Breeding of Cool-Season Forage Grasses for Abiotic and Biotic Stress Tolerance in the Southern United States

Eric Douglas Billman

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Breeding of cool-season forage grasses for abiotic and biotic stress tolerance in the southern United States

By

Eric Douglas Billman

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Submitted to the Faculty of Mississippi State University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Agronomy in the Department of Plant and Soil Sciences

Mississippi State, Mississippi

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Breeding of cool-season forage grasses for abiotic and biotic stress tolerance in the southern United States

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Abiotic stress tolerance and biotic stress resistance have long been targets for trait improvement in the field of plant breeding. To date, much of the target crop focus has been centered on commodity crops such as corn, soybean, wheat, and rice. However, little work has been conducted on improvement of these traits in forage grasses. This is due to a number of issues, particularly that most species are obligately outcrossing, the traits are governed by many genes at unknown loci, and are greatly affected by environmental variation. This creates major complications in successfully selecting and breeding populations of forage grasses tolerant to extreme high or low temperatures, as well as disease resistance. Recurrent phenotypic selection was used to select elite individuals of annual ryegrass (Lolium multiflorum Lam.) and orchardgrass (Dactylis glomerata L.) that expressed improved germination at high temperature. Selections were conducted within growth chambers at fixed temperature and light regimes (40/30°C, 12/12 hr, light/darkness) to eliminate environmental variation. Following three cycles of selection, we observed gains ($P < 0.001$) in selection over the base population for both species. Annual ryegrass mean cumulative germination for cycle 3 peaked at 45.8%, and
orchardgrass mean cumulative germination for cycle 3 peaked at 82.67%. Further
selection of annual ryegrass for freezing tolerance was also conducted. Flats of
unselected germplasm were grown to the three-leaf stage, then frozen for nine hours.
Significant differences \((P < 0.05)\) in freezing tolerance were observed between selected
germplasm in both cycle 1 (0.076%) and cycle 2 (0.125%) over the unselected cycle 0
(0.025%). Finally, initial stages of resistance breeding work were conducted involving
gray leaf spot (causal agent *Pyricularia grisea* Cke. [Sacc.]) on annual ryegrass. Isolates
of the pathogen were obtained and stored for future use. It was determined that the actual
pathogen species responsible was *Pyricularia oryzae* Cavara. Future work for annual
ryegrass and orchardgrass germplasm that germinates at high temperatures will involve
variety testing and cultivar release. Freezing tolerance and disease resistance work will
require larger-scale screening methodology that was able to be conducted in this work to
acquire sufficient population sizes for breeding.
DEDICATION

This work is dedicated to my mother and father, without whose love and support none of this would’ve been possible.
ACKNOWLEDGEMENTS

First and foremost, I would like to recognize and thank my major professor, Dr. Brian Baldwin. In my three years as his student he has not only taught me everything he knows about plant breeding, but has also helped me to vastly improve upon various aspects of my professional development. I am grateful for the time I have had as his student.

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Finally, I would like to extend my thanks to the Mississippi Agricultural and Forestry Experiment Station (MAFES) for providing the funding for both this research, as well as my education at Mississippi State University. Their generosity and contributions will not be forgotten.
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CHAPTER I
INTRODUCTION

Forage production in the southern United States is primarily comprised of a mixture of cool-season perennial grasses that are adapted to the transition zone as well as warm-season perennial grasses. Representative species include: tall fescue (Schedonorus arundinaceus (Schreb.) Dumort) for cool-season perennials, and bermudagrass (Cynodon dactylon (L.) Pers.), bahiagrass (Paspalum notatum F.), and dallisgrass (Paspalum dilatatum P.) for warm-season perennials (Ball et al., 2007). Unlike forage production in the humid-continental climates of the northern or mid-western United States, production and economically viable species in the southern USA are impeded largely by considerably different environmental conditions. This largely excludes the use of high quality, cool-season perennials such as perennial ryegrass (Lolium perenne L.), orchardgrass (Dactylis glomerata L.), and meadow fescue (Schedonorus pratensis (Huds.) P. Beauv.) that favor mild summers and can survive cold winter temperatures. However, what the South lacks in cool-season perennials it makes up for in terms of winter annual forage production. This is largely accounted for by a single cool-season forage species, annual ryegrass (L. multiflorum L.).

Production of annual ryegrass in the South is focused on providing a high quality, greater yielding forage in late winter and early spring (February to April). The crop is generally seeded in mid to late fall, and continues to grow during winter months. The
growth curves of warm-season grasses compared to cool-season grasses show that onset of forage growth in warm-season grasses occurs later in the growing season compared to cool-season grasses. Annual ryegrass allows growers to supplement their warm-season pastures, or tall fescue-based cool-season pastures with a crop that is easily established, and is capable of reseeding itself thus making the maintenance and management requirements much less strenuous on the producer than other species used in the South. Over-seeding annual ryegrass in toxic endophyte ‘Kentucky 31’ tall fescue can also dilute the proportion of toxic fescue consumed and reduce the risk of fescue toxicosis to grazing animals. Using annual ryegrass and tall fescue as the only viable cool-season species, southern pastures would be comparatively lower in available forage biomass during the spring months than northern pasture systems.

Currently, a major issue with annual ryegrass production is that it cannot be established until early to middle October in Mississippi. Ambient soil temperatures exceeding 20°C cause secondary dormancy in seed, preventing germination (Batlla et al., 2003). If an annual ryegrass cultivar could be developed that expresses limited secondary seed dormancy, germinates, and survives under high soil and air temperatures, earlier planting is a possibility. This would allow producers more time for biomass accumulation prior to grazing beef cattle stocker calves in winter and spring, potentially allowing greater weight gain.

Additionally, because annual ryegrass lacks winter dormancy it is susceptible to sudden bouts of cold weather and potentially devastating cold injury (Evers et al., 1997). Temperature threshold for cold damage in annual ryegrass is about -12°C (10°F), which is the minimum average temperature in USDA Plant Hardiness Zones 8 and 9 where the
crop is most commonly grown. However, the crop is also grown in Plant Hardiness Zone 7 which encompasses the northern part of Mississippi where average low temperatures fall between -12°C and -18°C. As such, this region of the state is where the greatest risk of cold injury on annual ryegrass is present. The cultivar Marshall, developed at the North Mississippi Branch Station in Holly Springs, MS (Mississippi Agriculture and Forestry Experiment Station; MAFES) is a cold-tolerant cultivar developed from 29 years of natural selection for cold tolerance (Arnold et al., 1981). However, there is concern regarding the selection method, and that cold tolerance may have declined over time. This necessitates using it as a source of germplasm for the development of a new, cold-hardy annual ryegrass cultivar.

In addition to the need for improving cold-hardiness, annual ryegrass is also very susceptible to the disease known as gray leaf spot (also known as ‘blast’) caused by the fungus Pyricularia oryzae [Sacc.] The disease has been reported to wipe out annual ryegrass stands in susceptible cultivars (Bain et al., 1972; Carver et al., 1972). Marshall, while more susceptible than ‘Gulf’, is still relatively poor in its resistance to blast (Trevathan et al., 1994). This necessitates the development of annual ryegrass cultivars that are resistant to blast, and again Marshall serves as a possible gene pool for selecting resistant individuals to constitute a new population.

Orchardgrass is another perennial cool-season grass that is used on occasion in the southern region of the United States, particularly in southern Tennessee, albeit to a much lesser scale than tall fescue. The species generally matures early compared to perennial ryegrass and tall fescue, complicating its management practices and reducing palatability (Kunelius, 1990; Van Santen and Sleper, 1996). It is capable of producing large dry
matter yields early in the growing season, and is able to tolerate drought and other conditions. However, the poor heat tolerance of orchardgrass is a limiting factor to wide scale production in areas where summer temperatures average greater than 32°C (Henning and Risner, 1993). Mississippi is also rooted in the production of annual ryegrass as their winter and spring grass forage for beef and dairy cattle, therefore, large scale adoption is unlikely without adapted cultivars. Additionally, orchardgrass seed is difficult to clean and process, making it an expensive investment to most producers, especially because of the unreliable/inconsistent stand establishment and poor persistence under high temperature stress. If an orchardgrass cultivar can be developed for more southerly latitudes where high temperatures and relative humidity are the norm, producers may be more willing to invest in establishing a perennial cool-season grass other than tall fescue to fill the need for early season grazing and hay production.

**Objectives**

Improvement of the cool-season bunchgrasses annual ryegrass and orchardgrass for use in Mississippi will offer southern producers improved forage availability with well-researched, high quality forage grass species. To that end, this proposed research has four objectives: 1.) screen populations of Marshall annual ryegrass for ability to germinate and grow at high temperatures (40°C) to develop a heat tolerant ryegrass cultivar that can be planted earlier in the growing season; 2.) develop an orchardgrass cultivar that is capable of surviving heat stress conditions in central Mississippi through screening for germination at 40°C; 3.) screen populations of Marshall annual ryegrass for cold tolerance to temperatures below -17°C and select individuals for new cultivar
development; and 4.) inoculate and screen populations of annual ryegrass for resistance to gray leaf spot (blast).
CHAPTER II
LITERATURE REVIEW

Abiotic Stresses

Abiotic stresses are one of the most prolific problems associated with the widespread cultivation and development of new crops. Abiotic stress refers to any event/phenomena associated with non-living factors. The major abiotic stress factors most commonly attributed to crop damage and loss are drought, flooding, and temperature extremes (either cold or hot) (Christiansen, 1982; Agrios, 2005). Unlike most biotic stresses (i.e. infectious diseases caused by fungi, bacteria, and viruses) abiotic stresses are not able to be circumvented by resistant cultivars. Rather, cultivars are selected for an increased level of tolerance, wherein they show some level of ability to withstand a particular abiotic stress (Zhang et al., 2006). The methods for identifying these tolerant individuals from a population are largely similar to identifying resistance to biotic stress; mass screenings of seedlings that are subjected to a particular stress, such as cold/hot temperatures, or lack of water, with the assumption that a tolerant seedling will equate to a tolerant mature plant.

Heat Stress

At one end of the spectrum are abiotic stresses related to very high temperatures, commonly referred to as heat stress. Wilt is the primary symptom of heat stress, resulting from a general increase in transpiration during times of elevated temperatures (Zhang et
al., 2006). Plants eventually close their stomata in order to prevent excessive transpirational loss and alleviate drought stress in the short term. However, prolonged periods of time of closed stomata prevent uptake of water, resulting in the wilt symptoms and overheating the plant due to there being no transpirational cooling. (Dipaola and Beard, 1992; Prasad et al., 2008). Degradation of vital plant cellular components such as chlorophyll, and cell walls are another subsequent effects (Wahid et al., 2007) Heat stress is generally accompanied by periods of severe drought, where the rate of transpiration is increased (lower concentration gradient of water in the atmosphere than in the plant, pulling water from soil reserves). Given recent worldwide climate change concerns, development of improved heat tolerant germplasm will be vital to sustaining agricultural production at its current levels of output. Grasses exhibiting heat tolerance have been found to have greater photosynthetic activity, as well as greater allocation of fixed carbon and increased nitrogen uptake than non-heat tolerant plants when subjected to high temperatures (Xu et al., 2011).

Similar to cold tolerance, the ability of a plant to tolerate heat stress is regulated by multiple genes, thus heat tolerance is a quantitative trait. Heritability is theoretically low, and field breeding efforts are complicated by the inability to control environmental conditions, along with confounding symptoms of heat stress with drought stress. Some summers might be exceedingly hot, whereas others may be mild, with temperatures not reaching significant heat stress thresholds for a given crop. Marshall (1982) states that a large degree of genetic diversity for heat tolerance is present in a number of grain crop species including sorghum [Sorghum bicolor (Linn.) Moench.], maize (Zea mays subsp. mays L.), soybeans (Glycine max L.) and oat (Avena sativa L.), and that said diversity is
indeed heritable. It therefore stands to reason that other crops, including cool-season forage grasses that are less adapted to high temperature stress, have some degree of genetic diversity that can be exploited through conventional breeding methods, and thus be adapted for use in more southerly latitudes. Recent work in cool-season turfgrass species such as creeping bentgrass (*Agrostis stolonifera* L.) has both shown phenotypic segregation of bentgrass populations for varying levels of heat tolerance, as well as identified multiple quantitative trait loci associated with heat tolerance in the species (Jespersen et al., 2016). Numerous studies have examined physiological mechanisms associated with cool-season forage and turfgrass species. Of particular note is the attributing of what are dubbed heat shock proteins (HSP’s) to improved heat tolerance (He and Huang, 2006; Xu et al., 2011). Kentucky bluegrass (*Poa pratensis* L.) cultivars were compared for expression of HSP’s, and it was found that cultivars exhibiting increased heat tolerance and survival had greater expression and accumulation of HSP’s (He and Huang, 2006; Peng et al., 2012). Work with creeping bentgrass has also shown similar effectiveness with the expression of HSP’s to providing overall improvement in heat tolerance (Jespersen et al., 2015). It was further deduced that these proteins are located in leaf membrane tissue, and thus affecting energy production, metabolism, and stress defenses, with their major effect being the stabilization of transcription messages in these areas (Jespersen et al., 2015).

Developing heat tolerant cultivars of cool-season forage grasses would greatly benefit producers in central and southern Mississippi. Cool-season forage grasses are generally high in nutritive value, produce adequate amounts of dry matter, and lack many of the detrimental attributes of warm-season grasses (poor germination, pubescence,
invasiveness, etc.) (Ball et al., 2007). Commercially available cultivars of these cool-season perennial forage grasses are most hindered in their ability to persist and perennate in the South primarily because of their inability to survive the longer duration of high summer temperatures. High nightly temperatures, in excess of 25°C, are a primary factor in lack of stand persistence (Baker and Jung, 1968).

Currently tall fescue is the only perennial cool-season grass that is commonly used for forage in the Southeast. Most of its production occurs in the southerly edges of the transition zone (Hoveland, 1993). However, many cultivars of tall fescue, particularly Kentucky 31, have a number of problems that make them less than ideal for widespread forage production. Of particular note is that endophyte-infected cultivars have a symbiotic relationship with the common toxic endophyte Epichloë coenophiala. This symbiosis is responsible for providing the plant with many of its beneficial agronomic traits, such as improved environmental stress resistance and greater yield capability (Schmidt and Osborn, 1993). However, presence of the endophyte produces some undesirable compounds, specifically ergot alkaloids such as ergovaline that are toxic to animals when consumed in large amounts. Kentucky 31 is the oldest North American cultivar of tall fescue, and has been distributed throughout a very wide geographic range (Asay, et al., 1979). It is also infected with the common toxic endophyte, making it less desirable for widespread forage production. By developing heat tolerant cultivars of other cool-season perennial forage grasses such as orchardgrass or annual ryegrass that lack toxic endophytes, these issues could be greatly alleviated.
Breeding for Heat-Tolerance

As previously mentioned, heat shock proteins have recently been linked to heat tolerance in a number of turf and forage grasses. Despite this, the mechanisms and specific genes related to heat stress are not as widely researched or understood as those governing cold-tolerance. Methods of selection of heat tolerant individuals from within a population vary, but there is a general consensus field testing is unreliable, largely due to the inability to control environmental temperature (Marshall, 1982). However, it is likely possible to attempt selection for heat tolerance on crops that are commonly adapted to general areas of cooler temperature. An example, and of interest to this work, is the introduction of cool-season perennial grasses into areas of the Deep South. Laboratory or greenhouse testing, where environmental conditions such as temperature and humidity can be easily regulated, have been shown to be more likely to yield consistent results (Sullivan and Ross, 1979). However, by limiting selections to only controlled environments, it can be argued that the complex interactions between heat tolerance and drought stress are not considered. Given that drought stress and heat tolerance almost always accompany each other in a natural environment, a heat tolerant plant that is bred under conditions where water is not a limiting factor would not simulate actual environmental conditions. Field selection allows breeders to simultaneously account for both heat, and possible drought stress, and results in cultivars that are better adapted to surviving under real-world stress conditions. However, the inability to control year to year environmental variation could necessitate multiple locations across a wide geographic range (Marshall, 1982). Cool-season grass seed is generally mature by late spring to early summer, when temperatures are only starting to reach annual peaks.
Therefore, more labor would be needed for seed to be harvested from each half-sib family, with phenotypic selections for heat tolerance occurring post-harvest and seed from selected plants being advanced to further cycles of selection. For this reason, screening seedlings in a growth chamber is more efficient than field selection.

**Cold Stress**

The abiotic stresses that are of most interest to this work are cold and heat tolerance. General symptoms of plants suffering from cold damage are wilt, and burn-like damage from frost (Zhang et al., 2006). Plants subjected to extreme cold temperatures experience the freezing of the water within their cells. This subsequently causes the water to expand and rupture the cells, causing heavy internal damage and dehydration to the plant tissue. This results in the necrotic tissue appearing, which resembles burning on the leaf margins in light frost conditions. Prolonged exposure to these conditions, particularly in a very young sward, will cause almost complete stand loss in an unadapted cultivar or species. However, these symptoms generally are not visible until about 24-48 hours post-freezing. Most plants suffering the greatest damage from cold do not go dormant over the colder winter months (Marshall, 1982). If the plant has not entered a period of winter dormancy, it will continue to actively grow and transpiration still occurs. Freezing temperatures ($\leq 0^\circ C$) will freeze water in the soil, making it unavailable to plants, which can further increase wilt conditions in addition to the internal damage already caused by freezing of the tissues. Certain widely cultivated perennial crops, particularly alfalfa (*Medicago sativa* L.), have been selected and bred utilizing variation within the species to enhance fall dormancy (McKenzie et al., 1988; Cunningham et al., 2001). This effectively allows plants with a greater dormancy rating to cease growth prior to the onset
of damaging cold temperatures. However, some annual crops lack winter dormancy and thus can be negatively impacted by freezing temperatures if a period of extreme cold occurs at any point in that crop’s lifespan. This is a major concern for any winter annual forage grass such as annual ryegrass. In the southern United States, the risk period for cold temperatures can last from 2-4 months, so the window for any stand reducing cold event is considerably long. Of particular concern in Mississippi is that sub-zero temperatures arrive as cold snaps – a sudden and brief duration of low temperatures, preceded and followed by warm temperatures.

**Breeding for Cold Tolerance**

Breeding for cold tolerance is a multi-faceted approach. There are a number of established methods which are actively utilized, however, there are inherent flaws with each of these methods. In the past many plant breeders conducted selection of various lines in a field setting, with the assumption that selection in the field would be more cold-hardy. One of the problems associated with this method, though, is the extreme environmental variability for temperature in most temperate climates over the winter months (Marshall, 1982). Northern Mississippi in particular exhibits large fluctuations in daily low temperatures, and it is not uncommon to have winter months with only a few days below freezing (Ward, 1969). This variability makes it difficult to effectively select for cold tolerance depending on location, given that plants are not being subjected to temperatures low enough to cause damage to them. Another method of breeding for cold tolerance is through years of natural selection. In perennial crops, this allows breeders to observe long-term effects over a longer period of time and observe which plants are persisting and surviving adverse weather conditions. The ryegrass cultivar Marshall was
developed in this manner (Arnold et al., 1981). However, this method is subject to the same problems as active field selection by breeders, in that the variation in weather might not provide adequate screening conditions (i.e. does not get cold enough) to determine if a plant is truly cold-hardy. The final established method of selecting for cold tolerant phenotypes is through controlled freezing tests (Marshall, 1982). In this method, breeders conduct mass screenings of populations through simulated freezing events. This usually entails the complete freezing of both the plant’s aboveground biomass, along with the soil/growth medium for a set period of time. Plants that survive and are able to regrow from this intensive stress are deemed cold tolerant. There are still inherent problems with this method; particularly that these tests often result in escapes, where seed have not germinated until after the freezing event has already occurred. This means for this method to be effective, great care must be taken by the breeder to ensure selected individuals are actually survivors of the freezing event, not simply escapes.

Previous work has indicated that cold tolerance is a quantitative trait, controlled by many genes and subject to a large amount of variability from genotype × environment interactions (Christiansen and Lewis, 1982; Stefaniak et al., 2008). Similar to yield, this has resulted in certain studies selecting for cold tolerance, per se, wherein selections are made for traits that have been correlated to cold tolerance. These include, but are not limited to: stand density, rate of spring green-up, leaf texture, and physiological components such as nonstructural carbohydrates (Humphreys and Eagles, 1988, Bertrand et al., 2013) and protein content (Zhang et al., 2006, Bertrand et al., 2013). Despite these efforts, it is still of interest to determine if freezing tests are capable of accurately selecting for cold tolerant phenotypes directly, without the need to select for it per se.
Cold tolerance in cool-season grasses has been further shown to be improved by plants undergoing a period of acclimation, wherein they are exposed to progressively lower temperatures (-1 to -3°C) (Bertrand et al., 2013), rather than a sudden and intense exposure to very low temperatures that might outright kill the plant. There is also a link to reduction of photoperiod and the initiation of cold tolerance (Hodgson, 1964).

Cold tolerance has been linked to a number of genes in multiple species that regulate its onset. These genes are expressed due to the binding of transcription factors with DNA of the plant upstream from the resistance genes (Zhang et al., 2005). Specifically, C-repeat binding factor (CBF) transcription factors have been the major component in altering gene expression upon exposure to cold temperatures (Kobayashi et al., 2005), and are found in nearly all tested plant species (Jaglo et al., 2001). However, the testing of the effects of CBFs in forage and turf grass species are limited, but have shown some signs of CBF regulation of cold tolerance genes in perennial ryegrass and annual ryegrass (Xiong and Fe, 2006; Xiong et al., 2007) as well as being conserved among members of Poaceae (Tamura and Yamada, 2007). This synteny indicates that CBFs likely are major regulators of cold tolerance gene expression in other species of grasses that can be improved for forage or turf use.

