J. Microbiol. Biotechnol. (1998), **8**(3), 237–244

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY

Effect of Environmental Factors on Flavonol Glycoside Production and Phenylalanine Ammonia-lyase Activity in Cell Suspension Cultures of *Ginkgo biloba*

KIM, MIN-SOO, WON-KYU LEE, HWA-YOUNG KIM, CHUL KIM, AND YEON-WOO RYU*

Division of Chemical Engineering and Biotechnology, College of Engineering, Ajou University, Suwon 442-749, Korea

Received: February 6, 1998

Abstract A study was carried out to elucidate the relation between the production of flavonol glycosides and the change of phenylalanine ammonia-lyase activity in cell suspension cultures of *Ginkgo biloba* by the unassisted and synergistic effects of various factors. The quercetin production showed a mixed-growth-associated pattern in cell suspension cultures. Fluorescent light and UV radiation increased phenylalanine ammonia-lyase (PAL) activity, and resulted in the increase of the production of quercetin and kaempferol ten- and four-fold, respectively, as compared to that obtained in the normal culture condition. The cell growth of *Ginkgo biloba* was enhanced at higher temperatures whereas the quercetin production was at its maximum at low temperatures. Moreover, the quercetin production was increased by temperature change during the culture period. In particular, the quercetin production was at the highest level when the culture temperature was elevated from 10°C to 30°C. The addition of phenylalanine as a precursor in the culture medium stimulated an 8-fold increase in the production of quercetin; the addition of naringenin caused a 10-fold increase. The quercetin production was also greatly increased by feeding enzyme cofactors such as 2-ketoglutarate and ascorbic acid in the culture medium, but specific PAL activity was not increased except with phenylalanine feeding. The synergistic effect of UV radiation and naringenin feeding was observed, resulting in the increase of flavonol glycoside production at a rate higher than in any other case investigated.

Key words: Flavonol glycosides, *Ginkgo biloba*, synergistic effect, PAL activity

Recently, attention has been paid to the secondary metabolites of plants due to their importance as drugs, flavours, and other industrial materials. Since many pharmaceuticals and other industrial products are based on plant raw materials, much effort has been made in the area of plant cell culture selection for high productivity and in the alteration of cell metabolism by environmental factors. However, such high-producing cultures often show a decline in productivity upon serial propagation. Another approach to improve the yield of production in plant cell cultures is the alteration of cell metabolism by external factors. Numerous studies have been carried out in an attempt to increase the production by plant cell culture of these useful secondary metabolites. However, industrial utilization of plant cell cultures for the production of biochemicals has been hampered by low yields of the target substances.

A mixture of flavonol glycosides have been extracted from the leaves of *Ginkgo biloba* and used as a pharmaceutical drug for certain circulatory diseases [20, 21]. More than three flavonol aglycones such as kaempferol, quercetin, and isorhamnetin have been separated from *Ginkgo biloba* leaves and identified by reverse phase HPLC and ultraviolet spectroscopy [21]. Cell cultures of *Ginkgo biloba* accumulate flavonol glycosides such as quercetin, kaempferol, and isorhamnetin glycosides. The specific yield, however, was much less than that in leaves [16].

The biosynthesis of flavonoids such as quercetin, kaempferol, and isorhamnetin glycosides are coordinately involved in two metabolic pathways (Fig. 1). The first pathway is a general phenylpropanoid pathway which contains three steps in the conversion of phenylalanine to 4-coumaroyl~CoA. The enzymes catalyzing the individual steps are phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase, and 4-coumarate~CoA ligase [22]. PAL, which exists as a tetramer with a MW range of 240,000~330,000 daltons, was discovered by Koukol [12] and is known as the key enzyme of the phenylpropanoid pathway. Various environmental stresses such as UV

*Corresponding author
Phone: 82-331-219-2449; Fax: 82-331-216-8777;
E-mail: ywryu@madang.ajou.ac.kr

