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Somatic Embryogenesis and Plant Regeneration from Immature Zygotic Embryo Culture in Pepper (*Capsicum annuum* L.)

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An efficient system of somatic embryogenesis was established for the red pepper plant (*Capsicum annuum* L. cv. Nokkwang) using immature zygotic embryos. The size of the immature zygotic embryos and the concentrations of 2,4-D and sucrose were found to be critical. Somatic embryos were induced *via* callus or directly from explants and regenerated into plantlets successfully. When zygotic embryos 1~2 mm long were cultured on the modified Murashige-Skoog (MS) medium supplemented with 2 mg/L 2,4-D for 3 weeks in the dark, somatic embryos were induced directly from the apical region of zygotic embryos with the highest frequency being approximately 90%. To mature the somatic embryos, ABA and an ethylene inhibitor $AgNO_3$ were used. The highest frequency of shoot regeneration (25% in each) resulted at 2 μM ABA or 20 μM $AgNO_3$ treatment at rates 3.7 and 1.6 times control, respectively. Shoots developed mainly from the cotyledonary node on $CoCl_2$-containing medium, and from the upper side of cotyledon on medium containing $AgNO_3$, while the embryos on the control medium produced shoots from both the cotyledonary node and the upper region of cotyledons both at frequencies of 50%. Indirect somatic embryogenesis *via* callus was induced at an efficiency of approximately 10% with zygotic embryos 3~4 mm long cultured on MS medium containing 5~10 mg/L 2,4-D for 5~7 weeks under a continuous light condition. The plants regenerated from the somatic embryos were morphologically normal. Using scanning electron microscopy, the direct and indirect somatic embryogeneses were observed to follow the globular, heart and torpedo stages, similar to zygotic embryogenesis. Also, suspensors appeared in the early globular and ovoid-shaped late globular embryos during indirect somatic embryogenesis.

Keywords: pepper (*Capsicum annuum* L.), immature zygotic embryo, somatic embryogenesis, ethylene inhibitor, scanning electron microscopy

Red pepper (*Capsicum annuum* L.) is a very important vegetable in Korea and other countries. Breeding of pepper is regarded to be difficult because of its interspecific incompatibility and the infertility of the F_1 hybrid (Harini and Lakshimi Sita, 1993). Therefore, traditional breeding technology has had difficulty establishing new cultivars envincing desirable genetic characteristics. Such challenges may soon be overcome by introducing the desired genes through the recent technology of genetic transformation. In peppers, the establishment of a dependable plant regeneration system is essential. In peppers, studies on regeneration *via* organogenesis have been actively pursued (Gunay and Rao, 1978; Saxena *et al.*, 1981; Phillips and Hubstenberger, 1985; Diaz *et al.*, 1988; Agrawal *et al.*, 1989; Liu *et al.*, 1990; Valera-Montero and Ochoa-Alejo, 1992; Ezura *et al.*, 1993; Lee *et al.*, 1993; Christopher and Rajam, 1994), but studies on somatic embryogenesis are rare (Harini and Lakshmi Sita, 1993; Jeong *et al.*, 1994). Somatic embryogenesis is a unique pathway originating from a single cell. Therefore, genetic transformation *via* somatic embryogenesis can avoid undesirable chimeric plants. In the course of maturing somatic embryos, ABA proved to be an efficacious regulator in caraway (Ammirato, 1977), soybean (Ranch *et al.*, 1985), carrot (Kitto and Jan-

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ick, 1985; Arnold and Hakman, 1988), and *Picea abies* (Arnold and Hackman, 1988). In addition, $CoCl_2$ and $AgNO_3$ are also considered to have some positive effects on embryonal maturation (Elmo and Beyer, 1976; Purnhauser *et al.*, 1987; Songstad *et al.*, 1988; Roustan *et al.*, 1989, 1990).

In this experiment, we have established an efficient system for somatic embryogenesis in red pepper by determining: the optimal length and stage of the immature zygotic embryos used; the optimal concentration of 2,4-D; and the effects of ABA, $CoCl_2$ and $AgNO_3$. Morphological examination with a scanning electron microscope revealed that somatic embryogenesis had close similarity to zygotic embryogenesis.

MATERIALS AND METHODS

Plant materials

F_1 hybrid pepper plants (*Capsicum annuum* L. cv. Nokkwang) were grown in greenhouse at 25°C±5°C under natural light, and green fruits of various sizes ranging 5~10 cm in length were harvested 2~4 weeks after anthesis and used for the experiment.

Direct induction of somatic embryos from zygotic embryos

Green fruits were surface-sterilized in 70% (v/v) ethanol and then 25% (v/v) common household bleach. Immature zygotic embryos in lengths of 1~2, 3~4, and 5~7 mm were excised from the fruits to be used for the experiment. Basal medium for the induction of somatic embryos was modified MS medium (Murashige and Skoog, 1962) containing 1/2 strength Fe-EDTA, and 0.5, 1.0, 2.0, 4.0, 8.0, or 16.0 mg/L of 2,4-D as a growth regulator. The immature zygotic embryos were transplanted onto the modified MS-agar (0.8%) solid media, and cultivated at 25°C under darkness.

