SEED PRIMING AS A BREEDING TOOL FOR PERENNIAL WARM-SEASON GRASSES: IMPROVING ABIOTIC STRESS TOLERANCE IN *PENNISETUM* AND DOUBLING THE CHROMOSOMES IN *SORGHUM*

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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August 2015

Major Subject: Plant Breeding

ABSTRACT

Warm-season perennial grasses are leading candidates for lignocellulosic biofuel feedstocks because of their relatively high photosynthetic rates, water use efficiency, and nutrient use efficiency. Interspecific F_1 hybrids between pearl millet (*Pennisetum glaucum* [L.] R. Br.) and napiergrass (*Pennisetum purpureum* Schumach.) (PMN) are "seeded-yet-sterile" triploids (2n=3x=21) with high biomass yield potential that are well adapted to the southern U.S. Similarly, sterile triploid hybrids can potentially be obtained by crossing annual diploid *Sorghum bicolor* (L.) Moench with perennial tetraploid *S. propinquum* (Kunth) Hitchcock to produce novel C_4 perennial sorghum biofuel feedstocks. The presowing hydration technique solid matrix priming (SMP) was utilized alone and in combination with the elicitor compounds 5-azacytidine (AZA) and chitosan on three PMN hybrids to improve soil emergence, growth, seedling yield, and chlorophyll fluorescence in ambient, heat, and heat plus drought stress environments. Because *S. propinquum* is a diploid, tetraploid germplasm is needed to produce triploid hybrids with *S. bicolor*. SMP and a standard moistening protocol were evaluated as techniques of applying colchicine rates with and without dimethyl sulfoxide (DMSO) to *S. propinquum* seed to induce chromosome doubling.

SMP usually reduced the time to maximum soil emergence in PMN from 4 to 2 d in all three environments. In the heat stress environment, SMP and AZA treatments increased tillering in the elite PMN hybrid 09TX04 relative to the control. The novel chlorophyll fluorescence method developed in this experiment successfully established

unstressed, moderate, and severe stress levels. In the heat plus drought stress environment, PMN 09TX04 trended as being less stressed than the other PMN hybrids. Seedling biomass yields for SMP-treated PMN were higher than the control in the ambient (38%) and the heat plus drought (43%) environments.

The treatment using SMP for 5 d with 0.1% colchicine plus 2% DMSO was the only one in which *S. propinquum* chromosome doubling occurred. Seven of 52 surviving plants in this treatment were tetraploids, a 13% success rate, which is a very high frequency of chromosome doubling.

DEDICATION

I would like to dedicate this work to my family, especially my beloved wife, my best friend Amy. Also I would like to dedicate this work to my children Chance, Skyler, Arianna, Seth, and Kael, and my granddaughter Payton. Being part of the land-grant mission at Texas A&M University means working as a part of a larger whole. It means having a role in providing for the present and future needs of our community. I hope that doing this work has provided a good example for my children. Lastly, I would like to thank my parents, Jane and Roy Richards, and my mother- and father-in-law, Joyce and Edward Young, for their incredible support and love.

ACKNOWLEDGEMENTS

I would like to respectfully thank my committee chair, Dr. Russell Jessup, for all of his encouragement and mentoring during the course of my doctoral program. Additionally, I am very grateful to my committee members, Drs. Burson, Klein, Tarpley, and Yates, for their analyses and perspectives. I would especially like to thank Dr. Burson for providing an outstanding example as a scientist and mentor. Lastly, I am grateful for having worked alongside and shared by experiences with my fellow graduate students Charlie Dowling, Andrea Fonseca, Mason Kearns, and Greg Wilson. I am grateful for their help in this project, particularly Mason Kearns, who provided invaluable help in the design and construction of the chlorophyll fluorescence instrument.

TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION iv

ACKNOWLEDGEMENTS v

TABLE OF CONTENTS vi

LIST OF FIGURES viii

LIST OF TABLES x

CHAPTER I INTRODUCTION AND LITERATURE REVIEW 1

- Seed Priming 1
- Pearl Millet-Napiergrass 8
- Priming Pearl Millet-Napiergrass 10
- Chlorophyll Autofluorescence 10
- Hybridization in *Sorghum* Species 12
- Colchicine-Induced Polyploidy 14
- Seed Priming as a Colchicine Seed Treatment 15

CHAPTER II SEED PRIMING EFFECTS ON HEAT AND DROUGHT STRESS DURING ESTABLISHMENT IN PEARL MILLET X NAPIERGRASS HYBRIDS: GROWTH, YIELD, AND CHLOROPHYLL AUTOFLUORESCENCE 17

- Introduction 17
- Materials and Methods 22
- Results and Discussion 27
- Conclusions 42

CHAPTER III SEED PRIMING EFFECTS UPON INDUCED CHROMOSOME DOUBLING OF *SORGHUM PROPINQUUM* 45

- Introduction 45
- Materials and Methods 48
- Results and Discussion 51
- Conclusions 53

CHAPTER IV SUMMARY 56
REFERENCES 58
APPENDIX A FIGURES 77
APPENDIX B TABLES 91

LIST OF FIGURES

Page

Figure 1. Weekly mean day and night (columns) and maximum and minimum (lines) temperatures in the daytime heat stress and heat and drought stress environments for SMP-primed PMN hybrids grown at the Perennial Grass Breeding Field Laboratory at Texas A&M University in College Station, Texas, USA from June 12 to September 4, 2014. 77

Figure 2. Morphological stage of development means by entry of three PMN hybrids treated with SMP in controlled growth chamber (CGC), daytime heat stress, and heat plus drought stress environments. Bars represent the standard error of the mean. 78

Figure 3. Morphological stage of development means by treatment† of SMP-primed PMN hybrids in controlled growth chamber (CGC), daytime heat stress, and heat plus drought stress environments. Bars represent the standard error of the mean. 79

Figure 4. Morphological stage of development means by PMN hybrid x treatment† in controlled growth chamber (CGC) conditions. Bars represent the standard error of the mean. 80

Figure 5. Morphological stage of development means by PMN hybrid x treatment† in a daytime heat stress environment. Bars represent the standard error of the mean. 81

Figure 6. Morphological stage of development means by PMN hybrid x treatment† in a heat plus drought stress environment. Bars represent the standard error of the mean. 82

Figure 7. Mean relative fluorescence by entry of three SMP-primed PMN hybrids in controlled growth chamber (CGC), daytime heat stress, and heat plus drought stress environments. Bars represent the standard error of the mean. . 83

Figure 8. Mean relative fluorescence by treatment† of three SMP-primed PMN hybrids in controlled growth chamber (CGC), daytime heat stress, and heat plus drought stress environments. Bars represent the standard error of the mean. 84

Figure 9. Relative fluorescence means by PMN hybrid x treatment† in controlled growth chamber (CGC) conditions. Bars represent the standard error of the mean. 85

Figure 10. Relative fluorescence means by PMN hybrid x treatment† in a daytime heat stress environment. Bars represent the standard error of the mean. 86

Figure 11. Relative fluorescence means by PMN hybrid x treatment† in a heat plus drought stress environment. Bars represent the standard error of the mean....87

Figure 12. Seedling emergence percentage of *Sorghum propinquum* plants exposed to colchicine and/or DMSO using germination pad or solid matrix priming (SMP) techniques.†.....88

Figure 13. Emergence rate† of *Sorghum propinquum* plants exposed to colchicine and/or DMSO using germination pad or solid matrix priming (SMP) techniques.†† 89

Figure 14. Example verification of chromosome doubling in Sorghum. X axis is the photon intensity associated with DNA content. Y axis is the particle or nuclei count. Diploid standard 2C and 4C peaks aligned at 100 and 200 respectively on the X axis. Tetraploid 2C and 4C peaks aligned at 200 and 400 respectively on the X axis (Wilson 2015, unpublished). 90

LIST OF TABLES

Page

Table 1. Significance of effects of entry, treatment (Trt), and entry × treatment (Entry × Trt) on seedling emergence percentage, emergence rate, and seedling yield of three PMN hybrids treated with SMP. 91

Table 2. Emergence percentage, emergence rate, and seedling yield means by entry of pearl millet and PMN treated with SMP in ambient, heat stress, and heat & drought stress conditions. 92

Table 3. Emergence percentage, emergence rate, and seedling yield means by treatment of three PMN hybrids treated with SMP in ambient, heat stress, and heat plus drought stress conditions. 93

Table 4. Entry × treatment emergence percentage means of three PMN hybrids† treated with SMP in ambient, heat stress, and heat & drought stress conditions....94

Table 5. Entry × treatment mean emergence rate of three PMN hybrids† treated with SMP in ambient, heat stress, and heat plus drought stress conditions....95

Table 6. Entry × treatment seedling yield means of three PMN hybrids† treated with SMP in ambient, heat stress, and heat plus drought stress conditions....96

Table 7. Significance of effects of entry, treatment (Trt), and entry × treatment (Entry × Trt) on seedling morphological development of three PMN hybrids treated with SMP in an ambient environment. 97

Table 8. Significance of orthogonal contrasts comparing seedling morphological development in three PMN hybrids† treated with SMP in ambient environmental conditions....98

Table 9. Significance of effects of entry, treatment (Trt), and entry × treatment (Entry × Trt) on seedling morphological development of three PMN hybrids treated with SMP in a heat stress environment....99

Table 10. Significance of orthogonal contrasts comparing seedling morphological development in three PMN hybrids† treated with SMP in heat stress conditions....100

Table 11. Significance of effects of entry, treatment (Trt), and entry × treatment (Entry × Trt) on seedling morphological development of three PMN hybrids treated with SMP in a heat plus drought stress environment....101

Table 12. Significance of orthogonal contrasts comparing seedling morphological development in three PMN hybrids† treated with SMP in heat plus drought stress conditions. 102

Table 13. Significance of effects of entry, treatment (Trt), and entry × treatment (Entry × Trt) on blue-light chlorophyll fluorescence of three PMN hybrids treated with SMP. 103

Table 14. Significance of orthogonal contrasts comparing blue-light chlorophyll fluorescence in three PMN hybrids† treated with SMP in ambient conditions. 104

Table 15. Significance of orthogonal contrasts comparing blue-light chlorophyll fluorescence in three PMN hybrids† treated with SMP in heat stress conditions. 105

Table 16. Significance of orthogonal contrasts comparing blue-light chlorophyll fluorescence in three PMN hybrids† treated with SMP in heat plus drought stress conditions. 106

Table 17. Number of confirmed tetraploid *Sorghum propinquum* plants exposed to colchicine and/or DMSO using germination pad or solid matrix priming (SMP) techniques 107

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Seed Priming

Overview

Seed priming is primarily utilized for the improvement of seed germination and seedling vigor. The basic method involves limited imbibition of seeds in water under conditions that initiate early metabolic events prior to germination. The period of imbibition is followed by drying seeds to their original moisture content for subsequent storage until time of planting (McDonald, 2000). The benefits of seed priming have been shown to include improvements in overall percent germination, uniformity of germination, seedling vigor, and growth under abiotic stresses such as chilling, heat, and drought (Bradford, 1986; Soeda et al., 2005; Chen et al., 2010). Seed priming has improved seedling performance in optimal and non-optimal environmental conditions in an array of plant species that includes wheat (*Triticum aestivum* L.) (Iqbal and Ashraf, 2007), maize (Farooq et al., 2008), pepper (*Capsicum anuum* L.) (Korkmaz and Korkmaz, 2009), sorghum, and pearl millet (Aune and Ousman, 2011).

Bewley (1997) identified three distinct phases of water uptake in germinating seeds: (I) a rapid, passive phase of water uptake due to the seed's low matric water potential; (II) a slow increase in seed moisture content that coincides with the activation of important metabolic events; and (III) a second rapid increase in seed water uptake during which radicle protrusion occurs. The goal of seed priming is to manipulate water

potential such that the seeds progress through stages I and II but are held at a seed moisture content that prevents radicle protrusion (Nonogaki et al., 2010). A key characteristic of primed seed is that water uptake is slower and more controlled than in typical germination conditions (Taylor et al., 1988).

Recently, Chen and Arora (2013) introduced a paradigm for explaining the performance improvements observed in primed seeds by outlining two strategies: (a) developmental advancement; and (b) the induction of stress cross-tolerance. Metabolic events that occur in both germinating and primed seeds are mitochondrial repair, DNA repair, mRNA synthesis, synthesis of components in protein translation machinery, and ATP synthesis (Varier et al., 2010). A transcriptome analysis in broccoli (*Brassica oleracea L.* var. *italica* Plenck) revealed that expression levels in primed seeds for cytochrome b, glutathione-*S*-transferase, and superoxide dismutase were intermediate between those in dry seeds and germinating seeds imbibed in water (Soeda et al., 2005). Proteome analyses in *Arabidopsis thaliana* (L.) Heynh., sugarbeet (*Beta vulgaris* L.), and spinach (*Spinacia oleracea* L.) have confirmed that, while water-imbibed and primed seeds exhibit similar patterns of protein expression, key differences exist (Catusse et al., 2011). Priming-specific protein ontologies included cell cycle components, enzymes in the glyoxylate cycle, translation initiation factors, ABA signaling elements, and heat shock proteins (Gallardo et al., 2001).

The lengthened period of phase II is perhaps the key difference between primed and water-imbibed seeds. An examination of energy metabolism during priming provides a different perspective for viewing potential developmental advancement.

Priming increases the ATP/ADP ratio, suggesting that primed seeds have more energy during germination (Corbineau et al., 2000). Chen and Arora (2013) proposed that improved efficiency of mitochondria integrity/activity via repair and reactivation of existing mitochondria and biogenesis could explain the energy advantage possessed by primed seeds.

Stress imprinting, or cross-tolerance, has been reported as a result of priming in a diverse array of crops and stress conditions. Seed priming induced drought stress tolerance in sugarcane (*Saccharum officinarum* L.) (Patade et al., 2009), heat and salinity stress in barley (*Hordeum vulgare* L.) (Mei and Song, 2008), and chilling stress in Kentucky bluegrass (*Poa pratensis* L.) and perennial ryegrass (*Lolium perenne* L.) (Yamamoto et al., 1997). Some priming techniques utilize moderate abiotic challenges such as osmotic, drought, or oxidative stress. Thus, the priming treatment itself can be regarded as a stress condition.

A second aspect of stress imprinting in primed seeds is the post-priming drying regime. During phases I and II of germination, late embryogenesis abundant (LEA) proteins are depleted and seeds rapidly lose desiccation tolerance (Tunnacliffe and Wise, 2007). These LEAs can accumulate once again during the post-priming drying phase, but the reacquisition of desiccation tolerance has been linked to drying rate. Soeda et al. (2005) found that genes expressed during seed maturation and slow drying post-priming were associated with stress and desiccation tolerance, but several key genes associated with desiccation tolerance were not upregulated when primed seeds were dried rapidly. Finally, stimulation of the antioxidant system and upregulation of ABA signaling

pathways have been proposed as mechanisms for stress cross-tolerance in primed seeds (Lopez-Molina et al., 2001; Bailly et al., 2008).

Techniques

The three predominant techniques used to prime seeds are: (1) hydropriming, typically a short duration (< 48 h) water soak; (2) osmopriming, which involves placing seeds in an aerated solution of either polyethylene glycol (PEG) or inorganic salts of known ψ; and (3) solid matrix priming (SMP), which involves using solid carriers of known matric potential to prime seeds (Taylor et al., 1988; Patanè, et al., 2006; Chen et al., 2010; Yadav et al., 2011). Hydropriming, though sometimes effective in germination enhancement, has often led to reduced seedling performance due to the uncontrolled rate of water uptake during priming (Yadav et al., 2011). Several workers have observed that the agent used in osmopriming, especially PEG, imposed an osmotic stress on seeds and led to oxidative injury (Moosavi et al., 2009; Balestrazzi et al., 2011). Further, the use of high concentrations or high molecular weight PEG has low oxygen solubility and diffusivity (Mexal et al., 1975).

In contrast to hydropriming, the solid carrier media used in SMP limits water uptake. The solid matrix environment is also more conducive for proper seed aeration than in osmopriming. Typically, homogeneous, mostly inert, solid carriers that remain friable at a wide range of moisture contents are used for SMP. Previous workers have used calcined clay, sand, peat moss, bituminous coal, and humic acid carriers (Taylor et al., 1988; Khan et al., 1992; Bosma et al., 2002; Ma et al., 2003). Because the water-

holding capacity and bulk density of the carriers differ greatly, the proportions of seed, solid matrix, and water used in SMP vary considerably. Taylor et al. (1988) noted that the optimum duration and temperature of priming varies not only among species but also among cultivars and even seed lots. Thus, assaying samples of different seed lots using varying matric potentials and priming duration is often used for optimizing SMP protocols used for larger quantities of seed.

Solid matrix priming has been employed to improve uniformity, velocity of germination, and subsequent stand establishment in a diverse array of crops. These species include bahiagrass (*Paspalum notatum* Flügge), bermudagrass (*Cynodon dactylon* [L.] Pers.), centipedegrass (*Eremochloa ophiuroides* [Munro] Hack.) (Hacisalihoglu, 2007), loblolly pine (*Pinus taeda* L.) (Wu et al., 1999), maize (Parera and Cantliffe, 1991), carrot (*Daucus carota* L.), pepper, and snapbean (Ph*aseolus vulgaris* L.) (Khan et al., 1992). Few studies have directly compared SMP to osmopriming; however, Taylor et al. (1988) found SMP was superior to PEG and salt priming in terms of overall germination percentage, velocity of germination, and dry weight per plant in carrot and onion (*Allium cepa* L.).

Amendments

The benefits of SMP can be extended by incorporating various chemicals or organic materials into the priming solution. During priming, seeds are metabolically active for approximately 1 to 14 d (depending on the species, cultivar, etc). This affords an opportunity to expose seeds to beneficial compounds, protectants, or microorganisms.

The biocontrol fungus *Trichoderma harzianum* provides protection against *Pythium* and *Alternaria* spp., and it has been used successfully in SMP-based crop protection (Harman et al., 1989). In fact, the term "biopriming" has come into use largely because of the success in developing *T. harzianum* strains used to inoculate seeds and impart beneficial effects. Biopriming using specifically developed salt-tolerant *T. harzianum* strains has improved salinity tolerance in rice (*Oryza sativa* L.) and wheat (Rawat et al., 2011, 2012).

Apart from beneficial microorganisms, synthetic or modified organic compounds, such as chitosan, possess antimicrobial and antifungal properties via activation of the plant's defense responses (Muzzarelli et al., 1990; Pospieszny et al., 1991). Chitosan is a deacetylated polymer derived from chitin. Chemically, it is an oligomer with glucosamine subunits with a degree of deacetylation that varies considerably in their exact composition, depending on the source and processing methodology (Freepons, 1991; Bautista-Baños et al., 2006). Foliar chitosan sprays are utilized to reduce transpiration losses, mainly due to ABA-dependent stomatal closure (Iriti et al., 2009; Ludwig et al., 2010). Short-duration (< 12 h) seed soaking of a chitosan-based product successfully controlled downy mildew as well as improved seed germination and seedling growth rate in pearl millet (Sharathchandra et al., 2004).

Chitosan treatments invoke specific mechanisms that differ from the physiological and biochemical responses typically found in seed priming. Molecular studies on seed and foliar applications have demonstrated that chitosan, which chemically mimic pathogens, elicit a cascade of responses such as protein

phosphorylation, ROS generation, jasmonic acid biosynthesis, and defense-related genes (Felix et al., 1993; Kuchitsu et al., 1995; Minami et al., 1996; Nojiri et al., 1996). Therefore, applying chitosan during or after seed priming can invoke a stress "memory" that may facilitate a more rapid response to subsequent biotic or abiotic stresses.

Evidence is accumulating that the capacity of seeds (or plants) to respond to stress is more complex than mere signaling cascades. Stress imprint has been defined as "a genetic or biochemical modification that occurs after stress exposure that causes future responses to future stresses to be different" (Bruce et al., 2007). Chilling stress in maize led to 5-methylcytosine demethylation in roots that was not reversed upon removal of the stress (Steward et al., 2002). Histone deacetylation was observed in submerged rice, resulting in a reversible activation of stress-response genes (Tsuji et al., 2006). Epigenetic changes are potential mechanisms for explaining stress imprinting (i.e. stress "memory"). These changes involve modification of DNA activity via histone modification, methylation, or chromatin remodeling (Madlung and Comai, 2002). DNA methylation and histone acetylation have an important role in the regulation of gene expression. Active genes tend to be under-methylated and associated with nucleosome having core histones that show increased acetylation (Loidl, 2004; Wolffe and Guschin, 2000).

