In vitro haploid production in sunflower genotypes and homozygosity assessment using microsatellite marker

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Abstract
Production of microspore-derived embryos from cultured anthers is now well established technique for isolation of homozygous lines in crop plants. Treatment of NAA 2.0mg/l +1.0mg/l BAP was superior giving maximum response for callus induction (90%) in Morden. Maximum shoot induction 10(1.15) was recorded in TSG-63 at 0.5 mg/l BAP and shoot elongation 5.33(0.88) in EC-16400 at 0.2 mg/l BAP. Seven SSR primers viz. ORS-05, ORS-662, ORS-1265, ORS-484, ORS-488, ORS-598 and ORS-811 were found useful for identification of haploids in sunflower.

The regenerated haploid lines had homozygous profiles with a single band while the donor parent had two bands that indicated allelic sites. The seven SSR markers exhibited 100% homozygosity. Haploid production and confirmation by molecular analysis will provide useful information for sunflower breeding programme.

Keywords: Sunflower, anther culture, callus, shoot, haploid, SSR.

Introduction

Sunflower (*Helianthus annuus* L.) belongs to the family *Asteraceae*. The chromosome number in sunflower is 2n=2x=34. Most species of the genus *Helianthus* are diploid (2n=34), tetraploid (2n=68) and hexaploid (2n=102) and an estimated genome size of 2871-3189 Mbp with narrow genetic base[12]. Sunflower is the third important major edible oil seed crop in the world after soybean and groundnut. In India sunflower becomes more popular oilseed crop due to high quality nutritional value, short duration crop, photo insensitivity, wide adaptability, drought and salt tolerance. Also it is being cultivated as cash crop at any season in India (www.wikipediasunflower.com).

Commercially available sunflower varieties contain 39% to 49% oil in the seed with very high calorific values. Seeds are highly nutritious containing 20% protein, cholesterol lowering factor constituting around 80-95% of total fatty acid, 60-70% linoleic acid with sufficient amount of calcium, iron and vitamin A, D, E and B complex. It supplies more vitamin E than any other vegetable oil. Sunflower oil is also rich in polyunsaturated fatty acids and is advised for heart patient. It can also be used as substitute for mineral oil in various applications such as a fuel, lubricant, or oil for hydraulic system[23,27]. Sunflower oil is considered as premium oil because of its high level of unsaturated fatty acids and lack of linolenic acid. It is a combination of 3 monounsaturated and polyunsaturated fats with low saturated fat levels. The primary fatty acids in the oil are oleic and linoleic constituting typically 90% unsaturated fatty acids (https://www.sunflowernsa.com).

The production of homozygotes is important both for genetic studies and hybrid seed production in highly cross pollinated crops like sunflower. Traditional breeding methods require minimum six generations for the development of near homozygous lines. Anther culture technology leading to production of homozygous diploids is a valuable tool in speeding up the progress of crop improvement. The potential of sunflower haplo-diploidization was first tested by Bohorova et al[6].

There are several methods available to obtain haploids and double haploids (DHs), of which *in vitro* anther or isolated microspore culture are the most effective and widely used[16]. Advantages of production of haploids and DH in plant breeding have been discussed by many workers[2,20,24]. Utilization of haploids and DHs in breeding program has been very well elucidated in several agronomically important dicots like maize[34], barley[18], rice[25], apple[41], brassica[31], capsicum[38], petunia[19] and sunflower[40].

SSRs are powerful DNA marker to easily differentiate between DH and diploid. Identification of haploids mostly includes SSR molecular markers because they are stable, co-dominant and allow separation of homozygotes from heterozygotes[5]. Presence of multiple alleles (polymorphic) will confirm diploid plant out of unwanted anther walls (somatic diploids) during anther culture and presence of monomorphic band will confirm pollen generated haploid and DHs plants. SSR analysis shows the uniformity as well confirms the origin of DH lines.

SSRs have been successfully used for identification of homozygous, spontaneous double haploids[7]. The present work was undertaken to develop true homozygous lines of sunflower genotypes for crop improvement programme.

Material and Methods

Plant material: The experimental material included in present study consists of sunflower genotypes Morden. SS-2038, EC-16400 and TSG-63 were collected from Oilseeds Research Station, Latur.

