Shoot Organogenesis and Plant Regeneration from Leaf Culture of *Rehmannia elata* L.

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Abstract: For the regeneration of shoots, leaf segments of *Rehmannia elata* were cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of 6-benzylaminopurine (BAP) (1, 2, or 4 mg L^{-1}) with or without 1-naphthalene acetic acid (NAA) used at 0.1 or 0.5 mg L^{-1}. Regenerated shoots were obtained from the leaf cultures at all BAP concentrations with or without both concentrations of NAA. However, the highest shoot regeneration frequency (92.23%) and shoot number per explant (4.03 shoots) were obtained on MS medium supplemented with BAP (4 mg L^{-1}) and NAA (0.1 mg L^{-1}) solidified with Gelrite (4 g L^{-1}). The survival rate was ~70% when the rooted plants were hardened and transferred to soil. The continuous production of *R. elata*-regenerated plants from leaf explants under these conditions could be used as a possible micropropagation system for this species.
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1. Introduction

Plant tissue culture is a useful tool that allows the rapid and large-scale production of new and true-to-type (i.e., genetically identical) plants using only a relatively small amount of space and supplies. Many plant species have been successfully cultured in vitro, such as *Tagetes erecta* L. (Vanegas et al., 2002), *Torenia fournieri* L. (Kanchanapoom et al., 2009) and *Kalanchoe blossfeldiana* Poelln. (Castelblanque et al., 2010) and they are suggesting the broad applicability and potential for plant tissue culture in plant propagation.

The genus *Rehmannia* of the family Scrophulariaceae contains 6 species of flowering plants that were originally discovered in China but that are also distributed in Japan and Korea. The root of this genus is particularly valued because of its medicinal properties. In particular, the root of *Rehmannia* contains a number of constituents, including beta-sitosterol; calcium; copper; glucose; glucosamine; histidine; mannitol; zinc; amino acids; and vitamins A, B, C, and D. Moreover, the root of *Rehmannia* has become popular for its usefulness in regulating deficient blood patterns (e.g., anemia), replenishing vitality, and strengthening the liver, kidney, and heart (Liang et al., 2009; Wang et al., 2009). In addition, this herbal root is effective for the treatment of a variety of ailments such as diabetes, constipation, urinary tract problems, and dizziness and for body temperature regulation. It has also been regarded as an effective fertility enhancer.

Rehmannia plants can be propagated from seeds or tuberous roots. However, propagation from seed is not an efficient method because the seeds show poor viability and are slow to germinate (15~30 days), consequently delaying the root harvesting time (Park et al., 2009). A few studies have reported on in vitro plant regeneration and micropropagation in several species of the *Rehmannia* genus (Zhang et al., 2008; Park et al., 2009). However, more studies are needed for an efficient plant propagation system for the production of beneficial medicinal compounds from plants in this genus.

Rehmannia elata, commonly known as Chinese Foxglove, possesses large flowers that provide a nice accent in the host. Hence, in addition to the medicinal root, *R. elata* has commercial value as an ornamental or cut flower. However, to date, no report has been published on the shoot regeneration from leaf explants of *R. elata*. The present investigation was undertaken with the objective of developing an efficient protocol for the regeneration of *R. elata* from in vitro lea f culture.

2. Material and Methods

Shoot Organogenesis

The plant materials for this study were obtained from *R. elata* plants maintained in the greenhouse. The leaves were thoroughly washed under tap water and surface sterilized with 70% (v/v)

ethanol for 30 s. Afterwards, the leaves were further disinfected with a 1% sodium hypochlorite solution or Clorox for 15 min. Next, the leaf explants were rinsed with sterilized distilled water to remove the residual Clorox. The explants were then cut aseptically into pieces approximately 7 × 7 mm^2 in size, and 7 explants were placed on medium in a petri dish (100 × 25 mm) containing 25 mL of Murashige and Skoog (MS) basal medium (1962). The MS basal medium contained mineral salts and vitamins and was supplemented with 3% (w/v) sucrose as a carbon source and 0.8% (w/v) Phytagar (Gibco) as a solidifying agent. The pH of the medium was adjusted to 5.7~5.8 before adding the Phytagar and was sterilized by autoclaving it at 121°C for 20 min.

