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Establishment of a Highly Efficient Micropropagation System of *Aquilaria crassna* Pierre ex Lecomte

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Abstract: *Aquilaria crassna* Pierre ex Lecomte is a principal species renowned for its production of agarwood. However, the active components of agarwood are not universally in compliance with the standards set by the Chinese Pharmacopoeia. We have identified an elite *A. crassna* tree with agarotetrol and alcohol extract levels that exceed these standards and have successfully established a stable *in vitro* micropropagation system using stem segments from this elite tree. The effects of auxins and minerals on axillary-bud induction, shoot multiplication, and rooting were investigated. The most effective medium for axillary-bud induction was a half-strength Murashige and Skoog (1/2MS) medium supplemented with 0.50 mg/L 6-benzylaminopurine (6-BA), achieving an induction rate of 53.33% with minimal hyperhydricity. The optimal shoot proliferation medium was an MS medium with 0.40 mg/L 6-BA, yielding a propagation coefficient of 2.96 without hyperhydricity. The best rooting medium comprised quarter-strength MS (1/4MS) macroelements and 1/2MS microelements with 0.10 mg/L naphthaleneacetic acid (NAA), resulting in an 82.54% rooting rate. Substrate effects on transplant survival and growth were also evaluated, and peat soil was identified as the best substrate, achieving a survival rate of 96.67%. This study introduces a straightforward and efficient *in vitro* micropropagation system utilizing mature *A. crassna* as explants. It holds significant importance for the consistent production of agarwood that complies with the standards of the Chinese Pharmacopoeia and provides a model for the targeted breeding of medicinal plants.

Keywords: *Aquilaria crassna* Pierre ex Lecomte; micropropagation; auxins; hyperhydricity; rooting

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1. Introduction

Aquilaria crassna Pierre ex Lecomte, a member of the Thymelaeaceae family, is naturally distributed in Southeast Asian nations including Cambodia, Laos, Thailand, and Vietnam, and holds significant medicinal and commercial importance [1,2]. The medicinal properties of *A. crassna* are derived from agarwood, a fragrant resinous heartwood that forms in response to injury, whether natural or induced. Agarwood possesses sedative and carminative properties, capable of alleviating gastric disorders, cough, rheumatism, and high fever [1,3,4]. It is a component of many traditional pharmacopoeias. This substance has been utilized in traditional Chinese medicine since the fifth century, with over 1500 formulations requiring agarwood [3]. Agarwood extracts commonly contain agarotetrol. Upon heating, agarotetrol generates a potent sedative effect and releases the distinctive aromatic compound benzylacetone, thereby indirectly reflecting the medicinal efficacy of agarwood. Consequently, the concentration of agarotetrol is regarded as one of the criteria for assessing the medicinal value of agarwood [4]. The 2020 edition of the Chinese Pharmacopoeia stipulates stringent criteria for the content of two key constituents in agarwood used in traditional Chinese medicine: the alcohol extract content must exceed 10%, and the agarotetrol content must be no less than 0.10% [5]. *A. crassna*, widely utilized in medicine, religious practices, high-end art, and premium spice industries for centuries,

has suffered extensive exploitation in natural forests. The IUCN Red List of Threatened Species classified *A. crassna* as critically endangered in 2006 [6].

Cultivation can alleviate the exploitation pressure on natural populations and supply the market with a substantial quantity of high-quality agarwood. As early as 1978, *A. crassna* was introduced to Guangdong Province, China. By 2011, approximately 40 hectares had been planted in southern China [7]. However, *A. crassna* plantation forests often face challenges, including difficulties in agarwood extraction, low content, or inferior quality. The value of agarwood is predicated on its quality, with the grade serving as a critical parameter for assessing both the agarwood and its products [1,3]. Varied grading models across nations reflect the diverse standards for agarwood quality [1]. In the context of medicinal use, the content of active compounds and resin are paramount, with agarotetrol being a key component in the quality assessment of agarwood as a medicinal material [3,4]. Different agarwood plants can exhibit varying agarotetrol levels [4]. Extraction techniques for agarwood encompass hydro-distillation, solvent extraction, solid-phase microextraction, and others, each presenting unique strengths and weaknesses, and potentially yielding different agarotetrol contents [1,4]. To address these issues, we have identified an elite *A. crassna* tree with an alcohol-soluble matter content of 24% and an agarotetrol content of 2.15%, which not only meets but also significantly exceeds the standards set by the Chinese Pharmacopoeia for agarwood. The task now is to propagate a large number of seedlings with the same genotype as this elite tree. Given the heterozygous nature of *A. crassna*, seed propagation would result in a significant proportion of offspring with genotypes differing from the mother plant [8]. Plant tissue culture, a form of asexual reproduction, offers the advantage of producing a large number of genetically identical plantlets while preserving the superior genetic traits of the mother plant [9]. Micropropagation based on tissue-culture technology is a pivotal method for the industrialization of agriculture and forestry, producing a significant quantity of medicinal and ornamental plants to meet market demands. This approach also aids in the conservation of species that are rare in the wild due to over-exploitation [10].

