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ORIGINAL ARTICLE

Efficient transgenic plantlet regeneration from hairy roots via somatic embryogenesis and hardening plantlets of *Panax vietnamensis* by iron nanoparticles-supplied culture

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Abstract

In this study, the plantlet regeneration of *Panax vietnamensis* via somatic embryogenesis derived from hairy root callus was investigated. The results showed that the percentage of callus-induced hairy roots was 100% on SH medium supplemented with 0.5 mg/L NAA combined with 2.0 mg/L BA and 30 g/L sucrose after 6 weeks of culture. Callus was transferred to SH medium supplemented with 1.0 mg/L 2.4-D combined with 2.0 mg/L BA to induce somatic embryogenesis. The percentage of somatic embryogenesis callus was 100%, and the average number of embryos per sample was 63.7 embryos after 6 weeks of culture. The somatic embryos were then transferred to the plant growth regulators-free SH medium for the maturation. They developed progressively through the globular, heart, torpedo, cotyledon stages, and finally formed plantlets. PCR analysis revealed that plantlets derived from hairy roots retained the Ri T-DNA. The morphology of these plantlets were not different from the non-transformed plantlets as a control, but the root growth was better. Adding iron nanoparticles to the culture medium had improved the in vitro rhizome proliferation and growth of plantlets derived from hairy root, which supported plantlets survival when planted in the soil. This is the first report of transgenic plantlets regeneration from hairy roots in *P. vietnamensis* that shows the possibility of regenerating transgenic plantlets derived from hairy roots via somatic embryogenesis in high valuable medicinal plants.

Key Message

Plantlet regeneration of Panax vietnamensis via somatic embryogenesis derived from hairy root callus Iron nanoparticles in culture medium improved the in vitro rhizome proliferation and growth of plantlets.

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Introduction

Panax vietnamensis, an endemic *Panax* species of Vietnam, is a well known Vietnamese ginseng rich in pharmaceutical compounds. It is used to support for anti-depression, anti-stress, anti-oxidant treatments, and to enhance immune system, etc. Saponin triterpenoid compounds, which are represented as G-Rb_1, G-Rg_1 and MR_2 are the main effective group of compounds in this species. Many saponin compounds of *P. vietnamensis* are common to *P. ginseng*, such as protopanaxadiol and protopanaxatriol saponins, however, high concentration in this species. Moreover, high concentration of MR_2 is also a characteristic of *P. vietnamensis*. In addition, *P. vietnamensis* contained the highest dammaran-frame saponin (12–15%) and saponin content among *Panax* genus. With these special features, *P. vietnamensis* is one of the most valuable species in Vietnam (Dong et al. 2007). However, the supply of *P. vietnamensis* is very limited due to its slow growth (it takes more than 5 years to harvest) and narrow distribution (mainly in Ngoc Linh Mountain area, Vietnam). Therefore, researchers are very interested in studies on propagation and biomass of this species.

Hairy root is a plant disease induced by *Agrobacterium rhizogenes* (Sharafi et al. 2014). It is used as a potential tool for the plantlet large-scale production of useful products or secondary metabolites because of its rapid growth rate, genetic stability, and ability to synthesize secondary metabolites (Shanks and Morgan 1999; Giri et al. 2001). To date, plant regeneration from hairy roots has been reported (Uozumi et al. 1996; Choi et al. 2004; He-Ping et al. 2011; Wu et al. 2012). Hairy root-derived plants are usually real transgenic because of the ability to retain the Ri T-DNA in their genome (Maknight et al. 1987; Gunjan et al. 2013). In this study, a regeneration protocol of *P. vietnamensis* from hairy roots via somatic embryogenesis was established. The protocol described here can be used as a basis for the future development of commercial-scale production of regenerated plants. Moreover, the use of hairy root offers the opportunity to introduce foreign genes into plant genomes that leads to an improvement in plant properties such as increased stress tolerance to arsenic, cadmium, and artemisinin (Pandey et al. 2021) or increased the ability to synthesize secondary compounds in plants (Xing et al. 2018).

Many studies of hairy root cultures have been reported on Panax ginseng (Inomata et al. 1993; Hwang et al. 1999; Yang and Choi 2000; Jeong et al. 2002; Woo et al. 2004), Panax hybrid (*P. ginseng* × *P. quinquefolium*) (Washida et al. 1998), and *P. quinquefolium* (Kochan et al. 2012, 2013). The hairy roots of *P. ginseng* synthesize the same saponins as those of the native root and up to twice as much as ordinary cultured roots (Yoshika and Furuya 1987). In 2017, Nhut et al. have successfully established a procedure for *A. rhizogenes*-mediated transformation of *P. vietnamensis* (Nhut et al. 2017a, b). Furthermore, the total saponin content of *P. vietnamensis* hairy roots was up to 70% of that of rhizomes from 6-year-old plants and the growth rate of hairy roots was much higher than that of field-cultivated ginseng producing 60 g of rhizome in 5 years (Ha et al. 2016).

