

Effect of Alternative Management Practices and the Fungal Endophyte (*Epichloë festucae* var. *lolii*) on Improving the Longevity of Perennial Ryegrass (*Lolium perenne* L.) in Minnesota

A THESIS
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

Garett C. Heineck

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE

Eric Watkins and Nancy J. Ehlke

December 2016

© Garrett Heineck 2016

Acknowledgements

I would like to thank the Department of Horticultural Science and the Department of Agronomy and Plant Genetics for the opportunity to pursue my M.S. degree over the past three years. The projects presented would not have been possible to complete without the collaboration of many students, faculty, staff and farmers.

I am appreciative of the advice and help received from my advisors Dr. Eric Watkins and Dr. Nancy Ehlke. I would also like to thank Dr. James Bradeen for serving on my committee and for the helpful advice received over the years.

For assistance in all of my endeavors in seed production research I am greatly indebted to Mr. Donn Vellekson and Dr. Dave Grafstrom. Their knowledge and dedication to grass seed production systems was critical to this project's success. For technical support on the endophyte project I am appreciative of the assistance that was given by Mr. Andrew Hollman. Finally, many thanks to Mr. Ian Lane for the helpful discussions and collaboration on statistical analyses.

Garett C. Heineck

Foreword

This thesis consists of three chapters. The first is a comprehensive literature review for the following two chapters. Chapters two and three include each, their own specific introduction, materials and methods, results, discussion, conclusion, tables and figures. Periodically, introductions for chapters two and three refer the reader to the introductory chapter for additional information on specific topics. A composite list of references is included at the conclusion of the thesis.

Table of Contents

ACKNOWLEDGMENTS.....	i
FOREWARD.....	ii
LIST OF TABLES.....	v
LIST OF FIGURES.....	vii
CHAPTER 1.....	1
Introduction.....	1
INTRODUCTION.....	2
Origins of <i>Lolium</i>	3
Identifying limitations to seed production in Minnesota.....	7
Winter stress and perennial ryegrass.....	8
Acclimation and deacclimation.....	9
Freezing tolerance and avoidance.....	12
Methods of improving winter survival.....	14
Sward management and winter hardiness.....	15
Clavicipitaceous endophytes role in abiotic stresses.....	17
Drought stress.....	20
Winter hardiness.....	22
Gleaning past research to improve perenniality.....	24
CHAPTER 2.....	25
Exploring alternative management options for multi-year seed production of perennial ryegrass (<i>Lolium perenne</i> L.).....	25
INTRODUCTION.....	25
Seed production in Oregon.....	28
Seed production in northern Minnesota.....	30
Rejuvenation of seed production fields.....	30
Maintaining seed purity in multi-year stands.....	32
Maintaining winter survival on multi-year stands.....	33
Objectives.....	35
MATERIALS AND METHODS.....	36
RESULTS.....	43
DISCUSSION.....	47
CONCLUSION.....	53
TABLES.....	54
FIGURES.....	63
CHAPTER 3.....	66
Effect of the fungal endophyte <i>Epichloë festucae</i> var. <i>lolii</i> on the freezing tolerance of perennial ryegrass (<i>Lolium perenne</i> L.).....	66
INTRODUCTION.....	67
Controlled environment screening for freezing tolerance.....	69
Method of population design.....	71
Objectives.....	76
MATERIALS AND METHODS.....	77
Germplasm.....	78
Endophyte detection methods.....	79

Non-isogenic population design.....	79
Isogenic population design.....	80
Isofrequent population design.....	81
Acclimation protocol.....	82
Freezing protocol.....	83
Experiment 1.....	84
Experiment 2.....	85
Experiment 3.....	85
Statistical analysis.....	86
RESULTS.....	88
Experiment 1.....	88
Experiment 2.....	90
Experiment 3.....	90
DISCUSSION.....	92
CONCLUSION.....	97
TABLES.....	98
FIGURES.....	111
REFERENCES.....	116

List of Tables

CHAPTER 2

Table 2.1 Field locations, soil types and soil test results for both location during trial years one, two and three.....	54
Table 2.2 Average monthly temperatures, extreme minimum winter temperature and number of days averaging a temperature below 0 °C for trial years one, two and three. Weather station provide all temperature data and was located at the GPS coordinates 48.655 N and 95.734 W.	55
Table 2.3 Analysis of variance for early season traits: spring plant count (SPC), relative chlorophyll index (RCI), and volunteer seedling emergence (VSE).	56
Table 2.4 Main effect means for spring plant count (SPC), relative chlorophyll index (RCI) and volunteer seedling emergence (VSE). Trial year were separated when there was a significant treatment by year interaction.	57
Table 2.5 Analysis of variance for late season traits: seed yield, aboveground biomass, harvest index (HI), plant height (PH) and lodging.	58
Table 2.6 Main effect means for late season traits: aboveground biomass, harvest index (HI), plant height (PH) and lodging. Trial years were separated when there was an interaction with treatment. There were no significant effects of plant growth regulator in the ANOVA, therefore it was excluded from means separation.	59
Table 2.7 Correlation among measured variables including spring plant count (SPC), relative chlorophyll index (RCI), harvest index (HI), lodging, volunteer seedling emergence (VSE), plant height (PH), aboveground biomass, seed yield over all three trial years and locations.	60
Table 2.8 Analysis of variance for seed quality characteristics: test weight (TW), thousand seed weight (TSW) and germination percentage.	61
Table 2.9 Main effect means for seed quality traits: test weight (TW), thousand seed weight (TSW) and germination percentage. Seed quality traits were only measured in trial year three.	62

CHAPTER 3

Table 3.1 List of entries included in Experiments 1, 2 and 3. Winter hardiness of entry is reported when possible.	98
Table 3.2 Population size for each of the seven entries in Experiment 1. E+ and E- fungicide populations are isogenic. E- congenital and E- control populations are isogenic. Endophyte infection frequency and the sample size of the test for each entry are reported.	99

Table 3.3 Population size for each of the six entries in Experiment 2. E+ and E- congenital populations are non-isogenic. Endophyte infection frequency and sample size is reported for all seven entries.	100
Table 3.4 Number of isofrequent E+ and E- half-sib families and population size for all seven entries in Experiment 3. Endophyte infection frequency and sample size for each entry is reported. Average germination percentage for each entry is reported.....	101
Table 3.5 Analysis of covariance on combined trials for Experiment 1. Isogenic E+ and E- populations consist of E+ and E- fungicide. Isogenic E- and E- populations consist of E- congenital and E- control. Non-isogenic E+ and E- populations consist of E+ and E- congenital.	102
Table 3.6 Analysis of covariance for plant survival of both trials one and two of Experiment 1. Isogenic E+ and E- populations consist of E+ and E- fungicide. Isogenic E- and E- populations consist of E- congenital and E- control. Non-isogenic E+ and E- populations consist of E+ and E- congenital. Isogenic and non-isogenic pairs were analyzed together and separately due to a significant interaction with population.....	103
Table 3.7 Main effect means for tiller accumulation for seven entries in Experiment 1 trials one and two.	104
Table 3.8 Analysis of variance for trial one and two of Experiment 1. Tiller accumulation of isogenic E- and E- consisting of E- congenital and E- control.....	105
Table 3.9 Analysis of covariance for plant survival in Experiment 2. Large non-isogenic E+ and E- populations consist of E+ and E- congenital.....	106
Table 3.10 Main effect means for tiller accumulation for six entries in Experiment 2.....	107
Table 3.11 Analysis of covariance for plant survival in Experiment 3. Entry consisted of Isofrequent E+ and E- half-sib populations.	108
Table 3.12 Analysis of covariance for plant survival of E+ and E- half-sib families in Experiment 3. Each of the seven entries is listed in a separate column.....	109
Table 3.13 Analysis of variance for Experiment 3. Tiller accumulation of isofrequent E+ and E- populations.	110

List of Figures

CHAPTER 2

Figure 2.1 Accumulation of growing degree days (GDD) for trial year one, two, and three calculated using a base temperature of 0° C from March 1st to August 7th. . Weather station provided all temperature data and was located at the GPS coordinates 48.655 N and 95.734 W.63

Figure 2.2 Effect of year and fall residue management on final seed yield reported as kg ha⁻¹. Fall residue management treatments were open burn, simulated bale and mow and control. Letters denote significant differences within years according to Tukey's HSD ($P \leq 0.05$). Treatment means are listed below letters.64

Figure 2.3 Effect of four plant growth regulators (PGR) and a control on spring plant count reported as plants per m². Trial years one two and three are combined. Letter denotes significant differences between PGRs according to Tukey's HSD ($P \leq 0.05$).....65

CHAPTER 3

Figure 3.1 Flow diagram of Experiments 1, 2 and 3. Green arrow specifies the same seed source for Experiment 1 was used in Experiment 2. Red arrow identifies that *E+* and *E-fungicide* populations were used as parents in Experiment 3.....111

Figure 3.2 Effect of endophyte across freezing treatments. A) All entries from *E+/-* isogenic populations from Experiment 1 trial one. B) All entries from *E+/-* isogenic populations from Experiment 1 trial two. C) All entries from *E+/-* isofrequent populations from Experiment 3. Solid lines represent the predicted survival curve with the shaded region reprinting the 95% CI.112

Figure 3.3 Effect of endophyte on an entry basis across freezing treatments. A) Entries from *E+* and *E-* non-isogenic populations from Experiment 1 trial one. B) Entries from *E+* and *E-* non-isogenic populations from Experiment 1 trial two. C) Entries from non-isogenic *E+* and *E-* populations from Experiment 2. Solid lines represent the predicted survival curve with the shaded region reprinting the 95% CI.113

Figure 3.4 Effect of endophyte on an entry basis across freezing treatments. A) Entries from *E-* and *E-* isogenic populations from Experiment 1 trial one. B) Entries from *E-* and *E-* isogenic populations from Experiment 1 trial two. Solid lines represent the predicted survival curve with the shaded region reprinting the 95% CI.....114

Figure 3.5 Effect of endophyte across entries in Experiment 2. Entries were combined due a lack of interaction between endophyte, entry and temperature. Dotted lines intersecting the solid line at 50 % survival is the LT₅₀ for *E+* and *E- congenital* populations.115

CHAPTER 1.

INTRODUCTION

Garett C. Heineck

Perennial ryegrass (*Lolium perenne* L.) is a widely used turf and forage grass. It is cultivated in North America, Europe, Australia and New Zealand. Its extensive use is due to its high seed production capacity, superior vigor and wear tolerance. As a turfgrass, perennial ryegrass is used on sports fields, home lawns and golf courses. The ability of perennial ryegrass to produce large quantities of seed with high seedling vigor is the impetus for its inclusion in many turfgrass mixes. The broad use of this species is not without limitations as swards grown in northern temperate regions are commonly subject to severe winterkill limiting its use as a turfgrass or forage (Beard, 1966 and Waldron et al., 1998a).

Major perennial ryegrass seed production regions in the United States are western Oregon and northern Minnesota. To meet demands of consumers, seed production capacity cannot be limited by winterkill, which is a common problem in northern Minnesota. Furthermore, increasing production capacity of perennial ryegrass in cold climates will not only help seed producers, but will also improve the utility of perennial ryegrass for turfgrass and forage managers in northern latitudes. Although the focus of this research pertains to perenniality of seed production systems the results could be applied to other areas of perennial ryegrass management.

The University of Minnesota breeding program has provided the grass seed consumers and producers of Minnesota with cultivars that exhibit satisfactory turf quality and produce economically viable seed yields. To this point, there has been a concentration of efforts on developing winter hardy cultivars that will survive one winter in seed production fields and will often survive multiple years when maintained as turf (Waldron et al., 1998b; Hulke et al., 2008 and Hulke et al., 2007). This goal, however, has been met with slow progress. Although varieties can commonly survive one winter, further survival is variable in both home lawns and production fields. This is likely due to slow gains from selection in breeding programs and

lack of variation in ryegrass germplasm (Iraba et al., 2013). Finding novel methods of increasing winter survival is essential for successful adaptation of perennial ryegrass.

Origins of Lolium

Understanding where a species or its family originated, environments it has adapted to, and how it spread to other regions is essential to understanding the agronomic potential and breeding limitations of the species. There are approximately 10,000 species of grass that cover an estimated 1/3 of the earth's land surface area however, only about 50 species have been utilized as turfgrasses (Turgeon, 2004; Beard, 2012). Early grass species evolved with woody perennials in savannas, which after becoming more arid, burned periodically removing most plant species save the grasses that evolved to survive burning by basal tillering (Beard, 2012). Grazing animals also sculpted the growth habit and range of perennial grasses.

A review by Stebbins (1981) offers insight into the global coevolution of grazing animals and grass species. Grazing animals such as those in the Bovidae family evolved unique anatomical morphology, such as high crowned teeth, to consume vast amounts of forage. In turn grasses had to further their ability to tolerate and avoid severe defoliation. It is interesting to note the observed traits of many predominant savannah grasses often align with those of turf and forage grasses. In fact, the term "grazing lawn" is used to describe the resulting dense prostrate growth of grasses in response to grazing gregarious animals (McNaughton, 1984). For example, density is a sought-after trait by turfgrass breeders and one of the common traits of savannah grasses is higher biomass per unit area disallowing the complete defoliation of the plant. Indeed, grasses have also developed many other distinctive mechanisms, besides growth habit, in defense against herbivory such as production of

indigestible silica bodies and infection of endophytes that produce toxic alkaloids (Gurevitch et al., 2006).

Lolium is a temperate grass genus consisting of about eight species (Terrell, 1968). Three of these species, *L. perenne* L., *L. multiflorum* Lam. and *L. rigidum* Gaud., are considered outcrossing species (Fearon et al., 1983). The other five species, *L. temulentum* L., *L. remotum* Schrank., *L. subulatum* Vis., *L. persicum* Boiss and Hohen and *L. canariense* Steud. are self pollinating species (Charmet and Balfourier, 1994). *Lolium* spp. probably originated in the Mediterranean region and spread into Asia, north Africa and Northern Europe (Beard, 2013). The genus *Lolium* is referred to in the Bible, likely due to its spread as a weed, in tandem, with that of wheat (Humphreys et al., 2010). In places such as Israel, darnel grass (*Lolium rigidum* Gaud.) is still a common weed in wheat fields.

Movement of *Lolium* spp. away from the point of origin had direct effects on perenniality and speciation because those that spread to warm regions are considered annuals (*L. rigidum*) and those that migrated to Europe evolved into perennials (*Lolium perenne*) (Balfourier et al., 2000). The origin of perennial ryegrass lends several clues as to the cause of common problems experienced by turfgrass and forage managers using perennial ryegrass in northern climates. The inability of perennial ryegrass to adapt to extreme temperatures precluded the transcontinental movement into North America without anthropological intervention. It is likely that *Lolium* spp. reached North America via early European settlement where it survived as a short-lived perennial or winter annual in northern climates (Beard, 2013).

Grasses have evolved several strategies to survive under intense grazing. The formation of the *Lolium* genus is a prime example of a species that evolved as a direct results of heavy animal grazing (Casler, 2006). In fact, grazing is a common requisite to long term survival of perennial ryegrass in natural settings (Casler et al., 1996). As grazing pressure increased,

several changes happened including later heading, lower growth habit and dense tillering. Many perennial grasses including perennial ryegrass are obligate outcrossing species. This permits rapid increases in favorable alleles that allowed populations to rapidly overcome environmental stresses. Increased seed production is one other adaptation that allowed perennial ryegrass to survive in adverse conditions where perennial growth is inhibited by stresses such as grazing or extreme temperatures (Sampoux et al., 2013). By producing seeds that are able to survive winter conditions many species are able to thrive in environments that would otherwise disallow continuation of the species (Burke et al., 1976). Many perennial grasses produce horizontal stems such as stolons or rhizomes to asexually reproduce. Perennial ryegrass has no such mechanism but instead forms a bunch by aggressively tillering from a primary meristem (Jewiss, 1972).

Perenniality of bunch-type grasses is centered on the formation of new tillers after old tillers have either flowered or died of other stresses (Hill and Watkin, 1975). The equilibrium between dying and emerging tillers must be kept static in order to extend sward longevity (Bahmani et al., 2000). Upon germination, an initial shoot is formed which consists of many compressed internodes ending in an apex and initially protected by a coleoptile (Jewiss, 1972). At each node there is a leaf primordium in which the collar and leaf blade originate. As the leaf expands it envelops the tiller bud that forms in the axil at each node. As buds are released from apical dominance, either because of increased carbohydrate allocation or due to a hormonal change, they develop into tillers (Jewiss, 1972). Tillers arising from the main stem are classified as primary tillers, tillers forming from primary tillers are secondary tillers and those formed from these are tertiary tillers (Bahmani et al., 2000).

As tillers age they are more likely to go from a vegetative state to a reproductive state (Hill and Watkin, 1975). The reproductive phase for most cool-season grasses is highly

dependent on day length and temperature. Within *Lolium* these requirements are highly variable. For example, species such as *Lolium temulentum* need only exposure to long days in order to flower whereas northern ecotypes of *Lolium perenne* require exposure to temperatures of 3 °C for 12-16 weeks in order to initiate primary induction (Evans, 1958; Evans, 1960). A secondary induction is also required in *Lolium perenne* and is cued by long day exposure that consequently initiates flowering (Evans, 1960). *Lolium* spp., in particular, clearly demonstrate the direct link between primary reproductive induction and perenniality in temperate grasses (Heide, 1994). This particular prerequisite aids in protecting the plant from precocious flowering in regions with severe winters. It also means that for species with dual requirements, seed production is limited by the number of tillers that have undergone primary induction. In any case, each tiller is considered monocarpic and will cease to exist post sexual reproduction. Therefore, to maintain perennial habit new vegetative tillers must succeed reproductive tillers after flowering. Management post defoliation, such as fall fertilizer application, can impact flowering in the spring due to increased numbers of fall tillers (Heide, 1994). Maintaining consistent production of vegetative tillers in perennial ryegrass swards often requires defoliation. This trait is linked to the aforementioned abiotic and biotic stresses and makes perennial ryegrass a principal species for use as a turf and forage grass assuming tillers can survive winter conditions.

Direct methodical selection for improved perennial ryegrass forage varieties has been underway for the past century (Stewart, 2006). As pressure under grazing is similar to stress under mowing and foot traffic, perennial ryegrass forage varieties were somewhat successfully used as turfgrass. Selection for turfgrass quality is a more contemporary practice and has only been conducted for the last 50 years (Sampoux et al., 2013). However, perennial ryegrass still has not been successfully adapted to cold climates. Recent climatological reports indicate that

northern latitudes will likely warm faster than near the equator (Change, 2013). Although this will likely aid in the adaptation of perennial ryegrass other challenges will still be present.

Larsen (1994) described winter hardiness as a supercharacter composed of many sub-components, each of which may or may not be present during a given winter. The trend towards gradual warming may help to alleviate some problems while intensifying others (Dalmannsdottir et al., 2015). For example, extreme winter minimum temperatures may rise, but reduced snow accumulation may result in reduced soil temperatures.

Identifying Limitations to Seed Production in Minnesota

Previously described was the evolutionary importance of stresses that have heavily influenced the growth habit and stress-tolerance of perennial grasses. This section focuses specifically on grass seed production. Burning and animal grazing both influenced grass speciation and spread. Contemporary management practices mimicking ancient processes also influence the persistence and productivity of grass swards. Challenges facing seed producers are different than those of turf and forage managers, as seed producers must obtain economically viable seed yield. In Oregon, perennial ryegrass seed production fields routinely maintain economically viable seed yield for four years when managed properly (Young et al., 1999). Field establishment for seed production is drastically different from turf swards. For example, perennial ryegrass seed production fields are seeded at a rate 6.7 kg ha^{-1} and turf swards are commonly seeded at 292.5 kg ha^{-1} (Ehlke and Vellekson, 2006; Christians, 2011). Seed is also drilled into 15 cm rows in production fields instead of broadcast seeding as is done to establish turfgrass swards. These two components are used specifically to reduce plant competition and monitor seed purity of perennial seed production swards (Hardison, 1980).

Oregon is the largest seed production region in the United States. In 2015, Oregon produced 92% of all perennial ryegrass seed in the US, although this percentage has been dropping steadily from nearly 100% since 2005 (NASS, 2016; NASS, 2006). Oregon State University has been publishing documents since the 1940s that focus on maintaining productive perennial seed fields (Hardison and others, 1949). Literature reviews have directly stated that the management of grass seed swards must mimic evolutionary stresses that sculpted grassland ecology and genetics (Hardison, 1980). Oregon seed producers have adopted several strategies to overcome increased stand density and straw accumulation post-harvest. Burning fields after harvest became a popular practice in the 1950s in Oregon, but fell out of use due to human health concerns (Hardison, 1980). In response to restrictions on burning, researchers have turned to alternative methods of residue control such as baling and/or chopping residue (Chastain et al., 1995). Both burning and chopping/baling fields are closely related to natural residue management practices grasses would have had to cope with in the distant past.

Seed producers in northern Minnesota have traditionally only been able to produce a single year of seed from perennial ryegrass fields. Therefore, residue management has not been a concern. Reasons for biennial seed production stem primarily from low inherent winter survival of perennial ryegrass in Minnesota (Waldron et al., 1998b). As both turf managers and seed producers would benefit from increased winter hardiness, novel breeding and management strategies should be explored. With that in mind multi-year seed production not only hinges on successful overwintering, but also residue management of perennial swards.

Winter Stress and Perennial Ryegrass

Winter conditions may be the most limiting factor of plant species distribution (Parker, 1963). In response, breeding for winter hardiness is the most commonly addressed abiotic

stress in grasses (Casler et al., 1996). Winter hardiness is often treated as a single stress, however, it should be considered a complex of many stresses that can be imposed simultaneously or individually depending on the year (Pearce, 2001; Kalberer et al., 2006; Gusta and Wisniewski, 2013; Larsen, 1994). For a species to successfully survive winter, plants must have a panoply of traits to overcome each subsidiary stress. The plant must overcome the combination of stresses of any given winter in order to be successful. Given that these individual stresses can happen at once or individually, it is not surprising that the adaptation to northern temperate regions has not been wholly successful for perennial ryegrass. To cope with adverse conditions, perennial ryegrass has evolved several mechanisms that allow for successful overwintering which can be combined into two broad categories 1) aspects of acclimation and deacclimation pre- and post-winter and 2) tolerance and avoidance mechanisms during winter.

Acclimation and Deacclimation

The ability of a plant to survive various winter stresses often depends on the status of acclimation going into winter. Acclimation can be defined as “an increase in tolerance over time to cold temperatures and cellular desiccation in response to inductive conditions such as cold temperature, short photoperiods, mild drought, and results from changes in gene expression and physiology” (Kalberer et al., 2006). The ability of perennial ryegrass to acclimate can be heavily influenced by day length, light intensity, nutrient availability and CO₂ concentration (Eagles and Williams, 1992; Webster and Ebdon, 2005; Harrison et al., 1997). These factors can affect acclimation analogously or inversely across different genetic backgrounds. Dalmannsdottir et al. (2015) tested the effect of temperature before acclimation in timothy (*Phleum pratense* L.), perennial ryegrass and red clover either adapted to northern or

southern Norway. Results demonstrated that, while northern collections were more apt to acclimate at higher temperatures, increasing light prior to acclimation decreases the ability to survive freezing temperatures. Environmental cues initiate acclimation, which in combination with inherent mechanisms of coping with winter stress, subsequently dictate freezing tolerance.

Cold acclimation induces many changes in the plant cell such as reduced water content, reduced growth, modification of lipid membrane, cell wall modification and osmotic regulation of amino acids and carbohydrates (Xin and Browse, 2000). Hoffman et al. (2010) measured biochemical and physiological changes in four perennial ryegrass accessions varying in winter hardiness when subjected to different acclimation treatments. Plants were acclimated at 2 °C for 21 days and tissue was sampled at 0, 7, 14 and 21 d for metabolic analysis. As the length of acclimation increased, significant changes in water soluble carbohydrates, proline accumulation, and leaf photochemical efficiency were recorded. Of these, only water soluble carbohydrates had a significant difference in accumulation between winter-hardy and non-hardy accessions. These changes were associated with significant increases in freezing tolerance.

Research also shows that initial acclimation is not enough to acclimate properly prior to winter. Some plants have the propensity to deacclimate during favorable conditions in early spring, but not before potential damaging conditions. Hoffman et al. (2014) quantified differences between two grass species testing deacclimation treatments and their ability to survive subsequent freezing. One accession each of non-winter hardy annual bluegrass and winter hardy creeping bentgrass were acclimated at -2 °C for 2 weeks. Subsequent deacclimation showed that creeping bentgrass had the ability to acclimate to a more favorable level of freezing tolerance than annual bluegrass. However, after five days of deacclimation at 12 °C, annual bluegrass and creeping bentgrass had the same freezing tolerance. This suggests

that without acclimation or with extensive deacclimation a freezing tolerant and non-tolerant species may reach the same level of freezing tolerance. Selection of plants with the ability to acclimate more quickly and remain in a state of dormancy for a longer period could be critical to the survival of perennial ryegrass in northern climates. Understanding pre-acclimation and acclimation is an important step in recognizing the fundamental mechanisms of winter survival.

Grasses have evolved several general survival mechanisms to overcome winter stresses once acclimated. Generally, two categories can explain the ability of a grass plant to survive abiotic aspects of winter: 1) level of dormancy or the ability of a plant to remain inactive when environmental conditions permit growth (Abe, 1980; Cooper, 1964) and 2) by either tolerating or avoiding freezing (Pearce, 2001; Burke et al., 1976). These two categories may exclude other stresses such as ice sheeting, winter desiccation or presence of diseases that can limit winter survival (Höglind et al., 2010; Humphreys, 1989; Casler and Van Santen, 2010). Some of these mechanisms, like dormancy, directly relate to acclimation but are not related to direct physiological changes. Rather, these mechanisms have already been pre-imposed during acclimation. Breaking down winter hardiness into groups of stresses and their known survival mechanisms offers key advantages. For example, identifying germplasm collections that can survive some stresses that other germplasm cannot and vice versa allows strategic recombination between plant collections.

In species such as alfalfa, germplasm is commonly aggregated by level of dormancy on a 1-9 scale (Undersander et al., 2005). Therefore, areas that are less prone to winter kill can support plants with lower levels of dormancy, or a higher dormancy rating. Although perennial ryegrass does not have a formal rating scale for dormancy, similar concepts can be applied. Cooper (1964) found that some collections of both perennial ryegrass and orchardgrass (*Dactylis glomerata* L.) displayed winter growth (lack of dormancy) in a manner negatively

correlated with winter hardiness. These specific physiological manifestations were related to the typical environmental conditions at the collection site. Overall, success in correlating subsidiary traits of winter hardiness is variable across experiments. For example, Hofgaard et al. (2003) correlated winter survival in the field with freezing tolerance, snow mold resistance, plant size and ice encasement using a full-sib family of perennial ryegrass. Freezing tolerance was correlated with plant size and winter survival. However, freezing tolerance was not correlated with snow mold resistance. Snow mold resistance was significantly correlated with winter survival. In this study ice encasement was not correlated with any other response, however there were significant differences between full siblings for ice encasement tolerance. The authors concluded that the genes for freezing tolerance and ice encasement are probably different and that there was likely no pressure in the field for ice encasement in the trial years. Humphreys (1989) found that using an indexing approach with 14 different factors gave the best correlation with winter survival and was thought to be associated with winter growth (lack of dormancy), winter hardiness and early spring green-up. Relevant literature makes it abundantly clear that in order to survive the winter, perennial ryegrass must possess the ability to successfully overcome many stresses. Furthermore, some of these stresses may not show up every year or only very rarely in particular environments. Finally, plants that acclimate and remain dormant may still succumb winter injury because of freezing conditions.

