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The efficacy of four gametocides for induction of pollen sterility in *Eragrostis tef* (Zucc.) Trotter

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Effective chemical hybridizing agents (CHAs) or male gametocides enhance cross pollination in plant breeding and genetic analysis of traits. The present study examined the efficacy and optimum concentration of four CHAs, namely: 2-chloroethyl phosphonic acid (Ethrel), ethyl 4'-fluorooxanilate (E4FO), 2, 4-dichlorphenoxy acetic acid (2, 4-D) and Promalin® (1.8% GA_{47} – gibberellins A_4+A_7 and 1.8% 6-BA–benzyladenine) on pollen sterility and seeding of a tef line (DZ-01-3186). Seed-derived and individually grown tef plants were treated with foliar applications of the four CHAs (at four levels each) sprayed once at the early booting stage, and bagged to control cross pollination. Female fertility was assessed by recording seed set following controlled pollinations. Although all the CHAs caused some pollen sterility, their efficacy levels varied from 9.77 to 99.50% in treated plants compared to the control (6.68 +/- 1.04%). Pollen sterility increased with increasing CHA concentration. Near-complete pollen sterility (99.50 ± 0.50%) was achieved by the application of E4FO at rates of 1500 to 3000 ppm, and Ethrel at 5000 ppm. All the CHAs significantly reduced seed yield, with E4FO, Ethrel and Promalin® at 5000 ppm causing the highest reduction, essentially yielding no seed. In the period following the application of the CHAs, plants treated with E4FO (1000 – 1500 ppm) exhibited stigmas that remained fertile. Hence, it is recommended that E4FO (at 1000 – 1500 ppm) can be used as a chemical emasculation agent for tef, with the least phytotoxicity and the highest female fertility.

Key words: *Eragrostis tef,* Ethrel, ethyl4-florooxanilate, female fertility, gametocides, pollen sterility.

INTRODUCTION

Tef [*Eragrostis tef* (Zucc.) Trotter] is an autogamous cereal food crop that has been cultivated in the Horn of Africa (Eritrea and Ethiopia) for over 2000 years (Ingram and Doyle, 2003). The grain is used to make a variety of food products, including a spongy fermented pancake called "*Enjera"* that regionally serves as a staple food. In Ethiopia, tef is considered the most important crop covering the greatest land area under cereal cultivation (Ketema, 1997). Despite a long history of cultivation, grain production is hampered by the low yield levels of

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the available cultivars, and vulnerability of the crop to lodging, which routinely causes yield reductions of 17 to 25% (Ketema, 1983). At the moment, the mean national grain yield of tef stands at 1.46 t ha^{-1} or 1465 $kg.ha^{-1}$ (Central Statistics Authority, 2014). Improved varieties of tef provide grain yields of 1700-2200 $kg.ha^{-1}$ on farmers' fields and 2200-2800 $kg.ha^{-1}$ under research-managed situations. Estimates are that the crop could yield as much as 6000 $kg.ha^{-1}$, had it received adequate research attention (Ketema, 1997; Teklu and Tefera, 2005).

Research on tef improvement began in the late 1950s in Ethiopia. This genetic improvement programme utilized mass and/or pure-line selection directly from the existing germplasm, hybridization, as well as the initiation of induced mutations (Belay et al., 2006). The major breeding objectives were to improve grain yield and to develop drought and lodging-resistant varieties of tef. Attempts to improve grain yield have resulted in a number of improved varieties. However, breeding efforts to develop lodging-resistant tef varieties have not yet succeeded due to the tedious and meticulous crossing technique of tef, attributed to its pollination process (Gugsa and Loerz, 2013). In tef, pollination occurs only during the early hours of the morning and requires the employment of an appropriate artificial hybridization technique. Under Ethiopian climatic conditions, tef flowers open and are fertilised only between 6:45 and 7:45 a.m. (Berhe, 1976; Ketema, 1997). In addition to this, the small size of its reproductive structures and its autogamous nature has made microscopic emasculation and cross pollination obligatory, which requires appropriate equipment and skilled personnel (Berhe and Miller, 1976; Ketema, 1997; Gugsa and Loerz, 2013). In a self-pollinated crop such as tef, with the male and female organs in the same flower (monoecious), the application of chemical hybridizing agents (CHAs) that selectively impair the male gamete would be of a great value in plant breeding or genetic analysis of traits, reducing the time invested in the laborious procedure of hand emasculation.

