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Evaluation of Effective Gametocides for Selective Induction of Male Sterility in Sorghum

Assefa AMELEWORK, Mark LAING and Hussein SHIMELIS

African Center for Crop Improvement, University of KwaZulu-Natal, Scottsville, Pietermaritzburg, South Africa

Abstract

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Effective gametocides can serve as alternative agents to induce male sterility in sorghum. The objectives of this study were to evaluate the effectiveness of selected male gametocides in sorghum, to determine the optimum concentration of the chemicals and to investigate its possible effect on female sterility. Ethyl 4-fluorooxanilate (E4FO) and ethrel were evaluated on three genotypes of sorghum. The gametocides were sprayed at five concentrations at the booting stage when the panicles were released halfway from the flag leaf. Pollen and spikelet sterility were assessed using light and scanning electron microscopes. Both chemicals were effective in inducing male sterility in sorghum. However, E4FO was the more potent chemical, inducing complete male sterility with high female fertility and relatively less phytotoxicity at lower concentrations, while higher concentrations of ethrel were required to give complete male sterility. E4FO induced on average of 99.5% male sterility at 2 mg/l in all the varieties, while with ethrel 97% male sterility was obtained at 3 ml/l. Therefore we recommend to use E4FO at 2 mg/l and ethrel at 3 ml/l as male sterilizing agents to facilitate controlled crosses in sorghum.

Keywords: chemical hybridizing agent; ethrel; ethyl 4-fluorooxanilate; gametocide; pollen sterility; spikelet sterility; *Sorghum bicolor*

Sorghum (*Sorghum bicolor* (L.) Moench) is a drought tolerant cereal crop feeding more than 500 million people in the semi-arid tropics where droughts cause frequent crop failures (FAO 2012). Sorghum has small and cleistogamous florets leading to a predominantly self-fertilization mating system. Therefore, controlled emasculation and subsequent crosses are cumbersome owing to the mating system and the small flower size. In sorghum, controlled crosses are essential for genetic analysis, breeding or hybrid seed production. Due to its self-fertilization, sorghum breeding has been largely restricted to development of cultivars through pure line, pedigree, or mass selection procedures (Rooney 2004).

The production of hybrid sorghum may offer an exciting opportunity for overcoming the stagnant grain yield plateau of sorghum in sub-Saharan Africa. Yield breakthrough may be possible in sorghum as with hybrid maize beginning in 1924 (Smith *et al*. 2004) through the exploitation of hybrid vigour or heterosis. Hybrid vigour has been reported through designed hybridization in sorghum (Pray *et al*. 1991). In sorghum, hybrid vigour can be commercially exploited only if effective male sterility systems are available and sufficient outcrossing can be obtained on male sterile plants to produce hybrid seeds. Several male sterility systems are found in sorghum that can be used by plant breeders to widen the range of possibilities for improving the crop and to develop different cultivar types (Praveen *et al*. 2015).

Cytoplasmic male sterility (CMS) is the most widely used system for the commercial production of sorghum hybrids. A number of difficulties exist with the use of CMS systems in sorghum. Firstly, not all the CMS systems give stable male sterility; secondly, not all stable CMS systems give stable CMS lines in all maintainer genotypes (Murty & Gangadhar 1990). Thus a large number of CMS systems and

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the potential maintainer lines are required so that a choice can be made to breed and select effective and stable CMS lines. Thirdly, it is difficult to ensure the expression of CMS across a range of environments (Li *et al*. 1981). Development of effective and appropriate maintainer and restorer lines presents a considerable obstacle to the effective and economic exploitation of the trait for the commercial hybrid seed production. Fourthly, the CMS parents (A, B and R lines) require deliberate and systematic maintenance to keep them pure. This, in turn, requires considerable expenditure of time and effort. Fifthly, breeding progress is limited to the male parents, reducing genetic gains per generation.

