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Research Article

Micropropagation from root segments to improve seedling quality in Chinese foxglove crops

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Abstract This is the first study to establish a complete protocol for micropropagation of *Rehmannia glutinosa* from root segments. The study involved investigating the effect of plant growth regulators on *in vitro* shoot regeneration and rooting and identifying substrates supporting survival and growth performance of *ex vitro* seedlings. A Murashige and Skoog (MS) medium containing 30 g/L sucrose for shoot induction and 0.2 mg/L indole-3-acetic acid (IAA), 1 mg/L 6-benzylaminopurine (BAP), and 1 g/L polyvinylpyrrolidone (PVP) for shoot multiplication resulted in the highest number of shoots per explant and shoot height. Applying a medium containing 0.5 mg/L IAA and 1 g/L PVP yielded optimal rooting of the shoots grown *in vitro*. Compost enriched with microbial inoculants and perlite enhanced seedling growth better than that with organic biofertilizer-free substrates (soil and sand). We recommend the continuous production of micropropagated *R. glutinosa* seedlings from root segments under the aforementioned conditions as a possible propagation technique for crops of this species.

Keywords IAA, Multiple shoots, NAA, *Rehmannia*, PVP, Root induction, Tissue culture

Introduction

Rehmannia glutinosa Libosch. belongs to the Scrophulariaceae family. It is one of the most important medicinal plants utilized in traditional Oriental medicine. Medicinal materials from this plant can be processed into different forms and used in different folk medicines. Rehmannia's fresh roots or dry rhizomes (< 15% moisture) were proved to have a variety of pharmacological functions on the improvement of human health systems such as the blood system, immune system, and cardiovascular system. Steam-prepared Rehmannia roots were remarkably highlighted with the properties of Yin-nourishing and blood enriching (Zhang et al. 2008). Recent studies have confirmed that active principles from *R. glutinosa* can support many functioning organs and also possesses anti-tumor and anti-senescence agents (Kang et al. 2011; Liu et al. 2012; Yu et al. 2006).

In Vietnam, the market demand for medicinal materials from *R. glutinosa* is considerably high and mostly imported from China by pharmaceutical companies. Whereas, the domestic agricultural sector contributed an insignificant account in the cultivation and provision of this medicinal plant for the market. The reasons for the limited production mostly associated with breeding, which often provided poor seedling quality for cultivation (Nguyen 2016). Seedlings were often originated from root segments that had been excised from small roots as the marketable size roots were often selected for further processing for steam-prepared Rehmannia. These cultivation habits were repeated over the years and caused trait degeneration and plant diseases, including virus-infected seedlings (Wu et al. 2018; Zhang et al. 2011, 2019). As a result, the yield and productivity of *R. glutinosa* crops were substantially declined. Therefore, the requirement of an alternative propagation technique to produce healthy seedlings while retaining superior growth and productivity traits is importantly needed.

Few studies developed micropropagation protocols that produced seedlings from leaf mesophyll protoplast (Xu and Davey 1983), leaves (Sang et al. 2009), shoot tips (Shoyama

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et al. 1983), auxiliary buds (Piątczak et al. 2014b). A recent study has developed a protocol to produced calli with subsequent regeneration of the plant, followed by genetic analysis of the regenerated plants (Piątczak et al. 2014a). The results demonstrated that the regenerative capability accumulation of bioactive metabolites to be reserved for four years of culture. So far, none of the developed *R. glutinosa in vitro* propagation has conducted using root segments effectively and an extended experiment to examine the effect of substrates on survival and growth of seedling in *ex vitro* stage. Therefore, the present study aimed to establish a complete micropropagation protocol started with shoot induction from the root segment, to shoot multiplication, rooting, and finished by substrates experiment to acclimatize new seedlings.

Materials and Methods

Plant materials and explant preparation

Rehmannia glutinosa Libosh. tuber roots collected from the plants, which were superior in growth and development in three provinces (Phu Tho, Vinh Phuc, and Tuyen Quang), were used for the experiments in the biotechnological laboratory of Insititute of Applied Research and Development, Hung Vuong University (Vietnam) from February to August 2019. The roots were thoroughly sterilized in 70% ethanol for 30 seconds, disinfected in 0.25% sodium hypochlorite twice for 15 minutes, and rinsed several times with microbial-free distilled water. As explants, root segments (1 ~ 2 cm long) excised from virus-free healthy plants were used.

