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# *Callus Induction and Production of Bilobalide and Ginkgolides by Callus and Cell Suspension Cultures of Ginkgo biloba Leaves*

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## *Abstract*

The aim of this study was to investigate the effect of growth hormone on biomass and production of bilobalide and ginkgolides A and B bycallus and cell suspension cultures of *Ginkgo biloba*leaves. Calluses were induced from *Ginkgo biloba* leaves on Murashige and Skoog (MS) medium supplemented with sucrose and combination of growth hormone in several concentrations. The production of bilobalide, ginkgolides A and B in callus with combination of 2 mg/L NAA and 0.1 mg/L kinetin showed the highest. To enhance the biomass and production of bilobalide, ginkgolides A and B, effects of growth hormone, $NO_3^-$, and carbon source were investigated in cell suspension cultures. Among several concentrations of kinetin + NAA and kinetin + 2,4-D, the highest content of bilobalide, ginkgolides A and B was observed in MS medium supplemented with 0.1 mg/L kinetin + 2 mg/L NAA. The production of bilobalide, ginkgolides A and B in the addition of glucose was higher than that of sucrose and fructose. Reducing 50% of $KNO_3$ in the medium decreased the biomass and the content of bilobalide, ginkgolides A and B.

***Keywords:****callus; Ginkgo biloba; bilobalide; ginkgolides.*

*Abbreviations:*

| | | |
|---|---|---|
| NAA | : | α-naphthaleneacetic acid |
| 2,4-D | : | 2,4-dichlorophenoxyacetic acid |
| $KNO_3$ | : | potassium nitrate |
| PCV | : | packed-cell volume |
| TMS | : | trimethylsilyl |

## 1. Introduction

*Ginkgo biloba* is one of the most used medicinal plants and has been of interest to mankind for more than 2,000 years, being commercialized as pharmaceutical oral solid dosage forms containing dried vegetal material or refined extracts, for the improvement of the peripheral and central blood circulation, arterial occlusive disease, vertigo and against demential disorders (memory impairment and concentration difficulties) [1][2]. In fact in China, the fruits and seeds of the *Ginkgo biloba* tree have been used in traditional Chinese medicine, with indications for the treatment of asthma, cough, and enuresis for over 5,000 years [3]. The ginkgo tree produces diterpenes (e.g. ginkgolides A, B, C and J), sesquiterpenes (e.g. bilobalide), ginkgo flavonol glycosides (e.g. the glycosides kaempferol, quercetin, and isorhamnetin), triterpenes (e.g. sterols), organic acids, polyprenols including ginkgolic acid, and tannic acid [4]. The ginkgolide, a unique $C_{20}$ cage molecule, is a naturally occuring platelet-activating factor (PAF) antagonist [5][6]. 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 [7][8][9]. 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 [10].

However, only trace amounts of ginkgolides are contained in the ginkgo leaves [11][12]. 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. Tissue culture of the ginkgo leaves was conducted as an alternative method of production. In this research, we investigated the effect of growth hormone on biomass and production of bilobalide, ginkgolides A and B in callus and cell suspension cultures of *G. biloba* leaves. The effects of growth hormone, $NO_3^-$, and carbon source in cell suspension culture on enhancement of cell growth and production of bilobalide, ginkgolides A and B were also investigated.

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## 2. Materials and methods

### 2.1. Plant material and the explant

*Ginkgo biloba* leaves were collected from the garden of the Faculty of Agriculture, Ehime University, Japan, on September 2012. The plant material was washed using neutral detergent two times and rinsing six to seven times with sterile distilled water. After surface sterilization with 70% ethanol (EtOH) for 1 min followed by a solution of 1% sodium hypochlorite for 15 min and two cleanings in sterilized distilled-water for 10 min, young leaves were cut and transferred to Murashige and Skoog (MS) medium.

