J Plant Biotechnol (2023) 50:089-095
DOI:https://doi.org/10.5010/JPB.2023.50.012.089

ISSN 1229-2818 (Print)
ISSN 2384-1397 (Online)

Research Article

Effect of carbon sources on somatic embryogenesis and cotyledon number variations in carrot (*Daucus carota* L.)

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Received: 7 April 2023 / Revised: 10 May 2023 / Accepted: 10 May 2023 / Published: 17 May 2023

Abstract In order to investigate the effect of carbon sources on somatic embryogenesis and cotyledon number variations in carrot, embryogenic callus were cultured in the medium supplemented with various concentrations of sucrose or glucose, and with combination of 2% sucrose and various concentrations of mannitol or sorbitol. The maximum number of somatic embryos formed per flask (1,555.70) was obtained in the medium supplemented with 2% sucrose rather than glucose alone or a combination of mannitol or sorbitol and 2% sucrose, and the number of somatic embryos was decreased with the increasing of mannitol or sorbitol concentration. The frequencies of somatic embryos with two cotyledons were 35.14% for sucrose, 19.88% for glucose, 32.55% for mannitol + 2% sucrose, and 38.59% for sorbitol + 2% sucrose, respectively, and the frequencies of abnormal somatic embryos having 3 or more cotyledons were 64.86% for sucrose, 80.12% for glucose, 67.44% for mannitol + 2% sucrose, and 61.41% for sorbitol + 2% sucrose, respectively. Particularly, the frequency of somatic embryos with two cotyledons (59.16%) was the highest in the 2% sucrose medium compared to the frequency of abnormal somatic embryogenesis with three or more cotyledons, and the frequency gradually decreased with increasing concentration of glucose, mannitol or sorbitol. According to these results, it was found that the ratio of abnormal somatic embryo was higher than the normal somatic embryo in carrot, and was shown that somatic embryogenesis and the cotyledon number was affected by the concentrations of sucrose, glucose as carbon source, and mannitol and sorbitol as osmotic agents in culture medium.

Keywords carbohydrates, carrot, cotyledon morphology, osmotic agents, somatic embryogenesis

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Introduction

Since dicotyledonous plants have been known to form a typical zygote embryo having two cotyledons through the fertilization process of pistil and stamens in their natural state, somatic embryos formed through in vitro culture have long been regarded as externally morphologically identical to zygote. However, as abnormal somatic embryos with a trumpet-shaped cotyledon were observed in vitro culture (Stuart et al. 1985), Buchheim et al. (1989) classified soybean embryos into 9 types according to the number and shape of cotyledons, and Goebel-Tourand et al. (1993) classified grape somatic embryos into 8 types according to their shape. Because these abnormal cotyledon types of somatic embryos *in vitro* culture have a lower rate of regeneration into plants compared to normal somatic embryos (Soh et al. 2001), normal somatic embryos are needed to be used in plant mass propagation for plant application studies such as artificial seed production.

Cotyledon development of somatic embryos in *in vitro* culture occurs when initial cells of cotyledon develop from globular stage to early heart stage. In general, cotyledon initial cells begin to differentiate from procambium cells, and then differentiate into two cotyledon in the case of two procambial strand, three cotyledon in the case of three procambial strand, four cotyledon in the case of four procambial strand, and cup-shaped cotyledon in the case of cylindrical procambial strand (Choi et al. 2005; Choi and Kwon 2013a, b). Like these, it is known that the cotyledon formation of somatic embryos is closely related to the development of procambial strand. In addition, the

development of a somatic embryo with three or more cotyledons is caused by BAP or zeatin or 2,4-D added in the culture medium (Choi et al. 1994; Lazzeri et al. 1987; Lee and Soh 1993). The frequency of normal somatic embryos having two cotyledons was high at a low concentration of sucrose, whereas the frequency of somatic embryos having abnormal cotyledons increased at a high concentration (more than 6%) of sucrose in the medium (Businge et al. 2013; Kageyama et al. 1990; Komatsuda 1992). Due to the fact that the morphological changes of somatic embryos is affected by various factors (Garcia et al. 2019; Vale et al. 2018), it is necessary to perform an investigation on optimal culture conditions including exogenous hormone or carbon source as energy and osmotic agents for the production of somatic embryo with normal cotyledons.