While molecular breeding is becoming more popular, conventional plant breeding methods have been shown to be able to effectively isolate and exploit genetic variation in a number of different grass species for cold hardiness (Bertrand et al., 2013). Conventional breeding strategies to select for cold tolerance in cool and warm-season grass species are focused on reducing the G × E interaction, and evaluating tolerance on a population level because the grass family is almost entirely obligately outcrossing (Casler
et al., 1996). This facet of breeding within the grass family significantly slows progress. In perennial species where vernalization is required this is further exacerbated because of the requirement of both cold temperatures and photoperiod to induce reproductive conversion. However, some species behave as annuals, such as annual ryegrass. This allows certain cultivars of this species to undergo multiple generations of selection within the same year, as not all annual ryegrasses require vernalization to induce seed-head formation (McLean & Watson, 1992).

**Biotic Stresses/Pathogens**

In addition to the above outlined risks from abiotic factors, plants are susceptible to a wide array of biotic pathogens, namely fungi, bacteria, and viruses. These organisms are responsible for causing diseases in every major class of agronomic and horticultural crop, resulting in roughly $33 billion per year in the United States in crop losses (Pimentel et al., 2001). Of these three plant pathogens, fungi account for an overwhelming majority of disease causing agents in the plant kingdom at roughly 85%, while bacterial and viral-based diseases comprise the remaining 15% (Agrios, 2005). Given the massive economic impact associated with plant pathogens and diseases, there is an ever-expanding effort to control infections, proliferation, and damage to crops through a variety of methods including cultural practices, chemical pesticides, and of most relevance for the purposes of this work, genetic resistance.

**Breeding for Disease Resistance**

The mechanisms of genetic resistance for crops to various plant pathogens are complex, but have been best quantified by the gene-for-gene theory (Flor, 1971). This
theory states that for every resistance gene (R-gene) in the host plant that confers resistance to a pathogen, there is a virulence gene (Vir-gene) in the pathogen that facilitates infection of the host (Flor, 1971; Wolfe et al., 1976; Agrios, 2005). Gene-for-gene theory forms the basis for the widely held idea that within a given outcrossing plant population, there is sufficient genetic variability to sexually produce recombinant individuals that possess an R-gene for a given pathogen and thus confer complete resistance to infection from that pathogen. This allows these plants to survive and form the new base for a resistant host plant population. However, this creates selection pressure upon the pathogen, and genetic variability within the pathogen’s population results in individuals with a new Vir-gene that allows them to overcome the newfound host resistance. This process is a prime example of the Red Queen hypothesis (Van Valen, 1973), wherein the aforementioned events are ongoing and vary in the time required for a host plant to develop new and widespread R-gene resistance, or the pathogen to overcome those R-genes. The above example represents the concept of vertical resistance, where a single R-gene is conferring resistance to the pathogen, and resistance is usually qualitative, with the host either being susceptible or resistant. However, the likelihood of a pathogen overcoming this resistance is both very probable, and likely to occur within a relatively short amount of time (Schumann and Wilkinson, 1992). Conversely, horizontal disease resistance is a quantitative variant of host-pathogen resistance (Vanderplank, 1963; Robinson, 1976). In this method, resistance is conferred through multiple R-genes in the host. No individual gene results in complete resistance, but cumulative effects are able to provide good mean resistance. Because horizontal resistance is quantitative, host phenotypes will vary, with some showing greater
symptoms/signs of infection, and others that are more resistant showing less. This form of host resistance inherently is harder for the pathogen to overcome, however, disease is never completely eradicated.

**Breeding Methods in Cool-Season Grasses**

**Phenotypic Selection**

Breeding in cool-season grasses can be conducted through a variety of methods. However, these methods are strictly catered to the unique challenges in breeding grasses; e.g. severe inbreeding depression, their obligate outcrossing nature, and working with a population that is in Hardy-Weinberg equilibrium. The overarching method is recurrent selection, in which favorable alleles and genotype frequency are increased over multiple cycles of selection for a particular trait. Furthermore, two forms of recurrent selection are practiced in forage grasses, mass selection and phenotypic selection (Casler et al., 1996). Mass selection implements screening of a large number of individuals (the population) and selecting individuals showing superior phenotypes for a desired trait. Seed would be harvested from selected individuals and bulked without pollen control to form the next generation for either progeny testing or further mass selection (Bernardo, 2014).

Recurrent phenotypic selection differs slightly from mass selection, in that pollen flow is limited to only allow selected individuals from the elite population to contribute pollen during intermating to form the next generation. This specific type of recurrent selection is referred to as restrictive recurrent phenotypic selection (RRPS). Vogel and Pedersen (1993) attest to the greater efficiency and ability of RRPS to develop cultivars that possess greater gains from selection than mass selection. This is due to the increased frequency of beneficial alleles resulting from controlling pollen flow. Recurrent
phenotypic selection is generally the most widely practiced breeding method in perennial forage grasses (Casler et al., 1996; Resende et al., 2013)

**Genotypic Selection**

Conversely to recurrent selection, there are a number of other selection methods, commonly referred to as progeny/genotypic selection. Under these methods the progeny of a plant are evaluated for fitness and other traits of interest in order to determine both general and specific combining ability. From these data specific parents are selected for polycross advancement to the next cycle of selection. While phenotypic selection is generally conducted on quantitative traits with relatively great heritability, genotypic selection is carried out when selecting for traits that are less heritable, but still quantitative in nature (Hallauer and Miranda, 1981; Bernardo, 2014). Examples of potential traits selected for under genotypic selection would be sugar content, flag leaf size, heading date/days to maturity, and yield (Paran and Zamir, 2003). However, recent work in native grasses has shown improvement of quantitative traits through recurrent phenotypic selection, specifically reduction in seed dormancy/increase in velocity of germination (Burson et al., 2009; Baldwin and Rushing, 2016). Multiple forms of genotypic selection exist, each with their own distinct methodology; advantages and disadvantages exist between them (Vogel and Pedersen, 1993). Half-sib family selection focuses on selection of individual mother plants based on desired traits. Because they are open-pollinated, only the contribution of the seed-bearing female parent is known, and pollen contribution is anonymous and could be any combination of other nearby plants in the crossing block. Seedlings from these plants are tested (progeny testing) and evaluated, then remnant seed is utilized to begin the next cycle of selection. Among-and-within-
family selection is slightly more complex, yet accurate to its name. Individual half-sib families are selected from amongst a large group following progeny testing, followed by selection of individual plants within each selected half-sib family (Casler, 2008; Resende et al., 2013). Thus one can effectively select both optimized half-sib families, as well as the most desirable individuals within that family. Finally, there is recurrent multi-step family selection. This genotypic selection method is identical to among-and-within-family selection, except that a second population is created using sub-samples consisting of the best genotypes from the primary selections. This second population is then evaluated for release as a potential cultivar (Vogel and Pedersen, 1993). A potential drawback of this method is bottlenecking, where the sub-population may not have enough genetic diversity to form an optimum phenotype.

**Synthetics**

Another breeding method common in cool-season forage grasses is the development of what are termed synthetic cultivars. Dubbed ‘synthetics’, they are commonly comprised of plants that are far along in the selection process. Selections of individual plants are based upon good general combining ability, rather than specific combining ability, and therefore the random-mating population is maintained through open-pollination of all selections. This creates a substantial degree of heterosis in the population, which is maintained with each successive synthetic generation progressively dubbed Syn1, Syn2, Syn3, etc. (Allard, 1960; Poehlman, 1983). Synthetic cultivars often are comprised of a narrower parental base than traditional mass phenotypic selection, often consisting of anywhere from 10-50 parents depending on the general combining ability of the individuals. These synthetic cultivars are designed to be released at the peak
of performance, where additive variance is maximized. Performance will progressively decrease the more generations a synthetic is carried out beyond the initial population intermating event. Therefore, synthetics allow breeders to release a cultivar quickly, but are only agronomically/economically viable for a few years. Because forage grasses are generally self-incompatible and maintained as a population, this method is often utilized for the development of forage grass cultivars.

**Mutation Breeding**

One last method of breeding that is taking a greater presence in modern science is the use of induced mutagenesis on selected germplasm of a particular crop. There is an array of techniques through which mutagenesis can be carried out, but exposure to variable rates of chemical radiation form the backbone of these techniques. The end-goal is to induce point mutations which alter a small segment of the genome without causing serious loss of functionality in the plant of other genes due to being over or under expressed. Ethyl methanesulfonate (EMS) is widely accepted as a strong candidate for inducing chemical mutagenesis in plant breeding. Seeds are soaked in the EMS solution at varying rates and duration, thus possibly resulting in viable mutant seedlings with a novel phenotype being expressed (Wang et al., 2014). These EMS treatments cause point mutations, which have a stronger likelihood of causing beneficial mutations without sacrificing vital growth and development functionality (Alcantara et al., 1996). While there is a marked increase in lethal mutations with increased rates of EMS treatment, there is also an increased efficiency in the development of beneficial mutations in individuals that survive these treatments (Devi and Mullainathan, 2011).
Cool Season Grasses

In the in the Transition Zone and Deep South, cool-season forage grasses comprise a significant percentage of total grass species present in pastures. The transition zone comprises a region stretching north to south from the lower Midwest to the upper Deep South, and east to west from the East Coast to the Great Plains (Upham, 2008). Tall fescue alone comprises roughly 71 thousand hectares of perennial forage pasture in northern Mississippi (Lemus, 2015), while annual ryegrass also constitutes a significant portion of pastureland as a winter annual. Perhaps not as prevalent in forage systems as the American Midwest, cool-season grasses in Mississippi provide early season growth for animal grazing and hay production until temperatures become adequate for warm-season grass production. Whereas warm-season forage grasses have Kranz anatomy and utilize the C₄ photosynthetic pathway (Anderson 2000), cool-season species use the C₃ photosynthetic pathway (Moser and Hoveland, 1996). Biologically this makes cool-season grasses less suited for greater temperatures and light intensity that occur during summer months due to photorespiration. Thus, perennial cool-season grasses exhibit a bi-modal pattern of growth throughout the growing season. Spring growth comprises the majority of forage growth and reproduction for the year, lasting from February to June. Growth slows by summer and is greatly reduced until fall, when temperatures return to more ideal ranges for growth to resume except under freezing temperatures in winter (Burns and Bagley, 1996). As a result, forage availability of cool-season grasses varies throughout the growing season and thus management strategies for grazing these grasses must be properly aligned with availability.
Other aspects of perennial grasses make management for forage production difficult. Each winter perennial grasses undergo vernalization, the process of exposure to long nights with low temperatures that results in the initiation of reproductive tillers (Fiil et al., 2011). These reproductive tillers emerge in the initial flush of spring growth. This is a key aspect to the maturity variation between different species and cultivars of grasses. Jointing of these reproductive tillers initiates the decline of forage quality due to a reduced leaf:stem ratio, and increased secondary cell wall fiber to support the seed head (Nelson and Moser, 1994; Baron et al., 2000). Earlier maturing cultivars often produce more seed, making them useful for breeding/seed increase, but lack in forage quality making them poorer in terms of animal performance.

Grasses also exhibit different morphological growth habits that lend themselves to greater or lesser amounts of regrowth following grazing or cutting for hay. Cespitose, or bunchgrass-type grasses originate from a crown and grow in a radial pattern away from the original point of emergence as more tillers are developed. Common cespitose forage species include orchardgrass and tall fescue. These types of cool-season forage grasses are most commonly used in forage production systems as bunchgrasses often have greater vegetative regrowth, particularly after first cutting, than stoloniferous or rhizomatous type grasses. Sod-type grasses proliferate laterally from their point of origin through the use of prostrate aboveground or belowground stems known as stolons or rhizomes, respectively. Kentucky bluegrass (*Poa pratensis* L.) is a common cool-season sod-type forage grass the spreads through the use of rhizomes (Fustec et al., 2005), but does not exhibit good regrowth following initial grazing or cutting. However, in Mississippi, these sod-type
cool-season grasses are unable to proliferate due to their inability to tolerate heat and drought stress.

**Annual Ryegrass**

Annual ryegrass is a cool-season grass known for its wide area of adaptation and cultivation for use as animal forage and fodder. Originally native to Italy (spawning the origin of its alternative common name, Italian ryegrass), the plant has spread across the entirety of Europe, the United States, and Canada, along with widespread distribution in Australia, New Zealand, and Japan (Jung et al., 1996). Annual ryegrass’ growth habit is that of a bunchgrass, with long, wide leaves, and taller height than that of perennial ryegrass. This makes the plant an ideal forage species due to increased yields over perennial ryegrass. The species exhibits a glossy sheen to its leaves common to the ryegrass genus (*Lolium*), and is often a favorite for interspecies hybridization with *Schedonorus* spp. in the development of festulolium [*Festulolium braunii* (K. Richt.) A. Camus] cultivars. Estimates of its use show that annual ryegrass is almost entirely used as forage, with a majority of that being as pasture for grazing animals, with limited use as hay (Evers et al., 1997). The primary method of planting annual ryegrass in the South is through over-seeding of warm-season grass pasture during mid to late October. At this time warm-season grass growth has been reduced as temperatures fall, allowing annual ryegrass seedlings to establish through the warm-season cover. Annual ryegrass is most commonly cultivated in the southern United States, in USDA Plant Hardiness Zones 8 and 9 (Evers et al., 1997). It can still grow in more northern regions, but its productivity is decreased. This is because the species is hampered by cold temperatures, showing decreased yields upon exposure to temperatures to below -12°C (USDA, 1990). Unlike
other cool-season forage grasses, annual ryegrass generally does not perenniate vegetatively. For this reason, the plant does not cease growth and become dormant during winter months. This makes annual ryegrass more susceptible to cold damage than other cool-season grass counterparts. In the Deep South, while the likelihood of these low temperatures are lessened, it only takes one such incidence to result in stand or yield losses.

Efforts to understand and alleviate this susceptibility to cold temperatures, and develop cold-hardy annual ryegrass germplasm have been a major focus by plant breeders working with annual ryegrass for many decades (Arnold et al., 1981; Watson et al., 1990; Prine, 1996; Nelson et al., 1992). Of this work, the most well-known cultivar to be released is known as Marshall. Originating from the North Mississippi Branch Station at Holly Springs, MS from in-field, natural selections from common reseeding annual ryegrass over 29 years, Marshall showed increased cold tolerance over other annual ryegrass cultivars being used for winter and spring forage in the South (Arnold et al., 1981). As such, it has served as the primary source of germplasm for imparting cold-hardiness into more recently developed cultivars, including Jackson (Watson et al., 1990), Surrey (Prine, 1996), and TAM 90 (Nelson et al., 1992). Of these cultivars, only TAM 90 was specifically selected for additional winter-hardiness at any point in development, but that was not the primary breeding objective. Over its 29 year history, Marshall’s parental germplasm was exposed to a wide array of winter temperatures, with some winters being mild, others well below average. Seed was collected and advanced only from surviving plants that naturally reseeded. However, NOAA (National Oceanic and Atmospheric Association) temperature history for northern Mississippi indicates that
the lowest temperatures at which Marshall would have been subjected to were in 1962 and 1963 at -20°C and -25°C, respectively. In addition, NOAA records (2016) show only nine other years during which temperatures dropped below the crucial -12°C temperature threshold at which freezing damage is most likely to occur. Given the conditions under which Marshall was selected, there is room for potential improvement of the cold-hardiness trait that was naturally selected for. By applying selection pressure on existing populations of Marshall through exposure to temperatures below -12°C, it should be possible to further increase the cold-hardiness of the cultivar and move its area of adaptation farther north, while further decreasing yield and stand-loss risks in Mississippi.

In addition to cold stress, there is a desire from producers to have an annual ryegrass cultivar that can be planted earlier in the growing season and survive high late summer or early fall temperatures in the South. However, planting prior to October in the South poses a risk because annual ryegrass is subject to the disease gray leaf spot (also known as blast). The causal agent is the fungal pathogen *Pyricularia grisea* Cke. [Sacc.] (Bain et al., 1972). It was first observed as a pathogen of annual ryegrass in the early 1970’s by researchers in Mississippi and Louisiana (Bain et al., 1972; Carver et al., 1972). The pathogen is an ascomycete, with its teleomorph (sexual stage) *Magnaporthe grisea* (T.T. Herbert) M.E. Barr not being found in nature (Agrios, 2005). Therefore, all infections are through the anamorph (asexual stage) *P. grisea* and the conidia it produces. In its first reported season of occurrence, an epidemic occurred, resulting in the moniker of ryegrass ‘blast’ (Trevathan, et al., 1994). *Pyricularia grisea* infects the host plant by entering through stomata, or from forming a structure known as appressoria and
penetrating through the leaf tissue, meaning that the fungus does not require a fresh
wound through which to infect. Symptoms of infection include brown or gray water-
soaked spots on leaves and stems, which develop into larger lesions with gray or blue-
gray centers and brown or purple margins. Older lesions also develop chlorotic borders
(Trevathan et al., 1994). If disease is allowed to progress it can result in thick mats of
slimy, necrotic ryegrass, and is particularly dangerous to adjacent seedlings because
infections can rapidly spread and overtake small plants with little photosynthetic tissue
(Nelson et al., 1997). The disease proliferates under prolonged periods of leaf wetness
and temperatures between 20 and 28 °C. The original source of the outbreak of blast on
ryegrass was never confirmed, but both Bain et al. (1972) and Carver et al. (1972)
speculate that spores of *P. grisea* were carried to Mississippi and Louisiana by the winds
of hurricane Edith in 1970. Because the conditions for infection are optimal when the
temperature is between 20 and 28°C, the disease is most prevalent in August and
September in Mississippi. This means that any heat tolerant cultivar developed for early
fall planting in Mississippi should be coupled with a blast resistance trait.

**Orchardgrass**

Orchardgrass has long been a popular cool-season grass used in forage productions
systems. Agronomically, the grass is adapted to shaded areas with slightly acidic to
neutral soil pH, and can also endure significant drought stress. It is not well adapted to
persistent and excessive soil moisture and flooding. It is very responsive to nitrogen
fertilizer application (Mortensen et al., 1964) and has rapid regrowth following grazing or
cutting for hay compared with some other forage species. Of the most common cool-
season forages used in the U.S., it is one of the earliest to initiate spring growth and this
makes it valuable as a forage early on in the growing season. However, this same aspect also means that the grass matures much earlier than other species and this can negatively impact forage quality and palatability to animals grazing it or consuming it for hay (Kunelius, 1990; Van Santen and Sleper, 1996). To rectify this problem, breeders have developed cultivars such as Profit, Harvestar, and Intensiv (Olson et al., 2015) that mature much slower than other cultivars and will maximize vegetative growth, thereby limiting secondary cell wall components that lower energy usage and intake by animals consuming that forage. Compared with tall fescue, another common forage in the U.S. that is arguably more widely used, it has the distinct advantage of lacking toxic endophytes that can produce harmful alkaloids that can be detrimental to animal performance over time upon repeated or continuous consumption (Clay, 1996). This makes it much more suitable for equine and dairy pasture and hay. The species also has softer textured and less rigid leaves than most cultivars of tall fescue that make it more palatable to grazing animals. Orchardgrass is considered to be fairly susceptible to disease, with brown patch, rusts, and leaf spot diseases commonly resulting in yield losses (Van Santen and Sleper, 1996).

One of the major problems with the adoption of orchardgrass in Mississippi is that the species does not survive and perenniate well due to its susceptibility to the very high temperatures and relative humidity of the Deep South. Orchardgrass is native to the temperate climates of central and eastern Europe, the Mediterranean, and central Asia (Renvoize, 1999). Therefore, the species is mostly adapted to areas of mild summers and cold winters. It can tolerate some heat stress, but prolonged periods of over 32°C can lead to large stand loss, particularly after grazing or hay harvest (Henning and Risner, 1993).
Because orchardgrass typically does not persist beyond the first summer in Mississippi, it is both uneconomical and inefficient for growers to plant it. The seed cost is fairly great, about $150 to $175 per 22.5Kg bag depending on cultivar (about 2-3x more expensive than the primary cool-season grass used in the South, annual ryegrass). If stands are drastically depleted after prolonged heat events growers would have to incur this cost every year, rather than only once if the crop actually survived more than one season as intended.
CHAPTER III
BREEDING A HEAT TOLERANT ANNUAL RYEGRASS FOR EARLIER FALL PLANTING IN MISSISSIPPI

Introduction

Annual ryegrass is a cool-season forage grass that is used as feed for beef cattle operations throughout much of the southeastern United States. The species has high forage nutritive value and grows rapidly in cool conditions during late fall and winter. Late summer or early fall planting of annual ryegrass is very desirable to producers, as it has the potential to increase available forage earlier in the season. However, soil temperatures during late summer and early fall in states such as Mississippi, Alabama, Georgia, and Texas often exceed 32°C. These temperatures impede annual ryegrass germination due to secondary seed dormancy. Secondary dormancy is associated with environmental factors, most commonly soil temperature, that limit germination even after primary dormancy has been broken (Batlla et al., 2003).

A heat tolerant annual ryegrass cultivar that can germinate under high temperatures and survive heat stress as a seedling would allow the crop to be planted earlier in the growing season. As such, producers can benefit from greater accumulation of forage biomass (rain permitting) prior to the onset of winter, allowing for possible fall and earlier winter or spring grazing. Currently in the South, most annual ryegrass is planted in October or November during which soil temperatures are usually less than
21°C (Evers et al., 1997). However, to benefit from the advantages of earlier planting
e.g., earlier cattle grazing, increased production time, and greater number of grazing
events, annual ryegrass should be planted in August and September, during which soil
temperatures often exceed 30°C.

**Objectives**

The purpose of this research was to develop a new cultivar of heat tolerant annual
ryegrass using recurrent phenotypic selection (RPS). To accomplish this the breeding
objectives were to i.) Eliminate secondary seed dormancy, ii.) Improve heat tolerance of
seedlings, and iii.) Examine potential effects of selection for heat tolerance on other
desirable phenotypic traits.

