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Research Note

In vitro plant regeneration and micropropagation of *Liriope platyphylla*

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Abstract

A rapid protocol for efficient shoot organogenesis and plant regeneration from meristem cultures of *Liriope platyphylla* Wang et Tang was developed. Meristem explants were cultured with different concentration of cytokinins. After that, 1 mg/L of each cytokinin was supplemented with various concentrations of auxins to determine the potential regeneration capacity and growth of shoots from excised meristem culture. Among the cytokinins, 6-benzylaminopurine and 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea showed the highest efficiency for shoot initiation, whereas kinetin and zeatin showed higher efficacy for shoot growth after 6 weeks in culture. In this study, zeatin (with any auxin) was highly effective for the initiation of shoot. Among the cytokinin/auxins combinations, zeatin with 0.1 mg/L indole-3-acetic acid proved optimal for yielding the highest number of shoots (4.2 per explant), followed by zeatin with 1 mg/L indole-3-butyric acid and zeatin with 0.1 mg/L naphthalene acetic acid. The combined application of zeatin and auxins might play a vital role in shoot organogenesis of *Liriope platyphylla*.

Keywords: *Liriope platyphylla*, organogenesis, cytokinin, auxins, *in vitro*, meristem.
Abbreviations: BAP: 6-benzylaminopurine; TDZ: 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea; IAA: indole-3-acetic acid; IBA: indole-3-butyric acid; NAA: naphthalene acetic acid.

Introduction

Liriope platyphylla Wang et Tang is an herbaceous perennial plant of the lily family (Liliaceae). In Korea, it has traditionally been used as a tonic, as an antitussive agent, and as an expectorant (Hur et al., 2004). *L. platyphylla* has various pharmacological and biological properties, including anti-bacterial (Kim et al., 2002), neuroprotective (Hur, et al., 2004), anti-inflammatory (Lee et al., 2005); it is also believed to improve learning and memory and to delay aging (Jiang et al., 2007a). Several steroidal saponins have been identified as the main active constituents of *L. platyphylla* (Jiang et al., 2007b; Watanabe et al., 1983). One of the major steroidal saponins, spicatoside A, has been reported to induce neurite outgrowth (Hur et al., 2009). Although most plants normally reproduce sexually, some species have the ability for vegetative propagation. Depend on plant species, various techniques are required for the vegetative propagation. However, for economically important plants, regeneration systems based on tissue culture have been recently developed (Chandra et al., 2010; Debnath et al., 2006). *In vitro* techniques considerably improve this system through the application of nutritional and hormonal components under aseptic conditions. Plant proliferation by this method is termed "micropropagation," because miniature shoots or plantlets are derived in the initial phase (Ekiert, 2000; Honda et al., 2001). Many methods for the regeneration of whole plants from excised plant tissue are available. Two principal techniques were considered in this study: plant regeneration from shoot organogenesis and somatic embryogenesis (Phillips and Hubstenberger, 1995). Organogenesis is a developmental system in which shoots or roots ("organs") are induced to differentiate from a single cell (or a group of cells). Plant regeneration by organogenesis typically involves induction and development of a shoot from explant tissue, followed by transfer to a different medium, and then induction of root formation and plant development (Boudaoud, 2010; Fleming, 2006). Research has shown that successful organogenesis in many plant species can be achieved, provided the correct medium components have been determined, a suitable explant has been selected, and there is appropriate control of the physical environment (Brown and Thorpe, 1986; Thorpe, 1990). The propagation of *L. platyphylla* is achieved either by division of the tuberous roots or by planting of seeds. Propagation from seed is difficult because of the low propagation rate and the delay implicit in root harvesting. This species is propagated conventionally through the division of roots (Han et al., 1993). Some studies have reported *in vitro* plant regeneration and micropropagation of *L. platyphylla* by somatic embryogenesis and the use of adventitious buds for multiple propagation (Kim et al., 2000; Mo et al., 2000). However, plant regeneration efficiency was low and not reliable. An efficient method for the regeneration of intact plants from tissue culture is essential to establish a multiple micropropagation system and a genetic transformation

Table 1. Effect of different concentrations of cytokinins on shoot regeneration and growth from meristem cultures of *Liriope platyphylla* after 6 weeks of culture.

