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TITLE: Development of a Molecular Detection Protocol for Ergot in Cool-Season Turf & Forage Grasses Grown for Seed

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SUMMARY: Ergot, caused by the fungal pathogen *Claviceps purpurea*, is a floral disease of grasses and a persistent problem in irrigated grass seed production. The current methods used to detect ergot ascospores utilizes Burkard volumetric 7-day recording spore traps, in which spores are collected on a sticky tape surface and the tape is examined under a microscope for the presence of ascospores (Fig. 1). 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 problem encountered during the microscopic examination of spore tapes 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. In addition, counting can be difficult if large numbers of spores have been trapped and 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 detection methods are more specific and sensitive than traditional microscopic identification. In addition, less time and labor are typically involved once a protocol is developed and technicians are trained. 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. DNA-based methods typically require only routine laboratory techniques which are performed by most scientists trained in molecular biology and specialized mycology skills are not necessarily required.

Molecular 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 which bond to a precise region of DNA only associated with the target fungus. When combined with enzymes called DNA polymerases under the appropriate conditions, copies of the DNA sequence of interest are synthesized. Quantitative PCR (qPCR) incorporates a fluorescent dye which is measured as the DNA is synthesized. When combined with 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. Quantitative PCR quantification has been used for many plant pathogenic fungi including *Claviceps* species such as *C. africana*, *C. sorghi*, and *C. sorghicola*; however, a similar protocol does not yet exist for *C. purpurea*. The objective of this research project was to develop a qPCR protocol for the detection of ergot 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 from spore traps.

OBJECTIVES: The objectives of this research project are to: 1) develop a quantitative PCR (qPCR) protocol for the detection of *C. purpurea* spores; and 2) validate the developed qPCR procedure using samples collected from spore traps.

PROCEDURES: Sclerotia of *C. purpurea* were collected from infected perennial ryegrass (*Lolium perenne*) in Oregon and infected Kentucky bluegrass (*Poa pratensis*) in Oregon and Washington. 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. Isolates were hyphal-tipped and stored on 1% potato dextrose agar slants until DNA extraction. Sclerotia from rye (*Secale cereale*) and smooth brome (*Bromus inermis*) were collected from field borders in Oregon and Washington, respectively, and pure cultures were obtained as described above. Freeze-dried 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 Tooley, USDA-ARS.

Genomic DNA was extracted from fresh mycelia grown in potato dextrose broth or from the tissue provided by Dr. Tooley. Genomic DNA was obtained using a phenol-chloroform extraction followed by a sodium acetate-ethanol precipitation. The quality and quantity of genomic DNA was determined with a NanoDrop 2000C spectrophotometer and Qubit fluorometer, respectively. Additionally, DNA extracts from *C. cynodontis* and *C. maximensis* were provided by Dr. Tooley.

A SYBR Green qPCR assay was developed to amplify a 96 base pair region of a *C. purpurea* species-specific RAPD amplicon (GenBank accession AJ252159). Forward and reverse primers were designed from this amplicon using Primer3Plus software. A standard curve ranging from

10 ng to 1 pg was achieved using ten-fold serial dilutions of genomic DNA that was quantified with a Qubit 3.0 fluorometer. A second standard curve was obtained using DNA extracts of known spore amounts (4, 40, 400, 4,000, and 40,000 spores) collected from pure cultures and quantified using a hemocytometer. Quantitative PCR reactions were performed in triplicate or duplicate using a StepOnePlus Real-Time PCR System and melt curve analysis was used to distinguish potential primer dimers and non-specific amplification products. A no-template water sample was included as a negative control in all amplifications. A cycle threshold (Ct) value < 40 was interpreted as a positive detection if the melt curve matched that of *C. purpurea*.

Spore trap tape samples 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 spore trap tape samples were collected from artificially-infested experimental plots located at the Central Oregon Agricultural Research Center (Madras, OR) and the Hermiston Agricultural Research and Extension Center (Hermiston, OR). Each spore trap tape sample was cut in half lengthwise and one half was used for microscopic quantification of spores while the other half was used 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).