Shoot regeneration by three different basal media

The sterilized root segments excised from *R. glutinosa* tubers were placed in three different basal media supplemented with 30 g/L sucrose to examine the effect of MS (Murashige and Skoog 1962), Knudson (1946), and VW (Vacin and Went 1949) on the shoot regeneration. The media were adjusted to pH 5.6 ~ 5.8 then autoclaved for 15 minutes at 121°C. Samples were incubated at 25 ± 2°C for 16 hours per day under a fluorescent light intensity of 2,000 lux. After four weeks, the number of explants with primary shoots, and the number of shoots per explant was recorded.

Effect of auxin and cytokinin combination on shoot multiplication

The shoot multiplication experiment was carried out after the success of the initiation phase. Initially, shoot tip excised from the explants were inoculated in MS medium supplemented with 30 g/L sucrose, 7 g/L agar, pH 5.8, and with various concentrations (0.0, 0.3, 0.5, 1.0, and 1.5 mg/L) of BAP (Bio Basic, Canada). The optimal BAP concentration was then selected to final shoot multiplication experiment in which, 1 g/L PVP (Fisher Chemicals, Germany) and various concentrations (0.0, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/L) of IAA (MERCH, Germany) were added to investigate the effect of auxin on shoot multiplication. After four weeks, shoot multiplication rate and shoot height were recorded.

Effect of auxin and PVP combination on *in vitro* rooting

The rooting experiment was carried out after the success of the shoot multiplication phase. Selected *in vitro* produced shoots were inoculated in ½ MS medium supplemented with 30 g/L sucrose, 7 g/L agar, pH 5.8, 1 g/L PVP, various concentrations of NAA (0.0, 0.3, 0.5, 1.0, and 1.5 mg/L), and 0.3, 0.5, 1.0, 1.5 mg/L of IAA. After four weeks, the number of rooting plantlets, the number of roots per plantlet, root length, and plantlet height were recorded.

Effect of substrates to survival rate and growth of *in vitro R. glutinosa* seedlings

Four-week-old rooted seedlings were transferred from rooting cultured environment into pots with five different growing substrate compositions, including 50% alluvium soil + 50% sand (ASS); 50% alluvium soil + 50% rice husk (ASRH); a compost including organic biofertilizer: a mixture of soil and sand (3:2) topped with 3 ~ 4 cm sand layer (COSS); 100% carbonized rice husk (RH); and 90% compost + 10% perlite (COP). Organic biofertilizer was based on chicken manure, sawdust, and coconut peat, in a ratio of 1: 4: 4 (v/v) with the addition of microbial inoculants including *Glomus intraradices, G. aggregatum, G. etunicatum, G. moseae* (CP, Thailand). Plantlets were acclimatized in a greenhouse under 25% ~ 50% shade with automatic water irrigation. The survival rate, number of leaves per plant, plant height and leaf length were recorded after four weeks of growth.

Statistical analysis

Observed data were analyzed using Microsoft Excel and Irristart 5.0 software. Means and standard errors were applied to assess the experiment results using the ANOVA test at $P < 0.05$. Treatments were designed randomly with three replicates.

Results and Discussion

Shoot multiplication

The results from Table 1 showed that all three types of basal media-induced multiple shoots effectively and were significantly different ($P \leq 0.05$). The highest percentage of shooting explants (56%) and the number of shoots per explant (5.05) were observed in MS medium. The lowest results were obtained for VW medium with 24.66 % explant with shoots and 3.69 shoots per explant (Fig. 1A). Some previous *in vitro* propagation studies on *R. glutinosa* also used MS as the basic medium, depending on the purpose of the culturing, some different growth regulators might be added. Since early, Xu and Davey (1983) and Mao et al. (1983) have succeeded in *in vitro* propagation of *R. glutinosa* from leaves and apical meristem tissues, respectively, using MS medium. *R. glutinosa* shoots were generated from leaves with the highest rate in MS medium containing 1 mg/L TDZ + 0.1 mg/L NAA + 3g/L gelrite (Sang et al. 2009). Shoot regeneration was also observed from callus of *R. glutinosa* in MS medium supplemented with 1.0 mg/L BAP and 0.1 mg/L IAA (Piątczak et al. 2014a). In the present study, 30 g/L sucrose was added to promote the shoot multiplication. The result was in line with a study in *Toddalia asatica* by Praveena and Veeresham (2014), where using sucrose at a concentration of 30 g/L was found to be optimal for growth and biomass accumulation.

