Detection of Multiple *Verticillium* Species in Soil Using Density Flotation and Real-Time Polymerase Chain Reaction

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Abstract

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Wet sieving of soil samples, followed by plating on semi-selective medium and microscopic analysis, is the most commonly used technique to quantify microsclerotia-forming *Verticillium* species in soil. However, the method is restricted to small samples, does not allow easy differentiation between species, and takes several weeks to complete. This study describes an alternative method to test 100-g soil samples for three *Verticillium* species (*V. tricornis*, *V. dahliae*, and *V. longisporum*) using density flotation-based extraction of microsclerotia followed by new real-time polymerase chain reaction (PCR) assays. Primers for these real-time PCR assays were designed to the ribosomal

DNA internal transcribed spacer for *V. tricornis* and the β -tubulin gene for *V. dahliae* + *V. longisporum* and *V. longisporum*. Tests with artificially and naturally infested soils showed that the new method is reproducible and sensitive (0.1 to 0.5 microsclerotia/g soil), allows differentiation among the three species, and can be completed in one day. The results of the new method and the wet-sieving method were highly correlated for *V. tricornis* ($R^2 = 0.78$), but not for *V. dahliae/V. longisporum*, probably due to the loss of germinability of *V. dahliae/V. longisporum* microsclerotia during prolonged dry storage of the soil.

Soils can contain multiple microsclerotia-forming *Verticillium* species, such as *V. dahliae* Kleb., *V. longisporum* (ex. *V. dahliae* var. *longisporum* Stark; comb. nov. Karapapa), and *V. tricornis* I. Isaac (15,22). *V. dahliae* infects a wide range of mainly noncruciferous hosts, including ornamentals, fruits, vegetables, and trees (22). *V. longisporum* almost exclusively infects crucifers, including cauliflower and oilseed rape. *V. longisporum* was described for the first time in 1997 by Karapapa et al. (21) based on distinctive morphological, molecular, and virulence characteristics compared to *V. dahliae* (e.g., *V. longisporum* has longer conidia, nearly double the DNA content, and mainly infects crucifers). It has been suggested that *V. longisporum* arose from a hybridization event between *V. dahliae* and *V. albo-atrum*, or *V. dahliae* and another (as yet unidentified) species (5,6,21). During the last 10 years, taxonomic controversy has evolved as to whether *V. longisporum* should be a separate species from *V. dahliae* (22). Therefore, *V. longisporum* isolates were also often referred to as “long-spored”, “near diploid”, or “amphihaploid” *V. dahliae* isolates (2,6,31). However, recent evidence indicates that *V. longisporum* is a distinct hybrid species (19). *V. tricornis* is a saprophyte or weak pathogen and suspected to be a biocontrol species (22). Specifically, reduced symptoms of *Verticillium* wilt of potato and cauliflower have been associated with the presence of *V. tricornis* in soil compared to soils with only pathogenic *Verticillium* species (8; S. C. França, M. Höfte, S. Pollet, and K. Spiessens, unpublished data). In addition,

greenhouse and field experiments have demonstrated that *V. tricornis* applied to soil can decrease symptoms caused by *V. dahliae* and *V. albo-atrum* on potato (8,32). Reduced symptoms of *Verticillium* wilt were also observed in lettuce in a greenhouse experiment when *V. dahliae* was co-inoculated with *V. tricornis* (30).

V. dahliae, *V. longisporum*, and *V. tricornis* form microsclerotia, and these three species have been observed simultaneously in a cauliflower field in Belgium (S. C. França, J. Debode, B. Declercq, and M. Höfte, unpublished data). Microsclerotia are resting structures, agglomerates of thick-walled, melanized cells that can survive for more than 13 years in soil (34). Even low numbers of microsclerotia of *V. dahliae* and *V. longisporum* can lead to high levels of disease (3). For example, 1 microsclerotium/g soil can result in infection of more than 5 to 15% of the plants in crops of strawberry (16), cauliflower (39), and artichoke (3). Moreover, a small increase in the inoculum density to about 3 microsclerotia/g resulted in about 40% of infected artichoke plants, an incidence high enough to reduce yield (3). No reliable curative measures exist to control these pathogens other than soil fumigation, which is either restricted in several countries, not economically viable for many crops, or both. This increases the importance of accurate, preplanting assessment of the inoculum potential of a soil, especially considering the difference in the host ranges of *V. dahliae* and *V. longisporum*. In addition, simultaneous quantitative detection of *V. tricornis* microsclerotia could further help in the evaluation of potential biological control activity by this species relative to the other two species in soil.

Several methods have been devised for quantifying *Verticillium* spp. in soil, including plating methods (dry and wet plating), bioassays, immunoassays, and molecular methods (reviewed by Goud and Termorshuizen [14]). The two semi-quantitative molecular methods for the detection of *Verticillium* spp. in soil are competitive polymerase chain reaction (PCR) (24,25) and a macroarray (23). Immunoassays and bioassays are not often used; the plating methods are most commonly used. These involve plating a small

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amount of soil on a semi-selective medium, incubating the plates for several weeks, and then microscopically analyzing the fungal colonies on the medium. With dry plating assays, a small number of dry soil particles are distributed onto agar plates. Wet plating assays include wet sieving as a step to remove particles smaller and larger than microsclerotia. One of the most commonly used wet sieving techniques, described by Harris et al. (17), includes plating the wet sieved soil onto semi-selective modified soil extract agar (MSEA) medium. One limitation of this conventional technique is that morphological differentiation of the microsclerotia from different *Verticillium* species on MSEA medium is very labor-intensive and difficult. Although the morphological differentiation between *V. tricorpus* and *V. dahliae* on the MSEA medium has been described (8,15), these two species may easily be confused by analysts. Moreover, microsclerotia from *V. longisporum* have no consistent morphological differences from those of typical *V. dahliae* strains (22). A second limitation is that the wet sieving method is usually restricted to small soil samples of 1.9 to 12.5 g (36), which limits the representativeness of the assay. A third limitation is that the assay includes air-drying of the soil, sieving the soil, and multi-week incubation of the soil on plates of agar medium, which together take 25 to 43 days to complete (36).

A DNA-based detection soil assay technique, such as real-time PCR, may offer an alternative for more specific and rapid detection of *Verticillium* species in soil than the wet sieving method. Recently, a real-time PCR assay specific for *V. dahliae* in potato tissue was developed (1), but its applicability to soil samples has not yet been demonstrated. To our knowledge, no real-time PCR detection method(s) specific for *V. tricorpus* and *V. longisporum* in soil has been described. PCR-based detection of *Verticillium* in soil requires DNA extraction from soil samples or from extracted microsclerotia. Commercially available DNA extraction kits can only process small soil sample volumes (usually 0.25 g and, exceptionally, up to 10 g of soil). These limited amounts are not representative for a whole field, and may result in false negative results, because as little as 1 microsclerotium/g of soil needs to be detected (3). Therefore, extraction of microsclerotia from soil, such as by the density flotation technique (18), should precede DNA extraction and may increase sensitivity of the assay.

The objectives of this study were: (i) to develop real-time PCR assays for specific detection and quantification of *V. tricorpus* and *V. longisporum*, and if need be, *V. dahliae*, that are applicable to soil samples, (ii) to develop a new method to detect and quantify microsclerotia of *V. dahliae*, *V. longisporum*, and *V. tricorpus* in soil using density flotation of soil followed by real-time PCR assays (DFS + real-time PCR), and (iii) to compare the results of the DFS + real-time PCR method with the results obtained after applying the conventional wet sieving method and after using real-time PCR analysis of DNA extracted directly from soil.

Materials and Methods

Fungal cultures and DNA extraction. DNA from 2 *V. albo-atrum*, 9 *V. dahliae*, 11 *V. longisporum*, and 5 *V. tricorpus* isolates, and 11 non-*Verticillium* isolates was used to design and test real-time PCR primers (Table 1). All individual fungal isolates were grown in potato dextrose broth (PDB) for 2 weeks, and DNA was extracted from approximately 100 mg fresh weight of mycelium per isolate (see below).

