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PLANT REGENERATION FROM MATURE ZYGOTIC EMBRYO EXPLANTS OF ACACIA CRASSICARPA A. CUNN EX BENTH. VIA ADVENTITOUS SHOOT ORGANOGENESIS

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Abstract

Acacia crassicarpa is a major species for fiber production in Southeast Asia. *In vitro* multiplication of this species could be valuable for expanding plantations. To explore new approaches to propagate *A. crassicarpa*, we developed system to regenerate plants from mature zygotic embryo explants via shoot organogenesis. Embryos were cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of TDZ and IAA. Combinations of TDZ at 4.54-9.81 μM and IAA at 0.57-5.71 μM were optimum to induce shoot bud formation. Medium containing 4.54 μM TDZ and 2.85 μM IAA yielded the highest rate of shoot buds (76.9%). The embryos with shoot buds were subcultured to fresh medium of the same composition for shoot development, and then proliferated into big green nodule-like callus. The callus was then transferred to the medium containing 2.89 μM GA_3 for shoot elongation induction. Elongated shoots rooted on half-strength medium with 2.46 μM IBA within 1month. Rooted plantlets were hardened and successfully established in soil with a 97.5% survival rate.

Key words: adventitious budding, indole-3-acetic acid, thidiazuron

INTRODUCTION

Acacia is a leguminous tree that is widely dispersed in tropical and sub-tropical regions. The planting area of *Acacia* species has increased, as the species have been used for reforestation, reclamation of wasteland, and industrial material production around Southeast Asia, especially Indonesia, as well as in some southern provinces of China (Zhigang and Yintian 1994, Minquan et al. 1995, Midgley 2000,). Many *Acacia* species are important for shelterbelts, soil improvement, and as sources of fuel and timber (Palmberg and Pasca 1981). *Acacia crassicarpa* has become increasingly important as a plantation species in various parts of Southeast Asia, where it is grown specifically for the production of pulp (Beilharz et al. 2004). It has become a preferred fiber source for the pulp and paper industry because of its rapid growth, high pulp yield, high fiber quality, and its ability to thrive in degraded soils (Zhigang and Yintian 1994). To meet the increasing requirements for fiber products, and because of its environmental importance, it would be useful to increase its yield and planting area. However, classical breeding programs for *Acacia* are strongly limited by its recalcitrant regeneration, its long life cycle, and the long delay until mature traits can be evaluated. Therefore, establishing a highly efficient *in vitro* regeneration system for *A. crassicarpa* is essential to meet the practical requirements for forestry production.

In natural habitats, the regeneration rate of leguminous trees is quite low (Dewan et al. 1992). There are several reports of *in vitro* regeneration of some *Acacia* species, including *Acacia catechu* (Rout et al. 1995), *Acacia auriculiformis* (Ranga Rao and Prasad 1991), *Acacia mangium* (Ahmad 1991, Bhaskar and Subhash 1996, Galiana et al. 1991a,b, Xie and Hong 2001a), *Acacia sinuate* (Vengadesan et al. 2002, Vengadesan et al. 2000), *Acacia nilotica* (Garg et al. 1996) and *Acacia farnesiana* (Ortiz et al. 2000).

Previously, we have reported a stable *in vitro* regeneration system through organogenesis based on the production of shoots and roots from phyllode (leaf) explants, rather than explants from cotyledons or hypocotyls (Yang et al. 2006). In the present study, we established a system for *in vitro* regeneration of *A. crassicarpa* from mature zygotic embryo explants *via* adventitious shoot organogenesis. This system was useful for producing high-quality regenerated plantlets of *A. crassicarpa*.

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MATERIALS AND METHODS

Plant material and surface disinfection

Mature seeds of *A. crassicarpa* were collected from 10-year old trees in a natural grove. The trees were 20-30 m in height and were located at a seed orchard in Guangzhou, China. The seeds were treated with water (100°C) for 1 min and then soaked in distilled water for 36 h. Seeds were surface disinfected by soaking in 70% ethanol for 1 min, then in 4% sodium hypochlorite for 5 min, followed by rinsing five times with sterile distilled water. The testa of each seed was cut manually and removed; embryos were then excised aseptically and prepared for primary shoot bud induction.

