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EVALUATION AND UTILIZATION OF A CHEMICAL MALE GAMETOCIDE IN SORGHUM

A Thesis

by

NICHOLAS ANDREW BOERMAN

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MASTER OF SCIENCE

Chair of Committee,	William L. Rooney
Committee Members,	Byron L. Burson
	David M. Stelly
Head of Department,	David D. Baltensperger

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ABSTRACT

Sorghum bicolor (L.) is an ancient grain and forage crop, grown from the tropics to temperate regions of the world. It is a self-pollinated species that can be grown as either a pure line cultivar or hybrid, depending on the region of production. Existing methods of hybridization of sorghum are useful but have some limitations. Methods of inducing temporal male sterility could enhance several aspects of sorghum breeding. One potential application of temporal male sterility would be to scale up cross-pollination in order to implement a doubled haploid (DH) breeding system in sorghum. A chemical male gametocide that reliably renders plants male-sterile might be ideal for this purpose.

Two studies were conducted to assess the utility of *trifluoromethanesulfonamide* (TFMSA) as a chemical gametocide of sorghum. The first study evaluated the amino acid composition of anther and glume tissues following application of TFMSA. To do so, anther and glume tissue were excised from florets of plants that had received an application of either 0 mg, 2 mg, 6 mg, or 20 mg TFMSA. After excision of the tissues, the amino acids were extracted in Milli-Q® water; then -high-pressure liquid chromatography (HPLC) was used to quantify the amino acids. Fold changes were calculated to look for shifts among treated and untreated plant tissues. In the anther tissues, several amino acids experienced drastic shifts, the most notable being a >10x decrease of proline and a >20x increase of asparagine. Similar shifts were not observed in glume tissue. Consequently, one, or, both of these shifts may be associated with the induction of male sterility.

The second study was conducted to evaluate the efficacy of TFMSA to induce male sterility in two field environments. Both environments were grown in College Station with planting dates that differed by 2 weeks. TFMSA was applied to three distinct genotypes using both hand application and backpack sprayer application in dosages ranging from 5 mg to 30 mg. Multiple applications of the 10 mg and 15 mg dosages were also evaluated. Results indicated that once a minimum dosage threshold was exceeded, specific dosages and number of applications had little overall effect on male sterility. Use of a backpack sprayer showed sterility induction albeit at slightly lower levels of male sterility. The results indicate that TFMSA can be used as an effective and durable chemical male gametocide in sorghum.

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Contributors

This work was supervised by a thesis committee consisting of Professor William L. Rooney-advisor, of the Department of Soil and Crop Science and Professor Byron L. Burson-committee member, of the Department of Soil and Crop Science and Professor David M. Stelly- committee member, of the Faculty of Genetics.

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NOMENCLATURE

DH	Doubled Haploid
TFMSA	Trifluoromethanesulfonamide
HPLC	High Pressure Liquid Chromatography
CMS	Cytoplasmic Male Sterility
GMS	Genetic Male Sterility
E4FO	Ethyl 4-fluorooxanilate
DAP	Days After Planting
GSA	Glutamate Semi Aldehyde
P5CS	Pyrroline-5-Carboxylate Synthetase
P5CR	Pyrroline-5-Carboxylate Reductase
CA	Carbonic Anhydrase

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1. INTRODUCTION

Sorghum bicolor (L.) is cereal grain crop grown worldwide for both grain and forage production. Between the 2013-2015 growing seasons, area devoted to grain sorghum production in the United States ranged from 7.1 million acres in 2014 to 8.5 million acres in 2015 (NASS, 2016). Although full forage sorghum production statistics are not available, silage sorghum production ranged from 306,000 acres in 2015 to 380,000 acres in 2013 (NASS, 2016). Recently, some have developed it to be an energy crop (Rooney et al., 2007).

Depending on the region in which sorghum is grown, it is either as a hybrid crop or pure line variety. The economics of each region are paramount in determining which type of sorghum is grown. In the case of hybrids, seed production requires an economic means of crossing to produce hybrid seed. However, cross-pollination methods are essential for breeding and improvement and could be even more important as new breeding technologies are developed.

While numerous crossing methodologies have been developed and will be reviewed herein, each of these systems has strengths and weaknesses. For example, the cytoplasmic male sterility (CMS) system is excellent for commercial hybrid seed production but is ill-suited for most breeding purposes. Likewise, manual emasculation is excellent for making a specific breeding cross, but the amount of seed produced is minimal and cannot be scaled.

New breeding methodologies, such as the production of doubled haploids, have revolutionized improvement in different crop species such as maize (*Zea maize* L.) and barley (*Hordeum vulgare* L.) (Baenziger, 1996). In maize, doubled haploid (DH) technology speeds the breeding process and allows breeders to effectively integrate new technologies, such as genomic selection, into the program. Deploying genomic selection in a DH breeding system is also thought to be more effective than in a field-based selection and inbreeding system. If using DH breeding systems in conjunction with genomic selection, they allow early generation performance testing and discarding of poor breeding populations prior to genotyping in cycle 0; therefore, allowing genomic selection to occur during cycles 1 and 2 of recombination (Bernardo and Yu, 2007).

There is interest in developing DH breeding systems in sorghum, but none of the current hybridization systems will effectively allow DH technology deployment. Consequently, there is a need to identify and develop a sterility induction system that is compatible with DH technology. The primary objectives of this study are i) to determine the effectiveness of the chemical gametocide TFMSA, to induce pollen sterility in sorghum under field conditions; ii) to determine if the composition of free amino acids in treated anthers are affected by TFMSA and if this is associated with male sterility induction.

2. LITERATURE REVIEW

Hybridazation is a basic requirement in most crop breeding programs. The number of crosses made depends on the species and the type(s) of end-products. For example, cotton (*Gossypium* L.) is grown as a pure-line cultivar in many parts of the world and crossing is mostly used to create breeding populations. In other parts of the world such as India and China, cotton is grown as an F_1 hybrid and cross-pollination is essential for the production of these varieties (Wang and Fok, 2014). Alternatively, other crops, such as sorghum, routinely require multiple types of crosses within both breeding and seed production systems. For these reasons, many methods to cross sorghum have been developed.

As a naturally self-pollinated crop, sorghum requires cross-pollination methods for the commercial production of hybrids. The current method for producing commercial hybrid seed is CMS. Genetic male sterility (GMS) was first considered as a means for hybrid production, but CMS works more efficiently because the restoration of fertility in the hybrid is logistically feasible with that system (Duvick, 1959). Also, the number of lines with GMS is limited, which constrains the genetic backgrounds readily available.

To produce breeding crosses, several different approaches, ranging from hand emasculation to hot water treatments, have been used. In addition, GMS has been used for specific work in population improvement, such as cyclical selection schemes. While these systems are effective, none are perfect as they are not applicable in all situations.

2.1 Cytoplasmic Male Sterility

Cytoplasmic male sterility has been found in at least 80 species, 25 genera, and 6 families within the plantae and animalia kingdoms of life (Edwardson, 1970). However, development of a functional CMS system for producing commercial hybrid seed can be complicated or stymied by any of several factors, such as unstable or lack of sufficient sterility, inability to consistently restore male fertility in the hybrid, inability to produce sufficient quantities of seed for commercial production, and the presence of undesirable pleiotropic effects of the CMS system used (McVetty, 1997).

Sorghum inbred genotypes are classified into A, B, and R lines based on their male fertility and ability of an individual to restore or maintain sterility when crossed with a cytoplasmic male sterile individual. Male-sterile genotypes possess A type cytoplasm and recessive fertility restorer (*rf*) genes (Rooney and Smith, 2000). Within sorghum, five distinct cytoplasmic male sterility systems have been identified: A_1, A_2, $A_{2'}$, A_3, and A_4 with A_1 being the most commonly used in modern hybrid production (Worstell et al., 1984; Klein et al., 2001). In contrast, male-fertile germplasm is classified as either a maintainer of sterility (B lines) or a restorer of fertility (R-lines). The classification of any fertile line is determined by crossing it to a CMS male-sterile and evaluating the hybrid progeny. If the testcross progeny is male-sterile or male-fertile, the tested germplasm is a B-line or R-line, respectively.

Breeding programs develop new B-lines for which an A-line must also be developed. New A-lines are produced through backcrossing (typically 5 to 6

backcrosses) using the method described originally by Stevens and Holland (1954). Compared to pollinator (R-line) development, sterilization adds an additional 3-4 years and greater expense to the development of new seed parents. Methods to evaluate these lines in hybrid combinations prior to sterilization would be valuable to minimize the number of lines being sterilized. Whereas the current system for A-line production requires CMS introduction prior to testing. A chemical gametocide could reduce the size of the sterilization block and allow the redistribution of resources to other projects.

Following sterilization and release of a respective line, seed increase is needed prior to commercial hybrid seed production. To perform a seed increase, the male sterile A-line is crossed with its paired B-line. To increase the R-line, the plants are self-pollinated, because they possess normal cytoplasm and R*f* genes. Following seed increase, hybrid seed is produced by pollinating the A-line with an R-line. The hybrid seed is then grown commercially and is fully fertile due to restoration of fertility to the hybrid.

2.2 Genetic Male Sterility

Genetic male sterility was first discovered by Karper in 1929 (Karper, 1936) in sudangrass. In 1935, a Texas Blackhull kafir plant with abnormal sterile anthers was found at the Texas Agricultural Experiment Station in Chillicothe, Texas (Stevens, 1937). Crosses were made onto the abnormal Blackhull kafir plant, and the F_1 generation progeny were self-pollinated, resulting in F_2 generation progeny that segregated in a 3:1 phenotypic ratio for

male fertility, indicating male sterility occurs when male sterility genes are recessive. Since those initial reports, numerous types of GMS have been described and characterized (Rooney, 2004).