A. crassna trees produce stable agarwood exclusively upon reaching maturity. The ability to form agarwood varies among *A. crassna* trees of different genotypes [8]. Therefore, establishing a rapid *in vitro* propagation system using stem segments of mature *A. crassna* trees has significant medicinal value and consequently also holds substantial commercial value. Although previous researchers have conducted tissue-culture studies using branches of mature *A. crassna* trees as explants, these studies have shown an extremely low rooting rate, which hinders the acquisition of a substantial number of tissue-cultured plantlets and fails to satisfy the demands of commercial production [8]. In this study, we utilized stem segments from an elite *A. crassna* tree as explants to assess the influence of various plant growth regulators and mineral nutrients on the axillary-bud induction, shoot multiplication, and rooting of *A. crassna* micropropagation. Additionally, we examined the effects of different substrates on the transplantation of *in vitro* propagated plantlets. The objective was to determine the optimal treatments for both the tissue-culture and transplanting processes, thereby establishing an efficient micropropagation system. This system demonstrates superior performance in multiplication rate, rooting rate, and transplant survival rate, effectively fulfilling the criteria for the efficient and rapid propagation of high-quality *A. crassna* plantlets. Furthermore, the establishment of an efficient and rapid micropropagation system is crucial for conserving *A. crassna*, an endangered species in natural forests.

2. Materials and Methods

2.1. Plant Materials

The elite *A. crassna* mother plant used in this study grows in the nursery of the Institute of Tropical Forestry, Chinese Academy of Forestry. For this study, we selected newly sprouted branches, no more than two months old, as explants during the spring season. On a sunny day, the branches were collected and defoliated. The twigs were sectioned into segments measuring 10–20 mm, each containing a single axillary bud or a

single shoot tip. Three hundred internodes and one hundred shoot tips were first rinsed three times with sterile distilled water, then immersed in a 0.10% (*w*/*v*) mercuric chloride solution for 7 min (internodes) or 5 min (shoot tips), and subsequently washed six times with sterile distilled water. The single-node explants were then cultured on a 1/2MS medium devoid of any plant growth regulators. One week later, all contaminated explants were removed, leaving only non-contaminated explants, including 140 internodes and 90 shoot tips, for the induction of bud sprouting.

2.2. Induction of Axillary Buds and Shoot Tips

A 1/2MS medium served as the basal medium. To investigate the impact of 6-BA on axillary-bud response, four concentrations were established: 0.00 mg/L (control), 0.20 mg/L, 0.50 mg/L, and 1.00 mg/L. Sterile explants were cultured in a 1/2MS medium supplemented with varying levels of 6-BA. Each culture vessel (200 mL) received a single explant, and the experiment was conducted in triplicate, with each replicate comprising 30 samples. After a 30-day cultivation period, the axillary-bud sprouting rates and hyperhydricity rates across the different 6-BA concentrations were assessed (bud sprouting rate = number of sprouting buds/number of explants for bud sprouting in each treatment; hyperhydricity ratio = number of hyperhydricity buds in sprouting buds/number of sprouting buds).

2.3. Shoot Multiplication of Subculture

All axillary buds that had sprouted were transferred to a fresh MS medium supplemented with 6-BA at a concentration of 0.20 mg/L, where they developed into shoot clusters. These newly regenerated shoots were then isolated from the clusters and subcultured to facilitate further multiplication.

After three months of continuous culture, with transfers occurring monthly, one hundred and fifty single regenerated shoots measuring 15–20 mm in length were transferred to an MS medium supplemented with varying concentrations of 6-BA at 0.00, 0.20, 0.40, 0.60, and 0.80 mg/L. There were four replicates for each concentration treatment, with 30 shoots per replicate. Following 40 days of culture on the multiplication medium, the propagation coefficient, the number of shoots, their lengths, and the hyperhydricity rate were assessed (propagation coefficient = number of newly regenerated shoots/original number of shoots transferred to medium; hyperhydricity ratio = number of hyperhydricity shoots in newly regenerated shoots/number of newly regenerated shoots).

2.4. Rooting

To investigate the impact of macroelements, microelements, and NAA on the adventitious root formation of shoots, a series of 12 experimental groups were established (Table 1). Groups 1 and 2 constituted the first set of experiments, groups 3 to 7 the second, and groups 8 to 12 the third. Individual shoots, 20–30 mm in length, were placed in distinct rooting media, with each group having five replicates. The rooting rate was assessed after five weeks (rooting rate = number of rooting shoots/number of shoots inoculated into the medium).

Table 1. Concentrations of microelements, macroelements, and NAA in different rooting induction media.

Group	Microelements	Macroelements	NAA (mg/L)
1	1/2MS	1/2MS	0.05
2	1MS	1/2MS	0.05
3	1/2MS	1/5MS	0.05
4	1/2MS	1/4MS	0.05
5	1/2MS	1/2MS	0.05
6	1/2MS	3/4MS	0.05
7	1/2MS	1MS	0.05
8	1/2MS	1/4MS	0.00
9	1/2MS	1/4MS	0.01
10	1/2MS	1/4MS	0.05
11	1/2MS	1/4MS	0.10
12	1/2MS	1/4MS	0.20

2.5. Culture Conditions

All hormones were added to the culture medium individually according to the experimental design, and all media were solidified with 7 g/L of carrageenan, and their pH was adjusted to 5.8 prior to autoclaving at 121 °C for 16 min. During the bud sprouting and proliferation stages, the sucrose concentration in the culture medium was 30 g/L, and during the rooting stage, it was 20 g/L. Each culture vessel contained a 30 mL culture medium. The cultures were maintained at 25 ± 1 °C with a 12 h photoperiod under a light intensity of 2000–2500 lux, provided by cool-white fluorescent lamps.

