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SHORT COMMUNICATION

Enhancement of centelloside production from cultured plants of *Centella asiatica* by combination of thidiazuron and methyl jasmonate

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Abstract In order to produce centellosides from whole plant cultures of *Centella asiatica* (L.) Urban, we evaluated the synergistic effects of thidiazuron (TDZ) and methyl jasmonate (MJ) on whole plant growth and centelloside production. After 4 weeks of treatment with 0.025 mg/L of TDZ coupled with 0.1 mM MJ, the production of madecassoside and asiaticoside from whole plant cultures was estimated to be 2.40- and 2.44-fold, respectively, above that of MJ elicitation alone. When whole plants were treated with a growth regulator and an elicitor, the growth of whole plants, as compared to the controls, did not differ. Additionally, total phytosyterol content in the leaves of whole plants co-treated with MJ and TDZ was 1.08-fold greater than those of MJ alone. These results demonstrate that combined treatments not only stimulate the accumulation of centellosides in the leaves but also inhibit the reduction of phytosterol levels caused by MJ elicitation.

Keywords Asiaticoside · *Centella asiatica* · Thidiazuron · Madecassoside · Methyl jasmonate · Phytosterols

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Introduction

The centellosides are important pharmacologically active components of the leaves of *Centella asiatica*, which have been utilized since ancient times in the treatment of skin disorders such as leprosy and syphilis. Among centellosides, madecassoside and asiaticoside are major compounds of *C. asiatica*. It has been reported that madecassoside exerts anti-rheumatoid effects and wound healing properties (Lui et al. 2008), while asiaticoside has been reported to induce type I collagen synthesis in human fibroblasts (Lee et al. 2006).

Gundlach et al. (1992) demonstrated that jasmonate and its derivatives may possibly function as signal compounds resulting in de novo transcription and translation and ultimately biosynthesis of secondary metabolites in plant cell cultures. In particular, methyl jasmonate (MJ) is a key signal molecule utilized extensively as an elicitor compound that strongly induces the accumulation of triterpene saponins in many plants. In order to increase the accumulation of secondary metabolites, as compared to that

obtained by MJ elicitation alone, chemical or plant growth regulators can be combined with MJ and added to the culture medium. The results of some previous reports have suggested that MJ combined with plant growth regulators or chemicals stimulated the production of ginsenosides from ginseng adventitious root cultures (Bae et al. 2006; Kim et al. 2007). These results may provide us with a greater understanding of problems associated with MJ elicitation such as reduction of plant growth, which negatively affect mass production of secondary metabolites. In addition, MJ stimulates secondary metabolites in plants, but concurrently inhibits phytosterol biosynthesis (Nkembo et al. 2005). In this study, we attempt to determine whether or not the use of TDZ as a plant growth regulator and MJ as an elicitor synergistically stimulate the production of centellosides from whole plant cultures of *C. asiatica*. We also discuss the positive effects of these compounds on phytosterol biosynthesis.

Materials and methods

Plant materials

For whole plant cultures of *C. asiatica*, whole plants were cultured as previously described by Kim et al. (2004a). In this work, instead of using B5 medium, three nodes were cultured in 250-ml Erlenmeyer flasks containing 50 ml of half-strength liquid MS medium with 3% sucrose under 16 h of light irradiation per day for 5 weeks at 100*g*. After 5 weeks of cultivation, whole plants derived from nodes were used as experimental material for elicitor treatment.

Addition of TDZ and MJ

After a 5-week precultivation period, whole plants were cultured on 1/2 MS liquid medium supplemented with 0.025 mg/l TDZ [thidiazuron, 1-phenyl-3-(1,2,3-thidiazol-5-yl)urea] alone, 0.1 mM MJ alone, or MJ plus TDZ, added to the above concentrations. Cultures were harvested every week for 28 days after elicitation. TDZ (Sigma, St. Louis, USA) was dissolved in dimethyl sulfoxide (DMSO) and was added to the medium prior to autoclaving. DMSO in the same quantity was added to the culture medium without TDZ to control for the effect of DMSO. At the end of the culture period, the whole plants were collected from the flasks and the biomass was freeze-dried, and dry weights determined.

