Influence of Medium and Auxin Concentration on *In vitro* Rooting of *Rehmannia glutinosa* L.

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Abstract: Plant tissue culture plays as an important role in plant biotechnology. Therefore, timesaving and cost-effective protocols have been needed to be constantly developed. Here, we investigated whether hormone-free media alone or in combination with auxins—influence *in vitro* rooting in *Rehmannia glutinosa* L. *In vitro* culture shoot explants were grown on different media for 4 weeks, after which rooting frequency, number of roots per explant, and root length were measured. Of the media tested, Schenk and Hildebrandt medium resulted in the highest rooting efficiency (100%), the highest number of roots (4.5 per explant), and the longest root length (32.2 mm). A 4-fold dilution of Schenk and Hildebrandt medium (¼ Schenk and Hildebrandt) was found to be the best condition providing the highest number of roots (5.3 per explant) and the longest root length (42.3 mm) compared to other Schenk and Hildebrandt medium dilutions. Supplementation of Schenk and Hildebrandt medium with different auxins at different concentrations did not improve root regeneration compared to controls. These findings provide useful information for future industrial scale root production.
[Thwe AA, Chae SC, Chung SO, Park SU. **Influence of Medium and Auxin Concentration on *In vitro* Rooting of *Rehmannia glutinosa* L.** *Life Sci J* 2013;10(3):685-688] (ISSN:1097-8135). http://www.lifesciencesite.com. 100

Keywords: *Rhemannia glutinosa*, root regeneration, auxin, excised stem

1. Introduction

Medicinal plants have been used extensively in cosmetics, agriculture, and food industry, and especially so in medicines. In fact, the World Health Organization estimates that up to 80% of people worldwide rely on traditional remedies such as herbs for their health care (Leena and Jaindra, 2003).

Rehmannia glutinosa L. (Scrophulariaceae) is a perennial herb autochthonous to China, Japan, and Korea. A number of ingredients such as iridoids, phenethyl alcohol, glycodides, cyclopentanoid monoterpenes, and noncarotenoids are extracted from fresh or processed roots of *R. glutinosa*. In Oriental medicine, *R. glutinosa* is used to replenish vitality; strengthen the liver, kidney, and heart; and cure diabetes, constipation, anemia, and some other diseases (Liang et al., 2009; Wang et al., 2009).

R. glutinosa can be propagated from seeds or by division of tuberous roots. However, this method can result in delayed root harvesting and low propagation rate. *In vitro* propagation of plants holds tremendous potential for the production of high-quality plant-based medicines (Murch et al., 2000). Many researchers have reported shoot regeneration from different plant organs of *R. glutinosa*. Recently, extensive research has been conducted on the development of shoot organogenesis and plant regeneration from leaf explants cultures of *R. glutinosa* (Park et al., 2009) for increased plantlet production. Since *Rehmannia* roots contain higher medical value compared to other plant parts, efficient *in vitro* techniques has become an important role to boost production of Rehmannia roots for medicinal purposes. Therefore, this study was conducted to determine the most suitable medium for rapid *in vitro* root proliferation from young stem explants of *R. glutinosa*.

2. Material and Methods

2.1 Plant Materials

Young shoots were collected from 1-year-old plants of *R. glutinosa* growing in the green house of the Chungnam National University, Daejeon, Korea. For establishment of *in vitro* shoot cultures, the leaves from young shoots were eliminated and later were cut around 50 mm in length. These explants were washed with tap water for 5–10 min and were surface-sterilized with 70% (v/v) ethanol for 30 sec and 1% sodium hypochlorite solution for 10 min. The explants were then rinsed thoroughly with sterilized distilled water and were incubated with 50 mL of hormone-free Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) in a magenta box under light conditions. The basal medium consisted of mineral salts and vitamins

supplemented with 30 g/L of sucrose and 8 g/L of Phytagar as a solidifying agent. The pH of the medium was adjusted to 5.8 before adding the Phytagar and sterilized by autoclaving at 121°C for 20 min. After 4 weeks of culture, elongated shoots were obtained and maintained under controlled environmental conditions until use.

