formae speciales of *F. oxysporum*, including those pathogenic to bean, basil, chickpea and gladiolus (García-Pedrajas *et al*., 1999; De Haan *et al*., 2000; Chiocchetti *et al*., 2001; Alves-Santos *et al*., 2002; Jiménez-Gasco and Jiménez-Díaz, 2003). In contrast to our study, most of these markers were developed for *in planta* detection, where in principle only pathogenic *F. oxysporum* isolates occur, and only tested for specificity using a relatively small collection of strains. Recently it was suggested that the avirulence gene *SIX1* may be specific for strains of the tomato pathogen *F. oxysporum* f. sp. *lycopersici* (Rep *et al*., 2004).

Preferably, markers to identify formae speciales are directly linked to traits conferring pathogenicity (Lievens and Thomma, 2005). In general, virulence of a plant pathogen may be attributed either to subtle nucleotide differences in homologous genes (Joosten *et al*., 1994), or to the unique presence of a gene or a set of genes that confer a specific trait to the pathogen, such as the production of host-specific toxins (Wolpert *et al*., 2002; Thomma, 2003; Friesen *et al*., 2006). However, the genetic basis of host specificity in *F. oxysporum* still remains unknown (Di Pietro *et al*., 2003). In our study, we have identified genetic markers that are associated with the ability to cause disease on specific hosts. The *F. oxysporum* f. sp. *radicis-cucumerinum* marker OPB-07_{277} was found to show high nucleotide similarity with the 5′ end of *Folyt1*, a 2615-bp transposon identified in *F. oxysporum* f. sp. *lycopersici* (Gómez-Gómez *et al*., 1999). This active transposable element is present in about 10 copies in the *F. oxysporum* f. sp. *lycopersici* genome, although the exact copy number and site of

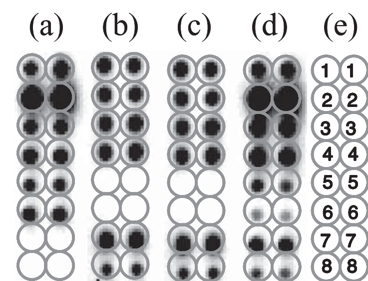

Fig. 4. DNA array-based detection and identification of the cucumber pathogens *Fusarium oxysporum* f. sp. *cucumerinum* and *F. oxysporum* f. sp. *radicis-cucumerinum* in environmental samples. Plant (roots) (a and b) and water (c and d) samples were collected from different commercial hydroponic systems with cucumber plants displaying typical *F. oxysporum* symptoms. While sample (a) was diagnosed with *F. oxysporum* f. sp. *cucumerinum*, samples (b) and (c) contained *F. oxysporum* f. sp. *radicis-cucumerinum.* In sample (d) both pathogens were detected. A scheme for the localization of the oligonucleotide detector oligonucleotides on the DNA array is shown in the panel at the right (e). The oligonucleotides, encompassing Dig1 (1), Uni1 (2), Fgn2 (3), Fox2 (4), Foc1 (5), Foc2 (6), Forc1 (7) and Forc2 (8), were horizontally spotted in duplicate.

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integration varied with strain. In addition, this transposon was found to be distributed among different formae speciales of *F. oxysporum*. In the *F. oxysporum* f. sp. *radicis-cucumerinum* genome, the *Folyt1*-related marker OPB-07_{277} is present in a single copy in all strains tested, irrespective of VCG or geographic origin (Table 3), suggesting that the transposon is inactive. Although a role of this transposon in host specificity remains obscure, in some other cases it has been found that pathogenicity of *F. oxysporum* isolates may be linked to the presence of transposons (Kelly *et al*., 1998; Mes *et al*., 2000; Jiménez-Gasco and Jiménez-Díaz, 2003). The *F. oxysporum* f. sp. *cucumerinum* marker OPZ-12_{865} was also found to be present in a single copy in the *F. oxysporum* f. sp. *cucumerinum* genome of all strains tested, irrespective of VCG, geographic region and grouping based on EF-1α sequences (Table 3). For this marker, no homology to any sequence present in public databases has been found. Further studies will have to reveal whether the diagnostic sequences identified in this study play a role in pathogenicity.

