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Design and development of a DNA array for rapid detection and identification of multiple tomato vascular wilt pathogens

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Abstract

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *lycopersici*, and Verticillium wilt, caused by either *Verticillium albo-atrum* or *Verticillium dahliae*, are devastating diseases of tomato (*Lycopersicon esculentum*) found worldwide. Monitoring is the cornerstone of integrated pest management of any disease. The lack of rapid, accurate, and reliable means by which plant pathogens can be detected and identified is one of the main limitations in integrated disease management. In this paper, we describe the development of a molecular detection system, based on DNA array technology, for rapid and efficient detection of these vascular wilt pathogens. We show the utility of this array for the sensitive detection of these pathogens from complex substrates like soil, plant tissues and irrigation water, and samples that are collected by tomato growers in their greenhouses.

Keywords: DNA array; Internal transcribed spacer region; *Fusarium*; Reverse dot blot; Tomato; Vascular wilt; *Verticillium*

1. Introduction

Fusarium and Verticillium wilts are the two most widely distributed and economically important vascular wilt diseases of tomato (*Lycopersicon esculentum* Mill.) worldwide. *Fusarium oxysporum* f. sp. *lycopersici*, the causative agent of Fusarium wilt is host-specific, but may also exist in symptomless alternate hosts representing many species [1]. Verticillium wilt is caused by either *Verticillium albo-atrum* or by *Verticillium dahliae*. Both of these *Verticillium* species are able to attack a broad range of plant species including cultivated crops and weeds [2]. These three soil-borne tomato wilt fungi are closely related in that they both invade the plant through either entrance at the root tip or at the site of lateral root formation [3,4]. Their entry is also facilitated by the presence of wounds, which can be due to nematode feeding. Each pathogen then grows intercellularly through the root cortex, and, subsequently rapidly grows into the xylem where it obstructs the upward movement of water and nutrients. As the vessels are plugged and collapse, the water supply to the leaves is blocked, thus causing wilt symptoms. For both diseases, brown vascular discoloration can be observed in stem tissue cross sections near the soil line, even though these stems remain firm and green on the outside.

Plants afflicted with Fusarium wilt first develop yellowing of the lowest leaves that is often restricted to one side of the plant or a single shoot. The affected leaves wilt and die. Wilting progresses up the stem until the foliage is killed and the stem decays. Conversely, Verticillium wilt causes uniform yellowing and wilting of lower leaves. Verticillium wilt generally does not result in death, but affected plants are frequently stunted and produce smaller fruit [5].

There are currently no fungicides available to control these wilts once plants have been infected. These vascular wilt fungi can survive in the soil or on decaying plant debris for years. Control of these fungi in infested soils can be established by reducing their presence through long crop rotations or soil fumigation. For *Verticillium*, having a broad host range, it is especially important that rota-

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Table 1
Fungal isolates used in this study

