In Vitro Cellular & Developmental Biology - Plant (2022) 58:437–446
https://doi.org/10.1007/s11627-021-10235-8

PLANT TISSUE CULTURE

Effective in vitro culture using dormant bud of nodal sections from a mature *Acacia* tree

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Received: 28 February 2021 / Accepted: 28 September 2021 / Published online: 12 November 2021 / Editor: Marco Buenrostro-Nava

Abstract

The micropropagation system described is a commercially viable method for asexual propagation of a mature 'elite' tree of *Acacia confusa*. The main stem of a mature tree was hardly pruned to induce new shoots for the explants. This study showed that PPM™ (Plant Preservative Mixture) was an effective agent for controlling the contamination of nodal explants and was added to the initial culture medium. This medium consisted of Murashige and Skoog (MS) basal salts and vitamins, supplemented with 4.0 mg L^{-1} BA, 0.1 mg L^{-1} IBA, 10 mg L^{-1} GA, 12 mg L^{-1} AS, 3.2 g L^{-1} PVP, and 2.0 mL L^{-1} PPM™, and was adequate for the establishment of in vitro mother stock for proliferation. The excellent growth response of nodal explants, obtained from May to July in 2019 to 2020, had shoot formation rate ranging from 15.8 to 18.5%. The optimal shoot multiplication was derived on MS medium supplemented with 1.0 mg L^{-1} BA, 0.5 mg L^{-1} TDZ, 12 mg L^{-1} AS, and 500 mg L^{-1} glutamine which could support feasible 23.7 ± 3.4 shoot proliferation per explant. Shoots generated in vitro with a length of 3 to 5 cm were excised and cultured on MS with 1.0 mg L^{-1} IBA for rooting. The rooting frequency could be increased to 72.2% within 6 wk. Rooted plantlets in vitro were acclimatized for 4 wk before transplanting to the pots in a greenhouse.

Keywords *Acacia confusa* · Micropropagation · Woody plant · Nodal culture · Seasonal effect · Rejuvenation

Introduction

The *Acacia* group (Leguminosae family, Mimosaceae subfamily) comprises more than 1250 known species of shrubs and trees (Simmons 1987), which are dispersed widely in tropical and subtropical regions of Australia, Africa, Asia, and South America (Beck and Dunlop 2001). Most species of *Acacia* have hard trunks and can be used as firewood, railway crosstie, pit timbers, a source of pulpwood production, furniture, farm tools, and especially as sawdust in polypropylene bags for mushroom farming. In the era of global environmental change, the pseudoleaf of *Acacia* trees evolved into the state of preventing water evapotranspiration and forms a self-protection mechanism. It adapts to the tropical soil in arid and humid areas, so that it can exist in extreme atmospheric conditions (Palmberg 1981). Because of its numerous and vigorous roots, it can hold onto soil and increase the soil fertility by symbiotic relationship with rhizobia and mycorrhizal fungi, which help improve the nitrogen deficiency of poor ridge land; withstand strong wind, drought, and poor soil quality; reduce soil erosion; and contribute to the stability of sand dunes (Skolmen 1986). *Acacia* trees are used for reforestation to reclaim wasteland and improve soil health, or as timber and shelter which are important for sustainable development of arid and tropical regions (Gantait *et al*. 2018).

The *Acacia* tree is naturally a cross-pollinated species. Only by grafting, cutting, layering, or tissue culture of asexual propagation can it have a great potential for clonal propagation of superior genotypes. The *Acacia* species have been given due importance in tree tissue culture owing to their proven wasteland reclamation ability, and ecological and economic significance. Micropropagation offers a rapid means to produce clonal planting stock for afforestation, woody biomass production, and conservation of elite germplasm.

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Generally, it is difficult to induce regeneration of mature woody plants in vitro. There were previously some reports on the development of plantlets from callus raised through shoot tip, cotyledonary buds, and nodal explant from new germination young seedlings in *Acacia* species (Mathur and Chandra 1983; Mittal *et al*. 1989; Darus 1990; Huang *et al*. 1994; Nandwani 1995; Nangia and Singh 1996; Vengadesan *et al*. 2000; Xie and Hong 2001a, b; Quoirin 2003; Rout *et al*. 2008; Monteuuis *et al*. 2013). According to the results of these studies, most of the explants were obtained from young seedlings with the best regeneration ability. However, the trunk character of young seedlings from a cross-pollinated woody tree is not known, which is different from obtaining explants from the elite mature plants or from young seedlings for micropropagation. When tissue culture is applied to the mature woody plants, there is a great difference in juvenile characters (Hackett 2011). Therefore, it is necessary to establish a culture system from mature plants as a source of explants and capable of efficient plant regeneration, to be widely used in the promotion of elite trees identified from their adult-stage phenotype. In vitro micrografting can also be an alternative for woody plant asexual propagation (Palma *et al*. 1996), but it is very time-consuming and is mostly used for special purposes, such as virus removal from contaminated elite mature mother stock plants.

One prominent aspect of the establishment of mother stock of woody perennials in microculture is "stabilization." The most obvious signs showing the stabilization stage are the successful disinfection, no lethal browning, and seasonal dormancy of the explants (McCown 2000; Chung and Chen 2018). When seedlings germinated in aseptic culture are used as explants, there is no contamination problem as well as no juvenility problem. However, the explants of mature woody plants grown in the field often have serious contamination and browning to overcome. The studies of melon, petunia, tobacco, and cauliflower examined 0 to 2.0 mL L^{-1} range of PPM™ (Plant Preservative Mixture) concentrations added in the first culture medium which was found to be optimal for controlling contamination without causing a reduction (Compton and Koch 2001; Miyazaki *et al*. 2010; Rihan *et al*. 2012). Browning of explants from woody plant species often was a problem, too. The aseptic culture system was established to overcome browning by using activated carbon (Thomas 2008) , vitamin C (Ko *et al*. 2009), PVP (Saxena and gill 1986; Reustle and Natter 1994), and citric acid (Huang *et al*. 2012), which was also the beginning step for a successful micropropagation (Singh *et al*. 1993).

