Production of Chlorogenic Acid and Its Derivatives in Hairy Root Cultures of *Stevia rebaudiana*

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**ABSTRACT:** Chlorogenic acid and its derivatives (CADs) are valuable bioactive plant secondary metabolites with many health benefits. In the present study, *Stevia rebaudiana* hairy root cultures were established, and the culture conditions for the production of CADs were optimized. The hairy roots were induced by coculture of *S. rebaudiana* leaves and *Agrobacterium rhizogenes* (C58C1) after infection, which were further verified by PCR detection of rolB and rolC genes. HPLC-MS and HPLC analysis showed that chlorogenic acid (3-cafeoylquinic acid, 3-CQA), 3,5-dicafeoylquinic acid (3,5-CQA), and 4,5-dicafeoylquinic acid (4,5-CQA) were the major CADs in the hairy roots. Eight single roots with rapid growth rate were selected. Among them, T3 had the highest yield of CADs. B5 medium supplemented with 40 g/L sucrose was more suitable for the production of CADs than others. Under optimal culture conditions, the total content of these three compounds reached 105.58 mg/g and total yield was 234.40 mg/100 mL.

**KEYWORDS:** *Stevia rebaudiana*, hairy root, *Agrobacterium rhizogenes*, chlorogenic acid, phenylpropanoid

**INTRODUCTION**

*Stevia rebaudiana* is a perennial herbaceous plant first found in South America.1 The leaves of *S. rebaudiana* contain large amounts of sweet diterpenoid secondary metabolites, named steviol glycosides. Stevioside and rebaudioside A are the main sweet components of *S. rebaudiana* leaf.2,3 With the increase of public concern with diabetes and obesity, *S. rebaudiana* has gradually become a main resource of commercial natural zero-calorie sweetener and is cultivated in many countries such as China, Korea, Brazil, the United States, Indonesia, and Canada.4,5 Besides steviol glycosides, secondary metabolites such as polyphenols,6,7 alkaloids,8 sterols,9 flavonoids,10 triterpenoids,11 and cardiac glycosides12 are also found in *S. rebaudiana*. Compared with the high content of steviol glycosides, the amount of these bioactive components is much lower. Karaköse et al. identified 24 hydroxycinnamic acid derivatives of quinic and shikimic acid in *S. rebaudiana* leaves by LC-MS, and the contents of 3-cafeoylquinic acid (3-CQA), 3,5-dicafeoylquinic acid (3,5-CQA), and 4,5-dicafeoylquinic acid (4,5-CQA) were 35.5, 145.6, and 37.2 μg/g, respectively.6

Compared to conventional plant cultivation, which suffers from the limitation of cultivated land and climate, tissue culture (e.g., hairy root and cell suspension culture) is a promising approach to produce high-value plant secondary metabolites.13 Hairy root cultures are genetically and biologically stable compared with cell suspension cultures and, furthermore, show vigorous growth in phytohormone-free medium.14–16 Moreover, hairy root cultures often have greater capacity to produce secondary metabolites compared to their parent plants.16 According to the research of Liu et al., *Echinacea purpurea* hairy root cultures were a possible way to produce caffeic acid derivatives, and the contents of cichoric acid, caftaric acid, and chlorogenic acid in these hairy roots were 19.21, 3.56, and 0.93 mg/g dry biomass, respectively.17 It has been reported that overexpression of the AtPAP1 gene in *Platycodon grandiflorum* hairy roots caused significant increase of chlorogenic acid accumulation; the chlorogenic acid content was 421.31 μg/100 mg dry weight, which was 9.89 times higher than that of the control hairy root (42.60 μg/100 mg).18

