Variation of Ginkgolides and Bilobalide Contents in Leaves and Cell Cultures of *Ginkgo biloba* L.

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Abstract Ginkgolides (GK) and bilobalide are valuable compounds that belong to the lactone terpene. The contents of these metabolites were determined by HPLC from female and male tree of *Ginkgo biloba* L. The productivity of *G. biloba* cells was also compared with the corresponding individual trees. High variations in the ginkgolides and bilobalide were observed from different individuals, plant parts, and cultured cells. The ginkgolides and bilobalide contents were different depending on the plant parts. Callus was obtained from various plant tissues, and NAA was better at callogenesis than 2,4-D in both the female and male trees. The plants and their corresponding cells showed considerable variation in their ginkgolides and bilobalide concentrations. The ginkgolides and bilobalide contents were not correlated with the production between dominant trees and their corresponding cells. Light irradiation enhanced the production of GK-A and GK-B, however, the concentration of bilobalide decreased under dark conditions.

Keywords: bilobalide, *Ginkgo biloba*, ginkgolides, sexuality, variation

INTRODUCTION

The ginkgo tree has various flavonoids (including flavanols, flavone, biflavones, tannins, and pro-anthocyanidins), terpenoids (including monoterpenes, sesquiterpenes, and diterpenes), and other compounds (including steroids, polyphenols, anacardoc acids, aromatic compounds, lignoids, phenylpropanoids, sugar alcohols, and amino acids) [1,2, 3]. Of those ingredients, the ginkgolides (GK) and bilobalide are believed to be especially beneficial to the human body [4]. The GK found in the extract are classified as A, B, C, J and M [5]. Ginkgo extract improves the microcirculation in small capillaries, and protects against oxidative cell damage from free radicals (antioxidant). In particular, GK-B has strong antagonist effects on platelet activating factors (PAF, "blood clotting"), which have been related to the development of a number of cardiovascular, renal, respiratory and central nervous system disorders [6].

In recent years, ginkgo preparation has gained high popularity in both Asia and the EU [7]. However, there remain several difficulties in its supply. Namely, it takes a long period to cultivate the trees, which are unstable due to difference in sexuality, seasonal variation, labor intensity in the leaf collection and difficulty of chemical synthesis [8].

For a sufficient supply, suitable strategies such as selection of high-yield trees, breeding programs and the development of *in vitro* culture are needed. However, over the past years, economically important plants have been brought into cell cultures, but in most cases the productivity was too low to allow for an economically feasible process [9]. Selection of dominant tree accumulating high content and introduction of the selected trees into *in vitro* cultures are expected to maintain the capability of high production. However, the concentrations of the secondary metabolites from mother plants did not agree with the cultured cells. Numerous researches have reported that the concentrations of the secondary metabolites in the cultured cells were lower than those in the mother plants. However, a few studies on the contents of useful secondary metabolites, between intact plants and cells cultured from mother plants, have been reported.

Therefore, in this paper, the GK and bilobalide contents, between the mother tree and *in vitro* cultured cells, from both female and male trees, were compared and the relation of the cell cultures content having originated from individual trees elucidated. The effect of light irradiation on the metabolites production was also investigated, which is one of the important factors that influence the accumulation of secondary metabolites.

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MATERIALS AND METHODS

Plant Materials

The ginkgo trees, approximately thirty-year old, used in this study were collected during October from those located on the campus of Kyungpook National University, Korea. For analyses of the GK and bilobalide contents, cell cultures of leaves, stems and stem bark of the ginkgo trees were prepared from four individual trees, with female and male tree. All the tree parts were prepared in triplicate from each individual tree.

Chemicals

Standard GK-A, GK-B, and bilobalide were provided by SunKyong Pharm (Korea). The organic solvents used were of analytical-reagent grade (Merck). All solvents used for the HPLC were filtered (0.45 μm) and ultrasonically degassed before use.