HPLC analysis of triterpenoids

For each sample, 100 mg of powder was extracted in 2 ml of solvent (90% methanol) by sonication for 30 min at room temperature. After centrifugation at 15,000*g* for 10 min, only the aqueous layer was collected and filtered through 0.45-μm PVDF membrane (Whatman, USA). Crude centelloside in the aqueous layer was used for HPLC analysis. Quantitative determinations of madecassoside, asiaticoside, madecassic acid, and asiatic acid were conducted by HPLC (Agilent 1100 series equipped with an auto sampler, a diode array detector, and a quaternary pump) using a Inertsil ODS-3 (3.0 × 150 mm, 5 μm) column (GL Sciences, Tokyo, Japan). The other HPLC conditions for the isolation of the four triterpenoids were as follows: flow rate, 1 ml/min; column temperature, 40°C; detector wave-length, 214 nm. The standards were purchased from ChromaDex (Santa Ana, USA).

Quantitative analysis of phytosterol

Quantitative analysis of phytosterols (cholesterol, campesterol, β-sitosterol, and stigmasterol) was conducted in accordance with the procedure described by Hartmann and Benveniste (1987). Freeze-dried leaves of *C. asiatica* (500 mg) were extracted twice with 20 ml of ethyl acetate at 100*g* on a gyrator for 6 h at room temperature. The extracts were chromatographed on a silica gel cartridge column (SepPak, Waters, USA). The eluent was extracted three times with 10 ml of aqueous 5% KOH and then extracted twice more with 10 ml of aqueous 5% HCl. The organic fraction was washed twice with 10 ml of water and water was eliminated with anhydrous sodium sulfate. The extract was evaporated and the residue was dissolved with 2 ml of hexane. Suspended particles were removed by 10 min of centrifugation at 6,000*g*. An aliquot of the solution was analyzed via GC–MS. GC–MS analysis was conducted on a mass spectrometer (Hewlett Packard, USA) connected to a gas chromatograph with an HP-1 capillary column (25 m × 0.25 mm, 0.33 μm methylpolysiloxane cross-linked capillary column; Hewlett Packard). The analytical conditions were as follows: carrier gas He (4 ml/min), and column temperature 100–250°C (20°C/min). The phytosterol contents were calculated from the ratio of the peak area of the respective compound to that of the standard. Authentic standards of cholesterol, campesterol, β-sitosterol, and stigmasterol were purchased from Sigma-Aldrich.

Results and discussion

Enhancement of centelloside production by TDZ plus MJ

In a previous paper, no differences in asiaticoside content were noted in *C. asiatica* on B5 medium supplemented

