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Effective Multiplication of Somatic Embryos Using Suspension Culture and Regeneration in Soybean

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Abstract

The use of liquid-medium-based procedure relative to the solid media led to a 4.5-fold increase in the number of cotyledon-stage embryos. The most efficient system for multiplication and regeneration of somatic embryos was CP6 procedure with the media MSD40/MSD20/ MSM6AC/FNL0S3S3GM. However, the rate of regeneration was lower. About 71% of the embryos with dicotyledon were continued to develop the roots after desiccation treatment and 92% of the germinated embryos produced shoots in 10 days. Of the four morphologically different types of embryos, dicotyledonous ones showed a high frequency of conversion, while only a few with fused and horn type cotyledon developed shoots. Mature somatic embryos were desiccated in empty petri dishes for 12-72 h. Embryo survival rate was the highest after 12 h of desiccation, but maximal germination was observed at 24 h. After desiccation, they were placed on MS medium without growth regulators for germination. Germinating embryos were transferred to small pots with vermiculite for plant regeneration. The etiolating the plants during the growth was resolved to add 1% activated charcoal on hormone-free MS medium.

Key words: somatic embryo, desiccation, suspension culture, regeneration, soybean

Introduction

For rapid growth of embryo, an embryogenic suspension culture system for soybean has been developed by Finer and Nagasawa (1988), where immature cotyledons were placed on a medium with very high concentration of 2,4-D. Samoylov et al. (1998) also developed a simple and quick procedure for histo-differentiation and maturation of soybean somatic embryos, which used a liquid-medium-based protocol and led to a 4-fold increase in the number of cotyledon-stage embryos. However, embryogenic suspension culture was difficult to establish and maintain, and the plants regenerated from these lines often showed reduced or complete loss of fertility (Hadi et al. 1996; Trick and Finer 1998). Moreover, the germination frequency of soybean somatic embryos was generally very low. In order to solve the problem, a number of variables such as desiccation conditions have been investigated in an attempt to improve the germination frequency of somatic embryos (Parrott et al. 1988: Buchheim et al. 1989; Durham and Parrott 1992). The objectives of this study was to establish efficient embryogenic suspension cultures and to investigate the effect of desiccation treatment and morphology of mature somatic embryos on plant regeneration.

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Materials and Methods

Histo-differentiation and development using liquid media

To induce embryo development, 40-50 mg of well-proliferating embryo clusters, derived from Pungsannamul-kong on MS (Murashige & Skoog, 1962), medium cantaining D20 (20 mg/L of 2, 4-D), were placed in 35 mL

of liquid medium in a 125 mL Erlenmeyer flask. Suspension cultures were agitated at 150 rpm on a rotary shaker under 10 $\mu molm^{-2}s^{-1}$ of cool white fluorescent light with a 16 h photoperiod at 27℃, and subcultured weekly. The media used for suspension culture were MS and FN medium (Table 1).

Desiccation treatment for embryo germination

After 4 weeks in suspension culture, embryos were placed in petri dish with small amount of FN Lite medium (about 2 mL), to prevent rapid dehydration, for 12, 24, 48 and 72 h. The number of embryos placed in the dish was adjusted depending on the size of embryos. Generally 20-30 embryos per petri dish are enough to maintain high

