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Plant Regeneration of Soybean Cultivars *via* Somatic Embryogenesis

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Effective plant regeneration from immature cotyledons of soybean [*Glycine max* (L.) Merr.] cultivars was achieved *via* somatic embryogenesis. Somatic embryogenesis was performed with the cotyledons of immature embryos 14-20 d after flowering. Immature cotyledons of cv. Whangkeum were placed abaxial or adaxial side down on modified MS medium containing 20 mg/L 2,4-D. The greatest number of somatic embryos, 1.2 per cotyledon, was produced from those of 4.0-4.9 mm in length which had been placed abaxial side down. Among cvs. Pecking, Whangkeum and Baekwoon, Pecking had the highest embryo induction efficiency with 4.3 somatic embryos per cotyledon in 20 mg/L 2,4-D treatment and with 1.0 embryo per cotyledon in 8 mg/L NAA treatment. Germinable globular somatic embryos were induced with the highest efficiency, 27.6%, in 20 mg/L 2,4-D and were proliferated efficiently on liquid medium containing 10 mg/L 2,4-D. The globular somatic embryos developed into germinable mature somatic embryos on medium containing 10 μM $CoCl_2$, 9% sucrose, and 0.5% activated charcoal. These mature somatic embryos germinated on hormone-free medium. After transfer to the soil, regenerated plants with seeds were obtained.

Keywords : soybean, immature cotyledon, somatic embryogenesis, plant regeneration

Soybean is the most important source of vegetable oil and protein in the world, but has shown difficult to regenerate *in vitro*. Recently there have been several reports concerning the successful regeneration of soybean after much effort. Since Christianson *et al.* (1983) produced cultures capable of regenerating plants from immature embryos of one genotype, immature embryos have been used as explants for induction of somatic embryos. It has been reported that the explanting orientation of these immature cotyledons affect the embryo induction because of differential response to growth regulators (Hartweck *et al.*, 1988). Somatic embryogenesis from immature cotyledon tissue also has been improved by high concentrations of auxin, 2,4-D at 5 to 40 mg/L or NAA at 0.8 to 10 mg/L (Lazzeri *et al.*, 1987a; Parrott *et al.*, 1989; Shoemaker *et al.*, 1991; Wright *et al.*, 1991; Liu *et al.*, 1992; Choi *et al.*, 1994). In addition, globular embryos derived from 2,4-D treatment proliferated in liquid culture medium supplemented with 2,4-D (Finer and Nagasawa, 1988; Choi *et al.*, 1991).

Maturation of soybean somatic embryos induced in medium containing 2,4-D was necessary to attain a high frequency of germination (Buchheim *et al.*, 1989). After 8 weeks on MS medium (Murashige and Skoog, 1962), supplemented with activated charcoal and high molar sucrose, soybean somatic embryos exhibited a high frequency of development to plantlets (Parrott *et al.*, 1988; Shoemaker and Hammond, 1988; Buchheim *et al.*, 1989). In spite of these approaches, germination of soybean somatic embryos into plants has been generally inefficient yet. The germination frequency of somatic embryos in soybean varied with the cotyledon morphology of mature somatic embryos without relation to exogeneous growth regulators (Lazzeri *et al.*, 1987b; Parrott *et al.*, 1988; Buchheim *et al.*, 1989). Buchheim *et al.* (1989) reported that soybean had been regenerated at higher frequencies from mature somatic embryos with mono-, di-, and poly-cotyledonous types than from those with others such as trumpet, fused, and

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fasciated cotyledonous types.

2,4-D was known to inhibit the meristematic development of carrot somatic embryos (Halperin and Wetherall, 1964) and to be closely correlated with a marked increase in endogeneous ethylene production (Pinfield *et al.*, 1991). And, in studies on the somatic embryogenesis of carrot using 2,4-D, the induction efficiency of germinable somatic embryos was improved by inhibiting ethylene synthesis (Roustan *et al.*, 1989, 1990).

In this study, somatic embryos were induced from immature soybean cotyledons, propagated, and successfully regenerated to whole plants. The effects of explant size, explanting orientation, growth regulators and genotype on embryo induction were investigated. Then, globular somatic embryos induced in medium containing 2,4-D were propagated and, especially, to attain a high frequency of germination, the possible correlation between $CoCl_2$, an inhibitor of ethylene biosynthesis, and maturation of propagated somatic embryos was examined.

