

RESEARCH

The Fungal Endophyte *Epichloë festucae* var. *lolii* Does Not Improve the Freezing Tolerance of Perennial Ryegrass

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ABSTRACT

A lack of winter hardiness limits the utility of perennial ryegrass (*Lolium perenne* L.) as a turf and forage grass in northern latitudes. The fungal endophyte *Epichloë festucae* var. *lolii* is commonly associated with perennial ryegrass and is believed to enhance stress tolerance in some environments. The effect of *E. festucae* var. *lolii* on freezing tolerance was assessed using seven diverse perennial ryegrass entries. Freezing tolerance was used as a proxy for winter hardiness, and most entries had been previously field tested to confirm this supposition. Three experiments were designed to isolate the effect of the endophyte from confounding effects from the grass host and the endophyte removal process. Experiment 1 compared hosts with (E+) or without (E-) endophytes that were either genetically identical or non-isogenic, but from the same entry. Isogenic E+ and E- plants did not differ in freezing tolerance; however, some non-isogenic populations differed in freezing tolerance. Experiment 2 used additional populations of non-isogenic hosts to confirm the association between freezing tolerant hosts and endophyte infection. Isogenic hosts were polycrossed and the half-sib E+ and E- progeny were used in Exp. 3, eliminating any effect of the endophyte removal process. Freezing tolerance did not vary between related families differing in endophyte infection, confirming that highly related hosts were not affected by endophyte. These results strongly suggest that native *E. festucae* var. *lolii* has no direct effect on freezing tolerance but may be found in higher frequencies in freezing-tolerant hosts.

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Abbreviations: E+, endophyte infected; E-, endophyte free; LT₅₀, lethal temperature 50.

FREEZING TEMPERATURES are a limiting factor of plant distribution and reduce the persistence of many perennial species in northern latitudes (Kreyling, 2010). Turf and forage grasses actively grow during both fall and spring, which increases the risk for winterkill because of fluctuating temperatures during acclimation and deacclimation. This leads to poor aesthetics and forage yields the following year (Bélanger et al., 2006). Perennial ryegrass (*Lolium perenne* L.), used worldwide for both turf and forage, is particularly susceptible to winterkill (Hofgaard et al., 2003). As a result, improved winter hardiness has been an important breeding objective in perennial ryegrass breeding programs (Casler et al., 1996).

Perennial ryegrass winter hardiness has been improved through breeding, albeit complete adaptation to northern latitudes is still lacking. Sampoux et al. (2013) compared turf-type perennial ryegrass cultivars released over a 30-yr period and observed that persistence and winter greenness of modern cultivars was only marginally increased compared with older cultivars. This slow genetic improvement in winter hardiness is likely due to the complex and irregular nature of winter stresses. Depending on the year, extreme low temperatures, fluctuating fall and spring conditions, ice accumulation, and psychrophilic plant pathogens can all cause winterkill (Larsen, 1994; Pearce, 2001; Kalberer

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et al., 2006; Gusta and Wisniewski, 2013). Hulke et al. (2007) found that although there was significant variation among 300 perennial ryegrass accessions for winter hardiness, high levels of mortality occurred across all accessions. Moreover, authors found substantial variation for winter-kill across years, leading to inconsistent selection pressure. Therefore, research has been dedicated to developing alternative controlled environment screening for various aspects of winter survival. The most commonly used proxy for winter hardiness is freezing tolerance, which has been shown to be heritable ($h^2 = 0.3\text{--}0.5$), repeatable, and an accurate predictor of winter survival in the field ($r = 0.35\text{--}0.75$) (Waldron et al., 1998; Hulke et al., 2008; Iraba et al., 2013; Rognli, 2013). Generally, the lethal median temperature 50 (LT_{50}) of a population, or the temperature at which 50% mortality is reached, is used as the sole indicator of freezing tolerance. However, interactions with temperature have resulted in using a combination of the LT_{50} , LT_{10} , or LT_{90} to predict winter survival (Wolf and Cook, 1994; Miranda et al., 2005).

Successful species adaptation to new climates, occurring either through natural selection or breeding, is dependent on many factors including reproductive strategy, phenotypic plasticity, and genetic variation in the founding or breeding population (Merilä and Hendry, 2014). Recently, research has shown that symbiotic associations with fungal endophytes can also play a significant role in grass adaptation to extreme abiotic environments (Márquez et al., 2007; Saikkonen et al., 2016). Specifically, tall fescue [*Schedonorus arundinaceus* (Schreb.) Dumort., syn. *Festuca arundinacea*] infected with the clavicipitaceous endophyte *Epichloë coenophialum* demonstrated increased drought tolerance and insect resistance, allowing successful integration and persistence of tall fescue into southern US pastures and grasslands (Clay and Holah, 1999; Saikkonen, 2000). Perennial ryegrass is commonly infected with *Epichloë festucae* var. *lolii*, which has also been shown to reduce abiotic and biotic stress (Clay and Schardl, 2002; Leuchtman et al., 2014).

Endophytes can drastically influence the transcriptome and metabolome of its host, which may lead to changes in abiotic stress response (Ambrose and Belanger, 2012). For example, Hahn et al. (2008) studied drought response by measuring biomass accumulation, water use efficiency, and relative water content and found that endophyte infection significantly decreased biomass production of both drought-treated and control plants. Reduced growth rate led endophyte-infected (E+) plants to take up less water and maintain a higher relative leaf water content, demonstrating that the impact of endophytes on growth rate can influence the response to abiotic stresses. Relating endophyte infection to winter survival, perennial ryegrass plants infected with novel *E. festucae* var. *lolii* had increased expression of cold response genes, suggesting that plants

would be better able to respond to temperature change during cold acclimation (Dupont et al., 2015).

Field studies focusing exclusively on Clavicipitaceous endophyte effects on winter survival are rare, and authors have reported variable effects (Ravel et al., 1995; Wäli et al., 2008; Bylin et al., 2014). Casler and van Santen (2008) performed two field experiments focused on the effect of *Neotyphodium coenophialum* on the winter hardiness of tall fescue and found no effect of endophyte on post-winter ground cover. Nonhardy and winter hardy germplasm were included; however, the winter stress may have been too extreme (95–100% loss in nonhardy germplasm) or too mild (0–12% loss in winter hardy germplasm) to damage plants in a manner that could characterize minor effects. In this case, effects could have been masked from detection unless the endophyte was the primary contributor of winter survival. Previous publications show that there is potential for small effects and endophyte \times host interactions between genotypes within a single population for stress tolerance (Hill et al., 1996; Assuero et al., 2000; Cheplick and Cho, 2003).