Maturation of somatic embryos

Clumps of somatic embryos induced from the combination of 1~2 mm long embryos and the 0.5 mg/L 2,4-D treatment were transplanted onto modified MS-Phytagel (0.4%) solid media containing 1, 2, or 4 μM ABA, and cultivated at 25°C under continuous light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) to mature the somatic embryos. After 2 weeks, the mature somatic embryos were transplanted onto germination media (MS media without any growth regulators). To examine the effects of $CoCl_2$ and $AgNO_3$ on the maturation of somatic embryos, globular shaped somatic embryos were transplanted onto maturation media containing 10, or 50 μM $CoCl_2$ and 10, 20, or 60 μM $AgNO_3$, respectively. $CoCl_2$ and $AgNO_3$-treated somatic embryos were incubated at 25°C under continuous light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 4 weeks, and then transplanted onto the germination media.

Indirect induction of somatic embryos *via* embryogenic calli from zygotic embryos

Immature zygotic embryos 3~4, or 5~7 mm length were excised and transplanted onto MS-Phytagel solid media containing 8 combinations of sucrose [3, 9% (w/v)] and 2,4-D (1, 5, 10, 20 mg/L), then incubated at 25°C under continuous light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for the induction of embryogenic callus. For the purpose of embryo induction and maturation, the MS-Phytagel solid media contained B5 vitamins, 1 g/L glutamine, and 0.5 μM ABA. The somatic embryos were induced and matured at 25°C under continuous light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The somatic embryos at the torpedo stage were transferred to 1/2 strength MS-Phytagel solid media containing 1/2 strength B5 vitamins, 1% sucrose, and 250 mg/L casein hydrolysate, then incubated at 25°C under continuous light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) until germination.

Scanning electron microscopy of somatic embryos

Somatic embryos were fixed by a double fixation method (prefixation in 5% glutaraldehyde and postfixation in 1% osmium tetroxide) in 20 mL glass vials. Fixed embryos were washed with 0.05 M phosphate buffer (pH 7.0), dehydrated through an ethanol series, and saturated with acetone. Acetone saturated samples were dried at room temperature then mounted on aluminum stubs. The stubs were coated with gold at 8 mA for 9 min (maximum thickness of 15 nm), then examined with a JSM 840A scanning electron microscope (JEOL, Japan) at 20 kV.

RESULTS

Direct induction of somatic embryos from zygotic embryos

Somatic embryos were produced from the apical and cotyledonary regions of zygotic embryos. The

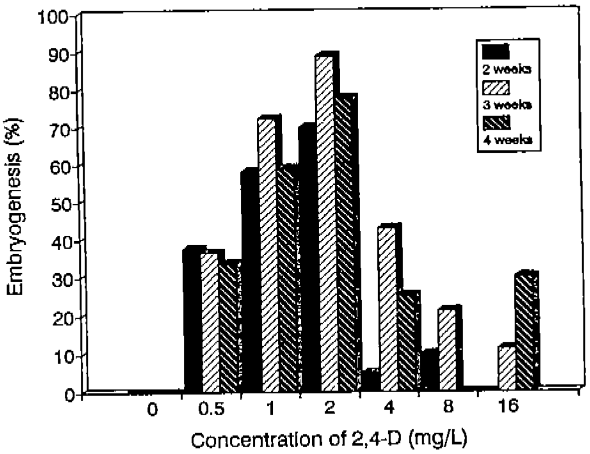

Fig. 1. Effects of 2,4-D concentration and embryo size on somatic embryogenesis (%) from apical region of immature zygotic embryos during various culture periods in pepper (*Capsicum annuum* L. cv. Nokkwang).

apical region of 1~2 mm long zygotic embryos showed the maximum embryogenecity of 88.9% after a 3 week incubation in the darkness (Fig. 1). But, most of those embryos did not develop normally if they did not pass the "maturation pathway" from the globular to the torpedo stages. Only torpedo-shaped somatic embryos developed to normal cotyledonary embryos (Fig. 2). Somatic embryos induced from the cotyledonary region of zygotic embryos generally had an amorphous, or fused, morphology therefore they could not develop into normal plants.

Maturation of somatic embryos

Shoot regeneration frequency was increased by the ABA treatment, and maximum efficiency (3.7 fold higher than control) was obtained at 2 μM ABA (Fig. 3). $AgNO_3$ was also effective in maturing somatic embryos, especially at the concentration of 20 μM. Shoot regeneration efficiency was slightly increased by $CoCl_2$ treatment, but optimal concentration range could not be determined. The region of shoot regeneration on the zygotic embryos varied according to whether they were treated with $CoCl_2$ or $AgNO_3$. In the case of $CoCl_2$, shoots formed on the cotyledonary node only (Figs. 4, 5), whereas $AgNO_3$ treatment changed the shoot primordial region from an even distribution to just the upper region of the cotyledon (Figs. 6, 7).

