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DEVELOPMENTAL BIOLOGY

Regeneration of soybean (*Glycine max* L. Merrill) through direct somatic embryogenesis from the immature embryonic shoot tip

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Abstract We describe here a simple and efficient system of soybean (*Glycine max* L. Merrill) regeneration through direct somatic embryogenesis by using immature embryonic shoot tips (IEST) as explants. The cultivar Kaohsiung 10 (cv. K10) used in this study did not show embryogenic response either from mature seed-derived explants (cotyledon, embryonic tip, leaf, shoot and root) or immature cotyledons. However, it showed a high percentage (55.8%) of somatic embryo (SEm) formation from the IEST excised 2–3 wk after flowering, thus indicating the crucial roles of type and age of explants. The IEST put forth primary SEm after 2 mo of culturing on Murashige and Skoog (MS) medium supplemented with 6% sucrose, 164.8 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 5 mM asparagine and 684 μM glutamine. Subsequently, secondary SEm were developed 1 mo after culturing on MS medium containing 123.6 μM 2,4-D and 3% sucrose. Cotyledonary embryos were induced on MS medium supplemented with 0.5% activated charcoal after 1 mo. The embryos were desiccated for 72–96 h on sterile Petri dishes and regenerated on hormone-free MS medium. Plantlets with well-developed shoots and roots were obtained within 5–6 mo of culturing of IEST. The SEm-derived plants were morphologically normal and fertile. Various parameters thought to be responsible for efficient regeneration of soybean through somatic embryogenesis are discussed. To our knowledge, this is the first report to employ IEST as explants for successful direct somatic embryogenesis in soybean.

Keywords Desiccation · Embryogenesis · *Glycine max* L. · Immature embryonic shoot tips · Somatic embryos

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Introduction

Soybean (*Glycine max* L. Merrill) is one of the major undisputable sources of vegetable protein and oil used for both human and animal consumption. It is an important food as well as green vegetable crop in Taiwan. Many laboratories throughout the world are striving to improve the nutritional qualities of soybean seed protein and tolerance of the plant towards biotic and abiotic stresses. Genetic improvement of commercially important soybean cultivars through classical breeding is laborious and time consuming. Alternatively, genetic engineering offers excellent opportunities for the improvement of existing soybean cultivars through transformation with agronomically important traits. However, the success of transformation methods (either particle bombardment or

Agrobacterium) highly relied on the plant regeneration system.

Primarily, soybean regeneration was achieved through two independent processes, *viz*. organogenesis (shoot morphogenesis) and somatic embryogenesis. Regeneration through organogenesis was successfully obtained using cotyledonary nodes (Paz et al. 2006), primary leaves (Wright et al. 1987), shoot meristems (McCabe et al. 1988) and mature seed-derived embryonic tips (Liu et al. 2004). Though genetic transformation and subsequent plant regeneration *via* organogenesis is well established in this valuable crop (Liu et al. 2004; Paz et al. 2006; Hong et al. 2007; Olhoft et al. 2007), somatic embryogenesis is still highly preferred over organogenesis, as the transformants derived through embryogenesis are more uniform and the chances for the occurrence of variation among individual clones are lesser (Terzi and Lo Schiavo 1990; Osuga et al. 1999). Furthermore, regeneration through somatic embryogenesis has certain advantages: (1) it is an efficient and high volume propagation system, hence less labour intensive, and (2) the embryogenic cultures developed from this system are pure and homogeneous, owing to their single cell origin (Jiménez 2001). Moreover, through exploitation of totipotency (regeneration from single cells), ability to induce dormancy and the technological advancement towards long-term storability of somatic embryo (SEm), they remain to be a valuable resource for synthetic seed technology (Gray et al. 1995).

