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Effects of ABA on Secondary Embryogenesis from Somatic Embryos Induced from Inflorescence Culture of *Aralia cordata* Thunb.

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In order to investigate the effect of ABA on secondary embryogenesis from somatic embryos in *Aralia cordata* Thunb., embryogenic callus and somatic embryos were induced from inflorescence on solid MS basal medium supplemented with 1.5 mg/L 2,4-D after eight weeks without subculture. For mass production of somatic embryos, embryogenic cell clumps were maintained in liquid MS medium supplemented with 1.0 mg/L 2,4-D, and then transferred to 2, 4-D-free medium. When developing embryos at various stages were cultured separately in liquid medium with ABA (0 to 2.0 mg/L) for three weeks, and then cultured in ABA-free liquid medium for two weeks, torpedo-shaped embryos exhibited secondary embryogenesis of 65.9% in only 0.2 mg/L ABA pretreatment. Cotyledonary embryos in cultures by 0.2, 0.5 and 1.0 mg/L ABA pretreatment also exhibited secondary embryogenesis (73%, 9.4% and 6.0%, respectively). However, globular and heart-shaped somatic embryos treated with ABA did not form secondary embryos on their hypocotyl surfaces. When cotyledonary embryos were cultured in ABA-free medium or 0.2 mg/L ABA treated medium for three weeks, and then in ABA-free liquid medium for 6 weeks, the germination frequency was lower in medium with 0.2 mg/L ABA (45.9%) than in hormone-free medium (56.8%). This result seems to be related to the high frequency of secondary embryogenesis. It is suggested that secondary embryogenesis by ABA application depends upon the stage of embryo cultured and the ABA concentration.

Keywords: secondary somatic embryogenesis, ABA, *Aralia cordata*

INTRODUCTION

A successful system for mass production of somatic embryos is required for the development of plant biotechnology as well as for scientific research. The conversion frequency of somatic embryos into plants is very low compared with zygotic embryos and the frequency is related to the morphology of somatic embryos (Soh, 1996; Soh *et al.*, 1996). It is known that exogenous ABA enhances the development of normal somatic embryos in caraway, *Daucus carota* and *Aralia cordata* (Ammirato, 1974, 1987; Kamada and Harada, 1981; Lee and Soh, 1994). However, it has been reported that the production of normal somatic embryos was dependent on the application period of ABA in *Daucus carota* and *Aralia cordata* (Soh *et al.*, 1997; Lee and Soh, 1998).

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ABA applied to embryogenic cell clumps in caraway prevented secondary somatic embryogenesis (Ammirato, 1987), but secondary embryogenesis from globular somatic embryos occurred in celery cell cultures (Nadel *et al.*, 1990) and from torpedo-shaped embryos in carrot cell cultures (Iida *et al.*, 1992). Therefore, the role of ABA on secondary embryogenesis is not consistent among plant species used in experiments. In *Aralia cordata* secondary somatic embryos was induced by cytokinins treatment (Lee and Soh, 1993b), however, secondary somatic embryogenesis by ABA treatment has not yet been reported. The present study was undertaken to clarify the effects of ABA on secondary embryogenesis.

MATERIALS AND METHODS

Plant Materials and Culture Condition

The immature inflorescences of *Aralia cordata*

Thunb., a perennial herbaceous plant were first sterilized with 70% ethanol for 1 min, 1% sodium hypochlorite solution for 10 min and then rinsed four times with sterile distilled water. Explants were cultured in petridishes (87 mm × 15 mm) containing MS (Murashige and Skoog, 1962) medium (pH 5.8) supplemented with 0.2 to 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g/L sucrose and 8 g/L agar. Abscisic acid (ABA) was applied after sterilization with membrane filter (pore size: 0.22 μm). All cultures were maintained at 25 ± 1°C and a 16 h photoperiod with cool white fluorescent light of 24 μmol m^{-2} s^{-1}.

Cell Suspension Culture and Somatic Embryogenesis

Embryogenic callus was cultured in liquid MS medium containing 1.0 mg/L 2,4-D on a gyrating shaker at 110 rpm and then subcultured at two-week intervals. The embryogenic cell clumps were filtered twice using 200 to 100 μm stainless steel sieves to remove large clumps, the supenatant which was allowed to settle for 10 min and then proliferated in the same medium for 4 weeks.

