Applied Soil Ecology 108 (2016) 176–186

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Assessment of *Verticillium* flax inoculum in agroecosystem soils using real-time PCR assay

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ARTICLE INFO

Article history:
Received 9 January 2016
Received in revised form 18 May 2016
Accepted 17 July 2016
Available online xxx

Keywords:
Real time PCR assay
Verticillium dahliae
Flax
Farming systems

ABSTRACT

Verticillium wilt, due to the soilborne fungus *Verticillium dahliae*, is a persistent disease affecting flax culture in Upper Normandy. This pathology has increased since the last decade, leading to yield losses for flax producers. In part due to the long survival of *V. dahliae* in soil and the difficulty of early diagnosis in affected plant, *Verticillium* flax wilt management remains problematic in the absence of efficient phytosanitary treatment. Pathogen avoidance and the reduction of soil inoculum through adapted cultural practices are the best alternatives to fight against *Verticillium* wilt. Therefore, the objective was here to optimize and validate a rapid and specific real-time PCR assay targeting the ribosomal DNA Internal Transcribed Spacers (ITS) to measure *V. dahliae* density in soil. This method was then used to assess and compare the pathogen density in fields from four diverse management systems: conventional, integrated with or without tillage, and organic. First, the real-time PCR assay provided sensitive and reliable quantification of *V. dahliae* in a range of artificially inoculated soils with known inoculum density. Then, this method was successfully applied in crop fields. Measured *V. dahliae* densities presented an intra-parcel heterogeneity, emphasizing the importance of an adapted sampling strategy to assess pathogen load in crop field. Furthermore, these densities appeared to be impacted by agricultural practices, particularly tillage. The influence of the previous crop on pathogen load had also to be considered with attention to manage efficiently this disease through crop rotation. Such knowledge of pathogen density in soils could provide critical information for stakeholders to identify infested fields and predict disease development. Molecular approach should be considered as a useful tool for *Verticillium* wilt management in flax culture.

1. Introduction

Flax (*Linum usitassimum*) has been an annually crop of great importance and interest, used as a source for both oil and high-quality fibers. Economic and commercial outlets are numerous and diversified from textile to composite materials in the car industry (http://www.coopdefrance.coop/fr/41/lin). According to agricultural reports (Arvalis report, 2015; FranceAgriMer report, 2013), France is the worldwide leader in flax fiber production. The main production area is localized in Normandy (France) which represents 64 % of the total French agricultural land sowed with flax (http://www.chambre-agriculture-normandie.fr/panorama-lin-normandie). A short growing cycle and low input make flax a very cost-effective crop for farmers. However, flax culture remains a risky choice because of its high sensitivity to external conditions, such as soil conditions and climate. Flax is also threatened by numerous disease caused by pathogenic microorganisms, leading to large yield loss, or even total destruction of the harvest.

Verticillium wilt, a monocyclic disease caused by the soilborne fungi *Verticillium dahliae*, is becoming more and more prevalent among flax culture according to monitoring organizations (as presented in the Arvalis report, 2015). This pathogen is found in the soil in its resistance form, called microsclerotia, which are spread freely or embedded within plant debris. In soil, the dormant microsclerotia remains viable for up to 14 years (Klosterman et al., 2009). Upon germination, hyphae from *V. dahliae* microsclerotia enter through the plant root and invade its vascular system, perturbing sap circulation and plant metabolism (Klosterman et al., 2009). Unfortunately, recognizable symptoms of *Verticillium* flax wilt are observed during the retting process when harvested plants are laid on soil to induce flax fibers separation (Henriksson

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http://dx.doi.org/10.1016/j.apsoil.2016.07.010
0929-1393/

et al., 1997). By identifying the disease this late, the use of any effective chemical treatments is impaired. Some studies have reported the direct relationship between *Verticillium* inoculum densities in soil, wilt development and disease severity on a number of economically important crops such as potato (Davis, 1994; Nagtzaam et al., 1997), eggplant (Cohen et al., 2005), cauliflower (Xiao and Subbarao, 1998), horseradish (Khan et al., 2000), cotton (Paplomatas, 1992), strawberry (Harris and Yang, 1996), olive (Lopez-Escudero and Blanco-Lopez, 2007), tomato (Grogan, 1979), artichoke (Berbegal et al., 2007) and pepper (Bhat et al., 2003). Therefore, knowledge on inoculum levels in the soil before planting could be a very useful method for crop protection.

In addition, the control of *Verticillium* wilt disease remains a primary issue because of the long viability of microsclerotia in soil and its broad host range (Fradin and Thomma, 2006). For monocyclic pathogens the main management strategy is to reduce the primary inoculum in the soil (Klosterman et al., 2009). For a long time, soil fumigation with chemicals has been extensively used (Ashworth and Zimmerman, 1976; Mckeen and Sayre, 1964; Triky-Dotan et al., 2007; Wilhelm et al., 1972). Nonetheless this practice often has only limited and temporary success on *Verticillium* wilt inoculum. Some fungicides have shown inhibitory effects on this fungus (Niu et al., 2006) but do not affect the pathogen once it has entered the plant vascular system (Fradin and Thomma, 2006). Because the distinguishable symptoms of *Verticillium* wilt in flax can rarely be detected early enough, the use of fungicide against this pathogen is not optimal. Moreover, the use of current chemical treatments becomes less and less attractive because of their deleterious impacts on the environment (Ceccherini et al., 2013; Wan, 2013). Growing awareness of the need to promote more sustainable agriculture has led to an increase in legislative constraints in order to reduce the chemical input (Gullino and Kuijpers, 1994), such as with the French action plan Ecophyto. This policy implies that farmers must search for alternative strategies in plant disease management. The most obvious solution, appreciated by the farmers, consists of developing resistant cultivars (Khaskheli et al., 2013). However, to our knowledge, no *Verticillium* wilt-resistant flax cultivar exists. Biological control is another prospected area for suppressing *Verticillium* wilt using fungal antagonists (Zheng et al., 2011), antagonistic rhizobacteria (Berg et al., 2000), mycorrhizal fungi (Azcón-Aguilar et al., 2002) and soil amendments (Lang et al., 2012). Nevertheless, interesting antagonist effects detected *in vitro* are often not reproduced in the field (Berg et al., 2000). Currently, crop rotation represents the only possibility for flax producers to manage *Verticillium* wilt. This practice relies on the expected natural reduction of the microsclerotia pool below the crop-specific threshold level by spacing in time through crop rotation the culture of host plants (Klosterman et al., 2009). However, for *V. dahliae*, the effects stay limited by its wide host range. The most efficient approach remains to be pathogen avoidance through the identification of infested fields. This evaluation must rely on an accurate method for pathogen detection and inoculum quantification in the soil. To our knowledge, no study has focused on the evaluation of *V. dahliae* inoculum density in soil for flax cultivation.

