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Embryogenesis and plant regeneration in anther culture of sunflower (*Helianthus annuus* L.)

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Summary

A protocol for high frequency callus induction and plant regeneration from sunflower (*Helianthus annuus* L.) anthers is described. Different variables using Murashige & Skoog (MS) basal medium supplemented with 2.0 mg/l α-naphthaleneacetic acid (NAA) and 1.0 mg/l N^6-benzyladenine (BA) were tested for their ability to enhance the frequency of anther callusing and subsequent embryogenesis. Of these, agar concentration, sucrose concentration, carbohydrate source had significant effect on callusing, while differences due to incubation under dark vs light conditions, cold pretreatment of capitula for 1 to 6 days prior to anther inoculation and genotype on callusing were non-significant. However, all these factors exerted highly significant influence on embryogenesis when calli from the various media were transferred to medium supplemented with 0.1 mg/l NAA and 0.5 mg/l BA. With the procedure developed, callusing as high as 100% and embryo formation at a frequency of 44% was achieved. Although complete embryos were formed the frequency of their conversion to whole plantlets was low (14.3%). Hence, the embryogenic pathway was bypassed to obtain multiple shoots by transferring embryogenic calli with developing embryos to MS medium supplemented with 0.5 mg/l BA. Elongated shoots rooted on half-strength MS medium supplemented with 0.5 mg/l NAA. Cytological analysis of embryogenic callus and somatic embryos revealed haploids at a frequency of 30% while that of rooted plants showed haploid regenerants at a frequency of 8.3%. Nevertheless, the frequency of putative haploid plants could be enhanced through mass multiplication using nodal explants of the regenerants.

Abbreviations: BA – N^6-benzyladenine, MS – Murashige & Skoog (1962), NAA – α-naphthaleneacetic acid

Introduction

The production of homozygotes is important both for genetic studies and hybrid seed production in highly cross pollinated crops like sunflower (*Helianthus annuus* L.). Traditional breeding methods require a minimum of six generations for the development of near homozygous lines. The potential of sunflower haplo-diploidization was first tested by Bohorova et al. (1980). Development of haploids in sunflower has been attempted through gynogenesis (Yang et al., 1985; Gelebart & San, 1987), anther culture (Bohorova et al., 1985; Gürel et al., 1991a; Pugliesi et al., 1993; Thengane et al., 1994; Zhong et al., 1994) and microspore culture (Gürel et al., 1991b; Coumans & Zhong, 1995).

Although attempts have been made, successful utilization of microspore-derived plants in sunflower breeding has not made much headway owing to their very low induction frequencies, poor reproducibility and doubtful ploidy status. Routine application of ovule culture is hindered by the low yields of the gynogenetic embryos besides, the tedium of the procedure (Yang et al., 1985; Gelebart & San, 1987). Research to define appropriate media for optimum androgenesis has been only partially successful in sunflower. Bohorova et al. (1985) obtained anther callusing at a frequency of 70–100% but failed to

achieve shoot regeneration. Gürel et al. (1991a) reported a low frequency of direct embryoid formation and shoot production (0.47 to 3.4%) from anther culture of sunflower and some interspecific hybrids but no entire plant has been recovered. Thengane et al. (1994) reported difficulties in embryo to plantlet conversion. Besides the low regeneration potential, the studies could not provide sufficient information on the ploidy of the produced embryos. Gürel et al. (1991b) and Coumans & Zhong (1995) assayed isolated microspore culture and achieved sustained division and microcallus formation but with no success in shoot regeneration.

Keeping in view the above limitations for large scale applicability of the technique, the present investigation has been undertaken with an objective to critically analyze the various factors which influence the anther response *in vitro* and enhance the induction frequency of anther plantlets in sunflower.

Materials and methods

Plant material

Immature capitula from sunflower (*Helianthus annuus* L.) cv. Morden – an open pollinated, dwarf, early maturing variety raised in the research farm at Directorate of Oilseeds Research, Hyderabad were used in all the experiments. For assessing the influence of donor genotypes, the cv. Morden was compared with the hybrids, KBSH-1, Jwalamukhi and MSFH-8.

