Effect of Biotic Elicitors on Dicentrine Production in Cell Suspension Cultures of *Stephania suberosa* and *Stephania venosa*

ผลจากสารกระตุ้นชีวภาพต่อการผลิตสารไดเซนทรีนจากเซลล์แขวนลอยบอระเพ็ดพุงช้างและบอระเพ็ดยางแดง

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ABSTRACT

The objective of this study was to investigate dicentrine production in cell suspension cultures of two *Stephania* species and effect of biotic elicitors on dicentrine production in the cultures. The results showed that dicentrine average range in *S. venosa* suspension cultures was higher than *S. suberosa* cultures (15-25 and 0.4-1.0 mg/g dry weight, respectively). Elicitation by methyl jasmonate was not effect on dicentrine production in cell suspension cultures of both species except at concentration 100 µM at day 6 that methyl jasmonate could induce dicentrine content in *S. venosa* suspension cultures. Yeast extract at lower concentration in long time exposure enhanced dicentrine production in *S. suberosa*. In contrast, yeast extract at high concentration in short time exposure could induce dicentrine production in *S. venosa*.

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาการผลิตสารไดเซนทรีนในเซลล์แขวนลอยของบอระเพ็ดพุงช้าง และบอระเพ็ดยางแดง รวมทั้งผลของการใช้สารกระตุ้นชีวภาพต่อการผลิตสารไดเซนทรีน จากการศึกษาพบว่า เซลล์แขวนลอยบอระเพ็ดยางแดงผลิตไดเซนทรีนได้ปริมาณที่สูงกว่าเซลล์แขวนลอยบอระเพ็ดพุงช้าง โดยเฉลี่ยในช่วง 15-25 และ 0.4-1.0 มิลลิกรัมต่อกรัมของน้ำหนักแห้ง ตามลำดับ การใช้สารกระตุ้นชีวภาพด้วยเมทิลจัสโมเนทไม่มีผลกระตุ้นการผลิตไดเซนทรีนในเซลล์แขวนลอยของพืชทั้งสองชนิด ยกเว้น ในวันที่ 6 ของการกระตุ้น เมทิลจัสโมเนทความเข้มข้น 100 ไมโครโมลาร์สามารถกระตุ้นการสร้างสารในเซลล์แขวนลอยบอระเพ็ดยางแดงได้ ส่วนการเติมสารสกัดยีสต์ความเข้มข้นต่ำ ในระยะยาว เซลล์แขวนลอยบอระเพ็ดพุงช้าง มีแนวโน้มกระตุ้นการผลิตสารไดเซนทรีนเพิ่มขึ้น ซึ่งตรงข้ามกับบอระเพ็ดยางแดงที่การเติมสารสกัดยีสต์ความเข้มข้นสูง ในระยะสั้น มีแนวโน้มกระตุ้นการผลิตสารไดเซนทรีน

Key Words: Dicentrine, Secondary metabolite production, Biotic elicitor

คำสำคัญ: ไดเซนทรีน การผลิตสารทุติยภูมิ สารกระตุ้นชีวภาพ

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Introduction

The plants of genus *Stephania* (Menispermaceae) have been several used in traditional medicine for treatment many ailments (Semwal et al., 2010). *Stephania suberosa* has been used as longevity and neurotonic in Thai traditional remedy while methanolic extract from tuber has been reported as AChE inhibitory activity (Ingkaninan et al., 2003). Tuber of *Stephania venosa* has been used for neuronal function, diabetes, appetizer and ethanol extract from tuber showed anticancer and antioxidative activities (Leewanich et al., 2011).

Dicentrine, aporphine alkaloids, is the bioactive compound with various pharmacological activities such as acetylcholinesterase inhibition (Ingkaninan et al., 2003), antitumor effect (Stevigny et al., 2005), antiarrhythmic effect (Young et al., 1994) and antiplatelet effect (Chen et al., 1996).

Treatment cell with biotic elicitors are the strategies to enhance the production of several bioactive secondary metabolites in plant tissue cultures (Sharma et al., 2011). Biotic elicitors are organism derived substances which stimulate the defense mechanism and also the secondary metabolites in plant cells (Angelova et al., 2006).

In this study, cell suspension cultures of *S. suberosa* and *S. venosa* were established and the effects of biotic elicitors; methyl jasmonate and yeast extract on dicentrine production in cell suspension cultures of *S. suberosa* and *S. venosa* were compared and investigated.

Objectives of the study

1. To obtain *S. suberosa* and *S.venosa* cell suspension cultures and their growth curves.

2. To study the effect of biotic elicitors on dicentrine production in *S. venosa* and *S. suberosa* cell suspension cultures.

3. To investigate the relationship between dicentrine production in *S. suberosa* and *S.venosa* cell suspension cultures.

