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Effect of Ammonium Ion on Morphogenesis from Cultured Cotyledon Explants of *Panax ginseng*

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Cotyledon explants of *Panax ginseng* were cultured on modified Murashige and Skoog medium with various concentrations of NH_4Cl and KNO_3. Morphogenesis such as somatic embryo, embryogenic callus, or adventitious root formation from cotyledon explants differently occurred according to the concentrations of NH_4^+ and NO_3^-. Somatic embryos were actively formed in a moderate concentration of NH_4^+ (20 mM) in combination of NO_3^-, but in a high concentration of NH_4^+ (60 mM), only embryogenic calli were formed. In little or no NH_4^+, adventitious roots were formed at a high rate. The influence of NO_3^- on those morphogenesis was slight but combination of NO_3^- with NH_4^+ was indispensable since the cotyledon explants were necrotized on medium containing only NH_4^+ as a nitrogen source. Histological observation revealed that somatic embryo and embryogenic callus formation occurred from the same origin (cotyledon epidermis), whereas, adventitious roots were originated from the cells near vascular strands.

Keywords: *Panax ginseng*, ammonium ion, somatic embryo, embryogenic callus, adventitious root

Panax ginseng is one of the most important medicinal plants. However, cultivation of ginseng is troublesome, since over 4 years of cultivation period require for seed harvest. Thus, tissue culture technology might be valuable for masspropagation. However, it has been known that plant regeneration from tissue culture of *Panax ginseng* were very recalcitrant (Chang and Hsing 1980; Arya *et al.* 1993). Interestingly, cotyledon explants of *Panax ginseng* can produce somatic embryos directly on medium without growth regulators (Choi and Soh, 1996), normal plant regeneration from those somatic embryos of ginseng was accomplished (Choi *et al.*, 1996). Plant regeneration on growth regulator-free medium may be useful for a regeneration system, since somaclonal variation is minimized. However, the embryogenesis using that culture system is likely to occur just temporarily from the cultured tissue, since growth regulators are omitted on the medium. Thus, regulation of somatic embryogenesis was the main obstacle.

It was reported that embryogenic callus of ginseng contains useful ginsenoside (Asaka, *et al.*, 1993a). Embryogenic callus production on growth regulator-free medium will have great advantage of not being contaminated with undesirable substance like synthetic growth regulators. Asaka *et al.* (1993a, b) induced the embryogenic tissues from multiple shoots of *Panax ginseng* by moderate high temperature treatment and produced the ginsenoside by culturing embryogenic tissues in bioreactors. By this method, edible powders of extract from ginseng embryogenic tissues was manufactured commercially. It is noteworthy for scientific and industrial application if continuous production of somatic embryos or embryogenic callus can be achieved on medium without growth regulators.

Besides the growth regulators, there are some reports that the reduced nitrogen compounds can regulate morphogenesis in plant tissue culture. In carrot and alfalfa embryogenic cell suspension culture, high concentration of NH_4^+ stimulated the somatic embryogenesis but low concentration of NH_4^+ promoted adventitious root formation (Halperin 1966; Walker and Sato, 1981). However, it has been no report that regulation of somatic embryogenesis was achieved by a solitary nitrogen treatment without the

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help of growth regulators.

In the present experiment, we describe that the regulation of morphogenesis (somatic embryo, embryogenic callus and adventitious root formation) according to the concentrations and combinations of NH_4Cl and KNO_3, and the way for continuous production of embryogenic callus on medium lacking growth regulators.