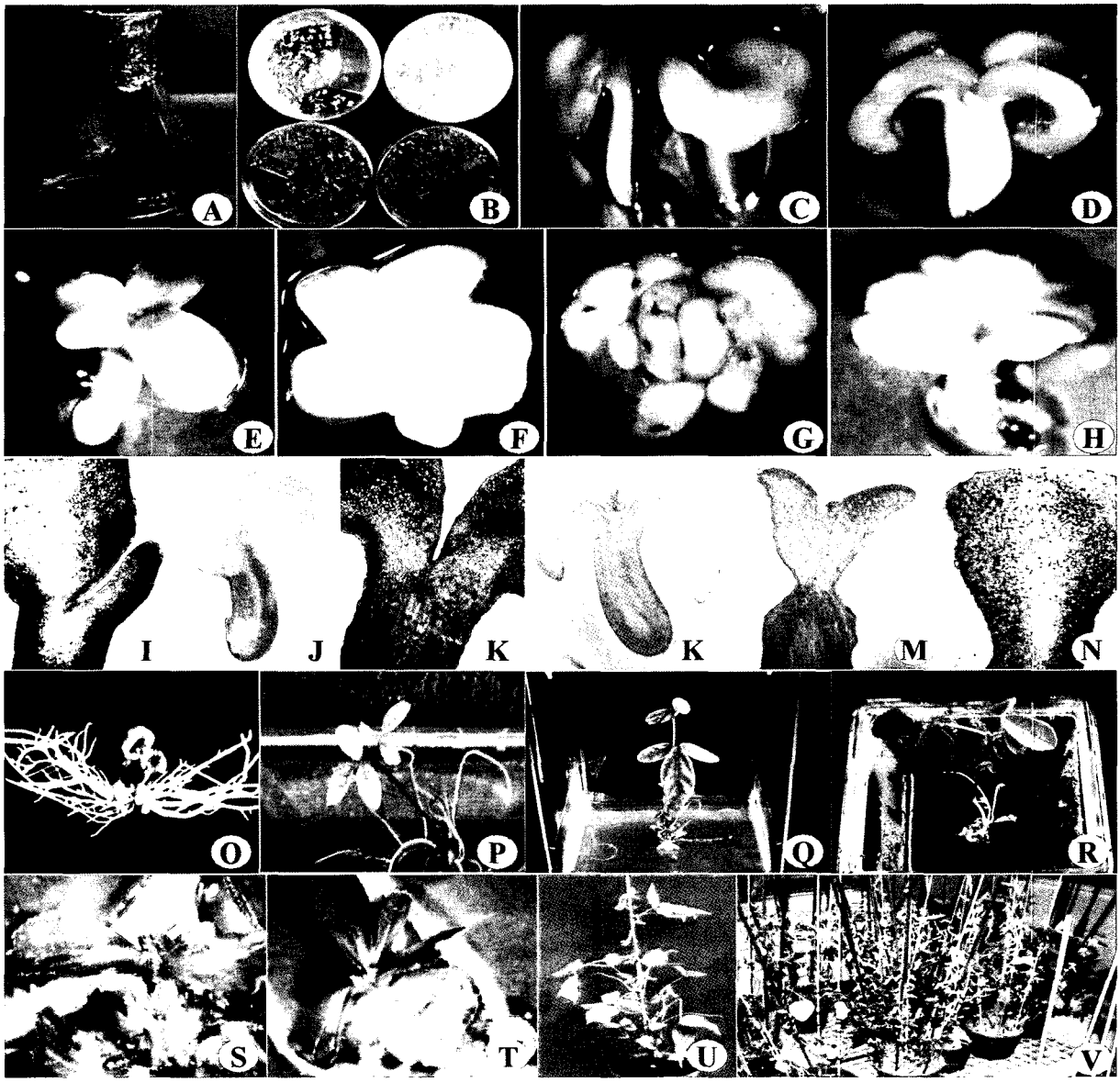

Figure 1. Regenerated plant and histological observation through somatic embryos from suspension culture of soybean. Suspension culture (A) and desiccation for embryo germination (B). Cotyledon shapes of somatic embryos in suspension culture (C-H); mono- (C), di- (D), polycotyledons (E, F), fused (G) and trumpet shaped cotyledon (H). Histological observation on the well-developed embryos with different shapes of cotyledon by longitudinal section in Alchankong (X50, X100); mono- (I, J), dicotyledon (K, L) with shoot apical meristem tissue. Trumpet shaped with (M) or without (N) shoot apical meristem. Germination of somatic embryos on hormone- free MS basal medium 10 days after the desiccation (O). Etiolated regenerants on MS0 medium (Q) and recovery on MS0AC (hormone-free MS basal medium plus 1% activated charcoal (R). Growth of regenerated soybeans. A few regenerants showed somaclonal variation in leaves (S, T), but they did not survive, however, most of the regenerants showed normal growth and set seed (U, V).

humidity inside the dish (Figure 1A, B). Germination occurred within 10 days after transfer to MS0 medium (Table 1). Regenerated plantlets were transferred to the same medium supplemented with 1% activated charcoal in Magenta GA7 box (Sigma) and in culture bottles and maintained at 27℃. To keep plants at vegetative stage, these were kept under 23 h photoperiod (Bailey et al. 1993). Embryo survival rate and germination rate were investigated 2 weeks after the desiccation.