Freezing Tolerance and Avoidance

Plants respond to freezing by either dying, avoiding stress, or tolerating the stress. It should be noted that plants must always avoid intracellular freezing, but not necessarily intercellular freezing. Therefore, the word “avoid” will be incorporated into mechanisms that involve freezing “tolerance”. Annual grass species may die as soon as a freezing event occurs;

however, their seeds may be able to survive at very low temperatures. Other grasses may avoid freezing temperatures by keeping their growing point at or below the soils surface where temperatures remain relatively warm. Some species of dogwood, which cannot avoid freezing temperatures, are able to survive temperatures of $-196\text{ }^{\circ}\text{C}$ by extensive cellular dehydration (Sakai and Yoshida, 1967). Other woody perennials avoid freezing by deep supercooling, or avoiding freezing below $0\text{ }^{\circ}\text{C}$. Pure water will not freeze until $-38.5\text{ }^{\circ}\text{C}$ when spontaneous ice nucleation occurs because of the lack of ice nucleators (Franks, 1985). Plants can further depress supercooling points by producing anti-freeze compounds. Anti-freeze compounds have been identified in cereals and have high degree of sequence similarity to pathogenesis related proteins (Yu and Griffith, 1999). Perennial ryegrass is able to tolerate low levels of freezing stress, but can avoid more extreme stress by keeping its meristem at or below the soils surface (Hulke et al., 2008; Burke et al., 1976). Most herbaceous plants, like perennial ryegrass, do not have a low supercooling point, but can tolerate intercellular freezing; for instance, winter wheat is able to tolerate intercellular freezing down to $-25\text{ }^{\circ}\text{C}$. When ice forms in intercellular spaces, but is excluded from intracellular spaces, water is drawn from inside the cell (Pearce, 2001). This is because ice has a lower water potential than that of water, furthermore as the temperature of the ice decreases so does the water potential (Guy, 1990). As water is drawn out of the cell the cellular contents become more viscous and the plant is at risk of damage from dehydration. Some plants are able to modify their cell membranes to accommodate or stop dehydration (Steponkus et al., 1988). This can result in plants having similar lethal freezing temperatures, but different lethal freezing durations.

Plants have many ways of coping with freezing conditions in order to avoid intracellular freezing. Freezing injury is critical to plant survival during winter and can be used as a proxy for winter hardiness. Significant correlations between controlled environment freezing

tolerance and winter survival have frequently been found and are considered an adequate alternative to measuring field survival (Fuller and Eagles, 1978; Humphreys and Eagles, 1988; Hulke et al., 2008).

Methods of Improving Winter Survival

Many attempts to increase the winter hardiness of perennial ryegrass through breeding have been documented. Sampoux et al. (2013) compared 31 turf-type perennial ryegrass varieties released over 30 years for the purpose of measuring changes in archetypal turfgrass traits. For each variety they measured general turf quality, wear tolerance, crown rust resistance, vertical growth and winter greenness (winter survival). All traits except winter greenness were improved substantially in contemporary varieties. This is a strong indicator that there is a lack of heritability and/or genetic variance for winter hardiness. A report by Hulke et al. (2007) examined 300 landrace perennial ryegrass accessions for winter hardiness over two years and found that although there was significant variation for winter hardiness high levels of winter kill were measured across all accessions. Moreover, the effect of year on accession performance was substantial. This adds to the proof that the stresses associated with winter hardiness change in nature and intensity from year to year. Iraba et al. (2013) used controlled freezing tests and recurrent selection to improve the freezing tolerance of two populations of turf type perennial ryegrass. Gains from selection were modest and inconsistent across cycles of selection, possibly because of variable acclimation treatments associated with the natural acclimation protocol used in this study. A common theme amongst studies is the lack of predictability for specific winter conditions from year to year and the effect of those conditions on specific populations of perennial ryegrass.

Strong correlation between freezing tolerance and winter hardiness has been proven across many studies. However, with low genetic variability and heritability in the known perennial ryegrass germplasm, only meager gains from long-term selection have been achieved. Using established screening techniques other options should be examined for mitigating winter stresses. Well-documented cases of plant-microbe interactions and management strategies exist for increasing persistency of perennial grasses. One of the most notable cases in which a plant-microbe interaction has allowed the successful survival of an organism in a extreme climate was documented by Márquez et al. (2007). Researchers found that a three-way interaction between a grass (*Dichanthelium lanuginosum* Torr.) a fungus (*Curvularia protuberate*) and a virus (*Curvularia* thermal tolerance virus) allowed the host plant to survive the extreme heat of Yellow Stone geothermal soils. Although this example does not portray microbe effects on winter stress it does show strong affects acting on abiotic stresses.

A substantial body of literature is available on the best management practices for optimizing a grass plants ability to overcome winter stress. Stand establishment, fertilizer rate, mowing and growth regulation all influence sward persistence and quality (Beard, 1972). Sward management as well as examining common endophytic relationships in combination with breeding could lead to a more ubiquitous solution to the quandary of winter hardiness.

Sward Management and Winter Hardiness

Perennial ryegrass is not only grown as a turf and forage in northern climates, it is also an economically important seed crop. Successful seed production in Minnesota, to some degree, is a function of winter survival. However, grass plants can survive the winter and not necessarily produce economic seed yields. Therefore, increasing winter hardiness is not the only consideration for seed producers. To maximize the genetic potential of perennial ryegrass

varieties in cold climates, correct management techniques must also be employed. Researchers from the University of Minnesota have developed several strategies for increasing winter survival of perennial ryegrass in seed production swards. Nutrient management can have a significant effect on winter survival. For example, the application of fall phosphorus significantly enhanced winter survival of first year seed production crops (Ehlke et al., 2014). Also, perennial ryegrass is commonly seeded with a spring wheat nurse crop. Perennial ryegrass grows beneath the spring wheat until harvest in late summer. Stubble left from the wheat acts as a snow catch that improves winter survival by insulating the grass over the winter. However, this effect is subsequently lost in the second year of seed production.

Nutrition rate and application timing can also play an important role in the freezing tolerance of perennial ryegrass when grown as a turf (Webster and Ebdon, 2005). Application of nitrogen has a large effect on the freezing tolerance of perennial ryegrass most likely due to the plant's inability to acclimate properly (Carroll, 1943). Potassium content and winter survival have also been shown to correlate: Brooks et al. (1980) found that the perennial ryegrass variety 'Manhattan' had an increased amount of winter kill if the nitrogen:potassium ratio exceeded 2:1 in the tillers. Considering both fertilizer composition and application timing is critical to maintaining winter hardiness.

The application of plant growth regulators (PGR) has also been extensively used in turfgrass management as well as seed production because of their ability to alter plant growth and physiology. The growth regulators prohexadione calcium and paclobutrazol have been used to increase seed production capacity in seed production fields (Koeritz et al., 2013; Hampton and Hebblethwaite, 1985). Both of these chemicals decrease gibberellic acid production by disrupting the synthesis pathway. Decreases in stem elongation during flowering subsequently reduce lodging, lower disease pressure, and improve seed yield.

Tiller survival has been associated with the production of seed heads in the spring. Emphasis should be placed on tillers produced prior to winter to improve seed production (Chastain and Young III, 1998; Hill and Watkin, 1975; Langer and Lambert, 1959). Several plant growth regulators have been linked with fall dormancy and winter survival. For example, fall applications of trinexapac-ethyl increased rate of dormancy of Bermuda grass (*Cynodon dactylon* L.), although it did not affect winter survival (Fagerness et al., 2002). Other plant growth regulators can alter carbohydrate source sink relationships within the plant (Cooper et al., 1988). As osmotic regulation of proteins and carbohydrates is an essential component of cold acclimation these PGRs may influence winter survival (Xin and Browse, 2000). A review by Jewiss (1972) summarizes that swards with high proportions of tillers in a reproductive state, such as a seed production field in late summer, are at risk of non-rejuvenation due to dominance held by reproductive tillers over intercalary buds. Seed heads are metabolic sinks restricting tillering during flowering (Phillips, 1975). More importantly, apical dominance is established and held by auxin produced in seed heads (Phillips, 1975). Applications of cytokinin can release grass tillers from their dormant state during flowering or immediately after flowering (Sachs and Thimann, 1964; Jewiss, 1972). Although not directly linked with winter survival sward longevity hinges on the successful replacement of reproductive tillers with vegetative tillers. The application of PGRs could facilitate the production of vegetative tillers.

Clavicipitaceous Endophytes Role in Abiotic Stresses

Symbiosis, or the close ecological interaction between two species was initially proposed by De Bary (1879). While ecological and evolutionary ramifications of symbiosis have been studied and debated for over a century, symbiotic relationships have only recently

been exploited for economic and agricultural benefit (Johnson et al., 2013; Kauppinen et al., 2016). The term endophyte, as used in this manuscript, was coined more recently and refers to a microbial organism living within a plant without causing symptoms (Wilson, 1995).

Endophytes can be fungal, bacterial, viral or a combination of these organisms (Márquez et al., 2007).

Class one endophytes are termed Clavicipitaceous endophytes, have a narrow host range and are found in grasses (Rodriguez et al., 2009). There are three other classes of endophytes that have a considerably wider host range and are associated with plants in all areas of the world (Rodriguez et al., 2009). Clavicipitaceous endophytes all infect grasses and do not invade host cells; rather they feed from extracellular nutrients and grow through the plant in an intercellular manner. These endophytes grow by intercalary hyphal extension through leaves from their point of origin in the grass meristem (Christensen et al., 2008). This is a highly regulated and delicate growth habit that accommodates the rapid extension of grass leaves.

Research has been shown that change to a single gene can turn an endophyte from a mutualist to a plant parasite, which indicates these endophytes may have at one time been pathogens (Tanaka et al., 2008). In fact, Clavicipitaceous endophytes are closely related to the common pathogen *Claviceps purpurea* (Jensen et al., 2011). There is diversity within fungal endophytes that infect grasses. Therefore, several distinct types have been categorized within Class 1 (Clavicipitaceous endophytes) to describe them based off of their ability to produce disease symptoms during certain life stages (Stier et al., 2013). Perennial ryegrass is infected with the anamorphic Type III endophyte *Epichloë festucae* var. *lolii* (Leuchtman et al., 2014). The agronomic importance of research on perennial ryegrasses native endophyte *Epichloë festucae* var. *lolii* has been quite extensive. Other symptomless fungal endophytes do infect perennial ryegrass such as *Gliocladium*-like and *Phialophora*-like endophytes. These species

are studied far less, likely due to the agronomic importance of the toxic alkaloids *Epichloë* spp. produce.

Intrinsic association with a particular fungal endophyte sometimes aids in the survival of abiotic and biotic stresses such drought and grazing (Casler et al., 1996). Research has shown that *E. lolii* produces toxic alkaloids that can deter vertebrate and invertebrate feeding (Eerens et al., 1998a; Patterson et al., 1991; Richmond et al., 2000). Depending on the location of coevolution it seems the mutualistic relationship may change based on what stresses the endophyte host has experienced. This relationship is not static and will often change over the course of time due to environmental stresses. Kane (2011) sampled perennial ryegrass plants from different Mediterranean regions and found that those that commonly underwent drought stress contained endophytes that helped alleviate negative effects of drought. Clay and Scharld (2002) found that varying the levels of animal herbivory and availability of water changed the infection frequency of fungal endophytes over time. This finding demonstrates that the importance of endophyte is linked with particular stresses. Often times no effect of endophyte is found when stresses are not present. To this end, it is likely that the endophyte imparts some kind of metabolic drain on the plant.

With respect to seed production, endophytes have also been known to alter the reproductive abilities of grass plants. Hesse et al., (2004) examined the effects of endophyte infection on perennial ryegrass seed yield. Initially, researchers reported that there was an increase in seed yield in infected genotypes. However, in many of the genotypes the relationship reversed in the second year of seed production. The authors conclude that in some cases the endophyte can be a hindrance to long-term seed yield, however with careful selection it may be possible to select for positive or at least neutral relationships.

Only modest efforts to ascertain the effect of endophyte on the winter hardiness of perennial ryegrass have been made in comparison to other traits such as drought stress. As endophyte effects on drought stress are the most extensively explored it is the most logical body of literature from which to build a foundation to research other abiotic stresses such as winter hardiness (Malinowski and Belesky, 2000).

Drought Stress

The plethora of information on endophyte effects on drought stress is perhaps equaled only by the ambiguity of the findings. In general, endophytes are thought to have positive effects on drought stress in both perennial ryegrass and tall fescue (*Festuca arundinacea* Schreb.) (Clay and Schardl, 2002). However, upon close examination of the literature there are few examples that conclusively find a wholly positive effect of endophyte in either species (Hahn et al., 2008). Reports have shown variable effects ranging from favorable to antagonistic (Hesse et al., 2003; Kane, 2011; Ravel et al., 1997), neutral (Barker et al., 1997; Cheplick et al., 2000; Lewis et al., 1997), and predominantly antagonistic (Eerens et al., 1998b; Cheplick, 2004).

Often, the effect of endophyte is detected in a narrow germplasm base and the response variables only measure the most rudimentary aspects of drought stress or recovery from drought stress. Kane (2011) subjected four diverse accessions of perennial ryegrass from the Mediterranean region and two commercial cultivars to drought. Some effects on traits related to drought tolerance were observed such as tiller length and shoot biomass. These measurements were only taken after drought occurred so tiller number prior to drought could not be ascertained. There was also a significant effect of trial and a significant accession by endophyte interaction. The authors concluded that endophytes were more apt to aid their host

if the interaction originated from regions of the Mediterranean that experienced drought. Ravel et al. (1995) studied four diverse collections of perennial ryegrass with and without endophyte and reported that effects on drought were sporadic and associated with particular populations of similar origin. Lewis et al. (1997) also proposed endophyte effects are linked with stresses present at the germplasm origin. This study did not directly measure the effect of drought on plants in any way; but instead correlated endophyte infection frequency in a large sample of accessions with areas that commonly experienced severe drought.

Similar pitfalls exist when studying winter hardiness. Like drought stress tolerance, winter hardiness is a complex trait and care is needed when ascertaining the proper response variables for the chosen explanatory variables. Hahn et al. (2008) offers a compelling explanation of the benefits of endophytes under drought stress. Their experiment extensively measured response variables related to drought including: herbage yield, leaf elongation, water uptake, water use efficiency, relative water content, osmotic potential, proline content and alkaloid profile. The authors found that the presence of the endophyte did not increase the biomass production of either drought or control plants. In fact, the endophyte may have acted as a metabolic drain on plants to some degree and in turn reduced growth rates. However, endophyte-infected plants seemed to take up less water and maintained higher relative water content. These findings indicate that the endophyte-infected plants are simply in a state of metabolic suspension under stressful conditions more so than uninfected plants. As effects of abiotic stresses are usually associated with oxidative stress it could be that endophyte infection acts as a pleiotropic gene helping to mitigate effects of several seemingly unrelated stresses (Xiong et al., 2002).

Winter Hardiness

Research on endophyte effects on drought stress collectively can be used to design an experiment to study endophyte effects on winter hardiness. As both drought tolerance and winter survival are complex traits, determining the correct response variables to measure them is critical. Literature describing endophyte effects on winter survival is scant and determining the effect of endophyte on winter survival is usually not the primary focus. Furthermore, these studies were also done in the field. This contrasts with most experiments examining endophyte effects on drought, especially those that found consistent significant effects.

Several examples are available in which at least one measured trait was related to winter survival. Ravel et al. (1995) tested several diverse collections of European origin perennial ryegrass and found a small effect of endophyte on frost susceptibility. However, this study was conducted in France and weather data provided suggests that plants were unlikely to be subjected to harsh winter conditions in any of the trial years. Wäli et al. (2008) studied two varieties of meadow fescue (*Schedonorus pratensis* Huds.) grown in a subarctic region. Two populations were selected for each variety, one being highly infected and one slightly infected with *Neotyphodium* spp. The authors found a three-way interaction between variety, environment and infection status for vegetative and reproductive tillers measured. For one of the varieties at some locations the endophyte had a significant effect on number of tillers per plant, number of panicles per plant, seed mass, reproductive shoot mass and harvest index. None of these measurements directly measure winter survival of the plants only that the environment in which the plants were trialed underwent cold winters and may have affected spring tiller numbers. Rochefort et al. (2007) tested the effect of endophyte-infected perennial ryegrass and tall fescue cultivars competitive abilities when inter-seeded into Kentucky bluegrass (*Poa pratensis* L.) in Canada. The authors found that the proportion of endophyte

infection did not increase over time suggesting infection does not aid in survival. It is very difficult to understand what the real relationship is in this example because the authors include data suggesting that the endophyte infection drops to 0% and then increases to 10 % later in the season. It is possible that researchers did not have a sufficiently large enough sample size to detect an accurate infection frequency. This paper also failed to ascertain if the cause of the reduction in the endophyte infection frequency was due to cold temperatures. Casler and van Santen (2008) performed two field experiments focused on the effect of the *Neotyphodium coenophialum* on the winter hardiness of tall fescue. The same four entries were included in both experiments: one winter susceptible experimental and three winter hardy varieties. No effect on percent ground cover post winter was found in either experiment, and the authors concluded that winter survival was unaffected by the presence of endophyte. This experiment included both non-winter hardy and winter hardy germplasm; however, winter selection pressure was too extreme or too mild to damage plants in a manner that could characterize endophyte effects. For example, the non-winter hardy variety suffered complete stand loss (100 to 95 %) whereas the winter hardy entries only suffered minor loss (0 to 12 %). In this case, effects would likely be masked unless the endophyte was the primary contributor of winter survival. Although radical endophyte effects on abiotic stresses are not unheard of they have never been reported in Clavicipitaceous endophytes (Márquez et al., 2007). Previous literature indicates that winter conditions and confounding variables make determining effects of endophyte difficult. It is clear that in order to gain a foothold on the endophyte effect on winter survival, a subsidiary trait of winter hardiness, such as freezing tolerance should be used. Furthermore, the experiment needs to be conducted in a controlled environment to reduce environmental noise and time to complete the project.

Although no published literature directly links infection of Clavicipitaceous endophytes with freezing tolerance in cool season grasses there is some literature that suggests this might be possible. Dupont et al. (2015) found that perennial ryegrass plants infected with *Epichloë festucae* had an increased expression of cold response genes suggesting that plants would be better able to respond to temperature changes. It is also possible that endophytes could produce anti-freeze compounds, which could lower the supercooling point of grass intercellular spaces where the endophyte is present.

Gleaning Past Research to Improve Perenniality

This review discussed the uses and the importance of perennial ryegrass seed production to the Minnesota economy. It is apparent that the limiting factor in successful perennial sward performance for any use is sward rejuvenation and adequate winter hardiness. Given the literature on the origins of perennial ryegrass and the complexities of breeding for winter hardiness it is not surprising that progress to date is met with only moderate success. In lieu of breeding for increased winter hardiness other approaches can be taken. In chapter II, I will explore alternative management options in seed production systems to subserve multi-year seed production. In chapter III, I will investigate the effect the native fungal endophyte *Epichloë festucae* var. *lolii* on the freezing tolerance of perennial ryegrass.

CHAPTER 2.

Exploring Alternative Management Options for Multi-Year Seed Production of Perennial Ryegrass

Garett C. Heineck

|

INTRODUCTION

Seed Production in Oregon

Almost two-thirds of the total cool-season grass seed in the United States is produced in Oregon's Willamette Valley. Seed production in this region started in the 1920s and has included many species of grass such as fine fescues (*Festuca* spp.), tall fescue (*Festuca arundinacea* Schreb.), Kentucky bluegrass (*Poa pratensis* L.) and perennial ryegrass (*Lolium perenne* L.). In 2015, there were 152,800 ha of grass seed produced in Oregon, with perennial ryegrass accounting for 39,000 ha (NASS, 2016). Although grass seed production in 2015 was the fifth most important commodity for the state, generating over 380 million dollars, it was not as prominent as in previous years. For example, in 2005 grass seed accounted for 206,000 ha of production area and ranked as the third most important commodity (NASS, 2006). Perennial ryegrass hectareage accounted for 78,100 ha of the total seed production area in 2005 (NASS, 2006). In ten years there was nearly a 40,000 ha reduction in perennial ryegrass seed production. Reasons for this reduction include production difficulties and displacement by other high value commodity crops such as hazelnuts. Decreases in production hectareage in Oregon open new possibilities for increased grass seed production in other areas of the US.

Oregon State University has supported grass seed producers with research in seed production agronomics since the late 1940s (Hardison et al., 1949). Maintaining perenniality, consistent yields and seed purity of a sward over successive years from a single planting has been heavily researched. Initially, scientists found that planting in rows as opposed to broadcasting seed reduced the need for residue management and helped prolong stand longevity (Hardison, 1980). However, straw and stubble eventually led to matting and reduction in sward yield capacity. To further sward longevity field burning was proposed as a method of residue control. By the early 1950s burning was adopted for species such as fine

fescues, tall fescue, Kentucky bluegrass and perennial ryegrass (Hardison, 1980). The success of this system comes as no surprise as Native Americans for millennia utilized burning, unintentionally or intentionally, to rejuvenate grasslands and subsequently attract herds of buffalo (Hardison, 1976). In fact, many native grasses, such as wiregrass (*Aristida stricta* Michx.), will not flower unless burning has removed old growth from sods (Hardison, 1976). Burning grass fields to maintain seed yield and reduce disease was a prominent management practice in Oregon until 1975. Changes in legislation due to concerns over the smoke produced by field burning led to the eventual elimination of this practice. Since 2007 field burning has been reduced to less than 10% of total seed production hectares (Hart et al., 2012). In response to restrictions on burning, researchers turned to alternative methods of residue control such as baling and/or chopping (Chastain et al., 1995). The introduction of new pesticides labeled for grass seed production also reduced the need for pest control through burning (Rolston et al., 2004; Mueller-Warrant et al., 1990). The baled product can then be exported to other countries such as Japan and Korea for use in their cattle industries. This market development was necessary in order to minimize economic penalties associated with the change in management, although open field burning is the most effective method of maintaining perennial seed production (Hart et al., 2012).

Consideration of Oregon's seed production capacity and agronomics are helpful in determining the future of perennial ryegrass seed production in Minnesota. As less perennial ryegrass seed is produced in Oregon increased market opportunities may present themselves to Minnesota producers. Through research and development, Oregon seed production systems have been successfully producing multi-year perennial ryegrass seed crops for decades. Although different environmental challenges exist for Minnesota seed producers similar approaches to maintaining stand longevity should be explored.

Seed Production in Northern Minnesota

There are several key differences between seed production in Oregon and Minnesota. First, more extreme winter conditions are a much greater obstacle to overcome in Minnesota than in Oregon. Minnesota seed production is carried out in a very rural area, which currently has more relaxed regulations on practices such as open burning. Northern Minnesota has a unique summer climate, with long-cool days and adequate rainfall making it conducive to grass seed production. However, seed yields in Minnesota are approximately 50 % of those in Oregon where yields as high as 3000 kg ha⁻¹ can be achieved (Pfender, 2009). Grass species with exceptional winter hardiness such Kentucky bluegrass have the inherent potential for multi-year seed production in Minnesota. Seed producers in northern Minnesota traditionally grew Kentucky bluegrass starting in the 1960s, but later switched to perennial ryegrass because of increased yield stability, ease of production and seed prices. By the mid 1990s substantial hectareage of perennial ryegrass seed was being produced in northern Minnesota. Perennial ryegrass has been traditionally grown as a biennial crop because of severe yield reductions after the first year of seed production.

The University of Minnesota has had a strong working relationship with grass seed producers dating back to the 1960s (Elling, 1976a). Annual progress reports and literature have been published as a result of these efforts. The first attempt to grow perennial ryegrass for seed in northern Minnesota was in 1972 and attempts continued annually until 1978 (Elling, 1975; Elling, 1981). Data from progress reports at this time give an indication of what the seed yields were like each year. Mean variety trial yields during the 1970s ranged from 12 to 1341 kg ha⁻¹ with a grand mean of 605 kg ha⁻¹ across years and trialed varieties. Notably, multi-year seed production was attempted twice, once in 1972 and again 1977 (Elling, 1976b; Elling, 1980).

Yield losses in the second year for the 1972 and 1977 plantings resulted in yield reductions of 80 and 60 %, respectively, compared to the first seed year yields. Inconsistent seed yields were usually associated with high levels of winterkill (Elling, 1979). Unacceptable second year seed yield loss and negligible improvement of first year seed yields eventually led the abandonment of the perennial ryegrass project (Elling, 1981).

Re-emergence of perennial ryegrass seed production developed in the mid 1990s due to increased demand for seed and subsequent increases in prices (Ehlke and Vellekson, 1997). Waldron et al. (1998b) used the winter hardy variety NK200, originally used in the 1970s program, as well as a topcross population between turf type perennial ryegrass varieties and NK200 as the launch point for a new perennial ryegrass breeding program. Traits important to successful turf and seed production are winter hardiness, turf quality, rust resistance, leaf texture and growth habit. The germplasm that was generated from this research eventually led to varieties with acceptable turfgrass quality and the ability to survive one winter in northern Minnesota. Data from progress reports show that first year seed yields from 1998 to 2012 steadily increased and became more consistent. Recently published research reported increased seed yield and reduced inputs by using split applications of growth regulators and nitrogen (N) fertilizer in the biennial system. Koeritz et al. (2013) used a split-split plot design to determine the effects of N rate and application method as well as plant growth regulators (PGR) on seed yield and other important seed characteristics. Optimizing N and PGR timing increased seed yields by 14 and 36 % respectively. Koeritz et al. (2015) tested different combinations of seeding rate, row spacing and N rate in a split-split plot design. A seeding rate of 7.8 kg ha⁻¹, row spacing of 10 cm and N rate of 157 kg ha⁻¹ nearly eliminated weeds. For every unit of N applied, seed yield increased by 4.7 kg ha⁻¹. Evidence suggests that the increasing and reproducible yields in biennial perennial ryegrass seed production are due to improvements in

seed genetics and management practices. This supposition gives credence to attempting multi-year seed production using current genetics and alternative management practices.

It has been over forty years since multi-year seed production has been empirically tested in northern Minnesota. Because of increased expertise in management and improved genetics an additional attempt is warranted. Perennial cropping systems have several benefits over annual cropping systems. Glover et al. (2010) in a review on perennial grasses and Asters for grain production, state that in general perennial systems have longer growing seasons, utilize more precipitation and accumulate additional photosynthates. Perennial crops also generally have deeper roots and greatly reduce water and nitrate runoff compared to annual systems (Randall et al., 1997). These advantages decrease inputs for the producer as well as the overall environmental impact of the system. The coupling of current perennial ryegrass germplasm and findings from agronomic research performed in Oregon is a logical starting point for a multi-year system in Minnesota.

Rejuvenation of Seed Production Fields

The perenniality of bunch grasses is derived from the development of new tillers (for more information please refer to Chapter 1). Vegetative tillers vernalize during the winter and subsequently joint, flower and produce seed. In species like perennial ryegrass this change requires vernalization that is usually caused by exposure to winter (Chastain and Young III, 1998). The number of seed heads produced in the spring is determined, to some degree, by the number of vegetative tillers present after the previous year's seed harvest (Chastain and Young III, 1998). The poor winter hardiness of perennial ryegrass makes it probable that many of the young tillers produced by second year plants after seed harvest die before jointing occurs (Hill and Watkin, 1975). High levels of tillering often decrease with plant age as the secondary and

tertiary tillers do not break bud as readily as primary tillers, possibly due to increased density (Bahmani et al., 2000). For multi-year seed production to be practical (i) new tillers need to develop after the first years harvest; (ii) newly produced tillers must subsequently survive the following winter; and (iii) volunteer seedlings from first year harvest must be controlled to maintain genetic purity of the seed field.

High yielding seed production fields are not only a function of genetic potential, but also appropriate management practices. Cultural practices such as fall residue management after seed harvest can influence seed production the following year (Young et al., 1999). In grass species such as Kentucky bluegrass, seed yield is greatly improved by complete residue removal (Ensign et al., 1982). Field burning after harvest was typically the method of choice for elimination of residue in perennial grass seed production fields in Oregon (Ensign et al., 1982). Removing residue on fields after harvest decreases disease the following year as well as increases tillering in the current growing season (Ensign et al., 1982; Young et al., 1999). Young et al. (1999) tested the effects of residue management post first-year harvest for three consecutive years on perennial ryegrass seed production fields in Oregon. Residue management treatments consisted of (i) straw removal by baling, flail chopping and leaving vegetative growth; (ii) baling straw and burning residue via propane torch; and (iii) uniform spread of straw and (iv) field burning. In the third and fourth year of seed production, field burning increased seed yields by 233 and 208 kg ha⁻¹ respectively. It was suggested that residue removal increased the light and light quality reaching plant crowns as well as biomass accumulation the following year.

