Monitoring of *Peronospora destructor* Primary and Secondary Inoculum by Real-Time qPCR

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Abstract

Onion downy mildew (ODM), caused by *Peronospora destructor*, is a serious threat for onion growers worldwide. In southwestern Québec, Canada, a steady increase in occurrence of ODM has been observed since the mid-2000s. On onion, *P. destructor* can develop local and systemic infections producing numerous sporangia which act as initial inoculum locally and also for neighboring areas. It also produces oospores capable of surviving in soils and tissues for a prolonged period of time. A recent study showed that ODM epidemics are strongly associated with weather conditions related to production and survival of overwintering inoculum, stressing the need to understand the role of primary (initial) and secondary inoculum. However, *P. destructor* is an obligate biotrophic pathogen, which complicates the study of inoculum sources. This study aimed at developing a molecular assay specific to *P. destructor*, allowing its quantification in environmental samples. In this study, a reliable and sensitive hydrolysis probe-based assay multiplexed with an internal control was developed on the internal transcribed spacer (ITS) region to quantify soil- and airborne inoculum of *P. destructor*. The assay specificity was tested against 17 isolates of *P. destructor* obtained from different locations worldwide, other members of the order Peronosporales, and various onion pathogens. Validation with artificially inoculated soil and air samples suggested a sensitivity of less than 10 sporangia g^{-1} of dry soil and 1 sporangium m^{-3} of air. Validation with environmental air samples shows a linear relationship between microscopic and real-time quantitative PCR counts. In naturally infested soils, inoculum ranged from 0 to 162 sporangia equivalent g^{-1} of dry soil, which supported the hypothesis of overwintering under northern climates. This assay will be useful for primary and secondary inoculum monitoring to help characterize ODM epidemiology and could be used for daily tactical and short-term strategic decision-making.

Keywords: network, oomycetes, surveillance

Among all vegetables grown in Canada, onion (*Allium cepa* L.) ranks third behind carrot and tomato, with a farm gate value of nearly CAD$95 million (Mailvaganam 2017). In Canada, 80% of the onion production is grown in the muck soils (chernozem) of Ontario and southwestern Québec. Typically, onion crops are sown at the end of April and harvested in September, with yields up to 60 tons/ha. During the growing season, the crop is highly susceptible to many airborne diseases, including Botrytis leaf blight (*Botrytis squamosa*), Stemphylium leaf blight (*Stemphylium vesicarium*), and onion downy mildew (ODM) (*Peronospora destructor*), the latter being one of the most preoccupant, with yield losses reaching up to 75% (de Araújo et al. 2017; Develash and Sugha 1997). The disease was reported in almost all production areas but is more severe in temperate climates (de Araújo et al. 2017; Develash and Sugha 1997; Fitz and O'Brien 1994; O'Brien 1992; Yarwood 1943). In southwestern Québec, the disease has been observed since the mid-2000s, with regional disease incidence reaching up to 33% of onion fields diseased in 2014 (Van der Heyden et al. 2020).

ODM is caused by the oomycete *P. destructor* (Berk.) Casp. ex Berk. The first symptoms appear as pale-green to yellow, elongated ovoid lesions harboring violet-gray sporulation, with infected leaves rapidly decaying, turning from green to pale green to yellow and eventually collapsing. Affected plants eventually dry standing, affecting yields and storage potential. The disease often begins in small foci and spreads rapidly to surrounding areas. Infection by *P. destructor* requires a continuous period of leaf wetness and temperatures between 5 and 27°C, with optimum temperatures being between 10 and 18°C (Hildebrand and Sutton 1984a). After infection, symptoms and new sporulation appear following a latency period of 13 to 17 days, depending on temperatures (Hildebrand and Sutton 1984b). The sporulation of *P. destructor* is a process dependent on photoperiod (Yarwood 1937), requiring relative humidity greater than 95% and night temperature between 4 and 25°C (Hildebrand and Sutton 1982). At maturity, sporangia can be vigorously discharged into the air in response to a reduction of relative humidity (hydrostatic movement) but the dispersal is mostly wind driven (Leach 1982; Leach et al. 1982). Hence, daily airborne concentrations of *P. destructor* sporangia generally follow a unimodal distribution, beginning early in the morning about 1.5 h after sunrise, and reaching their maximum as temperature increases and relative humidity decreases (Hildebrand and Sutton 1982).

Although *P. destructor* sporangia are largely responsible for seasonal development and spatial dispersion of the disease (secondary inoculum), information about sources of primary inoculum is limited. It was suggested that *P. destructor* can survive as mycelium on infected onion sets and volunteer plants (Hildebrand and Sutton 1980). However, like other oomycetes, *P. destructor* also reproduces sexually as oospores, although it is not known if survival in soil and crop debris is due to homothallism, heterothallism, or both. (Judelson 2008; Palti 1989). Actually, *P. destructor* oospores have been shown to remain viable for up to 25 years (McKay 1957), a record for the genus (Judelson 2008). In a recent study, Van der Heyden et al. (2020) showed that ODM epidemics in southwestern Québec were more frequent over the last decade and occurred earlier compared

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with earlier periods (1987 to 1996 and 1997 to 2006). The results of that study also suggested that ODM incidence is closely related to weather variables associated with the production and survival of overwintering structures. These findings emphasized the importance of inoculum sources and the need to better understand the role of both primary and secondary inoculum in the ODM epidemics.

Being able to detect and quantify inoculum is critical for effective disease management. Moreover, the study of primary inoculum sources (oospores) is difficult without appropriate detection tools. The only assay available so far for the detection of *P. destructor* sporangia is based on a monoclonal antibody used in a lateral flow device (Kennedy and Wakeham 2008). However, although its usefulness has been demonstrated for detection in planta, the assay has a detection limit of about 500 sporangia, which might be too high for adequate disease risk estimation. In addition, monoclonal techniques are time consuming and less cost effective than PCR-based methods. The measurement of plant pathogen inoculum in different epidemiological substrates (air, soil, seed, water, and plant) can be performed through classical methods such as direct microscopic observations or by direct plating on selective culture media (Hendrix and Campbell 1973; Lacey and West 2006). These methods have been proven to be limiting for airborne and soilborne diseases mainly because of the difficulties in identifying pathogens at the species level (Bilodeau 2011; Lievens and Thomma 2005; West et al. 2008). In addition, culture-dependent approaches rely on the capacity of the pathogen to grow on artificial media and, therefore, are not suitable for obligate biotrophic pathogens such as *P. destructor*.

