Acacia Mangium × A. Auriculiformis Micropropagation in a Non-Sterile Environment

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Research Article

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Abstract

Background: Autoclaving is used to eliminate contamination during tissue culturing, however, it is a complicated process, time-consuming and costly. Chemical sterilization of tissue culture can effectively eliminate contamination, is a simple procedure, and cost effective. However, studies on the chemical sterilization mostly focus on bud induction, while the effects of chemical sterilization overall process of tissue culture, including bud induction, proliferation, and rooting, remain to be determined. Here, we investigate the effect of chemical sterilization on bud induction, proliferation, and rooting of *Acacia mangium* × *A. auriculiformis*.

Results: The results showed that chlorothalonil (0.2 g/L) was a suitable chemical sterilant, and bud induction medium was 1/8 Murashige and Skoog medium + agar 7 g/L + chlorothalonil 0.2 g/L + 6-benzylaminopurine 0.5 mg/L The highest induction rate (99.54%) was observed in the third to fifth buds' stem segments collected in October treated with 0.8 g/L carbendazim for 3 min, with a contamination rate of 0. The rooting medium was agar 7 g/L + chlorothalonil 0.2 g/L+ indolebutyric acid 1.5 mg/L + naphthylacetic acid 0.5 mg/L, and the rooting rate was 97.62%. The proliferation rate and subculture duration showed a positive correlation, while the proliferation rate was 3.58 times higher at the fourth subculture rooting.

Conclusions: Our results suggest that chlorothalonil can effectively replace autoclaving during bud induction, proliferation, and rooting of *A. mangium* × *A. auriculiformis*. The findings of this study provide technical support for rapid seedlings propagation, accelerates the breeding process of *Acacia*, and can be applied in other tree species.

Background

Acacia mangium × *A. auriculiformis*, a hybrid of *A. mangium* and *A. auriculiformis* [1], is a promising tree species with superior characteristics of growth, adaptability, fiber quality, pulping properties, and insect and wind resistance compared to the parental species [2–6]. However, asexual propagation is the only way to maintain superiority [7, 8].

Tissue culture is a widely used method for the asexual propagation of trees [9, 10]. This method is characterized by fast reproduction speed and high reproduction coefficient, which can maintain the superiority of the variety [11, 12]. However, complicated and time-consuming operating procedures, as well as strict and meticulous technical requirements hinder the wide application of tissue culture [13]. For example, tissue culture requires a well-equipped laboratory, which requires high capital and operating costs [14]. Autoclaving is the most time-consuming process, which increases the production cost of tissue culture [15, 16]. However, it is essential for avoiding contamination in traditional tissue culture.

To identify an alternative method to autoclaving, researchers have proposed adding chemical sterilizing agents to the culture medium. This can mitigate contamination, while also ensuring the normal reproduction of seedlings [17]. Chemical sterilants include chlorine dioxide [18–20], sodium hypochlorite

[18, 21–23], peracetic acid [19], and hydrogen peroxide [24, 25] have been tested as potential candidates. However, most of these studies have only focused on bud induction. Whether antibacterial agents play the same role in proliferation and rooting remains to be determined. To make this method commercially viable, it is necessary to ensure that chemical sterilization can replace autoclaving to minimize contamination during bud induction, proliferation, and rooting.

Therefore, in this study, *A. mangium* × *A. auriculiformis* was used to identify the appropriate chemical sterilant, without the strict control requirements of traditional tissue culture, in a non-sterile environment. Most importantly, the feasibility in shoot induction, rooting, and subsequent proliferation was evaluated to provide an improved tissue culture method using chemical sterilization instead of autoclaving.

Methods

Materials

In 2014, some elite trees of *Acacia mangium* and *A. auriculiformis* were selected from plantations located at Shadui Town, Jiangmen City, Guangdong Province, China, and controlled their pollination. When the seeds were mature, we harvested and used them to establish cutting orchard at an interval of 50 × 50 cm, which were managed according to standard measures and were periodically fertilized, pruned, and treated with fungicides. The semi-lignified annual shoots containing axillary buds from the cutting orchard were used as explants.

We have independent intellectual property rights for the materials of this study.

Explants pretreatment

All of the experiments of this study were carried out in 2016. The semi-lignified annual shoots containing axillary buds of *A. mangium* × *A. auriculiformis* were collected from cutting orchard, washed in tap water with a soft brush, and then soaked in water with washing powder for 30 min followed by rinsing again in tap water for 10 min. The stems were trimmed into uninodal with axillary buds until the size ranged from 2.0 to 3.0 cm and then were rinsed with running water for 1 h.

Sterilization agent screening

In order to select the best bacteriostatic agent and its concentration, according to Huang et al.[32], we selected two sterilization agents, namely carbendazim or chlorothalonil, to set four concentration gradients ((0.2, 0.4, 0.6, and 0.8 g/L)). After pretreatment, 24 explants were inoculated in each treatment, repeated three times. The survival rate, contamination rate, and browning rate of explants in the experiment were recorded after 30 days of culture.

Disinfection of explants

According to Huang et al. [32], the collected stem segments were selected from the cutting orchard as explants in 2016, and the following three types were used: upper explants (the upper stem segment with axillary buds from the first to the second segments) with leaves (leaves were trimmed to approximately 1/4 of a whole leaf), and middle explants (from the third to the fifth stem segment containing axillary buds) with and without leaves.

The three types of explants after pretreatment were disinfected according to the protocol described in Table 4, the sterilized explants were then inoculated into a medium containing optimal concentrations of sterilizing agents, and 21 explants were inoculated for each treatment and repeated three times. After 30 days of culture, the growth status of explants for each trial was recorded (statistical survival, contamination, and browning rate). Finally, we screened out the best type of explants and disinfection strategy.

