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Caffeic Acid Derivatives Production by Hairy Root Cultures of *Echinacea purpurea*

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Inoculation of leaf explants of *Echinacea purpurea* (Moench) with *Agrobacterium rhizogenes* induced hairy roots with the capacity to produce biologically active caffeic acid derivatives (CADs), especially cichoric acid. The kinetics of growth, the uptake of macronutrients, and the accumulation of CADs were investigated in heterotrophically cultured hairy roots for a 50 day period. A maximum of 12.2 g L^{-1} dry biomass was achieved in MS nutrients supplemented with 30 g L^{-1} sucrose on day 40. The mathematical relationship between hairy root growth and conductivity was established during the exponential phase in Erlenmeyer flasks. HPLC analyses of methanolic (0.1% phosphoric acid; 70:30, v/v) extracts from hairy roots revealed the presence of important CADs: cichoric acid (19.21 mg g^{-1} dry biomass), caftaric acid (3.56 mg g^{-1} dry biomass), and chlorogenic acid (0.93 mg g^{-1} dry biomass). These results demonstrate that biotechnological production of CADs in hairy roots of *E. purpurea* is possible. Furthermore, these hairy root cultures offer, for the very first time, an excellent biological model to study the biosynthetic pathway of medicinally important CADs.

KEYWORDS: ***Echinacea purpurea*; *Agrobacterium rhizogenes*; hairy roots; caffeic acid derivatives; cichoric acid; HPLC**

INTRODUCTION

Echinacea purpurea (L.) Moench is a traditional North American perennial medicinal herb that has gained popularity in recent years throughout the world due to its immunostimulant activity (*1*, *2*). *Echinacea* herbal preparations are currently one of the best-selling herbs in North America and have gained great attention because of their increasing economic value (*3*, *4*). It has been reported that biological activities of *E. purpurea* products are due to the lipophilic and polar fractions of extracts, including caffeic acid derivatives (CADs), alkamides, glycoproteins, and polysaccharides (*1*, *5*−*8*). Among CADs, cichoric acid has been shown to inhibit the replication of hyaluronidase and human immunodeficiency virus type 1 integrase, to protect collagen-type free radical induced degradation, and to possess phagocytosis stimulatory activity in vitro and in vivo and antiviral activity (*9*−*14*).

Commercial production of *Echinacea* 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 (*15*). To address these issues, in vitro tissue culture techniques of plant cells have been exploited to obtain a standardized method for secondary metabolite production of *Echinacea* (*16*, *17*). Hairy root cultures are a promising in vitro source for a more standardized production of valuable phytochemicals in many medicinal plant species due to their biochemical and genetic stability (*18*−*20*). Very few reports on *Agrobacterium*-mediated transformation of *E. purpurea* are available (*21*−*23*), but none of them are related to establishment of hairy root cultures with the capacity to produce CADs. The main objective of the current study was to establish an efficient liquid culture system of *E. purpurea* hairy roots for biosynthesis of CADs, especially cichoric acid.

MATERIALS AND METHODS

Bacterial Strain. *Agrobacterium rhizogenes* strain ATCC 43057 used in the present study was purchased from ATCC, Manassas, VA. The strain was grown on YMB medium (*23*) and subcultured at monthly intervals.

Plant Materials. Seeds of *E. purpurea* were purchased from Richter's, Goodwood, Ontario, Canada. Surface-sterilized seeds were placed on MS (*24*) agar (6 g L^{-1}) solidified medium (pH 5.8)

