

E-P-METHOXYCINNAMIC ACID PRODUCTION IN HAIRY ROOT CULTURES OF SCROPHULARIA BUERGERIANA MIQUEL

SANG UN PARK¹, XIAOHUA LI¹, SEOK HYUN EOM², CHUNG YEOL LEE³ and SOOK YOUNG LEE⁴

¹Department of Crop Science, Chungnam National University, Daejeon, 305-764, Korea

²Department of Horticultural Biotechnology, Kyung Hee University, Yongin, Gyeonggi-do 446-701, Korea

³Department of Plant Bioscience, Pusan National University, Miryang, Gyeongsangnam-do, 627-706, Korea

⁴Research Center for Oral Disease Regulation of the Aged, Chosun University, Gwangju, 501-759, Korea

Abstract - E-p-methoxycinnamic acid (MCA) is one of the main active constituents of *Scrophularia buergeriana* Miquel and has hepatoprotective, anti-amnestic, and neuroprotective activities. For studying *in vitro* production of MCA, we established a hairy root culture of *S. buergeriana* by infecting leaf explants with *Agrobacterium rhizogenes* R1000, and tested the growth and MCA production of these cultures using different strengths of media and concentrations of auxins. Hairy roots grown in half-strength MS medium showed the highest levels of growth (10.3 g/l) as well as MCA production (0.83 mg/ 100g dry weight). Hairy root culture with the treatment of 0.5mg/l IBA produced the highest amount of dry weight (11.8 g/l) and MCA (1.26 mg/100g dry weight) production. These results demonstrate that the hairy root culture of *S. buergeriana* is a valuable alternative approach for the production of MCA.

Keywords: *Agrobacterium rhizogenes*, E-p-methoxycinnamic acid (MCA), hairy root, *Scrophularia buergeriana* Miquel

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INTRODUCTION

Scrophularia buergeriana Miquel of the Scrophulariaceae family, is a perennial herb widely distributed in East Asian countries. The root of *S. buergeriana* has been used in oriental traditional medicine for the treatment of fever, swelling, constipation, pharyngitis, neuritis and laryngitis (Duck and Ayensu, 1985). E-p-methoxycinnamic acid (MCA), a phenylpropanoid, is one of the main active constituents of *S. buergeriana* (Lee et al., 2007). The principal activities of E-p-methoxycinnamic acid are hepatoprotective (Lee et al., 2002), anti-amnestic (Kim et al., 2003), and neuroprotective (Kim et al., 2002; Kim and Kim, 2000).

A symptom of hairy root disease in a plant is the formation of adventitious roots at the site of infection with the soil bacterium *Agrobacterium rhizogenes*. Hairy root cultures of plants have been widely studied for the production of secondary metabolites as pharmaceuticals, cosmetics, and food

additives (Christey and Braun, 2005; Georgiev et al., 2007; Srivastava and Srivastava, 2007). The production of natural compounds using the hairy root culture of *S. buergeriana* has never been reported. Here we describe the production of E-p-methoxycinnamic acid by hairy root cultures of *S. buergeriana* transformed with *Agrobacterium rhizogenes*.

MATERIALS AND METHODS

Growth of Agrobacterium rhizogenes

A. rhizogenes strain R1000 cultures were grown to mid-log phase ($OD_{600} = 0.5$) at 28° C on a gyratory shaker (180 rev/min) in liquid Luria-Bertani (LB) medium. The bacterial cells were collected by centrifugation for 10 min at 224 g, 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 of *S. buergeriana* were taken from plants grown *in vitro* and were cut at the ends into sections 7 x 7 mm². The excised leaves were dipped into an *A. rhizogenes* culture in a liquid inoculation medium for 10 min, blotted dry on sterile filter paper, and incubated in the dark at 25°C on agar-solidified MS (Murashige and Skoog, 1962) medium. After 2 days of co-cultivation, the explant tissues were transferred to a hormone-free medium containing MS salts, 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 3 weeks. The hairy roots were separated from the explant tissue and subcultured in the dark at 25°C on agar-solidified MS medium.

Optimization of culture conditions

For the selection of optimal medium conditions for hairy root growth and E-p-methoxycinnamic acid (MCA) production, the effects of different strengths of MS (Murashige and Skoog) and SH (Schenk and Hildebrandt, 1972) media were tested. The addition of various concentrations (0.0, 0.1, 0.5 and 1.0 mg/L) of auxins, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) to the culture media were tested to promote the growth of hairy roots and MCA production. Isolated roots (0.5 g/l) were transferred to 30 ml of each liquid medium, containing 30 g/l sucrose, in 100 ml flasks. Root cultures were maintained in the dark at 25°C on a gyratory shaker (100 rev/min). After 21 days of culture, hairy roots were harvested and then the dry weights and E-p-methoxycinnamic acid contents were determined. Three flasks were used for each culture condition, and the experiments were performed in duplicate. After repeated transfer to fresh medium, rapidly growing hairy root cultures were obtained.

HPLC analysis of E-p-methoxycinnamic acid

Hairy roots of *S. buergeriana* were collected and fresh samples were stored in sealed clear polyethylene plastic bags at -80°C until use. The collected samples were dried using a freeze-dryer at -80°C for at least 48

h. Dried samples were ground into a fine powder using a mortar and pestle. Dried samples (0.1 g) were extracted with 3 mL pure methanol for 1 h at room temperature. The solution was filtered through a 0.45µm poly filter and then diluted two times with methanol prior to high performance liquid chromatography (HPLC) analysis.

