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ORIGINAL ARTICLE

Forecasting potato white mold by assessment of ascospores in Iran fields

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Abstract

Potato white mold caused by *Sclerotinia sclerotiorum* is an important plant disease occurring in many potato-producing areas throughout the world. In this study, a specific diagnostic method was used to detect and quantify *S. sclerotiorum* ascospores, and its forecasting ability was assessed in potato fields during flowering periods of 2011 to 2014 in Bahar County, Hamedan Province. Using GenEMBL database, a primer pair, HZSCREV and HZSCFOR, was designed and optimized for the pathogen. After testing the sensitivity of primers, DNA was extracted from samples of outdoor Burkard traps from potato fields. A linear association was observed between pathogen DNA and the number of ascospores using the quantitative PCR (qPCR) technique in the presence of SYBR dye. The qPCR could successfully detect DNA amounts representing two *S. sclerotiorum* ascospores and was not sensitive to a variety of tested fungi such as *Botrytis cinerea*, *Alternaria brassicae*, *Fusarium solani.* In contrast to the amount of rainfall, a direct relationship was found between ascospore numbers and the incidence of potato white mold from 2011 to 2014.

Key words: ascospores, forecasting, PCR, potato, *Sclerotinia sclerotiorum*

Introduction

Sclerotinia sclerotiorum is an ascomycetous phytopathogenic fungus which can infect over 400 plant species in the world (Boland and Hall 1994). The pathogen causes white mold in many potato-producing areas of Hamedan Province, Iran, mainly in fields irrigated by sprinkler systems (Ojaghian 2009). Although a few reports are available showing severe damage of white mold on potato, the pathogen frequently causes substantial yield loss in Hamedan fields (Partyka and Mai 1961; Ojaghian 2011). The sclerotia, overwintering structures of the fungus, are found in crop debris as well as in field soil and play an important role in the disease cycle of potato white mold. Epidemics of white mold start with the release of airborne ascospores produced from funnel-shaped apothecia formed on sclerotia (Clarkson *et al.* 2007). The first infection of potato plants by *S. sclerotiorum* is initiated by ascospores which can infect only senescent tissues and petals (Atallah and Johnson 2004). The secondary spread of the pathogen results from direct contact between healthy and infected tissues when colonized petals fall and transfer the pathogen onto lower branches and leaves (Abawi and Grogan 1979). Although several nonchemical methods such as biological control (Ojaghian 2010, 2011; Kowalska *et al.* 2017), UV irradiation (Ojaghian *et al.* 2017), induction of systemic acquired resistance (SAR) (Ojaghian *et al.* 2013) and biofumigation of *Brassica* crops (Ojaghian *et al.* 2012a, b)

have been reported against *Sclerotinia* diseases, most growers consider application of fungicides to be a major method against potato white mold which has led to severe pollution of underground water (data not presented).

Several forecasting systems have been reported to help make well-timed decisions against *Sclerotinia* diseases in different countries. Most of the forecasting methods have been based on climate conditions leading to spore release when the petals fall and contact the lower parts of host plants (Turkington *et al.* 1991a, b; Nordin *et al.* 1992; Twengstrom *et al.* 1998). However, Clarkson *et al.* (2007) showed that wide variation may be found in populations of the pathogen in response to warm and cool periods needed for carpogenic germination of the sclerotia and spore release. Consequently, forecasting schemes based on inoculum may be more precise in predicting the risk of epidemics.

The devices for air-sampling such as Burkard volumetric 7-day trap or rotating-arm sampler usually strike the air particles onto a thin layer of wax or grease coating on a plastic film (Lacey and West 2006). The grease-coated films are then evaluated under a light microscope to identify and count the articles of interest. Because ascospores of *S. sclerotiorum* are similar to ascospores of other closely related fungi such as *Botrytis* species, it is difficult to identify them by microscopy. In recent studies, researchers have integrated traditional air-sampling methods with molecular PCR-based techniques (Fraaije *et al.* 2005; West *et al.* 2006). Quantitative polymerase chain reaction (qPCR) can now be used to identify the replication of DNA during PCR to provide information about the abundance of target DNA rather than simply its existence. Quantitative PCR is a valuable method to measure phytopathogen growth in the tissues of plants in order to assess host resistance (Fraaije *et al.* 2001), and to determine the abundance of fungi inocula in other samples from the environment (Lievens *et al.* 2006; Chilvers *et al.* 2007). A PCR assay used for air samples has been reported by Freeman *et al.* (2002) to detect *S. sclerotiorum* ascospores. This method can be used in epidemiological and forecasting studies because it can quantify the absence or presence of ten ascospores in each reaction. However, in contrast to the other quantitative touch-down PCR techniques (Larsen *et al.* 2004), the touch-down PCR reported by Freeman *et al.* (2002) was not appropriate to be modified for qPCR which used the SYBR green system.