**Materials and Methods**

**Selection Methodology and Germination Testing**

Germplasm for this work was derived from ‘Marshall’ annual ryegrass (cycle 0).
Mass seed screening was conducted on 56 cm² stainless steel trays with two layers of
germination paper (Seedburo Equipment, Des Plaines, IL), covered with clear lucite.
Germination temperature and light conditions were 40/30°C and 12/12 hr, light/darkness
respectively, in a growth chamber (Percival Scientific, Series 101, Perry, IA) for 36 days.
Of the 255 survivors selected from this initial screening of approximately 30,000 seed, 64
random selections were transplanted into the field in fall of 2015 at the Mississippi State
H. H. Leveck Animal Research Center (33°26’16.11”, -88°47’53.09”). The field soil type
was a Catalpa silty clay loam (fine, smectitic, thermic Fluvaquentic Hapludolls). All
plants were fertilized with 160 mL of 107 ppm (mg/L) N, 23 ppm P, and 33 ppm K
solution (Peter’s 20-20-20) on a bi-weekly basis following transplanting until the end of February each season.

These elite selections formed a polycross block of cycle 1 plants. To establish this block, a 6.1 m² section of plastic (5 mm, white, low-density polyethylene) was placed (edges buried), with holes punched on an 8 x 8 grid on 61 cm centers. Selected plants were transplanted into these holes in late November 2015. A 1.5 m border of cereal rye (Secale cereale L.) was planted around the block to serve as a pollen barrier. Additionally, all fallow areas were mowed to further prevent pollen contamination from feral annual ryegrass. During each subsequent growing season, identical polycross blocks plants were established for phenotypic comparisons between the base population and any advanced cycles of selection. Seed from each cycle was harvested and bulked between May 31 and June 6. Each seed lot was conditioned using a belt thresher (ALMACO, Nevada, IA) to remove seed from spikes. Seed was sieved (1.64 x 9.53 mm mesh; Seedburo Equipment, Des Plaines, IL) and fractionally aspirated (Carter Day International, Inc. Minneapolis, MN) to remove chaff. Seed of cycles to be compared were always grown and tested during the same season to eliminate any differences in seed characteristics that ambient environment or storage conditions might produce.

Germination tests of cycle 1 to cycle 0 (AOSA, 2014) were conducted at recommended AOSA protocol for annual ryegrass (20/15°C, 12/12 hr, light/darkness); and at a 40/30°C (12/12 hr, light/darkness) test regime. Each seed lot was placed in Petri dishes (15.25 x 3cm) upon 15 ml of 1% water agar as the germination medium. Six hundred seed (6 reps of 100 seed) for each cycle of selection arranged as a completely randomized design were placed within each temperature regime. Germination testing
lasted for 14 days (AOSA, 2014). Observations were made every two days. Daily and cumulative germination for each cycle of selection was recorded and calculated. Velocity of germination at 8 days (VOGs) was also calculated. This is a measure of the speed at which germination occurs and was determined as the slope of the line over the first eight days of cumulative germination. Germination testing conditions were identical for the 2017 (cycles 0 and 2) and 2018 (cycles 0, 1, 2, and 3) test years.

To advance germplasm to the next cycle, a given cycle’s seed lot was screened identically to the antecedent generations. Seed from cycle 1 that germinated at 40°C were used as the elite parents of cycle 2. Sixty-four cycle 2 selections were transplanted to the field in November 2016. These plants were grown to maturity and harvested in late spring 2017. A cycle 0 polycross was also grown and harvested for seed. A cycle 1 polycross was not planted in 2016-2017. At maturity, sheaves were harvested and dried at 20°C. Seed from all blocks was conditioned as in years prior. Germination testing and screening was repeated each year to obtain the next cycle of selection (cycle 2→3 and cycle 3→4). For the 2017-2018 season, plants from all generations (cycles 0, 1, 2, and 3) were planted in individually isolated polycross nurseries for determination of selection progress.

**Additional Phenotyping of Selected Plants**

Selection for heat tolerance may impact other associated traits. Seasons over which traits were examined were fall 2015 through spring 2016 (2015-2016), fall 2016 through spring 2017 (2016-2017), and fall 2017 through spring 2018 (2017-2018). Other traits of interest were monitored within the advanced cycles of selection (cycles 1, 2, and 3). These traits included; days to maturity (Julian days) and reproductive tiller number.
Maturity was defined as the date of first seed head emergence from the leaf sheath of the plant. Observations for maturity were collected every two days on all plants in each polycross nursery. Reproductive tiller number was determined post-harvest by counting the number of stems per sheaf harvested from each plant. Additionally, possible changes in cold-hardiness on selections were examined. The 2015-2016 and 2016-2017 winters did not produce substantial sub-freezing temperatures to observe damage. However, during the 2017-2018 winter several freezing events occurred. Temperatures from -6 to -13°C persisted for three consecutive nights (19-21 Jan. 2018). Frost damage ratings were conducted approximately 5 weeks after the last freezing event. Each plant was assessed on a rating scale from 1-9 (1 = no damage, 9 = plant death).

**Determination of Heritability**

Heritability is the measure of the progress made due to selection that is due to genetic, rather than environmental effects (Briggs and Knowles, 1967). To determine heritability of selection for 40°C germination we applied the following equation to the datasets:

\[ h^2 = \frac{G}{R} \]  

(Eq. 3.1)

Where \( h^2 \) = realized heritability, \( G \) = gain, the number of observed individuals expressing a trait beyond that of the base germplasm (in this case germination at 40°C), and \( R \) = reach, the number of individuals selected for the trait of interest in the prior generation.
**Statistical Analysis**

Data from both the cumulative germination and the phenotypic trait assessment from each year of testing was analyzed as a completely randomized design using SAS 9.4 and PROC GLM to conduct ANOVA and mean separation. The statistical model was:

\[ Y_{ijk} = \mu + D_i + C_j + \varepsilon_{k(ij)} \]  

(Eq. 3.2)

in which \( Y \) = the response variable, \( \mu \) = mean, \( D \) = day of the test, \( C \) = cycle of selection, and \( \varepsilon \) = experimental error. An alpha level of 0.05 was used to determine significant differences.

Chi-square (\( \chi^2 \)) analysis was used as a secondary assessment of improvement for the second and third cycles of selection (2017 and 2018 for germination tests) compared to either the prior cycle of selection or the unselected population. The formula for chi-square analysis was:

\[ \chi^2 = \frac{(\text{observed} - \text{expected})^2}{\text{expected}} \]  

(Eq. 3.3)

Where, observed = the number of individuals seen to express a phenotype, and expected = the number of individuals predicted to express that phenotype. These expected values were then compared to the critical value of the \( \chi^2 \) table (Clewer and Scarisbrick, 2001), with degrees of freedom as \( n-1 \), and a significance level of \( \alpha=0.05 \). With 63 d.f., (64 total plants supplying seed), the critical value was 82.5.
Results

Germination Testing

For all germination tests, statistical analysis indicated an interaction between cycle and day \((P < 0.001)\). Therefore, the interactions were partitioned to compare mean cumulative germination between each cycle of selection at each day of the 14-day germination tests.

In the 2015-2016 season, germination percentage of cycles 0 and 1 at 40°C was not different \((P = 0.107)\) at any point in the 14-day test (Figure 3.1a). However, we observed a trend of the two populations diverging from one another after the 6-day observation. From this point, percentage germination of cycle 1 began to increase over cycle 0; however, this separation was not significant \((P = 0.48)\). Cycle 0 cumulative germination at 14 days was 3.27% and cycle 1 reached 5.52%. Cycle 1 VOG\(_8\) \((0.80x \text{ day}^{-1})\) was not greater than cycle 0 \((0.49x \text{ day}^{-1})\) within 8 days (Figure 3.1b). The lines fitted to VOG\(_8\) for cycle 1 and 0 were \(y = 0.80x – 1.03\) and \(y = 0.49x – 0.69\), respectively. While not significant, the slope of cycle 1’s VOG\(_8\) curve was 1.6 times that of cycle 0.

Although both seed lots were produced during the same year under the same environment, cycle 1 (71.5%) exhibited greater \((P < 0.001)\) percentage germination than cycle 0 (43.8%) within 4 days at 20°C (Figure 3.2a). Cycle 1 germination percentage remained significantly greater than cycle 0 for the duration of the 14-day test. Final (14-day at 20°C) germination of cycle 1 was 93.7%, compared to cycle 0 at 86.3%. Velocity of germination of cycle 1 \((30.8x \text{ day}^{-1})\) was also greater \((P < 0.001)\) than cycle 0 \((27.4x \text{ day}^{-1})\) (Figure 3.2b). The line fitted to VOG\(_8\) of cycle 1 at 20°C was defined by the equation \(y = 30.8x – 23.7\); the equation defining VOG\(_8\) of cycle 0 was \(y = 27.4x – 26.1\).
Figure 3.1  (a) Mean cumulative germination at 40°C of cycle 0 and cycle 1 annual ryegrass over a 14-day test period. Seed produced during the 2015-2016 production season; tested August 2016. No significant differences present ($P = 0.107$).

Figure 3.1  (b) Velocity of germination at 40°C of cycle 0 and cycle 1 annual ryegrass within the first 8 days of testing. Seed produced during the 2015-2016 season; tested August 2016.
Figure 3.2  (a) Mean cumulative germination at 20°C of cycle 0 and cycle 1 annual ryegrass over a 14-day test period. Seed was produced during the 2015-2016 production season; tested August 2016.
†Different letters indicate significant differences at P < 0.01.

Figure 3.2  (b) Velocity of germination at 20°C of cycle 0 and cycle 1 annual ryegrass within the first 8 days of testing Seed produced during the 2015-2016 season; tested August 2016.
Germination tests from the 2016-2017 season tested cycle 0 and cycle 2. At 40°C (Figure 3.3a), a greater ($P = 0.02$) germination percentage of cycle 2 (2.2%) seed was observed over cycle 0 (0.2%) by day 4. Germination of cycle 2 remained greater ($P < 0.001$) for the duration of the 14-day data collection period. Total mean 40°C cumulative germination for cycle 2 seed was 18.6% while germination of cycle 0 was 2.7%. In comparing cycle 2 VOG$_8$ (2.15x day$^{-1}$) we observed a greater ($P < 0.05$) value than cycle 0 (0.28x day$^{-1}$) (Figure 3.3b). The equations assessing VOG$_8$ for cycles 2 and 0 were $y = 2.15x - 2.08$ and $y = 0.28x - 0.33$, respectively. The rate at which cycle 2 seed germinated was 7.8 times that of the unselected control.

Under 20°C testing conditions (Figure 3.4a) greater ($P = 0.03$) germination of cycle 2 (17.6%) over cycle 0 (12.6%) was observed by day 4. This difference remained ($P < 0.001$) for the duration of the 14-day test. Final cumulative germination of cycle 2 seed was 60.8% while cycle 0 seed was 40.8%. Velocity of germination at 8 days of cycle 2 (15.2x day$^{-1}$) was greater ($P < 0.001$) than cycle 0 (10.4x day$^{-1}$) (Figure 3.4b). The equation for the line assessing cycle 2 VOG$_8$ was $y = 15.2x - 13.8$; the equation for cycle 0 was $y = 10.4x - 9.3$. The rate at which cycle 2 germinated was 1.5 times that of the unselected control.
Figure 3.3  (a) Mean cumulative germination at 40°C of cycle 0 and cycle 2 annual ryegrass over a 14-day test period. Seed produced during the 2016-2017 season; tested July 2017.
†Different letters indicate significant differences at P < 0.05.

Figure 3.3  (b) Velocity of germination at 40°C of cycle 0 and cycle 2 annual ryegrass within the first 8 days of testing. Seed produced during the 2016-2017 season; tested July 2017.
Figure 3.4  (a) Mean cumulative germination at 20°C of cycle 0 and cycle 2 annual ryegrass over a 14-day test period. Seed produced during the 2016-2017 season; tested July 2017.
†Different letters indicate significant differences at P < 0.05.

Figure 3.4  (b) Velocity of germination at 20°C of cycle 0 and cycle 2 annual ryegrass within the first 8 days of testing. Seed produced during the 2016-2017 season; tested July 2017.
The 2017-2018 season of germination testing compared seed from each cycle of selection (0, 1, 2, and 3). At 40°C (Figure 3.5a), cycle 3 germination was greater \((P = 0.02)\) than cycles 2, 1, and 0 by day 2 of the test. Beginning at day 2, cycle 3 germination was 0.67%, while all other cycles had no seeds germinate. At every following observation day, cycle 3 mean cumulative germination was greater \((P < 0.001)\) than cycle 2, cycle 1, and cycle 0. Total mean cumulative germination at 40°C for cycle 3 was 45.8%. Cycle 2 mean cumulative germination began to differentiate from cycle 1 and cycle 0 at day 8, and became greater \((P < 0.001)\) than those cycles by day 10. Mean cumulative germination of cycle 2 at day 14 was 26.3%. Cycle 1 and cycle 0 did not differ \((P > 0.05)\) from each other for the duration of the 14-day test; cycle 1 mean cumulative germination at 40°C peaked at 7.0% and cycle 0 reached 4.8%.

Velocity of germination at 40°C at eight days \((\text{VOG}_8)\) was compared among cycle 3, cycle 2, cycle 1, and cycle 0 (Figure 3.5b). Cycle 3 \(\text{VOG}_8\) (8.0x) was greater \((P < 0.001)\) than cycle 2 \(\text{VOG}_8\) (2.0x). Both cycle 3 and cycle 2 \(\text{VOG}_8\) were greater \((P < 0.001)\) than cycle 1 (0.72x) and cycle 0 (0.77x). The equations assessing \(\text{VOG}_8\) at 40°C were as follows: \(y = 8.0x - 5.1\) for cycle 3, \(y = 2.0x - 2.6\) for cycle 2, \(y = 0.72x - 0.75\) for cycle 1, and \(y = 0.77x - 0.83\) for cycle 0.

The control germination tests at 20°C resulted in mean cumulative germination of cycle 3 (78.0%) being greater \((P < 0.001)\) than cycle 2 (60.3%) by day 4 (Figure 3.6a). Both cycle 3 and cycle 2 were greater \((P < 0.001)\) than cycle 1 (19%), and cycle 0 (42.8%) by day 4. By day 8, cycle 3 (91.2%) and cycle 2 (85.5%) were not different from each other \((P > 0.05)\), but were greater \((P < 0.001)\) than cycle 1 (43.3%) and cycle 0 (74.3%). This trend remained for the duration of the 14-day test. Cycle 0 mean
cumulative germination was greater \( (P < 0.001) \) than cycle 1 from day 4 through the conclusion of the test period. Total mean cumulative germination at 20°C for day 14 was as follows: cycle 3 = 93.8%, cycle 2 = 89.0%, cycle 1 = 52.8%, and cycle 0 = 77.7%. At the 20°C temperature regime, velocity of germination at eight days (VOG₈) of cycle 3 (28.3x) and cycle 2 (27.6x) were greater \( (P < 0.001) \) than cycle 1 (14.6x) and cycle 0 (24.8x) (Figure 3.6b). Cycle 0 VOG₈ was greater \( (P < 0.001) \) than cycle 1. The equations assessing VOG₈ at 20°C were as follows: \( y = 28.3x - 6.6 \) for cycle 3; \( y = 27.6x - 12.6 \) for cycle 2; \( y = 14.6x - 12.2 \) for cycle 1; and \( y = 24.8x - 15.8 \) for cycle 0.
Figure 3.5  (a) Mean cumulative germination at 40°C of cycle 0, cycle 1, cycle 2, and cycle 3 annual ryegrass over a 14-day test period. Seed produced during the 2017-2018 season; tested July 2018. †Different letters indicate significant differences at P < 0.001.

Figure 3.5  (b) Velocity of germination at 40°C of cycle 0, cycle 1, cycle 2, and cycle 3 annual ryegrass within the first 8 days of testing. Seed produced during the 2017-2018 season; tested July 2018.
Figure 3.6  (a) Mean cumulative germination at 20°C of cycle 0, cycle 1, cycle 2, and cycle 3 annual ryegrass over a 14-day test period. Seed produced during the 2017-2018 season; tested July 2018. †Different letters indicate significant differences at P < 0.001.

Figure 3.6  (b) Velocity of germination at 20°C of cycle 0, cycle 1, cycle 2, and cycle 3 annual ryegrass within the first 8 days of testing. Seed produced during the 2017-2018 season; tested July 2018.
Chi-square Analysis

Chi-square tests were conducted in both 2017 and 2018 as an additional assessment of gains from selection for the 40°C germination tests (Table 3.1). Calculated $\chi^2$ values for the 2017 test year compared cycle 2 to cycle 1 (31.0) and cycle 0 (71.9). Neither value exceeded the critical value of the chi-square test, 82.53, therefore cycle 2 was not different as assessed through chi-square analysis ($P > 0.05$). In the 2018 test year, calculated $\chi^2$ values compared cycle 3 to cycle 2 (39.8), cycle 3 to cycle 1 (293.9), and cycle 3 to cycle 0 (688.0). The calculated value comparing cycle 3 to cycle 2 did not exceed the critical value of the test, and thus cycle 3 was not greater ($P > 0.05$) than cycle 2. The calculated values comparing cycle 3 to cycle 1 and cycle 3 to cycle 0 exceeded the critical value, and thus cycle 3 mean cumulative germination was greater ($P < 0.05$) than cycle 1 and cycle 0. The calculated values comparing cycle 2 to cycle 1 (78.2) did not exceed the critical value of the test, and thus cycle 2 was not greater than cycle 1 ($P > 0.05$). The calculated value comparing cycle 2 to cycle 0 (206.3) was greater than the critical value of the test, and thus cycle 2 mean cumulative germination was greater ($P < 0.05$) than cycle 0. Finally, the calculated value comparing cycle 1 to cycle 0 (6.8) did not exceed the critical value of the test, and thus cycle 1 mean cumulative germination did not differ ($P > 0.05$) from cycle 0.
Table 3.1  Chi-square ($\chi^2$) table for annual ryegrass germination at 40/30°C presenting observed, expected, and calculated values for each cycle of selection at each year of germination testing. No calculations were made for the 2016 test year, as no expected values were available.

<table>
<thead>
<tr>
<th>Year</th>
<th>Cycle</th>
<th>Observed</th>
<th>Expected</th>
<th>(Obs. - Exp.)</th>
<th>(Obs.-Exp.)^2</th>
<th>$\chi^2$ (Obs.-Exp.)^2 / Exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2016</td>
<td>0</td>
<td>3.3</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5.5</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>2017</td>
<td>0</td>
<td>2.7</td>
<td>3.3</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-----</td>
<td>5.52</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>18.6</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>0→2</td>
<td>18.6</td>
<td>3.3</td>
<td>15.3</td>
<td>234.1</td>
<td>70.9</td>
</tr>
<tr>
<td></td>
<td>1→2</td>
<td>18.6</td>
<td>5.5</td>
<td>13.1</td>
<td>171.61</td>
<td>31.2</td>
</tr>
<tr>
<td>2018</td>
<td>0</td>
<td>4.8</td>
<td>2.7</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>7</td>
<td>5.5</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>26.3</td>
<td>18.6</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>45.8</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>0→3</td>
<td>45.8</td>
<td>2.7</td>
<td>43.1</td>
<td>1857.6</td>
<td>688.0*</td>
</tr>
<tr>
<td></td>
<td>1→3</td>
<td>45.8</td>
<td>5.5</td>
<td>40.3</td>
<td>1624.1</td>
<td>295.3*</td>
</tr>
<tr>
<td></td>
<td>2→3</td>
<td>45.8</td>
<td>18.6</td>
<td>27.2</td>
<td>739.8</td>
<td>39.8</td>
</tr>
<tr>
<td></td>
<td>0→2</td>
<td>26.3</td>
<td>2.7</td>
<td>23.6</td>
<td>557.0</td>
<td>206.3*</td>
</tr>
<tr>
<td></td>
<td>1→2</td>
<td>26.3</td>
<td>5.5</td>
<td>20.8</td>
<td>432.6</td>
<td>78.7</td>
</tr>
<tr>
<td></td>
<td>0→1</td>
<td>7</td>
<td>2.7</td>
<td>4.3</td>
<td>18.5</td>
<td>6.8</td>
</tr>
</tbody>
</table>

Critical value = 82.5: $\alpha = 0.05$; d.f. = 63

* Significant at $\alpha = 0.05$

Phenotypic Trait Assessment

Analysis of phenotypic traits occurred during the 2016-2017 and the 2017-2018 growing seasons (Tables 3.2 and 3.3, respectively). During the 2016-2017 season, comparing cycle 0 with cycle 2 of selection no difference ($P > 0.05$) in reproductive tiller number existed between cycle 0 (100.7 tillers plant$^{-1}$) and cycle 2 (94.5 tillers plant$^{-1}$). An
increase ($P < 0.001$) was observed in cycle 2’s time to maturity (115.2 days) over cycle 0 (105.3 days).

Phenotypic data for the 2017-2018 season was collected and compared among all cycles (Table 3.3). An unusually cold winter allowed for cold hardiness assessment. Images of plants at each observed level of damage on the 1-9 rating scale are provided for context (Figure 3.5). Visual rating of mean frost damage indicated that cycle 3 ($\bar{x} = 3.40$) exhibited more frost damage ($P < 0.001$) than cycle 2 ($\bar{x} = 2.19$). Both cycle 3 and cycle 2 were more damaged than cycles 1 ($\bar{x} = 1.42$) and 0 ($\bar{x} = 1.61$). No plants were killed (9) and no plants were completely unaffected (1).

