Development of a TaqMan Real-Time PCR Assay for Quantification of Airborne Conidia of *Botrytis squamosa* and Management of Botrytis Leaf Blight of Onion

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ABSTRACT

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The use of a DNA-based method for quantifying airborne inoculum of *Botrytis squamosa*, a damaging pathogen of onion, was investigated. A method for purifying DNA from conidia collected using rotating-arm samplers and quantifying it using a TaqMan real-time quantitative polymerase chain reaction (qPCR) assay is described. The sensitivity of the qPCR assay was high, with a detection limit of 2 conidia/rod. A linear relationship between numbers of conidia counted with a compound microscope and those determined with the qPCR assay was obtained. Receiver operating characteristic curve analysis was used to evaluate the reliability of the two methods of conidia quantification (microscope examination and qPCR assay) to predict the risk of disease being below or above a damage threshold (D_{th}). In total, 142 field samples from

commercial onion fields were analyzed. At damage thresholds of 5 or 10 lesions/leaf, conidia quantification with the qPCR assay was more reliable at predicting disease risk than conidia quantification based on microscope counts. The proportion of decisions where the disease was present and predicted was higher for the qPCR assay than for the microscope counts, with values of 0.95 and 0.89 compared with 0.79 and 0.81 for D_{th} of 5 and 10 lesions/leaf, respectively. The proportion of decisions where the disease was present but not predicted was lower for the qPCR assay than for microscope counts, with values of 0.05 and 0.11 compared with 0.20 and 0.19 for D_{th} of 5 and 10 lesions/leaf, respectively. The results demonstrated that this new qPCR assay was reliable for quantifying *B. squamosa* airborne inoculum in commercial onion fields and that molecular conidia quantification could be used as a component of a risk management system for Botrytis leaf blight.

Additional keywords: disease management, spore trap.

Botrytis leaf blight (BLB) of onion (*Allium cepa* L.) is caused by *Botrytis squamosa* Walker (16). Disease development is characterized by small lesions on green leaves followed by leaf blight. BLB is a polycyclic disease and epidemics are initiated by different sources of inoculum. In warm growing regions, (e.g., Florida), conidia produced on leaves of infected volunteer onion plants or on onion cull piles serve as an inoculum source year round. In northern onion production areas (e.g., eastern Canada, New York, or Michigan), the main source of initial inoculum comes from conidia produced on sclerotia that overwintered in soil, on onion debris, or on onion bulbs in cull piles (12,13).

In most onion production areas, recommendations for managing BLB include a crop rotation of 2 to 3 years with nonsusceptible crops, the removal of volunteer plants and onion cull piles, the use of less susceptible cultivars, and the application of fungicides to reduce disease progress. In general, growers in eastern Canada practice only short-term rotation with carrot, lettuce, or celery. There are no resistant cultivars commercially available, even though differences in susceptibility between cultivars could be observed in the field. Nevertheless, most growers rely on fungicide applications for disease management.

Conventional fungicide spray programs for BLB management include application of fungicides every 7 to 10 days between the three- and four-leaf stage and shortly after the onion plants lodge (1,30). This conventional approach may result in unnecessary fungicide applications; however, there are alternative methods being used to optimize the number of fungicide applications. Some are based on biological monitoring of disease levels (29,33) or airborne inoculum (6). Other methods are weather based and provide disease risk prediction (21,30,34). In a recent study by Carisse et al. (7), monitoring-based and weather-based predictors were evaluated for their ability to discriminate between situations where BLB is below or above damage thresholds of 1, 5, or 10 lesions/leaf. This analysis showed that monitoring-based predictors were more reliable and, among them, airborne inoculum monitoring provided the most reliable estimation of BLB risk. Carisse et al. (6) used a critical concentration of airborne conidia of *B. squamosa* of 10 to 15 conidia/m³ of air to trigger a fungicide application. Despite the reliability of airborne inoculum monitoring as a tool to manage BLB, this method has limitations, which include the need for continuous operation of the samplers when deployed, maintenance and routine collection of rods, and limitations related to spore quantification.

Considering that most conidia are released between 10:00 a.m. and 12:00 p.m. (31), rods should be collected soon after 12:00 p.m. and warnings based on conidia quantification should be sent to advisors by the end of the day so that growers can make a decision whether to spray or not. However, this approach requires

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that spore counts are done accurately and in a timely fashion. Identification and quantification of spores is generally based on light-microscopy counts, a time-consuming technique that requires substantial expertise to make accurate identifications. Distinguishing target spores from soil particles on the sampling surface adds to the difficulty of making accurate quantification even when specific dyes are being used. DNA-based methods could be used to circumvent some of the limitations of microscopic counts.

