

**UNDERSTANDING INTERSPECIFIC HYBRIDIZATION BETWEEN  
*SORGHUM BICOLOR* AND ITS WEEDY CONGENER, *S. HALEPENSE***

A Thesis

by

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## ABSTRACT

The potential for hybridization between cultivated species and their weedy relatives poses agronomic and environmental concerns. *Sorghum bicolor* (sorghum), one of the most widely cultivated crops in Texas, is a prime example of a crop that has a weedy relative, *S. halepense* (johnsongrass), capable of exchanging genetic information. Previous crosses (sorghum as the female parent) have shown that the resulting triploid progenies typically collapse and only few of them develop into mature seed, whereas the majority of successful hybridizations involve tetraploid progenies, suggesting the production of 2n female gametes in sorghum. However, little is known on the effect of sorghum parental genotype and cytoplasmic male sterility type on 2n female gamete production in sorghum and consequently the frequency of hybridization. Furthermore, pollen fertility in sorghum (as a female parent) is expected to create a competitive environment where the johnsongrass pollen would have to outcompete the sorghum pollen, further reducing hybridization potential. The objectives of this research were to 1) determine the frequency of 2n female gamete production in sorghum and hybridization with johnsongrass (male parent) by sorghum parental genotype (12 lines) and cytoplasmic male sterility (CMS) type (A1, A2, and A3) under field conditions; and 2) quantify the frequency of hybridization between 12 different male-fertile sorghum parental genotypes (female) and johnsongrass under field conditions. Hybridization rates were as high as 1.28% under self-sterility (within the A1 CMS type, which is commonly used in breeding) and 0.045% under self-fertility across the 12 sorghum genotypes studied. Genotype\*sterility type interactions were also significant, with the greatest hybridization rates reaching at 2.2% in A3.Tx623. The knowledge generated from this study will be helpful for developing gene flow mitigation strategies.

Keywords:  $2n$  gamete production, crop-wild hybridization, evolution, gene flow, herbicide resistance, wild relatives

## **DEDICATION**

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## **CONTRIBUTORS AND FUNDING SOURCES**

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## CHAPTER I: INTRODUCTION AND REVIEW OF LITERATURE

### 1.1 Introduction

It is well understood that ecological processes such as gene flow and hybridization contribute to the genetic diversity that characterizes our agricultural landscapes (Morrell et al. 2005, Slatkin 1987). Gene flow is formally defined as, “the genetic differentiation of local populations, and the movement of gametes, individuals, and even entire populations” (Slatkin 1987). For plants, gene flow occurs through the movement of pollen, seed, and/or vegetative propagules (Beckie et al. 2019). However, gene flow is not only the movement of alleles, but also successful hybridization, resulting in the production of viable seed. There exist multiple factors (environmental, anthropogenic, etc.) that drive the rate of hybridization, and eventually evolution and speciation (McWhorter 1971).

Gene flow in agricultural systems has emerged as a topic of discussion and an area that requires further research attention because weedy relatives of a number of cultivated crops [e.g. sorghum (*Sorghum bicolor* (L.) Moench), rice (*Oryza sativa* (L.)), sunflower (*Helianthus annuus* (L.))] coexist in agricultural landscapes, and gene flow between them can lead to the exchange of traits of interest such as herbicide resistance (Ellstrand and Rieseberg 2016, Shivrain et al. 2007, Arias and Rieseberg 1994). For herbicide resistance traits, potential transfer of resistance from the cultivated species to weedy relatives can undermine our ability to control the weed species. As of now, several novel traits have been introduced in these crops through transgenic and non-transgenic means, making gene flow an important evolutionary process in agricultural landscapes (Fernandez-Cornejo and McBride 2000).

Crop, weedy, and feral relatives co-exist and interact in multiple areas, including production fields, ditch banks, roadsides, property lines, and fence lines, to include just a few. In agricultural landscapes of the US where sorghum is predominantly grown, johnsongrass [*S. halepense* (L.) Pers.] and shattercane [*S. bicolor* (L.) Monech ssp. *drummondii* (Nees ex Steud.) De Wet exDavidse] are two common weedy relatives of sorghum that are part of its genetic pool (Ohadi et al. 2018, Rooney 2004). Shattercane is predominantly found in crop fields and field edges, whereas johnsongrass is prevalent both in agricultural and natural habitats, especially in the environmental conditions of South Texas. Johnsongrass has indeterminate growth habit and exhibits flowering synchrony with sorghum. Due to genetic similarities with sorghum and a lack of selective control options, johnsongrass has become the primary weed in many sorghum production fields in the South Texas region.

Potential hybridization between sorghum and johnsongrass and transfer through gene flow of novel traits, including herbicide resistance, can have important agronomic and environmental consequences (Ohadi et al. 2017, 2018). Thus, the longevity of novel traits introduced through transgenic and non-transgenic technologies may depend on the extent of gene flow frequency between crops and compatible wild/weedy relatives in agricultural landscapes and the effectiveness of the measures implemented to mitigate gene flow. For sorghum, knowledge of the nature and characteristics of gene flow between cultivated and weedy biotypes can be beneficial for developing suitable gene flow mitigation strategies, including the selection of parental lines with limited hybridization potential with johnsongrass.

A well-known example of gene flow involves cultivated rice and its weedy relative *O. rufipogon*. In a study by Chen et al. (2004), hybridization between cultivated rice and *O. rufipogon* was up to 2.9% due to the high compatibility between the two species. Chen et al.

(2004) also contended that physical characteristics of weedy relatives such as flowering time, and plant height would shift over time to mimic the characteristics of the cultivated crop in order to increase the possibilities of cross-pollination and evade control. Shivrain et al. (2007) showed that hybrids between herbicide-resistant rice and weedy red rice were found at an average frequency of 435 hybrids ha<sup>-1</sup>, with the F<sub>1</sub> progenies showing weedy characteristics.

An issue analogous to the rice/weedy rice system described above, but with limited scientific investigation, is that of cultivated sorghum and its weedy relative johnsongrass. Sorghum, like rice, is a staple crop in some of the world's most important agricultural regions, particularly in parts of Africa and Asia. In the United States, sorghum is produced on a total of 2.3 million hectares, with Kansas, Texas, Colorado, and Oklahoma being the lead production states (USDA 2017). Johnsongrass is a tetraploid and shares a sub-genome in common with cultivated sorghum, which makes the two species very closely related (Fernández et al. 2013). As a result of this close genetic relationship there is no selective post-emergence chemical weed control option available for johnsongrass in sorghum fields, presenting a significant challenge to sorghum growers. Historically, the only commercial option available for johnsongrass control in sorghum is pre-emergence chemical control with an application of *S*-metolachlor with a seed safener treatment. Currently, non-transgenic herbicide-resistant sorghum lines [resistant to the acetolactate synthase-inhibitor herbicides nicosulfuron (Inzen<sup>TM</sup> sorghum) and imazamox (Igrowth<sup>TM</sup> sorghum), and to acetyl-CoA carboxylase-inhibitor quizalofop (Double Team<sup>TM</sup> sorghum)] are being developed by industry, and expected to be released in the near future. It is important to protect the longevity of these crop technologies through development of effective strategies for mitigating gene flow between sorghum and its weedy relative johnsongrass.

## **1.2 Review of Literature**

### **1.2.1 Sorghum: origin and its importance**

Grain sorghum is one of the most environmentally tolerant crops and therefore one of the most cultivated grain crops in the nation (Maunder 2002, USDA-NASS 2019). The center of origin for sorghum is known to be Eastern Africa where most of the genetic diversity is found (Dahlberg et al. 2001, Rooney 2004). Early diversification of cultivated sorghum is likely associated with the movement of people in Eastern Africa (Rooney 2004). The first recorded introduction of sorghum into the United States was in 1853 when the sweet variety ‘Chinese Amber’ was brought from France (Vinall et al. 1936). Since then, human selection and movement have led to a variety of uses of sorghum such as food particularly in Asia, Africa, and South America; animal fodder; as well as bioenergy production (Maqbool et al. 2001). Grain sorghum is one of the most environmentally tolerant crops and therefore an important grain crop in the US (Maunder 2002), with about 5.3 million acres planted in 2019 (USDA-NASS 2019). Due to its importance, efforts are devoted to improve sorghum using classical breeding as well as novel transgenic methods (Maqbool et al. 2001).

### **1.2.2 Johnsongrass: origin and distribution**

Johnsongrass originated in the Mediterranean region of Africa and Europe, and it is this Mediterranean ecotype that is believed to have been brought over to the Americas (McWhorter 1971). Although the epicenter of diversity for johnsongrass is considered to be Africa, similar to that of cultivated sorghum, it can be found anywhere from 55° N to 45° S latitudinal regions in the world (McWhorter 1971). The African epicenter, however, is where most of the natural hybridizations occur, which give rise to new ecotypes (de Wet 1978).

Since its introduction to the United States in the early 19<sup>th</sup> century, johnsongrass has spread throughout the country. Johnsongrass is now considered a noxious species in 33 states (NRCS 2015, USDA-AMS 2014), and has been reported to occur in every state of the country, except Alaska, Maine, and Minnesota (Kartesz 2015, USDA 2015). A study by Clements and DiTommaso (2011) investigated the evolutionary ability of johnsongrass, to advance northward longitudinally and establish in regions which were once considered uninhabitable for this species. This shift stems from wide climatic and environmental tolerance, a relatively short generation time, effective forms of reproduction and dispersal, and high competitive ability that allows for colonization in numerous environments (Holm et al. 1977, Warwick and Black 1983). As johnsongrass continues to spread and migrate to new regions, selection pressure may occur for multiple adaptive traits, sometimes concurrently (Clements and DiTommaso 2012).

### **1.2.3 Johnsongrass: ecology and biology**

Johnsongrass is among the most competitive weeds found in row crops, particularly so in sorghum production fields where selective chemical control options are minimal. Persistence of johnsongrass can largely be attributed to three major biological characteristics: staggered flowering and seed production, seed dormancy, and an ability to produce rhizomes (Monaghan 1979). Egley and Chandler (1978) found that 50% of johnsongrass seed was still viable after 25 years of burial at a depth of 38 cm below the soil surface. Dormancy is typically maintained until environmental conditions are favorable to induce germination. According to Krenchinski et al. (2015), a temperature of 32 °C is required to initiate johnsongrass seed germination under the absence of light. Hard seed coat can reduce germination, but can be overcome through scarification, allowing for imbibition of water (Taylorson and McWhorter 1969).

Johnsongrass plants grow 50 to 150 cm tall, with 10 to 35 cm long panicles (Monaghan 1979). Flowering begins approximately seven weeks after seedling emergence, and each plant is capable of continuous flowering (i.e. indeterminate flowering) throughout the growing season through production of multiple tillers (McWhorter 1971). The spikelets in the panicles form in pairs except for the terminal spikelet, which is a triplet structure. Each plant can produce an average of about 28,000 seeds (Horowitz 1973). At maturity, seed dispersal via shattering is another characteristic that enables the proliferation of johnsongrass. Shattering allows the seed to enter the soil seed bank and be stored until environmental conditions are better suited for germination. Shattering also allows for further dispersal through vectors such as wind, water, and animals to be transported to sites that might be better suited for germination (Monaghan 1979).