2.6. Acclimatization and Transplantation

Plantlets were acclimated in a glasshouse maintained at 25–30 °C under natural light conditions with an 80% shading rate. After a two-week hardening period, the plantlets were taken out from culture vessels, their roots were rinsed to eliminate residual medium, and they were individually transplanted into three types of substrates (yellow subsoil, vermiculite, and peat soil), then covered with polyethylene film. Each substrate received 30 plantlets with three replicates. For the first week post-transplantation, plantlets were misted six times daily to maintain a 90% relative humidity within the nursery bed, and the bed was covered with an 80% shadecloth. During the second and third weeks, plantlets were misted four times daily to maintain a humidity level of 70% in the nursery bed, and the bed was covered with a 70% shadecloth. By the fourth week, the plantlets no longer required film coverage but continued to receive 50% shading, and they were watered twice daily. After 45 days, the survival rate of the plantlets was assessed for each replicate across the three substrates, and both plant height and leaf count were recorded (survival rate = number of survival plantlets/number of transplanting plantlets).

The characteristics of peat soil are as follows: the pH is 5.9, the particle diameter ranges from 0–6 mm, and the manufacturer is Kekkilä Oy, Ayritie D, 01510 Vantaa, Finland. The characteristics of vermiculite are as follows: the pH is 6.5, the particle diameter ranges from 2–3 mm, and the manufacturer is Hainan Furui Xing Agricultural Trade Co., Ltd., Room 401, Building 2, Jinhai Community, Jinlong Road, Longhua District, Haikou City, Hainan Province, China. The characteristics of yellow subsoil are as follows: the pH is 5.4, the particle diameter ranges from 1–3 mm, and it is collected from a soil layer 1 m deep beside Huolu Mountain in Guangzhou City, China.

2.7. Statistical Analysis

All experiments were conducted using a completely randomized design. Hyperhydricity rates were analyzed using nonparametric tests with SPSS 26.0 software. The impact of microelements on adventitious root induction was assessed via independent-samples *t*-tests, also using SPSS 26.0. The remaining data were subjected to one-way ANOVA

with SPSS 26.0, followed by post-hoc Duncan's multiple range test. The threshold for statistical significance was set at $p < 0.05$. Statistical charts were generated using Origin 2019b software.

3. Results

3.1. Axillary Buds and Shoot Tip Buds Induction

Five days after the explants were transferred to the initial bud induction medium, all shoot tips exhibited severe browning and died. Only 120 internodes did not brown and could continue to be observed. After 10 days of inoculation on the induction medium, the axillary buds initiated sprouting (Figure 1A). Approximately 30 days post-inoculation, the base of these buds expanded to about 50 mm in diameter and began to produce leaves (Figure 1B). By 60 days, the base had further enlarged to approximately 0.8 cm in diameter, with new buds emerging from the swollen area. The length of the 60-day-old axillary buds approached 10 mm (Figure 1C). By day 90, the earliest emerging bud had reached approximately 20 mm in length, accompanied by the development of four new buds in the swollen regions, leading to the formation of shoot clusters. (Figure 1D). The 6-BA concentration significantly influenced axillary-bud sprouting. Shoot development was challenging to induce on a 6-BA-free MS medium, where the sprouting rate was a mere 3.33%. As the 6-BA concentration increased, so did the sprouting rate (Figure 2A), although this was accompanied by hyperhydricity in some buds. Higher 6-BA concentrations correlated with higher hyperhydricity rates among sprouting buds (Figure 2B). At a 6-BA concentration of 1 mg/L, the sprouting rate peaked at 61.11%, with a hyperhydricity rate of 21.62%. Conversely, at 0.50 mg/L, the sprouting rate was slightly lower at 53.33%, but the hyperhydricity rate was significantly reduced to 4.31%. Thus, the optimal 6-BA concentration for inducing axillary-bud sprouting in stem segments is 0.50 mg/L.

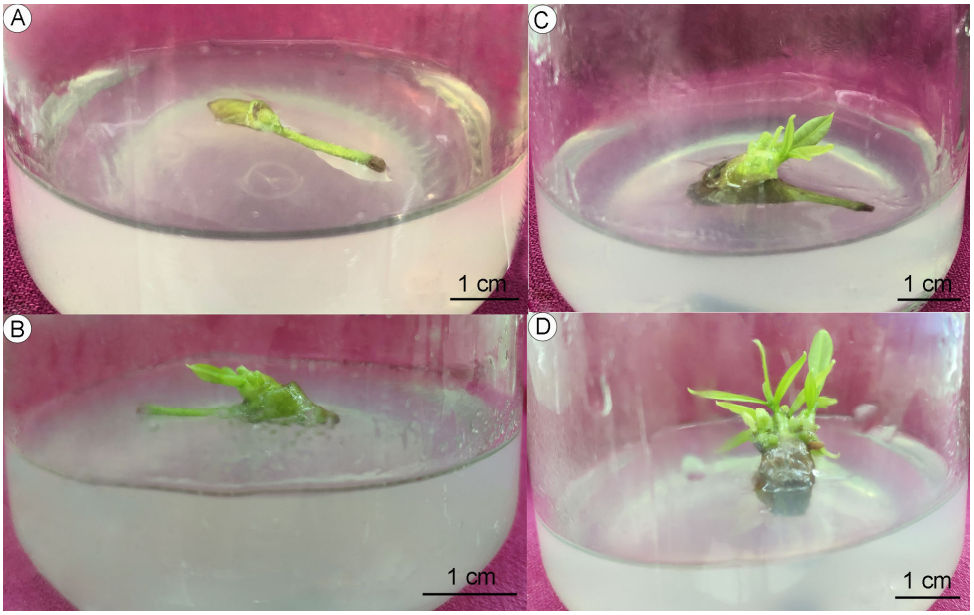

Figure 1. Growth status of *A. crassna* explants. (**A**) Stem segment with axillary buds after 10 days. (**B**) Stem segment with axillary buds producing leaves within 30 days. (**C**) Stem segment with axillary buds growing to 1 cm after 60 days. (**D**) Stem segment with axillary buds developing to shoot clusters after 90 days.

