

# Improvement of Pseudojубogenin Glycosides Production from Regenerated *Bacopa monnieri* (L.) Wettst. and Enhanced Yield by Elicitors

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*Bacopa monnieri* (L.) Wettst. was studied for shoot induction and regeneration on Murashige and Skoog (MS) medium supplemented with different plant growth regulators. Stem explants cultured on medium containing 0.1 mg/l thidiazuron (TDZ) resulted in the highest number of shoots (117 shoots/explant). Regenerated plants from medium with 0.5 mg/l TDZ contained the highest level of pseudojубogenin glycosides [(30.62 ± 1.29) mg/g dry wt] which was 2-fold higher than that of *in vitro* grown plants of the same age [(16.96 ± 1.49) mg/g dry wt]. Plantlets regenerated from 0.1 mg/l TDZ also showed a high level of pseudojубogenin glycosides [(27.94 ± 1.19) mg/g dry wt]. The effect of elicitor on pseudojубogenin glycosides accumulation in *B. monnieri* whole plant cultures was investigated. Chitosan at 150 mg/l and yeast extract at 2 mg/ml increased the pseudojубogenin glycosides production [(40.83 ± 2.24) mg/g dry wt and (40.05 ± 2.37) mg/g dry wt, respectively] after 7 days, which was 6-fold higher than in the control cultures.

**Key words:** Pseudojубogenin Glycosides, *Bacopa monnieri*, Elicitor

## Introduction

*Bacopa monnieri* (L.) Wettst. (Scrophulariaceae) has been used in traditional Ayurvedic medicine as a memory-enhancing, anti-inflammatory, analgesic, antipyretic and sedative drug. Recently, some pharmacological effects of *B. monnieri*, such as memory-enhancing (Das *et al.*, 2002; Sumathi *et al.*, 2002), antidepressant (Zhou *et al.*, 2007), antioxidant (Sinha and Saxena, 2006) and calcium antagonistic (Dar and Channa, 1999), have been reported. These pharmacological actions are mainly attributed to the saponin compounds present in the alcoholic extract of the plant. The major chemical constituents isolated and characterized from *B. monnieri* are dammaranes of triterpenoid saponins with pseudojубogenin glycosides or jубogenin glycosides (Chakravarty *et al.*, 2001, 2003; Garai *et al.*, 1996; Hou *et al.*, 2002).

*B. monnieri* has a great market demand due to its high medicinal values. Therefore, propagation of selected clones is necessary to guarantee the quality of the plant material. *In vitro* cultures of

*B. monnieri* for micropropagation have been reported previously (Shrivastava and Rajani, 1999; Tejavathi *et al.*, 2001; Mohapatra and Rath, 2005; Tiwari *et al.*, 2006), but there was no report on the saponin content from regenerated plants. In addition, the improvement of the pseudojубogenin glycosides production by means of tissue culture has not yet been reported. It would, therefore, be interesting to select plantlets yielding a high active compound through propagation. In this study, we investigated methods for multiple shoot regeneration and selection of regenerated *B. monnieri* with high yield of pseudojубogenin, including yield improvement of whole plant cultures by elicitors.

## Materials and Methods

### *Chemical and immunochemical*

Bacopaside I was purchased from Chromadex Inc. (Irvine, CA, USA). Peroxidase-labeled anti-mouse IgG was provided by ICN Pharmaceuticals (Costa Mesa, CA, USA). Human serum albumin (HSA),

6-benzyladenine (BA) and  $\alpha$ -naphthaleneacetic acid (NAA) were purchased from Fluka Chemical (Buchs, Switzerland). *N*-Phenyl-*N'*-1,2,3-thiadiazol-5'-yl urea (thidiazuron, TDZ) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were standard commercial products of analytical grade.

#### Plant material

*B. monnieri* was obtained from Faculty of Pharmaceutical Sciences, Naresuan University, Thailand. Plants were washed with sterile distilled water and surface-sterilized in 10% sodium hypochlorite for 15–20 min. Sterilized plants were washed with sterilized water three times and immersed in 70% ethanol for 1 min. Internode explants were cut and then cultured on hormone-free Murashige and Skoog (MS) medium containing 3% sucrose (w/v), pH 5.5, at  $(25 \pm 1)^\circ\text{C}$  under 16 h light/day. *In vitro* plants were subcultured on the same medium every 4 weeks.

#### Shoot induction and regeneration

Stem segments (0.5 cm) from *in vitro* plants were cultured on MS medium with plant growth regulators, *i. e.*, combinations of NAA (0.5–1 mg/l), BA (0.5–1 mg/l) and TDZ alone (0.1–1 mg/l). After 4 weeks, the regenerated shoots were subcultured on MS medium without hormones for elongation and rooting. The regenerated shoots were observed for rooting, and the pseudojubogenin glycosides content was determined after 4 weeks of culture. For each treatment, a total of 20 explants per treatment were tested. Analysis of variance was used to examine the effects of plant growth regulators on the induction of shoots. The data were collected using the least significant difference (LSD) test at  $P < 0.05$ .

