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Effects of Growth Regulators on Somatic Embryogenesis from Ginseng Zygotic Embryos

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인삼 접합자배로부터 체세포배의 발생에 미치는 생장조절제의 영향

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Intact mature zygotic embryos or their excised cotyledons of ginseng, were cultured on media containing various growth regulators such as auxin (2,4-D, IAA) and cytokinin (BAP, kinetin). In the culture of intact zygotic embryos, auxin inhibited germination but cytokinin did not. Somatic embryogenesis occurred only from those of ungerminated embryos. In the culture of cotyledon segments, medium without growth regulators was the most appropriate to somatic embryogenesis. Somatic embryos were produced sporadically over the surfaces of zygotic embryos on medium containing auxin, while on medium without growth regulators, or media containing cytokinin, somatic embryos formed only on the proximal region of cotyledon. on medium containing 2,4-D, somatic embryos originated from multiple cells which comprised epidermal and subepidermal layers of cotyledon, which resulted in poly-somatic embryogenesis. When these somatic embryos were cultured on the same medium, the primary somatic embryos produced secondary embryos, which arose from epidermal or subepidermal single cells.

Key words: cotyledon segment, secondary embryo, somatic embryo

In general exogenous growth regulators especially 2,4-D on culture medium was prerequisite for inducing somatic embryos in a tissue culture system (Ammirato, 1983: Borkird et al., 1986). However, in direct somatic embryogenesis, auxin only stimulated the process since potential producing somatic embryos had already been determined (Pence et al., 1980). On the other hand, somatic embryos could be formed on medium without any supplement of growth regulators (Hu and Sussex, 1971: Smith and Krikorian, 1989: Choi and Soh, 1994a, b). Wounding, excision (Smith and Krikorian, 1989: Choi and Soh, 1995b), osmotic or heavy metal stress (Kamada et al., 1989) of zygotic embryos stimulated somatic embryo production. In this culture system, it is not determined whether exogenously supplied growth regulators stimulate somatic embryogenesis.

Somatic embryos formed directly from cultured explants (William and Maheswaran, 1986: Choi and Soh, 1994b), originated from single cells (Konar and Nataraja, 1965: Thomas and Street, 1972: Jones and Rost, 1989), or from multiple cells (James et al., 1984). Multicellular origin appears to produce somatic embryos fused with the parent tissue over a broad area of the root pole or axis region, whereas a unicellular origin is more likely to produce embryos attached by a narrow suspensor-like organ (William and Maheswaran, 1986: Choi and Soh, 1994a, b). We reported that cotyledon segments of ginseng zygotic embryos cultured on MS basal medium actively produce somatic embryos and the origin and development of somatic embryos is different according as the tissue of the cultured cotyledon is meristematic or fully differentiated (Choi and Soh, 1994a, b). In the present experiment, we investigated the role of growth regulators by comparing somatic embryo development between medium free

of growth regulator and medium containing auxin or cytokinin.

MATERIALS AND METHODS

The seeds of Korean ginseng (*Panax ginseng* C.A. Meyer) have immature embryos two mm in length just after dehiscing. The embryos grew fully to 4 mm in length when stratified in moist sand for three months. The seeds after stratification were immersed in 70% alcohol for 1 minute, sterilized in 1% sodium hypochlorite solution for one hour, and then rinsed three times with distilled water. Intact zygotic embryos or excised cotyledon segments were placed on MS (Murashige and Skoog, 1962) basal salts containing 3% sucrose and 0.7% agar. Auxin (0.1 to 5.0 mg/L IAA and 1.0 mg/L 2,4-D) or cytokinin (1.0 mg/L BAP and kinetin) were added to the medium. The medium was adjusted to pH 5.8 before autoclaving at 120°C for 15 minutes. Cultures were performed using 10×1 cm glass Petridishes containing 30 ml of medium. The culture room was maintained at 24 ± 2°C with a 16-8 hour photoperiod under 1,900 lux cool white fluorescent illumination. The production rate of somatic embryos was evaluated by counting cotyledon explants showing somatic embryos from the total number of cultured explants. Thirty explants were cultured in each experiment which was repeated three times.

