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Culture-based Determination of *Verticillium* Densities in Soil Overestimates Disease Pressure of Verticillium Wilt of Potato in Manitoba

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Abstract
Verticillium wilt affects potato fields in Manitoba where 80% of the fields are planted to the moderately susceptible cultivar, Russet Burbank. An accurate determination of *Verticillium dahliae* inoculum in soil is critical for disease management. In this study, we investigated the presence of microsclerotia-producing *Verticillium* species in potato fields in Manitoba and compared published quantitative real-time PCR assays for *V. dahliae, V. tricorpus* and *V. longisporum* against a traditional plating method. Selected real-time PCR assays could differentiate and quantify the major microsclerotia-producing species, *V. dahliae*, *V. tricorpus* and *V. longisporum*. Results showed that the presence of *V. tricorpus* caused an overestimation of *V. dahliae* propagule density when using the plating method. As a result, Verticillium wilt severity was negatively related to cfu from traditional plating, while positive with the amounts of *V. dahliae* genomic DNA in soils.

Resumen
La marchitez por *Verticillium* afecta a los campos de papa en Manitoba, donde el 80% de los campos se plantan con la variedad moderadamente susceptible Russet Burbank. Una determinación precisa del inóculo de *Verticillium dahliae* en el suelo es crítico para el manejo de la enfermedad. En este estudio, investigamos la presencia de especies de *Verticillium* productoras de microesclerocios en campos de papa en Manitoba y comparamos los ensayos cuantitativos de PCR en tiempo real publicados para *V. dahliae, V. tricorpus y V. longisporum* con un método de siembra in vitro tradicional. Los ensayos de PCR en tiempo real seleccionados podrían diferenciar y cuantificar las principales especies productoras de microesclerocios, V. dahliae, V. tricorpus y V. longisporum. Los resultados mostraron que la presencia de *V. tricorpus* causó una sobreestimación de la densidad de propágulos de *V. dahliae* cuando se utilizó el método de placa. Como resultado, la gravedad del marchitamiento por verticilosis se relacionó negativamente con la UFC de las placas tradicionales, mientras que fue positiva con las cantidades de ADN genómico de *V. dahlia* en los suelos.

Keywords Propagule density · *Verticillium dahliae* · *Verticillium tricorpus* · Real-time PCR · *Verticillium longisporum* · *Verticillium wilt*

Introduction

Verticillium is a small genus, redefined with *V. dahliae* as the type species (Inderbitzin and Subbarao 2014), and comprised of 10 species, that differ by their morphological features, such as resting structures and capability to induce wilt disease in vascular plants (Inderbitzin et al. 2011). Resting structures in *Verticillium*, are the melanized hyphae, the short chains of rounded brown cells called chlamydospores, and the clusters of rounded and dark cells known as microsclerotia (Issac 1953; Pegg and Brady 2002). The presence of microsclerotia has been used to differentiate *V. dahliae* Kleb. and *V. longisporum* (C. Stark) Karapapa from other species in the genus, while *V. tricorpus* I. Isaac, *V. zaregamsianum* Inderb. et al. sp. nov., *V. isaacii* Inderb. et al. sp. nov., and *V. klebahnii* Inderb. et al. sp. nov. are recognized by the presence of resting mycelium, microsclerotia and chlamydospores simultaneously (Inderbitzin et al. 2011; Issac 1949; Karapapa et al. 1997).

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Verticillium dahliae can reside in soil as microsclerotia, which allows the fungus to survive for many years and serves as the main infective propagule under field conditions (Wilhelm 1955). The reduction of microsclerotia has become the most suitable means to mitigate this monocyclic pathogen's effect (Kowalska 2021). In a previous study, reduction of Verticillium wilt incidence in cv. Russet Burbank was associated to low *Verticillium* propagule density in soil treated with composted cattle manure. In contrast, untreated plots planted to wheat showed elevated *Verticillium* propagule levels, increased Verticillium wilt incidence in potato and lower potato yield (Molina et al. 2014).

Effective control strategies of *V. dahliae* rely on the precise detection and quantification of the propagule density of the pathogen in the soil (Cohen et al. 2012). Reliable knowledge of the levels of *V. dahliae* in soil that could affect future yields will contribute to more economical and environmentally-sound control. The wet plating method has been available to potato growers as a pre-planting inoculum level assessment. This test is based on the quantification of *Verticillium* microsclerotia forming colonies (cfu) growing on semi-selective medium (Goud and Termorshuizen 2003), which can originate from microsclerotia as well as from other different fungal structures (Butterfield and DeVay 1977). As the test relies upon the formation of microsclerotia colonies, it could overestimate the level of *V. dahliae* inoculum due to the presence of other *Verticillium* species in sampled soils which can produce similar microsclerotia colonies on the medium (Goud et al. 2003). Overestimation of *V. dahliae* could result in higher environmental impact on organisms not targeted and the costs associated to the use of soil fumigants for the control of Verticillium wilt.

A Verticillium wilt survey conducted in commercial cv. Russet Burbank fields in Manitoba indicated that Verticillium wilt incidence ranged from 37 to 79%, with severity from 11 to 33%, and propagule density, obtained with the wet plating method, from 0 to 228 cfu g^{-1} of soil (Bisht et al. 2021). Counts are relatively high compared to other potato growing regions where the propagules estimated through the wet plating method have shown less than 30 cfu g^{-1} soil (Omer et al. 2008; Smith and Rowe 1984). *Verticillium dahliae* can induce damage at relatively low inoculum densities. Soil propagule densities as low as 10 cfu g^{-1} soil have produced enough disease severity to reduce yield significantly (Davis and Sorensen 1986).

Although Manitoba potato fields have shown such high *Verticillium* propagule levels, severity of Verticillium wilt and yield loss have not been consistently related. There is no clear relation between low or high *Verticillium* propagule levels from the wet plating method and the severity of Verticillium wilt in potatoes (Molina et al. 2014). This situation has led us to suspect that *V. dahliae* propagule levels from the wet plating method in Manitoba might be inaccurate.

Advances in molecular biology offer new diagnostic tools to help manage *V. dahliae* in several different pathosystems. Real-time polymerase chain reaction (real-time PCR) (Atallah et al. 2007; Bilodeau et al. 2012; Tzelepis et al. 2017) and, more recently, droplet digital PCR (ddPCR) assays have been made available to quantify *V. dahliae* (Wang et al. 2022). Real-time PCR is a commonly adopted method of quantitative detection of plant pathogens in plant tissues and soil due to its rapid, reliable and sensitive performance (Gachon et al. 2004) and affordable operation compared to costly equipment used with ddPCR. Real-time PCR offers an alternative for more specific and rapid detection of *Verticillium* species in soil than traditional culture-based methods (Debode et al. 2011). Atallah et al. (2007) developed a real-time PCR assay to quantify *V. dahliae* in potato tissue using the designed primer pair VertBt-F and VertBt-R derived from the B-tubulin gene. Bilodeau et al. (2012) developed a TaqMan® real-time PCR assay base on the ribosomal DNA (rDNA) intergenic spacer (IGS) that was used for the quantitative detection of *V. dahliae* in soil samples.

This study aimed to: (i) investigate the presence of *Verticillium* species producing microsclerotia in commercial potato soil in Manitoba, (ii) to quantify *V. dahliae* and other microsclerotia-producing *Verticillium* species possibly present in soil using published real-time PCR assays, and (iii) to determine if the relation of disease severity and *Verticillium* propagule levels from the wet plating method are affected by *Verticillium* species producing microsclerotia.

Materials and Methods

I. Microsclerotia-producing *Verticillium* species in potato field soil

Commercial potato fields were sampled to quantify cfu of *Verticillium* and to identify *Verticillium* isolates using morphological characteristics.

Soil sampling

Commercial potato fields were selected randomly over a broad geographic area from eight Rural Municipalities (RMs) representing the major potato-productive regions of Manitoba, Canada. Sampled fields included three fields planted to the table potato cv. Standard Norland and 14 planted to the processing potato cv. Russet Burbank. Fifteen soil cores were taken from each field. Cores were collected to a depth of 20 cm and bulked into a single soil sample per site. Samples were passed through a 2 mm sieve, cleaned of roots and large plant debris, mixed thoroughly and air-dried at ambient temperature for seven days. For each sample,

200 g of air-dried soil was stored at 4 °C for up to four weeks before the soil was plated on a semi-selective medium to quantify *Verticillium* cfu.

Quantification of cfu of *Verticillium* in soils

The *Verticillium* propagule density for all sampled fields was determined by wet plating 1 g of air-dried soil on semi-selective Sorensen's NP-10 medium (Sorensen et al. 1991) supplemented with Pectin (polygalacturonic acid from orange, Sigma-Aldrich) to estimate *Verticillium* propagule density per gram of dried-soil (cfu) as described by Molina et al. (2014). The number of *Verticillium* colonies producing microsclerotia was counted using a stereo microscope and expressed as cfu.

