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CALLUS INDUCTION AND ESTABLISHMENT OF EFFICIENT REGENERATION SYSTEM FOR A NEWLY DEVELOPED LINE OF *Ananas comosus* (L.) MERRILL

Cheng-Li Liu[1], Fu-Rong Xu[1], Heng-Yu Huang[1,2]✉

[1] Yunnan Breeding and Cultivation Research and Development Center of Endangered and Daodi Chinese Medicinal Materials, Yunnan University of Chinese Medicine, Kunming, China

[2] Yunnan Fenghe-Shuli Technology Biological Co., Ltd.

ABSTRACT

In vitro culture method is developed for propagation of a new line of *Ananas comosus* (L.) Merrill selected from a spontaneous mutant of cultivar Yellow Mauritius. The stem with leaves base obtained from sucker buds was selected as explants. The effects of type and concentration of different plant growth regulators on callus induction, adventitious bud formation and plant regeneration were investigated by the single factor, complete combination and L_9 (3^4) orthogonal experiment. A large number of adventitious buds differentiated on Murashige and Skoog (MS) medium with 4.0 mg·L^{-1} 6-BA, 1.0 mg·L^{-1} NAA and 1.0 mg·L^{-1} KT, reaching differentiation coefficient over 12.8. Browning of callus after 2–3 subcultures was eliminated by the application of 1.0 g·L^{-1} of activated carbon in the optimal medium, which didn't significantly reduce differentiation coefficient. The main shoots in adventitious buds were higher in number and stronger suitable for rooting in culture. Hundred percent rooting *in vitro* was achieved on half-strength MS medium with 1.0 mg·L^{-1} NAA. The survival rate of the tissue culture-raised plants was 100%. The methods developed and defined can be used not only for improved the yield of *in vitro* plants, but also for shortening the whole culture cycle.

Keywords: activated carbon, adventitious bud, browning of callus, *in vitro*, rapid propagation

INTRODUCTION

Ananas comosus (L.) Merrill, a perennial herb of genus *Ananas* in Bromeliaceae, is commonly called pineapple. It is one of the four famous fruits in the tropics and subtropics [Editorial Board of Flora of China 1997]. Pineapple is not only delightful in colour, aroma and taste, but also rich in nutrition. Among them, vitamin C content is 5 times that of the apple. In addition, it is abundant in proteinase, which can help the human body digest protein [Ali et al. 2020]. Therefore, it has a broad planting prospect.

Pineapple seedlings are mainly bred by asexual reproduction, with occasional sexual reproduction. Sexual reproduction is performed with artificial assistance. Plant growth is usually slow and the first sexual cycle taking about 24 months in tropical conditions. In addition, due to the high heterozygosity of most parent varieties, most important traits are highly isolated in young plants and the selection cycle is longer, which makes them unsuitable for direct commercial cultivation [Reinhardt et al. 2018]. Traditionally, pineapple propagated asexually by various buds (buds derived from slips, hapas, suckers and crowns) obtained from the parent plant. On average, 2–3 propagules can be produced every year, and it takes 30 years to get

✉ hhyhhy96@163.com

10,000 m^2 of planting material from one plant, which cannot satisfy the growing demand for planting. Also, diseases which caused by bacteria and viruses carried by the mother strain continued to be spread in the propagules, thus posing serious problems for production [Sastry and Sastry 2013]. Moreover, due to vegetative propagation, most varieties of pineapple have strong self-compatibility, so it is very difficult to develop new varieties by traditional breeding methods. Pineapple is one of the few crops in which all cultivated varieties are derived from a spontaneous variant and natural evolution [Osei-Kofi et al. 1997].

Tissue culture technique is considered a better choice for breeding variant plants with excellent characters [Reinhardt et al. 2018]. Through tissue culture technology, not only the original excellent traits can be retained, but also the excellent single plant can be rapidly propagated into clones, and then a large number of promotions in the production, providing an experimental basis for molecular breeding and genetic engineering. Although there have been many reports on pineapple tissue culture [Soneji et al. 2002a, 2002b, Firoozabady and Gutterson 2003, Ibrahim et al. 2013], and many attempts have been made on various automated liquid culture systems for micropropagation [Escalona et al. 1999], the expanded application of tissue culture still needs to improve the regeneration coefficient and acclimation scheme [Reinhardt et al. 2018]. A high regeneration efficiency of pineapple, although expressed by bud aggregation in the existing reports [Soneji et al. 2002a, 2002b, Firoozabady and Gutterson 2003, Sripaoraya et al. 2003, Be and Debergh 2006, Zuraida et al. 2011, Ibrahim et al. 2013, Usman et al. 2013, Nelson et al. 2015, Scherer et al. 2015, Mendonça et al. 2017, Cacaï et al. 2023, Lakho et al. 2023, Torres Ruiz et al. 2023], the obtained adventitious buds were weak without regenerated plant morphology, which requires repeated rejuvenation culture [Soneji et al. 2002b, Firoozabady and Gutterson 2003, Reinhardt et al. 2018].

In this study, we removed the previous idea of inducing bud aggregation, and the plants of spontaneous mutant derived from *A. comosus* Yellow Mauritius were used as explants. The initial culture, callus induction, adventitious bud occurrence and proliferation, rooting *in vitro*, acclimation and field transplanting for pineapple were systematically studied. An efficient and stable artificial propagation technology system for a newly developed line of *A. comosus* was established. Although the proliferation coefficient was slightly lower than that reported by bud aggregation on the surface, the efficiency of propagation and plant formation was much higher than that reported by others. The results of this study can provide a theoretical basis and technical support for rapid propagation and industrial production of the plant *in vitro* of pineapple. Meanwhile, it can also provide experimental reference for other varieties of pineapple and artificial rapid propagation of variants with desirable characters.

MATERIALS AND METHODS

Plant materials and establishment of aseptic system

In May 2017, the variant of *Ananas comosus* (L.) Merrill plants were obtained from Longfei farm in Xishuangbanbanna, Yunnan, China (100°49'E, 22°01'N, 950–1,000 m a.s.l.). 30 sucker buds were selected as explants and returned to the laboratory for treatment within 48 h. These explants were washed with running water, and then the dirt on the surface and leaf axils was washed thoroughly with 10% washing powder solution (w/v). To facilitate further sterilization, the leaves of sucker bud were removed, leaving only the stem with 4–5 cm leaves base. Subsequently, they were transported into a sterile operating platform where they were treated with 75% ethanol solution (v/v) for 15 s, and then disinfected with 0.1% $HgCl_2$ (w/v) for 8, 10, 12, 14, 16, and 18 min. After that, each stem with leaves base was washed with sterile water for 6 times, each time no less than 3 min. The surface moisture was dried using a sterilized filter paper. Finally, the base of the leaf was removed by another 1 cm and then 3–4 cm stem with leaves base was used for initial culture.

Medium

Basic medium. Basic medium for all stages was Murashige and Skoog (MS) [Murashige and Skoog 1962] (1/2MS medium for rooting culture) with 3% sucrose (w/v) and 0.55% agar (w/v). Different concentrations of 6-benzylaminopurine (6-BA), zeatin (ZT), kinetin (KT), α-naphthaleneacetic acid (NAA), 2, 4-dichlorophenoxyacetic acid (2, 4-D) and α-naphthaleneacetic acid (NAA) were added according to

the actual requirement. The reagents and plant growth regulators (PGRs) used in the experiment were purchased from Dingguo Biotechnology (Beijing, China). The pH of the medium was adjusted to 5.6–5.8 with 1 N HCl before autoclaving (121°C, 22 min).