Microsclerotia of *V. dahliae* were obtained by collecting potato stems from a naturally infested field. Microsclerotia of *V. longisporum* strain O1 and *V. tricorpus* strain 305 were produced as described by Mol et al. (27) with modifications, then stored at 4°C. Briefly, conidial suspensions of these two isolates were prepared by flooding potato dextrose agar (PDA) cultures with sterile water and rubbing the surface of each plate with a glass rod. The suspensions were filtered through autoclaved cheesecloth and the spore concentration was determined using a hemocytometer. Five autoclaved segments (1.0 to 1.5 cm) of cauliflower petiole (cv. Cornell) were placed on MSEA medium, and each stem section was inoculated with 50 µl of a spore suspension of the appropriate *Verticil-*

lium species (10⁵ spores/ml). Twenty days after incubation at 22°C in the dark, a large amount of microsclerotia had formed on each segment. Both cauliflower and potato segments were air-dried at room temperature and gently ground with a mortar and pestle. Sterile water was added and the microsclerotia concentration in the suspension was determined using a hemocytometer. Subsequently, the suspensions were serially diluted resulting in 10, 50, 500, and 5,000 microsclerotia/ml, and 1 ml of each dilution was vacuum filtered (20 µm; Millipore). DNA was extracted from the microsclerotia retained on the filter.

DNA was extracted from mycelium and conidia with the Invitrogen Spin Plant Mini DNA extraction kit (Invitrogen) and from microsclerotia using the MoBio Power Soil DNA extraction kit (MoBio Laboratories) following the manufacturers' instructions. DNA concentrations of the samples were quantified using a Nanodrop spectrophotometer, and DNA was stored at -20°C until needed.

Primer design. The rDNA ITS regions and the partial β -tubulin genes of isolates STR3 (*V. albo-atrum*), Ve005 (*V. dahliae*), Pf1 (*V. longisporum*), and Ve010 (*V. tricorpus*) (Table 1) were amplified by PCR using the universal primers ITS1/ITS4 (38) and T1/T2 (29), respectively. PCR was performed with FastStart Taq DNA polymerase (Roche) in a GeneAmp PCR System 9700 (Applied Biosystems). An initial denaturation step at 94°C for 3 min was followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. After the last cycle, a final extension step at 72°C for 10 min was performed. The amplified fragments of expected size were purified using gel extraction with a NucleoSpin Extract II kit (Macherey-Nagel) and cloned in a pCRII-TOPO vector with the TOPO TA Cloning Kit (Invitrogen). DNA of the plasmid clones was extracted using a NucleoSpin plasmid DNA purification kit (Macherey-Nagel). The plasmid inserts were sequenced by MacroGen using primers Sp6 and T7.

The ITS sequences were deposited in GenBank (Table 1) and included in sequence alignments using Vector NTI's Align X module (Invitrogen), together with those of other *Verticillium* isolates already present in GenBank (*V. albo-atrum*: X60705, Z29508, Z29509, Z29523, and AF364014; *V. dahliae*: AJ865691, AJ970308, EF015891, Z29511, and Z29510; *V. longisporum*: AF364007, AY566605, and DQ266225; *V. tricorpus*: AF364017, AY555960, L28279, and Z29524). In addition, clones containing the partial β -tubulin gene were sequenced for *V. albo-atrum* (2 clones), *V. dahliae* (2 clones), *V. longisporum* (14 clones), and *V. tricorpus* (2 clones). For each species, a single sequence was obtained and deposited in GenBank, except for *V. longisporum*, for which two β -tubulin sequences were identified and deposited (Table 1). Other *Verticillium* β -tubulin sequences (DQ840570 to DQ840626 [5]) were included in the sequence alignments to design *V. longisporum*-specific primers.

Species-specific primers designed from areas polymorphic among species of the genus *Verticillium*, yet monomorphic within *V. tricorpus*, *V. longisporum*, or *V. dahliae*, were manually selected and evaluated for primer-dimer formation and self-annealing using the program Oligo Analyzer (Gene Link). Fifteen candidate primer pairs were tested in an initial screening through amplification and melting-curve analysis of real-time PCR reactions using DNA from 4 *Verticillium* isolates (STR3, Ve005, Pf1, and Ve010; Table 1). Subsequently, primer pairs VtF4/VtR2, VITubF2/VITubR1, and VdTubF2/VdTubR4 were selected for the detection of *V. tricorpus*, *V. longisporum*, and *V. dahliae* + *V. longisporum* (Table 2) and further evaluated for specificity using approximately 100 pg DNA from all the isolates listed in Table 1. Amplification efficiency ($= 10^{(1/\text{slope})} - 1$) (13) and sensitivity of each primer pair was determined using a triplicate 10-fold serial dilution of genomic DNA (10 to 1×10^6 fg gDNA/5 µl), and 5 µl (out of 100 µl) DNA extracts from two serial dilutions of microsclerotia (see above). In addition to the development of primers for *V. tricorpus*, *V. longisporum*, and *V. dahliae*, the specificity of primer pairs VertBt-F/VertBt-R (designed for *V. dahliae* detection in potato [1]) and OLG70/OLG71 (designed for *V. dahliae* and *V. longisporum* detection in oilseed rape [12]) were evaluated in this study.

Real-time PCR. Real-time PCR was performed in 50- μ l reactions containing 5 μ l template, 200 nM of each primer (or 300 nM of the *V. tricolor* primers VtF4/VtR2), and 25 μ l Maxima SYBR Green master mix (Fermentas). Real-time PCR and melting curve analysis was performed using an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). The thermocycling profile consisted of 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. Melting curve analysis was used to distinguish potential primer dimers and nonspecific amplification products.

Quantification was done using the standard curve technique with a 10-fold dilution series of gDNA in sterile water (10 to 1×10^6 fg/5 μ l) from reference strains Ve005 (*V. dahliae*), Pf1 (*V. longisporum*), and Ve010 (*V. tricolor*).

Conventional detection of *Verticillium* species in soil via the wet sieving method. To determine the number of viable microsclerotia in naturally infested soil samples, the wet sieving method described by Harris et al. (17) was used. Briefly, 12.5 g of dry soil was wet sieved, the 20 to 100 μ m fraction suspended in 0.08%

Table 1. Name, origin, accession number, and source of the fungal isolates and oomycetes used in this study and Ct values of the real-time polymerase chain reaction (PCR) assays showing the specificity of primer pairs VtF4/VtR2, VITubF2/VITubR1, and VdF2/VdR4 used to detect *Verticillium tricolor*, *V. longisporum*, and *V. dahliae*, respectively