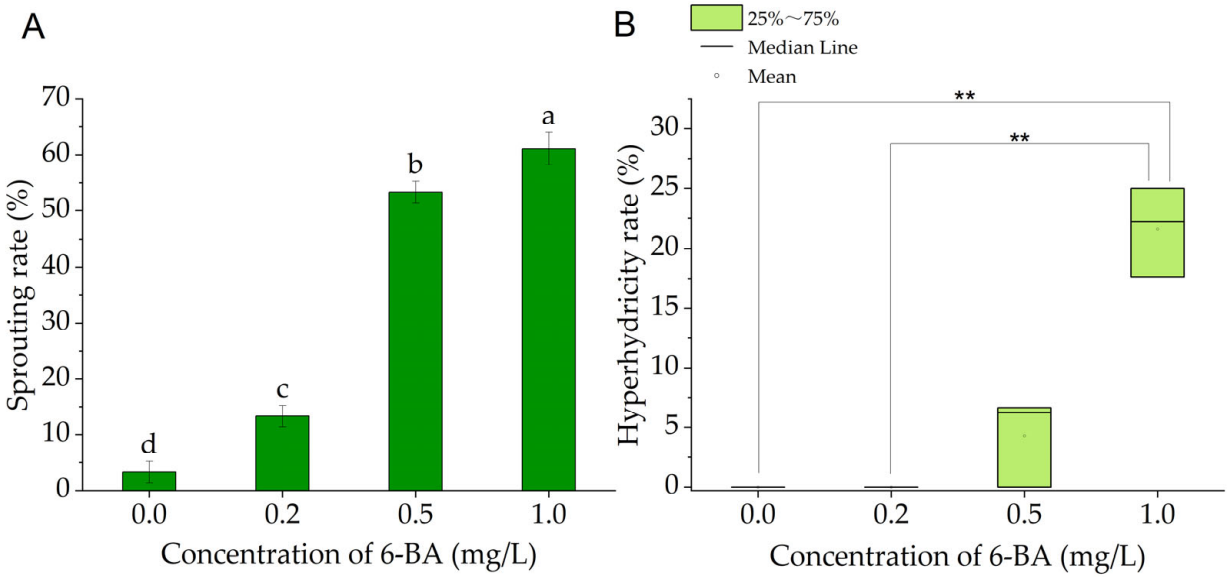

Figure 2. Sprouting of *A. crassna* axillary buds in media supplemented with varying concentrations of 6-BA. (**A**) Sprouting rate of axillary bud under different 6-BA concentrations. (**B**) Hyperhydricity rate of sprouting bud under different 6-BA concentrations. Different letters above the bars indicate statistically significant differences between concentrations at the $p < 0.05$ level according to Duncan's multiple range test. The symbol ** denotes that the character index is significantly different at the 0.01 level.

3.2. Subculture of Axillary Buds

One week after inoculation, the base of the shoots in the subculture medium began to expand. The base diameter of the shoots increased with both the duration of culture and the concentration of 6-BA (Figure 3). On the 6-BA-free MS medium, shoot growth was slow, with a proliferation coefficient of only 0.08 (Figure 4A). The addition of 0.20 mg/L 6-BA to the medium significantly increased the propagation coefficient to 2.19 (Figure 4A), and also accelerated shoot length (Figures 3A and 4B). Increasing the 6-BA concentration to 0.40 mg/L further enhanced the propagation coefficient to 2.96 (Figure 4A), but reduced shoot length (Figures 3C and 4B). A further increase to 0.60 mg/L 6-BA continued to raise the number of proliferating buds, but further decreased shoot length (Figures 3D and 4A,B). At a 6-BA concentration of 0.80 mg/L, the number of new shoots increased markedly (Figure 3E), with the propagation coefficient reaching 3.75 (Figure 4A), yet shoot length was significantly reduced (Figure 4B). Moreover, at 0.60 mg/L 6-BA, a certain proportion of hyperhydricity shoots began to appear (Figure 3F), with higher 6-BA concentrations correlating with a higher proportion of hyperhydric shoots (Figure 4C). There were extremely significant differences in shoot length, propagation coefficient, and hyperhydricity ratio across different 6-BA concentrations. Comparatively, the optimal subculture medium was found to be an MS medium supplemented with 0.40 mg/L 6-BA.

