Isolation and culture of *Epichloë sp.* for re-infection of endophyte-free southeastern wildrye (*Elymus glabriflorus*)

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Abstract
Mutualistic relationships between endophytic fungi and grasses have shown to improve the hardiness of the host. This relationship is common in grasses, including native cool-season grasses that are important in both forage and grassland ecosystems. Elymus genus members, such as Canada wildrye (CAWR), commonly host the endophytic fungi, Epichloë, while southeastern wildrye (SEWR) may not. In this study, seed of ten Elymus accessions and seed and leaves from local SEWR were assessed for endophyte infection. Infection status was confirmed via seed squash and leaf peel techniques and assessed using microscopy. Seed of one SEWR and nine CAWR accessions were assessed for endophyte infection by softening seeds in NaOH. Softened seeds were stained and squashed between a microscope slide and coverslip. Infection status was determined by scanning for mycelia within each seed. Leaf peels were excised from SEWR, stained and observed. Infection status was determined by scanning for mycelia in epidermal cells. Following assessment, all SEWR germplasm was E-, while six CAWR accessions were E+. Endophyte-infected seeds were germinated and pseudostems were used to isolate the endophyte by sterilizing the pseudostems and placing them on PDA in a dark germination chamber until endophyte growth. Isolated endophyte was used to infect E- SEWR. Infection status of SEWR was determined using leaf peels and PCR. This research will help determine if forced endophyte infection can be performed in SEWR.

Introduction
Native cool-season grasses (NCSG) play an important role in both forage and native grasslands throughout the southeastern United States. Southeastern wildrye (*Elymus glabriflorus*) (SEWR) is a native perennial cool-season grass that has shown potential as an alternative forage for southern pastures. This NCSG is adapted to a vast range of environmental conditions from partial shade to full sun, well drained or waterlogged soil, and neutral to acidic soils. When compared to commonly used forage grasses such as orchardgrass (*Dactylis glomerata*) and tall fescue (*Schedonorus arundinaceus*), SEWR has a high germination rate (>50%) and matures much later in the season (Belt et al. 2013). However, SEWR is less tolerant of frequent harvesting when compared to tall fescue (Richwine 2016). The combination of broad site tolerance and late maturity makes SEWR a quality candidate for improvement breeding for forage use.

Many grasses have a known mutualistic relationship with endophytic fungi in the genus *Epichloë*. Within the *Elymus* genus, both Virginia wildrye (*E. virginicus* L.) and Canada wildrye (*E. canadensis* L.) (CAWR) are hosts to *Epichloë*. However, it is currently unknown whether SEWR has a similar fungal mutualist. Studies have shown that the mutualistic relationship between tall fescue and *Epichloë* is highly beneficial to the grass and the fungi. Grasses infected with an endophyte have an (a) increased growth rate, (b) resistance to diseases, pathogens, and herbivores, (c) a higher reproductive rate, and (d) resistance to abiotic stresses such as heat, salinity, and drought (Márquez et al. 2012; Saikkonen et al. 2006). The potential to increase these hardiness characteristics in SEWR make endophyte infection a desirable option[1]. Endophyte-infection of SEWR could improve its tolerance to grazing and abiotic stressors, increasing its utility as a cool-season forage species.

While SEWR is vital to native landscapes and grasslands, it has limited use for forage production due to its inability to tolerate frequent harvesting. The objectives of this study are to improve SEWR germplasm for forage and grassland use by the forced infection of a stable endophyte from *E. canadensis*. This will be accomplished by

(1) Identifying and isolating an endophyte from *E. canadensis*.

[1] An often-cited example of endophyte-infection is KY-31 tall fescue in which the *Epichloë* endophytes can generate secondary metabolites that are harmful to livestock (i.e., fescue toxicosis). However, not all endophytes produce harmful secondary metabolites. These harmful metabolites have not been identified in *Elymus* associated *Epichloë*.

(2) Identifying E- SEWR (endophyte-free SEWR) germplasm and intentionally infecting E- SEWR with the stable endophyte.
(3) Confirming endophyte infection via histological analysis.
(4) Evaluating progeny of E+ SEWR (endophyte-infected SEWR) for tolerance to biotic and abiotic stress.

Methods

Seed of one SEWR and eight CAWR accessions were assessed for endophyte infection. In brief, each seed was soaked in 5% w/v NaOH for 12 hours, stained with aniline blue, gently squashed between a microscope slide and coverslip, then boiled slightly to set the stain. Infection status was determined by the presence or absence of fungal mycelia within the aleurone layer of each seed when observed via light microscopy (Bacon and White 1994). To determine infection status of mature SEWR plants, epidermal leaf peels were excised from mature leaves, stained, and observed in a similar fashion to the seeds. Infection status was determined by presence or absence of mycelia in the apoplast of epidermal cells (Bacon et al. 1977). Verified endophyte-infected CAWR seeds were germinated. Once matured, 1-centimeter pseudostem sections from each CAWR plant were collected for endophyte isolation. Pseudostem sections were sterilized with a 10% v/v bleach solution, placed on potato dextrose agar plates (PDA), and set in a dark 25°C germination chamber for 6 weeks or until endophyte growth was identified. Artificial infection of SEWR was performed using sterile techniques by germinating the E- seed on sterile water agar for nine days until the mesocotyl and coleoptile grew. A small incision was made in the junction between the mesocotyl and the coleoptile and a small amount of isolated endophyte was scrapped off the PDA and put into the incision. These seedlings were then allowed to grow for twelve weeks, and infection status was determined using leaf peels and DNA analysis (Latch and Christensen 1985). Confirmation of endophyte infection via DNA isolation, PCR, and gel electrophoresis is currently ongoing. Following forced infection, all surviving plants have been evaluated for positive endophyte infection using primers specific to the *Epichloë* endophyte (Table 1) (Charlton et al. 2012). Endophyte infected tall fescue and Canada wildrye donor plants were used as checks for DNA analysis.

