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AGROBACTERIUM-MEDIATED TRANSFORMATION OF *PANAX VIETNAMENSIS* HA *ET* GRUSHV.

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SUMMARY

In recent years, the *Agrobacterium*-mediated genetic transformation system has become the most useful method widely used for the introduction of foreign genes into plant cells followed by regeneration of genetically improved plants. *Panax vietnamensis* Ha *et* Grushv. is a highly valued medicinal plant native to Vietnam with limited area of distribution. This report illustrates the possibilities of biotechnology for genetic transformation aimed at establishing an effective production of secondary metabolites in *P. vietnamensis*. In the present investigation, 0.5 cm^2 leaf blades, 1 cm long leaf petioles and 0.5 cm^3 callus clusters were used for the hairy root induction. Results indicated that hairy roots were induced on *P. vietnamensis* callus clusters co-cultivated with *Agrobacterium rhizogenes* strain ATCC15834 at OD_{600} of 0.5 with an infection time of 20 min and a supplementation of 100 μM acetosyringone. PCR amplification of the DNA isolated from the resulting hairy roots was used to confirm the presence of *rol* genes. Compared to *in vitro* rhizome cultures, hairy root cultures appear to be potential for continuous production of valuable secondary metabolites with similar saponin profiles. The protocol described in this study is simple and rapid and therefore, can be used for large-scale experiments for the rapid production of valuable compounds.

Keywords: *Agrobacterium rhizogenes, gene transformation, hairy roots, Panax vietnamensis Ha et Grushv., saponins*

INTRODUCTION

Panax vietnamensis Ha *et* Grushv., a precious medicinal plant, mainly grows on high mountain of Ngoc Linh (Kontum and Quang Nam provinces) at an altitude of about 1,800 m. It was a secret medicinal plant of the Sedang ethnic group and used for the treatment of many serious diseases and enhancing body strength during long journeys on high mountains. According to numerous studies, *P. vietnamensis* has several medical effects such as anti-depressant, anti-aging, anti-cancer activities, memory improvement, liver protection, immune system stimulation, etc. (Nhut *et al*., 2012). A study that examined the chemical constituents in *P. vietnamensis* roots revealed that it contains up to 50 saponins. Moreover, *P. vietnamensis* accumulates saponins at higher levels compared to other species such as *P. ginseng, P. quinquefolium, P. notoginseng* (Duc *et al*., 1994).

P. vietnamensis takes long time to grow, and its ginsenoside level sufficiently accumulates until it reaches four to six years of age. At present, due to the limited supply of natural population, slow growth, and excessively harvesting, this ginseng is listed as one of the endangered species, at high risk of extinction in Vietnam (Vietnam' Red Data Book).

Genetic transformation is one the most widely used method for increasing the production of secondary metabolites in plants. Transformation using *Agrobacterium rhizogenes* of some economically important species to obtain transformed hairy roots have been reported. Since

the intact transformed plants are capable of synthesizing a large quantity of biologically active substances, the potential to metabolically engineer plants to significantly increase the production of these metabolites could be achieved (Georgiev *et al*., 2007; Guillon *et al*., 2006).

A. rhizogenes strains are the most frequently exploited gene transfer agents in a wide variety of plant species (Hooykaas, 1992). *A. rhizogenes*, a gram-negative soil bacterium, transfers DNA from its root inducing (Ri) plasmid into the genome of the infected plant (Chilton *et al*., 1982). Hairy roots induced by Ri plasmid have many advantages over normal roots, as they exhibit a vigorous growth and extensive lateral branching while growing in a simple phytohormone-free medium. The *rol*C gene of *A. rhizogenes* T-DNA plays an essential role in hairy root development and its expression in plants causes significant morphological and biochemical changes (Nilsson *et al*., 1996). Over the years, transformed root cultures from plants have received considerable attention because of their rapid growth rate, biochemical and genetic stability, ease of maintenance and ability to synthesize secondary metabolites (Shanks, Morgan 1999; Giri *et al*., 2001).

In this study, hairy roots were initiated from *P. vietnamensis* explants using *A. rhizogenes* strain ATCC15834. This is the first report on the development and optimization of an efficient transformation procedure for the establishment of transgenic *P. vietnamensis* root cultures using *A. rhizogenes*.