In 2018, relative time to maturity between the most advanced cycle (cycle 3) and the unselected cycle (cycle 0) did not differ ($P > 0.05$). Both had a mean relative time to maturity of 108.6 days. However, both cycle 3 and cycle 0 were earlier ($P < 0.001$) in time to maturity than cycles 2 (121.2 days) and 1 (124.7 days).

Reproductive tiller number in 2018 differed ($P < 0.001$) between cycles 0 (193.1 tillers plant$^{-1}$) and cycle 3 (128.9 tillers plant$^{-1}$). Both cycles 0 and 3 were greater ($P < 0.001$) than cycles 1 (69.9 tillers plant$^{-1}$) and cycle 2 (66.9 tillers plant$^{-1}$).
Table 3.2  Comparison of mean relative time to maturity (Maturity) and mean reproductive tiller number between cycles 0 and 2 polycrosses grown during the 2016-2017 season.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Maturity</th>
<th>Reproductive Tiller Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>---Julian Day---</td>
<td>------Tillers plant(^1)------</td>
</tr>
<tr>
<td>0</td>
<td>105.2b</td>
<td>100.7a</td>
</tr>
<tr>
<td>2</td>
<td>115.1a(^\dagger)</td>
<td>94.5a</td>
</tr>
<tr>
<td>Significance</td>
<td>***</td>
<td>NS(^\ddagger)</td>
</tr>
</tbody>
</table>

\(^\ddagger\) Within columns, different letters indicate significant differences.

\(^\dagger\) NS, Not significant.

*** Significant at \(P < 0.001\).

Table 3.3  Comparison of mean frost rating (Frost), relative time to maturity (Maturity) and reproductive tiller number among cycles 0-3 polycrosses grown during the 2017-2018 season.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Frost(^\dagger)</th>
<th>Maturity</th>
<th>Reproductive Tiller Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>---1-9---</td>
<td>---Julian Days---</td>
<td>------Tillers plant(^1)------</td>
</tr>
<tr>
<td>0</td>
<td>1.61c(^\ddagger)</td>
<td>108.6c</td>
<td>193.1a</td>
</tr>
<tr>
<td>1</td>
<td>1.42c</td>
<td>124.7a</td>
<td>69.9c</td>
</tr>
<tr>
<td>2</td>
<td>2.19b</td>
<td>121.2b</td>
<td>66.9c</td>
</tr>
<tr>
<td>3</td>
<td>3.40a</td>
<td>108.6c</td>
<td>128.9b</td>
</tr>
<tr>
<td>Significance</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
</tbody>
</table>

\(^\ddagger\) Frost damage on 1-9 scale; 1 no damage, 9 plant death.

\(^\dagger\) Within columns, different letters indicate significant differences.

*** Significant at \(P < 0.001\).
Figure 3.7 Images of each observed frost rating on annual ryegrass plants for winter 2017-2018 on a scale of 1-9; 1 = no damage, 9 = plant death. From left to right, top to bottom, are examples of plants rated: 2, 3, 4, 5, 6, and 7. Ratings of 1, 8, and 9 were not observed.

**Heritability Values**

Realized heritability was determined for each cycle of selection using cumulative germination under the high temperature screening conditions (Table 3.4). For the 2015-2016 season, cycle 1 heritability was calculated to be $h^2 = 0.022$. For the 2016-2017 seed
production season comparing cycle 2 to cycle 0, heritability was calculated as mean $h^2 = 0.08$ per generation, for a total of $h^2 = 0.16$ at cycle 2 of selection. In the 2017-2018 production season each cycle (0-3) was grown to provide estimates of $h^2$ as we advanced through each cycle of selection. Therefore, cycle 1 was compared to cycle 0, cycle 2 was compared to cycle 1, and cycle 3 was compared to cycle 2. Cycle 1 realized heritability was $h^2 = 0.022$, cycle 2 realized heritability was $h^2 = 0.19$, and cycle 3 realized heritability was $h^2 = 0.20$. Total accumulation of additive gene action was $h^2 = 0.41$.

Table 3.4 Comparison of annual ryegrass realized heritability ($h^2$) for germination (40°C). The 2015-2016 and 2016-2017 seasons compared the most advanced cycle of selection against the base population (cycle 0). The 2017-2018 season compared each cycle to the antecedent generation. Significance is based upon germination test differences, not heritability values.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>2015-2016</th>
<th>2016-2017</th>
<th>2017-2018</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.022</td>
<td>0.16</td>
<td>0.20</td>
</tr>
<tr>
<td>Total $h^2$</td>
<td>0.022</td>
<td>0.16</td>
<td>0.41</td>
</tr>
<tr>
<td>Significance</td>
<td>NS‡</td>
<td>*</td>
<td>***</td>
</tr>
</tbody>
</table>

* Significant at $P < 0.05$.
*** Significant at $P < 0.001$.
† Cycle 1 not planted in 2016-2017 field season.
‡ NS; not significant.
Discussion

Progression of Germination at 40°C and 20°C

The first cycle of selection tested in 2016 did not achieve significantly increased germination at high temperatures (40°C) over the unselected population (cycle 0); mean cumulative germination at 14 days for cycle 1 was 5.52% while cycle 0 was 3.27% (Figure 3.1a). This was likely due to a lack of accumulation of substantial additivity for the trait to significantly manifest following only one cycle of selection for germination at high temperature. Traits with lesser heritability require additive gene action (alleles) to be expressed and observed at levels that can be discerned through statistical analysis (Grama et al., 1984). Because these alleles occur at a lesser frequency and multiple alleles are present at many loci in the genome, several cycles of selection are often required to accumulate these levels of additivity. However, we were encouraged by the trend of divergence of cumulative germination between the two populations beginning at the 6-day observation period. At 20°C cycle 1 had greater germination than cycle 0 (Figure 3.2a). This difference was attributed to inadvertent selection, which increased VOGs; i.e., selection and propagation of the first seedling to germinate generates a population that germinates faster, while selection for germination at high temperatures alone would not influence speed of germination at normal temperatures.

With cycle 2 of selection for heat tolerance (tested in 2017) we observed a significant increase in germination at 40/30°C in the population (18.6%) compared to cycle 0 (2.7%) (Figure 3.3a). This 16% increase in germination at 14 days provided evidence that strictly controlling environmental variation can allow for significant selection of additive gene action observed from recurrent phenotypic selection. At 20°C
germination of cycle 2 seed compared to cycle 0 was also increased by about 20% (Figure 3.4a). This was attributed to inadvertent selection, which increased VOG₈. After-ripening effects may have contributed to an overall reduction in germination of seed at 20°C between testing years. After-ripening is predicated on several months of post-harvest storage with the effect of widening temperature range at which germination occurs and reducing secondary metabolites such as abscisic acid that impede germination (Allen and Meyer, 2002). Gramshaw (1972) found that germination of freshly harvested annual ryegrass seed resulted in maximum germination of 80%. Gallagher et al. (2004) found similar results; hydration of seed stored 1-6 weeks post-harvest did not significantly improve germination, but did at 12 weeks of post-harvest storage. Given that only four weeks of post-harvest storage was allowed in the 2017 test year while 8 weeks of storage occurred in 2016, after-ripening could account for the lower observed cumulative germination at 20°C that testing year.

Germination testing in 2018 indicated further improvements with cycle 3 annual ryegrass germination at 40/30°C (Figure 3.5a). Mean cumulative germination of cycle 3 (45.8%) was significantly greater than all previous cycles of selection (cycle 2 = 26.3%, cycle 1 = 7.0%, and cycle 0 = 4.8%). The increase in mean cumulative germination of cycle 3 of about 20% compared to cycle 2 lends support to the steady accumulation of additive alleles related to heat tolerance. The increases in mean cumulative germination at 40°C between cycles nearly doubled or tripled beginning from cycle 1→2. While we cannot directly compare results between years (because of strong environmental effects on this trait seed tested must be grown in the same testing year), it is important to note that mean cumulative germination trend lines are similar for relevant cycles tested in
previous years (Figure 3.1a, Figure 3.3a). This indicates that despite the lack of cycle 1 seed in the 2016-2017 production season, the genetics of the material remained stable. Thus, all seed lots should behave similarly when tested in identical environments and as long as we preclude pollen/seed contamination.

Germination tests in 2018 at 20°C behaved largely as expected; cycle 3 (93.8%) and cycle 2 (89.0%) mean cumulative germination was significantly greater than cycle 1 (52.8%) and cycle 0 (77.7%) (Figure 3.6a). The greater germination of cycle 3, cycle 2, and cycle 0 indicated that these seed lots are largely viable, and that post-harvest storage was not an issue. Curiously, cycle 1 germination was poor relative to the other cycles. This is possibly due to being harvested about a week later than the other cycles, which may have resulted in slightly less storage time (5-7 days), and not been sufficient to reduce after-ripening effects. In comparing the results from the 2018 germination tests at both 40°C and 20°C, we have determined that from the most advanced cycle of selection (cycle 3), roughly 48% of the population (~94% germination at 20°C - ~46% germination at 40°C) still needs to be able to germinate at 40°C. Therefore, further cycles of selection should be able to obtain gains and additive allele accumulation. However, as we have passed the 50% mark of expression in the population, gains may begin to decline with further cycles of selection.

Aside from improving germination at high temperatures, the selection methodology also resulted in an increase to VOG8. While we made selections for germination at 40°C, we simultaneously selected seedlings that germinated first. This effect has been present in similar work conducted to improve germination in native warm-season grasses. Springer (2017) found that as selections were made for improved
germination in little bluestem (*Schizachyrium scoparium* Nash) VOG also increased by factors of 97% and 260% for seed with no water stress and water stress, respectively. Work to improve stand establishment in bahiagrass through RPS resulted in significantly faster germination as well as greater cumulative germination than other cultivars (Anderson et al., 2009; Anderson et al., 2011). These results indicate similar findings.

During the 2016 germination tests, VOG₈ of cycle 1 was not improved over cycle 0 at 40°C (Figure 3.1b), but there was a difference at 20°C (3.4x day⁻¹) (Figure 3.2b). Velocity of germination at 40°C did not express due to overall poor germination from both cycles. This was attributed to a lack of sufficient additive gene action to allow either cycle to germinate well under hot conditions. The increased VOG₈ at 20°C was a direct result of selections unintentionally targeting the seedlings that germinated first in the screenings.

In the 2017 tests, however, VOG₈ of cycle 2 increased compared to cycle 0 at both testing temperature regimes (Figure 3.3b). The 40°C test indicated cycle 2 (2.15x day⁻¹) had an almost five-fold increase in VOG₈ when compared to cycle 0 (0.28x day⁻¹). This occurred in tandem with the increased cumulative germination for cycle 2 (18.6%) compared to cycle 0 (2.7%) at 40°C. These factors provided a strong indication that selection for germination at high temperature was successful. Also, VOG₈ acted congruently with cumulative germination at 40°C to provide both earlier and increased germination at high temperatures; at 20°C increased VOG₈ shifted germination earlier by a factor of 1.5x in the advanced cycles of selection over cycle 0 (Figure 3.4b).

Finally, the 2018 tests for VOG₈ at 40°C indicated that cycle 3 (8.0x) germinated four times as rapidly as cycle 2 (2.0x), which in turn was about two-and-a-half times as rapid as cycle 1 (0.72x) and cycle 0 (0.77x) (Figure 3.5b). The mean cumulative
The germination curve for cycle 3 is more linear than cycle 2, cycle 1, and cycle 0 trend lines. This is an effect of more seed germinating on days 2, 4, and 6 than previous cycles of selection. As with the 2017 tests, these results indicate that as we select for improved germination at 40°C we are steadily increasing VOG₈ due to inadvertent selection for seed that germinate earliest under those conditions. The 20°C VOG₈ tests for 2018 again indicated that more advanced cycles of selection (cycle 3 = 28.3x, cycle 2 = 27.6x) have significantly increased VOG₈ than the base population (cycle 0 = 24.8x) (Figure 3.6b). The lower VOG₈ of cycle 1 (14.6x) than all other cycles may be due to less storage time prior to testing due to being harvested 5-7 days later; cycle 1 VOG₈ was significantly greater than cycle 0 when previously compared at 20°C in 2016 (Figure 3.2b).

The improvement in germination of cycle 3 compared to cycle 0 as measured by realized heritability (Table 3.3) indicates a significant accumulation of additivity within the population germplasm. Current paradigms suggest use of recurrent phenotypic selection in breeding obligately outcrossing species (cool-season grasses) for quantitative traits (heat tolerance) is largely ineffective (Marshall, 1982; Semagn et al., 2010). The rationale for this is due to the large genotype x environment interaction. This is a factor of many genes at many loci being involved in expression of a trait (e.g., heat tolerance) when environmental variation is present. However, use of growth chambers with tightly controlled environmental conditions mitigated the latter, as evidenced by improved germination at very high temperatures within two generations. The outcrossing nature of most forage grass species also makes it difficult to fix a trait in a population due to recombination, often requiring several generations to be successful (Casler et al., 1996;
Jafari and Naseri 2007). Despite this aspect, we have observed significant improvements to germplasm performance at 40°C within 2-3 generations.

Significant progress has been made in increasing heat tolerance at the germination and seedling stages in the annual ryegrass germplasm (cycle 3 = 45.8%, cycle 2 = 26.3%, cycle 1 = 7.0%, and cycle 0 = 4.8%). This new population has putatively improved field germination over unselected germplasm when soil temperatures are above the critical threshold that would normally enforce secondary dormancy (21°C). In north-central Mississippi, this threshold is not met until late October (NOAA, 2018). This selected population could be planted and successfully germinate in mid to late August, when soil temperatures would still exceed this threshold. This germplasm also allows for seedlings to tolerate temperatures far greater than the species’ common acclimation range (10-25°C) (Evers et al., 1997), provided soil moisture is available. The screening protocol required elite seedlings be able to survive under 40°C conditions for a minimum of two weeks.

Early establishment of annual ryegrass may provide a benefit to beef cattle producers in the Deep South. Producers rely heavily on this forage for most of their winter biomass (Evers et al., 1997). The earlier a stand can be established and successfully grow, the more time there is for forage biomass accumulation prior to the onset of cold winter temperatures that impede rapid growth. This heat tolerant annual ryegrass has the potential to allow for late fall or early winter grazing to occur. This directly benefits beef producers raising fall stocker calves and could allow a greater period of grazing and live-weight gain prior to market. One necessary avenue for improvement in the germplasm is the incorporation of gray leaf spot (Pyricularia grisea
Cke. [Sacc.]) resistance. Earlier planting of annual ryegrass exposes it to large amounts of 
*P. grisea* inoculum. This disease proliferates in warm, humid conditions during August 
and September in Mississippi (Trevathan, 1994). Introgression and reselection with an 
established, resistant population would be the ideal method for incorporating the trait.

**Chi-Square Tests for Significance**

The chi-square tests conducted provided results that were more conservative than 
the mean separation conducted with ANOVA in SAS 9.4. This is because chi-square 
analysis is a one-tailed test used to detect improvements above a given alpha level, while 
ANOVA is a two-tailed test that determines if performance was above or below a given 
alpha level. Cycle 3 mean cumulative germination was found to only be greater (*P* < 
0.05) than cycle 1 and cycle 0 in 2018, and cycle 2 was only greater (*P* < 0.05) from 
cycle 0 in 2018 (Table 3.1). Comparatively, ANOVA analysis observed these 
comparisons to be different (*P* < 0.001 and *P* < 0.05, respectively) in both 2018 and 
2017. This was likely a factor of how chi-square is calculated. Because heat tolerance is 
quantitative, and is not inherited or expressed through simple Mendelian genetics, we 
could not predict its expression for each test year. Therefore, we used the single value of 
final mean cumulative germination from each previous year as the expected value in 
calculating *χ²* values. This means that chi-square analysis is not able to either a.) use the 
current year’s data, which may differ from previous years, or b.) include replications in 
its analysis. Both of these may explain the lack of detection of differences between cycle 
3 and cycle 2, and cycle 2 and cycle 1 in 2018 and 2017, respectively. However, chi-
-square analysis does provide a better estimate of overall population differences, as it is 
not comparing bi-daily differences in mean cumulative germination, as ANOVA analysis
does. However, the more conservative nature of chi-square analysis effectively makes it only able to detect differences between populations that differ greatly (Clewer and Scarisbrick, 2001). Therefore it should only be used when the calculated $\chi^2$ values exceed the degrees of freedom.

**Importance of Phenotypic Traits**

Several observations were made by monitoring phenotypic traits of plants in successive cycles of selection. Time to maturity, reproductive tiller number, and frost damage were assessed to determine if other important agronomic traits were altered by selection for heat tolerance. Hardy-Weinberg equilibrium states all alleles remain in equilibrium unless there is selection specifically for a trait, i.e., non-random mating. Therefore, if a significant change in any of these traits was observed, we could conclude either a.) alleles responsible for conferring heat tolerance could be closely linked to other traits of interest, or b.) changes were a result of inadvertent selection. Thus, selection for heat tolerance may have resulted in the unintentional selection of plants that either: a.) mature earlier or later, b.) have greater or fewer reproductive tillers, or c.) are more susceptible to freezing damage.

Regarding time to maturity, a comparison of cycle 2 to cycle 0 plants during the 2016-2017 season indicated a 10-day later time to maturity of cycle 2 than cycle 0 (Table 3.1). However, this is likely an artifact of differential fall establishment time for each population. Cycle 0 was transplanted to the field on November 14, 2016, whereas cycle 2 plants were transplanted two weeks later on November 29. Species within the ryegrass genus, including *L. multiflorum*, have been reported to induce flowering at cool temperatures and/or reduced photoperiod (Cooper, 1960; Aitken, 1966; Major, 1980;
McLean and Watson Jr., 1992). It is likely the additional time in the field of cycle 0 was sufficient to cause jointing, and likely accounts for the 10-day earlier time to maturity observed for cycle 0 compared to cycle 2 in 2017.

Concurrently, for the 2017-2018 season (Table 3.2) cycle 3 and cycle 0 plants were transplanted on the same day, September 4, 2017. The intermediary cycles 1 and 2 were not transplanted until November 13, 2017. Time to maturity of cycle 3 and cycle 0 were identical (108.6 days), while cycles 1 (124.7 days) and 2 (121.2 days) were significantly delayed by about 11-14 days. The delays in maturity observed were a direct consequence of the delayed planting time, subsequently causing reduced exposure to field growth conditions. These results suggest selection for heat tolerance at germination does not have an impact on days to maturity. This is crucial, as producers would not want a cultivar that matures faster in the spring. Additionally, delay of fall planting does impact time to maturity. Delays of 2-8 weeks at transplanting can impact maturity by a period of 10-14 days at the end of the season.

Reproductive tiller number was a trait of interest for two main reasons. First, the implications of greater or fewer numbers of reproductive tillers would have a direct impact on leaf:stem ratio, and thus nutritive value (Nelson and Moser, 1994). Nutritive value has been negatively correlated to reproductive tiller number in previous work (Mayland et al., 2000; Temu et al., 2014). Secondly, the ability of the population to produce seed for increase is critical for commercial release and distribution. A positive correlation between reproductive tiller number and seed production in grass species has been established. In many correlation studies reproductive tiller number was one of the
greatest contributing factors (Sukchain and Sidu, 1992; Lopes and Franke, 2011; Abel et al., 2017).

For the 2016-2017 season, reproductive tiller number did not differ between cycle 0 and cycle 2 (Table 3.1). However, results from the 2017-2018 season indicated several differences between cycles (Table 3.2). Cycle 0 averaged 193.1 tiller plant\(^{-1}\), which was significantly greater than cycle 3 (128.9 tillers plant\(^{-1}\)). Cycle 3 had significantly more tillers cycles 1 and 2 (69.9 and 66.9 tillers plant\(^{-1}\), respectively). This is due to several environmental, rather than genetic factors. First, the greater tiller numbers of cycles 0 and 3 over cycles 1 and 2 were a function of smaller end-of-season plant size due to delayed transplanting. Greater reproductive tiller number of cycle 0 over cycle 3 was also a function of plant size. Plants in cycle 0 were larger than those in cycle 3, despite uniform fertilization and identical planting times. This difference in size is likely due to cycle 3 having stunted growth from screening at 40°C. In annual ryegrass, each vegetative tiller converts to a reproductive tiller when critical photoperiod and temperature requirements are met (Casal et al., 1985). Thus, longer growing time or larger plant size will directly impact reproductive tiller number. We suspect that the differences in reproductive tiller number between cycle 0 and 3 observed in 2017-2018 polycrosses was due to a stressful seedling environment, rather than genotypic effects. The lower tiller numbers observed in cycles 1 and 2 are likely due to the delayed planting date, resulting in smaller plants with fewer tillers. Selection for heat tolerance at germination did not produce an identifiable change in tiller production.

An unusually cold winter (2017-2018) provided the opportunity to evaluate if selection for heat tolerance altered cold hardiness. Frost damage ratings provided
evidence of some loss of cold hardiness. Cycle 3 exhibited greater mean frost damage than cycle 2, cycle 1, and cycle 0 (Table 3.2). Cycle 2 also had greater frost damage than cycles 1 and 0. Unexpectedly, selection for heat tolerance seems to adversely impact cold hardiness. Hardy-Weinberg theory would suggest no change would occur. This was a record cold period for the production area (< -10°C), but this was a single event. Therefore, we cannot definitively conclude that the observed frost damage and apparent loss of cold hardiness was due to selection. The base germplasm, Marshall, is a cultivar selected for improved cold hardiness over other annual ryegrass cultivars at the time of its development in the 1980’s (Arnold et al., 1981). This apparent loss of cold hardiness in the advanced germplasm may be undesirable to producers farther north in Mississippi and the rest of the Deep South. Thus, further testing is needed and may involve additional screening of selected plants to freezing temperatures in controlled environments to determine if cold hardiness has truly declined as the population is moved away from the base Marshall germplasm.

