Detection and Quantification of Airborne *Claviceps purpurea* sensu lato Ascospores from Hirst-Type Spore Traps using Real-Time Quantitative PCR

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Abstract

The U.S. Pacific Northwest states of Oregon and Washington are major producers of cool-season grass seed. Ergot, caused by fungi in the *Claviceps purpurea* sensu lato group, is an important seed replacement disease of grass worldwide. Microscopic methods that are currently used to quantify airborne *Claviceps* ascospores captured by spore traps are not currently rapid enough to allow for detecting and reporting of spore numbers in a timely manner, hindering growers from using this information to help manage ergot. We developed a SYBR Green real-time quantitative polymerase chain reaction (qPCR)-based assay for fast and efficient detection and quantification of *C. purpurea* sensu lato ascospores from Hirst-type spore traps. Species-specificity of the qPCR assay was confirmed against 41 *C. purpurea* sensu lato isolates collected from six hosts and six other *Claviceps* spp. Significant relationships were observed between cycle threshold (Ct) values and

standard curves of serial dilutions of DNA ranging from 1 pg to 10 ng ($R^2 = -0.99$; $P = 0.0002$) and DNA extracted from a conidial suspension representing 8 to 80,000 conidia ($R^2 = -0.99$; $P = 0.0004$). Ct values from qPCR were significantly correlated with results from microscopic examination of spore trap samples from the field ($r = -0.68$; $P < 0.0001$) and the procedure was able to detect a single ascospore from spore trap tape samples. The qPCR procedure developed in this study provided a means for quantifying airborne *Claviceps* ascospores that was highly specific and useful over a wide range of spore densities, and could be performed in a matter of hours instead of days. The qPCR assay developed in this study could be part of an integrated pest management approach to help grass seed growers make risk-based fungicide application decisions for ergot management in grass grown for seed.

Cool-season grass seed is an important crop in the Pacific Northwest states of Oregon and Washington where, in 2012, over 300,000 acres were harvested, with a production value of over \$296 million. Kentucky bluegrass (*Poa pratensis* L.) and perennial ryegrass (*Lolium perenne* L.) are two of the major cool-season grass species grown in these two states. Ergot is an important seed replacement disease of grass and a persistent problem in grass seed production systems (Alderman 1991; Dung et al. 2016). Two species within the *Claviceps purpurea* sensu lato group, *C. purpurea* sensu stricto (Fr.) Tul. and *C. humidiphila* Pažoutová et M. Kolařík, can cause ergot in *P. pratensis*, whereas only *C. purpurea* sensu stricto has been reported to infect *L. perenne* (Douhan et al. 2008; Pažoutová et al. 2000; Scott et al. 2015). Soilborne sclerotia of the pathogen germinate in the spring to produce capitula which, in turn, release airborne ascospores that infect the unfertilized flowers. Once infected, the ovary is ramified by fungal hyphae and a sclerotium is formed instead of viable seed. Ergot causes economic losses to the cool-season grass seed industry due to reduced yields and the loss of viable seed and

increased labor costs during seed cleaning to remove sclerotia to meet certification standards.

Fungicides are an important component of an ergot management program and the majority of grass seed growers surveyed in Oregon and Washington apply fungicides at least once during anthesis (Walenta et al. 2015). However, the efficacy of fungicides depends upon the timing and coverage of the application. Fungicides that are applied after a major spore production event or do not reach the ovary will not be effective at preventing disease. Models can be used to predict when the majority of inoculum is likely to be present but pathogen populations vary daily depending on temperature, soil moisture, and other environmental factors (Dung et al. 2017). Methods to detect and quantify airborne ascospores during anthesis would provide an additional tool for growers to make more informed management decisions based upon the presence or absence of inoculum.

The ergot fungus requires unfertilized ovaries for infection; therefore, early detection and accurate quantification of airborne ascospores is imperative for successful management of ergot epidemics, especially when ascospore production coincides with anthesis. The current method used to detect and quantify *Claviceps* ascospores utilizes Hirst-type volumetric spore samplers, in which spores are collected on a sticky tape surface (Alderman 1993). Spore-trapping studies conducted over the past several years have proven to be useful in detecting airborne *C. purpurea* ascospores in grass seed fields (Alderman 1993; Alderman et al. 2010, 2015; Dung et al. 2017). Although spore trapping is effective at providing quantitative data on airborne spore numbers, the processing and examination of the tapes is time consuming (up to 8 h for a 7-day tape). The spore counting can be difficult and the amount of time and labor involved increases substantially when large numbers of ascospores are captured. This was the case during 2013, when over 9,000 ascospores were captured by a single spore trap on some days (Dung et al. 2017). Another problem encountered during the microscopic examination of spore tapes is that sections of the tape can be uncountable due to overlapping layers of pollen or high densities of soil

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particulates that can occur on dry, windy days. In addition, the potential for misidentification exists if other fungal spores with similar morphology are present, especially if the technician is not properly trained in the identification of *Claviceps* ascospores.

DNA-based detection methods are often considered to be more specific and sensitive than traditional microscopic identification, and less time and labor are typically involved once a protocol is developed and technicians are trained. The quantification of target DNA in the presence of extraneous spores from other species, pollen, insects, and airborne particulates can be performed more specifically and reliably. Molecular methods, mostly based on variations of polymerase chain reaction (PCR), have been used to detect many plant-pathogenic fungi (Schaad and Frederick 2002), and a real-time PCR assay was previously developed for *C. africana*, *C. sorghi*, and *C. sorghicola*, which cause ergot of sorghum (*Sorghum bicolor* (L.) Moench) (Tooley et al. 2010). Spore trapping has been successfully used in combination with PCR-based methods to detect and quantify plant pathogens in air samples (Falacy et al. 2007; Klosterman et al. 2014; Kunjeti et al. 2016), demonstrating the potential value of combining spore trapping with DNA-based assays to study and monitor the production and dispersal of airborne inoculum of plant pathogens affecting important crops.

