A Multiplex qPCR Assay for Detection and Quantification of *Plasmodiophora brassicae* in Soil

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Abstract

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Various physical and chemical factors in soil can inhibit the detection and quantification of soilborne plant pathogens using quantitative polymerase chain reaction (qPCR) assays. A multiplexed TaqMan qPCR assay, including a competitive internal positive control (CIPC), was developed to identify and (where necessary) compensate for inhibition in the quantification of resting spores of *Plasmodiophora brassicae* from soil. The CIPC amplicon was developed by modifying a sequence coding for green fluorescent protein so that it could be amplified with *P. brassicae*-specific primers. Addition of CIPC at 5 fg/μl to the singleplex qPCR assay designed to quantify *P. brassicae* genomic DNA did not reduce the sensitivity, specificity, or reproducibility of the assay. Each of the soil samples, either artificially inoculated or naturally infested with *P. brassicae*,

exhibited no amplification of the CIPC. When the samples were diluted and reassessed, the quantification cycle of the CIPC relative to the control (water only) was delayed in each sample. The magnitude of the delay was used to adjust the estimate of resting spore concentration. The corrected concentration estimates were significantly higher than the unadjusted estimate, which indicated the presence of DNA inhibitors in samples even after dilution. The only exception was a mineral soil sample inoculated with a low concentration (10^3 spores/g) of resting spores. The assay was optimized for use on a range of soil types. A sample of 0.25 g for mineral soil and 0.10 g for high-organic-matter soil was optimum for recovery of DNA of *P. brassicae*. The assay represents an improvement over existing assays for estimating resting spore concentration in infested fields.

Clubroot, caused by *Plasmodiophora brassicae* (Woronin), is one of the most destructive diseases of cruciferous vegetable and oilseed crops worldwide (7), causing losses of up to 100% in severely infested fields (8). Soilborne resting spores of this obligate pathogen are the sole primary source of inoculum. They are easily disseminated (24) and can remain dormant in soil for many years (28). As a result, accurate assessment of resting spore populations in soil is important for limiting the spread of the pathogen.

In Canada, clubroot has historically been a disease of Brassica vegetables (16). However, since the first report of clubroot on canola (*Brassica napus* L.) on the Canadian prairies in 2003, the pathogen has been spreading rapidly (24). In the last decade, the disease has been confirmed in more than 1,400 canola fields and represents a substantial threat to the canola industry across the region (24). Resting spore populations in soil build up quickly on susceptible canola cultivars. These resting spores are introduced to noninfested fields primarily on vehicles and farm equipment but may occasionally be transported over long distances by wind and water erosion (11).

There are several methods for assessing resting spore populations in soil but each method has significant drawbacks. The resting spores of *P. brassicae* are small (3 to 5 μm) and lack characteristic ornamentation that aids in quick, accurate identification. As a result, extracting and quantifying resting spores by microscopy is time consuming (31) and the results can be highly variable. Detection of *P. brassicae* in plant tissues by polymerase chain reaction (PCR) or quantitative (q)PCR assays is a standard practice that has been extended to quantification of resting spores in soil (10,21,28). However, several soil components such as clay particles, humic acids, pheonolic compounds, heavy metals, and assorted contaminants can inhibit PCR and reduce the reliability of quantification. Inhibitors are present at

different levels in soils and are difficult to eliminate (2). Therefore, an internal control (IC) is generally included in PCR assays to quantify inhibition in each sample, in a parallel reaction or together with the target sample (multiplexed). Most of the qPCR assays for *P. brassicae* lack an IC (4,9,17,21,29) or it is included in a parallel reaction (27). The advantage of multiplexing is that it saves time and resources. It also allows for reduced dilution of samples, which should increase the likelihood of detection of low-pathogen populations (3).

Multiplex TaqMan assays have been developed for quantification of various plant pathogens from seed, plants, and soil (2,25,26). In the majority of these assays, two separate primer pairs were used for amplification of the IC and the target region. A major drawback of these assays is primer interference, which can reduce amplification efficiency. To eliminate primer interference, a competitive internal positive control (CIPC) approach has been used for detection and quantification of human pathogens from environmental sources (12,13,19,23) and, recently, for quantification of *Verticillium dahliae* (3). In this approach, the CIPC is a nontarget amplicon of exogenous DNA, with primer-binding regions identical to those of the target sequence, an internal sequence similar to the target sequence in length and base composition, and a unique region where the probe binds to the amplicon that differentiates the CIPC from the target locus (15,22). These features ensure amplification of both the CIPC and the target locus with one primer pair only. Furthermore, a concentration of the CIPC is used such that it does not interfere with detection and quantification of target DNA.

The objective of our study was to develop a multiplex real-time qPCR assay for detection and accurate quantification of *P. brassicae*. It included development of a CIPC and validation of the method for use on field soils via assessment of soil samples inoculated with resting spores of *P. brassicae*.

Materials and Methods

Isolates of *P. brassicae*. A resting spore suspension of *P. brassicae* pathotype 6 was prepared from clubbed roots of canola ('46A76'; Pioneer Hi-Bred, Ontario, Canada) collected from the Muck Crop Research Station of the University of Guelph in fall 2011 and stored at −4°C until used. After grinding the roots using a commercial grinder, plant material was removed from the suspension by passage

through 16 layers of cheesecloth and then a 40-μm sieve, which yielded a clean suspension of resting spores. The spore suspension was used to prepare a serial dilution and artificially inoculate soil samples in subsequent experiments. Pathotyping of all of the isolates used in the study were based on Williams' differential system (32).

