In Vitro Propagation And Rejuvenation of Senescent Maternal Plant of *Ardisia Crenata* Var. *Bicolor* (Primulaceae)

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Abstract

Ardisia crenata var. *bicolor* is an ornamental shrub, owing to its declined wild population, recalcitrant seeds and few high-quality cuttings, the main objective of this study was to optimize an *in vitro* propagation protocol by using tip shoot and nodal segment as explants from senescent plant. Explants were sterilized and cultured on Muraghiye and Skoog medium contained $1.0 \text{ mg}\cdot\text{L}^{-1}$ benzylaminopurine and $0.05 \text{ mg}\cdot\text{L}^{-1}$ 1-naphthaleneacetic acid for shoot initiation. For shoot proliferation, explants were cultured on MS medium with $1.0 \text{ mg}\cdot\text{L}^{-1}$ BAP, $0.1 \text{ mg}\cdot\text{L}^{-1}$ NAA, and $0.5 \text{ mg}\cdot\text{L}^{-1}$ kinetin, and the proliferation coefficient were 3.1 and 2.5. Rooting was achieved by two explants in half-strength MS medium containing $0.5 \text{ mg}\cdot\text{L}^{-1}$ indole-3-butyric acid + $0.1 \text{ mg}\cdot\text{L}^{-1}$ or $0.2 \text{ mg}\cdot\text{L}^{-1}$ NAA, and $0.5 \text{ g}\cdot\text{L}^{-1}$ activated charcoal. The highest rooting rate were 72.7% and 65.1% with the highest mean number of roots (4.2 and 2.8, respectively). After acclimatization, 83.3% and 81.2% of plants were survived in the greenhouse. The plant can be rejuvenated via *in vitro* propagation and provide a reference for supplying the planting materials quickly with an uniform genotype.

Introduction

The genus *Ardisia* Swartz¹ was placed in Myrsinaceae in traditional taxonomy systems, but in APG (Ⅷ) and APG (IV), it was included in Primulaceae. *Ardisia crenata* Sims var. *bicolor* (Primulaceae) is an evergreen shrub that exists the transitional traits with *Ardisia crenata* Sims, which leads to no obvious distinction of them. The two plants forms clusters and glossy, drupe fruits² and produces red or white fruits^{3,4}, the berries could adhere to maternal plant for 12 months or longer in weak-light environment, which is one of the most popular ornamental plants grown in houses or as an undergrowth companion species in gardens. In addition, its phytochemical constituents possess anti-tumor, anti-cancer, and anti-inflammatory properties⁴⁻⁸. In China, it has been widely used as a traditional folk herbal medicine for detoxification. In recent years, wild sources of *A. crenata* var. *bicolor* have been heavily exploited, which has rapidly decreased natural populations, whereas the poor artificial selection and domestication methods resulted in low quality and mixed germplasm, which has become a bottleneck in its large-scale production.

Generally, the propagation of *A. crenata* var. *bicolor* is accomplished sexually by seed and asexually by cutting. The seeds are categorized as recalcitrant-type and thus are highly susceptible to desiccation which can't survive in dry storage⁹. Yang et al.¹⁰ also reported that seeds of *A. crenata* var. *bicolor* did not germinate under dry conditions for 90 days, but the germination rate reported by Tezuka et al.¹¹ was more rapid after long periods of storage at low temperature (approximately 5 °C). Roh et al.¹² used conventional sowing method, the seeds needed 13 weeks or longer to achieve 80% germination at 25.8 °C, and 4 years for obtaining commercial high-quality plants, while the cuttings required longer than 45 days to form 76% rooting, although the commercial seedlings were obtained in less than 2 years, only 31–40% of high quality plants. In order to protect *A. crenata* var. *bicolor* from extinction and utilize continually, although some researchers contributed to the species cultivation and propagation methods, few references reported on the *in vitro* culture of *A. crenata* var. *bicolor*.