### 2.2. Optimization of culture condition

The explants were cut into approximately 0.6 x 0.6 cm squares and placed on MS medium with sucrose (30 g/L) and gellan gum (2 g/L) on pH 5.6 and supplemented with combinations of α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin. The callus was sub-cultured every month and incubated at 24˚C in the dark.To induce cell suspension cultures, 2 g fresh weight (FW) of friable callus was transferred into 250 mL Erlenmeyer flask containing 40 mL MS liquid medium. In order to optimize cell growth, cells were cultured in different combination of growth regulators (auxin and cytokinin), carbon source, and of $NO_3^-$ in the medium. For auxin optimization, the cells were cultured on 2,4-D concentrations of 1 and 2 mg/L, and NAA at concentrations of 1 and 2 mg/L. Cytokinin concentration was optimized by using 0.1 and 1.0 mg/L of kinetin. The effect of carbon source of sucrose, glucose and fructose in similar concentration (3%) was also investigated. The effect of $NO_3^-$ was conducted by decreasing 50% of $KNO_3$ in the medium. The cell suspension cultures were shaken at 100 rpm and incubated at 25°C in the dark.

### 2.3. Measurement of growth

Callus growth was expressed as dry weight (gram). Callus (0.5-0.6 g of fresh weight) was transferred to MS medium supplemented with NAA, 2,4-D and kinetin in certain combination, then weighed after 30, 60 and 90 days. Individual flasks (in duplicate) were harvested at a regular interval of 1 week and analyzed for dry weight. The biomass of cell suspension cultures was measured using packed-cell volume (PCV). The biomass (in %PCV) was calculated using the equation:

$$[V_p/V_t] \times 100\%$$

where$V_p$ is precipitate volume, and $V_t$ is total volume.

### 2.4. Determination of bilobalide and ginkgolides

Fresh callus from leaves were harvested by filtering through filter paper (70 mm, Advantec) under reduced pressure. The remaining fresh weight cells were freezed in a refrigerator -30°C overnight and then freeze-dried for three days. The dried cells were ground with a mortar and pestle. Ten mL *n*-hexane was added and the samples were sonicated for 1h. After centrifugation, the *n*-hexane solution (upper layer) separated from the cells was removed by pipetting and samples of the cells were extracted with 10 ml ethyl acetate for 2 h in a sonicator. The cell extracts were then centrifuged at 12,000 rpm for 10 min, and the supernatant (ethyl acetate solution) was obtained and concentrated using a rotary vacuum evaporator under reduced pressure. The EtOAc soluble as ginkgolide fraction was separated using preparative high performance liquid chromatogram (HPLC), and each collected sample was analyzed by gas chromatography-mass spectrometry (GC-MS).

The ginkgolide fraction was separated by HPLC performed on a reverse phase column (Shimadzu VP Shimpak ODS RP column 250 x 4.6 mm i.d., 5 µm) in a Waters equipped with a UV (ultraviolet) detector (wavelength 220 nm) by isocratic elution with methanol-water-isopropanol (17.5-72.5-10) with a flow rate of 1.0 mL/min. The samples were dissolved in 300 µLMeOH (HPLC grade) and filtered through a pre-filter (0.2 µm pore size, Advantec) before subjected to HPLC. The individual retention times of bilobalide and ginkgolides were compared with that of standards. The amount of biobalide, ginkgolides A and B in cultured cells was determined by measuring the area of the corresponding peak and comparing this value to those in a standard curve.

For the analysis by GC-MS, each collected sample was trimethylsilylated by adding 40 µL of a BSA in pyridine. This mixture was vortexed and heated for 15 min at 70°C. Analysis of the TMS derivative of each collected sample was performed with a Shimadzu GC-MS QP2010 (Shimadzu, Japan) equipped with a TC-5 capillary column (30 m, id: 0.24 mm). The carrier gas was Helium delivered at a constant rate of 1.5 mL $min^{-1}$, with a column pressure of 100 Kpa and interface temperature of 280˚C. The temperature program was 60˚C, 10˚C $min^{-1}$ to 300˚C, and 300˚C for 18 min to allow the late eluting peak to exit the column. The injection volume was 1 µL and the injector temperature was maintained at 280˚C. The GC-MS conditions consisted of 40-700 mass ranges. Comparison of mass spectrum of each collected sample with that of each standard sample (bilobalide, ginkgolides A and B) was conducted.

## 3. Results and discussion

The effect of combinations of 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, several combinations and concentrations of α-naphthalene acetic acid (NAA) + kinetin and combination of 2,4-Dichlorophenoxyacetic acid (2,4D) + kinetin were added into the supplemented MS basal medium on which leaf explants and were cultured for 90 days in the dark. Among of the combination of NAA (1.0 and 2.0 mg/L) + kinetin (0.1 mg/L), the combination of NAA (2 mg/L) + kinetin (0.1 mg/L) was found to be the best for

the induction of callus of leaf explants after 60 days (Figure 1). The highest rate of induction was 100% in 60 days. Yellowish compact calluses were obtained after explants were placed on the medium for 30 days.

