



# High frequency regeneration in soybean [*Glycine max* (L.) Merrill.] through direct somatic embryogenesis from immature cotyledons

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**Abstract** A simple and efficient system of soybean regeneration protocol was standardized through direct somatic embryogenesis by using immature cotyledons as explant. Three genotypes, namely, DS 2706, Pusa 5 and PS 1477 were selected for the evaluation of their capacity for in vitro somatic embryogenesis and plantlet regeneration. The medium MSB5 (MS macro + micro + B5 organics) supplemented with different concentrations of plant growth regulators (2, 4-D, NAA, BAP and ABA) were evaluated for somatic embryo initiation and subsequent development. Somatic embryos were initiated in the shortest duration (25.1 days) with the highest response (86.5 %) on MSB5 medium supplemented with 40 mg l<sup>-1</sup> 2, 4-D compared to lower concentrations or combination treatments with NAA and BAP. Somatic embryos proliferation was highest (76.25 %) in the MSB5 medium supplemented with 20 mg l<sup>-1</sup> 2, 4-D and 13 mg l<sup>-1</sup> ABA. Somatic embryos and their morphological development were noted on medium supplemented with 6 % maltose and 0.5 % activated charcoal (79.47 %) compared to sucrose. Maltose at 6 % gave good maturation of somatic embryos (92.67 %). The highest germination (53.03 %) of somatic embryos into plantlets was achieved in the medium containing 1.5 % sucrose. Amongst the genotypes screened, DS 2706 was the most responsive. The protocol developed can be used for direct

regeneration via somatic embryogenesis in different genotypes, which could also be used for attempting genetic transformation.

**Keywords** *Glycine max* (L.) Merrill · Immature cotyledon · Regeneration · Somatic embryogenesis

## Introduction

Soybean [*Glycine max* (L.) Merrill] is one of the world's most important leguminous crops, being a major source of protein and edible oil. Soybean is cultivated for seed, which contains oil (18–20 %) and protein (38–40 %), primary ingredients in many processed foods. Several laboratories throughout the world are striving to improve the nutritional qualities of soybean seed protein and also tolerance towards biotic and abiotic stresses. Genetic improvement of commercially important soybean cultivars through classical breeding is laborious and time consuming (Loganathan et al. 2010). Alternatively, biotechnology offers excellent opportunities for the improvement of existing soybean cultivars through tissue culture techniques, *i.e.*, somatic embryogenesis. Further, in vitro techniques offer opportunity for creation of variation in otherwise self-pollinated crops like soybean, which have a limited genetic base.

The capacity for somatic embryogenesis is a heritable trait, a number of genes have been identified that control different aspects of somatic embryogenesis in soybean and other plants (Parrott et al. 1991; Tar'an and Bowley 1997). Moreover, several studies have shown differences among soybean genotypes for their capacity to respond to the different steps of somatic embryogenesis (Yang et al. 2009; Loganathan et al. 2010). Different plant tissues have been

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used as explant for regeneration of somatic embryos in soybean, *i.e.*, hypocotyl segment (Tripathi and Tiwari 2003), cotyledonary sections (Korbes and Droste 2005), embryonic axis (Kumari et al. 2006), immature embryo, immature cotyledon and immature embryogenic axis (Sharifi et al. 2006; Perez–Perez et al. 2009), and immature embryonic shoot tip (Loganathan et al. 2010). However, efficiency of soybean regeneration through somatic embryogenesis has been limited to a few genotypes that respond ideally to *in vitro* culture (Hiraga et al. 2007; Yang et al. 2009). Somatic embryogenesis is a highly preferred pathway over organogenesis, as the transformants derived are more uniform and the chances for the occurrence of variation among individual clones are lesser (Osuga et al. 1999).

Somatic embryogenesis from excised zygotic embryos was reported by Christianson et al. (1983). Later, Lazzeri et al. (1985) reported the use of immature cotyledons and, since then, immature cotyledons have been used as the sole explant system capable of regenerating into plantlets via somatic embryogenesis (Klink et al. 2008). Nevertheless, somatic embryo derived from immature cotyledons is highly genotype-dependent (Ko et al. 2004). The potential for embryogenesis can be improved to a certain extent by modification of tissue culture protocols for specific genotypes. The present investigation was undertaken to study the regeneration from immature cotyledons through somatic embryogenesis in selected soybean genotypes, so as to standardize a suitable protocol.

