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Enhancement of *in Vitro* Rooting By Gelling Agents and Activated Charcoal in *Rehmannia Glutinosa* L.

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Abstract: Plant tissue culture has proven to be commercially important for the replication of various plants species, including medicinal and ornamental plants. This technique has been used extensively in research for applications in agriculture and forestry. This study examines the effect of activated charcoal and the relative effectiveness of Gelrite compared to Phytagar. The number of roots per explant (8.2) and root growth (46.8 mm) was greater on Schenk and Hildebrandt medium containing 3 g/L Gelrite than that medium containing 7 g/L Phytagar which showed the number of roots per explant and root growth of 7.6 and 45.2 mm, respectively. Additionally, the highest regeneration rate (98%), the greatest number of roots per explant (8.8) and the longest root growth (71.5 mm) were achieved when 1 g/L of activated charcoal was added as a supplement to the medium. These findings provide useful information for future industrial-scale root production of *Rehmannia glutinosa.*

Keywords: Activated Charcoal, Gelrite, Phytagar, *Rehmannia Glutinosa*

Introduction

In a micro propagation system, the final culture stage prior to acclimatization is rooting (Ismail *et al.*, 2011; Millán-Orozco *et al.*, 2011). Survival of *in vitro* grown plantlets in the field depends on the development of a good rooting system to absorb water and nutrients from the soil (Benková and Bielach, 2010). It is well known that exogenously applied natural or synthetic auxins favor rooting (Osterc and Štampar, 2011) whereas little attention has been given to other variables, such as concentration of gelling agents and supplementation of medium with activated charcoal (Arthur *et al.*, 2006; Thomas, 2008).

Rehmannia glutinosa L. is a perennial herb belonging to the family Scrophulariaceae, distributed in China, Japan and Korea (Qi *et al.*, 2008). The herb can be propagated from seeds or by the division of tuberous roots. However, these methods can result in delayed root harvesting and a low propagation rate. Plant tissue culture techniques are useful for clonal propagation, genetic improvement and the conservation of rare species (Park *et al.*, 2009). A gelling agent, commonly agar or a bacterially synthesized polymer (Phytagel™), is often included to provide growth support to the culture. Romberger and Tabor (1971) reported that increasing the agar concentration of the medium resulted in restricted diffusion of macromolecules. Stoltz (1971) found that the decreased growth at higher agar concentrations was caused by reduced water availability. In a previous study, Park *et al*. (2009) reported *in vitro* shoot proliferation from leaf explants of *R. glutinosa* using MS basal medium with gelrite and phytagar as gelling agents.

The stimulatory effect of charcoal may involve the following: (1) the reduction of light intensity at the base of the shoots, providing an environment conducive to the accumulation of auxins, cofactors, or both; and (2) the adsorption of substances, such as inhibitory phenolics and excess auxins or cytokinins carried over from previous media (Arthur *et al*., 2006). Since the roots of *Rehmannia* are of a greater medical value compared to other plant parts, efficient *in vitro* techniques to boost production of the root portion of the herb are important. The purpose of this present study was to examine the effect of gelling agents and activated charcoal on the rooting ability of *in vitro* shoots of *R. glutinosa.*

Materials and Methods

Plant Materials

Young shoots were collected from one-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 by using a small scissor and were then cut to a length of approximately 50 mm. These explants were washed with tap water for 5-10 min and were surface-sterilized with 70% (v/v) ethanol for 30 sec followed by 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 addition of the Phytagar and sterilized by autoclaving at 121°C for 20 min. After 4 weeks of culture, elongated shoots were observed and maintained under controlled environmental conditions until ready for use.