The objectives of this study were to develop a specific and diagnostic method which would be able to accurately measure *S. sclerotiorum* DNA and to assess its role in a forecasting scheme in potato fields during flowering periods from 2011 to 2014 in Bahar County, Hamedan Province, Iran.

Materials and Methods

Designing primer

According to the data submitted to the GenEMBL database, reverse and forward primers were designed. The sequence SSU07553 obtained from the database was for a gene in *S. sclerotiorum* encoded ORF1 and the mitochondrial SSU rRNA intron. The sequence was subjected to BLASTN analysis and then aligned against the other related sequences. Reverse and forward sequences were then chosen over specificity regions for *S. sclerotiorum* and subjected to BLAST in order to assess the interactions with other sequences. Two primer sites including HZSCREV (5′-GAAGTCAGCATGCCTTTCTAGTTATAAGG-3′) and HZSCFOR (5′-ACGCTACAACTCAGATTTAGA TGATCGA-3′) were chosen because of the possible potential of specificity towards the pathogen but no other sequences in the database of GenEMBL.

Optimization of the primer

To determine the optimal annealing temperature, a PCR was conducted using a gradient block thermocycler machine (MJ Research Inc.) and the gradient was set to run between 43 and 55°C. In this experiment, the PCR was performed in a 20 µl volume containing 1 µl of DNA, 10 µl of 2 × PCR reaction buffer, 0.1 µM of primers and RedTaq DNA polymerase (1.5 mM Mg^{2+}). The following parameters were used in PCR amplifications: 94°C followed by 50 cycles of 93°C for 50 s, annealing temperature for 55 s and 73°C for 55 s, followed by a final extension phase of 73°C for 4 min. After cooling down to 4°C, the PCR products were loaded on 1.5% agarose gels in Tris-acetate-EDTA (TAE) buffer. The PCR reactions were replicated twice and *B. cinerea* was used as a template DNA for comparison.

qPCR optimization and sensitivity test

The amplifications of qPCR were done in a DNA Engine Opticon 4 System (MJ Research, MA, USA) using the SYRB Green I fluorescent dye detection (Sigma-Aldrich). The concentration of primers HZSCREV and HZSCFOR was optimized to 300 nM based on the manufacturer's recommendations. The amplifications were carried out in 20 µl volumes. The PCR parameters were set up as follows: an initial heating for 4 min at 95°C, followed by 50 cycles at 94°C for 20 s, 57°C for 26 s, 75°C for 35 s and finally 83°C for 3 s. After the first reaction showed that the PCR product had a peak at 82°C, a dissociation curve was produced. Thus, a further read step was added to the PCR at 80°C

to melt any exogenous materials like primer dimmers which might wrongly affect the calculation of target DNA. The reactions were conducted twice. The cycle threshold (CT) value was calculated and analyzed for each qPCR. In order to evaluate the influence of DNA extracted from *B. cinerea* on the sensitivity of the method, quantification of seven concentrations of *S. sclerotiorum* DNA (40 ng to 4×10^{-5} ng in each reaction) was compared alone and when mixed with two concentrations (5 or 0.5 ng) of *B. cinerea* DNA. In addition, the sensitivity of the technique was evaluated by comparing with the sensitivity of touch-down PCR described by Freeman *et al.* (2002) and by examining the detection of serial dilutions *S. sclerotiorum* DNA (40 ng to 4×10^{-5} ng in each reaction) alone and when mixed with two concentrations (5 or 0.5 ng) of *B. cinerea* DNA and observing the PCR products on agarose gel.