The compound 5-azacytidine (5-AC), a cytosine analogue and demethylating agent, has been used during seed imbibition to study chromosome organization in wheat (Santos et al., 2002). Neves et al. (1995) injected 5-AC into wheat × rye (*Secale cereale* L.) F_1 embryos 9 d after fertilization and conclusively showed that 5-AC reactivated

rRNA genes from the rye parent that normally are suppressed in this interspecific hybrid (Neves et al., 1995). Castilho et al. (1999) demonstrated that intermixing of the rye and wheat chromosomes in triticale (× *Triticale* Tscherm.-Seys. ex Müntzing) nuclei increased from 15 to 60% as a result of 5-AC-induced hypomethylation. In cell cultures using both 5-AC and a similar compound, 5-azadeoxycytidine, DNA replication was shifted to an earlier time frame, alterations in patterns of gene expression were observed, and frequencies of sister chromatid exchanges were increased (Haaf, 1995). Akimoto et al. (2007) induced dwarfism in rice after seeds were exposed to 5-azadeoxycytidine which was stably inherited for nine generations. Therefore, hypomethylation agents, such as 5-azacytidine, can be utilized in priming treatments specifically to induce epigenetic effects. Further, the combination of SMP and 5-AC in interspecific hybrids such as PMN has the potential to produce novel, stable phenotypes useful in breeding.

Pearl Millet-Napiergrass

Annual grain crops, particularly maize, account for the vast majority of the current biofuel usage in the U.S. Presently, more than 420,000 MT wk^{-1} of grain starch-based ethanol are produced in the U.S. (Renewable Fuels Association, 2015). The continued use of annual crops as biofuels that also are important as food and feed sources remains controversial. The USDA Economic Research Service issued a report specifically citing biofuels as one of the main contributing factors responsible for increases in food commodity prices, which in some cases rose 60% in a 2 yr timespan (Trostle, 2008). Efforts to examine usage of corn stover, a significant (51 gigaliters

ethanol yr^{-1}) potential source of lignocellulosic biomass, have raised concerns regarding the loss of soil carbon and soil erosion (Lal, 2005; Somerville et al., 2010).

In contrast, perennial C_4 plants such as sugarcane, Miscanthus, and napiergrass are high-biomass crops that possess a high net assimilation rate, WUE, and NUE. Additionally, perennial root systems can have a positive carbon balance and protect against erosion. Deployment of candidate lignocellulosic energy crops has been hampered by the need for vegetative propagation, risk of invasiveness, and poor establishment-year yields (Jessup, 2013). Thus, the need exists for a perennial biomass crop with high yield potential that can be successfully established using integrated agronomics limited predominantly to large-seeded, annual cereal crops. The interspecific hybrid between pearl millet and napiergrass (PMN) (*Pennisetum glaucum* R. Br. × *P. purpureum* Schumach), however, possesses these characteristics in a perennial crop. PMN, which is produced by crossing diploid pearl millet (2n=2x=14) × tetraploid napiergrass (2n=4x=28) parents and obtaining viable seed, is triploid and therefore sterile (Burton, 1944; Hanna, 1981). Hybrid seed production of PMN can exceed 1000 kg ha^{-1}, and the seed are large enough to be directly sown using existing planters, thereby reducing establishment costs relative to vegetatively propagated candidates such as energycane (*Saccharum* spp. L.) and Miscanthus x giganteus (Osgood et al., 1997). Yields of PMN are comparable to or even exceed that of energycane (Cuomo et al., 1996). In summary, PMN combines the advantages of high yields, sterility, perennialism, adaptability to marginal lands, low establishment costs, and non-invasiveness into a single crop.

Priming Pearl Millet-Napiergrass

The seeded-yet-sterile triploid biofuel feedstock PMN can be direct-seeded using existing agronomic equipment. The target regions for PMN deployment are subtropical and semiarid environments throughout the Gulf Coast plain of the southern U.S. SMP may be a valuable tool in ensuring successful PMN stand establishment and subsequent performance in potentially challenging environmental conditions.

While there are no published reports of SMP being used in pearl millet or PMN, pearl millet has been primed successfully using a hybrid hydro-/osmo-priming method using small doses of inorganic fertilizer as the osmoticum (Al-Mudaris and Jutzi, 1999; Aune and Ousman, 2011). Pearl millet also has been bioprimed using *Pseudomonas fluorescens* (Reddy, 2013). Therefore, a need exists for SMP protocols and subsequent performance to be established for PMN. Further, subjecting primed PMN seeds to abiotic stresses such as chilling, heat, and drought is not only an effective way to evaluate the efficacy of priming, it is also provides the means to evaluate PMN hybrids *per se*.

Chlorophyll Autofluorescence

Water and high temperature stress, which often co-occur, have major negative impacts on agricultural crop production due to reduced growth, inhibition of photosynthesis, and changes in metabolism (Lauer and Boyer, 1992; Tezara et al., 1999; Carmo-Silva et al., 2012). Seeds and seedlings tend to have greater thermosensitivity than later vegetative developmental stages (Levitt, 1980). Poor pearl millet seedling

establishment due to high soil- and air temperatures is one of the major causes of seedling thermal injury and death (O'Neill and Diaby, 1987; Peacock et al., 1993). In pearl millet, genotypic variation exists for thermosensitivity and both field- and laboratory-based screening methods such as electrolyte leakage, seedling regrowth potential, and protein synthesis capability (Peacock et al., 1993; Howarth et al., 1997). Water stress indicators, such as leaf water potential and relative water content, have been developed (Jackson et al., 1981; Hunt and Rock, 1989). The methods mentioned above often require more than 24 h for processing and some involve destructive tissue sampling.

A rapid, non-destructive system for plant stress assessment is therefore highly desirable. Chlorophyll fluorescence has been established as a sensitive indicator for plant water and temperature stress (Lang et al., 1996; Subhash et al., 2004). UV lights and lasers are commonly used as excitation sources (Takeuchi et al., 2002). Recently, advancement in LED lamp technology has improved the effectiveness of chlorophyll fluorescence by providing more energy-efficient, wavelength-specific excitation sources.

Recently, a fluorescence imaging system using high-intensity blue (460 nm) LED lamps was developed for evaluating water stress in cabbage (*Brassica oleracea* L. cabbage) seedlings, which showed significant differences in steady-state chlorophyll fluorescence for leaves in various stages of water stress (Hsiao et al., 2010). In that study, action spectra acquired under blue LED (460 nm) excitation were similar to those measured using UV and laser excitation methods (Heisel et al., 1996; Langsdorf et al., 2000). Further, LED lights had the advantage of illuminating the entire leaf while laser-

based methods typically illuminated relatively small areas within a leaf. An apparatus for measuring chlorophyll fluorescence requires a light-proof box for dark adaptation, a LED excitation source, a digital imaging instrument, and digital image analysis software (Hsiao et al., 2010).

Hybridization in *Sorghum* Species

Within the genus *Sorghum*, the subgenus *Eu-sorghum* contains the annual, non-rhizomatous species *S. bicolor* (2n=2x=20) and the perennial, rhizomatous species *S. propinquum* (2n=2x=20), which is native to Southeast Asia (de Wet, 1978). Even though *Sorghum* species are highly self-fertile, some natural outcrossing occurs. *Sorghum bicolor and S. propinquum* readily hybridize and is considered the widest euploid cross that can be made with *S. bicolor* using conventional methods (Kong et al., 2013). *Sorghum bicolor* is a major crop of tropical origin that carries out C_4 photosynthesis and has a genome size about one-fourth that of maize (Peterson et al., 2002). Therefore, it is an important model species in the study of other important, related species, including maize (Whitkus, et al., 1992) and sugarcane (Dufour et al., 1997). In addition to its importance as a model C_4 species, *S. bicolor* is the most drought-resistant of the world's top five cereal crops and is a leading biofuel candidate with the ability to produce high yields of starch, sugar and/or cellulose (Chittenden et al., 1994; Feltus et al., 2006; Rooney et al., 2007; Vandenbrink et al., 2013). *Sorghum propinquum* is a source of tall plant height and rhizomatousness, two traits of significant interest for development of *Sorghum* spp. as a biofuel crop (Paterson et al., 1995a).

Previous studies of *S. bicolor* × *S. propinquum* hybrids typically involved the development of recombinant inbred lines or F_{2-3} segregating populations to examine traits of interest (Kong et al., 2013; Tang et al., 2013). These lines and segregating populations were from diploid hybrids between *S. bicolor* and *S. propinquum*. Controlled wide crosses between *S. bicolor* and other *Sorghum* spp. with different ploidy levels have demonstrated that F_1 hybrids with a range of ploidy levels are often recovered. Crosses between *S. bicolor* and *S. halepense* (L.) Pers. (2n=4x=40) have produced rare diploid, triploid (2n=3x=30), and tetraploid hybrids (Bennett and Merwine, 1966; Sengupta and Weibel, 1971; Dweikat, 2005). Self-fertility in *S. bicolor* x *S. halepense* F_1 hybrids has varied across ploidy levels; 90% seed set was reported in diploid hybrids (Dweikat, 2005), but Endrizzi (1957) reported triploid and tetraploid hybrids as having 1.1% and 66% seed set, respectively.

The meiotic chromosome pairing behavior in *S. halepense* is quite variable and has from 7 to 20 bivalents, 0 to 5 quadrivalents, and rarely (2% of cells) a hexavalent or an octavalent in its pollen mother cells (PMC) (Hoang-Tang and Liang, 1988). Similar meiotic irregularities were observed in PMCs of colchicine-doubled (2n=4x=40) *S. bicolor*. Hoang-Tang and Liang (1988) reported as many as 9 quadrivalents in tetraploid *S. bicolor* with a mean of 3.79 per cell, more than double that in *S. halepense*. Thus, because of the irregular chromosome pairing behavior and range of chromosome associations in both tetraploid species, their hybrids tend to be aneuploids.

A *S. bicolor* × *S. halepense* breeding program carries an increased risk of invasiveness because *S. halepense* is a noxious, invasive weed (Southern Weed Science

Society, 1998). Even crossing strategies utilizing a rigorous examination of rhizomatousness and several generations of backcrossing involve a degree of risk due to the invasiveness potential of the *S. halepense* genomic material (Piper and Kulakow, 1994; Paterson et al., 1995b; Arriola and Ellstrand, 1996). Invasiveness is a serious concern in biofuel crop development, particularly when using *S. halepense* germplasm, due to its high annual seed production and elongate, creeping rhizomes (Jessup and Dowling, 2015).

In contrast, *S. bicolor* × *S. propinquum* hybrids are meiotically regular and pair as bivalents at metaphase I (Doggett, 1988). Thus, chromosome doubling of *S. propinquum* provides an opportunity to create novel triploid, seeded-yet-sterile *S. bicolor* × *S. propinquum* hybrids with desirable biomass characteristics. The interspecific *P. glaucum* × *P. purpureum* (PMN) hybrid and the reciprocal cross *P. purpureum* × *P. glaucum* (King grass) have been proposed as dedicated perennial, seeded-yet-sterile bioenergy feedstocks (Jessup, 2013). Thus, development of a triploid *S. bicolor* × *S. propinquum* crop would potentially capture the same ecological benefits of perennialism, reduced soil erosion, increased soil organic carbon, and reduced fertilizer and pesticide inputs, while limiting invasiveness potential (McLaughlin and Walsh, 1998; Lewandowski et al., 2003; Robertson et al., 2011).

Colchicine-Induced Polyploidy

Colchicine is a naturally occurring compound that can be extracted from the corm of *Colchicum autumnale* L., which may contain as much as 0.4 % colchicine on a

dry-weight basis (Blakeslee, 1939). It has been used to induce chromosome doubling in different species such as barley, portulaca (*Portulaca grandiflora* Hook.), pumpkin (*Cucurbita pepo* L.), spinach, and *S. bicolor* (Blakeslee, 1939; Chin, 1946; Bremer-Reinders and Bremer, 1952; Hoang-Tang and Liang, 1988). Colchicine application can be accomplished by packing colchicine-soaked cotton pads at the crown of young seedlings, coleoptile excision to expose meristematic tissue to the colchicine source, exposure during anther culture, or by soaking seeds in colchicine either alone or in combination with emulsifying agents such as lanolin (Chin, 1946; Schertz, 1962; Saisingtong et al., 1996; Sartor et al., 2009). Colchicine-based seed treatments can be optimized by using a dilution series in which colchicine concentration is varied from 0.01 to 0.4 (w/v) and/or addition of 2% dimethyl sulfoxide (DMSO) in the colchicine solution (Blakeslee, 1939; Sartor et al., 2009).

The ploidy level of colchicine-treated plants can be determined by cytological counting of the number of chromosomes in their root tip cells or by counting the chromosomes and observing the meiotic behavior of the chromosomes in developing PMCs (Schertz, 1962). The ploidy level also can be estimated by measuring the DNA content in somatic cells in leaf tissue using flow cytometry (DeLaat et al., 1987; Saisingtong et al., 1996; Doležel et al., 2007).

Seed Priming as a Colchicine Seed Treatment

Protocols for exposing seeds to colchicine typically involve placing seeds on moist germination paper such that the rate of imbibition is not controlled (Chin 1946;

Sartor et al., 2009). Thus, introduction of colchicine and/or DMSO over comparatively longer exposure periods allowed via priming solutions represents an opportunity to increase the chances of polyploid induction. Solid matrix priming (SMP) is well-suited for this purpose since it involves the admixture of seeds, a solid carrier such as calcined clay, and water to provide a specific matric potential for optimum priming (Khan et al., 1992). The concentration, duration, and temperature optima for colchicine seed treatment can therefore be established using low-cost materials and repeatable methods. Use of a controlled imbibition rate has the potential to improve the efficacy of polyploid induction. While not all colchicine trials report the total number of seeds or seedlings exposed to the treatment(s), Sartor et al. (2009) recovered only two tetraploid plants from 150 treated *Paspalum plicatulum* Michx. seeds. Colchicine treatment can be lethal to developing seedlings and this reduces the potential number of polyploids recovered (Sanders et al., 1959; Glowacka et al., 2010).

CHAPTER II

SEED PRIMING EFFECTS ON HEAT AND DROUGHT STRESS DURING ESTABLISHMENT IN PEARL MILLET X NAPIERGRASS HYBRIDS: GROWTH, YIELD, AND CHLOROPHYLL AUTOFLUORESCENCE

Introduction

Sustainable energy production from biomass requires a net shift away from starch-based sources because of finite grain supplies and the need for grain as a feed and food source, which represents a food security risk (Valentine et al., 2012). Lignocellulosic biomass sources such as perennial grasses can be produced without competing with food production. Pearl millet-napiergrass (PMN) hybrids have potential as a biomass candidate crop because of their high water and nutrient use efficiency, and they can be grown on more than 445 million ha of marginal and abandoned agricultural lands worldwide (Cox et al., 2002; Campbell et al., 2008). Utilization of marginal and abandoned lands as a biomass resource would not impact major food crop production, and it would reduce soil erosion, improve net carbon balance, and provide refuges for wildlife (Follett, 2001; Lemus and Lal, 2005).

Similar to seedless watermelon, PMN is produced by crossing diploid and tetraploid parents to obtain viable seed. Therefore, PMN is a strong biomass candidate crop because it is a 'seeded-yet-sterile' triploid, thus limiting the risk of invasiveness (Burton, 1944; Jessup and Dowling, 2015). Stands of PMN can be established using integrated agronomics typically limited to large-seeded annual row crops, unlike

vegetatively propagated biomass candidates such as Miscanthus and energycane. In addition, PMN yields approach or even surpass energycane (20 Mg ha^{-1}) in tropical environments (Cuomo et al., 1996).

Given the prospects for PMN cultivation in marginal and/or degraded subtropical or semi-arid lands, the need exists to develop agronomic strategies that will improve the rate of successful stand establishment. Seed priming, a presowing hydration treatment in which seeds are hydrated to a moisture content that initiates early physiological event of germination while preventing radicle protrusion, is potentially a valuable tool for PMN stand establishment. Priming increases germination rate and uniformity in perennial grasses (Yamamoto et al., 1997; Hacisalihoglu, 2007). Improved stand establishment may improve establishment-year biomass yields, which is a critical factor in the economic viability of dedicated bioenergy feedstocks (Perrin et al., 2008).

Solid matrix priming (SMP) is a priming technique in which known proportions of seed, a moist solid carrier, such as calcined clay or vermiculite, and a water-based solution are mixed to provide a low-matric potential medium (Taylor et al., 1988; Khan et al., 1992). SMP has been used successfully to improve germination and establishment in bermudagrass, bahiagrass, (Hacisalihoglu, 2007), pepper, and tomato (Khan et al., 1992). Typically, seed priming treatment occurs at a temperature (5° – 25°C) and duration (1 d to several wk) that depends on species, cultivar, and even seedlot (Khan et al., 1992; Capron et al., 2000; Ma et al., 2003). Following SMP, seeds are dried to the pre-priming moisture content for subsequent storage and/or sowing.

In addition to improving rate and uniformity of germination, seed priming can induce stress cross-tolerance, or improved performance, when seeds are subjected to abiotic stresses such as drought and salinity (Mei and Song, 2008; Patade et al., 2009). Because seed priming imposes a mild stress on seeds due to reduced matric potential, priming itself may constitute a stress which activates a stress-responsive system (Bruce et al., 2007). This 'stress imprinting' during priming can improve seed performance when subjected to subsequent stresses (Chen and Arora, 2013).

During SMP, seeds are hydrated and exhibit increases in metabolic activity. Synthetic compounds that potentially can result in further improvement in optimal and non-optimal conditions can be introduced in the priming solution. Epigenetic changes have been proposed as a mechanism for explaining 'stress imprint' in primed seeds (Bruce et al., 2007). These changes involve modification of DNA activity via histone modification, methylation, or chromatin remodeling (Madlung and Comai, 2002). Chilling stress in maize led to 5-methylcytosine demethylation in roots that was not reversed upon removal of the stress (Steward et al., 2002). The demethylating agent 5-azacytidine (5-AC) has been used during seed imbibition to study chromosome organization in wheat (Santos et al., 2002). In triticale, addition of 5-AC-induced hypomethylation reactivated rDNA genes from the rye genome (Neves et al., 1995), which increased the rate of wheat-rye chromosome intermixing in the nuclei from 15 to 60% (Castilho et al., 1999). Therefore, 5-AC is a strong candidate compound for inclusion in SMP protocols for the interspecific hybrid PMN.

Another candidate compound is chitosan, a deacetylated oligosaccharide polymer derived from chitin. The term 'chitosan' covers a heterogeneous assortment of oligomers having glucosamine subunits that vary considerably in their exact composition, depending on the source (Freepons, 1991; Bautista-Baños et al., 2006). Chitosan is often utilized in foliar applications as a antitranspirant that induces ABA-dependent stomatal closure (Ludwig et al., 2010). Also, chitosan seed soaks (< 12 h) have controlled downy mildew and improved germination and seedling growth rate in pearl millet (Sharathchandra et al., 2004).

PMN plantings are likely to occur in heat- and moisture-stress environments. In pearl millet, significant genotypic variation exists for thermosensitivity, and poor establishment has been attributed directly to high soil- and air temperatures (O'Neill and Diaby, 1987; Peacock et al., 1993). Preferably, the evaluation of primed PMN hybrids in daytime heat and/or drought stress conditions will be accomplished using non-destructive techniques. Chlorophyll fluorescence has been established as a sensitive indicator for plant water and temperature stress (Lang et al., 1996; Subhash et al., 2004). A fluorescence imaging system using high-intensity blue (460 nm) LED lamps has been developed to assess seedling water stress; differences in steady-state chlorophyll fluorescence were observed in cabbage seedlings in various stages of water stress (Hsiao et al., 2010). Blue LED (460 nm) excitation-based data were similar to those measured using UV and laser excitation methods while having the advantage of illuminating the entire leaf (Heisel et al., 1996; Langsdorf et al., 2000; Hsiao et al., 2010). In contrast, laser-based methods illuminate relatively small areas within a leaf.

Previous evaluations of the efficacy of seed priming have consisted of germination tests and/or short-term (1-6 wk post-planting) seedling emergence studies in soil plantings. While it was expected that SMP would provide abiotic stress tolerance during early establishment, a key question was whether this benefit would persist over the course of a longer time frame, such as over an entire growing season. The overall goal of the present research was to provide an in-depth assessment of the effect of SMP on pearl millet-napiergrass in daytime heat and/or drought stress environments in terms of stand establishment, chlorophyll fluorescence and biomass yield using a time frame of 12 wk that provided an approximation of one growing season. The duration of the study allowed for intensive chlorophyll fluorescence data collection, which was critical for its development as a breeding tool.

The objectives of this study were to:

1. Assess the efficacy of SMP, with and without the elicitor compounds 5-azacytidine and chitosan, on three PMN hybrids and one pearl millet check cultivar subject to (a) heat- and (b) heat- plus drought-stress environments. Specifically, the SMP treatments were assessed using whole-plant responses including emergence percentage, emergence rate, morphological development, and harvestable biomass.
2. Evaluate a blue-light LED excitation technique for quantifying chlorophyll steady-state fluorescence to determine plant stress response at the canopy level. The blue-light LED fluorescence method was used to evaluate both (i) the SMP treatments *per se* and (ii) to differentiate

among the three PMN hybrids in their heat- and/or drought-stress tolerance.