Initial medium. Due to the limited number of explants, it was not possible to use a variety of initial medium for experiments. Therefore, MS medium with 1.5 mg·L^{-1} ZT was used as the initial medium for the stem with leaves base culture. After the successful establishment of an aseptic system, the number of sterile shoots was insufficient for single factor experiment, and the explant accumulation culture was continued in MS medium with 1.5 mg·L^{-1} ZT. Single factor experiment was carried out after 3–5 subcultures in the initial medium.

Single factor medium. Different concentrations of 6-BA (1.0, 2.0, 3.0, and 4.0 mg·L^{-1}), KT (0.5, 1.0, and 2.0 mg·L^{-1}), 2, 4-D (0.01, 0.05, and 0.1 mg·L^{-1}) and NAA (0.5, 1.0, 1.5, and 2.0 mg·L^{-1}) were added to the basal medium, respectively. After 40 days, the growth status of each treatment was observed and analysed to screen out the suitable PGRs types and concentrations.

Medium for callus induction. A complete combination experiment was conducted with 6-BA (2.0, 3.0 and 4.0 mg·L^{-1}) and NAA (0.5, 1.0 and 1.5 mg·L^{-1}) as factors. The callus induction rate and regeneration coefficient were calculated after 40 days.

Simultaneous medium for callus induction, adventitious bud occurrence and proliferation. According to the results of a single factor and complete combination experiment, different concentrations of 6-BA (2.0, 3.0 and 4.0 mg·L^{-1}), NAA (0.5, 1.0 and 1.5 mg·L^{-1}), and KT (0.5, 1.0 and 1.5 mg·L^{-1}) were added to the MS medium for the design of the L_9 (3^4) orthogonal test. The orthogonal design was shown in Table 1. After 40 days, the adventitious bud occurrence coefficient (statistical standards: the height of adventitious bud not less than 0.5 cm) and regeneration coefficient were calculated.

Improved medium of browning. After 2–3 subcultures in the synchronous medium for callus proliferation, adventitious bud occurrence and proliferation, serious browning appeared in the callus. Therefore, 1 g·L^{-1} activated carbon (AC) was added to the optimal synchronous medium. After 40 days, adventitious buds occurrence coefficient and regeneration coefficient were calculated.

Medium for rooting. As the basic medium for rooting ½ MS with or without 1 g·L^{-1} AC was used, in which different concentrations of NAA (0.0, 0.5, 1.0, and 1.5 mg·L^{-1}) were added, respectively. After 45 days, the rooting rate and the average number of adventitious roots per plant were counted.

Inoculation method and culture conditions. To establish an aseptic system, 250 mL cans of one explant per bottle were used. Subsequently, 500 mL culture flasks were used for each subsequent stage. Among them, for single factor experiment, each treatment was inoculated with 5 vials and each vial contained 10 explants. Each treatment for complete combination and orthogonal experiment was inoculated with 10 bottles, each bottle of 12 explants. For rooting culture, each treatment was inoculated with 20 vials of 7 explants per vial. At the proliferation stage, callus was cut from the surrounding of the explant, divided into 0.7–1.0 × 0.7–1.0 cm (with 2–3 bud points) in size, and inserted into the medium. In the rooting stage, the main buds with a height of 2–3 cm and a strong basal stem were selected from cluster buds, and the callus were cleared and then inserted into the rooting medium. In addition to the establishment of a sterile system, the

Table 1. L_9 (3^4) orthogonal design for callus induction, adventitious buds occurrence and proliferation

Level	Factors (mg·L^{-1})		
	A (6-BA)	B (NAA)	C (KT)
1	2.0	0.5	0.5
2	3.0	1.0	1.0
3	4.0	1.5	1.5

Note: 6-BA – 6-benzylaminopurine; NAA – α-naphthaleneacetic acid; KT – kinetin

above treatment was repeated 3 times, if the explant was dead or contaminated, timely supplement.

The incubation temperature was controlled at 25 ±2 °C. The illumination intensity was maintained at 2,000–2,500 lx, and the illumination time was 12 $h \cdot d^{-1}$.

Acclimation and transplanting

Rooting in 1/2MS medium with 1.0 $mg \cdot L^{-1}$ NAA for 40 days, the height of *in vitro* plant was about 8–10 cm, with a well-developed root system; the culture bottle was moved from the culture room to the greenhouse and placed for 2–3 days. Next, the cap was removed and placed under natural light for 2–3 days, with a small amount of tap water added for another 1–2 days (prevented growth of bacteria in the medium). Afterwards, *in vitro* plants were gently removed with tweezers, followed by clean water to remove the culture medium attached to the roots, and soaked in 0.1% carbendazim solution (w/v) for 10–30 min. Finally, they were transplanted into plastic pots (10 cm in diameter) containing mixed soil in a ratio of peat soil : coconut bran : perlite : yellow mud (4 : 1 : 1 : 2). After 60 days, the survival rate was counted (temperature 20–25 °C and humidity about 70%).

For field transplanting, plantlets were planted in a health farm in Maguan County, Wenshan City, Yunnan Province, China (103°95'E, 22°89'N, 418–460 m a.s.l.). The long-term annual average temperature and total annual precipitation are 21.9 °C and 1,500 mm, respectively.

Statistical statistics

The collected data were processed and analyzed using SPSS 26.0 (IBM Corp., Armonk, NY) and Excel (MC Corp., Redmond, WA). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test to assess significant differences among treatments, with a significance threshold of $P < 0.05$. Adobe Photoshop (2021) and Origin (2021) software were conducted for image processing.). The calculation formula was as follows:

Contamination rate (%) = the number of contaminated explants/total number of explants × 100%

Survival rate of explant (%) = the number of surviving explants/total number of explants × 100%

Callus induction rate (%) = the number of explants with the effective callus/total number of initial inoculations × 100%

Adventitious bud occurrence coefficient = the number of effective adventitious buds/the number of calluses

Regeneration coefficient = the number of effective subculture/total number of initial inoculations

Rooting rate = the number of single shoots with adventitious roots/total number of single shoots inoculated × 100%

The survival rate = the number of surviving plants/total number of transplanting plants × 100%

RESULTS

Establishment of an aseptic system

A certain difference in the contamination and survival rate of explants treated with different disinfection time was revealed (Tab. 2). When sterilized for 8 min, the sterilization was insufficient, but the contamination rate was as high as 73.33% and only a few explants could survive. When disinfection time reached 18 min, the contamination rate was lowered to 6.67%, but the survival rate was only 26.67% due to a strong toxicity of $HgCl_2$ and the intolerance of explants. With the prolongation of sterilization time, the contamination rate of explants decreased, and the survival rate also decreased correspondingly. In each treatment, a certain number of explants were uncontaminated without the sign of growth, and then gradually died. No significant difference in explant survival between 12 min and 14 min disinfection ($P > 0.05$) but a significant difference in contamination rate was found ($P < 0.05$). Overall, optimal disinfection time was 14 min (33.3% contamination rate and 46.67% survival rate).

After 40 days of culture, signs of budding on the leaf base were found (Fig. 1A). After 60 days, green protuberance appeared (Fig. 1B). After 80 days, 3–4 buds appeared in each explant (Fig. 1C and D).

