Journal of Medicinal Plants Research Vol. 5(18), pp. 4560-4565, 16 September, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Rapid direct adventitious shoot organogenesis and plant regeneration from mature seed explants of *Phellodendron amurense* Rupr.

Jing Li Yang[1], Chen Guang Zhou[1], Bo Zhao[2], Chun Lian Jin[1]Teng Zhang and Cheng Hao Li[1]*

[1]State Key Laboratory of Tree Genetics and Breeding, Northeast Forestry University, Harbin, 150040, P. R. China.
[2]Pharmacy College, Heilongjiang University of Chinese Medicine, Harbin, 150086, P. R. China.

Accepted 11 August, 2011

Thidiazuron (TDZ) was more effective than 6-benzyladenine (6-BA) for adventitious shoot induction from mature seeds of *Phellodendron amurense* Rupr. Explants of hypocotyl + cotyledon produced more adventitious shoots than explants of cotyledon alone. Water rinsing pretreatment of dehusked seeds, optimally for two or three days, shortened the adventitious shoot induction period to 5 days. Adventitious shoot induction frequency (91.8%) and shoot number per explant (38.4) were highest on Murashige and Skoog (1962) medium (MS) with 1.0 mg/L TDZ and 0.2 mg/L α-naphthaleneacetic acid. Histological investigations indicated that adventitious shoots were induced directly from hypocotyl endodermal cells. 6-BA was more effective than TDZ for adventitious shoot elongation. Rooting frequency was highest (85%) with 1.0 mg/L indole-3-butyric acid. The rapid and high frequency adventitious shoot induction and plant regeneration method that we developed may be useful in future studies on *Agrobacterium*-mediated genetic transformation of *P. amurense*.

Key words: *Phellodendron amurense*, mature embryo, direct organogenesis, thidiazuron, ploidy analysis.

INTRODUCTION

Phellodendron amurense Rupr., in the family Rutaceae, is a deciduous tree native to northern China, Korea, and Japan. *P. amurense* is a traditional Chinese medicinal plant, producing many medicinal compounds such as biologically-active flavonoids. Alkaloids such as magnoflorine, berberine, palmatine, and phellodendrin have been isolated from *P. amurense* bark, and they have been used as anti-inflammatory, antipyretic, and antibacterial medicines (Ikuta et al., 1998). At present, large areas of wild *P. amurense* are rare and in severe danger due to overharvesting.

The extensive use of *P. amurense* in Asia has raised interest in seed propagation. Although propagation of these species through conventional breeding requires a long time to evaluate new genotypes, genetic engineering can quickly and efficiently produce specific genomic changes without substantially altering the original genetic integrity. The successful production of transgenic plants through genetic engineering depends on an efficient regeneration system (Magyar-Tábori et al., 2010). Compared to indirect organogenesis, direct organogenesis from differentiated tissue generally results in a lower frequency of somaclonal variation and a greater genetic and cytological uniformity (Annapurna and Rathore, 2010; Ghimire et al., 2010). Adventitious shoot regeneration can occur using interpetiolar buds, hypocotyls, internodes, or leaves as explants (Ariyoshi et al., 1986; Azad et al., 2004; Azad et al., 2005). Among these approaches, young leaves from *in vitro* shoots were highly efficient for regeneration (Azad et al., 2005); however, the overall culture procedure is too complex, requiring 12 weeks for callus induction and several subcultures for adventitious shoot formation.

To our knowledge, there are no reports describing the regeneration of *P. amurense* from mature zygotic embryos. In the current study, we have established a rapid and efficient method for direct adventitious shoot induction and plant regeneration from mature *P. amurense* seeds. We believe our method can be adapted

*Corresponding author. E-mail: chli0@163.com. Tel: +86-451-82190607-11. Fax: +86-451-82190607-11.

for genetic manipulation and functional genetic studies.

