In vitro propagation of *Stemona hutanguriana* W.Chuakul, an endangered medicinal plant

Sompop Prathanturarug · Rungthipwan Pheakkoet ·
Thaya Jenjittikul · Wongsatit Chuakul ·
Promchit Saralamp

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Abstract *In vitro* propagation of *Stemona hutanguriana* W.Chuakul, an endangered species of Stemonaceae, was established. Nodal and internodal explants were cultured on Murashige and Skoog (MS) medium supplemented with thidiazuron (TDZ) alone or in combination with 1-naphthaleneacetic acid (NAA). When cultured on MS medium supplemented with 18.16 μ M TDZ with 0.54 μ M NAA for 8 weeks and then on MS medium without plant growth regulator (PGR) for 8 weeks, nodal explants demonstrated a responding frequency of 91.67 % and a shoot regeneration rate of 5.46 shoots/responding explant. Furthermore, internodal explants demonstrated a responding frequency of 17.17 % with 11.17 shoots/responding explant. The regenerated shoots were rooted using a two-step protocol by culturing for 4 weeks on MS medium supplemented with various concentrations of NAA and Indole-3-butyric acid (IBA) and 8 weeks on MS medium without PGR. When 5.40 μ M NAA was used as a PGR, the maximum root induction rate was 69.45 %. Regenerated plantlets were successfully transferred to soil.

Keywords Conservation · Medicinal plant · Stemonaceae · *Stemona hutanguriana* · Tissue culture

Abbreviations

BA	6-Benzyladenine
IBA	Indole-3-butyric acid
MS medium	Murashige and Skoog (Murashige and Skoog 1962) medium
NAA	1-Naphthaleneacetic acid
PGR	Plant growth regulator
TDZ	Thidiazuron

Introduction

Stemona is a genus in the family Stemonaceae, with approximately 27 species occurring as subshrubs or twining herbs, mostly with perennial tuberous roots (Tsi and Duyfjes 2000). The root contains alkaloids with insecticidal, antitubercular, and antitussive activities (Pilli and de Oliveira 2000). *S. hutanguriana* W. Chuakul, an herb commonly known as Sam-Sib-Kip-Noi, occurs in restricted and vulnerable populations and was recently discovered in the Ubon Ratchathani Province of Thailand (Chuakul 2000). *S. hutanguriana* is ethnobotanically used for treatment of *Gnathostoma spinigerum* infections and head lice (Chuakul 2000). Since the plant was first discovered, the number of individual plants has dramatically decreased due to deforestation. *In vitro* propagation has been successfully applied for the conservation of a large number of endangered plant species that have limited reproductive capacity and exist in threatened habitats (Fay 1992; George et al. 2008; Pence 2010). Recently, there have been a number of established protocols for *in vitro* propagation of rare and threatened medicinal plants such as *Dysosma versipellis* (Jiang et al. 2012), *Polygonatum verticillatum* (Bisht et al. 2012), *Pseuderthria viscida* (Cheruvathur and Thomas 2011), and *Thymus lotocephalus* (Coelho et al. 2012).

S. Prathanturarug (✉) · R. Pheakkoet · W. Chuakul · P. Saralamp
Department of Pharmaceutical Botany, Faculty of Pharmacy,
Mahidol University,
447 Sri-ayudthaya Road,
Bangkok 10400, Thailand
e-mail: pyspr@mahidol.ac.th

T. Jenjittikul
Department of Plant Science, Faculty of Science, Mahidol
University,
272 Rama VI Road,
Bangkok 10400, Thailand

Stemona spp. can be propagated by planting tuberous roots with attached buds; however, this process takes time. Moreover, sexual propagation by seeds is very poor (Rungrojsakul 2001). Therefore, a number of *in vitro* propagation protocols based on organogenesis have been established for *Stemona* spp., including those for *S. collinsae* (Rungruchkanont and Pongrat 2008), *S. curtisii* (Montri et al. 2006), *S. tuberosa* (Singlaw et al. 2008), and *Stemona* sp. (Chotikadachanarong et al. 2005). In the present study, we aimed to develop an efficient protocol for *in vitro* mass propagation of *S. hutanguriana* using thidiazuron (TDZ) and 1-naphthaleneacetic acid (NAA) for conservation purposes.

