

Improving hairy root induction of *Urena lobata* L. by *Agrobacterium rhizogenes* ATCC 15834 by some factors

Vu Thi Bach Phuong, Pham Thi Anh Hong, Quach Ngo Diem Phuong*

ABSTRACT

Introduction: Our previous study showed that *Urena lobata* L. hairy root is a potential pharmaceutical source for type 2 diabetes treatment. In order to improve the transformation efficacy and the quality of hairy roots, this study examined the effects of several factors including age, parts of plants, infection time and culture medium in inducing hairy roots in *Urena lobata* L. **Methods:** In this study, we investigated four factors to improve the hairy root induction in *Urena lobata* L. These factors include: age of plant (15-day-old *in vitro* plants, 45-day-old *in vitro* plants and after two sub-culture generations plants), different parts of plant (roots, stems, and leaves), infection time (10, 20 and 30 minutes), and culture medium (Murashige and Skoog (MS), Gamborg B5 medium (GB5) and Woody plant medium (WPM)). All experiments were repeated three times, with uninfected leaf explants of 15-day-old *in vitro* as the negative control. The transformation frequency and the fresh biomass of hairy roots were recorded at four weeks after infection. **Results:** The results showed that the optimized procedure which used 15-day-old *in vitro* plants, the leafy part, the infection time of 10 minutes and culture in the WPM medium was better than the original procedure. The optimized procedure achieved a transformation frequency of 100%. In addition, the fresh biomass of hairy roots formed on an explant in the optimized procedure was 3.2 times higher than the ones induced by the original procedure. **Conclusion:** The results showed that the optimized procedure was more effective than the original procedure in inducing *Urena lobata* hairy roots.

Key words: *Agrobacterium rhizogenes*, Hairy root, Induction, Infection, rolB, rolC, virG, *Urena lobata* L.

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INTRODUCTION

Urena lobata L. belongs to the Malvaceae family, which is used in herbal medicine to treat a wide range of ailments such as colic, malaria, gonorrhoea, fever, wounds, toothache, rheumatism¹, and especially diabetes². Studies have shown that the extracts from *Urena lobata* L. have medicinal activities such as antioxidant, anti-inflammatory, antimicrobial, antidiarrheal, antidiabetic, anti-hyperlipidemic, and anti-diarrhoeal activities^{1,3}. In addition, studies on phytochemical have analyzed and identified different compounds from *Urena lobata* L. extracts such as alkaloids, falconoids, tannin, saponin, coumarin, steroid/triterpenoid, furocoumarin, mangiferin, quercetin, imperatorin, β -sitosterol, kaempferol, luteolin, hypolatin, gossypetin, and stigmasterol⁴⁻⁶.

In plant tissue culture, hairy root culture technique is a key step in the production of secondary compounds *in vitro*. Hairy roots are generated by infecting *Agrobacterium rhizogenes* T-DNA into the genome of the plant. The conditions of the gene transfer (the nature and age of the plants, the bacterial strain, the bacterial density, and the infection process) have a great influence on the frequency of gene

transfer as well as the growth and yield of the hairy roots. For examples, hairy root induction in *Agastache foeniculum*, Rosmarinic acid content in transformed roots (213.42 $\mu\text{g/g}$ dry wt) was significantly higher than non-transformed roots (52.28 $\mu\text{g/g}$ dry wt)⁷. Moreover, hairy root induction in *Hypericum perforatum* L., ATCC15834 strain and the excised seedling as explant produced the highest number of hairy roots⁸. After optimizing culturing parameters (medium composition, elicitor, precursor), hairy roots can be optimized to grow rapidly and produce valuable compounds⁹.

Due to the medicinal properties of *Urena lobata* L. and the advantage of hairy root culture technique, this study aimed to optimize hairy root induction in *in vitro* culture of *Urena lobata* L. to produce highly bioactive materials for the pharmaceutical industry. Therefore, this study focused on examining factors (age, plant parts, infection time, and culture medium) affecting hairy root induction to improve the hairy root induction and to increase the transformation frequency of *Urena lobata* L.