Primer and probe specificity. The forward primer (DC1F) was reported previously in a SYBR Green assay for *P. brassicae* (4) and the reverse primer (DC1mR) was slightly modified (the last nucleotide on the 3′ end was deleted) (Table 1). DC1F and DC1mR amplified a 90-bp fragment of the internal transcribed spacer 1 region of *P. brassicae*. A probe (PB1; Table 1) with the 5′ end reporter dye of FAM and 3′ quencher of NFQ-MGB was used. PB1 was selected based on a preliminary assessment of three probes designed with different annealing temperatures, lengths, and GC contents. Its annealing temperature was approximately 10°C higher than the PCR primers.

To test the specificity of the primers and probes to *P. brassicae*, pathotypes 2, 3, 5, 6, and 8 were tested using qPCR. These isolates were originally obtained from clubroot-infested canola roots from sites across Canada (6). The inoculum for these pathotypes was prepared from clubbed roots of Shanghai pak choy (*B. rapa* subsp. *chinensis* 'Mei Qing Choi') grown under controlled conditions (photoperiod of 16 and 8 h, day and night temperatures of 24 and 18°C, respectively)

and stored at −4°C. In addition, DNA from a number of fungal species was used to evaluate the specificity of the assay (Table 2). Each isolate was grown on commercial potato dextrose agar medium (Sigma-Aldrich, St. Louis). Genomic DNA (gDNA) was extracted from two replicates of each of the *P. brassicae* pathotypes (10^8 resting spores) and from a mycelial mat (2 by 2 cm) of each fungal species culture using a Power Soil DNA isolation kit according to the manufacturer's instructions (MO BIO Laboratories, Inc., Carlsbad, CA). Primer and probe specificity was further confirmed by conventional PCR analysis.

qPCR. The qPCR assays were conducted in a 48-well Step-One real-time PCR system (Applied Biosystems, CA) according to the manufacturer's instructions. Each 20-μl reaction mixture consisted of 10 μl of 2× TaqMan Universal PCR Master Mix (P/N 4304437; Applied Biosystems), 0.8 μl of 22.5 μM *P. brassicae* primer pairs, 0.8 μl of 6.25 μM *P. brassicae* probe, and 2 μl of target DNA. Two experimental replicates were used for each concentration of the standard series. There were two technical repetitions for each experimental replicate of each treatment in each assay, unless otherwise stated. A negative control sample (Hypure Molecular Biology Grade Water; Hyclone Laboratories Inc., Logan, UT) was also included in each assay. The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, followed by 62°C for 1 min.

Table 1. Primers and probe used in a multiplex TaqMan assay for detection and quantification of *Plasmodiophora brassicae*

Primer, probe name[a]	Sequence (5′ to 3′)[b]	Target region[c]	Amplified length (bp)
P. brassicae			
DC1F	CCT AGC GCT GCA TCC CAT AT	ITS1 of *P. brassicae*	90
DC1mR	CGG CTA GGA TGG TTC GAA A		
PB1	[6-FAM] CCA TGT GAA CCG GTG AC [NFQ-MGB]		
pDSK-GFPuv1			
PBGFPuv3F	CCT AGC GCT GCA TCC CAT AT**C GAT GGC CCT GTC CTT TTA C**	*GFPuv* gene in pDSK-GFPuv1 plasmid	116
PBGFPuv3R	CGG CTA GGA TGG TTC GAA A**GT GTA ATC CCA GCA GCA GTT A**		
GFP1	[VIC] ACC ATT ACC TGT CGA CAC AAT CTG CCC T [NFQ-MGB]		

[a] PBGFPuv3F and PBGFPuv3R are hybrid primers produced by combining a sequence from *P. brassicae* (indicated by regular letters) with the *GFPuv1* gene sequence (bold letters).
[b] The dye (6-carboxyfluorescein [6-FAM]) for each probe was located on the 5′ end and the quencher (nonfluorescent quencher-minor groove binder [NFQ-MGB]) on the 3′ end.
[c] ITS = internal transcribed spacer and GFP = green fluorescent protein.

Table 2. Evaluation of the specificity of primers and probes designed from the internal transcribed spacer 1 region of the *Plasmodiophora brassicae* genome using quantitative polymerase chain reaction (qPCR) and PCR

P. brassicae pathotype or fungal species	Isolation source	Accession number	Amplification outcome
P. brassicae P2	Shanghai pak choy, clubbed roots	…	Amplified
P. brassicae P3	Shanghai pak choy, clubbed roots	…	Amplified
P. brassicae P5	Shanghai pak choy, clubbed roots	…	Amplified
P. brassicae P6	Canola, clubbed roots	…	Amplified
P. brassicae P8	Shanghai pak choy, clubbed roots	…	Amplified
Epicoccum nigrum	Sclerotia of *Sclerotinia sclerotiorum*[a]	JN689342.1	Not amplified
Fusarium acuminatum	Sclerotia of *S. sclerotiorum*[a]	HM068320.1	Not amplified
F. equiseti	Sclerotia of *S. sclerotiorum*[a]	EU595566.1	Not amplified
F. oxysporum	Sclerotia of *S. sclerotiorum*[a]	DQ535184.1	Not amplified
Penicillium citrinum	Sclerotia of *S. sclerotiorum*[a]	FJ765031.1	Not amplified
S. sclerotiorum	Sclerotia of *S. sclerotiorum*[a]	…	Not amplified
Trichoderma hamatum	Sclerotia of *S. sclerotiorum*[a]	JQ040347.1	Not amplified
Alternaria brassicicola	Shanghai pak choy[b]	…	Not amplified
F. oxysporum	Celery[b]	…	Not amplified
Fusarium spp.	Carrot[b]	…	Not amplified
Phytophthora infestans	Tomato[b]	…	Not amplified
Rhizoctonia solani	Spinach[b]	…	Not amplified
S. cepivorum	Onion[b]	…	Not amplified
S. minor	Lettuce[b]	…	Not amplified
S. sclerotiorum	Carrot[b]	…	Not amplified

[a] Sclerotia of *S. sclerotiorum* were collected at the Muck Crop Research Station in 2011.
[b] Isolated from infected plants at the Muck Crop Research Station in 2013.