Cell Cultures

The tree leaves were harvested from 4 different male and female trees (#1 to #4). The leaf petioles of the ginkgo trees were sterilized using 1% (v/v) sodium hypochlorite solution, with several drops of triton X-100, by vigorously shaking for 25 min, and then rinsed several times in sterile distilled water. For induction of callus, leaf petioles were excised, and transferred to MS [10] medium, supplemented with 5~40 μM α-naphthalene acetic acid (NAA) or 2,4-dichlorophenoxy acetic acid (2,4-D), 3% (w/v) sucrose, 100 mg/L myo-inositol and 0.2% (w/v) phytagel.

Until a better maintenance medium can be found, 30% sedimented cell volume (SCV) of primary callus cultures were transferred into 70 mL of liquid MS medium containing 20 μM NAA in a 250 mL Erlenmeyer flask at 25 ± 1°C with a 16 h photoperiod, or under completely dark conditions. Cell suspension cultures were grown on a gyratory shaker at 100 rpm for 4 weeks. To measure the dry weight (DW), sampled cells were filtered through a Whatman No.1 filter paper on a Büchner funnel, under slight vacuum, for 30 min, and then placed in a dry oven at 40°C for 48 h.

Extraction and Purification of GK and Bilobalide

The ginkgo plant parts and cell cultures, from individual tree (#1 to #4), were oven dried, at 50°C for 72 h, and ground through a 20 mesh sieve using a Wiley mill. GK and bilobalide are thermo-stable, and the ginkgo tree materials and cell cultures were extracted and purified, with minor changes by the method of Huh and Staba [11]. The ginkgo materials and cell cultures (approximately, 500 mg) were extracted with 50% aqueous acetone (10 mL, 4×), with occasional stirring. After filtration through a Whatman No. 1 paper, the acetone was removed *in vacuo*. The resulting aqueous layer was washed with n-hexane (10 mL, 5×), its pH adjusted to 2.0, with HCl, and extracted with ethyl acetate (10 mL, 5×). The ethyl acetate layer was evaporated to dryness, and then suspended with water (10 mL). The water suspension was extracted with diethyl ether (10 mL, 5×), and the combined diethyl ether layer dehydrated with anhydrous sodium sulfate. The diethyl ether was then evaporated, the residue reconstituted in methanol, and analyzed by HPLC.

Quantitative Analysis by HPLC

GK and bilobalide were determined by injection of a 20 μL volume to an HPLC delivery system (TSP, USA), using a Lichrosorb R-18 (10 μm, 3.2 × 250 mm, Merck) column, a mobile phase consisting of methanol:distilled water (40:60), at a flow rate of 1.0 mL/min, and were detected by the absorbance at 220 nm using a UV detector (TSP, 3000HR). GK-A, GK-B, and bilobalide were identified by comparison of the retention times of the various peaks with those of authentic standards and co-chromatography. The correlation coefficient (R) of standards was 99.7% for GK-A, 99.9% for GK-B and 99.6% for bilobalide. The data were expressed as the average and the standard deviation (S.D.) of three separate experiments. The statistical significance between contrasting treatments was assessed by Duncan's multiple range test ($p = 0.05$).

RESULTS AND DISCUSSION

Comparison of GK and Bilobalide Contents Between Female and Male Trees

The ginkgo tree is a representative dioecism, which is classified as female and male trees. This feature gives rise to the question as to whether the contents, either primary or secondary metabolites, are affected by sexuality. For this reason, quantification of the GK and bilobalide was performed, depending on the sexuality of the individual trees.