MATERIALS AND METHODS

Korean ginseng (*Panax ginseng* C. A. Meyer) seeds were collected from experimental garden of the Korea Ginseng and Tobacco Research Institute. The seeds were stratified to mature in humidified sand for three months at a 10°C temperature since the zygotic embryos just after harvest were in an immature globular stage (at about 200 μm in length). After stratification, the seeds were kept in 4°C refrigerator for dormancy breaking. When zygotic embryos in seeds were matured into 4 mm in length, the seeds were immersed in 70% alcohol for 1 minute, sterilized in 1% sodium hypochlorite solution for one hour, and then rinsed three times with sterile distilled water. Cotyledon explants were cut from zygotic embryos and their adaxial surface were placed on the medium plane. All the components of the medium were the same as MS basal medium (Murashige and Skoog, 1962) except for the nitrogen source; 20 mM NH_4NO_3 was replaced by 4, 20 or 60 mM NH_4Cl; and the 19 mM KNO_3 was modified to 3.8 mM, 19 mM or 56 mM KNO_3. These were added to the medium independently or in varied combinations. The medium contained 3% sucrose and 0.7% agar and adjusted to pH 5.8 before autoclaving at 120°C for 15 minutes. Cultures were performed using 10×1 cm glass Petri dishes containing 30 ml of medium. The culture room was maintained at 24±2°C with a 16 hours illumination using a 1900 lux cool white fluorescent bulb. The frequency of somatic embryo, embryogenic callus and adventitious root formation was evaluated by counting cotyledon explants showing morphogenesis from the total number of cultured explants. Thirty explants were cultured in each experiment which was repeated three times.

The embryogenic callus was formed from near the basal excised portion of cotyledon on medium containing 60 mg/l NH_4Cl and 19-56 mg/l KNO_3, subcultured on the same medium as at culture initiation and maintained for over one years. To induce somatic embryos, embryogenic callus was pre-incubated on MS basal medium for different culture periods (from 2 to 10 weeks) and then subcultured on the same new medium. After two months of culture, development of somatic embryos from embryogenic callus was observed. Culture condition and culture vessel was the same to somatic embryo induction as mentioned above.

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

RESULTS

In nitrogen-free medium, the cotyledon explants slowly turned to brown. Cotyledon explants rapidly necrotized with dark brown color on medium containing 4, 20, and 60 mM NH_4Cl as the sole nitrogen source (Fig. 1D). Formation of somatic embryos, embryogenic callus and adventitious roots occurred according to the concentrations of NH_4Cl and KNO_3 shown in Table 1. These morphogenesis was highly influenced by the concentration of NH_4Cl. KNO_3 shows no considerable effect on morphogenesis. But addition of KNO_3 was essential, since cotyledon explants were necrotized on medium containing NH_4Cl as a sole nitrogen source (Fig. 1D). High frequency of somatic embryos was formed on medium containing 20 mM NH_4Cl and 19 mM KNO_3, in which the level of NH_4^+ was the same that of MS medium (Table 1). When these somatic embryos (Fig. 1A) were transferred to MS medium containing 10 mg/l GA_3, the embryos were matured into cotyledonary stage (Fig. 1H). In a higher concentration of NH_4Cl (60 mM), amorphous embryogenic tissues were formed (Fig. 1B, 2D), somatic embryo development from these tissue was not proceed, eventually resulting into embryogenic callus formation (Fig. 1B). Embryogenic callus was also formed when the concentration of KNO_3 was relatively low in comparison to NH_4Cl although the concentration of NH_4Cl was not notably high (20 mM).

In the culture of cotyledon explants on medium containing KNO_3 as the sole nitrogen source, adventitious roots formed at a high rate (Fig. 1C, Table 1). Adventitious roots and somatic embryos were formed on the same explants (Fig. 2B) or separately (Fig. 2C). In a low concentration of ammonium, maturation of somatic embryos was fast although the frequency of somatic embryos formation was low. A