MATERIALS AND METHODS

Plant materials

Leaf blades (cut into 0.5 cm^2 segments), leaf petioles (cut into 1 cm long) and callus clusters (cut into 0.5 cm^3 blocks) of 3-month old *in vitro* plants, cultured on 1/2 Murashige and Skoog (MS; 1962) medium containing 1.0 mg/l BA, 0.5 mg/l NAA, 50 g/l sucrose and 2.0 g/l AC (Nhut *et al*., 2013), were used for the hairy root induction.

Bacterial strains and plasmids

Wild-type strain of *A. rhizogenes* harboring the vector PTN289-GUS and *A. rhizogenes* strain ATCC15834 (obtained from Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Germany) was used for the hairy root induction.

Agrobacterium activation

Bacterial culture was maintained in Yeast Mannitol Broth (YMB) solid medium supplemented with 100 mg/l spectinomycine at 28°C for 48 hours. A single bacterial colony was inoculated in 5 ml of YMB liquid medium supplemented with 100 mg/l spectinomycine and the culture was grown on a rotary shaker at 220 rpm at 28°C for 16 – 18 hours. Subsequently, 45 ml of YMB liquid medium was added to the overnight bacterial culture and grown at 28°C for about 4 – 6 hours until it reached mid-log phase (OD_{600} = 0.5). The bacterial cells were harvested by centrifugation and re-suspended at a cell density OD_{600} of 1.0 in ½SH medium (Schenk, Hildebrandt, 1972).

Transformation procedure: (Four investigations for infection were tested)

(1) Explants were sliced into 0.5 cm^2 segments for leaf blades, 1 cm long for leaf petioles and 0.5 cm^3 blocks for callus clusters in ½SH liquid medium. Explants were dried on sterile filter papers and transferred onto a new plate containing 30 ml of overnight *Agrobacterium* suspension and incubated for 20 min with gentle shaking. The bacterial solution was discarded and the explants were dried on sterile filter paper and placed on SH solid medium for 2 – 3 days under low light intensity (specify the specific flux rate 35 – 40 µmol $m^{-2}s^{-1}$). After three days of inoculation with bacteria, the explants were placed onto new SH plates containing 500 mg/l cefotaxime. The explants were subcultured on new SH antibiotic plates every two weeks until hairy root tips appeared. Hairy root tips (4 – 5 cm) were then transferred to 50 ml flasks containing 10 ml of SH liquid medium and placed on a shaker at 60 rpm and 22 ± 2°C for further analysis.

The selected explant type, which was the most suitable for gene transformation, was used for subsequent experiments.

(2) Explants were infected with *Agrobacterium* suspension of varying OD_{600} (0, 0.3, 0.5, 0.7, 0.9) in order to investigate the effect of bacterial density on transformation efficiency.

(3) Explants were also submerged in the *Agrobacterium* suspension for different time durations (10, 20 and 30 min) in order to investigate

the optimal infection time for transformation process.

(4) Different concentrations of acetosyringone (50, 100, 150 and 200 µM) were added to the infection medium to determine its effect on transformation efficiency.

The percentage of *gus*-positive explants was prepared according to the procedure described by Jefferson (1987). Samples were immediately submerged in *gus* staining solution after harvest and placed under a vacuum for 10 min. The samples were incubated for 8-12 hours in darkness at 37°C. Chlorophyll was removed by submerging the stained tissues in 70% (v/v) ethanol. Plant material was placed on glass slides using 20% chloral (w/v) in 25% glycerol (v/v) for 10 min. *Gus* staining was visualized using a Leica MZ95 stereomicroscope with a color CCD camera. The *gus* staining solution contained 100 mM sodium phosphate buffer (pH 7.0), 10 mM Na_2EDTA, 1 mM $K_3[Fe(CN_6)]$, 1 mM $K_4[Fe(CN_6)]$, 0.5% (v/v) TritonX-100, 20% (v/v) methanol, and 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid (X-gluc).

All experiments were repeated 3 times with 15 explants for each explants type and the mean values are presented.

PCR analyses of transformation

To confirm the transformation of hairy root clones, genomic DNA from hairy roots was isolated according to method described by Khanuja *et al*. (1999). PCR analyses were used to evaluate transformed roots using *rol*C-specific primers (5′ ATGGCTGAAGACGACCTGTGTT 3′ and 5′ TTAGCCGATTGCAAACTTGCAC 3'). The cycling conditions were denaturation at 94ºC for 1 min, annealing at 53ºC for 30 sec and extension at 72ºC for 1 min, with samples being subjected to 30 cycles. The PCR products were analyzed on 1% (w/v) agarose gel stained with ethidium bromide (0.5 µg/ml).

