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# Effect of methyl jasmonate and salycilic acid synergism on enhancement of bilobalide and ginkgolide production by immobilized cell cultures of *Ginkgo biloba*

Agus Sukito[1*] and Sanro Tachibana[2]

## Abstract

**Background:** Bilobalide and ginkgolides are reported to be present only in *Ginkgo biloba*. However, only trace amounts of bilobalide and ginkgolides are contained in the ginkgo leaves. Nowadays, there has been considerable interest in plant cell cultures as a potential alternative to traditional agriculture for the industrial production of secondary metabolites. Much effort has been put into the use of in vitro cultures as one attractive biotechnological strategy for producing bilobalide and ginkgolides of commercial interest. The aim of this study was to enhance the production of bilobalide and ginkgolides A, B, and C in cell cultures of *G. biloba* using immobilized cell cultures and the process of elicitation.

**Results:** Based on the effect of the immobilization components on the free cell suspension cultures, it was considered that the jute fiber acted as an elicitor and forced the cells to release the product into the culture media. The resulting biomass was approximately 1.4 times higher than in the cell suspension cultures, and the production of bilobalide and ginkgolides A, B, and C increased 5.0, 3.3, 6.1, and 4.1 times, respectively. Eliciting with methyl jasmonate (MJ) and salicylic acid (SA) in the immobilized cells enhanced bilobalide and ginkgolides A, B, and C, compared with the unelicited controls. The highest accumulation was observed using a combination of 0.1 mM MJ + 0.1 mM SA in the immobilized cells, which produced 1.78, 1.95, 2.05, and 2.95 times more bilobalide and ginkgolides A, B, and C, respectively, than the controls.

**Conclusions:** The positive effects of immobilized cell cultures using jute fiber and the synergism effect of SA and MJ on immobilized cells of *G. biloba* appear to be the optimal conditions for continuous in vitro production for commercial purposes. This is the first report on analyzing the effects of jute fiber as immobilized cell material on *G. biloba* cell cultures and the synergism of MJ and SA on bilobalide and ginkgolide production.

**Keywords:** Immobilized cell, Elicitor, Bilobalide, Ginkgolide, *Ginkgo biloba*

## Background

Plants are a natural source of valuable secondary metabolites, which have been widely used around the world. Plants are used as flavors (Murakami et al. 1993), natural fragrances (Guterman et al. 2002), colors (Siva 2007), biopesticides (Gakuya et al. 2013), pharmaceuticals (Ramachandra Rao and Ravishankar 2002), and food additives (Amakura et al. 2002). Plant cell cultures have been used for more than 20 years to produce a variety of natural products (Sahai and Knuth 1985) and, more recently, to produce foreign gene products (Hiatt et al. 1989; Sijmons et al. 1990; James et al. 2000). Because of the importance of secondary metabolites, there is an increasing demand to obtain more of these metabolites through different plant tissue applications and cell

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culture methods. Some classical techniques, such as elicitor (Gundlach et al. 1992) and precursor (Mulabagal and Tsay 2004) additions, media composition (Yamada and Fujita 1983), and environmental conditions (Bais et al. 2002), are used to enhance secondary metabolite production. However, the problem of low secondary metabolite production caused by lack of cell-to-cell contact in plant cell cultures is still found with those techniques.

Immobilization of plant cells is a method used in plant cell cultures to induce secondary metabolite production. In this method, plant cells are immobilized in or on a supporting material such agar, agarose, calcium alginate, glass, or polyurethane foam (Lindsey and Yeoman 1985). Plant cell immobilization has a number of other processing advantages, such as re-use of cells (Brodelius et al. 1979), cultivation at higher cell densities, and separation of product and cells (Rosevear and Lambe 1983). Immobilization has been shown to improve the production levels of various secondary metabolites (Aoyagi et al. 1998; Villegas et al. 1999). Several studies have reported that immobilized plant cell cultures show an increase in secondary metabolite production compared with cell suspension cultures (Gilleta et al. 2000; Premjet and Tachibana 2004). Since immobilization of suspended cells potentially causes toxicity of polyurethane foam (Vorlop et al. 1992) and diffusional resistance of gel matrices (Ganguly et al. 2007), cells immobilized with loofa, sisal, and jute provided higher amounts of biomass with enhanced alizarin and purpurin content in immobilized *Rubia tinctorum* L. in a suspension culture (Nartop et al. 2013).

Jute (fibers of *Corchorus olitorius* leaves) is a natural lignocellulosic material (Tanobe et al. 2005). It has a stiff, biodegradable structure and can be supplied easily at low cost (Guimarães et al. 2009). Since ginkgolides were found as a naturally occurring platelet-activating factor (PAF) antagonist (Nunez et al. 1986; Braquet 1987), efforts have been made to enhance production through cell cultures. 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 (Küster et al. 1986; Dive et al. 1989). However, there is no previous report in the literature about its use as an immobilization material in plant cell cultures of *G. biloba* to enhance bilobalide and ginkgolide production. In the present study, jute fiber was used to immobilize suspended cells of *G. biloba*. The effects of methyl jasmonate (MJ) and salicylic acid (SA) as elicitors, separately and in combination, were investigated.