Over the last decade, attempts were made to develop DNA-based methods to detect and quantify airborne spores using various spore samplers (36). Recently, polymerase chain reaction (PCR) assays were developed to detect airborne spores (4,5, 15,37). PCR and hybridization techniques were also used to detect airborne spores from different environmental samples (38). However, conventional PCR assays do not allow precise quantification and, hence, could not be used to determine if airborne inoculum is above action thresholds. Recently, the introduction of real-time quantitative PCR (qPCR) has offered the ability of simultaneous detection and quantification of DNA of a specific organism (26,40). The advantages of qPCR technology are its quantitative properties and generally high sensitivity and specificity, which make this technique suitable for routine usage and disease management decisions (3,36).

There have been only a few attempts to use qPCR to quantify airborne inoculum (14,23,27,28,37). The aim of this study was to develop a rapid and sensitive method for detection and quantification of *B. squamosa* conidia from air samples and to evaluate the DNA-based quantification method to predict the risk of BLB being below or above a damage threshold.

MATERIALS AND METHODS

Development of the TaqMan qPCR assay. *Ultra Fast DNA extraction from air samples.* Single polystyrene rods from rotating-arm samplers were placed in a 2-ml screw cap tube containing ≈ 100 mg of 425- to 600- μ m glass beads (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and 75 μ l of ACS reagent-grade petroleum ether (Sigma-Aldrich Canada Ltd.). Tubes were shaken in a FastPrep FP120A Instrument (MP Biomedicals, Irvine, CA) at 4.0 m/s for 20 s to dislodge the conidia from the silicon grease present at the surface of the rods. Conidia were settled at the bottom of the tubes by centrifugation for 5 s at $10,000 \times g$. Petroleum ether was evaporated under vacuum using a Speedvac (Savant Instruments, Inc., Holbrook, NY) at 45°C for 15 min. Conidia were then resuspended in 300 μ l of DNA extraction buffer consisting of nuclease-free water (Integrated DNA Technologies, Inc., Coralville, IA), with salmon sperm DNA at 10 ng/ μ l (Invitrogen Corporation, Carlsbad, CA) as carrier DNA that mimics the complex environment of competitive DNA extracted from spores, 5% chelex 100 molecular-biology-grade resin (Bio-Rad Laboratories, Hercules, CA), and cDNA plasmid SH-poly (pSH-poly) at 5×10^4 copies/ μ l as an exogenous internal positive control (EIPC) (19). Tubes were placed in a dry bath at 110°C for 15 min to complete DNA extraction, agitated for 5 s using a vortex, and centrifuged at $15,000 \times g$ for 5 min at 4°C. Supernatants were maintained at 4°C for a maximum of 2 h and used as the DNA source for qPCR conidia assessment.

Primers and TaqMan probe design. Primers B_squa_up221 (5'-GCC GAA CCA ATA CAC CT-3'), B_squa_lo361 (5'-ATT GGA TCA CTT GGC GTG-3'), and the TaqMan probe B_squa_Taqman_up282 (5'-(6-FAM)TCT GTC CTG CAG AGA CGC CC(BHQ1)-3') (Integrated DNA Technologies Inc.) were designed based on the alignment of 64 RPB2 gene (RNA polymerase II largest second subunit) partial sequences and amplified a DNA fragment of 175 bp. The 64 sequences were from 25 fungal species, including eight *B. squamosa*, 22 *Botrytis* spp., one *Monilinia fructicola*, and one *Sclerotinia sclerotiorum* (Table 1).

All sequences were obtained from GenBank, except for isolates of *B. squamosa*, which were isolated from eight randomly selected onion fields from Quebec in 2002, 2003, and 2004. Those were cultivated on potato dextrose agar (PDA) prior to DNA extraction and sequences were obtained from RPB2 fragments amplified using fungal-specific RPB2 primers frpb2-5f and frpb2-7cr (22). All *B. squamosa* RPB2 sequences obtained were identical.

Construction of plasmid DNA of RPB2 gene. DNA of *B. squamosa* isolate 2R7 isolated from a diseased onion collected in an onion field in southwest Montreal (9) was extracted using the FastDNA kit (MP Biomedicals) according to the manufacturer's recommendations. PCR amplification was carried out in a 20- μ l reaction mixture containing 1 μ l of genomic DNA, 2 μ l of 10 $\times$ Titanium Taq PCR Buffer (Clontech Laboratories, Inc., Mountain View, CA), 200 μ M dNTP mix (Invitrogen Corporation), 300 nM each primers B_squa_up221 and B_squa_lo361 (Integrated DNA Technologies, Inc.), and 0.4 μ l of 50 $\times$ Titanium Taq PCR Polymerase (Clontech Laboratories, Inc.). The following PCR conditions were used for the amplification of the RPB2 fragment: 95°C for 3 min; followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s; and a final extension at 72°C for 8 min. PCR fragments of 175 bp were purified using the QIAquick PCR purification kit (Qiagen Inc., Mississauga, ON, Canada) according to the manufacturer's recommendations. Purified products were cloned into the pCR 2.1 TOPO vector using the TOPO TA Cloning Kit (Invitrogen Corporation) with electrocompetent cells following the manufacturer's recommendations. The recombinant plasmid stock was linearized by a 4-h digestion with *Bam*HI at 37°C, quantified using the NanoDrop spectrophotometer ND-1000 according to the manufacturer's instructions (NanoDrop Technologies Inc., Wilmington, DE) and converted into copy number, calculated as copy number = (concentration of linearized plasmid)/(molar mass) $\times (6.022 \times 10^{23})$. This DNA plasmid was used for optimization of TaqMan qPCR assay and generation of the plasmid standard curve.