DNA was extracted from each tape sample using the extraction and precipitation method described above. IGEPAL® CA-630, a nonionic and non-denaturing detergent, was added to the DNA extraction buffer to facilitate the release of spores from the tapes. Quantitative PCR reactions were performed as described above and each sample was subjected to 4 technical replicates. All reactions were repeated once (8 total reactions/sample).

SIGNIFICANT ACCOMPLISHMENTS: The primers developed in this study were tested against 41 *C. purpurea* 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 base pair 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 species-specific 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). In addition, the assay was highly sensitive and could detect as little as 1 pg (one trillionth of a gram) of *C. purpurea* DNA and as little as 4 spores. The qPCR reactions were highly efficient (97.57%), indicating a high degree of specificity and lack of PCR inhibition in the reactions. Melt curve analysis also 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 Ct values and the number of spores (R^2 = 0.99; P = 0.0004) used for standard curves (Figs. 2 and 3).

Microscopic examination of spore trap tapes detected ergot ascospores in 23 out of 26 samples collected from perennial ryegrass fields and 6 out of 8 samples collected from Kentucky bluegrass fields. In four cases, spores were detected on spore trap tapes from which sp120ores

were not observed using microscopic methods (Table 2); this 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 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 in the reaction, or other factors. Quantitative PCR was repeated using a 1:10 dilution of these 5 samples and resulted in a positive detection in 3 of the samples, indicating that PCR inhibitors were likely present. 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. Regardless, a significant correlation for Ct values and the number of spores from spore trap tapes was observed (r = -0.68; P < 0.0001) (Fig. 4).

BENEFITS & IMPACT: Current microscopic methods used to detect and quantify ergot spores captured by spore traps are 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. A fast and reliable detection protocol for the presence of airborne *C. purpurea* ascospores 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* ascospores would help growers decide if, and when, to spray protective fungicides. 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 *C. purpurea* spores and field populations. 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 hosts that are hosts of ergot.

ADDITIONAL FUNDING RECEIVED DURING PROJECT TERM: Additional funding ($29,981) for this project was awarded by the Western IPM Center 2015 Competitive Grants Program. These funds allowed for the validation of the technique with additional isolates and field samples. Funds were also used for the production of the Ergot Alert Newsletter, a weekly publication that was sent to grass seed growers and stakeholders in Jefferson County, OR, Union County, OR, and the Columbia Basin of OR and WA. The newsletter provided regional updates on spore counts and crop phenology for perennial ryegrass and Kentucky bluegrass as well as timely information on ergot epidemiology and control.

FUTURE FUNDING POSSIBILITIES: The qPCR detection and quantification protocol developed in this project is expected to be an important tool in a comprehensive Integrated Disease Management program for ergot. Current research efforts are also focused on the development of a prediction model for ergot spore production, screening new fungicides and testing novel fungicide application strategies, biocontrol, insect vectors, and population genetics. Our research group continues to receive funding from state commissions and regional grower groups to support field trials in the different grass production regions and produce the regional Ergot Alert Newsletters. A Project Initiation Grant totaling $29,707 was submitted to the

Western IPM Center to explore biocontrol options for ergot. Eventually, funds from the W-IPM Center Outreach and Implementation Grant program could be obtained to provide outreach and Extension and encourage the implementation of a fully developed Integrated Disease Management program for ergot in grass seed.

Table 1. Ergot species and isolates, grass hosts, cultivars, locations of origin, mean cycle thresholds (Ct), and cycle threshold standard deviations (Ct std. dev.) for the quantitative PCR assay developed in this study[1]