Phylum	Order	Species	Isolate[a]	Origin	Host or substrate
Ascomycota	Dothideales	*Didymella lycopersici*	CBS 378.67	The Netherlands	*Lycopersicon esculentum*
		Didymella lycopersici	CBS 101199	The Netherlands	*Solanum lycopersicon*
	Helotiales	*Sclerotinia minor*	MUCL 38484	unknown	unknown
		Sclerotinia minor	CBS 339.39	Italy	*Lactuca sativa*
		Sclerotinia sclerotiorum	DSM 1946	unknown	*Medicago sativa*
		Sclerotinia sclerotiorum	AED 1548	USA	*Glycine max*
	Hypocreales	*Nectria haematococca*	MUCL 20259	Belgium	Soil
	Phyllachorales	*Plectosphaerella cucumerina*	CBS 400.58	Canada	*Lycopersicon esculentum*
		Plectosphaerella cucumerina	CBS 101607	New Zealand	*Nicotiana tabacum*
Basidiomycota	Aphyllophorales	*Athelia rolfsii*	MUCL 19443	Belgium	Soil
		Athelia rolfsii	DSM 63030	Italy	*Solanum tuberosum*
Deuteromycota	Agonomycetales	*Rhizoctonia solani*	CBS 101761	The Netherlands	*Lactuca sativa*
		Rhizoctonia solani	CBS 101590	unknown	*Lycopersicon esculentum*
	Melanconiales	*Colletotrichum coccodes*	DSM 2492	unknown	*Lycopersicon esculentum*
		Colletotrichum coccodes	CBS 527.77	Bulgaria	*Lycopersicon esculentum*
	Moniliales	*Botrytis cinerea*	MUCL 18862	UK	*Lactuca* sp.
		Botrytis cinerea	CBS 676.89	The Netherlands	*Phaseolus vulgaris*
		Fusarium graminearum (lineage 7)	HCK PH1	unknown	unknown
		Fusarium oxysporum f. sp. *conglutinans*	HCK 81-4	unknown	unknown
		Fusarium oxysporum f. sp. *lycopersici* (race 1)	CBS 645.78	Morocco	*Lycopersicon esculentum*
		Fusarium oxysporum f. sp. *lycopersici* (race 1)	CBS 412.90	Israel	*Lycopersicon esculentum*
		Fusarium oxysporum f. sp. *lycopersici* (race 2)	CBS 646.78	Morocco	*Lycopersicon esculentum*
		Fusarium oxysporum f. sp. *lycopersici* (race 2)	CBS 413.90	Israel	*Lycopersicon esculentum*
		Fusarium oxysporum f. sp. *lycopersici* (race 1)	HCK FOL1	unknown	unknown
		Fusarium oxysporum f. sp. *lycopersici* (race 2)	HCK FOL2	unknown	unknown
		Fusarium oxysporum f. sp. *radicis-lycopersici*	MUCL 39789	Belgium	*Lycopersicon esculentum*
		Fusarium oxysporum f. sp. *radicis-lycopersici*	CBS 873.95	Israel	*Lycopersicon esculentum*
		Fusarium oxysporum f. sp. *radicis-lycopersici*	CBS 101587	unknown	*Lycopersicon esculentum*
		Fusarium oxysporum f. sp. *radicis-lycopersici*	HCK 0-1090/B	unknown	unknown
		Fusarium solani	CBS 165.87	Denmark	*Solanum tuberosum*
		Fusarium solani	CABI 17960	Brazil	*Solanum tuberosum*
		Fusarium solani	HCK S-66	unknown	unknown
		Trichoderma asperellum	MUCL 41923	unknown	unknown
		Trichoderma asperellum	MUCL 41924	unknown	unknown
		Trichoderma harzianum	MUCL 19412	unknown	unknown
		Trichoderma harzianum	MUCL 28446	unknown	unknown
		Verticillium albo-atrum	CBS 451.88	Belgium	unknown
		Verticillium albo-atrum	CBS 321.91	The Netherlands	*Lycopersicon esculentum*
		Verticillium albo-atrum	CBS 385.91	The Netherlands	*Lycopersicon esculentum*
		Verticillium dahliae	CBS 386.49	The Netherlands	*Solanum melongena*
		Verticillium dahliae	CBS 179.66	The Netherlands	*Lycopersicon esculentum*
		Verticillium dahliae	CBS 381.66	Canada	*Lycopersicon esculentum*
		Verticillium dahliae	RCR V44	USA	*Gossypium* sp.
		Verticillium dahliae	RCR PH	USA	*Pistacia* sp.
		Verticillium dahliae	RCR 115	Syria	*Gossypium* sp.
		Verticillium dahliae	RCR 70-21	USA	*Capsicum* sp.
		Verticillium dahliae	RCR BB	USA	*Solanum tuberosum*
		Verticillium dahliae	RCR S39	USA	Soil
		Verticillium nubilum	MUCL 8266	Germany	Soil
		Verticillium tricorpus	MUCL 9792	UK	*Lycopersicon esculentum*
	Sphaeropsidales	*Pyrenochaeta lycopersici*	DSM 62931	Germany	*Lycopersicon esculentum*
		Pyrenochaeta lycopersici	CBS 267.59	The Netherlands	*Lycopersicon esculentum*
Oomycota	Peronosporales	*Phytophthora capsici*	CBS 554.88	Argentina	*Lycopersicon esculentum*
		Phytophthora capsici	MUCL 43490	USA	*Capsicum annuum*
		Phytophthora cryprogea	CBS 113.19	Ireland	*Lycopersicon esculentum*
		Phytophthora cryptogea	MUCL 28777	The Netherlands	*Gerbera* sp.
		Phytophthora drechsleri	DSM 62679	Iran	*Beta vulgaris*
		Phytophthora drechsleri	CBS 359.52	Argentina	*Solanum tuberosum*
		Phytophthora infestans	MUCL 43257	unknown	*Solanum tuberosum*
		Phytophthora nicotianae	MUCL 40633	Zimbabwe	*Nicotiana tabacum*
		Phytophthora nicotianae	MUCL 28775	USA	*Nicotiana tabacum*
		Pythium aphanidermatum	CBS 634.70	Israel	*Lycopersicon esculentum*
		Pythium aphanidermatum	CBS 101589	Unknown	*Cucumis sativus*

Table 1 (*Continued*).

Phylum	Order	Species	Isolate[a]	Origin	Host or substrate
		Pythium dissotocum	CBS 166.68	USA	*Triticum aestivum*
		Pythium dissotocum	CBS 525.74	The Netherlands	*Iris* sp.
		Pythium irregulare	CBS 461.48	Australia	unknown
		Pythium irregulare	CBS 751.96	UK	*Lepidum sativum*
		Pythium polymastum	CBS 810.70	The Netherlands	*Lactuca sativa*
		Pythium polymastum	CBS 811.70	The Netherlands	*Lactuca sativa*
		Pythium sylvaticum	CBS 225.68	The Netherlands	Soil
		Pythium sylvaticum	CBS 226.68	The Netherlands	*Crocus* sp.
		Pythium ultimum	CBS 101588	unknown	*Cucumis sativus*
		Pythium ultimum var. *ultimum*	MUCL 16164	UK	*Pisum sativum*