Molecular approaches, especially PCR, can circumvent many of these limitations. In general, PCR-based methods are fast, sensitive, accurate, and, more importantly, they allow for detection of nonculturable plant pathogens. These methods include endpoint PCR, restriction fragment length polymorphism PCR, microarrays, sequencing, pyrosequencing, real-time quantitative PCR (qPCR) (SYBR green or probe based), and next-generation sequencing. Among the downsides of PCR-based approaches, they do not allow discrimination between viable and nonviable or between infectious and noninfectious propagules. However, for many reasons, probe-based real-time qPCR is considered to be the gold standard for specific pathogen quantitation: it takes significantly less processing time than the other PCR methods or sequencing; it is more specific than endpoint PCR or SYBR green real-time qPCR; and it can be multiplexed, which allows the use of internal controls (ICs) to measure reaction efficiency or inhibition (Crandall et al. 2018).

Research and development of real-time qPCR-based markers were conducted for several species belonging to the *Phytophthora* genus, especially following the finding of the genus-specific *atp9-nad9* loci (Bilodeau et al. 2014; Miles et al. 2017). Several assays were also developed for species belonging to the genus *Pythium*, although some species are indistinguishable from one another using the usual barcodes (Schroeder et al. 2013; Tambong et al. 2006). However, despite their economic importance in agriculture, only a few assays were developed for other members of the order Peronosporales such as *Bremia lactucae* (Kunjeti et al. 2016), *P. effusa* and *P. schachtii* (Klosterman et al. 2014), and *P. arborescens* (Montes-Borrego et al. 2011). These PCR-based assays were extremely sensitive (10 to 100 gene copy number) and specific to the target species.

The availability of a sensitive and specific molecular tool for detecting the primary and secondary inoculum of *P. destructor* in the environment is critical for the acquisition of knowledge of ODM epidemics and the pathogen's ability to overwinter. Therefore, this study aimed at developing a specific multiplex real-time hydrolysis probe qPCR assay for *P. destructor* identification and quantification in soil or air samples. The specificity and sensitivity of the developed assay was validated on fungal and oomycete DNA and artificially and naturally infested soil and air samples.

Materials and Methods

Isolate collection. *P. destructor* isolates were collected on diseased onion from fields primarily located in southwestern Québec (MRC Des Jardins de Napierville, 73°24′ to 73°37′ West, 45°10′ to 45°16′ North) and, to a lesser extent, from other regions, including France, California, and Ontario (Canada) (Table 1). Other Peronosporales isolates were collected from diseased plants in commercial fields, greenhouses, and gardens or obtained from collaborators. Samples consisted of sporangia harvested from the leaf surface using a BBL culture swab (Fisher Scientific, Mississauga, ON, Canada) and placed in a 2.0-ml microcentrifuge tube containing 300 µl of 100% isopropanol. Sporangia suspensions were kept at −20°C until DNA extraction. Nontarget fungal species were also collected from diseased onion plants and obtained from the Quebec provincial diagnostic laboratory (Laboratoire d'expertise et de diagnostic en Phytoprotection, MAPAQ, QC, Canada).

DNA extraction and sequencing. Total DNA was extracted from sporangial suspension using the DNA extraction procedure described by Carisse et al. (2009). Briefly, to disrupt the sporangia wall, 80 µl of the sporangial suspension was homogenized in a 2-ml microcentrifuge tube containing 100 mg of 425- to 600-µm glass beads (Sigma-Aldrich Canada Ltd., ON, Canada), in a Fastprep instrument (MP Biomedicals, OH, U.S.A.) for 40 s at a speed setting of 6.0 m s^{-1}, followed by a boiling step (20 min at 105°C) in 300 µl of a resuspension buffer containing 5% chelex100 (Bio-Rad Laboratories, ON, Canada) and a final centrifugation at 4°C for 5 min at 15,000 × g. Fungal DNA from cultured fungi was obtained using the FastDNA Spin Kit (MP Biomedicals) with the CLS-Y buffer, according to the manufacturer's instructions. Following DNA extraction, the high copy number internal transcribed spacer (ITS) region was amplified by PCR using the primers DC6 (specific for the orders Pythiales and Peronosporales) and ITS4 (Cooke et al. 2000; White et al. 1990). The quality and quantity of extracted DNA were assessed using a Nanodrop lite instrument (Thermo Fisher Scientific, ON, Canada). The PCR contained 1× Phusion High-Fidelity PCR Master Mix with HF Buffer (New England Biolab, MA, U.S.A.), 500 nM each primer, and 5 µl of template DNA. All PCR assays were conducted in a SureCycler 8800 (Agilent Technologies Canada Inc., ON, Canada) in a final volume of 30 µl, and the cycling conditions were 2 min at 98°C; followed by 35 cycles of 10 s at 98°C, 15 s at 60°C, and 30 s at 72°C; and a final extension step of 10 min at 72°C. PCR products were purified using the PureLink PCR purification kit (Thermo Fisher Scientific) and sent to the Centre de Recherche du CHUL/CHUQ at Laval University for Sanger sequencing. The DNA sequences obtained for *P. destructor* and other species of Peronosporales were aligned with a subset of nonredundant sequences selected from GenBank, using the MAFFT alignment method available in Geneious V9.1.5 (Katoh et al. 2002).

Primer and probe design. The DNA alignment was first examined to find regions highly conserved among *P. destructor* isolates and bearing the most difference from other species. The primers and hydrolysis probe were designed using Primer Express (V3.0.1) so that nucleotides specific to *P. destructor* were located at the 3′ end of the primers and in the middle of the hydrolysis probe (Bilodeau et al. 2012). Moreover, a difference of 10°C between the primer melting temperature (Tm) and the probe Tm was respected. A first evaluation of primer and probe specificity was conducted in silico by running the primer sequences against a nonredundant GenBank dataset with parameters set for the identification of short, nearly exact matches. Second, a neighbor-joining tree analysis using the Tamura-Nei substitution model with 100 bootstraps was used to illustrate the sequence dissimilarities in the *P. destructor* ITS1 region selected for the assay design, and identify the closest *Peronospora* spp. to be used in the validation assay. Then, the specificity of the primer and probe was assessed with DNA of *Peronospora* spp. closely related to *P. destructor*, other Peronosporales species, and pathogens isolated from onion.

