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DEVELOPMENTAL BIOLOGY/MORPHOGENESIS

High-frequency plant regeneration via adventitious shoot formation from deembryonated cotyledon explants of *Sesamum indicum* L.

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Abstract *Sesamum indicum* L. was used as an important oil crop in the world. An efficient protocol for *in vitro* plant regeneration via adventitious shoot formation from deembryonated cotyledon explants isolated from mature seeds of sesame is developed. Optimal medium for direct adventitious shoot formation was Murashige and Skoog (MS) medium with 22.2 μM 6-benzylaminopurin (BA) and 5.7 μM indole-3-acetic acid (IAA). Abscisic acid (3.8 μM ABA) and $AgNO_3$ (29.4 μM) were effective in enhancing the frequency of adventitious shoot formation. Preculture of cotyledon explants on high sucrose concentration (6–9%) for 2 wk and subsequent transfer to 3% sucrose enhanced the frequency of adventitious shoot induction. Root formation from the adventitious shoots was easily achieved on MS medium containing 2.7 μM of α-naphthalene acetic acid (NAA). Regenerated plantlets were acclimatized on sand and peat moss (1:1), showing 95% survival with subsequent flowering and seed set. We established the high-frequency plant regeneration via adventitious shoot formation in *S. indicum* L.

Keywords *Sesamum indicum* · Adventitious shoot · Plant regeneration · Sesame

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Introduction

Sesame (*Sesamum indicum* L.) belongs to family Pedaliaceae and is an important oil crop. In recent years, the annual worldwide production of sesame has amounted to about 2×10^6 tons. The seed contains about 50 to 60% oil, which is traditionally used for cooking and as a flavor additive in food products of Asian and Western countries (Pastorello et al. 2001).

Cultivation of sesame suffers from considerable yield loss because of pathogenic diseases like phytophthora blight and root/stem rot (Gangopadhyay et al. 1998). In addition, it is difficult to determine the time of harvest a sesame crop to maximize yield because plant growth is indeterminate and spontaneous capsules dehisce when mature (Day 2000). Thus, an interdisciplinary concerted effort with the participation of both conventional breeding technique and biotechnology is urgently required for genetic improvement of sesame.

Genetic transformation of sesame could be a good supplement for conventional breeding. For genetic breeding of sesame, a high reproducible plant regeneration protocol is necessary. Somatic embryogenesis from hypocotyl segments (Jeya Mary and Jayabalan 1997) and cotyledon and root and subapical hypocotyl segments from young seedlings (Zeevaart

and Creelman 1988) of *S. indicum* has been reported. However, Xu et al. (1997) reported that plant conversion rate from somatic embryos was very low (less than 12 to 13%). Multiple shoot induction from seedling shoot tips (George et al. 1987; Rao and Vidyanath 1997) and nodal segments with axillary buds (Gangopadhyay et al. 1998) was achieved; however, these protocols are hardly applicable for production of transgenic plants because of low transformation frequency. Plant regeneration via adventitious shoot formation from meristem-free organs is widely adopted for genetic transformation. In the present paper, we report the high-frequency plant regeneration through direct adventitious shoot formation from deembryonated cotyledon segments of *S. indicum* L.

Materials and Methods

Selection and sterilization of plant materials. Immature (40 d after flowering) and mature (60 d after flowering) seeds of nine cultivars of *S. indicum* L. (Annam, Dasak, Hwangbaek, Mankeum, Namsan, Pungna, Osan, Tainan 1, and Yangheuk) were harvested from the field of Honam Agricultural Research Institute, Korea. These seeds were aseptically sterilized by immersing in 70% ethanol for 1 min, followed by 2.5% sodium hypochlorite solution for 20 min, and finally washed three times with sterile distilled water.

