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# Characterisation and utilization of three distinct male sterile systems in marigold (*Tagetes erecta*)

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ABSTRACT

Three distinct male sterile systems were identified in marigold (*Tagetus erecta* L.) and classified as apetaloid, petaloid and gynomonoecious types based on their floral morphology. Detailed study indicated differences in their inheritance pattern, maintenance and utilization. Apetaloid male sterility was controlled by single recessive gene. Petaloid and gynomonoecious male sterile systems were under the control of cytoplasmic inheritance. Apetaloid sterile lines were maintained by intercrossing between sterile and fertile plants within the line. Petaloid and gynomonoecious lines were maintained by vegetative propagation. Besides utilizing male sterile system for hybrid seed production, the flowers of petaloid male sterile lines were found to be of commercial importance considering the flower head filled with all ray florets. SCAR marker was able to distinguish petaloid sterile types from apetaloid and gynomonoecious. For the first time, new male sterile systems are being reported in marigold with multiple sterile lines having significant commercial importance.

**Key words:** Apetaloid, Cytoplasmic male sterility, Hybrid, Male sterility, Marigold, Marker

Strategies and objectives in flower crop breeding remains entirely different from majority of other crops with the focus on end product as flowers and not fruit or seed as the case in most of the food crops. Flowers that can remain without senescence on plant for long duration and with longer shelf-life after harvest are the priority characters aimed in breeding of flower crops. Senescence of flowers is mainly attributed to ethylene, presence of which leads to shortening flower life and loss of bright colour (Jiang 2000). Flower senescence is regulated by increased amount of ethylene production following pollination and fertilization (Halevy *et al*. 1984, Serek *et al.* 1995). With no functional pollen, male sterile flowers in turn are expected to have longer life. Pollen production as well as fertilization leading to production of fruit and seeds is essential for food crops, including fruits and vegetables. On the contrary, fruit and seed are not required for a flower crop, and production of pollen is undesired investment for flower crop. Thus, male sterile line is advantageous and desired in flower crops not just as a parent for production of hybrid seeds; but as a variety itself provided the flowers are attractive. Male sterility as a mechanism to enhance flower longevity can be a viable approach in marigold (*Tagetes erecta* L.) breeding program besides its utility in hybrid seed production. Male sterile line reported in marigold consisted of deformed flowers without petals and are unattractive (He *et al*. 2009). Only one type of sterility is reported in marigold till date (Gupta *et al.* 1999, He *et al*. 2009, 2010, Ai *et al*. 2014) and identification of alternate male sterile systems with attractive floral forms will be of significance particularly considering its utility as flowers. The present work was carried out with an objective to identify different male sterile systems which can be utilized as variety itself besides their utility in hybrid seed production.

Apetaloid male sterile (flowers without petals) line identified in marigold is reported to be genetic male sterile system controlled by single recessive gene in the nucleus (Gupta *et al.* 1999, He *et al.* 2009). Utilization of genetic male sterility in commercial hybrid seed production has limitations considering the resource that need to be invested for maintenance of both, sterile and fertile plants till they come to flowering and are distinguishable. Alternate attempts were made to multiply sterile plants by vegetative propagation through *in vitro* culture so as to avoid the cost of rouging fertile plants (Kumar *et al.* 2004). The present work on identification of different male sterile system also had an objective of working out alternate propagation protocols for their efficient utilization.

Molecular marker assisted selection is suggested as an alternate approach in genetic male sterile system for identification of male sterility at early stage and rouging of fertile plants even before flowering. He *et al.* (2009, 2010)

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reported SCAR markers for monogenic apetaloid male sterile marigold line allowing for early selection of sterile plants. In our present work we have attempted to validate the utility of reported SCAR marker for characterisation of male sterile systems identified by us.

Identification of diverse source of male sterility within a crop species is essential to avoid inherent risks associated with single source in breeding programs. In this paper we report three morphologically distinct male sterile systems identified in our marigold breeding program at Indian Institute of Horticulture Research, Bengaline, India. Isolated male sterile lines have been characterized and efficient maintenance methods were worked out for utilization of selected lines.