A critical component and precursor of a risk-based, integrated pest management approach to ergot management is the development of a rapid and reliable means of early detection of *C. purpurea* sensu lato inoculum. Timely and accurate forecasting and warnings of the presence of *Claviceps* ascospores can help growers make more informed management decisions and is recognized as a major grower priority (Walenta et al. 2015). A fast and reliable detection protocol for the presence of airborne *C. purpurea* sensu lato ascospores would better enable grass seed growers to make more informed, risk-based decisions regarding fungicide applications, potentially improving fungicide timing and efficacy, reducing the number of fungicide applications, and contributing to reduced or delayed development of fungicide resistance. Quantification of ergot inoculum during the season may also allow growers to predict which seed lots will require additional cleaning after harvest, allowing them to plan their postharvest seed conditioning operations accordingly.

Because spore traps collect spores of both pathogenic and non-pathogenic fungi, species-specificity of the assay is required to accurately detect and quantify ergot inoculum. However, both *C. purpurea* sensu stricto and *C. humidiphila* can potentially cause ergot in cool-season grass grown for seed (Douhan et al. 2008; Pažoutová et al. 2000; Scott et al. 2015); therefore, the simultaneous quantification of both species in the *C. purpurea* sensu lato group would be a useful attribute of a quantitative (q)PCR assay for ergot monitoring in grass seed production systems. The objectives of this project were to (i) develop a qPCR assay to detect and quantify inoculum of *C. purpurea* sensu lato and (ii) validate the qPCR assay using spore trap tape samples collected from commercial Kentucky bluegrass and perennial ryegrass seed production fields.

Materials and Methods

Fungal material and DNA extraction. Sclerotia of *C. purpurea* sensu lato were collected from infected perennial ryegrass in Oregon and infected Kentucky bluegrass in Oregon and Washington. Sclerotia were surface sterilized in 70% ethanol for 10 s followed by 0.5% NaOCl for 3 min, rinsed in sterile distilled water (sdH₂O), and air dried in a laminar flow hood. Isolates were obtained in pure culture by breaking surface-sterilized sclerotia in half and plating them broken-side down onto 1.5% water agar amended with streptomycin sulfate (200 mg/liter). Isolates were hyphal tipped and stored on one-quarter-strength potato dextrose agar slants (Difco potato dextrose agar at 10 g/liter and Difco bacto agar at 15 g/liter; Becton, Dickinson, and Company, Sparks, MD). Sclerotia from rye (*Secale cereale*) and smooth brome (*Bromus inermis*) were collected from Oregon and Washington, respectively, and pure cultures were obtained as described above. Lyophilized tissue of *C. purpurea* from other states and countries and from other *Claviceps* spp. (*C. africana*, *C. fusiformis*, *C. paspali*, and *C. pusilla*) were kindly provided by Dr. Paul

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To obtain mycelium for the DNA extractions, isolates were grown in potato dextrose broth (Becton, Dickinson, and Company) on an orbital shaker at 150 rpm. Genomic DNA was extracted from lyophilized fungal tissue using a glass bead disruption protocol followed by phenol-chloroform extraction and sodium acetate-ethanol precipitation (Dobinson et al. 2000). The DNA pellet was subsequently resuspended in a buffered 10 mM Tris solution. Additional DNA extracts from *C. cynodontis* and *C. maximensis* were provided by Dr. Tooley. The quality and quantity of genomic DNA was determined with a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and Qubit 3.0 fluorometer (Thermo Fisher Scientific), respectively, according to the manufacturer's directions.

Identification of fungal isolates. PCR was used to amplify three loci for sequencing. Primers ITS1 and ITS4 (White et al. 1990) were used to amplify the internal transcribed spacer (ITS) region of ribosomal DNA. A portion of the β -tubulin gene was amplified using primers BT5 and BT3 (Annis and Panaccione 1998; Tooley et al. 2001) and partial translation elongation factor (EF)-1 α sequences were obtained using primers EF1-728F and EF1-986R (Carbone and Kohn 1999; Tooley et al. 2001). PCR were carried out in an Eppendorf Mastercycler nexus gradient Thermal Cycler or an Eppendorf Mastercycler Gradient 5331 Thermal Cycler (Eppendorf, Hauppauge, NY).

