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Grassland Research

SHORT COMMUNICATION

Determining the earliest growth stage to detect the presence of endophytes in tall fescue and perennial ryegrass seedlings using molecular markers

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Abstract

Background: Tall fescue (*Festuca arundinacea* [Schreb.], *Lolium arundinaceum* [Schreb.] Darbysh) and perennial ryegrass (*Lolium perenne*) are important cool-season forage and amenity grasses that have a mutualistic association with an endophytic fungus. Endophytes confer insect and drought resistance to plants but can produce mammalian toxins. Novel endophytes that do not produce mammalian toxins have been introduced to elite cultivars for commercial production. Seed companies need to maintain adequate levels of novel endophytes within the elite forage cultivars. Endophyte detection is performed using immunochemical and molecular techniques because of their speed and reliability. Early detection in seedlings is essential to evaluate the viability of the endophyte within seed lots.

Methods: This research aimed to identify the earliest growth stage in which immunochemical and molecular methods can detect viable endophyte in seedlings of tall fescue cultivars BarOptima (e34), Texoma MaxQII (584), and Jesup MaxQ (542), as well as the perennial ryegrass cultivar Remington (NEA2).

Results: Immunochemical testing detected endophytes in seedlings 14 days after germination (DAG), but the detection rate increased until 42 DAG in some cultivars tested. The molecular marker Tef1exon detected endophytes at a lower rate than the immunochemical method at 28–42 DAG. However, there was insufficient DNA to detect endophytes in 14 DAG seedlings using markers.

Conclusions: We conclude that the most accurate detection of viable endophytes in seedlings was 42 DAG, at which sufficient and consistent endophyte colonization occurred.

KEYWORDS

endophyte, immunoblot assay, molecular markers, perennial ryegrass, tall fescue

INTRODUCTION

Persistent perennial pasture systems are essential for modern livestock production. Tall fescue (*Festuca arundinacea* [Schreb.], *Lolium arundinaceum* [Schreb.] Darbysh) is widely grown in the United States and Europe for forage production and is planted on over 14.97 million ha of grazing land in the United States (Rogers & Locke, 2013). Tall fescue has a symbiotic relationship with an endophytic fungus (*Epichloë* spp.) (Young et al., 2014). The endophyte confers many benefits to the grass, such as biotic and abiotic stress tolerance. However, it can also be toxic to mammals. The different resistances and toxicities are due to alkaloids that the endophyte produces. Most *Epichloë* species can produce four classes of alkaloids: ergot alkaloids, indole diterpenes, lolines, and peramines, each associated with conferring protection to the host (Young et al., 2014). Ergot alkaloids can cause fescue toxicosis, a serious health issue that affects livestock feeding on endophyte-infected tall fescue, that leads to over $1 billion in losses annually for the US beef industry (Browning, 2003).

Perennial ryegrass (*Lolium perenne*) is another cool-season forage grass utilized in Japan, the United

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Kingdom, New Zealand, and Australia (Jung et al., 1996). Perennial ryegrass is commonly associated with the endophyte *Epichloë festucae* var. *lolii*. The perennial ryegrass endophyte has alkaloid makeup similar to tall fescue endophytes. Perennial ryegrass is known for causing a neurologic disorder in sheep called "ryegrass staggers," caused by the toxic compound lolitrem B. Lolitrem B is an indole-diterpene alkaloid and is not found in endophyte-infected tall fescue (Philippe, 2016).

Commercial ryegrass and tall fescue cultivars for use in regions with warmer, drier summers have been developed by screening for tall fescue endophytes that lack ergot alkaloids and for ryegrass endophytes lacking lolitrem alkaloids but still have alkaloids that act as insect deterrents and then inserting the selected endophyte strains into elite grass cultivars where the endophyte grows symbiotically. These nontoxic grass endophytes are often referred to as "novel." Examples of elite cultivar–novel endophyte strain combinations that are also used for this research are Jesup MaxQ (endophyte strain AR542), BarOptima (endophyte strain e34), and Texoma (endophyte strain AR584) tall fescues and Remington (endophyte strain NEA2) perennial ryegrass (Bluett et al., 2005; Bouton, 2009).

