Influence of Sodium Acetate on *Stemona* Alkaloid Production in Hydroponic Culture of *Stemona curtisii* Hook.f.

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ABSTRACT

To evaluate a potential method for the improvement of alkaloids production in *Stemona curtisii*, the influence of sodium acetate was studied in the modified hydroponic culture. Eight week–old *S. curtisii* plantlets from *in vitro* culture were used as plant materials. Different concentrations of sodium acetate at 0, 10, 20 and 50 mg/L were added into the culture medium. The samples of roots and culture medium in each treatment were collected on week 2 and week 4 after the elicitor addition and then analyzed for *Stemona* alkaloids production namely oxyprotostemonine, stemocurtisine and stemocurtisinol by high performance liquid chromatography. The HPLC result showed that the highest contents of total stemocurtisine (32.24 µg/g DW) and stemocurtisinol (89.46 µg/g DW) were detected in the treatment with 10 mg/L sodium acetate for 4 weeks, which were 1.8 and 1.4 fold significantly higher than the control, respectively. While, the treatment with 20 mg/L sodium acetate for 2 weeks had the highest content of total oxyprotostemonine, which was 233.65 µg/g DW (1.4 fold higher than the control). From this research, it was demonstrated that sodium acetate had a positive effect on the enhancement of these *Stemona* alkaloids production.

Keywords: *Elicitor, Hydroponic culture, Sodium acetate, Stemona alkaloid, Stemona curtisii*

INTRODUCTION

S. curtisii or "Non Tai Yak" (the Thai vernacular name) is one of the Thai insecticidal plants in the family Stemonaceae. This family is the only source of the unique alkaloids namely *Stemona* alkaloids (Zhou *et al*., 2006). The studies of biological activities of the *S. curtisii* root extracts or *Stemona* alkaloids have been reported by many researchers (Mungkornasawakul *et al*., 2004; Limtrakul *et al*., 2007). Kaltenegger *et al*. (2003) found that the root extracts of *S. curtisii* were shown to have insecticidal activity against *Spodoptera littoralis*. While, Sastraruji (2006) reported that the *S. curtisii* root extracts showed strong insect antifeedant activities on *Spodoptera littoralis*. Moreover, the utilization of *S. curtisii* extract as natural pesticide was examined. It was found that this pesticide was effective against leaf eating beetle (*Phyllotreta chontanica*), diamondback moth (*Plutella xylostella*), green aphid (*Lipaphis erysimi*), cabbage looper (*Trichoplusia ni*) and common cutworm (*Spodoptera littoralis*) in agricultural field trials (Sastraruji, 2006).

Hydroponic culture has been used for acclimatization of *in vitro* plantlets and improvement in the growth of various plants (Palee *et al*., 2012; Nhut *et al*, 2004). Moreover, Hydroponics is a new technique for the enhancement of secondary

metabolites production in plants such as genistein in *Lupinus luteus* (Kneer *et al*, 1999) and isoflavones in *Pueraria montana* (Kirakosyan *et al*., 2006). In hydroponic culture of *Datura innoxia*, Gontier *et al*. (2002) reported that high amount of tropane alkaloids was found in the culture medium. This result revealed that the secondary metabolites was not only extracted from the roots but also extracted from the medium. Thus, the plants could be continuously reused for secondary metabolites production. Furthermore, the addition of some elicitors in hydroponic cultures is one of the strategies employed to increase production of secondary metabolites. Elicitors could be used as enhancers for plant secondary metabolites production and play an important role in biosynthetic pathways to enhance production of commercially important compounds (Angelova *et al*, 2006). Sodium acetate is one of the elicitor which have been used to enhance secondary metabolites production in some plants, i.e. taxol in *Taxus chinensis* var. *mairei* (Wu *et al*., 1999), azadirachtin in *Azadirachta indica* (Balaji *et al*, 2003), artemisinin in *Artemisia annua* (Baldi and Dixit, 2008) and guggulsterone in *Commiphora wightii* (Mathur and Ramawat, 2008). In *Crocus sativus*, Zeng *et al*. (2003) found that the treatment with 1 mM sodium acetate increased crocin content in stigma-like-structure up to 6.00% above 2.21% in control medium. This is because sodium acetate is a potential precursor of crocin biosynthesis. In *S. curtisii* root cultures, Chotikadachanarong (2011) reported that the best contents of oxyprotostemonine and stemocurtisinol were detected in the treatment with 20 mg/L sodium acetate for 16 weeks while the treatment with 50 mg/L sodium acetate for 16 weeks yielded the highest content of stemocurtisine. For *Stemona* sp., Jadwadtanakul (2012) reported that the highest contents of stemofoline and 1′, 2′-didehydrostemofoline were observed in the treatment with 20 mg/L sodium acetate for one week. However, those reports have studied the effect of sodium acetate in tissue culture and no previous studies have examined their effects in hydroponics.

