

# DEVELOPMENT OF A DNA-BASED PROTOCOL TO DETECT AIRBORNE ERGOT SPORES IN COOL-SEASON GRASS SEED FIELDS

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## Introduction

Ergot, caused by the fungal pathogen *Claviceps purpurea*, is a floral disease of grasses and a persistent problem in irrigated grass seed production (Alderman et al., 1996; Alderman et al., 1998; Alderman et al., 2015). The pathogen infects the unfertilized flowers of grasses and grains and transforms seed into fungal structures called sclerotia. Sclerotia overwinter and germinate in the spring to produce fruiting bodies called capitula, which in turn release millions of airborne ascospores.

One commonly used method to detect airborne ergot spores (ascospores) utilizes Burkard volumetric 7-day recording spore traps, in which air samples are continuously collected on a sticky tape surface (Figure 1). The tape is then examined under a microscope for the presence of spores. Although spore trapping is effective in providing quantitative data on airborne spore numbers, processing and microscopic examination of the tapes is time consuming (up to 8 hours for a 7-day tape). Another issue encountered during the microscopic examination of spore tape samples is that sections of the tape can be uncountable because of overlapping layers of pollen or high densities of soil particulates that can occur on dry, windy days. Counting can also be difficult if large numbers of spores have been trapped. Finally, 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 *C. purpurea* spores.

DNA-based spore detection methods are more specific and sensitive than traditional microscopic identification. DNA-based methods, mostly based on variations of polymerase chain reaction (PCR), have been used to detect airborne spores of other plant pathogenic fungi. PCR utilizes short, specific fragments of DNA (called primers) that bond to a precise region of DNA associated only with the target fungus. When primers are combined with enzymes called DNA polymerases under the appropriate conditions, sufficient copies of the DNA sequence of interest are synthesized to allow confirmation of its presence in the sample. In quantitative PCR (qPCR), a fluorescent dye is used to quantify the amount of synthesized DNA. When

compared to a standard curve of known DNA quantities, qPCR can quantify the number of DNA copies that are present in a sample.

Quantitative PCR allows for the sensitive, specific, and reproducible detection and quantification of target DNA sequences. In addition, less time and labor are typically involved, providing faster results to support an IPM program with less expense, once a protocol is developed and technicians are trained. Quantitative PCR has been used for many plant pathogenic fungi, including *Claviceps* species such as *C. africana*, *C. sorghi*, and *C. sorghicola* (Tooley et al., 2010); however, a similar protocol does not yet exist for *C. purpurea*. The objective of this research project was to develop and validate a qPCR protocol for the detection of airborne ergot spores in cool-season grass seed crops, which would provide a more accurate and less time-consuming process to quantify the number of *C. purpurea* spores found in spore traps.

## Materials and Methods

Sclerotia of *C. purpurea* were collected from infected perennial ryegrass (*Lolium perenne*) in Oregon and infected Kentucky bluegrass (*Poa pratensis*) seed lots grown in Oregon and Washington between 2010 and 2014. Sclerotia from rye (*Secale cereale*) and smooth brome (*Bromus inermis*) were collected from



Figure 1. A Burkard volumetric 7-day recording spore trap and weather data logger deployed in a commercial Kentucky bluegrass seed production field (Benton County, WA).

field borders in Oregon and Washington, respectively. Freeze-dried tissue of *C. purpurea* from other states and countries and freeze-dried tissue from other *Claviceps* species (*C. africana*, *C. fusiformis*, *C. paspali*, and *C. pusilla*) were obtained. Isolates were obtained in pure culture by plating surface-sterilized sclerotia on potato dextrose agar. Pure culture isolates were maintained in pure culture until DNA extraction. Genomic DNA was obtained using a phenol-chloroform extraction followed by a sodium acetate-ethanol precipitation. Additional DNA extracts from *C. cynodontis* and *C. maximensis* were also obtained. The genomic DNA extracts obtained from the pure culture isolates were used to develop, test, and optimize the qPCR assay prior to validation with spore trap tape samples from the field.

Spore trap tape samples (ascospores) were obtained from commercial fields of perennial ryegrass (Umatilla County, OR) and Kentucky bluegrass (Jefferson County, OR and Union County, OR) in 2014 and 2015. Additional tape samples were collected from artificially infested experimental Kentucky bluegrass plots located at the Central Oregon Agricultural Research Center (Madras, OR) and perennial ryegrass plots at the Hermiston Agricultural Research and Extension Center (Hermiston, OR).

A qPCR assay was developed to amplify a species-specific 96 base pair region of the *C. purpurea* genome. A standard curve ranging from 10 nanograms to 1 picogram was achieved using 10-fold serial dilutions of genomic DNA. A second standard curve was obtained

using DNA extracts from known spore amounts (4, 40, 400, 4,000, and 40,000 spores) collected from pure cultures. Quantitative PCR reactions were performed in triplicate or duplicate, and melt curve analysis was used to distinguish potential nonspecific PCR products. A no-template water sample was included as a negative control in all PCR experiments. A cycle threshold (Ct) value < 40 was interpreted as a positive detection if the melt curve matched that of *C. purpurea*.

