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Research Article

Investigation of factors in improving *Agrobacterium*-mediated gene transfer in *Ruellia tuberosa* L. and evaluation of α-glucosidase inhibitory activity in established hairy roots

Dai M. CAO[1,2], Tram T.M. TRAN[1,2], Phuong N.D. QUACH[1,2*]

[1]*University of Sciences, Laboratory of Plant Biotechnology, Department of Plant Biotechnology and Biotransformation, Ho Chi Minh City, Vietnam; cmdai@hcmus.edu.vn; minhtram.clto@gmail.com; qndphuong@hcmus.edu.vn (*corresponding author)*
[2]*Vietnam National University, Ho Chi Minh City, Vietnam*

Abstract

Ruellia tuberosa (family Acanthaceae) is widely known in traditional medicine in Asian countries for the treatment of diabetes and other diseases. Its roots were demonstrated to possess a hypoglycemic ability in diabetic animal models. In this study, an original induced procedure was investigated to establish hairy root (HR) from *R. tuberosa*. With the aim of increasing the transformation rate, some induced factors (acetosyringone (AS) dosage, type of explant, age, infection time, bacterial density, co-cultivation duration) were individually examined. As a result, an improved procedure was implemented: ten-day-old *in vitro* cotyledon explants were injured and then immersed in the bacterial suspension ($OD_{600\ nm}$ = 0.4) added 200 μM AS during 10 min. The infected explants were co-cultivated for 4 days in the Murashige & Skoog (MS) medium before transferring to the medium containing cefotaxime for bacterial elimination. After thirty days of culture, the improved procedure revealed a synergistic effect by enhancing the rooting rate and number of secondary roots per explant up to 4.4- and 8.0-fold, respectively, in comparison with the original procedure. The *R. tuberosa* HR was then cultured in liquid MS medium and achieved the highest biomass production at the late exponential growth phase (3rd week). Its ethanol extract was also higher 2.0-fold in α-glucosidase inhibitory activity than that of the natural root. In conclusion, the α-glucosidase inhibitory activity of HR inducing by the improved procedure may offer an effective and reliable substitute for the utilization of this herbal plant.

Keywords: α-glucosidase; hairy root induction; induced factors; *R. tuberosa*; transformation rate

Abbreviations: AS: Acetosyringone; CTAB: Cetyltrimethylammonium bromide; DEPC: Diethylpyrocarbonate; E: enzyme commission number; HR: hairy root; MS: Murashige & Skoog; NSR: number of secondary roots per explant; OD: optical density; PCR : Polymerase chain reaction; rpm: revolutions per minute; RR: rooting rate; YMB: Yeast mannitol medium

Introduction

R. tuberosa originated from South America and also has a wide distribution of tropical areas. It is classified to Acanthaceae and known as a kind of herbal medicine possessed a potential in diabetic treatment

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in Asian countries. In 2011, different solvent fractions of *R. tuberosa* were investigated in alloxan-induced diabetic rabbits. There were significant decreases in lowering blood glucose levels from 15.17 to 28.64% in comparison with the control (Shahwar *et al*., 2011). Rajan *et al.* (2012) demonstrated that methanol extract of *R. tuberosa* could reduce blood glucose, lipid parameters, serum enzyme concentrations and also significantly increase density lipoprotein – cholesterol compared with glibenclamide (a standard drug). Recently, the roots of *R. tuberosa* have been evaluated and considered as an antidiabetic plant. Its extract could lower blood glucose levels by 60.3%, reduce the malondialdehyde (a type of free radical) level by around 50% and recover the function of the liver in diabetic rats in comparison with the untreated group (Kurniawati *et al*., 2018; Roosdiana *et al*., 2018; Safitri *et al*., 2019).

Hairy root (HR) or transformed root has been used as a successful model for many different studies in relevance to root physiology, phytoremediation, and secondary metabolite production. With the indirectly transferred genes of the *Agrobacterium rhizogenes* Ri (root-inducing) plasmid (Chilton *et al*., 1982), a non-geotropic branching HR was formed at the site of infection (Guillon *et al*., 2006). In Ri plasmid (pRi), a specific DNA fragment (T-DNA) was integrated to the host's genome. T-DNA takes up 5 – 10% of pRi which comprises of main regions are TL- and TR-DNA. T-DNA is classified based on the type of opine they synthesize. In there, TL-DNA contains *rol* genes promoting HR formation; TR-DNA carries *aux* genes are auxin-synthesis genes; *vir* genes support the integration of TL-DNA and TR-DNA into the plant genome; opine and opine catabolism are opine-making and opine-catalyze genes that transform opine into nutrients; oriV response for the replication of T-DNA (Sinkar *et al*., 1987). In the successful T-DNA transformation, the established HR is characterized by overgrowth, genetic stability, and secondary metabolite production with a quantity and quality equal to that of the natural root (Häkkinen and Oksman-Caldentey, 2018). However, the rate of HR induction is often low due to many endogenous and exogenous factors (age of plant, type of organ, co-cultivation time or the use of chemical signals) not being optimized. For example, by adding acetosyringone (AS) to the infection medium, there was a remarkable increase in the frequency of transformation of *Harpagophytum procumbens* up to 3.5 times in comparison with the control (Grabkowska *et al*., 2010). The transformation rate and the time required for HR induction also varied with the type of explant. The highest frequency of *Rhinacanthus nasutus* HR induction was confirmed with cotyledon explants (73% after 4 weeks), higher 1.5- and 2.7-fold of leaf and stem explants, respectively (Cheruvathur *et al*., 2015). Furthermore, new compounds can be produced by the introduction of *A. rhizogenes* genes into HR, enabling them to produce other secondary metabolites that possibly not explored in the non-transformed roots or parental plants (Veerasham, 2004).

The main purposes of our study were (1) to develop an efficient procedure of *R. tuberosa* HR induction and (2) to evaluate the α-glucosidase inhibitory activity of the established HR by comparing with the natural root.