Hence, the aim of this research was to investigate the effect of sodium acetate on the improvement of *Stemona* alkaloid production especially oxyprotostemonine stemocurtisine and stemocurtisinol in the modified hydroponic cultures of *S. curtisii* Hook.f. It was also hope that the findings from the present endeavor could lead to identifying a possible method for the production of secondary metabolites in *Stemona* plants.

METHODOLOGY

Plant Materials and Hydroponic Culture

The intact plants of *S. curtisii* (Figure 1A) were collected from Trang Province in Thailand. *In vitro* culture of *S. curtisii* was used to produce plantlets following the method described by Palee *et al*. (2012). *In vitro* shoots were cultured on MS (Murashige and Skoog, 1962) solid media supplemented with 1 mg/L naphthalene acetic acid for 8 weeks to induce roots. Then, eight week–old plantlets were used as plant materials (Figure 1B), which were transferred into the modified hydroponic culture (Figure 1C). These plantlets were washed with running tap water to remove agar on the roots, then soaked with 0.2% Carbendazim solution for 30 minutes, and then finally transplanted to hydroponic trays having a mixture of perlite and vermiculite in the ratio of 1:1 (w/w). Aeration of the nutrient solution was accomplished by aquarium pump.

Figure 1 (A) The intact plants of *S. curtisii* (B) *In vitro S. curtisii* plantlets (C) The modified hydroponic culture

Feeding of Sodium Acetate in Hydroponic Culture

The different concentrations of sodium acetate (10, 20 and 50 mg/L) were added to the culture medium. Non elicitor treatment was referred to control condition. All treatments were kept in the greenhouse. The samples of roots and culture medium in each treatment were harvested 2 times on week 2 and week 4 after sodium acetate addition. Then, the contents of Stemona alkaloids especially oxyprotostemonine stemocurtisine and stemocurtisinol in the roots and the medium were analyzed by HPLC.

Root and Medium Extraction

The dried roots of *S. curtisii* were ground and extracted three times with methanol (MeOH). While, the samples of culture medium were extracted three times with dichloromethane (DCM). The solution of roots and medium extracts was filtered and then evaporated to give a crude extract, which was dissolved in 1 mL of MeOH and 1 mL of water before extraction with DCM, and then evaporated to give a partially purified extract (DCM crude extract). After that, the DCM crude extract of roots and culture medium was analyzed by HPLC.

