

Review

Application of plant biotechnology in the medicinal plant, *Rehmannia glutinosa* Liboschitz

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***Rehmannia glutinosa* Liboschitz, a medicinal plant, is one of the 50 fundamental herbs used in traditional Chinese medicine. Chinese foxglove has a variety of beneficial effects and pharmacological actions on the blood, immune, endocrine, cardiovascular and nervous systems. Chinese foxglove is propagated conventionally through the roots. Therefore, a number of studies have reported *in vitro* plant regeneration, micropropagation and plant transformation of *R. glutinosa* from the culture of several explants for multiple propagations and plant improvement. This review summarizes previous and current information regarding the application of plant biotechnology (plant regeneration, micropropagation, hairy root culture and plant transformation) in *R. glutinosa* and provides new insights for future study in this discipline.**

Key words: Hairy root culture, micropropagation, plant regeneration, plant transformation, *Rehmannia glutinosa* Liboschitz.

INTRODUCTION

Rehmannia glutinosa Liboschitz (Figure 1), a widely used traditional oriental medicinal plant, is a member of the Scrophulariaceae family and is one of the 50 fundamental herbs used in traditional Chinese medicine. The common name of perennial flower *R. glutinosa* is Chinese foxglove, because the tubular flowers bear a resemblance to *Digitalis*. *Rehmannia radix* (the dried root of *R. glutinosa*) containing iridoids, saccharides, amino acid, inorganic ions, other trace elements, among others, is commonly used as a traditional Chinese medicine and has a very high medicinal value. *R. glutinosa* has been shown to have a variety of beneficial effects and pharmacological actions on the blood system, immune system, endocrine system, cardiovascular system and the nervous system (Bi et al., 2008; Hasegawa et al.,

1982; Liu et al., 2008; Zhang et al., 2008).

Catalpol (Figure 2) was confirmed to be the major iridoid constituent of the rhizomes in *R. glutinosa* (Albach et al., 2007). It exists broadly in many plants and has many biological functions such as anti-inflammation, promotion of sex hormones production, protection of liver damage and reduction of elevated blood sugar and neuroprotective effect (Jiang et al., 2008; Jiang et al., 2004; Li et al., 2008; Liang et al., 2009; Liu et al., 2006; Tian et al., 2007; Wang et al., 2009).

The propagation of Chinese foxglove is by the tuberous roots or planting seed. Propagation from seed is a difficult method due to poor seed viability, low propagation rate and delaying root harvest. Therefore, this plant is propagated conventionally through the roots. Some studies have reported *in vitro* plant regeneration, micropropagation and plant transformation of *R. glutinosa* L. from the culture of several explants for multiple propagations and plant improvement (Ling et al., 2009).

In this paper, various studies on the applications of

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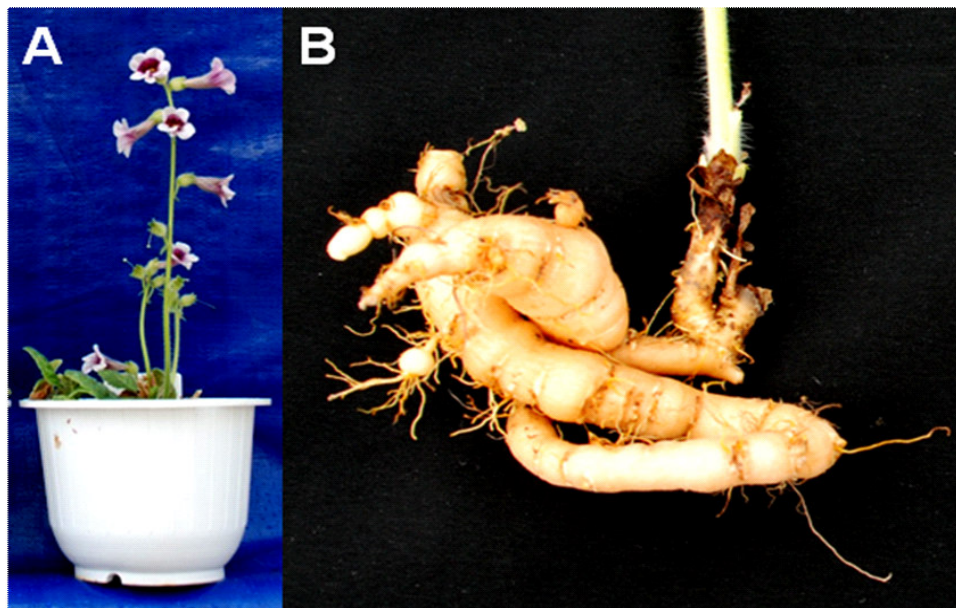


Figure 1. Plant in the pot (A) and root (B) of *Rehmannia glutinosa*.

