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# Improving root growth and cichoric acid derivatives production in hairy root culture of *Echinacea purpurea* by ultrasound treatment

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ABSTRACT

An efficient ultrasound-stimulation strategy was developed for improving the hairy root growth and caffeic acid derivatives (CADs) biosynthesis in the hairy root cultures of *Echinacea purpurea* L. The 15-day-old hairy roots stimulated every 5 days by ultrasound for 6 min produced the highest amount of CADs after 30 days of culture among all ultrasound treatment experiments. The obvious increase of CADs production in *E. purpurea* hairy roots stimulated by ultrasound was related to the increase of both *rol*B-regulated endogenous indole-3-acetic acid biosynthesis and phenylalanine ammonium lyase (PAL) activity. These results provided a basis for understanding of improving growth and secondary metabolism in the process of hairy root culture stimulated by ultrasound.



## 1. Introduction

*Echinacea purpurea* L. is a traditional perennial medicinal herb in North America. Caffeic acid derivatives (CADs) including cichoric acid, caftaric acid, chlorogenic acid and caffeic acid, the main bioactive components in *E. purpurea*, have the remarkable activities to inhibit the replication of hyaluronidase and improve human immunity [1–3]. Commercial production of *E. purpurea* has been limited by a range of issues including contamination of plant materials by microorganisms, pollution from the environment, variability of active components and lack of pure, standardized plant material for biochemical analysis [4]. To address these issues, *E. purpurea* hairy root culture techniques have been exploited as a promising source for CADs production [5–7].

The use of a low-energy ultrasound showed improved biological activity through enhancing mass transfer rate of gas and liquid nutrients in microbial fermentation [8]. Recently, ultrasound stimulation was found to significantly improve valuable secondary metabolites in plant cell cultures [9–14]. This enhanced secondary metabolite biosynthesis in plant cells was related to induce plant defense responses and change in physiological activity of the cells stimulated by ultrasound. It was also confirmed that sonication enhanced the pigment release from hairy root cultures of *Beta vulgaris* and CADs production from *E. purpurea* hairy root cultures [5,15]. However, the ultrasound-stimulation strategies in the hairy roots of *E. purpurea* have not been developed and the functional mechanism of ultrasound on the hairy roots is still not well elucidated.

The objective of the current study was to develop an efficient ultrasound-stimulation strategy on improving root growth and CADs production from the hairy root cultures of *E. purpurea*. The possible mechanism on improving root growth and CADs accumulation by ultrasound stimulation was also discussed.

## 2. Materials and methods

### 2.1. Hairy root cultures

Hairy root cultures were induced by transforming *Agrobacterium rhizogenes* into the leaf explants of *E. purpurea* as previously described [1]. The hairy roots were maintained in liquid MS medium (pH 5.8) supplemented with 30 g $L^{-1}$ sucrose, and subcultured every 21 days in 500 ml Erlenmeyer flasks. Each flask contained 200 ml liquid MS medium with 4 g fresh hairy roots. All ultrasound experiments were conducted in 250 ml Erlenmeyer flasks containing 100 ml MS medium with 2 g fresh hairy roots on a rotary shaker at 105 rpm and 25 ± 1 °C in dark.

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### 2.2. Ultrasound treatment

An ultrasonic cleaning bath (SB120D NINGBO SCIENTZ Biotechnology Co. Ltd., China) was used to sonicate *E. purpurea* hairy roots in flasks. The ultrasound frequency is 40 kHz, and its power is 240 W. The internal dimension of the bath is 30 ($L$) × 24($W$) × 14($H$) cm.

The hairy roots were exposed to ultrasound once for 6 min at different growth stages (day 10, 15, 20 and 25) in the process of shake flask culture. The 15-day-old hairy roots of *E. purpurea* were exposed to ultrasound once for a given time (0.5, 1, 3, 6, 8 and 10 min). The 15-day-old hairy roots of *E. purpurea* were repeatedly exposed to ultrasound for 6 min at different intervals (1, 3, 5, 7 and 15 days). After the ultrasound treatments, the flasks with the hairy roots were returned to the shaker for the further culture and harvested on day 30.

### 2.3. Analytical methods

Indole-3-acetic acid (IAA) was extracted from 1 g of fresh hairy roots grinded in 2 ml 80% methanol solution for 2 min and followed by ultrasound treatment for 30 min at room temperature. After the crude extracts were centrifuged at 12,000 rpm for 10 min, the supernatant was filtered through a 0.45 μm membrane for HPLC analysis. Agilent 1100 HPLC system equipped with fluorescence detector, a quaternary pump, an on-line solvent vacuum degasser and an auto-sampler with 20 μL injection loop was used to analyze IAA accumulation in the hairy roots [16,17]. An Alltech RP-C18 column (4.6 mm × 250 mm, 5 μm) fitted with an guard column (8 mm × 4.6 mm, 5 μm) was used, and 50 mM sodium phosphate buffer (A) and acetonitrile (B) were used as chromatographic eluents. The gradient elution was programmed as follows: 0–10 min, 10% B; 10–11 min, 10–30% B; 11–27 min, 30% B. The flow rate was 0.8 ml/min, and the injection volume was 10 μL. The excitation wavelength was 280 nm, and the emission wavelength was 348 nm. Reference standard of IAA was purchased from Sigma (Sigma–Aldrich Co., USA).