Male sterility is defined as the failure of a plant to produce functional anthers, pollen and/or male gametes during its reproductive stage. It can be triggered by multiple factors including adverse growth conditions such as temperature (Endo et al., 2009), diseases, inheritance, mutations or chemical agents (McRae, 1985; Budar and Pelletier, 2001). The deliberate elimination of the male gamete using CHAs has been established as a potentially viable approach in commercial hybrid seed production (Van Der Kley, 1954; Tu and Banga, 1998). It would solve many problems, such as the elimination of tedious hand emasculation, allowing for combinations of suitable parents, eliminating the need for expensive, complex and limiting cytoplasmic male sterility systems, increasing crossing choices in plant breeding and simplifying genetic analysis of self-pollinating crops. An effective CHA should induce an acceptable level of male sterility while retaining a high degree of female fertility (Cross and Ladyman, 1991). The major groups of chemicals screened so far for their possible CHA potential include auxins, antiauxins, growth regulators, arsenicals, ethylene-releasing compounds, halogenated aliphatic acids and several other patented chemicals (Batch et al., 1980). Several derivatives of oxalinates (Ali et al., 1999; Chakraborty and Devakumar, 2006a) and growth regulators (Badino, 1981; Praba and Thangaraj, 2005) were effective in inducing pollen sterility in various crops without negative effects on female fertility. The effectiveness of some CHAs (e.g. Ethrel) is genotype, dose and stage specific which limits their value (Bennet and Hughes, 1972).

Ali et al. (1999) examined the relative efficacy of three CHAs, including E4FO, on male sterility of rice (*Oryza sativa*). They reported that E4FO applied @1500 ppm at meiosis was the most effective CHA, causing the most pollen and spikelet sterility, the widest spectrum of action on multiple varieties and the least phytotoxicity. Foliar applications of Ethrel at rates ranging between 1000 and 4000 ppm also induced useful levels of male sterility in barley (*Hordeum vulgare*) (Kumar et al., 1976; Verma and Kuman, 1978), spring wheat (*Triticum aestivu*) (Rowell and Miller, 1971; Dotlacil and Apltauerova, 1978; Jan and Rowell, 1981), rice (*Oryza sativa*) (Parmar et al., 1979) and tef (Berhe and Miller, 1978). However, studies have also demonstrated that Ethrel induces significant levels of female sterility at the rates of application required for male sterility (Hughes et al., 1974; Berhe and Miller, 1978; Dotlacil and Apltauerova, 1978). There are also reports that Ethrel may cause defoliation in certain species (Morgan, 1969; Pedersen et al., 2006). As a selective CHA, 2, 4-dichlorphenoxy acetic acid (2, 4-D) is also one of the most widely studied CHAs (Helal and Zaiki, 1981). Foliar applications of 2, 4-D on tomato (Rehm, 1952), rice (Helal and Zaiki, 1981) and sesame (Prakash et al., 2001) induced high levels of male sterility. By contrast, the application of 0.4% 2, 4-D did not induce acceptable levels of pollen sterility in rice (Praba and Thangaraj, 2005).

The gametocidal property of gibberellic acid in inducing male sterility is also well studied in many corps including maize (Nelson and Rossman, 1958), common Onion (Van Der Meer and Van Bennekom, 1976; Badino, 1981), coriander (Kalidasu et al., 2009) and many other flowering plants (Sawhney and Shukula, 1994). Despite the volume of research on CHAs, there are no reports of male sterilizing CHAs being used for tef breeding (Berhe and Miller, 1976, 1978). In pursuit of a potential alternative for hand emasculation, the present study examined the efficacy and determined the optimum concentration of four CHAs on male sterility and subsequent seed set in tef. The chief objectives were 1) to identify an effective and suitable CHA for use in tef without adverse effects; 2) to standardize the concentration of the CHA for inducing an acceptable level of male sterility; 3) to investigate the morphological and physiological

changes induced by the CHA and; 4) to investigate post-treatment female fertility/sterility through the assessments of seed set after controlled pollination.