Many methods have been described for quantifying *V. dahliae* in the soil (for review: (Goud and Termorshuizen, 2003)). Real-time PCR technology provides new opportunities for the quantification of phytopathogenic targets in various environmental samples (Schena et al., 2004). PCR assays have emerged as a powerful tool for pathogen diagnosis due to higher sensitivity, specificity, reproducibility and rapidity compared with traditional methods. Some studies have presented real-time PCR methods to quantify *V. dahliae* in plant tissues [olive (Cohen et al., 2005; Mercado-Blanco et al., 2003); potato (Atallah et al., 2007; Pasche et al., 2013); hop (Maurer et al., 2013); spinach (Duressa et al., 2012)] and also in soil (Banno et al., 2011; Bilodeau et al., 2012; Ceccherini et al., 2013). These tools target different specific genomic regions (RAPD markers, β-tubuline gene or the intergenic spacer of ribosomal operon). Sequences of the internal transcribed spacer region (ITS) or the intergenic spacer (IGS) of ribosomal DNA are often used to design PCR primers to detect and/or quantify fungal pathogens (Bilodeau et al., 2012; Goud and Termorshuizen, 2003; Schena et al., 2004). Compared to single-copy targets, the presence of ITS and IGS in high copy number in the genome is expected to ease organisms detection and increase the sensitivity for their quantification, particularly in complex matrix such as soil (Bilodeau et al., 2012).

The objective of this study is to validate an alternative real-time PCR assay targeting Internal Transcribed Spacers to specifically detect and accurately quantify the microsclerotia pool of *V. dahliae* in soil. This real-time PCR assay was then applied to evaluate and compare inoculum density of *V. dahliae* in natural agricultural soils sampled in contrasted agrosystems. Assuming that pathogen density in the soil is correlated with disease severity on flax, such tool will be highly useful for disease monitoring and managing.

2. Materials and methods

2.1. Fungal strains maintenance

All *Verticillium* spp. used in this study are listed in the Table 1. These isolates were previously characterized by biological and molecular phenotyping (data not shown). Verticillium strains were obtained from the fungal collections of the French National Museum of Natural History (MNHN, Paris, France), the MIAE (Microorganisms of Agro-Environmental Interest) collection from the ERB ("Ensemble de Ressources Biological", or Biological Resource Bank) platform of the INRA research unit "Agroecologie" (Dijon, France), the French agricultural cooperative "Terre de Lin" (Saint Pierre le Viger, France) and the University of California (Davis, USA). All isolates were maintained on Potato Dextrose Agar (PDA) and stored at 4 °C. For fungal DNA extraction, a small agar plug from the fungal subculture in PDA were placed in a flask of Potato Dextrose Broth (PDB) and incubated in the dark for 2 weeks at 25 °C. Then the grown mycelium network was cut in pieces of 50-mg with a sterile scapel and stored at −20 °C until used.

2.2. Fungal DNA extraction and quantification

Total DNA was extracted from 50 mg of fungal mycelium using the PowerPlant DNA Isolation kit (MoBio Laboratories, 121 Carlsbad, CA, USA) according to the manufacturer's instructions with modifications. Briefly, each sample was first crushed with a sterile pestle and then digested overnight at 30 °C in 450 μl of the PowerPlant bead solution supplied by the manufacturer with addition of 400 U of lyticase solution (Sigma Aldrich, Saint-Louis, MO, États-Unis). All subsequent steps were performed according to the manufacturer's instructions. The DNA concentrations were assayed by fluorimetry using Quant-iT^{TM} dsDNA Broad-Range Assay Kit (Picogreen, Life Technologies, Carlsbad, CA, États-Unis) following the manufacturer's instructions. The DNA solutions were then stored at −20 °C until use.

2.3. Primers specificity to V. dahliae and PCR amplification

To allow for the easier detection of low inoculum quantity of the studied pathogen, the high-copy-number ITS (Internal Transcribed Spacers) from rDNA operon was chosen as a target for subsequent real-time qPCR assay. Species-specific primers for *V. dahliae* were used for PCR amplification (334 bp PCR amplicon): Vd-ITS1-45-F (5′- CCGGTCCATCAGTCTCTCTG-3′) and Vd-ITS2-379-R

Table 1
Isolates of *Verticillium* spp. used in this study and evaluation of the PCR amplification using *V. dahliae* primers set compared to the PCR amplification with universal fungi primer set.

Isolate	Species	Obtained from	*V. dahliae* specific PCR amplification	Universal fungal PCR amplification
05–837	*V. dahliae*	Terre de Lin	+	+
IN 1047	*V. dahliae*	Terre de Lin	+	+
DS 1072	*V. dahliae*	Terre de Lin	+	+
05–773	*V. dahliae*	Terre de Lin	+	+
05–883	*V. dahliae*	Terre de Lin	+	+
812.97	*V. dahliae*	CBS-KNAW fungal bank	+	+
1015	*V. dahliae*	National Museum of Natural History	+	+
05-828A	*V. dahliae*	Terre de Lin	+	+
PD589	*V. longisporum* A1/D3	University of California	+	+
09–2006	*Verticillium*.sp.	Terre de Lin	NA	+
05-828A	*Verticillium*.sp.	Terre de Lin	NA	+
05–836	*V. alboatrum*	Terre de Lin	NA	+
3243	*V. tricorpus*	National Museum of Natural History	NA	+
00344	*V. chlamydosporum*	MIAE (INRA Dijon)	NA	+
5126	*V. chlamydosporum*	National Museum of Natural History	NA	+
PD338	*V. alfalfae*	University of California	NA	+
PD592	*V. non alfalfae*	University of California	NA	+
PD348	*V. longisporum* A1/D1	University of California	NA	+
PD356	*V. longisporum* A1/D2	University of California	NA	+

+: amplification signal detected.
NA: no amplification.

(5′- ACTCCGATGCGAGCTGTAAC –3′). *In silico* specificity for *V. dahliae* of designed primers was checked after alignment of various ITS sequences of different *Verticillium* spp (*Verticillium alboatrum*, *Verticillium tricorpus*, *Verticillium chlamydosporium*, *Verticillium fungicola*, *Verticillium nigrescens*, *Verticillium nubilum*, *Verticillium longisporum* A1/D1, *V. longisporum* A1/D2, *V. longisporum* A1/D3, *Verticillium alfalfa*, *Verticillium non alfalfae*) from Genbank using Clustal Omega software (data not shown). *In vitro* species-specificity was checked by PCR amplification of each available *Verticillium* spp. isolates' DNA (*Verticillium* sp., *V. alboatrum*, *V. tricorpus*, *V. chlamydosporium*, *V. alfalfae*, *V. non alfalfae*, *V. longisporum* A1/D1, *V. longisporum* A1/D2, *V. longisporum* A1/D3). The PCR reaction was performed in a 25-μl volume containing 5 ng of fungal DNA, 0.4 μM of each primer (Vd-ITS-45-F, Vd-ITS-379-R) and 12.5 μl of GoTaq® Green Master Mix (Promega, Madison, Wisconsin, USA) in the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The cycling protocol was: 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 62 °C for 30 s and 72 °C for 20 s. A final extension step was carried out at 72 °C for 7 min. In parallel, each *Verticillium* isolate was checked for PCR amplification with the universal fungal PCR primer ITS1 and ITS4 (White et al., 1990). The PCR reaction was performed in a 25-μl volume containing 5 ng of fungal DNA, 0.5 μM of each primer (ITS1, ITS4), 1 X GoTaq® Green Master Mix (Promega, Madison, Wisconsin, USA) in the GeneAmp® PCR System 9700 (Applied Biosystem, Foster City, CA, USA). The cycling protocol used was 95 °C for 5 min, followed by 15 cycles at 95 °C for 30 s, a touchdown procedure from 65 °C to 58 °C for 30 s and 72 °C for 20 s, and then 20 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. A final extension step was carried out at 72 °C for 7 min. All PCR products were visualized by electrophoresis on agarose gel stained with ethidium bromide.

