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Cultures of *Ginkgo biloba*, Effect of Nutritional and Hormonal Factors on the Growth of Cultured Cells Derived from *Ginkgo biloba*

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Calli and suspension cultures were obtained following inoculation of the explant from leaves of *Ginkgo biloba* L. on the supplemented MS basal medium. The obtained calli and suspension cultured cells were able to produce detectable amounts of ginkgolides which are known as natural specific PAF antagonists. The production of ginkgolides in the calli and suspension cultured cells were identified using GC/MS, GC and HPLC with authentic compounds. Since the production of ginkgolides A and B in the calli and suspension cultured cells had been confirmed, effects of types and concentration of plant growth regulators, media and illumination on the induction and growth of the callus were studied. The concentrations of growth regulators for optimal callus induction were 1.0 to 2.0 mg/L for NAA and 0.1 mg/L for kinetin. The growth of the callus seemed to be more stimulated with the combination of NAA and kinetin than NAA and BA with illumination at all concentration ranges of 1.0 to 4.0 mg/L for NAA and 0.1 to 1.0 mg/L for kinetin or BA studied. Among 8 different media used, the induction rate of callus on Anderson, Eriksson, and Schenk and Hildebrant at 4 weeks after the innoculation was almost the same as that of MS. However, callus was rarely induced on Heller or White medium. Suspension cultures were easily initiated with 3 g of callus (fresh weight) derived from ginkgo leaves on supplemented MS medium. A typical growth curve of suspension cultured cells could be obtained by measuring the fresh weight of the suspension cultured cells at every 3 days. To improve the growth of suspension cultured cells of ginkgo, effects of concentrations of NAA, sucrose, phosphate ions and molar ratio of NH_4^+ to NO_3^- ions in the culture medium were studied. The maximum growth of the cells was achieved when the culture medium contained 1.0 mg/L of NAA, 30 g/L sucrose, 1.75 mM phosphate ions and 1 : 5 molar ratio of NH_4^+ to NO_3^- ions.

Key words: Callus, Cell growth, *Ginkgo biloba*, Ginkgolides, PAF, Plant growth regulator, Suspension cultured cells

INTRODUCTION

The leaves of *Ginkgo biloba* L. are of particular importance to provide only a unique C_{20} cage molecule, the ginkgolide, naturally occurring platelet-activating factor (PAF) antagonist (Braquet, 1984; Braquet, 1987; Nunez *et al.*, 1986). PAF is a potent mediator of anaphylaxis and inflammation and is also implicated in shock, graft rejection, renal diseases, ovoimplantation, and certain disorders of the central nervous system (Kuster *et al.*, 1986; Dive *et al.*, 1989; Wenche and Rolfsen, 1990). Indeed, the growing importance of PAF as a mediator of diverse pathologies increases the possible medicinal benefits that may be derived from ginkgolide, specific PAF antagonist (Schwabe, 1972; Schwabe, 1986; Braquet, 1988).

However, only trace amounts of ginkgolides are contained in the ginkgo leaves (Nakanishi, 1967; Teng, 1988). In addition, location, climate, and seasonal variations of the ginkgolides content are affecting factors for the production. Thus the constant and continuous commercial-scale supply of the ginkgolides from the field-grown leaves appears to be quite uneconomical and questionable.

Consequently, it is crucially important to come up with alternative ways to provide the ginkgolides. In the present study, as an alternative way of the ginkgolides production, tissue culture of the ginkgo leaves was conducted. As a result of the study, the produc-

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tion of ginkgolides in the cultured cells was identified, although smaller amounts than in the field-grown leaves. At first, the effects of media, plant growth regulators, nitrogen source, and phosphate ions on the growth of the cultured ginkgo cells were examined.

EXPERIMENTAL METHODS

Plant materials

The ginkgo leaves were collected from the field-grown trees in the campus of Seoul National University, Seoul, Korea between the middle of April and the end of June in 1989. The seedling leaves were obtained from the germinated ginkgo seeds in germination bed consisted of vermiculite and sand (1 : 1).