MATERIAL AND METHODS

Plant material

Soybean [*Glycine max* (L.) Merr.] was grown in the pot and/or the greenhouse. The cultivars used in this study were Baekwoon, Whangkeum and Pecking. Pods in age from 14 to 20 d after flowering were detached and surface sterilized in 70% (v/v) ethanol for 45 s and 2.5% (w/v) NaOCl for 10-15 min, and rinsed 4 times in sterile distilled water. Immature embryos were excised from surface-sterilized pods. The end of immature embryo containing the embryonic axis was removed and both cotyledons were used for somatic embryo induction.

Induction of somatic embryos

Immature cotyledons were placed on embryo induction medium (Table 1; Christianson *et al.*, 1983; Novak *et al.*, 1989) for 30 d at 27℃ in complete darkness. Three experiments were conducted to investigate embryo induction efficiency: First, for the effect of explant size and explanting orientation on embryo formation efficiency, immature embryo cotyledons of cv. Whangkeum ranging from 4 to 10 mm were divided into 5 groups in 1 mm size intervals except 2 mm interval of 8 to 10 mm. Fifty to one hundred cotyledons per treatment were placed on induction medium containing 20 mg/L 2,4-D adaxial or abaxial side down; Second, for the effects of 2,4-D and NAA concentration on the embryo induction of each cultivar, immture embryo cotyledons of cvs. Whangkeum, Baekwoon and Pecking ranging from 4 to 5 mm in size were abaxially cultured on medium containing 8 mg/L NAA, or 20 mg/L 2,4-D. In this study, 100-300 cotyledons per treatment were cultured; Third, for the effect of 2,4-D concentration on embryo formation, 70-100 cotyledons per treatment of cv. Whangkeum ranging from 4 to 5 mm

Table 1. Composition of medium used in soybean regeneration *via* somatic embryogenesis

Induction medium
modified MS salts[a] (1/2 KNO_3 and NH_4NO_3)
vitamins
B5 vitamins[b]
5 mg/L adenine sulfate
1 mg/L Ca-panthothenate
1 mg/L biotin
2 mg/L cysteine-HCl
1 mg/L glycine
100 mg/L *myo*-inositol
0.6% agar
3% sucrose+2,4-D
or
1.5% sucrose+NAA
Proliferation medium
salts and vitamins of induction medium
100 mg/L *myo*-inositol
1 g/L glutamine
3% sucrose+2,4-D
Maturation medium
MS salts
B5 vitamins
1 g/L glutamine
100 mg/L *myo*-inositol
$CoCl_2$ (filter sterilized)
9% sucrose
0.5% activated charcoal
0.8% agar
Regeneration medium
1/2 MS salts
1/2 B5 vitamins
250 mg/L casein hydrosylate
1% sucrose
0.6% agar
pH 5.8

[a]Murashige and Skoog (1962), [b]Gamborg *et al.* (1968).

in size were placed abaxially on medium supplemented with 1-40 mg/L 2,4-D. Each experiment was replicated more than 2-3 times.

Proliferation of somatic embryos

To examine the effect of 2,4-D concentration on globular somatic embryo proliferartion, 4-5 globular-stage embryos per treatment which had been induced on medium containing 20 mg/L 2,4-D were transferred into the embryo proliferation medium containing 1-20 mg/L 2,4-D (Table 1; Christianson *et al.*, 1983; Finer and Nagasawa, 1988; Novak *et al.*, 1989), and shaken at 80-100 rpm at 27℃ under darkness. After 50 d of culture, the proliferated globular-stage embryos were counted under a stereomicroscope. Proliferated globular embryos were subcultured for a 40-50 d interval.

Maturation of somatic embryos

The proliferated embryos were transferred to the maturation medium (Table 1; Buchheim *et al.*, 1989) under 24± 3℃, 50 $\mu E \cdot m^{-2} \cdot s^{-1}$, and 16 h photoperiod. The effect of $CoCl_2$ concentration on the transition of globular-stage to cotyledon-stage embryos was investigated on this medium containing 10 μM $CoCl_2$ or 5-30 μM $CoCl_2$. The embryos with mono-, di-, poly-, and fused-cotyledons were counted 50 d after transfer. Globular embryos were collected from the liquid culture which had been proliferated for 3 and 18 months, and tested for maturation.

