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Effect of Humidity on Somatic Embryogenesis in Cotyledon Explant Culture of Carrot

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In order to clarify the influence of low humidity culture on the structure and function of somatic embryo the cotyledon explants of *Daucus carrota* L. cv. Hongshim were cultured in the petridish whose lids had holes sealed with millipore filters. In the low humidity culture, the production of somatic embryos was enhanced and their maturation promoted but the cotyledon structure of somatic embryos were nearly similar to control. In addition, the low humidity culture improved the germination of somatic embryos. Especially, the germination frequency of jar-shaped embryos was much improved (68%) in comparison with that of jar-shaped embryos formed in constant humidity culture (23%). But low humidity culture at its extreme became an obstacle to normal plant regeneration in that precocious embryos were generated and the primary embryos turned into callus and formed secondary embryos. Therefore it is suggested that moderately low humidity culture (80-90% R.H.) is important to the higher production and better-quality of somatic embryos.

Keywords: low humidity, embryo production frequency, *Daucus carrota*

The production of somatic embryos has contributed to the development of plant embryology and synthetic seed technology and has been considered as being potentially identical with zygotic embryos. But, the structure of somatic embryos obtained from plant cells and tissue culture shows considerable differences from those of zygotic embryos (Ammirato *et al.*, 1971; Buchheim *et al.*, 1989; Soh, 1993; Choi *et al.*, 1994; Cho and Soh, 1995; Soh *et al.*, 1996). The abnormal somatic embryos show differences from normal somatic embryos not only in their structure, but also in their plant regeneration frequency (Soh, 1993; Wetzstein *et al.*, 1993; Cho and Soh, 1995; Soh *et al.*, 1996).

In *Daucus carota* and *Bupleurum falcatum* the abnormal somatic embryos either did not regenerate to plants or had low regeneration frequencies, which were in inverse proportion to their number of cotyledons (Cho and Soh, 1995; Soh *et al.*, 1996). In the case of *Aralia cordata*, however, a contrary result was reported that there was a proportional relationship between the regeneration frequency of the abnormal somatic embryos and the number of cotyledons (Lee, 1993). Thus the abnormality of somatic embryos is closely linked to the germination frequency, and remains an obstacle to plant regeneration from somatic embryos.

An efficacious method of plant regeneration was documented whereby water stress was applied in the process of somatic embryogenesis (Kermode 1985; Roberts *et al.*, 1991; Burns *et al.*, 1995). Since a protein associated with tolerance to aridity was synthesized in celery somatic embryos and those embryos showed a high plant regeneration frequency after a partial desiccation (Yehoshua *et al.*, 1992; Kazuko *et al.*, 1994), it has been accepted that partial desiccation may hold a key to more successful for production of synthetic seeds (Kermode, 1990). This study attempts to contribute to the enhancement of the production and regeneration of somatic embryos in a low humidity environment.

MATERIALS AND METHODS

The seeds of *Daucus carota* L. cv. Hongshim ochon were first sterilized in 70% ethanol for 1 min, with sodium hypochlorite solution for 15 min, then rinsed

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3 or 4 times with sterile distilled water. The seeds was aseptically germinated in half-strength MS basal medium (Murashige and Skoog, 1962) for 7 days. Callus was induced from cotyledon explants of *in vitro* seedlings. Explants were cultured in the petridish (87 mmφ × 15 mm), each contaning 25 mL of callus induction medium consisting of MS basal medium supplemented with 1 mg · L^{-1} 2,4-D, sucrose at 30 g · L^{-1} and agar at 8 g · L^{-1}. The pH was adjusted to 5.8 prior to autoclaving at 121°C and 1.2 kg · cm^{-2} for 15 min. The cultures were maintained in darkness at 25 ± 1°C for 3 weeks. Cultured petridish were sealed with parafilm.