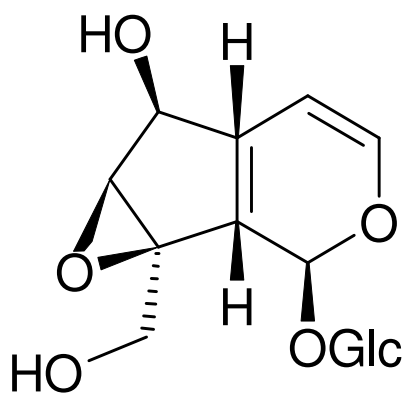


Figure 2. Chemical structure of catalpol.

plant biotechnology, *in vitro* plant regeneration, cell or hairy root cultures for bioactive compound production and genetic transformation for crop improvement of *R. glutinosa* is reviewed

APPLICATION OF PLANT BIOTECHNOLOGY IN *R. GLUTINOSA*

In vitro plant regeneration and micropropagation

Traditional skills are required in the vegetative propagation of plant species. However, the use of a regeneration system through tissue culture of economically important plants is a relatively new development. *In vitro* techniques considerably improve this potential by the application of nutritional and hormonal systems under aseptic condi-

tions. Plant proliferation by this method is termed micropropagation because miniature shoots or plantlets are initially derived. There are a number of pathways for the regeneration of whole plants from excised plant parts. Two main pathways can be considered, that is, generation through shoot organogenesis and somatic embryogenesis (Phillips et al., 1995).

Organogenesis is a developmental pathway in which shoots or roots (that is, organs) are induced to differentiate from a cell or group of cells. Plant regeneration through organogenesis generally involves induction and development of a shoot from explant tissue, followed by transfer to a different medium for the induction of root formation and development. Research has demonstrated that successful organogenesis in many plant species can be achieved by the correct establishment of medium components, selection of a suitable explant and control of the physical environment (Brown et al., 1986; Thorpe, 1990).

Steward et al. (1958) originally observed plant regeneration by somatic embryogenesis from cultured carrot cells. In somatic embryogenesis, somatic cells develop by division to form complete embryos analogous to zygotic embryos. The bipolar structure of the somatic embryo contains both shoot and root meristem. As the embryos develop, they progress through the distinct structural steps of the globular, heart, torpedo, cotyledonary and mature stages. Somatic embryogenesis can occur directly from cells of the explant tissue without an intervening callus phase. However, the indirect embryogenesis pathway, where somatic embryos are induced and develop from a proliferated callus, is generally more common (Pierik, 1987; Rashid, 1988). Many researchers have tried and some are still trying to develop *in vitro*

plant regeneration and micropropagation system of *R. glutinosa*.

Jiang et al. (1979) performed experiments demonstrating callus induction and *in vitro* plant regeneration of *R. glutinosa*. Usually, Chinese foxglove is asexually propagated by division of the tuberous roots. Seed propagation is a difficult method because of poor seed viability, low propagation rate and delaying root harvest. Therefore, *in vitro* micropropagation of *R. glutinosa* was developed (Matsumoto et al., 1986; Shoyama et al., 1983).

Shoot regeneration from mesophyll protoplast culture of *R. glutinosa* was reported by Xu et al. (1983). Mesophyll protoplasts obtained from leaves of *in vitro* grown Chinese foxglove shoots were cultured in Murashige et al. (1962) liquid or liquid-over-agar medium containing 2.0 mg L⁻¹ naphthaleneacetic acid (NAA) and 0.5 mg L⁻¹ benzylamino purine (BAP). Protoplast-derived colonies formed callus which readily regenerated shoots on transfer to MS based agar medium with 2.0 mg/l indoleacetic acid (IAA) and 1.0 mg/l BAP. Leaf explants also showed a marked capacity for shoot regeneration in *in vitro* culture.

Paek et al. (1995b) established micropropagation system of *R. glutinosa* by shoot tip and root segment culture. Shoot induction from the culture of shoot apical meristem and node-bud was achieved from the medium supplemented with 1 to 5 mg/l BA, 0.3 mg/L IAA and 3.0% sucrose with 0.6 – 1.2% bacto agar instead of gelrite which induced high frequency of vitrified shoots. Addition of activated charcoal at concentrations of 0.1 – 0.3%, markedly improved shoot regeneration and growth of roots and reduced the frequencies of verifications but inhibited shoot multiplication. Successful *in vivo* rooting from *in vitro* produced shoots was obtained and planting in rooting medium composed of 1:1 vermiculite and perlite. Field performance test of *in vitro* grown plants derived from apical meristem culture and conventionally propagated plants of *R. glutinosa* was conducted at two places to demonstrate the difference of growth, yield and the degree of virus infection. Usually *in vitro* grown plants showed better growth, yield and the resistant degree of virus infection than conventionally propagated plants (Paek et al., 1995a).