Species	Isolate	Host[2]	Cultivar	Location	Mean Ct	Ct std. dev.
Claviceps purpurea	**Cp002**	***Lolium perenne***	**Pavilion**	**OR, USA**	20.66	0.86
C. purpurea	**Cp003**	***L. perenne***	**Pavilion**	**OR, USA**	21.04	0.26
C. purpurea	**Cp006**	***L. perenne***	**Top Hat II**	**OR, USA**	20.14	0.43
C. purpurea	**Cp008**	***L. perenne***	**Unknown**	**OR, USA**	20.46	0.15
C. purpurea	**Cp012**	***L. perenne***	**Esquire**	**OR, USA**	20.79	0.24
C. purpurea	**Cp027**	***L. perenne***	**Unknown**	**OR, USA**	20.83	0.30
C. purpurea	**Cp036**	***L. perenne***	**Provocative**	**OR, USA**	20.50	0.47
C. purpurea	**Cp037**	***L. perenne***	**Zoom**	**OR, USA**	20.68	0.04
C. purpurea	**Cp040**	***L. perenne***	**Unknown**	**OR, USA**	20.33	0.41
C. purpurea	**Cp043**	***L. perenne***	**Casper**	**OR, USA**	26.77	0.15
C. purpurea	**Cp049**	***L. perenne***	**Frontier**	**OR, USA**	20.40	0.56
C. purpurea	**Cp055**	***L. perenne***	**Esquire**	**OR, USA**	20.69	0.34
C. purpurea	**Cp057**	***L. perenne***	**Esquire**	**OR, USA**	27.35	1.08
C. purpurea	**Cp064**	***L. perenne***	**PST-2M20**	**OR, USA**	28.10	0.24
C. purpurea	**Cp066**	***L. perenne***	**Unknown**	**OR, USA**	28.65	0.16
C. purpurea	**Cp072**	***L. perenne***	**Provocative**	**OR, USA**	20.62	0.14
C. purpurea	**Cp085**	***L. perenne***	**Unknown**	**OR, USA**	19.81	0.27
C. purpurea	**Cp032**	***Poa pratensis***	**Unknown**	**OR, USA**	22.13	0.21
C. purpurea	**Cp033**	***P. pratensis***	**Baron**	**OR, USA**	22.90	0.10
C. purpurea	**Cp052**	***P. pratensis***	**Unknown**	**OR, USA**	22.38	0.30
C. purpurea	**Cp053**	***P. pratensis***	**Unknown**	**OR, USA**	22.91	0.68
C. purpurea	**Cp054**	***P. pratensis***	**Unknown**	**OR, USA**	19.69	0.07
C. purpurea	**Cp065**	***P. pratensis***	**Baron**	**OR, USA**	25.98	0.71
C. purpurea	**Cp078**	***P. pratensis***	**Unknown**	**OR, USA**	24.05	0.75
C. purpurea	**Cp079**	***P. pratensis***	**Unknown**	**OR, USA**	19.82	0.08
C. purpurea	**Cp014**	***P. pratensis***	**Midnight**	**WA, USA**	22.70	0.05
C. purpurea	**Cp015**	***P. pratensis***	**Midnight**	**WA, USA**	23.27	0.38
C. purpurea	**Cp016**	***P. pratensis***	**Midnight**	**WA, USA**	22.36	0.78
C. purpurea	**Cp018**	***P. pratensis***	**Midnight**	**WA, USA**	22.63	0.26
C. purpurea	**Cp019**	***P. pratensis***	**Midnight**	**WA, USA**	22.18	0.82
C. purpurea	**Cp020**	***P. pratensis***	**Unknown**	**WA, USA**	22.00	0.60
C. purpurea	**Cp021**	***P. pratensis***	**Unknown**	**WA, USA**	22.95	0.76
C. purpurea	**Cp022**	***P. pratensis***	**Unknown**	**WA, USA**	22.64	0.21
C. purpurea	**Cp023**	***P. pratensis***	**Unknown**	**WA, USA**	22.22	0.47
C. purpurea	**Cp024**	***P. pratensis***	**Unknown**	**WA, USA**	22.34	0.72
C. purpurea	**Cp081**	***P. pratensis***	**Unknown**	**WA, USA**	21.41	0.56

