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BRIEF COMMUNICATION

Efficient regeneration of *Eucommia ulmoides* from hypocotyl explant

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Abstract

A rapid and efficient method for the regeneration of *Eucommia ulmoides* Oliver has been developed. The ability of hypocotyl segments to produce adventitious buds varied depending upon their position, seedling age and culture medium. The most adventitious buds were induced from the hypocotyl segments near to the root of 2-week-old seedlings on the Murashige and Skoog (MS) basal medium supplemented with 10 μM benzylaminopurine (BAP). However, the MS medium with 6 μM BAP and 6 μM gibberellic acid (GA_3) was most suitable for shoot multiplication and further growth. Shoots longer than 2 cm cultured on the half-strength MS medium supplemented with 0.5 μM naphthaleneacetic acid (NAA) produced a maximum number of roots per shoot. Regenerated plantlets could be successfully acclimatized.

Additional key words: auxin, cytokinin, gibberellic acid, histological analysis, shoot multiplication and elongation.

Eucommia ulmoides Oliver, a deciduous, dioecious woody plant, is one of the tertiary species surviving only in China. *E. ulmoides* is a living-fossil plant and its bark and leaves contain more than 30 kinds of medicinally important compounds (Xing *et al*. 2001). In addition, it produces *trans*-polyisoprene, called Eu-rubber (or Chinese gutta-percha) in the leaves, bark and pericarp (Bamba *et al*. 2002). Eu-rubber has several specific properties that differ from those of natural rubber (*cis*-polyisoprene) being an excellent nonconductor, and having an extremely low coefficient of thermal expansion/contraction. The native distribution of *E. ulmoides* is diminishing. In order to efficiently propagate the rare plant species or genetic alterations for medicinal compounds and Eu-rubber production, a regeneration system must be established. In this paper, we report a rapid and efficient method for the regeneration of a plant from hypocotyl explant of *E. ulmoides*.

Seeds of *Eucommia ulmoides* collected from Beijing Arboretum, China were surface sterilized by immersion in 70 % ethanol (v/v) for 1 min followed by immersion in 3 % (v/v) sodium hypocholorite solution (*Wako*, Osaka, Japan) for 25 min, and rinsed 5 times with sterile distilled water. After the seed coats were removed and the hard seed capsules were slit by a scalpel, they were placed on a germination medium containing 25 cm^3 of half-strength MS basal medium supplied with 15 g dm^{-3} sucrose in 90 × 15 mm Petri-dishes sealed with *Parafilm* (*Pechiney Plastic Packaging*, Menasha, USA) for germination. The hypocotyls elongated and attained an average length of 1.0 cm after 2 - 3 week culture.

The cotyledons and the shoot apexes of the 2-week-old seedlings were exscinded, below which, three hypocotyl segments about 3 - 4 mm in length were chosen as the explants. They were placed horizontally on various kinds of callus and adventitious bud induction media containing MS basal medium supplied with 30 g dm^{-3} sucrose, 3, 6, 10, and 15 μM BAP combined with 0, 0.1, 0.5, and 1 μM NAA (Table 1, CS1 - CS16), respectively. In order to determine the effects of hypocotyl segment position on adventitious bud induction, the three hypocotyl segments mentioned above, which were designated as H1, H2 and H3 (H1 being nearer to the cotyledon while H3 being nearer the root) were cultured separately on the MS medium containing 10 μM BAP (CS9, the best callus and adventitious bud induction medium). In addition, to

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Abbreviations: BAP - 6-benzylaminopurine; GA_3 - gibberellic acid; IAA - 3-indoleacetic acid; IBA - 3-indolebutyric acid; MS medium - Murashige and Skoog medium (1962); NAA - naphthaleneacetic acid.
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compare the effects of seedling age, some seedlings were maintained on the germination medium for 3 weeks and the hypocotyl segments were cultured on the medium CS9.

