Accumulation of Ferulic Acid in Cell Cultures of *Ajuga pyramidalis* Metallica Crispa

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Cell cultures of *Ajuga pyramidalis* Metallica Crispa accumulate ferulic acid, a potent antioxidant cinnamic acid. Most of the ferulic acid was found in a soluble form in the cultures. Maximum accumulation of ferulic acid was found in highly pigmented cultures (138 mg/100 g of fresh weight). The leaves from greenhouse-grown plants had lower levels of ferulic acid (24 mg/100 g of fresh weight) compared to the cultures. A close relationship was observed between anthocyanin and ferulic acid accumulation in the cultures. Supplementation with gibberellic acid, a specific anthocyanin inhibitor, at 10, 100, and 1000 μM reduced the levels of both anthocyanins and ferulic acid in the cultures.

Keywords: *Ajuga pyramidalis; cell culture; ferulic acid*

INTRODUCTION

Ferulic acid [3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid], a secondary plant metabolite, occurs primarily in seeds and leaves both in free form and covalently linked to mono- and disaccharides, glycoproteins, pigments, and insoluble cell wall polymers (Graf, 1992). In recent years, ferulic acid has gained popularity as an alternative to synthetic antioxidants in food preservation, and it is currently used as an active ingredient in many lotions and sunscreens for photoprotection (Graf, 1992). Ferulic acid has also been associated with antitumor, antihepatotoxic, antiestrogenic, and antimitotic activities (Graf, 1992; Harborne and Baxter, 1993). The high cost of ferulic acid production has limited its large-scale utilization in the food and pharmaceutical industries. Development of an *in vitro* system for the production of ferulic acid in an extractable form could be a viable alternative to chemical synthesis or extensive extraction protocols from insoluble plant constituents.

Only limited reports have appeared on the accumulation of ferulic acid in cell cultures. Cell suspension cultures of *Beta vulgaris*, *Zea mays*, and *Chenopodium rubrum* have been reported to accumulate ferulic acid esters (Bokern et al., 1991; Grabber et al., 1995; Strack et al., 1984). However, most of the ferulic acid was found in the insoluble form conjugated to cell wall constituents in these cultures. When ferulic acid is polymerized into the cell wall by linkages that resist acid or alkaline hydrolysis, only a fraction of the ferulates is extractable by conventional solvolytic methods (Grabber et al., 1995). We investigated the possibilities of establishing a cell line from another plant source capable of producing mainly soluble ferulic acid. The aerial parts of *Ajuga* species (Labiatae) have been reported to contain ferulic acid (Harborne and Baxter, 1993). Recent studies in our laboratory have revealed that cell cultures of *Ajuga pyramidalis* Metallica Crispa (bugle weed) accumulate ferulic acid as the major cinnamic acid conjugated to anthocyanins (Madhavi et al., 1996), which prompted us to carry out further qualitative and quantitative studies to evaluate the potential of these cell lines to produce soluble ferulic acid. This paper reports the accumulation of ferulic acid and explores the interrelationships between anthocyanin and ferulic acid accumulation in *A. pyramidalis* Metallica Crispa cell cultures.

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MATERIALS AND METHODS

A. pyramidalis Metallica Crispa plants were maintained in the greenhouse and as shoot cultures in woody plant medium (Lloyd and McCown, 1981) supplemented with 0.98 μM 6-(γ,γ-dimethylallylamino) purine.