Methodology

Cell suspension cultures

S. suberosa seeds were washed with distilled water and soaked in 2.4 % sodium hypochlorite for 10 - 20 min. After that the seeds were washed three times with sterile distilled water in sterile condition and then soaked in 70% ethanol for 1 min and cultured in hormone free Murashige and Skoog (MS) medium. *In vitro* plantlets were obtained and their leaf and stem segments were induced to callus formation in various combination of thidiazuron (TDZ) and 1-naphthaleneacetic acid (NAA) in MS medium. The optimal ratio of these plant growth regulators were chosen for culture *S. suberosa* cell suspension in liquid MS media. All of the above methods were also used to obtain *S. venosa* cell suspension culture. The cultures were agitated on a rotary shaker that operated at 100 rpm with temperature at 25°C, light for 16 hours per day and were subcultured every month into fresh medium.

Growth curve of cell suspension cultures

After subculture into fresh liquid media with optimal supplement ratio, *S. suberosa* and *S. venosa* cell suspension cultures were harvested every week for six weeks. The cultures of each plant species were dried at 50°C. The dry weight of cultures were determined and prepared for dicentrine content analysis by HPLC method.

Biotic elicitation

As the exponential phase, 25 days old of *S. suberosa* and *S. venosa* cell suspension cultures were used to elicit with methyl jasmonate and yeast extract. Biotic elicitor concentrations in the experiment were shown in Table 1. After the elicitation, the cultures were harvested on day 3, 6 and 9.

Table 1 Concentration of biotic elicitors

Biotic elicitors	Concentration
Methyl jasmonate	50, 100 and 200 µM
Yeast extract	0.1, 0.2 and 0.5 mg/ml

Sample extraction

Dry culture samples were grinded and weighed to 30.0 ± 0.5 mg per microtube. And 0.5 ml methanol was added then sonicated for 15 minutes. The extracts were centrifuged at 3,000 rpm for 3 minutes and collected the supernatant. Repeat the extraction step for 3 times and then the overall supernatant of each sample was evaporated and re-dissolved with 1.0 ml methanol.

HPLC analysis

The HPLC system was used RP-18 column and Hewlett Packard series 1100 with UV/VIS detector, working wavelength 308 nm, at flow rate 1.0 ml/min by solvent system contained 35% v/v acetonitrile and 0.1% v/v aqueous trifluoroacetic acid to determine the dicetrine contents. Each sample was examined in triplicate. The data were analyzed statistically by one-way analysis of variance (ANOVA) and comparison with Tukey's HSD at probability level of 0.01.

Results

Optimal TDZ and NAA ratio for cell suspension cultures

In liquid MS media, the concentration at 0.1 mg/l TDZ and 0.5 mg/l NAA are the optimal ratio for culture *S. suberosa* cell suspension. While supplement with 0.5 mg/l TDZ and 1.0 mg/l NAA are used for *S. venosa* cell suspension cultures.

Growth curves of cell suspension cultures

The growth curve of two culture plant species were shown in line chart to display trend of growth as dry weight of cultures over time and column chart to compare dicentrine content across time period (Fig.1). The exponential phase of two culture plant species were between 21–35 days. And 25 days after subculture of both culture species was selected for elicitation treatment.

Effect of biotic elicitors

In Figure 2, all of varied concentrations of methyl jasmonate and yeast extract in *S. suberosa* cultures reduced dicentrine production every time period except yeast extract at concentration 0.1 mg/ml significantly increased dicentrine content (1.84±0.17 mg/g dry weight) more than control (1.44±0.16 mg/g dry weight) in day 9.

For day 3 of elicitation by methyl jasmonate in *S. venosa* cell suspension cultures, all these elicitor concentrations reduced dicentrine production. However, methyl jasmonate at concentration 100 µM significantly increased dicentrine content (18.5±1.43 mg/g dry weight) more than control (12.8±0.74 mg/g dry weight) at day 6 of elicitation (Fig.3).

Yeast extract was not improve dicentrine content in *S. venosa cultures* except at concentration 0.5

mg/ml in day 3 which significantly increased dicentrine (17.2±0.24 mg/g dry weight) more than control (14.5±1.21 mg/g dry weight) (Fig.3).

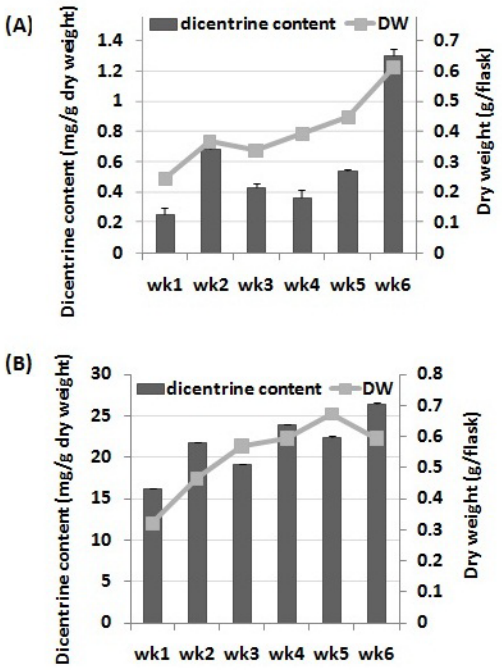

Fig. 1 Growth curve of (A) *S. suberosa* and (B) *S. venosa* cell suspension cultures