2.2 *In Vitro* Rooting by Using Different Media

Seven shoot explants were cultured each in a magenta box containing 50 mL of hormone-free full-strength basal MS mineral solution, Schenk and Hildebrandt (SH) mineral solution (Schenk and Hildebrand, 1972) and Gamborg (B5) medium (Gamborg et al., 1976), respectively. These basal media, containing salts and vitamins, were solidified with 0.3% Gelrite and supplemented with 3% (w/v) sucrose. The pH of the medium was adjusted to 5.8 prior to the addition of Gelrite and autoclaved at 121°C with the pressure of 1.1 kg/cm^2 for 20 min. Cultures were then incubated at 25 ± 1°C with a 16-hr photoperiod per day under the standard cool and white fluorescent tubes. All experiments were performed in triplicate. Rooting efficiency, average number of roots per explant, and root length were measured at 4 weeks after incubation.

2.3 Determination of SH Medium Concentration

The most suitable medium was selected from the preliminary experiment. Then, root regeneration was determined by using 4 different concentrations (¼ SH, ½ SH, SH and 2 SH) of the medium. About 10–20 mm of 7 shoot segments were cultured on the respective medium. The same procedure was followed for sterilizing the medium and culture conditions, as those mentioned above.

2.4 Promoting Root Regeneration with Auxin

Different auxins, i.e., 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), and indole-3-acetic acid (IBA) at different concentrations (0, 1, 3, and 5 µM) were used in combination with SH medium for efficient root regeneration in *R. glutinosa*. Five segments of 10–20 mm long shoots were cultured in a magenta box containing 50 mL of the respective medium. Media were sterilized by autoclaving following the same procedure mentioned above. Treatments were replicated 3 times and data were collected after 2 weeks of culture.

2.5 Transferring Plantlets to Green House Conditions

Regenerated plantlets were maintained continuously under *in vitro* conditions until the roots developed properly. After 8 weeks, the rooted plants were then transferred to the pots containing autoclaved vermiculite soil. Immediately after transplantation, the plants along with the pots were covered with a moistened polythene bag to prevent desiccation. To reduce sudden shock, the pots were kept in a growth-controlled room for 7–15 days. The polythene bags were gradually perforated to expose the plants to natural environment at 2–3-day intervals. The bags were completely removed (after 10–15 days) when the plantlets appeared to be self-supportive. At that stage, the plantlets were placed in natural environment for 3–10 hours daily. Finally, after 15–20 days, they were transferred to green house conditions.

2.6 Statistical Analysis

The data are presented as mean ± standard deviation from 50 shoot explants tested.

3. Results and Discussion

3.1 *In Vitro* Root Regeneration

The effect of different hormone-free basal media on root regeneration of *R. glutinosa* was investigated. Roots started to develop 1 week after the explants were cultured on different basal media. All the media used in this study demonstrated 100% rooting efficiency (Table 1). Among them, SH medium performed the best, with the highest number of roots (4.5/explant) and root length (32.2 mm), followed by B5 and MS basal medium. Therefore, SH medium was selected as a suitable basal medium for the root development and growth of *R. glutinosa*. The number of roots per explant and root length were found to be significantly different after 2 weeks of culture. In a previous study by Park et al., (2009), *R. glutinosa* shoot organogenesis was found to be more efficient when Gelrite was used as the gelling agent, with higher number of shoots and shoot growth per leaf explant compared to Phytagar.

Table 1. Effect of different media on root regeneration and growth from excised stems of *Rehmannia glutinosa* L. after 4 weeks of *in vitro* culture.

Medium	Regeneration frequency* (%)	No. of roots per explant**	Root length[a] (mm)
SH	100	4.5 ± 0.5	32.2 ± 1.4
B5	100	3.9 ± 0.4	28.5 ± 1.9
MS	100	2.5 ± 0.5	22.3 ± 1.3

* Regeneration frequency (%) = No. of explants with root differentiation/All explants × 100

** From a total of 100 stem explants.

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

Table 2. Effect of SH media at different concentrations on root regeneration and growth from excised stems of *Rehmannia glutinosa* L. after 4 weeks of *in vitro* culture.