## Methods

### Plant material and the explants

*Ginkgo biloba* leaves were collected from the garden of the Faculty of Agriculture, Ehime University, Japan, in August. The plant material was washed twice using neutral detergent and rinsed 6–7 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 cleaning twice in sterilized distilled water for 10 min, young leaves were cut and transferred to Murashige and Skoog (MS) medium.

### Optimization of culture condition

The explants were cut into approximately 0.6 × 0.6 cm squares and placed on MS medium with sucrose (30 g/L) and gellan gum (2 g) at pH 5.6 and supplemented with α-naphthaleneacetic acid (NAA) (2 mg/L) and kinetin (0.1 mg/L). The callus was sub-cultured every month and incubated at 25 °C in the dark. To induce cell suspension cultures, 2 g fresh weight (FW) of friable callus was transferred to 250-mL Erlenmeyer flasks containing 40 mL MS liquid medium and supplemented with sucrose (30 g/L) and growth hormone of NAA (2 mg/L) and kinetin (0.1 mg/L). The cell suspension cultures were shaken at 100 rpm and incubated at 25 °C in the dark.

### Preparation of immobilization materials

The jute fibers, cut transversely in 5-cm segments and weighing approximately 2 g, were placed into 250-mL flasks to fill the bottom area. They were washed for 15 min under tap water, soaked in boiling water for 30 min, and washed again three times with about 200 mL distilled water. After washing, they were placed in an incubator for drying and sterilization for 24 h at 60 °C. All materials were autoclaved for 20 min at 121 °C. Cells (2 g fresh weight) were transferred to the pre-sterilized immobilization jute fiber in 250-mL flasks with two replicates. They were cultivated on an orbital shaker at 100 rpm at room temperature in darkness.

### Elicitor preparation and treatment

MJ and SA solutions were purchased from Sigma-Aldrich. They were dissolved in distilled water and sterile filtered through a pre-filter (0.2 µm pore size; Advantec). The sterilized MJ and SA solutions at concentrations of 0.01 and 0.1 mM were added to the flasks of culture at 20 days incubation time. The synergism of the two elicitors was also studied by applying them as follows: "0.01 mM MJ + 0.01 mM SA" and "0.1 mM MJ + 0.1 mM SA". Control cultures were untreated during cultivation. Cell response was measured 12, 24, 48 and 72 h after adding MJ and SA, and compared with the control cultures.

### Determination of dry weight

Dry weight was determined by filtering the biomass through normal filter paper and the cell clumps were removed from the immobilization materials to obtain the

fresh weights of biomass for each flask. After freeze-drying, dry weights were calculated.

### Determination of bilobalide and ginkgolides

Fresh callus from leaves was harvested by filtering through filter paper (70 mm; Advantec) under reduced pressure. The remaining fresh weight cells were placed in a freezer at −30 °C overnight and then freeze-dried for 3 days. The dried cells were ground with a mortar and pestle. After adding 10 mL *n*-hexane, the samples were sonicated for 1 h. Following 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 fraction as a ginkgolide fraction was separated using preparative high-performance liquid chromatography (HPLC), and each collected sample was analyzed by gas chromatography–mass spectrometry (GC–MS).

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

For the analysis by GC–MS, each collected sample was trimethylsilylated by adding 40 μL of *N,O-Bis*(trimethylsilyl) acetamide (=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 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, B and C) was conducted.

## Results and discussion

### Cell growth in free cell suspension cultures and jute fiber immobilized cell cultures

The initial amount of cells in each flask was 2 g fresh weight. Cell growth was measured in terms of dry weight on days 5, 10, 15, 20, 25 and 30. As the results shown in Fig. 1, both free cell suspension cultures and immobilized cells had a sigmoid growth curve. In the free cell suspension cultures, the exponential phase was observed between 5 and 20 days of culturing. Cell growth increased slightly during the stationery phase between 20 and 30 days. This result may be due to the limiting of nutrients and dissolved oxygen.

The immobilized cells of *G. biloba* on jute fiber as lignocellulosic material provided around 1.4 times more biomass production than the suspension cultures. Maximum cell dry weight (238.1 mg/flask) was obtained by day 30. No sample turned brown, which indicated that the cells on jute fiber maintained their viability during the batch cultures. Almost all the biomass were obtained from cell aggregates immobilized on jute fiber, although a few suspended cells were also seen in the liquid media. The increasing cell growth and the absence of browning on immobilized cells showed that the fragile cells are more stable and expected to stay alive for a considerable time in the immobilized cells and thereby are capable of producing secondary metabolites. This is consistent with the results of Nartop et al. (2013) who reported that immobilizing cells of *R. tinctorum* L. on lignocellulose materials provided around three times more biomass production than suspension cultures.

### Accumulation of bilobalide and ginkgolides in *G. biloba* cells of immobilized cell cultures and free cell suspension cultures

To determine the bilobalide and ginkgolide production in the cultured cells derived from the leaves of *G. biloba*, the ginkgolide fraction was separated using preparative HPLC with a Shimadzu VP Shimpak ODS RP column ($H_2O$:MeOH:2-propanol = 72.5:17.5:10) to give bilobalide and ginkgolides A, B and C (data not shown), whose retention times were identical to those of authentic bilobalide and ginkgolides A, B and C. The HPLC results strongly indicated that bilobalide and ginkgolides A, B and C were produced in the callus derived from the leaves of *G. biloba*.