Cytokinin (mg/L)		Shoots per explants	Shoot length (mm)
BAP	0.1	3.6 ± 0.1	23.0 ± 2.3
	0.5	3.0 ± 0.2	18.4 ± 1.1
	1.0	3.4 ± 0.1	16.2 ± 1.9
	2.0	3.0 ± 0.1	15.0 ± 2.0
	4.0	2.8 ± 0.1	7.6 ± 1.5
Kinetin	0.1	1.8 ± 0.1	36.6 ± 3.3
	0.5	2.0 ± 0.1	37.8 ± 2.2
	1.0	1.8 ± 0.2	40.2 ± 3.8
	2.0	1.6 ± 0.1	35.8 ±1.8
	4.0	1.6 ± 0.1	30.0 ±1.6
TDZ	0.1	3.2 ± 0.3	21.2 ± 1.3
	0.5	3.6 ± 0.4	21.8 ± 1.6
	1.0	3.4 ± 0.4	22.4 ± 2.6
	2.0	3.8 ± 0.3	18.2 ± 1.9
	4.0	3.8 ± 0.1	10.0 ± 1.2
Zeatin	0.1	1.8 ± 0.1	35.4 ± 2.8
	0.5	2.0 ± 0.1	39.6 ± 2.6
	1.0	2.0 ± 0.1	30.2 ±2.9
	2.0	2.0 ± 0.1	29.0 ± 2.2
	4.0	1.8 ± 0.1	26.8 ± 2.4

protocol. In this study, we developed a method for plant regeneration and micropropagation from meristem cultures of *L. platyphylla.*

Results and discussion

Effect of cytokinins

A simple and effective protocol was developed for the *in vitro* plant regeneration and micropropagation of *L. platyphylla*. We investigated the effect of different cytokinins on the efficiency of shoot organogenesis. Shoot development from meristem culture was successful for all the cytokinin treatment regimes. The cytokinin 6-benzylaminopurine (BAP), at the lowest concentration (0.1 mg/L), produced the highest number of shoots from each explant, and resulted in the maximum shoot length at this concentration; with increasing BAP concentration, the number of shoots and shoot length decreased (Table 1). The variation in shoot number and shoot length was not significant within the range 0.1–1.0 mg/L of BAP. Kinetin (N^6-furfuryladenine) yielded similar results for the number of shoots from the explants; however, it did produce the longest shoots. For example, at 1 mg/L of kinetin, less than 2 shoots were initiated per explant, but the shoot length was 40.2 mm—almost double that obtained with BAP and 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ) over their concentration ranges. The trend of decreasing number of shoots with increasing concentrations was also evident with BAP, although TDZ showed a different behavior from BAP and kinetin: with increasing concentration, the number of shoots from explants increased. The variation in shoot number (at different concentration) was similar, and TDZ showed the best performance for shoot initiation among the cytokinins studied. The highest number of shoots (3.8 per explant) was initiated at both 2 and 4 mg/L. The trends for zeatin (for both initiating shoots and the shoot length) were almost identical to those for kinetin. In both the cases (and at different concentrations), a lower number of shoots of shoots were produced, but zeatin resulted in longer shoots. At 0.5 mg/L, the shoot number was only 2, but the shoot length was 39.6 mm. When *L. platyphylla* meristem was cultured on MS solid media supplemented with 2 mg/L TDZ, the various stages of the *L. platyphylla* shoot organogenesis were clearly observed. During the initial stage (up to 2 weeks of incubation), some expansion and proliferation of cells at the cut surface were observed, but callus growth was limited. After enlargement of the cut end of the meristem explant (within 3 weeks), formation of shoot primordia and small elongated shoots adjacent to the cut surface was observed (Figure 1A). We observed that cells of the epidermis had proliferated to produce shoots directly (without an intervening callus phase). Regenerated shoots had developed from shoot primordia within 4 to 5 weeks. After 6 weeks of culture, an average of 3.8 fully developed shoots of at least 18.2 mm in length were produced, presumably from the meristem explant (Figure 1B).