Validation of the qPCR assay. To develop a TaqMan assay, a range of *P. brassicae* resting spore concentrations (0, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, and 10^8) were added directly into the Microbead tubes provided with the Power Soil DNA isolation kit. There were three replicates for each treatment. DNA extraction and purification protocols were performed according to the manufacturer's instructions. A mechanical Bead Ruptor (Omni International, Inc., Kennesaw, GA) was used to homogenize each sample for 1 min at high speed. The gDNA derived from *P. brassicae* resting spore concentrations (from 10^3 to 10^8 spores) was used as a standard series in further qPCR assays and as treatments in validation of the multiplex assay. To determine the quantities of *P. brassicae* gDNA corresponding to the resting spore concentrations used in the standard series, the gDNA concentration derived from 10^8 *P. brassicae* resting spores was diluted in 10-fold increments down to the concentration equivalent to 10^2 resting spores. The gDNA concentration in the dilution series was measured using a Qubit fluorometer (Life Technologies, Eugene, OR).

Development of the CIPC. To identify changes in amplification efficiency resulting from PCR inhibitors, an IC was developed using a plasmid (pDSK-GFPuv1) containing a variant of the gene coding for green fluorescent protein (*GFPuv1*) (30). The origin of green fluorescent protein (GFP) is a jellyfish; therefore, it was assumed that this DNA would not be present in soil. For amplification of *GFPuv1*, forward (PBGFPuv3F) and reverse (PBGFPuv3R) hybrid primers were designed based on plasmid sequence (pDSK-GFPuv1), with binding sites for the forward and reverse amplification primers for *P. brassicae* ligated to the 5′ ends. Incorporation of the *P. brassicae* sites would generate an amplicon that can be amplified with DCIF and DC1mR primers for *P. brassicae*. The amplification conditions were 0.4 μM each hybrid primer (PBGFPuv3F and PBGFPuv3R; Table 1), 0.1 mM dNTPs, 2 mM MgCl₂, 1× reaction buffer, 1 U of DNA polymerase (JumpStart Taq DNA Polymerase; Sigma-Aldrich), and 5 μl of template DNA of plasmid pDSK-GFPuv1. PCR cycling conditions were 94°C for 2 min and 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and 72°C for 10 min in a reaction volume of 25 μl. Electrophoresis evaluation of this amplicon on 2% agarose gel (Ultrapure Agarose, Carlsbad, CA) and staining with ethidium bromide resulted in an amplicon (CIPC) of 116 bp (77 bp of pDSK-GFPuv1 + 39 bp of *P. brassicae* forward and reverse primers). The amplicon was treated with a Wizard SV Gel and PCR Clean-up kit (Promega Corp., Madison, WI) following the manufacturer's instructions to remove residual primers and dNTPs. The concentration of the purified CIPC amplicon was determined using a Qubit fluorometer.

Validation of the multiplex qPCR assay. To discriminate the CIPC from *P. brassicae* in the multiplex qPCR assay, a probe (GFP1) with a sequence complementary to a region of *GPFuv1* was used. The probe (GFP1) was labeled with the 5′ reporter fluorophore VIC and 3′ quencher dye NFQ-MGB (Applied Biosystems). To determine the amount of CIPC amplicon that does not interfere in a multiplex reaction, a 10-fold dilution series of CIPC ranging from 10^{-2} to 10^{-9} μg/μl was tested with concentrations of *P. brassicae* gDNA derived from 10^3 to 10^8 resting spores. The qPCR reaction mixture was the same as stated for the singleplex assay, except that 0.8 μl of 6.25 μM CIPC probe (GFP1) was added to the mastermix for the multiplex assay. Preliminary evaluations showed that 2 μl of the CIPC amplicon at 5×10^{-8} μg/μl (5 fg/μl) in 20 μl of reaction mix had a quantification cycle (C_q) value of 20.9 to 21.4 when multiplexed with concentrations of *P. brassicae* DNA derived from 10^3 to 10^8 resting spores.