Shoot Regeneration Procedure

The culture medium for testing the efficiency of plant hormones in shoot induction and growth consisted of MS salts (Murashige and Skoog, 1962) with 3% sucrose (w/v) and different combinations of 2 plant hormones at various concentrations. The first set of media contained 6-benzylaminopurine (BAP) alone in 3 different concentrations (1, 2, or 4 mg L^{-1}); the second set of media contained BAP (1, 2, or 4 mg L^{-1}) in combination with NAA (0.1 or 0.5 mg L^{-1}). Moreover, different concentrations of several gelling agents (6, 7, 8, or 9 g/L of Phytagar or 2, 3, 4, or 5 g/L of Gelrite) were examined to evaluate the effect on shoot regeneration and growth. Cultures were maintained at 25°C ± 1°C in a growth chamber with a 16-h photoperiod, under standard cool white fluorescent tubes (35 mmol s^{-1} m^{-2}) for 6 weeks. The cultures were transferred to new medium every 3 weeks.

Rooting of Regenerated Shoots

Regenerated shoots ~ 2 cm in length were transferred to a Magenta box containing 50 mL of MS medium solidified with 4 g L^{-1} Gelrite. The culture boxes were maintained in a growth chamber under controlled conditions for rooting. After 8 weeks, the rooted plants were washed with water to remove the Gelrite, transferred to pots containing autoclaved vermiculite soil, and covered with polyethylene bags for 1 week to maintain high humidity. These hardened plants were then transferred to the greenhouse for further use.

3. Results and Discussion

Enhancement of Shoot Organogenesis by Plant Growth Regulators

The ratio of auxin to cytokinin plays an important role in plant organogenesis and growth. In this study, we investigated the influence of various concentrations of BAP used alone or in combination with NAA and the effect of different gelling agents on the efficiency of shoot organogenesis. Regenerated shoots were observed from leaf cultures on MS medium containing BAP with or without NAA.

The shoot regeneration rate increased with increasing BAP concentrations. However, a decline in shoot regeneration was observed when the BAP concentration increased to 4 mg/L (Table 1). The highest regeneration rate (69.17%), number of shoots per explant (2.17), and shoot length (1.43 cm) were achieved with BAP at 2 mg L^{-1} (Table 1).

Table 2. Effect of Different Concentrations of BAP and NAA on Shoot Regeneration and growth from Leaf Explants of *Rehmannia elata* after 6 Weeks of Culture on MS medium

Plant hormone*		Regeneration rate** (%)	No. of shoots per explant**	Shoot length[a] (cm)
NAA (mg L^{-1})	BAP (mg L^{-1})			
0.1	1	76.63± 4.1	2.77± 0.3	1.33±0.1
	2	82.53± 5.2	3.27± 0.3	1.50±0.2
	4	86.40± 3.4	3.77± 0.2	1.73±0.2
0.5	1	75.00± 4.8	2.72± 0.1	1.20±0.3
	2	81.10± 2.8	2.83± 0.3	1.47±0.3
	4	82.67± 4.2	3.03± 0.2	1.57±0.2

-- No response

* Basal medium consisted of MS salts and vitamins and 30 g/L sucrose solidified with 8 g/L Phytagar.

** Based on 100 leaf explants per experimental condition.

[a] Values represent the mean ± standard deviation of 50 shoots.

No shoot organogenesis was observed in BAP-depleted MS medium, indicating that BAP is important for shoot organogenesis in *R. elata*. Eckardt (2003) reported that cytokinin deficiency results in a major reduction in shoot development, thereby leading to dwarfism, late flowering, plants, enhanced root growth, and alterations in reproductive development. Furthermore, cytokinins are required in plant tissue to maintain both cell division and leaf primordium formation. Shoot organogenesis and regeneration capacity increased significantly when BAP was used in combination with NAA (Table 2).

Table 1. Effect of Different BAP Concentrations on Shoot Regeneration from Leaf Explant of *R. alata* after 6 Weeks of Culture on MS medium.