As shown in Figs. 1 and 2, it seems that the variability, the GK and bilobalide contents of ginkgo were in line, not only with the sexuality, but the plant parts also. Both female and male trees have different production patterns in three parts (A) the leaves, (B) the stem bark, and (C) the stem. The GK-B and bilobalide were abundant in the leaves, whereas the GK-A was most abundant in the stem bark and stem, which contained only trace amounts (< 100 μg/g dry weight) of the GK-B and bilobalide (Fig. 1). GK-A existed relatively consistently from approximately 200~300 μg/g dry weight but in the cases of the GK-B and bilobalide there were high variation observed; from 800 (#4) and 1,000 (#2 and #3) to < 100 μg/g dry weight. The different distributions of GK-B and bilobalide in the different organs might be indicative of the potential compartmentation of biosynthetic enzyme loci and storage sites. Carrier *et al.* reported on the distribution of ginkgolide and terpenoid biosynthetic activities in *G. biloba*, and also suggested compartmentation, with

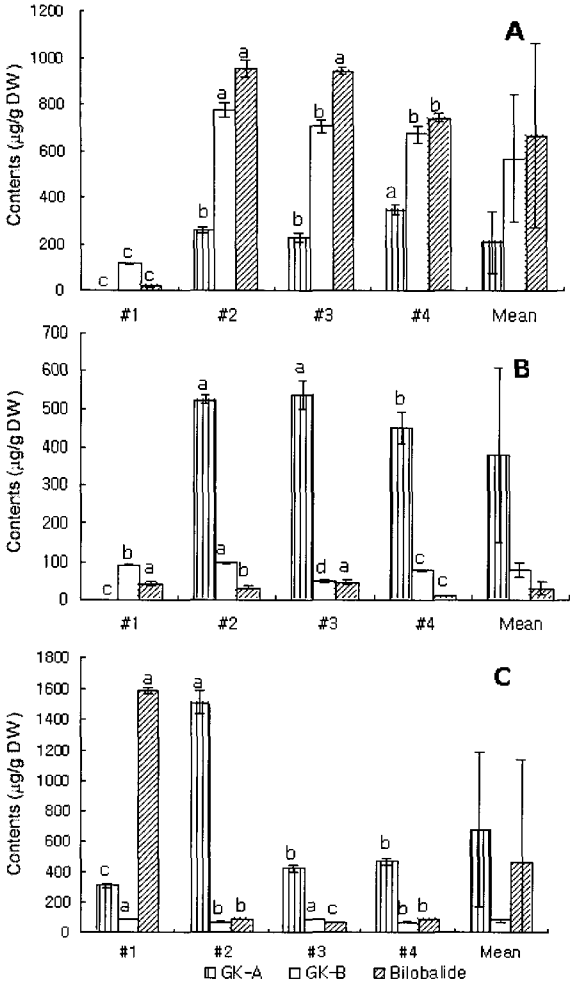

Fig. 1. The GK-A, GK-B and bilobalide contents, based on the various plant parts obtained from 30-year-old female ginkgo trees: (A) Leaves, (B) Stem bark, and (C) Stem. The error bars indicate standard deviation (S.D.) from the values of each replicate treatment separated from the same parts. Values without a common superscript letter are significantly different at $p < 0.05$.