In addition, effect of iron nanoparticles on the in vitro rhizome proliferation and growth of plantlets derived from hairy root of *P. vietnamensis* was also investigated in the present study. Nanomaterials are microscopic materials with a size range of 1–100 nm. Because of their small size, when exposed by roots, nanomaterials can easily migrate into the plant by symplastic or apoplastic pathways (Rico et al. 2011; Zhai et al. 2014; Tombuloglu et al. 2019a). In plants, iron is an indispensable element for many metabolic processes such as photosynthesis, respiration, and DNA synthesis (Vigani et al. 2013; Tripathi et al. 2018). Therefore, adding iron nanoparticles to the culture medium will effectively supply iron ions to the plantlets as an alternative to the traditional method, which iron is added to the culture medium in a chelate iron (i.e., Fe-EDTA).

Materials and methods

Plant material

The hairy root line HR2 which was provided by the Department of Molecular Biology and Plant Breeding, Tay Nguyen Institute for Scientific Research, Vietnam was used as the initial culture material in this study. This hairy root line derived from the infection of *P. vietnamensis* callus with *A.rhizogenes* strain ATCC 15834 that was reported in our previous study (Nhut et al. 2017a, b).

Medium culture

SH (Schenk and Hildebrandt 1972) medium supplemented with 30 g/L sucrose, 8 g/L agar was used in this study. Different plant growth regulators (PGRs) were added to the culture medium depending on the different experimental stages. All media were adjusted to pH 5.8 before autoclaving at 121 °C and 1 atm for 15 min.

Callus induction

The hairy root line HR2 was cultured on SH medium supplemented with 30 g/L sucrose, 8 g/L agar to proliferation

for 12 weeks of culture (Fig. 1A). Then, the hairy roots with a diameter of 0.1 cm were cut into segments with a length of 1.0 cm and were cultured on SH medium supplemented with PGRs (NAA combined with BA or KIN or Adenin), 30 g/L sucrose, 8 g/L agar to induce callus formation for 6 weeks.

Somatic embryogenesis and regeneration of plantlets from callus derived from hairy roots

Callus clusters (1.0 g fresh weight) derived from hairy roots line HR2 were cultured on SH medium supplemented with PGRs (BA, KIN, Adenine or BA combined with NAA, 2.4-D), 30 g/L sucrose, 8 g/L agar to induce somatic embryogenesis for 6 weeks. The somatic embryos were then transferred to the PGRs-free SH medium for the maturation of somatic embryos and plantlets regeneration. Plantlets with a height of 2.0 cm regenerated from somatic embryos were separated and transferred to new SH medium for continued growth.

PCR analysis

Total DNA was extracted from plantlets by cetyltrimethyl ammonium bromide (CTAB) method followed Doyle (1987). The DNA samples were then kept at − 20 °C.

Integration of the *rol A*, *B*, *C* and *aux1* genes in the genome of plantlet derived from hairy root of *P. vietnamensis* was determined by PCR amplification with gene-specific primers (Table 1). Plasmid DNA from *A. rhizogenes* strain