Inoculated and naturally infested soil samples. The efficacy of the TaqMan assay was assessed in two mineral soils and a high-organic-matter (OM) soil (approximately 80% OM) inoculated with known amounts of resting spores of *P. brassicae*. Samples of the mineral soils were obtained from the University of Guelph research farm at Elora, ON, Canada (London loam, aquic hapludalf) and from the Agriculture and Agri-Food research farm at Saskatoon, SK, Canada (Sutherland clay-loam, vertic haploborolls). The high-OM soil (Hemic histosol) was obtained from the Muck Crop Research Station. The physical characteristics and the pH of the soils were measured by an accredited laboratory (Agrifood Laboratories, Guelph, ON, Canada; Table 3). Clubroot had not previously been reported at any of the sampling locations. Each soil sample was ground to a fine powder using a mortar and pestle, passed through a 355-μm sieve, and dried at 37°C overnight. For the mineral soils, 0.25 g of powdered soil was added directly into the microbead tubes provided with the Power Soil DNA isolation kit. However, there was no DNA amplification from the high-OM soil when 0.25 g was assessed; therefore, a range of sample weights (0.25, 0.20, 0.15, 0.10, and 0.05 g) were tested and 0.10 g was selected for muck soil in this analysis. The soil samples—mineral (0.25 g) and muck (0.10 g)—were inoculated by replacing 0.1 ml of the solution in the microbead tubes with known concentrations of resting spores to produce concentrations of 0, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 spores. The soil samples added to the microbead tubes were subsamples of one sample for each soil type, to minimize potential heterogeneity of DNA inhibition among the soil samples. DNA extraction and purification processes were conducted as described above. Homogenization using the mechanical Bead Ruptor was optimized to 1 min at high speed based on a preliminary trial using the three soil types. There were three biological replicates for each treatment.

Soil samples naturally infested with *P. brassicae* were obtained from the Muck Crop Research Station; the Agriculture and Agri-Food Canada research farm at Normandin, QC, Canada (Labarre silty clay, humic gleysol); and the clubroot nursery site of Alberta Agriculture and Rural Development near Edmonton, AB, Canada (black Chernozemic loam). At each location, about 200 ml of soil was collected from soil cores extracted from the top 10 cm at each of three sites. From this bulked and mixed sample, a 0.50-g sample was drawn and divided into two 0.25-g samples for DNA extraction. These samples were processed as described above but were not inoculated with *P. brassicae*.

qPCR was employed to determine the quantity of *P. brassicae* gDNA in each soil sample. The *P. brassicae* gDNA from 10^3 to 10^8 resting spores was used to generate a standard curve. To accommodate as many target samples in a qPCR run as possible, the standard series consisted of two technical replicates for each concentration.

To determine the detection threshold of *P. brassicae* resting spores, DNA extracts of the Elora and high-OM soils were inoculated with 100 or 500 resting spores per 0.25 g, diluted 5- and 10-fold, and assessed as described previously.

Estimation of resting spores in a sample: The *P. brassicae* gDNA from 10^3 to 10^8 resting spores (in 10-fold increments) was used to generate a standard curve for the qPCR assays, as has been described previously for estimation of *Enterococcus* bacteria (13). Instead of using gDNA concentration directly, resting spore concentration was used to develop the standard curve (StepOne Software v.2.0; Applied Biosystems) and estimates of spore concentrations were generated as the output for C_q values. However, the software

Table 3. Physical characteristics (proportion of organic matter [OM], sand, silt, and clay), texture, and pH of the soil types used to test the efficacy of the multiplexed TaqMan assay

Location[a]	Soil texture	pH	OM (%)	Sand (%)	Silt (%)	Clay (%)
Elora, ON	Silt loam	7.3	2.8	35	54	11
Saskatoon, SK	Loam	5.3	4.5	44	43	13
MCRS, ON	Sandy loam	5.6	81.4	50	45	5

[a] Soil at Elora is a London loam (aquic hapludalf), at Saskatoon is a Sutherland clay-loam (vertic haploborolls), and at the Muck Crops Research Station (MCRS) is a muck soil (hemic histosol). The description of soil texture and mineral components from the MCRS refers only to the 18.6% mineral component of the soil.

generally did not estimate spore concentrations for samples with a C_q value >35. It is noteworthy that the lowest value on the standard curve was 10^3 spores (C_q 33.5) but the linear pattern of the curve continued to C_q 35. Therefore, a C_q value of 35 was selected as a cut-off point for accurately quantifiable data. For the samples with C_q value >35, the approximate estimate was calculated using following formula: $1,000/2^{(C_q\ sample\ -\ C_q\ 1,000\ spores)}$. The formula was generated based on the principle that a 100% efficient reaction in qPCR will yield a 10-fold increase in PCR amplicon every 3.32 cycles ($\log_2 10 = 3.32$) during the exponential phase of amplification (Applied Biosystems). For example, if the Cq of the sample was 35.5 and Cq for 1,000 spores was 33.5, the estimate would be $1,000/2^{(35.5\ -\ 33.5)} = 250$ spores; concentration is reduced by half with each 1-unit increased in Cq. Because these estimates were approximate quantifications, they were referred as "detected, approximately quantified" (DAQ).

Detection of inhibition and normalization of data. The presence of inhibitors in a sample was indicated when the C_q value of the CIPC rose above 21.4. If the C_q value was >24.5 or there was no DNA amplification, the level of inhibition in the sample was considered to be too high to provide an accurate estimate of resting spore concentration, and the sample was diluted and reassessed. However, if the C_q value of the CIPC was between 21.5 and 24.5, inhibition was considered to be low to moderate, and the estimate of resting spore concentration was corrected for inhibition using the formula of Bilodeau (2): correct concentration (n) = the estimate concentration based on the standard curve $x(E + 1)^{\Delta Cq}$, where E = efficiency and $\Delta C_q = C_q$ CIPC sample $- C_q$ CIPC control (water). In the initial assessments using undiluted extracts from soil, there was no amplification of DNA. Therefore, a 10× dilution with molecular-grade water was made for each gDNA sample. Because DNA was extracted from 0.25 and 0.10 g of the mineral and high-OM field soil samples, the initial estimates were multiplied by 4 and 10 for mineral and high-OM soil, respectively, to obtain estimates of resting spores concentration per gram.

