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Enhanced Development and Germination of Carrot Somatic Embryos on Modified Surface of Medium

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당근체세포배의 발생 및 발아에 미치는 배지표면의 물리적변화

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Carrot cotyledon explants cultured on MS medium with 1 mg/L 2,4-D were transferred to a hormone-free solid medium overlaid with filter paper in order to elucidate the effect of simple physical treatment on the development and germination of somatic embryos. Transfer of the explants cultured for one week on MS basal medium overlaid with 3 sheets of filter paper on to MS basal medium increased somatic embryo production 2-39 times over the one week culture on medium without filter paper overlay. Maturation and germination of somatic embryos was more prominent on medium overlaid with filter paper than on medium without filter paper. The explants cultured for one week on filter paper overlay added with liquid medium showed prominent decrease in somatic embryo formation compared to filter paper overlay only. It is suggested that the filter paper overlay affected the moisture environment of the somatic embryos developing on it.

Key words : *Daucus carota*, filter paper overlay, moisture content, somatic embryogenesis

Proembryonic masses in liquid medium can be cultured on filter paper or nylon mesh to facilitate retrieving the desired fraction of proembryonic masses for plating on solid medium (McKersie et al., 1989). Yellow-poplar proembryonic masses retrieved from liquid medium developed at high frequency into mature embryos directly on filter paper, with no underlying bed callus on solid medium (Merkle et al., 1990). On the other hand, proembryonic masses of pecan did not show a positive effect on embryo development on filter paper on medium and the filter paper served only as a convenient method for proembryonic mass collection (Bums and Wetzstein, 1995). Thus it is not consistent whether a physical change, such as filter paper overlay on medium, affects the development and germination of somatic embryos. Nor do we have sufficient information about the influence of the physical conditions of medium surface on embryo development and plantlet regeneration.

Reduced humidity in culture vessels enhanced the development and germination of carrot somatic embryos (Lee et al., 1997a). Filter paper overlay must alter the environment of the developing embryos due to the physical barrier between medium and the embryos. Thus, filter paper overlaid on a solid medium is necessary for maintaining low moisture during somatic embryo development. The tissues cultured on agar medium are subjected to more stress than those on liquid media (Bouniols, 1974). Somatic embryogenesis of carrot could be induced by osmotic or heavy metal iron stress, and the somatic embryos formed under such stresses, except under 2,4-D, grew readily to normal seedlings (Kamada et al., 1989). The development of somatic embryos was promoted in suspension culture in medium supplemented with high levels of mannitol, sucrose or polyethylene glycol (Litz, 1986: Nadel et al., 1989: Linossier et al., 1997). Thus it is assumed that stress induced somatic embryos can provide normal seedlings, and that development of somatic embryos may be controlled by modifying the environment of developing somatic embryos

by filter paper overlay. The present study attempts to elucidate whether simple physical treatment such as filter paper overlay on medium affects the development of somatic embryos and the conversion rate of somatic embryos into plantlets, and it reduces the moisture content of medium surface.

MATERIALS AND METHODS

The seeds of carrot (*Daucus carota* L. cv. Hongshim ochon) were sterilized in 70 % ethanol for 1 min, with sodium hypochlorite solution (1 %) for 15 min, and then rinsed 4 times with sterile distilled water. They were germinated aseptically in half-strength MS basal medium (Murashige and Skoog, 1962) for one week. Cotyledon segments of seedlings were cultured in petridishes (87 mm × 15 mm) containing 30 mL MS basal medium supplemented with 1 mg/L 2,4-D and 30 g/L sucrose and the medium was solidified with 8 g/L agar. The pH of the medium 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 a constant temperature of 25 ± 1°C. Callus was induced from the cotyledon explants after two weeks of culture and embryogenic callus appeared on the callus on MS medium with 1 mg/L 2.4-D. After culturing the explants with embryogenic callus on MS medium with 1 mg/L 2,4-D for 3 weeks, these were transferred to MS basal medium overlaid with Whatman filter paper (No. 2) or onto Whatman filter paper added with MS liquid medium for somatic embryo development for 1-4 weeks. Later the explants were moved to MS basal medium without filter paper for subsequent development and were observed for somatic embryo count under disecting microscope. The water content of filter paper, agar medium and somatic embryos during or after culture was determined as the moisture percentage by subtracting dry weight from fresh weight of the samples after drying at 60°C for one day. Humidity was measured by the method described in previous report (Lee et al., 1997a). Four cotyledon segments per petridish were cultured for 3 weeks, and each treatment was replicated in 5 petridishes.