Careful consideration should be given with respect to experimental design due to the complexity of confounding effects from host \times endophyte interactions. In the past, endophyte effect has been measured at an individual genotype, family, or cultivar scale (Clarke et al., 2006; Casler and van Santen, 2008; Hahn et al., 2008). However, the literature suggests that even closely related genotypes display varying degrees of symbiosis, ranging from mutualistic to antagonistic, for the same endophyte strain depending on the trait being measured (Bultman and Ganey, 1995; Cheplick et al., 2000). Grass breeding is often done on a population improvement scale, in which parents are selected based on the performance of their progeny (Vogel and Pedersen, 1993). Therefore, any effect of endophyte would need to be detectable on a population or family scale to be applicable to the development of a commercial cultivar. Designs for E+ and endophyte-free (E-) populations are generally divided into three types depending on how much the host plants differ in relatedness: (i) isogenic (Cheplick et al., 2000; Kane, 2011; Nagabhyru et al., 2013; Kaur et al., 2015), (ii) non-isogenic (West et al., 1993; Eerens et al., 1998), or (iii) isofrequent (Vaylay and van Santen, 1999; Casler and van Santen, 2008). Non-isogenic populations in outcrossing species are obtained by assembling different selections or cultivars with or without endophytes or by using a single genetically mixed cultivar and selecting E+ and E- plants from the bulked seed (Welty et al., 1991; Gwinn and Gavin 1992). Large sample sizes of 20+ individuals are generally needed to encompass sufficient variation for these designs (Bolaric et al., 2005). Unfortunately, the confounding effect of host genotype can become a limiting factor in this design, especially in populations of diverse origin

due to possible interactions between endophytes and host genotypes (Cheplick et al., 2000); therefore, isogenic host populations are advantageous. For example, Kaur et al. (2015) found that a single endophyte, when tested across a wide range of host genotypes, exhibited drastic variation in metabolomic profiles, resulting in higher levels of mammalian toxicity. Isogenic, or genetically identical, populations or genotypes are commonly used because any effect of host genotype is theoretically removed. Endophyte removal, accomplished by either using systemic fungicides such as benomyl or propiconazole or infection of several clones of a single host genotype, can produce isogenic E⁻ and E⁺ plants (Ravel et al., 1997; Kane, 2011; Hesse et al., 2003). The use of fungicides can alter plant response to stress (Ronchi et al., 1997), and few studies include a control testing the effect of fungicides on response variables. However, when studying native endophytes in fine fescue, Faeth and Sullivan (2003) included an E⁺ control and found no effect of fungicide on any growth or reproductive parameter. Nevertheless, confounding effects of fungicides should always be considered when designing populations focused on the effects of a fungus.

One way to circumvent possible effects of fungicides is to develop isofrequent populations (Casler and van Santen, 2008). Isofrequent populations are the progeny of isogenic or closely related parents and are congenitally E⁺ or E⁻, based on parental infection status. Therefore, allele frequencies are considered to be similar between E⁺ and E⁻ populations or families, which may offer substantial insight into the utility of the endophyte. Isofrequent populations have been used to study endophyte effects on red thread (caused by *Laetisaria fuciformis* McAlp.) disease in fine fescues (Bonos et al., 2005) and also to observe changes in the community composition of tall fescue swards (Clay et al., 2005).

Our objective was to examine the effect of *E. festucae* var. *lolii* on the freezing tolerance of a diverse collection of perennial ryegrass germplasm with a range of winter hardiness. Three experiments were designed to test the effect of endophyte in (i) small isogenic E⁺ and E⁻ and non-isogenic E⁺ and E⁻ populations ($n = \sim 10$); (ii) large non-isogenic E⁺ and E⁻ populations ($n = \sim 20$); and (iii) isofrequent E⁺ and E⁻ populations. Freezing tolerance was used as a proxy for winter hardiness, and both the LT₅₀ and the interactions between plant survival temperatures were used to determine the effect of *E. festucae* var. *lolii* in a perennial ryegrass host.

MATERIALS AND METHODS

Germplasm Selection

Entries consisted of three cultivars and four wild or landrace accessions for Exp. 1 and 3 (Table 1). Selected entries had diverse geographical origins and incorporated a wide range of winter survivability. Initially, seed of all entries tested positive

for endophyte with at least a 10% infection frequency (Table 1). One cultivar, 'NK200', was excluded from Exp. 2 due to complete loss of endophyte infection in bulk seed.

Endophyte Detection Methods

Entries were first tested for endophyte infection by tissue print immunoblot based on previous methods using a commercial kit (Phytoscreen Immunoblot Kit ENDO797-3, Agrinostics, <http://www.agrinostics.com>) (Hiatt et al., 1997). Scoring was done on a binomial scale, with any ambiguous blots removed from analysis. Additional screening was done because fungi related to *E. festucae* var. *lolii*, such as *Claviceps purpurea* which commonly infects perennial ryegrass, can confound the immunoblot results by indicating false positives (Jensen et al., 2011). Therefore, subsamples were also tested using light microscopy using methods described by Florea et al. (2015).

Non-isogenic Populations

Non-isogenic populations were developed for Exp. 1 and 2 (Fig. 1). Bulk seed from each entry was seeded into 12.7-cm × 12.7-cm trays with Sun Gro MVP (Sungro Horticulture) soilless media and placed into a 23°C greenhouse. Ten days after seeding, 100 randomly selected seedlings were transplanted into 50 cell trays with soilless media. Seedlings were watered daily and fertilized weekly with a 1:100 solution of 10 g N L⁻¹, 5 g P L⁻¹, and 20 g K L⁻¹, 48 g L⁻¹ ammonium sulfate, and 2 g L⁻¹ Sprint 330 (5 g Fe L⁻¹). Three tiller-stage plants were screened for endophyte using tissue print immunoblot, and infection frequency ranged from 10 to 89% (Supplemental Table S1).

Random E⁺ and E⁻ plants (hereafter referred to as E⁺ and E⁻ congenital) were chosen from each entry and pooled together to form the non-isogenic E⁺ and E⁻ populations. Each of the non-isogenic E⁺ and E⁻ populations was then clonally propagated to produce five clonal stock plants. Plants were grown in a greenhouse under a 16-h photoperiod at 23°C until each clone had produced a minimum of five tillers.

Isogenic Populations

Two pairs of isogenic populations (four total) were developed for each entry (Fig. 1). Two of these isogenic populations were derived from the original non-isogenic E⁺ plants (described above), and two were developed from original non-isogenic E⁻ congenital plants (described above). The first isogenic population (E⁺) consisted of plants propagated directly from the original E⁺ plants. The second isogenic population (E⁻ fungicide) was created by treating the original E⁺ congenital plants with fungicide to test for the effect of endophyte infection; five foliar applications of propiconazole (1-([2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl)-1H-1,2,4-triazole) (Kestrel MEX, Phoenix Environmental Care) were applied to the clones of the second set before tillering. The third population (E⁻ congenital) consisted of plants propagated directly from the original E⁻ congenital plants. The fourth population (E⁻ control) was created by treating the original E⁻ congenital plants with fungicide (as described above) to measure any possible effect of fungicide application on freezing tolerance. One week after the final fungicide application, the endophyte infection status of each population was tested via tissue print immunoblot.

Table 1. List of entries included in Exp. 1, 2, and 3. Winter hardiness and endophyte infection frequency of each entry are reported when possible.