MATERIALS AND METHODS

Plant material and adventitious shoot induction

Mature fruits of *P. amurense* Rupr. were collected from the Botanical Garden in Harbin, Heilongjiang province, P. R. China. The seeds were manually extracted, and the seed coat was removed. Dehusked seeds were agitated in 70% (v/v) ethanol for 30 s, surface-sterilized in a 1.0% (w/v) sodium hypochlorite solution for 10 min, and rinsed five times with sterile distilled water. The distal half of cotyledons of surface-sterilized seed explants were removed with a scalpel, and then slivered the hypocotyl + remaining half of cotyledon into 2 pieces (Figure 1a). The dissected cotyledon and hypocotyl + cotyledon explants were cultured on MS medium containing 3.0% (w/v) sucrose supplemented with 0.1, 0.5, 1.0, 4.0, 8.0 mg/L 6-benzyladenine (6-BA) or the same concentration of thidiazuron (TDZ), respectively together with 0.2 mg/L α-naphthaleneacetic acid (NAA). To evaluate the effect of water rinsing pretreatment on adventitious shoot induction, dehusked seeds were rinsed in running water for 0, 1, 2, 3, or 4 days, and then the dissected hypocotyl + cotyledon explants were cultured on MS medium containing 1.0 mg/L TDZ and 0.2 mg/L NAA. Cultures were incubated under a 16 h light/8 h dark photoperiod at 25°C under illumination with cool white fluorescent lights at 45 µmol/m^2/s.

Shoot elongation, rooting, and transplantation

Approximately 0.5 cm segments of adventitious shoots were transferred to ½ strength MS medium containing 2.0% (w/v) sucrose supplemented with 0.01, 0.1, 0.5, or 1.0 mg/L 6-BA, either alone or in combination with 0.2 mg/L NAA. Adventitious roots were induced by transferring > 3.0 cm high shoots to ½ strength MS medium containing 2.0% (w/v) sucrose supplemented with 0.1, 0.5, or 1.0 mg/L indole-3-butyric acid (IBA) alone or combine with 0.1, 0.5 mg/L NAA respectively. Regenerated plantlets were transferred to pots containing an autoclaved sand and soil mixture (1:3, v/v), covered with transparent plastic to maintain high humidity, and they were incubated in a growth chamber at 21°C with a 16 h light/ 8h dark photoperiod for 2 weeks.

Scanning electron microscope (SEM) and histological analyses

To determine the origin of the adventitious shoots, hypocotyl + cotyledon explants were collected at 0 and 5 days after culture on medium containing 1.0 mg/L TDZ and 0.2 mg/L NAA, and pre-cooled to –120°C using liquid nitrogen. The explants were viewed with a low-vacuum SEM (LV-SEM, S-3500N; Hitachi, Hitachinaka, Japan). For histological analysis, different induction period samples were fixed by immersion in a formalin:acetic acid:ethanol:water solution (FAA, 1:1:9:9 v/v) for 48 h and stored in 70% (v/v) ethanol at 4°C, then stained with hematoxylin solution for 2 days, dehydrated with increasing ethanol concentrations, and embedded in paraffin. Sections (8 µm) were cut with a microtome and fixed on glass slides. The sections were de-waxed in xylene and observed under a light microscope (Docuval, Carl Zeiss, Jena, Germany).

DNA ploidy analysis

Nuclear suspensions of *in vitro* and seed-propagated plants were prepared as previously described (Galbraith et al., 1983). Ploidy analysis was performed using the PA-I ploidy analyzer according to user manual (Partec, Munster, Germany).

Culture conditions and statistical analyses

All media used in experiments were adjusted to pH 5.8 before adding 0.55% (w/v) plant agar (Duchefa, Haarlem, The Netherlands) and autoclaving samples (1.1 kg/cm^2) at 121°C for 15 min. Explants were cultured in 150 ml Erlenmeyer flasks containing 30 ml medium and subcultures at 4 week intervals for: (i) adventitious shoot induction (20 explants/flask); or (ii) shoot elongation and plantlet regeneration (6 explants/ flask). Each experimental unit consisted of four flasks with three reduplications. Data were analyzed using analysis of variance (ANOVA) followed by Duncan's multiple range test at a 5% level.

RESULTS AND DISCUSSION

Adventitious shoot induction

After approximately 4 to 5 days of dark culture, hypocotyl explants began to swell, and the cotyledons became green but did not change shape. Small green spheroids appeared on the surface of the cotyledon axil region and hypocotyl on days 8 to 10, and the spheroids developed into adventitious shoots within the following 3 days (Figure 1b).