Locus	Primer name	Primer sequence	gDNA size (bp)
TefA	tef1-exon1d-1	GGG TAA GGA CGA AAA GAC TCA	860
	tef1-exon6u-1	CGG CAG CGA TAA TCA GGA TAG	
PER	per T2-F	TCTTCAGGCATCGCAGGAAC	600
	per T2-R	TCGGCCACCTCCAGCCTGATG	
LOL	lolC-3a	GGTCTAGTATTACGTTGCCAGGG	442
	lolC-5b	TCTAAACTTGACGCAGTTCGGC	
EAS	dmaW-F4	GTGTACTTTACTGTGTTCGGCATG	282
	dmaW-6R	GTGGAGATACACACTTAAATATGGC	
IDT	idtG-F	GAGCTTGAGAAGCTTACGAATCC	113
	idtG-R	GGGCAATGGAGCGATTCTCTC	

Results and Discussion

Preliminary work

Preliminary work was done on tall fescue to ensure the method of identifying and isolating endophyte from wildrye was successful. Tall fescue has a well-known fungal endophyte that can be easily identified through leaf peels, seed squashes, and on PDA once isolated. This preliminary work was used as a reference for later work performed on SEWR and CAWR. The convoluted structure of the mycelia under microscope and the appearance of isolate endophyte on PDA were referenced. All methods were successfully performed, and the results were recorded to support the research done on SEWR and CAWR.

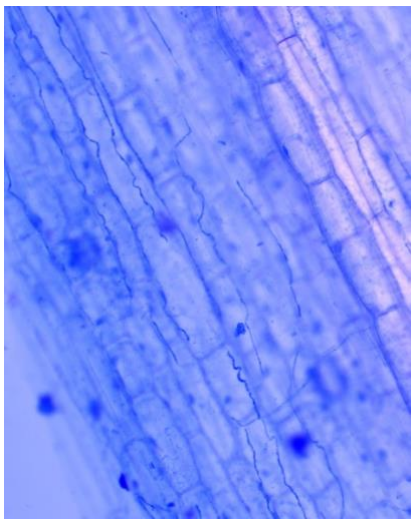

Figure 1: Leaf peels of endophyte-infected tall fescue at 100x magnification showing dark blue mycelia within the apoplast

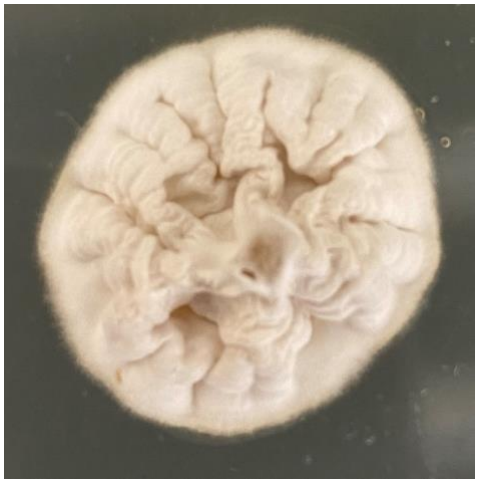

Figure 2: Potato agar petri dishes with *Epichloë* isolated from tall fescue pseudostem sections.

Wildrye germplasm endophyte assessment

All SEWR germplasm was endophyte-free. Both the seed squash and leaf peel assessments had negative results for endophyte mycelia presence. This indicates that the sampled populations of SEWR were most likely endophyte-free. For this assessment, three SEWR populations at Mississippi State University were sampled. Approximately 50 seeds from each population were assessed as well as 30 leaf peels from individual plants in each population. Each of these three SEWR populations had limited genetic diversity due to them being the progeny of few mother plants. If these mother plants were E-, then so would be the resulting progeny. This E- seed was artificially infected with endophyte from CAWR.

Seven out of the nine CAWR seeds were positive for endophyte infection. These seeds were obtained from the Germplasm Resource Information Network (GRIN) and Shooting Star Native Seeds (Spring Grove, Minnesota, USA). Ten seeds from each GRIN accession line and 30 seeds from Shooting Star Native Seeds ecotype were assessed for endophyte infection via seed squashes. Only one accession line from GRIN global was E-, the rest being E+. The E+ seeds were germinated in a climate control chamber for further use. Only one line from GRIN global and the seed from Shooting Star Native Seeds was successfully germinated. This very low germination rate could be due to the age of the seed, some of which were over 40 years old. The seed that successfully germinated were grown to maturity and then used for endophyte isolation.

Conclusions and/or Implications

If artificial infection of SEWR is successful, progeny testing on E+ and E- SEWR plants will be performed with various abiotic stressors such as heat, drought, and salinity to assess the impact of artificial endophyte infection on SEWR. Successful infection of SEWR with a stable endophyte could improve the forage capabilities of the native cool season grass, possibly allowing it to survive against higher levels of grazing and

abiotic stressors. The use of this artificially infected southeastern wildrye could greatly improve both forage and native grasslands across the southeastern United States.

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