Initial culture and single factor experiments

Obtained individual buds from an aseptic system were cut off and transferred to the initial medium. Af-

Table 2. Effects of different sterilization time on contamination and survival of *A. comosus* explants

Sterilization time (min)	Number of inoculated	Contamination rate (%)	Survival rate (%)
8	15	73.33 ±4.221 a	6.67 ±5.637 d
10	15	60.00 ±5.250 ab	20.00 ±11.547 c
12	15	46.67 ±6.667 b	40.00 ±9.667 a
14	15	33.33 ±5.267 c	46.67 ±6.667 a
16	15	13.33 ±6.258 d	33.33 ±13.333 b
18	15	6.67 ±5.756 e	26.67 ±5.543 c

Note: Different letters in the same column indicate significant differences at $P < 0.05$. Data are mean ± SE

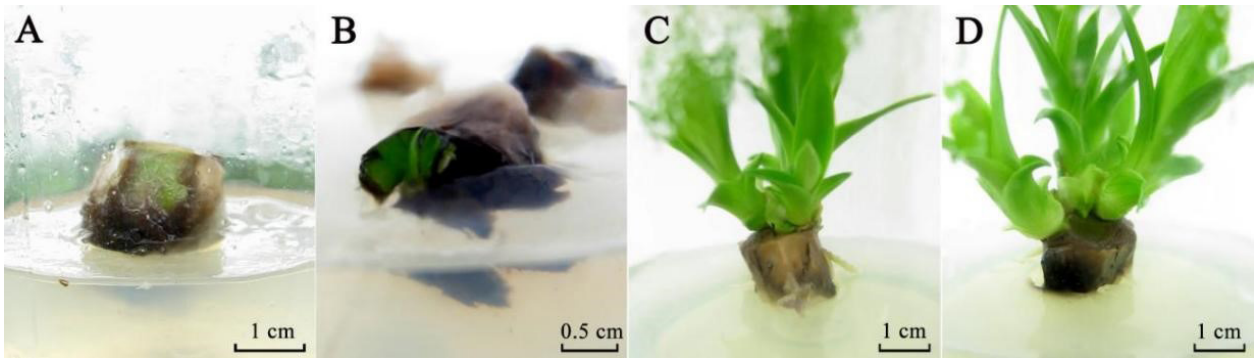

Fig. 1. Establishment of aseptic system

(A) The growth condition after 40 days of culture; (B) The growth condition after 60 days of culture; (C and D) The growth condition after 80 days of culture

ter 40 days, 2–3 lateral buds appeared at the base of the stem (Fig. 2A), or a small amount of callus was produced at the base, accompanied by the appearance of weak adventitious buds (Fig. 2B), with 2.25 regeneration coefficient (Tab. 3). Although ZT, added to initial culture, showed significant effect in promoting buds regeneration, due to its high cost, this study decided to replace it with some more affordable PGRs, such as 6-BA, KT, 2, 4-D, and NAA.

Growth of the bud of pineapple was observed in all treatments (Fig. 2 and Tab. 3). Among them, 6-BA treatment showed a significant effect on responses to callus induction as inducing a large amount of compact callus. However, almost no adventitious buds occurred, especially at the concentration of 2.0–4.0 mg·L^{-1}, with 68.5% callus induction rate (Fig. 2C). Of all KT treatments, a certain number of calluses and weak adventitious buds appeared at the base of main buds, and no significant difference among different levels was found (Fig. 2D). No callus was observed in 2, 4-D treatment with all levels. Compared with cytokinin treatment, buds in this treatment grew faster and clumped roots appeared at the base (Fig. 2E). NAA treatment significantly promoted the growth of buds, while a large number of adventitious roots occurred. *In vitro* shoots were well-grown with the strong roots, especially in 1.0–1.5 mg·L^{-1} NAA treatment (Fig. 2F).

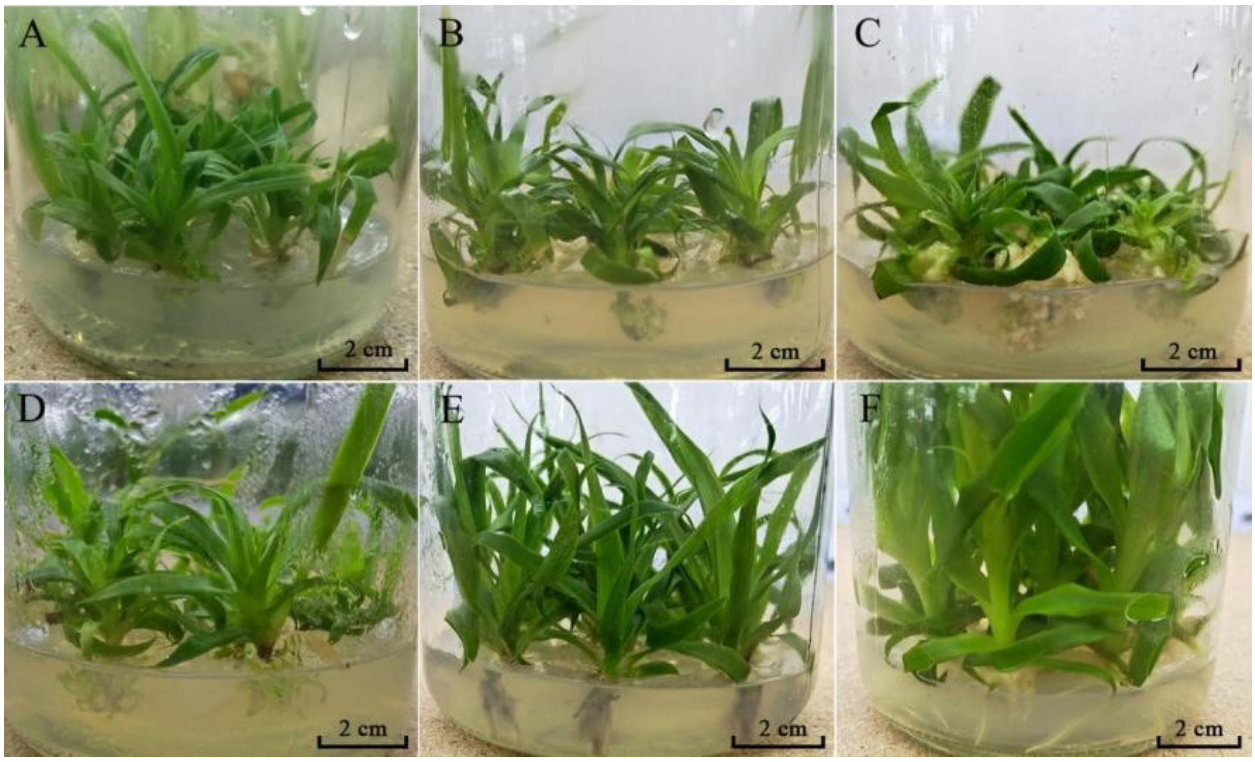

Fig. 2. Initial culture and single factor experiments

(A) After 40 days of initial culture, 2–3 new buds appeared in the base of a part of explants; (B) After 40 days of initial culture, a small amount of callus appeared in the base of a part of explants, accompanied by adventitious bud differentiation; (C) A large number of white calluses with compact in texture appeared in MS medium with 3.0 mg·L^{-1} 6-BA; (D) In MS medium with 1.0 mg·L^{-1} KT, a small amount of callus appeared in the base, with adventitious bud differentiation; (E) The growth potential in MS medium with 0.05 mg·L^{-1} 2, 4-D was perferable to that in cytokinin treatment, and the adventitious roots were clumped at the base; (F) In MS medium with 1.0 mg·L^{-1} NAA, the explant grew vigorously, adventitious roots developed, without the appearance of callus and lateral bud

Callus induction

As can be seen from Table 4, a significant difference between PGR treatment and blank control (CK) was revealed. Growth in CK treatment was not completely stopped; it is estimated to be that used explants were all from the single factor experiment, which had accumulated certain exogenous PGRs. In addition, the occurrence rate of callus was 100% in all treatments except CK, but a significant difference in the regeneration coefficient was observed. When the concentration of NAA remained constant, regeneration coefficient showed an upward trend within 2.0–3.0 mg·L^{-1} 6-BA. When the concentration of 6-BA was 3.0 mg·L^{-1}, this treatment had the highest regeneration coefficient, which was significantly different from other treatments ($P < 0.05$). The regeneration coefficient began to decrease when the ratio was above 3.0 mg·L^{-1}. When 6-BA concentration remained unchanged, the comparison of C_4 (3.0 mg·L^{-1} 6-BA+ 0.5 mg·L^{-1} NAA), C_5 (3.0 mg·L^{-1} 6-BA+ 1.0 mg·L^{-1} NAA) and C_6 (3.0 mg·L^{-1} 6-BA+ 1.5 mg·L^{-1} NAA) treatments showed that the regeneration coefficient of callus was related to the concentration of NAA, and a significant difference among treatments was revealed ($P < 0.05$). Thus, the MS medium containing 3.0 mg·L^{-1} 6-BA and 0.5 mg·L^{-1} NAA (C4) was optimal medium for callus induction.

