



Assessment of clonal fidelity of doubled haploid line of marigold (*Tagetes erecta*) using microsatellite markers

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ABSTRACT

In vitro multiplied plants of doubled haploid line of marigold (*Tagetes erecta* L.) derived through ovule culture, were subjected to evaluation of genetic uniformity using SSR markers in order to evaluate their genetic stability and/or detect likely existing variations among *in vitro* derived plantlets. Leaf samples were taken from randomly selected 13 plants at post acclimatization stage, for checking the clonal fidelity of the *in vitro* derived regenerants by comparing them with the mother plant from which the leaf explants were taken. Out of 36 primers, 12 showed amplification in which clear and reproducible PCR profiles. Analysis of individual primers revealed that SSR primers produced same banding pattern which were shared by the *in vitro* leaf derived regenerants and the donor mother plant, which indicated that there was no variation observed within the tissue culture raised plants. All the banding profiles produced were monomorphic and were similar to those of the mother plant, and hence, showed that plants were true to type. Information presented in the study indicated that the *in vitro* regenerated plants were identical to the mother plant.

Key words: Doubled haploid line, *In vitro* regeneration, SSR markers, *Tagetes erecta*

Amongst commercial loose flower crops, marigold (*Tagetes erecta* L.) belonging to the family Asteraceae, native of Mexico, has large spectrum of commercial potentialities in several sectors. Genus *Tagetes* is reported comprise of approximately 55 species (Godoy-Hernandez and Miranda-Ham 2007), out of which, *Tagetes erecta* L. (African marigold) and *Tagetes patula* L. (French marigold) are of commercial importance. Marigold ranks first amongst loose flower crops grown in India with respect to area and production. It is being cultivated in an area of 66.13 thousand hectares with the production of 603.18 thousand metric tonnes (Anonymous 2017). Marigold is a sexually propagated crop, asexual propagation is also successful using herbaceous shoot-tip cuttings. Conventionally, marigold is propagated by seed, produced after controlled pollination. Plant breeding can be done by various tools, of which tissue culture, particularly haploid and double haploid technology,

is extremely valuable in plant breeding and genetics. The potential of haploidy for plant breeding arose in 1964 with the achievement of haploid embryo formation from *in vitro* culture of datura anthers (Guha and Maheshwari 1964). In double haploid production system, homozygosity can be achieved in single generation, hence, eliminating several generations of self-pollination.

The plantlets regenerated through *in vitro* culture, appear no longer, to be the clonal copies of their donor genotype, probably due to various variations, which is understood to be generated via combination of genetic and/or epigenetic changes. The broader utility of any micropropagation system may be limited due to occurrence of cryptic genetic changes and development of somaclones (Rani and Raina 2000). Hence, it is important to check whether somaclonal variations of any kind have been induced. Such variations may multiply very fast and lead to loss of the chief characteristics of the parent plant. Furthermore, this genetic instability may be a risk associated with the application of *in vitro* culture techniques for handling and storage of germplasm (Ray *et al.* 2006). If the regenerants are lacking any phenotypic variation, it does not necessarily imply a concomitant lack of genetic (or epigenetic) change (Larkin and Scowcroft 1981) and therefore, it is of interest to assay the outcomes of *in vitro* raised plantlets at the genotypic level. The polymerase chain reaction (PCR) has previously been used in conjunction with SSR DNA primers to assess the genetic stability of micropropagated strawberry genotypes

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(Vandana *et al.* 2012), *Psidium guajava* L. plants (Rawls *et al.* 2015), coconut plantlets derived from unfertilized ovaries (Bandupriya *et al.* 2017). In the present study, microsatellite markers were used to confirm the genetic stability of micropropagated doubled haploid line of marigold (*Tagetes erecta* L.) derived through ovule culture.

MATERIALS AND METHODS

The present investigations were conducted during 2016-18 at Central Tissue Culture Laboratory and National Research Centre on Plant Biotechnology, ICAR-Indian Agricultural Research Institute, New Delhi. The doubled haploid line of African marigold variety Local Orange developed through ovule culture was used as the source of explant. The plants of the doubled haploid line were maintained under net house conditions at the farm of ICAR- Indian Agricultural Research Institute, New Delhi. Three different portions (Tip, middle and basal) of leaf were used as explants. After the *in vitro* regeneration, plants were taken outside where they were subjected to different VAM treatments. After about 15 days of establishment, leaf samples were taken from random 13 plants, post their *ex vitro* acclimatization, for checking the clonal fidelity of the *in vitro* derived regenerants by comparing them with the mother plant from which the leaf explants were taken.