## MATERIALS AND METHODS

Plant materials for the present study were selected from progeny population of ongoing marigold breeding program at Indian Institute of Horticulture Research, Bengaluru, India located at $13^0$58'North latitude and $78^0$East latitude at an altitude of 890M. Thousands of progeny plants resulting from distant hybridization program during 2010-2015 were screened and male sterile plants were identified, stabilized and charcterized.

Apetaloid, petaloid and gynomonoecious were the three types of male sterile systems identified in the breeding program based on floral morphology. Sterile plants isolated in the breeding program were multiplied by repeated selfing, crossing and stabilized into lines. Stabilized male sterile lines were classified into respective sterile system based on floral morphology and detailed study was taken up in comparison with fertile lines. Male sterile lines selected for the present study consisted of two apetaloid (flowers without petals) sterile lines, IIHR 10521 and IIHR 10572; two distinct petaloid sterile lines, IIHR 14 and IIHR 48; and a novel gynomonoecious line IIHR 5673. For comparison, two male fertile lines IIHRMYs1 and IIHRMY4 were used in the study. IIHRMYs1 had single row of ray florets and considered as single flower, whereas IIHRMY 4 had multiple rows of ray florets and considered as double flower.

Floral morphology was observed macroscopically as well as under light microscope. Sterile plants were crossed in different combinations for genetic analyzes of different male sterile systems. For apetaloid sterility, intercrossing between fertile and sterile plants within the line was attempted along with selfing of fertile plants of the same line. In case of petaloid and gynomonoeicious sterility, crossing with different fertile lines was attempted to study the inheritance of sterility. Number of fertile and sterile plants, was counted to assess the segregation pattern in progenies of self, cross and intercross to analyze the inheritance pattern. Frequency of sterile and fertile plants was compared using chi-square tests for contingency tables (Quinn and Keough 2002).

For marker studies, genomic DNA was extracted from young leaves of bulk samples using CTAB method (Doyle and Doyle 1990) with modifications. Bulk segregant analysis (BSA) with different male sterile systems in comparison with fertile was followed in the study. In case of apetaloid sterile system, sterile and their counterpart fertile plants in both IIHR10521 and IIHR10572 were bulked separately and used in the analysis. PCR was performed with 25µl reaction volume containing 60ng template DNA, 200 µM dNTPs, 1.5mM $MgCl_2$, 1X Buffer, 0.5U Taq DNA polymerase (Genei), 0.4µM of each forward and reverse SCAR primers SC4 and SCS48 (He *et al*. 2009*,* 2010). Amplified products resulting from the PCR protocol (2 min at 94°C; 30 cycles of 94°C for 1 min, 59°C for 1 min, 72°C for 45 sec; one cycle of 72°C for 10 min) were resolved on 2% agarose gel.

## RESULTS AND DISCUSSION

Identification of right male sterility system depending upon crop is essential for utilization in hybrid development (Saxena *et al.* 2010, Patel and Tikka 2014). Distinct types of morphological sterility have been characterized in different crops (Wolyn and Chahal 1998) and are in commercial use. In our study we are reporting three distinct male sterility types identified for the first time in marigold based on their floral morphology and inheritance pattern.

*Flower morphology and functionality*

Apetaloid male sterile lines: Sterile flower with no petals and all the floral organs turned into filament like structures was termed as apetaloid sterility. Homeotic conversion of floral organs into filament like structure forming apetaloid sterile flowers was reported in marigold. Apetaloid sterile lines IIHR10521 and IIHR10572 isolated in our breeding program with no petal were found to be morphologically similar to that reported earlier (He *et al.* 2009, 2010). Flower capitulum had degenerated ray and disc florets. Petals were completely absent in these flowers. All the florets appeared similar consisting of well-developed gynoecium with a style ending into well-formed stigmatic lobes. Androecium as well as petals was degenerated in to filament like structures. Flowers were male sterile with the absence of anther and devoid of pollen. Gynoecium was fully functional and was capable of setting seed when left for open pollination and crossed with pollen from fertile lines.

Sterile flowers are easily distinguishable because of its distinct morphology making it easier to rogue the fertile plants and to retain only male sterile plants. Distinct floral morphology makes the sterile system commercially feasible for utilization in hybrid seed production.