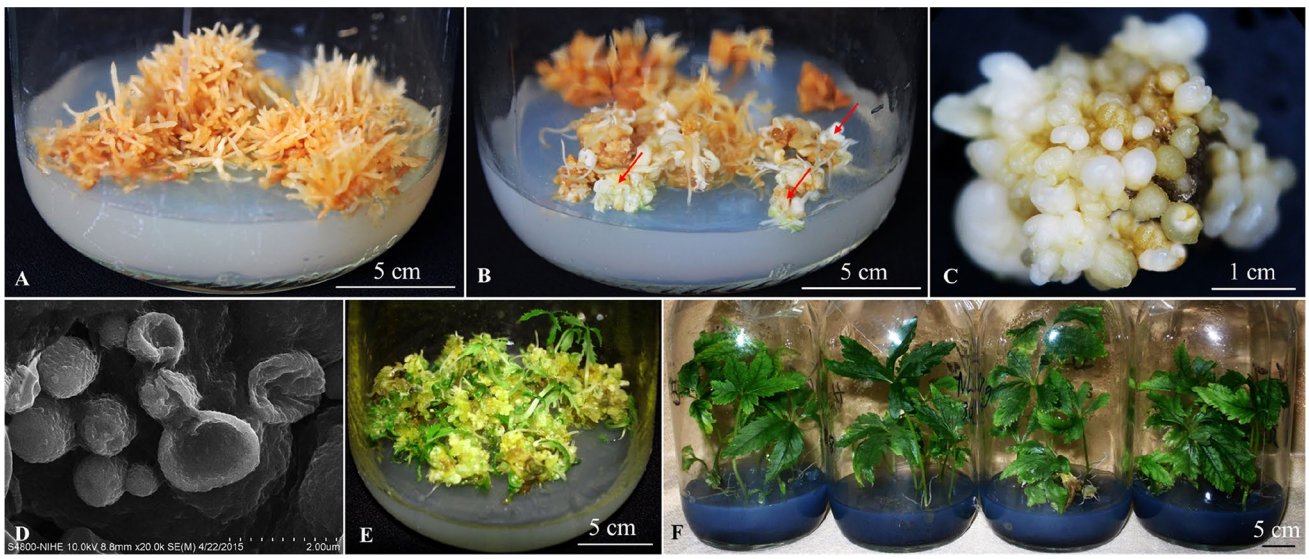

Fig. 1 Somatic embryogenesis, plant regeneration from hairy roots and hardening *P. vietnamensis* plantlets on medium containing iron nanoparticles. **A** 12 week-old hairy roots; **B** Somatic embryo from callus (arrows); **C** Somatic embryo; **D** Globular embryo was taken by scanning electron microscope (SEM); **E** Plantlets drived from somatic embryos; **F** 12 week-old hardened plantlets on medium containing iron nanoparticles

Table 1 Nucleotide sequence of the PCR primers used for detection of T-DNA integration in plantlet derived from hairy root of *P. vietnamensis*

Primer name	Nucleotide sequence	Product length (bp)	T_m (°C)	References
rolA for	5′-GTTAGGCGTGCAAAGGCCAAG-3′	239	60	Zdravković-Korać et al. (2003)
rolA rev	5′-TGCGTATTAATCCCGTAGGTC-3′			
rolB for	5′-AAAGTATGCTACCATTCCCCA-3′	393	61	Christey and Braun (2005)
rolB rev	5′- CCCATAAGCCACGACATCATA-3′			
rolC for	5′-CATTAGCCGATTGCAAACTTG-3′	600	58	Sevón et al. (1997)
rolC rev	5′-ATGGCTGAAGACGACCTG-3′			
aux1 for	5′-CCAAGCTTGTCAGAAAACTTCAGGG-3′	900	61	Alagarsamy et al. (2018)
aux1 rev	5′-CCGGATCCAATACCCAGCGCTTT-3′			
virD for	5′-ATGCCCGATCGAGCTCAAG-3′	338	62	Torkamani et al. (2014)
virD rev	5′-GACCCAAACATCTCGGCTG-3′			

ATCC 15834 was used as a positive control, and the non-transformed plantlet cultured in vitro was used as a negative control. The absence of residual *A. rhizogenes* was confirmed by PCR detection of *virD2* gene which is outside the T-DNA of Ri-plasmid with specific primers were designed according to Torkamani et al. (2014) (Table 1).

PCR reactions were performed under the following thermo cycle conditions: initial denaturation at 95 °C for 1 min, 35 cycles of denaturation at 95 °C (30 s), annealing at 60 °C (30 s) for *rolA*, 61 °C (30 s) for *rolB*, 58 °C (30 s) for *rolC*, 61 °C (30 s) for *aux1*, 62 °C (30 s) for *virD* genes, extension at 72 °C (30 s), and a final extension at 72 °C for 10 min. Amplification products were analyzed on agarose gels.

Enhance transformed plantlets growth and rhizome proliferation by supplement with iron nanoparticles

Transformed plantlets derived from hairy roots with a height of 2.0 cm were cultured on SH medium supplemented with 30 g/L sucrose and 1 g/L active charcoal. To improve the in vitro rhizome proliferation and growth of plantlets, iron nanoparticles (FeNPs) was added to the culture medium at different concentrations of 0.7, 1.4, 2.8, 5.6, 11.2 mg/L. The control was the medium SH without FeNPs. FeNPs with a size of 20–60 nm was provided by the Institute of Enviromental Technology (VAST, Hanoi, Vietnam) and FeNPs was synthesized by chemical method (Ngo et al. 2014). After 12 weeks, rooting rate (%), number of roots, diameter of rhizome (cm), rhizome length (cm), plantlet height (cm), fresh weight (g), chlorophyll contents in leaves-SPAD (nmol/cm^2) were be recorded.

