PHENOTYPIC AND CYTOLOGICAL CHARACTERIZATION OF INDUCED TETRAPLOID, PERENNIAL *Sorghum bicolor* x *S. propinquum* HYBRIDS

A Thesis

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

MASTER OF SCIENCE

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August 2020

Major Subject: Plant Breeding

ABSTRACT

The development and use of perennial alternatives to annual crops can help alleviate some of the challenges facing agriculture. Perennial sorghum has the potential of being a low maintenance, drought tolerant, high yielding forage or bioenergy crop.

A novel induced tetraploid (2*n*=4*x*=40), interspecific *Sorghum bicolor* (L.) Moench (annual) x *S. propinquum* (Kunth) Hitchc. (perennial) hybrid's progeny was characterized in the F_2 and F_3 generations.

Field trials were performed to determine their overwintering ability and evaluate various agronomic and morphological traits. The interspecific tetraploid population overwintered well in one evaluation environment (60-85%) and had promising yield potential. Tetraploid entries were intermediate to the parents for most morphological traits. Flowering in the tetraploid was mostly short-day photoperiodic - similar to *S. propinquum*. Some tetraploid individuals expressed positive transgressive segregation for plant height. Selections can be made for further breeding within the overwintered population based on forage or bioenergy characteristics.

The fertility and cytological behavior of the induced tetraploid *S. bicolor* x *S. propinquum* hybrid and its progeny were studied. The meiotic chromosome pairing behavior in these plants was relatively regular with an average of 0.45 univalents, 17.77 bivalents, 0.02 trivalents, and 0.98 quadrivalents per cell. Pollen stainability and seed set were lower than either diploid parent, but sufficiently high that the tetraploid hybrids can be propagated with seed. This germplasm has the potential of being developed into a commercial noninvasive perennial sorghum.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supported by a thesis committee consisting of Dr. Russell W. Jessup (advisor) and Dr. Byron L. Burson (USDA-ARS) of the Department of Soil and Crop Sciences, and Dr. David M. Stelly of the Faculty of Genetics.

All work for the thesis was completed by Nick Porter, under the advisement Dr. Russell W. Jessup, Dr. Byron L. Burson, and Dr. David M. Stelly. Additional assistance with labor and data collection was provided by Dr. Russell W. Jessup, Dr. Byron L. Burson, Tyler Foster, Heather Baldi, Xiaoqing Shen, and Caitlyn Turner.

Funding Sources

Graduate study was supported by the J. Roy Quinby Scholarship.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

I.1 RESEARCH PROBLEM

Agricultural land resources continue to decrease as the world population increases (Elliot et al., 2014). When water availability becomes limited, irrigation is primarily limited to the better farmlands and is used to grow the most profitable crops. Consequently, more farms will return to dryland cropping systems. As the percentage of dryland farming increases, the need for adapted, drought tolerant cultivars will increase. Forages are an important part of agriculture, and to continue to feed animals, forages will need to be developed that will produce in low input systems. This challenge can be partially addressed by two concepts - perennial cropping systems and improved plant water use efficiency (WUE).

This research characterized and evaluated germplasm for potential perennial forage and biomass sorghum cultivars. While developing the germplasm, interspecific hybrids between *Sorghum bicolor* (L.) Moench and *S. propinquum* (Kunth) Hitchc. were studied cytologically to better understand the relationship between the two species.

I.2 LITERATURE REVIEW

I.2.1 Perennial cropping system

There are numerous benefits of growing perennial crops. They may expend fewer resources than their annual counterparts in terms of capital assets, energy costs, and other inputs (Cox et al., 2006). They also help reduce soil erosion and improve both soil health and organic matter content. Perennial crops better utilize moisture and reduce water loss compared to annual crops (Crews and Cattani, 2018). Perennial crops also capture more solar energy because growth

begins earlier in the spring and continues until after harvest, leading to increased productivity (Glover et al., 2010).

Perennial cropping systems have deficiencies. Yield potential in perennial grain crops under single end-of-season harvest cropping systems are generally less than their annual counterparts; however, there are situations where the benefits from a perennial crop can offset the loss in yield potential (Cox et al., 2006). Most perennial grain crops are in the developmental phase, and the only commercially released perennial grass used for grain is Kernza™ - intermediate wheatgrass [*Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey] cultivars (The Land Institute, 2019).

Many perennial herbaceous crops are used as forages. Forages were grown on 25.5 million hectares in the United States in 2018 (Progressive Forage, 2019). Alfalfa (*Medicago sativa* L.), a perennial legume, consists of about 25% of nationwide forage production. Fifty-seven percent (14.5 million hectares) of national forage acreage is characterized as "other hay" meaning not alfalfa, silage or green chop. Other hay could include perennial or annual species. Many different perennial grasses (Bermudagrass for example) are used for hay, species used varies depending on climate. Texas has 1.9 million other hay hectares and only 57,000 alfalfa hectares. Annual sorghum can be used for forage grown as hay or silage. In 2017, 27,000 hectares of sorghum silage were harvested in Texas and 135,831 hectares were harvested nationally (USDA, 2019). Currently, the only commercially available perennial sorghums are a few Columbusgrass (*Sorghum* x *almum* Parodi) forage cultivars in Australia which resulted from crosses between *S. bicolor* and *S. halepense* (L.) Pers. (Jessup et al., 2017b). Progress has been made towards developing perennial cultivars in the United States. Two perennial genetic stocks were recently released for further development; one is a tetraploid hybrid between *S. bicolor* and

S. halepense and the other is a diploid hybrid between *S. bicolor* and *S. propinquum* (Jessup et al., 2017a, b). Additional research towards developing a tetraploid perennial grain sorghum is underway at The Land Institute, Salina, KS using *S. halepense* as the source of perenniality (Cox et al., 2018).

I.2.2 *Sorghum propinquum*

Sorghum propinquum is a perennial wild relative of *S. bicolor* that is native to Asia (de Wet, 1978). It has rhizomatous growth and is the only diploid sorghum species that produces rhizomes. It is fully interfertile with *S. bicolor*. *Sorghum propinquum* tillers readily and produces small seeds (Chittenden et al., 1994).

Cytologically *S. propinquum* is a diploid with 20 chromosomes that pair as 10 bivalents during meiosis I (Celarier, 1958). However, Magoon and Shambulingappa (1961) reported a dicentric bridge in about 3-4% of the anaphase I cells they observed, and 1-2 lagging bivalents also in 3-4% of the cells. They also noted the plant examined had very low seed set and 20-25% of the pollen was non-viable.

I.2.3 *Sorghum bicolor*

Sorghum bicolor is the most important species in the genus, and it is grown for food, feed, forage, syrup, and bioenergy. Sorghum is the third most grown cereal grain crop in the United States (USGC, 2019) and the fifth most grown cereal crop worldwide (Cox et al., 2018). *Sorghum bicolor* has better water use efficiency than maize (*Zea mays* L.) or any of the other major cereal grain crops, except for pearl millet [*Pennisetum glaucum* (L.) R. Br.] (Hadebe et al., 2017).

Cytoplasmic male sterility (CMS) is extensively used in *S. bicolor* breeding and for hybrid seed production. CMS enables hybrid seed to be easily produced in this highly self-

pollinated species. CMS involves a male sterile cytoplasm and nuclear fertility restoration genes. This allows a male sterile genotype to be reproduced by having an isogenic but fertile cytoplasm maintainer as well as the production of fertile hybrids (Quinby, 1974).

Sorghum bicolor is a diploid with 2*n*=2*x*=20 chromosomes. Cytological studies have shown that it has normal diploid chromosome paring of 10 bivalents during metaphase I of meiosis (Schertz, 1962). That said, multiple lines of evidence indicate remnant vestiges of ancient ancestral paleotetraploidization, including repetitive sequence distributions suggestive of tetraploidy or paleotetraploidy, the detection of duplicated linkage relationships among related markers, and cytogenetic behavior (Gomez et al. 1998; Wilson et al. 1999; Zwick et al. 2000; Kuhlman et al. 2008). Detailed sequence analyses and comparisons have confirmed the paleopolyploidization occurred approximately 70 MYA and was followed by extensive paleodiploidization (Paterson et al. 2009).

Tetraploid (2*n*=4*x*=40) germplasm has been developed, but these doubled diploids do not exhibit normal meiotic chromosome pairing. The average chromosome paring was 0.21 univalents, 12.19 bivalents, 0.09 trivalents, and 3.79 quadrivalents per cell. The range of chromosome pairing was 0-5 univalents, 2-20 bivalents, 0-4 trivalents, and 0-9 quadrivalents (Hoang and Liang, 1988).

I.2.4 Perennial Sorghum interspecific hybridizations

Attempts to develop perennial sorghum cultivars have involved using other *Sorghum* species, notably *S. halepense* and *S. propinquum*. *Sorghum halepense* is a tetraploid (2*n*=4*x*=40); whereas, *S. propinquum* is a diploid (2*n*=2*x*=20) (de Wet, 1978). Making the initial cross between *S. bicolor* and *S. halepense* usually involves using an induced tetraploid *S. bicolor* plant or by utilizing a male sterile diploid and screening progeny for the low occurrence of 2*n* gametes

(Cox et al., 2018). To its benefit, *S. halepense* is photoperiod neutral, and it flowers almost continually during the growing season. Therefore, timing is not a major issue in using it for hybridization purposes. Producing fertile hybrids between *S. bicolor* and *S. halepense* remains a difficult and laborious process.

Using *S. propinquum* is genetically simpler process because it will readily cross and is interfertile with *S. bicolor* (de Wet, 1978; Jessup et al., 2017). However, *S. propinquum* is photoperiod-sensitive and floral development is initiated only when the daylength is less than 12 hours and 20 min. This photoperiodic response introduces a minor complication in production of hybrids between *S. bicolor* and *S. propinquum.* Especially when crosses are made under field conditions because frosts during the fall tend to occur in most areas of the United States before seed can be produced in the field. Therefore, crosses must be made in a winter nursery in the tropics or in a greenhouse.

There is substantial evidence that *S. halepense* is a derivative of a natural interspecific hybrid between *S. bicolor* and *S. propinquum* followed by a polyploidy event (Cox et al., 2018, Paterson, 2008). There are no reports of any research attempting *de novo* creation of *S. halepense*, by crossing *S. bicolor* with *S. propinquum* and then inducing polyploidy. However, hybrids between *S. bicolor* and *S. propinquum* have been studied at the diploid level revealing that they are highly fertile and have normal meiotic chromosome pairing (Doggett, 1988; de Wet, 1978). Additional molecular studies of *S. bicolor* x *S. propinquum* hybrids have been conducted primarily for investigating genes associated with rhizome development (Vandenbrink et al., 2013; Washburn et al., 2013; Paterson et al., 1995; Kong et al., 2015), as well as enabled the first complete *S. bicolor* genome annotation (Paterson et al., 2009).

Sorghum halepense is a tetraploid with 40 chromosomes. Celarier (1958) summarized multiple cytological studies and reported that the average chromosome associations for *S. halepense* was 0.19 univalents, 17.4 bivalents, 0.03 trivalents, and 1.2 quadrivalents. Two other studies reported more irregularities with averages ranging as follows: 0.13-0.8 univalents, 13.19-14.74 bivalents, 0.06-0.25 trivalents, and 2.48-3.06 quadrivalents per cell, and the maximum number of quadrivalents observed was six (Endrizzi, 1957; Hadley, 1953). In these same studies, 40 chromosome hybrids between *S. bicolor* and *S. halepense* were examined cytologically, and those hybrids had average chromosome associations of 0.69-1.26 univalents, 10.18-12.71 bivalents, 0.02-0.41 trivalents, and 3.11-3.81 quadrivalents. The maximum number of quadrivalents observed was eight.

I.2.5 Progressive heterosis

Progressive heterosis is a concept where a tetraploid species expresses a higher degree of heterosis than a diploid species. This is because it is possible to have more than two different alleles at any given locus. This allows for a greater degree of heterosis because more than two beneficial alleles could be present at the same locus (Groose et al., 1989). This has been shown in induced tetraploid double cross maize hybrids where the tetraploid double cross hybrid had higher biomass yield than the tetraploid single cross hybrid (Washburn et al., 2019).