Below-ground, johnsongrass is a prolific producer of rhizomes or underground stems (Warwick and Black 1983). When aboveground biomass is accumulated, rhizomes also begin forming underground as a storage site for starch. Once autumn arrives, the rhizomes go dormant and the above ground vegetation dies due to winter frost (Warwick and Black 1983). During the following spring, when environmental conditions are favorable, the dormant rhizomes that survive the winter will generate new vegetative growth (Hartzler et al. 1991). In addition to rhizomes, johnsongrass plants are also capable of vegetative regeneration through crown reserves (Warwick and Black 1983). Thus, effective johnsongrass management requires season-long practices that include tillage, crop rotations, herbicide application and rotation, and other practices to reduce its ability for regeneration.

#### **1.2.4 Hybridization between sorghum and johnsongrass**

Hodnett et al. (2019) found 91% of hybrids recovered from controlled crosses between sorghum (female) and johnsongrass (male) in a greenhouse were tetraploid progeny. The

production of tetraploid progeny implies that  $2n$  gametes were derived from the *S. bicolor* parent. This study also suggested that the frequency at which  $2n$  gamete production occurs may be genotype dependent. In an earlier study, it was demonstrated that differences in ploidy level of sorghum x johnsongrass hybrid progeny can also cause variations in fitness (Walter Jay McClure 1962). Hadley (1958) observed the growth and reproduction of various interspecific hybrids and showed that 30-chromosome hybrids displayed weaker phenotypes, but reproduced through the formation of rhizomes. Furthermore, 40-chromosome hybrids were male sterile in some cases, but female fertile in others depending on the female with which the cross was made (Hadley 1958). Moreover, it is imperative to understand which sorghum genotypes are more likely to hybridize with johnsongrass. Sorghum and johnsongrass are capable of outcrossing due to similar genetic composition (Baker 1991). Further, occurrence of sorghum and johnsongrass in sympatry has allowed for hybridization and introgression (Morrell et al. 2005). Previous studies have produced hybrids between sorghum and johnsongrass, mostly under controlled greenhouse conditions and with various rates of success (Dweikat 2005, Hadley 1958, Hodnett et al. 2019). Recent work by Cox et al. (2017) has suggested that hybrids between diploid sorghum and tetraploid johnsongrass lead to production of diploid progenies that have atypical phenotypes. Although johnsongrass is predominantly a self-pollinated species, outcrossing between sorghum and johnsongrass does occur in nature and at higher rates than one would expect. Arriola and Ellstrand (1996) estimated that sorghum can pollinate johnsongrass at a rate of up to 15% in a field environment. Morrell et al. (2005) documented hybridization between sorghum and johnsongrass based on the presence of sorghum-specific alleles in johnsongrass. Ohadi et al. (2017) observed some degree of transgressive segregation in their study meaning that hybrid phenotypes reflected extreme characteristics when compared to their original parents. This could



potentially have implications in hybrid progeny that are found in the field and their ability to be either more or less weedy than the johnsongrass parent. Similar studies such as the one conducted by Piper and Kulakow (1993) observed similar patterns of segregation. In that particular study they observed that tetraploids continuously produced more above ground biomass and higher seed production than other ploidies which were not diploid. Thereby supporting the transgressive segregation idea that Ohadi et al. suggested with rhizome growth being a weedy trait.

### **1.2.5 Factors influencing interspecific hybridization**

The ability of johnsongrass to flower throughout the growing season, with the production of multiple tillers, increases the opportunities for cross pollination with its cultivated relative, sorghum. Different studies have determined that a variety of environmental factors will have an impact on the frequency of hybridization. Some of the key factors include distance between the two species, flowering synchrony, and the degree of pollen load/competition (Schmidt et al. 2018, Warwick et al. 2009). Arriola and Ellstrand (1996) showed that outcrossing between sorghum and johnsongrass was the greatest at close distances and gradually declined with increasing distance. Hanson et al. (2005) conducted a similar study in which they observed cross pollination between winter wheat (*Triticum aestivum* L.) cultivars. This study determined that environmental factors such as air temperature, light intensity, humidity, and other stressful factors could determine the amount of gene flow observed from cultivar to cultivar. Additionally, just like the Arriola and Ellstrand (1996) study, Hanson et al. (2005) determined that distance from pollen would be a major factor when determining rate of gene flow as they determined that in winter wheat, no gene flow was found beyond 30 meters from the pollen source.

Other studies focused on flowering timing and synchrony as a main driver for gene flow to occur. In a study conducted by Simard and Légère (2004) gene flow between canola and wild radish (*Rhaphanus raphanistrum*) was evaluated as a result of plant densities and flowering dates of the two species. The study found that hybridization is most likely to happen when there is uncontrolled early emerging flushes of weeds this may be different on a crop by crop basis.

Similarly, the available pollen load has also been characterized as one of the main drivers of gene flow. In Goggi et al. (2007), different densities of pollen load at 3 different distances were evaluated in maize (*Zea mays* L.) The study determined that when local pollen density (i.e. pollen load) is low this creates a competitive advantage for outcrossing for other incoming or non-local pollen which would give way to higher levels of outcrossing as compared to when the local pollen load is heavy and abundant. Goggi et al. (2007) also concluded that at further distances, the levels of outcrossing also decrease in maize- only reaching 0.1% and 0.03% under reduced pollen loads and normal pollen loads, respectively, at 100 meters from the pollen source.

Although all of these factors may contribute to gene flow, it is clear that outcrossing rates vary by species and can become difficult to truly calculate if there are multiple avenues for genes to be dispersed. Amand et al. (2000) showed that alfalfa (*Medicago sativa* L.) pollen spread by leaf cutter bees, outcrossing could be more precisely calculated since these bees use a directional and non-random path when pollinating fields. Movement of pollen in the field was much harder to detect and was only found at distance of 4 meters or less (Amand et al. 2000).

Through the smaller scope of gene flow within the sorghum genus, studies such as the one performed by Hadley (1958) as well as more recent work performed by Hodnett et al. (2019), it is clear that parental background as well as the source of sterility in the sorghum genotype are key factors in the hybridization potential between sorghum and johnsongrass.

However, it is possible that multiple factors such as environmental factors and other biological factors mentioned above may also be contributing to interspecific hybridization within sorghum. The more information we gather about these factors influencing gene flow, the more we understand that gene flow is nonrandom and is sometimes encouraged by certain genotypes (Edelaar and Bolnick 2012).

### **1.2.6 Implications of hybridization**

Gene flow and hybridization between sorghum and johnsongrass may affect our ability to control johnsongrass in a sorghum production field, if the trait transferred to johnsongrass provides an adaptive advantage such as resistance to herbicides that are used to control the species. Gene flow between sorghum and johnsongrass can occur in both directions (sorghum as the male parent and vice versa), with case specific implications (Arriola and Ellstrand 1997, Jessup et al. 2017). Once genes of interest are transferred into the weedy relative, the process is irreversible (Lu 2008). Gene flow with sorghum as the female parent has practical significance in regards to feral sorghum (i.e. sorghum establishing on roadsides and other non-cultivated habitats as a result of seed dispersal during commodity transport and other means) and volunteer sorghum (i.e. sorghum plants that establish in previous sorghum fields from seed lost during harvest).

Hybridization can occur between feral sorghum and johnsongrass when in close proximity to each other, which in turn may lead to the transfer of adaptive traits and enhance the dominance of johnsongrass in such habitats (Ohadi et al. 2018). Feral sorghum is typically founded by grain from production fields, which is essentially F<sub>2</sub> seed that segregates for male sterility; approximately 25% of these plants are expected to be male sterile and capable of being fertilized by pollen from surrounding johnsongrass. Ohadi et al. (2018) surveyed a total of 2,077

sites throughout South Texas and found that a total of 2.3% of the surveyed locations had the two species co-existing at close proximities, providing opportunities for gene flow to occur and the production and establishment of interspecific hybrids in natural habitats.

For volunteer sorghum, a similar scenario occurs in production fields after crop harvest. A proportion of the hybrids produced as a result of outcrossing (sorghum as the female parent) may return to soil as a component of harvest grain loss and establish a persistent population. When gene flow occurs in the other direction with johnsongrass present in sorghum production fields or in close proximity along field edges and roadsides serving as the female parent, the hybrid seed may shatter and establish more persistent populations if they acquire additional adaptive traits from sorghum. For example, Jessup et al. (2017) reported a perennial sorghum hybrid (*S. bicolor* (L.) x *S. halepense* (L.)) which was found to possess complete seed sterility and greater leaf numbers.

Arriola and Ellstrand (1997) determined that when a transgene is transferred to johnsongrass and benefits the weed, it will remain and persist in that population. As a result of crosses between *Sorghum vulgare* and *S. halepense*, Hadley (1958) observed multiple outcomes in chromosome numbers, stainable pollen, seed set numbers, and expression changes in rhizomes. These findings indicate that if a favorable gene is present in the population it is likely to spread and could be expected to migrate into the gene pool of the weedy relative.

For these reasons, gene flow between sorghum and johnsongrass has even more practical implications with the recent development of herbicide-resistant sorghum lines (Inzen™ sorghum, Igrowth™ sorghum, and Double Team™ sorghum). These technologies provide excellent control of grasses, including selective control of johnsongrass in sorghum fields. In a scenario where the resistance traits are transferred to the weedy biotype as a result of gene flow, it will

eventually lead to the loss of the technology in the absence of an appropriate mitigation plan.

The Inzen™ sorghum technology has already been approved for commercialization in the United States and the other two technologies mentioned above are in the approval process. An improved understanding of gene flow between sorghum and johnsongrass will allow for the development of proactive measures for mitigating gene flow and enhancing the longevity of these technologies.

### **1.2.7 Knowledge gaps**

Earlier studies have investigated gene flow from sorghum to johnsongrass (Arriola and Ellstrand 1996, Hadley 1958, Hadley and Mahan 1907), but a clear understanding is lacking on the rate at which this process occurs in agronomic settings and which factors influence hybridization. In this research, we are specifically focusing on three major knowledge gaps that need immediate attention. First, little is understood on the frequency of  $2n$  gamete production in sorghum and the influence of genetic background on this trait. Although seed can be produced after fertilization with johnsongrass pollen, the resulting progeny is usually triploid, which typically do not develop to maturity due to the failure or collapse of the endosperm (Hodnett et al. 2019). Under controlled and greenhouse conditions, however, it has been shown that other ploidy types such as tetraploids, pentaploids, and hexaploids are also forming, with tetraploid being the most common ploidy type among the fully developed hybrid seed, which is likely a result of  $2n$  gamete production (Hodnett et al. 2019). Production of  $2n$  gametes putatively increases the frequency of successful hybridization between the two species. Hodnett et al. (2019) also showed that the sorghum lines Tx623 and Tx631 yielded a different number of tetraploids, begging the question: how does  $2n$  gamete frequency and outcrossing potential differ among sorghum genotypes?

Second, given that hybrid sorghum is commonly grown and utilizes cytoplasmic male sterility (CMS) to produce the hybrid seed, it is possible that specific types of CMS systems could affect interspecific hybridization between sorghum and johnsongrass, which is not well understood. The use of CMS is essential to production of hybrid sorghum seed and is widely used in sorghum improvement programs (Schnable and Wise 1998). CMS is a sterility system that is maternally inherited and prevents self-fertilization of the seed parent (Schnable and Wise 1998, Dixon and Leaver 1982). CMS interferes with pollen production and is reversed with restorer genes that may restore fertility and suppress the male sterile phenotype (Dixon and Leaver 1982). The CMS system is the most economically feasible system used for large scale hybrid production (Rooney 2004).