In Vitro Rooting Using Different Gelling Agents

For root regeneration from shoot explants, 7 shoot explants were separately cultured in a magenta box containing 50 mL of hormone-free full-strength SH (Schenk and Hildebrandt, 1972) mineral solution with different gelling agents. The root regeneration SH medium containing salts and vitamins was solidified with 5, 6, 7, 8 and 9 g/L of Phytagar or 1, 2, 3, 4 and 5 g/L of Gelrite and supplemented with 3% (w/v) sucrose. The pH of the medium was adjusted to 5.8 prior to the addition of gelling agents and autoclaved at 121°C with a 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 standard cool and white fluorescent tubes for 4 weeks. All experiments were performed in triplicate. Rooting efficiency, average number of roots per explant and root length were measured at 4 weeks after incubation.

Promoting Root Regeneration with Activated Charcoal

The most suitable gelling agent and concentration was determined from the initial experiment. Root regeneration was promoted using different concentrations of activated charcoal (0, 0.1, 0.5, 1, 3 and 5 g/L). Different concentrations of activated charcoal were added to the medium before adjusting the pH of the medium. The same root regeneration SH medium solidified with 3 g/L of Gelrite supplemented with 3% (w/v) sucrose was prepared as described above. Media were sterilized by autoclaving following the same procedure described above. Five segments of shoots 10-20 mm in length were cultured in a magenta box containing 50 mL of the respective medium. Treatments were replicated 3 times and data were collected after 4 weeks of culture.

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 washed with sterile water to remove the Gelrite. Plantlets were then transferred to pots containing autoclaved vermiculite soil. The plants, along with the entire pot, were covered with polythene bags to prevent dehydration and maintain a high humidity. The pots were kept in a growth-controlled chamber for 7-15 days. The polythene bags were gradually perforated to expose the plants to the natural environment at 2-3-day intervals. The bags were completely removed after 10-15 days when the plantlets seemed to be acclimatizing to the natural environment. These hardened plants were then transferred to greenhouse conditions.

Statistical analysis: Data were collected from 3 replicates in each experiment. They are presented as mean ± standard deviation from 50 shoot explants tested.

Results

In vitro Rooting Using Different Gelling Agents

The effect of different gelling agents on hormone-free basal media for root regeneration of *R. glutinosa* was investigated. First, shoot explants were grown for 4 weeks in basal medium (MS salts and vitamins, 30 g/L sucrose) supplemented with 0.8% Phytagar for initial root establishment. Next, *in vitro* growing shoots were transferred to the SH medium with various concentrations of Phyagar and Gelrite (Table 1). Roots began to develop one week after the explants were cultured on media. Among the Phytagar treatments, increasing concentration of Phytagar from 5 to 7 g/L enhanced the rooting percentage, root number and root length. A further increase in concentration of agar (i.e., to 8 and 9 g/L) did not improve the rooting response. This result was similar to the *in vitro* rooting response of *Syzygium alternifolium* by Sha Valli Khan *et al*. (1999) where increasing the concentration of agar from 0% to 0.8% enhanced rooting, while no improvement in response was observed from 1.0% to 1.2% of agar. This can generally be say that increased agar concentration has adverse effect on rooting.

Promoting Root Regeneration with Activated Charcoal

To evaluate the effect of activated charcoal of different strengths on root regeneration of *R. glutinosa* excised stem cultures, stem explants were grown for 4 weeks on basal MS medium under a controlled environment.

Table 1. Effect of gelling agents on the regeneration and growth of roots from the excised stem of *Rehmannia glutinosa* L. *in vitro*

Gelling agent (g/L)		Regeneration frequency*(%)	No. of roots per explant*[1]	Root length*[1] (mm)
Phytagar	5.0	65	6.8±0.6	35.2±1.6
	6.0	68	7.3±0.5	40.4±1.7
	7.0	83	7.6±0.8	45.2±2.3
	8.0	75	7.1±0.5	41.5±1.9
	9.0	69	6.2±0.4	21.2±2.1
Gelrite	1.0	86	7.1±0.6	42.3±2.6
	2.0	92	8.0±0.6	43.2±1.9
	3.0	95	8.2±0.7	46.8±2.8
	4.0	78	6.4±0.5	41.2±2.3
	5.0	73	6.3±0.7	31.5±2.1

