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Full Length Research Paper

Highly efficient *in vitro* adventitious shoot regeneration of *Adenosma glutinosum* (Linn.) Druce using leaf explants

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***Adenosma glutinosum* (Linn.) Druce is an important aromatic plant, but no information is available regarding its regeneration, callus induction and proliferation from leaf explants. In this study, an *in vitro* shoot regeneration procedure was developed for native *A. glutinosum* using leaf explants. Callus induction and shoots regeneration from leaf explants was evaluated on Murashige and Skoog (MS) media supplemented with combinations of 6-benzylaminopurine (6-BA) and α-naphthaleneacetic acid (NAA). Callus induction in all 16 treatments exceeded 95%, and the highest adventitious shoot number per callus (7.22 shoots per explant) was obtained when leaf explants were cultured on MS medium supplemented with 0.5 mg• L^{-1} 6-BA, 0.1 mg• L^{-1} NAA, 3% sucrose and 0.72% agar. The highest shoots strengthening were obtained when adventitious buds were cultured on half-strength MS medium supplemented with 0.3 mg• L^{-1} NAA, 3% sucrose, 1.0 g• L^{-1} active carbon and 0.72% agar. The highest total root number (45.2) and root length (43.3 cm) were obtained when adventitious buds were cultured on half-strength MS medium supplemented with 0.0 mg• L^{-1} NAA, 3% sucrose, 1.0 g L^{-1} active carbon and 0.72% agar, while the highest total root surface area (4.1 cm^2) and total root volume (114.1 mm^3) were obtained when adventitious buds were cultured on half-strength MS medium supplemented with 0.5 mg• L^{-1} NAA, 3% sucrose, 1.0 g• L^{-1} active carbon and 0.72% agar. The efficient plant regeneration system developed here will be helpful for rapid micropropagation and further genetic improvement in *A. glutinosum*.**

Key words: *Adenoma glutinous*, plant growth regulator, plant regeneration.

INTRODUCTION

Adenosma is a genus of aromatic and ornamental perennial plants of Scrophulariaceae in the South and South-East Asia and Oceania, which include about 15 species. In southern China, there are four species including *Adenosma glutinosum*, *A. indianum*, *A. javanicum* and *A. retusilobum* (Hong et al., 1998). *Adenosma* contains eucalyptol, β-bisabolene, limoneneand various volatile oils (Wang and Wei, 2008) which play important role in pharmacological areas that are active against many diseases, especially rheumatism, stasis eliminating and detumescence analgesic (Ji and Pu, 1985; Liang and Zhong, 2005; Wu et al., 2010).

Micropropagation offers the potential to produce

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Abbreviations: MS, Murashige and Skoog; **6-BA,** 6-benzylaminopurine; **NAA,** α-naphthaleneacetic acid; **AC,** activated charcoal.

millions of clonal individuals through tissue culture via induction of morphogenesis from various plant tissues. This method is commonly employed for the mass propagation of plant species (Glowacka et al., 2010). To date, tissue culture of several aromatic plants has been reported with an emphasis on embryogenic callus formation, growth, and regeneration of *Pogostemon cablin* (Paul et al., 2010), *Tagetes patula* (Qi et al., 2011), *Eucalyptus* (Deepika et al., 2011), *Rosmarinus officinalis* (Deng et al., 2008), *Adenosma buchneroides* (Xu et al., 2008) and *A. glutinosum* (Liu et al., 2010). Qi et al. (2011) reported that callus induction from anther explants depended on genotype, temperature pretreatment, plant growth regulators, light regimes and sucrose concentration in *T. patula*. The highest callus induction rate and regeneration frequency of line 21605 was obtained when inflorescence buds were stored at 4℃ for 4 days, and anthers with microspores at the mid to late uninucleate stage were cultured on Murashige and Skoog (MS) basal medium containing 6-benzylaminopurine (BA, 2.2 μM) and α-naphthaleneacetic acid (NAA, 1.82 or 2.7 μM). Frequencies of callus induction and shoot regeneration were 100 and 70.5%, respectively with the whole regeneration procedure completed in 40 days under light. Deepika et al. (2011) also compared eight *Eucalyptus* genotypes for callus induction and shoot regeneration potential and concluded that browning of callus tissue and surrounding culture media is a common obstacle limiting regeneration of shoots in eucalypts and an optimum protocol for the generation of transgenic plants of clonal *Eucalyptus* genotypes was developed. Furthermore, Deng et al. (2008) studied the effects of different content of 6-BA and NAA on shoot tip culture using shoot tip from *R. officinalis* as explants and found that MS + 6-BA 1.0 mg• L^{-1} + NAA 0.02 mg• L^{-1} favored shoot tip culture and 1/2 MS + IAA 0.3 to 0.4 mg• L^{-1} was fit for root growth. There are relatively few reports on callus induction and plant regeneration in *A. glutinosum*.