The use of chemical hybridizing agents (CHA) presents an alternative method to produce male sterility (Cross & Ladyman 1991). The CHA acts as a gametocide selectively affecting the male gamete by inducing physiological abnormalities, which in turn prevent pollen development, pollen shed or pollen viability (Cross & Ladyman 1991). Research has been conducted to identify CHAs that induced male sterility without significant impairment of female fertility. Ethyl 4-fluorooxanilate (E4FO) and ethrel are the most widely studied gametocides. Ethrel induces male sterility in several crops, including wheat, rice, millet, barley, and tef (Jan & Rowell 1981; Colhoun & Steer 1983; Thakur & Rao 1988; Efisue *et al*. 2010; Ghebrehiwot *et al*. 2015). Similarly, a high level of pollen sterility has been reported using E4FO on rice, wheat and tef (Ali *et al*. 1999; Chakraborty & Devakumar 2006; Ghebrehiwot *et al*. 2015).

Most of the chemicals tested so far are either only partially effective, induce female sterility, or produce phytotoxic side effects. Very little information has been reported on chemical induction of male sterility in sorghum. In order to optimize the use of CHA in hybrid breeding, effective and safe chemical male gametocides need to be identified. This research was initiated with the objectives of evaluating the effectiveness of selected male gametocides in sorghum, to determine the optimum concentration of the chemicals and to investigate their possible effects on female fertility.

MATERIAL AND METHODS

Plant materials. In this study, three widely grown, dwarf and early maturing sorghum varieties (Kari Mtama, Dwarf Wonder and KAT 487 designated as AS-1, AS-71, and AS-72, in that order) were used. The varieties were planted in a tunnel at the University of KwaZulu-Natal, South Africa. Seeds from each variety were sown in plastic pots (300 mm diameter and 280 mm length) filled with Gromor potting media (http://www.gromor.co.za) and plants were fertilized with Agchem hydroponic water soluble fertilizer (http://www.agchem.co.za). The plants received optimum fertigation four times a day for 3 min. Four seeds per pot were sown, thinned-out to two plants per pot at three weeks after germination. Tillers were frequently removed in order to limit the plants to main stems. The control plots were maintained at an appropriate distance to avoid chemical drift and pollen contamination.

Chemical formulation and application. Ethyl 4-fluorooxanilate (Industricord, Beijing, China) and ethrel (Farmers Agri-Care, Pietermaritzburg, South Africa) were used for this study. The E4FO, formulated as a white powder emulsion, was prepared by first dissolving E4FO at a 1 : 6 w/v ratio with dimethyl sulfoxide (DMSO) and adding 2% Tween 80 as a surfactant. Spray emulsions of 1, 1.5, 2, 2.5 and 3 mg/l concentrations were prepared by diluting the solution with water. Ethrel (2-chloroethylphosphonic acid) being liquid and water soluble was directly made into aqueous solutions of 1, 2, 3, 4 and 5 ml/l. At all concentration levels of ethrel, 2% of Tween 80 was added as a surfactant. Chemical applications were performed when sorghum plants were at the booting stage, when the panicles were released halfway from the flag leaf. Chemical spraying was done with a hollow cone (HCX) 80° nozzle, using a CP3 knapsack sprayer. Spraying was done in the early morning. Each panicle was sprayed until runoff. The quantity of the liquid sprayed per plant was approximately 8 to 10 ml. The ethrel treated panicles, four days after spraying, were sprayed with promalin (Farmers Agri-Care) at 0.15 ml/l in order to promote ear emergence (Emongor *et al.* 2004). Distilled water was used to spray the control treatment.

Experimental design and data collection. The experiment was conducted in a controlled environment facility (CEF). The three varieties and two chemicals, with five concentration levels of each and the control, were arranged in a complete randomized block design with 36 treatments in five replications. For each treatment 25 pots and 50 plants (two plants per pot) were used. Forty plants were randomly tagged per treatment for further analysis. To prevent self-pollination, 20 of the randomly tagged panicles

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were covered with a paper bag while the remaining 20 were left uncovered for open pollination. Data for each replication was collected based on average measurements of four plants.