Characteristics in tissue cultured plants of Chinese foxglove for obtaining the basic breeding information were studied by Park et al. (1999). Effects of various plant growth regulators were investigated on leaf tissue for proliferation and most treatment easily produced callus. During the subculture, callus propagation rate recorded 15.4% with 0.2 mg/l NAA and 1.0 mg/l BA and plant regeneration improved on MS medium supplemented with 0.2 mg/l NAA and 0.5 mg/l kinetin. The number of shoot formed ranged from 1.7 on WPM medium to 3.4 on MS medium with 0.1 mg/l NAA and 0.5 mg /l BA treatment. Xue et al. (2002) induced *in vitro* Chinese foxglove microtuber on 1/2MS medium with 1 mg/l IBA. The best condition for microtuber production

was 2 mg/l BA, 0.1 mg/l NAA and 5% sucrose treatment on MS medium. The fittest incubation temperature was 25°C and the light length was (2,000 - 3,000 lx) 12 h/day. Jeong et al. (2002) established *in vitro* regeneration and plantlet formation of *R. glutinosa* using adventitious roots as explants.

Park et al. (2009) established an efficient and improved protocol for plant regeneration and micropropagation from leaf cultures of *R. glutinosa*. The regenerated shoots obtained from leaf cultures on solid MS medium contained different concentrations of TDZ. The highest number of shoots per explant (2.1) and shoot growth (1.2 cm) was obtained on MS medium containing 1 mg/l TDZ. The addition of auxins in MS medium containing 1 mg/l TDZ substantially improved the shoot regeneration of *R. glutinosa* and at the optimal concentration of 0.1 mg/l NAA was the most suitable auxin for the highest number of shoots per explant (3.8) and shoot growth (1.5 cm). Plant regeneration was found to be more efficient when gelrite was used as the gelling agent. The number of shoots produced per leaf explant was 15% higher and the growth of shoots was 12% greater, on 3 g/l gelrite compared to 6 g/l phytagar. The rooted plants were hardened and transferred to soil with a 73% survival rate. The continuous production of *R. glutinosa* regenerated plants could be used as a possible micropropagation system (Figure 3).

Some reports investigated the environmental condition during *in vitro* cultures of Chinese foxglove plantlets. Healthy *in vitro* Chinese foxglove plantlets, which had normal stomatal activity and high survival rates, were obtained by increasing the air exchange rates per hour in culture vessel. More than two times of leaf area and dry weight were observed compared with conventional tissue culture condition under increased air exchange rate, high PPF (photosynthetic photon flux) and CO₂ enriched environmental conditions. Although the treatment of sucrose led to maximal growth, the plantlets cultured on sucrose-free medium also grew well. This result clearly showed that plantlets grown under sucrose free, CO₂ enrichment and high PPF conditions had autotrophic growth characteristics (Seon et al., 1999). *R. glutinosa* plantlets were cultured for 4 weeks under different culture conditions to determine the optimum environment for *in vitro* growth and *ex vitro* survival. This study suggested that increased number of air exchanges of the culture vessel, decreased sucrose concentration and positive dark period temperatures (DIF) in combination with high PPF level enhanced growth and acclimatization of *R. glutinosa* plantlets (Cui et al., 2000). Seon et al. (2000) investigated the photosynthetic responses of *R. glutinosa* grown under photoautotrophic or heterotrophic conditions *in vitro* after transfer to greenhouse conditions. The control of transpiration during early stage after transplantation plays a key role in the acclimatization process and photoautotrophic conditions could be a solution to solve the problems associated with transplantation