Extracting DNA for testing the sensitivity of primers

Using a method described by Rogers *et al.* (2009), DNA was extracted from tape sections artificially coated with ascospores (described in the next part) in screw-top tubes (2 ml). MicroLYSIS (60 µl, Microzone) and Ballotini Beads (0.1 g, 400–600 µm diameter) were added to the tubes and shaken in a Fast Prep machine at 4 m · s^{-1} for 20 s. After transferring the liquid to a PCR tube (0.2 ml), PCR was conducted based on manufacturer's protocol (66°C for 14 min, 95°C for 2 min, 66°C for 5 min, 95°C for 2 min, 66°C for 2 min, 95°C for 30 s, 15°C hold). In addition, polyvinylpyrrolidone (2 mg) and TE buffer (40 µl, 10 mm, pH 8.0) were added and spun at 15,115 g for 14 min to eliminate polysaccharides, which may hinder the PCR reaction. A portion (60 µl) of the supernatant was transferred to a new tube (0.2 ml) to which ethanol (150 µl) and ammonium acetate (10 µl, 7.5 m, dissolved in water) were added and spun at 15,115 g for 14 min. After discarding the supernatant, the remaining pellets were air-dried, resuspended in sterile distilled water (SDW, 10 µl), kept at –25°C and 2.5 µl of it was used in each qPCR reaction.

Extracting DNA to test primers on air samples

According to Rogers *et al.* (2009), DNA was extracted from samples of outdoor Burkards from Hamedan fields in 2011, 2012, 2013 and 2014. Subsections of tape (50 × 7 mm) were cut into five pieces and located in an autoclaved 2-ml tube. In the next step, acid-washed Ballotini beads (400– 600 µm diameter), β-mercaptoethanol (15 µl) and CTAB buffer (500 µl, 100 mM Tris HCl, 2% CTAB, 20 mM EDTA, 1.4 mM NaCl) were added to each tube. The tubes were shaken in two 45-s Fast Prep cycles, incubated at 75°C for 55 min and centrifuged at 15,115 g for 20 min. The supernatant was collected and extracted using 400 µl chloroform : isoamyl alcohol (25 : 1). After being vortexed, the tubes were centrifuged for 16 min and the aqueous phase was collected (500 µl). In order to initiate the precipitation of DNA, 800 µl ice-cold ethanol (100%) and 50 µl of 3 M sodium acetate were added to each tube. After gentle inversion for mixing, the samples then remained at –25°C for 2 h. The DNA pellets were then precipitated after centrifugation for 10 min at 15,115 g and the supernatant was removed. Using ice-cold ethanol (70%), the pellets of DNA were then washed, dried at 36°C for 15–20 min and dissolved in SDW (100 µl). The DNA remained at –25°C. Subsamples (3 µl) of the remaining DNA were used to be tested by qPCR using the technique mentioned above for quantifying DNA of *S. sclerotiorum*.

Processing of air samples and observations in the field

According to Lacey and West (2006), the qPCR technique was first tested on the DNA extracted from ascospores of *S. sclerotiorum* collected on cellulose filters (Millipore Ltd.) which were artificially put onto the grease-coated tapes as observed in the Burkard spore sampler. As reported by Freeman *et al.* (2002), in order to collect ascospores onto cellulose filters, the surface-sterilized conditioned sclerotia were incubated in containers of sterile moistened perlite at 10°C in the dark. When stipes appeared, the containers were subjected to nUV light to instigate ascospore production. The ascospores were harvested on the cellulose filters using suction and then kept at –25°C. A suspension of ascospores was made in SDW (10 ml) after quarter sections of the cellulose filters were vortexed in autoclaved 50-ml centrifuge tubes. Using a haemocytometer, the concentration of ascospores was determined (approx. 2×10^5 ascospores · ml^{-1}) and a serial dilution (1/10 and 1/100) was made of the original concentration. A Chrom atomizer (CamLab) was used to spray a serial dilution of ascospore suspensions onto grease-coated plastic tapes joined to the collection drum of a Burkard trap. The tape sections became evenly coated with spray droplets, and before the tapes were removed from the collection drum they were air dried and cut into 50-mm sections which were similar in length to a 24-h Burkard trap sample. The grease-coated tapes which were not exposed to ascospores were used as negative controls.