MATERIALS AND METHODS

The CHAs and plant material

The tef variety - DZ-01-3186 was used for this particular study. Four CHAs were used in the study, Ethrel, ethyl 4'-fluorooxanilate (E4FO), Promalin® and 2, 4-D. The concentration levels tested were: Ethrel at 1000, 2000, 3000 and 5000 ppm; E4FO at 1000, 1500, 2000, 3000 ppm, Promalin® at 50, 100, 150 and 300 ppm; 2, 4-D at 10, 50, 100 and 500 ppm. At all concentration levels, 5% of Tween 80® (0.2%) was added as a wetting agent.

Growing conditions, treatments and experimental design

Experiments were conducted in an environmentally controlled glasshouse maintained at an air temperature of 28 ± 2.5°C. The tef plants were grown from seeds sown directly into plastic pots (250 mm in diameter and 210 mm in height) filled with 75% composted pine bark and 25% river sand. Ten seeds per pot were sown, thinned-out to one plant per pot at the trifoliate stage. All lateral tillers were constantly clipped off, allowing only one dominant tiller to grow to flowering. The tef plants received an optimal fertigation, scheduled twice a day and weeds were hand controlled. The CHA solutions were applied on once at the panicle initiation stage when the flower head (panicle) was 10-12 mm long and panicles were then bagged to avoid cross pollination. The sprays were applied with a small power sprayer and hand gun to thoroughly wet the inflorescence, leaf and stem surfaces. The quantity of liquid sprayed per plant was approximately 4 to 5 ml. The control plants were sprayed with same amount of tap-water. To test the effect of the wetter, a blank solution of Tween-80® (0.2% v/v) without CHA, was also applied. The four CHAs, with four concentration levels each, the water control (n=4) and the Tween 80® control (n= 4), were arranged in a completely randomized design with 18 treatments in four replications. Within the glasshouse, the pots were randomly rotated on a weekly basis to minimize positional effects. One week after the application of CHAs, pollen grains were sampled from each plant and analysed for viability/sterility. After screening the potency of the CHAs, to study the effect of the CHAs (that is, E4FO and Ethrel) on female fertility, four additional potted tef plants were sprayed with E4FO and Ethrel at 4 concentration levels each and the plants were subsequently hand cross pollinated, and left unbagged. At the end of the trial season, seeds produced from the mother plants were manually collected, counted and recorded. Difference in seed yield from panicles treated with CHAs followed by bagging to avoid cross pollination (SYPP) and seed set from panicles treated CHAs followed by artificial pollination (SSPP) would indicate the number of viable female organs (stigmas) remaining in the period following the CHAs treatment.

Pollen sterility test

A range of techniques were tested to visualise pollen grains (data not presented). The best technique for differentiating between viable and non-viable pollen grains of *tef* was achieved using an aniline blue lactophenol staining technique. The aniline blue lactophenol solution was prepared by mixing 5 ml of 1% aqueous aniline blue, 20 g phenol crystals ($C_6H_5O_4$), 40 ml glycerol, 20 ml lactic acid and 20 ml of distilled water (Kearms and Inouye, 1993).

A week after the application of the CHAs, plant inflorescences (spikes) were collected and stored in 70% ethanol until slide preparation. Pollen grains from newly dehisced florets were released onto a droplet of aniline blue lactophenol solution on a glass microscope slide. The droplet was covered with a cover slip and the pollen grains were allowed to stain for 3 h at 21°C before scoring. The preparation was then examined under a Zeiss Axio Scope A1 brightfield light microscope (Carl Zeiss, Ltd., Canada) equipped with an AxioCam camera and image captured using Axio Vision 2.05 software (Carl Zeiss Ltd., Canada). It was possible to vividly visualise the pollen grains at 40X magnification, with viable pollen grains appearing solid-blue and non-viable pollen grains appearing turquoise in colour (Figure 1a). A minimum of 300 pollen grains were counted from each sample by selecting random fields of view. Pollen grains from the control plants were also investigated to detect differences in pollen sterility/viability.