Indirect induction of somatic embryos *via* embryogenic calli from zygotic embryos

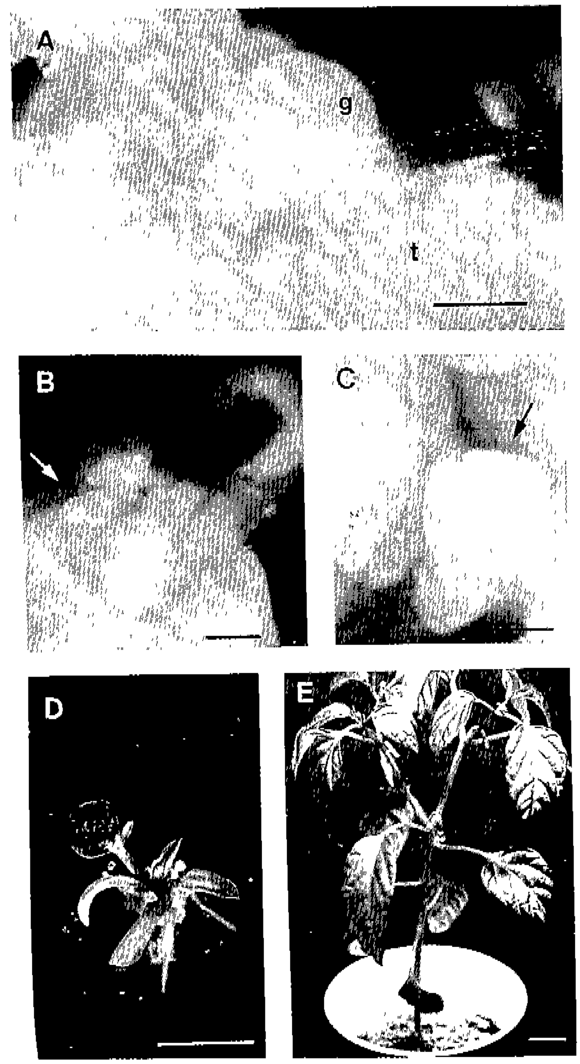

Fig. 2. Direct somatic embryogenesis and plant regeneration from apical region of immature zygotic embryo in pepper (*C. annuum* L. cv. Nokkwang). A, Somatic embryos at globular and torpedo stages were mixed. g, globular stage embryo: t, torpedo stage: B, Heart stage (arrow): C, Early torpedo stage (arrow): D, Germination of somatic embryo: E, Regenerated young plant with flowers. Bars in A, B, C indicate 0.5 mm and the other bars in D, E indicate 10 mm.

After a 5 week incubation on the treatment of 3% sucrose and 5 mg/L 2,4-D, globular somatic embryos were produced on the apical region of 3~4 mm long zygotic embryos. These globular embryos gradually changed to calli and finally turned to secondary embryos. In another case, secondary embryos appeared at a frequency of 9.1% after a 1 week incubation on media containing 0.5 μM ABA. These were 3~4 mm long zygotic embryos which

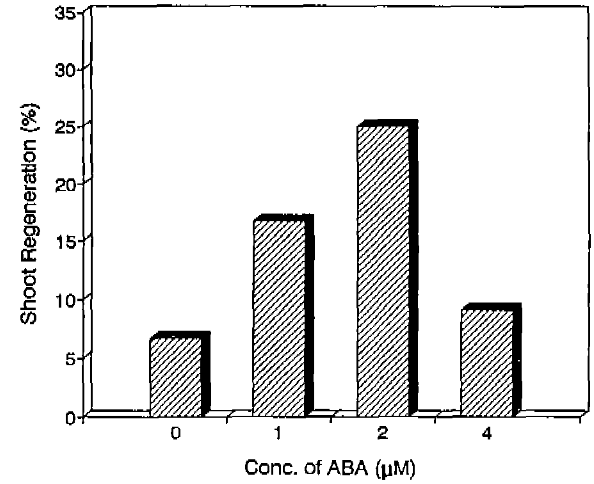

Fig. 3. Effect of ABA concentration on shoot regeneration (%) from the somatic embryos induced on modified MS medium supplemented with 0.5 mg/L 2,4-D, 3% sucrose and 0.3% Phytagel in pepper (*C. annuum* L. cv. Nokkwang). The basal medium used for maturation was modified MS medium supplemented with 3% sucrose, 0.4% Phytagel and 0.5% activated charcoal.

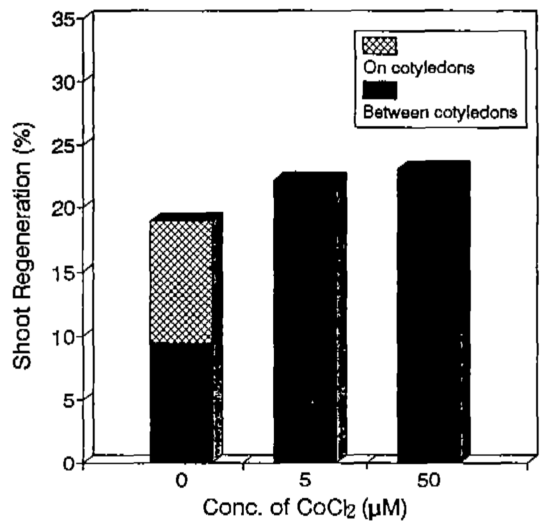

Fig. 4. Effect of $CoCl_2$ concentration on the frequencies (%) and the initial region of shoot development from the somatic embryos induced on MS medium supplemented with 2 mg/L 2,4-D, 3% sucrose and 0.3% Phytagel in pepper (*C. annuum* L. cv. Nokkwang). The frequency of shoot regeneration (%) was measured as [(numbers of shoot-forming explants)/(total numbers of explants)]. Shoots were developed on the upper side of the cotyledons, 'On cotyledons', or initiated from the cotyledonary nodes, 'Between cotyledons', in the control. The basal medium used for maturation was MS medium supplemented with 3% sucrose, 0.4% Phytagel and 0.5% activated charcoal.

