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Factors Affecting Somatic Embryogenesis from Immature Cotyledon of Soybean

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Abstract

Somatic embryos were induced from immature cotyledons and cultured on a MS medium containing 40 mg/L 2,4-D. The maximum induction of embryos was obtained from immature cotyledons in a size of 3-4 mm, and the highest frequency was obtained in the induction medium at pH 7.0. For embryo development, embryogenic tissues were transferred to a MSM6AC and MSM6 media. Developing embryos were placed at 27℃ with dim light (20 $\mu molm^{-2}s^{-1}$) provided by cool fluorescent tubes (3-D wavelength light is better than standard light). Somatic embryos were clearly developed from globular stage to cotyledonary stages. The color of embryo may be a useful parameter for estimation of embryo quality. When the embryo becomes mature, embryo will be ready for desiccation in order to induce roots and shoots of embryos.

Key words: somatic embryo, immature cotyledon, regeneration, soybean

Introduction

The application of advanced tissue culture techniques may lead to new avenues of crop improvement. For successful application of the tissue culture techniques to crop breeding, the callus induction and plant regeneration potential of each crop or cultivar must first be determined. Especially, organogenesis, somatic embryogenesis has been reliable method to multiply plant materials as proved in more than hundreds plant species from different families (Terzi and Loschiavo, 1990). Somatic embryos were formed either directly from cotyledons or indirectly via callus-like brown tissues derived from cotyledons of soybean (Komatsuda and Ohyama, 1988). For somatic embryogenesis, commonly used were the explants of the excised cotyledon (Liu et al. 1992), the intact zygotic embryo (Buchheim et al. 1989) and the excised embryo axis (Christianson et al. 1983) from immature seeds. In soybean, plant regeneration via somatic embryogenesis has been usually utilized cotyledons from immature embryos (Komatsuda et al. 1991; Liu et al. 1992), and it requires higher concentration of 2,4-dichlorophenoxy acetic acid (2,4-D) than any other species (Finer, 1988; Wright et al. 1991; Bailey et al. 1993). There were a few attempts to study factors affecting somatic embryogenesis such as pH, solidifying agent, wounding and silver nitrate ($AgNO_3$) treatment (Komatsuda and Ohyama, 1988; Parrott et al. 1989; Komatsuda et al. 1991; Santarém et al. 1997). However, embryogenic culture was difficult to establish and maintain. Moreover, the germination frequency of soybean somatic embryos was generally very low. Therefore, we tried to develop an effective procedure for induction, differentiation and maturation of somatic embryos in soybean.

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Materials and Methods

Explant preparation for somatic embryogenesis

Immature pods of soybean, cvs Alchankong, Ilpumgeomjeongkong, Sinpaldalkong #2, Pungsannamulkong, Duyukong, KW 530, Lx 5, Lx 15, Lx 16, Lx 17 and PI 96322 were collected from plants grown in a glass house under a 14 hr light photoperiod. They were 5 Korean

cultivars and 6 accessions that were 4 sprout soybeans and 7 large seed size soybeans. Somatic embryogenesis was developed with modified methods by Parrott et al. (1989), Finer and Nagasawa (1988), Santarém et al. (1997), and Samoylov et al. (1998). Pods were collected when the immature cotyledons were 2-6 mm long at about 2 weeks after flowering and surface-sterilized by immersion in 70% ethanol for 1 min followed by soaking in a 2% sodium hypochlorite (NaOCl) for 20 min. The pods were then rinsed three times in sterile distilled water. Immature seeds were aseptically removed from the pods and the end containing the embryonic axis was cut off and discarded. The two cotyledons were then removed from the seed coat, separated.