In addition, previous studies on *Acacia* reported that the evaluation of appropriate cytokinin, such as BA (Skolmen and Mapes 1976), TDZ (Huetteman and Preece 1993; Xie and Hong 2001a, b; Vengadesan *et al*. 2003), 2ip (Banerjee 2013), and kinetin (Nangia and Singh 1996), was carefully selected to break the apical dominance, avoid browning, and promote lateral bud proliferation, while GA was used to promote shoot elongation (Vengadesan *et al*. 2000, 2003). Finally, it is necessary to harden the plantlets in vitro in a proper way before transplanting, so as to facilitate the survival of plantlets transplanted.

There is no report on the micropropagation of *Acacia confusa* from mature trees. In the current study, in vitro cultures were initiated from the nodal sections taken from the adult plants in the field. The disinfection and juvenility of the explants, browning of tissues, and seasonal effect of materials will be the key factors for success. In this study, dormant buds from nodal segments of mature *Acacia* trees were used as explants for micropropagation and cultures were carried in different months of a year. The fact is that explants were derived from a large number of new vigorous branches after hard pruning; the value of explants can be used not only as the maintenance of elite trees for genetic fixation, but also as the rapid asexual propagation and the conservation of endangered tree species.

Materials and Methods

Source, juvenile treatment, and nodal section disinfection The elite trees of *Acacia confusa* used in this study spanned 15 y in age and were collected at the Chungpu stations of Taiwan Forest Research Institute and Da-Yeh University in Taiwan. For harvesting more juvenile explants for in vitro culture, the main stem of an adult tree was cut to 50 to 100 cm in height that would induce new shoots growing close to the ground. Explants were taken once every mo for in vitro culture to identify the time (month) on culture response.

Nodal sections were obtained by removing the leaves from new branches of field-grown *A. confusa*, washed with detergent for 3 times, put into a 150-mL flask containing RO water, and shaken with rotary shaker at 250 rpm for 2 h. These nodal sections were again shaken by hand in 50 mL of 70% ethanol adding 1 drop of acetic acid for 30 to 45 s. Finally, the explants were disinfected while swirling in 100 mL 20% Clorox™ (sodium hypochlorite 5.25%, Auckland, CA) bleach solution containing 1 drop of polyoxyethylene 20-sorbitan monolaurate (Tween 20) and with 40 kHz intensity of ultrasonic treatment for 15 min, then rinsed three times in sterile distilled water for 1 min each time.

PPM™ treatments in nodal culture Following the disinfection described above, the explants were inoculated on culture medium with or without 2.0 mL L^{-1} PPM™. The inoculated basal medium was that of Murashige and Skoog (MS; Murashige and Skoog 1962) with 3.2 g L^{-1} polyvinylpyrrolidone (PVP, Acros Organics, Geel, ANR), 30 g L^{-1} sucrose (Sigma-Aldrich, St. Louis, MO), and 7.0 g L^{-1} phytoagar (Sigma-Aldrich, St. Louis, MO) adjusted to a pH of 4.8 with

NaOH and HCl. PPM™ treatments were grouped into four tests (Table 1). Half a liter of medium was dispersed into a glass bottle and covered with plastic cap before autoclaving at 121 °C and 124 kPa for 15 min. Medium (25 mL) was dispersed into a 90 mm × 20 mm sterilized plastic Petri dish. Five explants were cultured in one Petri dish. Each treatment consisted of 50 explants. Cultures were first kept in dark at 25 ± 1 °C for 10 d. The disinfection tests were repeated four times. Percentage of sterilization of explants was calculated.

Nodal section culture of mature *A. confusa* trees In this process, the nodal sections of sterilized explants were cut into approximately 1-cm segments. The explants were cultured on basal media that were MS (Murashige and Skoog 1962), 1/2 MS, or Woody Plant Medium (WPM; Lloyd and McCown 1980) containing various combinations of growth regulators including 0.5, 1.0, 2.0, 4.0, and 6.0 mg L^{-1} of BA (Koch-Light Limited, Cambridge, UK); 0.5, 1.0, and 2.0 mg L^{-1} of TDZ (Chem Service, West Chester, PA); 0.5, 1.0, and 2.0 mg L^{-1} of 2iP (Phyto Technology Lab., Shawnee Mission, KS); 1.0 mg L^{-1} kinetin (Koch-Light, Buckinghamshire, UK); 12, 50, and 100 mg L^{-1} of AS (Sigma-Aldrich); 0.1, 0.25, 0.5, 1.0, and 1.5 mg L^{-1} of IBA (Koch-Light); or 4.0, 5.0, 8.0, 10, and 12 mg L^{-1} of GA (Koch-Light) (as shown in Table 2). All the testing media have the adjunctive 3.2 g L^{-1} PVP, 30 g L^{-1} sucrose (Sigma-Aldrich), 2.0 mL L^{-1} PPM™, and 7.0 g L^{-1} phytoagar, adjusted to a pH of 4.8 with NaOH and HCl. Twenty-five mL of medium was dispersed into each 90 mm × 20-mm plastic Petri dish after autoclaved at 121 °C and 124 kPa for 15 min. Each Petri dish was inoculated with 5 explants and there were 10 culture dishes in each treatment group. All of culture dishes were placed in the incubator (16-h light/8-h dark). According to the above disinfection and culture methods, the nodal section samplings of adult plants began in August 2019 and ends in July 2020 to test the effects of different times of the year in the field.

Effects of growth regulators on shoot proliferation After the aseptic culture of nodal explants was successfully established, the proliferation medium provided the axillary shoots for the next rooting stage. The explants were inoculated on MS medium supplemented with testing three types of cytokinin TDZ, BA, and AS in combination with IBA and glutamine (as shown in Table 3). Each culture bottle contained 50 mL medium in a 125-mL Erlenmeyer flask and inoculated 3 responsive explants. The number of explants with regenerated shoots and the number of newly formed axillary buds from the shoot on each responsive explant were recorded after 3 wk of culture. The regenerated shoots were subcultured at 45-d intervals onto medium of the same growth regulator composition for each test.

The percentage of the explants with shoots and the average number of axillary bud were calculated as per following formulae:

$$Percentageofexplantswithshoot(\%) = [(numberofexplantswithshoot)/(totalnumberofexplantsinoculated)] \times 100$$

$$Averagenumberofaxillarybudsperresponsiveexplant = (sumofthenumberofaxillarybudsineachexplant)/(numberofexplantswithshoot)$$

Microcutting rooting trials Well-grown shoots (3 to 5 cm in length) were collected as microcutting from proliferating shoot masses. Microcuttings were moved to 1/2 MS basal medium supplemented with 3.2 g L^{-1} PVP, and treated with either IBA or NAA (0.5, 1.0, and 2.0 mg L^{-1}) in combination with or without 100-g L^{-1} banana pulp. After the culture medium was finally prepared, the 30 g L^{-1} sugar and 7.0 g L^{-1} agar are added at a pH 4.8 for rooting. Medium of 25 mL in a 100-mL glass tube was inoculated with 1 microcutting for rooting.