In vitro propagation serves an important method to study shoot induction and initiation as it enables shoot to grow rapidly, genetic stability, and supply the planting materials quickly with an uniform genotype. The main

objective of this study was to optimize an efficient medium for the regeneration protocol of two types of explants (tip shoot and nodal segment), and include different concentrations of PGRs for obtaining the maximum frequency of shoot and root regeneration. This protocol will provide a reference for the efficient regeneration and contribute to its rapid propagation and germplasm preservation of *A. crenata* var. *bicolor*.

Results

Explants sterilization. Different sterilization methods significantly influenced the explants of *A. crenata* var. *bicolor*. The contamination rate of the tip shoot were slightly higher than that of the nodal segment using the same disinfection protocol, but the difference was no significant (Table 1). The highest contamination rate of the tip shoot was $58.9 \pm 0.1\%$ which was recorded in samples disinfected with 75% ethanol for 30 s and 0.1% HgCl_2 for 5 min (T1). With the extension of HgCl_2 disinfection time, the contamination rate of the two types of explants decreased (T1 to T6). *A. crenata* var. *bicolor* was disinfected with 75% ethanol (30 s) and 0.1% (w/v) HgCl_2 (10 min), obtaining contamination rate of $31.1 \pm 0.1\%$ and survival rate of $57.5 \pm 0.1\%$ for tip shoot explants sampled from 5-6 year old saplings. In addition, the survival rate of explants were also affected by the disinfectant types, the contamination rate of tip shoot and nodal segment decreased with the extension of NaClO disinfection time (T7 to T9). Among them, the highest survival rate of nodal segments was 56.1% and was disinfected in 75% ethanol for 45 s and in 3% NaClO for 10 min, while the other treatments were lower, especially T7, the tip shoot were only 19.5%.

Axillary shoot induction. The effects of different concentrations of plant regulators were studied on buds regeneration in MS medium and WPM medium. One week later, the first shoot was appeared in WPM media, and more shoots were observed after 15 days (Fig. 1C), this implies that the explants derived from *A. crenata* var. *bicolor* were suitable. However, the induction rate was significant differences among different concentrations of PGRs, some shoots were uneven, most of the buds or nodal segments showed browning.

Based on the phenomenon, three concentrations of BAP (0.5 , 1.0 , and $1.5 \text{ mg}\cdot\text{L}^{-1}$) and NAA (0.01 , 0.05 , and $0.1 \text{ mg}\cdot\text{L}^{-1}$) were further optimized for shoot regeneration (Table 2). The obtained results showed that MS medium supplemented with $1.0 \text{ mg}\cdot\text{L}^{-1}$ BAP recorded relatively higher shoot induction (T4~T6), which was found to exert the best response that fortified with $0.05 \text{ mg}\cdot\text{L}^{-1}$ NAA (T5), and the highest shoot induction rate of the tip shoot and the nodal segment were 68.58% and 70.53% respectively, followed by the T6, but there was no statistical difference between two treatments ($P > 0.05$). Although two types of explants cultured on WPM medium could also induce few shoots, the rate of shoot regeneration was much lower than that on MS medium ($P < 0.01$). The root necrosis with the extension of incubation time, and abnormal characteristics such as flower bud differentiation or albino leaves occurred in WPM medium (Fig. 1D).

Table 1 Effect of different disinfected methods on the explants of *Ardisia crenata* var. *Bicolor*