In the present work, we establish *S. rebaudiana* hairy root cultures. HPLC-MS analysis of hairy root extracts was carried out, and three compounds, namely, 3-CQA, 3,5-CQA, and 4,5-CQA, were detected (Table 1). It was reported that these compounds have various bioactivities. Shin et al. examined the in vivo anti-inflammatory effects of 3-CQA using dextran sulfate sodium induced colitis in mice and found that 3-CQA dramatically improved colitis histological scores and thus might be used in the diet to prevent intestinal inflammation.19 Cho et al. showed that 3-CQA was a potential bioactive compound for body weight reduction and lipid metabolism regulation in vivo.20 Watanabe et al. investigated the blood pressure-lowering effect and safety of CADs through a randomized clinical trial, and their results suggested that these compounds were effective in decreasing blood pressure and safe for patients with mild hypertension.21 According to Robinson et al., dicafeoylquinic acids inhibited HIV-1 replication by inhibiting the integrase activity.22 Xiong et al. reported that 3-CQA, 3,5-CQA, and 4,5-CQA all significantly suppressed the growth of *Staphylococcus aureus* and *Escherichia coli*.23 To the best of our knowledge, there is no literature on the production of CADs by *S. rebaudiana* hairy root cultures.
Our results indicated that hairy root cultures of *S. rebaudiana* would be a promising approach to produce these bioactive compounds.

MATERIALS AND METHODS

Chemicals and Reagents. 3-CQA (HPLC grade, ≥98%), 3,5-CQA (HPLC grade, ≥97%), and 4,5-CQA (HPLC grade, ≥98%) were purchased from Tautobiotech (Shanghai, China). An EasyPure Plant Genomic DNA Kit, an EasyPure Plasmid MiniPrep Kit, and 2× TransTaq High Fidelity PCR SuperMix were purchased from TransGen Biotech (Beijing, China). The HPLC grade methanol (MeOH) was purchased from TEDIA (Fairfield, OH, USA).

*Stevia rebaudiana*. The seeds of *S. rebaudiana* var. FengNong 3 were purchased from Suzhou, Anhui, China. The seeds were successively surface-sterilized with 70% ethanol for 30 s and 0.1% (w/v) mercuric chloride for 20 min. The sterile seeds were placed on 1/2MS medium (with 3% sucrose and 0.6% agar, pH 5.8 ± 0.2) and incubated at a 12/12 h (light/dark) photoperiod at 25 °C. After 2–3 weeks of incubation, the aseptic seedlings were obtained. The infected explants were placed on the surface of 1/2MS solid medium containing Cef (500 μg/mL). After bacteria were eliminated, hairy roots were transferred to 1/2MS solid medium containing 100 μg/mL of Kan and then cultured in a shaker (Sukun, Shanghai, China) at 180 rpm and 28 °C.

**Induction of Stevia rebaudiana Hairy Root Cultures.** The young leaves of aseptic *Stevia* seedlings were collected and precultured on the surface of 1/2MS solid medium containing 100 μmol/L acetylsyringone (AS) for 2 days. These precultured explants were immersed in the culture medium of *A. rhizogenes* for 20 min, and then the surface bacteria and moisture were removed with sterile filter paper. The infected explants were placed on the surface of 1/2MS solid medium containing 100 μmol/L AS and cultured at 28 °C in the dark. After 2 days of cocultivation, the explants were transferred to 1/2MS solid medium supplemented with 500 mg/L cefotaxime sodium (Cef) to eliminate the residual bacteria. Adventitious roots appeared in the wound sites of explants in 3 weeks after infection. A single root was excised and cultured on the 1/2MS solid medium containing Cef (500 mg/L). After bacteria were eliminated, hairy roots were transferred into 1/2MS liquid medium for subculture.

**Screening of Hairy Roots.** Hairy roots with high growth rate were selected for further studies. Half a gram of hairy roots was inoculated in a 250 mL Erlenmeyer flask containing 100 mL of 1/2MS liquid medium (with 3% sucrose, pH 5.8 ± 0.2) and then cultured in a shaker at 115 rpm and 28 ± 2 °C in the dark for 24 days. The hairy roots were collected after 24 days of culture, and the fresh weight (FW) was determined after the hairy roots were rinsed with tap water and the excessive water was removed with filter paper. The hairy roots were dried at 60 °C until a constant dry weight (DW) was obtained. CAD content was determined by HPLC. Hairy root lines that contained more TP-CADs (total production of chlorogenic acid and its derivatives) were selected for further experiments.