*Petaloid male sterile lines*: Attractive and distinct male sterile lines IIHR14 and IIHR 48 identified in the study had petaloid flowers. Stamens replaced by petals leading to petaloid sterility were reported even in carrot and is being efficiently utilized for production of hybrid seeds (Morelock *et al.* 1996, Wolyn and Chahal 1998). In the petaloid sterile lines, flower capitulum was devoid of disc florets and filled with ray florets. Each ray floret was like a petal with functional gynoecium having a style ending with well-formed stigmatic lobes and devoid of androecium. Devoid of androecium, flowers were completely male sterile. Petaloid

male sterile lines IIHR 14 and IIHR 48 produced attractive flowers, filled with only ray florets and were found to be ideal for flower production considering the reports on male sterility contributing for prolonged shelf life (Serek *et al*. 1995, Alan *et al.* 2004). With every ray floret having functional gynoecium, the flowers were capable of setting seed when left for open pollination and crossed with pollen from fertile lines. Beside their potential as seed parent for hybrid seed production this particular sterile system had added benefit of potential usage as a variety itself.

*Gynomonoecious line*: IIHR 5673 was a novel line with two types of flower capitulum, i.e. petaloid and hermaphrodite flower being present on the same plant. In the early stage of plant growth, petaloid male sterile flowers were produced by gynomonoeicous plants similar to petaloid male sterile lines. As the plant grows, the basal and side branches produced hermaphrodite flowers. Hermaphrodite flowers had ray florets with functional gynoecium and disc florets with functional androecium and gynoecium. Fully grown plant had both petaloid and hermaphrodite flowers existing on the same plant rendering the status of gynomonoeicous. Petaloid flowers were male sterile but capable of seed setting when cross pollinated. Hermaphrodite flowers having ray and disc florets, produced abundant pollen and were capable of seed setting when self or cross pollinated. Our observation of gynomonoecious plants adds marigold to the existing list of unique plant species that express sexual polymorphism (Charlesworth and Laporte 1998, Korpelainen 1998, Mathilde *et al.* 2010, Adam *et al.* 2011).

*Fertile lines***:** Male fertile plant had all hermaphrodite flowers with normal ray and disc florets. Each Ray floret had gynoecium having a style ending with well-formed stigmatic lobes and devoid of androecium. Disc florets had both androecium and gynoecium with well-developed anthers and stigmatic lobes respectively. Flowers with abundant pollen are capable of self and cross fertilization.

*Genetics and maintenance*

*Apetaloid male sterile lines*: These two-type system of apetaloid lines IIHR 10521 and IIHR 10572 were developed by selected intercrossing over generations and were stabilized with fertile and sterile plants consistently segregating in 1:1 ratio when intercrossed and selfing of fertile plants resulting in 3:1 ratio of fertile to sterile plants (Table 1).

Segregation pattern observed in intercross and selfed population from both IIHR 10521 and IIHR 10572 confirmed apetaloid sterility being controlled by single recessive gene. Apetaloid sterile plants were homozygous recessive (msms) and fertile plants of sterile line were heterozygous (Msms). They were maintained by allowing for intercrossing between sterile and fertile plants and collecting the seeds only from sterile plants ensuring segregating progeny of 1:1 fertile and sterile plants. Apetaloid sterility trait being under the control of single recessive gene was in confirmation with the earlier reports by various groups (Gupta *et al.* 1999).

*Petaloid male sterile lines*: Crossing of petaloid male sterile lines (IIHR 14 and IIHR 48) with pure fertile lines resulted in progenies of all petaloid sterile plants (100%) and indicated the inheritance being cytoplasmic male sterility. Similarly, petaloid male sterility being cytoplasmic male sterile type was reported in carrot (Wolyn and Chahal 1998). Cytoplasmic male sterility has been identified and characterized in over 150 plant species (Schnable and Wise 1998). Identification of appropriate fertile cytoplasm line for maintenance of cytoplasmic male sterile line is essential for seed propagated lines. However, both the lines (IIHR 14 and IIHR 48) identified in our breeding program could be maintained by vegetative propagation there by avoiding the necessity of maintainer line. Both the lines (IIHR 14 and IIHR 48) could be maintained by vegetative propagation through tip cuttings. Petaloid male sterility being cytoplasmic male sterile line is advantageous over apetaloid genetic male sterility overcoming the problem of roughing male fertile plants.