ITS reactions were performed in 25- μ l reactions containing 12.5 μ l of GoTaq Colorless Mastermix (2 $\times$) (Promega Corp., Madison, WI), 7.5 μ l of each primer (10 μ M), 8.5 μ l of water, and 2 μ l of DNA (2 ng/ μ l) using the following cycling parameters: denaturing at 95°C for 3 min, followed by 30 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. A final extension step was carried out at 72°C for 10 min. PCR for β -tubulin and EF-1 α sequences were performed in 50- μ l reactions containing 10 μ l of GoGreen PCR Buffer (5 $\times$) (Promega Corp.), 0.4 μ l of dNTP (10 mM) (Thermo Scientific), 7.5 μ l of each primer (10 μ M), 1 μ l of MgCl₂ (25 mM) (Bioline, Taunton, MA), 0.3 μ l of Taq polymerase (5 u/ μ l) (Promega Corp.), 0.1 μ l of Accuzyme (2 u/ μ l) (Bioline), 35.2 μ l of water, and 2 μ l of DNA template (2 ng/ μ l). In some cases, β -tubulin and EF-1 α amplicons were not obtained using the 50- μ l PCR mixture; when this occurred, β -tubulin and EF-1 α amplicons were obtained using the respective primers in 25- μ l PCR mixtures that were utilized to obtain ITS products as described above. PCR for β -tubulin and EF-1 α genes were performed using the following cycling parameters: denaturing at 95°C for 1 min, followed by 35 cycles of denaturing at 94°C for 15 s, annealing at 50°C (β -tubulin) or 58°C (EF-1 α) for 1 min, and extension at 72°C for 1 min. A final extension step was carried out at 72°C for 10 min. A 5- μ l subsample of the PCR product was analyzed by gel electrophoresis in a 1.25% agarose gel prepared with 1 $\times$ Tris-acetate-EDTA buffer and 1 $\times$ GelRed (Biotium, Inc., Fremont, CA).

For sequencing, a 3- μ l aliquot of a 10-fold dilution of each PCR product was treated with ExoSAP-IT (USB Corporation, Cleveland, OH), mixed with the appropriate primer, as previously described (Dung et al. 2011), and sequenced by Elim Biopharmaceuticals, Inc. (Hayward, CA). This was repeated with the corresponding primer of the pair. Paired forward and reverse sequences were quality checked, aligned, and manually edited using SeqTrace (Stucky 2012) to obtain consensus sequences.

Primer development and qPCR conditions. A SYBR Green qPCR assay was developed to amplify a 96-bp region of a *C. purpurea* sensu lato-specific random amplified polymorphic DNA (RAPD) amplicon (GenBank accession AJ252159) (Pažoutová and Tudzynski 1999; Pažoutová et al. 2000). Primer3Plus software (Untergasser et al. 2007) was used to design forward and reverse primers AJ252159-F3 (5'-AAGTGACCAAGTTGCACGTC-3') and AJ252159-R3 (5'-CAGACCGTGACAAATGGTTTCTG-3'), respectively. Primer specificity was tested against the 41 *C. purpurea* sensu lato isolates and 10 other *Claviceps* spp. described above (Table 1).

qPCR were performed in a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) in a total volume of 20 μ l containing 10 μ l of SYBR Select Master Mix (2 $\times$) (Thermo Fisher Scientific), 0.6 μ l

of each primer (10 μ M each), and 1 ng of DNA template. A no-template water sample was included in triplicate as a negative control in each run. Cycle conditions consisted of an initial denaturing step for 10 min at 95°C followed by 40 cycles of a denaturing step for 15 s at 95°C and annealing for 1 min at 60°C. A cycle threshold (Ct) value < 40 was interpreted as a positive detection if the melting curve of the amplicon matched that of *C. purpurea*. Melting curve analysis (from 60°C, gradually increasing 0.3°C/s to 95°C, with data acquisition every 1 s) was used to distinguish potential primer dimers and nonspecific amplification products. Melting peaks were obtained from melting curves by plotting the negative derivative of fluorescence against temperature. All samples were amplified using three

technical replicates within each run unless otherwise noted, and each run was repeated at least once.

A standard curve was developed using 10-fold serial dilutions of genomic DNA obtained from a pure culture of *C. purpurea* sensu stricto isolate Cp061. Isolate Cp061 was collected in 2013 from a commercial perennial ryegrass seed field in Umatilla County, OR. DNA was extracted from lyophilized tissue as described above. DNA was quantified with a Qubit 3.0 fluorometer and serial dilutions were performed in sdH₂O to achieve a dilution series ranging from 10 ng to 1 pg. qPCR were performed in triplicate as described above. The amplification efficiency was calculated for each run using a standard curve obtained from serial dilutions of DNA extracted from lyophilized tissue.

Table 1. *Claviceps* isolates and species, host and locations of origin, and mean cycle threshold (Ct) values ($\pm$ standard deviation [SD]) for the quantitative polymerase chain reaction assay developed in this study