In the C_4 treatment, after 10 days of culture, the base began to swell and white callus appeared (Fig. 3A). After 20 days, every base of verticillate leaf expanded to form white callus (Fig. 3B). After 30 days, callus grew and proliferated rapidly (Fig. 3C). After 40 days, most

Table 3. Effects of different types and concentrations of PGRs on buds, callus, and roots of *A. comosus* explants

PGRs (mg·L^{-1})		Regeneration coefficient	Callus induction rate (%)	Rooting rate (%)
Initial medium (MS with 1.5 mg·L^{-1} ZT)		2.25 ±0.09 a	13.33 ±3.33 e	0.00
6-BA	1.0	1.50 ±0.12 b	43.33 ±3.33 c	0.00
	2.0	1.03 ±0.03 cd	60.00 ±5.77 ab	0.00
	3.0	1.17 ±0.03 d	68.50 ±3.33 a	0.00
	4.0	1.03 ±0.03 d	53.33 ±3.33 b	0.00
KT	0.5	1.03 ±0.03 d	33.33 ±3.33 d	0.00
	1.0	1.10 ±0.06 d	36.67 ±3.33 cd	0.00
	2.0	1.07 ±0.07 d	30.00 ±3.33 d	0.00
2, 4-D	0.01	1.17 ±0.09 cd	0.00	33.33 ±3.33 d
	0.05	1.03 ±0.03 d	0.00	63.33 ±3.33 c
	0.1	1.30 ±0.01 c	0.00	40.00 ±5.78 d
NAA	0.5	1.03 ±0.03 d	0.00	70.00 ±5.78 bc
	1.0	1.00 ±0.00 d	0.00	100.00 ±0.00 a
	1.5	1.00 ±0.00 d	0.00	100.00 ±0.00 a
	2.0	1.00 ±0.00 d	0.00	76.67 ±3.33 b

Note: 6-BA – 6-benzylaminopurine; NAA – α-naphthaleneacetic acid; KT – kinetin. Different letters in the same column indicate significant differences at $P < 0.05$. Data are mean ± SE

Table 4. Effects of 6-BA and NAA combination on callus induction

Medium	PGRs (mg·L^{-1})		Regeneration coefficient
	6-BA	NAA	
CK	0	0	1.33 ±0.125 f
C_1	2.0	0.5	3.99 ±0.178 cd
C_2	2.0	1.0	3.51 ±0.245 cd
C_3	2.0	1.5	2.68 ±0.138 e
C_4	3.0	0.5	5.33 ±0.179 a
C_5	3.0	1.0	4.63 ±0.098 b
C_6	3.0	1.5	3.80 ±0.253 cd
C_7	4.0	0.5	4.18 ±0.283 bc
C_8	4.0	1.0	3.66 ±0.211 cd
C_9	4.0	1.5	2.86 ±0.169 e

Note: PGRs – plant growth regulators; 6-BA – 6-benzylaminopurine; NAA – α-naphthaleneacetic acid; CK – blank control. Different letters in the same column indicate significant differences at $P < 0.05$. Data are mean ± SE

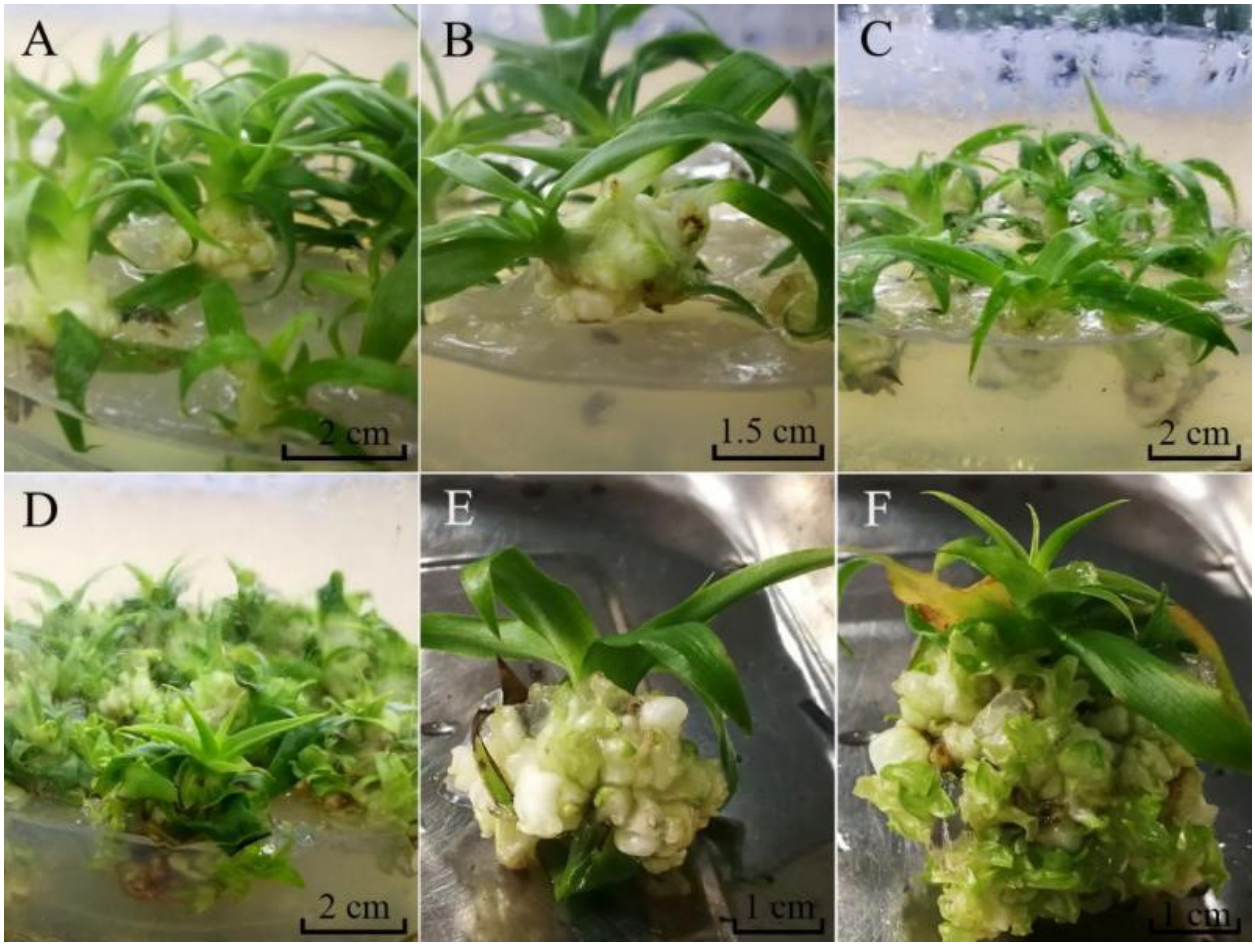

Fig. 3. Callus induction

(A) White enlargement callus appeared at the base of the bud after 10 days of culture; (B) After 20 days of culture, callus also appeared at the base of the leaf; (C) After 30 days of culture, the base of each bud expanded and new callus appeared, with a fast regeneration rate; (D) After 40 days of culture, the whole surface of the medium was basically covered by callus; (E and F) Layered callus with green protuberance

of the buds were covered by callus except for the top leaf (Fig. 3D). At this time, callus appeared at the axils of each leaf, presenting a unique stratified phenomenon (Fig. 3E, F).

