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Original Article

In vitro propagation of the aromatic herb *Strobilanthes tonkinensis* Lindau

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ABSTRACT

Strobilanthes tonkinensis Lindau is a rare aromatic herb belonging to the family Acanthaceae. Its plant extract has been confirmed as a major source of squalene. In this research, a protocol for micropropagation was developed that can support *ex situ* conservation and will benefit plant material production. Shoot explants were provided from plants grown in the greenhouse and trickle irrigated for 1 mth and then effectively sterilized by shaking in NaOCl at a concentration of 1.2% for 10 min, followed by 0.6% for 15 min, which produced 70% good-growing, healthy shoots. Increasing thidiazuron and N^6-benzyladenine (BA) concentrations did not promote shoot multiplication. Shoot multiplication was the best on Murashige and Skoog (MS) medium supplemented with 16 μM BA. The highest shoot number (12 shoots/explant) was obtained at 8 wk of culture. The highest shoot elongation was obtained on the medium added with 16 μM BA for 4 wk and subsequent subculturing to hormone-free MS medium for another 4 wk. High frequency rooting (21 roots/shoot) was obtained on MS medium fortified with 7.5 μM indole-3-butyric acid. Complete plantlets that were transferred to pots under greenhouse conditions produced healthy plants with 100% survival after 5 wk.

Introduction

Many aromatic plants containing large varieties of active organic compounds have been used as sources of traditional medicine (Fay, 1992). Estimates by the World Health Organization suggested that approximately 80% of people in developing countries still rely on traditional medicines for their primary health care needs, and about 85% of traditional medicine involves the use of plant extracts (Farnsworth et al., 1985).

Strobilanthes tonkinensis Lindau is a fragrant herb belonging to the Acanthaceae family (Fig. 7A). It is a medicinal and aromatic plant originating in Yunnan province, China and is also distributed in Thailand and Vietnam (Flora of China Editorial Committee, 2011). It has been reported that this plant contains a number of biological activities such as antiviral, antitumor, anti-inflammatory and anti-coagulant (Chen et al., 2013). It has also been confirmed to be rich in active compounds that provide the plant resource squalene (Yang et al., 2014).

Many medicinal plant species are considered endangered or threatened owing to rapid agricultural and urban development, deforestation and indiscriminate collection, so the tissue culture technique is the most effective tool for mass propagation and conservation of these rare and endangered medicinal plants (Fay, 1992; Hassan et al., 2011). There are only a few reports suggesting micropropagation of plants in the Acanthaceae family; furthermore, no report focuses on *S. tonkinensis* Lindau. Some of the *Strobilanthes* were induced to shoot formation by culturing on MS (Murashige and Skoog, 1962) medium supplemented with only N^6-benzyladenine (BA)—for example, *Strobilanthes flaccidifolious* (Deb and Arenmongla, 2011)—while some species multiplied shoots on MS medium supplemented with BA in combination with auxins such as indole-3-butyric acid (IBA)—*Strobilanthes hamiltoniana* (Shameer et al., 2008)—or 1-naphthalene acetic acid (NAA)—*S. flaccidifolious* (Deb and Arenmongla, 2012). For other species in Acanthaceae, the best shoot induction was reported on MS medium supplemented with BA in many plants such as *Hygrophila spinosa* (Varshney et al., 2009), *Adhatoda vasica* (Nath and Buragohain, 2005; Soni et al., 2012), *Justicia gendarussa* (Janarthanam and Sumathi, 2010) and *Crossandra infundibuliformis* (Girija et al., 1999). Some species were induced to produce roots by culturing single shoots on MS (*A. vasica*; Nath and Buragohain, 2005) or ½ MS (*J. gendarussa* Burm.f.; Janarthanam and Sumathi, 2010) without phytohormones. Additionally, MS medium supplemented with IBA has shown the best root induction results in many species such as

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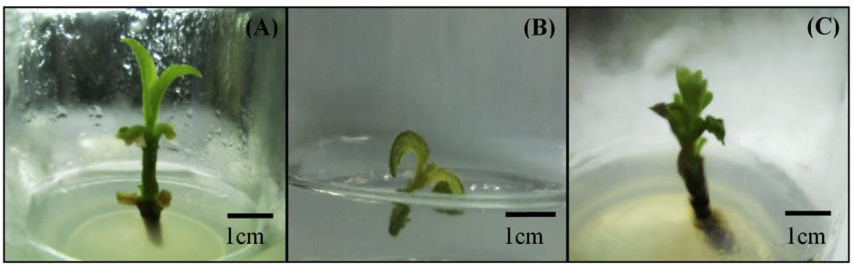

Fig. 1. Sterilized shoots of *Strobilanthes tonkinensis* Lindau after culturing on Murashige and Skoog (1962) medium for 4 wk: (A) healthy shoot; (B) stubborn shoot; (C) infected shoot.