Statistical analysis All experiments were performed in a completely randomized design. Data were subjected to analysis of variance using Statistical Product and Service Solutions (SPSS). Mean separation was achieved using one-way ANOVA analysis; $\rho < 0.05$ indicated significant difference.

Table 1 Using PPM™ to control contamination on *Acacia confusa* nodal explants after surface disinfection

Codes	Treatment processes	% of sterilization*
NNPPM	Explants were not soaked in PPM™ solution first and cultured on inoculated medium without adding PPM™	43.7b
NPPM	Explants were soaked in 500 mL L^{-1} PPM™ solution for 10 min before culturing on inoculated medium without adding PPM™	91.3a
PPM	Explants were not soaked in PPM™ solution before culturing on inoculated medium adding 2.0 mL L^{-1} PPM™	97.0a
PPPM	Explants were soaked in 500 mL L^{-1} PPM™ solution for 10 min, and cultured on inoculated medium adding 2.0 mL L^{-1} PPM™	97.8a

The meaning of the codes is shown in the description of the treatment processes in the table

PPM™, Plant Preservative Mixture

*Mean separation with different letters was achieved using one-way ANOVA analysis $\rho < 0.05$ indicated significant difference

Table 2 Necrosis and survivals in nodal culture of mature *Acacia confusa* trees at the initial establishment stage with different growth regulator combination

Media no	Basal medium*	Growth regulators (mg L^{-1})							Necrosis (%)**	Growth response
		BA	TDZ	2ip	Kinetin	AS	IBA	GA		
1	MS	-	-	-	-	-	-	-	100.0a	none
2	MS	0.5	0.5	-	-	-	-	-	96.5a	+
3	MS	0.5	1.0	-	-	-	-	-	90.6b	+
4	MS	0.5	2.0	-	-	-	-	-	84.8c	+ +
5	MS	0.5	-	-	1.0	-	-	-	98.2a	+
6	MS	1.0	-	-	1.0	-	-	-	90.0b	+
7	MS	2.0	-	-	1.0	-	-	-	85.2bc	+
8	MS	-	-	0.5	-	-	0.1	-	88.7b	+
9	MS	-	-	1.0	-	-	0.1	-	97.5a	+
10	MS	-	-	2.0	-	-	0.1	-	90.6b	+
11	MS	2.0	-	-	-	100	-	4.0	86.8bc	+
12	MS	2.0	-	-	-	100	-	8.0	94.6b	+
13	MS	2.0	-	-	-	100	-	12.0	92.8b	+
14	MS	1.0	-	-	-	50	1.0	-	100.0a	None
15	MS	1.0	-	-	-	50	0.25	-	94.6b	+
16	MS	1.0	-	-	-	50	0.5	-	88.7b	+
17	MS	4.0	-	-	-	12	1.0	5.0	75.8c	+ +
18	MS	4.0	-	-	-	12	0.1	10.0	81.5c	+ + +
19	MS	6.0	-	-	-	12	1.5	10.0	76.5c	+ + +
20	MS	1.0	0.5	-	-	50	-	-	89.0b	+
21	MS	1.0	1.0	-	-	50	-	-	92.5b	+
22	MS	2.0	0.5	-	-	50	-	-	86.8bc	+
23	MS	2.0	1.0	-	-	50	-	-	92.4b	
24	1/2 MS	-	-	-	-	-	-	-	100.0a	None
25	1/2 MS	1.0				12	0.1	10.0	84.8c	+ + +
26	1/2 MS	4.0				12	0.1	10.0	88.4b	+ + +
27	1/2 MS	6.0				12	1.5	10.0	91.2b	+ +
28	WPM	-	-	-	-	-	-	-	100.0a	None
29	WPM	1.0				12	0.1	10.0	93.2b	+ + +
30	WPM	4.0				12	0.1	10.0	84.1c	+ + +
31	WPM	6.0				12	1.5	10.0	93.2b	+ +

BA, 6-benzyl aminopurine; *TDZ*, thidiazuron; *2iP*, N6-(Δ2-isopentenyl) adenine; *AS*, adenine sulfate; *IBA*, indole-3-butyric acid; *GA*, gibberellin; *MS*, Murashige and Skoog (1962)

*Three types of basal medium have the adjunctive 3.2 g L^{-1} polyvinylpyrrolidone and 30 g L^{-1} sucrose and 7.0 g L^{-1} phytoagar adjusted to a pH of 4.8

**The data of necrosis were examined by 42 d after culturing. The same *letters* are not significantly different by one-way ANOVA analysis $\rho < 0.05$ indicated significant difference. Relative levels of growth response: + weak (callus), + + middle (callus, a few budding), + + + strong (callus, some budding)

Results and Discussion

Explant sources and disinfection procedure Most woody crop in vitro cultures are first established using shoot culture approaches where the stimulation of axillary buds from shoot tips is the prime objective (Chung and Chen 2018). The nature of the explant used considerably influences its multiplication. In the cases of mature *A. confusa* trees, many nodal section explants can be obtained, and the in vitro operation is convenient. However, clonal regeneration by somatic embryogenesis or organogenesis is still difficult for many woody species and is often limited to the use of juvenile explants (Bonga *et al*. 2010; Hackett 2011) which are derived mostly from in vitro seed germination. The problems of field-grown explants are contamination, necrosis, season, and juvenility. In this study, the main stem of an adult tree

Table 3 Effects of the basal medium containing various combinations of growth regulators and glutamine for nodal culture of mature *Acacia confusa* trees

