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Somatic Embryogenesis and Plantlet Regeneration from Hairy Roots Transformed by *Agrobacterium rhizogenes* in *Panax quinquefolium* L.

Shou-Jing Zhao • Jian-Hua Wang • Yan-Long Liang* • Li-Xin Xu

College of Biological and Agricultural Engineering, Jilin University, Changchun 130022, China

Corresponding author: * **liangyl@jlu.edu.cn**

ABSTRACT

Here, efficient plantlet regeneration via somatic embryogenesis from *Panax quinquefolium* hairy roots was reported. Callus development was optimal on MS medium containing 2,4-dichlorophenoxyacetic acid (1 mg l^{-1}) and α-naphthalene acetic acid (1 mg l^{-1}). After a 4-week growth, calli were transferred onto MS medium with different combinations of plant growth regulators (PGRs). The highest embryogenesis frequency (~55%) of the calli occurred with 0.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid after an additional 2-month culture. During embryos germination, different media with 3 mg l^{-1} gibberellin were evaluated for plantlet rooting. It was indicated that the suitable medium was 1/2 MS among the media tested. Furthermore, different PGRs were appraised for plantlet rooting using 1/2 MS medium. The data implicated that abscisic acid (0.5 mg l^{-1}) could play a role in promoting plantlet rooting.

Keywords: agricultural biostimulants, compost-stabilized waste, organic carbon fractions, humification indices, soil microbial populations

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **ABA**, abscisic acid; **BA**, N^6-benzyl amino purine; **GA_3**, gibberellic acid; **NAA**, α-naphthalene acetic acid; **KT**, kinetin

INTRODUCTION

American ginseng (*Panax quinquefolium*) is a perennial herbaceous plant, which is cultivated for its medicinally-valuable root (Chen and Punja 2002; Qi *et al.* 2010). Ginsenosides are the pharmalogically active compounds of this plant, which possesses many pharmacological effects, including anti-cancer and anti-diabetes (Corbit *et al.* 2006; Xie *et al.* 2009); therefore, it is now in high demand worldwide as an extremely valuable medicinal plant. Although it is native to North American, it has spread to North Asian. It has a long production cycle, as seeds are usually produced after a 3-year cultivation and must be vernalized for 12-18 months before germination. Consequently, its genetic improvement including yield increase and resistance to pathogens by conventional breeding way is difficult. Thus it is of investigative interest to develop *in vitro* clonal propagation ways, which would facilitate the genetic improvement of this plant, and would be useful for germplasm preservation. However, compared with the investigations on pharmacological effects of American ginseng, fewer studies focused on its clonal propagation.

American ginseng embryogenesis and plantlet regeneration have been reported from root, leaf, epicotyl, and cotyledon explants (Tirajoh *et al.* 1998; Zhou and Brown 2006). However, the microbial contamination is a problem associated with these explants (Tirajoh *et al.* 1998). Moreover, it is somewhat troublesome to collect these explants from field and to keep vitality of these explants. Hairy roots of *P. quinquefolium*, established by Ri plasmid transformation using *Agrobacterium rhizogenes*, can grow fast on hormone-free medium, and is easy to maintain, genetically-stable, and contaminant-free (Wang and Ding 1999; Mathur *et al.* 2009; Guillon *et al.* 2006). Additionally, plantlet regeneration frequency from hairy roots is high (Giri and Narasu 2000). Therefore, American ginseng hairy roots may serve as an alternative explant source for clonal propagation of this plant.

So far, there have been several reports on embryogenesis and plantlet regeneration by hairy roots of other plants, including *Armoracia rusticana* (Mano and Matsuhashi 1995), *Crotalaria juncea* (Ohara *et al.* 2000), *Panax ginseng* (Yang and Choi 2000), *Astragalus sinicus* (Cho and Widholm 2002), *Catharanthus roseus* (Choi *et al.* 2004), *Solanum khasianum* (Jacob and Malpathak 2005), *Coronilla varia* (Han *et al.* 2006), *Nicotiana tabacum* (Chintapakorn *et al.* 2007), *Centaurium erythraea* (Subotić *et al.* 2009a, 2009b), and *Beta vulgaris* (Ninković *et al.* 2010). While, according to our knowledge, there has been no report regarding embryogenesis and plantlet regeneration of *P. quinquefolium* from hairy roots. Herein, embryogenesis and plantlet regeneration from *P. quinquefolium* L. hairy roots were described, providing the opportunities for clonal propagation of this plant from hairy roots.