Somatic embryos of various developmental stages were induced from embryogenic cell clumps in 2,4-D-free medium (Fig. 3C-F). Embryos of each stage were obtained by sieving the cultures through stainless sieves (100 to 450 μm). The density of the cultures was approximately 0.2 mL (packed volume) per 100 mL Erlenmeyer-flask containing 20 mL liquid medium. ABA treatment (0.2 to 2.0 mg/L) was given to embryos of each stage for three weeks, and then the cultures were transferred to ABA-free liquid medium. In all experiments three replicates were used for each treatment and the experiment was repeated three times.

Germination in Liquid Medium

In this study, the term "germination" refers to the development of plantlets with a well established root and shoot with at least the first pair of leaves (Fig. 3 B and F). Cotyledonary embryos were cultured in liquid MS basal medium or with 0.2 mg/L ABA for three weeks, and then in ABA-free liquid medium for six weeks. Germination frequency was evaluated as the percentage of embryos showing germination. Embryos counted per replicate were approximately 200.

Histological Observation

Cotyledonary nodes with secondary embryos were fixed under vacuum for 20 minutes with FAA (formaldehyde : glacial acetic acid : ethanol : water = 2 : 1 : 10 : 7, v/v) and subsequently stored in FAA for 7 days at room temperature. The fixed tissues were dehydrated in a gradual ethanol-toluene series. Embedded materials in paraplast were sectioned at 10 μm using a rotary microtome. The sections, stained with hematoxylin, safranin and fast green, were mounted in Canada balsam for microscopic observations.

RESULTS

Somatic Embryogenesis

Callus was initiated from the basal part of inflorescence on MS medium with 0.1 to 2.0 mg/L 2,4-D after three weeks of culture (Fig. 2A). However, embryogenic callus and somatic embryos were induced from the explants on solid medium supplemented with 0.5 to 2.0 mg/L 2,4-D after eight weeks without subculture (Fig. 2B). The explants forming somatic embryos at the highest frequency (42.2%) were observed on the medium with 1.5 mg/L 2,4-D, but somatic embryos did not form on the medium with 0.1 mg/L 2,4-D (Fig. 1). Embryogenic callus induced from solid MS medium supplemented with 2,4-D (0.5 to 2.0 mg/L) was transferred to MS liquid medium containing 1.0 mg/L 2,4-D and

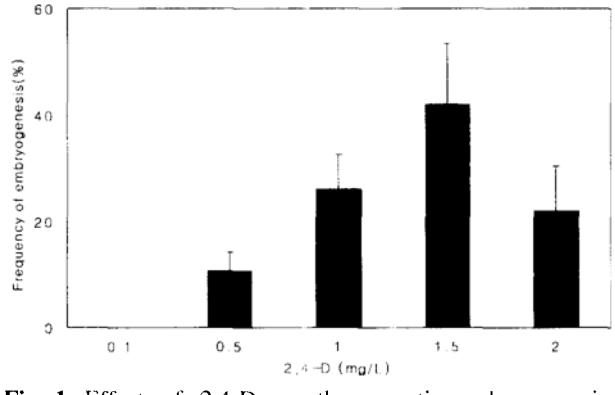

Fig. 1. Effect of 2,4-D on the somatic embryogenesis from the inflorescence of *Aralia cordata* after eight weeks without subculture. Data represent the mean ± SE of triplicates, was evaluated by No. of explants forming somatic embryos / No. of explants cultured. Explant number per replicate was 30, respectively.