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METHODS

Chemicals

Taq polymerase, 100 bp Plus Ladder were purchased from Bioline. *rolB*, *rolC*, *virG* were produced by Integrated DNA Technologies.

Sterilization of *in vitro* culture materials

Seeds of *Urena lobata* L. was locally collected in district 9, Ho Chi Minh City, Vietnam. The selected seeds had good quality and free from infection. The seeds were washed with 80% ethanol for two minutes. Then, the seeds were shaken and soaked in 2% sodium hypochlorite for 10 minutes and then washed with sterile distilled water. Seeds after sterilization were placed on the MS (Murashige and Skoog) medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) phytoagar (pH 5.8). The seeds germinated in a growth chamber at $25 \pm 2^\circ\text{C}$ under standard cool white fluorescent tubes with a 16-h/8-h photoperiod. Plantlets were collected at different ages depending on the purpose of the experiment.

Investigating factors affecting hairy root induction in *Urena lobata* L.

Preparation of *Agrobacterium rhizogenes*

Agrobacterium rhizogenes ATCC15834 strain was obtained from RIKEN bank (Japan) through the MEXT project. *A. rhizogenes* ATCC15834 cells were grown in a nutrient broth medium (beef extract 3 g/L, peptone 5 g/L, pH 7.0) for 48 hours in a shaking incubator (110 rpm, $25 \pm 1^\circ\text{C}$).

The original procedure for hairy root induction

The original procedure used for *Urena lobata* L. hairy root induction was as following¹⁰: the *in vitro* leaves from 15-day-old plants were injured on the surface to facilitate the infection process. These segments were soaked in the *A. rhizogenes* ATCC15834 suspension ($\text{OD}_{600} = 0.6$) for 20 minutes. After four days of co-cultivation, the explants were transferred to the MS medium (3% sucrose) supplemented with cefotaxime (250 mg/L) to eliminate the remained *A. rhizogenes* ATCC15834. The samples were grown under dark conditions at 25°C for hairy root induction. Within two weeks, numerous hairy roots emerged from the wounded sites on leaf explants. The number of responsive explants and number of hairy roots per explant were recorded 30 days after infection.

Besides the investigated factors, the remaining factors were similar to the original hairy root induction procedure. All experiments were repeated three times,

with the same negative control is leaf explants from 15-day-old *in vitro* seedlings that were not infected with *Agrobacterium rhizogenes*.

The effect of age and different parts of plant on hairy root induction

Roots, stems, leaves of three types of plants: 15-day-old *in vitro* plants, 45-day-old *in vitro* plants and after two subculture generations plants were infected with *Agrobacterium rhizogenes* to induce hairy roots.

The effect of infection time on hairy root induction

Samples were infected with *Agrobacterium rhizogenes* for 10 minutes, 20 minutes, and 30 minutes to determine the optimal infection time.

The effect of culture medium on hairy root induction

Three types of medium were used: MS, GB5 (Gamborg B5 medium) and WPM to determine the optimal culture medium.

The effect of the combined of improved factors on hairy root induction

The hairy roots were induced in two procedures: the original procedure and the optimized procedure with optimized conditions (age, plant parts, time of infection and medium of induction).

Confirmation of transgenic roots

Genomic DNA samples were extracted from *Urena lobata* L. hairy roots and *in vitro* roots (non-transgenic roots) by the CTAB method as described previously¹¹. The Ri-plasmid was isolated from *A. rhizogenes* ATCC 15834 by the method described by Curier and Nester¹². PCR reactions were performed using the genomic DNA from the hairy roots and non-transgenic roots as well as the Ri-plasmid with specific primer sets for *rolB*, *rolC*, and *virG* genes. The sequencing primers include F-*rolB* (5'-GCTCTTGCAGTGCTAGATT-3'), R-*rolB* (5'-GAAGGTGCAAGCTACCTCTC-3'); F-*rolC* (5'-CTCCTGACATCAAACCTCGTC-3'), R-*rolC* (5'-TGCTTCGAGTTATGGGTACA-3'); and F-*virG* (5'-TTATCTGAGTGAAGTCGTCAGG-3'), R-*virG* (5'-CGTCGCCTGAGATTAAGTGTC-3').

