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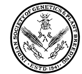

Anther culture of *Glycine max* (Merr.): Effect of media on callus induction and organogenesis

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Abstract

The present study was undertaken to standardize the doubled haploid regeneration protocol for soybean. Studies on association between floral bud size and pollen developmental stage revealed that the bud size of 2.5-4 mm containing microspore at uni-nucleate stage was most responsive for callus induction and organogenesis. Effect of different media on callus induction and plant regeneration in anther culture of three genotypes, namely, JS 335, SL 958 and SL 688 were studied. Combination of 10.0 mg l^{-1}2, 4-D and 0.5 mg l^{-1} BAP supplemented with 90 g l^{-1} sucrose and 10% v/v of coconut water in B5 medium was found best for callus induction (64.11%). Murashige and Skoog medium supplemented with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} thidiazuron gave shoot bud-like structures, in contrast, B5 medium promoted only root organogenesis (3.30-10.90%). Anther derived callus was confirmed through flow cytometry and root tip cell of anther callus derived roots to be haploid through root tip staining (chromosome number n = 20).

Key words: Anther culture, regeneration, *Glycine max* (L.) Merrilly, microspore, haploid

Introduction

Doubled haploid (DH) technology is a rapid method for developing homozygous lines, which can be used to accelerate the crop improvement programmes. Generation of homozygous lines following hybridization and anther culture of F_1 plants in one season as compared to six seasons in conventional plant breeding method is an established technique in many crop improvement programmes. Doubled haploids can be used in plant breeding for producing recombinant inbred lines (RIL's) which can be used for mapping quantitative trait loci, backcross breeding, bulked Segregant analysis, genetic maps, genomics, elite crossing and cultivar development. There are two hundred and fifty species where protocol for development of doubled haploids is available (Maluszynska 2003). In soybean, microspore embryogenesis has been tried since the early 1970s, mainly through anther culture (Tang et al. 1973; Ivers et al. 1974; Hu et al. 1996). However, a protocol for haploid and double-haploid plant production is not available so far in soybean (Rodrigues et al. 2006). The phenomenon of doubled haploid production through *in vitro* means is highly influenced by the factors like genotype and the culture media. In the present study, an attempt was made to develop a protocol for development of doubled haploids in soybean.

Materials and methods

Floral bud size conformation

Young flower buds of different sizes were harvested from polyhouse-grown plants of four genotypes, namely, G1, G2, GP 451 and PK 1347; ten buds from different size (2.0-4.0 mm) were sampled from each genotype. Floral buds were measured prior to fixation in 3:1 (100% ethanol: glacial acetic acid) at room temperature for 24 h and stored in 70% ethanol before analysis. At the time of analysis, anthers were dissected out from the buds and squashed in 1% aceto-carmine on glass slides and sealed under cover slips. Microspores were staged and classified under a microscope (Olympus, Japan). The microspore developmental phases were divided into four stages during the cytological examinations: pollen mother cell, tetrad, uni-nucleate microspore and multi-nucleate microspore (pollen).

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Anther culture

After anthesis, the unopened floral buds (2.0-3.0 mm) containing uni-nucleate to late uni-nucleate microspores were collected and buds were subjected to cold treatment at 4°C for 24h before culture. Thereafter, the buds were pre-treated by agitation in fungicide solution containing 0.2% carbendazim (Bavistin, BASF India), 0.2% Indofil-M (Rallis India) and 8-hydroxylquinnoline citrate (200 mg l^{-1}) for 1 hour. The buds were then sterilized by using mercuric chloride 0.1% $HgCl_2$ for 3.5 min. in laminar air-flow followed by three rinsing with sterilized double-distilled water and air-dried on sterile blotting paper cushion before inoculation.

The individual anther from surface-sterilized young floral buds was excised and inoculated aseptically in plastic petri dishes (90 mm in diameter, 60 anthers/dish) containing different callus induction on B5 medium supplemented with different concentrations of 2,4-D and BAP (Table 1). After inoculation, the cultures were incubated in dark at 26±1°C for seven day and later transferred to light condition (16/8 h photoperiod 47 µmol $m^{-2}s^{-1}$). For promoting callus induction, the B5 basal medium (Gamborg et al. 1968) supplemented with different concentrations of 2, 4-D (2.0-10.0 mg l^{-1}), IBA (0.5 mg l^{-1}) and BAP (0.5 mg l^{-1}) was used for different combination. In experiments to study the effect of growth hormones, the media without hormone was used as control and sucrose level was used at common dose 90 g l^{-1}. To estimate the most effective sucrose level for anther culture, four different concentration of sucrose (30, 60, 90 and 120 g l^{-1}) were added into basal media. Different concentrations of organic compounds, *viz.*, L-glutamine (200-400 mg l^{-1}), casein hydrolysate (100-200 mg l^{-1}) and coconut water (10-20% v/v) were added to study their influence on callus formation.