Quantification of *Stemoma* Alkaloids by HPLC

Analytical HPLC was performed using an Agilent 1200 series. Chromatographic separation was achieved with a C_{18} column (Inertsil ODS-3, 5 µm, 4.6 I.D. ×150 mm, GL sciences Inc., Japan). Data acquisition and analysis were performed by the Agilent ChemStation software. HPLC condition of three *Stemona* alkaloids namely oxyprotostemonine, stemocurtisine and stemocurtisinol was conducted as reported by Palee *et al.* (2013). The mobile phase was Milli-Q water and MeOH (30:70, v/v), at a flow rate of 1 mL/min with an injection volume of 20 µL and UV detection at 297 nm. The standards of oxyprotostemonine, stemocurtisine and stemocurtisinol were isolated from *S. curtisii* root extracts, which the purity of those *Stemona* alkaloid standards was shown in Table 1. Quantification was based on the external standard method using calibration curves. The standard solutions of three *Stemona* alkaloids were prepared in methanol. Each concentration of standard solution was injected onto the HPLC column in triplicate. Then, the mean peak areas for each

concentration were calculated and the standard calibration curves were constructed by plotting concentrations against the peak areas.

Table 1 The purity and retention time of *Stemona* alkaloid standards.

Stemona alkaloids	% Purity of standards (based on peak area)	Retention time of standards (minute)
Oxyprotostemonine	97.3%	2.9 min
Stemocurtisine	92.1%	3.4 min
Stemocurtisinol	96.3%	4.8 min

Statistical Analysis

The experiment was laid out in completely randomized design (CRD). All the experiments were conducted in three replicates with 15 plantlets per treatment. The values are expressed as the Mean±SD. The data were analysed using one-way analysis of variance by means comparison by Turkey test method. All statistical tests were considered significant at $P \leq 0.05$. Not – detected (ND) is defined as the *Stemona* alkaloids content was under the minimum detection of standard calibration curve.

RESULTS AND DISCUSSION

Effect of Sodium Acetate on the production of *Stemona* alkaloids in the roots

When 10 – 50 mg/L sodium acetate was added to the medium for 2 weeks, the HPLC result showed that all concentration levels of sodium acetate increased the production of oxyprotostemonine and stemocurtisine in the roots (Table 2). The content of stemocurtisine in the roots increased with increasing sodium acetate concentration from 10 to 50 mg/L. The highest content of stemocurtisine (24.24 ± 0.72 µg/g DW) was detected in the roots treated with 50 mg/L sodium acetate, which was 1.8 fold higher than the control. While, the roots treated with 20 mg/L sodium acetate showed the highest contents of oxyprotostemonine (228.94 µg/g DW) and stemocurtisinol (68.01 µg/g DW), which were 1.4 and 1.2 fold significantly higher than the control. After the roots were treated with sodium acetate for 4 weeks, it was found that the maximum contents of oxyprotostemonine (221.93 µg/g DW), stemocurtisine (32.24 µg/g DW) and stemocurtisinol (88.27 µg/g DW) in the roots were found in the treatment with 10 mg/L sodium acetate, which were 1.6, 1.8 and 1.4 fold significantly higher than the control, respectively. However, the content of oxyprotostemonine in the roots on week 2 was greater than that on week 4. These results revealed that sodium acetate had a positive effect on the production of these *Stemona* alkaloids in the roots, in agreement with the result reported by Chotikadachanarong (2011) in *S. curtisii* and Jadwadtanakul (2012) in *Stemona* sp.

Table 2 The effect of sodium acetate concentration on the production of three *Stemona* alkaloids in the roots.

week	Concentration of sodium acetate	*Stemona* alkaloid contents in the roots (µg/g DW)		
		Oxyprotostemonine	Stemocurtisine	Stemocurtisinol
2	Control	158.05 ± 3.28^{d}	13.34 ± 0.35^{e}	57.66 ± 1.19^{e}
	10 mg/L	193.46 ± 0.55^{b}	17.89 ± 0.32^{d}	61.62 ± 0.66^{de}
	20 mg/L	**228.94 ± 2.68^{a}**	19.44 ± 0.51^{d}	68.01 ± 0.65^{c}
	50 mg/L	161.86 ± 3.30^{d}	24.24 ± 0.72^{c}	47.86 ± 0.73^{f}
4	Control	135.44 ± 0.73^{e}	17.59 ± 0.59^{d}	64.20 ± 1.21^{cd}
	10 mg/L	**221.93 ± 1.69^{a}**	**32.24 ± 0.71^{a}**	**88.27 ± 1.37^{a}**
	20 mg/L	166.30 ± 0.44^{cd}	23.51 ± 0.36^{c}	67.30 ± 0.79^{c}
	50 mg/L	174.67 ± 2.80^{c}	27.11 ± 0.48^{b}	78.40 ± 1.28^{b}

Values expressing the Mean ± SD followed by similar letters in a column indicates as non-significantly difference at $p < 0.05$.

