Biotransformation and Impact of Ferulic Acid on Phenylpropanoid and Capsaicin Levels in *Capsicum annuum* L. cv. P1482 Cell Suspension Cultures

Seung-Mi Kang,[†] Hee-Young Jung,[†] Young-Min Kang,[†] Ji-Yun Min,[†]
C. S. Karigar,[‡] Jae-kyung Yang,[†] Sun-Won Kim,[§] Yeong-Rae Ha,[||]
Sung-Ho Lee,[§] and Myung-Suk Choi*,[†]

Divisions of Forest Science and Environmental Biotechnology National Core Research Center, and
Department of Food Science and Nutrition, Gyeongsang National University, Jinju, Korea, and
Department of Biochemistry, Bangalore University, Bangalore, 560001, India

Cell suspension cultures of *Capsicum annuum* L. cv. P1482 were fed with exogenous ferulic acid to monitor their biotransformation abilities. A portion of the ferulic acid was biotransformed into vanillin, a major natural flavor, and capsaicin, a principle secondary metabolite characteristic of *Capsicum* species. The cellular vanillin concentrations were relatively higher than capsaicin levels and were maximal (2 mg/g DW) 4 days after 0.6 mM ferulic acid feeding. Maximal vanillin levels in the culture medium were 10 mg/L at 4 and 3 days after feeding with 1.25 and 2.5 mM ferulic acid, respectively. With regard to capsaicin levels, the cellular levels were slightly decreased by ferulic acid feeding, whereas the levels in the culture medium were increased. Ferulic acid feeding not only enhanced vanillin and capsaicin production but also increased the concentrations of other phenylpropanoid metabolites.

KEYWORDS: Biotransformation; capsaicin; *Capsicum annuum* L.; cell suspension culture; ferulic acid; phenylpropanoids; vanillin

INTRODUCTION

Plants can be manipulated so that they produce novel products through biotransformation reactions (*1*). Such biotransformation reactions, along with precursor supplementation, have been used to produce secondary metabolites of economic interest. One such metabolite is vanillin (4-hydroxy-3-methoxybenzaldehyde), a major flavoring constituent of natural vanilla that is one of the most widely used flavoring substances the world over (**Figure 1**(*2*)). Vanillin is formed as an intermediate during the catabolism of eugenol (4-hydroxy-3-methoxy-phenyl-propene), ferulic acid (4-hydroxy-3-methoxy-*trans*-cinnamic acid), and lignin. Therefore, eugenol and ferulic acid are widely chosen as potential precursor substrates for biotransformation applications involving vanillin.

Ferulic acid is often found as a bound component of plant cell wall material and can be degraded into either vanillin or 4-vinyl-guaiacol (*2*). Therefore, ferulic acid serves as a major biosynthetic precursor of vanillin in plants (*3*). Most of these biotransformation studies have been carried out with microbial

systems, and the pathways by which precursors are biotransformed into valuable fine chemicals by plant cell cultures remain to be established.

Plant cell cultures can enzymatically transform various exogenously supplied precursor compounds. *Capsicum* species are the most suitable for such biotransformation studies since vanillin shares a common biosynthetic route involving phenylpropanoids. It is also possible to readily manipulate the secondary metabolic pathway of cultured *Capsicum annuum* cells (*4*). The major pungent metabolite of the *C. annuum* pepper is capsaicin, which is a derivative of vanillic acid (*5*). Two studies have described the production of vanillin through the biotransformation of suspended and immobilized cell cultures of *Capsicum* species (*6*, *7*). However, neither study examined the production kinetics of other phenylpropanoid compounds (cinnamic acid, ferulic acid, phenylalanine, and vanillic acid) or the production of capsaicin in relation to the production of vanillin. Ferulic acid in the culture medium can also influence the metabolism of other endogenous compounds of *C. annuum*. Therefore, we have investigated how the cinnamic acid, ferulic acid, phenylalanine, and vanillic acid levels in suspension cultures of *C. annuum* L. cv. P1482 vary in response to the exogenous precursor ferulic acid.

MATERIALS AND METHODS

Chemicals. Capsaicin, cinnamic acid, ferulic acid, phenylalanine, vanillin, and vanillic acid were purchased from Sigma Chemical Co.