Entry	Source	Origin	Winter hardiness	Infection frequency
			1–9 score†	%‡
PI 611044	Accession	Russian Federation	6.7	53
PI 610806	Accession	Romania	4.9	51
NK200§	Cultivar	University of Minnesota	4.8	89
W6 11256	Accession	Turkey	1.5	59
PI 223178	Accession	Greece	1.3	10
Green Emperor	Cultivar	University of Minnesota	NA	56
GrandSlam GLD	Cultivar	Peak Plant Genetics	NA	59

† Winter hardiness was measured on a 1–9 scale where 9 = 100% survival (Hulke et al., 2007); NA = not available.

‡ Endophyte infection frequency determined before Exp. 1

§ NK200 was not included in Exp. 2.

Ten genotypes from each of the four populations—E+, E- fungicide, E- congenital, and E- control (making isogenic E+ and E- and isogenic E- and E- populations) (Fig. 1)—were propagated and split into five clones and transplanted into 50 cell trays. Plants were grown in a greenhouse under a 16-h

photoperiod at 23°C until each clone had produced at least five tillers. There was a 60-d interim between chemical application and generation of final stock material to reduce any effect of fungicide. Previous studies have allowed varying amounts of

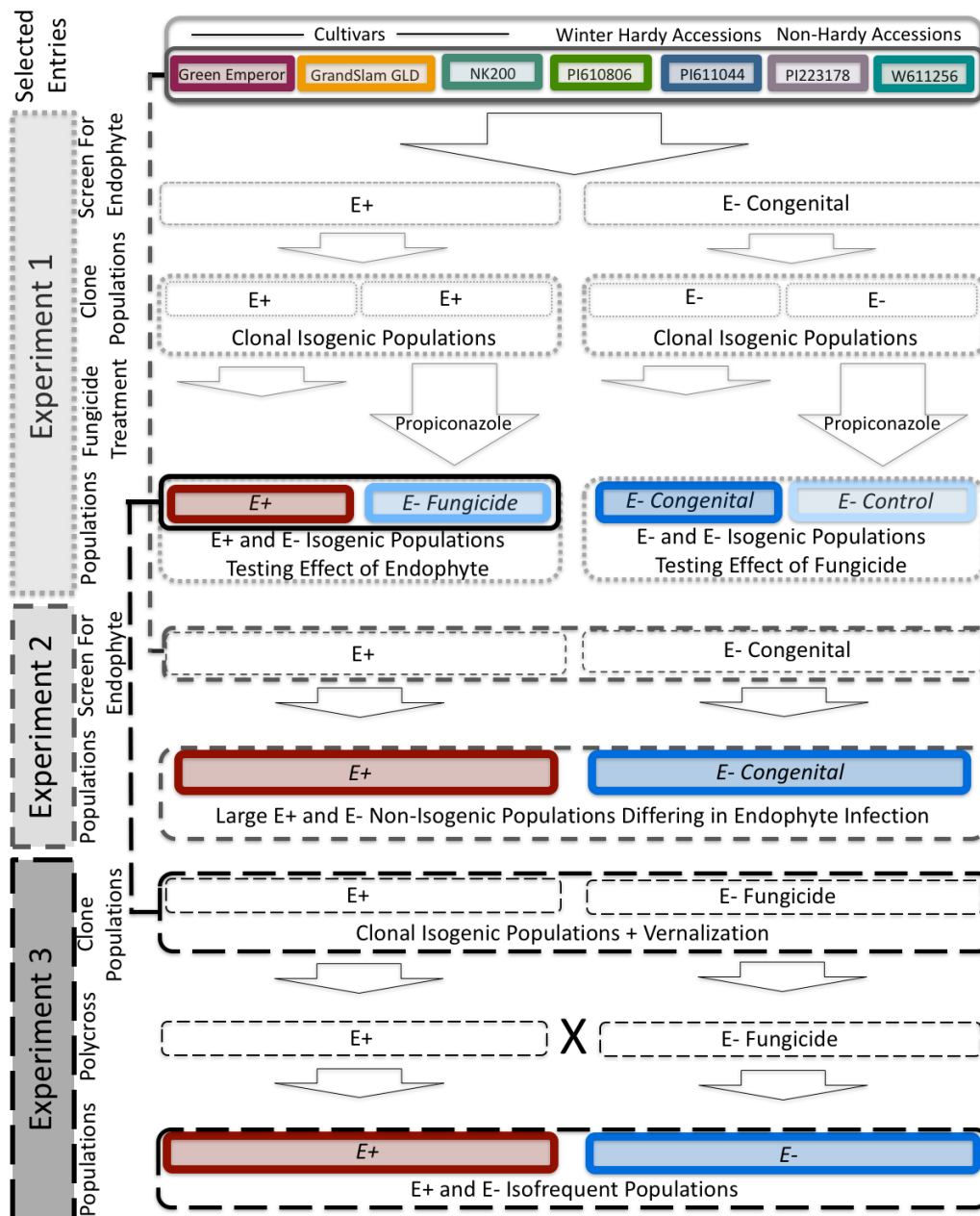


Fig. 1. Flow diagram for Exp. 1, 2, and 3. Each experiment has a different associated line pattern and gray scale color. Seven entries (three cultivars, two winter-hardy accessions, and two nonhardy accessions) were used throughout the experiments. Populations and entries for each experiment have a different encircling color used throughout the figure. Arrows and connecting lines between experiments denote the origin of the populations used. Experiment 1: endophyte-infected (E+), endophyte-free (E-) fungicide, E- congenital, and E- control; Exp. 2: E+, and E- congenital; Exp. 3: isofrequent E+ and E- families.

time for recovery after fungicide application ranging from a few weeks to 2 yr (Ravel et al., 1997; Cheplick et al., 2000; Hesse et al., 2003; He et al., 2013). After 60 d, endophyte status was confirmed for each genotype using light microscopy. Most E⁻ fungicide plants remained E⁻ for the duration of the study, and those that did not were removed. To balance the experiment, isogenic populations always contained the same genotypic pairs (Supplemental Table S2).

Isorefrequent Populations

Isogenic E⁺ and E⁻ fungicide populations were used to create isorefrequent populations (Fig. 1). Each of the 14 populations was propagated to create two clonal sets of plants. These clones were grown in 50 cell trays with Sun Gro MVP soilless media and allowed to root and grow to five or more tillers. The trays containing the two clonal sets of isogenic E⁺ and E⁻ populations were then placed into a walk-in cooler and allowed to vernalize for 114 d. The walk-in cooler was set to 3°C with a 10-h photoperiod. Light was provided by two 110-W cool white fluorescent bulbs hung ~20 cm above plants, providing 150 μmol m⁻² s⁻¹ photosynthetic photon flux at the plant canopy. During anthesis, entries were isolated in different greenhouses with no other flowering *Lolium* or *Festuca* spp. Seeds were collected and dried from each parent and were individually packaged to form the isorefrequent E⁺ and E⁻ populations. Overall, isorefrequent E⁺ and E⁻ populations had average infection frequencies of 98.9 and 1.4%, respectively (Supplemental Table S3). Deviation from 100 or 0% may have been due to harvesting or seed cleaning errors.