MATERIALS AND METHODS

Plant materials

Hairy roots of *P. quinquefolium* L. used in this study were previously established using root explants with *A. rhizogenes* A4 in our laboratory (Jia 2007) and maintained by subculture on solid half strength (1/2) Gamborg's B5 medium at 25°C and 4-week intervals. Unless otherwise stated, all the basal media used in this study were supplemented with 30 g l^{-1} sucrose, and the pH are adjusted to 5.8 prior to autoclaving at 120°C for 20 min. In this study, all cultures were performed in 500 ml Erlenmeyer flask containing 100 ml solid medium solidified by 9 g l^{-1} tissue culture agar, and all chemicals used are tissue culture grade (TaKaRa Dalian Biotechnology Co., Ltd.).

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Original Research Paper

Callus and embryo induction

Tips of 2-week old hairy roots cultured on 1/2 B5 medium were aseptically cut into 1-cm-long explants and plated onto solid Murashige and Skoog (MS) media comprising 9 g l^{-1} tissue culture agar and various PGRs combinations (concentrations are in mg l^{-1}): 2,4-dichlorophenoxyacetic acid (2,4-D , 0.5, 1, 1.5); 2,4-D (1) with α-naphthalene acetic acid (NAA, 0.5), NAA (1), NAA (1.5), abscisic acid (ABA, 0.5), N^6-benzyl amino purine (BA, 0.5), and kinetin (KT, 0.5), respectively. The cultures were maintained in a growth chamber at 23°C in the dark. Effects of regulator combinations on callus induction were evaluated based on callus induction frequency (percentage of explants with morphogenetic-competent calli) and appearance, after 4 weeks. For each regulator combination, three independent experiments were performed with 100 explants (~15 explants $flask^{-1}$).

After a 4-week culture, well developed calli were transferred by sterile steel pine (diameter, 8 mm) onto MS medium with different PGRs combinations (mg l^{-1}): 2,4-D (0.5), 2,4-D (1), NAA (0.5), NAA (1), 2,4-D (0.5) with NAA (0.5), for embryo induction. The cultures were carried out at 23°C and 16-h photoperiod provided by cold fluorescent lamps (2000 lux, 25 μmol m^{-2} S^{-1}), with transfers to fresh medium every 4 weeks. The effects of different regulators on embryogenesis were appraised according to the percentage of calli with abundant globular somatic embryos, after 2 months. For each regulator combination, triplicate experiments were carried out with 100 embryos (5-7 calli $flask^{-1}$).

Embryo germination

Initially, well developed embryos from 2-month old embryogenic calli were subcultured on MS medium with gibberellic acid (GA_3, 3 mg l^{-1}) for plantlet regeneration. To further optimize the medium conditions for regeneration, different media (MS, 1/2 MS, 1/4 MS, B5 and 1/2 B5), each with GA_3 of 3 mg l^{-1} were evaluated. Different PGRs (in mg l^{-1}): ABA (0.5/1), NAA (0.5/1), BA (0.5/1), and GA_3 (0.5/1), were also evaluated using 1/2 MS medium. All these media were supplemented with 1% activated charcoal, because of its positive effects on rooting (Tirajoh *et al*. 1998). The cultures were incubated under the same condition for embryogenesis induction. For each factor, three experiments were conducted by tracking 100 embryos (5-8 embryos per flask).

RESULTS AND DISCUSSION

Calli induction

To determine the optimal hormone combination for callus induction, experiments were performed with combinations of different PGRs. Callus development was optimal on MS medium containing 2,4-D (1 mg l^{-1}) and NAA (1 mg l^{-1}) with the highest calli frequency, and the resulted calli were pale yellow (**Fig. 1A**). The earliest formation of morphogenetic-competent callus appeared within ~2 weeks. Whereas callus development with all of the other PGRs combinations tested was less satisfactory; therefore these experiments were discontinued. For example, after a 4-week induction, the calli developed with KT turned brown, and concomitantly lost vitality (**Fig. 1B**), and adventitious roots were generated from the calli induced with combination of 2,4-D and (BA) or 2,4-D and ABA (**Fig. 1C** and **D**). Additionally, as expected, no morphogenetic-competent calli occurred on medium without any regulator. These data indicated the requirement of specific PGRs for efficient calli development from *P. quinquefolium* hairy roots. It was reported with *P. ginseng* hairy roots that 1.0 mg l^{-1} 2,4-D was efficient for calli induction (Yang and Choi 2000).