* To whom correspondence should be addressed. Tel: +82-55-751-5493. Fax: +82-55-753-6015. E-mail: mschoi@nongae.gsnu.ac.kr.
† Division of Forest Science, Gyeongsang National University.
‡ Bangalore University.
§ Department of Food Science and Nutrition, Gyeongsang National University.
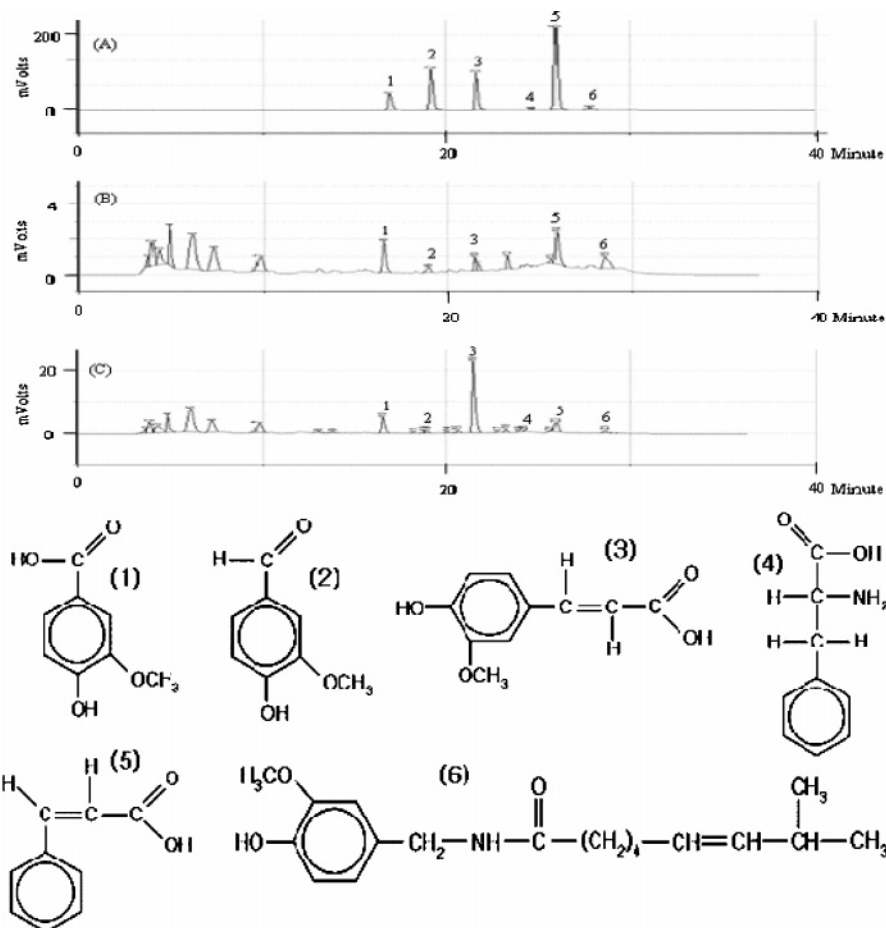|| Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju, Korea.

Figure 1. HPLC chromatography and chemical structures of the phenylpropanoids and capsaicin in the suspension culture of *Capsicum annuum* cv. P1482. (**a**) Chromatography of standard compounds, (**b**) chromatography of the extracts of the suspended cells, and (**c**) chromatography of the medium extract. Structures: 1, vanillic acid; 2, vanillin; 3, ferulic acid; 4, phenylalanine; 5, cinnamic acid; and 6, capsaicin.

(St. Louis, MO). All solvents used in high-performance liquid chromatography (HPLC) analysis were procured from Merck (Germany).

Plant Cell Culture. Calli of *C. annuum* L. cv. P1482 were induced in the dark at 25 °C on Murashige and Skoog's (MS) solid medium supplemented with 3% (w/v) sucrose and 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and subcultured every 4 weeks.

Cell Suspension Culture. *C. annuum* cells grown for 2 weeks on solid medium (1 g) were cultured in 100 mL Erlenmeyer flasks containing 30 mL of MS liquid medium supplemented with 3% (w/v) sucrose and 2 mg/L 2,4-D. The flasks were maintained in the dark at 25 °C in a rotary shaking incubator at 100 rpm.

Ferulic Acid Supplementation. Ferulic acid was dissolved in 50% (v/v) ethanol and added to the medium after filter sterilization. Ferulic acid was added to separate 2 week old *C. annuum* cell suspension cultures at concentrations of 0.6, 1.25, and 2.5 mM. The phenylpropanoid and capsaicin levels in the cells and spent culture medium were monitored every day for 6 days after the precursor feeding. All experiments were performed in triplicate in three independent experiments.

Extraction of Metabolites. Cells were collected from the spent culture medium by filtration using Whatman (no. 4) filter paper, and the culture medium was extracted twice with ethyl acetate (30 mL × 2) and pooled. The extracts were vortexed and evaporated to dryness under vacuum. The cells were thoroughly washed with distilled water and dried in an oven at 40 °C for 24 h and then weighed and powdered with a mortar and pestle. The amorphous powder of cells was extracted with ethyl acetate (10 mL) and centrifuged at 12000*g* for 10 min. The supernatant was concentrated by solvent extraction, vortexing, and evaporation to dryness under vacuum. The dry residue was dissolved in 50 μL of 80% (v/v) ethanol, and then, an aqueous solution was passed through Whatman (no. 2) filter paper. The extracts obtained from the

cells and spent culture mediums were analyzed separately for metabolites. All of the data presented are averages of at least three independent experiments.