There are other important considerations for tetraploid species that fit under this umbrella of progressive heterosis but not under the specific definition. The first is that additive gene action can be increased compared to a diploid as it becomes possible to have 4 copies of a gene instead of two. This can result in more of that gene's product being produced which can greatly increase some traits. If a plant or plant part, exhibits an increase in size because it is a tetraploid, this is called gigantism (Schertz, 1962).

Additionally, induced tetraploids can stabilize a phenotype even with generations of self-pollination, practically preserving a hybrid phenotype. Theoretically, this occurs in one of two ways. The first is if an induced tetraploid hybrid is an allotetraploid that exhibits strictly disomic inheritance, then the phenotype should be fixed and "breed true". The second possibility is if the induced tetraploid hybrid is an autotetraploid that exhibits tetrasomic segregation, then segregation will occur, i.e., it will not "breed true". However, the patterns of segregation ratios will not follow the same pattern as a diploid. In this scenario, the chance, when self-pollinated, of producing a homozygous allele across all four chromosomes for a single locus becomes much less. For example, a typical heterozygous "*Aa*" diploid hybrid is expected, following self-pollination, to have a genotypic segregation ratio of 1 *AA*: 2 *Aa*: 1 *aa* or a 50% loss in heterozygosity. For an induced autotetraploid of the same hybrid (now *AAaa*) following self-pollination, the expected genotypic segregation ratio (for centromere-linked loci) is 1 *AAAA*: 8 *AAAa*: 18 *AAaa*: 8 *Aaaa*: 1 *aaaa* which results in only a 5.55% loss in heterozygosity (Appels et al., 1998). Inbreeding depression resulting from homozygous recessive alleles will still occur but should be at a slower rate than that for a diploid. However, in autotetraploid plants (alfalfa and induced maize tetraploids) inbreeding depression has been shown to be similar to comparable diploids (Washburn et al., 2019). This may be due to a low beneficial allelic dosage that is quickly exposed with inbreeding. Natural tetraploid potatoes (*Solanum tuberosum* L.) also show intense inbreeding depression which is reportedly because of a high number of deleterious mutations that have accumulated over many generations of asexual reproduction (Lian et al., 2019). Without recombination events, the deleterious mutations could not be selected against. Deleterious mutations were found to be most concentrated in pericentromeric chromosomal

regions with low recombination rates (Lian et al., 2019). In an autotetraploid, it would be possible to mask three deleterious mutations at any locus without necessarily seeing a phenotype.

I.3 OBJECTIVES

The first objective of this study was to phenotypically characterize the F_2 and F_3 progeny derived from a single induced tetraploid *S. bicolor* x *S. propinquum* F_1 hybrid.

The second objective was to determine the reproductive fertility and cytology of both parents, the diploid *S. bicolor x S. propinquum* hybrid, and the induced tetraploid *S. bicolor* x *S. propinquum* hybrids. Fertility was determined by microscopically examining pollen stainability and determining percent seed set. For the cytological studies, chromosome pairing behavior in the diploid *S. bicolor* x *S. propinquum* hybrid, and the tetraploid *S. bicolor* x *S. propinquum* hybrids was compared with both parents to provide a better understanding of the relationship between *S. bicolor* and *S. propinquum*.

CHAPTER II

PHENOTYPIC CHARACTERIZATION

II.1 OBJECTIVE

Phenotypically characterize the F_2 and F_3 progeny derived from a single tetraploid plant induced from a *S. bicolor* x *S. propinquum* F_1 hybrid.

II.2 PLANT MATERIALS

II.2.1 *Sorghum bicolor* ATx623

A/BTx623 is a well-known CMS/maintainer inbred line, and the seed was provided by Dr. William Rooney. BTx623 was used to annotate the sorghum genome (Paterson et al., 2009).

II.2.2 *Sorghum propinquum*

The plant material used was grown from seed in 2017-2018. This unnamed accession was provided by USDA-ARS. This perennial *S. propinquum* accession has short rhizomatous growth, although the climatic range of perenniality is unknown (Jessup et al., 2017).

II.2.3 *Sorghum bicolor* x *Sorghum propinquum* 4*x* hybrid

The *S. bicolor* x *S. propinquum* F_1 hybrids were recovered from controlled hybridizations between ATx623 and *S. propinquum.* These F_1 hybrids, using tissue cultured shoots, were treated with 300 µM colchicine solution for 6 hours. One induced tetraploid plant was successfully recovered (Jessup, unpublished data, 2014). This plant was self-pollinated to produce F_2 seed. This seed was used in 2018 to evaluate and advance to the F_3 generation. The F_3 seed collected from plants grown in the greenhouse during the winter of 2018-2019 was used in 2019 for further evaluation. Figure 1 is a diagram showing how the F_2 and F_3 populations were created.

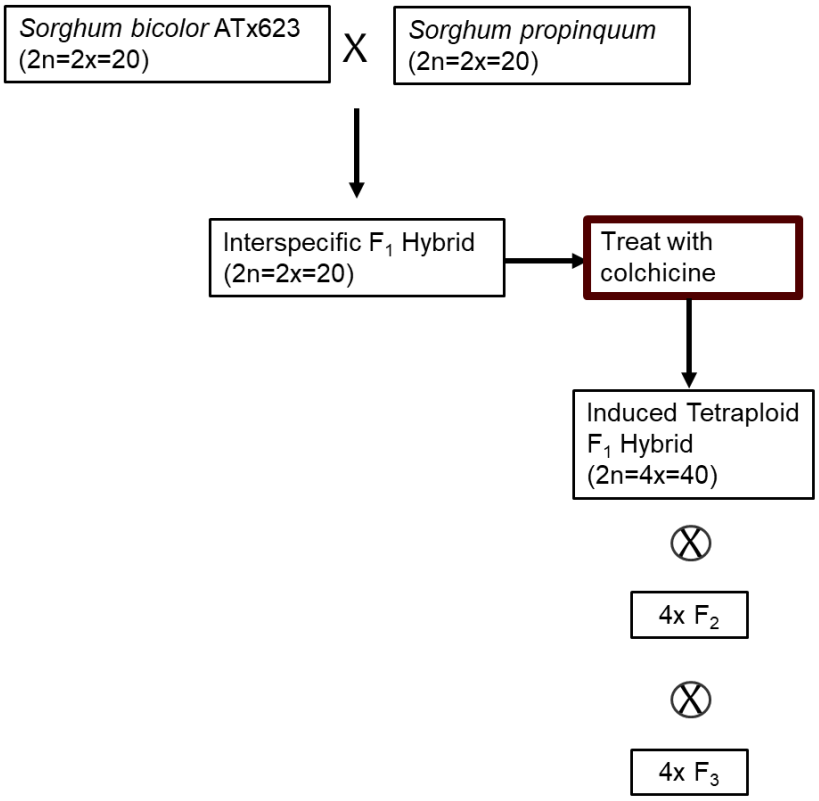


Figure 1. Schematic demonstrating the creation of the tetraploid interspecific population

II.3 METHODS

II.3.1 2018 field evaluation

F_2 4*x* hybrid plants (aprox. 120) were hand transplanted into a field nursery at College Station, Brazos County, TX (Chazos loamy fine sand) in June 2018. The 76 survivors were evaluated during the Fall of 2018. For comparison, 10 plants of each parent, BTx623 and *S. propinquum* were also included in the field trial, also transplanted in June. The experimental design was a Completely Randomized Design (CRD) with plant spacings of 0.5 m within rows and 1.25 m between rows. Data were recorded for maximum leaf height at 120 days after planting (DAP), tiller count at 120 DAP, and flowering date at mid-bloom. Additional data were recorded at boot stage regarding the main culm of each plant: number of green leaves, leaf width,

leaf length, and stem thickness. Overwintering was determined by the number of shoots that regrew from each plant in the spring of 2019. Selections were made from the surviving population.

II.3.2 2019 field evaluation

Fields were planted at College Station and Burleson County on April 11 and 17, 2019, respectively. Plants of the F_2 4*x* hybrid, F_3 4*x* hybrid, *S. propinquum*, and BTx623 were planted in single-row plots 3 m long. Plots were spaced 1.5 m apart within each row, and the distance between rows were 1.8 m. The experimental design was a CRD. F_2 4*x* hybrid plants were started from seed and grown in pots in the greenhouse in March and were transplanted into the field plots on the planting dates, with an in-row spacing of 10 cm. Transplants were approximately 20 cm tall at transplanting. All other entries were seed planted using a hand seeder with an in-row spacing of 10 cm. Due to small seed size, *S. propinquum* was hill-planted with 3 seeds per drop, and the other seeded entries had on average 1 seed per drop. Targeted seed depth for *S. propinquum* was 5 mm, whereas, the larger seeded entries were planted 12 mm deep. Replacement transplants were started for all entries in the greenhouse on the date of field planting. Emergence and stand establishment were evaluated for all seed planted entries one month following planting. At six weeks post planting, all plots were thinned, or transplants were planted to achieve a final stand of 16 plants per plot (approx. 20 cm spacing). Entries were replicated four times at two locations: College Station, Brazos County, TX (Chazos loamy fine sand) (30°37’47” N, 96°22’10” W) and Burleson County, TX (Weswood silty clay loam) (30°32’47.5” N, 96°26’6” W). A total of 8 replications and 128 plants were evaluated per entry. Data were recorded for maximum leaf height, tiller count, and flowering date at boot stage. Additional data were recorded for the main culm of each plant at boot stage: number of green

leaves, leaf width, leaf length, and stem thickness. Overwintering was evaluated by recording the number of plants that regrew (success/failure) in the spring of 2020 as well as recording the number of shoots (vigor) that regrew. With these data further selections were made.

Fertilizer was applied three times throughout the growing season (June 21, July 19, and September 9) at a rate of 56 kg N*ha^{-1} for a total nitrogen application of 168 kg N*ha^{-1}. At the Burleson County location flood irrigation was applied four times during the growing season (July 19, August 1, August 22, September 9), and at College Station, drip irrigation was applied weekly unless the soil was moist from precipitation events.

II.3.3 2019 clipping trial

A clipping trial was also conducted in 2019 at the Burleson County location. This trial included the following entries: F_3 4*x* hybrid, *S. propinquum*, Sorghum-Sudan grass (SP4105), and BTx623. These were also planted in single 3-m row plots with a final stand density of 16 plants per plot, similar to that described above. There were two replications per treatment, with treatments harvested at 45-day intervals (3 cuts per season), 90-day intervals (2 cuts per season), and at 180 days (single cut per season). Fresh weight was recorded immediately after each plot was harvested using all forage produced. Dry weight was determined by taking a sub-sample from each harvest and drying them at 60°C for several days. Plant material was taken from these dried sub-samples and were ground and used to determine the quality of each entry. Forage quality was determined by using Near Infrared Spectroscopy (NIR) contracted through the Texas A&M Forage Testing Laboratory. Forage assays included: Crude Protein (CP), Digestible Crude Protein, Acid Detergent Fiber (ADF), Neutral Detergent Fiber (NDF), Total Digestible Nutrients (TDN) (based on ADF), Net Energy (lactation, maintenance, and gain), Energy Est., in vitro true digestibility (IVTD), Ash, and Relative Feed Value (RFV). NIR mineral analysis was also

received for Phosphorus, Potassium, Calcium, and Magnesium. Overwintering was evaluated and recorded in the spring of 2020 following the same method used for the evaluation plots.

Fertilizer was applied three times throughout the growing season (June 21, July 19- after 1st cutting, and September 9 – after 2nd cutting) at a rate of 56 kg N*ha^{-1} for a total nitrogen application of 168 kg N*ha^{-1}. Flood irrigation was applied four times during the growing season (July 19, August 1, August 22, September 9.)

