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Production of Flavonoids and Terpene Lactones from Optimized *Ginkgo biloba* Tissue Culture

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Abstract

This study investigated the effects of various culture conditions on the growth and the production of flavonoids and terpene lactones in the callus of *Ginkgo biloba*. Callus induced from embryos displayed distinct morphological and physiological responses. MS medium with different plant growth regulators showed a significant effect on the quality and growth of callus. The optimal medium for inducing embryo-derived callus was MS with 2.0 mg/L naphthalene acetic acid (NAA) and 2.0 mg/L 6-benzylaminopurine (6-BA), and the culture medium MS+NAA (2.0 mg/L)+6-BA (1.0 mg/L) was better for the subculture of callus than other culture media tested in this study. In addition, both plant growth regulators and subculturing cycle strongly influenced the production of flavonoids and terpene lactones in the callus. The best subculturing cycle and the optimum culture medium for production of flavonoids and terpene lactones was 30 d and MS+NAA (2.0 mg/L)+6-BA (1.0 mg/L), respectively. These findings provided an important technical support for obtaining the callus cell line from *G. biloba* embryo that is the richest in flavonoids and terpene lactones.

Keywords: callus, culture medium, flavonoids, *Ginkgo biloba,* subculturing cycle, terpene lactones

Introduction

Ginkgo biloba L., also called maidenhair tree, is a deciduous gymnosperm that is valued in China. As one of the oldest tree species in the world, *G. biloba* enjoys the unique reputation of being a 'living fossil' (Bilia, 2002). *G. biloba* has great ornamental and medicinal value. The *Ginkgo* extract EGB761 contains two important active pharmaceutical components, flavonoids and terpene lactones (Van Beek and Montoro, 2009), which can promote blood circulation and cerebral metabolism. These compounds make EGB761 as an effective treatment for cerebrovascular diseases, such as coronary heart disease and high blood pressure (Mahadevan and Park, 2008; Lu *et al*., 2011; Nakanishi, 2005). However, the contents of flavonoids and terpene lactones are quite low in *Ginkgo* grown under natural conditions. In addition, the extraction procedure for these compounds is complicated. The development and utilization of the pharmaceutical ingredients of *Gingko* are greatly limited by the place where it grows and seasons (Camper *et al*., 1997). Therefore, industrialized production of flavonoids and terpene lactones through tissue culture is a significant direction in the study of *Ginkgo* (Sun *et al*., 2011).

In recent years, many studies on inducing the callus of *Ginkgo* and cell suspension culture have been conducted. Great progress has been achieved in cultivating *Ginkgo* organs and tissues for the production of secondary metabolites. Chen *et al.* (1997) have proposed the best culture medium and explants for inducing callus and producing flavonoids from the leaf, stem and root in *Ginkgo*. Kim *et al*. (1998, 1999) and Hao *et al*. (2009, 2010) have also reported that several exogenous inducing factors, including fungus, low temperature, ultraviolet light, abscisic acid, and heavy metal ions, can promote the accumulation of flavonoids in *Ginkgo* cell suspension cultures or callus tissue. Research has also been conducted on the *in vitro* culture of *Ginkgo* tissues for the production of terpene lactones. Carrier *et al*. (1991) first found ginkgolide A and traces of ginkgolide B in *G. biloba* suspension culture extracts. Subsequently, a more constant production of ginkgolide A, compared with intact trees, has been achieved using petiole-derived *G. biloba* cell cultures (Park *et al*., 2004). Recently, several biotic and abiotic elicitors have been used to increase the accumulation of bilobalide and ginkgolides in *G. biloba* cell suspensions (Kang *et al*., 2006a, 2009). Many studies have focused on producing flavonoids or terpene lactones respectively using *Ginkgo* tissue culture, little is known about the cultivation factors affecting both of

flavonoid and terpene lactone contents. Specifically, the effects of hormone variety and concentration on both of flavonoid and terpene lactone contents remain unclear. Therefore, the present study used *Ginkgo* embryo as the explant and investigated the effects of different hormone combinations on callus induction, subculture, and flavonoid and terpene lactone contents in the callus. This study aims to provide a theoretical foundation and optimum material for the fast and efficient production of flavonoids and terpene lactones from *Ginkgo* by selecting the callus cell line that is richest in these compounds.

Materials and methods

Plant materials

Seeds of *G. biloba* were harvested in China (Botanical Garden of Yangtze University, Jingzhou) in September and kept at 4 °C. The seeds were used after 2 and 5 months of storage when the embryos were at the cotyledonary stage. The seed surface was sterilized with 70% (v/v) ethanol for 1 min, followed by 3% (v/v) sodium hypochlorite for 15 min, and then rinsed six times with sterile distilled water. The embryo was separated from sterilized seed with sterile knife and forceps, cut longitudinally and then used for inducing callus.