[a]AED: collection of A.E. Dorrance, Ohio State University, Wooster, OH, USA; CABI: Centre for Agriculture and Bioscience International, Surrey, UK; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; HCK: collection of H.C. Kistler, University of Minnesota, St. Paul, MN, USA; MUCL: Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; RCR: collection of R.C. Rowe, Ohio State University, Wooster, OH, USA.

tional crops are free of alternate weed hosts. Furthermore, genetic resistance to each of these diseases has been identified [6,7]. However, fungal strains that are not restricted by these resistant host varieties do appear [8,9]. Alternatively, biocontrol measures against these vascular wilt diseases are being developed using either non-pathogenic *Fusarium* spp. [10] or other antagonistic microorganisms [11–14].

Until recently, pathogenic fungi were detected by growing them on selective media or by biochemical, chemical and immunological analyses. These methods are time-consuming, laborious, and require extensive knowledge of classical taxonomy. The advent of molecular biology and, in particular, polymerase chain reaction (PCR) has opened alternative ways for detection and identification of plant pathogens. For instance, random amplified polymorphic DNA analysis allows the characterization of *F. oxysporum* f. sp. *lycopersici*, *V. albo-atrum* and *V. dahliae* isolates [15,16]. In addition, real-time PCR technology has the advantage of accurate quantification capability for assessing the extent of pathogen biomass, and, with multiplex formats, enables simultaneous detection of different organisms. However, the total amount of PCR reactions in a single multiplex real-time PCR application is severely limited by the number of different fluorescent dyes available and the common use of a monochromatic energizing light source in the most common real-time PCR instruments [17]. These specific limitations do not apply to a conventional multiplex PCR, though the development of an efficient multiplex PCR requires extensive strategic planning and optimization of reaction conditions in order to discriminate at least several amplicons per reaction [18]. In contrast, DNA array technology may lead to unlimited multiplexing, that is detection and identification of numerous plant pathogens per assay. With this technology, oligonucleotide detectors are covalently bound to a solid support, such as a nylon membrane. The target DNA segment to be tested is amplified and simultaneously labeled, and subsequently hybridized to the membrane under stringent conditions. This technique, also called reverse dot blot technology, was originally developed as a technique to screen for human genetic disorders [19], but has also been successfully applied to detect and identify different microorganisms in clinical laboratories [20]. In plant pathology, DNA arrays have been successfully applied to discriminate and identify DNA samples isolated from specific oomycete, nematode and bacterial cultures [21–23]. Currently, however, this technology has not been used to detect and identify plant pathogens from multiple complex extracts isolated from artificially or naturally infested soils, plant material, or irrigation water.

In this paper we describe the design and development of a DNA array to specifically detect and identify the economically important tomato vascular wilt pathogens *F. oxysporum* f. sp. *lycopersici*, *V. albo-atrum*, and *V. dahliae* to the species level. The membrane was validated using samples from infected plants, contaminated water, and inoculated potting mixes, as well as naturally infected plant materials, and naturally infested soils, thus demonstrating the opportunities for utilization of the membrane in practice with extracts from complex substrates.

2. Materials and methods

2.1. Growth and maintenance of fungal isolates

Fungal isolates used in this study are listed in Table 1. The collection contains ascomycetous, basidiomycetous, deuteromycetous, and oomycetous species obtained from several sources. Stock cultures of oomycetes were maintained on corn meal agar slants under water at room temperature. Other fungal cultures were maintained on potato dextrose agar (PDA) slants in 25% glycerol at −80°C. All isolates were cultured on PDA containing 100 ppm streptomycin sulfate and incubated in darkness at 22°C.

2.2. DNA extraction of fungal cultures

DNA was extracted from 5- to 10-day-old cultures in

2-ml microcentrifuge tubes. A patch of mycelium (approximately 2 cm^2) was scraped from the margin of a colony and suspended in 300 μl lysis buffer (2.5 M LiCl, 50 mM Tris, 62.5 mM EDTA, and 4.0% Triton X-100, pH 8.0) together with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v) and approximately 75 μl of glass beads (212–300 μm). Cells were mechanically disrupted in a Fast Prep system (Thermo Savant, Holbrook, NY, USA) by reciprocal shaking of the samples for 30 s at maximum speed. The supernatant was collected after centrifugation (9300×*g*) and the DNA was precipitated by the addition of two volumes of absolute ethanol followed by incubation for 15 min at −20°C and subsequent centrifugation (5 min at 9300×*g*). The DNA pellet was washed with 70% ethanol, air-dried, and resuspended in 50 μl 10 mM Tris (pH 8.0). The amount of DNA was determined spectrophotometrically at 260 nm.