The scanning electron microscopy (SEM)

The embryos clusters were separated from the explant and incubated overnight after soaked in paraformaldehyde 4% solution at 4 °C. Next, the samples were rinsed three times in 25 mM sodium phosphate (pH 7) before dehydration by soaking in ethanol at the concentrations 10% (30 min), 30% (30 min), 50% (30 min), 65% (30 min), 75% (30 min), 90% (30 min), 95% (30 min), absolute ethanol (30 min, triplicates), absolute ethanol to incubate overnight at 4℃. The sample was processed to critical dryness via carbon dioxide, mounted on aluminum substrates and coated with a 60 nm palladium layer. Microscopic image was recorded with a scanning electron microscope (FE SEM S4800).

Culture conditions

The cultures were maintained in light at an intensity of 40–45 µmol.m^{-2} s^{-1} under a 12 h photoperiod at a temperature of 24 ± 2 °C.

Statistical analysis

Each experiment was repeated three times. All data were analyzed statistically using a statistical software SPSS (version 16.0). Significant differences among the treatments were determined using the LSD's multiple range test at $P < 0.05$ (Duncan 1955). The results were expressed as the mean of repeated experiments.

Results

Callus induction from hairy roots of P. vietnamensis

The addition of different PGRs during in vitro culture could prominently affect callus induction and production (Ashokhan et al. 2020). In this study, the PGRs NAA, BA, KIN and Adenine were used to induce the formation of callus of hairy roots of *P. vietnamensis*. The result showed that the initiation of callus formation did not occur in the control (free-PGRs medium SH). Meanwhile, callus induction was observed in all cultures supplemented with 0.5 mg/L NAA in combination with BA or KIN or Adenin. The callus formation rate increased with increasing concentration of BA, KIN and Adenine from 0.5 to 2.5 mg/L. It was also observed that the fresh weight of explant increased with increasing concentration of BA, KIN and Adenine from 0.5 to 2.0 mg/L, but decreased at concentration of 2.5 mg/L. The maximum rate of callus formation (100%) and explant fresh weight (4.15 g) were recorded on the SH medium supplemented with 2.0 mg/L BA in combination with 0.5 mg/L NAA (Table 2).

In plant cells, auxins and cytokinins are involved in regulating cell proliferation, differentiation and dedifferentiation (Ashokhan et al. 2020), which explains the formation of callus from the hairy roots of *P.vietnamensis* in this study. Cytokinin, such as BA and KIN, in combination with auxins were often used to promote callus initiation in plant species (Chai and Mariam 1998). The effect of combinations of PGRs on callus induction and proliferation have been reported in many previous studies such as a combination of 2.4-D with KIN in *Bunium persicum* (Valizaden et al. 2007), *Withania somnifera* (Chakraborty et al. 2013); or NAA combinated with BA in *Atropa acuminata* Royal ex Lindl (Dar et al. 2021); or 2.4-D combinated with BA in *Solanum tuberosum* L. (Elaleem et al. 2009), *Pinus koraiensis* (Gao et al. 2021); or NAA combinated with TDZ in Turkish *Crocus* species (Verma et al. 2016). The combination of auxin and cytokinin was also used for embryonic callus maintenance and proliferation of many species (Zuzana et al. 2011; Klimaszewska et al. 2016; Nunes et al. 2018). Those results are in agreement with the findings of our study, in which media supplemented with auxin NAA combination with cytokinin

Table 2 Effect of plant growth regulators (PGRs) on the callus induction from hairy roots after 6 weeks of culture

Concentrations of PGRs (mg/L)				Callus formation rate (%)	Explant fresh weight (g)
NAA	BA	KIN	Adenine		
–	–	–	–	0^{k*}	1.12^{k}
0.5	0.5	–	–	55.3^{f}	2.63^{i}
0.5	1.0	–	–	68.7^{e}	3.39^{f}
0.5	1.5	–	–	97.0^{b}	3.96^{c}
0.5	**2.0**	–	–	**100.0^{a}**	**4.15^{a}**
0.5	2.5	–	–	100.0^{a}	4.12^{ab}
0.5	–	0.5	–	25.3^{i}	1.61^{j}
0.5	–	1.0	–	48.0^{g}	2.77^{h}
0.5	–	1.5	–	86.3^{d}	3.93^{c}
0.5	–	2.0	–	95.7^{b}	3.49^{e}
0.5	–	2.5	–	100.0^{a}	3.42^{f}
0.5	–	–	0.5	5.3^{j}	1.62^{j}
0.5	–	–	1.0	45.3^{h}	3.07^{g}
0.5	–	–	1.5	86.0^{d}	3.62^{d}
0.5	–	–	2.0	92.7^{c}	4.07^{b}
0.5	–	–	2.5	100.0^{a}	4.07^{b}