Tissue Culture Conditions

The embryos were excised and transferred to the (MS) solid medium (pH 5.8) supplemented with 3% (w/v) sucrose, 0.3 (w/v) gelrite and two various concentrations of NAA and 6-BA (Murashige and Skoog, 1962). The concentration combinations of NAA and 6-BA were shown in Tab. 1. The cultures were incubated in the light (100 μmol $m^{-2} s^{-1}$) with a 16/8 h light/dark photoperiod at 24 ± 1 °C, and then subcultured after every 5 weeks. Callus induction including weight, colour and percentage of explants producing callus were recorded after 5 weeks.

Tab. 1. Different hormone concentration combination of MS medium

Culture medium Code	NAA (mg/L)	6-BA (mg/L)
A1	1.0	0.5
A2	1.0	1.0
A3	1.0	2.0
B1	2.0	0.5
B2	2.0	1.0
B3	2.0	2.0
C1	3.0	0.5
C2	3.0	1.0
C3	3.0	2.0

Extraction and Determination of Flavonoids and Terpene Lactones

The collected callus was immediately dried for 72 h at 40 °C and then ground into powder liquid nitrogen. The powder (0.1 g) of dried ginkgo callus was dissolved in 40 mL acidulated methanol by sonication at 70 °C for 90 min. The sample solution was then filtered through 0.2 μm filter membrane (Millipore, Nylon) filters for HPLC analysis. Flavonoid contents were quantified by HPLC using a previously described method (Xu *et al*., 2012). Quercetin, kaempferol, and isorhamnetin were selected as standard samples because many different flavonol glycosides in *G. biloba* are derivatives of these three flavonol aglycones. Flavonoid content was calculated by multiplying the total content of quercetin, kaempferol, and isorhamnetin (supplemental Tab. S1) by 2.51 (Van Beek, 2002) and expressed as percents of DW percentages. Ginkgolide A (GA), ginkgolide B (GB), ginkgolide C (GC) and bilobalide (BB) were extracted and quantified by gas chromatography with a wide bore capillary column (Liao *et al*., 2008). The content of terpene lactones was the sum of the contents of GA, GB, GC, and BB (supplemental Tab. S2) and expressed as DW percentages. All the tests were carried out in triplicate, and data represent the means ± standard errors (SE).

Statistical analysis

Data were analysed with one-way ANOVA using SPSS 11.0 (SPSS Inc., Chicago, Illinois) for Windows and means were compared with Duncan's multiple range test at $P \leq 0.05$.

Results

Effect of Different Concentrations of Naphthalene Acetic Acid (NAA) and 6-Benzylaminopurine (6-BA) on Callus Induction

The *Ginkgo* embryo was inoculated in a culture medium with different concentrations of NAA and 6-BA. A callus appeared in some explants after one week, and the shape of the callus was quite noticeable after 2 weeks. The callus (Fig. 1A) induced by the embryo was yellow green and had better dispersion and resistance to browning than the embryoidal cells that developed into dark green callus (Fig. 1B) with dark green. Our results showed that different culture media had different effects on the efficiency of callogenesis and callus quality. The efficiency of callogenesis and growth increment of callus after 3 weeks are shown in Tab. 2. The callus induced in culture medium B3 (MS + NAA 2.0 mg/L + 6-BA 2.0 mg/L) had the highest efficiency, maximum growth increment, greatest quantity, and best effect of yellow green and granulation with significant difference ($P \leq 0.05$) than those from other culture media. Thus, culture medium B3 is considered the best culture medium for inducing callus from *Ginkgo* embryo in this study.

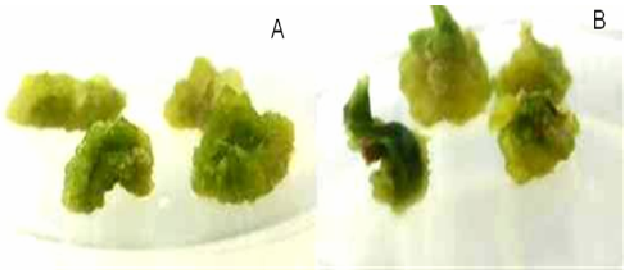

Fig. 1. The callus (A) derived from embryo and embryoidal cells developed into callus (B) of *Ginkgo biloba*

Effect of Different Concentrations of NAA and 6-BA on the Successive Growth of Callus

The calluses in culture media B2 and B3 at 2.0 mg/L NAA showed the best growth, with light yellow green color, loose texture, and obvious granulation (Fig. 2A). Most of the calluses in culture media C1, C2, and C3 at 3.0 mg/L NAA were light green with loose texture and obvious granulation, whereas some were dark green with compact texture and vigorous growth (Fig. 2B). Most of the calluses cultured in media A1, A2, and A3 were slightly green, whereas some were dark green with compact texture (Fig. 2C). Overall, the results showed that culture medium B2 (MS + NAA 2.0 mg/L + 6-BA 1.0 mg/L) is the best medium for callus subculture.