The type of explant significantly influenced the adventitious induction frequency and the shoot number/explant ($P < 0.0001$). Compared to the cotyledon explants, the hypocotyl + cotyledon explants produced a greater number of adventitious shoots and more shoots/explant when cultured on the same induction medium (Table 1). Similar effects of how the explant was oriented on the medium and what position the explant was taken from in the plant on the regeneration frequency were shown by Gow et al. (2009). TDZ was more effective than BA for adventitious shoot induction, with optimal results in MS medium containing 1.0 mg/L TDZ and 0.2 mg/L NAA (Table 1). In addition, while BA induced callus (Figure 1b), TDZ directly induced adventitious shoot formation (Figure 1c). We show highly efficient adventitious shoot production within only 12 days of mature embryo culture in induction medium containing TDZ.

Water rinsing pretreatment of dehusked seeds significantly improved adventitious shoot induction efficiency and shortened the induction period (from 12 to 5 days) when hypocotyls + cotyledon explants were cultured with 1.0 mg/L TDZ and 0.2 mg/L NAA. A two-day rinsing period was optimal for adventitious shoot induction, because rinsing for longer periods may damage the endosperm and the zygotic embryos during sterilization, resulting in decreased induction frequency (Table 2).

The rinsing pretreatment may stimulate water uptake and decrease the endogenous abscisic acid levels to break dormancy, thus improving adventitious shoot induction (Finch-Savage and Leubner-Metzger, 2006).

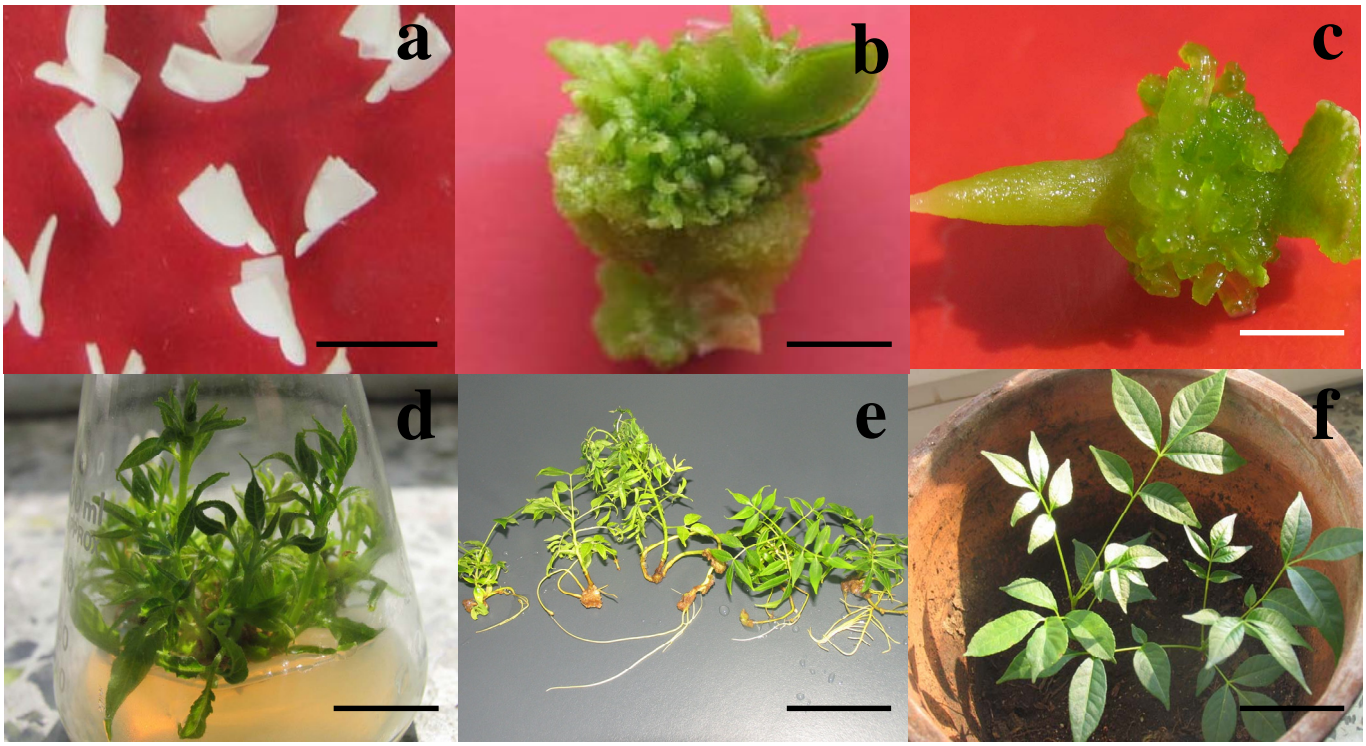


Figure 1. Organogenesis and plant regeneration of *P.* amurense by mature seeds. (a) Excised hypocotyls with cotyledon explants of mature embryos. Bar: 3 mm, (b) adventitious shoots induced from connection regions of hypocotyls and cotyledons. Bar: 2 mm, (c) adventitious shoots induced from connection regions of hypocotyls and cotyledons. Bar: 2 mm, (d) elongation of adventitious shoots. Bar: 20 mm, (e) regenerated plantlets. Bar: 40 mm, (f) plantlets transplantation in soil. Bar: 50 mm.