In addition, Liu et al. (2010) established a regeneration procedure of *A. glutinosum* using shoot tips and found that the cultural system for adventitious buds proliferation of *A. glutinosum* is the MS medium with 0.25 mg• L^{-1} of BA and 0.5 mg• L^{-1} of indole-3-butyric acid (IBA). However, it induced low shoot regeneration and required very long culture periods. Due to the small number of tissue culture studies in this species, embryogenic callus formation, callus growth, and plant regeneration in *A. glutinosum* are not well understood. Moreover, when considering the development of high-throughput transformation systems, an efficient and reliable regeneration system is needed for successful industrial production and also genetic transformation. Hence, the objective of the present study was to examine tissue culture propagation systems and determine suitable optimum growth conditions and culture medium for callus induction for *A. glutinosum*; we examined both establishments of adventtious buds and adventitious roots.

MATERIALS AND METHODS

Young *A. glutinosum* plants (2 to 3 cm long) used as explants were obtained from the botanical garden of the Zhejiang A and F University, Zhejiang, China. These plants were washed under running tap water followed by soaking in 10% liquid dishwasher detergent (P&G Co. Ltd, Cincinnati, OH) for 5 min. Thereafter, the plants were rinsed in distilled water for 8 to 10 min. They were followed by surface sterilization using mercuric chloride (0.1% w/v) in laminar flow cabinet and rinsed with sterilized distilled water 3 times. The apical shoots and axillary bud with internode were excised from the sterilized plants, cut to 8 mm and placed vertically into half strength of MS medium (Murashige and Skoog, 1962) containing various concentrations of 6-benzylaminopurine (6-BA) and α-naphthaleneacetic acid (NAA), with 3% (w/v) sucrose, 1.0 g• L^{-1} active carbon and 0.72% Type-A agar (Sigma Chemical Co., St. Louis, Mo., USA). Three weeks after initial culture, leaves enlarged enough to be used as leaf explants, were selected for regeneration experiment (Figure 1a). The pH of the medium was adjusted to 5.7 prior to addition of agar and was autoclaved at 105 kPa at 121℃ for 20 min. All cultures were kept in growth chambers at 25 ± 2℃ with 16-h photoperiod and a photon fluence of about 30 to 40 μmol m^{-2} s^{-1}.

Callus induction and adventitious buds from callus

Leaf-derived callus were cultured on MS medium for inducing callus or adventitious buds supplemented with 6-BA at 0.3, 0.5, 1.0 or 2.0 mg• L-1, NAA at 0.1, 0.3, 0.5 and 1.0 mg• L-1 in completely randomized design. The percentage of callus induction, the percentage of adventitious bud induction and the average number of adventitious buds per callus were observed. Three leaf explants were cultured per flask, and each experiment was repeated three times with 10 culture flasks per treatment.

Shoots strengthening and roots induction

After adventitious shoots reached about 2.0 cm in length with 2-pair of leaves, they were excised from the callus and placed in flasks on the half strength of MS medium containing various concentrations of NAA at 0.0, 0.1, 0.3, 0.5 or 1.0 mg• L-1 in combination with activated charcoal (AC) at 1.0 g• L^{-1}. Five shoots were cultured in each flask, and each experiment was repeated three times with 18 culture flasks per treatment.

Individual roots were dissected according to the branching order, starting from the distal end of the root system that was numbered as the first order and then increased sequentially with each branch from the first order to higher order roots. Following dissection, fine root samples were scanned by the Win-RHIZO system to analyze total root length (*L*), total root surface area (*SA*), total root volume (*V*) and total root number (*R*) per plantlet. Ten roots were scanned, and each treatment was repeated three times.