Evaluating transcript level of genes in ginseng hairy root transformants

RNA was isolated from 28-day-old hairy root cultures using Trizol according to the manufacturer's protocol and cleaned up using the RNeasy Mini kit and optional on-column DNase digestion with the RNase-free DNase set from Qiagen. RNA quality and quantity were verified by NanoPhotometer. First-strand cDNA was synthesized from 1 µg total RNA using the ImProm-II™ Reverse Transcription System according to the supplier's instructions. The relative level of auxin transcripts of the transformants was further quantified by real-time RT-PCR using actin as a housekeeping gene. The relative expression of auxin genes was established using REST software. Primers used were *rol*B.F:5' GGAGGATGATAGCAGACTTTGTTCTTC 3'; *rol*B.R: 5' CAGCATGGAGCCAGATAAA CCTATT 3' for *rol*B gene and actin.F: 3' GATGACATGGAAAAGATTTGGCAT 5', actin.R: 3' TGTTGTACGACCACTAGCATACAGG 5' for actin gene.

Saponin analysis

P. vietnamensis in vitro transformed hairy roots were used for saponin analysis. The procedures for saponin extraction, HPLC analysis were described by Zhai *et al*. (2001).

HPLC system: Supelco RP C18 column (250 mm x 4.6 mm; I.D. 5 mm), SPD-M20A-PDA detector. HPLC parameters: Volume injection of 20 ml; flow rate: 0.5 ml/min. Column temperature was held at 25°C.

Sample (0.5 g) was exhaustively extracted in methanol in sonicator (10 ml methanol x 6 times). The extracts were pooled together and concentrated by evaporator to give dried residues, dissolved the residues with 20 ml water and fractionated with ether ethylic and n-butanol, respectively. The ether ethylic fraction was discarded, and the n-butanol was collected and evaporated under vacuum pressure to yield the dried extract. The resulting dried extract was continuously dissolved with mixture of acetonitrile:water solvent (2:1, v/v) and fixed in 5 ml, passed through 0.45 µm membrane, the filtrate was finally injected to HPLC system for quantitative determination of saponins by using calibration curve method.

RESULTS AND DISCUSSION

Effect of explant types on gene transformation efficiency

When performing transformation and regeneration experiments, explant selection is a critical parameter because the different explant sources often have varying potential for transformation (Piqueras *et al*., 2010). In the present study, it can be seen that different explant types

resulted in different transformation efficiency (Fig. 1, Fig. 2). It was observed that callus produced the highest level of *gus* expression (86%) among the three explant types examined. Britton *et al*. (2008) demonstrated that *rolB* is one of the most important genes contributing to hairy root induction and the phenotypes of hairy root are affected by auxin. Therefore, when we used callus as the explant material for gene transformation, there was an accumulation of auxin in callus clusters, and the hairy root induction ability from callus (Fig. 2c) was significantly higher than those from leaf blades or petioles.

In 1997, Giri *et al*. published a paper in which among *A. rhizogenes* strains used, *A. rhizogenes* LBA 9402 and A4 treated on callus resulted in the greatest hairy root induction in *Aconitum heterophyllum* whereas leaf, stem and other explant sources were found to be unsuitable for gene transformation. Several studies have also reported the differential efficiency of various *A. rhizogenes* strains in promoting the formation and growth of hairy roots (Ionkova *et al*., 1997; Vanhala *et al*., 1995). *A. rhizogenes* strain 15834 was among the most effective at promoting hairy root growth and saponin synthesis. In the current study, *A. rhizogenes* strain 15834 used in experiments is not included in the list of biologically active agents and is not considered biological hazard, hence, obtained genetically modified organism is not harmful, and callus was used as the plant material for subsequent experiments.