**Confirmation of Hairy Roots.** Plasmid DNA of *A. rhizogenes* total DNA of hairy roots, and total DNA of untransformed roots were extracted with SDS reagent following by the manufacturer's instructions (TransGen Biotech, Beijing, China). PCR amplification was performed in a 50 μL reaction mixture containing 50 ng of templates DNA, 0.4 μM of each primer, and 25 μL of 2× EasyTaq PCR SuperMix (containing Taq DNA polymerase, MgCl₂, and dNTP). The forward and reverse primer sequences for rolB gene amplification were 5'-GCTCTTGCAGTCTAGATT-3' and 5'-GAAGGTGCAAGTACCTCTC-3', and those for rolC gene amplification were 5'-CTCCGTGAACATCCTGTC-3' and 5'-TGCTTCGAGTTATGGGTACA-3'. The procedure of PCR was as follows: the first cycle was performed at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 60 °C for 45 s, 72 °C for 60 s, and the final extension was 10 min at 72 °C. PCR products were analyzed by electrophoresis separation.

**Extraction of CADs.** Hairy roots were ground into fine powder after drying. Two hundred milligrams of hairy root powder was extracted by ultrasonics method in 4 mL of 70% ethanol twice. The supernatant was collected and combined after centrifugation for 10 min at 5000 rpm. Its volume was accurately adjusted to 10 mL with distilled water. After filtering through 0.22 μm pore size filter, the sample solution was used for HPLC and HPLC-ESI-MS analysis.

**Optimization of Culture Conditions.** Half a gram of T3 hairy roots was inoculated into 250 mL Erlenmeyer flasks that contained 100 mL of 1/2MS liquid medium supplemented with 30 g/L of different sugars (maltose, glucose, sucrose, or fructose) or 100 mL of different media (MS 1/2MS, B5, 1/2B5, WPM, or 1/2WPM) supplemented with 30 g/L of sucrose, or 100 mL of B5 liquid medium supplemented with different concentrations of sucrose (10, 20, 30, 40, or 50 g/L), respectively. All of the hairy roots were cultured under the same conditions described above, and FW, DW, and CAD content were determined.

**Dynamic Characteristics of Hairy Root Growth and CAD Accumulation.** The growth curve and CAD accumulation curve of T3 hairy root were established according to the change of FW, DW, and CAD content during a growth cycle. Half a gram of T3 hairy roots was inoculated in a 250 mL Erlenmeyer flask that contained 100 mL of B5 liquid medium supplemented with 40 g/L sucrose and then cultured for 36 days under the aforementioned conditions. FW, DW, and CAD content were determined every 3 days during the growth cycle.

**Data Statistics and Analysis.** The results were given as mean values ± standard deviations (SD). Data were analyzed by the DPS 6.55 statistical software, and statistical analysis was performed using the ANOVA followed by Tukey's test (at P ≤ 0.01).

### Table 1. Linear Relationships of HPLC Analysis of Chlorogenic Acids and Its Derivatives

<table>
<thead>
<tr>
<th>compound</th>
<th>regression eq</th>
<th>linearity correlation coefficient (R²)</th>
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<tbody>
<tr>
<td>3-cafeoylquinic acid</td>
<td>Y = 28.052X − 121.540</td>
<td>0.9997</td>
</tr>
<tr>
<td>3,5-dicaffeoylquinic acid</td>
<td>Y = 24.989X − 82.588</td>
<td>0.9985</td>
</tr>
<tr>
<td>4,5-dicaffeoylquinic acid</td>
<td>Y = 27.167X − 162.680</td>
<td>0.9982</td>
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</table>
RESULTS AND DISCUSSION

Establishment of Transgenic Hairy Root Lines.

Coculture is a method commonly used to induce hairy roots, by which hairy roots are induced from wounded explants when they were cocultured with *A. rhizogenes*. The sterilized *S. rebaudiana* explants (young leaves) were infected by *A. rhizogenes C58C1* after 2 days of preculture. Hairy root cultures were generated from the wounds of infected explants in the third week after infection, which showed high branching and no geotropism (Figure 1b). No similar root was observed in the

Figure 1. Establishment of *Stevia rebaudiana* hairy root cultures: (a) preculture of *S. rebaudiana* leaves on 1/2MS medium supplemented with 100 μmol/L of AS; (b) hairy roots grew from infected explants (1/2MS medium supplemented with 100 mg/L of Cef); (c) hairy roots cultured on 1/2MS solid medium; (d) hairy roots cultured in 1/2MS liquid medium.