Treatment details: MS basal media[30] with different combinations of NAA, 2,4-D and BAP were used for *in vitro*

induction of sunflower callus. MS media supplemented with NAA and BAP was used for *in vitro* shoot regeneration. MS media with only BAP[39] was used for elongation of *in vitro* regenerated shoots with some modifications.

Isolation of explant (anthers): Capitula was harvested prior to opening of ray florets when most of the microspores was at the mid to late uninucleate stage of development and the optimal pollen stage was determined from anther morphology.

Preparation of explant: The flower buds of 1.5 to 2.0cm in diameter with cream colored anthers possessing mostly uninucleate microspores were used. The flower buds were surface sterilized by immersing in 70% ethanol and 0.1% mercuric chloride for 2 minute each, then wash with double distilled water for 3 times to remove traces of chemicals and dried on blotting paper.

Inoculation of explants: The anthers which were at the mid to late uniuncleate stage were excised. All extraneous materials like papillae were removed. The white conical ovary portion at the bottom and the corolla joint at the top were cut with the help of a sharp scalpel to squeeze out the bundle of syngenesious anthers. Anthers were dissected from disc florets and inoculated onto solid media. In each plate, four anthers were inoculated as the development of disc florets was centripetal and anthers of different florets were likely to contain microspores at different stages of development.

Whenever possible, anthers from different florets were randomly distributed among plates of various media compositions to minimise variation in response to microspore development. The plates were incubated at 26-28ºC in the dark until macroscopic globular structures were seen. The efficiency of embryogenesis was calculated on the basis of embryo formation after 2-3 weeks incubation. Mature embryos were transferred to germination medium and incubated at 26-28ºC in light under a 16/8 hour photoperiod. Fresh calli were subcultured on 28 days.

Shoot regeneration and elongation: The induced green compact calli was aseptically inoculated on shoot induction media. Callus was transferred on to shoot regeneration medium containing basal MS medium supplemented with different concentration of BAP and NAA. The observations were statistically analysed using Completely Randomized factorial Design (CRD).

Haploid confirmation by using molecular markers: High quality genomic DNA was isolated from young and fresh leaves (6-8 days old), callus and regenerated shoots following Cetyl Trimethyl Ammonium Bromide (CTAB) DNA extraction method[11] with some modifications. The total reaction volume of 25µl contained 2.5µl *Taq* polymerase buffer, 0.2mM dNTPs, 1.7mM $MgCl_2$, 25pmol each primer (forward and reverse), 1U *Taq* DNA polymerase, 1µl of DNA (50ng/µl) and 18.75µl of sterile nuclease free water. The SSR primers specific for high oil content, high oleic acid content, test weight, high yield traits depending on the sunflower genotypes used in present study were selected for analysis of homozygosity in anther culture.

The sunflower genotypes were amplified by SSR markers in a 96 well thermal cycler (Sensoquest Labcycler, Germany) programmed for 35 cycles as follows: initial denaturation at 94ºC for 5 min., further denaturation at 94ºC for 30 sec, annealing at 57ºC for 50 sec., extension at 72ºC for 1 min. and final extension for 10 min at 72ºC followed by pause/hold at 4ºC

Results and Discussion

In vitro androgenesis via anther culture is most preferred technique for obtaining haploids plants. The production of haploids provides a particularly attractive biotechnological tool to produce homozygous plants which help plant breeders to develop new variety within a short span of time. In anther culture, some time the anther wall, which is diploid, can be regenerated into a diploid plant. These diploids need to be detected and rejected before development of DHs.

Anthers turned brown after 3-5 days from plating and callus initiations were observed on different days on the two ends of anthers (Figure 1). Callus formation starting from the filamentous ends of the anther was also observed by Goyal et al[17]. Days to callus initiation were varied with the genotype and media composition for Morden and EC-16400 ranged between 10 to 22 days, for SS-2038, 8 to 22 days and for TSG-63, 11 to 23 days. For all genotypes, the treatment T5 (2.0mg/l NAA+1.0mg/l BAP) recorded minimum time (8 days) for callus initiation and proliferation followed by T2 (0.5 mg/l NAA+0.5mg/l BAP).

