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Effects of Sodium Acetate and Sucrose on *in vitro* Alkaloid Production From *Stemona* Sp. Culture

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Abstract: Precursor feeding through plant tissue culture is provided to enhance plant secondary metabolite production. The effects of sodium acetate and sucrose on *Stemona* alkaloids were investigated. Shoot tips and auxiliary buds of *Stemona* sp. were cultured on MS agar medium supplemented with 3 mg L^{-1} benzyladenine for multiple shoot induction. Single shoots were then transferred to half-MS medium supplemented with 2 mg L^{-1} indolebutyric acid for root induction. *Stemona* plantlets were cultured on various concentrations of sodium acetate and sucrose and the alkaloids extract were determined. It was shown that both of them could enhance *Stemona* alkaloids production. The most effective precursor was 25 mg L^{-1} sodium acetate which gave the highest production of 1',2'-didehydrostemofoline and stemofoline at 2.35 and 2.04 folds higher than the control cultures, respectively. The study concluded that sodium acetate was the appropriate precursor than sucrose for enhancing *Stemona* alkaloids production.

Key words: 1', 2'-didehydrostemofoline, stemofoline, sodium acetate, sucrose, *Stemona* alkaloid production

INTRODUCTION

Thailand is a country that has an enormous range of plant diversity. The family Stemonaceae is one of the small monocotyledon families and it is a source of *Stemona* alkaloids which are valuable secondary metabolites produced from *Stemona* spp. The roots and leaves of the *Stemona* species possess pure alkaloid and extract derivatives that have been proven to be to natural insecticides (Tang *et al*., 2008). They also have antioxidant (Brem *et al*., 2004) and antitussive activities (Lin *et al*., 2008). There have been a variety *Stemona* alkaloids derived from *Stemona* spp. in Thailand that have been recorded, such as stemocurtisine, stemocurtisinol, oxyprotostemonine and stemokerrin (Pyne *et al*., 2007). A previously unidentified species of *Stemona* sp. had been found in Mae Moh District, Lampang Province, Thailand (a voucher specimen of this species was deposited at the Herbarium [Number 25375] of the Department of Biology, Chiang Mai University). An isolation of 1', 2'-didehydrostemofoline from this species was then recorded (Sastraruji *et al*., 2005). 1', 2'-didehydrostemofoline has been shown to have the highest level of inhibitory activity against acetylcholinesterase when it was compared to other *Stemona* alkaloids. This would indicate that this compound possesses significant potential as a therapeutic agent in the treatment of Alzheimer's disease (Baird *et al*., 2009). Stemofoline, another *Stemona* alkaloid that was derived from the *Stemona* sp., has also displayed inhibitory activity against acetylcholinesterase (Sastraruji *et al*., 2010). Additionally, stemofoline was shown to increase the potential of a variety of anticancer drugs which include vinblastine, paclitaxel and doxorubicin, in their capabilities to effectively be used to treat drug resistant cancer cells (Chanmahasathien *et al*., 2010). Therefore, *Stemona* alkaloids have been shown to be of significant interest for their potential to produce therapeutic anti-cancer medicines using plant tissue culture techniques. This is because up till now, alkaloid production that has been achieved from plants grown naturally has not always been reliable. The techniques of developing plant tissue cultures to increase the production of secondary metabolites, such as *Stemona* alkaloids from *Stemona curtisii* Hook, have been applied with a significant amount of success (Chotikadachanarong *et al*., 2011).

On the basis of biosynthetic pathways, several organic compounds have been added to the culture medium to enhance the synthesis of secondary metabolites. The overall yield of the desired product could potentially be increased by the exogenous supply of a biosynthetic precursor to the culture medium (Namdeo, 2007). There are many precursors that have been applied for the purpose of providing secondary metabolite production in plant cell cultures such as

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sodium acetate (Condori *et al*., 2010) and sucrose (Yu *et al*., 1996). In the present study, the effects of sodium acetate and sucrose on the growth of *Stemona* sp. and biosynthesis of *Stemona* alkaloids were examined.

MATERIALS AND METHODS

Plant materials: Before being washed three times with sterile distilled water, the shoot tips and auxiliary buds of *Stemona* sp. were surface sterilized with a 15% clorox solution for 20 min. They were then cultured on MS (Murashige and Skoog, 1962) agar medium supplemented with 3 mg L^{-1} benzyladenine for multiple shoot induction. Single shoots were then transferred to half-MS medium which was supplemented with 2 mg L^{-1} indolebutyric acid, for the purpose of root induction (Chaichana *et al*., 2011). Finally, the cultures were then moved to a growth room and kept at 25±2°C under a daily photo period of 16 h.