Table 4
Explant type and disinfection scheme for bud induction of *Acacia mangium* × *A. auriculiformis.*

Explant types	Disinfection strategy
Middle stem without leaves	0.8 g/L Carbendazim 3 min
	75% Alcohol 10 s
	75% Alcohol 15 s
Middle stem with leaves	0.8 g/L Carbendazim 3 min
	75% Alcohol 10 s
	75% Alcohol 15 s
Upper stem with leaves	0.8 g/L Carbendazim 1 min
	75% Alcohol 2 s
	75% Alcohol 5 s

Bud induction medium

In order to select the best bud induction medium for open tissue culture of *A. mangium* × *A. auriculiformis*, different types of media were examined: modified MS (a large number of MS elements were reduced to 50%), 1/2 MS, 1/4 MS, 1/8 MS (reducing all elements of MS to 1/2, 1/4, and 1/8, respectively), and blank as a control, containing different levels of 6-BA (0, 0.5, and 1.0 mg/L). The best type of explants were selected for pretreatment while the best disinfection strategy was used to disinfect the explants and inoculate the explants into different types of bud induction media containing 0.2 g/L chlorothalonil. Each treatment contained 24 explants and was repeated three times. After 30 d, the survival rate, contamination rate, browning rate, and budding rate were determined.

Explants collection season

Shoots with axillary buds were collected as explants in January, April, July, and October of 2016. The best type of explants were selected for pretreatment while the best disinfection strategy was used to disinfect the explants. The best induction medium screened was used to induce the bud under open conditions. Seventy-two explants were selected for induction in each season. The number of induced buds was recorded after 30 days of culture and the best month for explant collection was selected.

Rooting medium

In order to screen the best rooting medium for *A. mangium* × *A. auriculiformis*, well-growing buds selected from the bud induction medium were inoculated into a medium containing 0.2 g/L chlorothalonil and a combination of IBA and NAA at different concentrations for rooting (Table 5). Twenty-four induced buds were selected for each treatment; all treatments were repeated three times. The rooting of induced buds was observed after 15, 30, and 45 d, while the rooting rate was counted after 30 days of culture.

Table 5. Concentrations of IBA and NAA used for rooting medium.

Treatments	IBA (mg/L)	NAA (mg/L)
1	0	
2	0.75	
3	1.0	0.5
4	1.25	
5	1.5	
6		0
7		0.25
8	1.0	0.5
9		0.75
10		1

Proliferation by subculture rooting

Well-growing buds selected from the bud induction medium were inoculated into the best rooting medium containing 0.2 g/L chlorothalonil. After rooting for 45 d, the seedlings were cut into approximately 2.5 cm with at least one axillary bud for subculture rooting. The number of inoculated seedlings per generation

and the number of seedlings obtained after rooting subculture were recorded. Each subculture was inoculated with 16 plantlets; three replicates were used for all treatments.

Culture condition

Cultures were maintained under white fluorescent light with a photon flux of 30 μmol m^{-2} s^{-1} for a 12 h photoperiod at 25 ± 2°C. No sugar was added to the medium, and 7 g/L agar was added. The pH was adjusted to 5.8 ± 0.2. It did not require high temperature or autoclave sterilization, and 0.2 g/L chlorothalonil was added before the agar solidified. All operations of culture medium preparation were carried out in a non-sterile environment without using an ultra-clean bench. The explants were cultured in a culture flask throughout the experiment, without controlling the specific relative humidity.

Data analysis

SPSS program version 19.0 for Windows was used to perform the analysis of variance. The significant differences ($P < 0.05$) between the mean values of each treatment were determined using Duncan's multiple range test. The parameters were recorded as follows:

Survival rate = Number of survived explants/Total number of inoculated explants × 100%

Browning rate = Number of explants turned brown /Total number of inoculated explants × 100%

Contamination rate = Number of contaminated explants/Total number of inoculated explants × 100%

Bud induction rate = Number of explants with bud induction/Total number of surviving explants × 100%

Rooting rate = Number of seedlings with roots /Total number of survived seedlings × 100%

Proliferation = Number of proliferated seedlings/Total number of explants inoculated.

Results

Sterilization agent screening

The culture conditions and the different sterilants that were added to the medium are shown in Table 1. When the explants of *A. mangium* × *A. auriculiformis* were cultured under non-sterile conditions, significant differences were detected in the survival rate because of adding different sterilants to the medium. There was significantly higher contamination in the media with carbendazim than chlorothalonil ($P < 0.05$). As the chlorothalonil concentration increased, so did the browning rates, yet, the survival rates significantly decreased ($P < 0.05$). The contamination rates were not significantly different among different chlorothalonil concentrations. The highest survival rate was 80.55%, at a chlorothalonil concentration of 0.2 g/L. These results demonstrate the sterilization effect of adding 0.2 g/L chlorothalonil to the medium.

Table 1
Effects of sterilizing agents on bud induction of *Acacia mangium* × *A. auriculiformis.*

Sterilants	Concentration (g/L)	Survival rate (%)	Contamination rate (%)	Browning rate (%)
Chlorothalonil	0.2	$80.55^{a} \pm 4.81$	$6.95^{b} \pm 4.81$	$12.50^{b} \pm 0.00$
	0.4	$68.05^{a} \pm 8.67$	$6.94^{b} \pm 12.03$	$25.00^{b} \pm 4.17$
	0.6	$63.89^{a} \pm 8.67$	$9.72^{b} \pm 2.41$	$26.39^{b} \pm 10.48$
	0.8	$16.67^{b} \pm 4.17$	$20.83^{b} \pm 12.50$	$62.50^{a} \pm 8.33$
Carbendazim	0.2	$18.05^{b} \pm 24.41$	$56.94^{a} \pm 18.79$	$25.00^{b} \pm 8.33$
	0.4	$8.33^{b} \pm 8.34$	$62.50^{a} \pm 30.04$	$29.17^{b} \pm 22.05$
	0.6	$12.50^{b} \pm 14.43$	$65.28^{a} \pm 15.77$	$22.22^{b} \pm 13.39$
	0.8	$19.44^{b} \pm 6.36$	$59.72^{a} \pm 8.67$	$20.83^{b} \pm 8.34$
Control	0.0	$31.94^{b} \pm 6.36$	$54.17^{a} \pm 4.17$	$13.89^{b} \pm 2.41$

Different letters indicate significant difference using Duncan's test at 5% probability. Data are shown as the mean ± the standard error.