**Heritability of Heat Tolerance**

As previously discussed, the quantitative nature of the heat tolerance trait limits its heritability. The compounding effects of many loci acting in tandem in the expression of the trait caused heritability estimates to be non-significant in the first year of selection ($h^2 = 0.022$) (Table 3.3). However, we observed a significant increase to realized heritability between cycle 1 and cycle 2, with the value compared to the base germplasm of cycle 0 being $h^2 = 0.16$. An eightfold increase in heritability indicated that selections for heat tolerance (under controlled environmental conditions) were effective for improving the trait. It is important to note: calculated mean $h^2$ value per generation likely
resulted in an overestimation of cycle 1 realized heritability, and an underestimation of cycle 2 realized heritability. We would expect a greater proportion of additivity to be attributed to more advanced cycles of selection as desirable heat tolerance alleles accumulate.

This success provides support to the selection methodology, as we have increased additivity for the expression of heat tolerance as measured by $h^2$ over subsequent cycles of selection (cycles 0→1→2→3). We expect further cycles of selection will provide even greater increases in realized heritability of heat tolerance as additive loci continue to accumulate.

Realized heritability calculated for the 2017-2018 seed crop provided an assessment of each generation’s gain in heritability over three cycles of selection. The allelic accumulation of cycle 1 was very low, $h^2 = 0.022$. The increase in heritability from cycle 1 to 2 was greater, $h^2 = 0.19$. However, the increase in realized heritability from cycle 2 to cycle 3, $h^2 = 0.20$, was nearly identical to the gain from cycle 1 to cycle 2. This likely indicates that we may be nearing the end of our ability to increase the trait rapidly. While $h^2$ may have been identical from cycle 2 to cycle 3 as from cycle 1 to cycle 2, we still observed an increase in mean cumulative germination in the 2018 germination tests at 40°C. This gives further support to the additive nature of heat tolerance. Therefore, overall heritability from cycle 0 to cycle 3 for the 2017-2018 season was $h^2 = 0.41$ (cycle 1 $h^2 = 0.022 +$ cycle 2 $h^2 = 0.19 +$ cycle 3 $h^2 = 0.20$). These findings also support the notion that realized heritability estimates for previous years, where only the most advanced cycle and the base population were tested, were overestimating realized heritability for cycle 1, and underestimating it for cycle 2.
Conclusions

Over the course of three cycles of selection to improve germination at 40°C in annual ryegrass through RPS, we have made significant progress. Cycle 3 of selection exhibited mean cumulative germination of nearly 50% in the 2018 test year. The rate of increase from one cycle to the next has increased (~5% for cycle 1 in 2016 to ~20% for cycle 2 in 2017 and cycle 3 in 2018) and likely will continue to increase as we continue to accumulate heat tolerance additive alleles in the population. However, due to already increasing germination at 40°C in the population beyond 50% of total germination, the rate of increase may begin to slow with each cycle of selection beyond cycle 3. Chi-square analysis also indicated significant population improvement in cycle 3 over the base germplasm, Marshall, but was more conservative in its comparisons between intermediary cycles of selection. Chi-square is a useful analysis tool for plant breeders, but may only be useful several cycles away from the base-germplasm when differences are large enough to detect.

Velocity of germination within the first eight days after seeding has also been increased nearly eight times over the base germplasm. Through this process, we have effectively lowered secondary seed dormancy that would normally inhibit germination at 40°C in nearly 50% of the current population. Seedling heat tolerance has also been improved through selecting only germinated seed that are able to grow vigorously under 40/30°C day/night conditions that are greater than real-world environmental stresses they might encounter. These improvements will potentially allow producers in Mississippi and the rest of the Deep South to plant seed of the selected population earlier in the production season, ideally in late summer (August or early September). The reduced secondary
dormancy would allow seed to germinate despite high soil temperatures, germinate rapidly upon imbibition of water from late summer rains, and persist under high atmospheric temperatures at that time of the year post-germination. Ideally, this germplasm would be established into a prepared seedbed following a warm-season annual forage crop. This would prevent it being outcompeted by longer-lived warm-season perennial species such as bermudagrass. By establishing the crop earlier, producers could take advantage of greater biomass accumulation while fall and early winter temperatures are still ideal for cool-season grass production.

However, producers need to be cautious of several factors that could impede early season annual ryegrass production. There are three major concerns of early establishment, aside from high temperature: disease pressure, pest pressure, and drought. In terms of disease pressure, gray leaf spot (blast) (*Pyricularia grisea*) is a major disease of annual ryegrass during late summer months. *Pyricularia grisea* thrives under warm temperatures (20 to 28°C) and prolonged leaf wetness and humidity, making inoculum pressure greater. Fall armyworm (*Spodoptera frugiperda* J.E. Smith) is a particularly damaging insect pest to seedlings during late summer as well. This could necessitate denser seedings to overcome pest or pathogen load. Finally, drought conditions may be prevalent in late summer. While the germplasm developed in this work can germinate rapidly upon imbibition, and survive post-germination at high temperature, it cannot do so without water. This germplasm is reliant upon late summer rains, either from hurricanes and tropical storms or thunderstorms. Therefore, water availability should always be considered.
Through rigorous phenotyping of selected plants in the field, we have also determined that selecting for reduced secondary seed dormancy and improved seedling vigor under high temperature stress does not impact other desirable phenotypic traits. Over two testing seasons (2016-2017 and 2017-2018) we have concluded that environmental effects, rather than genotypic effects, were responsible for observed differences in time to maturity and reproductive tiller number. This means that available grazing time at the terminal end of the season, along with forage nutritive value components and seed production capability, should not differ from the base germplasm, Marshall. In these results, planting/transplanting date to the field heavily factored in observations of later time to maturity and fewer reproductive tillers. This was likely due to less exposure to ideal photoperiod conditions to induce flowering.

On a genetic level, estimations of realized heritability indicated germplasm at each cycle of selection was accumulating progressively greater amounts of additive gene action to the expression of heat tolerance. Realized heritability for cycle 2 to 3 (0.20) did not improve over the value for cycle 1 to 2 ($h^2 = 0.19$). However, the additive gene action allows a summation of all the heritability effects observed from cycle 0 to cycle 3, $h^2 = 0.41$. We expect this value to continue to increase with further cycles of selection as alleles responsible for conferring heat tolerance continue to accumulate. The success in developing this germplasm refutes the paradigm that breeding outcrossing species for quantitative traits is not feasible, with the caveat that we control environment under selection conditions. Moreover, because we have developed the population through bulk selection maintaining large population numbers rather than half-sib families and synthetic cultivar development, the final population at release will behave reliably without the loss
of performance from one generation to the next, as long as seed production is kept isolated from external wild-type ryegrass pollen flow.

Currently, seedlings forming the basis of cycle 4 have been selected, and are being grown to transplant in fall of 2018. Following this cycle, seed increase and variety testing will occur, with the intention of subsequent cultivar release.
CHAPTER IV

BREEDING A HEAT TOLERANT ORCHARDGRASS FOR PERENNIAL GROWTH IN HIGH TEMPERATURE STRESS ENVIRONMENTS OF THE DEEP SOUTH

Introduction

Forage production in the South is comprised primarily of warm-season perennial grasses and perennial or annual legumes. These are commonly supplemented by one of two cool-season forage grasses, either tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort) (a perennial) or annual ryegrass (*Lolium multiflorum* Lam.) (Rouquette et al., 1997). This management doctrine has been in place largely due to the environment of the Deep South. Issues include high relative humidity as well as high mean temperatures throughout the summer months, often exceeding 32°C for daily highs. Tall fescue is well adapted to these conditions, in part because of its symbiosis with common toxic and acrimonious endophyte, *Epichloë coenophiala* (Morgan-Jones & W. Gams) C. W. Bacon & Schardl. Novel endophytes also confer some measure of heat and drought stress tolerance to fescue plants (Schmidt and Osborn, 1993). Annual ryegrass is a winter annual adapted to growth in the mild winter conditions of the South (Nelson et al., 1997). This makes it useful as early season forage for many grazing operations. However, one of the major cool-season perennial grasses utilized for forage in the United States, orchardgrass (*Dactylis glomerata* L.), is noticeably absent from most southern pastures.
Orchardgrass is known for being a high-quality forage and is a staple forage grass in pastures north of the transition zone. It is quick to regrow following grazing or cutting for hay and responds very well to applications of nitrogen fertilizer (Mortensen et al., 1964; Ball et al., 2007). It is also commonly used as a companion crop in alfalfa stands for a grass/legume hay. The species is mostly adapted to areas of mild summers and cold winters. It can tolerate some heat stress, but prolonged periods of over 32°C can lead to stand loss, particularly after grazing or hay harvest (Baker and Jung, 1968). Repeated grazing or harvests can eventually cause permanent stand loss under these environmental stresses.

**Objectives**

The objective of this study was to develop a novel orchardgrass cultivar through recurrent phenotypic selection (RPS) that could tolerate the growing conditions (high temperatures and high relative humidity) in the South. The breeding objectives were to i.) alleviate secondary seed dormancy by selecting seed that germinate at high temperatures, ii.) improve heat tolerance of seedlings by screening at high temperatures, iii.) increase perennation of the species under high temperature stress environments, and iv.) determine if other important phenotypic traits were altered during selection for heat tolerance.

**Materials and Methods**

**Selection Methodology and Germination Testing**

Germplasm for this work was derived from an unknown orchardgrass cultivar planted 40 years ago at the MAFES Prairie Research Unit, Prairie, MS (33°47'48.25", -88°39'35.65”). The soil type was a Houston Clay (fine smectitic, thermic Udic)
Haplusterts). These plants underwent decades of natural selection for persistence at ambient high temperatures. Seed from these individuals was harvested in 2009 and stored for five years. From this population, a mother plant nursery consisting of 64 plants was established in fall of 2014 at the Mississippi State H. H. Leveck Animal Research Center (33°26’16.46”, -88°47’52.82”). The soil type at this location of the farm was a Catalpa silty clay loam (fine, smectitic, thermic Fluvaquentic Hapludolls). Plants were fertilized with 160 mL of 107 ppm N, 23 ppm P, and 33 ppm K solution (Peter’s 20-20-20) on a bi-weekly basis following transplanting until the end of February each season. Seed from this nursery was collected to serve as the base population, cycle 0, for the selection process.

In October 2015 seed from cycle 0 was screened in a growth chamber (Percival Scientific, Series 101, Perry, IA) for germination at 40/30°C (12/12 hr, light/darkness). Selections were made over a period of 36 days. Surviving seedlings were advanced to cycle 1. From these screenings, 95 plants were selected from a total of 828 plants. Of the 95 selections, 64 were transplanted into a 6.1 m² polycross nursery on an 8 x 8 grid on 61 cm centers at the Mississippi State H. H. Leveck Animal Research Center 33°25’24.02” N, -88°47’33.89” in fall 2015. A Marietta fine sandy loam (Fine-loamy, siliceous, active, thermic Fluvaquentic Eutrudepts) predominated at this location of the farm. Plastic (5 mm, white, low-density, polyethylene) was placed (edges buried) to reduce weed pressure. A 1.5 m border of cereal rye (Secale cereale L.) was planted around the block to serve as a pollen barrier. Each growing season (except for 2015-2016) an identical polycross of cycle 0 plants was established for phenotypic comparisons between the base population and any advanced cycles of selection. Cycle 1 seed was harvested and bulked
between May 31 and June 6, 2016. Seed was then conditioned using a belt thresher (ALMACO, Nevada, IA) to remove seeds from panicles. Seed was sieved (0.164 x 0.953 cm mesh, Seedburo Equipment, Des Plaines, IL) and fractionally aspirated (Carter Day International, Inc. Minneapolis, MN) to remove chaff. Cycles to be compared were always grown and tested during the same season to eliminate any differences in seed characteristics that ambient environment or storage conditions might produce.

Germination tests of cycle 1 seed (AOSA, 2014) were conducted at recommended AOSA protocol for orchardgrass (20/15°C, 12/12 hr, light/darkness) and at hot germination temperatures (40/30°C, 12/12 hr light/darkness). Seed was placed in Petri dishes (15.25 x 3cm) upon 15 ml of 1% water agar as the germination medium. Six-hundred seed (6 reps of 100 seed) for each cycle of selection arranged as a completely randomized design were placed in each temperature regime. The germination testing lasted for 22 days (AOSA, 2014). Observations were made every two days. Daily and cumulative germination was recorded and calculated for each cycle of selection. Velocity of germination at eight days (VOG₈) was also calculated. The slope of the germination trend-line within the first 8 days of the test was graphed and represented. Germination testing was conducted identically for the 2017 (cycles 0, 1, and 2) and 2018 (cycles 0, 1, 2, and 3) test years.

To advance germplasm to the next cycle of selection, a given cycle’s seed lot was screened in an identical manner as previous generations. Seed that germinated at 40°C were used to form the elite parents of the next cycle’s polycross population (i.e. seed from cycle 1 that germinated at 40°C were used as the elite parents of cycle 2). Sixty-four cycle 2 selections were transplanted in November 2016. These plants were grown to
maturity and seed was harvested in late spring 2017. Because orchardgrass is perennial, some plants established the previous year survived each season. Dead plants were replaced each year for comparison to the most advanced cycle of selection. Prior to harvest, tillers of each plant were tied in a sheaf and labeled for identification. At harvest, sheaves were dried at 20°C. Seed from all blocks was conditioned as in years prior. Germination testing and screening was repeated each year to obtain the next cycle of selection (cycle 2→3 and cycle 3→4). For the 2017-2018 season, polycrosses for each generation (cycles 0, 1, 2, and 3) were planted for determination of selection progress.

**Additional Phenotyping of Selected Plants**

Other phenotypic traits were monitored on plants in the polycross nurseries for each cycle of selection. Seasons over which traits were examined were fall 2015 through spring 2016 (2015-2016), fall 2016 through spring 2017 (2016-2017), and fall 2017 through spring 2018 (2017-2018). Traits included; days to maturity (in Julian days), reproductive tiller number, persistence, and frost damage. Maturity was defined as the day of the year at which the first seed head on a plant emerged from the leaf sheath on a reproductive tiller. Plants were monitored weekly until the first seed head in any polycross emerged, and then observed every two days until all plants in the nursery had at least one seed head visible. For reproductive tiller number, values were determined by counting the number of stems per sheaf from each harvested plant prior to seed conditioning. The perennation of selected plants was determined by counting surviving plants in each cycle’s polycross approximately 12 months after planting. Finally, during mid-January of 2018, several days of cold temperature in the range of -6°C to -13°C
caused frost damage to plants, enabling visual frost damage ratings. Each plant was assigned a rating on a 1-9 scale (1 = no damage, 9 = plant death) and ratings were averaged across each cycle of selection. Ratings were assessed six weeks after the frost period occurred (late February 2018).

**Determination of Heritability**

Heritability is the measure of the progress made due to selection that is due to genetic rather than environmental effects (Briggs and Knowles, 1967). To determine realized heritability of selection for 40°C germination we applied the following equation to the datasets:

\[ h^2 = \frac{G}{R} \]

(Eq. 4.1)

Where \( h^2 = \) realized heritability, \( G = \) gain, the number of observed individuals expressing a trait beyond that of the base germplasm (in this case germination at 40°C), and \( R = \) reach, the number of individuals previously selected for the trait of interest in the prior generation.

**Statistical Analysis**

Data from both the cumulative germination and the phenotypic trait assessment from each year of testing was analyzed as a completely randomized design using SAS 9.4 and PROC GLM to conduct ANOVA and mean separation. The statistical model was:

\[ Y_{ijk} = \mu + D_i + C_j + \xi_{k(ij)} \]

(Eq. 4.2)

in which \( Y = \) the response variable, in this case number of germinated seed, \( \mu = \) mean effects, \( D = \) day of the test, \( C = \) cycle of selection, and \( \xi = \) experimental error. An alpha level of \( \alpha=0.05 \) was used to determine significant differences.
Chi-square ($\chi^2$) analysis was used as a secondary assessment of improvement for the second and third cycles of selection (2017 and 2018 for germination tests, and 2018 for the phenotypic trait assessment) once expected values for each cycle of selection were determined from the previous year’s data. The formula for chi-square analysis was:

$$\chi^2 = \frac{(\text{observed}-\text{expected})^2}{\text{expected}}$$

(Eq. 4.3)

where observed = the number of individuals seen to express a phenotype, and expected = the number of individuals predicted to express that phenotype. These expected values were then compared to the critical value of the $\chi^2$ table (Clewer and Scarisbrick, 2001), with degrees of freedom as n-1, and a significance level of $\alpha=0.05$. With 63 d.f., (64 total plants supplying seed), the critical value was 82.5.

**Results**

**Germination Tests**

Germination test analysis of variance for each year resulted in a significant interaction ($P < 0.05$) between day and cycle. Therefore, each observation day of the 22-day test was partitioned to compare each cycle of selection within each day. For the initial year of testing (2016) cycle 1 was not compared to cycle 0. However, cumulative germination data was recorded for the cycle 1 seed lot. Cumulative germination of cycle 1 at 40/30°C peaked at 4.32% (Figure 4.1a). Velocity of germination of cycle 1 at 40/30°C was 0.15x day$^{-1}$ within the first eight days after (Figure 4.1b). The equation assessing VOG$_8$ of cycle 1 at 40/30°C was $y = 0.15x - 0.21$. At 20/15°C, cumulative germination peaked at 48.7% (Figure 4.2a). Cycle 1 VOG$_8$ at 20/15°C was 5.8x day$^{-1}$ during the first eight days of the test (Figure 4.2b). The line fitted to VOG$_8$ at 20°C was $y = 5.8x - 7.9$. 
Figure 4.1  
(a) Mean cumulative germination at 40°C of cycle 1 orchardgrass, over a 22-day test period. Seed produced during the 2015-2016 season; tested August 2016. No comparison to cycle 0.

Figure 4.1  
(b) Velocity of germination at 40°C of cycle 1 orchardgrass within the first 8 days of testing. Seed produced during the 2015-2016 season; tested August 2016. No comparison to cycle 0.
Figure 4.2  

(a) Mean cumulative germination at 20°C of cycle 1 orchardgrass, over a 22-day test period. Seed produced during the 2015-2016 season; tested August 2016. No comparison to cycle 0.

(b) Velocity of germination at 20°C of cycle 1 orchardgrass within the first 8 days of testing. Seed produced during the 2015-2016 season; tested August 2016. No comparison to cycle 0.
For the second year of testing (2017) cumulative germination of cycle 2 was compared among cycle 1 and cycle 0. At 40/30°C (Figure 4.3a) a increase in cumulative germination ($P < 0.01$) was observed by day 6; cycle 2 (4.00%) was greater than both cycle 1 (0.50%) and cycle 0 (1.33%). Cycle 2 remained significantly greater than both cycle 1 and cycle 0 for the remainder of the 22-day test period. Cycle 1 cumulative germination (1.00%) was less ($P < 0.001$) than cycle 0 (3.67%) by day 10. This observation remained for the duration of the test period. Total cumulative germination was: cycle 2 at 38.17%, cycle 1 at 3.67%, and cycle 0 at 23.3%. Velocity of germination results indicated that cycle 2 (2.4x day$^{-1}$) had greater ($P < 0.01$) VOG$_{8}$ than cycle 1 (0.16x day$^{-1}$) and cycle 0 (0.68x day$^{-1}$) (Figure 4.3b). The slope of cycle 2 VOG$_{8}$ was 10.8 times that of cycle 1, and 1.7 times the slope of cycle 0. The lines fitted to VOG$_{8}$ at 40°C were as follows: $y = 2.4x - 2.8$ for cycle 2; $y = 0.16x - 0.083$ for cycle 1; $y = 0.68x - 0.75$ for cycle 0.

At 20/15°C (Figure 4.4a) a difference ($P < 0.001$) in cumulative germination was observed among cycles 2, 1, and 0. Cycle 2 (22.4%) was greater than cycles 1 (9.33%) and 0 (14.5%) by day 4. However, by day 6 cycle 2 (46.8%) was not greater than cycle 0 (37.8%). Both were greater than cycle 1 (15.17%). This effect remained for the duration of the 22-day test period. Final cumulative germination at 20/15°C temperatures were as follows: cycle 2 at 75%, cycle 1 at 18.2%, and cycle 0 at 75%. Velocity of germination at the 20/15°C temperature regime indicated cycle 2 (21.0x day$^{-1}$) and cycle 0 (18.3x day$^{-1}$) did not differ ($P > 0.05$) (Figure 4.4b); Cycle 1 VOG$_{8}$ (5.6x day$^{-1}$) was less ($P < 0.001$) than both cycles 2 and 0. The equation lines fitted to VOG$_{8}$ at 20°C were as follows: $y = 21.0x - 19.8$ for cycle 2, $y = 5.6x - 3.8$ for cycle 1, and $y = 18.3x - 19.4$ for cycle 0.
Figure 4.3 a.) Mean cumulative germination at 40°C of orchardgrass cycles 0, 1, and 2 over a 22-day test period. Seed produced during the 2016-2017 season; tested July 2017.
†Different letters indicate significant differences at P < 0.01.

Figure 4.3 b.) Velocity of germination at 40°C of orchardgrass cycles 0, 1, and 2 within the first 8 days of testing. Seed produced during the 2016-2017 season; tested July 2017.
Figure 4.4  
a.) Mean cumulative germination at 20°C of orchardgrass cycles 0, 1, and 2 over a 22-day test period. Seed produced during the 2016-2017 season; tested July 2017.  
†Different letters indicate significant differences at P < 0.001.

