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In Vitro Plant Regeneration from Immature Leaflets Derived Callus of *Acacia confusa* Merr *via* Organogenesis

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An efficient plant regeneration system was established from immature leaflet-derived callus of *Acacia confusa* Merr, through organogenesis. Under optimized culture conditions, the high rate of callus induction and proliferation was obtained in 35 days on MMS medium supplemented with 2,4-D (3 mg l^{-1}) + NAA (0.01 mg l^{-1}) + Kin (0.05 mg l^{-1}). The highest percentage of shoot regeneration response (95%) and greatest number of shoots (52.9) were obtained after the 46-day transfer of green nodular calli onto the shoot regeneration medium (WPM) supplemented with the BA 3 mg 1^{-1} + NAA 0.05 mg l^{-1} + Zeatin 0.1 mg l^{-1} + $AdSO_4$ 5 mg l^{-1} combination. Efficient shoot elongation was achieved by transferring the clusters of adventitious shoot buds to medium (half-strength MS) containing GA_3 (1 mg l^{-1}) and BA (0.05 mg l^{-1}), within 30 days. The elongated shoots were rooted on half-strength MS medium supplemented with 4 mg l^{-1} IBA and 0.05 mg l^{-1} Kin in the 42-day culture. Rooted plantlets were hardened and successfully established in soil. The field-established plants were morphologically normal and fertile.

***Key words*:** *Acacia confusa,* immature leaflets callus*,* shoot regeneration, *in vitro* propagation.

Clonal propagation of high-value forest trees through organogenesis has the potential to rapidly capture the benefits of breeding or genetic engineering programs and to improve the quality and uniformity of nursery stock (1). *Acacia confusa* Merr, (Leguminosae) a species indigenous to Taiwan, is widely distributed over the hills and lowlands of Taiwan. It is an important commodity used for feedstock, charcoal-making, and construction material; and contributes significantly to conservation of soil and water (2,3). In addition, it has been used as Taiwan folk medicine for wound healing, antiblood stasis and antioxidant activity (4). In general, *A. confusa* propagation is through seeds present in the ground can germinate profusely after fire and spread through forest ornamental planting, also can be reproduced from cuttings. Conventional breeding of tree species is an inefficient and time-consuming process because of long life cycles and slow seed maturation. The regeneration rate of *Acacia* species in natural habitats is quite low (5). Thus, the expanding plantation of *A. confusa* requires clonal propagation of elite clones through *in vitro* propagation. Hence, it is important to develop suitable tools for improving the genetic quality and uniformity of the nursery planting stock. Further*, in vitro* culture techniques are required to develop genetic transformation systems, and to establish cell suspension for production of secondary metabolites. In this regard, vegetative propagation, particularly tissue culture, is worth special attention as a possible alternative to overcome the limited success of more conventional techniques (6,7).

Several *Acacia* species have been regenerated *in vitro*, including *A. catechu* (8), *A. auriculiformis* (9), *A. sinuata* (10,11), *A. mangium* (12), *A. farnesiana* (2, 13-17) and *A. nilotica* (18). To date, there has been no report on *in vitro* regeneration of *A. confusa*, and there remains much to be studied. Herein, we described the optimization of culture conditions and plant growth regulators required for callus induction, shoot regeneration, and rooting of plantlets from immature leaflets of *A. confusa.*

Materials and Methods

Plant materials and callus induction — Mature seeds of *Acacia confusa* seeds were immersed in water at 40°C (45 min), surface-sterilized with 70% ethanol (5 min), and 5% sodium hypochlorite solution (25 min). After every treatment,

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Abbreviations: ABA - abscisic acid; AA - Ascorbic acid; BA – 6-benzylaminopurine; CGN – compact greenish nodular; 2,4-D – 2,4- dichlorophenoxyacetic acid; GA_3 - Gibberellins IAA – indole-3-acetic acid; IBA - indole-3-butyric acid; *2ip* - isopentenyl adenine; Kin –Kinetin; MS – Murashige and Skoog medium; NAA – a-naphthaleneacetic acid; TDZ – thidiazuron, WPM – Woody Plant Medium, Zn – Zeatin.