The expected amplified fragment sizes were 423 bp for *rol* gene, 626 bp for *rolC* gene, 1030 bp for *virG* gene¹³. The PCR reactions were performed in a total volume of $25\mu\text{l}$ containing 100ng of plant genomic

DNA (or 40 ng of Ri-plasmid DNA), 5 µL Taq polymerase buffer (5X), 0.5 µM of each primer and 1U Taq polymerase (Bioline). PCR steps included initial denaturation at 95°C for 5 minutes, followed by 35 cycles of amplification (95°C for 30s, 54°C for 30s and 72°C for 60s) and a final extension at 72°C for 10 minutes. PCR products were visualized by agarose gel electrophoresis.

Statistical analysis

Each treatment included 20 explants in replicates of three. The transformation frequency was calculated at four weeks after infection. All data analyses were performed using the SPSS 16.0 (Copyright SPSS Inc.). Experimental results were shown as mean \pm standard deviation (SD). Differences between means were evaluated by Duncan's multiple range tests. Statistical significance was accepted at 0.05.

RESULTS

Sterilization of *in vitro* culture materials

The seeds of *Urena lobata* L. were sterilized by 2% sodium hypochlorite in 10 minutes. The germination rate was 90-100%. After two days on MS medium, germinated seeds developed into seedlings. 45-day-old plants were mature enough and suitable for subculture. Seedlings were collected at different ages depending on the specific purposes of each experiment (Figure 1).

Investigating important factors to improve the hairy root induction in *Urena lobata* L.

The effect of age and different parts of plants on hairy root induction

After four weeks after infection, *Urena lobata* L. leaves had the highest transformation frequency compared to the stems and roots at all ages (Table 1). The older plants had a lower transformation frequency than the younger plants. Specifically, leaves of 15-day-old *in vitro* plants had the highest transformation frequency (97.33%), and the second highest was stems (86.33%) of the same age plants (Figure 2). This result demonstrated that the younger tissues, especially cotyledon, positively correlate with the higher transformation frequency.

The effect of infection time on hairy root induction

The results indicated that the infection time of 10 and 20 minutes had a similar transformation frequency

(97.67% and 97.33%, respectively) (Table 2). However, when the infection time increased to 30 minutes, the transformation frequency significantly decreased (93.67%) (Table 2). In addition, the longer infection time correlates with a lower recovery rate of the wounded explants, and the induced hairy roots also developed to the lesser extent because of the bacterial overgrowth during the co-cultivation. Explants infected for 10 minutes have more hairy roots than the ones infected for 20 and 30 minutes (Figure 3).

Effect of culture medium on hairy root induction

The results showed that WPM and MS medium had a similar transformation frequency four weeks after infection (100% and 97.3%, respectively). GB5 medium had the lowest transformation frequency (91.67%) four weeks after infection (Table 3). However, WPM medium had higher hairy root development than the other two media (Figure 4). This observation indicated that the composition and the mineral content in WPM medium are better in supporting the growth of the *Urena lobata* L. hairy roots than the MS and GB5 medium.

The effect of the optimized conditions on hairy root induction

After identifying the optimal conditions for the hairy root induction in *Urena lobata* L., these conditions were combined and compared to the original procedure. Specifically, 15-day-old *in vitro* leaves were infected for 10 minutes and cultured in WPM medium. The results in the table 4 and the figure 5 showed that the optimized conditions had the transformation frequency of 100%, while the original procedure achieved a transformation frequency of 97.33% (Table 4, Figure 5). Importantly, the fresh biomass of hairy roots per explant in the optimized procedure (0.517g) was 3.2 times higher than the one of the original procedure (0.160g). In summary, this result showed that the optimized procedure is more effective than the original procedure in inducing and supporting the growth of hairy roots in *Urena lobata* L..

