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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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 1-pheny l-3-(thiadiazol-5-yl) urea (thidiazuron) (TDZ) and indole-3-acetic acid (IAA). Combinations of TDZ at 4.54-9.81 *μ*M and IAA at 0.57-5.71 *μ*M were optimum to induce shoot bud formation. Medium containing 4.54 *μ*M TDZ and 2.85 *μ*M 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 were then transferred to the media containing 2.89 *μ*Mgibberellic acid (GA3) for shoot elongation induction. Elongated shoots rooted on half-strength medium with 2.46 *μ*M indole-3-butyric acid (IBA) within 1month. Rooted plantlets were hardened and successfully established in soil with a 97.5% survival rate. 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, adventitious budding, thidiazuron, indole-3-acetic acid

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 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, 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 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 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), *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*.

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 (MS) basal medium 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 (PGR) 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 (GA3) 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 *μ*mol 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 *μ*M) and IAA (0.57, 2.85, or 5.71 *μ*M) 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, calli with shoot buds were transferred to MS medium supplemented with combinations of GA3 (0, 1.44, or 2.89 *μ*M) and TDZ (0, or 2.27, *μ*M) 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 *μ*MIBA for 1month and cultured in light. Each culture vessel contains 50ml 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-*μ*m 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 contained in the SPSS software suite (version 12.0; 2003) and p≤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. 1b) 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, shoot buds were transferred 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). 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 *μ*M TDZ and 2.85 *μ*M 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.

shoot differentiation and elongation

## 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 2.89 *μ*M GA3 (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 (98.9%) was obtained on medium containing 2.46 *μ*M 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](app:ds:vascular) [bundle](app:ds:bundle)s 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 shootorganogenesis. 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 (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 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* *auriculiformis* as explants to induce plant regeneration, multiple shoot induction was obtained through culturing on MS basal medium supplement with 10 *μ*M BA and 0.5 *μ*M 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 atmoderately 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 GA3 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 GA3 on shoot elongation, and found that it had a promoting effect (Table 2). This finding demonstrated that GA3 may have a significant role in the shoot elongation of *A. crassicarpa.*

## Shoots 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 (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 2.46 *μ*M IBA; the rooting frequency was greater than 98% (Table 3). The concentration of IBA is consistant 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](app:ds:in%20conclusion), 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 the 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, this was the limit of propagation via seeds in this report and needed further study in the future. 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 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 *μ*M TDZ and 2.85 *μ*M IAA. Note that shoot axes of embryo is enlarged. d. Shoot buds formed after 3 weeks of culture on MS medium containing 4.54 *μ*M TDZ and 2.85 *μ*M 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. l. Hardened plant.

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

a. Longitudinal section of emerging bud. Note that [vascular](app:ds:vascular) [bundle](app:ds:bundle)s of bud and maternal tissue were linked together (arrows) (10×). b. 20× Magnification, arrow shows ringed vessel of [vascular](app:ds:vascular) [bundle](app:ds:bundle).

**Table 1** Effect of TDZ and IAA on shoot bud regeneration from mature zygotic

embryo explants of *Acacia crassicarpa*

|  |  |  |  |
| --- | --- | --- | --- |
| **Plant growth regulators(*μ*M)** | | **Explants with shoot buds(%)** | **Mean No. shoots per explant** |
| **TDZ** | **IAA** |
| 2.27 | 0.57 | 65.5±1.1d | 24.5±0.9de |
| 2.27 | 2.85 | 64.5±0.8d | 26.3±0.7d |
| 2.27 | 5.71 | 64.6±0.9d | 23.5±0.7e |
| 4.54 | 0.57 | 73.2±1.1b | 46.5±3.9b |
| 4.54 | 2.85 | 76.9±1.5a | 56.5±1.0a |
| 4.54 | 5.71 | 69.6±1.4c | 46.3±0.8b |
| 9.81 | 0.57 | 53.0±1.0g | 33.8±0.7c |
| 9.81 | 2.85 | 59.4±1.4e | 34.4±0.7c |
| 9.81 | 5.71 | 55.3±0.9f | 33.1±0.2c |
| 18.16 | 0.57 | 48.9±0.7h | 25.9±0.8de |
| 18.16 | 2.85 | 47.6±0.5h | 24.2±0.9de |
| 18.16 | 5.71 | 41.9±1.4j | 19.5±0.5f |

Note: No. means number.

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* = 0.05 (Duncan’s multiple range test).

