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Seed Germination and *in vitro* Plant Regeneration through Callus Culture of Two *Lychnis* Species

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Abstract

Lychnis cognate Maxim and *Lychnis fulgens* Fish. Ex Spreng are two valued ornamental plants in Korea. Soaking of seeds in GA_3 solution remarkably promoted germination up to 60%, but the control (0 mg/l) was not effective (> 5%). To select an adequate temperature for seed germination, seeds, previously soaked in a 1000 mg/l GA_3 for 24 hrs, were incubated at 15, 20, 25, and 30°C. Seed germination of over 20% was obtained at 15, 20, and 25°C, but only 10% at 30°C. These results indicate that the seeds of *L. cognate* and *L. fulgens* are in a such dormant state that they hardly germinate even by dormancy breaker (GA_3) and low (15 - 25°C) temperature treatment. The highest callus induction was observed in the leaf explants of the seedlings on MS containing specific concentrations of 3.0 mg/l BA and 1.0 mg/l NAA. The adventitious shoot was formed < 90% of calli on 1/2 WPM medium. The height of *in vitro* propagated plantlet was no different media used for regeneration. This *in vitro* propagation protocol should be useful for conservation of endangered and ornamental plant.

Introduction

The genus *Lychnis* belongs to Caryophyllaceae and consists of about 30 species in the world. *Lychnis* spp. are distributed throughout the temperate regions of the Northern Hemisphere, from East Asia to Europe (Magnus et al. 2008), and 4 *Lychnis* genus (*Lychnis cognate* Max., *Lychnis wilfordii* Max., *Lychnis fulgens* Fisch., *Lychnis kiusiana* Makino) are native to Korea (Lee 1982, Lee 1997).

It grows in habitats from lowland to mountain, mostly in wet meadow, ditche, and light alder wood (Lee 1997). The average number of inflorescences per plant

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ranges from one up to 50 with 20 - 25 flowers on each inflorescence (Jennersten 1991, Wilson et al. 1995). The flowers are protandrous with ten anthers and five stigmas and normally carry five petals. Flowers are visited by a wide range of insects, among which bumble bees and butterflies are the most frequent (Jennersten 1991). It has a high ornamental value and cultivated as a pot or garden plants. Some species of *Lychnis* L. are frequently grown as horticultural plants for use as decorative herbs in Korea (Lee and Yoon 1998). However, there are a few variations in horticultural traits, such as flower color, flower form and plant form, within each species. According to our preliminary investigation on propagation of *L. wilfordii* via seed, we found that seed germination took 2 - 3 months and was sporadic from *ex vitro* or *in vitro* culture (Bae et al. 2014). Meanwhile, vegetative propagation was not possible as the process of rooting was too slow in this genus.

Also, previous phytochemical studies, several compounds have been identified and isolated from the different parts of the plant, including phytoecdysteroids (Girault et al. 1990, Ba´thori et al. 2001, Louden et al. 2001, Zibareva et al. 2003), saponins (Wagner 1941), triterpenoids (Bucharov et al. 1974) as well as phenolic acids (Ferry and Darbour 1979). Additionally, analysis of the volatiles of *L. flos-cuculi* flowers showed that the dominating compound classes are benzenoids, followed by fatty acid derivatives (Jurgens 2004). Polyphenolic compounds have not yet been investigated in this plant. Little is known about the presence of closely indefinable flavonoid compounds in *L. flos-cuculi* (Darmograi 1976). Pharmacological studies of the herb of *L. flos-cuculi* have focused on the potential cytostatic activity examined using Levan's test (Grzycka et al. 1978). Aqueous extracts of the plant were also tested for their antibacterial and antifungal activities (Chaumont and Bourgeois 1978, Gardan et al. 1978). Recently, the extract of *L. chalcedonica* was observed to improve hemorheological and cerebroprotective properties in rats with cerebral ischemia (Plotnikov et al. 2005). Therefore, the development of an *in vitro* protocol will be of great importance for conservation and sustainable utilization of this species. However, propagation protocols for this species *in vitro* have not yet been reported.