In soybean, Christianson et al. (1983) were the first to demonstrate somatic embryogenesis from excised zygotic embryos, though the system needed serial recurrent selection process to obtain embryogenic cultures. Later, Lazzeri et al. (1985) reported the use of immature cotyledons for the embryo induction and, since then, immature cotyledons have been used as the sole explant system capable of regenerating into plantlets *via* somatic embryogenesis (Finer and Nagasawa 1988; Bailey et al. 1993a; Ko and Korban 2004; Lim et al. 2005; Hiraga et al. 2007; Klink et al. 2008). Nevertheless, somatic embryogenesis from immature cotyledons is highly genotype-dependent (Komatsuda and Ohyama 1988; Bailey et al. 1993a; Tian et al. 1994; Meurer et al. 2001; Ko et al. 2004). The potential for embryogenesis can be improved to a certain extent by modification of tissue culture protocols for specific genotypes (Bailey et al. 1993b) or through conventional crossing between non-responsive cultivars and highly competent cultivars like Jack, as reported by Kita et al. (2007).

In the past, transgenic soybean plants were successfully developed using cell suspension culture-derived embryos as target tissues (Finer and McMullen 1991; Stewart et al. 1996). However, the cell culture system is time consuming and labour intensive (Droste et al. 2002). Hence, availability of an alternative system would greatly help the genetic manipulation of soybean cultivars that exhibit recalcitrance for regeneration through embryogenesis. Vasil (1987) suggested that the regenerants obtained through direct somatic embryogenesis are genetically uniform because of their unicellular origin.

In our laboratory, several attempts were made to optimise a routine and reliable regeneration system for an elite Taiwan soybean cv. K10, employing various explant types and through modification of tissue culture conditions. This paper reports a simple and efficient system for soybean regeneration through direct somatic embryogenesis from immature embryonic shoot tips (IEST).

Materials and Methods

Plant material. The commercial production soybean cv. K10 was used. The cultivar showed high recalcitrance for regeneration *via* somatic embryogenesis both from mature seed-derived explants (such as cotyledon, embryonic tip, leaf, shoot and root) and immature cotyledons (this study).

Isolation of Explants

Mature seed-derived explants. Soybean seeds were surface disinfected with 70% ethanol for 1 min, 4.0% sodium hypochlorite solution containing 0.1% Tween-20 for 15 min, followed by three washes with sterile distilled water. The seeds were germinated on half-strength B5 medium (Gamborg et al. 1968) supplemented with 6-benzylaminopurine (4.44 µM; Sigma, St. Louis, MO), sucrose (3%), agar (0.8%; Micropropagation/Plant Tissue Culture Grade, PhytoTechnology Laboratories, Shawnee Mission, KS), pH5.8. The cotyledons were excised after 5–6 d of seed germination by carefully removing the seed coat, hypocotyl and embryonic axis (Liu et al. 2004). The leaf, shoot and root segments were obtained from 14-d-old seedlings. Embryonic tips were isolated by soaking the surface sterilised seeds in sterile water for 24 h (Liu et al. 2004).

Immature seed-derived explants. Plants of the cv. K10 were established in a greenhouse under natural light conditions. Immature pods (4.0–5.0 cm) were harvested from plants after 1–4 wk of flowering and were surface sterilised, as described for mature seeds. Immature seeds (6–10 mm) were separated from the pods, and the IEST measuring 4–5 mm length were excised by dissecting the immature seeds (after carefully removing the seed coat) longitudinally between two cotyledons with sterile scalpels and cultured on embryo induction medium as shown in Fig. 1*a*.

Immature cotyledons were excised from pods collected 1–2 wk after flowering according to the published protocols (Finer and Nagasawa 1988; Bailey et al. 1993a).

Induction of primary (globular) SEm. All the explants were cultured on embryo induction medium containing Murashige and Skoog (MS) salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), sucrose (6%), different concentrations (41.2, 82.4, 123.6, 164.8 and 206.0 μM) of 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma), gellan gum (0.2%, Gelrite, Sigma), pH5.8. The explants were cultured at 25±1°C with 16 h photoperiod (20–30 μE $m^{-2}s^{-1}$) and subcultured onto the same fresh medium at 15-d interval until the primary SEm were induced. The percentage of embryogenesis was calculated after 2 mo by using the following formula: number of explants showing SEm induction/number of explants cultured×100.

