J. Plant Biol. 40(1) : 15~20 (1997)

Morphology of Carrot Somatic Embryos Formed in Medium with Abscisic Acid

Soh, Woong Young*, Duck Yee Cho[1], Kyung Sik Kim and Byung Yun Sun

Department of Biological Sciences, Chonbuk National University, Chonju 561-756
[1]Department of Biology, WooSuk University, Chonbuk 565-701, Korea

Somatic embryogenesis was induced from embryogenic cells derived from cotyledon explants cultured on MS medium supplemented with 1 mg/L 2,4-D. In order to clarify the effect of abscisic acid (ABA) on the morphology of somatic embryos, embryogenic cell clumps or developing somatic embryos were treated continuously, or briefly, with ABA during culture. When embryogenic cells in MS medium without 2,4-D were treated with 0.04 mg/L ABA for the first week, normal embryos with two cotyledons increased slightly and embryos with anomalous cotyledons decreased. However when cell clumps in 2,4-D-free medium were treated with ABA in the second week normal embryos with two cotyledons decreased prominently and this decrease of normal embryos also occurred in the continuous ABA treatment during culture. Thus the morphological abnormalities in somatic embryogenesis occurred by exogenous ABA treatment beyond globular stage or by continuous treatment. The length of somatic embryos with anomalous cotyledons was larger than that of normal embryos with two cotyledons in control but both the normal and anomalous somatic embryos treated with ABA were almost similar in length. Somatic embryos formed in medium with ABA were larger in size than those in control due mainly to enlarged cotyledons. The enlarged cotyledons were composed of a greater number of cells than those of control. Therefore the enlargement of cotyledon by exogenous ABA seems to be not due to the enlargement of cells in cotyledons.

Key words: somatic embryogenesis, cotyledon morphology, ABA, *Daucus carota*

A successful system for mass production of normal somatic embryos is required for the development of plant biotechnology as well as for scientific research. The conversion rate of somatic embryos into plants is very low compared with zygotic embryos and the rate is related to the morphology of the somatic embryos (Soh, 1993, 1996; Adriana *et al.*, 1994; Soh *et al.*, 1996). This phenomenon is the main limitation to the industrial scale application of somatic embryos. Carrot was the first species in which somatic embryogenesis was reported (Steward *et al.*, 1958). Since then, many reports have been published using carrot as experimental material (Halperin and Wethrell, 1964; Fujimura and Komamine, 1975; Kamada *et al.*, 1989; Smith and Krikorian, 1990; Nickle and Yeung, 1993; Soh *et al.*, 1996). However, there still remain the problematic phenomena such as the appearance of abnormal somatic embryos and low conversion rates into plant (Soh *et al.*, 1996). Structurally, carrot somatic embryos with anomalous cotyledons, multiple, and jar type cotyledons appear at fairly high frequency, and such abnormalities are functionally related to the low conversion rates.

It is known that exogenous ABA enhances the development of normal somatic embryos which are similar to zygotic embryos in caraway and in carrot (Ammirato, 1974, 1977, 1983; Kamada and Harada, 1981; Nickle and Yeung, 1993). However in celery (Kim *et al.*, 1987), the production of normal somatic embryos on medium containing ABA did not increase at a noteworthy rate. In cell clump cultures of *Aralia cordata*, when ABA added to 2,4-D-free medium for a brief period (one week) and then subcultured in 2,4-D-free medium without ABA, the development of normal somatic embryos was enhanced (Lee and Soh, 1994, 1997). But continuously treated cell clumps or briefly treated ones in 2,4-D-free medium during the last half of the culture

*Corresponding author: Fax +82-652-70-3362

period developed into abnormal somatic embryos at high frequencies compared to control.

Thus it is still necessary to elucidate the effect of exogenous ABA on the development of normal somatic embryos which would make them more similar to zygotic embryos. The purpose of the present study was to clarify whether the enhancing effect of exogenous ABA on the development of structurally normal somatic embryos is dependent on dvelopmental stage-specific. The focal point of our observation was adjusted to the cotyledon morphology of somatic embryos.