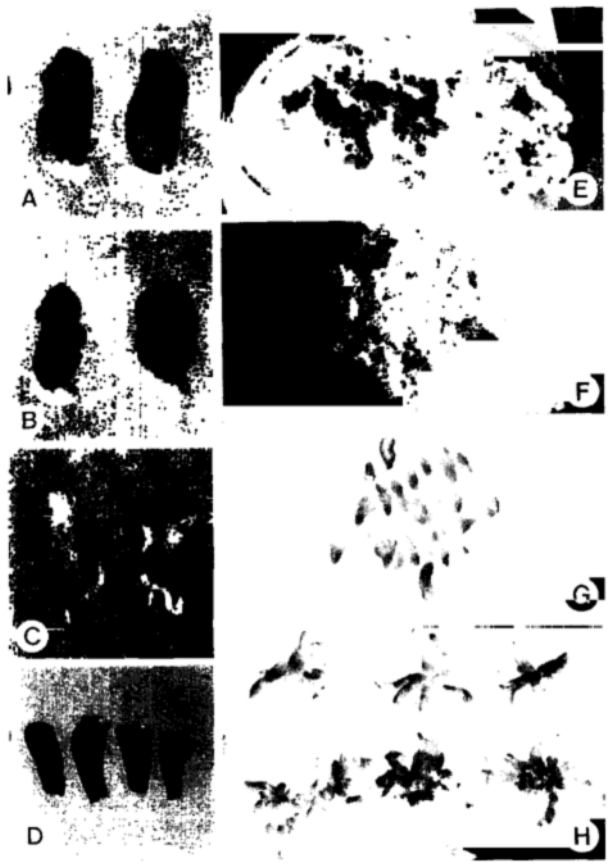

Fig. 1. Somatic embryo, embryogenic callus and adventitious root formation from cotyledon explants cultured on medium with various concentration of NH_4Cl and KNO_3 after 5 weeks of culture. A: somatic embryos (arrows) formed from cotyledon explants on medium containing 20 mM NH_4Cl and 19 mM KNO_3. B: embryogenic callus (arrows) formed on medium containing 60 mM NH_4Cl and 56 mM KNO_3. C: adventitious roots (arrows) formed on medium containing 3.8 mM KNO_3. D: necrotized cotyledon segments in medium containing NH_4Cl as sole nitrogen source. E: embryogenic callus maintained on medium containing 60 mM NH_4Cl and 19 mM KNO_3. F: embryogenic callus mixed with early globular embryos maintained by two weeks subculture on MS basal medium. G: cotyledonary somatic embryos (arrows) formed from aged embryogenic callus for 10 weeks on MS basal medium. H: cotyledonary somatic embryos on MS medium containing 10 mg/l GA_3.

low level of NH_4Cl (4 mM) did not hamper the production of adventitious roots but a high level of NH_4Cl (20 mM), as well as KNO_3 (60 mM) highly suppressed root formation (Table 1).

Histological observation revealed that the portions of cotyledons forming somatic embryos and embryogenic callus were equal (Fig. 2A, D). Both occurred on the epidermal cells near the basal excised

Table 1. Effects of NH_4^+ and NO_3^- on the formation of somatic embryos, embryogenic callus, and adventitious roots from cotyledon explants of *Panax ginseng* after 5 weeks of culture

Nitrogen source		Somatic embryo formation(%)	Embryogenic callus formation(%)	Adventitious root formation(%)
$NH_4Cl+KNO_3$ (mM)				
0	0	0	0	0
4	0	0	0	0
20	0	0	0	0
60	0	0	0	0
0	3.8	15±2.3[a]	0	55±4.7
0	19	15±3.5	0	8±2.8
0	56	20±2.5	0	0
4	3.8	43±2.5	0	40±5.6
4	19	42±5.3	0	3±1.2
20	3.8	0	33±4.6	0
20	19	76±5.6	0	0
20	56	68±7.7	0	0
60	19	0	65±5.1	0
60	56	0	84±7.2	0

[a]Data represent the mean values±S.D. from three independent experiments.

portion of cotyledon explants. However, the origin of adventitious roots was different from that of the somatic embryos or embryogenic callus since the roots occurred near the procambial strands of the cotyledon base (Fig. 2B, C).