*Different letters (a, b, …) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test)

Table 3 Effect of BA, KIN and Adenine on somatic embryogenesis from callus derived from hairy roots after 6 weeks of culture

Concentrations of PGRs (mg/L)			Somatic embryo formation rate (%)	Number of somatic embryos
BA	KIN	Adenine		
0.0	–	–	0^{i*}	0^{j}
0.5	–	–	45.3^{h}	15.3^{f}
1.0	–	–	83.3^{e}	23.0^{c}
1.5	–	–	96.3^{b}	25.0^{b}
2.0	–	–	100.0^{a}	30.0^{a}
2.5	–	–	100.0^{a}	28.7^{a}
–	0.5	–	75.3^{f}	4.7^{i}
–	1.0	–	86.0^{d}	11.0^{gh}
–	1.5	–	97.3^{b}	16.0^{f}
–	2.0	–	100.0^{a}	19.7^{de}
–	2.5	–	100^{a}	21.0^{d}
–	–	0.5	64.0^{g}	9.3^{h}
–	–	1.0	77.0^{f}	12.3^{g}
–	–	1.5	87.3^{d}	19.0^{e}
–	–	2.0	96.7^{b}	25.3^{b}
–	–	2.5	93.3^{c}	26.7^{b}

*Different letters (a, b, …) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test)

(BA, KIN, Adenin) induced formation of a compact, nodular embryogenic structure of the callus (Fig. 1B).

Somatic embryogenesis from callus derived from hairy roots

The cytokinins BA, KIN and Adenine were used singly to induce somatic embryogenesis from callus derived from hairy roots of *P. vietnamensis*. Our results revealed that the somatic embryogenesis was observed in the cultures except the control (Table 3). The rate of somatic embryo formation reached 100% on media supplemented with 2.0–2.5 mg/L BA or KIN, and the number of somatic embryos was the highest (30 embryos) at the concentration of 2.0 mg/L BA. Meanwhile, the maximum rate of somatic embryo formation on the medium supplemented with Adenine was 96.7% at the concentration of 2.0 mg/L (Table 3). Adenine is required during the cell division process and protein synthesis that take place during the somatic embryo development (Verma et al. 2016). However, based on the results, it was indicated that the ability of somatic embryogenesis of Adenine was lower than BA and KIN in *P. vietnamensis*.

Auxin and cytokinin are the key factors to determine the embryogenic response because of their participation in the cycle regulation and division of cell (Francis et al. 2001), and that a particular ratio of auxin and cytokinin in culture medium may be necessary for the induction of somatic embryogenesis. Different cultivars require different PGRs in the culture medium, and this requirement may need to be individually tested (Chaudhury and Qu 2000). In the present study, to improve the frequency of somatic embryogenesis, the combination of BA (at a concentration of 2.0 mg/L) with auxins NAA or 2.4-D was carried out. The results showed that the rate of somatic embryo formation was 100%, and the number of somatic embryos was 63.7 on the medium supplemented with 2.0 mg/L BA in combined with 1.0 mg/L 2.4-D (Table 4); these were higher than medium with supplemented only 2.0 mg/L BA (100% and 30 embyos, respectively) (Table 3). This data indicated that medium with a combination of BA and 2.4-D was more effective in somatic embryogenesis compared to single BA. Media with the combination of BA and NAA was less effective in somatic embryogenesis (particularly number of embryos) compared to the combination of BA and 2.4-D. On the media with the combination of BA and NAA, the highest number of somatic embryos was only 22.7 at the concentration of 2.0 mg/L BA in combined with 1.5 mg/L NAA (Table 4). Therefore, SH medium supplemented with 2.0 mg/L BA in combined with 1.0 mg/L 2.4-D was identified as the most optimum medium for the somatic embryogenesis of *P. vietnamensis*. This outcome was also supported by Gao et al. 2021 that suggested on the media contained 2.4-D + BA, embryogenic callus grew well and callus had more early somatic embryos on the surface in *Pinus koraiensis*. Morphological observations showed that most of the somatic embryos which were visible

Table 4 Effect of NAA or 2,4-D combined with BA on somatic embryogenesis from callus derived from hairy roots after 6 weeks of culture