In today's agricultural and horticultural practice, accurate detection and identification of plant pathogens before taking disease control measures is essential. The failure of classical techniques, based on morphological observations and bioassays, to quickly and efficiently identify plant pathogens has prompted the development of molecular diagnostics. In contrast to classical methods, molecular methods are generally faster, more specific, more sensitive, and do not require culturing of the microorganisms (Lievens *et al*., 2005a). One of the most important advantages of DNA array technology is that DNA arrays can be used, in principle, to detect an unlimited number of different pathogens in a single hybridization run, even if different genes are targeted (Lievens and Thomma, 2005). To date, however, most DNA arrays in plant pathology have been designed to qualitatively detect and identify pathogens to the species level (Lievens *et al*., 2003; Nicolaisen *et al*., 2005; Tambong *et al*., 2006). In this study, a previously designed multipathogen DNA array (Lievens *et al*., 2003), that was also adapted for quantitative detection (Lievens *et al*., 2005b), was enhanced to specifically detect pathogenic strains below the species level. In order to validate our assay under practical conditions, multiple environmental samples from diverse biological origins were assessed. In all cases, the pathogens were correctly identified, even at the presymptomatic stage of infection, demonstrating the high sensitivity and usefulness of the assay in practice. The ability to detect and identify multiple plant pathogens by DNA arrays has great potential for enhancing the throughput of detection and diagnostic procedures. This increased resolution power will further elevate the appeal of DNA arrays for diagnostics.

Experimental procedures

Fungal isolates and DNA extraction

A wide collection of *F. oxysporum* strains, obtained from several culture collections or kindly provided by colleagues, was assembled in this study. Seventy-eight *F. oxysporum* strains isolated from cucumber were used, encompassing three avirulent isolates, 46 isolates belonging to *F. oxysporum* f. sp. *cucumerinum* and 29 isolates belonging to *F. oxysporum* f. sp. *radicis-cucumerinum*, representing all known VCGs of these formae speciales (Table 1). Furthermore, two *F. oxysporum* f. sp. *radicis-cucumerinum* strains isolated from soil were added to the collection. In addition, the fungal strain collection used in this study contained 52 *F. oxysporum* isolates of other formae speciales and four isolates of two other *Fusarium* species (Table 1). Many of these isolates have been characterized with respect to pathogenicity, vegetative compatibility and genetic diversity in previous studies (e.g. O'Donnell *et al*., 1998; Vakalounakis and Fragkiadakis, 1999; Baayen *et al*., 2000; Punja and Parker, 2000; Skovgaard *et al*., 2001; Vakalounakis *et al*., 2004; Cafri *et al*., 2005). All isolates were grown in the dark at 22°C on potato dextrose agar containing 100 p.p.m. streptomycin sulfate. Genomic DNA from a patch of mycelium (approximately 2 cm^2) of 5- to 10-day-old cultures was extracted using the phenol-chloroform extraction method as described previously (Lievens *et al*., 2003). DNA yield was determined spectrophotometrically. As a control for DNA quality, all DNA samples were successfully subjected to PCR analysis using the universal primers ITS5 and ITS4, targeting the ribosomal RNA gene (White *et al*., 1990).

Gene genealogies

To study the genetic relationships of *F. oxysporum* f. sp. *cucumerinum* and *F. oxysporum* f. sp. *radicis-cucumerinum* within the *F. oxysporum* complex a cladogram was constructed using EF-1α sequences. Sequences were retrieved from GenBank (Benson *et al*., 2004) to represent the three clades of *F. oxysporum*. (O'Donnell *et al*., 1998). In addition, EF-1α sequences were determined for several isolates listed in Table 1. To this end, genomic DNA was amplified with primers EF-1 and EF-2 as described by O'Donnell and colleagues (1998). Subsequently, both amplicon strands were sequenced using the same primers. All DNA sequences were aligned using the ClustalX algorithm. Subsequently, a neighbour-joining tree (Saitou and Nei, 1987) was constructed and displayed by TreeView (v. 1.6.6; Page, 1996). The EF-1α sequence of *F. solani* (GenBank Accession number DQ247710) was used as outgroup. Support of internal nodes was determined by bootstrap analysis performed with 1000 replications. All sequences obtained in this study were deposited in GenBank (Accession numbers EF056744–EF056790) as denoted in Table 1.