For anatomical examination, the explants were fixed in FAA (formalin, acetic acid and ethyl alcohol), dehydrated in alcohol, then embedded in paraffin. Next the samples were cut to 10 μm thickness with a rotary microtome and the sections stained with hematoxylin.

Some samples fixed in 1% glutaraldehyde and dehydrated with ethyl alcohol were dried in a critical point drier. After being coated with gold, the samples were observed by scanning electron microscope (JSM T330A).

RESULTS

Intact Zygotic Embryo

Most intact zygotic embryos cultured on MS basal medium did not produce somatic embryos but germinated (Fig. 1, 2A). On medium containig 1.0 mg/l 2,4-D, germination of embryos was suppressed but the embryos swelled abnormally. After two to three weeks of culture, somatic embryos formed

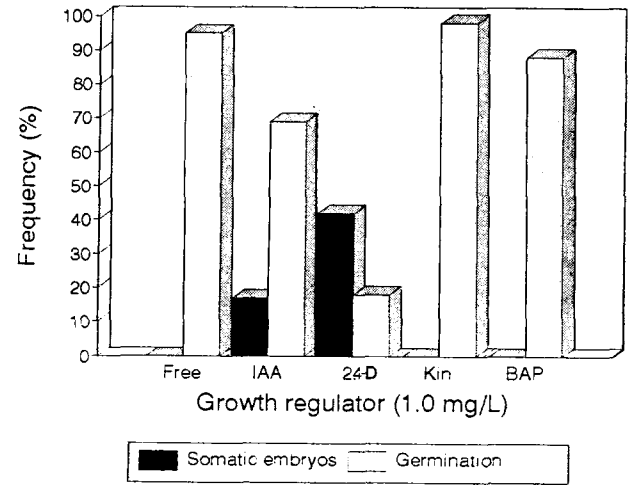

Figure 1. Somatic embryo formation from intact zygotic embryos cultured on medium with or without growth regulators.

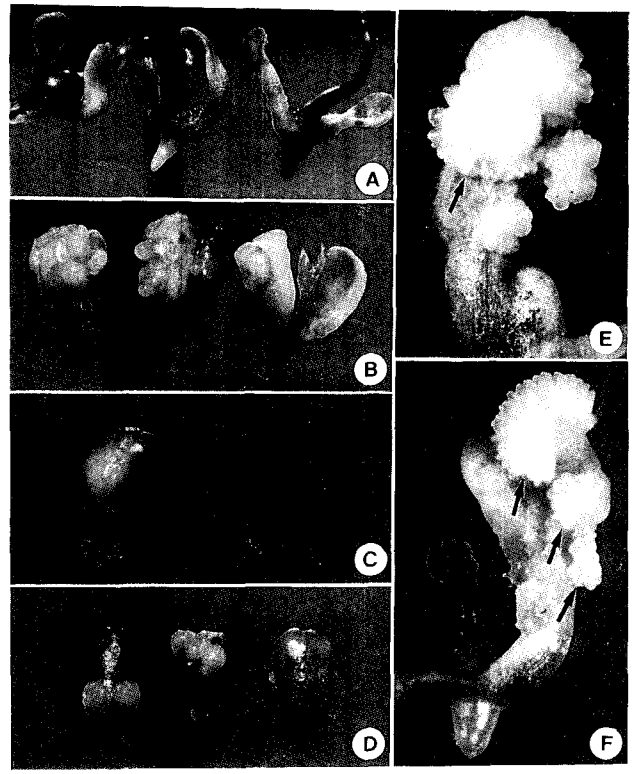

Figure 2. Somatic embryogenesis from intact mature zygotic embryos or excised cotyledons cultured on MS medium with or without 2,4-D. A: Germinating mature zygotic embryos cultured on MS basal medium: B: Sporadic somatic embryo production (arrows) from mature zygotic embryos cultured on MS medium containing 1.0 mg/L 2,4-D: C: Somatic embryogenesis from proximal portion (arrow) of cotyledon segments cultured on MS basal medium: D: Sporadic distribution of somatic embryo (arrows) on cotyledon segments cultured on medium containing 1.0 mg/L 2,4-D: E-F: Poly-somatic embryo (arrows) formation from the surface of mature zygotic embryos cultured on medium containing 1.0 mg/L 2,4-D.