IC and assay sensitivity. To detect any variation in the ability of the real-time qPCR assay to detect and quantify inoculum due to the presence of PCR inhibitors, an IC was multiplexed to the *P. destructor* assay (Bilodeau et al. 2012; Fall et al. 2015a; Van der Heyden et al. 2019). The IC consist of a random DNA sequence added at a constant concentration to the real-time qPCR mixture. Hence, a second set of primers and probes aimed at amplifying the IC (EIPC1MT)

was multiplexed with the principal assay (Fall et al. 2015a) to determine the highest concentration of IC to be added to the real-time qPCR mixture without affecting the principal real-time qPCR efficiency. The assessment of the assay sensitivity was conducted with a fivefold serial dilution of a *P. destructor* sporangia suspension ranging from 15,625 to 1 sporangium and with a gBlock-based standard curve of the ITS1 region ranging from 6×10^6 to 6×10^1, with and without the IC. The gBlock consist of a 178-bp synthetic DNA fragment (IDT DNA Technologies, IA, U.S.A.) that includes the region amplified by the primer and probe set and converted into copy number [(concentration of gBlock)/(molar mass) $\times (6.022 \times 1023)$] (Carisse et al. 2009). DNA extraction from sporangia was performed following the protocol described above, except that resuspension buffer contained 5% chlex100 (Bio-Rad Laboratories), UltraPure Salmon Sperm DNA (Thermo Fisher Scientific) at 10 ng μl^{-1}, and the EIPC1MT gBlock gene fragment at 2×10^2 copies μl^{-1}. The dilution buffer was the same as the extraction buffer without the chelex100 resin. Linear regression analysis was used to describe the relationship and best fit line for *P. destructor* sporangia and amplicon-based standard curves with and without the IC and a *t* test was used to compare the slopes and intercepts of the regression curves obtained.

Real-time qPCR conditions. The real-time qPCR assay was conducted in a Quantstudio 3 instrument (Thermo Fisher Scientific) with the following cycling conditions: 5 min at 95°C followed by 40 cycles of 30 s at 95°C and 30 s at 62°C. Each reaction consisted of 1× ECO master mix (Thermo Fisher Scientific), 250 nM PdestF and PdestR primers, 150 nM PdestP probe, 200 nM oEIPC 100R and EIPC 100F primers, 100 nM the EIPC probe (Table 2), bovine serum albumen at 0.2 μg/μl, and an additional 6 mM $MgCl_2$. For the soil samples, 600 copies of the IC were also added to the real-time qPCR master mix, unlike the air samples, where it was added to the extraction buffer.

Validation of the assay with artificially inoculated samples. The sensitivity of the designed assay for soil was evaluated by inoculating soil samples with a known quantity of *P. destructor* sporangia. These sporangia were collected from infected onion leaves bearing fresh sporulation using a BBL sampling swab (Fisher Scientific). Sporangia were resuspended in sterile distilled water and the suspension concentration was adjusted with an hemocytometer to obtain a concentration of 10,000 spores ml^{-1}. Then, sterilized soil samples, obtained after two heating cycles at 121°C for 20 min each, were inoculated with this suspension to obtain concentrations of 5,000, 1,000, 500, 100, 50, and 10 sporangia g^{-1} of dry soil. DNA extraction from soil samples were achieved with the FastDNA spin

Table 1. Isolates used to test the specificity of the designed assay

ID	SI[a]	Species	Host	Origin	DC6/ ITS4[b]	Pdest-F/R Pdest_P[b]
Qc1	1	*Peronospora destructor*	Dry bulb onion	Sherrington, Canada	+	+
Qc2	1	*P. destructor*	Dry bulb onion	Sainte-Clothilde, Canada	+	+
Qc3	1	*P. destructor*	Bunching onion	Sainte-Clothilde, Canada	+	+
Qc4	1	*P. destructor*	Bunching onion	Hemingford, Canada	+	+
Qc5	1	*P. destructor*	Dry bulb onion	Sherrington, Canada	+	+
Qc6	1	*P. destructor*	Bunching onion	Sainte-Clothilde, Canada	+	+
Qc7	1	*P. destructor*	Dry bulb onion	Sherrington, Canada	+	+
Qc8	1	*P. destructor*	Bunching onion	Sainte-Clothilde, Canada	+	+
Qc9	1	*P. destructor*	Dry bulb onion	Sherrington, Canada	+	+
Qc10	1	*P. destructor*	Dry bulb onion	Sherrington, Canada	+	+
Qc11	1	*P. destructor*	Dry bulb onion	Sainte-Clothilde, Canada	+	+
Qc12	1	*P. destructor*	Dry bulb onion	Sherrington, Canada	+	+
Qc13	1	*P. destructor*	Dry bulb onion	Sherrington, Canada	+	+
Qc14	1	*P. destructor*	Dry bulb onion	Napierville, Canada	+	+
Qc15	1	*P. destructor*	Dry bulb onion	Sherrington, Canada	+	+
Qc16	1	*P. destructor*	Bunching onion	Sainte-Clothilde, Canada	+	+
Qc17	1	*P. destructor*	Dry bulb onion	Sherrington, Canada	+	+
FR1	2	*P. destructor*	Shallots	Bretagne, France	+	+
FR2	2	*P. destructor*	Shallots	Bretagne, France	+	+
CA1	3	*P. destructor*	Dry bulb onion	California, U.S.A.	+	+
ON1	5	*P. destructor*	Dry bulb onion	Ontario, Canada	+	+
	4	*P. ducometi*	Buck wheat	Washington	+	−
	1	*P. farinosa*	Chenopodium	Napierville, Canada	+	−
	1	*P. effusa*	Spinach	Napierville, Canada	…	…
	1	*P. belbahrii*	Basilic	Ste-Hyacinthe, Canada	+	−
	2	*P. belbahrii*	Basilic	Bretagne, France	+	−
	1	*P. arborescens*	Poppy	Ste-Hyacinthe, Canada	+	−
	1	*P. lamii*	Salvia	Ste-Hyacinthe, Canada	+	−
	1	*Pseudoperonospora cubensis*	Cucumber	Leamington, Canada	…	…
	2	*Peronospora viciae*	Pea	Bretagne, France	+	−
	2	*Hyaloperonospora parasitica*	Radish	Bretagne, France	+	−
	1	*Plasmopara viticola*	Grape	Napierville, Canada	+	−
	1	*Bremia lactucae*	Lettuce	Napierville, Canada	+	−
	1	*Phytophthora infestans*	Potato	Napierville, Canada	+	−
	1	*P. capcici*	Cucurbit	Joliette, Canada	+	−
	1	*Aphanomyces euteiches*	Pea	Trois-Rivières, Canada	+	−
	1	*Botrytis cinerea*	Strawberry	Ste-Hyacinthe, Canada	+	−
	1	*B. squamosa*	Onion	Napierville, Canada	+	−
	1	*Stemphylium versicarium*	Onion	Napierville, Canada	+	−
	2	*Fusarium oxysporum*	Onion	Bretagne, France	+	−

[a] Sample isolates (SI) were obtained from 1: This study; 2: Dr. Celine Hamon, Vegenov, St-Pol de Leon, Bretagne, France; 3: Dr. Alexander Putman, University of California, Riverside, CA, U.S.A.; 4: Dr. Lindsay du Toit, Washington State University, Mount Vernon, WA, U.S.A.; and 5: Mr. Travis Cranmer, OMAFRA, Ontario, Canada.
[b] DC6/ITS4 and Pdest-F/R Pdest_P are primer/probe combinations.

kit for soil (MP Biomedicals) as described by Van der Heyden et al. (2019) and quantification of *P. destructor* sporangia was performed with the developed procedure. This experiment was repeated three times. The results of this experiment were used to predict the number of sporangia equivalent per gram of dry soil. To achieve this, the predicted number of sporangia per gram of dry soil was regressed against the predicted value of ITS copy number for quantification threshold (Cq) values of 16 to 33.