GC–MS spectrum of each collected sample after TMS derivatization was identical with that of each standard sample (bilobalide, ginkgolides A, B and C). Loss of methyl moiety from molecular ion $[M–CH_3]^+$ of each collected sample also showed at *m/z* 455, 537, 625 and 713, respectively, which are identical with that of authentic bilobalide, ginkgolides A, B and C. The HPLC and

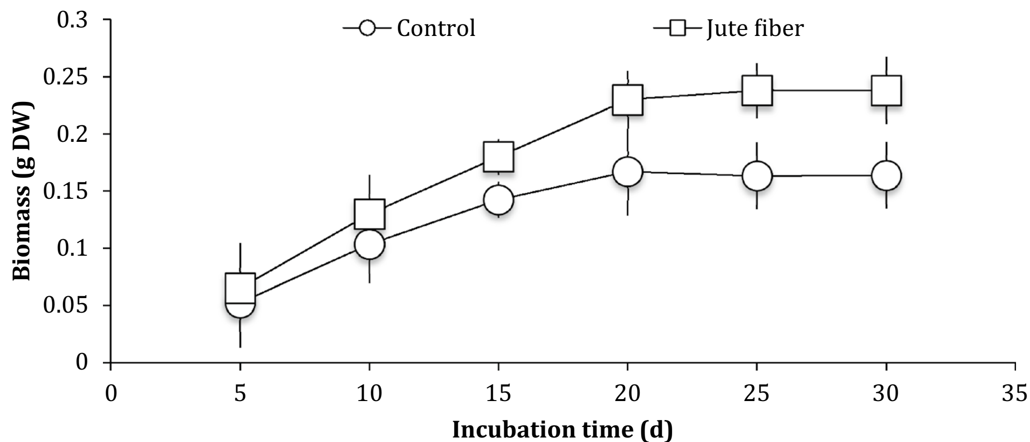


**Fig. 1** Biomass production of immobilized cells using jute fibers until 30-day incubation

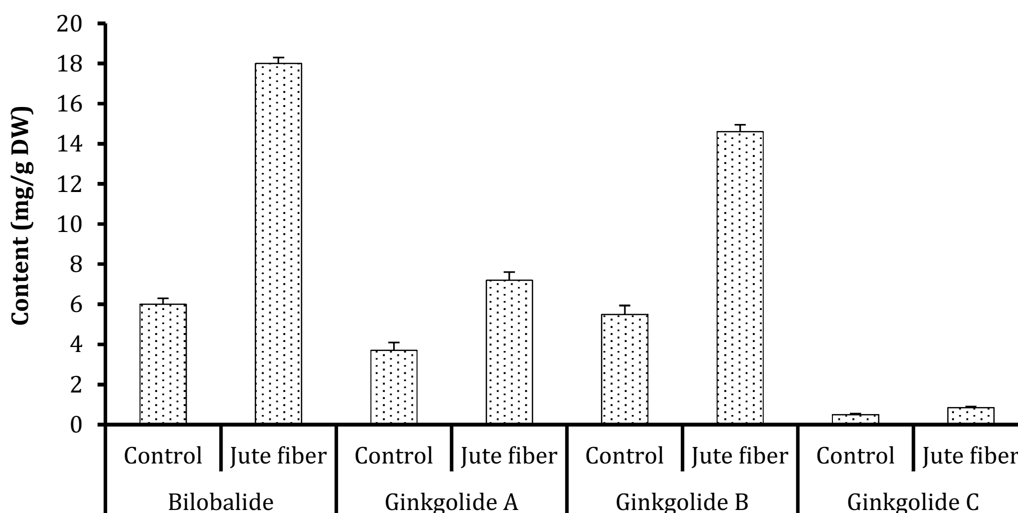


**Fig. 2** Production of bilobalide and ginkgolides A, B and C in immobilized cells using jute fibers after 30-day incubation

GC–MS spectra strongly indicate that bilobalide, ginkgolides A, B and C were produced in the callus derived from the leaves of *G. biloba*.

The effects of elicitation cells of *G. biloba* cell suspension cultures and immobilized cells were harvested at 30 days to determine bilobalide and ginkgolide levels. The results are given in Fig. 2. After 30-day incubation, the production of bilobalide and ginkgolides A, B and C in the immobilized cell cultures increased 3.0, 1.9, 2.7 and 1.7 times, respectively, compared with control in the free cell suspension cultures. According to this study, immobilization with jute fiber provided higher amounts of biomass with enhanced secondary metabolite content compared with suspended cells. Therefore, jute fiber could be used as an immobilization material in a cell culture of *G. biloba*.

### The effect of MJ, SA, and MJ + SA on cell growth in immobilized cell cultures

To enhance the production of bilobalide and ginkgolides in immobilized cell cultures of *G. biloba*, cells were grown in MJ, SA, and the combination MJ + SA in several concentrations. Figure 3 shows cell growth in the immobilized cell cultures during the period up to 72 h after treatment with 0.01 and 0.1 mM of MJ, SA, and MJ + SA as elicitors. Cell growth decreased with increased MJ and

