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# PHENOLIC COMPOUND PRODUCTION BY DIFFERENT MORPHOLOGICAL PHENOTYPES IN HAIRY ROOT CULTURES OF *FAGOPYRUM TATARICUM* GAERTN.

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*Abstract* - Hairy roots were obtained after inoculating sterile young stems of *Fagopyrum tataricum* with *Agrobacterium rhizogenes* R1000. The established roots displayed two morphological phenotypes when cultured on hormone-free medium containing Murashige-Skoog salts and vitamins. The thin phenotype had a higher growth rate than the thick phenotype. Further, the phenolic compound content of the thin phenotype was higher than that of the thick phenotype. In terms of their total dry weight, the thin phenotype produced an almost double amount of (-)-epigallocatechin as well as more than 51.5% caffeic acid, 65% chlorogenic acid, and 40% rutin compared to the thick phenotype after 21 days of culture. Therefore, selection of the optimal morphological phenotype of hairy roots of tartary buckwheat is an important factor for improved phenolic compound production.

*Key words*: *Fagopyrum tataricum* Gaertn., hairy root culture, phenolic compounds

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## INTRODUCTION

*Fagopyrum tataricum* Gaertn. (tartary buckwheat), belonging to the Polygonaceae family, is currently grown as a crop only in the mountainous regions of southwest China (Sichuan), northern India, Bhutan, Nepal, and a small part of northwest Europe (Bonafaccia et al., 2003; Xuan and Tsuzuki, 2004). Tartary buckwheat is an excellent medicinal and nutrient-rich plant. It is known to have higher contents of rutin and other phenolic compounds than common buckwheat (Fabjan et al., 2003). Furthermore, it reportedly exhibits various pharmacological and biological properties such as anticancer (Guo et al., 2007), antidiabetic (Yao et al., 2008), and antioxidant activities (Liu et al., 2008).

The different organs of buckwheat contain several phenolic compounds. Phenolic compounds constitute one of the main classes of secondary metabolites that are widely distributed in higher plants. Recently, research interest in these chemicals has been stimulated because of their potential health benefits. They protect the body's tissues against oxidative stress and its associated pathologies such as cancer, coronary heart disease, and inflammation (Croft, 1998; Karakaya, 2004; Linseisen and Rohrmann, 2008; Tapiero et al., 2002).

Hairy root cultures have been widely studied for the production of secondary metabolites useful as pharmaceuticals, cosmetics, and food additives (Christey and Braun, 2005; Georgiev et al., 2007). *In vitro* production of phenolic compounds in hairy root cultures of common buckwheat and tartary buckwheat has been reported (Kim et al., 2009; Lee et al., 2007; Trotin et al., 1993). Further, a previous paper has reported the phenotypic and growth variations observed among hairy root clones induced from an independent transformation event by using *Agrobacterium rhizogenes* (Alpizar et al., 2008).

In this paper, we describe phenolic compound production by different morphological phenotypes in hairy root cultures of *Fagopyrum tataricum* Gaertn.

## MATERIALS AND METHODS

### *Seed sterilization and germination*

Dehulled seeds of *Fagopyrum tataricum* were surface-sterilized with 70% (v/v) ethanol for 1 min and 4% (v/v) sodium hypochlorite solution for 10 min and then rinsed thrice in sterilized water. Six seeds were placed on 25 mL of agar-solidified culture medium in dishes (100 mm × 15 mm). The basal medium consisted of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and was solidified with 0.8% (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/(sm$^2$) and a 16-h photoperiod. For the wild-type roots, the *F. tataricum* mother plants were grown in a growth chamber at 25°C under standard cool white fluorescent tubes with a flux rate of 80 μmol/(sm$^2$) and a 16-h photoperiod.

### *Growth of Agrobacterium rhizogenes*

The culture of *A. rhizogenes* strain R1000 was initiated from glycerol stock and grown overnight at 28°C with shaking (180 rpm) in liquid Luria–Bertani medium [1% tryptone, 0.5% yeast extract, and 1% NaCl (pH 7.0)] to the mid-log phase ($OD_{600}$ = 0.5). The bacterial cells were collected by centrifugation at 224 ×*g* for 10 min and resuspended in liquid inoculation medium (MS medium containing 30 g/L sucrose). The cell density was then adjusted to give an $A_{600}$ of 1.0 for inoculation.