	Cp030	***Bromus inermis***	**Wild**	**WA, USA**	21.43	0.17
	Clp-1	***Hordeum vulgare***	**Unknown**	**MT, USA**	21.35	0.56
	Cp025	***Secale cereale***	**Wild**	**OR, USA**	20.85	0.05
	Clp-2	***S. cereale***	**Unknown**	**Unknown**	19.96	0.16
	Clp-3	***Spartina* sp.**	**Unknown**	**NJ, USA**	18.56	0.23
Claviceps africana	**Cls-4**	***Sorghum bicolor***	**Unknown**	**India**	NA[3]	ND[4]
	EAP-20	***S. bicolor***	**Unknown**	**India**	NA	ND
Claviceps cynodontis	**Ccyn**	***Cynodon* sp.**	**Unknown**	**Unknown**	NA	ND
Claviceps fusiformis	**Clf-1**	***Pennisetum typhoideum***	**Unknown**	**Africa**	NA	ND
	Clf-2	***P. typhoideum***	**Unknown**	**Africa**	NA	ND
	Clf-3	***P. typhoideum***	**Unknown**	**Africa**	NA	ND
Claviceps maximensis	**Cmax**	***Panicum maximum***	**Unknown**	**Unknown**	NA	ND
Claviceps paspali	**Cpas-1**	***Paspalum* sp.**	**Unknown**	**NC, USA**	NA	ND
	Cpas-2	***Paspalum* sp.**	**Unknown**	**GA, USA**	NA	ND
Claviceps pusilla	**Cpus-1**	***Bothriochloa* sp.**	**Unknown**	**Australia**	NA	ND
	Cpus-2	***Dicantium* sp.**	**Unknown**	**Australia**	NA	ND

[1] Data are mean values from two experimental replicates each containing two or three technical replicates.

[2] *Lolium perenne:* perennial ryegrass; *Poa pratensis*: Kentucky bluegrass; *Bromus inermis*: smooth brome; *Hordeum vulgare*: barley; *Secale cereale*: rye; *Spartina* sp.: cordgrass species; *Sorghum bicolor*: sorghum; *Cynodon* sp.: Bermuda grass species; *Pennisetum typhoideum*: pearl millet; *Panicum maximum*: Guinea grass; *Paspalum* sp.: paspalum species; *Bothriochloa* sp.: beardgrass species; *Dicantium* sp.: Dicantium species.

[3] NA: Not amplified.

[4] ND: Not determined.

Table 2. Cycle thresholds and detection results from quantitative PCR reactions using spore trap tapes collected from perennial ryegrass (*Lolium perenne*) and Kentucky bluegrass (*Poa pratensis*) seed crops[1]

Grass seed crop	No. spores on tape[2]	Cycle threshold[3]	Detected[4]
Lolium perenne	0	37.21	+
L. perenne	0	35.00	+
L. perenne	0	40.00	-
L. perenne	3	38.28	+
L. perenne	6	37.10	+
L. perenne	7	34.83	+
L. perenne	12	37.36	+
L. perenne	15	34.66	+
L. perenne	34	32.65	+
L. perenne	38	36.69	+
L. perenne	92	40.00	-
L. perenne	98	40.00	-
L. perenne	107	33.72	+
L. perenne	107	38.38	+
L. perenne	186	34.03	+
L. perenne	199	35.20	+
L. perenne	245	34.97	+
L. perenne	246	34.72	+
L. perenne	261	34.56	+
L. perenne	364	34.21	+
L. perenne	427	32.49	+
L. perenne	512	33.01	+
L. perenne	602	33.07	+
L. perenne	757	33.21	+
L. perenne	926	31.22	+
L. perenne	1054	31.73	+
Poa pratensis	0	34.99	+
P. pratensis	0	37.50	+
P. pratensis	3	34.08	+
P. pratensis	7	37.31	+
P. pratensis	9	37.75	+
P. pratensis	20	35.38	+
P. pratensis	69	38.05	+
P. pratensis	239	36.85	+

[1] Data are mean values from two experimental replicates each containing four technical replicates.

[2] Number of spores as determined using microscopic counting methods.

[3] A cycle threshold value < 40 was interpreted as a positive result.

[4] +: positive result; -: negative result.