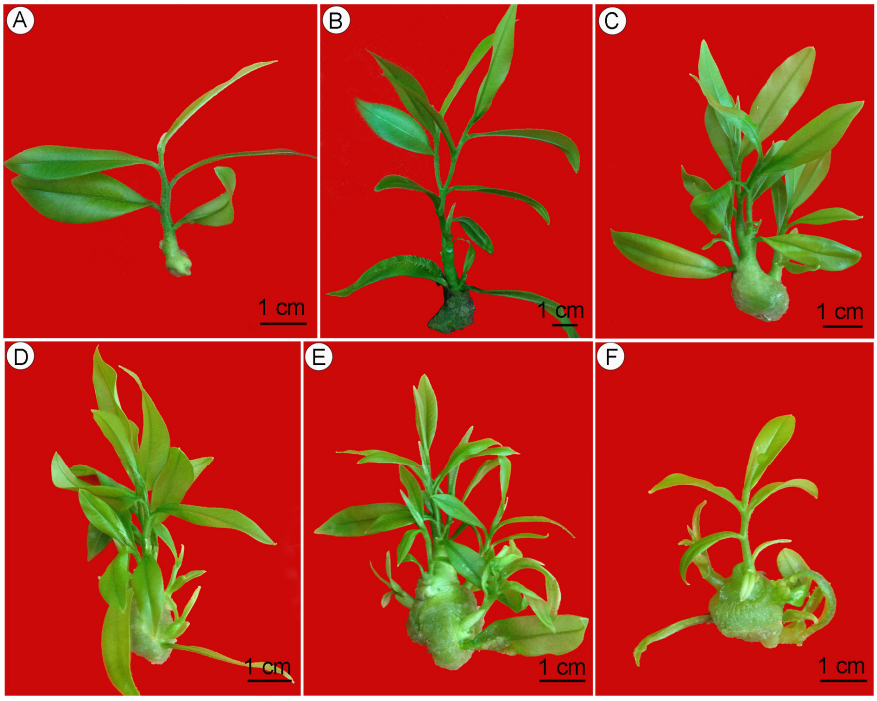

Figure 3. Growth status of regeneration shoots on MS medium supplemented with different concentrations of 6-BA in *A. crassna* (**A**) Shoots on 6-BA-free MS medium. (**B**) Shoots on MS medium supplemented with 0.20 mg/L 6-BA. (**C**) Shoots on MS medium supplemented with 0.40 mg/L 6-BA. (**D**) Shoots on MS medium supplemented with 0.60 mg/L 6-BA. (**E**) Shoots on MS medium supplemented with 0.80 mg/L 6-BA. (**F**) Hyperhydricity shoots.

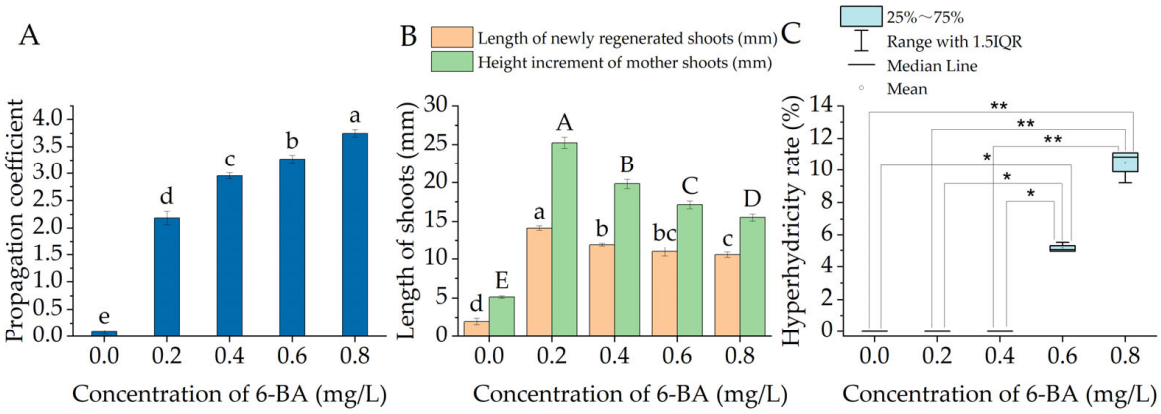

Figure 4. Growth of *A. crassna* regeneration shoots under different 6-BA concentrations. (**A**) Propagation coefficient of regeneration shoots under different 6-BA concentrations. (**B**) Length of shoots under different 6-BA concentrations. (**C**) Hyperhydricity rate of regeneration shoots under different 6-BA concentrations. Different letters above the bars indicate statistically significant differences between concentrations of the treatments at the $p < 0.05$ level according to Duncan's multiple range test. The symbols ** and * indicate that the character indexes are significantly different at the 0.01 and 0.05 levels, respectively.

3.3. Rooting Culture

Rooting is a pivotal phase in micropropagation protocols. Our experiments revealed that the concentrations of medium salts, encompassing macro- and microelements, significantly influenced root induction. Specifically, microelements at half the strength of the MS medium were more effective in promoting rhizogenesis than the full-strength MS (Figure 5A).

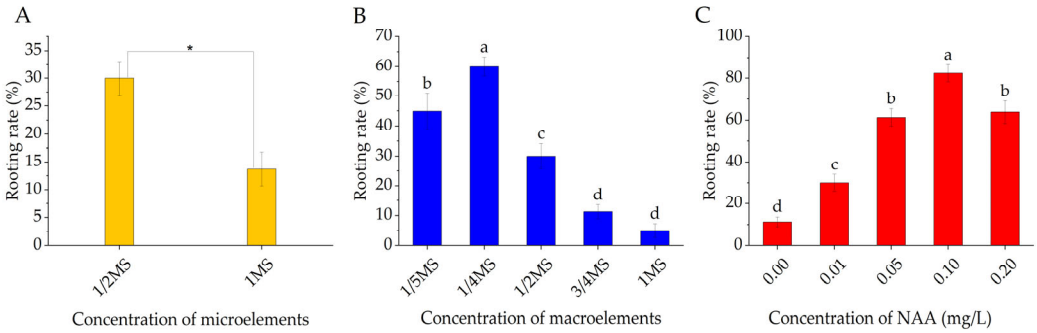

Figure 5. Root induction rate under different medium salt and NAA concentrations. (**A**) Rooting rate under different microelement concentrations. (**B**) Rooting rate under different macroelement concentrations. (**C**) Rooting rate under different NAA concentrations. Different letters above the bars indicate statistically significant differences between concentrations of the treatments at the $p < 0.05$ level according to Duncan's multiple range test. The symbol * denotes that the character indexes are significantly different at the 0.05 level.

