Detection of *Epichloë typhina* in Tall Fescue by Means of Enzyme-Linked Immunosorbent Assay

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Kentucky Agricultural Experiment Station Journal Series Paper 81-11-124.
We wish to thank S. D. Savage and M. A. Sall for making details of their procedures available prior to publication, R. C. Buckner and P. B. Burrus for providing fescue samples, and R. A. Chapman for valuable suggestions.
Accepted for publication 2 September 1981.

ABSTRACT

Johnson, M. C., Pirone, T. P., Siegel, M. R., and Varney, D. R. 1982. Detection of *Epichloë typhina* in tall fescue by means of enzyme-linked immunosorbent assay. Phytopathology 72:647-650.

An antiserum, prepared to homogenates of washed *Epichloë typhina* mycelium grown in a liquid medium, was used in an enzyme-linked immunosorbent assay (ELISA) to detect antigens of the fungus in tall fescue (*Festuca arundinacea*) tissue samples. With ELISA we could detect as little as 100 ng of freeze-dried *E. typhina* mycelium per milliliter, and could detect *E. typhina* in individual seeds of tall fescue. Of 14 fungal genera tested, including *Acremonium*, *Claviceps*, *Helminthosporium*, *Pythium*, *Rhizoctonia*, and *Sclerotium*, all showed reactivities less than 0.1% that of *E. typhina*.

Additional key words: fescue toxicity syndrome.

Epichloë typhina, a clavicipitaceous internal colonizer of tall fescue (*Festuca arundinacea* Schreber) is thought to be involved in the fescue toxicity syndrome of cattle (1). Because the infected fescue plants exhibit no external symptoms, detection of the fungus in tall fescue has been dependent on microscopic examination of culm pith tissue (1). This procedure requires sampling, staining, and examining stem sections of fertile culms from several tillers per plant during late spring and early summer in order to determine the presence of the fungus in an individual plant. A more rapid and sensitive technique was necessary that could easily handle large numbers of samples.

The enzyme-linked immunosorbent assay (ELISA) and radioimmunosorbent assay (RISA) techniques have proved valuable in the detection of plant viruses (2,5,8). Recently the use of immunosorbent assay has been expanded to detect fungal plant pathogens (3,11) and a fungal product (9) in host plant tissues.

This paper describes the application of the ELISA technique for detecting antigens of *E. typhina*.

MATERIALS AND METHODS

Antigen production. The isolate of *E. typhina* used in this study was obtained in 1980 from culm pith scrapings of a tall fescue plant in which the contorted mycelium typical of *E. typhina* had been observed. The culture was maintained on a cornmeal agar (CMA)

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0031-949X/82/06064704/$03.00/0

medium containing: cornmeal agar (Difco), 17.0 g; soytone (Difco), 0.2 g; dextrose, 0.5 g; and distilled water, 1,000 ml.

Mycelium for antigen production was grown in the liquid medium, M 43, used by Bacon et al (1). Cultures were incubated in 50 ml of medium for 3 wk on a gyratory shaker (100 rpm, 2.5-cm circular orbit) at room temperature (23 C) in 300-ml flasks. This was followed by another 3-wk incubation at room temperature during which the flasks remained stationary on the laboratory bench.

The entire contents of the flasks of 6-wk-old cultures were filtered on a Büchner funnel (Whatman #541 filter paper). The residue was washed several times with 0.02 M potassium phosphate buffered saline, pH 7.4 (PBS), removed from the filter paper and ground with a mortar and pestle. This preparation was then subjected to two centrifugations, each in 30 ml of PBS at 10,000 *g* for 10 min; the pellets were retained each time. The final pellet was suspended in a volume of PBS equal to three times the weight of the pellet and frozen in 1.0-ml portions in glass tubes.

Antiserum production. An antiserum to this antigen suspension was produced by three subcutaneous injections of a rabbit at 1-wk intervals. The injections consisted of 1 ml of the above antigen suspension emulsified with 1 ml of adjuvant; Freund's complete adjuvant was used as the emulsifier for the first injection, while Freund's incomplete adjuvant was used for the booster injections.

