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Genetic linkage between male sterility and non-spiny trait in safflower (*Carthamus tinctorius* L.)

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With 1 figure and 2 tables

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Abstract

Genetic male sterility (GMS) exists naturally in safflower (*Carthamus tinctorius* L.). In the existing safflower GMS lines, sterile and fertile plants are distinguishable at flowering. This causes delay in fertile plants rouging and reduction in hybrid purity. In this investigation, a cross between a spiny GMS parent 13-137 and a spiny non-GMS parent 'A1' was effected. One sib cross, SC-67, producing non-parental-type non-spiny sterile and spiny fertile plants in F_3 was advanced to F_9 through sib crossing between non-spiny sterile and spiny fertile plants. Mendelian digenic segregation was not observed for non-spiny trait and male sterility. The results revealed strong linkage between these traits. The linkage was confirmed in F_2 generations of crosses between a non-spiny marker-linked GMS line (MGMS) and five elite lines. Male sterility–linked non-spiny trait could distinguish sterile and fertile plants at elongation stage. The MGMS would be useful in production of pure F_1 hybrid seed and development of elite populations.

Key words: genetic male sterility — linkage — marker — non-spiny — safflower

Safflower (*Carthamus tinctorius* L.) is a multi-purpose crop grown in over 18 countries, mainly for seed oil. India ranks first in area and production of safflower (Damodaram and Hegde 2010). Argentina, Australia, China, Ethiopia, Mexico and USA are the other important safflower-growing countries. Safflower is predominantly a self-pollinated crop; however, natural crossing to the extent of 56% was reported (Claassen 1950). Honeybee is the major pollinator in safflower (Rubis et al. 1966, Mohan Rao et al. 1980). Hand emasculation to facilitate hybridization is tedious and time-consuming. The discovery of genetic male sterility (GMS), both dominant and recessive types (Heaton and Knowles 1982, Joshi et al. 1983), encouraged safflower breeders to go for commercial hybrids development. Recessive GMS system has been utilized in the development of four commercial hybrids available in India.

Genetic male sterile line produces male sterile and fertile plants (Singh 1996). Several GMS lines are available in safflower (Heaton and Knowles 1980, Anjani 1997b). In the existing GMS lines, the male sterile and fertile sister plants are distinguishable only at flowering (Carapetian 1994). Flowers in male sterile plants appear like a pinched brush and are devoid of pollen, whereas flowers in fertile plants have normal appearance and produce abundant pollen. Genetic male sterile lines are mainly being used in safflower breeding programme for commercial hybrid seed production and development of elite populations as well as in multiple crossing programmes. To avoid hand emasculation that is very labour-intensive and time-consuming, GMS lines are used in multiple crossing and population improvement programmes. In hybrid seed production plot, all fertile plants in the female parent (GMS line) rows have to be rouged out before flowering to avoid the possibility of female selfs in hybrid seed lot that would reduce hybrid purity and its yield. Similar problem was also encountered in population improvement and multiple crossing programmes (Deshmukh et al. 2008, Nandkhile et al. 2008). A preflowering stage marker linked to GMS would facilitate identification and removal of fertile plants prior to flowering, which would eventually overcome the problems indicated above. The objectives of this study were to investigate the linkage between GMS and non-spiny trait and its confirmation.

Materials and Methods

As a part of genetic improvement of GMS lines at the Directorate of Oilseeds Research, Hyderabad, India (17.36°N and 78.47°E), a cross between two spiny parents 13-137 and 'A1' was effected in 2000–2001. Schematic presentation of advancement of various filial generations of (13-137 x'A1') cross is depicted in Fig. 1. The female parent 13-137 is a recessive genetic male sterile line, derived from a cross between a recessive GMS line MS 6 (O) and a wild species, *C. oxyacantha* (2n = 24). *C. oxyacantha* is spiny in nature. The GMS line MS 6 (O) and 'A1' are spiny genotypes belonging to cultivated species (*C. tinctorius,* 2n = 24). F_1 generation of (13-137 × 'A1') was advanced to F_2 through self-pollination. All plants in F_1 were found to be fertile; these were backcrossed to the male sterile plants of 13-137. The F_2 generation was advanced to F_3 through sib crossing between male sterile and fertile sister plants. A total of 102 sib crosses effected in F_2 generation were advanced to F_3. Sib cross-wise planting was carried out in F_3 generation. In F_3 of a sib cross SC-67, all fertile plants were found to be spiny, and all sterile plants were non-spiny. This sib cross was advanced to F_4 generation through sib crossing between non-spiny sterile plants and spiny fertile plants. Two sib crosses, *viz.* F3-SC-6 and F3-SC-7, effected in F_3 of SC-67 were advanced to higher filial generations. F3-SC-6 was advanced up to F_6 through sib crossing, while F3-SC-7 was advanced up to F_9 through both sib crossing and self-pollination.

