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Stimulation of asiaticoside accumulation in the whole plant cultures of *Centella asiatica* (L.) Urban by elicitors

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Abstract The effects of a number of different elicitors on asiaticoside production in whole plant cultures of *Centella asiatica* were studied, including yeast extract, $CdCl_2$, $CuCl_2$ and methyl jasmonate (MJ). Only MJ and yeast extract stimulated asiaticoside production—1.53 and 1.41-fold, respectively. Maximum asiaticoside production was achieved following treatment with 0.1 m*M* MJ (116.8 mg/l). The highest asiaticoside production (342.72 mg/l) was obtained after 36 days of elicitation in cultures treated with 0.1 m*M* MJ and 0.025 mg/l 1-phenyl-3-(1,2,3-thidiazol-5-*yl*)urea (TDZ). Interestingly, MJ not only stimulated the production of asiaticoside but also had an important role in the senescence of *C. asiatica*. Although asiaticoside content did not change when TDZ was added to medium containing an elicitor, TDZ did increase shoot growth of *C. asiatica*. We discuss the interactive roles of MJ and TDZ in secondary metabolic production and biomass in whole plants of *C. asiatica*

Keywords Asiaticoside · *Centella asiatica* · Whole plant cultures · Methyl jasmonate

Abbreviations *DMSO:* Dimethyl sulfoxide · *HPLC:* High performance liquid chromatography · *MJ:* Methyl jasmonate · *TDZ:* 1-Phenyl-3-(1,2,3-thidiazol-5-*yl*)urea

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Introduction

Centella asiatica (L.) Urban has been used in traditional medicine in India for the treatment of leprosy, varicose veins, ulcer, lupus, certain eczemas and mental retardation (Sharma et al. 1985; Kartnig 1988). Asiaticoside, a trisaccharide triterpene, has been identified as the most active compound in the plant, has been associated with the healing of wounds and duodenal ulcers, while the triterpene saponins are reported to possess immunomodulatory properties (Plohmann et al. 1994). Asiaticoside derivatives can be regarded to be likely candidates for a therapeutic Alzheimer's disease drug because these have been shown to potentially protect cells against β-amyloid-induced cell death (Mook-Jung et al. 1999).

This interest in asiaticoside motivated us to investigate its production by in vitro cultures of *C. asiatica*. Plants of *C. asiatica* have been successfully regenerated from callus cultures and subsequently micropropagated (Patra et al. 1998; Tiwari et al. 2000), but few researchers have tried to enhance the synthesis rate of the principal secondary metabolites. Baek (1997) reported that the content of asiaticoside in micropropagated shoots of *C. asiatica* was 50% of that obtained from field-grown plants; in transformed hairy roots, the content was as low as 1/12 that of the field-grown plants. There was no detectable asiaticoside in undifferentiated cells, including cultured cell suspensions and calluses. Recently, experiments in our laboratory have shown that although whole plants derived from nodes are richer in asiaticoside than any other plant material, the levels in whole plants are generally low (Kim et al. 2002).

Elicitors have been found to induce secondary metabolite accumulation not only in intact plants but also in plant tissue cultures. Several studies have indicated that many plant tissue cultures are stimulated by elicitors and that secondary metabolites accumulate rapidly in response to treatment with elicitors (Dicosmo and Misawa 1985; Eilert et al. 1987; Mukundan and Hjortso 1990; Ning et al. 1994). However, the effect of elicitors on the accumulation of asiaticoside has not been described for whole