## Materials and methods

### Plant material

Three soybean genotypes, *viz.*, DS 2706, Pusa 5 and PS 1477 were used for the present study. The seeds were collected from the Soybean Laboratory, Division of Genetics, IARI, New Delhi. These genotypes were sown in Polyhouse Facility, IARI, New Delhi. After fruit set, the immature pods (15 days after of pod set) containing immature seeds (3.0–4.0 mm) of all the genotypes were harvested 1–2 weeks after flowering. These immature pods were collected in polybags and taken to laboratory for culture.

### Pretreatment and surface sterilization

The immature pods were pre-treated by agitation in fungicide solution containing 0.2 % carbendazim (Bavistin<sup>®</sup>, BASF India), 0.2 % indofil-M<sup>®</sup> (Rallis India) and 0.002 % 8-hydroxyquinoline citrate (Qualigens Chem., Mumbai) for 1 h, followed by rinsing in sterile water for

three times. Sterilized pods were shifted to laminar air-flow hood and surface sterilized with HgCl<sub>2</sub> 0.1 % for 4 min, followed by three rinsings with sterile double-distilled water. The immature seeds were separated from the pods and cotyledon explant (3.0–4.0 mm) was excised after removing the seed coat. The ends of the embryonic axis were removed with sterile scalpel and then cultured on embryo induction medium following the procedures outlined by Bailey et al. (1993a, b), Finer and Nagasawa (1988) and Huynh et al. (2013).

### Culture initiation, somatic embryo induction and germination

All the explants were cultured on embryo induction medium MSB5 (Murashige and Skoog 1962; Gamborg et al. 1968) supplemented with different concentrations of 2,4-D (10, 20, 40 mg l<sup>-1</sup>), BAP (3.0 mg l<sup>-1</sup>) and NAA (0.5, 10 mg l<sup>-1</sup>) in combinations with sucrose (3 %), gellan gum (0.2 %) (Gelrite<sup>™</sup>, Sigma Chem. Co. St. Louis, USA), with pH adjusted to 7.0 ± 0.01. The cultures were maintained at 26 ± 1 °C with 16/8 h photoperiod (39 μE m<sup>-2</sup> s<sup>-1</sup>) and sub-cultured onto the same fresh medium at 15-days interval until induction of somatic embryos. The percentage of embryogenesis was calculated after 4 weeks of culture.

After 30 days in somatic induction media, primary somatic embryos were transferred to MSB5 medium supplemented with 20 mg l<sup>-1</sup> 2,4-D alone or in combination with 13 mg l<sup>-1</sup> abscisic acid (ABA), 3 % sucrose and 0.2 % gellan gum, and pH adjusted to 5.8. After 20 more days, the percentage of embryogenic cotyledons, which got proliferated were scored and number of somatic embryos per cotyledon recorded. Globular somatic embryos cluster were then removed from cotyledon and transferred to MSB5 medium supplemented with different dosage of maltose and sucrose (3–6 %), activated charcoal (0.5 %) for further differentiation. The percentage of somatic embryos clusters, which got differentiated, was recorded after 30 days.

The cotyledonary stage embryos from the preceding step were separated individually and transferred onto MSB5 medium supplemented with different concentration of maltose and sucrose (3–6 %) to achieve somatic embryo maturation. The frequency of somatic embryos, which got matured were also calculated after 30 days. The matured cream-coloured cotyledonary somatic embryos were then desiccated in sterile disposable empty petridishes. After 7 days of desiccation, the embryos were transferred onto hormone-free MSB5 medium containing 1.5–3 % sucrose for germination. At this stage, a 23/1 h light/dark photoperiod was maintained. The frequency of the germinated embryos, shoot length and the root length was recorded after

4 weeks. After embryos converted into complete plant, they were transplanted into glass jar with PP cap containing peat, perlite and vermiculite (2:1:1; v/v). The hardened plants were maintained at  $26 \pm 1$  °C with 16/8 h photoperiod ( $97 \mu\text{E m}^{-2} \text{s}^{-1}$ ) and maintained under high relative humidity condition for 2 weeks. The hardened plantlets were then transferred to glasshouse under natural light conditions.