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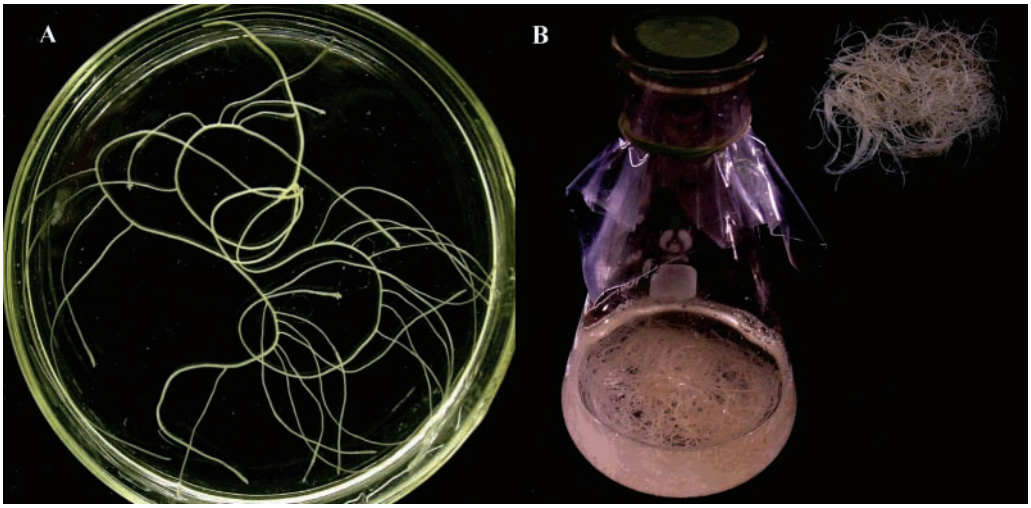

Figure 1. *E. purpurea* hairy roots in a Petri dish (**A**) and a liquid flask (**B**).

supplemented with 30 g L^{-1} sucrose. The seed cultures were incubated in light at 25 ± 1 °C for germination. After 4 weeks of growth, axenic leaves from the germinated seedlings were cut into explants and precultured on phytohormone free solid MS medium for 2 weeks.

Hairy Root Cultures. The leaf explants were infected by being dipped into an *A. rhizogenes* suspension for 30 min and then were washed three times with sterile distilled water to remove excess *Agrobacterium*. After 3 days of cocultivation with bacteria, the leaf explants were transferred to solid (agar, 6 g L^{-1}) MS medium supplemented with 30 g L^{-1} sucrose and 200 mg L^{-1} kanamycin and incubated in a growth chamber at 25 ± 1 °C to induce hairy roots. Five weeks after infection, the hairy roots were excised from infected *E. purpurea* leaf explants and cultured on phytohormone free MS medium supplemented with 30 g L^{-1} sucrose and 200 mg L^{-1} kanamycin. The procedure was repeated three times to ensure that no bacterial cell colony survived. Thereafter, the sterile hairy root cultures were maintained on phytohormone-free solid MS medium supplemented without kanamycin but with 30 g L^{-1} sucrose for further confirmation of bacterial cell colony eradication. A liquid culture was obtained by incubating the root tip explants in a 250 mL Erlenmeyer flask containing 50 mL of phytohormone-free MS medium supplemented with 30 g L^{-1} sucrose. Roots were then grown in the dark at 25 ± 1 °C on a rotary shaker (KeXue Yi Qi Chang-P270, Wuhan, China) at about 100−105 rpm. These roots were subcultured every 21 days and maintained for about 2 years in the liquid medium. The presence of the bacterial genes in the transgenic hairy roots was confirmed by Southern blotting techniques as described by Caraderelli et al. (*25*).

The kinetics of root growth were investigated in long-term, heterotrophically cultured transgenic roots (1 g fresh mass of inoculum per 50 mL of medium; pH adjusted to 5.8 prior to autoclave) grown for 50 days in the dark at 25 ± 1 °C. The hairy roots were sampled every 5 days to determine biomass accumulation, nutrient consumption, pH and conductivity of the medium, and the CADs production. Dry biomass was measured by oven-drying hairy roots at 60 °C for 24 h. The pH (ThermoOrion-818) and conductivity (Precision conductometer DDS 307, China) were measured in the biomass-free culture medium with digital instruments.