Figure 4.4  
b.) Velocity of germination at 20°C of orchardgrass cycles 0, 1, and 2 within the first 8 days of testing. Seed produced during the 2016-2017 season; tested July 2017.
Finally, germination testing during 2018 compared all cycles of selection (cycle 3, cycle 2, cycle 1, and cycle 0) (Figure 4.5a). Under the 40/30°C testing regime, cycle 3 mean cumulative germination (1.83%) was greater ($P < 0.01$) at day 4 than cycle 2 (0.50%), cycle 1 (0.17%), and cycle 0 (0.33%). Cycle 2, cycle 1, and cycle 0 did not differ at day 4 ($P > 0.05$). Cumulative germination of cycle 3 remained greater ($P < 0.001$) than all other cycles for the remainder of the 22-day test period. Cycle 2 mean cumulative germination (7.5%) became greater ($P < 0.001$) than cycle 1 (2.67%) and cycle 0 (4.33%) at day 8; this pattern remained for the duration of the 22-day test period. Cycle 1 germination (13.5%) was observed to be less ($P < 0.001$) than cycle 0 (21.17%) by day 14. This pattern also held for the duration of the test. Final mean cumulative germination at 22 days for each cycle was as follows: cycle 3 = 82.67%, cycle 2 = 55.33%, cycle 1 = 24.00%, and cycle 0 = 37.83%.

Velocity of germination within 8 days at 40/30°C indicated cycle 3 had a faster ($P < 0.001$) germination rate (8.2x) than cycle 2 (2.5x), cycle 1 (0.88x), and cycle 0 (1.4x) (Figure 4.5b). Cycle 2 VOG$_8$ was faster ($P < 0.001$) than cycle 1 and cycle 0; cycle 1 did not differ ($P > 0.05$) from cycle 0. The VOG$_8$ of cycle 3 was 3.38 times that of cycle 2, 9.32 times the VOG$_8$ of cycle 1, and 5.86 times that of cycle 0. The equations assessing the slope of VOG$_8$ for each cycle of selection were as follows: $y = 8.2x -11.8$ for cycle 3; $y = 2.5x - 3.5$ for cycle 2; $y = 0.88x - 1.3$ for cycle 1; and $y = 1.4x -2$ for cycle 0.

Germination testing at 20/15°C in 2018 also resulted in differences ($P < 0.001$) between cycles (Figure 4.6a). By day 4, cycle 3 germination (38.67%) was greater than cycle 2 (30.61%), which in-turn was greater than both cycle 1 (24.5%) and cycle 0 (23.17%). At day 8, cycle 3 (85%) did not differ from cycle 2 (80.5%) or cycle 1.
(82.5%), but all three were greater than cycle 0 (66.33%). From day 10 through the remainder of the 22-day test period cycle 3 (89.17%) was greater than cycle 2 (83.28%) and cycle 0 (72%), but was not greater than cycle 1 (86.5%). Cycle 2 and cycle 1 did not differ, but were significantly greater than cycle 0. Final mean cumulative germination at 22 days for each cycle was as follows: cycle 3 = 92.17%, cycle 2 = 84.94%, cycle 1 = 88.67%, and cycle 0 = 78.67%.

Velocity of germination within 8 days at 20/15°C also indicated that cycle 3 (29.3x), cycle 2 (28.9x), and cycle 1 (27.8x) did not differ from each other ($P > 0.05$) (Figure 4.6b). However, the VOG8 of these three cycles were all greater ($P < 0.001$) than cycle 0 (22.9x). The equations assessing the slope of VOG8 at 20/15°C for each cycle of selection were as follows: $y = 29.3x - 23.2$ for cycle 3; $y = 28.9x - 29$ for cycle 2; $y = 27.8x - 24.9$ for cycle 1; and $y = 22.9x - 21.6$ for cycle 0.
Figure 4.5 a.) Mean cumulative germination at 40°C of orchardgrass cycles 0, 1, 2, and 3 over a 22-day test period. Seed produced during the 2017-2018 season; tested July 2018.
†Different letters indicate significant differences at $P < 0.001$.

Figure 4.5 b.) Velocity of germination at 40°C of orchardgrass cycles 0, 1, 2, and 3 within the first 8 days of testing. Seed produced during the 2017-2018 season; tested July 2018.
Figure 4.6 a.) Mean cumulative germination at 20°C of orchardgrass cycles 0, 1, 2, and 3 over a 22-day test period. Seed produced during the 2017-2018 season; tested July 2018.
†Different letters indicate significant differences at P < 0.001.

Figure 4.6 b.) Velocity of germination at 20°C of orchardgrass cycles 0, 1, 2, and 3 within the first 8 days of testing. Seed produced during the 2017-2018 season; tested July 2018.
Chi-Square Analysis

Chi-square ($\chi^2$) analysis was conducted for the 2017 and 2018 40°C germination tests to compare population gains from selection between cycles (Table 4.1). In 2017, the calculated $\chi^2$ value between cycle 2 and cycle 1 (267.3) exceeded the critical value of the test (82.53, $\alpha = 0.05$; d.f. = 63). Therefore, $\chi^2$ analysis determined cycle 2 mean cumulative germination to be significantly greater than cycle 1. No comparison between cycle 2 and cycle 0 was possible, because of a lack of expected value from cycle 0 in 2016.

In 2018, all possible comparisons were made between all cycles. Calculated $\chi^2$ for cycle 3 exceeded the critical value of the test when compared to cycle 0 (151.4) and cycle 1 (1686.8). However, calculated $\chi^2$ for cycle 3 compared to cycle 2 (51.8) did not exceed the critical value. Therefore, cycle 3 mean cumulative germination was significantly greater than cycle 1 and cycle 0, but not significantly greater than cycle 2. The calculated $\chi^2$ for cycle 2 compared to cycle 1 (719.6) was also greater than the critical value, indicating cycle 2 mean cumulative germination was greater than that of cycle 1. However, calculated $\chi^2$ for cycle 2 compared to cycle 0 (43.9) did not exceed the critical value. Thus cycle 2 was not different from cycle 0. Finally, calculated $\chi^2$ for cycle 1 compared to cycle 0 (0.1) did not exceed the critical value of the test, and thus the two cycles were not different.
Table 4.1 Chi-square ($\chi^2$) table for orchardgrass germination at 40/30°C presenting observed, expected, and calculated values for each cycle of selection at each year of germination testing. No calculations were made for the 2016 test year, as no expected values were available.

<table>
<thead>
<tr>
<th>Year</th>
<th>Cycle</th>
<th>Observed</th>
<th>Expected</th>
<th>(Obs. - Exp.)</th>
<th>(Obs.-Exp.)$^2$</th>
<th>$\chi^2$ (Obs.-Exp.)$^2$ / Exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2016</td>
<td>0</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4.3</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>2017</td>
<td>0</td>
<td>23.3</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.7</td>
<td>4.3</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>38.2</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td></td>
<td>1→2</td>
<td>38.2</td>
<td>4.3</td>
<td>33.9</td>
<td>1149.21</td>
<td>267.3*</td>
</tr>
<tr>
<td>2018</td>
<td>0</td>
<td>37.3</td>
<td>23.3</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>24</td>
<td>3.7</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>55.3</td>
<td>38.2</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>82.7</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td></td>
<td>0→3</td>
<td>82.7</td>
<td>23.3</td>
<td>59.4</td>
<td>3528.4</td>
<td>151.4*</td>
</tr>
<tr>
<td></td>
<td>1→3</td>
<td>82.7</td>
<td>3.7</td>
<td>79.0</td>
<td>6241.0</td>
<td>1686.8*</td>
</tr>
<tr>
<td></td>
<td>2→3</td>
<td>82.7</td>
<td>38.2</td>
<td>44.5</td>
<td>1980.3</td>
<td>51.8</td>
</tr>
<tr>
<td></td>
<td>0→2</td>
<td>55.3</td>
<td>23.3</td>
<td>32.0</td>
<td>1024.0</td>
<td>43.9</td>
</tr>
<tr>
<td></td>
<td>1→2</td>
<td>55.3</td>
<td>3.7</td>
<td>51.6</td>
<td>2662.6</td>
<td>719.6*</td>
</tr>
<tr>
<td></td>
<td>0→1</td>
<td>24</td>
<td>23.3</td>
<td>0.7</td>
<td>0.5</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Critical value = 82.5: $\alpha = 0.05$; d.f. = 63

* Significant at $\alpha = 0.05$
Phenotypic Trait Assessment

For the 2016-2017 season, the traits of maturity, reproductive tiller number, and stand survival were observed and compared for cycle 0 and cycle 2 of selection (Table 4.2). There were no differences ($P > 0.05$) between cycle 0 and cycle 2 in regard to maturity, reproductive tiller number, or stand survival. Maturity dates observed for cycle 0 and cycle 2 were nearly identical at 83.6 days and 83.4 mean days to maturity, respectively. Reproductive tiller numbers were also very similar, with cycle 0 having 30.61 tillers plant$^{-1}$ and cycle 2 having 29.4 tillers plant$^{-1}$. Stand survival ratings indicated that there was no difference ($P = 0.0625$) between cycle 0 (25.0%) and cycle 2 (41.1%) 11 months post-planting.

For the 2017-2018 season (Table 4.3), visual frost ratings indicated no difference ($P > 0.05$) in frost damage between cycles 0 ($\bar{x} = 3.33$) and 3 ($\bar{x} = 3.28$), but did indicate the two cycles were more ($P < 0.001$) frost damaged than cycles 1 ($\bar{x} = 1.63$) and cycle 2 ($\bar{x} = 1.57$). Images of each observed level of the 1-9 rating scale are provided for context (Figure 4.5). No plants were completely killed (9).

Cycle 3 had an earlier ($P < 0.001$) time to maturity (65.1 days) than cycles 2 (79.1 days), cycle 1 (79.6 days), and cycle 0 (83.0 days). Mean reproductive tiller number indicated cycle 3 (44.1 tillers plant$^{-1}$) had more ($P < 0.001$) tillers than cycle 2 (31.8 tillers plant$^{-1}$), cycle 1 (31.2 tillers plant$^{-1}$), and cycle 0 (34.7 tillers plant$^{-1}$).

Finally, stand survival ratings indicated that cycle 3 (56.2%) and cycle 2 (54.7%) were greater ($P < 0.001$) in stand persistence after 10 months than cycle 1 (21.9%) and cycle 0 (27.0%).
Table 4.2  Comparison of orchardgrass mean reproductive tiller number, relative time to maturity (Maturity), and stand survival (11 months) of cycle 0 and cycle 2 polycrosses grown during the 2016-2017 season.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Maturity</th>
<th>Reproductive Tiller Number</th>
<th>Stand Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>---Julian Days---</td>
<td>-----Tillers plant(^{-1})------</td>
<td>---Percentage---</td>
</tr>
<tr>
<td>0</td>
<td>83.6</td>
<td>30.6</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>83.5</td>
<td>28.1</td>
<td>41.1</td>
</tr>
<tr>
<td>Significance</td>
<td>NS†</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

†Not significant, \(P > 0.05\).

Table 4.3  Comparison of orchardgrass mean frost rating (Frost), relative time to maturity (Maturity), reproductive tiller number, and stand survival (10 months) of cycles 0-3 polycrosses grown during the 2017-2018 season.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Frost</th>
<th>Maturity</th>
<th>Reproductive Tiller Number</th>
<th>Stand Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>---0-9--</td>
<td>---Julian Days---</td>
<td>-----Tillers plant(^{-1})------</td>
<td>---Percentage---</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0</td>
<td>3.33a†</td>
<td>83.0a</td>
<td>34.7b</td>
<td>27.0b</td>
</tr>
<tr>
<td>1</td>
<td>1.63b</td>
<td>79.6a</td>
<td>31.2b</td>
<td>21.9b</td>
</tr>
<tr>
<td>2</td>
<td>1.57b</td>
<td>79.1a</td>
<td>31.8b</td>
<td>54.7a</td>
</tr>
<tr>
<td>3</td>
<td>3.28a</td>
<td>65.1b</td>
<td>44.1a</td>
<td>56.2a</td>
</tr>
<tr>
<td>Significance</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
</tbody>
</table>

*** Significant at \(P < 0.001\).
† Within columns, different letters indicate significant differences.
Heritability Values

Calculation of realized heritability was conducted using cumulative germination under high temperature screening conditions during the 2016-2017 season (Table 4.4). Realized heritability comparing cycle 1 to cycle 0 was calculated as $h^2 = -0.197$. 

Figure 4.7 Images of each observed frost damage rating on orchardgrass during winter 2017-2018 on a scale of 1-9; 1 = no damage, 9 = plant death. From left to right, top to bottom, are examples of plants rated: 1, 2, 3, 4, 5, 6, 7, and 8. Rating 9 was not observed.
Comparison of cycle 2 to cycle 1 provided a calculated value of $h^2 = 0.345$. Total additive realized heritability for the 2016-2017 season was $h^2 = 0.148$. Calculated $h^2$ for the cycles of selection during the 2017-2018 season indicated cycle 1 with an $h^2 = -0.133$, cycle 2 with an $h^2 = 0.313$, and cycle 3 with an $h^2 = 0.273$. Total accumulation of additive gene action for the 2017-2018 season was $h^2 = 0.453$.

Table 4.4 Comparison of orchardgrass realized heritability ($h^2$) for germination at 40°C of cycle 1, cycle 2, and cycle 3 of selection compared to the base population (cycle 0). Significance is based upon germination testing, not heritability values.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>2016-2017 Realized Heritability</th>
<th>2017-2018 Realized Heritability</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>1</td>
<td>-0.197</td>
<td>-0.133</td>
</tr>
<tr>
<td>2</td>
<td>0.345</td>
<td>0.313</td>
</tr>
<tr>
<td>3</td>
<td>------</td>
<td>0.273</td>
</tr>
<tr>
<td>Total $h^2$</td>
<td>0.148</td>
<td>0.453</td>
</tr>
</tbody>
</table>

Significance

** Significant at $P < 0.01$

*** Significant at $P < 0.001$

Discussion

Improvement of Germination at 40°C and 20°C

Over three cycles of selection heat tolerance has been significantly improved in the advanced orchardgrass populations. The first cycle of selection, produced during the 2015-2016 season, was used only to advance the initial population and was not compared against cycle 0. Both the 40°C and 20°C germination tests during 2016 of seed from cycle 1 indicated that there may have been problems with seed viability; subsequent testing in
the high temperature (40/30°C) growth chamber resulted in germination between 4-5%. Germination in the control (20/15°C) growth chamber was also very poor (< 50%). Following the second cycle of selection, germination tests comparing cycle 2 to cycle 1 and cycle 0 further validated the poor quality of cycle 1 seed. The leading factor for this could be heavy stand loss of the population between the 2015-2016 season and the 2016-2017 season (only 5 plants survived to contributed pollen). Inbreeding depression was the likely reason for the poor germination that occurred for the 2016-2017 season. However, the poor germination of cycle 1 seed harvested from the initial 2015-2016 season of establishment cannot be explained by any collected data or observations; the population for cycle 1 consisted of 64 plants and seed was harvested at the proper time. It is possible the young age of cycle 1 mother plants (4 months) resulted in poor seed set (Lembicz et al., 2011), however we have not observed this phenomenon in the other polycrosses beyond this first year. Therefore, emphasis was on improvement of cycles 3 and 2 over cycle 0.

In the 2017 germination tests comparing cycles 0, 1, and 2, the most advanced population that season (cycle 2) exhibited significantly greater germination at 40°C. Mean cumulative germination of cycle 2 was 1.6 times that of cycle 0, 38.17% vs. 23.3%, respectively. However, cumulative germination of cycle 0 at 40°C was also substantially greater than expected. The germplasm for this work was sourced from orchardgrass plants growing for over four decades at the MAFES Prairie Research Unit in Prairie, MS. Additionally orchardgrass is a perennial species. The base germplasm had likely already been subjected to some form of natural selection for heat tolerance each summer over that extended period. This could account for the relatively large germination observed for
cycle 0 in the 2017 40°C germination tests. The greater presence of additive gene action prior to selection possibly allowed for a more rapid improvement of heat tolerance, thus accounting for 40% germination in cycle 2. At 20°C conditions, no differences were observed between cycles 2 and 0 beyond the first four days. This indicated that seed viability was not a factor germination of seed in these cycles.

The 2018 germination tests indicated that further improvement in selected germplasm; cycle 3 mean cumulative germination (82.67%) at 40°C was greater than cycle 2 (55.33%), cycle 1 (24%) and cycle 0 (37.34%). Most importantly, cycle 3 mean cumulative germination at 40°C reached values similar to the unselected germplasm (cycle 0) at 20°C (78.67%). This indicates that we are close to reaching the selection goal; germination at high temperatures over 22 days performed similar to germination under normal temperature conditions. However, the time required for germination at 40°C to reach similar performance as 20°C was substantially longer. Cycle 0 at 20°C exceeded 60% cumulative germination at day 8, while cycle 3 at 40°C did not exceed 60% germination until day 12. This suggests further cycles of selection should be focused on selecting seed that germinates within the first 8 days, and not selecting any individuals that germinate beyond this time-span. As with previous years, cycle 1 mean cumulative germination at 40°C was poor (< 25%). Poor performance during all three years, with germination at 40°C less than cycle 0 in both 2017 (Figure 4.3a) and 2018 (Figure 4.5a), further supports the assessment that this seed lot may have been improperly selected, or was contaminated with unselected orchardgrass pollen during its initial development. Cycle 1 seed was grown each year of the test, but was never reselected.
Lastly, seed lots tested in the 2018 germination tests at 20°C performed largely as expected. There was increased germination of cycle 3 (92.17%), cycle 2 (84.94%), and cycle 1 (88.67%) over the unselected cycle 0 population (78.67%). This indicates that seed viability under normal germination conditions was not a factor for these populations. All populations had similar germination curves at this temperature regime; they plateaued after reaching days 8-10 of the test. Cycle 3 germination at 20°C was about 10% greater than its germination at 40°C. This suggests that we may have reached close to the maximum germination potential under high temperature stress in this germplasm. Therefore, further cycles of selection should focus on improving germination velocity.

An unintended consequence of selection for improved germination at high temperature was the acceleration of VOG$_8$. This effect has been observed in similar work involving selecting seed at germination; inevitably the seed that are selected also are the seed that germinate first (Anderson et al., 2009; Anderson et al., 2011; Springer, 2017). Germination tests in 2016 indicated cycle 1 VOG$_8$ was very poor at both 40°C (0.37x day$^{-1}$) and 20°C (5.6x day$^{-1}$). Testing in 2017 indicated VOG$_8$ was significantly improved in cycle 2 (2.4x day$^{-1}$) over cycle 0 (0.68x day$^{-1}$) under 40°C conditions. However, no change in VOG$_8$ was present between cycle 2 and cycle 0 under 20°C conditions (21.0x day$^{-1}$ and 18.3x day$^{-1}$, respectively). Such a lack of difference in VOG$_8$ at 20°C indicates the populations differ purely in their ability to germinate at high temperatures, rather than under normal conditions. This also suggests that improvement of both VOG$_8$ and cumulative germination are essential to a successful heat tolerant cultivar. The 2018 VOG$_8$ tests indicated a very similar increase in rate of VOG$_8$ from cycle 2 to
cycle 3 (3.28 times increase) as was observed between cycle 0 and cycle 2 (a 3.85 times increase) in 2017. This further indicates that we have room to improve the germplasm’s VOGs. This would make the germplasm more competitive with seed grown under normal temperature conditions, and give it the unique advantage of being able to germinate rapidly at high temperature following imbibition.

These germination tests are evidence of the gains from selection that can occur when conducting RPS with an obligately outcrossed species to select for a quantitative trait. To achieve these gains, the environment must be kept uniform and stable, in this case, through use of growth chambers. The minimization of environmental variance in the genotype x environment interaction meant that selections were made without being confounded by environment. As a result, we have made significant improvements to the heat tolerance of orchardgrass germplasm as measured by germination at high temperatures.

The improvement of heat tolerance in the selected population putatively allows for the long-term adaptation of this germplasm to locations farther south than where orchardgrass has been historically cultivated. The main advantages of the species of other common cool-season forages already grown in the south are: a.) that it is a perennial and b.) while orchardgrass has an endophyte (*Epichloë typhina*) (Clay, 1996; Rozpadak et al., 2015). The former is in direct contrast to annual ryegrass. While annual ryegrass is high in nutritive value (Evers et al., 1997), the issue of having to plant it every season is considerable annual cost to producers. Conversely, the selected orchardgrass could potentially (germinate and) persist through extreme heat and survive for multiple growing seasons. Harboring a non-toxic endophyte contrasts with ‘Kentucky 31’, the most
commonly planted tall fescue cultivar in the South and the only other perennial cool-season forage grass common in the South (Baker and Jung, 1968; Nie and Norton, 2009). Toxic endophyte (*Epichloë coenophiala*) infection is a relatively common occurrence in tall fescue stands (Schmidt and Osborn, 1993). Because orchardgrass does not exhibit symbiosis with a toxic endophyte, there is little inherent risk to animals consuming it.

**Chi-Square Tests for Significance**

The chi-square analysis provided results that were largely similar to the ANOVA-based analysis. Examining the data from the 2017 germination tests, chi-square analysis detected a difference between cycle 2 and cycle 1. However, no comparison could be made between cycle 2 and cycle 0, due to the lack of an expected value for cycle 0 germination at 40°C from the 2016 test year. This is a major limitation of chi-square analysis, in that it requires predicted values. In traits governed by Mendelian inheritance, predictions can be made on the expected number of individuals expressing a trait in populations based upon observed allelic frequencies and phenotypes. However, with quantitative traits like heat tolerance such predictions are tentative due to multiple copies of causative alleles at different loci and the compounding effect of environment on each.