Treatment	75% Alcohol (s)	0.1% HgCl ₂ (min)	3% NaClO+0.1% tween20 (min)	Tip shoot		Nodal segment	
				Contamination rate (%)	Survival rate (%)	Contamination rate (%)	Survival rate (%)
T1	30	5	–	58.9 ± 0.1 a	26.6 ± 0.1 cd	45.6 ± 0.0 ab	32.5 ± 0.0 cd
T2	30	7	–	49.5 ± 0.0 b	29.6 ± 0.0 cd	40.5 ± 0.1 b	33.9 ± 0.0 cd
T3	30	10	–	31.1 ± 0.1c	57.5 ± 0.1 a	31.1 ± 0.1 c	39.2 ± 0.1 bc
T4	30	15	–	29.6 ± 0.1 c	44.4 ± 0.0 b	28.1 ± 0.1 c	36.6 ± 0.0 c
T5	45	7	–	46.9 ± 0.1 b	33.9 ± 0.0 c	40.5 ± 0.0 b	37.9 ± 0.1 bc
T6	45	10	–	31.1 ± 0.1 c	24.9 ± 0.0 d	26.6 ± 0.1 c	26.6 ± 0.1 de
T7	45	–	5	54.7 ± 0.1 ab	19.5 ± 0.0 d	49.5 ± 0.1 a	29.6 ± 0.0 d
T8	45	–	10	36.6 ± 0.0 c	32.5 ± 0.0 c	29.6 ± 0.0 c	56.1 ± 0.0 a
T9	45	–	15	35.3 ± 0.1 c	31.1 ± 0.1 cd	28.1 ± 0.0 c	41.8 ± 0.0 b

Contamination rate and survival rate indicated as means±SD and different lowercase letters in the same column indicated significant differences ($P < 0.05$)

Table 2 Effect of three concentrations of BAP and NAA on shoot induction from different explants of *Ardisia crenata* var. *bicolor*

Treatment	Initiation medium	Phytohormone (mg·L ⁻¹)		Shoot induction frequency (%)	
		BAP	NAA	Tip shoot	Nodal segment
T1	MS	0.5	0.01	56.1 ± 0.1 bc	61.9 ± 0.1 ab
T2	MS	0.5	0.05	61.9 ± 0.0 ab	65.1 ± 0.1 ab
T3	MS	0.5	0.1	60.4 ± 0.0 b	63.4 ± 0.1 ab
T4	MS	1.0	0.01	58.9 ± 0.1 b	60.4 ± 0.1 abc
T5	MS	1.0	0.05	68.6 ± 0.1 a	70.5 ± 0.1 a
T6	MS	1.0	0.1	66.8 ± 0.0 a	68.6 ± 0.1 a
T7	MS	1.5	0.01	46.9 ± 0.1 c	56.1 ± 0.1 bc
T8	MS	1.5	0.05	57.5 ± 0.0 b	58.9 ± 0.0 bc
T9	MS	1.5	0.1	50.8 ± 0.1 c	52.1 ± 0.0 c
T10	WPM	0.5	0.05	23.2 ± 0.0 d	21.4 ± 0.1 d
T11	WPM	1.0	0.1	32.5 ± 0.1 d	29.6 ± 0.0 d
T12	WPM	1.5	0.01	28.1 ± 0.1 d	24.9 ± 0.1 d

Average induction rate indicated means±SD and different lowercase letters in the same column indicate significant differences ($p < 0.05$), as determined by one-way analysis of variance (ANOVA) with Duncan's post-test.

Shoot multiplication. The explants of *A. crenata* var. *bicolor* were inoculated in culture medium with varying concentrations of BAP, NAA, and KT (Table 3). Among them, the highest multiplication coefficient of tip shoot and nodal segment in T4 were 3.1 and 2.5 respectively of BAP-containing (1.0 mg·L⁻¹) medium with NAA (0.1 mg·L⁻¹) and KT (0.5 mg·L⁻¹) (Fig. 1E), while BAP at the lowest and KT and NAA at their highest concentrations in T3 led to the least multiplication coefficient, 1.38 and 1.28 respectively, with yellowish-green shoots. With increasing of BAP concentrations, the multiplication coefficient of shoots first increased and then decreased, the lowest multiplication coefficient of shoots were inoculated in the medium containing the lowest concentration of BAP (0.5 mg·L⁻¹) and the highest concentrations of NAA (0.5 mg·L⁻¹) and KT (1.0 mg·L⁻¹). When the concentration of BAP was maintained constant, the proliferation coefficient of shoots were less at a lower or higher KT concentration than the optimal T4 medium (0.5 mg·L⁻¹), while NAA only affected induction of tip shoot ($P < 0.01$, Table 4), but had a weak influence on the nodal segments and the multiplication coefficient ($P > 0.05$). The proliferated shoots grew rapidly and healthily and elongated gradually within a month, but the average multiplication coefficient of the tip shoot and nodal segment were low (2.03 and 1.66). Based on the ANOVA (Table 4), which confirmed that an optimal concentration of BAP and KT plays a significant role in improving the shoot proliferation coefficient ($P < 0.01$), while NAA affects