Materials and Methods

Experimental Materials and Design

Seed of the F_1 hybrid pearl millet 'BMR-209' and three PMN hybrids –5098273 × 'Merkeron' (5098273), Tift 8577 × 'Merkeron' (Tift), and 09TX04 × 'Merkeron' (09TX04)– were used in these experiments. This experiment was conducted at the Perennial Grass Breeding Field Laboratory at Texas A&M University in College Station, Texas, USA (30.622° latitude, -96.359° longitude). The basic experimental unit was a set of 25 seeds planted individually into Deepots (Steuwe & Sons, Inc., Tangent, OR, USA) each having a depth of 18 cm and diameter 5 cm. Sunshine Redi-Earth growing media was used (Sunshine Horticulture Bellevue, WA). Planting depth was 6 mm. The seeds were planted on 12 June 2014. Three replicates of each treatment × entry were placed into each of three growing environments in a completely randomized design and grown for 12 wk. The growing environments were: (1) a control environment in a growth chamber with a 12 h photoperiod; (2) a greenhouse daytime heat stress environment; and (3) a greenhouse heat plus drought stress environment. In the growth chamber (controlled conditions), illumination was approximately 4300 lum m^{-2}, and mean light/dark temperatures were 32.0 ± 1.0 °C and 23.0 ± 1.0 °C, respectively. Illumination was 3.22×10^4 lum m^{-2} in both greenhouse environments. Weekly mean maximum and minimum temperatures and day/night mean temperatures for the

greenhouse environments are shown in Fig. 1. Temperature and illuminance data for all environments were collected using a HOBO® U12-011 Temperature/Relative Humidity Data Logger (Onset Computer Corp., Bourne, MA, U.S.A.) that was set to record one observation every 5 min. Imposition of the drought stress treatment was accomplished by twice-weekly watering to 50% field capacity during the first 4 wk of the study and thrice-weekly watering during the final 8 wk of the experiment.

Seed Priming Treatments

Prior to planting, 900 seeds of each genotype were exposed to the following treatments: (a) an untreated control; (b) a standard solid matrix priming (SMP) protocol (described below); (c) a SMP protocol using the demethylating agent 5-azacytidine (see below); and (d) a SMP protocol as per treatment (b) followed by a foliar application of a 1:19 (v/v) dilution of a liquid chitosan product (Elexa®4 Plant Defense Booster, Plant Defense Boosters Inc., Syracuse, NY, USA) at the fifth-leaf stage of development, which was on 11 July 2014, or 4 wk after planting.

The media used in all SMP treatments was a commercial calcined clay product, Agsorb® 40/100 LVM (Oil-Dri Corporation, Chicago, IL, USA). The standard solution for SMP in treatments (b) and (d) was 5 g L^{-1} KNO_3 and 1 g L^{-1} Banrot® 40WP fungicide (The Scotts Company LLC, Marysville, OH, USA). The solution used in treatment (c) above was 80 µM 5-azacytidine, as per Santos et al. (2002). Priming for each PMN hybrid was accomplished by placing 1.25 g seed and 10.00 g calcined clay media in a 10.2 x 15.2 cm resealable polypropylene bag and adding 3.5 mL of the

respective solution for each treatment. Once combined, the bag was shaken to obtain a homogeneous mixture. For pearl millet, a ratio of 10.00 g seed, 20.00 g clay, and 9.0 mL solution was used in all SMP treatments. Previous experiments (A. Watson, unpublished) established the ratio of seed:media:solution for PMN and pearl millet resulting in optimal performance for seedling vigor. SMP optimization was accomplished using a seed:clay ratio of 1:2 and using solution levels of 0.9, 1.0, and 1.1. Additionally, SMP durations of 3, 4, and 5 d were examined for each proportion of materials; therefore, the optimal SMP protocol was the result of nine distinct treatments.

The ratios of seed:clay:solution used in this trial resulted in a seed moisture content (MC) during priming of 29.0%. The initial, pre-priming MC was 6.0% for pearl millet and 5.9-6.7% for the PMN hybrids. After adding the solution, each bag was subsequently mixed thoroughly, sealed, and allowed to incubate at 18 °C for 5 d. Supplemental lighting was not used during SMP.

Every 24 h, each priming bag was opened to allow gas exchange, mixed thoroughly, and inspected for protrusion of radicles or pericarp cracking. After 48 h, solid matrix primed seeds typically equilibrated to a steady-state MC. Thus, daily moisture samples were taken from each bag beginning 48 h after initiating priming to determine if the seeds had reached the target MC of 29.0% ± 1.0 percentage unit. Seed MC samples were placed in a forced-air oven at 65 °C for 2 h to determine seed MC. Upon completion of priming, seeds were separated from the calcined clay media using a 30 mesh stainless steel screen and allowed to air-dry 48 h at 22 °C and 35% RH.

Thereafter, seeds were dried in a forced-air oven at 32 °C for 4 h to a final seed MC of 6.5%.

Data Collection and Statistical Analysis

Seedling emergence was recorded daily for 14 d after planting and weekly thereafter. Emergence rate was calculated according to Maguire's equation (Maguire, 1962): $M = n_1/t_1 + n_2/t_2 + \ldots n_{10}/t_{10}$; where n_1, n_2, …, n_{10} are the number of emerged seedlings at times t_1, t_2, …, t_{10}. Thus, the units for emergence rate are number of seedlings d^{-1}. Emergence rate was calculated using the first 10 d of emergence data because all experimental units had reached maximum emergence within 10 d after planting.

Every 7 d after emergence, each seedling was assessed for shoot morphological stage of development as per Gustavsson (2010). This evaluation system was developed principally as a forage crop management tool; it included vegetative, elongation, and reproductive phases of development. Twelve wk after planting, plants were harvested at a height of 1 cm and clippings were placed into a forced-air oven at 50 °C for 48 h to determine aboveground biomass. Yield, emergence percentage, and emergence rate data were analyzed as a completely randomized, fixed-effects model using SAS® statistical software (SAS Institute, 2011). Means separation procedures for emergence percentage, emergence rate, and yield were done using Tukey's test at an $\alpha = 0.05$ level of significance. Weekly morphological development and digital imaging data (described

below) were analyzed with the general linear models procedure using weekly data as a repeated measure, split-plot in time approach.

A set of orthogonal contrasts was used to elucidate entry and treatment effects on seedling morphological development and chlorophyll fluorescence. These contrasts were designed to accomplish two objectives. The first objective was to compare the performance of PMN 09TX04 to the other two entries for each treatment. PMN 09TX04 is an elite selection in biofuel yield trials because of its capacity to tiller profusely and achieve high biomass yields. The second objective was to compare the control and standard SMP for each of the three PMN hybrids. The contrasts also include a comparison of AZA vs. CH for 09TX04 to evaluate the efficacy of these treatments.

Imaging Analysis

Weekly imaging of the seedlings began 5 wk post-planting. For the image collection, plants dark adapted at least 30 min were placed in a light-proof box having horizontal dimensions of 70 × 102 cm. Throughout the experiment, the height of the box was adjusted so that images were taken 60 cm above the canopy height 20° from the vertical. After placement inside the box, seedlings were exposed to a 465 nm (13 W Advance Spectrum LED Grow Light Bulb) and a 430 nm (36 W Advanced Spectrum MAX ALL BLUE 3w-Chip LED Grow Bulb) bulb for 4 min. Images were taken using a Nikon® Cool Pix S560 camera in the JPEG (joint photographic experts group, .jpg) format at a 3 megapixel resolution. Captured images were downloaded to a personal computer for subsequent analysis.

Image analysis was performed using the software package ImageJ (version 1.48; Schneider et al., 2012). Prior to analysis, each image was cropped such that, for each image, the maximum gray level from each channel was determined by using the OTSU method for separation of background and foreground, thereby generating a region of interest from which to extract area and mean gray level data (Otsu, 1979). Mean gray level was used as the steady state fluorescence value (F_m) for each digital image. In the mean gray level system, 0 is black and 255 is white.

Results and Discussion

Climatological Summary

The light/dark temperature regime for seedlings grown in the controlled, growth chamber (CGC) conditions was 32 °/23 °C, respectively. Seedlings for both the heat stress and heat plus drought stress environments were grown on adjacent benches in the same greenhouse; therefore, the data summarized in Fig. 1 apply to both environments. Generally, the mean daytime temperature (the arithmetic mean of 144 observations taken from 0700 to 1900 daily) was more than 35 °C each wk of the study in the two heat stress-related environments. The grand mean daytime and nighttime temperatures were 37.2 ° and 28.9 °C, and the grand mean daily maximum and minimum temperatures were 43.6 °C and 26.9 °C, respectively.

Temperatures in the heat stress environments gradual warmed for the first 4 wk of the study followed by alternating warming and cooling from wk 4 to wk 8. Seedlings experienced the most extreme temperatures during wk 8 to wk 10. During this period

the daytime maximum temperature was more than 45.0 °C for 24 consecutive days. For nine consecutive days (wk 9-10) maximum temperatures were more than 50 °C, during which approximately 75% of the seedlings in the heat and drought stress treatment died. The highest single temperature measurement recorded during this experiment was 56.0 °C on 21 Aug 2014, approximately 10 wk after planting.

Chitosan foliar sprays for all seedlings were done 4 wk after planting. Foliar chitosan treatment reduces transpiration in plants by inducing stomatal closure (Bittelli et al., 2001; Iriti et al., 2009), but also tends to increase leaf temperature by 1 C° (Ludwig et al., 2010). The mean daily high temperature for the 5 d following chitosan treatment in the daytime heat stress and heat and drought stress environments was 45.3 °C, which may have offset the potential benefits of this treatment.

Seedling Emergence

The number of fully emerged seedlings 14 d after planting was used for determination of total emergence percentage. The emergence rate calculation utilized in this experiment is a function of both emergence percentage and uniformity, thus giving a useful indicator of whether the SMP treatments effectively improved PMN seedling emergence.

The ANOVA for emergence percentage and rate in the CGC environment indicated that the only significant ($p<0.01$) effect was the treatment effect on emergence rate (Table 1). Among entries in the growth chamber, there was a trend for PMN hybrids 5098273 and 09TX04 to have a slightly higher soil emergence percentage and

rate than PMN Tift (Table 2). Emergence rate of each SMP-related treatment was more than the control in the controlled growth chamber (Table 3). This was due mainly to the effect of SMP on rapid emergence in the first 4 d post-planting. Seedlings in control treatments reached 30-50% of maximum emergence within 2 d after planting while seedlings in the SMP, SMP + 5-azacytidine (AZA), and CH treatments were 50-70% emerged during the same period. In all environments, seedlings attained maximum soil emergence within 5 d after planting.

The ANOVA for the heat stress environment resulted in significant effects for emergence percentage by entry ($p<0.01$) and for emergence rate by treatment ($p<0.01$) and entry × treatment ($p<0.05$; Table 1). PMN hybrid 09TX04 had the highest overall emergence percentage in the daytime heat stress environment, and it was more than the pearl millet check hybrid (Table 2). Only SMP alone had emergence rate more than the control in the heat stress, well-watered environment (Table 3). This was due mainly to the slightly higher overall emergence percentage relative to the other SMP treatments. Even though the treatment effect was not significant, there was a general trend for SMP-treated seedlings having greater total emergence than the control. For PMN hybrid 5098273, emergence rate of SMP was more than the control and CH treatments (Table 5). Total soil emergence for the SMP- and CH-treated 5098273 seedlings was 90.7 and 80.0 %, respectively (Table 4), which resulted in the higher calculated emergence rate for SMP. The reason for the discrepancy in soil emergence between the SMP and CH treatments is unclear because both treatments were primed with the same protocol; the chitosan foliar spray was not applied until 4 wk post-planting. Two potential reasons for

the discrepancy between these two treatments are variability within the seedlots used and in the priming media itself.

In the heat plus drought stress environment, there was a significant ($p<0.01$) treatment effect for total emergence percentage and entry ($p<0.01$), treatment ($p<0.01$), and entry × treatment ($p<0.05$) for emergence rate (Table 1). Emergence rate for two of the three PMN hybrids was greater than the pearl millet check (Table 2). Among treatments in the heat plus drought environment, emergence rate of all SMP-related treatments were more than the control, and emergence percentage of SMP was more than the control (Table 3). Similar to the heat, well-watered environment, primed PMN seedlings reached approximately two-thirds maximum emergence within 48 h after planting. In addition, no seedlings had emerged in the first 24 h after planting in any experimental treatments. SMP reduced the time from 0 to maximum soil emergence from 96 to 48 h for PMN. This improvement in germination synchrony is potentially important for PMN stand establishment, particularly in challenging abiotic stress conditions. Standard SMP improved total emergence, on average, by 4.0 percentage units among the three PMN hybrids in the heat and drought stress regime (Table 4). Emergence rate was improved by standard SMP in two of the three PMN entries under heat and drought stress (Table 5).

Overall, the treatment that improved total seedling emergence and emergence rate the most consistently among the three environments was the standard SMP protocol. In the heat- and heat plus drought stress conditions, emergence percentage of SMP trended higher than controls for all four entries. Examination of the control emergence

rate data among PMN hybrids showed that Tift trended higher than the other entries in the daytime heat stress environment, and it was significantly ($p<0.05$) higher under heat and drought stress conditions.

Morphological Development

Morphological development was measured using a decimal scale developed for perennial forage grasses by Gustavsson (2010). In this scheme, stages 0-9 apply only to germination and were not utilized in this experiment; stages 10-19 describe leaf development (stage 11 = first fully expanded leaf, stage 12 = 2nd fully expanded leaf, etc.); stages 20-29 apply to tillering (stage 21 = main shoot + one tiller, stage 22 = main shoot + two tillers, etc.); and stages 30-39 correspond to stem elongation (stage 31 = first node palpable; stage 32 = two nodes palpable, etc.). Morphological stages 40-49 describe the various stages of booting; stages 50-59 describe inflorescence emergence (from the stage of the first spikelet being visible to the inflorescence bearing internode being visible above the flag leaf); and stages 60-69 describe anther development. In the experimental conditions used, fruit development and ripening (stages 71-97) did not occur in any of the entries used. Pearl millet, the only annual, did not reach the fertilization stage because of the extreme daytime heat stress experienced by the seedlings. Because PMN hybrids Tift and 5098273 produced few tillers, one might conclude that these entries were in the tillering stage during wk 7 to wk 12. For these entries, data points in the range of 20-29 represent a population consisting of seedlings in

the late vegetative (stages 15-19) and early elongation (stages 31-34) stages of development.

In the CGC, there was a significant ($p<0.05$) entry main effect across all sampling dates (Table 7). This was due primarily to the relatively rapid development of pearl millet, an annual, compared to the slower-developing perennial PMN hybrids. There were significant treatment main effects on seedling morphology at weeks 2, 5, 8, and 9. Additionally, there were significant entry × treatment interactions at weeks 2 and 7-9. During weeks 4-8, SMP- and AZA-treated PMN 09TX04 seedlings were more advanced developmentally than the other PMN entries in the growth chamber environment due to earlier and more frequent tillering (Table 8). For all three PMN hybrids in the CGC, SMP-treated seedlings were more advanced than the control for at least two sampling dates, but there were no significant control vs. SMP contrasts beyond wk 8. This is because, during the final 4 wk of the experiment, the PMN seedlings in the CGC were transitioning from vegetative to elongation stages of development. Typically, these PMN hybrids have a 2-3 wk lag period between the expansion of the uppermost fully expanded leaf and when the first node becomes palpable. Therefore, the treatments that tended to lag behind morphologically essentially "caught up" during the final 4 wk of the study.

PMN seedlings in the daytime heat stress environment showed the most uniformity in terms of morphological development among the three sets of environmental conditions (Table 9; Fig. 2). Similar to the CGC, the highly significant ($p<0.01$) entry main effect was due to the differences in growth patterns between pearl

millet and PMN. During the first 5 wk, PMN 09TX04 seedlings developed faster than the other PMN entries, mainly due to early tiller development (Table 10). Five wk post-planting, less than 3.0 % of PMN Tift and 5098273 seedlings had at least one secondary tiller in the heat stress regime. For PMN 09TX04, the percentage of seedlings with one or more secondary tillers was 7.1% for the control, 11.8% for SMP, 16.9% for AZA, and 8.6% for CH. Each PMN hybrid showed an advantage of approximately one growth stage for SMP compared to the control 4 wk after planting. Patterns of development for the control and SMP were similar for the remainder of the study for Tift and 5098273, but SMP-treated 09TX04 trended higher than all other treatments in wk 9-12 under daytime heat stress conditions. This is because the 09TX04 seedlings treated with SMP in this environment had a shorter vegetative-to-elongation lag period. Ten wk after planting, 64% of SMP-treated 09TX04 seedlings had reached the elongation stage, compared to 40, 45, and 37% for the control, AZA, and CH treatments, respectively (data not shown).

There was a significant ($p<0.05$) entry main effect on seedling morphology at wk 3 and a highly significant ($p<0.01$) entry effect in the heat plus drought stress environment (Table 11). In contrast to the CGC and daytime heat stress conditions, pearl millet seedlings did not advance beyond the vegetative stage of development. Generally, the development of PMN hybrids 09TX04 and 5098273 was more advanced than Tift from wk 3 to the end of the experiment in wk 9 (Fig. 2). As noted above, it was not possible to collect seedling morphology data in the heat plus drought stress environment beyond wk 9 due to seedling death. In contrast with the other two

environments, treatment differences between 09TX04 and the other two PMN hybrids tended to persist across sampling dates in the heat plus drought stress regime (Table 12). The advanced development of PMN 09TX04 vs. others was particularly noticeable in the control and CH treatments; the SMP-treated 09TX04 had a morphological stage score higher than the others at 2, 5 and 6 wk after planting. While there were no clear among-treatment differences for Tift and 5098273, development of AZA-treated 09TX04 lagged behind all other treatments throughout most of the experiment in heat plus drought stress (Fig. 6). Another trend was a slight edge in shoot development for CH-treated 09TX04 from wk 5-7, which corresponds to the three sampling dates immediately after chitosan application.

Shoot morphological development under the severe heat plus drought stress conditions imposed in this experiment was marked mostly by the inability of the seedlings to advance beyond the vegetative stage within 9 wk after planting. Compared to the heat, well-watered environment, senescence of the lower leaves was relatively common. PMN hybrid 09TX04 trended as being more morphologically advanced than the other hybrids. By the conclusion of morphology data collection, PMN hybrids Tift and 5098273 had 5 to 7 fully emerged, green leaves on the primary tiller compared to nearly 8 for PMN 09TX04. Finally, there were 5 entry × treatment combinations in which some seedlings survived the full 12 wk of the experiment. These were the control (7.7%) and SMP-treated (35.2%) seedlings for Tift and the SMP (25.4%), AZA (31.8%), and CH (3.3%) treatments for 09TX04. Thus, there is evidence that SMP treatment of PMN can induce abiotic stress-related mechanisms in plants that can make a difference

not only in terms of seedling growth but also in regard to survival. The seedling survival of AZA-treated 09TX04 under the extreme stress challenge imposed by the combination of temperatures more than 50 °C and water stress indicate that additional research is justified to continue evaluating demethylation agents and interspecific hybrids such as PMN to produce novel phenotypic effects.

Blue-Light Chlorophyll Fluorescence

For the mean relative fluorescence data, higher absolute numbers corresponded to a higher relative degree of plant stress. Yellowish foliage coloration tended to result in higher (i.e., closer to white) absolute scores, while green foliage typical of plants under minimal to moderate stress resulted in lower relative fluorescence. A comparison among entries for each of the three environments is given in Fig. 7. In the ANOVA (Table 13), the only entry main effects for relative fluorescence were during wk 9 in the CGC ($p<0.10$) and wk 6 in the heat plus drought environment ($p<0.01$). Fluorescence values at the various sampling dates followed similar trends in the CGC and daytime heat stress environments. During wk 4 and 5, mean fluorescence was approximately 100, and this value trended upward to 110-115 for the remaining sampling dates. The controlled growth chamber values were near 105 compared to 95-98 in the heat stress environment at wk 4. The slower-growing seedlings in the CGC environment had not attained canopy closure 4 wk after planting. Thus, the mean fluorescence value recorded at wk 4 was affected by background that was not completely removed from the foreground.

In CGC conditions, the fluorescence values for pearl millet trended higher than the three PMN hybrids from 8 to 10 wk after planting (Fig. 7). This is because the pearl millet seedlings had reached the reproductive stages of development; senescence of the lower leaves combined with the coloration of the inflorescence itself resulted in higher fluorescence values at those sampling dates. One of the tendencies of the blue-light imaging system utilized was that yellow-colored foliage (whether due to stress- or development-related leaf senescence) shifted fluorescence values upward relative to green, actively photosynthesizing tissue.

Unfortunately, there was a significant amount of seedling death in the CGC during the last 4 to 5 wk of the experiment, especially in PMN 09TX04. This resulted in the presence of a higher ratio of background (soil) to foreground (plant tissue), which interfered with the reliability of the results. Further refinement in the thresholding step during image processing could improve fluorescence values obtained from such images. Therefore, it is not possible to draw definite conclusions from the contrasts, treatment- and entry × treatment data presented in Table 14 and Figs. 8 and 9, respectively.

In contrast to the CGC, the fluorescence data from the daytime heat stress environment was more consistent and reliable. PMN 5098273 showed a trend toward being slightly more stressed than the other entries for most sampling dates, while 09TX04 was among the least stressed, particularly at wk 10 (Fig. 7). Chitosan-treated seedlings trended as being the most stressed among treatments from wk 7 to 10 when exposed to daytime heat stress, which is not surprising given the anticipated reduction in stomatal conductance and elevated leaf temperature associated with this compound (Fig.

