J. Plant Biol. 39(3) : 161~166 (1996)

Multiplication and Transformation of Medicinal Plants for Production of Useful Secondary Metabolites II. Establishment of Hairy Root Cultures of *Centella asiatica*

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The hairy root cultures of *Centella asiatica* were established by infection leaf explants with *Agrobacterium rhizogenes* A4, 15834 in 1/2 Murashige and skoog liquid medium supplemented with 50 μM acetosyringone. The induced hairy roots were subjected to paper electrophoresis for the detection of opine and opine-positive clones which were considered to have been transformed. Five hairy root clones were selected according to the different bacterial strains used, growth rate and pattern. Among media tested, MS basal medium substituted phosphate concentration by 2.5 mM K_2HPO_4 showed the highest growth rate in the dark condition.

Keywords : *Agrobacterium rhizogenes, Centella asiatica*, hairy root clones

Centella asiatica is a herbaceous plant of the Umbelliferae family. There are several biophamacologically useful substances extracted from *C. asiatica* such as, asiatic acid, madecassic acid, asiaticoside and alkaloid. Those substances have been used for treatment of ulcers, damaged skin tissue and mentally retarded children in France, Canada and U.S.A. (D'Amelio, 1987; Nalini *et al.*, 1992; Hausen., 1993; Rush *et al.*, 1993). Unfortunately, its existence in Korea has been identified in the limited area of southern islands of Korea and several trials to grow *C. asiatica* in Korea has appeared unsuitable economically. Therefore, hairy root culture has been focused to conserve the strain and to produce economically useful secondary metabolites from plants (Gränicher *et al.*, 1995; Ikenaga *et al.*, 1995; Arellano *et al.*, 1996; Tada *et al.*, 1996). Hairy root culture system has been known to have many advantages compared to cell culture (Hwang *et al.*, 1993). Furthermore, hairy root culture system has more advantage than the genetic improvement using conventional breeding method does because useful individual plants which has desirable trait can be generated in the short period of time using hairy root system (Uozumi *et al.*, 1992; Mano and Matsuhashi, 1995). In the hairy root system, elite transgenic plant can be generated without altering characteristics of hairy root clones which has desirable trait (Mano and Matsuhashi, 1995). Also, multiple shoot can be induced to extract much more substances from transgenic plants than from non-transformed plants (Tanaka *et al.*, 1995). In addition, mass culture can be performed by using mutual switching between hairy root and callus. In the present study, transformed hairy roots were induced by using *Agrobacterium rhizogenes*. Also, growth rate of hairy root in the various conditions was investigated.

MATERIALS AND METHODS

Seed germination and bacterial strains

The seeds of *Centella asiatica* used in this study was obtained from Jeju island in the middle of September. The seeds were treated with 50% H_2SO_4 for 15 min, 70% ethanol for 10 min, and 5% sodium hypochlorite for 10 min. After the treatment, the seed was washed three times with sterilized water. And then, the seed were scarificated and vernalization treatment for 2-9 weeks and GA_3 (0.1-15 mM) treatment were performed. After the treatment, 200

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pieces of the seed were placed on hormone free Murashige and Skoog (1962) medium (MSO). The germination rate of the seed was examined in triplicated experiment. For the hairy root induction, leaves of the seedling, and agropine type, *Agrobacterium rhizogenes* A4 and ATCC 15834 strain were used.

Induction and culture of hairy root

Some pieces of young leaves were placed into 1/2 MS liquid medium containing 50 μM acetosyringone (AS) and were cultured with *A. rhizogenes* A4 and 15834 for 24 hours. After washing with sterilized water, the pieces were placed into MS liquid medium containing 300 mg/L cefotaxime and were cultured in shaker for 48 hours. And then, the hairy roots were induced after placing the pieces of young leaves on MS medium with the lower epidermis facing upward. The induced hairy roots were cut and sorted according to their origin, and subcultured respectively. The other methods used for induction of hairy roots were identical with methods reported previously (Hwang *et al.*, 1993).

Opine analysis

The transformation of the hairy roots was examined by detecting the existence of opine using paper electrophoresis (Petit *et al.*, 1986).

Selection of hairy root clone

Five major clones were selected from 25 clones of hairy roots induced according to bacterial strains used, growth rate and growing morphology, and the clones were labeled with CA 1 through CA 5 respectively. The hairy root clone CA 1 was used for examination of growth rate in MS liquid medium.