Greenhouse acclimatization

The effects of three planting media on *ex vitro* plantlet acclimatization were studied. Plantlets with well-developed root systems (about 5 cm long) and shoots (6 to 8 cm long) were removed from the flasks and transplanted into plastic pots containing pre-watered mixtures. Three different mixtures were used, including common turf (Zhejiang Hongyue Seeds Co., Ltd, Zhejiang, China), Hawita turf (Hawita Gruppe GmbH, AWITA GRUPPE GmbH, Vechta, Germany) and medium containing a 1:1

Figure 1. *In vitro* adventitious shoot regeneration of *Adenosma glutinosum* (Linn.) Druce using leaf explants. (a) Shoot sprout on stem explants, bar =1 cm; (b) callus induction from leaf explants, bar =0.5 cm; (c) adventitious buds from callus, bar =1 cm; (d) shoots strengthening and roots induction, bar =2 cm; (e) transplant and acclimatization, bar = 5 cm; (f) flowering plant after acclimatization (90 days), bar =5 cm.

(v/v) mixture of common turf: perlite (Zhejiang Hongyue Seeds Co., Ltd, Zhejiang, China). The plants were transferred to a greenhouse at 25 ± 2°C, with a photon fluence of 800 μmol m^{-2} s^{-1}, humidity levels ranging from 70 to 98% and a 16-h photoperiod. Plantlets were watered at 2-day intervals. After 2 weeks of acclimatization, plantlets were fertilized weekly with 100 mg• L^{-1} fertilizer containing a 15:15:15 of nitrogen, phosphate and potassium. The percentage plantlet survival was recorded at 30 days after transplanting. Each treatment consisted of 50 plantlets in triplicate.

Statistical analysis

Callus induction was calculated using the following formula: (number of induced callus / total number of leaf explants) × 100%. Adventitious shoot number per callus was calculated using the

Table 1. Effects of different plant growth regulator combinations on the induction of callus and the differentiation of adventitious shoots form the leaf explants of *Adenoma glutinosum.*

6-BA (mg•L^{-1})	NAA (mg•L^{-1})	Number of inoculated	Callus induction (%)	Adventitious shoot number per callus
0.3	0.1	90	97.5 ± 3.0^{a}	4.5 ± 0.1^{bcd}
0.3	0.3	90	97.8 ± 3.9^{a}	5.3 ± 0.5^{b}
0.3	0.5	90	100.0 ± 0.0^{a}	2.5 ± 0.7^{g}
0.3	1.0	90	95.6 ± 5.1^{a}	0.8 ± 0.6^{h}
0.5	0.1	90	98.9 ± 1.9^{a}	**7.2 ± 1.0^{a}**
0.5	0.3	90	100.0 ± 0.0^{a}	5.0 ± 0.9^{bc}
0.5	0.5	90	98.9 ± 1.9^{a}	2.9 ± 0.5^{fg}
0.5	1.0	90	100.0 ± 0.0^{a}	2.9 ± 0.2^{fg}
1.0	0.1	90	97.8 ± 1.9^{a}	4.4 ± 0.5^{bcd}
1.0	0.3	90	100.0 ± 0.0^{a}	3.9 ± 0.4^{def}
1.0	0.5	90	98.9 ± 1.9^{a}	3.2 ± 0.1^{efg}
1.0	1.0	90	98.9 ± 1.9^{a}	1.4 ± 0.6^{h}
2.0	0.1	90	100.0 ± 0.0^{a}	4.8 ± 0.9^{bcd}
2.0	0.3	90	100.0 ± 0.0^{a}	4.2 ± 0.1^{cde}
2.0	0.5	90	100.0 ± 0.00^{a}	3.2 ± 0.4^{efg}
2.0	1.0	90	100.0 ± 0.0^{a}	0.7 ± 0.1^{h}

Values followed by different letters within a column are significantly different at P<0.05. Data of callus induction and adventitious shoots number per callus were obtained after 15 and 30 days culturing, respectively. BA, Benzylamino purine; NAA, naphthalene acetic acid.

following formula: (total number of adventitious shoots / total number of callus). Rooting percentage was calculated as (number of explants with rooting / total number of explants) × 100%. Percentage data were converted to relative proportions, arcsine transformed, and then analyzed for significant differences. The data were analyzed with DPS v2.0 for Windows (Microsoft Corp., Redmond, WA) using two-way analysis of variance with replications for different plant growth regulator combinations on callus induction and adventitious shoot number per callus, and means were separated using Duncan's multiple range test at P = 0.05. In the final analysis, all data was expressed as the mean of replicate ± standard error (SE).