There are many ways GMS can be used in population improvement. The common scheme is a true cyclical population improvement, better known as recurrent selection, using the sterile plants as female parents in specific crosses or open pollination, much like the process used in maize (Doggett and Eberhart, 1968).

Genetic male sterility has been useful for producing large backcross populations in sorghum introgression programs. To develop such populations, an R-line is crossed onto a sterile plant containing the recessive ms_3 male sterility gene. The resulting F_1 is self-pollinated and its F_2 seed grown out. The same R-line is then crossed onto the sterile F_2 plants most resembling the R-line. Generally, five back crosses are conducted in the same manner, then fertile individuals are crossed onto sterile individuals in the same row for two generations, producing a population that is segregating in a 1:1 ratio for GMS (Jordan et al., 2011).

Others have utilized GMS to produce randomly mating synthetic populations in sorghum. Zavala-Garcia et al. (1992) utilized two such populations, TP24D and KP9B, to evaluate the efficiency of selection for stress tolerance. In order to produce the TP24D population, they allowed random mating, then selected the plants that possess improved yield. Those individuals were then subjected to 3 generations of random mating, and then

200 S_1 families were derived. To produce the KP9B population, they crossed IAP2B, which is a GMS population, to 22 elite inbreds. The crosses were allowed to random mate with another population, YE Kafir, and then the seed were bulked. These GMS populations have also been utilized for introgression of unadapted germplasm (Menkir et al., 1994), and for evaluating the potential gain of selection for protein digestibility in sorghum (Bramel-Cox et al., 1990).

While GMS has many uses beyond those mentioned previously, use of GMS in breeding also entails several drawbacks. A key one is GMS is available in only a very limited number of genetic backgrounds. Another is that the sterility factor must be eliminated from any finished line.

2.3 Fertile x Fertile Crosses

In making breeding crosses, sorghum breeders typically use male-fertile lines to ensure the derived lines are fully male fertile (the exception is genetic male sterility systems). In this situation, one of the parents must be rendered male-sterile to some level to facilitate directed crossing. Several methods have been used to emasculate sorghum, and these include i) hot water, ii) plastic bag, and iii) hand emasculation.

Hot water emasculation was developed by Stevens & Quinby (1933). A plastic or rubber sleeve is placed over a panicle just prior to flowering, and then the panicle is immersed in water at 42-48°C for 10 minutes. After the panicle is removed from the water bath, a paper bag is placed over the inflorescence for 3-4 days and then controlled pollinations are made. This approach, when adjusted for genotype and environmental conditions, is

effective at sterilizing whole panicles. However, it is also difficult to deploy in the field, and the level of sterility is subject to both genotypic and environmental variation.

Plastic bag emasculation is easier to deploy in the field than hot water emasculation. In this method, plants that have initiated flowering, are used as females. The portion of the panicle that has flowered is removed, leaving about 3-5 cm of florets which will flower in the next 2-3 days. The remaining panicle is then covered with a plastic bag, and a weatherproof pollination bag. After 2-3 days the bags are removed, and because the plastic bag holds water, anthers that have exerted do not dehisce pollen. When the bags are removed, many of these anthers are also removed by tapping the panicle, then exogenous pollen is poured over the panicle (Schertz and Clark, 1967). Not all of the seed produced are hybrids; some self-pollinations occur but the hybrids are relatively easy to identify when the seed is planted. This method is simple and requires less precision, but it does produce a limited number of hybrid seed that are often mixed with some selfed seed.

Hand emasculation is only used when a small number of crosses and seed from those crosses are needed. It requires the anthers be coaxed out of the floret by inserting a fine pointed instrument or sharp pencil into the floret the day before anthesis, typically within 3 cm below the anthesis line (Rooney and Smith, 2000). It is important that all utensils and the panicle be rinsed and clean of pollen to prevent contamination. If these precautions are observed, the seeds that set are most certainly hybrid. However, only a limited number

of seed are produced, and hand emasculation is laborious, requiring skills derived from practice in the process.

2.4 Doubled Haploid Breeding Schemes

Doubled haploid breeding systems have become highly desirable for developing homozygous lines that can serve as parents of commercial hybrids (Forster and Thomas, 2005). They provide a faster pipeline for developing inbred lines, generally 2-4 years instead of 6 or more, by eliminating generations of inbreeding. While haploid induction removes the opportunities to select during the early generations, it increases selection efficiency because it is more efficient to select in homozygous, rather than heterozygous lines, due to the preservation of high linkage disequilibrium from the F_1 (Baenziger, 1996). One could also have a viewpoint such that selection among homozygous lines provides 100% control over the gametes that will be subsequently used when making the next cross. Whereas, selecting among heterozygous lines provides only 50% control over the genetic contribution provided to the next generation by the selected lines. A DH breeding scheme was outlined by Rober et al. (2005) for maize, which includes i) making a breeding cross, ii) cross the F_1 as a female parent with an "inducer" pollinator, iii) identify haploids and double the chromosome number to produce a diploid, iv) self-pollinate to increase seed, and v) make testcrosses and evaluate in yield trials. The remaining steps are similar to a conventional pedigreed hybrid breeding program.

If inducer lines can be identified in sorghum, it is logical to envision that a similar scheme could be deployed. However, sorghum forms perfect flowers, whereas, maize does not, so large-scale cross-pollination of sorghum F_1 hybrids (Step ii) would require that the fully fertile F_1 hybrids be at least temporally sterilized in order to complete step ii) as described by Rober et al. (2005). Neither CMS nor recessive GMS would suffice as male-sterilizing factors because all haploid/double-haploid progeny would be without possibility of fertility recovery. However, GMS could be used if the male-fertility were conferred through dominant sporophytic gene action, but introduction of the dominant male sterility would need to be through the female parent of the F_1 hybrid. Therefore, all of the maternal haploids and subsequent doubled lines would have the cytoplasmic genotype of the male - sterile line, and half of the derived offspring would also be genetically male-sterile, too, unless eliminated by some sort of artificial selection. The increases in throughput would likely be high and vastly offset functional loss of half of the products due to homozygosity for the dominant male-sterility gene. A survey of the literature did not reveal any reports of a dominant gene for sporophytically determined male sterility in sorghum, but they have been reported in other crops. They certainly could be created by genetic engineering methods, such as RNAi and CRISPR, and very likely also could be induced by chemical mutagenesis, as well. Hand emasculation would also be unsatisfactory for large-scale haploid production, because sorghum floret emasculation is time-consuming. Moreover, the frequency of haploids is

usually less than 10% in sorghum (Schertz, 1963), so the required numbers of emasculations would be excessively expensive. Given the considerations above for sorghum, methods that could be applied to render fertile lines temporarily sterile e.g. a chemical gametocide, seem essential for the deployment of a DH system on a large scale, and for implementing a breeding system based on doubled haploids

2.5 Chemical Gametocides

An effective chemical gametocide according to Parodi and Gaju (2009) should induce male sterility that is easily identified without the reduction of female fertility and progeny vigor. Also, phytotoxic effects of the treated plants should not be evident, and they should be effective in all genotypes, across a wide range of environments.

The search for effective chemical gametocides is not new. In 1949, Naylor (1950) evaluated maleic hydrazide on several plant species and noted a lack of flowering in some, whereas male sterility occurred in some others. Moore (1950) also reported male sterility in maize and several other species of plants that were treated with maleic hydrazide at the floral bud stage or prior to it. The first studies using a chemical to induce male sterility with the intent of producing hybrid plants occurred from 1955 through 1957 as a cooperative project on cotton, involving Texas A&M University and the University of California, Riverside (Eaton, 1957). In 1957, the use of gibberellin for the induction of male sterility in maize was studied at Michigan State University, resulting in some instances of male sterility (Nelson, 1958). It was observed in

The Netherlands that an herbicide FW-450, 2,3-dichloroisobutyricacid, caused moderate to high levels of male sterility in red clover (*Trifolium pretense* L.), white clover (*Trifolium repens* L.), and beets (*Beta* L.). It was also suggested that the level of sterility achieved by FW-450 could be enough for large-scale testing of varieties prior to introduction of CMS (Wit, 1960). Applying the chemical, Ethrel (active ingredient ethephon, i.e., 2-chloroethylphosphonic acid) to wheat (*Triticum aestivum* L.) prior to seed head emergence or during the early, mid, and late boot stages resulted in almost complete male sterility (Rowell and Miller, 1971). Metabolic processing of ethephon leads to the release of the plant hormone ethylene. Several chemicals were evaluated in sugarbeet (*Beta vulgaris* L.) with varying results; however, none were deemed feasible as a chemical gametocide (Hecker et al., 1972).

Three chemicals, including Ethrel, were studied using hexaploid triticale (*x Triticosecale* Wittm. ex A. camus), and they produced unsatisfactory levels of male sterility (Sapra et al., 1973). The gametocides RH-2956 and RH-532 produced satisfactory levels of male sterility for making breeding crosses in wheat. However, low natural outcrossing rates were observed; thus, indicating they are not useful for commercial hybrid development (Jan et al., 1976). Use of Ethephon as a chemical gametocide in tef (*Eragrostis tef* (Zuccagni) Trotter) resulted in male sterility, but practical ramifications in tef were muted by desiccation of the female gametes prior to fertilization in Ethephon-treated plants, as was also observed after hand emasculation (Berhe and Miller, 1978).