After embryogenic calli appeared from the explants were transferred to MS basal medium for somatic embryogenesis. For the culture of embryogenic calli in low humidity, ventilation was provided by a hole of 1 cm in diameter in each petridish lid covered with a millipore filter (pore size: 0.45 μm, Millipore Corporation, Bedford, MA, U.S.A.). The low humidity cultures were performed for semi-weekly, weekly, or for two, three or four weeks. Then, they were transferred back to 2,4-D free MS medium in petridish entirely sealed with parafilm where they were observed for the development frequency of somatic embryos.

The somatic embryos were germinated on solid half-strength MS basal media and cultured at 25°C under a photoperiod of 16 h with 46 μmol · s^{-1} · m^{-2} cool white fluorescent light. Germination was confirmed by the appearance of the primary leaf after rooting and germination frequency was measured 4 weeks later. The characteristics of at least 100 somatic embryos were observed in each trial which was repeated 3 times and germination frequency was acquired by calculating 50 somatic embryos, according to the number of cotyledons, for each of the treatment.

The water content of the somatic embryos was examined by measuring the fresh and dry weights of the cotyledonary embryos formed after the application of low humidity stress in the ventilative culture. The relative humidity in the petridishes was measured from semi-week to 4 weeks after transferring the explants with embryogenic callus to 2,4-D free medium using a Humicap humidity sensor, HMJ 32 (VAISALA, Helsinki, Finland). The sensor was quickly attached to a small plastic tube (1 cm × 4 cm) with 2 holes in its wall which had been placed on the culture medium within the petridishes for at least 3 days.

RESULTS

Relative humidity during culture

Table 1. Relative humidity (%) in space of untreated and ventilated culture vessels at different culture period and after ventilative culture water content (%) of fresh somatic embryos during somatic embryogenesis of *Daucus carota*

Treatment	Culture period (week)			
	1/2	1	2	4
Control	**94.0**-97.0	**95.6**-97.0	**97.0**-97.0	**97.0**-97.0
Ventilated	**90.0**-88.0	**85.3**-86.5	**80.3**-86.0	**67.6**-84.0

The relative humidity (bold number) in petridishes was measured from semi-week to 4 weeks after transferring the explants with embryogenic callus to 2,4-D free medium. Ventilated culture was conducted in petridish with a hole (1 cm) on lid sealed with a millipore filter.

The relative humidity in petridishes sealed with parafilm was higher than that in petridishes with a ventilative hole (Table 1), and remained nearly constant throughout culture. However the humidity in petridishes with ventilative holes decreased gradually after ventilation treatment. The water content of cotyledonary somatic embryos obtained from embryogenic calli on 2,4-D free medium was measured. The control group showed a constant water content of 97% throughout the culturing period. Embryos cultured in ventilated petridishes showed reduced water contents of 86% and 84% after 2 and 4 weeks of culture, respectively (Table 1). Therefore it was identified that the degree of humidity was lower in the ventilative culture compared to control.

Enhanced production of somatic embryos

When embryogenic calli were transferred on MS basal medium 20 embryos were produced in each dish. However, during the low humidity culture for a certain period of time, somatic embryos increased from 5 to 10 times compared to the control group (Fig. 1): 4-day culture produced 10 times as many somatic embryos as the control group, 1- and 2-week cultures, 7 and 5 times, but in 3- and 4-week cultures, 1/2 and 1/3 times (Fig. 1). Therefore, embryogenesis was fairly enhanced under a moderately low humidity culture, but the production of somatic embryos was severely inhibited under an extremely low humidity culture.

Cotyledon structure of somatic embryos

The somatic embryos of carrot, a dicotyledonous plant, showed considerable variation in the number of cotyledons (Fig. 2). In nonventilative cultures (control), the frequency of normal somatic embryos with 2 cotyledons was 44%. Among the abnormal embryos (56%), somatic embryos with a single cotyledon had