Materials and Methods

Plant material

R. tuberosa seeds and natural roots (3 months old) were obtained from the University of Science campus, Ho Chi Minh Vietnam National University, located in Thu Duc district, Ho Chi Minh City, Vietnam. The seeds were washed in ethanol 70% (v/v) for 45 s before planting in Murashige & Skoog (MS) medium (Sigma, America) (Murashige and Skoog, 1962) supplemented with 30 $g.L^{-1}$ sucrose and 8 $g.L^{-1}$ agar to produce explant tissues. The natural roots of *R. tuberosa* were washed rigorously and then dried on the desiccator (Ecocell, America) at 50 °C until the dry weight was unchanged.

Preparation of Agrobacterium rhizogenes

A. rhizogenes ATCC 15834 was obtained from RIKEN Bank through the MEXT project in Japan. The bacteria was cultured in liquid yeast mannitol medium (YMB) (Sigma, America) (yeast extract 10 $g.L^{-1}$, mannitol 10 $g.L^{-1}$, K_2HPO_4 0.5 $g.L^{-1}$, $MgSO_4.7H_2O$ 0.2 $g.L^{-1}$, NaCl 0.1 $g.L^{-1}$, pH 7.0) (Duta *et al.*, 2006) with shaking (110 rpm) at 25 ± 1 °C for 48 hours. The bacterial suspension was then measured at OD_{600nm} using a spectrophotometer and diluted until the value achieved approximately 0.6.

Transformation

In the original procedure of HR induction: ten-day-old *in vitro* leaves of *R. tuberosa* plantlets were injured. The explants were then immersed in the bacterial suspension (OD_{600nm} = 0.6) for 10 min without adding AS. After the time of infection, the infected explants were incubated in the dark on free-hormone MS medium for 4 d. They were subsequently washed rigorously before being transferred to the MS medium containing 200 $mg.L^{-1}$ cefotaxime for 30 d (Phuong *et al.*, 2018).

For the improvement of the frequency transformation, some factors in the original procedure were changed in four experiments:

(1) AS concentrations (0, 100, 200 and 300 μM)
(2) Types of explants (cotyledons, leaves and stems) were incorporated into the ages of plantlet (10-, 15-, 20- and 25-day-old)
(3) Concentrations of bacterial suspension (OD_{600nm} = 0,4, 0,6 and 1,0) were incorporated into the times of infection (5, 10, 15, 20 and 25 minutes)
(4) Times of co-cultivation (2, 3 and 4 d)

Finally, an optimized procedure comprised of the most improved factors was implemented and evaluated for the efficiency of transformation after 30 d in comparison with the original procedure and the control without inducing to *A. rhizogenes* suspension. The efficiency was evaluated by two parameters: (1) rooting rate (RR) is the percentage of explants induced new root per total explants, and (2) number of secondary roots per explant (NSR) is the average number of emerged roots per explant.

Rol genes detection

The integration of T-DNA into genomic plant DNA was confirmed by polymerase chain reaction (PCR) analysis using *rol*B, *rol*C, and *vir*G specific primers (GENEWIZ) (Table 1) (Kang *et al.*, 2006).

Table 1. Specific pairs of primers used for PCR analysis

Gene	Primer sequences	Amplified fragment (bp)
*rol*B	5'-GCTCTTGCAGTGCTAGATTT-3'	423
	5'-GAAGGTGCAAGCTACCTCTC-3'	
*rol*C	5'-CTCCTGACATCAAACTCGTC-3'	625
	5'-TGCTTCGAGTTATGGGTACA-3'	
*vir*G	5'-TTATCTGAGTGAAGTCGTCTCAGG-3'	1030
	5'-CGTCGCCTGAGATTAAGTGTC-3'	

Genomic DNA from non-transformed roots and transformed roots was extracted using the Cetyltrimethylammonium bromide (CTAB) method (Edwards *et al.*, 1991). Genomic DNA from *A. rhizogenes* ATCC 15834 was isolated using the modified CTAB method (William *et al.*, 2012) as the positive control. For amplification, the PCR program included a step of 5 minutes at 94 °C and 35 cycles (each consisting of 1 minute at 94 °C, 1 minute at 55 °C, and 1 minute at 72 °C), followed by a final extension at 72 °C for 10 minutes (Edwards *et al.*, 1991). PCR products were analyzed after electrophoresis in 1% agarose (Sigma-Aldrich, America) gel.

Hairy root culture and growth curve determination

After 30 days of infection, the HR were excised and transferred individually into liquid hormone-free MS medium containing sucrose 3% (w/v), pH = 5.7 ± 0.1. The HR (0.05 g fresh weight) was inoculated into 20 mL MS liquid medium in a 150 mL erlen and incubated in a rotary shaker (Model 6145, Eberbach) in the dark (80 rpm at 24 ± 1 °C). The HRs were harvested every week to determine fresh weight (FW – g), dry weight (DW – g), and α-glucosidase inhibitory activity ($1/IC_{50}$ – $(\mu g/mL)^{-1}$) in four weeks.

Preparation of ethanol extract

The dried *R. tuberosa* natural root (3 months old) and HR after three weeks cultured in liquid MS medium were ground into a fine powder using a blender. The coarse powder was then extracted with absolute ethanol (Cemaco, Vietnam) in a ratio of 1 g powder / 10 mL solvent using the maceration technique (Cujic *et al*., 2016). After every 24 hours, the mixture was filtered, and the extract was collected using a vacuum rotary evaporator (HS-2005S-N, Hahnshin Scientific Co.) at 50 °C. This process was repeated 3 times for all samples.