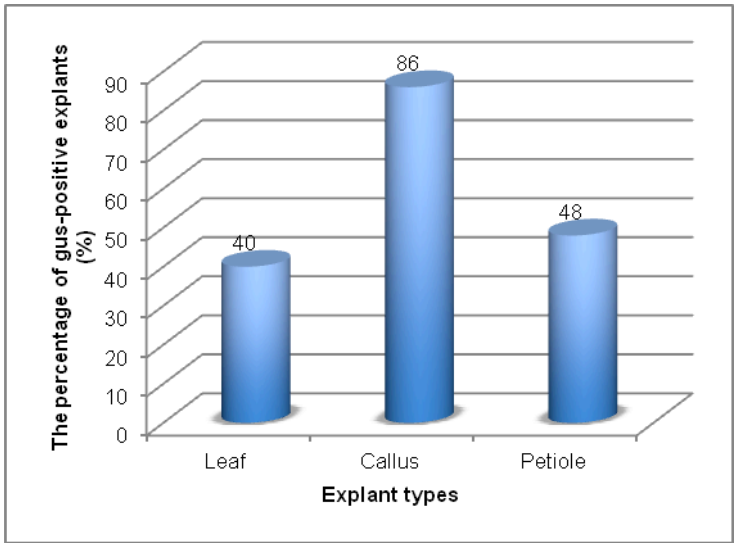

Figure 1. Effect of explant types on gene transformation efficiency.

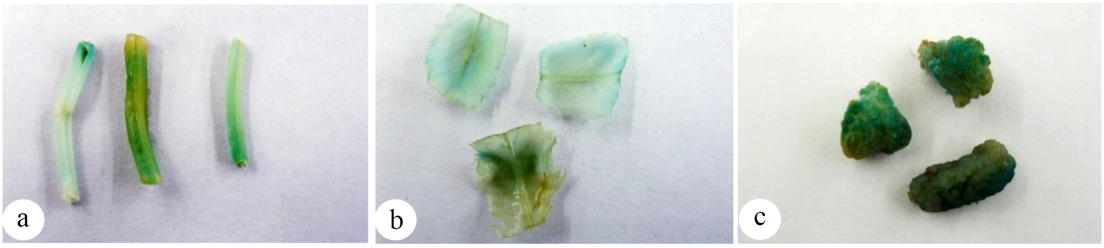

Figure 2. *Gus* expression in various *P. vietnamensis* explants following *A. rhizogenes* ATCC15834 infection. *Gus* expression in infected petioles (a); leaf (b); and callus clusters (c).

Effect of bacterial density on transformation efficiency

Bacterial density and the development of bacteria directly affect the transformation efficiency (Rashid *et al*., 2010). In this study, a correlation between bacterial density and the expression of *gus* was found. After 20 min of dipping the callus clusters in the *Agrobacterium* suspension, the percentage of *gus*-positive callus explants ranged from 25 to 61%, and the highest one was scored at the OD_{600} of 0.5 (Fig. 3). Previous studies reported that the suitable bacterial density for gene transformation is specific for the crop species and the bacterial strains used. For examples, Wahyu *et al.* (2012) found that when using *A. rhizogenes* ATCC15834 at OD_{600} of 0.5 for hairy root induction of *Lycopersicon esculentum* Mill, an induction rate of 33-59% was observed whilst a rate of 70% was obtained in *Pueraria phaseoloides* at OD_{600} of 1.0 (Shi, Kintizios, 2003).

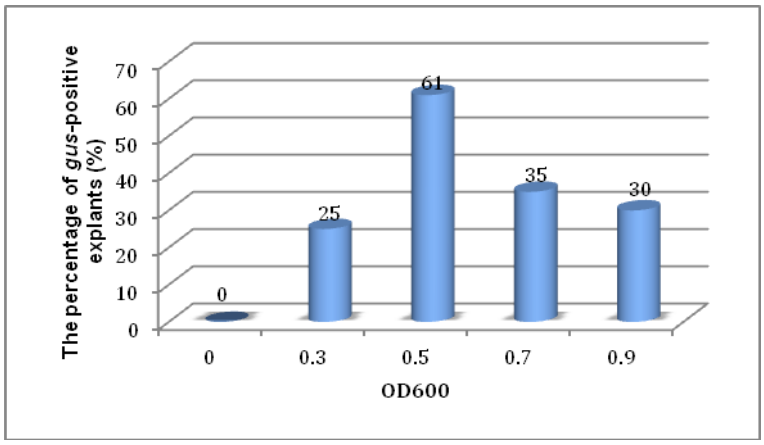

Figure 3. Effect of *A. rhizogenes* concentrations on transformation efficiency.

