BRIEF COMMUNICATION

Efficient regeneration of *Eucalyptus urophylla* from seedling-derived hypocotyls

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Abstract

Seedling hypocotyls were used as explants to establish a regeneration protocol for *Eucalyptus urophylla* and N-phenyl-N'-[6-(2-chlorobenzothiazol)-yl] urea (PBU), one kind of di-substituted urea, was found useful growth regulator. The hypocotyls incubated on a modified Murashige and Skoog medium (SPCa), supplemented with 6.6 μM PBU and 0.57 μM indole-3-acetic acid (IAA) dedifferentiated and form calli (100 % after 7 d). Compared with other growth regulator combinations, PBU stimulated more vigorous calli and restrained their darkening. In addition, the calli induced by PBU showed high frequency of adventitious buds formation (57 %). Shoot proliferation and elongation was then stimulated on SPCa medium containing 0.44 μM 6-benzyladenine (BA), 0.54 μM naphthalene acetic acid (NAA) and 0.3 μM gibberellic acid (GA_3). For rooting, shoots were cultivated on root induction medium containing 2.5 μM indole-3-butyric acid (IBA). Plantlets were then successfully transplanted to greenhouse.

Additional key words: bud induction, elongation, proliferation, substituted urea.

Conventional breeding of *Eucalyptus* is relatively slow, with a generation time of at least six years. Genetic transformation of *Eucalyptus* is also extremely difficult and its regeneration capacity is also low (Tournier *et al*. 2003). Transgenic eucalyptus expressing the cinnamyl alcohol dehydrogenase (CAD) gene (Tournier *et al*.2003) and plant expressing *cry3A* gene and the *bar* gene (Harcourt *et al*. 2000) were obtained, but with low regeneration and transformation efficiencies, and the published protocols could not be repeated in other laboratories. Synthetic phenylurea derivatives are potent plant growth regulators which exhibit cytokinin-like activity in various culture systems (Carra *et al*. 2006, Chung *et al*. 2007, Khan *et al*. 2006, Ricci *et al*. 2005, Turker *et al*. 2009). N-phenyl-N'-[6-(2-chlorobenzothiazol)-yl] urea (PBU; Fig. 1) was synthesized and purified in our laboratory (Li and Luo 2001). The aim of this study was to investigate effects of different factors on the regeneration of *Eucalyptus* hypocotyls and try PBU for efficient callus and bud induction.

Viable seeds of *Eucalyptus urophylla* S.T. Blake were kindly provided by China Eucalyptus Research Center. Seeds were dipped in distilled water for 3 h, surface-sterilized by 70 % (v/v) ethanol for 1 min, followed by 20 % (v/v) sodium hypochlorite for 10 min twice. The seeds were then rinsed 6 times in sterile distilled water and sown on 1/2 Murashige and Skoog (MS) medium for germination at 25 °C, in darkness.

Hypocotyls closed up to cotyledons (8 - 10 mm) were excised from 8-d-old seedlings and inoculated on SPCa medium supplemented with different plant regulator combinations (Table 1). SPCa medium was modified from SP medium (Barrueto *et al*. 1999) by increasing content of calcium chloride from 1.5 mM to 6.12 mM. They were incubated at 25 ± 2 °C in darkness for 2 weeks and then for another 2 weeks under a 16-h photoperiod with a photosynthetic photon flux density of approximately 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ emitted from cool fluorescent tubes (standard culture conditions). Each treatment contained 3 replications with 30 explants per replication.

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Abbreviations: BA - 6-benzyladenine; GA_3 - gibberellic acid; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog; NAA - naphthalene acetic acid; PBU - N-phenyl-N'-[6-(2-chlorobenzothiazol)-yl] urea; TDZ - 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron); 2,4-D - 2,4-dichlorophenoxyacetic acid.
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To induce adventitious bud, calli derived from hypocotyls were cultivated on SPCa medium containing different combinations of 6-benzyladenine (BA) and naphthalene acetic acid (NAA). There were 3 replications of each treatment with 24 calli per replication. The calli were maintained at standard culture conditions of photoperiod and temperature. After 40 d, adventitious bud formation was noted.

In order to stimulate shoot elongation and proliferation, calli with short adventitious bud were first transferred to SPCa medium supplemented with 0.44 μM BA, 0.54 μM NAA and 0.3 μM gibberellic acid (GA_3) for 40 d. After this period, the clusters of elongated shoots (approximately 25 mm long) were excised from the callus and cultivated separately in modified SP medium with 20 g dm^{-3} surcose but without plant growth regulators. After 30 d, the elongated shoots (approximately 35 mm long) were cultivated in modified SP medium containing 2.5 μM IBA for rooting. After 15 d under standard culture conditions, 5 - 9 adventitious roots were visible at the bottom of shoot. Another 10 d later, the roots were long 30 - 40 mm. Subsequently, rooted plantlets in conical flask covered with ventilate pellicle were transferred to greenhouse (25 ± 2 °C and 80 % relative humidity) for 7 d and then plantlets were planted in plastic pots, containing a mixture of fine sand : *Vermiculite* (1:1).