**Table 2** Effects of GA3 and TDZ on shoot elongation of *Acacia crassicarpa*

|  |  |  |
| --- | --- | --- |
| **Plant growth regulators(*μ*M)** | | **Mean length per shoot(cm)** |
| **TDZ** | **GA3** |
| 0 | 0 | 0.9±0.2c |
| 0 | 1.44 | 1.8±0.3b |
| 0 | 2.89 | 2.3±0.3a |
| 2.27 | 0 | 0.9±0.3c |
| 2.27 | 1.44 | 1.1±0.2c |
| 2.27 | 2.89 | 1.2±0.2c |

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 = 0.05 (Duncan’s multiple range test).

**Table 3** *In vitro* rooting of shoots on medium supplemented with indole-3-butyric acid (IBA).

|  |  |  |
| --- | --- | --- |
| **Plant growth regulators(*μ*M)** | **Rooted shoots(%)** | **Mean roots per shoot** |
| **IBA** |
| 0.49 | 97.2±2.0a | 19.7±1.6bc |
| 2.46 | 98.9±1.1a | 27.7±3.0a |
| 4.92 | 98.4±0.7a | 22.5±3.0b |
| 9.84 | 96.7±1.6a | 17.3±1.7c |

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 = 0.05 (Duncan’s multiple range test).

**Response to reviewer:**

**Response to the comments by Reviewer 1**

**C. Scientific quality**

**2.**Is the manuscript clearly written and well-organized? No. Comments: Manuscript needs English Editing. Episperm (line 89), dehulled (line 90), atmoderatly (line 173) are example of incorrect words. In addition text has a lot of type error (space between words is missing).

**Response:** The authors would like to thank the reviewer for his/her advice. The entire manuscript has been carefully checked and revised according to the suggestion.

**3.** Are the Abstract and the Key words adequate? No. Suggestions: Abstract: suppress section lines 40-46 that is a repetition from lines 32-39. Key words: missing Key words such as adventitious budding, zygotic embryo, plant regeneration. On the opposite suppress plant growth regulator names (TDZ, IAA), that have to be in abbreviations section (missing).

**Response:** As suggested by the reviewer, we have added “adventitious budding” as key word, but according to the chief editor’s suggestion, the words existing in the title should omit in the key words and the abbreviations will not be published (so we deleted the abbreviations).

**7.**Are the tables and figures titles and legends presented well and necessary? No. Comments: Fig 2: transverse section? longitudinal section

**Response:** “transverse section” has been changed to “longitudinal section” as suggested.

9. Have all relevant literature been citedNo. Suggestions: Introduction line 60: cited Palmberg et al 1981; in the reference list it is Palmberg and Pasca

**Response:** “Palmberg” has been changed to “Palmberg and Pasca” (introduction paragraph 1, line 51).

Please add further comments: As mentioned in the introduction (lines 65-67), for trees mature traits can be evaluated after a long period of time. In consequence, if using zygotic embryo has a lot of advantages for plant regeneration (conclusion lines 192-194), on the opposite one does not have any idea about the value of the plant regenerated. Clonal tests will have to be established. This is a limit of the propagation via seeds, point that has to be pointed out in the discussion/ conclusions.

**Response:** The reviewer’s concern is valid. The traits of mature trees should be evaluated after a long period of time, this was the limit of propagation via seeds in this report and needed further study in the future. We have added these sentences into the discussion (discussion paragraph 4, line 192).

**Response to the comments by Reviewer 2**

**C. Scientific quality**

**2.**Is the manuscript clearly written and well-organized? No. Comments:…See attached PDF; comments written directly on the manuscript. Check spacing throughout the paper, better to align text left. No justify.

**Response:** As suggested by the reviewer, we have checked spacing throughout the paper.

**3.** Are the Abstract and the Key words adequate? No. Suggestions:…… See attached PDF; comments written directly on the manuscript.

1) Report all plant growth regulators in micro molar (*μ*M) not milligrams per liter.

2) State concentration of GA3 used.

3) Use half-strength MS medium instead of 1/2 MS.

4) Line 27 “plantation” needs to be changed to “plantations”;

line 32 “The medium” needs to be changed to “Medium”;

line 33 “73.23%” needs to be changed to “73.2%”, here and through the manuscript; “in” needs to be changed to “to”;

line 34 “grew” needs to be changed to “proliferate”;

line 35 “Gibberellic” needs to be changed to “gibberellic”;

line 36 “The elongated” needs to be changed to “Elongated”, “3-indol ebutyric” needs to be changed to “indole-3-butyric”

5) Line 28 “an efficient”, line 34 “above”, line 36 “would be” and line 38-line 46 need to be deleted.