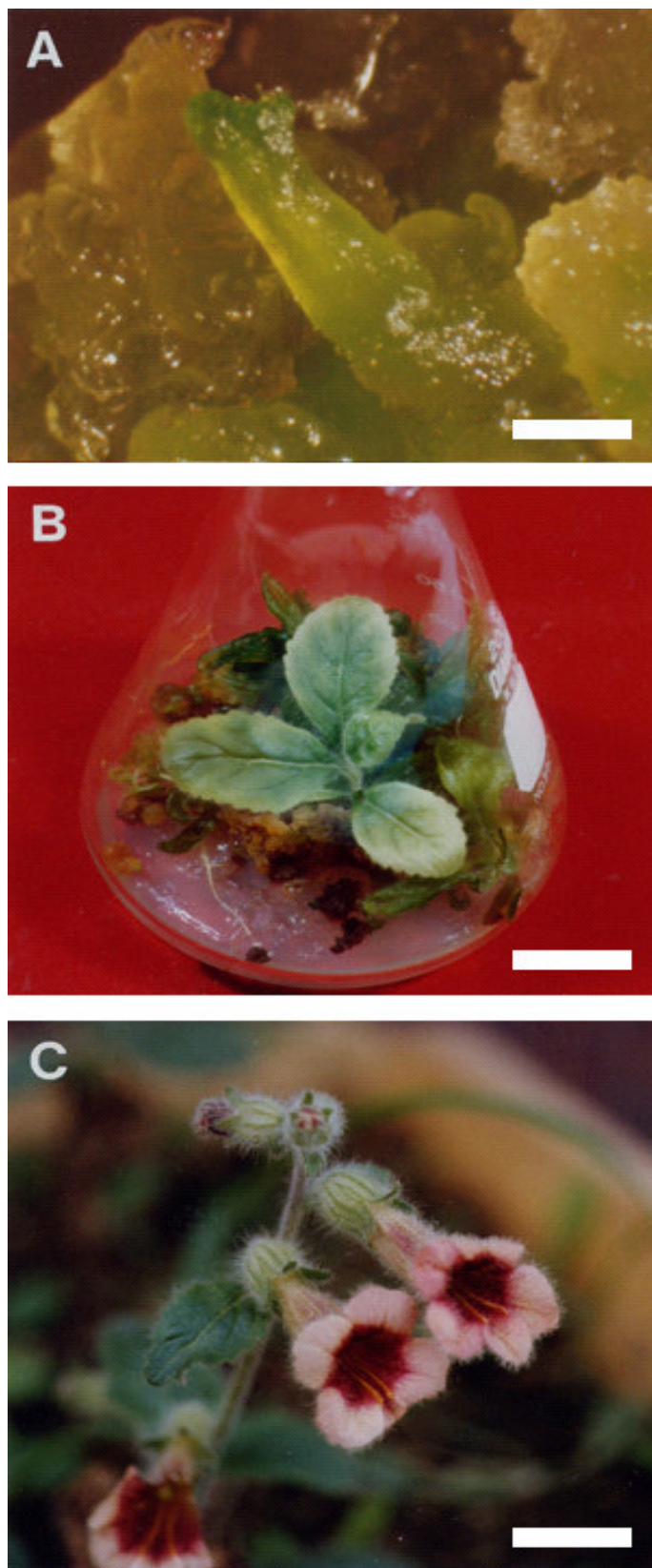


Figure 3. Plant regeneration and micropropagation of *Rehmannia glutinosa*: (A) initial shoot induction from leaf explants culture on MS media including 1 mg/l TDZ, (B) regenerated plant in the flask, (C) flowering regenerated plant in the green house.

stress.

***In vitro* cell cultures and hairy root culture for bioactive compound production**

Production of bioactive compounds through *in vitro* callus or cell cultures has been carried out in many plant species, especially in medicinal plant. Screening, selection, elicitation and media optimization are the methods applied for improving production of secondary metabolites using *in vitro* cultures (Mary, 2005). Some researchers tried to produce useful compound using cell culture of *R. glutinosa*. Four phenolic glycosides were isolated from callus culture of *R. glutinosa*. Two of the phenolic glycosides were identified as acteoside and forsythiaside and the structures of the other two were elucidated as 3,4-dihydroxy- β -phenethyl-*O*- β -D-glucopyranosyl-(1 \rightarrow 3) -4-*O*-calfeoyl- β -D-glucopyranoside and 3,4-dihydroxy- β -phenethyl-*O*- β -D-glucopyranosyl-(1 \rightarrow 3) -*O*- α -L-rhamnopyranosyl-(1 \rightarrow 6)-4-*O*-caffeoyl- β -D-glucopyranoside (Shoyama et al., 1986). When the calli were subcultured, embryogenesis occurred and regenerated shoot and root were obtained. Root tissues produced two iridoid glycosides, melittoside and rehmannioside D. A caffeoyl glycoside, acetoside and ethyl- β -d-glucose were detected in the regenerated shoots (Matsumoto et al., 1989).