Feeding precursors: Eight-week old *in vitro Stemona* plantlets were cultured for 1 and 2 weeks in liquid MS medium supplemented with 25, 50, 100 and 200 mg L^{-1} sodium acetate and 40, 50, 60 and 70 g L^{-1}sucrose. The control treatment was liquid MS medium with 30 g L^{-1} sucrose without sodium acetate. All the experiments described above were performed in triplicate. Both the roots as well as the cultured media from all treatments were extracted and then analyzed for the presence of *Stemona* alkaloids. The total amount of *Stemona* alkaloids that were produced was then expressed as the sum of the amount of alkaloids found to be present in both the roots and the medium.

***Stemona* alkaloids extraction and analysis:** The dry roots of *Stemona* sp. were ground and extraction was performed three times using methanol (Merck, HPLC grade, Germany). In order to obtain a crude extract which was further extracted with dichloromethane (DCM), the solution was filtered and then evaporated (Merck, HPLC grade Germany). In the next step, the crude DCM extract was dissolved and then passed through a 0.45 μm membrane filter. The quantification of *Stemona* alkaloids by HPLC (Agilent 1200 series, Palo Atto, CA, USA) was then performed. The Agilent Chemstation Software was used for the purposes of data acquisition analysis. This experimental stage utilized an Inertsil C18 ODS-3 5 μm particle size, 4.6×150 mm column (GL Sciences Inc., Japan). A methanol and water mixture was used in the mobile phase under gradient elution conditions (Table 1), at a flow rate of 0.5 mL min^{-1}. At 297 nm, the detection of compounds was achieved. An injection of 20 μL of the sample was made into the chromatography system. Within a period of 27 min, an analysis was achieved. After a comparison of the retention times with the *Stemona* alkaloid standards, *Stemona* alkaloids were able to be identified. Thus, it was revealed that the respective retention times of both the 1',2'-didehydrostemofoline and stemofoline standards were 11.5 and 18.2 min. By comparing the areas under the corresponding peaks of the standard curves that were prepared at a concentration range of 25-1000 ng L^{-1}, quantification was able to be achieved.

Table 1: HPLC condition gradient table of *Stemona* alkaloids separation

Time (min)	Methanol (%)	Water (%)	Flow rate (mL min^{-1})
7	70	30	0.5
7.5	10	90	0.5
12	10	90	0.5
13	70	30	0.5
20	70	30	0.5

Statistical analysis: Using the analysis of variance (ANOVA) with necessary adjustments for multiple comparisons with Turkey's test, statistical analysis was able to be determined. A p-value of less than 0.05 was used to determine and identify significant differences. All data have been expressed as an average of three independent experiments.

RESULTS AND DISCUSSION

Effect of sucrose on root growth and *Stemona* alkaloid production: Sucrose was efficient to promote high phytoestrogens production of *Psoralea corylifolia* suspension culture compared to other carbon sources (Shinde *et al*., 2009). It was therefore decided to select sucrose as carbon source for further studies. For the effect on root growth, it was shown that sucrose could increase root growth for 1 week after transfer. However, the root growth after transferring for 2 weeks decreased (Table 2). This was probably due to the influence of osmotic stress. Increasing the sucrose concentration also decreased the cell density in suspended cell cultures of strawberry for 13 days (Sato *et al*., 1996). For *Stemona* alkaloids production, the highest 1',2'-didehydrostemofoline and stemofoline production was found on 40 g L^{-1} sucrose for 1 week cultured at 1.98 and 2.03 folds of that in the control, respectively (Table 2). The effect of sucrose feeding on taxane accumulation in cell culture of *Taxus chinensis* was successfully achieved on day 7 and produced very high taxane of 274.4 mg L^{-1} (Wang *et al*., 1999). Sucrose also gave maximal total anthraquinone production by *Morinda elliptica* cell cultures (Abdullah *et al*., 1998). Moreover, saponin biosynthesis by suspension cultures of *Panax ginseng*

Table 2: Effects of various concentrations of sucrose on 1',2'-didehydrostemofoline and stemofoline production

