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Effect of biotic elicitors on the accumulation of bilobalide and ginkgolides in *Ginkgo biloba* cell cultures

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ABSTRACT

The effect of biotic elicitors on the production of bilobalide and ginkgolides in *Ginkgo biloba* cell suspension cultures was studied. The treatment of cell cultures with *Candida albicans* and *Staphylococcus aureus* as elicitors increased the amounts of bilobalide (BB), ginkgolide A (GA) and ginkgolide B (GB), with slight growth inhibition. The native bacterial elicitor was more effective for secondary metabolite accumulations both in cells and culture medium than autoclaved. However, exposure times of the cells to the elicitors strongly influenced the production of BB, GA and GB. This study suggests that biotic elicitors can regulate the production of BB, GA and GB either directly or indirectly. These results also describe the establishment of optimum conditions that determine the effects of biotic elicitors on secondary metabolism of bilobalides.

1. Introduction

An elicitor is an endogenous or exogenous substance that induces production of phytoalexins in higher plants. The biosynthesis of secondary metabolites in plants is under tight regulatory control during development. The accumulation of these metabolite, increase in response to stress and during microbial attack (Darvill and Albersheim, 1984). Elicitation is very attractive strategy for increasing the metabolite productivity in *in vitro* culture system. The elicitation of plant cells or tissue can lead to increased yields and shorter production times (Discosmo and Misawa, 1985). Both plant defense mechanism and metabolite production are interrelated via secondary metabolism (Sahai and Shuler, 1984). Thus, the activation of defense mechanism by biotic elicitor could lead to enhanced accumulation of secondary metabolites.

The Ginkgo tree is a source of various chemicals like flavonoids, terpenoids, and other compounds (Huh et al., 1992; Kang et al., 1990). Of these bilobalide and ginkgolides are beneficial to human health (Laurain et al., 1997). The ginkgolides are classified as A, B, C, J, and M (Carrier et al., 1998). Among different effects ginkgolide B (GB) in particular, shows potent antagonist effects on platelet activating factors (PAF), involved in the development of a number of cardiovascular, renal, respiratory and central nervous system disorders (Smith et al., 1996). This study investigates the effects of the biotic elicitors on production of BB, GA and GB in cell suspension cultures of *Ginkgo biloba*.

2. Methods

2.1. Callus induction and suspension cell cultures from G. biloba

The surfaces of the seeds were sterilized successively with 70% (v/v) ethanol for 1 min and sodium hypochlorite 3% (v/v) solution for 15 min. After seven times rinsing with sterile distilled water, the embryo was expelled from seeds with a knife and pincette. The resulting embryo were wounded and placed on MS solid medium supplemented with sucrose 3% (w/v), gelrite 0.3% (w/v),

Abbreviations: BB, bilobalide; GA, ginkgolide A; GB, ginkgolide B; KCTC, Korea Collection for Type Cultures.

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and 18.8 μM naphthalene-1-acetic acid (NAA) to induce callus formation. The pH of the medium was set to 5.8 before autoclaving at 121 °C for 15 min. The culture tubes were incubated in the dark at 25 °C and sub-cultured every 4th week. Callus (1 g cell fresh weigh (FW)/20 ml medium) was transferred into MS liquid medium supplemented with sucrose 3% (w/v) and 18.8 μM NAA to induce proliferation. The cell suspension cultures were shaken (100 rpm) in dark and on a incubator maintained at 25 °C.

2.2. Biotic elicitors and their application to cell culture

Staphylococcus aureus KCTC 1916 and *Candida albicans* KCTC 7121 were used as biotic elicitors. For elicitation, *S. aureus* and *C. albicans* were cultured on LB (Johnsen and Stanier, 1971) and YPD liquid medium respectively at 27 °C for 3 days, afterwards the bacterial culture was diluted to an OD of 1.0 at 600 nm and fungus (0.1 g/ml) was homogenized. The resulting homogeneous bacterial and yeast preparations were applied in the elicitation experiments as native and killed forms. In elicitation experiments 3 ml each of native bacterial or yeast cultures or their killed forms were incorporated into 2 weeks-old *G. biloba* cell suspension cultures.

2.3. Measurement of G. biloba cell growth

G. biloba cell suspension culture material weighing 1 g FW was transferred into a 100-ml conical flask containing 20 ml of culture medium. The cell growth was monitored at every 3rd day (up to 30 days) by determining the fresh weight. Briefly the cell cultures were harvested on every 3rd day to collect cells by filtration. The fresh weight of the cells was recorded with the help of a physical balance. Alternatively cell growth was measured, by sedimented cell volume (SCV) measurement. All data are expressed as an average of three separate experiments.

2.4. Extraction and quantification of BB, GA, and GB

The suspended cells were separated from the medium by filtration. The separated cells were washed with distilled water to remove any adhering medium to the cell surface to record the fresh weight ; the same cells were oven-dried at 50 °C for 24 h to obtain dry weight (DW). Dried cell samples (0.1 g) were ground with a mortar and pestle to extract the phytochemicals by solvent extractions. The various ginkgolides were extracted into 10 ml n-hexane by sonication for 1 h. After centrifugation the upper n-hexane layer was withdrawn with a pipette. The bottom layer of cells was extracted with 10 ml ethyl acetate for in an ultra-sonicator (Jinwoo Engineering, Korea) for 2 h. The resulting cell extracts were centrifuged at 12,000 × *g* for 10 min and the supernatant ethyl acetate layer was concentrated by rotary vacuum evaporation (EYELA, Japan). BB, GA, and GB in culture medium were directly extracted with equal volume of ethyl acetate. The combined ethyl acetate portion was concentrated using the rotary vacuum evaporator. The residue obtained was dissolved in 200 μl MeOH (HPLC grade), filtered through a pre-filter (0.2 μm pore size, Supelco, Bellefonte, PA) and analyzed by HPLC for quantitative determination of bilobalide and other ginkgolides.