TABLE 1. Enzyme-linked immunosorbent assay (ELISA) for a dilution series of mycelium of two *Epichloë typhina* isolates

	Absorbance at 405 nm[a,b]	
	Epichloë typhina isolate[c] from:	
Mycelial dry weight	Tall fescue	Bent grass
10 mg/ml	1.404	1.019
1 mg/ml	1.171	0.815
100 μg/ml	0.838	0.422
10 μg/ml	0.319	0.176
1 μg/ml	0.286	0.037
100 ng/ml	0.023	0.0[d]
10 ng/ml	0.013	0.0
PBS control	0.007	...

[a] Absorbance values were means of two adjacent wells.
[b] Absorbance values for both isolates were from the same ELISA plate.
[c] Isolates grown in liquid media, freeze-dried, ground in a mortar and pestle, and dilutions made in PBS-Tween-PVP.
[d] Zero indicates reading less than that of control.

TABLE 2. Enzyme-linked immunosorbent assay (ELISA) for mycelial preparations of various fungi[a]

	Absorbance at 405 nm[b] of a mycelial dilution	
Species	1:10	1:1,000
Epichloë typhina (Bent grass)	1.182	0.289
Thelephora terrestris	0.069	0.030
Rhizoctonia solani	0.026	0.0
Pythium aphanidermatum	0.0[c]	0.0
Pythium periplocum	0.0	0.0
Acremonium strictum	0.0	0.0
Gibberella zeae	0.0	0.0
Helminthosporium victoriae	0.0	0.0
Aphanomyces euteiches	0.0	0.0
Sclerotium rolfsii	0.0	0.0
Achlya americana	0.0	0.0
Neocosmospora sp.	0.0	0.0
Endothia parasitica	0.0	0.0
Phytophthora parasitica var. *nicotianae*	0.0	0.0
Pisolithus tinctorius	0.0	0.0
PBS control	0.005	0.0

[a] Mycelium of each species tested was scraped off agar slants, weighed, ground in a mortar and pestle, and appropriate dilutions made in PBS-Tween-PVP.
[b] Absorbance values were means of two adjacent wells.
[c] Zero indicates reading less than that of control.

The rabbit was bled 1 wk after the second booster injection. Reactivity of the serum to the antigen was tested initially using SDS-agar gel diffusion plates (6) and was characterized by the formation of a sharp precipitin band. The antiserum was stored frozen in 2.0-ml portions in glass tubes.

Ig preparation. Partial purification of immunoglobulin (Ig) involved diluting 2.0 ml of the antiserum to 20.0 ml with distilled water and adding 20.0 ml of saturated ammonium sulfate solution to precipitate the Ig. The Ig was collected by centrifugation and the resulting pellet was resuspended in 4.0 ml of half-strength PBS. After thorough dialysis against half-strength PBS, the Ig was eluted through DEAE cellulose (Whatman DE22), the unadsorbed fractions were collected and absorbance at 278 nm was determined. The Ig fractions were adjusted to approximately 1 mg/ml (E_{278} = 1.4) and stored frozen in 1.0-ml portions in glass tubes.

Enzyme-conjugated Ig was prepared by dissolving 2.5 mg of the enzyme, alkaline phosphatase (Type VII-S, Sigma), in 1.0 ml of the Ig preparation and dialyzing it extensively against PBS at 6 C. Glutaraldehyde was then added to a final concentration of 0.05% and this mixture was incubated at 23 C for 4 hr. The glutaraldehyde was removed by dialysis against several changes of PBS and the conjugate was stored at 6 C with bovine serum albumin added to a concentration of 1%.

ELISA. The enzyme-linked immunosorbent assay (ELISA) procedure as described by Clark and Adams (4) was followed. Antigen, prepared in the same manner as that used to produce the antiserum, was freeze-dried and used as standards in ELISA tests so that the sensitivity of the technique could be determined. In addition, a mycelial preparation of *Epichloë typhina* 9091, an isolate from bentgrass (*Agrostis perennans*) obtained from C. W. Bacon, was also freeze-dried and tested by using ELISA. Fourteen different fungal genera (supplied by J. W. Hendrix) were tested to establish the specificity of the assay.