Effect of carbon and nitrogen sources on SEm induction. The effect of carbon (sucrose and maltose) and nitrogen (asparagine and glutamine) sources on primary SEm induction was tested by culturing the IEST on embryo induction medium containing MS salts, B5 vitamin, 6% sucrose/maltose or a combination of both (each at 3%), asparagine (5 mM) or glutamine (684 μM) or a combination of both, 2,4-D (164.8 μM), gellan gum (0.2%), pH5.8. After 2 mo, the number of SEm induced from individual IEST was recorded.

Induction of secondary SEm. After 2 mo, primary SEm were cultured on a medium containing MS salts, B5 vitamins, asparagine (5 mM), glutamine (684 μM), different concentrations (82.4, 123.6 and 164.8 μM) of 2,4-D, sucrose (3%), gellan gum (0.2%), pH5.8 for the induction and multiplication of secondary SEm. Cultures were maintained at 25±1°C with 16 h light (20–30 μE $m^{-2}s^{-1}$). The

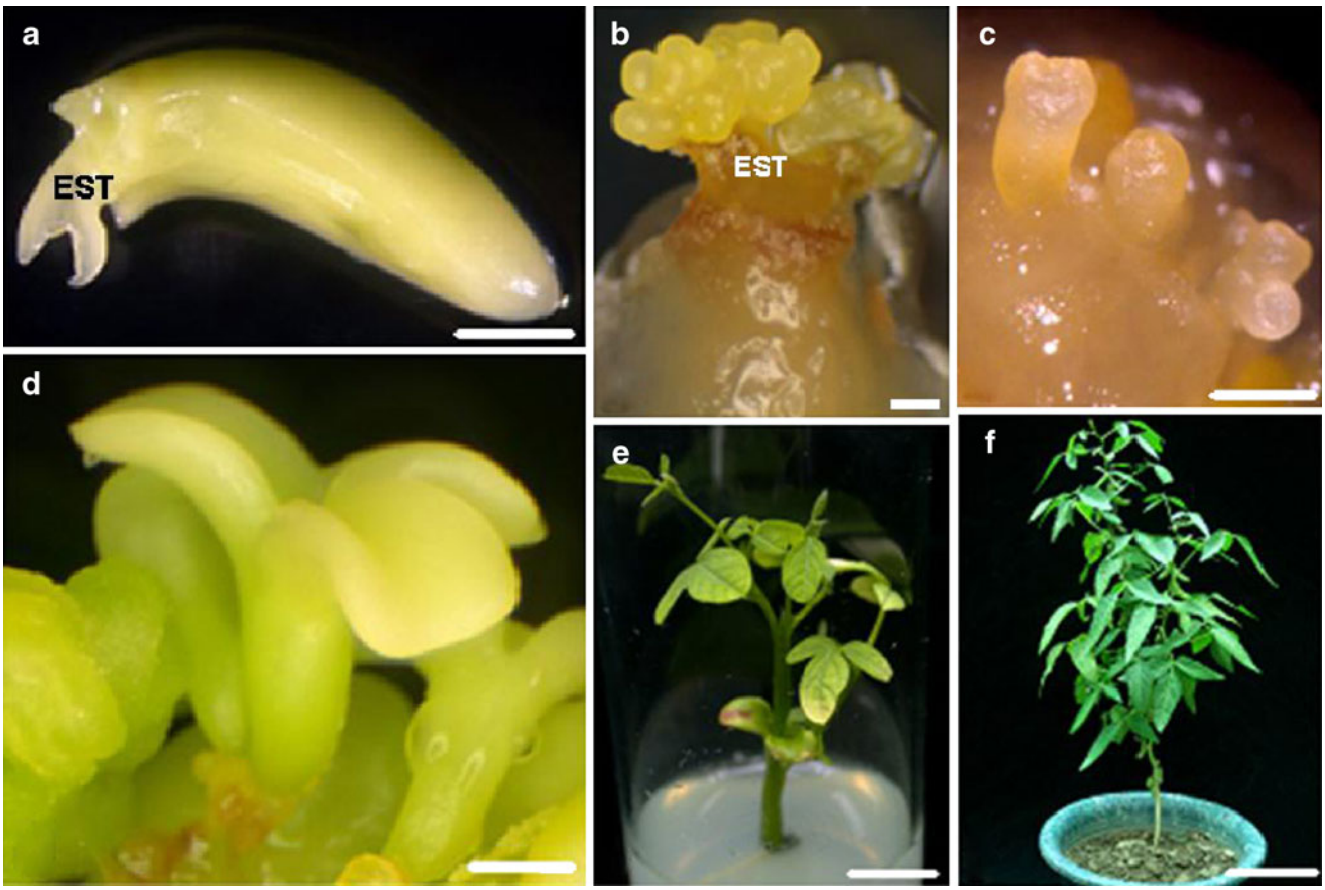

Figure 1. Regeneration of soybean cv. K10 through direct somatic embryogenesis from immature embryonic shoot tips (IEST). (*a*) IEST (4–5 mm length) plated on MSSD (MS salts, B5 vitamins, 6% sucrose, 5 mM asparagine, 684 μM glutamine, 164.8 μM 2,4-D, 0.2% gellan gum, pH5.8) medium. (*b*) Primary SEm induced from the apical region of embryonic shoot tip after 2 mo of culturing on MSSD medium. (*c*) Secondary SEm induced from primary SEm on MSD (MS salts, B5 vitamins, 3% sucrose, 5 mM asparagine, 684 μM glutamine, 123.6 μM 2,4-D, 0.2% gellan gum, pH5.8) medium. (*d*) Development of cotyledonary stage embryos on MSAC (MS salts, B5 vitamins, 6% maltose, 0.5% activated charcoal, 0.2% gellan gum, pH 5.8) medium. (*e*) Plantlet regeneration on MSB5 (MS salts, B5 vitamins, 3% sucrose, 0.8% agar, pH5.8) medium. (*f*) SEm derived plant at pod development stage. *Bars*: (*a*, *b*, *c*)=1.0 mm; (*d*)=2.0 mm; (*e*)=1.0 cm; (*f*)=10.0 cm. *EST* embryonic shoot tip.