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

Table 2. Effect of activated charcoal on the regeneration and growth of roots from the excised stem of Rehmannia glutinosa L. *in vitro*

Activated charcoal (g/L)	Regeneration frequency*(%)	No. of roots per explant*[1]	Root length*[1] (mm)
0.0	95	8.2±0.7	46.8±2.8
0.1	95	8.5±0.4	57.6±1.3
0.5	96	8.4±0.5	64.7±1.6
1.0	98	8.8±0.4	71.5±1.5
3.0	94	8.7±0.5	64.8±1.6
5.0	94	8.3±0.5	45.1±1.8

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

Then, root induction was performed on SH root regeneration medium containing different levels of activated charcoal. The result showed that 1 g/L of activated charcoal medium was the optimal condition for achieving the greatest number of roots (8.8 per explant) and root length (71.5 mm) (Table 2). Furthermore, the root length was nearly double compared with that of the charcoal-depleted control medium. It was observed that further increasing the amount of activated charcoal, i.e., more than 1 g/L, decreased the initiation of regeneration rate, root number and root growth. Indeed, root number, growth and regeneration rates were nearly identical with the control when the amount of charcoal was increased up to 5 g/L.

Discussion

Among the different concentrations of gelling agent, 3 g/L of Gelrite medium performed optimally, with the greatest number of roots (8.2 per explant) and root length (46.8 mm). The efficiency of root implantation was 12% greater on 3 g/L Gelrite compared to 7 g/L Phytagar. This result is consistent with a previous study (Park *et al*., 2009) in *R. glutinosa* which demonstrated that 3 g/L of Gelrite was found to be more efficient than 7 g/L Phytagar for shoot organogenesis. Moreover, Saito and Suzuki, 1999; Shrivastava and Rajani (1999) both reported that Gelrite has better performance than phytagar for the regeneration of shoot in apple. Surprisingly, Gelrite 1g/L in those studies was found to have a higher regeneration rate (86%) than 7 g/L of Phytagar (83%) in the present study. However, 1 g/L Gelrite is unsuitable for rooting since the medium is too soft, difficult to handle and is more susceptible to contamination. Therefore, our experiment was performed with optimized root regeneration medium consisting of SH salts and vitamins, 30 g/L sucrose and 3 g/L Gelrite as a solidifying agent. Shin *et al*. (2011) reported that the addition of 0.1 g/L activated charcoal in the absence of plant hormone in the modified Hyponex medium significantly accelerated the rate of seed germination. Activated charcoal treatment was beneficial for both root system development and shoot quality in *Quercus robur* and *Q. rubra* shoots (Sanchez *et al*., 1996). A similar effect of activated charcoal on the number and quality of *Pinus pinaster* roots were reported by Dumas and Monteuuis (1995). The addition of activated charcoal in the rooting expression medium was shown to improve the potential for adventitious rooting, not only in terms of rooting rates, but also in enhancement of the number and the length of the roots, as well as the root score. In this study, 1g/L of activated charcoal incorporated with 3 g/L of Gelrite containing SH medium is the appropriate condition for enhancing root regeneration in *R. glutinosa*.

Conclusion

Plant tissue culture plays a vital role in plant biotechnology, particularly for genetic transformation for plant improvement. 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 addition of activated charcoal, together with the Gelrite as a solidifying agent, was the most efficient protocol for *in vitro* rooting of *R. glutinosa*. Although further investigations are needed for an improved understanding of the roles of activated charcoal on the physiology of the explants in relation to adventitious rooting, the information from this study provides useful indications for future applications on commercial root production in the field of biotechnology.

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Author's Contributions

Aye Aye Thwe: Wrote the manuscript, performed the experiments and analyzed the data.

Hyun Ho Kim: Performed the experiments and analyzed the data.

Haeng Hoon Kim: Wrote the manuscript and analyzed the data.

Sang Un Park: Designed the experiments and analyzed the data.

Ethics

The authors declare that there is no conflict of interests regarding the publication of this paper.

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