α-glucosidase Inhibitory Activity of Ethanol Extract of R. tuberosa Hairy Root

The α-glucosidase (EC 3.2.1.20) inhibitory activity was assessed by the standard method (Dong *et al*., 2012) with some slight modifications. The ethanol extract was dissolved in 5% (v/v) dimethyl sulfoxide to achieve a sample solution (1 $mg.mL^{-1}$). The α-glucosidase (Sigma, America) solution (1.0 $U.mL^{-1}$) and 5 mM p-nitrophenyl-α-D-glucopyranoside (pNPG) (Sigma, America) solution were prepared in phosphate buffer (0.1 M and pH 6.8). A mixture containing 50 L of the sample solution and 40 L of the enzyme solution was incubated for 20 minutes, and then 40 L of pNPG solution was added. The reaction occurred for 20 minutes and was terminated by the addition of 130 L of 0.2 M Na_2CO_3 (Sigma, America). Finally, the reaction mixture was measured at 405 nm using an ELISA Microplate Reader (DAS, Italy). All steps of the enzyme experiment were implemented in the incubator (IN – IF Memmert) to maintain a temperature of 37 °C. Acarbose (Glucobay, India) was used as the standard. The inhibition of α-glucosidase (%) was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{(\text{OD control} - \text{OD blank control}) - (\text{OD sample} - \text{OD blank sample})}{(\text{OD control} - \text{OD blank control})} \times 100\%$$

OD control and sample represent the absorbance of the control and sample, respectively. OD blank control and blank sample represent the absorbance of the control and sample, respectively, without the addition of enzyme solution (replaced by phosphate buffer). The concentration of the ethanol extract required for inhibiting 50% of the α-glucosidase activity under the assay conditions was defined as the IC_{50} value ($\mu g.mL^{-1}$). In this study, we used the value of $1/IC_{50}$ ($(\mu g/mL)^{-1}$) to reveal the α-glucosidase inhibitory activity of the extract.

Statistical analysis

The RR, NSR, FW, DW and the α-glucosidase inhibitory activity ($1/IC_{50}$) were evaluated using one-way analysis of variance (ANOVA), and the least significant difference (LSD) at $p < 0.05$ level by the SPSS 16.0 statistical software (© 2007 SPSS Inc.). In the hairy root induction experiments, values are the mean of three replicates per experiment, with each replicate containing 30 samples. In the evaluation of α-glucosidase inhibitory activity, the experiment was repeated three times, with each repetition containing 3 samples.

Results

Effect of acetosyringone (AS) on the transformation efficiency

The addition of AS had a strong effect in improving the frequency transformation (Table 2). Using 200 μM AS could obtain the most significant HR formation with 3.6-fold RR (%) and 6.7-fold NSR of the control ($p < 0.05$).

Table 2. Effect of different AS concentrations on frequency of *R. tuberosa* hairy root formation

AS concentrations (μM)	RR after 30 days (%) ± SD	NSR after 30 days ± SD
0	16.20 ± 1.90 b	0.13 ± 0.03 b
100	24.43 ± 4.43 b	0.37 ± 0.12 b
200	**57.53 ± 1.32 a**	**0.87 ± 0.12 a**
300	70.90 ± 9.42 a	0.77 ± 0.09 a

The value in line marked with different lower cases denote significant differences between samples at $p < 0.05$ (Duncan's multiple range test). AS: acetosyringone; RR: rooting rate, NSR: number of secondary roots per explant; SD: standard deviations.

Effect of age and organ types on the transformation efficiency

Among three types of explants, the cotyledons were confirmed to be the optimal organ for HR induction (Table 3). Cotyledons with the youngest age (10 days) achieved the highest RR (47.77%) and NSR (1.21 roots) after 30 days of infection. At higher age of plantlet, the transformation efficiency of cotyledons dramatically plunged. Apart from leaves at 10 days of culture, there was no any emergence of roots from the infected sites of all older leaf and stem explants.

Table 3. Effect of types of explant and ages of plantlet on frequency of *R. tuberosa* hairy root formation

Types of explants	Ages of plantlet (day)	RR after 30 d (%) ± SD	NSR after 30 d ± SD
Cotyledons	**10**	**47.77 ± 7.79 a**	**1.21 ± 0.38 a**
	15	24.17 ± 8.21 b	0.24 ± 0.01 c
	20	24.50 ± 2.47 b	0.35 ± 0.13 c
	25	30.00 ± 5.77 b	0.33 ± 0.09 c
Leaves	10	34.43 ± 8.68 ab	0.78 ± 0.11 b
	15	ns	ns
	20	ns	ns
	25	ns	ns
Stems	20	ns	ns
	25	ns	ns

The value in line marked with different lower cases denote significant differences between samples at $P < 0.05$ (Duncan's multiple range test). RR: rooting rate, NSR: number of secondary roots per explant, SD: standard deviations, ns: non-significant.

Effect of concentrations of bacterial suspension and time of infection on the transformation efficiency

Although various concentrations of bacterial suspension and times of infection were tested for HR induction in *R. tuberosa*, the $OD_{600\ nm} = 0.4$ in 10 min showed significantly better results than others (Table 4). At this condition, RR and NSR were 72.23% and 1.1 roots, respectively.

Table 4. Effect of concentrations of bacterial suspension and times of infection on frequency of *R. tuberosa* hairy root formation

$OD_{600\ nm}$	Infection time (min)	RR after 30 d (%) ± SD	NSR after 30 d ± SD
0.4	5	64.46 ± 2.23 a	0.47 ± 0.16 b
	10	**72.23 ± 3.93 a**	**1.10 ± 0.21 a**
	15	76.67 ± 14.53 a	1.15 ± 0.27 a
	20	82.22 ± 1.12 a	1.44 ± 0.24 a
0.6	5	22.20 ± 7.74 bc	0.27 ± 0.12 b
	10	20.81 ± 6.36 bc	0.25 ± 0.05 b
	15	26.17 ± 4.93 bc	0.42 ± 0.18 b
	20	37.01 ± 3.70 b	0.56 ± 0.11 b
1.0	5	15.96 ± 2.18 c	0.28 ± 0.11 b
	10	19.44 ± 4.24 bc	0.23 ± 0.06 b
	15	15.80 ± 1.71 c	0.19 ± 0.04 b
	20	21.67 ± 4.17 bc	0.27 ± 0.06 b

The value in line marked with different lower cases denote significant differences between samples at $P < 0.05$ (Duncan's multiple range test). OD: optical density, RR: rooting rate, NSR: number of secondary roots per explant; SD: standard deviations.