Disinfection of explants

There was significant variation in the rates of browning, contamination, and survival for different disinfection strategies in *A. mangium* × *A. auriculiformis* explants (Table 2). When the upper stem with leaves and the middle stem without leaves were used as explants, the browning rates were significantly higher than those of the middle stem with leaves. However, the survival rates were significantly lower than of the middle stem with leaves, regardless of the disinfectant used. Therefore, the middle stem with leaves was suitable for the bud induction of *A. mangium* × *A. auriculiformis* (Fig. 1). The rates of browning, contamination, and survival were not significantly different for the different disinfection strategies. When the middle stem with leaves was used as explant, the explant's survival rate was approximately 96.83% after treatment with 0.8 g/L carbendazim for 3 min.

Table 2
Effects of explant type and disinfect strategy on *Acacia mangium* × *A. auriculiformis* bud induction.

Explant type	Disinfection strategy	Survival rate (%)	Contamination rate (%)	Browning rate (%)
Middle stem without leaves	0.8 g/L Carbendazim for 3 min	$28.57^{bc} \pm 14.29$	$9.52^{ab} \pm 0.00$	$61.90^{b} \pm 14.29$
	75% Alcohol for 10 s	$47.62^{b} \pm 14.29$	$0.00^{b} \pm 0.00$	$52.38^{bc} \pm 14.29$
	75% Alcohol for 15 s	$38.10^{b} \pm 9.53$	$0.00^{b} \pm 0.00$	$61.90^{b} \pm 9.53$
Middle stem with leaves	0.8 g/L Carbendazim for 3 min	$96.83^{a} \pm 2.75$	$0.00^{b} \pm 0.00$	$3.17^{d} \pm 2.75$
	75% Alcohol for 10 s	$76.19^{a} \pm 9.52$	$14.29^{ab} \pm 9.53$	$9.52^{d} \pm 0.00$
	75% Alcohol for 15 s	$90.48^{a} \pm 4.77$	$0.00^{b} \pm 0.00$	9.52d ± 4.77
Upper stem with leaves	0.8 g/L Carbendazim for 1 min	$38.10^{b} \pm 28.58$	$23.81^{a} \pm 19.05$	$38.10^{c} \pm 9.53$
	75% Alcohol for 2 s	$6.35^{c} \pm 2.75$	$4.76^{ab} \pm 4.76$	$88.89^{a} \pm 5.50$
	75% Alcohol for 5s	$4.76^{c} \pm 0.00$	$9.52^{ab} \pm 9.53$	$85.71^{a} \pm 9.53$

Different letters indicate significant difference using Duncan's test at 5% probability. Data are shown as the mean ± the standard error.

Bud induction medium

After adding chlorothalonil to the bud induction medium, the contamination rate increased. When the medium was 1/8 Murashige and Skoog medium (MS), there was no contamination of the explants, the survival rate reached > 90%, and the bud induction rate was higher than in other media. When the concentration of 6-benzylaminopurine (6-BA) was 0–1 mg/L, the germination rate first increased and then decreased. The germination rate was highest at 0.5 mg/L 6-BA (Table 3). The medium had a significant effect on the browning rate, and higher nutrient content in the medium led to a higher browning rate. Therefore, the best bud induction medium combination for *A. mangium* × *A. auriculiformis* is 1/8 MS + 6-BA 0.5 mg/L, with a maximum germination rate of 92.55%.

Table 3
Effects of medium and 6-BA concentration on bud induction of *Acacia mangium* × *A. auriculiformis*.

Treatment		Survival rate (%)	Contamination rate (%)	Browning rate (%)	Germination rate (%)
Medium type	6-BA (mg/L)				
1/2 MS	0	$11.11^{cd} \pm 4.82$	$8.33^{a} \pm 8.33$	$80.56^{a} \pm 12.73$	$0.00^{c} \pm 0.00$
	0.5	$56.94^{b} \pm 6.36$	$1.39^{a} \pm 2.41$	$41.67^{b} \pm 8.34$	$16.74^{c} \pm 10.54$
	1	$26.39^{c} \pm 9.62$	$1.39^{a} \pm 2.41$	$72.22^{a} \pm 8.67$	$6.67^{c} \pm 11.55$
1/4 MS	0	$55.56^{b} \pm 15.78$	$0.00^{a} \pm 0.00$	$44.44^{b} \pm 15.78$	$2.08^{c} \pm 3.61$
	0.5	$68.06^{b} \pm 14.63$	$2.78^{a} \pm 2.40$	$29.17^{bc} \pm 12.50$	$45.45^{b} \pm 8.18$
	1	$56.94^{b} \pm 6.36$	$1.39^{a} \pm 2.41$	$41.67^{b} \pm 8.34$	$2.22^{c} \pm 3.85$
1/8 MS	0	$90.28^{a} \pm 6.37$	$0.00^{a} \pm 0.00$	$9.72^{cd} \pm 6.37$	$92.12^{a} \pm 7.06$
	0.5	$94.45^{a} \pm 4.81$	$0.00^{a} \pm 0.00$	$5.55^{d} \pm 4.81$	$92.55^{a} \pm 2.84$
	1	$90.28^{a} \pm 6.37$	$0.00^{a} \pm 0.00$	$9.72^{cd} \pm 6.37$	$89.44^{a} \pm 4.83$
Modified MS	0	$5.55^{cd} \pm 4.81$	$4.17^{a} \pm 4.17$	$90.28^{a} \pm 2.41$	$0.00^{c} \pm 0.00$
	0.5	$23.61^{cd} \pm 13.40$	$4.17^{a} \pm 4.17$	$72.22^{a} \pm 14.63$	$4.76^{c} \pm 8.25$
	1	$1.39^{d} \pm 2.41$	$4.17^{a} \pm 7.22$	$94.44^{a} \pm 6.36$	$0.00^{c} \pm 0.00$
Control	0	$11.11^{cd} \pm 4.82$	$8.33^{a} \pm 8.33$	$0.00^{d} \pm 0.00$	$80.56^{a} \pm 12.73$
	0.5	$5.55^{cd} \pm 4.81$	$4.17^{a} \pm 4.17$	$3.03^{d} \pm 5.25$	$90.28^{a} \pm 2.41$
	1	$55.56^{b} \pm 15.78$	$0.00^{a} \pm 0.00$	$2.08^{d} \pm 3.61$	$44.44^{b} \pm 15.78$

