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Crop Science

ORIGINAL RESEARCH ARTICLE

Crop Breeding & Genetics

Breeding heat tolerant orchardgrass germplasm for summer persistence in high temperature stress environments of the southeastern United States

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Abstract

Orchardgrass (*Dactylis glomerata* L.) could serve as a cool-season perennial in southeastern production systems, but often does not behave as a true perennial under high temperature stress conditions of the region. This work sought to develop heat-tolerant orchardgrass germplasm through recurrent phenotypic selection (RPS) that would both reduce secondary seed dormancy caused by high soil temperatures and improve stand persistence over summer months. Selection was conducted in a growth chamber 40/30 ˚C (12/12 h, light/darkness), with germinated seedlings subjected to an additional 2–3 wk of 40/30 ˚C conditions. The base germplasm (Cycle 0) and selected individuals (Cycles 1–3) were transplanted into the field, then harvested for seed. Forty-degree germination tests compared mean cumulative germination, velocity of germination within 8 d (VOG_8), and realized heritability ($h^2_{Realized}$). Stand persistence was assessed 1 yr after transplanting. Results from 2018 tests indicated Cycle 3 seed germination was greater (82%) than all previous cycles of selection, and VOG_8 was eight times greater than that of Cycle 0. Additive gene action also increased, with final $h^2_{Realized}$ = .45, and preliminary data from Cycle 3 10-mo-stand persistence (56%) was double that of Cycle 0 (27%). These results indicated a significant improvement over the base germplasm for both germination at high temperatures and stand persistence in the field. This could lead to improved stand survival and greater adoption by southeastern forage producers.

1 | 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 southeastern United States (Mississippi, Louisiana, Alabama, and Georgia). Issues include high relative humidity as well as high mean temperatures throughout the summer months, often exceeding 32 ˚C for daily highs. Tall fescue tolerates these conditions in part because of its symbiosis with common toxic endophyte, *Epichloë coenophiala* (Morgan-Jones & W. Gams) C. W. Bacon & Schardl, which confers some measure of heat and drought stress tolerance to fescue plants (Schmidt & Osborn, 1993). Annual ryegrass is a winter annual adapted to growth in the

Abbreviations: $h^2_{Realized}$, realized heritability; RPS, recurrent phenotypic selection; VOG_8, velocity of germination within the first 8 d.

mild winter conditions of the U.S. 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 used 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 well to applications of nitrogen fertilizer (Ball et al., 2007; Mortensen et al., 1964). It is also commonly used as a companion crop in alfalfa (*Medicago* L.) stands for a grass and legume hay. Compared with tall fescue, orchardgrass lacks toxic endophytes that can produce harmful alkaloids that can be detrimental to animal performance (Clay, 1996). This makes orchardgrass more suitable for dairy and equine pasture or hay. Orchardgrass also has softer textured and less rigid leaves than most cultivars of tall fescue that make it more palatable to grazing animals (Baxter et al., 1986). While it lacks a toxic endophyte, this makes orchardgrass less tolerant to abiotic stresses such as heat or drought (Coblentz et al., 2006).

One of the major problems with using orchardgrass in the southeastern United States is that it does not persist well due to the stress it endures from high temperatures and high relative humidity of the region, making it more susceptible to disease. Orchardgrass is native to the temperate climates of central and eastern Europe, the Mediterranean, and central Asia (Renvoize, 1999). Therefore, the species is adapted to areas of mild summers and cold winters. It can tolerate brief instances of heat stress (3–7 d), but prolonged periods (1–4 wk) of over 32 ˚C and nightly lows above 25 ˚C can lead to large stand loss, particularly after grazing or hay harvest (Baker & Jung, 1968; Henning & Risner, 1993). Unfortunately, these conditions are common throughout much of the southeastern United States during summer months. Because orchardgrass typically does not persist beyond the first summer in the southeastern United States, it is not economical for growers to plant it. The seed cost is considerably greater (~$150–175 per 22.5 kg) than tall fescue and annual ryegrass seed (~$30–100 per 22.5 kg bag), depending on cultivar. If stands are significantly depleted after the first summer, growers would have to replant and incur this cost every year.

