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Research Article

Studies on the induction of basal stem cluster buds and nodes propagation of *Amomum villosum* Lour.

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Abstract

Amomum villosum Lour. as Chinese herbal medicine and seasoning, which has a huge potential economic value. This plant is increasing shortage of resources by the slow sexual reproduction and the low artificial reproduction rate. The plants with strong stress resistance and high yield were selected as the female parent, on the basis of single factor and complete combination, the orthogonal test of L_9 (3^4) and L_{16} (4^5) was further conducted. 6-BA induced basal stem cluster bud formation was obviously better than that of KT or ZT. 2, 4-D significantly induced callus occurrence and node enlargement, while NAA was more beneficial to adventitious root formation. The combination of 6-BA and NAA was more beneficial to induce the formation of cluster buds from basal stem, but the proliferation effect was not ideal. Thus, KT or 2, 4-D was introduced for two orthogonal tests of L_9 (3^4), and the highest proliferation coefficient was only 4.56. Then it was found that adding 0.5 mg·L^{-1} 2, 4-D to the above combination, appeared a unique phenomenon of node propagation. Next, L_{16} (4^5) orthogonal test was conducted using 4 plant growth regulators combinations of 6-BA, 2, 4-D, KT and NAA. The optimal medium for proliferation culture was the MS medium with 7.5 mg·L^{-1} 6-BA, 5.0 mg·L^{-1} NAA, 1.5 mg·L^{-1} KT, 0.5 mg·L^{-1} 2, 4-D, and the proliferation coefficient reached above 10.00. The optimal rooting medium was the 1/2 MS medium with 2.0 mg·L^{-1} NAA. With the node propagation, a rapid propagation system of *A. villosum* was established which provided a possible solution for improve the efficiency of artificial planting, solve the market demand and quality problems.

Keywords: *Amomum villosum* Lour.; node propagation; tissue culture; rhizomes; Zingiberaceae

Introduction

Fructus amomi (FA) is the dried ripe fruit of three species of the genus *Amumon* herbs in Zingiberaceae with *A. villosum* Lour., *A. villosum* Lour. var *xanthioides* T. L. Wu et Senjen or *A. longiligulare* T. L. Wu. FA as traditional Chinese medicine commonly used in prescriptions and certified that the nature is pungent and warm, which has the effect of helping with digestion, calming fetus, improving immunity. Furthermore, it also plays an important role in Xiangsha Liujun pill, Xiangsha Yangwei pill and other Chinese patent medicine

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(Chinese Pharmacopoeia Commission of the People's Republic of China, 2020). Modern pharmacological studies show that FA contain bomyl acetate, camphor, borneol and other volatile components, and has the pharmacological effect of protecting gastrointestinal, analgesia, resisting inflammatory, anti-diarrhea, bacteriostasis, regulating flora, hypoglycemic, anti-oxidant and so on (Li *et al.*, 2018). Found in the past two years, FA as main components of anti-dampness medicine can effectively treat Covid-19 (Wang *et al.*, 2020). Meanwhile, it is not only an important status in medicinal value, but also is used for seasoning, and as medicinal and edible plants to famous everywhere. FA usually grows in the mountainous wet place of Fujian, Guangdong, Guangxi and Yunnan province of China. The best quality of FA is in Yangchun county Guangdong province, also called Yang Chun Sha in Chinese phonetic writing (Flora of China Editorial Committee and Chinese Academy of Sciences, 1982).