Anther culture

Capitula were harvested prior to the opening of ray florets when the microspores were at the mid- to late-uninucleate stage of development. The capitula were thoroughly washed under running tap water and surface sterilized with sodium hypochlorite solution (1 to 1.2% available chlorine) for 15 min followed by three rinses in sterile distilled water. The anthers were excised under a stereo binocular microscope and plated with the filaments removed in 90 mm Petri dishes containing 25 ml of medium. For studying the effect of cold pretreatment on anther response, the capitula were stored at 4 °C for 1 to 6 days before anther harvest.

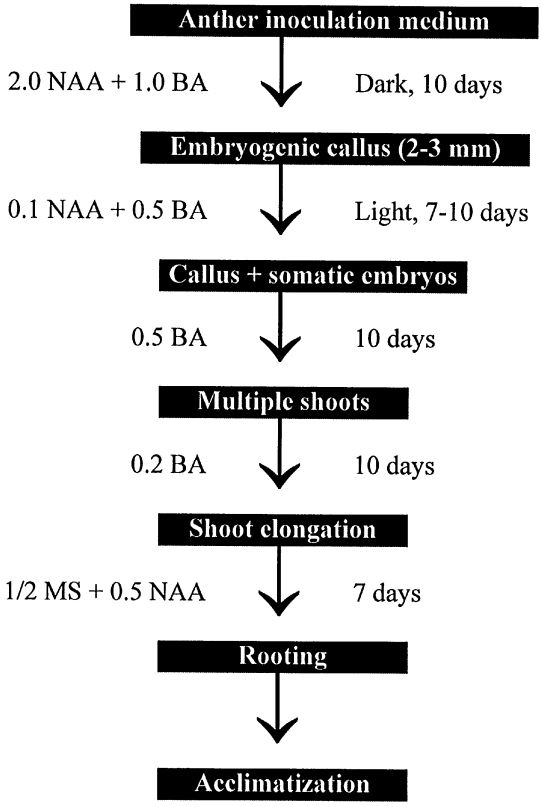

Figure 1. Experimental protocol used for embryogenesis and plant regeneration from *Helianthus annuus* anthers.

Culture media

The anther inoculation medium contained MS (Murashige & Skoog, 1962) salts and growth factors, 3% (w/v) sucrose, 0.7% (w/v) agar (Himedia, India) and supplemented with 2.0 mg/l NAA and 1.0 mg/l BA. For investigations on the effect of carbohydrate source, glucose and maltose at 3% were tested along with sucrose in the anther inoculation medium. Likewise, to study the effects of sucrose and agar, the concentrations were varied between 3 to 8% and 0.4 to 1.0%, respectively in the primary medium. After 10 days in culture, the anthers with embryogenic callus approximately 2 to 3 mm in diameter were transferred to MS medium supplemented with 0.1 mg/l NAA and 0.5 mg/l BA. Owing to problems in embryo to plantlet conversion, cotyledonary stage embryos were transferred to MS medium supplemented with 0.5 mg/l and 0.2 mg/l BA in 100 ml Erlenmeyer flasks for shoot proliferation and elongation, respectively. Rooting medium consisted of half-strength MS medium supplemented with 0.5 mg/l NAA in 25 × 150 mm

culture tubes. For all the media, pH was adjusted to 5.8 prior to addition of the gelling agent and autoclaving at 121 °C for 20 min. Details of media used and incubation periods for various transfers are presented in Figure 1.

Culture conditions

For all experiments, Petri dishes were sealed with Parafilm (Sigma, USA) and incubated at 25 ± 2 °C in dark in a growth room till callus induction. Subcultured calli and shoots were maintained under a 16 h photoperiod (30 μmol m^{-2} s^{-1}). To investigate the effect of light on anther callusing and embryogenesis the anthers were maintained in light under the same conditions.