To ameliorate this risk, a breeding project was conducted 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 southeastern United States. Similar work has already been conducted by this team, with success in improving the germination of annual ryegrass at high temperatures and reducing secondary dormancy (i.e., impairment of germination due to adverse environmental conditions; Billman et al., 2020). The breeding objectives were to (a) reduce secondary seed dormancy by selecting seed that germinate at high temperatures, (b) improve heat tolerance of seedlings by screening at high temperatures, (c) increase summer persistence of the species under high temperature stress environments, and (d) determine if other important phenotypic traits were altered during selection for heat tolerance.

Core Ideas

- Orchardgrass germination and yearly survival increased over three cycles of selection.
- Growth chambers limit environmental variation and improve selection efficiency.
- Germination and persistence were not linked to maturity, tillering, or cold tolerance.
- Static environments can be used to more reliably select traits of low heritability.

2 | MATERIALS AND METHODS

2.1 | Selection methodology and germination testing

Germplasm was derived from 160 parental plants of a summer dormant, tetraploid orchardgrass (cultivar unknown) planted 40 yr ago at the Mississippi Agricultural and Forestry Experiment Station 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). This foundation population underwent decades of natural selection for persistence at ambient high temperatures, but still exhibited substantial stand reductions over summer months (only 20–30% stand survival when initially tested). Seed from these individuals was harvested in 2009 and stored for 5 yr. Seed was rejuvenated by planting. A mother plant nursery consisting of 64 randomly selected 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 Peter's 20–20–20 (107 mg L^{-1}, N, 23 mg L^{-1} P, and 33 mg L^{-1} K) solution biweekly following transplanting until the end of February each season. All seed from this nursery was bulked to serve as the base population, Cycle 0, for the high temperature selection process.

In October 2015, seed from Cycle 0 was screened for high temperature germination in a growth chamber (Percival Scientific, Series 101, Perry, IA) at 40/30 ˚C (12/12 h, light/darkness). Selections were made over a period of 36 d.

Ninety-five plants were selected from a total of 828 germinated seedlings. Of these 95 seedlings, the 64 largest individuals were transplanted into a 6.1-m^2 polycross nursery on an 8-by-8 grid on 61-cm centers forming Cycle 1 of selection in fall 2015. White 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 to serve as a pollen barrier. All seed from the 64 parent plants in Cycle 1 were harvested and bulked the first week of June 2016. Seed was conditioned using a belt thresher (ALMACO, Nevada, IA), sieved (0.164-by-0.953-cm mesh, Seedburo Equipment, Des Plaines, IL), and fractionally aspirated (Carter Day International, Minneapolis, MN) to remove chaff.

To advance germplasm to the next cycle of selection, a given cycle's seed lot was screened in an identical manner to antecedent generations. Seed that germinated at 40 ˚C were used to form the elite parental population of the next cycle's polycross. Each growing season (except for 2015–2016) an identical polycross of Cycle 0 plants was established from the original Prairie Research Unit seedlot. As it became available, seed from the prior year's cycle was screened and advanced to the next cycle. Cycles were maintained and missing plants replaced appropriately, starting each growing season with 64 plants per polycross. This allowed for phenotypic comparisons of in-field parameters between the base population and any advanced cycles of selection in the same production year. This process repeated from 2015 to 2018 to reach Cycle 3 of selection. For the 2017–2018 season, polycrosses for each generation (Cycles 0, 1, 2, and 3) were planted for determination of selection progress.

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. Each year, only Cycle 0 and the most advanced cycle for each respective year were planted as entirely new polycross nurseries. Seed from the newly established Cycle 0, all intermediate cycles, and the most advanced cycle of selection were harvested and tested for germination annually.

Germination tests of relevant cycles of seed (AOSA, 2014) were conducted at recommended Association of Official Seed Analysis protocol for orchardgrass (20/15˚C, 12/12 h, light/darkness) and at hot germination temperatures (40/30 ˚C, 12/12 h, light/darkness). For both temperature regimes, seed were placed in Petri dishes (15.25 by 3 cm) 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 d (AOSA, 2014). Observations were recorded every 2 d. Two-d and cumulative germination were recorded and calculated for each cycle of selection. Velocity of germination at 8 d (VOG_8) was also calculated. For the most advanced cycle of selection, the slope of the germination trend-line within the first 8 d of the test was graphed against all previous cycles.