RESULTS

Callus induction and adventitious shoots from callus

In all treatments in which callus was produced, callus initiated first on the cut edge of the petiole and 4 days later on the cut blade surface. Callus was loosely packed, yellowish to green and friable (Figure 1b). There were no significant differences in callus induction between all treatments. Callus induction in all treatments exceeded 95%, and the percentage reached 100% in the most treatments with relatively high concentration of 6-BA (0.5, 1.0 and 2.0 mg• L-^{1}). All the treatments with 2.0 mg• L^{-1} 6-BA resulted in 100% callus formation in leaf explants (Table 1). The callus in leaf explants differentiated into meristemoid in 2 weeks under different treatments (Figure 1c).

Adventitious shoots were visible on the cut edge of the petiole in 2 to 3 weeks, and they were also visible on cut blade surface in 4 weeks. Variation in shooting response was observed due to exogenous level of plant growth regulator in the medium. Two-way analysis of variance showed that NAA, 6-BA and their interaction had obvious significant effect on adventitious shoot number per callus (P<0.01) (Table 2), while for callus induction, they had non-significance (data not shown). There was also a significant difference in average adventitious shoots number per callus observed. NAA was an important plant growth regulator for promoting shoot regeneration of leaf explants in this experiment. As the concentration of NAA increased (0.1 to 1.0 mg• L-^{1}) under any fixed 6-BA concentration, shoot number per leaf explant tended to decrease (Table 1 and Figure 2). When the concentration of NAA reached 1.0 mg• L-^{1}, fewer buds and shoots were produced from leaf explants. However, the treatment of combination of 6-BA (0.5 mg• L-^{1}) and NAA (0.1 mg• L-^{1}) resulted in the highest adventitious shoot number per callus nearly 100% (data not shown) and reached 7.2 shoots per explants (Figure 2e).

Shoots strengthening and rooting

Shoots about 2.0 cm long with 4 leaves (2 pairs) were excised and transferred to shoots strengthening and rooting medium. Leaf explants initiated roots and axillary bud produced in 5 to 7 days, and developed a cluster with well branched root system within 20 days (Tables 3 and 4; Figure d). 100% rooting frequency was recorded on all cultured explants of all treatments containing different concentration of NAA. NAA at concentration of 0.3 mg• L^{-1} resulted in the best strengthening effect. The average number of shoots and leaves per cluster reached

Table 2. Two-way analysis of variance with replications for different plant growth regulator combinations on adventitious shoot number per callus.

Source of variation	SS	df	MS	F	P-value
NAA factors	78.4	3	26.1	478.6	1.2 E-26
6-BA factors	20.2	3	6.7	123.3	1.2 E-17
NAA × 6-BA interaction	23.4	9	2.6	47.5	5.3 E-16
Error	1.7	32	0.1		
Total	123.7	47			

6-BA, 6-Benzylamino purine; NAA, naphthalene acetic acid; SS, sum of squares; df, degree of freedom; MS, mean of squares.