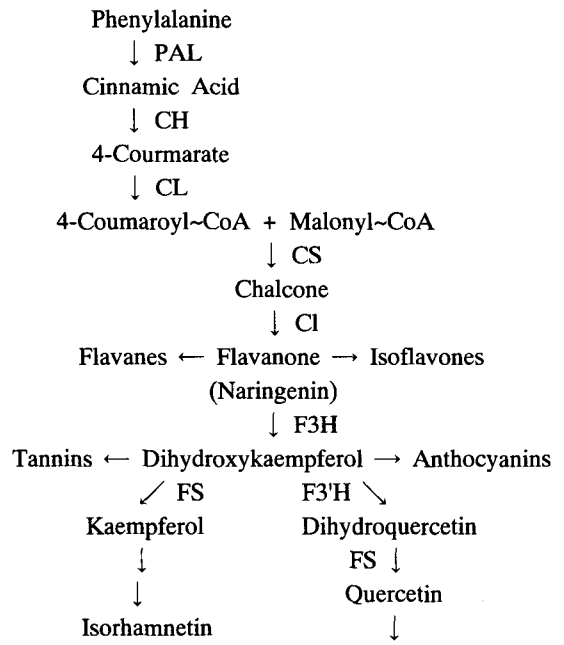

Fig. 1. The biosynthetic pathways of a general phenylpropanoid and flavonoid.
PAL: Phenylalanine ammonia-lyase, CH: Cinnamate 4-hydroxylase, CL: 4-Coumaroyl~CoA lyase, CS: Chalcone synthase, CI: Chalcone isomerase, F3H: Flavanone 3-hydroxylase, FS: Flavanol synthase, F3'H: Flavonoid 3'-hydroxylase.

radiation [15, 26] and fungal elicitors [5, 7] increase the PAL enzyme activity. UV light is a particularly effective abiotic stress for alkaloids [9], anthocyanins [11], and flavonoids production [8, 25, 26].

The following is a flavonoid pathway. The first product of the flavonoid pathway formed by chalcone synthase from 4-coumaloyl~CoA and malonyl~CoA is a chalcone [13]. The chalcone is further converted into flavanone (naringenin) by chalcone isomerase. This process has been reported in the literature in flavonoid-producing plants and plant tissues [14]. Forkmann *et al.* [4] hare shown that flowers of *Matthiola incana* contain a soluble flavanone 3-hydroxylase which catalyzes the 3-hydroxylation of flavanone to dihydroflavonols, and other soluble enzyme preparations (flavanone 3-hydroxylase and flavonol synthase activities) from cell suspension cultures of parsley catalyze not only the hydroxylation of flavanone in 3-position to dihydroflavonols but also the conversion of the dihydroflavonols to flavonols. All enzyme reactions require 2-oxoglutamate, ascorbate, and Fe^{2+} as cofactors [1]. Spribille and Forkman [24] have shown that a soluble enzyme preparation (flavonol synthase activity) from flower buds of *Matthiola incana* catalyzes the conversion of dihydrokaempferol to kaempferol and of dihydroquercetin to quercetin.

The major objective of this study is to establish a relation between the production of flavonol glycosides and the change of specific PAL activity by the unassisted and synergistic effects of environmental factors in suspension cultures of *Ginkgo biloba* cells.

MATERIALS AND METHODS

Chemicals

Quercetin, kaempferol, and isorhamnetin were supplied by Sigma Chemical Co. (St. Louis, U.S.A.). Methanol and water for HPLC were purchased from Fisher Scientific (Rochester, U.S.A.). All other chemicals used were reagent grades.

Cell Line and Cell Culture

Cell lines were originally developed from the leaves of *Ginkgo biloba* in Korea [16]. Murashige and Skoog (MS) medium [19] was modified by addition of 5 mg/l of NAA and 30 g/l of sucrose, and the modified MS medium was used in cell cultures. The pH was adjusted to 5.8 with 1 N KOH.

Callus subculture was carried out once every month by transferring a spoonful of healthy callus on to the solid medium. Suspension subculture was carried out every ten days by transferring one volume of culture broth to five volumes of fresh medium. Inoculum was prepared by growing cells in modified MS medium for 7 days and filtered with Whatman No. 1 filter paper. 3.5 g of fresh cell filtrate was inoculated in 40 ml culture medium. Suspension cultures were carried out in a 100 ml flask containing 40 ml modified MS medium at 25°C and at 45% humidity in a shaking incubator with 150 rpm agitation and illuminated under fluorescent light for 16 h per day (normal culture condition). All experiments were carried out with duplicate samples and the average values were used.

Determination of Cell Mass

Callus and suspension cells were filtered with Whatman No. 1 filter paper. The cells were washed with distilled water and the water was completely drained under vacuum until water drops no longer appeared. Fresh cell weight (FCW) was determined by quickly measuring the washed cells using a balance. After measuring the FCW, the cells on the pre-weighed aluminium tray were dried in an oven at 60°C to constant weight. Dry cell weight (DCW) is expressed as g/l.