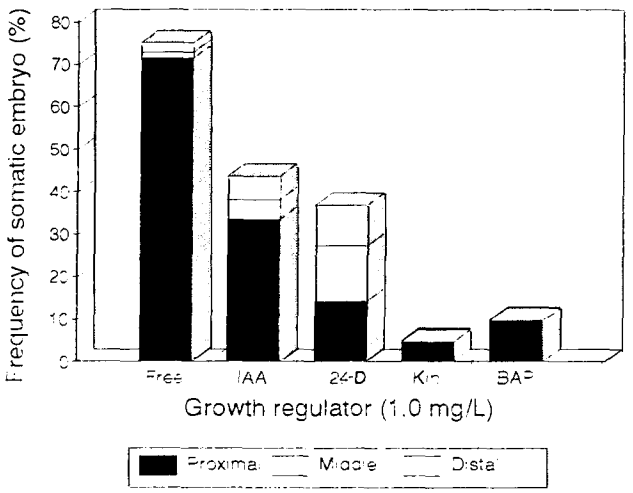

Figure 3. Somatic embryo formation from excised cotyledons cultured on medium with or without growth regulators

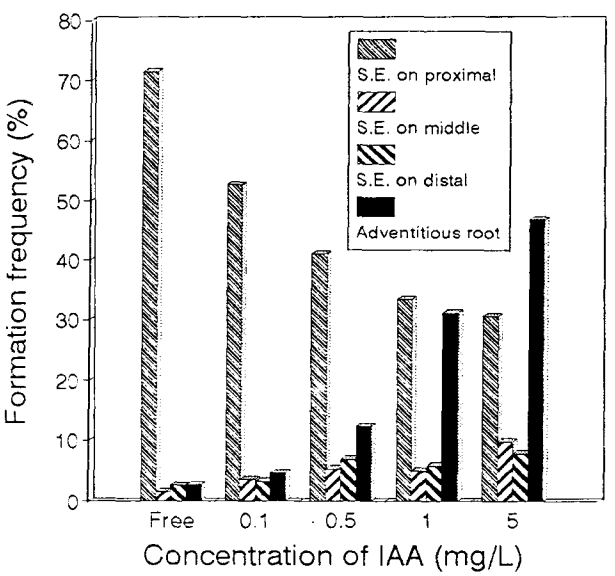

Figure 4. Somatic embryo (S.E.) and adventitious root production from cotyledon explants cultured on MS medium with various concentration of IAA.

mainly on the cotyledon surfaces of these ungerminated embryos (Fig. 1, 2B, E, F). Somatic embryogenesis occurred at a low frequency from the cotyledon surfaces on medium containing IAA (Fig. 1). On medium containing 1.0 mg/L BAP or 1.0 mg/L kinetin, most of the zygotic embryos germinated and somatic embryogenesis was not observed (Fig. 1).