### Statistical analysis

All the experiments were carried out in completely randomized design with five replications. Each treatment comprised of 25–30 units. Data analysis was done using IRRISTAT 5.0 software. The percentage data was subjected to Arc Sine  $\sqrt{\text{percentage}}$  transformation before calculating ANOVA.

### Results and discussion

The results showed that immature cotyledons cultured on MSB5 medium supplemented with different concentration of 2,4-D, BAP and NAA gave variable responses (Table 1). The medium containing  $40 \text{ mg l}^{-1}$  2,4-D gave good embryo induction in the shortest duration (25.07 days), followed by  $20 \text{ mg l}^{-1}$  2, 4-D and  $10 \text{ mg l}^{-1}$  2,4-D +  $10 \text{ mg l}^{-1}$  NAA, while medium supplemented with  $3 \text{ mg l}^{-1}$  BAP +  $0.5 \text{ mg l}^{-1}$  NAA gave delayed embryo induction (31.27 days). Among the three genotypes, DS 2706 was the most responsive genotype with the earliest response (28.56 days), followed by PS 1477 (28.65 days)

and Pusa 5 (28.09 days). The media standardized by Loganathan et al. (2010) was found efficient for different stages, *i.e.*, initiation to germination of somatic embryos. It was found that embryogenic potential was better using immature stage cotyledon explants and the medium containing  $40 \text{ mg l}^{-1}$  2, 4-D was most responsive. Embryogenic potential was highest (60–80 %) in the selected genotypes. This finding was in conformity with those of Loganathan et al. (2010). Use of auxin (2, 4-D) was found to have a prominent role in the induction and proliferation of somatic embryos. Earlier, several workers have advocated the beneficial role of 2, 4-D (Kim et al. 2000; Ponappa et al. 1999; Ugandhar et al. 2013). Further, it was reported that successful embryogenesis in soybean either takes place with low level of 2, 4-D (Orczyk and Orczyk 1994) or very high level of 2,4-D (Droste et al. 2002). This differential auxin requirement is attributed to the genotypic effect (Hiraga et al. 2007).

The frequency of somatic embryo initiation was highest (86.50 %) in MSB5 medium supplemented with  $40 \text{ mg l}^{-1}$  2, 4-D, followed  $10 \text{ mg l}^{-1}$  2,4-D +  $10 \text{ mg l}^{-1}$  NAA (59.63 %) and  $20 \text{ mg l}^{-1}$  2,4-D. The treatment MSB5 media supplemented with  $3.0 \text{ mg l}^{-1}$  BAP +  $0.5 \text{ mg l}^{-1}$  NAA gave the lowest frequency (14.60 %). Among three genotypes, DS 2706 gave the highest response (59.0 %), followed by Pusa 5, while lowest response was observed in PS 1477. Genotype dependent response for frequency of somatic embryogenesis has been documented in soybean (Loganathan et al. 2010; Yang et al. 2009).

The number of somatic embryo regeneration was highest in the treatment, where MSB5 medium was supplemented

**Table 1** Effect of MSB5 medium supplemented with different hormones on duration and frequency of somatic embryo (SE) induction in soybean

Treatment	Duration of somatic embryo induction (days)				Frequency of somatic embryogenesis (%)			
	DS 2706	Pusa 5	PS 1477	Mean	DS 2706	Pusa 5	PS 1477	Mean
Control (no hormone)	–	–	–	–	–	–	–	–
2,4-D ( $20 \text{ mg l}^{-1}$ )	26.80	29.60	28.20	28.20	56.00 (48.45)*	64.40 (53.37)	54.50 (47.58)	58.30 (49.78)
2,4-D ( $40 \text{ mg l}^{-1}$ )	26.20	24.30	24.70	25.07	84.00 (66.42)	88.20 (69.91)	87.30 (68.28)	86.50 (68.44)
2,4-D ( $10 \text{ mg l}^{-1}$ ) + NAA ( $10 \text{ mg l}^{-1}$ )	30.60	31.20	29.80	30.53	70.08 (56.85)	59.10 (50.24)	49.00 (44.43)	59.63 (50.53)
BAP ( $3.0 \text{ mg l}^{-1}$ ) + NAA ( $0.5 \text{ mg l}^{-1}$ )	30.80	31.20	31.90	31.27	25.20 (30.13)	12.04 (20.27)	6.20 (14.42)	14.60 (22.46)
Mean	28.58	29.08	28.65		59.00 (50.18)	56.00 (48.45)	49.25 (44.60)	
LSD ( $P \leq 0.05$ )								
Treatment (T)	1.06				6.53			
Genotype (G)	0.92				5.65			
Interaction (T × G)	1.84				11.30			
CV (%)	7.20				6.20			

