Journal of Plant Biology, December 2004, 47(4) : 361-365

Effect of Growth Regulators on Asiaticoside Production in Whole Plant Cultures of *Centella asiatica* (L.) Urban

Ok-Tae Kim[1], Min-Young Kim[1], Sun-Mi Huh[1], Jun-Cheul Ahn[2], Nak-Sul Seong[3], and Baik Hwang[1]*

[1]Department of Biology, Chonnam National University, Gwangju 500-757, Korea
[2]Department of Life Sciences, Seonam University, Namwon 590-170, Korea
[3]National Institute of Crop Science, RDA, Suwon 441-857, Korea

We investigated the effects of growth regulators on whole-plant cultures derived from nodes of *Centella asiatica*. A B5 liquid medium including 0.01 mg L^{-1} 2,4-D resulted in decreased growth and asiaticoside production. Among the cytokinins tested (TDZ, BA, zeatin, and kinetin), TDZ was the best supplement for the promotion of asiaticoside biosynthesis. To directly estimate this effect, we measured asiaticoside content in the leaf, the main organ for synthesis. The addition of TDZ did not affect asiaticoside accumulation. Nevertheless, our results suggest that treatment with exogenous TDZ may enhance the production of asiaticoside in cultures simply through an increase in biomass.

Keywords: asiaticoside, *Centella asiatica*, TDZ, triterpene saponins, whole-plant cultures

Centella asiatica L., a medicinal plant in the Umbelliferae family, grows in the tropical regions of Southeast Asia, Australia, and Southern and Central Africa (Brinkhaus et al., 2000). Since prehistoric times, it has been used in India as a traditional treatment for leprosy, varicose veins, ulcers, lupus and certain eczemas, and mental retardation (Sharma et al., 1985; Kartnig, 1988). This species contains madecassid acid and asiatic acid, as well as centellasaponins and three types of asiaticosides -- asiaticoside, asiaticoside A (madecassoside), and asiaticoside B (Brinkhaus et al., 2000; Matsuda et al., 2001). Of these, asiaticoside is the one that shows antibacterial and fungicidal activity (Mesnard, 1975).

Because of the great demand for these major compounds, researchers have worked to develop an *in vitro* propagation culturing system (Patra et al., 1998; Tiwari et al., 2000). Several tissue types have been analyzed for their asiaticoside content. For example, Baek (1997) found that, compared with levels measured in field-grown plants, the amount of asiaticosides from micropropagated shoots is one-half, while the level is only one-twelfth in *Agrobacterium rhizogenes*-mediated transformed hairy roots. In those tests, however, asiaticosides have not been detected in undifferentiated cell suspensions and calli. Recently, we established a whole-plant culture system for *C. asiatica* that is derived from nodes placed on B5 liquid media (Kim et al., 2002). Nevertheless, it has been possible to extract only very small amounts of asiaticosides from leaves, and success has been limited in attempting to enhance the accumulation of asiaticosides in various tissues.

An alternative method for increasing success involves exogenous applications of plant growth regulators in tissue culture (Lian et al., 2002). For example, the addition of IBA (indole-3-butyric acid) to hairy roots of *Panax ginseng* can increase saponin accumulation by 84%; enhancement can reach 179% with supplemental kinetin (6-furfurylaminopurine) (Yoshikawa and Furuya, 1987). Auxins also may play a direct or indirect role in regulatory levels of thiophene in the root tips of *Tagetes patula* (Croes et al., 1989).

The effect of growth regulators on asiaticoside accumulation has not been described for whole-plant cultures derived from nodes of *C. asiatica*. Therefore, the objective of this study was to determine if various plant hormones could influence biomass and asiaticoside production in that situation. We also investigated whether TDZ [thidiazuron, 1-phenyl-3-(1,2,3-thidiazol-5-yl)urea] directly or indirectly affected asiaticoside biosynthesis.