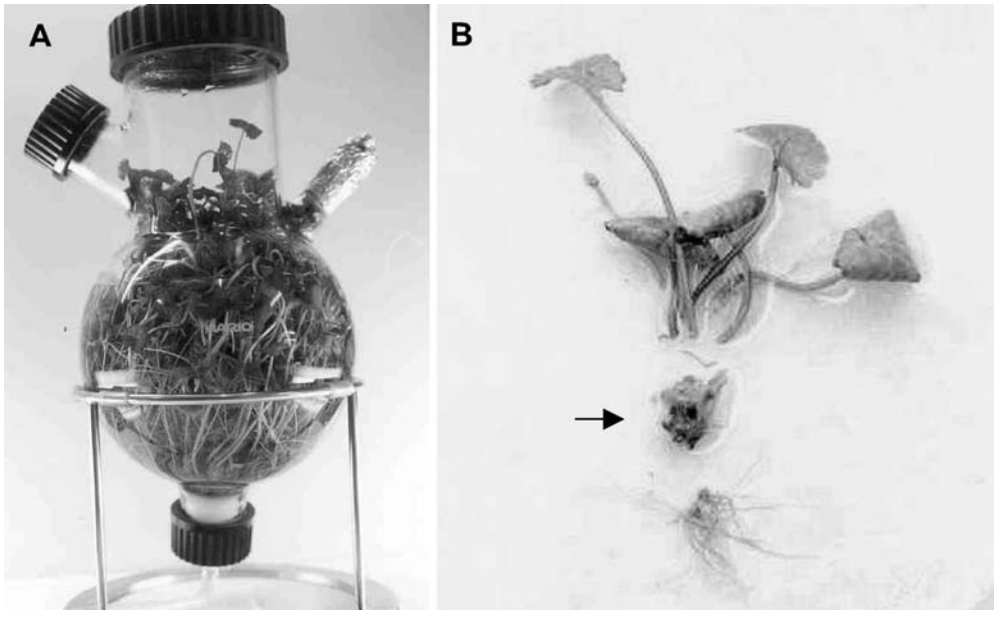

Fig. 1a,b A stock culture and inoculation region for whole-plant cultures. **a** A single stock culture grown in a 2-l bioreactor (air-lift type) under light at 25°C with air was used as inoculum for the experimental flasks. **b** After removal of the shoots and roots to ensure synchronous conditions, the nodes were inoculated into the flask. *Arrow* indicates region of inoculation

plant cultures from nodes of *C. asiatica*. Methyl jasmonate (MJ), however, has been shown to have physiological effects as a growth inhibitor in several plant species (Dathe et al. 1981). If MJ is applied exogenously to plants, it produces effects such as growth inhibition, induction of leaf senescence (Satler and Thimann 1981; Weidhase et al. 1987) and the promotion of ethylene production (Saniewski et al. 1987) and thus creates problems for the mass production of secondary metabolites in long-term cultures.

To investigate asiaticoside accumulation in whole plants derived from nodes, we screened several elicitors, including $CuCl_2$, $CdCl_2$, yeast extract and MJ. The optimal concentrations of yeast extract and MJ, which had the most effect on asiaticoside accumulation, were examined for their effect on production. In order to overcome the elicitation problem of growth inhibition and leaf senescence, TDZ (thidiazuron, 1-phenyl-3-(1,2,3-thidiazol-5-yl)urea), a cytokinin that plays a role as an antisenescence agent in several plants, was added to the medium with MJ. We then studied the effect of MJ plus TDZ on biomass production and asiaticoside concentration.

Materials and methods

Plant materials

Seeds of *Centella asiatica* from Jeju Island, Korea were sterilized with 3% sodium hypochlorite solution containing 0.1% Tween 20 for 10 min and then rinsed twice with sterile distilled water. Whether or not nodes are connected between the petiole and root has not been exactly defined, but Tiwari et al. (2000) described propagation by axillary bud proliferation in nodal segments isolated from mature plants. After 7 weeks of cultivation, nodes were removed from the whole plants. Liquid cultures of nodes for propagation were initiated as described by Kim et al. (2002) and established in 250-ml Erlenmeyer flasks containing 50 ml of culture medium. Among the various lines, one culture proved to be a fast-growing line. And we used this fast-growing line in subsequent experiments. Whole plants induced from nodes were maintained in 250-ml flasks containing 50 ml B5 liquid medium (Gamborg et al. 1968) supplemented with 3% sucrose on a rotary shaker (100 rpm) at 25±1°C under 16/8-h (light/dark) photoperiod. Subculture of whole plants was made at regular 6-week intervals.

Whole plant cultures

To obtain a number of nodes, we developed whole-plant cultures by introducing ten nodes into 2 l of B5 basal medium containing 3% sucrose in a bioreactor of the air-lift type (Hario, Tokyo, Japan) and culturing under a 16/8-h (light/dark) photoperiod with air for 10 weeks. A single stock culture grown in a 2-l bioreactor was used as inoculum for each experimental flask (Fig. 1a). Following removal of the shoots and roots (Fig. 1b), three nodes were transferred to 250-ml Erlenmeyer flasks containing 50 ml of B5 medium with 3% sucrose under the same conditions as described above. After 5 weeks of cultivation, the whole plants derived from the nodes were used as experimental material for elicitor treatment.

