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PRODUCTION OF GINKGOLIDE AND BILOBALIDE IN TRANSFORMED AND GAMETOPHYTE DERIVED CELL CULTURES OF *GINKGO BILOBA*

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Key Word Index—*Ginkgo biloba*; Ginkgoaceae; ginkgolide; bilobalide; cell cultures; *Agrobacterium rhizogenes*.

Abstract—Ginkgolide and bilobalide were detected by HPLC analysis in cell cultures established from various *G. biloba* explants. These secondary metabolites of pharmacological interest were found to occur in concentrations of 0.065 and 0.087% (dry weight) in two cell suspensions. One was derived from a female prothallus and the other one from putatively transformed embryos, by transfection via *Agrobacterium rhizogenes* agropine type strain CFBP 2409 (A4). © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The root bark and leaves of *Ginkgo biloba* L. contain diterpenoids (ginkgolides) and a sesquiterpenoid (bilobalide) [1, 2]. These secondary metabolites possess interesting pharmacological properties [3, 4]. In particular, ginkgolides are potent and selective antagonists of platelet activating factor (PAF) [5] and are at least partially responsible for the medicinal properties of *Ginkgo* extracts.

Some studies have been made of undifferentiated cell cultures of *Ginkgo biloba* with the aim of producing ginkgolides *in vitro* [6–8]. However, in these cultures, the terpenoid content was either not detectable or only small amounts of ginkgolide B were found. On the other hand, differentiated tissue cultures, especially hairy root cultures, could offer an attractive alternative means for the production of secondary metabolites that are associated with cellular differentiation [9].

The present paper reports on the terpenoid accumulating capacities in cell suspensions established from various explants of *G. biloba* [10–12]. In order to determine the production of these secondary metabolites, the ginkgolide and bilobalide profiles of suspensions derived from normal and putatively transformed zygotic embryos and two cell lines derived from gametophytic explants were compared by HPLC analysis.

RESULTS AND DISCUSSION

Transformation of Ginkgo biloba

Fifteen days after cutting off the roots from the mature zygotic embryos, one or two new roots developed on the control embryos while 2–10 short roots appeared at the site of the inoculation with *A. rhizogenes* (A4). Primary roots, as defined by Petit *et al.* [13], were cut off and transferred to shaken MT/2 liquid hormone-free medium in the presence of cefotaxime. This antibiotic substance was used in order to eliminate possible interference from bacteria. In the culture medium cells were released from small calli growing all along the 'transformed' roots which let to an albino cell suspension. In contrast, the control roots excised from normal embryos did not show similar cellular proliferation. The presence of opines, such as agropine and mannopine was detected by high voltage paper electrophoresis and showed that 'transformed' cells had most likely integrated T_R-DNA carrying genes responsible for opine synthesis. The opines, compounds never detected in normal cells, are specific biochemical markers for the transformation [13]. So, *Ginkgo biloba* showed a susceptibility to an agropine type *A. rhizogenes* strain, nevertheless no hairy roots appeared.

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Only cellular formations resembling primordium roots were observed on the calli. However, Petit *et al.* [13] reported that inoculations with the strain A4 induced tumours on *Kalanchoë tubiflora* from which roots emerged. In the same way the strain A4 inoculated to the *Allocasuarina verticillata* tree induced the formation of a small callus on which roots appeared later [14].

The ginkgolide and bilobalide production from cell cultures

In the present research four types of *Ginkgo biloba* cell cultures were analyzed: (A) cells 'transformed' by *A. rhizogenes*, (B) cell suspension derived from a prothallus, (c) callus from a zygotic embryo and (D) cell suspension derived from microspores. These cultures were cultured for 6–12 months prior to analysis of the secondary metabolites. Ginkgolide and bilobalide contents (μg g dry weight of cells^{-1}) are presented in Table 1 and in HPLC chromatograms (Fig. 1). Thus, 'transformed' cultures and a cell suspension from prothallus produced these secondary metabolites in 0.065 and 0.087% yield, respectively. Bilobalide was more concentrated in the 'transformed' cultures, while contents of ginkgolides A, B and C were higher in the culture derived from prothallus. In contrast, no ginkgolides and bilobalide could be detected in the microspore-derived cell suspension and in calli from immature zygotic embryos.