To assess the sensitivity of the designed assay for air samples, *P. destructor* sporangia were deposited on spore sampler rods and counted with a microscope at ×200 magnification. Rods containing 1, 2, 3, 7, 10, 15, 25, and 150 sporangia were submitted to the procedure developed in this study. DNA extraction was performed using the procedure described above, and sporangia counts was performed using the real-time qPCR assay. For this experiment, each concentration had three or four biological replicates and two technical replicates.

Validation of the assay with environmental samples. In total, 291 soil samples from commercial fields located in the Napierville County (QC, Canada) were collected in the spring from 2017 to 2019. Field selection was based on ODM frequency over the last decade and crop rotation length. Soil samples were collected and prepared following the protocol described by Van der Heyden et al. (2019). Briefly, soils were sampled according to a random pattern and taken from the first 20 cm from the surface, for a total weight of 250 g/sample. Each sample was air dried at room temperature for 24 to 48 h and homogenized using a mill grinder until a fine powder was obtained. DNA extraction was performed from 200 mg of soil using the method described above.

For airborne samples, a preliminary validation assay was conducted with nine rotating-arm impaction spore samplers (Carisse et al. 2009) installed from 15 June to 13 July 2017 in an onion field where ODM was present. The number of *P. destructor* sporangia in each sample was determined under a microscope at a magnification of ×250 and with the real-time qPCR procedure developed above.

In 2018 and 2019, a second validation was done using samples obtained from a network of spore samplers driven by fresh vegetable growers in Napierville County to estimate the risks associated with *Botrytis squamosa* (Carisse and Van der Heyden 2017; Carisse et al. 2012; Van der Heyden et al. 2012). In 2018, 16 rotating-arm impaction samplers were sampled from 24 May to 16 August, for a total of 560 analyzed samples. In 2019, 15 rotating-arm impaction samplers were used from 2 June to 20 August, for a total of 525 analyzed samples. In 2018 and 2019, the airborne *P. destructor* sporangia concentration was estimated using the real-time qPCR assay developed in this study. The samplers were in operation three times a week from 8:00 to 14:00 h, 50% of the time, for a total of 3,897 liters/air per sample. To investigate the relationship between airborne inoculum and disease incidence, ODM incidence expressed as percentage of onion plants diseased was assessed weekly in each field where a sampler was operated. In addition, the risk of *P. destructor* sporulation was calculated according to the DOWNCAST (DC) predictor (Sutton and Hildebrand 1985).

Statistical analysis. Linear regression analyses were used to describe the relationship and best fit line for *P. destructor* sporangia and gBlock-based standard curves with and without the IC. A one-sided *t* test was used to compare the slopes and intercepts of the regression curves obtained.

The data obtained in the sensitivity tests for soil and air samples were analyzed using an analysis of variance (ANOVA) followed by a least significant difference (LSD) multiple-comparison procedure, to determine whether the Cq values were different for each of the tested concentrations. All statistical analyses were performed using SAS/STAT software (version 9.4; SAS Institute Inc., Cary, NC, U.S.A.).

Results

Primer and probe development. The alignment of 108 sequences (ITS1 region) from 44 species allowed for the identification of single-nucleotide polymorphisms (SNPs) specific to *P. destructor* and for the design of specific primers and probes amplifying a 119-bp fragment (Table 1). Neighbor-joining tree analysis showed that the region chosen for the development of the assay was conserved among *P. destructor* isolates and helped identify *Peronospora* spp. that needed to be included in the validation assay (Fig. 1). These results showed that the portion of the ITS1 sequence of *P.* cf. *ducometii*, *P.* cf. *fagopyri*, and *P.* cf. *polygoni* had the highest percentage of similarity with *P. destructor*. Moreover, for these three species, the section of ITS1 used for the assay design was identical, facilitating the assessment of specificity, given the limited access to obligate biotroph DNA. *P. arborescens* and *P. farinosa* isolates also showed a high level of similarity compared with *P. destructor* (Fig. 1). For these species, the specificity of the assay was conferred by the presence of SNP on the probe and on the forward primer. Moreover, in order to avoid false-positive results, the reaction stringency was increased by optimizing the annealing and extension temperature to 62°C.

Assay specificity, reproducibility, sensitivity, and IC. As a control, all DNA were tested using the DC6-ITS4 (or ITS1-ITS4) primer set. This step was necessary because DNA extraction from *Peronospora* spp. was carried out using a chelex100-based method, and the concentration and purity of DNA obtained with this spectrophotometer-based method is less accurate. The PdestP probe and PdestF/R primers were able to repeatably and reliably amplify *P. destructor* isolates from different sampling years or geographic locations, whereas no amplification occurred with species showing higher sequence similitudes or with the other fungus species tested (Table 2). All isolates used in the validation assay were amplified with the DC6-ITS4 (or ITS1-ITS4) primer set (Table 2).

Both the sporangia-based and the gBlock-based standard curves displayed a linear dynamic range of amplification, suggesting the high sensitivity of the assay. For the sporangia-based standard curve, the slope of the linear regression was −3.531 and the amplification efficacy was 91.96%. For the gBlock-based standard curve, the slope of the linear regression was −3.525 and the amplification efficacy was 92.17% (Fig. 2A). The addition of the IC, optimized at 600 copies, did not affect the amplification efficiency or slope of the linear regression. The slope and amplification efficiency were still −3.528 and 92.06%, respectively, for the sporangia-based standard curve and −3.526 and 92.14% for the gBlock-based standard curve (Fig. 2B).

Table 2. Description of the primers and probes used in this study

Primer/probe names[a]	Nucleotide sequence	Reference
DC6	5′-GAGGGACTTTTGGGTAATCA-3′	Cooke et al. (2000)
ITS4	5′-TCCTCCGCTTATTGATATGC-3′	White et al. (1990)
ITS1	5′-TCCGTAGGTGAACCTGCGG-3′	…
PdestF	5′-CCAACCGAGGTCAGAACACTCG-3′	This study
PdestP (5′-FAM)	5′-CAAAAACATGCCACCAGCAGCCGCCAAGTAA-3′	…
PdestR	5′-CGTGAACCGTATCAACCCAATTAA-3′	…
EIPC100F	5′-AGGCTAGCTAGGACCGATCAATAGG-3′	Fall et al. (2015a)
EIPC100P (5′-HEX)	5′-CCTATGCGTTCCGAGGTGACGACCTTGCC-3′	…
EIPC100R	5′-AGTGCTTCGTTACGAAAGTGACCTTA-3′	…

[a] Each probe was quenched with QSY at the 3′ end.

