

Effects of genotype and culture medium on *in vitro* androgenesis in soybean (*Glycine max* Merr.)

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Anthers of ten genotypes of *Glycine max* were cultured on four fortified B₅ media supplemented with different levels of growth hormones, viz. B₅ DBIG (2.0 mg l⁻¹ 2,4-D+0.5 mg l⁻¹ IBA+100.0 mg l⁻¹ myo-Inositol+360.0 mg l⁻¹ L-glutamine), B₅ DB (2.0 mg l⁻¹ 2,4-D+0.5 mg l⁻¹ BA), B₅DK (2.0 mg l⁻¹ 2,4-D+0.5 mg l⁻¹ Kinetin) and B₅BKN (0.5 mg l⁻¹ BA+0.5 mg l⁻¹ Kinetin+1.0 mg l⁻¹ NAA). All the media were supplemented with 90.0 g l⁻¹ sucrose and 7.0 g l⁻¹ agar. Significant differences in the response of genotypes, culture medium and genotype x medium interactions were observed for callus initiation, formation of morphogenic calli and plantlet regeneration. Genotype JS 90-41 was found superior for *in vitro* androgenesis. Culture medium B₅DBIG exhibited higher response for androgenic callus formation and haploid plant regeneration.

Keywords: *Glycine max*, anther culture, androgenesis, *in vitro*, genotype x medium interactions, haploid plants

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Introduction

Soybean (*Glycine max*) is the world's most important plant protein source for human consumption and animal feeds, in addition to being one of the most important sources of a high quality, low cholesterol edible oil. Use of conventional breeding to produce new cultivars after hybridization is time-consuming. With anther culture, target genes can be introduced into soybean and released in pure lines in just one generation following hybridization. Haploid formation by anther culture is an established technique in developmental programmes of many crops. Anther culture is now being used extensively and is most suitable technique to obtain haploid plants for creating homozygosity, *in vitro* selection at haploid level, sporoplast and haploid protoplast isolation and for genetic transformation.

The recalcitrant *in vitro* androgenic nature of soybean makes progress in haploid plant induction sluggish. Tang *et al*¹ was first to report the callus induction from soybean anther culture. This was followed by other studies on anther culture² and sporadic haploid plantlets from Chinese soybean cultivars through organogenesis of microspore-derived calli³.

In soybean, factors influencing anther culture the most are genotype^{4,6} and nutrient medium^{4,7,8}. This report presents the genotypic influences and effects of medium composition on totipotent callus induction and plantlet regeneration from anthers of *G. max*.

Materials and Methods

All the genotypes of *G. max* (Table 1) were established cultivars from laboratory farm. Each treatment was replicated twice. A replication consisted of 200-400 anthers isolated from different flower buds of a single genotype, randomized and cultured on a specific culture medium. Readymade powdered B₅⁹ and MS basal media¹⁰ and all other additions were procured from HiMedia, Mumbai, India.

For excision of anthers, buds of predetermined size were selected at uninucleate microspore stage and selection was based on smear with 1% aceto carmine. Early-to mid-uninucleate microspores were observed in maximum frequency when the bracts were equal to 2/3-3/4 of the bud length (3.0-3.5 mm). Selected buds were stored for 3-5 days at 6±2°C for cold pretreatment. Before excision of anthers, selected buds were surface sterilized for one min in 70% ethanol. Anthers were excised aseptically and cultured on four fortified B₅ callus induction media [B₅ DBIG (2.0 mg l⁻¹ 2,4-D+0.5 mg l⁻¹ IBA+100 mg l⁻¹ myoinositol+360 mg l⁻¹ L-glutamine), B₅DB (2.0 mg l⁻¹ 2,4-