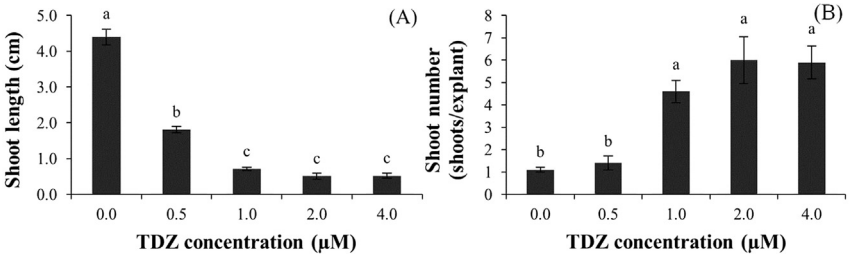

Fig. 2. Effects of TDZ on *Strobilanthes tonkinensis* Lindau after 8 wk culture on: (A) shoot length; (B) shoot number. Bars indicate standard error; n = 10; different lowercase letters above bars indicate significant differences ($p \leq 0.05$).

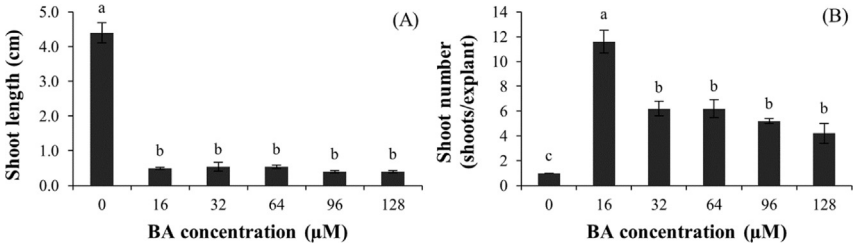

Fig. 3. Effects of N^6-benzyladenine (BA) on *Strobilanthes tonkinensis* Lindau after 8 wk culture on: (A) shoot length; (B) shoot number. Bars indicate standard error; n = 5; different lowercase letters above bars indicate significant differences ($p \leq 0.05$).

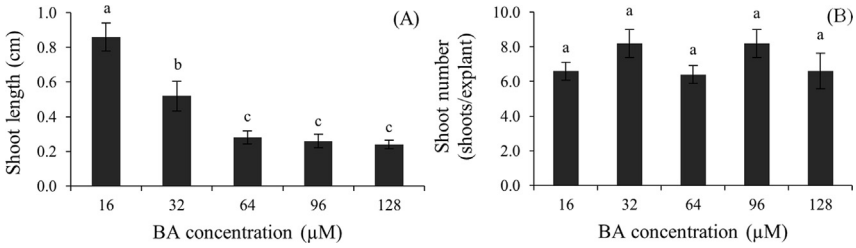

Fig. 4. Effects of N^6-benzyladenine (BA) on *Strobilanthes tonkinensis* Lindau after culturing for 4 wk and subsequently transferring to hormone-free Murashige and Skoog (1962) medium for another 4 wks on: (A) shoot length; (B) shoot number. Bars indicate standard error; n = 5; different lowercase letters above bars indicate significant differences ($p \leq 0.05$).

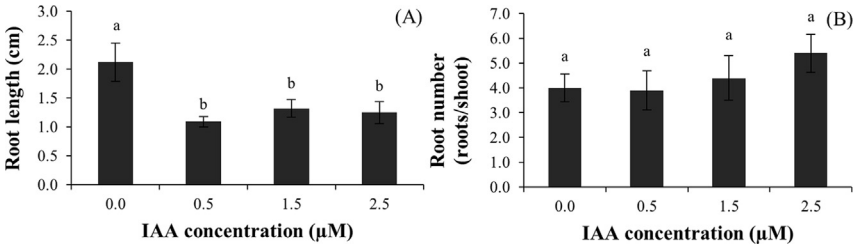

Fig. 5. Effects of indole acetic acid (IAA) on *Strobilanthes tonkinensis* Lindau after 4 wk culture on: (A) root length; (B) root number. Bars indicate standard error; n = 10; different lowercase letters above bars indicate significant differences ($p \leq 0.05$).