MATERIALS AND METHODS

Plant Materials and Subculturing by Inoculum of Nodes

Seeds of *C. asiatica* from Jeju Island, Korea, were

*Corresponding author; fax +82-62-530-3409
e-mail bhwang@chonnam.ac.kr

sterilized for 10 min with 3% sodium hypochlorite solution containing 0.1% Tween 20, then rinsed twice with sterile distilled water. Among the various cultures tested, the single fast-growing line was selected for our experiments. Although it is not well defined whether the nodes are connected between petiole and root, Tiwari et al. (2000) described successful propagation via axillary bud proliferation in nodal segments isolated from mature plants. After seven weeks of initial cultivation, we removed the nodes, and maintained the whole-plant cultures on a B5 liquid medium (Gamborg et al., 1968) supplemented with 3% (w/v) sucrose. Culture conditions in the 100-mL flasks, shaken at 100 rpm, included 25°C and a 16-h photoperiod. Subculturing was carried out by inoculating the shoot and root nodes removed from six-week-old whole plants (Fig. 1).

Addition of Growth Regulators

Eight culturing media were prepared from a B5 medium supplemented with 3% (w/v) sucrose and one of the following growth regulators (Sigma, USA): Control (no growth regulator added), or 0.01 mg L^{-1} each of IAA (indole-3-acetic acid), NAA (α-naphthaleneacetic acid), 2,4-D (2,4-dichlorophenoxyacetic acid), BA (6-benzylaminopurine), kinetin (6-furfurylamino purine), zeatin [6-(4-hydroxy-3-methylbut-2-enylamino)purine], or TDZ [thidiazuron, 1-phenyl-3-(1,2,3-thidiazol-5-yl)urea]. All media were adjusted to pH 5.7 before autoclaving. The nodes from our whole-plant cultures were used as inoculum, as described above. After six weeks, tissue samples were harvested, and their dry weights and asiaticoside contents were determined. For our second assay, we studied the effects of six TDZ concentrations (0, 0.005, 0.01, 0.025, 0.05, or 0.1 mg L^{-1}) on whole-plant growth and asiaticoside production.

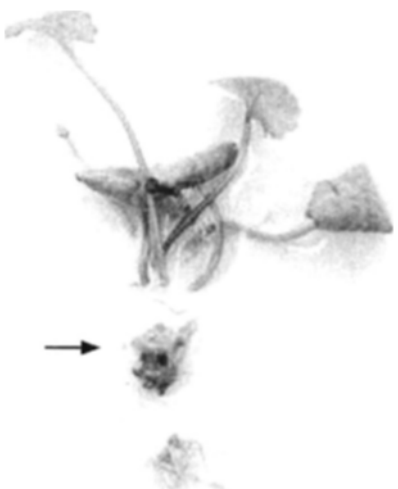

Figure 1. Inoculation region for whole-plant cultures, as indicated by arrow. After one shoot and root were removed for synchronous conditioning, a node induced from stolon was inoculated in a flask.

HPLC Analysis of Asiaticosides

Asiaticosides were extracted according to the method of Booncong (1989) with some modifications. Whole plants were removed from their flasks, frozen at −50°C, then freeze-dried for 24 h. For each sample, 100 mg of the powdered tissue was extracted for 20 min with 5 mL of a mixture of ethanol and H_2O (70:30). After filtering through Whatman #2 paper, the extracts were fractionated to petroleum ether. Cold acetone and diethyl ether were added to the collected water layer for an additional extraction. Only the aqueous layer was collected and filtered through a 0.45-μm membrane Millepore filter. Crude asiaticosides in this aqueous layer were subjected to HPLC analysis. Asiaticoside was quantified after separation on a Waters C_{18} μ-Bondapak column using a mixture of methanol and H_2O (60:40) and a mobile phase at a flow rate of 0.8 mL min^{-1}, then monitored at 214 nm. At least five flasks were used for each experiment.