Selection of explants and adventitious shoot formation. Cotyledon explants were aseptically isolated from the sterilized immature seeds, mature seeds, and 1- or 2-wk-old seedlings aseptically grown *in vitro* on half-strength Murashige and Skoog (MS) (Murashige and Skoog 1962) salts medium, including same dilution of organic compounds (vitamins, inositol, etc.) with 3% sucrose and 2.5 g/l of Phytagel (Sigma, St. Louis, MO). These cotyledon explants were dissected from the seeds in such a way that the embryonic axis was removed completely, thus having a cut at the proximal portion of the cotyledon (also known as deembryonated cotyledon). The deembryonated cotyledon explants were cultured on MS medium with various concentrations and combinations of 6-benzylaminopurin (BA) (0, 11.1, 22.2, and 44.4 μM) and/or indole-3-acetic acid (IAA) (0, 2.85, 5.7, and 11.4 μM) for adventitious shoot induction. Other cytokinins such as kinetin (9.3 μM) and zeatin (9.1 μM) were also studied in combination with IAA for adventitious shoot induction. The efficiency of adventitious shoot induction was studied by the addition of abscisic acid (ABA) (3.8 μM) and $AgNO_3$ (29.4 μM) as an additive with BA (22.2 μM) and IAA (5.7 μM) combinations.

To determine the effect of sucrose as a carbohydrate source, cotyledon explants were precultured on medium with 22.2 μM of BA and 5.7 μM of IAA or 22.2 μM of BA, 5.7 μM of IAA, 3.8 μM of ABA, and 29.4 μM $AgNO_3$ for 2 wk and subsequently transferred to medium with 3% sucrose. All media were solidified by 4 g/l of Phytagel and adjusted to pH 5.8 before autoclaving at 120°C for 15 min. The culture room was maintained at 24°C with a 16-h photoperiod and light intensity of 50 μmol m^{-2} s^{-1} using cool-white fluorescent tubes. About 15 cotyledon explants were cultured per treatment, and the experiment was repeated three times. After 6 wk of culture, adventitious shoot formation from total explants and/or number of adventitious shoots per explant were recorded.

Root induction and hardening. Adventitious shoots about 2–3 cm in height were transferred to Magenta plastic box containing hormone-free half-strength MS, or MS basal medium, or MS medium with 3% sucrose and 3.7 μM of α-naphthalene acetic acid (NAA) for rooting. Rooting and plant conversion rate were examined after 6 wk of culture. The rooted plantlets were transferred to plastic pots (5× 5 cm^2) containing clay soil, or sand, or sand and peat moss mixture (1:1) for acclimatization. The plantlets were covered with polyethylene cover for the first 3 wk and then exposed to external atmosphere in culture room for the next 5 wk at 25±2°C and 55% relative humidity. Survival rate of plants was counted after 2 mo. of culture. Later the surviving plants were transferred to large pots (15×15 cm^2) containing soil and maintained in a greenhouse with 30±2°C with a 16-h photoperiod and 70% relative humidity until seed set. The culture room was maintained at 24°C with a 16-h photoperiod and light intensity of 50 μmol m^{-2} s^{-1} using white fluorescent tubes.

Table 1. Effect of different concentrations of BA and IAA on adventitious shoot formation from deembryonated cotyledons of mature seeds in *S. indicum* L. cultivar Dasak on MS medium with 3% sucrose after 4 wk of culture

IAA (μM)	BA (μM)	Frequency of adventitious shoot formation (%)	No. of shoots/ explant
0	11.1	3±0.2 g	1.1±0.12 g
0	22.2	6±0.5 ef	2.3±0.16 f
0	44.4	0 h	0 h
2.85	11.1	7±0.3 e	2.3±0.34 f
2.85	22.2	12±0.7 b	4.3±0.52 bc
2.85	44.4	6±0.5 ef	3.0±0.43 e
5.7	11.1	9±0.5 d	2.0±0.19 f
5.7	22.2	17±1.2 a	5.3±0.64 a
5.7	44.4	10±1.1 cd	4.7±0.37 ab
11.4	11.1	5±0.3 f	3.3±0.26 de
11.4	22.2	11±0.9 bc	3.7±0.45 cd
11.4	44.4	7±0.4 e	2.3±0.32 f

Data represent mean±SE of three independent experiments. Mean values with different *letters* in a *column* are statistically different at $P=0.05$.