PAL Enzyme Activity

Measurement of PAL activity was carried out according to a modified method described by Hwang *et al.* [10]. One gram of fresh cells were homogenized with 2 ml of cold acetone for 2 min at 0°C. The homogenized cells were filtered with Whatman filter paper No. 1 and acetone

was evaporated from the filtrate under vacuum conditions for 24 h. Two ml of 0.1 M sodium borate buffer (pH 8.8) was added to the residual solution and incubated at 4°C for 1 h, then centrifuged at 4,000 rpm for 20 min. The supernatant was used as an enzyme solution. The reaction mixture contained 1.5 ml of enzyme solution and 1 ml of 0.05 M L-phenylalanine in sodium borate buffer (pH 8.8), and was incubated for 1 h at 30°C. Reaction was stopped by adding 0.1 ml of 5 N NaOH. The *trans*-cinnamate produced was determined by measuring absorbance at 269 nm. The specific activity of PAL was expressed in unit/g-protein. One unit of enzyme activity was defined as the amount of enzyme required for the formation of one μmole of *trans*-cinnamate during one minute under the assay condition. Protein was determined by the method of Lowry *et al.* [17] using bovine serum albumin as a standard.

Flavonol Aglycone and Sugar Analysis

Cells were harvested by vacuum filtration. For the measurement of intracellular flavonoid concentration, 2.0 g of cells (FCW) were extracted with 4 ml of distilled water by sonication at 70°C for 90 min and with 4 ml of methanol by homogenization at room temperature for 1 min. The extractant obtained was hydrolyzed with 2 ml of methanol and 0.5 ml of 35% HCl for 1.5 h. All extracts were filtered through 0.2 μm membrane filters and 20 μl of the solution was injected for HPLC analysis. The HPLC system was equipped with a Rainine C-18 (25 cm) column and a UV detector at 365 nm. A mixture of methanol and water which contains 1% phosphoric acid was used as a mobile phase. The content of methanol in the mixture was gradually changed from 35% to 70% in the time period of 20 min.

RESULTS AND DISCUSSION

Time Course of the Change in Cell Growth and Quercetin Production

Time course experiments were carried out in shake flasks and samples were taken every two days. Shown in Fig. 2 is the time course of the change in cell growth and quercetin production. At the end of the exponential growth phase, the dry cell weight (DCW) reached a maximum, typically at 8 days after inoculation to fresh medium. After the maximum DCW, 22.4 g/l, the DCW decreased slowly by cell lysis. The quercetin concentration was increased by raising the culture time up to a stationary growth phase, 10 days culture, and then decreased rapidly. Therefore, quercetin production showed a mixed-growth-associated pattern and it suggested that quercetin is an intermediate of secondary metabolism and not an end product of secondary metabolism in the *Ginkgo biloba* cell.

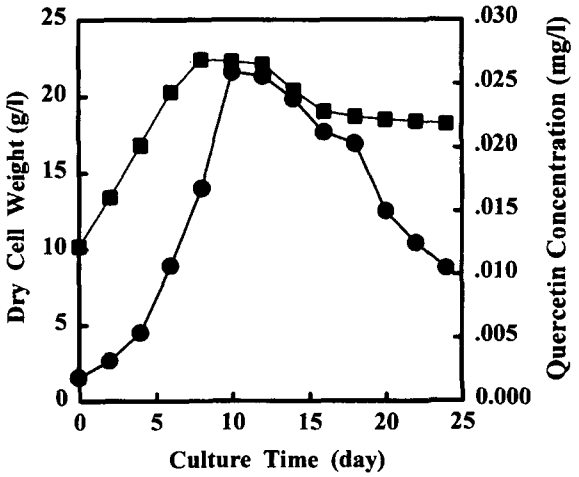

Fig. 2. Time course change of cell growth (■) and quercetin production (●).

Effect of Fluorescent Light Illumination

As light is a very important factor in the regulation of metabolism and during the differentiation processes of plants [8], studies on the effect of fluorescent light on the production of flavonol glycosides in cell suspension cultures of *Ginkgo biloba* were conducted. Suspension cultures were carried out in a 100 ml flask containing 40 ml modified MS medium for 10 days under continuous illumination; under 16-h illumination per day (control) and under dark conditions. The results are shown in Fig. 3. The best cell growth and quercetin production in the experimental conditions were obtained by 16-h illumination per day. These results confirm that fluorescent light is also involved in the induction of enzymes related to flavonoid biosynthesis in cell suspension culture of *Ginkgo biloba* as reported in cell suspension culture of

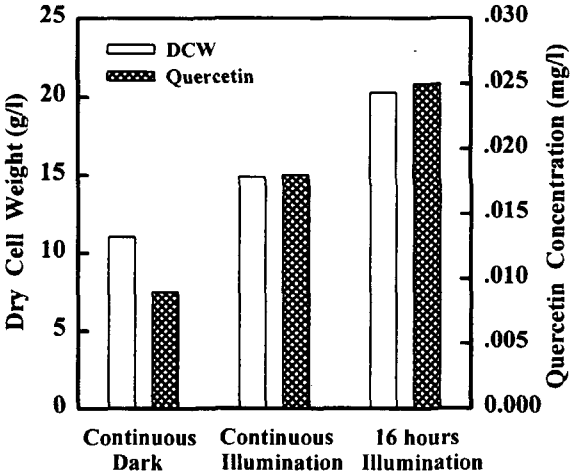

Fig. 3. Effect of fluorescent light on cell growth and quercetin production.

parsley by Heller *et al.* [8]. We found that fluorescent light illumination for 16 h per day is necessary for effective quercetin production in cell suspension cultures of *Ginkgo biloba*.

