Efficient Regeneration of the *Eucommia ulmoides* Oliver Plant from Hypocotyl Explant

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A rapid and efficient method for the regeneration of *Eucommia ulmoides* Oliver, a rare Tertiary species surviving only in China, has been developed. Hypocotyl segments excised from 2-week-old seedlings were chosen as explants. Multiple shoot buds were more significantly induced on medium containing MS basal medium supplemented with 10 μ M BAP alone than on other media. The addition of NAA resulted in callus enlargement, but it did not promote the production of multiple shoot buds. However, the adventitious shoots became cirrhotic and stunted after subculturing on an identical medium supplemented with BAP alone. It was found that the addition of 10 μ M GA3 to the medium was the most suitable for the stimulation of shoot elongation and further growth. Shoots longer than 2 cm cultured on the medium supplemented with 0.5 μ M NAA and 0.1 μ M ABA were highly responsive and produced the maximum number of roots per shoot. Regenerated plantlets with expanded leaves and well-developed roots could be successfully transferred to a pot containing vermiculite for acclimatization and then to a greenhouse. The regeneration protocol developed in this study provides the basis for *E. ulmoides* germplasm conservation, rapid propagation, and further genetic alteration for medicinal compound and EU-rubber production.

Key word: *Eucommia ulmoides*; hypocotyl culture; multiple shoot induction and elongation; plant regeneration

Introduction

Eucommia ulmoides Oliver, a deciduous dioecious woody plant, is a Tertiary species native to China. It is a living fossil plant, and the only species in both its genus and family^{1, 2)}. Its bark (cortex) and leaf contain more than 30 types of compounds, most of which are lignins and iridoid glycosides³⁻⁵⁾. A few of these compounds are known to have medical properties with pharmacological effects such as the lowering of blood pressure, nourishment of the liver and kidney, strengthening of muscles and bones, prevention of aging, and stimulation of protein synthesis in muscle. These compounds have been used as antihypertensives, diuretics, analgesics, and sedatives in traditional Chinese medicine since ancient times^{2, 4)}, and recently, an aqueous extract of *E. ulmoides* leaves has become a popular health beverage in Japan⁶⁾. In addition to producing bioactive substances, *E. ulmoides* is a higher plant known to produce a type of *trans*-polyisoprene termed EU-rubber in its leaves, bark, and pericarp^{1, 7, 8)}. EU-rubber has several specific properties that differ from those of natural rubber (*cis*-polyisoprene), including hard "plasticity," electrical insulation, and an extremely low coefficient of thermal expansion/contraction. These properties may be exploited for the manufacture of insulated cables, moulds, shoe soles, weapons, medical or science instruments, and sport utensils. Moreover, *E. ulmoi-*

des is also an oil- and hydrocarbon-rich plant, and as much as 27% edible oil can be extracted from its seeds¹⁾.

E. ulmoides is interesting in view of its economic potential. However, the native distribution of *E. ulmoides* is diminishing. Currently, it is becoming increasingly rare in China and is found only in arboretums. In order to efficiently propagate this rare plant species or perform genetic alterations for medicinal compound 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 plantlet originated from the hypocotyl explant of *E. ulmoides*.

Materials and Methods

Seed germination

E. ulmoides seeds were collected from Beijing Arboretum, China. The seeds were surface sterilized in a laminar flow chamber by immersion in 70% ethanol (v/v) for 1 minute followed by immersion in 3% (v/v) sodium hypochlorite solution for 25 minutes. Next, they were rinsed 5 times using sterile distilled water. After the seed coats were removed and the hard seed capsules slit using a scalpel, they were placed on a germination medium containing 25 ml of 1/2 MS (MS salts reduced to 1/2 strength) basal medium (Murashige and Skoog⁹⁾, 1962) supplied with 15 g/l sucrose in

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Abbreviations: BAP: 6-benzylaminopurine; NAA: naphthaleneacetic acid; GA3: gibberellic acid 3; ABA: abscisic acid; PPFD: photosynthetic photon flux density

Table 1. Effects of hormones on multiple shoot bud induction from the hypocotyl explant of *E. ulmoides*.

Medium No.	CS1	CS2	CS3	CS4	CS5	CS6	CS7	CS8
Hormones	BAP (μM)	3	3	3	3	6	6	6
	NAA (μM)	0	0.1	0.5	1	0	0.1	0.5
Shoots/Explants	0.57	0.86	0.05	0.05	1.67	1.38	0.76	0.67
Medium No.	CS9	CS10	CS11	CS12	CS13	CS14	CS15	CS16
Hormones	BAP (μM)	10	10	10	10	15	15	15
	NAA (μM)	0	0.1	0.5	1	0	0.1	0.5
Shoots/Explants	2.67	2.00	1.62	1.19	1.57	1.71	1.48	1.00

Table 2. Effects of hormones on multiple shoot elongation from the hypocotyl explant of *E. ulmoides*.

Medium No.	CS9	SL1	SL2	SL3	SL4	SL5	SL6
Hormones	BAP (μM)	10	6	6	6	6	6
	GA3 (μM)	0	1	3	6	10	20
Shoots/Explants	4.05	5.10	5.22	6.93	12.16	7.84	6.89
Shoot = 1-2 cm (%)	95.6	88.0	77.5	62.0	27.3	53.3	70.3
Shoot = 2-3 cm (%)	4.4	10.1	25.8	26.0	45.5	30.5	27.3
Shoot >3 cm (%)	0.0	1.9	6.6	12.0	27.3	16.2	2.5

Table 3. Effects of basal medium and hormones on root induction from the hypocotyl explant of *E. ulmoides*.