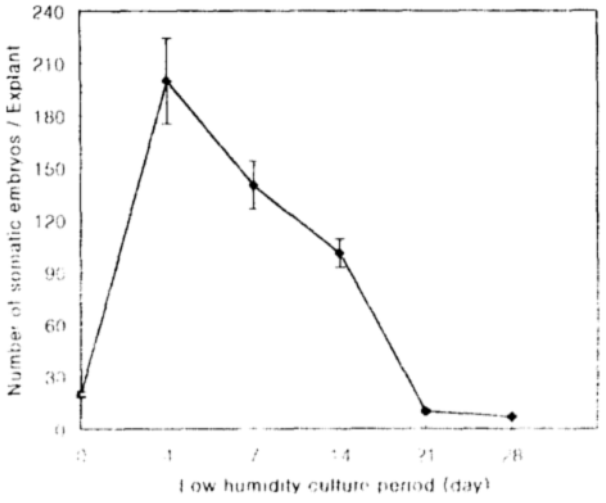

Fig. 1. Effect of low humidity on the formation of somatic embryo from cotyledon explants of *Daucus carota* L. The explants with embryogenic callus were cultured on MS agar medium without 2,4-D in petridish with lid having 1 cm hole sealed with ventilative filter for 4-28 days during somatic embryogenesis and transferred to MS agar mdeium without 2,4-D in petridish entirely sealed with parafilm. 0: Control. Vertical bars represent ± SE.

the highest frequency of occurrence with 33%, jar-shaped embryos occupied 7%, embryos with 3 cotyledons 14% and those with 4 or 5 cotyledons less than 1%. When the explants were cultured in the low humidity condition for 1 week and then transferred to a nonventilative condition, 2 cotyledon somatic embryos were 44% and jar-shaped cotyledons were 17% (Table 2). Therefore, in ventilative culture, the frequency of normal cotyledonary embryos was the same as that of control, even though the frequency of jar-shaped embryos become high by 10% point.

Improved embryo maturation

After embryogenic calli were cultured in the low humidity condition, somatic embryo maturation was observed. In the control group, more than 50% of somatic embryos still remained in the globular stage, but in the case of 4-day ventilative cultures, the cotyledon stage embryos were already green in color and the roots were developed, showing a rather fast maturation of somatic embryos. Somatic embryos were mostly in the cotyledon stage after 1 and 2 weeks of culture. Very little embryo development took place, and for the most part, remained as calli after 3 and 4 weeks of culture (Fig. 3). It has been observed so far that low humidity culture enhances embryogenesis, but extremely low humidity has a reverse, or inhibitive effect.

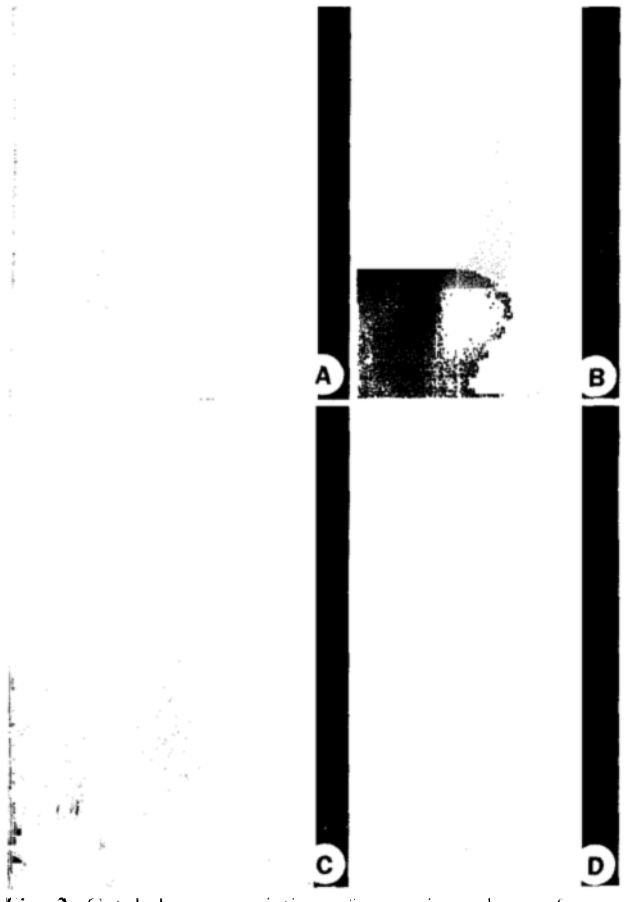

Fig. 2. Cotyledonary variation of somatic embryos formed from callus on cotyledon explants of *Daucus carota* L. on MS basal agar medium. A: One (M) and balling pin shape cotyledon (B); B: Jar-shaped cotyledon; C: Three cotyledons; D: Five cotyledons.