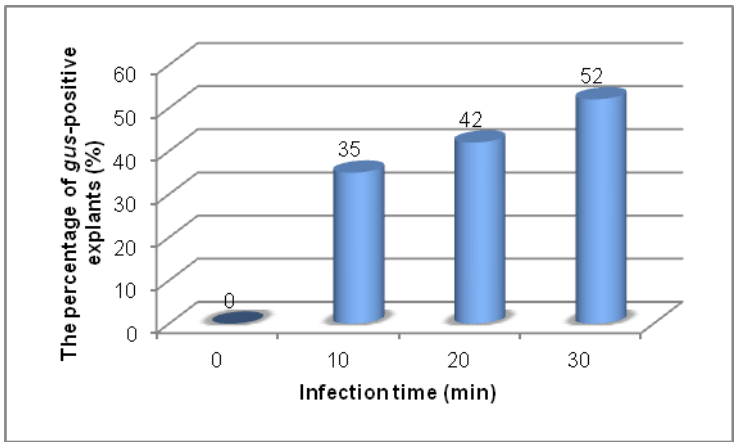

Figure 4. Effect of infection time on transformation efficiency.

Effect of infection time on transformation efficiency

Duration of infection time affects the transformation efficiency, survival and regeneration rates of explants. Increasing infection time directly affects the survival ability and development of plant tissues. The density of bacteria used during the infection process also influenced the development of bacteria during co-cultivation stage and elimination of bacteria. On the other hand, when using short time for bacterial infection, low bacterial density results in low transformation rate.

In this study, there was a significant correlation between infection time and the percentage of *gus*-positive explants (Fig. 4). However, longer time of infection caused necrotic explants (data not shown). Therefore, the time duration of 20 min was used for subsequent experiments.

In 2006, Tao, Li reported that 20 min was the optimal time duration for infection of *A. rhizogenes* in *Torenia fournieri* L. and obtained the best transformation efficiency as well as low necrosis rate.

Effect of acetosyringone concentrations on gene transformation efficiency

Acetosyringone (AS) acts as a chemotactic agent in very low concentrations and it activates the *vir* gene on the root inducing (Ri) plasmid, which initiates the infection process for the transfer of T-DNA (Huang *et al*., 2001). In the present study, ½SH medium supplemented with 100 µM acetosyringone gave the highest percentage of *gus*-positive explants (59%) compared with media with 50, 150, or 200 µM AS or AS-free medium (control) (Fig. 5). The results suggested that high concentrations of AS were not effective for infection efficiency.

After culturing 8 weeks on medium with antibiotic, hairy root formation was recorded. Hairy roots were excised from transformed callus clusters and subsequently cultured on SH medium supplymented with 30 g/l sucrose, 500 mg/l cefotaxime.

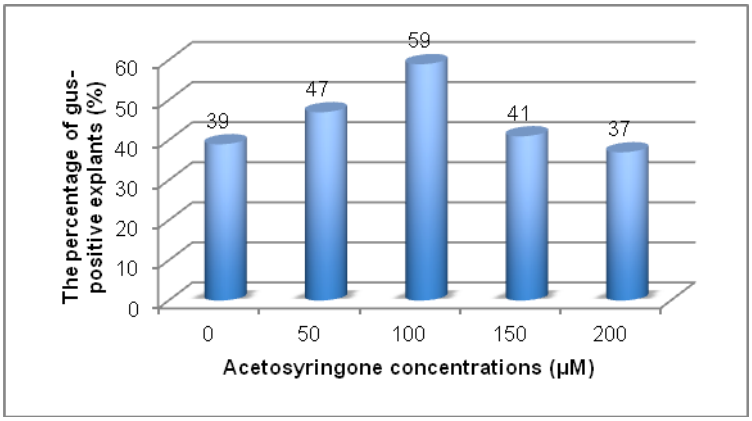

Figure 5 Effect of Acetosyringone concentrations on gene transformation.