Pollen sterility. Three to five days after chemical application, 10 spikelets were sampled from the middle of 5 randomly selected panicles per each treatment and fixed in 70% ethanol to avoid pollen desiccation. Anthers were extracted from the spikelets onto a glass slide and pollen was squeezed out with a needle into 2% iodine potassium iodide (I_2-KI) solution. Five microscopic slides were prepared and examined under a light microscope. Twenty microscopic fields were used to count sterile pollen grains. Pollen grains that were of normal size and shape and fully stained were assumed to be fertile and those that were non-stained or partially stained, or withered and shrivelled were considered to be sterile.

Percentage pollen sterility is computed as follows:

$$\% \text{ pollen sterility} = \frac{P_s}{P_s + P_f} \times 100$$

where:

P_s – number of sterile pollen grains

P_f – number of fertile pollen grains

Fresh pollen was collected from treated and control plants between 7:00 and 9:00 A.M., as the anthers began to dehisce. Pollen grains were released from the anthers by gentle tapping of the main stem of the plant just below the inflorescence. Pollen samples were collected on dried petri dishes and sieved through a 250-mm mesh to remove large floral particles. Dried pollen grains were mounted onto clean stubs using double-slide adhesive. The samples were then coated with a 30 nm layer of gold using an EIKO IB3 Ion Coater (EIKO Engineering CO., Ltd, Osaka, Japan) at an accelerated voltage of 15 kV Electron Microscope Unit of the University of KwaZulu-Natal. The coated pollen grains were then viewed and photographed with a ZEISS EVO LS 15 scanning electron microscope (Carl Zeiss, München, Germany). Pollen diameters were measured from treated and untreated spikelets.

Female sterility. To study the effect of the chemicals on female sterility, ovaries from the control and treated plants were fixed in 3% glutaraldehyde for 12 h. Fixed samples were washed with 0.05 M sodium cacodylate buffer for 30 min and post fixed with 2% osmium tetraoxide (OsO_4) in the same buffer for 1–2 h. Samples were washed with 0.05 M sodium cacodylate buffer for 30 min. This was followed by dehydration through an ethanol series of 10, 30, 50, 70, 90 and 100%. The samples were then rinsed twice in 100% propanol oxide and embedded in Spurr resin. Samples were sectioned using a LEICA UC7 Ultra microtome (Leica, Wetzlar, Germany) and sections were mounted on microscopic slides and stained with lactophenol cotton blue. The samples were viewed under a light microscope.

Spikelet sterility. Seeds from 20 bagged and unbagged panicles from each treatment, including the control, were harvested at maturity. Spikelets were threshed manually and the numbers of filled (fertile) and unfilled/shrivelled (sterile) grains were counted. Spikelet sterility induced in each variety was calculated using the following formula:

$$\% \text{ spikelet sterility} = \frac{S_c - S_f}{S_c} \times 100$$

where:

S_c – seeds per panicle in control plants

S_f – seeds per panicle in treated plants

Data analysis. Analysis of variance (ANOVA) was performed according to the randomized complete block design using GenStat, GenStat for Windows 17th edition (Payne *et al*. 2014). Based on the ANOVA, the least significant difference (LSD) at a 5% level of significance was computed for main and interaction effects.

RESULTS

Pollen sterility. The analysis of variance on pollen sterility and spikelet sterility on bagged and unbagged panicles is presented in Table 1. The fertile pollen grains were deeply stained, filled with starch grains while sterile pollen grains were transparent and contained little starch (Figure 1). With respect to pollen sterility, the ANOVA showed the highly significant mean sum of squares due to variety, chemical, concentration and all possible two and three factor interactions. A mean of 18% pollen sterility was observed in the untreated control plants. Both chemicals were significantly effective in the induction of pollen sterility in comparison with the control. The levels of pollen sterility increased with an increase in the concentration of chemicals (Table 2). Ethyl 4-fluorooxanilate induced the highest levels of pollen sterility (93%), considerably higher than ethrel (76%). The interaction of chemical with concentration showed significant differences in percentage sterility induction as a result of chemicals and varieties. E4FO induced high levels of pollen