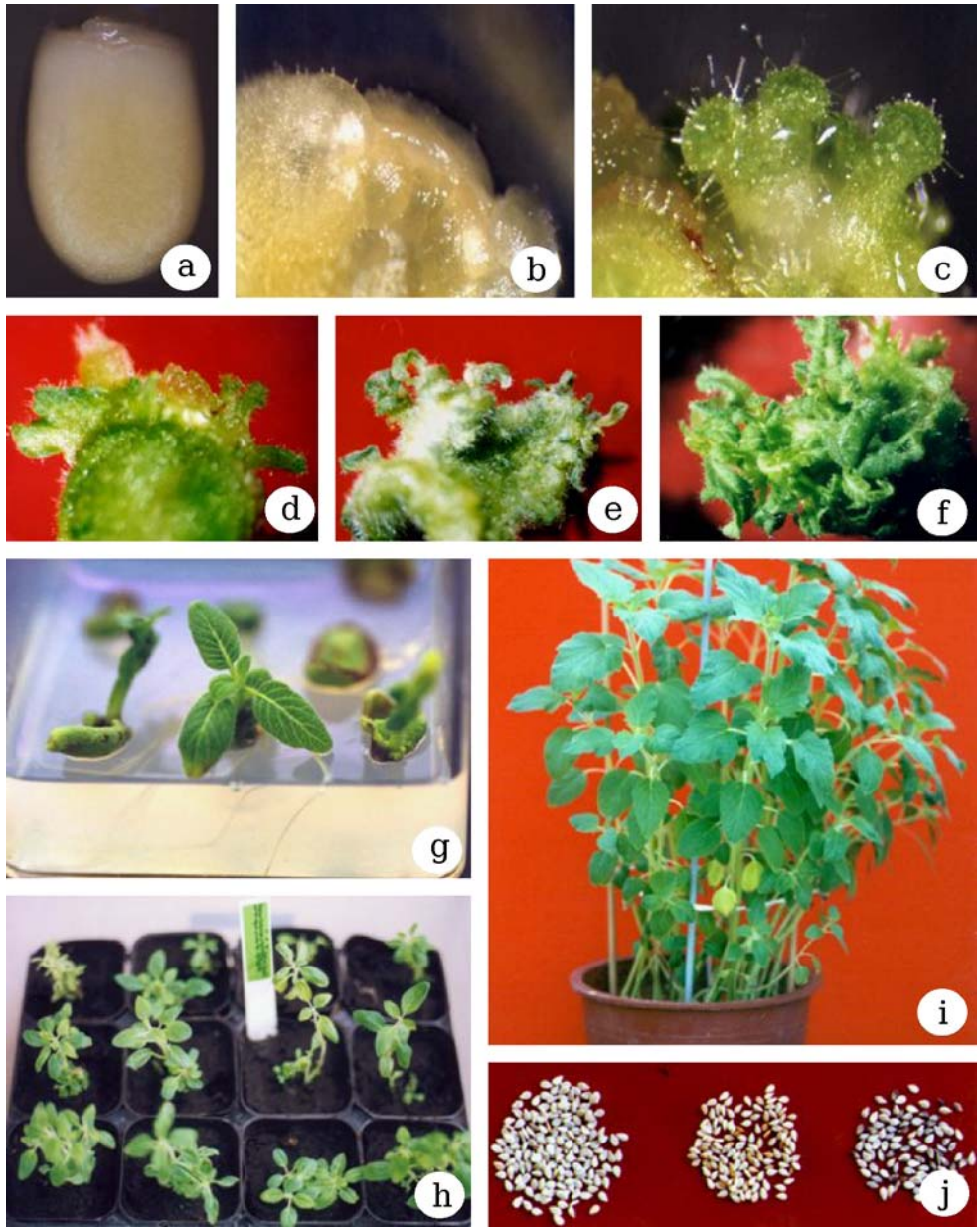

Figure 1. Plant regeneration of sesame via direct adventitious shoot formation. (*a*) Deembryonated cotyledon explants excised from mature seeds of *S indicum* cultivar Dasak. (*b*) Early callusing on proximal end of deembryonated cotyledon after 3 d of culture. (*c*) Adventitious shoot formation after 2 wk of culture. (*d–f*) Adventitious shoots on medium with 3% sucrose (*d*), 6% sucrose (*e*), and 9% sucrose (*f*). (*g*) Rooting of shoot on MS medium with NAA (2.7 μM). (*h*) Plants acclimatized in sand and peat moss (1:1). (*i*) Mature plants grown in greenhouse. (*j*) Harvested seeds.