Different letters indicate significant difference using Duncan's test at 5% probability. Data are shown as the mean ± the standard error.

Explants collection season

There were significant differences in the bud induction rates of explants collected during different growing season (Fig. 2). The bud induction rate was the lowest in January (66.21%) and reached its highest in October (99.54%). The bud induction rate of explants collected in April and July was slightly lower than in October but higher than 95%. Therefore, April to October is the most optimum period to collect explants for bud induction.

Rooting medium

Different concentrations Indolebutyric acid (IBA) and naphthylacetic acid (NAA) had significant effects on rooting (Fig. 3). In treatments 1 to 5, when the NAA concentration was 0.5 mg/L, the rooting rate increased with IBA concentration. For treatments 6 to 10, when the IBA concentration was 1 mg/L, increasing NAA concentration caused the rooting rate to increase and then decrease. The highest rooting rate was 97.62% when treated with IBA (1.5 mg/L) and NAA (0.5 mg/L; treatment 5). The results indicate that a high concentration of IBA improves rooting potential, and the optimal NAA concentration was 0.5–1.0 mg/L. The roots started to appear after culturing for 12 d (Fig. 4a), and root length increased up to 5 cm after culturing for 35 d (Fig. 4b).

Proliferation by subculture rooting

After rooting culture for 45 d, the roots of seedlings grew to approximately 5 cm, and then the seedlings were cut into 2–3 sections for subculture rooting. The multiplication rates increased significantly with the increase in subculture duration, and the proliferation rate of the fourth subculture was the highest at 3.58 (Fig. 5).

Discussion

Sterilization agent screening

Chemical sterilization has great applications and prospects and provides a new sterilization option for the development of micropropagation technology. Peiris et al. successfully used 0.003% sodium hypochlorite to control explant contamination in gerbera rooting in vitro [14]. Cardoso and Da Silva used ClO_2 for sterilization and reported an increase in the vigor of gerbera buds [26]. Meanwhile, Duan et al. found that hydrogen dioxide had a better sterilization effect on potato micropropagation and caused no damage to seedling growth and tuber induction [27]. These results indicate that different bacteriostatic agents are suitable for a range of explant sterilization methods.

There are many studies on *in vitro* propagation of *Acacia* species [28–31], but its regeneration system is inefficient, expensive, and time-consuming, which limits large-scale production. Chemical sterilization, instead of autoclaving, is an effective method for solving this problem [14]. Previous studies have shown that chlorothalonil can be used as a bacteriostatic agent for *A. auriculiformis* bud induction. Compared with other reported chemical disinfectants, chlorothalonil has the advantages of simple operation, low contamination, and high germination rate [32].

In this study, the sterilization effects of chlorothalonil and carbendazim were compared, and show that chlorothalonil had a good sterilization effect on A. *mangium* × *A. auriculiformis*. The antibacterial effects of chlorothalonil on bud induction, proliferation, and rooting were studied. These data clearly show that the addition of appropriate concentrations of chlorothalonil to the culture medium did not cause

phytotoxicity. In contrast, the appropriate concentration of chlorothalonil promoted the germination and rooting rate of explants. This further confirms the effectiveness of chlorothalonil as a chemical sterilant in the tissue culture process, and the feasibility of chemical sterilization in the process of bud induction, reproduction, and rooting.

Disinfection of explants

The *in vitro* establishment of explants varies according to type, size, sampling site [33], and disinfection method [34–36]. The source of the explants significantly influenced the contamination rate and subsequent axillary bud growth. Three types of explants were used in this study: leafy and leafless middle stem segments, and leafless stem tips. The semi-woody stem segment in the middle was the most suitable explant, consistent with previous studies [37, 38]. This may be due to the rich nutrients stored in the middle stem itself, which can promote the elongation and growth of the buds. The bottle caps used for cultivation were sealed; therefore, the presence of leaves can better ensure the energy supply of the explants, thereby improving the bud induction rate and growth.

Under natural conditions, the outside and inside of plant explants contain more bacteria and molds, which must be disinfected during tissue culture [4, 34, 39]. The pretreatment of explants can effectively reduce the contamination rate, and different types of explants require different disinfection methods. Mercury is commonly used as a disinfectant; however, it is highly toxic to explants, therefore, in the long-term, it is not suitable as a disinfectant for tissue culture.