The rooting rate increased with decreasing concentrations of macroelements. However, a reduction to one-fifth of the MS macroelements' concentration in the medium resulted in a decline in the rooting rate (Figure 5B). The optimal rooting media were identified with macroelements at a quarter strength of MS, outperforming both half- and full-strength MS (Figure 5B).

The concentration of NAA also played a crucial role in root formation. The absence of NAA resulted in a very low rooting rate, which significantly improved with the addition of 0.01 mg/L NAA. Further increments in NAA concentration led to a continuous increase in rooting rate, reaching a maximum at 0.10 mg/L before declining at 0.20 mg/L (Figure 5C). Consequently, the optimal combination for rooting medium was determined to be 1/4MS macroelements, 1/2MS microelements, and 0.10 mg/L NAA. Under these conditions, after 40 days of culture, over 80% of the shoots developed a substantial number of adventitious roots (Figure 6A).

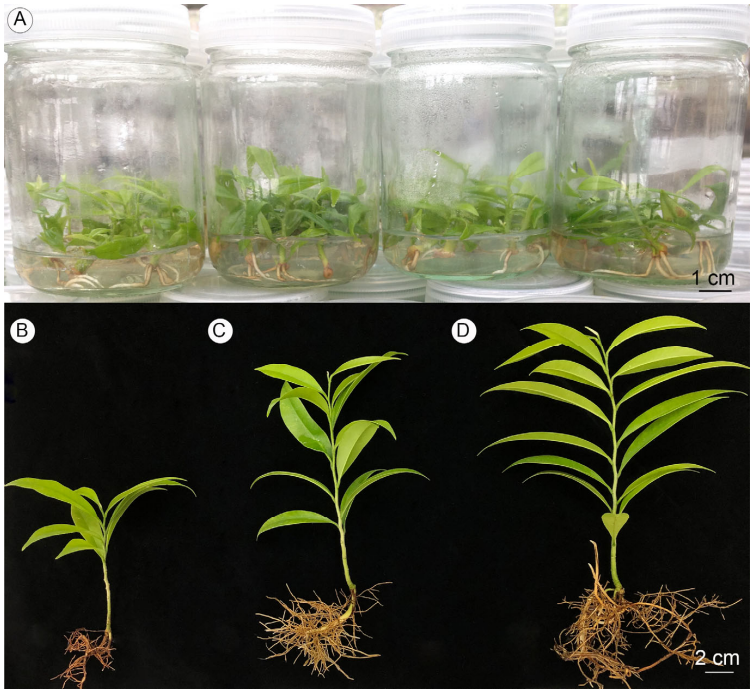

Figure 6. Phenotype of *A. crassna* plantlets. (**A**) Rooted plantlets in culture vessels. (**B**) Plantlet in yellow subsoil. (**C**) Plantlet in vermiculite. (**D**) Plantlet in peat soil.

3.4. Hardening and Acclimatization

After a two-week hardening period in the greenhouse, tissue-culture plantlets of *A. crassna* were transplanted into various substrates to evaluate their impact on plantlet survival and growth. The findings indicated that the substrate type significantly influenced both survival and growth (Table 2). Plantlets in yellow subsoil exhibited the lowest survival rate at 56.67%, and those in yellow subsoil had the least developed root systems and the fewest leaves (Figure 6B). Although there was no significant difference in survival rate between vermiculite and peat soil, there were notable differences in leaf count and plantlet height (Figure 6C,D, Table 2). Plantlets in peat soil not only had the highest survival rate but also the most developed root systems, and they had the greatest number of leaves and the tallest height, demonstrating significant advantages (Figure 6D). Consequently, peat soil was identified as the optimal substrate for transplanting tissue-culture plantlets of *A. crassna*.

Table 2. Effects of different substrates on transplanting of *A. crassna*.

Treatment Group	Transplanting Substrate	Survival Rate (%)	Height of Plantlets (cm)	Number of Leaves
1	yellow subsoil	56.67 ± 5.77 b	8.19 ± 0.27 c	8.00 ± 0.18 c
2	vermiculite	93.33 ± 1.93 a	15.98 ± 0.40 b	12.13 ± 0.16 b
3	peat soil	96.67 ± 1.93 a	18.33 ± 0.66 a	15.31 ± 0.41 a

Different letters within the same column indicate significant differences at the $p < 0.05$, while the same letters indicate no significant differences.

4. Discussion

We selected the young stem segments of *A. crassna* as explants due to the presence of dormant axillary meristems in their axils [11]. These meristems are typically inactive on the mother plant as a result of apical dominance [12]. After excision from the mother plant, these dormant axillary meristems can remain viable for a short period. Owing to the protective effect of the stem epidermis and petioles, they possess a certain level of resistance to disinfectants, preventing them from being killed by the disinfectants. Post-disinfection, on an appropriate medium, these axillary meristems can overcome dormancy, initiate growth in response to cytokinins, and ultimately emerge through the epidermis to form axillary buds [11,13]. This method of inducing axillary buds from plant stem segments for *in vitro* asexual reproduction is advantageous for maintaining the genetic stability of clones [14,15]. The use of stem segments as explants has been validated as an effective approach for micropropagation in numerous plant species [14–17].