Fig. 2. The growth of *Ginkgo* callus state after subculture on different culture medium. (A) The *Ginkgo* callus on culture medium B. (B) The *Ginkgo* callus on culture medium C. (C) The Ginkgo callus on culture medium A

The vigorous callus in culture medium B3 was selected for subculture in the culture media with different concentrations of hormones. The weight of the callus was recorded on the 15th, 30th and 45th days. The growth of the calluses in the different concentrations of hormones increased as the subculture period was prolonged (Fig. 3). The callus in culture medium B2 had the highest and significant higher ($P \leq 0.05$) dry weight on the 15th, 30th, and 45th days after the subculture than those calluses in other culture media. The results indicated that the culture medium B2 is the best subculture medium in this experiment.

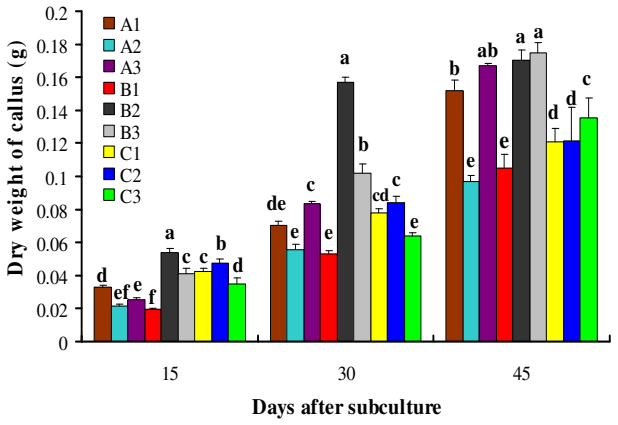

Fig. 3. The dry weight of *Ginkgo* callus after subculture on different culture media. Values are the mean of nine callus samples and bars represent standard errors. Means with the different letters are significantly difference at $P \leq 0.05$ by Duncan's multiple rang test

Effect of Different Concentrations of NAA and 6-BA on the Contents of Flavonoids and Terpene Lactones

The flavonoid content of the callus in the different culture media, except for culture medium C1, increased as the subculturing cycle was prolonged. After subculturing, the flavonoid content in the callus cultivated in medium B2 was significantly ($P \leq 0.05$) higher than that in the callus cultivated in the other media and peaked on the 45th day (1.25%, Fig. 4). Unlike the changes in flavonoid content, the terpene lactone content of the callus grown in each culture medium initially increased and then decreased after subculturing (Fig. 5). The terpene lactone content was the highest in culture medium B2, peaked on the 30th day at 0.078%, which was significantly ($P \leq 0.05$) higher than the terpene lactone contents in the other calluses. In terms of flavonoid and terpene lactone contents, the optimal subculture medium is B2 (MS + NAA: 2.0 mg/L + 6-BA 1.0 mg/L) and the best subculturing cycle is 30 d.

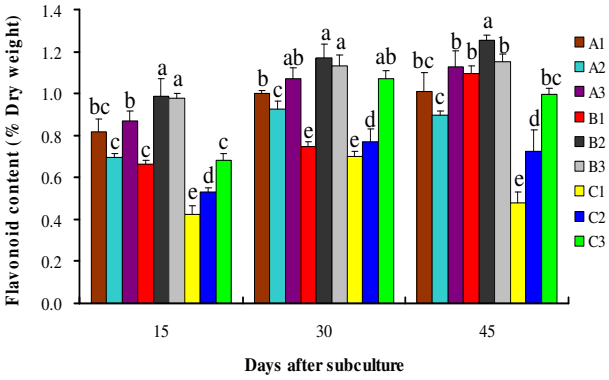

Fig. 4. The flavonoid content of *Ginkgo* callus after subculture on different culture media. Values are the mean of nine callus samples and bars represent standard errors. Means with the different letters are significantly difference at $P \leq 0.05$ by Duncan's multiple rang test