Comparison of the SYBR Green and multiplex TaqMan assays. Inoculated mineral soil from the Elora site was used to compare the efficiency of the standard SYBR Green assay (21) with the multiplex TaqMan assay for detection and quantification of *P. brassicae*. The qPCR reaction conditions for the multiplex assay were as described above, while the SYBR Green assay was run in a 10-μl volume containing 2 μl of template DNA solution, 2.5 μl of 3.2 mM primer mix, and 5.5 μl of SYBR Green PCR Master mix (Applied Biosystems)

as the detection dye. Reaction conditions consisted of an initial heat denaturation step at 95°C for 2 min followed by 35 cycles of 95°C for 15 s and 60°C for 60 s. The presence of a single amplification product at the end of each reaction was confirmed by a melting-point analysis.

Statistical analysis. Analysis of variance (ANOVA) and regression analyses were used to describe the relationship and best fit lines (standard curves) for *P. brassicae* gDNA obtained from 10^3 to 10^8 resting spores with or without the IC. The slopes and intercepts of the regression responses of standard curves were compared using Tukey's w procedure, and t tests and single-degree-of-freedom contrasts were used to compare treatment means of initial and corrected concentration estimates obtained in the multiplex assay and among SYBR Green and TaqMan assays. Differences were significant at $P \leq 0.05$ unless otherwise specified. All of the analyses were conducted using SAS software (version 9.3; SAS Inc.).

Results

Primer and probe specificity. The qPCR assay using primers DC1F and DC1mR and probe PB1 amplified gDNA of all of the pathotypes of *P. brassicae* and there was no amplification of gDNA from the fungi tested (Table 1). The results were confirmed by conventional PCR, where distinct 98-kb amplicons were observed for *P. brassicae* pathotypes, whereas no bands were observed with gDNA from the fungal species (data not shown).

Validation of the singleplex qPCR assay. Single-degree-of-freedom contrasts within ANOVA indicated that the standard curve for the gDNA concentrations extracted from suspensions of resting spores of *P. brassicae* (10^3 to 10^8 spores) in a singleplex TaqMan assay was linear (Fig. 1). The standard curve was validated in subsequent assays. Amplification efficiency ranged from 0.92 to 1.06 and there was a strong negative correlation (range 0.99 to 1.0) between C_q value and the $\log_{10}$ of *P. brassicae* resting spore concentration. Fluorometric analysis showed that the gDNA concentrations of the dilution series representing 10^8, 10^7, 10^6, 10^5, 10^4, and 10^3 resting spores was equivalent to 3×10^6, 3×10^5, 3×10^4, 3,000, 300, and 30 fg/μl, respectively ($n = 2$).

Similarly, a linear standard curve was obtained in the singleplex assay on a dilution series of CIPC amplicon concentrations using primers DC1F and DC1mR and CIPC-specific probe (GFP1). However, *P. brassicae* gDNA was not amplified using this combination of primers and probes (data not shown).
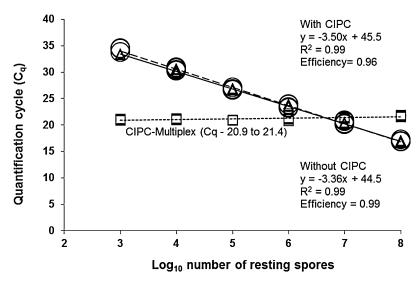

With CIPC
y = -3.50x + 45.5
R^2 = 0.99
Efficiency= 0.96

CIPC-Multiplex (Cq - 20.9 to 21.4)

Without CIPC
y = -3.36x + 44.5
R^2 = 0.99
Efficiency = 0.99

Fig. 1. Standard curves of a *Plasmodiophora brassicae* genomic DNA (gDNA) dilution series with quantification cycle (C_q) value plotted against the $\log_{10}$ number of resting spores used to extract *P. brassicae* gDNA. Values represent means of two experimental units with technical duplication of each unit ($n = 2$). Triangles and regression analysis represent amplification without the competitive internal positive control (CIPC) (standard error [SE] = 0.20), circles and regression analysis reflect amplification with CIPC (SE = 0.28), and the squares represent CIPC values (ranging from 20.9 to 21.4, SE = 0.16) when multiplexed with gDNA obtained from various concentrations of *P. brassicae* spores. The standard deviation of the C_q values of the *P. brassicae* gDNA concentrations for the technical duplicates corresponding to 10^8, 10^7, 10^6, and 10^5 resting spores ranged from 0.01 to 0.3, and from 10^4 and 10^3 resting spores ranged from 0.1 to 0.45, with and without CIPC.

Validation of the multiplex qPCR assay. The reaction conditions for coamplification of *P. brassicae* and the CIPC were determined after optimizing the reaction as a singleplex assay. Using these levels as a base, a range of concentrations of primers, probes, and nucleotides was assessed with a range of CIPC concentrations. Based on these assessments, CIPC DNA at 5 fg/μl per reaction provided consistent amplification (C_q value between 20.9 to 21.4) over a range of *P. brassicae* DNA concentrations from 10^3 to 10^8 spores, without reducing assay sensitivity or reproducibility. Inclusion of CIPC DNA did not affect the amplification efficiency, slope, or intercept of the *P. brassicae* standard curve ($n = 2$) (Fig. 1).

Inoculated and naturally infested soils. In the initial assessments of DNA extracted from inoculated soil samples without dilution, there was no DNA amplification. Even when diluted 10-fold, the C_q values for amplification of CIPC DNA in Elora and Saskatoon soil extracts were substantially higher than the control (Table 4). After normalization, based on ΔC_q, the corrected concentration estimates were significantly higher than the initial concentration estimates in diluted samples, except for one mineral soil sample inoculated with a low concentration (10^3 spores/g of soil) of resting spores. Because ΔC_q was never greater than 3.3 in the diluted samples, no further dilution was needed for the samples.