Plant Phenotype before Freezing

The number of tillers per plant was counted to gauge plant phenotypic differences that might be affected by endophyte infection during the growth period in the greenhouse. Before acclimation, tillers were counted for four random clones of each unique genotype comprising each population within each of the entries in Exp. 1 and 2. Tillers of six random genotypes per family were counted in Exp. 3.

Freezing Protocol

All experiments used the same acclimation protocol preceding freezing tests. Plants were randomized and then moved to a walk-in acclimation cooler set to 2°C with a 10-h photoperiod for 14 d (Hulke et al., 2008). Light was provided by two 110-W cool white, high-output fluorescent bulbs hung ~30 cm above plants. During acclimation, plants were rotated within the cooler three times per week to reduce random temperature heterogeneity. Thermocouples were placed on each shelf within the cooler and set to record ambient temperature once every 30 min to ensure acclimation repeatability (Watchdog A125, Spectrum Technologies). After the acclimation interval, plants were moved to a Tenney programmable freezer (Lunaire Environmental) for freeze testing. Thermocouples were placed on each of the four shelves within the freezer and set to record ambient temperature once every minute. Freezing tests for all three experiments included 11 temperature treatments each with two randomized blocks. Each genotype within each population within each entry was included in each block and freezing treatment.

Freezing treatments targeted a range from -20 to -10°C with ~1°C intervals; these treatment extremes were known from previous experiments to provide 100% death to 100% survival. Freezing treatments for each experiment had the following programmed conditions: (i) all treatments remained under -3°C for 23 h; (ii) the rate of temperature decrease was 1°C h⁻¹; (iii) the chamber remained at the target treatment temperature for 1 h; and (iv) each block was placed on the same shelves for each temperature treatment. After the 23 h of treatment, plants were removed and allowed to thaw for 3 d in the walk-in acclimation cooler. Plants were then moved into the greenhouse to recover for a minimum of 21 d (Hulke et al., 2008; Hoffman et al., 2014) and then scored for survival on a binomial scale, with 1 indicating survival and 0 indicating death.

Experiment 1: Comparisons between Isogenic and Non-isogenic Populations

Experiment 1 was trialed twice and included isogenic and non-isogenic E⁺ and E⁻ populations, as well as isogenic E⁻ and E⁻ populations (Fig. 1). The number of isogenic pairs ranged from 7 to 10 (Supplemental Table S1). Non-isogenic populations contained E⁺ and E⁻ congenital populations from each entry (Supplemental Table S1). Genotypes from each population were propagated to fulfill blocks and temperature treatments for freezing tests. Growing conditions in the greenhouse were very similar for both the first and second trials (mean [SD] of 23.3 [3.7] and 23.0 [4.4]°C respectively).

Experiment 2: Comparisons between Large Non-isogenic Populations

Experiment 2 included only non-isogenic populations with a target population size of 20 E⁺ and E⁻ congenital genotypes from each entry (Fig. 1). Endophyte infection frequencies remained sufficient to carry out this experiment, except for NK200, which was removed. Twenty genotypes of both non-isogenic E⁺ and E⁻ populations were identified for each entry, except for accession PI 223178 (Supplemental Table S2) because of both a high endophyte infection frequency and a low seed germination rate. Plants were grown similarly to those in Exp. 1.

Experiment 3: Comparisons between Isorefrequent Populations

Isorefrequent E⁺ and E⁻ seed underwent cool-moist stratification for at least 5 d to break dormancy and gain germination homogeneity (Fig. 1). Seeds were individually planted into 72 cell flats with Sun Gro MVP soilless media. Seeding was done as to stagger germination to account for the 10-d time lapse in freezing tests. Once seeds had begun germinating, nine random plants from each isorefrequent half-sib family were tested for endophyte infection using tissue print immunoblot. Only six half-sib families from each entry had high enough seed and germination percentages to be entered into this experiment. This resulted in the inclusion of a total of 84 families (42 isorefrequent E⁺ and E⁻ pairs) across the seven entries (Supplemental Table S3). Each entry's isorefrequent half-sib populations had the same parental background. Plants were randomized in 72 cell trays so that each block of each temperature treatment contained one genotype from each family. There were four blocks in each

of the nine freezing treatments in Exp. 3. This design allowed each entry to be represented by 36 genotypes per temperature treatment with 216 genotypes across temperature treatments.

Statistical Analysis

Plant survival data were analyzed using a generalized linear model for binomial data in R (version 3.3.2; R Core Team, 2016). Models included temperature as the only continuous variable to allow predictions of survival across temperature treatments. Entry and population were treated as fixed effects; block was treated as a random effect. Analysis of covariance was used to determine significance of main effects and interactions using a χ^2 test distribution ($\alpha = 0.05$). Significant interactions involving entry led to analysis of individual entries. Any interactions with temperature were visually represented using logistic regression. All figures were made using the R package ggplot2 (Wickham, 2016). Plotting a 95% confidence interval across all predicted points statistically differentiated regression lines. Effects at the LT_{50} were delimited using 95% confidence interval at the inflection point.

Tiller abundance was analyzed with linear fixed effects models using the R package AGRICOLAE (Mendiburu, 2016). Models included entry, endophyte, and genotype or half-sib family. Interactions and treatment effects were considered significant at $\alpha = 0.05$. Mean separations were performed on treatments showing significance in the ANOVA using the Tukey's honest significant difference method ($\alpha = 0.05$).

RESULTS

Experiment 1

Survival data were analyzed by pairing populations: isogenic E+ and E- fungicide, non-isogenic E+ and E- congenital, and isogenic E- congenital and E- control. These three pairings allowed us to examine the direct effect of endophyte on freezing tolerance, the association between endophyte infection and a freezing-tolerant host, and the influence of fungicide treatment on freezing tolerance, respectively. Based on several two- and three-way interactions with trial ($P < 0.05$), paired populations were analyzed individually for each of the two trials of Exp. 1. Overall, plants reached a lower LT_{50} and accumulated more tillers in Trial 1 than in Trial 2.

Analysis of covariance showed no significant effect of endophyte presence on plant survival for isogenic E+ and E- populations in Trials 1 and 2, and LT_{50} remained similar across trials (Table 2, Fig. 2). However, there was an effect of entry on freezing tolerance in both trials ($P < 0.001$) (Fig. 2). Entry ranking for isogenic E+ and E- populations was similar for Trials 1 and 2, with 'GrandSlam GLD' and 'Green Emperor' being the most freezing tolerant and W6 11256 and PI 223178 the least freezing tolerant (Fig. 2). There was no effect of endophyte on tiller abundance for isogenic E+ and E- populations in either trial (Table 3). Tillering was affected by entry ($P < 0.001$), with cultivars Green Emperor and GrandSlam GLD consistently accumulating more tillers in both trials (Table 3).