Establishment of Cell Cultures of *A. pyramidalis* Metallica Crispa. Leaf disks (~1.3 cm diameter) from shoot cultures were used for callus initiation. Explants were placed in the dark at 25 °C on a callus induction medium containing woody plant medium salts, supplemented with 100 μM Fe as $FeNa_2EDTA$, rose vitamins (Rogers and Smith, 1992), 0.1 g/L myoinositol, 0.15 g/L PVP-T (polyvinylpyrrolidone-10), 30 g/L sucrose, 0.05 g/L L-ascorbic acid, 2.26 μM (2,4-dichlorophenoxy)acetic acid (2,4-D), and 3.49 μM kinetin. Preparation of subcultures was performed at 2 week intervals. Pigmented suspension cultures were established by transferring colorless callus cultures to a pigment production medium containing 50 g/L sucrose, 2.26 μM indoleacetic acid, and 3.49 μM zeatin under a photosynthetic photon flux of 150 μmol m^{-2} s^{-1} in a gyratory shaker (New Brunswick, Edison, NJ) at 150 rpm. The cultures were harvested 7 days after pigment induction and analyzed for biomass, total anthocyanins and soluble and bound ferulic acid contents. Time-course studies for growth and accumulation of ferulic acid and anthocyanins were carried out over a period of 15 days. The results are the average of three replicates at each harvest date.

The interrelationships between anthocyanin and ferulic acid accumulation were studied by different treatments, viz., growing the cultures in the dark, developing a low-pigmented cell line by several subcultures in the callus induction medium followed by pigment induction, and inhibiting anthocyanin accumulation in the pigmented cultures by supplementing gibberellic acid (GA_3). GA_3 was added to the pigment production medium in varying amounts (0.0, 10, 100, and 1000 μM) after filter sterilization. There were three replicates per treatment, and the samples were analyzed as before.

Estimation of Total Anthocyanins and Ferulic Acid. For the estimation of total anthocyanins and ferulic acid, the samples were homogenized in ice cold acidified methanol (1%

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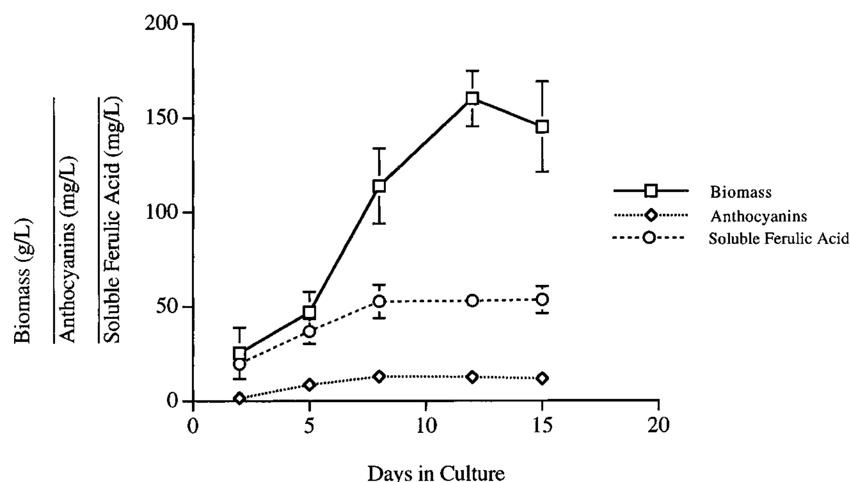

Figure 1. Time-course of growth and anthocyanin and ferulic acid accumulation in *A. pyramidalis* Metallica Crispa cell culture.