The C_q values of the gDNA obtained from soil samples inoculated with 10^8, 10^7, 10^6, 10^5, and 10^4 spores per 0.25 g and diluted 10-fold were <35. Similarly, samples of gDNA taken from soil inoculated with 10^3 spores per 0.25 g and diluted 10-fold also generally amplified successfully. The estimated concentration from the latter samples was about 20 spores (or 200 spores, after taking into account the dilution factor; Table 4). However, the C_q values of these samples were >35; therefore, the estimates were categorized as DAQ. There was no amplification of *P. brassicae* gDNA from diluted (5- or 10-fold) samples of mineral soil or high-OM soil inoculated with 100 spores per 0.25 g. Amplification of *P. brassicae* gDNA containing 500 spores per 0.25 g was observed inconsistently: five of six (in 5-fold dilutions) and two of six (in 10-fold dilutions) in mineral soil, and three of six (in 5-fold dilutions) and one of six (in 10-fold dilutions) in high-OM soil samples.

In the high-OM soil, gDNA was not amplified from 0.25-g samples (Table 5). At 10-fold dilution, the CIPC DNA was amplified with a high C_q value but *P. brassicae* gDNA was not amplified. When a range of sample sizes was assessed, *P. brassicae* gDNA was amplified from sample weights below 0.15 g in 10-fold diluted samples (Table 5). As a result, 0.10 g was selected as the sample weight for subsequent experiments with high-OM soil (Table 6). After normalization based on ΔC_q, the corrected concentration estimates were significantly higher than the initial estimates (Table 6).

The multiplex assay amplified *P. brassicae* gDNA from all three naturally infested field soils. As observed previously with inoculated soils, after normalization based on ΔC_q, the corrected concentration

estimates were significantly higher than the initial estimates (Table 7). No DNA amplification was observed in sample 23-1, which was collected from a field in Quebec where no clubroot was observed in a susceptible canola cultivar the previous year (Table 8) and, therefore, was believed to be free of resting spores of *P. brassicae*.

Comparison of SYBR Green and multiplex TaqMan assays. Based on single-degree-of-freedom contrasts, there were no differences in the unadjusted estimates of resting spore concentrations for most of the treatments between the SYBR Green and multiplex TaqMan assays (Table 8). After adjustment for inhibition, however, the corrected estimates from the multiplex assay were substantially higher than initial (unadjusted) estimates from the SYBR Green and TaqMan assays (Table 8). There was no difference in the detection threshold of the two assays.

Discussion

The multiplex qPCR TaqMan assay developed in this study provided sensitive quantification of resting spores populations of *P. brassicae* from a range of soil types. This is the first assay for *P. brassicae* that includes a multiplex CIPC. An amplicon of a plasmid containing a variant of the GFP gene (*GPFuv1*) was developed as the CIPC. The concentration of the CIPC in each sample is low to minimize competition for the primer and nucleotide pools. Inclusion of the CIPC in the multiplex reaction provided quantification of *P. brassicae* gDNA over a wide range (10^3 to 10^8) of resting spore concentrations and quantification of the level of inhibition in each sample based on the difference in the CIPC C_q values relative to a CIPC-only control. Quantification of the inhibition in each sample was used to improve estimates of resting spore populations, and

Table 5. Estimates of *Plasmodiophora brassicae* resting spore concentration from high organic matter (approximately 80%) soil from the Muck Crops Research Station, ON inoculated with 1.0×10^8 resting spores and assessed in a TaqMan quantitative polymerase chain reaction assay using primers DC1F and DC1mR

	Estimates of resting spore concentration[a]		
Weight of soil (g)	ΔC_q	Initial[b]	Corrected
0.25	0.8	NA	…
0.20	1.2	NA	…
0.15	1.3	0.29×10^8	$0.94 \times 10^{8*}$
0.10	1.1	0.36×10^8	$0.97 \times 10^{8*}$
0.05	1.5	0.27×10^8	$0.91 \times 10^{8*}$

[a] There was no amplification of DNA from the initial samples, so they were diluted 1:10. ΔC_q = quantification cycle (C_q) internal control (IC) sample – C_q IC control (water). NA = no amplification. Asterisk (*) indicates that the corrected concentration estimates differed at $P < 0.05$ from the initial concentration estimates based on single-degree-of-freedom contrasts.

[b] Each value is the mean of two biological replicates ($n = 2$), each with technical replicates, after taking into account the 10-fold dilution factor.

Table 4. Estimates of *Plasmodiophora brassicae* resting spore concentrations using a multiplexed TaqMan quantitative polymerase chain reaction assay (primers DC1F and DC1mR) in artificially inoculated mineral soil collected from Elora, ON and Saskatoon, SK

	Estimates of resting spore concentration[a]					
	Elora			Saskatoon		
Number of spores added[b]	ΔC_q	Initial[c]	Corrected	ΔC_q	Initial[c]	Corrected
1×10^8	2.3	0.23×10^8	$0.92 \times 10^{8*}$	1.2	0.32×10^8	$0.85 \times 10^{8*}$
1×10^7	2.9	0.16×10^7	$0.95 \times 10^{7*}$	0.6	0.57×10^7	$1.03 \times 10^{7*}$
1×10^6	1.5	0.42×10^6	$1.07 \times 10^{6*}$	0.8	0.45×10^6	0.88×10^6
1×10^5	2.1	0.30×10^5	$0.89 \times 10^{5*}$	1.2	0.35×10^5	$0.94 \times 10^{5*}$
1×10^4	1.4	0.41×10^4	$1.01 \times 10^{4*}$	1.0	0.42×10^4	$0.92 \times 10^{4*}$
1×10^3	2.2	215[d]	860*	0.9	369[d]	776*
1×10^2	2.3	ND	…	1.0	ND	…

[a] There was no amplification of DNA from the initial samples, so they were diluted 1:10. ΔC_q = quantification cycle (C_q) internal control (IC) sample – C_q IC control (water). An asterisk (*) indicates that the corrected concentration estimates differed at $P < 0.05$ from the initial concentration estimates based on single-degree-of-freedom contrasts. ND = not detected.