embryos were subcultured onto the same fresh medium at 15-d intervals until the secondary SEm were formed.

Embryo maturation, regeneration, plantlet recovery and acclimatisation. SEm showing active proliferation were plated onto maturation medium (MSAC) containing MS salts, B5 vitamins, maltose (6%), activated charcoal (0.5%), gellan gum (0.2%), pH5.8 (Bailey et al. 1993a). The plates were incubated at 25±1°C with 16 h light (20–30 μE $m^{-2}s^{-1}$) conditions. After 1–2 mo, cream coloured cotyledonary stage embryos were separated into individual embryos and desiccated for different periods (0, 24, 48, 72, 96 and 120 h) on sterile empty Petri dishes incubated at 25±1°C and 80% relative humidity. The partially dehydrated SEm were transferred to test tubes containing hormone-free MSB5 (MS salts, B5 vitamins, 3% sucrose, 0.2% gellan gum, pH5.8) medium. The plantlets were regenerated at 25±1°C with 16 h light (with a photon flux intensity of 140 μE $m^{-2}s^{-1}$) regime. After germination, the plantlets (5–6 cm) with well-developed shoots and roots were transferred to pots filled with a 3:1 mixture of sandy loam soil and farmyard manure (FYM), covered with polythene bags to maintain high relative humidity for 1 wk. Thereafter, the plantlets were gradually exposed to ambient humidity over a period of 1 wk, transplanted in 25-cm diameter pots filled with the above mixture and maintained in a greenhouse under natural light conditions until seed harvest. The morphological growth characteristics of the progenies from SEm-derived plants were compared with that of plants established from field-derived K10 seeds.