Media no.*	Growth regulators (mg L^{-1})					Number of buds per explant**	Description of axillary bud proliferation
	BA	IBA	TDZ	AS	Glutamine		
1	-	-	-	-	-	1.2 ± 0.2c	Obviously elongated
2	1.0	0.1	-	-	-	2.7 ± 0.6ab	Obviously elongated
3	2.0	0.1	-	-	-	2.6 ± 0.4ab	Obviously elongated
4	4.0	0.1	-	-	-	2.7 ± 1.0ab	Obviously elongated
5	1.0	-	0.5	12	-	2.2 ± 0.9ab	Clumpy and dwarf
6	1.0	-	1.0	12	-	2.0 ± 0.5abc	Clumpy and dwarf
7	2.0	-	0.5	12	-	2.2 ± 0.5ab	Clumpy and dwarf
8	2.0	-	1.0	12	-	1.9 ± 0.7bc	Clumpy and dwarf
9	1.0	-	0.5	12	500	2.9 ± 0.5a	Clumpy and dwarf
10	1.0	-	1.0	12	500	2.5 ± 0.3ab	Clumpy and dwarf
11	2.0	-	0.5	12	500	2.7 ± 0.3ab	Clumpy and dwarf
12	2.0	-	1.0	12	500	2.0 ± 0.4abc	Clumpy and dwarf

BA, 6-benzyl aminopurine; *IBA*, indole-3-butyric acid; *TDZ*, thidiazuron; *AS*, adenine sulfate

*The basal medium has the adjunctive 3.2 g L^{-1} polyvinylpyrrolidone and 30 g L^{-1} sucrose and 7.0 g L^{-1} phytoagar adjusted to a pH of 4.8

**The data of bud numbers were examined by 3 wk after culturing. Mean ± SD separation with different *letters* was achieved using one-way ANOVA analysis $\rho < 0.05$ indicated significant difference

was severely pruned to induce new shoots emerging close to the ground (Fig. 2*a*, *b*). Hard cutting back of main stem of a mature tree often induces the invigoration and possible rejuvenation, which can promote the regeneration ability of propagules (Hartman *et al*. 2014).

According to the general disinfection procedure (70 to 75% ethanol and 20% Clorox™), the contamination of *A. confusa* nodal segments had not been completely controlled and the follow-up experiments could not be carried out. We analyzed the effects of sodium hypochlorite and PPM™ on surface sterilization. No shoots were produced, either because of contamination of the explants, or loss of explants as a result of using 75% ethanol together with longer treatment of sodium hypochlorite. Results from this study showed that 2.0 mL L^{-1} PPM™ was an effective agent for controlling the contamination in *A. confusa* micropropagation system. After 10 d of culture, the control group not treated with PPM™ but by general disinfection procedure resulted in only 43.7% of surface sterilization. However, the PPM™ treatments showed high efficiency of more than 97.0% sterilization effect of explants from field-grown plants (Table 1).

The determination of the suitable concentration is an essential requirement for using PPM™ since the high concentrations can be harmful to plant cells (Rihan *et al*. 2012). It seems that there is a relationship between phenolic-like component exudation and the concentration of PPM™ used since there were no signs of the presence of these compounds when PPM™ concentrations were higher than 0.25 mL L^{-1} (Rihan *et al*. 2012). This accorded with Chamandoosti (2010) who mentioned that there was a relationship between chemical composition of the medium and phenolic compound production. The influence of PPM™ on plant regeneration depends on the plant species. Experiments of melon, petunia, tobacco, and cauliflower examined a 0 to 2.0 mL L^{-1} range of PPM™ concentrations when using it in the initial culture medium which was found to be optimal for controlling the contamination without causing a reduction (Compton and Koch 2001; Miyazaki *et al*. 2010; Rihan *et al*. 2012).

Table 1 shows there is no significant difference among the three treatment groups NPPM, PPM, and PPPM (with or without PPM™). The results indicated that it was not necessary to soak explants in PPM™ solution before culturing, but only 2.0 mL L^{-1} PPM™ added into the medium resulted in low contamination. Explants with low contamination rate were also derived from soaking in a 50% PPM solution (500 mL L^{-1} PPM™) for 10 min without adding PPM™ to the culture medium. But the PPM™ concentration used in this method is much higher and less economical. Therefore, the results revealed that adding 2.0 mL L^{-1} PPM™ in the medium was enough to achieve good sterilization of the explants (Table 1).

Mother stock establishment in *in vitro* culture When the sterile explants from field-grown nodal segments were cultured and examined, over 85.0% of explants remained green for 2 wk and gradually turned brown and died later when cultured on medium with combinations of BA, TDZ, 2iP, and kinetin. However, the combinations of BA, AS, IBA, and GA media were effective for decreasing the necrosis and provided with good growth response (Table 2). There are many factors affecting browning of explants, such as plant

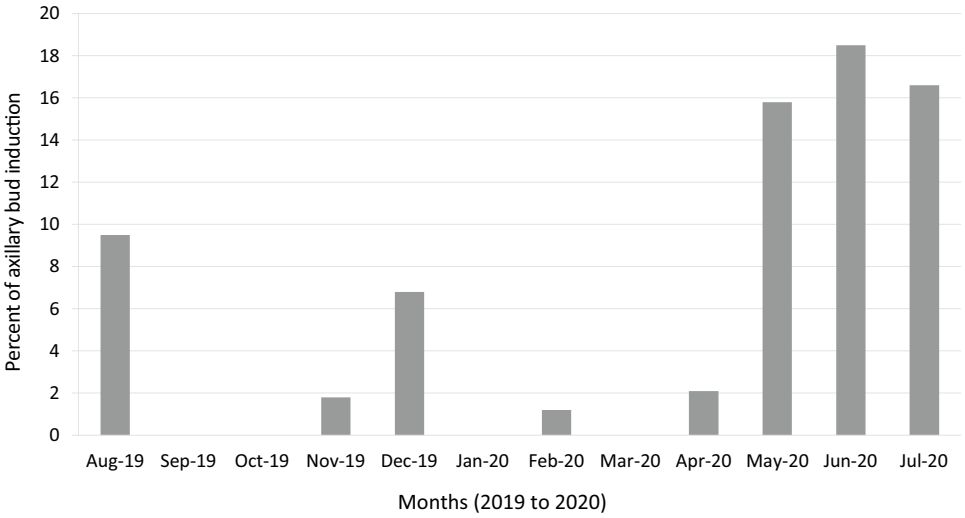

Figure 1. Percent of axillary bud induction from monthly sampling of mature *Acacia* tree nodal culture in 2019 to 2020

species and genotypes, physiological status, damage from sterilization, medium composition, culture conditions, and seasonal effect. The addition of browning inhibitors can alleviate the toxicity of phenolic substances and achieve the purpose of protecting explants. For example, activated charcoal and polyvinylpyrrolidone (PVP) are able to adsorb phenolic substance; ascorbic acid can be absorbed by the explants and have anti-oxidation effect (Saxena and Gill. 1986; Reustle and Natter 1994; Ko *et al*. 2009). A report on the research of *Cinnamomum kanehirae* in Taiwan demonstrated the browning of explants occurred after long-term culture could be reduced by adding 3.2 g L^{-1} PVP to the medium (Chang *et al*. 2002). In our study of *A. confusa*, PVP was used as anti-browning agent. It was also combined with different cytokinins and GA to induce outbreak of dormant buds to form multiple shoots (Table 2; Fig. 2*c*). In addition to the establishment of a stable mother stock in vitro, the purpose of mass reproduction was achieved in this study.