Examination of growth rate

B5 (Gamborg *et al.*, 1968), MS, SH (Schenk and Hildebrandt *et al.*, 1972), White (White, 1963) and WP (Lloyd and McCown, 1980) media were used for comparisons of growth rate on each medium. In addition, the growth rate of hairy roots on MS media with various conditions was compared. The conditions examined were different carbon sources, light (GRO-LUX® fluorescent, intensity 50 μmol m^{-2} s^{-1}, photoperiod 16-h) or dark conditions, various concentrations of phosphate and the various ratio of NH_4^+/NO_3^-. Briefly, 0.5 g (fr wt) hairy roots was placed into 100 mL flask containing 30 mL MS medium and was cultured in shaker (70 rpm) with or without light source at 25°C for 3 weeks. After washing with distilled water, the hairy roots were lyophilized at freeze dryer and their dry weight were measured.

RESULTS AND DISCUSSION

Germination

Germination was observed after 3-4 weeks of culture from sterilized seed cut to length (scarification) and placed on MSO while another seed which was treated for vernalization, the other seed treated with GA_3 and control seed did not show any germination. However, the germination rate was 18.2%, which needed *in vitro* micropropagation of *Centella asiatica* (Kim *et al.*, 1995).

Induction and culture of hairy root

A similar shape of hairy root was induced after three weeks of A4 and 15834 treatment on leaf explant while 15834 showed stronger activation than A4 did (Fig. 1a). The bacteria could be completely removed after 1-2 subcultures of induced hairy roots in MS media containing antibiotics for 4 weeks. The bacteria free hairy roots were subcultured in MS liquid or solid media which were free of antibiotics and hormone (Fig. 1b, c).

The acetosyringone (AS) used in the induction of hairy roots is one of the phenolic compounds and induces the expression of vir-gene of *A. rhizogenes* Ri-plasmid to facilitate the formation of hairy root of dicotyledon. However, it was also reported that AS can inhibit the formation of hairy roots in other strains of bacteria and plants (Vanhala *et al.*, 1995). In case of *C. asiatica*, the roots were induced from both AS treated leaf explants and control leaf explants. The roots induced from control leaf explants stopped growing after 4-5 weeks while AS treated root showed continuing growth. In addition, opine was detected from AS treated root, which indicates the important role of AS for induction of hairy roots.

Detection of opine

Opine can be detected only from transformed tissue which was treated with *A. rhizogenes* A4 and 15834. The bands for opine of hairy root clone CA

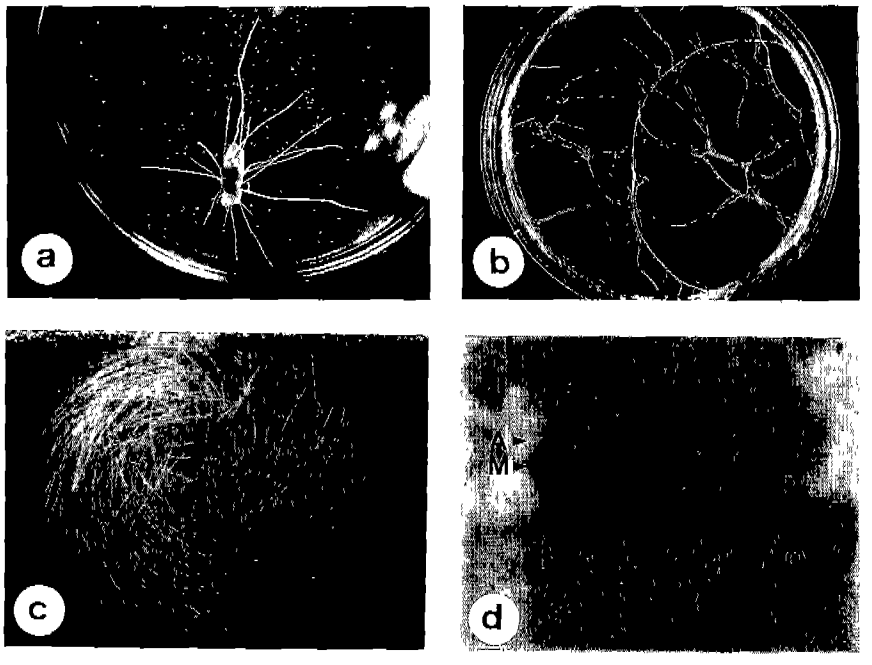

Fig. 1. a: hairy roots induced from leaf explants of *Centella asiatica*, b and c: hairy root cultured in hormone-free MS agar (b) and liquid(c) medium, d: opine assay. Paper electrophoretic of extracts from hairy root clones and ordinary root. A, agropine; M, authentic mannopine; Lane 1-5, hary root clone CA 1, 2, 3, 4, 5; Lane O, ordinary root.

1, 2, 3, 4, and 5 were found at the same distance with agropine and manopine on the paper after electrophoresis while no band was found from non-transformed tissue (Fig. 1d).