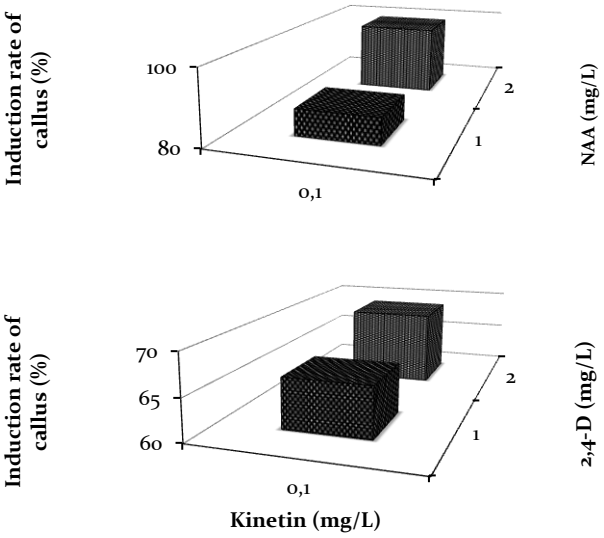


**Figure 1** Effect of combination of kinetin + NAA and kinetin + 2,4-D on the formation of callus from leaves of *G.biloba* in 60 days cultivation

Among the combination of 2,4-D + kinetin (1.0 + 0.4 and 2.0 + 0.4 mg/L), the combination of 2,4-D (2.0 mg/L) + kinetin (0.4 mg/L) was little bit higher than that of 2,4-D (1.0 mg/L) + kinetin (0.4 mg/L) after 60 day incubation. MS medium with combination of 2,4-D + kinetin was inhibited the formation of calluses.

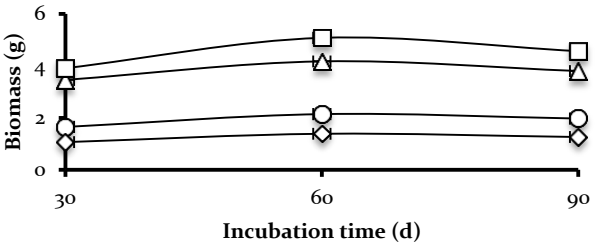


**Figure 2** Biomass of callus cultures in MS medium with addition of several combination of growth hormone

Note: △ = 0.1 mg/L Kinetin + 1 mg/L NAA,
□ = 0.1 mg/L Kinetin + 2 mg/L NAA,
◇ = 0.4 mg/L Kinetin + 1 mg/L 2,4-D,
○ = 0.4 mg/L Kinetin + 2 mg/L 2,4-D

To measure the biomass of callus, the callus was harvested at day 30, 60 and 90. The weight of dried callus was obtained by lyophilization. The callus was a compact yellowish mass during the culture period by 0-60 days, and had turned brownish by day 90. The time course of growth is shown in Figure 2. Calluscultures grew during 0-60 daysand tended to decline on day 90. Maximum fresh weight was 5.076 g, which was 1.3 fold that of the callus on 30 days. The highest dry weight was 0.668 g, 2 fold that of the initial callus. 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 [13]. 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. 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 [13].

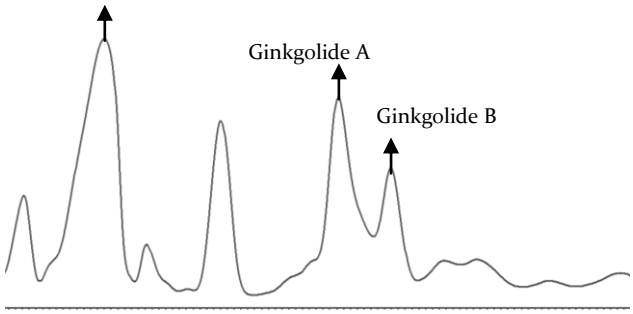


**Figure 3** HPLC profile of bilobalide, ginkgolides A and B from *G.biloba* callus culture

In order to determine the bilobalide and ginkgolides production in the cultured cells derived from the leaf of *G. biloba*, ginkgolide fraction was performed by HPLC and GC-MS analyses. The HPLC profile of bilobalide, ginkgolides A and B show the retention times of 5.947, 9.154 and 9.786 min respectively (Figure 3), which are identical to the retention time of authentic bilobalide, ginkgolides A and B. The HPLC chromatograms indicate the formation of bilobalide, ginkgolides A and B in the cultured cells.

