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Full Length Research Paper

Adventitious shoot regeneration from *in vitro* stem explants of *Phellodendron amurense*

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An efficient *in vitro* plant regeneration system from stem explants was established in *Phellodendron amurense*. Factors influencing shoot regeneration from stems including culture medium type, combinations of plant growth regulators and carbon source in the medium were investigated. Adventitious shoot regeneration was significantly influenced by the type of medium. Murashige and Skoog medium (MS) was the best for promoting shoot regeneration, followed by Gamborg medium (B5) and woody plant medium (WPM). The combination of 6-benzyladenine (BA) and naphthaleneacetic acid (NAA) produced better results for shoot regeneration. The optimum shoot regeneration frequency (74.5%) and number of shoots per explant (12.3) was achieved using MS medium supplemented with 29.7 µM BA and 5.8 µM NAA. High concentrations of BA and NAA in the medium inhibited shoot formation. Among the three sugars tested, 20 g dm^{-3} glucose was the optimum for shoot regeneration. Rooting of regenerated shoots was successful on 1/4-strength MS medium with the addition of 15.4 µM IBA. Almost 100% plantlets survived acclimatization after transferred to soil.

Key words: *Phellodendron amurense,* callus, shoot regeneration, stem explants.

INTRODUCTION

Highly efficient and reproducible *in vitro* regeneration system is a prerequisite for clonal propagation and for production of transgenic plants (Tang et al., 2006). *Phellodendron amurense* Rupr. belongs to the family *Rutaceae* and is native to northern China, Korea and Japan. It is an important rare endangered medicinal plant in China used as a crude drug for anti-stomachic, anti-inflammatory, and anti-pyreticagent (Yan et al., 2006; Ikuta et al., 1998). The wood of *P. amurense* is used for making furniture and industrial arts and the plant has potential as a source of industrial cork and yellow dyes (Azad et al., 2005). Commercial exploitation for production and conventional propagation of *P. amurense* is hampered due to its poor seed viability, low rate of germination, and poor rooting ability of vegetative cuttings (Azad et al., 2005). So far, several studies have been reported on micropropagation of *P. amurense* through axillary buds, adventitious shoot regeneration, and somatic embryogenesis (Ariyoshi, 1986; Azad et al., 2005, 2009). But there is no report on adventitious shoot regeneration from *in vitro* stem explants. The objective of this study was to determine the competency of *in vitro* stem tissue of *P. amurense* for regeneration and to develop an *in vitro* regeneration method for stem explants. Several factors affect shoot regeneration such as basal medium, carbon source and growth regulators were investigated. The method presented here will provide a regeneration method using a convenient and abundant tissue source that will facilitate the application of *Agrobacterium*-mediated transformation methods for improvement of this important tree species.

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Abbreviations: **BA**, 6-Benzyladenine; **B5**, gamborg medium; **IBA**, indole-3-butyric acid; **MS**, murashige and skoog medium; **NAA**, naphthaleneacetic acid; **WPM**, woody plant medium.

MATERIALS AND METHODS

Plant material

Stems were collected from *in vitro* cultured plantlets derived from dormancy buds of 20-years-old *P. amurense* grown in Northeast

Table 1. Effects of different concentrations and combinations of BA and NAA on adventitious shoot regeneration from stem - derived callus.