Statistical analysis

Levels of pollen sterility were computed by dividing the number of unstained (sterile pollen grains) by the total number of pollen grains (stained plus unstained) per field of view, expressed as a percentage. Data was normalised by square root transformation. The seed yield per panicle (SYPP) was estimated through approximation of the 1000-seed weight following the procedures described by Wiliam (1985). In variety DZ-01-3186, the 1000-seeds weight was found to be 294 mg. To obtain an estimate of SYPP, total harvested seed yield per panicle (weight) was divided by 294 and then multiplied by 1000. Data was subjected to one-way analysis of variance (ANOVA) using GenStat 14th edition Inc. (GenStat, 2011). Duncan's multiple range test procedure was used at $P < 0.05$ to compare the significance of differences among treatment means.

RESULTS

The effects of the four CHAs on levels of pollen sterility and subsequent SYPP are presented in Tables 1 and 2. A low level of pollen sterility (6.68 ± 1.04%) was observed in the untreated control plants. The wetter, Tween 80® had no significant effect on pollen sterility (5.05%). The treatments had a highly significant (P< 0.001) effect on pollen sterility (Table 1). Levels of pollen sterility increased with increases in CHA concentration (Table 3). Compared to the control, all the CHAs and at all concentration levels except 2, 4-D at 10 ppm to 50 ppm induced significant increases in pollen sterility, which ranged between 19.03 ± 1.00% to 99.50 ± 0.50%. Near complete male sterility percentage was achieved by the application of E4FO at rates ranging between 1500 to 3000 ppm and Ethrel at 5000 ppm. The CHAs also had a highly significant effect on SYPP (Table 2). Compared to the control (5564.28 ± 181.88), plants treated with the wetter, Tween 80®, did not exhibit any significant reduction in SYPP. In contrast, plants treated with all levels of E4FO, all levels of Ethrel, and Promalin® at 300 ppm, exhibited the greatest reduction in SYPP, essentially yielding no seed (Table 3). In CHA treated plants, relatively more SYPP was obtained from plants treated with 2, 4-D. Promalin® at rates ranging between 50 to 150 ppm showed a greater reduction in SYPP than those treated with 2, 4-D. Promalin® treated plants showed some phytotoxicity symptoms such as growth

Figure 1. A) Pollen grains of *Eragrostis tef* visualised under light microscope 40X magnification. Non-viable pollen grains (black arrow) and viable pollens (yellow arrows). **B)** Anther of *Eragrostis tef* filled with non-viable pollen grains. **C)** Growth deformity (coiling) caused by Promalin® treatment of tef. **D)** Premature senescence and floret collapse caused by Ethrel and Promalin® treatment.

Table 1. One-way analysis of variance (ANOVA) of the effect of the CHAs on percentage of pollen sterility in *Eragrostis tef.*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatments	17	83002.24	4882.48	225.74	<0.001
Residual	54	1167.97	21.63		
Total	71	84170.21			

Table 2. One-way analysis of variance (ANOVA) of the effect of the CHAs on seed yield per panicle (SYPP) of non-pollinated *Eragrostis tef* plants.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatments	17	345579304	20328194	37.08	<.001
Residual	54	29603056	548205		
Total	71	375182360			

deformity (coiling) and frequent dryness of inflorescences (Table 3 and Figure 1b). Promalin® at a rate of 5000 ppm caused an extreme reduction in seed set, due to floret collapse (Table 3 and Figure 1). At higher doses of 100 and 500 ppm, 2, 4-D treated plants exhibited excessive elongation of the inflorescence and drooping (Table 3). By contrast, treating the tef plants with all rates of Ethrel, and E4FO at a rate of 3000 ppm, led to early premature desiccation and ultimately to floret collapse. *t*-test comparison between the CHAs-treated-pollinated vs CHAs-treated-non-pollinated tef plants showed a highly significant (t = 5.496; P < 0.00) difference in seed set per panicle (SSPP). Artificial pollination of the plants previously treated with E4FO at rates of 1000 to 1500 ppm, showed a significant improvement in SSPP. By contrast, artificial pollination of the plants previously treated with all rates of Ethrel did not show any significant improvement in SSPP (Table 3).