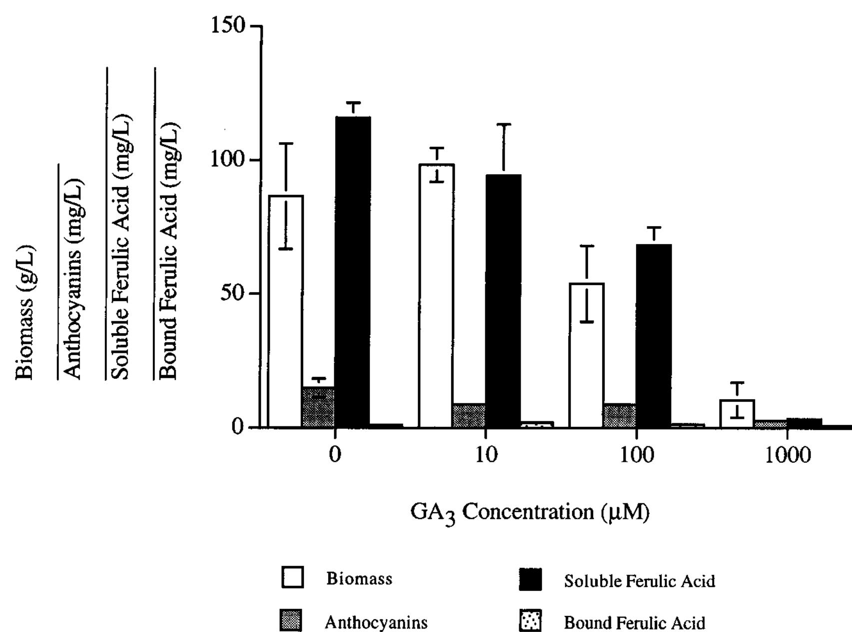

Figure 2. Effects of gibberellic acid on growth and anthocyanin and ferulic acid accumulation in *A. pyramidalis* Metallica Crispa cell culture.

Table 1. Anthocyanin and Ferulic Acid Accumulation in *A. pyramidalis* Metallica Crispa *in Vivo* and *in Vitro*

source	ferulic acid (mg/100 g of FW)	anthocyanins (mg/100 g of FW)	biomass (g/L of medium)
leaves	24.0 ± 2.4	11.0 ± 3.0	
high-pigmented cultures	138.48 ± 15.0	17.4 ± 2.5	86.58 ± 19.7
low-pigmented cultures	43.6 ± 3.9	5.85 ± 3.5	82.85 ± 7.4
dark grown cultures	39.97 ± 8.4	0	104.26 ± 10.3

HCl) (1:10 w/v) and extracted overnight at 4 °C. For the estimation of anthocyanins, the absorbance of the filtered solution was measured at 525 nm using a Beckman DU-65 spectrophotometer. The concentration of anthocyanins was calculated in terms of cyanidin 3,5-diglucoside using a molar absorption coefficient of 3.01×10^4 reported by Callebaut et al. (1990). Soluble and cell wall bound ferulic acid were estimated by alkaline hydrolysis (Markham, 1982; Strack et al., 1984). For the estimation of soluble ferulic acid, the methanol was evaporated under N_2 gas and the residue was hydrolyzed with 2 N NaOH overnight at room temperature. The hydrolysate was acidified with 2 N HCl, and the liberated ferulic acid was extracted with ethyl acetate. The ethyl acetate was evaporated *in vacuo* at 30 °C, and the residue was dissolved in 5 mL of methanol for the quantitative analysis of ferulic acid. Cell wall bound ferulic acid was extracted by hydrolysis as described by Strack et al. (1984). The tissue residue after methanol extraction was washed with methanol followed by acetone until the extract was colorless. The residue was treated with 2 N NaOH at 80 °C for 1 h and then allowed to stand at room temperature overnight. The hydrolysate was processed as before for the extraction of liberated ferulic acid.

The quantitative values were obtained using a reverse phase HPLC system and standard *trans*-ferulic acid (Sigma, St. Louis, MO). HPLC was performed using a Hitachi L-6200A Intelligent pump, a Hitachi diode array detector (Tokyo, Japan), and a Rheodyne (Cotiati, CA) 7125 injector. The column used was Whatman Partisil ODS-3 (250 × 4.6 mm, 5 μm) connected to a YMC-Pack S5 120A ODS-AM guard column. The solvents used were 10% formic acid (A) and 100% acetonitrile (B). Separation was obtained by an isocratic elution of 10% B in A at a flow rate of 1 mL/min, and the absorbance was monitored at 325 nm. Peak identification was based on the retention time and absorption spectra of standard ferulic acid. The presence of ferulic acid was further confirmed by the use of EI-MS. The peak corresponding to ferulic acid in the alkaline hydrolysates was isolated using semipreparative LC. EI-MS was performed using a VG 70-VSE mass spectrometer at 70 eV, employing a solid probe at 30 °C and an ion source temperature of 200 °C.