Table 2. Cotyledonary variation of carrot somatic embryos developed in ventilated conditions

Treatment \ Cotyledon number (%)	Jar	1	2	3	4	5
Control	7.1	33.3	44.0	14.3	0.7	0.6
Ventilated	16.9	15.6	44.2	16.9	5.6	0.9

Cotyledon explants with embryogenic calli were cultured on MS agar medium without 2,4-D in petridish with lid having hole (1 cm) sealed with milipore filter for 1 week and transferred on MS agar medium without 2,4-D in petridish entirely sealed with parafilm for 3 weeks.

Enhancement of germination frequency

Regardless of the number of cotyledons, the germination frequency of somatic embryos produced in ventilation culture increased (Fig. 4). An exception, normal somatic embryos with 2 cotyledons showed no improvement in germination frequency after the low humidity culture because they already had a high

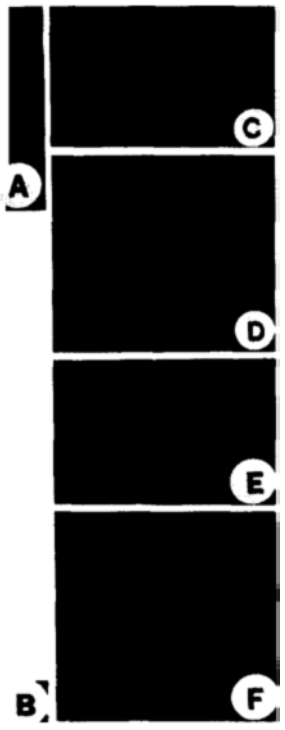

Fig. 3. Maturation of somatic embryos developed in low-humidity condition. Globular stage embryos (arrow) developing from callus on MS agar medium without 2,4-D (A), control. Somatic embryos with elongating root and greening cotyledon developed from callus on MS agar medium without 2,4-D in petridish with a hole sealed with ventilative filter for 4 days (B), for 1, 2 weeks (C, D), and 3, 4 weeks (E, F).

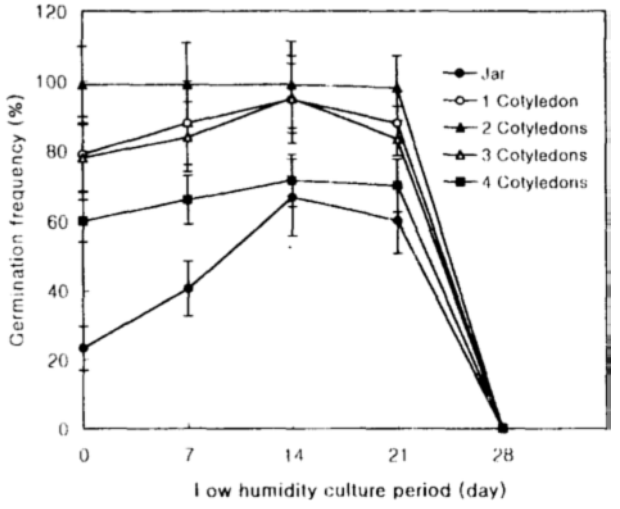

Fig. 4. Effect of low humidity on germination of somatic embryos of *Daucus carota* L. The explants with embryogenic callus were cultured on petridish with lid having a hole (1 cm) sealed with ventilative filter for 4- 28 days during somatic embryogenesis and transferred on MS agar medium in petridish entirely sealed with parafilm. 0: Control. Vertical bars represent ± SE.

germination frequency of 99% in the control. Thus there were remarkable germination improvements in the cases of abnormal embryos. Under ventilative culture, embryos produced in a 2-week period showed the highest germination frequency; in particular, jar-shaped embryos showed the most remarkable improvement from 23% in the control group to 68%. However, 4-week period showed 0% which was rather lower than the control (Fig. 4).