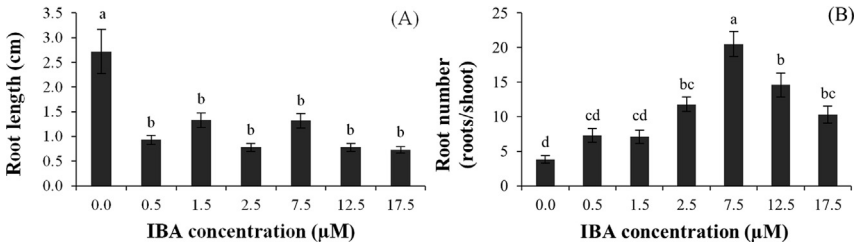

Fig. 6. Effects of indole-3-butyric acid (IBA) on *Strobilanthes tonkinensis* Lindau after 4 wk culture on: (A) root length; (B) root number. Bars indicate standard error; n = 10; different lowercase letters above bars indicate significant differences ($p \leq 0.05$).

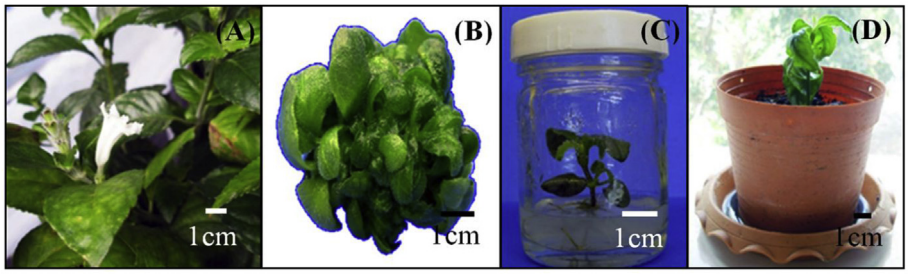

Fig. 7. Young mother plants of *Strobilanthes tonkinensis* Lindau grown in greenhouse for this research at different stages derived from tissue culture technique: (A) shoots used for surface sterilization showing terminal inflorescence of simple spikes; (B) multiple shoot formation on regeneration medium; (C) plantlet on rooting medium; (D) potted plant under natural conditions.

Rhinacanthus nasutus L. (Sundar et al., 2012), *A. vasica* (Soni et al., 2012) and *C. infundibuliformis* L. (Girija et al., 1999). The present study aimed to develop a protocol for *in vitro* propagation of the rare aromatic herb, *S. tonkinensis* Lindau. This plant material will be beneficial for bioactive compound production which may be useful for further application as a component of new medicines and cosmetic products. Notably, by using the raw material from the *in vitro* plant, it is possible to control both the quality and quantity and also help to reduce plant destruction and forest invasion.

Material and methods

Surface sterilization

Young plants of *S. tonkinensis* produced from cuttings were grown in a greenhouse and trickle irrigated for 1 mth. Shoots with 3–4 axillary buds were harvested and all leaves were removed. The shoots with buds were washed with tap water and subsequently shaken in 70% ethanol for 1 min. Shoots were surface sterilized by shaking in 1.2% sodium hypochlorite (NaOCl), followed by a lower concentration of 0.6% NaOCl for various durations of treatment.

The percentages of healthy, infected and stubborn shoots were recorded after culturing them on hormone-free MS medium for 4 wk.

Shoot multiplication

To provide the explants for the subsequent experiments, the surface-sterilized shoots from the first experiment (surface sterilization) were sub-cultured onto MS medium supplemented with 4 μM BA every 4 wk to multiply the shoots for 6 mth. These shoots were then sub-cultured to hormone-free MS medium for 5 wk to be used as explants.