The purpose of this study of two species of *Lychnis* (*L. cognate* and *L. fulgens*) was to improve the germination rates of seeds and develop a protocol for *in vitro* propagation and utilization.

Materials and Methods

Seeds of *Lychnis cognate* Max. and *Lychnis fulgens* Fish. Ex Spreng were collected from a wild population of Mt. Odae National Park in Korea at late September of 2010. Seeds were routinely cleaned by sieving, dried over silica gel, and stored in

air-tight screw-capped jars at 4°C chamber until used (Fig. 3a). Provided dark green seeds were plump and fully developed when picked, they ripened (and turned black) naturally postharvest, and germination rates were identical to those of fully mature seeds. Seeds for experiments described in this section (GA_3 and temperature effects) were spread evenly over filter paper (Whatman No. 2, Qualitative, Germany) in sterile, disposable Petri dishes (SPL, Korea, 120 mm diameter). Filter papers were pre-sterilized by autoclaving at 121°C and 120 kPa for 20 min. Dishes were moistened with 5 ml of distilled water per dish. Manipulations and germination assessments were performed under normal room lighting, and germinated seeds were removed from glass Petri dishes as they were recorded.

Seeds were soaked with GA_3 at 0, 200, 400, 600, 800, and 1000 mg/l solution while 24 hrs in darkness at 30°C. Four replicates of 100 seeds for each of above treatments were placed in 11 cm diameter glass Petri dishes with two layers of filter paper saturated with 10 ml distilled water. The dishes were placed in growth chamber (60% humidity, Sanyo, Japan) for 15 days. A seed was considered to have germinated when the radical extension was at least 2 mm.

After soaking in 1000 mg/l GA_3 solution for 24 hrs, the seeds were incubated at different temperature of 15, 20, 25, and 30°C in the incubators and photoperiod controls (12 : 12 hrs, light : dark intervals). A warm fluorescent light provided a photon flux density of 30 ± 2 μmol/m^2/s. Each replicate contained 100 seeds. A seed was considered to have germinated when the radicle emerged from the seed coat at least 5 mm. All treatments lasted two weeks and were replicated three times.

After cutting the leaf, stem and root explants into 10 mm in sizes (Fig. 3b), they were cultured on MS supplemented with auxins (0, 0.5, and 1.0 mg/l NAA, 0, 0.5, and 1.0 mg/l IAA) with or without 0, 1.0, and 3.0 mg/l BA. All media were supplemented with 30 g/l sucrose and solidified with 8.0 g/l plant agar, and then adjusted to pH 5.8 before autoclaving at 121°C for 20 min. Calli were maintained under cool white fluorescent lights (30 μmol/m^2/s) on a 16 hrs photoperiod at 25°C. The frequency of callus induction was evaluated after 12 weeks of culture. Thirty explants (leaf, stem and root) were incubated for each treatment and repeated five times.

Calli were transferred to WPM (Lloyd and McCown 1980), half-strength WPM, one-third strength WPM medium and MS, half-strength MS, and one-third strength MS for the growth of plantlets. The culture room was maintained at 25°C with a 16 hrs photoperiod under 30 μmol/m^2/s white fluorescent light. The adventitious shoot formation rate was evaluated by counting plantlets with well-developed shoot primordia after 4 weeks of culture.

All data were expressed as the mean ± standard error (SE) and ANOVA. Each experiment was replicated three times with at least 50 seeds per replication. Significant differences among the treatments were determined by performing multiple comparison tests using DMRT with $p < 0.05$ defined as significant (SAS 2003).