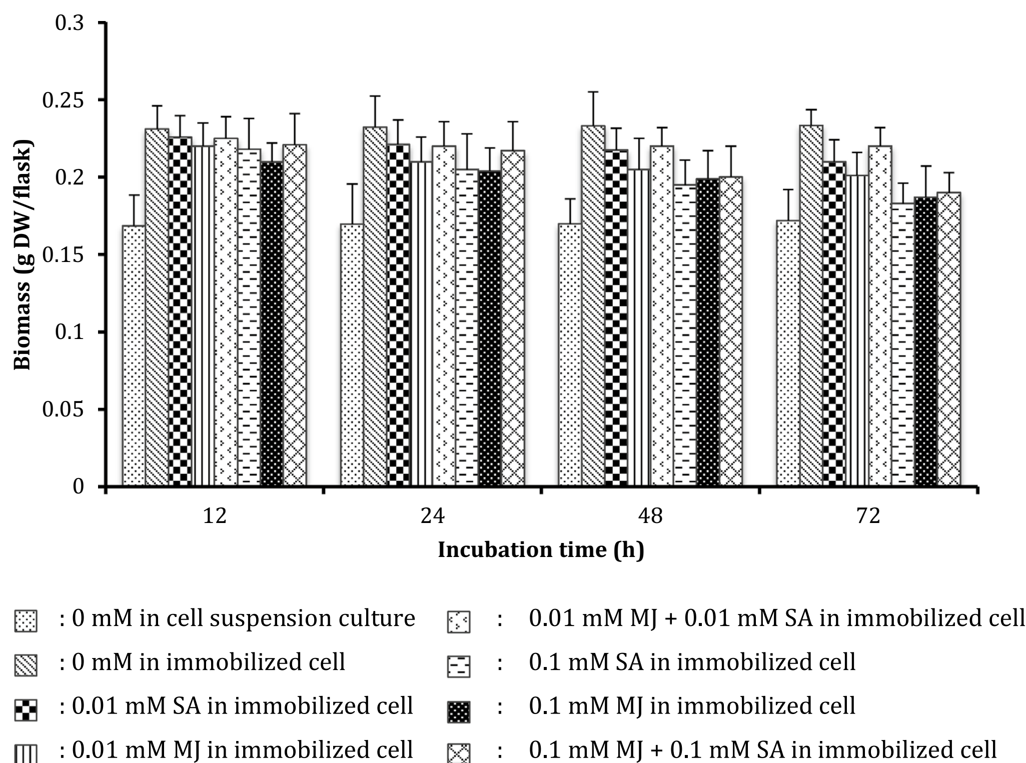


**Fig. 3** Biomass of immobilized cells using jute fiber after adding MJ, SA and MJ + SA

SA concentration. When the cultures were treated with 0.01 mM MJ, cell growth decreased by 13.9 %, relative to the control cultures after 72-h incubation, whereas 0.1 mM MJ decreased cell growth 19.9 % compared with control in the immobilized cells. Treatment with 0.01 mM SA decreased cell growth 10.1 % after 72-h incubation, whereas 0.1 mM SA decreased cell growth 21.6 %. Treatment with 0.01 and 0.1 mM MJ + 0.1 mM SA showed cell growth of 0.22 and 0.19 g DW, respectively, higher than both MJ and SA separately. However, 0.01 and 0.1 mM MJ + 0.1 mM SA decreased cell growth 5.8 and 18.6 %, respectively, compared with control in the immobilized cells after 72-h incubation.

Decreased growth following treatments with 0.01 and 0.1 mM MJ and SA is consistent with a toxic response to MJ and SA. Cosio et al. (1990) noted the inhibitory effect of MJ on plant growth. Concentrations above 0.01 mM of MJ were reported to inhibit root growth in some species (Lois et al. 1989). Treatment with 5 mM SA showed a 25 % reduction in the growth of *Vicia faba* L. after 7-day incubation (Manthe et al. 1992). Exogenously applied SA has been shown to affect a variety of processes in plants, including stomatal closure, seed germination, fruit yield, and glycolysis (Klessig and Malamy 1994). Furthermore, Kang et al. (2006) reported that treatment with MJ and SA decreased cell growth in a cell suspension culture of *G. biloba*.

The combination of MJ and SA shows less toxicity than the elicitors used separately. Although cell growth decreased after adding 0.01 and 0.1 mM MJ (0.201 and 0.187 g DW/flask, respectively), and also showed a similar effect with 0.01 and 0.1 mM SA (0.21 and 0.183 g DW/flask, respectively) after 72-h incubation, adding 0.01 mM MJ + 0.01 mM SA and 0.1 mM MJ + 0.1 mM SA showed cell growth of 0.22 and 0.19 g DW/flask, respectively. These results are in accordance with Largia et al. (2015) who have reported the synergistic effect of MJ and SA in combination.

### The effect of MJ, SA, and MJ + SA in enhancing bilobalide and ginkgolide production in immobilized cell cultures

The quantity of bilobalide and ginkgolide produced in the MJ-elicited immobilized cell cultures of *G. biloba* is depicted in Fig. 4. The results show that MJ influences the production of bilobalide and ginkgolides A, B, and C at all concentrations. Among them, 0.1 mM resulted in the highest content of bilobalide and ginkgolides A, B, and C. In the present study, we were able to produce 1.68 times