Cotyledon Segment

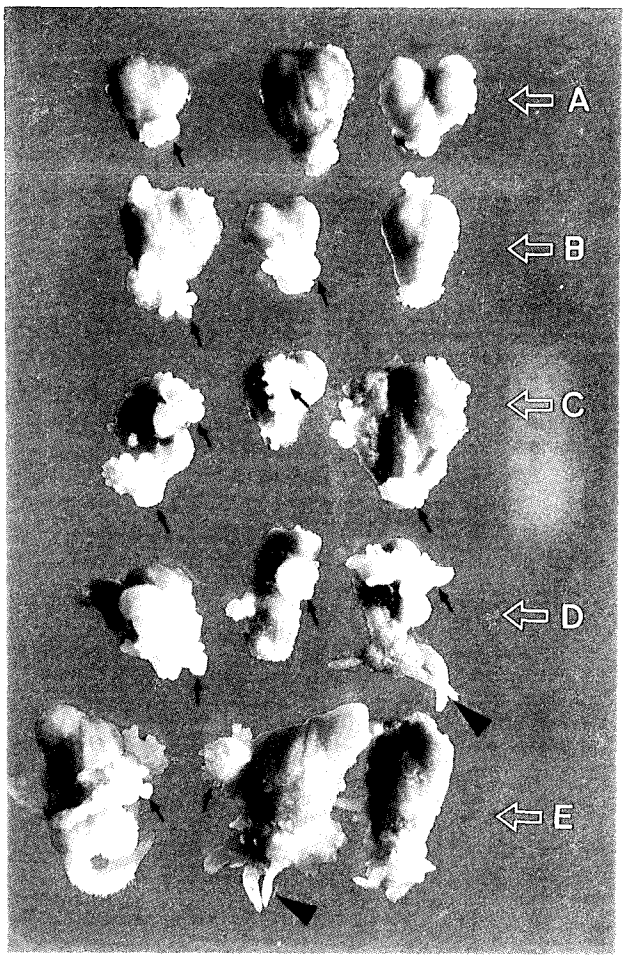

Figure 5. Somatic embryo (arrows) and adventitious root (arrow head) formation from cotyledon cultured on medium with various concentration of IAA. A: IAA-free: B: 0.1 mg/L IAA: C: 0.5 mg/L IAA: C: 1.0 mg/L IAA: D: 5.0 mg/L IAA.

On MS basal medium, the cultured cotyledon segments actively produced somatic embryos only on the basal excised portion of cotyledons (Fig. 2C). On medium containing 1.0 mg/L 2,4-D or 1.0 mg/L IAA, somatic embryos formed sporadically on cotyledons (Fig. 2D) and somatic embryo production was lower than on MS basal medium (Fig. 3). On medium containing various concentrations of IAA (Fig. 4), somatic embryo formation decreased but adventitious root formation increased (Fig. 5) as the concentration of IAA was increased. On medium containing kinetin or BAP, somatic embryos formed at a very low from near the basal excised portion (Fig. 3).

Origin of Somatic Embryo

On MS medium containing 1.0 mg/L 2,4-D, white nodulous

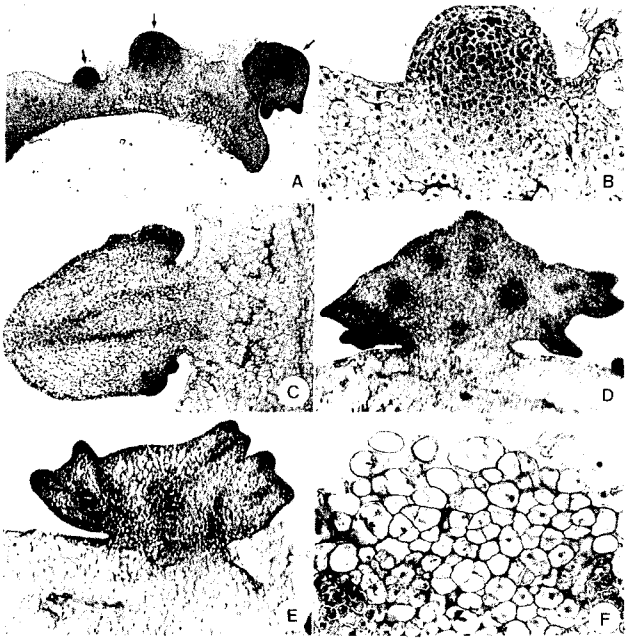

Figure 6. Histological observation of somatic embryogenesis from cotyledon segment cultured on medium containing 1.0 mg/L 2,4-D. A: Somatic embryos (arrows) formed sporadically from cotyledon; B: Nodular hemispherical embryo derived from the multiple cell of epidermis and subepidermis; C: Nodular embryos; D-E: Polyembryos formed from nodule; F: Nonembryogenic callus formed from excised portion of cotyledon.