Embryogenesis

As for embryo induction in this study, 2,4-D (0.5 mg l^{-1}) was superior to the other regulator combinations tested with the highest embryogenesis frequency (**Fig. 2**). This frequency was higher than those from other explants of *P. quinquefolium*, including root, leaf and epicotyl explants

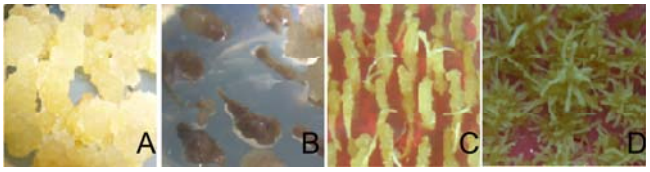

Fig. 1 *Panax quinquefolium* hairy root-derived calli on MS medium after 4-week culture. (**A**) Callus developed with 2,4-D (1 mg l^{-1}) and NAA (1 mg l^{-1}); (**B**) callus developed with 2,4-D (1 mg l^{-1}) and KT (0.5 mg l^{-1}); (**C**) callus induction with 2,4-D (1 mg l^{-1}) and BA (0.5 mg l^{-1}); (**D**) callus induction with 2,4-D (1 mg l^{-1}) and ABA (0.5 mg l^{-1}).

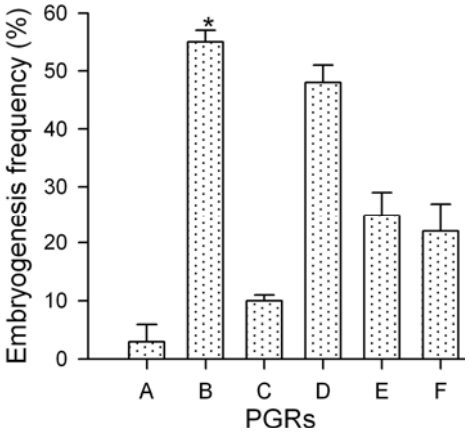

Fig. 2 Effects of different plant growth regulators (PGRs) on embryogenesis frequency. Embryogenesis frequency is expressed as the percentage of calli with abundant globular embryos recorded after a 2-month culture period. X-axis labels: A, absence of PGRs; B, 2,4-D, 0.5 mg l^{-1}; C, 2,4-D, 1 mg l^{-1}; D, NAA, 0.5 mg l^{-1}; E, NAA, 1 mg l^{-1}; F, combination of 2,4-D (0.5 mg l^{-1}) and NAA (0.5 mg l^{-1}). Each bar represents the mean value (±SE) from triplicate experiments, each with 100 calli. The asterisk above the bar indicates a statistically significant increase (Student's *t*-test, $P < 0.05$) between the frequency with 2,4-D of 0.5 mg l^{-1} and that with each of the other PGRs.

(Tirajoh *et al*. 1998), and also was higher than that reported from *P. ginseng* hairy roots (Yang and Choi 2000), where the highest embryogenesis frequency obtained was 37.4%. In *P. ginseng*, the closest relative of American ginseng, previous reports of callus growth and embryo formation mostly focused on root explants. The development of embryos from the root-derived calluses generally required 5 to 14 months, and the most frequently used PGRs combinations were: 2,4-D, 2,4-D/KT or 2,4-D/BA (Tiriajoh *et al.* 1998). It was also shown that efficient embryogenesis could be generated using calli from hairy roots of *P. ginseng* cultured with 0.5 mg l^{-1} 2,4-D (Yang and Choi 2000). Formation of abundant embryos were observed in ~6 weeks. This period is comparable with those from seedling leaf and cotyledon explants, but much shorter than that from root, mature leaf or epicotyl explants of *P. quinquefolium* (Tirajoh *et al*. 1998) while it takes a longer time (~5 months) for embryo formation using *P. ginseng* hairy roots, the closest relative to American ginseng (Yang and Choi 2000). The reason may be the genetic differences between American ginseng and *P. ginseng*, or the different cultural conditions. Surprisingly, although embryogenesis frequency (48%) achieved with NAA (0.5 mg l^{-1}) was also high, the embryogenesis induction rate (15 %) was significantly decreased with the combination of NAA (0.5 mg l^{-1}) and 2,4-D (0.5 mg l^{-1}). This may be due to an excess of auxins.

The typical embryogenic stages were observed in this study, namely globular, heart-shape, torpedo, and cotyledon embryo stages (**Fig. 3**). However, embryogenic development was not synchronous. This should be caused by the different micro-environment for different embryos.