Combined effects of cytokinins and different concentrations of auxins

Meristem explants were grown for 6 weeks in 1 mg/L of cytokinins (BAP, kinetin, TDZ, or zeatin) supplemented with varying concentrations of the different auxins (IAA, IBA, and NAA) to evaluate regeneration and shoot-growth capability from excised meristem culture. In every case, the combination of cytokinin and auxin produced more shoots from the explants, and their shoots were long, compared to the control (cytokinin alone). The optimal conditions for shoot number (4 per explant) were obtained with zeatin supplemented with 0.1 mg/L IAA (Table 2).The second and the third highest numbers of shoots (4 per explant) were also initiated by zeatin, but with 0.1 mg/L IBA 1 and 0.1 mg/L NAA, respectively. BAP showed better efficacy with 1 mg/L IBA than with other auxins, followed by 1 mg/L IAA. BAP with NAA produced almost the same number of shoots, regardless of auxin concentrations. At all concentrations of the auxins, kinetin did not yield good results in terms of the initiation of shoot/explants, but the growth of individual shoots improved with every kinetin/auxin combination. Among the tested auxins, IBA at 0.1 mg/L produced a higher number of shoots (3.4 shoots per explant). Kinetin along with 1 mg/L NAA, among other auxins, resulted

Table 2. Effect of different concentrations of cytokinins and auxins on shoot regeneration and growth from meristem cultures of *Liriope platyphylla* after 6 weeks of culture.

Cytokinin + Auxin (mg/L)			Shoots per explant	Shoot length (mm)
BAP	Control 0.0		3.4 ± 0.1	18.3 ± 1.5
	IAA	0.1	3.0 ± 0.2	21.7 ± 1.5
		0.5	3.3 ± 0.1	32.7 ± 1.5
		1.0	3.7 ± 0.2	25.3 ± 0.6
	IBA	0.1	3.8 ± 0.2	24.3 ± 1.5
		0.5	3.4 ± 0.1	31.3 ± 0.6
		1.0	2.9 ± 0.1	32.7 ± 1.5
	NAA	0.1	3.5 ± 0.1	29.3 ± 2.5
		0.5	3.4 ± 0.1	28.7 ± 2.5
		1.0	3.5 ± 0.1	25.3 ± 0.6
Kinetin	Control 0.0		1.7 ± 0.1	37.3 ± 1.5
	IAA	0.1	2.9 ± 0.1	29.5 ± 1.9
		0.5	2.5 ± 0.1	30.7 ± 1.5
		1.0	2.7 ± 0.1	27.0 ± 2.6
	IBA	0.1	3.4 ± 0.1	39.3 ± 2.5
		0.5	2.6 ± 0.1	35.3 ± 1.5
		1.0	2.3 ± 0.1	34.0 ± 3.0
	NAA	0.1	2.0 ± 0.1	30.3 ± 2.1
		0.5	2.1 ± 0.1	38.3 ± 2.2
		1.0	2.3 ± 0.1	42.3 ± 2.5
TDZ	Control 0.0		3.2 ± 0.2	27.3 ± 2.5
	IAA	0.1	3.2 ± 0.2	28.0 ± 2.6
		0.5	3.4 ± 0.2	27.3 ± 2.5
		1.0	3.8 ± 0.2	24.3 ± 1.5
	IBA	0.1	3.5 ± 0.1	41.3 ± 1.5
		0.5	3.5 ± 0.1	45.3 ± 2.5
		1.0	3.7 ± 0.1	48.7 ± 2.1
	NAA	0.1	3.1 ± 0.1	28.3 ± 2.1
		0.5	3.6 ± 0.1	36.5 ± 1.3
		1.0	3.5 ± 0.1	32.7 ± 1.5
Zeatin	Control 0.0		2.4 ± 0.1	25.7 ± 1.5
	IAA	0.1	4.2 ± 0.1	18.7 ± 1.5
		0.5	3.1 ± 0.2	26.0 ± 3.6
		1.0	2.7 ± 0.2	39.3 ± 2.2
	IBA	0.1	2.4 ± 0.1	29.0 ± 1.4
		0.5	2.8 ± 0.2	32.7 ± 1.5
		1	4.0 ± 0.2	35.3 ± 3.5
	NAA	0.1	3.9 ± 0.2	46.3 ± 1.5
		0.5	3.6 ± 0.1	44.7 ± 2.5
		1.0	3.3 ± 0.1	45.3 ± 1.5

in the maximum shoot length (42.3 mm). All auxins enhanced the initiation of shoot development with TDZ. The formation of new shoots from explants was observed when the explants were grown using TDZ combined with different concentrations of auxins. The highest number of shoots was observed with a combination of TDZ and 1 mg/L IAA, similar to that with TDZ and 1 mg/L IBA. Overall, the tallest shoot (48.7 mm) was observed with a combination of TDZ and 1 mg/L IBA.