Culture medium and conditions

The culture medium was Murashige and Skoog (1962) basal medium (MS) supplemented with 5% (v/v) coconut water (CW) (YiDa Food, China), 3% (w/v) sucrose (Real-Times Biotechnology, China), and solidified with 0.6% (w/v) agar (Real-Times Biotechnology, China). All media were adjusted to pH 5.8 with 1N NaOH. The plant growth regulators (PGRs) 1-pheny l-3-(thiadiazol-5-yl) urea (thidiazuron) (TDZ) and indole-3-butyric acid (IBA) were added at various concentrations before autoclaving media at 121°C for 15 min. Indole-3-acetic acid (IAA) and gibberellic acid (GA_3) were filter-sterilized through a 0.2-μm membrane filter, and added to autoclaved media. Unless otherwise mentioned, all cultures were maintained at 28°C under cool-white fluorescent lights at an irradiance of 26 μmol m^{-2} s^{-1} under a 16-h light/8-h dark photoperiod.

Shoot bud induction and elongation

Whole embryo explants were cultured on shoot bud induction medium containing combinations of TDZ (2.27, 4.54, 9.81, or 18.16 μM) and IAA (0.57, 2.85, or 5.71 μM) in dark in the first 3 weeks and then transferred to light. Each Petri dish (90 × 15 mm) contained 20ml of medium and 10 embryo explants. The experiment was duplicated with 100 explants per treatment and was repeated three times. The percentage of embryos that produced shoot buds was calculated after 2 months of culture. After 2 months, calluses with shoot buds were transferred to MS medium supplemented with combinations of GA_3 (0, 1.44, or 2.89 μM) and TDZ (0, or 2.27, μM) for shoot elongation. The experiment was duplicated with 50 explants per treatment and was repeated three times. The length of shoots was measured after 1 month of culture on shoot elongation medium.

Rooting of regenerated shoots and plantlet survival

Elongated shoots (≥ 2 cm) were removed from the callus and transferred to rooting induction medium supplemented with 0.49, 2.46, 4.92, or 9.84 μM IBA for 1month and cultured in light. Each culture vessel contains 50 ml half-strength MS medium and 5 shoots. After formation of lateral roots, plantlets were potted in disinfectant roseite, and then subsequently transplanted into 1 : 1 mixture of soil and roseite. The experiment was duplicated with 50 shoots per treatment and was repeated three times. The survival rate was determined 1 month after acclimatization. Subculturing to fresh treatment medium was conducted every 20 days.

Histological staining

For histological analysis, shoot bud tissues were fixed in FAA fixative (5% (v/v) formaldehyde, 5% (v/v) acetic acid, and 90% (v/v) alcohol) for 24 h. After dehydration through an alcohol–xylene series, the tissues were transferred to liquid paraffin at 60°C, and then embedded in paraffin blocks. Sections (5-μm thick) were cut using a LEICA RM 2135 rotary microtome (Germany). The sections were mounted on microslides, dewaxed and hydrated with xylene and decreasing concentrations of alcohol. The sections were stained with 1% safranin and 0.1% fast green, and dehydrated in xylene and increasing concentrations of alcohol. The slides were observed at 10 × and 20 × magnifications and photographed under a LEICA DM 2500 microscope (Germany).

Statistical analysis

Statistical analysis was performed by Duncan's multiple range test using SPSS software (version 12.0; 2003) and $p \leq 0.05$ was considered statistically significant.