Addition of elicitors

Yeast extract (Difco, Detroit, Mich.), $CuCl_2$ and $CdCl_2$ (Sigma, St Louis, Mo.) were dissolved in water and the pH adjusted to 5.8 before autoclaving. MJ (TCI, Tokyo, Japan) was prepared as a stock solution in ethanol and filter-sterilized. After a precultivation period of 5 weeks, whole plants were collected from the flasks and aseptically transferred to B5 liquid medium (50 ml) supplemented with MJ alone or MJ plus TDZ, which were added in suitable amounts. Cultures were harvested at 3, 5, 7, 12, 19, 26 and 36 days after elicitation. TDZ (Sigma, St Louis, Mo.) was dissolved in dimethyl sulfoxide (DMSO) and was added to the medium before autoclaving. DMSO in the same amount was added to culture medium without TDZ to insure synchronous conditions. At the end of a culture period, whole plants from flasks were collected, and the biomass was freeze-dried and the dry weight determined. The contents of asiaticoside from whole plants or leaf were determined.

Extraction and HPLC analysis of asiaticoside

Asiaticoside extraction was carried out using a modified method of Booncong (1989). Whole plants were removed from the flasks and freeze-dried for 24 h. For each sample, 100 mg of powder was extracted using 5 ml of solvent (70:30 ethanol/H_2O) for 20 min. After filtration through a filter the extract was fractionated into petroleum ether. Cold acetone and diethyl ether were added to the collected water layer. Only the aqueous layer was collected and filtered through 0.45-μm membrane. Crude asiaticoside in the aqueous layer was used for HPLC analysis. Quantitative determinations of asiaticoside were accomplished by HPLC using a C_{18}-Bondapak column. The composition of the mobile phase was optimized by varying the percentage of methanol in water. The following conditions were optimal: mobile phase, methanol/H_2O (60:40 v/v); flow rate, 0.8 ml/min; column temperature, 26°C; detector wave-length, 214 nm. The asiaticoside standard was purchased from ROTH (Karlsruhe, Germany). All experiments were performed in triplicate and the results expressed as mean values. There were no significant differences (5%) within the triplicate values.

Results and discussion

Asiaticoside contents in each tissue of whole-plant cultures

To elucidate tissue specificity of asiaticoside production in *C. asiatica*, we analyzed asiaticoside content in each tissue of the whole plants that were cultured for 8 weeks in a bioreactor containing 2 l of B5 medium. As shown in Table 1, 82.6% of the total asiaticoside content was produced from the leaf, with the petiole and root containing 17.4%. There was no detectable asiaticoside in the nodes. The biosynthesis of major secondary metabolites shows tissue specificity. For example, pyrrolizidine alkaloids (Toppel et al. 1987) and avenacins (Trojanowska et al. 2000) are produced in the roots, and vinblastine (Constabel et al. 1982), the essential oils (Hirata et al. 1990), and quinolizidine alkaloids (Pelosi et al. 1985) are produced in the shoots. Our results (see Table 1) suggest that asiaticoside production, which mainly occurs in the leaves of *C. asiatica*, is tissue-specific. This point is indirectly confirmed by the fact that it is the leaves of *C. asiatica* that are used as material for various medical products and health foods (Brinkhaus et al. 2000). Although whole-plant cultures also contained petioles, roots and nodes, where very little asiaticoside was synthesized, if tissue specificity of asiaticoside production is taken into account, whole-plant culture systems may be suitable for producing asiaticoside.

Table 1 Asiaticoside contents and distribution percentage of each tissue of the *C. asiatica* whole plant cultured after 8 weeks in the bioreactor under light culture conditions [16/8 h (light/dark)] (*ND* Not detected, *DW* dry weight)

Tissue	Asiaticoside	
	Contents (mg/g DW)	Distribution (%)
Leaves	9.56±0.91	82.6
Petioles	1.85±0.07	15.9
Roots	0.17±0.01	1.5
Nodes	ND	0
Whole plants	4.32±0.35	

Table 2 Asiaticoside production in whole plant of *C. asiatica* after a 7-day treatment with various elicitors. (*DW* dry weight)