MATERIALS AND METHODS

The soaked seeds of *Daucus carota* L. cultivar Obok were surface-disinfected in ethanol for 1 min and in 1% sodium hypochlorite for 15 min then rinsed four times with sterile-distilled water. Cotyledon segments of seedlings germinated on half-strength MS basal medium (Murashige and Skoog, 1962) were cultured on MS medium supplemented with 1 mg/L 2,4-D. All media were adjusted to pH 5.8, autoclaved at 121°C for 15 min and dispensed into 250 mL flask with 40 mL medium in each. Cultures were maintained at a constant temperature of 25°C with a low intensity of illumination (40 $\mu mol/m^2sec^{-1}$) from cool-white fluorescent light for 16 hr daily.

Callus was induced on cotyledon explants after two weeks of culture. Embryogenic callus was selected from the callus and cultured in MS liquid medium on a gyratory shaker at 100 rpm. Embryogenic cell suspensions of 40mL were transferred to 250 mL flask with 2,4-D-free liquid medium of 50 mL for somatic embryo formation. The counting of the embryos formed was made on the 14th day of culture in 2,4-D-free medium. For treatment with ABA, a filter-sterilized ABA solution was added to the MS media at concentrations ranging from 0.008 to 1 mg/L. The cell clumps or developing somatic embryos in 2,4-D-free medium during the first half or the last half periods were treated with ABA for one week or continuously during culture.

For anatomical study, five somatic embryos at cotyledonary stage were collected for each examination, fixed in FAA (formalin 5 : acetic acid 5 : alcohol 90 by volume) for over 48 hr at room temperature, dehydrated in a tertiary butyl alcohol series and embedded in paraffin. Embedded blocks were sectioned with a rotary microtome at 10 μm, the sections stained with hematoxylin, safranin and fast green, and mounted with Canada balsam.

RESULTS

In general carrot somatic embryos are routinely differentiated through globular and heart-shaped stages within one week in culture after embryogenic cell clumps in MS medium supplemented with 1 mg/L 2,4-D are transferred to medium without 2,4-D. The yield percent of the somatic embryos in this experiment were normal embryos with two cotyledons at 63%, and the remainder had anomalous cotyledons such as multiple, one and jar type cotyledons which were congruent with our previous report (Fig. 1; Soh *et al.*, 1996). To examine the effect of ABA on cotyledon morphology, ABA of four levels was tested on 2,4-D-free medium for the first week (Table 1), and in the result 0.04-0.2 mg/L was found to be favorable to normal cotyledon development of embryos.

In the case of ABA treatment to embryogenic cell clumps in MS medium with 2,4-D for one week normal somatic embryos with two cotyledons were formed at a similar frequency compared to control (Fig. 2). When embryogenic cells cultured in MS medium without 2,4-D were treated by ABA for the first week, normal embryos with two cotyledons increased slightly to 68% and total embryos with anomalous cotyledons decreased even though there was an increase of embryos with jar type cotyledons (Fig. 2). In another experiment, developing embryos in 2,4-D-free medium for embryo maturation were treated by ABA for the last week. The result was quite the opposite in that normal embryos with two cotyledons decreased prominently and embryos with anomalous cotyledons increased. And embryos with jar type cotyledons (Fig. 2) increased compared with control except cultures in MS medium with 2,4-D for one week. In the case of continuous ABA treatment during culture in 2,4-D-free medium for two weeks, the frequency of normal embryo development was almost the same as the above experiment-the last week treatment in 2,4-D-free medium (Fig. 2). However, somatic embryos with jar type cotyledons clearly increased in the cultures treated with ABA regardless of the period treated and in parallel to the length of treatment.