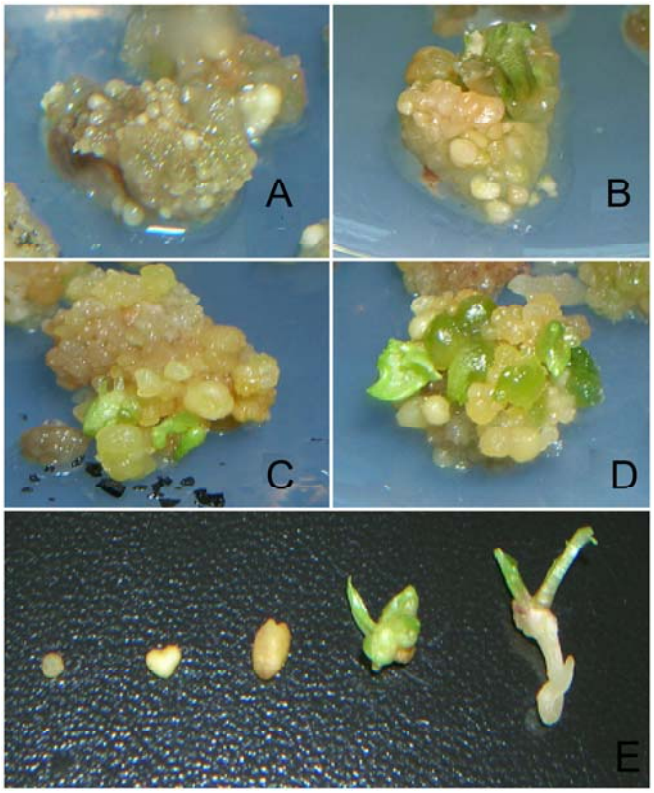

Fig. 3 Developmental stages of somatic embryos of *P. quinquefolium* hairy root-derived explants on MS medium. (**A**) Globular stage; (**B**) heart-shape stage; (**C**) torpedo stage; (**D**) cotyledon stage; (**D**) the whole cycle of embryogenesis.

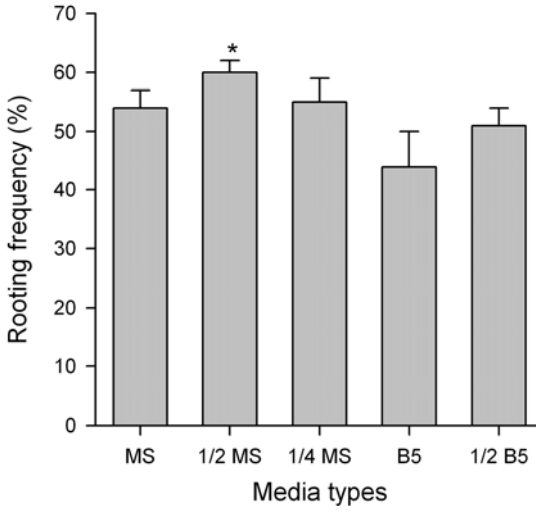

Fig. 4 Effects of different media on rooting frequency during embryo germination. Each medium was supplemented with GA_3 (3 mg l^{-1}) and activated charcoal (1%, w/v). Rooting frequencies were expressed as the ratio of the number of plantlets with well developed roots to the number of initial embryos used. The data were recorded after a 2-month culture period. Each bar represents the mean value (±SE) from triplicate experiments, each with 100 embryos. The asterisk above the bar indicates a statistically significant increase (Student's *t*-test, $P < 0.05$) between the frequency with 1/2 MS and that with each of the other media.

Embryo germination

In the present study, the embryos obtained on embryos induction medium must be transferred onto germination medium for efficient germination; otherwise, the most would be arrested in the torpedo or cotyledon stage. MS medium with 3 mg l^{-1} GA_3 was initially employed for embryo germination. After ~6 weeks, plantlets occurred. This period is comparable with that from other explants of *P. quinquefolium* (Tirajoh *et al*. 1998; Zhou and Brown 2006). However, the resulted percentage of plantlets without roots was high (50%). The difficulty in rooting is present in both Asian ginseng and American ginseng (Tirajoh *et al.* 1998). To relieve this symptom, different media (MS, 1/2 MS, 1/4 MS, B5 and 1/2 B5) containing 3 mg l^{-1} GA_3 were evaluated. As shown by **Fig. 4**, 1/2 MS medium was the optimal among the media tested, due to the resulted high rooting frequency (60%). Moreover, according to previous reports, BA, NAA and ABA could alleviate the difficulty in rooting (Chang and Hsing 1980; Lelu *et al*. 1994; Shoyama *et al*. 1988); so these regulators were tested for promoting rooting in this study. In agreement with the study by Lelu *et al*. (1994), our investigation showed that ABA (0.5 mg l^{-1}) could play a role on rooting (**Fig. 5**). In the case of plantlet regeneration from *P. ginseng* hairy roots, however, 10 mg l^{-1} of GA_3 was efficient for rooting (Yang and Choi 2000). **Fig. 6** shows the regenerated plantlet with ABA. In above-mentioned callus induction experiments, we also found that ABA could cause formation of abundant adventitious roots on the callus (**Fig. 1D**). Adversely, it was proposed that ABA had no obvious effects on rooting in regeneration of *P. quinquefolium* with root, leaf and epicotyl explants, while activated charcoal could alleviate the rooting problem (Tirajoh *et al.* 1998). This should be account for by the genotype difference of hairy roots from other kind of explants, which results in different response to ABA.