\* Arc Sin  $\sqrt{\text{percentage}}$  transformed data in parentheses

with 40 mg l<sup>-1</sup> 2,4-D (19.33 embryos cotyledon<sup>-1</sup>), followed 20 mg l<sup>-1</sup> 2, 4-D (13.27 embryos cotyledon<sup>-1</sup>), while the lowest number was in 3.0 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> NAA (4.53 embryos cotyledon<sup>-1</sup>). The average number of embryos produced by three genotypes differed non-significantly. The highest number of embryos was achieved in genotype DS 2706 (13.25 embryos cotyledon<sup>-1</sup>), followed (non-significantly) by Pusa 5 (11.75 embryos cotyledon<sup>-1</sup>) and PS 1477 (11.35 embryos cotyledon<sup>-1</sup>) (Table 2). In our study too, somatic embryogenesis from immature cotyledons was found to be highly genotype-dependent event as reported earlier (Hiraga et al. 2007; Ko et al. 2004).

Somatic embryos after formation were transferred onto MSB5 medium supplemented with different concentrations of 2, 4-D and ABA for proliferation. The result showed that the treatment supplemented with 20 mg l<sup>-1</sup> 2, 4-D + 13 mg l<sup>-1</sup> ABA gave significantly higher response (76.25 %) compared to treatment having 20 mg l<sup>-1</sup> 2,4-D alone (57.52 %). Genotype DS 2706 gave the highest somatic embryo

proliferation (71.79 %), followed significant by PS 1477 (65.47 %). Abscisic acid is known to promote embryo growth, development, maturation, and improved embryo germination, when used at the globular stage (Tian and Brown 2000), while ABA supplied during advanced maturation did not give increment in conversion frequencies (Schmidt et al. 2005). However, Weber et al. (2007) reported an increment in plant conversion when ABA was employed at both proliferation and maturation stages. Earlier, Droste et al. (2010) also reported that 50 µM ABA was optimum for somatic embryo proliferation in soybean genotypes (Table 3).

The globular embryos after proliferation were transferred to MSB5 medium supplemented with different concentrations of sucrose and maltose, along with 0.5 % activated charcoal. The results showed that after 3 weeks of incubation treatment supplemented with 6 % maltose gave highest differentiation (79.47 %), followed non-significantly by treatment containing 3 % of maltose (72.80 %), while 3 % sucrose gave the lowest differentiation frequency (Table 4). At this stage, genotype Pusa 5

**Table 2** Effect of MSB5 medium supplemented with different hormones on number of somatic embryos per explant initiation in soybean

Treatment	Number of somatic embryos per explant			
	DS 2706	Pusa 5	PS 1477	Mean
Control (no hormone)	–	–	–	–
2,4-D (20 mg l <sup>-1</sup> )	14.40	12.20	13.20	13.27
2,4-D (40 mg l <sup>-1</sup> )	21.80	18.60	17.60	19.33
2,4-D (10 mg l <sup>-1</sup> ) + NAA (10 mg l <sup>-1</sup> )	12.40	10.40	11.20	11.33
BAP (3.0 mg l <sup>-1</sup> ) + N AA (0.5 mg l <sup>-1</sup> )	4.40	5.80	3.40	4.53
Mean	13.25	11.75	11.35	
LSD ( $P \leq 0.05$ )				
Treatment (T)	5.16			
Genotype (G)	4.47			
Interaction (T × G)	8.94			
CV (%)	58.00			