F3-SC-7, which was advanced through sib crossing up to F_7 generation, is designated as marker-linked GMS line-7 (MGMS-7) from F_7 onwards. MGMS-7 was crossed to five elite spiny lines, *viz.* 'A1', '96-508-2-90', 'SFS-9947', '1706' and '11-103' in 2007–2008. All the five F_1 hybrids were planted in 2008–2009. The F_2 generation of each hybrid was derived through self-pollination of F_1 plants under net-caged condition. The F_2 generations were planted in 2009–2010.

Flower heads in all the experimental plants were covered with butter paper bags from bud stage to harvesting, and additionally, all experimental plots were covered with white nylon mosquito nets to avoid cross-pollination through honeybee, which is the major pollinating agent

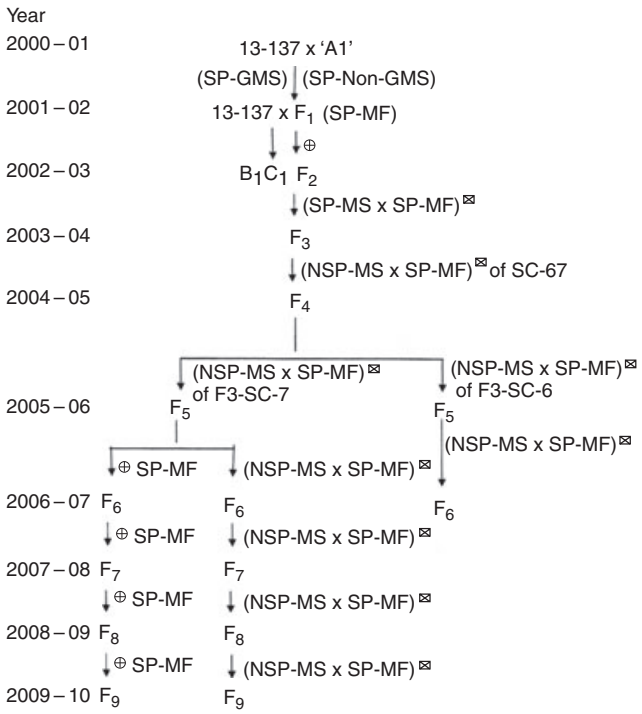

Fig. 1: Schematic presentation of advancement of various filial generations of (13-137 × 'A1') cross. GMS, genetic male sterile; SP–MF, spiny fertile; NSP–MS, non-spiny sterile; ⊠: sib cross; ⊕: self-pollination

in safflower. Seeds of each sib cross as well as of fertile plants were harvested and grown separately in each generation.

The seed of the parent 'A1' and other elite lines, *viz.* '96-508-2-90', 'SFS-9947', '1706' and '11-103', were produced by self-pollination under net cages. Male sterility in the female parent 13-137 is a monogenic recessive trait. It was maintained through sib crossing between male sterile (msms) and fertile (MSms) plants. The homozygous male fertile (MSMS) plants were eliminated from the population of 13-137 following the procedure standardized at the Directorate of Oilseeds Research (Anjani 2005). In this procedure, intensive pair-wise sib crosses will be effected initially between randomly selected male sterile (msms) and male fertile (MSms) plants of a GMS line where male sterility is monogenic recessive. Each sib cross progeny row and the fertile parent plant progeny rows will be grown in the subsequent year. The sib cross progeny rows and fertile parent progeny rows that produce only fertile plants will be discarded as the fertile plant used in the sib cross is a homozygous dominant (MSMS) for fertility. The sib cross progeny rows giving both sterile and fertile plants will be retained as the fertile plant used in the sib cross is heterozygous for fertility (MSms). In these rows, sib crossing will be effected between male sterile and fertile plants. Each sib cross progeny rows will be grown in the following year to confirm 1 : 1 segregation of male sterile and fertile plants so as to verify heterozygous nature of the fertile parental plants. Then the seed from sterile plants will be bulked to constitute the seed of GMS line. This seed will be further used for maintenance of GMS line as it comprises of male sterile (msms) and only heterozygous fertile (MSms) genotypes.