#### Effect of elicitors on the pseudojubogenin glycosides production

Elicitation was carried out with yeast extract (YE), methyl jasmonate (MJ) and chitosan (CH). Each elicitor in the following final concentration was added: 1) MJ, 50, 100 and 200  $\mu\text{M}$ ; 2) YE, 0.5, 1.0 and 2.0 mg/ml; 3) CH, 50, 100 and 150 mg/l. Internodes of *in vitro* stock cultures were cut and cultured on MS medium without hormones. After 4 weeks, plantlets were subcultured on liquid MS medium and kept on a rotary shaker at 100 rpm. Cultures were incubated at  $(25 \pm 1)^\circ\text{C}$  under 16 h

light/day. Various concentrations of elicitors were added to whole plant liquid cultures and were harvested after culture for 7 d. Each experiment was done in triplicate.

#### Plant sample preparation and analysis

Dried samples (50 mg) of *B. monnieri* were powdered, extracted five times with 500  $\mu\text{l}$  of methanol with sonication for 15 min and centrifugation at 3,000 rpm for 1 min. The extracts were evaporated at  $60^\circ\text{C}$  until dryness. The dried extracts were dissolved 1 ml of methanol. The concentration of pseudojubogenin glycosides in *B. monnieri* cultures was determined by ELISA using polyclonal antibodies (PABs) against bacopaside I as previously reported (Phrompittayarat *et al.*, 2007).

#### Results and Discussion

Shoot induction was evaluated on MS medium with combinations of NAA and BA or TDZ alone using stem explants. After 4 weeks of culture, we succeeded to induce shoot formation from all combinations of plant growth regulators. The best result concerning the shoot number per explant (117 shoots/explant) was obtained in MS medium supplemented with 0.1 mg/l TDZ after 4 weeks of culture (Table I, Fig. 1A). This result showed that a higher level of TDZ in the medium reduced the number of shoots per explant (Table I). The number of shoots per explant using a low concentration of TDZ (0.1 mg/l) was high compared with

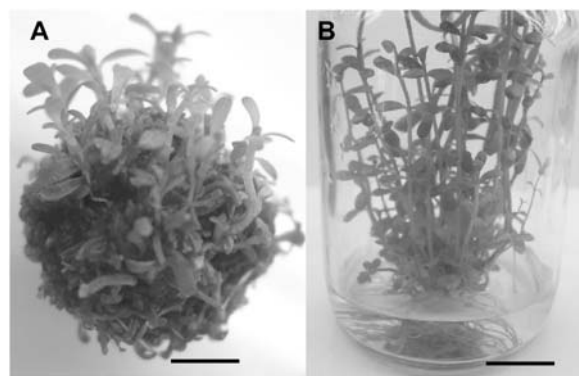


Fig. 1. Development of a regeneration system for *B. monnieri*. (A) Shoot organogenesis on MS medium with 0.1 mg/l TDZ. Bar: 5 mm. (B) Shoot growth after 4 weeks of subculture on MS medium without hormone. Bar: 20 mm.

Table I. Effect of plant growth regulators on the shoot induction from stem explants of *B. monnieri* and pseudojубogenin glycosides content in regenerated plants. Numbers with different letters are significantly different at  $P < 0.05$ .

Plant growth regulator [mg/l]			Number of shoots/explant*	Pseudojубogenin glycosides** [mg/g dry wt]
BA	NAA	TDZ		
–	–	0.1	116.8 <sup>a</sup>	27.94 ± 1.19
–	–	0.5	27.5 <sup>bc</sup>	30.62 ± 1.29
–	–	1	8.0 <sup>c</sup>	22.89 ± 2.17
0.5	1	–	11.0 <sup>c</sup>	4.29 ± 0.63
1	1	–	14.8 <sup>bc</sup>	6.87 ± 1.78
1	0.5	–	30.0 <sup>b</sup>	7.69 ± 1.74

\*  $n = 20$ .

\*\* Values represent the mean ± S.D. ( $n = 3$ ).

combinations of NAA and BA. Multiple shoots were elongated and rooted after being transferred to culture on MS medium without hormones for 4 weeks (Fig. 1B).

The effect of plant growth regulators on the pseudojубogenin glycosides production in regenerated plants has been studied. The pseudojубogenin glycosides content has been shown to increase significantly when TDZ was added under plant growth conditions (Table I). The pseudojубogenin glycosides content in plants regenerated from stem explants using 0.5 mg/l TDZ was (30.62 ± 1.29) mg/g dry wt which was 2-fold higher than that of *in vitro* grown plants of the same age [(16.96 ± 1.49) mg/g dry wt]. Plantlet regenerated from 0.1 mg/l TDZ also showed a high level of pseudojубogenin glycosides [(27.94 ± 1.19) mg/g dry wt]. On the other hand, plants regenerated from medium with NAA and BA contained a low level of pseudojубogenin glycosides (Table I).