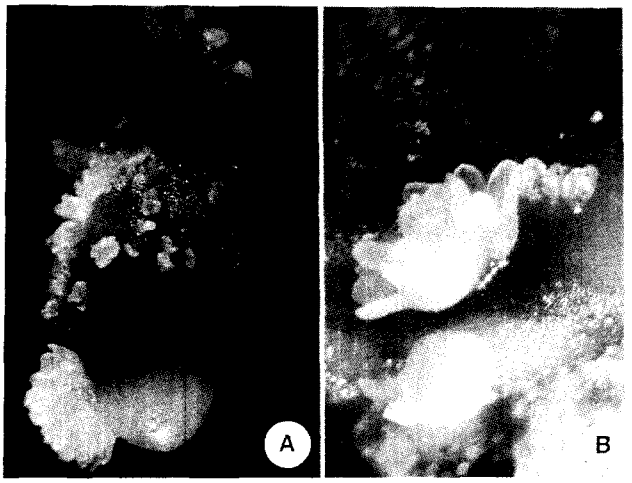

Figure 7. Secondary somatic embryogenesis from primary somatic embryos cultured on medium containing 1.0 mg/L 2,4-D. A: Secondary somatic embryos (arrows) developed from the surface of primary somatic embryos; B: Cotyledonary secondary somatic embryos.

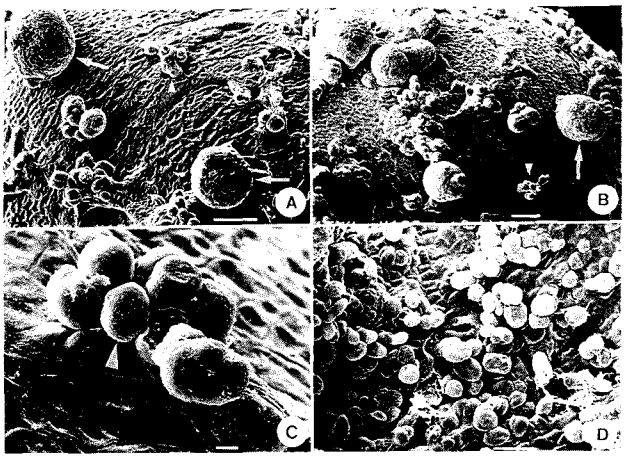

Figure 8. Scanning electron microscopic observation of secondary somatic embryos developed from the surface of primary somatic embryos cultured on medium containing 1.0 mg/L 2,4-D. A-B: Proembryogenic cell clumps (arrowheads) and globular somatic embryos (arrows) (bar: 100 μm); C: Proembryogenic cell complex (bar: 10 μm); D: Nonembryogenic callus formed from primary somatic embryos (100 μm).

tissue arose from the surfaces of cotyledons after 2 weeks of culture (Fig. 2B). Histological observation revealed that the nodulous tissue arose from division of massive cells in the epidermis and subepidermis of the cotyledons (Fig. 6A, B). After three weeks of culture these nodulous tissues grew much larger (Fig. 6C) and formed poly somatic embryos with fused radicle (Fig. 6D, E). Sometimes, friable callus constituted with vacuolated cells formed on the cotyledon surface from which somatic embryogenesis was not observed (Fig. 6F).

When cotyledonary somatic embryos were cultured on medium containing 1.0 mg/l 2,4-D, they were also swelled abnormally and their development was arrested. After three to five weeks culture, secondary somatic embryos were produced directly from the cotyledon and the embryo axis (Fig. 7). By scanning electron microscope (Fig. 8), protuberated proembryo-like structures composed of cells 20 μm in diameter were observed on the surface of the hypocotyl (Fig. 8A-C: arrowheads). They eventually developed into globular embryos (Fig. 8A, B). Sometimes, soft and greenish callus which was composed of cells 50 μm in diameter (Fig. 8D) was formed, but these calli did not produce somatic embryos. Histological observations revealed that secondary somatic embryos formed from cotyledons originated mainly from epidermal single cells, or, rarely from sub-epidermal single cells. In the former case, somatic embryogenesis initiated from periclinal division of epidermal single cells (Fig. 9A, B). Early globular somatic embryos with discernible structures of suspensor-like cells developed from these cells (Fig. 9C, D) and grew into cotyledonary embryos (Fig. 9F). Sometimes, somatic embryogenesis initiated from periclinal