2.2 | Additional phenotyping of selected plants

Other phenotypic traits were monitored on plants in the polycross nurseries for each cycle of selection. These traits were examined Fall 2017 through Spring 2018 (2017–2018) and included: days to maturity (in Julian days), reproductive tiller number, persistence, and frost damage. Within each 64-plant polycross, pseudo-replication (every two rows [16 plants] was treated as a replication, for 4 replications per polycross from which population means were determined) was used to determine preliminary values for these parameters. Maturity was defined as the Julian day at which the first seed head on a plant emerged from the leaf sheath on a reproductive tiller, with mean Julian date calculated across each cycle. Plants were monitored weekly until the first seed head in any polycross emerged, and then observed every 2 d 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 persistence of selected plants was determined by counting surviving plants in each cycle's polycross approximately 12 mo after planting, with no irrigation or amendments provided post seed harvest (May–Sept.). Finally, during mid-January of 2018, several days of cold temperature in the range of −6 to −13 ˚C caused frost damage to plants, enabling a one-time visual frost damage rating. 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 6 wk after the frost period occurred (late Feb. 2018).

2.3 | Determination of heritability

Genetic gain is the measure of the progress made due to selection that is due to genetic rather than environmental effects (Briggs & Knowles, 1967), and can be calculated as the mean of observed individuals expressing a trait minus the mean of the antecedent generation. To determine realized heritability of selection for 40 ˚C germination we applied the following equation to the datasets:

$$h^2_{Realized} = \frac{G}{R}$$

where $h^2_{Realized}$ = realized heritability; G = gain, the mean of observed individuals expressing a trait beyond that of the base germplasm (in this case germination at 40 ˚C); and R = reach, the selection differential for the trait of interest.

TABLE 1 Analysis of variance (ANOVA) and R^2 values for the models used with 2018 orchardgrass germinated and screened at 40 and 20 ˚C

Model Term	*df*	Type III SS[a]	Mean Square	*F*-Value	Pr > F	R^2
40 ˚C						
Rep	5	838.7	167.7	12.47	<.0001	.983
Cycle	3	53,538.4	17,846.1	13.26.53	<.0001	
Day	10	93,531.2	9,353.1	695.23	<.0001	
Cycle × Day	30	22,147.5	738.2	54.88	<.0001	
20 ˚C						
Rep	5	394.7	78.9	5.01	<.0001	.984
Cycle	3	7,069.2	2,356.4	149.5	<.0001	
Day	10	198,207.4	19,820.7	1,257.4	<.0001	
Cycle × Day	30	1,571.2	52.4	3.3	<.0001	

[a]SS, sum of squares.

2.4 | Statistical analysis

Data from the cumulative germination tests from each year of testing was analyzed as a completely randomized design using SAS 9.4 and PROC GLM to conduct analysis of variance (ANOVA) and mean separation. The statistical model was:

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

in which Y is the response variable, in this case number of germinated seed; μ is mean effects; D_i is the ith effect of the day of the test; C_j is the jth effect of cycle of selection; and $\varepsilon_{k(ij)}$ is the kth effect of experimental error associated with the ith effect of day and jth effect of selection. An α-level of .05 was used to determine significant differences. Because our phenotypic data was preliminary, we report the means but did not statistically analyze the data.

Chi-square (χ^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. It is the standard metric for determining differences among breeding populations, as it accounts for changes between populations based upon their performance in years prior. The formula for chi-square analysis was:

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

where observed = the number of individuals seen to express a phenotype and expected = the number of individuals predicted to express that phenotype. These calculated χ^2 values were then compared to the critical value of the χ^2 table (Clewer & Scarisbrick, 2001), with degrees of freedom as $n - 1$, and a significance level of $\alpha = .05$. With 63 *df* (64 total plants supplying seed); the critical value was 82.5.