Effect of UV Radiation

A study was carried out to find the optimal conditions of UV radiation for the production of flavonol glycosides in cell suspension cultures of *Ginkgo biloba*. Cells were grown for 7 days in normal culture conditions and irradiated with 365 nm and 254 nm UV wavelength for 6 h, then cultured for 49 h under fluorescent light illumination for 16 h per day, as well as under dark conditions. As shown in Fig. 4, the results showed that a shorter UV wavelength was more effective for quercetin production as also reported in anthocyanin production from *Cyanus centurea* by Kaneakwa *et al.* [11], although no considerable effect was observed on the change in cell concentration by UV radiation and wavelength. When irradiated with UV alone, little effect was observed. However, when white fluorescent light was used for 16 h per day after UV radiation, quercetin gradually accumulated, with a maximum level at 49-h culture. The content of quercetin was about 10-fold higher as compared to that obtained in the normal culture condition. Quercetin also accumulated under dark condition by UV radiation, though the content of quercetin was very low in the dark condition without UV radiation. Therefore, UV light is an effective stimulator for flavonoid production [2, 26], which in turn serve as important barriers to UV damage to the cell by strongly absorbing the UV radiation [6].

Suspension cultures were carried out under normal conditions for 7 days and irradiated with 254 nm UV for each radiation period, and then cultured under normal conditions. Samples were taken at 50 h of culture time including radiation periods. Results are shown in Fig. 5. Cell concentration was slightly decreased by increasing the period of UV radiation, but the quercetin production was first increased by increasing the UV radiation for the period up to 6 h, and was decreased over the 6-h radiation period. Therefore, the maximum concentration of quercetin can be obtained at 6 h of UV radiation.

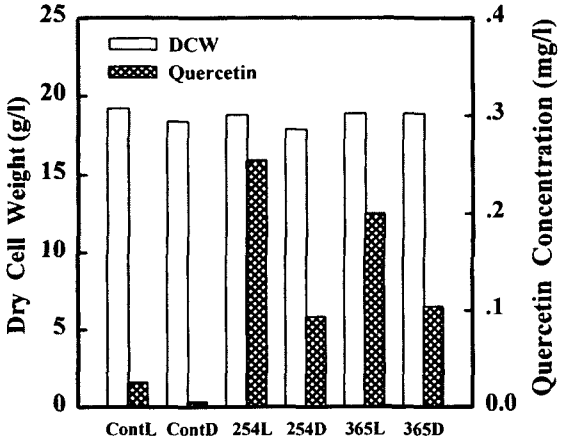

Fig. 4. Effect of UV radiation and fluorescent illumination on cell growth and quercetin production.
Cont: without UV radiation, 254 and 365: UV radiation of each wavelength (nm), L: fluorescent light illumination for 16 h per day, D: Continuous dark condition.

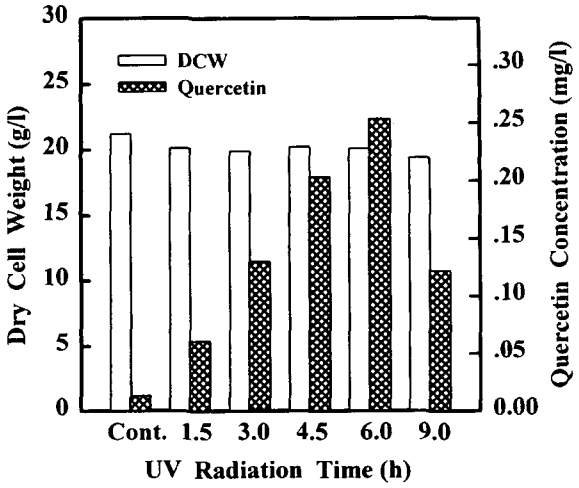

Fig. 5. Effect of UV radiation time on cell growth and quercetin production.