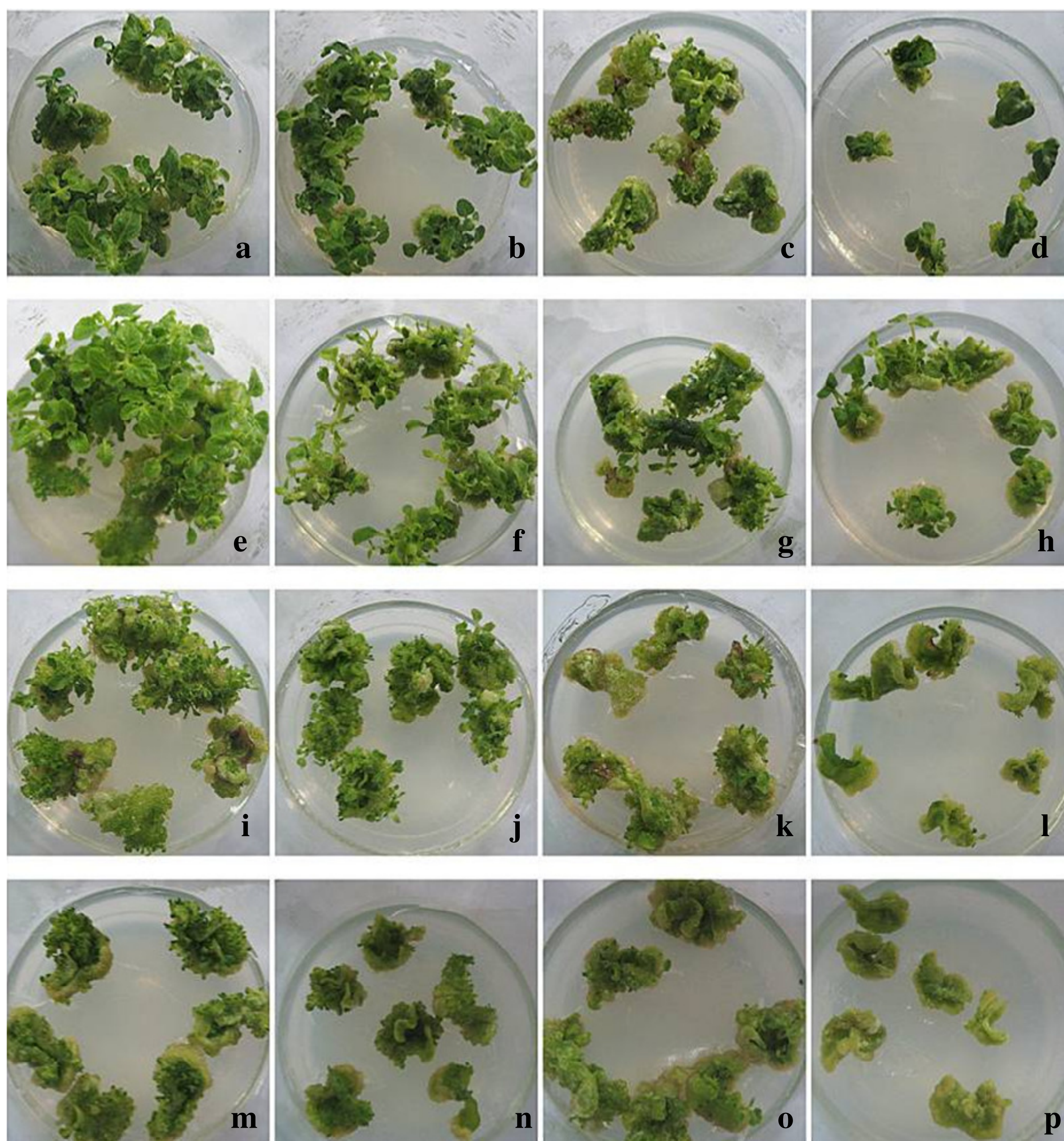

Figure 2. Effects of different plant growth regulator combinations on the induction of callus and the differentiation of adventitious shoots form the leaf explants of *Adenoma glutinosum*. The ordinal letters a to p match the treatments of Table 1 in series; photos were taken after 30 days culturing. Bottle diameter = 9 cm.

Table 3. Effects of different concentration of NAA on shoots strengthening and roots induction of *Adenoma glutinosum*.

NAA ($mg•L^{-1}$)	Average number of shoots per cluster	Average number of leaves per cluster	Average height of cluster (cm)	Frequency of rooting (%)
0.0	2.6 ± 0.3^{c}	10.2 ± 0.6^{a}	3.9 ± 0.4^{bc}	100.0
0.1	3.6 ± 0.6^{b}	10.3 ± 0.3^{a}	4.3 ± 0.4^{ab}	100.0
0.3	**4.6 ± 0.3^{a}**	**10.5 ± 0.5^{a}**	**4.6 ± 0.2^{a}**	100.0
0.5	3.7 ± 0.6^{b}	10.4 ± 0.2^{a}	3.7 ± 0.2^{c}	100.0
1.0	2.0 ± 0.2^{c}	9.1 ± 0.7^{b}	3.9 ± 0.5^{bc}	100.0

Values followed by different letters within a column are significantly different at P<0.05; Data were obtained after 20 days culturing.

Table 4. Effects of different concentration of NAA on root characteristics of regenerate plantlet of *Adenoma glutinosum.*

NAA ($mg•L^{-1}$)	Total root length (cm)	Total root surface area (cm^2)	Total root volume (mm^3)	Total root number
0.0	**43.3 ± 2.3^{a}**	3.6 ± 0.2^{bc}	48.8 ± 3.2^{b}	**45.2 ± 6.4^{a}**
0.1	39.0 ± 1.7^{bc}	2.6 ± 0.1^{d}	30.4 ± 2.6^{c}	22.5 ± 3.4^{b}
0.3	39.2 ± 1.4^{bc}	3.1 ± 0.2^{c}	42.9 ± 3.1^{b}	11.9 ± 2.0^{c}
0.5	35.8 ± 2.1^{bc}	**4.1 ± 0.2^{a}**	**114.1 ± 5.8^{a}**	21.2 ± 3.1^{b}
1.0	36.3 ± 2.6^{c}	3.1 ± 0.1^{c}	48.0 ± 2.4^{b}	14.5 ± 2.2^{c}

Values followed by different letters within a column are significantly different at P<0.05; Data were obtained after 20 days culturing.