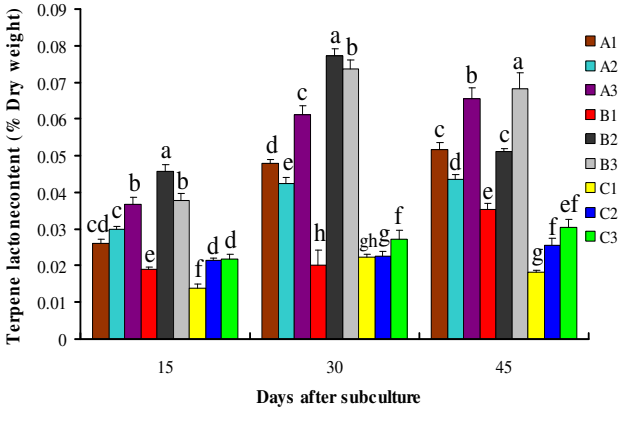

Fig. 5. The terpene lactone content of *Ginkgo* callus after subculture on different culture media. Values are the mean of nine callus samples and bars represent standard errors. Means with the different letters are significantly difference at $P \leq 0.05$ by Duncan's multiple rang test

Discussion

In this study, the embryo-derived callus of *Gingko* was treated with different concentrations of NAA and 6-BA. Comparison of the induction of the *Gingko* callus in each culture medium showed that the growth indices (including growth, vitality, color and granulation) of the callus was better in the culture medium composed of MS+NAA 2.0 mg/L + 6-BA 2.0 mg/L than in the other culture media. Thus, the culture medium composed of MS+NAA 2.0 mg/L+ 6-BA 2.0 mg/L may be considered optimal for inducing embryo-derived callus. The flavonoid and terpene lactone contents in the callus gradually increased as the subculture period was prolonged. This process is related to the growth of the callus being accelerated by the concentration of hormones (Fang *et al*., 2006; Sabater-Jara *et al*., 2013). The results also indicate that the best subculturing cycle is 30 d.

Numerous studies have demonstrated that exogenous hormones can regulate the accumulation of endogenous hormones and then induce differentiation in explants. Chen *et al*. (1997) showed that different combinations of hormones can affect the biological yield of leaf-derived callus. The present study showed that the interaction between NAA and 6-BA can influence callus production. High concentrations of 6-BA can increase the biological yield of embryo-derived callus when the concentration of NAA is 2 mg/L after 30 d of subculturing. Previous studies indicated that different exogenous hormones can affect secondary metabolites (Camper *et al*., 1997). In the present study, we found that the MS culture medium added with 2 mg/L NAA and 1 mg/L 6-BA has a greater effect on the accumulation of flavonoids and terpene lactones in the callus from ginkgo embryo compared with the other culture media. Our results showed that the concentration of NAA has a greater influence on the accumulation of secondary metabolites than that of 6-BA. Excessively high or low concentrations of NAA decrease the production of flavonoids and terpene lactones. High concentrations of NAA reduce the production of secondary metabolites to a greater degree than low concentrations of NAA. This result could be attributed to the fact that high concentrations of auxin inhibit the production of flavonoids and terpene lactones (Sun *et al*., 2011). Kim *et al*. (1999) reported that the content of flavonoids in the callus decreased as the biological production of callus increased. However, the present results showed that the flavonoid content increased and the terpene lactone content decreased as the production of callus increased. This finding may be caused by the different phytohormone concentrations or culture conditions.

Accumulated evidence suggested that the calluses induced from different *Ginkgo* explants might possess different contents of flavonoids and terpene lactones. For instance, Chen *et al*. (1997) demonstrated that the leaf-derived callus possessed the highest content of flavonoids. Subsequently, the flavonoid content of stem-derived calluses is significantly higher than that of the cotyledon-derived calluses. The flavonoid content in embryo-derived callus in the present study (1.254% highest in subculture medium on 45 d) is lower than that in the leaf-derived callus of *G. biloba* studied by Chen *et al*. (1997) [1.510% of dry weight (DW)], similar to that in the leaf-derived callus studied by Hao *et al*. (2009, 1.245% DW) and higher than that in the leaf-derived callus studied by Jiang *et al*. (2000, 0.050% DW). In fact, the leaf-derived calluses in the above-mentioned studies were obtained using different culture media and exogenous hormones. Therefore, the synergistic effect of explants and culture medium are effective in the production of secondary metabolites of *Ginkgo* callus. In terms of terpene lactones, most studies have investigated the effect of various elicitors on the production of bilobalide and ginkgolides in *G. biloba* cell cultures (Sabater-Jara *et al*., 2013). In spite of this, Boonkaew (2001) studied the effect of the source of explant and culture medium on the production of terpene lactones in *G. biloba* callus. The results showed that the total ginkgolide and bilobalide content in the root-derived callus was higher than that in the leaf-derived callus when grown on MSMO media. Although the highest terpene lactone content in the embryo-derived callus (0.077% DW) in the present study was higher than that in the root-derived (0.053% DW) and leaf-derived calluses (0.038% DW) studied by Boonkaew (2001) and Balz *et al*. (1999), respectively, but it was generally lower than that in the cell cultures studied by Laurain *et al*. (1997), Park *et al*. (2004) and Kang *et al*. (2006a, 2006b, 2010). These results indicated that the method of tissue culture is a crucial factor in accumulation of terpene lactone in *G. biloba* cultures.