The data presented here show a higher ginkgolide content than in the unorganized *G. biloba* cultures studied by Carrier *et al.* [6], Huh and Staba [7], and Jeon *et al.* [8]. In fact, Carrier *et al.* [6] showed that embryo-derived callus cultures did not produce terpene trilactones in sufficient amounts to be detectable by HPLC-RI. In spite of this, the regeneration of plantlets from microspore derived clusters could lead possibly to biochemical variants possessing high ginkgolide accumulating capacities. In *Datura innoxia* Mill, through the androgenesis pathway, some plantlets were obtained and these showed variations in their leaf alkaloid contents [16].

Many factors could explain these differences. In fact, the tissue cultures were initiated from four explants of different origin. These first results show that cultures putatively transformed by the strain A4 and the cell suspension from a prothallus of *G. biloba* could be an interesting source of ginkgolides and bilobalide. Hairy-roots can produce the same secondary metabolites as those synthesized by intact parent plants, sometimes in similar yields as for example in

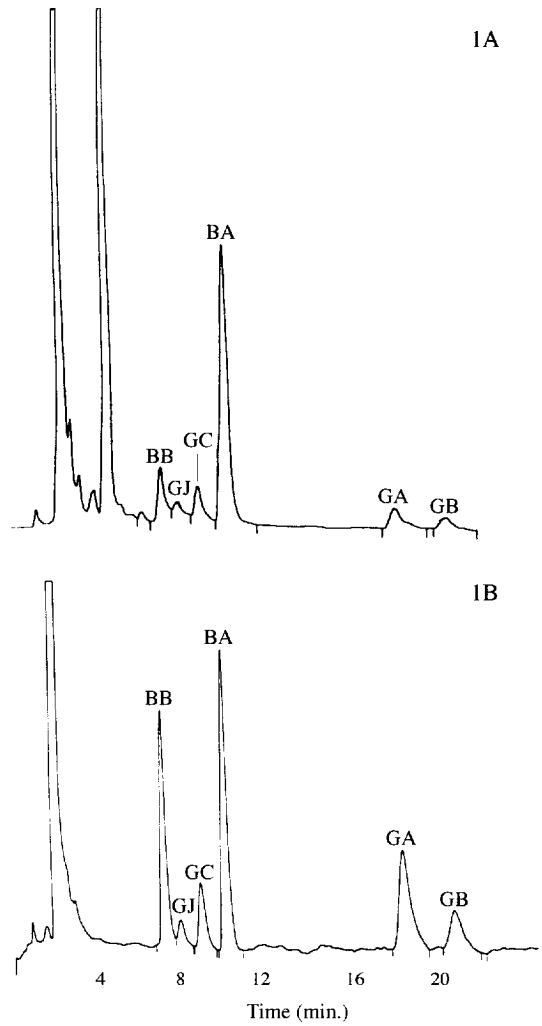

Fig. 1. (A) HPLC-RI trace of a purified extract of 1 g of lyophilized 'transformed' cell suspension. BB, bilobalide; GJ, ginkgolide J; GC, ginkgolide C; BA, benzyl alcohol; GA, ginkgolide A; GB, ginkgolide B. (B) HPLC-RI trace of standard mix of ginkgolides A, B, C and J, bilobalide and benzyl alcohol (internal standard).

Table 1. Ginkgolides (G) A, B, C and J and bilobalide (BB) content in *Ginkgo biloba* tissue cultures

Origin of tissue culture	Concentration in μg g dry tissue culture^{-1}					Total (% dry wt)
	BB	GJ	GC	GA	GB	
'Transformed' tissue culture	200±8	87±17	137±9	147±5	83±5	0.065
Female prothalli	160±3	70±14	213±5	270±1	160±1	0.087
Microspores	—	—	—	—	—	—
Immature zygotic embryos	—	—	—	—	—	—

—: not detectable.