HPLC quantification of the MCA was performed with a Futecs model NS-4000 HPLC apparatus (Daejeon, Korea). The analysis was monitored at 305 nm and performed using a C18 column (250 mm x 4.6 mm, 5 µm; RStech, Daejeon, Korea). The mobile phase was a gradient prepared from mixtures of acetonitrile and 0.15% acetic acid, the column was maintained at 25°C. The flow rate was set at 1.0 mL/min and the injection volume was 20 µL. The results were calculated using a standard curve. All samples were run in triplicate.

RESULTS

For inducing hairy roots from leaf explants of *S. buergeriana*, *A. rhizogenes* R1000 was used for its ability. Wounded *S. buergeriana* leaf explants were susceptible to infection by *A. rhizogenes* R1000. It infected more than 85% of the leaf explants 30 days after inoculation. Hairy root initials emerged from wound sites on the leaf explants within 15 days after inoculation. After 30 days, the hairy roots of *S. buergeriana* began to grow more rapidly. The rapidly growing hairy roots were excised from the explant tissues and transferred to agar-solidified MS medium containing 200 mg/l Timentin every two weeks. After repeated transfer to fresh medium for three months, the hairy roots were placed in MS liquid culture medium.

To examine the effect of different media on growth and E-p-methoxycinnamic acid (MCA) production, hairy roots of *S. buergeriana* were cultured for two weeks in full- and half-strengths of MS and SH basal media supplemented with 3% sucrose. As shown in Table 1, hairy roots grown in half-strength MS medium showed the highest levels of growth (10.3 g/l) as well as MCA production (0.83 mg/ 100g dry weight) as compared to other media.

Table 1. The effects of media on the growth and production of E-p-methoxycinnamic acid in hairy roots of *Scrophularia buergeriana* after 2 weeks in culture.

Media	Dry Weight (g/l)	Methoxycinnamic acid /D.W. (mg/100g)
1/2 MS	10.3 ± 1.1	0.83 ± 0.06
MS	8.7 ± 0.7	0.81 ± 0.02
1/2 SH	9.8 ± 0.9	0.78 ± 0.02
SH	9.9 ± 0.8	0.79 ± 0.01

Previous findings showed half-strength MS medium to be the best for the growth and production of MCA in hairy root culture of *S. buergeriana*. Therefore, half-strength MS medium was used to study the effects of different auxins on growth and MCA production in hairy root cultures. Hairy roots were grown for 2 weeks in half-strength MS medium supplemented with various concentrations (0.0, 0.1, 0.5, and 1.0 mg/l) of different auxins (IAA, IBA and NAA).

Our results revealed that all tested auxin treatments increased the growth rates of the hairy roots, and also increased the production of MCA under our experimental conditions (Table 2). The concentration of 0.5mg/l IBA produced the highest amount of dry weight (11.8 g/l) and the highest amount of MCA (1.26 mg/100 g dry weight). At the concentration of 0.5 mg/l IBA the dry weight and MCA production were around 15% and 52%, respectively, more than the control. Other auxin treatments slightly increased growth and MCA production in hairy root cultures of *S. buergeriana*.

DISCUSSION

A. rhizogenes infects wound sites of many plant species. The infections are characterized by the production of adventitious roots with numerous root hairs (Hamill et al. 1987; Giri and Narasu 2000). In general, hairy root cultures established by transformation with *A. rhizogenes* are attractive for the production of secondary metabolites as such cultures are genetically and biochemically stable,

show rapid growth rates, and have the ability to synthesize useful natural compounds at levels comparable to those produced by mother plants (Guillon et al. 2006). Therefore, hairy root cultures may thus be useful in studies on the production of important natural products.

For producing natural compounds in hairy root cultures, the optimization of the medium can play an important role in the growth of the roots as well as natural compound production. These findings are consistent with prior studies indicating the growth and natural compound biosynthesis in hairy root cultures of *Centranthus ruber* (Granicher, et al, 1995), *Fagopyrum esculentum* (Lee et al, 2007), and *Withania somnifera* (Murthy et al, 2008). It is well known that auxins have important roles in the promotion of plant growth and root development. The enhancement of hairy root growth and natural compounds is similar with previous reports that exogenous auxin treatments enhanced the growth and natural compound production in hairy root cultures of *Lippia dulcis* (Sauerwein et al., 1991), *Lobelia inflata* (Balvanyos et al., 2001), and Panax hybrid (Washida et al., 2004).

Table 2. The effects of auxins on the growth and production of E-p-methoxycinnamic acid in hairy roots of *Scrophularia buergeriana* after 2 weeks in culture.

Auxins	Dry Weight (g/l)	Methoxycinnamic acid /D.W. (mg/g)
Control 0.0	10.3 ± 1.1	0.83 ± 0.06
IAA 0.1	10.5 ± 1.0	0.85 ± 0.08
0.5	10.5 ± 0.9	0.89 ± 0.07
1.0	10.8 ± 1.0	0.91 ± 0.09
IBA 0.1	11.0 ± 1.1	0.87 ± 0.08
0.5	11.8 ± 1.0	1.26 ± 0.11
1.0	11.4 ± 1.2	0.84 ± 0.09
NAA 0.1	10.9 ± 1.1	0.90 ± 0.07
0.5	11.4 ± 1.2	0.84 ± 0.09
1.0	10.5 ± 1.0	0.84 ± 0.08

The results obtained in this research indicate that *S. buergeriana* hairy root culture can be a valuable alternative for the production of MCA. Using a selected culture medium (1/2 MS) and exogenous auxin treatments, a relatively high MCA production and improved root growth can be achieved. Further investigations for the improvement of MCA production in hairy root cultures of *S. buergeriana* are in progress at our laboratory.

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