II.4 RESULTS AND DISCUSSION

II.4.1 2018 field evaluation

In 2018, 76 4*x* F_2 plants growing in College Station were evaluated. These data provided general ranges of what would be expected. This trial cannot be compared to the 2019 trail because its experimental design was different. Additionally, it allowed for potential selection of individuals for breeding purposes. The means for the traits measured are reported in Table 1; however, some data were not collectable for BTx623 due to its early maturing. Generally, BTx623 has fewer leaves that are wider and shorter than the other entries. Additionally, its stem is normally thicker than the other entries. The most interesting finding was that only 13% of the 4*x* F_2 plants regrew in the spring of 2019 and neither perennial entry that overwintered was significantly different than zero.

Table 1. Mean values for various traits collected form the 2018 field evaluation

	Mean values							
Entry	Flowering mid-bloom	Height 120 DAP (cm)	Tiller count 120 DAP	# of leaves at boot	Stem thickness at boot (cm)	Leaf width at boot (cm)	Leaf length at boot (cm)	Overwintered %
BTx623	Aug 8^{a}	92.2^{a}	0.9^{a}	-	-	-	-	0%^{ns}
S. propinquum	Oct 20^{b}	209.8^{b}	32^{b}	13.3^{ns}	1.3^{a}	3.9^{a}	97.3^{a}	30.0%^{ns}
4*x* F_2	Oct 16^{b}	221.8^{b}	2^{a}	12.3^{ns}	1.7^{b}	6.8^{b}	89.8^{b}	13.2%^{ns}

Note: values not followed by the same letter are significantly different

II.4.2 2019 field evaluations

As shown in the following Tables (2 – 5), Entry was significant for every trait. Environment was significant for most traits except for leaf length and two early height measurements. Interactions between entry and environment were present for most traits as well.

Table 2. ANOVA significance levels for bi-weekly field plant heights in 2019

	Height							
	14-Jun	1-Jul	18-Jul	1-Aug	16-Aug	30-Aug	16-Sep	2-Oct
Entry	***	***	***	***	***	***	***	***
Environment	***	NS	NS	***	***	***	***	***
Entry*Environment	***	**	***	***	***	***	***	***

*** < .001, ** < .01, *< .05, NS=not significant

Table 3. ANOVA significance levels for monthly tiller counts in 2019

	Tiller number			
	14-Jun	July 18	16-Aug	16-Sep
Entry	***	***	***	***
Environment	*	***	***	***
Entry*Environment	NS	***	***	***

*** < .001, ** < .01, *< .05, NS=not significant

Table 4. ANOVA significance levels for five main culm traits in 2019

	Main culm				
	Boot date	# of Leaves	Stem Thickness	Leaf Length	Leaf Width
Entry	***	***	***	***	***
Environment	***	**	*	NS	**
Entry*Environment	NS	***	***	NS	NS

*** < .001, ** < .01, *< .05, NS=not significant

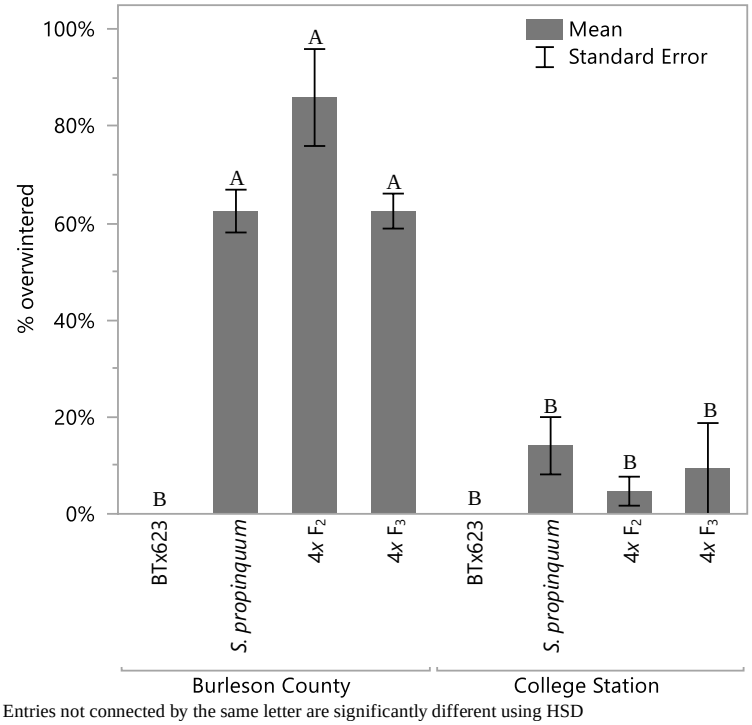
Table 5. ANOVA significance levels for field evaluation overwintering in 2019

	Overwintering	
	%	Vigor
Entry	***	***
Environment	***	***
Entry*Environment	***	***

*** < .001, ** < .01, *< .05, NS=not significant

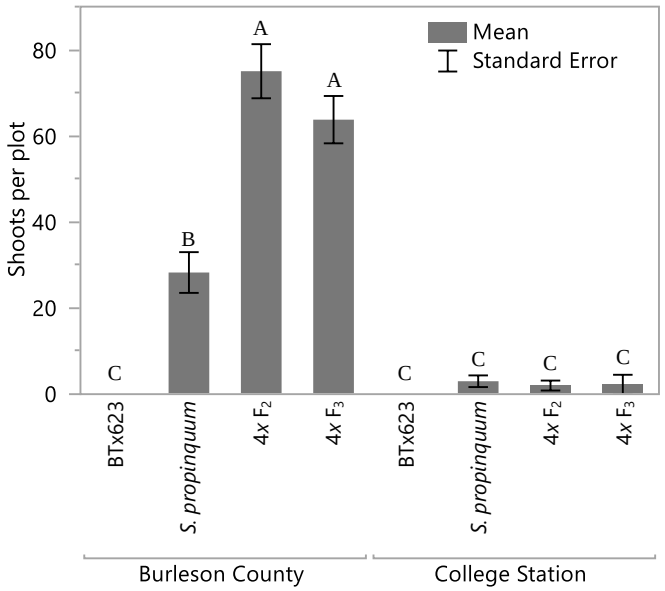
II.4.2.1 Overwintering

As expected, BTx623 did not overwinter at either location. The 4*x* hybrid and *S. propinquum* overwintered at statistically similar levels when comparing the percentage of plants that produced new shoots from crowns and short rhizomes (Figure 2). At the Burleson County location, overwintering was high (62-85%); however, at College Station overwintering was low (4-14%) and was not statistically different than zero. Overwintering vigor was measured by the number of shoots that regrew per plot (Figure 3). At the Burleson County location, the 4*x* hybrid had an average of 65-70 shoots/plot that regrew as compared to only 28 shoots/plot for *S. propinquum.* Again, at the College Station site, regrowth was low and not statistically different than zero. This was a surprising and promising finding because the tetraploid hybrid had more spring regrowth when compared to the perennial parent in at the Burleson County location.



Entries not connected by the same letter are significantly different using HSD

Figure 2. Percent of plants that overwintered by location and entry



Entries not connected by the same letter are significantly different using HSD

Figure 3. Plot regrowth by location and entry in 2020

Differences in soil types at the two locations undoubtedly played a major role in the plants ability to grow and overwinter. The Burleson County location has a deep silty clay loam soil with good drainage. However, at College Station, the topsoil is a loamy fine sand, but immediately below, at a depth ranging from 38 to 61 cm, is an undulating, highly impermeable clay pan. Because the sandy topsoil does not have a high-water holding capacity, this soil is detrimental to the plants during periods of drought. Unfortunately, the only water source available for supplemental irrigation at the College Station location is high in sodium, and with excessive irrigation, sodium accumulates on the impermeable hard pan which causes salinity issues to the plants. Another problem occurs when excessive rainfall occurs, especially during the winter. Because the highly impermeable clay pan prevents water from draining, this results in extended periods of standing water. This causes the topsoil to become waterlogged during the winter, and the underground crowns and rhizomes of the plants often die. The above-mentioned

soil conditions and their consequences are the primary reasons why the plants did not perform as well at the College Station location as in Burleson County.

II.4.2.2 Emergence

Emergence data were taken for all seed planted entries one month after planting in 2019. The 4*x* F_3 plants had the highest average emergence but not statistically different than BTx623. *Sorghum propinquum* did not establish from seed (only at low rates in 2 of 14 plots) and were completely replanted with transplants. Small seed size is probably the reason for lack of emergence. BTx623 has a large seed size (2.07 g/100 seed), the 4*x* F_3 seed was about half that size (0.99 g/100 seed), but *S. propinquum* seed is very small (0.10 g/100 seed). Being a tenth the size of the hybrid seed, *S. propinquum* likely lacked the energy to successfully establish in field conditions. It is important to recognize that even though the tetraploid hybrid seed is smaller than those of grain sorghum, the hybrid seed is large enough to obtain adequate stands in the field from planting seed. This is an important factor for commercial success.

II.4.2.3 Height

The inbred line, BTx623 performed similarly at both locations. It grew rapidly until flowering in early July. Conversely, *S. propinquum* grew very slowly at first at both locations, but it recovered and was the tallest entry at each location at the end of the study. This lag in growth was more pronounced at the Burleson County location than the College Station location. The primary reason for low seedling vigor is that *S. propinquum* seed is 20 times smaller than BTx623, and the smaller the seed size the less endosperm is available to nourish the seedling. BTx623 has ample endosperm which allows the seedling to become much larger in size before it is solely reliant on photosynthesis.

The tetraploid entries offset the height curves as they continued to grow (Figure 4). This is because the F_2 plants were transplanted rather than seeded at planting because there was a shortage of seed. Besides being offset, the curves are very similar for each location. Early in September there were high winds which caused lodging in the tetraploid entries and this is the reason for the decrease in plant height in the graph (Figure 4). None of the other entries lodged during that storm event. This is probably because *S. propinquum* had many tillers which provided more of a barrier to the wind. The tetraploid entries might perform better in larger plantings where there could be more strength with a higher population density.

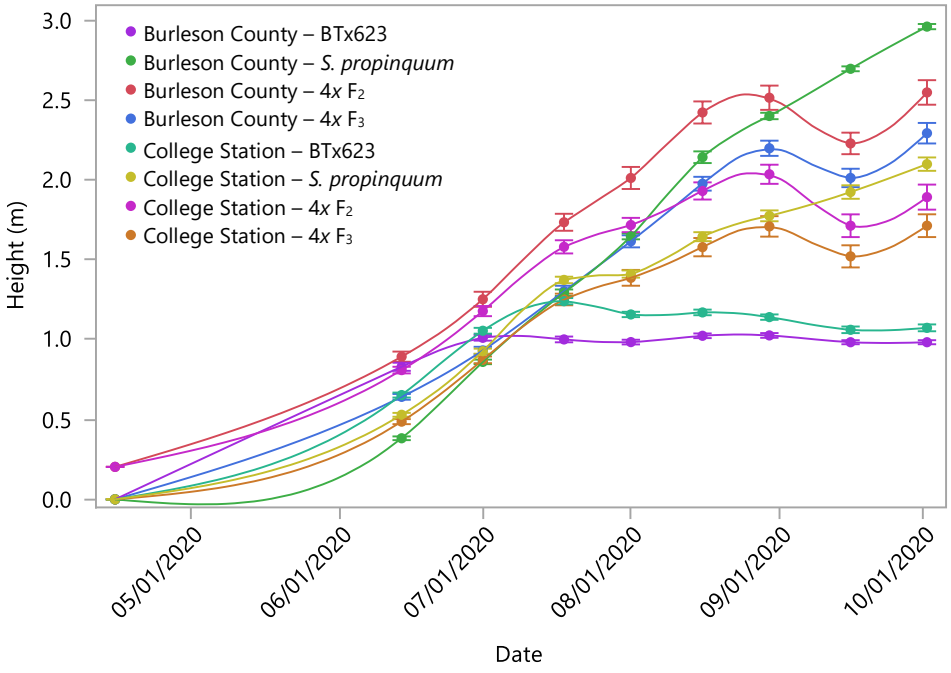
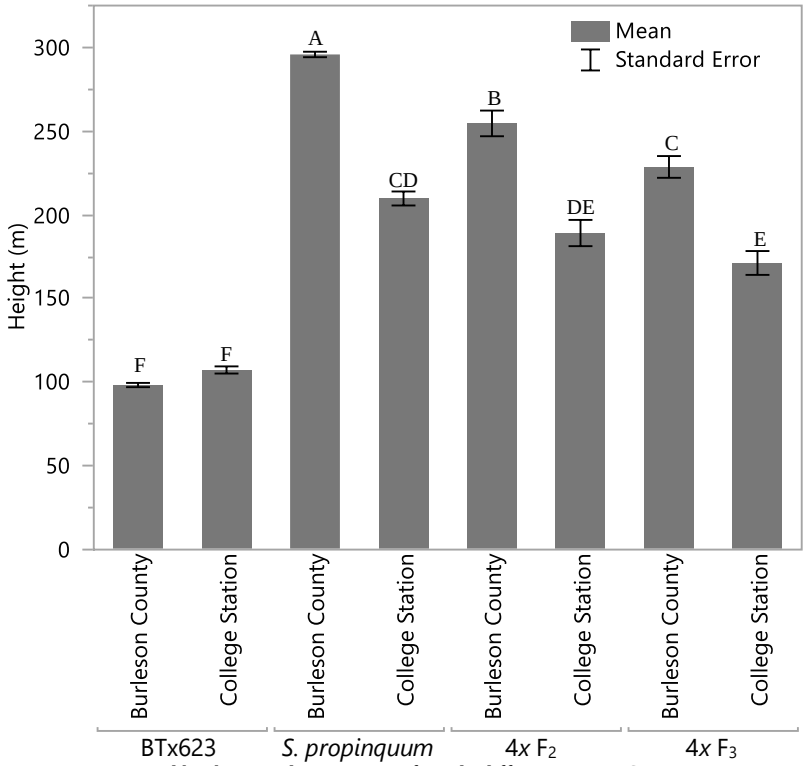