Callus induction and maintenance

The leaves were surface-sterilized by immersing them into 70% ethanol for 10 sec followed by a solution of 0.25% sodium hypochlorite for 8 min. After rinsing 4 times with sterilie water, the leaves were cut with a scalpel into approximately 0.7×0.7 cm squares. The excised explants were transferred to Murashige and Skoog basal salt medium supplemented with 3% sucrose, 0.8% agar, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxin-HCl, 0.1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 2 mg/L glycine, 1.0 mg/L 1-naphthalenacetic acid (NAA) and 0.1 mg/L kinetin (supplemented MS medium) to induce callus, and cultured at 25± 1℃ under the dark.

The induced callus was maintained at 25± 1℃ either under the dark or the illumination, and was subcultured every 3 weeks into fresh supplemented MS medium.

Suspension cultures

Cell suspension culture was initiated with 3.0 g of the callus derived from the leaves with 80 ml of the supplemented MS liquid medium in a 250 ml Erlenmeyer flask with agitation at the rate of 100-110 rpm on a reciprocal shaker at 25± 1℃ under the illumination. For kinetic studies of the growth of cultured cells, fresh weight of the cultured samples (3 replicates) were measured every 3 days during the culture period of 21 days.

Measurement of growth

The growth index of callus or suspension cultures was calculated by dividing the fresh weight of biomass at the time of collection with the initial inoculation weight. Fresh weight of callus or suspension cultured cells was measured aseptically after rinsing them with sterilized distilled water under the reduced pressure. Dry weight of callus or suspension cultured cells was determined after lyophilization.

Extraction and purification of ginkgolides from cultured cells derived from *Ginkgo biloba*