Treatment	Asiaticoside	
	Contents (mg/g DW)	Production (mg/l)
Control (without elicitor)	4.50±0.62	71.52±2.8
$CdCl_2$ (5 m*M*)	3.98±0.61	47.12±4.9
$CuCl_2$ (5 m*M*)	4.2±0.8	51.74±7.05
Yeast extract (0.1 g/l)	5.91±0.27	101.2±3.32
Methyl jasmonate (0.01 m*M*)	6.74±0.33	109.5±2.4

Screening of effective elicitors for promoting asiaticoside production

Elicitor treatment has been demonstrated to be effective for enhancing secondary metabolite biosynthesis in cell suspensions (Szabo et al. 1999; Lu et al. 2001) and root (Liu et al. 1999; Aoyagi et al. 2001), shoot and whole-plant cultures (Eilert 1989). In order to investigate asiaticoside biosynthesis in whole-plant cultures of *C. asiatica*, we treated 5-week-old whole plants derived from nodes with 0.1 g/l yeast extract, 5 m*M* $CuCl_2$, 5 m*M* $CdCl_2$ and 0.01 m*M* MJ for 7 days and then determined the asiaticoside content by HPLC (Table 2). After 7 days of treatment with $CuCl_2$ or $CdCl_2$, the level of asiaticoside production in whole plants declined. Lee et al. (1998) showed that 72-h treatments of transformed roots of Belladonna with 5 m*M* $CdCl_2$ and 5 m*M* $CuCl_2$ increased the release of alkaloid from tissue into the medium. However, no effect on the release of asiaticoside from tissue of *C. asiatica* into the medium was observed as a result of any of our treatments (data not shown).

In medium containing yeast extract (0.1 g/l), asiaticoside production increased 1.41-fold compared to the control; with MJ treatment (0.01 m*M*), asiaticoside production increased 1.53-fold. It was therefore quite evident that yeast extract and MJ markedly promoted asiaticoside production. MJ treatment has been found to significantly increase saikosaponin production in *Bupleurum falcatum* roots (Aoyagi et al. 2001), while the addition of yeast extract and MJ to the medium increased saponin content in a suspension culture of *Panax ginseng* cells (Lu et al. 2001). There are many reports of MJ inducing secondary metabolites, such as rosmarinic acid (Szabo et al. 1999), β-thujaplicin (Zhao et al. 2001) and sesquiterpene (Singh et al. 1998). In our study, MJ was slightly more effective in inducing asiaticoside production than yeast extract.

The effect of elicitors on the biosynthesis of secondary metabolites varies according to the concentration of the elicitor tested (Furze et al. 1991; Zhao et al. 2001) and the status of the cells or tissue (suspension cultures, shoot cultures and hydroponically grown whole plants) (Eilert 1989). To determine the optimal concentrations of elicitors for asiaticoside production, 0.1–0.8 g/l yeast extract

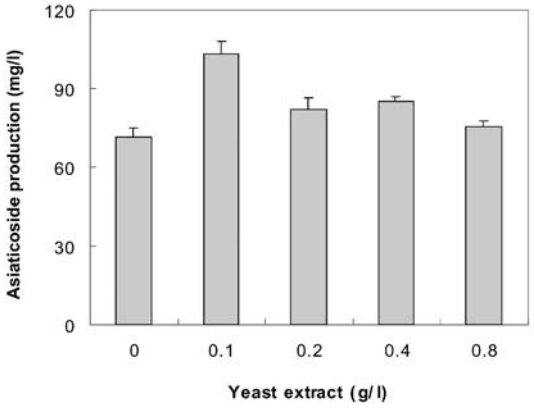

Fig. 2 Effect of yeast extract concentration on asiaticoside production in *C. asiatica* whole-plant cultures

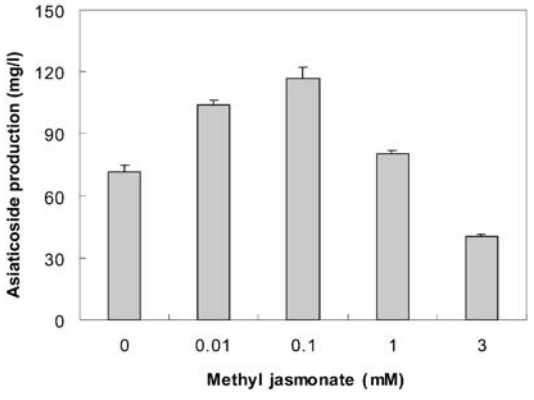

Fig. 3 Effect of MJ concentration on asiaticoside production in *C. asiatica* whole-plant cultures

and 0.01–3 m*M* MJ were added to the medium. After 7 days of elicitation, asiaticoside production by 0.1 g/l yeast extract and 0.1 m*M* MJ were 1.44- and 1.64-fold higher than that of the control, respectively (Figs. 2, 3). When 5-week-old whole plants were treated with 1 m*M* MJ for 5 days, browning of whole plants was observed. These results indicate that MJ apparently stimulates asiaticoside production more than other elicitors. The optimal concentration for elicitation was 0.1 m*M* MJ.