Histological study of embryos

Embryos with different shapes of cotyledon were sectioned at 20 μm thickness on a Digital Microtome Cryostat (Cryocut 1,800, Leica Co., Germany) at -25℃. Sections were stained with safranin-O and observed under a light microscope (×50, ×100).

Results and Discussion

Multiplication of somatic embryo using suspension culture

An alternative method for histo-differentiation and maturation of embryos is the suspension culture, in which callus multiplied and developed into embryos quickly. Although CP1 based solid medium had the highest rate of normal embryo formation and germination (Table 2, Figure 2), CP6-based-liquid medium was found to produce somatic embryos and their germination after desiccation treatment for 24 h. The number of embryos for med was 4.5 times higher and the culture time was shortened by about 20 days, compared to those of CP1.

After 5 weeks in liquid medium, somatic embryos could be classified into 4 morphological types by the modified Buchheim's classification (1989). The germination frequency of each morphological class varied significantly among the different culture conditions. Of the somatic embryos grown in the CP1 media, 83%, 5%, 8% and 4% had mono-cotyledons, dicotyledons, poly-cotyledons and trumpet shaped cotyledons, respectively (Figure 1C-H & 2).

The germination and shoot induction rate of the dicotyledonous embryos was 73% and 92%, respectively. However, the shoot induction frequencies of mono-cotyledons, poly-cotyledonous and trumpet shape embryos were lower than that of the dicotyledonous type embryos (Figure 3).

Histological examination revealed clear differences among different types embryos under light microscope. Sections of the embryos with mono and dicotyledon showed the presence of a well-developed shoot apical meristem (Figure 1I-M). However, no shoot apical meristem was observed in

Table 1. Composition of the media used for soybean somatic embryogenesis.

Medium[a]	Salts	Vitamins	Carbon source	pH	Growth regulators	Other nutrients
FN Lite	FN Lite macro, MS micro	B_5	1% sucrose	5.8	5 mg/L 2,4-D	1g/L asparagine
FNL0-S3S3GM	FN Lite macro, MS micro	B_5	3% sucrose, 3% sorbitol	5.8	-	30 mM glutamine, 2 mM methionine
MSM6AC	MS salts	B_5	6% sucrose	5.8	-	0.5% activated charcoal
MSM6	MS salts	B_5	6% maltose	5.8	-	-
MS0	MS salts	B_5	3% sucrose	5.8	-	-

[a]Solidifying agent is 0.2% Gelrite. FN Lite and FNL0S3S3GM are liquid media.

Table 2. Efficiency of proliferation and germination of embryos in different culture condition (mean±SE).

Medium	Inoculated embryo (mg)	Number of embryo (No./mg)[a]	Number of germinated embryo (%)	Time required (days)[b]
CP1	50±4.3	1.6±0.5	54± 7.5 (68)	97±4.4
CP2	51±5.2	4.7±1.2	135±13.5 (56)	105±9.2
CP3	48±4.1	7.1±1.9	174±11.9 (51)	86±9.5
CP4	53±3.8	6.6±1.4	160±16.5 (46)	80±4.1
CP5	51±3.4	7.1±0.6	183± 7.3 (51)	63±7.6
CP6	52±4.2	8.3±1.1	235±11.4 (55)	74±4.4

[a] Total number of embryos obtained after 5 weeks in suspension culture.
[b] From induction of embryo on MSD40 media to full development.
CP1, MSM6AC/MSM6; CP2, FN Lite/(MSD20)/MSM6AC/MSM6; CP3, FN Lite/FNL0S3S3GM/(MSM6); CP4, FNL0S3S3/(MSM6); CP5, FNL0S3S3GM/(MSM6); CP6, MSM6AC/FNL0S3S3GM.