Figure 2. PCR analysis of the *rolB* and *rolC* genes in the hairy root lines of *Stevia rebaudiana*. Lanes: M, marker (from 100 to 700 bp); 1 and 11, DNA from nontransformed roots (negative control); 2 and 12, Ri plasmid DNA (positive control); 3–10 and 13–20, DNA from T1, T3, T4, T5, T9, T14, T16, and T17 strains induced by *Agrobacterium rhizogenes*. *RolB* genes are shown in lanes 1–10, and *rolC* genes are shown in lanes 11–20.

Figure 3. HPLC chromatograms and mass spectra of hairy root extracts: (a) HPLC chromatogram of hairy root extracts; (b) mass spectrum of peak 1 shown in the HPLC chromatogram; (c) mass spectrum of peak 2 shown in the HPLC picture; (d) mass spectrum of peak 3 shown in the HPLC picture. Peaks 1 (6.877 min, *m/z* 353.1) was identified as 3-CQA (*C_{16}H_{18}O_{9})*, peak 2 (17.553 min, *m/z* 515.2) was identified as 3,5-CQA (*C_{25}H_{24}O_{12})*, and peak 3 (19.908 min, *m/z* 515.1) was identified as 4,5-CQA (*C_{25}H_{24}O_{12})*. HPLC chromatogram was obtained at 327 nm.
control. Sujatha et al. reported that *Artemisia vulgaris* hairy roots were generated in 2 weeks after co-cultivation, and no hairy roots appeared in the control group. According to Kúzma’s investigation, hairy roots grew from the wounded and infected sites of *Salvia australis* explants in 2 weeks after infection and continued to grow to the fourth week. Single root tips (2–3 cm long) were excised and cultured on 1/2MS hormone-free solid medium. The root tips grew rapidly (as shown in Figure 1c). After several successive subcultures, there was no visible bacterium on the surface of medium, indicating that the residual bacteria had been clearly removed. The bacteria-free root tips were transferred into 1/2MS liquid medium for suspension culture (Figure 1d).

**Confirmation of Hairy Roots by PCR.** Rol genes are the important Ri plasmid genes of *A. rhizogenes* and play a vital role in hairy root induction. Therefore, rol genes are commonly used as reference genes to confirm the hairy roots. In the present paper, eight hairy root samples, Ri plasmid (positive control), and uninduced root (negative control, from germ-free *S. rebaudiana* seedling) were analyzed by PCR. The results (Figure 2) showed that rolB (lanes 3–10 in Figure 2) and rolC (lanes 13–20) were present in all eight hairy root samples, as well as in the positive control (lanes 2 and 12). However, these genes were absent in the roots excised from nontransformed plants (lanes 1 and 11). The results indicated that the rol genes were successfully inserted into the genome of *S. rebaudiana*.

**Qualitative Analysis of CADs.** The extracts of *S. rebaudiana* hairy roots were analyzed by HPLC-ESI-MS and HPLC. The results indicated that there were three major compounds in the extracts (Figure 3a). These compounds were identified as chlorgenic acid (peak 1) and dicafeoylquinic acid (peaks 2 and 3) by their pseudomolecular ions at m/z 353 [M − H]−, 515 [M − H]−, and 515 [M − H]−, respectively. We further confirmed that the three compounds were 3-CQA (peak 1), 3,5-CQA (peak 2), and 4,5-CQA (peak 3), respectively, by standard addition of authentic standards.

It has been reported that the biosynthesis of steviol glycosides is limited to green tissues in *S. rebaudiana*. Steviol is produced in the plastids and endoplasmic reticulum and glycosylated by UDP-glucosyltransferases in the cytoplasm; the steviol glycosides are then stored in vacuoles. It is not surprising that no steviol glycoside was detected in the hairy root cultures because the biosynthetic genes were probably not expressed in roots. To overcome such problems, we will need to overexpress key genes of the biosynthetic pathway in the hairy roots. Certainly other parameters may also play roles for producing secondary metabolites, such as the available oxygen, nutrients, light, water, and pH. These growth conditions in the shake flask culture of hairy roots were dramatically different from those of field growth, and the notable difference may cause the silence of the critical genes of steviol glycoside synthesis. The insert of exogenous gene(s) from *S. rebaudiana* may also result in the change of gene expressing and thus lead to the absence of steviol glycoside.