In order to understand the effect of carbon source on the frequency of somatic embryogenesis and cotyledon number variations of somatic embryos, sucrose or glucose as energy sources, and mannitol or sorbitol as osmotic agents were treated, and then the formation of somatic embryos and the frequency of somatic embryos with three or more cotyledons were investigated in this study.

Materials and Methods

Plant materials

Carrot (*Daucus carota* L., cv. Sin Heuk Jeon 5 Chon), which is purchased from Asia Seed Korea was used in this study.

Induction of embryogenic callus

Carrot mature seeds were immersed in 70% alcohol for 1 min and 1% sodium hypochlorite solution for 15 min for surface sterilization, and then washed 3-5 times with sterile water. 15-20 of the sterilized seeds were cultured in each Petri dish containing MS basal medium (Murashige and Skoog 1962) for germination. After a culture for 4 weeks, hypocotyl explants (about 3.0-5.0 mm in length) were cut from the seedlings and cultured on MS medium with 1.0 mg/L 2,4-D for embryogenic callus induction. Six weeks later, embryogenic callus was selected under a dissecting microscope, and sub-cultured in the same fresh medium then for proliferation while. All media were adjusted to pH 5.8 and supplied with 0.8% agar before a autoclaving at 120° C and 1.2 atm for 15 min, and then each 25 ml of the autoclaved medium was dispensed in a petri-dish (Φ9 cm). All tissue culture was carried out at 25°C in dark conditions.

Synchronization of embryogenic callus and somatic embryogenesis

Synchronized culture of embryogenic callus was attempted using liquid culture medium containing 1.0 mg/L 2,4-D. Each 20 ml of the liquid medium was dispensed into 100 ml Erlenmeyer flasks, then about 1 g of embryogenic callus was added, and cultured in dark conditions in a shaking incubator rotating at 100 rpm. Embryogenic calluses in the liquid culture were proliferated for about 2 months or more periods, during which sub-culture was performed by a 2-week interval. In order to obtain a cell mass in same size from the embryogenic callus, the cell mass suspension for somatic embryogenesis was used by dispensing 5 ml per treatment after double filtering by a two mesh with a diameter of 250 μm and 500 μm. To investigate the number of somatic embryos formed from the embryogenic cell mass and the cotyledon number variations of somatic embryos, sucrose or glucose as carbon source was individually added to MS basal liquid medium at 0.5, 1.0, 2.0, 3.0, 6.0 and 9.0%, respectively. In order to investigate the effect of osmotic agents on somatic embryo induction frequency, 2% sucrose combined with 0.5, 1.0, 2.0, 3.0, 6.0, and 9.0% sorbitol or mannitol in concentrations were respectively used in culture medium for 2 weeks.

Observation of somatic embryos by scanning electron microscope

The cotyledon number variations during somatic embryogenesis was observed with a scanning electron microscope. Somatic embryos in cotyledonary stage were fixed in 2.5% glutaraldehyde solution at 4°C for 4 hours and then dehydrated in an alcohol series. After drying with a critical point dryer for 2 hours or more, the material was fixed on the Stab using double-sided tape and then silver pest was applied to the attached sample, and a 100 Å-thick gold skin was applied with an ion evaporator and observed with a scanning electron microscope.

Data collection and statistical analysis

All data were obtained from the average values of at least three independent experiments. The results are presented as mean values ± standard deviation (SD). Means between the treatments were compared at the 0.05 probability level.

