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Contributed Paper

Effects of BA and NAA on Micropropagation and *Stemona* Alkaloids Production of *Stemona curtisii* Hook.f.

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ABSTRACT

In vitro propagation of *Stemona curtisii* was explored for multiple shoot induction and root regeneration in different media. Shoot explants were cultured on solid MS media supplemented with 0, 1, 2 and 3 mg L^{-1} benzyladenine (BA) to produce multiple shoots. This research revealed that the medium containing 2 mg L^{-1} BA induced shoot formation at a rate of 100% with the highest number of 9.8 shoots per explant. Then, the elongated shoots were separated and cultured on MS solid and in liquid media supplemented with 0, 1, 2, 3 and 4 mg L^{-1} naphthalene acetic acid (NAA) to induce roots. It was found that the solid medium containing 1 mg L^{-1} NAA induced 100% root formation with the highest number of 21.5 roots per explant. For the *Stemona* alkaloids analysis from the *in vitro* roots, HPLC analysis showed the content of oxyprotostemonine 90 μg g^{-1} DW, stemocurtisine 7 μg g^{-1} DW and stemocurtisinol 9 μg g^{-1} DW.

Keywords: benzyladenine, naphthalene acetic acid, *stemona* alkaloids and *stemona curtisii*

1. INTRODUCTION

Stemona curtisii Hook.f. (Figure 1) or "Non Tai Yak" in the Thai vernacular, is one of the important monocotyledon plants belonging to the family Stemonaceae (order Pandanales) and is widely distributed in the southern region of Thailand. This family is of considerable interest because it is the only source of the unique alkaloids known as *Stemona* alkaloids [1], which have been found to have biological activity. *Stemona* alkaloids including stemocurtisine, stemocurtisinol and oxyprotostemonine (Figure 2) have been isolated from a root extract of *S. curtisii* [2-3]. Mungkornasawakul *et al.* [3] reported that the root extracts and the pure alkaloid of *S. curtisii* especially oxyprotostemonine were shown larvicidal activity against *Anopheles minimus*. While, Kaltenegger *et al.* [4] reported that the crude extracts of *S. curtisii* had insecticidal activities against *Spodoptera littoralis*. Whereas, the utilization of *S. curtisii* extract as a biopesticide has been reported by Sastraruji [5]. It was found that this pesticide was effective against some pests, which include *Phyllotreta chontanica*, *Plutella xylostella*, *Lipaphis erysimi*, *Trichoplusia ni* and *Spodoptera littoralis* in agricultural field trials. However, the process of sexual propagation has been found to be

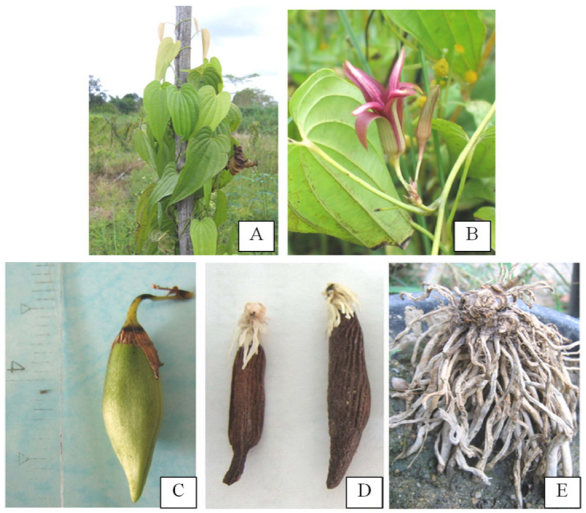

Figure 1. The botanical characteristics of *S. curtisii* Hook.f. (A) Leaves (B) Flowers (C) Fruits (D) Seeds and (E) Tuberous roots.

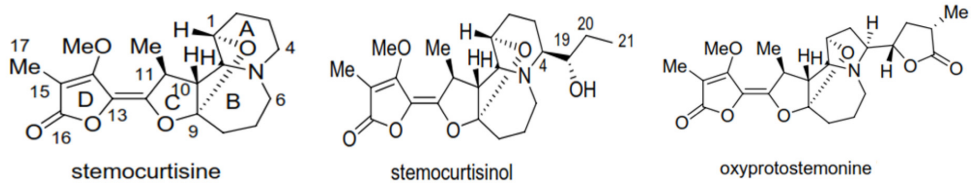

Figure 2. *Stemona* alkaloids isolated from the *S. curtisii* root extracts.

rather slow and results are difficult to come by because of a low germination rate and the fact that there are very few seed numbers in each pod. Furthermore, the plants could not be propagated in the winter because of its dormancy. As a result, the demand of *Stemona* roots and their *Stemona* alkaloids have increased on a commercial scale. *In vitro* propagation of *S. curtisii* is an alternative method that would allow the induction of more shoots and a higher yield of root formation, and ultimately produce the *Stemona* alkaloids in spite of the seasonal and environmental factors.