Medium	Regeneration frequency* (%)	No. of roots per explant**	Root length[a] (mm)
¼ SH	93	5.3 ± 0.4	42.3 ± 1.5
½ SH	90	4.8 ± 0.3	39.5 ± 1.9
SH	100	4.4 ± 0.5	35.5 ± 1.4
2 SH	82	2.6 ± 0.5	28.2 ±1.3

* Regeneration frequency (%) = No. of explants with root differentiation/All explants × 100
** From a total of 100 stem explants.
[a] Values represent the mean ± standard deviation of 50 roots.

3.2 Effect of SH Medium of Different Strengths

R. glutinosa stem explants were grown for 4 weeks on different SH medium strength. The result showed that ¼ SH medium was the best condition for achieving the highest number of roots (5.3/explant) and root length (42.3 mm)(Table 2). It was observed that increasing the strength of SH medium decreases the initiation of root number and root growth. Infact, root number and growth were reduced by ~2 fold in 2SH medium compared to ¼ SH medium.

3.3 Combined Effect of SH Medium and Auxins on Root Regeneration

In order to determine the combined effect of SH medium and auxins for efficient root regeneration, stem explants of *R. glutinosa* were cultured on SH basal medium supplemented with different auxin concentrations (0, 1, 3, and 5 µM of IAA, IBA, or NAA) (Table 3). The combination of SH medium and auxins was not favorable for root regeneration compared to control. However, SH medium with 1 µM of NAA induced the highest regeneration efficiency, number of roots per explant, and root length among the different concentrations and auxins used. Various growth regulators influence the induction of roots as well as their elongation (Balvanyos et al., 2001; Balestri and Bertini, 2003). However, roots mostly develop in the presence of auxins, particularly IAA, IBA, and NAA. In contrast, in the present study, auxin-supplemented SH medium did not induce root regeneration. Only NAA at 1 µM appeared to have some beneficial effects. Such effects had been previously observed by Taylor and Staden (1998) in tomatoes. Moreover, numerous studies have indicated that, among common auxins, NAA is the most effective auxin for induction of root regeneration (Johnson, 1978; Kitto and Young, 1981).

There were no previous studies on auxin-induced *R. glutinosa* root regeneration. In a classic series of experiments, Skoog and Miller (1975) showed that the ratio of cytokinin to auxin in nutrient media profoundly influences the morphogenesis of roots and shoots in plant tissue culture. Generally in organogenesis protocols, high cytokinin to auxin ratios induce shoots, high auxin to cytokinin ratios induce roots, and more equal concentrations of these phytohormones are found to cause callus proliferation. Dudits et al., (1975) reported that optimal root formation occurs in the presence of auxins and cytokinins in many plant species.

Table 3. Response of different concentrations of auxins on root regeneration and growth from excised stem of *Rehmannia glutinosa* L. after 4 weeks of *in vitro* culture.

Medium	Concentration (µM)	Regeneration frequency* (%)	No. of roots per explant**	Root length[a] (mm)
Control	0.0	100	4.3 ± 0.5	31.1 ± 1.5
SH + NAA	1	90	3.6 ± 0.5	26.3 ± 1.4
	3	40	2.8 ± 0.5	20.0 ±1.1
	5	–	–	–
SH + IBA	1	70	3.4 ± 0.5	26.0 ± 1.4
	3	60	2.3 ± 0.3	18.2 ±1.3
	5	20	1.8 ± 0.5	16.6 ± 1.2
SH + IAA	1	90	2.3 ± 0.4	24.3 ± 1.4
	3	–	–	–
	5	–	–	–

* Regeneration frequency (%) = No. of explants with root differentiation/All explants × 100
** From a total of 100 stem explants.
– No response
[a] Values represent the mean ± standard deviation of 50 roots.

In conclusion, plant tissue culture plays a vital role in plant biotechnology, particularly for genetic transformation leading to plant improvement. Therefore, efficient protocols are crucial for saving time and costs associated with molecular work. Establishment of reliable protocols for root regeneration is also an important factor for all plants whose roots have an economic value. In this study, we found that a 4-fold dilution of SH medium was the most efficient for *in vitro* rooting of *R. glutinosa*. This information provides useful indications for future applications on commercial root production via gene transformation. Nonetheless, further studies are needed to investigate the effect of other plant hormones on *R. glutinosa* root regeneration.

Acknowledgements:

This research was carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ906938)" Rural Development Administration, Republic of Korea.

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15/7/2013