2.3. Selection of detector probes and production of the DNA array

Species-specific detector probes were designed based on the internal transcribed spacer (ITS) region of fungal rDNA. The region between the small and the large subunit rDNA of all *F. oxysporum*, *V. albo-atrum* and *V. dahliae* isolates listed in Table 1 was amplified and sequenced using the universal primers ITS5 and ITS4 [24]. Amplification was carried out in 25 μl containing 10–15 ng genomic DNA using Platinum *Pfx* DNA polymerase (Invitrogen Corporation, San Diego, CA, USA) according to the manufacturer's protocol. The following thermal profile was used: 94°C for 2 min followed by 35 cycles of 45 s at 94°C, 45 s at 58°C, and 45 s at 68°C with a final 10-min extension step at 68°C. The PCR products were sequenced using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). GenBank was searched to find matching and closely related ITS sequences. This not only allowed the design of species-specific oligonucleotide detector probes but also confirmed the species identities of the isolates used in this study. All ITS sequences were aligned using the ClustalW algorithm and multiple 19- to 25-mer taxon-specific oligonucleotides were selected, attempting to respect optimal and uniform theoretical hybridization kinetics (Table 2). All oligonucleotides were synthesized with a 5′-C6-amino linker for covalent binding to a nylon membrane.

The selected oligonucleotides (40 μM) and the digoxigenin-labeled control (10 μM) were kept in duplicate in a microtiter plate in sodium bicarbonate buffer (0.5 M, pH 8.4). The oligonucleotides were spotted in duplicate on Immunodyne ABC membranes (PALL Europe Limited, Portsmouth, UK) using a pin replicator (V&P Scientific, Inc., San Diego, CA, USA). Printed membranes were air-dried for 10 min, transferred to blocking solution (2× saline sodium citrate (SSC), 0.5% casein, and 0.05% Tween 20), and gently agitated for 30 min [23]. Blots were stored in 2× SSC at 4°C until use.

2.4. Target amplification and hybridization

The target ITS regions of all fungal isolates listed in Table 1 were amplified and simultaneously labeled with alkaline-labile digoxigenin using the universal primers ITS5 and ITS4 [24]. The target samples (25 μl) containing 10–15 ng genomic DNA were amplified using 1.25 units Platinum *Taq* DNA polymerase (Invitrogen Corporation, San Diego, CA, USA) in the presence of 0.012 mM digoxigenin-11-d-UTP (Dig-dUTP; Roche Diagnostics GmbH, Mannheim, Germany) according to the same thermal profile as described above, with an elongation temperature of 72°C. The resulting Dig-dUTP-labeled amplicons were quantified by comparison to a DNA ladder after gel electrophoresis, calibrated, and subsequently used as target probes in hybridization reactions with the array.

Printed membranes were prehybridized for at least 1.5 h at 54°C in hybridization buffer (6× SSC, 0.1% sarcosine,

Table 2
Sequences of detector oligonucleotides used for the DNA array

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

[a]ITS4 primer [24].
[b]3′-end digoxigenin labeled.

and 0.02% sodium dodecyl sulfate (SDS)) plus 1% casein. Dig-dUTP-labeled amplicons (approximately 10 ng ml^{-1}) were denatured by boiling in hybridization buffer and hybridized overnight at 54°C. Membranes were subsequently washed twice with stringency buffer (6× SSC, and 0.01% SDS) at 54°C. Detection was performed using anti-digoxigenin alkaline phosphatase conjugate and CDP-Star substrate (both from Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. Chemiluminescence was detected using a highly sensitive digital CCD video camera (BioChemi System; UVP, Inc., Upland, CA, USA). Hybridization results were analyzed using Labworks 4.0 Image Acquisition and Analysis Software (UVP, Inc., Upland, CA, USA). All hybridizations were conducted at least twice.

2.5. Validation of the DNA array

The DNA array was validated using complex samples from different origins attempting to mimic samples obtained under current horticultural practices. To accomplish this, potting mix (DCM potting mix for house and garden; DCM, St.-Katelijne-Waver, Belgium) was pasteurized for 5 days at 60°C and subsequently inoculated with either *V. dahliae* or *F. oxysporum* f. sp. *lycopersici* or mock-inoculated as a negative control. Per gram dry weight of potting mix, 50 *V. dahliae* (CBS 179.66) microsclerotia [25] were added. For *F. oxysporum* f. sp. *lycopersici* (CBS 645.78) soil inoculum was prepared in chopped potato soil as described [26]. To inoculate, air-dried inoculum was ground with a mortar and pestle followed by sequential sieving through 2.0- and 1.0-mm sieves. Particles remaining on the 1.0-mm sieve were used to infest potting mix at 0.5 g inoculum per pot (800 ml). Directly after infestation with either pathogen, 10-day-old tomato seedlings (*L. esculentum* Mill. cv. Saint-Pierre) were transferred to both inoculated and mock-inoculated potting mixes. Plants were grown in a growth chamber with a 16-h photoperiod (225 μE m^{-2} s^{-1}) at 22°C. The night temperature was 18°C. Stems, roots and potting mix were collected 7 and 10 weeks after transplantation, at which time the plants had not yet developed symptoms. All plant tissues were surface sterilized (30 s submergence in 1% sodium hypochlorite, followed by three 30-s rinse