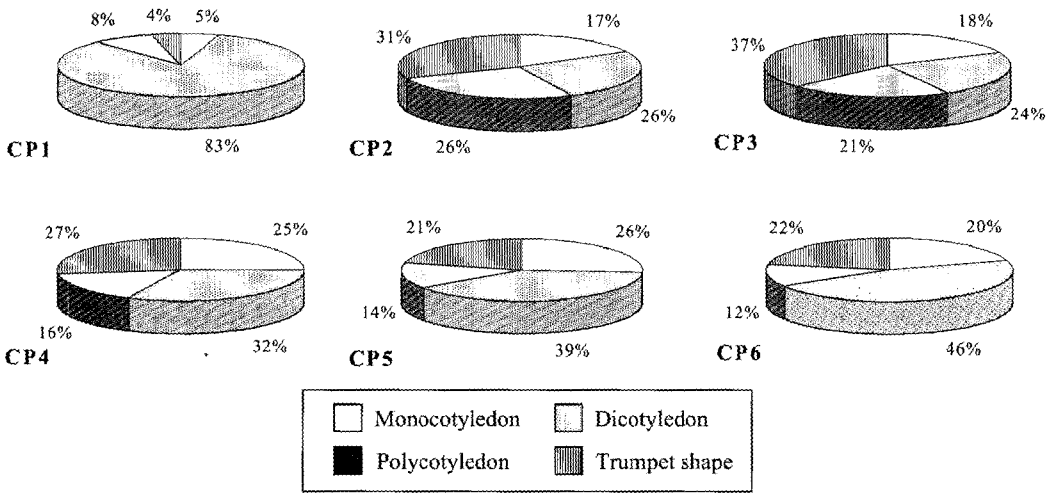

Figure 2. The distribution of somatic embryos by cotyledon types in various culture conditions in soybean (cv. Pungsannamulkong). CP1, MSM6AC/MSM6; CP2, FN Lite/(MSD20)/MSM6AC/MSM6; CP3, FN Lite/FNL0S3S3GM/(MSM6); CP4, FNL0S3S3/(MSM6); CP5, FNL0S3S3GM/(MSM6); CP6, MSM6AC/FNL0S3S3GM.

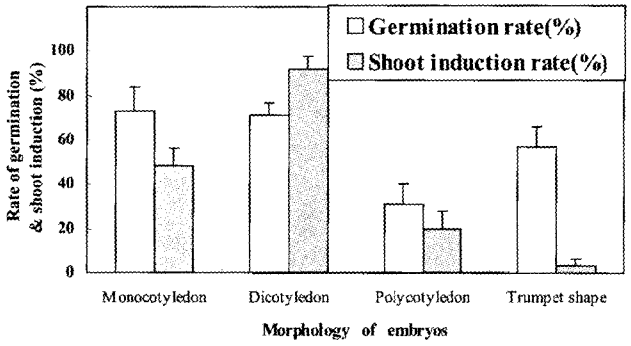

Figure 3. Variance of germination rate and shoot induction rate depending on somatic embryo morphology after desiccation treatment for one day Germination rate (%)=(number of germination/ number of embryos)×100, shoot induction rate (%)=(number of shoot induced/ number of embryos germinated)×100.

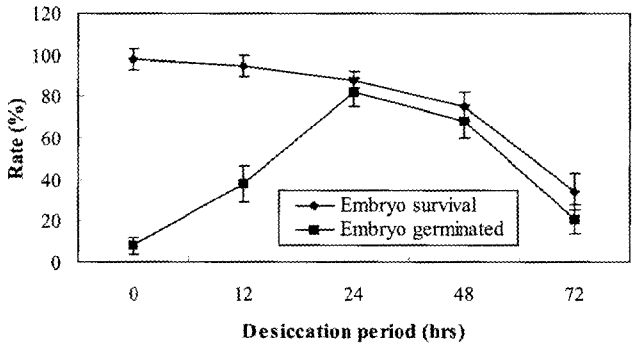

Figure 4. Effect of desiccation period on survival and germination of somatic embryos. Values are means ±SE of four replications.

the trumpet shaped embryos (Figure 1N). The results indicated that anatomical structure of developed embryos could provide a clear evidence for germination ability of the embryos.