Effect of co-cultivation time on the transformation efficiency

Among various duration time of co-cultivation, the best response was obtained in 4 day (Table 5) with the RR and the NSR was 31.10% and 0.48, respectively. At the treatment of 2 and 3 day, the infected explant could not develop any emerged hairy root.

Table 5. Effect of concentrations of cefotaxime and times of co-cultivation on frequency of *R. tuberosa* hairy root formation

Co-cultivation time (day)	RR after 30 d (%) ± SD	NSR after 30 d ± SD
2	0.00 ± 0.00 b	0.00 ± 0.00 b
3	0.00 ± 0.00 b	0.00 ± 0.00 b
4	**31.10 ± 5.88 a**	**0.48 ± 0.21 a**

The value in line marked with different lower cases denote significant differences between samples at $P < 0.05$ (Duncan's multiple range test). RR: rooting rate, NSR: number of secondary roots per explant; SD: standard deviations.

Combination effect of improved factors on the transformation efficiency

Here we examined whether the combination of improved factors could greatly increase the transformation efficiency. The optimized procedure had an outstanding effect. The RR was 4.4-fold and the NSR was 8.0-fold of the original procedure (Figure 1 and Table 6).

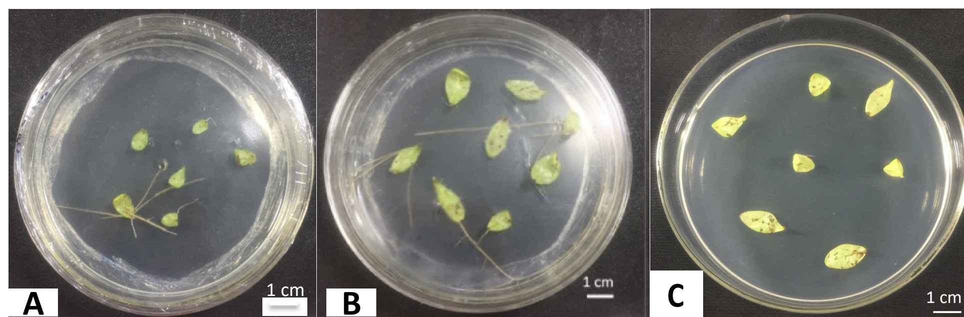

Figure 1. The formation of *R. tuberosa* hairy root. (A) Hairy root induction in the original procedure, (B) Hairy root induction in the optimized procedure, and (C) Control without inducing with *A. rhizogenes*

Table 6. Combination effect of improved factors on frequency of *R. tuberosa* hairy root formation

	RR after 30 d (%) ± SD	NSR after 30 d ± SD
Original procedure	21.40 ± 5.99 b	0.21 ± 0.06 b
Optimized procedure	**94.43 ± 5.57 a**	**1.69 ± 0.46 a**
Control procedure (non-infection of *A. rhizogenes*)	0.00 ± 0.00 c	0.00 ± 0.00 c

The value in line marked with different lower cases denote significant differences between samples at P < 0.05 (Duncan's multiple range test). RR: rooting rate, NSR: number of secondary roots per explant; SD: standard deviations.

Confirmation of transformation

The integration of Ri T-DNA into the genomes of plant cells caused the formation of HR, in which *rol* genes were harbored after 30 d (Figure 2). After infection with *A. rhizogenes* ATCC 15834, the genetic status of the established roots was assessed by using a PCR-based analysis of *rol*B and *rol*C genes. In addition, PCR analysis using gene-specific primers for *vir*G was applied to exclude bacterial contamination in the culture. The DNA of *A. rhizogenes* served as the positive control, and DNA from the non-transformed seedling roots served as the negative control. Our results showed that the PCR products at 423 and 626 bp for the *rol*B and *rol*C fragments, respectively, in positive control and HR, which indicated the successful genetic transformation (Figure 2A). In contrast, no product of PCR amplification was detected for the *vir*G gene in the same HR, indicating the absence of *A. rhizogenes* (Figure 2B).

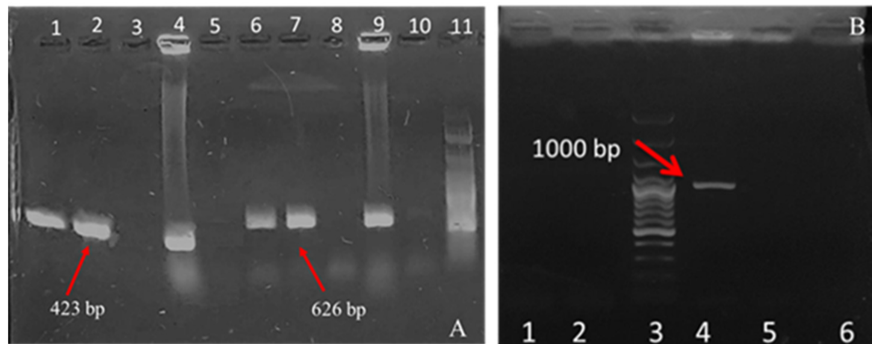

Figure 2. PCR detection of *rol*B, *rol*C, and *vir*G. (A) PCR amplification of 423 bp fragments of *rol*B and 626 bp fragments of *rol*C