Table 5. Survival rate of seedlings grown on different supporting mixtures after 30 days.

Transplanting condition	Survival rate after 20 day of transplanting (%)
Common turf	91.8 ± 4.2^{a}
Hawita turf	94.2 ± 4.5^{a}
Mixture media	96.2 ± 5.3^{a}

Values followed by different letters within a column are significantly different at P<0.05; Data were obtained after 20 d culturing.

4.6 and 10.5, respectively and the average height reached 4.6 cm.

For the effects of different concentration of NAA on root characteristics, MS medium without NAA treatment resulted in the optimum total root length (*L*) and total roots number (*R*) per regenerated plantlet, reached 43.3 cm and 45.2, respectively. However, for the total root surface area (*SA*) and total root volume (*V*) per regenerated plantlet, the optimum concentration of NAA was 0.5 mg• L^{-1} (Table 4).

Greenhouse acclimatization

The micropropagated plantlets were uniform and grew vigorously after 30 days of transplanting, with high plantlet survival percentages in greenhouse. In addition, no morphological abnormalities and variations were found when compared with the control. In all three media, plantlet survival percentage was more than 90% (Table 5 and Figure e). Ninety days later, plantlets grew well and flowered (Figure f). All the regenerated plantlets were successfully acclimatized and transferred to soil and can be used for conservation and ornamental purposes.

DISCUSSION

Supplement of various plant growth regulators in the medium at appropriate level may have effect on organogenesis and produce various types of cells. The ratio of auxin to cytokinin plays a pivotal role in plant growth and development. In this study, we determined the effects of different combinations of auxin and cytokinin on regeneration from leaf explants of *A. glutinosum*. Explants cultured on different concentrations of plant growth regulators showed various morphogenic regeneration responses. Regeneration of plants was affected by exogenous and endogenous plant growth regulator, particularly auxins and cytokinins, the explant type and culture conditions (Skoog and Miller, 1957). Cytokinin have been reported to be good plant growth regulators for shoot induction from leaf explants when cultured on MS medium (Poovaiah et al., 2006; Wang et al., 2009; Kumar et al., 2010), while Zhou et al. (2010)

and Raghu et al. (2011) reported that supplement of auxin in combination with cytokinin resulted in more number of shoot emergence.

Callus induction was higher than 95% in all treatments in the leaf explants of *A. glutinosum*. The highest adventitious shoot number per callus was observed in leaf explants cultured on media containing combinations of 0.5 mg• L-^{1} 6-BA and 0.1 mg• L-^{1} NAA. This was in agreement with the previous studies on micropropagation in the other aromatic plants: *P. cablin* (Paul et al., 2010), *T. patula* (Qi et al., 2011), *Eucalyptus* (Deepika et al., 2011), *R. officinalis* (Deng et al., 2008), and *A. glutinosum* (Liu et al., 2010). For *P. cablin*, Paul et al. (2010) reported that the highest number of shoots was obtained from leaf explants on MS medium containing 2.5 μM benzylaminopurine (BAP) and 0.5 μM NAA. Qi et al. (2011) reported that the highest callus induction rate of *T. patula* were obtained in explants cultured on MS medium containing 2.2 μM BA and 1.82 or 2.7 μM NAA. In addition, Deng et al. (2008) found that MS + 6-BA 1.0 mg• L-^{1} + NAA 0.02 mg• L-^{1} was favorable for shoot tip culture. However, Liu et al. (2010) found that the cultural system for adventitious buds proliferation of *A. glutinosum* is the MS medium with 0.25 mg• L-^{1} of BA and 0.5 mg• L-^{1} of IBA using shoot tips.

Through this study of tissue culture in *A. glutinosum*, methods for the induction of callus, adventitious bud and root proliferation, and plant regeneration were established. This new, highly efficient plant regeneration system would be helpful for industrial production and development of a genetic transformation protocol for *A. glutinosum*.

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