Growth regulator (μM)		Explants producing callus (%)	Shoot regeneration frequency (%)	Mean number of shoots/callus
BA	NAA			
0	0	0	0^{k}	0^{a}
9.9	2.9	100	63.8 ± 1.9^{c}	2.3 ± 0.2^{b}
19.8	2.9	100	69.5 ± 2.7^{efg}	3.7 ± 0.4^{c}
29.7	2.9	100	73.4 ± 1.2^{hi}	6.8 ± 0.5^{def}
39.6	2.9	100	72.1 ± 1.7^{ghi}	7.1 ± 1.1^{efg}
9.9	5.8	100	55.6 ± 1.7^{b}	1.2 ± 0.2^{ab}
19.8	5.8	100	70.1 ± 1.9^{fgh}	5.9 ± 1.0^{de}
29.7	5.8	100	74.5 ± 1.9^{j}	12.3 ± 0.7^{i}
39.6	5.8	100	66.3 ± 2.2^{cde}	9.8 ± 1.0^{h}
9.9	11.6	100	52.3 ± 2.0^{b}	7.2 ± 1.5^{efg}
19.8	11.6	100	65.3 ± 2.4^{cd}	7.8 ± 1.2^{fg}
29.7	11.6	100	67.8 ± 2.5^{def}	8.4 ± 0.4^{g}
39.6	11.6	100	34.5 ± 2.0^{a}	5.5 ± 0.4^{d}

The data were recorded after five weeks of culture and were presented as means ± SE; n = 90. Means followed by the same letter are not significantly different by Duncan's multiple comparison test at 0.05 probability level.

Forestry University experiment forest farm (45°20′N and 127°34′E). The dormancy buds were surface-sterilized for 30 s with 70% ethanol, 8 min in a 3% NaCLO, followed by three washes with sterilized-distilled water. Sterile dormancy buds were cultured on MS medium containing 20 g dm^{-3} sucrose and 7 g dm^{-3} agar. After four weeks culture, *in vitro* shoots were obtained from dormancy buds. Shoot multiplication was achieved by sub-culturing at four-weeks intervals on MS supplemented with BA 9.9 μM, 20 g dm^{-3} sucrose and 7 g dm^{-3} agar. Stems were collected from these *in vitro* shoots and used as explants.

Experimental design

Effect of hormonal composition of medium on adventitious shoot regeneration

To test the effect of plant growth regulators on shoot regeneration, stem explants were cultured on MS basal medium containing different concentrations and combinations of BA and NAA (Table 1) as well as 20 g dm^{-3} glucose and 7 g dm^{-3} agar. After five weeks of culture, the shoot regeneration frequency and the mean number of adventitious shoots per stem were recorded.

Effect of basal medium on adventitious shoot regeneration

To determine the effect of basal medium on shoot regeneration, the stems were cultured on MS, B5 and WPM medium containing BA 29.7 μM, NAA 5.8 μM as well as 20 g dm^{-3} glucose and 7 g dm^{-3} agar. After five weeks of culture, the shoot regeneration frequency and the mean number of adventitious shoots per stem were recorded.

Effect of carbon source on adventitious shoot regeneration

To examine the effect of carbon source on adventitious shoot regeneration, different sugars (glucose, fructose, sucrose) were applied at various concentrations (1, 2, 3 and 4%). Shoot regeneration frequency and the mean number of adventitious shoots per stem were recorded after five weeks of culture.

Shoot elongation

MS medium supplemented with BA 9.9 μM and 20g dm^{-3} sucrose was used for elongation of regenerated shoots (about 0.5 cm).

Rooting and transplanting

Elongated shoots (>4cm in height) were transferred to 1/4-strength MS medium supplemented with IBA 15.4 μM, as well as 20 g dm^{-3} sucrose, and 7 g dm^{-3} agar. After three weeks, the rooted plants were translated in the pots in the greenhouse. The pots were covered with transparent plastic films to ensure high humidity (>80%) during the acclimatization period of the first two weeks. After two weeks, the plastic films were removed.

Preparation of culture media and culture conditions

All media were adjusted to pH 5.8 prior to autoclaving at 121°C for 20 min. Cultures were incubated at 25 ± 2°C under a 16 h photoperiod of 40 μmol m^{-2} s^{-1} irradiance provided by white fluorescent tubes.