The nodal segments of mature *A. confusa* trees had the best growth response on MS medium supplemented with 4.0 to 6.0 mg L^{-1} BA, 0.1 to 1.5 mg L^{-1} IBA, 10 mg L^{-1} GA, and 12 mg L^{-1} AS (Table 2) resulting in 3 to 6 new buds on each node (Fig. 2*c*). Girijashankar (2011), in a survey of 18-mo-old *A. auriculiformis* trees, reported that MS medium with 2.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA supported high survival rate of explants, and nodal culture could induce 76% of shoot regeneration. Zhang *et al*. (1995) reported that shoot induction on MS basal medium supplemented with 10 µM BA and 0.5 µM IBA was obtained through culturing *A. auriculiformis* and *A. mangium* from 4-y-old mother trees, and the shoot regeneration rate of these two species was 66.7% and 55.0%, respectively. However, in our preliminary study, 15 y aged mature *A. confusa* trees were sampled and cultured without hard pruning. The survival rate of explants could not reach more than 5% in testing media (the data is not presented). After hard pruning (cutting the main stem down to 1 m above ground), the survival rate of the explants from new growth was increased to nearly 25% (Table 2) on medium with best nutrient combination, and the probability of axillary bud induction was about 19% in the initial culture (Fig. 1). The age of donor tree for explants may be counted on the different response in shoot regeneration in vitro. When the basal medium was concerned, 1/2 MS or WPM was used instead of MS medium (Table 2), the sprouting of new shoots showed 2 wk later than that of MS basal medium in this study. The differences in micropropagation in relation to various *Acacia* species, different physiological status of donor trees, or different applied basal medium were reviewed by Gantait *et al*. (2018).

Seasonal effect on survival and regeneration of explants In woody plants, nodal explants from seedlings had higher multiplication rates than the explants from adult plants. Cultures initiated at the beginning of May produced the highest percentage of shoot forming explants and multiplication rate in *Quercus euboica* (Kartsonas and Papafotiou 2007). In addition to seasonal bud dormancy, predetermined and limited periods of seasonal shoot growth and a marked change in growth characteristics as the plants progress from the juvenile to adult phases of their life cycle all complicate responsiveness and predictability in vitro. Therefore, in this experiment, explants taken from adult *A. confusa* trees for culture had been treated with rejuvenation by manipulating donor plants with hard pruning in advance (Fig. 2*a*, *b*).

For explants of mature *A. confusa* trees obtained in different seasons, inoculated on MS medium supplemented with 4.0 mg L^{-1} BA, 0.1 mg L^{-1} IBA, 10 mg L^{-1} GA, 12 mg L^{-1} AS, and 3.2 g L^{-1} PVP, growth response was mainly concentrated in the warm period of summer from May to July; the regeneration frequency of axillary buds ranged from 15.8