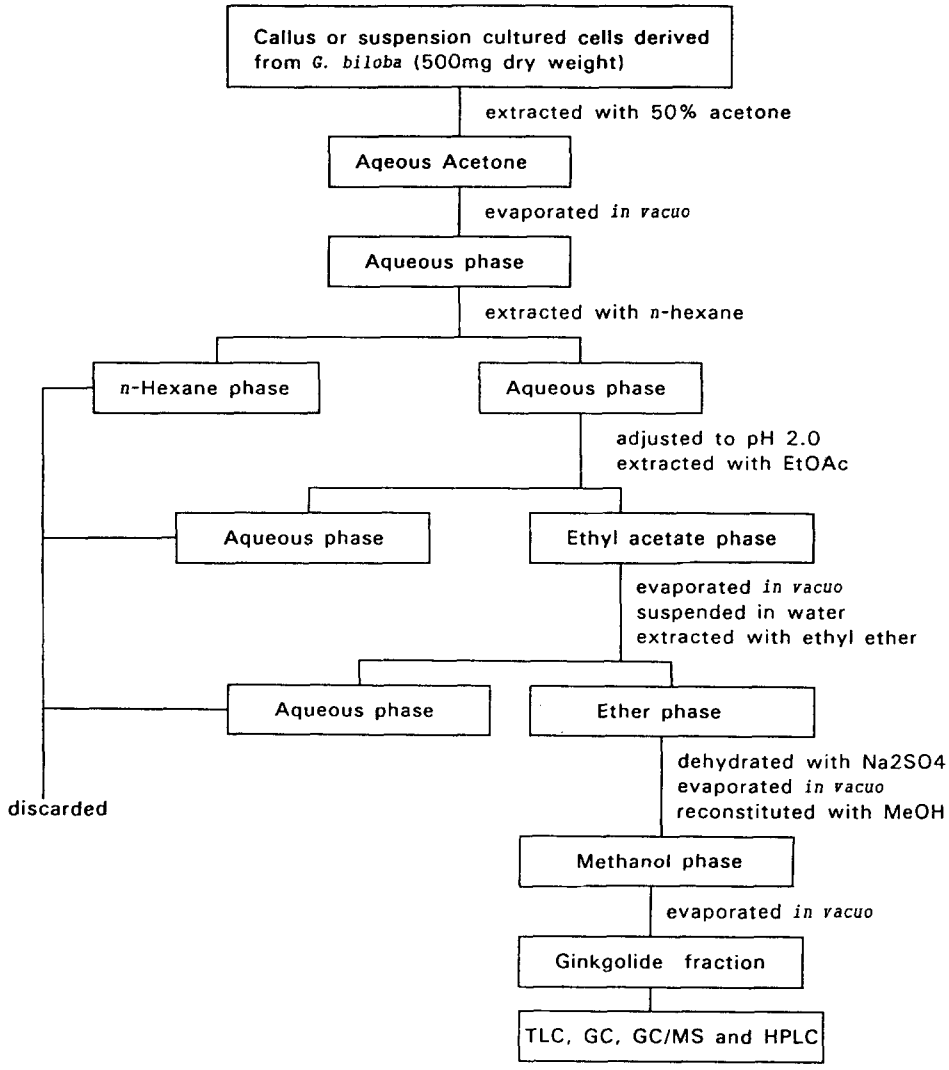
The harvested callus or suspension cultured cells were lyophilized and then pulverized. The resultant powder (500 mg) was extracted with 10 ml of acetone-water mixture (1 : 1, v/v) for 1 hr each 4 times using a sonicator at an ambient temperature. The combined extract was evaporated under vacuum until acetone is completely removed. Then, the remaining aqueous layer was extracted with 10 ml of n-hexane 5 times to remove non-polar substances. The partially purified remaning aqueous layer was adjusted to pH 2.0 with 1N HCl and extracted with 10 ml of ethyl acetate 5 times. The ethyl acetate layer was evaporated to dryness under vacuum. The residue was suspended in 5 ml of deionized water and extracted with 10 ml of diethyl ether 5 times. The combined ether extract was evaporated to dryness under vacuum. The residue was dissolved in 2 ml of methanol and passed through alumina column prewashed with methanol, at the rate of 0.8 ml/min with 50 ml of methanol. The eluent was evaporated to dryness under vacuum. The residue was reconstituted in 500 μl of methanol and then filtered through a 0.45 μm membrane (Lobstein, *et al.*, 1983; Tallevi and Kurz, 1991; van Beek, *et al.*, 1991).

GC analysis of ginkgolides

The identification and quantitation of ginkgolides from cultured ginkgo cells was accomplished by GC with authentic ginkgolides A, B and C. For the analysis with GC, the obtained ginkgolides fraction as above mentioned was reacted with silylating agent (Tri-Sil BSA in DMF, Pierce Chemical, Rockford, IL, USA) at 73℃ for 1 hr. GC analysis was performed with a Hewlett-Packard model HP 5985 series II equipped with a flame ionization detector (FID) and HP-1 capillary column (0.2 mm×35 m). The GC conditions were as follows: injection temperature of 295℃; column temperature of 280℃; detector temperature of 295℃ and at a flow rate of 0.5 ml/min using N_2 as a carrier gas. The amounts of ginkgolides were calculated from the measurement of the area of the corresponding peaks in the chromatogram.

GC/MS analysis of ginkgolides

The identification of ginkgolides in cultured ginkgo cells was also accomplished by GC/MS with authentic ginkgolides A, B and C. GC/MS analysis was performed with a Hewlett-Packard model HP 5985 series II GC directly interfaced to a VG Trio II mass spectrometer.



Scheme 1. Extraction and purification of ginkgolides.

The GC/MS conditions were as follows: SE-54 capillary column (0.2 mm×17 m); split ratio of 1 : 10; interphase temperature of 300℃; ion source temperature of 300℃ and electron-impact ionization of 70 eV. The column temperature was increased 20℃min from 100℃ to 300 ℃. Helium was used as the carrier gas at a flow rate of 0.89 ml/min. Chromatogram was acquired in the selected-ion monitoring mode.

