Rule Change Proposal No. 12

PURPOSE OF PROPOSAL

This proposal provides an alternative method for testing endophyte infection levels in tall fescue and perennial ryegrass.

PRESENT RULE

9. FUNGAL ENDOPHYTE TESTING

9.1 Method of preparation of aniline blue stain for use in testing grass seed and plant material for the presence of *Acremonium* spp.

a. Prepare a 1% w/v aqueous aniline blue solution in water (dilute 1 aniline blue to 100 ml water).
b. Prepare a solution of one part of 1% aniline blue solution with 2 parts of lactic acid (85%).
c. Use stain as-is or dilute with water if sections are too dark.

9.2 Procedure for determining levels of *Acremonium* spp. in grass seed

a. Take a sub-sample of seed (1 gram is sufficient) from the pure seed portion of the kind under consideration.
b. Digest seed at room temperature for 12-16 hours in a 5% sodium hydroxide solution or other temperature/time combination resulting in adequate seed softening.
c. Rinse thoroughly in running tap water.
d. De-glume seed and place on microscope slide in a drop of seed stain. Slightly crush seed. Use caution to prevent carryover hyphae of *Acremonium* from one seed to another.
e. Place coverglass on seed and squash with gentle pressure.
f. Examine with compound microscope at 100-400X magnification, scoring a seed as positive if any identifiable hyphae are present.
g. Various sample sizes may be used for this test. Precision changes with sample size; therefore, the test results must include the sample size tested.
h. Test tolerances — see section 5.6, Table 10.

9.3 Procedure for determining levels of *Acremonium* spp. in grass plant material

a. Tillers from field stands.
   (1) Tillers must be randomly collected; one tiller each from each clump.
   (2) Samples should be free of contaminating fungi and other grasses such as annual ryegrass, orchardgrass and crabgrass.
   (3) Freezing will preserve samples and make subsequent peeling of tissue easier.

b. Seedlings from seeds suspected to contain fungal endophyte.

(1) Select seeds at random and germinate.
(2) Examine seedlings from the sample germinated after growing for a minimum of 48 days.

c. Remove the outermost sheath from the tiller or seedling. Tissue should have no obvious discoloration from saprophytes and should have as little chlorophyll as possible.
d. Isolate a longitudinal section of sheath approximately 3-5mm in width.
e. Place the section on a microscope slide with the epidermis side down.
f. Stain immediately with aniline blue-lactic acid stain. Allow dye to remain at least 15 seconds but no more than one minute.
g. Blot off excess dye with tissue paper. Sections should remain on the slide, but may adhere to the tissue paper; (if so, remove and place on proper position on the slide).
h. Place a coverglass on the sections and flood with water.
i. Examine section 200X magnification. Score a section as positive if any identifiable hyphae are present.
j. Various sample sizes may be used for this test. Precision changes with sample size, therefore, the test results must include the sample size tested.
k. Test tolerances — see section 5.6, Table 10.

PROPOSED RULE

9. FUNGAL ENDOPHYTE TESTING

9.1 Microscopic Examination Procedure.

a. Method of preparation of aniline blue stain for use in testing grass seed and plant material for the presence of *Neotyphodium* spp. [*Acremonium* spp.]

(1) Prepare a 1% w/v aqueous aniline blue solution in water (dilute 1 aniline blue to 100 ml water).
(2) Prepare a solution of one part of 1% aniline blue solution with 2 parts of lactic acid (85%).
(3) Use stain as-is or dilute with water if sections are too dark.

b. Procedures for determining levels of *Neotyphodium* spp. [*Acremonium* spp.] in grass seed

(1) Take a sub-sample of seed (1 gram is sufficient) from the pure seed portion of the kind under consideration.
(2) Digest seed at room temperature for 12-16 hours in a 5% sodium hydroxide solution or other temperature/time combination resulting in adequate seed softening.
(3) Rinse thoroughly in running tap water.
(4) De-glume seed and place on microscope slide in a drop of seed stain. Slightly crush seed. Use caution to prevent carryover hyphae of *Acremonium* from one seed to another.
(5) Place coverglass on seed and squash with gentle pressure.

(6) Examine with compound microscope at 100-400X magnification, scoring a seed as positive if any identifiable hyphae are present.
(7) Various sample sizes may be used for this test. Precision changes with sample size; therefore, the test results must include the sample size tested.
(8) Test tolerances — see section 5.6, Table 10.

c. Procedure for determining levels of *Neotyphodium* spp. [*Acremonium* spp.] in grass plant material

(1) Tillers from field stands.
(a) Tillers must be randomly collected; one tiller each from each clump.
(b) Samples should be free of contaminating fungi and other grasses such as annual ryegrass, orchardgrass and crabgrass.
(c) Freezing will preserve samples and make subsequent peeling of tissue easier.

(2) Seedlings from seeds suspected to contain fungal endophyte.
(a)Select seeds at random and germinate.
(b) Examine seedlings from the sample germinated after growing for a minimum of 48 days.
(3) Remove the outermost sheath from the tiller or seedling. Tissue should have no obvious discoloration from saprophytes and should have as little chlorophyll as possible.
(4) Isolate a longitudinal section of sheath approximately 3-5mm in width.
(5) Place the section on a microscope slide with the epidermis side down.
(6) Stain immediately with aniline blue-lactic acid stain. Allow dye to remain at least 15 seconds but no more than one minute.
(7) Blot off excess dye with tissue paper. Sections should remain on the slide, but may adhere to the tissue paper; (if so, remove and place on proper position on the slide).
(8) Place a coverglass on the sections and flood with water.
(9) Examine section 200X magnification. Score a section as positive if any identifiable hyphae are present.
(10) Various sample sizes may be used for this test. Precision changes with sample size, therefore, the test results must include the sample size tested.
(11) Test tolerances — see section 5.6, Table 10.

9.2 Immunoblot Procedure for perennial ryegrass and tall fescue.

a. 100 seeds shall be tested. To avoid false positive results remove all ergot sclerotia.
b. Use Phytoscreen Immunoblot Kit (Cat. # ENDO7971) for detection of Neotyphodium in seeds of tall fescue and perennial ryegrass, Agrinostics, Ltd. Co., 2850 Elder Mill Rd., Watkinsville, GA 30677. Conduct test according to manufacturer's instructions.
c. Report the number of seeds tested and the percentage of positive or negative seeds.

9.3 Reporting Results

Report the test procedure used, number of seeds, seedlings or tillers examined and number or percentage of positive or negative results.

HARMONIZATION STATEMENT

This rule was accepted into the ISTA Rules July 2002. Canadian Methods and Procedures do not address endophyte testing. The present AOSA Rules and FSA are the same.

SUPPORTING EVIDENCE

The current rule for detection of endophyte fungus is subjective in relationship to the analysts' ability to recognize microscopic fungal bodies. Blind samples sent to various labs demonstrate that immunoblot and microscopic analytical procedures for endophyte detection provide comparable test results. Ring tests show that analysts are apt to underestimate endophyte detection using the microscopic method. The cost of the endophyte detection kit is $35 and takes less than 30 minutes of a technologist's time. The cost of the microscopic method is around $1 and takes a minimum of two hours of experienced technologist's time. The financial impact to the industry is explained in the supporting data.

The current rule incorporates harmful chemical use in the testing procedure. The proposed rule uses non-toxic reagents. This test method involves soaking the seed in 5% sodium hydroxide for one hour, followed by a thorough rinsing until all alkali is removed, and placing the seed on a nitrocellulose membrane supported on a sponge saturated with an extraction buffer. The seed are incubated overnight at 35 – 45 C, gently scraped from the membrane and the membrane dried. The dried membrane is placed in a protein solution for 30 minutes to block antigenic sites. The membrane is then placed into a series of 2 Neotyphodium specific antibody solutions (washing in antibody solutions) for 1 hour each, and finally a proteinA/alkaline phosphatase solution. After a final washing, a chromogenic reagent is added. After 20 minutes, each location on the membrane (where seed were present) is scored for positive or negative depending upon the color reaction.