However, the supply for FA is short of the demand due to its low-production. The reason for the low output is the special flower structure makes insects to be complicated for pollinating in narrow space, low rates of self-pollination and artificial pollination resulting in low rates of fruiting. Besides some businesses sell counterfeit product, causing confusion in the quality of *A. villosum* (Liu, 2019; He *et al.*, 2020). In nature, *A. villosum* mostly carries on sexual propagation (seed propagation) with occasional asexual propagation (rhizome propagation). Artificial multiplication is dominated by the above two methods, but is more common in asexual propagation. Though *A. villosum* have a great deal of seeds and easy to sow, obtaining a large number of seedlings requires high cost. The reason is that seeds have a very low natural germination rates during the dormant period. Dormant period is a physiological phenomenon of seed caused by immature embryo, defective internal structure and natural hormone inhibition. In addition, it can take three to five years or more to grow from seedings to commercial plants that produce flowers and fruit, so seed multiplication is too slow to be adopt. Although rhizome propagation faster to obtain young plants, a single rootstock produces only 2-3 buds per year. Meanwhile, the population growth decline and loss of genetic integrity were also observed. The large-scale cultivation and pharmaceutical use of *A. villosum* has been limited by its low reproductive rate (Chen *et al.*, 2015; Zhao *et al.*, 2020). Not only the tissue culture technology has fast growth, short cycle, strong repeatability and other advantages, but also can have single source of materials, and preserve plant genetic integrity from female parent. In addition, it can also obtain a large number of young plants with highly consistent genetic characteristics in a short period of time (Razdan, 1993). The development of plant culture *in vitro*, which provided an effective guarantee for medicinal plants with scarce resources. The germinal bud of rhizome is one of the most commonly used explants in Zingiberaceae (Mohanty, 2013). Meanwhile, it also is widely used in rhizomatous plant, such as: *Polygonatum cyrtonema* Hua (Zhao *et al.*, 2009), *Smilax glabra* Roxb (Dong *et al.*, 2014), *Paris polyphylla* Sm. Via (Raomai *et al.*, 2015) and so on. Zingiberaceae is widely studied in the world, such as *Curcuma kwangsiensis* Lindl. (Zhang *et al.*, 2011), *Curcuma aromatic* Salisb. (Mohanty *et al.*, 2008), *Curcuma attenuata* Wall (Kou *et al.*, 2013) and so on. However, the research on FA mainly focuses on pharmacology (Lu *et al.*, 2018; Kim *et al.*, 2020), identification (Wu *et al.*, 2007) and cultivation (Xu *et al.*, 2018). There are only a few reports on rapid propagation *in vitro*, and callus was successfully induced by explant to callus to adventitious bud method, but the callus did not differentiate into bud (Diao *et al.*, 2011; Li *et al.*, 2019). In this experiment, artificial propagation technology of *A. villosum* was studied by tissue culture technology, and an efficient plant regeneration system was established from organs directly. Then the young plants with genetic stability and excellent quality were cultivated, which expected to enhance the efficiency of artificial planting and solve the market movement faces problems with demand and quality. Meanwhile, it can also provide a foundation for artificial scale cultivation, germplasm conservation and transgenic technology.

Materials and Methods

Thirty plants samples were provided from the agricultural bureau of Mengla County of Xishuangbanna Dai Autonomous Prefecture in Yunnan Province of China, and were identified as *Amomum villosum* Lour. by Tao Sheng professor of Yunnan university in China. Subsequently, the plants were transplanted in the greenhouse of the Engineering Research Center for Reproducing Fine Varieties of Chinese Medicinal Plants Yunnan University of Chinese Traditional Medicine in April 2018. The sterilizing reagent ($HgCl_2$) needed for the experiment was purchased from Guizhou Tongren Research Institute of Chemical (Tongren, China), the Naphthalene acetic acid (NAA), Zeatin (ZT), 6-Furfurylaminopurine (KT) and absolute ethanol were purchased from Beijing Dingguo Changsheng Biotech Co., Ltd (Beijing, China), and the 6-Benzylaminopurine (6-BA) was purchased from Shanghai Ekear Biotech Co., Ltd (Shanghai, China), and the 2, 4 Dichlorophenoxyacetic acid (2, 4-D) was purchased from China Pharmaceutical Shanghai Chemical Reagent Co., Ltd (Shanghai, China).

Treatment of explants and establishment of germfree system

Choosing healthy rhizomes with buds of *A. villosum*, after clearing the surface soil immersed into 10 % (*w/v*) laundry detergent for 10 min, washed under running tap water for 30 min before shifted on a sterile operating platform. Furthermore, these materials were immersed into 75 % (*v/v*) ethyl alcohol for 15 s and surface sterilized using 0.1% (*w/v*) $HgCl_2$ for five times gradients (6, 8, 10, 12, and 15 min) and followed by treatment with sterile distilled water three times that was more than 3 min every time. Subsequently, the sterilized rhizomes with buds were placed on sterile blotting paper to dry the surface and the rhizome was cut about 1.0 cm^2 in size using a sterile scalpel. Finally, the cut explants were cultured (bud points up) in primary culture medium and then observed.