RESULTS AND DISCUSSION

Tissue culture of ginkgo leaves has long been a subject of many studies to clarify the biosynthetic pathway and production of bioactive substances, ginkgolides (Huh and Staba, 1992). There have been controversies as to whether ginkgolides are produced by cell cultures of ginkgo leaves. The cell culture of ginkgo leaves was first attempted by Nakanishi and Habaguchi (1971) with the objective of elucidating the biosynthetic pathway of ginkgolides. However, ginkgolides could not be detected with HPLC from their cultures. Since their pioneering work, no report has been published on ginkgolides production by cell cultures of ginkgo leaves until Carrier *et al.* (1990) established unorganized cell cultures from embryo. However, ginkgolide A among the five ginkgolides has been detected in a much lower yield than the field-grown plants. More recently, Huh and Staba (1993) briefly reported their results of ginkgolides production in plant tissue cultures. Yield of ginkgolide B was much higher in organ cultures than that of unorganized cultures. However, no detailed experimental descriptions of the results were provided. This study demonstrates the effects of several essential factors for the induction and maintenance of *G. biloba* cell cultures.

Callus induction

The effects of different types and concentrations of

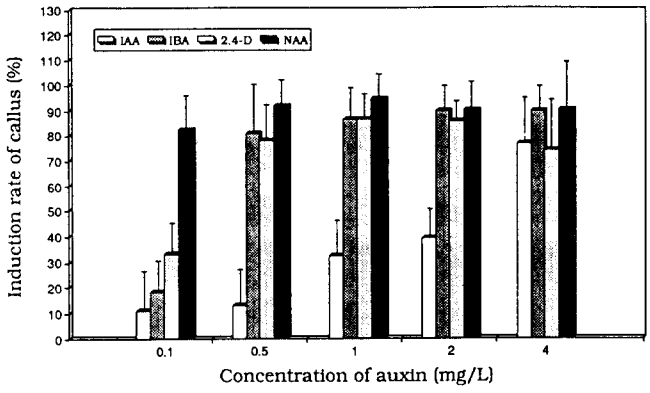


Fig. 1. Effects of types and concentrations of auxin on the induction of callus from ginkgo leaves.

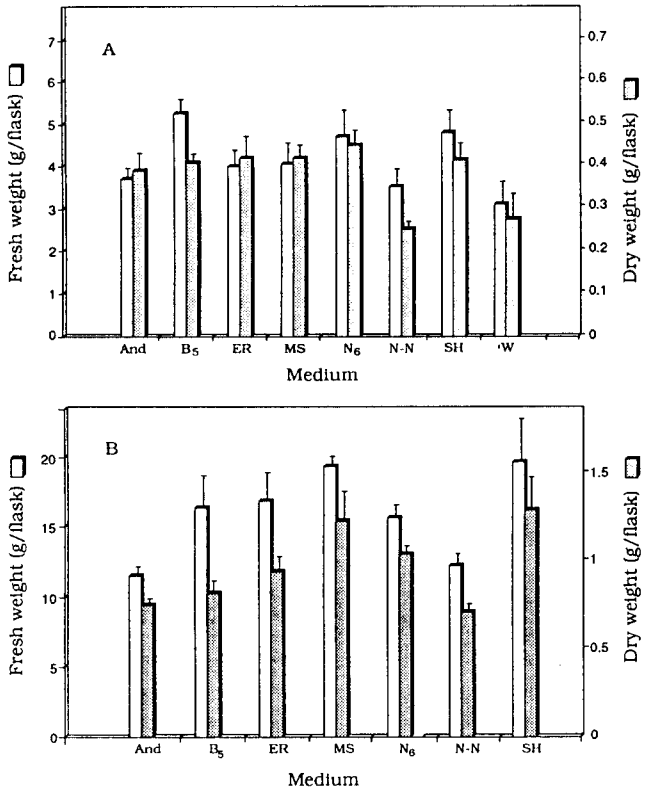


Fig. 2. (A) Effects of various types of culture medium on the growth of callus derived from ginkgo leaves.
(B) Effects of various types of culture medium on the growth of the suspension cultured cells from ginkgo leaves.