### *Establishment of hairy root cultures*

Young stems of *F. tataricum* were collected from plants grown *in vitro* and cut at the ends into 7-mm sections. The excised stems were dipped into the *A. rhizogenes* R1000 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 medium. After 2 days of co-cultivation, the explant tissues were transferred to a hormone-free medium containing MS salts and vitamins (0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine-HCl, 0.1 mg/L thiamine-HCl, and 2.0 mg/L glycine), 30 g/L sucrose, 500 mg/L cefotaxime, and 8 g/L agar. Numerous hairy roots were observed emerging from the wound sites within 2 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 transfer to fresh medium, rapidly growing hairy root cultures were obtained. Isolated roots (0.5 g/L) were transferred to 30 mL of MS liquid medium, containing 30 g/L sucrose, in 100-mL flasks. The root cultures were maintained at 25°C on a gyratory shaker (100 rev/min) in a growth chamber under standard cool white fluorescent tubes with a flux rate of 35 μmol/(s/m$^2$) and a 16-h photoperiod. After 30 days of culture, the hairy roots were harvested and their dry weight and phenolic compound contents were determined. Three flasks were used for each culture condition, and the experiments were performed in duplicate.

### *High-performance liquid chromatography analysis of phenolic compounds*

The wild-type roots and hairy roots were collected, and fresh samples were stored in sealed clear polyethylene plastic bags at -80°C until they were used. The collected samples were freeze-dried at -80°C for at least 48 h and then ground to a fine powder using a mortar and pestle. Rutin was extracted from the dried samples (0.1 g) with 3 mL of pure methanol at 60°C for 30 min; the remaining tested compounds were extracted twice with 3 mL of 80% methanol for 1 h at room temperature. The solutions were filtered through a 0.45-μm polyfilter and then diluted twofold with methanol prior to high performance liquid chromatography (HPLC) analysis.

HPLC was performed with a Futecs HPLC apparatus (model NS-4000; Daejeon, Korea). The analysis was monitored at 280 nm and performed by using a C18 column (250 mm × 4.6 mm, 5 μm; RStech, Daejeon, Korea) maintained at 30°C. The mobile phase was a gradient prepared from mixtures of acetonitrile and 0.15% acetic acid. 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

### *Establishment of hairy root cultures*

Hairy roots of *F. tataricum* were initiated by using stem explants inoculated with *A. rhizogenes* R1000. After 2 days of co-cultivation with *A. rhizogenes*, the explant tissues were transferred to agar-solidified MS medium containing 500 mg/L cefotaxime to eliminate *A. rhizogenes*. Hairy root initials emerged from