induction of tip shoot ($P < 0.01$), but a weak influence on nodal segment and the multiplication coefficient ($P > 0.05$), which may be related to the fact that auxin mainly plays a role in apical meristems.

Table 3. Orthogonal-array design for PGRs treatments and their effect on shoot proliferation of *Ardisia*

crenata var. *bicolor*

Treatment	Orthogonal array ^a			Phytohormone (mg·L ⁻¹)			Proliferation coefficient ^b	
	A	B	C	BAP	NAA	KT	Tip shoot	Nodal segment
T1	1	1	1	0.5	0.1	0.1	1.8 ± 0.2 d	1.4 ± 0.2 d
T2	1	2	2	0.5	0.2	0.5	2.0 ± 0.1 d	1.6 ± 0.0 cd
T3	1	3	3	0.5	0.5	1.0	1.4 ± 0.1 e	1.3 ± 0.3 e
T4	2	1	2	1.0	0.1	0.5	3.1 ± 0.1 a	2.5 ± 0.1 a
T5	2	2	3	1.0	0.2	1.0	2.5 ± 0.1 b	1.6 ± 0.2 cd
T6	2	3	1	1.0	0.5	0.1	1.9 ± 0.1 de	2.1 ± 0.1 b
T7	3	1	3	2.0	0.1	1.0	1.5 ± 0.1 e	1.3 ± 0.0 e
T8	3	2	1	2.0	0.2	0.1	1.8 ± 0.1 de	1.4 ± 0.1 d
T9	3	3	2	2.0	0.5	0.5	2.2 ± 0.1 c	1.8 ± 0.1 c
Mean	-	-	-	-	-	-	2.03	1.66
^a Numbers represent the levels in orthogonal array of 3×3 design								
^b values are mean±SD of three independent experiments and different lowercase letters in the same column indicated significant differences ($p < 0.05$), as determined by one-way analysis of variance (ANOVA) with Duncan's post-test								

Table 4
One-way analysis of variance of growth regulators influence on (A) shoot induction (%) and
(B) shoot multiplication coefficient

Explants	Factor	Sum of squares	df	Mean square	F	P-value
A. Effect on shoot induction						
Tip shoot	BAP	1606.127	2	803.064	12.853	0.000
	NAA	1169.364	2	584.682	7.247	0.003
Nodal segment	BAP	1119.707	2	559.854	10.018	0.001
	NAA	101.326	2	50.663	0.515	0.604
B. Effect on shoot multiplication coefficient						
Tip shoot	BAP	3.217	2	1.609	10.503	0.001
	KT	2.310	2	1.155	6.047	0.007
	NAA	0.480	2	0.240	0.897	0.421
Nodal segment	BAP	2.385	2	1.192	11.851	0.000
	KT	1.705	2	0.853	6.612	0.005
	NAA	0.322	2	0.161	0.863	0.435