Results and Discussion

Lychnis cognata seeds were extremely small dry seed weighs around 0.28 mg (weight of 100 seeds) and approximately spherical with a diameter of 1.8 mm (data not shown). It had been ellipse (oblong) shapes and blackish brown colored seed coat (Fig. 3a). To assess the effect of GA_3 on seed germination, seeds were soaked in various GA_3 solutions of 0, 200, 400, 600, 800, and 1000 mg/l for 24 hrs. After 30 day GA_3 treatment, the germination of < 200 mg/l GA_3 treated seed was < 20% while it was only 2% in the 0 mg/l GA_3 treatment (Fig. 1) from *L. cognate* seed. In *L. flugens*, the germination of < 200 mg/l GA_3 treated seed was < 25% while it was only 1% in the 0 mg/l GA_3 treatment (Fig. 1). Among the five different GA_3 levels, < 200 mg/l GA_3 was found to be the most effective to promote seed germination in *L. cognate* and *L. flugens*. Kong (2009) observed a similar result in inducing the onset and promotion of germination after 24 hrs presoaking in a 200 mg/l GA_3 solution in *Scrophularia takesimensis*. The effective

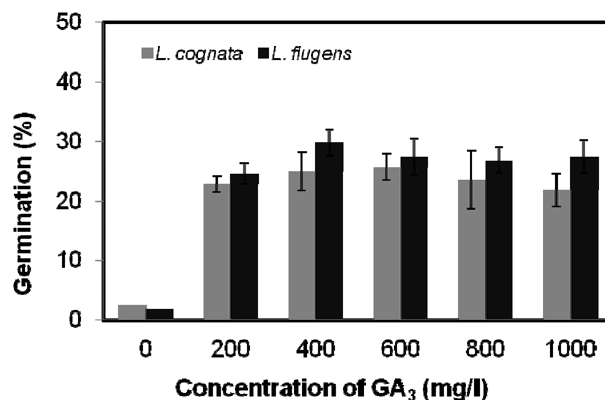

Fig. 1. Effect of GA_3 concentration on seed germination of *L. cognata* and *L. fulgens*. Final germination was determined at 30 days after sowing in a dark germinator. Vertical bars indicate mean ± SE of three replications.

concentration of GA_3 in promoting germination has been reported with a wide variation in many wild and cultivated plants: 250 - 500 mg/l for *Megaleranthis saniculifolia* (Lee et al. 2003); 250 mg/l for *Commelina communis*; 100 mg/l for *Belamcanda chinensis* (Park et al. 1995); 50 mg/l for *Dianthus superbus* (Shim et al.

1996); 5 mg/l for *Cyclaman persicum* (Lee et al. 1986). There are many reports that GA_3 could promote seed germination by reducing the period of dormancy or breaking dormancy (Kim and Um 1995, Kim et al. 1987). After soaking in 1000 mg/l GA_3 solution for 24 hrs, the seeds were incubated at different temperature regimes of 15, 20, 25, and 30°C in the incubators. Fig. 2 shows the time course of germination of *L. cognate* and *L. flugens* seeds incubated with different temperature (15, 20, 25, and 30°C) over 30 days. First germinants were observed on the five days of incubation, after which seeds germinated rapidly until the 20th day. Germination occurred most rapidly at incubation temperatures of 15, 20 and 25°C, and was slowest at 30°C (Fig. 2d). The germination rate reached < 30% when seeds were treated with 200 mg/l GA_3 and incubated in optimal temperature (20°C) (Fig. 2b). However, the germination rate was still not sufficient for production of the species by seed. Therefore, *in vitro* propagation of *L. cognate* and *L. flugens* were performed in the subsequent experiments.