RESULTS

The relative humidity in petridishes sealed with parafilm remains nearly constant throughout the culture period (Lee et al., 1997a). Water content of filter paper and medium surface without filter paper was nearly constant during the culture period: *ie* 68 % and 91 %, respectively (Figure 1). However, the water content of medium surface below filter paper decreased after the second week of culture. The filter paper overlaid on culture medium in the environment of culture vessel contained lower moisture than the medium surface without filter paper. The water content of somatic embryos formed on filter paper was low in comparison to that on solid medium. But the water content of somatic embryos formed on filter paper added with liquid medium or distilled water was constant throughout the culture period (Tables 1-3).

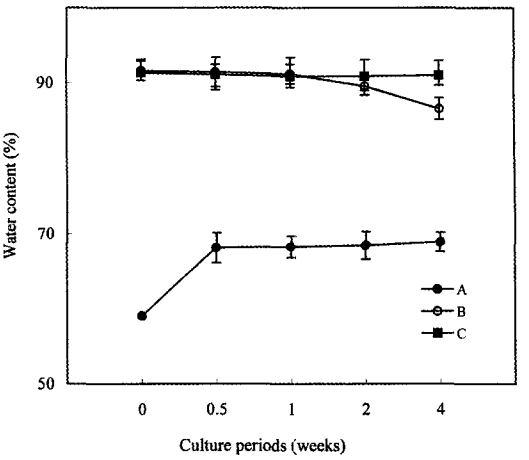

Figure 1. Water content of filiter paper overlaid on culture medium(A), surface culture medium below filter paper overlay(B) and surface culture medium without filter paper of 1 mm depth (C) during cotyledon explant cultures of *Daucus carota* for somatic embryogrnesis. Cotyledon explants were cultered on filer paper(Whatman No. 2) of 3 sheets on MS agar(0.8%) medium from half week to 4 weeks. Vertical bars represent means ± SD obtained three independent experiments

When cotyledon explants cultured on MS solid medium with 2,4-D were transferred to MS basal solid medium, 13 somatic embryos per explant were formed (Figure 3A). However, the explants cultured for one week on MS basal

Table 1. *Effects of filter paper overlaid on solid medium on somatic embryogensis in cotyledon explant cultures of Daucus carota* L

Filter paper explant	Embryos/ (sheet)	Developmental stages(%) Globular	Heart	Torpedo or Cotyledonary	Water content of embryos (%)
0	13 ± 1.04	17 ± 1.0	33 ± 2.18	50 ± 3.04	97.0 ± 1.42
1	96 ± 7.55	0	0.7 ± 0.55	99.3 ± 0.55	89.0 ± 2.09
3	512 ± 32.74	0	0.2 ± 0.12	99.8 ± 0.11	86.1 ± 1.49
6	157 ± 12.53	0	0	100 ± 2.29	84.7 ± 2.05

Cotyledon explants were cultured on filter paper (Whatman No. 2) overlaid on MS agar medium for 1 week and the explants were transferred to agar medium without filter paper overlay for 3 weeks and then somatic embryos were counted. Vertical bars represent means ± SD obtained from three independent experiments.

medium overlaid with 1, 3 or 6 sheets of filter paper were transferred on to the MS basal medium, somatic embryo production increased 2-39 times in comparison with the one cultured on medium without filter paper overlay (Table 1). In addition, somatic embryos on filter paper overlay matured earlier than those on medium without filter paper (Table 1).

To examine the effect of modified moisture and nutrients conditions by filter paper overlaid on medium on somatic embryo formation, 10 mL of distilled water and MS liquid

Table 2. Effects of MS liquid medium or distilled water added to filter paper overlaid on solid medium on somatic embryogenesis in cotyledon explant cultures of *Daucus carota* L.