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

Fungal isolate	Fgn1	Fgn2	Fox1	Fox2	Vgn1	Vgn2	Val1	Val2	Vda1	Vda2	Con1	Uni1	Dig1
Fusarium graminearum HCK PH1	○	○										○	○
Fusarium oxysporum f. sp. *conglutinans* HCK 81-4	○	○	○	○								○	○
Fusarium oxysporum f. sp. *lycopersici* CBS 645.78	○	○	○	○								○	○
Fusarium oxysporum f. sp. *lycopersici* CBS 412.90	○	○	○	○								○	○
Fusarium oxysporum f. sp. *lycopersici* CBS 646.78	○	○	○	○								○	○
Fusarium oxysporum f. sp. *lycopersici* CBS 413.90	○	○	○	○								○	○
Fusarium oxysporum f. sp. *lycopersici* HCK FOL1	○	○	○	○								○	○
Fusarium oxysporum f. sp. *lycopersici* HCK FOL2	○	○	○	○								○	○
Fusarium oxysporum f. sp. *radicis-lycopersici* MUCL 39789	○	○	○	○								○	○
Fusarium oxysporum f. sp. *radicis-lycopersici* CBS 873.95	○	○	○	○								○	○
Fusarium oxysporum f. sp. *radicis-lycopersici* CBS 101587	○	○	■	○								○	○
Fusarium oxysporum f. sp. *radicis-lycopersici* HCK 0-1090/B	○	○	○	○								○	○
Fusarium solani CBS 165.87		○										○	○
Fusarium solani CABI 17960		○										○	○
Fusarium solani HCK S-66		○										○	○
Nectria haematococca MUCL 20259		○										○	○
Verticillium albo-atrum CBS 451.88					■	○	○	○				○	○
Verticillium albo-atrum CBS 321.91					○	○	○	○				○	○
Verticillium albo-atrum CBS 385.91					■	■	■	■				○	○
Verticillium dahliae CBS 386.49					○	○			○	■		○	○
Verticillium dahliae CBS 179.66					■	○			○	○		○	○
Verticillium dahliae CBS 381.66					■	○			○	○		○	○
Verticillium dahliae RCR V44					■	○			○	○		○	○
Verticillium dahliae RCR PH					■	○			○	○		○	○
Verticillium dahliae RCR 115					○	○			○	○		○	○
Verticillium dahliae RCR 70-21					■	○			○	○		○	○
Verticillium dahliae RCR BB					■	○			○	■		○	○
Verticillium dahliae RCR S39					■	○			○	○		○	○
Verticillium nubilum MUCL 8266												○	○
Verticillium tricorpus MUCL 9792					■	○	○					○	○
Autoclaved ultrapure water													○

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

steps in sterile distilled water). Each sample of the surface-sterilized tissues as well as of the potting mix samples was plated on semi-selective medium [27,28] and incubated at 22°C in darkness. A parallel set of samples was retained for DNA extraction using the UltraClean Plant DNA Isolation Kit (for stems and roots) and the UltraClean Soil DNA Isolation Kit (for potting mix) as described by the manufacturer (Mo Bio Laboratories, Inc., Solana Beach, CA, USA).

To detect fungi in water-based samples, tap water was inoculated with conidia of *V. dahliae* CBS 179.66 (0.5 conidium ml^{-1}). DNA was extracted using the UltraClean Water DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA) as described by the manufacturer.

Scientia Terrae is a research institute that provides disease diagnostic services and advice to commercial tomato growers. Samples for which diagnosis from Scientia Terrae was requested by growers were assessed using the DNA array as well as classical disease diagnostic methods. DNA from those samples was isolated as described above.

For all complex biological samples, DNA was isolated as described above and amplified using the primers ITS1-F and ITS4 [24,29]. The target samples (20 μl) containing 1 μl genomic DNA were amplified using 1 unit Titanium *Taq* DNA polymerase (Clontech Laboratories, Inc., Palo Alto, CA, USA) in the presence of 0.0075 mM Dig-dUTP (Roche Diagnostics GmbH, Mannheim, Germany). Amplification was performed according to the following thermal profile: 94°C for 2 min followed by 35 cycles of 45 s at 94°C, 45 s at 59°C, and 45 s at 72°C, with a final 10-min extension step at 72°C. Hybridization of amplicons to the oligonucleotide array was performed as described above.

3. Results and discussion

3.1. Development of the oligonucleotide array

Based on the ITS sequences of *F. oxysporum*, *V. albo-atrum*, and *V. dahliae*, 10 taxon-specific oligonucleotide detectors with comparable theoretical hybridization kinetics were designed (Table 2). *Fusarium* is an anamorphic genus for which clear species boundaries are lacking [30]. Two genus-specific oligonucleotides were developed for *Fusarium* (Fgn1 and Fgn2), both covering a wide and largely overlapping range of *Fusarium* species. In addition, two species-specific oligonucleotides were designed for detection of *F. oxysporum* (Fox1 and Fox2). Also the genus *Verticillium* contains anamorphic species that are genetically very divergent. This diversity is reflected at the nucleotide level for the ITS region. Since a genus-specific oligonucleotide sequence shared by a wide range of *Verticillium* species could not be identified, two oligonucleotide sequences (Vgn1 and Vgn2) that are common for a subgroup of homologous *Verticillium* species including *V. albo-atrum*, *V. dahliae*, *V. longisporum*, and *V. tricorpus* were selected. Furthermore, species-specific oligonucleotides were designed for the detection of *V. albo-atrum* (Val1 and Val2) and for *V. dahliae* (Vda1 and Vda2).