The detection of *rol*B: Lanes 1 and 2: hairy roots, Lane 3: negative control, Lanes 4: positive control, Lanes 5: DEPC. Detection of *rol*C: Lanes 6 and 7: HRs, Lanes 8: negative control, Lane 9: positive control, Lane 10: DEPC, Lane 11: ladder 100 bp; (B) The absence of *vir*G in HRs: Lane 1: negative control, Lane 2: DEPC, Lane 3: ladder 1000 bp, Lane 4: positive control, Lanes 5 and 6: HRs

Growth curve and evaluation of α-glucosidase inhibitory activity of R. tuberosa hairy root

After 30 d of HR emergence, the HR of *R. tuberosa* was transferred to liquid MS medium to establish the growth curve. FW, DW and α-glucosidase inhibitory activity were examined during 4 weeks (Figure 3). The growth of HR showed a similar pattern between FW and DW. They increased significantly from week 1 to week 3 and then maintain stable biomass production till week 4. In the same way, 3-week-old HR achieved the highest α-glucosidase inhibitory activity but remarkably decreased to week 4. For these reasons, after 3 weeks culturing in liquid medium (Figure 4), HR was obtained and evaluated the α-glucosidase inhibitory activity in comparison with the natural root. The α-glucosidase inhibitory activity of HR ($1/IC_{50} = 1.024$ $(\mu g/mL)^{-1}$) was approximately 2.0-fold higher than the natural roots ($1/IC_{50} = 0.542$ $(\mu g/mL)^{-1}$) (Figure 5).

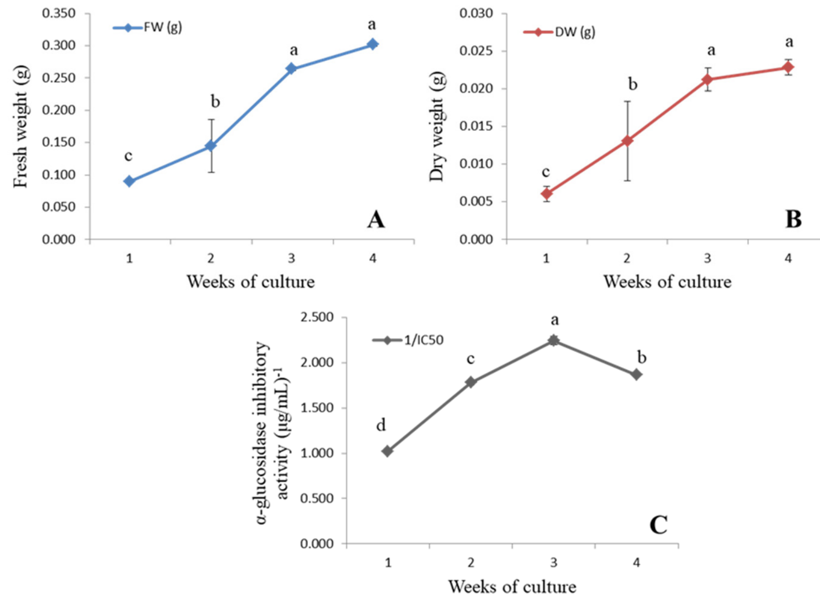

Figure 3. Growth and bioactivity of *R. tuberosa* HR during 4 weeks. (A) FW; (B) DW; (C) α-glucosidase inhibitory activity ($1/IC_{50}$)

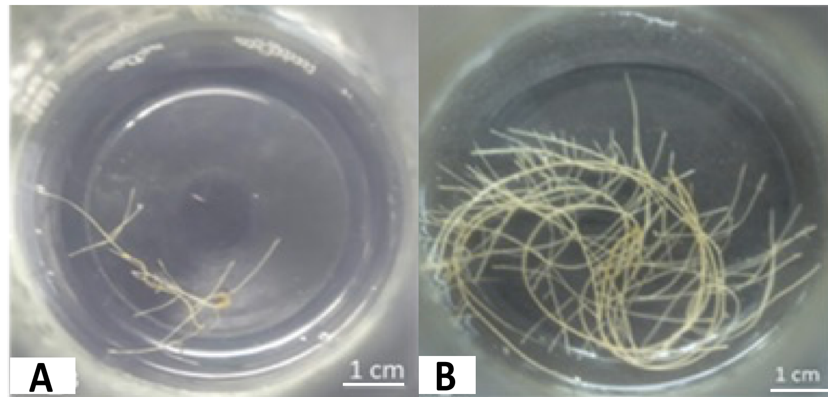

Figure 4. The growth of *R. tuberosa* hairy root. (A) HR cultured in liquid MS medium on the first day; (B) HR cultured in liquid MS medium after three weeks. Scale bar = 1 cm

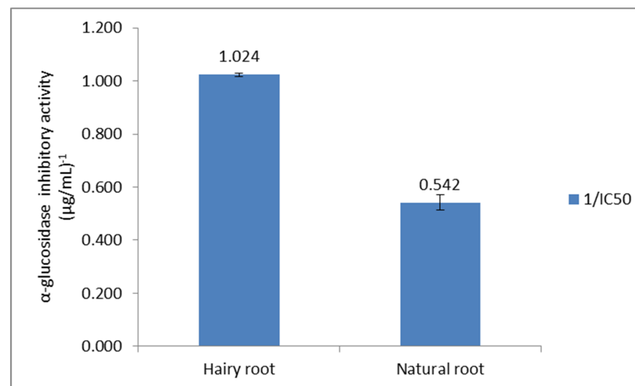

Figure 5. Evaluation of the α-glucosidase inhibitory activity of *R. tuberosa* HR. Error bars represent standard deviations

Discussion

Effect of AS on the transformation efficiency

AS is a chemical signal that belongs to phenolic compounds. It increases the efficiency of transformation by activating *vir* genes located on the Ri plasmid of bacteria (Hu and Alfermann, 1993). These genes stimulate the transportation of T-DNA into the plant genome, resulting in an acceleration in auxin production (Geng *et al*., 2012). In addition, AS was demonstrated to achieve the highest transformation efficiency with *A. rhizogenes* strain ATCC 15834 in many plant species. The HR transformation (%) was higher between 2.0- and 4.0-fold than other strains (Georgiev *et al.*, 2007; Saleh and Thuc, 2009). However, the AS concentration is over may decrease the transformation rate. The excessive amount of AS possibly has a harmful effect on the infected explants and therefore inhibits root growth. Similarly, in the HR establishment of *Citrullus lanatus*, Kavitah *et al.*, (2010) investigated different AS concentrations (0, 100, 200, 300, 400 μM) and found that 200 μM of AS elevated the transformation rate up to 5.5-fold of the control.