RESULTS AND DISCUSSION

Generally, secondary metabolites in a differentiated cellular organization are richer than those of undifferentiated cells. For a biosynthesis study, Ferreira and Janick (1996) successfully cultured differentiated shoots of *Artemisia annua* for artemisinin production. In addition, several attempts with shoot cultures have been made to induce secondary metabolites such as parthenolide (Stojakowska and Kisiel, 1997) and galanthamine (Sellés et al., 1997). Nevertheless, an efficient system for mass propagation was still required for asiaticoside production. In previous research, we developed a culture system for *C. asiatica* whole plants derived from nodes on a B5 liquid medium (Kim et al., 2002). In particular, the effects of media composition on biomass growth and asiaticoside content were examined, and conditions were opti-

mized to 1 mM NaH_2PO_4, 25 mM KNO_3, 10 mM $MgSO_4 \cdot 7H_2O$, and 1 mM $CaCl_2 \cdot 2H_2O$. This combination provided better results, in terms of asiaticoside amount, compared with the use of a standard B5 medium, but differences in productivity were not significant. We also concluded from those experiments that whole plants derived from nodes were best cultured on B5 basal media supplemented with 3% sucrose.

In the current study, we used our previously determined optimum conditions to examine how growth and asiaticoside production could be improved with supplemental auxins (IAA, NAA, and 2,4-D) and cytokinins (BA, kinetin, TDZ, and zeatin). In general, B5 liquid media that included 0.01 mg L^{-1} exogenous auxins did not change overall asiaticoside content (Fig. 2), even though both whole-plant growth rates and asiaticoside production were decreased. Ahn et al. (1999) also reported a potent inhibitory effect by IAA and IBA on saikosaponin biosynthesis from transformed hairy roots of *Bupleurum falcatum*. Likewise, media supplemented with 50 µM 2,4-D severely repressed furanocoumarin synthesis in micropropagated shoots of *Ruta graveolens* (Massot et al., 2000). This detrimental effect of auxins on secondary metabolite production in our *C. asiatica* whole plants may have been due to decreased asiaticoside accumulation in storage organs or to obstructed formation of shoots that normally contained abundant asiaticoside.

Among our cytokinin treatments, TDZ was the most effective for enhancing growth and asiaticoside production in whole plants during six weeks of cultivation (Fig. 3). However, none of the four cytokinin types dramatically changed whole-plant growth rates. To determine the optimum TDZ concentration, we tested a range from 0.005 to 0.1 mg L^{-1} (Fig. 4), and found that 0.025 mg L^{-1} promoted the highest level of asiaticoside. The biomass that resulted at that concentration was 1.37 times greater than from the control, reaching 0.372 g dry weight per flask; asiaticoside production was 50.1 mg L^{-1}, which was three times higher than that measured from the control samples. Finally, although whole-plant asiaticoside production was increased by BA treatment, zeatin and kinetin caused decreases.

TDZ is generally used as a potent stimulator of shoot organogenesis in woody species, e.g., *Malus* (Fasolo et al., 1989) and *Populus* (Russell and McCown,

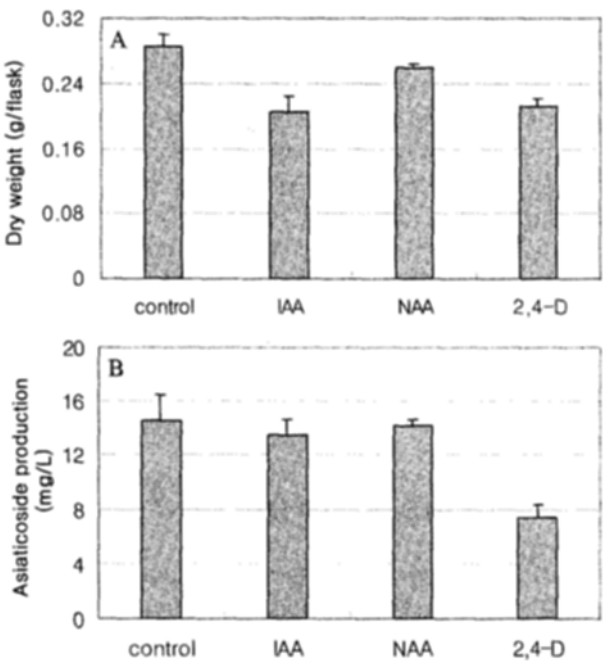

Figure 2. Effects of auxins (IAA, NAA, and 2,4-D) on *C. asiatica* after 6 weeks of inoculation. **A**, Whole-plant growth. Control was not supplemented with hormone; for each of the other treatments, 0.01 mg L^{-1} of one auxin type was added to B5 basal medium. **B**, Asiaticoside production. Bars indicate standard deviation (n=5).