Statistical analysis. The data collected were subjected to analysis of variance and the means were separated by Duncan's multiple range test (P=0.05).

Results and Discussion

Effect of growth regulator and explants stage on adventitious shoot formation. The deembryonated cotyledon explants from mature seeds of cultivar Dasak were cultured on MS medium with 3% sucrose and various concentrations of IAA and BA for adventitious shoot induction as shown in Table 1. The highest rate of adventitious shoot formation was obtained in the presence of 5.7 μM of IAA and 22.2 μM of BA (Table 1). Kinetin or zeatin was significantly less responsive than BA for adventitious shoot formation (data not shown). Time-lapsed observation of adventitious bud formation revealed that cut surfaces on the proximal region of the deembryonated cotyledon explants (Fig. 1*a*) became swollen after 3–5 d of culture (Fig. 1*b*). Adventitious shoots developed directly from these surfaces after 1 or 2 wk of culture (Fig. 1*c*). After 4 to 6 wk, shoot clusters on the proximal region of the explants were seen (Fig. 1*d–f*).

Specificity of explants. The potential of producing adventitious shoots was restricted to the deembryonated cotyledon explants from mature seeds and drastically declined after germination (Fig. 2). The frequency of adventitious

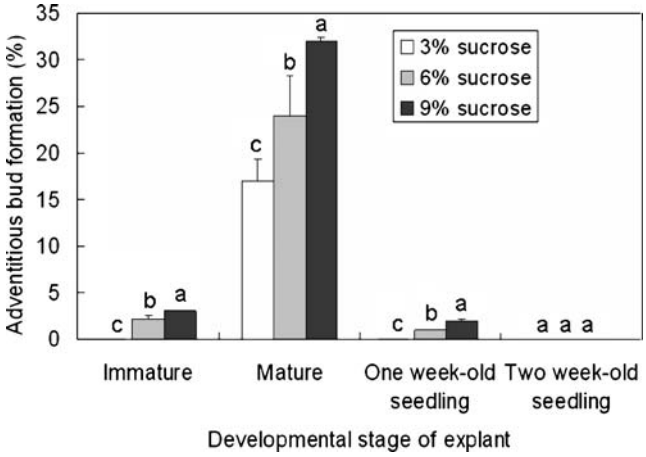

Figure 2. Adventitious shoot formation from deembryonated cotyledon explants isolated from immature, mature seeds, and 1- or 2-wk-old seedlings of *S indicum* cultivar Dasak on MS medium with 22.2 μM of BA and 5.7 μM of IAA. Cotyledon explants were cultured on medium with different sucrose concentration for 2 wk and subsequently transferred to 3% sucrose for further 4 wk of culture. Vertical bars indicate the mean values±SE.

shoot formation from deembryonated cotyledon explants isolated from immature seeds after 40 d of flowering was less than 3%. However, cotyledon explants from 2-wk-old seedlings never produced adventitious shoots (Fig. 2). Hypocotyls or root segments from 1- or 2-wk-old seedlings produced callus at high frequency but never produced adventitious shoots (data not shown). Deembryonated cotyledon explants from mature seeds were the best explants for adventitious shoot induction and the developmental stage of explants was critical for the induction of adventitious shoots in *S. indicum* (cultivar Dasak).

Effect of sucrose. Deembryonated cotyledon explants were cultured on medium with different concentration of sucrose (3, 6, and 9%). Prolonged culture of explants for 6 wk on 9% sucrose resulted in browning of explants. However, preculture of cotyledon explants for 2 wk on 9% sucrose and subsequent subculture to medium with 3% sucrose significantly enhanced adventitious shoot formation (Figs. 2 and 3). When cotyledon explants were precultured on 12% sucrose, the adventitious shoot induction frequency was zero and the explants browned and died (data not shown). Frequency of adventitious shoot bud induction was maximum when cotyledon explants were cultured for 2 wk on MS medium containing 9% sucrose followed by subculture to medium with 3% sucrose.