Statistical analysis. All the experiments were carried out in completely randomised design with three replications. Duncan's multiple range test was used to compare the treatment means using the software IRRISTAT Version 3.1 (Biometrices Unit, IRRI, Manila, The Philippines).

Results and Discussion

Induction of primary SEm. We observed a wide range of responses among mature and immature seed-derived explants of soybean cv. K10 in different culture concentrations (41.2, 82.4, 123.6, 164.8 and 206.0 μM) of 2,4-D (Table 1). Mature seed-derived explants such as shoot, leaf and root portions did not produce callus or SEm at any of the concentrations of 2,4-D tested, whereas cotyledons and embryonic tips were able to develop watery callus tissues at lower 2,4-D concentrations (41.2 and 82.4 μM), but at later stages, those calli failed to develop SEm (Table 1). However, IEST put forth primary SEm completely from the apical region of the embryonic shoot tips after 2 mo of culturing on MSSD medium with an embryogenic potential of 55.8% (Table 1 and Fig. 1*b*). The crucial factor at this stage was the 2,4-D concentration (164.8 μM), as the percent SEm induction was drastically reduced above 164.8 μM (Table 1). The advantage of this system is that SEm were induced directly from the IEST without any intermediate callus phase. The callus system needs repeated subculturing to select embryogenic callus portions from highly proliferating non-embryogenic tissues, leading to increased chances for somaclonal variation among the regenerants (Karp 1991).

Table 1. Embryogenic response of various explants of soybean cv. K10 on different concentrations of 2,4-D

Explant type	2,4-D (μM)[w]				
	41.2	82.4	123.6	164.8	206.0
Mature seed-derived explants					
Cotyledon[z]	0 c	0 c	0 c	0 c	0 c
Embryonic tip[z]	0 c	0 c	0 c	0 c	0 c
Leaf[y]	0 c	0 c	0 c	0 c	0 c
Shoot[y]	0 c	0 c	0 c	0 c	0 c
Root[y]	0 c	0 c	0 c	0 c	0 c
Immature seed-derived explants					
IEST	0 c	0 c	0 c	55.8 a	20.0 b
Immature cotyledon[x]	0 c	0 c	0 c	0.3 c	0.3 c

[z] Watery masses of callus tissues were observed up to 82.4 μM of 2,4-D, but at higher concentrations, the explants became necrotic

[y] The explants showed a little enlargement in size at 41.2 μM 2,4-D, but above that concentration, they showed no response

[x] The immature cotyledons developed calli on 41.2–123.6 μM 2,4-D but failed to put forth SEm at later stages

[w] Values in each *row* indicate the percentage of explants showing primary SEm formation; values in each *column* are means of three replications, each with 120 IEST; within a *column*, means followed by a common letter are not significantly different (P=0.05) by Duncan's multiple range test

The plant growth regulator 2,4-D plays a prominent role in the induction and proliferation of SEm in soybean (Shoemaker et al. 1991; Liu et al. 1992; Ponappa et al. 1999; Kim et al. 2000). Earlier reports indicated successful embryogenesis in soybean either with low (Christianson et al. 1983; Komatsuda and Ohyama 1988; Orczyk and Orczyk 1994) or high 2,4-D concentrations (Finer and Nagasawa 1988; Wright et al. 1991). This could be attributed to the differences in the sensitivity among genotypes to this synthetic auxin (Hiraga et al. 2007). Hence, the effective concentration of 2,4-D required for the induction of SEm needs to be optimised for individual cultivars. Previously, several laboratories have reported successful somatic embryogenesis in soybean using immature cotyledons on medium containing high concentration of 2,4-D (Bailey et al. 1993a, b; Droste et al. 2002). However, in our

Table 2. Embryo induction[z] from immature embryonic shoot tips of soybean cv. K10 on different carbon and nitrogen sources

