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Effect of Fungal Elicitor and Heavy Metals on the Production of Flavonol Glycosides in Cell Cultures of *Ginkgo biloba*

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Abstract The effect of fungal elicitor and heavy metal salts on the production of flavonol glycosides in cell cultures of *Ginkgo biloba* was investigated. Among the fungi tested, *Trichoderma longibrachiatum* ATCC 52326 was found to be the most efficient in the production of flavonol glycosides. Kaempferol production from the elicited callus increased ten-fold as compared to the unelicited callus, while quercetin concentration of elicited cells was nine-fold higher than that of unelicited cells in suspension cultures. The maximum quercetin concentration of 0.362 mg/l was obtained in 1.25 mg/l of the homogenate elicitor. Among the heavy metal salts tested, $CuSO_4$ showed a significant effect on quercetin accumulation, reaching to the concentration of 0.526 mg/l. Quercetin concentration increased to a maximum of 12-fold in response to $CuSO_4$ treatment as compared to that of untreated cells. The phenylalanine ammonia-lyase (PAL) activity and flavonol glycosides production simultaneously increased for 5 days of culture after fungal elicitor feeding, and their contents showed the same proportional patterns during the culture period. In contrast, PAL activity of cell cultures treated with $CuSO_4$ was almost constant during the culture period, although quercetin production increased remarkably.

Key words: Flavonol glycoside, *Ginkgo biloba*, fungal elicitor, heavy metals, PAL activity

Industrial utilization of plant cell cultures for the production of biochemicals has been hampered by low yields in the target substances. Cell cultures of *Ginkgo biloba* accumulate flavonol glycosides such as quercetin, kaempferol, and isorhamnetin. The specific yields of the flavonol glycosides in cell cultures, however, was much lower than those in leaves [12, 15]. Since many pharmaceuticals and other industrial products are based on plant raw materials, much effort has been made in the selection of plant cell cultures with high productivity and alteration of cell metabolism by environmental factors. In order to improve the yield of product, various techniques have been developed for the selection of high-producing plant cell cultures. However, such high-producing cultures often show a decline in productivity upon serial propagation. Another approach to improve the yield of product in plant cell cultures is alteration of cell metabolism by external factors. Cultured plant cells are, in principle, totipotent. Therefore, any product present in the parent plant should also be synthesized in cell culture under appropriate culture conditions. General methods for the induction of enzymes for secondary metabolism would be extremely valuable. It is well known that certain enzymes for secondary metabolism are induced in higher plants after treatment of elicitors [5, 9, 13, 14]. Hence, elicitors have been used as important tools for increasing the production of secondary metabolites [4, 7, 18, 22].

Biotic elicitors are the metabolites produced by plant pathogenic microorganisms, which stimulate the accumulation of phytoalexin in higher plants. Phytoalexins are low molecular weight compounds produced by higher plants in response to microbial infection [22]. During biotic elicitation, the phytoalexin accumulation may be due to increased rates of phytoalexin biosynthesis resulting from *de novo* transcription of DNA [5, 14, 22]. These phytoalexins accumulated at the site of infection inhibit the growth of invading microbes, and are thus believed to play a role in the plant defense mechanisms [1]. Rivera-Vargas *et al.* [19] reported that certain soybean flavonoids inhibited fungal growth. The treatment of plant cell cultures with biotic elicitors could increase the production of secondary metabolites resulting from the enzyme induced in secondary metabolism [3]. The plant-pathogen interaction, however, is most often species-specific. Elicitors are believed to play important roles as signal molecules in plant-pathogen

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interactions. It has been proposed that one of the first processes in the elicitation is the interaction between the elicitor molecules and the plasma membrane of plant cells [23]. However, little is known about the processes leading to gene activation after recognition of elicitor molecules by receptors.