Additional studies were conducted throughout the 1980s, '90s, and early 2000s with varying results. In wheat, the gametocide clofencet, a systemic chemical that is transported from the leaves to flowers, nearly meets the previous criteria for an effective gametocide. A genotypic effect was observed. It was recommended that doses be increased accordingly to overcome this effect and achieve a satisfactory level of male sterility greater than 95%, prospectively sufficing for commercial hybrid seed production (Parodi and Gaju, 2009).

Along with 24 other systemic chemicals, ethyl 4-fluorooxanilate (E4FO) was evaluated as a chemical gametocide in 29 diverse genotypes of wheat. The compound E4FO was the most effective (Chakraborty and Devakumar, 2006). Small negative impacts on agronomic traits such as plant height and spikelet number were noticed but female fertility was reduced by 4 percent. A single application was effective at the premeiotic stage, resulting in 99% male sterility.

Twenty chemicals were evaluated by Ali et al. (1999) using rice (*Oryza sativa* L.). Of the compounds studied, E4FO produced 91.5% male sterility which was one of the best performers. However, the carrier solvents butanol and acetophenone also caused relatively high male and female sterility. Thus, it was recommended that different carrier solutions be evaluated to find a combination that does not harm female fertility and improves plant absorption, resulting in a higher rate of male sterility.

In several vegetable species, applications of maleic hydrazide resulted in male sterility in a majority of them without detrimental effect on female fertility (Saimbhi and Brar, 1977). The chemical, Mendok (sodium 2,3-dichloroiso-butyrate), also known as FW-450 (Rustagi and Mohan, 1971), was evaluated in numerous vegetable species and complete male sterility was the result in most cases. A negative characteristic of Mendok is nearly all treatments resulted in high female sterility with lower fruit set and yield (Saimbhi and Brar, 1977).

In maize, applications of TFMSA resulted in complete male sterility while not affecting the female gametes or other plant functions (Loussaert, 2004). Laussaert (2004) also believed TFMSA to disrupt the transport of proline into the anthers, causing male sterility.

Trifluoromethanesulfonamide is unique within its class because it is water-soluble and is the most acidic sulfonamide. When the solution is at pH 7, it will remain stable for at least a month (Maren and Conroy, 1993). According to the safety data sheet, care should be taken to avoid contact with the skin, eyes and avoid inhalation. Use of gloves, eye protection, long sleeves and a dust mask is recommended when handling TFMSA. The LD_{50} in rabbits is 20 mg/kg when in a solution of pH 7. In comparison, when in a solution of pH 3.4 (TFMSA's pH in its natural unionized form) the LD_{50} is 4 mg/kg in rabbits (Maren and Conroy, 1993). Previously, TFMSA has been patented for use as a glaucoma eye drop (Kvam, 1989), in addition to many of its derivatives being used in

pharmaceuticals and as agricultural pesticides (Shainyan and Tolstikova, 2013).

Given the close phylogenetic relationship between maize and sorghum, Hodnett and Rooney (2017) evaluated TFMSA on sorghum under greenhouse conditions and observed male sterility, full female fertility and no obvious phytotoxic effects to the plant (Hodnett and Rooney, 2017). Also in sorghum, applications of E4FO and Ethrel have been evaluated. Pollen sterility ranged from 35-100%, depending on the dosage applied. However, as dosage increased, female fertility decreased, and other phytotoxic effects were observed (Amelework et al., 2016).

2.6 Proline Accumulation in Anthers

Pollen of some grasses contains 17 free amino acids and proline is the most abundant, representing 1.65% of the dry weight of perennial rye grass (*Lolium perenne* L.) pollen (Bathurst, 1954). In maize and sorghum, pollen abortion in cytoplasmic male-sterile lines appeared to be caused by low proline levels (Duvick, 1965; Kern and Atkins, 1972). It is also believed that certain storage proteins and enzymes crucial for pollen development require proline for their synthesis (Hong et al., 1982).

Proline is also associated with many stress mechanisms in plants, particularly for salt, water, and temperature stresses. Lansac et al. (1996) observed that proline levels increased to nearly 5% of the fresh weight of the anthers in grain sorghum plants grown in a cold environment (20/10˚ C day/night), as compared to a warm environment (27/22˚ C day/night).

Kern and Atkins (1972) evaluated amino acid levels in anther tissue of cytoplasmic male-sterile sorghum cultivars '*Combine Kafir 60*' and '*Martin*' A-lines and their respective B and R-lines. In field grown male-sterile lines, a slight decrease in proline level was observed from the tetrad to microspore stage, while male-fertile lines had a moderate increase in proline. During the transition from microsporogenesis to mature pollen in the fertile lines, a large amount of proline was accumulated. Contrastingly, greenhouse plants had strikingly greater increases in proline levels during the tetrad to microspore stages. Proline content in fertile lines was 40-60 times higher in mature pollen than at the tetrad stage.

According to Szabados and Savoure (2010) proline is synthesized from glutamate by a three-step process controlled by three genes in arabidopsis (*Arabidopsis thaliana* L.). The process is initiated when glutamate is reduced to glutamate-semialdehyde (GSA)by the pyrroline-5-carboxylate synthetase (P5CS) enzyme. Two genes, P5CS1 and P5CS2 encode for this. GSA is then spontaneously converted to pyrroline-5-carboxylate, which is then reduced to proline by pyrroline-5-carboxylate reductase (P5CR) that is encoded by a single gene. These processes occur in the cytosol, while proline is broken down in the mitochondria (Szabados and Savoure, 2010). Funck et al. (2012), working with arabidopsis, tagged P5CS1 and P5CS2 with green fluorescent protein and found that P5CS1-GFP was detected in the pollen grains and P5CS2-GFP was observed in the vegetative parts of the anthers. This suggests that P5CS2 expression in sporophytic anther tissue is crucial for male fertility.

Collapsed anthers were the most common anomaly observed at the time of flowering in P5CS2 mutant plants. It thus appears that blocking proline synthesis or transport is the likely mode of action for some gametocides such as TFMSA.

3. APPLICATION OF THE CHEMICAL MALE GAMETOCIDE TRIFLUOROMETHANESULFONAMIDE DISRUPTS THE FREE AMINO ACID PROFILE IN SORGHUM

3.1 Introduction

In any plant tissue, free amino acids are present due to the persistent need to produce proteins for cell and tissue function. However, the relative ratios of these free amino acids can vary due to biotic/abiotic stresses, and the respective developmental phase of a given tissue (Rai, 2002; Martinelli et al., 2007; Less and Galili, 2008; Kern and Atkins, 1972).

The same trends are observed in pollen of grass species. Among free amino acids within pollen of several grass species, proline was the most abundant, comprising over 1.5% of the total weight of perennial rye grass (*Lolium perenne* L.) pollen (Bathurst, 1954). The abundance of proline within pollen is presumably due to its role in storage proteins and enzymes crucial for pollen development (Hong et al., 1982) but other roles are also hypothesized. Mattioli et al (2009) proposed that proline protects gametes in pollen from spontaneous desiccation. Chiang and Dandekar (1995) observed the highest levels of proline in tissue containing the least amount of water in arabidopsis. For example, reproductive tissue such as seeds, siliques, and florets showed the least amount of water and the highest levels of proline; whereas, vegetative tissue such as rosette leaves and roots had the highest levels of water and the lowest levels of proline. In the primary roots of maize, proline increases greatly through deposition when the water potential is low (Verslues and Sharp, 1999). Alternatively, proline could provide energy for the growing pollen

tube, because catabolism of one proline molecule produces 30 adenosine triphosphate (ATP) (Mattioli et al., 2009). In maize and sorghum CMS lines, pollen abortion has been associated with low proline levels (Duvick, 1965; Kern & Atkins, 1972).

Given the interest in the induction of temporal male sterility in crop improvement programs, an understanding of the free amino acid changes associated with male sterility would be useful in both the development of such systems and their subsequent improvement. In many self-pollinated species, most methods of producing temporal male sterility involve the application of chemical gametocides that disrupt the development of the male gamete.

As a male gametocide in maize, TFMSA reduced proline concentrations in anthers and increased proline concentrations in the glumes, leading to the hypothesis that proline transport into the anthers was interrupted (Laussaert, 2004). Considering the phylogenetic similarities between sorghum and maize, TFMSA was recently evaluated in sorghum, and a similar male sterile phenotype was observed (Hodnett and Rooney, 2017). Given the effectiveness of TFMSA in sorghum and its reported effects on amino acid concentrations in maize, the objective of this study was to determine if the free amino acid composition in sorghum anthers are affected by TFMSA and if these changes can be associated with the induction of male sterility.

3.2 Materials and Methods

3.2.1 Genotypes

The two genotypes A/BTx623 and RTx437 were used to determine if the effects of TFMSA on male sterility and free amino acid content would be

consistent across selected genotypes. A/BTx623 is an inbred line with subtropical adaptation and a pedigree of BTx3197/SC170-6-4 (Burow et al., 2011). This line produces grain with a white pericarp on semi-compact panicles, and it has been commonly used as a seed parent in hybrids and as a genomic resource (Sorghum reference genome (RefSeq Accession NZ_ABXC00000000.1). The second parental line, RTx437, produces semi-compact panicles with white pericarp grain and is used as a pollinator parent in the production of grain sorghum hybrids (Rooney et al., 2003).