Using forceps each tape section artificially coated with ascospores was held at the edge and longitudinally cut along the centre line with scissors to provide two subsections (7 × 50 mm). One tape subsection was put into a 2-ml tube, tagged and stored at –25°C. The

second tape subsection was stained with trypan blue in polyvinyl alcohol on a microscope slide, and the numbers of ascospores were counted under a microscope (Lacey and West 2006). The number of ascospores was counted in two 200-µm longitudinal traverses of the tapes. Therefore, the counted area of tape was 5.7% of the subsection. In order to estimate the number of ascospores per tape subsection, the number of counted ascospores was multiplied by 17.5 because the number of ascospores per tape subsection divided by 7.2 equals the number of spores in each cubic meter of air as derived from a Burkard trap working at a constant flow rate of 10 l per minute).

Air sampling and incidence of potato white mold

Using Burkard 7-day spore samplers (Burkard Manufacturing Company Ltd.), air sampling was conducted based on standard methods described by Lacey and West (2006). The samples were taken from outdoor potato fields of Hamedan, Bahar in June and July 2011, 2012, 2013 and 2014. Daily sampling from Burkard traps was done similar to the method mentioned above for the tape sections artificially coated with ascospores in order to provide daily 7 × 50 mm subsections used for DNA extraction and observation under a microscope. A subset of microscope slides made of samples from Burkard traps was counted under a microscope. Elliptical spores ranging from 8 to 10 µm by 3–5 µm were counted as ascospores of *S. sclerotiorum*.

In this step, it was possible that dense masses of colorful spores and hyphal pieces masked the ascospores of *S. sclerotiorum*. In addition, spores of other fungi might have been counted as ascospores. Therefore, the number of ascospores was estimated from the mean DNA for each ascospore found on the tapes artificially inoculated with ascospores.

The incidence of white mold in potato fields at Bahar was yearly monitored within 1.5 km of the site of air sampling. Moreover, meteorological data was provided by the Hamedan meteorological station located approx. 2 km from the air samplers and field experiments in each season.

Results

Optimizing primers and qPCR

Using the primer pair HZSCREV and HZSCFOR, a PCR fragment of 150 bp was generated from all nine *S. sclerotiorum* isolates sampled in Hamedan (Iran) and Hangzhou (China). In addition, a smaller PCR product (90 bp) generated from *B. cinerea* DNA was observed. However, no PCR fingerprints were generated from DNA of other tested fungi (Table 1). In qPCR, the concentration of primers (300 nm per reaction) were optimized according to the instructions of the Engine Opticon qPCR machine, and a further step was added to the PCR (80°C for 30 s) to remove any exogenous materials which were not *S. sclerotiorum*. No PCR product was detected from the treatments containing only DNA of *B. cinerea* at this temperature, indicating that the primer pair can be used to detect *S. sclerotiorum* in mixed samples. In addition, the presence of 0.5 and 5 ng DNA of *B. cinerea* did not affect the amount of *S. sclerotiorum* DNA in a range from 40 ng to 4×10^{-5} ng, compared with the samples without DNA of *B. cinerea* (Fig. 1). However, 5 ng DNA of *B. cinerea* decreased the amount of *S. sclerotiorum* DNA at 4×10^{-5} ng, the lowest tested amount, which was near the limit for detecting pure DNA of *S. sclerotiorum*. Moreover, the presence of *B. cinerea* DNA did not influence the qPCR technique reported by Freeman *et al.* (2002), but its sensitivity was found to be less than the qPCR method described in this study because no amplification was observed for dilutions below 0.05 ng (data not presented). In order to confirm the specificity of qPCR, DNA was extracted from a variety of fungi shown in Table 1.