Most of the fungal elicitors used for increasing the secondary metabolites are cell wall constituents of fungi such as polysaccharides, glycoproteins, and peptides [1, 2, 20]. Numerous studies associated with production of secondary metabolites using phytopathogenic fungi have recently been reported. Hahlbrock *et al.* [9] reported that the increase of flavonoid compounds occurred in suspension-cultured parsley cells (*Petroselinum hortens*) treated with an elicitor preparation from *Phytophthora megasperma* var. *sojae*. They also found that the addition of heat-soluble cell wall fragments from several phytopathogenic fungi in the suspension culture of *Petroselinum crispum* induced the production of coumarin derivatives. The increases in coumarin derivatives and flavonol glycosides were due to increase in activities of PAL and 4-coumarate: CoA ligase, two key enzymes of general phenylpropanoid metabolism [9, 12, 13]. The changes of PAL activity in parsley cells were caused by the corresponding changes in mRNA activity for this enzyme [14]. Campbell and Ellis [5] showed that the activity of PAL in elicited cells was ten-fold higher than that in the corresponding control, and also showed an increase in other enzyme activities associated with lignin synthesis.

Secondary metabolites in plant cell cultures also accumulate in response to various agents termed abiotic elicitors such as heavy metal salts [18, 21, 22], UV light [10, 12, 14], detergents [22], organic solvents [8], metabolic inhibitors [2], and plant hormones [8]. Heavy metal salts are readily available, cheap, and easy to use, and are also chemically defined. It has been proposed that the treatment of plant cell cultures with heavy metals may be an ideal method of elicitation for commercial production of phytoalexins [21]. However, the effect of heavy metals on plant metabolism is complex and involves inhibition of many enzymes, disruption of membranes, and induction of certain enzymes. Parry *et al.* [18] reported that the accumulation of medicarpin in leaves of alfalfa (*Medicago sativa* L) increased six-fold in response to $CuCl_2$ treatment, resulting from modification of isoflavonoid metabolism. Yoshikawa [22] reported that an effective abiotic elicitor such as $CuSO_4$ for accumulating glyceollin strongly inhibited the constitutive glyceollin degrading enzymes.

The major objective of this study was to investigate the effect of fungal elicitors and heavy metal salts on the production of flavonol glycosides and change of PAL activity in cell cultures of *G. biloba*.

MATERIALS AND METHODS

Chemicals

Quercetin and kaempferol 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 of reagent grade.

Cell Line and Cell Culture

Cell lines were originally developed from leaves of *G. biloba* in Korea [15]. Modified Murashige and Skoog (MS) medium [17] containing 5 mg/l of NAA and 30 g/l of sucrose was used in cell cultures. The pH was adjusted to 5.8 with 1 N KOH.

Callus subculture was carried out every month by transferring a spoonful of healthy callus onto 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, filtered with Whatman No. 1 filter paper, and 3.5 g of fresh cell filtrate was inoculated in 40 ml culture medium. Suspension cultures were carried out in 100-ml flasks containing 40 ml modified MS medium at 25°C and at 45% humidity in a shaking incubator with 150 rpm agitation under fluorescent light for 16 h per day (normal culture condition). All experiments were carried out in duplicate and average values were used.

Selection of Fungal Strain and Elicitor Preparation

The fungal strain used as a biotic elicitor in this study was selected as follows: Fungi grown for 5 days in 500-ml flasks containing 200 ml malt extract (ME) medium were inoculated on modified MS solid medium and then incubated at 30°C for 5 days. *G. biloba* callus was inoculated on the plates with fungi grown and incubated at 25°C for 5 days. Calli were carefully collected without fungi, and quantitatively analyzed for flavonol glycosides by HPLC.