Random-amplified polymorphic DNA analysis and PCR amplification conditions

A total of 115 decamer oligonucleotides, randomly selected from the Operon primer kits (Operon Technologies, Alameda,

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CA, USA), was screened using a subset of 12 *F. oxysporum* isolates, encompassing three *F. oxysporum* f. sp. *cucumerinum* isolates, three *F. oxysporum* f. sp. *radicis-cucumerinum* isolates, and six isolates belonging to other formae speciales. Forty-two primers that led to the production of clear, distinct, reproducible and polymorphic bands for *F. oxysporum* f. sp. *cucumerinum* or *F. oxysporum* f. sp. *radicis-cucumerinum* were included in a second RAPD analysis using 32 selected isolates, of which eight isolates belonged to *F. oxysporum* f. sp. *cucumerinum*, eight to *F. oxysporum* f. sp. *radicis-cucumerinum* and 16 to other formae speciales. Finally, in order to identify target specific RAPD markers, the primers selected as the most suitable to discriminate the target formae speciales were screened against all isolates listed in Table 1. Amplification was performed using an Eppendorf Mastercycler® (Eppendorf GmbH, Hamburg, Germany) in a total volume of 25 μl containing 0.5 μM of single random primer, 0.15 mM of each deoxynucleoside triphosphate, 1.0 U Titanium *Taq* DNA polymerase (Clontech Laboratories, Palo Alto, CA, USA), and 5 ng of genomic DNA. Before amplification, DNA samples were denatured at 94°C for 2 min. Subsequently, 35 cycles were run of 1 min at 94°C, 1 min at 35°C, and 2 min at 72°C, with a final extension step at 72°C for 10 min. Random-amplified polymorphic DNA products were separated by loading 12.5 μl of the reaction volume on 1.5% agarose gels followed by electrophoresis in 1× Tris-acetate-EDTA (TAE) buffer, stained with ethidium bromide, and visualized with UV light. Both a 100-bp and a 1-kb DNA ladder (Smartladder, Eurogentec, Seraing, Belgium) were used as size markers for comparison. Gel images were acquired with the BioChemi System (UVP, Upland, CA, USA). All RAPD reactions were performed at least twice to check reproducibility.

Cloning and sequencing of RAPD amplicons

For several strains of each target forma specialis the identified markers were excised from agarose gels and DNA was purified using the QIAquick Gel Extraction Kit (Qiagen, Venlo, the Netherlands). Purified RAPD products were cloned into the vector pCR2.1-TOPO using the TOPO TA cloning kit (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. Both strands of the cloned fragments were sequenced using the universal primers M13 and T13. A search for sequence similarities was performed using the BLAST (Altschul *et al.*, 1990) algorithm to screen GenBank. In addition, the genome sequence of *Fusarium graminearum* (http://www-genome.wi.mit.edu/annotation/fungi/fusarium/) was screened to identify possible homologues.

Design of SCAR primers and PCR amplification conditions

Based on the sequences of the selected RAPD markers, SCAR primers were designed by elongating the original RAPD primer. The length of the primers was adjusted to obtain different SCAR primers with a similar melting temperature. In order to identify strains of *F. oxysporum* f. sp. *cucumerinum* primers FocF1 (5′-TCAACGGGACACTT TATGTTC-3′) and FocR2 (5′-TCAACGGGACTCCCTTCG-3′) were designed. For the identification of *F. oxysporum* f. sp. *radicis-cucumerinum* primers ForcF1 (5′-GGTGACGCA GCAGTCTAGA-3′) and ForcR2 (5′-GTGACGCAGGGT AGGCAT-3′) were designed. Specificity of the primers was tested using genomic DNA extracted from all isolates listed in Table 1. Genomic DNA was amplified in a reaction volume of 20 μl, containing 0.15 mM of each deoxynucleoside triphosphate, 0.5 μM of each primer, 1 U Titanium *Taq* DNA polymerase and 5 ng genomic DNA. Thermal cycling conditions comprised an initial denaturation at 94°C for 2 min, followed by a cycling protocol of 30 cycles of denaturation at 94°C for 45 s, annealing at 65°C for 45 s and elongation at 72°C for 45 s, and a final elongation step at 72°C for 10 min. Amplified products (1 μl) were resolved electrophoretically in a 1.5% agarose gel. All reactions were performed at least twice.

Real-time PCR analysis to determine the copy number of the RAPD markers

To assess the copy number of the selected markers, real-time PCR technology was used (Livak and Schmittgen, 2001; Li *et al.*, 2004). In order to normalize the number of DNA copies the single copy mating type gene (*MAT1*, idiomorph *MAT1-1*; Arie *et al.*, 2000) was used as a reference. Three primer pairs, one for each marker and one for the normalizer were designed to result in amplicons of similar length (Table 2). Real-time PCR amplifications were performed separately in a reaction volume of 25 μl using SYBR® Green I on a SmartCyclerII® (Cepheid, Sunnyvale, CA, USA). Each reaction mixture contained 2 μl genomic DNA (500 pg μl^{-1}), 12.5 μl of the QuantiTect™ SYBR® Green PCR Master Mix (Qiagen, Valencia, CA, USA), 5.0 mM $MgCl^2$, 0.625 μl of each primer (20 μM) and 9.25 μl of sterile distilled water. Thermal cycling conditions comprised an initial denaturation step of 15 min at 95°C followed by 45 cycles of 15 s at 94°C, 30 s at 62°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 previously (Brouwer *et al.*, 2003; Lievens *et al.*, 2006b). Standard curves to calculate PCR efficiencies ($E = 10^{-1/slope} - 1$) were generated for each primer pair by plotting the threshold cycle (C_T) of a 10-fold dilution series of standard DNA (two replicates) against the logarithm of the concentration. All real-time PCR reactions were performed at least twice in separate real-time PCR assays.