Shoot elongation, rooting, and transplantation

TDZ, a synthetic phenylurea derivative with cytokinin activity, is more active than 6-BA in inducing adventitious shoots and increasing shoot regeneration in many species (Guo et al., 2005; Annapurna and Rathore, 2010; Khurana-Kaul et al., 2010), possibly due to the stability of TDZ *in vitro* (Mok and Mok, 1985). Few shoots elongated, however, and the conversion number and frequency of adventitious shoots developing into seedlings were very low after long-term culture on TDZ, as previously reported for other species (Lyyra et al., 2006; Murthy et al., 1998). Shoots failed to elongate on *Salix nigra* inflorescence explants treated with either high TDZ concentrations or long-term exposure to TDZ (Lyyra et al., 2006). Over-exposure to TDZ can retard shoot and root development, which limits the recovery of functional plants (Murthy et al., 1998). Hence, in our study, the shoots induced by TDZ were transferred to media containing other plant growth regulators in order to induce rapid growth. 6-BA in combination with NAA enabled faster and enhanced elongation of adventitious shoots, with the longest shoots (5.0 cm) produced on medium with 0.5 mg/L 6-BA and 0.2 mg/L NAA (Table 3 and Figure 1d).

IBA was more conducive than NAA to rooting of *P. amurense* adventitious shoots, rooting frequency was highest (85%) with 1.0 mg/L IBA (Figure 1e). A total of 280 regenerated plantlets were obtained, and approximately 82% survived after acclimatization to greenhouse conditions (Figure 1f).

SEM and histological analyses

As compared to the smooth surface of controls (Figure 2a), several globular buds emerged on the hypocotyls regions after culture on induction medium containing TDZ (Figure 2b). These buds further developed into shoots after undergoing several divisions (Figure 2c). Although, Figure 2c shows simultaneous callus formation, shoots could not be induced from callus, suggesting that the adventitious shoots were induced directly from the zygotic embryo.

Histological observation of control seeds cultured on medium without growth regulators showed no adventitious shoot production (Figure 2d). In contrast, endodermal cells differentiated into shoots in the cotyledon axis region on induction medium containing TDZ (Figure 2e). The meristematic cells were closely arranged with a dense cytoplasm and conspicuous nucleus (Figure 2f). Continuous vascular strands from the explants to the shoots indicated a direct origin of shoots.

Table 1. Effect of 6-BA and TDZ on adventitious shoot induction of of *P. amurense.*

PGRs (mg L^{-1})			Cotyledon		Hypocotyls with cotyledon	
6-BA	TDZ	NAA	Shoot induction (%)[z]	Number of shoots per explant	Shoot induction (%)	Number of shoots per explant
0.1		0.2	0^{b}	0^{e}	52.1^{e}	3.3^{g}
0.5		0.2	4.8^{a}	7.1^{c}	60.9^{d}	8.3^{f}
1.0		0.2	4.2^{a}	7.9^{c}	63.2^{d}	9.2^{f}
4.0		0.2	4.2^{a}	10.5^{b}	78.0^{b}	14.2^{c}
8.0		0.2	4.6^{a}	7.2^{c}	71.4^{c}	11.4^{de}
	0.1	0.2	4.8^{a}	3.7^{d}	73.3^{bc}	12.5^{d}
	0.5	0.2	4.5^{a}	8.1^{c}	83.3^{a}	15.3^{c}
	1.0	0.2	4.4^{a}	11.0^{b}	87.1^{a}	21.1^{a}
	4.0	0.2	4.5^{a}	15.0^{a}	85.6^{a}	17.4^{b}
	8.0	0.2	4.3^{a}	7.0^{c}	73.9^{bc}	10.9^{e}

[z] Values with different letters in a column are significantly different according to Duncan's multiple range test at the 5% level.