There are different types of CMS available in sorghum. The A1 CMS system is most commonly used for hybrid seed production, whereas only a few hybrid lines have been produced using the A2 CMS system, and A3 is rarely utilized in breeding programs though is still available (Rooney 2004). More sources of CMS are known (Eckardt 2006), but the focus of this study was to determine the influence, if any, of A1, A2, and A3 CMS systems and their interactions with sorghum genotypes on the production of  $2n$  gametes. Additionally, hybrid seed production relies not only on these A lines (male sterile lines), but also needs B lines (male fertile lines) to maintain the A lines. A and B lines are identical genetically, except for the fact that A lines are male sterile and B lines are male fertile (Rooney 2004). R lines, or restoration lines, are used in hybrid seed production for fertility restoration of the A lines (Rooney 2004).

Third, the frequency of outcrossing may also be affected by differences in pollen load between sorghum and johnsongrass. In commercial production fields, the sorghum lines are male fertile and the overall load of sorghum pollen is typically orders of magnitude greater than that of

johnsongrass (variable depending on johnsongrass densities). Such differences in pollen load is expected to reduce the chances for johnsongrass pollen fertilizing sorghum (female), as shown in other systems (Amand et al. 2000). Under conditions of male sterility in sorghum, there is almost no competition from sorghum pollen, allowing for estimation of the upper level of outcrossing between the two species. However, the degree of impact of pollen fertility in sorghum on outcrossing potential with johnsongrass is not clear.

Overall, an understanding of the influence of sorghum genetic background and CMS type on the frequency of 2n gamete production and outcrossing will allow breeding programs to make knowledge-based decisions on the choice of parental backgrounds that are less likely to outcross with johnsongrass. Moreover, evaluation of the impact of self-pollen competition on outcrossing frequencies will help make case-specific assessments of outcrossing risk.

Just as de Jong and Rong (2013) stated, predicting the introgression of alleles from the crop to the weedy relative is research that is accompanied by great uncertainty. However, this information is necessary in order to be able to make conscious decisions that government organizations have to make every year.

### **1.3 Rationale**

Sorghum is an important field crop in Texas with a total of 1.5 million acres planted in 2019 (USDA-NASS 2019). In Texas and other parts of the southern US, the widespread occurrence of the weedy relative johnsongrass in sorghum production fields and surrounding areas demands an investigation of the nature and dynamics of gene flow. Understanding the frequency of gene flow and its determinants will not only help select sorghum lines with low risk for gene flow, but will also help develop other mitigation measures. Current weed management practices are mostly tailored as responses to weed pressure, but as we continue to understand

these evolutionary processes, management practices may shift towards preventative measures that help extend the life span of new technologies and encourage sustainability.

#### **1.4 Hypotheses**

The specific hypotheses of this research project were

- i) The frequency of 2n female gamete production in sorghum and subsequent rate of outcrossing with johnsongrass varies among different sorghum genotypes and sterility-inducing cytoplasm types
- ii) Pollen fertility in sorghum will greatly reduce outcrossing potential with johnsongrass as compared to sterile sorghum under field conditions

#### **1.5 Objectives**

The specific research objectives developed based on the hypotheses were to: 1) determine the frequency of 2n female gamete production in sorghum and hybridization with johnsongrass (male parent) as influenced by 12 different sorghum parental genotypes (all with sterile cytoplasm) and 3 CMS types (A1, A2, and A3) under field conditions; and 2) quantify the frequency of hybridization between 12 different male-fertile sorghum parental genotypes (female) and johnsongrass under field conditions.

In this regard, two separate field experiments were established in a production field naturally infested with johnsongrass. *Experiment I* consisted of 12 different seed parent lines with A1 CMS type (A-lines). Further, the *Experiment I* also included three seed parent lines each with A1, A2 and A3 CMS systems to assess the effect of sterility inducing cytoplasm type. The *Experiment II* consisted of the male fertile versions of the same 12 sorghum parent lines (B-lines) used in *Experiment I*.



## CHAPTER II: FREQUENCY OF INTERSPECIFIC HYBRIDIZATION BETWEEN SORGHUM BICOLOR AND SORGHUM HALEPENSE

### 2.1 Introduction

Gene flow is an important evolutionary force in agricultural landscapes (Morrell et al. 2005, Slatkin 1987). Gene flow is formally defined as, “the genetic differentiation of local populations and the movement of gametes, individuals, and even entire populations” (Slatkin 1987). For plants, gene flow occurs through the movement of pollen, seed, and/or vegetative propagules (Beckie et al. 2019). In the context of this research, the process of gene flow does not only refer to the movement of alleles, but also successful hybridization following gene flow, resulting in the production of viable seed. There exist multiple factors (environmental, anthropogenic, etc.) that drive the rate of gene flow and hybridization in natural and agricultural landscapes, and eventually evolution and speciation (McWhorter 1971).

Gene flow in agricultural systems has emerged as a topic of discussion in recent times and is an area that requires further research attention because weedy relatives of a number of cultivated crops [e.g. sorghum (*Sorghum bicolor* (L.) Moench), rice (*Oryza sativa* (L.)), sunflower (*Helianthus annuus* (L.))] coexist in agricultural landscapes, and gene flow between them can lead to the transfer of adaptive traits into the weedy forms (Ellstrand and Rieseberg 2016, Shivrain et al. 2007, Arias and Rieseberg 1994). As of now, several novel traits have been introduced in these crops through transgenic and non-transgenic means, making gene flow an important evolutionary force in agricultural landscapes with significant practical implications (Fernandez-Cornejo and McBride 2000).

Sorghum is an important grain crop in the world, and is a staple food crop in Africa and Asia (Rooney 2004). In the United States, sorghum is produced on a total of 2.08 million hectares with Kansas, Texas, Colorado, and Oklahoma being the lead production states (USDA

2019). Johnsongrass (*S. halepense*), a wild/weedy relative of cultivated sorghum, is a weed commonly found throughout the sorghum production areas in the world (Brown et al. 1988). Johnsongrass is a tetraploid ( $2n=4x=40$ ) that shares a sub-genome in common with cultivated sorghum ( $2n=2x=20$ ) (Fernández et al. 2013). Because of their close relationship, interspecific hybridization has been reported to occur between these two species, despite the ploidy differences (Arriola and Ellstrand 1997, Hodnett et al. 2019).

In agricultural landscapes, hybridization between sorghum and johnsongrass can result in the transfer of novel traits to the hybrids, with significant agronomic and environmental consequences depending on the nature of the trait being transferred (Ohadi et al. 2017, 2018). For instance, herbicide resistance is an important agronomic trait that has been introduced in cultivated sorghum (e.g. Kershner 2010). Given the genetic similarities between sorghum and johnsongrass, there has been no post-emergence chemical weed control option available for selectively controlling johnsongrass in sorghum fields, presenting a tremendous production challenge. Historically, the only commercial option available for johnsongrass control in sorghum is pre-emergence application of *S*-metolachlor with a seed safener treatment. Currently, non-transgenic herbicide-resistant sorghum lines [resistant to the acetolactate synthase-inhibitor herbicides nicosulfuron (Inzen<sup>TM</sup> sorghum) and imazamox (Igrowth<sup>TM</sup> sorghum), and acetyl-CoA carboxylase-inhibitor quizalofop (Double Team<sup>TM</sup> sorghum)] are being developed, and potential transfer of herbicide resistance from cultivated sorghum to johnsongrass has serious agronomic consequences since it might jeopardize effective control of johnsongrass. Thus, the level of gene flow between these two species may impact the longevity of these novel crop technologies.

Despite the practical significance of gene flow between sorghum and johnsongrass in agricultural systems, little has been understood on the factors governing gene flow rates. Arriola and Ellstrand (1996) studied the rate of gene flow between sorghum (male) and johnsongrass as influenced by distance and found that gene flow decreased as the distance between the two species increased and hybridization rates were as high as 2% when 100 meters apart. Gene flow between sorghum and johnsongrass can occur in both directions (i.e. sorghum as the male or female parent). The current research specifically focuses on gene flow when sorghum serves as the female parent. Here, no specific gene is tracked in the current research, thus the terms ‘outcrossing/hybridization’ are used hereafter *in lieu* of ‘gene flow’.

A recent greenhouse study by Hodnett et al. (2019) showed that  $2n$  gamete production in sorghum (female side) plays a key role in the rate of outcrossing because the majority of hybrids obtained in their crosses were tetraploids, rendering  $2n$  gamete production an important mechanism for outcrossing between the two species. In studies conducted by Hodnett et al. (2019), outcrossing level was heavily influenced by  $2n$  female gamete frequency in sorghum, leading to the production of tetraploid ( $4x$ ) and hexaploid ( $6x$ ) progenies, along with haploid ( $1x$ ), triploid ( $3x$ ), and pentaploid ( $5x$ ) progenies. However, factors affecting the frequency of  $2n$  gamete production in sorghum are yet to be understood. Hodnett et al. (2019) provided sufficient evidence to hypothesize that the frequency of  $2n$  gamete production varies by sorghum parental genotype.

The type of CMS system used in hybrid seed production might also influence the production of  $2n$  gametes and outcrossing. The CMS system is the only economically feasible system for hybrid seed production in sorghum (Rooney 2004). Several different types of CMS are known in sorghum. The A1 CMS system is most commonly used for hybrid seed production,

whereas only a few hybrid lines have been produced using the A2 CMS system. The A3 CMS system is rarely utilized in breeding programs though it is still available (Rooney 2004). Hybrid seed production relies not only on these A lines (male-sterile lines), but also needs B lines (male-fertile lines) to maintain the A lines. These A- and B-lines are identical genetically, differing only by alleles in the mitochondrial DNA (Rooney 2004). Hodnett et al. (2019) found that different types of sterility affected the amount of inter-specific hybrids produced with johnsongrass in lines such as Tx623, and it is therefore possible that the type of CMS system can influence inter-specific hybridization.

Pollen load and competition is another key factor that may govern outcrossing rates between plant populations. For example, St. Amand et al. (2000) demonstrated the effect of scale (i.e. pollen load) on the levels of outcrossing between genetically-modified (GM) and non-GM alfalfa fields. Likewise, Ghersa et al. (1994) showed in ryegrass that outcrossing levels by an undesirable biotype can be minimized by increased pollen load of the desirable biotype and altering flowering synchrony.

In inter-specific hybridization studies, male sterility in the female parent provides an estimation of the upper limit of hybridization between the two species due to the absence of pollen competition. Scenarios of male sterility do occur in sorghum under practical field conditions. During harvest and transportation, grain sorghum is often spilled in the fields and along roadsides. Given that the F<sub>2</sub> seed harvested from hybrid sorghum lines segregate for male sterility, about 25% of them are expected to be male sterile (Ohadi et al. 2017). Such sterile plants can co-exist with johnsongrass as volunteers within the production fields or as feral plants along roadsides (Ohadi et al. 2018), greatly improving the chances of outcrossing with johnsongrass due to the lack of self-pollen in sorghum.

Other factors such as air temperature, light intensity, humidity, and environmental stress can also play important roles in the level of outcrossing, as suggested by Hanson et al. (2005) in a study that investigated outcrossing between different winter wheat (*Triticum aestivum* L.) cultivars. However, this study focuses only on the influence of sorghum parental genotype, CMS type, and pollen competition on the rates of outcrossing with johnsongrass.

