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ScienceDirect
Colloids and Surfaces B: Biointerfaces 53 (2006) 101–104
COLLOIDS AND SURFACES B
www.elsevier.com/locate/colsurfb

Genetic transformation of *Echinacea purpurea* with *Agrobacterium rhizogenes* and bioactive ingredient analysis in transformed cultures

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Received 27 June 2006; received in revised form 23 July 2006; accepted 9 August 2006
Available online 17 August 2006

Abstract

A method of the transformed hairy roots cultures of *Echinacea purpurea* was established by infecting different types of explants with three type strains of *Agrobacterium rhizogenes* (A4, R1601 and R1000) in this paper. We obtained that the transformed percentage of *E. purpurea* leaves with A4, R1601 and R1000 were 80%, 60%, 40%, respectively and that of *E. purpurea* leafstalks were 10%, 30%, 45%, respectively. The contents of polysaccharides and phenolic compounds were measured in transformed hairy roots and non-transformed roots after 2 months in culture. For transformed hairy roots, the contents of polysaccharides and phenolic compounds were 236.0 and 18.9 mg g^{-1} DW, respectively. While the contents of polysaccharides and phenolic compounds in non-transformed roots were 161.5 and 33.3 mg g^{-1} DW, respectively.

Keywords: *Agrobacterium rhizogenes*; *Echinacea purpurea*; Hairy roots; Phenolic compounds; Polysaccharides

1. Introduction

Echinacea spp. (family Asteraceae) herbal medicines and dietary supplements are traditionally used as immunostimulants in the treatment of inflammatory and viral diseases. The species in common use are *E. Angustifolia* DC (roots), *E. Pallia* Nutt (roots), and *E. purpurea* (L.) Moench (roots and aerial parts) [1]. *Echinacea purpurea* (L.) Moench is an important commercial species. A great deal of research has indicated the chemical composition of *Echinacea* spp., including alkylamides, caffeinic derivatives, and polysaccharides attracting claims of beneficial pharmacological activity [2].

Transformed roots provide great potential as a production system of plant metabolites. Some high value pharmaceuticals, pigments, and cancer treatment medicines are known to be produced from plant tissue cultures. The transformed hairy roots by infecting with *Agrobacterium rhizogenes* and inserting Ri-plasmid into the chromosomes of host plant cells exhibit genetic and biochemical stability [3].

As the long growth cycle and the limitation of environment, it is rather difficult for pharmacy to transplant *E. purpurea* and breed it. However, hairy roots of medicinal plants, produced by infection with *A. rhizogenes*, have rapid growth and stability of physiology, biochemistry and transmissibility and are easy to control and operate. So it has developed into a system to obtain plant metabolites for pharmacy after the technique of cell culture [4]. The purpose of this study was to investigate the transformation sensitivity of *E. purpurea* through *A. rhizogenes* and synthesis of polysaccharides and phenolic compounds in the transformed hairy roots.

2. Materials and methods

2.1. A. rhizogenes and culture

A. rhizogenes strain A4 and R1000 were agro-pine type and *A. rhizogenes* strain R1601 was cucumopine type. This three type strains were used for infections. Bacteria from glycerol stock cultures (stored at −80 °C) were cultured on a LB solid medium [peptone (10 g L^{-1}), yeast extract (5.0 g L^{-1}), NaCl (10 g L^{-1}); pH 7.0] contains 50 mg L^{-1} (w/v) kanamycin for screening and selecting the desired colony. Bacterial cultures were prepared

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0927-7765/$ – see front matter © 2006 Published by Elsevier B.V.
doi:10.1016/j.colsurfb.2006.08.003

by incubation of a loop of bacteria in 20 mL liquid LB medium for 12 h at 28 °C with reciprocating shake at 130 rpm [5].

2.2. Plant materials

Seeds of *E. purpurea*, obtained from the plants growing in Canada, were surface sterilized in 70% (v/v) ethanol for 30–60 s, in 5% (v/v) clorox containing three drops of Tween-80 for 5–8 min, then in 0.1% $HgCl_2$ solution for 3–6 min, finally rinsed five times with sterile distilled water and inoculated on MS medium. Cultures were kept under 12/12 h light and dark cycles (2000 Lux) and at 25 °C. Germination started within 3–4 days. Explants like leaves and leafstalks of 10–15 days old seedlings were used for genetic transformation.

