Sampling Procedures for Determining Endophyte Content in Tall Fescue Seed Lots by ELISA

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ABSTRACT

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A two-stage sampling procedure was developed to test for endophyte content in tall fescue seed lots by means of enzyme-linked immunosorbent assay (ELISA). Of ten tall fescue seed lots assayed, four were rejected as unacceptable according to the two-stage sampling plan that was used. Antigens of the fungus were easily detected in samples consisting of only one endophyte-infected seed extracted along with as many as 19 endophyte-free seeds. Seed containing nonviable fungus also reacted positive in our ELISA system.

Additional key words: fescue toxicosis.

Tall fescue (*Festuca arundinacea* Schreber) has often been associated with poor cattle performance under certain pasture conditions, despite evidence that the grass is high in nutritional qualities (3,8,12,18). Recently, several lines of investigation have shown that tall fescue in pastures on which animals develop fescue toxicosis is infected with an endophytic fungus (1,6,16).

Investigators have considered the endophyte of tall fescue to be the same fungus, *Epichloë typhina* (Pers.) Tul., as that reported on many species of grasses (4,17). On hosts such as orchardgrass and bentgrass, the fungus develops into a dense stromatic sheath, completely enveloping the young inflorescence and causing it to degenerate (2,7). On tall fescue, however, *E. typhina* is apparently completely internal and cannot be observed by gross examination of the plant exterior (6).

There is some controversy concerning the nomenclature of the endophyte of tall fescue. Even though the sexual stage of the fungus has never been reported on tall fescue, it has often been referred to by the perfect state name, *Epichloë typhina*, in the literature (1,11). The imperfect state was named *Sphacelia typhina* Sacc. by Saccardo (13,14). However, in a recent report, Morgan-Jones and Gams (9) rejected *Sphacelia typhina* as a valid name for the imperfect state of *E. typhina*, and placed it and the endophyte of tall fescue under the genus *Acremonium*. Furthermore, they considered the imperfect state of *E. typhina* and the endophyte of tall fescue to be different species, ie, *Acremonium typhinum* Morgan-Jones & W. Gams and *Acremonium coenophialum* Morgan-Jones & W. Gams, respectively, but placed them together in a new section of *Acremonium*, Sect. *Albo-lanosa* Morgan-Jones & W. Gams (9).

Regardless of its taxonomic status, transmission of the endophyte of tall fescue appears to be limited to seed produced on infected plants (1,15). Other forms of transmission that may be important in nature have yet to be proved. Because infected seed is the primary if not the only means of spread, relatively clean pastures may be established by planting seed with little or no endophyte content. A seed certification program that identifies such seed sources would be desirable.

The development of an enzyme-linked immunosorbent assay (ELISA) for detecting antigens of the endophyte in tall fescue tissue samples (5) makes possible the determination of endophyte content in tall fescue seed lots. This paper describes the use of the ELISA technique related to seed sampling procedures for endophyte assay of tall fescue seed lots.

MATERIALS AND METHODS

ELISA. The enzyme-linked immunosorbent assay (ELISA) procedure as described by Johnson et al (5) was followed with only slight modifications. ELISA plates (Catalogue no. 76-381-04; Flow Laboratories, Inc., McLean, VA 22102) were coated with partially purified immunoglobulin (Ig) at a concentration of 0.5 μg per well. All samples for ELISA testing were ground in a mortar and pestle with PBS containing 0.05% Tween-20 and 2% polyvinylpyrrolidone, MW 40,000 (PBS-Tween-PVP). Each test sample was placed in two adjacent wells of an ELISA plate. Coating Ig, enzyme-conjugated Ig, and substrate were added to every well on the plate. However, plant test samples were not placed in columns one, two, and 12, but rather PBS-Tween-PVP was added to these wells. The Titertek® Multiskan photometer (Flow Laboratories) was blanked against column two of the ELISA plate when measuring absorbances.

Age of seed. After long-term storage, endophyte-infected tall fescue seed is known to give rise to plants that are free of the endophyte (11). This appears to be due to the loss of viability of the fungus in the seed with time. An experiment was conducted to determine if seed containing nonviable fungus still reacted in our ELISA system or whether the antigen disappeared concurrently with the loss of viability of the fungus. A seed lot stored at 10 C and 50% RH for 6 yr since harvest and another stored 4 mo since harvest were tested with ELISA on an individual seed basis. Both lots were obtained from plants diagnosed as infected with *E. typhina* by microscopic examination of pith scrapings (10). The single seed samples were ground in a mortar and pestle with 0.6 ml of PBS-Tween-PVP for assay. Seed from each of these two lots and from a certified tall fescue seed lot very low in endophyte content

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was sown in 7.5-cm-diameter plastic pots in the greenhouse. The resultant seedlings were assayed after 8–10 wk for the presence of the endophyte using ELISA. Stem sections approximately 2 cm long were collected from just above the soil line and dried in an oven at 60 C. A test sample consisted of two 2-cm lengths of stem from different tillers extracted in a mortar and pestle with 4.0 ml of PBS-Tween-PVP. The addition of 0.5 g of washed, sterilized sand facilitated the extraction of stem samples.