Embryo induction and development using solid media

For induction of somatic embryos, the cotyledons were placed on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962; Santarém et al. 1997) consisting of MS salts, B_5 vitamins, various concentration of 2,4-D, 1, 20, 40 and 50 mg/L with 0.2% Gelrite (Sigma) and 3% sucrose. The pHs of media were 6.5, 7.0, 7.5 and 8.0 and flat side of cotyledon was positioned as face-up. Induced embryos were transferred to MSD20 medium for 4 weeks. Ten clumps were transferred to MSM6AC medium for histodifferentiation. Development and maturation were conducted on MSM6 medium (Table 1). Light intensities were 10, 20 and 40 $\mu molm^{-2}s^{-1}$, and light sources used were cool-white fluorescent tubes (standard) and cool-red fluorescent tubes (three-dimensional wavelength, FL40PG) under a 16 hr photoperiod at 27℃. Each petri dish representing one replication contained 12 explants. The experiment was laid out in a randomized complete block design with 4 replications.

Results and Discussion

Pods containing immature cotyledons were collected about 2 weeks after flowering. The length of pods were about 4-5 cm. The best embryogenic cotyledons in pods for somatic embryogenesis were soft, translucent green, and easily squashy under finger pressure and 3-4 mm in size (Figure 1A-C). Once the cotyledon becomes dark-green and hard, it did not produce somatic embryos.

Immature cotyledons were placed on a MS medium containing 40 mg/L 2,4-D with the flat side up. The cotyledon gradually enlarged, swollen and turned brown after 7 days of culture. A few cotyledons turned dark brown after 3 weeks and died. Somatic embryos were observed on the surface of the cotyledon explants after 2-3 weeks of culture and they were light yellow, compact and at globular stage.

The size of immature zygotic embryo proves to be one of the major limiting factors for inducing somatic embryos. Usually, the optimal size of the cotyledon appears to be 4 mm-7 mm (Barwale et al. 1986; Parrott et al. 1988). However, we obtained a very interesting result suggesting causal relationship between the optimum size of immature cotyledon and the size of mature seed (Table 2). The optimal size of immature cotyledon for embryo induction was 3 mm in case of small seed size soybean (below 12 g in 100-seed weight) called sprout-soybean in Korea such as Pungsannamulkong, KW 530 and Lx 5, whereas 4 mm in large seed size soybean such as Ilpumgeomjeongkong, Sinpaldalkong #2, Duyukong, Lx 15 and Lx 16. And embryogenic potential was extremely low in the smaller ones (< 2 mm) or the larger ones (>5 mm) than the optimal size range.

Auxins such as 2,4-D, picloram and dicambar have been applied to induce and proliferate somatic embryos (Liu et al. 1992). When the concentration of 2,4-D in the MS medium was decreased to less than 20 mg/L, the formation of callus without embryos was increased. Among the treatments tested, MSD40 (MS medium containing 40 mg/L 2,4-D) appeared to be the best medium for direct induction of embryo from a minimum of 13.3% for Duyukong to a

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

Medium[a]	Salts	Vitamins	Carbon source	pH	Growth regulators	Other nutrients
MSD40	MS salts	B_5 vit.	3% sucrose	7.0	40mg/L 2,4-D	-
MSD20	MS salts	B_5 vit.	3% sucrose	5.8	20mg/L 2,4-D	-
MSM6AC	MS salts	B_5 vit.	6% sucrose	5.8	-	0.5% activated charcoal
MSM6	MS salts	B_5 vit.	6% maltose		-	-
MS0	MS salts	B_5 vit.	3% sucrose	5.8	-	-

[a] Solidifying agent is 0.2% Gelrite.

Table 2. The somatic embryo induction affected by the length of immature cotyledons in different soybean genotypes after 5 weeks culture on MSD40 medium.

Genotype	Immature cotyledon length (mm)					100-seed weight
	2	3	4	5	6	
Alchankong	- [a]	++	+	-	-	14.2
Ilpumgeomjeongkong	+	++	++++	+	+	25.6
Sinpaldalkong #2	-	++	+++	-	-	19.5
Pungsannamulkong	-	+++	+	-	-	10.7
Duyukong	-	++	++	-	-	17.6
KW 530	-	+++	+	-	-	10.1
Lx 5	+	+++	++	-	-	12.2
Lx 15	-	+++	+++	+	-	25.3
Lx 16	-	+++	+++	++	+	25.7
Lx 17	-	++	+	-	-	14.2
PI 96322	+	++++	+++	-	-	16.3

[a] Somatic embryo induction degree : - none, + rare, ++ moderate, +++ good, ++++ excellent.