In 2018, germination at 40°C of cycle 3 was significantly greater than cycle 0 and cycle 1. However, chi-square tests are more conservative than ANOVA (Clewer and Scarisbrick, 2001), and thus we did not detect a difference between cycle 3 and cycle 2, while ANOVA tests indicated a difference was present. Therefore, the chi-square test is more useful when large differences exist between generations, allowing for the critical value to be exceeded by the calculated $\chi^2$ value.
Impact of Other Phenotypic Traits

The other phenotypic traits assessed in this work are all vital to the economic importance of heat tolerant orchardgrass germplasm. These traits include: time to maturity, reproductive tiller number, perennation, and frost tolerance. These traits can either directly or indirectly affect many aspects of forage nutritive value, seed production, and stand survival that are critical to a successful forage grass cultivar.

Selection for high temperature germination did not affect maturity in the advanced cycles of selection during 2016-2017 (cycles 1 and 2). However, in the second year of testing (2017-2018) we did observe significantly earlier time to maturity in cycle 3 (65.1 days) compared to cycles 2 (79.1 days), cycle 1 (79.6 days), and cycle 0 (83.0 days). This difference was likely due to the short-long day regulation of orchardgrass flowering (Vince-Prue, 1975). Rather than induction, initiation, and development of flowers occurring under strictly short day or long day conditions, orchardgrass relies a combination of the two. Induction and initiation of flowering occurs under short day conditions, and development occurs under long day conditions. The screening protocol used photoperiods that would cause induction to occur. Plants selected for cycle 3 were kept in a growth chamber at an even split of 12/12 hrs light/darkness for the first several weeks post-germination, and then were placed in the greenhouse in mid-August 2018 where conditions were 13:20/11:40 hrs light/darkness. Compared to previous years, these selections took place about 2 months earlier to attempt fall planting in hot environmental conditions. Orchardgrass has a minimum photoperiod requirement of 12/12 hrs light/darkness to induce and initiate flowering (Salisbury and Ross, 1992). Thus, we unintentionally induced and initiated flowering in the growth chamber. Then,
development and maturation occurred more rapidly the following spring, as vernalization accelerates the process. This could have been avoided by either a.) making selections after the fall equinox so that the plants would not experience long day conditions, or b.) keeping the growth chamber under long day conditions to prevent the inducement or initiation of flowering until the plants were transplanted in the fall. Cycles 0, 1, and 2 were not exposed to these conditions in the growth chamber during the 2017-2018 season; their times to maturity did not differ.

Therefore, the observed earlier maturity of cycle 3 was likely due to screening methodology rather than true genotypic difference. An earlier time to maturity would negatively impact forage availability for prime grazing. Orchardgrass already joints earlier than most other cool-season grass species (Kunelius, 1990; Van Santen and Sleper, 1996), thus later maturation is crucial to a successful cultivar. These results also imply that the additive gene action (measured as realized heritability) we have been increasing in the selected populations has not altered time to maturity. This also holds to Hardy-Weinberg Theory, wherein alleles for unselected traits remained in equilibrium.

Reproductive tiller number serves as an assessment of both relative seed production capability (Sukchain and Sidu, 1992; Lopes and Franke, 2011; Abel et al., 2017), and as an assessment of forage nutritive value (Nelson and Moser, 1994; Mayland et al., 2000; Temu et al., 2014). If more reproductive tillers are present seed production capacity is increased, but nutritive value will be less due to the increased secondary cell wall components that occur in jointed stems. In the 2016-2017 season, reproductive tiller numbers did not significantly differ amongst the three cycles of selection tested (0, 1, and 2). However, in the 2017-2018 season, cycle 3 mean tiller number (44.1 tillers plant⁻¹)
was greater than the previous cycles (< 35 tillers plant⁻¹). Again, this effect is likely linked to the short-long day nature of the species. Earlier planting combined with growth chamber photoperiod induced more seed heads in cycle 3 plants than the antecedent populations. The observed increase in reproductive tillers was probably due to environmental effects, rather than any genotypic alterations caused by selecting for heat tolerance. This is supported by the lack of difference observed in the previous cycles of selection (0, 1, and 2).

Stand survival is one of the most crucial requirements of a successful heat tolerant orchardgrass cultivar for the Deep South. Unselected populations of the species are prone to heavy stand loss under high temperature (Drake et al., 1963) and water stress conditions (Orloff et al., 2016). However, orchardgrass is reported to be substantially less prone to stand loss than other perennial cool-season forage grasses such as perennial ryegrass under similar heat stress (Nie and Norton, 2009). Shaimi et al. (2008) report orchardgrass to be more summer dormant. Several Moroccan ecotypes have been used as the basis for improving drought stress and stand persistence in high temperature environments. Orchardgrass seed is expensive (US$100 - US$150 per 22.6 kg bag), with current market trends ranging in cost from as much as 1.5-2 times greater than annual ryegrass seed (US$40 - US$70 per 22.6 kg bag). Thus, for orchardgrass to be economically viable to producers in the Deep South, it must behave as a true perennial and persist.

In the first year (2016-2017) of comparison between cycle 2 and cycle 0 we did not observe significant improvements to stand survival; however, the \( P \)-value was 0.0625. Following a further cycle of selection, 10-month survival during the 2017-2018
season indicated that cycle 3 and cycle 2 populations exceeded 50% stand survival, while
cycle 1 and cycle 0 stand survival was lower ($P < 0.001$), ranging from 21-27%. While
stand survival cannot be entirely attributed to improved heat tolerance (due to potential
variation in rainfall and pathogen load among cycles), high temperature stress is a major
factor in failing orchardgrass stands (Drake et al., 1963). Stands were not irrigated,
sprayed, or provided prophylactic care post-seed harvest. Rather, they were left to endure
normal Mississippi environmental conditions (daily temperature highs frequently
exceeding 32°C, with high relative humidity). While isolated by distances of 275-450 m,
all cycles were grown in relatively close proximity to each other. Therefore, we can rule
out major differences in rainfall that might have contributed to increased stand survival of
cycles 2 and 3. Given cycle 0 and cycle 2 stand survival was similar to that of the 2016-
2017 populations (25% and 41.1%, respectively) we can conclude selections for high
temperature germination successfully increased stand survival of orchardgrass after 10
months in high temperature field conditions of Mississippi.

Finally, frost ratings conducted during the winter of 2017-2018 provided an
assessment of potential loss of cold hardiness due to selection for heat tolerance. If heat
tolerant germplasm was less cold tolerant than the base population a sudden winter
freezing event which occur in the South could wipe out a stand. Frost ratings indicated no
significant difference existed between cycle 3 ($\bar{x} = 3.28$) and cycle 0 ($\bar{x} = 3.33$).
However, cycle 3 and cycle 0 were significantly more frost damaged than the
intermediary populations (cycle 2 $\bar{x} = 1.57$; cycle 1 $\bar{x} = 1.63$). This was likely a factor of
microclimate as cycles 0 and 3 were located in fields of slightly lower elevation which
were more prone to frost than the locations of cycles 1 and 2. If there were a true genetic
effect, cycle 2 and cycle 3 would have been more frost damaged than cycles 0 and 1. The extreme cold of January 2018 was unexpected, and a single event. Further testing under more controlled cold temperatures will be necessary to confirm maintenance of cold hardiness in the advanced germplasm.

**Heritability of Heat Tolerance**

Because heat tolerance is a quantitative trait, heritability values were expected to be small in early cycles of selection (Marshall, 1982). However, realized heritability calculated comparing cycle 1 to cycle 0 was negative ($h^2 = -0.197$) (Table 4.4). This indicates an error in selection, lack of stringent selection, or contamination of the seed lot by unselected orchardgrass. This issue resolved in the next selection cycle when realized heritability of cycle 2 was $h^2 = 0.345$. This large heritability value was likely due to a more tightly monitored selection and in-field isolation in cycle 2 selections. However, overall accumulation of additive gene action was negatively impacted by the initial negative value observed from cycle 0 to cycle 1.

Realized heritability values for the 2017-2018 seed crop indicated large gains were achieved. Similar to the 2016-2017 testing, cycle 1 realized heritability was negative, $h^2 = -0.133$. Cycle 2 $h^2 = 0.313$, and indicated a substantial gain of desirable alleles between cycle 1 and cycle 2. Cycle 3 $h^2 = 0.273$, which indicated a further gain of additive gene action from cycle 2 to cycle 3. However, gain in this generation was slightly less than from cycle 1 to cycle 2. This decrease in $h^2$ suggests we may have reached the point of diminishing returns with continued selection for germination at high temperatures. Stagnation or decline in $h^2$ value during future selection will determine if further selection for high temperature germination is worthwhile, or if selection strictly
for increased VOG₈ at high temperatures should be the new focus of the germplasm. Current total additive gene action effects are expressed by an \( h^2 \) of 0.453. This value indicates an excellent response to selection, but less potential room for increased heat tolerance based solely on additivity.

**Conclusions**

Following three cycles of selection to increase germination at 40°C, results indicated a successful improvement for that trait in orchardgrass germplasm. The most advanced population (cycle 3) exhibited mean cumulative germination (> 80%) at 40°C by 22-days testing. This value is on par with mean cumulative germination of parental germplasm (cycle 0) at 20°C. However, cycle 3 germination at 40°C did not reach the peak value until day 12; germination at 20°C peaked at day 8. Additionally, \( h^2 \) data suggests continued high temperature selection will be met with diminishing returns. Further improvement of the germplasm should focus on enhancing VOG₈.

Additionally, observation of these populations has provided insight on the nature of important traits such as time to maturity, reproductive tiller number, cold tolerance, and stand survival. Based upon these observations, differences in maturity and reproductive tiller number among germplasm appears to be governed largely by environmental factors, most notably, date of transplanting/establishment in the field. Later time to maturity, and increased reproductive tiller number occurred in cycles that were field planted earlier in the fall. These differences resulted from exposure to a different photoperiod, closer to 12/12 hrs light/darkness. Earlier establishment enhanced the production of a greater number of reproductive tillers. Most importantly, stand
survival has putatively increased due to selection. This is the most significant aspect of this work, as the lack of persistence of orchardgrass in the Deep and Mid-South is a major factor in its limited acreage.

The final work conducted on this project was to make selections to advance cycle 3 to cycle 4, with an emphasis on improved VOGs. Those selections will be transplanted to the field in September 2018. Further germination testing and seed increases will be conducted in 2019 for variety testing and cultivar release.
CHAPTER V
SCREENING AN ANNUAL RYEGRASS BASE POPULATION FOR A POTENTIAL FREEZING TOLERANCE TRAIT

Introduction

Annual ryegrass is one of the most widespread cool-season forage grasses used for animal production in the Deep South. Its large yields and ability to be interseeded into established grass or legume swards make it ideal for forage in areas that are ill-suited to production of other cool-season grasses (Evers et al., 1997). However, annual ryegrass, despite being a cool-season grass, is susceptible to cold damage because it does not become dormant during winter months (Nelson et al., 1997). While the Deep South does not experience consistent cold temperatures, there is still risk of temperatures below -12°C occurring during intermittent cold fronts. These temperatures can cause substantial damage to annual ryegrass pasture stands (Evers et al., 1997). Therefore, the need to develop a cold-tolerant annual ryegrass cultivar for forage production is of great importance.

Marshall annual ryegrass was developed from 30 years of natural selection for cold-tolerance, but has been on the market since the early 1980s (Arnold et al., 1981). There is a strong likelihood that Marshall possibly was not as cold-tolerant as thought, due to being bred through natural selection where temperature extremes are inconsistent. Additionally, the mean minimum have declined in northern Mississippi over the past 30
years since Marshall’s release (NOAA, 2018). Also, “cold-tolerance” is a relative term, and what was considered cold tolerant 30 years ago may not be hardy enough today. With the growing issue of climate change, there is an expanding risk of temperature extremes, both greater and lesser than normal in areas where these temperatures are not commonly associated. In the northeast region of Mississippi, cold weather is brought on suddenly through cold fronts. A single instance of this could potentially be enough to suddenly cause temperatures drops that could kill an entire stand of young ryegrass. Thus, there is a risk of temperatures falling below -12°C in areas where these conditions have not frequently occurred previously. Therefore, development of new cold-tolerant cultivars will be advantageous to forage production in the South as they might confer more consistent winter growth, equating to greater yield or earlier grazing/harvest in spring.

Objectives

The aim of this work was to develop a novel freezing tolerant annual ryegrass cultivar for use in the northern areas of Mississippi, Alabama, and Georgia, and southern areas of Tennessee, with possible expansion father north. Recurrent phenotypic selection (RPS) was used to identify individuals able to survive freezing temperatures as seedlings. Survivors of this material served as germplasm for subsequent cycles of selection for cultivar development.

Materials and Methods

Marshall annual ryegrass served as the source of germplasm (cycle 0). Fifteen flats (dimensions: 52.5 x 36.5 x 7.62 cm) were mass seeded with Marshall on January 19, 2016, with roughly 2,250 seed flat¹ (34,000 total) being screened. Media used was a 1:1:2 (v:v:v)
ratio of coarse pine bark, potting soil (Sunshine #1) and fine play sand. Freezing tolerance screening occurred at Mississippi State’s R. R. Foil Plant Science Research Center 33°28’9.03”, -88°47’3.69”. Screening was conducted by taking each individual flat of seedlings at the three leaf stage (3 weeks after germination) and placing them in a chest freezer (-17.8°C, Frigidaire, Charlotte, NC) for 9 hours. Flats were watered prior freezing to simulate rain that occurs on the leading edge of cold fronts. Due to the effect of photoperiod on expression of cold tolerance screenings were conducted in a narrow window between late November and late February each winter. Initial screenings (cycle 0) occurred from February 9 to February 23, 2016. After removal from the freezer, flats were returned to the greenhouse at 18°C with ambient photoperiod (10.5-11 hrs light day⁻¹).

Dead leaf material was removed after three days. Surviving plants were identified by their ability to regrow following the freezing event. Escapes (post-freezing germination) were identified as seedlings with single leaf blades and no visible freezing damage, and were removed. Twenty-six selected individuals were advanced to cycle 1 of selection for freezing tolerance in February 2016.

Selected plants were moved outside in late March 2016. Plants were maintained in original flats augmented with additional flats of soil underneath to avoid transplanting stress. To avoid pollen contamination, these flats of plants were placed on maintained lawn. Seed harvest occurred between June 8, 2016, and June 12, 2016. All seed was bulked to form the germplasm for cycle 1 of selection.

Screenings of cycle 1 commenced in late fall and early winter 2016 for advancement to cycle 2. Screening was conducted twice, once in early December, 2016, and again in mid-January 2017. Each screening event used 14 flats. One thousand cycle 1
seed were sown in three rows on one end of the flat, and 1600 cycle 0 seed were mass sown on the remaining area of the flat to differentiate genotypes within each flat. Cycle 0 was screened alongside each successive cycle of selection to compare frequency of survivors and evaluate any gains from selection. Eleven survivors from cycle 1 were advanced to cycle 2 of selection. These plants were transplanted in late March 2017 into a polycross nursery lined with silage cover (plastic, 5 mm, white, low-density, polyethylene) to reduce weed pressure at the Mississippi State H. H. Leveck Animal Research Center 33°26’18.87”, -88°47’51.62”. The area was shielded with silt fencing to prevent external pollen contamination. Plants were allowed to mature, and seed was harvested in mid-June 2017.

Screenings for cycle 3 of selection occurred in an identical manner to those of cycle 2, except: a.) 10 flats were used for each screening instead of 14, and b.) the second screening used only 800 seed of cycle 2 per flat, rather than 1000. This was due to small seed yield from the previous season. Three selections from cycle 2 to cycle 3 were made during the first screening (December, 2017), while 12 selections were made from the second screening (February, 2018). Selections were maintained in flats in the greenhouse until transplanted into another polycross nursery. Plants were allowed to mature, and seed was harvested in mid-June 2018.

Statistical analysis

Data from both freezing tolerance screenings conducted on seedlings in winters of 2016-2017 and 2017-2018 was analyzed as a randomized complete block design using the PROC GLM command within SAS 9.4. Each flat of plants consisting of mass seeded cycle 0 seed, and two rows of either cycle 1 or cycle 2 seed was considered a replication
for this study. The following statistical model was used to analyze the datasets from both years of testing:

\[ Y_{ijk} = \mu + R_i + C_j + \xi_{k(ij)} \]  

(Eq. 5.1)

in which \( Y \) = the response variable, \( \mu \) = mean value, \( R \) = replication, \( C \) = cycle of selection, and \( \xi \) = experimental error.

**Results**

The screening process from the first cycle of selection in January of 2016 yielded a total of 26 plants from a screened population of about 34,000 seedlings. This calculates to a selection pressure of 0.076%. The following season, progeny tests compared cycle 1 to the base germplasm. Cycle 1 (0.076%) had a greater \( P = 0.033 \) rate of freezing survival than did cycle 0 (0.028%) (Figure 5.1). The most recent progeny tests compared cycle 2 seedlings from selections grown during the 2017 season to cycle 0. We observed an increase \( P = 0.04 \) in cycle 2 freezing survival (0.125%) over cycle 0 survival (0.025%) (Figure 5.2).
Figure 5.1  Comparison between cycle 0 and cycle 1 mean freezing survival (-17.8°C), winter 2017.
†Different letters indicate significant differences at P = 0.033.

Figure 5.2  Comparison between cycle 0 and cycle 2 mean freezing survival (-17.8°C), winter 2018.
†Different letters indicate significant differences at P = 0.04.
Discussion

The initial screenings conducted in January 2016 to establish cycle 1 provided evidence that much of the cycle 0 (Marshall) germplasm lacked the ability to withstand -17°C freezing events (survival percentage = 0.028%). Thousands of seedlings were killed by the freezing protocols, i.e. no regrowth was observed. While the intensity (both in temperature and duration) was substantially greater than what seedlings would be subjected to in nature, such intensive screening protocol is essential to identify individuals with adequate cold-tolerance in the germplasm. While very few in number, the identification of selected freezing survivors provided evidence that the base germplasm possessed enough genetic variability to withstand intensive freezing events. Through recurrent selection from cycle 0 to cycle 1, and from cycle 0 to cycle 2, we observed significant increases to -17°C freezing survival at the 3 week stage (0.028% vs. 0.076% and 0.025% vs 0.125%, respectively). However, due to the very small initial allelic frequency expression of freezing tolerance in cycle 0 germplasm (< 0.03%) gains from selection were relatively small. The maximum survival percentage observed was 0.125% for cycle 2 in 2018.

Similar work to improve cold hardiness has been conducted in related species. Findings by Iraba et al. (2013) with perennial ryegrass (L. perenne L.) support the observations that recurrent selection for cold hardiness was able to improve the trait. Through recurrent selection, they were able to generate populations that withstood much colder temperatures that we tested, as low as -30°C. However, that work implemented a step-wise acclimation protocol, wherein plants were gradually exposed to freezing temperatures. This was contrary to this experiment’s methodology, in which plants were
not acclimated to cooler temperatures prior to the actual freezing event. Characterization of creeping bentgrass (*Agrostis stolonifera* L.) populations for freezing tolerance supports the notion that acclimation greatly increases the temperature range at which plants can survive (Espevig et al., 2011). From this work, it is likely that small survival percentages were a product of no acclimation to cold prior to freezing. While reduced photoperiod does play a role in expression of cold tolerance, studies testing photoperiodic effects have implemented some form of step-wise exposure to freezing temperatures (Bertrand et al., 2017). However, this strategy does not reflect the usual pattern of freezing conditions in north-central Mississippi; frosts often occur as part of cold fronts, where sudden decline in temperature allows little time for plants to acclimate to cold temperatures. Additionally, these studies did not provide survival percentages, rather they reported the temperature at which 50% of the stand is killed (LT$_{50}$), making it more difficult to directly compare survival results.

Previous freezing tolerance studies conducted with cool-season grasses differ from this work in two major ways: i.) species tested were perennial, and ii.) accessions tested were turf-type ecotypes. Annual ryegrass does not enter dormancy during cold winter months (Nelson et al., 1997). This factor predisposes the species to a greater risk of damage to freezing conditions. Dionne et al. (2010) found results in annual bluegrass (*Poa annua* L.) supporting the notion that annuals are more susceptible to freezing than perennials. In tests comparing relative freezing tolerance between 42 ecotypes of turf-type annual bluegrass, perennial ecotypes performed better than their annual counterparts. Conversely, perennial ryegrass and creeping bentgrass (also perennial) reduce their growth during winter months, thus becoming less susceptible to freeze damage (Jung,
Turfgrass ecotypes are also less susceptible to frost damage, due to less biomass material for potential freezing damage (Zhang et al., 2006). These aspects likely impacted success rate of selecting for freezing tolerance in annual ryegrass.

The significant increase to freezing tolerance provides evidence of the ability to use RPS to select for the trait in this outcrossing species. Greater rates of expression of freezing tolerance are likely through successive generations. However, when such a small portion of the base germplasm possesses the genetic variability to regrow following intensive freezing events, it could take many generations to achieve a viable population. The solution to this is to screen a much larger population of seedlings, potentially allowing us to generate more selections in the same time period.

Aside from initial low expression of freezing tolerance, the major problem we observed with this work was limited numbers of selected individuals to constitute a population large enough for seed increases from one generation to the next. Each year, we were only able to acquire between 15-30 individuals. This creates two problems: i.) lack of sufficient genetic variability for reliable cross pollinating in an obligately outcrossed species, and ii.) fewer seed produced. The issue of seed production was the limiting factor in the advancement of freezing tolerance beyond the base germplasm. With freezing survival being less than 0.1% in all but the most recent cycle of selection, screening and selection without exhausting seed reserves was extremely difficult. This factor is compounded with the narrow window available for screening (November-February) due to increasing photoperiod effects on the loss of freezing tolerance. In the 2018 screenings, 15 individuals were selected from roughly 16,000 seedlings. In order to
acquire the minimum desired population for adequate seed production (50 plants) we would have to screen close to 60,000 seedlings. However, we were unable to screen this quantity, as we used about ¾ of the seed lot in screening 16,000 seedlings.