BAP concentration (mg L^{-1})*	Regeneration rate** (%)	No. of shoot/explant (shoots)**	Shoot length[a] (cm)
0	--	--	--
1	57.47 ± 4.6	1.57 ± 0.2	1.10 ± 0.1
2	69.17 ± 3.4	2.17 ± 0.3	1.43 ± 0.3
4	55.23 ± 4.9	1.83 ± 0.4	1.27 ± 0.2

* Basal medium consisted of MS salts and vitamins and 30 g/L sucrose solidified with 8 g/L Phytagar.
** Based on 100 leaf explants per experimental condition.
[a] Values represent the mean ± standard deviation of 50 shoots.

A high concentration of BAP combined with a low concentration of NAA resulted in the best conditions for maximum shoot organogenesis and regeneration. The highest regeneration rate (86.4%), number of shoots per explants (3.7) and greatest shoot length (1.73 cm) were obtained with BAP at 4 mg/L combined with NAA at 0.1 mg/L, so that using a combination of cytokinin and auxin was better than cytokinin alone. According to Skoog and Miller (1957), interactions between cytokinin and auxin play a crucial role in the control of plant morphogenesis. Generally, in organogenesis protocols, a high cytokinin-to-auxin ratio induces shoots, whereas a high auxin-to-cytokinin ratio produces roots. Meanwhile, equal or nearly equal concentrations of these phytohormones are found to cause callus proliferation.

Promoting Shoot Organogenesis by Gelling Agents

For investigating the effects of different concentration of gelling agents on the shoot regeneration of *R. elata*, leaf explants were grown for 6 weeks in MS basal medium containing mineral salts and vitamins, 30 g L^{-1} sucrose, 4 mg L^{-1} BAP, 0.1 mg L^{-1} NAA, and various concentrations of Phytagar or Gelrite (Table 3). The number of shoots per leaf explant and the shoot growth were ~10% and ~8% greater in media with Gelrite at 4 g/L than in that with Phytagar at 8 g/L.

Table 3. Effect of Different Gelling Agents on Shoot Regeneration and Growth from Leaf Explants of *R. elata* after 6 Weeks of Culture.

Gelling agent*	Concentration (g L^{-1})	Regeneration rate** (%)	No. of shoots per explant**	Shoot length[a] (cm)
Phytagar	6	54.27 ± 6.0	2.30 ± 0.2	1.23 ± 0.1
	7	78.57 ± 5.4	3.17 ± 0.3	1.50 ± 0.2
	8	84.33 ± 3.3	3.53 ± 0.2	1.77 ± 0.2
	9	81.67 ± 3.5	3.47 ± 0.2	1.63 ± 0.2
	10	77.83 ± 4.2	2.63 ± 0.2	1.33 ± 0.1
Gelrite	2	65.70 ± 4.5	3.34 ± 0.2	1.60 ± 0.2
	3	87.50 ± 3.2	3.60 ± 0.4	1.78 ± 0.3
	4	92.23 ± 2.9	4.03 ± 0.3	1.96 ± 0.2
	5	85.33 ± 3.1	3.74 ± 0.4	1.50 ± 0.2

* Basal medium consisted of MS salts and vitamins and 30 g/L sucrose.
** Based on 100 leaf explants per experimental condition.
[a] Values represent the mean ± standard deviation of 50 shoots.

This result is in agreement with a previous study by Park et al., (2009) who demonstrated that shoot organogenesis in *R. glutinosa* was more efficient on 3 g/L of Gelrite than on Phytagar 8 g/L used at the same concentration. In addition, a significant amount of research has shown that the use of Gelrite results in more efficient shoot organogenesis in *Bacopa monnieri* (L.) Pennell than the use of Phytagar (Shrivastava and Rajani, 1999). Moreover, Saito and Suzuki (1999) and Shrivastava and Rajani (1999) have reported the superiority of Gelrite over agar for the purposes of shoot regeneration in apples.

In this study, we were able to optimize the shoot regeneration medium in *R. elat*a by using MS salts and vitamins, 30 g L^{-1} sucrose, 4 mg L^{-1} BAP, 0.1 mg L^{-1} NAA, and 4 g L^{-1} of Gelrite as a gelling agent. This protocol can be useful for efficient shoot regeneration and micropropagation not only in plant tissue culture but also in other plant transformation approaches, even though further studies are still necessary for in vitro shoot organogenesis in *R. elata.*

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