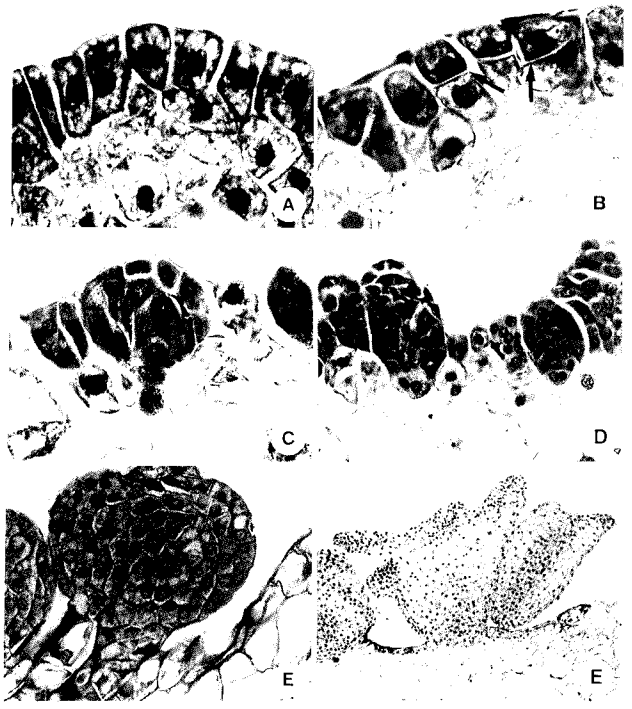

Figure 9. Ontogeny of secondary somatic embryogenesis from epidermal cells of primary somatic embryos. A: Epidermal cells before somatic embryogenesis: B: First periclinal division of embryonic development from epidermal single cells (arrows): C-D: Globular somatic embryos formed from single epidermal cells: E: Heart-shaped somatic embryos: F: Cotyledonary somatic embryos.

division of subepidermal single cells (Fig. 10). After some further division proembryos formed (Fig. 10B, C), and eventually became globular somatic embryos (Fig. 10D).

DISCUSSION

Effect of Auxin

In the present experiment, the effect of auxin especially 2,4-D on somatic embryognesis differed when the zygotic embryos were cultured in an intact state from when cultured in an excised state. In the culture of intact zygotic embryos, somatic embryogenesis occurred only on medium containing auxin, but in the culture of cotyledon segments, somatic embryogenesis was slightly suppressed by exogenous auxin. This contrary effect of auxin might be the result of whether the explants contained an embryo axis or not. Generally, somatic embryogenesis from zygotic embryos is often associated with poor growth or suppression of the main embryo axis (Hu and Sussex, 1971: Pence et al. 1980:

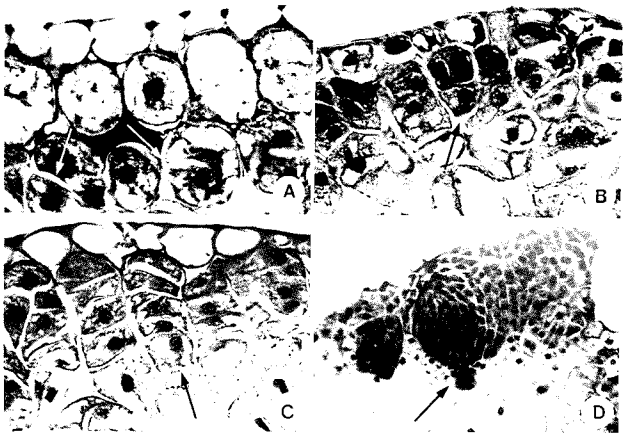

Figure 10. Ontogeny of secondary somatic embryogenesis from subepidermal cells of primary somatic embryos. A: First division of subepidermal cells: B-C: Proembryo formation (arrows) from repetitive transverse division of subepidermal cells: D: Globular somatic embryos (arrow).