Comparisons between traditional and molecular approaches to quantifying spore numbers were accomplished by cutting each tape sample in half lengthwise and using one half for microscopic quantification of spores and the other half for DNA extraction and qPCR. Samples used for qPCR were selected to represent a range of spore counts observed using microscopic methods (0 to 1,054 spores/half tape). Quantitative PCR reactions were performed as described above, and each sample was subjected to four technical replicates. All reactions were repeated once (eight total reactions/sample).

### Results and Discussion

Species specificity of the primers was confirmed against 41 *C. purpurea* isolates collected from six hosts, including perennial ryegrass (17 isolates), Kentucky bluegrass (19 isolates), barley (1 isolate), rye (2 isolates), smooth brome (1 isolate), and cordgrass (1 isolate). The species-specific primers did not amplify DNA from closely related isolates of *C. africana* (2 isolates), *C. cynodontis* (1 isolate), *C. fusiformis*

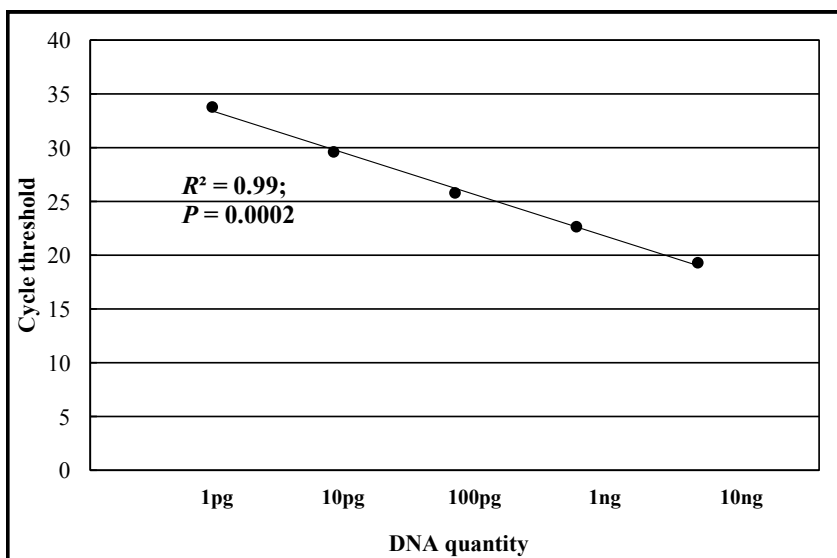


Figure 2. Standard curve of cycle threshold values calculated from serial dilutions of DNA from *Claviceps purpurea*.

(3 isolates), *C. maximensis* (1 isolate), *C. paspali* (2 isolates), or *C. pusilla* (2 isolates). In addition, the assay was highly sensitive and could detect as little as 1 picogram (one trillionth of a gram) of *C. purpurea* DNA and as few as four spores.

The qPCR reactions were highly efficient (97.6%), suggesting a high degree of specificity. Melt curve analysis confirmed that the qPCR reactions were highly specific and generated a single product (data not shown). Significant relationships were observed between Ct values and DNA quantity ( $R^2 = 0.99$ ;  $P = 0.0002$ ) and between Ct values and the number of spores ( $R^2 = 0.99$ ;  $P = 0.0004$ ) used for standard curves (Figures 2 and 3). These results indicated that the assay was applicable for samples containing a wide range of DNA (1 picogram to 10 nanograms) or spores (4 to 40,000 spores).

Microscopic examination of spore trap tape samples detected ergot spores in 23 out of 26 tape samples collected from perennial ryegrass fields and 6 out of 8 tape samples collected from Kentucky bluegrass fields. Quantitative PCR of tape samples detected ergot spores in 23 out of 26 tape samples collected from perennial ryegrass fields and all 8 tape samples collected from Kentucky bluegrass fields. Quantitative PCR detected spores on 2 KBG tape samples and 2 PRG tape samples from which spores were not observed using microscopic methods. This result could be due to the higher sensitivity of qPCR compared to traditional methods; difficulty in counting spores on tapes with large amounts of pollen, sand, or other debris; or unequal distribution of spores among the tape halves.

There were also five PRG tape samples in which spores were observed using microscopic methods but were not detected using qPCR. The reasons for these false negative results are not known, but it may have been due to excessive amounts of non-target DNA (e.g., pollen, other fungi), PCR inhibitors (e.g., humic acid, polysaccharides), or unequal distribution of spores among the tape halves. Quantitative PCR was repeated using a 1:10 dilution of these five samples, resulting in a positive detection in three of the samples, thus indicating that PCR inhibitors likely were present in these three samples. Inhibitors may be present in tape samples with excessive amounts of soil or other natural materials that are subsequently carried over into the DNA extraction. Regardless, a significant correlation for Ct values and the number of spores from spore trap tapes was observed ( $r = -0.68$ ;  $P < 0.0001$ ) (Figure 4).