Germination and regeneration

After cotyledons had been formed, embryos were transferred and regenerated on the regeneration medium (Table 1; Lazzeri *et al.*, 1987b) under 24± 3℃, 50 $\mu E \cdot m^{-2} \cdot s^{-1}$, and 16 h photoperiod. Regenerated plants were transplanted to 1 : 1 : 1 floratorf (Floragard product) : vermiculite : soil mixture.

Histological study

Globular embryos were fixed in FAA (formalin : glacial acetic acid : ethanol : water=2 : 1 : 10 : 7) for 1 h, dehydrated in tertiary butanol series, and subsequently infilterated and embedded in paraplast. Ten to twenty μm microtome sections were cut, and stained with hematoxylin. The sections were examined and photographed with an Olympus BHT system microscope.

Scanning electron microscopy

Somatic embryos were pre-fixed with 5% glutaraldehyde, and washed 3 times with phosphate buffered saline (PBS). These materials were fixed with 1% osmium tetroxide, washed, and dehydrated in ethanol series. Following dehydration, embryos were coated with gold. Coated samples were examined and photographed with a JEOL JSM-840A scanning electron microscope operated at 20 kV.

RESULTS AND DISCUSSION

Induction of somatic embryos

In order to investigate the effects of explant size and explanting orientation on somatic embryo induction, immature cotyledons of cv. Whangkeum ranging from 4 to 10 mm in size were classified by 1 mm length, and placed abaxial or adaxial side down on embryo induction medium containing 20 mg/L 2,4-D. After 30 d, somatic embryos were scored. The highest frequency of 1.2 embryos per cotyledon was obtained when the immature cotyledons of 4 to 4.9 mm in size were placed abaxial side down. Cotyledons which had been cultured adaxial side down formed embryos only in the 5 to 5.9 mm embryos (Fig. 1). However, when cotyledons of simi-

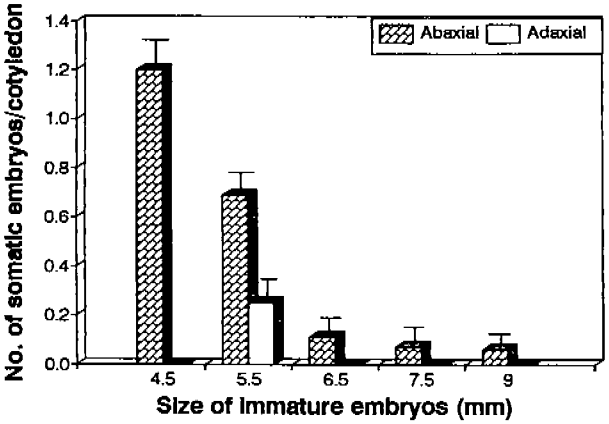

Fig. 1. Influences of immature embryo size and explanting orientation on somatic embryo induction in induction medium containing 20 mg/L 2,4-D in cv. Whangkeum.

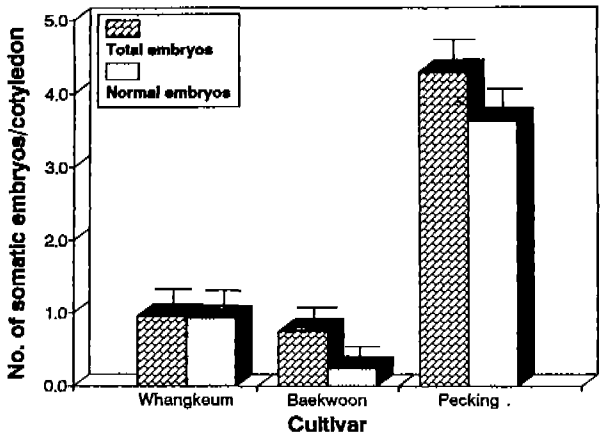

Fig. 2. Effect of genotype on somatic embryo induction in induction medium containing 20 mg/L 2,4-D. Normal embryos; globular, heart, and torpedo somatic embryos.