HPLC. The filtered samples were finally passed through Millipore (0.2 μm) microfilters and transferred with a syringe to the injection port of the HPLC system (Gilson, France) equipped with a TSK gel ODS-80 column (4.6 mm i.d. × 25 cm, 5 μm, Tosho) and a UV detector (Gilson, UV 3000) operating at a wavelength of 280 nm. The mobile phases were solvent A [a mixture of water and 5% (v/v) glacial acetic acid] and solvent B (methanol). After 10 μL of the sample was injected, the HPLC system was operated at a flow rate of 0.8 mL/min. The elution program was used as follows: first, the proportion of solvent B rose linearly from 0% at 0 min to 10% at 5 min, after which it rose to 30% at 10 min and 70% at 20 min, then finally decreasing to 10% at 30 min. For quantitative analysis, the system was calibrated with standards of capsaicin, cinnamic acid, ferulic acid, phenylalanine, vanillin, and vanillic acid. Quantification of the compounds was achieved by comparing the retention times with data obtained for the standards and a cochromatogram of the standards and samples. Data were expressed as averages of three separate experiments. The error bars indicate standard deviation (SD) from the mean of each replicate treatment. The statistical significance of the experimental results was assessed by Duncan's multiple range test ($p = 0.05$).

RESULTS AND DISSCUSION

The biotransformation of ferulic acid into vanillin was monitored every day for 6 days after feeding *C. annuum* L. cv. P1482 cell suspension cultures with various concentrations of the precursor. Johnson et al. (*8*) have reported that vanillin is not detected in *Capsicum* species cell cultures in the absence of the precursor. However, we observed trace amounts of

Changes in Phenylpropanoid and Capsaicin Levels

J. Agric. Food Chem., Vol. 53, No. 9, 2005 **3451**

Table 1. Changes in Cinnamic Acid, Phenylalanine, Vanillic Acid, and Capsaicin Levels in *C. annuum* L. cv. P1482 Suspension Cell Cultures upon Feeding with Various Concentrations of Ferulic Acid (Con, Control)

	days	cinnamic acid[a]	phenylalanine[a]	vanillic acid[a]	vanillin[a]	ferulic acid[a]	capsaicin[a]
Con	1	1.01 ± 0.05[d]	1.07 ± 0.01[n]	0.25 ± 0.02[kl]	0.17 ± 0.02[l]	0.2 ± 0.01[r]	1.62 ± 0.01[h]
	2	1.14 ± 0.06[c]	1.07 ± 0.03[n]	0.24 ± 0.02[kl]	0.16 ± 0.01[l]	0.23 ± 0.01[r]	1.84 ± 0.01[g]
	3	1.25 ± 0.02[b]	1.75 ± 0.02[i]	0.23 ± 0.01[l]	0.13 ± 0.01[m]	0.30 ± 0.02[r]	2.11 ± 0.02[a]
	4	1.50 ± 0.09[a]	1.62 ± 0.02[j]	0.27 ± 0.03[k]	0.18 ± 0.01[l]	0.32 ± 0.02[r]	2.06 ± 0.01[bc]
	5	1.19 ± 0.07[c]	1.71 ± 0.03[i]	0.26 ± 0.01[k]	0.13 ± 0.01[m]	0.30 ± 0.02[r]	2.05 ± 0.04[cd]
	6	0.87 ± 0.01[f]	1.19 ± 0.01[m]	0.23 ± 0.01[kl]	0.12 ± 0.01[m]	0.21 ± 0.01[r]	2.02 ± 0.02[cde]
0.6 mM	1	0.67 ± 0.03[ij]	3.43 ± 0.01[c]	1.67 ± 0.03[d]	0.38 ± 0.02[h]	11 ± 0.15[q]	1.34 ± 0.05[i]
	2	0.60 ± 0.01[k]	2.40 ± 0.01[f]	1.52 ± 0.04[f]	0.39 ± 0.02[h]	13 ± 0.21[p]	1.34 ± 0.03[i]
	3	0.52 ± 0.02[lm]	1.60 ± 0.03[j]	1.57 ± 0.02[e]	0.48 ± 0.01[f]	20 ± 0.30[o]	1.34 ± 0.02[i]
	4	0.56 ± 0.01[kl]	1.82 ± 0.05[h]	3.64 ± 0.02[a]	2.00 ± 0.03[a]	40 ± 0.32[k]	1.34 ± 0.03[i]
	5	0.72 ± 0.04[hi]	1.40 ± 0.03[l]	2.11 ± 0.01[b]	0.62 ± 0.01[d]	34 ± 0.25[m]	1.27 ± 0.03[j]
	6	0.46 ± 0.01[n]	1.04 ± 0.02[no]	2.13 ± 0.02[b]	0.76 ± 0.01[c]	37 ± 0.24[l]	1.34 ± 0.01[i]
1.25 mM	1	0.62 ± 0.02[ijk]	0.60 ± 0.05[p]	0.34 ± 0.04[j]	0.49 ± 0.02[f]	49 ± 0.32[h]	1.34 ± 0.05[i]
	2	0.57 ± 0.02[kl]	1.06 ± 0.03[n]	0.37 ± 0.02[j]	0.33 ± 0.02[ij]	48 ± 0.35[i]	1.34 ± 0.03[i]
	3	0.50 ± 0.01[mn]	0.99 ± 0.02[o]	0.68 ± 0.01[h]	0.52 ± 0.02[e]	53 ± 0.24[g]	1.34 ± 0.03[i]
	4	0.61 ± 0.03[ijk]	0.49 ± 0.01[q]	0.37 ± 0.02[j]	0.35 ± 0.02[i]	47 ± 0.09[j]	1.66 ± 0.03[h]
	5	0.62 ± 0.03[ijk]	0.53 ± 0.01[q]	0.37 ± 0.02[j]	0.30 ± 0.01[k]	30 ± 0.10[n]	2.10 ± 0.01[ab]
	6	0.67 ± 0.02[ij]	1.72 ± 0.08[i]	1.47 ± 0.02[g]	0.60 ± 0.01[d]	68 ± 0.52[e]	2.00 ± 0.05[de]
2.5 mM	1	0.81 ± 0.02[g]	3.11 ± 0.08[d]	0.27 ± 0.01[k]	0.30 ± 0.01[k]	75 ± 0.52[b]	1.99 ± 0.01[e]
	2	0.93 ± 0.03[e]	2.65 ± 0.07[e]	1.97 ± 0.02[c]	0.92 ± 0.02[b]	97 ± 0.58[a]	1.87 ± 0.01[g]
	3	0.80 ± 0.01[g]	6.61 ± 0.06[a]	0.42 ± 0.01[i]	0.74 ± 0.02[c]	72 ± 0.95[c]	2.02 ± 0.03[acd]
	4	0.74 ± 0.01[h]	1.51 ± 0.03[k]	0.26 ± 0.03[k]	0.38 ± 0.01[h]	68 ± 0.92[e]	2.00 ± 0.03[de]
	5	0.74 ± 0.02[h]	2.10 ± 0.03[g]	0.27 ± 0.02[k]	0.31 ± 0.01[kj]	64 ± 0.82[f]	1.83 ± 0.02[g]
	6	0.80 ± 0.01[g]	3.83 ± 0.02[b]	0.37 ± 0.02[j]	0.42 ± 0.01[g]	69 ± 0.52[d]	1.94 ± 0.02[f]

