Influence of Cytokinins and Auxins on Plant Regeneration from Hairy Roots of *Rehmannia elata*

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Abstract: In this study, we developed an efficient and simple protocol for plant regeneration from the hairy roots of *Rehmannia elata*. Excised hairy roots were cultured on media containing different concentrations of cytokinins and auxins. The cytokinin 6-benzylaminopurine at a concentration of 0.05 mg/L resulted in the maximum number of shoots and the longest shoots. This protocol generated 1.6 times as many shoots and 1.9-fold longer shoots than the control. Among the auxin treatments, naphthalene acetic acid at 0.01 mg/L and indole-3-butyric acid at 0.05 mg/L produced the highest number of shoots and produced 1.5 times more shoots than the control. The optimum protocol is a useful method for successful plant regeneration from the hairy roots of *R. elata*.
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1. Introduction

The genus *Rehmannia*, within the family Scrophulariaceae, includes six species of flowering plants that originated in China and that are also distributed in Japan and Korea. The roots of these plants are valued for their medicinal properties, particularly for regulating deficient blood patterns (e.g., anemia); replenishing vitality; and strengthening the liver, kidneys, and heart (Liang et al., 2009; Wang et al., 2009). In addition, *Rehmannia elata* is cultivated for ornamental purposes. It resembles a foxglove with large, bright, bell-shaped fuchsia flowers with yellow-spotted throats.

Rehmannia plants are propagated either from seeds or tuberous roots. Propagation from seeds is not an efficient method because the seeds show poor viability and are slow to germinate (taking 15–30 days), consequently delaying the root harvesting time (Park et al., 2009). *In vitro* plant regeneration and micropropagation has been conducted in several *Rehmannia* species (Zhang et al., 2008; Park et al., 2009; Kim et al., 2012). However, more studies are needed in order to develop an efficient plant propagation system for this genus.

Plants that are regenerated from hairy roots exhibit morphological alterations, such as reduced apical dominance, shortened internodes, wrinkled leaves, reduced leaf size, and extremely abundant and plagiotropic root systems (Tepfer, 1984). The altered phenotypes of some crops and ornamental plants that are regenerated from hairy roots may include qualities that make them useful in **plant** breeding programs (Christey, 2001; Casanova et al., 2005).

To date, no report has been published on shoot regeneration from the hairy roots of *R. elata*. The present investigation was undertaken to develop an efficient protocol for successful shoot organogenesis from the hairy roots of *R. elata*.

2. Material and Methods

2.1. Establishment of *R. elata* plant material on culture media

Shoot tips of *R. elata* were surface-sterilized with a 0.5% (v/v) sodium hypochlorite solution for 10 min, then rinsed three times in sterilized water. Three shoot tips were placed on 25 mL of agar-solidified culture medium in a culture vessel (100 × 150 mm). The basal medium consisted of salts and vitamins of MS (Murashige and Skoog, 1962) medium. The medium was adjusted to pH 5.8, solidified by adding 0.7% (w/v) agar, and then sterilized by autoclaving at 121°C for 20 min. The shoot tips were cultured in a growth chamber 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.

2.2. Preparation of *Agrobacterium rhizogenes*

Agrobacterium rhizogenes R1000 was grown to mid-log phase (OD_{600} = 0.5) at 28°C on a gyratory shaker at 180 rpm in liquid Luria-Bertani medium. The bacterial cells were collected by centrifugation for 10 min at 1500 rpm and resuspended at a cell density of A_{600} = 1.0 in a liquid inoculation medium (MS salts and vitamins containing 30 g L^{-1} sucrose).

2.3. Establishment of hairy root cultures

Excised leaves and stems from 10-day-old seedlings of *R. elata* were used as the explant material for cocultivation with *A. rhizogenes*. The excised leaves (0.7 × 0.7 cm) and stems (0.7 cm) were dipped into the *A. rhizogenes* culture in the liquid inoculation medium for 15 min, blotted dry on sterile filter paper, and incubated in the dark at 25°C on Phytagar-solidified MS medium. After 2 days of cocultivation, the explant tissues were transferred to a hormone-free medium containing MS salts and vitamins, 30 g L^{-1} sucrose, 500 mg L^{-1} carbenicillin, and 8 g L^{-1} Phytagar. Within 3 to 4 weeks, numerous hairy roots had emerged from the wound sites. The hairy roots were separated from the explant tissue and subcultured in the dark at 25°C on Phytagar-solidified MS medium. Repeated transfer of roots to fresh medium created a collection of rapidly growing hairy root cultures.

2.4. Shoot organogenesis

Hairy roots were cut at the ends into sections approximately 2 cm in length. Explants were placed on the medium in a Petri dish (100 × 25 mm). Each Petri dish contained approximately 25 mL of culture medium and seven explants. The basal medium consisted of salts and vitamins of Murashige and Skoog (MS) medium (1962), adjusted to a pH of 5.8, then solidified with 0.7% (w/v) Phytagar. The media were sterilized by autoclaving at 1.1 kg cm^{-2} at 121°C for 20 min. To determine the optimal cytokinin concentration for shoot regeneration from hairy root explants, we supplemented the MS medium with 0, 0.01, 0.05, and 0.1 mg/L 6-benzylaminopurine (BAP) and kinetin (N6-furfuryladenine). We also examined the effect of different concentrations of auxins, including 0, 0.01, 0.05, and 0.1 mg/L of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and naphthalene acetic acid (NAA), on shoot formation and growth in the culture media. Cultures were maintained at 25°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.

2.5. Statistical analysis

Data for the 30 tested leaf explants are expressed as mean ± standard deviation.