Based on our experiment, axillary buds from stem segments of *A. crassna in vitro* exhibited difficulty in sprouting without the addition of exogenous cytokinins to the medium (Figure 2A). However, the inclusion of 6-benzylaminopurine (6-BA) significantly promoted sprouting, which was similar to *Dendrocalamus strictus* (Roxb.) [15] and *Homalomena aromatica* Schott. [18]. In the case of gerbera, the sprouting rate peaked at a 6-BA concentration of 2.5 mg/L, declining thereafter with increasing concentrations [19]. In contrast, for *A. crassna*, the sprouting rate of axillary buds consistently increased with 6-BA concentrations up to 1 mg/L. At this concentration, however, hyperhydricity in the sprouting buds was notably high (21.62%). Conversely, gerbera shoots exhibited no hyperhydricity at a 6-BA concentration of 5 mg/L [19]. These findings indicate that plant explants respond variably to media supplemented with different concentrations of 6-BA.

6-BA is the predominant cytokinin utilized in the regeneration medium for micropropagation [20]. It has been demonstrated as the most efficacious cytokinin for certain plants [15–18,21]. Our research corroborates that exogenous 6-BA substantially enhances shoot multiplication during the subculture phase of *A. crassna*. At relatively low concentrations (0.2 mg/L), 6-BA not only stimulates shoot multiplication but also significantly promotes shoot growth. An increase in 6-BA concentration results in a notable rise in shoot number, although shoot length progressively diminishes, a pattern consistent with observations in *Calamintha cretica* [22] and *Clinopodium nepeta* [23].

Hyperhydricity is a prevalent issue in plant tissue culture [24]. In the axillary-bud sprouting and subculturing stages of *A. crassna*, hyperhydricity is an inescapable challenge, with higher 6-BA concentrations correlating with increased hyperhydricity rates. The literature consistently indicates a significant correlation between 6-BA concentration in the medium and shoot hyperhydricity during micropropagation [19,22,23,25–32]. However, the threshold 6-BA concentration that induces hyperhydricity varies among plant species. For instance, it is 7.5 mg/L for gerbera [19], 2 mg/L for *C. cretica* [22] and *C. nepeta* [23], 1 mg/L for *Populus* hybrid and *Malus domestica* [32], and 0.6 mg/L for *A. crassna*. This variation highlights the varying sensitivity of different plants to 6-BA. Additional research suggests that hyperhydricity induced by 6-BA is associated with the type of explants, with stem-bud explants being more prone to hyperhydricity than those from stem-apex explants [30,31]. Our attempts to use stem tips of *A. crassna* as explants resulted in 100% browning post-disinfection. Consequently, we can only conduct tissue-culture research using stem segments of *A. crassna* as explants. Hyperhydricity in micropropagation is believed to be an environmental stress response, partly due to high hormone concentrations in the medium that can lead to abnormal development [24,25]. Previous research has shown that exogenous 6-BA can increase endogenous cytokinin levels [24,32,33], thereby enhancing ethylene biosynthesis [24,28,34]. In *Vaccinium* spp. [35] and *Arabidopsis thaliana* [36], ethylene has been implicated in hyperhydricity. However, overcoming hyperhydricity induced by 6-BA is challenging, as this plant growth regulator is often essential for stem proliferation [27]. For *A. crassna*, avoiding hyperhydricity requires the use of lower 6-BA concentrations, albeit at the expense of reduced germination rates and proliferation multiples.

A high rooting frequency is a critical aspect of an effective micropropagation protocol. Many woody plants typically showed low rooting rates during *in vitro* micropropagation [37,38]. A strategy to enhance rooting *in vitro* is to reduce the salt concentration in the medium [37–39]. For instance, *A. crassna* showed an extremely low rooting rate (5%) on MS medium *in vitro*, and optimal rooting rates were achieved only when the macroelement concentration was reduced to one-fourth of the standard MS concentration, a finding consistent with studies on *Laburnum anagyroides* [40] and *Rosa Kazanlika* Top [41]. The MS medium's inorganic salts, comprising macroelements such as NH_4NO_3, $Ca(NO_3)_2$, and $MgSO_4$, as well as microelements, primarily influence rooting due to their high concentrations [37,39]. There is limited information on the impact of MS microelements on micropropagation rooting. Our study demonstrated that decreasing the concentration of microelements in the MS medium significantly increases the rooting percentage of *A. crassna in vitro*. Studies have indicated that an NH_4NO_3-free medium can improve *in vitro* rooting [39]. However, for *A. crassna*, very low concentrations of macroelements have been found to decrease the *in vitro* rooting rate, a phenomenon also observed in *Mentha spicata* [42], Gisela 5 cherry rootstock [43], and *Lycium chinense* [44]. This suggests that a certain salt concentration is essential for *in vitro* rooting. Indeed, $Ca(NO_3)_2$ and $MgSO_4$ have been shown to be beneficial for root induction [39]. For most plants, a medium with half the standard MS concentration was the most effective for rooting induction [37–39,42–47]. However, some species exhibited better *in vitro* rooting with a full-strength MS medium [48]. In the case of *Quercus suber*, no significant difference in rooting was observed between low and standard salt concentration media [49]. Thus, the optimal salt concentration for *in vitro* rooting is dependent on the plant species.