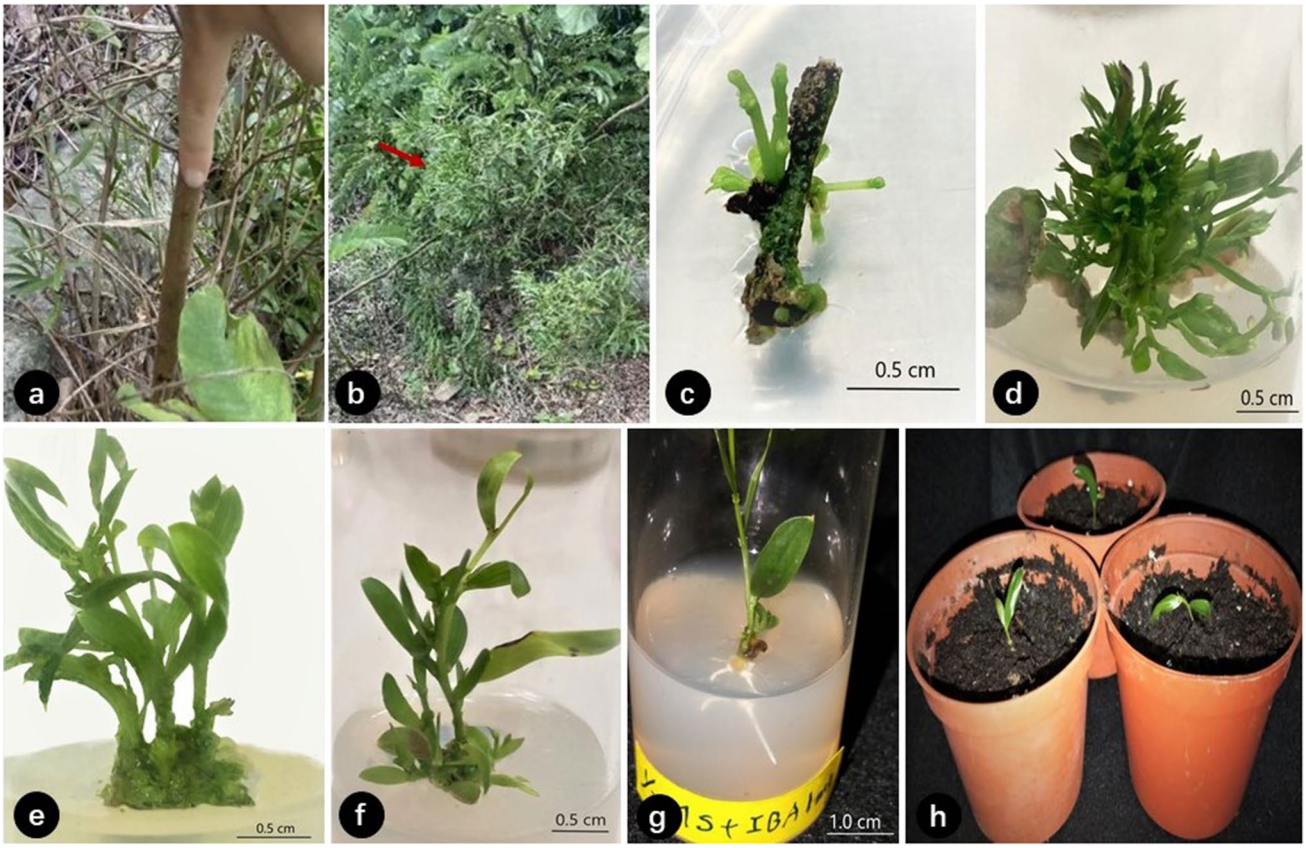

Figure 2. (*a*) The mature mother tree of *A. confusa* treated by strong cutting. The height of finger point was about 1.5 m from soil, and the diameter of the branch was about 2 cm. (*b*) The *red mark* shows that the rejuvenated shoots were growing out. (*c*) The establishment of a stable mother stock *in vitro*. (*d*) The combinations of 1 mg L^{-1} BA, 0.5 mg L^{-1} TDZ, 12 mg L^{-1} AS, and 500 mg L^{-1} glutamine could significantly improve the proliferation of adventitious buds. (*e*, *f*) Shoots generated in vitro with a length 3 to 5 cm would be excised to microcutting. (*g*) *In vitro* rooting. (*h*) Transplantation to soil

to 18.5%. Figure 1 also showed that axillary buds could be induced in August and December with a gradually decreased frequency to 9.5% and 6.8%. The outbreak of dormant buds could not be induced without GA addition (Table 2). In addition to the selection of medium and seasonal factors, the juvenile character of explants (Fig. 2 *a*, *b*) is the key to the sprouting of dormant buds on nodal segments. Chang *et al.* (2002) also mentioned seasonal effect that shoot tips taken in spring season gave the highest survival and multiplication rate on in vitro culture of *Cinnamomum kanehirae*.

Shoot multiplication The explants with in vitro response were subcultured in MS medium supplemented with three types of cytokinin TDZ, BA, and AS in combination with IBA and glutamine (as shown in Table 3). Table 3 indicates that the explants cultured in MS medium with 1.0 mg L^{-1} BA, 0.5 mg L^{-1} TDZ, 12 mg L^{-1} AS, and 500 mg L^{-1} glutamine initiated primarily clumpy and dwarf buds with approximately 2.9 axillary buds per responsive explant after 3 wk of culture. The number of shoots derived was significantly different at $\rho < 0.05$ by one-way ANOVA analysis. Only in the combinations of BA and IBA did the proliferation of axillary buds show obviously elongated shoots. The results showed that MS basal medium with the abovementioned plant growth regulators and glutamine affected the number of buds formed on subculturing nodal explant of *A. confusa* (Table 3). The regenerated shoots from nodal explant were transferred at a 45-d interval onto the same medium and growth regulator composition (Fig. 2*d*, Fig. 3).

Figure 3 shows that bud proliferation was significantly higher when explants were cultured on medium containing 0.5 mg L^{-1} TDZ compared to 1.0 mg L^{-1} (Table 3). Adding glutamine to the culture medium increased the number of shoots. Glutamine supports the growth of cells that have high energy demands and synthesizing large amounts of proteins and nucleic acids. It is an alternative energy source for rapidly dividing cells and cells that use glucose inefficiently (Greenwell and Ruter 2018). Higher percentage of nitrogen may improve the growth of plantlets and embryogenesis of cells (Ogita *et al.* 2001). In this study, 500 mg L^{-1} glutamine was added to the medium containing BA, TDZ, and AS at the same time; the multiplication of axillary buds decreased

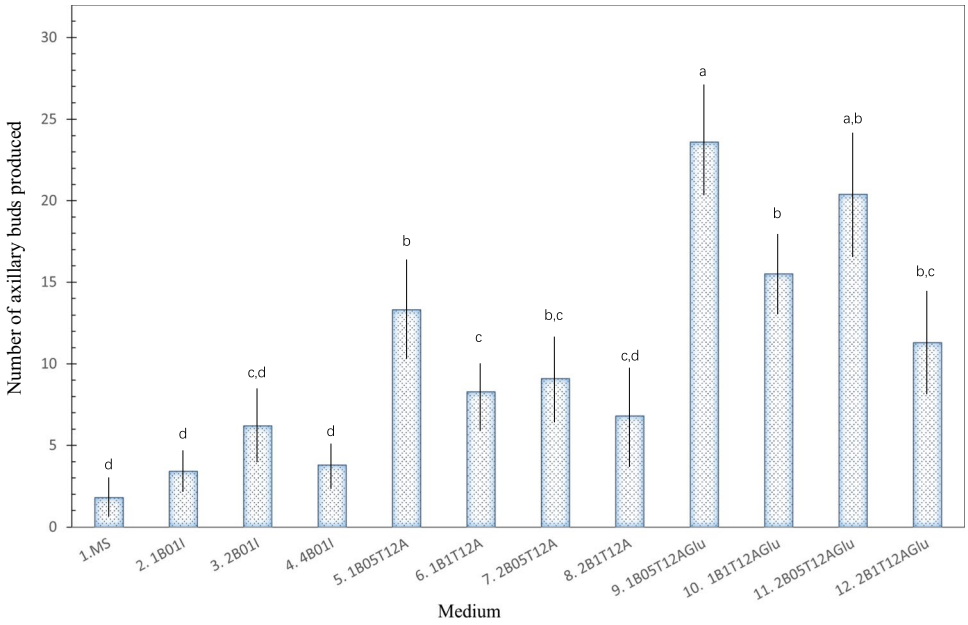

Figure 3. Effects of adventitious bud multiplication on subculturing medium after 45 d. The treatment groups indicated by the horizontal axis were in accordance with the medium code in Table 3. *Different letters* indicate statistically significant differences. The *bar line* represents the standard deviation of samples

with the increase of BA concentration. This may be due to the fact that BA, TDZ, and AS are cytokinins, and excessive cytokinin can inhibit the lateral bud proliferation (Huetteman and Preece 1993). In this study, 12 kinds of medium for inducing axillary bud regeneration were tested. The results showed that the combinations of 1.0 mg L^{-1} BA, 0.5 mg L^{-1} TDZ, 12 mg L^{-1} AS, and 500 mg L^{-1} glutamine could significantly improve the proliferation of multiple shoots (Fig. 2*d*; Fig. 3).