Some of the explants with calli and adventitious buds cultured on the medium CS9 for 3 weeks were embedded in the *Tissue-Tek 4583 OCT* (optimal cutting temperature) compound (*Miles Scientific*, Naperville, IL, USA) at -20 °C. They were cut to a 15 - 20 μm thickness using a *Cryocut CM1850* cryomicrotome (*Leica Microsystems*, Deerfield, IL, USA) and were mounted on *Vectashield* (mounting medium for fluorescence H-1000, *Vector Lab*, Burlingame, USA) coated slides. The slides were examined using a light microscope (*Nikon TE-2000U*, Tokyo, Japan).

The best-growing adventitious buds with explant cultured on the medium CS9 for 3 weeks were either sub-cultured to fresh medium of the same composition or transferred to the shoot multiplication and elongation media containing MS basal medium supplied with 30 g dm^{-3} sucrose, 0, 3, 6, or 10 μM BAP combined with 0, 6, 10 or 20 μM GA_3, respectively (Table 1, SL1 - SL16). They were sub-cultured to fresh medium at an interval of every three weeks.

The differentiated shoots longer than 2 cm were harvested and transplanted to the rooting media containing MS, ½ MS or ⅓ MS basal medium supplied with 30 g dm^{-3} sucrose and 0.1, 0.5, or 1 μM NAA (Table 1, R1 - R9). After the shoots produced roots, they were transplanted to pots containing *Vermiculite* (*Green Sangyo*, Niigata, Japan), were watered with tap water, and were placed in a culture-room for acclimatization. In the beginning, the pots were covered to prevent desiccation. The cover was then gradually opened week by week. After 4 weeks the cover was completely removed, and the plantlets were moved to the greenhouse.

All of the media mentioned above were adjusted to pH 5.8 prior to the addition of 5 g dm^{-3} *Agargel* (*Sigma-Aldrich*, St. Louis, USA). With the exception of the germination medium that was dispensed to Petri-dishes, the media were dissolved at 105 °C for 5 min, dispensed into 30 cm^{-3} per 100 cm^{-3} Erlenmeyer flasks, and capped with aluminum foil before being autoclaved at 121 °C for 15 min. The cultures were incubated at 25 °C under a 16-h photoperiod using cool, white fluorescent tubes providing irradiance of 50 μmol $m^{-2}s^{-1}$ PPFD (photosynthetic photon flux density). For each experiment a minimum of 5 flasks (7 explants per flask) were taken, and each experiment was repeated thrice. At the end of each culture stage, the numbers of differentiated buds (or elongated multiple shoots, or roots) per explant (or per shoot) on each medium or treatment was counted (Table 1), and their morphology was observed.

The hypocotyl segments on the callus and adventitious bud induction media remained green (Fig. 1*A*) and developed small nodular protuberances in cut ends during the first week. These protuberances then developed into calli in the second week. Adventitious buds appeared within 3 - 4 weeks (Fig. 1*B*). Significantly more adventitious buds were observed on the explants cultured on MS with 10 μM BAP alone, with an average of 2.67 adventitious buds per explant than on those cultured on other media. The number of adventitious buds formed decreased with lower (3 μM) or higher (15 μM) concentrations of BAP (Table 1). The stimulating effectiveness of BAP to induce adventitious meristem, whether directly from explanted tissues, or indirectly from callus, has been well documented in other plant species (Hiregoudar *et al*. 2006; Rao and Purohit 2006).

Addition of the auxin NAA resulted in callus enlargement and low bud initiation from the explant (Table 1), while the addition of GA_3 (1 - 10 μM) or coconut water (1 - 5 %) resulted in explant elongation but was not beneficial in producing more adventitious buds. On the media supplied with higher concentrations of GA_3 (6 - 10 μM), explants even elongated up to 2 - 3 cm but no buds formed (data are therefore not shown).

The ability of hypocotyl segments to produce adventitious buds varied depending upon their position on the seedling. The hypocotyl segments H1, which were nearer to cotyledonary nodes, produced the smallest number of adventitious buds (1.71 adventitious buds per explant). There was a gradual increase in the number of adventitious buds when explants were taken further from the cotyledonary node, the maximum being in H3 (3.64 adventitious buds per explant).

