Tissue Print-Immunoblot, an Accurate Method for the Detection of *Acremonium coenophialum* in Tall Fescue

K. D. Gwinn, M. H. Collins-Shepard, B. B. Reddick

Assistant professor, research associate, and associate professor, Department of Entomology and Plant Pathology, University of Tennessee, Knoxville 37901-1071.
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ABSTRACT

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Distribution of the endophytic fungus, *Acremonium coenophialum*, within tissues of infected tall fescue (*Festuca arundinacea*) can be determined with tissue print-immunoblot (TPIB); however, the accuracy of this method in the determination of endophyte infestation levels of pastures and seed lots has not been evaluated. The purpose of this study was to compare TPIB to other methods used for endophyte detection. Seed lots of known endophyte infestation levels were tested with TPIB. Percentage endophyte infestation did not differ significantly from the expected values. Also, no significant differences were found between results of protein A-sandwich ELISA and TPIB tests when both were used to estimate endophyte infestation levels in pastures. Therefore, accuracy of TPIB is comparable to other endophyte detection techniques and can be used for routine detection of endophyte in tall fescue tissues.

Additional keywords: endophyte, *Festuca arundinacea*

Fescue toxicosis in grazing animals is highly correlated with endophyte (*Acremonium coenophialum* Morgan-Jones & Gams) infestation levels in pastures of tall fescue (*Festuca arundinacea* Schreb.). Animals feeding on pastures with endophyte infestation levels of greater than 30% exhibit signs of fescue toxicosis (e.g., reduced reproductive capabilities, elevated body temperature, reduced lactation, and lowered average daily weight gain) (7,14). Since fescue toxicosis can result in high economic losses (an estimated $200 million annually in the United States) (13), accurate methods for the estimation of endophyte infestation levels of pastures and seed lots are essential.

The endophyte infestation level of pastures can be controlled at planting since the endophyte is primarily, if not exclusively, seed-disseminated (2). Endophyte infestation level of a seed lot can be determined by several methods. By microscopically examining stained squash mounts (5), estimates can be made of endophyte infestation levels in seed lots. This does not however, give any indication of the viability of the fungus. Since endophyte viability decreases rapidly with storage (16), estimates of viable endophyte are desired in most cases. One method for estimating viable endophyte levels is to place embryo explants on an auxin-supplemented tissue culture medium and examine the resulting calli for fungal hyphae (6). A more commonly used method, however, is to plant seeds and test tillers for endophyte with methods developed for pasture tiller analysis (1,12).

Estimates of endophyte infestation levels of existing pastures are frequently desired by researchers and producers. The most commonly used method is to examine stained epidermal peels of the tiller stem base (1); the hyphae of the endophyte are convoluted or serpentine and are considered diagnostic. Several antiserum-based assays have also been developed (8–10,12). Both double antibody sandwich (9,10) and protein-A sandwich enzyme-linked immunosorbent assay (PAS-ELISA) (12) methods are highly correlated with the microscopic method (9,12).

Tissue printing is the process of imprinting an image of a plant section onto a nitrocellulose membrane (NCM) (4,15). We have developed a tissue print-immunoblot (TPIB) technique for the detection of the endophyte proteins in tissue prints of tall fescue (8). The purpose of this study was to determine if the accuracy of TPIB was comparable to other methods for estimating endophyte infestation levels of pastures and seed lots.

MATERIALS AND METHODS

Seed and stem tissue sample preparation. Samples from tall fescue cv. Kentucky-31 seed lots of known endophyte infestation levels were provided by R. A. Shelby, Fescue Diagnostic Center, Auburn University, Auburn, AL. Seeds (100 per lot) were prepared for tissue imprinting by scarification and then allowed to imbibe water overnight (6) before being halved longitudinally. Pasture samples (20 samples per pasture) were selected from tall fescue cv. Kentucky-31 tiller base segments (approximately 7 cm) submitted to the University of Tennessee ELISA endophyte testing service. Two 1-mm cross sections were removed from each tiller segment for use in TPIB. The remainder of the tiller sample was tested with PAS-ELISA. Sections from plants grown and tested for endophyte as previously described (11) were used as controls.

Diagnostic tests. All chemicals, except Carnation nonfat dry milk, were purchased from Sigma Chemical, St. Louis, MO. PAS-ELISA was performed as described by Reddick and Collins (12).

TPIB. TPIB was performed as previously described (8). Sections were placed cut side down on NCM (TransBlot transfer medium, BIO-RAD, Richmond, CA). For tissue printing (4), materials were assembled as follows: glass plate, Whatman no. 1 filter paper, NCM, plant tissue section, waxed paper, and glass plate. Assembled materials were pressed manually for 20–30 sec. Plant material adhering to the NCM was removed and the NCM was then dried at 80 C for 1 h.