RESULTS AND DISCUSSION

The reverse phase HPLC analysis of the alkaline hydrolysate revealed ferulic acid as the major cinnamic acid in the cultures. The chromatographic conditions gave a clear separation of ferulic acid from other related cinnamic acids. Caffeic acid and *p*-coumaric acid eluted earlier (7.7 and 12.5 min, respectively) compared to ferulic acid (19.4 min) while sinapic acid did not elute from the column under the conditions employed. Quantitative analysis showed that the cultures accumulate mainly soluble ferulic acid [soluble, 40−138 mg/100 g of fresh weight (FW); bound, 1−1.9 mg/100 g of FW]. The UV absorption (UV_{max} 324 nm, shoulder 296 nm) and strong fluorescence properties indicated that the compound was *trans*-ferulic acid. The EI-MS of ferulic acid isolated from the alkaline hydrolysate showed the major molecular ion at *m/z* 194 ($C_{10}H_{10}O_4$), and the fragmentation pattern was identical to that of standard *trans*-ferulic acid. The time-course data revealed a close relationship between biomass, anthocyanin, and ferulic acid accumulation in the pigmented cultures (Figure 1). The biomass continued to increase through day 8 of culture, and anthocyanins and ferulic acid followed the same trend. Maximum accumulation was observed by 8 days of culture.

Ajuga cell cultures accumulate anthocyanins and other flavonoids in addition to cinnamic acids. It is a well-known fact that the biosyntheses of cinnamic acids and anthocyanins/flavonoids share a series of initial steps and intermediates before diverging from one another. It follows then that if the biosynthesis of anthocyanins and other flavonoids is inhibited, the cells may overaccumulate ferulic acid or its glycosides. We experimented with cultural conditions that would effectively inhibit anthocyanin/flavonoid accumulation and, in turn, lead to increased accumulation of ferulic acid in the cultures. Three means of anthocyanin inhibition were utilized: cultures grown in darkness; repeated subcultures in the presence of 2,4-D; and addition of GA_3 to the pigmented culture. Table 1 presents the relationship between anthocyanin and ferulic acid accumulation in some of the treatments. The maximum amount of ferulic acid was found in highly pigmented cell lines. In the dark grown cultures, anthocyanin accumulation was completely inhibited but the cultures accumulated lower levels of ferulic acid and had a higher growth rate compared to the results for the pigmented cell line.

Growth regulators 2,4-D and GA_3 have been reported to inhibit anthocyanin production in a number of cell lines including *Ajuga reptans* (Seitz and Hinderer, 1988; Callebaut et al., 1993). They inhibit chalcone synthase, the key enzyme responsible for the biosynthesis of the $C_6-C_3-C_6$ carbon skeleton of all flavonoids (Seitz and Hinderer, 1988). In *A. reptans* cell cultures, 2,4-D at 5.6 μM and GA_3 at 100 μM have been reported to exert pronounced negative effects on growth and anthocyanin production (Callebaut et al., 1993). The low-pigmented cell line derived in our studies by repeated subcultures in the callus induction medium containing 2.26 μM 2,4-D showed a reduction in both anthocyanin and ferulic acid accumulation as compared to the results for the high-pigmented culture (Table 1). Figure 2 presents the effects of supplementing increasing concentrations of GA_3 on growth and anthocyanin and ferulic acid accumulation in the cultures. At 100 and 1000 μM levels, GA_3 showed a strong inhibitory effect on all the parameters tested. At 10 μM, it had a slight stimulatory effect on growth, but the levels of ferulic acid and anthocyanins were reduced. None of the treatments altered the bound ferulic acid content in the cell lines. The results indicate that both growth regulators may inhibit some of the earlier enzymes in the phenylpropanoid pathway or the hydroxylating, methylating enzymes in the cinnamic acid biosynthetic pathway in the cultures. Our studies so far have indicated a close correlation between anthocyanin and ferulic acid accumulation in *A. pyramidalis* Metallica Crispa cultures. However, further examinations are needed to clarify cultural conditions for cell lines including media components and growth regulators, as well as irradiance conditions to enhance soluble ferulic acid accumulation.

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