**Table 3** Effect of MSB5 medium supplemented with different hormones on somatic embryo proliferation in soybean

Treatment	Somatic embryo proliferation (%)			
	DS 2706	Pusa 5	PS 1477	Mean
Control (no hormone)	–	–	–	–
2,4-D (20 mg l <sup>-1</sup> )	56.11 (48.50)*	55.12 (47.93)	61.33 (51.53)	57.52 (49.31)
2,4-D (20 mg l <sup>-1</sup> ) + ABA (13 mg l <sup>-1</sup> )	87.48 (69.30)	71.69 (57.86)	69.60 (56.54)	76.25 (60.87)
Mean	71.79 (57.92)	63.40 (52.77)	65.47 (54.03)	
LSD ( $P \leq 0.05$ )				
Treatment (T)	3.29			
Genotype (G)	4.03			
Interaction (T × G)	5.69			
CV (%)	6.50			

\* Arc Sin  $\sqrt{\%}$  transformed data in parentheses

**Table 4** Effect of MSB5 medium supplemented with different sugar levels on somatic embryo differentiation

Treatment	Somatic embryo differentiation (%)			
	DS 2706	Pusa 5	PS 1477	Mean
Control (no sugar)	–	–	–	–
Sucrose (3 %)	25.00 (30.00)*	36.00 (36.87)	35.00 (36.27)	32.00 (34.45)
Sucrose (6 %)	62.00 (51.94)	54.00 (47.29)	52.00 (46.15)	56.00 (48.45)
Maltose (3 %)	65.40 (53.97)	85.00 (67.21)	68.00 (55.55)	72.80 (58.56)
Maltose (6 %)	70.40 (57.04)	89.00 (70.63)	79.00 (62.72)	79.47(63.08)
Mean	55.70 (48.27)	66.00 (54.33)	58.50 (49.89)	
LSD ( $P \leq 0.05$ )				
Treatment (T)	8.20			
Genotype (G)	7.10			
Interaction (T × G)	14.20			
CV (%)	8.60			

\* Arc Sin  $\sqrt{\%}$  transformed data in parentheses**Table 5** Effect of MSB5 medium supplemented with different sugar levels on somatic embryo maturation in soybean

Treatment	Somatic embryo maturation (%)			
	DS 2706	Pusa 5	PS 1477	Mean
Control (no sugar)	–	–	–	–
Maltose (3 %)	72.00 (58.05)*	73.00 (58.69)	81.00 (64.16)	75.33 (60.20)
Maltose (6 %)	92.00 (73.57)	93.00 (74.66)	93.00 (74.66)	92.67 (74.32)
Sucrose (3 %)	83.00 (65.65)	83.00 (65.65)	79.00 (62.72)	81.67 (64.67)
Maltose (6 %) + ABA (13 mg l <sup>-1</sup> )	85.00 (67.21)	87.00 (68.87)	88.00 (69.73)	86.67 (68.61)
Maltose (6 %) + ABA (13 mg l <sup>-1</sup> ) + AC (0.5 %)	53.00 (46.72)	64.00 (53.13)	62.00 (51.94)	59.67 (50.59)
Mean	77.00 (81.34)	80.00 (63.44)	80.60 (63.87)	
LSD ( $P \leq 0.05$ )				
Treatment (T)	9.35			
Genotype (G)	7.24			
Interaction (T × G)	16.20			
CV (%)	6.20			

\* Arc Sin  $\sqrt{\%}$  transformed data in parentheses

was most responsive, which gave the highest differentiation (66.00 %), followed by (non-significantly) PS 1477 (58.50 %) and DS 2706 (55.70 %). Medium supplemented with activated charcoal presumably adsorbs auxin, auxin-derivatives and several phenols released from developing tissues and may promote a more normal morphology and increased germination ability (Merkle et al. 1995). Combination of ABA and activated charcoal has been observed to be beneficial for the development of *Picea abies* somatic embryos (Pullman et al. 2005) (Table 4).

Somatic embryo maturation was achieved upon transfer to MSB5 medium supplemented with different concentration of maltose, sucrose, ABA and 0.5 % activated charcoal. The medium supplemented with 6 % maltose gave the highest response frequency for somatic embryo maturation (92.67 %), followed by 6 % maltose + 13 mg l<sup>-1</sup> ABA (81.67 %) and

3 % sucrose (81.67 %), while the lowest response was noted in treatment with 6 % maltose + 13 mg l<sup>-1</sup> ABA + 0.5 % AC (59.67 %). Among three genotypes, PS 1477 was most responsive (80.6 %), followed by (non-significant) Pusa 5 (80.0 %) and DS 2706 (77.0 %). It has been shown that ABA used at advanced maturation stage of SE has negative effects (Schmidt et al. 2005) (Table 5).