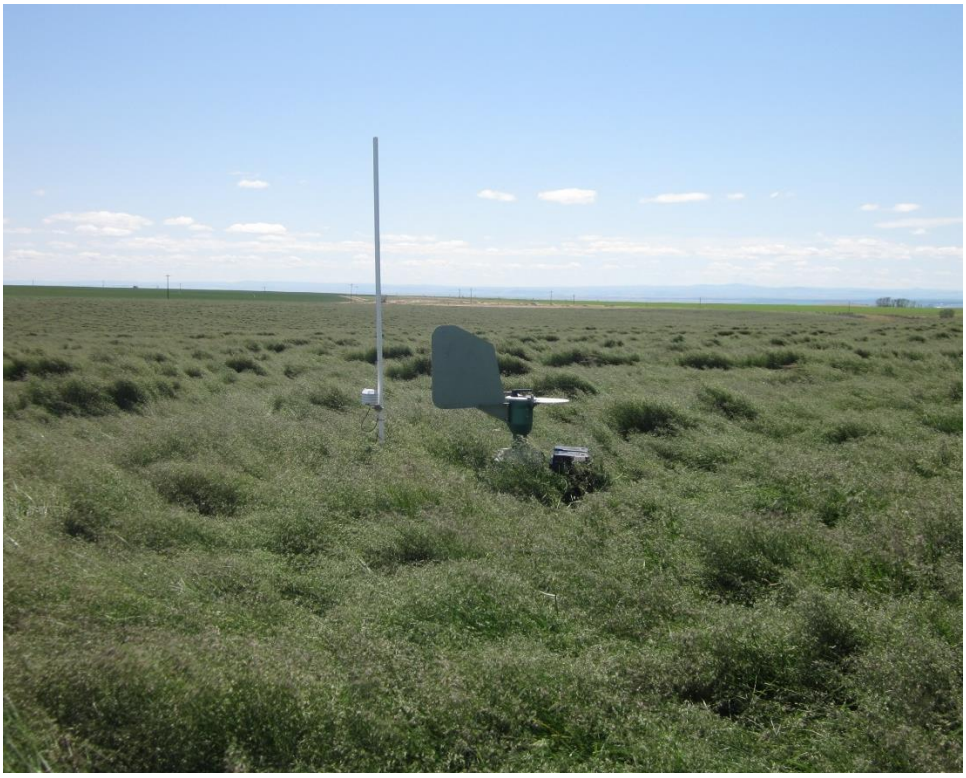

Fig. 1. A Burkard volumetric 7-day recording spore trap and weather data logger deployed in a commercial Kentucky bluegrass seed production field.

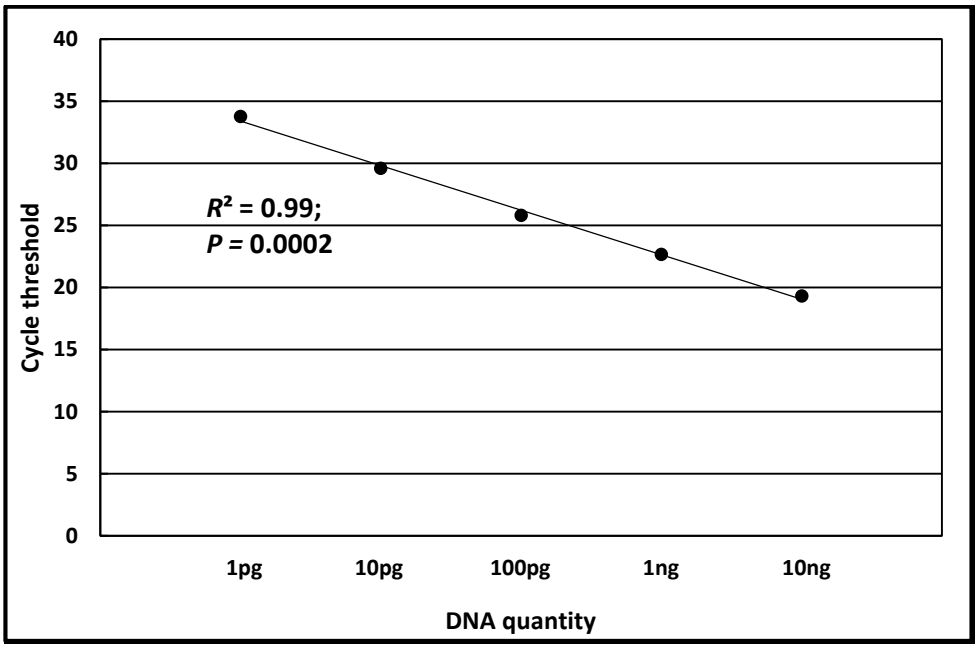

Fig. 2. Standard curve of cycle threshold values calculated from serial dilutions of DNA from *Claviceps purpurea*.