Preparation of conidia of *B. squamosa* for the qPCR standard curve. To prepare the conidia standard curve, dry conidia were harvested from sporulating sclerotia (32) and suspended in isopropanol (Sigma-Aldrich Canada Ltd.). A suspension of 5×10^4 conidia in 40 μ l of isopropanol was transferred into 2-ml screw caps tubes containing 100 mg of 425- to 600- μ m glass beads and a silicon-coated spore trap rod. Isopropanol was evaporated under vacuum using a Speedvac (Savant Instruments, Inc.) at 45°C for 15 min. DNA was extracted as described above, except that DNA was extracted from conidia suspension as opposed to conidia on spore sampler's rods coated with silicone. This DNA extraction was used to generate the conidia standard curve by qPCR.

Estimation of the number of conidia from the number of RPB2 copies. A plasmid standard curve was established by 10-fold serial dilutions of the linearized RPB2 plasmid, ranging from 1×10^7 to 1 copies/ μ l in nuclease-free water containing salmon sperm DNA at 10 ng/ μ l and cDNA pSH-poly at 5×10^4 copies/ μ l. A conidia standard curve was established from fourfold serial dilutions of 5×10^4 to 3.2 *B. squamosa* conidia/DNA extraction prepared in nuclease-free water containing salmon sperm DNA at 10 ng/ μ l and cDNA pSH-poly at 5×10^4 copies/ μ l. For both curves, the procedure was repeated five times. A three-step regression analysis was used to determine the number of copies of RPB2 per conidia. First, regression analysis was used to establish the relationship between the cycle threshold (Ct) value and the logarithm of the number of RPB2 plasmid. Second, regression analysis was used to establish the relationship between the Ct value and the logarithm of the number of conidia. Finally, the predicted number of conidia was regressed against the predicted number of plasmid for Ct values of 31 to 38.5. The resulting equation was used to determine the number of conidia from the number of plasmids obtained from the qPCR assay. The regres-

sion procedure (PROC REG) of SAS (version 9.1; SAS Institute Inc., Cary, NC.) was used to estimate the intercept and slope regression parameters.

EIPC. An EIPC was included in the assay to monitor the DNA extraction and qPCR amplification performed on each individual sample and to detect the possibility of false negative results resulting from inhibitors of the qPCR reaction or from experimental errors. The EIPC consisted of cDNA pSH-poly at 5×10^4 copies/ μ l previously added to DNA extraction buffer of the field samples. EIPC was amplified and detected separately from the *B. squamosa* quantification assay using the qPCR protocol slightly modified from Houde et al. (19). Reactions were performed in a 25- μ l final volume containing 3 μ l of DNA extract, 12.5 μ l of 2 $\times$ QTAQ PCR Buffer (Clontech Laboratories, Inc.), final concentrations of 300 nM each primer SH-Poly-A and SH-Poly-1, 200 nM TaqMan probe SH-Poly-Q (Integrated DNA Technologies, Inc.), and 0.5 μ l of 50 $\times$ QTAQ DNA Polymerase Mix (Clontech Laboratories, Inc.). The two-step PCR conditions were 95°C for 3 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Ct values for each sample EIPC within the same run were compared with the control EIPC of a negative sample control (silicon-coated rod). A variation of more than one Ct from the EIPC was used as a criterion to reject a sample result.

TaqMan qPCR assay. TaqMan qPCR amplifications were performed using the Stratagene Mx3005P QPCR System (Agilent, La Jolla, CA). Reactions were performed in 25 μ l containing 3 μ l of DNA extract, final concentrations of 12.5 μ l of 2 $\times$ QTAQ PCR Buffer (Clontech Laboratories, Inc.), 300 nM each primer

B_squa_up221 and B_squa_lo361, 200 nM TaqMan probe B_squa_Taqman_up282, and 0.5 μ l of 50 $\times$ QTAQ DNA Polymerase Mix (Clontech Laboratories, Inc.). The two-step PCR conditions were 95°C for 3 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Each qPCR run included 10-fold serial dilutions of the linearized RPB2 plasmid, ranging from 1×10^7 to 1 copies/ μ l in nuclease-free water containing salmon sperm DNA at 10 ng/ μ l, cDNA pSH-poly at 5×10^7 copies/ μ l, a no-template control, and the DNA samples. All reactions were performed in triplicates.