8). While there was some tendency for the relative fluorescence of CH to exceed other treatments under heat stress, this trend was prevalent for PMN Tift (Fig. 10).

The linear contrasts for the daytime heat stress environment showed that, for the PMN hybrids Tift and 5098273, SMP-treated seedlings were under greater stress than control plants (Table 15; Fig. 10), but only in the middle of the experiment. For the final sampling dates, these two treatments trended similarly. PMN 09TX04 treated with SMP was significantly less stressed at wk 7 and 10 relative to the other two hybrids. All PMN hybrids, at the final sampling date, had a mean fluorescence value for the SMP treatment approximately less than or equal to all other treatments. The most-stressed treatments in the daytime heat stress environment among all entries at wk 10 were either AZA or CH.

ANOVA for the fluorescence data in the heat plus drought stress environment showed a highly significant ($p<0.01$) entry main effect at wk 6 and significant ($p<0.05$) entry × treatment interactions at wk 6 and 7 (Table 13). The main entry effect was due to the higher fluorescence for pearl millet than the PMN hybrids. PMN 09TX04 trended as being less stressed than the other entries, particularly during wk 8-10 (Fig. 7). Therefore, primed 09TX04 seedlings were more advanced morphologically, under less stress in terms of the photosynthetic apparatus, and managed to survive to some extent under the acute thermal and moisture stress in this experiment. The general agreement among these variables also serves to validate the usefulness of the blue-light fluorescence method.

While there were no significant treatment main effects for the fluorescence data in any of the three environments, Fig. 8 serves to illustrate the differences in relative

fluorescence values among environments. In the daytime heat stress environment, mean fluorescence increased from 95-100 during wk 4-5 to 110-116 during wk 6-10. The latter sampling dates correspond to an extended period of mostly steadily rising daily mean and maximum temperatures (Fig. 1). In the combined heat plus drought stress environment, the values are slightly higher than in the heat environment, but the pattern is similar for wk 4-5 and again from wk 6 to 9. At wk 10, however, the period in which maximum temperatures were above 50 °C, a sharp increase in relative fluorescence was observed. These observations may be helpful in determination of threshold values for establishing benchmarks if the fluorescence technique is utilized in the field and/or for making breeding selections.

The PMN hybrid × treatment comparison in the heat plus drought stress environment (Fig. 11) showed that, with a few exceptions, control and SMP seedlings had similar relative fluorescence trends. Results of the linear contrasts for fluorescence in the heat plus drought stress regime are given in Table 16. The most consistent trend was CH-treated 09TX04 was less stressed than others at wk 6, 9-10. Thus, despite the CH-treated 09TX04 being **less stressed** than the other treatments during wk 8-10 based on fluorescence data, only **two seedlings survived** the extreme heat event at wk 10.

The PMN hybrids examined in this study tolerated the stress conditions imposed relatively well. Few differences among treatments and hybrids (within environments) were noted. Therefore, a more comprehensive survey of germplasm is needed to validate the method as a breeding tool. Each of the PMN hybrids in this research had the same male napiergrass parent. Analysis of PMN hybrids with different pedigrees as well

as a survey of other species would be highly beneficial in determining whether blue light steady-state fluorescence can be used to evaluate breeding lines. Obviously, field data needs to be collected. The other key element in continued evaluation of the method is determining if the differences in fluorescence are color-based (green- vs. yellow-colored foliage) or are truly an indicator of the relative efficiency of photosynthesis under stress conditions. Improvements in imaging analysis will be highly beneficial in improving data accuracy.

The blue-light fluorescence imaging technique utilized in this experiment demonstrated the potential efficacy of the method. The coefficient of variation (CV) ranged from 2.0 to 7.0% among environments and sampling dates. The CV was smaller at the earlier sampling dates, but environment- and treatment-related differences tended to become more noticeable toward the end of the experiment. Plant health status also has been assessed extensively with chlorophyll sensors such as the SPAD-502 (Minolta Corp., Ramsey, NJ) and the CCM-200 (Opti-Sciences, Tyngsboro, MA); CV values reported for the SPAD-502 have ranged from 10-17%, while the CV for the CCM-200 has exceeded 20% (Casa et al., 2014; Taskos et al., 2015). The blue-light fluorescence instrument used in this study utilized two relatively high-illumination sources. One difficulty in analyzing the canopy-level images was the glare on part of the upper leaf surface, which may have biased the relative fluorescence values recorded. An array of lower wattage LEDs placed in both the vertical and horizontal plane relative to the plants would improve the overall design. Another limitation was that the digital images were captured from above the canopy, which essentially is a record of the relative stress level

of the uppermost leaves. One of the main differences between the heat- and heat plus drought stressed seedlings was that the moisture stress resulted in senescence of the lower leaves. Thus, capturing images at an angle of 45° or less from the horizontal may have given a more complete depiction of plant health. Nevertheless, the blue light-based digital imaging system showed that relative steady-state fluorescence values were the highest (i.e., highest stress level) in the combined heat plus drought stress environment, and that PMN 09TX04's relative fluorescence was greater than the other PMN hybrids. Field evaluation of the PMN hybrids would likely be accomplished most efficiently during early- and mid-season time frames before the plants reach a size that prohibits effective sampling with an enclosed, light-proof frame. Depending on the prevailing environmental conditions, plant stress assessment can be timed to coincide with abiotic stress events such as extended periods of heat, chilling, and/or moisture stress.

Seedling Yield

ANOVA of dry matter seedling yield data showed a highly significant ($p<0.01$) entry main effect in the CGC and daytime heat stress environments, and a significant treatment main effect in the ambient ($p<0.10$) and heat plus drought ($p<0.05$) environments (Table 1). Also, there was a significant ($p<0.10$) entry × treatment interaction in the CGC and heat stress environments. Seedling yield data by entry for all environments are given in Table 2. In the controlled growth chamber environment, PMN 09TX04 yielded poorly across all treatments due to seedling death in the growth chamber, which negatively affected all PMN hybrids to a greater or lesser degree. PMN

Tift seedling yield was significantly more than the other PMN hybrids under daytime heat stress, and it also ranked as the highest-yielding PMN hybrid in the heat plus drought stress environment.

Even though the treatment main effect was not significant in the CGC environment, the mean seedling yield for all three SMP-related treatments was at least 38% higher than the control (Table 3). Seedling yields among treatments were generally similar in the daytime heat stress environment. In the combined heat plus drought environment, however, SMP was more than the control, representing a 43% increase in seedling yield.

Seedling yields of all entry × treatment combinations in each environment are in Table 6. All PMN hybrids in the CGC had SMP yield ranked higher than the control. Seedling survival for control and SMP of 09TX04 in the controlled growth chamber environment was approximately equal; seedling yield per plant was 0.17 and 0.28 g for the control and SMP treatments, respectively, a 65% increase when expressed on a per-plant basis (data not shown). Even though the overall entry × treatment interaction was significant in the heat stress environment, there were no significant differences among PMN hybrid × treatment combinations. While control- and SMP-treated Tift seedling yields were similar in the heat environment, yields of SMP-treated 5098273 and 09TX04 trended 39 and 29% higher, respectively, than the control. Seedling yield of AZA-treated PMN Tift ranked lowest in the heat stress environment, which was largely a factor of seedling survival. Among the three replications, only 54 AZA-treated plants survived in this treatment compared to 70 for the control. Similar to daytime heat stress

conditions, seedling yield for the control under heat plus drought stress was approximately equal to SMP for Tift but SMP ranked higher than the control in the other two hybrids. Seedling yield of SMP-treated 5098273 and 09TX04 was 38% higher than the control for both PMN hybrids under heat plus drought stress.

Conclusions

This research involved growing pearl millet and PMN seedlings in a greenhouse during the summer months in which air temperatures were often 10-12 C° higher during the day than the official meteorological observation at this location, and growing seedlings in a chamber that represented a control (abiotic-stress free) environment. The fact that PMN seedlings, particularly in the heat, well-watered stress regime, progressed through the vegetative, tillering, and elongation stages, clearly demonstrates the capability of this interspecific hybrid to withstand thermal stress. In particular, seedling survival of SMP- and AZA-treated PMN hybrid 09TX04 in the combination of extreme heat and limited water supply shows that breeding efforts utilizing this germplasm are justified. Solid matrix priming is an effective delivery technique for elicitor compounds such as 5-azacytidine (5-AC).

Optimization of SMP requires varying the proportions of seed:media:solution, temperature, and duration during the priming process. In these experiments, the same SMP protocol was used for all three PMN hybrids and pearl millet, mainly due to the small quantities of seed available. The benefits of SMP on PMN may be improved from additional experiments using different temperature regimes such as alternating

temperatures or moderate abiotic challenges during priming such as short-duration heat or chilling stress. Similarly, optimization of beneficial effects of the combination of SMP and 5-AC in PMN priming likely requires further research, especially regarding the concentration of 5-AC.

The blue light fluorescence data did not show clear, consistent trends of SMP-treated seedlings being less stressed than control plants. Chitosan-treated plants had higher relative fluorescence than all other treatments in the daytime heat stress, well-watered environment. Possibly the application of chitosan, by reduction of stomatal conductance and a concomitant increase in leaf temperature, imposed an additional stress on those seedlings. This trend was not noticed in the heat plus drought conditions, which may have been because seedlings under moisture deprivation had already reduced stomatal conductance, thus having an "equalizing" effect among treatments.

In terms of the PMN hybrid comparison using the fluorescence technique, it appeared that the three hybrids responded similarly with the exception of PMN 09TX04 exhibiting a lower relative fluorescence in the daytime heat plus drought stress regime, thus giving this hybrid some credibility as an elite selection especially considering its tendency to produce a high number of tillers. Despite this, the general conclusion for hybrid evaluation with the blue light technique is that three PMN selections that all have the same male parent tended to respond similarly to one another within each environment. It was thought that the heterozygous nature of the napiergrass parent 'Merkeron' and the general morphological differences (e.g., the tendency for PMN Tift to produce a single tiller and for 09TX04 to tiller somewhat profusely) would have been

enough diversity to result in among-hybrid fluorescence differences. While it would certainly be useful to collect additional fluorescence data from a wider germplasm base, whether PMN, related species, or other crops, the objective for the technique was to evaluate germplasm within the context of a specific breeding program. In summary, it was encouraging that when a moisture stress was imposed, it was possible to detect a difference among hybrids using the fluorescence technique. In both the CGC and daytime heat stress environments, in which the seedlings had adequate moisture, only transient differences were observed among entries.

The PMN hybrids studied were selections from a biomass breeding program. Therefore, the evaluation of SMP on seedling yield 12 wk after planting was critical. The main treatment effect in the CGC environment was not significant ($p = 0.08$), but SMP, AZA, and CH treatments had yields at least 38% higher than the control. Standard SMP treatment was greater than the control for all entries in heat plus drought stress conditions. This was because of improved emergence percentage due to SMP and higher biomass per plant. In heat and drought stress, total emergence of SMP was more than the control in Tift, 5098273, and 09TX04 by 9.3, 21.4, and 14.6 percentage units, respectively. Solid matrix priming increased Tift's, 5098273's, and 09TX04's mean biomass production per plant by 29.3, 100.4, and 24.3 %, respectively, in the combined heat plus drought stress trial. Clearly, SMP is a valuable tool for PMN stand establishment and in obtaining reliable first-year yield, a critical factor in the economics of biomass crops.

CHAPTER III

SEED PRIMING EFFECTS UPON INDUCED CHROMOSOME DOUBLING OF *SORGHUM PROPINQUUM*

Introduction

Annual *S. bicolor* and perennial, rhizomatous *S. propinquum* belong to the subgenus *Eu-sorghum* within the genus *Sorghum* and both species have 2n=2x=20 chromosomes (de Wet et al., 1976). *Sorghum propinquum*, a non-domesticated species, is native to southeast Asia (de Wet, 1978). These two species will hybridize readily; *S. bicolor* × *S. propinquum* hybrids are meiotically regular and their chromosomes pair as bivalents during metaphase I (Doggett, 1988).

Cultivated *S. bicolor* is the world's fifth most important cereal crop (FAOSTAT, 2015), and it is also a leading biofuel candidate because of its drought tolerance, high nitrogen use efficiency, and ability to produce relatively high yields of lignocellulosic biomass, grain, and/or stem sugar (Chittenden et al., 1994; Feltus et al., 2006; Rooney et al., 2007; Heaton et al., 2008). Dedicated biofuel crops are being developed to reduce carbon emissions from conventional, fossil fuel-based energy sources. *Sorghum bicolor* × *S. propinquum* hybrids have potential for sustainable biofuel production because of the combination of desirable biomass characteristics of *S. bicolor* together with the perennial, rhizomatous nature of *S. propinquum*.

Invasiveness potential is a serious concern when evaluating candidate biofuel crops. *Sorghum bicolor* x *S. halepense* hybrids have raised concerns regarding weediness

due to the aggressively rhizomatous nature of *S. halepense* (Jang et al., 2006). In contrast, the rhizomes of *S. propinquum* are relatively short, thus greatly reducing the weediness potential of this species (Washburn et al., 2013). Doubling the chromosome number of *S. propinquum* to produce a tetraploid (2n=4x=40) form of the species and then hybridizing diploid *S. bicolor* with the tetraploid *S. propinquum* makes possible an opportunity to produce a triploid, sterile bioenergy feedstock having removed seed- and reduced rhizome-invasiveness potential. Thus, hybridization of diploid *S. bicolor* with tetraploid *S. propinquum* would have the advantages of producing viable seed from the F_1 cross that is combined with sterility of the offspring. As a perennial bioenergy feedstock, these 'seeded-yet-sterile' hybrids also could provide a positive carbon balance, reduce soil erosion, and reduce fertilizer and pesticide inputs (McLaughlin and Walsh, 1998; Lewandowski et al., 2003; Robertson et al., 2011). Furthermore, triploid *S. bicolor* × *S. propinquum* hybrid stands can be established using the integrated agronomics typical of large-seeded, annual row crops, thereby giving this cropping system an advantage over vegetatively propagated biofuel crops such as *Miscanthus* × *giganteus* and energycane.

Chromosome doubling in *Sorghum* has been accomplished using plant- and seed-based colchicine applications (Chin, 1946; Schertz, 1962). Seed treatments with colchicine also have induced chromosome doubling in *Paspalum plicatulum* Michx. (Sartor et al., 2009), pepper (Pal and Ramanujam, 1939), and wheat (Müntzing and Runquist, 1939). Protocol development for optimum results in colchicine seed treatments requires varying concentration from 0.01 to 0.4% (w/v) and duration from 24

hr to 8 d (Blakeslee, 1939; Sartor et al., 2009). Pal and Ramanujam (1939) reported that chromosome doubling in pepper ranged from 6.9 to > 70% with the highest level of doubling occurring at a concentration of 0.2 and 0.4% colchicine. Colchicine-based treatments therefore require an effective minimum critical concentration while the duration must be sufficient to allow colchicine diffusion into target tissues without resulting in mortality (Dermen, 1940). The addition of dimethyl sulfoxide (DMSO) to colchicine solutions has improved the efficacy of chromosome doubling. In a doubled haploid experiment with barley using a 0.1% colchicine solution, Subrahmanyam and Kasha (1975) reported that the proportion of doubled tillers per plant was 55.2% and 66.3% when 2% and 4% DMSO was added, respectively, compared to 30.8% for the colchicine-only treatment. DMSO penetration into intracellular spaces can be limited if seeds are not hydrated and/or metabolically active (Pluenneke and Burson, 1973).

Seed priming is a preplanting, controlled hydration technique in which seeds imbibe water and become metabolically active, but water potential is held at a point that prevents radicle protrusion (McDonald, 2000). After completion of priming, seeds can either be planted immediately or dried back to their pre-priming moisture content for subsequent planting. Primed seeds typically have improved germination rate and uniformity (Bradford, 1986). During priming, seeds are metabolically active; important cellular functions such as DNA and mitochondrial repair, gene transcription and translation, cell cycle activity, and reserve mobilization occur (Chen and Arora, 2013). Solid matrix priming (SMP) is a technique that involves mixing seed, a solid carrier, and a water-based solution in known proportions at a specified temperature and duration

(Taylor et al., 1988). In SMP, matric potential is utilized to control seed hydration rate and it affords an opportunity to treat seeds with colchicine such that uptake occurs relatively slowly compared to uncontrolled imbibition on germination blotter or filter paper.

The objectives of this research were to: (1) determine if colchicine can successfully induce tetraploidy in *S. propinquum* seeds using SMP-based colchicine seed treatment; and (2) compare SMP-based colchicine seed treatment to a germination blotter paper-based method. Currently, there are no published reports of colchicine-based polyploid induction in *S. propinquum*. The recovery of tetraploid *S. propinquum* germplasm is essential for the development of triploid, 'seeded-yet-sterile' *S. bicolor* × *S. propinquum* hybrids and their subsequent evaluation as a biofuel candidate crop.

Materials and Methods

The germplasm source of *S. propinquum* used in this experiment was a single seed lot from the USDA-ARS germplasm collection at the Southern Plains Agricultural Research Center in College Station, TX. Prior to colchicine treatment, 900 seeds were surface sterilized with a 1:9 dilution of bleach:water, allowed to surface dry, and divided into nine sets of 100 seeds for each treatment. Each treatment was further divided into 4 replicates of 25 seeds each. One set of 100 seeds was used as a non-treated control. For each replication in non-SMP treatments, 25 seeds were placed on germination blotter paper in petri dishes 7.5 cm in diameter. In each petri dish, 25 mL of the following solutions were added: (1) 0.01% (w/v) colchicine; (2) 0.01% colchicine and 2% (v/v)

DMSO; (3) 0.1% (w/v) colchicine; and (4) 0.1% colchicine and 2% DMSO. Next, petri dishes were placed in a germination chamber for 24 h in a 12h/12h light/dark cycle using a light temperature of 30°C and 1690 lum m^{-2} of illumination followed by the dark period at 20°C. After completion of the colchicine treatment, seeds were placed in a stainless steel wire mesh strainer and rinsed with running water 5 min to remove colchicine residue.

Colchicine-based SMP treatment was done by placing each replicate of 25 seeds (100 seeds per treatment) into a 10.2 x 15.2 cm resealable polypropylene bag with 10.00 g of calcined clay (Agsorb® 40/100 LVM, Oil-Dri Corporation, Chicago, IL, USA). For each of the four colchicine and/or DMSO concentrations stated above, 3.5 mL of solution was added to each priming container and placed in a 20 °C environment for 5 d with no supplemental lighting. Once a solution was added, each bag was shaken to obtain a homogeneous mixture. Every 24 h, each priming bag was opened to allow gas exchange, mixed thoroughly, and inspected for protrusion of radicles or pericarp cracking. Upon completion of priming, seeds were separated from the clay media using a 30 mesh stainless steel screen followed by a 5 min water rinse as described above.

Upon completion of the colchicine and/or DMSO treatments and rinsing, seeds were allowed to air-dry on paper towels for 4 h for ease of planting. Thereafter, seeds were planted into Sunshine Redi-Earth growing media (Sunshine Horticulture Bellevue, WA) in 2.5 cm x 2.5 cm cell trays and placed in a greenhouse environment.

The number of emerged seedlings was recorded daily (emergence rate), and the emerged seedling percentage was calculated after 10 d. Seedlings were assessed 14 d

and 21 d post-planting to ensure that the reported final emergence percentage did not include abnormally developed seedlings and accounted for seedling death in the interim. Emergence rate was calculated according to Maguire's equation (Maguire, 1962): $M = n_1/t_1 + n_2/t_2 + \ldots n_{10}/t_{10}$; where $n_1, n_2, \ldots, n_{10}$ are the number of emerged seedlings at times $t_1, t_2, \ldots, t_{10}$. Germination and emergence data were analyzed as a completely randomized, general linear model using SAS® statistical software (SAS Institute, 2011). Means separation procedures were done using Tukey's test at an $\alpha = 0.05$ level of significance.

Twelve wk after planting, surviving plants from each treatment were transplanted into 3.0-L individual pots. Approximately 18 wk after planting, when the seedlings had reached at least the fourth-leaf stage of development, the ploidy levels of all the plants were determined using flow cytometry. Approximately 4 cm^2 of leaf tissue obtained from two adjacent and opposite leaves of each seedling was bulked and chopped with a razor blade in 0.6 mL of buffer for cell extraction. The buffer solution was prepared as per Galbraith (1989): 8.8 g L^{-1} sodium citrate dihydrate, 4.2 g L^{-1} MOPS, 4.26 mL L^{-1} $MgCl_2$, 1.0 mL L^{-1} Triton X-100, and 100 µL L^{-1} RNase A. After all the reagents were combined, the pH was adjusted to 7.2. Following 2 min of incubation, 1.0 mL of additional buffer was added to each sample, followed by filtration through a 30 µm mesh into a sample tube. Next, 50 µL of 1 mg mL^{-1} propidium iodide was added to each sample and the sample tubes were placed on ice for at least 15 min. Suspensions were analyzed with a CyFlow® flow cytometer (Partec GmbH, Münster, Germany). At least 2000 nuclei were counted for each sample. Data were analyzed using the Partec

FloMax® software. Both known diploid and tetraploid *Sorghum* samples were included to establish the relative positions of 2C and 4C peaks at both respective ploidy levels.

