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Full Length Research Paper

Rosmarinic acid production from transformed root cultures of *Nepeta cataria* L.

Sook Young Lee[1], Chung Yeol Lee[2], Seok Hyun Eom[3], Yong Kyoung Kim[4], Nam Il Park[4] and Sang Un Park[4]*

[1]Research Center for Oral Disease Regulation of the Aged, Chosun University, 375 Seosuk-Dong, Dong-Gu, Gwangju, 501-759, Korea.
[2]Department of Plant Bioscience, College of Natural Resourece and Life Science, Pusan National University, Miryang, Gyeongsangnam-do, 627-706, Korea.
[3]Department of Horticultural Biotechnology, College of Life Sciences, Kyung Hee University, 1 Seocheon-Dong, Giheung-Gu, Yongin, Gyeonggido 446-701, Korea.
[4]Department of Crop Science, Chungnam National University, 220 Gung-Dong, Yuseong-Gu, Daejeon, 305-754, Korea.

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Rosmarinic acid, a natural polyphenol antioxidant carboxylic acid, is an important secondary metabolite in ***Nepeta cataria*, a member of the mint family (Labiatae) and has antioxidant, anti-inflammatory and antimicrobial activities. To investigate, *in vitro* production of rosmarinic acid, hairy root cultures of *N. cataria* were established by infecting leaf and stem explants with *Agrobacterium rhizogenes* R1000, and tested the growth and rosmarinic acid production of these cultures. Hairy roots were cultured in Murashige and Skoog liquid medium and maximum growth (11.2 g dry wt/l) was attained after 15 days of culture, at which time the content of rosmarinic acid was 13.6 mg/g dry wt. These results demonstrate that hairy root culture of *N. cataria* is a valuable alternative approach for the production of rosmarinic acid.**

Key words: *Agrobacterium rhizogenes*, catnip, hairy root, *Nepeta cataria* L., rosmarinic acid.

INTRODUCTION

The perennial herb, *Nepeta cataria* L., commonly known as catnip, is a member of the mint family (Labiatae) and has a considerable folkloric reputation. In traditional use, catnip is believed to have sedative, carminative, and antispasmodic properties. It has also been used traditionally to treat colds, flu, and fevers (Tucker and Tucker, 1988; Grognet, 1990). The constituents of catnip have been investigated and several classes of secondary metabolites have been isolated such as flavonoids, phenolic compounds, essential oil-containing monoterpenes, terpenoids, and sterols (Ganzera et al., 2001; Chauhan et al., 2005; Klimek and Modnicki, 2005; Modnicki et al., 2007; Heuskin et al., 2009).

Rosmarinic acid (Figure 1), an ester of caffeic is found in big quantities in several plant species and it is one of the main active constituents of *N. cataria.* The principal activities of rosmarinic acid include an astringent property, antioxidant capacity, anti-inflammatory activity, antimutagenic ability, antimicrobial capacity, and an antiviral property (Parnham and Kesselring, 1985; Ly et al., 2006) (Figure 1).

Hairy root cultures of many plant species have been widely studied for the production of secondary metabolites useful as pharmaceuticals, cosmetics, and food additives (Guillon et al., 2006; Giri and Narasu, 2000). Biotechnological production of rosmarinic acid by plant cell culture and hairy root culture of several plant species has been reported (Park et al., 2008). Rosmarinic acid production by hairy root culture of *N. cataria* has never been reported. Here, we describe the production of rosmarinic acid by hairy root cultures of catnip transformed with *Agrobacterium rhizogenes.*

*Corresponding author. E-mail: supark@cnu.ac.kr.

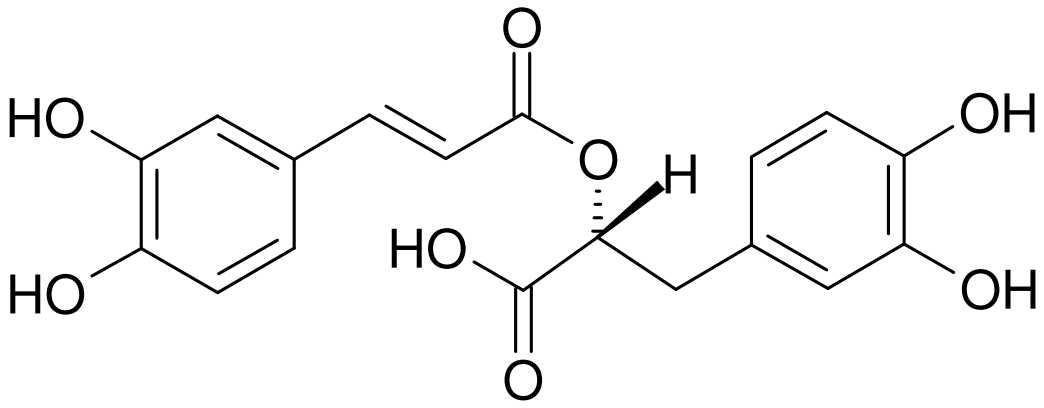

Figure 1. Chemical structure of rosmarinic acid.