Auxins are essential for *in vitro* root induction in many plants [37,39]. Rooting is challenging without exogenous auxin induction, or the rate is significantly low [38,48–50]. IBA and NAA are commonly recognized as the most effective auxins for promoting root formation. For certain plants, IBA is the most efficient auxin for root induction [43–46,48,51], while others require NAA [38,40,41,50,52]. In the case of *A. crassna*, NAA was particularly effective for *in vitro* root initiation. However, auxin concentrations in the rooting medium significantly affect the rooting rate in many plants [20]. High auxin concentrations have been reported to inhibit rooting [37,45,46,49,50]. Similarly, relatively high concentrations of NAA reduced the rooting rate in *A. crassna*. Nevertheless, for the *in vitro* rooting of

Kibandameno and *Taita* cultivars, there is no significant difference in rooting percentage between MS media with and without NAA supplementation [53]. Thus, the impact of auxins on *in vitro* rooting varies among different plant genotypes, and the optimal auxin type and concentration for rooting are genotype-dependent. In conclusion, the selection of auxin type and its concentration, tailored to the plant genotype, is pivotal for successful *in vitro* rooting in plant micropropagation.

Van Minh, T. also conducted tissue-culture research using branches from a mature *A. crassna* as explants, but the rooting rate was very low [8]. The NAA concentration used by Van Minh was relatively high (0.3 mg/L), and his study did not indicate any adjustment to the concentration of inorganic salts in the rooting medium, which may be the reason for the low rooting rate observed in tissue-culture shoots.

Transplanting rooted plantlets into a natural substrate and enabling their independent growth constitutes the final stage of successful micropropagation. The survival rate is a critical metric for assessing the success of this transplantation. The choice of transplanting substrate significantly impacts plantlet survival. Peat soil, vermiculite, and perlite are preferred substrates for this purpose [54]. Peat soil, in particular, is commonly utilized in plant micropropagation due to its superior chemical, physical, and biological properties, as well as its optimal aeration and drainage [23,47,54,55]. Vermiculite, with its high porosity and cation exchange capacity, also provides excellent aeration and drainage, making it a vital substrate in the seedling industry [56,57]. For the *A. crassna* plantlets, survival rates exceeded 90% when transplanted into both peat and vermiculite substrates. These substrates likely provided a favorable environment for the root growth of *A. crassna*. Although no significant difference in survival rates was observed between the two substrates, the growth of plantlets in peat was markedly superior to that in vermiculite, affecting both above-ground and below-ground parts. This superiority likely arises from the organic composition of peat, which has more favorable biological properties than vermiculite. Research indicates that peat soil hosts a more diverse and abundant microbial community [56]. Moreover, peat soil is rich in macro- and microelements, which are essential for plantlet growth [57]. While the germination rate of *Selenicereus undatus* was higher in vermiculite, the stem length was significantly greater in peat, suggesting that peat is more effective at enhancing plant biomass [58]. Conversely, it has been noted that peat soil fosters the development of above-ground soybean parts, whereas vermiculite supports root growth [59]. Yellow subsoil is a common type of soil in Guangdong Province, China. It is relatively easy to obtain and inexpensive, making it commonly used in the seedling industry in Guangdong Province. However, this substrate has strong water retention, a heavy texture, is prone to compaction, and has poor aeration [60]. Zhao et al. investigated the impact of different substrates on the rooting of cuttings from the *Manglietia conifera* [60]. Their study confirmed that among the three substrates—peat soil, vermiculite, and yellow subsoil—peat soil yielded the highest rooting rate, while yellow subsoil resulted in the lowest. These findings are similar to our research results.

Previous researchers used coconut fiber as a substrate for transplanting *A. crassna* tissue-culture plantlets, achieving high survival rates and demonstrating good adaptability of the plantlets in natural environments [8]. However, the application of coconut fiber may challenge "ecosystem quality" [55]. Despite being the optimal transplanting substrate for *A. crassna*, peat soil is a finite resource, and its excessive extraction can lead to environmental degradation [55]. Thus, exploring sustainable alternatives to peat for the transplantation of tissue-culture plants of *A. crassna* is imperative and will be the focus of our subsequent research.

5. Conclusions

In this study, we successfully developed an efficient *in vitro* regeneration system for an elite adult tree of *A. crassna*. The process encompasses disinfection of explants, induction of axillary buds, subculture for proliferation, *in vitro* rooting, and subsequent transplantation of plantlets. Young stem segments, less than two months old, were identified as the optimal

explants, preventing severe browning. Following collection, these explants were disinfected with 0.1% mercuric chloride ($HgCl_2$) for 7 min, rinsed six times with sterile water, and then cultured in a 1/2MS medium supplemented with 0.5 mg/L 6-BA to induce axillary-bud sprouting. The sprouted buds were subsequently subcultured monthly in an MS medium with 0.4 mg/L 6-BA to promote proliferation and expansion. The regenerated shoots were then transferred to a medium with 1/4MS macroelements and 1/2MS microelements, supplemented with 0.1 mg/L NAA to induce rooting, achieving an 82.54% rooting rate. Transplanted into peat soil, the plantlets exhibited a 96.67% survival rate. Considering the limited resources and slow regeneration rate of peat soil, we are exploring viable alternatives. Rice hulls, a byproduct of rice production in southern China, are under investigation due to their ready availability. Utilizing rice hulls poses no adverse effects on the ecological environment. Our research focuses on the use of fermented rice hulls blended with vermiculite or yellow subsoil. Rice hulls, enriched with organic matter and capable of enhancing soil aeration, show promise as substitutes for peat soil. Through using nodal stem segments from an elite tree as explants, a substantial number of genetically identical plantlets were produced. These plantlets are expected to contribute to the production of high-quality agarwood for Chinese medicine.

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