The results from Table 2 showed that the highest rate of multiplication and the average height of shoots were observed in explants treated by BAP 1.0 mg/L. However, the quality of shoots generated was under standard and characterized by the abnormal morphological shape of shoots, yellow color of leaves, and several symptoms of mosaic

Table 1 Differences in multiple shoot induction in *R. glutinosa* root explants by the basal medium used

Medium	Shooting explant	Shooting rate	Average number of shoots per explant
MS	28.00[a]	56.00%	5.02[a]
Knudson	15.33[b]	30.66%	4.12[a]
VW	12.33[c]	24.66%	3.69[c]

Each basal medium was supplemented with 30 g/L sucrose. Different letters (a, b, c) indicate significant differences in shooting between media ($P \leq 0.05$).

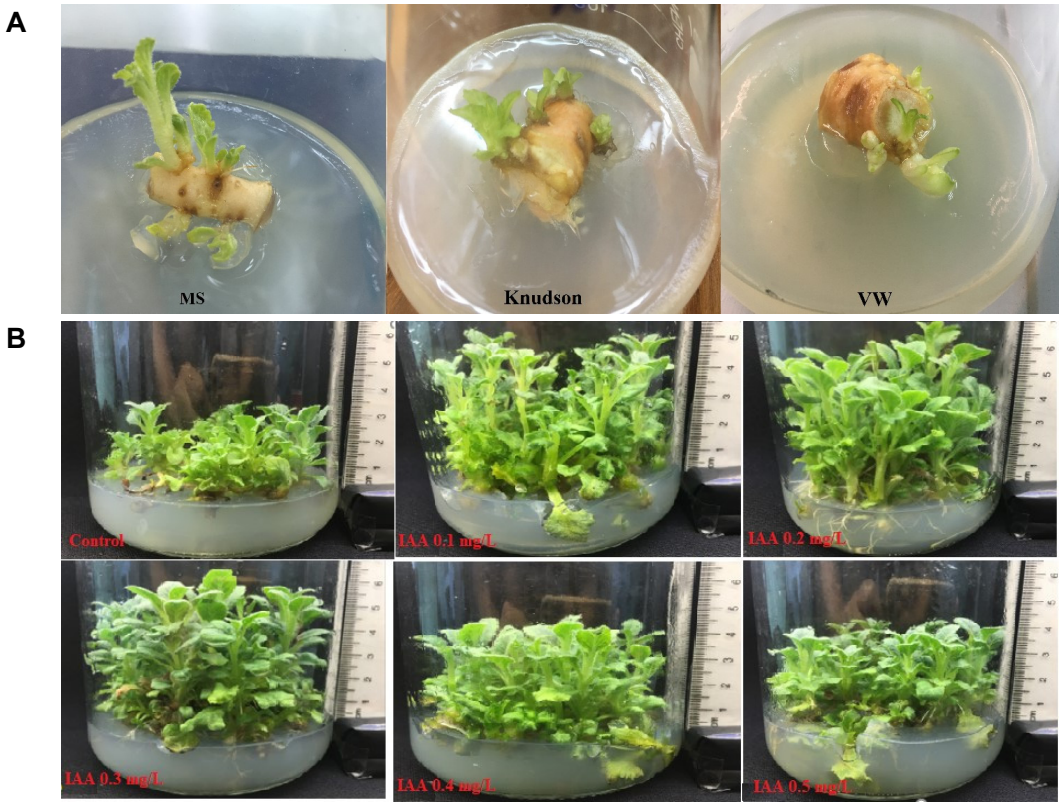

Fig. 1 **A** Shoot induction from root segments on MS, Knudson and VW medium; **B** Shoot multiplication from *in vitro* shoot tips of *R. glutinosa* cultured on MS medium containing various concentrations of IAA, 1 g/L PVP, and 1mg/L BAP