Transgenic roots were confirmed by PCR

The hairy root samples were analyzed by PCR to test whether the transgenic process was successful. DNA samples from the putative hairy roots and from *in vitro* non-transgenic roots were isolated and subjected to PCR analysis for the presence of *rolB*, *rolC*, and *virG* genes, which are present in Ri-plasmid. The Ri-plasmid of *A. rhizogenes* ATCC15834 was also included to serve as a positive control. The presence of

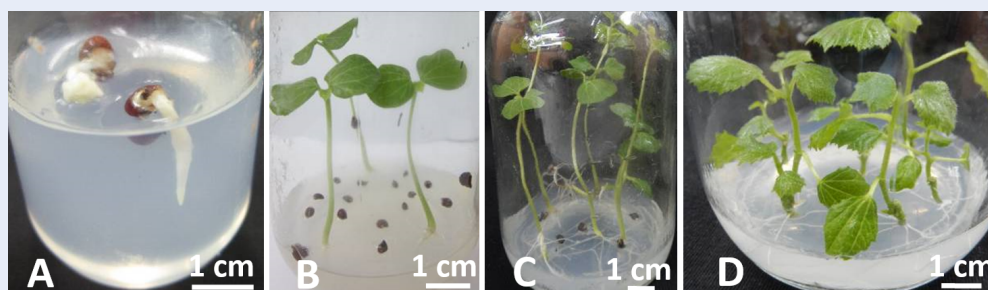


Figure 1: *In vitro* *Urena lobata* L. seedlings. (A) 5-day-old *in vitro* plant, (B) 15-day-old *in vitro* plant, (C) 45-day-old *in vitro* plant, (D) after two subculture generations plants.

Table 1: The effects of age and parts of plants on transformation frequency four weeks after infection (%)

Parts of <i>Urena lobata</i> L.		Transformation frequency (%)
After two subculture generations plants	Root	7.333 ^f ± 2.081
	Stem	6.333 ^f ± 1.528
	Leave	23.000 ^e ± 2.645
45-day-old <i>in vitro</i> plant	Root	8.333 ^f ± 2.081
	Stem	32.667 ^d ± 3.055
	Leave	40.000 ^c ± 3.000
15-day-old <i>in vitro</i> plant	Root	30.333 ^d ± 2.517
	Stem	86.333 ^b ± 2.517
	Leave	97.333 ^a ± 2.082

Data were shown as mean ± SD of three independent experiments. Letters a, b, c, d, e and f indicate significant differences at p < 0.05 according to Duncan's multiplerange-posthoc tests.

Table 2: The effect of infection time on hairy root induction after four weeks of infection

Infection time (minute)	Transformation frequency (%)
10	97.667 ^a ± 2.517
20	97.333 ^a ± 2.082
30	93.667 ^b ± 3.215

Data were shown as mean ± SD of three independent experiments. Letters a and b indicate significant differences at p < 0.05 according to Duncan's multiplerange-posthoc tests.

Table 3: The effect of culture medium on hairy root induction four weeks after infection

Culture media	Transformation frequency (%)
MS	97.333 ^a ± 2.082
WPM	100.000 ^a ± 0.000
GB5	91.667 ^b ± 1.528

Data were shown as mean ± SD of three independent experiments. Letters a and b indicate significant differences at p < 0.05 according to Duncan's multiplerange-posthoc tests.