After cells grown for 7 days under normal conditions were irradiated with 254 nm UV for 6 h, we investigated the time course changes of cell and quercetin concentration during cell suspension cultures in normal conditions. As shown in Fig. 6, no considerable change was observed in cell concentration up to 55 h culture, whereas quercetin concentration was increased by increasing the culture time up to 50 h and then it decreased by converting a

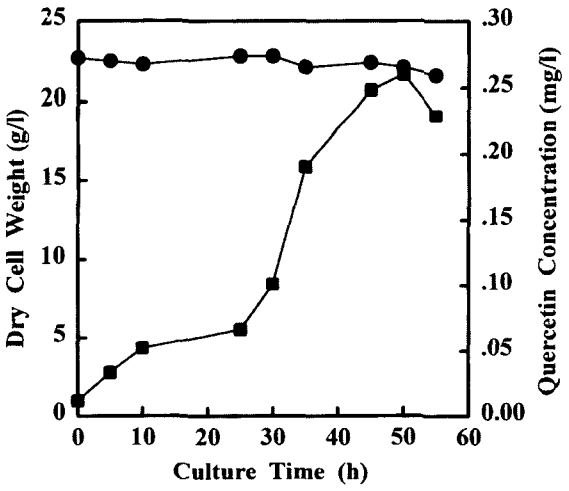

Fig. 6. Time course changes of cell growth (●) and quercetin production (■) after UV radiation for 6 h.

Table 1. The effect of UV radiation on cell growth, flavonol glycoside production, and specific PAL activity.

	Control	UV Radiation
Dry cell weight (g/l)	21.48	20.95
Quercetin (mg/l)	0.025	0.258
Kaempferol (mg/l)	0.015	0.056
Specific PAL activity (unit/g-protein)	1.105	2.531

quercetin to other compounds. In particular, after 25-h culture, the quercetin production was rapidly increased up to 50-h culture. It is believed that fluorescent and UV light induced the enzymes of flavonoid biosynthesis in cell suspension cultures of *Ginkgo biloba* as was reported for parsley [8, 26]. The quercetin reached a maximum concentration of 0.26 mg/l, at 50-h culture as reported by Chappell and Hahlbrock [2].

Ginkgo biloba cells were grown for 8 days in normal culture conditions and then irradiated with 254 nm UV for 6 h, then cultured for 2 days. As shown in Table 1, quercetin production increased approximately 10 fold, kaempferol by 3.8 and specific PAL activity by 2.3 as compared to normal culture condition. Hence, it was confirmed that PAL enzyme is induced by UV radiation in cell suspension cultures of *Ginkgo biloba*, and resulted in the increase of flavonol glycosides production as reported in parsley for Wellmann [26].

Effect of Culture Temperature

There has been little attention paid to the affect of culture temperature on cell growth and secondary metabolites production because the culture temperature was usually maintained at 25°C~30°C. It has been reported that the optimum temperature of cell growth does not coincide with secondary metabolite formation. Also, it is known that the secondary metabolic pathway comparably varies with culture temperature [3, 18]. To study the effect of culture temperature, suspension cultures of *Ginkgo biloba* cells were carried out at various culture temperatures in normal culture conditions. As shown in Fig. 7, the cell growth of *Ginkgo biloba* is favored at high temperatures but the quercetin exhibited its maximum content at 10°C as reported for *Catharanthus roseus* by Courtois and Guern [3], due to the decrease of conversion activity of quercetin to other compounds. And the kaempferol was only accumulated at low temperatures and could not be detected up to 20°C. In order to study the effect of abiotic stress of temperature change, after cells were grown for 7 days at 10°C, the culture temperatures were elevated to 20°C, 25°C, and 30°C, and cell cultures were continued for 5 days more in normal conditions. The control was maintained at 10°C for 12 days. As shown in Fig. 8, the quercetin production was increased by temperature change during

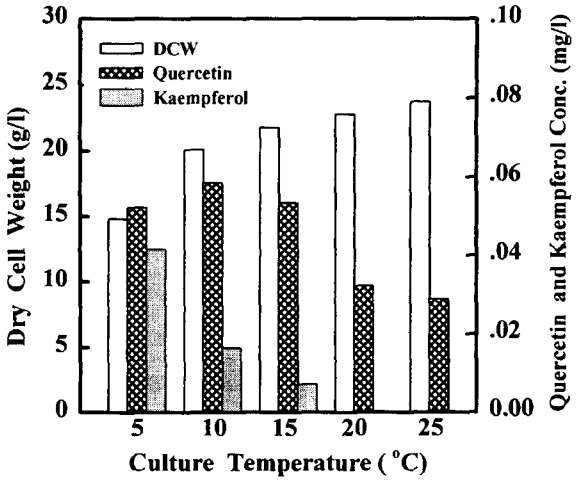

Fig. 7. Effect of culture temperature on cell growth and flavonol glycoside production.