As a control for the specificity of the detection system, a universal (non-discriminative) oligonucleotide (Uni1) supposed to detect the presence of any fungus, and another oligonucleotide with no known homology to fungal sequences (Con1), and not supposed to detect any fungus, were designed. The latter oligonucleotide was also synthesized with digoxigenin labeling (Dig1) as a positive control for detection.

3.2. Evaluation of the specificity of the oligonucleotide array

DNA was isolated from the different fungal isolates listed in Table 1 and the ITS regions (including the 5.8S rDNA region) of the rDNA were amplified and sequenced to confirm the identity of the isolates used in this study. PCR was performed to amplify and label the ITS regions and, subsequently, digoxigenin-labeled PCR amplicons directly generated from fungal DNA were hybridized to the array. Amplicons of the tested *F. oxysporum*, *V. albo-atrum* or *V. dahliae* isolates hybridized strongly to their respective detector oligonucleotides as shown in Table 3. Amplicons generated from fungal species listed in Table 1 other than from the genus *Fusarium* or *Verticillium* did not show hybridization (data not shown), demonstrating the specificity of the oligonucleotide detectors on the mem-

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

Fungal isolate mixture	Fgn2	Fox2	Vgn2	Val2	Vda1	Con1	Uni1	Dig1
F. oxysporum f. sp. *lycopersici* CBS 645.78; *V. albo-atrum* CBS 451.88; *V. dahliae* CBS 386.49	○	○	○	○	○		○	○
F. solani CBS 165.87; *V. albo-atrum* CBS 451.88; *V. dahliae* CBS 386.49	○		○	○	○		○	○
F. oxysporum f. sp. *lycopersici* CBS 645.78; *V. albo-atrum* CBS 451.88	○	○	○	○			○	○
F. oxysporum f. sp. *lycopersici* CBS 645.78; *V. dahliae* CBS 386.49	○	○	○		○		○	○
V. albo-atrum CBS 451.88; *V. dahliae* CBS 386.49			○	○	○		○	○
Autoclaved ultrapure water								○

[a]Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control and classified into three categories: blank = no signal; ■ = weak signal (not present here); ○ = strong signal.

brane. Differential hybridization strengths were observed for the two different oligonucleotides that were used to detect the genus *Verticillium* and the species *V. dahliae*. In general, hybridization to the oligonucleotides Vgn2 and Vda1 produced stronger signals than hybridization to the oligonucleotides Vgn1 and Vda2, respectively. For detection of *F. oxysporum* and *V. albo-atrum*, both oligonucleotides displayed similar hybridization strengths. Amplicons generated from *V. nubilum* DNA did not hybridize to any of the oligonucleotides designed for *Verticillium* detection while labeled amplicons from all *Fusarium solani* (*Nectria haematococca*, teleomorph) isolates tested only hybridized to Fgn2. This was anticipated because the designed genus-specific oligonucleotides do not cover all species within the genus due to the diversity at the nucleotide level. The species-specific oligonucleotide Val1, designed for the detection of *V. albo-atrum*, cross-hybridized with *V. tricorpus*. This latter fungus is, along with *V. dahliae* and *V. albo-atrum*, one of the three *Verticillium* pathogens of potato, but occasionally causes Verticillium wilt of tomato [31]. The oligonucleotide Val2 did not display this cross-hybridization. This difference in specificity cannot be explained by the number of mismatches between the *V. tricorpus* amplicon and the detector oligonucleotides, as two adjacent nucleotides do not match for both detectors (data not shown). However, while for Val2 the mismatch occurs in the central region of the oligonucleotide, it is at the extreme 3′ end for Val1. Thus the position of the mismatch explains the difference in specificity between the two detector oligonucleotides. Based on these findings the oligonucleotides Fgn2, Fox2, Vgn2, Val2, and Vda1 were selected for further experiments.

Within the species *F. oxysporum*, over 120 formae speciales have been defined, each consisting of strains with the ability to cause disease on a specific host [32]. Morphologically, these *F. oxysporum* strains are identical. Also discrimination based on nucleotide sequences (e.g. ITS, EF-1α, or β-tubulin genes) is not possible (data not shown). It was long believed that isolates with a shared host range are more closely related genetically than isolates with other host specificities (i.e. are monophyletic). However, for a number formae speciales, including *lycopersici*, polyphyletic origins were demonstrated, complicating molecular discrimination [33,34]. For some formae speciales, vegetative compatibility group (VCG) testing can be used to determine whether an isolate belongs to a given forma specialis. However, since relatively many strains within the f. sp. *lycopersici* are either self-incompatible or belong to single-member VCGs, this analysis is too complicated to identify unknown strains [35]. Therefore, the only remaining alternative currently is pathogenicity testing with host differentials [36].