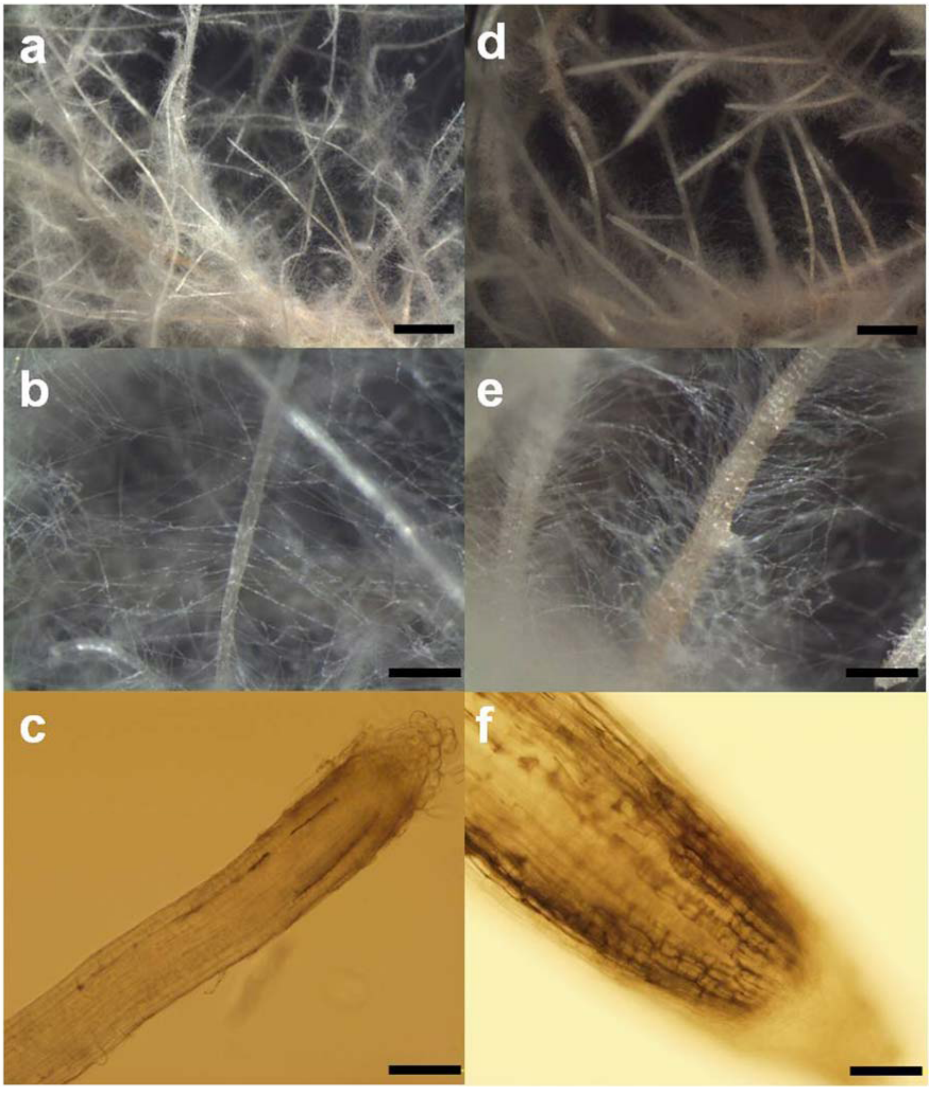


**Fig. 1.** Hairy roots of *F. tataricum* showing two different phenotypes within four weeks of inoculation with *A. rhizogenes* strain R1000. (a)–(c) Thin phenotype and (d)–(f) thick phenotype. Magnification: A and D, 1 mm; B and E, 400 μm; C and F, 100 μm.

wound sites on the explants within 5–7 days after inoculation. After 10–14 days, the roots began to grow more rapidly. About four weeks after co-cultivation with *A. rhizogenes*, the hairy roots were excised from the necrotic explant tissues and subcultured on fresh agar-solidified medium containing 500 mg/L cefotaxime.

Morphological changes that occurred during the induction and development of hairy roots from the stem explants were investigated by light microscopy. We found two different morphological phenotypes: around 20% of the hairy roots presented the characteristic traits of primary roots with a profusion of root hairs (thin phenotype) (Fig. 1a–c), and the remaining 80% presented very thick primary roots and several secondary roots (thick phenotype) (Fig. 1d–f). The thin phenotype had a higher number of hairy roots than the thick phenotype.

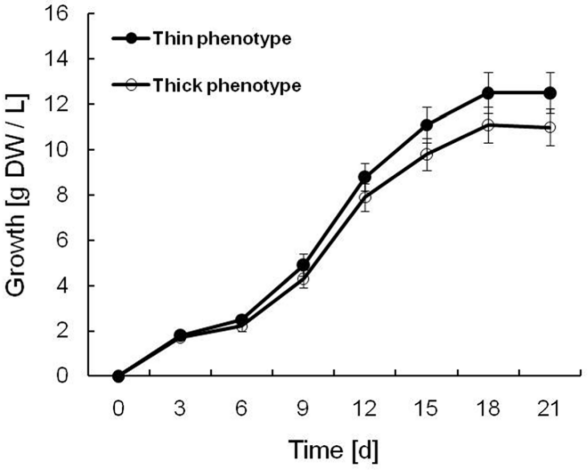


**Fig. 2.** Time course study of the growth of the two hairy root phenotypes of *F. tataricum* grown in MS medium for 21 days. The values represent the mean ± SD of six independent measurements. DW, dry weight.