Biotic elicitor was prepared as described by Campbell and Ellis [5]. *T. longibrachiatum* ATCC 52326 was grown in ME medium for 7 days at 30°C and 200 rpm in a shaking incubator. Elicitor was prepared by one of four ways. Whole culture broth elicitor was prepared by autoclaving at 121°C for 15 min. Homogenate elicitor was prepared by collecting mycelia on Whatman No. 1 filter paper by suction. Mycelia were washed with 100 ml sterile distilled water and dried in a dry oven at 60°C for 48 h. Dried mycelia were homogenized with a pestle and mortar, and suspended in sterile distilled water, depending on experimental conditions. Filtrate elicitor was prepared by filtering the spent medium of the cell culture broth through a Whatman No. 1 filter paper and sterilized by 0.2 μm millipore filtration or in an autoclave. Filtrate elicitor was used only in the experiments specifically designed to

determine elicitor effectiveness. All elicitors were stored at 4°C in a refrigerator until use.

Treatment of Heavy Metal Salts

Heavy metal salts reported as abiotic elicitors were selected from the literature [18, 21, 22]. The stock solution was autoclaved before feeding into the culture flask, or heat-unstable salts of the heavy metals were filtered with 0.2 μm membrane filter. The cells were grown for 8 days in normal conditions and then were incubated for 2 days after treatment with heavy metal salts.

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. Fresh cell weight (FCW) was determined by weighing the washed cells immediately after washing and water removal. After measuring the FCW, the cells were dried to constant weight on a pre-weighed aluminium tray in an oven at 60°C. Dry cell weight (DCW) is expressed as g/l.

PAL Enzyme Activity

PAL activity was measured by following the modified method described by Hwang *et al.* [11]. One gram of fresh cells was homogenized with 2 ml of cold acetone for 2 min on ice. The homogenized cells were filtered with Whatman filter paper No. 1 and acetone was evaporated from the filtrate *in vacuo* 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, followed by centrifugation at 4,000 rpm for 20 min. The supernatant was used as an enzyme source. The reaction mixture containing 1.5 ml of the enzyme solution and 1 ml of 0.05 M L-phenylalanine in sodium borate buffer (pH 8.8) was incubated for 1 h at 30°C. The reaction was stopped by adding 0.1 ml of 5 N NaOH, and *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 μmol of *trans*-cinnamate per min under the assay condition. Protein concentration was determined by the method of Lowry *et al.* [16] using bovine serum albumin as a standard.

Analysis of Flavonol Aglycone

Cells were harvested by vacuum filtration. For the measurement of intracellular flavonoid concentration, 2.0 g of cells (FCW) were homogenized at room temperature for 1 min after addition of 4 ml methanol and centrifuged at 4,000 rpm. The supernatant obtained was hydrolyzed with 2 ml of methanol and 0.5 ml of 35% HCl for 1.5 h. All samples 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 1% phosphoric acid in water was used as a mobile phase. The concentration of methanol in the mixture was gradually changed from 35% to 70% in the period of 20 min.

RESULTS AND DISCUSSION

Selection of Fungal Elicitor

Eight fungal strains were tested to increase the production of flavonol glycosides in cell cultures of *G. biloba*. The results are shown in Table 1. Among the fungi tested *T. longibrachiatum* was the most effective for the increase in the production of flavonol glycosides in callus cultures. In particular, kaempferol production in elicited callus was ten-fold higher than that in the corresponding control. Other fungi had either no effect or decreased in the production of flavonol glycosides, possibly indicating the species-

Table 1. Effect of various fungal strains on the production of flavonol glycosides.

Fungus strains	Quercetin (mg/g-DCW)	Kaempferol (mg/g-DCW)
Control	0.0015	0.0045
Fusarium moniliforme	0.0015	0.0004
Rhizoctonia solani	0.0042	0.0003
Physalospora baccae	0.0018	0.0005
Altenaria mali	ND	ND
Penicillium citrinum ATCC 9849	0.0008	0.0007
T. longibrachiatum ATCC 52326	0.0038	0.0440
Aspergillus niger ATCC 6275	0.0007	0.0035
Pyricularia oryzae	ND	ND

ND: Not detected.