The NCM was placed in a sealable plastic container. Blocking solution (BS) (0.5% Carnation nonfat dry milk in Tris buffered saline [0.02 M Tris + 0.05 M NaCl, pH 7.5]) was added to the container, which was shaken at 4 C overnight. BS was then replaced with antiendophyte serum (12) diluted 1:1000 in BS. After 2–4 h at room temperature (RT) with shaking, the antiserum was removed and the NCM was rinsed five times with BS, 6 min each rinse. After the final rinse, protein A-alkaline phosphatase (0.4 μg/ml in BS) was added and shaken 2–4 h at RT. The NCM was rinsed as before and developed in Fast Red solution (3) (equal volumes of Fast Red TR Salt-20% dye content [6 mg/ml] in 0.2 M Tris buffer, pH 8.2 and Naphthol AS-MX Phosphate [0.1%] in 0.2 M Tris buffer, pH 8.2). Maximum color development occurred within 15–30 min (prints of endophyte-infected tissues were deep red, whereas prints of endophyte-free tissues were brown or colorless). The reaction was terminated with a distilled water rinse, and the NCM was air-dried before examination.

TPIBs of seed lots were examined with a dissecting scope (×10). Each TPIB was evaluated by two individuals in a blind test; values

TABLE 1. Evaluation of *Acremonium coenophialum* infestation levels of fescue seed lots with tissue print-immunoblot (TPIB)

Lot number	Infected seeds (%)	
	Expected[a]	Observed[b]
1	10	18
2	15	13
3	16	17
4	20	16
5	28	28

[a] Seed lots tested at Fescue Diagnostic Center, Auburn University, Auburn, AL, by microscopic evaluation.

[b] Average of two independent microscopic evaluations of TPIB. For each seed lot, 100 seeds were tested. Longitudinal sections of seeds were imprinted onto nitrocellulose. Endophyte infection was determined with an immunoblot using antiendophyte antiserum. Observed values did not statistically differ from expected ($P = 0.05$, chi-square goodness of fit) values.

shown (Table 1) are the averages of these independent evaluations. Observed values for endophyte levels of seed lots were compared to expected values using a chi square goodness-of-fit test (ABSTAT, Anderson Bell, Parker, CO). Estimates of endophyte infestation levels from pasture samples were obtained with both PAS-ELISA and TPIB; data were compared with a paired *t* test (ABSTAT).

RESULTS

Estimated percentages of endophyte infestation of seed lots obtained when using TPIB did not differ significantly from expected values (Table 1). No significant differences were found between estimates of percent endophyte infestation of pastures obtained by PAS-ELISA and TPIB (Table 2). In pastures where percent infestation estimated by TPIB and PAS-ELISA were the same, endophyte status of individual tillers also did not differ. TPIB evaluation with tillers was not as variable as with seed.

DISCUSSION

Antiserum used in these experiments is specific for members of the family Claviciptaceae (12); thus, these methods do not detect *A. coenophialum* exclusively. However, the localized reactions in the TPIB suggest that we are detecting an endophytic fungus. When this antiserum was used in a double sandwich ELISA for determination of seed lot endophyte infestation, an unacceptable level of cross-reactivity, perhaps to a seed protein, resulted. These same cross reactions may have occurred to some extent with TPIB.

Using the TPIB method, we were able to accurately estimate percent endophyte in infected seed lots (Table 1); however, high variability between individual evaluators indicates a low precision for this method. Percent difference between evaluations ranged from 0 to 60% ($\bar{x} = 26.6$) (data not shown). Evaluations may differ because intensity of reaction at the embryo area varied within and among seed lots. Cross reactivity between antiserum and various seed proteins may have resulted in false positives. We believe, however, that precision can be increased with evaluator experience.

TPIB can also be used to detect endophyte in tall fescue tillers (8). Until now, however, the applicability of this technique for routine detection was not known. Estimates of percent endophyte in pasture samples using PAS-ELISA and staining techniques do not differ significantly (12). Since we routinely use PAS-ELISA for detection of endophyte in pasture samples, this method was chosen for comparison with TPIB. We determined that TPIB is as accurate as PAS-ELISA for evaluation of percent endophyte infestation in pastures (Table 2). This study was limited to fresh or frozen material, the applicability for hay samples is not known.

Accuracy of this technique does not exceed that of other published techniques, but TPIB may become the technique of choice in some laboratories. Mycological expertise is required to accurately evaluate samples by the microscopic technique, since large portions of the endophyte mycelium often lack diagnostic convolutions. Antiserum-based assays remove much of the subjective evaluation but many diagnostic laboratories do not have the necessary equipment to perform ELISA. The TPIB method makes it possible for diagnostic laboratories to obtain antiserum-specific results without the major equipment expenditures associated with ELISA detection methods.

TABLE 2. Comparison of Protein A-sandwich enzyme-linked immunosorbent assay (PAS-ELISA) and tissue print-immunoblot (TPIB) methods for evaluation of *Acremonium coenophialum* in tillers from pasture samples

Pasture	Endophyte infestation (%)	
	TPIB[a]	PAS-ELISA[b]
1	84	84, NS
2	72	90, NS
3	28	32, NS
4	88	92, NS
5	72	72, NS

[a] Cross sections of tiller bases were imprinted onto nitrocellulose. Endophyte infection was determined by immunoblot using antiendophyte antiserum. The remainder of the tiller base was used in PAS-ELISA.

[b] Tiller bases ground in buffer were added to microtiter plates pretreated with Protein A and antiendophyte antiserum. After incubation, antiendophyte antiserum was added followed by Protein A-alkaline phosphatase. Substrate was added, and absorbance was measured at 410 nm. Differences between methods were not significant (NS) ($P = 0.05$) according to a paired *t* test.

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