Results and Discussion

Induction of embryogenic callus from hypocotyl cultures of carrot

After 2 weeks of culture, callus began to form from wounded edge of the hypocotyl explants on the medium with 1.0 mg/L 2,4-D, and a light yellowish callus was newly grown on the surface of callus that was first appeared at the 4th week since the initiation culture. The light-yellowish callus could be regarded as embryogenic callus because somatic embryos were formed from them in MS basal medium without hormone, and then sub-cultured in the same solid medium at intervals of 4 weeks to proliferate in large quantities. These embryogenic callus were proliferated in the liquid medium at 2 week interval for 6 weeks and a synchronized cell mass was obtained. The synchronized cell masses were formed somatic embryos at the 2 weeks of culture in the liquid medium without the addition of hormones (Fig. 1). The embryogenic callus according to plants has various characteristics. In general, embryogenic callus is known to have high capacity for cell division, full cytoplasm, little vacuolization, many starch granules, and yellow callus surface, whereas non-embryogenic callus has a small nuclear to cytoplasmic ratio in the cell, a clear vacuolation, and the surface of the callus is less shiny and watery (Brown et al. 1989; Businge et al. 2013). In this study, the bright yellowish callus could be regarded as an embryogenic callus because it had a globular somatic embryo on the surface of them.

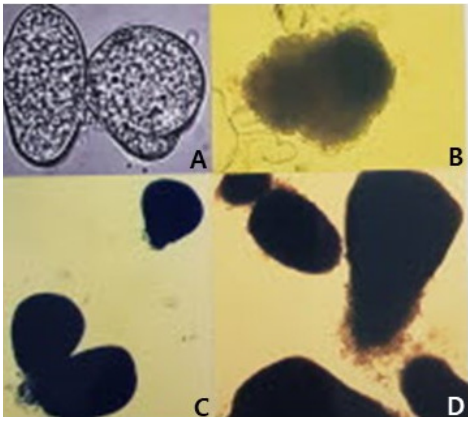

Fig. 1 Somatic embryogenesis from cell cultures of embryogenic callus in liquid medium without hormone for two weeks in *Daucus carota* L. A. Typical embryogenic cell; B: A clump of embryogenic cells; C: Somatic embryos in globular stage; D: Somatic embryos in late globular or torpedo stage

Effects of carbon sources on somatic embryogenesis

In the effects of sucrose or glucose on somatic embryogenesis using the liquid culture system, about 1,555.70 somatic embryos per flask were produced in 2% sucrose-added medium, and the number was gradually decreasing to 1,236.00 at 0.5% sucrose, 1,484.00 at 1.0% sucrose, 1,442.70 at 3.0% sucrose, 444.70 at 6.0% sucrose, and 42.33 at 9.0% sucrose. In the case of glucose, 87.30 somatic embryos were formed at 3.0%, 22.30 at 1.0%, 42.70 at 2.0%, 78.30 at 6.0%, and 40.70 at 9.0%, whereas somatic embryos were not formed in 0.5%.

When 2.0% sucrose and various concentrations of mannitol were combined, 299.30 somatic embryos formed at 0.5%, 280.70 at 1.0%, 155.70 at 2.0%, 83.70 at 3.0%, and somatic embryos were not formed at 6.0%. Like these, the number of somatic embryos per treatment showed a tendency of gradually decreased with the mannitol concentration increased. When 2.0% sucrose and various concentrations of sorbitol were combined, 735.70 somatic embryos formed at 0.5%, 543.70 at 1.0%, 262.70 at 2.0%, 113.30 at 3.0%, and 18.0 at 6.0%, and was not formed at 9.0% (Table 1). Based on these results, it was found that sucrose was more effective than glucose for somatic embryogenesis in carrot, and it's most effective concentration was at 2.0%. When the 2% sucrose was combined with various concentrations of mannitol or sorbitol, inhibitory effect on somatic embryogenesis was observed in all treatment groups, and the inhibition trend was increased with the concentration increasing of mannitol or sorbitol.