Somatic embryos formed in medium with ABA were larger in length and width than untreated ones (Fig. 1, 3), and the embryos also have heavier fresh weight than untreated ones. In the case of ABA treatment during culture in 2,4-D-free medium for the first week anomalous somatic embryos with multiple cotyledons were larger in size than normal embryos

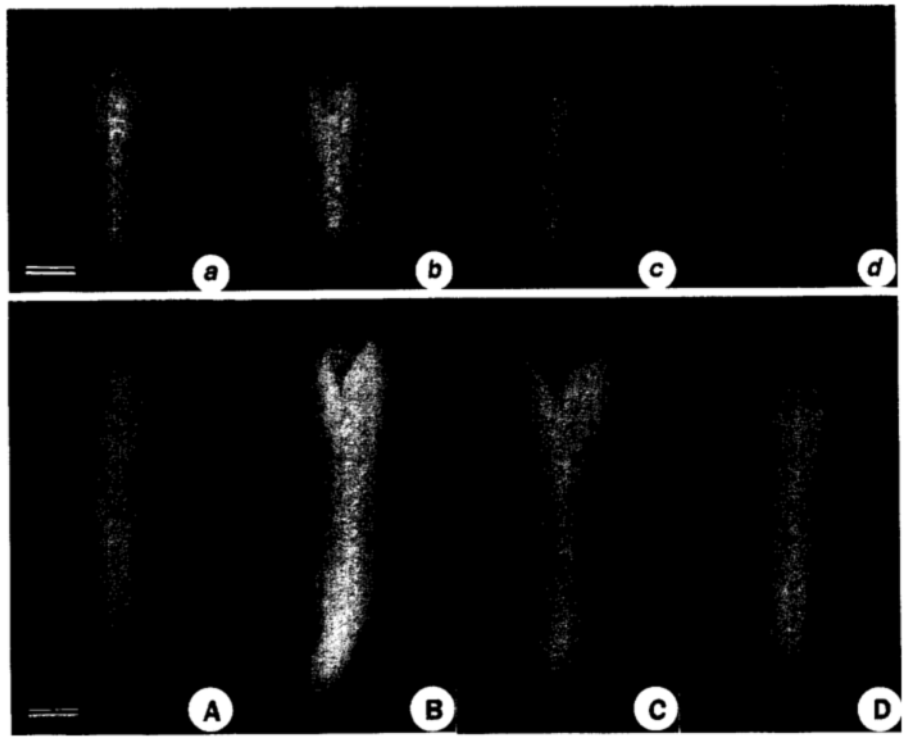

Fig. 1. Carrot somatic embryos with various type cotyledones formed in 2,4-D-free MS medium (a-d) and in the same medium treated with 0.04 mg/L ABA for the first week (A-D). The embryos have jar type (a, A), one (b, B), two (c, C), or three (d, D) cotyledons. ABA treated-embryos are larger in size than untreated ones. Scale bars=320 μm.

Table 1. Effect of ABA on the cotyledon number of somatic embryos in cell cultures of *Daucus carota* L.

Concentration (mg/L)	Cotyledon types(%)					
	Jar	1	2	3	4	5
0.000	1.0 ±0.1	4.5 ±0.3	63.0 ±1.1	21.0 ±1.3	6.0 ±0.4	4.5 ±0.3
0.008	3.0 ±0.3	2.7 ±0.1	62.0 ±1.6	27.0 ±1.9	4.0 ±0.3	1.3 ±0.2
0.04	3.1 ±0.2	2.0 ±0.8	70.0 ±2.2	19.1 ±2.5	3.6 ±0.4	2.2 ±1.5
0.2	2.6 ±0.6	2.0 ±0.4	68.0 ±1.4	19.0 ±2.8	6.0 ±2.8	2.4 ±0.5
1.0	4.3 ±0.6	4.4 ±1.1	58.0 ±1.4	23.0 ±2.6	8.0 ±3.7	2.3 ±0.4

Cultures were treated with ABA in MS medium without 2, 4-D for the first week. The values represent the mean± SE of at least three replicated experiments. Each treatment consisted of 200 embryos.