The phylogenetic identification of each *Verticillium* strain used in this study was confirmed by sequencing a 600 bp PCR product from PCR amplification using ITS1 and ITS4 primers (Genoscreen, Lille, France).

2.4. Real-time PCR assay on *verticillium isolates*

Real-time PCR amplification of the 334 bp (base pair) target sequence of *V. dahliae* was performed in a 25-μl reaction volume containing 4 ng of each *V. dahliae* isolate DNA, 0.4 μM of each primers (Vd-ITS1-45-F and Vd-ITS2-379-R), 4 μg ml^{-1} of Extreme Thermostable ssBinding Protein (EtSSB) (New England BioLabs, Ipswich, Massachusetts, USA) and 1 X LightCycler® 480 DNA SYBR Green I Master mix (Roche, Basel, Switzerland). Amplification was performed in a LightCycler 480 real-time PCR system (Roche, Basel, Switzerland). The PCR cycling conditions were 95 °C for 5 min and 50 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 20 s. After the final amplification cycle, the melting curve profiles were obtained by heating the samples to 95 °C, cooling to 60 °C and slowly heating to 97 °C, increasing 1.1 °C every 10 s with continuous measurement of fluorescence at 520 nm. DNA standard curves were obtained from 10-fold dilutions of *V. dahliae* TdL 05-833 isolate DNA (2 ng μl^{-1}). The PCR efficiency and DNA concentration were determined using the Roche second derivative maximum method. Real-time PCR reactions for the standard curve were repeated at least three times and PCR assays always include negative control (no DNA). *V. dahliae* 334 bp amplicons obtained were sequenced using Vd-ITS1-45-F by Genoscreen (Lille, France). In parallel, real-time PCR amplifications were performed as described above in a 25-μl reaction volume containing 4 ng of each other tested *Verticillium* isolate DNA.

2.5. Copy number of ITS *per* V. dahliae *genome*

To estimate ITS copy number in the nuclear genome of *V. dahliae*, three single-copy genes [endochitinase, glyceraldehyde-3-phosphate dehydrogenase (G3PD) and β-tubulin] were amplified and compared to ITS amplification as described by Bilodeau et al. (2012). The sequences of all primers used are listed in Table 2. Amplifications of the *V. dahliae* ITS species-specific sequence and the single-copy genes were performed in the same 96-well microplate and with the same real-time PCR amplification mix composition and cycling conditions, as described above. DNA templates from 8 *V. dahliae* isolates (Table 1) were used at a concentration of 2 ng μl^{-1}. The difference of mean Cq (quantification cycle) values between the ITS region and each single-copy sequence was calculated for all tested *V. dahliae* isolates. The mean ITS copy number per genome was then estimated using the following equation: copy number = $(1+\varepsilon_{ITS})^{\Delta Cq}$ (ε_{ITS} = amplification efficiency for ITS assay) (Bilodeau et al., 2012; Pfaffl, 2001).

Table 2
PCR primers used for amplification of single-copy genes to evaluate the copy number of the ribosomal DNA repeat unit in *Verticillium dahliae* strains.

Target gene	Primer name	Primer sequence	Reference
Endochitinase	Vd-endoch-1F	CTCGGAGGTGCCATGTACTG	Bilodeau et al. (2012)
	Vd-endoch-1R	ACTGCCTGGCCCAGGTTC	
β-tubulin	Vd-βtub-1F	GCGACCTTAACCACCTCGTT	Bilodeau et al. (2012)
	Vd-βtub-1R	CGCGGCTGGTCAGAGGA	
	VertBt-F	AACAACAGTCCGATGGATAATTC	Atallah et al. (2007)
	VertBt-R	GTACCGGGCTCGAGATCG	
G3PD	Vd-G3PD-2F	CACGGCGTCTTCAAGGGT	Bilodeau et al. (2012)
	Vd-G3PD-1R	CAGTGGACTCGACGACGTAC	

2.6. Production, preparation and quantification of V. dahliae microsclerotia inocula

For artificial soil inoculation, an agar plug of the *V. dahliae* TdL 05-833 isolate was transferred to an Erlenmeyer flask containing 50 ml of malt extract (10 g L^{-1}). The culture was incubated at 20 °C in the dark for 1 month to induce microsclerotia (msc) production. Mixed mycelium with msc were then harvested and crushed in a chilled microblender (Waring, New Hartford, CT, USA) with 25 ml PBS (Phosphate Buffered Saline: NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.76 mM) for 30 s at high speed and twice for 20 s at low speed. The milling was then filtered through several cell strainers (200 μm, 100 μm and 40 μm) to eliminate any residual mycelium and non-isolated msc. The final collected msc were between 40 μm and 100 μm in size. The mixed msc suspension was diluted to 20 ml with PBS, briefly vortexed and decanted for 15 min. The supernatant was then discarded to eliminate floating debris. The same decantation step was repeated a second time. The final msc suspension was diluted to 10 ml with PBS and stored at 4 °C until use. The predominance of isolated msc in the suspension was confirmed by microscopic observation (data not shown). Viable msc density in the suspension was estimated by dilution plating. Serial dilutions of msc suspension were plated onto PDA and incubated in the dark at 20 °C. After 3 days, the total number of germinated msc was counted.

2.7. Artificial inoculation of soil samples with V. dahliae microsclerotia suspension

Artificial inoculation was conducted on an autoclaved soil [3 × 20 min (121 °C)], previously sieved at 2-mm and assayed for the absence of *V. dahliae* (data not shown). A range of microsclerotia suspensions (1000, 100 and 10 msc g^{-1} soil) was inoculated in 40 g of soil dispensed in 180-ml plastic pot in parallel with a control modality without inoculation. Msc suspensions stored in PBS were diluted in sterile water (or only sterile water was added with PBS for control) to comply with a 70% soil water holding capacity (WHC). Soil WHC was determined by the methodology described by Cassel and Nielsen (1986) (Cassel and Nielsen, 1986). Immediately after the dilution of the msc suspensions, the msc inocula were carefully mixed with the soil as homogenously as much as possible. Each modality was repeated three times.