In order to determine the detection limit of the DNA array, a dilution series of DNA from *F. oxysporum* f. sp. *lycopersici* (CBS 645.78), *V. albo-atrum* (CBS 451.88), and *V. dahliae* (CBS 386.49) was made prior to PCR amplification. The amount of undiluted DNA in the PCR varied between 250 and 500 ng and was subsequently diluted in six 10-fold dilution steps. After PCR, samples were hybridized to the membrane and analyzed. For *V. dahliae*, 2.5 pg of DNA could still be detected (Fig. 1). For *V. albo-atrum* and *F. oxysporum*, however, a signal could easily be detected in the last dilution, which equaled to as little as 0.35 pg DNA for *V. albo-atrum* and 0.50 pg DNA for *F. oxysporum*. This reveals the high sensitivity of this technique (Fig. 1).

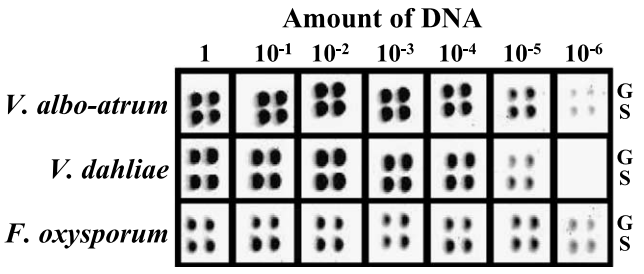

Fig. 1. Sensitivity of the DNA array. Blots are shown for detection of *V. albo-atrum*, *V. dahliae*, and *F. oxysporum* f. sp. *lycopersici* from a 10-fold serial dilution of fungal DNA template before PCR. The panels represent a genus-specific oligonucleotide detector (upper signal (G), Vgn2 and Fgn2 for *Verticillium* and *Fusarium*, respectively) and a species-specific oligonucleotide detector (lower signal (S), Val2, Vda1 and Fox2 for *V. albo-atrum*, *V. dahliae* and *F. oxysporum* f. sp. *lycopersici*, respectively) horizontally spotted in duplicate. The undiluted amount (1) of DNA template is 350 ng for *V. albo-atrum*, 250 ng for *V. dahliae*, and 500 ng for *F. oxysporum* f. sp. *lycopersici*.

To test the discriminatory potential of the oligonucleotide array, genomic DNA of *F. oxysporum* f. sp. *lycopersici* CBS 645.78, *V. albo-atrum* CBS 451.88, and *V. dahliae* CBS 386.49 were mixed in different combinations (10–15 ng for each isolate) prior to PCR amplification of the ITS region. As a control *F. solani* CBS 165.87 was incorporated also in some of these mixes. The expected hybridization pattern for each mixture was obtained, demonstrating that the desired amplicons were generated and detected from a fungal DNA mix (Table 4).

3.3. Validation of the DNA array using complex biological samples

To validate the DNA array with regard to biologically complex samples, pathogen infestation was assessed in substrate, plant material, and water. Tomato seedlings were transferred to potting mix that was pasteurized and inoculated with *V. dahliae* or *F. oxysporum* f. sp. *lycopersici*. Seven and 10 weeks after transfer, plant material and potting mix were sampled and evaluated for the presence of the pathogen. At this stage, plants did not show symptoms of disease, although some vascular discoloration was observed in stem cuttings. For PCR amplification of the ITS region, the primer ITS4 [24] was used in combination with ITS1-F [29]. In contrast to primer ITS5, which hybridizes to a rDNA sequence shared by all eukaryotes, ITS1-F hybridizes to a fungal-specific rDNA sequence.

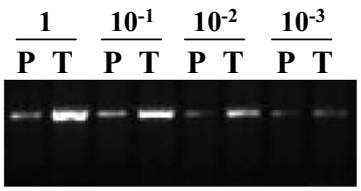

Fig. 2. Comparison of DNA polymerase enzymes. Comparison of Titanium *Taq* DNA polymerase (T) versus Platinum *Taq* DNA polymerase (P) for product yield in a PCR reaction with primers ITS5 and ITS4 in a 10-fold serial dilution of *F. oxysporum* f. sp. *lycopersici* DNA. Sample 1 is undiluted (350 ng).

