Review of JPOP732

Title page

PLANT REGENERATION FROM MATURE ZYGOTIC EMBRYO EXPLANTS OF *ACACIA CRASSICARPA* VIA ADVENTITOUS SHOOT ORGANOGENESIS

Running Title: Plant regeneration of *Acacia crassicarpa*

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** Report All plant growth regulators in ** micro Molar (µM) not milligrams per liter; here and throughout the manuscript.

Abstract:

Acacia crassicarpa is a major species for fiber production in Southeast Asia. *In vitro* multiplication of this species could be valuable for expanding plantation. To explore new approaches to propagate *A. crassicarpa*, we developed an ~~efficient~~ 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 1-pheny l-3-(thiadiazol-5-yl) urea (thidiazuron) (TDZ) and indole-3-acetic acid (IAA).Combinations of TDZ at 1-2 mg l^{-1} and IAA at 0.1-1.0 mg l^{-1} were optimum to induce shoot bud formation. ~~The~~ medium containing 1 mg l^{-1} TDZ and 0.5 mg l^{-1} IAA yielded the highest rate of shoot buds(73.23%). The embryos with shoot buds were subcultured in fresh medium of the same composition ~~above~~ for shoot development and then ~~grew~~ into ~~big~~ green nodule-like callus. The callus were then transferred to the media containing Gibberellic acid (GA_3) for shoot elongation induction. ~~The~~ elongated shoots ~~would be~~ rooted on 1/2 MS medium with 0.5 mg l^{-1} 3-indol ebutyric acid (IBA) within 1 month. Rooted plantlets were hardened and successfully established in soil with an 97.5 % survival rate. ~~The data from this study provided a useful technique with using mature zygotic embryos for *A. crassicarpa in vitro* regeneration, which might facilitate the expanding plantation of *A. crassicarpa* with high-quality regenerated plantlets. The highest rate of shoot bud induction (73.23%) was on medium containing 1 mg l^{-1} TDZ and 0.5 mg l^{-1} IAA. Embryos with shoot buds were subcultured on fresh medium with the same composition as that described above for shoot development. Large, green nodule calli grew from these embryos. The calli were then transferred to medium containing gibberellic acid (GA_3) to induce shoot elongation. The elongated shoots formed roots within 1 month when grown on 1/2 MS medium containing 0.5 mg l^{-1} 3-Indolebutyric acid (IBA). The rooted plantlets were hardened and successfully established in soil with a survival rate of 97.5%~~. This system, in which mature zygotic embryos were used to regenerate *A. crassicarpa* plants *in vitro*, will be useful for producing high-quality regenerated plantlets that could be used to expand plantations of this species.

Key words:Acacia crassicarpa, thidiazuron, indole-3-acetic acid

* Use half-strength MS medium instead of ½ MS * here and throughout the manuscript.

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State concentration of GA_3 used.

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 ~~they~~ these 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 (Midgley 2000, Minquan et al. 1995, Zhigang and Yintian 1994). Many *Acacia* species are important for shelterbelts ~~and~~, soil improvement, and as sources of fuel and timber (Palmberg ~~et al.~~ And Pasca 1981) . *A. 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 ~~been~~ become a preferred fiber source for the paper and pulp 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/or planting area. However, classical breeding programs of *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, ~~Xie and Hong 2001~~b), *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 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. The advantages of using embryos as the explant source include the ease of handling and ready availability of seeds, the high degree of physiological uniformity, and the ability to transport seeds and embryos long distances.

Materials and methods:

Plant material and surface ~~sterilization~~ disinfestation:

Mature seeds of *A. crassicarpa* were collected from ~~the~~ 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. ~~To sterilize the seed surface, the seeds were~~ Seeds were surface disinfested by soaking in 70% ethanol for 1 min, ~~and~~ then in 4% sodium hypochlorite for 5 min. ~~The sterilized seeds were rinsed~~ followed by rinsing five times with sterile distilled water. The episperm of each seed was cut manually, ~~with a sterile surgical blade. The~~ seeds were dehulled, and embryos were excised aseptically and prepared for primary shoot bud induction.