Concentrations of PGRs (mg/L)			Somatic embryo formation rate (%)	Number of somatic embryos
NAA	2,4-D	BA		
–	–	–	0[f*]	0[e]
0.5	–	2.0	75.0[e]	12.7[d]
1.0	–	2.0	88.0[d]	18.0[c]
1.5	–	2.0	87.3[d]	22.7[b]
2.0	–	2.0	100.0[a]	12.3[d]
2.5		2.0	100.0[a]	11.3[d]
–	0.2	2.0	88.3[d]	21.0[bc]
–	0.5	2.0	95.3[c]	32.7[c]
–	0.7	2.0	97.6[b]	48.3[b]
–	**1.0**	**2.0**	**100.0[a]**	**63.7[a]**
–	1.5	2.0	100.0[a]	61.0[a]

*Different letters (a, b, …) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test)

around the surface of the callus in the present study were globular shape.

Somatic embryos maturation and plantlets regeneration

Somatic embryos induced by callus derived from hairy roots were inoculated on the PGRs-free SH medium for the maturation and germination of somatic embryos (Fig. 1C, D). Because somatic embryos have a bipolar structure with both apical and basal meristem regions that are capable of forming shoot and root, respectively (Bhatia and Bera 2015), they developed into plantlets on this medium. The development of somatic embryos occured sequentially globular, heart, torpedo, cotyledon, and finally plantlet stages.

After 12 weeks of culture on the PGRs-free SH medium, plantlets with a height of 2.0 cm were separated and transferred to a fresh SH medium for subsequent growth. The morphology of these plantlets was not significantly different from the non-transformed plantlets but had more lateral roots (Fig. 1E). This is characteristic of the Ri syndrome because the plantlets derived from hairy roots may retain the Ri T-DNA, which will be confirmed by PCR analysis. On the other hand, this is also an advantage that can improve the acclimation of plantlets when they are planted in the soil because of their root system development. Similar to our study, Cui et al. (2020) reported that cotton plantlets derived from hairy roots showed Ri syndrome such emerged numerous adventitious roots, and morphologically similar to the non-transformed cotton plant. Beside that, plantlet regeneration from hairy root cultures via somatic embryogenesis has previously been reported for several plant species, including *Cucurbita pepo* L. (Balen et al. 2004), *Panax ginseng* C. A. Mey. (Wang et al. 2008), *Beta vulgaris* L. (Ninkovíc et al. 2010), *Gentiana macrophylla* Pall. (Wu et al. 2011), *Salvia miltiorrhiza* Bunge (Wang et al. 2013), and *Lopezia racemose* (Vargas-Morales et al. 2022). Moreover, plantlet regeneration directly from hairy root cultures has been reported several times, and may be dependent on the photoperiod. *Lotus corniculatus* L. (Petit et al. 1987), *Ophiorrhiza pumila* Champ. Ex Benth. (Watase et al. 2004), *Plumbago indica* L. (Gangopadhyay et al. 2010), and *Rauvolfia serpentina* (L.) Benth. ex. Kurz (Mehrotra et al. 2013) regenerated via direct organogenesis when the hairy roots. Through present study, we first time report on the development of an efficient and reliable *P. vietnamensis* plantlets regeneration method from hairy roots.

Molecular analysis of plantlets drived from hairy roots

A. rhizogenes strain ATCC 15834 belong to the group of agropine-type strains, have two T-DNA regions (T_L-DNA and T_R-DNA) on Ri-plasmid. These regions can be independently transferred to the genome of infected plant cell. The *rol A, B, C, D* genes localized in T_L-DNA are responsible for the induction and phenotype of hairy root, and *aux1,2* genes localized in T_R-DNA are responsible for the auxin biosynthesis (Nemoto et al. 2009; Nilsson and Olsson 1997). In present sutdy, in order to determine the presence of Ri T-DNA in plantlets derived from hairy roots of *P. vietnamensis*, total DNA extracted of plantlets derived from hairy roots was used as templates for PCR amplifying *rol A, B, C* and *aux1* genes. The results showed that the presence of the genes *rolA* (Fig. 2A), *rolB* (Fig. 2B), and *rolC* (Fig. 2C) and the *aux1* genes (Fig. 2A–C) in the plantlet derived from hairy root sample. To verify the absence of residual *A. rhizogenes* infection on plantlets derived from hairy roots, the *virD* gene outside the T-DNA region of Ri-plasmid was also studied. The result revealed the *virD* gene band was not observed in the plantlets derived from hairy roots (Fig. 2A–C). The finding clearly confirmed that the plantlets derived from hairy roots was not contaminated by *A. rhizogenes*. In addition, we also examined the presence of residual *A. rhizogenes* in the culture medium of the plantlets derived from hairy roots. The results also showed that *virD*, *rol A, B, C* and *aux1* gene bands were not observed in the culture medium sample (Fig. 2A–C). This demonstrated that *A. rhizogenes* was completely eliminated from the culture medium. From these results it concluded that plantlets derived from hairy roots retained the Ri T-DNA.