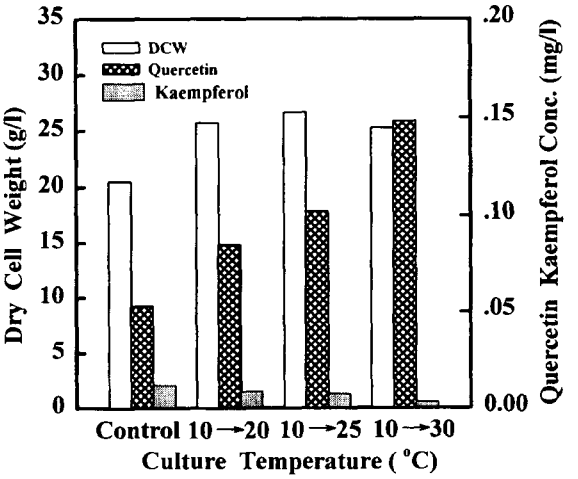

Fig. 8. Effect of temperature change on cell growth and flavonol glycoside production.
Control was maintained at the culture temperature of 10°C for 12 days.

the culture period. In particular, the quercetin production reached its highest level when the culture temperature was elevated from 10°C to 30°C. However, only kaempferol was produced at the low temperature. It seems that flavonoid 3'-hydroxylase which participates in the conversion of dihydrokaempferol to dihydroquercetin (Fig. 1) was activated not only at high temperatures, but also by the temperature change.

Effect of Precursor and Enzyme Cofactor Feeding

The addition of precursors overproduced the specific products by stimulating the specific biosynthetic pathway [23]. Phenylalanine is one of the most basic metabolites in the general phenylpropanoid pathway and naringenin also serves as a basic metabolite in flavonoid pathways in flavonol glycoside biosynthesis [6]. An

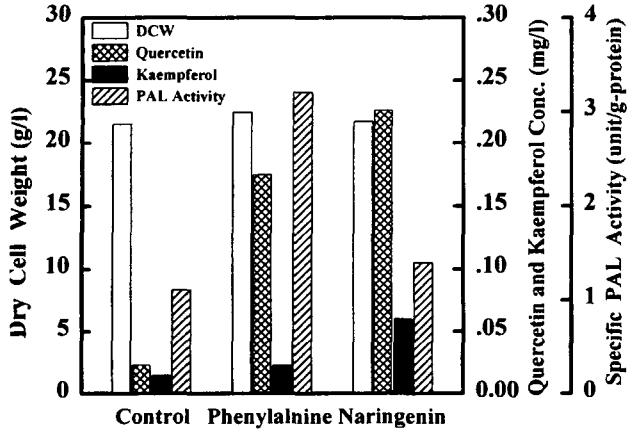

Fig. 9. The effect of phenylalnine and naringenin feeding on cell growth, flavonol glycoside production and specific PAL activity.

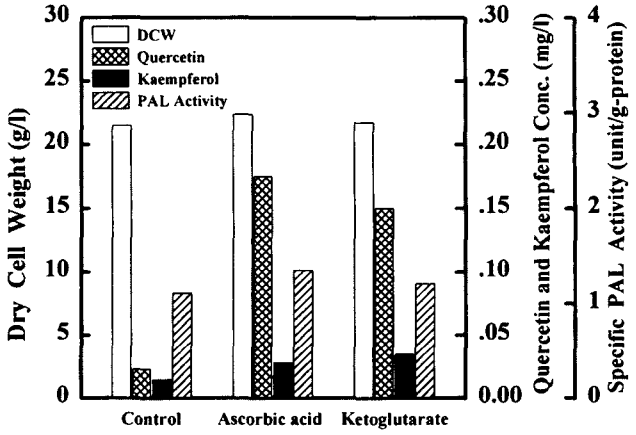

Fig. 10. The effect of each enzyme cofactor feeding on cell growth, flavonol glycoside production and specific PAL activity.

investigation was made on the effect of phenylalanine or naringenin on flavonol glycoside production related to the induction of PAL enzyme in cell suspension cultures of *Ginkgo biloba*. As shown in Fig. 9, no considerable effect was observed on the cell growth, while the production of flavonol glycosides and specific PAL activity showed conspicuous differences. The specific PAL activity increased about 2.9-fold when 200 mg/l phenylalanine was added. As a result, the production of quercetin and kaempferol increased by about 7.6- and 1.5-folds, respectively. The patterns of cell growth and flavonol glycoside production with naringenin feeding as a precursor were similar to those of phenylalanine feeding. In the feeding of 200 mg/l naringenin, the production of quercetin and kaempferol increased about 9.8- and 4.0-folds, while no considerable effect was observed in the specific PAL activity.

