

Artemisinin Production by Shoot Regeneration of *Artemisia annua* L. Using Thidiazuron

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An efficient *in vitro* method for multiple shoot bud induction and regeneration has been developed in *Artemisia annua* L. using leaf and stem explants in various concentrations and combinations of plant growth regulators to evaluate the frequency of regeneration. The sources of explants as well as plant growth regulators in the medium were found to influence the multiple shoot induction. The result shows that the stem segment cultured on Murashige and Skoog (MS) medium supplemented with 0.1 mg/l thidiazuron (TDZ) gave a perfect shoot formation (100%) and good shoot multiplication (57 shoots/explant) after 2 weeks of culture. Healthy regenerated shoots were elongated and rooted in MS medium without hormones. The artemisinin content in plants regenerated from stem explants using 0.1 mg/l TDZ was $(3.36 \pm 0.36) \mu\text{g}/\text{mg}$ dry weight and two-fold higher than that of *in vitro* grown plants of the same age $[(1.73 \pm 0.23) \mu\text{g}/\text{mg DW}]$. This system exhibited a potential for a rapid propagation of shoots from the stem explant and makes it possible to develop a clonal propagation of *A. annua*.

Key words: *Artemisia annua* L., Shoot Regeneration, Thidiazuron, Artemisinin

Introduction

Artemisia annua L. (wormwood or sweet wormwood), an important medicinal plant of the family Compositae, contains an antimalarial, artemisinin, which is effective against *Plasmodium falciparum* (Klayman, 1985). It is an annual herb native to China with a long history of use against hemorrhoids and fever including malaria (Bhakuni *et al.*, 2001). The low content [0.01–0.6% dry weight (DW)] of artemisinin in *A. annua* greatly limits its commercialization (Liersch *et al.*, 1986; Singh *et al.*, 1986). Numerous efforts focusing on enhancing the production of artemisinin have been made for a long time. However, a conventional breeding of high artemisinin yielding plants, the accumulation of artemisinin at different steps of plant development, the manipulation of culture conditions, the growth media and hormone levels to increase the yield of artemisinin in tissue and cell cultures have not been clear. At the present, the genes of the

key enzymes involved in the biosynthesis of artemisinin and the genes of the enzymes relevant to the biosynthesis of artemisinin have been cloned from *A. annua* (Chang *et al.*, 2000). Regarding genetic engineering, it is suggested that the overexpression of key enzymes involved in the biosynthesis of artemisinin or the inhibition of the enzymes involved in other pathways competing for their precursors promote the transgenic higher yielding *A. annua*. Shoot regeneration of *A. annua* from leaf has been demonstrated by Vergauwe *et al.* (1996) and Han *et al.* (2005). The regeneration media that have generally been seen to produce shoot regeneration in *A. annua* are Murashige and Skoog (MS) medium supplemented with 0.05 mg/l α -naphthaleneacetic acid (NAA) and 0.5 mg/l 6-benzyladenine (BA). Thidiazuron (TDZ) has been shown to be an efficient plant growth regulator for shoot regeneration in various plants (Faure *et al.*, 1998; Liu *et al.*, 2003; Mithila *et al.*, 2003; Landi and Mezzetti, 2006). However, TDZ has not yet

been tested for its ability to induce organogenesis in *A. annua*. In this study, we investigated the high efficiency of TDZ for the induction of shoot regeneration and artemisinin production in *A. annua*.

Materials and Methods

Chemicals

6-Benzyladenine (BA), α -naphthaleneacetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D), kinetin (Kin) and 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron, TDZ) were purchased from Sigma, St. Louis, MO, USA. All other chemicals were standard commercial products of analytical grade.

Plant materials

Artemisia annua L. seeds were obtained from the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. The seeds were washed with distilled water and were surface-sterilized in 10% sodium hypochlorite for 20 min. After being washed three times with sterilized water, the seeds were immersed in 70% ethanol for 1 min and then germinated on hormone-free MS medium containing 3% sucrose (w/v), pH 5.5. Germination started within 1 week and was carried out at $(25 \pm 1)^\circ\text{C}$ and 16 h light per day. Plantlets were subcultured on the same medium every 4 weeks.