Male sterility was assessed by visual observation of presence or absence of pollen and by squashing anthers prior to anthesis in acetocarmine for stainability of pollen grains under microscope. Male sterility percentage was calculated by using the formula:

$$(\text{Number of male sterile plants}/\text{Total plants}) \times 100.$$

Data were subjected to chi-square analysis to determine the goodness of fit to different genetic ratios in various generations, and recombination values (%) were estimated using INDOSTAT statistical software (INDOSTAT Services, Hyderabad, India).

Results

Both parents of (13-137 × 'A1') cross and the four elite lines, *viz.* '96-508-2-90', 'SFS-9947', '1706' and '11-103', bred true for all morphological traits including spiny trait. There was 1 : 1 segregation of male sterile and fertile plants in the maintenance block of the GMS line 13-137, and sterile plants were not observed in 'A1', '96-508-2-90', 'SFS-9947', '1706' and '11-103'.

Sterility in male sterile plants was absolute. All flower heads in male sterile plants were male sterile and remained sterile through to harvesting. Anthers were rudiment, and pollen was absent in male sterile plants, whereas abundant, well-stained, uniform-sized pollen grains were observed under microscope in fertile plants.

F_1, F_2 and backcross generations

All plants in F_1, F_2 and backcross generations of (13-137 × 'A1') cross were spiny. In F_1 generation, all plants were fertile, while in F_2, there were fertile and sterile plants in 3 : 1 ratio ($\chi^2 = 0.118$; P = 0.70–0.75). Of 102 sib crosses made in F_2, 81 produced male sterile and fertile plants in 1 : 1 ratio ($\chi^2 = 0.018$; P = 0.85–0.90) in F_3 generation; the remaining 21 sib crosses produced only fertile plants. Male sterile and fertile plants segregated in 1 : 1 ratio when F_1 was backcrossed as pollen parent to male sterile plants of 13-137.

F_3 and F_4 generations

Among 1223 F_3 plants of 102 sib crosses made in F_2 generation, 20 were of non-parental type (non-spiny). Two of these non-spiny plants were fertile and 18 were male sterile. These were the progenies of three sib crosses, *viz.* SC-67, SC-71 and SC-75. In F_3 of SC-67, there were sterile and fertile plants; all the sterile plants were non-spiny, while all the fertile plants were spiny. In F_3 of SC-71, sterile and fertile plants were both spiny and non-spiny (two spiny male fertile, two non-spiny male fertile, one spiny male sterile and seven non-spiny male sterile). All fertile plants in F_3 of SC-75 were spiny, whereas the sterile plants were both non-spiny and spiny (one spiny and one non-spiny male sterile).

Of the 12 sib crosses made among non-spiny male sterile and spiny fertile F_3 plants of SC-67, two sib crosses, *viz.* F3-SC-6 and F3-SC-7, produced only parental-type plants (non-spiny sterile and spiny fertile) in F_4 generation, while the remaining 10 sib crosses produced both parental and recombinant (spiny sterile and non-spiny fertile) types.

F_5–F_9 generations

Joint segregation of male sterility and non-spiny trait was observed in F_5 to F_9 generations of F3-SC-7 and in F_5 and F_6 generations of F3-SC-6 (Table 1). F_5 and F_6 generations of F3-SC-6 were derived through sib crossing between non-spiny male sterile and spiny fertile plants. F3-SC-6 produced parental types along with recombinant types (non-spiny fertile and spiny sterile) with $4.12 \pm 0.8\%$ recombination value in F_5 and $4.85 \pm 0.84\%$ in F_6. When different filial generations of F3-SC-

Table 1: Joint segregation of male sterility and non-spiny trait in various filial generations of F3-SC-6 and F3-SC-7

