Breeding Forage Grasses for Increased Heat Tolerance to Combat Climate Change

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Abstract
The onset of climate change brings many challenges for forage production in the southeastern United States, where it is projected to become hotter and dryer in the next century. To combat this climatic challenge, recurrent phenotypic selection was conducted in growth chambers on annual ryegrass (*Lolium multiflorum* Lam.) and orchardgrass (*Dactylis glomerata* L.) to select seedlings that can germinate and survive at temperatures of 40°C. Following three cycles of selection, germination was increased from < 5% to 45% in annual ryegrass, and from 20% to 80% in orchardgrass. The rate of germination also increased, in both species by a factor of 8x that of the base germplasm. Realized heritability also increased by 40 – 45% for each species by the end of the project. This work successfully improved a quantitative trait using recurrent phenotypic selection using growth chambers as a stable environment and provided the basis for combatting climate change in other outcrossing forage species.

Introduction
The southeastern United States is generally associated with long, hot summers, having temperatures commonly exceeding 32°C that often persist into early October. This is compounded by summer rainfall patterns in the region which often consist of intense, but infrequent rain events. Because of these climatic challenges, forage production in the southeastern United States has historically been reliant on warm-season perennial or cool-season annual species. However, warm-season forages, particularly grass species, are known to be of poorer nutritional value to grazing beef cattle, while cool-season annuals usually cannot be planted until October which delays growth and pushes grazing periods into the early spring. Additionally, most cool-season perennial forage species are generally not viable options due to lack of persistence under the high temperature stresses they are exposed to during summer months.
To alleviate these issues, genetic improvement of cool-season annual and perennial forage grasses could offer producers access to higher nutritive value forage either earlier in the growing season (annuals) or ensure their persistence over multiple growing seasons (perennials). The objective of this research was to develop novel heat-tolerant germplasm of a mainstay cool-season annual forage grass in the Southeast, annual ryegrass (ARG) and a cool-season perennial with widespread adoption in the Midwest and Northeast, orchardgrass (OG) using recurrent phenotypic selection. We hypothesized that recurrent phenotypic selection for germination at high temperatures in both species would be possible in a growth chamber due to the minimization of environmental variation that affects quantitative traits.

Materials and Methods
Germplasm Sources
Base germplasm of ARG for this breeding project was sourced from 'Marshall', a long-standing cultivar common throughout the southeast, particularly along the Gulf states (Mississippi, Alabama, Texas). For OG, the base germplasm originated from 160 parental plants of a summer dormant, tetraploid orchardgrass (cultivar unknown) planted 40 years ago at the Mississippi Agricultural and Forestry Experiment Station Prairie Research Unit, Prairie, MS. Each of these populations was subsequently referred to as Cycle 0 ARG or OG.
Selection Methodology

In August of 2015, Cycle 0 seed from each species were screened for the ability to germinate in a growth chamber (Percival Scientific, Series 101, Perry, IA) at 40:30°C (12:12 hr, light/darkness). Germinated seedlings were considered to express heat tolerance, and further kept in these conditions for 3 wks. The 64 most vigorous seedlings of each species were then selected, maintained in a greenhouse until they reached 4-6 true leaves, then transplanted into two 64-plant, 6.1 m^2 polycross nurseries on an 8 × 8 grid with 61-cm centers at the Mississippi State H. H. Leveck Animal Research Center. These selections were Designated Cycle 1 ARG and Cycle 1 OG. Plastic liner was used between plants to minimize weed pressure in the nurseries. Plants were maintained over the winter and spring, then hand-harvested for seed in May (OG) and June (ARG) of 2016. Harvested seed was bulked among all plants within each polycross. Seed was then sequentially conditioned with a belt thresher (ALMACO, Nevada, IA), sieved (1.64- by 9.53-mm mesh; Seedburo Equipment, Des Plaines, IL), and fractionally aspirated (Carter Day International, Minneapolis, MN). Following this, Cycle 1 seed was screened for selection of Cycle 2 ARG and Cycle 2 OG under identical conditions as the initial selections. This process repeated through Cycle 3 of selection in 2018.