It is known that sucrose was more effective than glucose in soybean embryogenesis, and the most effective concentration for somatic embryogenesis were 3% sucrose and 1.5% glucose, but the frequency of somatic embryogenesis decreased with the increasing of sucrose concentration (Lazzeri et al. 1987). Also, the medium supplemented with 4% sucrose is most effective for somatic embryogenesis in coconut (Ashburner et al. 1993). Compared to 3% sucrose, 12% sucrose was the most effective for somatic embryogenesis in sunflower (Finer 1987). While, sorbitol was known to be the most effective compared to other carbohydrates in corn (Businge et al. 2013; Swedlund and Locy 1993). According to the results of previous studies and this study, the appropriate carbon sources, and their concentrations required for somatic embryogenesis may vary depending on the plant species. In particular, the number of somatic embryos was decreased when 2% sucrose and mannitol or sorbitol were combined applied, which might be due to the negative osmotic effect of sorbitol and mannitol (Brown et al. 1989; Businge et al. 2013; Vale et al. 2018).

Table 1 The number of somatic embryos formed in liquid medium supplemented carbohydrates alone or/and with the combination of osmotic agents with 2% sucrose in suspension cell cultures of *Daucus carota* L.

Percentage (%)	[a]Total number of somatic embryos / flask			
	sucrose	glucose	mannitol + 2% sucrose	sorbitol + 2% sucrose
0.5	1,236.00 ± 3.50	0.00	299.30 ± 3.99	735.70 ± 5.88
1.0	1,484.00 ± 4.09	22.30 ± 4.85	280.70 ± 2.61	543.70 ± 3.86
2.0	1,555.70 ± 2.78	42.70 ± 2.67	155.70 ± 2.70	262.70 ± 2.48
3.0	1,442.70 ± 5.18	87.30 ± 3.19	83.70 ± 3.04	113.30 ± 2.15
6.0	444.70 ± 3.82	78.30 ± 5.02	0.00	18.00 ± 3.82
9.0	42.33 ± 1.03	40.70 ± 3.10	0.00	0.00

[a]Each value represents the mean ± standard deviation of at least three replicates.

Table 2 Effects of sucrose on the cotyledon number variations of somatic embryos formed in suspension cultures of *Daucus carota* L.

Sucrose conc. (%)	[a]Cotyledon morphology of somatic embryos (%)			
	two	three	four	five (<)
0.5	44.95 ± 1.02	20.06 ± 2.77	32.41 ± 1.22	2.58 ± 0.99
1.0	53.56 ± 3.75	14.95 ± 1.02	26.69 ± 2.31	4.80 ± 0.79
2.0	59.16 ± 1.95	12.16 ± 3.20	25.17 ± 2.10	3.51 ± 0.28
3.0	40.04 ± 2.66	15.59 ± 2.14	31.98 ± 2.18	12.39 ± 1.21
6.0	11.42 ± 3.60	21.16 ± 2.00	32.04 ± 4.18	35.38 ± 3.71
9.0	1.72 ± 2.39	4.31 ± 2.71	12.93 ± 2.51	81.04 ± 5.27
Total	**35.14**	14.70	26.87	23.28
			64.86	

[a]Each value represents the mean ± standard deviation of at least three replicates.

Effect of sucrose or glucose on cotyledon number variations of somatic embryos

From the investigation for the effect of sucrose or glucose on the cotyledon formation of somatic embryos, it was observed that the frequency of the somatic embryos with three or more cotyledons (61.41%-80.12%) compared to the frequency of somatic embryos with two cotyledons (19.88%-38.59%), which was found to be high at each concentration of carbohydrates. In the case of sucrose, the somatic embryos with two cotyledons accounted for 35.14% in the total somatic embryos, and the remaining 64.86% were abnormal somatic embryos having three or more cotyledons (Fig. 2).