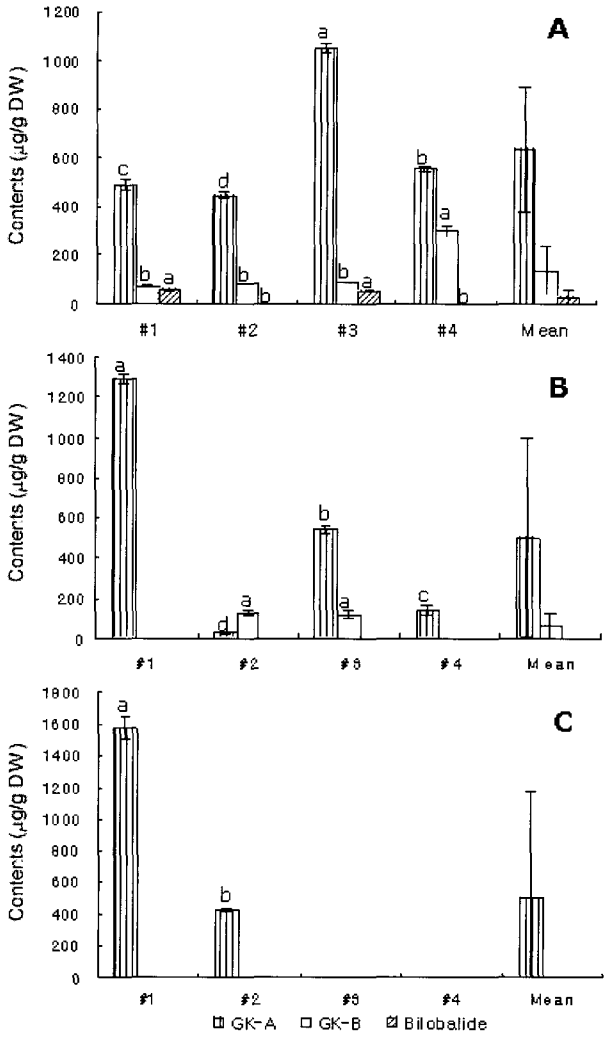

Fig. 2. The GK-A, GK-B and bilobalide contents, based on the various plant parts obtained from 30-year-old male ginkgo trees: (A) Leaves, (B) Stem bark, and (C) Stem. The error bars indicate standard deviation (S.D.) from the values of each replicate treatment separated from the same parts. Values without a common superscript letter are significantly different at $p < 0.05$.

synthesis in one region of the plant, which is then transported to a different region [5]. Tree #1 exhibited a considerable difference compared with the other trees; in particular, the GK-A was detected in trace amounts in all three parts. These results suggest that tree #1 was different to the other trees in its response to physiological, genetical and ecological states.

In the male ginkgo tree, GK-A existed predominantly in all the plant parts (Fig. 2). The GK-A content of the leaves was lower than the GK-B and bilobalide contents in the female tree (Fig. 1A), whereas their contents in the male tree was reversed. The GK-A contents of the stem bark (Fig. 2B) and stem (Fig. 2C) were major constituent, with the exception of tree #2. The GK-B and bilobalide, which accumulated in small amounts in the stem bark and stem of the female tree, were detected at different amounts in the male trees with exception of trees #2 and #3. In the stems, only the GK-A had accumulated. There were considerable variations, depending on the individual trees and plant parts.

Ginkgo Cell Culture

For the alternative productions of GK and bilobalide, ginkgo calli were induced from petiols of female and male trees. When various concentrations of NAA and 2,4-D were independently supplemented as growth regulators for the induction of calli, the NAA was more effective than 2,4-D in relation to the frequency of the callogenesis (data not shown).

When 30% (SCV) cells were inoculated into 70 mL of liquid medium in a 250-mL Erlenmeyer flask, the cells

Table 1. The GK-A contents with standard deviations in leaves of 30-year old trees and cells derived from the leaf petioles

Lines	Female		Male	
	Cells	Trees	Cells	Trees
#1	628.7 ± 15.6[b]	418.9 ± 16.1[a]	454.0 ± 19.1[c]	262.1 ± 14.1[b]
#2	604.1 ± 14.9[b]	233.7 ± 11.1[b]	677.0 ± 21.7[a]	5.31 ± 1.5[c]
#3	601.4 ± 16.9[b]	76.2 ± 6.2[d]	597.5 ± 11.1[b]	299.0 ± 16.5[b]
#4	786.2 ± 19.5[a]	137.1 ± 8.8[c]	263.0 ± 7.9[d]	1118.6 ± 90.0[a]
Mean	655.1 ± 81.1	216.5 ± 135.8	497.9 ± 165.0	421.2 ± 438.6

The cells were analyzed after 4-week culture.
Contents are expressed as μg per g dry weight. Values bearing different letters in a column are significantly different at $p < 0.05$.