Isolate name	Origin ^a	Geographic origin ^a	Accession number		Source ^a	Ct value (≈ 100 pg) ^{a,b,c,d}		
			ITS ^{a,e}	β -tubulin		VtF4/VtR2	VITubF2/VITubR1	VdF2/VdR4
<i>Verticillium albo-atrum</i> VA1	<i>Solanum lycopersicum</i> L.	Netherlands	AF364014	DQ840616-18	(7)	>40	>40	>40
<i>V. albo-atrum</i> STR3 (Ve002)	<i>Medicago sativa</i> L.	Canada	GQ495790	GU564540	(7)	>40	>40	>40
<i>V. dahliae</i> JKG1	<i>Solanum tuberosum</i> L.	Netherlands	/	/	(20)	>40	>40	26.7
<i>V. dahliae</i> JKG8	<i>S. tuberosum</i>	Netherlands	/	/	(20)	>40	>40	27.6
<i>V. dahliae</i> Ve005	Soil	Belgium	GQ495791	GU564541	ILVO	>40	>40	27.5
<i>V. dahliae</i> Ve012	Soil	Belgium	/	/	ILVO	>40	>40	27.6
<i>V. dahliae</i> VD73	<i>Linum usitatissimum</i> L.	Germany	/	/	(12)	>40	>40	24.3
<i>V. dahliae</i> ER1085	<i>Solanum melongena</i> L.	Italy	/	/	CRA-PAV	>40	>40	29.2
<i>V. dahliae</i> ER1180	<i>Hebe $\times$ franciscana</i> (Eastw.)	Italy	/	/	CRA-PAV	>40	>40	26.3
<i>V. dahliae</i> ER1606	<i>S. lycopersicum</i>	Italy	/	/	CRA-PAV	>40	>40	29.2
<i>V. dahliae</i> ER1352	<i>Olea europaea</i> L.	Italy	/	/	CRA-PAV	>40	>40	27.4
<i>V. longisporum</i> Be1	<i>Brassica oleracea</i> var. <i>botrytis</i> L.	Netherlands	AY566601	/	(10)	>40	27.6	25.9
<i>V. longisporum</i> Pf1 (Ve007)	<i>B. oleracea</i> var. <i>botrytis</i>	Germany	GQ495792	GU564542 GU564543	(10)	>40	28.5	27.4
<i>V. longisporum</i> K1	<i>B. oleracea</i> var. <i>botrytis</i>	Belgium	AY566600	/	(10)	>40	27.8	28.3
<i>V. longisporum</i> Wv12	<i>B. oleracea</i> var. <i>botrytis</i>	Belgium	AY566605	/	(10)	>40	27.0	27.2
<i>V. longisporum</i> O1	<i>B. oleracea</i> var. <i>botrytis</i>	Belgium	AY566606	/	(10)	>40	27.3	27.5
<i>V. longisporum</i> P4	<i>B. oleracea</i> var. <i>botrytis</i>	Belgium	AY566604	/	(10)	>40	25.5	25.6
<i>V. longisporum</i> S3	<i>B. oleracea</i> var. <i>botrytis</i>	Belgium	AY566603	/	(10)	>40	25.4	25.4
<i>V. longisporum</i> VL43	<i>B. napus</i> L. subsp. <i>oleifera</i> (Delile)	Germany	/	/	(12)	>40	29.6	25.3
<i>V. longisporum</i> VD1	<i>B. napus</i> ssp. <i>oleifera</i>	Sweden	/	/	(35)	>40	31.8	23.5
<i>V. longisporum</i> VD4	<i>B. napus</i> ssp. <i>oleifera</i>	Sweden	/	/	(35)	>40	27.6	23.9
<i>V. longisporum</i> VD11	<i>B. napus</i> ssp. <i>oleifera</i>	Sweden	/	/	(35)	>40	30.0	26.0
<i>V. tricolor</i> Ve010	<i>Chrysanthemum $\times$ morifolium</i>	Belgium	GQ495793	GU564544	ILVO	23.5	>40	>40
<i>V. tricolor</i> Vt1	NK	NK	/	/	ILVO	23.1	>40	>40
<i>V. tricolor</i> Vt2	NK	NK	/	/	ILVO	23.2	>40	>40
<i>V. tricolor</i> IIA	Soil	Belgium	/	/	ILVO	23.0	>40	>40
<i>V. tricolor</i> 305	Soil	Belgium	/	/	UGent	-	-	-
<i>Acremonium alternatum</i> PSKW01	<i>Capsicum annuum</i> L.	Belgium	/	/	ILVO	>40	>40	>40
<i>Alternaria solani</i> ALT	NK	Belgium	/	/	ILVO	>40	>40	>40
<i>Aspergillus westerdijkiae</i> ASP	<i>C. annuum</i>	Belgium	/	/	ILVO	>40	>40	>40
<i>Colletotrichum coccodes</i> PCF987	<i>Fragaria $\times$ ananassa</i> (Duch.)	Belgium	/	/	(37)	>40	>40	>40
<i>Fusarium oxysporum</i> dcp2283	<i>Apium graveolens</i> L. var. <i>rapaceum</i>	Belgium	/	/	ILVO	>40	>40	>40
<i>Galactomyces geotrichum</i> KHK64	<i>C. annuum</i>	Belgium	/	/	ILVO	>40	>40	>40
<i>Penicillium roqueforti</i> PEN	NK	Belgium	/	/	ILVO	>40	>40	>40
<i>Phytophthora cinnamoni</i> Ph226	Soil	Belgium	/	/	ILVO	>40	>40	>40
<i>Pythium ultimum</i> Py034	<i>Cucumis sativus</i> L.	Bulgaria	/	/	ILVO	>40	>40	>40
<i>Rhizoctonia solani</i> RS	<i>S. tuberosum</i>	ILVO	/	/	ILVO	>40	>40	>40
<i>Trichoderma harzianum</i> TRI	NK	Belgium	/	/	ILVO	>40	>40	>40

^a NK = not known; ITS = rDNA internal transcribed spacer region sequence; ILVO = Instituut voor Landbouwen Visserij Onderzoek, Merelbeke, Belgium; CRA-PAV = Centro di Ricerca per la Patologia Vegetale, Rome, Italy; Ct = threshold cycle; UGent = Ghent University.

^b - = not analyzed.

^c Real-time PCR amplifications were conducted using ≈ 100 pg DNA extracted from fungal mycelium of each isolate.

^d Ct > 40 was considered as the cut-off value.

^e / = not available in GenBank.

water agar (50 ml), and 0.8 ml of this suspension was spread on each of 10 petri dishes containing MSEA medium and incubated at 22°C. After 4 weeks, soil particles were carefully washed from the plates and clusters of *Verticillium* microsclerotia were counted under a dissecting microscope. Morphological differentiation between *V. tricorpus* and *V. dahliae*/*V. longisporum* microsclerotia was based on Davis et al. (8) and Goud et al. (15).

New method of detection of *Verticillium* species in soil via DFS + real-time PCR. One hundred grams of air-dried artificially or naturally infested soil were crumbled with a mortar and pestle and thoroughly mixed with 100 ml 70% (wt/wt) sucrose and centrifuged at $2,700 \times g$ for 20 min (24). The resulting supernatant was poured into a vacuum-filtration system (Gelman Sciences) over a 20- μ m nylon mesh filter (Millipore) and rinsed with sterile water. DNA was extracted from the material retained on the filter, using the MoBio Power Soil DNA isolation kit following the manufacturer's instructions. The material retained on the filter was always less than 0.25 g. DNA was eluted in 100 μ l buffer, and 5 μ l of the DNA extract was used in the real-time PCR assays. When wet soil was analyzed (without the air-drying step), the soil sample weight used in the test was increased to compensate for the water content. The moisture content was first determined by oven-drying a sub-sample (30 g) overnight at 110°C.

Analysis of inoculated soil samples using DFS + real time PCR. Inoculated soil samples were prepared by adding known numbers of *V. longisporum*, *V. tricorpus*, or *V. dahliae* microsclerotia to *Verticillium*-free soil. Microsclerotia prepared in aqueous suspensions (see above) were added to an autoclaved sandy-loam soil (pH-KCl 7.6, 1.8% C) collected from a corn field in Merelbeke, Belgium, to densities of 0.1, 0.5, 5.0, and 50.0 microsclerotia/g dry soil. Two replicates of each soil sample were analyzed separately using DFS + real-time PCR with primer pairs specific for *V. tricorpus*, *V. longisporum*, and *V. dahliae*.

Analysis of naturally infested soil samples using DFS + real-time PCR versus the wet sieving method. The DFS + real-time PCR method was compared with the wet sieving method using 25 naturally infested soil samples from three types of field soils that were cropped with a specific host during the sampling year. First, during the autumn of 2009, soil samples were collected from the top 0 to 30 cm depth of two Belgian cauliflower fields. Ten soil samples were taken per field, along two diagonal transects within each field. The first cauliflower field (denoted as PSKW) was located in the province of Antwerp, Belgium (sandy-loam soil, pH-KCl 5.5, 0.9% C), had been planted yearly for at least 7 years with cauliflower, and was associated with severe *Verticillium* wilt symptoms on cauliflower plants (cv. Cornell) during the growing season of 2007–2008. The second cauliflower field (denoted as POVLT) was located in the province of West Flanders, Belgium (sandy-loam soil, pH-KCl 5.6, 0.8% C). This field was associated with moderate *Verticillium* wilt symptoms on cauliflower plants (cv. Clapton) during the growing season of 2007–2008, and in the last 20 years had been rotated five times with potato. The cauliflower cultivars planted in these fields in 2007–2008 are of comparable susceptibility to *Verticillium* (K. Spiessens and S. Pollet, unpublished data). *Verticillium* wilt severity and incidence on cauliflower were evaluated in each field based on vascular discoloration of the stem. Both composite soil samples were analyzed for the presence of *Verticillium* species using the wet sieving method and the DFS + real-time PCR method. For the latter method, the experiment con-

sisted of two fixed factors: prehandling treatment and soil. Three prehandling treatments: (i) no drying of the soil, (ii) drying of the soil for 2 weeks, and (iii) storage of the soil for 2 weeks at 4°C, were applied to two soils (POVLT and PSKW), with three replicates per soil. One replicate consisted of the total DNA extracted from 100 g of soil using the DFS method. The prehandling treatments were included to evaluate whether drying the soil prior to analysis was necessary, and whether storing wet soil at 4°C had an influence on the amount of *Verticillium* DNA recovered. The primer pairs developed in this study that were specific for *V. tricorpus*, *V. longisporum*, and *V. dahliae* + *V. longisporum* were used in the real-time PCR analyses of these naturally infested soils.