Most of the studies in tissue culture of *Acacia* were used juvenile explants such as embryo, cotyledon, stem segment, and hypocotyl of seedling (Beck and Dunlop 2001; Vengadesan *et al*. 2002; Quoirin 2003; Gantait *et al*. 2018), and that only a few reports use materials from larger trees. Huang *et al*. (2012) reported that *A. mangium* 3- to 5-y-old stem segments had 20 to 30% shoot regeneration opportunities. Vengadesan *et al*. (2003) developed an *in vitro* propagation protocol using nodal explants from a 10-y-old 'elite' tree of *A. sinuata*. Maximum shoot proliferation was achieved from nodal explants collected during the December to March time frame. MS medium supplemented with 8.9 µmol BA, 2.5 µmol TDZ, and 135.7 µmol AS (60 d after inoculation) had the best induction effect on shoot proliferation (Vengadesan *et al*. 2003). The above report is not exactly similar to our current results on *A. confusa* which showed best seasonal effect in May to July, and next in December. The concentration of cytokinin used in culturing *A. sinuata* was approximately equal to 2.0 mg L^{-1} BA, 0.5 mg L^{-1} TDZ, and 50 mg L^{-1} AS, which was not comparable to our data derived from culturing *A. confusa*. In the current study, it is demonstrated that 500 mg glutamine and 3.2 g L^{-1} PVP added to the subculturing medium promote the multiple shoot proliferation and resist browning (Fig. 2*d*).

Plantlets rooting in vitro and transplanting to soil Shoots generated in vitro with a length of 3 to 5 cm were excised and cultured on 1/2 MS medium supplemented with IBA or NAA (Fig. 2*e*, *f*) for rooting *in vitro*. Of the two auxins tested for their ability to induce the root growth, MS medium supplemented with 1.0 mg L^{-1} IBA supported the best rooting response with 72.2% rooting frequency and an average of 3.5 roots per plant and 4.2 cm in root length within 6 wk (Fig. 2*g*). However, when the concentration of NAA or IBA was 2 mg L^{-1}, callus was induced at the basal part of the shoots. In *A. confusa* culture, adding 100 g L^{-1} banana pulp did not induce root formation, but banana pulp was effective for rooting in orchid plants (Souza *et al*. 2013).

In vitro rooted plantlets were hardened by moving culture glass tubes next to a window where oblique sunshine was available for more than 4 wk. Hardened plantlets were removed from the culture medium, washed thoroughly in water, and transferred to clay pots that contained a mixture of peat moss, vermiculite, and perlite in a 1:1:1 ratio. The potted plantlets were successfully acclimatized in a moist and shady place and eventually transferred to a greenhouse. Around 90% of the plantlets survived and initiated new growth after 2 mo (Fig. 2*h*).

Conclusion

The micropropagation system described above is a commercially viable method for propagating a mature 'elite' tree of *A. confusa*. This study shows that PPM™ can be an effective agent for controlling the contamination of explants. MS

medium containing 4.0 mg L^{-1} BA, 0.1 mg L^{-1} IBA, 10 mg L^{-1} GA, and 12 mg L^{-1} AS and the adjunctive 3.2 g L^{-1} PVP is sufficient for the establishment of mother stock in *in vitro* culture. Explants with shoots induced on the node in the first culture were subcultured on MS medium with the combination of 1.0 mg L^{-1} BA, 0.5 mg L^{-1} TDZ, 12 mg L^{-1} AS, and 500 mg L^{-1} glutamine for mass propagation of *A. confusa* plantlets *in vitro*.

In conclusion, (1) hard pruning of mature mother trees should be applied first to induce rejuvenated shoots for the explants, (2) donor plants should be kept away from pathogenic organisms to reduce contamination of explants, (3) seasonal effect for a successful micropropagation should be determined in the different areas, and (4) PPM™ and PVP in the initial culture medium are necessary for a clean and viable nodal culture of *Acacia* grown in the field.

Funding This research received financial support from the Forestry Bureau, Council of Agriculture, Executive Yuan, Taiwan (ROC) under Contract No. tfbc-1080504.

References

Banerjee P (2013) Rapid *in vitro* propagation of *Acacia auriculiformis* on solid and liquid media: role of organic additive, antioxidant and plant growth regulators. Cibtech J Bio Protocols 2:39–49

Beck SL, Dunlop RW (2001) Micropropagation of the *Acacia* species – a review. In Vitro Cell Dev Biol - Plant 37:531–538

Bonga JM, Klimaszewska KK, von Aderkas P (2010) Recalcitrance in clonal propagation, in particular of conifers. Plant Cell Tiss Org Cult 100:241–254

Chamandoosti F (2010) The relationship between plant growth regulators for organogenesis and phenolic compound in cotton (*Gossypium hirsutum* L.). Asian J Dev Biol 2:16–22

Chang SH, Ho CK, Tsay JY (2002) *In vitro* culture of *Cinnamomum kanehirae* Hay. Taiwan J for Sci 17:491–501 (**[in Chinese with English summary]**)

Chung JP, Chen KH (2018) Developing an *in vitro* quasi-symbiotic culture system of *Aquilaria malaccensis* with potentially beneficial microbes on agarwood resinous formation. Plant Cell Tiss Org Cult 133:193–202

Compton ME, Koch JM (2001) Influence of plant preservative mixture (PPM™) on adventitious organogenesis in melon, petunia, and tobacco. In Vitro Cell Dev Biol - Plant 37:259–261

Darus HA (1990) Micropropagation of *Acacia mangium* from aseptically germinated seedlings. J Trop for Sci 3:204–208

Gantait S, Kundu S, Das PK (2018) *Acacia*: an exclusive survey on *in vitro* propagation. J Saudi Soc Agric Sci 17:163–177

Girijashankar V (2011) Micropropagation of multipurpose medicinal tree *Acacia auriculiformis*. J Med Plants Res 5:462–466

Greenwell Z, Ruter J (2018) Effect of glutamine and arginine on growth of *Hibiscus moscheutos in vitro*. Ornam Hortic 24(4):393–399

Hackett WP (2011) Juvenility, maturation, and rejuvenation in woody plants. John Wiley & Sons, Ltd