All samples for ELISA testing were ground in a mortar and pestle with PBS containing 0.05% Tween-20 and 2% polyvinylpyrrolidone, MW 44,000 (PBS-Tween-PVP). Each test sample was placed in two adjacent wells of an ELISA plate (No. 1-223-29, Dynatech Laboratories, Inc., Alexandria, VA 22314), which previously had been coated with partially purified Ig at a concentration of 1 μg per well. Following incubation of the plate overnight at 6 C, 200 μl of enzyme-conjugated Ig (diluted 1:400 in PBS-Tween-PVP) were added to each well on the ELISA plate. The above concentrations of coating Ig and enzyme-conjugated Ig were found to be optimum. Approximately 40 min after the addition of the substrate solution, reactions were arrested with 3M NaOH. The absorbances at 405 nm were measured by using a Titertek® Multiskan photometer (Flow Laboratories, McLean, VA 22102).

Procedures for tall fescue samples. Eight individual tall fescue plants were collected in December 1980 from the lathhouse, potted in sterilized soil in 10.2-cm-diameter clay pots, and placed in the greenhouse at 25–28 C. These plants had been diagnosed as either healthy or infected with *E. typhina* by microscopic examination of their culm pith tissue the previous summer. (Pith tissue of plants diagnosed as healthy, from a minimum of three tillers per plant and from at least two internodes per tiller, was examined. Pith sections were cleared and stained using the ABLACID method [10].) After 5 wk in the greenhouse, the plants were tested by ELISA. A test sample consisted of two 2-cm lengths of stem from different tillers extracted in a mortar and pestle with 5.0 ml of PBS-Tween-PVP. Approximately 0.5 g of washed, sterilized sand was added to each sample to aid in extraction.

Mature seeds were collected in early summer 1980 from tall fescue plants diagnosed as either containing or not containing *E. typhina* by microscopic examination. Single seed samples were ground in a mortar and pestle with 0.5 ml of PBS-Tween-PVP and assayed on ELISA plates.

RESULTS

Sensitivity. By using ELISA of freeze-dried preparations of the homologous antigen (*E. typhina*, tall fescue isolate) we detected the fungus at concentrations as low as 100 ng/ml (Table 1). Freeze-

TABLE 3. Enzyme-linked immunosorbent assay (ELISA) for mycelia dry weight preparations of various fungi[a]

Mycelial dry weight	Absorbance at 405 nm[b,c]					
	E. typhina (fescue)	*Thelephora terrestris*	*Rhizoctonia solani*	*Pythium aphanidermatum*	*Acremonium strictum*	*Giberella zeae*
1 mg/ml	1.357	0.156	0.277	0.0	0.0	0.0
100 μg/ml	0.839	0.104	0.0[d]	0.0	0.0	...[e]
10 μg/ml	0.446	0.043	0.0	0.0	0.0	0.0
1 μg/ml	0.397	...	...	...	...	...
100 ng/ml	0.045	...	...	...	...	...
PBS control	0.010					

[a] Fungi grown in liquid media, freeze-dried, ground in a mortar and pestle, and dilutions made in PBS-Tween-PVP.
[b] Absorbance values were means of two adjacent wells.
[c] All absorbance values were from the same ELISA plate.
[d] Zero indicates reading less than that of control.
[e] ... = Not tested.

TABLE 4. Relationship of infection (based on microscopic examination) and enzyme-linked immunosorbent assay (ELISA) for *Epichloë typhina* in eight tall fescue plants

Fungus present by microscopic examination	ELISA test result	
	Visual	$A_{405\ nm}$[a]
−	−	0.017
+	+	0.432
−	−	0.026
+	+	0.513
−	−	0.023
+	+	0.206
−	−	0.0[b]
+	+	0.346
PBS control	−	0.010

[a] Absorbance values were means of two adjacent wells.
[b] Zero indicates reading less than that of control.