[b] Spores were inoculated in 0.25 g of soil.

[c] Each value is the mean of two biological replicates ($n = 2$), each with technical replicates, after taking into account the 10-fold dilution factor.

[d] These values lay outside of the optimized level of quantification, so they were considered "detected, approximately quantified".

could be used to rapidly identify samples that required further dilution or even reextraction to reduce inhibition.

Most studies on quantification of a pathogen using qPCR estimate DNA weight or copies of the pathogen in the target samples based on a standard curve derived from DNA concentrations expressed as weight or copies of the pathogen (1,3,14,20). This approach is required for pathogens that occur in soil or plant debris in two or more distinct forms (e.g., asexual and sexual spores) and for quantification of a pathogen in planta that has variable shape or size. However, *P. brassicae* occurs in dry soil only as uniform-sized, single-celled resting spores. For *P. brassicae*, resting spore concentration is directly equivalent to copy number or weight of amplified DNA. This equivalency was utilized to estimate *P. brassicae* concentration in soil samples based on a standard curve expressed as resting spore concentrations of *P. brassicae*. A similar methodology has been used to estimate populations of *Enterococcus* bacteria from fresh water samples (13).

One application of the multiplex assay with CIPC in each sample will be in research, where accurate estimates of the resting spore population may be as important as knowing that the pathogen is present. However, the most important application on the Northern Great Plains, where *P. brassicae* is spreading quickly (24), will be to reduce the potential for false-negative results when identifying infested fields. False-negative results are particularly damaging because they justify continuation of normal production at sites where the pathogen is present. This increases the likelihood that the pathogen population will increase and be dispersed before it is detected.

Inclusion of a CIPC in qPCR assays has been used to reduce false-negative results in assessments of pathogens where there is potential for inhibition (e.g., the target is often out-competed on media by fast-growing organisms) (1). False-negative results, which are a potential issue with the standard qPCR assay for *P. brassicae* in soil, could result from inhibition of PCR amplification by humic acids, enzymatic

Table 6. Estimates of *Plasmodiophora brassicae* resting spores from high-organic-matter (approximately 80%) soil from Muck Crops Research Station, ON from a TaqMan quantitative polymerase chain reaction assay using primers DC1F and DC1mR

Number of spores added[b]	Estimates of spore concentration[a]		
	ΔC_q	Initial[c]	Corrected
1×10^8	2.2	0.18×10^8	0.87×10^8*
1×10^7	1.8	0.29×10^7	1.07×10^7*
1×10^6	1.9	0.22×10^6	0.88×10^6*
1×10^5	2.1	0.20×10^5	0.92×10^5*
1×10^4	1.9	0.25×10^4	0.96×10^4*
1×10^3	2.4	196[d]	1,042*
1×10^2	2.1	ND	…

[a] There was no amplification of DNA from the initial samples, so they were diluted 1:10. ΔC_q = quantification cycle (C_q) internal control (IC) sample – C_q IC control (water). An asterisk (*) indicates that the corrected concentration estimates differed at $P < 0.05$ from the initial concentration estimates based on single-degree-of-freedom contrasts. ND= not detected.
[b] Spores were inoculated in 0.25 g of soil.
[c] Each value is the mean of two biological replicates ($n = 2$), each with technical replicates, after taking into account the 10-fold dilution factor
[d] These values were out of optimized level of quantification, so they were considered "detected, approximately quantified".

Table 7. Estimates of *Plasmodiophora brassicae* resting spore concentration from selected field soils using the multiplexed TaqMan assay with primers DC1F and DC1mR

Location (soil type)	Sample ID	Estimates of spore concentration[a]		
		ΔC_q	Initial[b]	Corrected
Normandin QC (Labarre silty clay)	23-1	1.2	ND	…
	23-4	1.9	2.66×10^6	8.78×10^6*
	28-3	2.6	1.23×10^6	6.15×10^6*
	29	2.3	5.51×10^4	2.64×10^5*
	Drapeau	0.6	3.13×10^3	3.64×10^3
Edmonton AB (Black Chernozemic loam)	1	2.1	4.72×10^4	1.42×10^5*
	2	2.3	7.64×10^4	3.06×10^5*
Muck Crops Research Station ON (Hemic Histosol)	Range 4-1	2.4	2.3×10^4	1.17×10^5*
	Range 4-2	1.7	8.1×10^4	2.10×10^5*

[a] There was no amplification of DNA from the initial samples, so they were diluted 1:10. ΔC_q = quantification cycle (C_q) internal control (IC) sample – C_q IC control (water). ND = not detected. An asterisk (*) indicates that the corrected concentration estimates differed at $P < 0.05$ from the initial concentration estimates based on single-degree-of-freedom contrasts.
[b] Each value is the mean of two biological replicates ($n = 2$), each with technical replicates, after taking into account the 10-fold dilution factor.