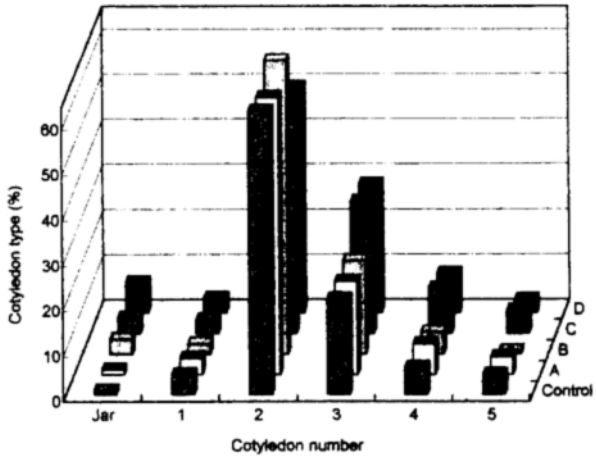

Fig. 2. Effect of ABA treatment stage on the cotyledon type of somatic embryos formed in cell cultures of *Daucus carota* L. Cultures were treated with 0.04 mg/L ABA in MS medium with 2,4-D for one week(A), in MS medium without 2,4-D for the first week (B), in 2,4-D-free medium for second week (C), and cotinuously in 2,4-D-free medium (D). The data represent the average of three replicates, and were obtained from the count of at least 500 embryos.

with two cotyledons. But the length of embryos with multiple cotyledons was similar to that of normal embryos with two cotyledons. In anatomical examination of organ, the cells of enlarged cotyledons and embryo axes was almost the same in size as those of untreated embryos (Fig. 3). Therefore, it was clear that the cotyledons of embryos formed in medium with ABA are composed of more cells than

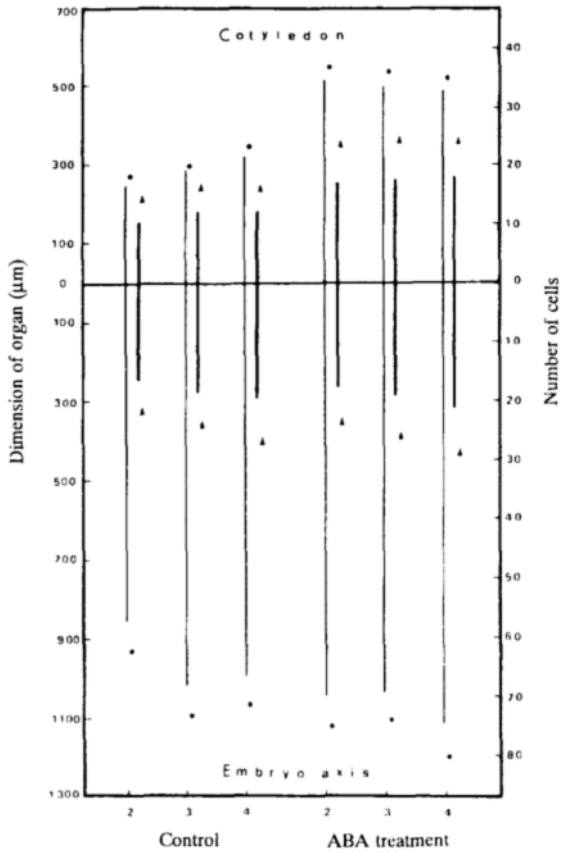

Fig. 3. Dimension of carrot somatic embryos with two, three and four cotyledons formed in MS medium (control) and in the same medium treated with 0.04 mg/L ABA for the first week. Vertical thin and thick lines represent the length and width of somatic embryos respectively. The number of cells arranged in longitudinal (●), and transverse (▲) direction of both organs was counted throughout whole extent of the organs, and was in parallel with the dimension of the organs. The data represent the average of three replicates, and were obtained from the count of at least 300 embryos for length and width measurement and from the anatomical examination of 15 embryos for cell number count.

those of control, and the cells are not enlarged.