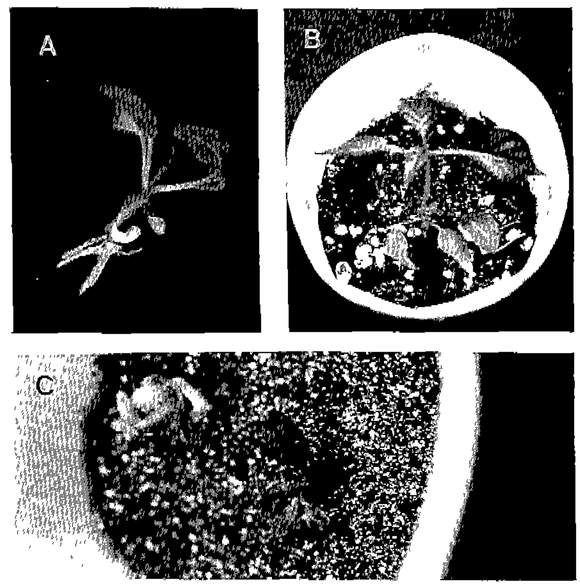

Fig. 5. Effect of $CoCl_2$ on the initial region of shoot development from the somatic embryo in pepper (*C. annuum* L. cv. Nokkwang). A, Control. (Note the shoot initiation on the cotyledon germinated from the somatic embryo): B, Shoot abnormally initiated in control (A) was growing vigorously in soil: C, 50 μM $CoCl_2$-treated somatic embryos normally developed shoots from the cotyledonary node.

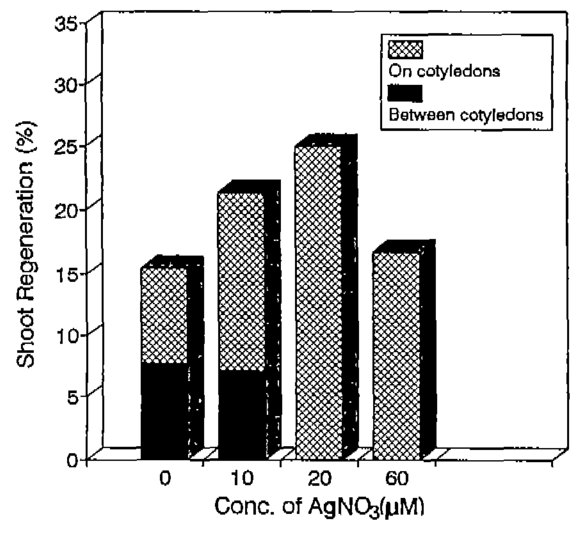

Fig. 6. Effect of $AgNO_3$ on the frequencies (%) and the initial region of shoot development from the somatic embryos induced on MS medium supplemented with 2 mg/L 2,4-D, 3% sucrose and 0.3% Phytagel in pepper (*C. annuum* L. cv. Nokkwang). Note the increasing concentration of $AgNO_3$ promoted the frequency of shoot initiation on the upper side of cotyledons, 'On cotyledons', to that from the cotyledonary nodes, 'Between cotyledons'. The basal medium used for maturation was MS medium supplemented with 3% sucrose, 0.4% Phytagel and 0.5% activated char-

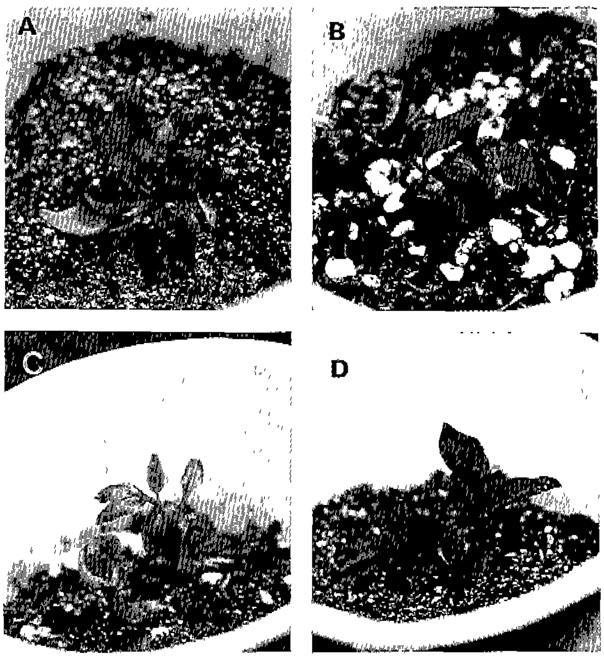

Fig. 7. Effect of $AgNO_3$ on the initial region of shoot development from the somatic embryo in pepper (*C. annuum* L. cv. Nokkwang). A, Normal development from the cotyledonary node from the 10 μM $AgNO_3$-treated somatic embryo: B and C, Abnormal initiation on the cotyledon germinated from the 10 μM and 20 μM $AgNO_3$-treated somatic embryos, respectively: D, 60 μM $AgNO_3$-treated somatic embryo showing vigorous shoot development on the cotyledons.