Endophyte was consistently associated with a freezing tolerant host in non-isogenic E+ and E- populations, meaning that E+ plants generally had a lower freezing tolerance than E- congenital plants. There was a significant interaction between population and entry in both trials (Table 2). The LT_{50} for all combinations of endophyte status (E+ and E- congenital) and entry revealed differences between populations for several entries (Fig. 3). In Trial 1, E+ populations of NK200 and PI 610806 achieved an LT_{50} of -15.3 and -14.9°C compared with the non-isogenic E- congenital counterparts' LT_{50} of -12.7 and -13.1°C , respectively. In Trial 2, E+ populations Green Emperor, NK200, PI 610806, and PI 611044 reached LT_{50} of -13.8 , -13.3 , -13.4 , and -13.3°C compared with the E- congenital LT_{50} of -12.8 , -11.7 , -12.1 , and -12.1°C , respectively.

Isogenic E- and E- populations measured the effect of fungicide application on freezing tolerance. The E- congenital and E- control populations did not differ for freezing tolerance in Trial 1 and there was no interaction with entry (Table 2, Fig. 4). Fungicide application affected plant survival in Trial 2 and interacted with temperature (Table 2, Fig. 4). Graphical analysis showed that E- control populations had a lower LT_{50} than E- congenital populations by 0.8°C , with increasing level of freezing tolerance at colder temperatures (Fig. 4). Entry ranking for isogenic E- and E- populations remained consistent in both Trials 1 and 2 (data not shown). The E- control plants accumulated fewer tillers than E- congenital plants despite having a lower freezing tolerance (Table 3).

Experiment 2

Analysis of covariance showed both a significant effect of population and an interaction between temperature and entry for plant survival (Table 2). The presence of endophytes in large non-isogenic E+ and E- populations affected plant survival ($P = 0.028$) and did not interact with entry. Non-isogenic E+ populations had a significantly lower LT_{50} than E- congenital populations by 0.2°C . There was an interaction between temperature and entry for plant survival, so the ranking of the entries changed considerably depending on temperature (Table 2, Fig. 5). GrandSlam GLD was still the most freezing tolerant entry, and W6 11256 and PI 223178 were the least freezing tolerant at the LT_{50} . Entry ranking for tiller abundance was similar to that of Exp. 1, with GrandSlam GLD and Green Emperor both having significantly more tillers than the other four entries (Table 3).

Experiment 3

Analysis of covariance showed no effect of endophyte presence on plant survival for isofrequent E+ and E- populations with LT_{50} of -13.4 and -13.5°C , respectively (Table 2). Additionally, there was no effect of endophyte presence at the LT_{50} for any of the 42 isofrequent E+ and

Table 2. Analysis of covariance for plant survival after freezing for Exp. 1 (Trials 1 and 2), Exp. 2, and Exp. 3. Isogenic endophyte-infected (E+) or endophyte-free (E-) populations consist of E+ and E- fungicide. Isogenic E-/- populations consist of E- congenital and E- control. Non-isogenic E+/- populations consist of E+ and E- congenital. Isofrequent E+/- populations are progeny of E+ and E- fungicide populations.

Source	df	Exp. 1						df	Exp. 2		Exp. 3	
		Trial 1			Trial 2				Non-isogenic	df	Isofrequent	E+/-
		Isogenic E+/-	Isogenic E-/-	Non-isogenic E+/-	Isogenic E+/-	Isogenic E-/-	Non-isogenic E+/-					
Block	1	*	NS†	NS	NS	NS	NS	1	NS	3	*	
Temperature (T)	1	***	***	***	***	***	***	1	***	1	***	
Entry (E)	6	***	***	***	***	***	***	5	***	6	***	
Population (P)	1	NS	NS	***	NS	***	***	1	*	1	NS	
T:E	6	NS	NS	NS	NS	NS	NS	5	***	6	*	
T:P	1	NS	NS	NS	NS	**	NS	1	NS	1	NS	
E:P	6	NS	NS	***	NS	NS	**	5	NS	6	NS	
T:P:E	6	NS	NS	NS	NS	NS	NS	5	NS	6	NS	

*, **, *** Significant at the 0.05, 0.01, and 0.001 $Pr(>\chi^2)$ levels, respectively

† NS, not significant at the 0.05 probability level.