2.8. DNA extraction from inoculated soil samples and microsclerotia suspension

Immediately after inoculation and homogenization, the total soil DNA was extracted from 0.5 g of moist soil using Fast DNA spin Kit for Soil (MP-Biomedicals, Santa Ana, CA, USA) according to manufacturer's instructions. The DNA was finally suspended in 50 μl of sterile molecular biology grade water (5Prime, Hamburg, Germany). Simultaneously, DNA extractions were performed on the msc suspension using the PowerPlant DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The soil and msc DNA concentrations were assessed by fluorimetry using Quant-iT™ dsDNA Broad-Range Assay Kit (Picogreen, life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions. The DNA solutions were then stored at −20 °C until use.

2.9. Optimization of real-time PCR assay for soil matrix

Reaction conditions, such as the annealing temperature of primers, the DNA concentration and the temperature when measuring the fluorescence signal of *V. dahliae* amplicon were optimized. Moreover, different concentrations of the following three PCR additives were tested to improve PCR sensitivity in the soil matrix: Bovine Serum Albumine (BSA) (New England BioLabs, Ipswich, Massachusetts, USA), EtSSB (New England BioLabs, Ipswich, Massachusetts, USA) and T4 bacteriophage Gene 32 product (T4 gp 32) (MP-Biomedicals, Santa Ana, CA, USA). The melting curve temperature profile and DNA standard curves were performed and obtained as previously described.

The potential influence of the presence of other components co-extracted from the soil on PCR efficiency and the quantification of the ITS copy numbers per gram of soil were also assessed. DNA extract from soil without *V. dahliae* was mix to the DNA extracted from serial dilutions of msc suspensions. Quantitative PCR amplifications with and without addition of the soil DNA background were performed in the same PCR microplate, and ITS copy numbers per gram of soil were compared. The DNA standard curves were obtained as described above.

2.10. Application of real-time PCR assay in native agricultural soils

2.10.1. Collection of agricultural soil samples

The used real-time PCR assay was applied in an agricultural context. Four diverse management systems were chosen: conventional (Conv) with statutory chemical input and occasional tillage; integrated with tillage and reduced chemical input (Int TRC), integrated with occasional tillage and reduced chemical input (Int RTRC); organic (Org) with tillage but no chemical input. Soil was sampled in field plots of interest, which presented comparable silt content, in the Normandy region of France. Locality and GPS coordinates of the chosen fields are presented in Table 3. The last flax or other *V. dahliae* host occurrence was highlighted for all fields (Table 3). Soil sampling was carried out in March 2012 for each crop management system in three similar wheat fields belonging to the same farmer. Wheat was cultivated in all fields at the time of sampling. A second soil sampling was repeated in same fields in October 2012. Similarly, soils from three permanent representative grasslands attached to the studied farms were also sampled. Physical and chemical characteristics of all these soils are summarised in Table 4. The standard soil analyses (texture, pH, total carbon and total nitrogen, cation exchange capacity) were performed using the standard methods as described in Forster (1999) (Forster, 1999). Soil texture was determined using a laser

Table 3
Localization and management of sampled fields. Occurrences of flax and other V. dahliae hosts species culture are pointed out for all fields.

Management system	Field	Locality	Geographic coordinates	Flax culture	Other known host crops
Conventional	1	Tourny	N 49° 10.454′ E 1° 32.151′	no flax since 10 years	Potatoes in 2009
	2	Tourny	N 49° 10.351′ E 1° 32.284′	in 2011	none
	3	Tourny	N 49° 10.924′ E 1° 33.162′	no flax since 10 years	none
Integrated RTRC	1	Guitry	N 49° 12.460′ E 1° 32.333′	in 2002	none
	2	Guitry	N 49° 12.355′ E 1° 32.435′	in 2007	Potatoes in 2005 and 2011
	3	Guitry	N 49° 12.207′ E 1° 32.484′	in 2007	Potatoes in 2011
Integrated TRC	1	Richeville	N 49° 15.742′ E 1° 32.357′	in 2004 and 2008	none
	2	Richeville	N 49° 15.810′ E 1° 32.475′	in 2006	none
	3	Richeville	N 49° 15.310′ E 1° 33.080′	in 2007	none
Organic	1	Combon	N 49° 06.491′ E 0° 51.185′	in 2011	none
	2	Sacquenville	N 49° 04.467′ E 1° 04.639′	no flax since 10 years	Potatoes in 2005 and 2011
	3	Sacquenville	N 49° 04.469′ E 1° 04.654′	in 2011	Potatoes in 2007

granulometer Malvern Mastersizer (Malvern Instruments, Malvern, UK). Soil pH was determined according the ISO standard NF ISO 10390. Soil total and organic carbon was measured using a TOC analyzer Shimadzu SSM-5000A/TOC-VCSH Carbone (Shimadzu, Kyoto, Japan). Soil total nitrogen was determined with the Kjeldahl method (Kjeldahl, 1883). Cation exchange capacity (CEC) was measured according to the Metson method (Metson, 1956). Soil was sampled from the surface horizon (0–10 cm) in 5 plots, located respectively at each corner and in the centre of a 30-m square, to take into consideration spatial variability. Each sample consisted of bulk composite soil with a combination of 5 replicates. The fall repeated sampling was done at the exact same GPS plot coordinates than the spring one. Moist soils were then sieved to 2 mm particle size the day of sampling date and immediately used for analyses.

2.10.2. DNA extraction from soil samples and quantification of V. dahliae ITS copy number by real-time PCR

The total soil DNA was extracted from 0.5 g of moist soil using the Fast DNA spin Kit for Soil (MP-Biomedicals, Santa Ana, CA, USA) according to manufacturer's instructions. The DNA was finally suspended in 50 μl of sterile molecular biology grade water. The soil DNA concentrations was assessed by fluorimetry using the Fluorescent DNA quantitation Kit Hoechst 33258 (Biorad, Hercules, CA, USA), following the manufacturer's instructions. The DNA solutions were stored at −20 °C until use.

dahliae ITS copy numbers per gram of dry soil were estimated for each sample (for both sampling campaigns) using the real-time PCR conditions as determined above. The soil DNA optimal concentration to use for the real-time PCR assay was also tested. DNA standard curves were obtained from 10-fold serial dilutions of *V. dahliae* TdL 05-833 DNA (2 ng μl^{-1}) and were performed in triplicate for each run.

2.11. Data analyses

Cq values for each qPCR reaction were calculated using the second derivative maximum method developed by Rasmussen (2001). The *V. dahliae* ITS copy number was then determined by constructing the calibration curve that related Cq to known quantities of *V. dahliae* DNA. Amplification efficiencies (ε) were calculated from the slope (s) of the standard curve using $\varepsilon = (10^{-1/s^{-1}})$. Distribution of the *V. dahliae* ITS copy number was tested for normality using Shapiro-Wilk test and for variance homoscedasticity using the Levene test. To compare the *V. dahliae* ITS copies number quantified in artificially inoculated soil with the expected values from msc suspensions, Student's *t*-test or a non-parametric Wilcoxon test was used depending of the results of normality and homoscedasticity tests. Comparison of *V. dahliae* ITS copies number between farming systems were performed using a non-parametric Kruskal-Wallis test; between sampling date with a non-parametric Wilcoxon test for apparied data. Spearman's rho correlation was used to describe associations between pathogen densities and soil chemical characteristics. All tests were performed with R software (R Development Core Team, 2004) and statistical significance was set at $p < 0.05$.