Figure 4. Mean canopy height by entry and location over time

As shown by the final height bar graph in Figure 5, except for BTx623, all entries were shorter at the College Station location than at the Burleson County location. This shorter growth

at College Station is probably because of the soil type, and the issues that arise from the low water holding capacity and potential salinity issues therein.

The tetraploid entries included individuals that showed transgressive segregation. The maximum heights for the F_3 population at the Burleson County location was 3.35 m, compared to 3.05 m at the College Station location and the maximum heights for the F_2 population were 3.66 m (Burleson County location) and 3.56 m (College Station location). Whereas, the maximum heights for *S. propinquum* were 3.2 m (Burleson County location) and 2.84 m (College Station location).

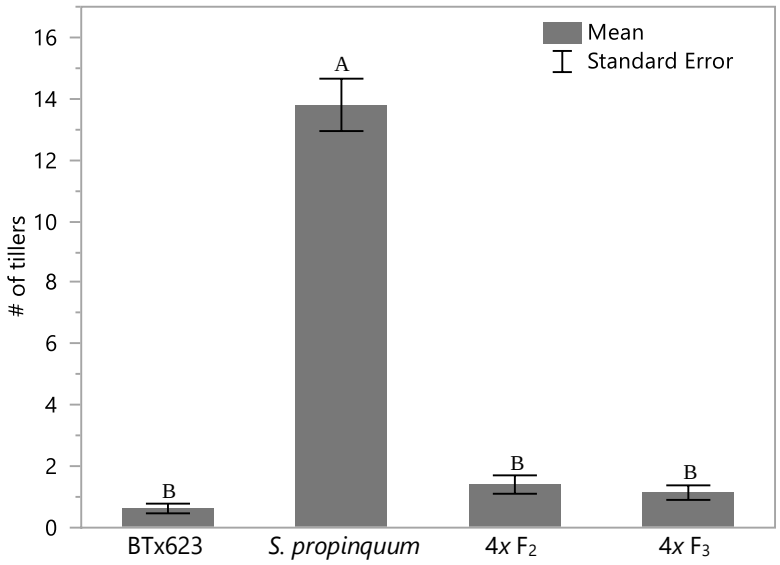


Entries not connected by the same letter are significantly different using HSD

Figure 5. Mean height on October 2, 2019 by entry and location

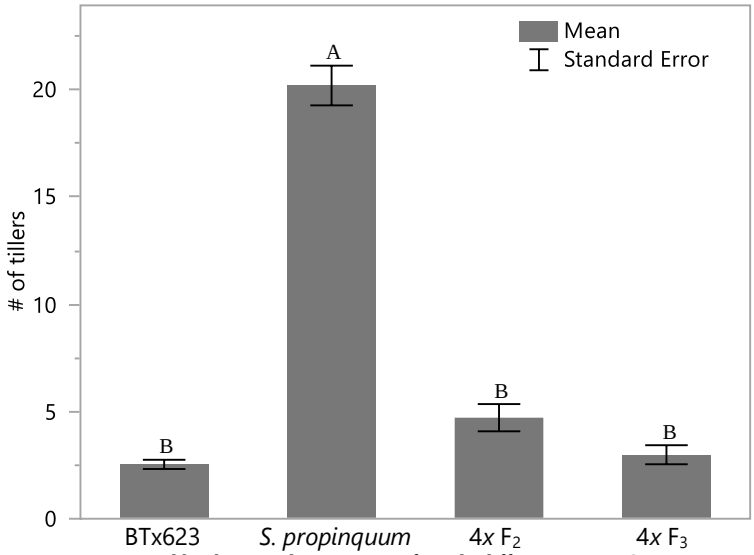
I.4.2.4 Tillering

Sorghum propinquum was the most profusely tillering entry at both locations. This is clearly shown in Figures 6 and 7. Although the mean tiller number was not significantly different between the F_2 and F_3 tetraploids and BTx623, the distributions for these three entries were all right skewed. The tetraploid entries had an increased percentage of individuals with more than 5 tillers compared to BTx623 at the same location (Table 6). The largest difference occurred at the Burleson County location. These findings indicate that progress could be made in selecting for more tillering in the tetraploid population which should improve both regrowth after harvest and forage yield.



Entries not connected by the same letter are significantly different using HSD

Figure 6. Mean tiller count per plant by entry at College Station on Sept 16, 2019



Entries not connected by the same letter are significantly different using HSD

Figure 7. Mean tiller count per plant by entry at Burleson County on Sept 16, 2019

Table 6. Tillering of entries by location in 2019 according to three measures

	% zero tillers	% >5 tillers	Max tiller count
BTx623 - College Station	69%	3%	6
S. propinquum - College Station	3%	94%	34
4*x* F_2 - College Station	50%	9%	13
4*x* F_3 - College Station	52%	5%	10
BTx623 - Burleson County	7%	9%	8
S. propinquum - Burleson County	0%	100%	40
4*x* F_2 - Burleson County	19%	39%	22
4*x* F_3 - Burleson County	23%	25%	24

II.4.2.5 Flowering

At the Burleson County location, most plants had grown to boot stage by October 31, 2019. Besides BTx623, a reduced percentage of plants had grown to the boot stage at College Station before the first killing frost which occurred on November 1, 2019 as shown by Figure 8. Because of this reduced rate, flowering times were only compared for the Burleson County

location as shown in Figure 9. Both parents behaved as expected, BTx623 reached boot stage near July 1st (74 DAP), and *S. propinquum* was completely photoperiodic reaching boot stage at or near October 12th (179 DAP). Both parents had little variation for time of flowering. At Burleson County, all BTx623 plants reached boot stage within a 15-day period, and all *S. propinquum* plants reached boot stage within an 8-day period. Additionally, while photoperiod is a critical factor for flowering for photoperiod sensitive sorghums (including *S. propinquum*), it is not the only factor as evidenced by the difference in flowering at each location.

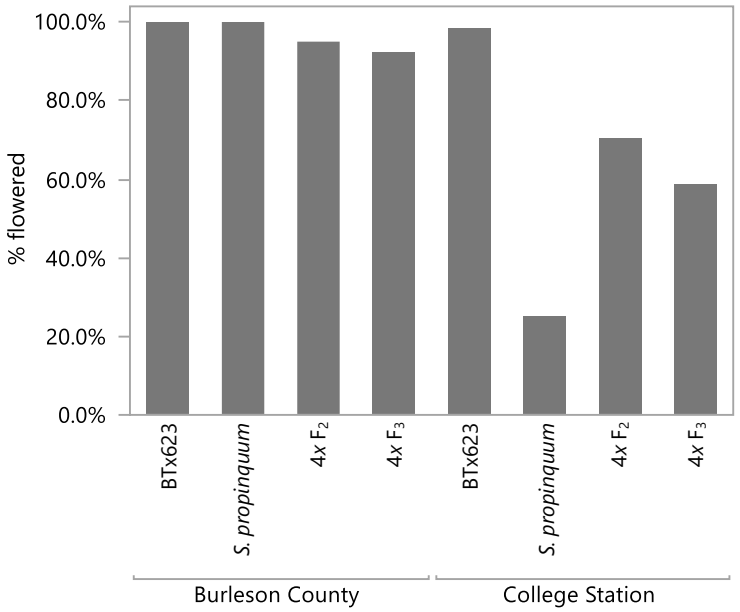
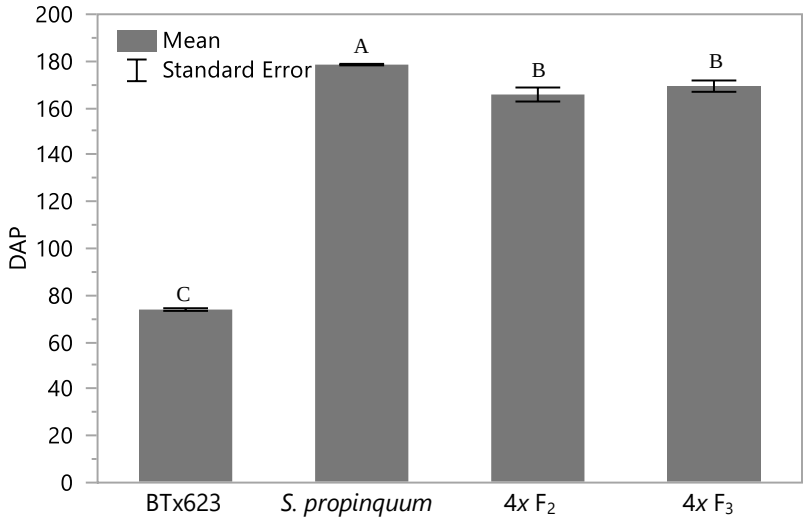


Figure 8. Percent of plants that reached boot stage in 2019 by entry and location



Entries not connected by the same letter are significantly different using HSD

Figure 9. Mean days after planting (DAP) that plants reached boot stage in 2019 by entry at Burleson County

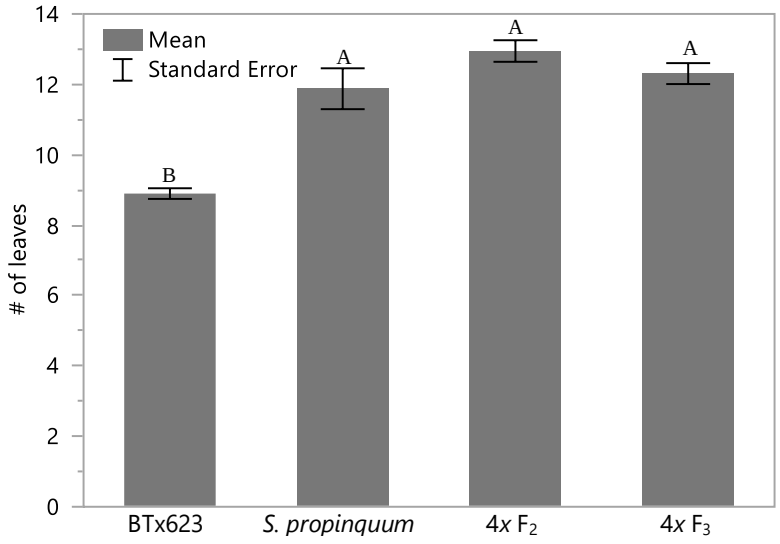
Both tetraploid F_2 and F_3 entries reached boot stage near October 1st (166 and 169 DAP, respectively), even though the median boot date was 171-172 DAP. In both entries 75% or more of the individuals reached boot stage after the mean date, and 3% of the plants segregated for early flowering, such that they flowered at nearly the same time as BTx623. Most of the tetraploid plants maintained photoperiodic flowering behavior with a few segregates (8.6%) that flowered before the fall equinox. These early flowering individuals can be easily removed from the population because they are easily recognized.