Carbon/nitrogen source	Number of SEm induced/explant[y]			
	No asparagine and glutamine	Asparagine (5mM)	Glutamine (684 µM)	Asparagine (5mM)+glutamine (684 µM)
Sucrose (6%)	3.0 c	4.0 b	3.0 c	8.3 a
Maltose (6%)	1.2 f	2.2 d	3.0 c	2.4 d
Sucrose (3%)+maltose (3%)	1.3 f	1.8 e	2.0 e	3.3 c

[z] The embryo induction medium consisted of MS basal salts, B5 vitamins, 164.8 µM 2,4-D along with the indicated levels of carbon and nitrogen sources

[y] Values in each *column* are means of three replications, each with 30 immature embryonic shoot tips; within a *column*, means followed by a common letter are not significantly different (P=0.05) by Duncan's multiple range test

study, the immature cotyledons did not show embryogenic response over a wide range (41.2, 82.4, 123.6, 164.8 and 206.0 µM) of 2,4-D concentrations (Table 1), possibly because somatic embryogenesis from immature cotyledons is highly genotype-dependent (Bailey et al. 1993a; Meurer et al. 2001; Ko et al. 2004; Hiraga et al. 2007).

Effect of carbon and nitrogen sources on SEm induction. We studied the effect of different carbon (sucrose and maltose) and nitrogen (asparagine and glutamine) sources on the SEm induction ability (number of SEm induced/explant) of IEST. Among different carbon and nitrogen sources evaluated, the medium supplemented with sucrose (6%), asparagine (5 mM) and glutamine (684 µM) proved to be effective in terms of number of SEm induced from individual IEST (Table 2). Maltose, also used in this study either alone or together with sucrose, did not improve the SEm induction (Table 2). The carbohydrates are not only the source of carbon but they also act as an osmotic regulator in the tissue culture medium. Several studies have indicated that osmotic stress can promote somatic embryogenesis and plant regeneration (Jain et al. 1997). However, the mechanism by which the osmotic stress promotes embryogenesis is still a subject of investigation. Furthermore, the choice of the carbon source largely depends on the crop and genotype used. For example, maltose was found superior over sucrose for embryogenesis in rice (Jain et al. 1997), but effectiveness of sucrose (6%) has been demonstrated for the induction of SEm from immature cotyledons of soybean (Bailey et al. 1993a; Jang et al. 2001).

Similarly, asparagine and glutamine were found to play important roles in the development of soybean embryogenic cell suspensions (Finer and Nagasawa 1988). Jang et al. (2001) reported that application of asparagine in cell suspension medium had greater effect on formation of embryogenic clumps. Likewise, many researchers emphasised the addition of glutamine during embryo development mainly to increase the size of the embryos (Dyer et al. 1987; Lippmann and Lippmann 1993). Previously, in soybean, these two amino acids were tested only in cell suspension cultures i.e. after the SEm induction have been achieved (Finer and Nagasawa 1988; Schmidt et al. 2005). In contrast, we added these two amino acids in the SEm induction medium itself and found the addition to be highly productive, as evaluated based on the number of primary SEm developed from the IEST. There was a twofold increase in the number of globular embryos induced from a single IEST on medium containing both asparagine (5 mM) and glutamine (684 µM), when compared to IEST

Table 3. Influence of the age of the immature embryonic shoot tips on the SEm induction

Age (weeks after flowering)	Number of SEm induced[z]	SEm induction (%)
1	0.7	2.3 a
2	23.0	76.6 c
3	23.7	78.8 c
4	3.3	11.0 b

[z] Values are means of three replications, each with 30 immature embryonic shoot tips; within a *column*, means followed by a common letter are not significantly different (P=0.05) by Duncan's multiple range test