DNA extraction: DNA was extracted from the mother plant and all 13 leaf regenerated marigold plants grown in open field by using CTAB (Cetyl Trimethyl Ammonium Bromide) method as described by Murray and Thomson (1980) with a few modifications. Young leaves were collected from the plants and wrapped in aluminum foil, labeled and then kept at -80°C ultra-low temperature freezer. Three grams of leaf material was placed in a pre-chilled mortar, frozen by adding liquid nitrogen and the tissue was ground vigorously with a pestle to a very fine powder. The finely powdered leaf tissue was immediately transferred to autoclaved oak ridge centrifuge tubes containing 15 ml of pre-heated (at 65°C) CTAB extraction buffer. 30 μl of β -mercaptoethanol (0.2%) was added to the mixture in centrifuge tubes. The mixture was vortexed thoroughly and incubated at 65°C in water bath for one hour with continuous shaking. Followed by this, the centrifuge tubes were cooled down to room temperature and then equal volume of chloroform and isoamyl alcohol (24:1) was added. The contents were mixed by gentle swirling and then tubes were centrifuged at 11,000 rpm for 20 min in the centrifuge at 4°C . The aqueous part was transferred to fresh, sterile oak ridge tubes and then re extraction was done with 15 ml of chloroform and isoamyl alcohol (24:1) and the aqueous phase was transferred to another sterile centrifuge tube and equal volume of chilled isopropanol was added and mixed by gentle inversions. The tubes were left for overnight at -20°C . To pellet down the DNA, the tubes were centrifuged at 11,000 rpm at 4°C for 20 minutes. Then the aqueous part was decanted and the pellet was washed twice with 3 ml of 70% ethanol and centrifuged at 11,000 rpm for 10 min at 4°C . The aqueous part was

decanted and the pellet was dried free of ethanol and then the pellet was dissolved in 1.6 ml of TE buffer after which, the tubes were stored at 4°C .

DNA purification: Samples were treated with 30 μl of RNase A (stock 10 mg/ml) and were incubated in water bath at 3°C for an hour. Followed by this, 800 μl of RNase A treated sample was transferred to 2 ml centrifuge tubes and 55 μl of proteinase K, 13 μl of 10% sodium dodecyl sulfate was added (SDS) and incubated at 60°C for an hour. Equal volume of phenol was added to the DNA solution and mixed by swirling for 2-3 min. Sample tubes were centrifuged at 11,000 rpm for 15 minutes and supernatant was collected in a fresh sterile tube. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) mixture was added to the DNA solution and mixed by gentle swirling for 2-3 minutes. Samples were centrifuged at 11,000 rpm for 15 minutes and supernatant was collected in a fresh tube. This was followed by extraction with chloroform: isoamyl alcohol (24:1). Finally the supernatant was collected in a fresh sterile tube and purified DNA was precipitated by adding double the volume of chilled absolute ethanol and kept at -20°C overnight. Then centrifuging was done at 11,000 rpm for 20 minutes to pellet down the DNA. Extra salts were removed by giving two washes with 70% ethanol and the pellet was properly dried till the removal of ethanol odour. Finally the pellet was dissolved in 150-200 μl of TE buffer at room temperature and stored at -20°C temperature.

Gel preparation: Initially 1X TAE buffer was prepared by dissolving 2 ml of 50X TAE buffer in 98 ml of distilled water. 0.8 gm of agarose was weighed and dissolved in 100 ml 1X TAE buffer and was melted in a microwave oven. Molten agarose was cooled down to $50-55^{\circ}\text{C}$ temperature and 1 μl of ethidium bromide was added and mixed well. Then the agarose was poured into a gel mould having comb in place. After solidifying, the gel was transferred to an electrophoresis apparatus having appropriate quantity of 1X TAE buffer and the comb was removed carefully.

DNA quantification: For DNA quantification, 30, 40, 50 and 60 ng markers were used to quantify the DNA amount by visual observation. For gel analysis method, 2 μl of sample DNA, 3 μl of loading dye and 5 μl of nuclease free water were mixed and loaded to each well of the agarose gel. 100bp ladder was loaded in one well and the gel was run at 80 V for one 45 minutes. An approximate estimate of DNA quantity (40-50 ng) was obtained by comparing the band intensities of the samples. DNA working stocks were prepared for all the samples by diluting with appropriate amount of TE buffer to yield a working concentration of 25 ng/ μl and stored at 4°C .