Table 8. Comparison of the estimates from SYBR Green and multiplexed TaqMan assays (using the same primers, DC1F and DC1mR) of *Plasmodiophora brassicae* resting spore concentration using an inoculated mineral soil from Elora ON

Number of spores added[b]	SYBR Green (no CIPC)[c]	TaqMan with CIPC[a]		
		ΔC_q	Initial[c]	Corrected
1×10^8	0.22×10^8	1.9	0.24×10^8	0.89×10^8**
1×10^7	0.36×10^7	1.2	0.32×10^7	0.96×10^7**
1×10^6	0.31×10^6	1.8	0.23×10^6*	0.85×10^6**
1×10^5	0.16×10^5	1.3	0.31×10^5*	0.93×10^5**
1×10^4	0.23×10^4	2.1	0.19×10^4	0.88×10^4**
1×10^3	324[d]	1.5	246[d]	812*
1×10^2	ND	1.7	ND	…

[a] CIPC = competitive internal positive control. ΔC_q = quantification cycle (C_q) internal control (IC) sample – C_q IC control (water). A single asterisk (*) indicates that the values obtained in the TaqMan assay differed at $P < 0.05$ from the SYBR Green based on single-degree-of-freedom contrasts. A double asterisk (**) indicates that the corrected concentration estimates differed at $P < 0.05$ from the initial concentration estimates in both assays based on single-degree-of-freedom contrasts. ND = not detected.
[b] Spores were inoculated into 0.25 g of soil.
[c] There was no amplification of DNA from the initial samples, so they were diluted 1:10. Each value is the mean of two biological replicates ($n = 2$), each with technical replicates, after taking into account the 10-fold dilution factor.
[d] These values were outside of the optimized level of quantification, so they should be considered "detected, approximately quantified.

inhibitors, phenolic compounds, heavy metals, nontarget DNA, and contaminants, and even clay particles in soil (2,18). Dilution of the extracted DNA reduces the effect of inhibitors in many samples. However even after dilution, inhibition of *P. brassicae* gDNA amplification occurred in samples from all of the soil types assessed. Use of the CIPC to calibrate the inhibition provided a more accurate assessment of the level of *P. brassicae* in each sample but also identified samples where additional purification steps are required to produce a reliable assessment.

A CIPC competes with the amplification target for all the resources in a reaction mix, including primers (3); therefore, choosing the correct C_q for CIPC to detect DNA amplification inhibition due to PCR inhibitors in the sample is important. A range of C_q values for CIPC varying from 20 to 30 has been used by previous researchers in multiplex detection assays (3,12,13,19,23). In the current assay, C_q values for CIPC concentrations from 21 to 27 could be used to detect DNA amplification inhibition in a sample (data not shown) but changing the C_q value may affect the corrected estimate of spore concentration.

One disadvantage of diluting a sample is that, at very low levels of inoculum, dilution may take the sample below the level of detection (2). In the current study, pathogen detection was inconsistent for samples containing 100 or 500 spores per 0.25 g of soil. Reducing dilution as a strategy to increase the frequency of detection of low concentrations of resting spores might have a negative impact on quantification, because samples with 5-fold dilution generally had a higher ΔC_q value than 10-fold dilution. A high ΔC_q indicates that confidence in the quantification is reduced. Similarly, this approach would not be effective for samples where the target gDNA amplification is completely inhibited. Additional purification steps (e.g., magnetic beads and columns) could be included to maximize removal of PCR inhibitors (3) and increase detection sensitivity. In high-OM soils, humic acid is reported as a major cause of the DNA inhibition and specific procedures are often required to extract and amplify DNA from these samples (33). In the initial assessments of resting spore populations at the heavily infested OM site, no amplification of target gDNA was observed. However, the CIPC was amplified, which ruled out that humic acid as the cause of inhibition of DNA amplification. By assessing factors such as time and speed of the homogenization and weight of the sample, it was determined that the weight of the sample had an important impact on DNA amplification in the high-OM samples. High-OM soils are lighter and have a larger volume per gram than mineral soils; therefore, the quantity of buffer (0.5 ml) provided in the DNA isolation kit was not adequate for this large volume of soil. We concluded that use of a smaller (0.1 g) amount of high-OM soil is more suitable for the amount of the buffer provided in the extraction tubes. Kits that allow larger amounts of soil to be processed are available but would substantially increase the cost of the assay.

In the comparison of the SYBR Green and multiplex TaqMan assays, the initial estimate of gDNA from inoculated mineral soil was similar. The difference in the assays was the presence or absence of a CIPC. Estimates from the multiplex assay after correction for inhibition were substantially and consistently higher (two to seven times) than from the SYBR Green assay. Without a CIPC, resting spore concentrations could be underestimated because of the presence of inhibitors, even when the samples were diluted (12). There was no difference in the detection limit of the two assays, likely as a result of using the same primer set.

In this study, an accurate and sensitive one-step multiplex TaqMan assay was developed for detection and quantification of *P. brassicae* gDNA in mineral soils. Also, a modification of the assay was developed for use in soils with high OM content. This one-step multiplex assay represents an improvement over previous assays in that the presence of a CIPC in the multiplex saves time and resources by permitting correction of estimates in a single run when inhibition is low to moderate. Inclusion of the CIPC in this assay provides more accurate estimates of resting spore populations in soil but should also reduce the potential for false negatives caused by DNA amplification inhibitors, which can have important consequences for subsequent clubroot management strategies.

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