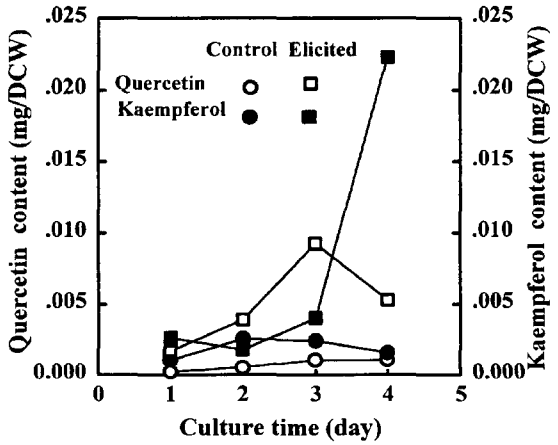

Fig. 1. Time course of changes in flavonol glycosides production in unelicited and elicited calli.

specificity of phytopathogen interactions. *T. longibrachiatum*, therefore, was selected as an effective elicitor and used in subsequent elicitation experiments.

After inoculating the callus on the plate of *T. longibrachiatum* grown for 5 days in modified MS medium, the time course of changes in flavonol glycosides production was investigated. As shown in Fig. 1, a significant change in flavonol glycosides content in unelicited callus (control) was not observed up to 4 days culture. Quercetin content in elicited callus was slightly increased in 3 days of the culture period and thereafter decreased. In particular, a remarkable increase of kaempferol in elicited calli was observed as compared with unelicited calli. The production of flavonol glycosides was observed almost in the same patterns in the simultaneous inoculation with *T. longibrachiatum* and *G. biloba* callus on the plate of modified MS medium (data not shown). From the above results, it was concluded that some constituents of *T. longibrachiatum* induced the enzyme expression in flavonoid metabolism.

Effect of Fungal Elicitor in Cell Suspension Cultures

In general, fungal elicitors include intact fungal mycelium [13], cell homogenate [5], cell wall [9, 13], enzyme [4] and cell wall carbohydrate, such as glucan [7], and glycoprotein [2, 20]. Hence, an investigation of the effect of fungal elicitator on cell growth and flavonol glycoside production in suspension cultures of *G. biloba* cells was carried out. *T. longibrachiatum* was grown in malt extract medium for 5 days, and elicitor solutions were prepared from whole culture broth and cell-free culture broth as described in Materials and Methods. Experiments were carried out on the suspension cultures of *G. biloba* cells in normal culture conditions for 8 days and were continued for 5 days after addition of 1 ml of each elicitor solution to 40 ml cell cultures.

As shown in Fig. 2, the production of quercetin was remarkably increased in suspension cultures by elicitation, while elicited calli showed a remarkable increase in kaempferol concentration (Table 1). The cell concentrations increased slightly. The quercetin concentration increased by nine-fold in cells treated with whole culture broth elicitor and by five-fold in cells treated with filtrate elicitors as compared to that in the control. Two kinds of filtrate elicitors showed almost the same effect on the production of quercetin and cell growth. Fresh culture media had no effect on cell growth and flavonol glycosides production. These results indicate that fungal elicitor may be considered as heat stable cell wall components of *T. longibrachiatum*.

Elicitor concentration in cell cultures strongly affects the intensity of flavonoid production because these compounds act as inhibitors or fungicides to fungal growth [19, 23]. To find an optimal concentration of elicitor for the production of flavonol glycosides, eight-days-old cell cultures were treated with a whole culture broth elicitor and incubated for 5 days. Cell and kaempferol showed almost constant concentrations at each elicitor volume (Fig. 3), while quercetin concentration increased by increasing elicitor volume up to 20 ml. This result indicates that the quercetin accumulated in the cells were strongly dependent on the amount of elicitor added, as reported by Kombrink and Hahlbrock [13].