Each tissue culture of *Ginkgo biloba* was extracted once or twice depending on the available amount. Each extract obtained was injected three times for HPLC analysis.

Catharanthus trichophyllus [17] or even in higher yields [18]. The ginkgolide and bilobalide content in the 'transformed' cells from *Ginkgo* roots was found to be higher (0.065%) (Table 1) than in roots of 3-month-old seedlings controls (0.021%) [7]. By comparison with some batches of *G. biloba* leaves, the ginkgolide content in 'transformed' cultures was similar to that found in leaves from an average tree. In contrast, the bilobalide content (Table 1) was relatively low [19].

These first results indicate the ginkgolide and bilobalide synthesis by various ginkgo cell cultures varies with the nature of the initial explant and that transformation with *Agrobacterium rhizogenes* A4 is favourable for the production of *Ginkgo biloba* terpene trilactones. It is known that the synthesis of secondary metabolites is related to the cell physiological state which is affected by the regulation of the environmental culture [9]. In view of the optimization of the ginkgolide accumulation, it will be necessary to know the most suitable interval of time for subculturing cell suspensions. The literature reports that the subculture regime seems to be crucial for increasing both growth and metabolite production. Likewise, it will be interesting to verify whether the ginkgolide production is changed during a long period of culture. In fact, the loss of secondary metabolite accumulating capacities in some cell suspension cultures maintained during long periods of subculturing has been reported by several authors [20]. However, it is possible to recover the metabolite production ability by a cloning process [20]. Furthermore, studies on the influence of culture conditions on the ginkgolide and bilobalide production in the genetically transformed cultures and cell suspension derived from prothallus are obviously indicated.

EXPERIMENTAL

'Transformed' suspension cultures. Agrobacterium rhizogenes agropine type strain CFBP 2409 or A4 was used (L. Gardan, INRA, Angers, France). This strain grew at 28° on the solid LPGA medium (5 g l^{-1} biogelytone, 5 g l^{-1} of yeast extract, 10 g l^{-1} of glucose and 15 g l^{-1} of bacto-agar). The Ri plasmid of agropine type strain contains two independently transferred T-DNA regions (T_L and T_R) which can be integrated in the plant genome. The genes that encode for the opine synthesis are located on the T_R-DNA [14].

'Transformed' cultures were induced from *G. biloba* mature zygotic embryos which were carefully taken out of ovules harvested in China. These mature zygotic embryos, after cutting off their roots with a sterile scalpel, were cultured on Murashige and Tucker solid medium [21] (MT) supplemented with sucrose 30 g l^{-1} and agar 10 g l^{-1}. A freshly grown *A. rhizogenes* strain was used to inoculate the wounded mature zygotic embryos. Then, the cocultures were plated at 28° in the dark for 72 hr. As control intact embryos were cultured under the conditions previously described. Three months after the bacterial inoculation some roots that had developed from the wound area were cut and then cultured in the dark at 24±1° in Erlenmeyer flasks containing 50 ml of modified Murashige and Tucker [21] liquid medium, termed MT/2 containing half-strength mineral salts of MT and 350 μg ml^{-1} cefotaxime. (Claforan, Roussel Laboratories, France). From these roots, some proliferating calli permitted to initiate cell suspensions which grew routinely in agitated (75 rpm) MT/2 liquid medium at 24±1° in the dark. In liquid medium, all cell suspensions were subcultured at weekly intervals and after four subcultures the cefotaxime was omitted.

From bacteria-free transformed calli, the opines (i.e. mannopine, agropine) were extracted and analysed by high voltage paper electrophoresis and detection with alkaline silver-nitrate staining according to the method described by Petit *et al.* [13]. Non-transformed callus was treated in parallel as controls.