Treatment	Embryos/explant	Developmental stages(%)		Water content of embryos (%)
		Heart	Torpedo or Cotyledonary	
Filter paper overlay	512 ± 39.59	0.2 ± 0.1	99.8 ± 0.1	86.1 ± 3.26
Distilled water	391 ± 19.97	0	24(76) ± 1.98	97.7 ± 2.49
Liquid medium	236 ± 9.64	0	47(53) ± 1.82	97.6 ± 1.9

Cotyledon explants were cultured on filter paper (Whatman No. 2) of 3 sheets overlaid on MS agar medium or on filter paper overlaid on medium added with 10 mL MS liquid medium or 10 mL distilled water for 1 week and the explants were transferred to agar medium without filter paper overlay for 3 weeks and then somatic embryos were counted. Some cotyledonary embryos (in parenthesis) showed greening of cotyledon and elongation of hypocotyl. Vertical bars represent means ± SD obtainedfrom three independent experiments.

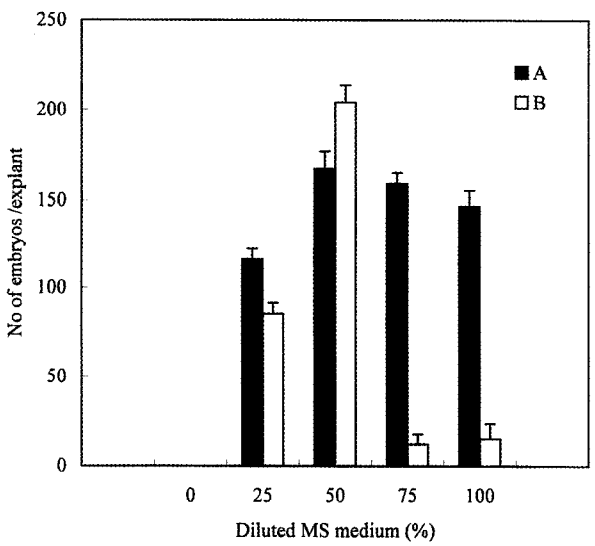

Figure 2. Effects of diluted MS medium added to filter paper overaid on an empty petridish on somatic embryogenesis in cotyledon explant culture of *Daucus carota* L. Cotyledon explants were cultured on filter paper (Whatman NO. 2) of 3 sheets added with diluted MS liquid medium for 1 week and the explants were transferred to agar medium without filter paper overlay for 3 weeks and then somatic embryos were counted. Vertical bars reperesent means ± SD obtained from three independent experiments. A: Cotyledonary embryos B: Cotyledonary embryos showing hypocotyl elongation.

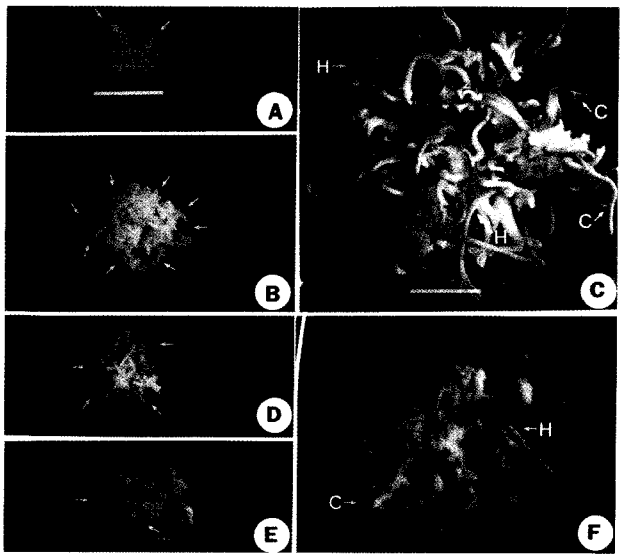

Figure 3. Somatic embryogenesis from cotyledon explant culture of carrot on MS basal medium. The explant cultured on MS medium with 1 mg/L 2,4-D was transferred to MS basal medium and then the explant formed in part somatic embryo (arrows) (A). On MS basal medium somatic embryos (arrows) were actively formed from the explant (B) subcultured on filter papers of 3 sheets overlaid on MS basal agar medium for one week after culture on MS medium with 2,4-D. And culture of the explant on filture paper of 3 sheets overlaid on MS agar medium added with 10 ml distilled water showed less somatic embryos than in (B) but the embryos fastly matured showing the prominent elongation of hypocotyl (H) and cotyledon (C) (C). The explant cultured on 3 sheets filter paper added with 5 mL (D), 10 mL (E) and 15 mL (F) of MS liquid medium formed less or more somatic embryos than in (B) and (A) but the embryos had the elongation of hypocotyl and cotyledon in (F). Bars=10 mm