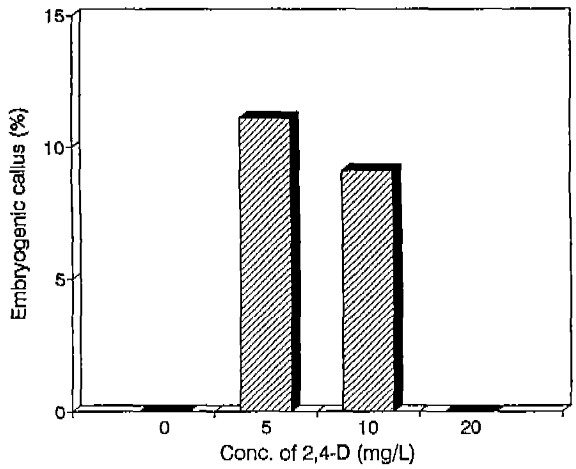

Fig. 8. Effect of 2,4-D concentration on the induction of embryogenic callus from the somatic embryo induced from immature zygotic embryo 3~4 mm long. The basal medium used was MS medium supplemented with 3% sucrose and 0.4% Phytagel. The frequency (%) of embryogenic callus induction was measured after 7 week-culture in the continuous light.

had been incubated for 6 weeks on embryo induction media containing 3% sucrose and 10 mg/L 2,4-D (Fig. 8). They could regenerate to normal plants without the aid of maturation media, and bore flowers (Fig. 9).

Scanning electron microscopy of somatic embryos

Somatic embryos induced directly from the apical region of zygotic embryos proved to follow the normal embryogenic pathway from the globular to the torpedo stage the same as zygotic embryos (Fig. 10). Indirect secondary somatic embryos from embryogenic calli seemed to be connected to the primordial tissue by suspensor-like structures (Fig. 11A, B), and some torpedo stage embryos were found to have radicle-like structures (Fig. 11C, D). In addition, ovoid embryos having suspensor-like structures appeared, seemingly an intermediate form of globular-to-heart stage. Both types of embryos, direct or indirect somatic embryos, appeared to have a more compact surface structure than the surrounding tissues (Fig. 11).

DISCUSSION

Immature zygotic embryos at the torpedo stage showed the greatest responsiveness in bell pepper (Harini and Lakshimi Sita, 1993), but heart or globular stage embryos did not show any viability cultured *in vitro*. This indicates that overly young zygotic embryos can not survive *in vitro*, therefore *in vitro* viability is the critical point for regeneration competence. The shoot primordial regions on zygotic embryos were confined to the apical or cotyledonary regions. And, according to the primordial region, the optimal 2,4-D concentration and the maximal frequency of shoot induction varied. It has already been reported that ABA has some positive effects on the maturation of somatic embryos (Ranch *et al.*, 1985). ABA is known to induce adventitious embryos or to inhibit premature germination of an immature embryo (Ammirato, 1977). In addition, it is proved to catalyze the accumulation of lipids essential to maturation and to strengthen the surface tissue of somatic embryos (Kitto and Janick, 1985; Arnold and Hakman, 1988). In this experiment, 2 μM ABA increased the frequency of shoot regeneration considerably (3.7 fold higher than control). Therefore, ABA proved to stimulate matura-