To identify ploidy level of anther derived callus, flow cytometry method was used. The anther derived callus and leaf sample (control) of genotype SL 688 was sent to Genetics Laboratory, Delhi University, South Campus, New Delhi for confirming ploidy status following standard procedure.

Plant organogenesis

To promote shoot organogenesis, 30-45 day-old anther derived callus was subjected to the Murashige and Skoog (1962) (MS) or B5 media supplemented with different concentrations of cytokinin [BAP (0.1-3.0 mg l^{-1}), TDZ (0.1 mg l^{-1}), kinetin (3.0 mg l^{-1})] and auxin [NAA (0.04-0.8 mg l^{-1})]. The callus mass with shoot bud-like structure were taken for routine microtome and observed at 10 x magnification for confirmation of organogenesis.

Data analysis

All the experiments were carried out in completely randomized design with five replications. Each replication comprised of 60 anthers. Data analysis was done using IRRISTAT 5.0 software. The percentage data was subjected to Arc Sine percentage transformation before calculating the ANOVA.

Results and discussion

Association between floral bud size and pollen developmental stage

The results are presented in Figs. 1 and 2. It is evident

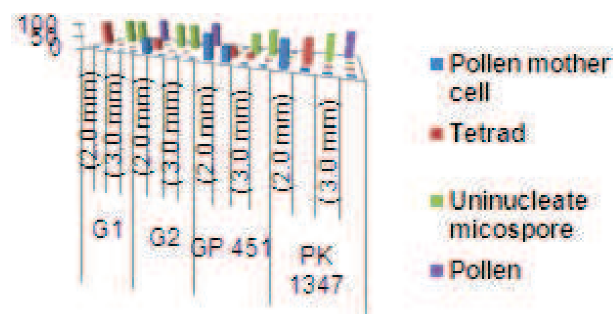


Fig. 1. Association between flower bud size and pollen development stages in soybean

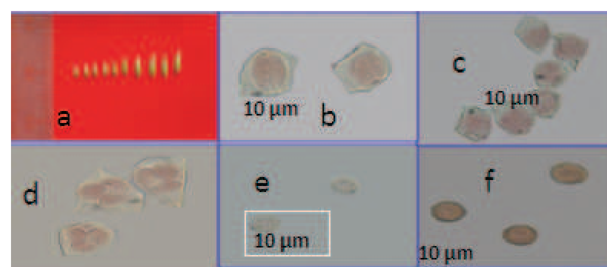


Fig. 2. Floral bud at different sizes and pollen grains at different development stages in soybean. (a) floral buds at different size; b & c) pollen mother cell; d) tetrad; d) uni-nucleate microspore, and f) pollen

that there were differences in association between bud size and microspore development stage for each genotype. Bud size of 2.5-3 mm for genotype, G1 and G2, 3 mm for PK 1347 and 4 mm for GP 451 were observed to contain microspore at uni-nucleate stage.

According to Lauxen et al. (2003), microspores from the early to late uni-nucleate stages are the most responsive for the anther culture.

Effect of plant growth regulators on callus induction

The results indicated that the soybean anther cultured on B5 medium supplemented with different concentrations of 2,4-D and BAP gave variable response (Table 1). The effect of plant growth regulators on days to callusing as well as frequency of callus induction was significant (Table 1). Among the treatments, the treatment 10 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP was most effective on callus formation. Irrespective of the combinations, the treatments having 2,4-D in combination with 0.5 mg l^{-1} BAP was most responsive. There were significant differences for days to callusing among the genotypes but the differences for frequency of callus induction among genotypes were non-significant. Data obtained in the present study showed that plant growth regulators have important role in callus induction in soybean anther culture. The best response for anther callusing at 2.0 mg l^{-1} 2,4-D combined with 0.5 mg l^{-1} 6-benzyladenine (BA) was also noted by Jian et al. (1986), Kaltchuk-Santos et al. (1997) and Oliveira et al. (1998) on soybean. However, Rodrigues et al. (2004) concluded that higher 2,4-D concentration (10 mg l^{-1}) significantly increased callogenic response with no effect on embryogenesis. High concentration of auxin (40 mg l^{-1} 2,4-D) was found to induce somatic embryogenesis from immature soybean cotyledons (Bailey et al. 1993; Droste et al. 2001). High concentration of auxin (40 mg l^{-1} 2,4-D) gave similar results in our experiments as well. Combination of 2,4-D and BAP gave better response compared to IBA. Among three genotypes, SL 688 was most responsive genotype with the shortest duration of response (12.52 days).