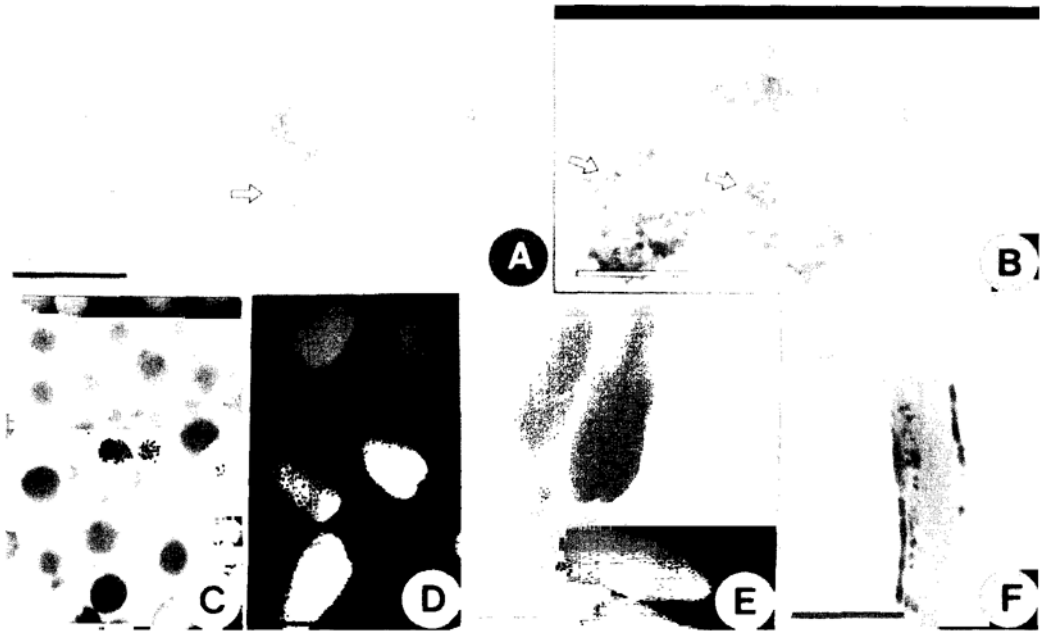

Fig. 2. Callus induction and somatic embryogenesis from inflorescence culture. Callus (A, arrow) and somatic embryos (B, arrows) induced on solid MS medium supplemented with 1.5 mg/L 2,4-D after three (A) and eight weeks (B) of culture. Globular embryos (C), heart (D), torpedo-shaped embryos (E) and cotyledonary embryo (F) induced from embryogenic cell clumps in liquid MS basal medium. Bar=1 cm (A & B). Bar=1,700 μm (C, D, E & F).

subcultured at two-week intervals. For mass production of somatic embryos, the embryogenic cell clumps selected using a stainless steel sieve were transferred to 2,4-D-free liquid medium. After about two weeks of culture, globular embryos were induced (Fig. 2C). The globular embryos were matured to cotyledonary embryos after one or two weeks of culture (Fig. 2D-F).

Secondary Embryogenesis

When somatic embryos at various stages induced in hormone-free liquid medium were cultured separately in liquid medium with ABA (0 to 2.0 mg/L) for three weeks, and then transferred to ABA-free liquid medium for two weeks, torpedo-shaped embryos exhibited secondary embryogenesis of 65.9% in only the 0.2 mg/L ABA pretreatment whereas cotyledonary embryos in cultures pretreated with 0.2, 0.5 and 1.0 mg/L ABA exhibited secondary embryogenesis (73%, 9.4% and 6.0%, respectively). However, secondary embryos did not form in somatic embryos treated at the globular and heart-shaped stages in all ABA pretreatments (Table 1).

The secondary embryos formed from only the upper hypocotyl region of primary embryos (Fig. 3C, F). Histological analysis revealed that the secondary embryos had a multicellular origin from both epidermal and subepidermal layers (Fig. 3D-E).

Germination in Liquid Medium

Cotyledonary embryos were separately cultured in liquid MS basal medium or with 0.2 mg/L ABA for three weeks, and then in ABA-free liquid medium for 6 weeks. Germination frequency was lower in medium with 0.2 mg/L ABA (45.9%) than in hormone-free medium (56.8%) (Table 2, Fig. 3 B and F). In order to elucidate the reason for this inhibitory effect of ABA, a scrutinous observation was carried out. Primary embryos with secondary embryos showed only a 52.2% germination frequency (Table 3, Fig. 3F).

DISCUSSION

Induction of somatic embryogenesis has been successful in 42.2% of inflorescence explants of *Aralia cordata* on MS medium supplemented with 1.5 mg/L 2.4-D. This frequency was lower than those of leaf, petiole, cotyledon explants and regenerants from tissue cultures (Lee and Soh, 1993a). However, zygotic embryos as an explant