The key enzymes such as flavanone 3-hydroxylase and flavonol synthase in flavonoid pathway required 2-oxoglutamate, ascorbic acid, and Fe^{2+} as cofactors [1, 4, 24]. An investigation was made on the effect of enzyme cofactors feeding on flavonol glycoside production related to specific PAL activity in cell suspension cultures of *Ginkgo biloba*. Cells were grown for 8 days under normal culture conditions. 0.01 mM of 2-ketoglutarate or L-ascorbic acid were added as enzyme cofactors, and then cultured for 2 days in normal culture conditions. As shown in Fig. 10, quercetin and kaempferol production increased about 7.9- and 1.9-folds, respectively, when L-ascorbic acid was added, and 6.5- and 2.3-folds when 2-ketoglutarate was added. However, there was no noticeable effect of specific PAL activity by the addition of enzyme cofactors. Hence, an increase in the production of flavonol glycosides is possible without increasing the PAL enzyme activity, by feeding of enzyme cofactor and precursor related to flavonoid pathway.

Synergistic Effect of Various Environmental Factors

The synergistic effect of a combination of UV radiation and precursors feeding on flavonol glycoside production and its relationship to the induction of PAL enzyme in cell suspension cultures of *Ginkgo biloba* were investigated (Table 2). Cells were grown for 8 days in normal culture conditions and irradiated with 254 nm UV for 6 h, then cultured for 2 days in the medium containing 200 mg/l of phenylalanine or naringenin. In the synergistic effect of UV radiation and phenylalanine feeding, quercetin and kaempferol production were very similar as compared to UV radiation only, but specific PAL activity showed a 1.6-fold higher value. It was suspected that PAL enzyme would be induced by the

Table 2. The synergistic effect of UV radiation (524 nm) and precursors on cell growth, flavonol glycoside production, and specific PAL activity.

	Control	UV	UV	
			Phenylalanine	Naringenin
Dry cell weight (g/l)	21.48	20.95	21.63	20.75
Quercetin (mg/l)	0.023	0.241	0.255	0.316
Kaempferol (mg/l)	0.015	0.056	0.042	0.078
Specific PAL activity (unit/g-protein)	1.105	2.531	3.943	2.423

Table 3. The synergistic effect of precusor and enzyme cofactor on cell growth, flavonol glycoside production, and specific PAL activity.

	Control	PHE	NAR	PHE		NAR	
				AA	KTG	AA	KTG
Dry cell weight (g/l)	21.48	22.45	21.66	21.14	22.48	22.17	22.78
Quercetin (mg/l)	0.023	0.175	0.226	0.215	0.238	0.269	0.264
Kaempferol (mg/l)	0.0146	0.0225	0.0595	0.035	0.0512	0.0435	0.0694
Specific PAL acivity (unit/g-protein)	1.105	3.203	1.403	3.405	3.127	1.228	1.120

PHE: phenylalanine (200 mg/l); NAR: naringenin (200 mg/l); AA: L-ascorbic acid (0.01 mM); KTG: 2-ketoglutarate (0.01 mM).

synergistic effect of UV radiation and phenylalanine precursor. However, in the synergistic effect of UV radiation and naringenin feeding, the production of quercetin and kaempferol were increased as compared to UV radiation only. In contrast, specific PAL activity was slightly decreased.

The synergistic effect of enzyme cofactors and precursor feeding were also investigated (Table 3). When 200 mg/l phenylalanine and either 0.01 mM L-ascorbic acid or 2-ketoglutarate were fed simultaneously in 8-day cultured cells, quercetin production increased slightly in comparison with the case of unassisted feeding of each factor. Specific PAL activity did not increase as compared to the phenylalanine-only feeding. In case of the simultaneous feeding of 200 mg/l naringenin and 0.01 mM L-ascorbic acid or 2-ketoglutarate, quercetin production increased more than in the instance using the naringenin as a precursor, but specific PAL activity remained unchanged. Therefore, the synergistic effect of two factors are effective in increasing flavonol glycoside by simultaneously increasing the enzymes concerned with phenylpropanoid and flavonoid pathways.

Acknowledgments

This work was supported by the Korea Science and Engineering Foundation (KOSEF) through the Research Center for New Bio-Materials in Agriculture at Seoul National University.