Isolate	<i>Claviceps</i> spp.	Host	Origin	Ct $\pm$ SD ^a
Cp016	<i>Claviceps humidiphila</i>	<i>Poa pratensis</i>	Washington	22.36 $\pm$ 0.78
Cp018	<i>C. humidiphila</i>	<i>P. pratensis</i>	Washington	22.63 $\pm$ 0.26
Cp019	<i>C. humidiphila</i>	<i>P. pratensis</i>	Washington	22.18 $\pm$ 0.82
Cp020	<i>C. humidiphila</i>	<i>P. pratensis</i>	Washington	22.00 $\pm$ 0.60
Cp021	<i>C. humidiphila</i>	<i>P. pratensis</i>	Washington	22.95 $\pm$ 0.76
Cp022	<i>C. humidiphila</i>	<i>P. pratensis</i>	Washington	22.64 $\pm$ 0.21
Cp024	<i>C. humidiphila</i>	<i>P. pratensis</i>	Washington	22.34 $\pm$ 0.72
Cp032	<i>C. humidiphila</i>	<i>P. pratensis</i>	Oregon	22.13 $\pm$ 0.21
Cp081	<i>C. humidiphila</i>	<i>P. pratensis</i>	Washington	21.41 $\pm$ 0.56
Cp002	<i>C. purpurea</i> sensu stricto	<i>Lolium perenne</i>	Oregon	20.66 $\pm$ 0.86
Cp003	<i>C. purpurea</i> sensu stricto	<i>L. perenne</i>	Oregon	21.04 $\pm$ 0.26
Cp006	<i>C. purpurea</i> sensu stricto	<i>L. perenne</i>	Oregon	20.14 $\pm$ 0.43
Cp008	<i>C. purpurea</i> sensu stricto	<i>L. perenne</i>	Oregon	20.46 $\pm$ 0.15
Cp012	<i>C. purpurea</i> sensu stricto	<i>L. perenne</i>	Oregon	20.79 $\pm$ 0.24
Cp027	<i>C. purpurea</i> sensu stricto	<i>L. perenne</i>	Oregon	20.83 $\pm$ 0.30
Cp036	<i>C. purpurea</i> sensu stricto	<i>L. perenne</i>	Oregon	20.50 $\pm$ 0.47
Cp037	<i>C. purpurea</i> sensu stricto	<i>L. perenne</i>	Oregon	20.68 $\pm$ 0.04
Cp040	<i>C. purpurea</i> sensu stricto	<i>L. perenne</i>	Oregon	20.33 $\pm$ 0.41
Cp043	<i>C. purpurea</i> sensu stricto	<i>L. perenne</i>	Oregon	26.77 $\pm$ 0.15
Cp049	<i>C. purpurea</i> sensu stricto	<i>L. perenne</i>	Oregon	20.40 $\pm$ 0.56
Cp055	<i>C. purpurea</i> sensu stricto	<i>L. perenne</i>	Oregon	20.69 $\pm$ 0.34
Cp057	<i>C. purpurea</i> sensu stricto	<i>L. perenne</i>	Oregon	27.35 $\pm$ 1.08
Cp064	<i>C. purpurea</i> sensu stricto	<i>L. perenne</i>	Oregon	28.10 $\pm$ 0.24
Cp072	<i>C. purpurea</i> sensu stricto	<i>L. perenne</i>	Oregon	20.62 $\pm$ 0.14
Cp085	<i>C. purpurea</i> sensu stricto	<i>L. perenne</i>	Oregon	19.84 $\pm$ 0.27
Cp033	<i>C. purpurea</i> sensu stricto	<i>P. pratensis</i>	Oregon	22.90 $\pm$ 0.10
Cp052	<i>C. purpurea</i> sensu stricto	<i>P. pratensis</i>	Oregon	22.38 $\pm$ 0.30
Cp053	<i>C. purpurea</i> sensu stricto	<i>P. pratensis</i>	Oregon	22.91 $\pm$ 0.68
Cp054	<i>C. purpurea</i> sensu stricto	<i>P. pratensis</i>	Oregon	19.69 $\pm$ 0.07
Cp065	<i>C. purpurea</i> sensu stricto	<i>P. pratensis</i>	Oregon	25.98 $\pm$ 0.71
Cp079	<i>C. purpurea</i> sensu stricto	<i>P. pratensis</i>	Oregon	19.82 $\pm$ 0.08
Cp030	<i>C. purpurea</i> sensu stricto	<i>Bromus inermis</i>	Washington	21.43 $\pm$ 0.17
Clp-1	<i>C. purpurea</i> sensu stricto	<i>Hordeum vulgare</i>	Montana	21.35 $\pm$ 0.56
Clp-2	<i>C. purpurea</i> sensu stricto	<i>Secale cereale</i>	Unknown	19.96 $\pm$ 0.16
Cp025	<i>C. purpurea</i> sensu stricto	<i>S. cereale</i>	Oregon	20.85 $\pm$ 0.05
Cp066	<i>C. purpurea</i> sensu lato	<i>L. perenne</i>	Oregon	28.65 $\pm$ 0.16
Cp014	<i>C. purpurea</i> sensu lato	<i>P. pratensis</i>	Washington	22.70 $\pm$ 0.05
Cp015	<i>C. purpurea</i> sensu lato	<i>P. pratensis</i>	Washington	23.27 $\pm$ 0.38
Cp023	<i>C. purpurea</i> sensu lato	<i>P. pratensis</i>	Washington	22.22 $\pm$ 0.47
Cp078	<i>C. purpurea</i> sensu lato	<i>P. pratensis</i>	Oregon	24.05 $\pm$ 0.75
Clp-3	<i>C. spartinae</i>	<i>Spartina</i> sp.	New Jersey	18.56 $\pm$ 0.23
ClS-4	<i>C. africana</i>	<i>Sorghum bicolor</i>	India	NA
EAP-20	<i>C. africana</i>	<i>S. bicolor</i>	India	NA
Ccyn	<i>C. cynodontis</i>	<i>Cynodon</i> sp.	Unknown	NA
Clf-1	<i>C. fusiformis</i>	<i>Pennisetum typhoideum</i>	Africa	NA
Clf-2	<i>C. fusiformis</i>	<i>P. typhoideum</i>	Africa	NA
Clf-3	<i>C. fusiformis</i>	<i>P. typhoideum</i>	Africa	NA
Cmax	<i>C. maximensis</i>	<i>Panicum maximum</i>	Unknown	NA
Cpas-1	<i>C. paspali</i>	<i>Paspalum</i> sp.	North Carolina	NA
Cpas-2	<i>C. paspali</i>	<i>Paspalum</i> sp.	Georgia	NA
Cpus-1	<i>C. pusilla</i>	<i>Bothriochloa</i> sp.	Australia	NA
Cpus-2	<i>C. pusilla</i>	<i>Dicantium</i> sp.	Australia	NA

^a NA = not amplified.