Table 2. The GK-B contents with standard deviations in leaves of 30-year-old trees and cells derived from the leaf petioles

Lines	Female		Male	
	Cells	Trees	Cells	Trees
#1	10.0 ± 1.6[b]	70.6 ± 3.1[b]	5.0 ± 1.0[a]	138.1 ± 9.7[a]
#2	10.0 ± 2.1[b]	68.1 ± 5.6[b]	3.0 ± 0.9[b]	69.2 ± 7.2[c]
#3	35.0 ± 4.4[a]	77.7 ± 4.5[b]	3.0 ± 0.7[b]	117.3 ± 8.0[b]
#4	10.0 ± 1.2[b]	98.9 ± 6.5[a]	3.0 ± 0.4[b]	69.6 ± 3.8[c]
Mean	16.3 ± 11.5	78.8 ± 13.4	3.5 ± 1.1	98.5 ± 32.0

The cells were analyzed after 4-week culture.
Contents are expressed as μg per g dry weight. Values bearing different letters in a column are significantly different at $p < 0.05$.

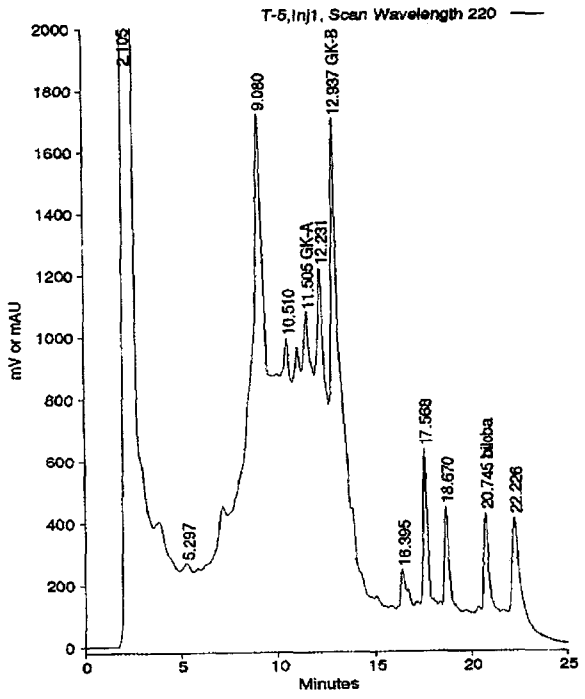

Fig. 3. HPLC chromatogram of the GK-A, GK-B and bilobalide (biloba) in female ginkgo cells. The cells were cultured for 4 weeks under dark condition. The GK and bilobalide were detected by monitoring the absorbance at 220 nm using a UV detector.

reached stationary phase in 4 weeks and analyzed the contents of GK and bilobalide (data not shown). GK-A, GK-B and bilobalide have also been detected in cell suspension cultures (Fig. 3). GK-A was the major constituent in suspension cultures of both female and male trees. The production profile of the female cells was similar to that of the males, with the lactone terpenes being abundant in the order: GK-A > bilobalide > GK-B (data not shown). GK-B had hardly accumulated, while much bilobalide was found in the male suspension cultures, close to the levels of GK-A.

Comparison of GK and Bilobalide Contents between Tree and Cell

A relatively constant production of GK-A was achieved by cell cultures, whereas an extremely variable content was revealed by intact trees (Table 1). Interestingly, tree #4 gave significant production, approximately 1,200 μg/g dry weight. The GK-A content in tree #4 was more than that of the cell culture of #4, while the others (#1~#3) were more abundant in both the female and male cells. Consequently, the dedifferentiated cells were more stable for GK-A production than the differentiated trees. These results suggest that GK-A production is extremely influenced by several environmental factors, and the stable production of GK-A can be achieved by *in vitro* cultures by control of external factors.