II.4.2.6 Leafiness

Because these data were taken at boot stage, many data points were not collected from plants that did not grow to the boot stage. As with the flowering data, only the Burleson County data were presented in Figure 10. The tetraploid entries had the highest mean number of leaves (12.3, 12.9), but they were not different from *S. propinquum* (11.9). BTx623 had a significantly lower number of green leaves at 8.9. It is important to remember that data were taken from only

the main culm. Additionally, these data were the number of green leaves still on the culm, not the number of nodes.

There was substantial variability in all entries for this trait as shown by the error bars in Figure 10. Greater leafiness would be a desirable trait for forage production and improvement is possible. However, as the variation is large even in the inbred line (BTx623), there is a large error, and without testing, it could be assumed to have low heritability. This would imply that improvement by selection for this trait may be relatively slow.



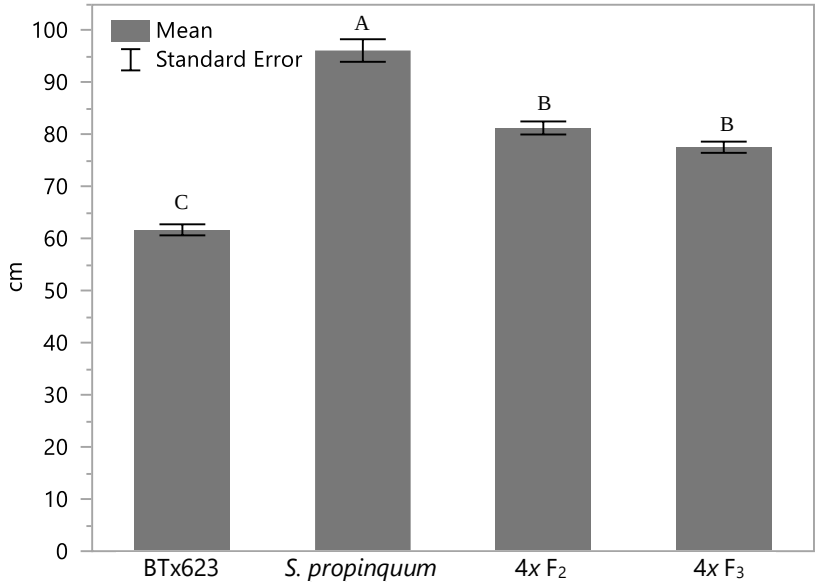
Entries not connected by the same letter are significantly different using HSD

Figure 10. Mean number of green leaves on main culm at boot stage by entry at Burleson County

II.4.2.7 Leaf length and leaf width

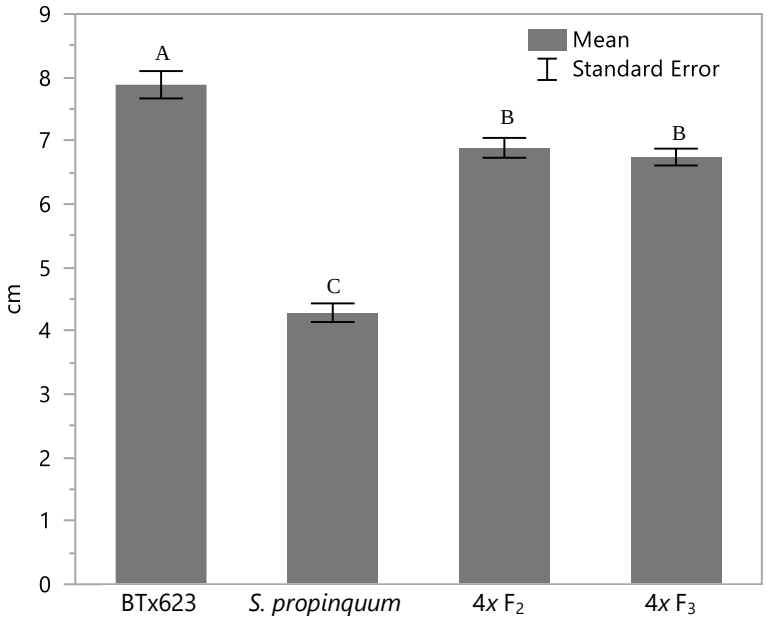
Data regarding leaf length and width were also collected at the boot stage and data only from the Burleson County location are included in Figures 11 and 12. Btx623 had the shortest leaves at 62 cm, the tetraploid F_2 and F_3 entries were intermediate (81 and 78 cm), and *S. propinquum* had the longest leaves at 96 cm.

BTx623 had the widest leaves with a mean of 7.9 cm. The tetraploid plants were again intermediate with 6.7-6.9 cm leaf width, and *S. propinquum* had the narrowest leaves at 4.3 cm. Data from the parents suggest there may be a negative correlation between leaf width and leaf length, such that the longer a leaf is the narrower it is. Fortunately, when tested, no correlation was found between leaf length and leaf width. Ideal forage leaf morphology would be wide, long leaves which combines traits from both parents. The tetraploid entries were intermediate in both traits but as there was no correlation selection can be made for individuals with wide, long leaves resulting in a superior leaf morphology.



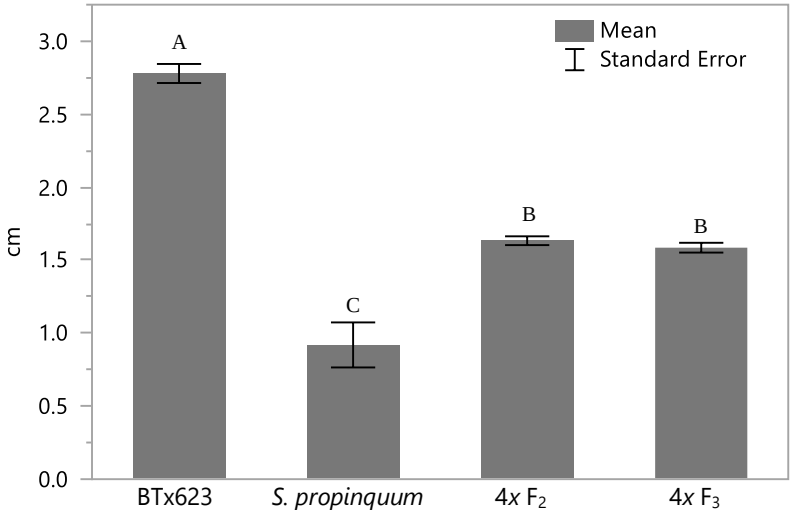
Entries not connected by the same letter are significantly different using HSD

Figure 11 Mean leaf length of the seventh leaf below the flag leaf on the main culm at boot stage by entry at Burleson County



Entries not connected by the same letter are significantly different using HSD

Figure 12. Mean leaf width of the seventh leaf below the flag leaf on the main culm at boot stage by entry at Burleson County



Entries not connected by the same letter are significantly different using HSD

Figure 13. Mean stem thickness (diameter) on the internode between the 7th and 8th leaves below the flag leaf on the main culm at boot stage by entry at Burleson County

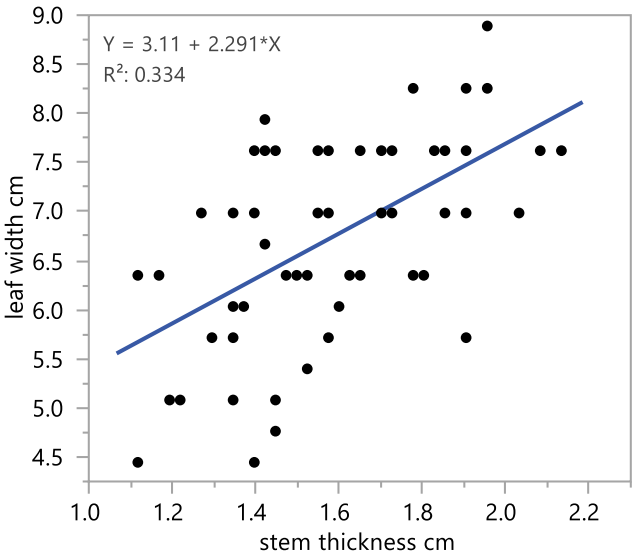


Figure 14 Linear regression for 4*x* F_3 at Burleson County of leaf width and stem thickness

II.4.2.8 Stem thickness

These data were also taken at boot stage, and data from only from the Burleson County location are included in Figure 13. BTx623 had the largest stem diameter (2.77 cm). The tetraploid F_2 and F_3 entries were intermediate (1.63 and 1.59 cm), and *S. propinquum* had the smallest stem diameter with a mean of 0.92 cm.

When taking the data, it appeared that leaf width and stem diameter were roughly corelated by approximately pi (the ratio of circumference to diameter of a circle). The best correlation of mid leaf width and stem thickness at Burleson County was for the 4*x* F_3 entry (Figure 14). However, this correlation has an R^2 value of 0.334, and the slope of the fit line is 2.291 not pi. Although slightly correlated for this entry, it should be possible to select for both thin stems and wide leaves which would be ideal for a hay type forage.

II.4.3 2019 Clipping trial

To facilitate comparisons dry yields were combined across multiple harvests, and the quality data were averaged over all harvests for each plot using a weighted average to account

for the portion of total dry yield each harvest contributed. As shown in Tables 7 and 8, entry, treatment, and their interaction had significant effects on yield. Additionally, entry was significant for all quality traits, except for Net energy gain. Treatment was significant for all quality traits, except for Ash and Mg, and interactions were present for most quality traits, excluding NDF, Ash, Net energy gain, Ca, and Mg.

For most of the traits, the treatment effect (sum of squares) was larger than the entry effect. Entry had a larger effect than treatment for the following traits: Dry yield, NDF, Ash, K, Ca, and Mg.

Table 7. ANOVA significance levels for nine clipping trial traits in 2019(1 of 2)

		multiple cuttings averaged - weighed by portion of yield							
	Dry yield total (kg)	CP %	CP dig. %	ADF %	NDF %	TDN %	IVTD %	Ash %	RFV
Entry	***	**	**	**	***	**	***	**	***
Treatment	***	***	***	***	***	***	***	NS	***
Entry*treatment	***	**	**	**	NS	*	*	NS	*

*** < .001, ** < .01, *< .05, NS=not significant

Table 8. ANOVA significance levels for eight clipping trial traits in 2019(2 of 2)

	multiple cuttings averaged - weighed by portion of yield							
	Net Energy Lact Mcal/lb	Net Energy maint Mcal/lb	Net energy gain Mcal/lb	Energy est. therms/cwt	P %	K %	Ca %	Mg %
Entry	**	*	NS	**	**	***	***	*
Treatment	***	***	**	***	**	**	**	NS
Entry*treatment	**	*	NS	*	**	*	NS	NS