The GC-MS patterns of the bilobalide and ginkgolides extracted from the ginkgolide fraction showed the bilobalide, ginkgolides A and B molecular weight values of [M-$CH_3$] 455, 537 and 625, respectively, after TMS derivatization, which are identical to the molecular weight values of authentic bilobalide, ginkgolides A and B (Figure 4). The production of ginkgolides in the callus cultures was identified by HPLC and GC-MS by comparing the molecular ion and the fragmentation pattern with that of authentic ginkgolides. The HPLC and GC-MS spectra strongly indicate that bilobalide, ginkgolides A and B were produced in the callus derived from the leaves of *G. biloba*.

Figure 5 shows the production of bilobalide, ginkgolides A and B in the callus cultures after addition of several growth hormones combination of NAA + kinetin. The production of bilobalide, ginkgolides A and B was found to be highest after the addition of NAA + kinetin (2.0 and 0.1 mg/L). The bilobalide, ginkgolides A and B increased 1.5, 1.3 and 1.1folds, respectively, than those of the addition of NAA + kinetin (1 and 0.1 mg/L).

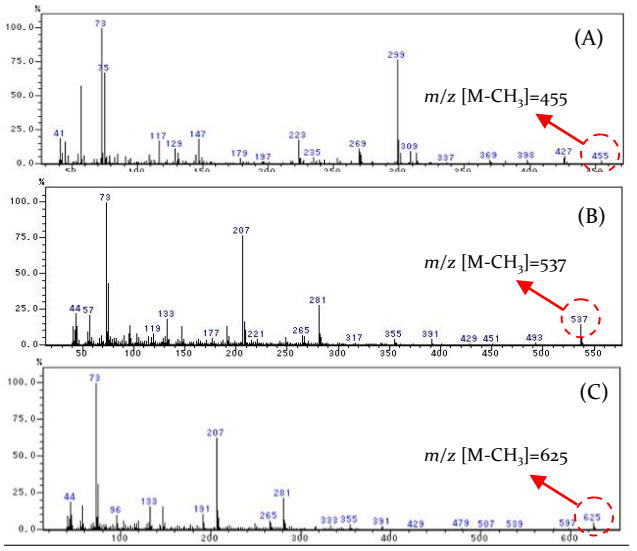


**Figure 4** GC-MS spectra of bilobalide, ginkgolides A and B after TMS derivatives of callus extract collected by preparative HPLC

Note: (A) Mass spectrum of bilobalide; (B) Mass spectrum of ginkgolide A; and (C) Mass spectrum of ginkgolide B

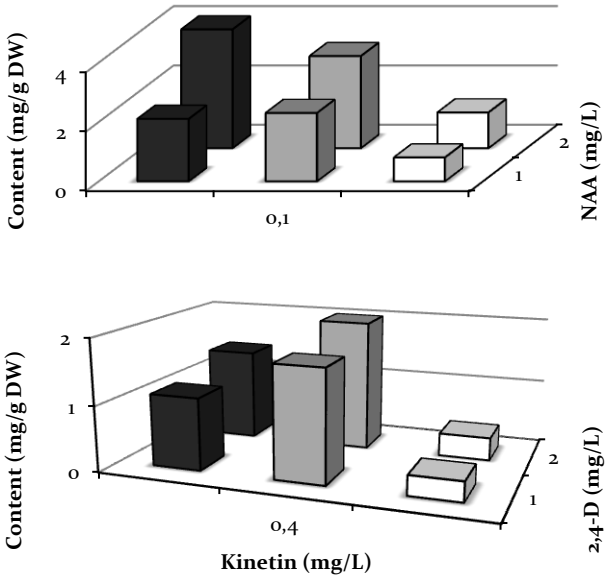


**Figure 5** Content of bilobalide, ginkgolides A and B in *G.biloba* callus cultures with combination of kinetin + NAA and kinetin + 2,4-D after 60 days incubation

Note: ■ = Bilobalide; ▒ = Ginkgolide A; □ = Ginkgolide B

Among the growth hormones combination of 2,4-D + kinetin, the production of bilobalide, ginkgolides A and B was found to be the highest after the addition of 2 mg/L 2,4-D + 0.4 mg/L kinetin. The maximum production of bilobalide, ginkgolides A and B was around 1.2, 1.2 and 1.1 folds, respectively.