with TDZ, but the growth of the whole plants was enhanced (Kim et al. 2004b). It has been suggested that TDZ, used as a plant growth regulator, contributes to the development of shoots rather than the stimulation of secondary metabolites. MJ elicitation has been employed as an effective strategy to enhance the production of triterpene saponins from cell suspension, hairy root, and adventitious root cultures. We have also shown earlier that MJ stimulates asiaticoside production in whole plant cultures of *C. asiatica* (Kim et al. 2004a). In this paper, in order to characterize the effects of TDZ coupled with MJ elicitation on four triterpenoids, whole plant cultures of *C. asiatica* were elicited after 5 weeks of pre-culture. Leaf contents of four triterpenoids (madecassoside, asiaticoside, madecassic acid, and asiatic acid) in whole plant cultures were monitored for 28 days by HPLC (Fig. 1). Whole plant cultures grown on 1/2 MS medium supplemented with 0.025 mg/l TDZ alone had higher leaf content of two centellosides and higher growth rates than those of the controls after 21 days of treatment; however, no differences in leaf content of two centellosides were observed after 28 days (Fig. 1a, b). The biological activity of TDZ is generally higher than or similar to that of the most active adenine-type cytokinins (Mok et al. 1982). Additionally, it has been noted that TDZ treatment results in an increase in the levels of hypericins in *Hypericum perforatum*, suggesting that it can affect secondary metabolites via modifications of plant growth and development (Liu et al. 2007). After 28 days of elicitation with 0.025 mg/L TDZ plus 0.1 mM MJ, madecassoside and asiaticoside contents in the leaves of whole plant cultures were 2.40- and 2.44-fold, respectively, above those of MJ elicitation alone. Consistent with this result, the madecassic acid and asiatic acid contents were also higher in cultures treated with MJ and TDZ than in those treated with MJ alone (Fig. 1c, d). In this study, these results were different from those previously reported. Kim et al. (2004b, 2005a) reported that asiaticoside content did not change when TDZ was added to medium containing MJ. They suggested that the production of asiaticoside caused by adding MJ with TDZ was due to an increase in shoot growth rather than a stimulation of asiaticoside accumulation. However, our current results demonstrate that a combination of TDZ and MJ increases centelloside content in leaves of *C. asiatica* whole plant cultures. Additionally, the combination of TDZ and MJ did not cause any damage to whole plant growth, in contrast to elicitation by MJ alone. As is demonstrated in Table 1, the total centelloside yield (the sum of madecassoside and asiaticoside) from whole plant cultures treated with TDZ plus MJ was 3.76-fold greater than that of the controls and 2.43-fold greater than that of MJ treatment alone. Similar results from other plants have been previously reported by Kim et al. (2007), who demonstrated enhancements of ginsenoside production from ginseng adventitious root cultures elicited by MJ treatment, together with indole-3-butyric acid (IBA). In that study, histological observation