[a] mg/g DW.

vanillin in the *C. annuum* control cultures, as evidenced by our HPLC analysis of the cells (**Table 1**). In these control cells, the maximum level of vanillin produced was 0.18 mg/g DW (**Table 1**). As vanillin shares the same metabolic route as that of the phenylpropanoids of *C. annuum*, this may lead to its accumulation in *C. annuum* control cells. In fact, vanillin occurs in trace amounts in other plants, including commercial products such as tobacco (*9*). Vanillin has also been reported to be present in many fruits and fruit products. In mango, it is found either as "free" vanillin or as vanillyl glucoside (*10*). However, when the *C. annuum* cells were fed with ferulic acid, their vanillin levels increased by 10-fold. The cellular vanillin levels were highest 4 days after 0.6 mM ferulic acid feeding, as quantities up to 2 mg/g DW were obtained.

The increased accumulation of vanillin in *C. annuum* cells after feeding with ferulic acid suggests that the externally provided ferulic acid is being converted to vanillin. However, not all of the ferulic acid taken up by the cells in culture may be converted to vanillin, as when the cells were fed with 2.5 mM ferulic acid, the cells contained considerably more ferulic acid than when they were fed with lower amounts. This may be due to the ability of high substrate concentrations to inhibit the biotransformation process. We found that the vanillin levels were maximal when the cells were fed with only 0.6 mM ferulic acid. This indicates that this may be the optimal or near optimal concentration of the biotransformation reaction component. Hence, it is important to determine the optimum ferulic acid concentration with which to feed the cultured cells, as this determines the efficiency of the biotransformation of ferulic acid to vanillin.

The vanillin levels in the spent culture medium of the control cells remained unchanged up to 5 days but increased on the sixth day (**Table 2**). Upon feeding the cells with ferulic acid, the maximal vanillin level in the culture medium was found to be 10 mg/L after 4 days of feeding 1.25 mM ferulic acid, which differs from the patterns oberved within the cells. The untransformed ferulic acid was determined to range between 180 and 350 mg/L at 1.25 mM and between 370 and 460 mg/L at 2.5 mM. However, both 1.25 and 2.5 mM ferulic acid fed cultures

exhibited similar vanillin contents (approximately 10 mg/L) in the spent culture medium. This again suggests that high doses of ferulic acid may inhibit the enzyme required for vanillin biotransformation.