Cell suspension derived from female prothallus. Mature prothalli (i.e. megagamethophytes) were taken out of the ovules sterilized as described by Laurain *et al.* [10], each cut into half, and placed on various MT solid media supplemented with sucrose and naphthaleneacetic acid (NAA) (2 mg l^{-1}) associated with kinetine (KIN) (0.2 mg l^{-1}) or 6-benzyladenine (BA) (2 mg l^{-1}). After 2 months of culture, a callus was subcultured in an Erlenmeyer flask containing 100 ml of MT solid medium supplemented with 2 mg l^{-1} NAA and 0.2 mg l^{-1} KIN, kept at 24±1° in the dark and subcultured at monthly intervals. On 15 November 1993 a cell suspension was initiated by introducing 0.65 g of friable embryogenic prothallus derived callus into 25 ml of agitated MT liquid medium (75 rpm) in the dark at 24±1° and subcultured every 14 days.

Cell suspension derived from microspores. A white cell suspension was established from 0.92 g of embryogenic clusters, obtained from uninucleate microspores of *G. biloba* as described in a previous report [10], and introduced into 25 ml of MT liquid medium supplemented with NAA (2 mg l^{-1})+KIN (0.2 mg l^{-1}). The suspension was shaken at 75 rpm and stored at 24±1° in the dark. Cell suspensions were subcultured in the same way as the primary culture and transferred at 14 day intervals [12].

Calli issued from immature zygotic embryos. Calli, of which the obtaining from immature zygotic embryos has been described previously [11], were cultured on the MT medium supplemented with NAA (2 mg l^{-1}) and KIN (0.2 mg l^{-1}) with a light–dark cycle of 16:8.

Standards. Ginkgolides A, B and C and bilobalide were extracted and purified in our own laboratory (Wageningen, The Netherlands) starting from commercially available standardized *Ginkgo biloba* leaf extract (6% terpene trilactones).

Analysis of ginkgolides and bilobalide. Call and suspension cultures were lyophilized and then pulverized with a pestil and a mortar. Solvents, instrumentation, chromatographic conditions for HPLC analysis (elu-

ent used: methanol–water 31.5:68.8, flow rate 0.95 ml min^{-1}) and quantitation have been described in a previous paper [2].

Extracation and purification procedure. Lyophilized powder (1 g) was mixed with 10 ml of 10% MeOH in water in a 25 ml conical flask and refluxed for 15 min in an oil-bath of 140°. Then the condenser was removed and the hot aq. extract was filtered under vacuum over a Büchner funnel with filter-paper (Schut, Heelsum, The Netherlands). 10% aq. MeOH (2 ml) was used for washing the flask. After filtration the tissue culture was returned to the flask and extracted a second time in the same way with 5 ml of 10% aq. MeOH. After filtration the combined filtrates were transferred to a 25 ml conical flask and evaporated to dryness with a rotary evaporator. Distilled water (5 ml) was added to the dried extract and the soln was refluxed for 15 min on an oil-bath at 140° and then transferred to a silica gel column [10 g of silica gel (0.063–0.200 mm, Merck) in a column of 2 cm internal diameter and 14 cm long]. The flask was washed with 2 × 1 ml of distilled water and the washings were transferred to the column. The column was eluted with such a quantity of methyl acetate–hexane (75:25) that 40 ml eluate were obtained. The eluate was dried by passing it through 4 g anhydrous Na_2SO_4 (Merck) in a SPE column (6.5 cm long). The solvent was evapd and the residue was dissolved in 250 μl of methanol, 500 μl of benzyl alcohol soln (60 mg/30 ml MeOH–H_2O 1:1) and followed by 3 × 250 μl of water. The benzyl alcohol was used as an int. standard and was added by means of a volumetric pipette. The soln was transferred to a little flask which was stoppered and ready for injection in the HPLC system. During the analysis the flask was placed on a water bath at 50° to prevent any crystallization of ginkgolides. The extraction of transformed cultures led to the formation of a gelatinous filtrate that needed purification. The gelatinous filtrate was dissolved in 20 ml of water and 2 g of NaCl were added to the soln. The soln was extracted 3 × with 25 ml of EtOAc. The solvent was dried with Na_2SO_4, then filtered over paper and evapd. The residue was then ready to be dissolved in the solvents for the HPLC analysis (see above). The different tissue cultures of *Ginkgo biloba* were analysed once or twice depending on the available amount. Each extract was injected three times for HPLC analysis.

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