The seedling age also influenced the adventitious bud induction. In comparison with those of hypocotyl segments taken from 2-week-old seedlings, the hypocotyl segments taken from 3-week-old seedlings led to a decrease in the number of adventitious buds (1.71 adventitious buds per explant). It is not clear why the hypocotyl segments taken from nearer to cotyledonary nodes, or from 3-week-old seedlings produced the least number of adventitious buds. In general, this may be related to explant position, polarity or age effects, but one hypothesis that may also explain this phenotype is that *E. ulmoides* is one of the few woody plants producing hard fibrous rubber, which mainly consists of *trans*-polyisoprene repeating units in intercellular spaces (Tangpakdee *et al*. 1997, Bamba *et al*. 2002), and which may disturb cell growth. We found that the *trans*-polyisoprene began to accumulate obviously in 3-week-old seedlings, and its accumulation gradually decreased from cotyledonary nodes to roots (unpublished data). In such circumstances, it would be necessary to select the explant type that has the highest capacity for regeneration.

Histological analysis of responsive hypocotyl segments showed that a characteristic ring of callus tissue was formed along the whole cut surface of the explants, and then several meristemoids were initiated. These meristemoids differentiated and developed into adventitious buds after 3 weeks of culture (Fig. 1*C*).

After 3 weeks of culture, the best-growing adventitious buds with explants cultured on the medium CS9 were either sub-cultured to fresh medium of the same composition or transferred to 16 kinds of shoot multiplication and elongation media supplied with GA_3 to