Simultaneous culture for callus proliferation, adventitious bud differentiation and proliferation

In order to further explore an effective method of callus redifferentiation into adventitious cluster buds and improve proliferation efficiency, another PGR (KT) was introduced for orthogonal experiment according to the results of single factor and complete combination experiment, and the results were shown in Table 5.

The range (R) analysis in Table 5 shows that $R_{KT} > R_{6\text{-BA}} > R_{blank} > R_{NAA}$, indicating that the response of KT and 6-BA were effective in adventitious buds occurrence coefficient, among which KT had the best response, while NAA had no significant response. According to the results of variance analysis (Tab. 6), KT showed a significant response on adventitious bud occurrence coefficient ($P < 0.05$), while 6-BA and NAA had no significant response ($P > 0.05$). Duncan test (Tab. 7) for the three levels of KT shows that level 2 (1.0 mg·L^{-1}) had the greatest response on the adventitious bud occurrence coefficient, which was significantly different from level 1 (0.5 mg·L^{-1}) and level 3 (1.5 mg·L^{-1}). According to the levels of each factor

Table 5. Results of L_9 (3^4) orthogonal experiment on callus proliferation, adventitious bud differentiation and proliferation

Medium		PGRs (mg·L^{-1})				ABOC	RC
		A (6-BA)	B (NAA)	C (KT)	D (Error)		
1		2.0	0.5	0.5	(1)	6.11 ±0.015	5.98 ±0.103
2		2.0	1.0	1.0	(2)	10.02 ±0.105	7.97 ±0.053
3		2.0	1.5	1.5	(3)	8.12 ±0.372	6.96 ±0.061
4		3.0	0.5	1.0	(3)	11.76 ±0.040	8.72 ±0.046
5		3.0	1.0	1.5	(1)	9.86 ±0.050	8.02 ±0.031
6		3.0	1.5	0.5	(2)	7.64 ±0.101	7.21 ±0.100
7		4.0	0.5	1.5	(2)	8.99 ±0.208	8.93 ±0.131
8		4.0	1.0	0.5	(3)	9.63 ±0.036	8.68 ±0.174
9		4.0	1.5	1.0	(1)	12.53 ±0.026	10.44 ±0.079
ABOC	*K*	8.083	8.953	7.793	9.500	–	–
	K	9.753	9.837	11.437	8.883	–	–
	K	10.383	9.430	8.990	9.837	–	–
	R	2.300	0.884	3.644	0.954	–	–
RC	*K*	6.970	7.877	7.290	8.147	–	–
	K	7.983	8.223	9.043	8.037	–	–
	K	9.350	8.203	7.970	8.120	–	–
	R	2.380	0.346	1.753	0.110	–	–

Note: PGRs – plant growth regulators; 6-BA – 6-benzylaminopurine; NAA – α-naphthaleneacetic acid; KT – Kinetin; ABOC – adventitious bud occurrence coefficient; RC – regeneration coefficient; *K* – average; *R* – range

in the L_9 (3^4) orthogonal design table (Tab. 1) and the average value in L_9 (3^4) orthogonal experiment results (Tab. 5), the optimal combination of PGRs for adventitious bud occurrence was $A_3B_2C_2$ (level 3 of 6-BA, level 2 of NAA, and level 2 of KT), namely 4.0 mg·L^{-1} 6-BA + 1.0 mg·L^{-1} NAA + 1.0 mg·L^{-1} KT.

The descending order of regeneration coefficient was shown in Table 5: $R_{6\text{-BA}} > R_{KT} > R_{NAA} > R_{blank}$, and the *R* values of 3 PGRs were all higher than the blank column, indicating that three PGRs were effective in proliferation effect, of which 6-BA had the best effect, followed by KT and NAA. According to the results of variance analysis (Tab. 6), 6-BA had a significant effect on the regeneration coefficient ($P < 0.05$), while KT and NAA had no significant effect ($P > 0.05$). Duncan test (Tab. 7) was carried out on three levels of 6-BA. For the regeneration coefficient, level 3 (4.0 mg·L^{-1}) had the best influence, which was significantly different from that of level 1 (2.0 mg·L^{-1}) and level 2 (3.0 mg·L^{-1}). By means of average value analysis, the optimal combination of PGRs for regeneration coefficient was also $A_3B_2C_2$, namely 4.0 mg·L^{-1} 6-BA + 1.0 mg·L^{-1} NAA + 1.0 mg·L^{-1} KT.

The optimal combination was obtained by repeating the orthogonal experiment for 3 times. After 10 days of culture, adventitious buds began to appear on the surface of callus (Fig. 4A and B). After 20 days of culture, with the continuous differentiation of adventitious buds, the callus proliferated rapidly (Fig. 4C and D). After 30 days, early adventitious buds grew vigorously, and new adventitious buds were constantly differentiated (Fig. 4E and F). After 40 days,

Table 6. Variance analysis of occurrence coefficient of adventitious bud and regeneration coefficient

Factor	Sources	Type III sum of square	DF	Mean square	F value	Significance
ABOC	A (6-BA)	8.476	2	4.238	1.093	$P > 0.05$
	B (NAA)	1.173	2	0.586	0.115	$P > 0.05$
	C (KT)	20.692	2	10.346	5.617	$P < 0.05$
	D (Error)	1.402	2	0.701		
RC	A (6-BA)	8.559	2	4.280	5.202	$P < 0.05$
	B (NAA)	0.227	2	0.114	0.051	$P > 0.05$
	C (KT)	4.689	2	2.344	1.597	$P > 0.05$
	D (Error)	0.020	2	0.010		

Note: ABOC – adventitious bud occurrence coefficient; RC – regeneration coefficient; DF – degree of freedom; 6-BA – 6-benzylaminopurine; NAA – α-naphthaleneacetic acid; KT – kinetin

Table 7. Three levels of KT and 6-BA Duncan test

Factor	PGRs	Level	Mean
ABOC	KT	2 (1.0 mg·L^{-1})	11.437 a
		3 (1.5 mg·L^{-1})	8.990 ab
		1 (0.5 mg·L^{-1})	7.793 b
RC	6-BA	3 (4.0 mg·L^{-1})	9.350 a
		2 (3.0 mg·L^{-1})	7.983 ab
		1 (2.0 mg·L^{-1})	6.970 b

Note: ABOC – adventitious bud occurrence coefficient; RC – regeneration coefficient; 6-BA – 6-benzylaminopurine; KT – kinetin; PGRs – plant growth regulators. The different letters following the mean indicate a significant difference ($P < 0.05$) between the levels of KT or BA corresponding to the mean

the surface of callus was covered by adventitious buds (Fig. 4G and H). At this point, adventitious buds occurrence coefficient was 12.80, and regeneration coefficient of subculture was 10.38.