Table 2 Comparison of shooting *in vitro* of *R. glutinosa* at various BAP concentrations

Cytokinin (mg/L)	Shoot multiplication	Shoot height (cm)
Control (BAP-free)	2.05^{g}	2.32^{f}
BAP 0.3	2.37^{f}	2.84^{d}
BAP 0.5	2.87^{bc}	3.08^{c}
BAP 1.0	3.09^{a}	3.50^{a}
BAP 1.5	2.90^{b}	3.22^{b}

The MS medium was supplemented with 30 g/L sucrose, 7 g/L agar, and with various concentrations (0.0, 0.3, 0.5, 1.0 and 1.5 mg/L) of BAP and maintained at a pH of 5.8. Different letters (a, b, c) indicate significant differences between media ($P \leq 0.05$).

Table 3 Comparison of shooting *in vitro* of *R. glutinosa* at various concentrations of IAA with 1.0 mg/L BAP

Auxin (mg/L)	Shoot multiplication	Shoot height (cm)
Control (auxin-free)	2.86^{f}	2.94^{f}
IAA 0.1	3.95^{c}	4.46^{c}
IAA 0.2	4.74^{a}	5.16^{a}
IAA 0.3	4.39^{b}	5.03^{b}
IAA 0.4	3.79^{d}	4.26^{d}
IAA 0.5	3.60^{e}	3.81^{e}

The control included MS + 1 g/L PVP + 1mg/L BAP. Different letters (a, b, c) indicate significant differences between media ($P \leq 0.05$).

phenomena. This event was probably due to phytotoxins (some phenolic acids) released during the explant excising process (Du et al. 2009). Therefore, PVP was added to the medium to eliminate the toxicity of possible phenolic acids. The results showed a positive effect on the growth and quality of regenerated shoots, as shown in Table 3.

Shoot tips were excised from shooting explants and cultured on MS medium with various concentrations of IAA (0.1, 0.2, 0.3, 0.4, and 0.5 mg/L) in combination with 1.0 mg/L BAP containing 1 g/L PVP to promote regeneration of adventitious shoots. The results showed that the effect of IAA with low concentrations (0.1, 0.2, and 0.3 mg/L) substantially induced multiple shoots. In contrast, the higher concentration of IAA (0.4 and 0.5 mg/L) significantly decreased the shoot multiplication (Fig. 1B). The highest number of shoots (4.74) and shoot height (5.16 cm) was recorded when shoot tip explants were supplemented with 0.2 mg/L IAA, whereas the lowest number of shoots (2.86), as well as shoot height (2.94 cm), were observed in the auxin-free medium. These above variations were statistically different ($P \leq 0.05$). The results indicated that plant growth regulators proved their essential role in promoting multiple shoots. The effect of a combination of BAP and IAA (1.0 mg/L and 0.2 mg/L, respectively) was more effective in shoot multiplication in *R. glutinosa* than BAP as used individually (Table 2 and Table 3). Auxin and cytokinin in combination were found to create a synergistic action on shoot regeneration and multiplication from plant tissues in some plant species, such as *Ruta graveolens* (Faisal et al. 2018) and *Moringa oleifera* (Gupta et al. 2020). Similar results were also observed in an earlier study by (Thi et al. 2012) when the highest shoot regeneration frequency (92.23%) and shoot number per explant (4.03 shoots) were obtained on MS medium supplemented with BAP (4 mg/L), and NAA (0.1 mg/L) solidified with Gelrite (4 g/L). Since both auxin and cytokinin directly regulate the growth and development of plants, therefore, a species-specific balance between them is required. For instance, high concentrations of cytokinin combined with low concentrations of auxins synergistically promote the cell division and in vitro shoot multiplication (Piątczak et al. 2014a). The supplementation of PVP was supposed to reduce phenolic oxidation and media browning by binding to phenols and therefore prevents oxidation. The present study's results showed that this type of antioxidant was effective on promoting shoot multiplication, as observed in other studies by Getnet (2017), Podwyszyńska et al. (2017), and Srikanth et al. (2019).