Effect of age and organ types on the transformation efficiency

Ages of plantlets and types of organs are important factors closely involved to the formation of root (Hoque and Mansfield, 2004). According to Pawar and Maheshwari (2004), the transformation rate depends on the physiological state of plant cells. Young explants have more differentiated cells that get better results in HR induction than older explants. In addition, leaves are responsible for auxin production that facilitates the emergence of roots. However, in the first stage of growth, cotyledons possibly accumulate higher endogenous auxin levels than leaves, resulting in the highest transformation rate. For the HR induction of *R. tuberosa*, ten-day-old cotyledons have shown a remarkable effect in improving RR and NSR. *Rhinacanthus nasutus* (also belong to Acanthaceae) had revealed a similar result. The RR of young cotyledons was 1.5- and 2.7-fold of the leaf and stem explants, respectively (Cheruvathur *et al*., 2015).

Effect of concentrations of bacterial suspension and times of infection on the transformation efficiency

The concentration of bacterial suspension may indirectly affect the transformation rate through the interaction between bacteria and injured explants. Low concentration ($OD_{600\ nm}$ = 0.3 – 0.6) will limit the transport of T-DNA into the host cell. By contrast, higher concentrations of bacterial suspension ($OD_{600\ nm}$ = 1.0) caused overgrowth of bacteria which may lead to re-infection in the cultural medium even in the presence of high concentrations of anti-biotics. The bacterial re-infection negatively affected the regenerative capacity of the plant tissues, leading to low efficiency of transformation (Phan *et al*., 2016). *R. tuberosa* is quietly susceptible to *A. rhizogenes* due to the browning of explants after being induced with a high concentration ($OD_{600\ nm}$ = 1.0) of bacteria. The initiation of *R. tuberosa* HR was observed at a value of $OD_{600\ nm}$ = 0.4. The infection time influenced the frequency of HR formation. Petrova *et al.,* (2013) reported increased transformation frequency as the infection time increased from 5 to 20 minutes. Short infection time may result in low availability of bacteria for transforming the plant cells while long durations may lead to a decline in the transformation rate by competitive inhibition (Kumar *et al*., 1991). The transformation rate of *R. tuberosa* HR achieved an outstanding effect when incubating explants and bacteria suspension for 10 min.

Effect of co-cultivation time on the transformation efficiency

Co-cultivation is a crucial step in the transformation process. Bacteria attachment, T-DNA transfer and integration are realized during this stage (Su *et al*., 2002). The process can be accelerated by prolonging co-cultivation time. In the HR induction of *Arnica montana*, a co-cultivation of 1-2 d was insufficient to complete the transformation process, but a period longer than 5 d resulted in necrosis of explants, which prevented root

induction (Petrova *et al*., 2013). Therefore, 4 d of co-cultivation is an adequate duration of T-DNA transformation to the host plant cells.

Combination effect of improved factors on the transformation efficiency

After examining individual factors, we investigated whether the combination of improved factors could greatly increase the transformation rate. The optimized procedure had a remarkable effect in improving RR and NSR in comparison with the original procedure. According to Petrova *et al*., (2013), optimizing these endogenous (types of organ and ages of plantlet) and exogenous factors (AS concentrations, bacterial concentrations, infection time, and co-cultivation time) was crucial for the efficiency of gene transfer. In the HR induction of *A. montana*, the collaboration of improved factors (types of explants, infection time, and bacterial concentrations) has increased the transformation frequency up to 2.5-fold compared with the original procedure (Petrova *et al*., 2013). According to this study, if both endogenous and exogenous factors associated with the hairy root induction procedure were improved, the transformation frequency could remarkably increase. The improved endogenous factors place the focus on developing the dedifferentiated level of a cell. The plant cell is young at this stage, which not only facilitated the emergence of hairy roots but also maintained the cell's tolerability in the face of injured stress, and bacterial impact. For the success of T-DNA integration, the improved exogenous factors concentrated on creating favorable conditions through (1) increasing the recognition of *A. rhizogenes* and plant cell response (AS addition), and (2) selecting the appropriate concentration, time of bacterial infection and integration for each plant species.

Growth curve and evaluation of α-glucosidase inhibitory activity of R. tuberosa HR

Many previous studies have shown that the integration of *rol* genes indirectly affected the expression of enzymes belonging to the biosynthesis pathway of secondary metabolites, leading to an increase in the bioactivity level higher than the control (Bulgakov, 2008). For example, Tusevski *et al.,* (2019) investigated the antioxidant ability and phenolic production in 15 HR clones of *Hypericum perforatum*. The results have shown that the bioactivity and secondary metabolite content of these clones are significantly higher than the non-transformed root. A study of Bergier *et al*., (2012) found that the integration of *rol*B into plant cells through the *A. rhizogenes* transformation process activated the phenylalanine ammonia lyase activity of *H. perforatum*, leading to increased flavonoids production. Root of *R. tuberosa* possesses lupeol which is the product of the mevalonate pathway (Mohan *et al*., 2014; Shibuya *et al*., 2007). This compound exhibits a broad spectrum of biological activities and can be utilized as a chemopreventive to avoid several diseases. According to Ali *et al*., (2006), lupeol reduced the activity of α-amylase which was also demonstrated in the *R. tuberosa* methanol root extract (Wulan *et al*., 2015).