Table 2. Effect of water rinsing pretreatment on adventitious shoot induction of of *P. amurense* mature seeds explants.

Water rinsing period (days)	Time of adventitious shoots induction (days)[&]	Adventitious shoot induction (%)[z]	Number of shoots per explant
0	12	87.1^{a}	21.1^{c}
1	7	85.6^{a}	33.5^{b}
2	5	91.8^{a}	38.4^{a}
3	5	64.3^{b}	37.5^{a}
4	10	32.4^{c}	9.3^{d}

[&]The dissected hypocotyls with cotyledon explants were cultured on MS medium supplemented with 1.0 mg L^{-1} TDZ and 0.2 mg L^{-1} NAA. [z] Values with different letters in a column are significantly different according to Duncan's multiple range test at the 5% level.

Ploidy stability analysis

All samples analyzed had the same ploidy level (2n) as control seedlings, and no polyploidy was observed (Figures 3a and b).

The ploidy stability among the regenerated plants likely resulted from plant regeneration through direct organogenesis, which generally reduces variation caused by the lack of a callus phase (Annapurna and Rathore, 2010; Ghimire et al., 2010). Further analyses are therefore needed to determine whether such genetic alterations occur among the regenerated plants.

Conclusion

In conclusion, this study developed a rapid and high frequency direct adventitious induction method from mature seeds of *P. amurense* on culture media containing TDZ alone or in combination with NAA. SEM and histological analyses revealed that the adventitious shoots were indeed initiated directly from the cotyledon axis of the mature embryonic explants.

This method may be useful for future studies on *Agrobacterium*-mediated genetic transformation of

Table 3. Effect of 6-BA and NAA on elongation of adventitious shoots of *P. amurense*.

6-BA (mg L^{-1})	NAA (mg L^{-1})	Length of shoots (cm) [z]
0.01		3.4[d]
0.01	0.02	3.6[cd]
0.01	0.2	3.7[cd]
0.1		3.8[cd]
0.1	0.02	3.7[cd]
0.1	0.2	3.7[cd]
0.5		3.8[cd]
0.5	0.02	4.8[ab]
0.5	0.2	5.0[a]
1.0		3.4[d]
1.0	0.02	3.9[cd]
1.0	0.2	4.3[bc]

[z] Values with different letters in a column are significantly different according to Duncan's multiple range test at the 5% level.