Verticillium isolates

Verticillium isolates were recovered from soil and symptomatic potato plants collected from 17 commercial potato fields in Manitoba, Canada. To recover isolates from soil, samples were wet-plated on semi-selective Sorensen's NP-10 medium (Sorensen et al. 1991). A single microsclerotia from randomly selected colonies was harvested and placed onto potato dextrose agar (PDA; Difco Laboratories, Spark, MD) plates supplemented with 0.02% streptomycin sulfate. To recover *Verticillium* isolates from potato plants, potato stems sections of 15–20 cm were washed with distilled water and surface sterilized 1 min with 5% household bleach (0.053% Na hypochlorite), dipped 1 min in 95% ethanol, rinsed in distilled water, and blotted dry on sterile absorbent paper. The stems were cut into small 3–4 mm width discs, placed onto the semi-selective medium (Sorensen et al. 1991) and incubated at 23 ± 1 °C in darkness. After seven days, a single microsclerotia was harvested and placed onto PDA plates supplemented with 0.02% streptomycin sulfate. Ten days later monosporic cultures were established from each isolate recovered from soil or potato plant material by sub-culturing single conidia onto a PDA medium.

Morphological characterization and identification of *Verticillium* isolates

Six *V. dahliae*, one *V. tricorpus,* six *V. albo-atrum*, two *Gibellulopsis nigrescens* (formerly known as *V. nigrescens*), and six *V. longisporum* reference isolates, obtained from laboratories across Canada and United States (Table 1), were used to become acquainted with the different *Verticillium* species and to verify the specificity of real-time PCR assays for *V. dahliae*, *V. tricorpus* and *V. longisporum*.

The morphological features of the isolates obtained from soil or plant were examined and compared with the reference isolates *V. dahliae* (Vd1396-9), *V. tricorpus* (DVt3) and *V. longisporum* (VD624). The morphological features examined were the presence of dark melanized mycelium growing throughout the plate, the size (length and width) and shape (round or oval) of microsclerotia, presence and size (length and width) of chlamydospores and the colour, size and shape of the conidia for each isolate. Colony characteristics like colour and occurrence of yellow discoloration of medium were recorded. Measurements were done using the Image-Pro program (Media Cybernetics, Rockville, MD) after taking pictures with a Megapixel Q-Color 3 imaging system (Olympus, Melville, NY) on either a compound microscope or a stereo microscope.

ii. Quantification of V. dahliae and other microsclerotia-producing Verticillium species using real-time PCR assays

Microsclerotia-producing *Verticillium* species found in potato fields in Manitoba were identified and quantified using selected published quantitative real-time PCR assays. A test with soil spiked with *V. dahliae* microsclerotia was used to test the efficiency of the selected real-time PCR assay to detect gDNA extracted from *V. dahliae* microsclerotia in soil.

Soil genomic DNA extraction

Total soil gDNA was extracted from 500 mg of pulverized air-dried soil using the MoBio PowerSoil® DNA Isolation extraction Kit (now called DNeasy PowerSoil™ PRO, QIAGEN). Each sample was processed with this kit using a modified protocol from that of kit instruction to improve DNA yield. The cell disruption step was modified using a mini-bead beater-24 (BioSpec Products, Bartlesville, OK) at 3450 strokes min^{-1} for three minutes. The quality and concentration of gDNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). Extracted gDNA was then kept at -20 °C until used in PCR amplifications.

The identification of the isolated fungi based on morphological characteristics was confirmed using the ribosomal RNA intergenic spacer (IGS) sequences. Genomic DNA (gDNA) extraction from 300 mg of mycelium followed the protocol for fungal gDNA extraction described by Mahuku and Platt (2002). IGS region of each isolate was compared with those previously submitted to the National Center for Biotechnology Information (NCBI) (Qin et al. 2006). The IGS sequences were amplified by PCR using the primers VdIGSF1 (5' GGGTCCTGTAAG CAGTAG 3') and VdIGSR1 (5' GAGCCATTCGCAGTT TCG 3') (Qin et al. 2006). Primers were synthesized by IDT (Integrated DNA Technologies, Coralville, IA).

Table 1 Reference isolates of *Verticillium* species used in this study, and verification of specificity of real-time PCR assays for *V. dahliae*, *V. tricorpus* and *V. longisporum*

Code	Host/ Source	Province/State	Year isolated	Real-time PCR assays [f]			Morphological and molecular identity verification [g]
				V. dahliae	*V. tricorpus*	*V. longisporum*	
V_104b[a]	Potato	PEI, CA	2006	-	-	-	*V. albo-atrum*
107724[b]	Potato	Ontario, CA	1965	-	-	-	*V. albo-atrum*
177115[b]	Potato	PEI, CA	1980	-	-	-	*V. albo-atrum*
216604[b]	Potato	PEI, CA	1992	-	-	-	*V. albo-atrum*
AT3[c]	Potato	Ontario, CA	1986	-	-	-	*V. albo-atrum*
Vd1396-9[a]	Potato	Manitoba, CA	2006	+	-	-	*V. dahliae*
241194[b]	Tomato	BC, CA	2010	+	-	-	*V. dahliae*
Dvd-E6[c]	Eggplant	Ontario, CA	1993	+	-	-	*V. dahliae*
Dvd-3[c]	Potato	Ontario, CA	1993	+	-	-	*V. dahliae*
Dvd-P2[c]	Potato	Ontario, CA	1995	+	-	-	*V. dahliae*
Dvd-S100[c]	Soil	Ontario, CA	1996	+	-	-	*V. dahliae*
226890[b]	Soil	Ontario, CA	1998	-	-	-	*G. nigrescens*
226891[b]	Soil	Ontario, CA	1998	-	-	-	*G. nigrescens*
Dvt-3[c]	Unknown	Ontario, CA	1999	-	+	-	*V. tricorpus*
VD624[d]	Cauliflower	California, USA	Unknown	-	-	+	*V. longisporum*
VD348[d]	Cauliflower	California, USA	Unknown	-	-	+	*V. longisporum*
243377D[e]	Canola	Manitoba, CA	2014	-	-	+	*V. longisporum*
243377E[e]	Canola	Manitoba, CA	2014	-	-	+	*V. longisporum*
243378D[e]	Canola	Manitoba, CA	2014	-	-	+	*V. longisporum*
243378E[e]	Canola	Manitoba, CA	2014	-	-	+	*V. longisporum*

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[f]Evaluation of species specificity of the real-time PCR assays for *V. dahliae (*Bilodeau et al. 2012*)*, *V. tricorpus* (Debode et al. 2011) and *V. longisporum* (Banno et al. 2011). (+) indicates isolate amplified with assay, and (-) indicates not amplification

[g]Identity of the reference isolates was confirmed after morphological attributes (Presence of microsclerotia, resting mycelium and chlamydospores, and conidia size) were analyzed (Description is presented in Supplemental Table 1)

Reactions included 1 × Thermo Scientific DreamTaq Green Buffer (Thermo Fisher Scientific, Waltham, MA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 uM of each of the primers VdIGSF1 and VdIGSR1, 1 U of Thermo Scientific DreamTaq (Thermo Fisher Scientific, Waltham, MA) and 10 ng of gDNA template. PCR amplification was carried out in a C-1000 thermal cycler (Bio-Rad Laboratories, Hercules, CA) programmed for 5 min at 95C, 35 cycles of 1 min at 95 C, 50 s at 60 °C and 2 min at 72 C, followed by 72 °C for 10 min. Aliquotes (5ul) of each PCR product was visualized by electrophoresis using a 1.2% agarose gel containing 0.5 ul of 10,000X GelRed (Biotium, Hayward, CA, USA) dye and visualized on a UV transilluminator GBox (Syngene, Cambridge, UK). PCR products were sent to Macrogen Sequencing Service (Macrogen, Maryland, MD) for sequencing.

DNA sequences were edited and aligned using the DNAStar computer software package (DNAStar Lasergene, Inc., Madison, WI). A BLASTN search of the National Center for Biotechnology Information (NCBI) database was performed to compare the IGS sequences of the isolates with those available online. Sequence contigs were assembled using the SeqMan Pro module of the DNAStar computer software package (DNAStar Lasergene, Inc., Madison, WI).

Real-time PCR primers and assay conditions

Two published real-time PCR assays were evaluated for each of the *Verticillium* species *V. dahliae*, *V. tricorpus* and *V. longisporum* to select the most sensitive and specific real-time PCR assays for the quantification of the *Verticillium*

species (Table 2). The evaluated real-time PCR assays were selected based on their sensitivity and specificity for identification and quantification of the target species in soil and/or potato tissue samples. Real-time PCR amplification was carried out in a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Real-time PCR amplifications were performed using the 5 Prime Real Master Mix without rox (Fisher Scientific, Waltham, MA) for the TaqMan® assays and SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA) for the SYBR® Green assays. TaqMan® probes were synthesized by BioSearch (LGC Biosearch Technologies, Inc., Novato, CA, USA) and primers by IDT (Integrated DNA Technologies, Coralville, IA).