In special, the somatic embryo number with two cotyledons was the highest to 59.16% in 2.0% sucrose concentration, and was the lowest to1.72% in 9.0% sucrose concentration. On the other hand, the abnormal somatic embryos with three or four cotyledons did not differ significantly from 0.5% to 6.0% sucrose concentration, but they were significantly lowered to 4.31% and 12.93% in the high concentration of 9% sucrose, respectively. Somatic embryos with five or more cotyledons significantly increased with the increasing of sucrose concentration, and it was up to 81.04% in 9% sucrose concentration (Table 2). In the case of glucose, the frequency of somatic embryos having two

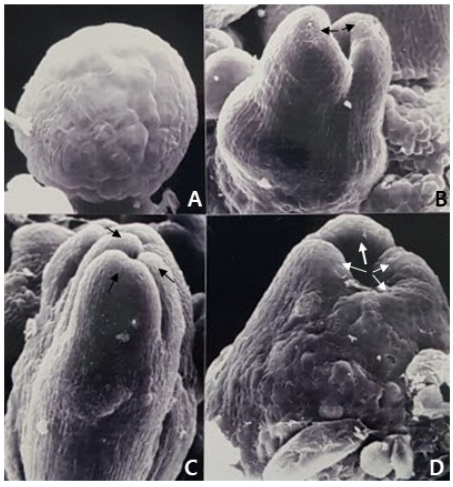

Fig. 2 Somatic embryos in globular stage and with cotyledon variations obtained from cell cultures of *Daucus carota* L. A: Somatic embryo in globular stage; B: Somatic embryo with two cotyledons; C: Somatic embryo with three cotyledons; D: Somatic embryos with four cotyledons

Table 3 Effects of glucose on the cotyledon number variations of somatic embryos formed in suspension cultures of *Daucus carota* L.

Glucose conc. (%)	[a]Cotyledon morphology of somatic embryos (%)			
	two	three	four	five (<)
0.5	0.00	0.00	0.00	0.00
1.0	48.39 ± 4.77	24.19 ± 3.28	14.52 ± 2.18	12.90 ± 1.62
2.0	23.14 ± 2.56	19.01 ± 1.02	33.06 ± 2.82	24.79 ± 1.50
3.0	17.50 ± 2.64	17.50 ± 1.80	31.25 ± 2.01	33.75 ± 2.19
6.0	7.77 ± 1.02	11.40 ± 1.29	44.56 ± 3.67	36.27 ± 2.71
9.0	2.61 ± 0.56	4.58 ± 0.33	35.29 ± 1.30	57.52 ± 1.85
Total	**19.88**	15.33	31.73	33.04
			80.12	

[a]Each value represents the mean ± standard deviation of at least three replicates.

Table 4 Effects of mannitol on the cotyledon number variations of somatic embryos formed in suspension cultures of *Daucus carota* L.

Mannitol conc. (%) + 2% sucrose	[a]Cotyledon morphology of somatic embryos (%)			
	two	three	four	five (<)
0.5	36.01 ± 2.50	26.30 ± 2.19	26.00 ± 3.17	11.69 ± 0.99
1.0	34.17 ± 3.11	27.08 ± 2.10	24.86 ± 2.55	13.89 ± 3.10
2.0	32.52 ± 2.86	20.19 ± 4.18	32.52 ± 0.77	14.77 ± 1.33
3.0	27.53 ± 2.00	23.08 ± 3.60	36.44 ± 3.90	12.95 ± 1.05
6.0 -9.0	0.00	0.00	0.00	0.00
Total	**32.55**	24.16	29.95	13.32
			67.44	

[a]Each value represents the mean ± standard deviation of at least three replicates.

cotyledons in total somatic embryos was 19.88%, and the frequency of abnormal somatic embryos having three or more cotyledons was 80.12%. The frequency of somatic embryos with two (48.39%) or three (24.19%) cotyledons reached the highest in 1.0% glucose concentration, and the frequency decreased with the increasing of glucose concentration. The frequency of the somatic embryos with four or five or more cotyledons gradually increased with the increasing of glucose concentration, and it reached the highest to 35.29% and 57.52% in 9% glucose concentration, respectively (Table 3).