Plant micropropagation

Regenerated shoots (~1cm) were transferred to MS medium without any exogenous plant hormone in each culture vessel. After 5 weeks, the regenerated shoots induced roots (Figure 1C). The rooted plants were transferred to pots containing autoclaved vermiculite, and covered with polyethylene bags for 1 week to maintain high humidity. The regenerated plants were hardened and transferred to soil (90% survival rate) where they grew normally in a greenhouse (Figure 1D). Previous studies have shown *L. platyphylla* plant regeneration by somatic embryogenesis. Mature zygotic embryos produced embryogenic calli on MS medium supplemented with 4.52 μM 2,4-dichlorophenoxyacetic acid. Upon transfer to half-strength MS basal medium, embryogenic calli gave rise to numerous somatic embryos, which then developed into plantlets (Kim et al., 2000). A study showed that adventitious buds of *L. platyphylla* were obtained by culturing the buds of root stocks on MS medium supplemented with 1.5 mg/L BA + 0.5 mg/L NAA; adventitious buds could then be subcultured once a month; on the other hand, MS medium supplemented with 0.25 mg/L IBA proved suitable for rooting. After a period of hardening, the roots could be planted in soil (Mo et al., 2000). Our study optimized the development of a rapid protocol for efficient shoot organogenesis and normal plant regeneration from meristem cultures of *L. platyphylla.* The continuous production of *L. platyphylla* regenerated plants without the loss of morphogenetic capacity is important, as this technique could potentially be used in micropropagation systems and for the

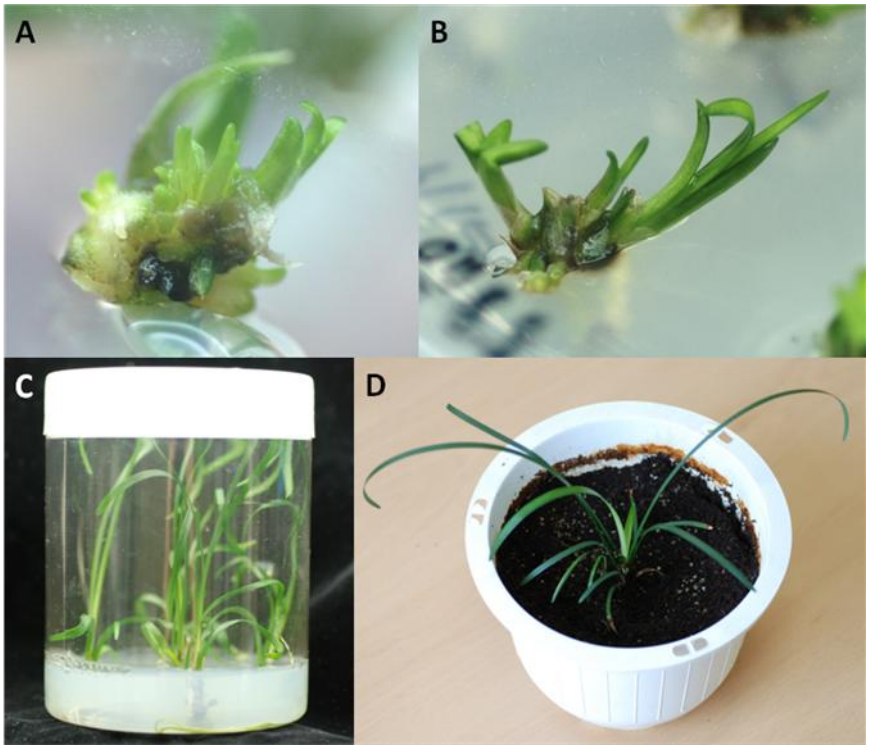

Fig 1. *In vitro* plant regeneration and micropropagation of *Liriope platyphylla*. (A) Shoot primordia emerging from a meristem explant of *L. platyphylla* 3 weeks after cultivation on MS solid media supplemented with 2 mg/L TDZ (6×). (B) After 6 weeks of culture, fully developed shoots were produced from the meristem culture (1.5×). (C) The rooted plants in a culture vessel. (0.5×). (D) The regenerated plant in a pot (0.3×).

regeneration of transgenic plants. Every year, conventional cultivators remove some tuberous roots from the harvest for the purpose of future mass propagation; however, our system can produce approximately 4 plantlets *in vitro* from 1 meristem explant culture of *L. platyphylla*, and the system can be used at any time in the year.