TABLE 5. Enzyme-linked immunosorbent assay (ELISA) for 30 individual tall fescue seeds

Seed no.	Absorbance at 405 nm[a] seed lot[b,c]		
	1	2	3
1	0.0[d]	0.0	0.283
2	0.008	0.382	0.654
3	0.010	0.244	0.322
4	0.0	0.416	0.312
5	0.0	0.554	0.320
6	0.0	0.196	0.580
7	0.0	0.0	0.474
8	0.0	0.0	0.498
9	0.0	0.267	0.349
10	0.0	0.307	0.385
PBS control		0.007	

[a] Absorbance values were means of two adjacent wells.
[b] Seed lots were pooled collections from two or more plants.
[c] Seed lot 1 was collected from plants that were free of *E. typhina*, while seed lots 2 and 3 were collected from plants that were all infected with *E. typhina* as determined by microscopic examination of culm pith tissue.
[d] Zero indicates reading less than that of control.

dried preparations of the bentgrass isolate, however, were less reactive, the limits of detection being about 1 μg/ml (Table 1). In two additional experiments (*unpublished*), the fescue isolate again appeared to be approximately 10 times more reactive than the bentgrass isolate in ELISA tests.

Specificity. Of 14 different genera tested, excluding *Epichloë*, only three (*Thelephora*, *Rhizoctonia*, and *Claviceps*) reacted to any extent in our ELISA system (Tables 2 and 3). Freeze-dried preparations of *Thelephora* and *Rhizoctonia* at 1 mg/ml elicited absorbance values comparable to those elicited by *E. typhina* antigen at 1 μg/ml (Table 3). A high concentration (50 mg/ml) of sclerotia of *Claviceps* gave an absorbance value of 0.043, roughly equivalent to that of *E. typhina* at 100 ng/ml.

Detection of *E. typhina* in tall fescue samples. Stem extracts of the four plants shown to be infected by microscopic examination elicited strongly positive absorbance values in ELISA tests (Table 4). Stem extracts of the four plants considered to be healthy, based on microscopic examination, resulted in absorbance values close to background readings (PBS control) (Table 4). Antigens of *E. typhina* were readily detected in single tall fescue seeds collected from plants diagnosed as infected by microscopic examination of their culm pith tissue. ELISA tests indicated that in seed lots 2 and 3, 70 and 100% of the seeds, respectively, contained *E. typhina* (Table 5). Antigens of *E. typhina* were not present in seeds collected from plants diagnosed as not containing *E. typhina* (seed lot 1) as indicated by the low ELISA absorbance readings (Table 5).

DISCUSSION

The high (100 ng/ml) sensitivity and high degree of specificity of the ELISA procedure for *E. typhina* make it extremely useful in assaying tall fescue tissue samples. Savage and Sall (1) in their development of a radioimmunosorbent assay (RISA) for detection of the fungal plant pathogen, *Botrytis cinerea*, also obtained a sensitivity of 100 ng/ml. In our ELISA system the ease with which we detected *E. typhina* in stems and seeds of tall fescue testifies to the superiority of ELISA over microscopic examination of culm pith tissue in identifying infected plants. The greater reactivity of the fescue isolate in ELISA tests as compared to the bentgrass isolate is not surprising, since the antiserum was prepared against the fescue isolate and ELISA is known to enhance any antigenic differences between strains or isolates (7).

The ELISA technique has great potential to help answer a number of questions concerning *E. typhina* and its relationship to tall fescue. Most important is the role of *E. typhina* in alkaloid accumulation in the plant and fescue toxicity of cattle. Means of spread, existence of hosts other than tall fescue, and environmental effects on *E. typhina* also are being investigated. Studies on the effectiveness of heat and chemical treatments as control measures will be facilitated with the use of ELISA. Extensive surveys and seed certification programs should now be possible with the help of this assay.

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