plant growth regulators on the callus induction from the excised leaf segments of *G. biloba* were examined to find out the optimal conditions for the callus induction. For the study, four different auxins, such as 3-indoleacetic acid (IAA), 3-indolebutyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthalene acetic acid (NAA) were added at the concentrations of 0.1, 0.5, 1.0, 2.0 or 4.0 mg/L into the supplemented MS basal medium on which the leaf explants were

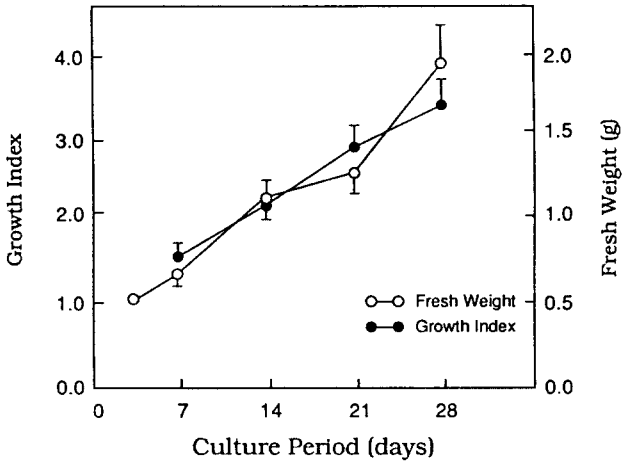


Fig. 3. Growth profile of the callus derived from ginkgo leaves.

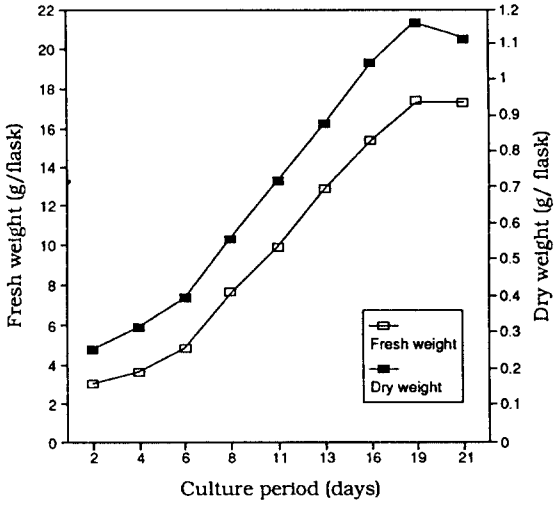


Fig. 4. Time course of the growth of suspension cultured cell from ginkgo leaves.

inoculated and cultured 4 weeks under the dark (Fig. 1). In terms of the types of auxin employed, NAA had the highest induction rate of callus and IAA had the lowest over a concentration range from 0.1 to 4.0 mg/L. The induction rate was concentration dependent for IAA. In contrast to IAA, NAA did not show concentration-dependency and was the most efficient at the concentration of 1.0 mg/L showing 95% induction rate. Although NAA itself could induce the callus as much as 95% from the excised leaf segments of *G. biloba* at the concentration of 1.0 mg/L, the effect of kinetin on the callus induction was examined in various combinations with NAA. The combination of 0.1 mg/L of kinetin to 1.0 mg/L of NAA induced the callus more efficiently than NAA alone. The hormonal concentrations for optimal callus induction were 1.0 to 2.0 mg/L for NAA and 0.1 mg/L for kinetin.