**Response:** The manuscript has been revised according to the reviewer’s suggestion and the concentration of GA3 had been stated.

**5.** Materials, methods and study design. Improvement needed. Suggestions:… See attached PDF; comments written directly on the manuscript.

1) Line 83 “sterilization” needs to be changed to “disinfestation”;

line 86 “To sterilize the seed surface ,the seed were” needs to be changed to “Seeds were surface disinfested by”;

line 87 “To sterilize the seed” needs to be changed to “Seeds were surface”;

line 88 “The sterilized seeds were rinsed” needs to be changed to “followed by rinsing”;

line 89 “with a sterile surgical blade” should be deleted;

line 92 “MS basal medium” needs to be changed to “Murashige and Skoog (MS) basal medium”

line 97 “*μ*mol s-1m-1” needs to be changed to “*μ*mol m-2 s-1”;

line 99 “shoot buds” needs to be changed to “shoot bud”;

line 100 ,line 104, line 107 “placed onto” needs to be changed to “cultured on”, “media” needs to be changed to “medium”;

line 104 “different” needs to be changed to “with”;

line 108 “from” needs to be changed to “of”;

line 111 “explants” needs to be changed to “shoots”;

line 112 “ratio” needs to be changed to “rate”; “onto” needs to be changed to “to”;

2) The comma after all tittles needs be deleted;

line 84 “the”, line 87,line 93 “and”, line 98 (PAR),line 100 “different”, line 109 “base”, line 113 “of the same composition”, line 119 “and then” need to be deleted.

3) Line 92, need state brand of co. of CW and agar.

4) Line 100 “Embryo explants” needs describe briefly (whole embryo? Partial? etc)

5) Line 101, what was the volume of medium?

6) Spell out all words then abbreviate when first used in the text.

7) On IBA for how long? Cultured in dark or light? Cultured vessel? ml of medium?

8) Acclimatization process? Soil mix?

9) Need a paragraph on statistical procedure.

10)Line TDZ (0.5,1.0,2.0,4.0) should be changed to (0.5,1.0,2.0, or 4.0), here and through the manuscript.

**Response:**1-2), 6),10) The manuscript has been revised according to the reviewer’s suggestion.

3) According to the reviewer’s suggestion, we have added the brand of CW and agar.

4) “Embryo explants” has been changed to “Whole embryo explants”.

5) The volume of medium was 50ml per culture vessel.

7) On IBA for 1month, cultured in light, 50ml medium per culture vessel.

8) As suggested, we added acclimatization process and soil mix.

9) A paragraph on statistical procedure have been added into the materials and methods in the revised manuscript.

6.Results and Discussion .Should be adjusted – Suggestions:

1) Line 127 “one end of ” , what end of embryos? Short or root axes?

2) Line 128 “when exposed to light ” ,were cultures kept in the dark prior or light?

3) In the discussion, need to compare or contrast your results to results in the literature for *Acacia* or other woody species.

4) Compare or contrast the concentrations used for organogenesis of rooting.

5) Line 131, “the addition” needs to be changed to “The addition of”;

line 132, “to” needs to be changed to “in”;

line 137, “The explants” needs to be changed to “Explant”;

line 143, “98.87” needs to be changed to “98.9”;

line 170, “the” needs to be changed to “The”.

6) Line124, “from mature zygotic embryos”; line 131, “As shown in Table 1”; line 136, “Plant growth regulators”; line 158 , “material”, line 166, “and redifferentiation”, line169, “Xie reported that”, line 174, “the phytohormone” , line 187 “In this system, mature zygotic embryos were used as explants materials, and were cultured on MS basal medium containing various PGRs”; line 192, “Using this method, the entire process from mature seeds to regeneration plantlets took less than 5 months” need to be deleted.

7) Line 132, “(Table 1)”should be added after “shoot bud formation”;

line161, “of” should be added after “a high rate”;

**Response:**1) The swelling end of embryos is shoot axes, this have been revised.

2) The embryos were cultured in dark for 3 weeks and then transferred to light. We have added related information to the revised manuscript.

3) To address the reviewer’s concern, we have compared plant regeneration of *Acacia crassicapa* with *Acacia* *auriculiformis* and *A. mangium* in discussion (discussion paragraph 2, line 163)*.*

4) According to the reviewer’s suggestion, we have discussed the IBA concentration for rooting (discussion paragraph 3, line 184).