An experiment was also carried out to determine an optimal concentration for the homogenate elicitor as described in Materials and Methods. *G. biloba* cells were strongly responsive to the broad range of elicitor concentrations as shown in Fig. 4. The optimal concentration of homogenate elicitor was 1.25 mg/l with a maximum quercetin concentration of 0.362 mg/l. This is 13.6 times higher than the unelicitated cells (control), although elicitor concentrations lower or

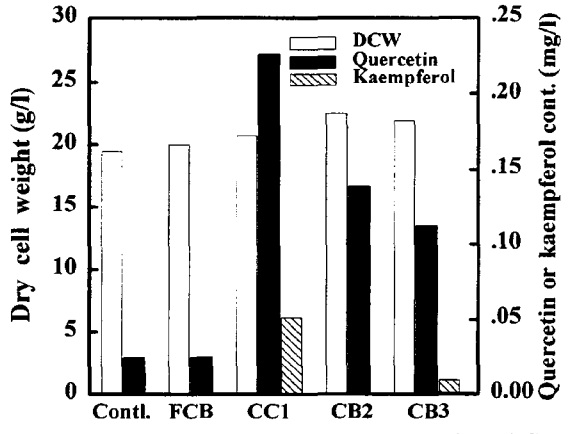

Fig. 2. Effect of fungal elicitors on cell growth and flavonol glycosides production.
Contl: without elicitor; FCB: fresh ME medium; CC1: whole culture broth elicitor; CB2: filtrate elicitor filtered; CB3: filtrate elicitor autoclaved.

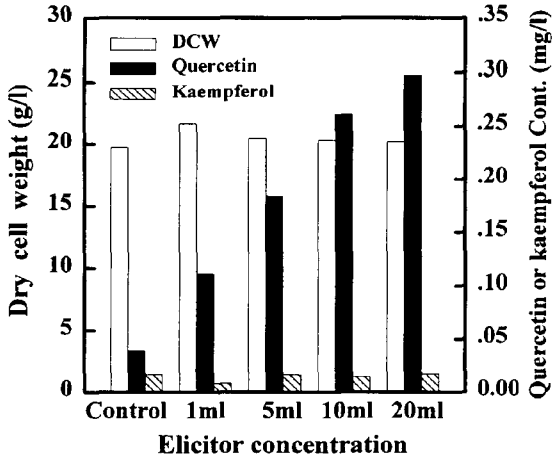

Fig. 3. Effect of the elicitor volume on cell growth and flavonol glycosides production.
Elicitor solution was prepared by an autoclave of the whole cell culture broth.

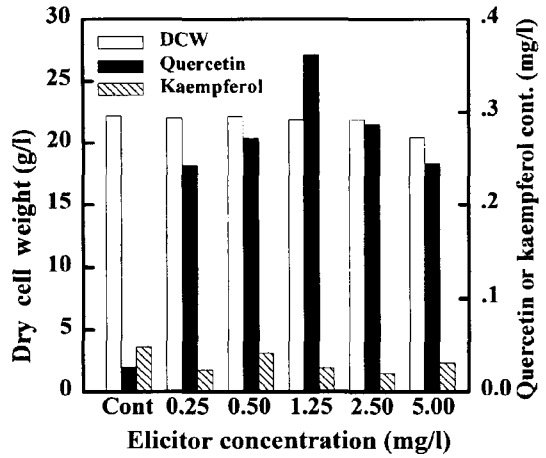

Fig. 4. Effect of the concentration of homogenate elicitor on cell growth and flavonol glycosides production.