Sensitivity of the qPCR assay. Drops of a *B. squamosa* conidia solution in isopropanol were deposited onto glass rods coated with silicon vacuum grease. The rods were kept at room temperature for 5 min to allow the evaporation of isopropanol. The number of conidia per rod was counted under a light microscope at $\times 250$ magnification and it was determined that conidia were deposited at 0 to 116 conidia/rod. DNA was extracted from conidia as described above, and the number of conidia was estimated with the qPCR assay and standard curve as described above. The experiment was repeated three times. Regression analysis was used to establish the relationship between the number of conidia estimated by qPCR and by microscopy. The analysis was performed using the regression procedure (PROC REG) of SAS.

Specificity of the qPCR assay. The specificity of the TaqMan qPCR assay was tested against eight species from the family Sclerotiniaceae (*B. aclada*, *B. porri*, *B. cinerea*, *B. elliptica*, *B. ficarium*, *B. ranunculi*, *Sclerotium cepivorum*, and *Sclerotinia*

TABLE 1. Alignment of the RPB2 partial sequences from GenBank used for primer and probe design^a

Species	No. of seq. ^b	Access. no. ^c	B_squa_up221	B_squa_Taqman_up282	B_squa_lo361 ^d
			GCCGAACCAATACACCT	TCTGTCCTGCAGAGACGCC	CACGCCAAGTGATCCAAT
<i>Botrytis squamosa</i> 01R4	1	FJ870989			
<i>B. squamosa</i> 15R1	1	FJ870990			
<i>B. squamosa</i> 18R1	1	FJ870991			
<i>B. squamosa</i> 18R6	1	FJ870992			
<i>B. squamosa</i> 26R7	1	FJ870993			
<i>B. squamosa</i> 26R8	1	FJ870994			
<i>B. squamosa</i> 29R1	1	FJ870995			
<i>B. squamosa</i> 33R4	1	FJ870996			
<i>B. squamosa</i> PRI026	1	AJ745707			
<i>B. squamosa</i> MUCL1107	1	AJ745710			
<i>B. squamosa</i> MUCL9112	1	AJ745711			
<i>B. cinerea</i>	6	AJ745674	C	G	T.....
<i>B. porri</i>	2	AJ745704	C	C..G.....A..	T.....
<i>B. aclada</i>	3	AJ745663	C.....C	C.....C	T..A.....
<i>B. allii</i>	4	AJ745666	C.....C	C.....C	T..A.....
<i>B. byssoidea</i>	1	AJ745670	C	C..G.....	T.....G.....
<i>B. calthae</i>	3	AJ745671	C	G.....	T.....
<i>B. convoluta</i>	2	AJ745679	C	C..G.....	T.....
<i>B. croci</i>	1	AJ745681	G..C	C..G.....	T.....G.....
<i>B. fabae</i>	2	AJ745685	C	G.....	T.....
<i>B. elliptica</i>	3	AJ745682			T.....
<i>B. ficarium</i>	2	AJ745688	C	C.....	T.....
<i>B. galanthina</i>	2	AJ745689	C	G.....	T.....G.....
<i>B. gladiolorum</i>	2	AJ745691	C	C..G.....	T.....G.....
<i>B. globosa</i>	2	AJ745693	C	C..G.....	T.....G..C.....
<i>B. hyacinthi</i>	2	AJ745695	G..C	C..G.....	T.....G.....
<i>B. narcissicola</i>	2	AJ745697	G.....C	C..G.....	T.....G.....
<i>B. paeoniae</i>	2	AJ745699	C	C..G.....	T.....
<i>B. pelargonii</i>	2	AJ745662	C	G.....	T.....
<i>B. polyblastis</i>	2	AJ745702	A.....C	C..G.....	T.....G.....
<i>B. ranunculi</i>	1	AJ745706	C	C.....	T.....
<i>B. sphaerosperma</i>	2	AJ745708	C	C..G.....	T.....G..C.....
<i>B. tulipae</i>	3	AJ745712	C	C..G.....	T.....G.....
<i>Monilinia fructigena</i>	1	AJ745715	C	..A.....G.....	T.....C..T..
<i>Sclerotinia sclerotiorum</i>	1	AJ745716	G...	..T.....A.....A	T.....

^a Dots represent matching nucleotides to the primers and probe sequences.

^b Number of identical sequences used for the design of primers and probe.

^c GenBank accession numbers.