The results showed a very similar trend to the achievements in previous studies in which the frequency of the normal somatic embryos with two cotyledons was increased at low concentrations of sucrose (< 2.0%), and the frequency of the abnormal somatic embryos with multi-cotyledons was increased at concentrations of sucrose (higher than 3.0%) in soybean somatic embryogenesis (Businge et al. 2013; Kageyama et al. 1990; Lazzeri et al. 1988; Vale et al. 2018). Therefore, it was found that sucrose was more effective than glucose as a carbon source for the somatic embryogenesis in carrots, and was confirmed that low concentrations of sucrose was more effective than high-concentration sucrose in the development of normal somatic embryos.

Effect of mannitol or sorbitol on cotyledon number variations of somatic embryos

When mannitol was combined applied with 2% sucrose by concentration, the frequency of the somatic embryos with two cotyledons in total somatic embryos was 32.55%, and the frequency of the abnormal somatic embryos with three or more cotyledons was 67.44%. The frequency of the somatic embryos with two or three cotyledons slightly decreased with the increasing of mannitol concentration, whereas the frequency of the somatic embryos with four cotyledons slightly increased. The frequency of the abnormal somatic embryos with five or more cotyledons did not change significantly by the increase of mannitol concentration (Table 4). When sorbitol was combined used with 2% sucrose by concentration, the frequency of the somatic embryos having two cotyledons was 38.59%, and that of the abnormal somatic embryos having three or more cotyledons was

Table 5 Effects of sorbitol on the cotyledon number variations of somatic embryos formed in suspension cultures of *Daucus carota* L.

Sorbitol conc. (%) + 2% sucrose	[a]Cotyledon morphology of somatic embryos (%)			
	two	three	four	five (<)
0.5	49.55 ± 3.18	31.18 ± 2.45	14.79 ± 0.72	4.88 ± 0.33
1.0	50.42 ± 2.87	31.59 ± 3.77	15.26 ± 1.88	2.73 ± 0.20
2.0	36.83 ± 1.37	30.21 ± 2.53	23.35 ± 2.31	9.30 ± 1.77
3.0	29.39 ± 2.92	25.12 ± 3.70	25.71 ± 2.53	19.78 ± 2.18
6.0	26.76 ± 2.64	25.35 ± 2.67	30.99 ± 3.42	16.90 ± 3.73
9.0	0.00	0.00	0.00	0.00
Total	**38.59**	28.69	22.02	10.71
			61.41	

[a]Each value represents the mean ± standard deviation of at least three replicates.

61.41%. The frequency of the somatic embryos with two or three cotyledons decreased with the increasing of sorbitol concentration, whereas the frequency of the abnormal somatic embryos with four or more cotyledons increased (Table 5).

The application of the medium supplemented with high concentration of maltose not only reduced the frequency of the somatic embryos but also led morphological changes in somatic embryos (Strickland et al. 1986). In addition, the morphological changes of somatic embryos was also caused by exogenous hormones such as BAP, Zeatin, 2,4-D added to medium (Businge et al. 2013; Garcia et al. 2019; Lazzeri et al. 1987; Lee and Soh 1993; Strickland et al. 1986; Vale et al. 2018). Like this, our results indicated that the sucrose was more effective than monosaccharide such as glucose or the combination of sucrose and mannitol or sorbitol as osmotic agents in somatic embryogenesis in carrot, and high concentration of sucrose or osmotic agent reduced the formation of somatic embryo due to high osmotic effect. A phenomenon can be presumed that the occurrence of the abnormal somatic embryo with three or more cotyledons is caused by several major components (exogenous hormones and carbon sources) added to the medium. In the future, it is judged that research on culture medium optimization to reduce the frequency of the abnormal somatic embryos with three or more cotyledons, and to increase the frequency of the normal somatic embryos with two cotyledons should be conducted.

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