*Gynomonoecious line*: Crossing of petaloid sterile flowers of gynomonoecious line (IIHR5673) with fertile line resulted in progeny of all petaloid sterile plants (100%) indicating possible cytoplasmic inheritance of male sterility. Intercrossing between sterile and hermaphrodite flowers within the plant as well as selfing of hermaphrodite flowers resulted in a progeny of petaloid sterile (66.5%), fertile (19.3%) and gynomonoecious (14.2%) plants. The ratios between sterile, fertile and gynomonoecious plant types were found to be varying generation to generation and no fixed ratio could be attained from efforts on selfing and intercrossing. Varying segregation ratios of sterile, fertile and gynomonpecious plants observed in progenies resulting from selfing, crossing and intercrossing indicated cytoplasmic inheritance of male sterility along with presence of incomplete fertility restorer genes in gynomonoecious system. Varying ratios of fertile and sterile plants are reported to occur in cytoplasmic inheritance of sterility being under the influence of incomplete restorer genes (Haan *et al.* 1997). The gynomonoecious line IIHR5673 could be maintained by vegetative propagation through tip cuttings.

Table 1 Segregation of fertile and sterile plants in crosses involving male sterile lines of marigold

| Cross | Generation | Fertile plants (no) | Sterile plants (no) | Segregation ratio (fertile:sterile) | Chi-square value | P value |
|---|---|---|---|---|---|---|
| IIHR 10521(s) × IIHR 10521(f) | F1 | 83 | 74 | 1:1 | 0.52 | > 0.05 |
| Self of IIHR 10521(f) | F2 | 70 | 19 | 3:1 | 0.63 | > 0.05 |
| IIHR 10572(s) × IIHR 10572(f) | F1 | 134 | 145 | 1:1 | 0.43 | > 0.05 |
| Self of IIHR 10572(f) | F2 | 13 | 35 | 3:1 | 0.11 | > 0.05 |

Amenability of plants for vegetative propagation was limited to petaloid and gynomonoecious line, and we could not succeed in our attempts to propagate either apetaloid sterile lines or fertile lines through vegetative propagation. Physiological and biochemical factors underlying the ability of certain lines to propagate by vegetative propagation and their association with sterility will be of interest and can be of future line of research.

The standard explanation of gynomonoecious plants is that they represent the developmental outcome of an incomplete restoration by nuclear male fertility genes of male-sterilizing cytoplasm (Koelewijn and Van-Damme 1995). Heteroplasma had also been suggested as the possible reason for intra-individual variation in gender expressions (Anderson 1999). Hormones known to be intricately involved in gender expression may be acting as a switching mechanism (Sarah 1999). It would be of interest to investigate further, the cause and effect responsible for changing gender within a plant so that mechanism can be effectively utilized in breeding program.

*Fertile lines*: IIHRMYs1 and IIHRMY4 were stabilized homozygous fertile lines, resulted in 100% of fertile plants when selfed. They are seed propagated and maintained by selfing.

*Analysis of SCAR markers linked to sterility*

Apetaloid sterile lines isolated in our breeding program were observed to be morphologically similar to that reported earlier along with the inheritance governed by single recessive gene (He *et al.* 2009). With the similarity in morphology and inheritance, we expected the reported markers (He *et al*. 2009, 2010) to distinguish our apetaloid sterile lines IIHR 10521 and IIHR 10572 from that of others. The first set of SCAR primer (SCS48F and SCS48R) derived from SRAP primers (He *et al.* 2009) gave a monomorphic banding pattern without any differentiation among the lines studied (Fig 1). SCS48 primer set showed no polymorphism and produced monomorphic band of 460bp irrespective of sample.