medium each were added to the filter paper. The explants cultured on such filter paper for one week were then transferred to MS basal medium for subsequent development of somatic embryos (Table 2). Cotyledon explants cultured for one week on filter paper overlay supplemented with distilled water or liquid medium showed prominent decrease in the rate of somatic embryo formation in comparison to filter paper overlay only. However, the maturation and hypocotyl elongation of somatic embryo were promoted in one week culture on filter paper supplemented with distilled water or liquid medium (Table 2, Figure 3C). These organ elongation and maturation proceeded with germination even though the first leaf of embryos hardly appeared.

To examine somatic embryo formation on filter paper supplemented with liquid nutrient medium, cotyledon explants were cultured on 3 sheets of filter papers with 5 ml to 20 ml liquid medium, but not on MS solid medium, for one week, and then the explants were transferred to MS solid medium for subsequent embryo development (Table 3). Rate of

somatic embryo formation decreased on filter paper according to the amount of liquid nutrient medium used. Hypocotyl elongation and maturation of somatic embryo leading to germination was inversely proportional to the amount of the nutrient liquid medium (Table 3, Figure 3D, 3E, 3F).

Table 3. Effects of filter paper added with diluted liquid medium on somatic embryogensis in cotyledon explant cultures of *Daucus carota L.*

Liquid medium (ml)	Embryos/explant	Developmental stages(%)		Water content of embryos (%)
		Heart	Torpedo or Cotyledonary	
5	233 ± 9.17	0.3 ± 0.11	99.7 ± 0.11	87.6 ± 2.76
10	162 ± 17.69	0	91(9) ± 2.46	97.5 ± 1.21
15	91 ± 3.56	0	41(59) ± 1.68	97.65 ± 1.82
20	53 ± 2.57	0	22(78) ± 1.28	97.6 ± 1.32

Cotyledon explants were cultured on filter paper (Whatman No. 2) of 3 sheets with 5-20 mL MS liquid medium for 1 week and the explants were transferred to agar medium without filter paper overlay for 3 weeks and then somatic embryos were counted. Some cotyledonary embryos (in parenthesis) showed greening of cotyledon and elongation of hypocotyl. Vertical bars represent means ± SD obtainedfrom three independent experiments.

Cotyledon explants on the filter paper supplemented with distilled water showed meager somatic embryogenesis. Explants were cultured on filter paper supplemented with 10 mL MS liquid medium of the concentrations of 75 % to 25 % for one week prior to MS basal medium culture (Figure 2). Somatic embryos were formed more on filter paper with diluted liquid medium than with 100 % liquid medium. Embryo formation on filter paper added with 50 % liquid medium was double than that with the addition of 100 % liquid medium (Figure 2). In addition, hypocotyl elongation and maturation of somatic embryos were rapid on filter paper supplemented with 50 % liquid medium as on filter paper with 25 % liquid medium (Figure 2). The embryos matured very slowly on filter paper with 100 % and 75 % liquid medium (Figure 2). Thus, the diluted liquid medium added to filter paper was more suitable for somatic embryo formation and maturation than the full strength medium (100 %). The diluted liquid medium added to filter paper was also favorable for somatic embryo maturation (Figure 2) in comparison with the explants cultured for one week on filter paper overlay only (Table 1).