Materials and methods

S. hutanguriana used in this experiment was collected from Ubon Ratchathani Province, Thailand, the same area that the plant was found and firstly described as a new species (Chuakul 2000), and identified by Professor Wongsatit Chuakul. Shoots of *S. hutanguriana* were surface sterilized with 1 % sodium hypochlorite solution for 20 min and then rinsed with sterilized distilled water three times. The shoots were cultured on MS medium (Murashige and Skoog 1962) supplemented with 3 % sucrose and 0.55 % Agargel®. Nodal and internodal explants of two-month-old *in vitro* plants were used as initial explants.

An optimum combination of cytokinin and auxin are important for shoot induction (George et al. 2008), therefore we used TDZ as cytokinin with various concentrations (1.14–

18.16 μM) in combination with a selected concentration (0.54 μM) of NAA as auxin. The explants were cultured on MS medium supplemented with TDZ alone (1.14–18.16 μM) or in combination with 0.54 μM NAA for 8 weeks followed by MS medium without plant growth regulators (PGRs) for 8 weeks. Regenerated shoots were cut and rooted on MS medium supplemented with NAA (5.40–43.20 μM) or IBA (4.92–39.36 μM) for 4 weeks followed by MS medium without PGRs for 8 weeks.

For all experiments, the medium contained 3 % sucrose and 0.55 % Agargel®, and the pH was adjusted to 5.8. A total of 12 replicates were performed for each treatment, and each experiment was repeated three times. The cultures were maintained at 25 °C with a 16-hr photoperiod with a light intensity of 37.5 $\mu\text{E m}^{-2} \text{s}^{-1}$. After rooting, the regenerated plantlets were transferred to plastic bags containing soil under greenhouse conditions.

Calli with new shoot primordia were free-hand sectioned and directly observed under a microscope.

Data are presented as mean of three repeated experiments and were analyzed using one-way ANOVA. The means were compared using Duncan's Multiple Range Test (DMRT) at a confidence level of 95 %.

Results and discussion

Using the sterilization conditions of 1 % sodium hypochlorite for 20 minutes, no microbial contamination was observed. *In vitro* nodal and internodal explants were cultured on MS medium supplemented with various

Table 1 Effects of TDZ and NAA on *S. hutanguriana* nodal explants after 8 weeks in MS medium supplemented with PGR and 8 weeks in MS medium without PGR

Plant growth regulator		Explant response (%)		Shoots/responding explant		Shoot length (cm)		Callus induction (%)	
TDZ (μM)	NAA (μM)	8 weeks in MS+PGR	8 weeks in PGR- free MS	8 weeks in MS+PGR	8 weeks in PGR- free MS	8 weeks in MS+PGR	8 weeks in PGR- free MS	8 weeks in MS+PGR	8 weeks in PGR- free MS
0	0	91.67	94.45	1.06c	1.35d	2.65a	2.63abcd	0c	0e
1.14	0	94.45	94.45	1.26bc	1.50d	2.03b	2.29abcd	2.78c	8.33de
2.27	0	88.89	88.89	1.25bc	1.71d	2.19ab	2.41abcd	5.55c	5.55de
4.54	0	80.56	83.33	1.27bc	2.05d	1.87b	1.95d	19.44bc	22.22cde
9.08	0	97.22	97.22	1.65ab	3.88abc	1.83b	2.29abcd	36.11ab	50.00a
18.16	0	91.67	94.45	1.86a	4.49ab	1.85b	2.10cd	36.11ab	58.33a
1.14	0.54	91.67	91.67	1.24bc	1.85d	1.98b	2.20bcd	16.67bc	25.00bcd
2.27	0.54	88.89	88.89	1.36bc	2.27cd	2.32ab	2.93a	28.54ab	38.89abc
4.54	0.54	88.89	94.45	1.36bc	2.79cd	1.94b	2.28abcd	19.44bc	47.22ab
9.08	0.54	77.78	83.33	1.50abc	2.87bcd	2.17ab	2.85ab	36.11ab	50.00a
18.16	0.54	88.89	91.67	1.85a	5.46a	1.99b	2.71abc	45.45a	52.78a

Each value represents the mean of 3 repeated experiments. Means within each column with different letters are significantly different at $p=0.05$ (DMRT)

concentrations of TDZ alone (1.14–18.16 μM) or in combination with 0.54 μM NAA. The frequencies of responding nodal explants were 77.78–97.22 % and 83.33–97.22 % after 8 weeks and 16 weeks of culture, respectively (Table 1).