Results and Discussion

Colchicine-Based Induction of Tetraploidy

Results from the colchicine treatments are listed in Table 17. Germination of seed on pads moistened with colchicine did not result in doubling the number of chromosomes in any *S. propinquum* plants. Solid matrix priming at a 0.1% colchicine concentration with DMSO resulted in seven tetraploids, a 13% success rate among surviving plants, while the remaining SMP treatments did not produce any 4x plants. Because of the limited availability of *S. propinquum* seed, it was not possible to germinate more seed on pads treated with colchicine for longer durations of time. The seven tetraploid plants were analyzed again 14 d later using a different set of opposite leaves to confirm the earlier results.

The shorter duration (24 hr) of the germination pad treatments compared to 5 d of SMP could explain why polyploidization was unsuccessful with the former method. Pad-treated seeds may not have received sufficient colchicine exposure for polyploidization to occur. Also, the colchicine concentrations used in this experiment may have been insufficient, particularly with the germination pad technique. Pal and Ramanujam (1939) reported more than 50% of surviving pepper plants were polyploid using 0.2% and 0.4% colchicine concentrations for 24 and 48 hr exposure periods, respectively.

Colchicine seed treatment can reduce seedling survival and result in abnormally developing seedlings with rough, thickened leaves and fasciation of the stems (Pal and Ramanujam, 1939; Sanders et al., 1959; Sartor et al., 2009). Only 63 of 384 (16%) pad-treated seeds survived while 149 of 384 (39%) of the SMP-treated seeds survived. The slower, controlled rate of water uptake during seed priming may have influenced seedling survival. During uncontrolled water imbibition, as in the germination pad treatment, seeds typically have a moisture content more than 50% within 4 to 6 h. Moisture content of solid matrix primed *S. bicolor* seeds (using the same materials and protocol in this experiment) was 18% 4 h after the start of priming, 22% at 8 hr, and gradually rose to a steady-state moisture content of 27% at 72 h (Watson, unpublished). During priming, embryo cells advanced from the G_1 to G_2 phase of the cell cycle whereupon the cycle was arrested, allowing synchronization to occur (Ozbingol et al., 1999). Cell division occurs earlier and to a greater extent in primed seeds as compared to control seeds (Varier et al., 2010). Thus, even though it is difficult to compare the efficacy of SMP to the germination pad method because of the difference in the duration time of colchicine exposure; SMP potentially can improve the overall efficacy of colchicine seed treatments.

Seedling Final Emergence and Emergence Rate

Final emergence percentages are given in Fig. 12. The overall trend in this experiment was higher total soil emergence for SMP-treated compared to pad-treated seeds. Only the solid matrix primed, 0.1% colchicine with DMSO treatment had

significantly ($\alpha = 0.05$) greater emergence than the pad treatments. Increasing colchicine rates can improve polyploid induction, but often is associated with reduced seedling survival. In this experiment, the colchicine concentrations used may not have been high enough to have a more detrimental effect on overall seedling emergence percentage.

Emergence rate, given in Fig. 13, shows a clear improvement in uniformity of emergence in all treatments using the SMP method. Interestingly, the SMP, high colchicine with DMSO treatment, the only treatment having any tetraploid plants, also had the highest overall emergence and emergence rate score. This may be due to the relatively high variability within the *S. propinquum* seed lot used, and not necessarily a function of the colchicine and DMSO concentration. The Maguire (1962) emergence rate calculation is affected by both the *earliness* of emergence and the *total number* of seedlings, which explains why the primed, high colchicine with DMSO rate was significantly higher than in the other SMP treatments. Essentially, all the pad-treated seeds emerged gradually from 5 to 10 d after planting. In contrast, the SMP seeds emerged more uniformly from 4 to 7 d after planting. Specifically, SMP *S. propinquum* emergence increased from 0 to approximately 50% of maximum emergence during a 24 hr period, from 3 to 4 d post-planting.

Conclusions

This study demonstrated that SMP is an effective technique in successfully inducing polyploidy in *S. propinquum* using colchicine and DMSO. Due to the limited availability of *S. propinquum* seed, examination of a wider range of colchicine

concentrations and exposure times was not possible in this experiment. Nevertheless, a SMP-based colchicine seed treatment has been validated. This may be beneficial in polyploid induction efforts in other taxa, particularly if an improvement in germination can be obtained. The tetraploid *S. propinquum* plants recovered from this research can be used subsequently in breeding efforts, including hybridization attempts with diploid *S. bicolor* to produce triploid, sterile hybrids as biomass feedstocks. Previous research has demonstrated that, while *S. propinquum* and *S. bicolor* readily intermate, the nucleolus is organized on a different chromosomes (Magoon, 1961) and inheritance of rhizome formation is polygenic in nature (Paterson et al., 1995b). Therefore, it remains to be determined if the effective transfer of perennialism will occur in diploid *S. bicolor* crosses with tetraploid *S. propinquum*.

The slower, more controlled water uptake rate during SMP may have been responsible for the increase in plant survival after colchicine treatment. Seed and seedling mortality is a critical limiting factor in colchicine-based polyploidization experiments. Simply stated, the greater the number of plants screened, the greater the potential for recovering doubled plants. Finally, it is important to note that no chimeric plants were found in this study during the evaluation period as described. Subsequent testing is likely needed to fully validate the findings in this experiment, as chimeric tissue can develop during subsequent plant development. An example of flow cytometric analysis depicting doubling of chromosomes in *Sorghum* is given in Fig. 14 (Wilson 2015, unpublished). While there is a possibility that the controlled hydration and relatively long exposure time of the SMP-based method of colchicine delivery

improved colchicine penetration into embryo tissue, additional research would be required to conclusively demonstrate this.

CHAPTER IV

SUMMARY

Pearl millet-napiergrass, a leading candidate biofuel crop for the southern U.S., was exposed to severe thermal stress temperatures routinely exceeding 45 °C with and without water stress and was established successfully. Among the variables measured, seedling yield improvement by SMP treatment was clear and, especially in the heat plus drought stress regime, consistent. The demethylating agent 5-azacytidine (AZA) showed potential in improving tillering in the elite hybrid 09TX04 x 'Merkeron'. Additional research is needed to establish the optimal AZA concentration in SMP-based seed treatment. Also, DNA methylation analysis should be done to verify the effects of AZA on PMN. The blue light-based fluorescence device can be modified for deployment in field conditions; this research established general benchmarks useful for assessing plant stress status and showed strong repeatability. Further research is needed to evaluate SMP-treated PMN in the field and to examine the expression of genes of interest, such as transcription factors located on biomass QTL and stress-response genes such as ROS scavenging enzymes and heat shock proteins.

The success in doubling *S. propinquum* chromosomes using an SMP-based approach for delivering colchicine opens novel breeding approaches for this species, either alone or in interspecific crosses. In particular, the cross of diploid *S. bicolor* x tetraploid *S. propinquum* for biomass purposes is an intriguing possibility if viable

progeny can be obtained. Furthermore, the SMP-based technique can be utilized in chromosome doubling studies of related species.

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APPENDIX A

FIGURES

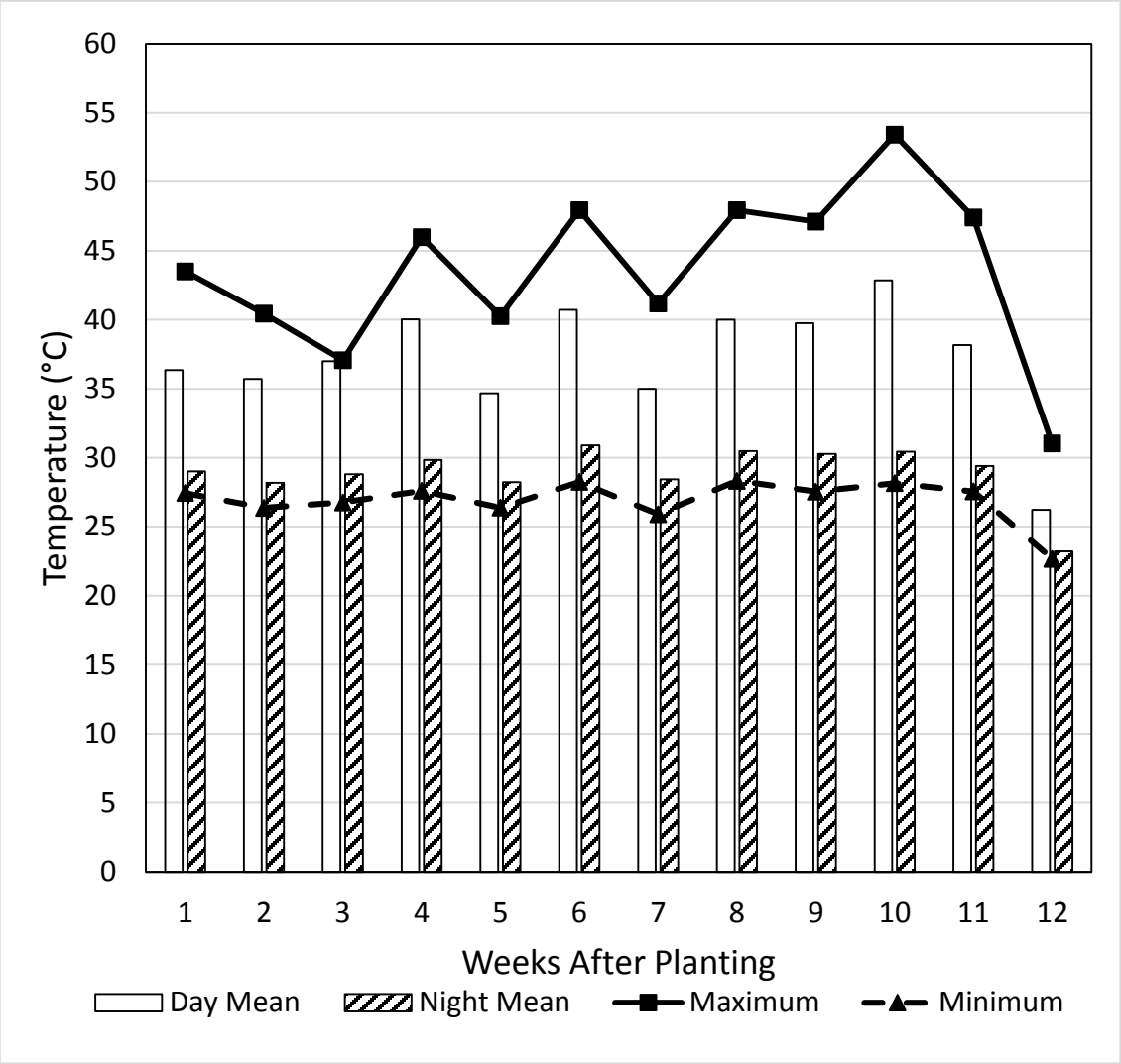

Figure 1. Weekly mean day and night (columns) and maximum and minimum (lines) temperatures in the daytime heat stress and heat and drought stress environments for SMP-primed PMN hybrids grown at the Perennial Grass Breeding Field Laboratory at Texas A&M University in College Station, Texas, USA from June 12 to September 4, 2014.

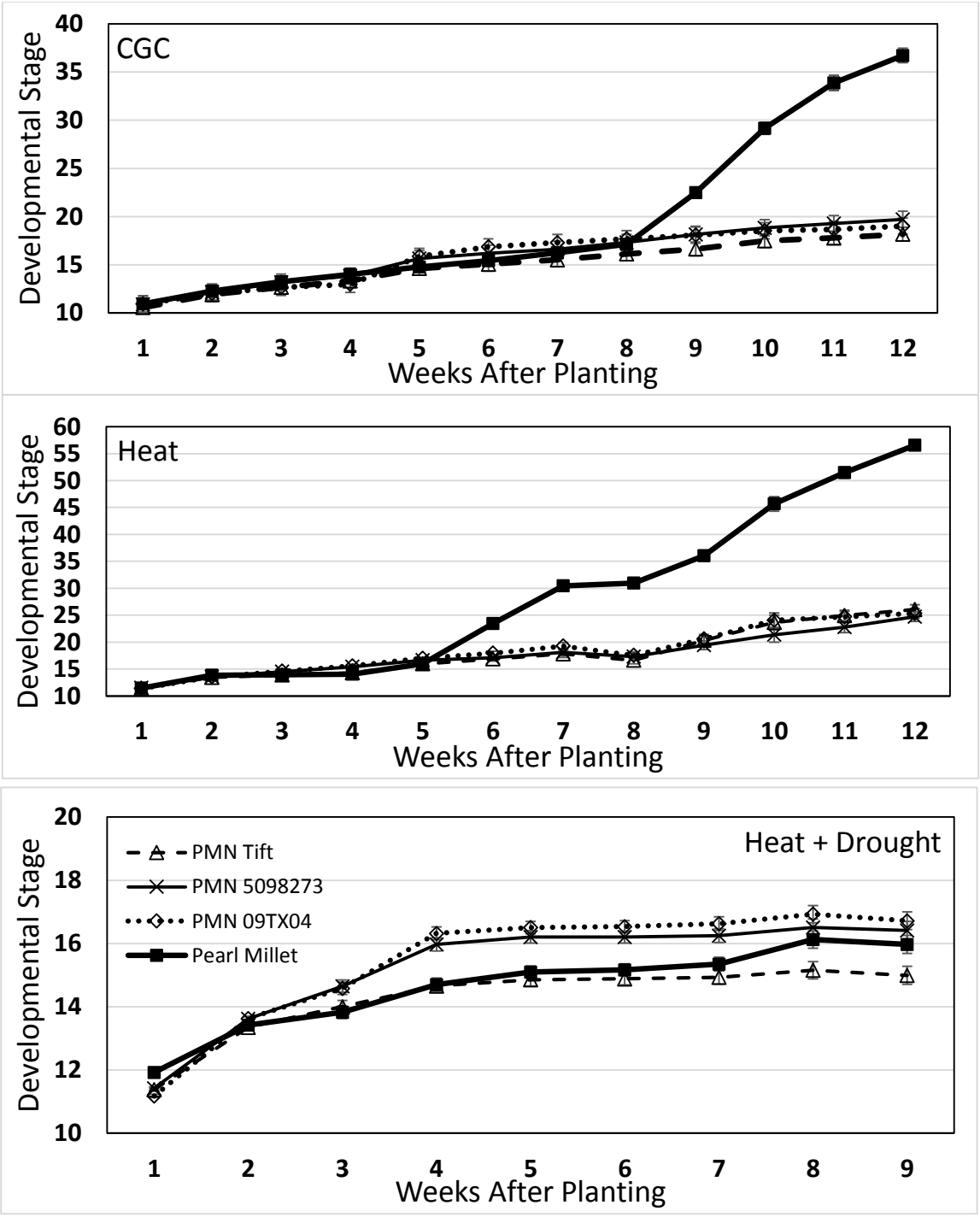

Figure 2. Morphological stage of development means by entry of three PMN hybrids treated with SMP in controlled growth chamber (CGC), daytime heat stress, and heat plus drought stress environments. Bars represent the standard error of the mean.

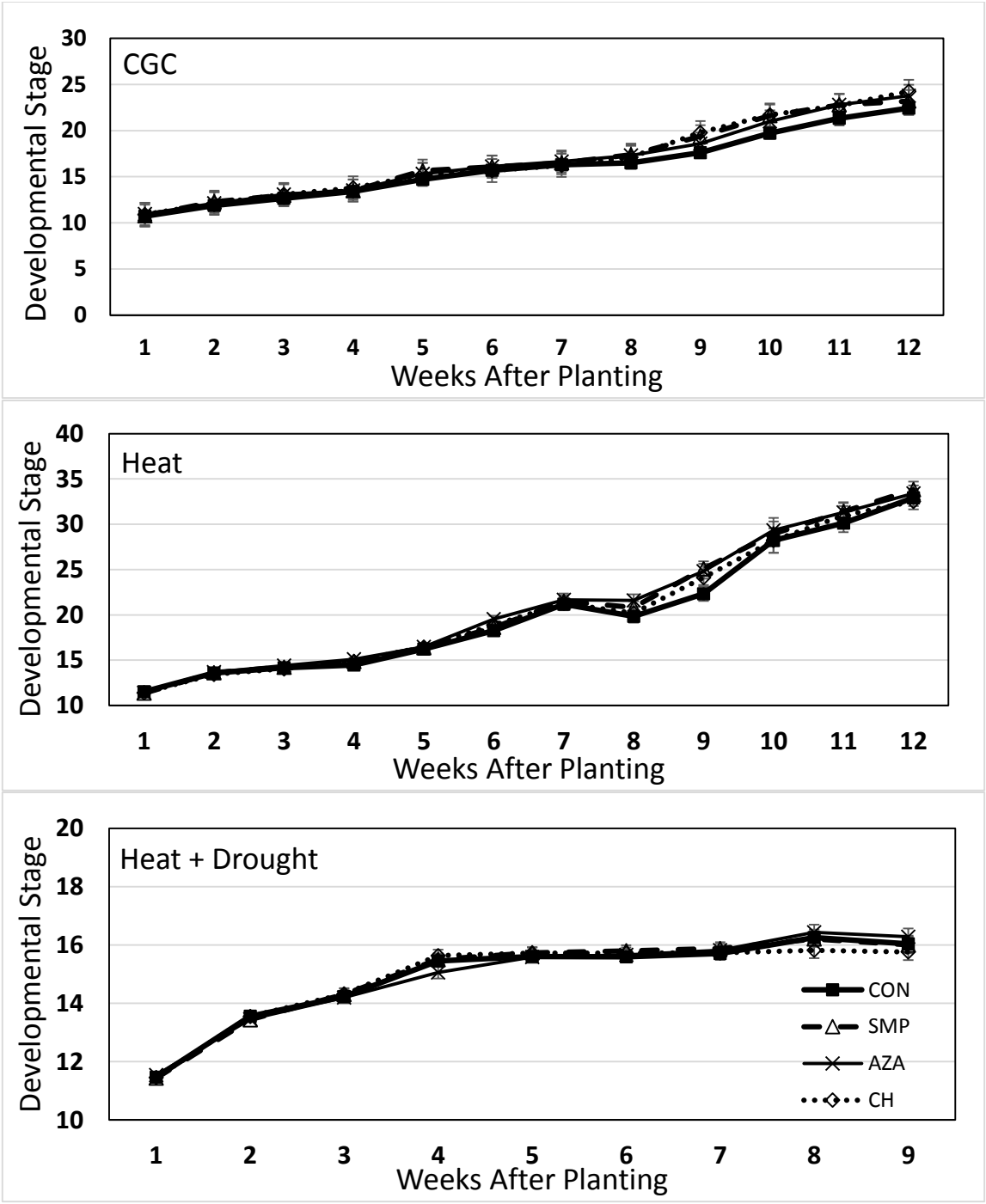

Figure 3. Morphological stage of development means by treatment† of SMP-primed PMN hybrids in controlled growth chamber (CGC), daytime heat stress, and heat plus drought stress environments. Bars represent the standard error of the mean.
† CON = Control; AZA = SMP + 5-Azacytidine; CH = SMP + Chitosan treatments

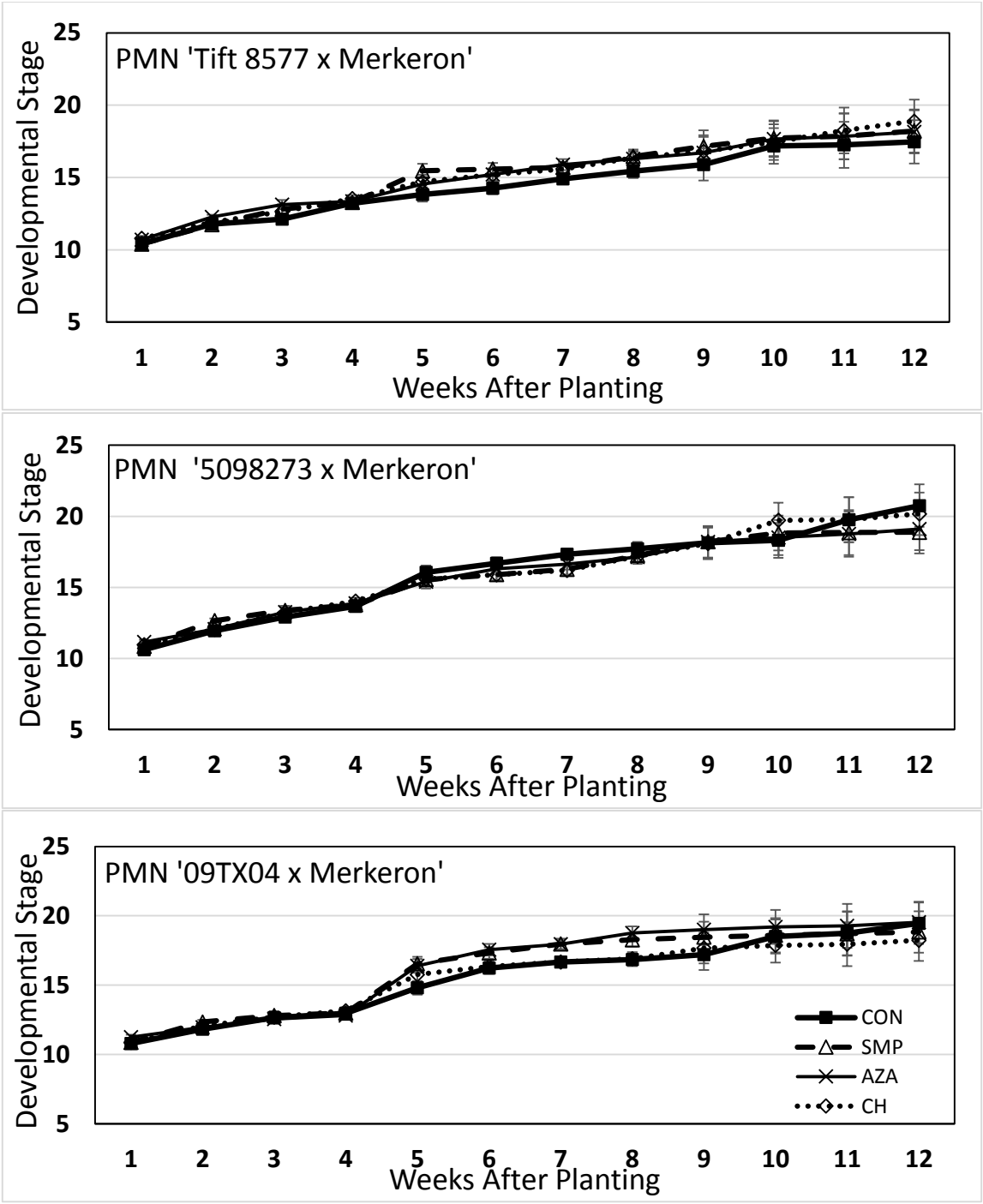

Figure 4. Morphological stage of development means by PMN hybrid x treatment† in controlled growth chamber (CGC) conditions. Bars represent the standard error of the mean.