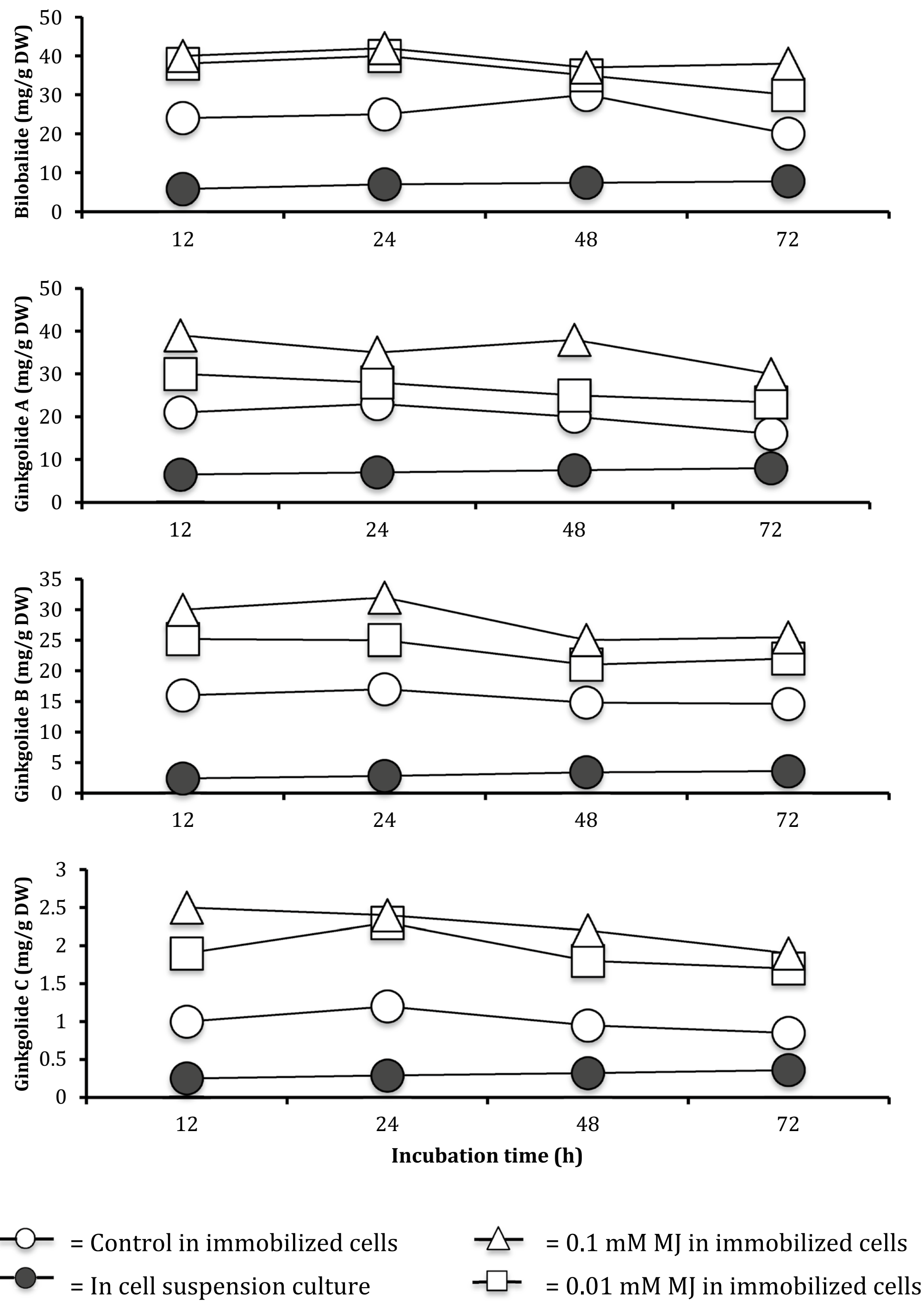


**Fig. 4** Production of bilobalide and ginkgolides A, B and C in immobilized cells using jute fiber after adding methyl jasmonate (*MJ*)

the amount of bilobalide after 24 h and 1.86, 1.88 and 2.5 times the amount of ginkgolides A, B, and C, respectively, after 12-h incubation following application of 0.1 mM MJ, compared with the unelicited control in the immobilized cells. This result indicates that elicitor concentration and incubation time played a major role in the

increased production of bilobalide and ginkgolides A, B, and C. Therefore, to achieve maximum stimulation of the synthesis of bilobalide and ginkgolides A, B, and C, both the concentrations and the incubation times of the elicitors need to be optimized. This conclusion agrees with those reached for several other metabolite and plant cell systems: the levels of ginkgolides A and B after treatment with 1.0 mM MJ increased 4.3 and 8.2 times over control after 12 h (Kang et al. 2006) and isoflavone production was enhanced by adding 100 μM of MJ for about 72 h in hairy root cultures of soybean (Theboral et al. 2014).

With SA elicitation, 0.1 mM increased the production of bilobalide and ginkgolides A, B and C by 1.8, 1.7, 1.8 and 2.2 times, respectively, higher than the quantity produced using unelicited cultures (Fig. 5). Almost all the highest production occurred after 24-h incubation except for bilobalide (the highest production came after 12 h incubation). Elicitation with 0.01 mM SA increased the production of bilobalide and ginkgolides A, B and C up to 1.6, 1.5, 1.5 and 1.8 times, respectively, compared with control in the immobilized cells. The most common explanation for increased production is that the decreased growth rate after elicitation in immobilized cells changes the metabolic pathways, thereby increasing the production of some metabolites. Since most secondary metabolites are associated with stress responses, increased production of these products is often associated with decreased cell growth.