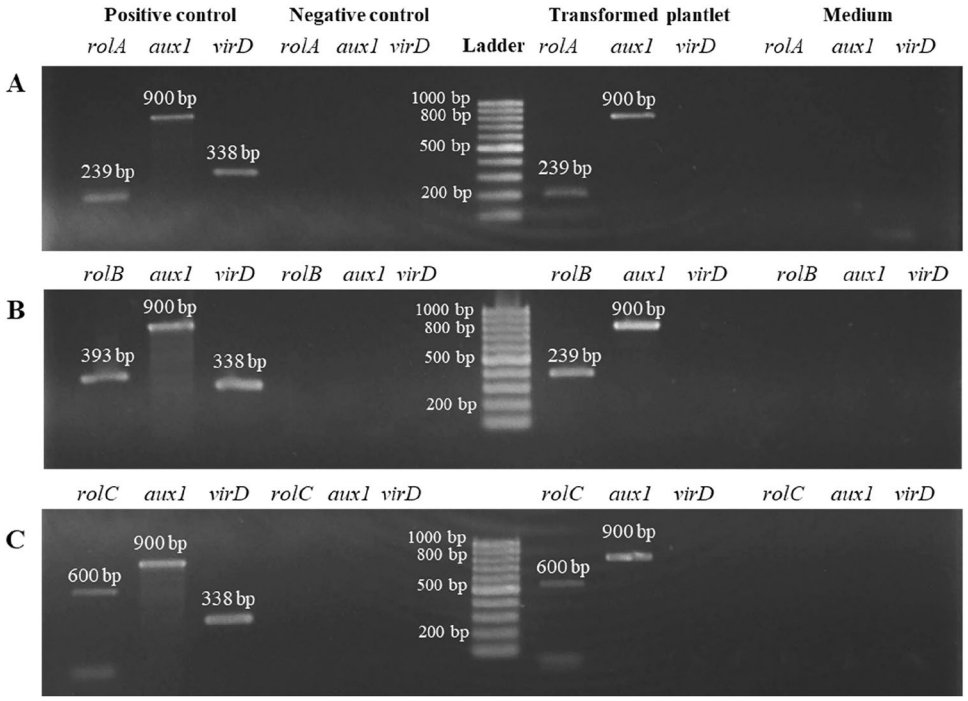

Fig. 2 Detection of integration of the *rol A, B, C* and *aux1* genes in plantlets derived from the hairy root of *P. vietnamensis* transformed by *A. rhizogenes* strain ATCC 15834. **Positive control** *A. rhizogenes* strain ATCC 15834 harboring Ri-plasmid. **Negative control** The non-transformed *P. vietnamensis* cultured in vitro. **Transformed plantlet** Planlet drived from hairy root. **Medium** Culture medium of hairy root. **A–C** Detection of *rol A, B, C* genes

The role of iron nanoparticles on the rhizome proliferation and growth of plantlets derived from hairy roots

After 12 weeks of culture, the results showed that parameters such as the chlorophyll content, fresh weight, rhizome diameter and length increased with increasing concentration of iron nanoparticles (FeNPs) from 0.7 to 5.6 mg/L (Table 5). Iron is an important component of chlorophyll, cytochromes and many vital enzymes, and it plays critical role in metabolic processes such as photosynthesis, respiration, and DNA synthesis (Vigani et al. 2013; Tripathi et al. 2018). In this study, the addition of FeNPs to the culture medium increased the chlorohyll content, this was due to the increased iron content in the plantlets and resulted in the promotion of chlorophyll synthesis. This had been demonstrated in some previous studies that iron oxide nanoparticles supplement increased the iron content of the plant tissues and translocated to the leaves (Pariona et al. 2017; Palmquist et al. 2017; Tombolo et al. 2019a, 2019b). The increased chlorophyll content stimulated the photosynthetic machinery, which led to an increase in plantlets growth and biomass accumulation (Fig. 1F). As a result, plantlet height, plantlet fresh weight, rhizome diameter and length of FeNP-treated plantlets were increased in the present study. Similarly, as in our experiment, Amri et al. 2020 reported that Fe_2O_3 nanomaterials dramatically increased the content of chlorophyll, and it can be attributed to the increased content of iron elements present in the structure of nanomaterials. Therefore, it may have increased photosynthetic activities, plant biomass and growth. As a result, root length, plant height, biomass of wheat were increased and our result was similar to the study have been reported by Martínezballesta et al. (2016).