Table 3. Number of cotyledons forming somatic embryo (%)[a] from immature cotyledons of different soybean genotypes on different media after 4 weeks of cultures.

Genotype	2,4-D concentrations (mg/L)			
	MSD1	MSD20	MSD40[b]	MSD50
Alchankong	2 (6.7)	7 (23.3)	18 (60.0)	4 (13.3)
Ilpumgeomjeongkong	0 (0.0)	6 (20.0)	21 (70.0)	14 (46.7)
Sinpaldalkong #2	4 (13.3)	9 (30.0)	20 (66.7)	11 (36.7)
Pungsannamulkong	0 (0.0)	5 (16.7)	14 (46.7)	7 (23.3)
Duyukong	0 (0.0)	3 (10.0)	4 (13.3)	2 (6.7)
KW 530	0 (0.0)	4 (13.3)	6 (20.0)	4 (13.3)
Lx 5	3 (10.0)	5 (16.7)	14 (46.7)	7 (23.3)
Lx 15	0 (0.0)	6 (20.0)	21 (70.0)	14 (46.7)
Lx 16	2 (6.7)	8 (26.7)	20 (66.7)	8 (26.7)
Lx 17	0 (0.0)	2 (6.7)	5 (16.7)	3 (10.0)
PI 96322	2 (6.7)	9 (30.0)	26 (86.7)	15 (50.0)

[a] Number of cotyledons inoculated is 30 in each treatment. Media on pH 7.0. [b] Fisher's $LSD_{0.05}$ = 3.2 on MSD40 medium.

maximum of 86.7% for PI 96322 (Table 3). The ability to induce somatic embryos from immature cotyledons was genotype-dependent. Similar results were observed by Parrott et al. (1989), Komatsuda et al. (1991), Bailey et al. (1993), Li and Grabau (1996). In this study, PI 96322 showed the best potential for embryogenic response.

Another important factor in embryo induction was pH condition in the medium.

A higher formation rate of embryos showed in most of the genotypes on pH 7.0, whereas the best induction of embryos was on pH 7.5 in Alchankong, Ilpumgeomjeongkong and Pungsannamulkong (Table 4). Similar results were reported by Komatsuda and Ohyama (1988) and Santarém et al. (1997) who supposed that the enhancement of embryo induction at pH 7.0 for soybean might result from a slower and more gradual uptake of 2,4-D. Edwards and Goldsmith (1980) found a similar results in maize coleoptiles.

Some embryos formed directly from cotyledon without callus formation in immature cotyledon explants on MSD40 medium and secondary embryos immediately appeared on primary embryos (Figure 1D). The average number of primary globular-stage embryos per cotyledon after 4 weeks

Table 4. The effect of pH conditions on somatic embryo induction from immature cotyledons of different soybean genotypes after 4 weeks of cultures on MSD40 medium.

Genotype	Number of cotyledons inoculated	Number of cotyledons forming somatic embryo (%)			
		pH 6.5	pH 7.0[a]	pH 7.5	pH 8.0
Alchankong	40	9 (22.5)	12 (30.0)	14 (35.0)	17 (37.5)
Ilpumgeomjeongkong	30	14 (46.7)	16 (53.3)	23 (76.7)	6 (20.0)
Sinpaldalkong #2	40	7 (17.5)	21 (52.5)	23 (57.5)	11 (27.5)
Pungsannamulkong	40	4 (10.0)	10 (25.0)	7 (17.5)	6 (15.0)
Duyukong	30	3 (10.0)	8 (26.7)	4 (13.3)	2 (6.7)
KW 530	40	7 (17.5)	12 (30.0)	4 (10.0)	0 (0.0)
Lx 5	40	14 (35.0)	24 (60.0)	18 (45.0)	7 (17.5)
Lx 15	30	11 (36.7)	16 (53.3)	14 (46.7)	10 (30.0)
Lx 16	30	14 (46.7)	18 (60.0)	15 (50.0)	10 (30.0)
Lx 17	40	10 (25.0)	11 (27.5)	8 (20.0)	14 (35.0)
PI 96322	40	24 (60.0)	32 (80.0)	30 (75.0)	13 (32.5)

[a] Fisher's $LSD_{0.05}$ = 3.7 at pH 7.0 on MSD40 medium.