The real-time assay reaction mixture with the TaqMan® probe (25 µL total volume) for *V. dahliae* and *V. tricorpus* was performed as described by Bilodeau et al. (2012). Cycling condition for the TaqMan® assay used with *V. dahliae* was optimized experimentally to increase the specificity of the assay with 2 min at 98 °C followed by 40 cycles of 15 s at 95 °C and 60 s at 68 °C (Table 2). The reaction mixture of the real-time assays performed with the intercalating dye SYBR® Green (20 µl total volume) contained 2 ul of template gDNA dilution (1:10), 200 nM of each primer and 10 µl of SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The conditions for the real-time PCR assays are shown in Table 2. The reactions were run in triplicate for each sample (Technical replication).

Specificity and sensitivity of real-time PCR assays using reference *Verticillium* isolates

The specificity of the real-time PCR assays was assessed using gDNA extracted from 20 reference fungal isolates (Table 1). All fungal isolates used for specificity testing were cultured and subjected to DNA extraction using the abovementioned methods. In order to determine the sensitivity of each real-time PCR assay, the concentration of purified gDNA of the reference isolates Vd1396-9 (*V. dahliae*), DVt3 (*V. tricorpus*), PD624 (*V. longisporum*) was measured individually, using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). Then, ten-fold serial dilutions of the extract were prepared, ranging from 10 or 20 ng μl^{-1} to 10 or 20 fg μl^{-1} of gDNA to test the PCR assay's sensitivity. Each standard curve was generated by plotting the cycle threshold (C_T) values, which are inversely proportional to detected gDNA content, versus the log of concentrations of gDNA (tenfold dilution series) from cultured mycelia. The amount of gDNA for unknown samples was extrapolated from the C_T value and the value obtained from the standard curve.

Once the real-time PCR assays that showed the best sensitivity and efficiency were selected for each *Verticillium* specie, all reference isolates and isolates recovered from soil and potato plants were run with the selected assays. When soil samples were subjected to real-time PCR, relative values for

Table 2 Real-time PCR assays screened for detection of the microsclerotia-producing *Verticillium* species *V. dahliae*, *V. tricorpus* and *V. longisporum*

Target	Chemistry	Primers	Protocol	Reference
VD[a]	SYBR® Green	VertBt-F AACAACAGTCCGATGGAT AATTC VertBt-R GTACCGGGCTCGAGATCG	2 min at 98 °C, 40 cycles of 5 s at 98 °C and 5 s at 60 °C	Atallah et al. (2007)
	TaqMan®	Vd-F929-947 CGTTTCCCGTTACTCTTCT Vd-R1076-1094 GGATTTCGGCCCAGA AACT Probe: [5′ 6-FAM] CACCGCAAGCAGACT CTTGAAAGCCA [3′ BHQ1]	2 min at 98 °C, 40 cycles of 15 s at 95 °C and 1 min at 68 °C	Bilodeau et al. (2012)
VT	SYBR® Green	VtF4 CCGGTGTTGGGGATCTACT VtR2 GTAGGGGGTTTAGAGGCTG	2 min at 95 °C and 40 cycles of 5 s at 95 °C and 5 s at 60 °C	Debode et al. (2011)
	TaqMan®	IGS_VtF1 TAGTAGAATACTAGATARCTAG IGS_VtR1 AGCCTAGGTCTTTATAGCTAG Probe: [CAL FluorRed 610] TCCTACTAA TACCTTACTATAACTCTTAAGGTA [BHQ2]	90 s at 95 °C and 40 cycles at 15 s at 95 °C and 30 s at 57 °C	(Bilodeau et al. 2012)
VL	SYBR® Green	VlTubF2 GCAAAACCCTACCGGGTTATG VlTubR1 AGATATCCATCGGACTGT TCGTA	10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C	Debode et al. (2011)
	SYBR® Green	Vlsp-F1 AGCCTGAGTCACGAGAGATAT GGG Vlsp-R4 CAAACCACGCCACTGCATTCT CGT	2 min at 98 °C and 40 cycles of 5 s at 98 °C and 5 s at 60 °C	(Banno et al. 2011)

[a]Target organisms: *Verticillium dahliae* (VD), *V. tricorpus* (VT), *V. longisporum* (VL)

target abundance in each soil sample were extrapolated from the standard curve generated from gDNA extracted from reference isolates. All real-time PCR assays were monitored on a CFX96™ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). All real-time PCR reactions were repeated three times for each sample and always included a positive control accordingly (DNA from either *V. dahliae* or *V. tricorpus* or *V. longisporum*), and a negative control (NTC: no template control). In order to confirm the presence and interference of non-target fungal gDNA and PCR inhibitors in the real-time PCR assays, any soil gDNA sample of C_T value higher than 36 (TaqMan®) or 34 (SYBR® Green) or had no amplification at all was spiked with gDNA of the target organism at a concentration of 1 ng μl^{-1} and re-run.

Efficiency of real-time PCR assay to detect gDNA *V. dahliae* microsclerotia in soil

To determine whether the presence of total soil gDNA affected the efficiency of the selected real-time PCR assay to detect gDNA extracted from soil with *V. dahliae* microsclerotia, a test was performed using spiked soil with microsclerotia of *V. dahliae*. For this purpose, microsclerotia of *V. dahliae* was produced in the laboratory using the reference isolate Vd1396-9. The fungus was grown on semisolid Czapek- Dox medium supplemented with Pectin (polygalacturonic acid from orange, Sigma-Aldrich) in the dark at 24 °C (Hawke and Lazarovits 1994). After three weeks of incubation, the culture turned black due to the presence of microsclerotia, then it was poured through mesh screens to obtain microsclerotia 75 to 106 µm in diameter. Then, microsclerotia was spread and separated with the help of a micro-spatula on a Petri plate to help individual counts under a stereo microscope. Soil with no detectable endogenous populations of *V. dahliae* was autoclaved and air-dried for 7 days. Then, 5, 25, 50, 100, 150, and 250 individual *V. dahliae* microsclerotia per gram (Vppg) were thoroughly mixed with the autoclaved field soil. Total gDNA from each spiked and non-spiked soil was extracted from triplicate 500-mg samples using the PowerSoil® DNA Isolation Kit. Real-time PCR reactions were conducted in triplicate using the real-time PCR assay that best performed in the specificity and sensitivity test for *V. dahliae*. A regression was generated to analyze the association between the *V. dahliae* microsclerotia Vppg and the amount of *V. dahliae* gDNA (pg g^{-1}) extracted from the soil with and without microsclerotia.

iii. Relationship between disease severity and *Verticillium* propagule levels from the wet plating method

Soils collected from two commercial fields were used to compare inoculum quantification methods, before two replicated field studies were used to determine the relationship between *V. dahliae* inoculum and Verticillium wilt, respectively.

Wet plating method and real-time PCR quantification of microsclerotia-producing *Verticillium* species in soil

The traditional soil wet plating method was compared against the more specific and sensitive real-time PCR assays examined previously for their species specificity and capability to detect *V. dahliae*, *V. tricorpus* and *V. longisporum*. This study used ten soil samples collected in two commercial potato fields with 30 to 50% incidence of Verticillium wilt. Additionally, a soil sample (Control soil) with no detectable endogenous population of *V. dahliae*, *V. tricorpus* or *V. longisporum*, was included. Quantification of *Verticillium* propagule density (cfu) and extraction of total gDNA from each soil sample were performed four times using the methods described previously. Real-time PCR reactions were conducted in triplicate for each extracted sample.

Relationship between gDNA of *V. dahliae* and development of Verticillium wilt

To determine if the level of *V. dahliae* quantified by the selected real-time PCR assay can be used to predict the severity of Verticillium wilt in the processing cultivar cv. Russet Burbank, two replicated field sites, located on the RM of North Cypress (Site D) and North Folk (Site E), with plots treated with composted pig slurry solids at 20 Mg ha^{-1} (CSS20); 40 Mg ha^{-1} (CSS40) and 80 Mg ha^{-1} (CSS80), supplemental fertilizer and untreated were used. Each site was replicated four times and set up on two different commercial potato fields. Soil samples were collected two weeks before potato seed planting. Propagule density of *V. longisporum*, *V. dahliae* and *V. tricorpus* was estimated as pg of gDNA g^{-1} of soil, using the selected real-time PCR assays described above. Soil gDNA extractions and real-time PCR assays were conducted as described above.

Disease severity was assessed 10 weeks after planting and recorded weekly thereafter until crop was harvested. Symptoms of Verticillium wilt were assessed on a scale 0 to 100%, where 0 = no symptoms and 100% = all foliage senescent or wilted. Fifteen plants were rated individually in each plot. Severity values were converted to the area under the wilt progress curve (AUWPC) using the equation $\sum_i^{n-1} [Y_i + Y_{i+1})/2](t_{i+1} - t_i)$, where Y_i=cumulative disease severity at the i^{th} observation, t_i = time (days after planting) at the i^{th} observation, and n = number of observations (Shaner and Finney 1977).