RESULTS

Induction of adventitious shoot buds and nodule-like callus

The zygotic embryo explants (Fig. 1A) excised from mature seeds which cultured in dark started to swell (Fig. 1A) after 2 days in culture. After 2 weeks in culture, shoot axes of the swelling embryos enlarged faster than root axes (Fig. 1C). Shoot buds formed from the surface of the shoot axes end after approximately 3 weeks in culture on medium containing TDZ and IAA (Fig. 1D) and then turned green when exposed to light (3 weeks later) (Fig. 1F). One month later, the parts of buds contacting medium gradually formed green nodule-like calluses with potential regeneration capacity (Fig. 1G). The addition of a moderately low concentration of TDZ in the medium resulted in shoot bud formation (Table 1). The highest rate of shoot bud induction (76.9%) was on medium supplemented with 4.54 μM TDZ and 2.85 μM IAA. At higher concentrations of IAA or TDZ, fewer calluses formed, and those that did form showed a poor regeneration capacity and a withered appearance.

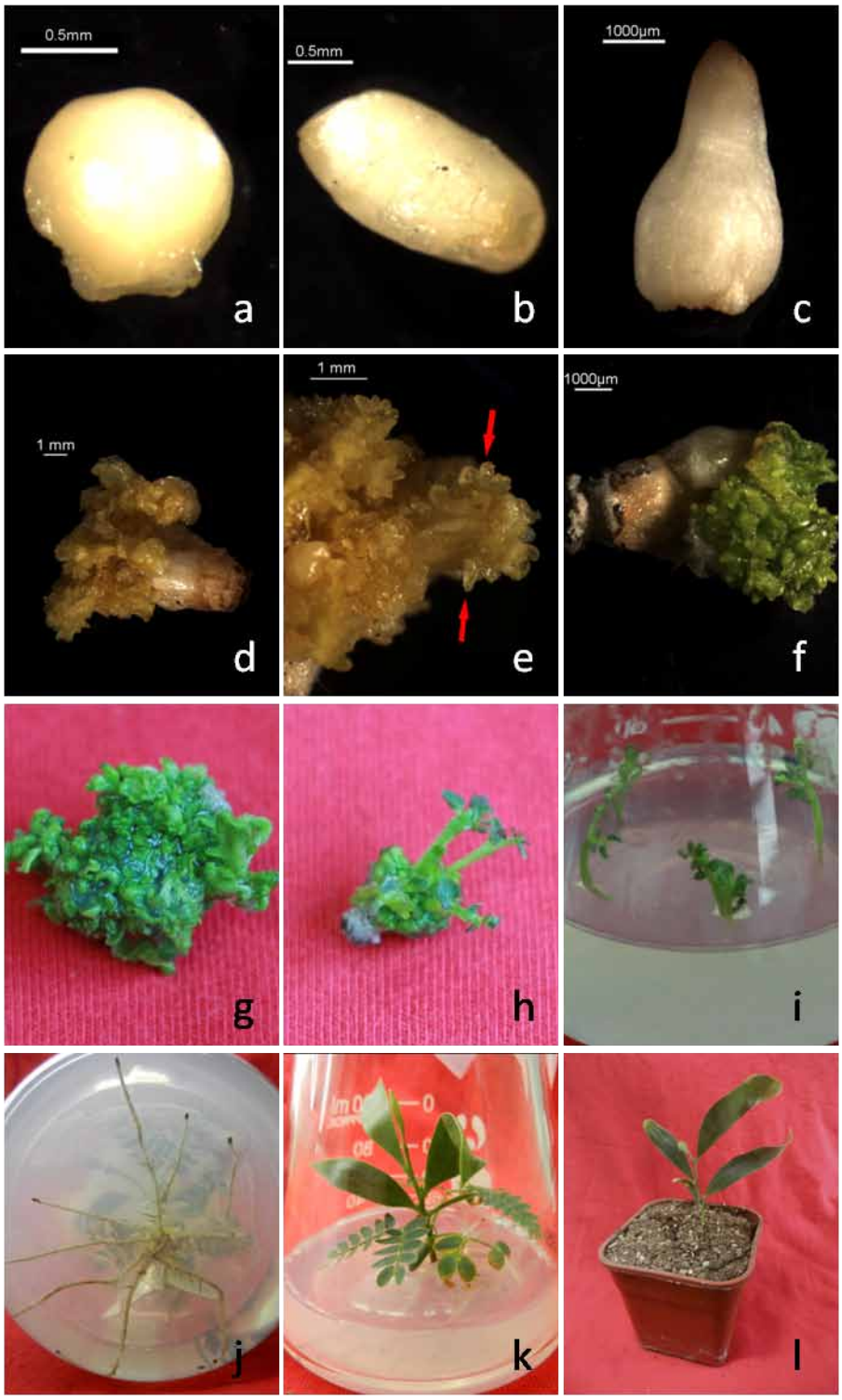

Fig. 1. Shoot bud induction and plant regeneration from mature zygotic embryos of *Acacia crassicarpa.* A) Zygotic embryos excised from the mature seeds, B) Swelling of zygotic embryos after 2 days of culture on MS medium, C) Differentiation of zygotic embryos after 2 weeks of culture on MS medium containing 4.54 µM TDZ and 2.85 µM IAA. Note that shoot axes of embryo is enlarged, D) Shoot buds formed after 3 weeks of culture on MS medium containing 4.54 µM TDZ and 2.85 µM IAA, E) 50 × magnification of buds under stereomicroscope (red arrows), F) Greening of shoot buds after exposure to light, G) Callus with shoot buds, H) Elongated shoots, I) Shoots on rooting medium, J)Roots formed from shoots, K) Regenerated plant, and L) Hardened plant.

Table 1. Effect of TDZ and IAA on shoot bud regeneration from mature zygotic embryo explan.