Second, in the spring of 2010, soil samples were collected from the top 0 to 30 cm depth of an agricultural field in Merelbeke, Belgium (sandy-loam soil, pH-KCl 6.0, 0.8% C). This field (denoted as Me) had been rotated with monocotyls (ryegrass and wheat) in 2006 and 2007 and later with flax (2008) and potatoes (2009). In 2010, it was divided into 32 experimental plots of 11×15 m. Per plot, 8 soil samples were taken, thoroughly mixed, dried for 2 weeks, and stored at room temperature. In a preliminary experiment, the non-species-specific number of *Verticillium* microsclerotia was analyzed in samples from each plot using the wet sieving method. In January 2011, 11 plots (A1, A4, B5, B6, C5, C7, C8, D1, D3, D4, and D7) with varying levels of total numbers of *Verticillium* microsclerotia were selected for further analysis using the wet sieving method as well as the DFS + real-time PCR method. In total, 10 replicate plates were used for the wet sieving method (12.5 g total), and two replicate samples of 100 g were used for DFS + real-time PCR.

The third set of soil samples was collected in July to September 2009 from the top (0 to 30 cm) and the deeper (30 to 60 cm) soil layers of two flax fields in France at three time points/field. Four soil samples were taken per field, time point, and depth, thoroughly mixed, and stored at room temperature until analysis. In the first field (located in the province of Calvados; denoted as S3), no *Verticillium* symptoms were observed in 2009. In the second field (located in the province of Oise and denoted as S8), severe *Verticillium* symptoms were observed in 2009. The soil samples were analyzed using the wet sieving and DFS + real-time PCR methods in January 2011. In total, 10 replicate plates were used for the wet sieving method (12.5 g total), and two replicate samples of 100 g were used for the density flotation method followed by real-time PCR.

Analysis of naturally infested soil samples using DFS + real-time PCR versus direct DNA extraction + real-time PCR. In March 2011, soil samples were collected from the top 0 to 10 cm depth of each plot of the Me field (see above). Soil samples from 5 plots were selected (A1, A6, A8, C5, and D7) and analyzed by real-time PCR using primer pair VdTubF2/VdTubR4, and DNA extracted (i) directly from 0.025 g of soil using the MoBio Power Soil kit, (ii) directly from 10 g of soil using the MoBio UltraClean Mega Soil kit, and (iii) from the material retrieved on the filter after density flotation of 100 g of soil using the MoBio Power Soil kit (the DFS + real-time PCR method, see above). For each method, duplicate samples were analyzed and 5 μ l of the resulting DNA samples was used for real-time PCR analysis.

Assessment of PCR inhibitors. The presence of PCR inhibitors in DNA samples extracted from soil samples collected in 2010 from the Me field was assessed by: (i) 10-fold diluting the DNA

Table 2. Names, sequences, and location of the primers developed to detect and quantify *Verticillium* spp. in soil

Target organism	Gene target	Primer	Sequence (5' to 3')	Location ^a	Amplicon size (bp)
<i>V. tricorpus</i>	rDNA ITS	VtF4	CCGGTGTGGGGATCTACT	337-355	123
		VtR2	GTAGGGGGTTTAGAGGCTG	441-459	
<i>V. longisporum</i>	β -tubulin	VITubF2	GCAAAACCCCTACCGGGTTATG	374-394	143
		VITubR1	AGATATCCATCGGACTGTTCGTA	494-516	
<i>V. dahliae</i> + <i>V. longisporum</i>	β -tubulin	VdTubF2	GGCCAGTGCCTAAGTTATTCT	215-235	82
		VdTubR4	ATCTGGTTACCCGTTCATCC	276-296	

^a Based on accession numbers GQ495793, GU564543, and GU564541, for *V. tricorpus*, *V. longisporum*, and *V. dahliae* + *V. longisporum*, respectively.

samples and (ii) spiking each DNA sample and a water control with 10^4 pDNA copies containing the rDNA ITS region of *Colletotrichum acutatum* isolate NI90, and analyzing them using a *C. acutatum*-specific real-time PCR assay (11). The shift in Ct value after diluting ($\Delta C_{t\text{dilution}} = C_{t1/10} - C_{t1/1}$) was compared with the theoretical shift in the absence of PCR-inhibition, being the slope of the standard curve based on pure *V. dahliae* gDNA. For the spiked samples, the Ct values attributed to the *C. acutatum* DNA in the sample background versus in water were compared. If no PCR inhibiting compounds are present in the samples, then $\Delta C_{t\text{spiked}} = C_{t\text{sample}} - C_{t\text{water control}}$ is expected to be not significantly different from zero.

Statistical analysis. The relationship between the results of the wet sieving and the DFS + real-time PCR method was analyzed using linear regression. The effects of pretreatments and soil origin (cauliflower fields POVL T versus PSKW) were analyzed using ANOVA. The data relating to the presence of inhibitors were analyzed using *t* tests. The effects of soil origin (flax fields S3 versus S8) were also analyzed using a *t* test, after log-transformation of the amounts of *Verticillium* DNA/g soil to normalize the data. All tests were conducted using STATISTICA 9.0 (Statsoft).

Results

Primer design and evaluation. The ITS2 regions of the *V. tricornis* isolates were clearly divergent from the ITS2 regions of isolates of the three other *Verticillium* species evaluated (*V. tricornis* versus *V. albo-atrum*, *V. dahliae*, and *V. longisporum*: 2.6, 3.5, and 2.6% divergent or 12/456 bp, 16/459 bp, and 12/459 bp differences, respectively) and therefore were used to design specific primers for *V. tricornis*. Four primer pairs were tested. Primer pair VtF4/VtR2 (Table 2) was selected for further research, as it produced the lowest Ct values, resulted in a single fragment based on melting curve analysis, and showed a linear relationship with near-theoretical slope between the Ct values and the log values of the DNA concentration ($y = -3.37x + 40.54$, $R^2 = 0.98$, amplification efficiency = 0.98) when using a triplicate 10-fold serial dilution of gDNA (10 to 1×10^6 fg) of *V. tricornis* strain Ve010. Subsequently, approximately 100 pg of gDNA of 37 isolates were included in the SYBR Green real-time PCR assay to further test specificity of the selected primer pair. Primer pair VtF4/VtR2 allowed the detection of the four *V. tricornis* strains only, with a mean Ct value $\pm$ SE of 23.2 ± 0.1 (Table 1).

The β -tubulin gene of the different *Verticillium* species was sequenced partially. Per strain, the clones had identical sequences, except for *V. longisporum* strain Pf1, for which the 14 clones had two sequences. The sequences of 7 of the 14 clones were identical to the *V. dahliae* sequence, and the sequence of the other 7 clones was specific for *V. longisporum*. A total of four primer pairs was tested, but only primer pair VITubF2/VITubR1 was selected for further analysis (Table 2), as this was the only primer pair specific for *V. longisporum*; furthermore, the melting curve analysis indicated the presence of a single amplification product. The relation between Ct values and log values of the DNA concentration was linear ($y = -3.32x + 44.98$, $R^2 = 0.99$, amplification efficiency = 1.00), using a triplicate 10-fold serial dilution of gDNA from 100 to 1×10^6 fg from *V. longisporum* strain Pf1. Further specificity testing, with approximately 100 pg gDNA of the 37 isolates listed in Table 1, showed that primer pair VITubF2/VITubR1 allowed specific detection of each of the 11 *V. longisporum* strains, with a mean Ct of 28.0 ± 0.2 (Table 1).