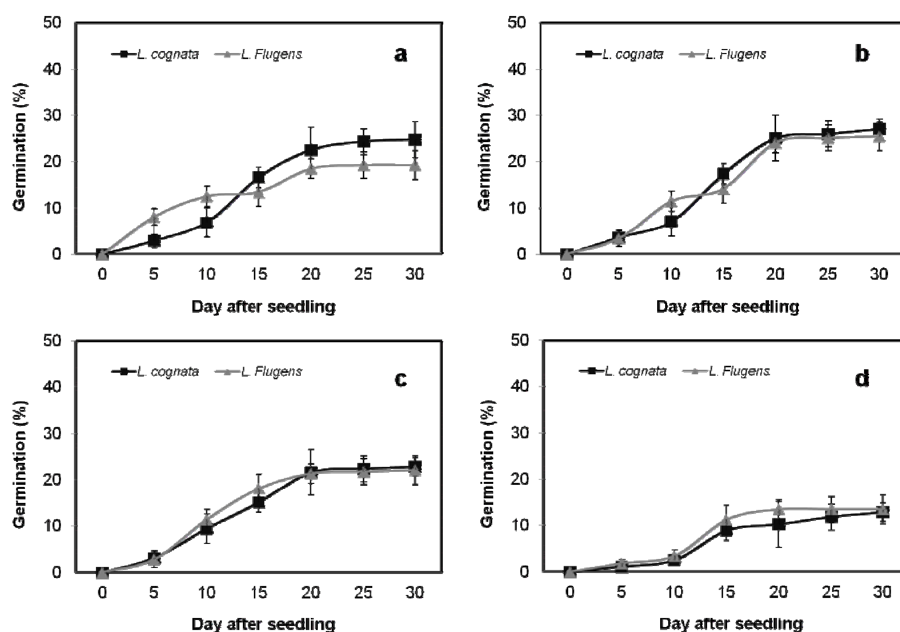

Fig. 2. Effects of various temperature on seed germination of *L. cognata and L. fulgens* incubated in 15°C (a); 20°C (b); 25°C (c); 30°C (d). The GA_3 treatment was conducted by soaking in a 1000 mg/l GA_3 solution for 24 hrs before incubating at various temperatures. Final germination was determined at 30 days after sowing in a dark germinator. Vertical bars indicate mean ± SE of three replications.

Callus formation varied significantly depending on the kind of explants and plant growth regulator (PGR) in *L. cognate* and *L. flugens* (Tables 1 and 2). When explants were cultured on the medium with BA and/or NAA and IAA, callus

formation was more efficient compared to the explants without PGR's treatment (Tables 1 and 2). The leaf explants formed callus after 8 weeks of culture, but stem

Table 1. Effects of NAA and IAA in combination with BA on callus formation from leaf, stem, and leaf explants of *L. cognata*. MS containing 30 g/l sucrose. Data were collected after 12 weeks of culture.

PGR's (mg/l)			Callus formation (%)		
BA	NAA	IAA	Leaf	Stem	Root
0	0	0	0	0	0
	0.5	0	33.3 ± 4.3	24.7 ± 6.3	14.6 ± 4.8
	1.0	0	38.7 ± 3.1	28.0 ± 3.3	18.3 ± 3.6
	0	0.5	22.0 ± 3.3	14.7 ± 2.3	13.6 ± 4.2
	0	1.0	28.3 ± 4.2	21.7 ± 4.8	15.8 ± 5.3
1.0	0.5	0	81.7 ± 4.5	54.3 ± 7.7	22.3 ± 5.8
	1.0	0	87.6 ± 3.3	59.3 ± 8.8	28.7 ± 4.9
	0	0.5	48.0 ± 7.6	41.0 ± 2.8	15.8 ± 1.7
	0	1.0	52.3 ± 6.5	40.7 ± 4.6	19.9 ± 5.9
3.0	0.5	0	91.7 ± 6.5	66.3 ± 4.7	31.4 ± 5.8
	1.0	0	94.6 ± 5.2	71.8 ± 6.8	29.8 ± 3.9
	0	0.5	58.0 ± 4.4	49.2 ± 3.8	21.4 ± 1.7
	0	1.0	61.3 ± 3.2	51.6 ± 4.6	25.8 ± 3.9

Data are the means ± SD, of five time experiments, n = 30.

Table 2. Effects of NAA and IAA in combination with BA on callus formation from leaf, stem and leaf explants of *L. fulgens*. MS containing 30 g/l sucrose. Data were collected after 12 weeks of culture.