After maturation the somatic embryos were individually transferred to hormone-free MSB5 medium supplement with different concentrations of sucrose for germination. The highest percentage of normal plantlet germination was noted in the media supplemented with 1.5 % sucrose (53.03 %), followed by 3 % sucrose (43.87 %). However, when no sugar source was added in the medium, the percentage of germinated plantlets was lowest (5.27 %). Among three genotypes, PS 1477 gave the highest

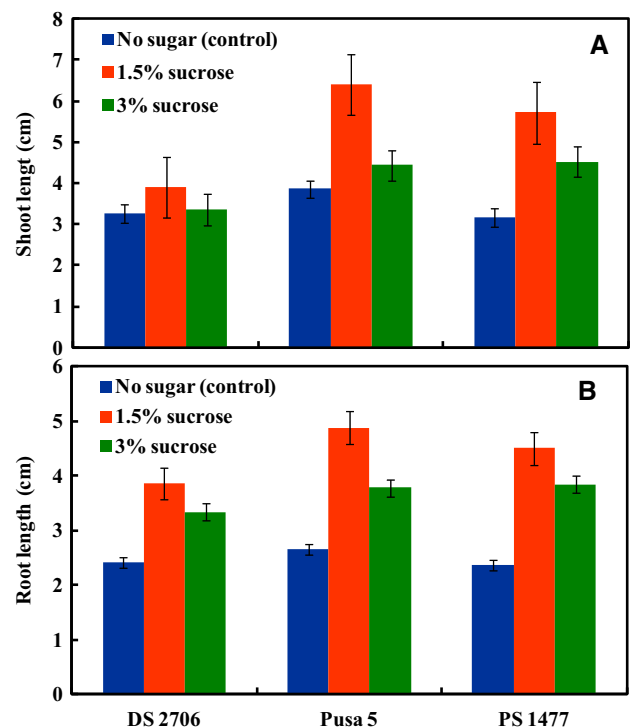
**Table 6** Effect of MSB5 medium supplemented with sucrose levels on somatic embryo germination in soybean

Treatment	Normal plantlet germination (%)			
	DS 2706	Pusa 5	PS 1477	Mean
Control (no sugar)	8.40 (16.85)*	4.80 (12.66)	2.60 (9.28)	5.27 (13.31)
Sucrose (1.5 %)	47.00 (43.28)	49.00 (44.43)	63.10 (52.59)	53.03 (46.72)
Sucrose (3 %)	33.20 (35.18)	41.10 (39.87)	54.30 (47.47)	43.87 (41.50)
Mean	29.53 (31.90)	32.63 (34.82)	40.00 (39.23)	
LSD ( $P \leq 0.05$ )				
Treatment (T)	5.25			
Genotype (G)	5.25			
Interaction (T × G)	9.09			
CV (%)	20.8			