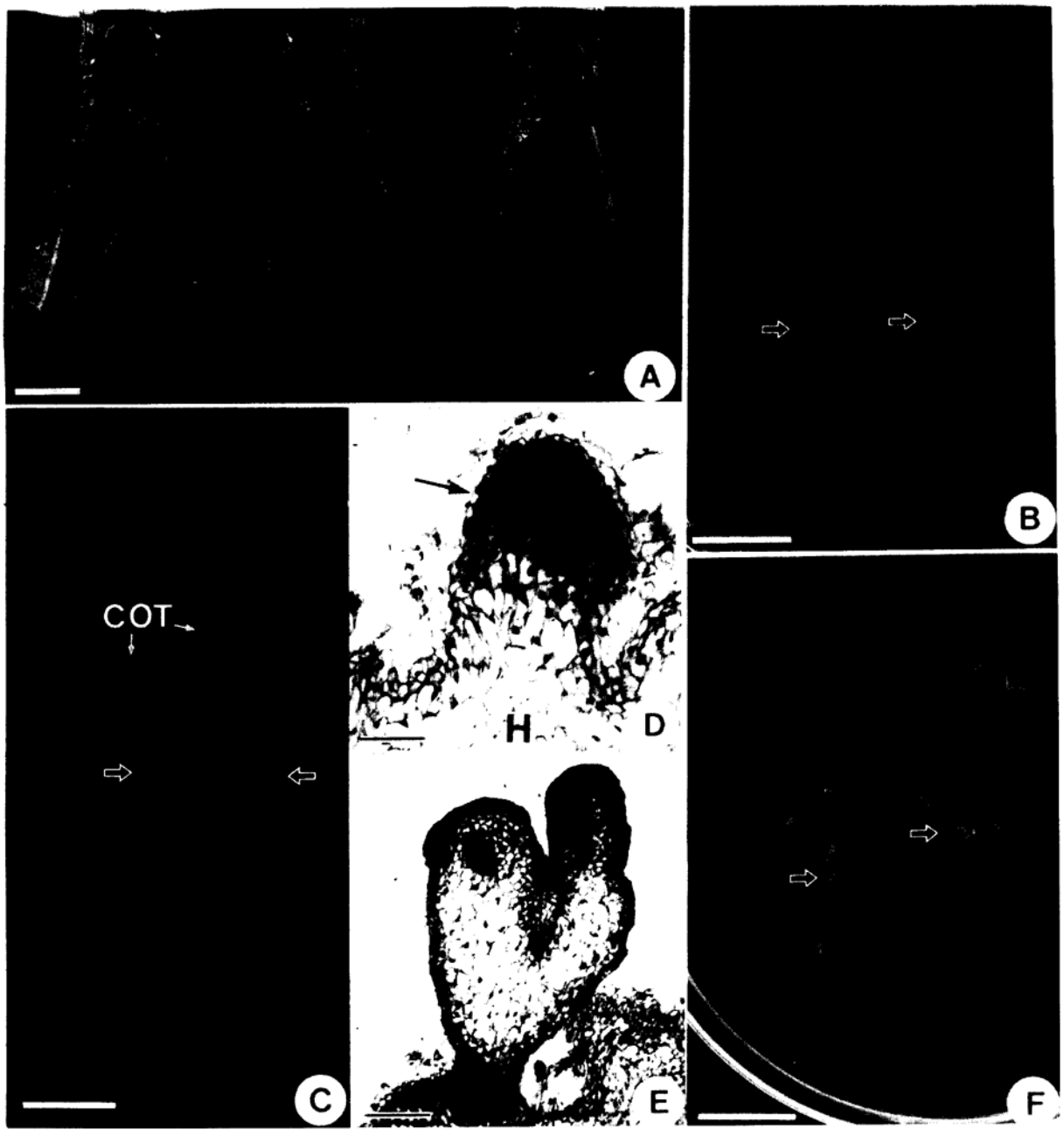

Fig. 3. Germination and secondary embryogenesis from embryos induced in liquid MS basal medium or with ABA. A, Plantlets regenerated from cotyledonary embryos. Bar=1.8 cm. B, Enlarged view of A. Arrows=cotyledons. Bar=1 cm. C, Secondary embryos (arrows) formed on hypocotyl, COT=cotyledon. Bar=5 mm. Cross section of a globular embryo (D, arrow) and torpedo-shaped embryo (E) formed on hypocotyl surface. Bar=145 μm (D, E). F, Germinating primary embryos with secondary embryos (arrows). Bar=1 cm.

source for somatic embryogenesis are inconvenient, because the zygotic embryos in seeds are immature immediately after harvest and it takes long period, of about 6 months, for maturation of the seeds, also embryo maturation frequency is low (Lee and Soh, 1993a). Therefore, inflorescences seem to be a useful material for somatic embryogenesis even if the harvest time is limited.

A successful system for mass production of normal somatic embryos is required for the development of plant biotechnology as well as for scientific research. Thus, ABA has been used for the

Table 1. Relationship between (0 to 2.0 mg/L) ABA treatment to developing somatic embryos at various stages of *Aralia cordata* in MS medium and secondary embryogenesis in the developing embryos

Stages of somatic embryo	ABA (mg/L)				
	0	0.2	0.5	1.0	2.0
Globular, Heart	0	0	0	0	0
Torpedo	0	65.9±7.8	0	0	0
Cotyledonary	0	73.0±5.9	9.4±2.3	6.0±1.2	0

All embryos were cultured in liquid MS medium with (0 to 2.0 mg/L) ABA for three weeks and then transferred to liqiud ABA-free medium for two weeks. Data represent the mean+SE of triplicates. Embryos counted per replicate were 200, respectively.

development of normal somatic embryos in caraway, carrot and *Aralia cordata* (Ammirato 1974, 1987; Kamada and Harada, 1981, Soh *et al.*, 1997; Lee and Soh, 1994, 1998). In *Aralia cordata*, the frequency of normal somatic embryos with two cotyledons was increased only after ABA treatment to embryogenic cell clumps, but was decreased in the case of globular stages treated with ABA (Lee and Soh, 1994, 1998).