Polymerase chain reaction: Whankaew *et al.* (2014) developed 36 polymorphic SSR markers and carried out molecular characterization and genetic relationship of African and French marigold. These microsatellite or SSR markers were used to study the clonal fidelity of *in vitro* raised plants. Out of 36 primers, 12 showed amplification in which clear and reproducible PCR profiles were obtained (Table 1). PCR amplifications were carried out in a total

Table 1 Details of Simple Sequence Repeats (SSR) markers used in the study

Primer code	Forward Primer Sequence	Reverse Primer Sequence	Annealing temperature (°C)
T14	GACAGACACACGAGGTTTATTC	AGACAACATCATTAGCATAGTCC	56
T22	CACACACACTCGCCTTCACTG	ACACACTAAACCCTAACC	54
T31	GAGAGAGGTTTAGAAATAGGG	GGTAGGTTTGAAAGTATTTTGG	54
T52	CACACCCACAGCCACAGC	CACACACACCATTTTCATC	54
T101	GGGTGAGGATCTACAATCAG	GGTGGGGTGTGGTCAAAGG	56
T105	GGGTGGGGTGGGGTGCAACC	GGTGGGGTGTATCCATAAC	56
TE11	CGCCTAATTTGTTGCATGG	ATGTCACCGCCAAAGGATT	54
TE12	TTGAAGGCGAAAGTTGCAG	GAACGAGCAAATCGAAGCA	54
TE20	CAGGCCATCAAACCATCTG	CGACCCACACAAACTCCAC	56
TE27	CGGTTTTCTGTCTCCAAAA	TTCCTTCTCCTTCATCCCTG	54
TE35	ACCCTCCTTGACCCTGTTG	GGTGTTGTTGCTGCTTGCT	56
TE41	GTTGAATACCTCGCTCGCA	CCCTCTCATTTGTTTCCGC	54

volume of 25 µl containing 5 µl (25 ng/µl) genomic DNA, 12.5 µl of PCR master mix (10X PCR buffer + MgCl₂ (3Mm) + dNTPs (10mM each + DNA Taq polymerase (1 U)), 2.5 µl of both forward and reverse primers and 5 µl of nuclease free water. PCR amplification was performed in DNA thermal cycler which was programmed for initial DNA denaturation at 94°C for 4 min followed by 35 cycles of 30 sec denaturation at 94°C, 30 sec annealing (temperatures specific to different primers, mentioned separately in Table 1) and 30 sec extension at 72°C with a final extension at 72°C for 7 min and finally storage temperature at 4°C. PCR tubes were taken out from the machine and stored at 4°C.

Electrophoresis: 3 µl loading dye (6X) was added to each of the PCR product and was loaded in the wells of agarose gel (3.0%). 5 µl Gene ruler (100 bp) was loaded in the first lane of each gel to determine the size of bands amplified. For genetic fidelity testing, the second lane was loaded with mother plant DNA and the subsequent wells were loaded with amplified products of clones derived from leaf explants. Electrophoresis was carried out in 1X TAE buffer at 120 V for 2 h. The resolved amplified products were visualized under UV light on a UV-transilluminator and photographed by using gel documentation system.

RESULTS AND DISCUSSION

The advantage of *in vitro* multiplication of doubled haploids is that large number of quality regenerants per explant are obtained and moreover it is a year round activity. The main drawback for mass multiplication of planting material is the occurrence of undesirable somaclonal variations. Hence, a mechanism is needed to detect this variation at an early stage. Among various tools DNA based markers are more efficient for assessment of genetic fidelity of *in vitro* raised plants due to their ability to detect low level of polymorphism and because of the fact that these are independent of environment and developmental changes (De Laia *et al.* 2000, Rahman and Rajora 2001 and Alizadeh *et al.* 2015). In the present study, microsatellite

(SSR) markers were used to check the clonal fidelity of leaf derived regenerants. SSR markers were used because of their high reproducibility and simplicity, requirement of small quantity of DNA sample, high sensitivity, co-dominant nature and having strong discriminatory power among the genotypes (Reddy *et al.* 2002). Recently, 36 polymorphic SSR markers, specific to marigold, were developed using ISSR technique and a SSR-enriched genomic DNA library (Whankaew *et al.* 2014).