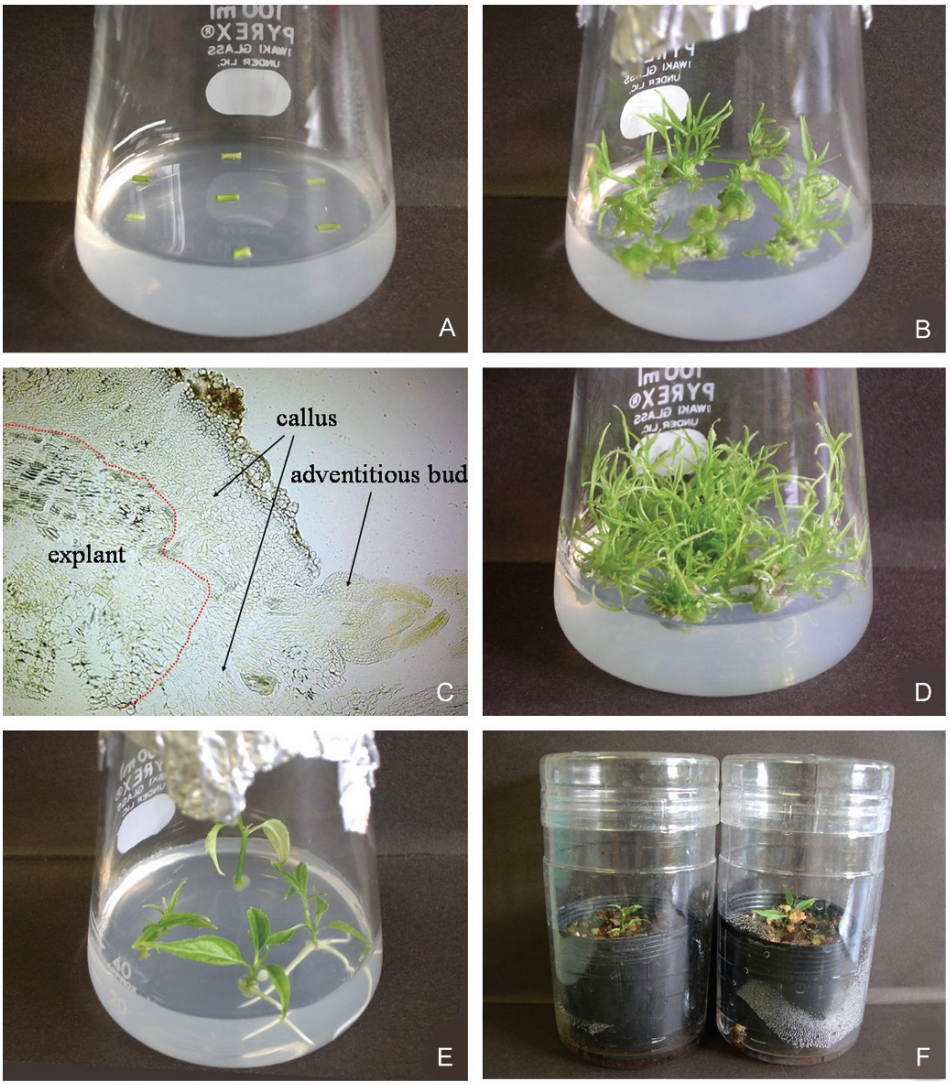

Fig. 1. Regeneration of *E. ulmoides* plant from hypocotyl explant. *A* - hypocotyl segments from 2-week-old seedlings chosen as the explants; *B* - adventitious buds induced from hypocotyls segments on MS medium supplemented with 10 µM BAP (medium CS9) for 3 weeks; *C* - histological analysis showing the adventitious buds developed from the calli induced in explant surfaces; *D* - shoot multiplication and elongation on MS medium containing 6 µM BAP supplemented with 6 µM GA_3 (medium SL10) for 3 weeks; *E* - rooting of shoots cultured on ½ strength MS medium supplemented with 0.5 µM NAA (medium R5) for 3 weeks; *F* - plantlets transferred to pot containing *Vermiculite* for acclimatization.

stimulate further growth (Table 1). On the medium CS9, the adventitious buds became cirrhotic and stunted, especially after being sub-cultured several times. This problem seemed to be the bottleneck for the regeneration of *E. ulmoides* (Zhu *et al*. 1997). In marked contrast, on all the media supplied with GA_3, the green adventitious buds developed into multiple shoots and became elongated during 2 - 3 weeks of continuous culture. It was found that the medium SL10, which contained 6 µM BAP (in a little lower concentration than in the adventitious bud induction stage) supplemented with 6 µM GA_3, was most suitable for shoot multiplication and elongation. GA_3 can influence plant growth and development in a variety of ways. Occasionally, shoots are treated with GA_3 to increase their length during multiplication, or prior to rooting. The treatment may be beneficial where a high level of cytokinin has resulted in many short shoots (e.g. in rosa, Valles and Boxus 1987). On the medium SL10, the multiple shoots grew healthier and developed the highest rate of long shoots (> 2 cm) about 2 - 50 times more than those cultured on the other shoot multiplication and elongation media (Table 1, Fig. 1*D*). There were also a lot of adventitious buds and/or multiple shoots produced, but they were too small to be counted properly and are excluded in Table 1. Shoots longer than 2 cm were harvested for rooting and the residual explants with multiple shoot buds (shorter than 2 cm) were maintained on the medium SL10 and were sub-cultured to fresh

Table 1. Effects of hormones on adventitious bud induction (CS1 - 16), shoot elongation (SL1 - 16), and rooting (R1 - 9) from hypocotyl explant of *E. ulmoides*. Means ± SE, n = 15.