^d Real sequence of this reverse primer is 5'-ATTGGATCACTTGGCGTG-3'.

sclerotiorum) and 36 unidentified airborne fungal contaminants isolated from 16 rods randomly selected from onion field samplings in June and July 2008. Isolates of *B. aclada*, *B. cinerea*, *B. porri*, *B. elliptica*, *B. ficariarum*, and *B. ranunculi* were kindly provided by Dr. K Seifert (eastern cereal and oilseed research centre, Agriculture and Agri-Food Canada). Isolates of *Sclerotium cepivorum* and *Sclerotinia sclerotiorum* were isolated from onion and carrot fields. All isolates were cultured on PDA (Difco Laboratories, Detroit) and DNA was extracted as described previously. The TaqMan qPCR assay was performed as described previously using 10 pg of genomic DNA added to the 25- μ l assay reaction. In addition, electrophoresis on 2% agarose gel and sequencing of post qPCR amplicons were performed with 19 different positive environmental samples from 4 June to 30 July to confirm specificity of the TaqMan qPCR assay upon environmental samples.

Reliability of qPCR and microscopic quantification of *B. squamosa* conidia as a tool to predict risk of BLB. *Air sampling.* Data were collected in 15 commercial onion fields located in Sherrington County, $\approx$ 50 km southwest of Montreal. The onion fields were 10 to 30 ha and onion seed were sown with susceptible cultivars (Norstar, Frontier, Fortress, Flagship, Nebula, Champlain, and Infinity) in late May at a rate of 30 seeds/m within rows with a row spacing of 0.36 m. A preemergence herbicide (Prowl at 7.5 liters/ha; pendimethaline, BASF Canada Inc.) was applied, and weeding was done four to five times by hand during the course of the experiments. The plots were treated with insecticide when necessary. Airborne concentrations of *B. squamosa* conidia were measured in the center of each field using rotating-arm impaction spore samplers running from 10:00 a.m. to 12:00 p.m. (6). These samplers consist of two vertical arms (sampling surface of 1.65 by 20 mm) separated by 83 mm. Airborne particles are impacted onto the leading edges of the rotation rods coated with silicone grease (Dow Corning, Midland, MI). After exposure, the number of conidia on one rod was counted under a light microscope at $\times$ 250 magnification and the counts were transformed into conidia per cubic meter (number of conidia per rod $\times$ 1,000 liters/m³)/(20.65 liters/min rod $\times$ 60 min/h $\times$ 2 h). Conidia of *B. squamosa* were identified based on size and morphology (35). Spore samplers ran three times per week for a total of 615 samples (41 sampling dates $\times$ 15 fields), among which 165 were selected for comparing the spore quantification methods. These were selected to represent the commercial period of monitoring for BLB and included weekly sampling in June and biweekly sampling in July, for a total of 11 sampling dates of 15 onion fields (6). For each sample, one rod was randomly selected for counts under the microscope and the same rod was processed with the TaqMan qPCR assay as described above.

Disease assessment. Disease intensity, expressed as the number of lesions per leaf, was estimated three times per week. At each sampling date, all leaves of 20 randomly selected plants per field were assessed for the number of *B. squamosa* lesions. Data on disease intensity were simultaneously collected in fields where airborne samples were collected and in adjacent plots that were not sprayed for at least 15 days (unsprayed plots).

Receiver operating characteristic curve analysis. Conidia quantifications based on microscopic counts and those obtained from the TaqMan qPCR assays were compared for their accuracy in identifying risk of BLB. Data from 142 of the 165 air samplings were divided into two groups retrospectively, based on disease intensity observed in the unsprayed plots 5 days later (it was not possible to quantify conidia by either method for 23 samples). The “cases” were defined as samplings associated with a BLB severity observed in unsprayed plots above the damage threshold (D_{th}), and the “controls” were defined as the samples associated with BLB severity below the D_{th} (20,39). The analysis was conducted for D_{th} of 5 and 10 lesions/leaf (7). Each quanti-

fication method was evaluated for its ability to classify the risk of BLB being above or below the D_{th} by recording the number of cases and controls that were correctly classified as such for each number of estimated conidia.

Receiver operating characteristic (ROC) curves were used to illustrate the relationship between the true-positive proportion (TPP; sensitivity) and the false-positive proportion (FPP; 1-specificity) across all possible action threshold values of the predictor (25). Here, the microscope counts and the Ct values were converted to the number of conidia per cubic meter of air as predictor values. The TPP was calculated by dividing the number of true positive decisions by the total number of cases (i.e., sampling declared above D_{th} by the tests which were actually above D_{th}). The true-negative proportion (TNP; specificity) was calculated by dividing the number of true negatives by the total number of controls (i.e., sampling declared below D_{th} by the tests which were actually below D_{th}). The FPP was calculated as 1 – TNP and the false-negative proportion (FNP) was calculated as 1 – TPP (20,39). The area under the ROC curve (AUC) was used to evaluate the accuracy of each conidia quantification method (17, 18). A quantification method that provides no discrimination between disease below or above the D_{th} will have a ROC curve that follows the no information line (the straight line where TPP = FPP and AUC = 0.5). A method that provides a perfect discrimination will have an AUC = 1. The nonparametric approach of Delong and Delong (10) was used to compare the AUCs for the two tests.