Primer pair VertBt-F/VertBt-R, published by Atallah et al. (1), amplified part of the β -tubulin gene of both *V. dahliae* Ve005 and *V. longisporum* Pf1, but not of *V. albo-atrum* STR3 or *V. tricornis* Ve010. As this primer pair only amplified *V. dahliae* and *V. longisporum* gDNA, it was initially selected for detection of *V. dahliae* + *V. longisporum* in our assays. However, in a later stage of the project, occasional nonspecific amplification was observed when using this primer pair with DNA extracted from soil samples. Specifically, melting curve analysis consistently showed the presence of a second amplicon when DNA extracted from some of the samples

of the naturally infested soil from the Me field was used as template. Cloning and sequencing of this fragment revealed that primer VertBt-R by itself was able to amplify a 450-bp fragment of DNA of an unknown organism, which apparently limits the usefulness of the primers of Atallah et al. (1) for some soils. Primer pair OLG70/OLG71, published by Eyneck et al. (12), amplified DNA of *V. albo-atrum* STR3, *V. dahliae* Ve005, *V. longisporum* Pf1, and *V. tricornis* Ve010. As this primer pair was not species-specific, it was not a useful alternative. We therefore developed new primers based on the β -tubulin sequence alignment of the different *Verticillium* species. Out of seven candidate primer pairs, we selected primer VdTubF2/VdTubR4 based on specific and selective amplification of *V. dahliae* Ve005 and *V. longisporum* Ve Pf1 DNA (Table 2). The relation between Ct values and log values of the DNA concentration was linear ($y = -3.41x + 41.28$, $R^2 = 0.99$, amplification efficiency = 0.96), using a triplicate 10-fold serial dilution of gDNA from 10 to 1×10^6 fg from *V. dahliae* strain Ve005. Further specificity tests showed that primer pair VdTubF2/VdTubR4 allowed detection of the 9 *V. dahliae* and 11 *V. longisporum* strains with a mean Ct value of 27.3 ± 0.1 and 26.0 ± 0.1 , respectively (Table 1), but not the other fungal species tested.

Detection limits. Assays including small amounts of template DNA (10 to 10^6 fg) and DNA extracted from serial dilutions of *V. tricornis* microsclerotia (10, 50, 500, and 5,000 microsclerotia) revealed that the primer pair VtF4/VtR2 reliably detected as little as 10 fg gDNA, or 0.5 microsclerotia per PCR reaction (mean Ct values of 38.1 ± 0.2 and 32.0 ± 0.1 , respectively) using 1/20 of the total DNA extracted from 10 microsclerotia (5 μ l out of 100 μ l). Similar assays with *V. longisporum* revealed that primer pair VITubF2/VITubR1 reliably detected 100 fg DNA, or 2.5 microsclerotia of *V. longisporum* per PCR reaction (mean Ct values of 39.4 ± 0.2 and 34.1 ± 0.2 , respectively). Primer pair VdTubF2/VdTubR4 reliably detected 100 fg gDNA or 0.5 microsclerotia (mean Ct values of 36.4 ± 0.2 and 33.8 ± 0.2 , respectively).

Detection of *Verticillium* in artificially inoculated soil samples. Soil samples inoculated with *V. tricornis* microsclerotia were analyzed using DFS + real-time PCR and primer pair VtF4/VtR2. Detection was successful starting from 0.1 microsclerotia/g soil (Ct of 34.9), and a linear relationship was observed between the log concentration of *V. tricornis* microsclerotia added to the soil and the log concentration of *V. tricornis* DNA detected (Fig. 1). The theoretical detection limit (at a Ct of 40) was at 0.002 microsclerotia/g soil. Soil samples inoculated with *V. longisporum* and *V.*

Fig. 1. Quantitative detection of *Verticillium* in soil inoculated with microsclerotia and then assayed using the real-time polymerase chain reaction (PCR) assays with primer sets VtF4/VtR2 for *Verticillium tricornis*, VITubF2/VITubR1 for *V. longisporum*, and VdTubF2/VdTubR4 for *V. dahliae*. Log-transformed concentrations of *Verticillium* DNA detected are plotted versus the log-transformed concentrations of *Verticillium* microsclerotia/g soil for each species. Error bars indicate standard errors of the mean. Each data point is the mean of two independent observations.

dahliae microsclerotia were also analyzed using DFS + real-time PCR, but with primer pairs VITubF2/VITubR1 and VdTubF2/VdTubR4, respectively. Detection of target DNA started at 0.5 microsclerotia/g soil (Ct of 37.2 and 36.2, respectively), and the log concentration of microsclerotia added to the soil and the log concentration of *Verticillium* DNA were highly correlated (Fig. 1). The theoretical detection limit (at a Ct of 40) was 0.02 and 0.04 microsclerotia/g soil, respectively. Using the linear regression lines shown in Figure 1 to convert fg DNA/g soil into microsclerotia/g soil, it was calculated that 1 microsclerotium/g soil corresponded to 41.7 fg DNA/g soil for *V. longisporum*, 117.5 fg DNA/g soil for *V. dahliae*, and 162.2 fg DNA/g soil for *V. tricorpus*.

Detection of *Verticillium* in naturally infested soil samples using DFS + real-time PCR versus direct DNA extraction from soil + real-time PCR. Five naturally infested soil samples were analyzed by real-time PCR using primer pair VdTubF2/VdTubR4 and DNA extracted directly from 0.025 g and 10 g of soil (i and ii, respectively) and DNA extracted from material left on the filter after density flotation of 100 g of soil (DFS + real-time PCR method) (iii).

For the MoBio Power Soil kit (methods i and iii), DNA was eluted in 100 µl, of which 5 µl was used per PCR reaction. Thus, 0.0125 g and 5.0 g soil equivalent was analyzed per PCR reaction (Table 3). For the UltraClean Mega Soil kit (method ii), DNA of 10 g of soil was eluted in 8 ml. As 5 µl also was used in the PCR reaction, the soil equivalent analyzed was 0.00625 g (Table 3). Real-time PCR melting curve analysis of all 5 soil samples showed that only the target sequences were amplified.

For method (i), there was detection in only two of the five samples with a range of <800 to 5,329 fg *V. dahliae* + *V. longisporum* DNA/g soil (Table 3). The mean coefficient of variation (CV) ± SE for the two soil samples was 0.99 ± 0.43. For method (ii), there was only detection in three of the five samples with a range of <1,600 to 11,071 fg *V. dahliae* + *V. longisporum* DNA/g soil (Table 3), and the mean CV ± SE for the 3 soil samples was 1.28 ± 0.23. For method (iii), there was detection in all six soil samples with a range of 40 to 353 fg *V. dahliae* + *V. longisporum* DNA/g soil (Table 3). The mean CV ± SE for the five soil samples was 0.60 ± 0.44.

Detection of *Verticillium* in naturally infested soil samples using DFS + real-time PCR versus the wet sieving method. Analysis of 25 naturally infested soil samples from three types of fields using the wet sieving method showed that the range of microsclerotia/g of naturally infested soil was 0.0 to 86.9 for *V. tricorpus* and 0.0 to 62.7 for *V. dahliae* + *V. longisporum* (Table 4). Using DFS + real-time PCR, the range of fg DNA/g soil was 1.9 to 86.7 for *V. tricorpus* and 39.0 to 9932.7 for *V. dahliae* + *V. longisporum* (Table 4). In addition, there was a significant linear relationship between the number of *V. tricorpus* microsclerotia/g soil and the amount of *V. tricorpus* DNA/g soil ($P = 0.0001$, $R^2 = 0.78$). This contrasted with *V. dahliae* + *V. longisporum*, where no linear relationship was found between the wet sieving method and the DFS + real-time PCR method ($P = 0.79$, $R^2 = 0.0032$). Melting curve analysis of all 25 soil samples showed that only the target sequences were amplified. The mean CV ± SE for the 25 soil samples was 0.30 ± 0.03

for *V. tricorpus* and 0.41 ± 0.05 for *V. dahliae*. *V. longisporum* was detected in only 1 of the 25 soil samples, with a CV of 0.10.