Hartman HT, Kester DE, Davies FT Jr, Geneve RL (2014) Hartmann & Kester's plant propagation: principles and practices, 8th edn. Pearson Education Inc, Upper Saddle River

Huang FH, Al-Khayri JM, Gbur EE (1994) Micropropagation of *Acacia mearnsii*. In Vitro Cell Dev Biol - Plant 30:70–74

Huang LJ, Chen ZX, Zhang SQ, Liang RG (2012) Tissue culture technique of *Acacia mangium* elite trees. For Res 25:227–230

Huetteman CA, Preece JE (1993) TDZ: a potent cytokinin for woody plant tissue. Plant Cell Tiss Org Cult 33:105–119

Lloyd G, McCown B (1980) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Comb Proc Int Plant Prop Soc 30:421–427

Kartsonas E, Papafotiou M (2007) Mother plant age and seasonal influence on *in vitro* propagation of *Quercus euboica* Pap., an endemic, rare and endangered oak species of Greece. Plant Cell Tiss Org Cult 90:111–116

Ko W, Su C, Chen C, Chao C (2009) Control of lethal browning of tissue culture plantlets of Cavendish banana cv. formosana with ascorbic acid. Plant Cell Tiss Org Cult 96:141–145

Mathur I, Chandra N (1983) Induced regeneration in stem explants of *Acacia nilotica*. Curr Sci 52:882–883

McCown BH (2000) Woody shrubs and trees. In: Smith RH (ed) Plant tissue culture techniques and experiments 2nd. Academic Press, New York, pp 123–134

Mittal A, Agrawal R, Gupta SC (1989) *In vitro* development of plantlets from axillary buds of *Acacia auriculiformis*. Plant Cell Tiss Org Cult 19:65–70

Miyazaki J, Tan BH, Errington SG (2010) Eradication of endophytic bacteria via treatment for axillary buds of *Petunia hybrida* using Plant Preservative Mixture (PPM™). Plant Cell Tiss Org Cult 102:365–372

Monteuuis O, Galiana A, Goh D (2013) *In vitro* propagation of *Acacia mangium,* and *A. mangium* x *A. auriculiformis*. In: Lambardi M, Ozudogru EA, Jain SM (eds) Protocols for micropropagation of selected economically-important horticultural plants. Springer, New York, pp 199–211

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nandwani D (1995) *In vitro* micropropagation of a tree legume adapted to arid lands: *Acacia tortilis* subsp. *radiana*. Ann for Sci 52:183–189. https://doi.org/10.1051/forest:19950208

Nangia S, Singh R (1996) Micropropagation of *Acacia tortilis* Hayne (Umbrella thorn) through cotyledonary node culture. Indian J Exp Physiol 1:77–79

Ogita S, Sasamoto H, Yeung EC, Thorpe TA (2001) The effects of glutamine of the maintenance of embryogenic cultures of *Cryptomeria japonica*. In Vitro Cell Dev Biol - Plant 37:268–273

Palma B, Vogt GF, Neville P (1996) A combined *in vitro*/*in vivo* method for improved grafting of *Acacia senegal* (L.) Willd. Hort Sci 3:379–381

Palmberg C (1981) A vital fuelwood gene pool is in danger. Unasylva 33:22–30

Quoirin M (2003) Micropropagation of *Acacia* species. In: Jain SM, Ishii K (eds) Micropropagation of woody trees and fruits. Forestry Sciences, vol 75. Springer, Dordrecht, pp 245–268. https://doi.org/10.1007/978-94-010-0125-0_9

Reustle G, Natter I (1994) Effect of polyvinylpyrrolidone and activated charcoal on formation of microcallus from grapevine protoplasts (*Vitis* sp.). Vitis 33:117–121

Rihan HZ, Al-Issawi M, Al-swedi F, Fuller MP (2012) The effect of using PPM (plant preservative mixture) on the development of cauliflower microshoots and the quality of artificial seed produced. Sci Hort 141:47–52

Rout GR, Senapati SK, Aparajeta S (2008) Micropropagation of Acacia chundra (Roxb.) DC. Hort Sci 35:22–26. https://doi.org/10.17221/648-HORTSCI

Saxena PK, Gill R (1986) Removal of browning and growth enhancement by polyvinylpolypyrrolidone in protoplast cultures of *Cyamopsis tetragonoloba* L. Biol Plant 28:313–315

Simmons MH (1987) The genus *Acacia*. In: Simmons MH (ed) *Acacias* of Australia, vol 1. Nelson, South Melbourne, pp 7–10

Singh HP, Singh S, Saxena RP, Singh RK (1993) *In vitro* bud break in axillary nodal segments of mature trees of *Acacia nilotica*. Indian J Plant Physiol 36:21–24

Skolmen RG (1986) Acacia (*Acacia koa* Gray). In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 1. trees 1. Springer-Verlag, Berlin, pp 3755–4384

Skolmen RG, Mapes MO (1976) *Acacia koa* Gray plantlets from somatic callus tissue. J Hered 67:114–115

Souza G, Lone A, Faria R, Oliveira K (2013) Pulp fruit added to culture medium for *in vitro* orchid development. Semin Cienc Agrar 34:1141–1146

Thomas TD (2008) The role of activated charcoal in plant tissue culture. Biotechnol Adv 26:618–631

Vengadesan G, Ganapathi A, Anand RP, Anbazhagan VR (2000) *In vitro* organogenesis and plant formation in *Acacia sinuata* (Lour.) Merr. Plant Cell Tiss Org Cult 6:23–28

Vengadesan G, Ganapathi A, Anand RP, Selvaraj N (2003) *In vitro* propagation of *Acacia sinuata* (Lour.) Merr. from nodal segments of a 10- year-old tree. In Vitro Cell Dev Biol - Plant 39:409–414

Vengadesan G, Ganapathi A, Amutha S, Selvaraj N (2002) *In vitro* propagation of *Acacia* species-a review. Plant Sci 163:663–671

Xie D, Hong Y (2001a) *In vitro* regeneration of *Acacia mangium* via organogenesis. Plant Cell Tiss Org Cult 66:167–173

Xie D, Hong Y (2001b) Regeneration of *Acacia mangium* through somatic embryogenesis. Plant Cell Rep 20:34–40

Zhang HW, Huand XL, Fu JR, Yang MQ, Chen CQ (1995) Axillary bud culture and plantlet regeneration of *Acacia auriculiformis* and *A. mangium*. J Trop Subtrop Bot 3:62–68