Effect of sucrose level on callus formation

Depending on the results obtained in the first experiment, the medium comprising of B5 basal salts supplemented with 10.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BAP was selected as the most effective medium and was used for further experiment by addition of sucrose (0 to 120 g l^{-1}).

The effect of different doses of sucrose was measured in terms of days to callus induction as well as frequency of callus formed. The effect of different doses of sucrose on days to callus induction as well as frequency of response was highly significant (Table 2); 90 g l^{-1} sucrose was most effective for callus formation for anther culture in soybean. There were significant differences for days to callusing when 90 g l^{-1} sucrose (9.7 days) was used as compared to the

Table 1. Effect of different hormone levels on callusing of anther

Treatment	Day to callusing (days)				Callusing (%)			
	JS 335	SL 958	SL 688	Mean	JS 335	SL 958	SL 688	Mean
No hormone (control)	18.20	18.80	18.20	18.40	1.00 (5.74)*	0.32 (3.14)	0.43 (3.63)	0.59 (4.44)
2 mg l^{-1} 2,4-D + 0.5 mg l^{-1} IBA	12.80	15.00	12.40	13.40	16.44 (23.89)	27.10 (31.37)	20.87 (27.20)	21.47 (27.63)
10 mg l^{-1} 2,4-D + 0.5 mg l^{-1} IBA	12.40	11.60	11.20	11.73	29.83 33.09)	32.01 (34.45)	29.20 (32.71)	30.35 (33.46)
2 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP	10.00	11.00	10.60	10.53	56.17 (48.56)	54.22 (47.41)	39.00 (38.65)	49.80 (44.80)
10 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP	9.400	10.80	10.20	10.13	73.19 (58.82)	61.79 (51.83)	66.89 (54.88)	67.29 (55.12)
Mean	12.56	13.44	12.52		35.33 (36.45)	35.09 (36.33)	31.28 (34.02)	
LSD ($P \leq 0.05$)								
Treatment (A)	0.73				9.09			
Genotype (B)	0.56				7.04			
Interaction (AxB)	1.26				15.75			
CV (%)	7.80				3.67			

*Arc Sin $\sqrt{\%}$ transformed data; Full-strength B5 + 3% sucrose

Table 2. Effect of different sucrose concentrations on callus induction in soybean anther culture

Treatment (g l^{-1})	Duration of response (Days)				Frequency of response (%)			
	JS 335	SL 958	SL 688	Mean	JS 335	SL 958	SL 688	Mean
0.0 (control)	18.60	20.80	18.80	19.40	10.52 (18.91)*	2.07 (8.33)	5.67 (13.81)	6.09 (14.30)
30	10.60	11.80	9.800	10.73	71.93 (57.99)	44.57 (41.90)	48.83 (44.51)	55.11 (47.93)
60	9.600	10.80	10.00	10.13	71.33 (57.61)	54.02 (47.29)	53.22 (46.83)	59.52 (50.48)
90	9.200	10.00	9.200	9.47	71.08 (57.48)	59.40 (50.42)	55.84 (48.33)	62.11 (52.00)
120	11.20	11.40	10.20	10.93	54.25 (47.47)	43.97 (41.55)	68.52 (55.86)	55.58 (48.22)
Mean	11.84	12.96	11.60		55.82 (48.33)	40.81 (39.70)	46.42 (42.94)	
LSD ($P \leq 0.05$)								
Treatment (A)	0.52				8.73			
Genotype (B)	0.40				6.76			
Interaction (AxB)	0.89				15.11			
CV (%)	5.80				2.51			

*Arc Sin √% transformed data; Full-strength B5 + 10 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP

Table 3. Effect of different organic compounds on callusing in anthers of different soybean genotypes