Maheswaran and Williams, 1985). In our previous experiment, the plumule and the radicle of mature ginseng zygotic embryos contained a factor suppressing somatic embryogenesis, therefore removal of the embryo axis was required to induce somatic embryogenesis from the cotyledon segments on MS basal medium (Choi and Soh, 1995). From the above results, the role of auxin on somatic embryogenesis of intact zygotic embryos might be related to the suppression of normal growth of the embryo axis although the mechanism remains to be determined.

In the culture of cotyledon segments, somatic embryogenesis actively occurred on medium without growth regulators and was suppressed by exogenous auxin. This result represents stimulus treatment such as excision or wounding of explants which was enough to induce somatic embryos but the exogenous auxin was inhibitory to somatic embryo production. A similar experiment has been reported in the culture of *Quercus rubra* cotyledon segments (Gingas and Lineberger, 1989).

In the present experiment, somatic embryogenesis occurred near the basal excised portions of cotyledon segments in growth regulator-free medium but formed sporadically on cotyledons in media containing auxin. This result indicates that the sporadic embryogenesis on cotyledon segments occurred because of disturbance to the polar somatic embryogenesis by exogenous auxin. Polar growth or development was suppressed by high concentrations of exogenous auxin (Smulders et al. 1988).

Effect of Cytokinin

On medium containing cytokinin, the somatic embryo production rate was highly suppressed but the portion producing somatic embryos was similar to that of the growth regulator-free medium. In others reports, cytokinin stimulated germination of ginseng somatic embryos (Kwon et al., 1986) but suppressed somatic embryogenesis (Arya et al., 1993). These results mean that cytokinin suppresses the somatic embryogenesis but cytokinin does not disturb polar somatic embryogenesis in ginseng.

Origin of the Somatic Embryo

In general, direct somatic embryogenesis was associated with two distinct developmental patterns. The first was single cell origin from epidermal cells. The second was multiple cell origin from internal meristematic cells (Pence et. al., 1980: Hu and Sussex, 1971). In our previous experiments, the origin and development of somatic embryos from ginseng zygotic embryos on MS basal medium differed according to the degree of maturity of the zygotic embryos used (Choi and Soh 1994a, b). But on medium containing 1.0 mg/L 2,4-D, most of the somatic embryos originated from multiple cells. We supposed that the 2,4-D transformed the cotyledon tissue into a meristematic state which in turn induced somatic embryogenesis from multiple cells. On the other hand, in the culture of primary somatic embryos on medium containing 2,4-D, secondary somatic embryos originated mainly from single cells. Generally, the epidermis of somatic embryos was not intact and showed an irregular arrangement of cells (Ammirato, 1985). This condition could promote somatic embryo development from single cells.

적 요

온전한 접합자배 또는 자엽절편을 오옥신(2,4-D, IAA)과 사이토카이닌(BA, kinetin)이 첨가된 배지에 배양하였다. 접합자배를 온전한 상태로 배양했을 경우, 오옥신은 발아를 억제하였으나 사이토카이닌은 억제하지 않았고, 체세포배의 발생은 발아되지 못하는 배에서만 유도되었다. 자엽절편을 배양한 경우, 기본배지에서 체세포배발생률이 가장 높았다. 오옥신을 첨가한 배지에서는 체세포배가 자엽의 표면에서 산재하여 발생되었는데 비해서 기본배지에서는 자엽의 기부에서만 발생되었다. 2,4-D를 첨가한 배지에서는 체세포배가 자엽의 표피 및 하표피를 포함한 다수의 세포로부터 기원되어 다배로 발생되었다. 이 체세포배를 같은 배지에 배양했을 때 일차배의 표면으로부터 이차배가 발생되었는데 이들의 경우는 주로 표피 또는 하표피의 단세포로부터 유래되었음을 조직학적으로 밝혔다.