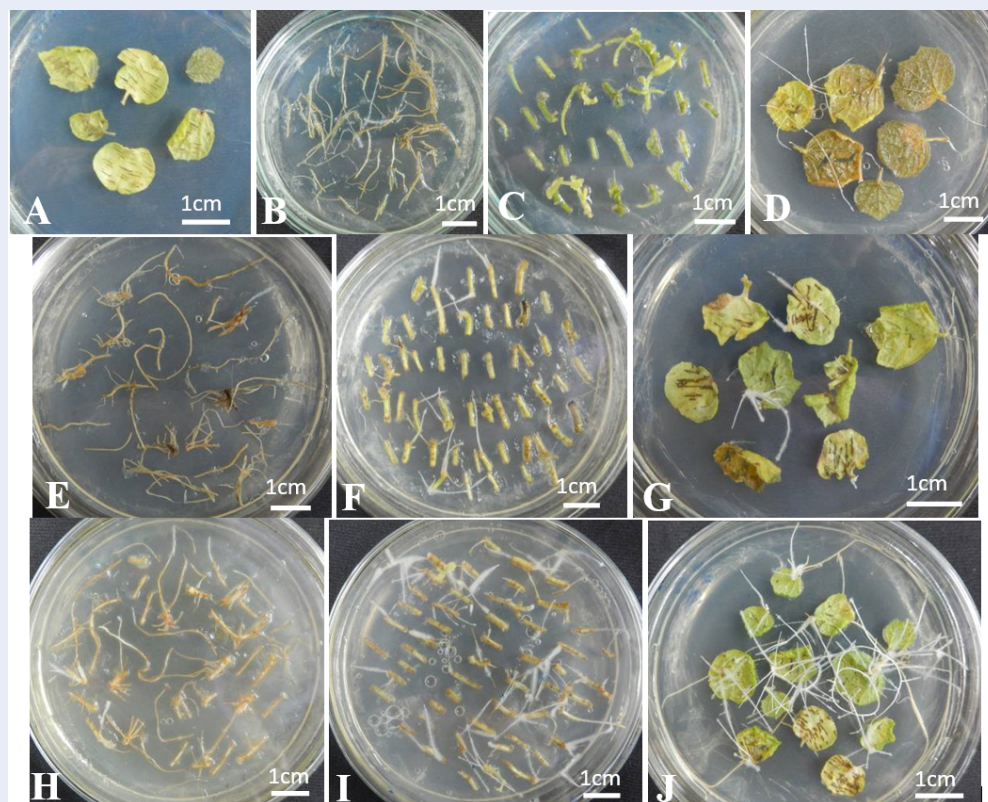


Figure 2: The effect of age and parts of plants on hairy root induction four weeks after infection. (A) uninfected leaf explants (negative control). **(B, C, D)** explants form roots, stems, leaves of after two subculture generations, **(E, F, G)** explants form roots, stems, leaves of 45-day-old *in vitro* plant, **(H, I, J)** explants form roots, stems, leaves of 15-day-old *in vitro* plant.

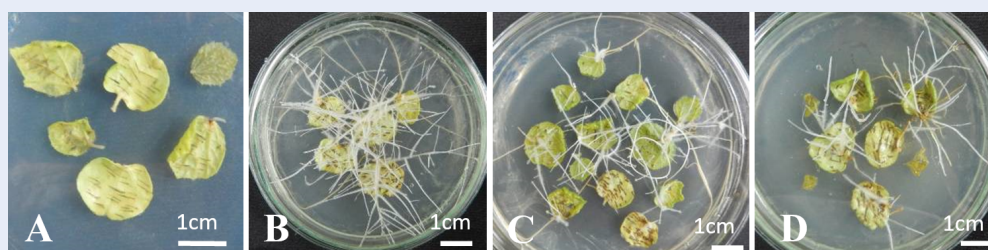


Figure 3: The effect of infection time on hairy root induction four weeks after infection. (A) uninfected leaf explants (negative control), **(B, C, D)** infected with *A. rhizogenes* ATCC 15834 for 10, 20, and 30 minutes, respectively.

Table 4: The combined effect of the optimized condition on hairy root induction four weeks after infection

Induced process	Transformation frequency (%)	Fresh weigh/explant (g)
Original condition	97.333 ± 2.082	0.160 ± 0.053
Improved condition	100.000 ± 0.00	0.517 ± 0.076