Statistical analysis Linear regression analyses were performed to determine whether significant relationships

existed between *V. dahliae* gDNA (pg g^{-1} soil) and propagule density (cfu g^{-1} soil) from soil plating and *V. dahliae* microsclerotia g^{-1} spiked-soil (Vppg). The efficiency of each real-time PCR assay, slope and the coefficient of determination (R^2) were automatically calculated by the Bio-Rad CFX manager software v3.0 (Bio-Rad Laboratories, Hercules, CA). The slope and R^2 were determined by quantifying the standards described earlier. R^2 was considered as suitable when no lower than 0.96. The efficiency was considered satisfactory when higher than 90% and lower than 105% (González-Salgado et al. 2009; Pfaffl et al. 2009). The efficiency was used as an indicator of the reproducibility of the real-time PCR assay and it was determined from the slope of the standard curve using the formula: $E = 10^{(-1/\text{slope})}$-1 (González-Salgado et al. 2009). Verticillium wilt severity variability and deviation of plate counting and real-time PCR quantification of *V. dahliae* between samples were analyzed using analysis of variance (ANOVA) with the PROC Mixed procedure (SAS Institute, release 9.2, Cary, NC). The data were analyzed as a one-way ANOVA, with replication as a random effect and treatment as fixed effect. Site means were separated using the Bonferroni's procedure if the F-test was significant ($P < 0.05$). Where data were not normally distributed, appropriate transformations were performed prior to analysis. The relationship between Verticillium wilt severity, *Verticillium* propagule density (cfu), and gDNA quantity of *V. dahliae* and *V. tricorpus* was analyzed using simple regression. All analyses were performed using the Statistical Software SAS (SAS Institute, release 9.2, Cary, NC).

Results

i. Microsclerotia-producing *Verticillium* species in potato field soil

Three microsclerotia-producing *Verticillium* species were found among 82 monosporic cultures from soil, and potato plants from 17 potato fields in Manitoba (Table 3). Direct identification of the *Verticillium* isolates on Sorensen's NP-10 medium plated with soil was difficult due to the similitude in the microsclerotia forming colonies, or for the presence of other fungal and bacterial colonies interfering with the visualization, or the physical perturbation that some colonies suffer before the examination under the microscope from washing the excess of soil from the surface of the agar plates. According to the morphological characterization on PDA medium, the dominant species were *V. dahliae* and *V. tricorpus*, which compromised 68 and 28% of the total isolates (n = 82). *Verticillium dahliae* was frequently recovered from soils and potato plants and represented between 24 to 100% (mean = 78%) of the total isolates obtained from each RM (Table 3). *Verticillium dahliae* isolates were cosmopolitan at all sampled fields. *Verticillium tricorpus* was found in the RMs of Elton, North Cypress and North Norfolk, while *V. klebahnii* and *G. nigrescens* were recovered from fields in the RMs of North Norfolk and Thompson, respectively (Table 3). Interestingly, 68% of the 21 recovered isolates in the North Norfolk RM were *V. tricorpus,* while 24% were *V. dahliae*, 5% *V. klebahnii* and 5% *G. nigrescens*. *Verticillium longisporum* was not found in any of the sampled fields.

Table 3 Number of isolates recovered from potato plants and soil from the eight major potato producing Rural Municipalities in Manitoba

Rural Municipality	*V. dahliae*		*V. tricorpus*		*V. klebahnii*		*G. nigrescens* [a]		*Total*
	Plant	Soil	Plant	Soil	Plant	Soil	Plant	Soil	
Elton	4	3	2	-	-	-	-	-	9
North Cypress	3	17	2	5	-	-	-	-	27
North Norfolk	2	3	2	12	-	1	-	1	21
Portage la Prairie	4	2	-	-	-	-	-	-	6
Dufferin	1	1	-	-	-	-	-	-	2
Thompson	1	-	-	-	-	-	-	1	2
Stanley	1	10	-	-	-	-	-	-	11
Rhineland	1	3	-	-	-	-	-	-	4
Total	17	39	6	17	0	1	0	2	

[a]Not a *Verticillium* species (formerly called *V. nigrescens*)

Morphological characterization and identification of *Verticillium* isolates

Colonies of *V. dahliae* isolates produced on PDA, were initially white and then black, due to the development of microsclerotia (Fig. 1a, d). Microsclerotia were usually globose, oval to elongate (Fig. 1d, e). Hypha was hyaline, and there was not presence of dark resting mycelium (Fig. 1a, b). The microsclerotial size (length x width) ranged from 49.6 to 101 by 35.6 to 78 µm, with average size of 50.4 by 84 µm (Table S1). Conidia were hyaline with rounded apices to oval (Fig. 1b, c, f). The size of the conidia ranged from 1.6 to 4 by 2.5 to 11 µm, with a mean of 2.7 by 5.4 µm (Fig. 1f, Table S1). Chlamydospores were absent.

Colonies of *Verticillium tricorpus* on PDA were initially white and turned to dark brown due to the development of microsclerotia and melanized mycelia (Fig. 2a, b, c). The isolates showed a yellow-orange discoloration around the edges of the colony when growing on PDA after two weeks (Fig. 2a, d). Such discoloration was not always present in colonies growing on Sorensen's NP-10 medium. The presence of dark mycelia was very noticeable (Fig. 2a, d) connecting or not with the microsclerotia when growing on PDA (Fig. 2c). Microsclerotia were usually rounded or elongated ranged from 43.3 to 94.5 µm by 73 to 154 µm, with an average size of 64.7 by 100.8 µm (Fig. 2c, f). Conidia was hyaline and rounded to oval, ranging from 2.3 to 4.6 µm by 3.4 by 11.2 µm, with a mean size of 3.3 by 5.7 µm (Fig. 2c, d, Table S1). Chlamydospores were present with a mean size of 6.6 by 7.9 µm (Table S1) and were usually found at the same time with microsclerotia and dark hyphae (Fig. 2c, e, f).

Although *V. tricorpus* isolates were morphologically similar, one was identified as *V. klebahnii* after sequencing (Table 2, S1). A 1.7–1.9 kb fragment of the IGS region of each monosporic isolate was sequenced. The sequences were compared with sequences deposited in the GenBank ID database. The comparison with the database confirmed the identification of the *V. dahliae* and *V. tricorpus* isolates, and allowed the identification of those ambiguous isolates, morphologically identical, such as *V. tricorpus* and *V. klebahnii*. *Verticillium klebahnii* colonies produced on PDA were initially white and then dark brown, due to microsclerotia, chlamydospores and melanized resting mycelium, similar to the *V. tricorpus* isolates. However, the yellow-orange ring on the edges of the colony was not observed. Chlamydospores ranged from 4.5 to 7.6 µm by 8.8 to 10.3 µm, with a mean of 6.6 by 9.5 µm. The conidial size ranged from 2.4 to 3.1 by 3.6 to 8.2, with an average size of 2.8 by 5.6 µm

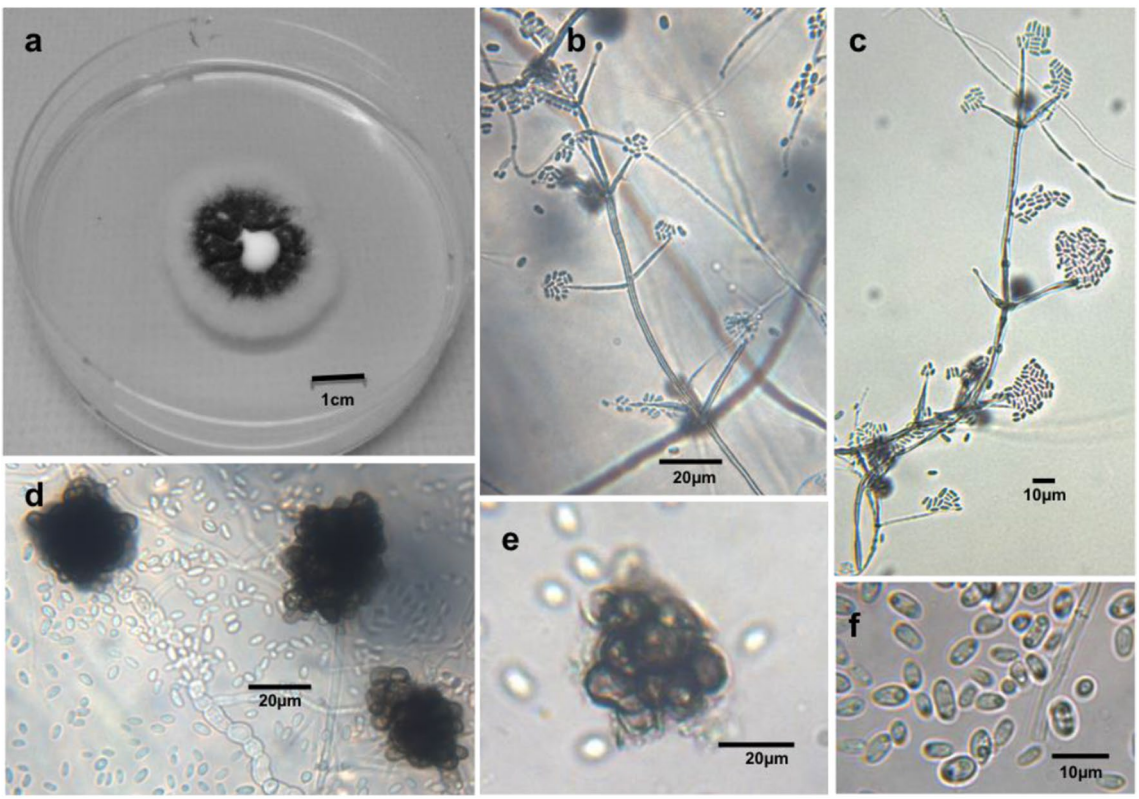

Fig. 1 Morphology of *V. dahliae* isolated from Manitoba fields. (**a**) Colony after 18 days on PDA. (**b**), (**c**) Conidiophore and whorl phialide with conidia. (**d**), (**e**) Microsclerotia. (**f**) Conidia