DISCUSSION

The low humidity culture in somatic embryogenesis of *Daucus carrota* resulted in a 10-time higher frequency of embryo formation than the control group. Therefore it is suggested that moderately low humidity culture using the ventilative petridish operates as a stressor inducing somatic embryogenesis (Kermode *et al.*, 1989). In carrot and palm, higher production frequencies and better-quality somatic embryos were gained by such stressors as the treatment of high-level sucrose, sodium hypochlorite and cadmium ions (Kamada *et al.*, 1989), or by the blocking of sucrose provision (Veramendi and Navarro, 1996).

In low humidity culture, cotyledonary abnormalities of somatic embryos were not prominent but the formation of jar-shaped embryos increased. In varied sucrose level, multicotyledonary embryos were reportedly formed in *Codonopsis lanceolata* (Soh, 1993). The formation of abnormal cotyledon embryos was dependent on the cell density in cell cultures of caraway (Ammirrato, 1983). In the case of *Glycine max*, the number of jar-shaped somatic embryos increased as the 2,4-D level increased (Choi *et al.*, 1994a). Multicotyledonary somatic embryos of *Aralia cordata*, were produced when embryogenic calli were cultured in MS medium supplemented with 2,4-D, cytokinins and ABA (Lee *et al.*, 1993a,b, 1994). Therefore, it was confirmed that the variation in the number of cotyledons are affected by plant growth regulators and other factors (osmoticum) but not by low humidity.

It has been observed in the present experiment that the ventilative culture improved embryo maturation. In the somatic embryogenesis of pecan, dehydration promoted embryo development as indicated by an increase in embryo size (Burns and Wetzstein, 1995). In contrast to these examples, the application of low humidity culture to spruce embryos decreased the germination time relative to the maturation of the somatic embryos, and improved the synchrony of root

and shoot elongation compared to untreated somatic embryos (Attress *et al.*, 1991; Roberts *et al.*, 1991).

In ventilative culture germination of somatic embryos was promoted more than in control (Fig. 4), especially, germination of jar-shaped embryos improved remarkably. Although jar-shaped somatic embryos produced in liquid culture did not germinate at all (Soh *et al.*, 1996), their germination frequency improved to 23% on solid medium in nonventilative culture and to 67% in ventilated culture. In addition, from the estimation of total abnormal embryos formed by low humidity treatment, the germination frequencies in control were 83% but that in ventilative culture were 91%. Therefore it is clear that germination increased overall in somatic embryos formed in low humidity culture. When the drying treatment was applied to somatic embryos of cassava, the germination frequency increased more than 40%, compared with that of the control group (Mathews *et al.*, 1993). In pecan, only the root growth of somatic embryos took place and normal germination did not occur, but after the drying treatment, the germination frequency increased to 8.1% (Burns and Wetzstein, 1995). It was reported in the case of asparagus somatic embryo culture that germination was better in medium whose culture vessels were capped with Mili wrap than those with caps of aluminum foil (Saito *et al.*, 1991).

On the other hand, in extremely low humidity culture, secondary embryo and callus formation were observed in the present experiment which therefore became an obstacle to normal embryo development (Burns and Wetzstein, 1995; Mathews *et al.*, 1993), but moderate low humidity was efficient for the enhancement of somatic embryo development. There are some reports that high-density ethylene and CO_2 restrain the formation of the somatic embryo in carrot and *Zea mays* (Philippe *et al.*, 1989; Roustan *et al.*, 1989). As we have seen here, the relative humidity of the culture environment is deeply involved in somatic embryo formation. Also, other gases in the culture vessel are important for somatic embryogenesis. So, studies are in progress to determine whether, in the ventilative culture, the improvement of somatic embryo development and germination is also attributable to other gases, such as ethylene.

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