Cytology

For cytological analysis, calli/roots of embryogenic calli, somatic embryos and rooted shoots were fixed in Carnoy's fluid II (1:3:6 – glacial acetic acid:chloroform:absolute alcohol) at 4 °C for 24 h. After acid hydrolysis (1 N HCl – 10 min at 60 °C) and enzyme digestion (10% Pectinase – 10 min at 37 °C) the material was stained with Feulgen (Sharma & Sharma, 1980) and squashed with 0.1% aceto-orcein.

Data analysis

Each experiment had 3 replicates comprising of 3 Petri dishes containing 80–100 anthers each and was repeated at least twice. Data on the number of anthers forming callus and number of calli differentiating into embryos were recorded. Results were analyzed by one-way analysis of variance after arcsin transformation of the percentages and means were separated by LSD at α = 0.05 using MSTATC statistical package.

Results and discussion

The anthers turned brown within 48 to 72 h of culture. Browning of anther wall did not hinder callogenesis and callus proliferation was visualized in more than 90% of the plated anthers as small white protuberances within three to five days of culture. Callus initially watery in appearance turned hard, compact, opaque white to pale yellow with globular structures after 7–10 d in culture. Occasionally, embryoids and roots were observed at frequencies of 1.5 to 2.4 and 3.0 to 13.7%, respectively, depending on the genotype and the culture medium.

Anther callus was typically embryogenic and embryos started to differentiate within one week after subculture onto medium supplemented with 0.1 mg/l NAA and 0.5 mg/l BA (Figures 2a and 2b). Number of embryos per calli varied from 1 to 8 with a mean of 2.4 ± 2.8. However, the frequency of conversion of embryos to complete plants (Figure 2c) was very low (0 to 14.3%) following subculture to several media varying in type, concentration and ratio of auxins and cytokinins including growth regulator free media. In most cases there was suppression of the radicular end which resulted in vitrification of the cotyledons and or callusing of the embryos (Figure 2d). Similar problems were encountered by Thengane et al. (1994) and they could obtain germination of 10–15% of the cotyledonary stage embryos upon transfer to medium containing a combination of cytokinins (0.5 mg/l Kn and 0.5 mg/l BA) supplemented with 100 mg/l casein hydrolysate and 2.5 mg/l silver nitrate. To circumvent this problem and in order to have more plantlets, an organogenic pathway was preferred in the present investigation. Subculture of embryogenic callus along with developing embryos onto medium supplemented with 0.5 mg/l BA facilitated greening and proliferation of multiple shoots from the cotyledonary embryos within 10 days after transfer.

Effect of different variables

Genotype

Comparison of the cv. Morden with the hybrids indicated a better response for the hybrids, KBSH-1 and MSFH-8 both for anther callusing and embryogenesis (Figure 3a). Anthers of these two hybrids readily produced embryos on the anther inoculation medium itself but at a low frequency of 1.5 to 2.4%. On subculture medium, frequency of embryogenesis varied with the genotype and the maximum embryogenesis (44%) was recorded for KBSH-1. Differences in callusing due to genotype were non-significant while the influence of genotype was highly significant on embryo formation. Differences in embryogenic response could be due to innate differences in the endogenous levels of growth regulators in different genotypes. The study simply indicates that genotype plays a significant role in anther culture of sunflower also like in many crop species but detailed investigations are necessary to understand the nature of genetic control and the gene recombinations favouring androgenic capacity.