5-7) The manuscript has been revised as suggested.

7. Are the tables and figures titles and legends presented well and necessary? Improvement needed. Suggestions:…… See attached PDF; comments written directly on the manuscript

1) The arrows on Fig.1 were missed.

2) Line 284, “one end of”, what do you call this end? shoot axes? Root axes?

3) The magnification times of Fig.1e was missing.

4) What was the concentration of PGR.

5) Data of tables should be reported to one decimal place.

6) Line 297 “*A*. crassicarpa”, should be spell out.

7) Describe abbreviations in foot note.

8) Table title should be within the table size.

9) Tables need to stand alone from the text.

10) Line 281, “buds” needs to be changed to “bud”, “regenation” needs to be changed to “regeneration”;

line 292, 302,310,321, “are” needs to be changed to “were”;

line 293,b should be “20× magnification, arrow shows ringed vessel of vascular bundle”;

line 297, “buds” needs to be changed to “bud”, “embryos explant” needs to be changed to “embryo explants”

line 299, “Mean number of shoots per explant” needs to be changed to “Mean No. shoots per explant”;

line 305, “in MS medium” needs to be changed to “of *Acacia crassicarpa*”;

line 314, “different concentrations of IBA” needs to be changed to “IBA”;

11) Line 301, “in”; line 314, “1/2 MS”, should deleted.

**Response:**1) The arrows has been added into Fig.1.

2) “one end of” means shoot axes.

3)The Fig.1e was 50× magnified. This has been added into the figure legend.

4) The concentration of PGR has been stated.

5-11) The manuscript has been revised according to the reviewer’s suggestion.

8.Data and statistical treatment. Improvement needed. Comments:… See attached PDF; comments written directly on the manuscript

Need a paragraph below line122 on statistical procedure.

**Response:** As suggested, a paragraph of Statistical analysis has been added.

9. Have all relevant literature been cited. No. Suggestions:…

1) Need to compare and contrast previous literature on *Acacia* to current results.

2) Italics: words “Passalora perplexa, indica, Bradyrhizobium, Acacia” should be italics

3) Line 211, “journal of experimental biology” needs to be changed to “Journal of Experimental Biology;

line 218,220, “soil” needs to be changed to “Soil”;

line 225,226,239,258, “&” needs to be changed to “and”;

line236, “plantarum” needs to be changed to “Plantarum”;

line246, “Regeneration, Hypocotyl, Callus, Multipurpose, Tree Legume” needs to be changed to “regeneration, hypocotyl, callus, multipurpose, tree legume”.

4) Line 241-242, this is a book, give editors and publisher.

**Response:** 1) According to the reviewer’s suggestion, we have compared plant regeneration of *Acacia crassicapa* with *Acacia* *auriculiformis* and *A. mangium*

2-4) The manuscript has been revised according to the reviewer’s suggestion.

**8.** Please add further comments.

Introduction:

1) Line 55, “they” needs to be changed to “these”;

line 58, “and” needs to be changed to “,”;

line 59, “A.” needs to be spell out;

line64, “and/or” needs to be changed to “and”.

2) line 53, “:”, line 61 “been”, line72 “Xie and Hong 2001b” should be deleted.

**Response:**1-2) The manuscript has been revised as suggested.

**Response to the comments by Prof. Ivan Iliev:**

1. Key words: please delete the words, existing in the title.

**Response:** Thanks to the editor-in-chief’s suggestion, we have deleted the words existed in the title. According to the key words requirements of PJOP: “Latin names of the studied main species covered in the paper must be included here”. We kept the word “*Acacia crassicarpa*”.

1. In the end of introduction you should introduce the real aim of your study. It is not necessary to explain what you have established, what are the advantages of this method etc.

**Response:** As suggested, we have added the aim of our study: “This system was useful for producing high-quality regenerated plantlets of *A. crassicarpa*.” (introduction paragraph 3, line 70).

1. Please use “disinfection” instead “sterilization” everywhere on the text.

**Response:** It has been instead.

1. Please use mg l-1 instead mg/l in table 1,2, and 3 and everywhere on the text

**Response:** According to the reviewer 2’s suggestion, we have used *μ*M instead of mg l-1 in the revised manuscript.

5.Your fig 2 is pale. Please provide better and more contrast photograph

**Response:** We have uploaded better and more contrast photograph.