Statistical analysis

Each treatment consisted of 30 plants and was done in triplicate. After two weeks of culture, the percentage of explants producing callus was recorded. The frequency of shoot induction and average number of shoots per culture were determined and recorded after five weeks of culture. Data were subjected to ANOVA, and were analyzed by SPSS18.0, when the ANOVA indicated statistical

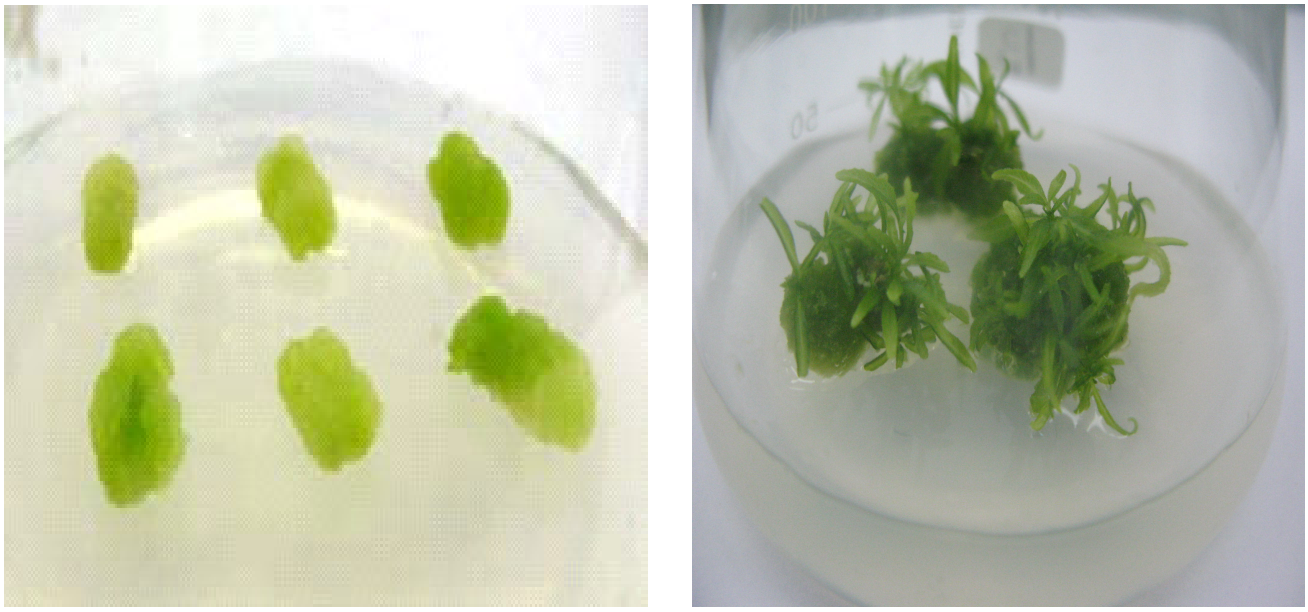

Figure 1. A, Callus induction on stem explants on the medium containing 29.7 µM BA and 5.8 µM NAA after two weeks of culture; B**,** Shoots regeneration from stem-derived callus after five weeks of culture on the medium containing 29.7 µM BA and 5.8µM NAA .

significance, and Duncan's multiple comparison tests was used to distinguish the difference between treatments.

RESULTS AND DISCUSSION

Effect of growth regulators on shoot regeneration

In the conducted experiments, stem explants were incubated on MS medium supplemented with a broad range of BA concentrations in combination with NAA (Table 1). Explants cultured on media without growth regulators did not develop callus or any shoot. On the medium containing BA and NAA, initially, the leaf explants gradually enlarged. After one week of culture, almost all the stem explants began to produce callus at the cutting surface (Figure 1a). The following two weeks, the explants began to produce adventitious shoots. Shoot regeneration frequency varied with different BA and NAA combinations. The medium containing BA 29.7 µM and NAA 5.8 µM was the most effective, providing high shoot regeneration frequency (74.5%) with 12.3 shoots per explants. On this medium, the regenerated shoots grew vigorously (Figure 1b). High concentration of BA (39.6 µM), not only decreased the regeneration frequency, but also reduced the number of shoots per callus. On the other hand, the presence of NAA concentration at 11.6 µM was found to inhibit shoot regeneration.