Shoot induction and regeneration

Leaf and stem segments (1 cm) from 4 weeks fully grown *in vitro* plantlets were cultured on MS medium supplemented with TDZ (0.1–1.5 mg/l) either alone or in combination with NAA (0.05–1.0 mg/l) or BA (0.5–1.0 mg/l). Regenerated shoots were transferred to MS medium without hormone for elongation and rooting. For the determination of the optimal duration of exposure of the explants to the medium containing TDZ, stem segments were cultured on MS medium supplemented with 0.1 mg/l TDZ for 1, 2, 3, 4 and 5 weeks followed by transfer to MS medium without hormone for 4 weeks. For each treatment, a total of 24 explants was tested. Analysis of variance was used to analyze the effects of plant growth regulators and duration of exposure of the explants on induction of shoot. The data were carried out using the least significant difference (LSD) test at $P < 0.05$.

Sample preparation and analysis

Leaves from regenerated plants and normally *in vitro* grown 4-week-old plants were dried and ground. Leaf powders (30 mg) were extracted with 400 μl of petroleum ether five times using an ultrasonic bath for 15 min. After filtration and evaporation to dryness, the residue was resuspended in 1 ml of methanol. Artemisinin equivalents in sample solutions were determined by a competitive ELISA using monoclonal antibodies (MAb) against artemisinin and its derivatives as previously reported (Putalun *et al.*, 2006): Artemisinin-HSA (100 μl of 1 $\mu\text{g/ml}$) was adsorbed onto a 96-well immunoplate and then treated with 300 μl phosphate buffered saline (PBS) containing 5% (w/v) skim milk (S-PBS) for 1 h. 50 μl of various concentrations of artemisinin or samples that had been diluted in 20% (v/v) methanol were incubated with 50 μl monoclonal antibody solution for 1 h. The plate was washed three times with PBS containing 0.05% (v/v) Tween 20 (T-PBS), and then incubated with 100 μl of a 1,000-fold dilution of peroxidase-labeled goat anti-mouse IgG for 1 h. After washing the plate with T-PBS, 100 μl of substrate solution [0.1 M citrate buffer, pH 4.0, containing 0.003% (v/v) H_2O_2 and 0.3 mg/ml ABTS] were added to each well and incubated for 15 min. The absorbance was measured by a microplate reader at 405 nm. All reactions were carried out at 37°C .

Results and Discussion

A. annua seeds were germinated on MS medium in the absence of growth hormones resulting in a single shoot which can be used for the explant for regeneration. TDZ had a highly significant effect on the production of shoots when stem explants were treated. A perfect shoot regeneration ratio (100%) and the best result of shoots number per explant [(57.33 ± 4.09) shoots/explant] were obtained in MS medium supplemented with 0.1 mg/l TDZ after 2 weeks of culture (Table I). The effect of the duration of induction on TDZ-supplemented medium on the number of shoot regeneration from stem explants was investigated. The shoots number per explant was (22.16 ± 14.28) in 7 days of expose. Cultures grown on medium supplemented with TDZ for 2 weeks produced a maximum number of shoots [(56.45 ± 8.12) shoots/explant], however, there was no significant difference in the number of shoots formed when the

Table I. Effect of the TDZ concentration on the percentage of shoot regeneration and shoot induction from stem and leaf explants of *A. annua*. Columns with different letters are significantly different at $P < 0.05$.

TDZ [mg/l]	Shoot regeneration (%)		Number of shoots per explant	
	Stem	Leaf	Stem	Leaf
0.1	100.0	100.0	$57.33 \pm 4.09a$	$4.29 \pm 1.20c$
1.0	50.0	6.2	$23.21 \pm 1.91b$	$1.17 \pm 0.87c$
1.5	66.7	31.2	$21.00 \pm 2.19b$	$2.25 \pm 0.61c$