Validation of the assay with artificially inoculated samples. The first validation step consisted of inoculating sterilized chernozem soils with different concentrations of *P. destructor* sporangia (Fig. 3). The linear regression conducted with the obtained standard curve showed a negative correlation between the Cq values and *P. destructor* sporangia concentration ($y = -3.08x + 36.32$, $R^2 = 0.98$) and provided a significant fit of the data ($P < 0.0001$). In addition, the results of the ANOVA followed by a multiple comparison test (LSD) suggested that the Cq values for each concentration were different from each other ($P < 0.0001$). The Cq values ranged from 24.39 Cq for 5,000 sporangia/g of dry soil to 32.87 Cq for 10 sporangia/g of dry soil (Fig. 3). From this superimposition of standard curves, a conversion factor of 0.81 was calculated to obtain the sporangia equivalent from the ITS1 copy number.

A significant linear relationship between the log-transformed sporangia concentration on impaction trap sample rods and Cq values was also observed (Fig. 4). The correlation was negative ($y = -3.59x + 31.52$, $R^2 = 0.93$) and provided a significantly good fit of the data ($P < 0.0001$). Cq values ranged from 31.46 Cq for 1 sporangium to 24.44 Cq for 150 sporangia. The majority of the mean Cq values were within the 95% confidence interval, except for 2 and 10 sporangia/sample rods (Fig. 4).

Validation of the assay with environmental samples. Among the 290 samples collected from onion grower's fields, concentrations ranged from 0 to 810 sporangia-equivalent per gram of soil. In all, 58 samples (20.0%) were positive for *P. destructor*, and 9.0% of the samples were positive but considered to be under the quantification limit. For the other samples, 3.4% were between 10 and 15 sporangia-equivalent per gram of soil and 6.6% were above 15 sporangia-equivalent per gram of dry soil (Fig. 5). Soil samples collected from fields where onion has never been grown were all negative for *P. destructor*. The use of sterile soil samples verified that there was no nonspecific amplification. However, because no other detection methods were available, type I and type II error could not be assessed. In general, the average soilborne inoculum concentration tended to be correlated with the crop rotation length ($P = 0.053$) (Fig. 5).

For airborne samples, validation was first performed by comparing microscopic counts with real-time qPCR counts. During this first step, nine spore samplers were placed in an infected field in 2017 and 58 samples were collected and analyzed. The relationship between microscopic and real-time qPCR counts was linear and the linear regression provided a significantly good fit of the data ($Sporangia_{qPCR} = 1.01 \times Sporangia_{microscope} + -0.99$; $R^2 = 0.96$, $P < 0.0001$). The regression slope was not significantly different from 1 and the intercept was not significantly different from 0 (Fig. 6).

In 2018, airborne sporangia concentrations were relatively low, with only 24 positive samples out of the 560 tested. The majority of the sporangia were caught between 24 June 24 and 1 July and the highest concentration (4 sporangia m^{-3} of air) was measured on 1 July (Fig. 7A). Sporangia have also been caught between 19 July and 2 August and correspond to the first ODM observation date (Fig. 7A). In 2018, the disease was only observed in three of the monitored fields on 7, 9, and 16 August (Fig. 7A). In 2019, airborne sporangia concentration was greater than in 2018, with 42 positive samples out of the 525 tested. Sporangia concentrations were irregular at the beginning of the growing season and increased toward the end of it (Fig. 7B). Airborne sporangia concentration varied from 0 to 33 sporangia m^{-3} of air. Sporangia were caught on 6, 11, 25, and 27 June as well as 2 and 9 July. The first ODM observation was made on 10 July and incidence increased until 14 August (Fig. 7B). In 2018, 11 DC periods were observed (6 between 25 May and 1 July, only 1 between 1 July and 10 August, and 4 between 10 and 20 August) (Fig. 7A). In 2019, 14 DC periods were observed (6 between 25 May and 1 July, 4 between 1 July and 10 August, and 4 between 10 and 20 August) (Fig. 7B).

Discussion

In this study, a real-time qPCR assay was developed for the detection and monitoring of *P. destructor* primary inoculum in soil and secondary inoculum from spore trap samples. Despite the importance

of ODM for onion growers in southwestern Quebec and elsewhere, knowledge of its epidemiology remains limited, especially with respect to sources of inoculum. *P. destructor* can develop systemic infections in plant tissues, producing thousands of short-lasting sporangia and several long-lasting oospores (Palti 1989). The sporangia are known to be dispersed over distances ranging from a few meters to several kilometers. Locally, they are responsible for the
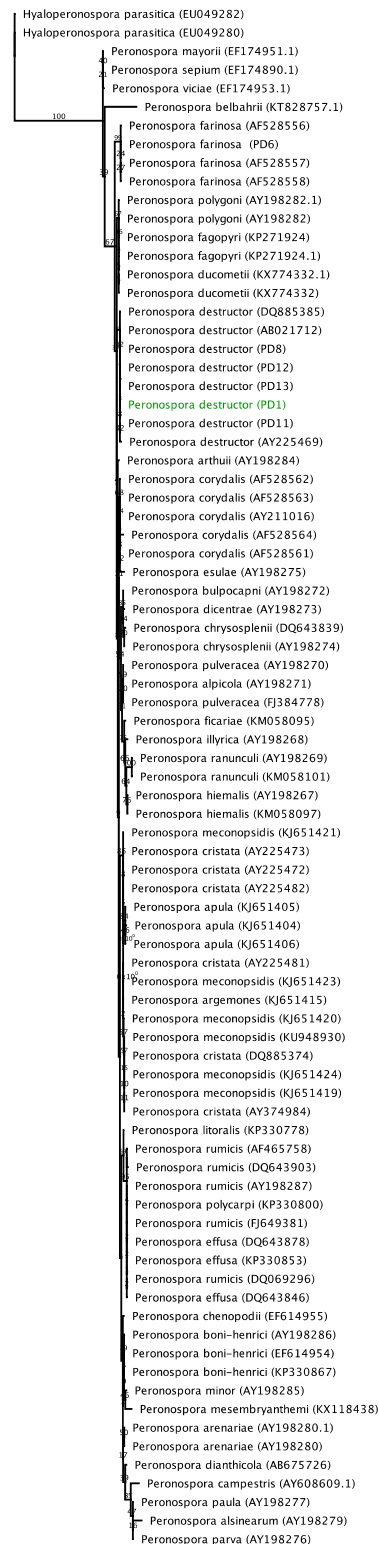

Fig. 1. One of the most parsimonious trees based on the internal transcribed spacer sequences from 83 *Peronospora* spp. obtained in this study and gathered from the GenBank database.

majority of secondary infections and, as the disease spreads, they are also responsible for initial infections in neighboring regions (Aylor 2017). Sources of primary inoculum are believed to be systemically infected onion bulbs, plant parts, and soil containing oospores (Palti 1989). Oospores of *P. destructor* are known to remain viable in soil for up to 25 years (McKay 1957) but their role in the epidemiology of the disease remains unclear. In theory, *P. destructor* is not known to survive North American winters even if there is no evidence to confirm or disprove this hypothesis. In a recent study, however, the frequency and intensity of seasonal ODM epidemics in southwestern Quebec were closely correlated to variables associated with the production and survival of overwintering inoculum (Van der Heyden et al. 2020).