Germination and plant regeneration from somatic embryos

The germination rate of somatic embryos was generally very low. A number of studies have been attempted to improve the germination frequency of somatic embryos by desiccation for breaking their dormancy (Parrott et al. 1988; Buchheim et al. 1989). To evaluate the effect of desiccation of mature somatic embryos, survival and germination rate of somatic embryos were investigated 10 days after the treatment. When somatic embryos were desiccated for 24 h, their germination dramatically increased up to approximately 80%, while only 8% of the non-desiccated somatic embryos germinated. In contrast, viability of embryos after desiccation for 12 h was 95% and it decreased gradually by increasing the desiccation period. After 48 h of desiccation treatment, survival and germination of the embryos were drastically reduced (Figure 4).

Using empty petri dishes for desiccation had a disadvantage of too rapid dehydration and low survival of embryos. Slow dehydration of embryos showed high frequency of germination (Buchheim et al. 1989). Therefore, 15 to 30 embryos were placed in a petri dish with 2 mL of media in order to maintain the viability of embryo after desiccation.

Finally, somatic embryos after the desiccation treatment

Table 3. Comparison of the morphological characteristics between the control plants and regenerants *in vitro* via somatic embryogenesis.

Genotype	Stem height (cm)		No. of pods/plant		Days to maturity[a]		Ratio of pod set (%)		Seed yield (g/plant)		100-seed weight (g)	
	CT[b]	PR	CT	PR	CT	PR	CT	PR	CT	PR	CT	PR
Pungsannamulkong	51	49	13	14	125	108	46.3	34.5	8.5	7.9	10.4	9.8
Alchankong	55	54	18	16	120	107	51.8	49.2	15.3	13.7	13.2	13.0
Sinpaldalkong #2	51	49	13	10	117	110	35.3	34.8	14.7	14.5	18.5	17.7
PI 96322	56	45	12	9	120	101	24.6	20.3	7.2	6.8	13.8	12.3

[a] Days to maturity from seeding in control plant, and from transplating to soil in regenerated plants. [b] CT: Control, PR: Plantlets regenerated *in vitro*.

were transferred to regeneration medium (MS0, pH 5.8) containing MS salts, B_5 vitamins, 3% sucrose, 0.2% Gelrite, lacking growth regulators in order to induce roots and shoots. The germinated embryos (Figure 1O), turned pale yellow to green and produced shoots (Figure 1P, Q). Many researchers found that the conversion of embryos to form roots and shoots was genotype-dependent (Ranch et al. 1986; Komatsuda & Ohyama, 1988; Bailey et al. 1993). However, Li and Grabau (1996) reported little genotype dependence. In this study, the most critical factors for germination of embryos were morphological shape and desiccation treatment rather than genotypes. After germination of embryos, plantlets were regenerated on the same medium.

Activated charcoal and polyvinyl pyrrolidone (PVP) were used to absorb unknown toxic phenolics or melanin compounds produced from plantlets. In addition, activated charcoal has often been used to absorb auxin such as 2,4-D and aromatic compounds which are inhibitory to growth or development of plantlets (Fridborg et al. 1987). The etiolation of the plants during growth was overcome by adding 1% activated charcoal on hormone-free MS medium (Figure 1R & 5).

Somaclonal variation of plantlets regenerated *in vitro* from cultured immature cotyledons was also studied. Regenerants of Pungsannamulkong and Alchankong showed early maturation, and a little decrease in plant height, pod set, ratio of pod set, seed yield and 100-seed weight, compared to normal plants (Table 3).

A few regenerated plantlets showed somaclonal variation in the leaves but they did not grow into mature plants (Figure 1S, T), however, most of the regenerants showed normal growth and seed set (Figure 1U, V). Similar results were reported by Hildebrand et al. (1989). They found that the progeny from first generation of regenerants showed greater phenotypic variation than the control population, but the variations for fatty acid and agronomic characters were not observed in the second generation. Other deleterious somaclonal variants were not observed in the regenerants. Therefore, somatic embryogenesis system established in this study can also be used for genetic transformation of soybean.

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