TDZ has been shown to be an efficient plant growth regulator for shoot regeneration in various plants (Landi and Mezzetti, 2006; Lualon *et al.*, 2008). These results suggested that 0.1 mg/l TDZ shows high efficiency for the induction of shoot regeneration and improves production of pseudojубogenin glycosides in *B. monnieri*. Lualon *et al.* (2008) showed that addition of TDZ to the culture medium enhances artemisinin production in regenerated *Artemisia annua*. Similarly, in our study, the results showed that plantlets obtained from TDZ cultures have an increased level of pseudojубogenin glycosides. Therefore, the plant growth regulator TDZ may hold a potential to improve the production of pseudojубogenin glycosides.

We also studied the acclimation of *in vitro* plantlets to grow in open field culture. Plantlets regenerated from 0.1 mg/l and 0.5 mg/l TDZ were selected to be transferred for culture in the open field. Plants were harvested to determine their pseudojубogenin glycosides content by ELISA after 4 weeks of culture. After grown in the field, the pseudojубogenin glycosides contents of plants regenerated from 0.1 mg/l and 0.5 mg/l TDZ were (29.65 ± 2.93) mg/g dry wt and (43.87 ± 4.39) mg/g dry wt, respectively, compared with the intact plant (16-week-old) which had (12.33 ± 3.74) mg/g dry wt. The pseudojубogenin glycosides content of the *in vitro* plantlets and the open field-grown plants appeared to be correlated. It is suggested that the pseudojубogenin glycosides content in regenerated *B. monnieri* plantlets is maintained during transfer from *in vitro* to the open field culture. Therefore, the regeneration system using TDZ (0.1–0.5 mg/l) was optimal for plantlets yielding a higher content of pseudojубogenin glycosides.

We investigated the effect of elicitors on the pseudojубogenin glycosides accumulation in whole plant cultures of *B. monnieri* as shown in Fig. 2. Chitosan at 150 mg/l and yeast extract at 2 mg/ml gave a high content of pseudojубogenin glycosides [(40.83 ± 2.24) mg/g dry wt and (40.05 ± 2.37) mg/g dry wt, respectively] after 7 days, which was 6-fold higher than that of the control culture. In addition, yeast increased the pseudojубogenin glycosides production after 7 days in a dose-dependent manner (Fig. 2). The treatment with methyl jasmonate indicated no effect on the level of pseudojубogenin glycosides. Our results are similar to those of Palazon *et al.* (2003)

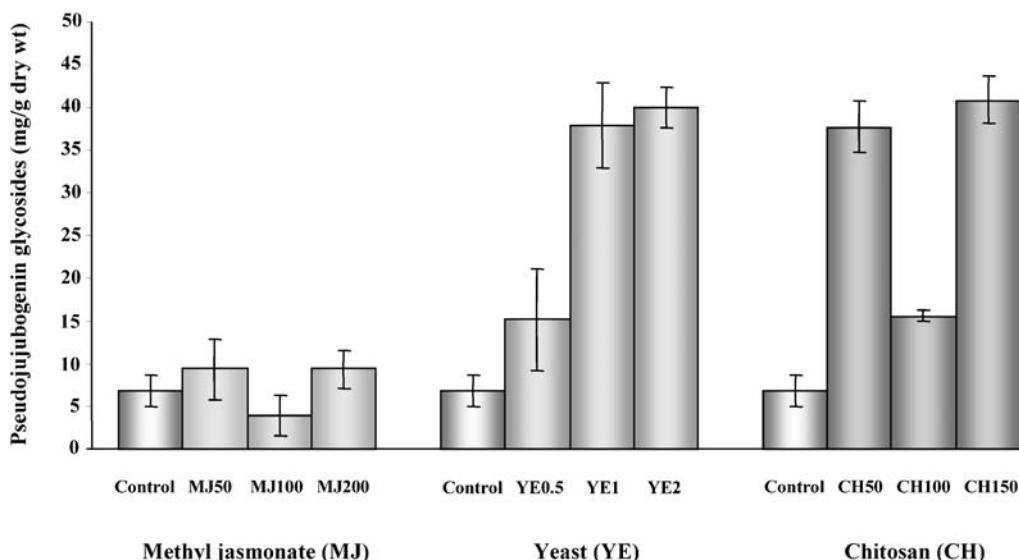


Fig. 2. Effect of elicitors on pseudojубogenin glycosides production in whole-plant cultures. Various concentrations of elicitors were added to whole-plant cultures of *B. monnieri* to treat them for 7 days. The values are means of triplicate results and error bars show standard deviations.

who have reported the stimulation of ginsenosides (triterpenoid saponin) in *Panax ginseng* hairy roots by chitosan. In our study, chitosan and yeast extract were effective in inducing of the pseudojубogenin glycosides production in whole plant cultures of *B. monnieri*.

In conclusion, our results make evident that MS medium supplemented with 0.1 mg/l TDZ can be used for the induction of high shoot formation and increased yields of pseudojубogenin glycosides

in *B. monnieri*. In addition, chitosan and yeast extract are suitable as elicitors for increased accumulation of pseudojубogenin glycosides in *B. monnieri* whole plant cultures.

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