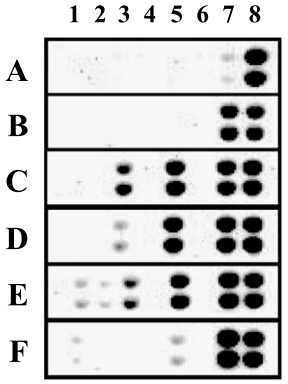

Fig. 3. Diagnosis of greenhouse samples. DNA was isolated from greenhouse samples gathered from commercial tomato growers, amplified and hybridized to the DNA array. Detector oligonucleotides are vertically spotted in duplicate: Fgn2 (1), Fox2 (2), Vgn2 (3), Val2 (4), Vda1 (5), Con1 (6), Uni1 (7) and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

Furthermore, based on a test with different DNA polymerases, we observed that the yield of the PCR product was highest when using Titanium *Taq* DNA polymerase (Fig. 2). Therefore, this DNA polymerase was used for PCR on DNA samples from complex biological substrates. The presence of both pathogens could be detected in roots and stems from plants that were grown in inoculated potting mixes when using the DNA array, demonstrating that detection of the pathogen was successful in plants even at the pre-symptomatic stage of infection (Table 5). Furthermore, the pathogen was also detected in the potting mix. The pathogen was not detected in plant or potting mix samples of non-inoculated control treatments. The signal obtained with the universal detector probe (Uni1) in roots and potting mix for mock-inoculated potting mix indicates the presence of fungi that associate with the roots but are not removed by pasteurization. In addition, we were also able to detect all three pathogens from water samples that were inoculated with 0.5 spores ml^{-1} water. All of the results above were confirmed by selective plating methods.

Finally, we evaluated whether the DNA array could also be used for diagnosis on biological samples gathered from commercial tomato growers. Out of 15 plant samples analyzed, one was diagnosed with *V. dahliae* and one with *F. oxysporum*, while four carried *V. dahliae* as well as a *Fusarium*, though not *F. oxysporum*. From eight soil samples tested, all contained *Fusarium* (in three samples *F. oxysporum*) and two contained *V. dahliae*. Finally, in two irrigation water samples, one was contaminated with *F. oxysporum* and the other with *V. dahliae* (See Fig. 3 for representative examples). For all samples, results from the DNA array were corroborated by classical plating and taxonomy techniques, demonstrating that both the classical and the DNA array approaches were equally reliable. However, the major advantage of the DNA array is that the diagnosis is completed much faster, generally within 24 h, whereas plating for these organisms takes at least 1 to 2 weeks.

The results from all of these tests illustrate the power of DNA arrays for routine analysis of samples from different biological sources. This technology can easily be implemented for the detection of tomato vascular wilt pathogens in planta. For this purpose, detection at the forma specialis level for *F. oxysporum* is not crucial since healthy tomato plants are only susceptible to wilt by *F. oxysporum* f. sp. *lycopersici*. Successful implementation of this technology for soil diagnosis will require additional effort. Once *F. oxysporum* is detected in a soil, pathogenicity tests with host differentials will have to be performed to determine whether the isolate is a pathogen of tomato (either

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

		Fgn2	Fox2	Vgn2	Val2	Vda1	Con1	Uni1	Dig1
F. oxysporum f. sp. *lycopersici* CBS 645.78	stem	○	○					○	○
	roots	○	○					○	○
	potting mix	○	○					○	○
V. dahliae CBS 179.66	stem			○		○		○	○
	roots			○		○		○	○
	potting mix			○		○		○	○
Mock-inoculated	stem								○
	roots							○	○
	potting mix							■	○

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

f. sp. *lycopersici* causing wilt or f. sp. *radicis-lycopersici*, the causal agent of crown and root rot) or not. Furthermore, sampling strategies will have to be developed to account for possible spatial variability of inoculum. Finally, a correlation between inoculum density and disease development will have to be calculated to aid integrated disease management. Potentially, this array could be enlarged to include other tomato pathogens of fungal, bacterial, and viral origin as well as parasitic nematodes. Ultimately, this may lead to a complete pathogen assessment for a specific crop plant in a single assay. Alternatively, this array could be enlarged to include vascular wilt pathogens of other crops in order to obtain a universal vascular wilt detection array.

There are currently no effective curative treatments for plants afflicted with vascular wilt diseases, so preventive disease management is an important strategy to deploy wherever possible. Detection of the presence of the pathogen is necessary before culturing susceptible crops. Traditional detection and identification methods are time-consuming, laborious, and require extensive knowledge of classical taxonomy. In this study, we developed a DNA array to specifically detect and identify within 24 h the most important tomato vascular wilt pathogens *F. oxysporum* f. sp. *lycopersici*, *V. albo-atrum*, and *V. dahliae* to the species level based on the fungal ITS region from different sample matrices. An advantage of this technique is the ability to detect organisms that cannot be cultured in vitro, a specific requirement for most classical methods. In contrast to previously published papers on the use of DNA arrays in plant pathology [20–22], we showed the applicability of such a membrane to current horticultural practice. We demonstrated first that by using such a membrane the pathogen can be detected from complex substrates such as infested soil, plant material (pre-symptomatic), and irrigation water. Second, we demonstrated that the sensitivity of this technology is high enough to detect these pathogens at densities in which they are likely to occur in the field. Finally, we showed that this membrane can be used successfully for diagnostics of diseased plants that are submitted to a plant disease clinic by commercial tomato growers.

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