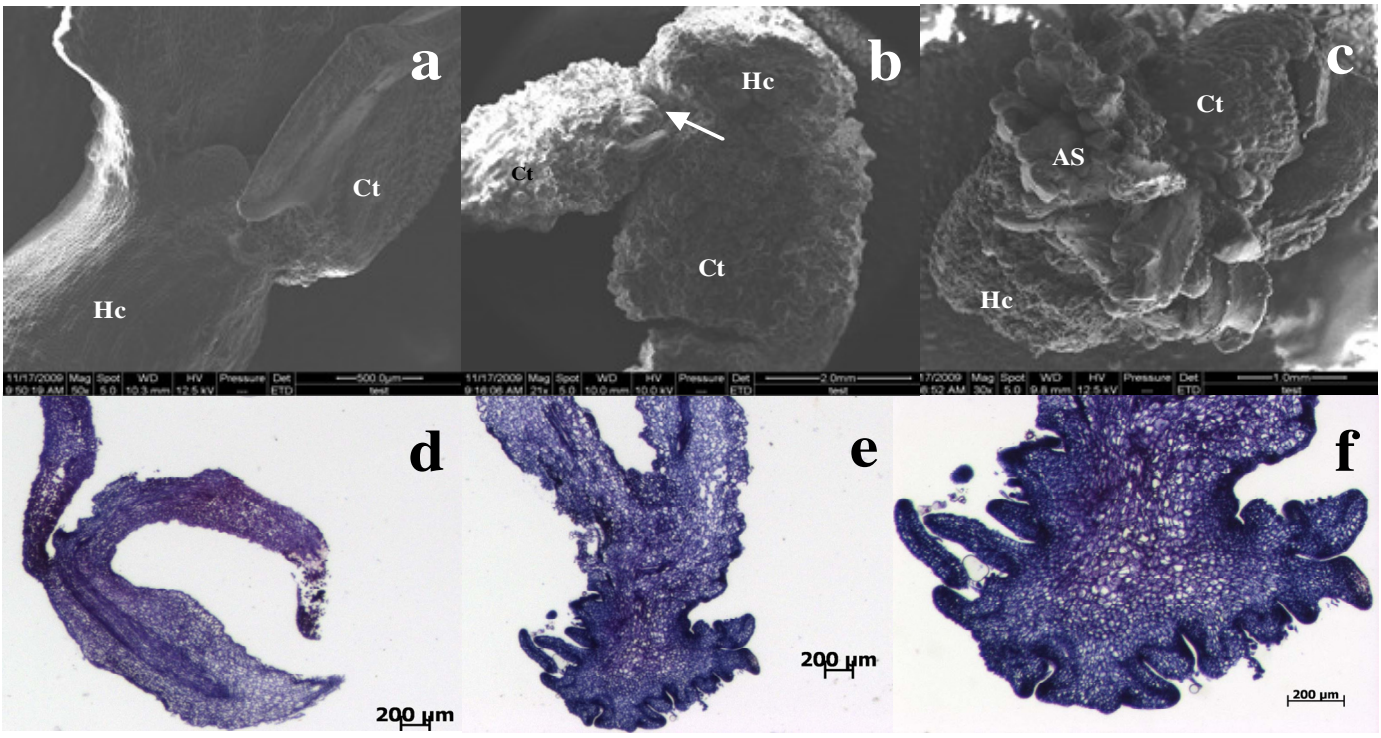
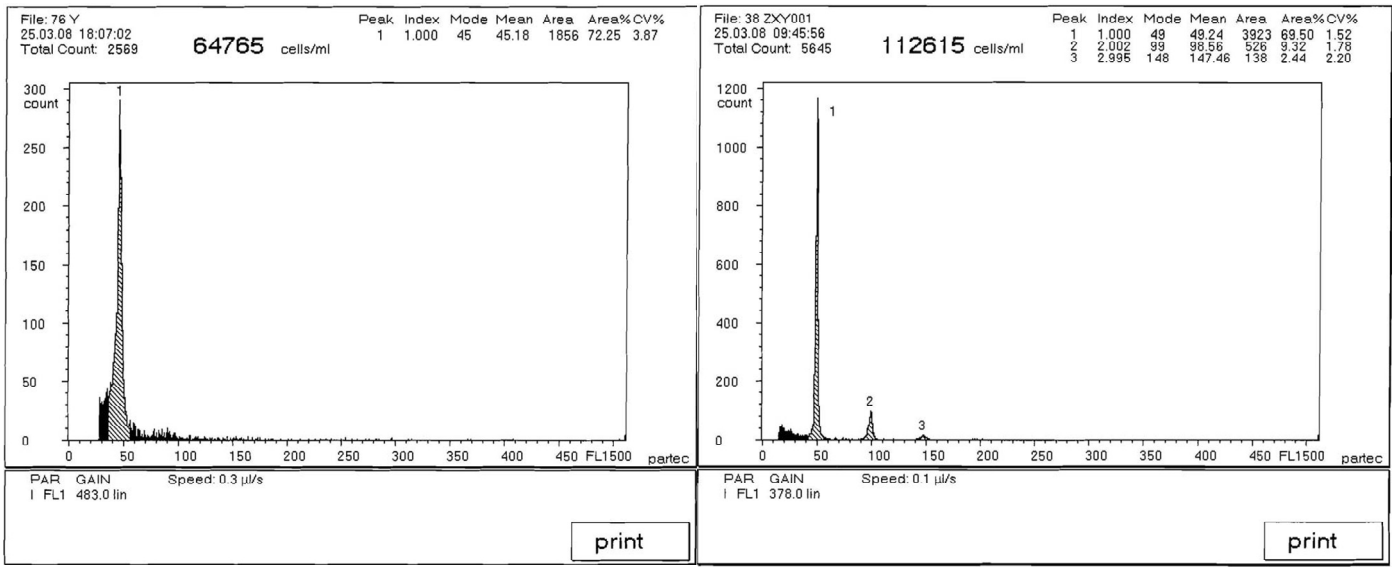


Figure 2. Scanning electron microscopy (a, b, c) and histological observation (d, e, f) of adventitious shoots induction of *P. amurense*. (a) Untreated control seed. (b) Surface of hypocotyls + cotyledon cultured for 3 days on induction medium, adventitious shoots (arrow) initiated at this time. (c) Numerous adventitious shoots developed following 7 days of culture. (d) Germinated control seed. (e) Adventitious shoots induced from hypocotyls of explants. (f) Adventitious shoots directly originated from vascular strands of hypocotyls of explants.