Tab. 2. Effect of different growth regulators on callus induction and growth from *Ginkgo* embryo. Values of growth increment are the mean of nine callus samples and bars represent standard errors

Medium Code	% explants producing callus	Growth increment (cm)		
		<0.2	0.2-0.5	>0.5
A1	55.6	3.24 ± 0.19 c	1.50 ± 0.31 f	0
A2	58.3	2.56 ± 0.57 cd	4.35 ± 0.28 c	0.18 ± 0.02 e
A3	63.9	2.60 ± 0.21 cd	5.38 ± 0.35 b	0
B1	79.2	3.96 ± 0.72 b	3.72 ± 0.23 d	0.51 ± 0.03 d
B2	84.7	4.15 ± 0.55 ab	6.57 ± 0.36 b	1.27 ± 0.07 b
B3	94.4	2.93 ± 0.30 c	11.84 ± 0.41 a	2.50 ± 0.14 a
C1	83.3	4.42 ± 0.19 a	3.86 ± 0.60 d	0.85 ± 0.12 c
C2	80.6	1.55 ± 0.24 e	3.10 ± 0.49 e	0
C3	81.9	2.29 ± 0.15 d	3.06 ± 0.33 e	0

The study of the molecular biology involved in the biosynthetic pathway of flavonoids and terpene lactones in *Gingko* has made breakthroughs in recent years. Many key enzyme genes of flavonoids and terpene lactones have been isolated and identified from *Gingko*. Our group and Tang's group have cloned and characterized several genes involved in the flavonoid biosynthetic pathway. These genes include phenylalanine ammonia-lyase (Xu *et al*., 2008a), chaconne synthase (Pang *et al*., 2005; Xu *et al*., 2007), flavanone 3-hydroxylase (Shen *et al*., 2006), chalcone isomerase (Cheng *et al*., 2011), flavonol synthase (Xu *et al*., 2012), dihydroflavonol-4-reductase (Hua *et al*., 2013), and anthocyanidin synthase (Xu *et al*., 2008b). Besides, most of genes related to the biosynthetic pathway of terpene lactones have also been isolated and identified. These genes

include levopimaradiene synthase (Schepmann *et al*., 2001), mevalonate diphosphate (Pang *et al*., 2006), 1-deoxy-D-xylulose 5-phosphate synthase (Kim *et al*., 2006a), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Kim *et al*., 2006a), 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase (Kim and Kim, 2010), 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase (Kim *et al*., 2008a), 2-*C*-methyl-D-erythritiol 4-phosphate cytidyltransferase (Kim *et al*., 2006b), 2-*C*-methyl-D-erythritiol 2,4-cyclodiphosphate synthase (Kim *et al*., 2006c), and 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase (Kim *et al*., 2008b). The time and spatial expression patterns of certain key genes indicated that the expression level of these key genes had significantly positive correlation with the flavonoid or terpene lactone contents of *Gingko*. However, these key genes have not been used in the genetic transformation of *Gingko*. A callus culturing system was established in this study, which provides important technical support for the genetic transformation of *Gingko* embryo for key genes involved in secondary metabolism, inducing transgenic callus, and then obtaining the callus cell line, the richest in flavonoids and terpene lactones.

Conclusions

This study showed that the optimal media for induced *G. biloba* embryo callus and subculture of callus were MS + NAA 2.0 mg/L + 6-BA 2.0 mg/L, and culture medium MS + NAA 2.0mg/L + 6-BA 1.0 mg/L, respectively. The flavonoid and terpene lactone contents in the callus gradually increased as the subculture period was prolonged. However, the best subculturing cycle and the optimum culture medium for production of flavonoids and terpene lactones were 30 d, and MS+NAA (2.0 mg/L) + 6-BA (1.0 mg/L), respectively. The protocol in the present work may have valuable application for obtaining the callus cell line from *G. biloba* embryo that is the richest in flavonoids and terpene lactones.

Acknowledgments

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