To use the TaqMan qPCR assay for conidia quantification and decision making, it is essential to know the best cutoff point to use as an action threshold for fungicide applications. If sensitivity and specificity are considered equally important (in other words, if the objective is to minimize the overall error rate), then the cutoff point could be calculated from Youden’s index, which is the point on the ROC curve farthest from random (or the line of no information). Youden’s index was calculated as $J = \text{sensitivity} + \text{specificity} - 1$ (2,7). The statistical package ROCtools Beta.1 (Institut Philippe-Pinel de Montréal, Montréal) was used to produce the ROC curves and to calculate the AUC and associated statistics.

RESULTS

Development of the TaqMan qPCR assay. *Estimation of the number of conidia from the number of RPB2 copies.* The five plasmid standard curves generated by 10-fold serial dilutions of plasmids were found to be linear, with efficiency and R^2 values of 96.5 to 99.7% and 0.99 to 1.00, respectively. Similarly, the conidia standard curves generated by fivefold serial dilutions of conidia DNA extractions were found to be linear, with efficiency and R^2 values of 93.5 to 101.6% and 0.99 to 1.00, respectively. Based on the pooled data (five replications), the best linear equation relating the logarithm of the plasmid number per reaction was $\log(\text{plasmid number}) = 11.177 - (0.296 \times Ct)$, $R^2 = 0.99$ (Fig. 1). Similarly, the best linear equation relating the number of *B. squamosa* conidia per sample was $\log(\text{number of conidia}) = 11.401 - (0.288 \times Ct)$, with $R^2 = 0.99$ (Fig. 1). From these equations, a final equation relating the number of *B. squamosa* spores per sample to the number of plasmids per reaction was also found to be linear and of the form $\text{conidia} = 1.445 + (2.973 \times \text{plasmid number})$, $R^2 = 0.99$.

Sensitivity and species specificity studies. On all rods coated with silicon grease, no signals were detected from rods free of conidia. TaqMan qPCR signals were obtained from all rods from which 2 to 116 conidia were deposited. There was a linear relationship between the number of conidia deposited onto the rods and the number of conidia estimated with the TaqMan qPCR assay ($R^2 = 0.97$) (Fig. 2). At 10 pg of genomic DNA, specificity tests with all *B. squamosa* and *B. elliptica* isolates resulted in

signals at Ct values of 30.35 and 34.97, respectively, meaning that this test will detect conidia of *B. elliptica* impacted on samplers. Specificity assays with the other species from the family *Sclerotiniaceae* and with the 36 airborne fungal contaminants produced no signals. No signals were detected from negative PCR controls. Post-qPCR analysis confirmed a 175-pb fragment and 100% homology with all *B. squamosa* isolates used for experiment design.

EIPC evaluation in qPCR assay. Among the original 165 samples, 8 were considered to be missing data because the sample EIPC Ct values varied >1 Ct value relative to the Ct value of the control EIPC within the same qPCR run. For six other samples, no EIPC Ct values were obtained, indicating complete inhibition of the qPCR reaction or that failure in the DNA extraction had occurred and, hence, these samples were considered to be missing data. Overall, the TaqMan qPCR assay failed for 14 of 165 samples, whereas microscopic counts were not possible for 23 of 165 rods (mainly due to the presence of dust). These included the six samples for which no qPCR signal was detected. Overall, 142 samples for which quantification was possible by both methods were retained for the ROC analysis.

Fig. 1. Relationship between log(plasmid number) and cycle threshold (Ct) value and between log(number of conidia) and Ct value. Solid and open symbols represent the logarithms of plasmid number and numbers of conidia, respectively.

Fig. 2. Relationship between the numbers of conidia of *Botrytis squamosa* deposited onto silicon-coated glass rods estimated based on the polymerase chain reaction assay and on microscopic counts.

Reliability of qPCR and microscopic quantification of *B. squamosa* conidia as tool to predict risk of BLB. *Detection and quantification of airborne conidia of *B. squamosa* in air samples.* Airborne conidia of *B. squamosa* were detected earlier in the season when quantified based on microscope counts than with the TaqMan qPCR assay (Fig. 3). The estimated concentrations of airborne conidia based on microscope counts were higher than estimated concentrations based on amount of *B. squamosa* DNA until 14 July (Fig. 3). Nevertheless, with both methods, the concentration of airborne conidia remained below the action threshold of 15 conidia/m³ of air until 14 July (Fig. 3). Toward the end of the sampling period, the concentration of airborne conidia was estimated as above the action threshold more often when concentrations were calculated with the TaqMan qPCR assay (Fig. 3).