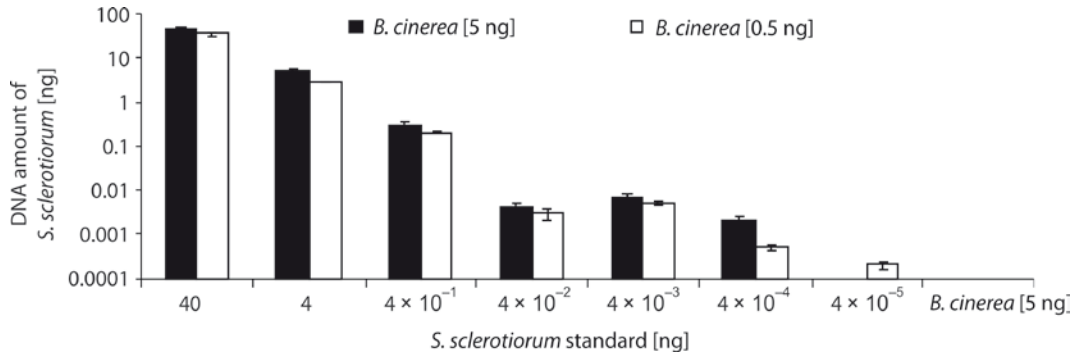

Fig. 1. Quantification of different amounts of *Sclerotinia sclerotiorum* DNA (standard) in the presence of *Botrytis cinerea* DNA (5 or 0.5 ng). A sample of only *B. cinerea* DNA (50 ng) was also used as control. The standard errors of means have been represented by bars

Table 1. DNA detection from different fungi tested by qPCR and PCR using the primer pair HZSCREV and HZSCFOR

Fungal and plant species (isolate)	Detection with PCR	Detection with qPCR
Sclerotinia sclerotiorum (SSIC12)	+	+
S. sclerotiorum (SDJ14)	+	+
S. sclerotiorum (SSG23)	+	+
S. sclerotiorum (SLOI4)	+	+
S. sclerotiorum (SSWA7)	+	+
S. sclerotiorum (HB14)	+	+
S. sclerotiorum (HBD29)	+	+
S. sclerotiorum (HLO8)	+	+
S. sclerotiorum (SSIU6)	+	+
Botrytis cinerea (HM1254)	+	–
B. cinerea (isolated from carrot)	+	–
B. cinerea (FD1204)	+	–
B. cinerea (PIG835)	+	–
B. cinerea (isolated from grapes)	+	–
B. cinerea (MNA932)	+	–
S. minor (PL21)	–	–
S. minor (KJH7)	–	–
S. trifoliorum (CL4J)	–	–
Alternaria brassicae (FDD2)	–	–
Verticillium dahliae (isolated from cotton)	–	–
Leptosphaeria maculans (FRT78)	–	–
Fusarium camptoceras (RTI7)	–	–
Phytophthora parasitica (HM75)	–	–
Fusarium solani (MKC42)	–	–

"–" – DNA was not detected within 50 cycles of qPCR

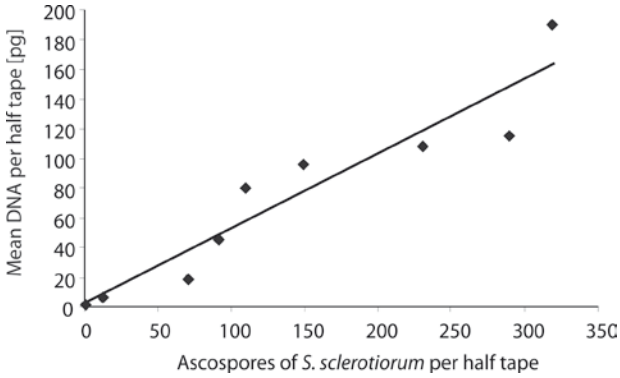

Fig. 2. The correlation between ascospore numbers counted by microscopy (estimated according to the mean number of ascospores of two longitudinal traverses per 1/2 tape) on artificially grease-coated tapes and DNA quantification of *Sclerotinia sclerotiorum* from the opposite half of the tape section determined by qPCR (equation of fitted line is: y = 0.5071x + 1.8841; R2 = 0.902)

Inoculated sections of tape

The results (Fig. 2) showed that there was a direct relationship between the number of ascospores microscopically estimated in each tape subsection and DNA of *S. sclerotiorum* determined by qPCR at the ratio of 0.35 pg DNA/spore ($R^2 = 0.902$).

Outdoor samples of Burkard traps, weather and white mold incidence

The results showed low numbers of *S. sclerotiorum* ascospores · m^{-3} · day^{-1} in June and July (flowering period) of 2011 and 2013 compared with 2012 and 2014 (Fig. 3). The number of ascospores was estimated from the mean DNA per ascospore obtained from inoculated tapes within 1.5 km of the potato plants (Fig. 2). The highest number of five and four ascospores were detected in early July in 2011 and 2013, respectively. Moreover, white mold incidence was reported as 5 and 4% for 2011 and 2013, respectively. In contrast, the pattern of airborne DNA of the pathogen at Bahar in 2012 showed that a high number (>6) of ascospores was frequently observed between mid-June and mid-July, peaking at 12 ascospores · m^{-3} · day^{-1} and the incidence of potato white mold was found to be approximately 21%. In 2014, the maximal number of 11 ascospores was detected in early July and mid-July, and disease incidence was found to be 19% (Fig. 3). The examination of fields in 2014 showed that the incidence of white mold was 9% due to application of fungicide fluazinam in early June compared with disease incidence in untreated plots (19%). In addition, no differences were observed between different cultivars toward white mold. These results were obtained from sampling 2,000 plants from each untreated or treated plots ($p < 0.005$).