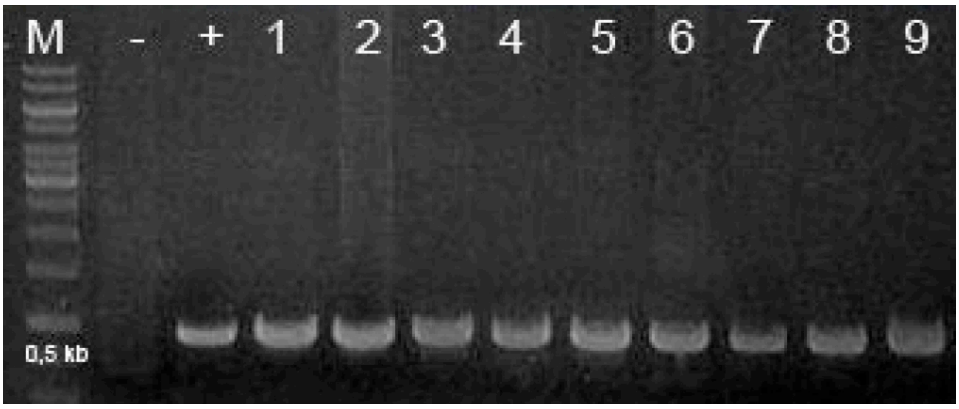

Figure 6. PCR analysis of the *rol*C gene in the *P. vietnamensis* transformed roots. (**-**) negative control (PCR mixture without plant DNA); (**+**) positive control; (1-9) transformed hairy root samples.

PCR amplification for checking T-DNA integration into the genome

The *rolC* gene of *A. rhizogenes* T-DNA plays an important role in the formation of hairy roots (Gorpenchenko *et al*., 2006). In order to examine the integration of T-DNA into the genome of *P. vietnamensis* hairy root clones, total DNA extracted

of root clones were used as templates for PCR amplifying *rolC* gene). It was clear that all root clones represent *rolC* gene bands that are approximately 0.6 kb, similar to positive control band as shown in fig. 6. The presence of *rolC* gene in the samples proved that the transgenic cultures were actually transformed with *rol* genes.

Morphological variability among hairy root clones

Morphological differences have been observed among hairy root clones in various plant sources (Wang *et al*., 2001). These differences might be caused by the non-specific integration of T-DNA into the host genome. The number of T-DNA copies and the integrative position of each transgenic clone are different. If the T-DNA is inserted into the active transcription regions, the expression of T-DNA may be much better than that of inactive transcription regions (Ngoc *et al*., 2012). In this study, the hairy root clones showed four different phenotypes such as roots that displayed the characteristic trait of hairy root with primary roots and some long lateral root branching (HR1) (Fig. 7a); roots that produced considerable lateral roots on primary roots (HR2) (Fig. 7b); roots that showed callus like phenotype with thick and short primary and lateral roots (HR3) (Fig. 7c) and roots which formed root clusters containing numerous short, thick root tips (HR4) (Fig. 7d).

These results are similar with those of Mallol *et al*. (2001) who described three morphological phenotypes observed when infected *P. ginseng* explants with *A. rhizogenes* strain A4 – an agropine-type similar to ATCC15834. When cultured on phytohormone-free SH medium, HR1 did not induced new root tips and had only primary or initial root tips developed without branching. Meanwhile, HR3 which formed callus-like phenotypes hardly developed hairy roots, and that the number and the length of root tips grew extremely slowly even after prolonged period of culturing on phytohormone-free SH medium. Similarly, HR4 also developed slowly in phytohormone-free SH medium. After 3 - 4 weeks, roots in the callus clusters began to elongate, but the number of clusters remained unchanged. In contrast, HR2 phenotype roots displayed the most rapid growth kinetic (Fig. 8). For example, after transfer to new media for one to two weeks, HR2 roots regenerated new lateral roots that elongated rapidly and exhibited prolific branching.

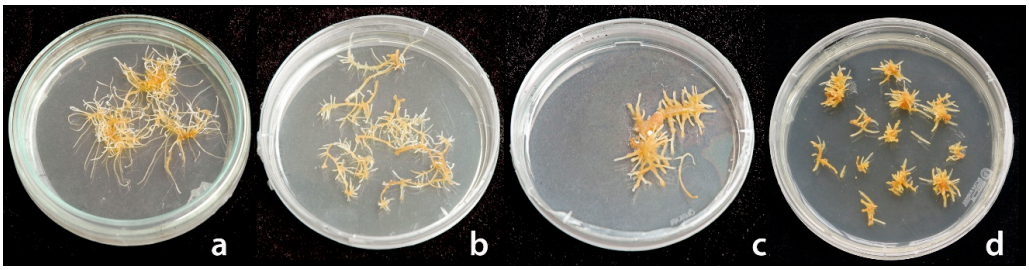

Figure 7. *P. vietnamensis* hairy root morphologies. a; b; c; d: HR1, HR2, HR3, HR4 phenotypes.