Selection of hairy roots clone

Five hairy roots were selected among the 25 clones of hairy roots induced according to bacteria used, morphological feature and growth rate. The selected hairy root clones CA 1 and 2 were induced by bacteria A4 and clone CA 3, 4, and 5 were induced by bacteria 15834. There was no morphological difference according to bacteria used. CA 1 and 2 showed white thick roots and grew to length. Also, CA 1 and 2 showed high growth rate and lateral branching rate while growth rate of CA 1 was higher than that of CA 2. By the way, CA 3 showed low growth rate and had brown thick roots with low lateral branching rate. However, CA 4 showed lateral branching and grew to length. In case of CA 5, 2 mm-long calli with black spots were induced because of the sensitive response to wounding such as disrupted balance of phytohormones (Fig. 2).

Paek *et al.* (1993) reported that different clones of red beet showed significant differences in the morphology, the ratio of betacyanin/betaxanthin and the

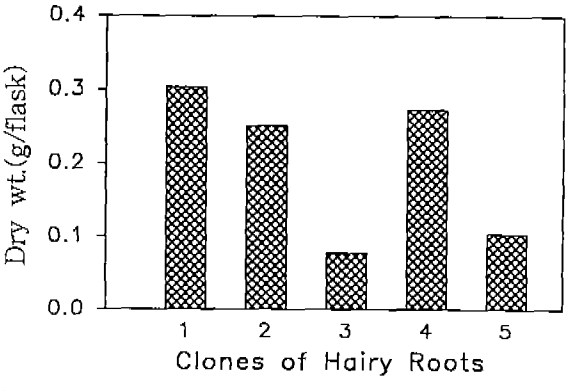

Fig. 2. Growth from clones of hairy roots (CA 1-CA 5) of *Centella asiatica* after 3 weeks culture, Initial inoculum: 0.5 g (fr wt).

capacity to produce those pigments. Also, Gränicher *et al.* (1995) reported that hairy roots of *Centranthus ruber* enhanced the metabolism in quantity and quality so that more secondary products could be produced than from parent plant. Therefore, the selection of hairy root clone is an essential step in the mass production of secondary metabolites. In addition, the hairy root transformation system appeared excellent method for the selection of cell lines which can yield high amount of secondary metabolites.

Examination of hairy root growth rate

The growth rate and synthesis of secondary metabolites can be affected by the culture conditions, culture time, and culture cycle (Hirasuna *et al.*, 1996). In the present study, carbon sources, concentrations of phosphate and the ratio of nitrate sources were examined by using MS medium. Also, B5, MS, SH, White and WP media were used for the examination of growth rate. MS and B5 media appeared suitable media while White and WP media appeared unsuitable (Fig. 3).

Hairy roots which were cultured in dark condition showed high growth rate having white long roots (Fig. 3). On the other hand, green hairy root which might be considered to have an ability of photosynthesis were induced when the hairy root were grown with lights. This observation raised the possibility of production of substances which may be generated during chloroplast-dependent reaction (Signs and Flores 1990; Flores *et al.*, 1993).

Sucrose is a widely used carbon source in plant cell and tissue culture. The disaccharide sucrose may be hydrolyzed to glucose and fructose during high temperature sterilization or can be hydrolyzed by invertase located in the cell wall. In general, glucose has been reported as a preferred form of monosaccharide taken by cell (Fowler, 1982; Kino-Oka *et al.*, 1994). In the present experiment using 30 g/L sucrose, 30 g/L glucose and 30 g/L fructose. however, high growth rate was obtained when sucrose or fructose were used (Fig. 4). This observation indicated that toxic compounds such as 5-(hydroxymethyl)-2-furaldehyde (HMP) and phenolic compounds may be easily generated from glucose rather than fructose during high temperature sterilization to inhibit the growth (Schenk *et al.*, 1991).

Phosphate has been known to control the activation of various metabolism as well as to be substance for the synthesis of bio-macro molecules or phospholipids (Brodelius and Vogel, 1985). In the present study, high growth rate was obtained with increased concentrations of phosphate in MS media. Three fold increase in concentration of phosphate in MS basal media showed increased volume of root indicating the storage of phosphate, which was not suitable for long-term culture. By the way, two fold increase in concentration of phosphate in MS basal media appeared suitable for tissue culture (Fig. 5).