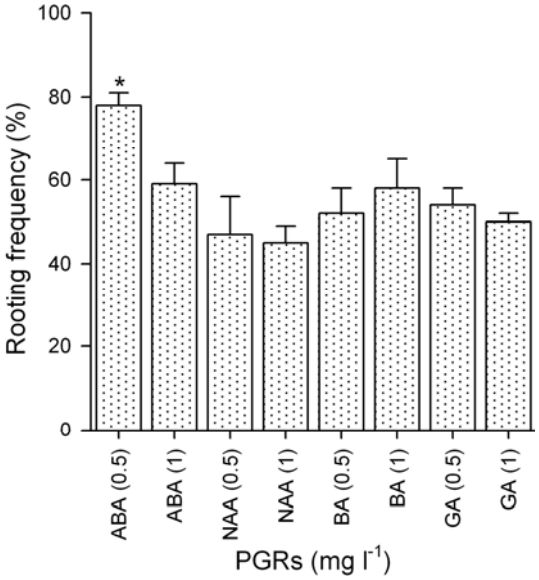

Fig. 5 Effects of different plant growth regulators (PGRs) on rooting frequency during embryo germination. The data were obtained after a 2-month culture period. The PGR concentrations are in mg l^{-1}. Each bar represents the mean value (±SE) from triplicate experiments, each with 100 embryos. The asterisk above the bar indicates a statistically significant increase (Student's *t*-test, $P < 0.05$) between the frequency with ABA of 0.5 mg l^{-1} and that with each of the other media.

Fig. 6 Plantlets regenerated with ABA (0.5 mg l^{-1}) after a 2-month incubation period.

Table 1 Optimized conditions used in this study.

Stages	Mediums	Culture conditions	PGR combinations	Frequencies
Calli introduction	MS with 30 g l^{-1} sucrose	23°C in the dark	2,4-D (1 mg l^{-1}) + NAA (1 mg l^{-1})	87%
Embryogenesis	MS with 30 g l^{-1} sucrose	23°C under 16-h photoperiod (25 μmol m^{-2} s^{-1})	2,4-D (0.5 mg l^{-1})	55%
Embryo germination	1/2 MS with 1% activated charcoal	23°C and 16-h photoperiod (25 μmol m^{-2} s^{-1})	ABA (0.5 mg l^{-1})	78.7%

CONCLUSIONS

The results of this study demonstrated the possibility for embryogenesis and plantlet regeneration from *P. quinquefolium* hairy roots. The methods and data presented in this study are briefly summarized in **Table 1**. This system was characterized as relatively high regeneration frequency and short regeneration period. The hairy roots explants are contaminant-free, and easy to maintain. Therefore, they can serve as a desire alternative explant source for plantlet regeneration. In addition, plants regenerated from hairy roots generally have abundant root mass (Choi *et al*. 2004). This was potentially-useful for breeding of *P. quinquefolium*, of which the roots are utilized in medicine. Recently, introduction of exogenous gene into hairy roots has been achieved for several plant species (Hu and Du 2006; Guillon *et al*. 2006), especially for Asian ginseng (Liang *et al*. 2009), the closest relative to Americian ginseng. Transgenic plants could be attained by regeneration from the according hairy roots, which are established by transformation of Ri plasmid harboring the desired genes using *A. rhizogenes*, without requirement of selection agents, which often inhibit shoot regeneration; for the reason that hairy root-phenotype could work as a selection marker. By this way, the achievement of transgenic plants was facilitated. As such, regeneration from *P. quinquefolium* hairy roots could provide opportunities for development of new efficent breeding and micropropagation ways for this valuable plant in the future. Nevertheless, there is the possibility that *Agrobacterium*-based transformation might not be stable. More research will be necessary to evaluate the stability of the transgenic plants.

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