Nutrient Consumption. The residual sucrose concentration was determined by a method using phenol and concentrated sulfuric acid, using glucose solution as the standard (*26*), and the nitrate (NO_3^-), ammonium (NH_4^+), and extracellular phosphate (P_i) were determined according to the methods of Wang et al. (*27*), Weatherburn et al., (*28*), and Chen et al., (*29*), respectively.

Extraction and Determination of CADs and Alkamides. Extraction and quantification of hairy roots was carried out using the method described by Luo et al. (*30*) with minor modification. Briefly, a 20 mg dry root sample was ground and ultrasonicated in methanol and 0.1% phosphoric acid (70:30, v/v; 1 mL for each) for 30 min. After centrifugation (10000 rpm, 5 min), a 0.1 mL aliquot of the extract was filtered through a 0.45 μm membrane into an HPLC vial for analysis.

An Agilent 1100 HPLC system equipped with variable-wavelength UV detector, a quaternary pump, an on-line solvent vacuum degasser, and an autosampler with a 20 L injection loop was employed in the current study. An Alltech analytical column (250 mm × 4.6 mm i.d., 5 μm) fitted with an Alltech C18 guard cartridge (8 mm × 4.6 mm i.d., 5um) was used at a column temperature of 25 °C. Ultrapure water containing 0.1% phosphoric acid (A) and acetonitrile (B) were 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. For alkamides, ultrapure-water-containing acetonitrile (55:45) was used isocratically with a flow rate of 1 mL/min and an injection volume of 5 μL. UV spectra were recorded in the range of 200−400 nm, while 330 nm was used for quantification of CADs and 254 nm for alkamides. Reference standards of cichoric acid, caftaric acid, chlorogenic acid, caffeic acid, and dodeca-2(*E*),4(*E*),8(*Z*),10(*E*/*Z*)-tetraenoic acid isobutylamide were purchased from Chromadex (California). The quantification of caftaric acid (t_R, retention time, of 12.1 min), chlorogenic acid (t_R = 12.4 min), caffeic acid (t_R = 16.33 min), and cichoric acid (t_R = 33.91 min) was determined on the basis of the peak area of the UV absorption at 330 nm with comparison to the above-mentioned CADs standards, and dodeca-2(*E*),4(*E*),8(*Z*),10(*E*/*Z*)-tetraenoic acid isobutylamide (t_R = 16.33 min) was determined at 254 nm with comparison to the reference standard. Stock standard solutions of CADs and dodeca-2(*E*),4(*E*),8(*Z*),10(*E*/*Z*)-tetraenoic acid isobutylamide standards (500 μg mL^{-1}) were prepared in methanol and stored at −20 °C. The standard working solutions at the concentration of the calibration range were prepared by serial dilutions of stock solutions with methanol. To determine the precision of the method, three samples of hairy root cultures (at low, middle, and high levels of the calibration range) were analyzed three times on the same day. The accuracy of the method was evaluated by analyzing recovery percentages. Moreover, recoveries were calculated by using the ratio of the detected to the added.

Data Analysis. Triplicate flasks were used in all experiments, and the experiments were repeated twice. All data were the mean ± SD.

RESULTS AND DISCUSSION

Growth Kinetics of Hairy Roots in Conical Flasks. Leaf explants developed roots 5 weeks after infection with *A. rhizogenes*. About 65% of the inoculated leaves developed roots either directly or through callus formation. The hairy roots showed typical morphological traits, with primary roots producing extensive lateral branching and an abundance of root hairs (**Figure 1A**). The hairy roots cultured on liquid MS medium supplemented with 30 g L^{-1} sucrose showed rapid growth with a lag phase of 5 days during a 50 day period of the study, and the maximum dry biomass of 12.2 g L^{-1} was obtained at day 40 (**Figures 1B** and **2**A). Similar growth rates were also