**Screening of High-Producing Hairy Root Lines.** Eight hairy root lines with typical phenotype and high growth rate were selected from the induced hairy roots at the beginning. These hairy root lines were further confirmed by PCR detection of rolB and rolC genes. To get the lines with high production of CADs, the eight roots were further screened by the determination of FW, DW, and CAD content after 24 days of culture (Figure 4). Among the eight roots, T3 exhibited the highest growth rate, followed by T17. The FW and DW of T3 were 15.23 and 1.54 g/100 mL, respectively, which increased over 30 times after 24 days of culture. According to the results of HPLC analysis, T3 hairy roots had the highest contents of 3-CQA (39.41 mg/g) and 3,5-CQA (48.10 mg/g), whereas T9 contained more 4,5-CQA (4.29 mg/g). For each line, 3,5-CQA was the most abundant compound, followed by 3-CQA. The content of 4,5-CQA was much lower than that of 3,5-CQA and 3-CQA. In terms of TP-CADs, T3 was the optimal line. Its TP-CADs (140.36 mg/100 mL) is significantly higher than that of other lines. Therefore, T3 was selected as the optimal one for further experiments.

**Optimization of Culture Conditions on Biomass Accumulation and TP-CADs.** It is widely believed that sucrose is a suitable carbon source for the biosynthesis of secondary metabolites in the cultivation of plant in vitro systems. In the present paper, the effects of maltose, glucose, sucrose, and fructose on biomass accumulation and TP-CADs of T3 hairy root were compared. Our results confirmed that sucrose was the most suitable carbon source. Furthermore, the effect of sucrose concentration (10–60 g/L) on biomass accumulation and TP-CADs of T3 hairy roots was also investigated. The highest biomass accumulation was achieved in 40 g/L of sucrose, and the FW and DW were 17.93 and 2.34 g/100 mL, respectively (Figure 5a). The slow growth of hairy roots in the medium with sucrose lower than 40 g/L might be caused by the lack of energy substance. However, the inhibition of growth in higher concentration of sucrose might result from the high osmotic stress. Similar to our study, the
highest biomass accumulation of *A. vulgaris* hairy roots was obtained in 1/2MS medium supplemented with 40 g/L sucrose. The highest content of 3-CQA (39.45 mg/g) was achieved in B5 medium with 30 g/L sucrose. However, for 3,5-CQA and 4,5-CQA, the highest contents were obtained with 20 g/L sucrose, which were 69.45 and 8.63 mg/g, respectively (Figure 5).

In consideration of both biomass and metabolite content, the highest TP-CADs (233.54 mg/100 mL) were achieved in 40 g/L sucrose (Figure 5c). The results indicated that 40 g/L sucrose was the optimal concentration for both growth and TP-CADs of *S. rebaudiana* hairy roots. It has been reported that high content of caffeic acid derivatives was obtained in MS medium supplemented with 30 g/L sucrose by *E. purpurea* hairy root cultures. Wu et al. used 1/2MS medium with 50 g/L sucrose to cultivate adventitious roots of *E. purpurea* in a 1000 L airlift bioreactor and obtained 5 mg of chlorogenic acid, 22 mg of chichoric acid, and 4 mg of caftaric acids per gram of roots (DW).

Culture medium was one of the most important factors in the accumulation of secondary metabolites of in vitro cultures. In the present study, the effects of three commonly used media with two different concentrations on the production of CADs were compared (Figure 6). The results showed that 1/2MS, B5, and WPM were all suitable for T3 hairy root growth; although the highest DW (1.69 g/100 mL) was obtained in B5 medium, no significant difference was observed among these three media (Figure 6a). The highest contents of 3-CQA, 3,5-CQA, and 4,5-CQA were obtained with 20 g/L sucrose, which were 69.45 and 8.63 mg/g, respectively (Figure 6b). For TP-CADs, B5 was the most suitable culture medium with production of 167.78 mg/100 mL (Figure 6c). Therefore, B5 was selected as the optimal culture medium for *S. rebaudiana* hairy root growth and CAD production. Similar to our study, B5 medium was found to be fit for *Scutellaria baicalensis* hairy root growth, and the baicalin content of dry root cultured in this medium was 14.1−30.0%, which was significantly higher than that of the control (field-grown roots).