A possible alternative to selection through artificial freezing events would be to utilize large-scale field screening in clines farther north than the testing location. Mass planting of large areas of annual ryegrass would allow for a large screening population many times larger than what artificial screening procedure could accommodate. This would overcome the problem of small number of selections constituting the next generation. Secondly, a location north of the transition zone would allow for more frequent and longer duration freezing events to occur than experienced in Mississippi. This would overcome a shortcoming in the development of Marshall. It was selected for cold tolerance through natural selection in Mississippi, but minimum temperatures rarely fell below the critical threshold (-12°C) necessary for plant death to occur (NOAA, 2016). While we cannot guarantee the occurrence or severity of freezing events in a natural environment, the ability to screen large populations could potentially offset this issue. However, the large environmental variation could slow progress and lower heritability estimates. Larger freezers and greater greenhouse space would be necessary to screen the requisite amount of individuals under current methodology, while still maintaining less environmental variability.

**Conclusions**

Through three cycles of selection for improvement of freezing tolerance in annual ryegrass using RPS, we have observed a significant increase in the trait. Additionally, expression of the trait in the base population appears to be nearly identical between
testing years. This is promising for the testing methodology, which differed in a number of crucial ways from other freezing/cold tolerance tests. Primarily, we did not subject seedlings to a gradual or step-wise cold temperature acclimation regime prior to freezing. This practice is common amongst many other studies examining freezing tolerance, and allows the plant to gradually adapt to the change in temperature. However, we were attempting to isolate individuals in the Marshall germplasm that could withstand intensive freezing with no acclimation period. In this manner, we more reliably isolated freezing tolerance alleles. This methodology put heavy selection pressure on the population, resulting in a small number of selected individuals constituting the population each generation. While we observed improvements at each cycle of selection, the limited seed available for testing limited the ability to generate large populations for outcrossing. This aspect will need to be addressed in future breeding work, either via seed increases at each generation, or through screening larger populations.
CHAPTER VI
ISOLATION AND INITIAL SCREENING TO GENERATE GRAY LEAF SPOT
(PYRICULARIA ORYZAE) RESISTANT ANNUAL RYEGRASS

Introduction

Annual ryegrass (Lolium multiflorum L.) is susceptible to the disease gray leaf spot (also known as ryegrass blast), caused by the fungal pathogen Pyricularia grisea Cke. [Sacc.] (Bain et al., 1972). Pyricularia grisea was first observed as a pathogen of annual ryegrass in the early 1970’s by researchers in Mississippi and Louisiana (Bain, et al., 1972; Carver et al., 1972). The pathogen is a deuteromycete with its teleomorph (sexual stage) Magnaporthe grisea (T.T. Herbert) M.E. Barr is not commonly found in nature (Agrios, 2005, Smiley et al., 2005). Therefore, infection is incited by the anamorph (asexual stage), P. grisea via conidia. This pathogen can infect a wide range of both cool and warm-season turf and forage grasses including those of the genera Lolium, Schedonorus, Festuca, Stenotaphrum, Cynodon, and Paspalum. It also can infect rice (Oryza sativa L.) and cereal grains such as wheat (Triticum aestivum L.) (Smiley et al., 2005). In the first season of occurrence on annual ryegrass, an epidemic occurred, resulting in a report that branded the disease with the layman term ‘ryegrass blast’ (Bain et al., 1972; Carver et al., 1972).

P. grisea infects the host plant by entering through stomata or direct penetration through the leaf tissue via appressoria, and therefore does not require a fresh wound to
infect (Harmon and Latin, 2003). Symptoms of infection include brown or gray water-soaked spots on leaves and stems, which develop into larger lesions with gray or tan-gray centers and brown or purple margins. Older lesions also develop chlorotic borders (Trevathan et al., 1994). A common symptom known as “fish-hooking” may also be observed if the pathogen affects the apical portion of the blade (Harmon and Latin, 2003). As the disease progresses it can result in thick mats of slimy, necrotic plant tissue. Such an infection is particularly dangerous to annual ryegrass because it can rapidly spread on seedlings and overtake the sward (Nelson et al., 1997). The pathogen proliferates under prolonged periods of leaf wetness and temperatures between 20°C and 28°C, but is capable of infection at greater temperatures. The original source of the outbreak of blast on ryegrass was never confirmed, but both Bain et al., (1972) and Carver et al., (1972) speculate that spores of *P. grisea* were carried to Mississippi and Louisiana by the winds of a hurricane. Because the conditions for infection are optimal when the temperature is between 20°C and 28°C, disease outbreaks are most prevalent in August and September in Mississippi.

Work is currently being conducted to establish an annual ryegrass cultivar that germinates and can be established in late summer (August) or early fall (September) in Mississippi. However, this would potentially expose the cultivar to suitable environmental conditions and inoculum from *P. grisea*. Due to these risks, resistance breeding work is extremely important in early establishment of annual ryegrass cultivars.
Objectives

The purpose of this research was to successfully acquire isolates of *P. grisea* for use in inoculation and screening of annual ryegrass germplasm for potential resistance. We also wanted to use these isolates to conduct initial screenings of ryegrass germplasm for resistance to *P. grisea*. Koch’s postulates served as the basis for this process. Therefore, the objectives were to: i.) Identify and isolate *P. grisea* from an infected annual ryegrass plant, ii.) Successfully culture and induce sporulation of isolates on nutrient media, iii.) Develop a conidial suspension for inoculation of healthy grasses, and iv.) Inoculate healthy plants to reproduce symptoms of gray leaf spot, and re-isolate *P. grisea* from infected plant tissue. Beyond Koch’s postulates, the final objective was to identify resistant individuals in annual ryegrass germplasm through mass screening.

Materials and Methods

Initial *Pyricularia* spp. samples were obtained in late August 2017 from annual ryegrass plants growing in the greenhouse. This incidence of disease was serendipitous. Ten individual leaf samples with large lesions were collected and placed into a clear Ziploc® bag with a moist paper towel. Samples were transferred to Petri plate lined with moist filter paper and incubated in the dark at 30°C for 48 hours to induce sporulation (Figure 6.1). Using methods adapted from Harmon and Latin (2003), each of the ten leaves were cut into four subsamples, each with a single lesion (Figure 6.2). Each subsample was then affixed to the lid of a Petri dish (15.25 x 3cm) using double-sided tape. When the lid was placed onto the dish spores diffused and germinated on V8 agar (V8A) [10 % V8A (1.5%) containing 0.03% CaCO₃]. A total of 40 leaf subsamples were plated in this manner. Lids containing sporulating leaf samples were transferred to fresh
V8A every 24 hours for three days. Cultures grew under constant fluorescent light at 20°C for three to five days until individual colonies were visible. Individual colonies were then transferred to fresh media. Isolates showing the most profuse growth were again transferred to fresh V8A and placed in an incubation chamber [constant 25°C, 12/12 hrs light(ultraviolet & fluorescent)/dark] for 3 weeks to induce sporulation (Figure 6.3). Three isolates that produced large amounts of conidia were selected, with four replications of each isolate.

During this incubation time, four flats of 32 plants (128 total plants) of the annual ryegrass cultivar, Marshall, were grown and transferred to the greenhouse to perform the final steps of Koch’s postulates: inoculation of healthy plants, reproduce symptoms of the disease, and pathogen re-isolation. This number of plants (128) was replicated for each successive inoculation and resistance screening (five screenings, 640 total plants screened).

On 8 September 2017 six isolates of *P. grisea* were removed from the incubation chamber and conidia suspensions for each were obtained. The desired conidial suspension for inoculating annual ryegrass was $2.0 \times 10^5$ conidia mL$^{-1}$ (Moss and Trevathan, 1987). With the use of a hemacytometer, conidial suspensions were calculated using the following equation:

\[
(Mean \text{ conidia number})(2500)(\text{Dilution factor}) \quad (\text{Eq. 6.1})
\]

Where dilution factor was the total volume from which the sample was taken. The outer four 4x4 squares of the hemacytometer were counted and summed to obtain a total count. This was repeated 3 times to obtain mean conidia count for the combined replicates. To obtain a concentration of $2.0 \times 10^5$ conidia mL$^{-1}$, the following equation was used:
In this case, desired concentration was $2.0 \times 10^5$ conidia mL$^{-1}$, and the current concentration was $2.0475 \times 10^6$ conidia mL$^{-1}$. It was determined that 0.0977 µL of conidia solution added to 0.9023 µL of 0.1x (2.4 g L$^{-1}$) PD (potato dextrose) broth was needed to make a $2.0 \times 10^5$ conidia mL$^{-1}$ solution. A final spray volume of 30 mL was needed to apply this $2.0 \times 10^5$ conidia mL$^{-1}$ solution, thus 0.0977 µL of solution and 0.9023 µL of PD broth were multiplied by 30 mL to get a ratio of 2.931 mL solution added to 27.069 mL of PD broth. This solution was then applied to the 128 Marshall annual ryegrass plants using a CO$\text{₂}$ pressurized spray tank at 20 PSI with an XR teejet 8002-VS nozzle.

Plants were then placed in the greenhouse (ambient temperature 25-35°C) in a mist chamber that used a humidifier to augment humidity. Symptoms of gray leaf spot were observed within 72 hours. Disease was allowed to progress an additional 24 hours after inoculation, at which point leaf samples showing characteristic gray leaf spot lesions were taken from infected plants. These samples were placed on moist filter paper in a Petri dish and incubated at 25°C for 24 hours to induce sporulation, similar to the initial isolation protocol. Samples were prepared in an identical manner as described for the initial isolation. Single colony isolates were transferred to fresh V8A and allowed to grow until sporulation occurred for identification.

Confirmation of *Pyricularia* spp. was then conducted. First, *Pyricularia* spp. isolates were grown in pure culture. Then, genomic DNA extraction was conducted from fresh mycelium using a Fungi/Yeast Genomic DNA Isolation Kit (Norgen Biotek Corp., Thorold, ON, Canada). Polymerase chain reaction (PCR) was performed using the
ITS1/ITS4 primer set to amplify the internal transcribed spacer (ITS) region of the ribosomal nucleic acid (rRNA) genes (White et al., 1990). Amplicons were sequenced at Eurofins Genomics LLC, Louisville, KY. The resultant ITS sequences were then compared in the National Center for Biotechnology Information (NCBI) BLASTN database for comparison and species identification.

To prepare samples for long-term storage, transfers of re-isolated *Pyricularia* spp. were placed into a Petri dish on a piece of sterile fiberglass filter paper, on 3.9% potato dextrose agar (PDA). Cultures colonized the filter paper for two weeks under constant fluorescent light at 20°C. Filter paper pieces colonized with mycelium of each isolate were cut into small triangular pieces and placed in plastic bags for long-term storage at 0°C.

Screening of 128 inoculated ryegrass seedlings was conducted four times in addition to the initial inoculation described (five total inoculations). The first three inoculations (September, October, and November 2017) occurred in a greenhouse (Mississippi State University R. R. Foil Plant Science Research Center 33°28’9.03”, -88°47’3.69”). A humidity chamber was constructed, using fine cheesecloth and clear plastic to retain moisture, generated by two humidifiers running 8 hours day⁻¹. This created an environment at 100% humidity. At each screening Marshall seedlings were at the three to four-week growth stage. Each population of 128 seedlings was screened post-inoculation for 7-10 days to observe plants displaying no incidence of disease. The final two screenings (December 2017, and March 2018) were conducted in growth chambers [29/25°C, 12/12 hr, light/(fluorescent)/darkness] due to unsuitable ambient greenhouse conditions.
Figure 6.1  Initial Identification of gray leaf spot symptoms (lesions) on annual ryegrass (August, 2017).

Figure 6.2  Symptoms and signs of *Pyricularia* spp. on infected ryegrass leaf. Conidia on conidiophores
Results

Koch’s postulates were successfully achieved. The pathogen, *Pyricularia* spp., was: i.) Identified on an infected plant showing symptoms and signs of infection; ii.) Isolated from the infected plant and grown in pure culture; iii.) Used to inoculate a healthy annual ryegrass plant; and iv.) Re-isolated from the gray leaf spot symptomatic plant and grown in pure culture (Figure 6.4; Figure 6.5). Samples were successfully
retained in long-term storage, and reconstituted following six months storage to verify viability. However, sporulation in these samples was more difficult to induce than from fresh samples.

Figure 6.4 Marshall ryegrass inoculated with an isolate of *Pyricularia* spp. isolated from annual ryegrass displaying symptoms of gray leaf spot; later confirmed by DNA sequencing.
External genotyping of the *Pyricularia* spp. isolates from Mississippi indicated the isolate was *Pyricularia oryzae* Cavara (Figure 6.5). This is contradictory to what was initially expected in this work.

Regarding the five inoculations of 128 Marshall seedlings, only the first two inoculations (September and October 2017) were successful. After the first screening in September, all plants exhibited symptoms of gray leaf spot. We attempted to lower inoculum levels for the October inoculation to $1.0 \times 10^5$ conidia mL$^{-1}$ to potentially identify resistant ryegrass genotypes; however, only two of the flats (64 plants) showed
symptoms, and all 64 plants were infected. Symptoms were not observed during any other inoculation attempt (November and December 2017, and March 2018).

**Discussion**

**Koch’s Postulates**

The success in performing Koch’s Postulates provided first-hand experience in plant pathology methods and techniques associated with *P. oryzae*. By following the exact methods previously described by Harmon and Latin (2003), isolation and culturing of the pathogen proceeded successfully. The repeatability of their results validates their methodology in handling the pathogen in vivo and in vitro.

Regarding sporulation of isolates and replications of *P. oryzae*, there did appear to be some variability between isolates in relation to quantity of spores produced in pure culture. As a facultative saprophyte, *P. oryzae* often requires a living host, but can be propagated on nutrient media if necessary (Smiley et al., 2005). This could explain differences observed between isolates in terms of both degree of sporulation, and of mycelial growth. When using V8A we observed a much greater degree of sporulation compared to PDA, possibly due to the greater free sugar content along with presence of trace elements such as copper (Kent et al., 2008). Poor sporulation was the reason why only three isolates were chosen for the initial inoculation in September, 2017. Had cultures with poor sporulation been selected, use of the hemacytometer to quantify conidia concentration and subsequent formulation of inoculum solution would have been challenging.
Samples frozen for long-term storage will potentially allow for further research on screening annual ryegrass. The six month post-storage test indicated these isolates remained viable. However, reduction in sporulation may limit their practical use, and may necessitate isolating *Pyricularia* spp. from infected leaf material to initiate new cultures.

**Identification of Pathogen Species**

During the initial stages of this work, we believed the pathogen to be *P. grisea*, a very similar species to *P. oryzae* (Klaubuf et al., 2014). This was due to much of the previous literature citing *P. grisea* as the causal organism (Bain et al., 1972; Carver et al., 1972; Moss and Trevathan, 1987; Trevathan et al., 1994). However, sequencing of the internal transcribed spacer (ITS) region revealed the pathogen we initially isolated in Mississippi to be *P. oryzae*. Minor differences in conidia tip shape observed in the Mississippi and North Carolina isolates confirmed the species as *P. oryzae* through phenotypic means. These samples possessed more rounded tips consistent with *P. oryzae*, while *P. grisea* typically displays more pointed tips (Klaubuf et al., 2014). Recent phylogenetic work has also established that *P. oryzae* has a much wider host range (Klaubuf et al., 2014). This makes it more likely to be found in samples than *P. grisea*. While the practical implications of this may be minor (both infect the same host species and cause nearly identical symptoms), it is important to phylogenetically distinguish the predominant species in an area or region in case resistance to fungicides is lost or host-plant resistance declines.
Resistance Screening Attempts

In the five attempts over a seven-month period to identify resistant annual ryegrass plants post-inoculation of *P. oryzae*, two of the five attempts were successful for inoculation and expression of symptoms, but no wholly resistant ryegrass individuals were observed. Thus, no selections were made. There were some individuals expressing slightly less incidence of disease, but no individuals were observed free of gray leaf spot symptoms. We speculate the failure of inoculations in November 2017, December 2017, and March 2018 were due to inadequate environmental conditions necessary for *P. oryzae* to infect and parasitize host plants. An abnormally cold winter of 2017-2018 resulted in greenhouse temperatures for the November inoculations approximately 10-15°C. Best conditions for *P. oryzae* proliferation are 20-28°C conditions. These cooler temperatures also favor robust growth of annual ryegrass, which is a cool-season species. Lower stress on the host would make parasitism by the pathogen more difficult.

Because of the cool greenhouse environment during the winter, we attempted to use growth chambers to simulate infection conditions. However, these growth chambers could not be maintained at 100% relative humidity and constant leaf wetness required for favorable infection conditions (Harmon and Latin, 2003). Thus, December 2017 and March 2018 inoculations were unsuccessful.

These observations indicate the best time for screening annual ryegrass germplasm for potential resistance to gray leaf spot is during the late summer or early fall months in Mississippi. During these periods, ambient temperature and relative humidity are greater than the rest of the annual ryegrass growing season, thus allowing infection by *P. oryzae* to occur more readily. Ideal environmental conditions would allow large scale
intensive screening with a suitable selection pressure able to detect resistant individuals in large populations. Screenings were limited by the ability to only screen 128 plants per inoculation event. Flats of 32 individual plants were necessary to provide seedlings enough space to observe individual lesions. However, this severely limited the effective number of individuals that could be transplanted into flats and grown prior to inoculation. Flats or pots of mass seeded ryegrass could be effective, but identifying resistant individuals grown in close proximity would be challenging.

Conclusions

This work was largely preliminary to further potential screening and phenotypic selection of gray leaf spot-resistant individuals within Marshall annual ryegrass germplasm. By performing Koch’s postulates several times, we were able to reliably isolate and store \textit{P. oryzae} samples obtained from annual ryegrass hosts for future use. These isolates can potentially serve as inoculum to augment this work. Various projects have previously identified both \textit{P. oryzae} (Makaju et al., 2016) and \textit{P. grisea} (Trevathan et al., 1994) as causal organisms for gray leaf spot (ryegrass blast). This work indicated \textit{P. oryzae}, not \textit{P. grisea}, was the causal pathogen on annual ryegrass in these samples. A single year of experimentation was conducted on this pathology project. Drought conditions during normally peak infection times in August and September 2016 prevented suitable conditions critical to allow development of the disease. This, combined with unfavorable inoculation conditions during winter 2017-2018, precluded success of this project.

Recurrent phenotypic selection has been used to select for disease resistant ryegrass cultivars (Reith and Blount, 2003; Studer et al., 2006; Studer et al., 2007) and
thus remains a viable option for development of gray leaf spot resistant germplasm. A potential avenue for improving current methodology involves planting larger numbers of ryegrass, followed by mass inoculation with *P. oryzae* and containment in clear plastic. This would allow for the identification of potentially resistant genotypes in the base population.
CHAPTER VII
CONCLUSIONS

Breeding for improved environmental stress tolerance in outcrossing, cool-season grass species through RPS has long been thought impractical. Common citations of large environmental variation, coupled with traits exhibiting additive gene action (quantitative genetics), led many plant breeders and geneticists to forgo attempts at conventional selection for traits that fall into this category. However, work with both annual ryegrass and orchardgrass has provided results that contradict these assumptions. If environmental variation is removed from the selection process it is possible to make progress based purely on phenotypically observed additive genetic effects. Gains from selection are evident over the unselected germplasm in both species. These gains will continue to increase with further cycles of selection, though with diminishing returns. As the population nears or exceeds 50% mean cumulative germination, it becomes more difficult to shift the remaining segment (declining in numbers) of the population to greater heat tolerance.

In regard to future goals for each species, both have been advanced to cycle 4. The annual ryegrass germplasm will likely undergo one or two additional cycles of selection in an attempt to maximize the percentage of the population for germination at 40°C. Germination rate of cycle 3 annual ryegrass exceeded unselected germplasm, even in the 20°C tests. With the orchardgrass germplasm, mean cumulative germination at
40°C at the end of 22 days exceeded 80%, which was similar to the unselected population’s germination at 20°C. However, germination does not peak until about day 12 of testing. Future cycles of selection, therefore, should focus on improving velocity of germination under 40°C conditions to allow for more rapid establishment following individual rain events.

Freezing tolerance work, while similar to breeding for heat tolerance, has not progressed as well. This is likely due to screening of plants post-germination, where more physiological factors are responsible for conferring the trait (gradual exposure to shorter photoperiod and lower temperature). We attempted to select individual seedlings that expressed freezing tolerance without being subjected to gradual cold acclimatization to simulate passing of a cold front. However, the ratio of expression of the trait was very small (0.03%) in Marshall annual ryegrass germplasm. The few selections made provided only small gains in expression (~0.1%) after two cycles of selection. This was likely due to the inability to stabilize and define the optimum selection environment. Future work will likely involve large-scale freezing of tens or hundreds of thousands of plants in large freezers to generate sufficient population sizes under fixed photoperiod and temperature.

Finally, the disease resistance work with gray leaf spot (causal agent *Pyricularia oryzae*) successfully acquired isolates of the pathogen from both locally in Mississippi and eastern North Carolina. These have been placed in long-term cold storage in the Mississippi State Turfgrass Pathology Laboratory freezer and can be accessed for use in future resistance breeding work. We were unsuccessful in inoculation attempts in these resistance screenings. This was attributed to poor or inadequate environmental conditions for the formation of disease in host ryegrass plants. Future work will focus on large-scale
inoculations under high temperature and high humidity conditions ideal for *P. oryzae* infection of annual ryegrass.

These four breeding projects have provided novel positive results without the use of DNA fingerprinting that is extremely difficult to apply to obligately outcrossing species. Conventionally bred crops lack the gratuitous stigma of genetically modified germplasm, and provide avenues for organic as well as conventional producers to benefit. Successive generations of seed increases, followed by large-scale field testing will be the focus for the most advanced cycles of selection of heat tolerant annual ryegrass and orchardgrass.
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