REFERENCES

1. Britscht, L., W. Heller, and H. Grisebach. 1981. Conversion of flavanone to flavone, dihydroflavonol and flavonol with an enzyme system from cell cultures of parsley. *Z. Naturforsch.* **36C:** 742–750.
2. Chappell, J. and K. Hahlbrock. 1984. Transcription of plant defense genes in response to UV light or fungal elicitor. *Nature* **311:** 76–78.
3. Courtois, D. and J. Guern. 1980. Temperature response of *Catharanthus roseus* cells cultivated in liquid medium. *Plant Sci. Lett.* **17:** 473–482.
4. Forkmann, G., W. Heller, and H. Grisebach. 1980. Anthocyanin biosynthesis in flowers of *Matthiola incana* flavanone 3- and flavonoid 3'-hydroxylases. *Z. Naturforsch.* **35C:** 691–695.
5. Hahlbrock, K., C. J. Lamb, C. Purwin, J. Ebel, E. Fautz, and E. Schäfer. 1981. Rapid response of suspension-cultured parsley cells to the elicitor from *Phytophthora megasperma* var. *sojae*: Induction of the enzymes of general phenylpropanoid metabolism. *Plant Physiol.* **67:** 768–773.
6. Harborne, J. B. 1988. The *Flavonoides - Advances in Research Since 1980.* pp. 399–425. Chapman and Hall, London and N.Y.
7. Hattori, T. and Y. Ohta. 1985. Induction of phenylalanine ammonia lyase activation and isoflavon glycosides accumulation in suspension cultured cells of red beans, *Vigna angularis*, by phytoalexin elicitors, vanadate, and elevation of medium pH. *Plant Cell Physiol.* **26:** 1101–1110.
8. Heller, W., B. Egin-Bühler, S. E. Gardiner, K. H. Knobloch, U. Matern, J. Ebel, and K. Hahlbrock. 1979. Enzymes of general phenylpropanoid metabolism and of flavonoid glycoside biosynthesis in parsley: Differential inducibility by light during the growth of cell suspension cultures. *Plant Physiol.* **64:** 371–373.
9. Hirata, K., M. Asada, E. Yatani, K. Miyamoto, and Y. Miura. 1993. Effect of near-ultra violet light on alkaloid production in *Catharanthus roseus* plants. *Planta Med.* **59:** 46–50.
10. Hwang, J. H., M. W. Kim, and Y. H. Kang 1994. Effects of ethylene and Ca^{2+} on activity of phenylalanine ammonia-lyase in glucan-treated *Daucus carata. J. Plant. Biol.* **37:** 263–269.
11. Kaneakwa, K., Y. Kaneco, K. Hattori, K. Koike, and K. Takeda. 1987. Cell cultures of *Centurea cyanus* produce malonated anthocyanin in UV light. *Phytochemistry* **26:** 2261–2263.
12. Koukol, J. 1961 The metabolism of aromatic compounds in higher plants: IV. Purification and properties of the phenylalanine deaminase in *Hordeum vulgare. J. Biol. Chem.* **236:** 2692–2698.
13. Kreuzaler, F. and K. Hahlbrock. 1975. Enzymatic synthesis of an aromatic ring from acetate units. Partial purification and some properties of flavanone synthesis from cell suspension cultures of *Petroselinum hortense. Eur. J. Biochem.* **56:** 205–213.

14. Kühn, B., G. Forkmann, and W. Seyffert. 1978. Genetic control of chalcone-flavanone isomerase activity in *Callistephus chinesis. Planta* **138:** 199–203.
15. Lee, A. H. and M. E. Schwochau. 1971. Ultraviolet light-induced formation of pisatin and phenylalanine ammonia-lyase. *Plant Physiol.* **47:** 588–590.
16. Lee, W. K., Y. W. Ryu, S. Y. Byun, and H. G. Chung. 1993. Effects of nutrients and culture conditions on the cell growth and flavonol glycosides production in cell culture of *Ginkgo biloba. Kor. J. Biotechnol. Bioeng.* **8:** 55–61.
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
18. Morris, P. 1986. Regulation of product synthesis in cell cultures of *Catharanthus roseus*. Effect of culture temperature. *Plant Cell Rep.* **5:** 427–429.
19. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant* **15:** 473–497.
20. Peter, K. and J. Hermann. 1972. Vasoactive drugs from *Ginkgo biloba* leaves. *Ger. Offen.* 2, 117, 429.
21. Peter, V. R., J. Fisel, and W. Weisser. 1966. Pharmacological study of the active substance of *Ginkgo biloba. Arzenimittel-Forsch.* **16:** 719–725.
22. Shirley, B. W. 1996. Flavonoid biosynthesis: 'new' functions for an 'old' pathway. *Trends Plant Sci.* **1:** 377–382.
23. Simola, L. K., A. Martisen, A. Huhtikangas, R. Jokela, and M. Lounasma. 1989. Feeding experiments with precursor of tropan alkaloid using suspension culture of *Atropa belladonna. Acta Chemica Scandinavica* **43:** 702–705.
24. Spribille, R. and G. Forkmann. 1984. Conversion of dihydroflavonols to flavonols with enzyme extracts from flower buds of *Matthiola incana* R. Br. *Z. Naturforsch.* **9C:** 714–719.
25. Vogt, T., P. G. Gulz, and H. Reznik. 1991. UV irradiation dependent flavonoid accumulation of *Citrus laurifolius* L. *Z. Naturforsch.* **46C:** 37–42.
26. Wellmann, E. 1975. UV dose-dependent induction of enzymes related to flavonoid biosynthesis in cell suspension cultures of parsley. *FEBS Lett.* **51:** 105–107.