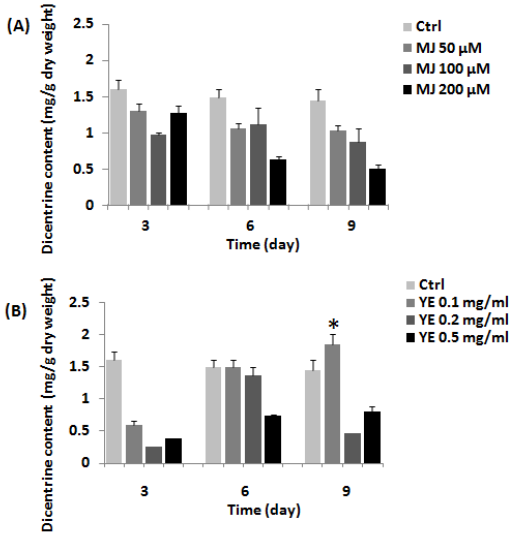

Fig. 2 Effect of (A) methyl jasmonate and (B) yeast extract on dicentrine production in *S. suberosa* cell suspension cultures

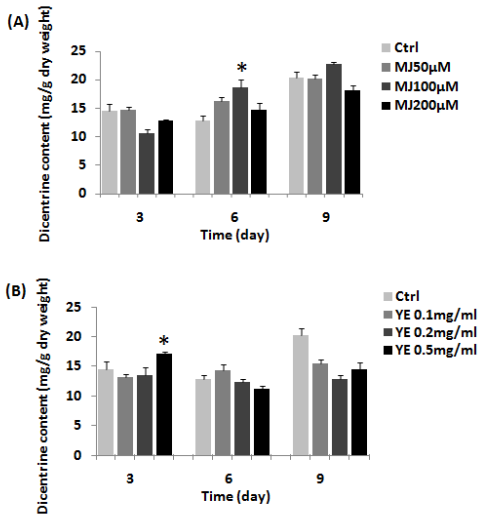

Fig. 3 Effect of (A) methyl jasmonate and (B) yeast extract on dicentrine production in *S. venosa* cell suspension cultures

Discussion and Conclusions

Plants in Menispermaceae and Lauraceae family are also founded dicentrine production such as flower buds and peduncles of *Lindera megaphylla* (Chen et al., 1995), fruits of *Ocotea puberula* (Montrucchio et al., 2012) and leaves of *Cissampelos capensis* (Wet et al., 2011) but those yields were lower than *S. venosa* cell suspension cultures (25 mg/g dry weight). And to compare dicentrine yield against the natural tubers of *S. venosa* and *S. suberosa* that are the part of used, dicentrine content in the tubers were investigated. The results showed that dicentrine in natural tubers of both *Stephania* species were also lower than *S. venosa* suspension cultures (data not shown). The range of dicentrine content on exponential phase of *S. suberosa* and *S. venosa* cell suspension are 0.4-1.0 and 15-25 mg/g dry weight, respectively. This study suggested that *S. venosa* cell suspension culture has potential to be alternative source of dicentrine production more than *S. suberosa.*

Exponential phase is the suitable period for elicit the plant cells because of enrich the precursors and ready to produce the secondary metabolites that why growth curve of two culture species are necessary in the study. (Savitha et al., 2006; Vasconsuelo and Boland, 2007)

Methyl jasmonate is the hormone regulating the defense mechanism in plant cell that act as signal transducer to induce secondary metabolites formation (Memelink et al. 2001) and has stimulated the expression of some common methyltransferases in benzylisoquinoline alkaloids pathway lead to increase its production (Frick and Kutchan 1999; Cho et al. 2008) In this work, methyl jasmonate could only induce dicentrine production in *S. venosa* cell suspension at concentration 100 µM at day 6 whereas in *S. suberosa*, all concentrations of this elicitor suppressed dicentrine production at all of time period, probably due to it could not induce specific enzymes related to dicentrine (aporphine alkaloids) biosynthesis in *Stephania* species such as (*S*)-corytuberine synthase; CYP80G2 (Ziegler et al., 2009).

The lowest concentration of yeast extract in long term exposure could increase dicentrine production in *S. suberosa* in contrast to *S. venosa* which was induced dicentrine content by highest concentration of yeast extract in short term exposure. This could be explained by spatio-temporal regulation of alkaloid biosynthesis (Facchini, 2001), therefore different plant species could be different specific enzyme activities such as norcoclaurine synthase activity in *Papaver rhoeas* and *Papaver somniferum* (Liscombe et al., 2005).

In conclusion, the pattern of elicitation between different plant species gave different results and *S. venosa* cell suspension cultures are the potential source for dicentrine production. Further study might investigate other effective biotic elicitors for more improvement on dicentrine production such as elicitors that derived from pathogen or endophytic microbe of *S. venosa*.

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