By inactivating the reaction of α-amylase or α-glucosidase, starch or other carbohydrates cannot be further transformed into excess glucose in the blood. In this study, HR of *R. tuberosa* had higher α-glucosidase inhibitory activity than natural root may lead to a hypothesis. The integrated *rol* genes possibly accelerated the expression of enzymes belonging to the mevalonate pathway, resulting in an increase in lupeol or other triterpenoid compound production. Consequently, in our further study, investigating the lupeol compound in *R. tuberosa* HR is absolutely crucial for applying the strategy of secondary metabolites and biological activity enhancement.

Conclusions

We first used *A. rhizogenes* ATCC 15834 to induce HR formation from *R. tuberosa* explants and assessed the conditions affecting the transformation process. By improving induced factors, an optimized

procedure was established: ten-day-old *in vitro* cotyledons of *R. tuberosa* plantlets were injured. The explants were then immersed in the bacterial culture ($OD_{600\ nm}$ = 0.4) for 10 min containing 200 μM AS. After the time of infection, the infected explants were incubated in the dark on MS medium for 4 days. These explants were subsequently washed rigorously before being transferred to the MS medium containing 250 $mg.L^{-1}$ cefotaxime. The established *R. tuberosa* HR showed higher α-glucosidase inhibitory activity than natural roots. This study (1) provided a useful protocol for hairy root induction of *R. tuberosa* to enhance the efficiency of transformation and also (2) was a first step to creating a potential resource for diabetes treatment.

Authors' Contributions

Conceptualization, Writing – original draft, Visualization, Methodology: DMC; Data curation, Formal analysis, Investigation, Resources: TTMT; Writing – review and editing, Supervision, Validation, Project administration: PNDQ. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Ali H, Houghton PJ, Soumyanath A (2006). α-amylase inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to *Phyllanthus amarus*. Journal of Ethnopharmacology 107(3):449-455. https://doi.org/10.1016/j.jep.2006.04.004

Bergier K, Kuzniak E, Sklodowska M (2012). Antioxidant potential of Agrobacterium-transformed and non-transformed *Physalis ixocarpa* plants grown *in vitro* and *ex vitro*. Advances in Hygiene and Experimental Medicine 66:976-982. https://doi.org/10.5604/17322693.1023086

Bulgakov VP (2008). Functions of *rol* genes in plant secondary metabolism. Biotechnology Advances 26(4):318-324. https://doi.org/10.1016/j.biotechadv.2008.03.001

Cheruvathur MK, Jose B, Thomas TD (2015). Rhinacanthin production from hairy root cultures of *Rhinacanthus nasutus* (L.) Kurz, In Vitro Cellular & Development Biology-Plant 51:420-427. https://doi.org/10.1007/s11627-015-9694-9

Chilton MD, Tepfer DA, Petit A, David C, Casse Delbart F, Tempé J (1982). *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells. Nature 295:432-434. https://doi.org/10.1038/295432a0

Cujic N, Savikin K, Jankovic T, Pljevljakusic D, Zdunic G, Ibric S (2016). Optimization of polyphenols extraction from dried chokeberry using maceration as traditional technique. Food Chemistry 194:135-142. *https://doi.org/10.1016/j.foodchem.2015.08.008*

Dong HQ, Li M, Zhu F, Huang JB (2012). Inhibitory potential of trilobatin from *Lithocarpus polystachyus* Rehd against α-glucosidase and α-amylase linked to type 2 diabetes. Food Chemistry 130(2):261-266. *https://doi.org/10.1016/j.foodchem.2011.07.030*

Duta FP, França FP, Lopes LMA (2006). Optimization of culture conditions for exopolysaccharides production in *Rhizobium* sp. using the response surface method. Electronic Journal of Biotechnology 9(4). *https://doi.org/10.2225/vol9-issue4-fulltext-7*

Edwards K, Johnstone C, Thompson C (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Research 19(6):1349. *https://doi.org/10.1093/nar/19.6.1349*

Geng L, Niu L, Gresshoff PM, Shu C, Song F, Huang D, Zhang J (2012). Efficient production of *Agrobacterium rhizogenes*-transformed roots and composite plants in peanut (*Arachis hypogaea* L.). Plant Cell, Tissue and Organ Culture 109:491-500. *https://doi.org/10.1007/s11240-012-0113-1*

Georgiev MI, Pavlov AI, Bley T (2007). Hairy root type plant *in vitro* systems as sources of bioactive substances. Applied Microbiology and Biotechnology 74:1175-1185. *https://doi.org/10.1007/s00253-007-0856-5*

Grabkowska R, Królicka A, Mielicki W, Wielanek M, Wysokinska H (2010). Genetic transformation of *Harpagophytum procumbens* by *Agrobacterium rhizogenes*: iridoid and phenylethanoid glycoside accumulation in hairy root cultures. Acta Physiologiae Plantarum 32:665-673. *https://doi.org/10.1007/s11738-009-0445-6*

Guillon S, Trémouillaux-Guiller J, Pati PK, Rideau M, Gantet P (2006). Harnessing the potential of hairy roots: dawn of a new era. Trends in Biotechnology 24(9):403-409. *https://doi.org/10.1016/j.tibtech.2006.07.002*

Häkkinen ST, Oksman-Caldentey KM (2018). Progress and prospects of hairy root research. In: Hairy Roots: An Effective Tool of Plant Biotechnology. Springer, Singapore pp 3-19.