Thirty-six plants of each genotype were grown in the greenhouse during the Spring of 2017. Each plant was grown in an 8-inch pot using Fafard® 52 growing media (Sun Gro Horticulture) and 3 tablespoons of Osmocote® Pro 19-5-8 (ICL Specialty Fertilizers) were incorporated into the growing media at the time of planting. An additional 3 tablespoons of Osmocote® were added at the 8-leaf stage of growth. Pots were watered as needed to avoid water stress.

3.2.2 Gametocide Application

A total of eight chemical treatment applications were evaluated in this study. The compound, TFMSA, was applied at four dosages; 0 mg (control), 2 mg, 6 mg, and 20 mg of TFMSA per plant) using two different carriers which were i) 5% glycerol, 0.25% Tween 20, and deionized water, and ii) 1% Destiny® methylated soy oil (Winfield Solutions), 0.7% R-11® spreader activator (Wilbur Ellis), and deionized water. For each treatment, a micropipette was used to place the solution onto a leaf on each side of the

plant, which was then spread with a small paintbrush. Each combination was replicated three times; a single plant was considered an experimental unit.

3.2.3 Sample Preparation and Analysis

To assay amino acid profiles, rachis branches were harvested the day prior to anthesis from each panicle directly below the last spikelet with a floret that had undergone anthesis (sorghum flowers from the top of the panicle downward). To identify the precise area of the panicle to use, the physiological maturity in relation to flag leaf emergence was considered. For example, if the flag leaf emerged 55 days after planting (DAP), then upper rachis branches were sampled, but if the flag leaf emerged 59 DAP, then sampling occurred on the lower rachis branches. This approach assured all samples were harvested when the anthers were at a similar physiological growth stage.

Immediately after rachis branch removal, the anthers and glumes were excised from the florets and kept separate. The samples were dried in a forced-air oven for 24 hours at 55˚ C. After drying, the samples were frozen at -80˚ C until all samples were collected and processed. The anther and glume samples were removed from the freezer and ground using a FastPrep® 24 (MP Biomedical) Homogenizer, and then the entire sample was diluted in 250ul Milli-Q® water. The samples were centrifuged at 5,100 rpm for 5 min at 24˚ C to pelletize insoluble debris. Following centrifugation, ≈ 200ul of supernatant of each sample was transferred into a new micro-centrifuge tube and used for

quantification of free amino acids by high-pressure liquid chromatography (HPLC).

The free amino acid assay consisted of 1 nmole internal standard – Norvaline for primary amino acids, and Sarcosine for secondary amino acids - in 20 ul Milli-Q® water. Each sample consisted of 20 ul 1nmole internal standard, 20 ul of the amino acid sample, and 20 ul of 0.4 N borate buffer. Samples were run in duplicate using fluorescent detection on a G1321B fluorometric detector (Agilent Technologies, Inc.), with excitation/emission levels of 340/450 nm for the primary amino acids and 266/305 nm for the secondary amino acids. Analysis was conducted using ChemStation® software. Calibration of the assay was done using a standard (Agilent #5061-3331) and it underwent the same treatment as the samples. A blank sample of Milli-Q® water, which underwent the same procedure as the amino acid samples, was included as a control for analysis.

3.2.4 Phenotyping

During anthesis, fertility of each panicle was evaluated daily. Fertility was rated by visually observing the anthers including the presence or absence of pollen shed and later, seed set on the panicle. If the anthers were pale, shrunken with no evidence of pollen shed, the plant was classified as sterile for that day. If a panicle produced sterile anthers throughout anthesis, the panicle was fully sterile.

3.2.5 Statistical Analysis

Fold changes for 19 free amino acids were calculated on an individual plant basis, using genotype, dose, and carrier as blocking terms. Positive fold changes were calculated by dividing the mean percent nmol composition for each dose by the mean percent nmol composition of the 0 mg control. Whereas, negative fold changes were calculated by dividing the mean percent nmol composition of the 0mg control by each respective dosage. JMP Pro 13.0.0 software (SAS Institute, Cary, NC, USA) was then used to conduct Single Factor ANOVAs comparing the means at alpha = 0.05.

3.3 Results and Discussion

3.3.1 Panicle Phenotypes and Amino Acid Profiles of Sterile Plants

Panicles of the control group (0 mg TFMSA) were fully fertile. A majority of the panicles treated with TFMSA were completely sterile (27/33). The remainder of the treated panicles were partially sterile (6/33). These panicles were initially sterile, but as flowering progressed, the remainder of the florets were fertile.

These plants are subsequently referred to as revertants. The TFMSA dosage appeared to influence the level of sterility; all of the revertant panicles arose from the 2mg dosage class

while all panicles in the 6mg and 20mg dosage classes remained male-sterile. Revertant panicles were observed in both genotypes, but the frequency of reversion was non-significantly more frequent in BTx623 (5 revertants) than RTx437 (1 revertant).

No major differences in fold changes in amino acid content were observed for either genotype, dose or carrier, but the tissue type was significant with major shifts in relative proportions of specific amino acids between anther and glume tissue. In this situation, the shifts occurred primarily in the anther tissue (Table 1) with minimal changes occurring in the glume tissue (Table 2). Based on these findings, the focus will be on relative fold changes within anther tissue.

Table 1. Fold changes of the percent composition of free amino acids in anthers for nine amino acids with significant shifts in concentrations following application of TFMSA. Differing letters within rows indicate statistical significance at $p<0.05$.

Dosage	Asparagine	Arginine	Glutamate	Glutamine	Histidine	Phenylalanine	Proline	Tryptophan	Tyrosine
2 mg	18.0 a	-2.0 c	4.4 b	3.2 b	5.3 b	3.4 b	-8.6 d	5.7 b	-1.7c
6 mg	24.5 a	-3.1 f	2.7 bc	3.2 bcd	4.8 bc	3.2 bcd	-18.6 g	5.4 b	-2.0 ef
20 mg	19.0 a	-2.7 d	6.6 b	2.9 bc	4.8 bc	3.4 bc	-14.0 e	7.0 b	-2.5 d
2 mg reverted	25.3 a	-2.4 c	3.3 bc	4.1 b	3.0 bc	2.3 bc	-12.3 d	5.0 b	-2.6 c

Table 2. Fold changes of the percent composition of free amino acids in glumes for nine amino acids with significant shifts in concentrations following application of TFMSA. Differing letters within rows indicate statistical significance at $p<0.05$.

Dosage	Asparagine	Arginine	Glutamate	Glutamine	Histidine	Phenylalanine	Proline	Tryptophan	Tyrosine
2 mg	1.1 a	-1.4 bc	0.7 a	-1.7 c	0.9 a	0.8 a	-0.7 ab	0.9 a	0.7 a
6 mg	-0.1 ab	-1.2 c	0 ab	-1.2 c	0.9 ab	0.8 a	-0.1 b	0 b	0.7 ab
20 mg	0.7 a	-1.3 c	0.4 a	-1.7 c	0.8 a	0.7 ab	-0.8 bc	0.5 a	0.6 a
2 mg reverted	0.5 a	-1.7 a	-1.2 a	-1.4 a	-1.5 a	-0.5 a	-0.6 a	-0.7 a	-0.6 a

Because amino acids can be grouped into families based on chemical structure and similarity, it is likely that TFMSA may affect some biosynthetic pathways more than others. In this study, the largest shifts in amino acid content occurred in the glutamate family which consists of glutamate, glutamine, arginine, and proline. However, shifts of interest also occurred in asparagine of the aspartate family, histidine, and the aromatic family which consists of tyrosine, tryptophan, and phenylalanine. It is important to note that a secondary peak eluted quite close to tryptophan in some individuals, making a precise quantification difficult; this likely imparts some variation to the mean fold changes reported. Other amino acid families were affected less.

In the glutamate family, both glutamate and glutamine levels increased 3-6x and 3x, respectively, following treatment with TFMSA (Table 1). Alternatively, arginine and proline content decreased by factors of 3x and 10-18x, respectively (Table 1). If some of these amino acids changed, then shifts of the others within the family would be expected given that proline is synthesized from glutamate in a three-step process in plants. Synthesis is initiated when glutamate is reduced to GSA by P5CS. In arabidopsis and sorghum, there are two P5CS genes, P5CS1 and P5CS2 (Szabados and Savoure, 2010; Su et al., 2011). Following the reduction of glutamate to GSA, spontaneous conversion to pyrroline-5-carboxylate occurs, followed by further reduction catalyzed by P5CR to proline (Szabados and Savoure, 2010). The proline-glutamate shift could indicate P5CS function is being disrupted, given its location in the proline biosynthetic pathway. Another key indicator for the disruption of P5CS is

that P5CS2 function has produced male-sterile plants possessing shrunken anthers (Funck et al., 2012).

While P5CS is a sensible target of TFMSA, another possibility that should be considered is the targeting of carbonic anhydrases (CAs). Sulfonamides, specifically, TFMSA have been shown to be inhibitors of CAs in plant and animal species (Maren and Conroy, 1993; Maren and Sanyal, 1983). Moreover, expression of α and γ CAs have been found solely in anther tissue and pollen grains of sorghum and maize (DiMario et al., 2017); whereas, βCAs have been found primarily in photosynthetic tissues of sorghum and maize (Dimario et al., 2017). However, in arabidopsis, βCAs have been associated with tapetal cell development (Huang et al., 2017). Huang et al. (2017) have also associated a decrease in proline levels with disruption of these βCAs. While it is purely speculative that TFMSA is targeting CAs in sorghum and maize, the drastic decrease of proline in anther tissue and the lack of shifts within glume tissue (Tables 1 and 2) could be indicative of either α or γ CA being specifically targeted.