*** < .001, ** < .01, *< .05, NS=not significant

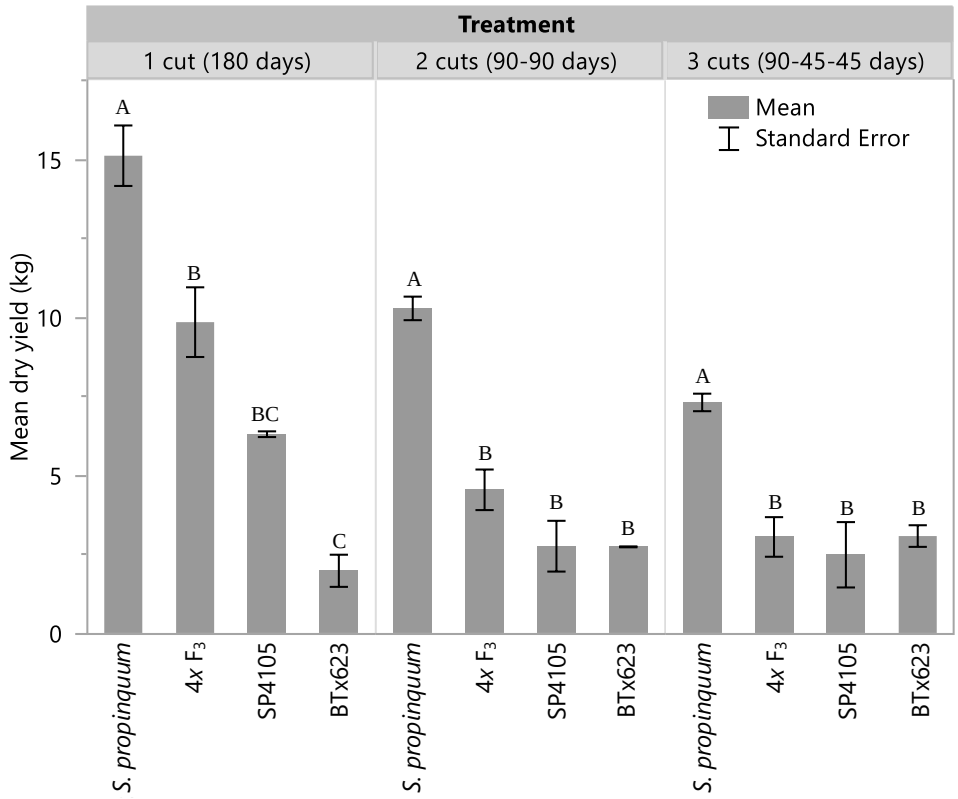
Overwintering for the clipping trial was measured but only a few *S. propinquum* plants regrew, and because of this, no comparisons were performed for overwintering in the clipping

trial (not listed in Tables 7 and 8). It was unfortunate that these plots did not regrow, especially considering the vigorous regrowth of the F_2 and F_3 tetraploid entries in the evaluation plots at the same location. Additional trials should be conducted to determine a proper harvesting regime for successful overwintering. A potential scheme for the Burleson County location would be to harvest twice a growing season. The first harvest would be at 90-120 DAP, but the second cutting would not occur until after the first killing frost. This would reduce forage quality for the second cutting, but it might improve chances of overwintering. Once successful establishment year practices are perfected, then multiyear trials could be conducted.

II.4.3.1 Yield

It was expected that the commercial Sorghum-Sudan grass cultivar (SP4105) would produce the most forage; however, *S. propinquum* was the most productive across all treatments (Figure 15). Additionally, the tetraploid F_3 hybrid produced as much forage as or more than either commercial entry, SP4105 or BTx623.

Only limited conclusions can be drawn from these yield data because the data were taken from small plots with only two replications. However, these findings do indicate that the tetraploid hybrid has a high forage yield potential and additional forage trials are merited.



Entries not connected by the same letter are significantly different using HSD

Figure 15. Total dry forage yield by entry and treatment

II.4.3.2 Forage Quality

Forage quality results, when evaluated by treatment, showed less significant differences that evaluated across treatments, and the mean values are presented in Tables 9 and 10. Also, mineral contents, even if significantly different, do not have practical differences. For the single cut treatment (180 days), only CP, dig CP, Ca, and Mg had significant differences. In this treatment, BTx623 and the commercial check had higher CP than the 4*x* F_3 entry. The two-cut treatment (90-90 days) had the most significantly different traits, with all but Ash, Net energy maint, and K being non-significant. For the three-cut treatment (90-45-45 days), almost half of the traits were significant.

Table 9. Mean values for eight quality traits by entry and treatment (1 of 2)

	Mean							
	CP %	CP dig. %	ADF %	NDF %	TDN %	IVTD %	Ash %	RFV
1 cut (180 days)								
BTx623	9.35[a]	5.2[a]	37.36	58.14	55.58	62.79	9.53	95.85
S. propinquum	8.25[ab]	4.15[ab]						
SP4105	8.95[a]	4.8[ab]						
4*x* F_3	6.35[b]	2.45[b]						
2 cuts (90-90 days)								
BTx623	9.86[ab]	5.66[ab]	30.04[b]	50.59[b]	61.80[a]	73.07[a]	9.29	120.49[a]
S. propinquum	8.22[b]	4.09[b]	36.18[a]	59.50[a]	56.63[c]	63.68[b]		95.03[c]
SP4105	12.67[a]	8.27[a]	33.74[a]	52.51[b]	59.93[b]	78.40[a]		110.89[b]
4*x* F_3	11.94[a]	7.60[a]	33.74[a]	52.72[b]	59.71[b]	75.61[a]		110.70[b]
3 cuts (90-45-45 days)								
BTx623	10.58	6.34	33.04	53.06[b]	59.75	73.70[a]	7.18[b]	114.76[a]
S. propinquum				59.76[a]		69.36[b]	8.35[b]	97.25[c]
SP4105				53.16[b]		74.84[a]	10.65[a]	108.69[ab]
4*x* F_3				57.74[a]		74.34[a]	8.50[b]	101.76[bc]

Values not followed by the same letter are significantly different, entries were evaluated within each treatment using HSD

Entries with no significant differences are shown with a combined mean

Table 10. Mean values for eight quality traits by entry and treatment (2 of 2)

	Mean							
	Net Energy Lact Mcal/lb	Net Energy maint Mcal/lb	Net energy gain Mcal/lb	Energy est. therms/cwt	P %	K %	Ca %	Mg %
1 cut (180 days)								
BTx623	0.56	0.60	0.28	46.61	0.18	1.36	0.35[b]	0.19[b]
S. propinquum							0.33[b]	0.21[b]
SP4105							0.49[a]	0.26[a]
4*x* F_3							0.32[b]	0.23[ab]
2 cuts (90-90 days)								
BTx623	0.63[a]	0.64	0.35[a]	52.32[a]	0.22	1.30[b]	0.37[b]	0.22
S. propinquum	0.57[c]		0.29[c]	47.46[c]		1.54[b]	0.37[b]	
SP4105	0.61[b]		0.33[b]	50.63[b]		1.92[a]	0.53[a]	
4*x* F_3	0.61[b]		0.33[b]	50.43[b]		1.61[ab]	0.44[ab]	
3 cuts (90-45-45 days)								
BTx623	0.61	0.65	0.35	50.44	0.19[b]	1.20[b]	0.36[b]	0.21
S. propinquum					0.21[ab]	1.21[b]	0.33[b]	
SP4105					0.24[a]	1.83[a]	0.46[a]	
4*x* F_3					0.20[ab]	1.45[b]	0.39[ab]	

Values not followed by the same letter are significantly different, entries were evaluated within each treatment using HSD

Entries with no significant differences are shown with a combined mean

In the two-cut treatment, CP was different than for the single cut treatment with the 4*x* F_3 entry being similar to the commercial check (SP4105). Also, in the two-cut treatment, there was a difference in fiber content. ADF in BTx623 was lower than in all the other entries, and the NDF content of *S. propinquum* was higher than all the other entries. Both the two and three cut treatments had the same significance pattern for IVTD with *S. propinquum* being lower than the other entries.

While the findings of different forage quality traits offer limited conclusions, the quality results from these trials are similar to reported means from the Texas A&M Sorghum Silage Trials done performed annually in Bushland, Texas (Bell et al., 2014-2019). The data listed above had very similar CP to the silage trials from 2019, however the other quality measures were slightly poorer than the silage trials results in 2019. The six year (2014-2019) mean ranges from the silage trials reported by Bell et al. are as follows: CP 6.6-8.1%, ADF 29.8-37.4%, NDF 44.6-54.3%, and RFV 98-128. This indicates that the quality of this germplasm is acceptable for forage sorghum.

In future experiments, harvest times should be adjusted, as mentioned previously, to identify the proper management practice for successful overwintering. After that is determined, management practices for best quality could then be established. These results indicate that if a single end-of-season harvest is utilized, the quality of all entries will be similar because, all will have poorer quality than a multi-cut harvesting regime. However, when considering the yield data, the small reduction in quality probably is not worth the major reduction in yield. It would also be expected that harvest intervals will change between the establishment year and seasons following overwintering.

CHAPTER III

CYTOLOGICAL CHARACTERIZATION

III.1 OBJECTIVE

Determine the reproductive fertility and cytology of both parents, the diploid *S. bicolor* x *S. propinquum* hybrid, and the induced tetraploid *S. bicolor* x *S. propinquum* hybrids. Fertility was determined by microscopically examining pollen stainability and determining percent seed set. For the cytological studies, chromosome pairing behavior in the diploid *S. bicolor* x *S. propinquum* hybrid, and the tetraploid *S. bicolor* x *S. propinquum* hybrids was compared with both parents to provide a better understanding of the relationship between *S. bicolor* and *S. propinquum*.

III.2 PLANT MATERIALS

The plant materials used were the same as listed in Chapter II, with the exception of the diploid *S. bicolor* x *S. propinquum* hybrid. The diploid *S. bicolor* x *S. propinquum* plants used were selected F_2 individuals that were used for another research project and were growing in large planters (40-gallon plastic drums cut in half).

BTx623 and the 4*x S. bicolor* x *S. propinquum* hybrid material used for the pollen stainability and seed set studies was collected from the 2019 field evaluation plots. However, *S. propinquum*, due to its very late flowering, was nearly all collected from plants started outside in large planters that were moved to a greenhouse before the first frost. Plant materials used for the PMC analyses were collected primarily from plants growing in the greenhouse.

III.3 CYTOLOGICAL METHODS

III.3.1 Reproductive Fertility

Fertility was determined by measuring pollen stainability and seed set. Pollen was stained using a 1% potassium iodide (I_2-KI) solution. Some pollen was stained with 1% aceto-carmine stain instead of I_2-KI. The spikelets were collected in the morning prior to anthesis, and they were immediately transported to the laboratory. The florets were removed from inside the spikelets, and the anthers were dissected from the florets. Then the anthers were placed on a microscope slide into a drop of I_2-KI and macerated with a needle to release the pollen. Anther debris was removed from the solution on the slide using the tip of a dissection needle. A 22 x 22 mm glass coverslip was gently placed over the solution, and the pollen grains were then examined using a light microscope at 100 X magnification. A minimum of 500 pollen grains were examined for each plant, and each grain was counted and recorded as to whether it was stained or unstained. At least 10 different plants were used for each entry (BTx623, *S. propinquum*, 2*x* hybrid, and 4*x* hybrid).

Seed set was determined by counting the total number of florets (excluding sessile or unformed florets) on a panicle and comparing it to the number of seeds on that panicle. Data were taken for one panicle from 10 different individual plants per entry (BTx623, *S. propinquum* and 4*x* hybrid).

Analysis of means was performed with ANOVA, Fishers LSD, and Turkey's HSD. JMP Pro 14 (SAS Institute) was used to perform the analysis.

III.3.2 Meiotic evaluation – Pollen Mother Cells (PMC)

Panicles were collected at the young boot stage shortly after the flag leaf had emerged. The objective was to locate florets in which the microsporocytes, commonly called pollen

mother cells (PMC), were undergoing meiosis. Considerable trial and error was used to determine the appropriate developmental stage for collection. The collected panicles were fixed in Carnoy's solution (6 parts absolute ethanol, 3 parts chloroform, and 1 part glacial acetic acid) for one day and then transferred into 70% ethanol for storage.

Upon examining the fixed panicles, individual florets were dissected, and the anthers were transferred on to a microscope slide. A drop of 1% aceto-carmine stain was added, and the anthers were macerated in the stain to release the PMCs. Anther debris were removed, and a cover slip was placed over the solution. The slide was heated slightly over a low flame. The slide then was placed between the sides of a folded piece of filter paper and pressure was applied to the cover slip to flatten the cells and spread the chromosomes.