Rooting induction. After shoot multiplication, healthy and well-grown shoots were selected and inoculated into the rooting medium. The effects of different concentrations of IBA and NAA, and $0.5 \text{ g}\cdot\text{L}^{-1}$ AC were analyzed to determine the optimal rooting medium. Table 5 shows a broad spectrum of rooting responses. Root regeneration was produced from the base of a bud directly but not from the callus (Fig. 1F). Of the three concentrations of IBA tested, the half-strength MS medium supplemented with $0.5 \text{ mg}\cdot\text{L}^{-1}$ IBA was found to exert the relatively better response on root induction, from which three concentrations of NAA were investigated, half-strength MS medium fortified with $0.1 \text{ mg}\cdot\text{L}^{-1}$ NAA was found to produce the best response from the tip shoot and the highest rooting rate was 72.7% with the mean root number (4.2) in T4 media, while the nodal segment in T5 medium with $0.2 \text{ mg}\cdot\text{L}^{-1}$ NAA obtained the highest rooting rate of 65.1% and mean root number of 2.6, which were no significant difference with T4 medium (2.8) ($P > 0.05$).

Table 5. Effect of three concentrations of IBA and NAA on root induction from different explants of *Ardisia crenata* var. *bicolor*

Treatment	Rooting medium	Phytohormone (mg·L ⁻¹)		Tip shoot		Nodal segment	
		IBA	NAA	Rooting rate (%)	Root number	Rooting rate (%)	Root number
T1	1/2 MS	0.2	0.1	66.8 ± 0.0 ab	2.7 ± 0.2 c	61.9 ± 0.1 ab	1.8 ± 0.4 bc
T2	1/2 MS	0.2	0.2	61.9 ± 0.8 bc	2.3 ± 0.3 d	60.4 ± 0.8 ab	2.0 ± 0.1 b
T3	1/2 MS	0.2	0.5	58.9 ± 0.7 c	1.9 ± 0.5 de	54.7 ± 0.7 b	1.6 ± 0.1 c
T4	1/2 MS	0.5	0.1	72.7 ± 0.9 a	4.2 ± 0.1 a	63.4 ± 0.8 ab	2.8 ± 0.3 a
T5	1/2 MS	0.5	0.2	68.6 ± 0.9 ab	3.8 ± 0.2 ab	65.1 ± 0.8 a	2.6 ± 0.5 a
T6	1/2 MS	0.5	0.5	63.4 ± 0.8 bc	3.3 ± 0.6 bc	57.5 ± 0.7 ab	2.1 ± 0.2 b
T7	1/2 MS	1	0.1	58.9 ± 0.7 c	2.4 ± 0.1 cd	58.9 ± 0.7 ab	1.9 ± 0.2 b
T8	1/2 MS	1	0.2	57.5 ± 0.7 cd	2.1 ± 0.2 de	54.7 ± 0.7 b	1.6 ± 0.3 c
T9	1/2 MS	1	0.5	53.4 ± 0.6 d	1.5 ± 0.2 e	50.8 ± 0.6 b	1.2 ± 0.1 c

Average rooting rate indicated as means±SD. Different lowercase letters in the same column indicate significant differences ($P < 0.05$), as determined by one-way analysis of variance (ANOVA) with Duncan's post-test.

Transplanting. After 5 weeks, all aseptic rooted shoots were planted in the four different proportions of the disinfected matrix (Table 6). Among them, the highest survival rate from tip shoot and nodal segment were 83.33% and 81.2% that planted in peat: vermiculite: perlite = 3:2:1 (v/v) at 75% shading condition (Fig. 1G), which were higher than other treatments.

Table 6
Effects of four different substrates on aseptic plantlets of *Ardisia crenata* var. *bicolor*

Treatment	Substrate mixture peat: vermiculite: perlite	Shade rate	Survival rate (%)	
			Tip shoot	Nodal segment
□	1:1:1	75%	77.78%	75.56%
□	2:1:1	50%	67.78%	63.35%
□	3:2:1	75%	83.33%	81.15%
□	4:2:1	50%	70.00%	64.44%

Discussion

Type of explants, genotype, exogenous PGRs and culture conditions (e.g. light intensity and light quality) could affect the growth of axillary shoots and roots and their physiological characteristics^{13–17}. However, the availability of explants and their aseptic nature, the ability to respond to induction and propagation methods are crucial for the successful establishment of *Aidisia* regeneration system. The result provided evidence that the tip shoot and the nodal segment subjected to different disinfection methods could be used as inoculated materials, but explant type have a great influence on the surface sterilization, cell permeability could increase in 75% ethanol for 30–45 s, whereas the cell injury increase and survival rate of explants decrease along the HgCl₂ immersion time.