PGR's (mg/l)			Callus formation (%)		
BA	NAA	IAA	Leaf	Stem	Root
0	0	0	0	0	0
	0.5	0	34.5 ± 5.1	28.4 ± 3.6	11.4 ± 3.2
	1.0	0	42.8 ± 2.8	29.5 ± 2.8	12.5 ± 1.8
	0	0.5	33.8 ± 2.9	18.4 ± 1.3	11.4 ± 1.2
	0	1.0	32.8 ± 3.8	20.6 ± 3.8	13.1 ± 3.5
1.0	0.5	0	87.5 ± 3.6	59.2 ± 6.5	28.2 ± 3.4
	1.0	0	88.7 ± 5.4	62.3 ± 5.6	32.8 ± 4.1
	0	0.5	52.6 ± 6.4	45.3 ± 3.4	18.6 ± 2.1
	0	1.0	58.1 ± 4.5	41.8 ± 2.6	22.3 ± 4.8
3.0	0.5	0	89.5 ± 2.3	69.3 ± 4.1	36.6 ± 2.3
	1.0	0	92.7 ± 4.2	72.3 ± 5.3	39.4 ± 5.2
	0	0.5	60.4 ± 5.2	52.8 ± 4.1	18.6 ± 2.1
	0	1.0	64.2 ± 2.8	53.6 ± 5.9	22.9 ± 3.4

Data are the means ± SD, of five time experiments, n = 30.

and root generated callus from cut surfaces after 10 weeks of culture. In *L. cognate*, the calli of the leaf (Fig. 4a), stem (Fig. 4b) and root (Fig. 4c) were compact, globular and yellowish on MS with both 3.0 mg/l BA and 1.0 mg/l NAA after 12 weeks of culture, but the control (non-treated PGR) did not form callus (Table 1). However, the stem and root explants exhibited 71.8 and 29.8% callus formation, respectively (Table 1). In *L. flugens*, calli of leaf (Fig. 4d), stem (Fig. 4e) and root (Fig. 4f) were compact, globular and yellowish on MS with both 3.0 mg/l BA and 1.0 mg/l NAA after 12 weeks of culture, but control (non-treated PGR) did not form callus (Table 1). However, the stem and root explants exhibited 72.3 and 39.4% callus formation, respectively (Table 1). It was reported that the induction of callus was difficult and the proliferation of initiated callus was very slow and somehow difficult to maintain in other species (Zheng et al. 1998, Luciani et al. 2006).

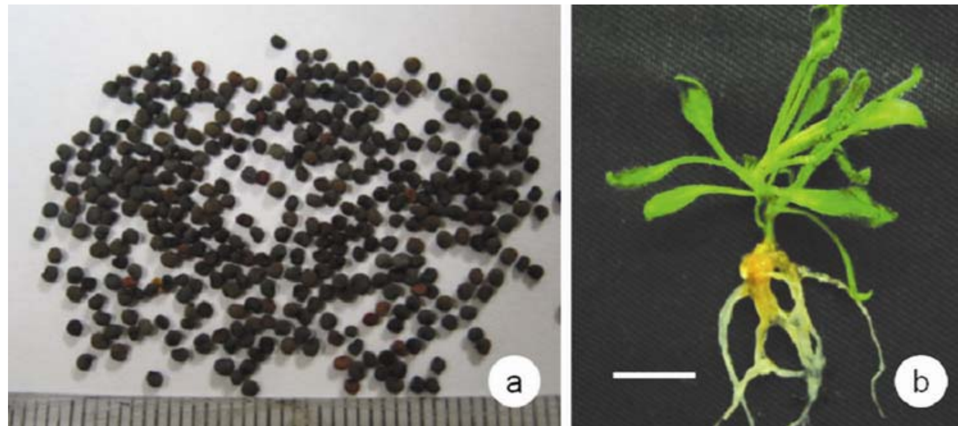

Fig. 3. Seeds and seedling of *L. cognata*. (a) Ripened (and turned black) seeds after 8 weeks of 4°C chamber, (b) *In vitro* seedling from seed after 4 weeks, Scale bar = 10 mm.