Germination Testing and Validation

Germination testing occurred annually following seed harvest and conditioning, with each cycle being compared to the antecedent generations. The design for this test was a completely randomized design, with Cycles 0 -3 ARG and OG tested and compared during summer of 2018. Following the Association of Official Seed Analysts (AOSA) guide, six replications of 100 seed from each tested cycle of selection. were placed in petri dishes on 15 ml of 1% water agar as the germination medium. Germination testing lasted for 14-d for ARG and 22-d for ORG (AOSA, 2018). Baseline germination under normal temperatures was conducted at 20:15°C, while hot germination was set at 40:30°C (12/:12 hr, light/darkness). Germinated seed were counted every two days. Cumulative germination and velocity of germination within the first eight days (VOG_8) were assessed.

Additionally, the OG germplasm was assessed for persistence by annually monitoring the number of plants that survived in the polycross nurseries from each cycle of selection.

Statistical Analyses

Data were analyzed using SAS 9.4 (Cary, NC) with the PROC GLM command to conduct ANOVA and mean separation. We also assessed realized heritability of the heat tolerance trait using the equation, $h^2_{realized} = \frac{G}{R}$, where $h^2_{realized}$ is realized heritability, G is gain (the number of individuals expressing the trait), and R is reach (the selection differential). Significant differences were determined at $P < .05$.

Results and Discussion

Cumulative Germination

From the 2018 germination tests that compared Cycle 0 – 3 ARG and Cycle 0 – 3 OG, improvement of germination at 40°C in advanced cycles of selection of ARG (Fig. 1a) and OG (Fig. 1b).

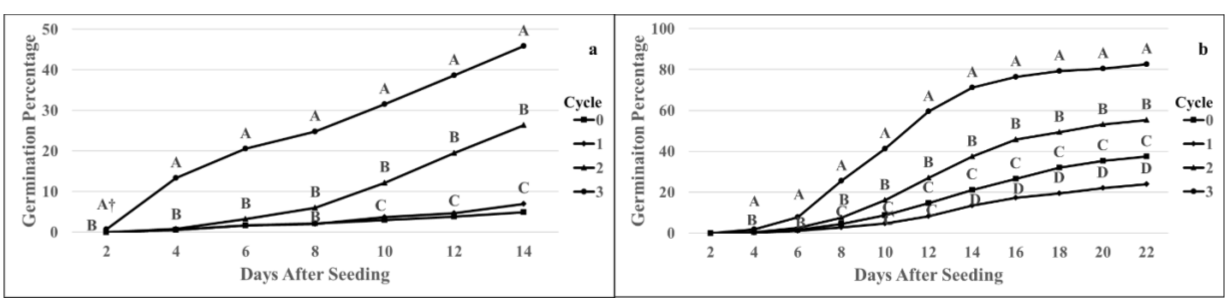

Figure 1. Cumulative germination of a) Cycles 0 – 3 annual ryegrass at 40:30°C, and b) Cycles 0 – 3 orchardgrass at 40:30°C. † Different letters indicate significant differences at *P* < .05.

For both ARG and OG, there were steady increases in cumulative germination with successive cycles of selection. Cycle 0 ARG began at < 5% cumulative germination under 40:30°C, but by Cycle 2 there was a

5x increase in germination, and Cycle 3 ARG reached nearly 50%, with germination appearing nearly linear. Orchardgrass germination also increased but took longer into the germination testing period to become evident. By the time we reached Cycle 3 OG, germination appeared to peak around 12 – 14-d after seeding and accruing over 80% cumulative germination.

Velocity of Germination

For both the ARG and OG germplasm, there were steady increases in VOG_8 with successive cycles above the base Cycle 0 germplasm. For ARG, Cycle 3 had an 8x increase over Cycle 0, and was 4x that of Cycle 2 (Fig. 2a). These patterns were nearly identical among cycles in the OG germplasm, but most of this increase was attributed to increases in germination on day 8 (Fig. 2b).