As mentioned above, embryogenic callus formed on medium containing 60 mM NH_4Cl in combination with 19 or 56 mM KNO_3, it showed opaque white, and was friable (Table 1, Fig. 1B). After separation of this callus from parent cotyledon explants, the callus was subcultured on the same medium as callus initiation. Proliferation of callus achieved and maintained by one month of subculture (Fig. 1E). When the callus were optimally maintained, they show a 5 to 7 fold increase in fresh weigth on growth regulator-free MS medium after one month of culture. The embryogenic callus was maintained for over one year. Once the embryogenic callus were maintained, the embryogenic callus was also actively proliferated on growth regulator-free MS basal medium without ammonium modification (Fig. 1F). To investigate whether the callus maintained for one year was habituated nonmorphogenic callus or still retained embryogenic potentials, callus was transferred to medium containing a low concentrations of 4 mM NH_4Cl and 3.8 mM KNO_3, which concentration fostered the maturation of somatic embryos in the culture of cotyledon explants. But on this medium, the callus became transparent, mucil-

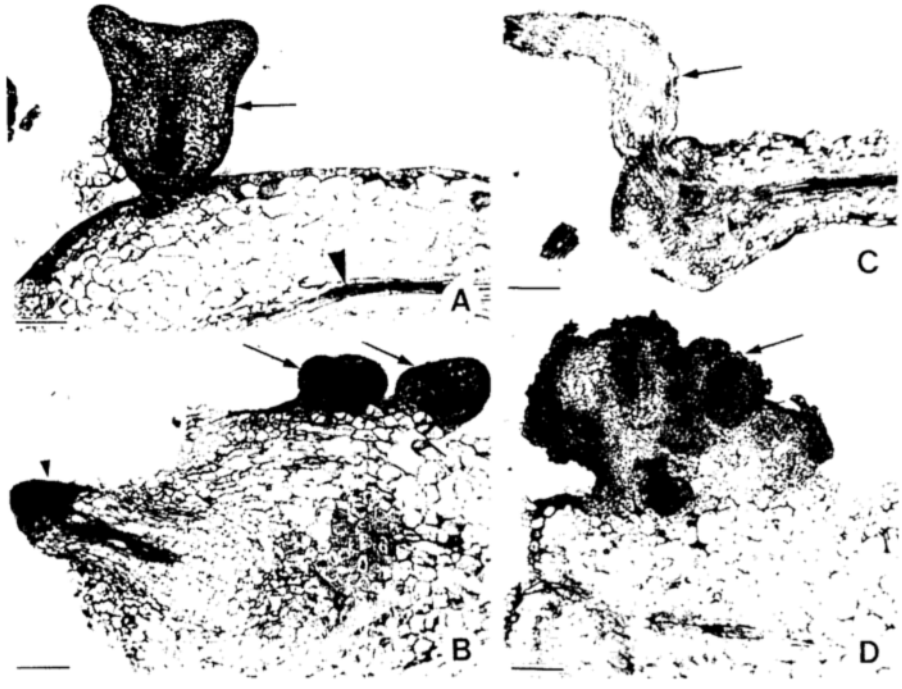

Fig. 2. Median longitudinal sections of cotyledon explants having somatic embryos, embryogenic callus, and adventitious roots. A: somatic embryos (arrow) formed from epidermal cells of cotyledon base cultured on medium containing 20 mM NH_4Cl and 19 mM KNO_3, arrowhead indicating procambial strends (Bar: 230 μm). B: both somatic embryo (arrows) and adventitious root (arrowhead) formed from a cotyledon explants on medium containing 4 mM NH_4Cl and 3.8 mM KNO_3 (Bar: 460 μm). C: adventitious root (arrow) formed from mesophyl cells near vascular strand (arrowhead) on medium containing 3.8 mM KNO_3 (Bar: 230 μm). D: embryogenic callus (arrow) formed from the surface of cotyledon base on medium containing 60 mM NH_4Cl and 56 mM KNO_3 (Bar: 230 μm).

aginous, from which further somatic embryogenesis was not observed (data not shown). Somatic embryo development from callus was achieved by the aging of callus. After the callus was preincubated in growth regulator-free MS basal medium for 1 to 5 months, then the callus was transferred to the fresh same MS basal medium. When embryogenic callus was sustained for over 3 months without transferring to fresh medium, somatic embryos were developed from the callus, although many of them had structures without an intact epidermis or, were slightly callused (Fig. 1G).