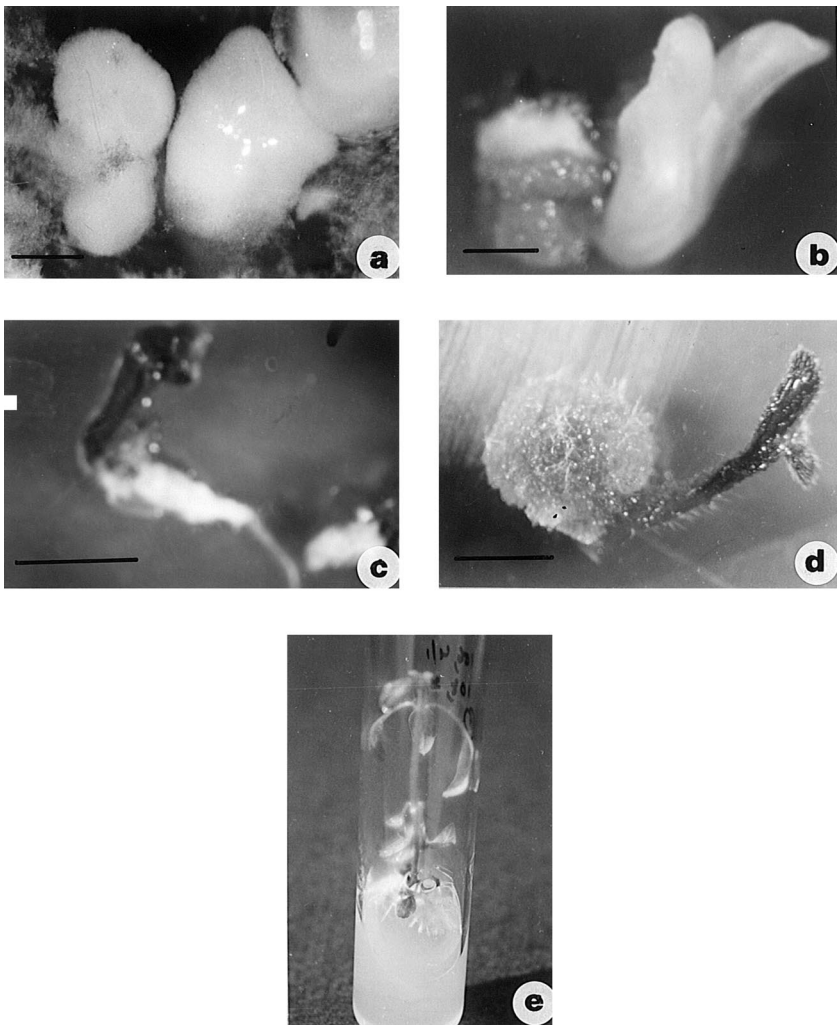

Figure 2. Differentiation of embryos from anther callus of *Helianthus annuus* on MS medium supplemented with 0.1 mg/l NAA and 0.5 mg/l BA after 10 d in culture. a) Globular and early heart stage embryos (bar = 0.2 mm). b) Cotyledonary stage embryo (bar = 0.2 mm). c) Complete plantlet (bar = 1.2 mm). d) Callusing of the radicular end of a cotyledonary embryo (bar = 0.8 mm). e) Rooting of shoots on half-strength MS medium supplemented with 0.5 mg/l NAA.

Light vs dark incubation

Callus induced under dark was white or light yellow and compact, while that induced under light was prolific, green and less compact. The effect of dark incubation was more pronounced in embryoid formation than callus induction (Figure 3b). Callus developed under dark conditions differentiated embryoids whereas that developed under light conditions lacked morphogenic competence. Thus, cultivation of the anthers in the dark until the development of callus seems to be a crucial factor favouring androgenesis in sunflower.

Experiments of Paterson & Everett (1985) revealed the stimulatory effect of light pretreatment on regeneration from seedling explants. In agreement with our study, Gürel et al. (1991a) also could not regenerate shoots from anther callus induced on medium supplemented with 5.0 mg/l zeatin and with incubation under a 16 h photoperiod. This contradictory result could be due to the differences in the ploidy of the tissues under study and or the exogenous growth regulators used. It has been reported by many investigators that light is not a prerequisite for the induction process *per se* but is essential for post-induction growth (Sangwan-Norreel, 1977; Sunderland & Roberts, 1977; Nair et al., 1983).