3. Results and Discussion

3.1. Hairy root induction from leaf explants of *R. elata*

Hairy roots were induced from leaf explants of *R. elata.* After 2 days of cocultivation with *A. rhizogenes* R1000, explant tissues were transferred to agar-solidified MS medium containing 200 mg Timentin L^{-1} to kill *A. rhizogenes.* Young hairy roots emerged from wound sites on leaves within 2 weeks after bacterial inoculation. After 4 weeks of exposure to the bacteria, the hairy roots began to grow more rapidly. Rapidly growing hairy roots were excised from the necrotic explant tissues and subcultured on fresh agar-solidified MS medium containing 200 mg/L Timentin. Mature hairy roots were generally thicker and exhibited more prolific branching after repeated transfer to fresh selection medium for 2 to 3 months.

3.2. Effect of cytokinins on shoot regeneration from hairy roots

We compared different techniques for *in vitro* shoot regeneration from the hairy roots of *R. elata*. After growing roots on MS basal medium supplemented with different concentrations of cytokinins (Table 1), we measured the number of shoots and the length of the shoots. Shoot growth was enhanced by both BAP and kinetin treatment, relative to growth in the control group. Shoot growth increased as the concentrations of BAP and kinetin increased from 0.01 to 0.05 mg/L, after which it declined as the concentrations of both cytokinins increased. The concentration 0.05 mg/L produced the highest number of shoots and greatest shoot length for BAP and kinetin. BAP at this concentration generated 3.5 shoots per explant and the longest shoot was 2.3 cm. This was 1.6 times more shoots and a 1.9-fold longer shoot length than the control treatment (Table 1). Kinetin at a concentration of 0.05 mg/L resulted in 1.5 times more shoots than the control group, whereas the longest shoot was obtained at a concentration of 0.1 mg/L, which was 1.8 times as long as the control (Table 1).

Our results are consistent with those of previous studies, which reported that the plant hormone BAP was the most effective treatment for shoot organogenesis of *Nicotiana tobacum* (Marcotrigiano, 1986), *Cucumis melo* (Marta and Andrés, 2009), and *R. elata* (Thi et al., 2012). Another study reported that cytokinin deficiency strongly limits shoot development, leading to dwarfism, late flowering, enhanced root growth, and alterations in reproductive development (Eckardt, 2003). Clearly, cytokinin is an important plant hormone for growth.

3.3. Effect of auxins on shoot regeneration from hairy roots

Previously cultured *R. elata* hairy roots were grown for 5 weeks on MS basal medium supplemented with various concentrations (0, 0.01, 0.05, and 0.1 mg/L) of different auxins (IAA, IBA, and NAA). Among the treatments, NAA at 0.01 mg/L and IBA at 0.05 mg/L produced the highest number of shoots (3.3 shoots per explant), each generating 1.5 times more shoots than the control (Table 2). Auxin at a concentration of 0.01 mg/L resulted in the longest shoot (1.9 cm), which was 1.6 as long as the control (Table 2). This study demonstrates that cytokinins and

auxins play an important role in shoot regeneration of *R. elata*. It is well known that cytokinins stimulate plant cell division, play a role in the cell cycle, and participate in the release of lateral bud dormancy, the formation of adventitious buds, and the growth of lateral buds. Auxins exert a strong influence on various cell activities, including the initiation of cell division, meristem organization that gives rise to unorganized tissue (callus) or defined organs (shoots), cell expansion, cell wall acidification, promotion of apical dominance, vascular differentiation, and formation of roots (Gaspar et al. 1996, 2003). Therefore, the manipulation of the exogenous cytokinin: auxin balance could favor a certain developmental pattern or orient a particular organogenic program (Gaspar et al., 2003). Our findings indicate that cytokinins and auxins can be a valuable alternative approach for plant regeneration from hairy roots of *R. elata.*

Table 1. Effect of different concentrations of cytokinins on shoot regeneration from hairy roots of *Rehmannia elata* after 5 weeks on MS medium. Each value is the mean ± standard error of three repeated experiments with 30 explants used in each treatment.

Cytokinin (mg/L)	Number of shoots per explant ± SE	Shoot length ±SE (cm)
Control (0)	2.2 ± 0.1	1.2 ± 0.1
BAP (0.01)	2.5 ± 0.2	1.7 ± 0.1
BAP (0.05)	3.5 ± 0.2	2.3 ± 0.2
BAP (0.1)	3.1 ± 0.2	2.1 ± 0.1
Kinetin (0.01)	2.3 ± 0.2	1.5 ± 0.1
Kinetin (0.05)	3.2 ± 0.3	1.9 ± 0.1
Kinetin (0.1)	2.8 ± 0.2	2.1 ± 0.2

Each value is the mean ± standard error of three repeated experiments with 30 explants used in each treatment.

Table 2. Effect of different concentrations of auxins on shoot regeneration from hairy roots of *Rehmannia elata* after 5 weeks on MS medium.

Auxin (mg/L)	Mean number of shoots per explant ± SE	Shoot length ± SE (cm)
Control (0)	2.2 ± 0.1	1.2 ± 0.1
IAA (0.01)	2.1 ± 0.2	1.9 ± 0.1
IAA (0.05)	2.6 ± 0.1	1.7 ± 0.1
IAA (0.1)	2.5 ± 0.2	1.3 ± 0.1
IBA (0.01)	2.5 ± 0.4	1.3 ± 0.1
IBA (0.05)	3.3 ± 0.3	1.6 ± 0.1
IBA (0.1)	3.1 ± 0.2	1.5 ± 0.1
NAA (0.01)	3.3 ± 0.2	1.3 ± 0.2
NAA (0.05)	2.8 ± 0.2	1.1 ± 0.1
NAA (0.1)	2.7 ± 0.2	0.8 ± 0.1

Each value is the mean ± standard error of three repeated experiments with 30 explants used in each treatment.

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