In order to find out the effect of culture medium

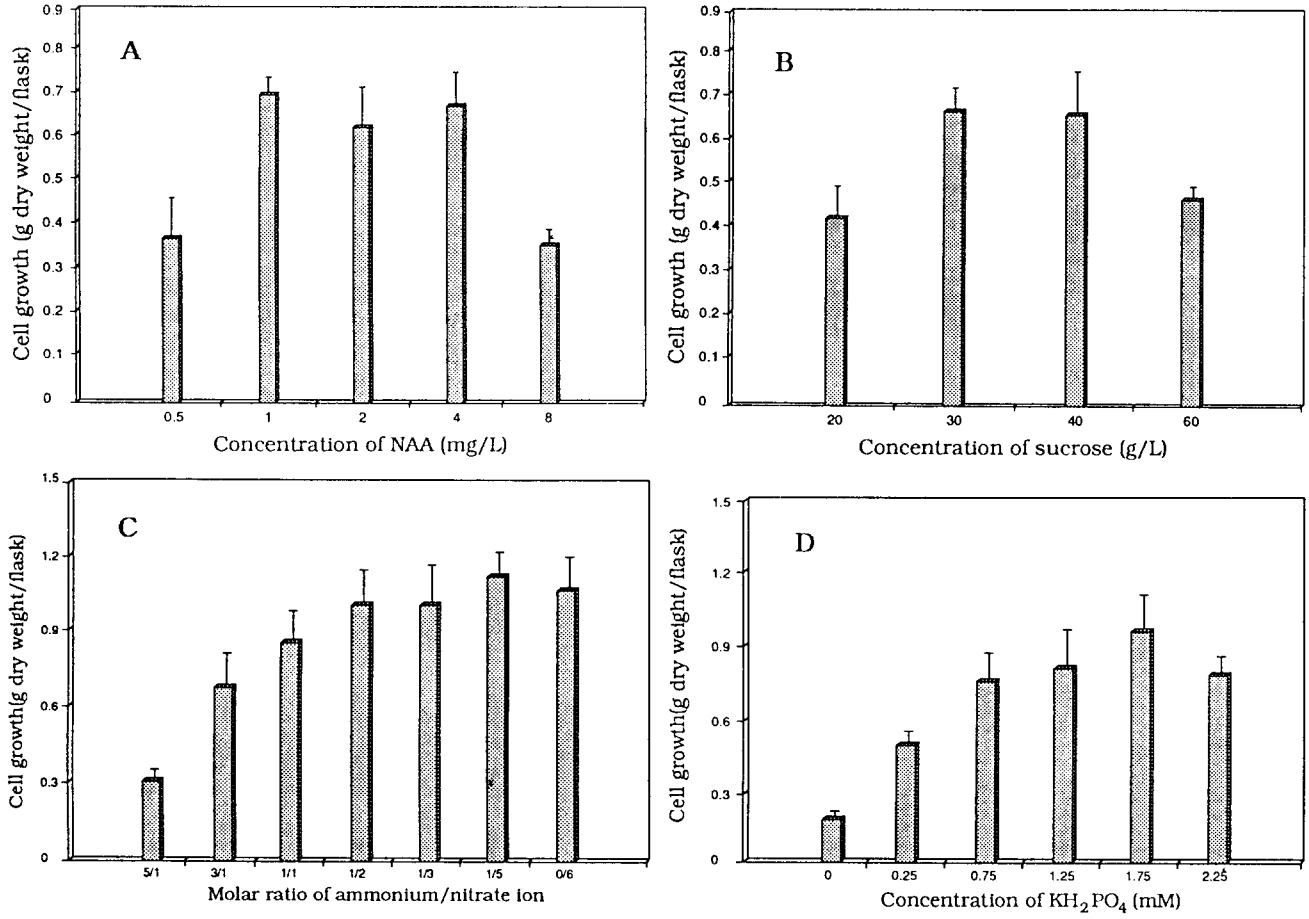


Fig. 5. (A) Effects of NAA concentration on the growth of the suspension cultured cells from ginkgo leaves. (B) Effects of sucrose concentration on the growth of the suspension cultured cells from ginkgo leaves. (C) Effects of molar ratio of NH_4^+ to NO_3^- on the growth of the suspension cultured cells from ginkgo leaves. (D) Effects of phosphate concentration on the growth of the suspension cultured cells from ginkgo leaves.