† CON = Control; AZA = SMP + 5-Azacytidine; CH = SMP + Chitosan treatments.

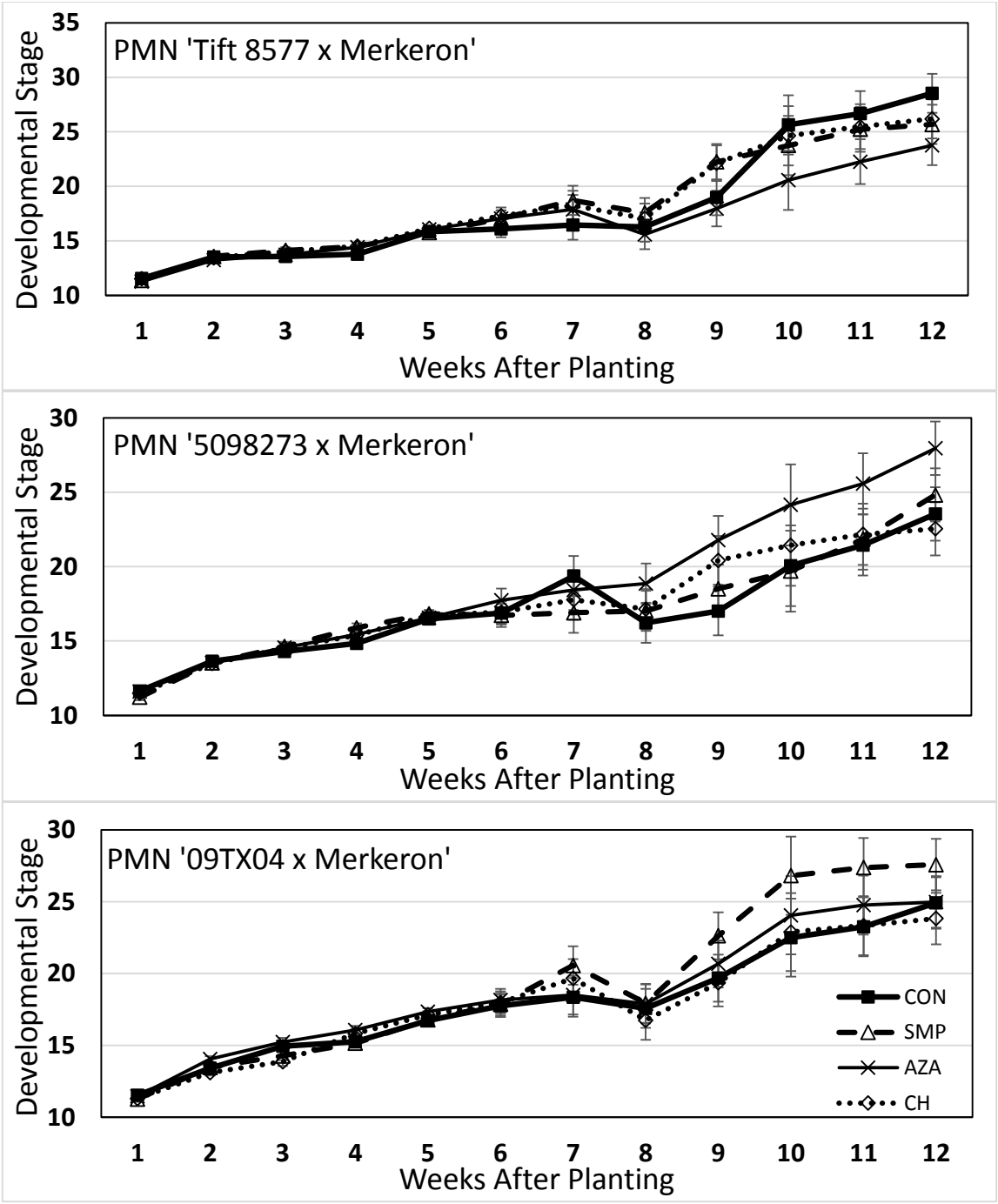

Figure 5. Morphological stage of development means by PMN hybrid x treatment† in a daytime heat stress environment. Bars represent the standard error of the mean.
† CON = Control; AZA = SMP + 5-Azacytidine; CH = SMP + Chitosan treatments.

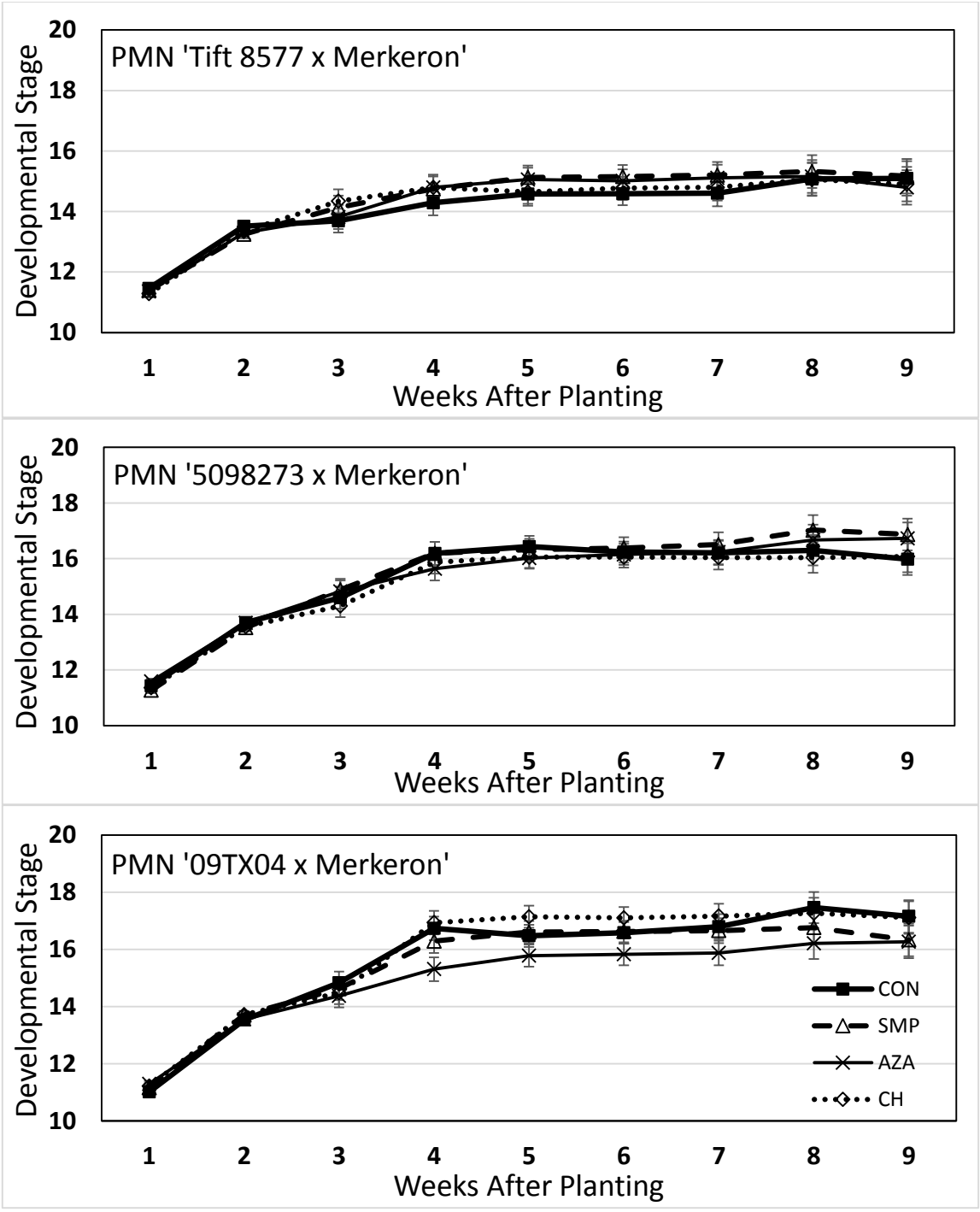

Figure 6. Morphological stage of development means by PMN hybrid x treatment† in a heat plus drought stress environment. Bars represent the standard error of the mean.
† CON = Control; AZA = SMP + 5-Azacytidine; CH = SMP + Chitosan treatments.

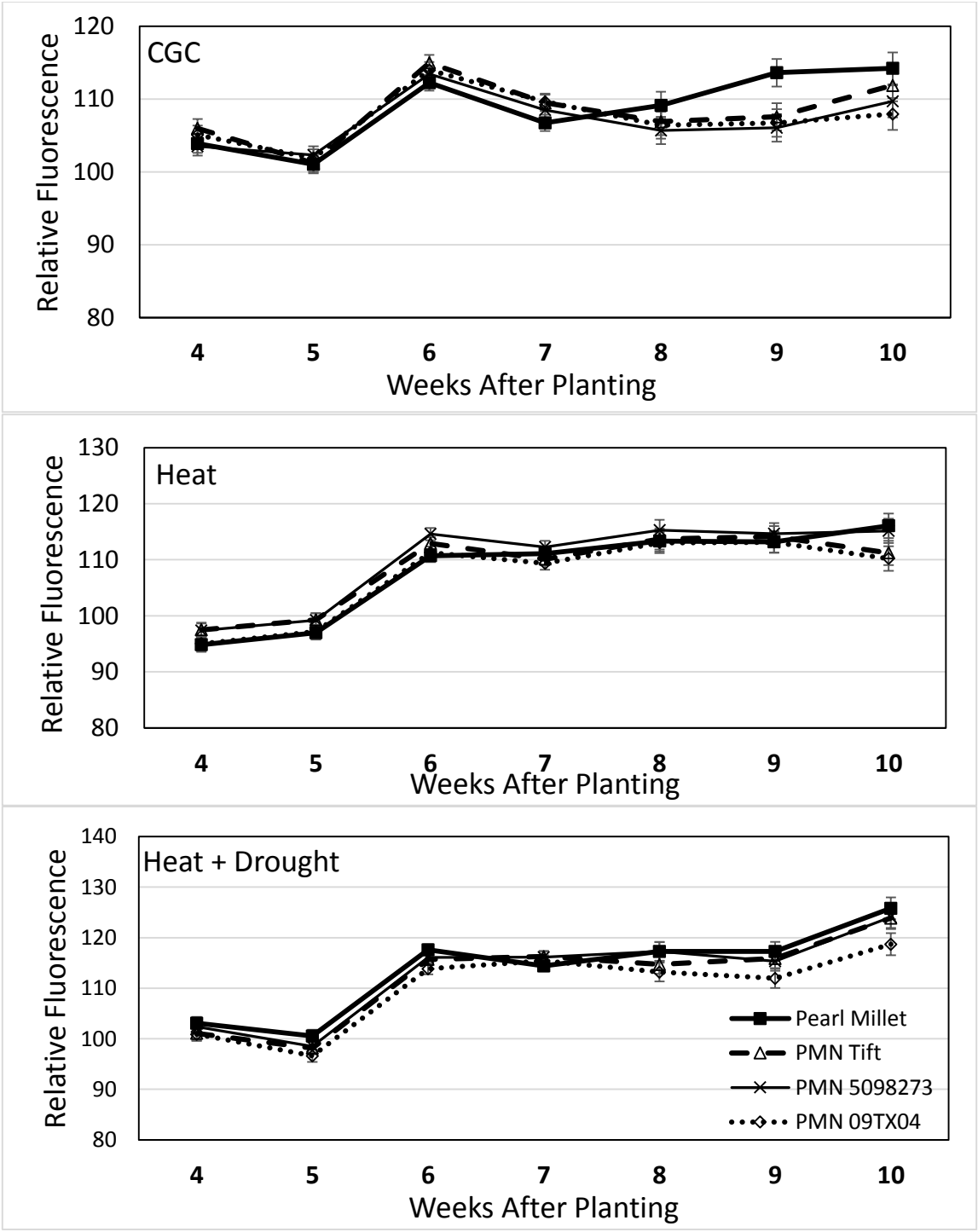

Figure 7. Mean relative fluorescence by entry of three SMP-primed PMN hybrids in controlled growth chamber (CGC), daytime heat stress, and heat plus drought stress environments. Bars represent the standard error of the mean.

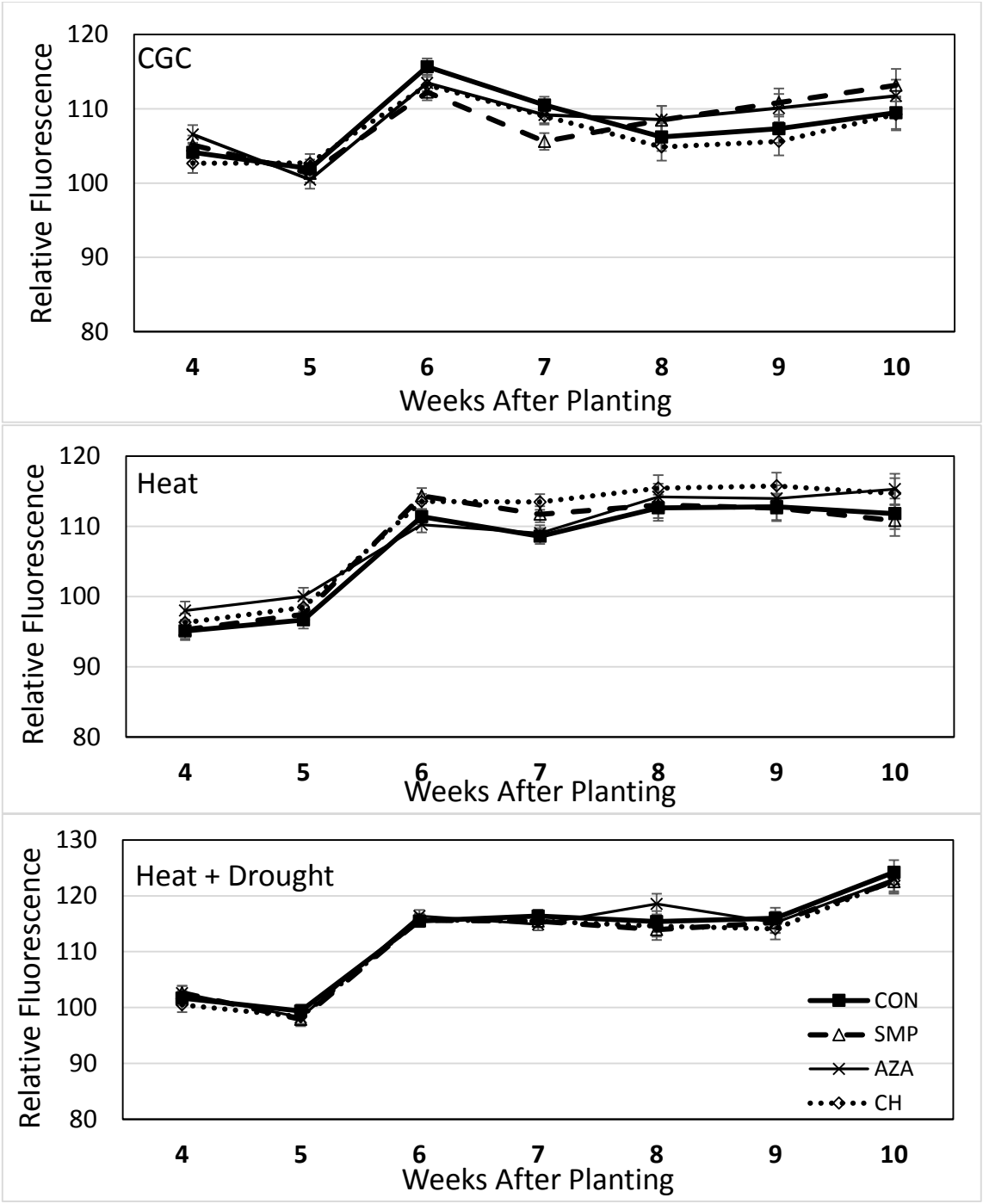

Figure 8. Mean relative fluorescence by treatment† of three SMP-primed PMN hybrids in controlled growth chamber (CGC), daytime heat stress, and heat plus drought stress environments. Bars represent the standard error of the mean.
† CON = Control; AZA = SMP + 5-Azacytidine; CH = SMP + Chitosan treatments.

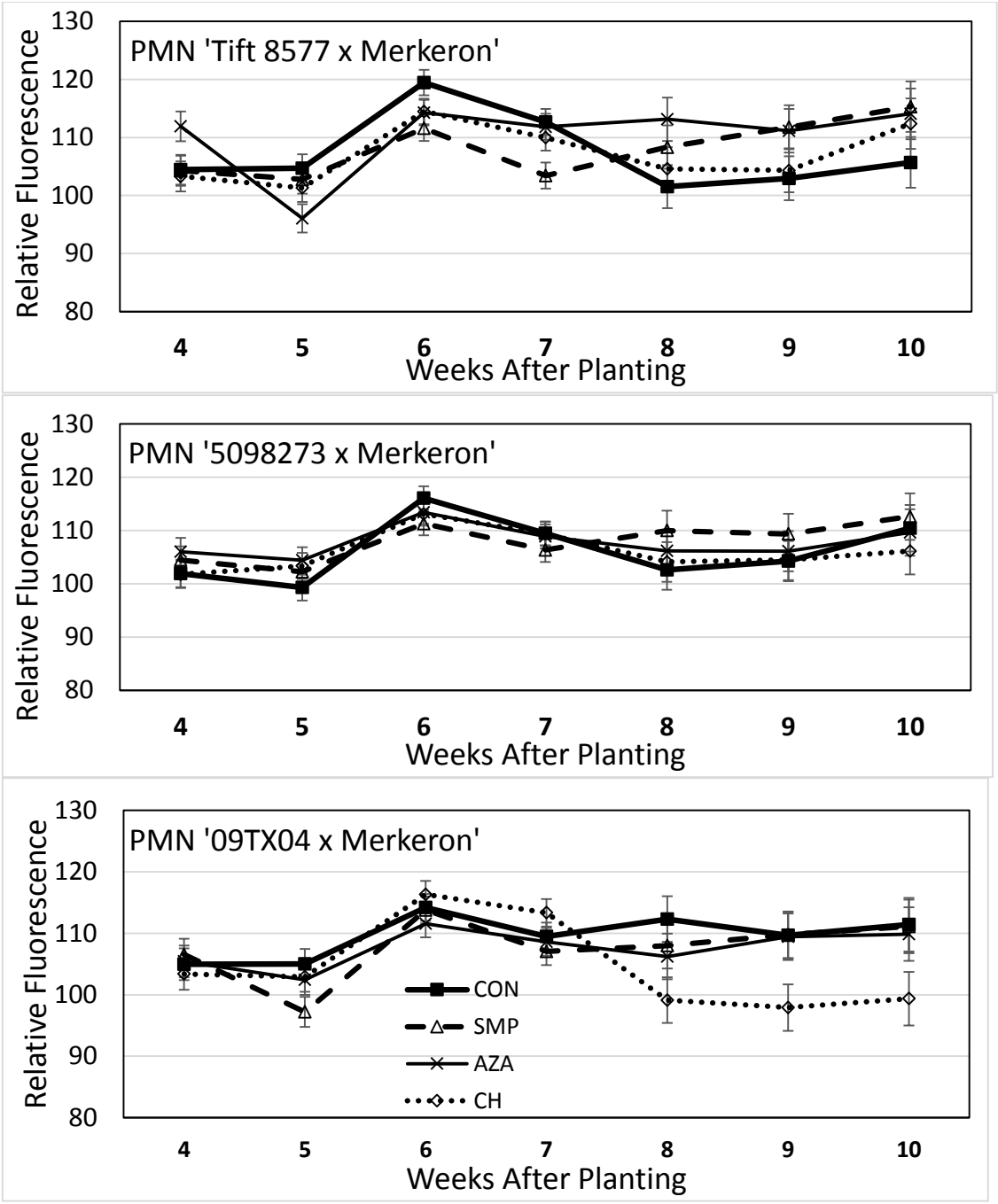

Figure 9. Relative fluorescence means by PMN hybrid x treatment† in controlled growth chamber (CGC) conditions. Bars represent the standard error of the mean.
† CON = Control; AZA = SMP + 5-Azacytidine; CH = SMP + Chitosan treatments.