Soil samples collected from two cauliflower fields naturally infested with *Verticillium* spp. were analyzed using the wet sieving method and the DFS + real-time PCR method. Using this latter method, three soil pretreatments (drying, no drying, and storage at 4°C) were applied to both soil samples. Analysis of variance showed that there was no significant pretreatment effect and no significant pretreatment × soil interaction effect using the real-time PCR assays for *V. tricorpus*, *V. longisporum*, and *V. dahliae* + *V. longisporum* (Table 5). Therefore, the results of the prehandling treatments were pooled. Both the wet sieving and DFS + real-time PCR method showed that in the POVLT field, significantly more *V. tricorpus* and *V. dahliae*/*V. longisporum* propagules were present than in the PSKW field (Tables 3 and 5). In addition, real-time PCR analysis showed that the 4.4 microsclerotia/g soil detected with the wet sieving method in the PSKW soil were *V. longisporum* microsclerotia, whereas the 62.7 microsclerotia/g POVLT soil were *V. dahliae* microsclerotia (Table 4).

Analysis of naturally infested soil samples from the Me field using the wet sieving and the DFS + real-time PCR method showed that both *V. dahliae* and *V. tricorpus* were present in these samples. All DNA detected using real-time PCR and the *V. dahliae* + *V. longisporum* primers (VdTubF2/VdTubR4) could be attributed to *V. dahliae*, as there was no detection with the *V. longisporum* primers (VITubF2/VITubR1). Using the *V. dahliae* primers, the mean ± SE $\Delta C_{t\text{dilution}}$ of the soil samples was 3.0 ± 0.3 and not significantly different from the slope of the standard curve ($P = 0.53$). The mean $\Delta C_{t\text{spiked}}$ was very low: 0.2 ± 0.1, but significantly different from 0 ($P = 0.001$).

Analysis of naturally infested soil samples from the French flax fields showed that both *V. dahliae* and *V. tricorpus* were present in both soils, whereas no *V. longisporum* DNA was detected (Table 3). The amount of *V. tricorpus* found in these soils using both the wet sieving and the DFS + real-time PCR method was very low (1.1 microsclerotia/g soil and 11.3 fg DNA/g soil on average). Remarkably, for *V. dahliae*, the wet sieving method gave very low microsclerotia counts (mean of 1.3 microsclerotia/g soil), especially compared to the amount of *V. dahliae* DNA detected with the molecular test (mean of 1314.1 fg DNA/g soil) (Table 3). Statistical analysis of the molecular data showed that the amount of *V. dahliae* DNA in the symptomatic S8 field was significantly higher than in the nonsymptomatic S3 field ($P = 0.001$). No significant differences were found between the S3 and S8 fields in the amount of *V. tricorpus* DNA/g soil ($P = 0.35$).

Discussion

In this study, we developed a new method for quantifying microsclerotia-forming *Verticillium* species in soil that is specific, sensitive, and faster than the widely used wet sieving method. One of the main advantages of the new method is that it allows differentiation between *V. dahliae* and *V. longisporum*. This differentiation has not been possible previously, neither with conventional plating methods nor with previously published real-time PCR assays (1,12). Although historically there has been a lack of agreement on

Table 3. Quantities of *Verticillium dahliae* + *V. longisporum* in five naturally infested soil samples (labeled A1 through D7) using direct DNA extraction from 0.25 and 10.0 g of soil and density flotation of 100 g of soil, all followed by real-time polymerase chain reaction analysis using primer pair VdTubF2/VdTubR4

Method ^a	Soil per sample (g)	Soil equivalent per PCR reaction (g)	Detection limit (fg/g soil)	DNA quantity (fg Vd+Vl DNA/g soil) ± SE				
				A1	A6	A8	C5	D7
MoBio Power Soil	0.25	0.0125	800	ND ^b	5,189 ± 5,189 ^c	5,329 ± 2,131	ND	ND
MoBio UltraClean Mega Soil	10	0.00625	1,600	ND	11,071 ± 7,949	1,625 ± 1,625*	ND	1,184 ± 1,184*
DFS + MoBio Power Soil	100	5	2	353 ± 269	61 ± 23	220 ± 70	269 ± 70	40 ± 1

^a DFS = density flotation of soil.

^b ND = No detection (Ct > 40 for both replicate samples).

^c * = One of the two replicate samples had a Ct value > 40 (under the detection limit). A value of 0 fg Vd+Vl DNA/g soil was used for this sample.

whether the long-spored *V. longisporum* strains are distinct species (22), recent analyses have established that they are of hybrid origin and most likely belong to a distinct species (19). Especially from a pathological point of view, it is very important to be able to differentiate between these species. This was illustrated in the present study in the experiment with naturally infested soil collected from two cauliflower fields. Real-time PCR analysis showed that the large amount of *V. dahliae*/*V. longisporum* microsclerotia found in the POVLT soil with the wet sieving method was *V. dahliae*. This species was probably introduced with or stimulated by the potato crop and is less pathogenic to cauliflower than *V. longisporum* (9), resulting in a lower disease incidence. The number of *V. dahliae* + *V. longisporum* microsclerotia and the total amount of *Verticillium* DNA was much lower in the PSKW soil than in the POVLT soil, but these microsclerotia could be attributed to *V. longisporum*, which is much more pathogenic to crucifers (19). The lower disease incidence in the POVLT soil than in the PSKW soil can also be attributed to the high amounts of *V. tricorpus* microsclerotia detected, which were probably also introduced with or stimulated by the potato crop (8). *V. tricorpus* is nonpathogenic for many crops, including cauliflower (22; S. C. França, L. Tyvaert, and M. Höfte, unpublished data), and has potential biocontrol activity against pathogenic *Verticillium* species (8,30,32).

Because of the hybrid nature of *V. longisporum* isolates (19), development of primers for *V. longisporum* was not easily done without also detecting isolates of *V. dahliae*. We sequenced 14 clones containing a part of the β -tubulin gene of one *V. longisporum* isolate, and our findings are in agreement with Clewes et al. (5) and Collado-Romero et al. (6), who reported the presence of multiple (two or three) sequences of the β -tubulin gene in *V. longisporum* isolates, but not in *V. dahliae* isolates. One of these sequences is similar to that of *V. dahliae*, while the other sequence presumably originated from an as-yet-unknown species. The *V. longisporum* primers were designed based on the sequence of the β -tubulin gene of this unknown species. Given the lack of knowledge about this unknown species and its potential pathogenicity, one can state that there might be a risk that the PCR primers developed in this study could also amplify DNA of this unknown species as well as those of *V. longisporum*, which could lead to overestimation of the amount of this species in soils. However, there are no indications that this unknown species is present in soil at concentrations that would interfere with the detection of *V. longisporum*. In the naturally infested soils analyzed in this study as well as in individual samples from several other soils (data not shown), we never encountered a detection signal with the *V. longisporum* primers in the absence of a signal with the *V. dahliae* primers. Once the primers

Table 4. Quantities of *Verticillium tricorpus* (Vt), *V. dahliae* (Vd), and *V. longisporum* (Vl) in 25 naturally infested soil samples using the wet sieving method and the density flotation of soil (DFS) + real-time polymerase chain reaction (PCR) method using primers sets VtF4/VtR2, VdTubF2/VdTubR4, and VITubF2/VITubR1, respectively