MATERIALS AND METHODS

Seed sterilization and germination

Seeds of *N. cataria* were purchased from Otto Richter and Sons Limited (Goodwood, Canada) and stored at 4°C. The seeds were surface-sterilized with 70% (v/v) ethanol for 30 s and 2% (v/v) sodium hypochlorite solution for 10 min, then rinsed three times in sterilized water. Ten seeds were placed on 25 ml of agar-solidified culture medium in Petri dishes (100 x 15 mm).

The basal medium consisted of salts and vitamins of MS (Murashige and Skoog, 1962) medium and solidified with 0.7% (w/v) agar. The medium was adjusted to pH 5.8 before adding agar, and then sterilized by autoclaving at 121°C for 20 min. The seeds were germinated in a growth chamber at 25°C under standard cool white fluorescent tubes with a flux rate of 35 μmol s^{-1} m^{-2} and a 16 h photoperiod.

Culture of *A. rhizogenes*

A. rhizogenes R1000 were grown to mid-log phase (OD_{600} = 0.5) at 28°C on a gyratory shaker (180 rpm) in liquid Luria-Bertani (LB) medium. The bacterial cells were collected by centrifugation for 10 min at 2000 rpm, and resuspended at a cell density of A_{600} = 0.5 in liquid inoculation medium (MS salts and vitamins, with 30 g/l sucrose).

Establishment of hairy root cultures

Young leaves and stems of *N. cataria* were taken from plants grown in *vitro.* Excised leaves and stems were dipped into *A. rhizogenes* culture in liquid inoculation medium for 10 min, blotted dry on sterile filter paper, and incubated in the dark at 25°C on agar-solidified MS medium. After 2 days of co-cultivation, the explant tissues were transferred to a hormone-free medium containing MS salts and vitamins, 30 g/l sucrose, 200 mg/l timentin, and 8 g/l agar.

Numerous hairy roots were observed emerging from the wound sites within 4 weeks. The hairy roots were separated from the explant tissues and subcultured in the dark at 25°C on agar-solidified MS medium. After repeated and transfer to fresh medium, rapidly growing hairy root cultures were obtained. Isolated roots (0.5 g dry weight/l) were transferred to 30 ml of MS liquid medium, containing 30 g/l sucrose, in 100 ml flasks. Root cultures were maintained at 25°C on a gyratory shaker (100 rpm) in a growth chamber under standard cool white fluorescent tubes with a flux rate of 35 μmol s^{-1} m^{-2} and a 16 h photoperiod. After 18 days of culture, hairy roots were harvested and dry weights and rosmarinic acid contents were determined. Three flasks were used for each culture condition, and experiments were performed in duplicate.

HPLC analysis of rosmarinic acid

Harvested hairy roots of *N. cataria* (1 g) were frozen in liquid N_2, ground to a fine powder using a mortar and pestle, and extracted twice with methanol (10 ml) for 24 h at 25°C. Extracts were reduced to dryness under vacuum dried, and dissolved in methanol. The extracts were analyzed by high performance liquid chromatography (HPLC) on a C_{18} reverse phase column (4.6 x 250 mm; Ultrasphere, Beckman-Coulter) at room temperature.

The solvent gradient used in this study was formed through with an initial proportion of mix of 70% solvent A (3% acetic acid in water) and to 30% solvent B (methanol). After 50 min, the solvent gradient had reached 100% solvent B. The flow rate of the solvent was kept constant at 1.0 ml/min. Samples (20 µl) were detected at wave lengths of 280 nm. We identified the rosmarinic acid in solution A by matching the retention times and spectral characteristics to those from single HPLC run of a known rosmarinic acid standard.

RESULTS

Hairy root formation and culture system of catnip (*N. cataria* L.) for rosmarinic acid production was established using *A. rhizogenes* R1000. Two different explants, leaf and stem, were investigated for hairy root induction. Leaf and stem explants of *N. cataria* were inoculated with *A. rhizogenes* R1000. After 2 days of co-cultivation with *A. rhizogenes,* two explant tissues were transferred to agar-solidified MS medium containing 200 mg/l timentin, for removing *A. rhizogenes.* Initially, callus developed within 10-15 days of bacterial inoculation at the wounded edges of stem (Figure 2a) and leaf (Figure 2b) explants and then, hairy roots emerged within 4 weeks.