The effect of exogenous ferulic acid feeding on the levels of other phenylpropanoid compounds and capsaicin in the cell suspension cultures (**Table 1**) and in the spent medium (**Table 2**) was then investigated. In the cells, the capsaicin concentrations were slightly decreased by ferulic acid feeding and did not accumulate to significant levels. In contrast, the capsaicin levels in the culture medium were increased in the 1.25 and 2.5 mM ferulic acid fed cultures, with the maximal level being 3.78 mg/L in the 2.5 mM ferulic acid fed culture after 1 day. Holden et al. (*11*) have reported that the sink of bound phenolics in the cell wall of plants may turnover and thus could serve as substrates for the capsaicin biosynthetic pathway. Other studies have indicated that cell cultures derived from *C. annuum* (*12*, *13*) or *C. frutescens* (*14*) produce capsaicin that is excreted into the medium. Capsaicin is a small molecule with a low melting point and moderate hydrophobicity. These properties facilitate the penetration of the skin stratum corneum by capsaicin from topical applications. Capsaicin has been used for many years as a topical treatment for chronic pain conditions, including posttherpetic neuralgia, painful diabetic neuropathy, and osteoarthritis. (*15*, *16*) In addition, capsaicin is an incredibly powerful and stable alkaloid seemingly unaffected by cold or heat, as it retains its original potency despite age, cooking, or freezing (*17*). Therefore, capsaicin is also a popular food ingredient. Consequently, it is interesting that capsaicin levels as well as vanillin levels in the culture medium are increased by ferulic acid feeding.

The vanillic acid levels in the cells increased rapidly after 4 days of 0.6 mM ferulic acid feeding; this is similar to that of other concentrations of ferulic acid. Vanillic acid is the principal intermediate in the ferulic acid degradation pathway and, unlike vanillin, often accumulates in substantial amounts (*18*, *19*). Barghini et al. (*20*) have reported a yield of 95% vanillic acid (6 g/L) from ferulic acid within 5 h with *Pseudomonas fluorescens* BF13. Therefore, vanillin accumulation can be

3452 *J. Agric. Food Chem.,* Vol. 53, No. 9, 2005

Kang et al.

Table 2. Changes in Cinnamic Acid, Phenylalanine, Vanillic Acid, and Capsaicin Levels in the Spent Culture Medium of *C. annuum* L. cv. P1482 Suspension Cell Cultures upon Feeding with Various Concentrations of Ferulic Acid (Con, Control)

		cinnamic acid[a]	phenylalanine[a]	vanillic acid[a]	vanillin[a]	ferulic acid[a]	capsaicin[a]
Con	1	0.98 ± 0.01^f	2.34 ± 0.01^r	0.27 ± 0.01^d	0.42 ± 0.02^t	1.7 ± 0.02^o	1.06 ± 0.02^m
	2	1.13 ± 0.02^e	1.11 ± 0.02^u	0.20 ± 0.04^d	0.29 ± 0.01^v	1.3 ± 0.02^o	1.99 ± 0.02^f
	3	0.49 ± 0.02^h	2.62 ± 0.01^q	0.20 ± 0.05^d	0.40 ± 0.02^t	1.4 ± 0.01^o	1.60 ± 0.03^h
	4	1.64 ± 0.02^d	1.82 ± 0.02^t	0.26 ± 0.07^d	0.25 ± 0.01^w	1.2 ± 0.01^o	1.45 ± 0.06^{ik}
	5	0.83 ± 0.01^g	2.35 ± 0.01^r	0.27 ± 0.01^d	0.37 ± 0.02^u	1.3 ± 0.01^o	1.87 ± 0.05^g
	6	1.56 ± 0.01^d	2.05 ± 0.04^s	2.81 ± 0.01^b	1.01 ± 0.02^s	3.0 ± 0.03^o	1.48 ± 0.03^{ij}
0.6 mM	1	4.35 ± 0.02^c	7.33 ± 0.02^i	2.67 ± 0.04^{bc}	5.00 ± 0.03^o	95 ± 1.2^l	1.38 ± 0.05^{kl}
	2	4.34 ± 0.03^c	5.46 ± 0.01^l	2.66 ± 0.02^{bc}	4.97 ± 0.02^p	88 ± 1.2^m	1.34 ± 0.03^l
	3	4.34 ± 0.03^c	4.31 ± 0.02^m	2.61 ± 0.02^c	3.71 ± 0.03^q	62 ± 0.9^n	1.59 ± 0.05^h
	4	4.33 ± 0.04^c	2.79 ± 0.05^p	2.76 ± 0.01^{bc}	5.51 ± 0.01^m	117 ± 1.5^k	1.38 ± 0.06^{kl}
	5	4.32 ± 0.09^c	2.95 ± 0.08^o	2.77 ± 0.01^{bc}	5.83 ± 0.01^l	122 ± 1.9^i	1.34 ± 0.01^l
	6	4.31 ± 0.12^c	3.45 ± 0.09^n	2.67 ± 0.08^{bc}	3.19 ± 0.02^r	119 ± 1.8^{ik}	1.55 ± 0.01^{hi}
1.25 mM	1	8.69 ± 0.04^b	8.63 ± 0.09^e	5.34 ± 0.09^a	7.70 ± 0.03^g	240 ± 2.8^f	2.75 ± 0.01^c
	2	8.69 ± 0.15^b	8.01 ± 0.07^h	5.30 ± 0.02^a	5.98 ± 0.02^k	180 ± 2.1^i	2.67 ± 0.04^d
	3	8.69 ± 0.03^b	8.98 ± 0.07^d	5.30 ± 0.09^a	5.03 ± 0.02^n	213 ± 2.9^h	2.68 ± 0.03^{cd}
	4	8.69 ± 0.21^b	7.32 ± 0.07^i	5.44 ± 0.09^a	10.01 ± 0.01^a	225 ± 2.8^g	2.65 ± 0.03^d
	5	8.69 ± 0.22^b	6.19 ± 0.06^k	5.39 ± 0.15^a	9.39 ± 0.01^c	331 ± 1.5^e	2.91 ± 0.03^b
	6	8.69 ± 0.02^b	6.64 ± 0.06^j	5.31 ± 0.02^a	8.54 ± 0.01^d	350 ± 1.2^d	2.51 ± 0.03^e
2.5 mM	1	8.68 ± 0.05^b	10.98 ± 0.02^a	5.34 ± 0.14^a	7.74 ± 0.01^f	383 ± 1.5^b	3.78 ± 0.03^a
	2	8.67 ± 0.08^b	9.44 ± 0.04^b	5.33 ± 0.12^a	7.00 ± 0.01^i	370 ± 2.5^c	2.91 ± 0.03^b
	3	8.69 ± 0.06^b	9.33 ± 0.03^c	5.34 ± 0.16^a	9.91 ± 0.01^b	386 ± 2.5^b	2.68 ± 0.03^{cd}
	4	10.50 ± 0.06^a	8.27 ± 0.01^f	5.33 ± 0.08^a	8.44 ± 0.01^e	384 ± 2.9^b	2.67 ± 0.06^d
	5	8.68 ± 0.04^b	8.12 ± 0.12^g	5.34 ± 0.04^a	6.44 ± 0.02^j	456 ± 2.8^a	2.66 ± 0.09^d
	6	8.69 ± 0.12^b	8.62 ± 0.09^e	5.35 ± 0.22^a	7.34 ± 0.01^h	460 ± 2.8^a	2.68 ± 0.08^{cd}