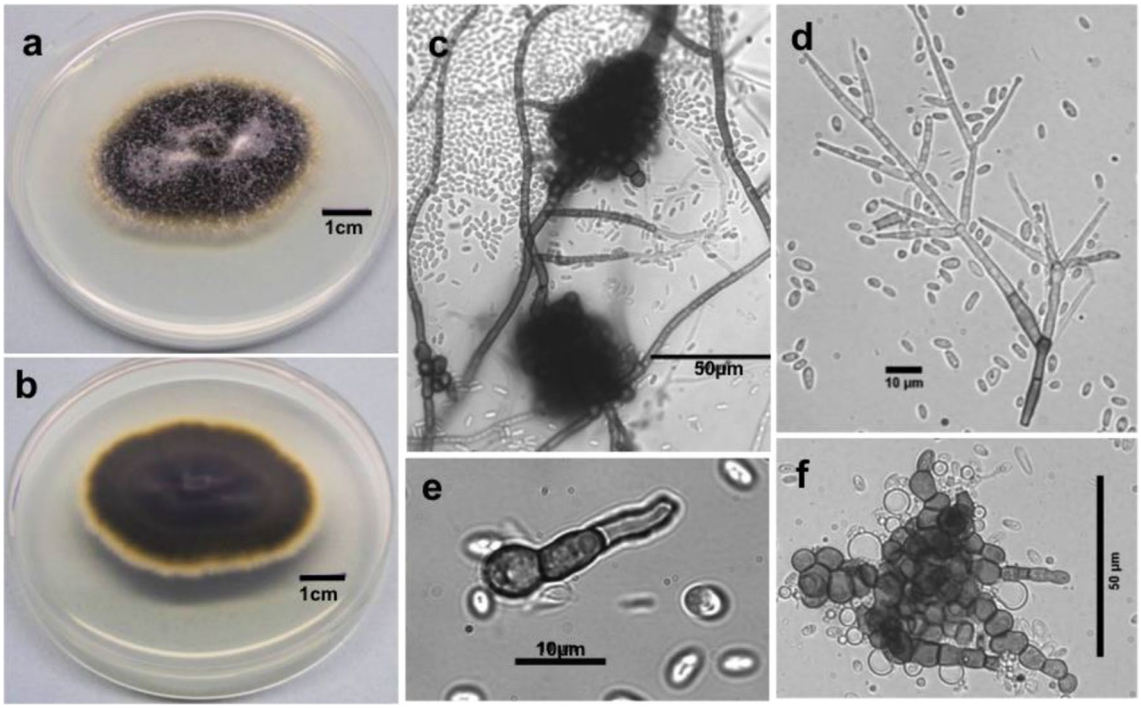

Fig. 2 Morphology of *V. tricorpus* isolated from Manitoba fields. (**a**) Colony after 18 days on PDA, frontal view. (**b**) Colony after 18 days on PDA, reverse view. (**c**) Microsclerotia and dark mycelium. (**d**) Conidiophore, whorl phialide and conidia. (**e**) Chlamydospore. (**f**) Microsclerotia

(Table S1). The size of the microsclerotia of the one isolate found ranged from 57.9 to 67 by 93.9 to 111 µm, with a mean size of 61.5 by 99.8 µm (Table S1).

Gibellulopsis nigrescens isolates were recovered while trying to pick single microsclerotia from colonies growing on semi-selective medium plated with soil. These isolates did not produce microsclerotia when growing on Sorensen's NP-10 or PDA medium. *Gibellulopsis nigrescens* isolates were initially white and soon became brown when growing on PDA. Conidia size ranged from 2.3 to 3.6 µm by 3.7 to 9.2 µm, with an average size of 3.1 by 6.3 µm (Table S1). Chlamydospores were also found as single or in short chains, with an average size of 5.6 by 7.8 µm.

ii. Quantification of *V. dahliae* and other microsclerotia-producing *Verticillium* species using real-time PCR assays

Specificity and sensitivity of real-time PCR assays using reference *Verticillium* isolates

The specificity of the real-time PCR assays (Table 2) was evaluated using a panel of gDNAs from related and unrelated fungi (Table 1). The real-time PCR assays with the primers VertBt-F/VertBt-R and Vd-F929-947/Vd-R1076-1094 for *V. dahliae*, VtF4/VtR2 and IGS-VtF1/IGS-VtR1 for *V. tricorpus,* and VlTubF2/VITubR1 and Vlsp-F1/Vlsp-R4 for *V. longisporum* were specific for the intended species, respectively. None of the primer sets showed cross-amplification with other *Verticillium* species (Table 1).

Although the real-time PCR assays using the primers VertBt-F/VertBt-R, IGS-VtF1/IGS-VtR1, and VlTubF2/VITubR1 showed species specificity (Table 1), low sensibility of amplification occurred after four orders of magnitude of gDNA concentration (0.0002 ng ml^{-1} or lower) of Vd1396-9 (*V. dahliae*), DVt3 (*V. tricorpus*), PD624 (*V. longisporum*), respectively (data not shown). The real-time PCR assays with the primers Vd-F929-947/Vd-R1076-1094 for *V. dahliae*, VtF4/VtR2 for *V. tricorpus,* and Vlsp-F1/Vlsp-R4 for *V. longisporum* showed high sensitivity when screened with the respective reference isolates through serial dilutions of gDNA (Fig. 3). The standard curves generated using a range of gDNA demonstrated that the selected assays have good reproducibility of amplification with 99, 94 and 98% efficiency with six orders of magnitude of purified gDNA of *V. dahliae*, *V. tricorpus* and *V. longisporum*, respectively (Fig. 3). Quantification showed a linear regression ($R^2 = 0.96$, 0.97 and 0.99, for *V. dahliae*, *V. tricorpus* and *V. longisporum*, respectively) between the log of the gDNA concentration and the C_T value over the range of gDNA concentrations evaluated (Fig. 3).

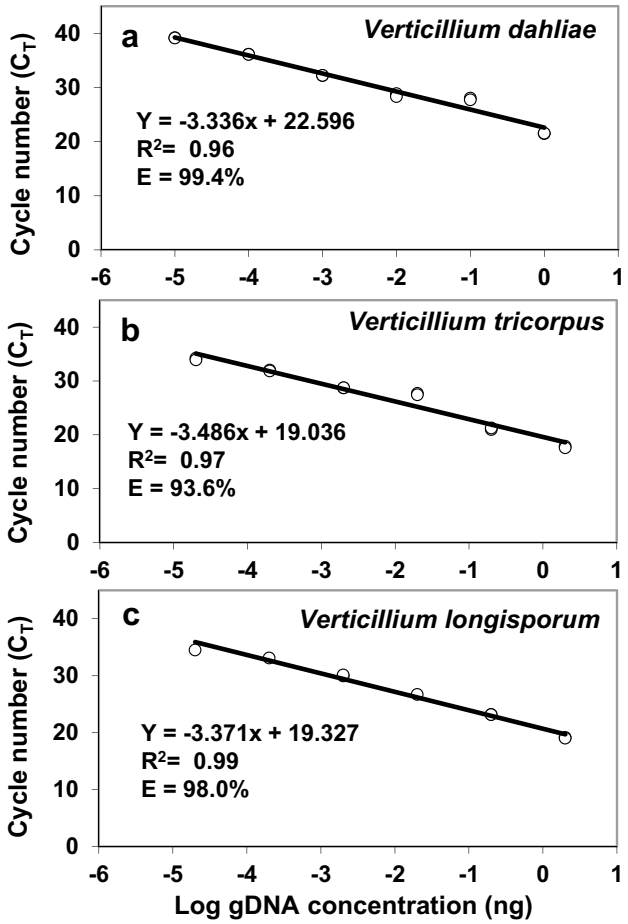

Fig. 3 Standard curves showing amplification of successive tenfold dilutions of (**a**) *V. dahliae* (Isolate Vd1396-9), (**b**) *V. tricorpus* (Isolate DVt3) and (**c**) *V. longisporum* (Isolate PD624) genomic DNA (gDNA)