DISCUSSION

Although it is expected that somatic embryos will appear identical to zygotic embryos, in practice there are many abnormal somatic embryos included in the obtained populations from the present experiment and others (Table 1; Ammirato, 1987; Suhasini *et al.*, 1990; Soh, 1993, 1996; Wetzstein and Baker, 1993). It is known that ABA treatment to embryogenic cells of caraway in 2,4-D-free medium promoted the development of normal somatic embryos similar to zygotic embryos (Ammirato, 1974, 1977, 1983). Such an enhanced production of normal somatic embryos by ABA treatment would be very useful for plant regeneration, because somatic embryos with abnormal structures especially, cotyledon abnormalities show low germinability (Soh, 1996; Soh *et al.*, 1996).

In the present experiments where embryogenic cell clumps in 2,4-D-free medium were treated with ABA for the first week the normal embryos increased and abnormal embryos decreased. These results are similar to those of *Aralia cordata* (Lee and Soh, 1994) but different from those of *Daucus carrota* L. cv. us-Harumaki gosun (Kamada and Harada, 1981). Thus it is suggested that the response to ABA treatment in the same species differs depending on genotype or the dissimilarity of observation point: root growth vs. cotyledon number. In the next experimental results where embryogenic cells in 2,4-D-free medium were treated with ABA for the last week, somatic embryos unexpectedly had anomalous cotyledons at a prominently high frequency. Because embryogenic cell clumps in medium treated with ABA during the first week developed into late globular stage embryos, it is clear that the late globular stage, at which cotyledon primordium is initiated, is the controllable stage of cotyledon development in our material. Therefore it is supposed that the most positive response of ABA treatment for normal embryogenesis would occur by targeting the initiation stage of the cotyledon primordium. However, in the case of *Sapindus trifoliatus* the response was different from our results: the enhanced development of normal somatic embryo occurred by ABA treatment after heart-shaped stage (Unnikrishnan *et al.*, 1990). In the continuous treatment of ABA during culture, the results were almost the same as in those of ABA treatment for the last week in 2,4-D free-medium. From these results it is suggested that normal cotyledon development is scarcely controlled by ABA treatment to 2,4-D-free medium during the last week, from the heart-shaped stage to the cotyledonary stage. However this response to exogenous ABA differs depending on species because normal cotyledon development was promoted by continuous ABA treatment in caraway cell culture (Ammirato, 1974, 1977).

The enhanced development of normal somatic embryos by exogenous ABA occurred at the globular stage in 2,4-D-free medium (carrot: present study, Fig. 2; *Aralia cordata*: Lee and Soh, 1994), after the

heart-shaped stage (*Sapindus trifoliatus*: Unnikrishnan *et al.*, 1990), and ABA treatment beyond such stages induced anomalous embryogenesis. Therefore it is suggested that the enhancement of morphological normalities in somatic embryogenesis by exogenous ABA is dependent upon species or genotype, and late globular stage-specific in our material. There is one instance, in celery (Kim *et al.*, 1987), where morphological abnormalities during somatic embryogenesis were not only exacerbated by ABA treatment, but also without enhanced nomality.

Other growth regulators also effected the development of somatic embryos with anomalous cotyledons. Embryogenic callus of *Aralia cordata* cultured continuously on 2,4-D-containing medium developed into abnormal somatic embryos with jar or bowling pin type cotyledons at a high frequency (Lee and Soh, 1997). Somatic embryos with anomalous cotyledons of soybean formed under the same conditions (Choi *et al.*, 1994b). More abnormal somatic embryos were obtained from the cultures of pecan initiated with 2,4-D than with NAA (Adriana *et al.*, 1994). In the culture of melon embryogenic callus on BA-containing medium, somatic embryos with jar type cotyledons appeared at a three times higher rate than two cotyledon ones (Choi *et al.*, 1994a). In addition BA and other cytokinins influenced the development of somatic embryos with anomalous cotyledons in *Aralia cordata* (Lee and Soh, 1993). Therefore it is suggested that the disturbance of endogenous hormone balance by exogenous growth regulator treatment might result in the initiation of abnormal cotyledon primordia in turn causing cotyledon abnormalities to appear.