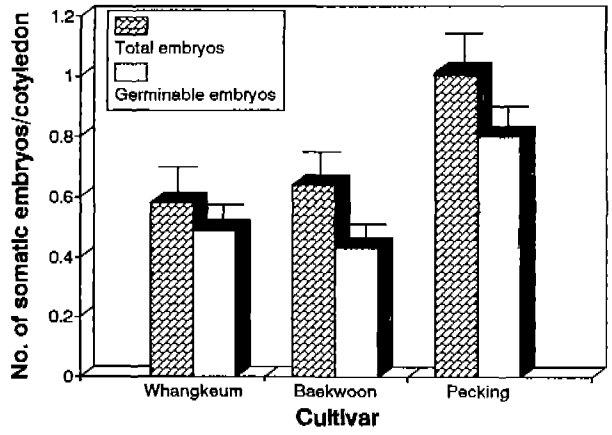

Fig. 3. Effect of genotype on somatic embryo induction in induction medium containing 8 mg/L NAA. Germinable embryos; mono-, di- and poly-cotyledonous somatic embryos.

lar size but different developmental stage were cultured concurrently, embryo induction efficiency was significantly different from each other (data not shown). These results showed that induction of somatic embryos was dependent upon the developmental stage of cotyledons. The data showing embryo induction efficiency higher in abaxial explants than in adaxial explants was consistent with the report that the explanting orientation influenced hormone interaction (Hartweck *et al.*, 1988). Such effects presumably involve differences in auxin transport or conjugation or both, as well as differences due to cell type or age.

To determine if embryo induction efficiency depended on the auxin type or soybean genotype, immature cotyledons of cvs. Whangkeum, Baekwoon and Pecking were exposed to 20 mg/L 2,4-D and 8 mg/L NAA, respectively. The somatic embryos with normal and abnormal morphology were scored in 2,4-D treated-medium, and the embryos with mono-, di-, poly- and fused-cotyledons were counted in NAA treatment. Cultivar Pecking gave 3.9 and 5 times higher induction efficiency than those of cvs. Baekwoon and Whangkeum in 2,4-D treatment (Fig. 2); and 1.6 and 1.9 times in NAA treatment, respectively (Fig. 3). Explants which had been treated with 2,4-D formed yellowish globular embryos which were composed of either large embryos or clumps (Fig. 4A). These embryos could be separated easily from the other parts. On the medium containing NAA, mature somatic embryos with mono-, di-, poly-, and fused-cotyledons were obtained 30 d

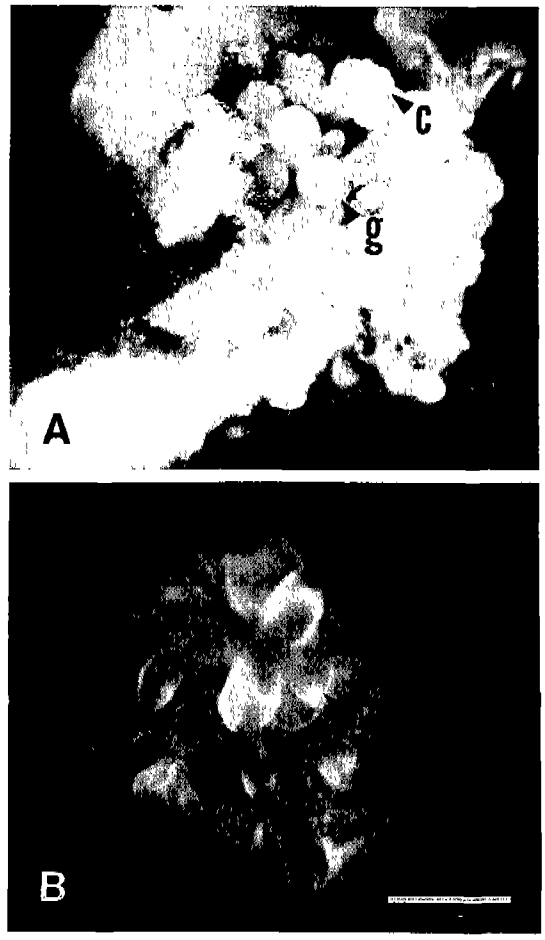

Fig. 4. Morphology of somatic embryos. (A) Somatic embryos induced by 20 mg/L 2,4-D; g, globular somatic embryo; c, clump containing 3-15 globular-stage embryos. (B) Somatic embryos induced by 8 mg/L NAA; m, mono-cotyledonous; d, di-cotyledonous; p, poly-cotyledonous somatic embryos. Bars indicate 5 mm.