Effects of MJ plus TDZ on plant growth and asiaticoside production

Figure 4 shows the dry biomass of cultures that had been subjected to 0.1 m*M* MJ alone or to 0.1 m*M* MJ with 0.025 mg/l TDZ for 36 days compared with untreated cultures as controls. The whole plant grew under all conditions. After 26 days of elicitation the growth of the whole plants was observed, and we found that whole plants treated with 0.1 m*M* MJ plus 0.025 mg/l TDZ grew better than those treated with MJ alone. This result shows that TDZ prevented the negative effects of MJ on whole-plant growth. The production of asiaticoside in whole

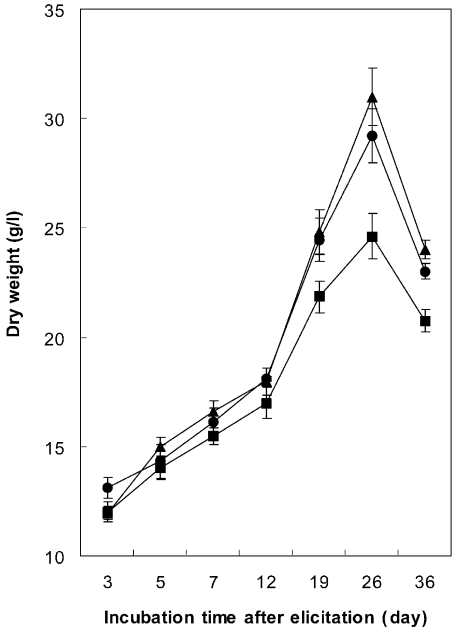

Fig. 4 Time course of growth of *C. asiatica* whole-plant cultures treated with 0.1 m*M* MJ (*filled square*), 0.1 m*M* MJ with 0.025 mg/l TDZ (*filled triangle*) and control without elicitor (*filled circle*)

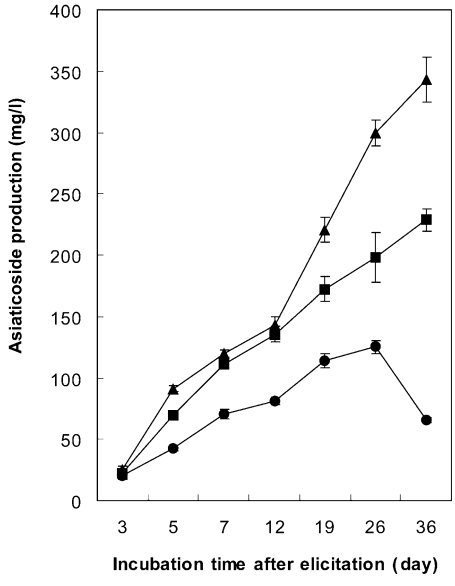

Fig. 5 Time course of asiaticoside production of *C. asiatica* whole-plant cultures treated with 0.1 m*M* MJ (*filled square*), 0.1 m*M* MJ with 0.025 mg/l TDZ (*filled triangle*) and control without elicitor (*filled circle*)

plants treated with MJ or MJ plus TDZ increased with increasing incubation time (Fig. 5). Although a decrease in growth after 36 days of elicitation was observed, the highest asiaticoside production was obtained in cultures treated with MJ plus TDZ. The additions of TDZ to cultures treated with MJ activated asiaticoside production more than MJ alone or no treatment. These results indicate that treatment with MJ plus TDZ seems to have a

synergistic effect on asiaticoside production in whole-plant cultures. We did not analyze the growth and asiaticoside content of whole plants after 36 days of elicitation because of the growth decrease caused by nutrient depletion and the stress of elicitation. Weidhase et al. (1987) found that cytokinins counteracted the degradation of ribulose-1,5-biphosphate carboxylase and chlorophyll in senescing barley leaf segments triggered by MJ. Exogenous application of cytokinins inhibited the degradation of chlorophyll and photosynthetic proteins (Richmond and Lang 1957; Badenoch-Jones et al. 1996). Senescence was also delayed in transgenic plants expressing a bacterial gene encoding IPT, the enzyme catalyzing the first step of cytokinin synthesis (Smart et al. 1991; Gan and Amasino 1995).