[a] mg/L.

important to avoid degradation of exogenously fed ferulic acid to vanillic acid. In our results, the accumulation patterns of vanillic acid by *C. annuum* cells resembled those of vanillin and ferulic acid.

Phenylalanine and cinnamic acid are further upstream in the biotransformation of ferulic acid to vanillin. However, phenylalanine is a precursor of capsaicin and proteins. Cinnamic acid, the product of the reaction catalyzed by phenylalanine ammonialyase (PAL, EC 4.3.1.5.), would also be expected to be incorporated into capsaicin, although this has not been demonstrated previously in cultured cells. Cinnamic acid has been reported to be involved in cell wall metabolism, and this may proceed in competition with capsaicin synthesis (*21*). Therefore, we investigated the effect of ferulic acid feeding on phenylalanine and cinnamic acid levels in the cells and culture medium. We found that phenylalanine levels in the cell were increased by ferulic acid feeding as compared to the control cultures while cinnamic acid levels were slightly decreased. However, the levels of both phenylalanine and cinnamic acid in the spent medium were markedly increased by ferulic acid feeding, with the highest levels being observed (10.98 and 10.50 mg/L, respectively) when the cells had been fed with the highest concentration of ferulic acid tested (2.5 mM). It is not clear how feeding cell cultures with precursors influences this capsaicinoid content. Dixon and Paiva (*22*) reported that stress may influence phenylpropanoid metabolism and may indirectly affect capsaicinoid synthesis. Thus, it may be that vanillin and capsaicin levels in cultured *C. annuum* cells become altered due to changes in phenylpropanoid metabolism because of the biotransformation of ferulic acid to vanillin and that the stress promoted by these changes may eventually induce capsaicin synthesis.

We assessed the efficiency with which exogenously fed ferulic was converted into vanillin on the basis of our biotransformation results by calculating the biotransformation ratio (**Figure 2**). In cells, vanillin accumulation was highest on the fourth day when the cultures were fed with 0.6 mM ferulic acid (**Figure 2a**). Although 1.25 and 2.5 mM ferulic acid fed cultures contained more ferulic acid (2 and 4 times) than the 0.6 mM

fed culture, their vanillin contents were only 30 and 50% of those in the 0.6 mM fed culture, respectively. In the spent culture medium, the vanillin levels produced by both the 1.25 and the 2.5 mM ferulic acid fed cultures were similar (10 mg/L). However, the bioconversion of ferulic acid into vanillin in the culture medium was considerably enhanced in the 1.25 mM ferulic acid fed cultures (**Figure 2b**).
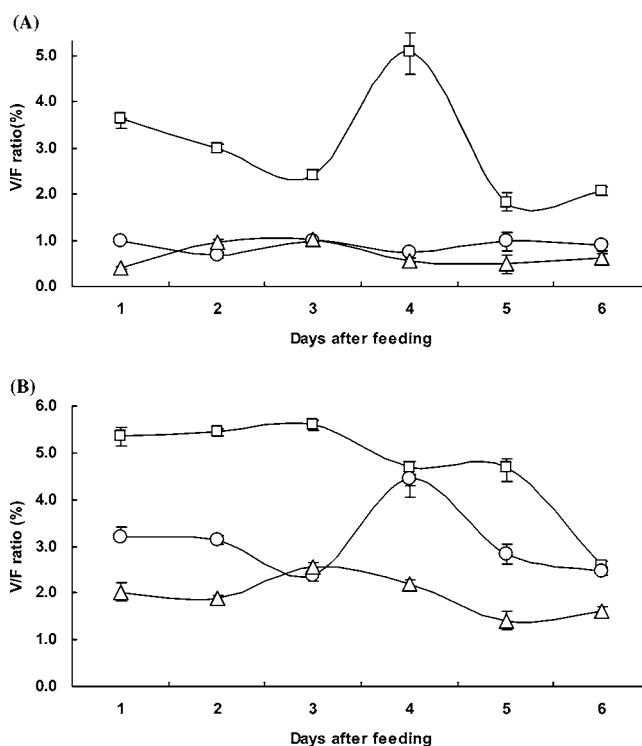