Table 1. Callus induction from immature leaflets of *Acacia confusa* grown on MMS containing different combinations of growth hormones

Growth hormones (mg l^{-1})			Callus proliferation/ explant	Nature of callus
2,4-D	NAA	Kin	(Percentage Mean ± SE)	
0.5			20.0 ± 1.05^{i}	WF
1.0			42.5 ± 1.20^{h}	WF
3.0			65.0 ± 1.86^{f}	YF
5.0			62.5 ± 1.35^{g}	YG
3.0	0.001		70.0 ± 1.31^{e}	CY
3.0	0.01		95.0 ± 2.72^{b}	CN
3.0	0.05		80.0 ± 2.15^{d}	CN
3.0	0.1		70.0 ± 2.40^{e}	NB
3.0	0.01	0.01	92.5 ± 2.11^{c}	CYN
3.0	0.01	0.05	100.0 ± 0.00^{a}	CGN
3.0	0.01	0.1	95.0 ± 1.85^{b}	CGN
3.0	0.01	0.5	92.5 ± 2.44^{c}	CBN

Note: Data were recorded after 35days of culture when the callus can be seen clearly by the naked eye. The experiment was repeated twice with 40 explants per treatments. WF=whitish friable; YF=yellowish friable; YG=yellowish granular; CY=compact yellowish; CN=compact nodular; NB=nodular brownish; CGN=compact greenish nodular; CYN=compact yellowish nodular; CBN=compact brownish nodular. Within columns, values followed by the same letter are not significantly different using Duncan's new multiple range test at 5% level.

the seeds were rinsed 4-5 times with sterilized distilled water. The sterilized seeds were placed on half-strength MS medium (19) for seed germination. For induction of callus, the immature leaflets were excised from 15-day-old *in vitro* grown seedlings and cut into small pieces (0.3-0.5 cm); cultured on MMS medium (modified MS salts + B5 vitamins) (19, 20) containing 2,4-D (0.5-5.0 mg l^{-1}) alone and the combinations of 2,4-D (3 mg l^{-1}) + NAA (0.001-0.1 mg l^{-1}); and 2,4-D (3.0 mg l^{-1}) + NAA (0.01 mg l^{-1}) + Kin (0.01-0.5 mg l^{-1}) (Table 1). After 3 days of culture, the percentage of explants, color and nature of callus were noted and three subcultures were performed in all treatments.

Shoot regeneration and development from callus — To promote shoot bud regeneration, the compact greenish nodular calli (CGN) were transferred onto the fresh shoot regeneration medium woody plant medium (21) supplemented with various concentrations and combinations of growth regulators as follows: BA (0.5, 1.0, 2.0, 3.0, 4.0 mg l^{-1}) alone; BA (3 mg l^{-1}) + NAA (0.01, 0.05, 0.1, 0.5 mg l^{-1}); BA (3 mg l^{-1}) + NAA (0.05 mg l^{-1}) + Kin (0.01, 0.05, 0.1, 0.5 mg l^{-1}); and BA (3 mg l^{-1}) + NAA (0.05 mg l^{-1}) + Zeatin (Zn) (0.01, 0.05, 0.1, 0.5 mg l^{-1}). In addition, $AdSO_4$ (adenine sulphate) (5 mg l^{-1}) was added to all treatments to enhance regeneration of healthy shoot buds. Subculture was carried out in fresh medium of the same composition at an interval of 14 days. The data were recorded after 46 days of culture when the shoots could be seen clearly with the naked eye. Each treatment described for shoot regeneration here was 40 CGN callus per piece and repeated twice.

Shoot elongation and rooting — The regenerated shoot buds (0.1-0.5 cm) were excised and transferred to half-strength MS medium supplemented with the combination of GA_3 1 mg l^{-1} + BA 0.05 mg l^{-1} for shoot elongation. This 30-day treatment with two subcultures was repeated twice. Then shoots of 2-5.5 cm in length were transferred to the rooting medium (half-strength MS) containing various concentrations of IBA (-3-indolebutyric acid) (1, 2, 3, 4 mg l^{-1}) and IAA (1, 2, 3, 4 mg l^{-1}) as individual treatments, and with IBA 4 mg l^{-1} combined with various concentrations of Kin (0.1, 0.05, 0.1, 0.5 mg l^{-1}) for 42 days of culture.

Hardening and acclimatization — After formation of lateral roots, plantlets were removed from culture vessels, washed gently under slow running tap water and transferred to pots containing a mixture of sand, vermiculite, garden soil at a ratio of 1:1:2 (v/v) and watered every 3 days. Rooted plants were enhanced in a growth chamber with 80 per cent relative humidity under cool-white fluorescent light at an irradiance of 26 µmol m^{-2} s^{-1} (PAR) for a 16h day photoperiod at 24-26°C.