Plant growth regulators (μM)		Explants with shoot buds (%)	Mean number of shoots
TDZ	IAA		
2.27	0.57	65.5 ± 1.1 d	24.5 ± 0.9 de
2.27	2.85	64.5 ± 0.8 d	26.3 ± 0.7 d
2.27	5.71	64.6 ± 0.9 d	23.5 ± 0.7 e
4.54	0.57	73.2 ± 1.1 b	46.5 ± 3.9 b
4.54	2.85	76.9 ± 1.5 a	56.5 ± 1.0 a
4.54	5.71	69.6 ± 1.4 c	46.3 ± 0.8 b
9.81	0.57	53.0 ± 1.0 g	33.8 ± 0.7 c
9.81	2.85	59.4 ± 1.4 e	34.4 ± 0.7 c
9.81	5.71	55.3 ± 0.9 f	33.1 ± 0.2 c
18.16	0.57	48.9 ± 0.7 h	25.9 ± 0.8 de
18.16	2.85	47.6 ± 0.5 h	24.2 ± 0.9 de
18.16	5.71	41.9 ± 1.4 i	19.5 ± 0.5 f

Data were recorded after 2 months on culture when shoots were clearly visible. The experiment was duplicated with 100 explants per treatment. Means followed by same letters within a column were not significantly different at $p \leq 0.05$ (Duncan's multiple range test).

Table 2. Effects of GA_3 and TDZ on shoot elongation of *Acacia crassicarpa.*

Plant growth regulators (μM)		Mean length per shoot (cm)
TDZ	GA_3	
0.00	0.00	0.9 ± 0.2 c
0.00	1.44	1.8 ± 0.3 b
0.00	2.89	2.3 ± 0.3 a
2.27	0.00	0.9 ± 0.3 c
2.27	1.44	1.1 ± 0.2 c
2.27	2.89	1.2 ± 0.2 c

Data were recorded after 1 month of culture on elongation medium. The experiment was duplicated with 50 explants per treatment. Means followed by the same letters within a column were not significantly different at $p \leq 0.05$ (Duncan's multiple range test).

Table 3. *In vitro* rooting of shoots on medium supplemented with IBA.

IBA (μM)	Rooted shoots (%)	Mean roots per shoot
0.49	97.2 ± 2.0 a	19.7 ± 1.6 bc
2.46	98.9 ± 1.1 a	27.7 ± 3.0 a
4.92	98.4 ± 0.7 a	22.5 ± 3.0 b
9.84	96.7 ± 1.6 a	17.3 ± 1.7 c

Data were recorded after 1 month in culture, when root initiation and development could be clearly visualized. The experiment was conducted in duplicate with 50 explants per treatment. Means followed by the same letters within a column were not significantly different at $p \leq 0.05$ (Duncan's multiple range test).