ROC curve analysis. The frequency distribution of airborne conidia concentration estimated by either microscopic counts or TaqMan PCR are shown for both case and control plots of BLB at threshold values of 5 and 10 lesions/leaf (Fig. 4). Ideally, all controls would be on the left side of the action threshold so that the probability of falsely treating a control is zero. Similarly, all cases should ideally be on the right side of the action threshold so that the probability of not spraying when the disease is above the D_{th} is zero.

For the microscope counts, most controls were encountered when conidia counts were below the action threshold of 10 to 15 conidia/m³ of air (Fig. 4). However, cases were frequently encountered when the conidia counts were below the action threshold. The distribution of controls and cases was similar for the TaqMan qPCR assay, except that no controls had conidia counts

Fig. 3. Number of conidia of *Botrytis squamosa* estimated by **A**, microscope counts and **B**, the TaqMan quantitative polymerase chain reaction (qPCR) assay from 142 air samples collected in onion fields located southwest of Montreal (Quebec, Canada).

above the action threshold. The frequency of cases where conidia counts were below the action threshold was lower than for microscope counts (Fig. 4).

ROC curves presented in Figure 5 are grouped by D_{th} to facilitate comparison between the quantification methods. For both airborne conidia quantification methods, the AUCs were significantly different from 0.5 (Table 2). The AUCs were significantly higher for the TaqMan qPCR assay with AUC = 0.94 and 0.95 for D_{th} = 5 and 10 lesions/leaf, respectively, than for the microscope counts with AUC = 0.85 and 0.84 for D_{th} = 5 and 10 lesions/leaf, respectively (P = 0.0029 and 0.0016 for D_{th} = 5 and 10 lesions/leaf, respectively). For both methods of quantification, the best cutoff point was lower for the D_{th} of 5 lesions/leaf than for D_{th} of 10 lesions/leaf (Table 2). For microscope counts, the best cutoff points according to Youden's index (expressed in units of the predictor values) for the two D_{th} s were 14.7 and 15.3 conidia/ m^3 of air. For DNA method, the cutoff points for the two D_{th} s were 7.2 and 12.5 conidia/ m^3 of air.

DISCUSSION

This study reports the development and evaluation of a TaqMan qPCR assay to detect and quantify *B. squamosa* spores from the collecting surface (rods) of rotating-arm samplers. This method was developed to serve as an alternative to the conventional method, which relies on counting spores with a microscope (6,7).

When developing the qPCR assay, it was necessary to ensure that DNA could be extracted efficiently from conidia impacted on the rods, the limit of detection was below the conidia action threshold, and the procedure was fast (ideally, could be completed within a few hours). The whole procedure from collection and preparation of the field samples to running the assay and evaluation of results took less than 3 h for 15 samples. The analytical sensitivity evaluation showed that DNA from *B. squamosa* spores could be efficiently extracted from silicone-coated rods, in at least two cases with as few as 2 spores, meaning that the method of conidia extraction from field samples was appropriate and the qPCR detection sensitivity was high. Within the range of conidia per rod generally observed in commercial onion fields (7), the numbers of conidia deposited artificially on silicone-coated rods determined with the qPCR assay were linearly correlated with the actual number of conidia per rod, and no signals were detected from rods free of *B. squamosa* conidia. The specificity of the qPCR assay was tested with DNA from eight species from the *Sclerotiniaceae* family and 36 airborne fungal contaminants isolated from the collecting surface (rods) of rotating-arm samplers. It was shown to be generally specific to *B. squamosa* but the test could detect conidia of *B. elliptica*. However, *B. elliptica* is a lily pathogen (11) and is unlikely to be found in large onion fields, though it could be a concern in other production areas. From the 165 air samples, 14 could not be assessed by the qPCR assay, which corresponded to 8% of the samples. This error rate is

Fig. 4. Frequency of controls (solid bars) and cases (dashed bars) for Botrytis leaf blight based on **A** and **B**, microscope and **C** and **D**, DNA quantification of airborne conidia of *Botrytis squamosa*. The frequencies were calculated for a damage threshold of **A** and **C**, 5 and **B** and **D**, 10 lesions/leaf. The dashed vertical line represents the proposed action threshold for fungicide application (6).

common for airborne samples because rods are often improperly greased or recovered by soil dust (24). Overall, it was concluded that the qPCR assay can serve as a tool for quantifying airborne *B. squamosa* conidia from field samples.

From a grower's standpoint, the precise quantification of airborne inoculum may not be of foremost importance. In most cases, it is sufficient to know if disease is above or below the predetermined D_{th} s. The ROC curve analysis clearly showed that the qPCR method was reliable in identifying the risk of BLB. From the ROC curve analysis, the best action thresholds when airborne conidia concentration is estimated with the qPCR assay were 7.2 and 12.5 conidia/ m^3 of air for D_{th} of 5 and 10 lesions/leaf, respectively.