To determine adventitious shoot induction from callus, both types of callus (compact and friable ones; Fig. 5a) were transferred onto media (WPM, 1/2WPM, 1/3WPM, MS, 1/2MS, and 1/3MS). After 20 to 25 days of culture, the callus turned greenish (Fig. 5a) and several adventitious shoots regenerated on 1/2MS medium (Fig. 5b). The highest adventitious shoot induction rate was obtained with 1/2MS medium (94.3%) (Table 3). However, there was not a remarkable difference in the growth of plantlets among the six media (Table 3). The height of *in vitro* propagated plants in the pot was around 12 - 18 cm (Fig. 5c-d). Adventitious shoot regeneration on different media may be due to the differences of NO^{3-}/NH^{4+} ratio, an important factor in nitrogen uptake and pH regulation during plant tissue culture (Fracago and Echeverrigaray 2001). Lower NO^{3-}/NH^{4+} ratio in

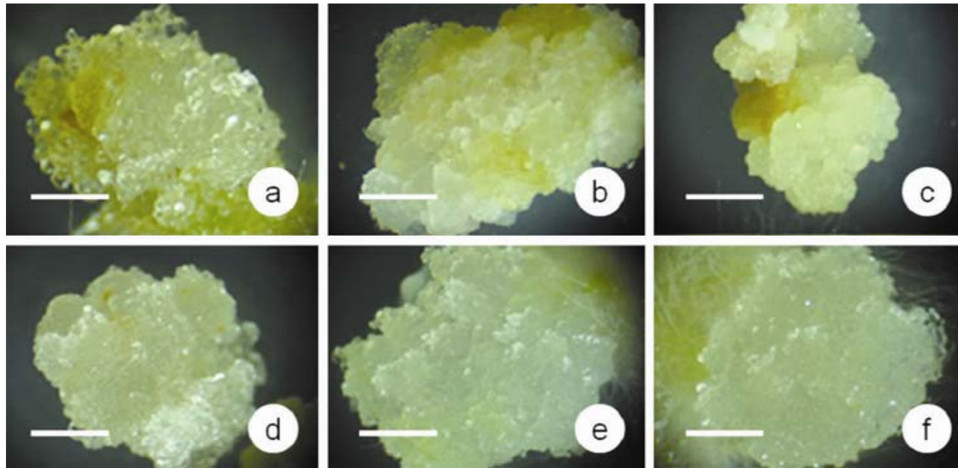

Fig. 4. Various type of callus from *L. cognata* and *L. fulgens*. Initiation of callus induction from *L. cognate* leaf (a), stem (b), and root (c) explants on MS with 0.5 mg/l BA and 3.0 mg/l NAA ; Initiation of callus induction from *L. fulgens* leaf (d), stem (e), and root (f) explants on MS with 0.5 mg/l BA and 3.0 mg/l NAA, Scale bar = 0.3 mm.