Table 5. Effect of light intensity and quality on induction and development of somatic embryos.

Somatic embryos	Light intensity ($\mu molm^{-2}s^{-1}$)[a]			Light quality	
	10	20	40	SD[c]	3D
Embryo induction	+++[b]	++++	++	++	+++
Embryo development	++	+++	+++	++	++++

[a] Light provided by cool-white fluorescent tubes
[b] Degree : - none, + rare, ++ moderate, +++ good, ++++ excellent
[c] SD : standard light, 3D : 3-D wavelength light.

of culture ranged from 3 to 24 among the genotypes. A similar result was observed by Barwale et al. (1986). While few explants induced callus first and embryos later with very low frequency of 0.1% (Figure 1E). In many cases, it is known that, if non-embryogenic callus was induced from cotyledon first, it overgrows against embryos and prohibits embryo induction (Figure 1F).

Culture conditions such as light intensity affect induction and development of embryos. Best embryo induction was observed when explants were exposed to 20 $\mu molm^{-2}s^{-1}$. However, no difference in embryo development was obtained between 20 and 40 $\mu molm^{-2}s^{-1}$. Induction and development of embryos were also affected by light sources. The best result was obtained under the light condition provided by cool-white fluorescent tubes with 3-D wavelength (Table 5).

In case that embryonic axis was not thoroughly cut off from cotyledon, root or shoot-like tissues were induced from the cut edge of the cotyledons (Figure 1G,H). However, they did not develop into regenerated plantlets.

After 4 weeks of culture, embryos induced on MSD40 medium were transferred to MSD20 medium containing 20 mg/L 2,4-D, pH 5.8. They were friable, bright, and vigorously proliferated on the medium and constituted of clusters of globular somatic embryos with pale yellow (Figure 1I). They were subcultured every 2 weeks for 2-3 times. Clumps of proliferative globular-stage embryos were transferred to solid medium (MSM6AC) or liquid medium (FN Lite or FNL0S3S3GM) for histodifferentiation and development (Figure 1J).

Somatic embryo clusters including cotyledon were transferred to development medium (MSM6AC) for 4 weeks. Activated charcoal was added in the medium to absorb residual 2,4-D to prevent the hormone effect for blocking of histodifferentiation and development of somatic embryos.

In the culture, globular somatic embryos were developed into heart, torpedo and cotyledonary stage embryos, and their color also turned pale yellow to light green (Figure 1K-M).

For maturation, embryos were transferred to maturation medium (MSM6; Finer and Nagasawa, 1988). They shows