Somatic embryos were larger in size than zygotic ones in our materials, and caraway also showed a similar tendency (Ammirato, 1974). The sizes of stress-induced somatic embryos were variable in carrot showing high germinability in large ones (Kamada *et al.*, 1989). Because in anatomical examination embryos developed in medium with ABA composed of a larger number of cells, it is suspected that the enlargement of somatic embryos in medium with ABA are due to a proliferation of cells in embryos as in the case of *Aralia cordata* (Lee and Soh, 1997) and Jerusalem artichoke and tobacco (Minocha, 1979; Kocchar, 1980). It is clear in the present experiment that the cotyledon morphology of somatic embryos are more susceptible to exogenous ABA during its early morphogenesis because cotyledons were more enlarged than embryo axes.

In conclusion, the cotyledon morphology of somatic embryos formed in ABA-free medium can be influenced depending on the stage treated. It is deduced that this phenomenon is related to the inconsistency of hormonal requirement during somatic embryogenesis. Thus, a more thorough understanding of this inconsistent requirement of growth hormone is necessary for the production of normal somatic embryos.

ACKNOWLEDGEMENT

The present studies were supported by the Basic Science Research Institute Program, Ministry of Education, 1995, Project No, BSRI-95-4427. The authors are thankful to Professor S. S. Hong, Department of Biology Education, Chonnam National University for his helping anatomical examination, and Misses E. K. Lee and N. S. Kim, Department of Biology, Graduate School, Chonbuk National University for their technical assistance and for typing manuscript.

LITERATURES CITED

Adriana, P.M. Rodriguez and Hazel Y. Wetzstein. 1994. The effect of auxin type and concentration on pecan (*Carya illinoinensis*) somatic embryo morphology and subsequent conversion into plant. *Plant Cell Reports*, **13**: 607-611

Ammirato, P.V. 1974. The effect of abscisic acid on the development of somatic embryos from cells of caraway (*Carum carvi* L.). *Bot. Gaz.* **135**: 328-337.

Ammirato, P.V. 1977. Hormonal control of somatic embryo development from cultured cells of caraway. Interactions of abscisic acid, zeatin, and gibberellic acid. *Plant Physiol.* **59**: 579-586.

Ammirato P.V. 1983. The regulation of somatic embryo development in plant cell cultures: Suspension culture techniques and hormone requirements. *Bio/Technology* **1**: 68-74.

Ammirato P.V. 1987. Organizational events during somatic embryogenesis. In: Plant Tissue and Cell Culture, C. E. Green *et al.*, eds, Alan R. Liss Inc, New York, pp. 57-81.

Choi, P.S., W.Y. Soh, D.Y. Cho and J.R. Liu. 1994a. High frequency somatic embryogenesis and plant regenreation in seedling explant cultures of melon (*Cucumis melo* L.). *Korean J. Plant Tissue Cult.* **21**: 1-6.

Choi, P.S., W.Y. Soh, D.Y. Cho and J.R. Liu. 1994b. Somatic embryogenesis in immature zygotic embryo cultures of Korean soybean (*Glycine max* L.) cultivars and effect of 2,4-dichlorophenoxy acetic acid on somatic embryo morphology. *Korean J. Plant Tissue Cult.* **21**: 7-13.

Fujimura, T. and A. Komamine. 1975. Effects of vari-

ous growth regulators on the embryogenesis in a carrot cell suspension culture. *Plant Sci. Lett.* **5**: 359-364.

Halperin, W. and D.F. Wethrell. 1964. Adventive embryony of tissue cultures of the wild carrot, *Daucus carota*. *Amer. J. Bot.* **51**: 274-283.

Kamada, H. and H. Harada. 1979. Studies on the organogenesis in carrot tissue cultures I. Effects of growth regulators on somatic embryogenesis and root formation. *Z. Pflanenphysiol* **91**: 255-268.