Casal et al. (1985) studied the effects of light quality on tillering in annual ryegrass and found that as canopy density increased, the interception of red/far-red light by the crown was reduced, which led to the subsequent decline in new tiller production. Economically viable

seed production often relies on the successful control of residue after first-year harvest. However, unlike many cool season grasses, such as Kentucky bluegrass and orchardgrass, perennial ryegrass seed yield potential does not greatly depend on tiller size or number going into winter (Chastain and Young III, 1998). Chastain et al. (1998) studied the effects of post harvest drought on perennial ryegrass seed yield and researchers found that plants could completely compensate for the decrease in stand density the following spring even when stand recovery was limited by lack of water assuming some amount of plant material survived. In summary, elimination of old growth and the initiation and survival of some new growth is critical to multi-year perennial ryegrass seed production.

Maintaining Seed Purity in Multi-Year Systems

Aside from the yield benefits, seed producers in Oregon use field burning as an effective method of controlling volunteer seedlings (Hardison, 1980). Control of volunteer seedlings and grassy weed species is important in maintaining seed purity for seed certification (Mueller-Warrant et al., 1994). In situations where burning is not used some herbicides may be useful in decreasing volunteer seedlings. Mueller-Warrant et al. (1994) studied the effects of various pre- and post-emergent herbicide treatments on seedling emergence in perennial ryegrass seed production fields with and with out field burning. They found that a pre-emergent application of pendimethalin and post-emergent application of oxyfluorfen sufficiently controlled volunteer seedlings and grassy weeds. The pre- and post-emergent herbicide applications combined with burning achieved the highest efficacy. For example, in relation to a control, the herbicide treatments combined with open burning controlled 97.4 % of seedlings compared to the 84.2 % controlled by the combination of baling residue with herbicide treatment. Successful multi-year perennial ryegrass seed production in Minnesota not

only depends on seed yield potential but also production of pure seed. Control of volunteer seedlings through field burning and herbicides have worked for producers in Oregon and should be investigated in Minnesota.

Maintaining Winter Survival in Multi-Year Systems

Winter survival of first year perennial ryegrass stands in Minnesota, in part, is credited to spring wheat stubble that acts as a snow catch and consequently insulates plants (Ehlke and Vellekson, 1998). Continual and even snow cover throughout winter maintains higher soil temperatures and decreases temperature fluctuation (Leep et al., 2001). This benefit is lost in the second year of seed production and plants have to depend on inherent winter hardiness to survive.

Winter survival is dependent on several factors including environmental, such as minimum temperature and snow-fall, as well as genetic factors such as dormancy and freezing tolerance (Williams et al., 2015). Carbohydrate sequestration within the root system is one of the components to successful overwintering (Humphreys et al., 2010). One of the effects of fall acclimation is an increase in stored carbohydrates in the crown and roots. Ball et al. (2002) examined differences in soluble carbohydrate composition in two buffalograss (*Buchloe dactyloides* Nutt.) cultivars in relation to their level of freezing tolerance and found that the more freezing tolerant cultivar also accumulated more carbohydrates in the form of raffinose.

Breeding has successfully increased the winter hardiness of perennial ryegrass, although gains from selection have been slow. Exploring alternative management practices such as applications of plant growth regulators that alter plant physiology may influence winter survival. Producers in northern Minnesota have typically used plant growth regulators (PGRs) to reduce lodging by shortening plant height. For example, seed producers commonly employ

a single application of prohexadione Ca to perennial ryegrass fields to decrease lodging and increase yields (Koeritz et al., 2013). However, PGRs can also have an effect on the overwintering capacity of cool season grasses. Steinke and Stier (2003) applied the PGR trinexapac-ethyl, an inhibitor of gibberellic acid (GA), in the late summer and fall to supina bluegrass (*Poa supine* Schrad.). Over two years, they found that applications of the PGR increased winter survival by 80 %. It is possible that by inhibiting GA the plant internodes were shortened and were subject to less temperature variation due to the meristematic tissue being better protected. Subcrown internode length, or deep crowns, has been associated with increased winter survival in perennial ryegrass as well as winter cereals (Wood and Cohen, 1984). Dofing and Schmidt (1985) found a negative correlation ($r = -0.57$, $P < 0.01$) between subcrown internode length and mean winter survival in 29 lines of winter barley, indicating that a deeper, less elongated stem increased winter survival. Similar results have also been recorded in winter wheat (Gul and Allan, 1978).

In a greenhouse study, Cooper et al. (1988) applied the PGR mefluidide to annual bluegrass (*Poa annua* L.) plants and measured carbohydrate distribution in relation to a control. Applications resulted in decreased seed head production and subsequent redistribution of carbohydrates to the root system. Carbohydrates were subsequently redistributed to shoot growth post seed-head inhibition. As carbohydrates are important to successful overwintering increasing carbohydrate accumulation in the roots might affect winter survival. Auxins and cytokinins are important plant hormones involved in carbohydrate sink strength by controlling the import and export of photosynthates. Maslova et al. (2007) examined accumulations of auxin and cytokinin in the rhizomes of reed canarygrass (*Phalaris arundinacea* L.) during the fall and found that cytokinin/ABA ratio increased as temperatures decreased. Cytokinins are

essential for the formation of stress proteins important for overwintering capacity and the authors postulate that the increase in cytokinin accumulation may aid in early spring greenup.

Testing fall applications of a variety of PGRs that target tiller elongation and carbohydrate storage may increase the winter survival of perennial ryegrass. Plant growth regulators applied to seed production fields will likely have a short half-life, and therefore the benefits of any PGR would be negligible in the following growing season (Rademacher, 2000). Although the effects of PGRs would not persist into the following year effects on winter survival may impact seed yield. Growth regulators can increase carbohydrate accumulation in plant roots, shorten plant internodes and increase tillering. Although these traits can also be altered through traditional plant breeding some PGRs are routinely used in seed production systems.

Objectives

Our objective was to assess the impact of fall residue management (FRM) practices and various PGRs post first-year harvest on winter survival and second year seed yield of perennial ryegrass seed production fields in northern Minnesota.

MATERIALS AND METHODS

The experiment spanned three trial years from August 2013 to August 2016. Each trial year included two locations, which changed each year giving a total of six location-year combinations or environments. Research was done on-farm under non-irrigated conditions. Plot locations were established in cooperation with experienced seed producers in perennial ryegrass swards that had already undergone one full cycle of seed production. Plot locations were chosen based on stand uniformity and convenience to the seed producer. This meant that plots were generally located near the edge of fields or near a field road. The varieties used for the experiment changed with the preference of the producer and year, although the varieties were the same within years. The variety ‘Arctic Green’ was used in trial year one and ‘Royal Green’ was used in trial years two and three. ‘Royal Green’ is related to ‘Arctic Green’ through the common parent ‘Ragnar II.’ These varieties are commonly used by growers in the region because of their winter hardiness and tolerance to the herbicide quizalofop-P (aryloxyphenoxypropionate). The commercial varieties ‘Arctic Green’ and ‘Ragnar II’ have also recently been used for agronomic research in northern Minnesota (Koeritz et al., 2015; Kurcinka et al., 2009).

All six locations were located around Roseau, MN, in Roseau County. Specific details on soil type and other details are listed in Table 2.1. The common soil types of the seed production region are well represented by the six different environments (Koeritz et al., 2013). Weather data for all locations were collected at the University of Minnesota sponsored weather station south of Roseau, MN at the GPS coordinates 48.655 N and 95.734 W. Growing degree days (GDD) were calculated using a base temp of 0 °C. Data were taken on soil temperature 10

cm below the soil surface. Cumulative growing degree days (GDD) were tracked from March 1st through the first week of August, which is the typical harvest time for perennial ryegrass in northern Minnesota.

Best management practices were used and mimicked those for typical single year perennial ryegrass seed production in northern Minnesota. Previous research showed that fall applications of nitrogen (N), phosphorus (P) and potassium (K) increased yield in spring seeded first year plots when followed by an additional spring application of N (Ehlke and Vellekson, 2011). Fall fertilizer (20-10-10) was applied three weeks post-harvest (August 21st to August 27th) after FRM treatments were applied using a Gandy drop spreader (Gandy Company, Owatonna, MN) at a rate of 33.5 kg N ha⁻¹, 16.7 kg P ha⁻¹, 16.7 kg K ha⁻¹. Spring fertilizer (46-0-0) was applied in May at a rate of 123.1 kg N ha⁻¹. Broadleaf weeds were controlled between 600-700 GDD with an application of 2, 4-D, (2, 4-dichlorophenoxyacetic acid) at a rate of 0.27 kg a.i. ha⁻¹ and dicamba, (3,6-dichloro-*o*-aniscic acid) at a rate of 0.27 kg a.i. ha⁻¹ at each environment. Grassy weeds were controlled 800-850 GDD by applying quizalofop P-ethyl, ({ethyl (R)-2-[4-(6-chloroquinoxalin-2-yloxy)-phenoxy] propionate}) (Assure II, Dupont, Wilmington, DE) ('Arctic Green' and 'Royal Green' are tolerant to Assure II (Gronwald et al., 1992)) at a rate of 0.075 kg a.i. ha⁻¹ at each environment. Lodging was controlled at the two-node growth stage by a single application of prohexadione calcium, ([calcium 3-oxido-5-oxo-4-proponylcyclohex-3-enecarboxylate]) (Apogee, BASF corp., Research Triangle Park, NC) at a rate of 0.15 kg a.i. ha⁻¹ at each environment. Rust (*Puccinia graminis* subsp. *graminicola* and *Puccinia coronata* f. sp. *Lolii*) was controlled between 1,100 and 1,300 GDD by a single application of propiconazole, (1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1,2,4-triazole) (Quilt Excel, Syngenta Crop Protection, Greensboro NC) and Azoxystrobin, (Methyl (2*E*)-2-(2-{[6-(2-cyanophenoxy)pyrimidin-4-yl]oxy}phenyl)-3-methoxyacrylate),

(Quilt Excel, Syngenta Crop Protection, Greensboro NC) at rates of 0.059 and 0.15 kg a.i. ha⁻¹, respectively, at each environment. Stem rust does not always threaten seed crops. For example, yield losses in the Oregon and Minnesota seed production fields range from 0 - 98 % and <10 - 50 % respectively (Pfender, 2009; Ehlke and Vellekson, 2011; Ehlke and Vellekson, 2016). However, because seed losses due to stem rust can be as great as 98% preventative measures were taken to ensure treatment effects were not confounded by heavy rust infestation. All general management chemical applications were done using a CO₂ powered bicycle sprayer equipped with a 2.75 m boom and eight 1002 TurboTeeJet® (TeeJet, Springfield, IL) nozzles operating at 186 kPa in water equivalent to 117 L ha⁻¹.

The experimental design was a randomized block design with four replicates in a split-plot treatment arrangement. Residue management was the whole plot factor and PGR was the split-plot factor. Each experimental unit (split-plot) measured 1.8 m wide x 6 m long. A 1.8 m perennial ryegrass border separated each whole plot to minimize edge effects. There was no border between split-plots.

Fall residue management treatments were applied two weeks after harvesting the first-year stand with treatments being straw bale and mow (SBM), chemical and physical open burn (OB) and a control where all residue and plant material was left on the plot. Simulated bale and mow treatment was done using riding lawn mower (John Deere, Moline, IL) set at a 7.6 cm height of cut. This removed much of the post harvest vegetative growth as well as the straw residue. The residue was raked up and removed to simulate baling.

Open burn treatments were carried out by applying Paraquat, (1,1'-Dimethyl-4,4'-bipyridinium dichloride) (Gramoxome, Syngenta Crop Protection, Greensboro NC) at a rate of 0.24 kg a.i. ha⁻¹ at each environment. The canopy was allowed to desiccate for one week or

longer until humidity allowed a physical burn to take place. Physical burning was initiated via a standard torch fueled by a 50:50 mixture of gasoline and diesel.

Plant growth regulator treatments were applied 14 d post burning to allow new vegetation to grow prior to application. Plant growth regulator treatments consisted of ethephon, [(2-chloroethyl)phosphoric acid]] (Proxy, BASF corp., Research Triangle Park, NC) at a rate of 0.37 kg a.i. ha⁻¹ at each environment; IBA and Cytokinin, (3-Indolebutyric acid and Kinetin) (Radiate, Loveland Products, Greeley, CO) at a rate of 0.03 and .0057 kg a.i. ha⁻¹ respectively, at each environment; mefluidide, (N-[2,4-dimethyl-5-[[trifluoromethyl)sulfonyl]amino)phenyl]acetamide) (Embark, PBI/Gordon Corporation, Kansas City, MO) at a rate of 0.37 kg a.i. ha⁻¹ at each environment; Prohexadione calcium, ([calcium 3-oxido-5-oxo-4-proponylcyclohex-3-enecarboxylate]) (Apogee, BASF corp., Research Triangle Park, NC) at a rate of 0.15 kg a.i. ha⁻¹ at each environment; and a control. Chemical treatments were applied using a CO₂ powered backpack sprayer with a 1.8 m boom and four 1002 TurboTeeJet® (TeeJet, Springfield, IL) nozzles operating at 186 kPa in water equivalent to 117 L ha⁻¹.

Data were collected on three categories of response variables: 1) winter survival and spring greenup, 2) seed yield and yield components and 3) seed quality characteristics. Seed quality characteristics were only measured in the third trial year.

Winter survival was measured by a spring plant count (SPC), which was the number of plants that survived the winter in a 1 m x 1 m area after spring greenup, but before individual plants within rows grew together. Spring plant count was measured after at least 250 GDD were accumulated in each trial year. Spring greenup as measured as relative chlorophyll index (RCI) and was conducted using a FieldScout® CM 1000 Chlorophyll Meter, which estimates the quantity of chlorophyll in leaves. Each plot was measured at five random points, which

were then averaged. Measurements were not taken at the same accumulation of GDD units from one trial year to the next so treatment effects could not be extended across years. Relative chlorophyll index readings were measured on May 30th, 12th and 15th for trial years one, two and three respectively. Volunteer seedling emergence (VSE) is typically measured in binomial fashion with either the presence or absence of seedlings being recorded in a defined area (Mueller-Warrant et al., 1994). Volunteer seedling emergence data were estimated by the percent of each plot covered by seedlings. Seedlings emerged uniformly across plots and, consequently, could be easily estimated visually.

Seed yield and aboveground biomass was determined by harvesting 1 m x 1 m swath of perennial ryegrass per experimental unit. Seed was harvested at 40 % moisture and was dried for 5 days at 95 °C in a forced air drier. Dried biomass was then weighed and passed through a small plot thresher. Seed and chaff were then separated by passing seed through a 1.625 mm x 9.525 mm sieve twice. Light seed was removed by aspiration using a Superior® Fractionating Aspirator (Carter-Day Int. Inc., Minneapolis, MN). Seed was sieved one more time using a 1.190 mm x 9.525 mm sieve. Seed was weighed and its mass subtracted from the initial biomass sample. Harvest index (HI) was calculated as the ratio of seed weight to total aboveground biomass. Plant height (PH) was measured at harvest by measuring plants from the ground to the end of the spike in random 0.25 m x 0.25 m area in each experimental unit. Lodging was measured at harvest in a qualitative manner by visually estimating the percent of each plot lodging where 0 = no lodging and all plants are standing erect, and 100 = all plant are lying completely prostrate (Koeritz et al., 2013).

Seed quality characteristics were only measured for trial year three and included test weight, thousand seed weight and percent germination. Test weight, a measure of seed density, was determined by a Perton Instruments AM 5200 and is reported in kg hL⁻¹. Thousand seed

weight (TSW) was calculated by counting 500 randomly selected seeds from each rep of each split plot. Seeds were weighed on a micro scale to the nearest 10^{-5} g and used to estimate the weight of 1000 seeds⁻¹. Percent germination was estimated by selecting 100 random seeds from each split-plot using the Association of Official Seed Analysis (AOSA) standards for perennial ryegrass (Association of Official Seed Analysis, 2015). The procedure included placing seeds on blue blotter paper inside 10 cm x 10 cm plastic germination containers and wetting with a 0.2 % solution of KNO₃. Treated seeds were stratified for 5 d at 10 °C. After this seeds were allowed to germinate for 14 d in a germination chamber set to 25 °C d and 10 °C night temperatures with LED bulbs providing 8 h photoperiod. Germinating seeds were checked and counted at days 5 and 14. The total number of germinated seeds was then divided by 100 to calculate the germination percentage for each treatment.

Statistical Analysis

For all measurements, excluding seed quality characteristics, analysis of variance (ANOVA) and analysis of co-variance (ANCOVA) of the data from the three trial years were evaluated using a linear mixed effects model from LME4 package of Project R (Bates et al., 2015). Main plot was nested within location and was considered random. Year was not included as a random term because weather data, including soil temperature, helped in explaining a significant amount of the variation between trial years. The Satterthwaite approximation was used to calculate residual degrees of freedom for fixed effects. Treatment effects were considered significant at $\alpha = 0.05$. For main effects showing significance in the ANOVA, pair-wise comparisons were performed using the Tukey-Kramer method. The package LSMEANS was used for the post hoc analysis of treatment means and package MULTCOMP was used to obtain letter groupings on the Tukey-Kramer HSD test (Lenth, 2016

; Hothorn et al., 2008). Data were combined for treatments across years only when no significant interaction existed. Pearson correlations were calculated on an individual plot basis between all continuous variables using HMISC package in Project R (Harrell, 2016).

Occasionally the amount of variation explained by a given response variable was of interest. R^2 was calculated using a linear fixed effects model and the `lm` function in the `core` package of project R 3.3.1. The linear model consisted of environment, treatments and their interactions when significant with main plot included in the error term to avoid pseudo replication.

Seed quality characteristics were analyzed using ANOVA of the data from the third trial year using the `AGRICOLAE` package of Project R (Mendiburu, 2016). The linear model consisted of environment, treatments and their interactions with main plot included in the error term to avoid pseudo replication. Environments were separated if the interaction was significant. Means separation was done using the Tukey-Kramer method and package `TUKEYC` of project R (Faria et al., 2016). Treatment effects were considered significant at $\alpha = 0.05$.

RESULTS

Growing degree day accumulation for trial years one, two and three were 1744, 1933 and 2052 respectively (Figure 2.1). Spring GDD accumulation in years two and three were much higher than in year one (Figure 2.1). Total precipitation from March 1st through August 7th for trial years one, two and three was 226.3, 294.6 and 354.3 mm respectively. The ten-year average precipitation for the growing season is 220.6 mm. No data on snowfall accumulation or persistence over time were recorded for any trial year. Mean daily air temps for each month from September 1 through April are listed in Table 2.2. Extreme minimum air temperatures for trial years one, two and three were -33.2, -31.0 and -28.8 °C respectively. Soil temperature data are reported in Table 2.2. The coldest monthly mean soil temperatures were recorded at -2.8, -4.1 and -0.8 °C for the months of February in trial year one, December in trial year two and January for trial year three respectively. Extreme minimum soil temperatures for trial years one, two and three were -4.6, -11.2 and -2.1 °C respectively. The most extreme minimum air and soil temperatures did not occur within the same trial years most likely due to differing amounts of snow cover. Total number of days with soil temperatures below 0 °C for trial years one, two and three were 127, 131 and 92 d respectively (Table 2.2). Soil temperatures were above freezing by March 1st for trial year three, but not until April 1st for trial years one and two.

Year and FRM affected seed yield and there was an interaction between year and FRM (Table 2.5). Average yields for trial years one, two and three were 1156.6, 586.8 and 927.9 kg ha⁻¹ respectively. There was no effect of PGR on yield in any trial year (Table 2.5). Managing residue by OB or SBM resulted in statistically similar seed yields in trial year one and three (Figure 2.2). Open burn resulted a statistically superior seed yield in trial year two (Figure 2.2).

Leaving residue in the field (control) post first-year harvest consistently resulted in the lowest seed yield although it was only significantly less than SBM in trial years two and three (Figure 2.2). Leaving residue resulted in a lower seed yield than OB in all three trial years (Figure 2.2). In trial year two control plots yielded 259.5 kg ha⁻¹ whereas OB average 866.0 kg ha⁻¹, more than a threefold increase in seed yield. All response variables were correlated with yield except PH (Table 2.7). Biomass was most closely correlated with seed yield ($r = 0.66$, $P < 0.001$).

There were significant effects of year, residue management and PGR on SPC (Table 2.3). There was also a significant interaction between year and FRM, but not between year and PGR (Table 2.3). The average SPC in trial years one, two and three were 11.9, 5.4 and 27.8 plants per m² respectively (Table 2.4). There was no effect of FRM on plant survival in trial year one. Simulated bale and mow significantly increased the SPC in trial year two and three when compared to the control. Open burn did not increase living plants in any trial year. In fact, in trial year two less than one plant per m² was recorded on average at 250 GDD. Plots treated with fall applications of the PGRs Radiate and Proxy had nearly the same number plants as the control, but had significantly more plants than those plots treated with Embark (Figure 2.3). No PGR treatment resulted in a significantly different number of plants when compared to the control. Spring plant count was correlated with all variables, most of all RCI, lodging and biomass (Table 2.7). It is hard to compare RCI between years because they were taken between 400 and 700 GDD. Fall residue management and year had an effect on RCI (Table 2.3). In trial year two OB and SBM resulted in higher RCI (Table 2.4). In trial year three SBM increased RCI by 200 units when compared to control and OB, which only averaged 550.2 units. There was no effect of PGR on RCI. Relative chlorophyll index was most highly correlated with SPC and biomass (Table 2.7). There was also a strong negative correlation between RCI and HI (Table 2.7).

Volunteer seedling emergence was estimated by the percent of plots covered by seedlings. OB and SBM increased the amount of volunteer seedlings compared to the control (Table 2.4). Volunteer seedling emergence was only affected by FRM and there was no interaction with any other explanatory variable (Table 2.3). Typically, control plots had the least number of volunteers, possibly due to residue and old plant growth covering soil surface. However, it is unlikely that volunteer seedlings dramatically contributed to yield. Analysis of covariance shows that there was an effect of year, FRM and VSE on seed yield. The model predicts 58.5 % of the variation for seed yield. The term including volunteer seedlings contributes 8.3 % to the total prediction for seed yield. Volunteer seedling emergence was not highly correlated with any variable, but was significantly correlated with yield and biomass (Table 2.7).

There were differences between trial years for both aboveground biomass accumulation and HI (Table 2.5). Plant growth regulator had no effect on aboveground biomass or HI (Table 2.5). There was an interaction between trial year and FRM for biomass, but not for HI (Table 2.5). There was no effect of FRM on biomass in trial years one and three, although these years had the highest accumulation of biomass. In trial year three OB accumulated 4249.1 kg ha⁻¹ of biomass (Table 2.6). This was significantly more than either SBM or the control, which accumulated 2952.9 and 1896.6 kg ha⁻¹ respectively (Table 2.6). Open burn and SBM resulted in an increased HI compared to the control in all trial years (Table 2.6). Increases over the control for HI were 4.0 and 4.7 % for SBM and OB respectively. The effect of year on HI was much more drastic as the average HI in trial year one was 28.2 % compared to 15.6 and 19.5 % in trial years one and two respectively. There were no treatment effects on lodging or PH (Table 2.5). Harvest index was negatively correlated with every response variable except yield (Table 2.7).

Seed quality characteristics were only measured in trial year three. Test weight, a measure of seed density, was not affected by any treatment (Table 2.8). There was an interaction between residue management and location for TSW (Table 2.8). Burning and SBM resulted in similar TSW of 1.64 and 1.69 g respectively. The average control TSW was 1.70 and 1.55 g at environments one and two respectively. There was no effect of FRM or PGR on seed germination. The average seed germination was 95.1 % across treatments.

DISCUSSION

Fall residue management after harvest in first and second year seed production swards has been heavily researched in Oregon for several cool season grasses including perennial ryegrass. Appropriate control of residue leads to increased vegetative growth, tillering, interception of light by the crown and control of volunteer seedlings (Canode and Law, 1979, Casal et al., 1985; Mueller-Warrant et al., 1994). Until now the primary focus of plant breeders and agronomists in Minnesota has been on increasing winter survival and maximizing agronomics of perennial ryegrass fields to support consistent first-year seed yields (Waldron et al., 1998; Koeritz et al., 2013; Koeritz et al., 2015). As this has been achieved, attention towards managing residue and maintaining winter hardiness after first year harvest in preparation of a second harvest year is crucial. The objective of this study was to test the common residue management techniques long proven in Oregon and experiment with PGRs that might alter plant physiology to maintain or improve plant survival.

The most important variable to grass seed producers is final seed yield. As seed production fields age they inevitably decrease in yield capacity. Although seed producers in Oregon can commonly achieve three years of seed production, seed producers in Minnesota do not usually attempt more than one year. In this experiment, year and residue management both affected seed yield. Trial year two had the coldest extreme minimum bare soil temperature compared to trial years one and three (-11.2 vs. -4.6 and -2.1 °C, respectively) and was much colder than the ten-year average of -6.4 °C. Trial year two also had a much lower average seed yield (Figure 2.2). The general reduction in seed yield across treatments in trial year two most likely resulted from extreme cold near the crown, which can cause death in other species such as alfalfa (Leep et al., 2001). In trial years one and three OB and SBM did not differ

significantly and treatments averaged 1242.3 and 1058.8 kg ha⁻¹ respectively. In trial year two OB yielded significantly more than any other treatment and average 866.5 kg ha⁻¹. The mean for OB over trial years one, two and three was 1064.4 kg ha⁻¹.

Estimating yield reduction due to stand age is important before making recommendations. The NASS has no published information on perennial ryegrass seed yields in Minnesota. Alternatively, typical first year yields can be approximated from recently published papers and theses. Kurcinka et al. (2009) conducted two experiments at two locations in 2005 and 2006 testing N application rate and timing on winter hardy perennial ryegrass varieties in northern Minnesota. Location one and two were located on Zippel very fine sandy loam and Percy fine sandy loam respectively. Split applications of N resulted in an average seed yield of 1634 and 930 kg ha⁻¹ over N rates at locations one and two respectively. A single spring application of N resulted in average seed yields of 1461 and 1016 kg ha⁻¹ across N rates at locations one and two respectively. Koeritz et al. (2013) studied effects of N and PGR rate over two locations and years on the perennial ryegrass variety 'Arctic Green' in northern Minnesota. Soil types for location one was a Wabunica silt loam and a Zippel very fine sandy loam, plots here received no fall N application. Soil type for location two was a Zippel very fine sandy loam and a Percy fine sandy loam, plots received 56, 10 and 20 kg ha⁻¹ N, P and K. Seed yields at locations differed substantially more than between years with location one and two averaging 1,222 and 781 kg ha⁻¹ respectively across years. Koeritz et al. (2015) studied effects of row spacing, seeding and N rate on the perennial ryegrass variety 'Arctic Green' in northern Minnesota. The experiment spanned two years each with two unique locations. Each year plots were established on both a Zippel fine sandy loam and a Garnes fine sandy loam. The effect of year was more pronounced than the effect of location in this study, with regards to seed yield. Mean seed yields for years one and two were 1351 and 1080 kg ha⁻¹

respectively. Of the three examples the first two cited soil type as being an important interacting factor and the latter observed a larger effect of year. Seed yields over six trial years and four soil types ranged from 781 to 1684 kg ha⁻¹. The current study spanned three trial years and three soil types commonly reported in the other studies and resulted in average yields 1257, 586 and 972 kg ha⁻¹ over trial years one two and three respectively. Open burn treatment yields ranged from 866.5 to 1280.7 kg ha⁻¹ over years, well within the previously established range.

Spring plant count was measured after 250 GDD were accumulated. Fall residue management and PGR had an effect on SPC. There were no differences between any treatment for SPC in trial year one. In trial years two and three the SBM treatment increased plant survival when compared to the control by 10.3 and 10.9 plant per m² respectively. Open burn never increased SPC compared to the control. It is likely that some plants counted as dead at the time of measurement may have eventually started tillering, especially in trial year two. Plant growth regulator had an effect on SPC in trial year two, although no PGR was significantly different than the control. Both ethephon and ABA/kinetin applications increased the number of living plants compared to mefluidide. Gerrish and Dougherty (1983) found that effect of mefluidide on tall fescue growth was limited to 6 to 8 weeks with a subsequent flush of growth once the effect dissipated. In this study application timing of PGRs varied between trial years because of timing of OB after first year harvest. Effect of mefluidide would have likely started dissipating in late October, mid November and late November for trial years one, two and three respectively. Average October temperatures in trial year one was 5.1 °C and average November temperatures were -7.3 and 0.1 °C for trial years two and three. Low temperatures coupled with short days stimulate fall acclimation in perennial ryegrass. It is possible that the dissipation of mefluidide effect triggered a flush of growth while plants should

have been reducing growth. Simulated bale and mow generally increased RCI compared to the control and OB, although RCI and seed yield were not strongly correlated ($r = 0.25$, $P < 0.001$).