on the callus induction from the leaf segments of *G. biloba*, eight different culture media were examined. Under the experimental conditions used, callus formation was mainly observed on Murashige and Skoog (MS), Gamborg (B_5), Eriksson (ER), Anderson, Chu (N_6), and Schenk and Hildebrant (SH) media, and rarely on White or Heller media. The calli induced on MS medium became light brown and were rather compact than those induced on ER medium. It is of interest to note that adventituous buds began to form within three weeks following the inoculation of explants on SH medium.

Therefore, the supplemented MS basal medium with 1.0 mg/L of NAA and 0.1 mg/L of kinetin was selected as a medium of choice for the callus induction.

Callus maintenance

To determine the effect of different types and concentrations of cytokinin in combination with NAA on the growth of the callus derived from the leaf segments of *G. biloba*, 500 mg of induced callus was transferred to the supplemented MS medium differing the concentrations of kinetin or 6-benzyladenine (BA), and cultured for 4 weeks under the dark or illumination (data not shown). Growth of the callus seemed to be more stimulated with the combination of NAA with kinetin than NAA with BA under the illumination at all concentrations studied. However, significant difference in fresh weight of callus after 4 weeks of the culture could not be obtained. From the study, it could be postulated that the growth of the callus induced from the leaf segments of *G. biloba* may be depended on the types and concentrations of auxin rather than that of cytokinin employed.

To determine the effect of different culture medium on the growth of the callus, 1.2 g of callus induced on the supplemented MS basal medium with 1.0 mg/L of NAA and 0.1 mg/L of kinetin was transferred to 8 different media, MS, B5, ER, White, Anderson, N_6, Nitsch and Nitsch (N-N) and SH medium, respectively, and cultured for 4 weeks. The rapid growth of callus was obtained on MS medium. The growth of callus

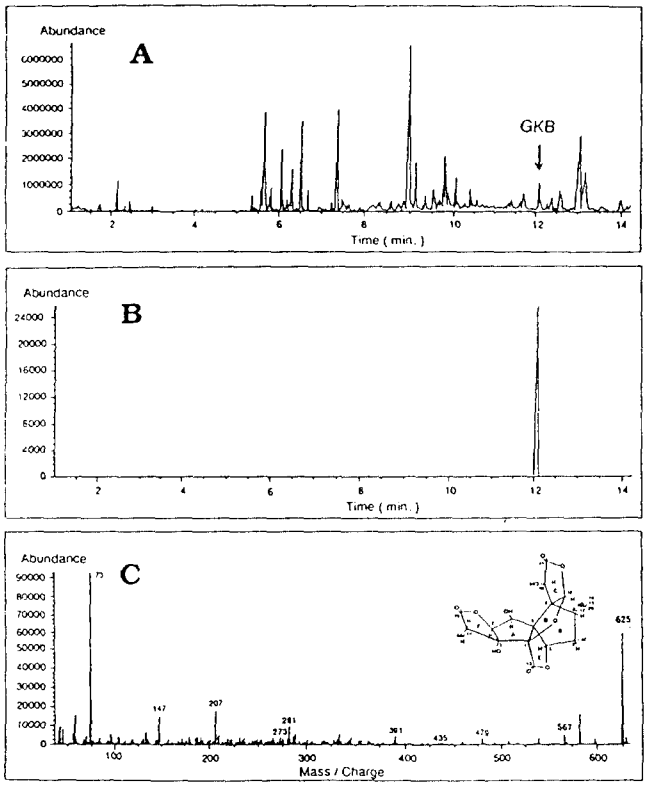


Fig. 6. GC/MS spectrum of the ginkgolides fraction from the suspension cultured cells from ginkgo leaves.
A; chromatogram of purified fraction from cultured cells.
B; selective ion monitering chromatogram.
C; mass spectrum of the arrowed peak in A.

was fairly good on Anderson, N_6, B_5 or SH medium and rarely on Anderson, White or N-N medium (Fig. 2A).