Culture medium and conditions:

The culture medium was ~~MS basal medium~~ Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) supplemented with 5% (v/v) coconut water (CW), ~~and~~ 3% (w/v) sucrose, and solidified with 0.6% (w/v) agar. All media were adjusted to pH 5.8 with 1N NaOH.The plant growth regulators (PGR) TDZ and IBA were added at various concentrations before autoclaving media at 121°C for 15 min. IAA and 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 $s^{-1}m^{-1}$ ~~(PAR)~~ μmol m^{-2} s^{-1} under a 16-h light/8-h dark photoperiod.

State brand + Co. of CW. + AGAR

see below *

Shoot buds induction and elongation:

describe briefly (whole embryo? partial? etc)

Embryo explants were ~~placed onto~~ cultured on shoot bud induction ~~media~~ medium containing ~~different~~ combinations of TDZ (0.5, 1.0, 2.0, or 4.0 mg l^{-1}) and IAA (0.1, 0.5, or 1.0 mg l^{-1}). Each Petri dish (90 × 15 mm) contained 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, calli with shoot buds were transferred to MS ~~media~~ medium supplemented ~~different~~ with combinations of GA_3 (0, or 0.5 mg l^{-1}) and TDZ (0, 0.5, or 1 mg l^{-1}) 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 ~~media~~ medium.

ml. of medium?

? |

*Spell out all words then abbreviate when * first used in the text. Abstract does not count.

Rooting ~~from~~ regenerated shoots and plantlet survival

Elongated shoots (≥2 cm) were removed from the callus ~~base~~ and transferred to ~~rooting medium (½~~ MS supplemented with 0.1,0.5,1.0,2.0 mg l^{-1}IBA~~)~~. After formation of lateral roots, plantlets were transferred into pots.The experiment was duplicated with 50 ~~explants~~ per treatment and was repeated three times.The survival ~~ratio~~ was determined 1 month after acclimatization. Subculturing ~~onto~~ fresh medium ~~of the same composition~~ 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, ~~and then~~ 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).

Results

Induction of adventitious shoot buds and nodule-like callus ~~from mature zygotic embryos~~

The zygotic embryo explants (Fig. 1a) excised from mature seeds started to swell (Fig. 1b) after 2 days in culture. After 2 weeks in culture, one end of the swelling embryos enlarged faster than the other (Fig. 1c). Shoot buds formed from the surface of the enlarged end after approximately 3 weeks in culture on medium containing TDZ and IAA (Fig. 1d) and then turned green when exposed to light (Fig. 1f). One month later, the parts of buds contacting medium gradually formed green nodule-like calli with potential regeneration capacity (Fig. 1g). ~~As shown in Table 1~~, the addition a moderately low concentration of TDZ to the medium resulted in shoot bud formation. The highest rate of shoot bud induction (73.23%) was on medium supplemented with 1 mg l^{-1} TDZ and 0.5 mg l^{-1} IAA. At higher

concentrations of IAA or TDZ, fewer calli formed, and those that did form showed a poor regeneration capacity and a withered appearance.

~~Plant growth regulators promoted~~ Shoot differentiation and elongation

The explants were transferred to differentiation medium after 2 months in culture. The nodule-like calli 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 1 mg l^{-1} GA_3 (Table 2).

Rooting and plantlet survival

When shoots reached 2 cm, they were removed from the base of the calli and transferred to rooting medium (Fig. 1i). The highest rooting rate (~~98.87%~~ 98.9%) was obtained on medium containing 0.5 mg l^{-1} IBA (Fig. 1j; 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

Need to compare & contrast your results to results in the literature for Acacia & other woody species.

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 explant material to study regeneration in various plant species (Hosseini Tafreshi et al. 2011, Muñoz-Concha et al. 2012, Zhang et al. 2010). 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 ~~de~~differentiation ~~and redifferentiation~~ 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). 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). ~~Xie reported that~~ 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 ~~the phytohormone~~ 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 may have a significant role in the shoot elongation of *A. crassicarpa.*

Compare + Contrast the concentrations used for organogenesis + Rooting.