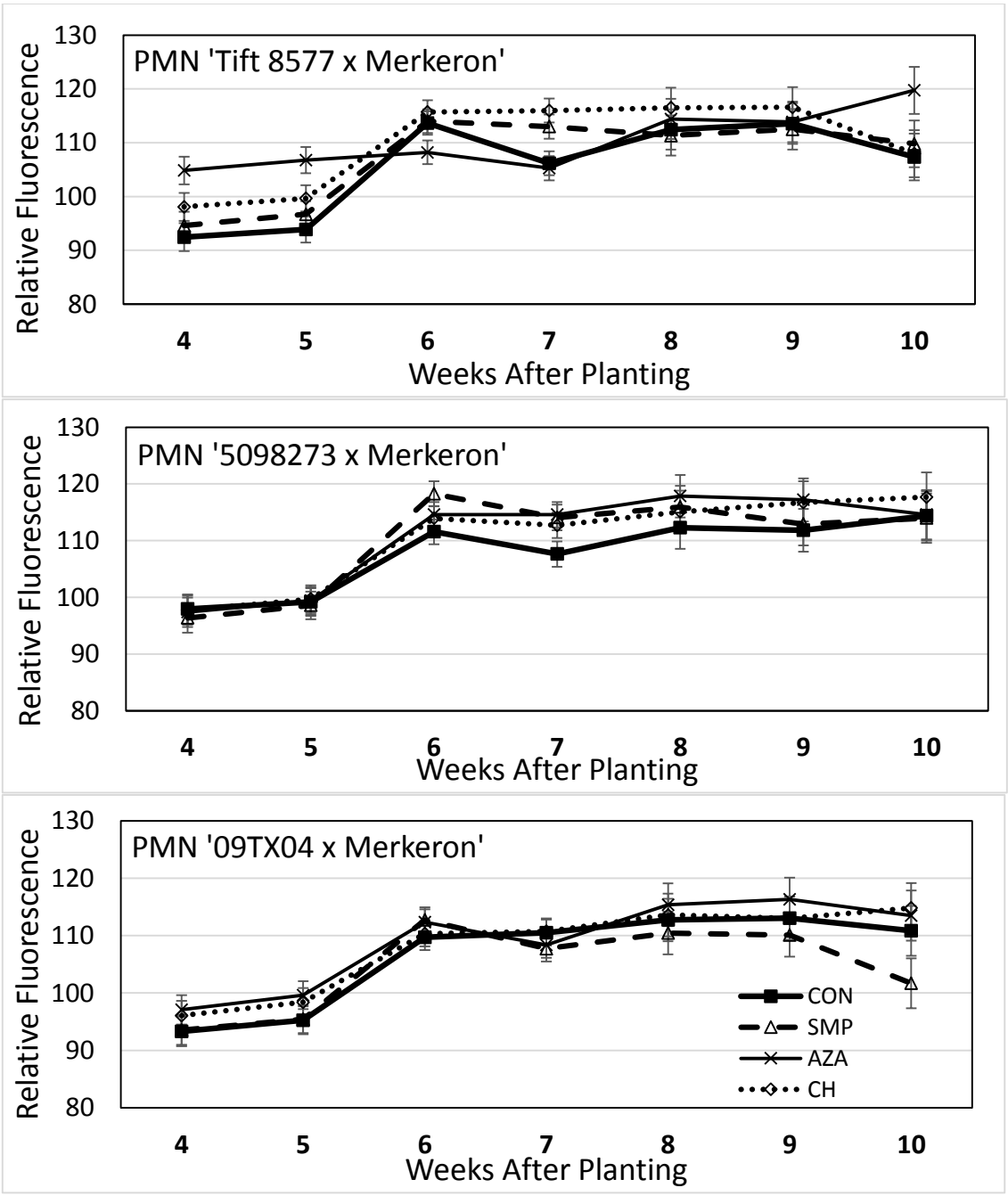

Figure 10. Relative fluorescence means by PMN hybrid x treatment† in a daytime heat stress environment. Bars represent the standard error of the mean.
† CON = Control; AZA = SMP + 5-Azacytidine; CH = SMP + Chitosan treatments.

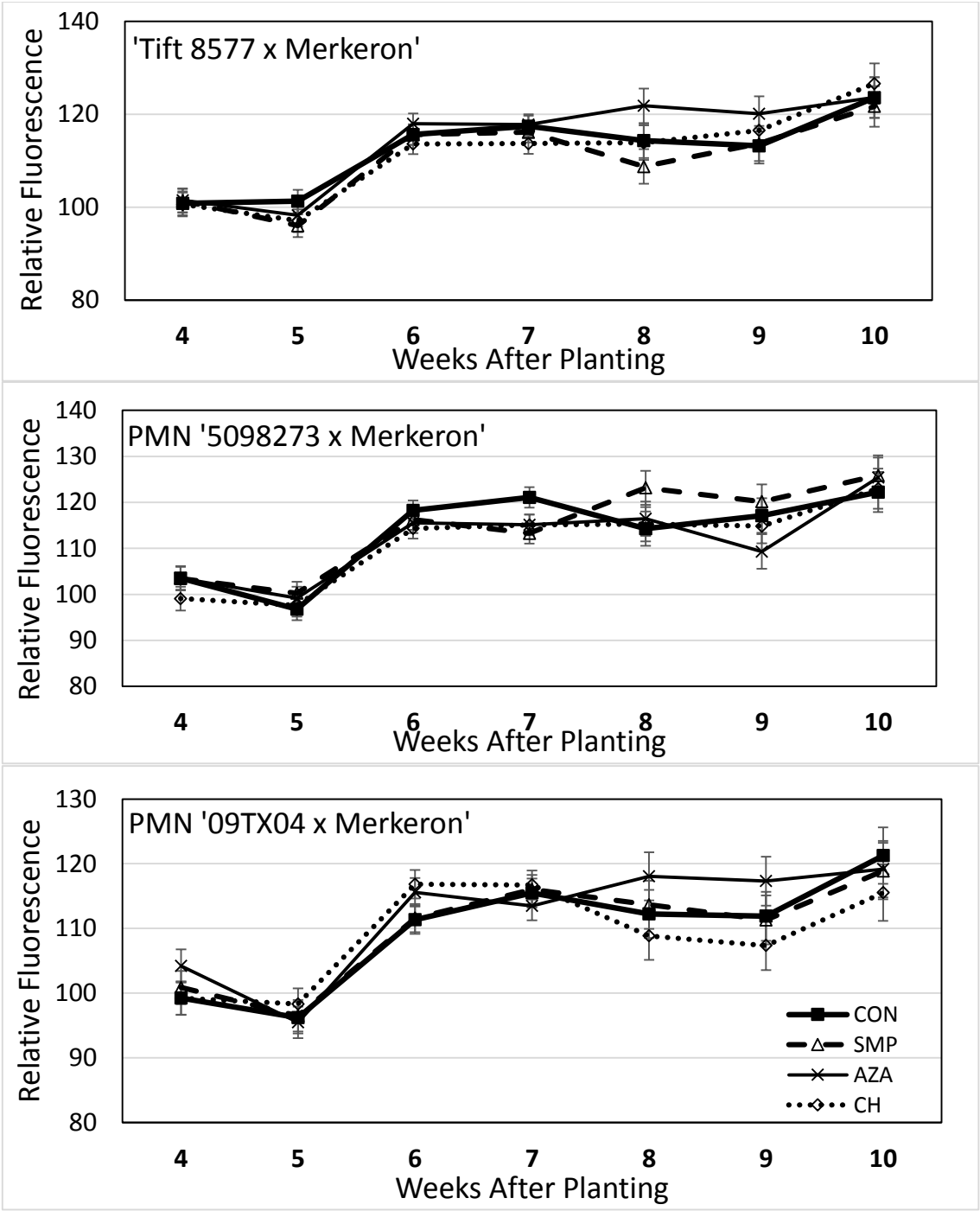

Figure 11. Relative fluorescence means by PMN hybrid x treatment† in a heat plus drought stress environment. Bars represent the standard error of the mean.
† CON = Control; AZA = SMP + 5-Azacytidine; CH = SMP + Chitosan treatments.

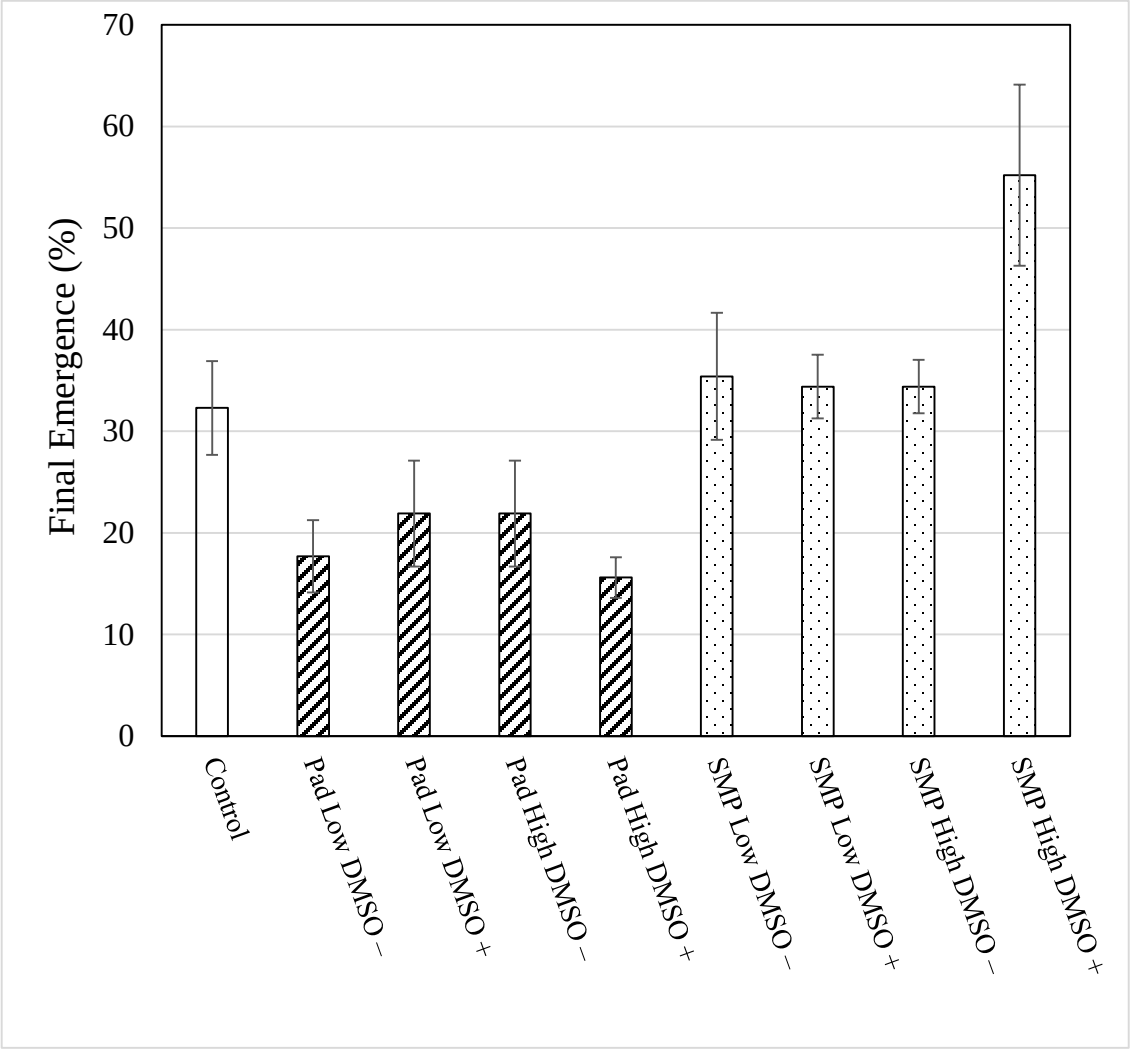

Figure 12. Seedling emergence percentage of *Sorghum propinquum* plants exposed to colchicine and/or DMSO using germination pad or solid matrix priming (SMP) techniques.†

† Treatment abbreviations are as follows: pad = germination pad method; low = 0.01% and high = 0.1% colchicine, respectively; and +/– refers to the presence/absence of DMSO, respectively. Bars represent the standard error of the mean. Means not sharing the same letter are significantly different (Tukey MSD, $p < 0.05$).

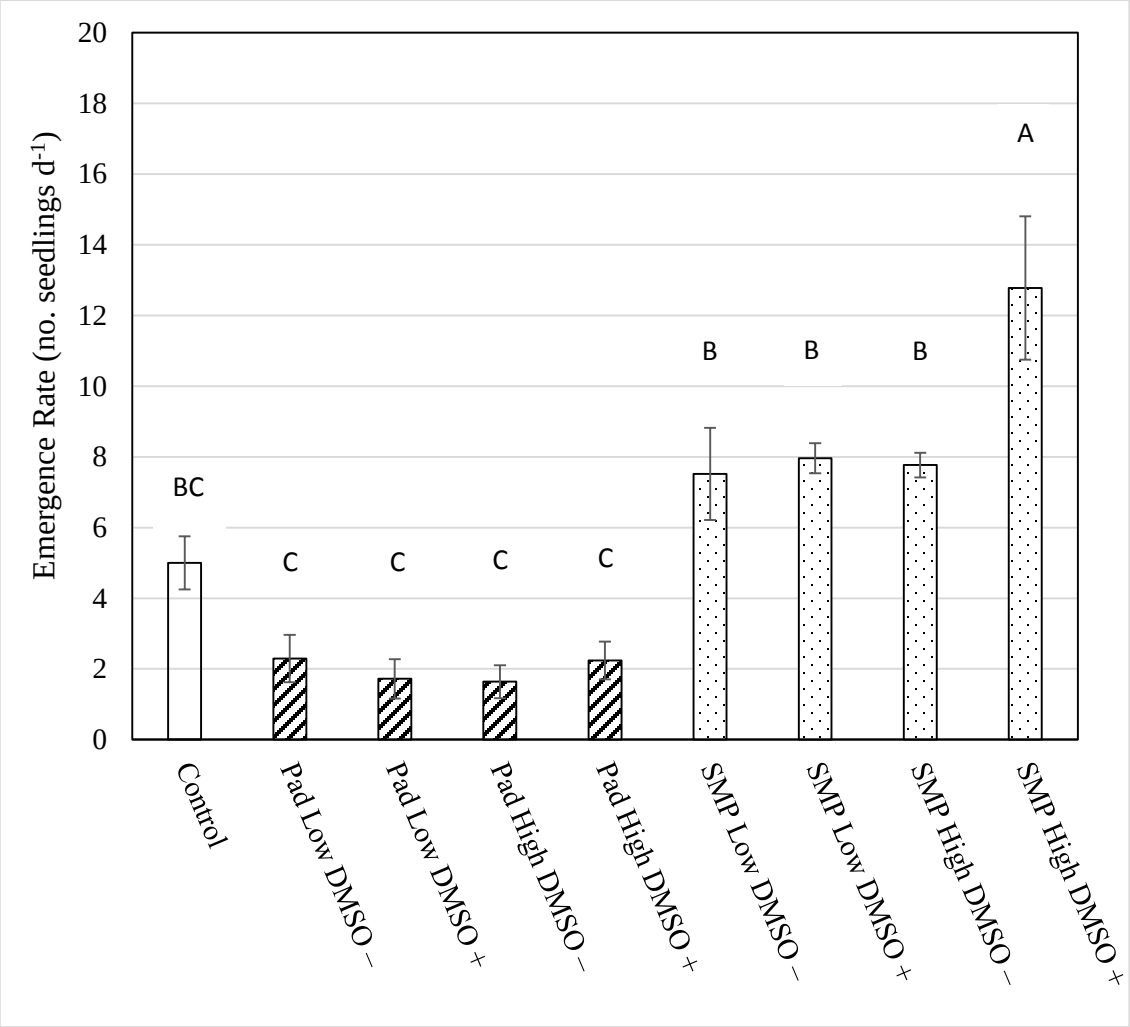

Figure 13. Emergence rate† of *Sorghum propinquum* plants exposed to colchicine and/or DMSO using germination pad or solid matrix priming (SMP) techniques.††

† Emergence rate was calculated as per Maguire (1962).

†† Treatment abbreviations are as follows: pad = germination pad method; low = 0.01% and high = 0.1% colchicine, respectively; and +/– refers to the presence/absence of DMSO, respectively. Bars represent the standard error of the mean. Means not sharing the same letter are significantly different (Tukey's MSD, $p < 0.05$).

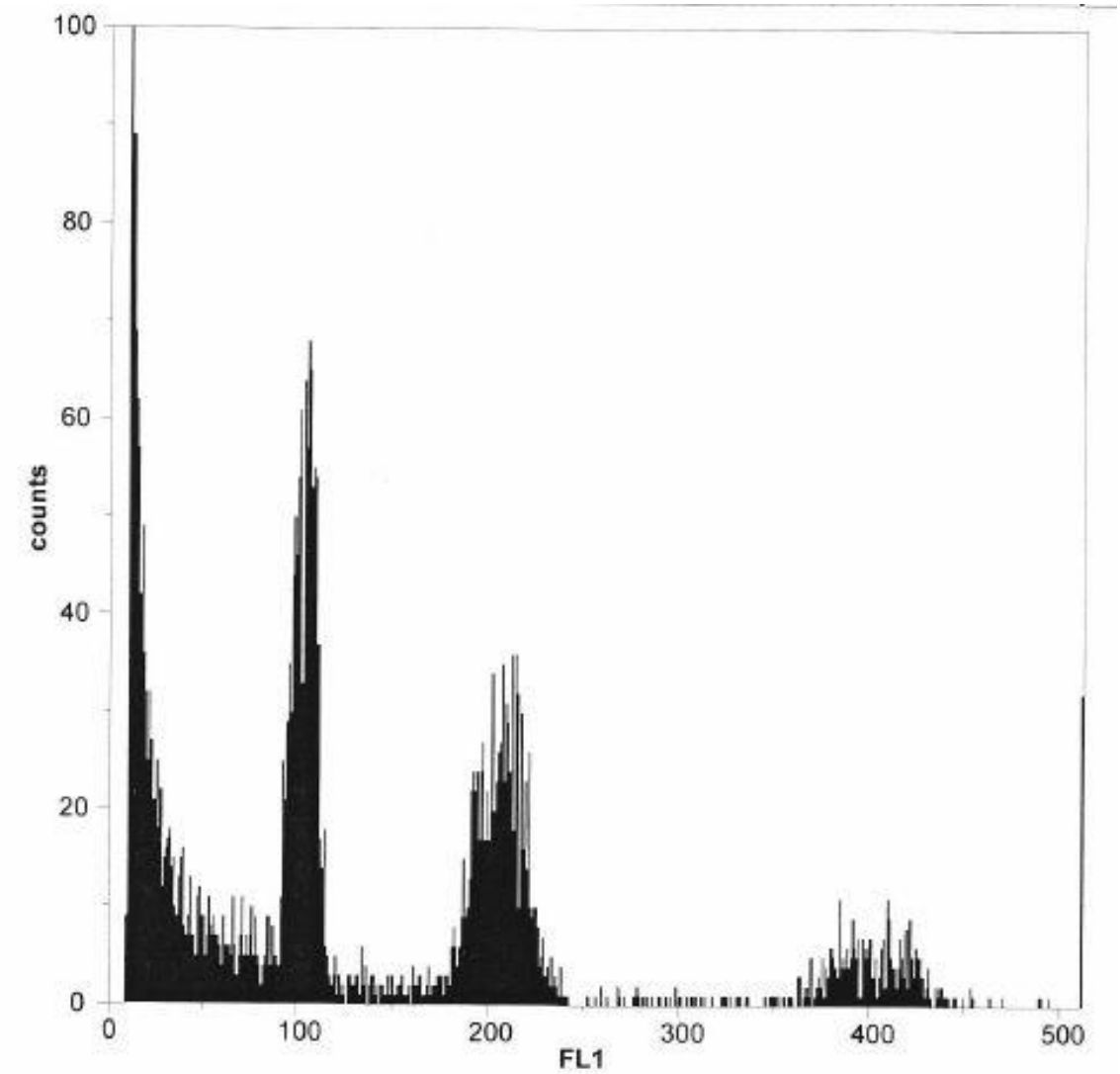

Figure 14. Example verification of chromosome doubling in Sorghum. X axis is the photon intensity associated with DNA content. Y axis is the particle or nuclei count. Diploid standard 2C and 4C peaks aligned at 100 and 200 respectively on the X axis. Tetraploid 2C and 4C peaks aligned at 200 and 400 respectively on the X axis (Wilson 2015, unpublished).