Determination of sensitivity. Determination of endophyte content in seed lots at low levels (ie, 5%) by ELISA testing of individual seeds is impractical because of the large number of single seed samples required. By increasing the number of seeds per sample, the total number of samples can be decreased if the assay is sufficiently sensitive to detect samples that contain at least one infected seed. Several experiments were designed to determine the sensitivity of the assay in detecting endophyte-infected seed in the presence of endophyte-free seed.

Sources of essentially endophyte-free seed (≤0.81%) and 100% endophyte-infected seed were identified. Tests were conducted using various ratios of infected:uninfected seed. A sample contained either 10 or 20 seeds depending on the experiment. Seeds were ground in mortar and pestle both at 10 seeds per 1.0 ml PBS-Tween-PVP and at 10 seeds per 2.0 ml to compare different concentrations of test sample.

Statistical information. Sampling procedures were devised for maximum efficiency and for incorporation into a certification program to test for endophyte content in tall fescue seed lots. Methods were desired that would have a high probability of rejecting lots with an endophyte content >5%. However, it was essential that any certification scheme also protect the seed producer and ensure that seed with an actual endophyte content of 5% or less would have a small probability of being rejected by the certifier. A two-stage sampling procedure was developed.

An additional sampling procedure was desired which would be a much shortened version of the above sampling procedure and which could be used for experimental and research purposes in identifying lots high in endophyte content. It was hoped that such a method would be helpful when screening large numbers of seed lots. A short or limited sampling procedure was developed.

ELISA testing of seed lots. Ten seed lots of cultivar Kenhy tall fescue, representing nine different fields of nine different growers from Oregon, were tested with ELISA for endophyte content. All 10 lots were tested initially with the short version of the sampling procedure. Those lots that the results indicated were high in endophyte content were also tested on an individual seed basis. Finally, each of the 10 lots was tested by using the two-stage sampling procedure.

RESULTS

Seed age. Antigens of *E. typhina* were readily detected in individual tall fescue seeds from a 6-yr-old seed lot that only gave rise to endophyte-free seedlings (Table 1). Seed from the endophyte-infected lot, harvested 4 mo previously, gave rise to 26 infected seedlings of 30 seedlings tested (Table 1). Thus, the ELISA system failed to distinguish between seed that results in endophyte-infected seedlings and seed containing antigen(s) but nonviable fungus and hence that results in endophyte-free seedlings.

Sensitivity. An individual endophyte-infected seed could be detected in the presence of 19 endophyte-free seeds when extracted together as a single sample (Table 2). Samples containing a higher proportion of infected seeds usually elicited greater absorbance values than those with lower proportions of infected seeds (Table 2).

Results of additional experiments indicated that greater absorbances were obtained when samples were ground at 10 seeds per 1.0 ml PBS-Tween-PVP compared to 10 seeds per 2.0 ml PBS-Tween-PVP (Table 3). Furthermore, as expected, individual infected seeds were more easily detected (elicited higher absorbances) in samples consisting of 10 seeds than in samples consisting of 20 seeds (Table 4).

Two-stage sampling procedure. The two-stage sampling procedure may be described as follows: the first stage of the procedure consists of examining n samples of k seeds each and declaring a given sample as positive if one or more endophyte-infected seeds are present in that sample (ie, a positive ELISA reading is obtained). A seed lot is accepted if the number of

TABLE 1. Relationship of seed age and enzyme-linked immunosorbent assay (ELISA) for tall fescue seeds and seedlings

		ELISA test result[a]	
Age of seed	Fescue type	Seed	Seedlings
6 yr	G1-307[b]	44/45[c]	0/30
4 mo	1-805[b]	55/55	26/30
4 mo	Kenhy	0/30	0/30

[a] Seeds and seedlings tested on an individual basis.
[b] Seed obtained from plants previously diagnosed as infected as determined by microscopic examination of pith tissue.
[c] Number of positive samples per number tested.