Fig. 5. Receiver operating characteristic curves for estimating *Botrytis* leaf blight based on microscope counts (broken line) and DNA quantification (solid line) of airborne conidia of *Botrytis squamosa*. The analysis was conducted for a damage thresholds of **A**, 5 and **B**, 10 lesions/leaf.

It is important to know the accuracy and the risk associated with any management decision tool. Accuracy could be defined as the proportion of correct decisions, which includes both true-positive and -negative decisions. In other words, an accurate decision tool advises a fungicide application only when needed. One measure of risk, and perhaps the most important from a grower's perspective, is the proportion of decisions where the disease is present but not predicted (false negative). In this case, a fungicide spray was needed but not advised, resulting in potentially high yield losses. The results suggest that estimating *B. squamosa* airborne inoculum with the qPCR assay was more reliable than based on microscope counts (Table 3). In addition, at both D_{th} s, the risk associated with the qPCR assay was lower than with the microscope counts.

Previous studies on the aerobiology of BLB showed that monitoring the presence and quantity of airborne secondary inoculum (conidia) could provide a direct measure of vulnerability of onion to infections (6,7). An action threshold of 10 to 15 conidia/ m^3 has been shown to be reliable in determining the need for fungicide application (6). Furthermore, experiments on the spatial distribution of airborne inoculum showed that airborne inoculum of *B. squamosa* is randomly distributed when disease is low or within disease levels generally observed in commercial onion fields (8). Hence, monitoring of airborne inoculum could be achieved with one or few samplers per onion field. Unfortunately, the difficulties in quantifying airborne inoculum using conventional methods (microscopy) have limited the practicality of inoculum-based disease risk indicators.

In this study, a rapid and reliable method of detection and quantification of airborne conidia was developed. There have been several studies where airborne inoculum has been quantified with PCR (4,14,15,23,37). In this study, we went one step further and tested the reliability of the qPCR assay as a decision tool using field data. BLB is a polycyclic inoculum-limited disease and one of the few diseases for which aerobiological data are available (6–8,34). The results of this study demonstrated that spore trapping combined with qPCR assays is suitable for assessing risk of BLB at the field level. Despite the reliability of the qPCR assay, it is not advisable to use airborne conidia concentration as sole indicator to determine whether a fungicide spray is needed. Airborne inoculum concentration is influenced

TABLE 3. Proportion of true positive (TPP) and true negative (TNP) predictions at the action thresholds (cutoff points) identified by Youden's index for both methods of spore quantification at damage thresholds (D_{th}) of 5 and 10 lesions/leaf

Method	$D_{th} = 5$		$D_{th} = 10$	
	TPP	TNP	TPP	TNP
Microscope	0.79	0.82	0.81	0.78
DNA	0.95	0.82	0.89	0.93

TABLE 2. Prevalence of cases, area under the receiver operating characteristic curve (AUC) with associated confidence interval (CI), and P value for a two-tailed test of the AUC being significantly different than 0.5 for two methods of conidial quantification at two damage thresholds (D_{th})

Method	D_{th}	Prevalence ^a	Cutoff (conidia/ m^3) ^b	AUC ^c	95% CI of AUC ^d		Significance ^e
					Lower limit	Upper limit	
Microscope	5 lesions	44	14.7	0.85	0.79	0.91	<0.0000
DNA	5 lesions	44	15.3	0.94	0.90	0.98	<0.0000
Microscope	10 lesions	36	7.2	0.84	0.78	0.91	<0.0000
DNA	10 lesions	36	12.5	0.95	0.91	0.99	<0.0000

^a Prevalence of cases (sample size = 142).

^b Best cutoff point expressed in conidia per cubic meter of air was calculated from Youden's index.

^c AUC curve and its standard error were calculated as described by Hanley and McNeil (16). For a perfect diagnostic test, the AUC = 1 and for tests that have no power to discriminate, the AUC = 0.5.

^d The 95% CI around AUC.

^e Significance is the P value for a two-tailed test (AUC = 0.5).

by various factors, including the microclimate, crop rotation, and susceptibility of onion cultivars. In addition, for practical purposes, sampling was restricted to 2 h (10:00 a.m. to 12:00 p.m.), which means that inoculum quantification may not be reliable on days when peak of spore release occurs in the afternoon (31). To circumvent these limitations, it is proposed to assess airborne inoculum of *B. squamosa* three times per week (7). Furthermore, the information of airborne inoculum is intended to be used in combination with disease monitoring data and weather-based disease forecasts. The method developed in this study could be implemented at the regional or farm levels provided that a suitable network of spore traps is established and samples could be processed quickly and centrally. Such a network of spore traps is being implemented in the organic soil area of southwest Montreal.

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