After isolation, hairy roots of both phenotypes were cultured in MS liquid medium for 21 days and their growth rate was investigated by harvesting six flasks at 3-day intervals (Fig. 2). During the 21-day culture period, the dry weight of the thin phenotype increased from the original level of 0.5 g/L to 12.5 g/L. On the other hand, the growth of the thick phenotype increased (from 0.5 g/L to 11.1 g/L) up to 18 days and then started to decline. At all times, the growth rate was higher for the thin phenotype

Table 1. Phenolic compound contents of the thin and thick phenotypes of hairy roots of *F. tataricum*.

| Phenolic compound | Thin phenotype (mg/g DW) | Thick phenotype (mg/g DW) |
|---|---|---|
| Caffeic acid | 0.08 ± 0.01 | 0.06 ± 0.01 |
| (-)-Catechin hydrate | 0.36 ± 0.01 | 0.34 ± 0.07 |
| Chlorogenic acid | 0.16 ± 0.01 | 0.11 ± 0.01 |
| (-)-Epicatechin | 5.33 ± 0.25 | 5.04 ± 0.11 |
| (-)-Epigallocatechin | 0.21 ± 0.04 | 0.11 ± 0.01 |
| Gallic acid | 0.03 ± 0.00 | 0.03 ± 0.01 |
| Rutin | 1.63 ± 0.07 | 1.32 ± 0.08 |

The values represent the mean ± SD of six independent measurements.
DW – dry weight.

than the thick phenotype. After 21 days of culture, the hairy roots turned brown, and a brown pigment was released into the culture medium.

*Analysis of phenolic compound contents*

Seven phenolic compounds were determined by HPLC analysis (Table 1). Irrespective of the phenotype, the content of epicatechin was the highest followed by rutin and that of gallic acid was the lowest. However, the thin phenotype produced almost a double amount of (-)-epigallocatechin than the thick phenotype. Furthermore, in terms of their total dry weight, the thin phenotype produced more than 51.5%, 20%, 65%, 20%, 21%, and 40% of caffeic acid, (-)-catechin hydrate, chlorogenic acid, (-)-epicatechin, gallic acid, and rutin, respectively, than the thick phenotype at 21 days of culture.

## DISCUSSION

When *A. rhizogenes* infects the wounds of plant species, adventitious roots with numerous hairs are produced (Giri and Narasu, 2000; Hamill et al., 1987). In general, hairy root cultures established by transformation with *A. rhizogenes* are attractive tools for the production of secondary metabolites because such cultures are genetically and biochemically stable, show rapid growth rates, and can synthesize useful natural compounds at levels comparable to those produced by wild-type roots. Therefore, such cultures may be useful in studies into the production of important natural products (Guillon et al., 2006; Srivastava and Srivastava, 2007).

In this study, the two morphological phenotypes of tartary buckwheat hairy roots showed variations in growth rate and phenolic compound content. Similar studies have reported that individual hairy roots often show different phenotypes and, in most cases, a variability that generally affects the branching intensity, root diameter, growth rate, and secondary metabolite production. For example, in one study, transformed ginseng hairy roots displayed three morphological phenotypes, and their ginsenoside pattern and growth rate varied according to their morphology (Mallol et al., 2001). Twenty-nine isolated hairy root clones of *Scopolia japonica* displayed various phenotypes characterized by different growth rates and levels of tropane alkaloid production (Mano et al., 1989). In addition, the characterization of the established hairy root clones of *Duboisia leichhardtii* F. Muell showed that there was considerable variation in the growth rate, alkaloid content, and productivity from clone to clone (Mano et al., 1989).

Our results indicate that hairy root culture is a valuable alternative approach for obtaining phenolic compounds from *F. tataricum*. Selection of the optimal morphological phenotype of hairy roots is an important factor for improved secondary metabolite production *in vitro*.

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