After the regenerants were raised from leaf explant of doubled haploid line of African marigold variety Local Orange derived through ovule culture, the micro shoots obtained were given rooting and *in vitro* hardening treatments and after maintaining the raised plantlets, they were hardened *ex vitro* using VAM consortia. After 10-15 days of their establishment, a set of thirteen plants were randomly selected for assessment of genetic fidelity by Simple Sequence Repeat (SSR) markers. The PCR based microsatellite markers were used for screening of 13 leaf regenerants along with the control mother plant. Out of 36 primers, 12 showed amplification in which clear, reproducible PCR profiles were obtained. By using gradient PCR, annealing temperature (AT°C) was standardized for each primer. The annealing temperature for SSR primers varied from 54 to 56°C (Table 1) and 100 bp ladder was used in the study to locate the band size of the *in vitro* leaf derived regenerants and the donor mother plant. Analysis of individual primers revealed that SSR primers produced same banding pattern which were shared by the *in vitro* leaf derived regenerants and the donor mother plant, which indicated that there was no variation observed within the tissue culture raised plants. The 12 SSR primers revealed the same banding pattern i.e. monomorphism among all the *in vitro* leaf derived regenerants and the donor mother plant (Fig. 1-4). In the present study, 12 SSR polymorphic markers were used to detect the somaclonal variation. No polymorphic bands were detected during the study hence, indicating that the *in vitro* raised plants were similar to mother plant and showed no variation. Similar results were also shown by Mallaya *et al.* (2013) in egg plant. Bandupriya

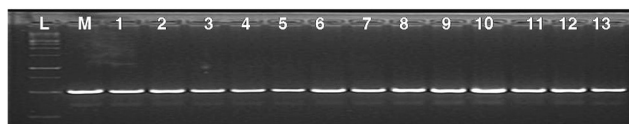


Fig 1. PCR amplification products obtained with SSR primer (T 101). Lane L represents 100 bp ladder, lane M represents the doubled haploid line “Local Orange”, lanes 1-13 represents the *in vitro* raised regenerants derived from leaf explant.

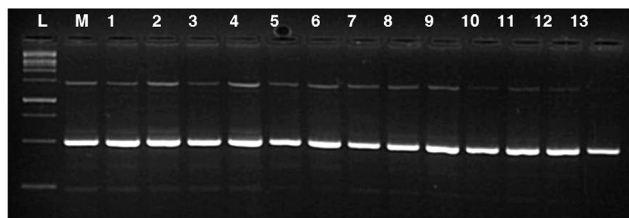


Fig 2. PCR amplification products obtained with SSR primer (T 52). Lane L represents 100 bp ladder, lane M represents the doubled haploid line “Local Orange”, lanes 1-13 represents the *in vitro* raised regenerants derived from leaf explant.

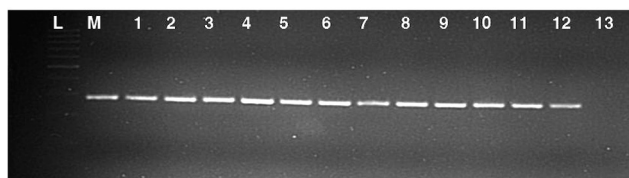


Fig 3. PCR amplification products obtained with SSR primer (TE 27). Lane L represents 100 bp ladder, lane M represents the doubled haploid line “Local Orange”, lanes 1-13 represents the *in vitro* raised regenerants derived from leaf explant.

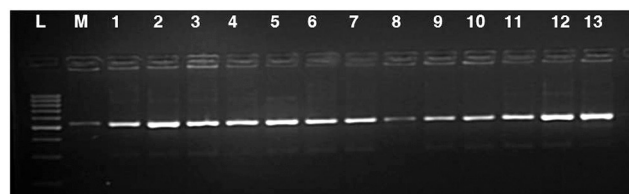


Fig 4. PCR amplification products obtained with SSR primer (T 22). Lane L represents 100 bp ladder, lane M represents the doubled haploid line “Local Orange”, lanes 1-13 represents the *in vitro* raised regenerants derived from leaf explant.

et al. (2017) on their study on *Cocos nucifera* showed that the plantlets regenerated from unfertilized ovaries showed no apparent differences among themselves. Rawls *et al.* (2015) used SSR markers to assess the genetic fidelity of micropropagated guava plants. Ravindra *et al.* (2018), after developing *in vitro* protocol for mass multiplication of commercially popular African and French marigold cultivars, detected the somaclonal variation and found no variation. In an experiment conducted by Vandana *et al.* (2012), micropropagated plants of two commercially important genotypes of strawberry (*Fragaria × ananassa* Duch.), viz. Festival and Sweet Charlie were subjected to evaluation of genetic uniformity using EST-SSR markers. Pandey *et al.* (2012) did an experiment for the early

assessment of genetic fidelity of micropropagated plants of *Saccharum officinarum* regenerated through direct organogenesis using RAPD and SSR markers and found no evidence for somaclonal variation.

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