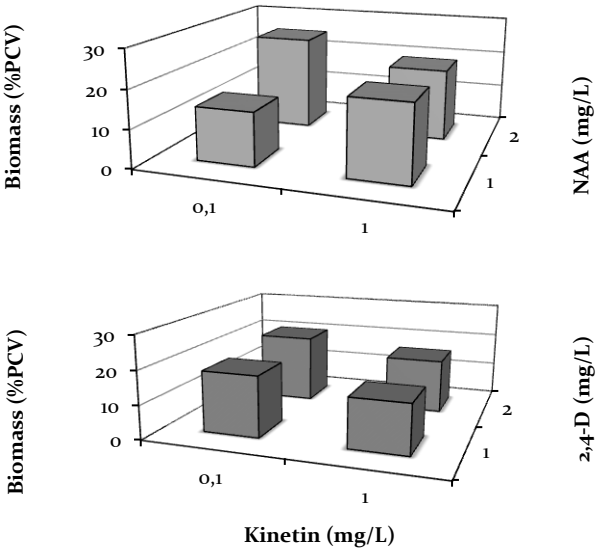


**Figure 6** Biomass amount of cell suspension cultures with combination of kinetin + NAA and kinetin + 2,4-D as hormone growth after 30 days incubation

The manipulation of physical aspects and nutritional components in in vitro cultures is one of the most fundamental approaches to optimize their productivity. *Invitro* culture conditions such as basal salt media, temperature and light irradiation have been optimized for ginkgolides and bilobalide production from *G. biloba*. Plant cell culture media widely used to support growth of cells are normally not the optimum for the production of ginkgolides and bilobalide. To enhance the biomass and the production of bilobalide, ginkgolides A and B, cell suspension culture from *G.biloba*with combinations of growth regulators after 30 days incubation was investigated. Figure 6 shows the biomass from cell suspension cultures with combination of kinetin + NAA and kinetin + 2,4-D after 30 days incubation. In terms of the types of auxin employed, NAA had the highest effect of cell growth and 2,4-D had the lowest over a concentration range from 0.1 to 2 mg/L. The hormonal concentrations for optimal cell growth (25% PCV) were 2 mg/L for NAA and 0.1 mg/L for kinetin. The optimal growth regulator for the cell growth in cell suspension cultures was consistent with that of previously reported [13][14][15].

Quantification of bilobalide and ginkgolides content in cell suspension cultures with several combination of kinetin + NAA and kinetin + 2,4-D was investigated by HPLC analysis as shown in Figure 7. Among several growth hormone combinations, the combination of 0.1 mg/L kinetin + 2mg/L NAA was found to be the best. The production of bilobalide dramatically increased around 2 folds in combination of 0.1 mg/L kinetin + 2 mg/L NAA, whereas the production of ginkgolides A and B increased slightly compared with another treatment. This result

indicates that the supplemented MS basal medium with 2 mg/L of NAA and 0.1 mg/L of kinetin seems to be quite suitable for both callus and cell suspension cultures in producing the bilobalide, ginkgolides A and B.

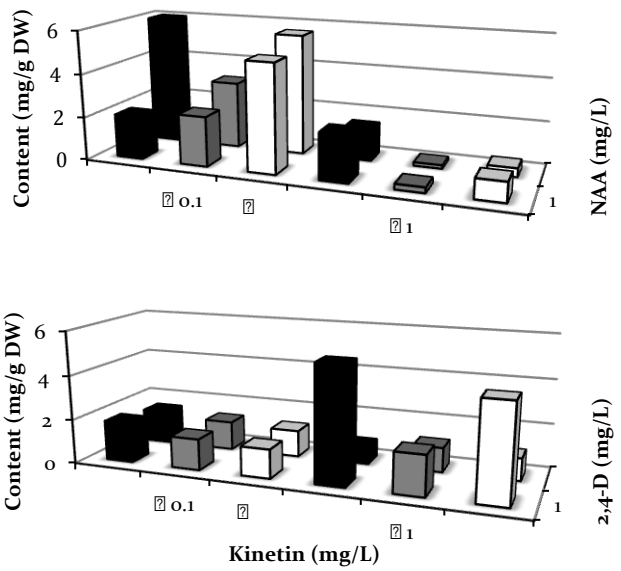


**Figure 7** Content of bilobalide, ginkgolides A and B in cell suspension cultures with combination of kinetin + NAA and kinetin + 2,4-D as hormone growth after 30 days incubation