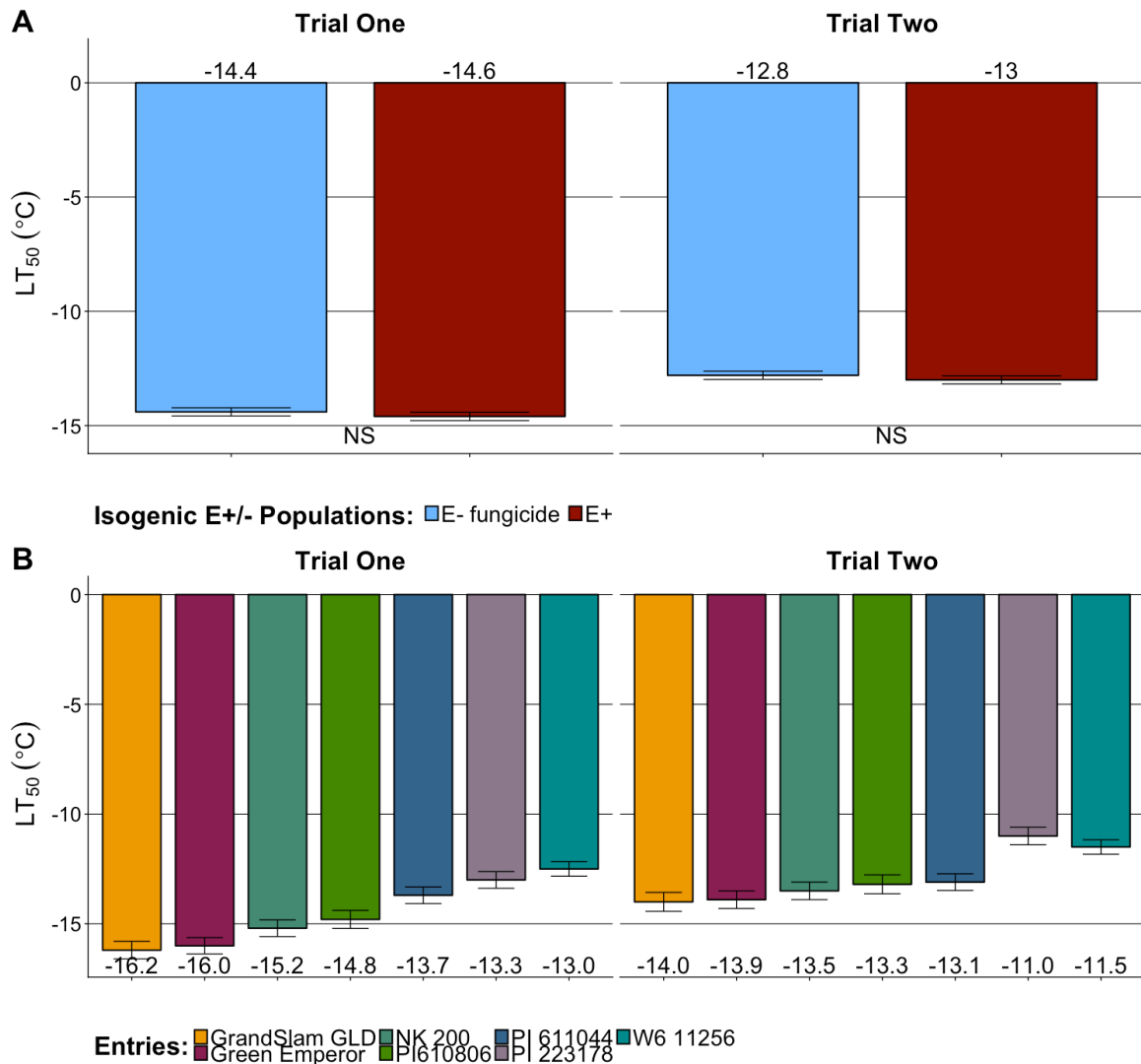


Fig. 2. Lethal temperature 50 (LT₅₀) for Trials 1 and 2 of Exp. 1. (A) Freezing tolerance of endophyte-infected (E+) and endophyte-free (E-) isogenic populations directly tests the effect of endophyte on freezing tolerance. (B) Freezing tolerance of entries combining E+ and E- fungicide populations. LT₅₀ values are given with error bars representing 95% confidence intervals. NS designates no significant differences at the 0.05 probability level.

Table 3. Tiller accumulation by entry and population for Exp. 1, 2, and 3. Experiment 2 only consisted of endophyte-infected (E+) or endophyte-free (E-) congenital populations. Experiment 3 consisted of E+ and E- isofrequent populations from the same maternal sources.

Entry	Mean tiller count			
	Exp. 1		Exp. 2	Exp. 3
	Trial 1	Trial 2		
Green Emperor	5.6a†	3.8a	6.5a	12.5a
GrandSlam GLD	6.1a	4.1a	6.6a	12.5a
PI 611044	4.3b	2.8b	5.2b	10.1bc
PI 223178	4.2b	2.6b	4.9b	7.1d
NK200	4.2b	2.9b	NA‡	10.8ab
W6 11256	4.1b	2.6b	4.7b	9.5bc
PI 610806	3.8b	2.6b	4.7b	9.1c
Population	Mean tiller count			
E+	4.8a	3.7a	5.3a	9.8b
E- fungicide	5.0a	4.3a		
E- congenital	3.7ab	2.4b	5.6a	10.3a§
E- control	3.2b	1.2c		

† Means followed by the same letter within entry and population columns are not statistically different according to Tukey's honest significant difference. ($P < 0.05$).

‡ NK200 was removed from Exp. 2 due to loss of endophyte viability.

§ E- population was isofrequent to the E+ population in Exp. 3.

E- half-sib family pairs, and there were very few effects of family on LT_{50} within entry (data not shown). There was a significant interaction between temperature and entry leading to a few rank changes across temperatures ($P = 0.03$). The LT_{50} of the top two entries (GrandSlam GLD and Green Emperor) and bottom two entries (W6 11256 and PI 223178) remained the same as the isogenic E+ and

E- populations in Exp. 1 (Fig. 5). The presence of endophyte decreased tiller abundance by an average negative effect of 0.5 tillers. Entry ranking for tiller abundance was similar to those of Exp. 1 and 2 (Table 3).

DISCUSSION

Endophytic associations have existed for hundreds of millions of years and are thought to increase plant persistence in stressful environments (Rodriguez and Redman, 2008). The de facto evidence defining the anamorphic *E. festucae* var. *lolii* as a mutualist of perennial ryegrass emanates from the high infection frequencies observed across many ecotypes (Latch et al., 1987; Lewis et al., 1997). A reasonable hypothesis is that endophytic associations persist because the endophyte contributes to plant fitness by either directly contributing favorable metabolites, or changing the host gene expression, increasing fitness in stressful environments (Clay and Schardl, 2002), although this is debatable and not omnipresent (Richmond et al., 2003; Cheplick, 2004). Research has shown that endophyte infection can increase production of the secondary metabolite anthocyanin, which could increase freezing tolerance (Dupont et al., 2015). To elucidate any favorable effects *E. festucae* var. *lolii* may have on the freezing tolerance of perennial ryegrass, we examined wild and cultivated perennial ryegrass germplasm previously described as having a wide array of winter hardiness ranging from poor to excellent and harboring native endophytes.

Morphological development as measured by crown width and tiller number is correlated with winter survival and