3. Results

3.1. ITS primers specificity and real-time PCR assay sensitivity on pure strains

Based on alignment and comparison of ITS sequences from the different *Verticillium* species (from Genbank, NCBI), 2 distinct regions specific to *V. dahliae* were identified and used to generate PCR primers [Vd-ITS1-45-F and Vd-ITS2-379-R]. *In silico* analysis showed that the designed ITS primers were specific enough to distinguish *V. dahliae* from others *Verticillium* species, particularly from *V. alboatrum*, *V. tricorpus*, *V. alfalfae*, *V. non alfalfae* and *V. longisporum*, excepting lineage A1/D3 described as containing *V. dahliae* ITS region (Diehl et al., 2013; Inderbitzin et al., 2011). To confirm, the possibility of cross-amplification for some *Verticillium* spp. isolates was also checked by PCR amplification with the Vd-ITS1-45-F and Vd-ITS2-379-R primer set for available strains. Only *V. dahliae* strains showed positive amplification of a 334-bp amplicon with *V. dahliae* ITS primers, whereas all strains were amplified with the universal primer set ITS1-ITS4 (Table 1).

As previously described, all *V. dahliae* strains were positively amplified when performing the real-time PCR assay. As expected, a similar positive amplification was noticed only for *V. longisporum* A1/D3. The melt curve analysis of *V. dahliae* amplicon indicated the presence of a single amplification product with a specific melting temperature (Tm) of 88 °C. PCR efficiency always exceeded 90% over six orders of magnitude of *V. dahliae* DNA concentration with a linear relationship between log of the DNA concentration and Cq value with a coefficient of determination $R^2 = 0.9993$ and a $P < 10^{-6}$. The lowest *V. dahliae* DNA concentration accurately and reproductively detected was 2 fg μl^{-1}, i.e. 4 fg of DNA by PCR reaction.

3.2. Estimation of rDNA copy number in V. dahliae strains

As described by Herrera et al. (2009) or Bilodeau et al. (2012), the estimation of the rDNA copy number in fungal strains could be based on the comparison of the mean Cq value obtained with the real-time PCR amplification of single-copy genes and the multi-copy target region. Several genes in *V. dahliae* genome have been considered to be reliable single-copy, such as endochitinase, glyceraldehyde-3-phosphate dehydrogenase (G3PD) and β-tubulin (Atallah et al., 2007; Bilodeau et al., 2012). Because amplification efficiencies obtained for these PCR targets [91.1%, 98.7%, 93.5%, 90.5% and 85.5% for ITS, endochitinase, G3PD and the two

Table 4
Mean values of the basic physico-chemical characteristics for the studied soils. Total (C) and organic carbon (C_{org}), nitrogen (N), pH and cation exchange capacity (CEC) were determined according to Forster (1999).

	C_{org} (mg g^{-1} of dry soil)	N (mg g^{-1} of dry soil)	C/N	pH	CEC ($cmol^{+}$ kg^{-1} of dry soil)
Grassland	27.82	2.40	13.31	6.03	13.76
Crop field					
Conv	10.40	1.04	10.16	7.17	11.66
Int RTRC	10.25	1.01	10.34	7.93	11.51
Int TRC	10.07	0.95	11.01	7.70	10.27
Org	9.56	0.90	10.94	7.00	9.89

β-tubulin regions, respectively] showed some differences, all copy numbers calculated are only approximations. The average Cq differences between the ITS region and each individual single-copy gene were between 6.06 and 6.82 (Table 5), with a mean of 6.34 and a low standard variation of 0.68. The ITS copy number was thus estimated to 64 copies per genome of *V. dahliae*. The number of copies per genome ranged from 31 to 99 (Table 5). The estimated ITS copy number for the *V. dahliae* strains 05-837, IN1047, DS1072, 05-773, 05-833 and 05-828A isolated from flax tissues tended to be higher than for the 2 other strains (respectively 31 and 39 for isolates 1015 and 812.97) (Table 5).

3.3. Real-time PCR assay in soil and validation of PCR conditions

To determine the optimal PCR parameters to accurately quantify *V. dahliae* microsclerotia in the soil, artificial inoculations of soil with a known quantity of microsclerotia were performed. A step-by-step process was then applied to optimize the procedure. Because microsclerotia are multicellular, a direct link between the number of microsclerotia and the number ITS copies cannot be *a priori* postulated. Therefore, the ITS copy number of *V. dahliae* measured for inoculated soil samples were compared with that measured for an identical quantity of microsclerotia from the same suspensions used for inoculation. All real-time PCR assays were performed using DNA standard curves obtained from tenfold dilutions of *V. dahliae* 05-833 isolate which was used for artificial inoculation.

The final optimized PCR reaction mixture consisted of the following (at a final volume of 25 μl): 2 μl DNA, 1 X LightCycler® 480 DNA SYBR Green I Master (Roche, Basel, Switzerland), 0.4 μM of each primer, 0.5 mg ml^{-1} BSA and 4 ng μl^{-1} T4 gp 32. The optimal amplification protocol consisted in an initial denaturation step at 95 °C for 5 min, followed by 50 cycles of 30 s at 95 °C, 30 s at 60 °C, 20 s at 72 °C and a final step of 30 s at 82 °C for fluorescence measurement. Using these PCR conditions, efficiency always exceeded 89 % over a six orders of magnitude of *V. dahliae* DNA concentration. The method proved to be highly reproducible between sample replicates and between users. No significant differences were found between *V. dahliae* ITS copy number quantified for the DNA extracted from each inoculation modality and the DNA directly extracted from microsclerotia suspension ($P = 0.888$, 0.306 and 1 respectively for 1000, 100 and 10 microsclerotia g^{-1} of soil) (Table 6). Similarly, the addition of DNA soil background to the DNA extracted from the microsclerotia suspension did influence the result ($P > 0.2$) (data not shown).

3.4. Application of the real-time PCR assay in agricultural soils

In March, *V. dahliae* was detected in only one or two soil samples per field (Fig. 1) for Conv and Int RTRC. *V. dahliae* inoculum densities were slightly higher in Conv (between 1.21 10^4 and 1.83 10^5 ITS copy number g^{-1} dry soil) than in Int RTRC (between 2.45 10^3 and 7.17 10^4 ITS copy number g^{-1} dry soil). *V. dahliae* could not be detected in one conventional field (Fig. 1, field 1). In Org and Int TRC, *V. dahliae* was detected more consistently in all sampled fields (from one to five soil samples). Measured *V. dahliae* inoculum densities were respectively between 7.73 10^3 to 1.12 10^5 and 1.02 10^4 to 2.85 10^5 ITS copy number g^{-1} dry soil for Int TRC and Org. The density of *V. dahliae* inoculum tended to higher in Org, with a significant difference when compared to Int RTRC ($P = 0.024$). In grassland, the density of the pathogen was low and detected only in one soil sample per site. *V. dahliae* inoculum were 9.34 10^1, 4.57 10^3 and 1.11 10^4 ITS copy number g^{-1} dry soil for integrated, conventional and organic systems, respectively (Fig. 1). No significant difference was seen between the repeated soil sampling in October and the first one in March.