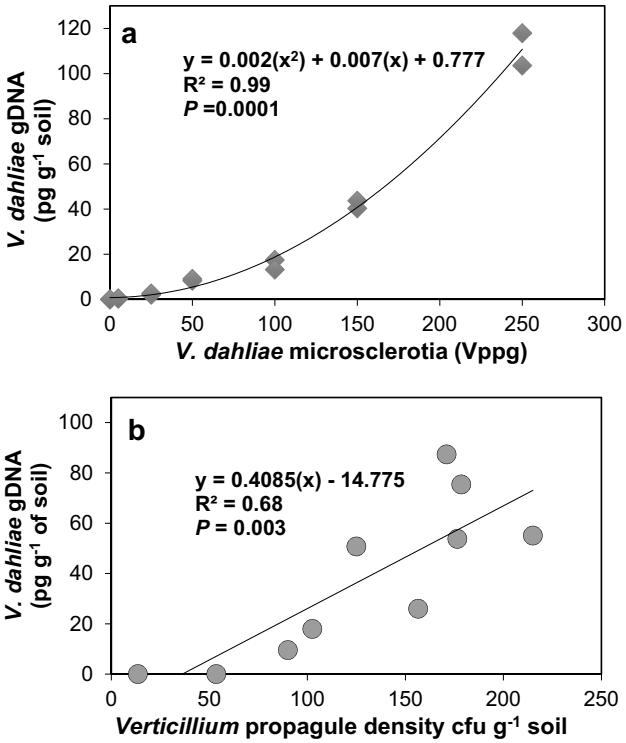

Fig. 4 (**a**) Relationship between the number of *V. dahliae* (Vd1396-9) microsclerotia per gram of soil (Vppg) and amount of *V. dahliae* genomic DNA (gDNA) from soil with known quantities of *V dahliae* (Vd1396-9) microsclerotia. (**b**) Relationship between *Verticillium* propagule density (cfu) in soils naturally infested with *V. dahliae* and amount of *V. dahliae* genomic DNA (gDNA) in soil

The minimum starting concentration of *V. dahliae* gDNA that could be accurately quantified with the real-time PCR assay using the primers Vd-F929-947/Vd-R1076-1094 was of 0.1 pg, which corresponds to a C_T value of 36 (Fig. 3a). The real-time PCR assays with the primers VtF4/VtR2 for *V. tricorpus* and Vlsp-F1/Vlsp-R4 for *V. longisporum* quantified gDNA up to a concentration of 0.02 pg, which correspond to C_T values of 34 for each assay (Fig. 3b, c). Therefore, the detection limits were fixed at the estimated C_T values corresponding to those concentrations at 36 for *V. dahliae*, and 34 for *V. tricorpus* and *V. longisporum*. The selected real-time PCR assays for *V. dahliae* with primers Vd-F929-947/Vd-R1076-1094, and for *V. tricorpus* with primers VtF4/VtR2, were then tested for their ability to identify the *Verticillium* isolates collected from potato soil and plant material from Manitoba. The real-time PCR assays effectively confirmed the identity of the isolates given by the morphological and molecular analysis (Table 3, S2, Fig. 1, 2).

Efficiency of real-time PCR assay to detect gDNA *V. dahliae* microsclerotia in soil

In artificially spiked soils with *V. dahliae* microsclerotia, gDNA of *V. dahliae* extracted from the spiked soil samples ranged from 0.3 to 118 pg g^{-1}, for densities of *V. dahliae* from 5 to 250 Vppg (Fig. 4a). There was a significant quadratic relationship ($R^2 = 0.99$ $P = 0.0001$) between the amount of *V. dahliae* gDNA extracted from the spiked soil and the number of microsclerotia of *V. dahliae* added (Vppg) (Fig. 4a).

iii. Relationship between disease severity and *Verticillium* propagule levels from the wet plating method

Wet plating method and real-time PCR quantification of microsclerotia-producing *Verticillium* species in soil

The wet plating method did not allow specific quantification of either *V. dahliae* or *V. tricorpus* in samples from commercial potato fields due to the similarity in the colony morphology. Propagule density (cfu) in the 10 soil samples ranged

from 14 to 215 cfu. The real-time PCR assays for *V. dahliae* (primers Vd-F929-947/Vd-R1076-1094), *V. tricorpus* (primers VtF4/VtR2) and *V. longisporum* (primers Vlsp-F1/Vlsp-R4) confirmed the presence of *V. dahliae* or *V. tricorpus*, or both species producing microsclerotia in the subject soils, except in the control soil. The concentration of *V. dahliae* gDNA ranged from 0 to 87 pg g^{-1} of soil (Table 4). *Verticillium tricorpus* gDNA was only found in five soil samples (Soil 522, 524, 531, 534, and 536) with levels varying from 6.0 to 21.1 pg gDNA g^{-1} of soil (Table 4). *Verticillium longisporum* was not found in either site (Table 4). There was a relationship between *Verticillium* cfu estimated by the traditional plating method and the amount of *V. dahliae* gDNA estimated through the real-time PCR assay ($R^2 = 0.68$ $P = 0.003$, Fig. 4b).

Relationship between gDNA of *V. dahliae* and development of Verticillium wilt

The compost and fertility treatments did not affect *Verticillium* propagule density in soil compared to the control (data is not presented). The average propagule density as cfu measured before plots were planted to potato was two times higher in Site E than Site D ($P = 0.001$, Fig. 5a). Real-time PCR assays detected *V. dahliae* and *V. tricorpus* in both sites. However, the levels of *V. dahliae* and *V. tricorpus* before potato planting were significantly different between sites ($P = 0.0001$, Fig. 5b). DNA detection levels for *V. dahliae*

Table 4 *Verticillium* propagule density estimated by wet plating (cfu g^{-1} soil) and genomic DNA concentration of microsclerotia forming *Verticillium* species in some Manitoba soils by real-time PCR assays

Soil ID[a]	cfu[b]	Concentration pg genomic DNA g^{-1} soil [b]		
		V. dahliae	*V. tricorpus*	*V. longisporum*
Soil 522	54 (37)	0[c] (0)	15.1 (2.4)	0[d] (0)
Soil 524	90 (27)	9.5 (3)	21.1 (8.6)	0 (0)
Soil 531	14 (3)	0 (0)	10.7 (2.3)	0 (0)
Soil 534	179 (36)	75.4 (11.8)	6.8 (3.5)	0 (0)
Soil 536	215 (83)	55.1 (15.1)	6.0 (1.9)	0 (0)
Soil 566	157 (31)	26 (7.6)	0[d] (0)	0 (0)
Soil 570	171 (53)	87.4 (19.8)	0 (0)	0 (0)
Soil 571	103 (17)	17.9 (5.4)	0 (0)	0 (0)
Soil 594	177 (14)	53.8(14.7)	0 (0)	0 (0)
Soil 603	125 (25)	50.7 (11)	0 (0)	0 (0)
Control soil[e]	0 (0)	0 (0)	0 (0)	0 (0)

[a]Soil samples from two commercial potato fields

[b]Soils were plated and extracted four times (n = 4). Mean (standard deviation)

[c]C_T value for this soil was > 37

[d]C_T value for this soil was > 35

[e]Control soil, no *Verticillium* sp present

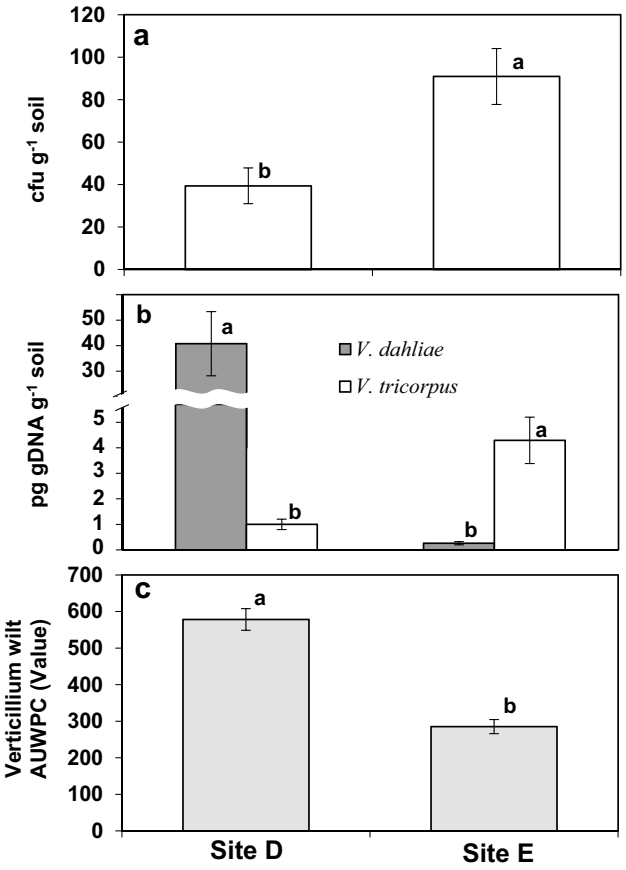

Fig. 5 (**a**) Propagule density of *Verticillium* sp as cfu g^{-1} soil. (**b**) Propagule density of *V. dahliae* and *V. tricorpus* as pg genomic DNA (gDNA) g^{-1} soil. Mean comparison within each site. (**c**) Area under the wilt progress curve (AUWPC) in sites D and E. Means followed by the same letter are not significant different according to Bonferroni's multiple comparison test ($P > 0.05$). Error bars are ± 1 standard error of the mean

and *V. tricorpus* in Site D ranged from 0.2 to 62.3 pg g^{-1}, and 0.4 to 8.5 pg g^{-1} of soil, respectively. While in Site E, the levels were 0.2 to 0.4 pg gDNA g^{-1} of soil for *V. dahliae*, and 2 to 8.5 pg g^{-1} of soil for *V. tricorpus* (Fig. 5b). Severity of Verticillium wilt, measured as AUWPC, was significantly different between sites ($P = 0.001$, Fig. 5c).