Single shoot tips 1.0 cm in length were cultured on the MS medium supplemented with various concentrations of BA (0.0 μM, 16.0 μM, 32.0 μM, 64.0 μM and 96.0 μM) or thidiazuron (TDZ) (0.0 μM, 0.5 μM, 1.0 μM, 2.0 μM and 4.0 μM) for 8 wk. After 4 wk, half of all the BA treatments were sub-cultured to MS medium without BA for another 4 wk. The number of shoots and their length were measured after 8 wk culture.

Root induction and transfer to soil

Single shoots 1.0 cm in length were excised and cultured on the MS medium containing 0.0 μM, 1.5 μM and 3.0 μM indole acetic acid (IAA) or 0.0 μM, 0.5 μM, 1.5 μM, 2.5 μM, 7.5 μM, 12.5 μM and 17.5 μM IBA for root induction. The numbers of roots and their lengths were recorded after 4 wk of culture. Rooted plants were acclimatized by uncapping the culture vessels and placing in a greenhouse for 3 wk. Subsequently, they were transferred to pots containing soil, sand and rice husk charcoal (1:1:1 volume per volume) and kept for 2 wk in a mist chamber with 75–90% humidity, followed by 4 wk on benches with 45–65% humidity. Survival plantlets were scored 5 wk after being transferred onto the open benches.

Statistical analysis

A completely randomized design with 10 replicates was performed to determine the effect of BA and TDZ on shoot multiplication and the effect of IAA and IBA on root induction. Equal variances were tested using Levene's method. Significant differences ($p \leq 0.05$) among treatments were separated using Tukey's B multiple range test. All analyses were performed using the PASW Statistics 18 software package (SPSS Inc.; Quarry Bay, Hong Kong).

Results and discussion

Surface sterilization

Shoots of *S. tonkinensis* Lindau were harvested from young plants grown in a greenhouse. They were disinfected for 1 min by shaking in 70% ethanol which was recommended as a mild disinfectant for initial general use (Maroti et al., 1982; Badoni and Chauhan, 2010). In general, the effective technique for shoot

Table 1
Percentage of healthy, stubborn and infected shoots of *Strobilanthes tonkinensis* Lindau after 4 wk following sterilizing using sodium hypochlorite at two concentrations for various periods.

NaOCl		Shoot result		
1.2%	0.6%	Healthy (%)	Stubborn (%)	Infected (%)
15 min	20 min	40	30	30
10 min	15 min	70	0	30
10 min	5 min	20	0	80
5 min	15 min	40	0	60

surface sterilization should be a low concentration of sterilant with a shorter exposure time (Smith, 2000). The suitable sterilization treatment was 10 min, followed by 15 min of NaOCl giving 70% healthy shoots which grew very well and showed expanded leaves after 1 wk (Table 1; Fig. 1A). A high concentration of NaOCl (1.2%), followed by a low concentration of 0.6% could reduce the toxicity of the sterilant to avoid plant tissue damage. The longest duration treatment at 15 min, followed by 20 min resulted in a low number of healthy shoots and 30% stubborn shoots which grew very slowly and turned brown after culturing on MS medium for 1 mth (Table 1; Fig. 1B). The short duration treatment of 15–20 min produced a high level of infected shoots though they did not appear to be damaged (Table 1; Fig. 1C).

Shoot multiplication

In general, TDZ and BA are more commonly used for shoot proliferation (Smith, 2000). Shoot multiplication of *S. tonkinensis* Lindau was induced by culturing shoot tips on MS medium supplemented with TDZ or BA for 8 wk. Shoots cultured on the medium without cytokinins appeared long but were low in number (Figs. 2B and 3B). There were 4–6 shoots/explant induced by 1–4 μM TDZ which was close to the shoot number derived from 32 to 128 μM BA (Figs. 2B and 3B). High shoot numbers were found using 1.0–4.0 μM TDZ with no significant differences among these concentrations. Maintaining shoots on TDZ for the long term (up to 8 wk) could induce 5–6 shoots (Fig. 2B). Using only TDZ or in combination with other plant growth regulators has been suggested to promote shoot proliferation of Acanthaceae (Nath and Buragohain, 2005). An amount of 0.9 μM TDZ produced the highest shoot number of Dwarf hygro (*Hygrophila polysperma*) according to Karataş et al. (2013) and 1.35 μM TDZ was best for shoot production of *A. vasica* (Nath and Buragohain, 2005).