Carbendazim and penicillin have been used to control contamination, while carbendazim effectively killed fungi and reduced the contamination rate of explants [40]. Pretreating explants with 0.1% (carbendazim) + 0.1% (Syngenta) for 1 h resulted in the lowest microbial contamination (40.60%) [41]. A mixture of carbendazim, polyvinylpyrrolidone, ascorbic acid, citric acid, and benzylpenicillin as the pretreatment agent reduced the contamination rate of *Madhuca hainanensis* explants [42]. Silver and copper nanocolloids are new non-toxic sterilization agents with good bactericidal effects [43]. The selection of a suitable disinfectant and disinfection time for explant pretreatment is key to the success of the experiment. The explants in this study soaked with 0.8 g/L carbendazim for 3 min were free of contamination, and the survival rate reached 96.83%. Therefore, to achieve the ideal disinfection effect, the disinfection strategy must be determined according to the type, location, size, disinfectant, and disinfection time of the material.

Bud induction medium

The optimal type and concentration of antimicrobial agent may vary, depending on the explant. A total active chlorine concentration ≥ 0.0003% can completely sterilize the medium: a total active chlorine concentration of 0.0003% caused the biomass of pineapple buds and the number of new buds to more than double [21]. In the establishment of *Eucalyptus benthamii* in a non-autoclaved medium *in vitro*, the number and total length of sprouts supplemented with 0.001% and 0.003% active chlorine were most similar to the traditional medium [44]. Chlorothalonil can effectively control the aseptic environment,

reduce the contamination rate, and increase the survival rate of explants for *A. auficuliformis* bud induction [32]. In this study, chlorothalonil achieved a good effect in the bud induction stage of *A. mangium* × *A. auriculiformis*: using 1/8 MS + 0.5 mg/L 6-BA as the bud induction media, the bud induction rate was 92.55%.

The composition of the culture medium also affects *Acacia* in vitro culture. The media used here for bud induction differed from those of traditional tissue culture. In the traditional tissue culture method of *A. auriculiformis*, a medium of MS + sucrose 20 g/L yields a bud induction rate of 97.33% [45]. In this study, 1/8 MS medium was most suitable for bud induction of *A. mangium* × *A. auficuliformis*, indicating that the induction stage of *A. mangium* × *A. auficuliformis* is more suitable for medium with low nutrient concentrations. The explants can survive and grow on medium without sucrose, indicating that these seedlings possessed photoautotrophic growth and could synthesize the energy needed for their growth. In terms of the bud induction rate, survival rate, and contamination rate, adding 0.5 mg/L 6-BA to the culture medium produced better results. This is different from the optimal concentration of traditional tissue culture, which may be related to bactericide in the culture medium.

The addition of chlorothalonil in this study controlled the growth of microorganisms in the medium and reduced the contamination rate. Compared with traditional tissue culture, it maintained a high induction while simplifying the operation and saving cost. This is consistent with previous findings [32].

Rooting Medium

There are many relevant studies on the choice of rooting medium type, sucrose concentration, and hormone type and concentration [46–48]. There are also some reports on autotrophic micropropagation technology [49–51], which have shown that sucrose, as a carbohydrate in the culture medium, is essential in traditional micropropagation. However, sucrose may not be used in the photoautotrophic micropropagation system, but CO_2 will be used as the plant's carbon source to replace sucrose so that it can carry out photosynthetic autotrophic micropropagation. In this study, due to the non-sterile culture environment, sucrose was not added to the medium to prevent contamination of large amounts of explants. However, no carbon substitute was added, which would help the seedlings better adapt to the environment, greatly reducing the time of domestication and transplanting. The addition of an appropriate concentration of plant growth regulators ensured an optimum-rooting rate of explants. The results show that hormone concentration had a significant effect on rooting. The higher rooting rate also shows that the addition of chlorothalonil does not negatively impact plant growth regulators. In future research, the exact point where the hormone concentration promotes the growth of explants will be determined, and sucrose substitutes will be identified that can provide a carbon source and inhibit bacterial growth.

The addition of chemical disinfectants can significantly reduce the contamination rate, but few studies have focused on rooting. Ding et al. [51] reported that using Tuzet as a fungicide for rooting culture of *Aspergillus niger*, the rooting rate was 85%, and the survival rate of seedlings was higher than that of

traditional tissue culture. In this study, 0.2 g/L chlorothalonil was added to the medium for chemical sterilization. Compared with autoclaved medium, the addition of chemical sterilants can increase the rooting rate to up to 97.62%. Although this rate is lower than the traditional tissue culture (99.43%) [38], the difference is not significant, indicating that chemical sterilization can effectively replace high-pressure sterilization as, not only does it not inhibit rooting, but it also reduces the contamination rate. However, the effect of chemical sterilization on explant growth was not obvious from the rooting rate analysis alone. It is necessary to observe the growth performance after transplantation and other aspects.

Proliferation by subculture rooting

Few reports have described the successful proliferation of explants after chemical sterilization of the culture medium [52, 53]. Sodium hypochlorite and sugar were added to the culture medium for banana tissue culture, and high toxicity of the sterilization agent was found [53]. Most of the cluster buds showed a high level of browning in the early stage of inoculation, with no success in the proliferation of bananas. H198 was used as a sterilization agent in the tissue culture of Konjak, and most of the explants were observed to brown during the proliferation culture [52].

In this study, 0.2 g/L chlorothalonil was added to the culture medium without sucrose during the proliferation culture, and there was no differentiation or proliferation. However, no browning or contamination was observed. Sucrose was added to the culture medium with 0.2 g/L chlorothalonil, and explants showed high level of contamination at the early stage of culture, resulting in culture failure. Sucrose plays an important role in the proliferation process as a carbon source and maintaining osmotic pressure. Sucrose affects cell differentiation and the nutritional status of explants and affects their proliferation. If sucrose were not added to the medium, the explants would not differentiate and proliferate. If, conversely, sucrose was added, it would cause contamination and failure of culture.