The second set of SCAR primers (SC4F and SC4R) derived from AFLP (He *et al*. 2010) showed polymorphism. However unlike as it was reported, the SC4 primer set failed to distinguish apetaloid sterile lines, but produced distinct banding pattern for petaloid sterile lines IIHR 14 and IIHR 48. SC4 primer set produced two bands of 500bp and 300bp differentiating petaloid sterile types from fertile and apetaloid sterile types (Fig 2). 500 band was present in all except in petaloid varieties. 300bp band was obtained in petaloid male sterile varieties as well as in one of the fertile lines. Genes governing sterility may differ over populations. Male sterile lines isolated were result of the breeding program that had several of the genes intermingled. Wide hybridization might have been a reason for occurrence of several recombinants and there by resulting in markers being different for different sterility population. For instance, male sterility in marigold is reported to be governed by monogenic (Gupta *et al.* 1999, He *et al.* 2009) in case of *Tagetes erecta*

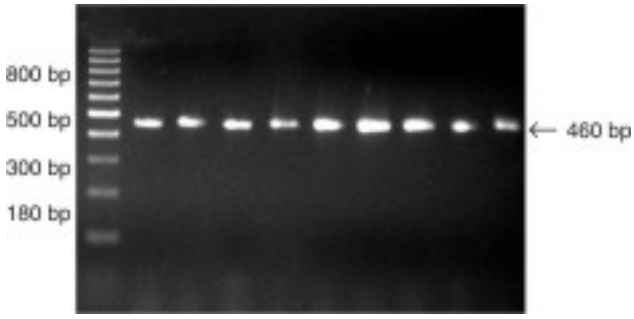


Fig 1 DNA profile of different male sterility systems using SCAR marker (SCS48). Lane1-100bp Ladder; Lane 2- Sterile plants bulk of Apetaloid sterile line IIHR10521; Lane 3- Fertile plants bulk of Apetaloid sterile line 10521; Lane 4- Sterile plants bulk of Apetaloid sterile line IIHR10572; Lane 5- Fertile plants bulk of Apetaloid sterile line IIHR10572; Lane 6- Bulk of Petaloid sterile line IIHR14; Lane 7- Bulk of Petaloid sterile line IIHR48; Lane 8- Bulk of Gynomonoecious line 5673; Lane 9- Bulk of fertile line IIHRMYs-1; Lane 10- Bulk of fertile line IIHRMY-4. The arrow indicates monomorphic band (460bp) present in all the samples.

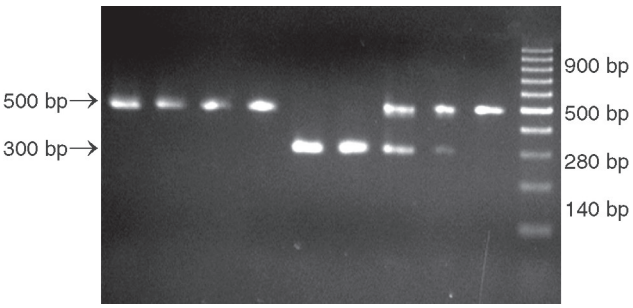


Fig 2 DNA profile of different male sterility systems using SCAR marker (SC4).; Lane 1- Sterile plants bulk of Apetaloid sterile line IIHR10521; Lane 2- Fertile plants bulk of Apetaloid sterile line 10521 ; Lane 3- Sterile plants bulk of Apetaloid sterile line IIHR10572; Lane 4- Fertile plants bulk of Apetaloid sterile line IIHR10572; Lane 5- Bulk of Petaloid sterile line IIHR14; Lane 6- Bulk of Petaloid sterile line IIHR48; Lane 7- Bulk of Gynomonoecious line 5673; Lane 8- Bulk of fertile line IIHRMYs-1; Lane 9- Bulk of fertile line IIHRMY-4. Lane10-100bp Ladder. The arrow indicates polymorphic 500bp band absent in petaloid sterile plants but present in all other lines.

and digenic in case of *Tagetes patula* (Ai *et al.* 2014).

SCS48 and SC4 markers reported to be molecular marker for sterility (He *et al*. 2009, 2010) could not be used in apetaloid male sterilie lines isolated in our program as these markers failed to differentiate apetaloid sterile plants from their fertile type. Further investigation in identification of right molecular marker for apetaloid sterile lines will be of significance for efficient exploitation of apetaloid sterility in marigold breeding program.

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