Kamada, H. and H. Harada. 1981. Changes in the endogeneous level and effects of abscisic acid during somatic embryogenesis of *Daucus carota* L. *Plant & Cell Physiol.* **22**: 1423-1429

Kamada, H., K. Kobayashi, T. Kiyosue and H. Harada. 1989. Stress induced somatic embryogenesis in carrot and its application to synthetic seed production. *In Vitro Cell. Dev. Biol.* **25**: 1163-1166.

Kim Y.H., Chung T.Y. and Choi W.Y. 1987. Effects of sucrose and abscisic acid on somatic embryogenesis in celery (*Apium graveolens* L.). *Korean J. Plant Tissue Cult.* **14**: 123-130.

Kocchar, T.C. 1980. Effect of abscisic acid and auxins on the growth of tobacco callus. *Z. Pflanzenphysiol.* **97**: 1-4.

Lee K.S. and Soh W.Y. 1993. Effect of cytokinins on the number of cotyledons of somatic embryos from cultured cells of *Aralia cordata* Thunb. *Korean J. Plant Tissue Cult.* **20**: 171-175.

Lee K.S. and Soh W.Y. 1994. Effects of ABA on the number of somatic embryo cotyledons in tissue cultures of *Aralia cordata* Thunb. *Korean J. Plant Tissue Cult.* **21**: 287-291.

Lee, K.S. and W.Y. Soh. 1997. Structural aspects of somatic embryos developed from *Aralia cordata* cells cultured in medium with ABA. *Phytomorphology* (in press).

Minocha, S.C. 1979. Abscisic acid promotion of cell division and DNA synthesis in jerusalem artichoke tuber tissue cultured *in vitro*. *Z. Pflanzenphysiol.* **92**: 327-339.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**: 473-495.

Nickle, T.C. and Yeung, E.C. 1993. Failure to establish a functional shoot meristem may be a cause of conversion failure in somatic embryos of *Daucus carota* (Apiaceae). *Amer. J. Bot.* **80**: 1284-1291.

Smith, D.L. and A.D. Krikorian. 1990. pH control of carrot somatic embryogenesis. In, Nijkamp, H.J.J., L. H.W. Van der Plas, J. van Aatrijk, eds., Porgress in Plant Cellular and Molecular Biology, Kluwer Academic Publishers, Dordrecht, pp 449-453.

Soh W.Y. 1993. Developmental and structural diversity of regenerated plants in cell and tissue cultures. Mol. Appro. Plant Cell Differ., Proc. 7th Symp. Plant Biotich., Bot. Soc. Kor. 1-35.

Soh W.Y. 1996. Germinability and cotyledon structure of somatic embryos in some dicotyledonous plants. Proc. 2nd Asia-Pacific Conf. Plant Cell & Tiss. Cult., Beijing pp. 51-59.

Soh W.Y., Cho D.Y. and Lee E.K. 1996. Multicotyledonary structure of somatic embryos formed from cell cultures of *Daucus carota* L. *J. Plant Biol.* **39**: 71-77.

Steward, F.C., Mapes M.O. and Mears K. 1958. Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. *Amer. J. Bot.* **45**: 705-708.

Suhasini, K., A.P. Sagare and K.B. Krishnamurthy. 1990. Study of aberrant morphologies and lack of conversion of somatic embryos of chickpea (*Cicer arietinum* L.). *In Vitro Cell. Dev. Biol.-Plant* **32**: 6-10.

Unnikrishnan, S.K. and A.R. Mehta. 1990. Abscisic acid induced high frequency embryogenesis from *Sapindus trifoliatus* leaves. Acta Horticulturae. In Vitro Culture and Horticultural Breeding. Janik, J. and R.H. Zimmerman eds., pp 89-94.

Wetzstein, H.Y. and C.M. Baker. 1993. The relationship between somatic embryo morphology and conversion in peanut (*Arachis hypogaea* L.). *Plant Sci.* **92**: 81-89.

(Received November 27, 1996)