3 | RESULTS

3.1 | Germination tests

For all germination tests, ANOVA indicated an interaction between cycle and day ($P < .001$; Table 1). Thus, these interactions were examined to compare each cycle of selection at each day of the 21-d germination testing period. Cumulative germination testing for the 2017–2018 season compared all cycles of selection (Cycle 3, Cycle 2, Cycle 1, and Cycle 0; Figure 1). Under the 40/30 ˚C testing regime (Figure 1a), Cycle 3 mean cumulative germination (1.83%) was greater ($P < .01$) 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 ($P > .05$). Cumulative germination of Cycle 3 remained greater ($P < .001$) than all other cycles for the remainder of the 22-d test period. Cycle 2 mean cumulative germination (7.5%) became greater ($P < .001$) than Cycle 1 (2.67%) and Cycle 0 (4.33%) at Day 8; this pattern remained for the duration of the 22-d test period. Cycle 1 germination (13.5%) was observed to be less ($P < .001$) than Cycle 0 (21.17%) by Day 14. This pattern was consistent for the duration of the test. Final mean cumulative germination at 22 d 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 d at 40/30 ˚C indicated Cycle 3 had a faster ($P < .001$) germination rate (8.2x) than Cycle 2 (2.5x), Cycle 1 (0.88x), and Cycle 0 (1.4x; Figure 1b). Cycle 2 VOG_8 was faster ($P < .001$) than Cycle 1 and Cycle 0; Cycle 1 did not differ ($P > .05$) 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 linear slopes of VOG_8 for each cycle of selection

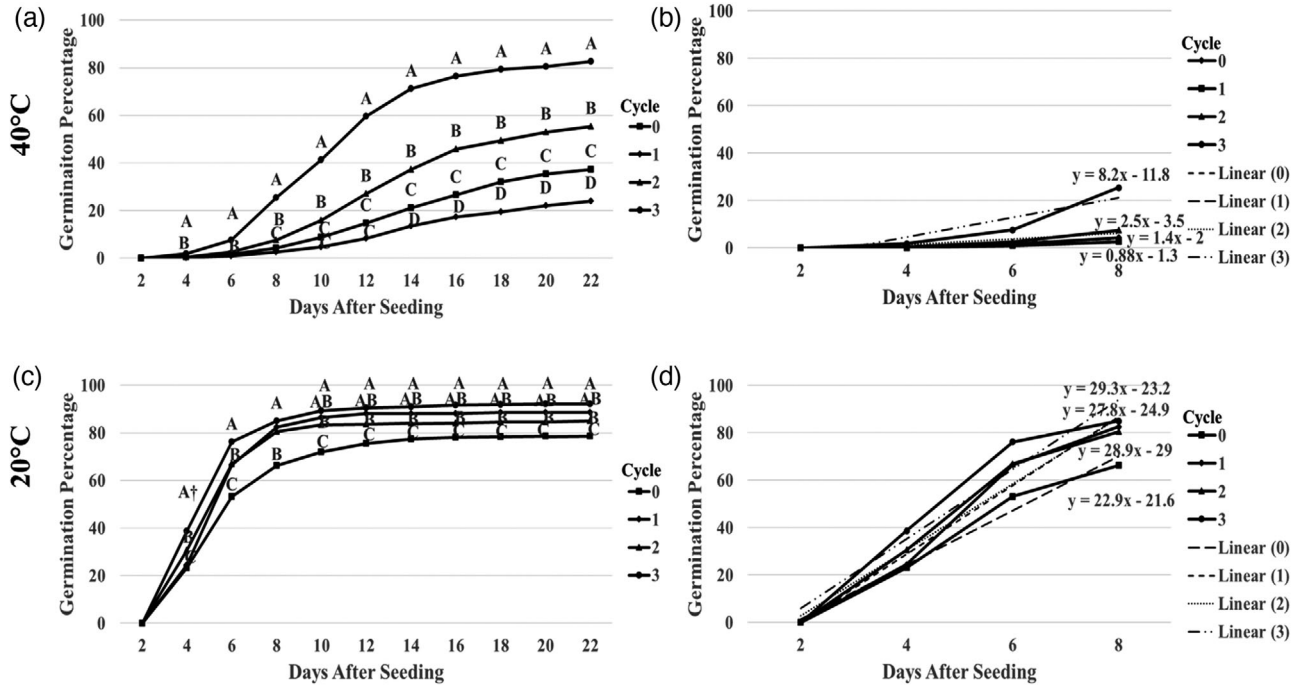

FIGURE 1 Germination tests comparing Cycle 0, Cycle 1, Cycle 2, and Cycle 3 orchardgrass over a 22-d test period, (a) mean cumulative germination at 40 ˚C, (b) velocity of germination at 40 ˚C within the first 8 d of testing, (c) mean cumulative germination at 20 ˚C, and (d) velocity of germination at 20 ˚C within the first 8 d of testing. Seed was produced during the 2017–2018 season, and tested July 2018. Means not sharing a common letter are significantly different at $P < .001$