For the most perennial plants, the quality of explants decreased with decrepit plant, which can be rejuvenated via *in vitro* culture^{18,19}. Tip shoot or nodal segment, in most cases, have differentiated first into callus, and then take longer into adventitious shoots^{20,21}. *Ardisia crenata* var. *bicolor* is a perennial plant, it is difficult to induce shoots from the tip shoot or nodal segment, but the bud propagation is time-saving and more reliable way to obtain plantlets from tissue culture, which could shorten the period of propagation. The WPM medium is known for good results with subcultured of woody species and produced more shoots²². Zhang et al.²³ reported maximum response for induction frequency, mean number of shoots and rooting rate of two dogwoods achieved on WPM medium, but this was not the case for *A. crenata* var. *bicolor*. In our study, WPM medium performances proved to be inferior to MS medium and MS medium gave better results. MS medium was used to induce the shoot initiation and proliferation of the tip shoot and the nodal segment, and half-strength MS medium was beneficial for rooting. So, the composition of media and suitable concentration plays important role in establishing tissue culture regeneration system in *A. crenata* var. *bicolor*.

Plant growth regulators play important roles in regulating cell differentiation, division, and morphogenesis²⁴. In most shoot induction studies, high level of cytokinin has been used together with low level of auxin, which induced synergistic effect on shoot regeneration and leaf induction²⁵. In this protocol, explant types (tip shoot and nodal segment) of *A. crenata* var. *bicolor* showed different responses to different combinations of PGRs (BAP, KT and NAA), and regeneration ability of plant depends on explant types or age²⁶. Shoot induction of tip shoot and the nodal segment were higher in media containing BAP (1.0 mg·L⁻¹) and NAA (0.05 mg·L⁻¹). Wang et al.²⁷ reported that NAA has a weak influence on buds, which suggested a significant role of optimal

KT in increasing the proliferation rate. However, with increasing cytokinin concentrations negatively affected the shoot regeneration frequency, which was consistent with Rejthar et al.²⁸. In addition, it has been confirmed that a synergistic effect of different cytokinins can also result in effective regeneration rate²⁹, which indicates that KT and BAP in combination were necessary to induce shoot proliferation.

The quality of the root induction is affected by the composition and concentration of the media, most species require auxin to induce rooting and shoot promotion³⁰, but the root induction rate was decreased with increasing concentration of IAA³¹. Some research showed that IBA was found to be more effective in rooting induction compared to NAA and IAA^{25,32}. Our results showed that the rooting rate and the root number of tip shoot and nodal segment increased first and then decreased with an increase in the IBA concentration, the appropriate concentration of IBA and NAA could promote rooting significantly. The best concentration optimized for root induction was on half-strength MS medium augmented with IBA 0.5 mg L⁻¹ + NAA 0.1 or 0.2 mg L⁻¹ showed 72.7% and 65.1% root induction efficiency of tip shoot and nodal segment respectively. The highest survival rate of transplanting plantlets were 83.3% and 81.2% which were slightly lower than its related species of *A. crenata* (86.6%)³³, which might be related to the difference of the genetic characteristics and culture environments of *A. crenata* var. *bicolor*, but the aseptic seedlings grew well. Approximately, 6-months duration was required from initial culture to regenerate into whole plant and provided a supply of propagule free of main pathogens. We established a successful regeneration protocol of *A. crenata* var. *bicolor* by tip shoot and nodal segment, which might need to be further optimization, but it is a far more efficient method of propagating commercial seedlings than by conventional cuttings.