REFERENCES

Ammirato PV (1985) *In* RR, Henke, KW Hughes, MP Constantin, A Hollarender, eds, Tissue Culture in Forestry and Agriculture, Plenum Press Cooperation, New York, pp 9-29

Ammirato PV (1983) *In* DA Evans, WR Sharp, PV Ammirato and Y Yamada, eds, Hand Book of Plant Cell Culture, Vol. 1. Techniques for propagation and breeding, Macmillan Publishing Co., New York, pp 82-123

Arya S, Arya ID, Eriksson T (1993) Rapid multiplication of adventitious somatic embryos of *Panax ginseng*. Plant Cell, Tissue and Organ Culture **34**: 157-163

Borkird C, Choi JH, Sung ZR (1986) Effect of 2,4-dichlorophenoxyacetic acid on the expression of embryogenic program in carrot. Plant Physiol **81**: 1143-1146

Choi YE, Soh WY (1994a) Origin of somatic embryos from excised cotyledons of ginseng. Korean J Plant Tissue Cult **21**: 177-182

Choi YE, Soh WY (1994b) Origin of somatic embryo induced from cotyledons of zygotic embryos at various developmental stages of ginseng. J Plant Biol **37**: 365-370

Choi YE, Soh WY (1995a) Effect of plumule and radicle on somatic embryogenesis in the cultures of ginseng zygotic embryos. Plant Cell Tissue and Organ Cult (submitted)

Choi YE, Soh WY (1995b) Polar somatic embryogenesis on embryonal cotyledon segments of ginseng cultured on MS basal medium. J Plant Biol (submitted)

Gingas VM, Lineberger RD (1989) Asexual embryogenesis and plant regeneration in *Quercus*. Plant Cell Tissue and Organ Cult **17**: 191-203

Hu CY, Sussex IM (1971) *In vitro* development of embryoids on cotyledons of *Ilex aquifolium*. Phytomorphology **21**: 103-107

James DJ, Passey AJ, Deeming DC (1984) Adventitious embryogenesis and the in vitro culture of apple seed parts. J Plant Physiol **115**: 217-229

Jones TD, Rost TL (1989) The developmental anatomy and ultrastructure of somatic embryos from the rice (*Oryza sativa* L.) scutellium epidermal cells. Bot Gaz **150**: 41-49

Kamada H, Kobayashi K, Kiyosue T, Harada H (1989) Stress induced somatic embryogenesis in carrot and its application to synthetic seed production. In Vitro Cell Dev Biol **25**: 1163-1166

Konar RN, Nataraja K (1965) Experimental studies in *Ranunculus sceleratus* L. Development of embryos from the stem epidermis. Phytomorphology **15**: 132-137

Kwon WS, Jung CM, Ahn SD, Choi KT (1986) Effects of growth regulators on the germination of *Panax ginseng* C. A. Meyer. Korean J Ginseng Sci **10**: 159-166

Maheswaran G, Williams EG (1985) Origin and development of somatic embryos formed directly on immature embryos of *Trifolium repens* in vitro. Ann Bot **56**: 619-630

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue. Physiol Plant **15**: 473-497

Pence VC, Hasegawa PM, Janick J (1980) Initiation and development of asexual embryos of *Theobroma cacao* L. *in vitro*. Z Pflanzenphysiol **98**: 1-14

Smith DL, Krikorian AD (1989) Release of somatic embryogenic potential from excised zygotic embryos of carrot and maintenance of proembryonic cultures in hormone-free medium. Amer J Bot **76**: 1832-1843

Smulders MJM, Croes AF, Wullems GJ (1988) Polar transport of 1-napthalenacetic acid determines the distribution of flower buds on explants of tobacco. Plant Physiol **88**: 752-756

Williams EG, Maheswaran G (1986) Somatic embryogenesis: factors influencing coordinated behavior of cells as an embryogenic group. Ann Bot **57**: 443-462

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