TABLE 2. Enzyme-linked immunosorbent assay (ELISA) for 30 tall fescue seed samples each consisting of 20 seeds[a] and representing various ratios of endophyte-infected:endophyte-free seed

Absorbance at 405 nm[b]					
0:20[c]	1:19	2:18	5:15	10:10	20:0
0.0[d]	0.155	0.221	0.463	0.592	1.252
0.010	0.154	0.276	0.394	0.539	0.910
0.0	0.053	0.218	0.432	0.433	0.937
0.015	0.208	0.139	0.329	0.565	0.907
0.0	0.121	0.206	0.371	0.605	0.951

[a] Each sample was ground in a mortar and pestle in 2.0 ml PBS-Tween-PVP.
[b] All absorbance values were from the same ELISA plate and were means of two adjacent wells.
[c] Ratio of endophyte-infected:endophyte-free seed in sample.
[d] Zero indicates reading less than that of control.

TABLE 3. Enzyme-linked immunosorbent assay (ELISA) for tall fescue seed samples each consisting of one endophyte-infected (EI) seed plus nine endophyte-free (EF) seeds ground in 1.0 ml or 2.0 ml of buffer

		ELISA test result[ab]	
Sample	Volume (ml)	$A_{405\ nm}$	Range
1EI + 9 EF	1.0	0.269	(0.197–0.439)
1EI + 9 EF	2.0	0.185	(0.106–0.268)
Controls			
10 EF	1.0	0.025	
10 EF	2.0	0.025	

[a] Absorbance values were means of two adjacent wells. Numbers in parentheses show range of values for six determinations.
[b] All absorbance values were from the same ELISA plate. Samples were randomized on the plate.

TABLE 4. The detection of antigens of the endophyte in samples consisting of only one endophyte-infected (EI) tall fescue seed extracted together with nine or 19 endophyte-free (EF) seeds by enzyme-linked immunosorbent assay (ELISA)[a]

	ELISA test result[bc]	
Sample	$A_{405\ nm}$	Range
1EI + 9 EF	0.155	(0.104–0.244)
1EI + 19 EF	0.115	(0.070–0.201)
Controls		
10 EF	0.008	
20 EF	0.000	

[a] Samples extracted at the rate of 10 seeds per 1.0 ml of PBS-Tween-PVP.
[b] Absorbance values were means of two adjacent wells. Numbers in parentheses show range of values for 10 determinations.
[c] All absorbance values were from the same ELISA plate. Samples were randomized on the plate.

observed positive samples in the first stage is less than or equal to r_1 while the seed lot is rejected if the number of observed positive samples is greater than or equal to $r_2 > r_1$. If the number of positive samples in the first stage is between r_1 and r_2, then another set of n samples of k seeds each is examined in the second stage of sampling. In that case the seed lot is accepted if the total number of positive samples for the two stages combined is less than or equal to s.

Given that the portion of endophyte-infected seed in a seed lot is p ($100p\%$), it follows that the probability, Θ, of observing one or more endophyte-infected seeds in a sample of k seeds is

$$\Theta = 1-(1-p)^k.$$

This quantity can be used to compute the probability that the two-stage sampling procedure will accept a seed lot with $100p\%$ endophyte-infected seeds. This probability is

$$\alpha = \sum_{x=0}^{r_1} \binom{n}{x} \Theta^x (1-\Theta)^{n-x} + \sum_{x=r_1+1}^{m} \binom{n}{x} \Theta^x (1-\Theta)^{n-x} \sum_{y=0}^{s-x} \binom{n}{y} \Theta^y (1-\Theta)^{n-y},$$

where m is the smaller of s and r_2-1. Note that the first term on the right side of the definition of α represents the probability of accepting the lot in the first stage of sampling, while the second term represents the probability of accepting the lot in the second stage of sampling. Also, notice that in this second term the value of y, the number of positive samples in the second stage of sampling is constrained by the value of x, the number of positive samples in the first stage.

TABLE 5. Characteristics of some desirable two-stage sampling plans having high probability (α_A) of accepting tall fescue seed lots with 5% endophyte content ($P_A = 0.05$) and low probability (α_U) of accepting lots with 10% endophyte content ($P_U = 0.10$)

n[a]	k[b]	r_1[c]	r_2[d]	r[e]	s[f]	α_A	α_U
30	10	11	21	12–20	31	0.9737	0.0220
30	20	16	26	17–25	46	0.9831	0.0110

[a] Number of samples examined per stage.
[b] Number of seeds per sample.
[c] Maximum number of positive samples in the first stage that allows for immediate acceptance of a lot.
[d] Minimum number of positive samples in the first stage that allows for immediate rejection of a lot.
[e] Number of positive samples obtained from the first stage that indicates a second stage of testing is required.
[f] Maximum number of positive samples from the two stages combined that allows for acceptance of a lot.