P. amurense.

ACKNOWLEDGEMENTS

This research was supported by the "Sponsoring the excellent doctor degree dissertation in Northeast Forestry University (OPTP10-NEFU)", "Sponsoring the postgraduate degree dissertation in Northeast Forestry University (Gram09)" and "Fundamental Research Funds for the Central Universities (DL09CA12)".



a **b**

Figure 3. Histograms of relative fluorescence intensity obtained through flow cytometry. (a) Leaves from *in vitro* regenerated plants, (b) leaves from seed propagated plants.

REFERENCES

Annapurna D, Rathore TS (2010). Direct adventitious shoot induction and plant regeneration of *Embelia ribes* Burm F. Plant Cell Tiss. Org. Cult., 101: 269-277.

Ariyoshi K (1986). Plantlet formation by intrapetiolar bud culture of *Phellodendron amurense* Rupr. For. Tree Breed, 138: 24-26.

Azad MAK, Yokota S, Ohkubo T, Andoh Y, Yahara S, Yoshizawa N (2005). *In vitro* regeneration of the medicinal woody plant *Phellodendron amurense* Rupr. through excised leaves. Plant Cell Tiss. Org. Cult., 80: 43-50.

Azad MAK, Yokota S, Ohkubo T, Yahara S, Yoshizawa N (2004). Micropropagation of plantlets through callus in Kihada (*Phellodendron amurense* Rupr.). J. S.H.I.T.A, 16: 122-130.

Finch-Savage WE, Leubner-Metzger G (2006). Seed dormancy and the control of germination. New Phytol., 171: 501-523.

Ghimire BK, Seong ES, Goh EJ, Kim NY, Kang WH, Kim EH, Yu CY, Chung IM (2010). High-frequency direct shoot regeneration from *Drymaria cordata* Willd. leaves. Plant Cell Tiss. Org. Cult., 100: 209-217.

Gow WP, Chen JT, Chang WC (2009). Effects of genotype, light regime, explant position and orientation on direct somatic embryogenesis from leaf explants of *Phalaenopsis orchids*. Acta Physiol. Plant, 31: 363-369.

Guo D, Zhu Z, Hu X, Zheng S (2005). Effect of cytokinins on shoot regeneration from cotyledon and leaf segment of stem mustard (*Brassica juncea* var. tsatsai). Plant Cell Tiss. Org. Cult., 83: 123-127.

Ikuta A, Hakamura T, Urabe H (1998). Indolopyridoquinazoline, furoquinoline and canthinone type alkaloids from *Phellodendron amurense* callus tissues. Phytochemistry, 48: 285-291.

Khurana-Kaul V, Kachhwaha S, Kothari SL (2010). Direct shoot regeneration from leaf explants of *Jatropha curcas* in response to thidiazuron and high copper contents in the medium. Biol. Plant, 54: 369-372.

Lyyra S, Lima A, Merkle SA (2006). *In vitro* regeneration of *Salix nigra* from adventitious shoots. Tree physiol., 26: 969-975.

Magyar-Tábori K, Dobránszki J, Teixeira D, Silva JA, Bulley SM, Hudák I (2010). The role of cytokinins in shoot organogenesis in apple. Plant Cell Tiss. Org. Cult., 101: 251-267.

Khurana-Kaul V, Kachhwaha S, Kothari SL (2010). Direct shoot regeneration from leaf explants of *Jatropha curcas* in response to thidiazuron and high copper contents in the medium. Biol. Plant, 54: 369-372.

Lyyra S, Lima A, Merkle SA (2006). *In vitro* regeneration of *Salix nigra* from adventitious shoots. Tree physiol., 26: 969-975.

Magyar-Tábori K, Dobránszki J, Teixeira D, Silva JA, Bulley SM, Hudák I (2010). The role of cytokinins in shoot organogenesis in apple. Plant Cell Tiss. Org. Cult., 101: 251-267.

Mok MC, Mok DWS (1985). The metabolism of [14C]-thidiazuron in callus tissue of *Phaseolus lunatus* L. Plant Physiol., 65: 427-432.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant, 15: 473-497.

Murthy BNS, Murch SJ, Saxena PK (1998). Thidiazuron: a potent regulator of *in vitro* plant morphogenesis. *In Vitro* Cell. Dev. Biol. Plant, 34: 267-275.