The dynamic range of amplification of the developed assay was linear over six orders of magnitude and the real-time qPCR efficiency obtained with the gBlock standard curve and through a dilution of sporangia suspension were 92.06 and 92.14%, respectively. However, using a standard curve based on a dilution of DNA extracted

from a suspension of sporangia is a good approach to assess the assay sensitivity and efficiency (Carisse et al. 2009; Fall et al. 2015a); it also important to perform a standard curve that more accurately reflects reality. Hence, the validation conducted with increasing number of sporangia deposited on greased sample rods showed similar linearity and efficiency.

Many studies described the development of real-time qPCR assays for the detection and quantification of oomycetes, especially for *Pythium* and *Phytophthora* spp. (Bilodeau et al. 2009, 2014; Cullen et al. 2007; Fall et al. 2015a; Hussain et al. 2005; Kernaghan et al. 2008; Kunjeti et al. 2016; Lees et al. 2019; Li et al. 2010, 2014; Lievens et al. 2006; Miles et al. 2017; Schroeder et al. 2006; Spies et al. 2011; Than et al. 2013; Van der Heyden et al. 2019). However, fewer assays were developed for obligate biotrophic *Peronospora* spp. causing downy mildew diseases (Belbahri et al. 2005; Gent et al. 2009; Klosterman et al. 2014; Montes-Borrego et al. 2011; Mota et al. 2011), while the only published assay aiming at detecting *P. destructor* inoculum was based on the use of monoclonal
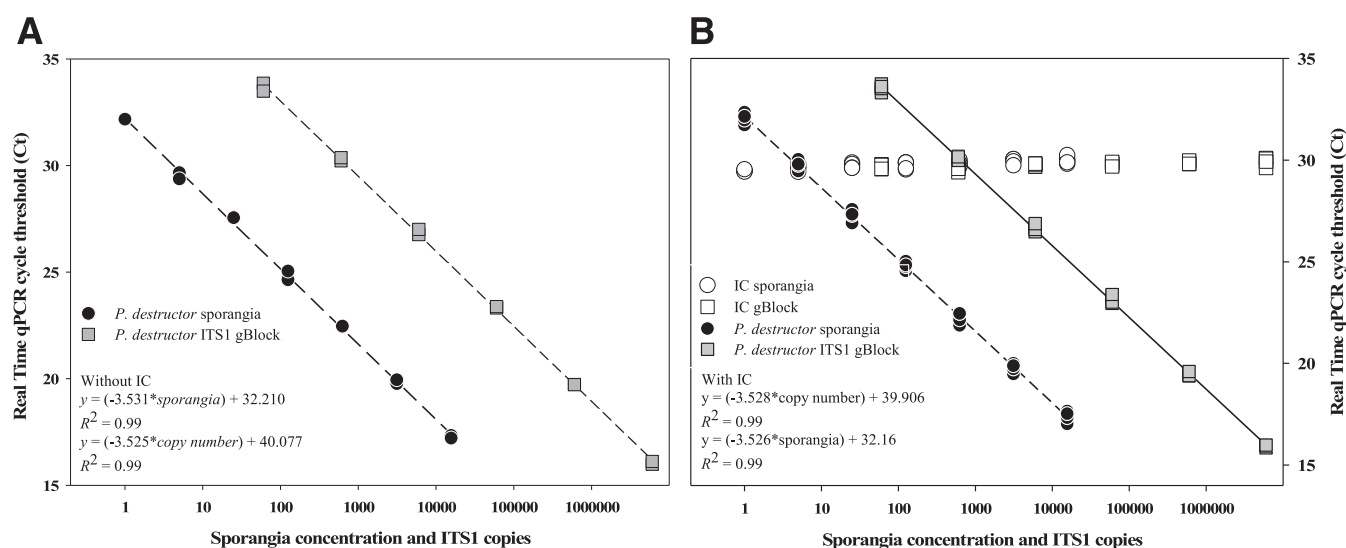
Fig. 2. Sporangia- and gBlock-based standard curves of *Peronospora destructor*. Quantitative PCR (qPCR) quantification threshold (Cq) values of *P. destructor* internal transcribed spacer (ITS)2 were plotted against the log10 starting quantity **A**, without and **B**, with the addition of the internal control (IC). Results were obtained from five independent real-time qPCR runs. Black circles represent amplification of the sporangia-based standard curves while the gray squares represent amplification of the gBlock-based standard curves with the addition of the internal control.
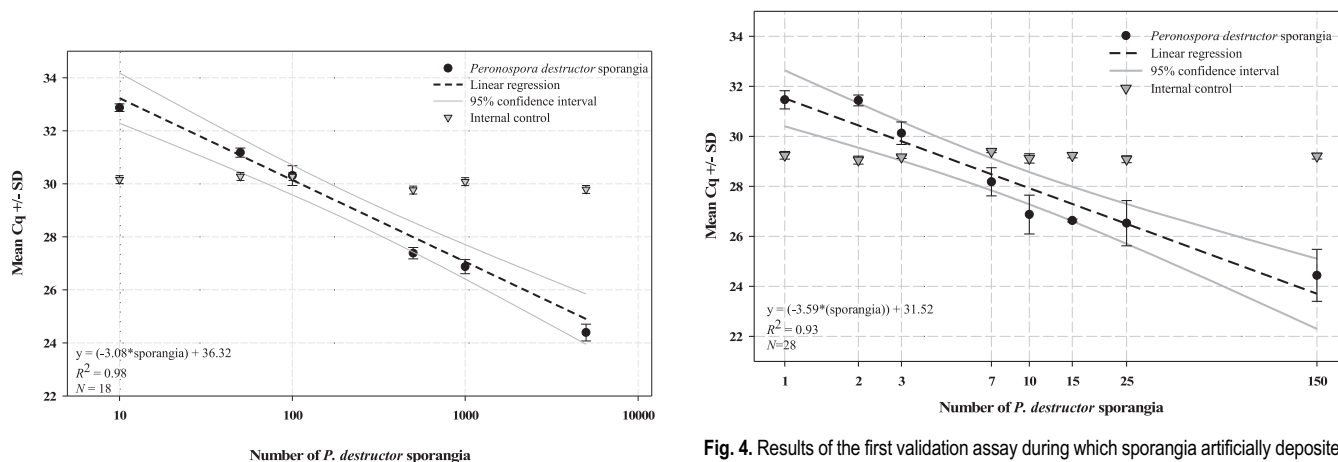
Fig. 3. Standard curves of *Peronospora destructor* sporangia concentration obtained in the validation assay in soil, with quantification threshold (Cq) value plotted against the log10 starting quantity. SD = standard deviation. For this validation assay, *P. destructor* sporangia were artificially inoculated in sterilized soils at concentrations ranging from 5,000 to 10 sporangia g^{-1} of dry soil.