After 2–3 generations of culture in the synchronous medium for callus proliferation, adventitious bud occurrence and proliferation, callus showed serious browning (Fig. 5A). At this time, adding 1.0 mg·L^{-1} AC could completely eliminate callus browning. After 10 days, callus began to differentiate adventitious buds (Fig. 5B). After 20 days, adventitious buds grew rapidly, and callus also proliferated rapidly (Fig. 5C and D). After 30 days, callus was further proliferated, and adventitious buds on the callus grew vigorously (Fig. 5E and F). After 40 days, the whole medium was covered by adventitious buds, and 3–5 robust main buds appeared on each callus, which was conducive to the next step of rooting culture (Fig. 5G and H). At this time, the occurrence coefficient and the proliferation coefficient of adventitious bud didn't significantly reduce. Browning did not appear in the subsequent subculture proliferation.

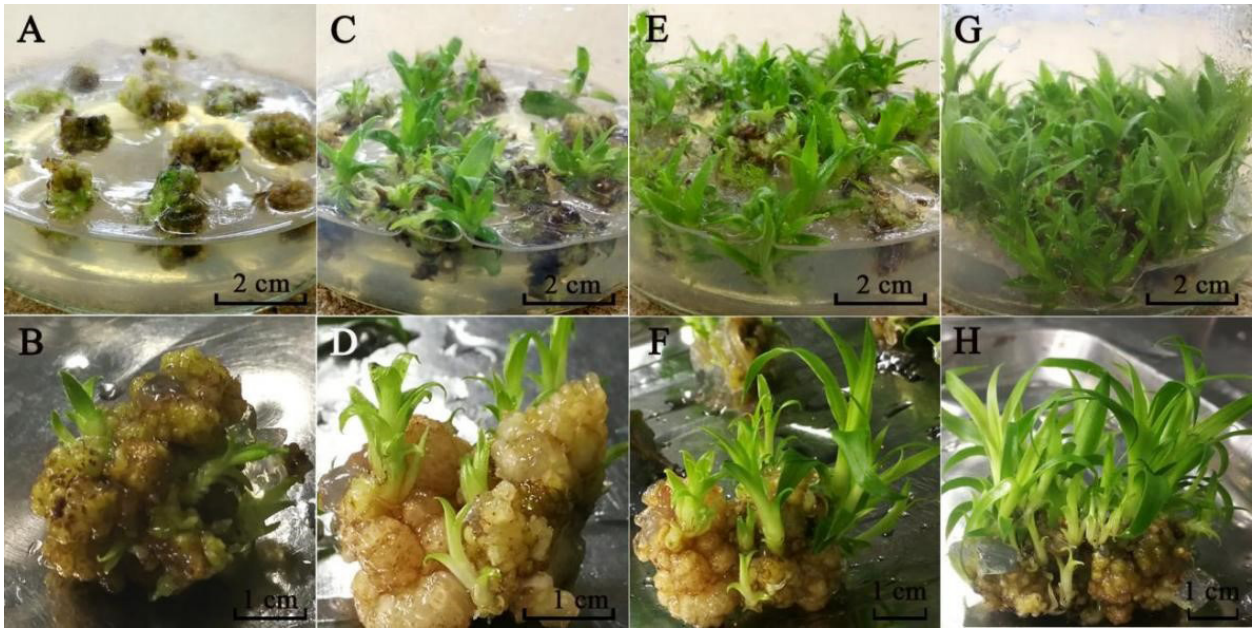

Fig. 4. Synchronous culture for callus proliferation, adventitious bud differentiation and proliferation

(A and B) After 10 days of culture, green adventitious buds appeared on the surface of callus; (C and D) After 20 days, callus proliferated rapidly with the increase of the number of adventitious buds; (E and F) After 30 days, early adventitious buds had grown into plantlets, and new adventitious buds were constantly differentiated from callus; (G and H) After 40 days, the whole surface of callus was covered by adventitious buds

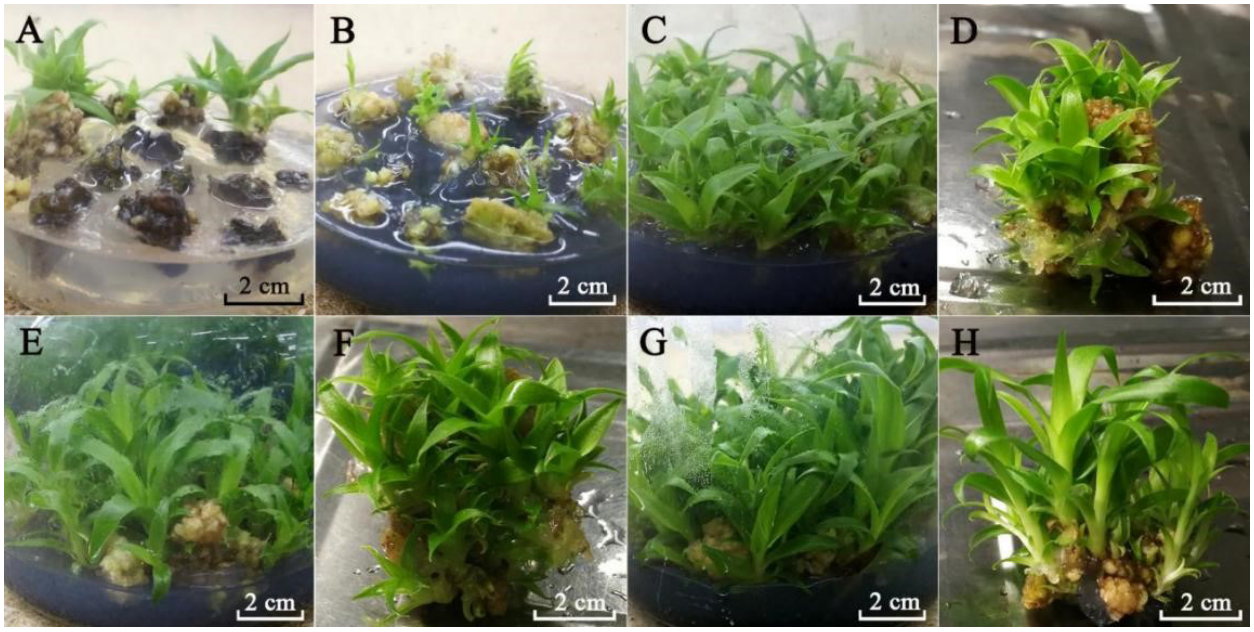

Fig. 5. Improvement of callus browning and proliferation culture

(A) Browning callus that lost the ability to proliferate and redifferentiation; (B) To alleviate the problem of callus browning, 1.0 mg·L^{-1} AC was added to the culture medium; After 10 days of culture, adventitious buds began to differentiate on the surface of callus; (C and D) After 20 days, callus also proliferated rapidly to adapt to the rapid growth of adventitious buds; (E and F) After 30 days, adventitious buds grew vigorously and stimulated callus to proliferate further; (G and H) After 40 days, adventitious buds occupied the whole surface of the medium, with 3–5 healthy main buds per cluster buds

Rooting culture

The rooting rate of all treatments including CK was 100%. It can be seen from Table 8 that the treatment without AC was more suitable for induction and growth of adventitious roots than the treatment with AC. The number of adventitious roots per plant in NAA treatment was significantly higher than that in CK ($P < 0.05$), which proved that auxin NAA had a significant effect on root induction. The number and growth of adventitious roots increased in a positive ratio with the increase of the concentration in 0.5–1.0 mg·L^{-1} NAA. However, the number of adventitious roots decreased and the growth trends of *in vitro* shoot became weaker as over 1.0 mg·L^{-1} NAA. In other words, a higher concentration of auxin not only inhibited the occurrence of adventitious roots, but also was not