Field ^f	Plot ^g	Crop ^h	Sampling date (d/m/y) ⁱ	Analyses date (m/y) ⁱ	Verticillium wilt symptoms ^{a,b,c}					Depth ^j	Wet sieving ^d (micro-sclerotia/g soil) ± SE		DFS + real-time PCR ^e (fg DNA/g soil) ± SE		
					Incidence		Severity				Vt	Vd+Vl	Vt	Vd+Vl	Vl
					2007	2008	2007	2008	2009						
POVLT	-	<i>Brassica oleracea</i> var. <i>botrytis</i>	27/08/09	08-09/09	30%	30%	60%	70%	-	1	53.9 ± 9.4	62.7 ± 9.4	43.4 ± 3.7	1816.2 ± 485.0	<20.0
PSKW	-	<i>B. oleracea</i> var. <i>botrytis</i>	10/09/09	09/09	60%	70%	90%	100%	-	1	<0.6	4.4 ± 2.7	1.9 ± 0.9	272.7 ± 21.0	204.5 ± 7.8
Me	A1	<i>Solanum tuberosum</i>	13/04/10	01/11	-	-	-	-	-	1	12.1 ± 1.9	0.6 ± 0.3	5.8 ± 0.4	82.0 ± 10.1	<20.0
Me	A4	<i>S. tuberosum</i>	13/04/10	01/11	-	-	-	-	-	1	29.7 ± 2.8	2.8 ± 1.3	8.5 ± 1.3	199.9 ± 11.9	<20.0
Me	B5	<i>S. tuberosum</i>	13/04/10	01/11	-	-	-	-	-	1	8.3 ± 1.9	6.1 ± 2.8	25.5 ± 0.3	127.4 ± 2.1	<20.0
Me	B6	<i>S. tuberosum</i>	13/04/10	01/11	-	-	-	-	-	1	5.0 ± 1.3	1.1 ± 0.6	30.6 ± 11.3	166.4 ± 48.6	<20.0
Me	C5	<i>S. tuberosum</i>	13/04/10	01/11	-	-	-	-	-	1	40.2 ± 6.6	7.7 ± 2.2	43.1 ± 8.0	492.0 ± 202.1	<20.0
Me	C7	<i>S. tuberosum</i>	13/04/10	01/11	-	-	-	-	-	1	64.9 ± 3.8	20.6 ± 3.4	38.2 ± 1.4	80.6 ± 5.9	<20.0
Me	C8	<i>S. tuberosum</i>	13/04/10	01/11	-	-	-	-	-	1	49.5 ± 4.1	3.9 ± 1.6	47.4 ± 11.3	484.6 ± 61.7	<20.0
Me	D1	<i>S. tuberosum</i>	13/04/10	01/11	-	-	-	-	-	1	86.9 ± 5.0	33.0 ± 3.4	76.1 ± 13.3	386.5 ± 41.9	<20.0
Me	D3	<i>S. tuberosum</i>	13/04/10	01/11	-	-	-	-	-	1	66.1 ± 7.8	6.6 ± 2.2	86.7 ± 9.0	376.5 ± 227.7	<20.0
Me	D4	<i>S. tuberosum</i>	13/04/10	01/11	-	-	-	-	-	1	47.8 ± 6.6	3.9 ± 1.3	71.1 ± 9.1	325.4 ± 76.7	<20.0
Me	D7	<i>S. tuberosum</i>	13/04/10	01/11	-	-	-	-	-	1	64.4 ± 5.3	19.3 ± 4.1	67.8 ± 18.6	190.9 ± 28.9	<20.0
S3	-	<i>Linum usitatissimum</i>	27/07/09	01/11	-	-	-	-	1	1	<0.6	<0.6	8.4 ± 2.7	66.9 ± 38.6	<20.0
S3	-	<i>L. usitatissimum</i>	27/07/09	01/11	-	-	-	-	1	2	0.6 ± 0.3	1.1 ± 0.6	15.4 ± 6.6	39.0 ± 26.0	<20.0
S3	-	<i>L. usitatissimum</i>	31/08/09	01/11	-	-	-	-	1	1	<0.6	3.3 ± 1.3	9.9 ± 4.1	156.2 ± 79.1	<20.0
S3	-	<i>L. usitatissimum</i>	31/08/09	01/11	-	-	-	-	1	2	<0.6	1.1 ± 0.6	18.3 ± 2.1	197.6 ± 42.7	<20.0
S3	-	<i>L. usitatissimum</i>	21/09/09	01/11	-	-	-	-	1	1	<0.6	<0.6	9.0 ± 1.9	260.7 ± 119.7	<20.0
S3	-	<i>L. usitatissimum</i>	21/09/09	01/11	-	-	-	-	1	2	<0.6	<0.6	14.5 ± 5.6	462.7 ± 15.4	<20.0
S8	-	<i>L. usitatissimum</i>	28/07/09	01/11	-	-	-	-	3	1	<0.6	0.6 ± 0.3	10.9 ± 2.1	794.2 ± 56.3	<20.0
S8	-	<i>L. usitatissimum</i>	28/07/09	01/11	-	-	-	-	3	2	1.7 ± 0.9	2.8 ± 1.3	10.6 ± 0.1	211.1 ± 32.7	<20.0
S8	-	<i>L. usitatissimum</i>	18/09/09	01/11	-	-	-	-	3	1	0.6 ± 3.1	1.1 ± 0.6	6.9 ± 3.0	2360.0 ± 456.7	<20.0
S8	-	<i>L. usitatissimum</i>	18/09/09	01/11	-	-	-	-	3	2	8.9 ± 1.9	4.5 ± 1.6	11.5 ± 2.7	9932.7 ± 2491.6	<20.0
S8	-	<i>L. usitatissimum</i>	28/09/09	01/11	-	-	-	-	3	1	1.1 ± 0.6	<0.6	11.9 ± 3.0	800.1 ± 89.4	<20.0
S8	-	<i>L. usitatissimum</i>	28/09/09	01/11	-	-	-	-	3	2	<0.6	1.1 ± 0.6	8.3 ± 0.3	488.3 ± 283.3	<20.0

^a Verticillium wilt severity and incidence of cauliflower was based on vascular discoloration of the stem. Specifically, the stems of 30 plants/field were cut lengthwise after harvest of the cauliflower heads, and scored for severity using a scale of 0 to 3, in which 0 = no discoloration, 1 = <30%, 2 = 30 to 70%, and 3 = >70% of the stem length (= approximately 20 cm) showing vascular discoloration. A disease index (severity) was calculated per field where: severity = $100 \times (\sum(i \times x_i)) / (3 \times \text{number of plants})$ with i = 0 to 3 severity rating and x_i is the number of plants with score i .

^b Verticillium wilt severity of flax was evaluated in each field based on visual evaluation of the stems with 1 = no symptoms and 4 = highly symptomatic.

^c - = not determined.

^d The wet sieving method as described by Harris et al. (17).

^e The DFS + real-time PCR method was developed in this study and included density flotation of 100 g soil, filtration, DNA extraction, and real-time PCR analysis.

^f POVLT and PSKW = Belgian cauliflower fields, Me = Belgian experimental field, S3 and S8 = French flax fields.

^g - = not applicable.

^h Most recent crop.

ⁱ d = day, m = month, y = year.

^j 1 = 0 – 30 cm; 2 = 30 – 60 cm.

have been more widely used, it will become possible to state whether the assay is not confounding isolates of the unknown species with isolates of *V. longisporum*. Also, the β -tubulin sequence of the unknown species does not show a match with environmental samples currently present in GenBank. Thus, the limited importance of this unknown species is so far supported by the lack of DNA sequence records of this species.

Although morphological differentiation between *V. dahliae*/*V. longisporum* and *V. tricornis* isolates is possible (but sometimes very difficult) using the wet sieving method (15), we also designed primers for specific detection of *V. tricornis*. Similar to the primers of Moukhamedov et al. (28) for a conventional PCR assay, the real-time PCR primers developed in this study for *V. tricornis* were designed based on the ITS region. Using the DFS + real-time PCR method, we demonstrated that *V. tricornis* was present in all naturally infested fields tested. The role of *V. tricornis* in these naturally infested soils needs to be further elucidated, and the real-time PCR assay developed in this study may help to evaluate the possible association of this species with disease-suppressive soils and/or the potential of isolates of this species to serve as biological control agents.