Conclusion

The over exploitation of resources and the reduction of wild populations forces the search for rapid propagation techniques to meet the growing needs, compensate for habitat destruction and conserve natural populations of plants. For the perennial shrubs, *in vitro* propagation of *A. crenata* var. *bicolor* by tissue culture methods is feasible and multiple plantlets can be regenerated from a single tip shoot and nodal segment explants in spite of some difficulties. Statistical analysis revealed that there was a significant difference among PGR combinations treatments applied in both shoot multiplication and rooting experiments, but did not show organogenic changes. The application of NAA and KT combinations influenced greatly shoots proliferation in *A. crenata* var. *bicolor*; 0.1 mg L⁻¹ BAP + 0.1 mg L⁻¹ NAA combined with 0.5 mg L⁻¹ KT was found to be optimal in producing the maximum mean number of shoots per explant. Application of NAA at a concentration of 0.1 mg L⁻¹ and 0.2 mg L⁻¹ NAA combined with 0.5 mg L⁻¹ IBA was more effective for root induction of shoot tip and nodal segment, suggesting that this protocol enables the large-scale propagation of *A. crenata* var. *bicolor* from two explants within a short time, which is contributing to its rapid propagation and germplasm preservation, but the caulogenesis score was relatively low in the plants, thus may requires to be further optimized of this protocol by adjusting or replacing the media compositions or the other techniques like culturing of explants in liquid medium.

Materials And Methods

Plant materials. Few seedlings of *A. crenata* var. *bicolor* were collected from the southern part of Gaoligong Mountain (24°56'39"N–24°57'03"N, 98°45'17"E–98°49'19"E), Yunnan Province, Southwest China, in September 2014–2015, the voucher specimen of this material has been deposited in the herbarium of Southwest Forestry University and identified by Prof. Fan Du and Juan Wang (ID: 00033070), which planted in the nursery of the College of Landscape Architecture and Horticulture Sciences, Southwest Forestry University, Yunnan, China. The maternal plants were gradually senescent after six to seven years and collected its tip shoot and nodal segment as materials. All methods as follows were performed in accordance with the relevant guidelines and regulations by including the collection of plant materials.

Experimental method. The roots of six to seven year old maternal plants grown in pots were irrigated with 0.5% carbendazim solution, and kept them in a dry environment 3–5 days, then excised the tip shoot and nodal segment from plants as starting materials for *in vitro* propagation (Fig. 1A, B), soaked in 10% (w/w) detergent solution (common laundry detergent) for 15 min, and washed for 60 min under flowing water. The washed buds were surface disinfected in 75% ethanol for 30–45 s, rinsed three times with sterile distilled water, and disinfected in 0.1% (w/w) mercury bichloride (HgCl₂) containing 0.1% (v/v) Tween 20 for 5min, 10min and 15 min respectively, then rinsed three times in distilled water to remove HgCl₂ completely. Alternatively, the explants were disinfected with 75% ethanol for 45 s, 3% sodium hypochlorite (NaClO) containing 0.1% (v/v) Tween 20 for 5min, 10min and 15 min respectively, and rinsed thrice with distilled water. Single-node explants of approximately 1.0 to 1.5 cm in length were excised and cultured in nutrient media mentioned below.

Culture establishment and shoot induction. Murashige and Skoog (MS)³⁴ basal medium and Woody Plant Medium (WPM)²² containing salt and vitamins were obtained from PhytoTech (Lenexa, Kansas, USA) and Chemik Co. Ltd. (Ningbo, China), respectively. For shoot induction, the basal medium was added of 3% sucrose, 0.7% agar and different concentrations of BAP and NAA. All medium were adjusted to a pH of 5.8 with NaOH or HCl (1 N) before adding the agar. Subsequently, media, Petri dishes (90 mm), and forceps were autoclaved at 121°C for 20 min and inoculated in a laminar cabinet. Stock growth regulators were disinfected by filtration through 0.22 µm membrane filter and then added into the autoclaved medium, which was then placed at 4°C in a disinfected environment. Twelve media compositions were tested to select the optimum culture induction medium, during which single factor experiments were investigated the effect of BAP and NAA combinations on the growth of *A. crenata* var. *bicolor* plantlets *in vitro*. Cultures were kept at 25°C ± 2°C under cool white fluorescent lights (27 µmol·s⁻¹·m⁻², about 2000 lux light intensity) with a 16/8 h photoperiod. After 4–5 weeks of culturing, an explant that induced shoots was considered to have successfully regenerated.