Agrobacterium rhizogenes is a genus of gram-negative soil bacteria belonging to the Rhizobiaceae responsible for hairy root formation at the site of infection of plant (Giri et al., 2000; Guillon et al., 2006). Hairy root cultures from plants are getting considerable attention because of their genetic and biochemical stability, rapid growth rate and ability to synthesize secondary products at levels comparable to the original plants (Christey et al., 2005; Georgiev et al., 2007; Srivastava et al., 2007).

Hwang (2005, 2009) reported the production of catalpol in Chinese foxglove (*R. glutinosa*) hairy roots transformed with *A. rhizogenes* ATCC15834. The optimization of culture conditions for both root growth and catalpol production, effects of various combinations of seven basal media, pH and carbon sources were investigated. The best condition of root growth was obtained in an SH medium containing 4% sucrose and the highest catalpol content (0.54% of dry weight) was achieved in a WPM medium supplemented with 4% sucrose. Effects of plant growth regulators and chitosan were also studied. The treatment of 2 mg/l IAA significantly increased root lengths and the frequency of lateral roots. Chitosan (50 mg/l) and GA₃ (0.5 mg/l) increased catalpol production, with contents calculated at 0.7 and 0.65% dry weight, respectively. Zhou et al. (2007) achieved hairy root induction and culture of *R. glutinosa* for catalpol production. The content of catalpol in a transformed hairy root clone was 0.557 mg/g. FW by means of high performance liquid chromatography (HPLC), gave 48.5 and 18% of fresh and dried *Rehmannia* root, respectively.

Plant transformation and biotechnology

An efficient transformation protocol was developed for *R. glutinosa* using *Agrobacterium tumefaciens*. Putative transgenic plants were induced from leaf explants co-cultivated with *Agrobacterium*. Detection of the neomycin phosphotransferase gene, high activity of β -glucuronidase (GUS) transcripts and histochemical localization of GUS confirmed the integrative transformation of Chinese foxglove. This result demonstrated the potential for using *A. tumefaciens* to transfer important genes into commercial Chinese foxglove cultivars (Park et al., 2002). An efficient system of genetic transformation and plant regeneration from hairy roots by infecting the leaf sections and stem segments of *in vitro* *R. glutinosa* Libosch. plantlets have been developed. Hairy roots were induced after co-culturing with *A. rhizogenes* ATCC 15834 (Hwang, 2008; Zhou et al., 2009).

R. glutinosa is one of the most important medicinal plants. However, various plant pathogens, including *Fusarium* spp., cause damage on *R. glutinosa*. Lim et al. (2003) reported the introduction of antifungal gene (Rs-AFP) into *R. glutinosa* to breed *Fusarium*-resistant plants. Detection of Southern blot and AFP protein analyses confirmed the integrative transformation in transgenic plants. Lim et al. (2005) performed metabolic engineering of resveratrol biosynthesis in *R. glutinosa*. To increase production of resveratrol, they attempted ectopic expression of peanut resveratrol synthase gene (*AhR5S3*) in *R. glutinosa*. The transgenic plants produced new compounds identified as resveratrol and 3'-H-resveratrol-3-O- β -D-glucoside (R-gluc). The contents of resveratrol compounds in the transgenic plants were further increased by abiotic stresses and transgenic plants were highly resistant to *Fusarium oxysporum* infection. They demonstrated that these results showed diverse benefits for human and plant health and could provide a new opportunity for the improvement of *R. glutinosa* products.

CONCLUSION

Plant cell and tissue culture play important roles in the manipulation of plants for improved crop varieties. Plant regeneration system is an essential part of micropropagation and molecular approaches leading to plant improvement in *R. glutinosa*. Plant tissues of Chinese foxglove are still difficult to culture and to establish optimal growing conditions *in vitro*. Therefore, there continues to be an urgent need for extensive work in the field of basic tissue culture protocols for *R. glutinosa* plants before any practical utilization of molecular biology approaches can be achieved.

Plant transformation acts as a core research tool in a basic research for plant biology and a practical tool for plant improvement. Genetic engineering using plant gene transformation system has already been responsible for the production of transgenic plants with enhancement in

a range of desirable traits. For the establishment of genetic engineering of *R. glutinosa*, plant transformation system is essential. Though the transformation system of *R. glutinosa* has been developed, it is still difficult to produce transgenic plant easily. Therefore, there is a need to make an optimal protocol for Chinese foxglove transformation. The purpose of this review is to collect all possible information regarding applications of plant biotechnology in *R. glutinosa*. It is hoped that it will help students and scientists to take action for future study in this discipline.

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