DISCUSSION

It was clarified in the previous report (Lee et al., 1997a) that the formation and maturation of somatic embryos on solid medium was more prominently promoted in low humidity by ventilated cultures than in high humidity in tightly closed culture vessels. In present study, filter paper overlay on solid medium showed 39 times greater somatic embryo production and faster maturation than in control (Table 1, Figure 1A, 1B). The water content of somatic embryos formed on filter paper was lower than that on solid medium without filter paper overlay (Table 2). These results suggest that low moisture on filter paper (Figure 1) operates as an appropriate inducer for somatic embryo formation and maturation. In suspension cultures of pecan, a mild dehydration of embryo aggregates suppressed recurrent embryogenesis, promoted development of globular embryos into cotyledonary embryos and enhanced plant regeneration (Bums and Wetzstein, 1995) as in other plants (Merkle et al., 1993: Nickle and Yeung, 1993: Wetzstein and Baker, 1993). In cell cultures of *Carcia*, celery and *Hevea brasiliensis*, somatic embryogenesis was also promoted in medium supplemented with high level of osmoticum, such as manitol, sucrose, and polyethylene glycol (Litz, 1986: Nadel et al., 1989: Linossier et al., 1997).

Addition of 10 mL distilled water or liquid medium to the filter paper overlay reduced somatic embryo formation compared to that on filter paper overlay without distilled water (Table 2). Thus, reduced somatic embryo formation on filter paper with distilled water or liquid medium seems to be due to surplus moisture on the filter paper overlay. On the other hand, addition of 10 mL MS liquid medium to the filter paper overlay hastened hypocotyl elongation and maturation of somatic embryo on being transferred to solid medium. Thus the formation of somatic embryos required low moisture as physical condition while high moisture retarded embryo formation and promoted the maturation. This maturation led to germination even though it was not clarified whether the growth of plumule occurs. In liquid medium cultures, most somatic embryos of *Liriodendron tulipifera* appeared to skip normal processes of maturation and show elongation of both radicle and cotyledon (Merkle et al., 1990). Somatic embryos of *Bupleurum falcatum* L. in liquid medium showed elongation of embryo axis but poor cotyledon formation compared to embryos on solid medium (Lee et al., 1997b), as well as somatic embryos of carrot and caraway (Ammirato, 1971, 1983).

Somatic embryo formation on filter paper not overlaid on solid medium but added with liquid medium decreased in accordance with the amount of medium but embryo maturation was inversely affected by the amount of liquid medium (Table 3, Figure 3D, 3E, 3F). Thus, it may be

concluded that somatic embryo formation was enhanced by low moisture on filter paper, by the appropriate exposure of embryogenic cells to filter paper overlay for gas exchange. In date palm tissue cultures, physical conditions of liquid culture were shown to affect somatic embryogenesis and the promotion of embryo formation by continuous shaking of the cultures for gas exchange (Veramendi and Navarro, 1996).

In cultures on filter paper added with diluted liquid medium, the half strength liquid medium was most favorable for somatic embryo formation and maturation (Figure 2). Therefore, it seems that lowering of moisture by filter paper overlay retards nutrient absorption and this is suitable for embryo formation and maturation. Eventually somatic embryo formation and maturation are meager in full strength liquid medium.

In summary, filter paper overlay on solid medium not only facilitates retrieving of desired cultures but also enhances formation of somatic embryos. This technique possibly alters the environment of the developing embryos by changing the moisture conditions of the culture support. Therefore, enhanced formation of somatic embryos on filter paper seems to result from sufficient oxygen supply for respiration of the developing embryos because filter paper may contribute to the gas exchange around developing embryos. An experiment on exposure of developing embryos to air by temporary immersion in liquid culture is in progress for clarifying the effect of lowered moisture and exposure of cultures on somatic embryo formation and maturation.

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적 요

배지상에 여과지를 덮어놓는 간단한 물리적 처리가 체세포배의 발생 및 발아에 미치는 영향을 밝히기 위하여 본 실험관찰을 수행하였다. 배지상에 덮어놓은 세장의 여과지위에서 일주일 동안 당근 자엽절편을 배양한 후 MS 기본배지에 계대배양을 하면 여과지 덮개 없는 배지에서의 체세포배발생 보다 2배에서 39배의 배발생을 나타내었고, 체세포배의 성숙 및 발아도 증진되었다. 배지상의 여과지에 배양액을 첨가한 다음 배양을 하면 여과지에 배양액을 첨가하지 않은 경우보다 배발생이 감소되었다. 그러므로 여과지 덮개는 자엽절편을 치상한 배양환경의 습기를 변화시켜 체세포배의 발생을 증진시킨 것으로 생각된다.

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