The ratio of NH_4^+/NO_3 has been well known to affect the growth and the production of substrates. In case of madder hairy roots, only NO_3^- was used as a nitrogen source and in case of carrot, and red beet hairy roots, NH_4^+ inhibited the growth of hairy root by blocking the absorption of NO_3^- and sub-

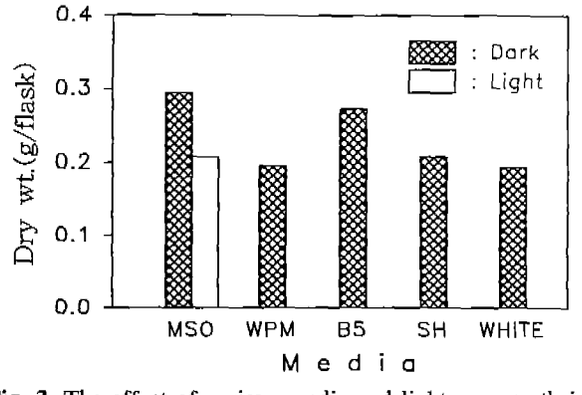

Fig. 3. The effect of various media and light on growth in clone of hairy root CA 1 after 3 weeks culture

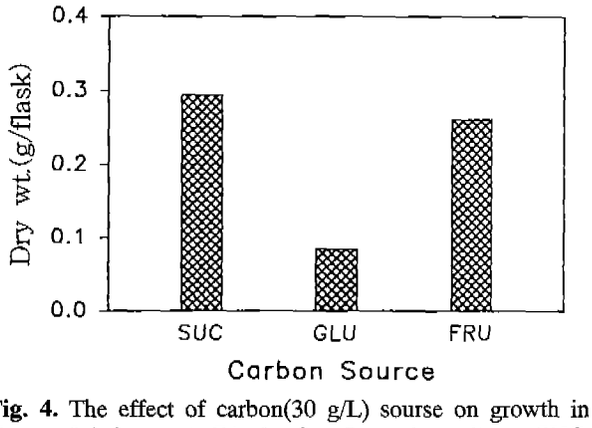

Fig. 4. The effect of carbon(30 g/L) sourse on growth in clone of hairy root CA 1 after 3 weeks culture. SUC, sucrose; GLU, glucose; FRU, fructose.

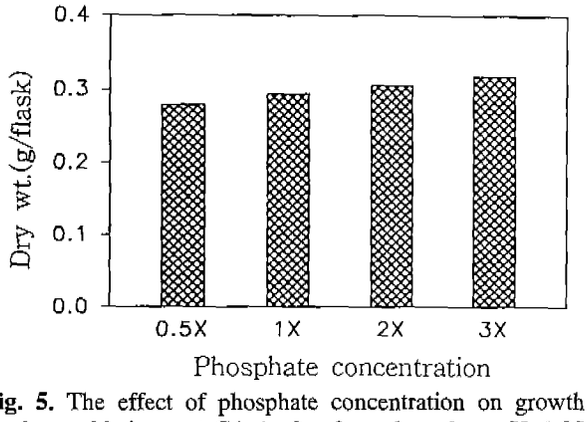

Fig. 5. The effect of phosphate concentration on growth in clone of hairy root CA 1 after 3 weeks culture. X=1.25 mM KH_2PO_4.

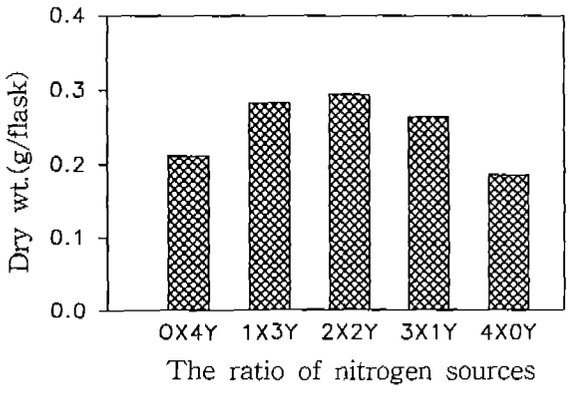

Fig. 6. The effect of nitrogen sourse on growth in clone of hairy root CA 1 after 3 weeks culture. X=20.6 mM NH_4NO_3, Y=18.8 mM KNO_3.

sequent assimilation of nitrogen in the hairy root cells (Kino-Oka *et al.*, 1993). In present study, the highest growth rate was oserved when same ratio (2X2Y) of NH_4^+/NO_3^- in MS basal medium were used. A significant inhibition of growth by NH_4^+ was not observed as Kino-Oka *et al.* (1993) reported. However, 0X4Y appeared to be suitable for growth of hairy root rather than 4X0Y because higher growth rate was observed when NO_3^- (0X4Y) was used as a nitrogen source than when NH_4^+ (4X0Y) was used (Fig. 6).

ACKNOWLEDGMENTS

We thank the Ministry of Education who gracefully provided a grant (BSRI 95-4424) for this investigation.

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(Received August 12, 1996)