Medium

This study except the rooting stage, the basic medium of other cultures of *A. villosum* was Murashige and Skoog (MS) supplemented with different type and concentration of plant growth regulators (PGRs) as needed, 3% saccharose and 0.47% agar. The pH of all media was adjusted to 5.4-5.8 and then autoclaved at 121 °C and 0.105 MPa for 20 min. In this experiment, the PGR concentration refers to the mass concentration.

Primary culture medium

The number of explants was too little to use multiple starting media for experiments. According to other literature of rhizome with buds were induced (Nayak, 2000; Li *et al.*, 2017), the medium was MS medium containing 2.0 $mg{\cdot}L^{-1}$ 6-BA was used as primary culture medium of rhizome with buds. After the successful establishment of germfree system, continue cultured for 3-5 generations on the above medium to obtain sufficient sterile plants, then performed on experiment of single factor.

Single factor experiment

The sterilized rhizomes with buds were cultured in MS medium containing different type and concentration PGRs which included 6-BA (1.5, 2.5, 3.5, 4.5, 5.5, 6.5, and 7.5 $mg{\cdot}L^{-1}$), 2, 4-D (0.05, 0.1, 0.5, 1.0, and 1.5 $mg{\cdot}L^{-1}$), KT (0.05, 0.5, 1.5, 2.5, and 3.5 $mg{\cdot}L^{-1}$), NAA (1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 $mg{\cdot}L^{-1}$) or ZT (1.0, 2.0, 3.0, 4.0, and 5.0 $mg{\cdot}L^{-1}$). The growth conditions were recorded and the types and concentration ranges of PGRs with suitable for the growth of *A. villosum* were selected after 60 days.

Complete combination experiment

Based on the results of chapter single factor experiment, three groups of complete combination experiments were designed. The first experiment was KT (0.5, 1.5, and 2.5 $mg{\cdot}L^{-1}$) and 2, 4-D (0.5, 1.0, and 1.5

mg·L^{-1}); the second experiment was 6-BA (6.5, 7.5, and 8.5 mg·L^{-1}) and 2, 4-D (0.5, 1.0, and 1.5 mg·L^{-1}); the third experiment was 6-BA (6.5, 7.5, and 8.5 mg·L^{-1}) and NAA (3.0, 4.0, and 5.0 mg·L^{-1}). To test the effect of rhizomatic adventitious bud induction from different combinations of PGR concentrations in two kinds of factors, and each group of the proliferation coefficient of bud was calculated 60 days later.

The medium of basal stem cluster bud occurrence and the propagation from nodes

According to the results of experiments with single factor and complete combination, A (6-BA), B (KT), C (2, 4-D), D (NAA) were used as factor (Table 1). After 60 days, the proliferation coefficient of bud was calculated.

Table 1. Orthogonal design L_{16} (4^5) for the proliferation of cluster buds induced

Level	Factor (mg·L^{-1})			
	6-BA	KT	2, 4-D	NAA
1	5.5	0.5	0.5	3.0
2	6.5	1.5	1.0	4.0
3	7.5	2.5	1.5	5.0
4	8.5	3.5	2.0	6.0

Note: 6-BA: 6-Benzylaminopurine; KT: 6-Furfurylaminopurine; 2, 4-D: 2, 4 Dichlorophenoxyacetic acid; NAA: Naphthalene acetic acid.

The medium of rooting

The basic medium was half-strength MS, on the basis of repeating NAA single factor, then performed on complete combination experiment of supplying with 6-BA (0.05, 0.10, and 0.50 mg·L^{-1}). Furthermore, each treatment group supplemented with 1.0 g·L^{-1} Activated charcoal (AC) alone to control the effect of AC on adventitious root induction of *A. villosum*. The experiment group without adding AC was called A group and with adding AC was called B group. The rooting rate and adventitious roots per plant were calculated 60 days after cultured.