Maximum time (23 days) was recorded in T8, T9 and T10. Treatment 5 is significantly superior over rest of all the treatments in all genotypes. The treatment T5 also gave maximum response for callus induction, 90% in Morden, 86% in SS-2038, 80% in EC-16400 and 58% in TSG-63 (Table 1). The genotypes produced friable to compact calli and the colour ranged from light white, cream colour to light yellow callus. Anther calli showed embryogenesis in T1, T2, T3, T4 and T5 treatments. The embryogenic calli were friable green with both embryogenic and nonembryogenic portions and required 20-30 days for appearance.

The treatment T5 contained major quantities of NAA (2.0 mg/l) and BAP (1.0 mg/l) manifested high callus induction for most of the genotypes and required less days for callus initiation. Similar results for callus induction were also reported by Patil[32], Thengane et al[40] and Zhong et al[44]. However, increase in the concentration of auxin and cytokinin (NAA and BAP) does not have significant contribution to callus production[36].

Figure 1: Embryogenic callus induction from Morden genotype.

Table 1
Effect of different concentration of growth regulators on callus induction.

Genotype	Treatment									
	T1	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**	**T10**
Morden	73	88	83	72	90	57	41	35	36	28
SS-2038	58	82	66	67	86	49	54	19	21	29
EC-16400	76	77	69	59	80	53	42	32	21	16
TSG-63	48	65	65	57	58	44	40	31	20	25

Hormone concentration (mg/l): T1-0.3NAA+0.3 BAP; T2-0.5NAA+0.5BAP; T3-1NAA+0.5 BAP; T4-1NAA+1BAP; T5-2NAA+1BAP; T6-2 2,4-D+1BAP; T7-2 2,4-D; T8-3 2,4-D; T9-4 2,4-D: T10-5 2,4-D.

Table 2
Effect of different concentration of growth regulators on shoot induction.

Genotype	Treatment									
	T1	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**	**T10**
Morden	4 ±0.57	1.33 ±0.33	2±0.57	7 ±0.57	1.33 ±0.33	9.33 ±1.20	1.66 ±0.66	1.66 ±0.66	2 ±0.57	1±0
SS-2038	1.66 ±0.33	2±0.57	2.33± 0.33	6.33 ±0.88	1.33 ±0.33	9.66 ±1.20	2.33 ±0.88	1.66 ±0.66	1.3 ±0.33	1.33 ±0.33
EC-16400	1±0	2±0.57	1.66± 0.33	5 ±0.57	0.66 ±0.33	8.66 ±0.33	1.33 ±0.33	2±0.57	2 ±0.57	0.33 ±0.33
TSG-63	1.33 ±0.33	1.33 ±0.88	1.66± 0.33	5.33 ±0.33	1±0	10 ±1.15	2.33 ±0.33	1.66 ±0.66	1.3 ±0.33	1.66 ±1.20

Hormone concentration (mg/l): T1-0.5NAA+0.5BAP; T2-1NAA+1BAP; T3-1.0 NAA+2BAP; T4-2NAA+1BAP; T5-1NAA+3BAP; T6-0.5BAP; T7-1BAP; T8-2BAP; T9-3BAP: T10-4BAP.

Callus induction was also observed in the treatment T7, T8 and T9 that had the auxin 2,4-D alone in increased amounts[1,37]. It may be because 2,4-D is a powerful suppressant of organogenesis and often induces callus even in the absence of any endogenous cytokinin[10]. In treatment 5 (2.0mg/l NAA+1.0mg/l BAP), anther callus produced direct pink coloured root only in Morden genotype. Similar results were reported by Vijaya et al[42] during the study of anther culture in sunflower genotypes (*H. annuus*×*H. tuberosus*). The pink colour roots were produced from callus in the medium supplemented with 2.0 mg/l NAA+0.1mg/l 2,4-D+1 mg/l BAP.

Anther callus was typically embryogenic and embryos started to differentiate within two weeks after sub culturing on medium supplemented with different combinations of

growth regulators for shoot induction. Treatment 6 (0.5 mg/l BAP) is significantly superior recording minimum time (20-25 days) for shoot induction. Maximum time (30-35days) was required in T1, T9 and T10. However, the efficiency of conversion of embryos to shoot induction was very low among ten treatments. The best shoot induction response 10(1.15) was recorded in TSG-63 (Figure 2) followed by SS-2038 9.66(1.20), Morden 9.33(1.20) and EC-16400 8.66**(**0.33) in treatment 6 (Table 2).