Treatment	Day to callusing (days)				Callusing (%)			
	JS 335	SL 958	SL 688	Mean	JS 335	SL 958	SL 688	Mean
200 mg l^{-1} L-glutamine	10.80	10.60	10.60	10.67	63.08 (52.59)*	49.23 (44.54)	64.66 (53.55)	58.99 (50.18)
400 mg l^{-1} L-glutamine	11.80	11.60	11.20	11.53	50.26 (45.17)	22.29 (28.18)	55.29 (48.04)	42.61 (40.74)
100 mg l^{-1} casein hydrolysate	10.20	11.60	10.40	10.73	50.97 (45.57)	54.40 (47.52)	73.40 (58.95)	59.59 (50.53)
200 mg l^{-1} casein hydrolysate	9.80	10.20	10.00	10.00	59.80 (50.65)	42.70 (40.80)	69.59 (56.54)	57.36 (49.26)
100 mg l^{-1} casein hydrolysate + 200 mg l^{-1} L-glutamine	9.60	9.80	9.60	9.67	58.49 (49.89)	51.67 (45.97)	61.98 (51.94)	57.38 (49.26)
200 mg l^{-1} casein hydrolysate 400 mg l^{-1} L-glutamine	9.80	10.80	10.20	10.27	46.39 (42.94)	39.39 (38.88)	41.67 (40.22)	42.48+ (40.69)
10% coconut water	9.40	10.40	9.40	9.73	68.18 (55.67)	58.10 (49.66)	66.06 (54.39)	64.11 (53.19)
20% coconut water	9.80	10.40	10.20	10.13	44.25 (41.73)	47.52 (43.57)	60.21 (50.89)	50.66 (45.40)
Mean	10.15	10.68	10.20		54.93 (47.81)	45.41 (42.36)	61.36 (51.59)	
LSD (*P* d" 0.05)								
Treatment (A)	0.60				9.32			
Genotype (B)	0.37				5.71			
Interaction (AxB)	1.05				16.15			
CV (%)	8.10				2.39			

*Arc Sin √% transformed data; Full-strength B5 + 10 mg l^{-1} 2,4 D + 0.5 mg l^{-1} BAP + 9% sucrose

control (19.4 days). Significant differences were noticed for frequency of response as well (62.11 Vs 6.09) among the three genotypes. Among the three genotypes tested, JS 335 was most responsive. The treatment without sucrose gave very low callusing frequency (6.87%) with delayed response (19.40 days).

This showed that sucrose had a role for callus formation. Sugar is considered as the source of carbon and energy and also acts as an osmotic regulator in the induction medium (Ferrie et al. 1995). Earlier, Kumar and Murthy (2004) in their study on *Cucumis sativus* found that enhanced sucrose from 0.15 to 0.25 M (90 g l^{-1}) resulted in increase in number of embryos on anther callus. However, further increase beyond 0.25M resulted in decline in response.

Effect of organics addenda to callus formation

It is evident from the data shown in Table 3, casein hydrolysate and L-glutamine showed some influence on callus induction. The highest frequency for callusing was recorded in treatment containing 10% of coconut water (64.11%). The treatment containing only 200 mg l^{-1} casein hydrolysate + 400 mg l^{-1} L-glutamine gave low response frequency. Among three genotypes, there was no significant difference in duration of response. The anther derived callus of genotype SL 688 was confirmed as haploid using flow cytometry method (Fig. 4). Flow cytometry has been used in identifying the ploidy level in plant due to its accuracy, quickly and reliability. Dang et al. (2011) used this tool to detect false positives in bulks of maize seeds and Mohammadi et al. (2007), Kleiber et al. (2012) and Couto et al. (2013) to identify haploid maize.

Effect of media composition on organogenesis

The 30-45 day-old callus was transferred to the regeneration medium containing MS or B5 salts supplemented with different concentrations of cytokinins (BAP, TDZ, kinetin) and auxin (NAA). The results show that shoot bud-like structures could not grow further on MS medium supplemented with higher levels of BAP and NAA from 0.2 to 0.8 mg l^{-1}. Some improvement was observed when medium was supplemented with 2.0 mg l^{-1} BAP and 1.0 mg l^{-1} TDZ, as callus turned green but with restricted growth having shoot bud like structures on the periphery of the callus masses. The shoot buds had restricted growth and did not differentiate into full grown shoots even after 2-3 sub-cultures when confirmed through microtome (Fig. 3). However, when callus was placed on B5 salts (4 times of micro salts) medium having combinations of BAP (0.04-3.0 mg l^{-1}), TDZ (0.1 mg l^{-1}), kinetin (3.0 mg l^{-1}) and NAA (0.04 mg l^{-1}), there was an appearance of roots (Table 4 and 5; Fig. 5 a). It was evident that B5 medium had promoted root organogenesis compared to MS medium.