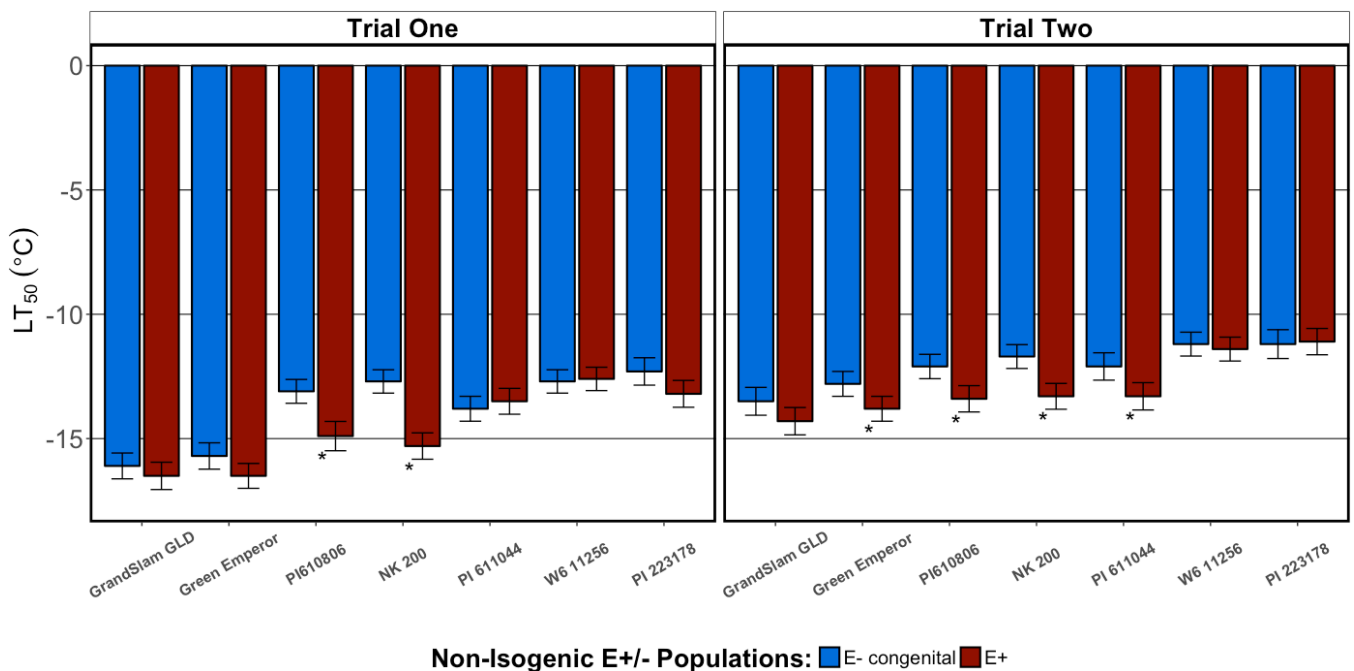


Fig. 3. Lethal temperature 50 (LT_{50}) of non-isogenic endophyte-infected (E+) and endophyte-free (E-) populations for Trials 1 and 2 of Exp. 1. Populations for each entry are shown due to the interaction between entry and E+ and E- congenital populations. Error bars represent 95% confidence intervals. Bars with an asterisk are significantly different for endophyte population at the 0.05 probability level. Differences between populations suggest a relationship between infection frequency and host freezing tolerance.

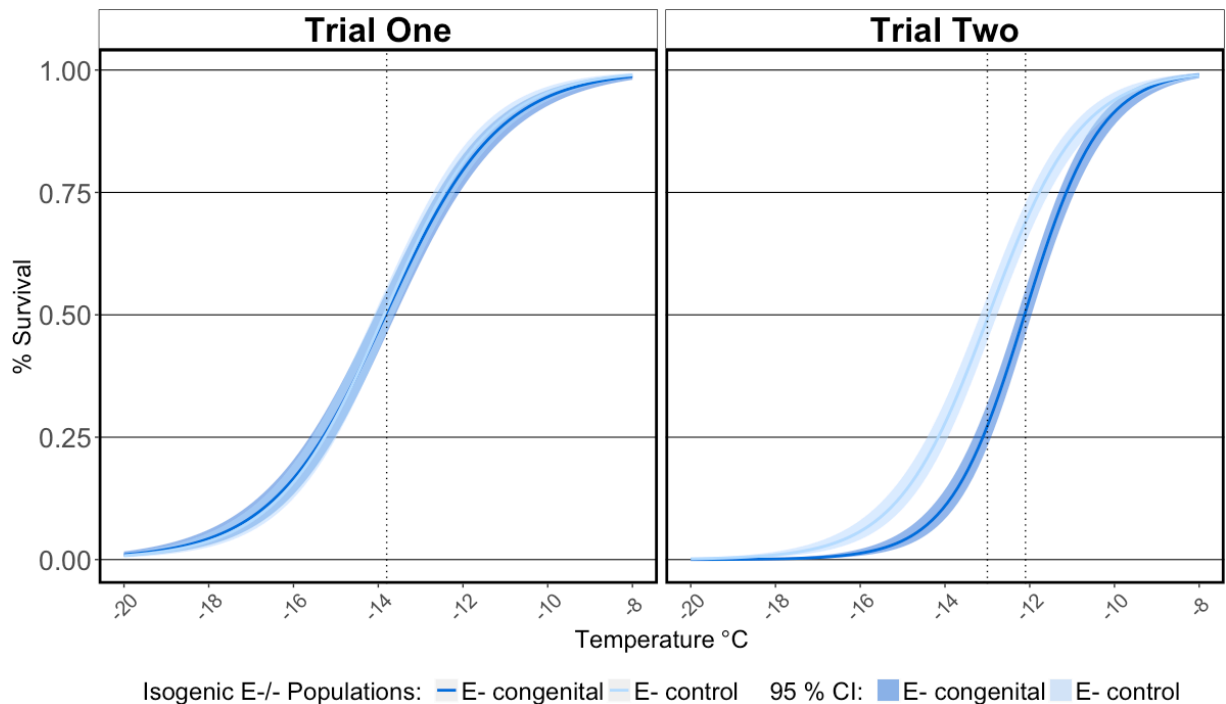


Fig. 4. Plant survival of isogenic endophyte-free (E⁻) and E⁻ populations across freezing temperatures in Exp. 1. Entries could be combined within each E⁻ congenital and E⁻ control population due to a lack of interaction. Dotted lines intersect the logistic regression line at the lethal temperature LT_{50} . Population interacting with temperature caused rank changes outside the inflection point. Error ribbons around predicted lines represent 95% confidence intervals (CI). Differences across temperatures indicate the effect of fungicide on freezing tolerance.

freezing tolerance (Fuller and Eagles, 1978; Hulke et al., 2007). Endophyte infection has been shown to affect many aspects of plant development, including tiller development. For example, Rozpądek et al. (2015) reported that E⁺ plants accumulated more biomass and had increased photosynthetic efficiency, which was likely due to endophyte suppressing seed production. Tiller abundance was measured in each experiment of this study to determine if plant development was altered by endophyte infection. Mean tiller abundance varied between trials, but in Exp. 1 and 2, entry rankings for the number of tillers were very similar ($r = 0.98$, $P < 0.001$). The presence of endophyte in isogenic E⁺ and E⁻ populations had no effect on tillering in Exp. 1 Trials 1 and 2. However, E⁻ control plants accumulated fewer tillers than E⁻ congenital plants, suggesting that fungicides may have affected plant growth. In Exp. 3, endophyte infection had a significant but minor negative effect on tiller abundance (Table 3). Reduction in tillering due to endophyte infection has been reported, especially in cases when stress is applied to the plant (Cheplick et al., 2000).

Results revealed that there was often an interaction between entry and temperature for plant survival; however, the ranking of entries remained fairly consistent across experiments (Fig. 2 and 5). Entry rankings for LT_{50} from these experiments generally agree with Hulke et al. (2008), although direct comparisons are difficult due to the association between endophyte infection- and freezing-tolerant hosts in non-isogenic E⁺ and E⁻ populations. For example, PI 610806 attained a lower LT_{50} than

NK200 in published work (-12.8 and -13.6°C , respectively), but NK200 either ranked above or below the LT_{50} of PI 610806 in Exp. 1, depending on endophyte status of the non-isogenic population (Fig. 3). These results show that entries previously described as winter hardy indeed had a lower freezing tolerance in this study, but they also connote the implications of endophyte infection in non-isogenic hosts.

Although only seven entries were included in this study, this germplasm represented a wide range of adaptation to freezing conditions. Previous literature suggests that stresses imposed on endophyte host associations in their native range can influence stress tolerances conferred by the endophyte (Hesse et al., 2003; Kane, 2011). Two of our accessions, such as PI 610806 and PI 611044, originated from cold environments and have been shown to have exceptional winter hardiness (Table 1). Two of the cultivars, Green Emperor and NK200, were bred specifically for cold climates and have maintained endophyte infection without direct selection for E⁺ parents. Therefore, it would be reasonable to assume germplasm included would be more likely to have associations with an endophyte that confers freezing tolerance, as cold temperatures limit this species distribution.