Taking into account the morphological characters, we found that supplementing the medium with both MJ and TDZ led to inhibitory effects on root formation (compared to controls). This effect became more evident with the longer incubation time. We also observed that the diameter of petioles of whole plants treated with TDZ and MJ after 26 days of elicitation were thicker than those of plants treated with MJ alone (data not shown). Although the roots of 5-week-old whole plants were browned by MJ and TDZ treatment, a negative effect on the shoot development of whole plants treated with both was not observed.

Time course of asiaticoside accumulation in the leaf

Because asiaticoside accumulates mainly in the leaves of *C. asiatica*, we investigated the pattern of asiaticoside accumulation in the leaves in order to address whether or not treatment with MJ plus TDZ shows a synergistic effect on asiaticoside production in whole-plant cultures. Following the addition of 0.1 m*M* MJ to whole-plant cultures of *C. asiatica* at week 5 of the culture period, asiaticoside content in the leaves was monitored for 36 days. After the addition of MJ, the asiaticoside content in the leaf continuously increased after elicitation in comparison to the control cultures (Fig. 6). The maximum asiaticoside concentration was 50.38 mg/g dry weight 36 days after elicitation. This concentration was 3.5-fold greater than in the control. Also, we added 0.025 mg/l TDZ into culture medium containing 0.1 m*M* MJ to enhance asiaticoside production. In this experiment, asiaticoside content did not change after elicitation in the medium containing TDZ, but the level of asiaticoside was higher than that of the control. Asiaticoside accumulated in whole-plant cultures treated by MJ with TDZ in the Fig. 5 due to an increase in shoot growth rather than a stimulation of secondary metabolites. Thus, MJ and exogenous cytokinin treatment did not show a synergistic effect on asiaticoside content in *C. asiatica*. As we wished to determine whether TDZ affects the inhibition of senescence during treatment of whole-plant cultures treated with MJ plus TDZ, we assayed the leaf chlorophyll content, since senescence as induced by MJ is characterized by a drastic loss of chlorophyll (Weidhase et al. 1987). In this experiment, the content of chlorophyll a and b in the leaves of whole plants treated with MJ plus TDZ for 26 days was higher than in the leaves of plants treated with MJ alone but less than that found in the leaves of the untreated controls (unpublished). Therefore, inhibition of senescence as induced by MJ may be caused by the addition of cytokinin. In conclusion, these results indicate that asiaticoside production is enhanced by MJ treatment and that TDZ addition sustains plant growth by inhibiting the senescence caused by MJ treatment.

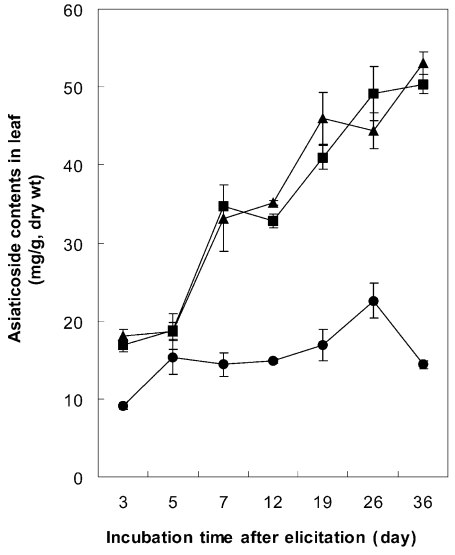

Fig. 6 Time course of asiaticoside content in leaves of *C. asiatica* following the addition of 0.1 m*M* MJ (*filled square*) and 0.1 m*M* MJ with 0.025 mg/l TDZ (*filled triangle*) in comparison to control cultures (*filled circle*)

On the basis of our results we conclude that the accumulation of asiaticoside in whole plants is best enhanced by a system using both MJ and TDZ, which may result from both the increased growth of shoots having a high asiaticoside content and the inhibition of root development.

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