Discussion

In our previous studies, antifungal and SAR efficacy of crude plant extracts and E-cinnamaldehyde was tested against *Sclerotinia* diseases on potato and carrot (Ojaghian *et al.* 2014a, b, 2105, 2016). In addition, mycelial compatibility groups of the pathogen were identified in potato fields of Hamedan Province (Ojaghian and Xie 2012c). Based on previous reports (Turkington *et al.* 1991a; McCartney *et al.* 1999), it is known that the risk of *Sclerotinia* white mold is related to the concentration of air borne ascospores during the flowering period of plants or the infestation percentage of petals at early bloom. In this study, qPCR was shown to be an accurate and rapid technique to detect and quantify DNA of *S. sclerotiorum* from natural samples. The sensitivity of the method mentioned in this study was higher than

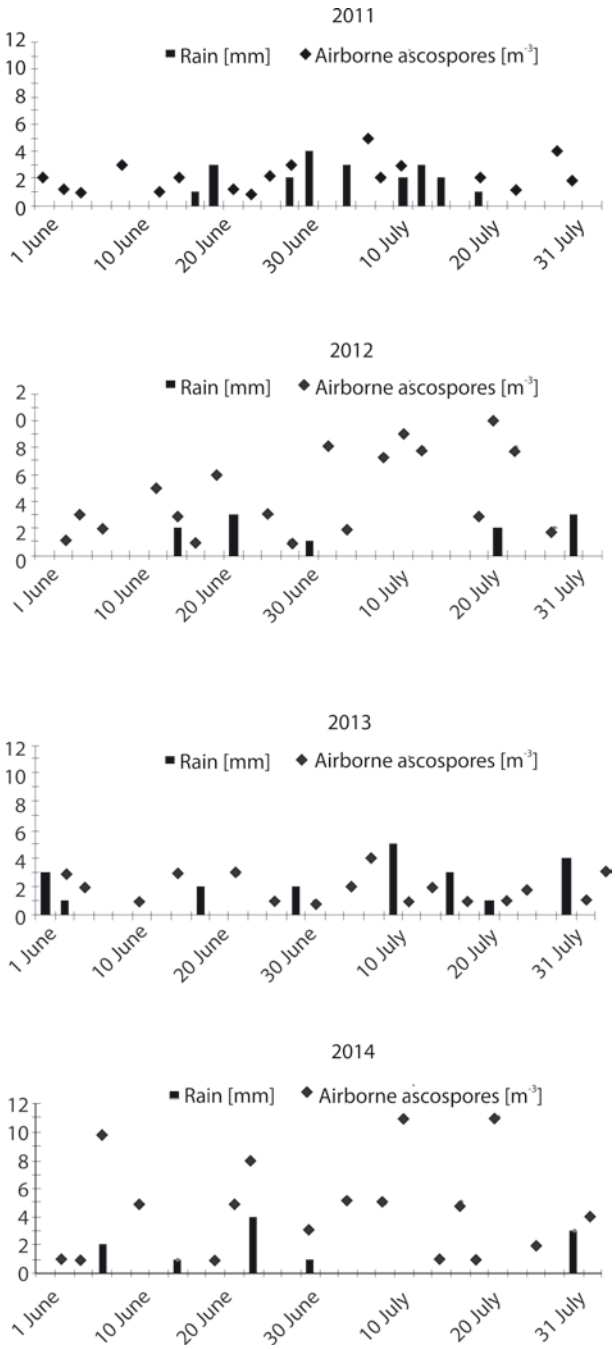

Fig. 3. The rainfall level and number of airborne ascospores leading to incidence of potato white mold caused by *Sclerotinia sclerotiorum* by 5, 21, 4 and 19% in 2011, 2012, 2013 and 2014, respectively. The number of ascospores was estimated from mean DNA per ascospore obtained from inoculated tapes within 1.5 km of the potato plants