Verticillium propagule density, measured as cfu, was a weak predictor of the development of Verticillium wilt symptoms (Fig. 6a). AUWPC followed a significant negative linear relationship ($R^2 = 0.37$, $P = 0.04$, Fig. 6a) in response to the number cfu. In contrast, there was a significant quadratic relationship ($R^2 = 0.75$, $P = 0.003$) between the amount of *V. dahliae* gDNA in soil using the real-time PCR assay and AUWPC (Fig. 6b). The AUWPC seems to stay lower than 400 when the amount of *V. dahliae* gDNA in soil was lower than 0.5 pg g^{-1}. However, when the amount of *V. dahliae* in soil was higher than 10 pg g^{-1}, AUWPC increased to 505, and stayed within a range from 505 to 721 even after

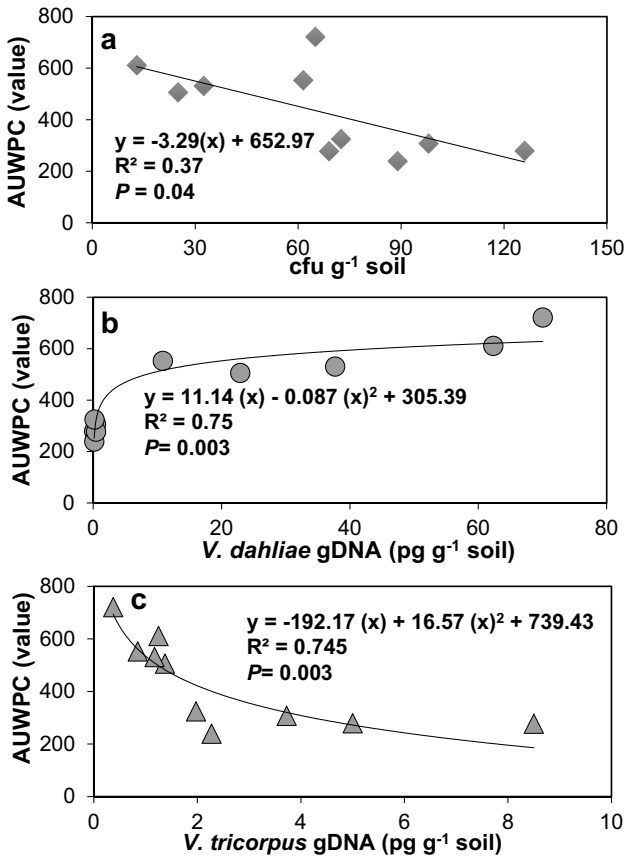

Fig. 6 Relationships between AUWPC and: (**a**) *Verticillium* propagule density as cfu g^{-1} soil, (**b**) *V. dahliae* genomic DNA (gDNA) in soil (pg g^{-1} soil), and (**c**) *V. tricorpus* genomic DNA (gDNA) in soil (pg g^{-1} soil)

the *V. dahliae* gDNA increased up to 70 pg g^{-1} (Fig. 6b). In contrast, there was a negative significant quadratic relationship between the level of *V. tricorpus* and AUWPC ($R^2 = 0.74$, $P = 0.003$, Fig. 6c). Potato plants planted in soils with more than 2 pg g^{-1} of *V. tricorpus* gDNA exhibited lower AUWPC, than in soils with lower levels of *V. tricorpus* (Fig. 6c).

Discussion

Verticillium dahliae is the major causal agent of Verticillium wilt (Powelson and Rowe 1993), which causes premature senescence resulting into 30–50% yield reduction and low tuber quality (Johnson 1988). Therefore, accurate *V. dahliae* identification and quantification is crucial for effectively preventing and managing Verticillium wilt (Cohen et al. 2012). The traditional estimation method, which is based on the number of microsclerotia forming colonies on a semi-selective culture medium, considers all the *Verticillium* species that produce microsclerotia and does not quantify or differentiate them individually (Termorshuizen et al. 1998). In the current study, three fungal species were found producing microsclerotia in potato soil samples collected from eight rural municipalities in Manitoba, representing the major productive potato regions in the province. In previous studies conducted in Manitoba, 91% of the recovery isolates from plant tissue or tubers were identified as *V. dahliae* (Uppal et al. 2007). In the current study, morphological and molecular characterization confirmed the presence of two dominant species, *V. dahliae* and *V. tricorpus* representing 97% of the isolate's recovery from soil or plant. Seventy six percent of the isolates produced microsclerotia as the only resting structure and failed to produce dark mycelia and chlamydospores, which is consistent with the description given to *V. dahliae* isolates (Inderbitzin et al. 2011; Jing et al. 2018; Pegg and Brady 2002; Termorshuizen et al. 1998). Resting structures are important for the biology of *Verticillium* and its taxonomy. *Verticillium* species reproduce only asexually, as no sexual state is known (Inderbitzin et al. 2011; Usami et al. 2009). Microsclerotia is a very important persistent dormant structure for *V. dahliae*, because it is the only mean besides the mitotically-produced spores that allow the fungus to reproduce and survive prolonged periods (Milgroom et al. 2014; Mol and Scholte 1995). Resting structures are traditionally used as the primary characteristic to distinguish *Verticillium* species. In fact, *V. dahliae* has been defined based on microsclerotia, the only resting structure produced by the pathogen (Inderbitzin et al. 2011; Issac 1949).

The second most commonly found microsclerotia-producing *Verticillium* specie in Manitoba was *V. tricorpus*, with a frequency of occurrence of 21% of the recovered isolates. It was detected and recovered from 3 of 8 major productive potato regions in Manitoba, which indicates that *V. tricorpus* is somewhat dispersed in Manitoba. Another *Verticillium* species found in Manitoba was *V. klebahnii*, for which identification was only confirmed after the IGS sequence was compared with sequences of *V. tricorpus*, *V. klebahnii* and *V. isaacii* strains placed in the NCBI.

Detection and further identification of *V. dahliae* species using the classical isolation and growth on semi-selective medium methods can be laborious and challenging. For instance, microsclerotia-formed colonies of *V. tricorpus* can be very similar to those of *V. dahliae*. Despite that, microsclerotia in *V. tricorpus* colonies form a scattered pattern with a few microsclerotia, compared with a more prolific and radial microsclerotia pattern for *V. dahliae* (Goud et al. 2003). Colonies can be misidentified and counted or not as *V. dahliae*, since small colonies with fewer than 25 microsclerotia are very difficult to identify as either *V. dahliae* or *V. tricorpus* (Goud et al. 2003). Additionally, most characteristics of these *Verticillium* species are less pronounced when growing on soil-plated medium (Goud et al. 2003).

Therefore, in the case of those species morphologically similar, like *V. longisporum* and *V. dahliae*, individual quantification using the soil plating method will only be possible after growing the isolates in pure culture, by looking at morphological traits like the size of the conidia, which is larger in *V. longisporum*, 8 × 2.5 µm (Bilodeau et al. 2012; Karapapa et al. 1997). Nevertheless, culturing *Verticillium* isolates does not guarantee the identification of the specie, as some of the morphological characteristics could be compromised. For instance, *V. tricorpus* can produce smaller microsclerotia and fewer dark resting mycelia and chlamydospores and restrict the production of yellow-orange pigmentation when grown on PDA (Qin et al. 2008).

Verticillium longisporum was not found in the soils analyzed in the current study, but it is another important *Verticillium* specie present in Manitoba fields (Desjardins et al. 2015; Zou et al. 2020). *Verticillium longisporum* is an important pathogen producing significant economic losses in canola (Karapapa et al. 1997), a crop commonly used in the rotation programs with wheat and potato in Manitoba (Mohr et al. 2011). The presence of other *Verticillium* species producing microsclerotia in the soil, like *V. longisporum*, will contribute to overestimate the levels of *V. dahliae* when using the traditional plating method on semi-selective medium, particularly because the colony morphology of the *V. longisporum* cannot be differentiated from that in *V. dahliae* when growing on NP-medium (Bilodeau et al. 2012), which is the same medium used in the wet plating method. *Verticillium tricorpus* is mainly known as a saprophyte that thrives on decaying organic matter and, causal agent of opportunistic infections on weakened plant host (Goud et al. 2003). Indeed, *V. tricorpus* is able to prompt wilt symptoms when a host plant has been challenged with other abiotic stresses, such as high soil nitrogen levels and waterlogging conditions (Isaac 1956), or biotic, such as wilting by *V. dahliae* (Robinson et al. 2007) This could explain the presence of *V. tricorpus* in potato plants severely affected by Verticillium wilt, as plants were wilted and individual leaflets and stems were already dying, common symptoms of advanced stages of the disease (Rowe and Powelson 2002). The lack of accurate identification and quantification of *V. dahliae* propagule density can contribute to higher potato production cost. The results presented in the current study suggest that the current wet plating method is not sufficiently specific to differentiate propagule density of *V. dahliae* from other *Verticillium* species producing microsclerotia, currently present in Manitoba soils, such as *V. tricorpus* and *V. klebahnii* (current study) and *V. longisporum* (Desjardins et al. 2015; Zou et al. 2020).