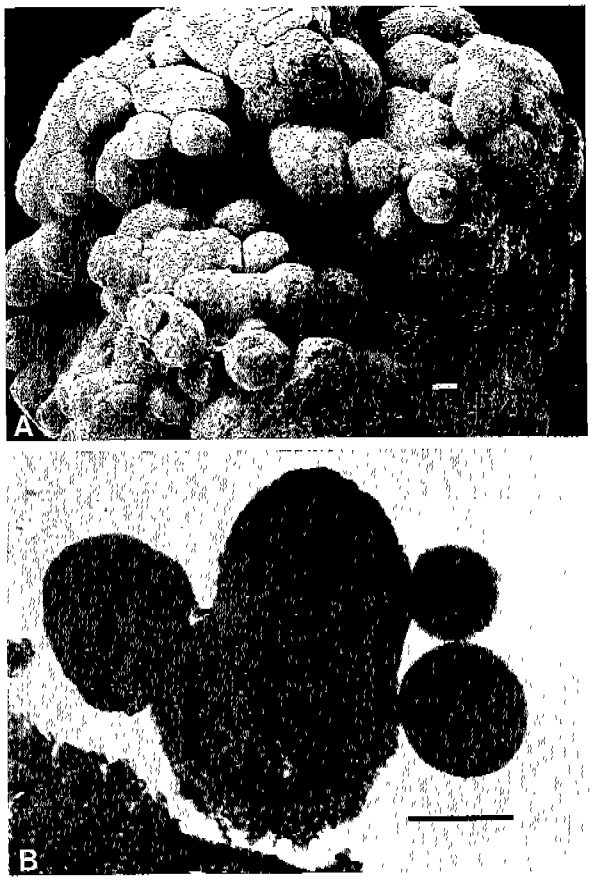

Fig. 7. Proliferating globular somatic embryos in liquid medium containing 10 mg/L 2,4-D. A, clump of globular somatic embryos; B, secondary globular embryos forming on the primary embryo. Bars indicate 1 mm.

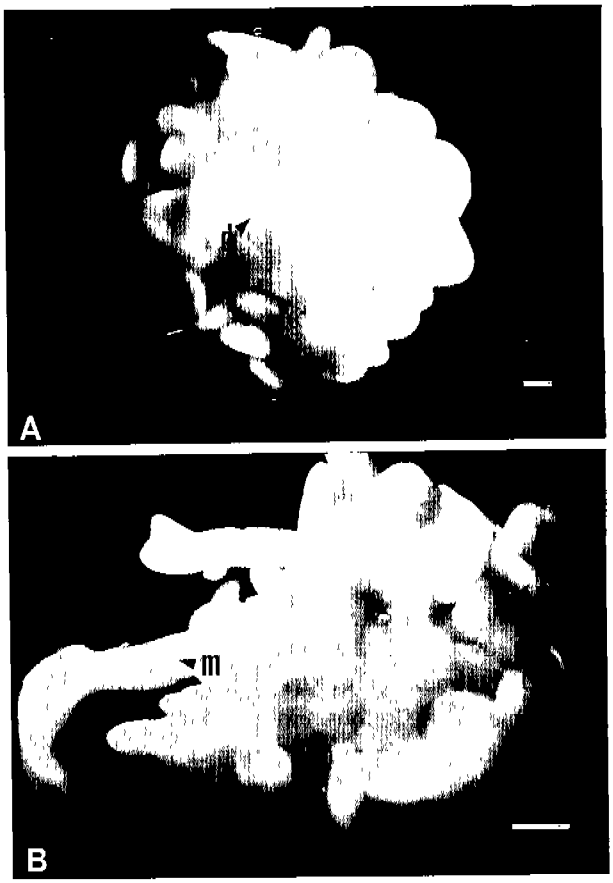

Fig. 8. Various morphology of mature somatic embryos in maturation medium containing 9% sucrose, 0.5% activated charcoal and 10 μM $CoCl_2$ (A) or not (B). m, monocotyledonous; d, di-cotyledonous; f, fused-cotyledonous somatic embryos. Bars indicate 1 mm.

mg/L (Fig. 6). Single globular embryo had been proliferated to 35 and 25 embryos after 50 d of culture in 5 and 10 mg/L 2,4-D treatment, respectively, but nonembryogenic callus and rescued embryos were frequently formed in 5 mg/L 2,4-D treatment. In this study, the medium containing 10 mg/L 2,4-D was used for proliferation in order to arrest the globular embryos. Secondary somatic embryos were formed on the epidermal surfaces of single, cluster embryos (Fig. 7). However, it was not identified if they had originated from single cells.