In this study, the height of the seedlings was approximately 5 cm or higher after the rooting culture of 45 d and the seedlings could be cut into 2–3 segments, and then successfully proliferated through the rooting subculture. The proliferation of the fourth subculture was 3.58. The proliferation rate of traditional tissue culture was 2.52% (proliferation by inducing cluster buds). This study was superior, in terms of proliferation rate, to traditional tissue culture. The explants developed roots more easily after the first rooting subculture, and the number of days for rooting gradually decreased with increasing subcultures. The average day for one-time subculture rooting was approximately 40 d. As the rooting culture environment was similar to that of the nursery culture, the seedlings could be transplanted directly without acclimatization. For traditional tissue culture, the proliferation days were 40 d, the rooting days were 15 d, and the acclimatization period was 5–10 d [38]. Therefore, this study was superior to traditional tissue culture and could save seedling time and improve the utilization rate of seedlings.

Conclusions

In this study, the antibacterial effect of chlorothalonil was verified, and a novel method for *A. mangium* × *A. auriculiformis* micropropagation is proposed. The suitable bud induction medium is 1/8 MS + agar 7

g/L + chlorothalonil 0.2 g/L + 6-BA 0.5 g/L for *A. mangium* × *A. auriculiformis*, with a bud inducing rate of 99.54%. After bud induction for 20 d, buds of approximately 3 cm were used as explants for rooting culture. The suitable rooting medium was agar (7 g/L + chlorothalonil 0.2 g/L + IBA 1.5 mg/L + NAA 0.5 mg/L) with a rooting rate of 97%. The roots appeared after culturing for 12 d with rooting medium. The seedlings with roots grew to approximately 5 cm (or higher) after culturing with rooting medium for 45 d and then were cut into 2–3 cm for subculture rooting. Successful proliferation was achieved through this method, and the proliferation rate was 3.58 of the fourth subculture. These results indicate that chlorothalonil can effectively control explant contamination in different media with no toxic effects on the three stages of tissue culture of *Acacia* hybrids, namely bud induction, proliferation, and rooting; hence, it can be a good substitute for autoclaving, reducing the cost of autoclaves and ultra-clean workbenches for large facilities, simplifying the operation process, and improving the efficiency of tissue culture.

Abbreviations

MS: Murashige and Skoog medium; 6-BA: 6-benzylaminopurine; IBA: indolebutyric acid; NAA: naphthaleneacetic acid.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LH conceived and designed the experiments. HW performed the experiments. YL, LH, HW, and MQS analyzed the data. YL and LH wrote the paper. YL, LH, and MQS revised the paper. All authors read and approved the final manuscript.

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Figures

Figure 1

Bud induction of the middle explants with leaves of Acacia mangium × A. auriculiformis.

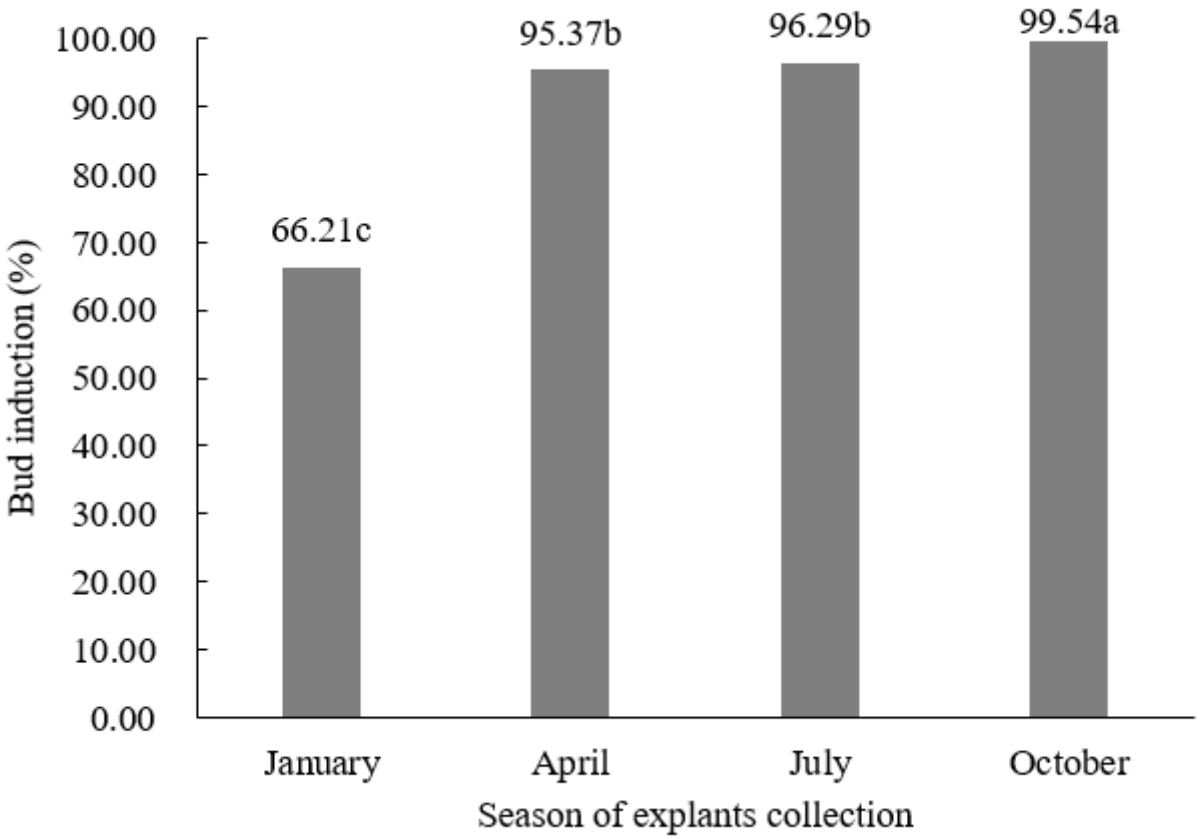

Figure 2

Bud induction rate of Acacia mangium × A. auriculiformis explants collected from different months. Different letters indicate significant differences at $P < 0.05$ using Duncan's multiple range test.