Open burn and SBM treatments accumulated more volunteer seedlings than the control in the spring even after a fall application of pendimethalin. Although it is unlikely that these seedlings represent any substantial amount of seed yield ($R^2 = 0.07$, $P < 0.05$), they as vegetative grass plants would be using available N. Volunteer seedling emergence was measured in the spring after any effects of burning and pre-emergent herbicide would have dissipated. Mueller-Warrant et al. (1994) found that untreated checks ranged from 56.9 to 73.2 % coverage in perennial ryegrass fields in Oregon. Open burning alone reduced volunteer seedlings in late winter by between 66.6 to 88.9 % compared to the control. The combination of the pre-emergent herbicide pendimethalin with open burning increased the control of volunteer seedlings 86.0 to 95.2 % compared to the control. Results of the current study show that there was no difference between OB and SBM treatments with combination of pendimethalin (VSE was 27.6 and 28 % respectively). Volunteer seedling emergence was less than was previously reported for untreated bale and mow check most likely due to application of pendimethalin (Mueller-Warrant et al. 1994). On the basis of previous literature a minimum of 81 % control is necessary if fields were certified in Oregon (Mueller-Warrant et al., 1994). This means that plots in this study were slightly over the standard for acceptable control. It could be possible for producers to better time their paraquat and subsequent field burn. Furthermore, harrowing fields in the spring could further reduce volunteer seedlings to meet standards for seed certification.

Open burn had a higher HI in years two and three when compared to the control and always a higher seed yield. However, OB never had a higher SPC than the control and only had a high RCI in trial year two when the control plots had almost no green cover. This is in

agreement with Silsbury (1965) who observed that perennial ryegrass plants had low levels of juvenility and could produce seed at an early stage of vegetative development. Hill and Watkin (1975) found that perennial ryegrass plants that tillered earlier had more florets and heavier seeds. However, a review by Chastain and Young (1998) reported that there was no relationship between the number or size of tillers a perennial ryegrass plant produced in the fall and the seed yield the following year. This would indicate that the complete lack of residue and fewer plants with younger tissue associated with OB treatment was the cause of increased seed yield and HI.

Harvest index and aboveground biomass were negatively correlated ($r = -0.32$, $P < 0.001$) and biomass and yield positively correlated ($r = 0.66$, $P < 0.001$). Except in trial year two there were no differences between FRM treatments for aboveground biomass. In trial year three OB had more than three times the yield as the control (866.5 vs. 259.8 kg. ha^{-1}). This would indicate that plots with no residue removal contain plants that are unable to shift from a vegetative phase to a reproductive phase. This supposition is further supported by the correlation between RCI and biomass accumulation ($r = 0.73$, $P < 0.001$) and a much lower correlation with yield ($r = 0.25$, $P < 0.001$). This suggests that even when plants have early spring greenup, they do not necessarily produce high seed yields.

No management strategy had an effect on either PH or lodging (Table 2.5). It is likely the differences in PH and lodging between trial years was due to precipitation accumulation as trial years two and three were 74.0 and 133.7 mm above the ten-year rainfall average during the perennial ryegrass growing season. In our study, trial year one had less lodging and more favorable HI, thereby confirming a previous conclusion by Koeritz et al. (2013) who found that less lodging increased HI.

No effect of germination was found for either FRM or PGR. The mean germination was 95.1 %, which is a commonly reported germination percentage (Koeritz et al., 2013; Koeritz et al., 2015). Thousand seed weight and TW were positively correlated ($r = 0.56$, $P < 0.01$). There was no difference between any treatment for seed TW. Test weight is generally a trait reported in small grain production and is a measure of seed density. Increased density is associated with large, plump, durable kernels (Czarnecki and Evans, 1986). Both grass seed producers, seed cleaning and packaging plants must be aware of test weight as seed that has a low density will not fit into the standard pre-sized 50-pound bags. Koeritz et al. (2013) found that perennial ryegrass TSW ranged from 1.56 to 1.69 g and that TSW and seedling vigor were positively correlated. Open burn and SBM treatments produced statistically similar TSW of 1.63 and 1.69 g respectively (Table 2.9). On the basis of the TSW averages, seedling vigor will likely be acceptable.

Conclusion

Economically viable second year perennial ryegrass seed production is possible in northern Minnesota using current winter hardy commercial varieties, proven agronomics, and appropriate residue management. In this study, environmental conditions varied greatly across years and a wide range of common soil types were trialed. Over the three trial years extreme minimum soil temperatures ranged from above average to the coldest observed in ten years. Although trial year two suffered an overall yield reduction from winter kill OB still provided adequate yields (Kurcinka et al., 2009; Koeritz et al., 2013; Koeritz et al., 2015).

This research indicates that PGRs, although having a varying effect on SPC, do not affect the overall seed yield. Furthermore, SPC and RCI, although positively correlated with seed yield, were not strong predictors of this trait. It is likely that perennial ryegrass plants have an extraordinary ability to recover from injury, and from just a few plants and tillers produce an acceptable seed yield. It is also clear that the removal of old growth post first year harvest is essential. No treatments affected plant height or lodging. Seed quality characteristics were relatively unaffected by any treatment. Thousand seed weight and seed germination were in agreement with previously published literature. Additionally, the TSW indicates that seedling vigor would be acceptable. It is unlikely that volunteer seedlings accounted for a substantial proportion of seed yield, however there were high percentages of volunteer seedlings in both SBM and OB. Seed producers would likely benefit from future research testing fall fertilizer and pre-emergent herbicide application rates and timing

Table 2.1 Field locations, soil types and soil test results for each location during trial years one, two and three.

Trial year	Location	Lat.	Long.	Soil type †	OM‡	pH
2013-14	1	48°48'6.2"N	95°45'7.85"W	Borup silt loam, 0-1 % slope	5.0	7.7
2013-14	2	48°50'40.57"N	95°48'5.84"W	Borup silt loam, 0-1 % slope	3.8	8.1
2014-15	1	48°52'44.22"N	95°51'22.44"W	Zippel very fine sandy loam, 0-2% slope	3.3	8.1
2013-14	2	48°48'10.64"N	95°48'4.40"W	Borup silt loam, 0-1 % slope	2.9	8.1
2015-16	1	48°52'32.38"N	95°52'30.38"W	Warroad fine sandy loam, 0-2% slope	3.0	8.2
2015-16	2	48°48'42.14"N	95°46'11.47"W	Borup silt loam, 0-1 % slope	2.8	8.1

† Soil type determined by the National Resource Conservation System Web Soil Survey.

‡ Organic matter presented in percent of a 6" soil samples.

Table 2.2 Average monthly temperatures, extreme minimum winter temperature and number of days averaging a temperature below 0 °C for trial years one, two and three. Weather station provided all temperature data and was located at the GPS coordinates 48.655 N and 95.734 W.

Month	Trial 1 (2013-14)	Trial 2 (2014-15)	Trial 3 (2015-16)
	Air temperature		
	Mean (SD) °C †		
Sept.	15.0 (3.8)	13.8 (4.4)	16.3 (4.8)
Oct.	4.8 (5.2)	7.3 (4.9)	8.1 (4.1)
Nov.	-5.6 (7)	-7.5 (7.5)	0.4 (6.4)
Dec.	-20.1 (8)	-8.0 (7.5)	-6.3 (7.2)
Jan.	-19.6 (8.4)	-13.6 (8.6)	-13.6 (8.8)
Feb.	-19.0 (5.8)	-18.0 (5.5)	-9.8 (7.1)
Mar.	-10.8 (8.1)	-1.9 (8.1)	-0.3 (6.5)
Apr.	1.0 (5.3)	6.0 (5.8)	3.8 (6)
Extreme min.‡	-33.2	-31.0	-28.8
Days < 0 °C	155.0	129.0	116.0
	Soil temperature §		
Sept.	17.5 (2.8)	15.1 (2.6)	17.0 (3.3)
Oct.	6.7 (3.7)	7.5 (2.9)	7.8 (2.8)
Nov.	0.6 (1.2)	-1.8 (3.1)	2.9 (2.4)
Dec.	-0.7 (0.6)	-4.1 (3.1)	-0.1 (0.4)
Jan.	-2.3 (0.7)	-2.7 (1.3)	-0.8 (0.6)
Feb.	-2.8 (0.6)	-3.3 (0.8)	-0.7 (0.5)
Mar.	-2.1 (1.2)	-0.5 (3.1)	1.3 (2.1)
Apr.	2.5 (2.5)	6.2 (4)	4.7 (3.5)
Extreme min.	-4.6	-11.2	-2.1
Days < 0 °C	127.0	131.0	92.0

† Mean temperature and standard deviation reported in degrees Celsius.

‡ Extreme minimum winter temperature based on average daily means.

§ Soil temperature was measured 10 cm below the surface of bare soil.

Table 2.3 Analysis of variance for early season traits: spring plant count (SPC), relative chlorophyll index (RCI), and volunteer seedling emergence (VSE).

Source	df	SPC	RCI	VSE
		Pr(>F)	Pr(>F)	Pr(>F)
Year	2	*	***	NS
FRM ‡	2	***	***	***
PGR §	4	*	NS	NS
Year:FRM	4	*	*	NS
Year:PGR	8	NS †	NS	NS
FRM:PGR	8	NS	NS	NS
Year:FRM:PGR	16	NS	NS	NS

* Significantly different at the 0.05 probability level.

** Significantly different at the 0.01 probability level.

*** Significantly different at the 0.001 probability level.

† Main plot treatment, fall residue management.

‡ Split plot, plant growth regulator.

§ NS not significant at the 0.05 probability level.

Table 2.4 Main effect means for spring plant count (SPC), relative chlorophyll index (RCI) and volunteer seedling emergence (VSE). Trial year were separated when there was a significant treatment by year interaction.

Treatment	SPC			RCI			VSE
	Trial year 1	Trial year 2	Trial year 3	Trial year 1	Trial year 2	Trial year 3	
FRM ‡	no. m ⁻²			Relative chlorophyll index (0-999)			%
OB ‡	10.6 a †	0.9 b	28.9 ab	135.8 a	123.3 a	313.1 b	27.6 a
SBM	15.7 a	12.8 a	32.7 a	177.8 a	119.7 a	403.2 a	28.0 a
Control	9.5 a	2.5 b	21.8 b	182.8 a	86.9 b	317.4 b	9.4 b
PGR §							
Embark	12 a	3.6 b	26.3 a	180.5 a	100.7 a	575.8 a	19.4 a
Apogee	12.6 a	4.8 ab	25.7 a	152.9 a	107.5 a	591.7 a	23.2 a
Control	11.8 a	5.1 ab	28.2 a	163.8 a	111 a	608.5 a	21.5 a
Proxy	11.3 a	6.6 a	29.7 a	172.3 a	111.8 a	602.2 a	21.2 a
Radiate	11.8 a	7.0 a	29.0 a	157.5 a	119.0 a	602.1 a	22.9 a
Mean	11.9 B	5.4 B	27.8 A	109.9 B	165.4 B	596.1 A	21.7 -

† Means followed by the same letter within a column are not statistically different according to Tukey's HSD ($P \leq 0.05$).

‡ Main plot treatment, fall residue management.

§ Split plot, plant growth regulator.

Table 2.5 Analysis of variance for late season traits: seed yield, aboveground biomass, harvest index (HI), plant height (PH) and lodging.

Source	df	Seed yield	Biomass	HI	PH	Lodging
		Pr(>F)	Pr(>F)	Pr(>F)	Pr(>F)	Pr(>F)
Year	2	***	***	***	***	***
FRM †	2	***	***	***	NS	NS
PGR ‡	4	NS§	NS	NS	NS	NS
Year:FRM	10	*	*	NS	NS	***
Year:PGR	20	NS	NS	NS	NS	NS
FRM:PGR	8	NS	NS	NS	NS	NS
Year:FRM:PGR	40	NS	NS	NS	NS	NS

* Significantly different at the 0.05 probability level.

** Significantly different at the 0.01 probability level.

*** Significantly different at the 0.001 probability level.

† Main plot treatment, fall residue management.

‡ Split plot, plant growth regulator.

§ NS not significant at the 0.05 probability level.

Table 2.6 Main effect means for late season traits: aboveground biomass, harvest index (HI), plant height (PH) and lodging. Trial years were separated when there was an interaction with treatment. There were no significant effects of plant growth regulator in the ANOVA, therefore it was excluded from means separation.

Treatment	Biomass			HI			Lodging			PH		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
FRM †	kg ha ⁻¹			%								
OB	4617.2 a	4249.1 a	6064.1 a	29.0 a	21.7 a	18.1 a	17.1 a	32.8 a	45.5 a	47.5 b	59.6 a	57.5 a
SBM	4425.7 a	2952.9 b	6901.9 a	28.4 a	22.6 a	15.5 ab	9.4 a	13.0 a	66.0 a	46.1 a	54.8 a	59.6 a
Control	3749.0 a	1896.6 b	5998.4 a	27.3 a	14.0 b	13.3 b	5.7 a	0.2 a	67.3 a	44.2 ab	54.4 a	59.7 a
Mean	4264.0 B§	3032.8 C	6342 A	28.2 A	19.5 B	15.6 C	10.8 A	15.4 A	59.6 B	45.9 A	56.3 B	58.9 B

† Main plot treatment, fall residue management.

‡ Means followed by the same lowercase letter within a column are not statistically different according to Tukey's HSD ($P \leq 0.05$).

§ Means followed by the same upper case letter between trial years are not statistically different according to Tukey's HSD ($P \leq 0.05$).

Table 2.7 Correlation among measured variables including spring plant count, relative chlorophyll index (RCI), harvest index (HI), lodging, volunteer seedling emergence (VSE), plant height (PH), aboveground biomass, seed yield over all three trial years and locations.

Trait	SPC	RCI	Lodging	VSE	PH	Biomass	HI	Seed yield
SPC	1	0.83 ***	0.47 ***	0.12 *	0.18 **	0.63 ***	-0.21 ***	0.36 ***
RCI		1	0.67 ***	0.15 **	0.36 ***	0.73 ***	-0.43 ***	0.25 ***
Lodging			1	0.15 **	0.55 ***	0.73 ***	-0.46 ***	0.25 ***
VSE				1	0.08 NS †	0.22 ***	0.09 NS	0.27 ***
PH					1	0.36 ***	-0.50 ***	-0.08 NS
Biomass						1	-0.32 ***	0.66 ***
HI							1	0.42 ***
Seed yield								1

*Significant at the 0.05 probability level.

**Significant at the 0.01 probability level.

***Significant at the 0.001 probability level.

Pearson correlation coefficient calculated on an individual split plot basis.

† NS not significant at the 0.05 probability level.

Table 2.8 Analysis of variance for seed quality characteristics: test weight (TW), thousand seed weight (TSW) and germination percentage.

Source	df	TW	TSW	Germination
Environment	1	*	**	NS
FRM †	2	NS §	***	NS
PGR ‡	4	NS	NS	NS
Environment:FRM	2	NS	***	NS
Environment:PGR	4	NS	NS	NS
FRM:PGR	8	NS	NS	NS
Environment:FRM:PGR	8	NS	NS	NS

* Significantly different at the 0.05 probability level.

** Significantly different at the 0.01 probability level.

*** Significantly different at the 0.001 probability level.

† Main plot treatment, fall residue management.

‡ Split plot, plant growth regulator.

§ NS not significant at the 0.05 probability level.

Table 2.9 Main effect means for seed quality traits: test weight (TW), thousand seed weight (TSW) and germination percentage. Seed quality traits were only measured in trial year three.

Treatment	TW	TSW		Germination
	kg hL ⁻¹	Environment 1	Environment 2	%
		— g 1000 seeds ⁻¹ —		
FRM †				
OB	33.4 a ‡	1.61 b	1.65 a	95.5 a
SBM	34.0 a	1.69 ab	1.69 a	94.6 a
Control	32.9 a	1.70 a	1.55 b	95.3 a

† Main plot treatment, fall residue management.

‡ Means followed by the same letter within a column are not statistically different according to Tukey's HSD ($P \leq 0.05$).

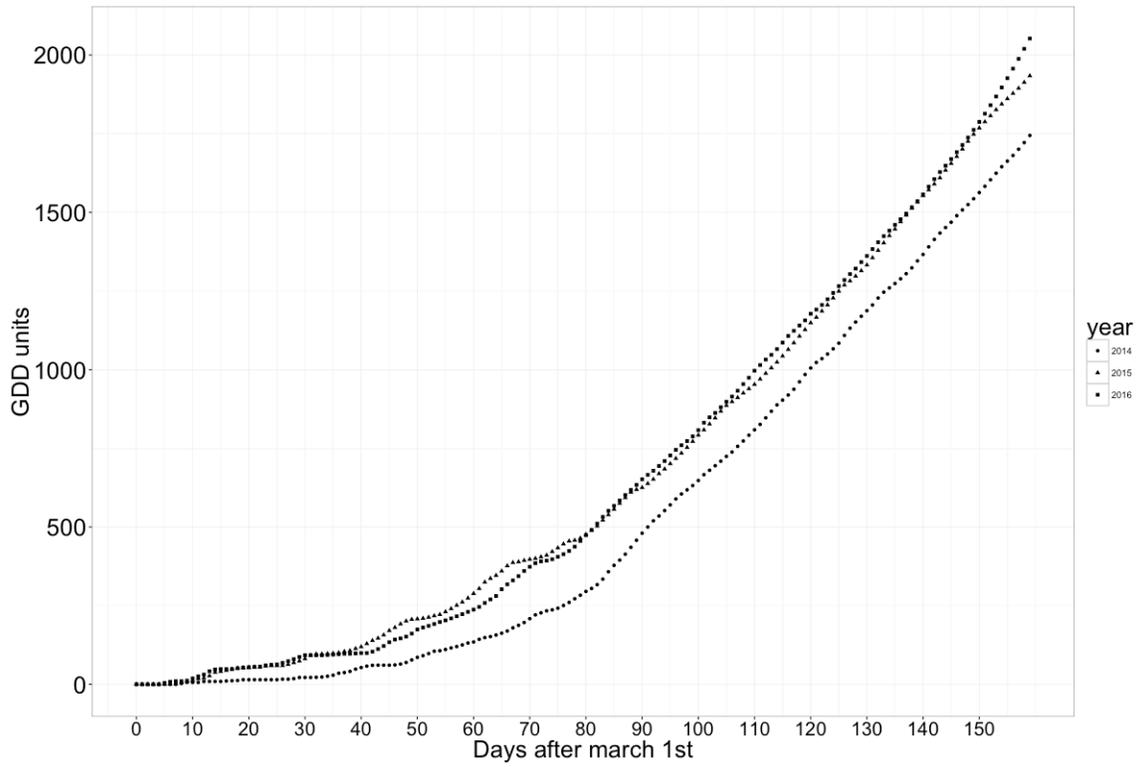


Figure 2.1 Accumulation of growing degree days (GDD) for trial year one, two and three calculated using a base temperature of 0 °C from March 1st to August 7th. . Weather station provided all temperature data and was located at the GPS coordinates 48.655 N and 95.734 W.

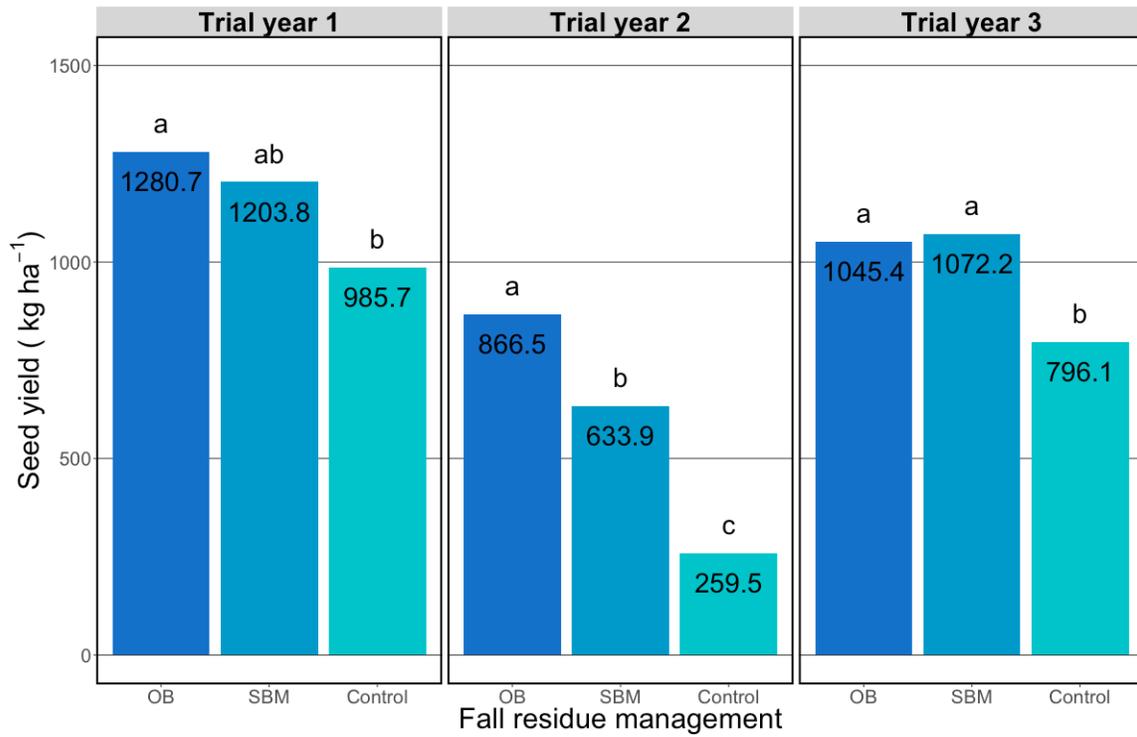


Figure 2.2 Effect of year and fall residue management on final seed yield reported as kg ha⁻¹. Fall residue management treatments were open burn, simulated bale and mow and control. Letters denote significant differences within years according to Tukey's HSD ($P \leq 0.05$). Treatment means are listed below letters.

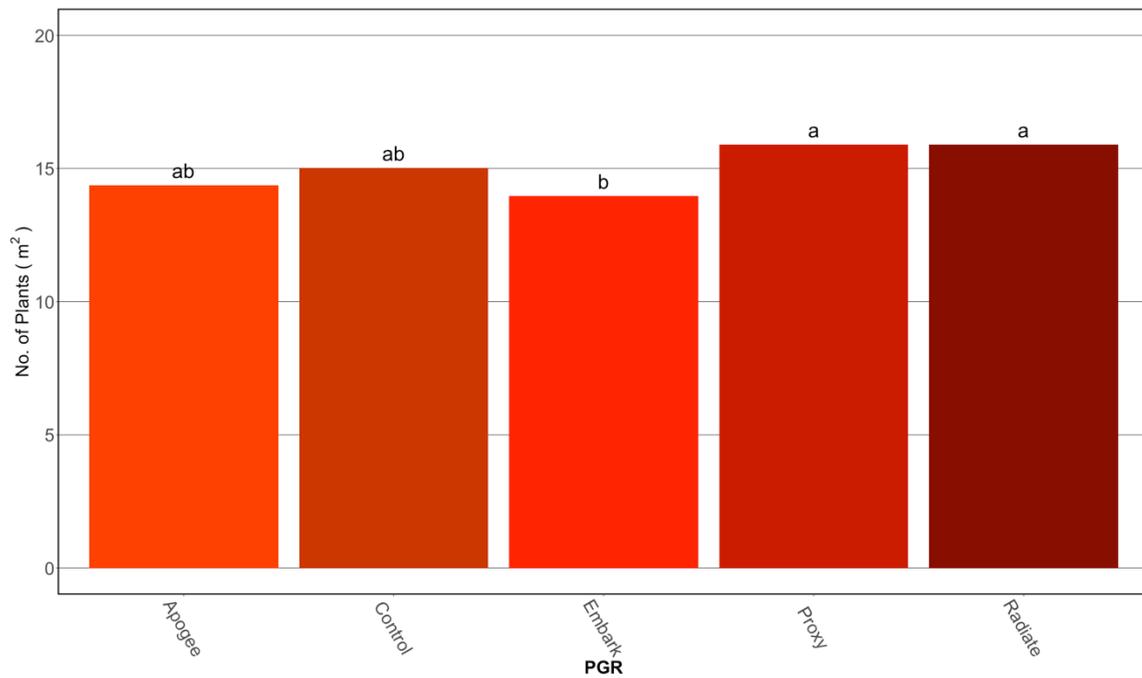


Figure 2.3 Effect of four plant growth regulators (PGR) and a control on spring plant count reported as plants per m². Trial years one, two and three are combined. Letter denotes significant differences between PGRs according to Tukey's HSD ($P \leq 0.05$).

CHAPTER 3.

Effect of the fungal endophyte *Epichloë festucae* var. *lolii* on the freezing tolerance of perennial ryegrass (*Lolium perenne*)

Garett C. Heineck

INTRODUCTION

Among the most heavily researched fungal endophytes are the agriculturally important Clavicipitaceous endophytes encompassing the genre *Epichloë* and *Neotyphodium*. Effects of these maternally inherited asexual endophytes range from production of mammalian toxic alkaloids to enhancing host drought tolerance. Incidence of the species *Epichloë festucae* var. *lolii* is widespread and it commonly inhabits perennial ryegrass irrespective of its geographic origin (Latch et al., 1987; Lewis et al., 1997). A review of the literature on both the well-understood and debated effects of *E. lolii* is essential in the development of testable hypotheses to determine their possible effects on perennial ryegrass winter survival in northern latitudes.

Diversity and functional roles of fungal endophytes have been thoroughly studied and results offer a broad perspective on the breadth of lifestyles and host ranges of fungal endophytes (Rodriguez et al., 2009; Clay and Schardl, 2002; Johnson et al., 2013; Wani et al., 2015). This review will only focus on Class 1 Clavicipitaceous endophytes. These particular endophytes were first observed in the late 19th Century on *Lolium* spp. (Vogl, 1898). Within this group three types of endophytes have been recognized based on the range of symptoms each imparts on their grass host (Clay and Schardl, 2002). Perennial ryegrass is infected with the Type III endophyte *Epichloë festucae* var. *lolii* (formerly *Acremonium lolii* and *Neotyphodium lolii*) (Leuchtmann et al., 2014). Type III endophytes are characterized by their asymptomatic and anamorphic life style suggesting that they evolved to be mutualistic (Clay and Schardl, 2002). Like other Clavicipitaceous endophytes, *E. lolii* grows in intercellular spaces of the plant apoplast, has a narrow host range and is restricted to above ground tissue (Sampson, 1933). *E. lolii* usually produces several toxic alkaloids including: ergovaline, lolitrem and peramine (Clay and Schardl, 2002). Of these three alkaloids, ergovaline and

lolitrem are thought to be associated with neurotoxic effects in mammals (Siegel et al., 1987; Siegel and Bush, 1997). These alkaloids are the cause of disorders in livestock and reduced rates of gain in feeding trials (Paterson et al., 1995 and Eerens et al., 1998a). Peramine is a desirable alkaloid in the turfgrass and forage industry because of its known toxicity to a wide range of invertebrates (Rudgers and Clay, 2007; Bush et al., 1997).

Because some endophytes are beneficial in some systems and not others research has identified endophyte strains with differing alkaloid profiles. Several commercial endophyte strains for forage type perennial ryegrass have been identified and include: SE (Christensen et al., 1993), AR1 (Young et al., 2009), AR37 (Young et al., 2009), NEA2 (van Zijl de Jong et al., 2008) and NEA6 (van Zijl de Jong et al., 2008). Some of these commercial strains are used in place of the wild type (SE) strain especially in production areas such as New Zealand (Agriseeds Superior Pasture, 2013). The alkaloid profiles of each of these strains is not static and depends on several other factors including environment (Lane et al., 1997), host genotype (Easton et al., 2002) and amount of host tissue colonized (Easton et al., 2002). Commercial endophyte strains tested in different host genotypes of a single variety can result in different alkaloid profiles and host phenotypes (Tian et al., 2013a). These discoveries give credence to the testing of any new perennial ryegrass variety infected with novel or wild type endophytes on a population scale due to phenotypic plasticity.