Caffeic acid derivatives (CADs) were extracted from the dry root mass by methanol and 0.1% phosphoric acid (70:30, v/v). The extracting solution was centrifuged at 12,000 rpm for 10 min, and the supernatant was filtered through a 0.45 μm membrane for HPLC analysis. Agilent 1100 HPLC system equipped with variable-wavelength UV detector and an Alltech RP-C18 column with the guard column was used for CADs analysis. Ultra pure water containing 0.1% phosphoric acid (A) and acetonitrile (B) was used as chromatographic eluents. The gradient elution for CADs was programmed as follows: 0–30 min, 10–20% B; 30–110 min, 20–80% B. The flow rate was 0.8 ml/min and the injection volume was 5 μL. UV spectra were recorded in the range of 200–400 nm, while 330 nm was used for quantification of CADs. Reference standards of cichoric acid, caftaric acid, chlorogenic acid and caffeic acid were purchased from Chromadex (CA, USA).

To determine the cell viability of the hairy roots, approximately 0.2 g fresh hairy roots were incubated in 50 mM potassium phosphate buffer (pH 7.4) containing 0.8% (w/v) 2,3,5-triphenyltetrazolium chloride at 25 °C, under darkness for 5 h and then washed three times with distilled water. The intracellular insoluble formanzan was extracted from the hairy roots with 5 ml 95% ethanol at 60 °C for 30 min. The absorbance of the ethanol extract was determined at 485 nm. The root cell viability was expressed as the percent of the absorption of ultrasound-treated roots to that of the control [18,19].

Phenylalanine ammonium lyase (PAL) was extracted from 0.5 g fresh hairy roots by 5 ml 0.2 M borate buffer (pH 8.8) containing 5 mM β-mercaptoethanol and 0.5 g $L^{-1}$ polyvinyl pyrrolidone. After centrifuged at 12,000 rpm for 10 min at 4 °C, the cell-free extract was mixed with 20 mM substrate phenylalanine and reacted for 30 min at 30 °C. One unit of the PAL activity (U) is defined as the amount of absorbance variation of 0.01 per minute at 290 nm detected by UV-Spectrometer [7].

For fresh weight (FW) determination, the hairy roots were gently pressed on filter paper to remove excess water and weighed. Subsequently, the roots were dried in an oven at 60 °C for 24 h and dry weight (DW) was recorded. The root length and number of laterals were measured after 15 days. The root growth-unit was calculated by the following equation [20]:

$$\text{Root growth-unit (cm)} = \frac{\text{Total root length (cm)}}{\text{Number of inoculated root tips}} \quad (1)$$

To detect *rol*B expression with real-time RT-PCR, total RNA was extracted from the hairy roots with RNA extraction Kit (TIANGEN, China). Potential DNA contamination was removed by RNase-free DNase I (TaKaRa, Japan). For cDNA first-strand synthesis, 1 μg of RNA was used as a template for cDNA synthesis with reverse transcriptase system (Promega, USA). Quantitative RT-PCR was performed on DNA-Engine OPTICON 2 detection system (BIO-RAD, CA). 18S rRNA was considered as house-keeping gene to quantify *rol*B gene expression. A pair of 24-mer primers corresponding to *rol*B sequences 5′-TGGCGACAACGATTCAACCATATC-3′ and 5′-GTGCCGCAAGCTACAACATC-ATAG-3′ were used for PCR amplification of a 174-bp fragment from *rol*B cDNA. The primers of 18S cDNA were 5′-AGGGCTTGTGCTGTTATG-3′ and 5′-ACACCAAGTATCGCATTTT-3′ for amplification of a 195-bp fragment. Each reaction mixture consisted of 7.2 μL dd $H_2O$, 10 μL Fluorescence mix, 0.8 μL primers (forward + reverse) of *rol*B, or 18S and 2 μL cDNA. The PCR conditions were as follows: one cycle at 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 25 s, and 1 cycle at 72 °C for 10 min.

### 2.4. Statistical analysis

Each experimental condition was repeated three times, and all estimations of the samples were carried out in triplicate. The values were analyzed using one-way ANOVA followed by Duncan's multiple range test. $P$-value ≤ 0.05 is regarded as significant, and all values are presented as the means in triplicate ± S.D.

## 3. Results and discussion

### 3.1. Hairy root growth and CADs production treated once by ultrasound

To investigate the timing effect of ultrasound exposure on the CADs production, the hairy root cultures were exposed to ultrasound for a given time on various cultivation days (starting from

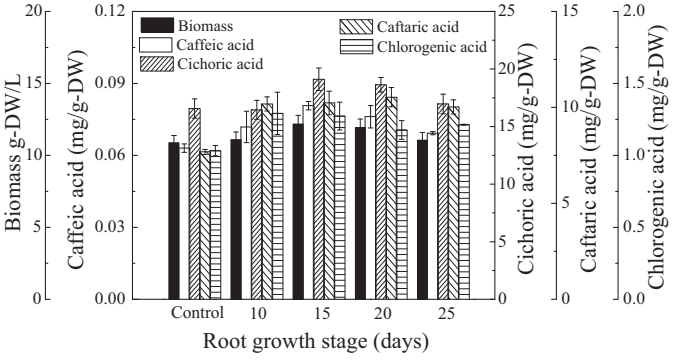


**Fig. 1.** Effects of ultrasound treatment at different growth stages on root growth and CADs accumulation in the hairy roots of *E. purpurea*. Values are means ± S.D.