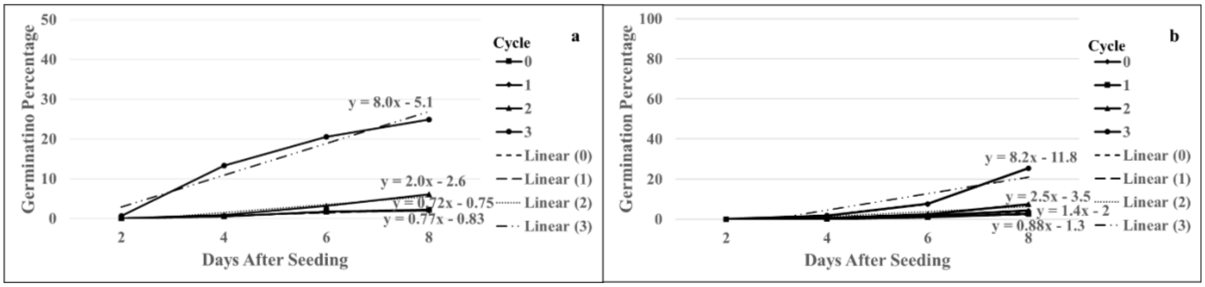

Figure 2. Velocity of germination within the first eight days (VOG_8) of a) Cycles 0 – 3 annual ryegrass at 40:30°C, and b) Cycles 0 – 3 orchardgrass at 40:30°C. Linear regression trendlines are included for each cycle of selection.

The increased VOG_8 was attributed to inadvertent, but beneficial, selection for the earliest germinating seeds during the selection process. This is a well-established phenomenon that has been documented in other recurrent phenotypic selection with seedlings (Anderson et al., 2009; Springer et al., 2017). Faster germination allows seedlings of either ARG or OG to germinate and become established earlier in the growing season, while the accompanying tolerance to high temperature stress observed would aid in survival if high temperatures were still present.

Orchardgrass Persistence

Aside from germination at 40°C, orchardgrass persistence was evaluated over the course of the breeding work (Table 1).

Table 1. Comparison of orchardgrass stand survival (10 months) of Cycles 0-3 polycrosses grown during the 2017-2018 season.

OG Cycle	Stand Survival
	Percentage
0	27.0b[a]
1	21.9b
2	54.7a
3	56.2a
Significance	***

*** Significant at $P < .001$

[a] Different letters indicate significant differences

Results indicated that orchardgrass persistence doubled from Cycle 1 – 2, topping at 56.2% for Cycle 3. It is possible that orchardgrass allele frequency may have reached its maximum, given the small change between Cycles 2 – 3 and the peak cumulative germination of ~80%.

Realized Heritability
In assessing realized heritability, both ARG and OG germplasm increased from Cycle 0 – 3 (Table 2). Both ARG and OG germplasm reached realized heritability levels of over 40%, indicating a substantial shift in allele frequency for the ability to germinate at high temperatures.

Table 2. Realized heritability of annual ryegrass (ARG) and orchardgrass (OG) germplasm.

	ARG		OG	
Cycle	Realized Heritability	22-d Cumulative Germination at 40°C	Realized Heritability	22-d Cumulative Germination at 40°C
	$h^2_{Realized}$	%	$h^2_{Realized}$	%
0	------[a]	4.8c[b]	------	27.8d
1	0.022	7.0c	-0.133	24.0c
2	0.19	26.3b	0.313	55.3b
3	0.20	45.8a	0.273	82.6a
Total $h^2_{Realized}$	0.41	------	0.453	------
Significance	------	***	------	***

*** Significant at *P* < 0.001

[a] Dashes indicate values that could not be calculated, due to requiring a comparison between cycles

[b] Within columns, different letters indicate significant differences

Conclusions
Following three cycles of selection in annual (ARG) and perennial (OG) forage grasses, it is evident that germination at temperatures ranging from 30 - 40°C was substantially increased. Despite the quantitative nature of heat tolerance, the controlled environment of a growth chamber appeared to minimize environment interaction with genotypes. To view the complete findings of this research, see the full articles recently published in Crop Science (Billman et al., 2020; Billman et al., 2021). These results have provided both a methodology for improving the heat tolerance of forage species through traditional breeding methods, as well as germplasm of two widely-used forage species in the country. As of 2022, the ARG germplasm has advanced to Cycle 5, and is currently being increased to a second batch of breeder seed and licensing for upcoming PVP certification and variety release. There has been further interest in the OG germplasm for use in the Transition Zone as summer temperatures continue to increase.

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