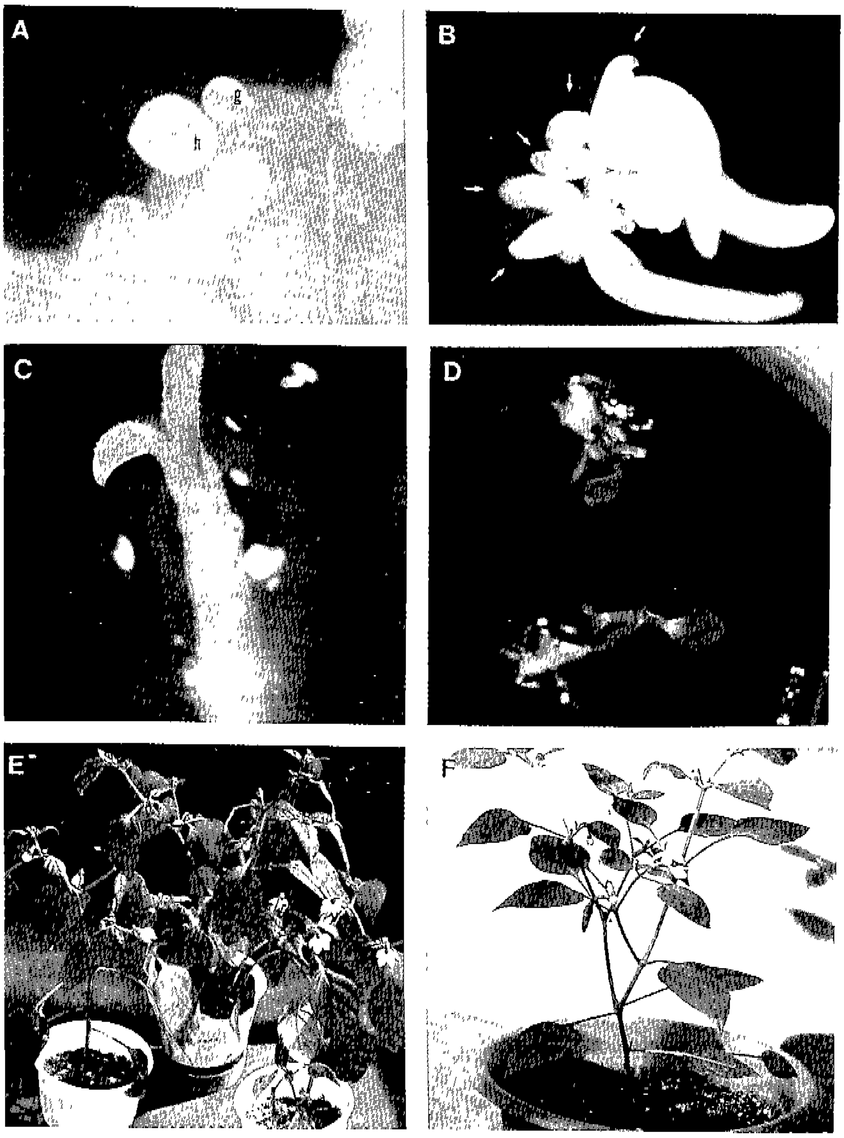

Fig. 9. Indirect somatic embryogenesis *via* callus and the plant regeneration in pepper (*C. annuum* L. cv. Nokkwang). A, The secondary somatic embryos at globular and early heart stage were produced from the surface of callus, which had been induced from the first somatic embryo at apical region of zygotic embryo. g, globular stage embryo: h, heart stage: B, Mass of somatic embryos on the surface of embryogenic callus (arrows): C, Torpedo stage somatic embryos (arrows) developed in bulk from the surface of embryogenic callus: D, Two cotyledonary somatic embryo: E, Germinated somatic embryos: F, Regenerated plants with flowers: G, Regenerated plant bearing green immature fruits.

tion of the somatic embryos in pepper as in other species. $CoCl_2$, as an inhibitor of ethylene biosynthesis, showed some effect on maturation, but did not induce distinguishable enhancement. All of the shoot primordial regions were confined to the cotyledonary node, whereas a non-$CoCl_2$ treatment showed an even distribution between the upper region of the cotyledon and cotyledonary node. But, considering that most of the somatic embryos from the upper region showed normal development, $CoCl_2$ seems to elicit a soundness in the somatic embryos of pepper. $AgNO_3$, as an inhibitor of ethylene action, showed distinguishable effects (frequency of shoot induction 1.6 fold higher than control). In contrast to $CoCl_2$ treatment, a gradual increase of $AgNO_3$ caused the shoot primordial region to migrate from an even distribution to just the upper region of cotyledon. This implies that ethylene moves from radicle to cotyledon through embryonal axis and negatively affects somatic embryogenesis. In the case

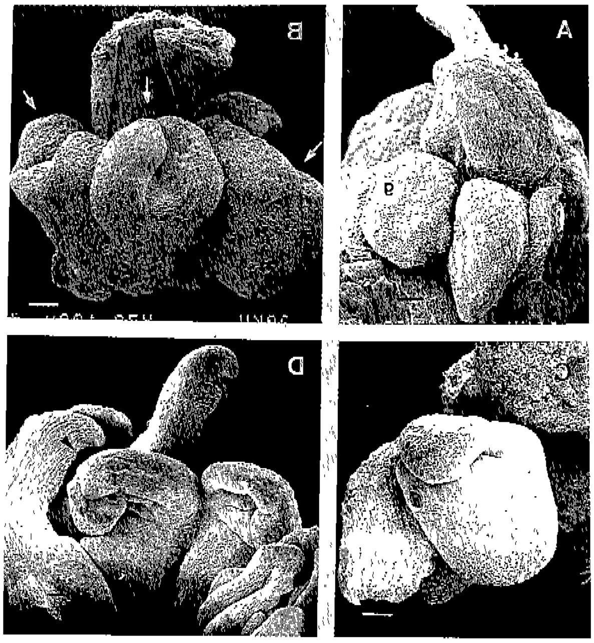

Fig. 10. SEM study on the direct somatic embryogenesis in pepper (*C. annuum* L. cv. Nokkwang). A, Globular somatic embryo (g): B, Heart-shaped somatic embryo (h) and three torpedo-shaped somatic embryos (arrow) developed at the apical region of the zygotic embryo: C, Asymmetrical growth of cotyledons in torpedo-shaped embryo: D, Late torpedo stage showing cotyledon bending (arrow head). Bars indicate 100 μm.

of indirect somatic embryogenesis *via* embryogenic calli, primarily induced somatic embryos turned to embryogenic calli, and then to much more secondary embryos than primary ones. Most of the secondary embryos passed through a normal embryogenic pathway as zygotic embryos do (Lindsey and Topping, 1993; Goldberg *et al.*, 1994). This means that secondary embryos can be proliferated successfully from confined primary embryos.