were as follows:

$$y = 8.2x - 11.8 \text{ for Cycle 3}$$

$$y = 2.5x - 3.5 \text{ for Cycle 2}$$

$$y = 0.88x - 1.3 \text{ for Cycle 1}$$

$$y = 1.4x - 2 \text{ for Cycle 0}$$

Germination testing at 20/15 ˚C in 2018 also resulted in differences ($P < .001$) between cycles (Figure 1c). 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-d 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 d 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 d 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 ($P > .05$; Figure 1d). However, the VOG_8 of these three cycles were all greater ($P < .001$) than Cycle 0 (22.9x). The equations assessing the slope of VOG_8 at 20/15 ˚C for each cycle of selection were as follows:

$$y = 29.3x - 23.2 \text{ for Cycle 3}$$

$$y = 28.9x - 29 \text{ for Cycle 2}$$

$$y = 27.8x - 24.9 \text{ for Cycle 1}$$

$$y = 22.9x - 21.6 \text{ for Cycle 0}$$

3.2 | Chi-square analysis

Chi-square (χ^2) analysis was conducted for the 2018, 40 ˚C germination tests to compare population gains from selection between cycles (Table 2). Expected values for Cycles 0–2 (rows 1–3 in Table 2) were determined using the mean values of those cycles in the 2017 germination tests (data not shown). In 2018, calculated χ^2 for Cycle 3 exceeded the critical value of the test when compared to Cycle 0 (151.4) and Cycle 1 (1,686.8). However, calculated χ^2 for Cycle 3 compared to Cycle 2 (51.8) did not differ. Therefore, Cycle 3 mean

TABLE 2 2018 Chi-square (χ^2) values for orchardgrass germination at 40/30 ˚C presenting observed, expected, and calculated values for each cycle of selection

Cycle	Observed	Expected	(Obs. − Exp.)	(Obs. − Exp.)^2	χ^2 (Obs. − Exp.)^2 / Exp.
0	37.3	23.3	——	——	——
1	24	3.7	——	——	——
2	55.3	38.2	——	——	——
3	82.7	——	——	——	——
0→3	82.7	23.3	59.4	3,528.4	151.4*
1→3	82.7	3.7	79.0	6,241.0	1,686.8*
2→3	82.7	38.2	44.5	1,980.3	51.8
0→2	55.3	23.3	32.0	1,024.0	43.9
1→2	55.3	3.7	51.6	2,662.6	719.6*
0→1	24	23.3	0.7	0.5	0.1

Note: Critical value = 82.5: α = .05; *df* = 63.
*Significant at the .05 probability level.

TABLE 3 Comparison of orchardgrass cycles' mean frost rating (Frost), relative time to maturity (Maturity), reproductive tiller number, and stand survival (10 mo) of Cycles 0–3 polycrosses grown during the 2017–2018 season

Cycle	Frost 0–9	Maturity Julian Days	Reproductive Tiller Number Tillers plant^{-1}	Stand Survival %
0	3.33	83.0	34.7	27.0
1	1.63	79.6	31.2	21.9
2	1.57	79.1	31.8	54.7
3	3.28	65.1	44.1	56.2

cumulative germination was significantly greater than Cycle 1 and Cycle 0, but not significantly greater than Cycle 2. The calculated χ^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 χ^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 χ^2 for Cycle 1 compared to Cycle 0 (0.1) did not exceed the critical value of the test, and thus the populations were not different.

3.3 | Phenotypic trait assessment

For the 2017–2018 season (Table 3), visual frost ratings in frost damage between Cycles 0 ($\bar{x}$ = 3.33) and 3 ($\bar{x}$ = 3.28) were numerically similar, whereas cycles 1 ($\bar{x}$ = 1.63) and 2 ($\bar{x}$ = 1.57) were closer in value. Images of each observed level of the 1–9 rating scale are provided for context (Figure 2), with plants being completely killed (9). Maturity dates ranged from 65.1 Julian Days for Cycle 3 to 83.0 d for