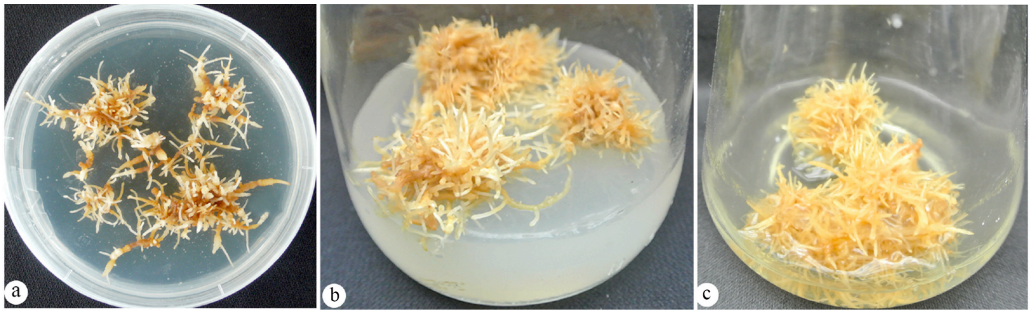

Figure 8. The growth of HR1 on solid medium after 8 weeks (a); 24 weeks (b) and liquid shaking medium after 12 weeks (c).

Estimate oncogenes expression through Real time PCR

Real time PCR has become a key technique to measure changes in expression of target genes due to its accuracy, sensitivity and reproducibility. The expression of target genes in different conditions treatment is estimated using references genes that constitutively express in every tissue. Actin gene is commonly used as a reference gene for estimating gene expression in plants. The transcription level of target and reference genes are calculated based on the determination of fluorescence signal in real time PCR system. In this study, the expressions of oncogenes (*rol*B, *aux*1) are quantified via actin gene using 2^{-ddCt} calculating method.

It is evident that there was a relevant correlation between development ability and transgene expression in hairy root phenotypes. For instance, HR3 phenotype developed slowest and had the lowest gene expression in both *rol*B and aux1 gene. Meanwhile, HR2 phenotype that proliferated outstandingly among the various root morphologies showed the highest *rol*B and aux1 expression, 8.72 and 5.52 times compared to HR3 (Fig. 9). The transgene expression in HR2 was almost double than that of HR4 and approximately four times HR1 (Fig. 9). Overall, *rol*B and aux1 play an important role in hairy roots formation and development. In this study, the increase in *rol*B expression coincided with the rise of hairy roots growth ability.

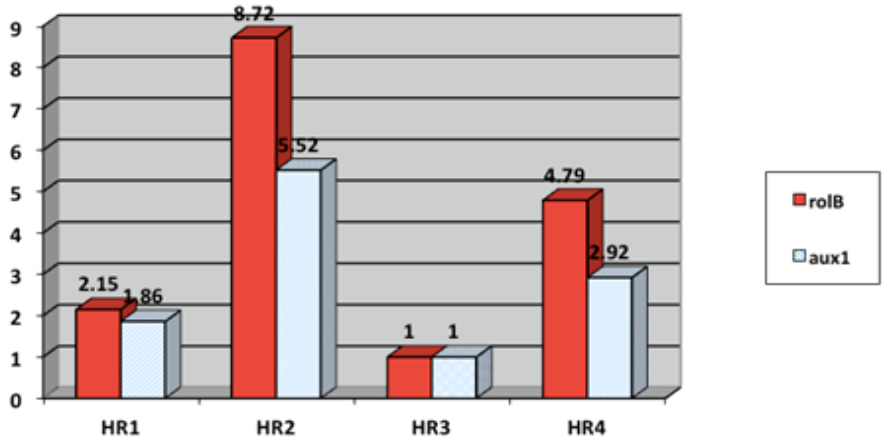

Figure 9. Oncogenes expression level in HR1, HR2, HR3 and HR4.