Hence, the purposes of this research were to investigate the effects of various BA concentrations on multiple shoot induction and various NAA concentrations on root induction from *in vitro* shoot cultures of *S. curtisii*. Moreover, the roots from *in vitro* plantlets were analyzed for *Stemona* alkaloids production, especially stemocurtisine, stemocurtisinol and oxyprotostemonine.

2. MATERIALS AND METHODS

2.1 Plant Materials

The intact plants of *S. curtisii* were collected at Tambol Kaunmao, Amphor Rasda, Trang Province, Thailand. A voucher specimen (No. 17581) was deposited at the herbarium of the Department of Biology, Faculty of Science, Chiang Mai University (CMU) and identified by Mr. James F. Maxwell from the Department of Biology, CMU.

2.2 Tissue Culture Methods

Shoot tips and axillary buds were excised and washed with running tap water to remove any remaining particles and soil. They were then surface-sterilized with a 15% clorox solution for 15 minutes and then rinsed three times with sterile distilled water. Finally, shoot

tips and axillary buds were cultured on Murashige and Skoog (MS, 1962) [6] media supplemented with 3% (w/v) sucrose, and 0.2% (w/v) gelrite™ and adjusted to a pH of 5.8 before being autoclaved. The cultures were maintained at 25±2°C for a 16 h photoperiod with fluorescent light at 2,500 lux. Regenerating shoots were subcultured to the fresh medium (solid MS medium without growth regulators) every 4 weeks, for 12 weeks (3 subcultures) to produce shoot materials for multiple shoot and root induction.

To investigate the effect of benzyladenine (BA) on shoot multiplication rate, individual shoots (from *in vitro* culture) were used as explant materials and cultured on solid MS media supplemented with 0, 1, 2 and 3 mg L^{-1} BA. All cultures were maintained at 25±2°C over a 16 h photoperiod with a light intensity of 2,500 lux for 4 weeks. The percentage of shoot induction and average number of shoots per explant were recorded.

For root induction, each single shoot was transferred to MS solid and liquid media supplemented with 0, 1, 2, 3 and 4 mg L^{-1} naphthalene acetic acid (NAA). All cultures were maintained under the same conditions of multiple shoot induction for 12 weeks. The percentage of root and callus induction and average number of roots per shoot were recorded. After that, the roots from *in vitro* plantlets were collected and analyzed for *Stemona* alkaloids production.

2.3 Root Extraction and Analysis of *Stemoma* Alkaloids

Fresh *in vitro* roots, from the best medium of root induction, were cut into small pieces and dried in an oven at 40°C. Then, the dried *in vitro* root (1 g) was macerated sequentially with 3×50 ml methanol (MeOH) at room temperature over 3 days. The 150 ml of MeOH solution was filtered and evaporated to give a methanol crude extract, which was dissolved in 1 ml of MeOH and 1 ml of water before extraction with dichloromethane (DCM). The solution was then evaporated to give a partially purified extract (DCM crude extract). After that, DCM crude extract was analyzed by high performance liquid chromatography (HPLC), Agilent 1200 series. Chromatographic separation was achieved on 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. 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 standard solutions of stemocurtisine, stemocurtisinol and oxyprotostemonine were prepared in methanol at concentration range 0.31-10.00 μg mL^{-1}. Each concentration of standard solution was injected into 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.

2.4 Experimental Layout and Data Analysis

The experiment was laid out in completely randomized design (CRD). Each treatment was repeated thrice with 10 replicates. The data was analyzed using the one-way analysis of variance (ANOVA) and then followed by the Turkey test. All statistical tests were considered significant at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Effect of BA Concentration on Multiple Shoot Induction