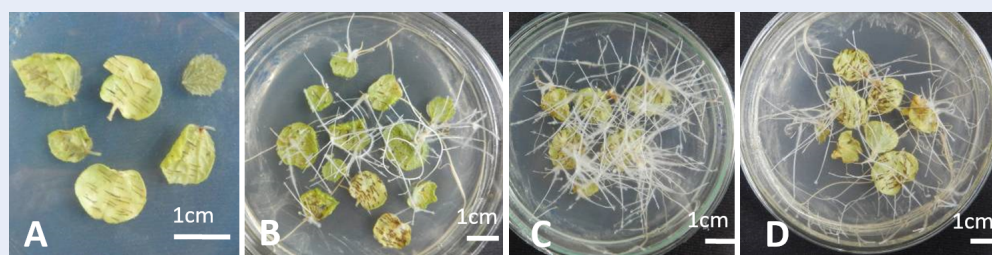


Figure 4: The effect of culture mediums on hairy root induction after three weeks after infection. (A) uninfected leaf explants (negative control), **(B, C, D)** explants infected with *A. rhizogenes* ATCC15834 in MS, WPM, GB5 medium, respectively.

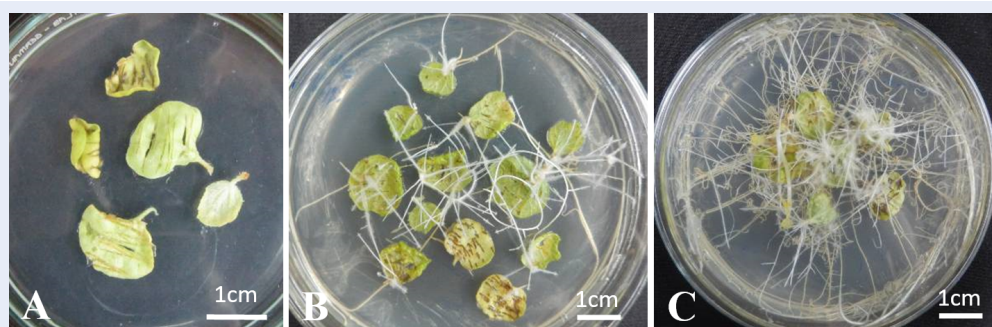


Figure 5: The combined effect of improved factors on hairy root induction after four weeks of infection. (A) uninfected leaf explants (negative control). **(B)** Hairy roots are induced by the original process. **(C)** Hairy roots are induced by the optimized process.

rolB and *rolC*, as well as the absence of *virG* from these hairy roots confirmed that *Ri*-plasmid was integrated successfully into the plant genome (Figure 6).

DISCUSSION

The age and parts of plants are important factors influencing the transformation of *Agrobacterium rhizogenes* into plants. Young plants and seedlings have higher transformation efficacy than tissues and organs from mature plants. Young plant tissues such as hypocotyl, cotyledon, and young leaves are often used for infection to induce hairy roots¹⁴. Our data in *Urena lobata* L. also showed that leaves from 15-day-old plants have the highest rate of hairy root induction. In 15-day-old *Urena lobata* L., the leaves are cotyledons, and the stem is hypocotyl. Hence, they are good materials for infection. In addition, many studies also showed that leaves have the highest rate of hairy root induction. This observation was attributed to the ability of leaves to produce a large number of cells in wound healing response. Wound healing response is the most important factor in inducing hairy roots.

The optimal infection time to induce hairy root for each plant species is different. For example, the optimal infection time to induce hairy root in *Urena lobata* L. is 10 minutes. Likewise, the optimal infection time for *Berberis aristata* DC is 3 hours¹⁵ and *Arachis hypogaea* L. is 20 minutes¹⁶. Therefore, it is necessary to optimize the infection time for each plant species to achieve the highest hairy root induction.

The culture medium is one of the most important factors influencing the induction as well as the growth of hairy roots. In this experiment, WPM medium was the optimal media to support the induction and growth of hairy roots in *Urena lobata* L., which indicates that the composition and the mineral content in WPM medium were suitable for the growth of the *Urena lobata* L. hairy roots. WPM medium is frequently used to support the growth of woody species, which may support the growth of a subshrub plant like *Urena lobata* L. better than MS and GB5 medium.