A limited amount of proline synthesis could also be occurring through a secondary pathway utilizing arginine as a starting substrate, this is unlikely because of the large decrease in arginine and concomitant increase in glutamate. If proline production had shifted to this pathway, and because arginine is also synthesized from glutamate (Slocum, 2005), glutamate would be expected to decrease or at least remain stable. Others have observed low proline levels in CMS lines as compared to their fertile counter parts in many species including sorghum, wheat, and peppers (Kern and Atkins, 1969; Rai and Stoskopf, 1974; Fang et al., 2016). Cumulatively, these results,

at a minimum, show a correlation between negative shifts in proline content and male sterility induction using TFMSA.

Asparagine content in the anthers increased >20x following application of TFMSA (Table 1). This drastic shift in asparagine is likely the result of either an increase in glutamate, or the application of fertilizer at the 8-leaf stage, given both glutamate and ammonia can act as a starting substrate for asparagine (Gaufichon et al., 2010). The increase of glutamate is the likely reason for this shift, given asparagine levels increased 1x in revertant plants; whereas, glutamate levels decreased 1x in revertants, in comparison to their sterile 2 mg counterparts. In this case, asparagine is likely acting as a nitrogen transporter to the anthers; because both asparagine and arginine act as nitrogen transporters. Asparagine is commonly the primary nitrogen transporter in plant vascular tissue (Gaufichon et al., 2010).

In anther tissues, concentrations of histidine increased 4x following application of TFMSA (Table 1). The biosynthetic pathway of histidine is unique because it is larger than most amino acid pathways, requiring 11 steps, and shares no known metabolic links to other amino acid biosynthetic pathways (Stepansky and Leustek, 2006). Therefore, the positive shift observed is likely a result of greater demand, instead of an increase of starting substrate or precursor. Blockage of the active sites of CAs could be a possible reason for the positive increase, because it has been observed that both γ and β CAs have histidine molecules located at the active site (DiMario et al., 2017; Maren and Conroy, 1993). Furtermore, free histidine is thought of to be a chelator of metals, and key in resistance to nickel toxicity; plants that uptake abnormally high quantities of nickel have also been found to have abnormally high

quantities of histidine (Kerkeb and Kramer, 2003). Given this, the increase in free histidine following application of TFMSA could be an attempt to bind and remove the TFMSA from the plant's vascular system. This could be key to why fertile revertants occurred in some instances, assuming histidine is successful in binding to TFMSA. Histidine has many other functions, which include incorporation into proteins, and acting as precursors for important compounds relative to reproductive function and vegetative growth.

Similar to histidine, phenylalanine, tyrosine, and tryptophan play important roles as precursors for many hormones and compounds used during reproductive development, vegetative growth, and times of stress (Tzin and Galili, 2010). These amino acids shifted following TFMSA application, with tyrosine decreasing 2x, phenylalanine increasing 3x, and tryptophan increasing 5-7x (Table 1). Expression of some genes regulating tryptophan biosynthesis are known to increase in response to wounding and pathogen attack (Maeda and Dudareva, 2012). Therefore, the increase of tryptophan could be in response to the TFMSA. Whereas, the fold changes of phenylalanine and tyrosine could indicate partitioning of carbon through an alternative pathway for phenylalanine biosynthesis, that utilizes phenylpyruvate, instead of arogenate, as a precursor. While this is purely speculative, the fold change trends indicate there could be some form of dependence of phenylalanine to tyrosine. Yoo et al. (2013) observed in petunia (*Petunia hybrida* (*Petunia* x *atkinsiana* D. Don ex Loudon) that down-regulating phenylpyruvate aminotransferase (PPY) using RNAi, resulted in decreased levels of phenylalanine. Whereas upregulation of PPY resulted in increased levels of phenylalanine. Biochemical characterization suggested that

phenylalanine was preferentially synthesized from phenylpyruvate over arogenate when stress factors reduce the ability to use the main arogenate pathway, and tyrosine was the preferred amino donor during transamination. In the arogenate pathway, the enzymes involved with phenylalanine biosynthesis are under feedback inhibition, thus controlling phenylalanine levels. Contrastingly, the phenylpyruvate pathway is much less regulated, allowing greater production of phenylalanine, with tyrosine being the only limiting factor (Yoo et al., 2013). Of further relevance, the phenylpyruvate pathway was observed in floral tissue. In plants where fertility reversion occurred, the respective shifts did not change for tyrosine and phenylalanine (Table 1). This indicates these shifts are not factoring into sterility induction or responding to the proline shift. However, they do occur in response to TFMSA application.

3.3.2 Amino Acid Profiles of Revertants vs. Fully Sterile

Of the six panicles that reverted to fertility at some point during anthesis, fold changes in free amino acid concentrations were, for the most part, similar to the panicles that remained fully sterile. The largest shift was in proline, which decreased 12x for the reverted 2 mg plants, compared to a decrease of 8x for the sterile 2 mg plants (Table 1). The fold change in glutamate increased (1x) in the reverted plants, compared to a 4x increase for fully sterile panicles in the 2 mg dosage treatment (Table 1). There was a 7x increase in asparagine concentrations indicating the level of asparagine is likely not dependent of changes in glutamate (Table 1). Finally, histidine changes were lower (3x) in the reverted plants compared to a fold change of 5x in the fully sterile plants of the same dosage (Table 1).

3.4 Conclusions

Concomitant with its dramatic effects on male fertility, TFMSA was found to alter the free amino acid pool within anthers on a relatively broad scale. The application of TFMSA significantly affected several amino acid pathways; specifically, the aspartate (higher in anther, but not in glume), aromatic (tyrosine, tryptophan, phenylalanine), and glutamate families of amino acids. Within the glutamate family (arginine, glutamate, glutamine, proline), the largest shift and greatest anther-specificity involved a decrease of proline in anthers. TMFSA strongly reduced the anther/glume ratio for tyrosine, but raised it for tryptophan, phenylalanine. Whereas TFMSA most strongly *increased* asparagine levels (overall and anther/glume ratio), it most strongly *decreased* anther proline levels, which parallels reports on reduced proline levels in anthers of CMS lines in sorghum and other crops . Although, at least some CMS and GMS lines reported in the literature indicate proline to be important. The data here are concordant with hypothesized roles for high proline in anthers as prerequisite for male fertility, but do not prove a cause-effect relationship. Similarly, the concordance of sterility and reduced proline effects is concordant with the hypothesis that the reduction of anther proline is the mechanism by which TFMSA induces male sterility but it does not prove it. Revertants to fertility after 2 mg treatments exhibited increased histidine, but the observations on fertility reversion were based on fewer samples and so inferences on associated trends must be considered tentative. However, the proline and histidine levels in both sterile and revertant plants do lead to the possibility of α and specifically γ CAs being targeted by TFMSA in maize and sorghum.

4. EFFICACY OF THE CHEMICAL MALE GAMETOCIDE TRIFLUOROMETHANESULFONAMIDE IN GRAIN SORGHUM GROWN UNDER FIELD CONDITIONS

4.1 Introduction

Sorghum is a cereal grain crop grown from the tropics to temperate regions for both grain and forage production. Sorghum has also been utilized as an energy crop (Rooney et al., 2007).

Sorghum is a self-pollinated species grown as either a pure line variety or a hybrid crop, primarily depending on the production economics of the production region. In the case of hybrids, seed production requires an economic means of crossing to produce hybrid seed. However, for breeding and improvement purposes, cross-pollination methods are essential, and perhaps becoming even more important as new breeding technologies are developed.

While numerous crossing methodologies have been developed, each has strengths and weaknesses. For example, the CMS system is excellent for commercial hybrid seed production but cannot be used for breeding purposes. Conversely, hand-emasculation is excellent for making a specific breeding cross, but the amount of seed produced is minimal and cannot be scaled.

Recently, there has been great interest in deploying a DH breeding system in sorghum because of the great increase in efficiency it has provided to maize and barley breeding programs. The DH breeding system provides more efficient populations for genomic selection than in a conventional selection

and inbreeding system due to the production of homozygous lines in the early generations (Bernardo and Yu, 2007). While it seems that DH technology is possible in sorghum, none of the current hybridization systems in sorghum is genetically suitable, and practically robust enough to enable effective large-scale deployment of a DH technology. CMS and conventional recessive GMS would be genetically unsuitable. Using CMS, the logistics of fertility restoration would be impossible. Using GMS, the chromosome-doubled maternal haploid progeny would be homozygous recessive for the GMS-conferring gene and thus phenotypically male-sterile, precluding sexual maintenance and increase. Were a dominant GMS trait available, it would be usable, albeit plagued by male-sterility of 50% of the chromosome-doubled maternal haploid progeny, unless tweaked genetically or procedurally to preclude their formation and/or recovery. Also, mechanical methods of temporal male sterility such as hand emasculation, hot water emasculation, and plastic bag emasculation are not suitable. All of these methods yield too low a quantity of hybrid seed and only the plastic bag emasculation system would allow the large quantities of cross-pollinations necessary for a DH system. The requirement for large numbers of cross pollinations in sorghum will be inversely related to the frequency of haploids and chromosome doubling, i.e., the frequency of doubled-haploid derivatives. Haploids in sorghum have been reported to occur at a rate of less than 1% (Schertz, 1963). If the typical F1 hybrid panicle averages 800 seed and the rate of haploid recovery is 1%, then about 8 haploids would be obtained per cross-pollinated

panicle. It seems likely that "haploid inducers" can be found or created in sorghum mutants, as in a number of other crops and model organisms (Ravi and Chan, 2010). For example, maize haploids now typically occur at a frequency greater than 10% in a contemporary DH system (Wu et al., 2017). Thus, there is a need to develop a sterility induction system in sorghum that is compatible with DH technology, so breeding work can be conducted to increase sorghum's haploid induction frequency.