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D+0.5 mg l⁻¹ BA), B₅DK (2.0 mg l⁻¹ 2,4-D+0.5 mg l⁻¹ kinetin) and B₅BKN (0.5 mg l⁻¹ BA+0.5 mg l⁻¹ kinetin+1.0 mg l⁻¹ NAA)]. Such fortifications of culture media were used since earlier it has been proved superior for soybean anther culture^{4,5,7}. These media were supplemented with 90.0 g l⁻¹ sucrose and 7.0 g l⁻¹ agar. The pH of culture medium was adjusted to 5.6 with 1N KOH solution prior to the addition of agar. Each 90×15 mm Petridishes were plated with 60-70 anthers evenly distributed to four different callus induction media. All the petridishes were sealed with Para film[®] and incubated at 26±1°C for 7 days in absence of light. Later, cultured dishes were placed under 16 hrs light/8 hrs dark photoperiod cycle (1200 lux) at 24±2°C under dim light.

After 45-50 days, calli (72 mm diam) were transferred to the regeneration medium, consisting of MS basal medium fortified with 0.4 mg l⁻¹ BA, 0.4 mg l⁻¹ NAA, 20.0 g l⁻¹ sucrose and 8.0 g l⁻¹ agar for regeneration. After 35-40 days of subculturing on regeneration medium, plantlets were counted and transferred to the glass house for hardening.

The data were analyzed in factorial completely randomized design and data, recorded in percentage, were transformed according to *Arc-sine* transformation.

Results and Discussion

Isolated soybean anthers from ten genotypes were cultured on four fortified B₅ media after cold pretreatment. In our preliminary experiment, anthers pretreated for 3-5 days at 6±2°C exhibited better androgenic response as compared to fresh anther culture. Cold pretreatment of 4-8°C for few days have been found to be beneficial for callus induction^{6,11}. Cold pretreatment is known to maintain the viability of embryogenic microspores¹². Basal medium B₅ was used in all the four induction media since earlier it had been proved superior for soybean anther culture^{3-5,11}. Sucrose concentration in the medium is another important factor for soybean androgenesis. Earlier studies indicated that high sucrose contents of 9-12% were crucial to initiate soybean microspore divisions followed by callus development^{3,6,13}. It has been further suggested that higher sucrose concentrations in medium were capable of inhibiting somatic callus formation^{4,6}.

After 8-10 days of culture, the first response of the anthers was similar and mostly independent of genotypes and media. These anthers became swollen

and no callus proliferation was observed during first few days. By the third week of culture all the anthers turned brown in colour and callus initiation was observed from upper portion of the anther lobes (Fig. 1a). During the present investigation, cultured anthers turned brown after 10 days and such anthers usually proliferated callus and/or embryoids from the upper portion of anther lobes (not in contact with culture media). In no case, callus development was observed from residues of filamentous tissues connecting tissues or various layers of microsporangium. A histological analysis⁸ on cultured anthers of soybean showed cell proliferation from pollen in anthers locule and formation of androgenic tissue (callus and/or embryoids).

After four weeks of culture, initiated calli were distinguished on the basis of their phenotypic appearances. The final count on the basis of callus characteristics was made after 40-50 days of culture. Three main types of callus cultures were observed: (a) early appearing undifferentiated calli, cream in colour and soft friable in texture (b) late appearing, compact and light green and (c) mixture of creamish and light green calli dense and glossy in texture. After 6-7 weeks 'b' type calli were counted as morphogenic calli (Fig. 1b; Table 1). The increase of the cell number causes the rupture of the microsporangium and callus proliferation projecting outside⁸. Apparent embryogenesis in the form of bipolar embryo like structures was not observed during the present investigation. However, earlier reports^{4,6} indicated the formation of embryo like structures from cultured anthers of Chinese soybean cultivars.