Maturation of somatic embryos

It has been reported that cobalt ions are an inhibitor of ethylene biosynthesis (Yang and Hoffmann, 1984) and stimulate somatic embryogenesis in carrot (Roustan *et al.*, 1989). In order to identify the effect of Co^{2+} on the stimulation of soybean somatic embryogenesis, globular embryos, 3 month after suspension culture, were placed on the maturation medium containing 10 μM $CoCl_2$. The production of mono-, di-, and poly-cotyledonous embryos per total embryos was 1.7 times higher in the $CoCl_2$ treatment than that in control, and the proportion of dicotyledonous embryos was 4 times higher (Figs. 8 and 9). The effect of $CoCl_2$ concentration on somatic embryo maturation was highest at 10 and 20 μM (Table 2). In these concentrations. the proportion of germinable embryos per total embryos was significantly increased. The higher the concentration of $CoCl_2$ was, however, the less the total embryos were. The reduction of cotyledonous embryos in higher $CoCl_2$ concentration was due to the rapid conversion of globular embryos to cotyledonous embryos. These results gave the conclusion that the addition of 10 μM $CoCl_2$ was suitable for a maturation process.

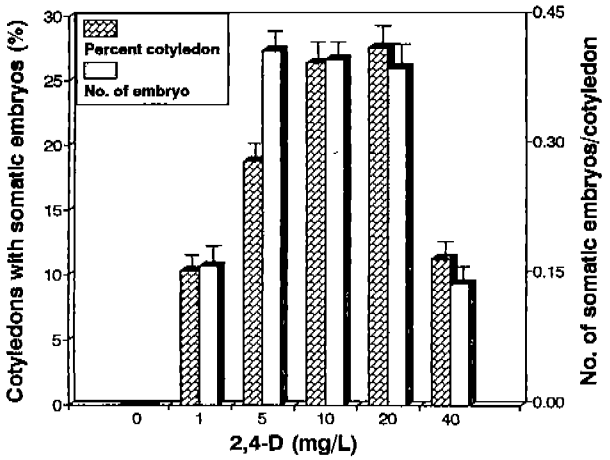

Fig. 5. Effect of 2,4-D concentration on globular somatic embryo induction in cv. Whangkeum.

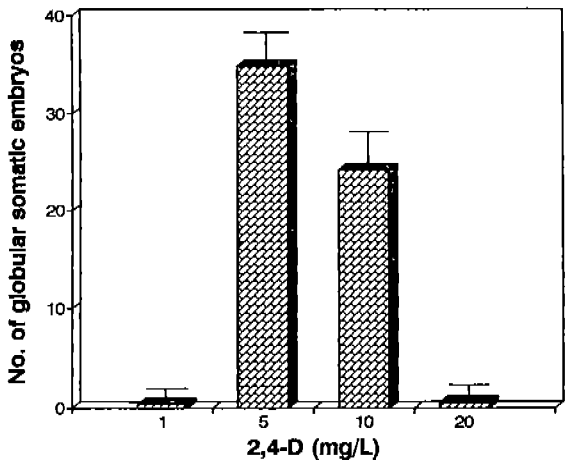

Fig. 6. Effect of 2,4-D concentration on proliferation of globular somatic embryos. Y axis indicates the number of globular somatic embryos proliferated per initial globular embryo after 50 d suspension culture.

after explanting (Fig. 4B). Induction efficiency was relatively low in NAA treatment compared with that in 2,4-D treatment (Figs. 2 and 3), but the ratio of germinable cotyledonous embryos per total cotyledon-stage embryos was much higher in NAA treatment than that in 2,4-D treatment devoid of $CoCl_2$ (Table 2 and Fig. 3). The result that germinable mono-, di-, and poly-cotyledonary embryos were induced at higher proportions in NAA treatment conformed to the view that embryos formed on medium containing NAA were advantageous for regeneration (Lazzeri *et al.*, 1987a). Explants which had been placed on the medium containing NAA formed hairy roots around the marginal surface.