This showed that high concentrations of cytokinin and auxin in the medium inhibited shoot regeneration. Our results reveal that the addition of BA with NAA was essential in inducing adventitious shoots from the stem explants of *P. amurense*. Results obtained here showed consistency with other studies where BA and NAA were beneficial for callus culture and plant regeneration (Belarmino et al., 1992; Zhang et al., 2003; Agrawal and Sardar, 2006; Chen and Chang, 2006; Koroch et al., 2002). In this study, shoot regeneration was significantly affected by the different combinations of BA and NAA. As with other studies, a suitable BA and NAA combination is important for improvement in shoot regeneration for *P. amurense*. The optimum combination of BA and NAA for shoot regeneration may vary with the plant species.

Effect of basal medium on shoot regeneration

The mineral salt composition of the basal medium used is known to affect the regeneration capacity of various species (Kai et al., 2008). In this study, the type of media had no effect on callus induction; almost all the explants produced callus in two weeks culture (Table 2). However for further shoot regeneration from the callus, MS was the most effective, followed by B5 and WPM, which was similar in *Isatis indigotica* (Zhang et al., 2004). On MS medium, the shoot regeneration frequency was 72.1% with an average shoot number per explant being 11.7 within five weeks of culture. On WPM medium, the adventitious shoots occurred more slowly than those on the other two media. Many reports described the effects of medium composition on the shoot regeneration of different explants (Kai et al., 2008; Haliloglu, 2006;

Table 2. Effect of basal medium on adventitious shoot regeneration from stem-derived callus of *P. amurense*.

Basal medium	Explants producing callus (%)	Shoot regeneration frequency (%)	Mean number of shoots/callus
MS	100	72.1 ± 1.7^{c}	11.7 ± 0.9^{c}
B5	100	67.9 ± 1.5^{b}	9.1 ± 0.7^{b}
WPM	100	36.3 ± 1.0^{a}	3.3 ± 0.4^{a}

The data were recorded after five weeks of culture and were presented as means ± SE; *n* = 90. Means followed by the same letter are not significantly different by Duncan's multiple comparison test at 0.05 probability level.

Pretto and Santarem, 2000; Don Palmer and Keller, 2011). MS, B5 and WPM are the three standard types of most widely used media for the shoot regeneration of many species. It was inferred that the difference in their ionic strength might be the primary factor affecting shoot regeneration of many species. When we compared the three media, (MS, B5 and WPM), the contents of phosphorus and calcium were similar, while the content of nitrogen was different. The total nitrogen content in WPM medium (14.7 µM) was much lower than that in MS (60.03 µM) and B5 (27.0 3µM) (Shi et al., 2000). In this study, it appears that the content of total nitrogen might be an important factor that affected shoot regeneration of *P. amurense*. Knittel et al. (1991) also showed that the nitrogen supply had a decisive influence on shoot regeneration from sunflower cotyledons.