Culture conditions and statistical analysis — All the above media were supplied with 30 g l^{-1} sucrose and solidified with 0.8% (w/v) agar and autoclaved at 121°C for 15 min. All media were adjusted to pH 5.8 with 1 N NaOH and HCl. Subculture was done in fresh medium of the same composition at the required intervals in all the above experiments. Unless otherwise specified, all the cultures were maintained at 24-26°C under cool-white fluorescent light at an irradiance of 26 µmol m^{-2} s^{-1} (PAR) for a 16h day photoperiod. Data on adventitious shoot and root induction were statistically analyzed and means values were separated by using Duncan's new multiple range test (DMRT) at 5% probability level of significance.

Results and Discussion

Callus Induction — The induction of compact nodular calli with shoot bud formation competence is a prerequisite in

organogenesis for the development of complete plantlets. Several factors such as choice of explants, culture environments, hormonal and non-hormonal regulators act synergistically in determining the proper induction, proliferation of compact nodular calli and regeneration into complete plants. In the present investigation, the cut end segments of immature leaflets were cultured on MMS medium supplemented with 2,4-D (3 mg l^{-1}) alone, or with 2,4-D (3 mg l^{-1}) + NAA (0.01 mg l^{-1}) combination produced friable callus (65%) and compact nodular callus (95%), respectively after 35 days of culture with three subcultures. Further, the compact greenish nodular (CGN) calli (Fig.1 A) were induced on MMS medium containing the 2,4-D (3 mg l^{-1}) + NAA (0.01 mg l^{-1}) + Kin (0.05 mg l^{-1}) combination and yielded higher response (100%) of CGN callus proliferation after 35 days of culture with three subcultures (Table 1). Similarly, Yang et al. (22) produced compact greenish nodule callus in *Acacia crassicarpa* on medium (MS) containing NAA and TDZ (thidiazuron) in combination after 40 days of culture. Thus, the other treatments in this experiment could also induce calli of different natures with low callusing response. However, there were noticeable differences observed when compared with that induced under optimum concentration of the 2,4-D + NAA + Kin combination. The present study reveals that the callus induction rate declined gradually when the concentrations of cytokinin and auxin were either increasing or decreasing from the optimum in all treatments (Table 1). Previous reports described similar pattern of compact nodular calli induction in *Acacia catechu* (23), and *Aegle marmelos* (24). Therefore, the present result was in contrast to the formation of friable, loose and white yellowish calli in *A. mangium* (16).

Shoot regeneration from CGN callus — The compact greenish nodular calli generated from the immature leaflets were transferred to the shoot regeneration medium WPM supplemented with various concentrations of BA alone, as well as the BA + NAA; BA + NAA + Zn; and BA + NAA + Kin combinations. Each treatment involved $AdSO_4$ 5 mg 1^{-1} as an additional supplement to enhance the development of healthy shoots. Regeneration of shoot bud was observed in the 46-day callus culture (Fig. 1B & B1). Though calli of different natures, obtained from different hormonal treatments, were cultured onto the shoot regeneration medium, the results showed low response of shoot bud regeneration except for CGN calli (data not shown). Of the four different kinds of hormonal treatments, the BA (3 mg l^{-1}) + NAA (0.05 mg l^{-1}) + Zeatin (0.1 mg l^{-1}) combination gave rise to the highest shoot induction response (95%) and greatest average number of shoots (52.9) obtained in per culture (Table 2). In addition, the BA (3 mg l^{-1}) + NAA (0.05 mg l^{-1}) + Kin (0.05 mg l^{-1}) combination produced a shoot regeneration response rate of 85% and an average of 25.1 shoots per culture. These results suggest that zeatin was more suitable for shoot regeneration than kinetin when the medium contains the BA + NAA combination. Previous research reveals differentiation of multiple shoots on medium supplemented with cytokinins like BA, Kin or Zeatin (5) in *A. nilotica*. Furthermore, the present study had $AdSO_4$ added (for all treatments) to the shoot regeneration medium and obtained healthy and vigorous shoots regenerated from CGN callus compared with treatments without $AdSO_4$ added. Similar observations have been reported in other tree species, such as *Aeagle marmelos* (24), and *Acacia catechu* (23), thus supporting that addition of $AdSO_4$ could be responsible for enhancement of healthy shoot regeneration.