A second standard curve was obtained using 10-fold serial dilutions of a DNA extract from a conidial suspension of isolate Cp061. Conidia were washed from pure cultures grown on *Claviceps* medium (Gilmore et al. 2016) using sterile distilled water and quantified with a hemacytometer. DNA was extracted from the conidial suspension as described above and 10-fold serial dilutions were performed to achieve extractions representing 8×10^3 to 8×10^7 conidia $25 \mu\text{l}^{-1}$. Each extraction ($1 \mu\text{l}$, representing 8 to 80,000 conidia) was used for developing the standard curve.

Spore trap samples and qPCR validation. The qPCR assay was validated using spore trap samples from Kentucky bluegrass and perennial ryegrass fields in Oregon and Washington. Hirst-type Burkard 7-day recording volumetric spore samplers (Burkard Scientific Ltd., Uxbridge, Middlesex, UK) were used to collect airborne ascospores of *C. purpurea* sensu lato in commercial fields of perennial ryegrass (Umatilla County, OR) and Kentucky bluegrass (Jefferson County, OR; Union County, OR; and Benton County, WA). Spore samplers were also placed in artificially infested experimental plots located at the Central Oregon Agricultural Research Center (Jefferson County, OR) and the Hermiston Agricultural Research and Extension Center (Umatilla County, OR). Spore samplers were situated approximately 150 m from the border of commercial fields and in the center of experimental plots, with the air intake orifice located approximately at the top of the crop canopy (approximately 0.5 m above ground level). Spore traps were calibrated weekly to confirm that they were sampling air at 10 liters min^{-1} . Spores and other airborne particulates were impacted onto clear Melinex tape (supplied by Burkard Scientific Ltd., Uxbridge, Middlesex, UK) coated with silicone grease (Ted Brown Associates, Los Altos, CA). In all, 64 spore trap samples obtained from April through June 2014 ($n = 17$), 2015 ($n = 17$), and 2017 ($n = 30$) were included in this study.

Tape samples from spore traps were collected and replaced every 7 days and processed as described by Alderman (1993), with minor modifications. Tapes were partitioned into daily segments and divided into hourly sections according to the manufacturer's instructions. Each daily segment of spore trap tape was cut in half lengthwise and one-half was used for microscopic quantification of ascospores while the other half was used for DNA extraction and qPCR.

Fig. 1. Standard curve of cycle threshold values calculated from serial dilutions of DNA from **A**, lyophilized tissue and **B**, conidia from pure cultures of *Claviceps purpurea* sensu stricto.

Daily half-tape segments were mounted onto microscope slides and ascospores were stained with aniline blue solution (30 mg of aniline blue, 20 ml of deionized water, 10 ml of glycerol, and 10 ml of 80% lactic acid). The number of ascospores trapped per hour was counted under a microscope at $\times 300$ magnification for each 24-h period (12:00 A.M. to 11:59 P.M.) and summed to determine daily ascospore counts.

DNA was extracted from each daily half-tape segment using the extraction and precipitation method described above, with minor modifications. A 0.1% solution of IGEAL CA-630 (MilliporeSigma, St. Louis), a nonionic and nondenaturing detergent, was added to the DNA extraction buffer (23.9% vol/vol) to facilitate the release of spores from the tapes (Calderon et al. 2002). β -Mercaptoethanol (0.3% vol/vol) was also included in the extraction buffer to aid in the denaturing of proteins and removal of tannins and polyphenols. qPCR were performed as described above and the DNA extracted from each daily half-tape served as the template in each of four technical replicates per qPCR run. All qPCR runs were repeated once, such that eight total qPCR were performed for each daily half-tape sample.

Data analyses. Data from qPCR runs were analyzed using StepOne software (version 2.3; Thermo Fisher Scientific). Regression analysis was performed on data collected from DNA and conidia standard curve runs using PROC REG in SAS 9.4 (SAS Institute, Cary, NC). A Pearson correlation coefficient was calculated for spore counts obtained using microscopy and Ct values obtained from qPCR using PROC CORR in SAS. Normality and heteroscedasticity of data were tested prior to regression and correlation analyses with the Shapiro-Wilk test ($P > 0.05$) and by plotting studentized residuals using PROC UNIVARIATE and PROC PLOT in SAS.

Receiver operating characteristic (ROC) curve analysis was performed and the limit of quantification (LOQ) was determined to estimate the performance of the qPCR validation assay, as described by Nutz et al. (2011). Briefly, an ROC curve was generated by plotting sensitivity versus $1 - \text{specificity}$, wherein sensitivity is the fraction of true-positive samples that score positive and specificity is the fraction of true-negative samples that score negative. For each qPCR cycle (from 28 to 40 in the dataset), the number true-positive samples with an equal or lower Ct value and the number of true-negative samples with higher Ct values were used to calculate sensitivity and specificity. The optimal cutoff point was defined as the qPCR cycle with the highest Youden index value, which was defined as sensitivity + specificity – 1 (Fluss et al. 2005). ROC analysis and Youden index calculations were performed with a custom R script, v3.4.0 (R Core Team 2017). LOQ was determined as the amount of ascospores or DNA corresponding to the optimal cutoff point in the calibration curve. The quantitative relationship between the ascospore counts on spore trap tapes and Ct values was analyzed using logistic regression (PROC LOGISTIC) in SAS and the fitted predicted probability curves were plotted using the results from the logistic regression analysis. The limit of detection (LOD) was not calculated because of an insufficient number of data points with small quantities of ascospores in this study.