Seed production systems in northern Minnesota can benefit from endophyte enhanced varieties. As of 2013 the estimated contribution of novel endophyte strains to the forage industry in New Zealand was \$200 million annually (Johnson et al., 2013). Revenue is generated from patented endophyte strains, not simply advertising varieties as “endophyte enhanced.” As a vast majority of the seed produced in Minnesota is used for turfgrass and not for forage, potential toxic effects on livestock are somewhat irrelevant. However, producers

that also graze beef cattle have observed toxic effects of commercial turfgrass varieties during fall grazing (Vellekson, personal communication). Focus on researching effects on important abiotic stresses is more valuable in this system. Considering the adaptation of a non-winter hardy species such as perennial ryegrass in northern latitudes, focus should rest on *E. lolii* effects on winter hardiness. Known abiotic effects of endophytes in cool season grasses include alleviation of drought stress, soil mineral imbalance and soil acidity (Malinowski and Belesky, 2000).

Controlled Environment Screening for Freezing Tolerance

Winter conditions often vary across years in relation to severity and the specific stresses imposed. For example, presence or absence of ice sheeting, winter disease, lack of snow cover and freeze thaw events in early or late winter can all impact winter survival (Humphreys, 1989). Open environment screening wherein data are collected over multiple locations and years is probably the most accurate, albeit an expensive and time consuming method. To reduce cycle time, Waldron et al. (1998) suggested the use of controlled environment screening for more rapid gains in perennial ryegrass winter hardiness. Correlations between freezing tolerance and winter survival in the field were significant and acceptable ($r = 0.35$ to 0.54), especially when considering the benefits in time to make selections. Unfortunately, additional complexities emerge when using indirect selection methods to predict winter survival in the field.

Variables such as plant age, growing conditions, acclimation length and temperature, freezing temperature and duration all impact the ability to mimic winter damage (for specific details on freezing injury refer to Chapter 1) (Fuller and Eagles, 1978; Dalmannsdottir et al., 2015; Iraba et al., 2013). For example, Fuller and Eagles (1978) found that freezing tolerance

of perennial ryegrass seedlings were comparable to that of a mature plants. However, Hides (1979) found that young plant freezing tolerance of annual ryegrass (*Lolium multiflorum* Lam.) was not correlated with winter survival in the field. In fact, selecting for more freezing tolerant young plants led to a reduction in winter hardiness of mature plants in some cases. This discrepancy could be due to species differences or indirect selection pressure for seedling vigor. Growing conditions prior to acclimation can result in a change in freezing tolerance and must be considered when interpreting results (for specific details on acclimation refer to Chapter 1) (Dalmannsdottir et al., 2015). Lawrence et al. (1973) found that varying the light intensity during pre-treatment resulted in an interacting effect on freezing tolerance. Lowering the light intensity during acclimation reduced the freezing tolerance of some accessions, especially those with inherently low freezing tolerance. Eagles and Williams (1992) discovered that differences in diurnal temperature influenced the ability of plants to properly acclimate, meaning that length of hardening temperatures in the presence or absence of light influenced the overall freezing tolerance. Due to the complexities of correlating winter hardiness with an artificial screening method, great care must be taken when using anything other than field testing over many years to determine differences in winter hardiness.

Hulke et al. (2008) developed a method of screening that used a programmable freezer to measure plant survival on a binomial scale that could then be used to calculate lethal temperature 50 (LT_{50}) of a plant population. Data from previous research on the same selected entries tested over two years in the field was used to verify findings from freezing tests (Hulke et al., 2007). Using specific growing conditions and acclimation protocol, the predicted LT_{50} had a significant and acceptable predictive ability ($r = -0.72$, $P < 0.001$) for winter survival across a wide range of perennial ryegrass germplasm grown in the field. Although no method will ever perfectly predict plant survival in the field, previous research has proven that with the

appropriate experimental design and data analysis reliable estimates can be achieved. More importantly, with regards to measuring endophyte effects on winter survival, freezing test temperatures can be adjusted to meet the needs of a diverse range of germplasm.

Methods of Population Design

Several different experimental designs are commonly used to study effects of endophytes in cool season grasses with regard to population design. Careful consideration on several points is needed due to the complexity of confounding effects from host genotypes. Effect of endophyte has been measured on either an individual genotype basis or on a population scale (Hahn et al., 2008; Casler and van Santen, 2008). Literature suggests that even closely related genotypes display varying degrees of symbiosis for the same endophyte strain. For example, Cheplick et al. (2000) found variable effects of endophyte infection on drought stress across ten genotypes of perennial ryegrass derived from a single variety. Some host genotypes showed no effect of endophyte while others displayed reduced vigor from infection. Contrary to these findings, a reduction in insect herbivory is seen across populations infected with endophyte when compared to uninfected populations (Bultman and Ganey, 1995). Endophyte effects on insect herbivory are well documented and even commercialized. It may be that many other hypothesized effects are dependent on very specific conditions and may be easily masked by statistical noise making inference across populations more difficult.

Perennial ryegrass is considered as an obligate outcrossing species with highly diverse populations. Grass breeding is often done on a population scale, in which parents are selected based off of the performance of their progeny (Vogel and Pedersen, 1993). Therefore, any effect of endophyte would need to be detectable on a population scale to be applied in an agricultural system. The high prevalence of endophyte across many ecotypes of perennial

ryegrass combined with the anamorphic nature of many grass endophytes suggests a common benefit is realized in nature for one or several traits on a population scale (Clay and Schardl, 2002). Endophyte infected (E+) and endophyte uninfected (E-) populations have been generated several ways. Plants in E+ and E- populations can either be isogenic, non-isogenic or isofrequent (Eerens et al., 1998b; Eerens et al., 1998c; West et al., 1993; Cheplick et al., 2000; Kane, 2011; Casler and van Santen, 2008).

Non-isogenic populations are derived by several means. Welty et al. (1991) selected 20 cultivars of tall fescue ranging from 0 to 86 % endophyte infection to determine the effect of *Neotyphodium coenophialum* on stem rust (*Puccinia graminis* subsp. *graminicola*). Endophyte had no effect on stem rust resistance; however, even with a large collection of varieties effect of endophyte could be masked by effects of variety. In contrast, Gwinn and Gavin (1992) used multiple seed lots of a single variety of tall fescue infected with varying infection frequencies of *Neotyphodium coenophialum* to examine any effect on *Rhizoctonia zae* infection. A high correlation between endophyte infection frequency and disease severity was observed indicating a favorable effect of endophyte. The authors conclude that although the correlation between endophyte infection and disease resistance is compelling there may be confounding effects of host population. The benefits of non-isogenic populations are twofold: 1) plants can be tested and aggregated by infection status easily and 2) large populations of diverse material can be tested quickly. Unfortunately, the confounding effects of host genotype are limiting, especially in populations of diverse origin.

Isogenic, or genetically identical, populations are a common experimental unit because any effect of host genotype is theoretically removed. Isogenic E+ and E- plants can be produced by removing or infecting one of two clones of a single genotype. Removal of the endophyte is done using the systemic fungicides benomyl or propiconazole (Cheplick et al.,

2000; Kane, 2011; Ravel et al., 1997; Hesse et al., 2003). One possible drawback of using fungicides is the possibility of the fungicide itself causing an effect that subsequently attribute to the endophyte. Faeth and Sullivan (2003) conducted an experiment with four E+ fine fescue genotypes that were cloned and half were treated with propiconazole. The resulting populations consisted of isogenic E+, E- and E+ control plants. E+ control was the result of the fungicide not removing the endophyte from an E+ clone. No effect of fungicide was observed for any growth or reproductive parameter measured. There are several examples of fungicide effects on abiotic and biotic stresses, albeit not related to endophytic fungi per se. Ronchi et al. (1997) examined the effect of tetraconazole, a triazole inhibitor, on transcript abundance in maize. The authors found that increased levels of anthocyanin were produced, cell wall structures were enhanced and increased resistance to drought stress was observed. However, this study conducted the drought stress 8 d post application so dissipation of effect could be possible if more time had passed. Herms et al. (2002) examined the effect of the strobilurins (pyraclostrobin) on pathogenesis related (PR) proteins on tobacco and used tobacco mosaic virus to test if a modified defense response was seen on treated plants. Inoculation of the virus was done 24 h post fungicide treatment. The authors found that tobacco mosaic virus had reduced virulence on treated plants even though fungicides are not toxic to viruses. This result was due to an increase in PR proteins in response to the fungicide application. Very few studies include a control testing effect of fungicides on response variables. As fungicides can impact both abiotic and biotic stresses care is need to avoid confounding effects.

Isogenic populations created via endophyte inoculation can be done at the seedling stage or in individual tillers of a mature plants (Latch and Christensen, 1985). Introduction of an endophyte drastically alters plant metabolome, transcriptome and morphology (Ambrose and Belanger, 2012; Dupont et al., 2015). However, it is unclear if, when the endophyte is

transmitted vertically to progeny, the effect holds constant throughout generations, as the endophyte would be then maternally inherited if present. Drawbacks of these approaches include the large amount of time required to develop even small isogenic populations. Results could also be biased because of either fungicide application or introduction of a new organism into the plant, either of which could result in an effect not true to the typical maternal inheritance of the organism.

Finally, effects of endophytes have been measured at a population level by creating isofrequent populations (Casler and van Santen, 2008). Isofrequent populations are the progeny of isogenic, or closely related parents. Therefore, gene frequencies are equal between E+ and E- populations. Bonos et al. (2005) created isofrequent populations from three Chewings fescue (*Festuca rubra* ssp. *Commutate* Thuill.) and two strong creeping red fescue (*Festuca rubra* ssp. *rubra* Gaudin) parents infected with the same four strains of endophyte (*Epichloë festucae* and *Neotyphodium* spp.). Parents were polycrossed in isolation producing several populations with approximately equal allele frequencies but differing in endophyte infection. This population design was successful in estimating endophyte effect on disease resistance. Clay et al. (2005) also developed isofrequent populations of tall fescue differing in infection status by intermating large numbers of E- seed left out of cold storage with that of E+ seed kept in cold storage to study heritable symbiosis. Creation of isofrequent populations first take the initiation of isogenic populations, then vernalization and finally producing seed from crossing populations. This is a time-consuming process and care is needed to account for the variation within each E+ and E- population. For example, perennial ryegrass typically requires 15 weeks of vernalization and the consequent anthesis can be irregular across genotypes (Heide, 1994). The advantage of isofrequent populations is that any effect of endophyte removal or introduction in the isogenic populations can be tested in the following

generation. This is because progeny of the isogenic populations have the same host allelic frequencies and are congenitally endophyte infected or endophyte free, based on parental populations.

This review demonstrated the importance of endophytic fungi in cool season grass systems. Effects of Clavicipitaceous endophytes on reducing animal herbivory are a model example because it has been well documented and commercialized. Other effects on abiotic stresses such as drought tolerance are more disputed and care is needed when drawing inference from results (refer to Chapter 1). In the context of perennial ryegrass seed production and turfgrass in northern latitudes the question of *E. lolii* effects on winter hardiness is of higher import than any other abiotic stress. Previous research on this subject has led to mixed results because of the confounding nature of field experiments leading to inconsistent or extreme results. Controlled environment testing done in a manner that will mimic winter stress will produce more repeatable and sound results. Because perennial ryegrass exists as diverse populations in both nature and commercial varieties the effect of endophyte should be measured on a population or family basis. Moreover, a wide range of germplasm should be tested because of the known variation between host and endophyte. To account for confounding effects of host genotype isogenic E+ and E- (from now on referred to as isogenic E+ and E-) populations should be used with controls included for effect of endophyte removal on E- populations. These will be manifested in the form of *E- control* (from now on referred to as isogenic E- and E-). Finally, heritability of any effect on freezing tolerance will be confirmed by crossing isogenic E+ and E- populations to produce isofrequent E+ and E- progeny.

Objectives

Our objective was to examine the effect of *Epichloe festucae* var. *lolii* on freezing tolerance of a diverse collection of perennial ryegrass. Experiments were designed to test the effect of endophytes in 1) small isogenic E+ and E- populations, 2) small non-isogenic E+ and E- populations, 3) large non-isogenic E+ and E- populations and 4) large isofrequent E+ and E- populations. Freezing tolerance was used as a proxy for winter hardiness with both the lethal temperature 50 (LT₅₀) and interaction between plant survival temperature determining effect of *E. lolii*.

MATERIALS AND METHODS

Germplasm

Experimental subjects, hereafter termed entries, consisted of three varieties and four wild or landrace accessions for Experiments 1 and 3 (Table 3.1). One variety, ‘NK200’, was excluded from Experiment 2 due to complete loss of endophyte infection in bulk seed (Table 3.3). The four accessions (PI 611044, PI 610806, W6 11256 and PI 223178) were chosen based their winter hardiness characteristics that range from excellent to poor; winter hardiness was previously reported by Hulke et al. (2007) (Table 3.1). The commercial variety ‘Green Emperor’ was chosen because it has shown adequate winter hardiness and turfgrass quality (4.9 on a 1 - 9 scale) (Qu et al., 2014; Ehlke and Vellekson, 2014). The commercial variety ‘GrandSlam GLD’ (Peak Plant Genetics-PR 164) was chosen for its exceptional turfgrass quality (6.0 on a 1 - 9 scale) (Qu et al., 2014). The forage variety ‘NK200’ has poor turfgrass quality but was bred for cold climate perennial ryegrass seed production and has been used as a reliable check variety at the University of Minnesota for several decades (Elling, 1975). ‘NK200’ also scored moderately well for winter hardiness when compared to the four accessions (Hulke et al., 2007) (Table 3.1). Seed from accessions was obtained from the Germplasm Resource Information Network (<http://www.ars-grin.gov/>). The same seed lots were used for Experiments 1 and 2. Breeder’s seed of the varieties ‘NK200’ and ‘Green Emperor’ was from the University of Minnesota turfgrass breeding program. Certified seed of the variety ‘GrandSlam GLD’ was obtained from Peak Plant Genetics (Turner, OR). All entries were diploid ($2n=2x=14$) and tested positive for endophyte with at least a 10 % infection frequency (Table 3.2).

Endophyte Detection Methods

Entries were tested for endophyte infection by tissue print immunoblot and light microscopy. Individual sample sizes for each population and experiment will be discussed later. Protocol and materials for tissue print immunoblot were provided by a commercial kit (Phytoscreen Immunoblot Kit #ENDO797-3, Agrinostics, Watkinsville, Georgia, USA, <http://www.agrinostics.com>). This particular immunoblot employs both monoclonal and polyclonal antibodies combined with a chromogenic solution to detect Clavicipitaceous endophyte growing in the grass plant pseudostem (Hiatt et al., 1997; Hiatt et al., 1999). Scoring was done on a binomial scale with any ambiguous blots removed from analysis.

Detection of endophytes via microscopy was adopted from the protocol established by Florea et al. (2015). Fresh pseudostem tissue was harvested from plants with at least three tillers. Sheath tissue was separated from each plant and cut into 0.5 cm sections. Two pieces were submerged in 95 % ethanol for 10 - 20 min to clear chlorophyll. Cleared tissue was placed on a glass slide and covered with several drops of distilled water. A second glass slide was placed on top of the first glass slide to trap the tissue and water. Water and plant material was then frozen by applying dry ice for five min above and below the glass slides. Frozen tissue was then sectioned starting halfway down the length of the tissue using a razor. The thawed product resulted in translucent tissue with a decreasing amount of tissue when traversing the length of the sheath exposing many different tissue types. Two drops of aniline blue-lactic acid solution were used as the staining agent. Stain absorption was increased by covering tissue and stain with a small piece of parafilm and heating at 70 °C for five min in a small oven. Stain was cleared with distilled water and visualized under a light microscope at

100 to 400X magnification. Visual scoring for the presence of endophyte hyphae was done on a binomial scale.

Both tissue print immunoblot and microscopy have been proven to accurately detect Clavicipitaceous endophytes (Trento et al., 2007). Microscopic evaluation takes longer and requires a higher skill level than tissue print immunoblot, but is less prone to false negatives or positives. For example, fungi related to *Epichloë festucae* var. *lolii*, such as *Claviceps purpurea* that commonly infects perennial ryegrass grown in the field, can confound results by indicating false positives when using tissue print immunoblot (Jensen et al., 2011). This is why along with the broad testing of germplasm using tissue print immunoblot small samples of final material were also tested using light microscopy.

Non-Isogenic Population Design

Non-isogenic populations were developed for Experiments 1 and 2. Bulk seed from each entry (three commercials and four accessions) was seeded into 12.7 cm x 12.7 cm trays (08:01) with Sun Gro® MVP soilless media and placed into a 23 °C greenhouse. Ten days post seeding, 100 randomly selected seedlings were transplanted into 50 cell trays with Sun Gro® MVP soilless media. Seedlings were watered daily and fertilized weekly with a 1:100 solution of 50 g L⁻¹ 20-10-20, 48 g L⁻¹ ammonium sulfate and 2 g L⁻¹ Sprint 330 (0-0-0, Fe 10). Once the seedlings had reached the three tiller stage plants were screened for endophyte using tissue print immunoblot, infection frequency ranged from 10-89 % (Table 3.1).

Randomly, E+ and E- plants (these E- plants will be referred to as '*E- congenital*') were chosen from each entry. These plants, although grouped by entry, may or may not be progeny from the same parent and are therefore considered non-isogenic E+ and E- populations. Each of the non-isogenic E + and E- populations was then propagated to produce five clonal stock

plants. Plants were grown in the greenhouse under 16 h photoperiod at 23 °C until each clone had produced at least five tillers. After this, one clone from each *E+* and *E- congenital* genotype was tested to verify endophyte infection.

Isogenic Population Design

Isogenic populations were developed for use in Experiment 1. The non-isogenic *E+* and *E-* populations selected from each entry (described above) were the starting point for the development of two isogenic populations. The first isogenic population was derived from the *E+* population and tested the direct effect of endophyte. The second was derived from the *E- congenital* population and tested for any effect of the endophyte removal process.

Each *E+* and *E- congenital* population was propagated to make two identical population pairs. The first population remained untreated then each genotype in the second set was split into 5 clones and treated with fungicide. Five full rate foliar applications of the systemic fungicide propiconazole (1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]Methyl]-1H-1,2,4-triazole) (Kestel MEX, Phoenix Environmental Care, LLC) were applied to the clones of the second set once rooted, but before tillering had commenced (Latch et al., 1985).

Endophytes were removed via fungicide application from the second *E+* set of clones making an *E- fungicide* population. Similarly, the second *E-* population (*E-congenital*) would remain *E-* yielding an *E- control*. The *E- control* populations were created to measure any effect of fungicide on freezing tolerance (Faeth and Sullivan, 2003).

One week after the final fungicide application the *E- fungicide* and the *E- control* populations were confirmed to lack endophyte infection via tissue print immunoblot. About 60 % of the *E- fungicide* plants initially tested negative for infection. All four populations, *E+*, *E- fungicide*, *E- congenital* and *E- control* (making isogenic *E+* and *E-* and isogenic *E-* and *E-*

populations) were then propagated out one more time and split into five clones and transplanted into 50 cell trays. Plants were grown in the greenhouse under 16 h photoperiod at 23 °C until each clone had produced at least five tillers. This step was completed when 60 d had passed between chemical application and generation of final stock material to reduce any effect of fungicide. Previous studies have allowed varying amounts of time for recovery after fungicide application ranging from a few weeks to two years (He et al., 2013; Cheplick et al., 2000; Hesse et al., 2003; Ravel et al., 1997). Initially in this study, there were obvious signs of phytotoxicity and changes in growth habit after fungicide treatment, including deepening of color and root trimming.

After 60 d stock plants had visually reverted to original phenotype and endophyte status was then confirmed on a single genotype using light microscopy. The majority of the *E-fungicide* plants remained endophyte free, but not all. This resulted in several entries having fewer genotypes per isogenic E+ and E- population than originally selected. However, isogenic populations always contained the same number of plants. Furthermore, genotypes within entry and isogenic group were always clones and only differed in exposure to fungicide and endophyte status.

Isofrequent Population Design

Isogenic E+ and E- populations (*E+* and *E-fungicide*) for each entry were used to create isofrequent populations for Experiment 3. Each of the 14 populations were 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 5 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 100 and 114 d. The 2 wk difference

allowed for even pollination and consistent seed set. The walk in cooler was set to 3 °C with 10 h photoperiod. Light was provided by two 110-W cool white fluorescent bulbs hung approximately 20 cm above plants, providing 150 $\mu\text{mol s}^{-1} \text{m}^{-2}$ irradiance at the plant canopy. After this, the trays were transplanted into large 7.6 cm containers with Sun Gro® 852 RSi soilless media and grown in a 23 °C greenhouse with a 16 h photoperiod. Plants were sub irrigated daily and fertilized twice a week with 1:100 solution of 50 g L⁻¹ 20-10-20, 48 g L⁻¹ ammonium sulfate and 2 g L⁻¹ Sprint 330 (0-0-0, Fe 10).

Upon jointing, containers were separated by entry. Each entry was isolated in its own greenhouse with no other flowering *Lolium* spp. or *Festuca* spp., which controlled pollen flow and allowed isogenic E+ and E- plants from each entry to inter-mate randomly. All entries transitioned from a vegetative stage to a reproductive stage uniformly 18 d post acclimation. Seed maturation was highly variable between entries and genotypes within entries so care was needed to select ripe seed. Harvest began and ended 60 and 80 d post vernalization respectively. Seed was harvested when spikelets lost green color but before shattering occurred. The amount of seed collected was unaffected by endophyte infection (F(1,251), P > 0.1) and there was no interaction with entry (F(6,251), P > 0.1), although this study did not robustly test effect of endophyte on seed production. Seed yield was significantly affected by entry (F(6,258), P < 0.001). Seed collected from each parent was individually packaged and made up the isofrequent E+ and E- populations.

Acclimation Protocol

All experiments used the same acclimation protocol preceding freezing tests. Prior to acclimation, clones from each population were completely randomized within block for each freezing treatment. Plants were then moved to a walk-in acclimation cooler set a 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 approximately 30 cm above plants and providing 75, 170 and 150 $\mu\text{mol s}^{-1} \text{m}^{-2}$ irradiance at plant canopy for Experiments 1, 2 and 3 respectively. Changes in light intensity were caused by fluorescent bulb replacement. During acclimation, plants were rotated within the cooler three times a 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, Plainfield, IL, USA).

Freezing Protocol

After acclimation plants were moved to a Tenney programmable freezer (Lunaire Environmental, New Columbia, PA, USA) for freeze testing. All experiments used the same freezing protocol. Thermocouples were placed on each of the four shelves within the freezer and set to record ambient temperature once every minute (Watchdog A125, Spectrum Technologies, Plainfield, IL, USA). Although the programmed temperatures were consistent across experiments, the mean temperature across the four shelves was used as the true temperature treatment. Freezing tests included 11 temperature treatments each with two 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.

At the beginning of each treatment the freezer was set to reduce and hold temperature at -3 °C. Freezing treatments had the following conditions: 1) all treatments remained under -3 °C for 23 h, 2) the rate of temperature decrease was 1 °C h⁻¹, 3) the chamber remained at the target treatment temperature for 1 h, and 4) each block was placed on the same shelves for each

temperature treatment. After the 23 h treatment plants were removed and allowed to de-acclimate for 3 d in the walk-in acclimation cooler. Plants were then moved into the greenhouse to recover for at least 21 d (Hulke et al., 2008; Hoffman et al., 2010), after which they were scored for survival on a binomial scale with 1 indicating survival and a 0 indicating death. Temperature treatments used were known to provide 100 % death to 100 % survival. Plants from all 11 temperature treatments were included in the final model.

Experiment 1: Comparisons Between Isogenic and Non-Isogenic Populations

Experiment 1 included isogenic and non-isogenic E+ and E- populations as well as isogenic E- and E- populations (Figure 3.1). Isogenic E+ and E- populations contained between eight and ten genotypic pairs (Table 3.2). Isogenic E- and E- populations contained between seven and ten genotypic pairs (Table 3.2). Non-isogenic populations contained E+ and E- *congenital* populations from each entry (Table 3.2). Each isogenic pair consisted of the same genotypes. The target number for each pair was ten genotypes, however this was reduced in some cases because of the fungicide not completely removing the endophyte.

Ten plants would not wholly encompass the genetic diversity within each variety and therefore may not accurately predict the true freezing tolerance of the entry (Bolaric et al., 2005). However, it would give a strong indication of the effect of the endophyte on a subset of the population.

Genotypes from each population were propagated to fulfill blocks and temperature treatments for freezing tests. Flats in the greenhouse were shuffled halfway through the experiment to reduce random climate differences in the greenhouse. Tillers were counted for four random clones of each of the 262 unique genotypes comprising the four populations within each of the seven entries. Experiment 1 was trialed twice using clonal stock material for each

trial. Growing conditions in the greenhouse during trial one and trial two were very similar (mean (SD) of 23.3 (3.7) and 23.0 (4.4) °C for trials one and two respectively).

Experiment 2: Comparisons Between Large Non-Isogenic Populations

Experiment 2 included only non-isogenic populations with a population size of 20 *E+* and *E- congenital* genotypes from each entry. Populations were derived from the same seed sources as in Experiment 1 (Figure 3.1). Endophyte infection frequencies remained sufficient to carry out this experiment except for NK200, which was removed from this experiment (Table 3.3). Twenty genotypes per non-isogenic *E+* and *E-* populations were produced for each entry except for accession PI 223178 (Table 3.3). Fifteen genotypes were used for PI 223178 because of both a high endophyte infection frequency and low seed germination.

Genotypes from each population were propagated to fulfill blocks and temperature treatments for freezing tests. Flats in the greenhouse were shuffled halfway through the experiment to reduce random climate heterogeneity in the greenhouse. Tillers were counted for four random clones of each of the 230 unique genotypes comprising the two populations within each of the six entries (Table 3.3).

Experiment 3: Comparisons Between Isofrequent Populations

Seed produced from polycrossing isogenic *E+* and *E-* populations making up each isofrequent *E+* and *E-* population underwent cool-moist stratification for at least 5 d to break dormancy (Figure 3.1). Seeds were individually planted into 72 cell flats with Sun Gro® MVP soilless media. Seed germination was highly variable and ranged from 24 to 76 % (Table 3.4). 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

isofrequent half-sib family were tested for endophyte infection using tissue print immunoblot. Infection status of a single plant from each family was then confirmed using light microscopy. All families retained parental endophyte status with only a very small proportion resulting in incorrect status (Table 3.4).

Only six half-sib families from each entry had both enough seed and high enough germination percentages to be entered into this experiment. This resulted in the inclusion of a total of 84 (42 isofrequent E+ and E- pairs) families across the seven entries (Table 3.4). Each entry had the same isofrequent populations. For example, progeny from the same exact twelve isogenic E+ and E- parents were included for each entry. Seedlings can respond differently than mature plants to freezing temperatures, therefore seedlings were allowed to grow to least 5 tillers each before acclimation (Hides, 1979). Tillers of ten random genotypes per family were counted prior to freezing. 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 Experiment 3. The decreased number of freezing treatments was a result of low germination and seed yield. This design allowed each entry to be represented by 36 genotypes per temperature treatment and 216 genotypes across temperature treatments (Table 3.4). Future experiments should use cool-moist stratification at 10° C for 5 d with the addition of a 0.2 % KNO₃ solution (Association of Official Seed Analysis, 2015).

Statistical Analysis

Plant survival data was analyzed using a generalized linear model with family designated as binomial in Program R (Version 3.3.2). Model included temperature as the only covariate unless block was significant. Entry and population were treated as fixed effects.

Analysis of covariance was used to determine significance of main effects and interactions using a Chi square test distribution ($\alpha = 0.05$). Significant interactions with entry led to analysis of individual entries. Individual genotypes were included as a random effect. Half-sib family was included as a fixed effect in Experiment 3.

Any interactions with temperature were visually represented using logistic regression and the R package ggplot2 (Wickham H., 2009). Regression lines were statistically differentiated by a 95 % confidence interval calculated by multiplying standard error of the coefficient by 1.96 across all predicted points. Effects at the LT_{50} are delimited by using 95 % confidence intervals.

Tiller counts were analyzed with linear fixed effects models using the R package AGRICOLAE (Mendiburu, 2016). The genotypic samples ($n = 4$) in Experiments 1 and 2 were averaged for each genotype within population. Genotypic samples in Experiment 3 ($n = 10$) were not averaged because there was no clonal replication. Models included entry, endophyte and genotype or half-sib family. Insignificant terms were removed and any interacting terms were separated and analyzed. Treatment effects were considered significant at $\alpha = 0.05$. Means separations were performed on treatments showing significance in the ANOVA using the Tukey-Kramer method ($\alpha = 0.05$).

