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Micro-propagation and 1', 2'-didehydrostemofoline Production from *Stemona* Sp.

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Abstract: Plant propagation through tissue culture technique is required to produce plant secondary metabolite. The objective of this experiment was to obtain the optimum media composition for *in vitro* shoot and root formation of *Stemona* sp. It was shown that Murashige and Skoog (MS) medium supplement with 3 mg L^{-1} benzyladenine and half-MS medium supplemented with 2 mg L^{-1} indolebutyric acid were the appropriate media for shoot and root induction, respectively. The media could produce 100% of shoot induction and 80% of root induction. The increase or decrease in 1',2'-didehydrostemofoline was related to the root growth. The highest 1', 2'-didehydrostemofoline production of 31.04 mg g^{-1} root dry weight was observed in 16 week old root.

Key words: *Stemona* sp., micropropagation, benzyladenine, indolebutyric acid, 1',2'-didehydrostemofoline

INTRODUCTION

Useful secondary metabolites from plants have been of interest in recent years for their flavors, fragrances, dyes, pharmaceuticals and pesticide properties (Oksman-Caldentey and Inze, 2004). Valuable secondary metabolites from plants under cultivation or grown in nature are not always satisfactory. It is often restricted to species or genus and might be activated only during a particular growth and developmental stage or under specific season, stress or nutrient availability. For these reasons in the past several decades, a lot of effort has been put into plant cell cultures as a possible production method for plant secondary metabolites (Murthy *et al*., 2008; Verpoorte *et al*., 2002; Ramachadra-Rao and Ravishankar, 2002).

Stemona alkaloids are valuable secondary metabolites from *Stemona* spp. The pure alkaloids and extracts derived from leaves and roots of *Stemona* species have been shown to be toxic to insects (Tang *et al*., 2008). They also have antioxidant (Brem *et al*., 2004) and antitussive activities (Lin *et al*., 2008). Sastraruji *et al*. (2005) reported the isolation of 1',2'-didehydrostemofoline from an unidentified *Stemona* sp. Baird *et al*. (2009) reported that 1',2'-didehydrostemofoline from *Stemona* sp. has shown the highest inhibitory activity of acetylchacetylcholinesterase and also has a potential in the relieve of Alzheimer's disease.

The presences of cytokinin and auxin composition media have been shown to influence shoot and root production, respectively. Palee *et al*. (2006) reported that *in vitro* culture of *Stemona curtisii* root induction was highest when cultured on MS medium supplemented with 1 mg L^{-1} Naphthalene Acetic Acid (NAA). Moreover, other plant growth regulators are considered for micropropagation in many plants. Sharifi and Ebrahimzadeh (2010) studied the effects of indolebutyric acid (IBA) and NAA at different concentrations added to the solidified (Murashige and Skoog, 1962) (MS) or Gamborg B5 (B5) medium for root induction of saffron (*Crocus sativus* L.). The percentage of root formation on B5 medium containing 2.46 μM IBA was highest. However, the maximum number of root per explant was observed on MS medium with 19.6 μM IBA. This study was conducted to investigate the *in vitro* culture for multiple shoot and root induction of *Stemona* sp. *Stemona* alkaloid biosynthesis at each time period of *in vitro* culture was also performed.

MATERIALS AND METHODS

Plant materials and culture medium: *Stemona* sp. was originally obtained from Lampang province, Thailand. This voucher specimen was deposited at the Herbarium (number 25375) of the Department of Biology, Chiang Mai University. Shoot tips and axillary buds were surface sterilized with 15% clorox solution for 20 min followed by washing 3 times with sterile distilled water. They were then cultured on MS medium consisting of mineral salts, vitamins and sucrose as the basal medium. Sucrose was added to the medium as carbon source at 30 g L^{-1}. Medium pH was adjusted to 5.8 with 1M KOH. Gelrite agar at 2 g L^{-1} (Sigma-Aldrich, USA) was melted and added for solidification. Ten milliliters of the medium was

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poured in each of the culture bottles, closed with plastic caps and autoclaved. The cultures were placed in the growth room at 25±2°C under 16 h/d photoperiod.