The specific objectives of this study were to 1) estimate the frequency of 2n gamete production and frequency of outcrossing with johnsongrass as influenced by a) sorghum parental genetic background, and b) CMS type in sorghum; and 2) elucidate the influence of pollen fertility in sorghum on rates of outcrossing with johnsongrass.

## **2.2 Materials and Methods**

Field experiments were established in 2018 and 2019 at the Texas A&M field research facility near College Station, TX. The mean annual temperature for this location is 20.6°C and average annual precipitation is 1018 mm. The aim of *experiment I* was to determine if the frequency of outcrossing between sorghum (female) and johnsongrass varies among 12 different male-sterile sorghum seed parent lines (i.e. absence of sorghum pollen). Utilization of male sterile lines allowed for convenient estimation of 2n female gamete frequencies in sorghum lines and also for estimating the upper limits of successful interspecific hybridization between the two species. In addition, three of these seed parent lines were present in three different forms of cytoplasmic male sterility. These were used to assess if sterility type influences the frequency of 2n gametes or outcrossing rates. In *experiment II*, the frequency of outcrossing between 12 male-fertile sorghum lines with johnsongrass was evaluated. In this situation, pollen competition between sorghum and johnsongrass is expected to mitigate outcrossing. This experiment allowed

for more realistic estimations of outcrossing levels under normal agronomic conditions. The two field experiments were conducted parallelly in adjacent research sites.

<b>Table 1.</b> Details of the 12 sorghum parental genotypes and specific CMS types utilized in the experiments I and II.				
<b>S. No.</b>	<b>Sorghum genotype</b>	<b>Pedigree</b>	<b>CMS type</b>	<b>Reference</b>
1	B/ATx2752	(BTx399*(BTx378*(4dwTx378*KS30)))	A1	Stephens and Karper (1965)
2	B/ATx2921	(74C5462-1 x BTx615)	A1	Miller (1977)
3	B/ATx2928	(RS4906 x BTx399) x RS4906)	A1	Miller (1986a)
4	B/ATx3408	Tx631/08PR047	A1	Miller (1986b)
5	B/ATx378	SA378	A1, A2, A3	Miller et al. (1992)
6	B/ATx623	(BTx3197*SC170-6-4-4)	A1, A2, A3	TAMU (2020)
7	B/ATx626	(BTx378*SC110-6)	A1	TAMU (2020)
8	B/ATx631	((BTx378*SC110-9)*BTx615)	A1, A2, A3	Johnson et al. (1982)
9	B/ATx642	(BC1 derivative of SC35)	A1	Rooney (2003)
10	B/ATx645	BTx623*(BTx625*BTx642)	A1	Rooney (2003)
11	B/ATx ARG-1	MR807*BTx624	A1	Mbulwe et al. (2016)
12	B/AHF14	BTx643*BTx635	A1	Stephens and Karper (1965)

## 2.2.1 Experiment I

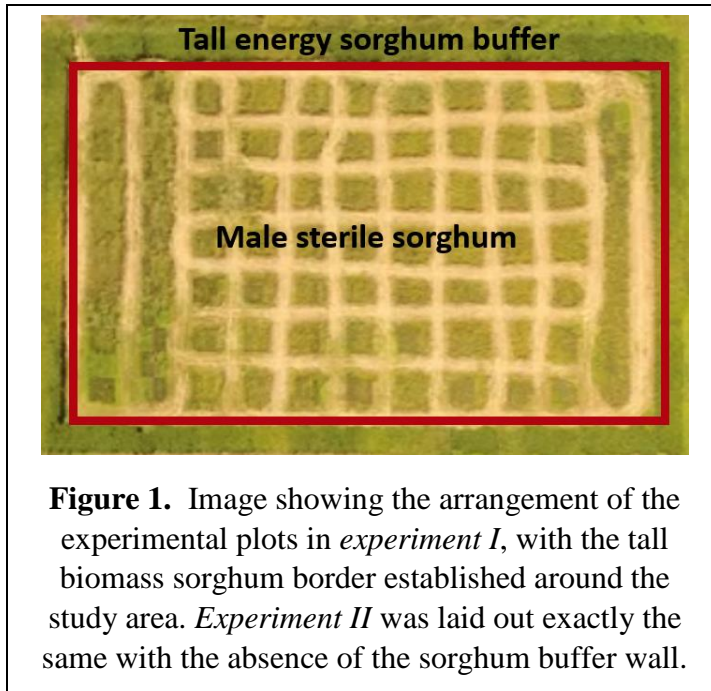
### 2.2.1.1 Plant materials

A total of 12 different male-sterile sorghum seed parent lines and three types of CMS (A1, A2, and A3) were tested in this experiment. All 12 genotypes contained the A1 CMS type (Table 1). Three seed parent lines (Tx378, Tx631, and Tx623) contained three different CMS types (A1, A2, and A3) (Table 1). Combined, there were a total of 18 entries in this field trial.

### **2.2.1.2 Experimental setup and field maintenance**

The experimental units were laid out in a randomized complete block design with four replications (Figure 1). Each plot was eight rows wide (0.76 m row spacing) and 6.7 m long; there was a total of 72 plots (i.e. 18 individual plots\*4 replications). The sorghum genotypes were planted into the johnsongrass infested plot area on April 20<sup>th</sup>, 2018 and April 20<sup>th</sup> 2019. The natural johnsongrass infestation served as the pollinator parent.

Because sorghum production does occur in this region, the tall growing bioenergy sorghum hybrid ES5200 was planted around the test as a buffer to eliminate extraneous sources of *S. bicolor* pollen (Figure 1). ES5200 is a photoperiod-sensitive energy sorghum that has dense growth and does not flower in Central Texas, meaning it did not shed any pollen during the life of the study.



**Figure 1.** Image showing the arrangement of the experimental plots in *experiment I*, with the tall biomass sorghum border established around the study area. *Experiment II* was laid out exactly the same with the absence of the sorghum buffer wall.

A pre-emergence application of pendimethalin (Prowl H<sub>2</sub>O<sup>®</sup>) was made at 2,130 g ai/ha immediately after sorghum planting. An early-postemergence application of *S*-metolachlor+ atrazine (Bicep II Magnum<sup>®</sup>) at a rate of 1,736 + 2,242 g ai/ha and a mid-postemergence application of a premix of 79 g ai/ha pyrasulfotole and 445 g ai/ha bromoxynil (Huskie<sup>®</sup>) were applied for additional weed control. These applications were made mainly to control morning-glories (*Ipomoea* spp.) and other broadleaved weeds and had little activity on johnsongrass. Insecticide [(Prevathon<sup>®</sup>) (56.5 g ai/ha), Silencer<sup>®</sup> (17.5 g ai/ha), and Sivanto<sup>®</sup> (153.6 g ai/ha)] applications were made to control sorghum midge, sugarcane aphid, armyworm, and other insect pests. Further, a premix of propiconazole 127.6 g ai/ha and 76 g ai/ha azoxystrobin (Quilt<sup>®</sup>) was applied to pre-emptively control ergot infestations during seed development.



### **2.2.1.3 Data collection**

Plots were monitored during anthesis to verify that sufficient *S. halepense* pollen was present on all plots. A low frequency (< 0.1%) of male fertility revertants is known to occur in male sterile sorghums, which could result in successful fertilization of the panicle and others nearby. Such panicles were visually identified at tip flowering and removed. At maturity or at the post-hard dough stage (~35 days after flowering), at least 50 mature panicles were randomly selected and manually harvested from the center of each plot. Because fertility revertants may have escaped rouging, the presence of panicles with full seed set were assumed to be fertile and were excluded along with adjacent panicles. If sterility breaks were high across the plot, then the entire plot was excluded from the harvest and terminated. Sterility revertants were found in two plots (A3.Tx378 and A2.Tx738) in 2018, and none in 2019.

The harvested panicles were stored in mesh bags and air dried under room temperature. Aluminum phosphide (Weevil-cide<sup>®</sup>) was applied at a rate of 145 tablets per 28.31 m<sup>3</sup> (9.2 g ai/m<sup>3</sup>) to control moths and other pests during seed storage. Each panicle was individually threshed using an Almaco BT14 Belt thresher. Threshed seed were cleaned using an aspirator to remove chaff and other foreign material. The total number of mature seed produced in each panicle was determined using a seed counter. Total number of florets produced by each panicle was estimated based on the maximum plot average value obtained from the male fertile version of each sorghum line (50 panicles/line) in *experiment II*.

### **2.2.1.4 Quantification of outcrossing**

To quantify outcrossing frequency, true hybrids in the harvested F<sub>1</sub> seed were confirmed based on ploidy level. Since contamination with *S. bicolor* pollen (due to either sterility break or external sources) was possible, these contaminants must be identified and excluded from

calculations of outcrossing frequency. Because these were *S. bicolor* self- or cross-pollinations, they would be diploid. Ploidy of all progeny was determined through the flow cytometry assay (see section 2.2.2.5), conducted using the ACURI C6 flow cytometer (Becton Dickinson and Co., Franklin Lakes, NJ). Any progeny with triploid or higher ploidy level was considered an interspecific hybrid. For confirmation of assignment using ploidy levels, all diploid seedlings from the flow cytometry assay were transplanted to the field to confirm an *S. bicolor* phenotype.

The outcrossing rate was evaluated using samples from two of the four field replications. For each genotype, a sub-sample (3 g, about 150 total seed) of the F<sub>1</sub> putative hybrid seed was drawn. Half of that seed was utilized for flow cytometry analysis to collect ploidy distribution data, and the other half was planted in the field for evaluating progeny fitness (see section 2.2.1.5).

#### **2.2.1.5 Quantifying progeny fitness**

Hybrid progeny fitness was assessed via successful emergence and seedling establishment the following summer (i.e. summer 2019 for the 2018 harvests). A sub-sample of the F<sub>1</sub> seed from each sorghum genotype was direct planted in the field on April 2019 and April 2020; in 2018, 1.5 g of the F<sub>1</sub> seed was drawn, and in 2019 the actual number of seed in the 1.5 g sub-sample was counted prior to planting. Once seedlings were established from this planting, total number of plants was counted and the ploidy level of each plant was determined following the flow cytometry procedure described in section 2.2.2.5. The genomic status (ploidy level) of successfully established field progeny was compared with the seedling emergence and ploidy data obtained from the Petri dish and greenhouse assays (considered ideal germination and establishment conditions) (section 2.2.2.5). The rationale is that any reduction in plant

establishment level in the field environment, compared to the greenhouse environment, reflects a lack of fitness for the specific ploidy types and the associated genotypes.

## **2.2.2 Experiment II**

### **2.2.2.1 Plant materials**

In this evaluation, the male-fertile versions (B-lines) of the 12 parental lines evaluated in *Experiment I* were used (Table 1).

### **2.2.2.2 Experimental setup and field maintenance**

In both years, the study was conducted in a randomized complete block design, with 4 replications (Figure 1), thus making a total of 48 plots. Plot sizes, planting dates, and general field establishment and maintenance were identical to those described in *experiment I*.

While the test was planted in a field with a dense infestation of johnsongrass, a no-sorghum buffer equivalent to the plot size was maintained around each plot to provide sufficient johnsongrass pollen. This field was adjacent to the *experiment I*, but a biomass sorghum border was not required.

### **2.2.2.3 Data collection**

Field data collection was identical to that of *experiment I* until threshing and seed counting. In this experiment, the total number of florets produced by each panicle was estimated based on the average seedset value obtained from each plot (50 panicles/genotype).