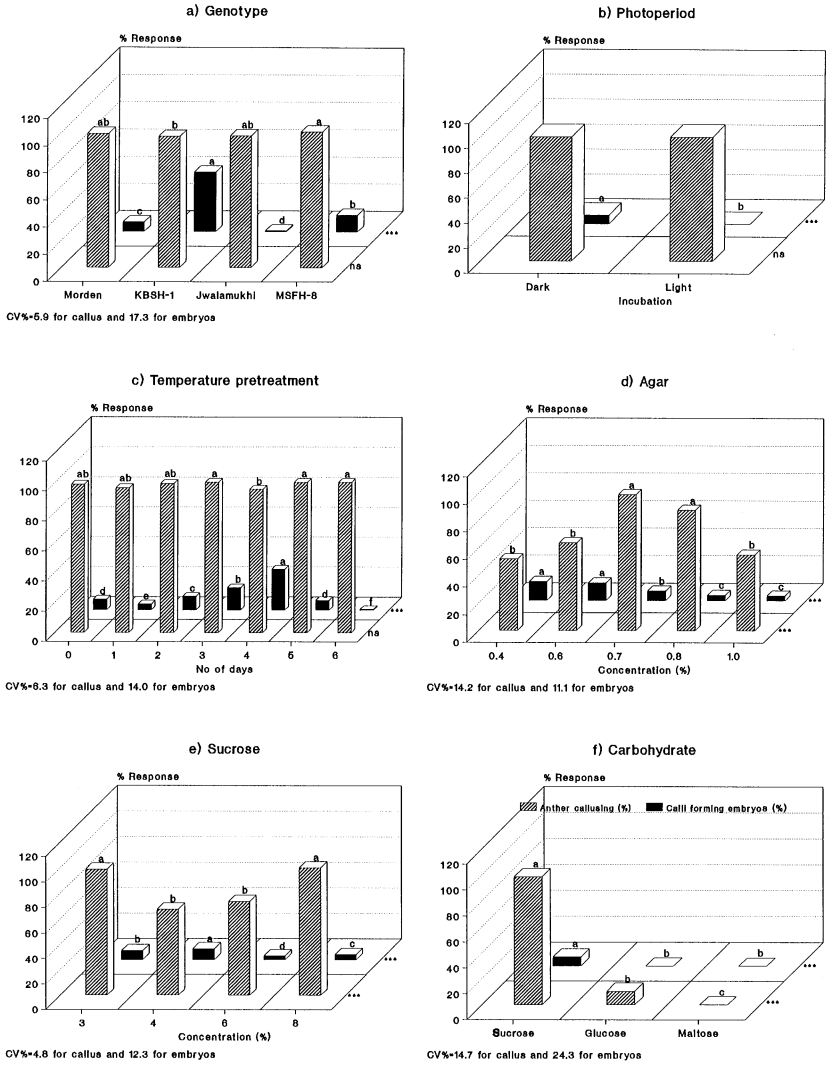

Figure 3. Effect of different variables on callusing and embryo formation from *Helianthus annuus* anthers. Bars for each parameter denoted by same letters are not significantly different according to LSD at $\alpha = 0.05$ (ns = non-significant, *** = significant at $p \leq 0.1\%$).

Cold pretreatment of capitula

Application of a 4 °C pretreatment to the capitula after harvest for different incubation times revealed no significant differences for callusing but exhibited strong influence on embryogenesis (Figure 3c). Chilling pretreatment for 4 days resulted in a four-fold increase (27.1%) and for 3 days in a two-fold increase (14.7%) in embryoid formation when compared with the control (7.0%).

Contradictory results exist in literature about the temperature pretreatment of anthers for obtaining optimal response in sunflower. A pretreatment at 32 or 35 °C has been shown favorable for anther culture by Jonard & Mezzarobba (1990). Studies of Coumans & Zhong (1995) showed complete loss of microspore viability after 3 days of culture when precultured at 32 °C in the dark. Thengane et al. (1994) reported a stimulatory effect of cold pretreatment on embryo induction in sunflower. The effect of cold treatment was found to be indirect. In tobacco, the increase in androgenesis was mainly attributed to the fact that low temperature (3–5 °C) retains the pollen viability longer, delays senescence, and prevents the abortion of pollen and thereby increases the number of available viable pollen which are destined to form embryos (Bajaj, 1978).

Table 1. Frequency of haploids during various subcultures in anther culture of *Helianthus annuus*

Material studied	Number of samples*	Number of haploids	Number of diploids	Frequency of haploids (%)
Embryogenic callus	23	7	16	30.4
Somatic embryos	60	18	42	30.0
Rooted shoots	24	2	22	8.3

* indicates number of calli or roots observed. In each sample, 50–180 metaphase plates were scored.