Further supporting evidence as attached:

1. Immunoblot and Microscopic Analysis of Red- and Tall fescue Seed Lots: A Ring Test Comparing Accuracy of Data For Commercial Applications, Dr. Nicholas S. Hill Dept. Crop and Soil Sciences, University of Georgia, Athens, GA, USA
2. Seed Testing for Endophytes by Microscopic and Immunoblot Procedures
N.S. Hill, E.E. Hiatt, III, J. P. De Battista, M.C. Costa, C.H. Griffiths, J. Klap, D. Thorogood, and J.H. Reeves
3. ISTA Protocol for Comparing Endophyte Infection Status in Perennial Ryegrass and Tall Fescue Using Microscopic Analysis vs. Immunoblot Analysis. Dr. Nicholas S. Hill, Dept. Crop and Soil Sciences, University of Georgia, Athens, GA 30602 USA
4. Quality Assurance/Quality Control Statement. Dr. Nicholas S. Hill, Agrinostics Ltd. Co.

SUBMITTED BY: Sharon K Davidson, RST
Agri Seed Testing, Inc. 1930 Davcor CT SE, Salem, OR 97302
Ph 503-585-1440, fax 503-588-0733, sdagriseed@aol.com

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IMMUNOBLOT AND MICROSCOPIC ANALYSIS OF RED- AND TALL FESCUE SEED LOTS: A RING TEST COMPARING ACCURACY OF DATA FOR COMMERCIAL APPLICATIONS

Nick Hill Dept. Crop and Soil Sciences, University of Georgia, Athens, GA, USA

BACKGROUND:

In April 2002 Barenbrug, USA submitted three red fescue seed lots to the Oregon Department of Agriculture and Agrinostics Ltd. Co. for endophyte (Neotyphodium) analysis. Each seed lot was replicated (blind replicate) three times so as to examine variability of the results within and between laboratories; and compare the results of different analytical procedures used by these labs. One of the seed lots was bred for high endophyte infection frequency, one was known to be low in endophyte infection frequency, and one was known to have an endophyte infection frequency of zero or near zero. Each laboratory was instructed to analyze 50 seeds from each seed lot using the methods employed in their laboratory. It is important to distinguish that the Oregon Department of Agriculture utilizes the microscopic staining method for endophyte analysis, while Agrinostics Ltd. Co. utilizes an antibody-based immunoblot method. The results (Table 1) indicated that Agrinostics Ltd. Co. tended to find more endophyte with the immunoblot method than did the Oregon Dept. of Agriculture with the microscopic method. This raised the question as to the accuracy of the methods or

Table 1. Endophyte infection frequencies of three red fescue seed lots as measured by antibody-based immunoblot methodology (Agrinostics Ltd.) or microscopic staining of histological samples (Oregon Dept. of Ag.).

Cultivar	Seed Lot I.D.	Agrinostics	Oregon Dep. Ag.
	---------- % Infection Frequency (Std. Dev.) ----------		
Bridgeport	L177-1-Bridgeport	9.33 (4.2)	0.00 (0.00)
FIE	2000 Harvest	81.33 (9.9)	16.67 (17.0)
Hardtop	2000 Breeder Seed	0.67 (1.1)	0.00 (0.0)

whether one laboratory was more capable of detecting Endophyte. The scope of this study was limited and no conclusive evidence for either hypothesis could be established. Therefore, Barenbrug, USA solicited Agrinostics Ltd. Co. to design an experiment which would test the capabilities of the two diagnostic methods and laboratories which utilize those methods. The ultimate goal of Barenbrug was to employ the best technique in a research project at Barenbrug West Coast Research Center. Agrinostics subsequently solicited the Pennington Seed Company (Oregon Division) to assist with implementation of a plan.

METHODS:

Three tall fescue seed lots were used for the study. The seed lots were selected based upon a previous knowledge of endophyte frequency as determined by 1) seed analysis of endophyte, 2) confirmation by growing seedling plants and analyzing them for presence of viable endophyte, and 3) analysis of seed for frequency of endophyte-derived ergot alkaloids. The seed lots were assigned a fictitious seed lot number so laboratories included in the test could not identify the seed lot to be analyzed. Seed lots were provided to Barenbrug USA and Pennington Seed Co. who subsequently submitted the samples to the laboratories for analysis.

The laboratories receiving seeds for analysis were selected based upon the method of endophyte detection used. The Oregon Department of Agriculture and Oregon State University Seed Laboratories were selected because each uses microscopic examination of stained histological samples for analysis. Agri Seed Testing (Salem, OR) and Agrinostics Ltd. Co. (Watkinsville, GA) were selected because each uses the antibody-based immunoblot method for endophyte detection. It should be noted here that OSU and ODA were not informed of this study, rather fees were paid for their services. Agri Seed Testing was unaware of the identity of the samples but volunteered to be a part of the study. Seed samples analyzed by Agrinostics Ltd. Co. were provided to a laboratory technician to analyze without knowledge of the study or identity of the samples. Each laboratory was instructed to analyze 50 seeds for endophyte presence using the methods employed in their laboratories. Data were reported back to the seed companies who submitted the samples, and all data compiled by Barenbrug, USA.

Data were analyzed using analysis of variance procedures. Treatments were assigned to a fixed effects model in a completely random design.

RESULTS:

All laboratories correctly identified the seedlot which had no endophyte regardless of the method used (Table 2). Agrinostics Ltd. Co. and the Oregon State University Seed Laboratory had similar results for the seed lot with 'medium' endophyte infection frequencies, while Agri Seed testing found slightly more than either Agrinostics or the OSU lab. The Oregon Department of Agriculture Laboratory, on the other hand, found few seeds that were infected with endophyte. Similarly, the Oregon State University Seed Laboratory found the greatest frequency of endophyte in the 'high' while Agrinostics and Agri Seed Testing found slightly lower infection frequencies of the 'high' seed lot. The Oregon Department of Agriculture Laboratory found considerably less endophyte in the 'high' seed lot.

INTERPRETATION:

Microscopic evaluation of Endophyte in seed samples is dependent upon having adequately trained and conscientious technical staff. The success of the test is totally dependent upon their ability to accurately detect Endophyte because there is no known system of validating the results of any given sample. This is evident from the results in tables 1 and 2 since the Oregon Department of Agriculture unknowingly miss-diagnosed the samples. Thus, it can be deduced from this study the Oregon Department of Agriculture was incapable of detecting Endophyte when present in tall fescue seed samples. This is not to say microscopic evaluation does not have its merits. The Oregon State University Seed Lab appeared to have correctly identified endophytes in the seed samples and had the highest infection rates among the 'high' seed lot. Thus, microscopic evaluation of stained histological samples can provide and accurate assessment of endophyte status when appropriately conducted.

Table 2. Infection frequencies of seed lots testing either low, medium, or high for Endophyte when tested by the Oregon State University Seed Laboratory (OSU), Oregon Department of Agriculture (ODA), Agrinostics Ltd. Co., or Agri Seed Testing. OSU and ODA used the AOSA approved microscopic evaluation of stained histological samples, Agrinostics Ltd. and Agri Seed Testing used the immunoblot method.

Lab	Analytical Method	Infection frequency class of seed lots		
		Low	Medium	High
		---------- % Infection Frequency ----------		
OSU	Microscope	0.0	22.0	94.0
Agrinostics	Immunoblot	0.0	26.7	86.7
Agri Seed	Immunoblot	0.0	35.3	82.7
ODA	Microscope	0.0	4.0	12.7
LSD (p=0.05)		NS	8.5	6.6

The immunoblot method of endophyte detection provided more consistent results than the microscopic method (Table 3). In fact, the inter-laboratory standard deviation (a measure of repeatability) for the microscopic analysis was equal to or greater than the inter-laboratory mean for the two seed lots containing Endophyte. Conversely, the inter-laboratory mean for the two seed lots containing Endophyte were significantly lower when the immunoblot procedure was used. Therefore, the immunoblot method provided superior analysis to that of the microscope in this study.

Table 3. Mean endophyte infection frequency for microscopic evaluation vs. immunoblot evaluation for all seed lots.

Method of analysis	Seed lot	Mean Endophyte infection	Between Lab Variation
		------- % -------	(Standard deviation)
Microscope	High	53.4	57.5
	Medium	13.0	12.7
	Low	0.0	0.0
Immunoblot	High	84.7	2.8
	Medium	31.0	6.1
	Low	0.0	0.0

IMPLICATIONS:

Seed companies rely on certifiable data from testing laboratories for a variety of traits associated with seed quality. Testing for Endophyte is one such quality parameter not because it affects the seed, but because it has the capability of producing livestock toxicoses. Seed lots that test less than 5% Endophyte frequency are considered to be safe for livestock use. However, if test results indicate a 4% infection frequency and the true infection rate is 30% (as in this study) then livestock toxicoses are likely to occur even if a certified seed label indicates less than 5% infection. The implications go beyond whether livestock grazing pastures from this seed lot suffer from toxicoses. In all probability, a producer with livestock experiencing toxicosis syndromes will test pastures for Endophyte infection frequencies. When pasture tests results indicate a potentially toxic condition in the pasture, it is highly probably a financial resolution will be sought with the seed company. The producer will likely re-test the seed for Endophyte and, if found to contain an unacceptable Endophyte infection frequency, this raises the question of the integrity of the company. The question of integrity may be in the form of 1) companies attempting to deceive the customer, or 2) a perception that the company is incapable of keeping seed lots separate during bagging and labeling. In either scenario the seed company risks losing credibility with the customer base. Thus, it is the seed company who suffers most from inaccurate or misleading Endophyte testing.