Whatever the crop management considered, *V. dahliae* was always detected in fields when flax or potatoes were the previous crop (Fig. 1). No clear pattern of inoculum density could be emphasized according only to the occurrence of flax or other susceptible hosts in crop rotation (Table 3). For example, in the field 2 for Int RTRC, a low density of *V. dahliae* was detected in only

Table 5
Determination of ribosomal DNA (rDNA) copy number per genome for *V. dahliae* isolates. In bold, *V. dahliae* isolate used for artificial inoculation of soil and for standard in all real-time PCR reactions.

Isolate	Difference between Cq obtained for *V. dahliae* ITS and for single-copy gene region				Mean C_t diff (± SD)	Copy number
	Endoch-ITS	βtub1-ITS	βtub2-ITS	G3PD-ITS		
05−837	6.14	6.26	7.02	6.79	6.55 ± 0.42	70
IN 1047	5.77	5.8	6.54	6.3	6.1 ± 0.38	52
DS 1072	6.64	6.45	7.18	6.84	6.78 ± 0.31	81
05-773	6.76	6.86	7.72	7.06	7.1 ± 0.43	99
05-883	**6.56**	**6.6**	**7.59**	**6.8**	**6.89 ± 0.48**	**86**
05-828A	5.9	6.11	6.96	6.2	6.29 ± 0.46	59
1015	4.92	5.33	5.71	5.34	5.32 ± 0.32	31
812.97	5.82	5.68	5.87	5.27	5.66 ± 0.27	39
Between isolates Mean Ct diff (± SD)	6.06 ± 0.6	6.14 ± 0.5	6.82 ± 0.74	6.33 ± 0.69	6.34 ± 0.68	64.6

Table 6
V. dahliae ITS copy number recovery for *n* microsclerotia (msc) after artificial inoculation in soil compared to same quantity of msc in suspension, determined with the final optimized real-time PCR assay.

Modality (*n* msc)	Mean *V. dahliae* ITS copy number for *n* msc (± SD)		P
	Soil sample	Msc suspension	
1000	$1.3\ 10^6 \pm 1.83\ 10^5$	$1.29\ 10^6 \pm 2.03\ 10^5$	0.888 (ns)
100	$2.86\ 10^5 \pm 6.69\ 10^4$	$3.01\ 10^5 \pm 1.45\ 10^4$	0.306 (ns)
10	$1.03\ 10^4 \pm 9.85\ 10^3$	$1.06\ 10^4 \pm 2.61\ 10^3$	1.000 (ns)

P: p value calculated from Student's *t*-test or a non-parametric Wilcoxon test.
ns: not significant.

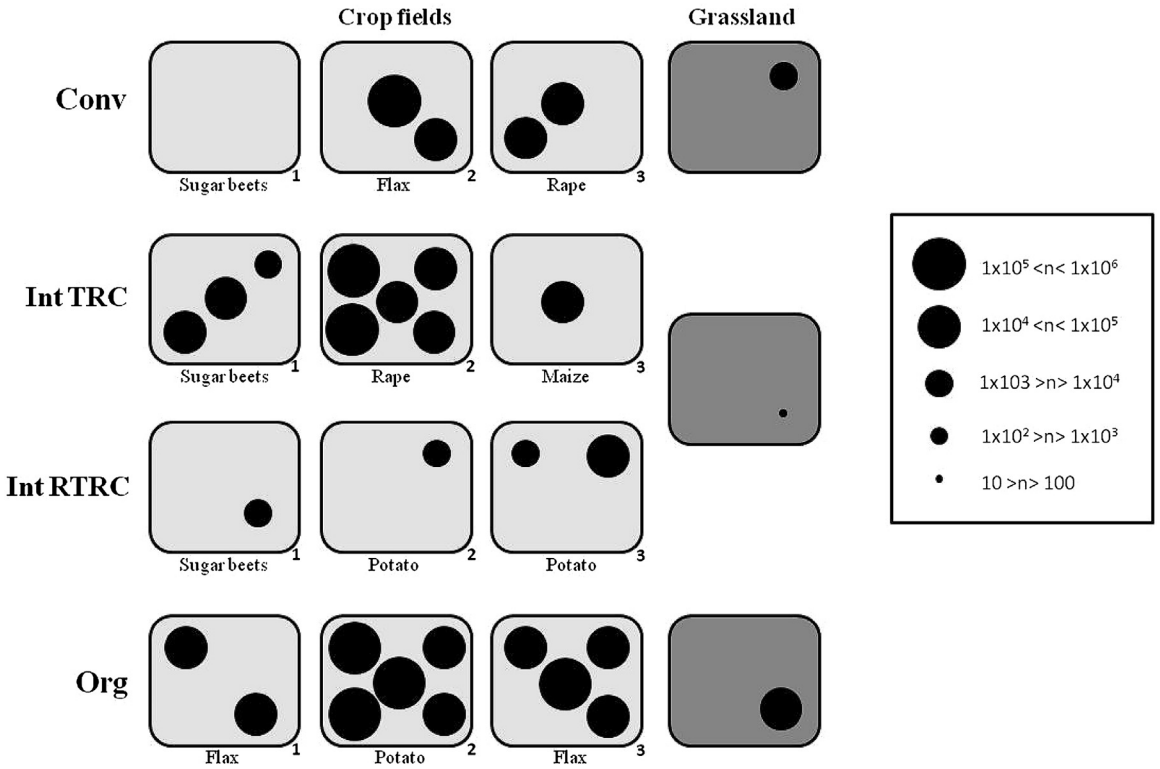

Fig. 1. Schematic representation of *V. dahliae* inoculum density (ITS copy number g^{-1} of dry soil) measured in soil sampled in March 2012, in 3 crop fields and permanent grassland, for each studied agricultural management: conventionnal (Conv), integrated with tillage (Int TRC) and without tillage (Int RTRC), organic (Org). Previous crop is pointed out under each depicted field. Black circles are proportional to ITS copy number per gram of dry soil as showed in the legend.

one sample (Fig. 1), despite three susceptible host plants were cultivated in the last 10 years (Table 3). Similarly, *V. dahliae* was not detected or in low quantity in the field 3 for Int RTRC (Fig. 1) while two susceptible plants were cultivated in 2007 and 2011 (Table 3). On the contrary, fields 1 and 2 for Int TRC presented higher pathogen densities for a similar scenario (with respectively two and one previous occurrence of flax culture in the rotation). No clear link could be highlighted between measured density and time elapsing since the last host culture. *V. dahliae* was also detected in a plot without any known host culture since 10 years (Fig. 1, Conv, field 3).