Seed companies producing novel endophyte products have established quality control standards within the Alliance for Grassland Renewal (https://grasslandrenewal.org). Their minimum standard is to provide seed with no more than 5% toxic types and no less than 70% viable endophyte within commercial seed lots (Pirelli et al., 2016). The interval between harvest (mid to late July in Oregon, USA) and fall seed sales (early September) provides a challenge for seed lots to be cleaned and tested for endophyte viability. Typically, testing for endophyte viability requires testing 6-week-old seedling plants for endophyte presence (Missaoui & Hill, 2015). A rapid procedure that accurately assesses seedling endophyte viability is, therefore, needed to minimize the time necessary for detection.

Methods to determine endophyte presence within a plant include microscopy, immunoblot tissue print testing, and polymerase chain reaction (PCR) testing for endophyte-derived molecular markers (Ahmad et al., 2019) and a plate-based assay, enzyme-linked immunosorbent assay. High-performance liquid chromatography can be used to identify alkaloid content (Hiatt et al., 1999). The International Seed Testing Association has approved microscopic and immunoblot methods for endophyte detection (Hill et al., 2002). The tissue print immunoblot procedure is preferred because multiple samples can be tested at one time, the method requires less training, and is more rapid and reliable (Trento et al., 2007).

Molecular markers that target different genes in the endophyte have also been developed via targeted sequencing. Markers can be used to identify the alkaloid profile of the endophyte as well as endophyte presence. Gene clusters have been identified for ergot alkaloids, lolines, and indole diterpenes. Tef1exon is a marker for the gene translation elongation factor 1-α (*tefA*), a housekeeping gene that functions to transport aminoacyl transfer RNA to ribosomes. It is commonly used in fungi for identification and has served as a good presence/absence marker for endophyte detection in grasses (Song & Nan, 2015; Wang et al., 2022).

The objective of this study was to determine the best time point between 14, 28, and 42 days after germination (DAG) at which novel endophytes can be detected in tall fescue and perennial ryegrass seedlings using molecular and immunoblot assays.

MATERIALS AND METHODS

Plant material and sampling

Jesup E−, Jesup MaxQ (542), BarOptima Plus (e34), and Texoma MaxQII (584) tall fescue commercial cultivars and perennial ryegrass cultivars Albion and Remington (NEA2) were planted in germination pouches (Mega International) in the lab. Albion and Jesup E− are endophyte-free and serve as controls. All seeds were purchased from commercial retailers. Three replications of 50 seedlings each were sampled 14, 28, and 42 DAG for a total of 108 germination pouches used. Approximately 1-cm sections of the psuedostem bases of the 14-day-old seedlings were longitudinally split, with one-half tested using the immunoblot and one-half tested using molecular markers. Cross-sections of two 3-mm pseudostem sections were harvested from 28- and 42-day-old seedlings and randomly assigned to immunoblot or molecular marker analysis. Samples assigned to the immunoblot analysis were immediately tested for endophyte. Those samples assigned to molecular detection were freeze-dried and stored in a −80°C freezer for later DNA extraction and PCR.

Endophyte immunoblot testing

All materials used to test for endophyte were within a commercial kit for testing endophyte in seedling tillers (Cat# ENDO797-2; Agrinostics Ltd., Co.) (Figure 1b). Excised pseudostem pieces from the 14-day-old seedlings were gently crushed on top of a nitrocellulose membrane to express plant sap from the tissue. Cut surfaces of the cross sections from the 28- and 42-day-old seedlings were placed onto a nitrocellulose membrane saturated with extraction buffer by standing the pseudostem pieces with one end down. All samples were tested using the test kit components as per technological instructions included in the test kit.

Endophyte marker testing

Individual freeze-dried pseudostems were ground in a 2010 GenoGrinder (Spex Sample Prep) and DNA was extracted using a 2% cetyltrimethyl ammonium bromide (CTAB) method modified from Doyle and Doyle (1987). The DNA was diluted to 5000 ng μL^{-1} and tested for endophyte using primer pairs for the tef1exon (Table 1). PCR reagent mix was for a 20 µL total reaction: 2 µL of Taq standard reaction buffer, 1.2 µL of $MgCl_2$, 0.4 µL of deoxynucleoside triphosphates (dNTPs), 0.5 µL of