The effect of 2,4-D concentration on globular embryo induction was investigated in cv. Whangkeum. Embryo production per cotyledon was not significantly different in the range of 5 to 20 mg/L 2,4-D, and also the number of cotyledons with embryos was similar (Fig. 5). But the embryos formed in the medium containing 20 mg/L 2,4-D were compact and fragile, and suitable for proliferation by liquid culture.

Proliferation of globular embryos

The correlation of 2,4-D concentration to globular embryo proliferation appeared highest at 5 and 10

Table 2. Effect of $CoCl_2$ on somatic embryo maturation in cv. Whangkeum

Cotyledonous type		Embryos with each cotyledonous type of total embryos (%)				
		$CoCl_2$ (μM)				
		0	5	10	20	30
Mono-cotyledon	a[a]	18.9± 3.2	27.5± 3.7	34.2± 2.7	33.3± 2.2	29.8± 1.4
	b[b]	38.3± 5.5	52.0± 8.7	46.2± 5.4	50.1± 5.3	55.0± 6.5
Di-cotyledon	a	19.4± 2.4	17.1± 2.7	34.5± 2.6	36.1± 3.7	31.7± 2.0
	b	29.6± 3.6	32.8± 4.1	35.7± 5.3	37.4± 3.2	30.6± 3.2
Poly-cotyledon	a	3.6± 0.1	2.1± 0.4	4.5± 0.5	6.7± 0.7	6.0± 0.8
	b	2.0± 0.7	2.6± 0.4	4.0± 0.4	4.9± 0.9	4.0± 0.2
Fused-cotyleon	a	58.2± 5.4	53.2± 5.6	26.8± 2.4	23.8± 1.2	32.6± 1.4
	b	30.1± 5.7	12.6± 2.7	14.1± 2.5	7.6± 0.9	10.4± 1.2
Total No.	a	392	327	336	252	218
of embryos	b	325	307	426	312	175

[a]Using globular somatic embryos after 3 months suspension culture. [b]Using globular somatic embryos after 18 months suspension culture.

Also, the effect of $CoCl_2$ on somatic embryo maturation was reduced after 18 months of selective subculture (Table 2). The time being necessary for maturation was 35 to 50 days. 2,4-D was known to inhibit the meristematic development of carrot somatic embryos (Halperin and Wetherall, 1964) and to be closely correlated with a marked increase in endogenous ethylene production (Pinfield *et al.*, 1991). Also, Roustan *et al.* (1989) reported that endogeneous ethylene production had been strongly inhibited by $CoCl_2$ in carrot cell suspensions, demonstrating that the number of somatic embryos was increased con-

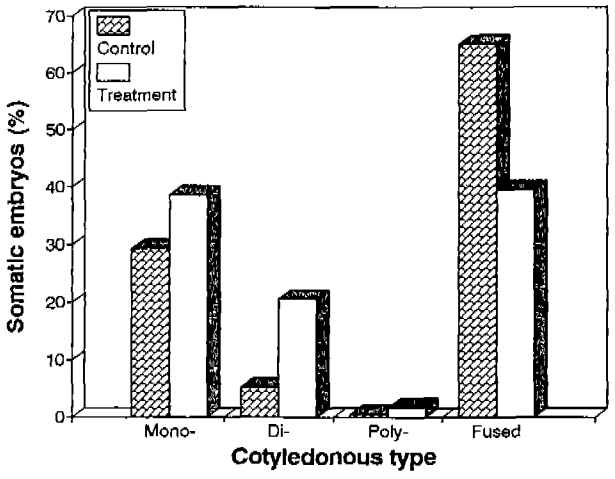

Fig. 9. Effect of 10 μM $CoCl_2$ on the maturation of somatic embryos in cv. Whangkeum.

Fig. 10. A, Plantlets derived from somatic embryos; B, Regenerated soybean plants *via* somatic embryogenesis.