### **2.2.2.4 Quantification of outcrossing**

After threshing, 300 g of seed (~ 30,000 seed) was drawn from each plot after bulking seed threshed from all 50 panicles within the plot. Thus, approximately 120,000 F<sub>1</sub> progeny were screened for each sorghum genotype across the four field replications. This seed was planted in the field on April 18, 2019. At mid-anthesis, the rows were visually screened and every potential

interspecific hybrid (ie, off-type) within the row was identified by their phenotype. The typical hybrid phenotype was > 2 meters tall, with more open panicles and smaller seed florets than that of an F<sub>1</sub> *S. bicolor* x *S. bicolor*. Additionally, the actual number of all established plants in the progeny rows (among the ~30,000 seed planted for each plot) were also counted for calculating outcrossing frequency. Further, the ploidy status of the off-types was determined using flow cytometry analysis (see section 2.2.2.5). Diploids were considered either fertility revertants or outcrosses within *S. bicolor*. Progenies with triploid or higher ploidy level were considered interspecific hybrids.

#### **2.2.2.5 The flow cytometry process for ploidy determination**

Ploidy of the F<sub>1</sub> progeny for both *experiment I* and *experiment II* was determined through the flow cytometry assay, using the ACURI C6 flow cytometer (Becton Dickinson and Co., Franklin Lakes, NJ). The process of flow cytometry begins with germinating the seed in Petri dishes after treating with a fungicide mix to prevent fungal contamination in the growth media. The fungicide mixture consisted of 5 ml MaximXL<sup>®</sup> (fludioxonil and mefenoxam) and 19 ml ApronXL<sup>®</sup> (mefenoxam). After germination data was recorded, seedlings were transferred to plastic cells filled with potting soil (LC1 potting soil mix, Sungro Horticulture, Canada) and placed in a controlled environment greenhouse maintained at 32/28° C day/night temperature regime and a 14-hour photoperiod.

The following flow cytometry protocol (Loureiro et al. 2007) was followed with minor modifications: When seedlings reached about 15-cm height, a small piece of the newest leaf tissue (approx. 1 cm<sup>2</sup>) was harvested from young leaves of the progeny and chopped with a single-edged razor blade in cold, modified woody plant nuclei isolation buffer (WPB). The WPB is an aqueous solution consisting of 20 mM tris (hydroxymethyl)aminomethane

(C<sub>4</sub>H<sub>11</sub>NO<sub>3</sub>), 4 mM magnesium chloride 6-hydrate (MgCl<sub>2</sub> 6H<sub>2</sub>O), 2 mM ethylenedinitrilo-tetraacetic acid ([HO<sub>2</sub>CCH<sub>2</sub>]2N[CH<sub>2</sub>]2N[CH<sub>2</sub>CO<sub>2</sub>-Na]2H<sub>2</sub>O), 86 mM sodium chloride (NaCl), 10 mM sodium metabisulfite (Na<sub>2</sub>SO<sub>3</sub>), and 1% polyvinylpyrrolidone (PVP-12310), and 0.5% (v/v) Triton X-100 at pH 7.5. Further, RNase A (PureLink™, Invitrogen, Carlsbad, CA) was added to WPB just prior to use at 5 mg L<sup>-1</sup> to prevent staining of double stranded RNA. The slurry of each plant sample was filtered through a 30 µm CellTrics disposable filter (Partec, Munster, Germany) and the nuclei in the filtered buffer was stained with 50 µg ml<sup>-1</sup> propidium iodide (Sigma-Aldrich, St. Louis, MO). Samples were placed on ice until they were analyzed. The propidium iodide labeled nuclei in each sample were analyzed for ploidy in the flow cytometer with an air-cooled laser operating at 488 nm; the fluorescence collected through a 585/20 band pass filter (Becton Dickinson and Co., Franklin Lakes, NJ) was quantified. The nuclei of a sorghum-sugarcane hybrid with known ploidy was used as a standard for comparison during the evaluation, and the ploidy level was determined by the ratio of their respective G1 peaks.

## **2.3 Statistical analysis**

### **2.3.1 Calculation of 2n gamete and outcrossing frequency**

#### **2.3.1.1 Experiment I**

The frequency of 2n gametes was calculated for each genotype using the following formula:

$$2n \text{ gamete frequency} = [\text{sum of tetraploids} + \text{hexaploids per panicle} / \text{total number of florets per panicle}] * 100$$

and the outcrossing frequency was calculated for each genotype (at an individual panicle level) using the following formula:

$$\text{Outcrossing frequency} = (\text{number of hybrids produced per panicle} / \text{avg number of total florets per panicle}) * 100$$

where the total florets per panicle were estimated based on maximum plot average seed set for the fertile version of the same parental line in *Experiment II*.

### **2.3.1.2 Experiment II**

The outcrossing frequency was calculated for each genotype (at an individual plot level) using the following formula:

$$\text{Outcrossing frequency} = (\text{number of hybrids produced per plot} / \text{total number of plants established in progeny rows for the plot}) * 100$$

## **2.3.2 Statistical model and means comparison**

### **2.3.2.1 Genotype effects**

For both *experiment I* and *II*, the frequency of 2n gamete production and outcrossing rate as influenced by different sorghum parental backgrounds was analyzed using the GLIMMIX procedure of the Statistical Analysis Software (SAS Institute Inc., Cary, NC). The GLIMMIX procedure was used because routine evaluation of the data revealed a non-normal distribution and GLIMMIX is a robust procedure that is able to handle non-normal data in experiments with multiple environments. Prior to GLIMMIX, data transformations were considered and explored, but no transformation was sufficient to normalize the data. The statistical model for analysis was  $\text{genotype}_i + \text{year}_k + \text{replication} + \text{genotype} * \text{year} + \text{error}$ , with genotype considered as the fixed effect and year and replication as random effects. Because this analysis revealed a statistically significant and meaningful genotype\*year interaction, the two years were analyzed separately using the model  $\text{genotype}_i + \text{replication} + \text{error}$ . Following ANOVA, mean separations were

carried out using the Fisher's protected LSD method ( $\alpha=0.05$ ). Further, a t-test was also conducted to compare  $F_1$  hybrid establishment data between greenhouse and field environments, using PROC TTEST in SAS.

### **2.3.2.2 Cytoplasm effects**

In *experiment I*, the frequency of  $2n$  gamete production and outcrossing rate as influenced by cytoplasm type for the three sorghum parental lines was analyzed using the GLIMMIX procedure in SAS. The statistical model for analysis was  $\text{genotype}_i + \text{cytoplasm}_j + \text{year}_k + \text{replication} + \text{genotype}*\text{cytoplasm} + \text{genotype}*\text{year} + \text{genotype}*\text{cytoplasm}*\text{year} + \text{error}$ , with genotype and cytoplasm considered as the fixed effects and year and replication as random effects. The model analysis revealed a statistically significant and meaningful  $\text{genotype}*\text{year}$  interaction, and thus the two years were analyzed separately using the model  $\text{genotype}_i + \text{cytoplasm}_j + \text{genotype}*\text{cytoplasm} + \text{replication} + \text{error}$ . Mean separations were carried out as a post-hoc test using the Fisher's protected LSD method ( $\alpha=0.05$ ). Further, a t-test was conducted to compare differences in  $F_1$  progeny establishment between the greenhouse and field environments, using PROC TTEST in SAS.

## **2.4 Results**

The johnsongrass infestation was uniform across the plots and exhibited good flowering synchrony with sorghum, and sufficient johnsongrass pollen was available in the experiment (Figures 2, 3).



**Figure 2.** Image showing natural infestation of johnsongrass in the experimental site where male sterile sorghum lines were established to estimate hybridization. The tall biomass sorghum border is in the background.



**Figure 3.** Image showing natural heavy infestation of johnsongrass in the experimental site where male sterile sorghum lines were established.



In most plots, fertilization of the male-sterile lines was evident approximately seven days post anthesis as small seed began to appear between the glumes of each floret. However, between 15-20 days post-anthesis the vast majority of seed collapsed, indicating that the endosperm development had failed and the embryo had died. This indicates that there was a significant amount of interspecific hybridization, but endosperm failure, which is common in these cases, resulted in most of the seed being lost. Endosperm failure is common in triploid type progeny (Hodnett et al. 2019, Marks 1966, McClure 1965), and indicates that most of these failed seed were likely triploid interspecific hybrids.

#### **2.4.1 Phenotypic descriptions of interspecific hybrids**

Interspecific hybrids were observed to be phenotypically different than the regular diploid sorghum and regular weedy johnsongrass. First, hybrids were much taller than the diploid grain sorghum seed parents used in this study; these hybrids reached heights of up to 2 meters or higher. Their panicles were much more open, with larger florets than that of johnsongrass (personal observations, Figure 4). Their leaves were typically wider and the culms were thicker than that of johnsongrass, but not as wide or thick as



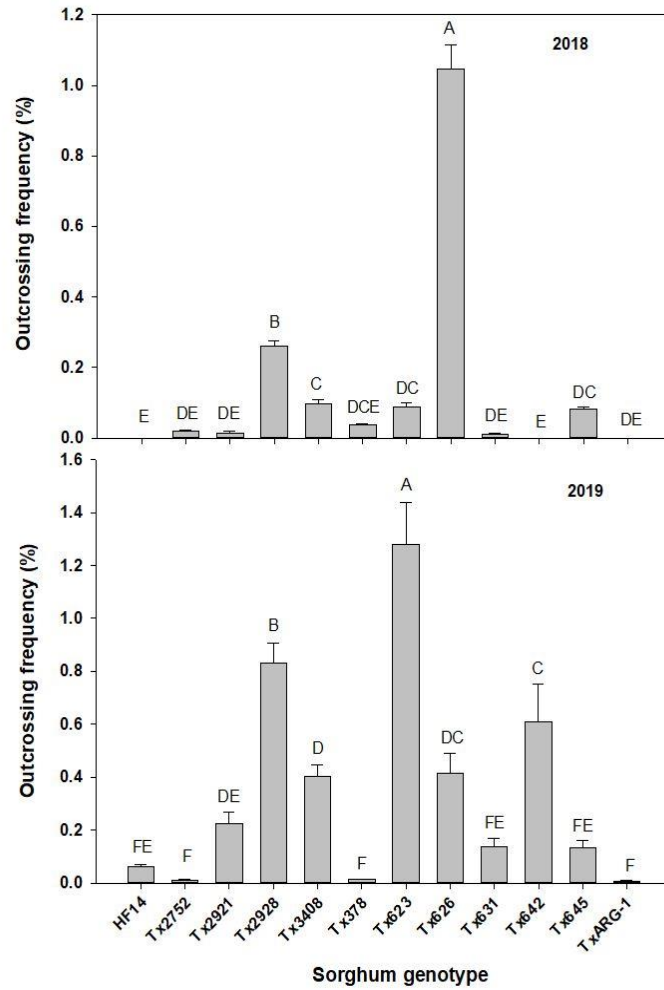
**Figure 4.** Image reflecting the phenotype of an F<sub>1</sub> interspecific hybrid (tall plant), compared to regular diploid *Sorghum bicolor* plants surrounding it. Hybrids are taller, with intermediate leaf width, and open panicles that do not resemble either a johnsongrass or a grain sorghum panicle (picture taken in late summer 2019).

leaves and stems in regular *S. bicolor* (personal observations, data not shown). Prior reports and observations of phenotypic characteristics of hybrids describe similar characteristics (Dweikat 2005, Hadley 1958, Morrell et al. 2005).