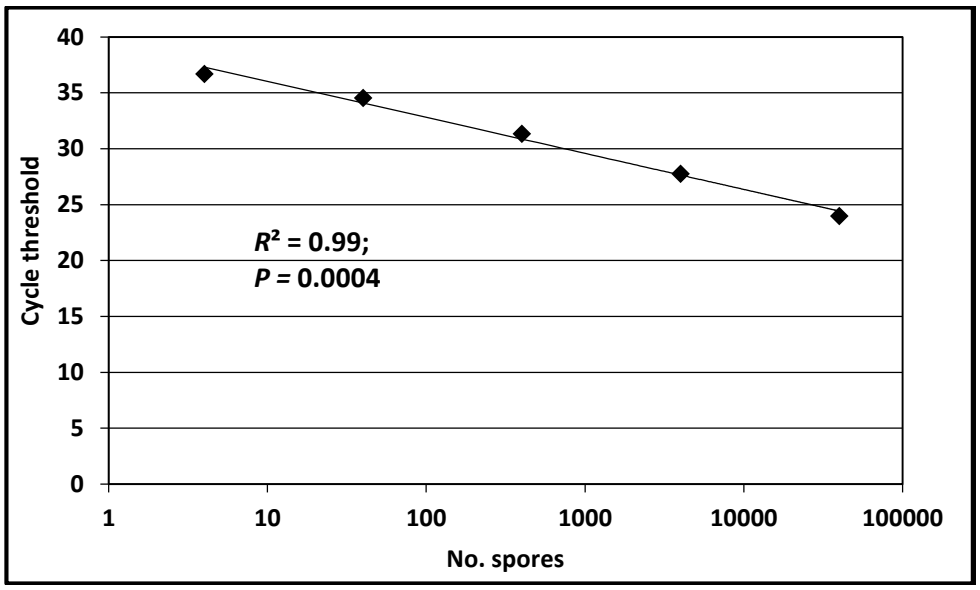

Fig. 3. Standard curve of cycle threshold values calculated from serial dilutions of spores from *Claviceps purpurea*.

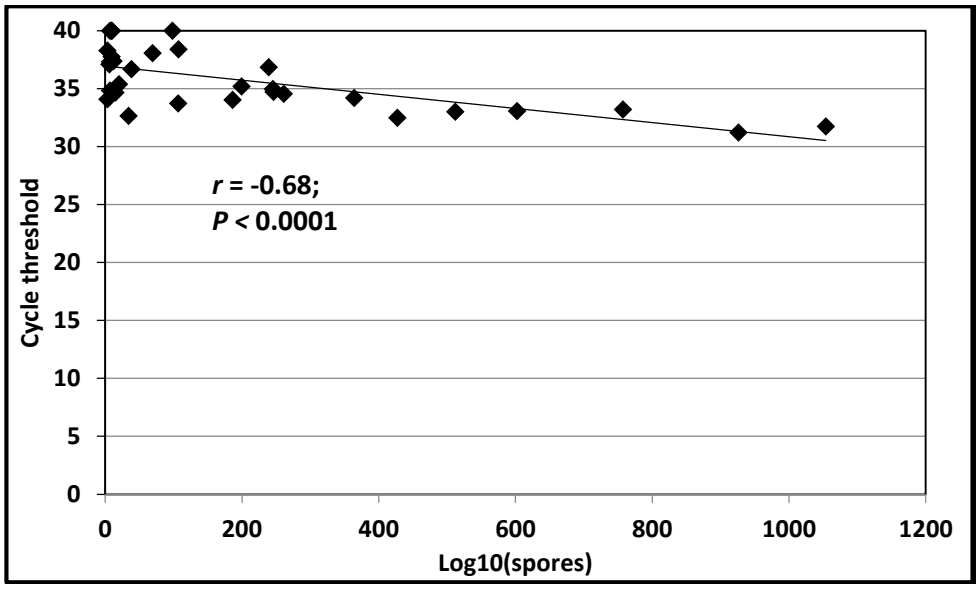

Fig. 4. Correlation between cycle threshold values and $\log_{10}$-transformed counts of *Claviceps purpurea* ascospores obtained using traditional microscopic methods.