No significant correlation was observed between *V. dahliae* densities and each soil chemical characteristics (data not shown)

4. Discussion

4.1. Real-time PCR assay

In this study, a real-time PCR assay based on a multi-copy genetic target has been used to evaluate the stock of *V. dahliae* microsclerotia in soil. PCR primers designed in the ITS region and the stringency of PCR procedure led to species-specific amplification when tested against a range of different *Verticillium* species as well as other fungi commonly found in soil. This procedure appeared to be specific enough to amplify *V. dahliae* ITS, excluding a majority of close species. In particular, no PCR amplification was observed with *V. alboatrum* and *V. tricorpus*, 2 species known to be difficult to distinguish from *V. dahliae* according to the colony morphology when grown on artificial medium (Debode et al., 2011). *V. dahliae* and *V. longisporum* are hardly distinguishable since the first is the parent of two of the three lineages of the second, and thus show sequence homology (Inderbitzin et al., 2013). No PCR amplification was observed for *V. longisporum*, except for the lineage A1/D3 as expected. This has already been described and accepted in Diehl et al. (2013), who successfully used ITS targets.

The developed SYBR Green PCR assay allowed for the quantification of as few as 4 fg of *V. dahliae* isolate DNA under the theoretical threshold of one *V. dahliae* genome. This threshold is comparable or even lower than those observed in the literature (Atallah et al., 2007; Banno et al., 2011; Bilodeau et al., 2012). This depth of analysis could not have been reached with a mono-copy target. Therefore, this real-time PCR assay represents an acceptable

specificity towards *V. dahliae* and an interesting sensitivity potential. When targeting ITS for quantification, prior knowledge of their number of copies per genome and the possible variation among isolates of the studied species are both required. By comparing PCR amplification of ITS regions and those for single-copy genes, 31–99 copies of ITS were estimated per haploid genome according to the eight isolates used in this study. Such an approach to determine the approximation of isolate rDNA copy number have already been used for *V. dahliae* (Bilodeau et al., 2012), other fungi ((Herrera et al., 2009; Howlett et al., 1997)) and oomycete (Gangneux et al., 2014). The observed variation range is similar to what is described for other fungal species. The ITS copy numbers of strains isolated directly from flax were higher (between 52 and 99 copies) than those observed for strains isolated from other plants (31 and 39 copies). These results confirm that ITS copy numbers can vary substantially between isolates, as has already been described for *V. dahliae* by Bilodeau et al. (2012). *V. dahliae* ITS copy numbers found in the present study reached higher values than those observed by Bilodeau et al. (2012), who worked on 8 isolates originated from United States, 1 from Israel and 2 from Spain. This difference may be due to the geographic and/or plant host origin of *V. dahliae* isolates. Bilodeau et al. (2012) showed that the impact of the difference among isolates in ITS copy number per genome on the accuracy of the quantification procedure are negligible because of the high correlation observed between the molecular quantification and microsclerotial plate counts of *V. dahliae* in field soils. Similarly, Gangneux et al. (2014) showed a low predicted statistical error margin despite possible 3-fold variation in ITS copy number. Observed differences in pathogen densities discussed here as significant presented generally a log-fold difference, while observed variation of the copy number between local isolates could reached at most a 2-fold difference (from 52 to 99 ITS copy numbers). However, this variation has to be taken in consideration when pathogen densities are compared. It could be interesting to study more precisely and to a broader scale the genetic diversity, and particularly the copy number of ITS regions, of local *V. dahliae* strains isolated from contaminated flax in Normandy.

4.2. Validation of the method for soil matrix

Applying real-time PCR assay in soil involves taking into account specific characteristics and difficulties linked to this complex matrix with the possible presence of many enzyme inhibitors. During DNA extraction, various additional substances may be co-extracted, e.g. humic acids, leading to inhibition of PCR amplification (Matheson et al., 2010). However, real-time PCR seems to be more robust compared to classic PCR in dealing with soil inhibitors, which primarily affect the later cycles of amplification (Mumford et al., 2006). Numerous optimizations are often necessary on the composition of the PCR reaction mix or on amplification cycles to obtain a method for the accurate quantification in soil. Here we used an approach through the artificial inoculation of soil. Because *V. dahliae* was stored in soil in microsclerotia form, these specific fungal structures were used for artificial inoculation to closely mimic possible environmental conditions. Because of the multicellular nature of microsclerotia, the measured ITS copy number in the soil was always compared to expected values obtained from microsclerotia suspension. The validated reaction mixture included the use of two PCR additives, BSA and T4 gene 32, known to optimize PCR efficiency, specificity and capacity to remove PCR inhibition (Matheson et al., 2010). Regarding thermal cycling conditions, a supplemental final step was added because melting profiles occasionally presented another peak with a melting temperature (Tm) of 79 °C compared to the target amplicon with a Tm of 88 °C. This unspecific product was most likely a primer dimer because it could not be detected by agarose gel electrophoresis (data not shown). Furthermore, such peaks were only observed for samples without or with a very low concentration of *V. dahliae* DNA template. To avoid taking account of this unspecific product in the quantification assay, the fluorescence signal of the target amplicon was measured during the last additional step at 82 °C. At this temperature, the unspecific product is denatured, and only the *V. dahliae* amplicon can be detected. Such observations and the supplemental step for fluorescence measurement have already been described and used in many studies (Jiménez-Fernández et al., 2010; Mercado-Blanco et al., 2003; Weng et al., 2005). Final real-time PCR procedure allowed detecting *V. dahliae* densities in inoculated soil comparable to expected values from microsclerotia suspension. The procedure showed high reproducibility with acceptable efficiencies (>89%).

In addition to the PCR assay, the DNA extraction procedure from the soil also represents an important step for accurate quantification. According to our results, the Fast DNA spin Kit for Soil (MP-Biomedicals, Santa Ana, CA, USA) was adequate in recovering the majority of the inoculum. In native soil samples, combining direct DNA extraction from soil and real-time PCR assay could also lead to possible overestimation through the quantification of DNA from degraded cells, non infective hyphae or non viable microsclerotia. Nevertheless, it is generally assumed that extracellular DNA is rapidly degraded in moist soil by microorganisms (Lievens et al., 2005). Same postulates could be formed regarding *V. dahliae* hyphae, assuming that only microsclerotia are considered to be persistent in soil. Similarly, a correlation observed between the molecular results and plate counts of viable microsclerotia suggests that quantification of non-viable structures must be considered as negligible (Bilodeau et al., 2012). Most DNA extraction kits require a low quantity of soil, generally 0.5 g per extraction. With low inoculum densities, such a small amount of soil may actually affect the accuracy of the quantification, strictly due to the statistic likelihood of being confronted with the inoculum. Combining our approach with the concentration of microsclerotia by density flotation (Debode et al., 2011) or a method of DNA extraction processing from greater amounts of soil should be considered to improve pathogen quantification in case of low density inocula.