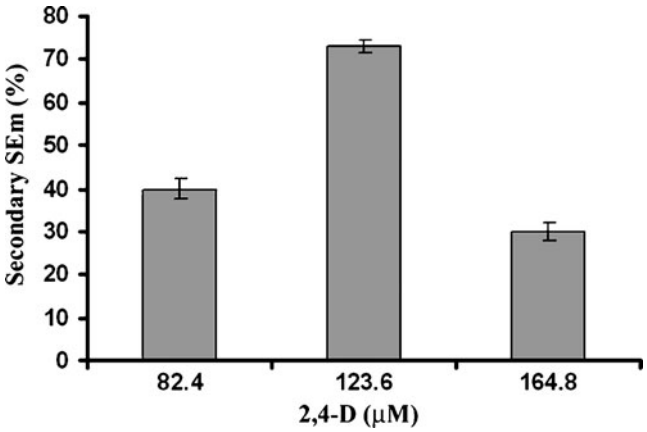

Figure 2. Effect of 2,4-D concentration on the induction of secondary SEm. Values were obtained from mean of three replications (n=30 embryos/replication). *Bars* represent the standard deviations.

Table 4. Effect of desiccation period on SEm germination

Desiccation period (h)	Number of SEm germinated[z]	Germination of SEm (%)
0	0.3	1.5 a
24	2.0	10.0 b
48	14.3	71.5 c
72	19.0	95.0 d
96	18.7	93.5 d
120	0.7	3.5 a

[z] Values are means of three replications, each with 20 SEm; within a *column*, means followed by a common letter are not significantly different (P=0.05) by Duncan's multiple range test

cultured on either of these carbon sources (Table 2). This will be an added advantage, especially for the genetic improvement of cultivars showing low frequency of transformation. As most soybean cultivars are highly resistant to transformation methods (Kita et al. 2007), generally large number of embryos are required for the genetic transformation experiments to generate reasonable number of independent transformants.

Age of the explants. It was evident in the present study that the potential for embryogenesis also varies with the age of the explants. Significantly, higher percentage (76–79.0%) of SEm induction was achieved from IEST excised from immature pods collected 2–3 wk after flowering (Table 3). This was in close accordance with the immature cotyledonary explant system (Finer and Nagasawa 1988; Bailey et al. 1993a).

Induction of secondary SEm. Somatic embryogenesis is considered successful, only when it produces single cell SEm. Otherwise, it may result in chimeric transformants due to multicellular origin of primary SEm, as was evidenced in soybean immature cotyledonary system (Parrott et al. 1989). Single cell SEm can be induced through the establishment of cell suspension cultures (Finer and Nagasawa 1988) or by the induction of secondary SEm (Santarém et al. 1997). In a cell suspension system, a more complex medium with additional factors is required to induce dedifferentiation and initiation of cell division in the explants for developing competence to undergo embryogenesis (Williams and Maheshwaran 1986). As an alternative to the liquid culture system, Wright et al. (1991) described a procedure for proliferation of SEm derived from immature cotyledons on a semisolid medium. Moon and Hildebrand (2003) reported a better regeneration rate in SEm that are proliferating on solid state medium. This procedure was successfully used to develop fertile transgenic plants and it seems to be a rapid and less labour-intensive process than embryogenic suspension culture systems (Trick et al. 1997; Droste et al. 2002). In the present study with a view to producing single cell

Stage	Medium[A]	Duration	Light condition
IEST (2-3 wk-old)	-	-	-
↓			
Primary SEm	MSSD	2 mo.	16 h light (20- 30 µE m^{-2} s^{-1})
↓			
Secondary SEm	MSD	1 mo.	as above
↓			
Embryo maturation and induction of cotyledonary embryos	MSAC	1-2 mo.	as above
↓			
Embryo desiccation	Sterile empty Petri dishes	72-96 h	-
↓			
Plantlet regeneration	MSB5	1 mo.	16 h light (140 µE m^{-2} s^{-1})
↓			
Hardening	Sandy loam soil + FYM (3:1)	2 wk	as above
↓			
Maintenance in a greenhouse	as above	Until seed harvest	Natural light

Figure 3. Summary of the different steps for direct somatic embryogenesis in soybean cv. K10 from immature embryonic shoot tips. *A*: The compositions of the medium used at different stages of the embryogenesis are detailed in Fig. 1.