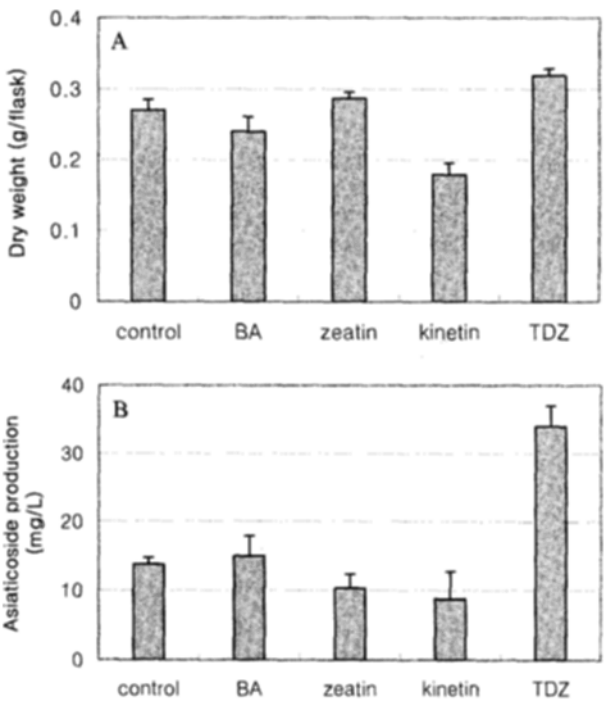

Figure 3. Effects of cytokinins (BA, zeatin, kinetin, and TDZ) on *C. asiatica* after 6 weeks of inoculation. **A**, Whole-plant growth. Control was not supplemented with hormone; for each of the other treatments, 0.01 mg L^{-1} of one cytokinin type was added to B5 basal medium. **B**, Asiaticoside production. Bars indicate standard deviation (n=5).

1988). Its biological activity is usually higher than or similar to that of the most active adenine-type cytokinins (Mork et al., 1987; Huetteman and Preece, 1993), but its biochemical action is not completely understood. Certainly, our evaluation of TDZ demonstrated that this growth regulator was the most effective for improving both growth and asiaticoside production in *C. asiatica* cultures.

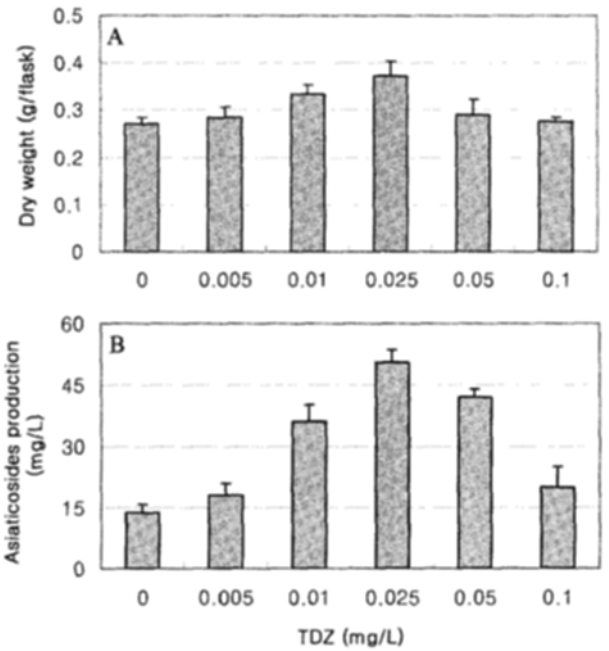

Figure 4. Effects of various TDZ concentrations on *C. asiatica* after 6 weeks of inoculation. **A**, Whole-plant growth. Control had no supplemental hormone in B5 basal medium. **B**, Asiaticoside production. Bars indicate standard deviation (n=5).