Table 2. Multiple shoot regeneration from immature leaflets derived callus (CGN) of *Acacia confusa* on WPM supplemented with various concentrations of growth hormones

Growth hormones (mg l^{-1})			% of response (Mean ± SE)	No.of Shoots/callus (Mean ± SE)
BA	NAA	Zn		
0.5			22.5 ± 1.30^{o}	3.1 ± 0.48^{m}
1.0			30.0 ± 1.15^{n}	5.4 ± 1.16^{l}
2.0			37.5 ± 1.60^{m}	6.5 ± 1.05^{k}
3.0			52.0 ± 1.70^{k}	8.3 ± 0.95^{j}
4.0			50.0 ± 1.64^{l}	8.0 ± 0.21^{j}
3.0	0.01		55.0 ± 1.21^{j}	14.6 ± 0.16^{i}
3.0	0.05		67.5 ± 1.92^{i}	25.9 ± 0.23^{e}
3.0	0.1		70.0 ± 2.30^{h}	24.7 ± 1.04^{f}
3.0	0.5		70.0 ± 2.11^{h}	20.3 ± 0.21^{h}
3.0	0.05	0.01	72.5 ± 2.41^{g}	26.5 ± 1.30^{d}
3.0	0.05	0.05	77.5 ± 2.86^{e}	38.8 ± 0.66^{c}
3.0	0.05	0.1	95.0 ± 2.54^{a}	52.9 ± 0.95^{a}
3.0	0.05	0.5	92.5 ± 2.40^{b}	47.0 ± 1.17^{b}
3.0	0.05	0.01	75.0 ± 2.41^{f}	22.2 ± 0.46^{g}
3.0	0.05	0.05	85.0 ± 2.72^{c}	25.1 ± 0.48^{e}
3.0	0.05	0.1	85.0 ± 2.55^{c}	24.5 ± 0.67^{f}
3.0	0.05	0.5	82.5 ± 2.18^{d}	20.7 ± 0.15^{h}

Note: Data were recorded after 46 days for shoot multiplication and developments in culture when the shoots can be seen clearly by the naked eye. The experiment was repeated twice with 40 callus piece per treatment. Within columns, values followed by the same letter are not significantly different using Duncan's new multiple range test at 5% level.

The data show that other hormonal treatments could also induce shoot bud regeneration from CGN callus but there were noticeable differences observed when compared with optimized hormonal treatments. Similarly, shoot regeneration was attained from callus on medium containing cytokinins BA and Kin in combination and low concentration of NAA in *Aeagle marmelos* (24). Taken together, the present investigation reveals that the shoot regeneration rate was reduced gradually when the concentrations of cytokinin and auxin were either increasing or decreasing from the optimum in all treatments (Table 2). Therefore, the present study reveals that the synergetic combination of auxin and cytokinin promoted shoot regeneration. Yang *et al* (22) reported that MS medium containing cytokinin (thidiazuron) alone and in combination of auxin (NAA) induced shoot regeneration in *A. crassicarpa*. Moreover, earlier reports suggested that the promoting effect of auxin and cytokinin in combination on organogenic differentiation has been well established in several systems (25-26). Similar shoot development patterns were reported for *A. mangium* (16). The present results indicated that a high concentration of auxin and cytokinin inhibited shoot regeneration, as was also reported for the leguminous tree, *Dalbergia sisso* Roxb. (27).

Shoot elongation — Efficient shoot elongation with the response rate of 85% was achieved (data not shown) on half-strength MS medium containing GA_3 (1 mg l^{-1}) and BA (0.05 mg l^{-1}), which was then employed to determine the optimal elongation conditions for adventitious shoots in *A. confusa.* Adventitious shoots elongated to an average of 2-5 cm per shoot and developed a visual stem (Fig.1C) after 30 days of culture. Yang and his coworkers (22) reported that GA_3 in combination with cytokinin (thidiazuron) enhanced maximum shoot elongation in *Acacia crassicarpa* compared with GA_3 alone. The present study highlighted that GA_3 and BA in combination could enhance shoot elongation, suggesting the involvement of BA in the modulation of endogenous growth regulators, especially auxins and cytokinins (24, 28).

Rooting and hardening of in vitro raised plants — The elongated shoots were transferred to half-strength MS medium containing IBA or IAA of various concentrations for rooting. The observation shows that IBA 4 mg l^{-1} induced a rooting response of 55% and an average of 8.7 roots compared with IAA (3 mg l^{-1}), which yielded a rooting response of 25% and an average of 3.7 roots per shoot after 42 days of culture. Further, the optimum concentration of IBA (4 mg l^{-1}) in combination with Kin (0.05 mg l^{-1}) promoted the highest rooting response (100%) and greatest number of roots (32.0) obtained per shoot after 42 days of culture (Table 3; Fig.1D & D1). Similar response of root formation was attained on half-strength MS medium containing auxin (IBA) in combination with low concentrations of cytokinin (Kin) in *Aegle marmelos* (24). The earlier study on *A. nilotica* reported that IAA alone produced greater rooting response compared with IBA in the present study (5). Previous reports achieved the highest root induction rate in half-strength MS medium containing auxin alone in *A. catechu* (23, 29) and *A. crassicarpa* (22). Thus, the results indicated that IBA is a more suitable auxin than IAA for root induction, and the addition of low concentrations of cytokinin with optimum concentration of auxin had more significant influence when compared with auxin alone. Overall, the present investigation reveals that the root induction rate declined gradually when the concentrations of auxin and cytokinin were either increasing or decreasing from the optimum in all treatments (Table 3). The rooted plants were transferred into pots. Eighty five percent of the regenerated plantlets survived and showed vigorous growth and normal phenotypes in the growth chamber and *in vivo* (Fig.1E).