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Table 1. Analysis of variance for the response of three sorghum varieties to two chemical gametocides with six concentrations in relation to induced pollen and spikelet sterility

Source of variation	df	Pollen	Spikelet (mean squares)	
			bagged	unbagged
Replication	4	3	11	81
Variety (V)	2	26***	70***	104**
Gametocides (G)	1	8 764***	284***	738***
Concentration of gametocides (C)	5	29 802***	46 587***	42 769***
V × G	2	50***	129***	448***
V × C	10	38***	56***	47*
G × C	5	1 532***	20	76**
V × G × C	10	130***	42***	113***
Residual	140	3	11	25
Total	179			

df – degree of freedom; significant at * P =0.05; ** significant at P = 0.01, *** significant at $P < 0.001$

sterility at high and low concentrations that ranged from 65% to 100%, whereas ethrel was effective only at higher concentrations. Complete pollen sterility was caused by E4FO at concentrations ranging between 2 to 3 mg/l and ethrel at 5 ml/l.

Microscopic observation of the pollen grain revealed morphological differences between the pollen grains obtained from chemically treated plants and untreated control. Significant differences were observed in pollen diameter between treated and untreated plants (Table 3). Pollen grains from untreated plants were round and had a significantly larger mean diameter (48 µm), while pollen grains from treated plants were wrinkled with a mean pollen diameter of 44 µm (Figure 2). A significant chemical × variety interaction was obtained. Pollen grains collected from KAT 487 were more sensitive to chemical treatments than those from Dwarf Wonder.

Female fertility. Female fertility was significantly reduced as the concentration levels increased. Microscopic views of individual ovaries showed that the number of cells in the central parts of the ovary decreased with increasing concentration levels (Figure 3). At 3 mg/l, the cells in the ovary were dead, indicating that the female was sterile due to the chemical effect. Other phytotoxic effects were observed on plants treated with E4FO at 2.5–3 mg/l and ethrel 4–5 ml/l.

Spikelet sterility. Spikelet sterility was examined under bagged and unbagged conditions. The ANOVA for spikelet sterility revealed a significant difference between bagged and unbagged panicles. Significant differences in seeds per spikelet between bagged and unbagged panicles occurred for both chemicals and concentrations, indicating the presence of outcrosses. Seeds per spike on bagged plants were 5% lower than on unbagged plants. In the bagged panicles, a highly

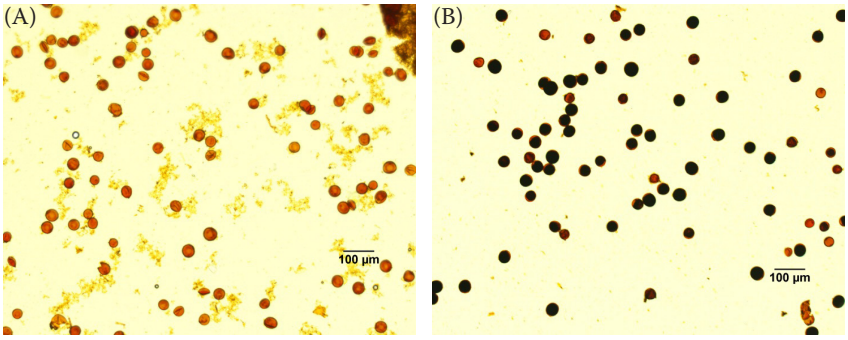

Figure 1. Pollen of *Sorghum bicolor* visualized under a light microscope after I_2-KI stain: (A) – sterile pollen following ethyl 4-fluorooxanilate treatment, (B) – fertile pollen collected from an untreated plant; scale bar 100 µm

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Table 2. Percent pollen and spikelet sterility in three sorghum genotypes (AS-1, AS-71, and AS-72) after treatment with two gametocides at six concentrations