Results

Primer specificity, reaction efficiency, and LOD. The primers developed in this study were tested against 41 *C. purpurea* sensu lato isolates collected from six hosts, including perennial ryegrass ($n = 17$), Kentucky bluegrass ($n = 19$), barley ($n = 1$), rye ($n = 2$), smooth brome ($n = 1$), and cordgrass ($n = 1$), and amplified a 96-bp product with a melting temperature between 80.7 and 81.9°C. The mean Ct value was 22.11 and ranged between 18.56 and 28.65 (Table 1). The primers were specific to *C. purpurea* sensu lato, including *C. purpurea* sensu stricto, *C. humidiphila*, and *C. spartinae*, and did not amplify products from closely related isolates of *C. africana* ($n = 2$), *C. cynodontis* ($n = 1$), *C. fusiformis* ($n = 3$), *C. maximensis* ($n = 1$), *C. paspali* ($n = 2$), or *C. pusilla* ($n = 2$).

A significant relationship was observed between Ct values and serial dilutions of DNA derived from pure culture of isolate Cp061 ($R^2 = 0.99$; $P = 0.0002$), with Ct values ranging from 18.50 (10 ng of DNA) to 32.01 (1 pg of DNA). Ct values were also significantly related to DNA extracted from conidial suspensions of isolate

Table 2. Detection and amount of *Claviceps purpurea* sensu lato ascospores observed using microscopy compared with detection and mean cycle threshold (Ct) values ($\pm$ standard deviation [SD]) obtained from quantitative polymerase chain reaction (qPCR) analysis of spore trap tapes collected from Kentucky bluegrass (*Poa pratensis*) and perennial ryegrass (*Lolium perenne*) seed fields

Year, county, state	Grass host	Microscopy	<i>Claviceps</i> ascospores (<i>n</i>)	qPCR	Ct $\pm$ SD
2014					
Union County, OR	<i>P. pratensis</i>	—	0	+	39.37 $\pm$ 1.77
Umatilla County, OR	<i>L. perenne</i>	—	0	—	40.00 $\pm$ 0.00
Umatilla County, OR	<i>L. perenne</i>	+	6	+	38.83 $\pm$ 1.28
Umatilla County, OR	<i>L. perenne</i>	+	7	+	38.71 $\pm$ 2.70
Jefferson County, OR	<i>P. pratensis</i>	+	9	+	39.44 $\pm$ 1.06
Umatilla County, OR	<i>L. perenne</i>	+	12	—	40.00 $\pm$ 0.00
Umatilla County, OR	<i>L. perenne</i>	+	34	+	39.08 $\pm$ 2.60
Umatilla County, OR	<i>L. perenne</i>	+	38	+	38.16 $\pm$ 2.69
Umatilla County, OR	<i>L. perenne</i>	+	92	—	40.00 $\pm$ 0.00
Umatilla County, OR	<i>L. perenne</i>	+	98	—	40.00 $\pm$ 0.00
Umatilla County, OR	<i>L. perenne</i>	+	186	+	34.77 $\pm$ 3.05
Umatilla County, OR	<i>L. perenne</i>	+	199	+	35.20 $\pm$ 1.42
Umatilla County, OR	<i>L. perenne</i>	+	246	+	36.70 $\pm$ 3.21
Umatilla County, OR	<i>L. perenne</i>	+	261	+	34.56 $\pm$ 1.27
Umatilla County, OR	<i>L. perenne</i>	+	427	+	32.49 $\pm$ 0.78
Umatilla County, OR	<i>L. perenne</i>	+	602	+	33.07 $\pm$ 1.00
Umatilla County, OR	<i>L. perenne</i>	+	926	+	31.22 $\pm$ 0.60
2015					
Umatilla County, OR	<i>L. perenne</i>	—	0	+	38.75 $\pm$ 2.33
Umatilla County, OR	<i>L. perenne</i>	—	0	+	38.95 $\pm$ 1.66
Jefferson County, OR	<i>P. pratensis</i>	—	0	+	39.69 $\pm$ 0.88
Jefferson County, OR	<i>P. pratensis</i>	+	3	+	37.04 $\pm$ 3.33
Umatilla County, OR	<i>L. perenne</i>	+	3	+	39.78 $\pm$ 0.61
Jefferson County, OR	<i>P. pratensis</i>	+	7	+	37.98 $\pm$ 1.79
Umatilla County, OR	<i>L. perenne</i>	+	15	+	36.00 $\pm$ 2.97
Jefferson County, OR	<i>P. pratensis</i>	+	20	+	35.96 $\pm$ 2.00
Jefferson County, OR	<i>P. pratensis</i>	+	69	+	38.39 $\pm$ 1.99
Umatilla County, OR	<i>L. perenne</i>	+	107	+	36.86 $\pm$ 6.17
Umatilla County, OR	<i>L. perenne</i>	+	107	+	39.60 $\pm$ 0.83
Jefferson County, OR	<i>P. pratensis</i>	+	239	+	38.42 $\pm$ 1.98
Umatilla County, OR	<i>L. perenne</i>	+	245	+	37.48 $\pm$ 2.78
Umatilla County, OR	<i>L. perenne</i>	+	364	+	38.55 $\pm$ 2.73
Umatilla County, OR	<i>L. perenne</i>	+	512	+	34.76 $\pm$ 3.27
Umatilla County, OR	<i>L. perenne</i>	+	757	+	33.21 $\pm$ 1.67
Umatilla County, OR	<i>L. perenne</i>	+	1054	+	31.73 $\pm$ 0.50
2017					
Benton County, WA	<i>P. pratensis</i>	—	0	+	37.30 $\pm$ 0.78
Union County, OR	<i>P. pratensis</i>	+	1	+	37.59 $\pm$ 0.92
Union County, OR	<i>P. pratensis</i>	+	1	+	38.36 $\pm$ 1.71
Benton County, WA	<i>P. pratensis</i>	+	1	+	38.87 $\pm$ 1.60
Benton County, WA	<i>P. pratensis</i>	+	1	+	39.37 $\pm$ 0.89
Umatilla County, OR	<i>L. perenne</i>	+	2	+	36.76 $\pm$ 0.33
Umatilla County, OR	<i>L. perenne</i>	+	2	+	37.96 $\pm$ 2.88
Umatilla County, OR	<i>L. perenne</i>	+	3	+	37.13 $\pm$ 0.02
Jefferson County, OR	<i>P. pratensis</i>	+	5	+	36.81 $\pm$ 1.24
Umatilla County, OR	<i>L. perenne</i>	+	6	+	35.47 $\pm$ 1.66
Jefferson County, OR	<i>P. pratensis</i>	+	7	+	35.86 $\pm$ 0.49
Umatilla County, OR	<i>L. perenne</i>	+	8	+	39.02 $\pm$ 1.39
Umatilla County, OR	<i>L. perenne</i>	+	9	+	35.77 $\pm$ 3.19
Umatilla County, OR	<i>L. perenne</i>	+	13	+	36.63 $\pm$ 1.95
Union County, OR	<i>P. pratensis</i>	+	13	+	37.60 $\pm$ 2.16
Jefferson County, OR	<i>P. pratensis</i>	+	16	+	34.72 $\pm$ 1.06
Union County, OR	<i>P. pratensis</i>	+	17	+	36.53 $\pm$ 1.55
Union County, OR	<i>P. pratensis</i>	+	24	+	36.17 $\pm$ 0.06
Jefferson County, OR	<i>P. pratensis</i>	+	25	+	35.03 $\pm$ 2.66
Jefferson County, OR	<i>P. pratensis</i>	+	29	+	38.00 $\pm$ 2.18
Umatilla County, OR	<i>L. perenne</i>	+	29	+	38.16 $\pm$ 2.60
Jefferson County, OR	<i>P. pratensis</i>	+	31	+	37.44 $\pm$ 3.61
Jefferson County, OR	<i>P. pratensis</i>	+	37	+	37.35 $\pm$ 3.75
Jefferson County, OR	<i>P. pratensis</i>	+	74	+	37.93 $\pm$ 2.59
Jefferson County, OR	<i>P. pratensis</i>	+	79	+	36.24 $\pm$ 2.06
Umatilla County, OR	<i>L. perenne</i>	+	100	+	36.82 $\pm$ 0.35
Jefferson County, OR	<i>P. pratensis</i>	+	103	+	33.55 $\pm$ 1.26
Jefferson County, OR	<i>P. pratensis</i>	+	125	+	35.93 $\pm$ 1.40
Umatilla County, OR	<i>L. perenne</i>	+	215	+	34.85 $\pm$ 1.07
Umatilla County, OR	<i>L. perenne</i>	+	266	+	35.42 $\pm$ 0.51

