Enhancement of the *In Vitro* Root Regeneration Efficiency of *Rehmannia glutinosa* Libosch. Stem Explants by Different Carbon Sources

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Abstract: The success of plant tissue cultures is influenced by plant growth regulators, nutrient supply, and the carbon source in the medium. We investigated the most suitable carbon source and concentration for the *in vitro* rooting of *Rehmannia glutinosa*. Stem segments of *R. glutinosa* were cultured *in vitro* with 30 g/L of seven different carbon sources (sucrose, dextrose, mannose, glucose, sucrose, galactose, fructose, and maltose) for adventitious root regeneration. Subsequently, the best carbon source for regeneration was selected, and the optimal concentration (0%, 0.5%, 1.0%, 2.0%, 3.0%, 4.0%, or 5.0%) for promoting root regeneration was determined. Three percent sucrose was found to be the most suitable carbon source and concentration for adventitious root regeneration.
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1. Introduction

In vitro culture is a key tool of plant biotechnology that exploits the totipotent nature of plant cells (Haberlandt, 1902). *In vitro* culture facilitates the rapid multiplication of superior clones, and is essential for improving plants via genetic engineering techniques (Kothari et al., 2010). *Rehmannia glutinosa* is a herb of Scrophulariaceae family. The species name "glutinosa" originates from glutinous, referring to the sticky nature of the *R. glutinosa* root. This species is an economically important medicinal plant that is widely used in traditional Chinese medicines, as the tuberous root contains various pharmacological properties. (Wen et al., 2002). The *Rehmannia* root is used to (i) reduce blood temperature, (ii) promote the production of body fluids, (iii) address autoimmune conditions of the adrenal and thyroid glands, and (iv) reduce hypertension (Bi et al., 2008; Hasegawa et al., 1982; Liu et al., 2008; Zhang et al., 2008).

Cultures of the plant cells, tissues, or organs normally require the incorporation of a carbon source into the culture medium (Karhu, 1997). An important component of tissue culture media is the carbon source. This is because carbon supplies energy to plants, particularly during the early stages of tissue culture when they are not ready to photosynthesize their own food (Al-Khateeb, 2008). In general, sucrose is the carbohydrate of choice for *in vitro* studies of callus and shoot induction, and in the development of various species; however, this compound is not always the most effective carbon source for these purposes (Thompson and Thorpe, 1987). Species-specific carbon sources and carbon concentrations for optimal growth rates have been identified for various culture systems. For instance, one study found that different patterns of morphogenesis were attributable to the type of carbohydrate and its concentration (Romano et al., 1995). Here, we examined the performance of *R. glutinosa* root growth from stem segments on SH medium using various carbon sources and concentrations. The findings of this study will provide information for economically efficient plant regeneration systems that facilitate rapid *R. glutinosa* root development for medicinal use.

2. Material and Methods

2.1 Establishment of Plant Material

Plant material for the establishment of *in vitro* shoot cultures was initiated from juvenile shoots of *ex vitro R. glutinosa* plants that were grown in a greenhouse at Chungnam National University, Daejeon, Korea. To prepare samples, the shoot tips were first washed with tap water for 15 min. These shoot tips were then disinfected with 70% (v/v) ethanol for 1 min and 2% sodium hypochlorite solution (NaClO) with a few drops of Tween 20 solution for 10 min. The explants were then rinsed thoroughly with autoclaved distilled water under

sterilized conditions, and were then cultured in a magenta box containing 50 mL of hormone-free Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) under light conditions. The basal medium, containing mineral salts and vitamins supplemented with 30 g/L of sucrose and 8 g/L of Phytagar as a solidifying agent, was sterilized by autoclaving at 121°C for 20 min. After 6 weeks of culture, elongated shoots were obtained and used as explants in this experiment.

2.2 Root Regeneration Condition

To investigate the best type of sugar for root regeneration, seven carbon sources were examined: dextrose, mannose, glucose, sucrose, galactose, fructose, and maltose. The carbon sources were added at a concentration of 3% (w/v) to Schenk & Hildebrandt medium (SH; Schenk & Hildebrandt, 1972), which was supplemented with 1 mg/L of myo-inositol, 5 mg/L of nicotinic acid, 0.5 mg/L of pyridoxine HCl, and 5 mg/L of thiamine HCl. The medium was solidified with 3 g/L of Gelrite (SIGMA, USA) and the pH of all media was adjusted to between 5.7 and 5.8 using 1 N KOH or HCl before autoclaving. After identifying the best type of sugar, the effect of various concentrations (0.5%, 1%, 2%, 3%, 4%, and 5% w/v) on root regeneration was tested. The experiments were repeated three times. The culture vessels were incubated at 25 ± 2°C under cool white fluorescent light (35μmol photons m^{-2} s^{-1}), with a 16-h photoperiod. SH medium without sucrose was used as the control. Observations on rooting efficiency, the number of roots, and root length were scored after 4 weeks of culture.

2.3 Statistical analysis

Data collected from 50 root explants of the two experiments, with three replicates, are presented as mean ± standard deviation.