TABLE 6. Number of positive samples found in 10 tall fescue seed lots tested by enzyme-linked immunosorbent assay (ELISA) using various sampling plans

	Sampling plans			
Lot	$n = 5$[a] $k = 10$	$n = 30$[b] $k = 1$	$n = 60$[b] $k = 1$	$n = 30$[c] $k = 10$
A	0			4
B	1			4
C	0			0
D	0			0
E	5	12 (40%)		30
F	5	12 (40%)		30
G	5	11 (37%)		30
H	0			0
I	5		7 (12%)	26
J	0			2

[a] Limited sampling procedure. ELISA testing of $n = 5$ samples and $k = 10$ seeds per sample from each seed lot.
[b] ELISA testing of $n = 30$ or $n = 60$ single ($k = 1$) seed samples. Numbers in parentheses indicate percent infected seed.
[c] First stage of two-stage sampling procedure. ELISA testing of $n = 30$ samples and $k = 10$ seeds per sample from each seed lot.

A computer program was written to evaluate α for given choices of n and k and two choices of p, one that typifies acceptable lots and the other which typifies unacceptable lots. The program examined the properties of all two-stage sampling plans defined by varying r_1, r_2, and s and listed those that are the most reasonable.

We desired sampling plans for $n = 30$, and $k = 10$ or 20, with a high probability α_A of accepting lots with endophyte contents of $p_A = 0.05$ and a low probability α_U of accepting lots with endophyte contents of $p_U = 0.10$, (total error ≤ 0.05). In addition, we wanted the value of $r_2 - r_1$ to be as small a number as possible to reduce the number of times the second stage of sampling would be required. The two-stage sampling plans that best fit these criteria are shown in Table 5.

Limited sampling procedure. The ELISA testing of only $n = 5$ samples and $k = 10$ seeds per sample from a given seed lot provided a means of identifying seed lots that contain more than 5% endophyte-infected seed if all five samples were found to be positive (one or more endophyte-infected seeds in each sample). Again, this was derived from the probability Θ of observing one or more endophyte-infected seeds in one sample of $k = 10$ seeds

$$\Theta = 1-(1-p)^{10}$$

The probability of finding all five samples positive for any given p is this expression raised to the fifth power or

$$[1-(1-p)^{10}]^5.$$

Thus, it was determined that the probability of finding all five samples positive if the actual endophyte content is 5%, ($p = 0.05$), would be 1.04%. In contrast, the probability of finding all five samples positive if the actual endophyte content is 40%, ($p = 0.4$), would be 97.01%.

Assay of seed lots. The two-stage sampling procedure of $n = 30$, $k = 10$ (Table 5) was used. Of the 10 seed lots tested, four (E, F, G, and I) were rejected as containing more than 5% endophyte-infected seed (Table 6). The second stage of sampling was not required for any of the lots tested because the number of positive samples after the first stage was either less than r_1 or greater than r_2. The limited sampling procedure of $n = 5$, $k = 10$ was effective in identifying the seed lots with higher proportions of endophyte-infected seed (Table 6). Information from the Oregon State University Seed Laboratory indicated that seed lots F and G were from the same grower's field.

The ELISA testing of seed lots A, C, D, H, and J by the limited sampling procedure ($n = 5$, $k = 10$) resulted in no positive samples being observed (Table 6). Furthermore, these five lots were all determined to be in the acceptable category following the first stage of the two-stage sampling procedure (Table 6). If the actual endophyte content of a given seed lot is 10% ($p = 0.10$), then the probability of no endophyte-infected seed being observed by the limited sampling procedure is calculated to be:

$$(1-p)^{nk} = (1-0.1)^{5 \cdot 10} = (0.9)^{50} = 0.0051538$$

or 0.5%. Thus, lots assayed by the limited sampling procedure in which none of the five samples is declared positive can be stated with at least 99.5% confidence to contain not more than 10% endophyte-infected seed.

DISCUSSION

If evidence continues to indicate a causal relationship between the endophyte of tall fescue and fescue toxicosis, cattle producers may find it advantageous to renovate infested pastures with seed that will produce a low percentage of infected plants. In theory, there are two methods that could be used in obtaining such seed. One is manipulation of endophyte-infected seed, whether by chemical treatments, physical treatments (temperature), or long-term storage, to kill the endophyte. It may be difficult to accomplish this and still retain acceptable levels of seed

germination and seedling vigor. The other method relies on initially determining that a given seed source is relatively free of the endophyte.

The two-stage sampling procedure was effective in identifying seed lots which contain low percentages of endophyte-infected seed. Incorporation of the two-stage sampling procedure into a seed certification program would represent the testing by ELISA of a total of 300–1,200 tall fescue seeds per lot, depending on the size of k and the need for a second stage. The tagging of acceptable lots would inform a buyer that tagged seed has a very high probability of being low in endophyte content.

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