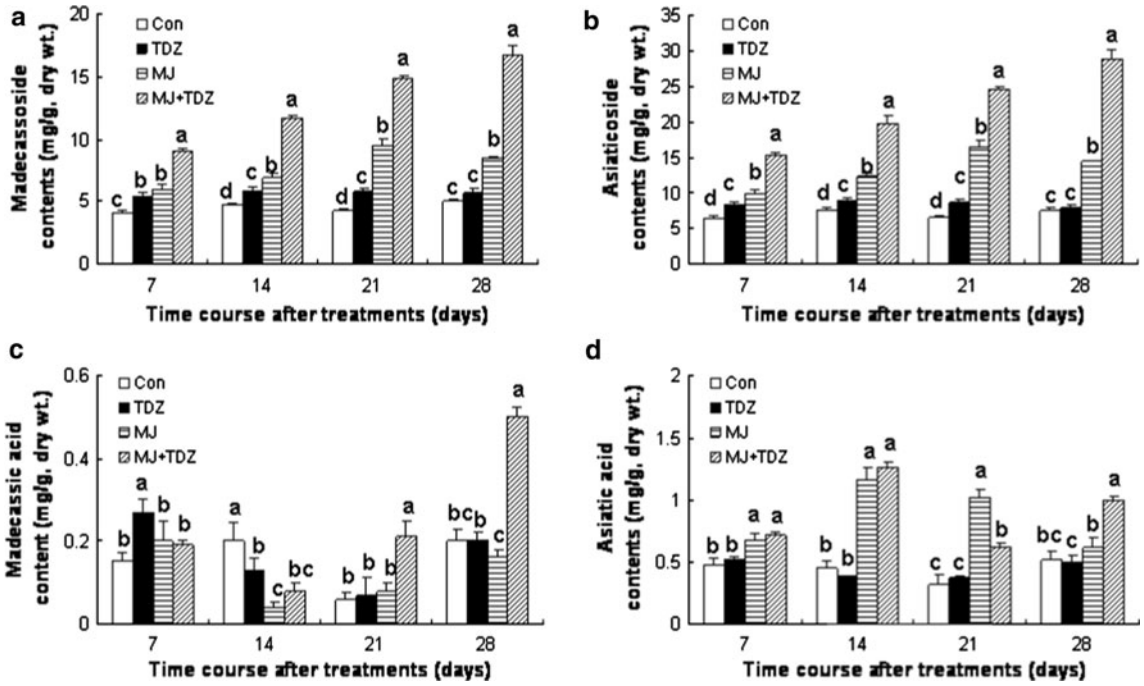

Fig. 1 Time course of madecassoside (**a**), asiaticoside (**b**), madecassic acid (**c**), and asiatic acid (**d**) contents in leaves of *C. asiatica* whole plant cultures treated with 0.1 mM MJ and 0.025 mg/L TDZ. *Vertical bars* indicate the mean values ± SE of three independent samples. Means with the *same letters* do not differ significantly at 5%, according to the Duncan's multiple range test

Table 1 Production of four triterpenoids from whole plant cultures of *C. asiatica* after 4 weeks of elicitation

Treatments	Dry weight (g/L)	Triterpenoid yields (mg/L)			
		Madecassoside	Asiaticoside	Madecassic acid	Asiatic acid
Con	29.2 ± 2.56 a	145.0 ± 6.01 c	219.0 ± 10.53 c	5.55 ± 0.77 b	15.08 ± 1.32 b
TDZ	29.7 ± 1.67 a	156.4 ± 7.76 c	232.6 ± 11.72 c	6.13 ± 0.56 b	14.75 ± 1.57 b
MJ	24.6 ± 2.99 c	209.1 ± 2.46 b	355.1 ± 21.45 b	3.93 ± 0.49 c	15.17 ± 1.67 b
MJ + TDZ	30.1 ± 1.98 a	503.7 ± 22.98 a	865.9 ± 39.28 a	14.85 ± 0.63 a	30.20 ± 1.06 a

After 5 weeks of cultivation, TDZ, MJ, or MJ + TDZ were added to *C. asiatica* whole plant cultures. Means with the same letter are not significantly different at 5% by the Duncan's multiple range test

also demonstrated that IBA combined with MJ actively stimulated cell division, relative to that seen with MJ alone. Therefore, current results show that a combination of TDZ and MJ not only maintains whole plant growth without the damage induced by elicitation, but also increases centelloside accumulation.

TDZ maintains phytosterol biosynthesis at normal levels and prevents MJ-induced reduction of plant growth

When MJ is applied exogenously to plants, it stimulates the production of secondary metabolites from cell and organ cultures, and also exerts physiological effects including growth inhibition, the induction of leaf senescence (Weidhase et al. 1987) and the promotion of ethylene production (Saniewski et al. 1987) in several plant species. Therefore, MJ elicitation reduces plant growth, which is problematic for mass production of secondary metabolites. The phytosterol profile in the majority of plants consists of cholesterol, campesterol, β-sitosterol, and stigmasterol. It has been well established that these sterols reinforce the phospholipid bilayer of the plasma membranes of eukaryotic cells. Among these sterols, campesterol is the precursor of the brassinosteroids, which perform critical functions in the regulation of cell expansion, morphogenesis, apical dominance, and leaf senescence (He et al. 2003). In this study, MJ together with TDZ was applied to whole plant cultures of *C. asiatica* to determine whether or not TDZ positively affects centelloside production and whole plant growth in plants treated with MJ. We determined phytosterol contents of the leaves of whole plants treated with MJ plus TDZ. As is shown in Fig. 2a, b, the addition of MJ alone reduced the total phytosterol contents below control levels. This result verifies the negative effect of MJ, which suppresses the accumulation of phytosterols (campesterol, β-sitosterol, and stigmasterol) in *C. asiatica* cultured plants (Mangas et al. 2006). Nkembo et al. (2005) also reported that MJ inhibited the accumulation of chlorophyll, carotenoids, and β-sitosterol in the leaves of *Scoparia dulcis*. However, campesterol and β-sitosterol contents in the