Table 3. Root induction from immature leaflets callus derived shoots of *Acacia confusa* grown on half strength MS medium supplemented with IBA alone and IBA in combination with Kin

Growth hormones (mg l^{-1})		% of response (Mean ± SE)	No.of roots/shoot (Mean ± SE)
IBA	Kin		
1.0		20.0 ± 0.95^{i}	5.7 ± 1.09^{f}
2.0		22.5 ± 0.7^{h}	6.5 ± 0.93^{e}
3.0		55.0 ± 1.87^{e}	8.7 ± 1.02^{d}
4.0		47.5 ± 1.82^{f}	8.2 ± 0.76^{d}
1.0		15.0 ± 0.85^{j}	1.3 ± 0.94^{h}
2.0		22.5 ± 1.04^{h}	3.0 ± 0.17^{g}
3.0		25.0 ± 1.08^{g}	3.7 ± 1.24^{g}
4.0		22.5 ± 1.10^{h}	3.1 ± 1.16^{g}
4.0	0.01	75.0 ± 2.12^{d}	23.7 ± 1.21^{c}
4.0	0.05	100.0 ± 0.00^{a}	28.6 ± 0.98^{b}
4.0	0.1	95.0 ± 2.15^{b}	32.8 ± 1.08^{a}
4.0	0.5	92.0 ± 2.60^{c}	28.5 ± 0.95^{b}

Note: Data were recorded after 42 days for root initiation and development of response in culture and when the roots can be seen clearly by the naked eye. The experiment was repeated twice with 40 shoots per treatment. Within columns, values followed by the same letter are not significantly different using Duncan's new multiple range test at 5% level.

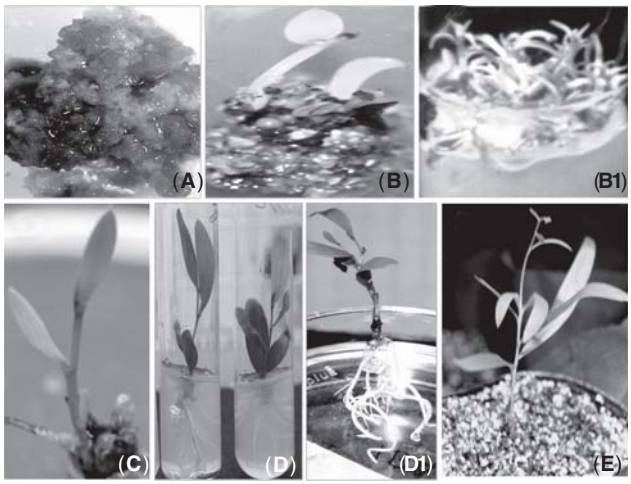

Fig. 1. Callus induction, shoot regeneration, shoot elongation, rooting and hardening of *in vitro* raised plantlets from immature leaflets of *Acacia confusa*. (**A**) Compact greenish nodular calli from immature leaflets on MMS medium supplemented with 2,4-D (3 mg l^{-1}) + NAA (0.01 mg l^{-1}) + Kin (0.05 mg l^{-1}); (**B** & **B1**) Multiple shoots regeneration from green nodular calli. on WPM medium supplemented with BA 3 mg l^{-1}, NAA 0.05 mg l^{-1}, Zn 0.1 mg l^{-1} and $AdSO_4$ 5 mg l^{-1}; (**C**) Shoot elongation on half-strength MS medium containing GA_3 (1 mg l^{-1}) and BA (0.05 mg l^{-1}); (**D** & **D1**) Root induction on half-strength MS medium supplemented with 4 mg l^{-1} IBA and 0.05 mg l^{-1} Kin, and (**E**) Acclimatized plantlet in pot.

The plant regeneration protocol established in this investigation will facilitate research in genetic transformation and *in vitro* propagation of *A. confusa*. The present study showed that it is possible to induce high-frequency plant regeneration through organogenesis in immature leaflet-derived calli using the synergistic combination of BA, NAA and Zeatin with $AdSO_4$ added. Using this protocol, it is estimated that a single immature leaflets can produce approximately 52 viable plants within 5 months.

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