In addition, the most effective plant growth regulator (PGR) reported for *R. nasutus* (L.) Kurz was 4 μM TDZ in combination with 0.8 μM NAA (Cheruvathur and Thomas, 2014) and the best PGR for *Barleria prionitis* L. was 1.8 μM TDZ in combination with 6.66 μM BA (Lone et al., 2011).

The highest shoot number was obtained when cultured on the medium supplemented with 16 μM BA. Shoot number were lower with a higher concentration of BA from 32 μM to 128 μM but were not significantly different (Fig. 3B).

Increasing the concentration of BA did not promote shoot multiplication of *S. tonkinensis*. It has been suggested that BA at high concentration might inhibit shoot production (Wang et al., 2011).

Additionally, transferring shoots from BA containing media to hormone-free MS medium promoted shoot elongation (Fig. 4A). On the other hand, subculturing shoots onto fresh medium with the same composition could induce a number of multiple shoots (Fig. 3B). After transferring shoot buds to the media without BA, they became elongated causing the shoot numbers of each treatment to increase by about 2–3 shoots, with no significant difference among treatments. The shoot length obtained on medium containing 16 μM BA was significantly higher than for other treatments (Fig. 4A and B). In addition, after 8 wk culture, the medium supplemented with 16 μM BA produced the highest numbers of shoots (12 shoots/explant; Figs. 3B and 7B) while at all of the higher concentrations of BA (32–128 μM), there was no variation in shoot numbers (Fig. 3B). Many reports have suggested that BA at various concentrations is suitable for shoot multiplication in Acanthaceae such as 6 μM BA for *S. flaccidifolious* (Deb and Arenmongla, 2011), 8.88 μM BA for *A. vasica* (Nath and Buragohain, 2005) and 4.44 μM BA for *C. infundibuliformis*, *J. gendarussa* and *A. vasica* (Girija et al., 1999; Janarthanam and Sumathi, 2010; Soni et al., 2012).

Root induction and transfer to soil

Individual shoots of *S. tonkinensis* Lindau were cultured on MS medium supplemented with IAA and IBA for root induction. The longest root length was produced using the MS medium without IAA or IBA while there were no significant differences among the other media (Figs. 5A and 6A). Rooting on IAA containing media at concentrations of 0–2.5 μM produced 4–5 roots with no significant difference among concentrations (Fig. 5B). Root induction on IBA containing medium at a concentration of 7.5 μM gave the highest root number (21 roots/shoot; Figs. 6B and 7C). The number of roots derived on 12.5 μM IBA was not significantly different from that of 2.5 μM and 17.5 μM IBA. The least effective levels of IBA for rooting were 0–1.5 μM (Fig. 6B). Comparing the effect of IAA and IBA at 0–2.5 μM, the number of roots derived from IBA (12 roots/shoot) was higher than that obtained from IAA (5.4 roots/shoot; Figs. 5B and 6B). The results indicated that IBA was suitable for root induction of *S. tonkinensis* Lindau. High root induction using IBA was also reported in some species in Acanthaceae, such as 7.4 μM IBA for *R. nasutus* L. (Sundar et al., 2012) and 4.9 μM IBA for *C. infundibuliformis* L. (Girija et al., 1999) and *A. vasica* (Soni et al., 2012).

Shoots of *S. tonkinensis* were cultured on MS supplemented with 7.5 μM IBA for root induction. They rooted at 100% *in vitro* and had a 100% survival rate at 5 wk after transfer to greenhouse conditions. All plantlets were healthy, grew well and showed normal characteristics (Fig. 7D).

Young plants of *S. tonkinensis* were grown in the greenhouse to prepare shoot explants. Shoots were effectively surface sterilized by shaking in 1.2% NaOCl for 10 min followed by 0.6% NaOCl for 15 min. This procedure produced 70% healthy shoots without stubborn shoots that were stunted and turned brown 1 wk after culturing. MS medium supplemented with 16 μM BA produced the highest shoot number (12 shoots/explant) at 8 wk culture. In addition, shoots initiated on the medium added with 16 μM BA for 4 wk with subsequent transfer to hormone-free MS medium for another 4 wk produced the greatest elongation when compared to other concentrations of BA. The best conditions for root induction were on the MS medium fortified with 7.5 μM IBA. Transferring complete plantlets to pots under greenhouse conditions resulted in a 100% survival rate of healthy plants after 5 wk.

Conflict of interest

None.

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