### 2.4.2 Male sterile parental line effects

Data analysis revealed significant effects for both parent and year as well as a parent\*year interaction, for both 2n gamete frequency and outcrossing rate (Table 2). Because of the significant interaction term, further analysis of parent effects was conducted by year to minimize the effect of these interactions (Figure 5).

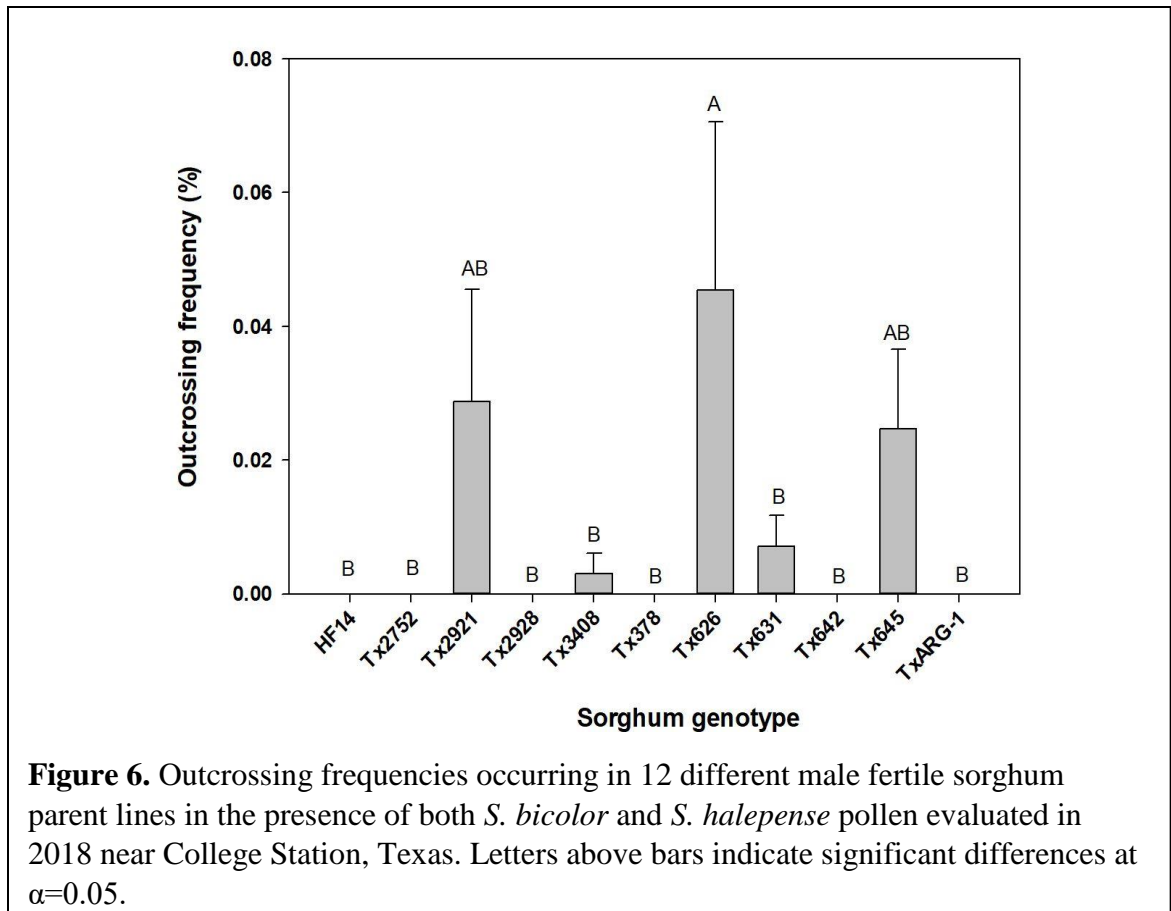
<b>Table 2.</b> <i>P</i> values for 2n gamete and outcrossing frequency for the evaluation of 12 male sterile sorghum seed parent lines pollinated with <i>S. halepense</i> in College Station in 2018 and 2019 ( $\alpha=0.05$ ).			
<b>Effect</b>	<b>Df</b>	<b>2n gamete frequency</b>	<b>Outcrossing frequency</b>
Year	1	<0.0001	<0.0001
Parent	11	<0.0001	<0.0001
Year*Parent	11	<0.0001	<0.0001



**Figure 5.** Outcrossing frequencies occurring in 12 different male sterile (A1 cytoplasmic male sterility) sorghum parent lines only in the presence of *S. halepense* pollen evaluated in a) 2018 and b) 2019 near College Station, Texas. Letters above bars indicate significant differences at  $\alpha=0.05$ .

In 2018, outcrossing frequency ranged from 0% to 1.04% (average: 0.14%) across the 12 parent lines. In 2019, the outcrossing frequency had a wider range and was slightly higher than in 2018 (0.007% to 1.27%; average, 0.34%) (Figure 5). Of the genotypes evaluated, Tx626 had the greatest level of outcrossing at 1.04% in 2018, while Tx623 was the highest in 2019 (1.27%) (Figure 5). The shifts in response across years for two lines are one reason why the interaction term was significant. However, it should be noted that there was consistency in the outcrossing frequency of some of the genotypes across years. For example, several lines were consistently low and others high (Figure 5). This strongly indicates that there are differences in seed parent lines for the rate of outcrossing. Although there are small differences across the two years, the genotypes that are consistently on top tend to remain as the greatest outcrossers.

In the fertile version of these lines, the outcrossing frequencies were orders of magnitude lower than in the male sterile lines (Figure 6).



As data were only available for one year, an interaction was not available, but there were significant differences found in the outcrossing frequencies (Figure 6) across parental backgrounds ( $P=0.029$ ). In 2018, the greatest outcrossing frequency was reported in Tx626 at 0.04%, with most genotypes at levels significantly lower than that (Figure 6). This indicates that the presence of *S. bicolor* pollen severely reduces the possibility of pollination by *S. halepense* pollen, but it does not exclude it. It is important to note that trends in the rate of outcrossing rate

were similar between the *Experiment I* (sterile lines) and *II* (fertile lines), albeit at orders of magnitude lower in the latter (Figures 5, 6). This implies that factors inherent to the parent genotypes could be involved in the differential response.

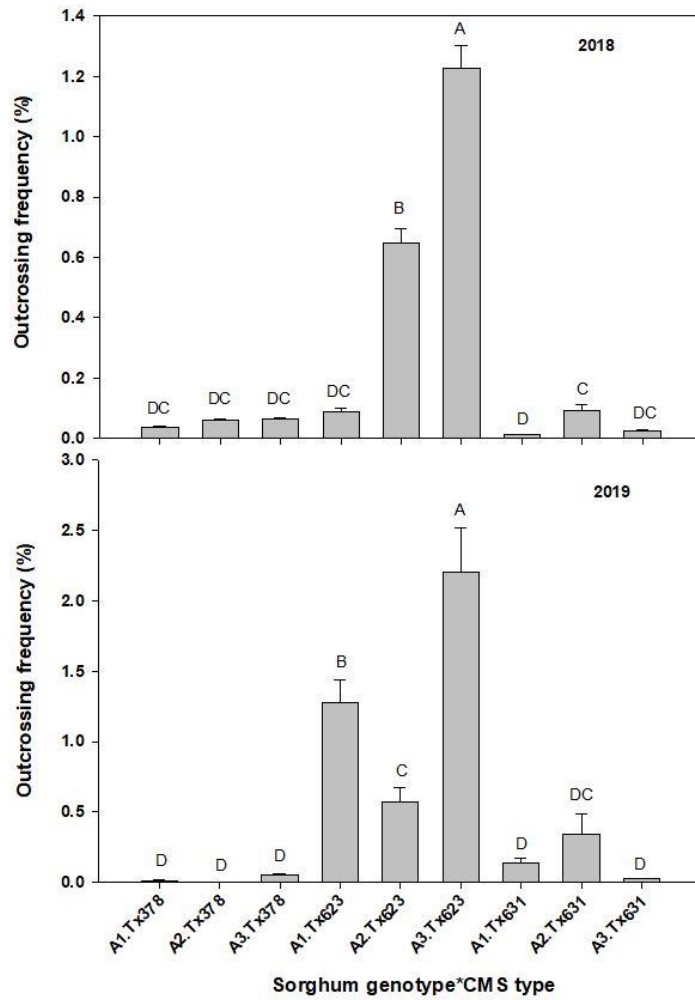
### 2.4.3 Cytoplasm effect

In comparisons of cytoplasm, the effects of year, parent, cytoplasm, and their interactions were all highly significant (Table 3). Consequently, the data were analyzed by year to minimize the effect of these interactions.

<b>Table 3.</b> <i>P</i> values for 2n gamete and outcrossing frequency for the evaluation of 3 cytoplasm types in 3 male sterile sorghum seed parent lines, pollinated with <i>S. halepense</i> in College Station in 2018 and 2019 ( $\alpha=0.05$ ).			
<b>Effect</b>	<b>Df</b>	<b>2n gamete frequency</b>	<b>Outcrossing frequency</b>
Year	1	<0.0001	<0.0001
Parent	2	<0.0001	<0.0001
Year*Parent	2	<0.0001	<0.0001
Cytoplasm	2	<0.0001	<0.0001
Cytoplasm*Year	2	0.0003	0.0022
Cytoplasm*Parent	4	<0.0001	<0.0001
Cytoplasm*Year*Parent	4	<0.0001	<0.0001

The effect of parent line closely followed the trends observed in the survey of the 12 parents described above (as in Figure 5). Most notably, Tx623 (all cytoplasm types) had significantly higher outcrossing frequencies than either Tx378 or Tx631 (Figure 7). In evaluating the specific cytoplasm effect, it is clear that A3 results in higher levels of outcrossing frequencies, in the Tx623 genetic background (Figure 7). Hodnett et al. (2019) noted a higher outcrossing rate in Tx623 than in Tx631 and the data presented herein support that observation.





**Figure 7.** Outcrossing frequencies occurring in 3 different male sterile parent lines (Tx378, Tx623, and Tx631) utilizing three different sources of cytoplasmic male sterility (A1, A2, and A3) only in the presence of *S. halepense* pollen evaluated in a) 2018 and b) 2019 near College Station, Texas. Letters above bars indicate significant differences at  $\alpha=0.05$ .

Given that this is the first report of the effect of cytoplasm on outcrossing rates, the observation that the A3 cytoplasm has a higher rate of outcrossing is subject to verification. Further, given the strong interaction with parent line, it is possible that this response is genotype specific, which warrants further investigation.

#### **2.4.4 Ploidy status of the interspecific hybrids**

True interspecific hybrids were produced in most seed parent lines, although the numbers varied across parents and years. The distribution of different ploidy types in the F<sub>1</sub> progenies was relatively consistent across years and genotypes. By far, the most commonly observed ploidy was tetraploidy (Tables 4-8) and triploid progeny were a distant second. The same trend was also observed in the F<sub>1</sub> progenies obtained in the fertile sorghum lines (*experiment II*) (Table 5), and the cytoplasm study (Tables 7 and 8). There was also one rare pentaploid produced in the cytoplasm study (Table 7).

**Table 4.** Distribution of different ploidy types<sup>a</sup> in the F<sub>1</sub> progeny derived from male sterile *S. bicolor* pollinated with *S. halepense* in College Station in 2018. A total of 12 different male sterile parent lines with A1 cytoplasm were evaluated.