DNA array hybridization

A number of detector oligonucleotides used in this study (Table 4) was designed previously, including the ITS-based detectors to detect the genus *Fusarium* (Fgn2) and the species *F. oxysporum* (Fox2), as well as the control oligonucleotides (Uni1 and Dig1) (Lievens *et al.*, 2003). In addition, oligonucleotides to detect the target formae speciales *cucumerinum* (Foc1 and Foc2) and *radicis-cucumerinum* (Forc1 and Forc2) were designed as described previously (Lievens *et al.*, 2003; 2006a) based on the sequence of the selected markers. All oligonucleotides were spotted in duplicate on nylon membranes generating an array of 16 spots.

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DNA arrays were produced as reported previously (Lievens *et al*., 2003; 2006a).

For DNA array analysis, separate amplification reactions were performed. In a first reaction, the ITS regions were amplified using the universal fungal primers ITS1-F and ITS4 (Gardes and Bruns, 1993) and simultaneously labelled with alkaline-labile digoxigenin (0.15 mM digoxigenin-11-dUTP mix; Roche Diagnostics GmbH, Mannheim, Germany). In addition, SCAR markers were amplified in separate PCR reactions and simultaneously labelled with alkaline-labile digoxigenin using the selected SCAR primers. DNA samples were amplified according to the same thermal profile as described above, except for amplifying the ITS regions a 59°C annealing temperature was used (Lievens *et al*., 2003; 2005b; 2006a). The resulting labelled amplicons were subsequently combined and used for DNA array hybridization as previously described (Lievens *et al*., 2003; 2005b; 2006a). All hybridizations were performed at least twice on separate DNA arrays.

Detection and identification of the F. oxysporum *cucumber pathogens by DNA array hybridization in environmental samples*

To test the potential use of the diagnostic assay for detection and identification of *F. oxysporum* strains in environmental samples, several experiments were performed. First, cucumber (*C. sativus* L. cv. 'straight Eight') seedlings were inoculated at the first-true-leaf stage with either *F. oxysporum* f. sp. *cucumerinum* (NETH 11179) or *F. oxysporum* f. sp. *radicis-cucumerinum* [Afu-68(A)]. Roots were dipped into a conidial suspension (10^7 microconidia ml^{-1}) of the test isolate for 30 min. Subsequently, seedlings were transplanted into 400 ml of dark Sphagnum peat mix (DCM, Grobbendonk, Belgium) which was pasteurized for 5 days at 60°C. Just before transplanting, slow release fertilizer (8-5-7, 1:1 Ecomix 1-Ecomix 4 blend, DCM, Grobbendonk, Belgium) was incorporated into the mixes at a rate of 1 g per pot. Plants were grown in a growth chamber with a 12 h photoperiod (225 $\mu E\ m^{-2}\ s^{-1}$) at 22°C. Root, stem and potting mix samples were taken 7 and 20 days after inoculation at which time the plants did not or did display clear symptoms respectively. Sub-samples were used for DNA array analysis and for plating on semi-selective medium (Komada, 1975). Genomic DNA was isolated from 0.75 g plant material and 0.5 g potting mix using the UltraClean Plant DNA Isolation Kit and the UltraClean Soil DNA Isolation Kit, respectively, according to the manufacturer's instructions (Mo Bio Laboratories, Solana Beach, CA, USA).

In addition, at commercial cucumber growers, water and root samples collected from hydroponic growing systems with cucumber plants showing *F. oxysporum* symptoms as well as no disease symptoms, were analysed by the DNA array. Bulk DNA was obtained from a 200-ml water sample or from 0.75 g plant tissue using the UltraClean Water DNA Isolation Kit and the UltraClean Plant DNA Isolation Kit respectively. DNA amplification, labelling and hybridization were performed as described above. In addition, a parallel set of samples was retained for classical plating on semi-selective medium (Komada, 1975) followed by microscopic examination (Nelson *et al*., 1983).

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