### Conclusions

Microscopic methods used to detect and quantify ergot spores captured by spore traps are usually not rapid enough to allow for the detection and reporting of results in a timely manner, preventing growers from using this information in the current season or in an IPM system. A fast and reliable detection protocol for the presence of airborne *C. purpurea* spores will enable grass seed growers to make better-informed decisions regarding fungicide applications. When used in conjunction with predictive models, a qPCR detection protocol for airborne *C. purpurea* spores could help growers decide if, and when, to spray protective fungicides (Dung et al., 2013).

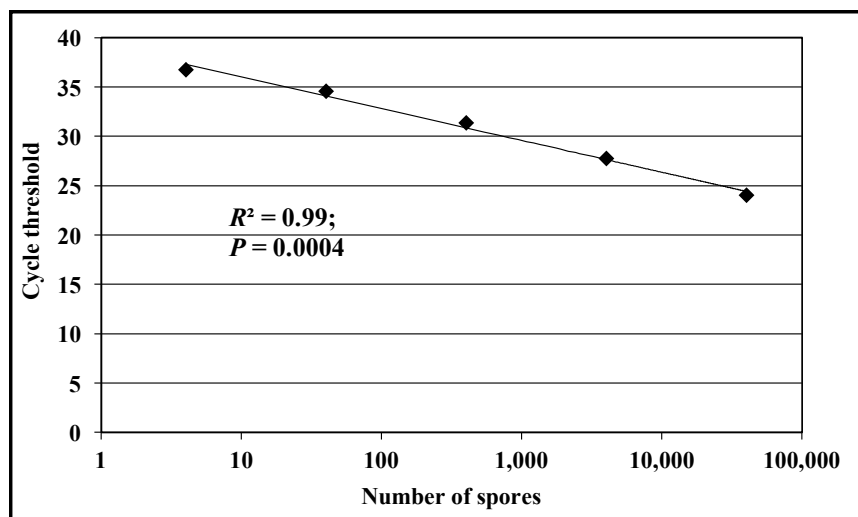


Figure 3. Standard curve of cycle threshold values calculated from serial dilutions of spores from *Claviceps purpurea*.

Quantification of ergot inoculum during the season may also enable growers to predict if seed lots may require additional cleaning after harvest, allowing them to plan their postharvest operations accordingly.

This assay provides a means for detecting and monitoring airborne *C. purpurea* spores in the field and in experimental plots. It was highly specific, was useful over a wide range of spore densities, and could be performed in a matter of hours instead of days. The protocol could be useful not only for ergot detection in cool-season grasses, but also for important grain crops (e.g., barley, rye) and wild or weedy grass hosts of ergot.

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### Acknowledgments

This project was funded in part by the USDA National Institute of Food and Agriculture, through the Western Integrated Pest Management Center. Funding was also provided by the Agricultural Research Foundation. The authors thank Paul Tooley (USDA-ARS) for providing tissue and DNA of *Claviceps* species. The authors would like to also acknowledge the grower cooperators, seed conditioners, and stakeholders who participated in spore trapping and sample collection.

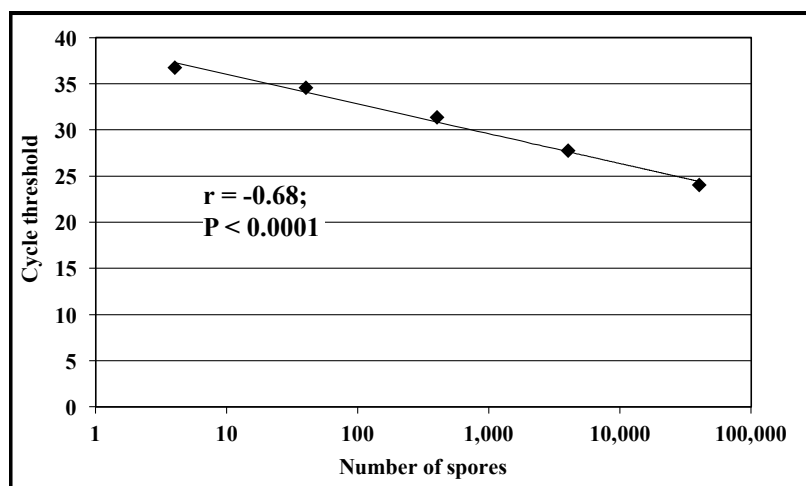


Figure 4. Correlation between cycle threshold values and  $\log_{10}$ -transformed counts of *Claviceps purpurea* spores obtained using traditional microscopic methods.