Morphological studies by scanning electron microscope indicated that both types of somatic embryos passed through the normal developmental pathway. Compactness of the surface structure compared to surrounding tissues was found in both types of somatic embryos. This means that surface compactness is closely related to the viability and morphogenic potential of tissue (Kitto and Janick, 1985). Indirect somatic embryos appeared to have identical features to zygotic embryos, that is, they were connected to surrounding tissues by suspensor-like structures. Suspensor-like structures appeared from ovoid somatic embryos to later cotyledonary stage, but they are known to be nonessential in other species

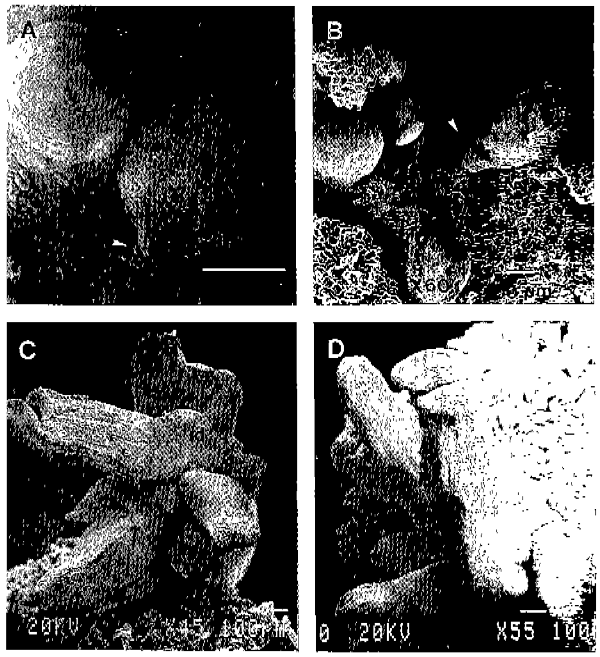

Fig. 11. SEM study on the indirect somatic embryogenesis *via* embryogenic callus induced from the apical region of zygotic embryos in pepper (*C. annuum* L. cv. Nokkwang). A, Early globular stage. Somatic embryos were round. Note the suspensor-like structure (arrow head) at bottom of embryo: B, Elongated and egg-shaped late globular embryo with suspensor-like structure (arrow head): C, Late torpedo-shaped embyos showing the formation of radicle (rd) and the initiation of cotyledon elongation. Note the mass of embryos grown from exterior of callus: D, Side view of the torpedo-shaped embryo (C). Bars indicate 100 μm.

(Halperin and Wetherell, 1964, Xu and Bewley, 1992; Zimmerman 1993). According to the former reports, exogenous auxin stimulates only the polarization of specific cells, but inhibits further embryogenic development (Schiavone and Cooke, 1987; Michalczuk *et al.*, 1992a, b). That is to say, embryo transition from the globular stage to the heart stage requires the removal of exogenous auxin. If auxin is removed, the gene expression involved in the morphogenesis of the heart stage embryo may be activated (Zimmerman, 1993). Therefore, the reason for the structural intactness of indirect somatic embryos may result from the effects of 2,4-D concentration, because secondary somatic embryos were induced on the media containing no 2,4-D. In conclusion, indirect somatic embryogenesis *via* embryogenic callus is more effective in pepper than a direct one. The addition of ABA, $CoCl_2$, $AgNO_3$, and the removal of auxins at the appropriate time are the critical points in pepper somatic embryogenesis. For a more efficient induction of embryogenic calli, we are trying to screen several carbon sources.

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LITERATURE CITED

Agrawal, S., N. Chandra and S.L. Kothari. 1989. Plant regeneration in tissue cultures of pepper (*Capsicum annuum* L.). *Plant Cell Tiss. Org. Cult.* **16**: 47-55.

Ammirato, P.V. 1977. Hormonal control of somatic embryo development from cultured cells of caraway (*Carum carvi* L.). *Plant Physiol.* **59**: 579-586.

Arnold, S.V. and I. Hakman. 1988. Regulation of somatic embryo development in *Picea abies* by abscisic acid. *J. Plant Physiol.* **132**: 164-169.

Christopher, T. and M.V. Rajam. 1994. *In vitro* clonal propagation of *Capsicum* spp. *Plant Cell Tiss. Org. Cult.* **38**: 25-29.

Diaz, L., R. Moreno and J.B. Power. 1988. Plant regeneration from protoplasts of *Capsicum annuum. Plant Cell Rep.* **7**: 210-212.

Elmo, M. and Jr. Beyer. 1976. A potent inhibitor of ethylene action in plants. *Plant Physiol.* **58**: 268-271.

Ezura, H., S.S. Nishimiya and M. Kasumi. 1993. Efficient regeneration of plants independent of exogenous growth regulators in bell pepper (*Capsicum annuum* L.). *Plant Cell Rep.* **12**: 676-680.

Goldberg, R.B., G. de Paiva and R. Yadegari. 1994. Plant embryogenesis: Zygote to seed. *Science* **266**: 605-614.