Table 3. The effect of four different chemical hybridizing agents (CHAs) applied at four different concentration levels on pollen sterility, seed yield per panicle (SYPP) and seed set per panicle (SSPP) of *Eragrostis tef* (Zucc.) Trotter, cultivar Etsub (Var: DZ-01-3186). Phytotoxicity symptoms observed on CHA treated plants compared to untreated controls.

CHAs	CHAs concentration (ppm)	Pollen sterility (%)	SYPP+SE of self-pollinated plants	SSPP+SE of artificially pollinated plants	Observations
E4FO	1000	86.41 ± 2.86^{b}	0.00 ± 0.00^{f}	569.7 ± 39.97^{b}	No phytotoxicity symptoms observed
	1500	95.83 ± 4.17^{a}	0.00 ± 0.00^{f}	281.00 ± 27.41^{c}	No phytotoxicity symptoms observed
	2000	98.61 ± 1.39^{a}	0.00 ± 0.00^{f}	241.20 ± 17.17^{cd}	No phytotoxicity symptoms observed
	3000	99.50 ± 0.50^{a}	0.00 ± 0.00^{f}	34.20 ± 5.49^{d}	Floret dryness and early premature senescence
Ethrel	1000	79.00 ± 2.68^{cd}	0.00 ± 0.00^{f}	9.50 ± 3.88^{d}	Floret dryness and early premature senescence
	2000	82.00 ± 6.38^{bc}	0.00 ± 0.00^{f}	3.00 ± 1.91^{d}	Floret dryness and early premature senescence
	3000	83.00 ± 1.47^{bc}	0.00 ± 0.00^{f}	00.00 ± 0.00^{d}	Floret dryness and early premature senescence
	5000	94.12 ± 0.58^{a}	0.00 ± 0.00^{f}	00.00 ± 0.00^{d}	Floret dryness and early premature senescence
2, 4-D	10	9.77 ± 0.47^{i}	5592.17 ± 749.69^{a}	*	Panicle elongation and drooping
	50	13.53 ± 1.31^{hi}	4377.45 ± 976.26^{b}	*	Panicle elongation and drooping
	100	19.03 ± 1.00^{h}	4031.60 ± 308.38^{b}	*	Panicle elongation and lightly seeded panicle
	500	29.94 ± 1.76^{g}	2933.87 ± 673.58^{c}	*	Panicle elongation and lightly seeded panicle
Promalin®	50	54.83 ± 0.73^{f}	218.40 ± 23.78^{e}	*	Twisting inflorescence and leaves, week and premature senescence
	100	57.19 ± 0.94^{f}	197.95 ± 26.99^{e}	*	Twisting inflorescence and leaves week and premature senescence
	150	64.58 ± 1.20^{e}	1390.00 ± 348.24^{d}	*	Twisting inflorescence and leaves week and premature senescence
	300	74.40 ± 2.98^{d}	0.00 ± 0.00^{f}	*	Twisting inflorescence and leaves week and premature senescence
Control I (Tap water spray)	0	6.86 ± 1.04^{i}	5572.64 ± 170.34^{a}	5572.64 ± 170.34^{a}	No phytotoxicity symptoms observed
Control II (Tween 80®)	(0.02% v/v)	6.05 ± 1.64^{i}	5564.28 ± 181.88^{a}	5664.28 ± 181.88^{a}	No phytotoxicity symptoms observed

Values in the same column with shared letter(s) are not statistically different according to Duncan's multiple range test at the 5% level of significance.

DISCUSSION

Hybridization in tef is a tedious and difficult undertaking that relies on hand emasculation. Hand emasculated tef often fail to yield any seed (Berhe and Miller, 1978). Currently, a large number of CHAs are being developed to induce mass emasculation in a number of crops for the purpose of hybrid seed production, or as a substitute for hand emasculation (Tu and Banga, 1998; Praba and Thangaraj, 2005; Chakraborty and Devakumar, 2006b). Results of the present study cast some light on the possibility of using CHAs for tef breeding.