Table 5 Effect of FeNPs on the rhizome proliferation and growth of plantlets derived from hairy root after 12 weeks of culture

Nano FeNPs (mg/L)	Rooting rate (%)	Number of roots	Diameter of rhizome (cm)	Rhizome length (cm)	Plantlet height (cm)	Fresh weight (g)	Chlorophyll (nmol/cm^2)
0.0	100.0	2.67^{cd*}	0.38^{c}	1.51^{c}	3.93^{d}	0.86^{c}	12.77^{e}
0.7	100.0	3.67^{bc}	0.52^{bc}	1.63^{bc}	5.34^{b}	0.89^{c}	16.50^{d}
1.4	100.0	4.33^{b}	0.58^{b}	1.65^{bc}	5.54^{b}	1.17^{b}	17.80^{c}
2.8	100.0	4.67^{b}	0.60^{b}	1.67^{b}	6.79^{a}	1.20^{b}	19.90^{b}
5.6	100.0	6.00^{a}	0.87^{a}	1.93^{a}	4.59^{c}	1.36^{a}	21.42^{a}
11.2	100.0	2.33^{d}	0.58^{b}	1.6b^{c}	2.91^{e}	0.73^{d}	9.70^{f}

*Different letters (a, b, …) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test)

In micropropagation of *P. vietnamensis*, the transfer of seedlings grown in soil or greenhouses is difficult because of the low survival rate. In present study, FeNPs enhanced the rhizome proliferation and growth of plantlets derived from hairy root. This will improve the acclimatization and increase the survival rate of the plantlets when planted in soil. The maximum number of root (6.0), rhizome diameter (0.87 cm), rhizome length (1.93 cm), plantlets height (4.59 cm) and fresh weight (1.36 g) were recorded on the SH medium supplemented with 5.6 mg/L FeNPs (Table 5). Numerous mechanisms for the uptake of nanoparticles by plant tissues have been suggested. When exposed by roots, nanoparticles can use either symplastic or apoplastic pathways to migrate into the plant (Rico et al. 2011; Zhai et al. 2014; Tombuloglu et al. 2019a). It has been assumed that due to the great surface area, nanoparticles can attach to organic chemicals or to carrier proteins existing in the cell and hence could be interact with them (Xu et al. 2011).

FeNPs enhanced the rhizome proliferation and growth of plantlets, however, FeNPs at concentrations of 11.2 mg/L significantly decreased number of root, rhizome diameter and length, plantlets height and fesh weight of *P. vietnamensis* (Table 5). It is because iron is considered as a plant micronutrient, iron high concentrations inhibited plant growth. Some reports previously indicated that nanoparticles could cause positive or negative effect on root elongation and plant growth (Ma et al. 2011, 2010; Rico et al. 2015; Wang et al. 2011). At high concentration of iron oxide nanoparticles, the cell viability decreased (Marcus et al. 2016). The excess of an increase iron content can disturbed the physiological functions and formed reactive oxygen species (ROS) that could cause damages to the plant cells. Iron may transmit electrons to O_2 to form H_2O_2 or other chemical products, forming the extraordinary reactive OH radical (Konate et al. 2017). The formation and accumulation of OH radical lead to the degradation of pectin polysaccharides in cell walls, which in turn result in cell wall loosening (Schopfer 2001; Kim et al. 2014).

Conclusion

To the best of our knowledge, this is the first report of plantlet regeneration from hairy root of *P. vietnamensis*. Plantlets derived from hairy roots retained the Ri T-DNA, and they have more lateral roots than non-transformed plants. Addition of FeNPs to the culture medium enhanced the rhizome proliferation and growth of plantlets. Our plantlet regeneration system offers many advantages because it not only contributes to the improvement of plantlet survival when planted in soil, but also offers suitable prospects for enhancing the productivity of ginsenosides, a valuable compounds that are concentrated mainly in the roots of this medicinal species.

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Author contributions DTN and TTH conceptualized and designed the study. DTN, HHD and TTH performed experiments. NHH, HTMN, LTD, HTT, HDK, NTNM, DMC, VQL, TTT, DDG, NNK, NVB, CHH, PBN participated in interpretation of data and revision for intellectual content acquired data wrote the manuscript.

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Declarations

Competing interest The authors declare no conflict of interest.

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