Conversely, the amount of GK-B was higher in the trees (Table 2). Relatively constant amounts of GK-B

Table 3. The bilobalide contents with standard deviations in leaves of 30-year-old trees and cells derived from the leaf petioles

Lines	Female		Male	
	Cells	Trees	Cells	Trees
#1	150.0 ± 9.5[a]	10.0 ± 1.2[b]	424.3 ± 11.6[b]	231.4 ± 15.5[c]
#2	22.9 ± 2.6[c]	11.6 ± 1.1[b]	2413.3 ± 83.2[a]	542.5 ± 20.0[a]
#3	8.0 ± 0.8[d]	10.0 ± 0.6[b]	445.7 ± 16.8[b]	566.7 ± 14.1[a]
#4	55.0 ± 4.5[b]	50.0 ± 3.0[a]	283.3 ± 7.6[c]	401.3 ± 15.7[b]
Mean	59.0 ± 58.1	20.4 ± 17.9	891.7 ± 920.6	435.5 ± 140.3

The cells were analyzed after 4-week culture.
Contents are expressed as μg per g dry weight. Values bearing different letters in a column are significantly different at $p < 0.05$.

Table 4. The GK-A, GK-B, and bilobalide contents with standard deviations based on the lighting conditions in 4-week-old ginkgo cells

Lines	Female		Male	
	Light	Dark	Light	Dark
GK-A	619.0 ± 81.6[b]	184.0 ± 35.2[d]	398.0 ± 18.3[c]	1210.0 ± 59.0[a]
GK-B	976.0 ± 41.8[a]	32.0 ± 4.6[b]	13.0 ± 2.0[b]	19.0 ± 4.0[b]
Bilobalide	198.0 ± 23.4[d]	597.0 ± 17.6[b]	301.0 ± 17.1[c]	1399.0 ± 67.4[a]

Contents are expressed as μg per g dry weight. Values bearing different letters in a column are significantly different at $p < 0.05$.

were observed in the female trees, but the contents of male trees varied; for instance tree #1 was 2-fold higher than #2 or #4. The biosynthesis of GK-B was inhibited in the ginkgo cells. In particular, the male tree derived cells accumulated trace amounts of GK-B. This result might indicate that the biosynthesis of GK-B could be related to differentiation. Besides it would seem that no correlation exists between a high-producing tree and its cultured cells. In other words, although a tree may accumulate the highest GK-B content, the cultured cells derived from the dominant tree could not produce the same levels. Thus this suggests that the ability of an intact plant to produce a high amount of metabolites is not exactly transmitted to its dedifferentiated cells.

With the bilobalide content, considerable variation was observed among the individuals of both tree and cell (Table 3). For instance, the content of the female tree #1 cell was 15-fold greater than that of tree #1. Both the female and male trees of #3 accumulated increased bilobalide contents compared with their cells. The capabilities of production in the intact trees #3 and #4 were similar to those of their cells, whereas the cells of trees #1 and #2, on the contrary, produced increased amounts of bilobalide than their corresponding female and male trees. Consequently, the accumulation of bilobalide is speculated to be dependent on the individual variations rather than the physiological states of dedifferentiation or differentiation.

The Effect of Light Irradiation on GK and Bilobalide Accumulation

The synthesis of secondary metabolites is known to be related to the physiological state of a cell, which is affected by regulation of the environment. Among the regulatory factors, light is important which can lead to increased biosynthesis, including the possibility of stimulatory effect on key enzyme expression, although the precise mechanism of the biosynthetic metabolism remains to be elucidated.

When the ginkgo cells were cultivated in dark and light conditions, the GK-A, GK-B and bilobalide showed different production profiles (Table 4). The production of GK-A differed from its original sexuality, which was more abundant under light conditions in the female cells, with the result reversed, in the male cells. As deduced from this result, the capability for GK-A synthesis may be related to the original sexuality in response to light irradiation. Conversely, GK-B was rarely produced in the male cell under either of the conditions (Table 4). Peculiarly, in the female cells, the production of GK-B was significantly increased, by approximately 1mg/g dry weight, in response to light. This result demonstrates that on the basis of individual variation, disturbance of metabolism by light irradiation can also be expected. The same effect was revealed for the bilobalide in both the female and male cells, with dark conditions being better than light, by increasing the production up to 3-, and 4.7-fold, respectively.