Gametocide	Concentration	AS-1			AS-71			AS-72		
		pollen	spikelet		pollen	spikelet		pollen	spikelet	
			bagged	unbagged		bagged	unbagged		bagged	unbagged
E4FO (mg/l)	control	12.8[a]	0.0[a]	0.0[a]	22.0[a]	0.0[a]	0.0[a]	21.8[a]	0.00[a]	0.00[a]
	1.0	71.3[b]	92.7[b]	80.5[b]	70.2[b]	90.1[b]	84.5[b]	68.5[b]	88.37[b]	83.26[b]
	1.5	99.9[c]	93.5[b]	84.6[b]	89.0[c]	99.7[c]	96.5[c]	94.5[c]	100.00[c]	95.74[c]
	2.0	100.0[c]	98.5[c]	89.2[bc]	96.2[d]	100.0[c]	97.0[c]	97.6[d]	100.00[c]	97.49[c]
	2.5	100.0[c]	99.8[c]	98.3[d]	100.0[e]	100.0[c]	98.1[c]	100.0[e]	100.00[c]	95.59[c]
	3.0	100.0[c]	100.0[c]	99.6[d]	100.0[e]	100.0[c]	99.7[c]	100.0[e]	100.00[c]	100.00[c]
Ethrel (ml/l)	control	17.0[a]	0.0[a]	0.0[a]	18.1[a]	0.0[a]	0.0[a]	17.8[a]	0.00[a]	0.00[a]
	1.0	35.9[b]	95.7[b]	84.9[b]	38.2[b]	79.1[b]	63.9[b]	46.8[b]	83.03[b]	70.81[b]
	2.0	52.2[c]	98.1[b]	92.4[c]	69.2[c]	87.9[c]	83.1[c]	63.3[c]	94.73[c]	86.57[c]
	3.0	86.4[d]	97.4[b]	94.9[c]	82.7[d]	93.7[d]	86.5[c]	83.4[d]	100.00[d]	95.22[d]
	4.0	97.2[e]	97.0[b]	91.8[c]	91.8[e]	96.6[e]	91.1[c]	93.6[e]	100.00[d]	99.18[d]
	5.0	99.0[e]	96.1[b]	94.8[c]	100.0[f]	98.0[e]	92.2[cd]	100.0[f]	100.00[d]	100.00[d]
Grand mean		73.2	80.0	75.8						
CV (%)		2.4	4.1	6.4						
LSD (5%)		2.2	4.1	6.2						
R^2		0.997	0.992	0.980						

[a]Means in a column followed by the same letter(s) are not significantly different at a 5% probability level; E4FO – ethyl 4-fluorooxanilate

significant difference was observed between varieties, chemicals, concentrations, and all the two and three factor interactions except for the interaction of chemical with concentration (Table 1).

The ANOVA of spikelet sterility on the unbagged panicles reflected significant differences between varieties and in their reaction with concentration. The

Table 3. Analysis of variance for the effects of two sorghum varieties and two gametocides on pollen diameter

Source of variation	df	SS	MS	VR	*F*
Replication	4	253.7	63.4	2.8	
Variety (V)	1	0.13	0.1	0.01	0.939
Gametocide (G)	1	2 478.3	2 478.3	110.4	< 0.001
V × G	1	330.8	330.8	17.47	< 0.001
Residual	672	15 085.3	22.5		
Total	679	18 748.14			

df – degree of freedom; SS – sum of squares; MS – mean square; VR – variance ratio; *F* – *F*-probability

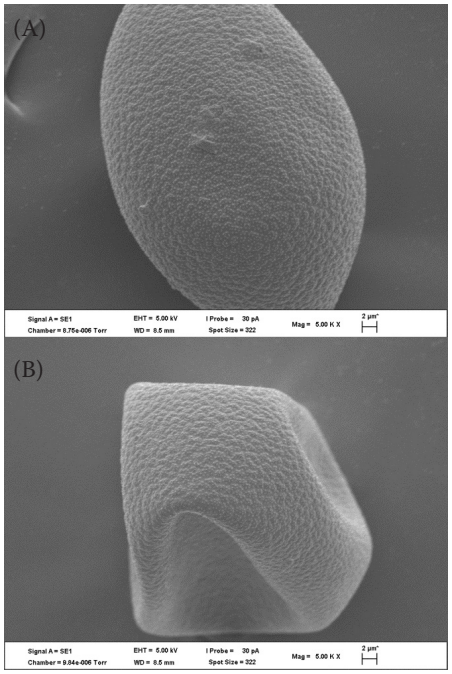

Figure 2. Sorghum pollen grains from AS-72 observed under a scanning electronic microscope: (A) – untreated plant, (B) – treated plant; scale bar 2 μm