Figure 2. Biotransformation of ferulic acid into vanillin in (**A**) suspension cultures of *C. annuum* L. cv. P1482 cells and (**B**) their culture medium in response to feeding with ferulic acid. The V/F ratio is the percentage of vanillin/ferulic acid contents (□, 0.6 mM ferulic acid; ○, 1.25 mM ferulic acid; and △, 2.5 mM ferulic acid).

Changes in Phenylpropanoid and Capsaicin Levels

J. Agric. Food Chem., Vol. 53, No. 9, 2005 **3453**

Thus, in our study, vanillin accumulation was transiently increased in the presence of ferulic acid. Vanillin may have formed as an intermediate during ferulic acid degradation. The pathways involved in the conversion of ferulic acid to vanillin in this plant systems were not investigated in detail. However, a β-oxidation mechanism analogous to the β-oxidation pathway of fatty acid catabolism has been proposed for the degradation of substituted cinnamic acids in wheat shoots (*23*). There are also several reports on the occurrence of vanillin as an intermediate of the microbial degradation of ferulic acid (*24–27*).

In conclusion, the unique metabolism of *C. annuum* produces vanillin as the metabolic overflow product during the degradation of exogenously fed ferulic acid since the feeding of cell cultures with ferulic acid elevated the cellular concentrations of ferulic acid, vanillin, and vanillic acid. Moreover, ferulic acid feeding also varies the accumulation of other phenylpropanoid compounds and capsaicin in *C. annuum* since supplementation of the culture medium with the precursor increased the levels of not only ferulic acid, vanillin, and vanillic acid but also cinnamic acid and phenylalanine. The maximal levels of vanillin in the cultured cells and the spent culture medium were 2 mg/g DW and 10 mg/L, respectively, which are lower as compared to those produced by bacterial cultures (*28*). However, the dramatic enhancement of phenylpropanoid and capsaicin levels produced by *C. annuum* suspension cultures upon the exogenous feeding of ferulic acid is particularly interesting. It suggests that ferulic acid feeding may be able to modify the production of the pungent molecule capsaicin and therefore could have a variety of applications in the medicinal and food industry.

LITERATURE CITED

(1) Pras, N. Bioconversion of naturally occurring precursors and related synthetic compounds using plant cell cultures. *J. Biotechnol.* **1992**, *26*, 29–62.

(2) Peleg, H.; Naim, M.; Zehavi, U.; Rouseff, R. L.; Nagy, S. Pathways of 4-vinylguaiacol formation from ferulic acid in model solutions of orange juice. *J. Agric. Food Chem.* **1992**, *40*, 764–767.

(3) Goodner, K. L.; Jella, P.; Rouseff, R. L. Determination of vanillin in orange, grapefruit, tangerine, lemon, and lime juices using GC–olfactometry and GC-MS/MS. *J. Agric. Food Chem.* **2000**, *48*, 2882–2886.

(4) Lindsey, K. Incorporation of [^{14}C] phenylalanine and [^{14}C] cinnamic acid into capsaicin in cultured cells of *Capsicum frutescens*. *Phytochemistry* **1986**, *25*, 2793–2801.

(5) Bennett, D. J.; Kirby, G. W. Constitution and biosynthesis of capsaicin. *J. Chem. Soc.* **1968**, *C*, 442–445.

(6) Rao, S. R.; Ravishankar, G. A. Biotransformation of protocatechuic aldehyde and caffeic acid to vanillin and capsaicin in freely suspended and immobilized cell cultures of Capsicum frutescens. *J. Biotechnol.* **2000**, *76*, 137–146.

(7) Suresh, B.; Ritu, T.; Ravishankar, G. A. Vanillia flavour production through biotransformation using *Capsicum frutescens* culture. *Biocatal. Biotransform.* **2003**, *121*, 333–340.

(8) Johnson, T. S.; Ravishankar, G. A.; Venkataraman, L. V. Biotransformation of ferulic acid and vanillylamine to capsaicin and vanillin in immobilized cell culture of *Capsicum frutescens*. *Plant Cell, Tissue Organ Cult.* **1996**, *44*, 117–121.

(9) Makkar, H. P. S.; Becker, K. Vanillin-HCl method for condensed tannins. *J. Chem. Ecol.* **1993**, *19*, 613–621.