The shoot induction rates increased gradually when TDZ levels were elevated. The highest shoot induction rates of 1.86 and 1.85 shoots/responding explant were achieved in nodal explants cultured on MS medium supplemented with 18.16 μM TDZ alone and in combination with 0.54 μM NAA, respectively. When the nodal explants were transferred to MS media without PGRs, the shoot induction rates increased. The maximum shoot induction rate of 5.46 shoots/responding explant was achieved when explants were cultured on MS medium supplemented with 18.16 μM TDZ and 0.54 μM NAA (Table 1 and Fig. 1a and b).

In internodal explants, lower responding rates were observed, with values between 3.03 and 17.17 % after 16 weeks of culture (Table 2). During the 8 weeks of TDZ treatment, shoot induction rates were quite low, ranging between 0.33 and 1.00 shoots/responding explant. The maximum shoot induction rate of 11.17 shoots/responding explant was obtained from explants that were cultured on MS medium supplemented with 18.16 μM TDZ and 0.54 μM NAA and transferred to MS medium without PGRs (Table 2 and Fig. 1c and d).

Calli were induced from both nodal and internodal explants, especially when both TDZ and NAA were included in the culture medium (Tables 1 and 2). Some calli further developed into new emerging shoots. Figure 2 shows an example of a callus with new shoots. These results indicate that the regenerated shoots of *S. hutanguriana* were formed via direct and indirect organogenesis.

At high TDZ concentrations with or without NAA, the shoot induction rates increased more than 2 times after the explants were transferred to MS medium without PGRs, in both explant types. This indicates that TDZ, a potent PGR, might be needed as a trigger for shoot primordial. Thus, incubation in MS medium without PGRs was needed for shoot elongation, consistent with our previous findings in *Curcuma longa* (Prathanaturug et al. 2003; Prathanaturug et al. 2005), *Gagnepainia godefroyi*, and *G. thoreliana* (Prathanaturug et al. 2007). TDZ has also been used for shoot induction in *Stemona* (Montri et al. 2006; Singlaw et al. 2008). Singlaw et al. (2008) reported a shoot induction rate of 3.1 shoots/explant in *S. tuberosa*, using 4.54 μM TDZ. In *S. curtisii*, TDZ was shown to induce a number of shoots with stunting and thickening of stems (Montri et al. 2006).

Rungruchkanont and Pongrat (2008) reported a shoot induction in *S. collinsae* using 22.22 μM BA in nodal, but

Fig. 1 Nodal (a–b) and Internodal explants (c–d) of *S. hutanguriana* were cultured in MS medium supplemented with 18.16 μM TDZ in combination with 0.54 μM NAA for 8 weeks (a, c) and then transferred to MS medium without PGR for 8 weeks (b, d) (Bar=1 cm)

not internodal, explants. Chotikadachanarong et al. (2005) reported low frequencies of shoot induction in terminal buds obtained from *Stemona* sp. in MS medium supplemented with BA.

We report, for the first time, that shoot organogenesis can be induced from somatic cells of internodal explants of the genus, *Stemona*, which is difficult to induce (Rungruchkanont and Pongrat 2008). All previously published papers used meristematic tissues as initial explants, including terminal bud explants in *Stemona* sp. (Chotikadachanarong et al. 2005), nodal explants in *S. collinsae* (Rungruchkanont and Pongrat 2008), shoot tip explants in *S. tuberosa* (Singlaw et al. 2008), and shoot explants in *S. curtisii* (Montri et al. 2006). We used TDZ, which has been shown to be one of the most

Table 2 Effects of TDZ and NAA on *S. hutanguriana* internodal explants after 8 weeks in MS medium supplemented with PGR and 8 weeks in MS medium without PGR