Gunay, A.L. and P.S. Rao. 1978. *In vitro* plant regeneration from hypocotyl and cotyledon explants of red pepper *Capsicum. Plant Sci. Lett.* **11**: 365-372.

Halperin, W. and D.F. Wetherell. 1964. Adventive embryony in tissue cultures of wild carrot, *Daucus carota. Am. J. Bot.* **512**: 274-283.

Harini, I. and G. Lakshmi Sita. 1993. Direct somatic embryogenesis and plant regeneration from immature embryos of chilli (*Capsicum annuum* L). *Plant Sci.* **89**: 107-112.

Jeong, W.J., S.R. Min, J.R. Liu, Y.J. Park and K.W. Cho. 1994. Somatic embryogenesis and plant regeneration in immature zygotic embryo cultures of hot pepper (*Capsicum annuum* L.). *Korean J. Plant Tissue Cult.* **21**: 299-302.

Kitto, S.L. and J. Janick. 1985. Hardening treatments increase survival of synthetically-coated asexual embryos of carrot. *J. Amer. Soc. Hort. Sci.* **110**: 283-286.

Lee, S.J., B.D. Kim and K.H. Paek. 1993. *In vitro* plant regeneration and *Agrobacterium*-mediated transformation from cotyledon explants of hot pepper (*Capsicum annuum* L. cv. Golden Tower). *Korean J. Plant Tissue Cult.* **20**: 289-294.

Lindsey, K. and J.F. Topping. 1993. Embryogenesis: A question of pattern. *J. Exp. Bot.* **44**: 359-374.

Liu, W., W.A. Parrott, D.F. Hildebrand, G.B. Collins and E.G. Williams. 1990. *Agrobacterium*-induced gall formation in bell pepper (*Capsicum annuum* L.) and formation of shoot-like structures expressing introduced genes. *Plant Cell Rep.* **9**: 360-364.

Michalczuk, L., T.J. Cooke and J.D. Cohen. 1992a. Auxin levels at different stages of carrot somatic embryogenesis. *Phytochem.* **31**: 1097-1103.

Michalczuk, L., D.M. Ribnicky, T.J. Cooke and J.D. Cohen. 1992b. Regulation of indole-3-acetic acid biosynthetic pathways in carrot cell cultures. *Plant Physiol.* **100**: 1346-1353.

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

Phillips, G.C. and J.F. Hubstenberger. 1985. Organogenesis in pepper tissue culture. *Plant Cell Tiss. Org. Cult.* **4**: 261-269.

Purnhauser, L., P. Medgyesy, M. Czakó, P.J. Dix and L. Márton. 1987. Stimulation of shoot regeneration in *Triticum aestivum* and *Nicotiana plumbaginifolia* Viv. tissue cultures using the ethylene inhibitor $AgNO_3$. *Plant Cell Rep.* **6**: 1-4.

Ranch, J.P., L. Oglesby and A.C. Zielinski. 1985. Plant regeneration from embryo-derived tissue cultures of soybeans. *In Vitro* **21**: 653-658.

Roustan, J.P., A. Latche and J. Fallot. 1989. Stimulation of *Daucus carota* somatic embryogenesis by inhibitors of ethylene synthesis: Cobalt and nickel. *Plant Cell Rep.* **8**: 182-185.

Roustan, J.P., A. Latche and J. Fallot. 1990. Control of carrot somatic embryogenesis by $AgNO_3$, an inhibitor of ethylene action: Effect on arginine decarboxylase activity. *Plant Sci.* **67**: 89-95.

Saxena, P.K., R. Gill, A. Rashid and S.C. Maheshwari. 1981. Isolation and culture of protoplasts of *Capsicum annuum* L. and their regeneration into plants flowering *in vitro. Protoplasma* **108**: 357-360.

Schiavone, F.M. and T.J. Cooke. 1987. Unusual patterns of somatic embryogenesis in the domesticated carrot: Developmental effects of exogenous auxins and auxin transport inhibitors. *Cell Differ.* **21**: 53-62.

Songstad, D.D., D.R. Duncan and J.M. Widholm. 1988. Effect of 1-aminocyclopropane-1-carboxylic acid, silver nitrate, and norbornadiene on plant regeneration from maize callus cultures. *Plant Cell Rep.* **7**: 262-265.

Valera-Montero, L.L. and N. Ochoa-Alejo. 1992. A novel approach for chilli pepper (*Capsicum annuum* L.) plant regeneration: Shoot induction in rooted hypocotyls. *Plant Sci.* **84**: 215-219.

Xu, N., K.M. Coulter and J.D. Bewley. 1990. Abscisic acid and osmoticum prevent germination of developing alfalfa embryos, but only osmoticum maintains the synthesis of developmental proteins. *Planta*

182: 382-390.
Xu, N. and J.D. Bewley. 1992. Contrasting pattern of somatic and zygotic embryo development in alfalfa (*Medicago sativa* L.) as revealed by scanning electron microscopy. *Plant Cell Rep.* **11**: 279-284.
Zimmerman, J.L. 1993. Somatic embryogenesis: A model for early development in higher plants. *Plant Cell* **5**: 1411-1423.

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