The PMCs were briefly examined using light microscopy at 100 to 200X magnification to determine if the cells were undergoing meiosis. If they were, the slide was moved to a microscope equipped for phase contrast microscopy, and individual cells were examined at 1000 to 2000X magnification to interpret chromosome pairing behavior. Each evaluable PMC was characterized by the number of various chromosome configurations (univalent, bivalent, trivalent, and quadrivalent) present at diakinesis, prometaphase I, and metaphase I. The majority of data were collected at metaphase I. A minimum of 20 PMCs was characterized per plant, and at least 10 plants were evaluated for the 4*x* hybrid and *S. propinquum.* Only one plant (10 replications of 20 PMC's minimum) was evaluated for the inbred line BTx623. Even though the chromosome pairing behavior of *S. bicolor* is well documented, meiosis was observed in BTx623. Only two plants of the diploid hybrid were evaluated because limited germplasm of this hybrid was available.

Analysis of means were performed using ANOVA and Fisher's LSD. JMP Pro 14 (SAS Institute) was used to perform the analysis.

III.4 RESULTS AND DISCUSSION

III.4.1 Fertility

The 4*x S. bicolor* x *S. propinquum* hybrid had significantly lower fertility than either parent or the diploid hybrid, for both pollen stainability and seed set. Pollen stainability was significantly different between entries (Table 11). Pollen stainability was high (85-90%) for BTx623, *S. propinquum*, and the male fertile diploid hybrids (since the cross was made using ATx623 there was segregation for male sterility in the hybrids). Male sterile segregates from the diploid F_2 germplasm were not included in this percentage because, as expected, none of their pollen stained. Pollen stainability in the 4*x S. bicolor* x *S. propinquum* F_2 hybrids was much lower – 57% than the parents. These differences are shown in the images in Figure 16 and graphically in Figure 17. The variation between individuals was quite large ranging from 17% to 77%, and pollen stainability in 9 of the 10 plants examined was above 50%.

The assumed to be non-viable pollen does not stain or is only partially stained (Figure 16) because they are devoid of starch. The unstained or lightly stained pollen grains also tends to be smaller than the stained pollen (Figure 16), which was expected because viable grains continue to enlarge as they mature. The tetraploid hybrid's pollen grains were larger than those of the diploids examined, and interestingly *S. propinquum* had noticeably smaller pollen than BTx623 (Figure 16). The diameter of the stained pollen in Figure 16 are roughly as follows: 45 µm for BTx623, 39 µm for *S. propinquum*, and 55 µm for the 4*x* hybrid. The diameter of the tetraploid pollen is roughly 1.25x (twice the volume of a sphere) that of BTx623 which was expected because induced tetraploids often have larger pollen grains than their diploid counterparts. The

smaller pollen of *S. propinquum* verses BTx623 was unexpected since their the genome sizes are similar: this could be an aberration because these observations were from single individuals. However, if the difference is real, one explanation could be that large pollen (greater starch content) could have been inadvertently selected for in BTx623 during the improvement process.

Table 11. Analysis of variance for pollen stainability

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	3	0.732929	0.24431	13.4506
Error	36	0.653886	0.018164	Prob > F
C. Total	39	1.386816		<.0001

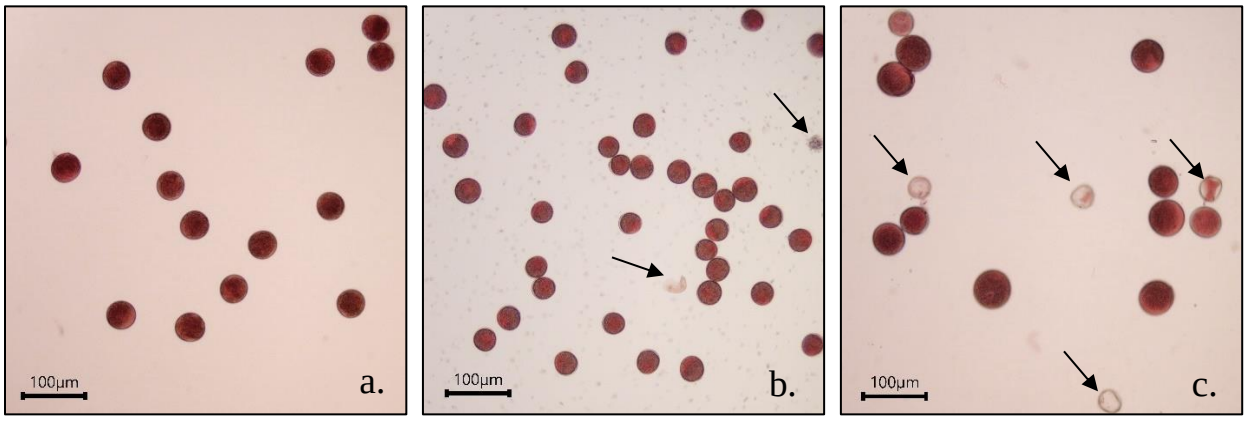
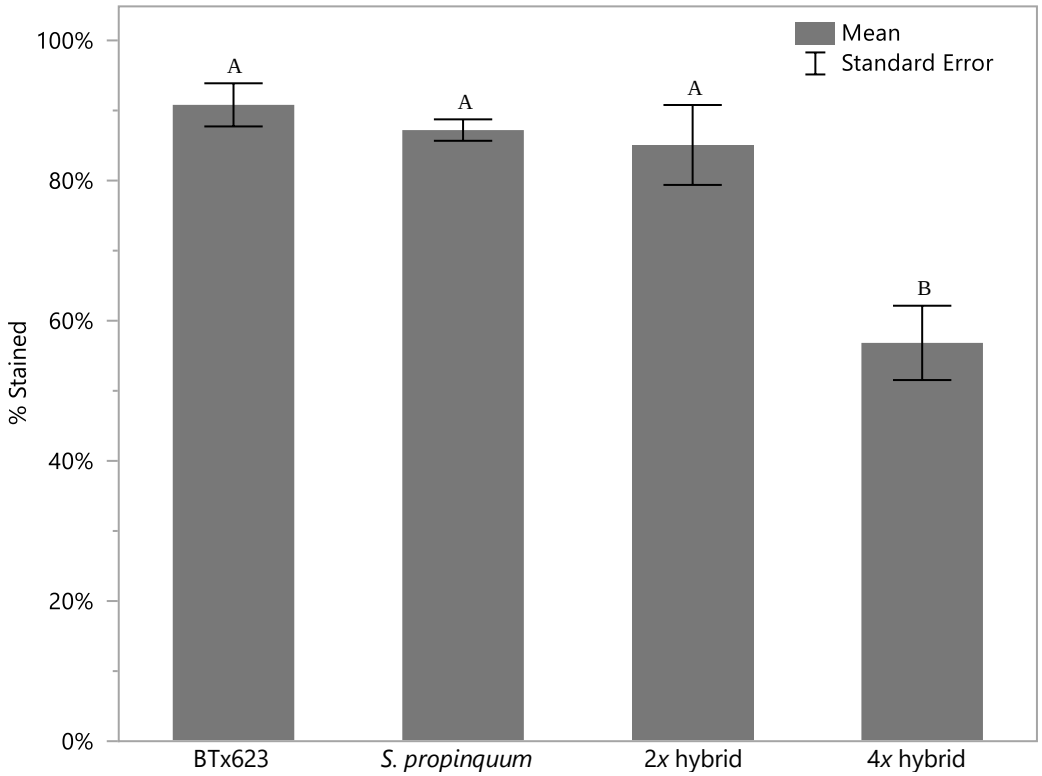


Figure 16. Stained pollen of BTx623 (a.), *Sorghum propinquum* (b.), and 4*x* hybrid (c.). Non-viable pollen grains are indicated by arrows



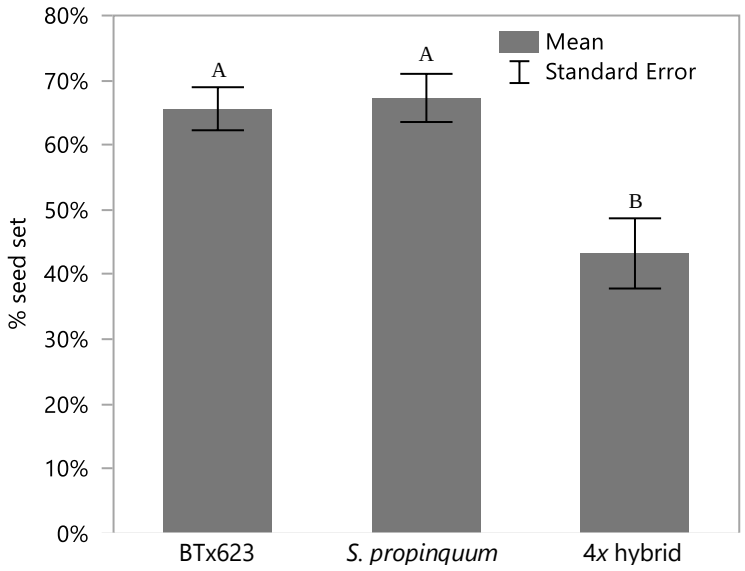
Entries not connected by the same letter are significantly different using HSD

Figure 17. Mean pollen stainability by entry

Seed set was also significantly different among entries (Table 12), similar to pollen stainability. Both diploid parents had similar seed set percentages (65-67%); whereas, the tetraploid hybrid had reduced seed set (43%) as shown in Figure 18. As observed with pollen stainability, there was a considerable variation in seed set among the 4*x* hybrids, ranging from 14% to 68%. It should be noted the panicles that were collected, matured late in the growing season to minimize environmental error between early and late flowering types. The BTx623 panicles were collected from basal tillers produced after the main culm had matured. Therefore, it could be expected that BTx623 would have a higher seed set if the seed on the main culm had been used.

Table 12. Analysis of variance for seed set

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	2	0.359203	0.179601	9.9428
Error	27	0.487715	0.018064	Prob > F
C. Total	29	0.846918		0.0006



Entries not connected by the same letter are significantly different using HSD

Figure 18. Mean percent seed set by entry

Without actually calculating seed set, it could be expected that BTx623 would have higher seed set than *S. propinquum*, because BTx623 has a very compact panicle and large seed size. This inbred line appears to have high seed set, whereas, the opposite is true of *S. propinquum* as illustrated in Figure 19.

While the hybrid has reduced seed set, its actual seed production capacity under field conditions has not been determined. The tetraploid panicle (far right) in Figure 19 was produced by an early flowering off type that produces many tillers (>10) with similar panicle sizes. This individual had high seed set (68%) with ~2300 seed on the panicle examined. If selected plants have similar seed set capacities as this individual, then seed production should not be an issue.

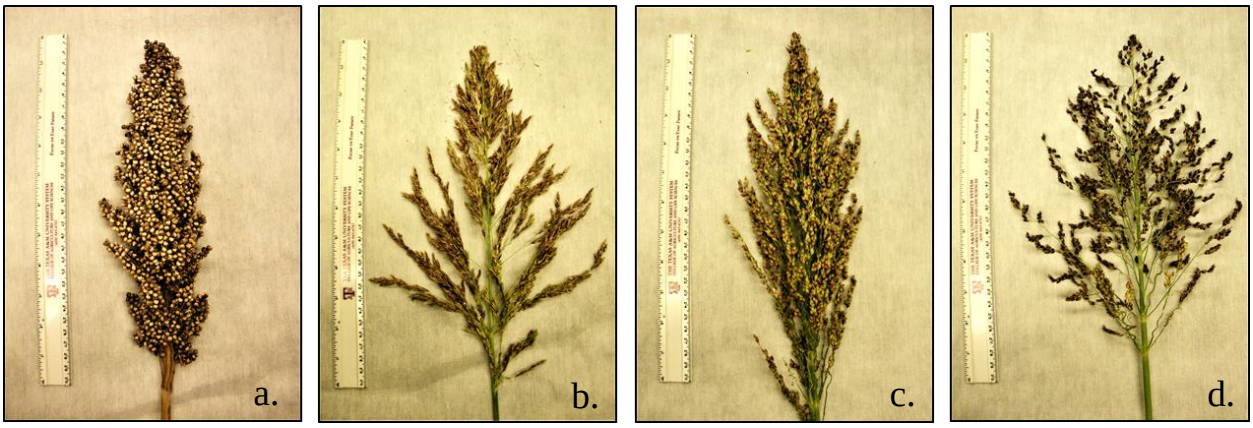


Figure 19. Panicles left to right: BTx623 (a.), *S. propinquum* (b.), 4*x* hybrid (c. and d.)