How does a testing facility know if it is providing accurate data? In the case of a laboratory conducting a microscopic analysis they often do not. When analyzing with the microscope, the underlying assumption is there is no error in detection. However, there is no means of determining whether a particular analysis is an accurate assessment of the Endophyte status of the seed because there is no system of checks or controls to determine whether the technician is performing to standards. On the other hand, there are two systems of controls by which performance of the immunoblot can be evaluated. First, positive and negative control antigens are dotted onto the membrane. This establishes whether or not the reagents for the test are performing as expected by examining each for a color reaction, or lack thereof. Secondly, seeds from seed lots known to test highly positive or negative for Endophyte are included to determine whether extraction of the antigens from the seed was successful. Additionally, the color reactions from the Endophyte-infected and the Endophyte-free seeds can be used to score the seeds from the sample. Furthermore, the membranes can be dried and placed into storage for a permanent record of the analysis. If there is a need to re-examine the membrane when an analysis is in question, the membrane can be retrieved from the file, re-wetted, and scored again. The membrane does not necessarily have to be scored by the technician who conducted the analysis, but a second technician or even multiple technicians can score it. The membrane can also be used as evidence in litigious circumstances.

The conclusion from this study is the immunoblot method is a superior analytical tool to the AOSA approved microscopic method for Endophyte analysis.

Seed Testing for Endophytes by Microscopic and Immunoblot Procedures

N.S. Hill[1], E.E. Hiatt[1], III, J. P. De Battista[2], M.C. Costa[2], C.H. Griffiths[3], J. Klap[4], D. Thorogood[3], and J.H. Reeves[5]

[1]Dept. Crop and Soil Sciences, University of Georgia, Athens, GA, USA; [2]Instituto Nacional de Tecnologia Agropecuaria, EEA Concepcion del Uruguay, Entre Rios, Argentina; [3]Dept. Plant Genetics and Breeding, Institute of Grassland and Environmental Research, Aberystwyth, United Kingdom; [4]Barenbrug Research, Duitsekampweg 60, Wolfheze, The Netherlands; [5]Dept. Statistics, University of Georgia, Athens, GA, USA.

Abstract

The presence of alkaloid-producing endophytes in forage grasses are generally considered detrimental, but are beneficial to turf grasses. Endophyte technology is changing to where they are now viewed positively for forages as well. Therefore, endophyte testing is likely to occur more frequently in the future. To do so will require a less laborious method than current histological/microscopic techniques. The objective of this project was to compare an immunological test kit with the histological/microscopic procedure for endophyte (*Neotyphodium coenophialum* and *N. lolii*) detection among independent laboratories. Three tall fescue and perennial ryegrass seed lots testing low, medium, and high for endophyte were sent in three blind replicates to laboratories in The United States, Netherlands, United Kingdom, and Argentina. Commercial immunochemical test kits were sent to each laboratory, as well as the International Seed Testing Association/Association of Official Seed Analysts histochemical protocol for endophyte detection. All laboratories analyzed fifty seeds with both procedures. Within and between laboratory variability were compared and data from the two procedures regressed to determine specificity across the range of data. Endophyte values from one laboratory were outliers compared to values from the other three laboratories for both methods. Data from that lab was omitted from the test. Intra-laboratory variability ranged from 0.0 to 9.6 percentage units while inter-laboratory variability ranged from 0.0 to 7.7 percentage units. Intra- and inter-laboratory variability was similar for both endophyte detection methods. When microscopic and immunoblot data were regressed with each other the intercepts of the regression lines were not different from 0.0, slopes were not different from 1.0, and regression coefficients were 0.88 or higher. The immunoblot procedure is an acceptable alternative to microscopic analysis of tall fescue and perennial ryegrass seed for endophyte.

The presence of alkaloid-producing endophytes in cool season grasses are considered a detriment in forage grasses, but have beneficial uses in turfgrasses because of superior persistence, insect resistance, and growth of the grass. Development of non-toxic forage type germplasms and cultivars (producing little or no ergot alkaloids) of cool season grasses has been successful (Bouton et al., 1998; Adcock et al., 1997). Therefore, it is likely that endophytes will be successfully manipulated with beneficial traits for forages grasses as well. As endophyte technology progresses, it will be necessary to have an approved rapid and reliable method of testing for its presence.

The approved International Seed Testing Association (ISTA) and Association of Official Seed Analysts (AOSA) method for determining endophyte presence is to histologically stain and microscopically examine seed tissue for presence of the serpentine hyphae characteristic of *Neotyphodium* spp. (formerly *Acremonium*) endophytes (Clark et al., 1983; Welty et al., 1986; Hiatt et al., 1999). Microscopic examination of numerous seed lots is impractical because it is laborious, tedious, and demoralizing to laboratory technicians. ELISA technology has been used to detect endophytes in tall fescue, but often give spurious reactions when polyclonal antibodies are used. Hiatt et al. (1999) reported that a commercially available monoclonal antibody immunological test kit gave nearly identical results as microscopic examination of stained seed and leaf sheath tissue. The immunoblot test was repeatable with intra-laboratory technicians but was not tested among laboratories. Immunological procedures have an advantage over microscopic methods in they can be extremely specific, more rapid, and easy to use (Hiatt et al., 1997). The objective of this research was to compare microscopic and immunological endophyte detection procedures among independent laboratories.

Materials and Methods

Six seed lots were used in this study, three tall fescue (*Festuca arundinacea* Schreb.) and three perennial ryegrass (*Lolium perenne* L.). The seeds were tested for endophyte presence by immunoblot (see procedure below) and were low, medium, and highly infected within each grass species. The lots were sampled in triplicate and randomly assigned a seed lot number for histological detection, and independently randomized for immunological

detection. The seed lots were sent to four testing laboratories in The United Kingdom, The Netherlands, Argentina, and The United States. Seed lots were re-randomized for each laboratory prior to shipping in order to prevent comparisons in the unlikely event they had common knowledge of the test. All laboratories had histories of utilizing histological staining for endophyte determination, but only two had used the immunological procedure prior to the test

A copy of the International Seed Testing Association (Welty and Rennie, ISTA Working Sheet No. 55) and Association of Official Seed Analysts (Anonymous, 1998) worksheets (same methods) for histological endophyte detection was included with the seed lots with strict instructions that each laboratory was to use only that method. Briefly, the 18 seed samples identified for histological evaluation had 500 to1000 seeds soaked in 30 mL of 5% sodium hydroxide containing 0.1% tryptan blue for approximately 15 hours. After washing the seed with clean tap water, the endophyte mycelium in the seed were stained by boiling the seed in an aqueous solution containing 2 parts of 1% aqueous aniline blue and 1 part of an 85% lactic acid:15% distilled water solution. Fifty seeds from each lot were placed on a glass slide in 1 - 2 drops of a solution of glycerine:deionized water (1:2 v/v), crushed with a cover slip, and microscopically examined at 100x to 400x magnification for hyphae detection. Commercially available *Neotyphodium*-specific test kits (Agrinostics Ltd. Co., Watkinsville, GA, USA) were sent to each laboratory for evaluation of the 18 seed samples. With the exception of a 5% sodium hydroxide solution, all reagents used for immunological detection of endophyte were made by diluting test kit reagents with distilled water as describe by instruction booklets include in the kits. Briefly, 100 to 500 seed were soaked in 30 mL of 5% sodium hydroxide for 60 minutes, after which they were thoroughly washed in clean tap water to remove all sodium hydroxide. A cellulose sponge was placed in a plastic food storage container, saturated with an extraction solution, a piece of blotting paper placed on top of the sponge, and a 60-mm by 60-mm piece of nitrocellulose membrane placed on top of the blotting paper. Purified proteins from *Neotyphodium* spp. were spotted on each membrane as a positive control to establish whether the chemical reagents worked properly. Known endophyte-infected and endophyte-free seeds (included in the kits) were also prepared as positive and negative controls to determine whether the extraction procedure was properly conducted. Fifty intact seed from each seed sample were placed on top of the saturated nitrocellulose membrane and incubated for 15 hours at 45^{o}C. The seed surface placed on the membrane was random without attention as to whether the rachilla was facing up or down. The membrane was removed from the extraction buffer with forceps, placed on top of a dry piece of blotting paper, the seed gently brushed from the membrane, and the membrane dried for 15 minutes in a laboratory oven set at 70^{o}C or for 1 hour at room temperature. The dry membranes were placed in 15-cm diameter polycarbonate petri dishes and placed on an orbital shaker with 10 mL of blocking solution for 30 minutes. The blocking solution was poured off and a solution containing *Neotyphodium*-specific monoclonal antibodies was added and placed on the orbital shaker for 60 minutes. The antibody solution was poured off and the membrane washed twice with a washing solution. A secondary antibody solution was added and placed on the orbital shaker for 60 minutes. The secondary antibody solution was poured off, the membrane washed twice, a chromophore solution added, and the petri dish returned to the shaker for 30 minutes. The chromophore solution was poured off, the membrane washed twice, and a chromogenic solution added. The petri plate was immediately covered with aluminum foil and returned to the shaker. After 20 minutes, the chromogenic solution was poured off, the membrane washed twice in clean tap water, and scored immediately for endophyte presence by calculating the percentage of seeds which stained a dark color.