secondary SEm, 2-mo-old primary SEm were cultured on solid MS-based medium containing different concentrations (82.4–164.8 μM) of 2,4-D. After 1 mo, 70% of the embryos developed secondary embryos on MSD medium containing 123.6 μM 2,4-D (Fig. 1*c* and 2), a conversion rate approximately two times higher than on 82.4 and 164.8 μM 2,4-D (Fig. 2). Previous reports indicated the requirement for high levels (82.4 μM) of 2,4-D for the multiplication of SEm on solid medium (Wright et al. 1991), while a low level (20.6 μM) of 2,4-D was found beneficial in cell suspension cultures (Finer and Nagasawa 1988; Bailey et al. 1993a). These data together with our results indicate that proliferation of SEm on solid state medium requires high concentration of 2,4-D to prevent actively proliferating non-embryogenic tissues which severely hamper the secondary SEm formation. Importantly, we could maintain the viability of secondary SEm even beyond a year by repeated subculturing of SEm onto MSD medium at 15-d interval.

Embryo maturation, regeneration, plantlet recovery and acclimatisation. Embryo maturation was performed essentially as reported by Bailey et al. (1993a). Cream coloured cotyledonary stage embryos were formed 1 mo after culturing of secondary SEm on MSAC medium containing 0.5% activated charcoal (Fig.1*d*). The cotyledonary stage embryos produced in this system were uniform, two to three lobed and symmetrical in nature unlike abnormal embryos, which had fused cotyledons, long hypocotyl vestigial cotyledons and cup-shaped cotyledons as observed in immature cotyledons (Hiraga et al. 2007). In general, the germination frequency of soybean embryos is very low (Jang et al. 2001), and therefore, partial desiccation of SEm was emphasised with a view to improving the germination frequency in soybean (Parrott et al. 1988; Buchheim et al. 1989; Durham and Parrott 1992). Hence, in the current study, the 45–60-d-old cotyledonary stage embryos were desiccated for different time periods on sterile empty Petri dishes, so as to improve their germination frequency. The study showed that the duration of desiccation was very crucial for the germination of embryos wherein, short (0–48 h) and high (120 h) desiccation has drastically reduced the embryo germination, while 72–96 h desiccation induced 95% germination (Table 4). Jang et al. (2001) reported 96 h as the optimum desiccation period for better germination (90%) of SEm derived from immature cotyledons. Kermode (1990) showed the existence of a positive relationship between water loss and seed germination in monocot and dicot plants. Desiccation is an important process for the developmental metabolic arrest (off-regulation of developmental mRNAs) and induction of germination (on-regulation of germination associated mRNA synthesis) in many plants (Kermode 1990). Liu et al. (1994) observed the expression of maturation-associated genes, *Mat*1 and germination associated lipoxygenase genes (*SC*514 and *LOXB2*) in soybean SEm desiccated for 72 h. This finding indicates that desiccation is associated with the expression of transcripts necessary for transition of SEm from maturation phase to germination.

The embryos desiccated for 72–96 h were regenerated with normal shoot and root growth within 1 mo of culturing on hormone-free MSB5 medium (Fig. 1*e*). The plants showed a normal growth pattern in the greenhouse and produced viable seeds under natural light conditions (Fig. 1*f*). The progenies of SEm-derived plants exhibited morphological growth characteristics comparable to those of plants established from field-derived seeds (data not shown).

Conclusion

We have successfully regenerated fertile soybean plants through direct somatic embryogenesis by using IEST as the explant (Fig. 3). The present regeneration system is simple, efficient and highly reproducible in terms of SEm induction, multiplication and regeneration into plantlets. We found the crucial factors for successful embryogenesis to be the type and age of explants, 2,4-D concentration and the carbon and nitrogen sources during embryo induction, and the desiccation period after embryo maturation. We are hopeful that through a judicious manipulation of the above factors, embryogenesis can be induced in other soybean cultivars that exhibit recalcitrance to regeneration.

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