Fig. 4. Results of the first validation assay during which sporangia artificially deposited on plastic rods and processed using the quantitative PCR assay developed in this study. Cq = quantification threshold and SD = standard deviation. The eight-point standard curve is based on the amplification of purified DNA from 1 to 150 sporangial count. Each point represents the average and standard deviation of three or four biological replicates. The linear regression was conducted using all the points.

antibodies combined with a lateral flow device (Kennedy and Wakeham 2008). It is generally accepted that action thresholds for downy mildew are relatively low, in the range of 10 sporangia m^{-3} of air (Dhar et al. 2020; Fall et al. 2015a, b). However, the monoclonal antibody assay developed for detecting *P. destructor* sporangia from air samples had a limit of detection of 500 sporangia, which limits its practical applications (Kennedy and Wakeham 2008). From this perspective, the present study represents a significant improvement on the approach because it allows the detection of a single sporangium per impaction trap sample rod.

The sensitivity of the present assay is similar to that obtained in various studies. For example, assays developed for *Bremia lactucae* (Kunjeti et al. 2016) or *Phytophthora infestans* (Fall et al. 2015a) were also sensitive enough to detect one sporangium per impaction

sampler rod. In this study, primers and probes targeting the *Peronospora destructor* ITS1 region were used for the development of the assay. The use of high-copy-number regions such as ITS is essential to achieve low sensitivity (Belbahri et al. 2005; Gent et al. 2009; Klosterman et al. 2014; Montes-Borrego et al. 2011; Mota et al. 2011). The utilization of a unique locus in the mtDNA region also showed high sensitivity in addition to high specificity (Bilodeau et al. 2014; Kunjeti et al. 2016; Miles et al. 2017). High-throughput sequencing approaches are increasingly being used, including for the development of specific DNA markers and identification of plant pathogen in environmental samples. However, the associated costs are still high, and time required to process the samples is too long for disease management decisions. Given the availability of DNA sequences in public databases, the use of a directed sequencing approach for the identification of species-specific regions remains an effective and affordable approach for the development of real-time qPCR assays, when possible.

The results obtained in this study suggested that *P. destructor* can be detected in air samples using rotating-arm impaction samplers, as described in other studies (Carisse et al. 2009, 2012; Dhar et al. 2020; Fall et al. 2015c; Klosterman et al. 2014; Kunjeti et al. 2016; Van der
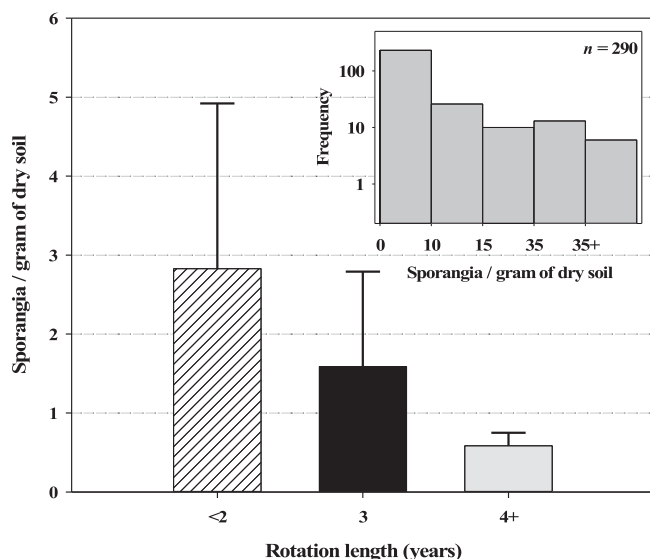

Fig. 5. Field validation of the real-time quantitative PCR assay for soil samples. The embedded histogram represents the frequency distribution of the 291 soil samples collected and tested in this study. Results are also presented according to a classification based on rotation length. The dashed bar represents the average soilborne sporangia concentration for a rotation length of 2 years or less, the black bar represents a rotation length of 3 years, and the gray bar a rotation length of 4 years or more. The error bar represents the standard deviation for the category.
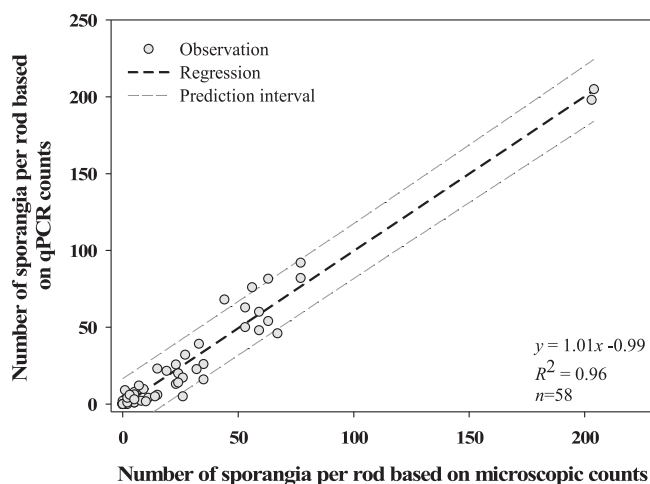