Discussion of any effect of entry or endophyte on seed yield or germination percentage should be considered with care. However, cognizance of any variables effects on seed production is important to the understanding of the final population design. Analysis of seed yield was completed using ANOVA in the AGRICOLAE package of Project R (Mendiburu, 2016). The linear model consisted of endophyte, accession and replication. Means separations were performed on treatments showing significance in the ANOVA using the Tukey-Kramer method ($\alpha = 0.05$).

RESULTS

Experiment 1

Clonal material was used for both trials one and two of Experiment 1. Analyzing main effects from all combined data in trials one and two was not possible due to several significant two and three way interactions (Table 3.5). Splitting isogenic populations into separate groups (isogenic E+ and E-, isogenic E- and E- and non-isogenic E+ and E- populations) resulted in fewer interactions when trials were combined (Table 3.5). There was no significant effect of endophyte on plant survival or any interacting variable with endophyte for isogenic E+ and E- populations (Table 3.5). Both non-isogenic E+ and E- and isogenic E- and E- populations had several interacting variables with trial making analysis on these populations difficult while trials were combined. Trials were separated to analyze isogenic E- and E- and non-isogenic E+ and E- populations.

Results from trial one revealed significant effect of population (n=4) on freezing tolerance and an interaction between entry and population. Entries were separated into isogenic E+ and E- and isogenic E- and E- populations and non-isogenic E+ and E- populations (Table 3.6). This removed the effect of population in isogenic E+ and E- populations for plant survival (Table 3.6). There was no effect of endophyte in isogenic E+ and E- population for any entry at the LT_{50} or any other temperature (Figure 3.2). In trial one, non-isogenic E+ and E- populations showed a significant effect of endophyte on plant survival with a significant interaction between entry and population (Figure 3.3). Analyzing entries individually revealed significant differences between non-isogenic E+ and E- populations for 'NK200' and PI 610806 (Figure 3.3). E+ populations of 'NK200' and PI 610806 achieved an LT_{50} of -15.6 and -15.1 °C compared to the non-isogenic E- congenital LT_{50} -13.2 and -13.4 °C respectively.

There was no interaction between population and temperature for non-isogenic E+ and E- populations. Entry LT₅₀ in trial one ranged from -16.1 to -12.3 for ‘GrandSlam GLD’ and PI 223178 respectively.

Trial two had similar interacting effects as trial one and so populations were analyzed separately as before. There was no effect of endophyte on the LT₅₀ of isogenic E+ and E- populations for any entry with no interacting effects between temperature and population (Figure 3.2). There was a strong effect of population on isogenic E- and E- populations in trial two for plant survival with an interaction between entry and population (Table 3.6). On an individual entry basis *E- control* populations of ‘NK200’ and PI 610806 had a lower LT₅₀ than *E- congenital* populations (Figure 3.4). Non-isogenic E+ and E- populations differed significantly for plant survival with no significant interaction with entry (Table 3.6). Comparing non-isogenic E+ and E- populations on an entry basis revealed significant differences between ‘Green Emperor’, ‘NK200’, PI 610806 and PI 611044 (Figure 3.3). Endophyte infected populations reached an LT₅₀ of -13.9, -13.8, -13.1 and -12.8 °C compared to the *E- congenital* LT₅₀ of -12.8, -12.2, -12.6 and -12.1 °C for ‘Green Emperor’, ‘NK200’, PI 610806 and PI 611044 respectively. There was no interaction between population and entry in the *E- control* and *E- congenital* groups for plant survival (Table 3.6). This indicates similar effect across all entries for the isogenic E- and E- populations. All control populations, except PI 223178, achieved a lower LT₅₀ than their isogenic counterparts (Figure 3.4). Entry rankings derived from LT₅₀ for trial two ranged from -14.0 to -10.9 °C for ‘GrandSlam GLD’ and PI 223178 respectively. This was slightly higher than trial one however entry rankings remained similar.

There was a significant effect of trial on tiller accumulation. Plants in trial one accumulated significantly more tillers than those in trial two ($P < 0.001$); however ranking and

significant differences between entries remained consistent between trials (Table 3.7). ‘GrandSlam GLD’ and ‘Green Emperor’ consistently had significantly more tillers than the other five entries while PI 610806 had the fewest. Entry and endophyte had a significant and interacting effect on tiller accumulation. There was never a significant difference between isogenic E+ and E- populations for any entry. Tiller number was significantly increased in ‘NK200’ non-isogenic E+ and E- populations in both trials one and two. There was no effect of endophyte on isogenic E- and E- populations in trial one or two, but there was an interaction between endophyte and entry in trial two (Table 3.8).

Experiment 2

‘NK200’ was not included in experiment two due to loss of endophyte in bulk seed. ANCOVA results show a significant effect of entry and endophyte on plant survival (Table 3.9). Endophyte had a moderately significant effect on plant survival ($P = 0.028$). There was no significant interaction between entry and endophyte. Non-isogenic E+ populations had a significantly lower LT_{50} than E- populations, -14.1 vs. -13.9 °C respectively ($P \leq 0.05$) (Figure 3.5). Separating entries and using 95 % CI resulted in the elimination of differences between all entries (Figure 3.4). There was significant interaction between temperature and entry so the ranking of the entries based on plant survival changes depending on the temperature (Table 3.9).

There was no effect of endophyte on tiller accumulation in Experiment 2. Entry ranking for tiller accumulation was similar to Experiment 1 (Table 3.10). ‘GrandSlam GLD’ and ‘Green Emperor’ both had significantly more tillers than the other four entries.

Experiment 3

There was a significant effect of temperature, entry and block on plant survival in Experiment 3 (Table 3.11). A significant interaction between temperature and entry suggests

that the rankings of entries change based on plant survival across temperatures. There was no significant effect of population on plant survival or a significant interaction between population and temperature for isofrequent E+ and E- populations. Considering each entry separately there was never significant effect of population on plant survival, but there was one instance of an interaction ($P = 0.016$) between temperature and population for accession PI 610806 at -12°C (Figure 3.2). There was no effect of endophyte on LT_{50} for any of the 42 isofrequent E+ and E- half-sib family pairs (Table 3.12). There were very few effects of family on LT_{50} within any entry (Table 3.12). PI 611044 ranked more favorably for LT_{50} in Experiment 3 than in E+ and E- isogenic populations in Experiment 1, although it still had still significantly higher LT_{50} than the top two entries and a significantly lower LT_{50} than the least favorable entry in Experiment 1 trial two. The LT_{50} of the top two and bottom two entries ‘GrandSlam GLD/Green Emperor’ and W6 11256/PI 223178 respectively remained the same as isogenic E+ and E- populations in Experiment 1.

There was a significant effect of population on tiller accumulation ($P = 0.004$) and no significant interaction between population and entry ($P \leq 0.05$) in isofrequent E+ and E- populations. The presence of endophyte decreases tiller number and had an average negative average effect of 1.7 tillers. This effect was most pronounced in ‘Green Emperor’ where E+ and E- families averaged 11.0 and 9.2 tillers respectively. There was a large increase in tillering in Experiment 3 when compared with the other two experiments. However, the top to entries ‘Green Emperor’ and ‘GrandSlam GLD’ had statistically more tillers than any other entry and PI 610806 had the fewest, which corresponds to Experiments 1 and 2. Increased tillering may have been a function of growing the plants for a longer period of time to avoid any juvenile effects on freezing tolerance.

DISCUSSION

Symbiotic relationships are common across several kingdoms such as gut bacteria in ruminant animals and rhizobia in leguminous plants (Paracer and Ahmadjian, 2000).

Endophytic associations in plants have existed for hundreds of millions of years (Rodriguez and Redman, 2008). The de facto evidence defining *E. lolii* as a mutualist emanate from the high infection frequencies observed across many ecotypes within perennial ryegrass (Latch et al., 1987; Lewis et al., 1997). It is reasonable to assume that endophytic associations are made and persist because the endophytes contribute to plants fitness by either directly contributing favorable metabolites, such as toxic alkaloids, or changing the host gene expression, such as up regulation of defense genes (Clay and Schardl, 2002; Dupont et al., 2015).

This study examined the effect of *E. lolii* on the freezing tolerance of a diverse array of perennial ryegrass germplasm. Five of the seven entries were previously described as having a wide array of winter hardiness ranging from poor to excellent (Hulke et al., 2007). Infection frequencies across entries ranged from 10 - 89 % in Experiment 1. Infection frequency decreased substantially for most entries in Experiment 2 despite seed being stored at 5 °C (Table 3.3). Previous reports have shown that seeds infected with endophyte, even when in cold storage can lose endophyte viability over long periods of time (Tian et al., 2013b). Because seed was sampled from the same seed lots for both Experiments 1 and 2, the frequency of families sampled at random could have changed. Isorefrequent E+ and E- populations showed almost perfect inheritance of parental endophyte status (Table 3.4). Overall, isorefrequent E+ and E- populations averaged 98.9 and 1.4 % infection frequency respectively. Deviation from 100 or 0 % may have been due to harvesting or seed cleaning errors.

There was often an interaction between entry and temperature for plant survival. Graphing the plant survival curve of each population within entry allows observation of any statistical difference using 95 % CI. Entry LT_{50} differed significantly between experiments; however, the ranking of entries remained reasonably consistent across experiments. Entry rankings for LT_{50} from these experiments generally agree with Hulke et al. (2008) although direct comparisons are difficult due to effect of endophyte on the freezing tolerance in non-isogenic E+ and E- populations. For example, ‘NK200’ achieved a lower LT_{50} than PI 610806 in published work, but it either ranked above or below the LT_{50} of PI 610806 in Experiment 1 depending on endophyte status in non-isogenic E+ and E- populations.

No significant direct effect of endophyte was observed at the LT_{50} or for any other survival proportion across temperatures in Experiments 1 and 3 (Figure 3.2). No direct effect of endophyte on plant survival or interaction with temperature or entry was observed in either trials of Experiment 1. Furthermore, there was no significant effect at the LT_{50} for any half-sib family in Experiment 3. These findings provide compelling evidence that *E. lolii* has no direct effect on the freezing tolerance of perennial ryegrass. Although only seven entries were included in this study, those included represent a wide range of germplasm adapted to freezing conditions. Previous literature suggest that stresses imposed at the point of coevolution can influence stress tolerances conferred by the endophyte (Kane, 2011). PI 610806 (collected from N (46.46666667), E (24.08333333)) and PI 611044 (collected from N (43.71916667), E (41.59583333)), originated from cold environments and have been shown to have exceptional winter hardiness. Two of the varieties, ‘Green Emperor’ and ‘NK200’, were bred specifically for cold climates. Therefore it would be reasonable to assume germplasm included would be more likely to have associations with an endophyte that confers freezing tolerance.

In Experiment 1 consistent association between endophyte and a freezing tolerant hosts were observed in non-isogenic E+ and E- populations (Figure 3.3). This association was confirmed in Experiment 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 Experiment 1 on an entry basis (Figure 3.3). In particular, the E+ population for PI 610806, in Experiment 2 did not achieve a significantly lower LT_{50} than the E- congenital population. This may be due to a reduction in endophyte viability in the seed from Experiment 1 to Experiment 2 (Tables 3.2 and 3.3). Researchers have offered caution with regards to the use of non-isogenic populations when drawing inference on effects between E+ and E- populations (Faeth and Sullivan, 2003). The difficulty in determining the reason for the association is the inability of separating effects of random loss of endophyte in seed, if it is indeed random, or endophytes associating themselves with more freezing tolerant hosts. This is a complex problem that could be resolved by temporal observation of a selected population, for example the accession PI 610806, that showed consistent associated effect of endophyte in Experiment 1. 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 utilized by Gwinn and Gavin (1992) when studying the effect of *Acremonium coenophialum* on the infectivity of *Rhizoctonia zeae* in tall fescue. Researchers observed an increased disease severity in seedlings from seed lots of the same variety but differing in endophyte infection frequency; they suggested the correlation between disease severity and infection frequency was likely the direct cause of endophyte presence. However, they also acknowledged that different host genetics and seedling vigor between seed lots might have confounded results. Using isofrequent E+ and E- populations could eliminate this problem if conducted in a similar manner, but using the E-

population as a control. If used in tandem with data provided in Experiment 1 and 3, a similar outcome would be highly indicative of an association between plants with inherently superior freezing tolerance and endophyte infection.

In relation to the presence of endophytes in the winter hardy commercial varieties 'NK200' and 'Green Emperor' further questions are raised. Infection frequency in bulk breeders seed was 10 and 56 % in 'NK200' and 'Green Emperor' respectively (Table 3.2). For either variety it is impossible to know the original infection frequency of the parental population and difficult to determine the quantity lost in seed storage. Without temporal observation of infection frequencies it is not easy to postulate the selective advantage for either the plant or endophyte in a cold climate, where both of these varieties were bred. The fact that endophyte infection is present to some degree suggests that there could be an advantage to endophyte infection (but see Faeth and Sullivan (2003)). Clay et al. (2005) designed an experiment to answer this question with regards to *Neotyphodium coenophialum* effect on tall fescue in plots with continual or absent animal herbivory from both invertebrates and mammals. Results strongly indicated that presence of herbivory increased the proportion of tillers infected with endophyte over 60 months of sampling. Conversely, endophyte infection frequency decreased in plots that received no stress.

The fitness of a strictly vertically transmitted, anamorphic fungus, such as *E. lolii*, depends primarily on the fitness of its host (Saikkonen et al., 2004; Clay and Schardl, 2002). 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 survival in winter it would be unlikely that a purely symbiotic organism would be prevalent after many cycles of selection. However, if the endophyte aids in other traits important to superior fitness in a breeding program such as disease or insect resistance endophyte infection may be favorable. For example, infected plants

may have increased seed production capacity or turfgrass quality and subsequently be selected more frequently and therein maintain infection frequency of the endophyte (Majidi and Mirlohi, 2016; Bonos et al., 2005).

Tiller production has not been associated with freezing tolerance, however it is a useful trait to measure to plant development (Yamada et al., 2004). Tiller accumulation varied between trials one and two of Experiment 1, but entry rankings were not significantly different. Entries in Experiment 2 also ranked in a similar fashion. This suggests plant phenotypes were similar within and across experiments with respect to tiller accumulation. The presence of endophyte in isogenic E+ and E- populations had no effect on tillering in Experiment 1 trials one and two. Endophyte infection seemed to have a direct effect on tiller accumulation in Experiment 3 by decreasing the mean number of tillers by 1.7. This could indicate that 1) there is an effect of endophyte on growth and development at a young age or 2) there was an effect of fungicide on the growth and development of plants in Experiment 1. Faeth and Sullivan, (2003) included a control for fungicide treatment in a study examining the effect of endophyte on wild fine fescue in Arizona and reported no effect of fungicide on plant growth and development. Reduction in tillering due to endophyte infection has been reported, especially in cases when stress is applied to the plant (Clay and Schardl, 2002). Cheplick et al., (2000) reported that E+ plants accumulated fewer tillers than isogenic E- plants after imposing a 2 wk drought. Richmond et al. (2003) also found endophyte infection decreased tillering when plants were grown in a mixture with *Digitaria sanguinalis* L. These findings however do not explain the effect of reduced tillering by grasses harboring endophytes in stress free environment such as the growing conditions used for Experiment 3.

CONCLUSION

The presence of *E. lolii* never affected freezing tolerance of any entry in either Experiments 1 or 3, which were made up of isogenic and isofrequent E+ and E- populations respectively. Moreover, none of the 42 isofrequent E+ and E- family pairs showed an effect of endophyte on plant survival in Experiment 3. These results suggest the endophyte *E. lolii* has no direct effect on the freezing tolerance of perennial ryegrass and a subsequent limited effect on winter hardiness. Although it should be noted that freezing tolerance is not perfectly correlated with winter survival.

There were several consistent associations between freezing tolerant hosts and the presence of endophyte. Growth rates as measured by tillering were not affected by endophytes in any isogenic E+ and E- population. However, tillering was decreased in isofrequent E+ populations for six of the seven entries compared to E- populations. Possible effects of fungicide were detected for freezing tolerance in one of two trial runs in Experiment 1. Further research could be done to understand the association between freezing tolerant hosts and endophyte infection.

Table 3.1 List of entries included in Experiments 1, 2 and 3. Winter hardiness of entry is reported when possible.

Entry	Source	Origin	Winter hardiness †
PI 611044	Landrace	Russian Federation	6.7
PI 610806	Landrace	Romania	4.9
NK200‡	Cultivar	University of Minnesota Check	4.8
W6 11256	Landrace	Turkey	1.5
PI 223178	Landrace	Greece	1.3
Green Emperor	Cultivar	University of Minnesota	NA
GrandSlam GLD	Cultivar	Peak Plant Genetics	NA

† Winter hardiness 1 - 9 scale 9 = 100 % survival (Hulke et al., 2008).

‡ NK200 not included in Experiment 2.

Table 3.2 Population size for each of the seven entries in Experiment 1. *E+* and *E- fungicide* populations are isogenic. *E- congenital* and *E- control* populations are isogenic. Endophyte infection frequency and the sample size of the test for each entry are reported.

Entry	Isogenic E+ and E- populations		Isogenic E- and E- populations		Infection frequency †	
	<i>E+</i>	<i>E- Fungicide</i>	<i>E- Congenital</i>	<i>E- Control</i>	%	n
PI 610806	8	8	10	10	53	36
PI 611044	9	9	10	10	51	74
PI 223178	8	8	7	7	89	54
W6 11256	10	10	10	10	59	41
NK200	10	10	10	10	10	122
Green Emperor	10	10	10	10	56	45
Grand Slam GLD	9	9	10	10	59	46

† Endophyte infection frequency determined by tissue print immunoblot.

Table 3.3 Population size for each of the six entries in Experiment 2. *E+* and *E- congenital* populations are non-isogenic. Endophyte infection frequency and sample size is reported for all seven entries.

Entry	Non-isogenic populations		Infection frequency †	
	<i>E+</i>	<i>E- congenital</i>	%	n
PI 610806	20	20	39	90
PI 611044	20	20	14	200
PI 223178	15	15	90	120
W6 11256	20	20	42	50
NK200	NA	NA	0	306
Green Emperor	20	20	22	90
Grand Slam	20	20	51	69
GLD				

† Endophyte infection frequency determined by tissue print immunoblot.

Table 3.4 Number of isofrequent E+ and E- half-sib families and population size for all seven entries in Experiment 3. Endophyte infection frequency and sample size for each entry is reported. Average germination percentage for each entry is reported.

Entry	Isofrequent HS families		Isofrequent populations		Infection frequency E+†		Infection frequency E-		Germination
	E+	E-	E+	E-	%	n	%	n	%
PI 610806	6	6	216	216	100	54	0	54	74
PI 611044	6	6	216	216	100	54	4	54	76
PI 223178	6	6	72	72	100	54	0	54	24
W6 11256	6	6	216	216	96	54	4	54	64
NK200	6	6	216	216	96	54	0	54	59
Green Emperor	6	6	216	216	100	54	0	54	69
GrandSlam GLD	6	6	216	216	100	54	2	54	61

† Endophyte infection frequency determined by tissue print immunoblot.

Table 3.5 Analysis of covariance on combined trials for Experiment 1. Isogenic E+ and E- populations consist of *E+* and *E- fungicide*. Isogenic E- and E- populations consist of *E- congenital* and *E- control*. Non-isogenic E+ and E- populations consist of *E+* and *E- congenital*.

Source	Isogenic E+ and E-; isogenic E- and E-		Isogenic E+ and E-		Isogenic E- and E-		Non-isogenic E+ and E-	
	df	Pr(>Chi)	df	Pr(>Chi)	Pr(>Chi)	Pr(>Chi)	Pr(>Chi)	
trial	1	***	1	***	***	***	***	
temp	1	***	1	***	***	***	***	
entry	6	***	6	***	***	***	***	
population (pop)	3	***	1	NS	**	***	***	
trial:temp	1	***	1	***	***	***	***	
trial:entry	6	***	6	NS	NS	**	**	
temp:entry	6	***	6	*	NS	NS	NS	
trial:pop	3	**	1	NS	**	NS	NS	
temp:pop	3	NS†	1	NS	NS	NS	NS	
entry:pop	18	***	6	NS	NS	**	**	
trial:temp:entry	6	NS	6	NS	NS	NS	NS	
trial:temp:pop	3	NS	1	NS	NS	NS	NS	
trial:entry:pop	18	*	6	NS	NS	*	*	
temp:entry:pop	18	NS	6	NS	NS	NS	NS	
trial:temp:entry:pop	18	NS	6	NS	NS	NS	NS	

*, **, *** Significant at the 0.05, 0.01 and 0.001 probability levels, respectively.

† NS, non-significant at the 0.05 probability level.

Table 3.6 Analysis of covariance for plant survival of both trials one and two of Experiment 1. Isogenic E+ and E- populations consist of *E+* and *E- fungicide*. Isogenic E- and E- populations consist of *E- congenital* and *E- control*. Non-isogenic E+ and E- populations consist of *E+* and *E- congenital*. Isogenic and non-isogenic pairs were analyzed together and separately due to a significant interaction with population.

Source	Trial one		Trial two		Trial one			Trial two		
	Isogenic E+ and E-; isogenic E- and E-		Isogenic E+ and E-; isogenic E- and E-		Isogenic E+ and E-	Isogenic E- and E-	Non-isogenic E+ and E-	Isogenic E+ and E-	Isogenic E- and E-	Non-isogenic E+ and E-
	df	Pr(>Chi)	df	Pr(>Chi)	df	Pr(>Chi)	df	Pr(>Chi)	df	Pr(>Chi)
temp	1	***	1	***	1	***	1	***	1	***
entry	6	***	6	***	6	***	6	***	6	***
population(pop)	3	***	1	***	1	NS	1	NS	1	***
temp:entry	6	*	6	**	6	NS	6	NS	6	NS
temp:pop	3	NS†	1	*	1	NS	1	NS	1	**
entry:pop	18	***	6	***	6	NS	6	NS	6	NS
temp:pop:entry	18	NS	6	NS	6	NS	6	NS	6	NS

*, **, *** Significant at the 0.05, 0.01 and 0.001 probability levels, respectively.

† NS = Not significant at the 0.05 probability level.

Table 3.7 Main effect means for tiller accumulation for seven entries in Experiment 1 trials one and two.

Entry	Trial One Tillering‡	Trial Two Tillering
Green Emperor	5.6 a†	3.8 a
GrandSlam	6.1 a	4.1 a
GLD		
PI611044	4.3 b	2.8 b
PI223178	4.2 b	2.6 b
NK200	4.2 b	2.9 b
W611256	4.1 b	2.6 b
PI610806	3.8 b	2.6 b

† Means followed by the same letter within a column are not statistically different according to Tukey's HSD ($P \leq 0.05$).

‡ Tillers of clones (n = 4) for each genotype were counted prior to freezing.

Table 3.8 Analysis of variance for trial one and two of Experiment 1. Tiller accumulation of isogenic E- and E- consisting of *E- congenital* and *E- control*.

Source	df	Trial one	Trial two
		Tiller accumulation	Tiller accumulation
		Pr(>F)	Pr(>F)
entry	6	***	***
population	1	NS†	NS
entry:population	6	NS	**

*, **, *** Significant at the 0.05, 0.01 and 0.001 probability levels, respectively.

† NS = Not significant at the 0.05 probability level.

Table 3.9 Analysis of covariance for plant survival in Experiment 2. Large non-isogenic E+ and E- populations consist of *E+* and *E- congenital*.

Source	df	Non-isogenic E+ and E-
		Pr(>Chi)
temp	1	***
entry	5	***
population	1	*
block	1	NS†
temp:entry	5	***
temp:population	1	NS
entry:population	5	NS
temp:population:entry	5	NS

*, **, *** Significant at the 0.05, 0.01 and 0.001 probability levels, respectively.

† NS = Not significant at the 0.05 probability level.

Table 3.10 Main effect means for tiller accumulation for six entries in Experiment 2.

Entry†	Tillering §
Green Emperor	6.5 a‡
GrandSlam GLD	6.6 a
PI 611044	5.2 b
PI 223178	4.9 b
PI 610806	4.7 b
W6 11256	4.7 b

† NK200 removed from Experiment 2.

‡ Means followed by the same letter within a column are not statistically different according to Tukey's HSD ($P < 0.05$).

§ Tillers of clones ($n=4$) for each genotype were counted prior to freezing.

Table 3.11 Analysis of covariance for plant survival in Experiment 3. Entry consisted of Isofrequent E+ and E- half-sib populations.

Source	Isofrequent E+ and E-	
	df	Pr(>Chi)
temp	1	***
entry	6	***
population	1	NS†
block	3	*
temp:entry	6	*
temp:population	1	NS
entry:population	6	NS
temp:population:entry	6	NS

*, **, *** Significant at the 0.05, 0.01 and 0.001 probability levels, respectively.

† NS = Not significant at the 0.05 probability level.

Table 3.12 Analysis of covariance for plant survival of E+ and E- half-sib families in Experiment 3. Each of the seven entries is listed in a separate column.

Source	df	Green Emperor	GrandSlam GLD	NK200	PI 610806	PI 611044	W6 11256	PI 223178
		Pr(>Chi)	Pr(>Chi)	Pr(>Chi)	Pr(>Chi)	Pr(>Chi)	Pr(>Chi)	Pr(>Chi)
temp	1	***	***	***	***	***	***	***
HS family	5	NS†	*	NS	NS	NS	NS	NS
endophyte	1	NS	NS	NS	NS	NS	NS	NS
temp:HS family	5	*	***	NS	NS	NS	NS	NS
temp:endophyte	1	NS	NS	NS	*	NS	NS	NS
HS family:endophyte	5	**	NS	NS	NS	NS	NS	NS
temp:endophyte:HS family	5	NS	NS	*	NS	NS	NS	NS

*, **, *** Significant at the 0.05, 0.01 and 0.001 probability levels, respectively.

† NS = Not significant at the 0.05 probability level.

Table 3.13 Analysis of variance for Experiment 3. Tiller accumulation of isofrequent E+ and E- populations.

Source	df	Pr(>F)
entry	5	***
population	1	**
entry:population	5	NS†

*, **, *** Significant at the 0.05, 0.01 and 0.001 probability levels, respectively.

† NS = Not significant at the 0.05 probability level.

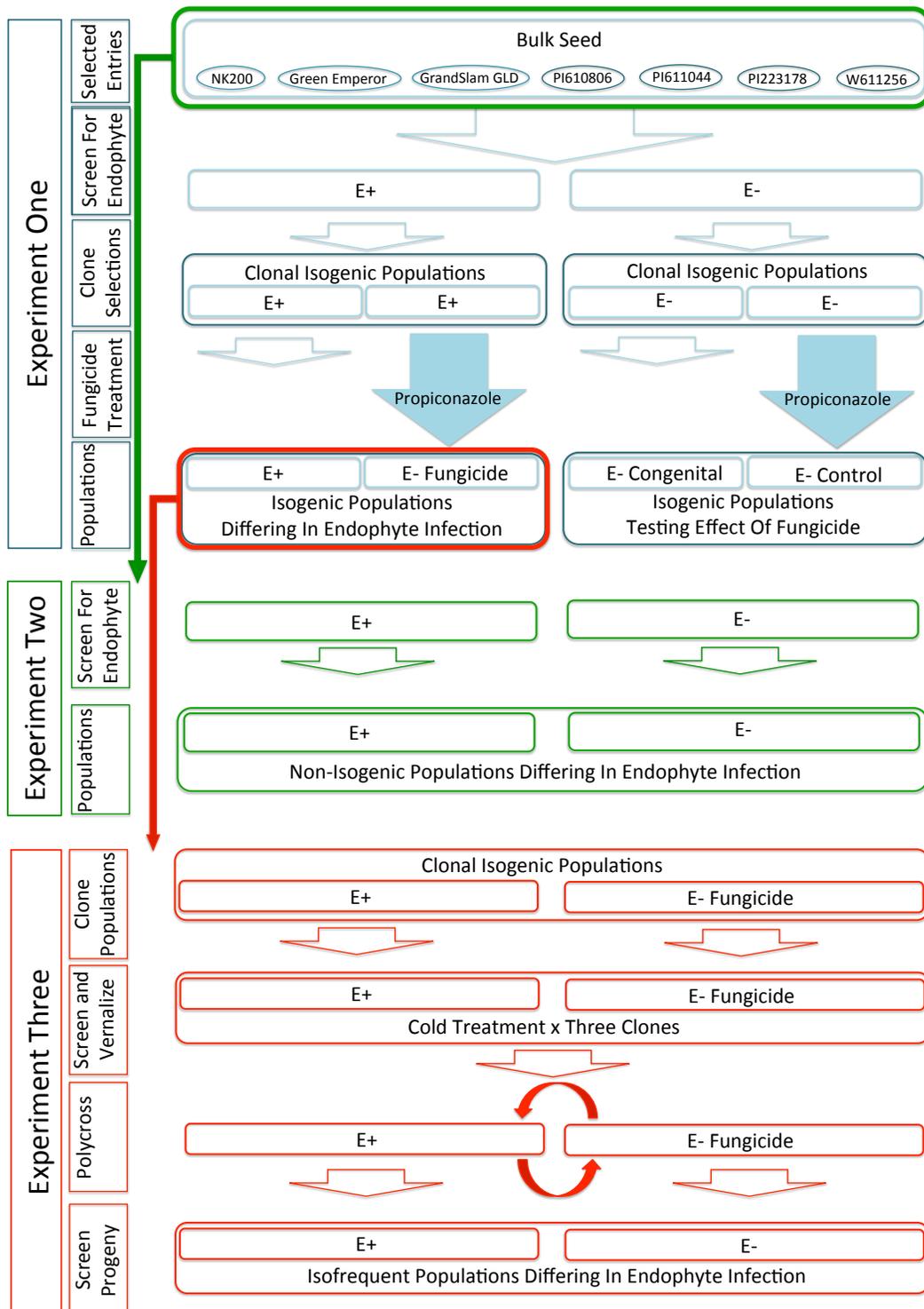


Figure 3.1 Flow diagram of Experiments 1, 2 and 3. Green arrow specifies the same seed source for Experiment 1 was used in Experiment 2. Red arrow identifies that *E+* and *E-fungicide* populations were used as parents in Experiment 3.