The quantification of secondary metabolites was carried out with HPLC (Gilson, Villiers-le-Bel, France) system fitted with a Lichrospher® 100 RP-18 (4.6 mm × 25 cm, 5 μm, Merck, Darmstadt, Germany) column and a UV detector (Gilson, UV 3000) operating at a wavelength of 250 nm. Isocratic mobile phase consisted of a mixture of MeOH and H_2O (50:50, v/v). After the injection of 20 μl of the sample solution, the column was operated with a flow rate (0.5 ml min^{-1}) (Kang et al., 2006a, 2006b).

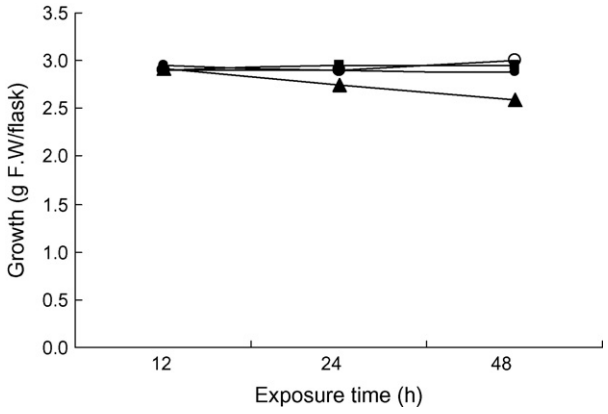

Fig. 1. Effects of *C. albicans* elicitor on growth of *G. biloba* suspension cell cultures (●: killed *C. albicans*, ▲: native *C. albicans*, ■: YPD medium, ○: control).

2.5. Statistical analysis

All experiments were conducted in replications. The data generated was subjected to statistical analysis by using SAS for Window Version 6.12 (SAS Institute Inc., 2001).

3. Results and discussion

3.1. The influence of C. albicans as an elicitor

The treatment of plant cell cultures with *C. albicans* as an elicitor was detrimental to growth (Fig. 1). After 24 h of native elicitor treatment, moderate browning of the cells as well as decrease in growth was observed. The native elicitor exposure to growing cells decreased the growth by 8% compared to control. The observed cell damage could be attributed to possible rupture of the cell membrane or due to cellular lysis by the attacking *C. albicans* in *G. biloba*. To avoid the cell damage and infection the same elicitor in the killed form was employed. When treated with this type of growth of cell cultures did not decrease; however, the physical appearance of the cultures was un-healthy.

The effect of elicitation by native and killed *C. albicans* on BB, GA and GB accumulation in cells is shown in Table 1. Native *C. albicans* as an elicitor produced more of BB, GA and GB after 24 h exposure. In particular, the native state of *C. albicans* enhanced GA and

Table 1
Influence of elicitor exposure time on production of bilobalide and ginkgolides by *G. biloba* cell cultures.

Treatments	Exposure time	Bilobalide and ginkgolides (mg/g D.W)		
		BB	GA	GB
Killed *C. albicans*	12 h	$8.5 \pm 0.6^{d*}$	11.8 ± 0.4^{c}	3.0 ± 0.4^{cb}
	24 h	6.2 ± 0.3^{fe}	12.0 ± 0.4^{c}	3.03 ± 0.4^{cb}
	48 h	8.3 ± 0.3^{d}	11.9 ± 0.4^{c}	2.9 ± 0.4^{cbd}
Native *C. albicans*	12 h	13.1 ± 0.3^{b}	11.8 ± 0.4^{c}	3.4 ± 0.3^{b}
	24 h	14.2 ± 0.9^{a}	20.3 ± 0.4^{a}	5.2 ± 1.1^{a}
	48 h	10.1 ± 0.6^{c}	18.3 ± 1.1^{b}	2.1 ± 0.4^{ced}
YPD medium feeding	12 h	6.9 ± 0.3^{e}	8.9 ± 0.4^{d}	2.2 ± 0.4^{ced}
	24 h	6.2 ± 0.2^{fe}	8.2 ± 0.4^{ed}	2.1 ± 0.4^{ced}
	48 h	8.2 ± 0.3^{d}	8.5 ± 0.4^{d}	2.1 ± 0.5^{ced}
Control	12 h	5.9 ± 0.2^{f}	7.5 ± 0.3^{e}	1.9 ± 0.4^{e}
	24 h	8.0 ± 0.1^{d}	8.1 ± 0.4^{ed}	1.9 ± 0.3^{e}
	48 h	8.1 ± 0.2^{d}	8.5 ± 0.5^{d}	2.0 ± 0.4^{ed}

*Data are expressed as an average of at least three separate experiments. The statistical significance of the results was assessed by Duncan's multiple range test ($p = 0.05$).