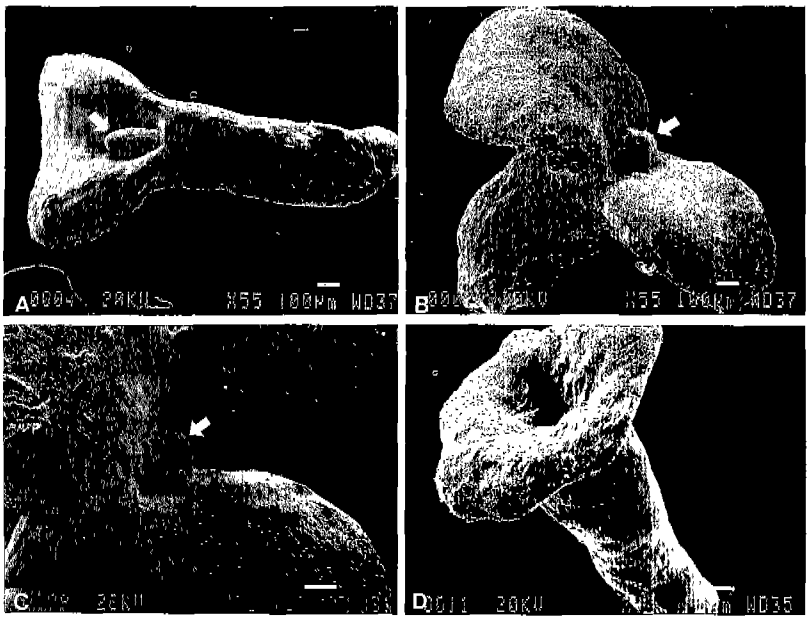

Fig. 11. Mature somatic embryos of various cotyledonous types. Arrows indicate shoot apical meristem just emerging. A, mono-cotyledonous; B, di-cotyledonous; C, poly-cotyledonous; D, fused-cotyledonous. Bars indicate 100 μm.

currently. In this study, we did not test the inhibition effect of $CoCl_2$ on ethylene synthesis, but it could be inferred that cobalt ions had caused the increase of germinable somatic embryos.

Germination and regeneration of somatic embryos

Mature embryos which had been transferred and cultured on the regeneration medium for 45 d formed shoots and roots simultaneously (Fig. 10A). There was a close relationship between the morphology of cotyledon and germination effiency. The germination effiency of mono-, di-, and poly-cotyledonous embryos amounted to 60% or greater, but fused-cotyledonous embryos did not quite germinate (Fig. 11). The regenerated plants were acclimated and grown to whole plants. We could harvest the seeds from all tested cultivars. These plants apparently had no morphological abnormality (Fig. 10B).

In conclusion, we have achieved effective plant regeneration from immature cotyledons of soybean cultivars *via* somatic embryogenesis. This system will be beneficial for verifying molecular mechanism of embryogenesis.

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體細胞胚 發生을 통한 콩 品種의 植物體 再分化

文 倍 煥 · 金 世 圭 · 崔 相 烽 · 李 光 雄*
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적 요

개화 후 14-20일이 경과한 콩의 미성숙 자엽을 재료로 하여 효율적인 체세포배 발생 기법을 규명하고자 하였다. 황금 품종에 대해 크기가 4.0-10.0 mm인 미성숙 종자의 자엽을 20 mg/L 2,4-D를 포함하는 변형된 MS 배지에 배축성 또는 향축성 방향으로 배양한 결과, 크기가 4.0-4.9 mm인 미성숙 종자의 자엽을 배축성 방향으로 배양하였을 때 자엽당 형성된 체세포배의 수가 1.2개로 가장 많았다. 20 mg/L 2,4-D 처리구 및 8 mg/L NAA 처리구에 있어서, Pecking 품종이 자엽당 형성된 체세포배의 수가 각각 4.3개와 1.0개로, 황금 품종에 비하여 각각 4.5배 및 1.7배, 백운 품종에 비하여 5.7배 및 1.6배로 체세포배 유도 효율이 높았다. 또한 2,4-D 농도 1에서 40 mg/L까지의 범위 중 20 mg/L 처리구에서 27.6%로 가장 높은 체세포배 유도 효율을 보였고, 액체 배양을 통한 구형 체세포배의 증식에는 10 mg/L 2,4-D의 사용이 적합하였다. 이들 체세포배를 10 μM $CoCl_2$, 9% sucrose, 0.5% 활성 탄소를 포함하는 배지에서 성숙시키고, 호르몬이 첨가되지 않은 배지에서 발아시켜 재분화 개체 및 종자를 획득하였다.

주요어 : 콩, 미성숙 자엽, 체세포배 발생, 식물체 재분화

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