Data were statistically analyzed using a completely random model with the PROC GENMOD subroutine of SAS (Cary, NC). All treatment variables were considered fixed effects in the models, and a factorial of all possible interactions of the main effects (test protocol, laboratory, level of infection of seed lots, and plant species) were tested for significance at the 0.05 level of probability using a Pearson's chi square analysis. The analysis indicated that data from laboratory 2 was different from the other three laboratories for both the immunoblot and microscopic methods. Hence, data from laboratory 2 was omitted and the remaining data re-analyzed using the GENMOD procedure. The analysis indicated a significant plant species (tall fescue, perennial ryegrass) by endophyhte level (high, medium, low) interaction. Therefore, the data was sorted by plant species and endophyte level and the analysis re-run using the GENMOD procedure.

The International Seed Testing Association and International Seed Health Initiative guide for comparative testing of methods for detection of seed-borne pathogens state that accuracy, precision, and specificity of the protocols must be compared. To obtain an estimate of accuracy of the immunoblot test, the means of each seed lot were compared for each test protocol using the ANOVA procedure of SAS. Precision of the two methods of endophyte analysis was calculated by comparing within- and between-lab variability for the seed lots for each detection method. To test within-laboratory variability, the standard deviations for each method were calculated for the low, medium and high endophyte seed for each grass species within each laboratory. These standard deviations represented the within laboratory variability for each seed lot, so mean standard deviations (among laboratories)

were calculated for each test procedure and seed lot, and compared for similarity. To test between-laboratory variability, means were calculated for each laboratory for both the immunoblot and microscopic method for each of the seed lots. The laboratory means were used to calculate standard deviations for each endophyte analytical procedure to give an estimate of between laboratory variability. Specificity was determined by regressing the immunoblot (independent variable, n=54) vs. microscopic data (dependent variable, n=54) to determine whether intercepts and slopes were different from 0 and 1, respectively, and screening for goodness of fit of the data (high R^2).

Results and Discussion

Mean values from laboratory 2 clearly had lower endophyte infection values regardless of the method used to analyze for endophyte (Table 1). One of the limitations of the microscopic method is that there is no way of determining why one lab might differ from another. Conversely, the immunoblot procedure has built-in controls to provide insight as to potential failures. The purified *Neotyphodium* spp. proteins spotted on the membranes gave positive results for the immunoblot tests for all laboratories. Therefore, the chemical reagents in the kit performed as designed in all cases. Laboratory 2 reported that the endophyte-infected positive control seed gave similar stains as the endophyte-free control seed. All other labs found the positive control seed gave the characteristic color reaction for endophyte presence. Inasmuch as the positive control seed were not different from the negative control seed for laboratory 2, it appears that the procedure in which endophyte proteins were extracted from the seeds failed in this case. Our experience has determined there are three possible reasons why the extraction procedure may have failed. The first example is the time seed is soaked in the 5% sodium hydroxide solution. If the seed are not soaked for exactly one hour it is likely that the results will vary from those which are soaked for one hour, usually with weak color reactions for endophyte-infected seed. Secondly, seed will have a soapy feel if the sodium hydroxide is not completely rinsed from the seed and the attachment of the *Neotyphodium* proteins to the membrane can be compromised. Thirdly, saturation conditions may not be met for the extraction procedure if the sponge used for the extraction process is too large. In such cases there is insufficient buffer solution to extract the *Neotyphodium* proteins from the seed. Inasmuch as values for both microscopic and immunoblot were low for laboratory 2, this lab was considered an outlier, their data was eliminated from the data set, and statistical analysis was re-run using data from the remaining laboratories.

Probability values of the Pearson's chi square comparing microscopic vs. immunoblot data within each seed lot indicated that there was a difference among methods only in the tall fescue seed lot testing high in endophyte (Table 2). All other seed lots had chi-square values with probabilities of greater than 0.05, thus indicating no differences among endophyte detection methods. Since there were no differences among detection methods among the seed lots with low endophyte levels, it is unlikely that phylogenetically related organisms, such as ergot, resulted in false positives in these seed lots.

Probability values for the Pearson's chi-square indicate that endophyte values for the perennial ryegrass lot with low endophyte were different for laboratory 4 than laboratories 1 and 3 (Table 2). The mean endophyte values for the low endophyte perennial ryegrass seed lot were 4.33 for laboratory 1, 4.00 for laboratory 3, and 9.67 for laboratory 4. There was no difference for endophyte values due to test methods within laboratories.

The intra-laboratory variation was compared by calculating standard deviations for each analytical method for the seed lots within each lab, and averaging the standard deviations from the three labs. Standard deviations of the test means were similar for both endophyte detection methods suggesting the intra-laboratory error was the same for both test methods (Figure 1)). The inter-laboratory variability was examined by calculating the mean endophyte infection rate for each seed lot within each analytical method, and using the means to calculate a standard deviation among the laboratories. Inter-laboratory means and standard deviations were similar among test methods regardless of plant species or level of endophyte infection, suggesting the laboratory-to-laboratory variation of the test methods were similar (Figure 2). There tended to be more inter- and intra-laboratory variability among seed lots with medium levels of endophyte and less variability when there were either high or low endophyte levels in the seed regardless of the method used to detect endophytes (Figures 1 and 2). This is expected since the response variable is a proportion, with a theoretical SD given calculated by:

[1] $SD = \sqrt{(p*(1-P)/50)}$.

Thus, standard deviations are likely to be larger when the P value is near 0.5 than when near 0.0 (low infection) or 1.0 (high infection). More importantly, the intra- and inter-laboratory standard deviations of both analytical methods were below permissible tolerances established by the Association of Official Seed Analysts specifically for endophyte testing (Anonymous, 1998).

A major concern when using immunodetection procedures for fungal organisms is the potential for spurious cross reactions with other fungi that provide false data. For example, if protein extracts used to vaccinate

animals to generate polyclonal antibodies are not highly purified and specific only to the organism of interest, erroneous diagnostic values are likely to result (Hill et al., 1998). Similarly, cross reactions occur if antibodies from monoclonal hybridoma cell lines have not been adequately screened for specificity to the target organisms. Therefore, if cross contamination were a problem, immunoblot values would consistently be higher than microscopic values, thus lending bias to the data. Hence, the specificity of the immunoblot procedure was determined by conducting a linear regression analysis between the immunoblot and microscopic analytical methods. If the immunoblot procedure consistently gave results different from the microscopic method, the data would then contain a bias and the intercept would be different from 0.0. If the immunoblot procedure were accurate at one end of the range of the data but lost accuracy and repeatability at the other, then the slopes would be different from 0.0, the standard errors of the estimates of intercept and slope would be inflated, and the regression coefficient would be low. Regardless of whether data were pooled into one large data set, or regression conducted within each plant spcecies, the regression analyses had intercepts not different from 0.0, slopes not different from 1.0, and regression coefficients (R^2) of 0.88 or greater (Table 3). Standard errors of the estimates of the intercepts and slopes were also low.