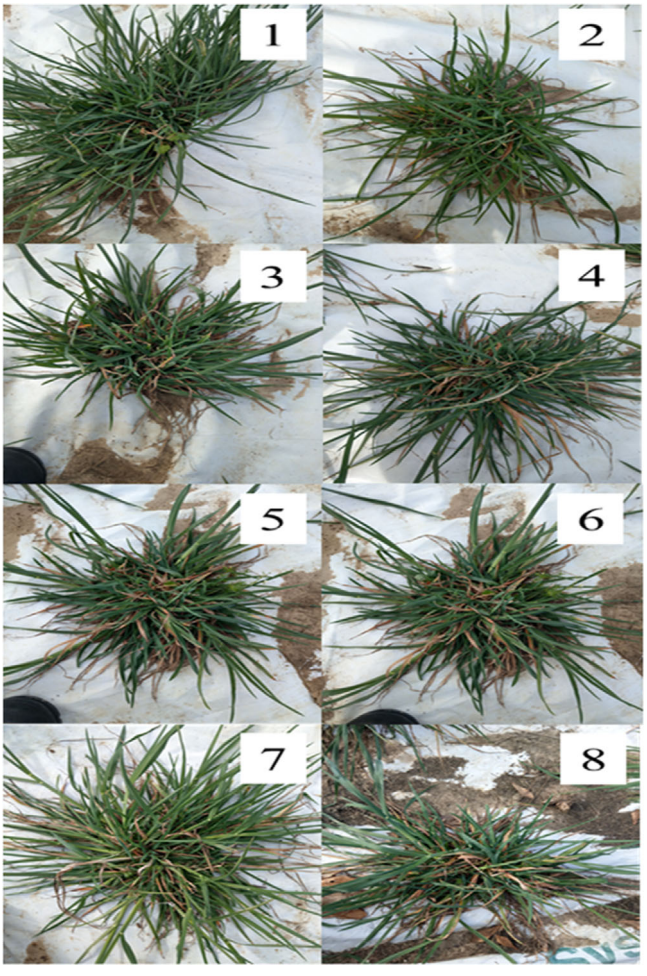

FIGURE 2 Images of each observed frost damage rating on orchardgrass during winter 2017–2018 on a scale of 1–9; 1 = no damage, 9 = plant death. From left to right, top to bottom, are examples of plants rated: 1, 2, 3, 4, 5, 6, 7, and 8. Rating 9 was not observed

TABLE 4 Comparison of orchardgrass realized heritability ($h^2_{Realized}$) 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

	2017–2018	
	Realized Heritability	22-d Cumulative Germination at 40 ˚C
Cycle	$h^2_{Realized}$	%
0	—— [a]	27.8d
1	−0.133	24.0c
2	0.313	55.3b
3	0.273	82.6a
Total $h^2_{Realized}$	0.453	——
Significance	——	***

Note: Within columns, no shared letters indicate significant differences.
[a]Dashes indicate values that could not be calculated due to requiring a comparison between cycles.
***Significant at the .001 probability level.

Cycle 0. Mean reproductive tiller number ranged from 31.2 tillers plant^{-1} for Cycle 1 to 44.1 tillers plant^{-1} for Cycle 3, with Cycle 3 having approximately 10 more tillers per plant than the next closest cycle. Finally, stand survival ratings indicated that Cycle 3 (56.2%) and Cycle 2 (54.7%) persistence values were approximately twice those of Cycle 1 (21.9%) and Cycle 0 (27.0%).

3.4 | Heritability values

Calculation of realized heritability was conducted using cumulative germination under high temperature screening conditions during the 2017–2018 season (Table 4). Calculated $h^2_{Realized}$ for the cycles of selection indicated Cycle 1 had a $h^2_{Realized} = -.133$, Cycle 2 had a $h^2_{Realized} = .313$, and Cycle 3 had a $h^2_{Realized} = .273$. Between each cycle of selection, these increases in $h^2_{Realized}$ correspond to subsequent increases ($P < .001$) in cumulative germination at 40 ˚C, ranging from 27.8% for Cycle 0 to 82.6% germination for Cycle 3. Total accumulation of additive gene action measured in the 2017–2018 season for Cycle 0–3 was $h^2_{Realized} = .453$.