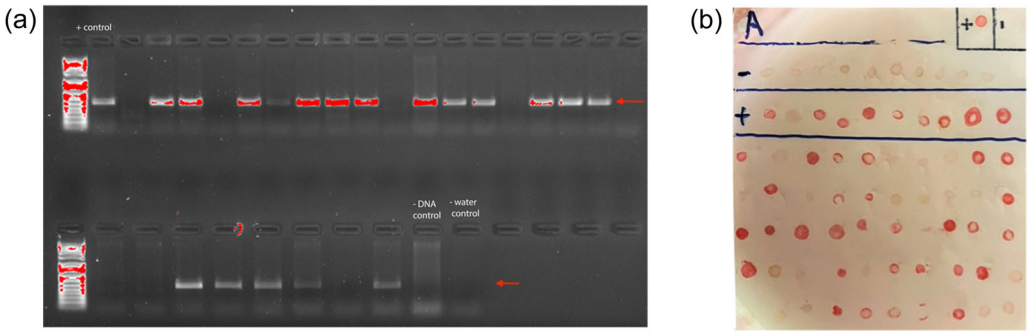

FIGURE 1 Examples of the endophyte testing utilized. (a) An ethidium bromide agarose electrophoresis gel showing PCR products of the marker Tef1exon. The strong bands present around 1000 base pairs in each of the two rows of gels (shown by red arrows) indicate the presence of the endophyte. (b) An immunoblot nitrocellulose membrane with internal positive and negative controls (upper right-hand corner) and negative and positive seedling plant controls (the rows marked − and +, respectively, at top). PCR, polymerase chain reaction.

TABLE 1 Mean endophyte detection rate for IB and polymerase chain reaction testing (Tef1) for the endophyte-derived "Tef1" molecular marker at 14, 28, and 42 days after germination.

Detection method	Days after germination			LSD
	14	28	42	
IB	39.5	50a	53.6a	3.95
Tef1	–	41.6b	44.2b	7.1
LSD	–	8.68	3.98	–

Note: The LSD is reported for each time point and each detection method. Letters after the mean indicate significance levels calculated using log-transformed data for each detection method (columns).

Abbreviations: IB, immunoblot; LSD, least significant difference; Tef1, translation elongation factor 1.

reverse primer (10 mM), 0.5 µL of forward primer (10 mM), 0.08 µL of Taq polymerase, 14.32 µL of water, and 1 µL of DNA. End-point PCR was carried out in a Bio-Rad C1000 thermal cycler under the following conditions in this order: 95°C for 15 min, 65°C for 30 s with a decrease of 1°C per cycle, 72°C for 2 min, 65°C for 30 s for nine additional cycles decreasing by 1°C per cycle, 95°C for 30 s, 55°C for 30 s, 72°C for 2 min, 55°C for 30 s for 24 cycles, and finally, 72°C for 7 min. The primer sequences used for tefA were forward primer Tef1-exon1d-1 GGGTAAGGACGAAAAGACT CA and reverse primer Tef1-exon 6u-1 CGGCAGCGA TAATCAGGATAG. Known E− DNA from tall fescue was used as a negative control in each PCR test. A 1-kb ladder was included for every gel that was processed. Results were run on a 2% EtBr agarose electrophoresis gel for 45 min at 100 V and 400 W. The gels were visualized in a Gel Doc EZ Image UV light reader (Bio-Rad) (Figure 1a).

Data analysis

The percent of endophytes detected was analyzed using a detection method (marker or immunoblot), time point, cultivar, and grass species. All zeroes in the data set were changed to 1.1. All data were then log-transformed before analysis. A three-way analysis of variance (ANOVA) was initially carried out in RStudio (v. 2022.120+353) using the R package *stats version 4.2.2* to analyze the results of the immunoblot assay and marker testing (RStudio, 2022). Replication was considered a random effect and cultivar, detection method, and time point were considered fixed effect. Two-way interactions were then parsed by ANOVA and means were separated using the least significance difference (comparison of two means), and Duncan's marginal rank tests (separation of more than two means) with R package *agricolae* version 1.3-5. The precision of analyses was calculated for each variety within each detection method and time period by calculating the coefficients of variation (CV).

RESULTS

The ANOVA indicated that the detection method, cultivar, and time all had significant effects on the percentage of seeds in which endophyte was identified ($p < 0.001$; Table S1). Significant two-way interactions were also observed between the cultivar and detection method, time and detection method, and cultivar and time ($p < 0.001$). ANOVAs were carried out for each cultivar as well as for each time point. The percentage of seeds indicated as containing endophyte differed between the two detection methods for all the E+ cultivars ($p < 0.01$). The two detection methods also differed in the percentage of seedlings detected as E+ at all time points ($p < 0.001$). DAG was the only treatment factor for which endophyte detection differed significantly for each E+ cultivar (all $p > 0.001$, except Remington $p < 0.05$). Similarly, the percent endophyte detected at 14 DAG, 28 DAG, and 42 DAG differed significantly between cultivars ($p < 0.001$).