Shoot differentiation and elongation

Explants were transferred to differentiation medium after 2 months in culture. The nodule-like calluses with shoot buds developed into clustered shoots. Clustered shoots elongated to 1-2 cm and developed a stem in 3 months (Fig. 1H). Efficient shoot elongation was achieved on medium containing 2.89 μM GA_3 (Table 2).

Rooting and plantlet survival

When shoots reached 2 cm, they were removed from the base of the calluses and transferred to rooting medium (Fig. 1I). The highest rooting rate (98.9%) was obtained on medium containing 2.46 μM IBA (Fig. 1, Table 3). When the plantlets reached 4-5 cm in height 1 month later (Fig. 1K), they were transplanted into pots. The transplanted plantlets showed a 97.5% survival rate, vigorous growth, and normal phenotypes (Fig. 1L).

Histological analyses

Analysis of histological sections of 40-day-old shoot buds showed that the growing tip and young leaves had already formed (Fig. 2). The vascular bundles of the bud and maternal tissues were linked together and were not independently differentiated. The meristematic regions had sieve tubes and ringed vessels (Fig. 2A, and amplified in Fig. 2B) and were connected to the maternal tissues. These data and observations indicated that the *A. crassicarpa* plantlets had regenerated via shoot organogenesis.

DISCUSSION

In this study, we tested a new protocol for plant regeneration of *A. crassicarpa* via adventitious shoot organogenesis. The success of tissue culture largely relies on the selection of a suitable explant for use as the starting material (Sahin-Demirbag et al. 2010). Recently, there has been increasing use of zygotic embryos as the experimental explants to study regeneration in various plant species (Zhang et al. 2010, Hosseini Tafreshi et al. 2011, Muñoz-Concha et al. 2012). The use of mature zygotic embryos for *A. crassicarpa* regeneration eliminates the need for immature explant materials. In this study, we obtained a high rate of shoot bud production *in vitro* by culturing embryo explants on MS medium containing TDZ and IAA. After shoot elongation and rooting treatments, the regenerated plantlets with healthy roots were hardened and successfully established in soil, with a 97.5% survival rate. The entire regeneration process took less than 5 months.

Plant growth regulators that are known to play a key role in organogenesis can regulate the differentiation of plant cells. Many previous reports showed that low concentrations of auxins and cytokinins play a critical role in plant regeneration (Carra et al. 2012, Yang et al. 2012). Zhang used axillary buds of *Acacia*