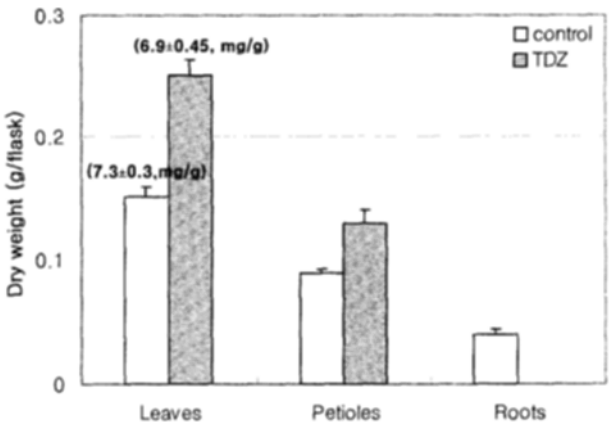

Figure 5. Effect of 0.025 mg L^{-1} TDZ on growth of *C. asiatica* after 6 weeks of cultivation. Values in parentheses show asiaticoside content (mg g^{-1} dry wt.) in leaves from cultured whole plants. Bars indicate standard deviation (n=5).

When morphological characters were examined, however, we noted that a high concentration of TDZ (0.025 mg L^{-1}) promoted leaf expansion and thicker petioles, but strongly inhibited root formation. Supplemental auxins also induced an inhibitory effect on shoots and roots compared with the controls (data not shown). These results suggest two possible explanations for the asiaticoside enhancement seen with TDZ. One is that the greater biomass was more capable of synthesis, because 82.6% of all asiaticosides were synthesized in the leaf. The other reason may be that TDZ directly affected its biosynthesis in that organ. To solve this question, we cultured whole plants on a medium containing 0.025 mg L^{-1} TDZ. This growth regulator enhanced shoot growth during the six weeks of cultivation, but inhibited root development (Fig. 5). Leaf dry weights were especially affected positively by TDZ. Although asiaticoside contents in leaves did not increase (Fig. 5), overall asiaticoside production was still higher than that from the control plants. Therefore, we believe the enhancing effect of TDZ on treated plants may have resulted from their higher biomass rather than from greater biosynthetic activity, thereby proving that exogenously applied TDZ does not directly affect asiaticoside biosynthesis in *C. asiatica*. Similar results have been reported with shoot cultures of *Artemisia annua*, in which artemisinin production was only indirectly affected by growth regulators because of their inhibition of root development (Ferreira and Janick, 1996).

This is the first report on the enhancement of asiaticoside production by supplemental growth regulators in whole-plant cultures of *C. asiatica*. Further research to estimate the influence of growth regulators on secondary metabolite synthesis may provide useful analytical data on the regulation of these glycosided triterpenes.

Received August 23, 2004; accepted October 25, 2004.

ACKNOWLEDGEMENT

This work was supported by a grant from the BioGreen 21 Program, Rural Development Administration, Korea.

LITERATURE CITED

Ahn JC, Kim ES, Lee HJ, Hwang B (1999) Effects of IAA, IBA, and media on growth and saikosaponin biosynthe-

sis in *Bupleurum falcatum* hairy root culture. Kor J Plant Tiss Cult 26: 171-175

Baek YW (1997) Micropropagation of *Centella asiatica* (L.) Urban by *in vitro* cultures and production of triterpene glycosides. Chonnam National University Press, Gwangju

Booncong P (1989) A pharmacognostic and taxonomic study of *Centella asiatica* (Apiaceae). Miami University Press, Miami

Brinkhaus B, Lindner M, Schuppan D, Hahn EG (2000) Chemical, pharmacological and clinical profile of the East Asian medical plant *Centella asiatica*. Phytomed 7: 427-448