Effect of Sodium Acetate on the secretion of *Stemona* alkaloids into the medium

The contents of three *Stemona* alkaloids were also observed in the culture medium (Table 3). When adding sodium acetate to the medium for 2 weeks, it was found that sodium acetate did not have a positive effect on the secretion of three *Stemona* alkaloids into the medium. On the other hand, after 4 weeks of elicitation, the treatment with sodium acetate stimulated the excretion of oxyprotostemonine and stemocurtisinol from roots into the culture medium. The maximum content of oxyprotostemonine (9.72 µg/g DW) was detected in the treatment with 20 mg/L sodium acetate, which was 1.8 fold higher than the control. Meanwhile, the elicitation by 50 mg/L sodium acetate showed the maximum content of stemocurtisinol (1.83 µg/g DW), which was 1.5 fold higher than the control. However, sodium acetate in concentration of 10 – 50 mg/L did not have positive effect on the secretion of stemocurtisine into the medium.

Table 3 The effect of sodium acetate concentration on the secretion of three *Stemona* alkaloids into the medium.

week	Concentration of sodium acetate	*Stemona* alkaloid contents in the medium (µg/g DW)		
		Oxyprotostemonine	Stemocurtisine	Stemocurtisinol
2	Control	5.17 ± 0.03^{f}	ND*	1.45 ± 0.03^{b}
	10 mg/L	6.10 ± 0.06^{c}	ND	0.66 ± 0.02^{e}
	20 mg/L	4.71 ± 0.06^{g}	ND	0.91 ± 0.03^{d}
	50 mg/L	3.78 ± 0.03^{h}	ND	0.57 ± 0.02^{e}
4	Control	5.50 ± 0.05^{e}	ND	1.21 ± 0.04^{c}
	10 mg/L	5.81 ± 0.07^{d}	ND	1.19 ± 0.04^{c}
	20 mg/L	**9.72 ± 0.04^{a}**	ND	1.58 ± 0.03^{b}
	50 mg/L	7.59 ± 0.05^{b}	ND	**1.83 ± 0.03^{a}**

Values expressing the Mean ± SD followed by similar letters in a column indicates as non-significantly difference at $p < 0.05$. * ND = Not-detected.

Effect of Sodium Acetate on the enhancement of *Stemona* alkaloids production

Total contents of three *Stemona* alkaloids are shown in Table 4. The results displayed that the elicitation by 20 mg/L sodium acetate for 2 weeks resulted in the maximum content of total oxyprotostemonine (233.65 µg/g DW), which was 1.4 fold significantly higher than the control. While, the highest contents of total stemocurtisine (32.24 µg/g DW) and stemocurtisinol (89.46 µg/g DW) were found in the treatment with 10 mg/L sodium acetate for 4 weeks, which were 1.8 and 1.4 fold significantly higher than the control, respectively. A positive effect of sodium acetate on *Stemona* alkaloids production was also reported by Jadwadtanakul (2012) in tissue culture of *Stemona* sp. It was found that sodium acetate enhanced the production of stemofoline and 1′, 2′- didehydrostemofoline in the roots and also stimulated the secretion of these alkaloids into the medium. The highest contents of total stemofoline and 1′, 2′-didehydrostemofoline were observed in the treatment with 20 mg/L sodium acetate for one week. In *S. curtisii*, sodium acetate increased *Stemona* alkaloid content in the roots but it did not stimulate the secretion of these alkaloids into the medium. The highest contents of oxyprotostemonine and stemocurtisinol were detected in the treatment with 20 mg/L sodium acetate for 16 weeks. While the treatment with 50 mg/L sodium acetate for 16 weeks revealed the highest content of stemocurtisine (Chotikadachanarong, 2011).