Carbohydrate was demonstrated to act as an energy source and an osmoticum in shoot formation (Brown et al. 1979). The precise physiological role of sucrose in the induction of shoots was determined by many workers (Brown et al. 1979; Ramage and Leung 1996; Strickland et al. 1987). An exogenous supply of sucrose is essential for at least the first 4 d of culture in hypocotyl explants of *Capsicum annuum* (Ramage and Leung 1996). An ultrastructural study showed that starch grains in tissue was accumulated during early shoot meristem formation and utilized during shoot formation process (Ross et al. 1973). Enhanced adventitious shoot formation by high sucrose pretreatment in the present study might be resulted in high uptake of sucrose in explants, which in turn favors the adventitious shoot formation.

Effect of ABA and $AgNO_3$ on adventitious shoot formation. Abscisic acid and $AgNO_3$ treatment were effective for the induction of adventitious shoots on MS medium with 3% sucrose (Table 2). The frequency of adventitious shoot formation was 17% on medium without ABA and $AgNO_3$ treatment. Individual ABA or $AgNO_3$ treatment was increased twofold in adventitious shoot formation. Combined supplementation of ABA (3.8 μM) and $AgNO_3$ (29.4 μM) induced maximum frequency of adventitious shoots up to 50% (threefold increase) (Table 2).

Abscisic acid plays a role in fostering seed maturation and inhibiting the germination of seeds (Zeevaart and Creelman 1988). The influence of ABA is well investigated in somatic embryogenesis. Abscisic acid treatment inhibits the precocious germination of somatic embryos and reduces secondary embryo formation (Choi and Jeong 2002; García-Martín et al. 2005). In the moss *Funaria hygrometrica*, adventitious shoot formation is inhibited by ABA (Christianson 2000). Temporary pretreatment of ABA stimulated adventitious shoot proliferation in sesame. It was known that ABA promotes sucrose uptake *Beta vulgaris* root tissue (Saftner and Wyse 1984). In addition, high sucrose treatment increases the endogenous ABA content

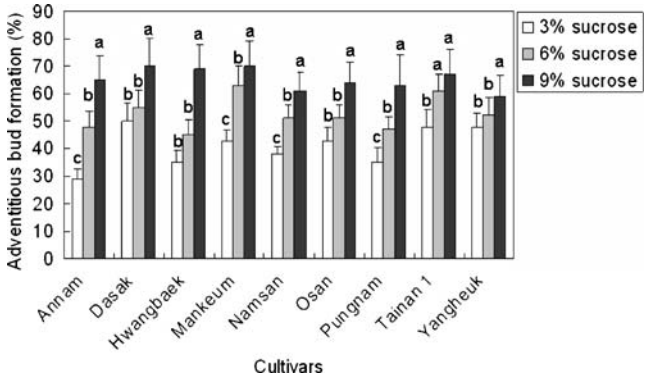

Figure 3. Adventitious shoot formation from cotyledon explants of mature zygotic embryos in various sesame cultivars. Explants were cultured on MS medium with different concentration of sucrose, and 22.2 μM of BA, 5.7 μM of IAA, 3.8 μM of ABA, and 29.4 μM of $AgNO_3$. Cotyledon explants were cultured on medium with a different sucrose concentration for 2 wk and subsequently transferred to 3% sucrose for further 4 wk of culture. *Vertical bars* indicate the mean values±SE.

Table 2. Effect of BA, ABA, and $AgNO_3$ on adventitious shoot formation from deembryonated cotyledons of mature seeds of *S. indicum* L. cultivar Dasak after 6 wk of culture

Treatments				Adventitious shoot formation (%)
IAA (μM)	BA (μM)	ABA (μM)	$AgNO_3$ (μM)	
5.7	11.1	0	0	12±1.4 c
5.7	22.2	0	0	17±2.1 c
5.7	44.4	0	0	11±0.9 c
5.7	11.1	3.8	0	33±4.2 b
5.7	22.2	3.8	0	34±3.7 b
5.7	44.4	3.8	0	26±5.3 b
5.7	11.1	3.8	29.4	47±5.3 a
5.7	22.2	3.8	29.4	50±6.7 a
5.7	44.4	3.8	29.4	29±4.5 b

Data represent mean±SE of three independent experiments.
Mean values with different *letters* in a *column* are statistically different at P=0.05.