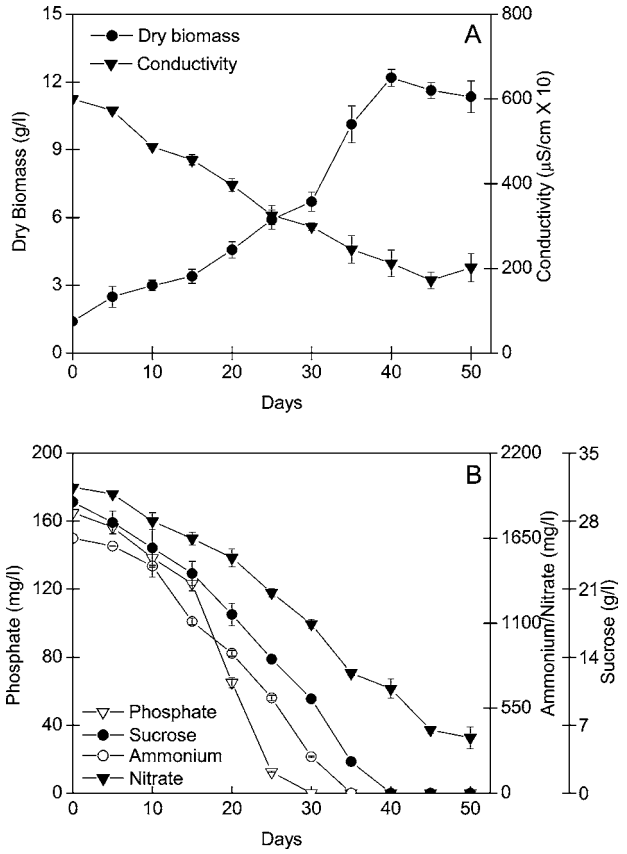

Figure 2. Time course of growth and conductivity change (**A**) and nutrient consumption (**B**) in an *E. purpurea* hairy root culture. Values are means ± SD.

observed in our previous study with hairy roots of *Artemisia annua* L (*31*). The growth rate of transformed roots depended on linear root extension, the formation of new growing points, and a secondary increase in root diameter (*32*). From a kinetic perspective, hairy roots are divided into two cell types: tip cells or cells in the apical meristem, which continuously divide, and the remaining cells that do not divide (*33*). Meristematic cells at the base of tip cells divide to form a new tip, which results in the formation of a lateral branch (*19*). The conductivity of the culture medium decreased with an increase of the dry mass between day 0 and day 40 and increased after 45 days because of the decay of the root cultures (**Figure 2A**). A linear relationship with an intercept between the increase in dry mass and the decrease in medium conductivity is shown in the following equation: $\Delta X = K\Delta C + \beta$, where X is the dry biomass (g L^{-1}), K is the empirical coefficient (g cm^{-1} ms^{-1}), C is the conductivity (μs cm^{-1}), and β is an empirical constant (the intercept). The parameters K and β were determined to be 621.2 g cm^{-1} ms^{-1} and -37.8 g L^{-1}, with the correlation coefficient R^2 being 0.99. Similar results have also been observed in other suspension cell cultures of medicinal plant species (*33*−*36*).