It is interesting that E4FO and Ethrel induced the highest levels of male (pollen) sterility in tef. These two CHAs also induced the greatest reduction in the SYPP of the non-pollinated (bagged) plants. The lack of seed production, together with the high levels of pollen sterility found in plants treated with E4FO and Ethrel indicates the effectiveness of these CHAs in mass emasculation of the male gametes in tef. However, it is not sufficient that a CHA should cause a high level of male sterility, but it should not cause any adverse side effects to the rest of the plant (Chakraborty and Devakumar, 2006a). Therefore, determining the effects of the CHAs on phytotoxicity and female fertility were equally important. Artificial pollination of the plants previously treated with E4FO (at a rate of 1000 – 1500 ppm) resulted in significant increases in seed set per panicle (SSPP). By contrast, Ethrel sprayed plants did not show significant increases in SSPP after artificial pollination. Tef is a predominantly self-pollinating, monogamous plant and the degree of natural outcrossing is less than 1% (Ketema, 1997). These results indicated that the plants sprayed with E4FO at 1000 to 1500 ppm had female organs (stigmas) that remained fertile to alien pollination, but all rates of Ethrel diminished the female fertility in the period following the application of the CHAs.

In the phytotoxicity study the tef plants treated with E4FO at 1000 to 2000 ppm had no detectable adverse effects on growth of tef. By contrast, all levels of Ethrel and E4FO at the highest level of 3000 ppm led to early premature desiccation and ultimately floret collapse. Similar deleterious effects of Ethrel on tef were reported by Berhe and Miller (1978). These authors examined the gametocidal effect of Ethrel on two varieties of tef. In this study, foliar applications of 600, 900, and 1,200 ppm were applied three times during heading and all the treatments were effective in reducing seed set with no signs of phytotoxicity. However, ovary and ovular tissue in sterilized florets underwent premature desiccation and ultimately lead to female sterility. Due to its deleterious effects on female fertility, Ethrel was not recommended as a useful CHA for tef (Berhe and Miller, 1978). The tef plants treated with all levels of 2, 4-D exhibited low levels of male sterility and only a slight reduction in SYPP. This treatment also caused certain undesirable phytotoxic symptoms including panicle elongation and drooping. The tef plants sprayed with all levels of Promalin® showed more pollen sterility and reduction in seed yield, but there were also detectable growth deformities such as stem weakness, coiling and low plant vigour. Therefore, these two CHAs cannot be recommended as viable CHAs for tef breeding.

The lactophenol cotton blue staining technique provided an excellent technique for the visualisation of pollen sterility/viability in tef. E4FO was the most effective CHA, inducing the highest levels of pollen sterility, with the least phytotoxicity. The good seed set in artificially pollinated plants previously treated with E4FO (at 1000 – 2000 ppm) indicates that the plants had viable female reproductive structures that remained receptive to pollen in the period following the application of the CHAs. Hence, it can be recommended that E4FO applied once at a booting stage, and at rates of 1000 to 1500 ppm (a relatively wide application window), can be used as an efficient CHA for inducing high levels of male sterility without adverse effects on female fertility in tef. Further studies are needed to determine the potential application of E4FO in commercial hybrid seed production of tef and for its use in breeding programmes and fine-tune concentrations of E4FO. Given that the effect of CHAs on plant development, growth, morphology and female fertility often shows a characteristic of genotype specificity (Kaul, 1988), further studies are required to screen responses of multiple tef varieties and gametocides at a gradient of concentration levels.

Conclusion

Amongst all the Chemical Hybridizing Agents tested, a single spray of E4FO (1000 - 1500 ppm) induced the highest level of pollen sterility in tef without significant damage on female fertility and least phytotoxicity. Hence, it is recommended that E4FO (at 1000 - 2000 ppm) can be used as a potent chemical emasculation agent for tef. Given that CHAs are genotype specific; the results are applicable for this particular tef variety only.

Conflict of interest

The authors have not declared any conflict of interest.

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