The combined application of MJ and SA resulted in the highest production of bilobalide and ginkgolides A, B and C in the immobilized cells of *G. biloba* (Fig. 6). Of the two different concentrations, treatment with 0.1 mM MJ + SA resulted in the highest improvement in bilobalide and ginkgolide production. When the immobilized cells were treated with 0.1 mM MJ + 0.1 mM SA, the production of bilobalide and ginkgolides A and B increased up to 2.0, 1.8, and 2.1 times, respectively, after 24-h incubation, whereas ginkgolide C increased up to 2.9 times after 48-h incubation compared with control.

The pattern of release of bilobalide and ginkgolides into the liquid medium of immobilized cell cultures of *G. biloba* is shown in Table 1. Treatment with 0.01 mM MJ increased the releasing of bilobalide and ginkgolides A, B, and C into the medium up to 60, 75, 61 and 6.2 mg/g DW, respectively, whereas 0.1 mM MJ increased the release up to 70, 79, 64 and 6.4 mg/g DW, respectively, higher than that of control in the immobilized cells. This result indicates that MJ may be related to the release of bilobalide and ginkgolides A, B and C into the liquid medium of immobilized cells.

Treatment with SA not only increased the production of bilobalide and ginkgolides A, B and C in cells, but also increased their release into the culture medium. The 0.01-mM SA treatment produced a maximal release of bilobalide after 72-h incubation and increased the concentration of bilobalide and ginkgolides A, B and C in the culture medium up to 69, 68, 58 and 6.2 mg/g DW, respectively, whereas 0.1 mM SA stimulated the release of bilobalide and ginkgolides A, B and C, which reached the highest levels after 72 h (72, 70, 63 and 6.8 mg/g DW, respectively). Many plant products produced by cell cultures have been reported to accumulate intracellularly, but some compounds were secreted into the media. *Chinchona ledgeriana* cells excreted anthraquinones into liquid medium (Robins and Rhodes 1986). Berberine was released continuously from cells into a liquid medium of *Thalictrum minus* cell suspension cultures (Nakagawa et al. 1984).

Similar to the MJ and SA results, treatment with MJ + SA also increased the production of bilobalide and ginkgolides. Treatment with 0.1 mM MJ + 0.1 mM SA stimulated the release of bilobalide and ginkgolides A, B and C at the highest level after 72-h incubation up to 78, 79, 71 and 7.5 mg/g DW, respectively, compared with all treatments in immobilized cells, whereas 0.01 mM MJ + 0.01 mM SA stimulated the release of bilobalide and ginkgolides A, B and C up to 70, 75, 68 and 6.6 mg/g DW, respectively. Biotic elicitors such as MJ are considered signal molecules that act as key compounds of the signal transduction pathway involved in the induction of the biosynthesis of secondary metabolites, which takes part in plant defense reactions (Almagro et al. 2009). In addition, SA is also a well-known compound that induces a rapid and local activation of defense responses that are mediated by several resistance genes against some pathogens and other stress factors by producing certain compounds and proteins, such as pathogenesis-related proteins (Li et al. 2004). In this research, we found that the synergism of MJ and SA increased the release of bilobalide and ginkgolides A, B and C into the liquid medium of immobilized cells. The secretion of compounds from cells into liquid media allows the generation of higher production quantities, because a product accumulated intracellularly sometimes inhibits its own synthesis by regulating mechanisms such as product inhibition and repression. Kang et al. (2006) reported that releasing ginkgolides and bilobalide through MJ and SA treatment was caused by medium acidification. MJ and SA treatment changes medium acidification, which could perturb the equilibrium between the intracellular and extracellular compartments. The releasing of secondary metabolites to the medium may be the result of many factors, including feedback inhibition of membrane transport, biosynthesis, gene activity, degradation of the product by enzymatic or non-enzymatic processes in the medium or cells, and volatility of substances produced