The sterilization was efficient and only few seeds were polluted. On the third day, all seeds germinated and on the eighth day, seedlings were long 40 mm. The explants were important for subsequent callus induction. The larger were the seeds the more vigorous were the seedlings. The nearer the explants were to cotyledons, the easier they began to differentiate. If the original explants contained meristems of axillary buds the shoots would outgrow directly.

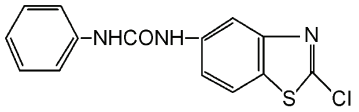

Fig. 1. Structures of the N - phenyl-N'-[6-(2-chlorobenzothiazol) yl] urea (PBU).

The calli at the cut surface were obtained at 100 % at the seventh day when the explants were cultured on SPCa medium containing PBU and IAA (Table 1). The calli were fast-growing, compact and greenish-yellow (Fig. 2). The calli could maintain growing ability for 30 - 40 d if supplied with fresh SPCa medium. Compared with TDZ or the combination of BA and 2, 4-D, PBU stimulated more vigorous callus and restrained calli from browning. In addition, the calli induced by PBU showed high frequency on adventitious buds induction after transferring to adventitious buds inducing medium.

The adventitious buds were observed 40 d after the calli were cultivated on SPCa medium supplemented with NAA and BA. The percentage of shoot induction ranged from 9.7 to 56.9 % (Table 2). If calli derived from hypocotyls were cultivated on SPCa medium without any plant growth regulators, adventitious shoots could not be produced. The results showed that the best combination of hormone for adventitious bud induction was 3.52 μM BA combined with 0.28 μM NAA (Table 2).

Table 1. Callus induction and growth from *E. urophylla* hypocotyls excised from 8-d-old seedlings and incubated on SPCa medium. The number was counted at 30th day. Each treatment contained 3 replications with 30 explants per replication. Values are means ± SD. Means within a column followed by different letter are significantly different by the Duncan's multiple range test at 1 % probability level.

Growth regulators [μM]	Number of calli [explant^{-1}]	Number of calli bigger than 5 mm in diameter [explant^{-1}]	Number of browning calli [explant^{-1}]
TDZ 1.5 + IAA 0.57	19.0 ± 1.0^{e}	7.7 ± 0.6^{cd}	15.7 ± 0.6^{a}
TDZ 2.0 + IAA 0.57	21.0 ± 0.6^{d}	9.7 ± 0.0^{c}	13.7 ± 0.6^{b}
TDZ 2.5 + IAA 0.57	18.7 ± 0.6^{e}	8.3 ± 0.6^{cd}	13.3 ± 0.6^{b}
PBU 4.9 + IAA 0.57	30.0 ± 0.0^{a}	19.0 ± 1.0^{b}	0^{d}
PBU 6.6 + IAA 0.57	29.7 ± 0.6^{a}	22.7 ± 0.6^{a}	0^{d}
PBU 8.2 + IAA 0.57	30.0 ± 0.0^{a}	19.0 ± 1.0^{b}	0^{d}
BA 0.9 + 2,4-D 0.45	27.0 ± 1.0^{c}	7.3 ± 1.5^{d}	0.7 ± 0.6^{cd}
BA 1.8 + 2,4-D 0.45	28.7 ± 0.6^{ab}	7.0 ± 1.0^{d}	1.3 ± 0.6^{c}
BA 2.7 + 2,4-D 0.45	28.3 ± 0.6^{bc}	6.3 ± 0.6^{d}	1.3 ± 0.6^{c}

Table 2. The effect of different combinations BA with NAA on adventitious buds induction from calli of *E. urophylla*. The results were analyzed at 40th day. Each treatment contained 3 replications with 24 calli per replication. Values are means ± SD. Means within a column followed by the different letter are significantly different by the Duncan's multiple range test at 1 % probability level.

Growth regulators [μM]	Rate of buds differentiation [%]	Bud number [callus^{-1}]
BA 2.64 + NAA 0.28	9.7 ± 2.4^{c}	1.7 ± 0.6^{c}
BA 3.08 + NAA 0.28	36.1 ± 2.4^{b}	3.3 ± 0.6^{b}
BA 3.52 + NAA 0.28	56.9 ± 6.4^{a}	8.0 ± 1.0^{a}
BA 3.96 + NAA 0.28	29.2 ± 4.2^{b}	2.7 ± 0.6^{b}
BA 4.40 + NAA 0.28	9.7 ± 2.4^{c}	1.7 ± 0.6^{c}

An increase in calcium concentration gave higher total protein and sugar contents in the callus tissue of *Eucalyptus*. It was possible to verify that calcium stimulated organogenesis at concentration of 6.12 mM (Arruda *et al*. 2000). To our experience, if the Ca^{2+} content was decreased to 1.5 mM (Ca^{2+} content of SP medium), the rate of adventitious buds formation decreased. And so did the number and the vigorousness of adventitious buds. What is more, the differentiation was delayed.