Plant growth regulator		Explant response (%)		Shoots/responding explant		Shoot length (cm)		Callus induction (%)	
TDZ (μ M)	NAA (μ M)	8 weeks in MS+PGR	8 weeks in PGR- free MS	8 weeks in MS+PGR	8 weeks in PGR- free MS	TDZ (μ M)	NAA (μ M)	8 weeks in MS+PGR	8 weeks in PGR- free MS
0	0	0	0c	0	0b	0	0b	0e	0d
1.14	0	0	0c	0	0b	0	0b	0e	0d
2.27	0	0	0c	0	0b	0	0b	0e	0d
4.54	0	0	3.03c	0	1.33ab	0	0.43b	3.03e	8.59d
9.08	0	3.03	3.03c	0.33	3.67ab	0.47	0.92b	3.03e	3.03d
18.16	0	0	0c	0	0b	0	0b	0e	3.03d
1.14	0.54	0	0c	0	0b	0	0b	12.73de	12.73cd
2.27	0.54	0	6.67bc	0	0.83ab	0	0.58b	29.09bc	29.09b
4.54	0.54	0	27.42a	0	6.89ab	0	2.70a	44.60a	47.37a
9.08	0.54	0	3.03c	0	2.67ab	0	0.85b	22.12cd	25.15bc
18.16	0.54	6.06	17.17ab	1.00	11.17a	0.56	1.47ab	38.74ab	38.74ab

Each value represents the mean of 3 repeated experiments. Means within each column with different letters are significantly different at $p=0.05$ (DMRT)

potent plant growth regulators for woody species (Huetteman and Preece 1993). In addition to shoot induction from meristematic tissues, TDZ also induces adventitious bud regeneration from internodal explants of *Fagus sylvatica* L. (Cuenca et al. 2000) and *Prunus avium* L. (Matt and Jehle 2005). Our results demonstrate that internodal tissue of *S. hutanguriana* is capable of forming adventitious shoots.

Using a two-step rooting protocol, we achieved a maximum rooting frequency of 69.45 % when regenerated shoots were incubated in MS with 5.40 μ M NAA for 4 weeks and in PGR-free MS for 8 weeks (Fig. 3a and b). For IBA treatments, the maximum rooting frequency was 66.67 %, achieved at 4.92 μ M IBA (Table 3). Root induction appeared to be difficult to achieve in *S. hutanguriana* compared to other *Stemona* spp. (Montri et al. 2006; Rungruchkanont and Pongrat 2008). There-

fore, a two-step protocol which increased culture period and time taken to produce plantlets was needed for *S. hutanguriana*. Figure 3c pictures a healthy regenerated *S. hutanguriana* plant, which was successfully transferred to soil and grown under greenhouse conditions compared to the mother plant grew in the natural habitat (Fig. 3d).

In conclusion, we described an *in vitro* propagation protocol of *S. hutanguriana* using nodal and internodal explants, by culturing on MS medium supplemented with 18.16 μ M TDZ and 0.54 μ M NAA for 8 weeks and then MS medium without PGR for 8 weeks. Rooting was optimal when regenerated shoots were incubated on MS medium with 5.40 μ M NAA for 4 weeks and then cultured on MS medium without PGR for 8 weeks. Regenerated plantlets were successfully transferred to soil. This established protocol will be applied for propagation and conservation of the plant.

Fig. 2 A callus of *S. hutanguriana* was induced from a nodal explant on MS medium supplemented with 18.16 μ M TDZ in combination with 0.54 μ M NAA for 8 weeks (a). A cross section demonstrates that a new shoot primordium (arrow) has developed (b)

Fig. 3 Rooting is shown from a regenerated shoot of *S. hutanguriana* cultured on 5.37 μ M NAA for 4 weeks (a) and then transferred to MS medium without PGR for 8 more weeks (b). A three-month old regenerated plant (c) is shown compared to a natural plant (d) (Bar=1 cm)

Table 3 Effects of NAA or IBA on rooting of *S. hutanguriana* regenerated shoots after 4 weeks in MS medium supplemented with PGR and 8 weeks in MS medium without PGR

Plant growth regulator		Rooting (%)		Roots/regenerated shoot	
NAA (μ M)	IBA (μ M)	4 weeks in MS+PGR	8 weeks in PGR- free MS	4 weeks in MS+PGR	8 weeks in PGR- free MS
0	0	16.67abc	41.67bc	0.67b	1.36c
5.40	0	2.78bc	69.45a	0.33b	2.88bc
10.80	0	2.78bc	50.00abc	0.67b	2.44bc
21.60	0	0c	25.50c	0b	2.42bc
43.20	0	0c	41.66bc	0b	2.43bc
0	4.92	25.00ab	66.67ab	1.72ab	2.70bc
0	9.84	27.78a	50.00abc	2.83a	4.36ab
0	19.68	2.78bc	58.33ab	0.30b	5.28a
0	39.36	2.78bc	52.78ab	0.67b	3.18bc

Each value represents the mean of 3 repeated experiments. Means within each column with different letters are significantly different at $p=0.05$ (DMRT)

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