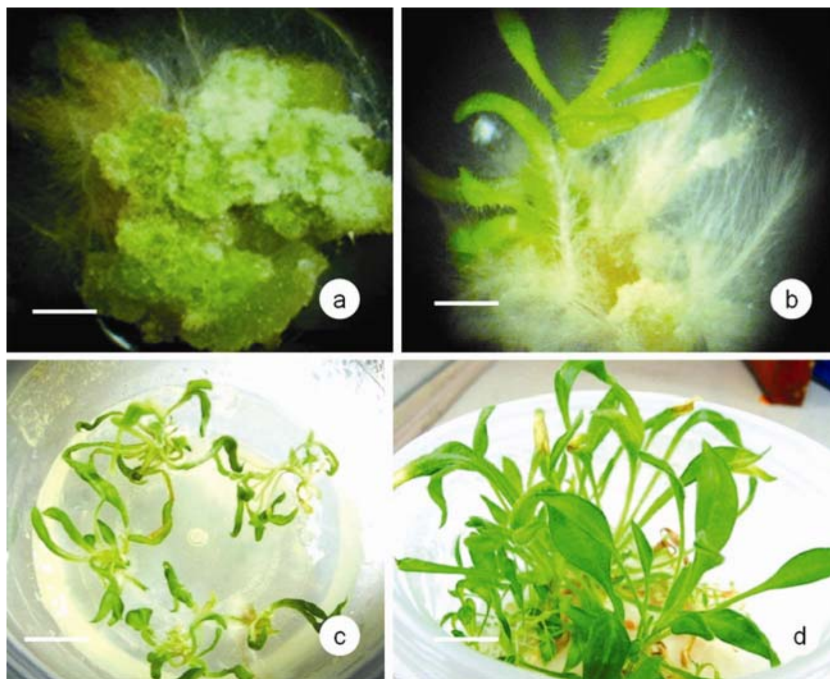

Fig. 5. Plant regeneration from callus derived from *L. cognata*. Initiation of callus induction from leaf explants on MS with 0.5 mg/l BA and 3.0 mg/l NAA after 12 weeks of culture (a), scale bar = 0.3 mm; Conversion of shoot and root on 1/2 MS without PGR's after 4 weeks of culture (b), scale bar = 1.0 mm; Proliferation of shoots on 1/2 MS without PGR's after 8 weeks of culture (c), scale bar = 30 mm. Plantlet conversion after 12 weeks of culture (d) scale bar 30 = mm.

B5 medium stimulated shoot conversion and growth of some *Allium* species (Gamborg et al. 1968, Chu et al. 1975, Luciani et al. 2006). *In vitro* culture technique is an alternative way for germplasm conservation and micropropagation of valuable endangered plants. It has the advantages of preserving healthy plant material in a small space, and for easy and rapid multiplication of material for exchange as well as cost reduction. Generally, an addition of BA to the medium could induce the formation of adventitious buds (Ayabe et al. 1995, Ayabe et al. 1998, Guo et al. 2005, Xu et al. 2008).

Table 3. Effect of various kinds of media on adventitious shoot formation and plantlet conversion of *L. cognata* and *L. fulgens* from callus. Adventitious shoot formation (%) was collected after 8 weeks of culture. Plantlet conversion was collected after 12 weeks of culture.

Media	Adventitious shoot formation (%)		Length of shoot (cm)		Length of root (cm)	
	L. cognata	*L. fulgens*	*L. cognata*	*L. fulgens*	*L. cognata*	*L. fulgens*
WPM	81.0 ± 3.5d	74.1 ± 4.5de	11.0 ± 1.6a	2.9 ± 0.7a	12.4 ± 1.2a	3.1 ± 0.4a
1/2WPM	89.3 ± 2.5b	77.3 ± 2.5d	12.4 ± 1.0a	2.7 ± 0.8a	12.8 ± 1.0a	3.2 ± 0.8a
1/3WPM	79.7 ± 3.7de	71.7 ± 3.7f	11.6 ± 1.5a	3.1 ± 0.8a	11.4 ± 1.5a	2.9 ± 0.8a
MS	88.7 ± 2.3bc	91.7 ± 3.3b	12.0 ± 1.0a	3.3 ± 1.1a	12.8 ± 1.0a	3.2 ± 1.1a
1/2MS	94.3 ± 3.3a	98.2 ± 3.3a	13.4 ± 1.1a	3.1 ± 0.7a	12.7 ± 1.1a	3.1 ± 0.7a
1/3MS	71.3 ± 2.3f	88.2 ± 3.6c	12.0 ± 1.0a	3.2 ± 0.9a	12.5 ± 1.0a	3.2 ± 0.9a

*Data are the means ± SD, of five time experiments. Different alphabetical letters are significantly different according to DMRT at $p < 0.05$.

In conclusion, we established the tissue culture system to propagate *L. cognate* and *L. flugens* and this technique may be applicable to commercially propagated endangered and ornamental plants.

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