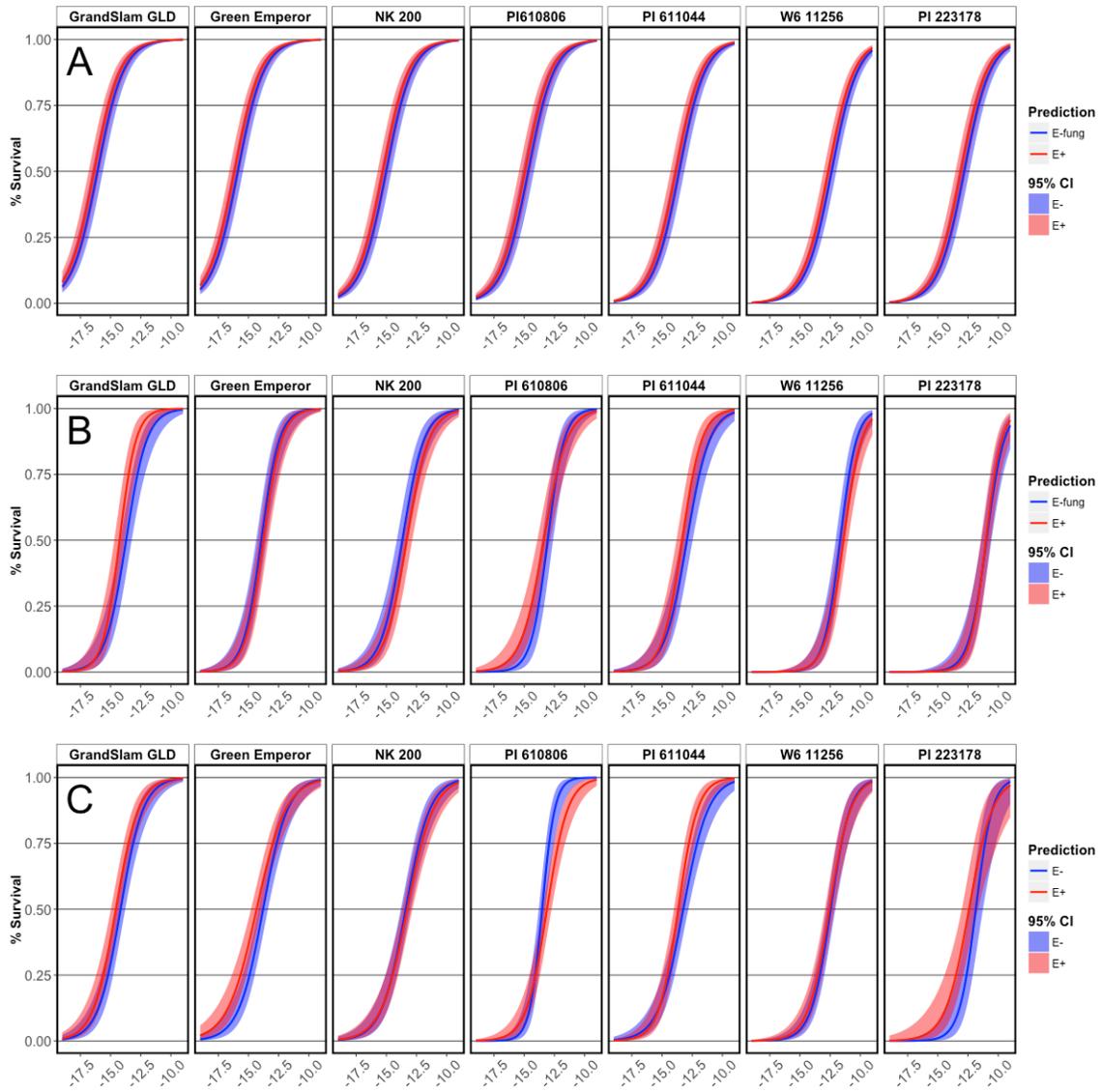


Figure 3.2 Effect of endophyte across freezing treatments. A) All entries from E+/- isogenic populations from Experiment 1 trial one. B) All entries from E+/- isogenic populations from Experiment 1 trial two. C) All entries from E+/- isofrequent populations from Experiment 3. Solid lines represent the predicted survival curve with the shaded region reprinting the 95% CI.

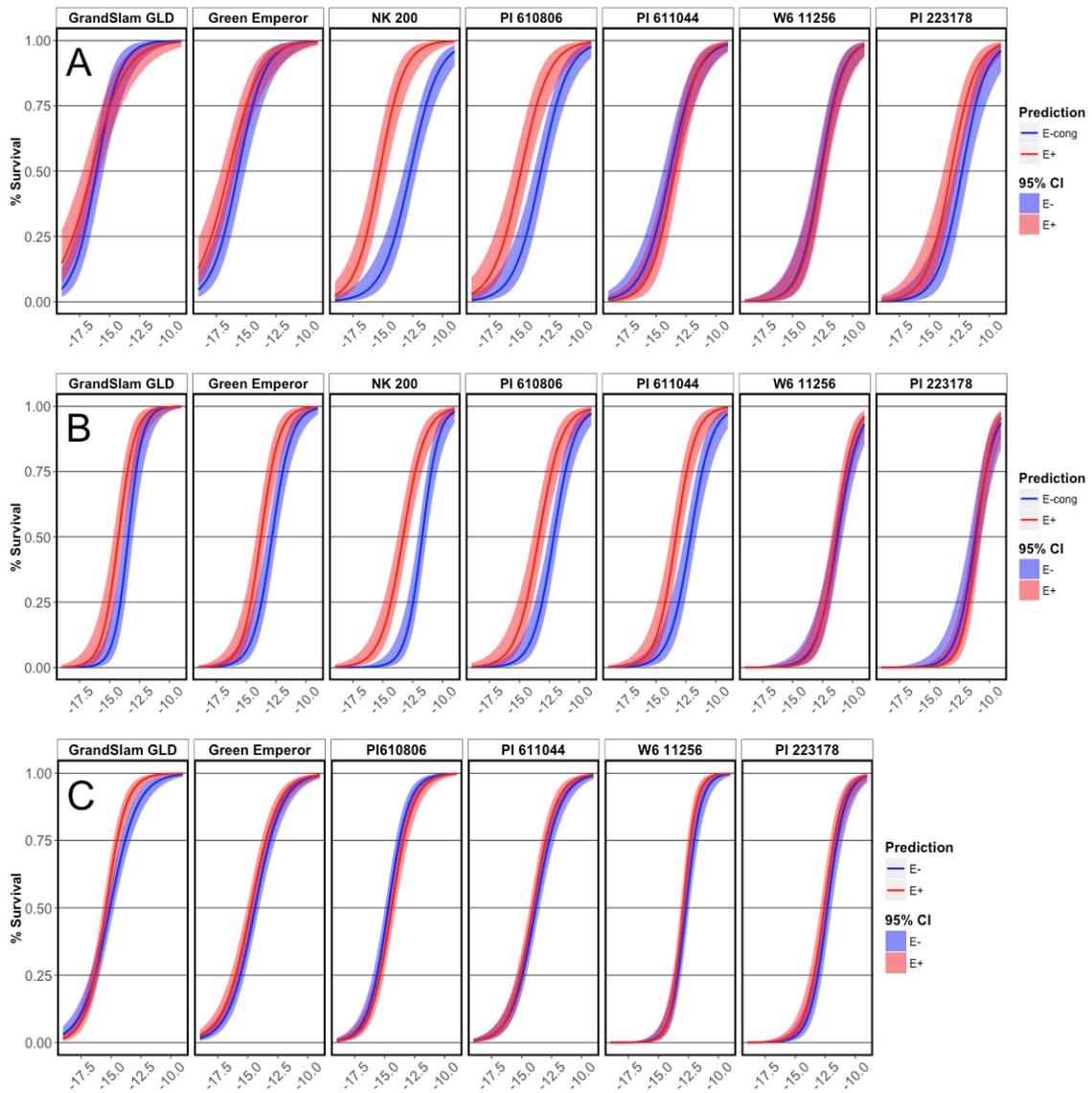


Figure 3.3 Effect of endophyte on an entry basis across freezing treatments. **A)** Entries from non-isogenic E+ and E- populations from Experiment 1 trial one. **B)** Entries from non-isogenic E+ and E- populations from Experiment 1 trial two. **C)** Entries from non-isogenic E+ and E- populations from Experiment 2. Solid lines represent the predicted survival curve with the shaded region reprinting the 95% CI.

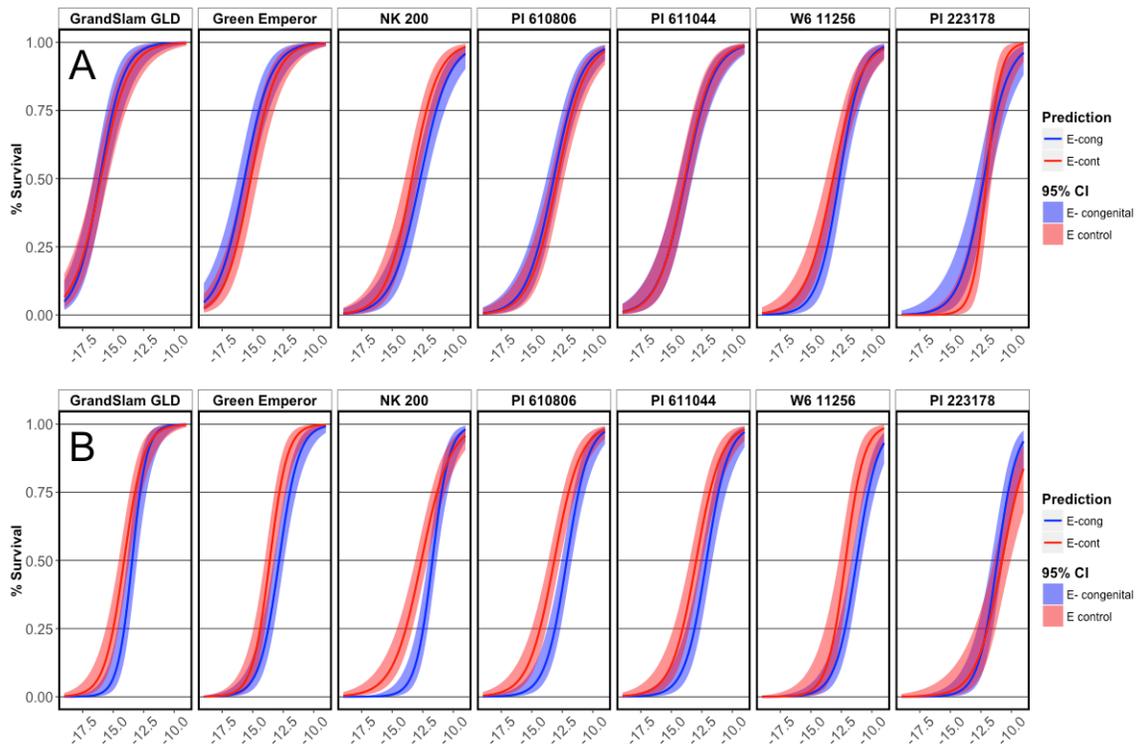


Figure 3.4 Effect of fungicide on an entry basis across freezing treatments. A) Entries from E- and E- isogenic populations from Experiment 1 trial one. B) Entries from E- and E- isogenic populations from Experiment 1 trial two. Solid lines represent the predicted survival curve with the shaded region reprinting the 95% CI.

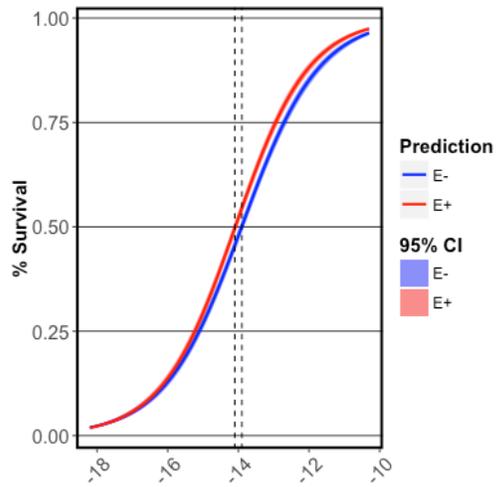


Figure 3.5 Effect of endophyte across entries in Experiment 2. Entries were combined due a lack of interaction between endophyte, entry and temperature. Dotted lines intersecting the solid line at 50 % survival is the LT₅₀ for *E+* and *E-* congenital populations.

REFERENCES

- Abe, J. 1980. Winter hardiness in Turkish populations of cocksfoot, *Dactylis glomerata* L. *Euphytica* 29(3): 531–538.
- Agriseeds Superior Pasture. 2013. Trojan perennial ryegrass. www.argiseeds.co.nz (accessed 20 Nov. 2016).
- Ambrose, K.V., and F.C. Belanger. 2012. SOLiD-SAGE of endophyte-infected red fescue reveals numerous effects on host transcriptome and an abundance of highly expressed fungal secreted proteins. *PloS one* 7(12): e53214.
- Association of Official Seed Analysis. 2015. AOSA rules for testing seeds volume 1. principles and procedures. AOSA, Ithica, NY.
- Bahmani, I., L. Hazard, C. Varlet-Grancher, M. Betin, G. Lemaire, C. Matthew, and E.R. Thom. 2000. Differences in tillering of long-and short-leaved perennial ryegrass genetic lines under full light and shade treatments. *Crop Sci.* 40(4): 1095–1102.
- Balfourier, F., C. Imbert, and G. Charmet. 2000. Evidence for phylogeographic structure in *Lolium* species related to the spread of agriculture in Europe. A cpDNA study. *Theor. Appl. Genet.* 101(1–2): 131–138.
- Ball, S., Y.L. Qian, and C. Stushnoff. 2002. Soluble carbohydrates in two buffalograss cultivars with contrasting freezing tolerance. *J Am. Soc. Hortic. Sci.* 127(1): 45–49.
- Barker, D.J., D.E. Hume, and P.E. Quigley. 1997. Negligible physiological responses to water deficit in endophyte-infected and uninfected perennial ryegrass. p. 137–139. *Neotyphodium/grass interactions*. Springer.
- Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using {lme4}. *J. Stat. Softw.* 67(1): 1–48.
- Beard, J.B. 1966. Direct low temperature injury of nineteen turfgrasses. *Mich. Agric. Exp. Stn. Q. Bull* 48(3): 377–383.
- Beard, J.B. 2012. Origin, biogeographical migrations and diversifications of turfgrasses. *Turfgrass History and Literature*. Michigan State University Press. PP1-26. <http://pesticidetruths.com/wp-content/uploads/2011/12/Reference-Turf-Ornamentals-2011-00-00-Origin-of-Turfgrasses-Beard.pdf> (accessed 4 Dec. 2015).
- Beard, J.B. 2013. Origins of North American turfgrasses. *Agronomy Monograph* 56. *Turfgrass: biology, use, and management*: 1–35.

- Beard, J.B., and others. 1972. Turfgrass: science and culture. Available at <http://agris.fao.org/agris-search/search.do?recordID=US201300490411>.
- Bolaric, S., S. Barth, A.E. Melchinger, and U.K. Posselt. 2005. Genetic diversity in European perennial ryegrass cultivars investigated with RAPD markers. *Plant Breeding* 124(2): 161–166.
- Bonos, S.A., M.M. Wilson, W.A. Meyer, and C. Reed Funk. 2005. Suppression of red thread in fine fescues through endophyte-mediated resistance. *App. Turfgrass Sci.* 2(1): 0–0.
- Brooks, C.C., K.A. Hurto, J. Troll, and others. 1980. The influence of nitrogen: potassium ratios on nutrient content and low temperature hardiness of perennial ryegrass. *Agronomy Abstracts*. 72nd annual meeting, American Society of Agronomy.
- Bultman, T.L., and D.T. Ganey. 1995. Induced resistance to fall armyworm (Lepidoptera: Noctuidae) mediated by a fungal endophyte. *Environ. Entomol.* 24(5): 1196–1200.
- Burke, M.J., L.V. Gusta, H.A. Quamme, C.J. Weiser, and P.H. Li. 1976. Freezing and injury in plants. *Annu. Rev. of Plant Physiol.* 27(1): 507–528.
- Bush, L.P., H.H. Wilkinson, and C.L. Schardl. 1997. Bioprotective alkaloids of grass-fungal endophyte symbioses. *Plant Physiol.* 114(1): 1.
- Canode, C.L., and A.G. Law. 1979. Thatch and tiller size as influenced by residue management in Kentucky bluegrass seed production. *Agronomy J.* 71(2): 289–291.
- Carroll, J.C. 1943. Effects of drought, temperature and nitrogen on turf grasses. *Plant Physiol.* 18(1): 19.
- Casal, J.J., V.A. Deregibus, and R.A. Sanchez. 1985. Variations in tiller dynamics and morphology in *Lolium multiflorum* Lam. vegetative and reproductive plants as affected by differences in red/far-red irradiation. *Ann. Bot.* 56(4): 553–559.
- Casler, M.D. 2006. Perennial grasses for turf, sport and amenity uses: evolution of form, function and fitness for human benefit. *J. Agric. Sci.* 144(3): 189–203.
- Casler, M.D., J.F. Pedersen, G.C. Eizenga, and S.D. Stratton. 1996. Germplasm and cultivar development. *Cool-season forage grasses (coolseasonforag)*: 413–469.
- Casler, M.D., and E. van Santen. 2008. Fungal endophyte removal does not reduce cold tolerance of tall fescue. *Crop Sci.* 48(5): 2033–2039.
- Casler, M.D., and E. Van Santen. 2010. Breeding objectives in forages. p. 115–136. *Fodder Crops and Amenity Grasses*. Springer.
- Change, I.C. 2013. The physical science basis. Contribution of working group I to the fifth assessment report of the intergovernmental panel on climate change. K., Tignor, M., Allen, SK, Boschung, J., Nauels, A., Xia, Y., Bex, V., Midgley, PM, Eds: 1535.

- Charmet, G., and F. Balfourier. 1994. Isozyme variation and species relationships in the genus *Lolium* L.(ryegrasses, Graminaceae). *Theor. App. Genet.* 87(6): 641–649.
- Chastain, T.G., W.C. Vellooza, W.C. Young III, M.E. Mellbye, C.J. Garbacik, and T.B. Silberstein. 1998. Dieback of perennial ryegrass does not reduce seed yield.
- Chastain, T.G., and W.C. Young. 1998. Vegetative plant development and seed production in cool-season perennial grasses. *Seed Sci. Res.* 8(2): 295–301.
- Chastain, T.G., and W.C. Young III. 1998. Vegetative plant development and seed production in cool-season perennial grasses. *Seed Sci. Res.* 8(2): 295–302.
- Chastain, T.G., W.C. Young III, C.J. Garbacik, and B.M. Quebbeman. 1995. Residue management practices for grass seed crops grown in the Willamette Valley. Seed production research at Oregon State University, USDA-ARS cooperating. *Ext/CrS* 102: 1–4.
- Cheplick, G.P. 2004. Recovery from drought stress in *Lolium perenne* (Poaceae): are fungal endophytes detrimental? *Am. J. Bot.* 91(12): 1960–1968.
- Cheplick, G.P., A. Perera, and K. Koulouris. 2000. Effect of drought on the growth of *Lolium perenne* genotypes with and without fungal endophytes. *Funct. Ecol.* 14(6): 657–667.
- Christensen, M.J., R.J. Bennett, H.A. Ansari, H. Koga, R.D. Johnson, G.T. Bryan, W.R. Simpson, J.P. Koolaard, E.M. Nickless, and C.R. Voisey. 2008. *Epichloë* endophytes grow by intercalary hyphal extension in elongating grass leaves. *Fungal Genet. Bio.* 45(2): 84–93.
- Christensen, M.J., A. Leuchtmann, D.D. Rowan, and B.A. Tapper. 1993. Taxonomy of *Acremonium* endophytes of tall fescue (*Festuca arundinacea*), meadow fescue (*F. pratensis*) and perennial ryegrass (*Lolium perenne*). *Mycol. Res.* 97(9): 1083–1092.
- Christians, N. 2011. *Fundamentals of turfgrass management*. John Wiley & Sons.
- Clay, K., J. Holah, and J.A. Rudgers. 2005. Herbivores cause a rapid increase in hereditary symbiosis and alter plant community composition. *Proc. Nat. Acad. Sci. U.S.A.* 102(35): 12465–12470.
- Clay, K., and C. Schardl. 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *Am. Nat.* 160(S4): S99–S127.
- Cooper, J.P. 1964. Climatic variation in forage grasses. I. Leaf development in climatic races of *Lolium* and *Dactylis*. *J. Appl. Ecol.*: 45–61.
- Cooper, R.J., J.R. Street, P.R. Henderlong, and A.J. Koski. 1988. An analysis of the carbohydrate status of mefluidide-treated annual bluegrass. *Agronomy J.* 80(3): 410–414.

- Czarnecki, E., and L.E. Evans. 1986. Effect of weathering during delayed harvest on test weight, seed size, and grain hardness of wheat. *Can. J. Plant Sci.* 66(3): 473–482.
- Dalmanndottir, S., M. Rapacz, M. Jørgensen, L. Østrem, A. Larsen, R. Rødven, and O.A. Rognli. 2015. Temperature before cold acclimation affects cold tolerance and photoacclimation in Timothy (*Phleum pratense* L.), perennial ryegrass (*Lolium perenne* L.) and red clover (*Trifolium pratense* L.). *J. Agron. Crop Sci.*
- De Bary, A. 1879. *The phenomenon of symbiosis*. Karl J. Trubner, Strasbourg, Germany.
- Dofing, S.M., and J.W. Schmidt. 1985. Relationship between subcrown internode length and winter survival in winter barley. *Crop Sci.* 25(4): 690–692.
- Dupont, P.-Y., C.J. Eaton, J.J. Wargent, S. Fechtner, P. Solomon, J. Schmid, R.C. Day, B. Scott, and M.P. Cox. 2015. Fungal endophyte infection of ryegrass reprograms host metabolism and alters development. *New Phytol.* 208(4): 1227–1240.
- Eagles, C.F., and J. Williams. 1992. Hardening and dehardening of *Lolium perenne* in response to fluctuating temperatures. *Ann. Bot.* 70(4): 333–338.
- Easton, H.S., G.C.M. Latch, B.A. Tapper, and O.-P. Ball. 2002. Ryegrass host genetic control of concentrations of endophyte-derived alkaloids. *Crop Sci.* 42(1): 51–57.
- Eerens, J.P.J., R.J. Lucas, H.S. Easton, and J.G.H. White. 1998a. Influence of the ryegrass endophyte (*Neotyphodium lolii*) in a cool-moist environment II. Sheep production. *New. Zeal. J. Agr. Res.* 41(2): 191–199.
- Eerens, J.P.J., R.J. Lucas, S. Easton, and J.G.H. White. 1998b. Influence of the endophyte (*Neotyphodium lolii*) on morphology, physiology, and alkaloid synthesis of perennial ryegrass during high temperature and water stress. *New. Zeal. J. Agr. Res.* 41(2): 219–226.
- Eerens, J.P.J., R.J. Lucas, H.S. Easton, and J.G.H. White. 1998c. Influence of the ryegrass endophyte (*Neotyphodium lolii*) in a cool moist environment. I. Pasture production. *New. Zeal. J. Agr. Res.* 41(1): 39–48.
- Ehlke, N.J., and D.J. Vellekson. 1997. Progress report on seed production research. Prepared for: Minnesota Turf Seed Council. <http://www.mnturfseed.org/2014.pdf> (accessed 10 Nov. 2016).
- Ehlke, N.J., and D.J. Vellekson. 1998. Progress report on seed production research. Prepared for: Minnesota Turf Seed Council. Prepared <http://www.mnturfseed.org/2014.pdf> (accessed 10 Nov. 2016).
- Ehlke, N.J., and D.J. Vellekson. 2006. Progress report on seed production research. Prepared for: Minnesota Turf Seed Council. <http://www.mnturfseed.org/2014.pdf> (accessed 10 Nov. 2016).

- Ehlke, N.J., and D.J. Vellekson. 2011. Progress report on grass seed production research. Prepared for: Minnesota Turf Seed Council. <http://www.mnturfseed.org/2014.pdf> (accessed 10 Nov. 2016).
- Ehlke, N.J., and D.J. Vellekson. 2014. Progress report on grass seed production research. Prepared for: Minnesota Turf Seed Council. <http://www.mnturfseed.org/2014.pdf> (accessed 10 Nov. 2016).
- Ehlke, N.J., and D.J. Vellekson. 2016. Progress report on grass seed production research. Prepared for: Minnesota Turf Seed Council. <http://www.mnturfseed.org/2014.pdf> (accessed 10 Nov. 2016).
- Ehlke, N.J., D.J. Vellekson, and D. Grafstrom. 2014. Progress report on grass seed production research. Prepared for: Minnesota Turf Seed Council. <http://www.mnturfseed.org/2014.pdf> (accessed 10 Nov. 2016).
- Elling, L. 1975. Progress report on grass seed production research. Prepared for: Minnesota Turf Seed Council. <http://www.mnturfseed.org/2014.pdf> (accessed 10 Nov. 2016).
- Elling, L. 1976a. Kentucky bluegrass: A summary of residue management studies in northern Minnesota. Prepared for: Minnesota Turf Seed Council. <http://www.mnturfseed.org/2014.pdf> (accessed 10 Nov. 2016).
- Elling, L. 1976b. Progress report on grass seed production research. Prepared for: Minnesota Turf Seed Council. <http://www.mnturfseed.org/2014.pdf> (accessed 10 Nov. 2016).
- Elling, L. 1979. Progress report on grass seed production research. Prepared for: Minnesota Turf Seed Council. <http://www.mnturfseed.org/2014.pdf> (accessed 10 Nov. 2016).
- Elling, L. 1980. Progress report on grass seed production research. Prepared for: Minnesota Turf Seed Council. <http://www.mnturfseed.org/2014.pdf> (accessed 10 Nov. 2016).
- Elling, L. 1981. Progress report on grass seed production research. Prepared for: Minnesota Turf Seed Council. <http://www.mnturfseed.org/2014.pdf> (accessed 10 Nov. 2016).
- Ensign, R.D., V.G. Hickey, and M.D. Bernardo. 1982. Effects of sunlight reduction and post-harvest residue accumulations on seed yields of Kentucky bluegrass. *J. Appl. Seed Prod.*: 19-21.
- Evans, L.T. 1958. *Lolium temulentum* L., a long-day plant requiring only one inductive photoperiod. *Nature* 182: 197-198.
- Evans, L.T. 1960. The influence of temperature on flowering in species of *Lolium* and in *Poa pratensis*. *J. Agric. Sci.* 54(3): 410-416.