Calli with adventitious bud, which were cultivated on SPCa medium supplemented with 0.44 μM BA, 0.54 μM NAA and 0.3 μM GA_3 for a period of 30 to 40 d, produced vigorous and healthy green shoots (Fig. 2). There were

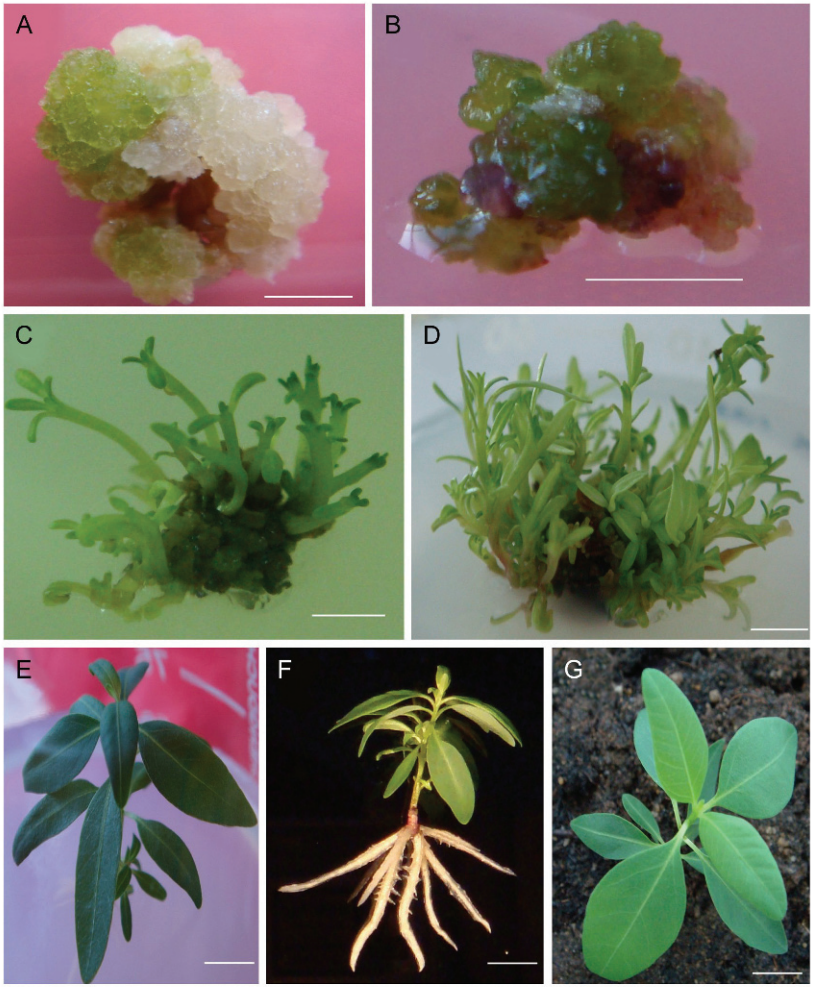

Fig. 2. Regeneration of *E. urophylla* from seedling-derived hypocotyls. *A* - Callus induced from 8-d-old seedling hypocotyls inoculated on SPCa medium with 6.6 µM PBU and 0.57 µM IAA, *bar* = 6 mm. *B*,*C* - Differentiation of callus and adventitious buds after transfer on SPCa medium with 3.52 µM BA and 0.28 µM NAA, *bar* = 6 mm; *D* - Elongation and proliferation of adventitious buds inoculated on SPCa medium supplemented with 0.44 µM BA, 0.54 µM NAA and 0.3 µM GA_3, *bar* = 10 mm; *E* - Shoot elongation on modified SP medium without plant growth regulators, *bar* = 5 mm; *F* - Rooting of elongated shoot cultivated in modified SP medium supplemented with 2.5 µM IBA, *bar* = 30 mm; G - plant in plastic pot in greenhouse, *bar* = 20 mm.

20 to 30 shoots per callus. Excised from calli and cultivated individually on SP medium, the shoots continued to elongate and leaves began to form.

Shoots transferred to rooting medium with 2.5 µM IBA had a good response, with the rooting rate between 95 to 100 % at fifteenth day. Additionally, the IBA treated shoots exhibited a superior rooting capacity (the average of 7 roots per shoot, 30 - 40 mm long), compared with the cultures in the SP medium without hormones (2 to 3 roots per shoot and only 10 mm long). The results showed that IBA effectively increased the rooting efficiency. Almost all of the rooted plantlets transferred to plastic pots, containing a mixture of sand and *Vermiculite*, survived in greenhouse and developed into normal plants.

We had developed a simple, reliable and efficient protocol to regenerate *E. urophylla* plants from seedling-derived explants. PBU, as one kind of di-substituted urea, was more efficient to induce *Eucalyptus* calli than thidiazuron or the combination BA and 2,4-D. To our knowledge, we firstly used PBU to induce *Eucalyptus* calli. This class of synthetic cytokinins probably acts by activating the cytokinin response pathway (Yamada *et al*. 2001).

This protocol had four stages consisting of a callus induction on a PBU-containing medium, an adventitious shoot formation stage on medium containing BA and NAA, an elongation stage in presence of BA, NAA and GA_3 and a final rooting stage on medium with IBA. It lasted about 5 months to get regenerated plant from seedling-derived hypocotyl. The protocol can be helpful to *E. urophylla* genetic engineering.

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