The immunoblot procedure gave similar endophyte values as the microscopic method with the exception of the high-endophyte tall fescue seed lot (Figures 1 and 2). Differing results among the endophyte detection methods is paradoxical as to which is more exact. Hiatt et al. (1999) conducted a similar study, using the same immunoblot test kits as in this study, to compare microscopic and immunoblot procedures for detection of endophytes within grass tillers. They too found that immunblot gave slightly higher endophyte frequencies than microscopic analysis. However, their study differed from this study in that re-testing of grass tillers was feasible when the two detection methods did not consistently detect endophyte. They found errors occurred when the immunoblot gave a positive test and the microscope gave a negative test for endophyte. Upon re-examination of the tiller in question with both methods, both gave positive results. Therefore, it was the microscope that erred in that case and it was assumed to be associated with fatigue that is common among laboratory technicians who use microscopic examination of seeds and stems for endophytes. Hence, it is not unreasonable that endophytes were missed during microscopic examination in this study. Another source of variation could be cross reaction with *Claviceps*. However, if *Claviceps* caused higher values among the immunoblot tests, one would expect means of the low-endophyte seed lots to be more adversely affected than those testing for high endophyte. This was not the case in this study. Even though, the effects of false positives from ergotized seed can be minimized by removing ergot sclerotia from a seed sample prior to immunoblot analysis.

The results from this study demonstrate that immunoblot and microscopic analytical procedures for endophyte detection provide comparable test results. This is evidenced by similarity of intra- and inter-laboratory variability between the two analytical methods and that the intra- and inter-laboratory standard deviations were below permissible tolerances (Anonymous, 1998). Also, regression of the data from the two methods gave a linear response surface with high regression coefficients and intercepts and slopes not different from 0.0 or 1.0. Hence, immunoblot endophyte test kits provide an acceptable alternative to microscopic examination for endophyte analysis. It should be kept in mind that seed-born endophytes, such as *N. coenophialum*, suffer mortality when seed are stored in warm or humid conditions (Welty and Azevedo, 1985). Currently, the only way to estimate viability of endophytes is to grow seed and examine seedling plants.

Table 1. Mean laboratory values for endophyte concentration among all seed lots and probability values (P-values) of paired Pearson's chi-square analysis to test for similarity between laboratories. Values are based upon numerical data using both immunoblot and microscopic examination of seed lots.

Seed lab	Mean infection rate	Pairwise test between labs (P-values)			
	---- % ----	Lab 1	Lab 2	Lab 3	Lab 4
Lab 1	42.22	--	0.001	0.441	0.096
Lab 2	30.72	0.001	--	0.001	0.001
Lab 3	46.27	0.441	0.001	--	0.367
Lab 4	46.27	0.096	0.001	0.367	--

Table 2. Probability values for significance of the Pearson's chi-square analysis for comparison of the microscopic vs. immunoblot methods of endophyte detection and comparison of laboratories 1 and 3 vs. laboratory 4 calculated within perennial ryegrass and tall fescue endophyte levels using the GENMOD procedure.

	Perennial ryegrass			Tall fescue		
	low	medium	high	low	medium	high
Scope vs. blot	0.327	0.087	0.325	0.502	0.496	0.037
Lab 1 vs 4	0.031	0.067	0.289	0.981	0.489	0.475
Lab 1 vs 3	0.682	0.098	0.487	0.981	0.294	0.635
Lab 3 vs 4	0.021	0.281	0.947	1.000	0.577	0.892

Table 3. Linear regression slopes, intercepts, and coefficients from regressions between immunoblot (independent variable) and microscopic (dependent variable) analyses of endophytes. Regression statistics are presented for the entire data set and within each crop species. Standard deviations for each statistic are in parentheses.

Data set	Intercept (S. E.)	Slope (S. E.)	R^2
All data	3.33 (2.12)	1.00 (0.04)	0.93
Ryegrass	4.62 (3.76)	0.99 (0.07)	0.88
Tall Fescue	2.14 (2.39)	1.01 (0.04)	0.96

Titles for Figures

Figure 1. Mean intra-laboratory variation (dotted lines) when testing for endophyte using the immunoblot procedure (dark bars) or microscopic procedure (light bars). Acceptable intra-laboratory variation is represented by the solid line immediately next to the dotted lines.

Figure 2. Mean inter-laboratory variation (dotted lines) when testing for endophyte using the immunoblot procedure (dark bars) or microscopic procedure (light bars). Acceptable intra-laboratory variation is represented by the solid line immediately next to the dotted lines.

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ISTA Protocol for Comparing Endophyte Infection Status in Perennial Ryegrass and Tall Fescue Using Microscopic Analysis vs. Immunoblot Analysis.

Crops: Tall fescue and perennial ryegrass

Organism: Endophytes (*Neothyphodium coenophialum* and *N. lolli*)

Test Coordinator
Dr. Nicholas S. Hill
Dept. Crop and Soil Sciences
University of Georgia,
Athens, GA 30602 USA
706-542-0923
nhill@arches.uga.edu

1. Description of test protocol:

The standard method of testing for endophytes in grasses is to soak overnight in a 5% sodium hydroxide solution, removing the glumes, and squashing the seed in an analine blue dye solution, and microscopically examining each seed for presence of the characteristic circuitous mycelium (Ref 1, 3, 4, 5, 6). A copy of the procedure from the ISTA "Handbook on Seed health Testing" (Working sheet no. 55) is included for further details (See Schedule 1).

A new immunologically based detection system has been developed (Agrinostics Ltd. Co., Watkinsville, GA, USA) (Ref. 2,3) which is based upon previous immunochemical techniques (Ref 4, 5). This test involves soaking the seed in 5% sodium hydroxide for one hour, followed by a thorough rinsing until all alkali is removed, and placing the seed on a nitrocellulose membrane supported on a sponge saturated with an extraction buffer. The seed are incubated overnight at 35-45^{o} C, gently scraped from the membrane, and the membrane dried. The dried membrane is placed in a protein solution for 30 minutes to block antigenic sites. The membrane is then placed into a series of 2 *Neotyphodium*-specific antibody solutions (washing in between antibody solutions) for 1 hour each, and finally a protein-A/alkaline phosphatase solution. After a final washing, a chromogenic reagent is added. After 20 minutes, each location on the membrane (where seed were present) is scored for positive or negative depending upon the color reaction. See the attached technical information from the company for further details.

2. Samples:

a. Number of samples:

Three tall fescue and three perennial ryegrass seed lots have been selected for this study.

b. Characterization of samples.

All seed samples were from the 1998 seed harvest. Some seed lots were from foundation fields, others represent commercial seed lots. All were conditioned to remove weed seed and provide uniform seed size. Endophyte infection was approximately 1, 45, and 90% infection within each grass species. The seed lots were replicated three times, for a total of 18 samples in the study.

c. Sample storage and handling.

Because germination was not a requirement and presence of endophyte is independent of storage conditions, seed were stored at room temperature.

3. Materials - Handling and Preparation:

a. Samples

Each seed sample was tested by examining 50 seeds using the AOSA/ISTA procedure for microscopic analysis and 50 seeds using the Agrinostics Ltd. Co. (Watkinsville, GA) endophyte test kit. Four laboratories examined the seed samples. To guarantee there was no associated with the analyses, the seed samples (6 lots x 3 replications) were randomly assigned laboratory ID numbers (eg. Sample 1, Sample 2, ... Sample 18) within laboratories, and within sampling procedures. That is, the randomization procedure was conducted independently for the samples to be analyzed microscopically and the randomization procedure for the immunoblot analysis

performed independently. Similarly, the randomization process for each laboratory were independent from the other laboratories. Therefore replications were blind to laboratory technicians and, in the event one lab determines that another is conducting the test, the laboratory numbers did not correspond between laboratories. Hence, a completely random factorial model was used for analysis of variance.

b. Cultures

Endophytes are constitutive and, therefore, culturing is not required.

c. Reagents

All immunoblot reagents were provided by Agrinostics Ltd. Co. in a test kit format. These reagents were stored under refrigerated conditions at 4^{o} C All laboratories selected for this study produce their own staining reagents for microscopic testing for endophyhte. Hence, these reagents were not provided.