When the shoot explants were cultured on MS media supplemented with 0, 1, 2 and 3 mg L^{-1} BA for 4 weeks, it was found that

the shoot induction percentage increased with increasing of the BA concentration from 0 to 3 mg L^{-1} (Table 1). The solid MS media containing 2 and 3 mg L^{-1} BA induced 100% shoot formation, followed by those treated with 1 mg L^{-1} BA. Furthermore, the highest average number of shoots per explant (9.8 shoots, 4-fold higher than that of the control) was obtained from the solid MS media supplemented with 2 mg L^{-1} BA (Figure 3A). But in the other species of the *Stemona* plants, Chidburee *et al.* [7] found that the maximum number of shoots (3.3 shoots) in *S. tuberosa* was produced in the MS medium containing 4 mg L^{-1} BA and 1 mg L^{-1} NAA. While, Chaichana *et al.* [8] reported the best results for multiple shoot induction of *Stemona* sp. (4.4 shoots) were obtained from the MS medium supplemented with 3 mg L^{-1} BA. However, similar results of 2 mg L^{-1} BA on multiple shoot induction were found in *Solanum capsicoides* [9], *Spilanthes acmella* [10] and *Sansevieria trifasciata* [11].

In addition, the study of BA concentrations on shoot formation was also reported in various plant species. For example in *Citrus halimii*, Normah *et al.* [12] reported that the MS containing 1 mg L^{-1} BA induced the maximum number of 4.3 shoots per explant, which is also in line with the findings of Dheeranupattana *et al.* [13] in *Curcuma aeruginosa* and Arimura *et al.* [14] in *Zingiber officinale*. Thus, this study revealed that the number of shoots per plant did not increase with an increase of BA or cytokinin concentrations, which is in accordance with those results reported by Chaichana *et al.* [8] in *Stemona* sp., Rauf *et al.* [15] and Jorge *et al.* [16] in *Gossypium hirsutum*.

Table 1. Effect of BA on multiple shoot induction of *S. curtisii* after 4 weeks of culturing.

BA concentration (mg L^{-1})	Shoot induction (%)	Number of shoots per explants (mean ± SD)
0	90	2.4 ± 1.1 ^{d}
1	95	5.3 ± 0.9 ^{c}
2	100	9.8 ± 1.3 ^{a}
3	100	8.1 ± 1.0 ^{b}

Values showing the mean ± SD in a column followed by similar letters do not differ significantly at $p < 0.05$

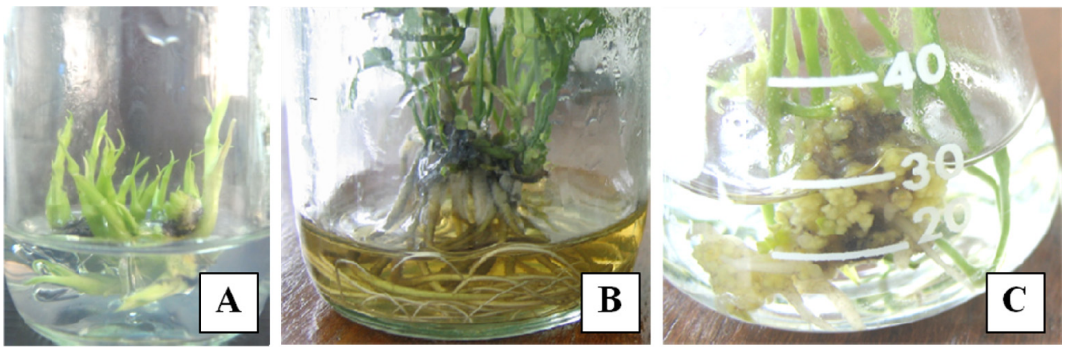

Figure 3. Micropropagation of *S. curtisii* (A) Mutiple shoot formation in solid MS medium supplemented with 2 mg L^{-1} BA (B) Root formation in solid MS medium supplemented with 1 mg L^{-1} NAA and (C) Root formation in liquid MS medium containing 3 mg L^{-1} NAA.

3.2 Effect of NAA Concentration on Root Induction

Root induction of *S. curtisii* was examined in MS solid and liquid media containing 0, 1, 2, 3 and 4 mg L^{-1} NAA for 12 weeks. It was found that the percentage of root induction and the number of roots per plant in the liquid media were less than those found in the solid media (Table 2). Each concentration of NAA in MS solid media produced 100% root formation. The solid MS medium containing 1 mg L^{-1} NAA gave the highest average number of 21.5 roots per plant. The roots were produced at the base and the nodal segments of the plantlets (Figure 3B). Similar results of 1 mg L^{-1} NAA on root induction were reported by Chidburee *et al.* [7] in *S. tuberosa*, Mukhtar *et al*. [17] in *Citrus reticulata* and Priyakumari and Sheela [18] in *Gladiolus grandiflorus*. But in the same species of *Stemona*, Montri *et al.* [19] reported that the highest number of roots per plant was found in the solid MS medium containing 10 μM IAA (*ca.* 1.75 mg L^{-1}). For the liquid media, the medium containing 3 mg L^{-1} NAA produced root formation at a rate of 100% with the highest average number of 17.4 roots per plant and induced 70% callus formation. These roots were produced at the base of the plantlets and were developed from the callus (Figure 3C). However, the number of roots per plant in both the solid and liquid media was only counted from the roots that were produced at the base of the plantlets.