DISCUSSION

Cotyledon explants were necrotized on medium containing ammonium as the sole nitrogen source. But in combination with nitrate, ammonium toxicity was not observed. This indicates that the nitrate combination makes up for the detrimental effect of ammonium. It has been reported that ammonium toxicity in medium containing ammonium as the sole nitrogen source results from problems of NH_4^+ ion transport (Gamborg and Shyluk, 1970), assimilation (Behrend and Mateles, 1976) and acidification of the medium (Dougall and Verma, 1978).

In ginseng cotyledon culture, the requirement of ammonium ion for somatic embryo and adventitious root formation was highly different. A high level of ammonium fostered the formation of somatic embryos and embryogenic calli but a low level of ammonium stimulated the formation of adventitious roots. Similar results has been reported that the presence of reduced nitrogen compounds was highly effective for somatic embryogenesis (Wetherell and Dougall, 1976). In suspension cultures of carrot (Halperin, 1966), adventitious root and somatic embryo formation were observed as alternative morphogenetic events depending on the concentration of ammonium. In the present experiment, regulation of somatic embryogenesis by the nitrogen treatment has special importance, since the regulation of morphogenesis from ginseng cotyledons was achieved on medium lacking growth regulators.

In the present experiment, maturation of somatic embryos from ginseng cotyledon explants was influenced by the level of ammonium ion. In a low or morderate concentration of ammonium, maturation of somatic embryos was normally proceed, but in high concentration of ammonium, only embryogenic callus formation was achieved. This indicates that high concentration of ammonium salts suppressed the maturation of somatic embryos but fostered the proliferation of embryogenic cells. When the embryogenic callus were subcultured repetitively on the fresh same medium, the callus was proliferated continuously for over one year. This culture system can be applied to a new method for embryogenic callus production on medium lacking growth regulators. Generally callus maintained on growth regulator-free medium shows habituated characteristics which do not have morphogenic potential (Bhojwani and Raazdan, 1983; Meins, 1981). However, the ginseng embryogenic callus maintained in this experiment can produce somatic embryos, although the frequency of somatic embryo formation from the maintained ginseng embryogenic callus was very low. A similar result was observed in carrot zygotic embryo culture, that proembryogenic cell clumps were formed and maintained without losing of embryogenic competency in medium containing 1~5 mM ammonium as the sole nitrogen source (Smith and Krikorian, 1989).

In ginseng, callus production by tissue culture can be special importance, since ginseng callus also contains useful ginsenoside. In addition, callus production on growth regulator-free medium will have better advantage of not being contaminated with any toxic or undesirable substance like synthetic growth regulators. Asaka *et al.* (1993a,b) induced the somatic embryos from *in vitro* multiple shoots of *Panax ginseng* by moderate high temperature treatment in the prescence of kinetin, and these ginseng embryogenic tissue had been used as an industrial source of ginseng saponins and a ginseng extract. In the present experiment, high level of ammonium salt treatment can be a new methods for production of ginseng callus on growth regulator-free medium.

In the present experiment, the tissue forming somatic embryos and adventitious roots were different although the portion forming morphogenesis was toward the basal cut end. Somatic embryos formed from epidermal cells while adventitious root formation occurred from near the procambial strands. In carrot cell suspension culture (Halperin, 1966), somatic embryogenesis occurred from the surface of cell clumps but adventitious root formation occurred from the cells within the cell clumps. These results might indicate that there is different morphogenic potential between the inside and outside cells of cotyledon tissues or cell clumps.

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