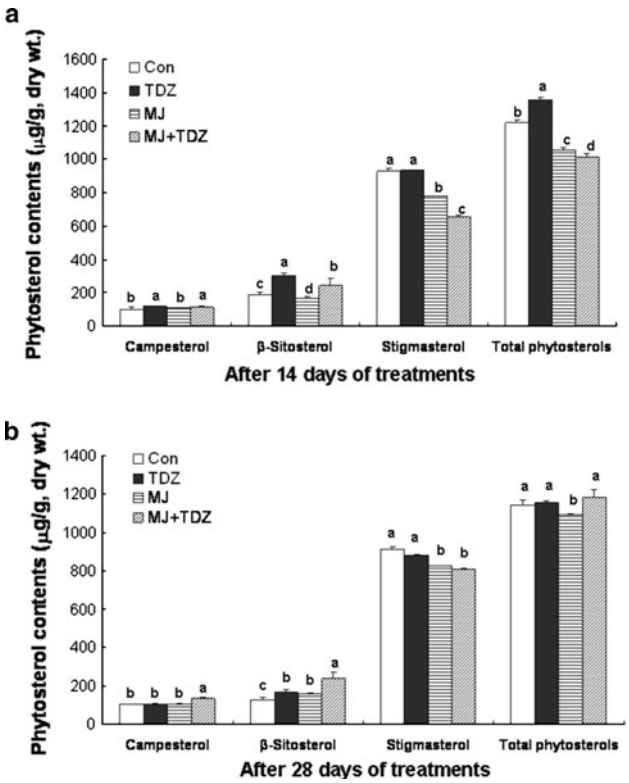

Fig. 2 Phytosterol contents in the leaves of *C. asiatica* whole plant cultures treated with 0.1 mM MJ and 0.025 mg/L TDZ after 14 days (**a**) and 28 days (**b**). *Vertical bars* indicate the mean values ± SE of three independent samples. Means with the *same letter* do not differ significantly at 5%, according to Duncan's multiple range test

leaves of whole plants co-treated with MJ and TDZ were greater than those of the controls and MJ-alone groups. After 28 days of treatment with MJ plus TDZ, no differences in total phytosterol contents, compared to the controls, were noted. Dry weight values of these samples were consistent with these findings Kim et al. (2007) reported a similar result, that the combination of IBA and MJ enhanced the growth of *Panax ginseng* adventitious roots. Therefore, our results suggest that the exogenous application of TDZ prevents the inhibition of phytosterol biosynthesis caused by elicitation stress. Our previous results

suggest that TDZ has an effect on phytosterol biosynthesis. In that study, we reported that the level of *CaCYS* (*C. asiatica* cycloartenol synthase) mRNA, an oxidosqualene cyclase involved in phytosterol biosynthesis, was higher 5 days after MJ plus TDZ treatment than in plants treated with MJ alone (Kim et al. 2005b). This may be evidence that adding TDZ with MJ permits sustained shoot growth despite MJ elicitation.

In conclusion, it can be inferred that madecassoside and asiaticoside production from whole plant cultures under the control of MJ plus TDZ was enhanced. The centelloside yield compared to that observed with MJ alone was increased by 2.43-fold. Furthermore, the addition of TDZ to the medium containing MJ contributed to whole plant growth without the damage caused by elicitation stress. This was supported by the finding that the total phytosterol content was maintained at the level of the controls despite MJ elicitation. This study demonstrates the suitability of a plant growth regulator and elicitor feeding strategy for the improvement of centelloside production in whole plant cultures of *C. asiatica*. Additionally, the effects of MJ plus TDZ may prove to be a valuable tool for understanding the mechanisms and biosynthesis enzymes relevant to the induction and overproduction of centellosides.

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