Shoots formed roots on ~~1/2~~ half-strength MS medium supplemented with IBA, which has been reported to be the optimum PGR 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 (Rafique et al. 2012, Sahin-Demirbag et al. 2010, Zhang et al. 2010). In our study, plantlets showed strong root growth after 15 days on medium containing 0.5 mg l^{-1} IBA; the rooting frequency was greater than 98% (Table 3). 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. ~~In this system, mature zygotic embryos were used as explant materials, and were cultured on MS basal medium containing various PGRs~~. Previously, we reported regeneration from phyllode explants of *A. crassicarpa,* with a shoot bud induction rate of 56% (Yang et al. 2006). The

Repetitive

system reported here represents a more efficient (73.23% bud induction rate) and convenient method of regeneration via shoot organogenesis. Using mature seeds as the explant source has several advantages, including the ease of handling and the year-around availability of seeds. ~~Using this method, the entire process from mature seeds to regenerated plantlets took less than 5 months~~. 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*.

Acknowledgment

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Figure legends:

Fig. 1 Shoot buds induction and plant regenation 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 TDZ and IAA. Note that one end of embryo is enlarged. d. Shoot buds formed after 3 weeks of culture on MS medium containing TDZ and IAA. e. 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. l. Hardened plant.

Fig.2 Histological sections of 40-day-old shoot buds of *Acacia crassicarpa*

a.Transverse section of emerging bud. Note that vascular bundles of bud and maternal tissue ~~are~~ were linked together (arrows) (10×). b. Magnification ~~of fig. 2a.~~ ,arrow shows ringed vessel of vascular bundle ~~(20×)~~.

Table 1 Effect of TDZ and IAA on shoot buds regeneration from mature zygotic embryos explant of *A. crassicarpa*

Plant growth regegulators(mg/l)		Explants with shoot buds(%)	Mean number of shoots per explant
TDZ	IAA		
0.5	0.1	65.53±1.11d	24.50±0.87de
0.5	0.5	64.50±0.79d	26.33±0.65d
0.5	1	64.60±0.90d	23.50±0.66e
1	0.1	73.23±1.10b	46.47±3.89b
1	0.5	76.87±1.50a	56.53±0.96a
1	1	69.60±1.42c	46.27±0.83b
2	0.1	53.00±1.00g	33.83±0.74c
2	0.5	59.43±1.37e	34.37±0.72c
2	1	55.27±0.87f	33.07±0.15c
4	0.1	48.87±0.74h	25.90±0.80de
4	0.5	47.63±0.47h	24.20±0.91de
4	1	41.93±1.36j	19.50±0.53f

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

* Describe abbreviations in a footnote.

* Table title should be within the table size.

Table 1. Effect of .....

Plant growth Regulator (μM)

* Do this for all tables. Tables need to stand alone from the text. (all of the above)

of ACACIA CRASSICARPA.

Table 2 Effects of GA_3 and TDZ on shoot elongation ~~in MS medium~~

Plant growth regegulators(mg/l)		Mean length per shoot(cm)
TDZ	**GA_3**	
0	0	0.87±0.19c
0	0.5	1.76±0.27b
0	1	2.32±0.34a
0.5	0	0.92±0.32c
0.5	0.5	1.14±0.19c
0.5	1	1.21±0.16c

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 ~~are~~ were not significantly different at at P = 0.05 (Duncan's multiple range test).

Table 3 *In vitro* rooting of shoots on ~~1/2 MS~~ medium supplemented with ~~different concentrations of IBA~~.

Plant growth regegulators(mg/l) IBA	~~Rooted shoots~~(%)	Mean roots per shoot
0.1	97.17±1.98a	19.7±1.61bc
0.5	98.87±1.06a	27.67±2.99a
1	98.37±0.71a	22.5±3.01b
2	96.67±1.53a	17.27±1.70c

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 ~~are~~ were not significantly different at P = 0.05 (Duncan's multiple range test).