\* Arc Sin  $\sqrt{\%}$  transformed data in parentheses

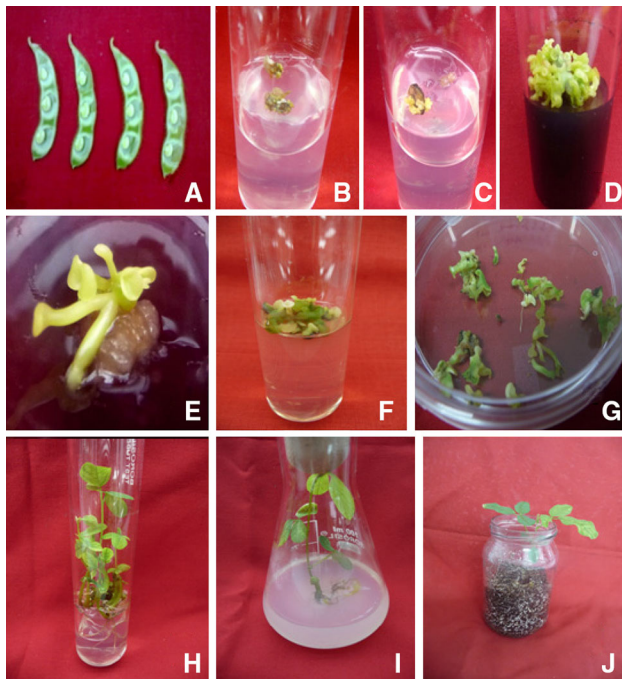
germination (40.0 %) of somatic embryos, followed by Pusa 5 (32.63 %), while lowest germination was observed in DS 2706 (29.53 %) (Table 6). Efficacy of carbon sources on somatic embryogenesis has been well documented. The carbohydrates are not only the carbon source but they also act as an osmotic regulator in the tissue culture medium. Jain et al. (1997) have shown that osmotic stress can promote somatic embryogenesis and plant regeneration. Accordingly, high sucrose level has been advocated for somatic embryogenesis in soybean (Jang et al. 2001; Walker and Parrott 2001). Embryo maturation was successfully achieved by Bailey et al. (1993a), after 1 month on 0.5 % AC supplemented medium. It was observed that the germination frequency of soybean embryos was quite low (Jang et al. 2001), and accordingly short period desiccation treatment was found effective for improving the germination frequency in soybean (Mariashibu et al. 2013). Kermode (1990) observed positive relationship between water loss and seed germination in monocot and dicot plants. The carbon source also played critical role in somatic embryos germination. In this study, we found that the medium containing 1.5 % sucrose was most effective in enhancing the germination of embryos into plantlets compared to higher dose (3 %) or no sucrose source (control).

After 30 days of germination, the average shoot length was highest in the treatment having 1.5 % sucrose (5.34 cm), followed by 3 % sucrose (4.11 cm). The shortest shoot length was noted in control treatment devoid of sucrose (3.43 cm), however, it was non-significantly different from treatment 3 % of sucrose. The genotype Pusa 5 gave the highest shoot length (4.90 cm), followed by (non-significantly) PS 1477 (4.47 cm), while shortest shoots (3.51 cm) were recorded in genotype DS 2706 (Fig. 1A). Similarly, the medium supplemented with 1.5 % sucrose also gave the longest roots (4.41 cm), followed by (non-significantly) medium supplemented with 3 % sucrose (3.65 cm), while shortest roots were noted in control

**Fig. 1** Effect of different sucrose levels on **A** shoot length and **B** root length of germinated plantlets. Vertical bars represent  $\pm$  standard deviations of mean

treatment (2.48 cm). However, there were non-significant differences among the three genotypes with regard to root length ( $>3.0$  cm) (Fig. 1B).

Rooted plantlets (after 4 weeks) were dipped in sterile double-distilled water to wash-off the agar–agar, followed by two rinsings in sterile double-distilled water and a final dip in 0.2 % carbendazim. The plantlets were then, transplanted into potting medium comprising peat, perlite and vermiculite (2:1:1) filled in glass jar with polypropylene (PP) cap and moistened with 1/4th MS salt solution. The



**Fig. 2** Somatic embryo induction, proliferation, differentiation, maturation and plantlet regeneration in soybean cultivar DS 2706: **A** 10-day-old pod, **B** induction of embryogenic calli, **C** multiplication of somatic embryo (SE), **D** differentiation of SE on callus mass, **E** macro view of the SE, **F** maturation of SE, **G** desiccations of SE, **H** germination of SE, **I** plantlet conversion, and **J** hardening of plantlet

plantlets were maintained at  $26 \pm 1$  °C with 16/8 h photoperiod ( $150 \text{ E} \mu \text{ m}^{-2} \text{ s}^{-1}$ ) for 4 weeks. The hardened plantlets were then successfully transferred to glasshouse ( $27 \pm 2$  °C; 85 % RH), which gave over 80 % survival after 3 weeks of ex vitro transfer (Fig. 2).

It can thus be concluded that efficient in vitro regeneration in soybean could be achieved using immature cotyledon segments (Fig. 2). The carbon source and plant growth regulator played important roles in somatic embryos induction, proliferation, differentiation, maturation and germination into plantlets. There were slight genotypic variations in the frequency of response, which confirms the variability in soybean genotypes for in vitro regeneration via direct somatic embryogenesis. The protocol reported here could be successfully used for developing transgenic plants and seems rapid and also less labour intensive than other regeneration pathways.

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