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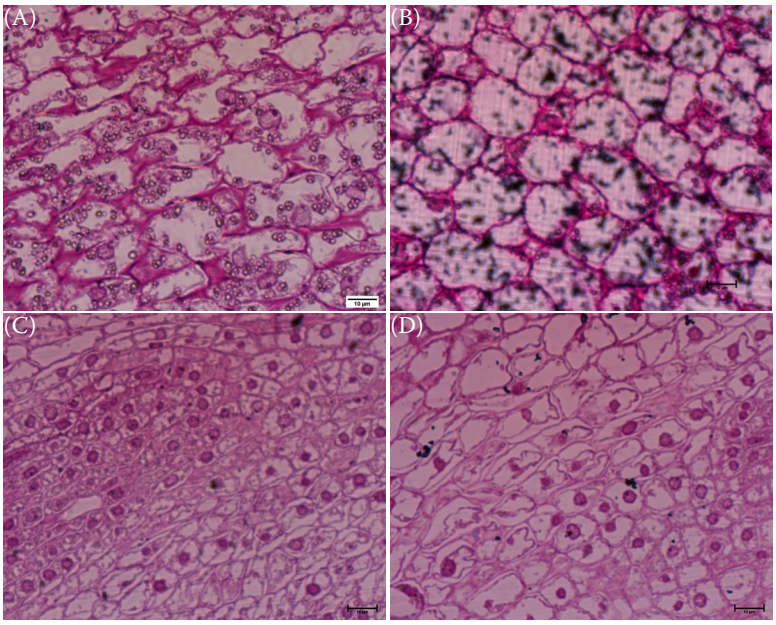

Figure 3. Microscopic view of the central parts of sorghum ovaries of AS-72: (A) ovary from an untreated plant, (B): ovary from a plant treated with E4FO at 2 mg/l, (C) ovary from a plant treated with E4FO at 2.5 mg/l, (D) ovary from a plant treated with E4FO at 3 mg/l; E4FO – ethyl 4-fluorooxanilate; scale bar 10 μm

mean sum of squares due to chemicals, concentrations and all their interactions was highly significant (Table 1). Plants treated with both E4FO and ethrel exhibited significant reductions in numbers of seeds per spikelet compared to the control. Nearly complete spikelet sterility was attained on plants treated with E4FO at rates ranging between 2 to 3 mg/l and ethrel at 5 ml/l (Table 2). However, plants treated with high concentrations of both gametocides showed a complete senescence of inflorescences.

DISCUSSION

Sorghum being a self-pollinated crop, selective sterilization of pollen is of paramount importance. Hybridization in sorghum has mainly relied on hand emasculation or the use of CMS lines. The complexity of developing and maintaining the A, B and R lines in the use of CMS makes the technique less appealing. As an alternative, the use of CHA for the induction of male sterility through suppression of viable pollen formation can be used as a quick and flexible method for hybrid production (Lu *et al.* 1994). An effective CHA can be used to generate many parental cross combinations for genetic evaluation and for the production of hybrids. Therefore two-line breeding through chemical emasculation may be regarded as the best option, provided that the chemical is safe, consistent in its effects and has few phytotoxic effects.