3. Results and Discussion

3.1 Determination of a Suitable Carbon Source

The effect of different carbon source supplements to the SH medium were examined in *in vitro* root cultures. Each carbohydrate was to the medium to a final concentration of 30 g/L (or 3%). The rooting conditions of each carbohydrate are shown in Table 1. Of the 7 types of sugar tested, sucrose, galactose, and glucose produced higher root regeneration rates, number of roots per explant, and average root length (95%; 15.3 ± 0.5 and 14.96 ± 0.8 mm), (91%, 14.0 ± 0.6, 12.90 ± 0.6 mm), and (90%, 11.8 ± 0.4, 10.79 ± 0.8 mm) respectively. The remaining carbon sources (i.e., mannose, maltose, fructose, and dextrose) produced regeneration rates of 85%, 79%, 78%, and 75%, respectively. These carbon sources produced the number of roots per explant of 10.5, 6.5, 9.0, 8.0, and root length of 9.57 mm, 7.25 mm, 10.18 mm, and 10.77 mm, respectively. Of note, the root growth of stem segments was lowest in media without sucrose, producing a regeneration efficiency of just 60%. This result implies that carbon is required as an energy source, and that it plays an important role during tissue development in plant regeneration.

George (1993) observed that the cultures of some plants grow better in media containing autoclaved (rather than filter-sterilized) sucrose. This finding indicates that cells benefit from the availability of glucose and fructose, which arise as a result of the autoclaving process. Paiva Neto and Otoni (2003) reported interesting information about changes in pH values of liquid media after autoclaving. The authors mentioned that the pH value (5.7) of medium containing sucrose solution was maintained after autoclaving, whereas the pH of medium containing glucose solution fell to 4.65. Owen et al. (1991) noted that media autoclaved with sucrose generally have a slightly lower pH than those autoclaved without sucrose. In contrast, the post-autoclaved pH of media was significantly reduced if maltose, glucose, or fructose was added instead of sucrose. In our experiment, sucrose was the best and most effective carbon source for root growth and development.

3.2 Optimal Sucrose Concentration for Promoting Root Regeneration

An experiment was carried out to identify the optimum level of sucrose or efficient root regeneration and growth of *R. glutinosa* in media. To evaluate the optimum strength of sucrose for use in the root induction medium, seven different concentrations of sucrose (0%, 0.5%, 1%, 2%, 3%, 4%, and 5%) were added to SH media that was solidified with 3 g/L Gelrite.

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Data on rooting efficiency, average root number, and root length were recorded after 4 weeks of culture in the respective medium. Of the different sucrose concentrations tested, the medium containing 3% sucrose was the best for rooting capacity (Table 2). This concentration exhibited the highest rooting efficiency (98%), greatest root number per explant (12.5 ± 0.3), and longest root length (16.04 ± 0.2 mm). Furthermore, the number of roots per explant and root length were nearly 3 times and 4 times higher in 3% sucrose medium compared to medium without sucrose.

Table 1. Influence of different carbon sources on the regeneration and growth of roots from the excised stem of *Rehmannia glutinosa* Libosch.

Carbon sources	Regeneration rate[1] (%)	No. of roots/explant*	Root length* (mm)
Control	60	3.0 ± 0.4	6.84 ± 0.5
Dextrose	75	8.0 ± 0.4	10.77 ± 0.6
Mannose	85	10.5 ± 0.3	9.57 ± 0.4
Glucose	90	11.8 ± 0.4	10.79 ± 0.8
Sucrose	95	15.3 ± 0.5	14.96 ± 0.8
Galactose	91	14.0 ± 0.6	12.90 ± 0.6
Fructose	78	9.0 ± 0.7	10.18 ± 0.6
Maltose	79	6.5 ± 0.3	7.25 ± 0.6

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

Table 2. Effect of different sucrose concentrations on the regeneration and growth of roots from the excised stem of *Rehmannia glutinosa* Libosch.

Sucrose concentration (%)	Regeneration rate[1] (%)	No. of roots/explant*	Root length* (mm)
0	60	3.0 ± 0.4	6.84 ± 0.5
0.5	75	3.8 ± 0.5	8.85 ± 0.1
1	80	7.0 ± 0.1	11.00 ± 0.3
2	90	8.0 ± 0.2	13.58 ± 0.2
3	98	12.5 ± 0.3	16.04 ± 0.2
4	85	5.5 ± 0.2	10.71 ± 0.3
5	65	5.3 ± 0.4	8.95 ± 0.5

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

The root development performance of the other sucrose strengths was higher compared to the control. It was observed that root development increased with increasing sucrose concentrations in the medium, i.e., from 0.5% to 3%. However, there was a gradual decline in root growth above a concentration of 3% sucrose. The osmotic contribution of the carbon source has an inverse relationship with carbon source concentration. Consequently, this relationship causes an initial increase followed by a reduction in the values of the assessed parameter. This reduction can be caused by an excessive osmotic contribution or by the toxicity of the carbohydrate (Slesak et al., 2004). Therefore, it is important to determine the optimum carbon source concentration when developing plant regeneration techniques.

Our experiment determined the suitable type of carbon source and concentration for use in the rooting medium of *R. glutinosa*. These results, together with an efficient protocol for root regeneration, provide information for the development of different carbohydrate supplements used in culture media.

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