A chemical male gametocide could circumvent the issues presented by the current hybridization methods. According to Parodi and Gaju (2009) an effective chemical gametocide should induce easily recognizable male sterility without reducing female fertility or vigor of the progeny. It should also not produce any phytotoxic effects in the treated plants and be effective in all genotypes across a wide range of environments. While having easily recognizable male sterility is important in some situations. In sorghum, if using a gametocide for haploid development or testing of female lines this is not a necessity if both endosperm and embryo markers are present, allowing for selection of hybrids and tossing of inbred individuals.

Several compounds have been evaluated using sorghum. Applications of E4FO and Ethrel produced male sterility ranging from 35-100%. However, as dosage increased, sterility in the female gametes and other detrimental phytotoxic effects to the plants increased (Amelework et al., 2016).

Use of TFMSA as a gametocide was first reported in maize and showed great promise (Loussaert, 2004). Therefore, due to the similarities between

maize and sorghum, its efficacy was tested in the greenhouse using sorghum (Hodnett and Rooney, 2017). It was determined that a single application of 2 mg/200 ul TFMSA solution was required to achieve complete panicle sterility (Hodnett and Rooney, 2017). This concentration was 1000x higher than the minimum efficacy level in maize. Even with this increase, and much like Loussaert (2004) observed in maize, there were no phytotoxic effects to the plant, or reduction in female gamete fertility (Hodnett and Rooney, 2017). Therefore, the objective of this study is to determine the effectiveness of multiple dosages and applications of TFMSA and their ability to induce pollen sterility in multiple genotypes grown in the field.

4.2 Materials and Methods

4.2.1 Environments

Field studies of TFMSA were conducted at the Texas A&M Agrilife Research Farm near College Station, Texas. The plots were grown under two environments, defined by different planting dates and fields, at College Station Texas using a completely randomized design. The planting dates were 23 March and 6 April, the two fields were approximately 1 km apart. Each genotype was grown in adjacent production rows that were planted on the same day using agronomic practices standard for growing production grain sorghum.

4.2.2 Dosages and Number of Applications

Trifluoromethanesulfonamide (TFMSA) was applied using four different dosages; 10 mg, 15 mg, 20 mg, and 30 mg per plant. In all cases, the dosage

was administered in a single application. To evaluate the effect of multiple applications, the 10 mg dose was also applied to specific plants at weekly intervals over 1, 2 and 3 applications (Fig. 1). These applications produced a 10 mg, 20 mg, and 30 mg dosage over a period of 8-9 days. A 15 mg dose was applied both once and twice to produce accumulated dosages of 15 and 30 mg, respectively (Fig. 1).

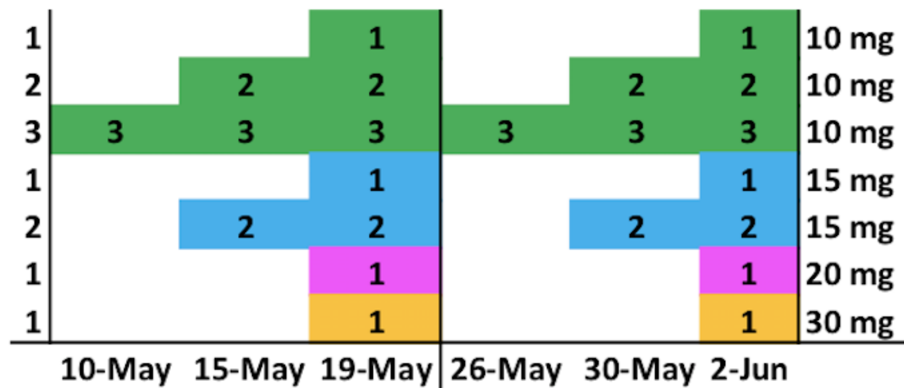

Figure 1. Diagram of the combination of dose applied and number of applications. The number of applications is depicted on the left-hand Y-axis and numerically within each cell. The dose applied is depicted on the right-hand Y-axis and as color fill of each cell: 10 mg (green), 15 mg (blue), 20 mg (pink) and 30 mg (orange). The X-axis indicates the dates of applications.

4.2.3 Genotypes

These dosages and combinations of applications were applied to three grain sorghum inbred lines: RTx437, BTxARG-1, and BTx2928. These three lines have the following breeding history. RTx437 was developed and released by Texas A&M Agrilife Research in 1998 (Rooney et al., 2003). It

produces semi-compact panicles with white seed on tan plants that fully restore fertility in the A1 cytoplasm. It is resistant to head smut and both systemic and local lesion downy mildew. RTx437 produces high-yielding grain sorghum hybrids adapted primarily to South Texas and Northeastern Mexico. A/BTxARG-1 is a seed parent inbred line released in 1990 (Miller et al., 1992). It is a temperately adapted, white-seeded, waxy endosperm seed parent line. It produces hybrids short in stature with good yield, general disease resistance, and drought resistance. A/BTx2928 is a seed-parent sorghum germplasm released in 2001 (Rooney, 2003). It is a short-statured tan plant with white seed. When used as a female parent in hybrid combination, ATx2928 produces high-yielding hybrids with high agronomic desirability and the potential to produce food grade grain, depending on the male parent used.

4.2.4 Carrier Solution and Method of Application

The carrier solution for TFMSA consisted of 1% Destiny® methylated soy oil (Winfield Solutions), 0.7% R-11® spreader activator (Wilbur Ellis), and deionized water. Solution was mixed no more than 24 hours prior to treatment at a base concentration of 100 mg/ml TFMSA. Then a varying volume, dependent of the desired dosage was applied to each plant. A micropipette was used to apply the solution to the upper surface of a leaf blade on each side of the plant, and was spread with a small paintbrush.

The plants used for the TFMSA experiments were planted on 23 March, 2017 and 6 April, 2017. TFMSA was applied to the plants in the early planted environment on

10 May, 15 May and 19 May in the late morning (Fig. 1). The single application of all dosages and final applications of the multiple application portion were applied on 19 May. In all cases, the chemical was applied after the morning dew had evaporated from the leaves. In the late-planted environment, TFMSA was applied on 26 May, 30 May and 2 June (Fig. 1). The applications were made in the late morning on 26 May; however, on 30 May and 2 June, the applications were made in the mid-late afternoon. As can be seen in Figure 1, the final dosage of the multiple-application treatments and the single application of single-application treatments was applied on the last day of treatment. Within each environment, 15 plants were treated for each dosage and combination of application number, totaling 105 plants per genotype.

4.2.5 Data Collection and Phenotyping

The date of flag leaf emergence was recorded in Julian Days. Approximately one day prior to flowering, the panicle was covered with a weatherproof pollinating bag to prevent contamination by exogenous pollen. Approximately five days after bagging, two treated and bagged plants within each dosage and combination of application number were pollinated with either RTx430, RTx433, or RTx434 to confirm the fecundity of the seed parent, the viability of the progeny, and the frequency of self- and cross-pollinated seed from the cross. The remaining 13 treated panicles were phenotyped for male sterility by measuring seed set.

To estimate seed set in the 13 panicles, approximately 25 days following bagging the bags were removed, and the panicles evaluated for percent seed set by visual estimation. If a panicle set any number of seed, it was given a

binary rating of 0 to denote fertility, whereas, if no seed were present, a rating of 1 denoting sterility was given. Panicle length was measured in centimeters because this can potentially be an indicator of the amount of time required for the panicle to flower. Sorghum flowers from the top of the panicle downward over a period of several days.

To evaluate the progeny from the testcrossed panicles, each testcrossed panicle was threshed individually. Seed samples from each panicle were evaluated using dominant phenotypic markers from the pollinator parent. The presence of that phenotype in the seed/plant was used to determine if that plant was the result of cross-pollination. For the testcrosses from the inbred lines BTx2928 and RTx437, the dominant phenotypic marker was plant pigmentation as both pollinator parents were pigmented and both seed parents were tan (non-pigmented). For both, seed were grown in the greenhouse in 72 well propagation trays. A tray of RTx437 (tan)and BTx623 (pigmented) were grown as checks. Approximately 4 weeks after planting, each plant was evaluated for presence of pigmentation. Plants that were pigmented resulted from cross-pollination; whereas, plants that were tan resulted from self-pollination.

For the testcrosses from BTxARG-1, the phenotypic marker was endosperm type. Since BTxArg-1 has a waxy endosperm and both pollinators had non-waxy endosperm, testcross seed were cracked and stained with I_2-KI, which stains non-waxy starch blackish blue and waxy starch red, which represented cross- and self-pollinations, respectively.

4.2.6 Backpack Sprayer Study

The efficacy of TFMSA applications using a backpack sprayer was evaluated using late-planted material. TFMSA treatments consisted of approximately 5 and 30 mg dosages, using the same carrier solution as described above, except with ¾ the quantity of Destiny® and R11® to prevent burning of the whorl. Each plot was subdivided into 0.9144 m sections and 100 ml of solution applied to each subsection. The sprayer was calibrated to spray 100 mL of solution consistent with each dosage at 15 PSI. Pollination bags were placed on the treated panicles approximately one day prior to anthesis. Testcrosses were also made with two plants within each sprayed section. The same procedures were used for acquiring phenotypic data; however, the first and last day of flag leaf emergence date for each subplot was recorded and the average was computed, in lieu of recording the flag leaf emergence date of each plant within the plot.