4. Test Parameters

Summary of sources of variability

2 seed species
3 seed lots within each species
3 replications
4 cooperating laboratories
2 methods of endophyte analysis

The Analysis of Variance Table for the Proposed Method Analysis

Source	d.f.
Total	143
Seed Species (Species)	1
Endophyte level (Endophyte)	2
Laboratory (Lab)	3
Method of analysis (Test)	1
Species x Endophyte	2
Species x Lab	3
Species x Test	1
Endophyte x Lab	6
Endophyte x Test	2
Lab x Test	3
Species x Endophyte x Lab	6
Species x Endophyte x Test	2
Species x Lab x Test	3
Endophyte x Lab x Test	6
Species x Endophyte x Lab x Test	6
Error	96

This statistical design permitted a direct comparison for the two methods as well as all possible combinations.

a. Accuracy

The ability of the immunoblot method to accurately detect endophyte was compared with that of microscopic evaluations via the seedlot x method of analysis interaction in the analysis of variance table.

b. Limits of detection

Lower limits of detection were examined by comparing accuracy and precision of the immunoblot results among the seed lots with low infection rates. Upper limits of detection were similarly examined among the seed lots with high infection rates.

c. Precision

The precision of each method was placed under scrutiny by examining within lab and between lab variability for the seed lots.

d. Sensitivity

Sensitivity was determined by comparing performance of the immunoblot and microscopic endophyte detection methods over all infection levels of all seed lots.

e. Specificity

The major concern with use of immunodetection of fungal organisms is the potential for cross reaction with other fungi. Therefore, if cross contamination were a problem, immunoblot values will be consistently higher than microscopic values. Hence, similarity of values with microscopic techniques were compared using regression analysis (microscopic data dependent values and immunoblot data independent values). Slope and intercept were compared. If slope was not different from 1 and intercept not different from 0, then the data were interpreted to mean the immunoblot test is specific for endophtye.

5. Characterization of the Method

a. Interferences

The *Neotyphodium* antibodies cross react with *Claviceps purpurea*. Therefore, the instruction booklet specifically directs users to remove ergot sclerotia from the sample. Otherwise, there are no additional concerns about interferences.

b. Performance specifications

The immunoblot method is a proprietary product. Therefore, the reagents are provided by the company in a test kit form. All components of the kit are generic (to provide proprietary protection) and laboratory equipment requirements are listed without specificity to vendors.

c. Suitability tests

Standard endophyte-infected and endophyte-free seed lots are included into the test kit for comparison purposes to aid interpretation of the results. Positive and negative controls are placed on the nitrocellose membranes during manufacture to provide assurances the test kit is properly working.

d. Critical steps or parameters.

Critical steps are clearly identified in the technical booklet provided by the company.

e. Comparison with other methods.

This study compares the new immunoblot method with the standard microscopic technique by design.

6. Safety Considerations.

All safety considerations for use of the immunoblot test kit are identified in the technical bulleting provided by the company. Use of rubber gloves when handling sodium hydroxide and analine blue stain is necessary for the microscopic method.

7. Data Record Sheets

See attached Schedule 1.

8. Proposed Statistical Analysis

See section 4.

9. Test Timetable

Testing will be performed between 1 June 1999 and 1 January 2000.

10. Obligations of participants

See attached Schedule 2.

11. Bibliography

1. Clark, E.M., J.F. White, and R.M. Patterson. 1983. Improved histochemical techniques for the detection of Acremonium coenophialum in tall fescue and methods for in vitro culture of the fungus. J. Microbiol. Meth. 1:149-155.

2. Hiatt, E.E., N.S. Hill, J.H. Bouton, and C.W. Mims. 1997. Monoclonal antibodies for detection of Neotyphodium coenophialum. Crop Science 37:1265-1269.

3. Hiatt, E.E., N.S. Hill, J.H. Bouton, and J.A. Stuedemann. 1999. Tall fescue endophyte detection: Commercial immunoblot test kit compared with microscopic analysis. Crop Science 39:796-799.

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5. Welty, R.E., G.M. Milbrath, D. Faulkenberry, M.D. Azevedo, L. Meek, and K. Hall. 1986. Endophtye detection in tall fescue seed by staining and ELISA. Seed Sci. Tech. 14:105-116.

6. Welty, R.E. and M.D. Azevedo. 1985. Survival of endophyte hyphae in seeds of tall fescue stored one year. Phytopathology 75:1331-1331.

Schedule 1
Grass Endophyte Testing as Outlined by ISTA Handbook on Seed Health Testing
Working Sheet No. 55

Working Method:

A sample of 500 seeds is soaked overnight (about 15 hr) at room temperature in 20-30 mL of 5% sodium hydroxide containing 0.1% trypan blue. Seeds are washed for 1 min in clean tapwater or distilled water, stained by boiling gently on a hot plate in a fume hood for about 15 min in 20-30 mL of lactophenol (1:1:1:5 v/v lactic acid-85%:phenol:glycerine:deionized water) containing 0.1% trypan blue and then rinsed in tap water as before. Analine blue may be substituted for trypan blue (Ref. 1), and stain may be omitted from sodium hydroxide digestion if desired (Ref. 1). A solution without phenol can be substituted: 2 parts 1% aqueous aniline blue: 1 part 85% lactic acid:15 parts water (Ref 1).

Examination:

Each seed is placed on a glass slide in 1-2 drops of a solution of glycerine:deionized water (1:2 v/v), then crushed with a coverslip. Microscopic examination is done at x100 - x400 magnification. Hyphae typical of *Acremonium coenophialum* (now called *Neotyphodium coenophialum*) are easily detected in seeds by microscopic examination. The hyphae appear to be between the aleurone layer (which also stains blue) and the nucellar tissue. The hyphae may be found near the embryo, but can also be found in other areas with and without the aleuronic cells. The density of hyphae in individual seeds varies considerably and may range from a few to numerous strands. Hyphae of other seedborne fungi can also be seen, but with practice can easily be distinguished from *A. coenophialum* (*N. coenophialum*). Fifty seeds may be examined in approximately 90 minutes.

References:

1. Clark, E.M., J.F. White, and R.M. Patterson. 1983. Improved histochemical techniques for the detection of Acremonium coenophialum in tall fescue and methods for in vitro culture of the fungus. J. Microbiol. Meth. 1:149-155.

Schedule 2
Data Record Sheet

Assay type: **Immunoblot**					
Lot #	Date tested	# positive blots	# negative blots	% endophyte infection	Signature of Evaluator
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					

Assay type: **Microscopic**					
Lot #	Date tested	# positive stains	# negative stains	% endophyte infection	Signature of Evaluator
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					

Schedule 3.
Obligations of Participating Laboratories

Dear Cooperating Laboratory:

Read the following materials **thoroughly** before proceeding with endophyte evaluations.

Inspect the contents of this box and make sure the materials listed in the "Contents of box" below are present. Upon completing the inspection and making sure all materials are in good condition, fill out the self-addressed return post-card for receipt of materials and return it immediately. If any materials are in poor condition, contact Nick Hill immediately (Nick Hill, Dept. of Crop and Soil Sciences, University of Georgia, Athens, GA, 30602 USA; Tel. 706-542-0923; FAX 706-542-0914; e-mail nhill@arches.uga.edu) and arrange for replacement materials.

Contents of box:

1. Self addressed return post-card for receipt of materials.

2. 18 seed samples (designated Lot 1, Lot 2, Lot 3, ..., Lot 18) to be tested using immunoblot (marked "Blot") and 18 seed samples to be tested using microscopic assay (marked "Microscope"). **Therefore there are a total of 36 seed samples**.

3. 9 Zip-lock plastic bags containing endophyte detection kits.

4. Two "Data Record Sheets" on which to log your data (one for microscope and one for immunoblot).

5. Directions for handling Test Kits and Seed Storage prior to testing (read immediately and store kits and seed accordingly).

6. One "Analytical Protocol" information sheet. This must be read and understood prior to conducting endophyte analyses.

7. One "ISTA Microscopic Technique Sheet" for testing endophytes in grasses. This is a verbatim reproduction of the methods as described in "ISTA Working Sheet No. 55".

Thank you for your cooperation and good luck with your analyses.

Sincerely,

Nicholas S. Hill

Directions for Test Kit and Seed Storage Prior to Testing

Keep the test kits in their zip-lock bags and store them refrigerated at 4^{o} C until they are used. Pay attention to the expiration date of the test kits as the shelf life is 6 months.

Endophytes in seeds die during storage, but the ability to detect them is not dependent upon viability of the organism. Therefore, seed can be stored at ambient conditions in the laboratory providing the humidity level is low enough to prevent growth of ancillary fungi or seed pathogens.

Analytical Protocol

NOTE: THE ANALYTICAL PROTOCOL MUST BE FOLLOWED EXACTLY AS DESCRIBED OR THE TEST WILL BE INVALID!!!