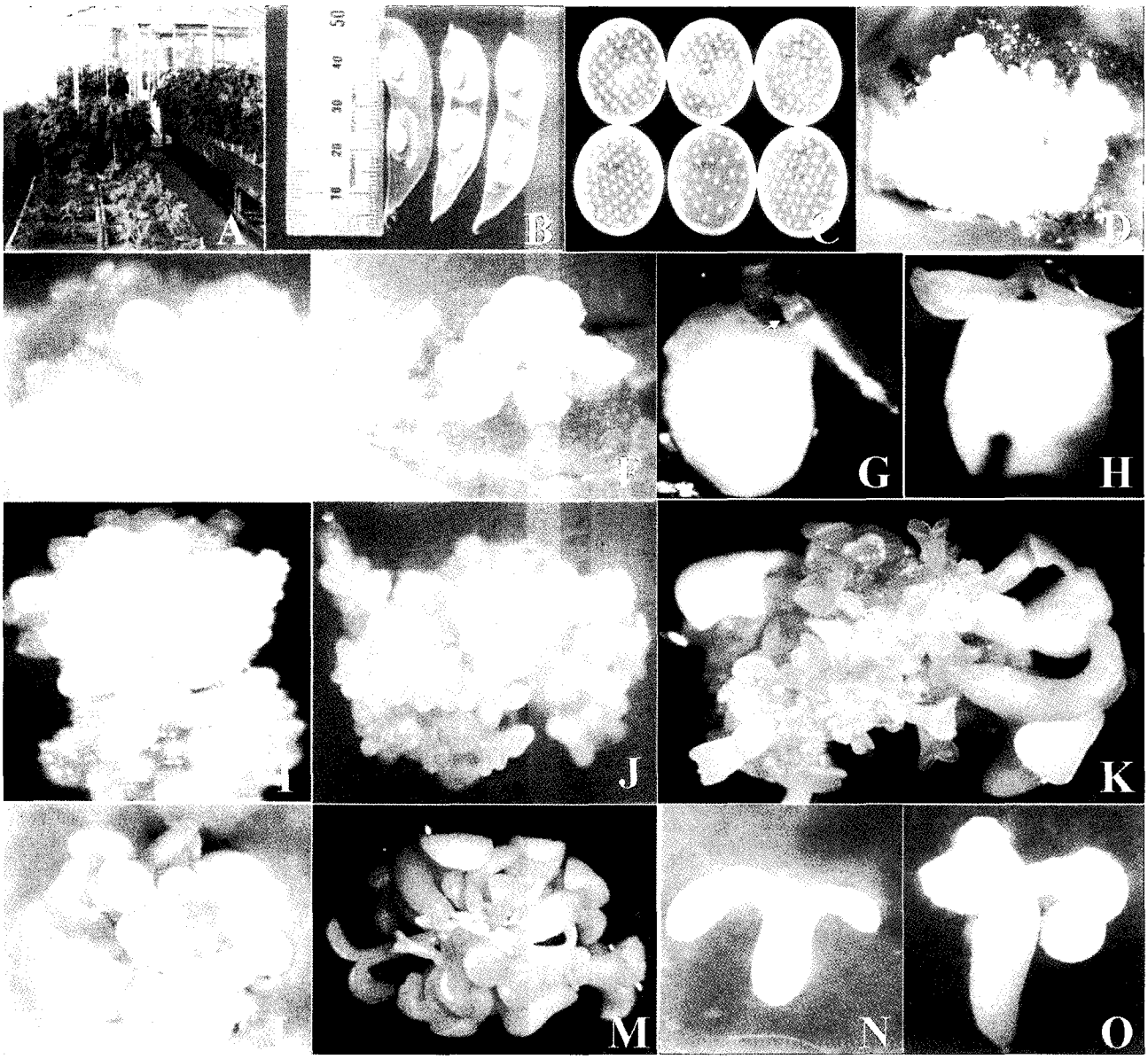

Figure 1. Development somatic embryo from immature cotyledon. A: Plant materials used in a glass house. B: Immature cotyledons containing pods. C: Inoculated cotyledon explants. D, E: Formation of somatic embryos and callus from immature cotyledon on MS salt + B_5 Vitamin + 3% sucrose + 40 mg/L 2,4-D, pH 7.0 (MSD40) medium. Embryo formed directly (D) from cotyledon explants. Embryos produced indirectly (E) from callus. F: non-embryogenic callus induced from cotyledon. G, H: Embryo and shoot developed from the cotyledon from which plumules were not thoroughly cut off. I, J: Numerous globular somatic embryos formed from excised cotyledon on MS salt+ B_5 Vitamin + 3% sucrose + 20 mg/L 2,4-D, pH 5.0 (MSD20) medium. K-M: Developmental stages of embryo clumps on MS salt+ B_5 Vitamin + 6% sucrose + 0.5% activated charcoal, pH5.0 (MSM6AC) solid medium. Globular (Ka), heart (Kb), torpedo (Kc), and cotyledonary stage embryos (Kd). N: Developing embryo. O: Fully mature embryo with well-developed cotyledon.

a typical shape of normal mature embryos, which can be easily germinated and regenerated. Somatic embryos were clearly developed from globular stage to cotyledonary stages. The color of embryo may be a useful parameter for estimation of embryo quality. When the embryo becomes mature, it loses its green color, and acquires a creamy yellow color (Figure 1N,O). At this time, the fully matured cotyledonous embryo will be ready for desiccation in order to induce roots and shoots of embryo.

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