There was, invariably, no significant direct effect of endophyte on freezing tolerance in this study. Endophyte infection did not interact with temperature or entry in either trials of Exp. 1 (Table 2). However, there was a significant

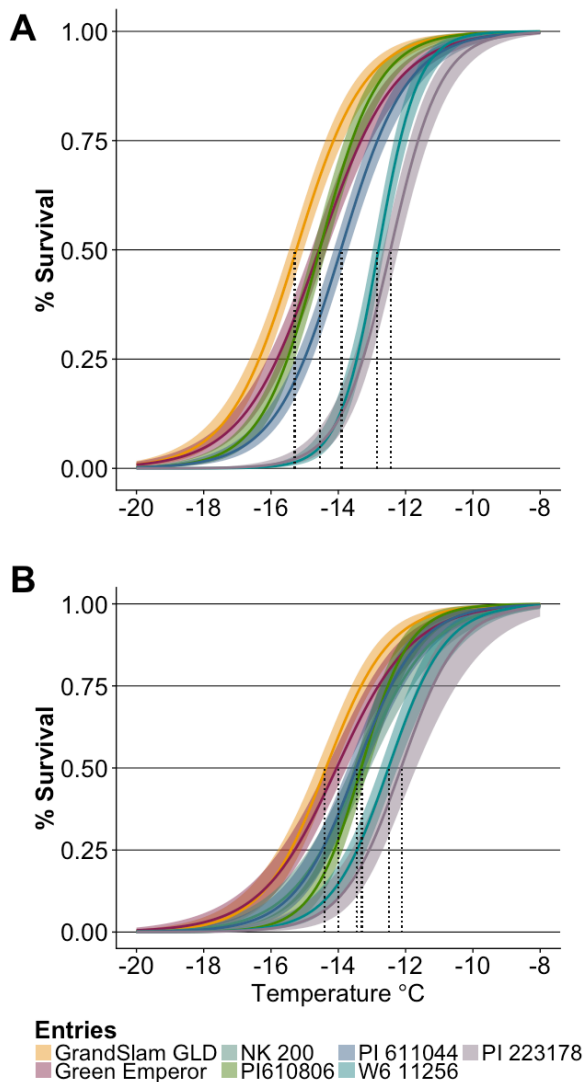


Fig. 5. Plant survival plotted across temperatures for all entries in (A) Exp. 2 and (B) Exp. 3. Interaction with temperature caused ranks changes outside the inflection point. Dotted lines intersect the logistic regression line at the lethal temperature 50. Error ribbons around predicted lines represent 95% confidence intervals.

effect of fungicide application on the freezing tolerance of E⁻ congenital and E⁻ control populations in Trial 2 across all entries (Fig. 4). Therefore, Exp. 3 was designed specifically for the purpose of testing the effect of *E. festucae* var. *lolii* while removing any potential effect from fungicide. Again, no significant differences were detected at the LT₅₀ for any entry or between any isofrequent E⁺ and E⁻ half-sib family pair ($P > 0.05$, data not shown). Considerable variation exists between endophyte strains, and therefore relationships may exist that confer increased freezing tolerance on a genotype basis; however, no individual effects altered freezing tolerance on a population or family level in this study (Johnson et al., 2013). Taken together, our findings provide compelling evidence that *E. festucae* var. *lolii* has no direct effect on the freezing tolerance of perennial ryegrass.

In Exp. 1, consistent associations between endophyte infection and a freezing-tolerant host were observed in

non-isogenic E⁺ and E⁻ populations (Fig. 3). This association was confirmed in Exp. 2 using larger non-isogenic E⁺ and E⁻ populations from the same seed sources ($P = 0.028$); however, effects were not as pronounced as in Exp. 1. It is also interesting to note that significant differences between E⁺ and E⁻ congenital populations are found only in winter-hardy germplasm in Exp. 1, although the effect was seen across all entries in Exp. 2. This may have been due to the reduction in endophyte viability in the seed from Exp. 1 to Exp. 2 and the subsequent loss of the winter-hardy cultivar NK200. These experiments cannot explain this association due to the inability of separating effects of random loss of endophyte in seed, if it is indeed random, or endophytes associating with freezing tolerant hosts. The latter is a complex problem that could be resolved by temporal observation of a selected population—for example, the cultivar NK200, which showed consistent increased freezing tolerance in E⁺ compared with E⁻ congenital populations. One might consider the effect of the loss of endophyte infection in isofrequent E⁺ and E⁻ families over time, and therefore determine if those individual plants that lost endophyte viability first were those that inherently had a lower freezing tolerance. A similar approach was used by Gwinn and Gavin (1992) when studying the effect of *E. coenophialum* on the infectivity of *Rhizoctonia zeae* in non-isogenic populations of tall fescue. Authors found a negative correlation between disease severity and infection frequency in non-isogenic populations. Using isofrequent E⁺ and E⁻ populations could eliminate this problem if conducted in a similar manner, but with using the E⁻ population as a control.

The fitness of a strictly vertically transmitted, anamorphic fungus, such as *E. festucae* var. *lolii*, depends primarily on the fitness of its host (Clay and Schardl, 2002; Saikkonen et al., 2004). If the primary selection pressure imposed by plant breeders in a cold climate is for superior winter hardiness and endophytes do not aid in host overwintering capacity, it would be unlikely that a purely asexual symbiotic organism would be prevalent after many cycles of selection. In this case, the endophyte would likely not be behaving as a mutualist. Clay et al. (2005) observed *Neotyphodium coenophialum* frequency in tall fescue in plots with continual or limited animal herbivory and water stress over 54 mo. Results strongly indicated that presence of herbivory increased the proportion of tillers infected with endophyte over 60 mo of sampling compared with plots receiving limited stress, most likely due to increased competition from E⁻ plants in the stress limited environment. Although *E. festucae* var. *lolii* did not have any direct effect on freezing tolerance in our study, it could be possible that other traits important to fitness in northern latitudes could be enhanced. For example, infected plants may have increased seed production capacity or turfgrass quality

through reduction of disease incidence and subsequently be selected more frequently and therein maintain infection frequency in breeding populations or a natural environment (Wiewióra et al., 2015; Majidi and Mirlohi, 2016).

CONCLUSION

The effect of *E. festucae* var. *lolii* on freezing tolerance was determined through three experiments that isolated the effect of the endophyte from confounding effects including endophyte removal via fungicides and plant host interactions. Endophyte infection had no direct effect on the freezing tolerance on any entry, suggesting limited potential impact on winter hardiness. It should be noted that although freezing tolerance is a major component of winter survival, there are other abiotic stresses that contribute to this trait. Interestingly, there were several consistent associations between freezing-tolerant hosts and endophyte infection. This association may give further insight into the relationship between host fitness and endophyte infection.

Conflict of Interest

The authors declare that there is no conflict of interest.

Supplemental Material Available

Supplemental material for this article is available online.

Acknowledgments

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