4.2.7 Statistical Analysis of Fertility/Sterility of Panicles

Statistical analysis for the testcross data were conducted using JMP PRO 13.0.0 (SAS Institute, Cary, NC, USA). For the hand applied TFMSA testcross data, the dependent variable, *Percent Self-Pollinations*, was fit to the linear regression model: $PERCENT\ SELF\ POLLINATIONS = Dosage + Genotype + Environment + Dosage * Genotype + Dosage * Environment + Genotype * Environment + Dosage * Genotype * Environment + Error$ where all effects were considered fixed. A second model was fit replacing *Dosage* with *Number of Applications*. Both models were then re-fit to the same data,

following exclusion of the controls 0 mg and 0 applications, respectively. Data were not of a normal distribution according to a Shapiro Wilk test, even after removing the control data. An arcsine transformation was conducted; however, data were still not from a normal distribution.

Testcross data from the sprayer applied TFMSA were analyzed in a similar fashion. The model developed was $PERCENT\ SELF\ POLLINATIONS = Dosage + Genotype + Dosage * Genotype + Error$ where all effects were considered fixed. The model was run again following removal of the 0 mg control. Data including the control were not of a normal distribution according to a Shapiro Wilk test; however, following removal of the control data, they were of a normal distribution.

For the non-testcross individuals, logistic regression was conducted using R *stats* package (R Core Team, 2017). Logistic regression is a method of regression using dichotomous response variables and either categorical or continuous explanatory variables (Peng et al., 2002). A basic logistic regression equation is depicted as $logit(Y) = natural\ \log(odds) = \ln\left(\frac{\pi}{1-\pi}\right) = \alpha + \beta X$. Where the odds are calculated by dividing the probability (π) of achieving a “success” by one minus the probability of achieving a “success” (Peng et al., 2002); which in the instance of this study is complete male sterility. The Y intercept is denoted by α, and the regression coefficient(s) is/are depicted by βX (Peng et al., 2002); in some instances, the regression coefficients are referred to as beta coefficients. The beta coefficients can also be presented in the form of an odds ratio, which is a score that explains the degree of

association between an explanatory variable and the desired outcome. The odds ratio is calculated by raising base *e* to the power of each beta coefficient estimate.

By taking the natural log of the odds when calculating beta coefficients, the relationship is linear between the response variable and explanatory variables. Therefore, the null hypothesis for such a model says there is not a linear relationship among the explanatory variable(s) (Peng et al., 2002). The null hypothesis in logistic regression can be tested using a Likelihood Ratio Test, which uses an Analysis of Variance (ANOVA) to compare a full "alternative" model to a reduced "null" model. A deviance statistic is produced which can then be used in a Chi-square test, using the number of models tested as the degrees of freedom, to produce a p-value that describes whether one can reject the null hypothesis.

Each genotype was subset and fitted to the following logistic regression model at the 95% confidence level: $Phenotype = Dosage + Number\ of\ Applications + Date\ of\ Flag\ Leaf\ Emergence + Panicle\ Length$ where phenotype is a binary response variable.

A likelihood ratio test conducted to determine whether *Dosage* and *Number of Applications* have an overall statistical significance; thus, influencing the number of sterile panicles produced. The same procedure was taken for the analysis of the backpack sprayer study, with the exception of the model: $Phenotype = Dosage + Panicle\ Length + Avereage\ Flag\ Leaf\ Emergence\ Date$.

4.3 Results and Discussion

4.3.1 Linear Regression Analysis of Testcrosses

Within -hand-applied TFMSA testcrosses, dosage was the only main effect with significance in the dosage model (Table 3). None of the first- or second-order interactions were significant (Table 3). Similarly, in the model for number of applications, the main effect, number of applications was the only effect with significance (Table 4). Again, no first or second order interactions were significant (Table 4).

Table 3. Mean squares for dosage from the analysis of variance, considering dosage, genotype, environment, and their interactions of hand applied TFMSA, including the control dosage of 0 mg.

Source	Degrees of Freedom	Mean Square
Dosage	4	13336.01***
Genotype	2	35.99
Environment	1	1.25
Dosage*Genotype	8	24.95
Dosage*Environment	4	22.16
Genotype*Environment	2	14.03
Dosage*Genotype*Environment	8	28.9
Error	58	98.71

*** = $p<0.001$

Table 4. Mean squares for number of applications from the analysis of variance, considering number of applications, genotype, environment, and their interactions of hand applied TFMSA, including the control, 0 application of TFMSA.

Source	Degrees of Freedom	Mean Square
Number of Applications	3	17775.63***
Genotype	2	34.60
Environment	1	0.67
Number of Applications*Genotype	6	18.00
Number of Applications*Environment	3	11.74
Genotype*Environment	2	16.14
Number of Applications*Genotype*Environment	6	9.20
Error	64	96.13

*** = $p<0.001$

There was limited statistical significance in the hand-applied models, the only difference was between the control of 0 mg TFMSA and the dosages where TFMSA was applied (Table 5). These results indicate that application of TFMSA itself is most important for reducing the number of self-pollinations; however, once a dosage threshold for minimum efficacy is reached, number of applications and dosage are of minimal importance. Although statistical significance was minimal, some numeric differences did occur between dosages. Dosages of 10 and 15 mg produced essentially the same level of self-pollinations, while 20 and 30 mg produced fewer, with 30 mg producing the fewest (Table 5). BTxARG-1 produced the highest levels of self-pollinations, while RTx437 and BTx2928 produced essentially the same level of self-pollinations in both models (Table 5). There was little environmental influence, as both fields produced similar levels of self-pollinations in both models (Table 5). Similarly, when considering the number of applications used, the only statistical significance occurred between the control of 0 applications and any application

number of TFMSA (Table 6). Some numeric differences were also observed, where the percent self-pollinations decreased as the number of applications increased. Three applications produced the lowest percent self-pollinations, while a single and two applications produced essentially the same percentage (Table 6).

Table 5. Summary of least squares means for the percent self-pollinations of testcrosses, showing the effect of dosage, genotype, and environment. Means followed by the same letter within a column are not significantly different by Student's T ($p < 0.05$).

Main Effect	Comparison	Least Square Mean
Dosage	0 mg	100.00 a
Dosage	10 mg	3.13 b
Dosage	15 mg	3.13 b
Dosage	20 mg	0.58 b
Dosage	30 mg	0.28 b
Genotype	BTx2928	20.72 a
Genotype	BTxARG-1	22.96 a
Genotype	RTx437	20.59 a
Environment	Early	21.57 a
Environment	Late	21.28 a

Table 6. Summary of least squares means for the percent self-pollinations of testcrosses, showing the effect of number of applications, genotype, and environment. Means followed by the same letter within a column are not significantly different by Student's T ($p < 0.05$).

Main Effect	Comparison	Least Square Mean
Number of Applications	0	100.00 a
Number of Applications	1	2.64 b
Number of Applications	2	2.32 b
Number of Applications	3	1.26 b
Genotype	BTx2928	26.33 a
Genotype	BTxARG-1	28.12 a
Genotype	RTx437	25.33 a
Environment	Early	26.67 a
Environment	Late	26.44 a

Within sprayer applied TFMSA testcrosses, dosage was the only main effect with significance (Table 7). The first order interaction with genotype was not significant (Table 7). Much like was observed in the hand applied TFMSA, the only significance occurred between the control dosage of 0 mg TFMSA and the dosages where TFMSA was applied (Table 8). Numeric differences were minimal within the testcrosses of the sprayer application. Interestingly, the 5 mg dosage produced the numerically lowest percent self-pollinations (Table 8). Similar to the hand applied models, BTxARG-1 produced the highest numerical percentage of self-pollinations, while RTx437 and BTx2928 produced the same percentage (Table 8).

Table 7. Mean squares from the analysis of variance for the percent self-pollinations from the sprayer study, considering dosage, genotype, and their interaction. The control of 0 mg TFMSA is included in the dosage effect.

Source	Degrees of Freedom	Mean Square
Dosage	2	15908.02***
Genotype	2	4.20
Dosage*Genotype	4	1.23
Error	15	3.02

*** = $p<0.001$

Table 8. Summary of least squares means for the percent self-pollinations of the sprayer study, showing the effect of dosage and genotype. Means followed by the same letter within a column are not significantly different by Student's T ($p < 0.05$).

Main Effect	Comparison	Least Square Mean
Dosage	0 mg	100.00 a
Dosage	5 mg	0.97 b
Dosage	30 mg	1.84 b
Genotype	BTx2928	34.87 a
Genotype	BTxARG-1	33.44 a
Genotype	RTx437	34.50 a

These results indicate that application of TFMSA is important for reducing the number of self-pollinations. However, once a dosage threshold for minimum efficacy is breached, it does not matter how many applications are used or the dosage applied. The occurrence of self-pollinations also indicate that fertility reversion occurs, which we believe is mostly due to the timing of application in regard to the physiological maturity of the plants being treated. In greenhouse studies, Hodnett and Rooney (2017) determined that TFMSA was active in the plant for only a certain period of time, and the dosage applied played a role in the duration of said activity. The numeric differences in the dosage model also indicated this within field conditions. Increasing the number of applications could safeguard against within plot variation of individual plant maturity; thus, decreasing self-pollinations, as was indicated by the numeric differences within the model for number of applications.