1. Description of seeds.

The seeds selected for analysis are a combination of tall fescue and perennial ryegrass seed lots. Some are commercial seed fields while others are foundation seed fields. There are a total of 18 seed samples, labeled as Lot 1, Lot 2, Lot 3, ..., Lot 18. Note that some envelopes with the seed are labeled "Microscope" and others are labeled "Blot". Those labeled "Microscope" **must be analyzed using the microscopic technique**, and those labeled "Blot" **must be analyzed using the immunoblot technique**. The reason for this is that the seed samples have been assigned different lot numbers within each testing procedure to avoid comparisons of one test with the other during the analysis process. This keeps evaluators from biasing their data from one method to the next. Hence, keep the packets of seed marked "Microscope"or "Blot" separate and analyze them accordingly.

2. Testing of seeds.

Make sure to follow directions exactly as directed. If you have questions, please contact Nick Hill immediately (Dr. Nicholas S. Hill, Dept. Crop and Soil Sciences, University of Georgia, Athens, GA 30602; Tel. 706-542-0923; FAX 706-542-0914;
email: nhill@arches.uga.edu). Testing must be completed by 1 January, 2000. Those of you in North America will want to complete your testing prior to the summer rush resulting from the new seed harvest.

You will test 50 seeds from each sample endophyte. The immunoblot method is designed to test 100 seeds per kit. You will have enough kits if you analyze two seed lots per kit. There are two microcentrifuge tubes in each kit, one containing 90% endophyte-infected seed (there will be some that will test non-infected) and one containing endophyte-free seed (<1% infected). These are to be used as positive and negative comparisons when scoring the seeds for endophyte presence. **Make sure you analyze the samples marked "Blot" on the envelopes with this method**. Specific instructions on immunoblot testing are provided in the technical bulletin included in the test kit. Make sure and read the technical from cover to cover before running the analysis.

You will also test 50 seeds for endophyte using the microscopic procedure. **Make sure you analyze the samples marked "Microscope" on the envelopes with this method.** Specific instructions on microscopic testing have been reproduced verbatim from the AOSA "Rules for Testing Seeds" publication in case you are in need of a refresher.

Do not rush your analyses. Conduct only as many as you can perform without feeling stressed, hurried, or fatigued. Remember, the objective here is to get quality data from each method. This can only be done if you are relaxed and take your time.

3. Recording the data.

Use the "Data Record Sheets" to record your data. The sheets have Lot Numbers on them to correspond with the numbers on the seed envelopes. Note there are two Data Record Sheets: one data sheet for immunoblot and one data sheet for microscopic techniques. Report the data from the immunoblot technique on the Data Record Sheet designated "Immunoblot" and record the data from the microscopic technique on the Data Record Sheet designated "Microscope". As you record the data, record the date of the test, the number seeds testing positive for endophyte in the seed lot, the number of seeds testing negative for endophyte in the seed lot, calculate the percentage of endophyte infection for the seed lot and enter it into the data sheet, and have the evaluator who conducted the test on that seed lot sign the data sheet on the line for the seed lot.

Make copies of the data and keep them in separate locations so in case of a fire, misplacement, or accident the data can be easily reproduced. Once the data is complete, make a copy of the final data set. Send the original Data Record Sheets to Nick Hill, Department of Crop and Soil Sciences, 3111 Miller Plant Sciences Building, University of Georgia, Athens, GA 30602, USA. Notify Nick Hill by e-mail (nhill@arches.uga.edu) when you send your Data Record Sheets to let him know when to expect delivery (in case the mail system loses the data). Keep a copy of the data in your laboratory/office in case the data is lost during delivery.

Quality Assurance/Quality Control Statement.

Agrinostics Ltd. Co.
2850 Elder Mill Rd.
Watkinsville, GA 30677
USA
706-769-2397

1. Statement of approved written policy defining the critical elements of the quality assurance program.

The written policy for quality control states that:

A. All buffers have a defined salt content, stabilizers, and optimum pH necessary for extraction of endophyte proteins from seed, and in which antibodies and chromogens must be diluted for reagents to work. Each of the buffers is made in batches and tested on a monthly basis to guarantee they work.

B. The monoclonal antibodies are produced in batch quantities and tested for affinity to a known standard, as well as tested for cross-reactivity with taxonomic relatives of Neotyphodium to guarantee specificity for the target organism. Antibodies are tested in the kit format on a monthly basis to guarantee performance.

2. Statement that product is manufactured in compliance with the written quality policy. The product is manufactured according to the written policy statement.

3. Quality Policy.

a. Kits are constructed by dispensing precise volumes of standard stock solutions (buffers and antibodies) into reagent vessels. Vessels are calibrated for volumes where necessary.

b. Each kit is coded with a product number and lot number.

c. Internal records are maintained for 1) quality of lot numbers of products provided by suppliers and 2) identification of which batch numbers of each reagent has been used for each coded kit. Therefore, problematic test kits can be traced from our lot numbers to the specific source in case product recall is necessary.

d. Batch records are maintained. The company policy, thus far, has been to keep all records regardless of batch expiration in case of changes in production practices by suppliers that might affect product performance.

e. Batches of reagents are tested according to statement 1.

f. Unique lot numbers are maintained as described in 3c.

g. Each test kit is marked with an expiration date. This date is a conservative estimate of shelf life of the product. In fact, shelf life of the product has been tested to be at least three times as long as the expiration date marked on the product.

h. Quality audits are conducted by having multiple laboratory technicians test the products for performance.

i. Non-conforming materials are discarded immediately.

Phytoscreen

Immunoblot Kit
Cat. # ENDO7971

Neotyphodium in SEED 4H2:5C7:15D7

AGRINOSTICS, Ltd. Co.
2850 ELDER MILL RD.
WATKINSVILLE, GA 30677 USA
Tel./FAX(706) 769-2397

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INTENDED USE

The Agrinostics, Inc. Phytoscreen *Neotyphodium* immunoblot assay is to be used for the qualitative determination of extracted *Neotyphodium* spp. cell wall proteins from grass seed. The assay will detect presence of endophyte in tall fescue and perennial ryegrass.

READ THE ENTIRE PROTOCOL BEFORE USE.

PRINCIPLE OF THE METHOD

The Agrinostics Phytoscreen kit is a solid phase stacked Immunoblot assay. When properly conducted, proteins from the *Neotyphodium* endophyte are extracted by the Phytoscreen process, attaching them to a solid membrane support. Monoclonal antibodies specific to different epitopes of the *Neotyphodium* protein are added and bind to the proteins.

The first step is to surface sterilize the seed in 5% NaOH and place the sterilized seed on the membrane support while it sits on top an extraction buffer. After incubation overnight at 45°C, the membrane is blocked and pooled monoclonal antibodies are added. After washing, a second antibody specific for the monoclonal antibodies is added, hence resulting in a stacking of antibodies. Excess anti-monoclonal antibody is removed, the membrane washed, and protein- A with an alkaline phosphatase enzyme conjugate is added to complete the stacking effect.

After incubation and washing off excess protein-A alkaline phosphatase, a chromogen solution is added and color develops wherever membrane bound *Neotyphodium* protein is present. The presence of chromogen is usually in the shape of the seed.

REAGENTS PROVIDED

Store kits and reagents at 4°C.

1. *Nitrocellulose membrane.* One 60 x 60 mm square piece of nitrocellulose membrane sandwiched between two disposable red and white sheets of membrane support paper.

2. *Blotting paper.* Two 70 x 70 mm square pieces of blotting paper.

3. *Extraction buffer* (EB), to be diluted to 200 mL in distilled water prior to use.

4. *Blocking/washing/working reagent A,* (BWW-A) add to 150 mL distilled water as the first step to making the blocking/ washing/working solution (BWW).

5. *Blocking/washing/working reagent B*, (BWW-B) add to solution containing BWW-A to complete making the blocking/washing/ working solution (BWW)

6. *Pooled monoclonal antibodies 4H2, 5C7, and 15D7* (MAB) to be diluted to 10 mL with BWW.

7. *Rabbit anti-mouse antibody* (RAM) to be diluted to 10 mL with BWW.

8. *Protein A with alkaline phosphatase conjugate* (PA) to be diluted to 10 mL with BWW.

9. *BCIP/NBT Chromogen* (BN) use undiluted directly from container.