When considering only E+ cultivars, there was a significant interaction between time points and the detection method. The immunoblot detection method resulted in a nonsignificant but numerically higher percentage of endophytes detected than the marker testing method at all time points, significantly so at 14 DAG and 42 DAG ($p < 0.05$) (Table S1).

Insufficient DNA within 14 DAG seedling plants resulted in zero endophyte presence detected using the PCR method. While the endophyte was testable at the later time points, the immunoblot test continued to result in a higher percentage of endophytes detected. The immunoblot test also proved more consistent across time points than the marker testing (Table 1). However, overall, there was an increase in the consistency of endophyte detection as time from germination increased, regardless of the cultivar or detection method. The immunoblot method yielded a significantly higher percentage of endophyte detection for cultivars Texoma 584, Jesup 542, and Remington ($p < 0.05$). Both testing methods were comparable for BarOptima. Testing methods were comparable for both the E− cultivars, Albion and Jesup E−. Which cultivar had the highest percentage of endophytes detected varied at every time point, but Jesup 542 and Texoma 584 were consistently high (Figure 2). Jesup 542 and Texoma 584 were also proven to have the lowest CV, showing the consistency of the detected endophyte infection rate (Table 2). The endophyte detection percentage was higher at 42 DAG than at 14 or 28 DAG for Texoma 584, BarOptima, and Remington (86.13%, 76.3%, and 52.6%, respectively) (Figure 2).

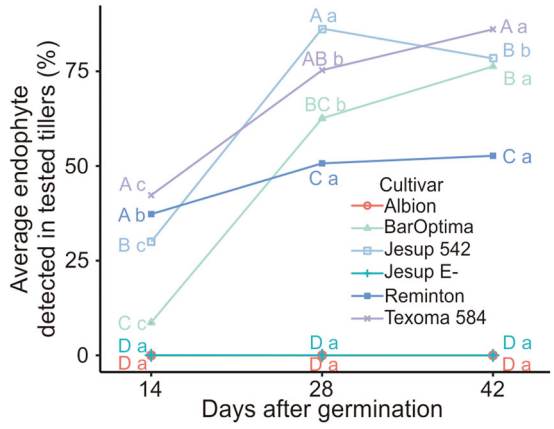

FIGURE 2 Average percent of endophyte infection of tested seeds detected at 14, 28, and 42 days after germination. Capital letters indicate significance groups within each time point among cultivars. Lowercase letters indicate significance groups within each cultivar among time points. Line shapes and colors differ by grass cultivar.

DISCUSSION

The objective of this study was to determine at what stage after germination novel endophytes can be detected in tall fescue and perennial ryegrass cultivars using molecular and immunoblot assays with the hope of reducing the necessary time for determining endophyte viability in novel endophyte-infected seed. Tef1exon was chosen as it is a highly utilized and tested marker that is used for phylogenetic work and identification of *Epichloe* sp. and is used widely throughout the literature (Charlton et al., 2014; Song & Nan, 2015; Takach & Young, 2014). The results showed that endophyte colonization of seedling plants is not synchronized among all individuals within a plant population. Some seedling plants were colonized by their respective endophytes within 14 days. However, the seedling colonization process continued over the 42-day growth period, thus increasing endophyte frequency in the seedling plants as their age progressed (Figure 2). It would be useful if data from early seedling data could be used to predict the 42-day infection frequency, thus artificially shortening the time necessary to test endophyte viability. However, accurate prediction of endophyte infection based upon infection frequency of younger seedling plants would be difficult based on the variation in endophyte percent detected between time points within a cultivar and even within a cultivar; endophyte colonization varies between plants and even between tillers within a plant. For example, seedling infection of BarOptima was the least among all cultivars tested at 14 DAG and yet, was similar to the Jesup at 42 DAG. Conversely, endophyte infection of Remington was greater than that of BarOptima at 14 DAG but less than BarOptima at 42 DAG. Variations among a cultivar at a single time point show that endophyte colonization varies between plants. Thus, using endophyte infection frequencies at early growth stages to broadly model or predict the frequency of endophyte infection at 42 DAG does not seem to be feasible as of yet. Further work based on cultivars and inclusion of more time points would need to be done to develop such prediction models.