CONCLUSIONS

This study identified important factors that can improve the hairy root induction in *Urena lobata* L. by

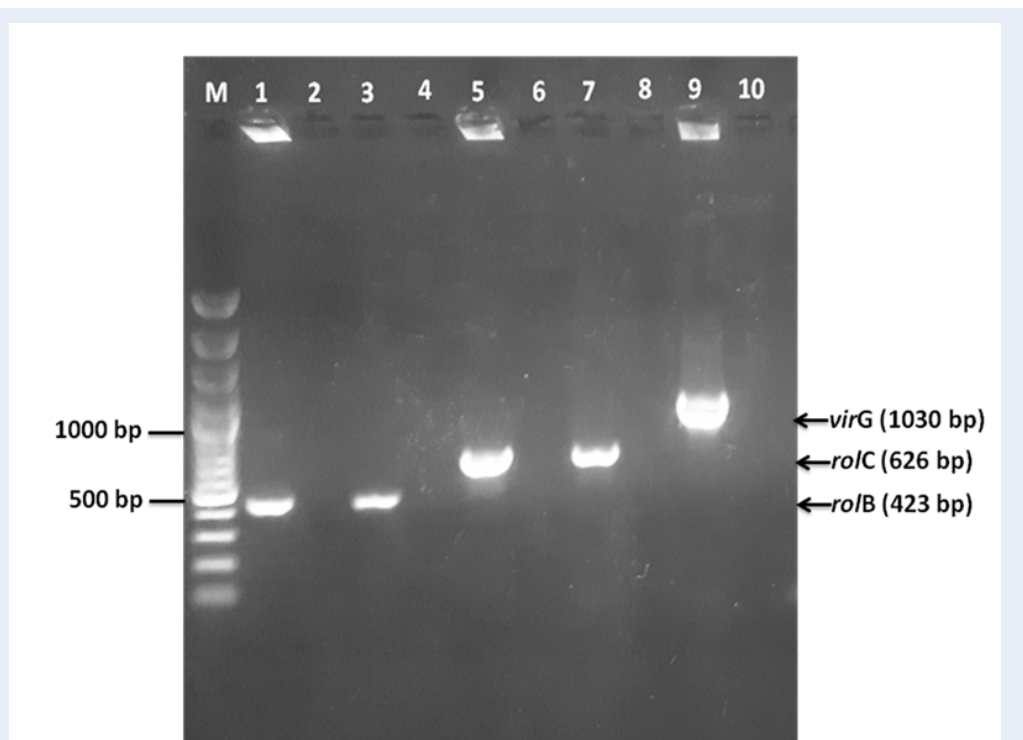


Figure 6: Confirmation of transformation by PCR. PCR amplification of *ro/B* (lanes 1, 2, 3), *ro/C* (lanes 5, 6, 7), and *virG* (lanes 8, 9, 10) genes from *Urena lobata* L. roots and hairy roots, and *Agrobacterium rhizogenes* ATCC15834. Lane M: molecular weight marker (100 bp plus ladder); lanes 1, 5, 9: *Agrobacterium rhizogenes* ATCC15834 DNA (positive control); lanes 2, 6, 8: DNA from *in vitro* non-transformed roots (negative control); lanes 3, 7, 10: DNA of transformed roots obtained after *Agrobacterium rhizogenes* ATCC15834 infection; lane 4: negative control of PCR amplification.

Agrobacterium rhizogenes (ATCC 15834). These factors include parts of plants, age of plants, infection time and culture medium. By using the optimized conditions (leaves of 15-day-old plants, infection time of 10 minutes, WPM medium), 100% transformation efficacy was achieved, and the fresh weight of hairy roots per explant was 3.2 times higher than that of the original procedure.

COMPETING INTERESTS

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

Vu Thi Bach Phuong implemented the experiment and wrote the manuscript. Quach Ngo Diem Phuong proposed ideas and reviewed. Pham Thi Anh Hong is the advisor.