Hoque ME, Mansfield JW (2004). Effect of genotype and explant age on callus induction and subsequent plant regeneration from root-derived callus of Indica rice genotypes. Plant Cell, Tissue and Organ Culture 78:217-223. *https://doi.org/10.1023/B:TICU.0000025640.75168.2d*

Hu ZB, Alfermann AW (1993). Diterpenoid production in hairy root cultures of *Salvia miltiorrhiza*. Phytochemistry 32(3):699-703. *https://doi.org/10.1016/S0031-9422(00)95156-2*

Kang HJ, Anbazhagan VR, You XL, Moon HK, Yi JS, Choi YE (2006). Production of transgenic *Aralia elata* regenerated from *Agrobacterium rhizogenes*-mediated transformed roots. Plant Cell, Tissue and Organ Culture 85:187-196. *https://doi.org/10.1007/s11240-005-9070-2*

Kavitah G, Taghipour F, Huyop F (2010). Investigation of factors in optimizing Agrobacterium-mediated gene transfer in *Citrullus lanatus* cv. Round Dragon. Journal of Biological Sciences 10(3):209-216.

Kumar V, Jones B, Davey MR (1991). Transformation by *Agrobacterium rhizogenes* and regeneration of transgenic shoots of the wild soybean *Glycine argyrea*. Plant Cell Reports 10:135-138. *https://doi.org/10.1007/BF00232044*

Kurniawati ANL, Aulanni'am, Srihardyastutie A, Safitri A (2018). The effect of root extract *Ruellia tuberosa* L on histopathology and Malondialdehyde levels on the liver of diabetic rats. IOP Conference Series: Materials Science and Engineering 299(1):012022. https://doi.org/*10.1088/1757-899X/299/1/012022*

Mohan VR, Rajendra KN, Vasantha K (2014). GC-MS analysis of bioactive components of tubers of *Ruellia tuberosa* L. (Acanthaceae). American Journal of phytomedicine and clinical therapeutics 2(2):209-216.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15:473-497.

Pawar PK, Maheshwari VL (2004). *Agrobacterium rhizogenes* mediated hairy root induction in two medicinally important members of family Solanaceae. Indian Journal of Biotechnology 3:414-417. *http://nopr.niscair.res.in/handle/123456789/5875*

Petrova M, Zayova E, Vlahova M (2013). Induction of hairy root in *Arnica montana* L. by *Agrobacterium rhizogenes*. Open Life Sciences 8(5):470-479. *https://doi.org/10.2478/s11535-013-0157-6*

Phan HT, Quach PND, Nguyen NN (2016). Hairy induction from *Impatiens balsamina* L. using fourteen *Agrobacterium rhizogenes* strains. Science and Technology Development Journal 19(2):38-47. *https://doi.org/10.32508/stdj.v19i2.801*

Phuong VTB, Hong PTA, Phuong QND (2018). Improving hairy root induction of *Urena lobata* L. by *Agrobacterium rhizogenes* ATCC 15834 by some factors. Science and Technology Development Journal, 21(3-4):90-97. *https://doi.org/10.32508/stdj.v21i3.430*

Rajan M, Kumar VK, Kumar PS, Swathi KR, Haritha S (2012). Antidiabetic, antihyperlipidaemic and hepatoprotective activity of methanolic extract of *Ruellia tuberosa* Linn leaves in normal and alloxan induced diabetic rats. Journal of Chemical and Pharmaceutical Research 4(6):2860-2868.

Roosdiana A, Mahdi C, Safitri A (2018, December). The influence of ethanolic root extracts of *Ruellia tuberosa* L. on pancreatic protease activity and MDA level of rats (*Rattus norvegicus*) induced by MLD-STZ. In: IOP Conference Series: Earth and Environmental Science 217(1):012041.

Safitri A, Roosdiana A, Evindasari CA (2019). Hypoglycaemic activity of hydroethanolic root extracts of *Ruellia tuberosa* L in diabetic rats. In: Journal of Physics: Conference Series 1146(1):012020.

Saleh NM, Thuc LV (2009). Assessment of hairy roots induction in *Solenostemon scutellarioides* leaves by different strains of *Agrobacterium rhizogenes*. African Journal of Biotechnology 8(15):3519-3523.

Shahwar D, Ullaha S, Ahmad M, Ullah S, Ahmad N, Khan MA (2011). Hypoglycemic Activity of *Ruellia tuberosa* Linn (Acanthaceae) in Normal and Alloxan-Induced Diabetic Rabbits. Iranian Journal of Pharmaceutical Sciences 7(2):107-115.

Shibuya M, Xiang T, Katsube Y, Otsuka M, Zhang H, Yutaka EY (2007). Origin of structural diversity in natural triterpenes: direct synthesis of seco-triterpene skeletons by oxidosqualene cyclase. Journal of the American Chemical Society 129(5):1450-1455. *https://doi.org/10.1021/ja066873w*

Sinkar VP, White FF, Gordon MP (1987). Molecular biology of Ri-plasmid—a review. Journal of Biosciences, 11(1):47-57. *https://doi.org/10.1007/BF02704657*

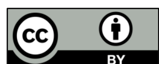
Su J, Duan RQ, Hu CQ, Li YP, Wang F (2002). Regeneration and Agrobacterium-mediated transformation for Chinese cabbage. Fujian Journal of Agriculture Science 17(4):241-243.

Tusevski O, Stanoeva JP, Stefova M, Spasenoski M, Simic SG (2019). State of antioxidant systems and phenolic compounds' production in *Hypericum perforatum* L. hairy roots. Acta Physiologiae Plantarum 41:132. *https://doi.org/10.1007/s11738-019-2919-5*

Veerasham C (2004). Medicinal plant biotechnology. CBS, New Delhi, pp 377-419.

William S, Feil H, Copeland A (2012). Bacterial genomic DNA isolation using CTAB. Sigma 50(6876).

Wulan DR, Utomo EP, Mahdi C (2015). Antidiabetic Activity of *Ruellia tuberosa* L., Role of α-amylase Inhibitor: *In Silico*, *In Vitro*, and *In Vivo* Approaches. Biochemistry Research International 1-9. *https://doi.org/10.1155/2015/349261*



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