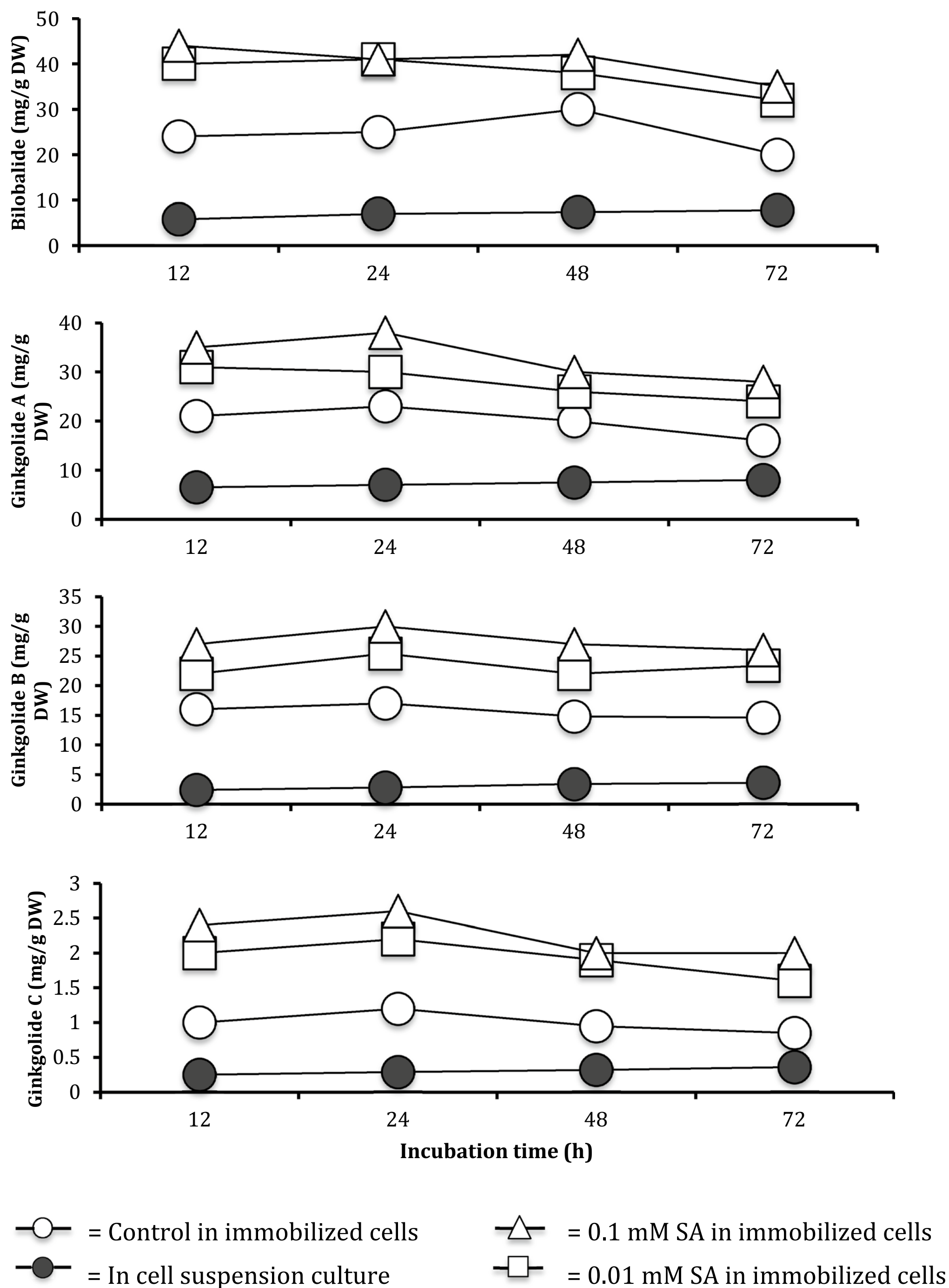


**Fig. 5** Production of bilobalide and ginkgolides A, B and C in immobilized cells using jute fiber after adding salicylic acid (*SA*)

(Luckner 1990). However, detailed research on the releasing mechanism of ginkgo cell cultures is required.

Optimization of culture conditions is one of the importance approaches to enhance and improve the production of bilobalide and ginkgolide in *G. biloba* using in vitro cultures (Park et al. 2004; Sabater-jara et al. 2013). In the other hand, elicitation is very attractive strategy for increasing the metabolite productivity in in vitro culture