Subculture of embryogenic callus along with developing embryo onto medium supplemented with 0.5 mg/l BAP facilitated greening and proliferation of shoots from the cotyledonary embryos within 20 days after transfer. Similar results were observed by Saji and Sujatha[39] when cotyledonary stage embryos from the embryogenic callus were transferred to medium supplemented with 0.5 mg/l BA on which an average of 4 to 6 shoots from each embryo was obtained with 2.8 mean.

However, the frequency of conversion of embryos to complete plants was very low (0 to 14.3%). Similar results were also observed by Vijaya et al[42] where *H. annuus*×*H. tuberosus* hybrid anther calli showed embryogenesis in AR4 (0.2 mg/l BAP) and AR8 (0.5 mg/l BAP) treatments. Days to shoot elongation ranged between 39 to 47 days. It varied with the genotype and the media composition. Treatment 2 (0.2 mg/l BAP) was significantly superior recording minimum time (39 days) response for shoot elongation recording 5.33(0.88) in EC-16400, 5(0.57) in TSG-63, 4.66**(**0.33) in Morden and 4.33(0.33) in SS-2038 (Table 3). Maximum time (45-47 days) was recorded in T4 and T5 treatments.

The efficiency of shoot elongation was very low among five treatments. Leaf differentiation was observed after 20 days of sub culturing the shoots on shoot elongation medium. On the 39th day in regeneration medium T2, plantlets developed with 2-3 leaves and 2.00 cm height (Figure 3).

Figure 2: Shoot induction from callus in TSG-63 genotype.

Figure 3: Shoot elongation in EC-16400 genotype.

Table 3
Effect of different concentration of growth regulators on shoot elongation.

Genotype	Treatment				
	T1	**T2**	**T3**	**T4**	**T5**
Morden	1.33±0.33	4.66±0.33	1.33±0.33	1.66±0.33	1±0
SS-2038	0.66±0.33	4.33±0.33	1.33±0.33	1±0	1.66±0.33
EC-16400	1±0	5.33±0.88	1.66±0.33	1.66±0.66	1±0.57
TSG-63	1.33±0.33	5±0.57	1±0	1.66±0.33	0.33±0.33

Hormone concentration (mg/l): T1-0.1BAP; T2-0.2BAP; T3-0.3 BAP; T4-0.4BAP; T5- 0.5BAP; T6-1BAP.