In short, extreme variations existed in terms of the sexuality and individual trees. For this reason, it may be difficult to obtain stable yields of GK and/or bilobalide by attempting their production by direct extraction from intact ginkgo trees. Furthermore, no correlation was found between the high-producing trees and cells induced from these dominant trees. The effect of light irradiation dif-

fered depending on the metabolites; only GK (GK-A and GK-B) showed an enhanced production with light irradiation of the female cells.

CONCLUSION

Ginkgolides and bilobalide are valuable compounds produced from *G. biloba*. For the *in vitro* production, and further mass production by scale-up, it is necessary to know the relation between intact plants and their corresponding *in vitro* cells. Female and male ginkgo trees had different GK-A, GK-B and bilobalide production profiles. There were also different production patterns shown in the leaves, stem bark and stem parts. There were considerable variations between the production from trees and that of their cells. The GK content in the cell cultures that originated from high-producing dominant trees were not coincident. The production of GK in *G. biloba* was affected by individual variation, differentiation and environmental factors. Light irradiation was selectively effective on the production of GK-A and GK-B in the cell cultures of the female trees. The introduction of several biotechnological strategies, such as high producing cell line selection and light irradiation, may provide an alternative approach to the stable and enhanced production.

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REFERENCES

[1] Huh, H., E. J. Staba, and J. Singh (1992) Supercritical fluid chromatographic analysis of polyphenols in *Ginkgo biloba*. *J. Chromatogr.* 600: 364-369.

[2] Kang, S. S., J. S. Kim, W. J. Kwak, and K. H. Kim (1990) Flavonoids from the leaves of *Ginkgo biloba*. *Kor. J. Pharmacogn.* 21: 111-120.

[3] Kang, S. S., J. S. Kim, W. J. Kwak, and K. H. Kim (1990) Identification and quantitative analysis of flavanol glycosides from *Ginkgo biloba* leaves by high performance liquid chromatography. *Kor. J. Pharmacogn.* 21: 148-152.

[4] Laurain, D., J. T. Guiller, J. C. Chenieux, and T. A. van Beek (1997) Production of ginkgolide and bilobalide in transformed and gametophyte derived cell cultures of *Ginkgo biloba*. *Phytochemistry* 46: 127-130.

[5] Carrier, D. J., T. A. van Beek, R. van der Heijden, and R. Veroorte (1998) Distribution of ginkgolides and terpenoids biosynthetic activity in *Ginkgo biloba*. *Phytochemistry* 48: 89-92.

[6] Smith, P. F., K. Maclennan, and C. L. Darlington (1996) The neuroprotective properties of the *Ginkgo biloba* leaf: a review of the possible relationship to platelet-activating factor (PAF). *J. Ethnopharmacol.* 50: 131-139.

[7] Son, Y. H. and H. W. Kim (1998) Above-ground biomass and nutrient distribution in a 15-year-old ginkgo (*Ginkgo biloba*) plantation in central Korea. *Bioresource Technol.* 63: 173-177.

[8] Kim, G. S., Y. W. Paek, K. M. Ko, S. J. Hwang, Y. J. Kim, S. J. Chung, and B. Hwang (1996) Detection of flavonoid compounds by cell culture of *Ginkgo biloba* L. *Kor. J. Biotechnol. Bioeng.* 11: 1-7.

[9] Misawa, M. and T. M. Nakanishio (1998) Antitumor compounds; Production by plant cell culture. pp. 199-207. In: Y. P. S. Bajaj (ed.). *Biotechnology in Agriculture and Forestry*. Vol. 4. *Medicinal and Aromatic Plants*. Springer-Verlag, Berlin, Germany.

[10] Murashige, T. and F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* 15: 473-497.

[11] Huh, H. and J. Staba (1992) Ontogenic aspects of ginkgolide production in *Ginkgo biloba*. *Planta Med.* 59: 232-239.

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