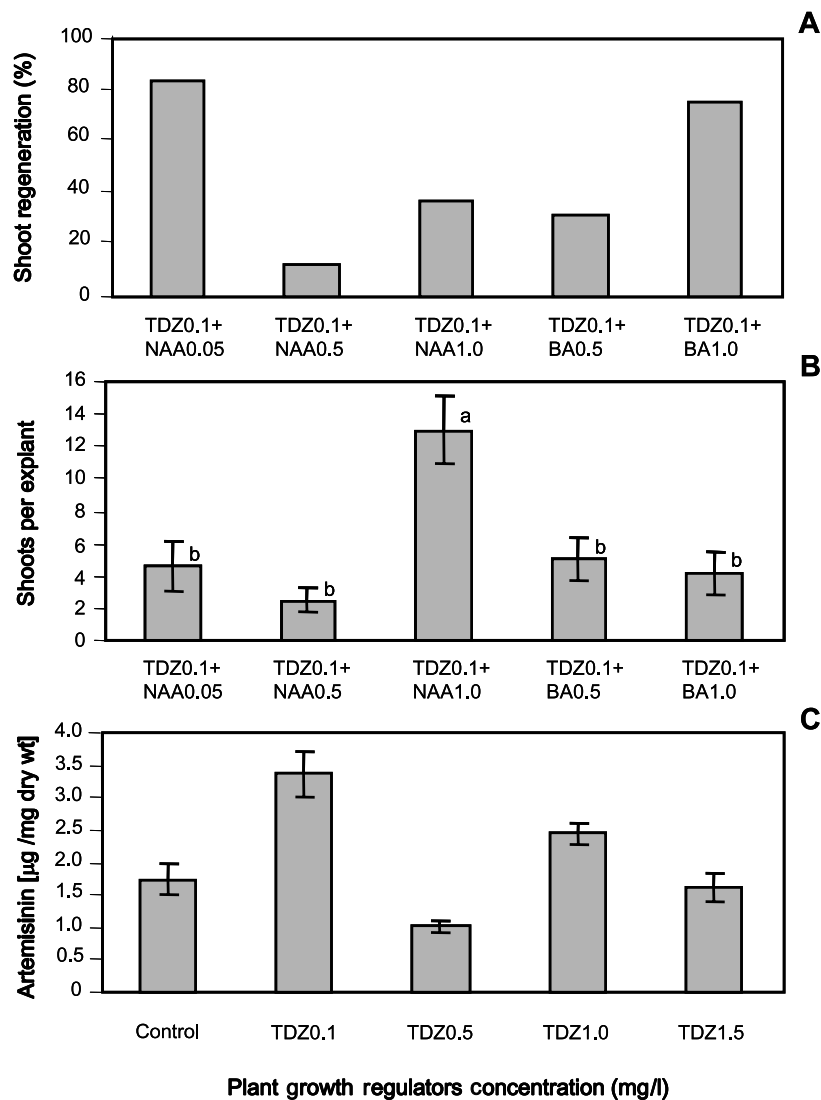


Fig. 1. Effect of TDZ in combination with NAA or BA on the percentage of (A) shoot regeneration and (B) shoot induction of stem explants of *A. annua*. Columns with different letters are significantly different at $P < 0.05$. (C) Artemisinin content in leaves of 4-week-old regenerated plants from TDZ-supplemented medium and normal *in vitro* grown plants.

duration of exposure to TDZ was increased to 21 and 35 days. Since the highest ratio of shoot regeneration was observed in MS medium supplemented with 0.1 mg/l TDZ after 2 weeks of culture, we therefore used 0.1 mg/l TDZ-containing medium for further experiment. Various combinations of 0.1 mg/l TDZ and NAA or BA were tested for shoot induction of *A. annua*. The maximum frequency of shoots regeneration (83.3%) from the combinations of plant growth regulators was found in stem explants cultured on MS medium with 0.1 mg/l TDZ and 0.05 mg/l NAA (Fig. 1A). The shoot number per explant was (13 ± 2.11) at 0.1 mg/l TDZ and 1 mg/l NAA after 6 weeks of culture (Fig. 1B). All combinations of TDZ and NAA or BA in leaf explant culture could not induce shoot regeneration.