4.3.2 Logistic Regression Analysis of Bagged Panicles

The efficacy of both TFMSA dose and application number varies depending on genotype (Table 9). For example, chi-square tests indicate that dosage and number of applications do not significantly influence the number of male

sterile panicles observed in BTx2928 (Table 9). Whereas, for both BTxARG-1 and RTx437, dosage and number of applications significantly influenced the number of male sterile panicles observed (Table 9). Collectively, these results indicate that the genotypic response to TFMSA should be evaluated to ensure efficacy at a specific dosage. Regardless, the results also indicate that TFMSA is effective at inducing sterility under field conditions and that the dosage applied, or the timing of the applications used herein are effective, considering they occurred within a physiologically reasonable period of growth.

Table 9. Chi-square p-values following likelihood ratio test of dosage and number of applications for each genotype within individuals receiving hand applied TFMSA.

Genotype	p-value
BTx2928	0.063
BTxARG-1	0.001***
RTx437	0.010**

*** = p<0.001
** = p<0.01

Both BTx2928 and RTx437 did not have any significant beta coefficients. It should be mentioned that in RTx437 there were only 7 fertile plants in the logistic regression portion and all of which were in the single application class (data not shown). This lack of dispersion of fertile plants among application classes leads the model to begin “guessing” when evaluating the effect of number of applications; thus, resulting in a large estimate, standard error and

subsequently, odds ratio (Table 10). The lack of significance within BTx2928 and RTx437, indicate either that there is no genotypic specificity of the measured parameters; or, more likely indicating TFMSA was applied at the appropriate physiological growth stage to achieve complete sterility; therefore, not allowing for proper discernment between treatments.

In BTxARG-1, the beta coefficients: dosage, number of applications, date of flag leaf emergence, and panicle length were significant (Table 10). The odds ratio for dosage (1.3) indicates there is a 1.3x greater odds that sterility will occur for each milligram increase in dose when all other coefficients are kept constant. Similarly, increased numbers of applications, with all other variables constant, would increase the odds of sterility by 6.1x. The odds ratio for the date of flag leaf emergence (0.74) indicates that as the number of days for flag leaf emergence increases, it becomes less likely to produce completely sterile panicles; indicating it is important to pay attention to the physiological growth stage of the treated plants.

Table 10. Estimates, standard error and odds ratios for beta coefficients of the logistic regression model that were significant within at least one genotype receiving hand applied TFMSA.

Genotype	Parameters	Dosage	# of Applications	Fl ag leaf Emergence	Panicle Length
	Estimate	-0.04	1.01	0.09	0.09
BTx2928	Std. Error	0.04	0.76	0.12	0.09
	Odds Ratio	1.3	6.1	0.74	0.74
	Estimate	0.26***	1.81**	-0.30**	-0.29**
BTxARG-1	Std. Error	0.07	0.58	0.09	0.11
	Odds Ratio	1.0	2.8	1.1	1.1
	Estimate	0.10	17.47	-0/7	0.03
RTx437	Std. Error	0.07	1606.71	0.13	0.13
	Odds Ratio	1.1	38825894.3	1.0	1.0

*** = $p<0.001$
** = $p<0.01$

As Hodnett and Rooney (2017) observed, TFMSA must be applied a specific number of days prior to flag leaf emergence. Flag leaf emergence appears to be a phenotypic indicator of an underlying stage of development in the male reproductive cycle. It has been observed in sorghum that free microspores were present in anthers in the tip of the panicle when it neared the flag leaf (Kern and Atkins, 1972). Similarly, as panicle length increases, it becomes less likely of achieving complete sterility, according to its odds ratio (0.74). This implies that for genotypes, or individuals, with longer panicles, a higher dose is needed to provide those plants a time range large enough to completely flower (sorghum flowers from the top of the panicle down, over a period of several days). Given the large odds ratio for number of applications, a second or third application of 20 or 30 mg could therefore increase the likelihood of achieving complete panicle sterility. More plants than are needed for the desired number of crosses should be treated. Then following panicle emergence, the plants with a shorter panicle length should be identified and chosen to use as the seed parent because they likely will require fewer days to flower and therefore should not run the risk of flowering beyond the window of chemical activity within the plant.

The logistic regression output for the sprayer TFMSA did not indicate any beta coefficients were significant for all three genotypes (Table 11).
The chi-square test for Tx2928 was not significant (Table 12), indicating the dosage applied had no effect on whether a plant was sterile. Similarly, chi-square test results were observed in TxARG-1 (Table 12); again, indicating dosage had little overall effect on the number of sterile panicles observed. In RTx437, the chi-square test again showed dose had no significant effect on the number of sterile panicles produced (Table 12). These results, in conjunction with the test cross results, indicate the parameters surrounding application of TFMSA are not important; given, the application occurs during an appropriate physiological growth period and the dosage applied is above a minimum efficacy threshold.

Table 11. Estimates, standard error and odds ratios for beta coefficients of the logistic regression model within each genotype of sprayer applied TFMSA.

Genotype	Parameters	Dosage	Panicle Length	Avg. Flag leaf Emergence	Plot
BTx2928	Estimate	0.07	-0.23	1.28	-0.48
	Std. Error	0.09	0.12	0.69	0.68
	Odds Ratio	1.1	0.8	3.6	0.6
BTxARG-1	Estimate	-0.09	-0.12	1.41	0.09
	Std. Error	0.07	0.08	1.08	0.56
	Odds Ratio	0.9	0.9	4.1	1.1
RTx437	Estimate	0.04	-0.14	0.22	0.04
	Std. Error	0.06	0.09	0.32	0.46
	Odds Ratio	1.0	0.9	1.2	1.0

Table 12. Chi-square p-values following likelihood ratio test of dosage for each genotype within individuals receiving sprayer applied TFMSA.

Genotype	p-value
BTx2928	0.447
BTxARG-1	0.224
RTx437	0.514

4.4 Conclusions

The induction of male sterility in field conditions using TFMSA was successful but details to ensure that treated plants are fully sterile must be further clarified. For example, treatment timing appears critical; it must occur within a physiological window prior to flag leaf emergence and with a high enough dosage to remain active during the critical period of male gamete development. The results also confirm that once an effective dosage threshold of TFMSA is crossed, the number of applications are less important. Both hand applied and backpack sprayer applied methods repeated these trends. Additional studies should be conducted using a sprayer to perfect its use, much like was done using the hand application method. These studies should also include a measure to evaluate whether TFMSA has any negative environmental impacts.

Further testing to evaluate whether TFMSA can be used to effectively test female lines prior to sterilization should be conducted. Using at least 30 well

known grain and forage type A/B pairs possessing traits such as: waxy endosperm, non-waxy endosperm, black pericarp, yellow endosperm and brown midrib. Crosses should be made using at least 2 common R lines as pollinators to each A line and then to its paired B line which would have been treated with TFMSA. Seed from each cross should then be grown in replicated field trials in at least 4 locations across the state of Texas. This will allow for evaluating the agronomic performance and uniformity of hybrids derived from sterilization by TFMSA in comparison to their CMS counterparts; indicating whether using TFMSA for female line testing prior to introduction of CMS is a feasible approach to develop new female lines. The use of TFMSA on an F_1 hybrid should also be tested, since temporal male sterility will have to be induced in F_1's within a haploid breeding system. Using the F_1 hybrids derived from the previous B/R test crosses would allow a thorough test of TFMSA's ability to induce male sterility across a wide range of genetically diverse F_1 hybrids.

5. CONCLUSIONS

In the course of these two studies, the data indicate TFMSA is effective at inducing male sterility in multiple field conditions. It also disrupts the amino acid profile within anthers.

It is seems that TFMSA disrupts the amino acid profile within the anthers, while minimally influencing the glume amino acid profile. Interestingly, the amino acid profiles in CMS lines of many species including sorghum, are similar to what was observed in this study. However, the amino acid profile of several revertant plants provides evidence that CAs, specifically α and γ CAs, may be the target of TFMSA in sorghum and maize; instead of specific enzymes critical for amino acid synthesis, or transporters of amino acids.

In field environments, TFMSA is effective at producing completely male-sterile panicles to varying degrees from both hand and sprayer applications. Moreover, testcrosses confirmed that female fecundity is not affected, and the rate of self-pollination is low enough that it may be of use in a breeding program, e.g., for testing female lines prior to sterilization, making breeding crosses, or if there is ever a need to make only a couple of testcrosses. Available data suggest TFMSA can be used to deploy a DH system in sorghum, given reasonable rates of haploidy and chromosome-doubling efficiency.

Further testing is needed to establish the range of genotypes to which TFMSA is effective, including inbreds and hybrids. Pairwise testing is needed to establish if TFMSA can be applied to non-CMS female lines to easily create conventional F1 hybrids, and if their performance enables accurate prediction of performance by F1

hybrids that would be produced after cytoplasmic conversion of the same maternal lines. The ability of TFMSA to induce temporal male sterility in F_1 hybrids would also need direct evaluation, since F1 hybrids could respond differently than inbreds to TFMSA, and are requisite to a DH breeding system.

The results of this study show TFMSA to be an effective and easily adaptable male gametocide, if certain parameters are followed during application. Therefore, TFMSA could revolutionize the sorghum breeding industry and could very well be the harbinger of DH technology and genomic selection, among other advancements.

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