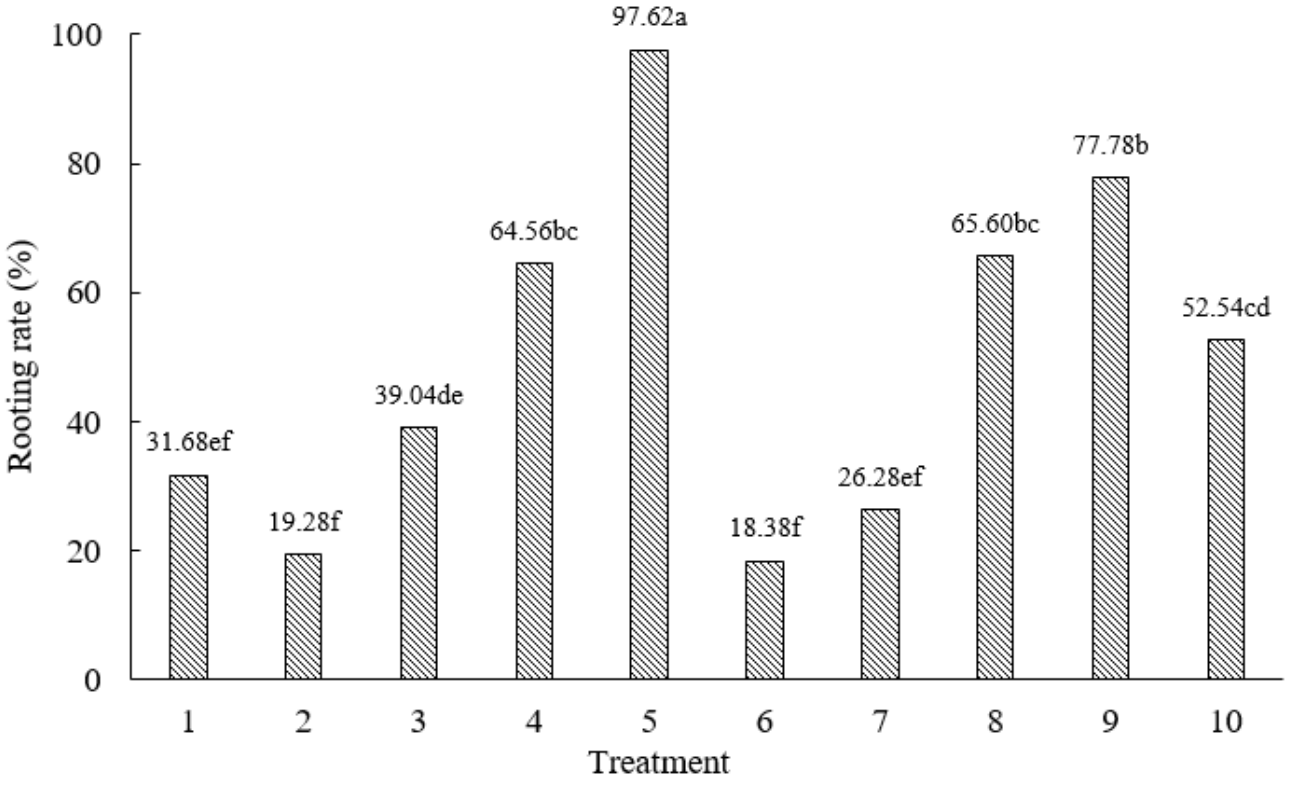

Figure 3

Effects of different concentrations of IBA and NAA on rooting of Acacia mangium × A. auriculiformis. Different letters indicate significant differences at $P < 0.05$ using Duncan's multiple range test.