Among the different explants used, leaf explant showed

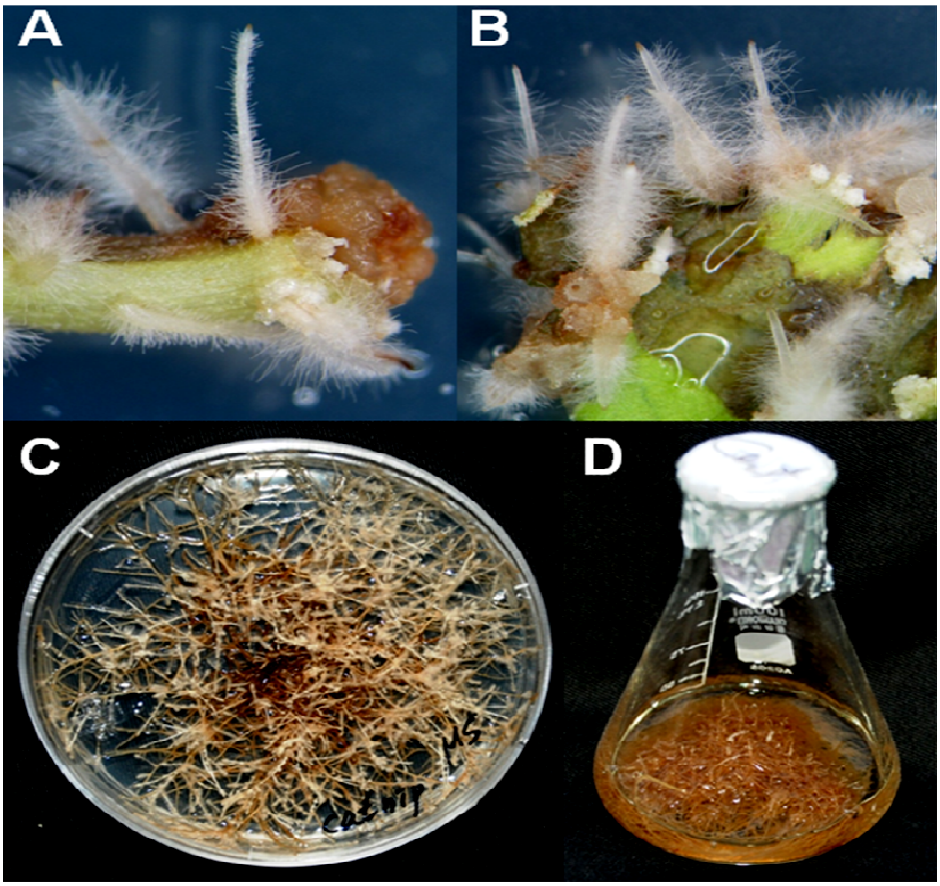

Figure 2. Hairy root induction and cultures of catnip, *Nepeta cataria.*
Note: Development of hairy roots from catnip stem (A, x13) and leaf (B, x13) after inoculation with *Agrobacterium rhizogenes* strain R1000; Rapidly growing hairy roots (C, x0.5) on agar-solidified MS medium and hairy root culture in MS liquid culture medium (D, x0.5).

Table 1. Effect of different explant tissues of *Nepeta cataria* on the frequency of infection and the growth of hairy root cultures.

Explant tissues	Infection frequency (%)	Number of hairy roots per explant	Root length(cm)
Leaf	71	6.6 ± 0.9	2.7 ± 0.3
Stem	56	4.8 ± 0.6	2.5 ± 0.4

Note: Values represent means ± SD values of three independent measurements made 30 days after bacterial inoculation. Approximately 50 explants were examined for each measurement.

slightly better response in terms of transformation and initiation of hairy roots with the infection frequency ranging from 56 - 71%. Leaf explant was chosen, however, as the optimal explant tissue for co-cultivation with *A. rhizogenes*, as the frequency of bacterial infection was higher than that seen in stem explants and the resulting hairy roots grew more rapidly than those derived from stem (Table 1).

After 30 days of exposure to the bacteria, the hairy roots began to grow more rapidly. Rapidly growing hairy roots were excised from the necrotic explant tissues and subculture on fresh agar-solidified MS medium containing 200 mg/l timentin. Mature hairy roots generally became thicker following subculture. After repeated, transfer to fresh agar-solidified MS medium for 2 - 3 months (Figure 2c), rapidly growing hairy root cultures of catnip were transferred to liquid culture medium (Figure 2d) (Table 1).