Organogenesis potential also depends upon the genotype. Out of three genotypes tried, JS 335 and SL 688 were more responsive to root organogenesis compared to SL 958. The ploidy level of root tip cell from anther callus derived roots was confirmed as haploid with chromosome number n = 20 (Figs. 5b-c).

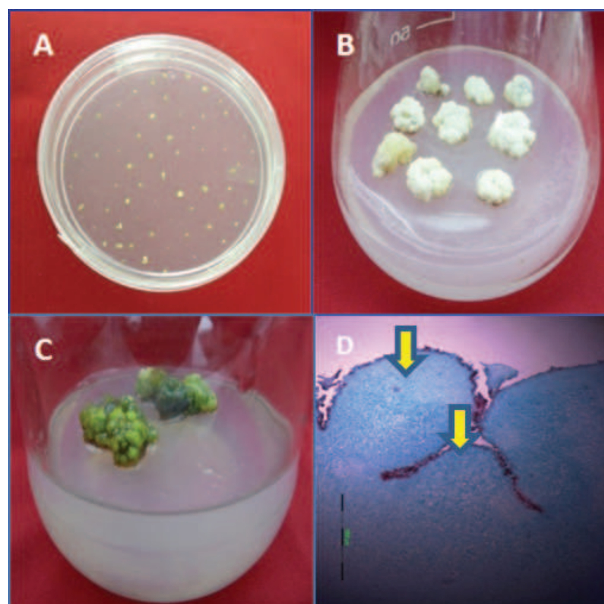
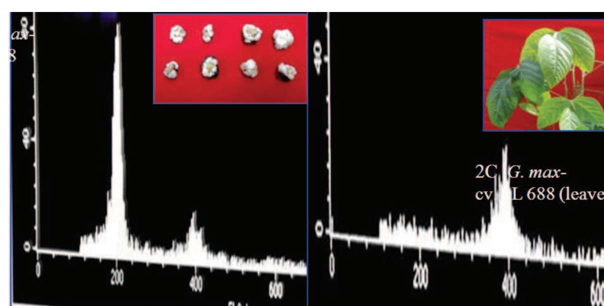


Fig. 3. Anther culture and shoot-bud like structure formation in soybean genotype JS355 (a) Callus induction on B5 medium supplemented with 10 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP + 10% coconut water + 9% sucrose; (b) anther callus at 45-days, (c) : anther callusi derived shoot bud like structure on medium MS + 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} TDZ, and (d) microtome section of shoot bud-like structure differentiation (10 X)



A: Haploid callus **B: Diploid plants**

Fig. 4. Histograms of flow cytometric analysis of PI-stained nuclear suspensions of anther derived callus. (A) G0/G1 Nuclei peak of the sample *G. max* anther callus cv. SL 688 (1C = 1.134 pg) was positioned in channel 200; and (B) Nuclei peak of the diploid sample *G. max* (leave) (2C = 2.268 pg) was positioned in channel 400, showing anther callus ploidy level as n = x = 20 chromosomes

Table 4. Effects of PGRs on root organogenesis (%) in soybean anther culture

Treatment	JS 335	SL 958	SL 688
MS + No hormone (control)	-	-	-
MS + 0.2 mg l^{-1} BAP + 0.2 mg l^{-1} NAA	-	-	-
MS + 0.4 mg l^{-1} BAP + 0.4 mg l^{-1} NAA	-	-	-
MS + 0.8 mg l^{-1} BAP + 0.8 mg l^{-1} NAA	-	-	-
MS + 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} TDZ	-	-	-
B5 + 3.0 mg l^{-1} BAP + 0.04 mg l^{-1} NAA	8.89 (17.38)*	-	-
B5 + 0.1 mg l^{-1} BAP + 0.1 mg l^{-1} TDZ	10.90 (19.28)	-	10.90 (19.28)
B5 + 3.0 mg l^{-1} Kinetin + 0.04 mg l^{-1} NAA	10.30 (18.72)	-	10.30 (18.72)
B5 + 1.0 mg l^{-1} BAP + 0.1 mg l^{-1} NAA	3.30 (10.47)	3.30 (10.47)	-

*Arc Sin √% transformed data

Table 5. Effect of media composition on organogenesis in anther culture of different soybean genotypes