Medium No.	R1	R2	R3	R4	R5	R6
Hormones	NAA (μM)	0.1	0.1	0.5	0.5	1
	ABA (μM)	0	0.1	0	0.1	0
Roots/Explants	0.2	0.4	3.2	3.4	0.6	2.4

90 mm $\times$ 15 mm petri dishes for germination.

Callus and multiple shoot bud induction

The cotyledons and shoot apices of 2-week-old seedlings were excised, and 3 hypocotyl segments below the excision that were approximately 3–4 mm in length were chosen as initial explants. They were placed horizontally on different callus and multiple shoot bud induction media containing an MS basal medium supplied with 30 g/l sucrose and 3, 6, 10, and 15 μM BAP combined with 0, 0.1, 0.5, and 1 μM NAA (Table 1), respectively.

Shoot elongation

The best growing multiple shoot buds developed from explants cultured on medium CS9 for 3 weeks were either subcultured to a fresh medium of identical composition or transferred to the shoot elongation media containing MS basal medium supplied with 30 g/l sucrose and 6 μM BAP combined with 1, 3, 6, 10, 15, or 20 μM GA3 (Table 2). They were subcultured to fresh medium every 3 weeks.

Rooting

Differentiated shoots longer than 2 cm were harvested and transplanted to rooting media containing MS basal medium supplied with 30 g/l sucrose and 0.1, 0.5, or 1 μM NAA alone or combined with 0.1 μM ABA (Table 3).

Plantlet acclimatization

After the shoots produced roots, they were transplanted

to pots (ϕ 100 mm $\times$ 150 mm) containing vermiculite. They were watered using tap water and placed in a culture room for acclimatization. In the beginning, the pots were covered to prevent desiccation. The cover was then gradually opened each week. After 4 weeks, the cover was completely removed and the plantlets moved to a greenhouse.

All the above mentioned media were adjusted to pH 5.8 prior to the addition of 5 g/l Agargel (Sigma, USA). They were autoclaved at 121 $^{\circ}\text{C}$ for 15 minutes. The cultures were incubated at 25 $^{\circ}\text{C}$ under a 16-hour photoperiod of 50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD. At the end of each culture stage, the number of differentiated multiple shoot buds (or elongated shoots, or roots) per explant (or per shoot) on each medium or by each treatment was counted (Tables 1–3).

Results and Discussion

Effects of hormones on multiple shoot bud induction

The hypocotyl segments on the callus and multiple shoot bud induction media remained green and developed small nodular protuberances at the cut ends during the first week. These protuberances then developed into calli in the second week. Adventitious buds appeared within 3–4 weeks and then gradually formed multiple shoot buds. A significantly higher number of multiple shoot buds were observed on explants cultured on 10 μM BAP alone (medium CS9), with an average of 2.67 multiple shoot buds per explant, than on those cultured on other media. The number of multiple shoot buds formed decreased at lower (3 μM) or higher (15 μM)

concentrations of BAP (Table 1). Although addition of the auxin NAA resulted in callus enlargement, it did not promote the production of a higher number of multiple shoot buds (Table 1).

Effects of hormones on multiple shoot elongation

After 3 weeks of culture, the best growing multiple shoots that developed from the explant cultured on medium CS9 were either subcultured to a fresh medium having identical composition or transferred to 16 different types of multiple shoot elongation media containing GA3 to stimulate elongation and further growth (Table 2). On the medium CS9, the multiple shoot buds became cirrhotic and stunted, particularly after repeated subculturing. In marked contrast, on all the media containing GA3, the green multiple shoot buds developed into multiple shoots and became elongated during the 2-3 week continuous culture. Medium SL4, which contained 6 μ M BAP (at a slightly lower concentration than at the multiple shoot bud induction stage) supplemented with 10 μ M GA3, was observed to be the most suitable medium for multiple shoot elongation. The shoots that grew on this medium were healthier and developed at the highest rate for long shoots (>2 cm), i.e., approximately 2-15 times more than those that were cultured in other multiple shoot elongation media. Shoots longer than 2 cm were harvested for rooting, and the residual explants with multiple shoot buds (shorter than 2 cm) were maintained on medium SL4 and subcultured to a fresh medium with identical composition after an interval of 3 weeks. New multiple shoots were developed, and these were also excised at the end of the culture period. Through this procedure of shoot excision and reculturing of the original explant, an average of 10-20 shoots could be obtained per explant.

Effects of hormones on rooting

Shoots longer than 2 cm were harvested and transplanted to the rooting media. Those cultured on the medium containing MS basal medium supplemented with 0.5 μ M NAA and 0.1 μ M ABA (medium R4) afforded the highest rooting rate and were healthy with an average of 3.4 roots per shoot after 4 weeks. Supplementing the medium with a single dose of 0.5 μ M NAA also induced rooting; however, the frequency of rooting decreased slightly to 3.2 roots per shoot; these roots invariably developed calli at their apical ends. In contrast, medium supplemented with 0.1 or 1 μ M NAA rarely induced rooting (Table 3).

Plants (3-5 cm in height) with 6-8 expanded leaves and well-developed roots could be successfully transferred to a pot containing vermiculite for a 4-week acclimatization. These plants were then transferred to a soil mixture. After 8 weeks of transplantation, 66.6% plantlets survived.

The data presented above clearly demonstrates that *E. ulmoides* plants can be successfully regenerated from hypocotyl segments obtained from seedlings through direct organogenesis. 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 compound and EU-rubber production. *E.*

ulmoides tissue culture continues to be difficult and rather inefficient. To our knowledge, this is the first report describing the regeneration of *E. ulmoides* plants from hypocotyl explants in the absence of a pre-existing meristem.

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