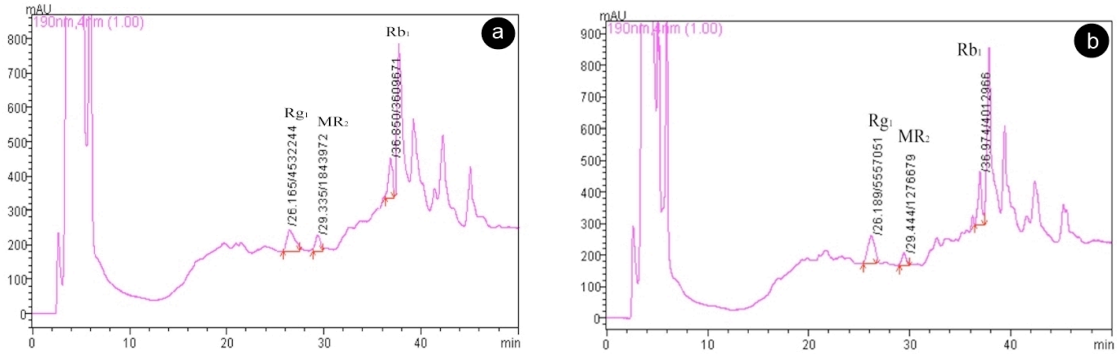

Figure 10. Diagram of the basic components of secondary metabolites of (a) *P. vietnamensis in vitro* rhizomes (control); and (b) transformed roots.

Saponin analysis

Saponin accumulation of transformed hairy roots and rhizomes of *in vitro P. vietnamensis* is shown in HPLC diagram (Fig. 10). Rg_1, Rb_1 and MR_2 were detected at the 26th, 30th and 37th minutes, respectively (Fig. 10). These results indicated that there were no significant differences between the

number of ginsenosides of transformed hairy roots of *P. vietnamensis* and that of the *in vitro* rhizomes.

CONCLUSION

In conclusion, we have successfully established a procedure for *A. rhizogenes*-mediated transformation of *P. vietnamensis*. Other factors affecting the hairy root induction and the transformation efficiency were also investigated. The protocol described in this study is simple and rapid and therefore, can be used for large-scale experiments for the rapid production of valuable compounds.

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CHUYỂN GEN QUA TRUNG GIAN VI KHUẨN *AGROBACTERIUM* Ở CÂY SÂM NGỌC LINH (*PANAX VIETNAMENSIS* HA *ET* GRUSHV.)

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TÓM TẮT

Trong những năm gần đây, chuyển gen qua trung gian vi khuẩn *Agrobacterium* đã được sử dụng rộng rãi để đưa gen ngoại lai vào tế bào thực vật và sau đó tái sinh thành các cây biến đổi di truyền. Sâm Ngọc Linh là cây thuốc có giá trị cao của Việt Nam nhưng diện tích phân bố của loài này bị giới hạn. Nghiên cứu này trình bày quy trình chuyển gen thu nhận rễ tơ sâm Ngọc Linh, tạo nguồn nguyên liệu cho nuôi cấy sinh khối thu nhận saponin một cách hiệu quả. Trong nghiên cứu này, mẫu lá (có diện tích 0,5 cm^2), cuống lá (dài 1 cm) và cụm mô sẹo (thể tích 0,5 cm^3) được sử dụng để chuyển gen cảm ứng tạo rễ tơ. Kết quả thu được cho thấy, rễ tơ sâm Ngọc Linh được cảm ứng từ việc lây nhiễm mô sẹo với vi khuẩn *Agrobacterium rhizogenes* chủng ATCC15834 ở OD_{600} = 0,5 với thời gian lây nhiễm là 20 phút và có bổ sung 100 μM acetosyringon. PCR được sử dụng để kiểm tra sự hiện diện của gen *rol*. So sánh với rễ sâm Ngọc Linh nuôi cấy *in vitro* cho thấy, rễ tơ có tiềm năng sản xuất liên tục các chất chuyển hóa thứ cấp và có lượng saponin tương tự với rễ nuôi cấy *in vitro*. Quy trình chuyển gen thu nhận rễ tơ sâm Ngọc Linh mô tả trong nghiên cứu này đơn giản và nhanh chóng, do đó có thể được sử dụng cho quy mô lớn để sản xuất nhanh các hợp chất thứ cấp có giá trị.

Từ khoá: *Agrobacterium rhizogenes, chuyển gen, rễ tơ, saponin, sâm Ngọc Linh*