Table 8. Rooting culture

Medium	NAA (mg·L^{-1})	The number of adventitious roots	Explant characteristics
Without AC			
CK-1	0.0	2.36 ±0.39 d	pale green leaves; few roots with slender; *in vitro* shoot was weak with a slow growth rate; a small amount of callus
R_1	0.5	4.67 ±0.52 b	plenty of bright green leaves; stout and long roots; a good growth trend of *in vitro* shoot
R_2	1.0	7.35 ±0.44 a	dark green leaves; plenty of stout roots; a good growth trend of *in vitro* shoot
R_3	1.5	4.34 ±0.47 b	green leaves; stout and short roots with a poor toughness; a slow growth trend
With AC (1.0 g·L^{-1})			
CK-2	0.0	1.31 ±0.51 e	small number of green leaves; a few slender roots; slender *in vitro* shoot; a poor growth trend; a small amount of callus
R_4	0.5	3.35 ±0.37 cd	green leaves; a few slender roots
R_5	1.0	3.69 ±0.61 c	leaflet; a slow growth trend; underdeveloped root; a poor growth trend
R_6	1.5	2.68 ±0.46 d	green leaflet; slow to take root; a poor growth trend

Note: AC – Activated carbon; NAA – α-naphthaleneacetic acid. Different letters in the same column indicate significant differences at $P < 0.05$. Data are mean ±SE

conducive to the growth of *in vitro* shoot. Therefore, the optimal induction medium for adventitious roots of pineapple was 1/2MS medium with 1.0 mg·L^{-1} NAA. In this medium, after 15 days of culture, white root tips appeared around the base of *in vitro* shoot (Fig. 6A and B). After 25 days, leaves were extended, new leaves appeared continuously, plant height was obvious, and the growth of adventitious root was visible with the elongation of adventitious root (Fig. 6C and D). After 35 days, the basal stem of *in vitro* shoots became thick, adventitious roots became thick and elongated with obvious root hairs (Fig. 6E and F). After 45 days, *in vitro* shoots grew robust, with developed and robust adventitious roots at the bottom, which was suitable for acclimation and transplanting (Fig. 6G and H).

Acclimation, transplanting and variation

After acclimation, the survival rate of *in vitro* plant was 100% (Fig. 7A and B). After 4 months, the plant grew healthily, new leaves were constantly appeared, old leaves were wider and longer, and the height of the plant was about 15–20 cm, which met the requirements of field planting (Fig. 7C and D). After 8 months of field planting, inflorescence expanded with the appearance of the fruit (Fig. 7E). There was no significant difference in the size and taste of the fruit between the tissue culture seedings and the mother plants after acclimation and transplanting after 12 months (Fig. 7F).

Notably, the variation of about 0.01% leaves of *in vitro* shoots was observed during proliferation or rooting (Fig. 8A and B). Due to a small number, the variation was not tracked in this study and was discarded immediately after discovery. In addition, no similar phenomenon was observed in the process of acclimation and transplanting.

DISCUSSION

In this study, direct plantlet regeneration from leaf base had poor problems, including a low reproduction coefficient and a weak plant. In addition, to obtain re-

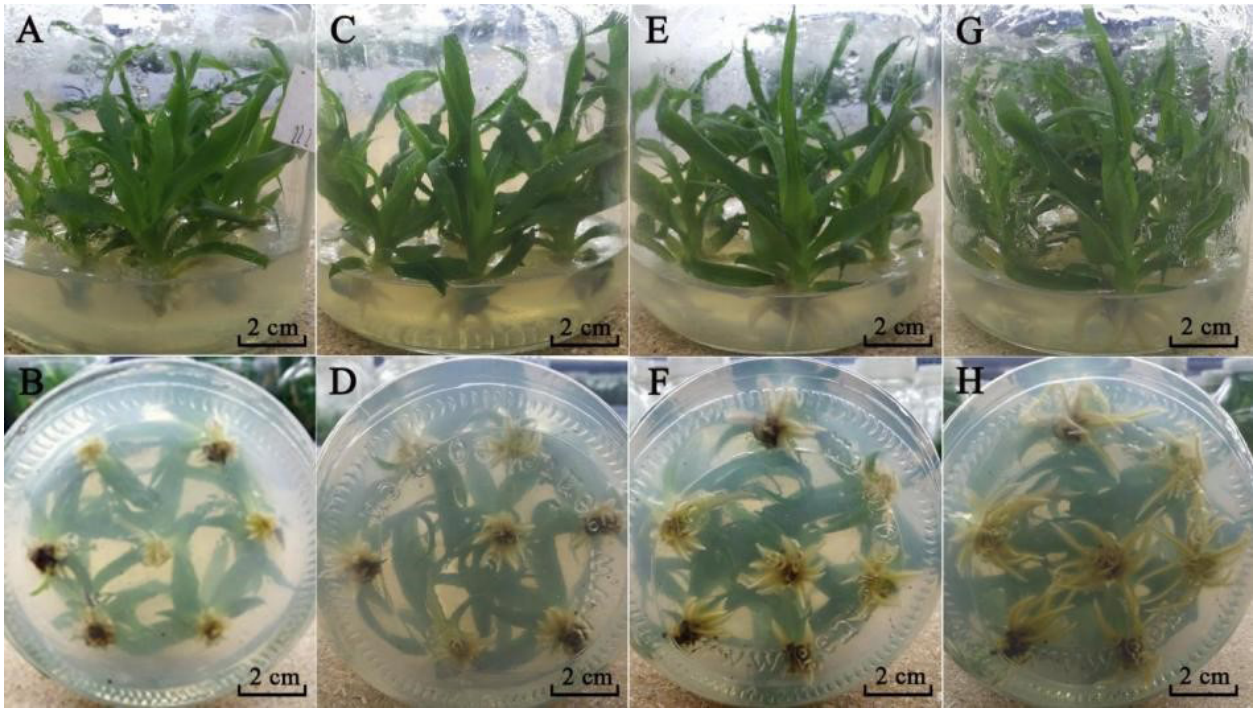

Fig. 6. Rooting culture

(A and B) After culture for 15 days, *in vitro* shoot grew obviously, and adventitious roots grew in a wheel shape at the bottom of the bottle; (C and D) After 25 days, *in vitro* shoots grew further and adventitious roots also grew rapidly; (E and F) After 35 days, *in vitro* shoots were thickened and adventitious roots had developed root hairs; (G and H) After 45 days, *in vitro* shoots with strong and thick roots were the best acclimation material

Fig. 7. Acclimation, transplanting and variation

(A) *In vitro* plant acclimation for 15 days; (B) After 60 days of acclimation, the roots of *in vitro* plant were developed; (C) Plant after 4 months; (D) After 5 months of acclimation, the leaves were red because of low temperature; (E) After 8 months of transplanting, fruit began to appear; (F) After 12 months of transplanting, the fruit was ripe

generated plants on a large scale, it could only be done on the premise of sufficient number of explants, and it was quite difficult to operate. Thus, this method was only suitable for the initial culture stage. For indirect organogenesis, it was found that the appearance of callus was closely related to the rupture of leaf base, that was, the expansion of callus from the bottom to the top resulted in the rupture of leaf base of each whorl, which resulted in the emergence of new callus. After proliferation, each leaf could be wrapped up, presenting a unique stratified phenomenon. Because of this phenomenon, the number of calluses was significantly increased, which also proved that the leaf base of pineapple has a strong potential for dedifferentiation [Firoozabady and Moy 2004]. The leaf base located near the axillary meristem may contain meristem regions or newly developed meristem, which can rapidly divide cells and construct organ morphology during culture. Interestingly, in the existing reports of *in vitro* rapid propagation of pineapple [Soneji et al. 2002a, 2002b, Firoozabady and Gutterson 2003, Sripaoraya et al. 2003, Be and Debergh 2006, Zuraida et al. 2011, Ibrahim et al. 2013, Usman et al. 2013, Nelson et al. 2015, Scherer et al. 2015, Cacaï et al. 2023, Mendonça et al. 2017, Lakho et al. 2023, Torres Ruiz et al. 2023], callus stage was rarely mentioned, and most of them