When embryos of different stages were treated with 0.2 mg/L ABA, torpedo-shaped and cotyledonary embryos exhibited profuse secondary embryogenesis (Table 1). Our observations on secondary embryogenesis by ABA treatment substantiate the findings of Iida *et al.* (1992) on torpedo-shaped embryos of carrot. Similar results were also obtained for globular embryos of celery (Nadel *et al.*, 1990). However, in caraway, secondary embryogenesis was prevented by embryogenic cell clump culture in ABA treated medium (Ammirato, 1987). Therefore, it is suspected that secondary embryogenesis by ABA treatment depends on the plant species or the stage of the developing embryos.

In the present study secondary embryogenesis developed only on the upper hypocotyl of primary embryos (Fig. 3 C and F). This differs from the cases where secondary embryos developed on the root apices of primary embryos in celery (Nadel *et al.*, 1990), and both cotyledons and hypocotyls, of primary embryos in *Juglans regia* (Polito *et al.*, 1989). Our histological analysis revealed that these embryos had a multicellular origin from both epidermal and subepidermal layers on the hypocotyl of primary embryos (Fig. 3D) as in the situation of *Camellia reticulata* (Plata *et al.*, 1991).

When cotyledonary embryos were cultured in liquid MS basal medium or with 0.2 mg/L ABA, and then in ABA-free liquid medium for 6 weeks, germination frequency was lower in medium with 0.2 mg/L ABA than in hormone-free medium. This result seems to be related to the high frequency of secondary embryogenesis caused by ABA treatment, because primary embryos with secondary embryos showed only 52.2% germination (Table 3). This suggests that secondary embryogenesis could be occurred when germination frequency of primary embryos was low. Primary embryos of *Aralia cordata* treated with cytokinins showed a morphological abnormality by swelling of hypocotyl part when secondary embryos were induced from primary embryos (Lee and Soh, 1993b). Therefore, secondary embryos seem to be a kind of morphologically abnormal structure on primary embryos caused by exogenous hormone such as ABA or cytokinins.

Desiccation stress increased plant germination from somatic embryos in carrot, it is suggested to be related to endogenous ABA content (Lee *et al.*, 1997). Moreover, it has been reported that a high frequency of plant regeneration on solid MS basal

Table 2. Effect of ABA on germination from cotyledonary embryos of *Aralia cordata* in liquid MS medium

ABA(mg/L)	Germination frequency(%)
0	56.8±1.1
0.2	45.9±2.9

Cotyledonary embryos were cultured in liquid MS basal medium or with 0.2 mg/L ABA for three weeks, respectively, and then they were cultured in liquid ABA-free medium for 6 weeks. Data represent the mean±SE of triplicates. Embryos counted per replicate were 200.

Table 3. Effects of ABA on germination and secondary embryogenesis from cotyledonary embryos of *Aralia cordata*

Secondary embryos	Frequency (%) of embryos		Total No. of embryos
	Germinated	Nongerminated	
with	198(38.4%) (52.2%)[a]	181(35.1%) (47.8%)[a]	379
without	39 (7.6%)	98(19.0%)	137
Total No. of embryos	237 (45.9%)	279 (54.1%)	516

Cotyledonary embryos were cultured in the MS liquid medium containing 0.2 mg/L ABA for three weeks, and then transferred to ABA-free liquid medium for 6 weeks.
[a]No. of germinated or nongerminated embryos/No. of primary embryos with secondary embryos×100.

medium was achieved from heart or torpedo-shaped embryos grown in liquid MS medium with ABA in *Aralia cordata* and carrot (Lee *et al.*, 1998: Nickle and Yeung, 1993). The results mentioned above are contrary to our results. Therefore, it is considered that plant regeneration through somatic embryos using ABA application depends on culture conditions such as solid or liquid medium, or on the stages of the developing embryos.

The present investigation revealed that a high frequency of secondary embryos was induced from suspension cultures of *Aralia cordata* by ABA treatment. We are currently developing a system for plant regeneration from secondary embryos.

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