**Growth Kinetics of Hairy Root Cultures and TP-CADs.** The time course of *S. rebaudiana* hairy root growth in B5 liquid medium is shown in Figure 7a. The whole growth cycle could be divided into four stages: adaptive phase (0−6th day), exponential phase (6−24th day), stationary phase (24−30th day), and decline phase (after the 30th day). The highest biomass accumulation appeared in the 24−27th days, and the DW reached 2.22 g/100 mL, which was over 48 times that in inoculation. The hairy roots changed to brown, and the liquid culture medium gradually darkened after 30 days. Similar results were reported by Cesar et al., and they obtained the highest DW of muscadine grape hairy roots at the 21st day with was obtained by inoculating 0.5 g of hairy roots in 100 mL of B5 liquid medium, which was supplemented with 10, 20, 30, 40, 50, or 60 g/L sucrose. Each experiment was repeated five times.

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**Figure 5.** Effect of sucrose concentrations on biomass and CAD accumulation of T3: (a) biomass accumulation of T3 cultured in medium supplemented with 10, 20, 30, 40, 50, or 60 g/L sucrose, respectively; (b) content of 3-CQA, 3,5-CQA, and 4,5-CQA of T3 cultured in medium supplemented with 10, 20, 30, 40, 50, or 60 g/L sucrose, respectively; (c) TP-CADs of T3 cultured in medium supplemented with 10, 20, 30, 40, 50, or 60 g/L sucrose, respectively. Half a gram of hairy roots was inoculated in 250 mL Erlenmeyer flasks containing 100 mL of B5 liquid medium, which were supplemented with 10, 20, 30, 40, 50, or 60 g/L sucrose, respectively. Each experiment was repeated five times.

**Figure 6.** Effect of medium on biomass and CAD accumulation of T3: (a) biomass accumulation of T3 cultured in different media; (b) content of 3-CQA, 3,5-CQA, and 4,5-CQA of T3 cultured in different media; (c) TP-CADs of T3 cultured in different media. Half a gram of hairy roots was inoculated in 250 mL Erlenmeyer flasks containing 100 mL of MS, 1/2MS, B5, 1/2B5, WPM, or 1/2WPM liquid medium, respectively. All of the media were supplemented with 30 g/L sucrose. Each experiment was repeated five times.

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cultures of day after inoculation. Our results suggest that the hairy root production, and the best harvest date of hairy root was the 27th with 40 g/L sucrose was the best culture condition for CAD and quantification of the content of 3-CQA, 3,5-CQA, and 4,5-CQA of hairy roots: (a) time course of growth of hairy roots; (b) time course of the contents of 3-CQA, 3,5-CQA, and 4,5-CQA of hairy roots; (c) time course of TP-CADs of hairy roots. Half a gram of hairy roots was collected every 3 days. Each experiment was repeated three times.

BDS medium and at the 27th day with BDS+GR medium. The highest contents of 3-CQA (40.85 mg/g) and 3,5-CQA (61.03 mg/g) were achieved at the 27th day after inoculation. However, the highest 4,5-CQA content (9.81 mg/g) was achieved at the 36th day (Figure 7b). We obtained the highest TP-CADs at the 27th day after inoculation (Figure 7c), which was 234.40 mg/100 mL. Therefore, hairy root cultures of S. rebaudiana provide an alternative and sustainable approach to produce CADs.

In conclusion, the hairy root cultures of S. rebaudiana for the production of CADs were first reported. Three secondary metabolites, 3-CQA, 3,5-CQA, and 4,5-CQA, were identified and quantified in the hairy roots. BS medium supplemented with 40 g/L sucrose was the best culture condition for CAD production, and the best harvest date of hairy root was the 27th day after inoculation. Our results suggest that the hairy root cultures of S. rebaudiana are promising for the large-scale production of CADs.

**REFERENCES**