Table 4 Total contents of three *Stemona* alkaloids.

week	Concentration of sodium acetate	Total contents of *Stemona* alkaloid (Roots + Medium) (µg/g DW)		
		Oxyprotostemonine	Stemocurtisine	Stemocurtisinol
2	Control	163.22 ± 3.26^{e}	13.34 ± 0.35^{e}	59.11 ± 1.19^{e}
	10 mg/L	199.56 ± 0.55^{b}	17.89 ± 0.32^{d}	62.28 ± 0.65^{de}
	20 mg/L	**233.65 ± 2.64^{a}**	19.44 ± 0.51^{d}	68.92 ± 0.64^{c}
	50 mg/L	165.64 ± 3.29^{de}	24.24 ± 0.72^{c}	48.43 ± 0.71^{f}
4	Control	140.94 ± 0.78^{f}	17.59 ± 0.59^{d}	65.41 ± 1.25^{cd}
	10 mg/L	**227.74 ± 1.64^{a}**	**32.24 ± 0.71^{a}**	**89.46 ± 1.35^{a}**
	20 mg/L	176.02 ± 0.47^{cd}	23.51 ± 0.36^{c}	68.88 ± 0.81^{c}
	50 mg/L	182.26 ± 2.81^{c}	27.11 ± 0.48^{b}	80.23 ± 1.31^{b}

Values expressing the Mean ± SD followed by similar letters in a column indicates as non-significantly difference at $p < 0.05$.

The utilization of sodium acetate as an elicitor on the improvement of secondary metabolite production has been successfully in shoot cultures of *Hypericum perforatum* (Rao *et al*., 2011), cell cultures of *Azadirachta indica* (Balaji *et al*., 2003) and *Artemisia annua* (Baldi and Dixit, 2008). Chitturi and co-workers (Chitturi *et al*., 2010) reported that the elicitation by sodium acetate increased the production of withaferin A in *Withania somnifera* callus cultures with the best result found in the treatment with 50 mg/L sodium acetate. Zeng *et al*. (2003) indicated that sodium acetate improved crocin content in stigma-like-structure of *Crocus sativus*. This is because sodium acetate is a potential precursor of crocin biosynthesis. Similarly in hairy root cultures of *Arachis hypogaea*, Medina-Bolivar *et al*. (2007) reported that the treatment

with 10.2 mM sodium acetate for 24 h resulted in a 60-fold induction and secretion of trans-resveratrol into the medium. This is because malonyl-CoA, one of the main precursors of resveratrol, also utilizes acetate as precursor in its biosynthesis and therefore acetate may play a precursor role in addition to elicitor of resveratrol. As a reason, this study proposed the use of sodium acetate as a precursor in the biosynthesis pathway of *Stemona* alkaloids to increase three *Stemona* alkaloids contents.

CONCLUSION

This experiment demonstrated that sodium acetate had a positive effect on the enhancement of three *Stemona* alkaloids production in hydroponic culture of *S. curtisii*. Sodium acetate increased the production of oxyprotostemonine, stemocurtisine and stemocurtisinol in the roots and also stimulated the secretion of some alkaloids such as oxyprotostemonine and stemocurtisinol from roots into the medium. The maximum content of total oxyprotostemonine was found in the treatment with 20 mg/L sodium acetate for 2 weeks, which was 1.4 fold significantly higher than the control. While, the maximum contents of total stemocurtisine and stemocurtisinol were observed in the treatment with 10 mg/L sodium acetate for 4 weeks, which were 1.8 and 1.4 fold significantly higher than the control, respectively.

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