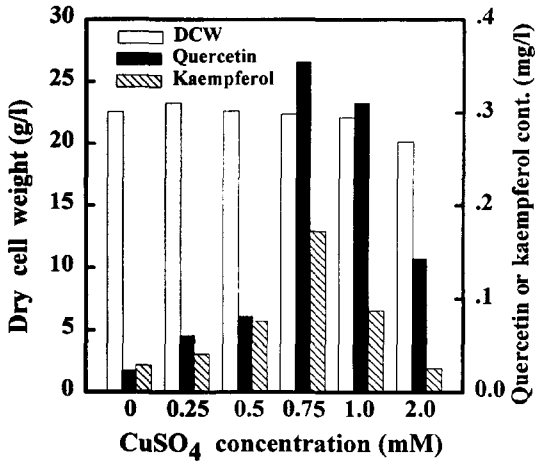

Fig. 5. Effect of $CuSO_4$ concentration on cell growth and flavonol glycosides production.

higher than 1.25 mg/l showed about the same effect, having an increase of 9.6-10.8 times that of the control. Kombrink and Hahlbrock [13] showed a similar response of coumarin production in parsley cells, depending on the elicitor concentration.

Effect of Heavy Metal Salts in Cell Suspension Cultures

Eight heavy metal salts selected from the literature were tested for possible increase in the production of flavonol glycosides in suspension culture of *G. biloba* cells. Cells were grown for 8 days under normal culture conditions. One mM of heavy metal salts was added and growth was allowed to continue for 2 days. The results are shown in Table 2. Among the heavy metal salts tested, four metal salts, namely, $PbCl_2$, $FeCl_3$, $CrCl_3$, and $CuSO_4$, were effective for increasing in the production of flavonol glycosides. $CuSO_4$, in particular, showed a significant effect on quercetin accumulation of up to 0.526 mg/l, which is 12-fold higher as compared to that obtained with untreated cells, while $PbCl_2$ and $CrCl_3$ were effective elicitors for the production of kaempferol and quercetin. Other heavy metal salts had no effect on flavonol glycoside production, and $HgCl_2$ rather inhibited the cell growth and flavonol glycoside production as reported by Yoshikawa [22]. These results indicated that the production of flavonol glycosides in cell culture of *G. biloba* was considerably dependent on heavy metals, as reported in the literatures [10, 22]. Hence, it could be presumed that each heavy metal acted on flavonoid metabolism of *G. biloba* cells in different modes.

Table 2. Effect of heavy metal salts on cell growth and flavonol glycoside production.

Heavy metal salts[a]	Dry cell weight (g/l)	Quercetin (mg/l)	Kaempferol (mg/l)
Control	22.0	0.044	0.022
$HgCl_2$	16.7	0.020	0.001
$PbCl_2$	20.1	0.220	0.189
$SnCl_2$	21.5	0.061	0.061
$FeCl_3$	20.9	0.197	0.027
$CrCl_3$	19.1	0.146	0.101
K_2CrO_7	18.1	0.049	0.024
$CuSO_4$	18.4	0.526	0.026
$CoCl_2$	18.0	0.056	0.063

[a]The concentration of heavy metal salts was 1 mM.

Based on the result shown in Table 2, $CuSO_4$ was selected as an abiotic elicitor and further investigation was made to determine the optimal concentration of $CuSO_4$ for maximizing flavonol glycosides production. As shown in Fig. 5, the production of flavonol glycosides increased with the increase of $CuSO_4$ concentration up to 0.75 mM and then decreased, while the cell concentration decreased gradually with the increase of $CuSO_4$ concentration. This indicated that a high concentration of $CuSO_4$ had a toxic influence on plant cells. The optimal concentration of $CuSO_4$ was found to be 0.75 mM with maximum quercetin and kaempferol productions of 0.354 mg/l and 0.172 mg/l, respectively. These results were 15- and 6-fold higher as compared to those obtained from untreated cells.

Elicitor Effectiveness and PAL Activity

A study on the relationship between flavonol glycosides production and PAL activity was made. Experiments were carried out on the suspension cells cultured under normal culture conditions for 8 days and were continued for 7 days after addition of 1.25 mg/l of homogenate fungal elicitor and 0.74 mM of $CuSO_4$ to 40-ml cell cultures.