Effect of carbon source on shoot regeneration

To investigate the effects of carbohydrate sources on shoot regeneration, different concentrations of sucrose, glucose or fructose were added to MS medium containing 29.7 µM BA and 5.8 µM NAA. The results indicate that a continuous supply of carbohydrate from the medium is necessary for shoot regeneration. Stem explants grown on a medium without carbohydrates did not form any callus and shoot. Among the three sugars tested, glucose was the best for callus shoot regeneration, followed by sucrose and fructose. The best result was obtained on the medium containing 20 g dm^{-3} glucose, shoot regeneration frequency been 73.9% with 11.5 shoots per explant (Table 3). Further increase in glucose concentration (>20 g dm^{-3}) brought about the formation of more friable callus, as well as decrease in shoot regeneration frequency and shoot number per explant. This showed that higher glucose concentration inhibited shoot regeneration for *P. amurense*. Similar results were found on media containing sucrose and fructose. The superiority of glucose in promoting shoot regeneration from stem explants of *P. amurense* is consistent with the results obtained with Beech, Rosa hybrid and *chinensis minima* (Cuenca and Vieitez, 2000; Hsia and Korban, 1996; Lemos and Baker, 1998). This may be because that glucose is more efficiently uptake and transported in tissues or more immediately used to provide energy for shoot organogenesis. The lesser suitability of sucrose for adventitious bud initiation may also be due to the partial breakdown of sucrose into glucose and fructose as the result of autoclaving (Cuenca and Vieitez, 2000).

Shoot elongation

After five weeks of culture, regenerated shoots were subcultured on MS medium with 9.9 µM BA and 20 g dm^{-3} for shoot elongation and proliferation. When subcultured onto fresh medium, regenerated shoots excised from callus continued to produce shoots providing a potential method for further multiplication of shoots and an increase in regeneration efficiency (data not shown).

Rooting and acclimatization of the regenerated shoots

Elongated shoots (>4 cm in height) were transferred to 1/4 - strength MS medium supplemented with 15.4 µM IBA, and 20 g dm^{-3} sucrose. After ten days of culture, root formation occurred from basal cutting of shoots. Root frequency reached 90.6% with 4.3 roots per shoot. After three weeks culture on rooting medium, the rooted plantlets were transferred to the soil in the greenhouse. During the first two weeks in the greenhouse, air relative humidity >80% was maintained. Under these conditions, 100% of the plantlets survived and resumed normal growth.

Conclusion

In this study, we achieved a higher shoot regeneration frequency from stem explants of *P. amurense*. MS medium with suitable BA and NAA combination are necessary for the desirable shoot regeneration from stem-derived callus. Among different sugars, glucose was found to be best for promoting shoot regeneration. This research demonstrates that *in vitro* stem tissue of *P. amurense* is competent for adventitious shoot organogenesis which will be most useful for future *P. amurense* genetic transformation.

Table 3. Effect of carbon source on adventitious shoot regeneration from stem-derived callus of *P. amurense.*

Carbon Source (g dm^{-3})	Explants producing callus (%)	Shoot regeneration frequency (%)	Mean number of shoots/callus
0	0	0^{g}	0^{g}
Glucose (10)	100	34.1 ± 1.9^{b}	3.5 ± 1.0^{ab}
Glucose (20)	100	73.9 ± 2.8^{ef}	11.5 ± 0.4^{f}
Glucose (30)	100	65.2 ± 2.6^{d}	9.1 ± 0.3^{e}
Glucose (40)	100	57.3 ± 3.2^{c}	8.8 ± 1.1^{e}
Sucrose (10)	100	23.1 ± 1.7^{a}	3.2 ± 0.8^{ab}
Sucrose (20)	100	70.4 ± 2.7^{e}	8.7 ± 0.5^{e}
Sucrose (30)	100	75.4 ± 2.8^{f}	8.3 ± 0.9^{de}
Sucrose (40)	100	63.1 ± 1.7^{d}	7.2 ± 0.8^{d}
Fructose (10)	100	77.5 ± 2.6^{f}	4.2 ± 0.7^{bc}
Fructose (20)	100	66.0 ± 2.4^{d}	4.9 ± 0.3^{c}
Fructose (30)	100	36.3 ± 2.4^{b}	3.5 ± 0.5^{ab}
Fructose (40)	100	33.2 ± 1.8^{b}	2.7 ± 0.6^{a}

The data were recorded after 5 weeks of culture and were presented as means ± SE, $n = 90$. Means followed by the same letter are not significantly different by Duncan's multiple comparison test at 0.05 probability level.

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