	1 week cultured period				2 week cultured period			
Sucrose	Growth (g DW)	Root extract (μg g^{-1} DW)	Medium extract (μg g^{-1} DW)	Total (μg g^{-1} DW)	Growth (g DW)	Root extract (μg g^{-1} DW)	Medium extract (μg g^{-1} DW)	Total (μg g^{-1} DW)
1',2'-didehydrostemofoline								
Control	0.152±0.065^{a}	23.573±0.750^{ab}	0.777±0.056	24.350±0.663^{ab}	0.221±0.019^{a}	22.500±0.670^{a}	0.342±0.068	22.842±0.572^{a}
40 g L^{-1}	0.188±0.009^{bc}	48.320±0.452^{a}	ND*	48.320±0.452^{a}	0.160±0.009^{a}	64.175±0.164^{a}	ND	41.525±0.164^{a}
50 g L^{-1}	0.232±0.009^{a}	22.630±0.344^{ab}	ND	22.630±0.344^{ab}	0.170±0.009^{a}	37.797±0.298^{a}	ND	37.797±0.298^{a}
60 g L^{-1}	0.216±0.016^{ab}	12.467±0.163^{b}	ND	12.467±0.163^{b}	0.184±0.011^{a}	15.470±0.572^{a}	ND	15.470±0.572^{a}
70 g L^{-1}	0.200±0.009^{ab}	42.293±1.063^{ab}	ND	42.293±1.063^{ab}	0.202±0.008^{a}	21.780±0.276^{a}	ND	21.780±0.276^{a}
Stemofoline								
Control	0.152±0.065^{a}	57.600±0.612^{b}	11.580±0.318^{a}	69.180±0.634^{b}	0.221±0.019^{a}	78.167±0.559^{ab}	4.476±0.032^{b}	82.642±0.594^{ab}
40 g L^{-1}	0.188±0.009^{bc}	137.067±1.271^{a}	3.514±0.011^{b}	140.581±1.056^{a}	0.160±0.009^{a}	107.242±0.651^{ab}	7.373±0.072^{ab}	114.615±0.606^{ab}
50 g L^{-1}	0.232±0.009^{a}	87.420±0.610^{ab}	3.859±0.073^{b}	91.279±0.602^{ab}	0.170±0.009^{a}	98.600±0.631^{ab}	10.460±0.021^{a}	109.060±0.668^{ab}
60 g L^{-1}	0.216±0.016^{ab}	106.608±1.282^{ab}	4.462±0.568^{b}	111.070±1.645^{ab}	0.184±0.011^{a}	117.910±0.904^{ab}	5.216±0.038^{b}	123.126±0.908^{a}
70 g L^{-1}	0.200±0.009^{ab}	75.132±0.702^{ab}	5.388±0.808^{b}	80.520±1.325^{ab}	0.202±0.008^{a}	72.180±0.883^{b}	4.577±0.096^{b}	76.757±0.837^{b}

ND: Non-detected, the data are Means±SD of triplicates, similar letters in a column indicate as significant difference at $p<0.05$

Table 3: Effects of various concentrations of sodium acetate on 1',2'-didehydrostemofoline and stemofoline production

	1 week cultured period				2 week cultured period			
Sodium acetate	Growth (g DW)	Root extract (μg g^{-1} DW)	Medium extract (μg g^{-1} DW)	Total (μg g^{-1} DW)	Growth (g DW)	Root extract (μg g^{-1} DW)	Medium extract (μg g^{-1} DW)	Total (μg g^{-1} DW)
1',2'-didehydrostemofoline								
Control	0.152±0.065^{a}	23.573±0.750^{ab}	0.777±0.056	24.350±0.663^{ab}	0.221±0.019^{a}	22.500±0.670^{a}	0.342±0.068	22.842±0.572^{a}
25 mg L^{-1}	0.189±0.011^{ab}	57.323±0.103^{a}	ND*	57.323±0.103^{a}	0.154±0.012^{ab}	8.680±0.069^{a}	ND	8.680±0.069^{a}
50 mg L^{-1}	0.186±0.012^{ab}	15.925±0.298^{b}	ND	15.925±0.298^{b}	0.179±0.011^{ab}	9.765±0.088^{a}	ND	9.765±0.088^{a}
100 mg L^{-1}	0.191±0.009^{ab}	30.140±0.865^{b}	ND	30.140±0.865^{b}	0.126±0.008^{b}	16.427±0.123^{a}	ND	16.427±0.123^{a}
200 mg L^{-1}	0.218±0.010^{a}	36.180±0.957^{b}	ND	36.180±0.957^{b}	0.175±0.010^{ab}	18.900±0.168^{a}	ND	18.900±0.168^{a}
Stemofoline								
Control	0.152±0.065^{a}	57.600±0.612^{b}	11.580±0.318^{a}	69.180±0.634^{b}	0.221±0.019^{a}	78.167±0.559^{ab}	4.476±0.032^{b}	82.642±0.594^{ab}
25 mg L^{-1}	0.189±0.011^{ab}	139.297±0.940^{a}	2.026±0.277^{b}	141.323±1.209^{a}	0.154±0.012^{ab}	68.787±0.313^{b}	8.095±0.094^{b}	76.881±0.337^{b}
50 mg L^{-1}	0.186±0.012^{ab}	93.600±1.238^{b}	2.590±0.080^{b}	96.190±1.017^{b}	0.179±0.011^{ab}	57.330±0.408^{b}	3.085±0.723^{d}	60.415±1.029^{b}
100 mg L^{-1}	0.191±0.009^{ab}	87.487±0.790^{bc}	2.036±0.284^{b}	89.523±0.889^{b}	0.126±0.008^{b}	104.207±1.120^{a}	12.490±0.032^{a}	116.697±1.088^{a}
200 mg L^{-1}	0.218±0.010^{a}	86.940±1.383^{bc}	2.865±0.024^{b}	89.085±1.415^{b}	0.175±0.010^{ab}	72.500±0.978^{b}	6.347±0.009^{bc}	78.847±1.020^{b}