Cp061 ($R^2 = 0.99$; $P = 0.0004$) and Ct values ranged from 23.99 (8×10^4 conidia) to 36.71 (8×10^0 conidia) (Fig. 1). A strong relationship was observed between Ct values and serial dilutions of DNA used for standard curves among all runs ($R^2 = 0.979 \pm 0.005$) and the qPCR were highly efficient among all runs ($97.6 \pm 1.5\%$), indicating specificity and lack of PCR inhibition in the reactions. In addition, the assay was highly sensitive and could detect as little as 1 pg of *C. purpurea* sensu lato DNA and DNA obtained from as few as 8 conidia.

Melting curve analysis also confirmed that the qPCR were highly specific and generated a single product (data not shown). The melting temperature of the product amplified from isolate Cp061 was 80.72 ± 0.10 but differences in melting temperatures were observed among *C. purpurea* sensu lato isolates. The mean melting temperature of the product amplified from *C. purpurea* sensu stricto isolates was greater ($81.13 \pm 0.08^\circ\text{C}$) than the melting temperature observed for amplicons from *C. humidiphila* isolates ($80.99 \pm 0.02^\circ\text{C}$); the melting temperature for the lone *C. spartinae* isolate included in this study was $81.62 \pm 0.08^\circ\text{C}$.

qPCR validation using spore trap samples. The qPCR assay detected *C. purpurea* sensu lato in 60 of 64 samples compared with microscopic examination of slides that detected *Claviceps* ascospores in

58 of 64 samples (Table 2). The range of detection for the qPCR assay was between 1 and 1,054 ascospores, with Ct values ranging between 39.78 and 31.22. The qPCR assay detected ascospores in five of six spore trap tape samples from which no ascospores were observed by microscopy but failed to detect ascospores in six spore trap tape samples from which ascospores were observed by microscopy; performing qPCR on 1:10 dilutions of DNA from these samples resulted in positive detections in three of the six samples. A significant negative correlation between Ct values and the number of ascospores counted using microscopy was observed when data from all three years' were combined ($r = -0.68$; $P < 0.0001$). Among individual years, significant negative correlations were observed in 2014 ($r = -0.89$; $P < 0.0001$), 2015 ($r = -0.81$; $P < 0.0001$), and 2017 ($r = -0.46$; $P = 0.01$).