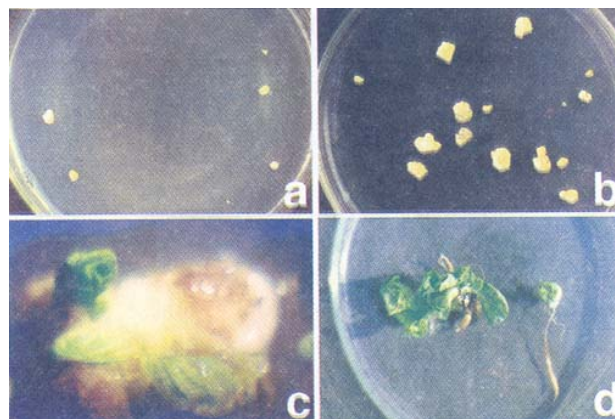


Fig. 1—*In vitro* androgenesis in soybean: a. cultured anthers with callus initiation; b. callus proliferation; c. shoot formation & d. regenerants.

Table 1—Callus initiation and embryogenic callus formation from soybean anthers of 10 genotypes on four induction media

Genotype ▼ Culture media ►	Callus forming anthers (%)					Morphogenic calli (%)				
	B ₅ DBIG	B ₅ DB	B ₅ DK	B ₅ BKN	Mean	B ₅ DBIG	B ₅ DB	B ₅ DK	B ₅ BKN	Mean
Bragg	3.07	3.19	0.00	1.82	2.02	1.38	0.00	0.00	0.00	0.34
JS 72-44	5.60	4.06	3.94	3.01	4.15	3.40	0.00	2.20	2.84	2.11
JS 72-280	3.87	4.43	3.04	2.00	3.33	1.35	2.65	2.76	2.60	2.34
JS 75-46	5.74	4.28	4.91	4.31	4.81	2.47	3.37	2.55	1.99	2.59
JS 90-29	4.79	5.07	4.02	4.37	4.56	3.61	2.65	2.78	2.93	2.99
JS 90-41	7.27	3.43	2.97	4.12	4.44	3.76	3.46	2.28	4.09	3.39
JS 335	2.99	2.83	2.19	0.00	2.00	1.03	1.62	1.39	1.98	1.50
NRC 2	3.52	2.76	3.12	3.94	3.33	2.47	2.68	1.61	2.92	2.42
Panjab 1	5.60	4.06	3.94	3.01	4.15	3.40	0.00	2.20	2.84	2.11
PK 472	4.06	4.47	3.46	2.61	3.65	2.47	2.60	1.69	0.00	1.69
Mean	4.40	3.73	2.96	2.85	3.43	2.27	1.90	1.76	1.93	1.95
CD (0.5)										
Genotypes					0.78					0.55
Media					0.66					0.43
Genotype x medium					0.61					0.46

Callus forming anthers were counted after 40-50 days of culture. Callus formation was recorded in all the genotypes except for Bragg on culture medium B₅DK and JS 335 on B₅BKN. The mean sum of squares due to genotypes, culture media and genotypes x culture media interactions for callus cultures and formation of morphogenic calli was significant at 5% probability level (Table 1). This indicated the presence of considerable amount of variability among the genotypes, culture media and their interactions.

Cultured anthers of genotype JS 75-46 (4.81%) followed by JS 90-29 (4.56%) and JS 90-41 (4.44%) showed maximum callus initiation. However, these results did not differ significantly with each other. These three genotypes also exhibited higher morphogenic callus formation as compared to remaining seven genotypes. The wide variations in androgenic response among cultivars of the same species have brought into focus the role of genotypes¹⁴. A considerable variability among genotypes recorded for *in vitro* response during the present investigation may be correlated to variation in the endogenous hormone levels¹⁵.