Filial generations of F3-SC-6 and F3-SC-7[1]	Number of plants[2]				Male sterility (%)	Recombination value (%)	Filial generations of F3-SC-7[3]	Number of plants[4]				Male sterility (%)	Recombination value (%)
	MF		MS					MF		MS			
	SP	NSP	SP	NSP				SP	NSP	SP	NSP		
	P	R	R	P				P	R	R	P		
F_5 generation							F_5 generation						
F3-SC-6	319	23	5	331	49.5	4.12 ± 0.80							
F3-SC-7	25	0	0	33	56.8	0.0	F3-SC-7	157	0	0	51	24.5	0.0
F_6 generation							F_6 generation						
F3-SC-6	831	61	24	834	49.0	4.85 ± 0.84							
F3-SC-7	362	0	0	355	49.5	0.0	F3-SC-7	187	0	1	67	26.6	0.39 ± 0.12
F_7 generation[5]							F_7 generation						
F3-SC-7	1262	0	3	1305	50.8	0.11 ± 0.09	F3-SC-7	178	1	0	59	25.1	0.41 ± 0.16
F_8 generation							F_8 generation						
F3-SC-7	1391	0	4	1410	50.4	0.14 ± 0.06	F3-SC-7	242	1	1	79	24.7	0.30 ± 0.11
F_9 generation							F_9 generation						
F3-SC-7	1287	0	4	1321	50.7	0.15 ± 0.09	F3-SC-7	342	1	2	118	25.9	0.64 ± 0.18

MF, male fertile; MS, male sterile; SP, spiny; NSP, non-spiny; P, parental type; R, recombinant type.
[1]Filial generations were derived through sib crossing between non-spiny sterile and spiny fertile plants.
[2]Combined data of all sib crosses in each generation.
[3]Filial generations derived through self-pollination of fertile plants.
[4]Combined data of all fertile plants in each generation.
[5]F3-SC-7 derived through sib crossing was designated as MGMS-7 from F_7 onwards.

7 were derived through sib crossing, recombinant-type plants were absent in F_5 and F_6, but appeared at negligible frequency in F_7 to F_9 (3–4 plants) with 0.0 to 0.15 ± 0.09% recombination values in various generations. Male sterility percentage in F_5–F_9 generations of F3-SC-7 was 49.5% to 56.8%, while it was 49.5% in F_5 and 49% in F_6 generation of F3-SC-6. Independent Mendelian digenic segregation for male sterility and non-spiny trait was not found in any filial generations of F3-SC-6 and F3-SC-7.

When filial generations (F_5–F_9) of F3-SC-7 were derived through self-pollination of spiny fertile plants, the recombination value was 0.0 to 0.64 ± 0.18 in various generations. Digenic segregation for male sterility and spiny traits was not observed in any self-generations of fertile plants. Parental types appeared significantly in higher frequency than recombinant types.

Hybrids

All five F_1 hybrids of the crosses between non-spiny marker-linked genetic male sterile line MGMS-7 and five elite lines, *viz.* '96-508-2-90', 'SFS-9947', '1706', '11-103' and 'A1', were spiny and fertile. In F_2 generation of the hybrids, male sterility and non-spiny trait showed joint segregation; Mendelian digenic segregation was not observed for these traits ($\chi^2 > 0.001$). Recombination value in F_2 generations ranged from 0.007 ± 0.001 to 0.031 ± 0.019% (Table 2).

Characteristics of non-spiny trait-linked male sterile plants

Spines appeared on leaves of 40- to 45-day-old fertile plants but not on leaves of male sterile plants, thus male sterile plants could be differentiated from fertile plants at 40–45 days after planting that was about 45–50 days prior to flowering. The non-spiny marker-linked male sterile plants morphologically resembled their sister fertile plants in all aspects, except for non-spiny trait and male sterility. Flower heads exhibited pinched brush–like appearance. Plant height, flower shape, opening of flower heads and florets and seed hull of sterile plants were not altered due to the linkage. Germination of seeds from sterile plants was normal and 100%. Pollen was absent in sterile flower heads as anthers were either not developed or rudimentary. There was no reversion of male sterility to fertility in any generation. Male sterile plants failed to set seed when flower heads were bagged. Sterile and fertile plants flowered in 80–90 days and matured in 120–130 days after planting.

Discussion

Natural occurrence of GMS was reported in cultivated safflower (Deshpande 1940, Carapetian and Knowles 1976, Hamadan et al. 2008). Joshi et al. (1983) reported gamma-rays induced dominant GMS. The first commercial safflower hybrid in India (Anjani 1997a) was developed using a recessive GMS line (Anjani 1997b). Recessive GMS was also found in pigeon pea (Reddy et al. 1978), cotton (Singh and Kumar 1993), barley (Hockett and Eslick 1967), wheat (Athwal et al. 1967) and in many other crops.

The 1 : 1 segregation of male fertile and sterile plants in the maintenance block of the genetic male sterile line 13-137 was because of elimination of homozygous (MSMS) fertile plants of 13-137. Absence of sterile plants in 'A1', '96-508-2-90', 'SFS-9947', '1706' and '11-103' indicates homozygous state (MSMS) of fertility in these non-GMS genotypes.