Multiple shoot and root induction: Each single shoot was cultured on MS medium supplemented with 1, 2, 3 and 4 mg L^{-1} of benzyladenine (BA) or 0.1, 0.2, 0.3, 0.4, 0.5 mg L^{-1} of thidiazuron (TDZ) for multiple shoot induction. The result was recorded for 8 weeks after transferring into shoot induction media. The percentage of shoot induction and average shoot per explant were determined. For root induction, single shoot were transferred to MS and half-MS medium supplemented with 1, 2, 3 and 4 mg L^{-1} of NAA or IBA for 8 weeks. The cultures were placed in the growth room at 25±2°C under 16 h/d photoperiod. Results were recorded as percentage of root induction and average root per explants to determine the appropriate culture medium for *in vitro* culture of *Stemona* sp., respectively. Shoot with root was cultured for 4, 6, 8, 12, 16, 20 and 24 weeks. Root growth from each period was recorded and the alkaloid production was also quantified.

Extraction method and HPLC condition: Dry roots of *Stemona* sp. from each period were ground and extracted 3 times with methanol (Merck, HPLC grade, Germany). The solution was filtered and evaporated to get crude extract which was extracted again with dichloromethane (DCM) (Merck, HPLC grade Germany). The extract was concentrated to get crude DCM extracts and the weight of crude extracts was also recorded. The crude DCM extract was dissolved in methanol and filtered with 0.45 μM membrane filter (Filtrex syringe membrane filtration). Finally, *Stemona* alkaloid was quantified by HPLC (Agilent 1200 series). Data acquisition and analysis were performed by the agilent chemstation software. HPLC condition development was performed to investigate the appropriate column, mobile phase, flow rate and UV detection wavelength. 1',2'-didehydrostemofoline was used as standard.

RESULTS AND DISCUSSION

Multiple shoot and root induction: Multiple shoot induction was observed at 8 weeks. It was found that BA was more effective for shoot induction than TDZ. MS medium supplemented with 3 mg L^{-1} BA gave 100% shoot induction with 4.4 shoots per explant (Table 1, Fig. 1a). BA is widely used to provide multiple shoot in several plants. For example in cotton, an average of 3.4 shoots per explants was the obtained when cultured on the medium supplemented with 3 mg L^{-1} BA (Morre *et al*., 1998). For micro propagation of *Vitis vinifera*, it was found that MS medium with 5 μM BA was observed on better for shoot induction with 80% of shoot proliferation and maximum rooting (80%) occurred on the medium with 10 μM IBA (Jaskani *et al*., 2008). IBA also resulted in the highest root induction of *Stemona* sp. Haft-MS medium supplemented with 2 mg L^{-1} IBA was the best selection medium over than any MS medium or NAA supplementation. The medium could provide 80% of root induction and could produce an average of 20.63 roots per explant after being transferred to the medium for 8 weeks (Table 2 and Fig. 1b) while MS medium supplemented with 1 mg L^{-1} NAA gave 60% of root induction and produced an average of 20.00 roots per explant. IBA was the cytokinin plant growth regulator that is successfully provides root induction in many plants. It was found that MS medium supplemented with 3.0 mg L^{-1} IBA could provide the highest root induction (26.4%) of *Helianthus annus* L. (Elavazhagan *et al*., 2009) and half-strength MS medium supplemented with 0.5 IBA and 0.2 mg L^{-1} NAA resulted in 89% root induction of *Decalepis arayalpathra*. It also produced maximum 2-hydroxy-4-methoxy benzaldehyde (0.16%) compound after 6 weeks of culture (Sudha and Seeni, 2001).

Table 1: Percentage of shoot induction and average shoot per explant after 8 weeks

Medium	Shoot induction (%)	Shoots per explant
MS	50	1.8
MS+ 1 mg L^{-1} BA	90	2.4
MS+ 2 mg L^{-1} BA	90	3.4
MS+ 3 mg L^{-1} BA	100	4.4
MS+ 4 mg L^{-1} BA	90	4.4
MS+ 0.1 mg L^{-1} TDZ	80	2.4
MS+ 0.2 mg L^{-1} TDZ	90	2.6
MS+ 0.3 mg L^{-1} TDZ	100	2.9
MS+ 0.4 mg L^{-1} TDZ	80	2.3
MS+0.5 mg L^{-1} TDZ	100	3.4