- Faeth, S.H., and T.J. Sullivan. 2003. Mutualistic asexual endophytes in a native grass are usually parasitic. *Am. Nat.* 161(2): 310–325.
- Fagerness, M.J., F.H. Yelverton, D.P. Livingston, and T.W. Rufty. 2002. Temperature and trinexapac-ethyl effects on bermudagrass growth, dormancy, and freezing tolerance. *Crop Sci.* 42(3): 853–858.
- Faria, J., E. Jelihovschi, and I. Allaman. 2016. Conventional Tukey test.
- Fearon, C.H., M.D. Hayward, and M.J. Lawrence. 1983. Self incompatibility in ryegrass. V. Genetic control, linkage, and seed set in diploid *Lolium multiflorum* Lam. *Heredity* 50: 35–46.
- Florea, S., C.L. Schardl, and W. Hollin. 2015. Detection and isolation of *Epichloë* species, fungal endophytes of grasses. *Curr. Protoc. Microbiol.* 19A.1-24.
- Franks, F. 1985. *Biophysics and biochemistry at low temperatures.* Cambridge University Press.
- Fuller, M.P., and C.F. Eagles. 1978. A seedling test for cold hardiness in *Lolium perenne* L. *J. Agri. Sci.* 91(1): 217–222.
- Gerrish, J.R., and C.T. Dougherty. 1983. Tall fescue sward response to mefluidide and nitrogen. *Agronomy J.* 75(6): 895–898.
- Glover, J.D., J.P. Reganold, L.W. Bell, J. Borevitz, E.C. Brummer, E.S. Buckler, C.M. Cox, T.S. Cox, T.E. Crews, S.W. Culman, and others. 2010. Increasing food and ecosystem security through perennial grain breeding. *Science* 328(5986)
- Gronwald, J.W., C.V. Eberlein, K.J. Betts, R.J. Baerg, N.J. Ehlke, and D.L. Wyse. 1992. Mechanism of diclofop resistance in an Italian ryegrass (*Lolium multiflorum* Lam.) biotype. *Pestic. Biochem. Physiol.* 44(2): 126–139.
- Gul, A., and R.E. Allan. 1978. Inheritance of subcrown internode length, crown depth, and crown-tissue regrowth in a winter wheat cross. *Crop Sci.* 18(3): 438–440.
- Gurevitch, J., S.M. Scheiner, G.A. Fox, and others. 2006. *The ecology of plants.* Sinauer Associates Sunderland.
- Gusta, L.V., and M. Wisniewski. 2013. Understanding plant cold hardiness: an opinion. *Physiol. Plant* 147(1): 4–14.
- Guy, C.L. 1990. Cold acclimation and freezing stress tolerance: role of protein metabolism. *Ann. Rev. Plant Bio.* 41(1): 187–223.
- Gwinn, K.D., and A.M. Gavin. 1992. Relationship between endophyte infestation level of tall fescue seed lots and *Rhizoctonia zeae* seedling disease. *Plant Dis.* 76(9): 911–914.

- Hahn, H., M.T. McManus, K. Warnstorff, B.J. Monahan, C.A. Young, E. Davies, B.A. Tapper, and B. Scott. 2008. Neotyphodium fungal endophytes confer physiological protection to perennial ryegrass (*Lolium perenne* L.) subjected to a water deficit. *Environ. Exp. Bot.* 63(1): 183–199.
- Hampton, J.G., and P.D. Hebblethwaite. 1985. The effect of the growth regulator paclobutrazol (PP333) on the growth, development and yield of *Lolium perenne* grown for seed. *Grass and forage Sci.* 40(1): 93–101.
- Hardison, J.R. 1976. Fire and flame for plant disease control. *Ann. Rev. Phytopath.* 14(1): 355–379.
- Hardison, J.R. 1980. Role of fire for disease control in grass seed production. *Plant Dis.* 64(7): 641–645.
- Hardison, J.R., and others. 1949. Blind seed disease of perennial ryegrass. Oregon Agricultural Experiment Station.
- Harrell, F. 2016. Hmisc: Harrel Miscellaneous. R package version 3.17-4. <https://CRAN.R-project.org/package=Hmisc> (accessed 4 Nov. 2016).
- Harrison, J., C. Tonkinson, C. Eagles, and C. Foyer. 1997. Acclimation to freezing temperatures in perennial ryegrass (*Lolium perenne*). *Acta Physiol. Plant.* 19(4): 505–515.
- Hart, J.M., N.P. Anderson, A.G. Hulting, T.G. Chastain, M.E. Mellbye, W.C. Young, T.B. Silberstein, and others. 2012. Postharvest residue management for grass seed production in western Oregon. Corvallis, Or.: Extension Service, Oregon State University.
- He, L., J.H. Hatier, S.D. Card, and C. Matthew. 2013. Endophyte-infection reduces leaf dehydration of ryegrass and tall fescue plants under moderate water deficit. p. 151–156. *Proc. New Zealand Grassland Association.*
- Heide, O.M. 1994. Control of flowering and reproduction in temperate grasses. *New Phytol.* 128(2): 347–362.
- Hermes, S., K. Seehaus, H. Koehle, and U. Conrath. 2002. A strobilurin fungicide enhances the resistance of tobacco against tobacco mosaic virus and *Pseudomonas syringae* pv. tabaci. *Plant Physiol.* 130(1): 120–127.
- Hesse, U., H. Hahn, K. Andreeva, K. Förster, K. Warnstorff, W. Schöberlein, and W. Diepenbrock. 2004. Investigations on the influence of endophytes on plant growth and seed yield of genotypes. *Crop Sci.* 44(5): 1689–1695.
- Hesse, U., W. Schöberlein, L. Wittenmayer, K. Förster, K. Warnstorff, W. Diepenbrock, and W. Merbach. 2003. Effects of *Neotyphodium* endophytes on growth, reproduction and drought-stress tolerance of three *Lolium perenne* L. genotypes. *Grass and Forage Sci.* 58(4): 407–415.

- Hiatt, E.E., N.S. Hill, J.H. Bouton, and C.W. Mims. 1997. Monoclonal antibodies for detection of *Neotyphodium coenophialum*. *Crop Sci.* 37(4): 1265–1269.
- Hiatt, E.E., N.S. Hill, J.H. Bouton, and J.A. Stuedemann. 1999. Tall fescue endophyte detection: commercial immunoblot test kit compared with microscopic analysis. *Crop Sci.* 39(3): 796–799.
- Hides, D.H. 1979. Winter hardiness in *Lolium multiflorum* Lam. III. Selection for improved cold tolerance and its effect on agronomic performance. *Grass and Forage Sci.* 34(2): 119–124.
- Hill, M.J., and B.R. Watkin. 1975. Seed production studies on perennial ryegrass, timothy and prairie grass. *Grass and Forage Sci.* 30(1): 63–71.
- Hoffman, L., M. DaCosta, and J.S. Ebdon. 2014. Examination of cold deacclimation sensitivity of annual bluegrass and creeping bentgrass. *Crop Sci.* 54(1): 413–420.
- Hoffman, L., M. DaCosta, J.S. Ebdon, and E. Watkins. 2010. Physiological changes during cold acclimation of perennial ryegrass accessions differing in freeze tolerance. *Crop Sci.* 50(3): 1037–1047.
- Hofgaard, I.S., A.V. Vollsnes, P. Marum, A. Larsen, and A.M. Tronsmo. 2003. Variation in resistance to different winter stress factors within a full-sib family of perennial ryegrass. *Euphytica* 134(1): 61–75.
- Höglind, M., A.K. Bakken, M. Jørgensen, and L. Østrem. 2010. Tolerance to frost and ice encasement in cultivars of timothy and perennial ryegrass during winter. *Grass and Forage Sci.* 65(4): 431–445.
- Hothorn, T., Frank Bretz, and P. Westfall. 2008. Simultaneous inference in general parametric models. *Biom. J.* 50(3): 346–363.
- Hulke, B.S., E. Watkins, D. Wyse, and N. Ehlke. 2007. Winterhardiness and turf quality of accessions of perennial ryegrass (*L.*) from public collections. *Crop Sci.* 47(4): 1596–1602.
- Hulke, B.S., E. Watkins, D.L. Wyse, and N.J. Ehlke. 2008. Freezing tolerance of selected perennial ryegrass (*Lolium perenne* L.) accessions and its association with field winterhardiness and turf traits. *Euphytica* 163(1): 131–141.
- Humphreys, M.O. 1989. Assessment of perennial ryegrass (*Lolium perenne* L.) for breeding. II. Components of winter hardiness. *Euphytica* 41(1–2): 99–106.
- Humphreys, M.O., and C.F. Eagles. 1988. Assessment of perennial ryegrass (*Lolium perenne* L.) for breeding. I Freezing tolerance. *Euphytica* 38(1): 75–84.
- Humphreys, M., U. Feuerstein, M. Vandewalle, and J. Baert. 2010. Ryegrasses. p. 211–260. *Fodder crops and amenity grasses*. Springer.

- Iraba, A., Y. Castonguay, A. Bertrand, D.J. Floyd, J. Cloutier, and F. Belzile. 2013. Characterization of populations of turf-type perennial ryegrass recurrently selected for superior freezing tolerance. *Crop Sci.* 53:2225-2238.
- Jensen, J.B., V.T. Gonzalez, D.U. Guevara, T.V. Bhuvaneswari, P.R. Wäli, M.V. Tejesvi, A.M. Pirttilä, D. Bazely, M. Vicari, and K.A. Brathen. 2011. Kit for detection of fungal endophytes of grasses yields inconsistent results. *Methods Ecol. Evol.* 2(2): 197–201.
- Jewiss, O.R. 1972. Tillering in grasses—Its significance and control. *Grass and Forage Sci.* 27(2): 65–82.
- John C Stier, Brian P Horgan, Stacy A Bonos. 2013. Turfgrass: biology, use, and management. American Society of Agronomy, Madison, WI.
- Johnson, L.J., A.C. de Bonth, L.R. Briggs, J.R. Caradus, S.C. Finch, D.J. Fleetwood, L.R. Fletcher, D.E. Hume, R.D. Johnson, A.J. Popay, and others. 2013. The exploitation of epichloae endophytes for agricultural benefit. *Fungal Divers.* 60(1): 171–188.
- Kalberer, S.R., M. Wisniewski, and R. Arora. 2006. Deacclimation and reacclimation of cold-hardy plants: current understanding and emerging concepts. *Plant Science* 171(1): 3–16.
- Kane, K.H. 2011. Effects of endophyte infection on drought stress tolerance of *Lolium perenne* accessions from the Mediterranean region. *Environ. Exp. Bot.* 71(3): 337–344.
- Kauppinen, M., K. Saikkonen, M. Helander, A.M. Pirttilä, and P.R. Wäli. 2016. Epichloë grass endophytes in sustainable agriculture. *Nature Plants* 2: 15224.
- Koeritz, E.J., E. Watkins, and N.J. Ehlke. 2013. A split application approach to nitrogen and growth regulator management for perennial ryegrass seed production. *Crop Sci.* 53(4): 1762–1777.
- Koeritz, E.J., E. Watkins, and N.J. Ehlke. 2015. Seeding rate, row spacing, and nitrogen rate effects on perennial ryegrass seed production. *Crop Sci.* 55(5): 2319–2333.
- Kurcinka, E., N.J. Ehlke, and D.L. Wyse. 2009. Nitrogen fertilizer source and application timing in perennial ryegrass (*Lolium perenne* L.) and Kentucky bluegrass (*Poa Pratensis* L.) grown for seed. M.S. thesis, University of Minnesota, Twin Cities.
- Lane, G.A., O.J.P. Ball, E. Davies, and C. Davidson. 1997. Ergovaline distribution in perennial ryegrass naturally infected with endophyte. p. 65–67. *Neotyphodium/Grass Interactions*. Springer.
- Langer, R.H.M., and D.A. Lambert. 1959. Ear-bearing capacity of tillers arising at different times in herbage grasses grown for seed. *Grass and Forage Sci.* 14(2): 137–140.

- Larsen, A. 1994. Breeding winter hardy grasses. p. 149–158. *Breeding Fodder Crops for Marginal Conditions*. Springer.
- Latch, G.C.M., W.F. Hunt, and D.R. Musgrave. 1985. Endophytic fungi affect growth of perennial ryegrass. *New Zealand J. Agric. Rese* 28(1): 165–168.
- Latch, G.C.M., L.R. Potter, and B.F. Tyler. 1987. Incidence of endophytes in seeds from collections of *Lolium* and *Festuca* species. *Ann. Appl. Bio.* 111(1): 59–64.
- LATCHs, G.C.M., and M.J. Christensen. 1985. Artificial infection of grasses with endophytes. *Ann. Appl. Bio* 107(1): 17–24.
- Lawrence, T., J.P. Cooper, and E.L. Breese. 1973. Cold tolerance and winter hardiness in *Lolium perenne*: II. Influence of light and temperature during growth and hardening. *J. Agric. Sci.* 80(2): 341–348.
- Leep, R.H., J.A. Andresen, and P. Jeranyama. 2001. Fall dormancy and snow depth effects on winterkill of alfalfa. *Agronomy J.* 93(5): 1142–1148.
- Lenth, R. 2016. Least-squares means: The {R} package {lsmeans}. *J. Statis. Softw.* 69(1): 1–33.
- Leuchtmann, A., C.W. Bacon, C.L. Schardl, J.F. White, and M. Tadych. 2014. Nomenclatural realignment of *Neotyphodium* species with genus *Epichloë*. *Mycologia* 106(2): 202–215.
- Lewis, G.C., C. Ravel, W. Naffaa, C. Astier, and G. Charmet. 1997. Occurrence of *Acremonium* endophytes in wild populations of *Lolium* spp. in European countries and a relationship between level of infection and climate in France. *Ann. Appl. Bio.* 130(2): 227–238.
- Majidi, M.M., and A. Mirlohi. 2016. Impact of endophytic fungi on seed and seedling characteristics in tall and meadow fescues. *IJPP* 10: 4.
- Malinowski, D.P., and D.P. Belesky. 2000. Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. *Crop Sci.* 40(4): 923–940.
- Márquez, L.M., R.S. Redman, R.J. Rodriguez, and M.J. Roossinck. 2007. A virus in a fungus in a plant: three-way symbiosis required for thermal tolerance. *Science* 315(5811): 513–515.
- Maslova, S.P., G.N. Tabalenkova, S.V. Kurenkova, and S.N. Plusnina. 2007. Seasonal changes in anatomical and morphological structure and the content of phytohormones and sugars in underground shoots of a long-rhizome perennial grass *Phalaroides arundinacea*. *Russ. J. Plant Physiol.* 54(4): 491–497.
- McNaughton, S.J. 1984. Grazing lawns: animals in herds, plant form, and coevolution. *Am. Nat.*: 863–886.

- Mendiburu, F. 2016. agricolae: Statistical procedures for agricultural Research. R package version 1.2-4. <https://CRAN.R-project.org/package=agricolae>.
- Mueller-Warrant, G.W., M.E. Mellbye, and W.C. Young. 1990. Effect of herbicide treatment and crop residue removal method on volunteer weed control and yield in tall fescue and perennial ryegrass. <http://ir.library.oregonstate.edu/xmlui/bitstream/handle/1957/15039/1990.pdf?sequence=1>.
- Mueller-Warrant, G.W., W.C. Young, and M.E. Mellbye. 1994. Influence of residue removal method and herbicides on perennial ryegrass seed production: I. Weed control. *Agronomy J.* 86(4): 677–684.
- NASS. 2006. Oregon agriculture facts and figures. <http://library.state.or.us/repository/2010/201010121527412/2005.pdf> (accessed 10 Nov 2016).
- NASS. 2016. Oregon Agriculture Facts and Figures. https://www.nass.usda.gov/Statistics_by_State/Oregon/Publications/facts_and_figures/facts_and_figures.pdf (accessed 10 Nov 2016).
- Paracer, S., and V. Ahmadjian. 2000. *Symbiosis: an introduction to biological associations*. Oxford University Press.
- Parker, J. 1963. Cold resistance in woody plants. *The botanical review* 29(2): 123–201.
- Paterson, J., B.L. Chris Forcherio, M. Samfordt, and M. Kerleyf. 1995. The effects of fescue toxicosis on beef cattle productivity. *J. Anim. Sci.* 73: 889–898.
- Patterson, C.G., D.A. Potter, and F.F. Fannin. 1991. Feeding deterrence of alkaloids from endophyte-infected grasses to Japanese beetle grubs. *Entomol. Exp. App.* 61(3): 285–289.
- Pearce, R.S. 2001. Plant freezing and damage. *Ann. Bot.* 87(4): 417–424.
- Pfender, W. 2009. A damage function for stem rust of perennial ryegrass seed crops. *Phytopathology* 99(5): 498–505.
- Phillips, Idj. 1975. Apical dominance. *Annual review of plant physiology* 26(1): 341–367.
- Qu, Y., E. Kock, R. Bara, D. Smith, E. Szerszen, S. Bonos, and W. Meyer. 2014. Performance of perennial ryegrass cultivars and selections in New Jersey trials. <http://turf.rutgers.edu/research/reports/2014/131.pdf> (accessed 4 Sept. 2016).
- Rademacher, W. 2000. Growth retardants: effects on gibberellin biosynthesis and other metabolic pathways. *Annual review of plant biology* 51(1): 501–531.

- Randall, G.W., D.R. Huggins, M.P. Russelle, D.J. Fuchs, W.W. Nelson, and J.L. Anderson. 1997. Nitrate losses through subsurface tile drainage in conservation reserve program, alfalfa, and row crop systems. *J. Environ. Qual.* 26(5): 1240–1247.
- Ravel, C., G. Charmet, and F. Balfourier. 1995. Influence of the fungal endophyte *Acremonium lolii* on agronomic traits of perennial ryegrass in France. *Grass and Forage Sci.* 50(1): 75–80.
- Ravel, C., C. Courty, A. Coudret, and G. Charmet. 1997. Beneficial effects of *Neotyphodium lolii* on the growth and the water status in perennial ryegrass cultivated under nitrogen deficiency or drought stress. *Agronomie* 17(3): 173–181.
- Richmond, D.S., P.S. Grewal, and J. Cardina. 2003. Competition between *Lolium perenne* and *Digitaria sanguinalis*: Ecological consequences for harbouring an endosymbiotic fungus. *J. Veg. Sci.* 14(6): 835–840.
- Richmond, D.S., H.D. Niemczyk, and D.J. Shetlar. 2000. Overseeding endophytic perennial ryegrass into stands of Kentucky bluegrass to manage bluegrass billbug (Coleoptera: Curculionidae). *J. Econ. Entomol.* 93(6): 1662–1668.
- Rocheffort, S., Y. Desjardins, D.J. Shetlar, and J. Brodeur. 2007. Establishment and survival of endophyte-infected and uninfected tall fescue and perennial ryegrass overseeded into existing Kentucky bluegrass lawns in northeastern north America. *HortSci.* 42(3): 682–687.
- Rodriguez, R., and R. Redman. 2008. More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. *J. Exp. Bot.* 59(5): 1109–1114.
- Rodriguez, R.J., J.F. White Jr, A.E. Arnold, and R.S. Redman. 2009. Fungal endophytes: diversity and functional roles. *New Phytol.* 182(2): 314–330.
- Rolston, M.P., B.L. McCloy, and N.B. Pyke. 2004. Grass seed yields increased with plant growth regulators and fungicides. p. 127–132. *Proceedings of the New Zealand Grassland Association.*
- Ronchi, A., G. Farina, F. Gozzo, and C. Tonelli. 1997. Effects of a triazolic fungicide on maize plant metabolism: modifications of transcript abundance in resistance-related pathways. *Plant Sci.* 130(1): 51–62.
- Rudgers, J.A., and K. Clay. 2007. Endophyte symbiosis with tall fescue: how strong are the impacts on communities and ecosystems? *Fungal Biol. Rev.* 21(2): 107–124.
- Sachs, T., and K.V. Thimann. 1964. Release of lateral buds from apical dominance. *Nature* 201, 939-940.
- Saikkonen, K., P. Wäli, M. Helander, and S.H. Faeth. 2004. Evolution of endophyte–plant symbioses. *Trends Plant Sci.* 9(6): 275–280.

- Sakai, A., and S. Yoshida. 1967. Survival of plant tissue at super-low temperature VI. Effects of cooling and rewarming rates on survival. *Plant Physiol.* 42(12): 1695–1701.
- Sampoux, J.P., P. Baudouin, B. Bayle, V. Béguier, P. Bourdon, J.F. Chosson, K. Bruijn, F. Deneufbourg, C. Galbrun, M. Ghesquière, and others. 2013. Breeding perennial ryegrass (*Lolium perenne* L.) for turf usage: an assessment of genetic improvements in cultivars released in Europe, 1974–2004. *Grass Forage Sci.* 68(1): 33–48.
- Sampson, K. 1933. The systemic infection of grasses by *Epichloe typhina* (Pers.) Tul. *Transactions of the British Mycological Society* 18(1): 30–IN3.
- Siegel, M.R., and L.P. Bush. 1997. Toxin production in grass/endophyte associations. p. 185–207. *Plant Relationships*. Springer.
- Siegel, M.R., G.C.M. Latch, and M.C. Johnson. 1987. Fungal endophytes of grasses. *Annual review of phytopathology* 25(1): 293–315.
- Silisbury, J.H. 1965. Interrelations in the growth and development of *Lolium*. I. Some effects of vernalization on growth and development. *Crop and Pasture Sci.* 16(6): 903–913.
- Stebbins, G.L. 1981. Coevolution of grasses and herbivores. *Annals of the Missouri Botanical Garden*: 75–86.
- Steinke, K., and J.C. Stier. 2003. Influence of trinexapac-ethyl on cold tolerance and nonstructural carbohydrates of shaded supina bluegrass. p. 207–215. *In* I International Conference on Turfgrass Management and Science for Sports Fields 661.
- Steponkus, P.L., M. Uemura, R.A. Balsamo, T. Arvinte, and D.V. Lynch. 1988. Transformation of the cryobehavior of rye protoplasts by modification of the plasma membrane lipid composition. *Proceedings of National Academy of Sciences* 85(23): 9026–9030.
- Stewart, A.V. 2006. Genetic origins of perennial ryegrass (*Lolium perenne*) for New Zealand pastures. *Grassland Research and Practice Series* 12: 55–62.
- Tanaka, A., D. Takemoto, G.-S. Hyon, P. Park, and B. Scott. 2008. NoxA activation by the small GTPase RacA is required to maintain a mutualistic symbiotic association between *Epichloë festucae* and perennial ryegrass. *Molecular microbiology* 68(5): 1165–1178.
- Terrell, E.E. 1968. A taxonomic revision of the genus *Lolium*. US Dept. of Agriculture.
- Tian, P., T.-N. Le, E.J. Ludlow, K.F. Smith, J.W. Forster, K.M. Guthridge, and G.C. Spangenberg. 2013a. Characterization of novel perennial ryegrass host–*Neotyphodium* endophyte associations. *Crop Pasture Sci.* 64(7): 716–725.

- Tian, P., T.-N. Le, K.F. Smith, J.W. Forster, K.M. Guthridge, and G.C. Spangenberg. 2013b. Stability and viability of novel perennial ryegrass host–Neotyphodium endophyte associations. *Crop Pasture Sci.* 64(1): 39–50.
- Trento, S., S. Elias, A. Garay, and J. Zavala. 2007. Comparison of endophyte detection in fescue and ryegrass seeds using an immunoblot assay and a microscopic method. *Seed Science and Technology* 35(1): 65–74.
- Tugeon, A.J. 2004. *Turfgrass management* (7th Edition). Prentice Hall 2004.
- Undersander, D.J., M.G. Bertram, J.R. Clark, A.E. Crooks, M.C. Rankin, K.G. Silveira, and T.M. Wood. 2005. Forage variety update for Wisconsin. Univ. Wis. Coop. Ext. Publ. A 1525. <http://www.uwex.edu/ces/forage/pubs/a1525-2006.pdf> (accessed 20 November 2016).
- Vogel, K.P., and J.F. Pedersen. 1993. Breeding systems for cross-pollinated perennial grasses. *Plant Breeding Reviews*, Volume 11: 251–274.
- Vogl, A. 1898. Mehl und die anderen Mehlprodukte der Cerealien und Leguminosen. *Nahrungsm Unters Hyg Warenk* 12: 25–29.
- Waldron, B.L., N.J. Ehlke, D.J. Vellekson, and D.B. White. 1998a. Controlled freezing as an indirect selection method for field winterhardness in turf-type perennial ryegrass. *Crop Sci.* 38(3): 811–816.
- Waldron, B.L., N.J. Ehlke, D.J. Vellekson, and D.B. White. 1998b. Controlled freezing as an indirect selection method for field winterhardness in turf-type perennial ryegrass. *Crop Sci.* 38(3): 811–816.
- Waldron, B.L., N.J. Ehlke, D.L. Wyse, and D.J. Vellekson. 1998c. Genetic variation and predicted gain from selection for winterhardness and turf quality in a perennial ryegrass topcross population. *Crop Sci.* 38(3): 817–822.
- Wäli, P.R., M. Helander, O. Nissinen, P. Lehtonen, and K. Saikkonen. 2008. Endophyte infection, nutrient status of the soil and duration of snow cover influence the performance of meadow fescue in sub-arctic conditions. *Grass Forage Sci.* 63(3): 324–330.
- Wani, Z.A., N. Ashraf, T. Mohiuddin, and S. Riyaz-Ul-Hassan. 2015. Plant-endophyte symbiosis, an ecological perspective. *App. Microbiol. Biotechnol.* 99(7): 2955–2965.
- Webster, D.E., and J.S. Ebdon. 2005. Effects of nitrogen and potassium fertilization on perennial ryegrass cold tolerance during deacclimation in late winter and early spring. *HortSci.* 40(3): 842–849.
- Welty, R.E., R.E. Barker, and M.D. Azevedo. 1991. Reaction of tall fescue infected and noninfected by *Acremonium coenophialum* to *Puccinia graminis* subsp. *graminicola*. *Plant Dis.* 75(9): 883–886.

- West, C.P., E. Izekor, K.E. Turner, and A.A. Elmi. 1993. Endophyte effects on growth and persistence of tall fescue along a water-supply gradient. *Agronomy J.* 85(2): 264–270.
- Williams, C.M., H.A. Henry, and B.J. Sinclair. 2015. Cold truths: how winter drives responses of terrestrial organisms to climate change. *Biol. Rev.* 90(1): 214–235.
- Wilson, D. 1995. Endophyte: the evolution of a term, and clarification of its use and definition. *Oikos*: 73(2):274–276.
- Wood, G.M., and R.P. Cohen. 1984. Predicting cold tolerance in perennial ryegrass from subcrown internode length. *Agronomy J.* 76(4): 516–517.
- Xin, Z., and J. Browse. 2000. Cold comfort farm: the acclimation of plants to freezing temperatures. *Plant, Cell & Environment* 23(9): 893–902.
- Xiong, L., K.S. Schumaker, and J.-K. Zhu. 2002. Cell signaling during cold, drought, and salt stress. *The plant cell* 14(suppl 1): S165–S183.
- Yamada, T., E.S. Jones, N.O.I. Cogan, A.C. Vecchies, T. Nomura, H. Hisano, Y. Shimamoto, K.F. Smith, M.D. Hayward, and J.W. Forster. 2004. QTL analysis of morphological, developmental, and winter hardiness-associated traits in perennial ryegrass. *Crop Sci.* 44(3): 925–935.
- Young, W.C., M.E. Mellbye, and T.B. Silberstein. 1999. Residue management of perennial ryegrass and tall fescue seed crops. *Agronomy J.* 91(4): 671–675.
- Young, C.A., B.A. Tapper, K. May, C.D. Moon, C.L. Schardl, and B. Scott. 2009. Indole-diterpene biosynthetic capability of *Epichloë* endophytes as predicted by *ltn* gene analysis. *Applied and environmental microbiology* 75(7): 2200–2211.
- Yu, X.-M., and M. Griffith. 1999. Antifreeze proteins in winter rye leaves form oligomeric complexes. *Plant Physiol.* 119(4): 1361–1370.
- van Zijll de Jong, E., M.P. Dobrowolski, N.R. Bannan, A.V. Stewart, K.F. Smith, G.C. Spangenberg, and J.W. Forster. 2008. Global genetic diversity of the perennial ryegrass fungal endophyte. *Crop Sci.* 48(4): 1487–1501.