TDZ, a non-purine cytokinin-like compound has been reported to promote shoot initiation in many plants (Prathantharug *et al.*, 2003; Liu *et al.*, 2003; Ledbetter and Preece, 2004). In these reports, the results show that the stem segment cultured with different concentrations of TDZ (0.1–1.5 mg/l) is effective for shoot induction. It has been reported that induction of cytokinin accumulation and also enhancement of accumulation and translocation of auxin within TDZ-exposed tissue could form a large number of shoots (Prathantharug *et al.*, 2003). Low concentration of TDZ (0.1 mg/l) stimulated the highest number of shoots [(57.33 ± 4.09) shoots/explant] compared to those found in higher concentrations of TDZ. Moreover shoots appeared in earlier time. The period of shoot induction was 2 weeks, shorter in this system than that in the combination of plant growth regulators. The combinations of TDZ with NAA and BA were investigated for shoot regeneration in *A. annua*. The frequency of shoot regeneration and the number of shoots per explant using only TDZ were higher than in combinations of TDZ with NAA and BA. These results indicated that TDZ is enough for simple and rapid shoot regeneration of *A. annua*. These results resembled also the observation of Liu *et al.* (2003) who showed that TDZ induces shoot organogenesis in *A. judaica* L. Rooting was observed after transfer to MS medium without hormones for 4 weeks. The regenerated plants were not different in appearance from the normally grown *A. annua* plants (Fig. 2).

The artemisinin contents in leaves of regenerated plants compared with contents found in normal *in vitro* grown plants are shown in Fig. 1C.

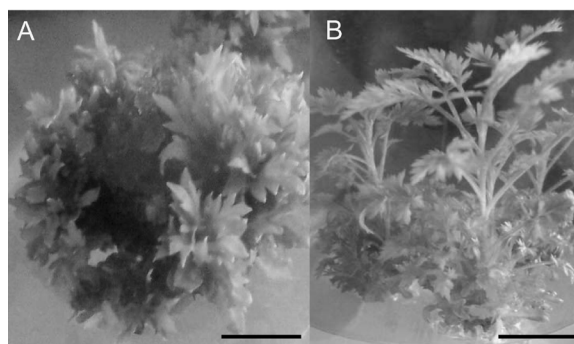


Fig. 2. Development of a regeneration system for *A. annua*. (A) Shoot organogenesis on MS medium with 0.1 mg/l TDZ. Bar 5 mm. (B) Shoot growth after 4 weeks of subculture on MS medium without hormone. Bar 10 mm.

This result indicated that plants regenerated from 0.1 mg/l TDZ contain the highest level of artemisinin in comparison with plants regenerated from other contents. The artemisinin contents in plants regenerated from stem explants using 0.1 mg/l TDZ was $(3.36 \pm 0.36) \mu\text{g/mg DW}$ and 2-fold higher than that of normal *in vitro* grown plants of the same age [$(1.73 \pm 0.23) \mu\text{g/mg DW}$]. This result resembled to the observation of Kim *et al.* (2005) who showed that addition of TDZ to the culture medium enhanced the asiaticoside production of whole plant cultures of *Centella asiatica* (L.) Urban. On the other hand, regarding artemisinin, Vergauwe *et al.* (1996) reported that the artemisinin content changed to 0.17% in an *A. annua* plant regenerated from 0.11% in normal *in vitro* plant.

Vergauwe *et al.* (1996) reported the shoot regeneration from leaf explants of *A. annua* on MS medium with 0.05 mg/l NAA and 0.5 mg/l BA after 5 weeks of culture. However, we established an improved protocol for shoot regeneration of *A. annua* using stem explants on MS medium supplemented with TDZ resulting in a rapid and high frequency of shoot regeneration and a high number of shoots per explant in this study. Therefore, this regeneration system might be a useful method for high regeneration efficiency and has commercial advantage due to the shoot regeneration period over a combination of several plant growth regulators.

In conclusion, our results make evident that stem explants can be used for the induction of high shoot formation under the influence of TDZ. An optimal content of TDZ was achieved from stem

explants cultured on medium supplemented with 0.1 mg/l TDZ for 2 weeks. The regeneration system developed in this study will be useful for plant improvement through micropropagation and genetic engineering of *A. annua*. Moreover, this system can be available for the clonal propagation in order to obtain the strain containing a constant concentration of artemisinin in *A. annua*.

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