Hairy roots were cultured in MS liquid medium for 18 days. Both hairy root growth and rosmarinic acid production were investigated by harvesting six flasks at intervals of 3 days (Figure 3). During the 18 days culture period, the dry weight of the hairy root culture increased from the original inoculum level of 0.5 g dry wt/l to attain 11.7 g dry wt/l. The maximum growth (11.2 g dry wt/l) was

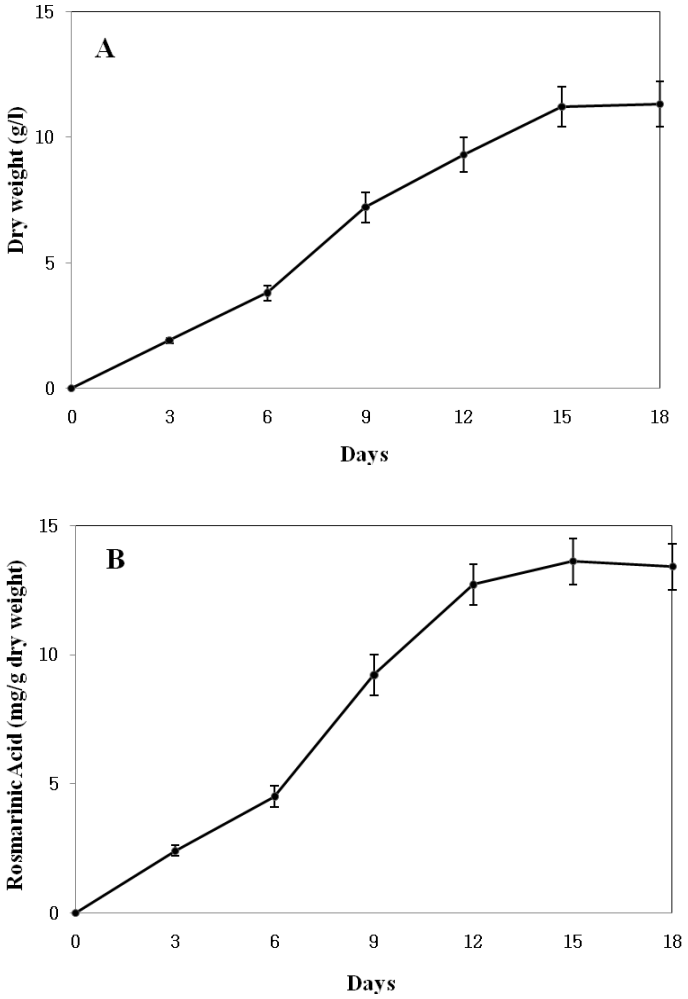

Figure 3. Time course studies of growth (A) and rosmarinic acid production (B) in hairy root culture of *Nepeta cataria* grown in MS medium for 18 days. Values represent the mean ± SD of six independent measurements.

attained after 15 days of culture when the content of rosmarinic acid was 13.6 mg/g dry wt. After 18 days of culture, the hairy roots turned brown and a brown pigment was released into the culture medium.

DISCUSSION

A. rhizogenes, a soil pathogen can infect wounds of plant and induces hairy roots in a number of plant species. Hairy root cultures established by transformation with *A. rhizogenes* are attractive system for the production of plant secondary metabolites, because of it's independence of seasonal and geographical conditions, biochemical and genetic stability, rapid growth rates, and ability to produce secondary metabolites at levels comparable to the mother plants (Christey and Braun, 2005; Georgiev et al., 2007; Srivastava and Srivastava, 2007).

Rosmarinic acid production by hairy root culture of several plant species in the mint family (*Lamiaceae*), *Ocimum basilicum* (Tada et al., 1996), *Salvia miltiorrhiza* (Chen et al., 2001), *Coleus forskohlii* (Li et al., 2005), *Salvia officinalis* (Grzegorczyk et al., 2006), and *Agastache rugosa* (Lee et al., 2008) has been reported. Here, we describe, for the first time, an efficient *A. rhizogenes*-mediated transformation protocol for the establishment of catnip hairy root cultures. Specifically, we achieved rosmarinic acid production from transformed roots of *N. cataria* using *A. rhizogenes* R1000. Our results indicate that hairy root culture is a biotechnological and valuable alternative approach for the production of rosmarinic acid from *N. cataria*. Our current laboratory efforts are aimed at further improving rosmarinic acid production in hairy root cultures of *N. cataria*.

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