ROC curve analysis was conducted to determine the sensitivity and specificity of the qPCR assay and determine the LOQ value of the assay. The area under the ROC curve was 0.73 (Fig. 2A) and the optimal cutoff point was a Ct value of 37. The LOQ was calculated to be approximately 45 fg of DNA and one conidium based on standard curves of DNA extracted from lyophilized tissue and conidial suspensions, respectively. The fitted probability curves of the relationship between ascospore counts from spore trap tapes and Ct values is shown in Figure 2B.

Discussion

A SYBR Green qPCR assay was developed for the detection and quantification of *C. purpurea* sensu lato, including *C. purpurea* sensu stricto and *C. humidiphila*, both of which can cause ergot in grass seed production systems of the Pacific Northwest. The assay could also be used to detect and quantify *C. spartinae*, a pathogen of *Spartina* and *Distichlis* grass species in coastal salt marshes (Duncan et al. 2002; Pažoutová et al. 2000, 2015).

The assay developed in this study targeted a multicopy sequence-characterized RAPD amplicon and was highly sensitive, detecting as little as 1 pg of DNA from pure culture and a single ascospore from spore trap tape samples collected from commercial fields. Although the multicopy nature of the target sequence can increase the sensitivity of the assay, it may also limit absolute quantification in pathogen populations with variable copy numbers of the sequence. Variation in copy number of the target sequence may have been reflected in the range of Ct values observed among isolates.

The qPCR method developed in this study offers many advantages over microscopic methods previously used to detect *Claviceps* from spore traps, including greater sensitivity, higher specificity, and shorter assay times. A conventional PCR assay was previously developed for the molecular detection of *C. purpurea* sensu lato but the pathogen could not be quantified (Tooley et al. 2001). Real-time qPCR assays have been described for *C. africana*, *C. sorghi*, and *C. sorghicola* (Tooley et al. 2010), causal agents of ergot in sorghum; but, to our knowledge, this is the first qPCR assay developed for the detection and quantification of *C. purpurea* sensu lato.

Microscopic examination of spore trap tape samples detected ergot ascospores in 58 of 64 samples collected from perennial ryegrass and Kentucky bluegrass seed fields. *Claviceps* ascospores were detected on five spore trap tapes from which ascospores were not observed using microscopic methods (Table 2); this could be due to the higher sensitivity of qPCR compared with traditional methods; difficulty in counting ascospores on tapes with large amounts of pollen, sand, or other debris; or unequal distribution of ascospores among the tape halves.

Based on ROC curve analysis and Youden's index, a Ct value of 37 was determined to be the optimal cutoff point for the detection of *C. purpurea* ascospores from spore trap tape samples. The sensitivity of this assay increased when Ct values greater than 37 were allowed but with a corresponding reduction in assay specificity. The SYBR Green assay developed in this study allows for the analysis of melt curves, which can be used to screen out nonspecific reactions when spore levels are low and increased sensitivity is needed. More research on ergot inoculum thresholds in grass seed crops would aid in establishing thresholds and decision-making guidelines when using this assay.

Fig. 2. A, Receiver operating characteristic curve and B, fitted predicted probability curves showing the relationship of real-time quantitative polymerase chain reaction cycle threshold (Ct) values to *Claviceps purpurea* sensu lato ascospores counted on spore trap tapes.

Inhibitors may be present in spore trap tape samples with excessive amounts of soil or other natural materials that are subsequently carried over into the DNA extraction (Mahaffee and Stoll 2016). There were six spore trap tape samples from which ascospores were observed using microscopic methods but were not detected using qPCR. The reasons for these false-negative results are not known but they may have been due to excessive amounts of nontarget DNA (e.g., pollen or other fungi), PCR inhibitors in the reaction, unequal distribution of ascospores on spore trap tapes, or other factors. qPCR was repeated using a 1:10 dilution of all samples and resulted in a positive detection in three additional samples, indicating that PCR inhibitors were likely present. Regardless, a significant correlation between Ct values and the number of ascospores from spore trap half-tapes was observed.

Scouting for ergot with the goal of making timely management decisions is problematic because of the relatively long latent period (approximately 7 days) that occurs after infection and before the onset of symptoms (Tudzynski and Scheffer 2004). Additionally, once ergot symptoms occur, it is often too late to treat for the disease because fungicides can only prevent ovary infection by the fungus. As a result, early detection of *Claviceps* inoculum is important for ergot management. The ability to use molecular methods to detect low levels of inoculum (e.g., 1 to 10 spores) can help to overcome limitations inherent to spore trapping (e.g., low air sampling volumes or large amounts of soil and pollen that may make visual detection of spores difficult). Molecular methods also often require less time and labor, and the techniques used in this study are commonly used and easily transferrable to other labs. The use of qPCR to quantify *Claviceps* ascospores from spore traps could enable the sampling of a large number of sites over longer periods, allowing for region- or state-wide detection and forecasting programs that would otherwise be difficult to accomplish using traditional microscopic methods. If collected daily, spore trap tape samples could be processed within 24 h, providing growers with near real-time information on ergot inoculum production and disease risk prior to and during anthesis. When used in conjunction with scouting (Dung et al. 2016) and predictive models (Dung et al. 2017), this qPCR assay for airborne *C. purpurea* sensu lato ascospores could be a component of an integrated pest management approach and help grass seed growers make risk-based fungicide application decisions for ergot management.

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