In vitro rooting in *R. glutinosa*

In rooting experiments, PVP was also supplemented to the media to eliminate browning and abnormal morphological characteristics of plantlets caused by phenolic acids and other toxins from excised explants. Each shoots induced in ½ MS medium with 30 g/L sucrose, 7 g/L agarose, pH 5.8, 1 g/L PVP, and with either NAA or IAA. Root induction was observed after three weeks. The results presented in Table 4 showed that the addition of growth regulators and PVP positively affected to the rooting of *R. glutinosa in vitro* shoots. Plantlets were not observed with any signs of browning or abnormal appearance. Optimal rooting was obtained in culture media supplemented with 0.5 mg/L IAA (Fig. 2). Planlets developed in this optimal medium were healthy, formed 26.78 roots with an average of 1.76 cm in length and 4.05 cm in plant height. These results are consistent and corroborated with the findings of an earlier work by Zamir et al. (2017) and Jiménez-Mariña et al. (2019), who demonstrated that IAA at 0.5 mg/L was the most effective exogenous auxin in triggering rooting

Table 4 Comparison of *in vitro* rooting in *R. glutinosa* after a 4-week culture by the type and concentration of auxin used with PVP

Auxin (mg/L)	Number of rooting plantlets	Number of roots per plantlet	Root length (cm)	Plant height (cm)
Control (growth regulator-free)	43[b]	10.37[f]	1.36[d]	3.52[d]
NAA 0.3	45[a]	22.24[e]	1.59[c]	3.33[e]
NAA 0.5	45[a]	22.60[e]	1.74[b]	4.25[a]
NAA 1.0	45[a]	23.33[d]	2.09[a]	3.60[d]
NAA 1.5	45[a]	22.00[e]	2.11[a]	3.82[c]
IAA 0.3	45[a]	24.32[c]	1.52[c]	3.21[f]
IAA 0.5	45[a]	26.78[a]	1.76[b]	4.05[b]
IAA 1.0	45[a]	25.68[b]	1.25[e]	3.28[ef]
IAA 1.5	45[a]	24.72[c]	1.14[f]	3.12[f]

The control included half-strength MS + 1 g/L PVP. Different letters (a, b, c) indicated significant differences between media ($P \leq 0.05$).

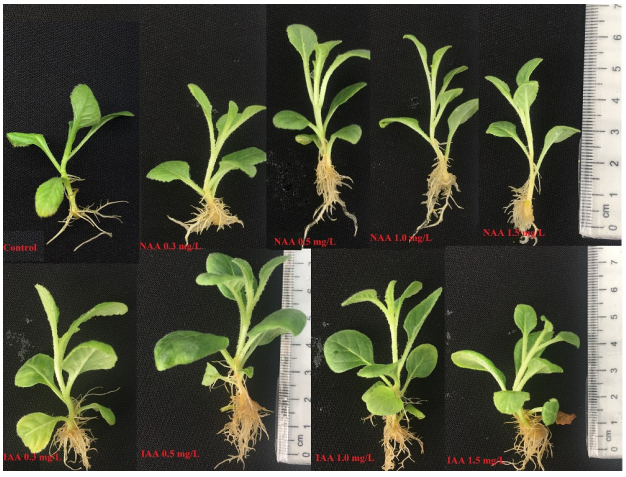

Fig. 2 Root induction from shoots cultured on half-strength MS containing various concentrations of NAA and IAA supplemented with 1 g/L PVP

from *in vitro* induced shoots. Similar results regarding the effect of IAA on *R. glutinosa* rooting performance were also confirmed by Piątczak et al. 2014b). Although plantlets treated with NAA produced relatively high growth parameters, the quality of roots were unhealthy and subsequently affected the later stages of plant development. Auxin-containing media has normally been used to induce rooting from *in vitro* shoots but have found to be differently effective in various species. For example, Cuenca and Amo-Marco (2000) suggested that, the addition of the auxins IAA or IBA did not improve the rooting while the supplementation of NAA showed an inhibitory effect. In contrast, Hunková and Gajdošová (2019) found that NAA was superior for rooting compared to IBA and IAA in *Amelanchier alnifolia.*