(Choi and Jeong 2002; Zeevaart and Creelman 1988). Sucrose and ABA work synergistically, which was shown by *Arabidopsis* mutants that are insensitive to both ABA and sucrose (Huijser et al. 2000; Laby et al. 2000). The enhanced shoot bud induction by high sucrose and ABA treatment might be corelated with the effective sucrose uptake and related with the enhanced shoot formation.

In the present study, combined treatment of $AgNO_3$ (an ethylene inhibitor) with ABA enhanced the frequency of adventitious shoot proliferation (Table 2). Ethylene is produced during *in vitro* plant tissue culture (Chi and Pua 1989). It was reported that the production of ethylene is associated with poor regeneration or recalcitrant behavior of culture materials (Chi and Pua 1989). $AgNO_3$ treatment was effective for the conversion of somatic embryos into plants in sesame (Xu et al. 1997). $AgNO_3$ treatment stimulates organogenesis in *Triticum aestivum* (Purnhauster et al. 1987), *Brassica campestris* (Chi and Pua 1989), *Helianthus annuus* (Chraibi et al. 1991), *C. annuum* (Hyde and Phillips 1996), *Cucumis sativus* L. (Mohiuddin et al. 1997).

Response of different cultivars on adventitious shoot formation. Based on the results obtained on adventitious shoot formation from deembryonated cotyledons isolated from mature seeds of sesame cultivar Dasak, a total of nine cultivars of sesame were studied. Deembryonated cotyledon explants were cultured on medium with 22.2 μM of BA, 5.7 μM of IAA, 3.8 μM of ABA, and 29.4 μM of $AgNO_3$ and with different concentrations of sucrose (3, 6, and 9%) for 2 wk and subsequently transferred to medium with 3% sucrose. In all nine cultivars, the frequency of adventitious shoot bud formation was 29 to 50% in 3% sucrose, and increased markedly (64 to 70%) on 9% sucrose pretreatment (Fig. 3). It is clear that high sucrose pretreatment enhanced adventitious shoot formation in all nine cultivars of *S. indicum*.

Table 3. Effect of MS medium strength and NAA on rooting of adventitious shoots and plant conversion with well-developed shoot and roots in *S. indicum* L. cultivar Dasak after 6 wk of culture

Culture medium	Rooting (%)	Plant conversion (%)
1/2 MS	90±12.3 a	58±5.4 a
MS	85±13.5 a	62±8.2 a
MS+2.7 μM of NAA	98±22.1 a	57±7.6 a

Plant conversion indicates the plantlets with well-developed shoot and root.
Data represent mean±SE of three independent experiments.
Mean values with different *letters* in a *column* are statistically different at P=0.05.

Rooting and soil transfer. When adventitious shoots at 2 to 3 cm in height were transferred to hormone-free half-strength or full-strength MS or MS medium with 2.7 μM of NAA rooting was induced within 6 wk (Fig. 1*g*). The rooting frequency was slightly increased on full strength MS with 2.7 μM of NAA (98%), followed by half-strength MS (90%) and MS (85%), although the values were not statistically significant (Table 3). The conversion rate of adventitious buds into plantlets with shoot and roots was also not statistically different among treatments (Table 3).

Plantlets about 10 cm in height were acclimatized in different soil conditions (clay soil, sand, or 1:1 soil and peat moss mixture; Fig. 1*h*). Only 20% of plantlets survived in clay soil and 55% in sand (Table 4). In sand and peat moss mixture (1:1), 95% of plantlets survived and subsequently flowered and set seed (Fig. 1*i*, *j* and Table 4).

In conclusion, we established a high-frequency plant regeneration system via direct adventitious shoot formation from deembryonated cotyledon explants of mature seeds, and the converted plantlets were successfully transferred to soil.

Table 4. Acclimatization effect of soil types after 2 mo. of transplanting

Soil type	Survival rate (%)
Clay soil	20±4.2 c
Sand	55±5.7 b
Sand and peat moss mixture	95±11.5 a

Data represent mean±SE of three independent experiments.
Mean values with different *letters* in a *column* are statistically different at P=0.05.

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