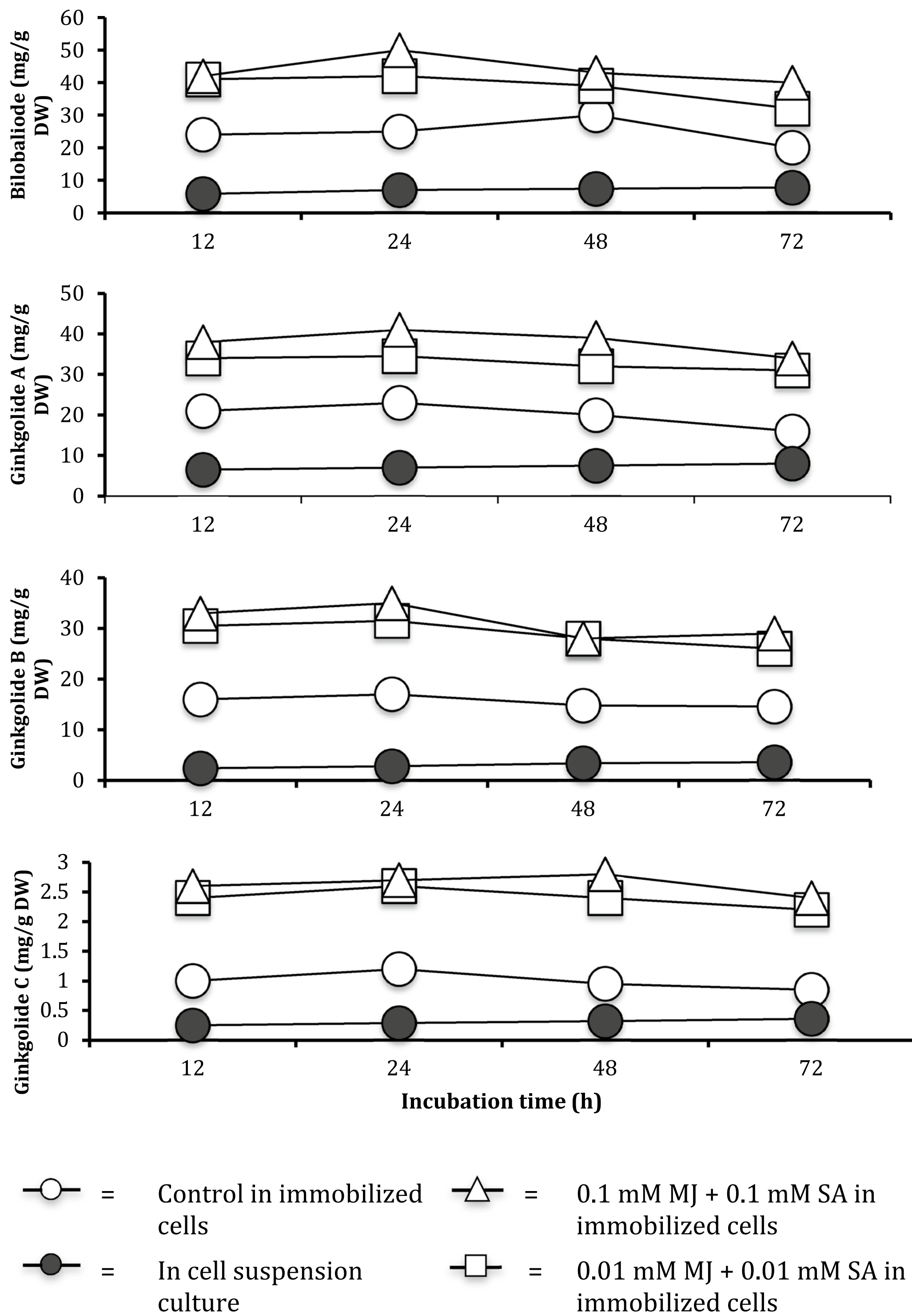


**Fig. 6** Production of bilobalide and ginkgolides A, B and C in immobilized cells using jute fiber after adding methyl jasmonate + salicylic acid

**Table 1 Effect of 72-h incubation with different concentrations of elicitors in immobilized cell cultures of *G. biloba* in MS liquid medium**

| Concentration of elicitor (mM) | | Production in liquid medium (mg/g DW) | | | |
|---|---|---|---|---|---|
| MJ | SA | Bilobalide | Ginkgolide A | Ginkgolide B | Ginkgolide C |
| 0 | 0 | 22 ± 1.78 | 62 ± 2.81 | 43 ± 2.01 | 4.0 ± 0.51 |
| 0.01 | 0 | 60 ± 2.72 | 75 ± 2.01 | 61 ± 1.63 | 6.2 ± 0.27 |
| 0.1 | 0 | 70 ± 1.86 | 79 ± 2.21 | 64 ± 1.52 | 6.4 ± 0.20 |
| 0 | 0.01 | 69 ± 1.75 | 68 ± 1.88 | 58 ± 1.56 | 6.2 ± 0.89 |
| 0 | 0.1 | 72 ± 2.23 | 70 ± 2.02 | 63 ± 1.81 | 6.8 ± 0.43 |
| 0.01 | 0.01 | 70 ± 1.79 | 75 ± 2.23 | 68 ± 1.98 | 6.6 ± 0.13 |
| 0.1 | 0.1 | 78 ± 1.99 | 79 ± 2.31 | 71 ± 1.65 | 7.5 ± 0.18 |

system. However, addition of elicitor decreased the cell growth due to medium acidification (Kang et al. 2006), moderate browning or rupture of cell membrane (Kang et al. 2009) caused by cell-to-cell contact. In this experiment, immobilization of cell culture using jute fiber was conducted to repair cell-to-cell contact and increase the cell growth before elicitation. The natural characteristics of jute fiber as immobilization materials caused no negative effect on cells and did not prevent biomass increase and secondary metabolite production. This experiment found that immobilization with jute fiber increased the amounts of biomass with enhanced bilobalide and ginkgolide production. Our finding is in accordance with Nartop et al. (2013) who have found that loofa, sisal and jute provided higher amounts of biomass with enhanced the alizarin and purpurin content compared to suspended cells.

According to Khosroushahi et al. (2006), the combination of elicitors and secondary signaling molecules often appears to be very effective in secondary metabolite accumulation in larger quantities. Cui et al. (2012) highlighted the importance of synergism of two elicitors on increased valtrate production in the adventitious root cultures of *Valeriana amurensis*. Recently, Largia et al. (2015) also reported a spectacular improvement in bacoside A elicited by a combination of 25 µM MJ and 25 µM SA for about 3 weeks. The combined application of MJ, SA, and yeast extract resulted in the increased production of rosmarinic acid (twofold) in the whole plant culture of *Solenostemon scutellarioides* (Sahu et al. 2013).

To the best of our knowledge, this is the first report on analyzing the effects of jute fiber as immobilized cell material on *G. biloba* cell cultures and the synergism of MJ and SA on bilobalide and ginkgolide production. The positive effects of immobilized cell cultures using jute fiber and the synergism effect of SA and MJ on immobilized cells of *G. biloba* appear to be the optimal conditions for continuous in vitro production for commercial purposes.

**Authors' contributions**

AS designed the research concept, designed and conducted all experiment (preparation, extraction and instrumental analysis), analyzed data, and made manuscript. ST designed the research concept, designed experiment, prepared all chemical/material for experiment, advised on experiment, and corrected manuscript. Both authors read and approved the final manuscript.

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**Competing interests**

The authors declare that they have no competing interests.

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