III.4.2 Meiotic chromosome behavior

Findings from the pollen mother cell analyses are summarized in Table 13. Meiotic chromosome pairing in both diploid parents revealed almost complete 10 bivalent pairing at metaphase I (Figures 20a, b). *Sorghum bicolor* and *S. propinquum* had a mean chromosome pairing behavior of 9.987 bivalents and 0.026 univalents and 9.991 bivalents and 0.017 univalents per cell, respectively (Table 13). Both had a maximum of 2 univalents per cell at metaphase I. Three of 232 cells observed in *S. bicolor* and 2 of 228 cells observed in *S. propinquum* had two univalents. Additionally, bivalents of BTx623 were almost always ring bivalents (having at least two chiasmata) with the mean occurrence of rod bivalents was 0.14 per cell (Table 14). *Sorghum propinquum* had a mean of 0.5 rod bivalents per cell (Table 14) which was significantly higher than that of BTx623. Laggards and uneven distribution of chromosomes at anaphase I were rarely observed in *S. propinquum*, and the number of lagging chromosomes were not quantitively characterized. One bivalent in BTx623 occasionally tended to separate precociously at metaphase I (Figure 20a).

The diploid hybrid had the expected chromosome number of $2n=2x=20$, and its chromosomes paired primarily as 10 bivalents at metaphase I (Figure 20c) similar to that

observed in both parents (Table 13). There was a low frequency of two univalents in some cells which was slightly higher than that observed in the parents (Table 13). The frequency of rod bivalents was higher in the diploid hybrid compared to the parents with an average of 1.07 rod bivalents per cell (Table 14). These findings demonstrate (Figure 20c) that *S. bicolor* and *S. propinquum* have homologous chromosomes which indicates both species have a similar genome and are closely related. Although closely related, there is significant divergence as evidenced by the increased incidence of rod bivalents. This agrees with what Celarier (1958) reported in hybrids between these two species. Because the *S. propinquum* accession used in this study differs phenotypically from the *S. propinquum* accessions presently in the USDA National Plant Germplasm System collection, we were interested in determining if the accession used is actually this species. Similarities in the chromosome pairing behavior in the *S. bicolor* x *S. propinquum* F_1 hybrids reported by Celarier (1958) and those observed in this study, provides support that the accessions are similar, and the accession used in this study is *S. propinquum.*

Table 13. Chromosome pairing at metaphase I of meiosis for *S. bicolor*, *S. propinquum*, 2*x* hybrid, and 4*x* hybrid

	No. of plants	2*n*	No. of PMCs	Range				Average per cell			
				I	II	III	IV	I	II	III	IV
S. bicolor - Btx623	1	20	235	0-2	9-10	-	-	0.026[b]	9.987[b]	-	-
S. propinquum	10	20	230	0-2	9-10	-	-	0.017[b]	9.991[b]	-	-
2*x S. bicolor x S. propinquum*	2	20	54	0-2	9-10	-	-	0.074[b]	9.963[b]	-	-
4*x S. bicolor x S. propinquum*	10	40	232	0-4	10-20	0-2	0-5	0.45[a]	17.77[a]	0.02	0.98

Values not followed by the same letter are significantly different

Table 14 Frequencies of ring vs. rod bivalent paring of *S. bicolor*, *S. propinquum*, 2*x* hybrid, and 4*x* hybrid

	No. of plants	2*n*	No. of PMCs	Average II per cell		
				Rings	Rods	Total
S. bicolor - Btx623	1	20	235	9.85[b]	0.14[d]	9.987[b]
S. propinquum	6	20	133	9.48[c]	0.5[c]	9.985[b]
2*x S. bicolor x S. propinquum*	2	20	54	8.89[d]	1.07[b]	9.963[b]
4*x S. bicolor x S. propinquum*	10	40	232	14.96[a]	2.81[a]	17.77[a]

Values not followed by the same letter are significantly different

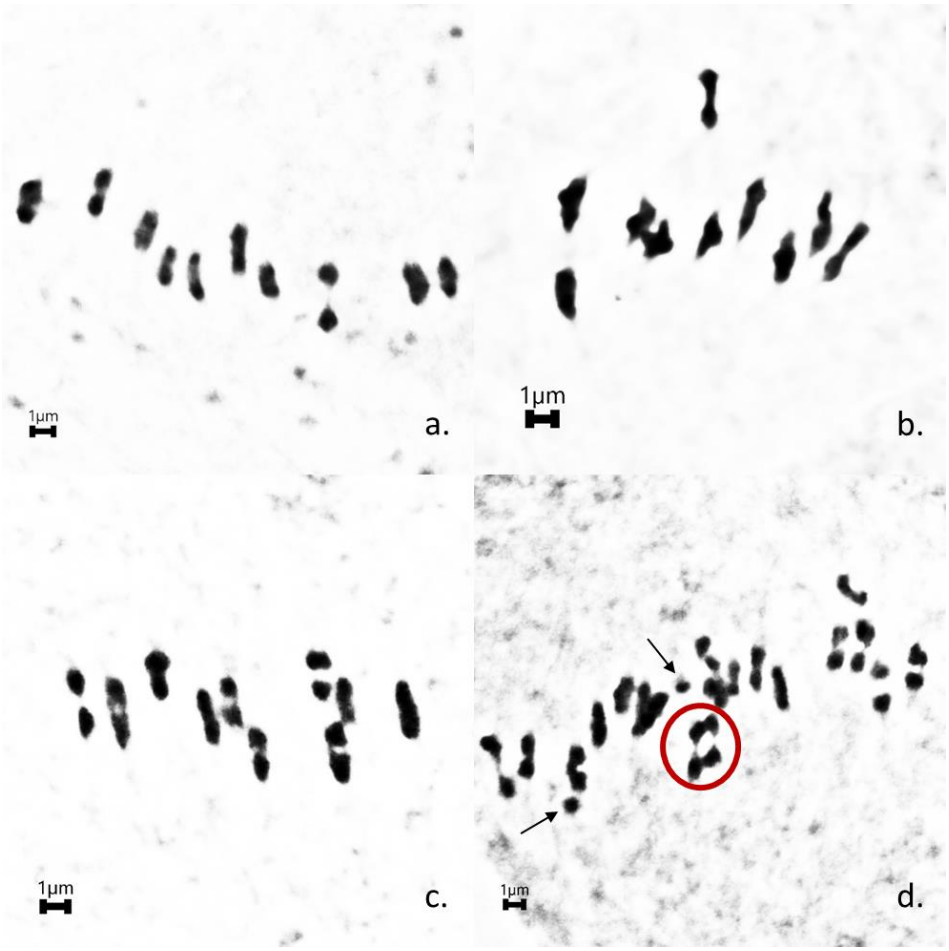


Figure 20. Chromosome spreads for a. BTx623, b. *S. propinquum*, c. 2*x S. bicolor* x *S. propinquum* hybrid, d. 4*x S. bicolor x S. propinquum* hybrid; arrows indicate univalents, a quadrivalent is circled

The induced tetraploid *S. bicolor* x *S. propinquum* hybrid had a relatively high frequency of bivalents (Figure 20d). The average chromosome association for 232 PMCs examined was 0.45 univalents, 17.77 bivalents, 0.02 trivalents, and 0.98 quadrivalents per cell (Table 13). The maximum number of quadrivalents observed was 5 and these were seen in only one PMC. Twenty bivalents were present in 27.5% of the cells examined. Rod bivalents had a mean occurrence of 2.81 per cell in the tetraploid hybrid (Table 14). Figure 20 shows the metaphase I chromosome pairing behavior in PMCs of both parents and the diploid and tetraploid *S. bicolor* x *S. propinquum* hybrids.

Celarier (1958) suggested that *S. propinquum* is a diploid ancestor of a tetraploid rhizomatous taxa, but de Wet (1978) indicated that comparative morphology refutes such an assumption. Because *S. propinquum* crosses readily with *S. bicolor* to produce fertile hybrids, Harlan and de Wet (1972) proposed that *S. propinquum* belongs to the *S. bicolor* taxon. However, because the natural distribution of the two species are very different, de Wet (1978) proposed this perennial species is a separate taxon. He also indicated *S. propinquum* had affinities with *S. halepense* rather than *S. bicolor.* The average meiotic chromosome pairing configuration in four tetraploid *S. halepense* accessions reported by Celarier (1958) was 0.19 univalents, 17.40 bivalents, 0.03 trivalents, 1.21 quadrivalents, and 0.01 pentavalents. This was somewhat similar to that observed in the tetraploid *S. bicolor* x *S. propinquum* (Table 13). However, the pairing behavior in *S. halepense* as reported by Endrizzi (1957) and Hadley (1953) was more irregular than that observed in the tetraploid *S. bicolor* x *S. propinquum* hybrid and its progeny. This suggests there are similarities in the chromosome composition of this hybrid and *S. halepense*.

Studies that look at the genome content in the diploid and tetraploid hybrid, as well as *S. halepense* would be helpful to further understand the genomic relationship. Patterson (1995) reported on one such study that showed that *S. halepense* contained restriction fragment alleles specific to *S. propinquum* or *S. bicolor* as well as alleles common to both. The study conducted by Patterson was limited, including only 125 alleles. Future studies should have increased coverage and should include an inducted tetraploid *S. bicolor* x *S. propinquum* F_1 hybrid, segregates from the F_2 or subsequent generations, as well as *S. halepense*. Studies that include these components would provide understanding of genome content as well as deepening understanding of neo-polyploid development.

Morphologically the 4*x S. bicolor* x *S. propinquum* population does not currently resemble *S. halepense.* It is, however, possible to theorize how it could – through natural selection – become more similar to *S. halepense.* This tetraploid shows mostly photoperiodic flowering (which is desired); however, there is the occurrence of non-photoperiodic segregation so these individuals would be selected for immediately in an environment that freezes before photoperiodic flowering can set seed. Additionally, as the 4*x* hybrid is a neo-polyploid, there is reduced fertility and seed set; however, as generations advance high fertility would quickly be selected for. Any other traits that would cause this population to be a successful weed (long rhizomes, additional cold tolerance, etc.) would accumulate over time.

CHAPTER IV

CONCLUSION

The interspecific tetraploid *S. bicolor* x *S. propinquum* population performed well in initial evaluations and further trials are merited. This germplasm has promising overwintering capability with improved vigor over *S. propinquum*. The tetraploid also had comparable yield potential to a commercial Sorghum-Sudan grass cultivar. Although segregation did not increase much from the F_2 to the F_3 generation, there is enough variability that selection should prove successful at improving performance.

One logical next step would be to make selections to establish a recurrent selection crossing block from which synthetic cultivars could be developed. Besides a recurrent selection breeding program, agronomic trials would be merited to determine best management practices.

The decreased fertility in the 4*x* population is of no concern for production as the resulting cultivars would be for forage or bioenergy production and targeted for regions where they would not flower. A challenge may occur for seed production, and a trial plot in a climate amenable to flowering would be merited to estimate seed yield. If a recurrent selection program were established, one target would understandably be fertility.

The tetraploid hybrid had an average chromosome pairing of essentially 18 bivalents and 1 quadrivalent which is similar to previous reports for *S. halepense*. However, the current population does not pose a weediness threat. The tetraploid *S. bicolor* x *S. propinquum* hybrid has photoperiodic flowering, non-shattering seed, and short rhizomes. Care must be taken in development of cultivars to ensure all early flowering, shattering, or invasive rhizome segregates

are promptly removed. These characteristics are easily recognized and should not pose an issue in control.

With future research indicated this population could give rise to a commercial non-invasive perennial sorghum forage. Perennial forage sorghum can provide environmental benefits coupled with reduced inputs and maintain high forage yield.

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