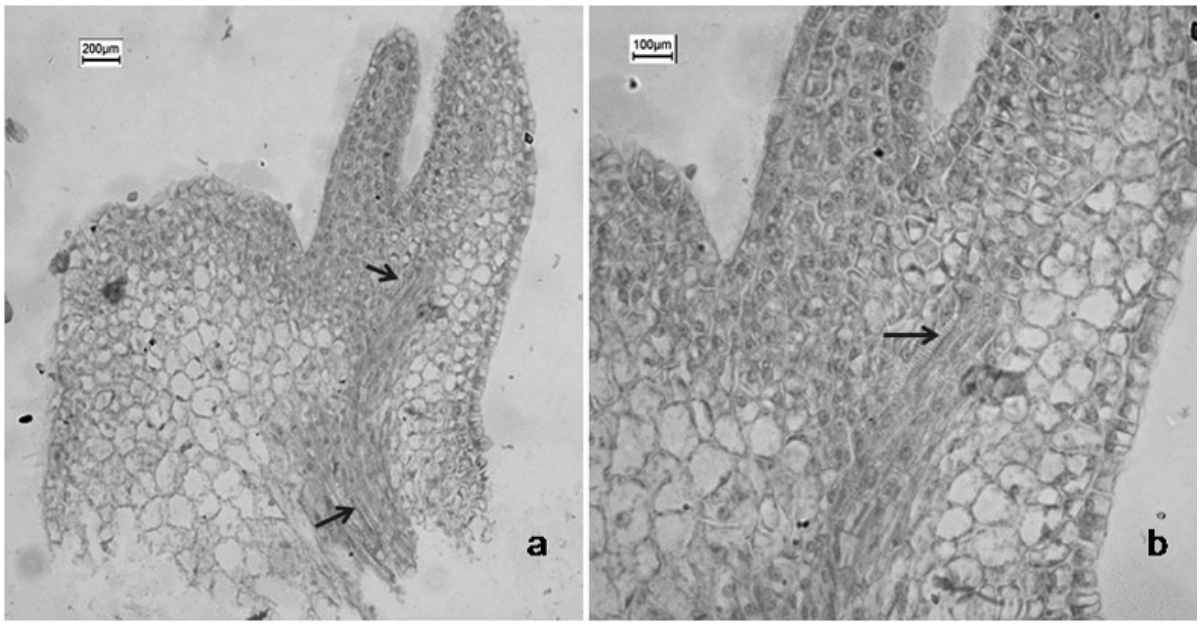

Fig. 2. Histological sections of 40-day old shoot buds of *Acacia crassicarpa*. A) Longitudinal section of emerging bud. Note that vascular bundles of bud and maternal tissue were linked together (arrows) (10 ×) and B) 20 × magnification, arrow shows ringed vessel of vascular bundle.

auriculiformis as explants to induce plant regeneration, multiple shoot induction was obtained through culturing on MS basal medium supplement with 10 μM BA and 0.5 μM IBA. 66.7% of axillary buds of *Acacia auriculiformis* explants produced multiple shoots, the largest shoot number per explants reached 55 (Zhang et al. 1995). Our experiments choose zygotic embryos as explants and the culture medium contain TDZ and IAA obtained 76.9% budding rate. TDZ has been used widely to promote shoot regeneration in many plant species, and generally gives better results than other cytokinins (Xie and Hong 2001b, Abu-Qaoud 2012). The combination of TDZ and IAA promoted the differentiation of green-yellowish and friable embryogenic callus and its development into plantlets *via* somatic embryogenesis in *A. mangium* (Xie and Hong 2001b). TDZ at moderately low concentrations was useful for micropropagation of woody species (Lu 1993). Our results showed that TDZ effectively induced adventitious bud formation in *A. crassicarpa.* It was reported that GA_3 promoted plant growth by stimulating cellular expansion (Sauret-Güeto et al. 2012) and induced shoot elongation in species that were normally recalcitrant to elongation *in vitro* (Phinney 1984). We tested the effects of GA_3 on shoot elongation, and found that it had a promoting effect (Table 2). This finding demonstrated that GA_3 mayShoots formed roots on half-strength MS medium supplemented with IBA, which has been reported to be the optimum plant growth regulators to induce the root-forming response of shoots (Wakhlu and Barna 1989). IBA has been used to induce rooting in the regeneration process for many species (Sahin-Demirbag et al. 2010, Zhang et al. 2010, Rafique et al. 2012). In our study, plantlets showed strong root growth after 15 days on medium containing 2.46 μM IBA; the rooting frequency was greater than 98% (Table 3). The concentration of IBA is consistent with our previous results (Yang et al. 2006). The survival rate of rooted plantlets transferred to soil and grown in the greenhouse was 97.5% and the rooted plants grew normally after acclimatization.

In conclusion, we have established a stable and effective regeneration system to produce *A. crassicarpa* plantlets. Previously, we reported regeneration from phyllode explants of *A. crassicarpa,* with a shoot bud induction rate of 56% (Yang et al. 2006). The system reported here represents a more efficient (76.9% bud induction rate) and convenient method of regeneration *via* shoot organogenesis. Using mature seeds as explant source has several advantages, including the ease of handling and the year-around availability of seeds. However, the traits of mature trees should be evaluated after a long period of time, since we believe to report the limit of propagation via seeds and hence, further study is necessary to clarify the issue. Our protocol can offer an alternative strategy for *A. crassicarpa* propagation, which will be useful for expanding plantations of this species. The described method for shoot proliferation and regeneration of *A. crassicarpa* is not only suitable for rapid micropropagation but also for further molecular biology research of *A. crassicarpa.*

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