Materials and methods

Seed sterilization and germination

For preparing plant materials, seeds of *L. platyphylla* were collected from the experimental farm in Chungnam National University (Daejeon, Korea) and stored at 4°C. The seeds were surface-sterilized with 70% (v/v) ethanol for 30 s and 2% (v/v) sodium hypochlorite (Sigma, St. Louis, Mo. USA) solution for 10 min, then rinsed three times in sterilized water. Seven seeds were placed on 25 mL of agar-solidified culture medium in Petri dishes (100 × 15 mm). The basal medium consisted of MS (Murashige and Skoog, 1962) salt and vitamin medium (Sigma, St. Louis, Mo. USA) solidified with 0.7% (w/v) agar. The MS salt and vitamin medium was adjusted to pH 5.8 before adding the agar and was then sterilized by autoclaving at 121°C for 20 min. The seeds were germinated after 2 weeks culture in a growth chamber about 70-80% humidity at 25°C under standard cool white fluorescent tubes with a flux rate of 35 μmol s^{-1} m^{-2} and a 16-h photoperiod.

In vitro plant regeneration

Meristems of *L. platyphylla* were cut into pieces approximately 0.7 × 0.7 cm in size, respectively, from plants grown *in vitro* that had been cut aseptically at the ends. Explants were placed on medium (approximately 25 mL) in 100 ×25 mm Petri dishes. Explants were placed on the medium in the Petridish (100 x 25 mm). Petridish contained approximately 25ml of culture medium. Seven explants were cultured in each petri dish. The basal medium consisted of salts and vitamins of Murashige and Skoog (MS) medium (1962) and solidified with 0.7% (w/v) Phytagar (Sigma, St. Louis, Mo. USA). The pH of medium was adjusted to 5.8 before adding Phytagar. The media were sterilised by autoclaving at 1.1 kg cm^{-2} (121 °C) for 20 min. For shoot regeneration from meristem explants, the MS medium was supplemented with 0, 0.1, 0.5, 1, 2, and 4 mg/l BAP (6-benzylaminopurine), Kinetin (N^6-furfuryladenine), TDZ (1- phenyl-3-(1,2,3-thiadiazol-5-yl) urea; thidiazuron), and zeatin. For improvement of shoot regeneration, the medium was optimized by testing the effect of different concentrations of 0.0, 0.1, 0.5 and 1.0 mg/l IAA (indole-3-acetic acid), IBA (indole-3-butyric acid) and NAA (naphthalene acetic acid) on shoot formation and growth. Plant hormones were purchased from Sigma chemical Corporation (St. Louis, MO, USA). Cultures were maintained at 25±1 °C in a growth chamber with a 16-h photoperiod under standard cool white fluorescent tubes (35 μmol s^{-1} m^{-2}) for 6 weeks.

Rooting of regenerated shoots

Regenerated shoots (around 1cm long) were placed in MS medium. The medium was solidified with 3g/l Gelrite (Sigma, St. Louis, Mo. USA) and dispensed at 30ml per culture vessel and four shoots were cultured in each culture vessel. Regenerated shoots were incubated at 25±1 °C in a growth chamber with a 16-h photoperiod under standard cool white fluorescent tubes (35 μmol s^{-1} m^{-2}) for 5 weeks. After five weeks, the rooted plants were washed with sterile water to

remove Gelrite, transferred to pots containing autoclaved vermiculite, and covered with polyethylene bags for one week to maintain high humidity. The plants were then transferred to soil and maintained in a growth chamber with a 16-h photoperiod, and a night/day temperature of 18/20°C for 2 weeks. These hardened plants were then transferred to the greenhouse.

Statistical analysis

The data obtained was expressed as mean ± standard deviation from 50 meristems tested.

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