10. Control seeds in microcentrifuge tubes marked E+ and E-.

Handling note: This kit contains small quantities of sodium azide to stabilize the reagents. While the concentrations in the kits are below established toxic levels, sodium azide can be toxic to humans. Therefore, care should be taken to prevent exposure to skin, eyes, and mucous membranes. Use of rubber gloves when conducting this procedure is one way to minimize exposure to sodium azide. Reagents should **never** be ingested.

Disposal: Sodium azide can react with lead or copper plumbing to form explosive metal halides. Flush drains with a large volume of water to prevent azide accumulation.

SUPPLIES - NOT PROVIDED

1. Graduated cylinders in various sizes.

2. Deionized or distilled water.

3. 5% solution of sodium hydroxide in distilled water.

4. Glass or plastic tubes for diluting and dispensing reagents.

5. 45°C incubator.

6. 70°C oven.

7. A 4 x 6 inch plastic container with lid for incubating seeds.

8. Cellulose sponge for incubating seeds .

9. Petri dish for reaction vessel.

10. Aluminum foil or a suitable opaque wrap to cover reaction vessel during chromogen development.

11. Orbital or wrist-action shaker.

PROCEDURAL NOTES/QUALITY CONTROL

1. Keep kit components refrigerated when not in use. Warm reagents to room temperature prior to use.

2. Always handle the nitrocellulose membrane with extreme care. Nitrocellulose is fragile and will rip or tear easily.

3. DO NOT touch the nitrocellulose membrane with bare skin. Always wear latex gloves to prevent contamination by fingerprints.

4. Label all vessels containing solutions to avoid improper mixing.

5. Avoid ergot sclerotia at all times. Remove the sclerotia from seed prior to surface sterilization. *Claviceps purpurea* has a soluble protein similar to that in *Neotyphodium* and can give false positives if seed is contaminated. Surface sterilization and incubation temperature are critical to avoiding false positives from *Claviceps* **mycelia** found in seed. Be sure to follow procedures as stated to avoid false positive readings.

6. Use a rotary shaker set at 50 rpm to keep reagents agitated

when reacting with the membrane.

7. *Neotyphodium* protein has been "spotted" inside the black box marked "+" on the membrane. A positive reaction means the test was properly conducted.

8. Cover or cap all reagents when not in use.

9. Do not mix or use reagents from different kits.

10. Check color reaction (final step) every 5 minutes to prevent overexposure. Stop the reaction when the positive controls (or seed) have a dark color.

11. The chromogen is light sensitive. Be sure and cover the reaction vessels with aluminum foil or a suitable opaque wrap to prevent light from entering the reaction and causing chromogen coloration.

REAGENT PREPARATION AND STORAGE

1. Extraction solution should be diluted to 200 mL with **distilled** water only (not spring water). If you have no access to distilled water in your laboratory, gallon containers of distilled water can be purchased at your local supermarket.

2. Dissolve the BWW reagents in 150 mL **distilled** water using a clean low density polyethylene plastic or glass container. The solution should be made immediately prior to assaying samples but can be stored if refrigerated at 4°C. Discard BWW solution after 3 days.

3. Dilute MAB, RAM, and PA with 10 mL of BWW solution just prior to each step using the respective reagents.

4. DO NOT dilute the chromogen reagent. Use it at full strength. Keep chromogen reagent **OUT OF LIGHT** during storage and use.

ASSAY PROCEDURE

Be sure to read this entire booklet prior to carrying out the assay.

Allow reagents to reach room temperature prior to use. Gently mix all reagents before using.

1. Remove ergot sclerotia from seed.

2. Surface sterilize seed by placing approximately one teaspoon of seed into a 50 mL beaker. Add 40 mL of 5% sodium hydroxide to the seed and stir thoroughly, making sure all seed are wet. Leave seed in the sodium hydroxide solution for

60 minutes, stirring every 15 minutes. Rinse seed with copious amounts of water to remove residual sodium hydroxide from the seed. Drain seed of excess water. Seed can be used damp or permitted to air dry and used dry. Do the same with the E+ and E- control seeds.

3. Place a 3.5 x 5.0 x 0.75 inch **cellulose** sponge on the bottom of a 4.0 x 6.0 inch plastic container. Add EB solution to the container to wet (not cover) the sponge. Place one piece of the blotting paper on top of the sponge. Using forceps or tweezers, place the nitrocellulose membrane on top of the blotting paper. Make sure no air bubbles are between the membrane and blotting paper. Use caution not to touch the membrane with a naked hand. Use latex gloves. Place seed close together on the membrane. The membrane is sufficiently large enough to handle 100 seed. Place 5 E+ and E- seeds at the bottom of the blot for comparison purposes and aid in blot interpretation. Place cover on the container and put seed into a 45°C incubator overnight.

5. Remove plastic container from incubator. Peel the nitrocellulose membrane from the blotting paper and place it onto a dry piece of blotting paper. Gently remove the seeds or excess debris from the nitrocellulose membrane with a lab brush.

6. Dry the membrane in a lab oven at 70°C for 15 minutes or at room temperature for an hour.

7. Place the membrane into the bottom of the petri dish. Add 10 mL of BWW solution, place cover on dish, and place on orbital shaker for 30 min.

8. Remove petri dish from shaker and gently pour off the solution.

9. Add 10 mL of BWW solution to the MAB tube and mix by shaking. Pour MAB solution over the membrane in the petri dish. Place onto shaker for 1 hour.

10. Remove petri dish from shaker and gently pour off the antibody solution.

11. Add 10 mL of BWW solution and return petri dish to the shaker for 6 minutes. Pour off BWW solution. Repeat this step one more time.

12. Add 10 mL BWW solution to the RAM tube and mix by shaking. Pour RAM solution over the membrane in the petri dish. Place onto shaker for 1 hour.

13. Remove petri dish from shaker and gently pour off the exactly

antibody solution. Add 10 mL of BWW solution and return petri dish to the shaker for 6 minutes. Pour off BWW solution. Repeat this step one more time.

14. Add BWW solution to the PA tube to reach the 10 mL mark. Mix by shaking. Pour PA solution over the membrane and cover the petri dish. Place onto shaker for 30 min.

15. Remove petri dish from shaker and pour off solution. Add 10 mL of BWW solution and return petri dish to the shaker for 6 minutes. Pour off BWW solution. Repeat this step one more time.

16. Add BN chromogen, replace lid on petri dish, return dish to the shaker, and cover with aluminum foil. After color has developed, pour of chromogen solution and stop the reaction by rinsing the membrane in 20 mL distilled water twice. Score membrane for positive *Neotyphodium* seed (see "Limitations to Procedure).

LIMITATIONS TO PROCEDURE

The test is somewhat subjective. Any location expressing even partial dark color development where the seed contacted the membrane is positive for *Neotyphodium.* Seed that do not develop more than a light gray color are negative for *Neotyphodium.*

PERFORMANCE CHARACTERISTICS

SENSITIVITY

The theoretical minimum detectable amount of endophyte of *Neotyphodium* in the seed is 50 ng/seed. This was determined by adding serially diluted extracts from cultures of *Neotyphodium* directly to the membrane and viewing the color reaction.

PRECISION AND ACCURACY

Seed samples with varying percentages of *Neotyphodium* infection were tested for infection in multiple assays to determine precision and accuracy of the assays. Immunoblots were scored by two independent technicians to determine variability due to seed sampling vs. variability due to individual scoring the sample.

	Lot 1	Lot 2	Lot 3	Lot 4
Mean infection rate	2.2	29.2	72.3	95.3
SD sampling error	2.4	2.3	3.0	3.0
SD technician error	0.0	2.6	3.0	3.0

SD = Standard Deviation

Notes

Warranty

This Agrinostics Phytoscreen seed test is guaranteed to provide accurate assessments of *Neotyphodium* spp. (formerly *Acremonium* and commonly called "endophyte") infection rates in seed when the enclosed protocol is followed. If you are dissatisfied with the results of your test, Agrinostics will, at its option, provide test kits to replace faulty kits or reimburse the price of the kit. Inasmuch as endophyte dies at a differential rate as seed, and the rate of endophyte death is dependent upon storage temperature and humidity as well as age of the seed, this seed test may not be indicative of the infection rate of plants in fields established from improperly stored seed. Therefore, Agrinostics Ltd. Co. makes no claim as to the accuracy of the test beyond the presence of endophyte (viable or not) in the seed. Growing seedling plants from seed lots and screening for *Neotyphodium* using Agrinostics Phytoscreen test kit # ENDO7972 will provide accurate assessment of viable endophyte.