Table 2. Effect of NAA on callus and root formation of *S. curtisii* after 12 weeks of culturing.

	NAA concentration (mg L^{-1})	Root induction (%)	Number of roots per plant (mean ± SD)	Callus induction (%)
Solid MS Medium	0	100	12.4 ± 1.3 ^{c}	0
	1	100	21.5 ± 2.0 ^{a}	0
	2	100	14.0 ± 2.1 ^{c}	0
	3	100	13.6 ± 1.6 ^{c}	0
	4	100	12.0 ± 2.0 ^{cd}	0
Liquid MS Medium	0	60	4.2 ± 1.1 ^{g}	20
	1	80	8.2 ± 1.3 ^{ef}	30
	2	60	6.7 ± 1.3 ^{f}	10
	3	100	17.4 ± 1.2 ^{b}	70
	4	60	10.0 ± 1.2 ^{de}	10

Values showing the mean ± SD in a column followed by similar letters do not differ significantly at $p < 0.05$

Thus, the results revealed that the solid medium was more effective for the root induction of *S. curtisii* than the liquid medium. These results are contrary to the findings of Aslam and Khan [20] in *Phoenix dactylifera* and Kumar *et al.* [21] in *Aloe vera*. Thakur and Kanwar [22] reported that the poor rooting response of *Pyrus pyrifolia* in the liquid medium may be attributed to the higher degree of basal callusing. This reason may then explain the failure of root formation of *S. curtisii* in the liquid medium in the present studies. On the other hand, Babu *et al.* [23] showed that the liquid medium was more effective for the adventitious root production of *Zingiber officinale* than the solid medium.

3.3 Analysis of *Stemona* Alkaloids from the *in vitro* Roots

Twelve-week-old roots, from *in vitro* plantlets grown in MS solid medium containing 1 mg L^{-1} NAA, were analyzed for *Stemona* alkaloids production, especially oxyprotostemonine, stemocurtisine and stemocurtisinol production by HPLC. The results revealed that the production of oxyprotostemonine 90 μg g^{-1} DW, stemocurtisine 7 μg g^{-1} DW and stemocurtisinol 9 μg g^{-1} DW were presented in the *in vitro* roots. However, the content of three *Stemona* alkaloids from the *in vitro* roots was less than those found to be present in the natural roots, in which oxyprotostemonine 1074 μg g^{-1} DW, stemocurtisine 968 μg g^{-1} DW and stemocurtisinol 703 μg g^{-1} DW were recorded. This result may be explained by the effect of the root age on *Stemona* alkaloids accumulation because the natural roots had grown for more than five years and were older than the *in vitro* roots (twelve weeks). The influence of the plant's age on the content of the secondary metabolites in the plants has been reported by many researchers. Shi *et al.* [24] reported that the content of ginsenosides in the root and root-hair increased with an increase in the age of *Panax ginseng* from 1 to 5 years. While, Court *et al.* [25] showed that *Panax quinquefolium* roots harvested after 3 years contained lower amounts of ginsenosides than roots harvested after 4 years. However, according to other studies, the secondary metabolite production from the *in vitro* culture have been successfully produced in numerous plants, *i.e.* ginsenoside production in *Panax notoginseng* [26] and tropane alkaloid in *Datura stramonium* [27].

Although *in vitro* culture produces low *Stemona* alkaloids content when compared to the natural roots, the production of numerous plantlets by this method was found to be faster than the natural propagation process and this method could also prevent the extinction of *Stemona* plants in nature.

CONCLUSIONS

This report has presented an efficient protocol for the mass multiplication of *S. curtisii*. The solid MS medium containing 2 mg L^{-1} BA gave the highest number of shoots per explant, while the optimum concentration of NAA for maximum root production was found to be 1 mg L^{-1}. For *Stemona* alkaloids analysis, the contents of oxyprotostemonine, stemocurtisine and stemocurtisinol originating from the *in vitro* roots were found to be less than those obtained in the natural roots. Thus, in the future research work, the enhancement of *Stemona* alkaloids production in *S. curtisii* roots will be studied.

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