The sensitivity and accuracy of molecular analyses provided encouragement that using the tef1exon combined with PCR techniques could detect endophytes in preference to the antibody-based immunoblot system.

TABLE 2 Coefficients of variation for endophyte detection within each cultivar for each sampling period and detection method.

Days after germination	Method	Texoma 584	Jesup 542	BarOptima	Remington	Mean
14	IB	1.36	11.5	94.2	4.2	27.82
	Marker	–	–	–	–	–
28	IB	3.2	5.5	2.3	5.6	4.15
	Marker	30.4	5.2	48.1	41.6	31.33
42	IB	4.8	5.2	1.9	19.3	7.8
	Marker	9.9	10	12	18.3	12.5
Mean		8.27	6.23	26.42	14.83	–

However, the data herein suggest that insufficient DNA was present in 14 DAG seedling plants to detect endophytes. Furthermore, endophyte frequencies within 28 and 42 DAG seedlings were less when using the tef1 marker than endophyte detection using the immunoblot procedure (Table 1). All cultivars with novel endophytes met the established criteria of 70% endophyte infection when tested with the immunoblot, but Jesup and Remington failed to meet 70% infection in seedlings 42 DAG when assessed using the molecular method. In addition, the immunoblot procedure had greater precision in determining endophyte infection of seedling plants, as indicated by the CV values. These findings suggest that, for now, the immunoblot procedure provides more accurate and precise data than molecular analysis.

Regardless of the method of endophyte detection, endophyte frequency increased in seedling plants with seedling plant age. This suggests that neither system can overcome the biology of the endophyte colonization process in seedling plants. Understanding the endophyte colonization process may lead to methods by which the process can be synchronized by adjusting environmental variables or treatment with growth regulators. Additionally, further work that characterizes the mechanism of endophyte–host interactions genetically could provide useful tools. Such treatments are speculation at this time as there is much that remains unknown about these interactions, but research to understand the colonization process may lead to technologies that will synchronize or enhance the fungal colonization process. Such research has been done to investigate the ways in which plants and fungi interact to promote fungal colonization. However, further work is needed to be applicable (Barker & Tagu, 2000; Hedden & Phillips, 2000).

Immunoblot testing using commercial kits is very user-friendly and does not require extensive training. The process is done over 2 days. A typical immunoblot assay kit costs about $100 for 100 samples, resulting in a low cost of approximately $1 per sample (https://www.agrinostics.com/shop/). Using molecular markers has a high startup cost, but in an operational lab, it is affordable. DNA extraction of plant and fungal tissue can be performed in many different ways, which may change the cost. When calculated for our standard 2% CTAB DNA extraction protocol, the cost was approximately $710 for 500 extractions. This is only $1.42 per extraction. PCR reagents cost approximately $150 for taq polymerase 500 reactions, $40 for primer pairs, and $40 dNTPs for 500 reactions. This results in PCR costing less than 50 cents per reaction. In the end, it can cost less than $2 to test for endophyte presence using molecular markers, depending on specific protocols and reagents purchased. Markers can also be more helpful for gathering specific information. Primers can be used to target specific endophyte genes to identify the alkaloid content. Multiple markers can be multiplexed together to do this quickly. DNA extraction and PCR require trained personnel to carry out the testing. The whole process takes 2–3 days with freeze-drying, DNA extraction, and PCR.

Which method of testing for endophytes in tall fescue is best is an individual decision made by the producer based on their specific goals. Both markers and immunoblot testing are highly reliable detection methods, each with their own benefits and downsides.

CONCLUSIONS

We conclude that the early detection of viable endophytes in seedling plants was not dependent upon the detection method used but rather the time necessary for the endophyte to colonize the seedling plants. Due to the level of infection not being fully revealed until after 28 days and to cultivars varying in endophyte colonization time, we would advise seed producers to wait until 42 DAG to test seedlings, whichever method is used.

AUTHOR CONTRIBUTIONS

Kendall Lee: Conceptualization; data curation; formal analysis; investigation; methodology; project administration; writing—original draft. **Nicholas Hill**: Conceptualization; investigation; methodology; project administration; resources; supervision; validation; writing—original draft; writing—review and editing. **Chloe Dela Cerna**: Investigation. **Ali Missaoui**: Conceptualization; project administration; resources; supervision; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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