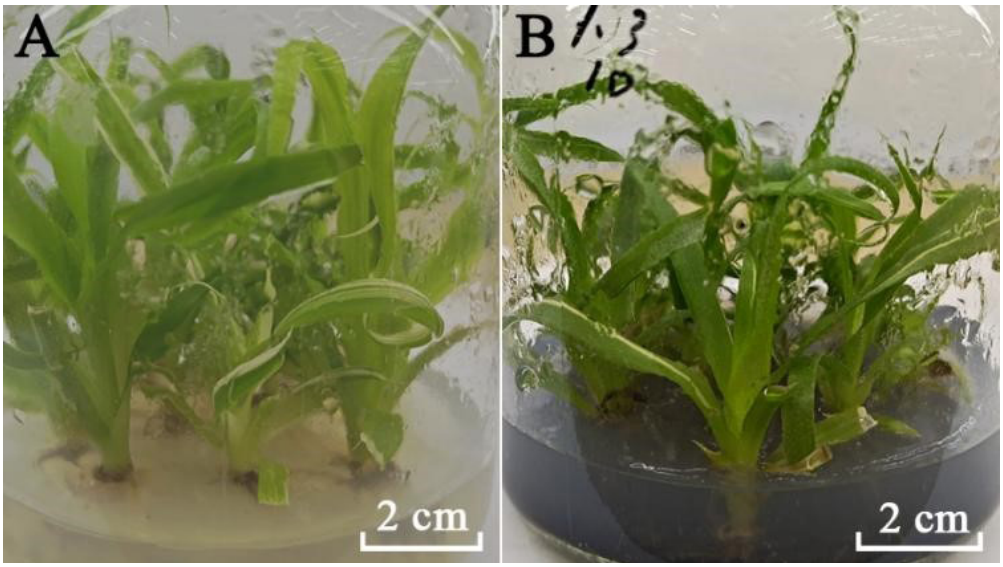

Fig. 8. Phenotypic variation; (A and B) Albino striped variegated foliage

were replaced by bud aggregate. However, in an early stage of this study, callus had obvious and strong proliferative effect, and only after the adventitious cluster buds appeared did the bud aggregates with numerous buds formed on the carrier of callus. Another difference was that adventitious buds produced from callus in this study grew well. In the period of proliferation, these adventitious buds showed a complete morphology of regenerated plants. However, according to the descriptions or pictures of other researchers, adventitious buds were weak and basically had no regenerated plant morphology, which requires repeated rejuvenation culture [Soneji et al. 2002b, Firoozabady and Gutterson 2003, Reinhardt et al. 2018]. Also, the callus in this study proliferated quickly, and high propagation coefficient could be obtained through a subculture in a short period of time, which reduced the high labour cost and production cost caused by repeated rejuvenation. In addition, it has been reported that in *in vitro* rapid propagation of pineapple, genotype had a strong influence on the regeneration method [Da Silva et al. 2016]. Whether this view is applicable to the object of this study still needs to be verified by similar studies on other varieties or variants of pineapple.

It was reported that there was no ideal method for *in vitro* propagation of specific plant species at present, and micropropagation needed to be adjusted and improved for each specific explant in order to achieve higher efficiency [Reinhardt et al. 2018]. The methods of callus induction, adventitious bud occurrence and proliferation, and plant regeneration for a variant pineapple were established in this study. Current reports in different varieties of pineapple, adventitious buds occurrence and proliferation could be achieved by using high concentration of 6-BA alone (5.0–8.0 mg·L^{-1}) or in combination with auxin such as NAA, IAA or IBA [Soneji et al. 2002b, Firoozabady and Gutterson 2003, Sripaoraya et al. 2003, Be and Debergh 2006, Zuraida et al. 2011, Ibrahim et al. 2013, Usman et al. 2013, Nelson et al. 2015, Scherer et al. 2015, Da Silva et al. 2016, Mendonça et al. 2017, Cacaï et al. 2023, Lakho et al. 2023, Torres Ruiz et al. 2023]. Different from the results of previous studies, only part of the explants used cytokinin in this study produced callus, and the culture effect was far from expected. Auxin alone showed a significant effect on the rooting rejuvenation of the explant. When 6-BA and NAA were combined, the callus induction rate significantly increased, and the best treatment could reach 100%. However, adventitious bud differentiation was less, even if differentiation, there were signs of growth inhibition. Thus, KT was selected for orthogonal experiment. Not only callus proliferation was greatly increased, but also differentiation coefficient of adventitious buds was remarkably increased. These results indicated that KT could not only promote cell division, but also induce bud differentiation and

development. It also proved that the synergistic effect produced by multiple factors was more conducive to the proliferation culture of the study object.

Serious browning appeared after further propagation culture. Surprisingly, although pineapple tissue culture has been widely reported, only Soneji et al. [2002a] have mentioned this phenomenon and no improvement measures have been taken. In addition, according to related reports, the addition of AC in tissue culture could not only prevent oxidation, but also remove most inhibitory and toxic substances, even substances released during culture, thus reducing browning [Lizeth and María de Lourdes 2018]. Therefore, in this study, AC was added at this stage, and browning was basically eliminated, which indicated that AC had a positive role in reducing the browning of this variety of pineapple.

In the culture after the elimination of browning, the proliferation of callus and adventitious buds was complementary to each other. The proliferation of callus clearly occurred after the appearance of adventitious buds. In a certain culture space, the more adventitious buds, the faster the proliferation of callus. Generally, plant endogenous auxin is produced at the tip of the stem, while cytokinin is produced at the tip of the root. In this study, the concentration of cytokinin was much higher than that of auxin. It was estimated that after the occurrence of adventitious buds, the growth hormone synthesized by bud tip might be transported down rapidly and combine with the exogenous cytokinin accumulated in callus, thereby promoting callus proliferation. At this time, the process of callus induction could be removed in this study, and callus proliferation and adventitious bud occurrence were carried out simultaneously in 6-BA, NAA and KT combinations, with a proliferation period of only 40–45 days. This kind of simultaneous culture was not only effective but also has a high regeneration coefficient, which achieved the goal of efficient and rapid propagation.

CONCLUSIONS

This study successfully established methods for callus induction, adventitious bud formation, and plant regeneration in the new line of *A. comosus* selected from a spontaneous mutant of cultivar Yellow Mauritius. Callus formation was closely linked to the rupture of the leaf base, leading to increased callus generation. The stratified phenomenon of leaf wrapping further enhanced callus proliferation, demonstrating the significant dedifferentiation potential of the pineapple leaf base. In this study, the addition of activated carbon (AC) effectively mitigated browning during propagation, highlighting its beneficial role in this process. Ultimately, the study achieved high callus proliferation and a high propagation coefficient through rapid subculture, thereby reducing labour and production costs associated with repeated rejuvenation.

AUTHORS' CONTRIBUTIONS

The author confirms their contributions to the manuscript as follows: Writing-Original Draft Preparation, Validation, Visualization: CL; Formal analysis, Funding acquisition, Project administration: FX; Writing-review & editing, Conceptualization, Methodology, Supervision, Writing-review & editing: HH. All authors reviewed the results and approved the final version of the manuscript.

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CONFLICT OF INTERESTS

All authors declare no conflict of interest.

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