Finally, the method used in this study appears to be another accurate and rapid way to evaluate the *V. dahliae* microsclerotia reservoir in agricultural soil. This tool could be helpful for assessing *Verticillium* risk before sowing as a decision support for flax growers. However, establishing if a significant direct correlation between the abundance of *V. dahliae* populations in soil and disease development, as establish for other crops, would be essential. The challenge would consist of determining a threshold for *V. dahliae* inoculum density above which *Verticillium* wilt development is inexorable. This diagnostic test could also allow for the following of *V. dahliae* dynamics in soil, for example in response to crop management or to evaluate the effectiveness of control measures, such as crop rotation.

4.3. Comparison of V. dahliae densities in 4 contrasted crop management

The used quantification method has been applied to a variety of agricultural soils sampled from 4 contrasted management systems: Conv, Int RTRC, Int TRC and Org.

Measured *V. dahliae* inoculum densities presented an intra-parcel heterogeneity across the 5 soil samples collected in each studied field. Spatial patterns of soilborne pathogens are often more or less aggregated, according to the considered organisms (Campbell and Noe, 1985). Some studies showed the aggregated

distribution of plants affected by *Verticillium* wilt in natural fields, postulating same pattern for inoculum in soil (Johnson et al., 1988; Navas-Cortes et al., 2008). Johnson et al. (1988) and Xiao et al. (1998) demonstrated that microsclerotia of *V. dahliae* occur in clustered aggregated patterns in potato and cauliflower fields, respectively. In the case of a monocyclic pathogen, the knowledge of the amount and the spatial distribution of soilborne inoculum are essential for disease management. This characteristic should be considered for designing sampling strategy to determine the "health status" of soil using real-time PCR assay.

Observed levels of *V. dahliae* density in studied fields were probably due in part to random events but also as a possible result of crop management. Agricultural practices are well known to influence pathogen abundance and disease development (Ghorbani et al., 2010; Krupinsky et al., 2002; Smiley et al., 2013). Results showed a difference of the inoculum load between cropping systems with or without repeated deep tillage. Several studies reported that tillage could have an influence on incidence and severity of some soilborne diseases, and/or inoculum densities. The final effect of tillage on plant disease widely depends on the pathosystem considered, according to its life cycle and infection processes, but also because of competition with other microbial populations or possible release of phytotoxins during plant residue degradation (Duressa et al., 2012; Mueller et al., 2002; Paulitz, 2000; Quiroz et al., 2008; Sturz et al., 1997; Tinline and Spurr, 1991). Quiroz et al. (2008) measured lower density of *V. dahliae* microsclerotia in parcels with no tillage. They argued that the increase of the pathogen pool in system with tillage could result from the burial of infected plant residues, with high concentration of inoculum. Considering the life cycle of *V. dahliae*, burial of infected crop residues would also favor pathogen development contrary to other fungal pathogen such as *Sclerotinia* (Mueller et al., 2002) or *Fusarium* (Dill-Macky and Jones, 2000).

The previous crop species had also to be considered with attention and was probably the main factor influencing pathogen density. Obviously, the cultivation of a susceptible host may lead to an increase of *V. dahliae* inoculum (Duressa et al., 2012; Mol et al., 1995; Toledo-Souza et al., 2008). But in a broader vision, it is rather the entire crop succession that has to be considered. Crop rotation including several known hosts of the pathogen presents a higher risk to increase an initial *V. dahliae* inoculum. Using crop sequence as a tool for disease management has drawn increasing attention (Hao and Subbarao, 2006; Xiao et al., 1998). Long rotations, avoiding *V. dahliae* host crops, were often reported to reduce both level of pathogen in soil and subsequent infection of susceptible crops (Diehl et al., 2013; Harrington and Dobinson, 2000; Mol et al., 1996). The four cropping systems studied here used long crop rotations with flax cultures every 5–10 years and could also include potatoes cultures. The high observed pathogen load in two fields for Int TRC, although previous crops (sugar beet and colza) were not susceptible hosts to our knowledge, could illustrated the long survival of *V. dahliae* microsclerotia in soil (up to 14 years, Klosterman et al., 2009) after flax culture. However, neither occurrence nor time interval since the last culture of a susceptible host (flax or potatoes) allowed explaining alone the *V. dahliae* density pattern observed in this study. Application of systematic broad-spectrum fungicides in conventional and integrated systems could also have an impact on *V. dahliae* load. Even if the fungicide is used towards other targets, it could also affect this pathogen. Finally, pathogen load observed in all fields was probably the results of all combined factors from soil characteristics, crop management techniques, such as tillage, to crop rotation.

In this study, the presence of non-negligible density of *V. dahliae* was proved in a field where no host plants had been cultivated since 10 years. This pathogen load illustrates the cosmopolitan aspect of this species and again the long survival of microsclerotia in soil, sometimes even at levels that will cause significant damages (Fradin and Thomma, 2006; Klosterman et al., 2009; López-Escudero et al., 2007; Xiao et al., 1998). It may also be the consequence of a possible introduction of the pathogen from field to field via contaminated farm equipment with infected plant residue or seeds (Göre et al., 2011).

The authors are aware that all conclusions on *V. dahliae* densities according to crop management systems have to be considered very carefully. The particular cases of each agricultural management types studied here may not necessarily reflect what would be observed in other farms practicing same system. Furthermore, because each farmer had their own specific crop management techniques, some differences in field history were inevitable, particularly concerning crop rotation and so the nature of the previous year's crop.

5. Conclusion

Crop yield losses caused by disease due to *V. dahliae* could have important economic effects on growers of fiber flax. Because neither chemical solutions nor flax resistant varieties are available to date, pathogen avoidance appears to be as the most pertinent to limit disease incidence on flax culture. When diseases are caused by telluric microorganisms, accurate quantification of pathogen inocula in soil would represent a timely and appropriate way to give guidance regarding following cultivation practices (avoidance, choice of resistant crops or adaptation of plant protection treatments when available). So this study presented an optimized real-time PCR method for direct detection and quantification of *V. dahliae* in soil samples. This bioassay was successfully applied for the estimation of inoculum load in natural fields for the comparison of four crop management systems. This real-time PCR method, measuring inoculum density in soil, may be useful in order to estimate the "health status" of soil and so risk of *Verticillium* wilt on flax. The relationship between *V. dahliae* density in soil and *Verticillium* wilt severity on flax has to be further studied now. Besides risk diagnosis and disease prediction, this tool may also be used to evaluate the effect of specific disease management policies on inoculum load. In particular, it could be useful to be able to determine optimized crop rotation resulting in a reduction of microsclerotia in soil and a concomitant reduction of wilt development on flax. In the long term, the objective would be to propose preventive diagnosis as an alternative for pathogen management and a decision support tool for farmers.

Acknowledgements

This study was supported by the project AlterAgro, call of the "Réseau de Recherche VASI" funded by the "Conseil Régional de Haute-Normandie" and the French "Ministère de l'Enseignement Supérieure et de la Recherche" through a post-doctoral grant. This study was also funded by The European Regional Development Fund (ERDF) allocated by the European Union. The authors are grateful to the French agricultural cooperative "Terre de Lin", INRA Dijon (UMR Agroécologie) and University of California for free donation of *Verticillium* isolates. We would also generously thank all farmers that participated to our study by providing access to their fields and crop history.

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