Acclimatization of *R. glutinosa* seedlings

Nutrients are the most important factor when transplanting plantlets from *in vitro* media to *ex vitro* environments. In the present study, five different substrates were assessed for the first time, their effect on micro-propagated plantlet growth under greenhouse conditions. The results demonstrated the effectiveness of biofertilizers with the addition of microbial inoculants (included in COSS and COP) over the sand and soil substrates, individually. After four weeks of acclimatization, in treatments with the COSS, *R. glutinosa* seedlings showed the highest rate of survival (88%) and the best vegetative growth performance with seven leaves per seedling, 3.32 cm in leaf length, and 8.26 cm in plant height, followed by COP (Fig. 3). Biofertilizer-free substrates, including RH, ASS, and ASRH promoted a much lower rate of survival and plant growth of *R. glutinosa* seedlings (Table 5). These differences were statistically different ($p \leq 0,05$). The less effective substrates (without organic biofertilizers) probably provided an insufficient level of water and minerals for plant growth in *ex vitro* conditions. The role of compost supplemented with beneficial microbes was confirmed by earlier studies as significantly increased the percentage of survival rate and promoted seedling growth in acclimatized conditions (Abohatem 2011; Barje et al. 2016; Schultz 2001). Their used composts not only increased the soil fertility by promoting the availability of N, P, K, and organic matter amount but also eliminate sodium (an undesirable element) (Sarwar et al. 2008). As a result, the addition of fertilizer is not necessary for the healthy growth of plants. The combination of compost and additional microbial inoculants was confirmed to improve plant growth by increase nutrition supply in many species such as *Argania spinosa* (Mrabet et al. 2014) *Trifolium alexandrium* (Jan et al. 2014). Some other studies also found the correlation between biomass production and compost rate, which were from 5% to 75% to improve soil fertility (Akhzari et al. 2015; Prayudyaningsih and Sari 2016). In the present ex-

Table 5 Comparison of survival rate and growth of plantlets by the acclimatization medium used

Acclimatization medium	Survival rate (%)	Average number of leaves per plant	Leaf length (cm)	Plant height (cm)
50% alluvium soil + 50% sand	51.66e	4.95e	2.18e	6.81e
50% alluvium soil + 50% rice husk	54.33d	5.17d	2.32d	7.25d
Compost : soil + sand (3:2)*	74.67b	5.61c	2.65c	7.56c
100% rice husk	65.00c	6.27b	3.08b	8.00b
90% Compost + 10% perlite	88.00a	7.00a	3.32a	8.26a

*Mixture of organic biofertilizer (compost): a mixture of soil and sand (3:2), topped with a 3 ~ 4 cm layer of sand. The organic biofertilizer was based on chicken manure, sawdust, and coconut peat in a ratio of 1:4:4 (v/v) with the addition of microbial inoculants (CP, Thailand). Different letters (a, b, c) indicate significant differences between media ($P \leq 0.05$).

Fig. 3 Growth of plants after 4 weeks of acclimatization in containers containing 90% compost + 10% perlite (COP)

periment, the most effective substrate for high survival rate and seedling growth appeared to be BP. This compost mixture based on 90% compost (organic fertilizer: chicken manure, sawdust, and coconut peat, in a ratio of 1: 4: 4 (v/v), with additional microbial inoculants (CP, Thailand)) and 10% perlite.

Conclusion

The present study assessed for the first time, the effect of principal factors involved in a complete process of *R. glutinosa* micropropagation from shoot regeneration from excised root segments, shoot multiplication, root induction, and seedling acclimatization. The results revealed that MS medium with 30 g/L sucrose, IAA 0.2 mg/L in combination with 1.0 mg/L BAP containing 1 g/L PVP, and 0.5 mg/L IAA with 1mg/L PVP were found to be optimal *in vitro* regulators for primary shooting, multiple shooting, and rooting, respectively. The compost enriched with microbial inoculants (CP, Thailand) and perlite enhanced the seedling growth compared with organic biofertilizer-free substrates (with soil and sand). The result also indicated that PVP clearly played an important role in protecting in vitro tissues from autotoxins released from the excised explants. These findings constitute support for field experiments for reliable and efficient production of *R. glutinosa* crops.

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