Response of various culture media for callus initiation differed significantly being highest on media B₅DBIG (4.40%) followed by B₅DB (3.73%), B₅DK (2.96%) and B₅BKN (2.85%). Different culture media also exhibited considerable variation in their response to morphogenic calli formation (Table 1). An auxin in combinations with a cytokinin i.e. 2,4-D and BAP, respectively facilitated callus formation

from cultured anthers. Higher *in vitro* response on B₅DBIG with myoinositol and L-glutamine in the medium suggested their synergetic effects on *in vitro* phenomena when compared with B₅DB medium without these supplements. B₅DK and B₅BKN performed further inferior for *in vitro* androgenesis. The great differences in the response can be associated with the presence/absence of 2,4-D and BAP in combination during the induction period. These results are in accordance with other findings⁴ where the importance of 2,4-D in combination with BAP was emphasized for significantly increasing the androgenic callus frequency.

Composition of basal medium does not play a deciding role in the *in vitro* response as compared to the type and concentration of the growth hormones¹⁶. The endogenous levels of hormones in the cells probably determine sensitivity to growth hormones¹⁷. Thus, the genetic basis of variability in tissue culture response and androgenesis is most likely due to differences in hormone metabolism within the explant, which is established by the level of gene expressions for individual hormones by the genotypes.

Genotypes interacted differently with various culture media for callus induction ability. Genotypes JS 90-41 performed the most excellent on B₅DBIG (7.27%), JS 90-41, JS 75-46 and JS 72-44 performed higher on B₅DBIG; JS 90-29, JS 72-280 and PK 472 on B₅DB whereas, NRC 2 initiated maximum calli on B₅BKN medium. Similar trend was followed by genotypes for morphogenic calli formation on various

Table 2—Number of plants regenerated on regeneration medium from androgenic calli of ten soybean genotypes raised on four induction media

Genotype ▼ Induction media ►	Plantlets regenerated (Number of anthers cultured)				
	B ₅ DBIG	B ₅ DB	B ₅ DK	B ₅ BKN	Total
Bragg	0 (610)	0 (398)	0 (480)	0 (564)	0 (2052)
JS 72-44	0 (730)	0 (468)	0 (630)	0 (380)	0 (2216)
JS 72-280	0 (730)	0 (763)	0 (390)	0 (410)	0 (2293)
JS 75-46	1 (390)	1 (368)	0 (690)	0 (530)	2 (1978)
JS 90-29	0 (430)	0 (456)	0 (368)	0 (398)	0 (1652)
JS 90-41	4 (560)	0 (360)	0 (640)	1 (390)	5 (1950)
JS 335	0 (490)	0 (430)	0 (538)	0 (410)	0 (1868)
NRC 2	0 (455)	0 (630)	0 (530)	0 (483)	0 (2098)
Panjab 1	0 (430)	0 (538)	0 (438)	0 (580)	0 (1986)
PK 472	0 (408)	0 (563)	0 (588)	0 (450)	0 (2009)
Total	5 (5145)	1 (4974)	0 (5300)	1 (4595)	7 (20102)

culture media, as the extents of results were different for various interactions. Significant differences in genotype x medium interaction, observed in the present investigation, revealed that *in vitro* response could be improved further by modifying the culture medium ingredients.

Both genetic and environmental factors affect callus formation and plant regeneration in soybean anther culture, often significantly. This usually makes comparison of work from various laboratories difficult as genotypes; media and culture conditions differ considerably. The results of current study indicate the importance of considering such interactions in the development of protocols with locally adopted genotypes and methodology.

Plant regeneration occurred in the form of gamogenesis and leaf like structures appeared on the surface of morphogenic calli (Fig. 1c). Only few plants were obtained with induced roots (Fig. 1d). Only 7 regenerants were obtained from a total of 20102 anthers with maximum of 4 plants from genotype JS 90-41 on B₅DBIG medium (Table 2). Whereas, if compared with many cereals, scanty results suggest that the medium composition currently used for anther culture is by no means optimal for haploid plant production. It can be further improved through adjustments in the medium components such as concentration and type of growth hormones, sugars, gelling agents etc. In conclusion, it was revealed that some response of recalcitrant species *G. max* could be obtained by selecting responsive genotypes and suitable anther culture medium.

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