The growth index of callus induced on the supplemented MS medium with 1.0 mg/L of NAA and 0.1 mg/L of kinetin was determined by measuring fresh weight of the callus over a total culture period of 28 days at the 7 days interval. The growth index increased about 0.6 unit every week over the culture period of 4 weeks (Fig. 3).

The results indicate that the supplemented MS basal medium with 1.0 mg/L of NAA and 0.1 mg/L of kinetin seems to be quite suitable for both callus induction and maintenance. The optimal concentration of growth regulator for the induction and maintenance of the callus on the solid medium was consistent with that of previously reported (Carrier *et al.*, 1990; Huh and Staba, 1993).

Cell suspension cultures

Suspension cultures were easily initiated with 3 g of the callus in the supplemented MS liquid medium with 1.0 mg/L of NAA and 0.1 mg/L of kinetin. The culture was subsequently subcultured every other week. To obtain the growth curve of the suspension cultured cells, the fresh weight of the suspension cultured cells was measured every 3 days. The growth pattern of the cells showed typical sigmoidal curve. After 19 days of the culture, the cell growth reached at the stationary phase (Fig. 4).

To determine the effects of different culture media on the growth of the suspension cultured cells, 3 g of the cells was transferred to MS, B_5, ER, Anderson, N_6, N-N and SH medium, respectively, and cultured for 2 weeks. The rapid growth of the cells was obtained with SH or MS medium. The growth of the cells was fairly good with N_6, ER or B_5 and rarely with Anderson or N-N (Fig. 2B). To improve the cell growth, effects of the concentration of NAA, sucrose, and phosphate ions and the molar ratio of NH_4^+ to NO_3^- ions on the growth were studied. The concentration of NAA in the medium was varied in the ranges from 0.5 mg/L to 8.0 mg/L. The dry weight value of the cells showed NAA concentrations in the ranges from 1.0 to 4.0 mg/L were favorable to cell growth exhibiting no significant differences. However, the growth of the cells became declined at higher NAA concentrations than 4.0 mg/L (Fig. 5A). The sucrose concentration in the medium was varied in the ranges from 20 g/L to 60 g/L. The maximal cell growth was achieved in sucrose concentration ranges from 30 g/L to 40 g/L (Fig. 5B). At the sucrose concentration of 60 g/L, the declined cell growth was obtained. The molar ratio of NH_4^+ to NO_3^- ions in the medium was varied in the ranges from 5 : 1 to 0 : 1. The total amounts of nitrogen were fixed to 0.06 M as that of supplemented MS basal medium. The cell growth (g dry weight/flask) at the 14th day of the culture was increased as nitrate ion concentration increased (Fig. 5C). The concentration of KH_2PO_4 in the medium was varied in the ranges from 0.00 mM to 2.25 mM. The optimal phosphate ions concentration for the growth in the medium was found to be 1.75 mM (Fig. 5D).

Ginkgolides production in culture

In order to determine the ginkgolides production in the cultured cells derived from the leaf segments of *G. biloba*, ginkgolides were extracted and purified as described in scheme I, and TLC, GC, GC/MS and HPLC analyses were performed. The TLC patterns of the ginkgolides extracted from the lyophilized suspension cultured cells showed ginkgolides A and B spots at the R_f values of 0.30 and 0.34, respectively, which are identical to the Rf values of authentic ginkgolides A and B.

The ginkgolides production in the callus or suspension cultured cells was identified by GC/MS by comparing the molecular ion and the fragmentation pattern with that of authentic ginkgolides. GC/MS spectra

strongly indicate the production of ginkgolides A and B in the suspension cultured cells as well as the callus derived from the leaf segments of *G. biloba* (Fig. 6). The HPLC chromatograms also indicate the formation of ginkgolides A and B in the cultured cells (data not shown).

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