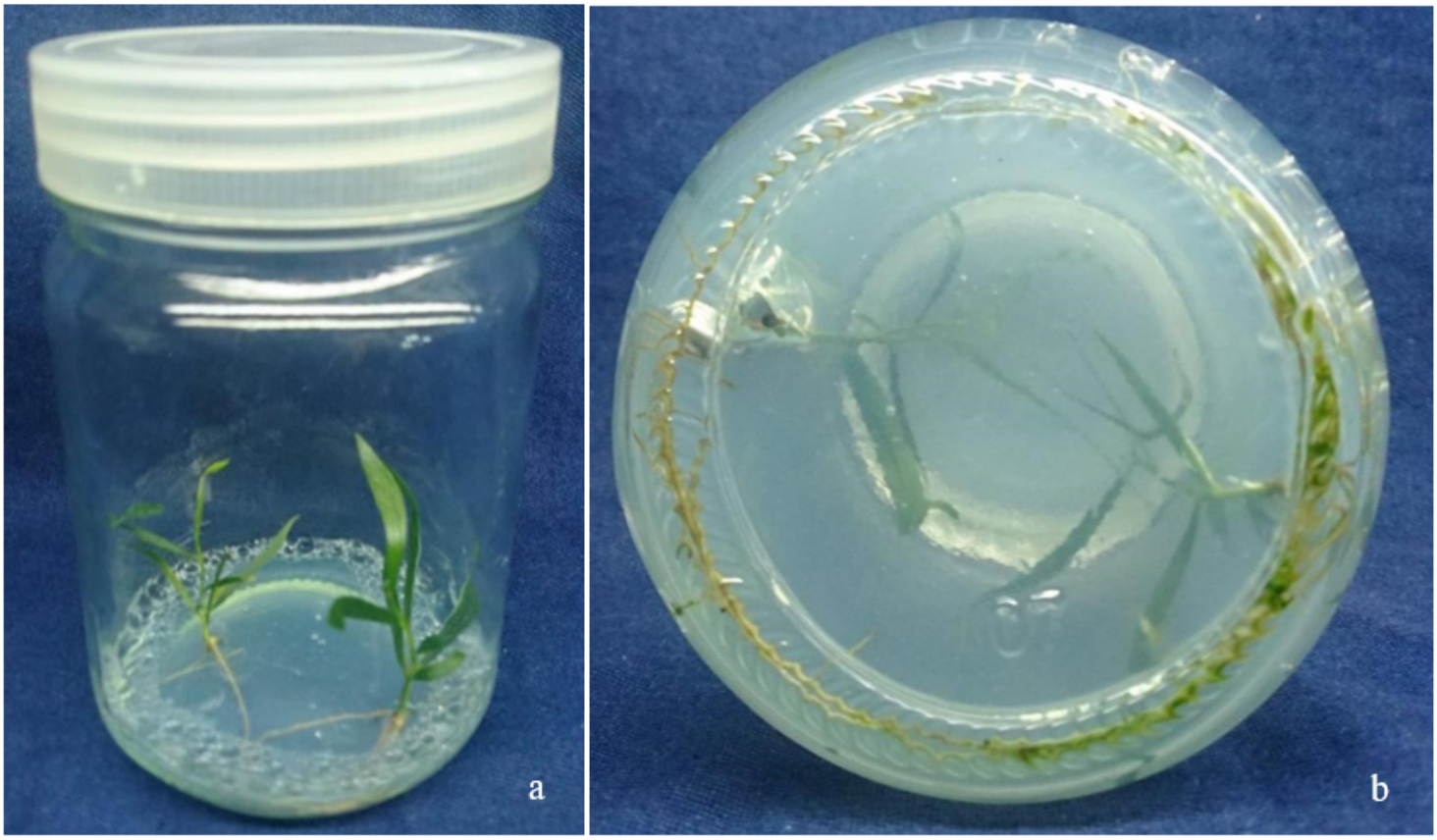

Figure 4

Rooting culture of Acacia mangium × A. auriculiformis. Rooting culture after a: 12 d; b: 30 d.

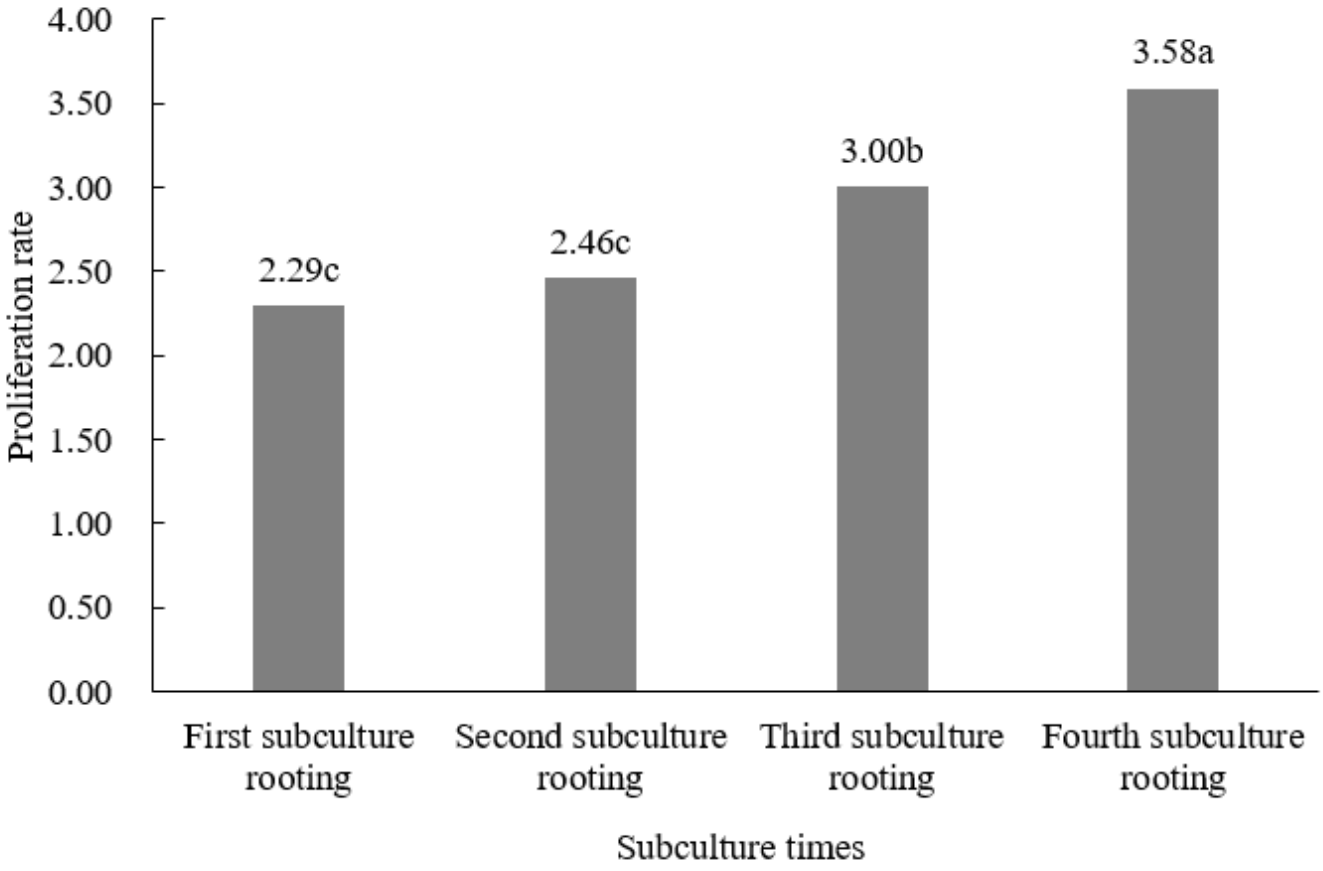

Figure 5

Proliferation rate of subculture rooting of Acacia mangium × A. auriculiformis. Different letters indicate significant differences at $P < 0.05$ using Duncan's multiple range test.