The time course of cell and flavonol glycosides concentrations and PAL activity are shown in Fig. 6. Unelicited cells showed a slight decrease in flavonol glycosides content, and specific PAL activity remained almost constant throughout the culture period, while elicitor

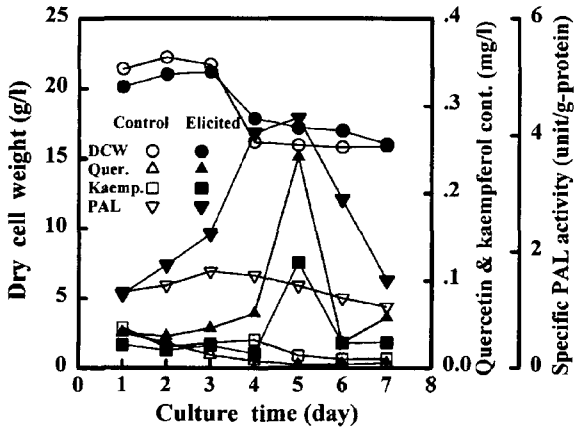

Fig. 6. Profiles of cell growth, flavonol glycoside production, and specific PAL activity after addition of fungal elicitor.

treated cells reached maximum quercetin and kaempferol concentrations of 0.242 mg/l and 0.121 mg/l, respectively, at 5 days culture and then decreased sharply to the initial level. Five days of culture was needed to obtain the maximum production of flavonol glycosides. The profile of specific PAL activity of elicitor-treated cells showed a similar pattern in the production of flavonol glycosides. Cells showed signs of necrosis (browning of cells), resulting in decrease of cell concentration after 3 days of culture. Therefore, it was confirmed that fungal elicitor stimulated the induction of PAL enzyme of phenylpropanoid metabolism and resulted in an increase of flavonol glycosides production. The above results show that plant cells have a narrow and specific range of fungal elicitors, whereas the fungi selected act as a biotic elicitor in a broad range of elicitor concentrations.

The time course of changes in concentrations of cell, flavonol glycosides, and specific PAL activity in cells treated with 0.75 mM $CuSO_4$ are shown in Fig. 7. In the cells treated with $CuSO_4$, the concentrations of quercetin

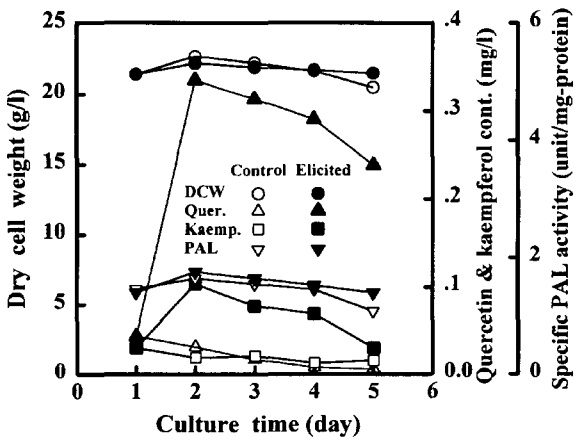

Fig. 7. Profiles of cell growth, flavonol glycosides production, and specific PAL activity after treatment with 0.75 mM $CuSO_4$.

and kaempferol reached the maximum values of 0.354 mg/l and 0.172 mg/l, respectively, at 2 days after addition of $CuSO_4$. Specific PAL activities of treated and untreated cell cultures were not significantly different during the culture period. It can be postulated from this experimental observation that $CuSO_4$ strongly inhibited flavonoid or flavanone degrading enzymes in flavonoid metabolism, resulting in the accumulation of flavonol glycosides. Hence, the use of heavy metals as one of the abiotic elicitors may be a preferable tool to increase secondary metabolites in plant cell cultures, although mechanisms of accumulation of secondary metabolites by heavy metals are not clear.

From a biotechnological point of view, an increase in productivity after the treatment with an elicitor is of great interest. In the present study, a possible increase in productivity of flavonol glycosides by more than ten-fold was achieved without extending the cultivation time. However, the final concentration of flavonol glycoside obtained in the present study was still too low as compared to leaves of *G. biloba*.

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