Treatment	Duration for rooting (days)				No. of roots/callus			
	JS 335	SL 958	SL 688	Mean	JS 335	SL 958	SL 688	Mean
MS + No hormone (control)	-	-	-		-		-	
MS + 0.2 mg l^{-1} BAP + 0.2 mg l^{-1} NAA	-	-	-		-	-	-	
MS + 0.4 mg l^{-1} BAP + 0.4 mg l^{-1} NAA	-	-	-		-	-	-	
MS + 0.8 mg l^{-1} BAP + 0.8 mg l^{-1} NAA	-	-	-		-	-	-	
MS + 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} TDZ	-	-	-		-	-	-	
B5 + 3.0 mg l^{-1} BAP + 0.04 mg l^{-1} NAA	11.33	-	-		2.67	-	-	
B5 + 0.1 mg l^{-1} BAP + 0.1 mg l^{-1} TDZ	14.67	-	12.33		5.67	-	3.33	
B5 + 3.0 mg l^{-1} Kinetin + 0.04 mg l^{-1} NAA	17.33	-	16.00		3.00	-	1.67	
B5 + 1.0 mg l^{-1} BAP + 0.1 mg l^{-1} NAA	7.00	20.00	-		1.00	1.00	-	

Tiwari et al. (2004) reported that the culture medium B_5 DBIG (2 mg l^{-1} 2,4-D + 0.5 mg l^{-1} IBA + 100 mg l^{-1} of *myo*-inositol + 360 mg l^{-1} L-glutamine) exhibited higher response for androgenic callus formation. Haploid plant regeneration in genotype JS 90-41 was reported on MS medium supplemented 0.4 mg l^{-1} BAP, 0.4 mg l^{-1} NAA and 20 g l^{-1} sucrose (Tiwari et al. 2004). We can conclude that, MS medium to be more

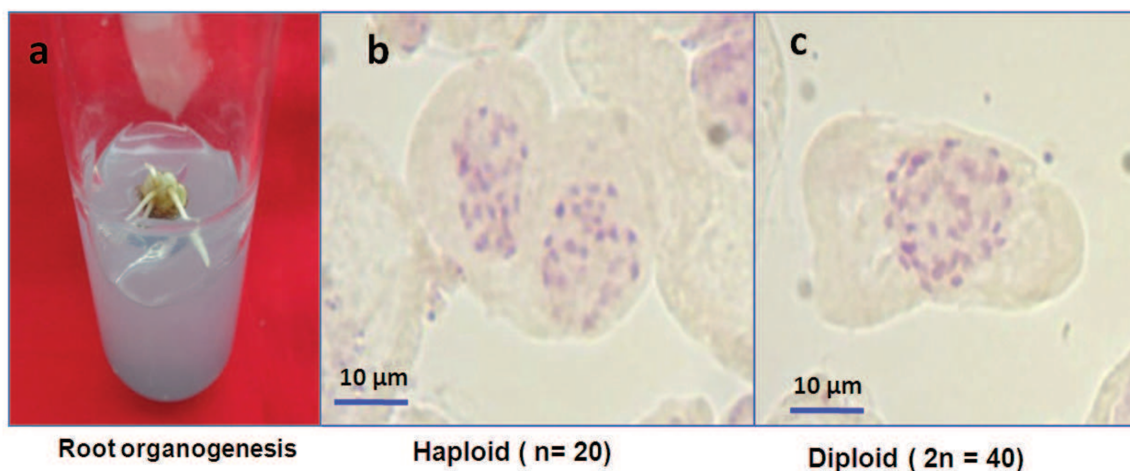


Fig. 5. Root organogenesis on anther derived callus and cytogentical observation of root tips in soybean genotype JS 335. (a) root organogenesis on B5 + 0.1 mg l^{-1} BAP + 0.1 mg l^{-1} TDZ; (b) Hapoid cell (n = 20); and (c) Diploid cell (2n = 40) observed at 100 X

effective for androgenesis in soybean though the PGRs had critical role in organogenesis. The shoot-bud regeneration can be enhanced by modification of culture medium composition, hormonal levels, physical manipulations etc. in soybean anther culture to develop repetitive protocol for doubled-haploid plantlet production.

Author's contributions

Conceptualization of research (SLK, SKS, NHH); Designing of the experiments (SKL, SKS, NHH, Vinod, AT); Contribution of experimental materials (SKL); Execution of field/lab experiments and data collection (NHH, SKL, SKS); Analysis of data and interpretation (SKL, SKS, NHH); Preparation of the manuscript (SKL, SKS, NHH, AT).

Declaration

The authors declare no conflict of interest.

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