Consumption of Nutrients and Change in pH. P_i was the first nutrient to be depleted, within 30 days, from an initial level of 170 mg L^{-1}. NH_4^+ and sucrose were depleted from initial levels of 1650 mg L^{-1} and 30 g L^{-1}, respectively, in 35 and 40 days (**Figure 2B**). A drop in pH value to 4.5 and lower values and the subsequent increase to 5.3 (data not shown) were studied for 50 day hairy root cultures. The pH value during the course of an experiment is a good indicator of the condition of the hairy root culture. At first, the pH value decreases because the culture uses NH_4^+ ions and hence releases H^+ ions into the medium (*35*). Later, the medium is depleted of NH_4^+, and the hairy root culture metabolism changes to use NO_3^- ions and release OH^- ions in exchange for the negatively charged nitrate with a rise in pH (*37*, *38*). The relative order of uptake of P_i, sucrose, and NH_4^+ may have implications for optimization of biomass production in long-term batch or continuous plant culture bioprocesses such as nutrient feeding during an initial semicontinuous operation (*33*). The rapid consumption of NH_4^+ causes strong membrane depolarization that can block the anion/H^+ cotransport of NO_3^- (*39*). Similarly, uptake of phosphate that proceeds with a proton symport stoichiometry of $4H^+/H_2PO_4^-$ can be inhibited by NH_4^+ uptake (*40*). In the current study, these interactions among NH_4^+, P_i, and NO_3^- at the plasma membrane are possibly reflected by the initial acidification and subsequent alkalization of the extracellular medium of *E. purpurea* hairy root cultures.

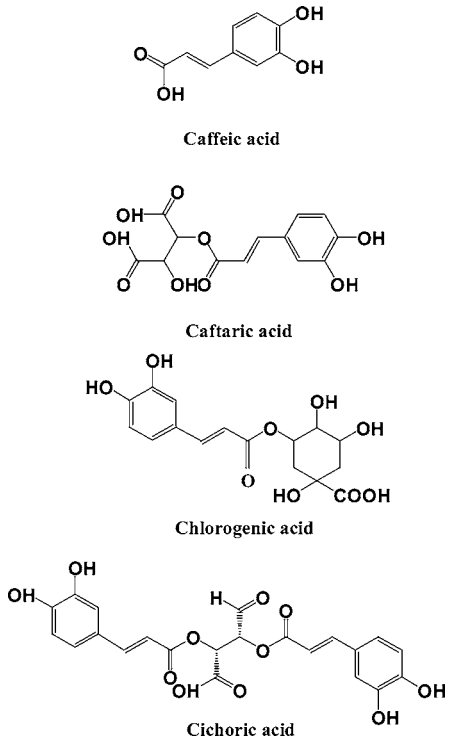

Figure 3. Chemical structures of caffeic acid derivatives detected in hairy root samples of *E. purpurea* by HPLC.

Caffeic Acid Derivatives Production. The main CADs, including caffeic acid, cichoric acid, caftaric acid, and chlorogenic acid (**Figure 3**), were detected by the valuable HPLC method in *E. purpurea* hairy root samples. Detection limits for caftaric acid, chlorogenic acid, caffeic acid, cichoric acid, and dodeca-2(*E*),4(*E*),8(*Z*),10(*E*/*Z*)-tetraenoic acid isobutylamide were 0.02, 0.009, 0.02, 0.002, and 0.01 μg mL^{-1}, respectively. The calculated recovery (%) value ranges from 96.79 to 104.46, and the precision (%) ranges from 0.10 to 2.90 from repeated measurements as summarized in **Table 1**.

As shown in **Figure 4**, the accumulation of cichoric acid and caftaric acid occurred to maximum levels of 19.21 and 3.56 mg g^{-1} dry biomass after 45 days, respectively. Chlorogenic acid has shown a maximum accumulation of 0.93 mg g^{-1} dry biomass after 40 days, and caffeic acid was detected in our long-term culture but synthesized at very low levels (<0.1 mg g^{-1} dry biomass). The most striking aspect of this work was to discover major CADs production (cichoric acid, caftaric acid, and chlorogenic acid) in the hairy roots at levels comparative to those in the source mother plants, and the highest content of cichoric acid was found especially in the hairy root cultures