Medium No.	CS1	CS2	CS3	CS4	CS5	CS6	CS7	CS8	
BAP [μM]	3	3	3	3	6	6	6	6	
NAA [μM]	0	0.1	0.5	1	0	0.1	0.5	1	
Adventitious buds [explants^{-1}]	0.57±0.14	0.86±0.65	0.05±0.05	0.05±0.05	1.67±0.72	1.38±0.70	0.76±0.50	0.67±0.41	
Medium No.	CS9	CS10	CS11	CS12	CS13	CS14	CS15	CS16	
BAP [μM]	10	10	10	10	15	15	15	15	
NAA[μM]	0	0.1	0.5	1	0	0.1	0.5	1	
Adventitious buds [explants^{-1}]	2.67±0.30	2.00±0.38	1.62±0.73	1.19±0.92	1.57±0.80	1.71±0.14	1.48±0.16	1.00±0.29	
Medium No.	SL1	SL2	SL3	SL4	SL5	SL6	SL7	SL8	
BAP[μM]	0	0	0	0	3	3	3	3	
GA_3 [μM]	0	6	10	20	0	6	10	20	
Shoots (>2 cm) [explants^{-1}]	0±0	0.21±0.21	0.07±0.07	0.36±0.30	0±0	0±0	0.43±0.40	0.14±0	
Medium No.	SL9	SL10	SL11	SL12	SL13=CS9	SL14	SL15	SL16	
BAP[μM]	6	6	6	6	10	10	10	10	
GA_3 [μM]	0	6	10	20	0	6	10	20	
Shoots (>2cm) [explants^{-1}]	0.07±0.07	3.64±0.10	1.93±0.30	0.43±0.40	0±0	0.14±0.14	0.14±0.14	0.21±0.21	
Medium No.	R1	R2	R3	R4	R5	R6	R7	R8	R9
Basal medium	MS	MS	MS	½ MS	½ MS	½ MS	⅓ MS	⅓ MS	⅓ MS
NAA[μM]	0.1	0.5	1	0.1	0.5	1	0.1	0.5	1
Roots [explants^{-1}]	0±0	0.83±0.24	1.67±1.41	0±0	8.50±3.06	5.67±0.47	0±0	5.17±0.71	5.17±2.59

medium of the same composition at an interval of every three weeks. New multiple shoots were developed again and were also excised at the end of the culture period. Through this procedure of shoot excision and re-culturing of the original explant up to six sub-cultures, an average of 20 - 30 shoots could be obtained per explant.

Shoots longer than 2 cm were harvested and transplanted to the rooting media. The shoots cultured on the medium containing ½ MS basal medium supplemented with 0.5 μM NAA exhibited the highest rate, with an average of 8.50 roots per shoot after four weeks (Fig. 1*E*). Supplementation of 1μM NAA also induced roots, but the frequency of roots decreased to 5.67 roots per shoot and invariably developed callus at their apical ends, while medium supplemented with 0.1 μM NAA rarely induced roots. Shoots cultured on the medium containing MS or ⅓ MS basal medium sharply or slightly decreased the frequency of root induction (Table 1). This result is similar to the results of Chen *et al*. (1995), who reported that reducing the MS salts to ⅓ strength could promote the induction of roots from shoots obtained from 1-month-old *E. ulmoides* seedlings. No roots were observed from regenerated shoots maintained on the medium supplemented with various concentration of IAA or IBA (data not shown). It seemed that the root formation was not affected by the IAA or IBA concentration.

Plants (3 - 5 cm height) with 6 - 8 expanded leaves and well-developed roots could be successfully transplanted to pots containing *Vermiculite* for acclimatization for 4 weeks (Fig. 1*F*), then were transferred to a mixture of soils (*Takii*, Kyoto, Japan). After 8 weeks of transplantation, 66.6 % of plantlets survived.

The data presented above demonstrate clearly that *E. ulmoides* plants can be successfully regenerated from hypocotyl segments obtained from seedlings. By using this protocol, a number of *E. ulmoides* plants can be produced at any time of the year. The protocol provides a basis for *E. ulmoides* germplasm conservation, rapid propagation, and further genetic alteration for medicinal compounds and Eu-rubber production. Several studies in the past reported only on callus culture and failed in redifferentiation of full plantlet. Zhu *et al*. (1997) used endosperm from *E. ulmoides* seeds to induce callus in hopes of establishing a new regeneration system. Although shoot redifferentiation was achieved 2 - 5 %, most were abnormal shoots. Normal shoot redifferen-

tiation occurred at a rate of 0.1 - 0.01 %. The standardization of a highly reproducible regeneration system in *E. ulmoides* using hypocotyl segments during the present investigation may be useful in genetic improvement programs of this rare tertiary species plant.

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