Fig. 6. Field validation of the quantitative PCR (qPCR) assay. Aerial samples were collected in 2017 from an infected onion field, using nine impaction samplers. Sporangia enumeration was conducted with the qPCR assay and compared with microscope counts. The figure shows linear regression between the number of *Peronospora destructor* sporangia obtained from microscope counts compared with the estimation obtained with the qPCR assay.
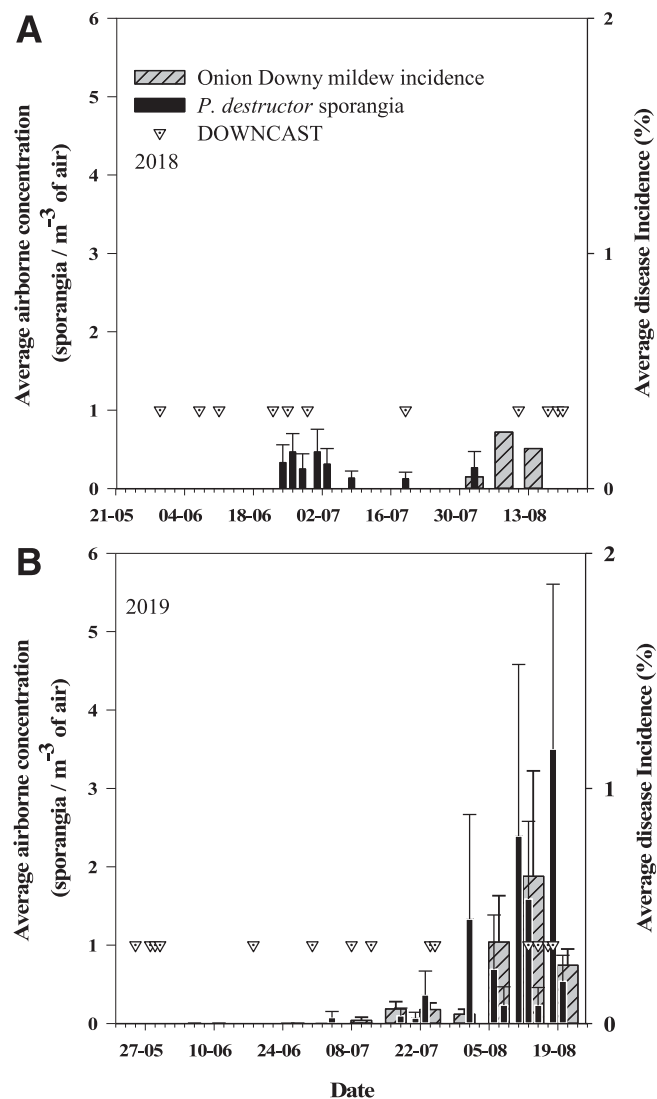

Fig. 7. Field validation of the quantitative PCR assay conducted in commercial fields in **A,** 2018 and **B,** 2019 using a network of impaction traps. In both panels, the black dot represents the average airborne *Peronospora destructor* concentration and the error bars represent the standard deviation. Gray bars represent the average onion downy mildew incidence and the error bars represent the standard deviation. In both panels, the triangles indicate the risk of downy mildew sporulation according to DOWNCAST.

Heyden et al. 2012, 2014). The validation of the assay under commercial conditions was carried out in 2018 and 2019, two different years in regards to regional disease incidence and severity. Airborne *P. destructor* sporangia concentrations showed a high level of seasonal variation. However, in both years, *P. destructor* sporangia were caught between 5 and 15 days prior to symptom observations. Similar observations were also made for other oomycetes. In the New Brunswick province, *P. infestans* sporangia were detected 6 to 7 days before the appearance of the first potato late blight symptoms (Fall et al. 2015c) while, for *B. lactucae* sporangia, airborne inoculum concentrations were used effectively to trigger fungicide applications in lettuce (Dhar et al. 2020). The cumulative risk indices for 2018 were almost 25% less than for 2019. In addition, the risk was mainly distributed at the beginning and end of the season. This could help explain the difference in disease incidence between the two seasons.

Although molecular markers have been developed to detect different *Peronospora* spp. in air and plant samples, few markers are available to investigate their presence and abundance in soil. The assay developed in this study was sensitive enough to detect less than 10 sporangia/g of dry soil, with a linear dynamic range of amplification from 5,000 to 10 sporangia/g of dry soil. No such quantitative assay was described in the literature for *Peronospora* spp. in soil but similar assays were developed for soilborne oomycetes, mainly *Pythium*, *Phytophthora*, and *Aphanomyces* spp., with sensitivity ranging between 1 and 50 oospores/g of soil (Almquist et al. 2016; Gangneux et al. 2014; Van der Heyden et al. 2019). In this study, however, sporangia were used instead of oospores due to the difficulty of producing them in vitro. However, to confirm that it is possible to detect primary inoculum, soil samples were collected in the spring. Thus, it was assumed that short-lived sporangia or mycelia were absent at that time of year (after a 7-month delay between onion harvest and sampling) and that detected inoculum consisted mainly if not solely of oospores. Soil samples taken from fields with no onion cultivation history were all negative for *P. destructor*. In addition, among the 290 soil samples from onion fields analyzed in this study, almost 20% were positive for *P. destructor* (up to 162 sporangia equivalent per gram of dry soil), supporting to some extent the hypothesis of overwintering in northern regions such as eastern Canada.

Oospores produced by most *Peronospora* spp. go through a variable period of constitutive dormancy that can last many years (Judelson 2008). This dormant state can be caused by barriers to nutrient entry or autoinhibitors and, as a result, oospore germination in the population does not take place at the same time, even if environmental conditions are favorable (Judelson 2008). For *P. viciae*, oospores with a maturation period of less than 3 years were considered to be ineffective (Van Der Gaag and Frinking 1997b,c), while *P. destructor* oospores needed a maturation period of at least 4 years (McKay 1937, 1957). For these reasons, the correlation between oospore density and disease incidence has hardly been demonstrated. For example, concentrations between 2 and 21 oospores/g of soil of *P. viciae* were associated with disease incidence between 17 and 75% (Van Der Gaag and Frinking 1997a). This pathogen's strategy, which aims to prevent the simultaneous germination of all of the oospores of a given population, could somehow promote the establishment and maintenance of the disease in a specific area by allowing for the accumulation of inoculum from one cropping season to the next. The results obtained in this study suggest higher soilborne inoculum levels in samples collected from fields with shorter rotation length, supporting the hypothesis of inoculum build-up.

Knowledge of both primary and secondary inoculum concentration is essential for tactical and strategic decision making, and precise monitoring tools are fundamental in achieving accurate decision. Spore sampling coupled with real-time qPCR quantification assays has proven to be efficient in improving tactical decisions in onion and other crops (Carisse and Van der Heyden 2017; Carisse et al. 2012; Dhar et al. 2020; Fall et al. 2015c; Klosterman et al. 2014; Kunjeti et al. 2016; Van der Heyden et al. 2012), while quantification of soilborne inoculum allows for strategic short-term decisions (Bilodeau et al. 2012; Cullen et al. 2005; Lees et al. 2002; Sauvageau et al. 2019; Van der Heyden et al. 2019). The assay developed in this study

will contribute to the development of integrated disease management strategies and provides a new tool for monitoring *P. destructor* populations. Several questions certainly continue to arise. Could climate change alone be responsible for the increase in the frequency of seasonal epidemics or is this a genetic-driven adaptation? With increased trade, are mixed populations capable of surviving our winters? Hence, in addition to better understanding the role of oospores in downy mildew epidemics, it seems essential to improve our knowledge of population genetics of *P. destructor*.

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