Table 1. Precision and Accuracy Data for Caffeic Acid Derivatives and Dodeca-2(*E*),4(*E*),8(*Z*),10(*E*/*Z*)-tetraenoic Acid Isobutylamide

sample	added concn (μg mL^{-1})	found concn (μg mL^{-1})	recovery[a] (%)	precision[b] (%)
caftaric acid	20	20.31	101.54	0.38
	4	4.02	100.52	0.18
	2	2.04	100.19	0.10
chlorogenic acid	20	19.61	98.04	1.36
	4	3.94	97.99	2.90
	2	2.05	102.44	0.91
caffeic acid	20	19.36	96.79	0.27
	4	4.08	101.87	0.73
	2	2.06	102.83	0.74
cichoric acid	20	20.74	103.69	0.95
	4	4.17	104.46	0.32
	2	2.01	100.46	0.20
dodeca-2(*E*),4(*E*),8(*Z*),10(*E*/*Z*)-tetraenoic acid isobutylamide	0.1	0.103	102.98	0.53
	1.0	1.024	102.39	0.48
	10.0	10.42	104.18	0.20

[a] Recovery = CAD or alkamide concentration found in the sample/CAD or alkamide added concentration. [b] Precision = coefficient of variation.

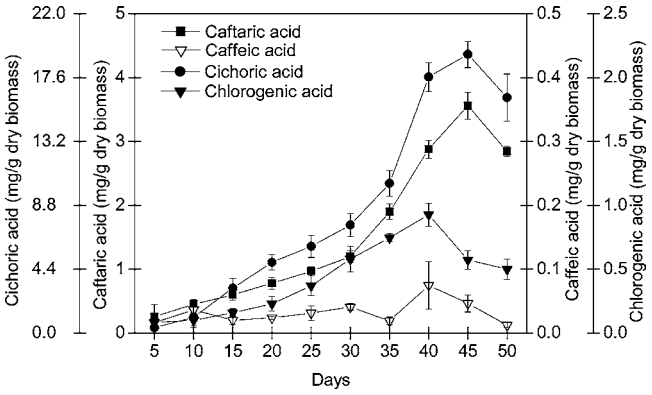

Figure 4. Time course of cichoric acid, caftaric acid, chlorogenic acid, and caffeic acid accumulation in an *E. purpurea* hairy root culture. Values are means ± SD.

among all parts of the mother plant material tested (data not shown). The dodeca-2(*E*),4(*E*),8(*Z*),10(*E*/*Z*)-tetraenoic acid isobutylamide was observed at lower levels in plant materials from the source mother plant, but was not detected in the hairy roots induced from leaf explants of the source mother plants. The results in the current study with the hairy roots showed an obvious difference from those in a previous report in which biologically active dodeca-2(*E*),4(*E*),8(*Z*),10(*E*/*Z*)-tetraenoic acid isobutylamide was observed in the limited-growth hairy root cultures induced from hypocotyls of *E. purpurea* germinated seedlings (*21*). This might be due to cultivation conditions, the *A. rhizogenes* strain types exploited for transformation, genetic diversity existing in the seed lot (*41*), the type of explants infected by *A. rhizogenes*, or the age/stage (*42*) of the source plant exploited for the purpose of induction of hairy roots.

Considerable variation has been observed among different populations of *E. purpurea* grown and cultivated in different regions of the world (*30*, *43*−*45*). Studies have shown that CADs production is affected by seasonal changes, the cultivation time, the growing site, the harvest period, and genetic diversity in the seeds (*46*). Recently, it has been inferred that the development of a high-quality *E. purpurea* crop requires a detailed understanding of the physiology and metabolism of the species (*41*). Therefore, the hairy root cultures developed in the current study will provide a good system to study CADs biosynthesis further.

In conclusion, hairy root culture is a promising alternative for the production of secondary metabolites because they are genetically stable and can grow in hormone-free medium and produce valuable secondary metabolites at a rate comparable to that of the original plants. To the best of our knowledge, this is the first report on significant CADs production in hairy root cultures of *E. purpurea*. Our results showed that the established hairy root cultures are a promising and continuous source for important CADs in the species, especially for cichoric acid, which is gaining interest due to its antiviral and inhibition activity against the integrase of HIV-1. Further investigations are being conducted to improve CADs production to commercially interesting levels by different strategies.

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