4 | DISCUSSION

4.1 | Improvement of germination at 40 and 20 ˚C

The 2018 germination tests indicated that selection improved high temperature germination (Figure 1a); 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%; Figure 1c). However, the time required for germination at 40 ˚C to reach similar performance as 20 ˚C was substantially longer. As high temperatures interrupt metabolism in C_3 species, Cycle 0 at 20 ˚C exceeded 60% cumulative germination at Day 8, whereas Cycle 3 at 40 ˚C did not exceed 60% germination until Day 12. Subsequent cycles of selection should focus on selecting seed that germinates within the first 8 d, excluding any individuals that germinate beyond this timespan. Poor performance of Cycle 1, with germination at 40 ˚C being less than Cycle 0 in 2018, indicates that Cycle 1 may have been contaminated with unselected orchardgrass pollen during its initial selection in 2015, or inconsistent timing of pollen shed among plants resulted in underdeveloped seed. 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 (Figure 1c). There was increased percentage 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 suggest that we may have reached close to the maximum germination potential under high temperature stress in this germplasm. These findings are consistent with similar work conducted on annual ryegrass germination at high temperatures (Billman et al., 2020). Therefore, further cycles of selection should focus on improving germination velocity.

A serendipitous consequence of selection for improved germination at high temperature was the acceleration of VOG_8 (Figure 1b). This change in VOG_8 is an artifact of impatience from the those conducting selection. It has been observed in similar work involving selecting seed at germination; inevitably the seed that were selected were the seed that germinated first (Anderson et al., 2009, 2011; Billman et al., 2020; Springer, 2017). The 2018 germination tests indicated an increase in VOG_8 from Cycle 2 to Cycle 3 (3.28x). This further indicates that we have room to improve the germplasm's VOG_8. 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. This rapid germination also provides an advantage of this orchardgrass germplasm over weedy species that may germinate in the late summer or early fall months (Sanderson & Elwinger, 2002).

These germination tests express 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 screening environment must be kept uniform and stable, in this case, through use of growth chambers. The minimum effect of genotype × environment interaction indicates that selections for these quantitative traits were made without being confounded by environment. As a result, significant improvements for heat tolerance of orchardgrass germplasm as measured by germination at high temperatures have been achieved.

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 orchardgrass over other common cool season forages already grown in the southern United States are that (a) it is a perennial and (b) it has a nontoxic endophyte (*Epichloë typhina*; Clay, 1996; Rozpadek 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 annual sowing every season represents a 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 nontoxic endophyte contrasts with 'Kentucky 31′, the most commonly cultivated tall fescue cultivar in the South and, effectively, the only perennial cool-season forage grass common in the region (Baker & Jung, 1968; Nie & Norton, 2009). Toxic endophyte (*Epichloë coenophiala*) infection is a relatively common occurrence in tall fescue stands (Schmidt & Osborn, 1993). Because orchardgrass does not exhibit symbiosis with a toxic endophyte, there is little inherent risk to animals consuming it.

4.2 | Chi-square tests for significance

The chi-square analysis provided results that were largely similar to the ANOVA-based analysis. In 2018, germination at 40 ˚C of Cycle 3 was significantly greater than Cycle 0 and Cycle 1 (Table 2). However, chi-square tests are more conservative than ANOVA (Clewer & Scarisbrick, 2001), and thus we did not detect a difference between Cycle 3 and Cycle 2, whereas ANOVA tests indicated a difference was present. This is likely due to the chi-square test only being able to detect differences based upon the final mean of each whole population, as it does not account for differences in cumulative germination on an every-other-day basis. 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 χ^2 value. However, this test confirmed that Cycle 3 was different than Cycle 0, the base germplasm, which is a crucial step toward final cultivar release.

4.3 | Notes on 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, persistence, 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.

Preliminary phenotypic data resulting from selection for high temperature germination (2017–2018) resulted in times to maturity that appeared to be due to the handling of our most advanced germplasm (Cycle 3) being affected by the short–long day regulation of orchardgrass flowering. Rather than induction, initiation, and development of flowers occurring under strictly short day or long day conditions, orchardgrass relies on 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 12/12 h light/darkness for the first several weeks postgermination, and then were placed in the greenhouse in mid-August 2018 where ambient conditions were 13:20/11:40 h light/darkness. Compared to previous years, these selections took place about 2 mo earlier to attempt fall planting in hot environmental conditions. Orchardgrass has a minimum photoperiod requirement of 12/12 h light/darkness to induce and initiate flowering (Salisbury & Ross, 1992). Thus, we unintentionally initiated flowering in the growth chamber. Then, development and maturation occurred more rapidly the following spring, as vernalization accelerated 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 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, thus resulting in maturity dates 15–20 d later.