The results confirmed the monogenic recessive inheritance of both male sterility and non-spiny trait. It was in accordance with the earlier findings (Heaton and Knowles 1982, Singh 1996, Venkoba Rao 1943). The present study revealed strong linkage between male sterility and non-spiny trait. Absence or occurrence of very few recombinant types indicates the linkage is in coupling phase. Strong linkage between nuclear genes governing male sterility and high linoleic acid content was reported in safflower (Hamadan et al. 2008). Singh (1997) reported recombination value as low as 0.72 ± 0.73% and as high as 7.97 ± 1.97% between nuclear genes governing male sterility and dwarfness in safflower. Strong linkages were also reported in other crops like cotton (Rhyne 1957), rice (Yakoo and Fujimaki 1971), turnip rape (Hawk 1982, Stringam 1977), cucumber (Vakalounakis 1993), tomato (Vakalounakis et al. 1997) and black gram (Arshad et al. 2005).

A few attempts were made earlier to identify male sterility–linked seed or seedling marker in safflower. Ebert (1964) reported thin hull–associated structural sterility. This sterility could not be exploited due to occurrence of 10–33% female selfs in harvested seed lots of hybrids (Urie and Zimmer 1970). Carapetian and Knowles (1993) reported genetic linkage between trigenic male–female sterility and oil quality alleles in safflower. This linkage was of no use as both male and female were affected. Hamadan et al. (2008) reported repulsion-phase linkage between high linoleic acid (*Li*) and GMS (*Ms*) loci with 0.09 recombination value. However, oil quality allele–linked male sterility cannot be feasible and economical for commercial hybrid

Table 2: Joint segregation of male sterility and non-spiny trait in F_2 generations of crosses between non-spiny marker-linked GMS line-7 (MGMS-7) and five elite lines

	Number of plants in F_2 generation					
	MF		MS			
	SP	NSP	SP	NSP		
F_1 hybrid	P	R	R	P	χ^2 (9 SP-MF:3 NSP-MF: 3SP-MS:1NSP-MS)	Recombination value (%)
MGMS-7 × 96-508-2-90	147	1	1	51	200 (>0.001)	0.010 ± 0.008
MGMS-7 × SFS-9947	139	2	1	45	170 (>0.001)	0.016 ± 0.004
MGMS-7 × 1706	94	3	1	29	81 (>0.001)	0.031 ± 0.019
MGMS-7 × 11-103	201	1	1	66	257 (>0.001)	0.007 ± 0.001
MGMS-7 × 'A1'	212	0	0	65	255 (>0.001)	0.0

MF, male fertile; MS, male sterile; SP, spiny; NSP, non-spiny.
Figures in parentheses are probability values.

seed production. Weisker (1995) patented a genetic dwarf male sterility system for hybrid safflower production. In this system, a male sterility gene has pleiotropic effect on plant morphology that dwarfs the plant, which allows early and effective removal of normal fertile plants in hybrid seed production. No commercial hybrids produced using this system are available to date. Similar male sterility system reported by Singh (1996) was not found useful for hybrid seed production because of the occurrence of high number of dwarf fertile plants in F_1 resulting in reduction in hybrid purity and seed yield (Anjani 2003).

The non-spiny trait-linked male sterility reported here is a natural mutant. Natural mutations in safflower have been reported earlier also. Two natural mutations, thin hull and striped, and a natural sterile mutant lacking normal development of floral parts have been isolated in safflower (Rubis 1967). Deshpande (1940) described a natural sterile mutant in safflower that failed to produce flowers or seeds. The dwarf sterile safflower mutant is also a natural mutant. Ebert and Knowles (1966) suggested that hybridization between certain safflower strains may stimulate mutations.

In safflower, spines appear on margins and tips of leaves on stems and branches at elongation stage when plants are 40–45 days old depending on genotype (Deokar et al. 1974). As the non-spiny marker could distinguish sterile and fertile plants at elongation stage, it qualifies as an early growth stage marker. Non-spiny marker facilitates visual identification of male sterile plants and provides enough time for rouging of spiny fertile plants before flowering. Thus, genetically pure F_1 hybrid seed could be produced and full potential of hybrids could be realized due to absence of sterile plants and female parent selfs in F_1 hybrid. All these factors and normal plant type of male sterile plants make the non-spiny marker-linked GMS advantageous over the other reported GMS systems. This can also be used in the development of elite populations in population improvement programmes.

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