2.3. Transformation of hairy roots

Laminas were excised from 10 to 15 days old seedlings and cut into pieces of approximately 2 cm in length. The cotyledon explants were immersed in a suspension of Agro infection for 3–8 min and then blotted on sterilized filter paper. As antitheses, explants were immersed similarly in LB liquid medium without bacteria. All explants were then placed in plate that contained hormone free MS solid medium for co-cultivation. After 2 days of co-cultivation, explants were transferred to fresh solid MS medium that contained 500 mg L^{-1} cefotaxime and they were sub-cultured at 2-week intervals to eliminate the bacteria. Bacteria-free explants of laminas that had produced hairy roots after several subcultures were transferred to MS medium without cefotaxime [6]. We excised roots tips of approximately 1 cm in length from hairy roots that had grown vigorously on this medium and transferred them to fresh MS medium. Hairy roots were sub-cultured similarly at 1-month intervals. Cultures were incubated at 28 °C with 16 h photoperiod light at 2000 Lux. Then the hairy roots were transferred into liquid cultures.

2.4. PCR analysis

DNA was extracted from transformed hairy roots using a simplified CTAB mini-prep method [7]. PCR analysis was conducted with the following rolB primers: 5′-GCTCTTGCAGTGCTAGATTT-3′, 5′-GAAGGTGCAAGCTACCTCTC-3′ with a predicted product size of 423 bp. Amplification conditions for rolB were 1 cycle at 94 °C for 3 min followed by 1 cycle at 94 °C for 50 s, 1 cycle at 56 °C for 45 s, 34 cycles at 72 °C for 1 min and 1 cycle at 72 °C for 10 min. PCR products were analyzed by electrophoresis in a 1% (w/v) agarose–ethidium bromide gel. Meanwhile the Ri-plasmid of *A. rhizogenes* was extracted as positive control.

2.5. Determination of the content of polysaccharides and phenolic compounds in hairy roots

2.5.1. Extraction of polysaccharides and phenolic compounds

Some fresh hairy roots or non-transformed roots in liquid medium were chosen and washed with distilled water, and then filter paper was used to absorb the water on their surface. The roots were weighted and dried at 80 °C in vacuum box until constant weight, then weighted again. After that, the roots were cut up, put into triangle bottles, added 50% ethanol in the bottles and sealed them up with membrane. Under the condition of 28 kHz ultrasonic, they were extracted by 40% ethanol for 15 min twice [8]. The extracted liquid was merged and its volume is determined. The samples were filtered through 0.22 μm micro-filtration membrane, to determine the content of phenolic compounds; 25 mL of it was also filtered, sank in 85% ethanol for a night and centrifuged. Collected the deposition, volume is determined to 25 mL, to determine the content of polysaccharides.

2.5.2. Determination

The content of phenolic compounds was measured by spectrophotometry at 650 nm with pyrogallol acid as standard sample [9]. And the content of polysaccharides was measured by spectrophotometry at 490 nm using phenol–sulfuric method and glucose standard sample [10].

3. Results and discussion

A. rhizogenes infectivity was determined by observing the frequency of hairy roots formation on leaves and leafstalks. No hairy roots formation was observed in control explants.

3.1. Hairy roots formation

Different types of *A. rhizogenes* and different explants had great influence on the induction of hairy root (see Table 1). In this study, three kinds of *A. rhizogenes* were used to infect leafstalks and leaves, respectively. It was found that leaves were comparatively easier to produce hairy roots than leafstalks, which may attribute to their better ability to regenerate and the cells around the cut were easier to dedifferentiation to be competent cell, in favor of production of hairy roots. A4 had higher transformed percentage of *E. purpurea*, especially had high transformed percentage of leaves (80%), while the transformed percentage of leafstalks was only 10%. R1601 had better ability to induce leaves to produce hairy roots than leafstalks. Moreover, R1000 had better ability to induce leafstalks to produce hairy roots (the percentage was 45%) than leaves, turned out to

Table 1
Effect of strain types and type of explants on the frequency (%) of hairy roots induction

Explants	Bacteria	Number of inoculation	Number of inducement	Rate of inducement (%)
Leaves	A4	20	16	80
	R1601	20	12	60
	R1000	20	8	40
	Control	20	0	0
Leafstalks	A4	20	2	10
	R1601	20	6	30
	R1000	20	9	45
	Control	20	0	0