Molecular methods, such as real-time PCR, have overcome the drawbacks of traditional methods for detecting and identifying plant pathogens. Several studies have successfully used real-time PCR methods for the diagnostic and management of several potato-infecting fungal species, due to the high level of sensitivity and specificity that the assays offer (Brierley et al. 2009; Cullen et al. 2001, 2002; Schaad and Frederick 2002; Ward et al. 2004). Real-time PCR assays for detecting and quantifying *V. dahliae, V. tricorpus* and *V, longisporum* have been developed (Atallah et al. 2007; Banno et al. 2011; Bilodeau et al. 2012; Borza et al. 2018; Debode et al. 2011; Gramaje et al. 2013). In the current study, we evaluated several real-time PCR assays and selected three assays for their specificity, sensitivity and reproducibility to quantify *V. dahliae*, *V. tricorpus* and *V. longisporum*, respectively. The real-time PCR assay selected for *V. dahliae* was the TaqMan® using primer set Vd-F929-947/R1076-1094 and probe 5′6-FAM (Bilodeau et al. 2012). The indicated assay was confirmed specific to *V. dahliae* and achieved high amplification sensitivity with high efficiency over five orders of magnitude of gDNA concentration up to 0.1 pg gDNA of *V. dahliae*. In the current study, the analysis of field soils with spiked microsclerotia of *V. dahliae* indicated that the real-time PCR in conjunction with the soil pulverizing step performed before soil DNA extraction using the MoBio PowerSoil DNA Isolation system, detected up to 5 microsclerotia per gram of soil, which corresponded to 0.3 pg of gDNA of *V. dahliae*. Although the indicated real-time PCR assay was not tested with a lower number of microsclerotia, the strong relationship between the added microsclerotia and the detected *V. dahliae* gDNA demonstrated accuracy in detecting the density of the pathogen in soil. This is particularly important for potato producers because the reported disease thresholds for *V. dahliae* in North America range between 8 to 20 microsclerotia per gram of soil (Davis and Sorensen 1986; Nicot and Rouse 1987). Therefore, the selected real-time PCR can quantify *V. dahliae* microsclerotia at levels below those producing significant disease losses.

Nevertheless, the indicated real-time PCR assay has the potential to detect up to one microsclerotia of *V. dahliae* (3 fg gDNA) (Bilodeau et al. 2012). However, this will likely depend on improving the soil DNA extraction process. If the DNA extraction method can increase yield DNA, without increasing PCR inhibitors in the DNA, detection limits will likely improve. The real-time PCR assays selected for *V. tricorpus* and *V. longisporum* were the SYBR® Green based-protocols using the primer pair VtF4/VtR2 (Debode et al. 2011) and Vlsp-F1/Vlsp-R4 (Banno et al. 2011), respectively. Real-time PCR assays were very sensitive as both quantified gDNA of their corresponding pathogen up to a concentration of 0.02 pg, for *V. tricorpus* and *V. longisporum*, respectively. The sensitivity of the indicated real-time PCR assays was similar to the sensitivity achieved in previous studies (Banno et al. 2011; Debode et al. 2011). The evaluated real-time PCR assays detected and quantified *V. dahliae* in artificially and naturally infested soils in Manitoba. Additionally, real-time PCR assays were successfully

evaluated for the detection and quantification of *V. tricorpus* in naturally infested soil from Manitoba.

A relationship ($R^2 = 0.68$) between the amount of *V. dahliae* gDNA in soil and the number of cfu estimated using the wet plating method was observed in this study (Fig. 4b). However, a stronger relationship was achieved by Bilodeau et al. (2012) between cfu g^{-1} of *V. dahliae*, and the C_T values obtained from the same real-time PCR assay. Although different soil sample size, sampling method, and microsclerotia distribution in soil could had contributed to the different relationship, in the current study, the weaker relationship was perhaps due to the presence of *V. tricorpus* propagules as the real-time PCR assay using the primer set VtF4/VtR2 detected gDNA of *V. tricorpus* in some of the soil samples used in the analysis. In contrast, when soil with no *Verticillium* was spiked with microsclerotia of *V. dahliae*, there was a significant relationship ($R^2 = 0.91$) between the amount of *V. dahliae* gDNA detected and the number of *V. dahliae* microsclerotia added. Therefore, the weaker relationship between the *V. dahliae* gDNA and cfu counts in naturally infected soils is probably due to the presence of resting structures of *V. tricorpus*, as previously hypothesized. The presence of microsclerotia-formed colonies of *V. tricorpus* could contribute to overestimating the level of *V. dahliae* in soil when measured as cfu g^{-1} of soil. Another factor that has been considered in previous studies is the presence of nonviable microsclerotia that could contribute to overestimation of the soil inoculum. Nevertheless, it is generally assumed that gDNA originated from dead cells degrades fairly rapidly in natural moist soil conditions due to microbial activity, suggesting that the bias due to presence of dead microsclerotia might be negligible in the assay (Bilodeau et al. 2012).

A study by Tzelepis et al. (2017) suggested that the relationship between the amount of *V. dahliae* gDNA in soil and the number of cfu must be examined cautiously since the number of cells and thereby the amount of gDNA in individual microsclerotium varies substantially. Quantification of pathogen's DNA in soil using real-time PCR assays has been a good predictor of diseases in potatoes (Brierley et al. 2009; van de Graaf et al. 2003). This study compared the capability of the real-time PCR assay of *V. dahliae* gDNA in soil and the cfu number to predict Verticillium wilt severity in two Manitoba fields naturally infested with *V. dahliae*, and the history of Verticillium wilt. The two fields had very different *Verticillium* propagule density. A quadratic relationship ($R^2 = 0.75$) was observed between the amounts of *V. dahliae* gDNA in soil and Verticillium wilt severity in cv. Russet Burbank. In contrast, cfu counts have a weaker relationship with disease severity. Interestingly, the field with the highest level of cfu in soil was the same site with the lowest level of *V. dahliae* gDNA, but with the highest level of *V. tricorpus*.

Production of cfu from resting mycelia, chlamydospores and microsclerotia of *V. tricorpus*, must likely contribute to the higher numbers of cfu in soil and ultimately to the negative relationship between the number of cfu in soil and the disease development. Unfortunately, the role of the *V. tricorpus* on the development of Verticillium wilt in Manitoba is unknown and needs further study. Negative relationships between *V. tricorpus* in soil and Verticillium wilt have been previously reported in potatoes (Davis et al. 2000; Davis and Sorensen 1985), suggesting that *V. tricorpus* could be a potential biological control against *V. dahliae*. However, *V. tricorpus* has been found to cause Verticillium wilt in potato as well (Nair et al. 2015; Robinson et al. 2007), which indicate the possible presence of several pathotypes within this specie (Ebihara et al. 2003).

In conclusion, a specific, sensitive and reproducible real-time PCR assay using the primer pair Vd-F929-947/Vd-R1076-1094 and probe 5′6-FAM (Bilodeau et al. 2012) was selected to quantify *V. dahliae* gDNA in soil. The real-time PCR assay was optimized with a soil-pulverizing step performed before soil DNA extraction. Quantification of *V. dahliae* using the real-time PCR was a better predictor of Verticillium wilt severity than cfu from the wet plating method. For accurate detection and quantification of *V. tricorpus* and *V. longisporum*, specific and reproducible real-time PCR assays using the primer pair VtF4/VtR2 (Debode et al. 2011) and Vlsp-F1/Vlsp-R4 (Banno et al. 2011) were selected for the two species respectively. Both real-time PCR assays were sensitive to a concentration of 0.02 pg of gDNA. In this study, the *V. tricorpus* was confirmed in Manitoba soils producing microsclerotia that contribute to overestimating the propagule density of *V. dahliae* measured as cfu, using the wet plating method. Considering the presence of *Verticillium* species producing microsclerotia, the relatively low propagule density, patchiness of the *V. dahliae* soil inoculum and the inaccuracy of the wet plating method, real-time PCR methods should be considered for the detection and quantification of *V. dahliae* propagule density in soil. The traditional wet plating method does not seem to properly estimate the level of *V. dahliae* in soil or predict disease severity, due to other *Verticillium* species producing microsclerotia, particularly *V. tricorpus*.

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Data Availability All the data to support this study is included in the manuscript.

Declarations

Conflicts of Interest The authors have not conflicts of interest.

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