Regarding the sensitivity of the assay, we developed a method that was able to detect *Verticillium* in soils inoculated with as few as 0.1 *V. tricornis* and 0.5 *V. dahliae* and *V. longisporum* microsclerotia/g soil. This is more sensitive than the DNA microarray method described by Lievens et al. (23), which had a detection limit of 6.7 *V. dahliae* microsclerotia/g soil. The sensitivity of this new method is mainly attributed to the density flotation, as the amount of soil that was sampled was 8 times greater than with the wet sieving method (100.0 versus 12.5 g) and at least 10 times greater than when DNA was extracted directly from soil (100.0 versus 0.25 to 10.0 g). Moreover, the amount of soil equivalent that was analyzed was 2.8 times greater than with the wet sieving method (5.0 versus 1.8 g) and at least 400 times greater than when DNA was directly extracted from soil (5.0 versus 0.00625 to 0.0125 g). This was illustrated in the present study with the experiment in which *V. dahliae* + *V. longisporum* could be detected in five naturally infested soil samples when density flotation was used and only in two to three soil samples when DNA was extracted directly from 0.25 g and 10 g of soil, respectively. Due to the small amount of soil equivalent per PCR reaction in the samples from which the DNA was directly extracted, the calculation of the amount of *Verticillium* DNA per g of soil uses a very large multiplication factor, resulting in overestimated amounts if there is detection. Thus, density flotation makes the new method more sensitive and more reproducible than when DNA is directly extracted from soil.

Table 5. Analysis of variance for effect of three soil prehandling treatments (drying, no drying, and storage at 4°C) and two soils (POVLT and PSKW) on fg DNA/g soil for *Verticillium tricornis*, *V. longisporum*, and *V. dahliae* + *V. longisporum* obtained with real-time polymerase chain reaction (PCR) using primer set pairs VtF4/VtR2, VITubF2/VITubR1, and VdTubF2/VdTubR4, respectively

Species	Source of variation	df	MS ^a	P ^b
<i>V. tricornis</i>	Soil	1	711E5	0.009*
	Prehandling	2	139E5	0.192
	Soil × prehandling	2	145E5	0.181
	Error	12	73E5	
<i>V. longisporum</i>	Soil	1	166E5	0.001*
	Prehandling	2	235E4	0.097
	Soil × prehandling	2	335E4	0.050
	Error	12	82E4	
<i>V. dahliae</i> + <i>V. longisporum</i>	Soil	1	311E7	0.015*
	Prehandling	2	986E6	0.117
	Soil × prehandling	2	957E6	0.127
	Error	12	38E7	

^a MS = mean of squares.

^b P = Probabilities associated with corresponding F tests. Values marked with * indicate a significant difference (P < 0.05).

Sensitivity of the assay also is determined by the real-time PCR target region and primers. The detection of *V. tricornis* was at least 10 times more sensitive than that of *V. longisporum* and *V. dahliae*. This difference was due to the multi-copy versus single-copy nature of the respective targets: *V. tricornis* primers were designed based on the multi-copy rDNA ITS region, whereas the *V. longisporum* and *V. dahliae* primers were designed based on the single-copy β -tubulin gene for specificity reasons. A few studies have shown that the number of rDNA copies can fluctuate with the age and stage of growth of the fungus, and nonorthologous copies of the ITS region among strains of the same species have been described (1). Given that such inconsistencies may affect the quantification of pathogen propagule numbers and potential comparisons among samples, we have determined the copy number of five *V. tricornis* isolates from different origins as described by Debode et al. (11) for *C. acutatum* and showed that the difference in copy number between the five tested isolates was small (mean copy number ± SE = 18.60 ± 1.28). Based on these results, the ITS primers were preferred when analyzing soil samples.

The correlation between the results of the wet sieving and the DFS + real-time PCR methods for 25 naturally infested soils was very high for *V. tricornis*, but there was no correlation for *V. dahliae* + *V. longisporum*. The reason for this species-related difference is that for *V. dahliae* + *V. longisporum* there was a very low recovery using the wet sieving method, which was not observed for *V. tricornis*. Our results indicate that the viability or germinability of *V. dahliae* microsclerotia may be reduced during extended dry storage, while the viability and germinability of the *V. tricornis* microsclerotia may be less affected by dry storage. More specifically, the Me and flax soil samples had been stored dry for 6 months and 1.5 years, respectively. Presumably the viability or germinability of the *V. dahliae* microsclerotia decreased over these time periods, while their DNA stayed intact due to the limited microbial activity in these dried samples. Therefore, this DNA was measured with the molecular method and represents the more appropriate inoculum level at the time of sampling. In contrast, the recovery of *V. tricornis* in these soils was higher and correlated with the amount of *V. tricornis* DNA detected. Further research is necessary to determine the effect of dry storage on the germinability and viability of microsclerotia of different *Verticillium* species. The inter-species difference should be less for fresh samples or samples stored under normal moisture conditions, and this was observed with the inoculated soil samples.

As real-time PCR also detects DNA from dead or dormant cells in dry-stored soils, the question arises as to the possibility of an overestimation of the inoculum using this real-time PCR method. Although it is generally assumed that DNA from dead cells usually degrades fairly rapidly in natural moist soil due to microbial activity (23), experimental data concerning DNA degradation speed in dead microsclerotia in soil would be welcome to further rule out the possibility of bias due to detection of dead microsclerotia.

Using the wet sieving method, seasonal fluctuations in the concentration of recoverable *V. longisporum* microsclerotia has been detected in the field, even in uncultivated soils (S. C. França, M. Höfte, and K. Spiessens, unpublished data). It is unclear whether these seasonal fluctuations are attributed to dormancy of microsclerotia or the weakness of microsclerotia in the soil after a period of sporulation (26,33). The real-time PCR (DNA-based) method incorporates this dormant/weak inoculum into the soil assay. Analysis of soil samples from cultivated as well as uncultivated soils using both the wet sieving and real-time PCR method at multiple time points throughout the year may help to clarify this issue.

Another factor to consider when using real-time PCR is that DNA is extracted from a soil environment. One of the problems encountered when amplifying templates collected from soil for PCR-based quantification of a pathogen is the presence of other compounds extracted along with the DNA that can influence amplification efficiency. Analysis of 25 naturally infested soil samples showed that the CV of the real-time PCR results was 0.34. This is

quite low given the transformation from a log scale and well within the amount of variability observed with the conventional plating methods (36). Therefore, the results can be considered reproducible. In addition, the DNA extracted from the Me soil contained low amounts of PCR inhibitors, as $\Delta C_{T_{\text{dilution}}}$ was not significantly different from the slope of the standard curve. Although $\Delta C_{T_{\text{spiked}}}$ was significantly different from 0, it was very low (0.2) and ranged within the variability of the real-time PCR system (1). The strongly reduced amount of PCR inhibitors in the DNA of these soil samples can be attributed to the sucrose density gradient approach used in this study and the MoBio Power Soil DNA extraction system, which is especially designed to extract DNA from environmental samples containing a high humic acid content. However, for soils that are very rich in organic matter, the 100 g sample can be split in half, with only a limited reduction in detection limit.

This study showed that the time needed for analysis of a soil sample for *Verticillium* species can be reduced from at least 25 days with the wet sieving method to only 1 day with the DFS + real-time PCR method. Using the wet sieving method, air-drying of the soil is necessary to eliminate undesired propagules, such as conidia and mycelial fragments sensitive to desiccation (4), and thus increase specificity of the assay. Density flotation eliminates the need to dry the soil samples for 2 weeks before real-time PCR analysis, making it possible to perform the whole assay (sucrose flotation, filtering, DNA extraction, and real-time PCR assay) in 1 day. If it is not possible to perform the analysis directly after soil sampling, the wet soil can be stored at 4°C for at least 14 days before analysis.

Finally, in the present study, data were included on disease incidence and severity with the cauliflower and the flax soils. For the cauliflower soils, it was demonstrated that the extent of the symptoms was correlated with the identity of the specific *Verticillium* species present, and not with the total amount of microsclerotia detected. For the flax soils, it was demonstrated that the highest amount of *V. dahliae* DNA was found in the flax field with *Verticillium* symptoms (S8), and that this difference was statistically significant. These results indicate that the new method developed in this study can help in evaluating the risk of soils for host-specific development of *Verticillium* wilt and contribute to sustainable disease control strategies.

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