Shoot multiplication. Aseptic shoots induced on the above-mentioned medium compositions were used as explants for multiplication and MS was selected as basal medium, during which a L9 (3³) orthogonal experiment was designed to test the proliferation effect of different concentrations of BAP (0.5–2.0 mg·L⁻¹), NAA (0.1–0.5 mg·L⁻¹) and KT (0.1–1.0 mg·L⁻¹) with the aim to further optimize the medium for shoot multiplication.

Root induction. Elongated shoots, dissected to approximately 1.5 cm in length, were cultured in the rooting medium, which were designed based on 3×3 orthogonal array and comprised different concentrations of IBA (0.2, 0.5 and 1.0 mg·L⁻¹) and NAA (0.1, 0.2, 0.5 mg·L⁻¹). All of these combinations were added to a half-strength MS medium consisting of 2% sucrose, 0.8% agar, and 0.5 g·L⁻¹ AC to prevent browning. Statistics were made according to rooting rate and shoot growth.

Transplanting. The *in vitro* plantlets were moved to a greenhouse before transplanting, opened each tube cap and added 2 ml aseptic water for 3 to 4 days. Subsequently, the plantlets were allowed to adjust to new growth conditions for 2 days and were carefully removed from the tubes and rinsed with sterile water to eliminate media remnants on roots. The clean plantlets were transplanted into seedling- raising plates or bags which were filled with four different proportions of the mixed matrix (peat: vermiculite: perlite=1:1:1; 2:1:1; 3:2:1; 4:2:1) and were then sprayed with 0.5% carbendazim solution. The seedbed was covered with transparent plastic film and 75% or 50% sunshade nets to maintain a constant temperature (25 ± 2°C) and relative humidity (approximately 85%). The survival of transplanting was determined after 5 weeks.

Statistical analysis. All experiments data were recorded by visual observation of successful shoot and root induction and were subjected to statistical analysis. All percentage data converted to arcsine square root ($\sqrt{P}$)³⁵ to normalize error distribution. Analysis of variance (ANOVA) was used to analyze the data, and means were compared using Duncan's post-test at a 5% probability level. All analyses were conducted using the SPSS (version 21.0) software. All data are presented as means ± standard deviations.

Declarations

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Author's contributions

X.M.A. identified problems, conceived the concept, analyzed the data, and prepared manuscript; X.M.A. and Y.H.W. collected the material, designed the study, performed the laboratory experiments; C.W. provided validation, supervision and revision, prepared the manuscript. All authors have read and approved the manuscript.

Competing interests

No potential conflict of interest was reported by the authors.

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Figures

Figure 1

In vitro propagation via organogenesis of *A. crenata* var. *bicolor* A-B: explants from *A. crenata* var. *bicolor*; C: shoot induction; D: abnormal flower bud differentiation; E: shoot proliferation; F: different concentrations of IBA and NAA for root induction; ① - ⑤ show the rooting of tip shoots: ① 0.2 mg·L⁻¹ IBA + 0.1 mg·L⁻¹ NAA ② 0.5 mg·L⁻¹ IBA + 0.1 mg·L⁻¹ NAA ③ 0.5 mg·L⁻¹ IBA + 0.2 mg·L⁻¹ NAA ④ 1.0 mg·L⁻¹ IBA + 0.1 mg·L⁻¹ NAA ⑤ shows the rooting of nodal segment: 0.5 mg·L⁻¹ IBA + 0.1 mg·L⁻¹ NAA G: aseptic plantlets transplanted in the seedling-raising bags