Encouraging results have been obtained on the gametocidal properties of a wide range of chemicals on different crop species (Cross & Ladyman 1991). In this study, E4FO and ethrel induced high levels of male sterility in all the sorghum varieties studied. E4FO caused nearly complete pollen sterility (97–100%) at 2 mg/l with no negative effect on female fertility. Ali *et al.* (1999) and Chakraborty and Devakumar (2006) reported that E4FO induced 99.7% and 100% male sterility at 1.5mg/l in wheat and rice, respectively. Ghebrehiwot *et al.* (2015) reported that 96–99% male sterility was achieved without a significant reduction in female fertility in tef using E4FO at 1–1.5 mg/l.

Female fertility determined by microscopic observation of plant ovaries indicated that the number of cells in the central parts of ovary was greatly reduced as the concentration levels of both gametocides increased. These cells later develop into endosperm cells to constitute the sorghum kernel (Yang *et al.* 2009). The complete damage to these cells by the chemicals reflects female sterility and the abnormal kernel development. Plants sprayed with higher concentrations of E4FO, i.e. 2.5 and 3 mg/l, showed some undesirable phytotoxic effects such as death of panicles and premature senescence of the top leaves.

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There was an increase in the degree of sterility with increasing ethrel concentration until complete sterility was reached. In this particular study, nearly complete pollen and spikelet sterility (99–100%) was achieved at an ethrel concentration of 5 ml/l. Similar results were reported by Ghebrehiwot *et al*. (2015) on tef. Chan and Cheah (1983) reported 100% spikelet sterility in bagged panicles of rice at 3 ml/l of ethrel. The highest ethrel concentrations (4–5 ml/l) caused a significant reduction in female fertility and plant phytotoxic effects such as premature spikelet desiccation and leaf senescence. Ethrel at high concentrations can also severely affect plant height and panicle emergence (Rowell & Miller 1971). To reverse this side effect here, plants were sprayed with promalin at 0.15 ml/l (Emongor *et al*. 2004). No abnormal spikelets were observed, suggesting that this is an effective treatment that could increase the level of outcrossing in female plants due to the proper emergence of panicles from the sheath of the flag leaf.

A relatively high level (18%) of spikelet sterility was observed in the control plants due to the excessively high summer temperatures encountered. Growth temperatures ≥36°C significantly decrease pollen production, pollen viability, seed-set, seed yield and harvest index (Prasad *et al*. 2006). Within treatments there were significant differences in the amounts of seeds set per spikelet between bagged and unbagged spikelets, which suggests the presence of outcrossing or female fertility. Significant variation was observed between varieties in unbagged panicles while the differences in seed set in the bagged panicles were not significant. This confirms the effectiveness of the chemicals in inducing male sterility and that the ovary remained receptive to outcrossing. Similarly, significant variations in seeds per spikelet in bagged and unbagged panicles were observed for the chemicals between the varieties. Complete male sterility was achieved in Dwarf Wonder and KAT 487 at 2 mg/l of E4FO while Kari Mtama required 3 mg/l. This may result from the difference in their panicle morphology because Dwarf Wonder and KAT 487 are semi-compact varieties while Kari Mtama has very loose panicles. This morphological variation, in turn, may affect the impact of surfactants that favour the penetration of gametocides into the plant (Parodi & Gaju 2009). In addition, E4FO and ethrel are known to have systemic activity (Ali *et al*. 1999; Chakraborty & Devakumar 2006).

In conclusion, E4FO and ethrel were effective in inducing male sterility in sorghum. E4FO induced a mean of 99.5% male sterility at 2 mg/l in all the varieties, while using ethrel at 3 ml/l 97% male sterility was obtained in the bagged panicles. Levels of male sterility equal or higher than 95% are considered satisfactory for the production of hybrid seed (Parodi & Gaju 2009). However, their effectiveness may be genotype specific if the varieties have different panicle morphologies. Further studies are needed to establish the mode of action of the chemicals, selection of effective surfactants and evaluation of their effects on a large number of sorghum varieties with a range of morphotypes.

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Corresponding author:

Assefa Amelework, Ph.D., University of KwaZulu-Natal, African Center for Crop Improvement, Private Bag X01, Scottsville 3209, Pietermaritzburg, South Africa; e-mail: amele_g@yahoo.com; assefa@ukzn.ac.za