Note: = Bilobalide; = Ginkgolide A; = Ginkgolide B

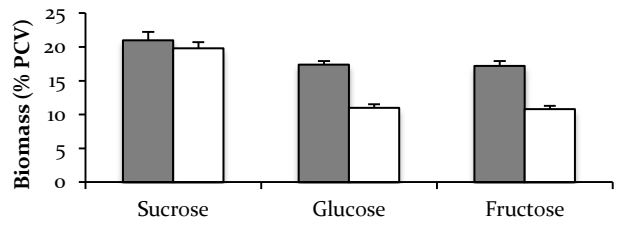


**Figure 8** Biomass of cell suspension cultures with the combination of $NO_3^-$ ratio and carbon source after 30 days incubation

Note: = 3.8% $KNO_3$; = 1.9% $KNO_3$

In order to determine the effect of carbon source and $NO_3^-$ in MS medium for the production of bilobalide, ginkgolides A and B, several carbon sources such as sucrose, glucose and fructose in combination with the decreasing of $KNO_3$ were used as shown in Figure 8. Sucrose was found to be the best as the carbon source in MS medium. The decreasing of $KNO_3$ also decreased the biomass of cells in all treatments. Maximal growth of the cells was achieved in sucrose concentration ranges from 30 g/L to 40 g/L, and the biomass increased with the increasing of nitrate ion until the molar ratio of ammonium/nitrate ion 1:5 [13].

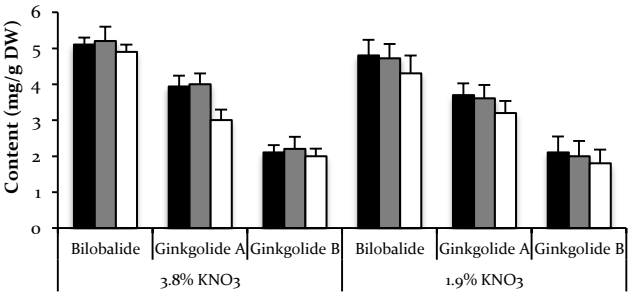


**Figure 9** Content of bilobalide, ginkgolides A and B in cell suspension cultures with the combination of $NO_3^-$ ratio and carbon source 30 days incubation

Note: = Sucrose; = Glucose; = Fructose

The nitrogen source, supplied as $NO_3^-$, greatly influenced the production of bilobalide, ginkgolides A and B. Figure 9 shows the production of bilobalide, ginkgolides A and B in cell suspension culture with the decreasing of $KNO_3$ and combination of carbon source after 30 days incubation. Production of bilobalide, ginkgolides A and B with the addition of glucose was higher than that of sucrose and fructose. Reducing 50% of $KNO_3$ in the medium slightly decreased the content of bilobalide, ginkgolides A and B. These results agree with those previously reported where the decreasing of nitrate ion caused a decline in ginkgolides A and B production [14].

## 4. Conclusions

This study shows that the combination of 0.1 mg/L kinetin + 2 mg/L NAA was found to be the best for the induction of callus. The highest biomass and production of bilobalide, ginkgolides A and B were found in this combination after 60 days incubation. The bilobalide, ginkgolides A and B increased 1.5, 1.3 and 1.1 times, respectively, than those of other growth hormone combinations. Treatment with 3% sucrose resulted in the highest biomass compared with glucose and fructose. However, the production of bilobalide, ginkgolides A and B in the treatment of glucose was higher than that of sucrose and fructose. Reducing 50% of $KNO_3$ slightly decreased the biomass and the content of bilobalide, ginkgolides A and B. However, the production of bilobalide, ginkgolides A and B with 3% sucrose were higher compared with glucose and fructose.

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