Agar

The gelling strength of the medium had a significant influence on anther callusing and subsequent embryoid differentiation (Figure 3d). Callusing was maximum (87.4 to 98.7%) when medium was gelled with 0.7 to 0.8% agar while embryoid formation was maximum (12.6 to 13.7%) from calli derived on medium gelled with 0.4 or 0.6% agar.

Studies of Sopory & Maheshwari (1976) showed that supply of oxygen is critical for obtaining optimal response and they achieved highest embryo yields when anthers were placed on the surface of the solidified medium, avoiding submersion. However, higher concentrations of agar are known to restrict diffusion of macromolecules (Romberger & Tabor, 1971) and reduce water availability (Stoltz, 1971), thereby, limiting access to both nutrients and hormones. The present study indicates the importance of consistency of the medium for callusing and subsequent embryogenesis from cultured anthers of sunflower.

Sucrose

The effect of sucrose on anther response was not well pronounced. Optimal concentration of sucrose for callus induction was 3 or 8% while a 4% concentration was required for eliciting a better embryogenic response (Figure 3e). The observation is in agreement with the studies of Thengane et al. (1994) where the optimal androgenesis was obtained with 40 g/l sucrose. The differential requirement of sucrose during developmental stages is not precisely known, although an osmoregulatory role has been suggested (Binding, 1972). The role of sucrose in embryogenesis and callus proliferation has been widely studied and it was suggested that anthers should be cultured on high sucrose media in the beginning and then transferred to low levels of sucrose for optimal post-induction growth (Keller et al., 1987).

Carbohydrate

Anther response in sunflower was greatly influenced by the carbohydrate source. Of the three tested sugars, sucrose proved to be better in promoting maximum callusing as well as embryoid formation (Figure 3f). Maltose was completely inhibitory in the present study although it was reported to enhance the asymmetrical and symmetrical divisions in the isolated microspores (Coumans & Zhong, 1995). Medium supplemented with glucose supported callusing at a low frequency (10.4%) but failed to promote embryogenesis.

Shoot multiplication

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 were obtained. Rapid shoot elongation was stimulated by transferring the proliferating shoots to medium supplemented with 0.2 mg/l BA. All the elongated shoots (approximately 2–4 cm) exhibited precocious flowering on multiplication and proliferation media. Flowers were small with few ray (6–10) and disc (10–22) florets; and in most capitula, only the outer whorl of disc florets emerged out. Flowering is one of the most frequently occurring morphogenetic processes in tissue cultures of sunflower (Greco et al., 1984; Paterson & Everett, 1985).

Cytological studies revealed a decline in the frequency of haploids from 30% in the primary culture to 8.3% in the rooted shoots (Table 1). The calli scored as haploids showed diploid cells as well at frequencies varying between 2.3 to 68.2%. Appearance of diploid cells could be due to differentiation not only from the microspores but also from various other parts of the anthers. The putative haploids were further multiplied on medium supplemented with 0.5 mg/l BA using nodal sections since the apices possessed floral meristems.

Well elongated shoots rooted within a week on transfer to the rooting medium (Figure 2e). The rooted shoots were washed with sterile water to remove traces of agar sticking to the roots and dipped in 0.2% (w/v) of a broad spectrum fungicide solution (Bavistin, BASF, India) for 8 min. Shoots were transferred to sterile vermiculite in 6 cm diameter plastic pots kept in a tray and maintained at high humidity by covering with a glass plate for 1 week followed by gradual exposure to sunlight.

In conclusion, the protocol for the establishment of plant regeneration system from cultured anthers described in this paper is simple, efficient (anther callusing $\geq$ 90%; frequency of calli differentiating into embryos = 44%) and rapid (plantlet recovery within seven weeks starting from anther inoculation). The results also contribute important information about the factors that promote callusing and subsequent embryogenesis from sunflower anthers. Further attempts of developing methods to double the chromosomes and avoid premature flowering in addition to circumventing the dominance of diploid cells through microspore culture are under progress.

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