Reproductive tiller number serves as an assessment of both relative seed production capability (Abel et al., 2017; Lopes & Franke, 2011; Sukhchain & Sidhu, 1992), and as an assessment of forage nutritive value (Mayland et al., 2000; Nelson & Moser, 1994; Temu et al., 2014). However, there is an inverse relationship between tiller number/seed production and forage nutritive value. In the 2017–2018 season, Cycle 3 (44.1 tillers plant^{-1}) had at least 10 more tillers plant^{-1} than the previous cycles (<35 tillers plant^{-1}) (Table 3), which is potentially linked to the short–long day nature of the species. However, full variety testing is necessary to further quantify this.

Stand survival is one of the most crucial requirements of a successful heat-tolerant orchardgrass cultivar for the southeastern United States. 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 & Norton, 2009). Shaimi et al. (2008) reported 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 (U.S. $100–150 per 22.6 kg), with current market trends ranging in cost from as much as 1.5–2 times greater than annual ryegrass seed ($40–70 per 22.6 kg). Thus, for orchardgrass to be economically viable to producers in the southeastern United States, it must behave as a true perennial and persist.

Following three cycles of selection, 10-mo survival during the 2017–2018 season indicated that Cycle 3 and Cycle 2 populations exceeded 50% stand survival, whereas Cycle 1 and Cycle 0 stand survival approximately half that level, ranging from 21 to 27% (Table 3). 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 contributing factor to the decline and potential failing of orchardgrass stands (Drake et al., 1963). Stands were not irrigated, sprayed, or provided other prophylactic care post-seed harvest. Rather, they were left to endure normal Mississippi environmental conditions (daily temperature highs consistently exceed 32 ˚C and nightly temperatures exceed 28 ˚C, with high relative humidity). While isolated by cereal rye and distances of 275–450 m, all cycles were grown in relative 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. Future testing with fully replicated field trials with stands evaluated over at least 3 yr will be necessary to fully quantify improvements to persistence.

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 (Table 3; Figure 2). Frost rating similarities between Cycles 0 and 3 were likely a factor of microclimate as Cycles 0 and 3 were located in fields of slightly lower elevation which, due to cold air drainage, caused slightly lower temperatures than the locations of Cycles 1 and 2. The extreme cold event of January 2018 (72 h of −6 to −13 ˚C) was unexpected, and a single event that is not easily replicated in a Mississippi climate. Further testing under more consistent cold temperatures will be necessary to confirm maintenance of cold hardiness in the advanced germplasm.

4.4 | Realized heritability of heat tolerance

Because heat tolerance is a quantitative trait, realized heritability values were expected to be small in early cycles of selection (Marshall, 1982). However, realized heritability values for the 2017–2018 seed crop indicated large gains were achieved (Table 4). Cycle 1 realized heritability was negative, $h^2_{Realized} = -.133$. Cycle 2 $h^2_{Realized} = .313$ and indicated a substantial gain of desirable alleles between Cycle 1 and Cycle 2. Cycle 3 $h^2_{Realized} = .273$, 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 per-generation $h^2_{Realized}$ suggests we may have reached the point of diminishing returns with continued selection for germination at high temperatures. Stagnation or decline in $h^2_{Realized}$ value during future selection will determine if further selection for high temperature germination is worthwhile, or if selection strictly for increased VOG_8 at high temperatures should be the new focus of the germplasm. Current total additive gene action effects are expressed by an $h^2_{Realized}$ of .453. This value indicates an excellent response to selection, but less potential room for increased heat tolerance based solely on additivity.

5 | 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-d 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 and germination at 20 ˚C peaked at Day 8. Additionally, $h^2_{Realized}$ data suggests continued high temperature selection may be met with diminishing returns. Further improvement of the germplasm should focus on enhancing VOG_8. Future variety testing over several years will be necessary to validate the preliminary observations found with persistence, maturity, reproductive tiller number, and susceptibility to freezing damage. Currently, seed increases are ongoing for subsequent field-testing for persistence under various harvest frequencies and assessment of forage productivity compared to other germplasm and cultivars.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

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AUTHOR CONTRIBUTIONS

Eric D. Billman: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing-original draft; Writing-review & editing. Jesse I. Morrison: Methodology; Supervision; Visualization. Brian S. Baldwin: Conceptualization; Funding acquisition; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing-review & editing

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