Parent ID	Progeny No.	%haploid*	%triploid	%tetraploid
HF14	0	0	0	0
Tx2752	6	17	17	67
Tx2921	6	0	0	100
Tx2928	11	0	0	100
Tx3408	3	0	33	67
Tx378	9	0	0	100
Tx623	15	0	0	100
Tx626	34	3	0	97
Tx631	2	0	50	50
Tx642	0	0	0	0
Tx645	20	0	0	100
TxARG-1	0	0	0	0
<b>Total/Average</b>	106	2	3	95

<sup>a</sup>Values rounded to the nearest whole number

\*Haploids are not considered interspecific hybrids

Results from the t-test also showed that the establishment potential of the hybrid progeny was much lower when the F<sub>1</sub> seed were planted under field conditions, compared to establishment in a greenhouse. On average across the different sorghum lines and CMS types, successful seedling establishment was only 3.35% of total hybrid seed planted, a 52% reduction (P<0.001) compared to when the seedlings were established under favorable growth conditions in a greenhouse.

**Table 5.** Distribution of different ploidy types<sup>a</sup> in the F<sub>1</sub> progeny derived from male sterile *S. bicolor* pollinated with *S. halepense* in College Station in 2019. A total of 12 different male sterile parent lines with A1 cytoplasm were evaluated.

Parent ID	Progeny No.	%haploid*	%triploid	%tetraploid
HF14	2	50	0	50
Tx2752	3	33	67	0
Tx2921	19	11	5	74
Tx2928	25	0	4	96
Tx3408	5	0	40	60
Tx378	5	0	80	20
Tx623	57	0	0	100
Tx626	15	0	0	100
Tx631	5	0	80	20
Tx642	34	0	21	79
Tx645	10	0	10	90
TxARG-1	3	0	33	67
<b>Total/Average</b>	183	2	13	85

<sup>a</sup>Values rounded to the nearest whole number

\*Haploids are not considered interspecific hybrids

**Table 6.** Distribution of different ploidy types<sup>a</sup> in the F<sub>1</sub> progeny derived from male fertile *S. bicolor* parent lines pollinated with *S. halepense* in College Station in 2018. A total of 12 different male fertile parent lines were evaluated.

<b>Parent ID</b>	<b>Progeny No.</b>	<b>%haploid</b>	<b>%triploid</b>	<b>%tetraploid</b>
HF14	0	0	0	0
Tx2752	0	0	0	0
Tx3408	0	0	0	0
Tx378	0	0	0	0
TxARG-1	0	0	0	0
Tx2921	4	0	75	25
Tx2928	2	0	0	100
Tx623	1	0	0	100
Tx626	11	0	0	100
Tx631	1	0	0	100
Tx642	2	0	100	0
Tx645	0	0	0	0
<b>Total/Average</b>	21	0	24	76

<sup>a</sup>Values rounded to the nearest whole number

\*Haploids are not considered interspecific hybrids

**Table 7.** Distribution of different ploidy types<sup>a</sup> in the F<sub>1</sub> progeny derived from three different sorghum lines with male-sterility induced by three different cytoplasms. These lines were pollinated with *S. halepense* in College Station in 2018.

<b>Cytoplasm</b>	<b>Parent ID</b>	<b>Progeny No.</b>	<b>%haploid</b>	<b>%triploid</b>	<b>%tetraploid</b>
A1	Tx378	9	0	0	100
A2	Tx378	9	11	0	89
A3	Tx378	12	0	0	100
A1	Tx623	15	0	0	100
A2	Tx623	14	7	0	93
A3	Tx623	23	0	0	100
A1	Tx626	2	3	0	97
A1	Tx631	1	0	50	50
A2	Tx631	5	0	0	100
A3	Tx631	9	0	0	100
<b>Total/Average</b>		90	2	1	97

<sup>a</sup>Values rounded to the nearest whole number

\*Haploids are not considered interspecific hybrids

**Table 8.** Distribution of different ploidy types<sup>a</sup> in the F<sub>1</sub> progeny derived from three different sorghum lines with male-sterility induced by three different cytoplasms. These lines were pollinated with *S. halepense* in College Station in 2019.

<b>Cytoplasm</b>	<b>Parent ID</b>	<b>Progeny No.</b>	<b>%haploids</b>	<b>%triploids</b>	<b>%tetraploids</b>	<b>%pentaploids</b>
A1	Tx378	5	0	80	20	0
A2	Tx378	5	0	80	20	0
A3	Tx378	6	0	50	50	0
A1	Tx623	57	0	0	100	0
A2	Tx623	45	0	2	98	0
A3	Tx623	103	2	7	90	1
A1	Tx631	5	0	80	20	0
A2	Tx631	29	3	38	59	0
A3	Tx631	5	0	60	40	0
<b>Total/Average</b>		260	1	14	84	0.4

<sup>a</sup>Values rounded to the nearest whole number

\*Haploids are not considered interspecific hybrids

#### 2.4.5 2n gamete frequencies

Based on the results reported here, the frequency of 2n gamete formation was as high as 1.026% (Tx626) in 2018 and 1.28% (Tx623) in 2019 (Table 9). These two lines consistently had high levels of outcrossing as well. Parental lines such as TxARG-1, HF14, and Tx2752 were consistently on the lower end of the 2n gamete production scale during both experimental years (Table 8).

**Table 9.** The estimated 2n gamete frequency observed in 12 male sterile sorghum seed parent lines sterilized in A1 cytoplasm and pollinated exclusively by *S. halepense* in 2018 and 2019.

Genotype	2n gamete frequency (%)*	
	2018	2019
Tx626	1.02 A	0.42 DC
Tx2928	0.26 B	0.80 B
Tx623	0.09 C	1.28 A
Tx645	0.08 C	0.12 FE
Tx3408	0.05 DC	0.26 DE
Tx378	0.04 DC	0.003 F
Tx2921	0.02 DC	0.16 FE
Tx2752	0.01 D	0 F
Tx631	0.01 D	0.12 FE
TxARG-1	0.0 D	0.01 F
Tx642	0.0 D	0.51 C
HF14	0.0 D	0.03 F

\*Means separated by letters within a column were significantly different at  $\alpha=0.05$ .

Similar to the outcrossing frequencies observed in this research, Tx623 with the A3 CMS type showed the highest levels of 2n gamete production in both 2018 and 2019 field experiments (Table 10, Figure 7). The genotype\*sterility interaction was also very evident for the 2n gamete frequency. Additionally, most of these 2n gametes are leading to tetraploid hybrids (Tables 6 and 7) that produce viable progenies.



**Table 10.** The estimated 2n gamete frequency observed in 3 male-sterile sorghum seed parent lines sterilized in three different cytoplasm types and pollinated exclusively by *S. halepense* in 2018 and 2019.

Genotype	CMS type	2n gamete frequency (%)*	
		2018	2019
Tx623	A1	0.09 DC	1.28 B
	A2	0.61 B	0.53 C
	A3	1.23 A	2.03 A
Tx631	A1	0.01 D	0.12 D
	A2	0.10 C	0.21 D
	A3	0.03 DC	0.01 D
Tx378	A1	0.04 DC	0.003 D
	A2	0.06 DC	0 D
	A3	0.06 DC	0.04 D

\*Means separated by letters within a column were significantly different at  $\alpha=0.05$ .

## 2.5 Discussion

A total of 12 different sorghum genotypes and 3 CMS types were assessed in this study for the frequency of 2n gamete production and outcrossing potential with johnsongrass (male parent). To our knowledge, this is the first detailed study where such a range of sorghum genetic backgrounds and CMS types were compared under natural field conditions. Furthermore, outcrossing frequencies were determined for the presence or absence of self-pollen competition in sorghum; this allowed for estimation of outcrossing frequencies in both a normal and a worst-case scenario. Research by Hodnett et al. (2019) was conducted using selected male-sterile sorghum (female) genotypes under controlled environmental conditions in the greenhouse, which laid the foundation for this work.

### 2.5.1. Types of progeny

The majority of the progeny produced in the male sterile lines were interspecific hybrids. These progeny phenotypically exhibited the characteristics expected of an interspecific hybrid between *S. bicolor* and *S. halepense*. These characteristics include tall growth (>2 m in height), with thicker stems and wider leaves than that of johnsongrass, but thinner and narrower than that of sorghum. The panicles were also much less compacted and multiple panicles per plant were observed. These hybrids were commonly tetraploid, but triploids were found as well. Tetraploids tend to have more weedy and aggressive characteristics than triploids do. Additionally, the hybrids produced in this study were all male sterile (personal observations). Aguna and Bekele (2013) recorded that crosses of crop (female)-wild (male) sorghum were fertile, which indicates that fertility status of the F<sub>1</sub> hybrids cannot be generalized and that fertility of any hybrid must be confirmed.

Diploids were also observed in the pool of progeny. These progeny were likely the result of *S. bicolor* pollen in the experimental area from either outside sources or sterility breaks that can occur even at individual floret levels, although the CMS system is very efficient and widely used (Papathanasiou and Lessman 1969). Moreover, the biomass sorghum planted around the experimental area in 2019 did not establish as tall as it was in 2018, leading to relatively more production of diploid F<sub>1</sub> progeny in 2019 (62%) than in 2018 (55%). Field observations of a subsample of the diploid progeny showed a *S. bicolor* phenotype and thus the diploid progeny data were excluded from calculation of outcrossing frequency (data not shown).

A significant amount of these seed never germinated. While dormancy is well known in johnsongrass (Taylorson and McWhorter 1969), it is not common in *S. bicolor*, especially in

types bred for grain sorghum production. Consequently, seed that did not germinate were likely not viable, but some have been saved to confirm that dormancy was not a contributing factor.

### **2.5.2. Understanding outcrossing frequencies as a result of genotype and CMS**

Outcrossing frequencies ranged from 0 to 0.04% in the male fertile seed parent lines and between 0 to 1.27% in the male sterile parental lines. These results have two implications. First, there are sterility conditions and genotypes that are clearly less inclined to cross pollinate with johnsongrass under any conditions and others that are more inclined toward interspecific hybridization. Second, male fertility in *S. bicolor* is likely the single most important means of reducing interspecific hybridization with johnsongrass; at the high ranges reported herein, outcrossing frequencies in the absence of *S. bicolor* pollen were between two and three orders of magnitude higher than when *S. bicolor* pollen was present.

Evaluation of the male sterile systems pollinated only with *S. halepense* provide direct insight into the factors causing interspecific hybridization between *S. bicolor* and *S. halepense*. In this study, significant variation for outcrossing frequency was caused by all three main effects (parent, year, and cytoplasm). Further, these main effects were confounded by interactions. However, some trends emerged when analysis was completed by year, which was done to avoid the complexity of these interactions. Environmental conditions recorded for 2018 and 2019 at the Texas A&M research farm are included in Table 11.

In fact, environmental factors can certainly affect outcrossing rates. Hanson et al. (2005) showed in wheat that several environmental factors affected the ability of pollen to cross-fertilize. Some of these environmental factors include relative humidity, rainfall, air temperature, and light intensity.

**Table 11.** Total monthly rainfall and monthly average temperature for April-August 2018 and 2019 collected at the Texas A&M research station near College Station, TX where the *experiments I and II* were conducted.