ND: Non-detected, the data are Means±SD of triplicates, similar letters in a column indicate as significant difference at $p<0.05$

was stimulated by sucrose with the maximum saponin production of 275 mg L^{-1} (Akalezi *et al*., 1999). For exogenous accumulation, sucrose had no effect on the discharge 1',2'-didehydrostemofoline into the medium but affective for stemofoline (Table 2). Stemofoline was found to release into the medium. Similar experiment revealed that plant secondary metabolites increased with sucrose supplement. For example, it could produce tropine alkaloids in hairy root cultures of *Datura stramonium* (Jaziri *et al*., 1988) and anthocyanin production from *Perilla frutescens* cell culture (Zhong and Yoshida, 1995).

Effect of sodium acetate on root growth and *Stemona* alkaloids production: It was found that the root growth increased when cultured at 1 week but decreased at 2 weeks (Table 3). It was shown that 25 mg L^{-1} sodium acetate at 1 week culture was an optimal concentration for *Stemona* alkaloids biosynthesis. 1',2'-didehydrostemofoline and stemofoline content reached 2.35 and 2.04 folds of that in the control, respectively (Table 3). Sodium acetate was also found to increase phenylethanoid glycosides production of *Cistanche deserticola* cell culture. The highest production was 1.5 folds of the control. On the other hand, sodium acetate had slightly negative effects on cell growth (Ouyang *et al*., 2005). Sodium acetate was also found to enhance phytoecdysteroids biosynthesis in *Ajuga turkestanica* hairy root cultures approximately 2 folds (19.9 μg mg^{-1}) compared to control cultures (Cheng *et al*., 2008). Moreover, sodium acetate is known to be a potential precursor for various secondary metabolites such as stevioside (Dheeranupattana *et al*., 2007) and hinokitiol (Fujii *et al*., 1995). It was found to stimulate *Stemona* alkaloids into the media (Table 3). Similarly, addition of sodium acetate into *Morinda elliptica* cell cultures for 6 days not only increased intracellular anthraquinones production (124 mg DW^{-1}) but also increased extracellular production (14.3 mg L^{-1}) (Chiang and Abdullah, 2007). The high concentration of sodium acetate gave negative effect on 1',2'-didehydrostemofoline and stemofoline accumulation. It is noted that excess precursors may cause feedback inhibition to the metabolic pathway. Therefore, the minimum sodium acetate concentration of 25 mg L^{-1} was appropriate. In addition, feeding of sodium acetate might increase the production of *Stemona* alkaloids by

increasing the levels of acetyl-Co A which may ultimately lead to enhance the alkaloids production. Sodium acetate may effect the enzyme for metabolite production as investigated before. It enhanced β-glucosidase activity involving in the scent compound formation of *Delphinium elatum* L. (Yang *et al*., 2009).

CONCLUSION

Both sodium acetate and sucrose significantly promote in *Stemona* alkaloids production. Addition of sodium acetate at 25 mg L^{-1} into the medium provided higher *Stemona* alkaloids production than addition of sucrose. It could produce 1',2'-didehydrostemofoline and stemofoline 2.35 and 2.04 folds respectively higher amounts than the control.

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