the PCR technique described by Freeman *et al.* (2002) because it was possible to detect *S. sclerotiorum* DNA as low as 4×10^{-4} ng (0.4 pg) as *B. cinerea* DNA was present. It is known that small numbers of ascospores present in air for a long period of time may cause a considerable epidemic of *Sclerotinia* disease (McCartney *et al.* 1999). However previous techniques were not able to detect low numbers of airborne ascospores. The primer used in the qPCR technique produced a DNA product from *B. cinerea* as used in an end point PCR. However due to different melting points and sizes of PCR products of *S. sclerotiorum* and *B. cinerea*, the qPCR conditions were optimized to prevent any quantification of *B. cinerea* DNA and primer dimmers which had potential to cause fluorescence in the presence of SYBR dye. Therefore, the qPCR used in this research was specific to quantify *S. sclerotiorum* DNA. When different amounts of DNA of two closely related fungi species were mixed, the detection sensitivity of *S. sclerotiorum* was not reduced, except for very high amounts of *B. cinerea* DNA (5 ng) mixed with very slight amounts of *S. sclerotiorum* (4×10^{-5} ng). It is nearly impossible that such a high amount of *B. cinerea* DNA occurs in the air. In addition, high amounts of *B. cinerea* DNA decreased sensitivity of *S. sclerotiorum* detection only in a point very near to the detection limit of pure DNA of *S. sclerotiorum*. The qPCR performed on tape sections artificially inoculated with ascospores under laboratory conditions quantified 0.4 pg DNA per ascospore of *S. sclerotiorum*. Furthermore, there was a variability ($R^2 = 0.902$) in the relationship which could have been due to rough distribution of the ascospores sprayed on tape surfaces, resulting in small differences between microscopically counted ascospores on half of the tape and ascospores detected by the qPCR method on the other half. The DNA amount determined for each ascospore was used to measure the number of ascospores from DNA calculated from outdoor samples using a Burkard trap which was able to detect 1.5 ascospores daily from air samples (0.1 ascospore per m^3 air). In comparison, quantification accuracy of ascospores using microscopy was less than the qPCR method due to difficulty in ascospore identification. Moreover, counting ascospores was done in two traverses of tape under a microscope representing only 3% of the entire tape surface, which means it was usually impossible to detect less than 40–50 ascospores per air sample representing 3–5 ascospores $\cdot$ m^{-3} $\cdot$ day^{-1}.

The copy number of the DNA target sequence can affect the sensitivity of the primer set used in detection of airborne ascospores. However, there is currently no information about the copy numbers of the target DNA sequence present in each ascospore. In addition, the method used in DNA extraction may influence sensitivity of the primer pair. Both methods used to extract DNA in this research were shown to be similarly reliable (data not presented). Although there may be natural products present in the airborne ascospores to act as PCR inhibitors and cause false negative results, the methods of DNA extraction and serial dilution used in this research were not found to cause any false negative results from previously examined air samples.

The results obtained from outdoor samples indicate that this technique has potential to be used in

forecasting schemes based on inoculum similar to those used for soybean rust (http://www.ces.ncsu.edu/depts/pp/soybeanrust/howtoreadforecast.php) and tobacco blue mold (http://www.ces.ncsu.edu/depts/pp/bluemold). There are several forecasting studies on *Sclerotinia* diseases which have included a rainfall component in the process of decision (Nordin *et al.* 1992; Turkington and Morrall 1993; Twengstrom *et al.* 1998; Koch *et al.* 2007). However, this study showed that the high number of ascospores present in the air during the flowering time plays a more important role than the amount of rainfall in the incidence of potato white mold. For example, no significant rainfall was observed in 2012, but disease incidence was found to be approximately 21%. In this research, the association between airborne ascospores and incidence of potato white mold was studied as an example for application of the qPCR technique which can be used to assess the quantity and presence of *S. sclerotiorum* on seeds, soil and petals. In 2014, application of fluazinam as a protectant fungicide significantly reduced disease incidence in potato fields which may have been caused by protecting leaves and stems from infected petals, confirming the disease cycle. Further research is necessary to assess the effect of sprinkling irrigation on releasing ascospores from apothecia and evaluate conditions favorable to petals sticking to stems and leaves in different crops.

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