APPENDIX B

TABLES

Table 1. Significance of effects of entry, treatment (Trt), and entry × treatment (Entry × Trt) on seedling emergence percentage, emergence rate, and seedling yield of three PMN hybrids treated with SMP.

Source	Emergence %	Emergence Rate	Seedling Yield
		Controlled Growth Chamber	
Entry	NS	NS	***
Trt	NS	***	NS
Entry × Trt	NS	NS	*
		Heat	
Entry	***	NS	***
Trt	NS	***	NS
Entry × Trt	NS	**	*
		Heat + Drought	
Entry	NS	***	*
Trt	***	***	**
Entry × Trt	NS	**	NS

NS = Not significant.
*, **, and *** indicate significant effects at the 0.10, 0.05, and 0.01 level, respectively.

Table 2. Emergence percentage, emergence rate, and seedling yield means by entry of pearl millet and PMN treated with SMP in controlled growth chamber (CGC), daytime heat stress, and heat & drought stress conditions.

Entry	Emergence		Emergence Rate		Seedling Yield	
	%		No. Seedlings d^{-1}		g	
			CGC			
Pearl Millet	79.0		27.8		24.45	A
PMN ‘Tift 8577’	77.0		26.5		13.75	B
PMN ‘8095273’	83.0		28.7		10.87	BC
PMN ‘09TX04’	85.3		28.2		7.92	C
Tukey’s $MSD_{0.05}$	NS		NS		5.52	
			Heat			
Pearl Millet	83.0	B	30.3		48.68	A
PMN ‘Tift 8577’	90.3	AB	31.3		40.93	A
PMN ‘8095273’	86.3	AB	29.2		26.39	B
PMN ‘09TX04’	94.0	A	30.4		27.76	B
Tukey’s $MSD_{0.05}$	7.7		NS		11.31	
			Heat + Drought			
Pearl Millet	84.7		26.1	B	16.45	
PMN ‘Tift 8577’	88.3		29.0	A	18.06	
PMN ‘8095273’	85.7		27.6	AB	13.30	
PMN ‘09TX04’	86.0		28.0	A	15.77	
Tukey’s $MSD_{0.05}$	NS		2.1		NS	

Table 3. Emergence percentage, emergence rate, and seedling yield means by treatment of three PMN hybrids treated with SMP in controlled growth chamber (CGC), daytime heat stress, and heat plus drought stress conditions.

Treatment†	Emergence		Emergence Rate		Seedling Yield	
	%		No. seedlings d^{-1}		g	
			CGC			
CON	80.0		24.4	B	10.89	
SMP	82.0		28.5	A	15.02	
AZA	78.7		28.2	A	15.61	
CH	83.7		30.1	A	15.48	
Tukey's $MSD_{0.05}$	NS		3.5		NS	
			Heat			
CON	85.7		28.8	B	33.86	
SMP	90.7		32.6	A	39.55	
AZA	89.3		31.1	AB	36.47	
CH	88.0		28.7	B	33.89	
Tukey's $MSD_{0.05}$	NS		2.6		NS	
			Heat + Drought			
CON	80.0	B	24.2	B	12.69	B
SMP	91.3	A	28.1	A	18.11	A
AZA	86.3	AB	29.2	A	17.34	AB
CH	87.0	AB	29.2	A	15.43	AB
Tukey's $MSD_{0.05}$	7.8		2.1		4.93	

† CON = Control; AZA = SMP + 5-Azacytidine; CH = SMP + Chitosan treatments.

Table 4. Entry × treatment emergence percentage means of three PMN hybrids† treated with SMP in controlled growth chamber (CGC), daytime heat stress, and heat plus drought stress conditions.

Entry	Trt††	Emergence		
		CGC	Heat	Heat + Drought
		%	%	%
Pearl Millet	CON	70.7	76.0	81.3
Pearl Millet	SMP	82.7	84.0	81.3
Pearl Millet	AZA	81.3	88.0	86.7
Pearl Millet	CH	81.3	84.0	89.3
PMN A	CON	81.3	89.3	86.7
PMN A	SMP	77.3	96.0	96.0
PMN A	AZA	74.7	84.0	86.7
PMN A	CH	74.7	92.0	84.0
PMN B	CON	77.3	86.7	73.3
PMN B	SMP	85.3	90.7	94.7
PMN B	AZA	85.3	88.0	88.0
PMN B	CH	84.0	80.0	86.7
PMN C	CON	90.7	90.7	78.7
PMN C	SMP	82.7	92.0	93.3
PMN C	AZA	73.3	97.3	84.0
PMN C	CH	94.7	96.0	88.0
Tukey's $MSD_{0.05}$		NS	NS	NS

†PMN A = 'Tift 8577' × 'Merkeron'; PMN B = '5098273' × 'Merkeron'; and PMN C = '09TX04' × 'Merkeron'.

†† Trt = Treatment; CON = Control; AZA = SMP + 5-Azacytidine; CH = SMP + Chitosan treatments.

Table 5. Entry × treatment mean emergence rate of three PMN hybrids† treated with SMP in controlled growth chamber (CGC), daytime heat stress, and heat plus drought stress conditions.

Entry	Trt††	Emergence Rate (No. seedlings d^{-1})					
		CGC		Heat		Heat + Drought	
Pearl Millet	CON	21.7	28.1	ABC	22.1	D
Pearl Millet	SMP	29.4	30.2	ABC	26.0	ABCD
Pearl Millet	AZA	29.6	32.5	ABC	27.9	ABCD
Pearl Millet	CH	30.4	30.3	ABC	28.6	ABC
PMN A	CON	24.7	32.4	ABC	25.4	BCD
PMN A	SMP	25.6	34.0	AB	31.8	A
PMN A	AZA	27.5	29.5	ABC	30.4	AB
PMN A	CH	28.2	29.4	ABC	28.3	ABC
PMN B	CON	25.3	26.9	BC	25.0	BCD
PMN B	SMP	30.1	34.2	A	26.5	ABCD
PMN B	AZA	30.0	30.2	ABC	29.6	AB
PMN B	CH	29.3	25.6	C	29.4	AB
PMN C	CON	25.9	28.0	ABC	22.9	CD
PMN C	SMP	29.0	31.9	ABC	29.6	AB
PMN C	AZA	25.8	32.2	ABC	29.0	AB
PMN C	CH	32.3	29.3	ABC	30.7	AB
Tukey's $MSD_{0.05}$		NS	7.2		5.8	

†PMN A = 'Tift 8577' × 'Merkeron'; PMN B = '5098273' × 'Merkeron'; and PMN C = '09TX04' × 'Merkeron'.

†† Trt = Treatment; CON = Control; AZA = SMP + 5-Azacytidine; CH = SMP + Chitosan treatments

Table 6. Entry × treatment seedling yield means of three PMN hybrids† treated with SMP in controlled growth chamber (CGC), daytime heat stress, and heat plus drought stress conditions.

Entry	Trt††	Seedling Yield (g)			
		CGC	Heat		Heat + Drought
Pearl Millet	CON	19.32	37.22	ABC	9.89
Pearl Millet	SMP	25.26	61.10	A	22.39
Pearl Millet	AZA	24.66	58.76	AB	18.41
Pearl Millet	CH	28.56	37.65	ABC	15.10
PMN A	CON	11.76	44.07	ABC	16.75
PMN A	SMP	13.93	44.58	ABC	16.73
PMN A	AZA	15.18	28.98	BC	20.89
PMN A	CH	14.13	46.08	ABC	17.86
PMN B	CON	7.12	21.82	C	9.84
PMN B	SMP	11.20	30.39	ABC	13.60
PMN B	AZA	11.68	27.21	C	15.08
PMN B	CH	13.46	26.15	C	14.67
PMN C	CON	5.35	23.75	C	14.29
PMN C	SMP	9.68	30.70	ABC	19.72
PMN C	AZA	10.91	30.92	ABC	14.99
PMN C	CH	5.75	25.68	C	14.07
Tukey's $MSD_{0.05}$		NS	30.96		NS

†PMN A = 'Tift 8577' × 'Merkeron'; PMN B = '5098273' × 'Merkeron'; and PMN C = '09TX04' × 'Merkeron'.

†† Trt = Treatment; CON = Control; AZA = SMP + 5-Azacytidine; CH = SMP + Chitosan treatments.

Table 7. Significance of effects of entry, treatment (Trt), and entry × treatment (Entry × Trt) on seedling morphological development of three PMN hybrids treated with SMP in a controlled growth chamber environment.

	Weeks After Planting					
Source	1	2	3	4	5	6
Entry	**	**	**	***	***	***
Trt	NS	***	NS	NS	*	NS
Entry x Trt	NS	**	NS	NS	NS	NS

	Weeks After Planting					
Source	7	8	9	10	11	12
Entry	***	***	***	***	***	***
Trt	NS	*	**	NS	NS	NS
Entry x Trt	*	*	**	NS	NS	NS

NS = Not significant.
*, **, and *** indicate significant effects at the 0.10, 0.05, and 0.01 level, respectively.

Table 8. Significance of orthogonal contrasts comparing seedling morphological development in three PMN hybrids† treated with SMP in a controlled growth chamber (CGC) environment.

	Weeks After Planting					
Contrast††	1	2	3	4	5	6
PMN C Con vs. PMN A, B Con	NS	NS	NS	NS	NS	NS
PMN C SMP vs. PMN A,B SMP	NS	NS	NS	*	*	***
PMN C AZA vs. PMN A,B AZA	NS	NS	NS	**	**	***
PMN C CH vs. PMN A, B CH	NS	NS	NS	**	NS	NS
PMN A Con vs. SMP	NS	NS	NS	NS	**	**
PMN B Con vs. SMP	NS	***	NS	NS	NS	NS
PMN C Con vs. SMP	NS	**	NS	NS	**	*
PMN C AZA vs. CH	NS	NS	NS	NS	NS	**

	Weeks After Planting					
Contrast	7	8	9	10	11	12
PMN C Con vs. PMN A, B Con	NS	NS	NS	NS	NS	NS
PMN C SMP vs. PMN A,B SMP	***	**	NS	NS	NS	NS
PMN C AZA vs. PMN A,B AZA	***	***	NS	NS	NS	NS
PMN C CH vs. PMN A, B CH	NS	NS	NS	NS	NS	NS
PMN A Con vs. SMP	NS	NS	NS	NS	NS	NS
PMN B Con vs. SMP	*	NS	NS	NS	NS	NS
PMN C Con vs. SMP	**	**	NS	NS	NS	NS
PMN C AZA vs. CH	**	***	NS	NS	NS	NS

† PMN A = Tift 8577; PMN B = 5098273; and PMN C = 09TX04.
†† CON = Control; AZA = SMP + 5-Azacytidine; CH = SMP + Chitosan treatments.
NS = Not significant.
*, **, and *** indicate significant effects at the 0.10, 0.05, and 0.01 level, respectively.

Table 9. Significance of effects of entry, treatment (Trt), and entry × treatment (Entry × Trt) on seedling morphological development of three PMN hybrids treated with SMP in a daytime heat stress environment.

	Weeks After Planting					
Source	1	2	3	4	5	6
Entry	NS	*	***	***	***	***
Trt	NS	NS	NS	**	NS	NS
Entry x Trt	NS	NS	NS	NS	NS	NS

	Weeks After Planting					
Source	7	8	9	10	11	12
Entry	***	***	***	***	***	***
Trt	NS	NS	*	NS	NS	NS
Entry x Trt	NS	NS	NS	NS	NS	NS

NS = Not significant.
*, **, and *** indicate significant effects at the 0.10, 0.05, and 0.01 level, respectively.

Table 10. Significance of orthogonal contrasts comparing seedling morphological development in three PMN hybrids† treated with SMP in daytime heat stress conditions.

	Weeks After Planting					
Contrast††	1	2	3	4	5	6
PMN C Con vs. PMN A, B Con	NS	NS	***	**	*	NS
PMN C SMP vs. PMN A,B SMP	NS	NS	NS	NS	NS	NS
PMN C AZA vs. PMN A,B AZA	NS	**	***	***	***	NS
PMN C CH vs. PMN A, B CH	NS	NS	NS	**	**	NS
PMN A Con vs. SMP	NS	NS	NS	*	NS	NS
PMN B Con vs. SMP	NS	NS	NS	**	NS	NS
PMN C Con vs. SMP	NS	NS	NS	**	NS	NS
PMN C AZA vs. CH	NS	***	***	NS	NS	NS

	Weeks After Planting					
Contrast	7	8	9	10	11	12
PMN C Con vs. PMN A, B Con	NS	NS	NS	NS	NS	NS
PMN C SMP vs. PMN A,B SMP	NS	NS	NS	NS	NS	NS
PMN C AZA vs. PMN A,B AZA	NS	NS	NS	NS	NS	NS
PMN C CH vs. PMN A, B CH	NS	NS	NS	NS	NS	NS
PMN A Con vs. SMP	NS	NS	NS	NS	NS	NS
PMN B Con vs. SMP	NS	NS	NS	NS	NS	NS
PMN C Con vs. SMP	NS	NS	NS	NS	NS	NS
PMN C AZA vs. CH	NS	NS	NS	NS	NS	NS

† PMN A = Tift 8577; PMN B = 5098273; and PMN C = 09TX04.
†† CON = Control; AZA = SMP + 5-Azacytidine; CH = SMP + Chitosan treatments.
NS = Not significant.
*, **, and *** indicate significant effects at the 0.10, 0.05, and 0.01 level, respectively.

Table 11. Significance of effects of entry, treatment (Trt), and entry × treatment (Entry × Trt) on seedling morphological development of three PMN hybrids treated with SMP in a daytime heat plus drought stress environment.

	Weeks After Planting					
Source	1	2	3	4	5	6
Entry	***	***	**	***	***	***
Trt	NS	NS	NS	NS	NS	NS
Entry x Trt	NS	NS	NS	NS	NS	NS

	Weeks After Planting		
Source	7	8	9
Entry	***	***	***
Trt	NS	NS	NS
Entry x Trt	NS	NS	NS

NS = Not significant.
*, **, and *** indicate significant effects at the 0.10, 0.05, and 0.01 level, respectively.

Table 12. Significance of orthogonal contrasts comparing seedling morphological development in three PMN hybrids† treated with SMP in daytime heat plus drought stress conditions.

Contrast††	Weeks After Planting								
	1	2	3	4	5	6	7	8	9
PMN C CON vs. PMN A,B CON	***	NS	NS	***	*	**	**	**	**
PMN C SMP vs. PMN A,B SMP	NS	**	NS	NS	*	*	NS	NS	NS
PMN C AZA vs. PMN A,B AZA	NS	NS	NS	NS	NS	NS	NS	NS	NS
PMN C CH vs. PMN A, B CH	NS	*	NS	***	***	***	***	**	**
PMN A CON vs. SMP	NS	NS	NS	NS	NS	NS	NS	NS	NS
PMN B CON vs. SMP	NS	NS	NS	NS	NS	NS	NS	NS	NS
PMN C CON vs. SMP	NS	NS	NS	NS	NS	NS	NS	NS	NS
PMN C AZA vs. CH	NS	NS	NS	***	**	**	**	NS	NS

† PMN A = Tift 8577; PMN B = 5098273; and PMN C = 09TX04.
†† CON = Control; AZA = SMP + 5-Azacytidine; CH = SMP + Chitosan treatments.
NS = Not significant.
*, **, and *** indicate significant effects at the 0.10, 0.05, and 0.01 level, respectively.

Table 13. Significance of effects of entry, treatment (Trt), and entry × treatment (Entry × Trt) on blue-light chlorophyll fluorescence of three PMN hybrids treated with SMP.

Source	Weeks After Planting						
	4	5	6	7	8	9	10
	Controlled Growth Chamber						
Entry	NS	NS	NS	NS	NS	*	NS
Trt	NS	NS	NS	NS	NS	NS	NS
Entry × Trt	NS	NS	NS	NS	NS	NS	NS
	Heat						
Entry	NS	NS	NS	NS	NS	NS	NS
Trt	NS	NS	NS	NS	NS	NS	NS
Entry × Trt	**	**	NS	NS	NS	NS	NS
	Heat + Drought						
Entry	NS	NS	***	NS	NS	NS	NS
Trt	NS	NS	NS	NS	NS	NS	NS
Entry × Trt	NS	NS	**	**	NS	NS	NS

NS = Not significant.
*, **, and *** indicate significant effects at the 0.10, 0.05, and 0.01 level, respectively.

Table 14. Significance of orthogonal contrasts comparing blue-light chlorophyll fluorescence in three PMN hybrids† treated with SMP in controlled growth chamber conditions.

Contrast††	Weeks After Planting						
	4	5	6	7	8	9	10
PMN C CON vs. PMN A,B CON	NS	NS	NS	NS	NS	NS	NS
PMN C SMP vs. PMN A,B SMP	NS	NS	NS	NS	NS	NS	NS
PMN C AZA vs. PMN A,B AZA	NS	NS	NS	NS	NS	NS	NS
PMN C CH vs. PMN A, B CH	NS	NS	NS	NS	NS	NS	**
PMN A CON vs. SMP	NS	NS	**	**	NS	NS	NS
PMN B CON vs. SMP	NS	NS	NS	NS	NS	NS	NS
PMN C CON vs. SMP	NS	*	NS	NS	NS	NS	NS
PMN C AZA vs. CH	NS	NS	NS	NS	NS	*	*

† PMN A = Tift 8577; PMN B = 5098273; and PMN C = 09TX04.
†† CON = Control; AZA = SMP + 5-Azacytidine; CH = SMP + Chitosan treatments.
NS = Not significant.
*, **, and *** indicate significant effects at the 0.10, 0.05, and 0.01 level, respectively.

Table 15. Significance of orthogonal contrasts comparing blue-light chlorophyll fluorescence in three PMN hybrids† treated with SMP in daytime heat stress conditions.

Contrast††	Weeks After Planting						
	4	5	6	7	8	9	10
PMN C CON vs. PMN A,B CON	NS	NS	NS	NS	NS	NS	NS
PMN C SMP vs. PMN A,B SMP	NS	NS	NS	**	NS	NS	*
PMN C AZA vs. PMN A,B AZA	NS	NS	NS	NS	NS	NS	NS
PMN C CH vs. PMN A, B CH	NS	NS	NS	NS	NS	NS	NS
PMN A CON vs. SMP	NS	NS	NS	**	NS	NS	NS
PMN B CON vs. SMP	NS	NS	*	**	NS	NS	NS
PMN C CON vs. SMP	NS	NS	NS	NS	NS	NS	NS
PMN C AZA vs. CH	NS	NS	NS	NS	NS	NS	NS

† PMN A = Tift 8577; PMN B = 5098273; and PMN C = 09TX04.
†† CON = Control; AZA = SMP + 5-Azacytidine; CH = SMP + Chitosan treatments.
NS = Not significant.
*, **, and *** indicate significant effects at the 0.10, 0.05, and 0.01 level, respectively.

Table 16. Significance of orthogonal contrasts comparing blue-light chlorophyll fluorescence in three PMN hybrids† treated with SMP in daytime heat plus drought stress conditions.

Contrast††	Weeks After Planting						
	4	5	6	7	8	9	10
PMN C CON vs. PMN A,B CON	NS	NS	***	*	NS	NS	NS
PMN C SMP vs. PMN A,B SMP	NS	NS	***	NS	NS	NS	NS
PMN C AZA vs. PMN A,B AZA	NS	NS	NS	NS	NS	NS	NS
PMN C CH vs. PMN A, B CH	NS	NS	**	NS	NS	*	*
PMN A CON vs. SMP	NS	*	NS	NS	NS	NS	NS
PMN B CON vs. SMP	NS	NS	NS	***	NS	NS	NS
PMN C CON vs. SMP	NS	NS	NS	NS	NS	NS	NS
PMN C AZA vs. CH	NS	NS	NS	NS	NS	*	NS

† PMN A = Tift 8577; PMN B = 5098273; and PMN C = 09TX04.

†† CON = Control; AZA = SMP + 5-Azacytidine; CH = SMP + Chitosan treatments.

NS = Not significant.

*, **, and *** indicate significant effects at the 0.10, 0.05, and 0.01 level, respectively.

Table 17. Number of confirmed tetraploid *Sorghum propinquum* plants exposed to colchicine and/or DMSO using germination pad or solid matrix priming (SMP) techniques.

Method†	Colchicine‡	DMSO§	No. Tetraploids	No. Plants
Control	N/A	N/A	0	32
Pad	L	–	0	17
Pad	L	+	0	20
Pad	H	–	0	13
Pad	H	+	0	13
SMP	L	–	0	35
SMP	L	+	0	30
SMP	H	–	0	32
SMP	H	+	7	52

† Pad = germination blotter paper method; ‡ L = 0.01% and H = 0.1% colchicine, respectively; § + and – signify the presence and absence of DMSO in solution, respectively.