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REFERENCES

1. Ali SL, Babu SS, Madhuri DB. A pharmacological review of *Urena lobata* plant. *Asian Journal Pharmaceutical and Clinical Research*. 2016;9:20–22.
2. Purnomo Y, Soeatmadji DW, Sumitro SB, Widodo MA. Anti-diabetic potential of *Urena lobata* leaf extract through inhibition of dipeptidyl peptidase IV activity. *Asian Pacific Journal of Tropical Biomedicine*. 2015;5:645–9. Available from: DOI:10.1016/j.apjtb.2015.05.014.
3. Choi EM, Hwang JK. Screening of Indonesian medicinal plants for inhibitor activity on nitric oxide production of RAW264.7 cells and antioxidant activity. *Fitoterapia*. 2005;76:194–203. Available from: DOI:10.1016/j.fitote.2004.11.010.
4. Ghosh K. A furocoumarin, imperatorin isolated from *Urena lobata* (Malvaceae). *Molbank*. 2004;2004:382. Available from: Doi:10.3390/m382.
5. Morelli CF, Cairolì P, Speranza G, Alamgir M, Rajia S. Triglycerides from *Urena lobata*. *Fitoterapia*. 2006;77:296–9. Available from: DOI:10.1016/j.fitote.2006.03.010.
6. Mshelia IY, Dalori BM, Hamman LL, Garba SH. Effect of the aqueous root extract of *Urena lobata* (Linn) on the Liver of Albino Rat. *Res J Appl Sci Engine Technol*. 2013;5:01–06.
7. Nourozi E, Hosseini B, Hassani A. Influences of various factors on hairy root induction in *Agastache foeniculum* (Pursh) Kuntze. *Acta Agriculturae Slovenica*. 2016;107:45–54. Available from: DOI:10.14720/aas.2016.107.1.05.

8. Bivadi V, Zakaria RA, Zare N, Yazdani B. Effects of different tissue culture conditions in Hairy root induction in *Hypericum perforatum* L. *International Journal of Agriculture and Crop Sciences*. 2014;7:646–53.
9. Bensaddek L, Villarreal ML, Fliniaux MA. Induction and growth of hairy roots for the production of medicinal compounds. *Electronic Journal of Integrative Biosciences*. 2008;3:2–9.
10. Dai CM, Minh HTT, Phuong VTB, Phuong QND. In vitro and Hydroponic Hairy Root Culture of *Urena lobata* to Obtain α -glucosidase Inhibitory Materials. *Bull Env Pharmacol Life Sci*. 2016;5:72–80.
11. Porebski S, Bailey LG, Baum BR. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Molecular Biology Reporter*. 1997;15:8–15. Available from: [Doi:10.1007/bf02772108](https://doi.org/10.1007/bf02772108).
12. Currier TC, Nester EW. Isolation of covalently closed circular DNA of high molecular weight from bacteria. *Analytical Biochemistry*. 1976;76:431–41. Available from: [Doi:10.1016/0003-2697\(76\)90338-9](https://doi.org/10.1016/0003-2697(76)90338-9).
13. Lan X, Quan H. Hairy root culture of *Przewalskia tangutica* for enhanced production of pharmaceutical tropane alkaloids. *Journal of Medicinal Plants Research*. 2010;4:1477–81.
14. Tomilov A, Tomilova N, Yoder JI. *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* transformed roots of the parasitic plant *Triphysaria versicolor* retain parasitic competence. *Planta*. 2007;225:1059–71. Available from: [DOI:10.1007/s00425-006-0415-9](https://doi.org/10.1007/s00425-006-0415-9).
15. Brijwal L, Tamta S. *Agrobacterium rhizogenes* mediated hairy root induction in endangered *Berberis aristata* DC. *Springer-Plus*. 2015;4:443. Available from: [DOI:10.1186/s40064-015-1222-1](https://doi.org/10.1186/s40064-015-1222-1).
16. Minh HT, Phuong QN, Le BV. Development of an efficient protocol for resveratrol production in hairy root of *Arachis hypogaea* L. using *Agrobacterium rhizogenes* mediated transformation. *Journal of Biotechnology*. 2011;9:665–72.