(10) Sakho, M.; Chassagne, D.; Crouzet, J. African mango glycosidically bound volatile compounds. *J. Agric. Food Chem.* **1997**, *45*, 883–888.

(11) Holden, M. A.; Hall, R. D.; Lindsey, K.; Yeoman, M. M. Capsaicin biosynthesis in cell cultures of *Capsicum frutescens*. In *Process Possibilities for Plant and Animal Cell Culture*; Webb, C., Mavituna, F., Faria, J. J., Eds.; Institute of Chemical Engineers Publications: Ellis Horwood: Chichester, J. K., 1987.

(12) Salgado-Carciglia, R.; Ochoa-Alejo, N. Increased capsaicin content in PFP-resistant cells of chilli pepper (*Capsicum annuum* L.). *Plant Cell Rep.* **1990**, *8*, 617–620.

(13) Sudhakar Johnson, T.; Ravishankar, G. A.; Venkataraman, L. V. Separation of capsaicin from phenylpropanoid compounds by high-performance liquid chromatography to determine the biosynthetic status of cells and tissues of *Capsicum frutescens* Mill. in vivo and in vitro. *J. Agric. Food Chem.* **1992**, *40*, 2461–2463.

(14) Sudhakar Johnson, T.; Ravishankar, G. A. Precursor biotransformation in immobilized placental tissues of *Capsicum frutescens* Mill II. Influence of feeding intermediates of the capsaicinoid pathway in combination with l-valine on capsaicin and dihydrocapsaicin accumulation. *J. Plant Physiol.* **1998**, *153*, 240–243.

(15) Szallasi, A.; Blumberg, P. M. Vanilloid (Capsaicin) receptors and mechanisms. *Pharmacol. Rev.* **1999**, *51*, 159–212.

(16) Katritzky, A. R.; Xu, Y. J.; Vakulenko, A. Model compounds of caged capsaicin, synthesis, and photoreactivity. *J. Org. Chem.* **2003**, *68*, 9100–9104.

(17) Ochi, T.; Takaishi, Y.; Kogure, K.; Yamauti, I. Antioxidant activity of a new capsaicin derivative from Capsicum annuum. *J. Nat. Prod.* **2003**, *66*, 1094–1096.

(18) Andreoni, V.; Bernasconi, S.; Bestetti, G. Biotransformation of ferulic acid and related compounds by mutant strains of *Pseudomonas fluorescens. Appl. Microbiol. Biotechnol.* **1995**, *42*, 830–835.

(19) Eggeling, L.; Sahm, H. Degradation of coniferyl alcohol and other lignin-related aromatic compounds by *Nacardia* sp DSM 1069. *Arch. Microbiol.* **1980**, *126*, 141–148.

(20) Barghini, P.; Montebove, F.; Ruzzi, M.; Schiesser, A. Optimal conditions for bioconversion of ferulic acid into vanillic acid by *Pseudomonas fluorescens* BF13 cells. *Appl. Microbiol. Biotechnol.* **1998**, *49*, 309–314.

(21) Priefert, H.; Rabenhorst, J.; Steinbiichel, A. Biotechnological production of vanillin. *Appl. Microbiol. Biotechnol.* **2001**, *56*, 296–314.

(22) Dixon, R.; Paiva, N. Stress-induced phenylpropanoid metabolism. *Plant Cell* **1995**, *7*, 1085–1097.

(23) Vollmer, K. O.; Reisener, H. J.; Grisebach, H. The formation of acetic acid from *p*-hydroxycinnamic acid during its degradation to *p*-hydroxybenzoic acid in wheat shoots. *Biochem. Biophys. Res. Commun.* **1965**, *21*, 221–225.

(24) Achterholt, S.; Priefert, H.; Steinbuchel, A. Identification of *Amycolatopsis* sp. Strain HR167 genes, involved in the bioconversion of ferulic acid to vanillin. *Appl. Microbiol. Biotechnol.* **2000**, *54*, 799–807.

(25) Edlin, D. A. N.; Narbad, A.; Dickinson, J. R.; Llyod, D. The biotransformation of phenolics compounds by *Brettanomyces anomalus. FEMS Microbiol. Lett.* **1995**, *125*, 311–316.

(26) Falconnier, B.; Lapierre, C.; Lesage-Meessen, L.; Yonnet, G.; Brunerie, P.; Ceccaldi, B. C.; Corrieu, G.; Asther, M. Vanillin as a product of ferulic acid biotransformation by the white rot fungus *Pycnoporus cinnabarinus* I-37: Identification of metabolic pathways. *J. Biotechnol.* **1994**, *37*, 123–132.

(27) Gurujeyalakshmi, G.; Mahadevan, A. Dissimilation of ferulic acid by *Bacillus subtilis. Curr. Microbiol.* **1987**, *16*, 69–73.

(28) Narbad, A.; Gasson, M. J. Metabolism of ferulic acid via vanillin using a novel CoA-dependent pathway in a newly isolated strain a *Psedomonas fluorescens. Microbiology* **1998**, *144*, 1397–1405.