Croes AF, van den Berg AJR, Bosveld M, Breteler H, Wullems GJ (1989) Thiophene accumulation in relation to morphology in roots of *Tagetes patula*. Effects of auxin and transformation by *Agrobacterium*. Planta 179: 43-50

Fasolo F, Zimmerman RH, Fordham I (1989) Adventitious shoot formation on excised leaves of *in vitro* grown shoots of apple cultivars. Plant Cell Tiss Org Cult 16: 75-87

Ferreira JFS, Janick J (1996) Roots as an enhancing factor for the production of artemisinin in shoot cultures of *Artemisia annua*. Plant Cell Tiss Org Cult 44: 211-217

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension culture of soybean root cells. Exp Cell Res 50: 195-202

Huetteman CA, Preece JE (1993) Thidiazuron: A potent cytokinin for woody plant tissue culture. Plant Cell Tiss Org Cult 33: 105-119

Kartnig T (1988) Clinical application of *Centella asiatica* (L.) Urb. *In* LE Craker, JE Simon, eds, Recent Advance in Botany, Horticulture and Pharmacology, Vol. 3. Oryx Press, Phoenix

Kim OT, Kim MY, Hong MH, Ahn JC, Oh MH, Hwang B (2002) Production of triterpene glycosides from whole plant cultures of *Centella asiatica* (L.) Urban. Kor J Plant Biotechnol 29: 275-279

Lian ML, Chakrabarty D, Peak DY (2002) Effect of plant growth regulators and medium composition on cell growth and saponin production during cell-suspension culture of Mountain Ginseng (*Panax ginseng* C.A. Mayer). J Plant Biol 45: 201-206

Massot B, Milesi S, Gontier E, Bourgaud F, Guckert A (2000) Optimized culture conditions for production of furanocoumarins by micropropagated shoots of *Ruta graveolens*. Plant Cell Tiss Org Cult 62: 11-19

Matsuda H, Morikawa T, Ueda H, Yoshikawa M (2001) Medicinal foodstuffs. XXVII. Saponin constituents Gotu Kola (2): Structures of new ursane- and oleanane-type triterpene oligoglycosides, centellasaponin B, C, and D, from *Centella asiatica* cultivated in Sri Lanka. Chem Pharm Bull 49: 1368-1371

Mesnard P (1975) Venotonic drugs with terpene active principles - three recent acquisitions. Israel Pharmaceut J 18: 330-337

Mork MC, Mork DWS, Turner JE, Mujer CV (1987) Biological and biochemical effects of cytokinin-active phenylurea derivatives in tissue culture system. HortSci 22: 1194-1197

Patra A, Rai B, Rout GR, Das P (1998) Successful plant regeneration from callus culture of *Centella asiatica* (L.) Urban. Plant Growth Regul 24: 13-16

Russell JA, McCown BH (1988) Recovery of plants from leaf protoplasts of hybrid-poplar and aspen clones. Plant Cell Rep 7: 59-62

Sélles M, Bergoñón S, Viladomat F, Bastida J, Codina C (1997) Effect of sucrose on growth and galanthamine production in shoot-clump cultures of *Narcissus confusus* in liquid-shake medium. Plant Cell Tiss Org Cult 49: 129-136

Sharma R, Jaiswal AN, Kumar S, Chaturvedi C, Tiwari PV (1985) Role of brahmi in educable mentally retarded children. J Res Educ India Med 1-6: 55-77

Stojakowska A, Kisiel W (1997) Production of parthenolide in organ cultures of feverfew. Plant Cell Tiss Org Cult 47: 159-162

Tiwari KN, Sharma NC, Tiwari V, Singh BD (2000) Micropropagation of *Centella asiatica* (L.), a valuable medicinal herb. Plant Cell Tiss Org Cult 63: 179-185

Yoshikawa T, Furuya T (1987) Saponin production by cultures of *Panax ginseng* with *Agrobacterium rhizogenes*. Plant Cell Rep 6: 449-453