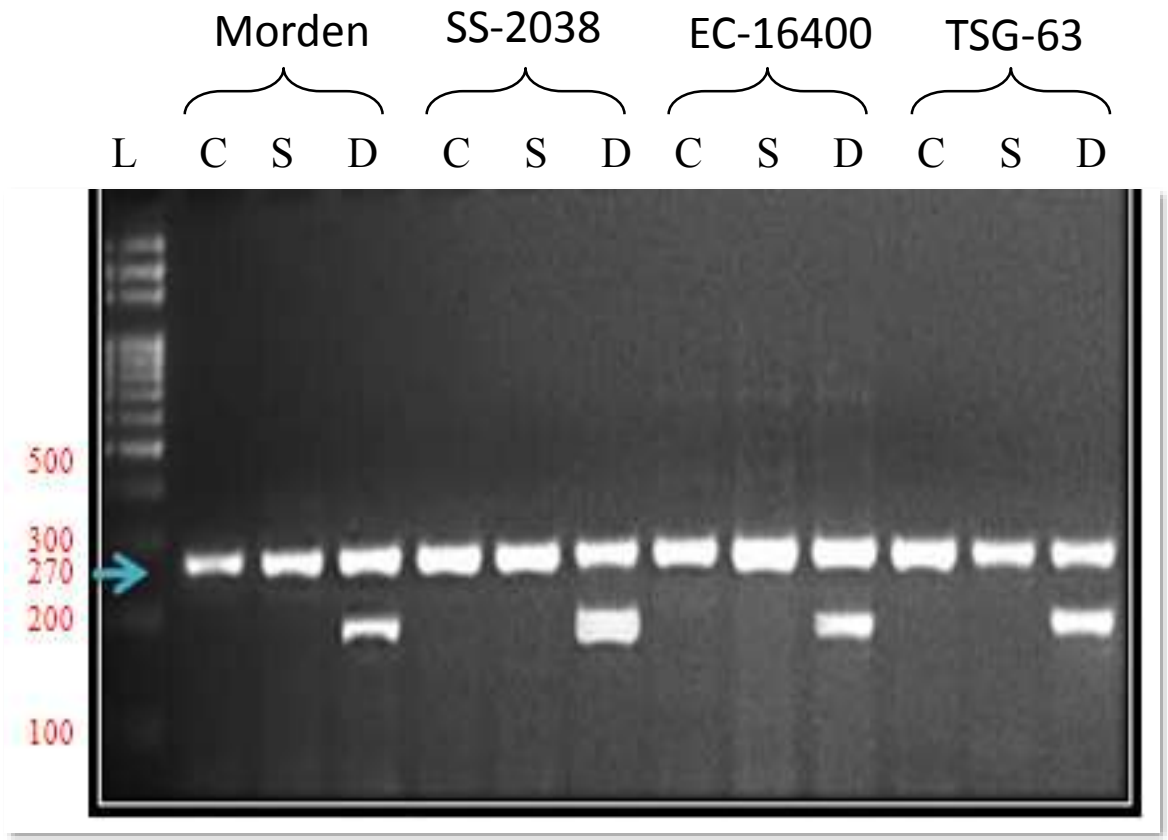

Figure 4: Banding Profile of haploids and parents obtained by Primer ORS-811 L-Ladder, C-Callus; S-Shoot; D-Donor plant (diploid).

SSR analysis would be very useful in breeding for rapid and early verification of haploid in large population. SSR markers have been widely used in ploidy analysis and chromosome duplication by various scientists.[3,5,9,21,28,29,33,35,43] The haploid confirmation discussed here was done by comparing banding patterns of haploids with their respective donor parents. For haploid analysis, two types of banding patterns was observed in the donor parents and haploids. Similar findings were reported for analysis and identification of haploids by using SSR markers in maize by Couto et al[8] and Battistelli et al[4].

Among 28 SSR primers screened, seven primers *viz*., ORS-05, ORS-662, ORS-1265, ORS-484, ORS-488, ORS-598 and ORS-811 showed polymorphism and were used to confirm the haploid identification of regenerated calli and shoots with their donor parent line. The regenerated haploid lines had homozygous profiles with a single band while the donor parent had two bands that indicated allelic sites. The banding patterns of the markers ORS-05(300bp), ORS-662(314bp), ORS-1265(222bp), ORS-484(400bp), ORS-488(200bp), ORS-598(370bp) and ORS-811(270bp) appeared in the antheral calli lines, regenerated line and the donor parent line (Figure 4). The seven SSR markers showed 100% homozygosity in callus and regenerated shoots. Among 117 regenerants, 10 shoots and 08 calli were true haploids.

Belicuas et al[5] identified four haploids among 462 plants obtained from cross between the line W23 and the hybrid BRS1010 of sunflower. Two polymorphic primers mmc0022 and mmc0081were used on the parents. Microsatellites have also been employed to characterize regenerants obtained from citrus anther culture[13-15] and to

assess homozygosity in apple[22] and pear[7]. The single multi-allelic self incompatibility gene has been used in apple by Verdoodt et al[41] to discriminate homozygous from heterozygous individuals obtained by anther culture as well as by parthenogenesis *in situ*.

Extra unique allele did not appear in the regenerated calli and shoots at a locus other than diagnostic heterozygous allele present in donar sunflower genotypes indicating absence of somaclonal variation in the regenerated calli and shoots. However, Krutovsky et al[26] found that only one locus (UAKLly6) was heterozygous for the maternal tree no. 30 and therefore could serve as a diagnostic locus for haploid cell lines (CLs) and was likely to have a mutational origin indicating a very high rate of somaclonal variation.

Conclusion

In vitro regeneration protocol for haploid production in sunflower genotypes Morden, SS-2038, EC-16400 and TSG-63 successfully developed using anther as an explant. The plantlets thus regenerated are confirmed as haploid by SSR markers. This will be helpful for developing homozygous lines, haploid and double haploid plants for plant breeding programme.

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