2018			2019		
month	rain (mm)	temperature (°C)	month	rain (mm)	temperature (°C)
April	30.5	18.5	April	95.2	19.9
May	25.4	25.9	May	123.6	24.8
June	39.5	28.7	June	58.9	27.1
July	48.0	28.9	July	25.8	28.6
August	12.2	29.3	August	54.9	30.1
<b>Total</b>	155.5	26.3	<b>Total</b>	358.3	26.1

Outcrossing and hybridization frequencies for other species and their weedy relatives have been reported at various levels: an average of 10% hybridization between *Brassica napus* L. lines and the wild relatives, *B. rapa* L. and *Raphanus raphanistrum* L. (Halfhill et al. 2004), up to 89% mean value at distances up to 400 meters between *R. raphanistrum* L. and cultivated radish (*R. sativus* L.) (Klinger et al. 1992), and as high as 2.3% between indica rice (*Oryza sativa* L.) and Costa Rican weedy rice (*O. sativa*) (Olguin et al. 2009). More closely related to this study, Arriola and Ellstrand (1996) documented hybridization rates as high as 2% at 100 meter distance from between sorghum and johnsongrass.

While interactions caused some shifts in magnitude, some genotypes were consistently more likely to outcross (notably Tx2928, Tx626, and Tx623) while some others were consistently less likely to do so (notably HF14, Tx2752, and TxARG-1). The differences between the parent lines implies that there is, at a minimum a genotypic effect with a likely genetic component underlying these differences. Given that these seed parents are common and

elite, and are used directly as seed parents in commercial hybrids and as breeding parents to create new hybrids, this information is relevant to applied sorghum breeding (Pfeiffer et al. 2019). A myriad of factors could be causing these differences – they range from floral structure to range of stigma receptivity and ovule viability to unreduced or 2n gametes (Moran et al. 2003, Cisneros-López et al. 2012, Nguyen et al. 2013). For instance, Tx623 was consistently one of the parental lines that contained the highest outcrossing frequencies. Tx623 is a parental line that is known to possess stigmas that can remain receptive for longer periods of time after the onset of anthesis (Moran et al. 2003). While all of these factors should be considered and evaluated, several factors point to the likelihood that the frequency of unreduced or 2n gametes are a significant factor.

The first and most important observation supporting the role of 2n gametes is the type of interspecific hybrids recovered. With amazing consistency, the majority of hybrids were tetraploids. Hodnett et al. (2019) reported the same results with regard to ploidy frequency of progeny. Given these results, it is not surprising that there is a strong correlation between outcrossing frequency and the estimated frequency of 2n gamete formation in different sorghum parental lines.

The preponderance of tetraploid progeny indicates that 2n gamete production is a critical factor in the production of interspecific hybrids between *S. bicolor* and *S. halepense*. As noted earlier, most *S. bicolor* florets in the male sterile lines appeared to have been fertilized by *S. halepense* pollen, but then the seed collapsed due to the failure of the endosperm. This failure is common in triploid progeny resulting from crosses involving unbalanced ploidies (McClure 1965, Marks 1966, Hodnett et al. 2019).

Given that a triploid progeny would be expected from the union of a normal  $n=x=10$  gamete from *S. bicolor* and a normal  $n=2x=20$  gamete from *S. halepense*, most of the florets would form a triploid progeny and the vast majority will fail before maturity. While a few survive, the majority that survive are tetraploids that are formed when a  $2n$  gamete ( $2n=2x=20$ ) in the sorghum female forms a zygote with a normal  $n=2x=20$  gamete from johnsongrass. Because chromosome number and ploidy are balanced in this case, the endosperm is less likely to fail and these seeds develop to maturity to produce viable seedlings. Consequently, the formation of  $2n$  gametes appears to be the key component for successful interspecific hybridization between sorghum and johnsongrass.

While relatively new to sorghum,  $2n$  gamete formation is known and used in breeding for other crops such as potato and alfalfa (De Maine 1982, Veronesi et al. 1986). Additionally, Griffiths et al. (1971) noted unsatisfactory endosperm establishment in triploid progeny of ryegrass hybrids; tissue culture embryo recovery was required for successful interspecific hybridization. Given that hybrids between *S. bicolor* (diploid) and *S. halepense* (tetraploid) are typically expected to be triploids, consistent recovery of tetraploids indicates that the vast majority of the triploid progeny collapse during seed development due to unbalanced chromosome sets in the endosperm while most tetraploids fully develop (Cox et al. 2018).

Overall the genotypic differences observed for both outcrossing frequency and  $2n$  gamete formation implies an underlying genetic control of  $2n$  gamete formation. The patterns observed of frequencies of  $2n$  gamete formation and the abundance of tetraploid progenies are not only reserved for the sorghum genera. Unreduced gamete formation has been observed in other genera such as *Lilium* (van Tuyl et al. 1989), *Lotus* (Negri and Lemmi 1998), *Citrus* (Xie et al. 2019), *Avena* (Nikoloudakis et al. 2018), *Populus* (Zhao et al. 2017), to name a few.

The exploitation of 2n gametes has been widely used in plant breeding and there are even approaches to investigate the potential to stimulate 2n gamete formation (Younis et al. 2014). There are currently efforts to further discover how to induce and more efficiently use these 2n gametes, but this study has focused on the natural formation of 2n gamete as a result of genotypic backgrounds. Other studies such as the one conducted by Fakhri et al. (2016) also found that frequency of unreduced gamete production was a trait that is genetically controlled in hybrids produced by *Triticum aestivum* X *Aegilops triuncialis* which would agree with our results that indicate unreduced gamete frequencies differ as a result of genotype\*sterility interactions.

Ultimately, genotypic differences in 2n gamete production could potentially lead to the selection of genotypes that are less likely to produce interspecific hybrids. It is apparent that further research to assess the underlying genetic factors leading to 2n gametes in sorghum is needed. If confirmed and heritable, selection against their presence in seed parents could be an effective means of mitigating outcrossing.

Study of the effect of cytoplasm on this trait was motivated by prior research that indicated cytoplasm has mixed effects on hybrid progeny. Although Hoffman and Rooney (2013) and Vacek and Rooney (2018) found no effect on biomass productivity as a result of type of CMS, significant yield reductions were observed when A3 CMS was used in grain sorghum development when compared to A1 and A2 (Moran and Rooney 2003). Although it is not known why A3 CMS reduces yield potential, it is reported that it is not due to fertility restoration (Moran and Rooney 2003). This same study determined that A1 and A2 do not yield significantly different, but our results show that when combined with certain genotypes (Tx623

and Tx631), they are more likely to hybridize, which can be a useful information for sorghum improvement programs.

In addition to the genotypic effects discovered herein, the effect of cytoplasm resulted in some of the highest outcrossing frequencies recorded in this study. Herein, the A3 cytoplasm had higher outcrossing frequency than either A1 or A2 in both 2018 and 2019. Specifically, Tx623 in combination with A3 cytoplasm produced abnormally high levels of outcrossing and 2n gamete frequency. Hadley (1958) observed that ploidy ratios of triploids to tetraploids of interspecific hybrids changed depending on the type of induced sterility. Similarly, Hodnett et al. (2019) noted differences in hybrid ploidy ratios when different sterility induction systems were used (genetic vs. chemical gametocides). While there is not an obvious reason as to why the sterility induction system could cause these differences, the fact that they have consistently been observed in the limited number of studies completed indicates that something is occurring that merits further study. As previously mentioned, the A1 CMS type is the most common type of CMS used in sorghum improvement programs, but A3 CMS has had application in the production of male sterile forage hybrids. Given that the outcrossing frequencies are especially high in A3Tx623, avoidance of this seed parent in those types of hybrids is recommended. Regardless of the cause of the effect, this knowledge has practical applications in sorghum improvement and the mitigation of interspecific hybridization.

### **2.5.3 Applications of the interspecific hybrids**

Other progeny traits have been documented to be ploidy related such as was concluded in Dweikat (2005) where several traits from johnsongrass such as greenbug resistance and cold tolerance were being expressed in resulting progenies. Further studies are underway to add to this body of research where we will evaluate the phenotypic differences that are resulting due to



the differences in genotype, CMS, and ploidy. Similarly, Hadley (1958) determined that rhizome vigor differed as a result of 30 and 40 chromosome phenotypes in sorghum by johnsongrass hybrids. Although other ploidies such as pentaploids and even some haploids were found in our study, their relative proportions were miniscule and not attributed to  $2n$  gamete production.

In terms of fitness observations, only a fraction of the total hybrid seed established successfully and there was a significant reduction in establishment potential in field plantings compared to greenhouse plantings. Up to 6.4% of all seed planted in the greenhouse was able to successfully establish while only 3.3% of seed was able to establish in the field. These establishment differences as a result of environment have implications because they indicate that overall outcrossing risks will be much lower since only a small proportion of the  $F_1$  seed is capable of establishing under field and competitive conditions. The ability to establish will have long-term implications in population dynamics as Johnson and Coble (1986) observed in large crabgrass and broadleaf signal grass, and as Palmblad (1968) described in their observations of intra-specific weed competition.

Further studies will focus on the phenotypic characteristics of these  $F_1$  hybrids and their performance in the field. Previous studies have shown that outcrossing rates are variable depending on morphological characteristics of hybrids (Ellstrand and Foster 1983). Therefore, further studies are necessary to quantify outcrossing at the  $F_1$  stage and determine what the potential factors affecting outcrossing at this stage are.

## CHAPTER III CONCLUSIONS AND SUMMARY

### 3.1 Conclusions

Results are consistently showing that the frequency of outcrossing significantly differs throughout the 12 different sorghum genotypes and 3 different types of CMS. The results also indicate that the presence of self-pollen competition decreases outcrossing frequencies by two orders of magnitude.

It is unknown how the different ploidy types govern adaptive characteristics of the hybrids and influence their fate and long-term dynamics. Future research will further expand on what has been documented here, and potentially focus on backcrossing to further understand progeny characteristics and fitness potentials, as well as quantifying gene flow in the opposite direction when johnsongrass is the pollen recipient.

Furthermore, research on gene flow mitigation through management and genetic means, and developing suitable stewardship protocols using the information generated from this study should be undertaken. By further understanding the biology of these crop-weed systems, we can make more conscious decisions as far as the management practices that are being put in place beginning as early as the seed variety developmental stage.

The implications of this research indicate that outcrossing can be reduced and mitigated through appropriate selection of sorghum parental backgrounds that are less likely to hybridize with the weedy relative. This information could potentially lead to more conscious decisions on behalf of the sorghum improvement programs and extend the longevity of new seed technologies by reducing the potential for weedy relatives to obtain genes of interest (i.e. resistance genes).

### 3.2 Summary

The current sorghum production systems do not have any post-emergence chemical options to control grass weed species that may affect grain sorghum yields. The potential for hybridization between these two species makes it a challenge to incorporate new seed technologies due to fear of transferring genes of interest from the cultivated crop into the weedy relative. The potential for the transfer of 2n gametes makes it possible for emergence of vigorous and competitive tetraploid progenies. Now that further research has been conducted concerning gene flow and hybridization between these two species, we further understand the potential for gene flow in true agronomic conditions. Further research is necessary to better understand fitness differences and phenotypic disadvantages in the field. However, we now understand that selection of appropriate sorghum genetic backgrounds may be advantageous to reduce the potential for hybridization once a released variety reaches a grower. Every season we are concerned about valuable herbicides being lost due to weed resistance. It is imperative that we use every aspect of integrated weed management in order to reduce the potential for novel technologies to be lost to resistance after just a few growing seasons.

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