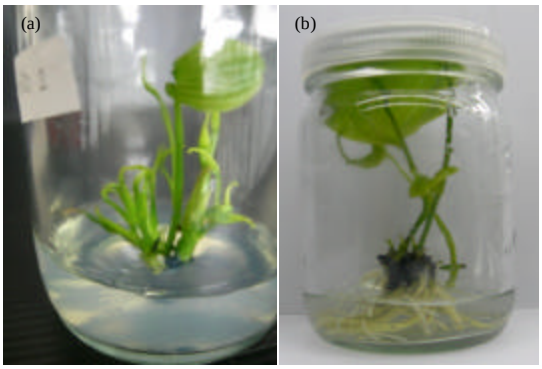

Fig. 1(a-b): (a) Mutiple shoot induction on MS supplemented with 3 mg L^{-1} BA medium and (b) root induction on haft-MS supplemented with 2 mg L^{-1} IBA medium

Table 2: Percentage of root induction and average root per explant after 8 weeks

Medium	Root induction (%)	Roots per explants
MS	10	15.00
MS+1 mg L^{-1} NAA	60	20.00
MS+2 mg L^{-1} NAA	10	17.00
MS+3 mg L^{-1} NAA	30	18.33
MS+4 mg L^{-1} NAA	10	18.00
MS+1 mg L^{-1} IBA	10	18.00
MS+2 mg L^{-1} IBA	10	19.00
MS+3 mg L^{-1} IBA	10	18.00
MS+4 mg L^{-1} IBA	20	18.50
1/2MS	10	18.00
1/2MS+1 mg L^{-1} NAA	40	18.75
1/2MS+2 mg L^{-1} NAA	10	18.00
1/2MS+3 mg L^{-1} NAA	10	16.00
1/2MS+4 mg L^{-1} NAA	10	17.00
1/2MS+1 mg L^{-1} IBA	40	19.25
1/2MS+2 mg L^{-1} IBA	80	20.63
1/2MS+3 mg L^{-1} IBA	40	18.25
1/2MS+4 mg L^{-1} IBA	70	19.00

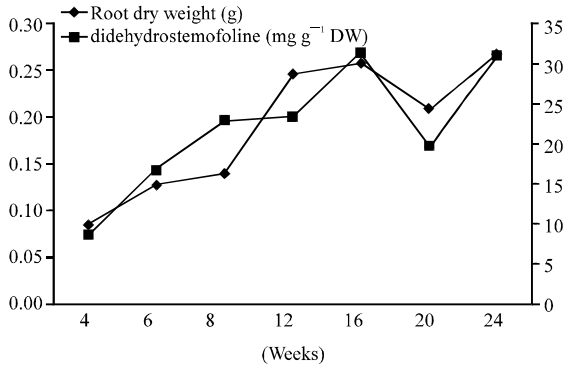

Fig. 2: Root growth and alkaloid content in root extract from *in vitro* culture of *Stemona* sp.

HPLC condition and *Stemona* alkaloid production: Inertsil C18 ODS-3 5 μM particle size, 4.6×150 mm column (GL sciences Inc., Japan) was used. A mixture of methanol and water (90:10, v/v) was used as a mobile phase in isocratic condition at flow a rate of 0.3 mL min^{-1}. Detection of compound was accomplished at 297 nm. A 20 μL of sample was injected into chromatography system. The analysis was achieved within 15 min. The retention time of 1',2'-didehydrostemofoline standards was 8 min. Growth and the alkaloid production of 1',2'-didehydrostemofoline is shown in Fig. 2. The highest growth rate and *Stemona* alkaloids production were observed on 16 week old root. It was 31.04 milligram per gram dry weight (mg/g DW) of 1',2'-didehydrostemofoline. However, the production of this alkaloid at this time was not statistically different from that of 24 weeks old root. It seems that 1',2'-didehydrostemofoline production increased or decreased with the root growth. The pattern of root growth and alkaloid production was similar to *Salvia miltiorrhiza* hairy roots culture. The increase in total tanshinone content and the root dry weight went together (Wu and Shi, 2008). Further study will investigate the increase in *Stemona* alkaloids production *in vitro* culture. In many plants, elicitors were added into the medium to increase the production of secondary metabolites such as salicylic acid and methyl jasmonate (Kanga *et al*., 2004), chitosan (Gaid *et al*., 2009) and yeast extract (Yan *et al*., 2006).

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