The method of inoculating

Using 50 ml conical bottles to establish the aseptic system and one bottle of a material. The single factor experiment needs ten bottles of per treatment and one bottle of ten material. At the stage of adventitious bud occurrence and the culture of proliferation (the experiment of complete combination and orthogonal) and the culture of rooting need ten bottles of per treatment and one bottle of twelve materials. At the stage of proliferation, the materials were cut into plantlet that on average with 2-3 cm length and each plant had 2-3 leaves or similar size rhizome with buds (each root had 2-3 shoots). Meanwhile, the materials of nodes propagation were separately cultured in new medium. The strong basal stem cluster buds were selected, and subsequent cut out the bud's leaf blade of upper part to become separate bud with 2-3 cm length, which were used as materials in the rooting stage. In addition, the obtained shoots from nodes propagation can also be used as rooting materials. The above each experimental group was repeated three times and if the plant was death or pollution then to supply with same experiment in bottles timely.

Culture conditions

The cultivation temperature was maintained between 21 °C and 23 °C. The photoperiod was 12 h with 1500~2000 lx.

Acclimatization and Transplanting

When the rooted plants in the culture bottle grow to 6-8 cm in height and the root had become strong, they were exposed to natural light for 5 days, subsequent took down the sealing film of culture bottles to place

in light for 2 days. Then the plantlets were removed from the culture bottle, carefully washed the agar of root with water, and immersed into 0.1% (*w/v*) chlorothalonil for five minutes. Subsequently, transplanted them into humus soil with high temperature sterilization to culture in constant temperature (20 to 25 °C) and humidity (about 70%) for 60 days, the survival rate was recorded after 60 days.

Statistical index

The obtained data were processed and analyzed by SPSS 22.0 (IBM Corp, Armonk, USA) and Excel (MC Corp, Redmond, USA) software.

Contamination rate (%) = (the number of pollution bottles/the number of bottles of initial inoculations) × 100%;

The survival rate (%) = (the number of survival plants/the total number of plants free from contamination) × 100%;

Basal stem cluster bud occurrence rate (%) = (the number of materials with two or more adventitious buds/the number of materials of initial inoculations) × 100%;

Occurrence rate of nodes propagation (%) = (the number of adventitious buds produced from nodes/the number of initial inoculations) × 100%;

Rooting rate (%) = (the total number of materials of adventitious roots produced/the total number of initial inoculations) × 100%;

The survival rate of transplanting (%) = (the total number of survival plants/the total number of transplanting plants) × 100%;

Proliferation coefficient = (the number of materials of effective inoculated/the number of initial inoculations) × 100%;

The average number of adventitious roots per plant = (the total number of adventitious roots produced/the total number of materials of inoculations) × 100%.

Results

Establishment of aseptic system and the initial proliferation of material

The results showed that there were some differences in contamination and survival rate of explant with different disinfection time (Table 2). When explants were disinfected for 6 minutes, reached to the highest pollution rate (90.00%) because of inadequate sterilization, but uncontaminated explants were mostly survival. The explants' pollution rate dropped to 31.00% at sterilization time of 15 minutes, but survival rate was only 37.59% because mercuric chloride toxicity is so strong that the tolerance of explants is reduced. In the whole, pollution and survival rate of explants were reduced with increasing sterilization time. Although explants' survival rate of sterilization for 8 minutes was higher than that for 10 minutes, there was no significant difference in between them ($P > 0.05$) and the latter had a lower pollution rate than the former. Therefore, the best sterilization time of rhizome with buds of *A. villosum* was 10 minutes in this study.

Table 2. Effects of different sterilization time on pollution and survival of explants

Sterilization time (min)	Number of inoculated (plant)	Pollution rate (%)	The survival rates after 60 days
6	20	90.00±1.443a	93.33±6.085a
8	20	72.00±2.327b	87.67±4.753a
10	20	62.00±2.327c	84.84±5.723a
12	20	47.00±2.327d	57.15±3.702b
14	20	31.00±1.708e	37.59±1.670c

Note: Different letters in the table indicate significant differences in the 5% level; ± Standard error, same below.

The buds of rhizome began to show signs of sprout after 15 days of inoculate (Figure 1A). After 30 days of culture, the buds of rhizome began to grow rapidly (Figure 1B). After 45 days of culture, the petiole gradually elongated, the leaves began to differentiation and new bud occurrence from basal (Figure 1B). After 60 days of culture, the petiole of old buds continues to grow and leaf blade stretched, and the new buds also had a certain growth, so the aseptic system was successfully established (Figure 1C). The proliferation coefficient was about 1.73 of each generation in primary culture medium and sterile materials were gradually accumulated after 3-5 generations of culture.

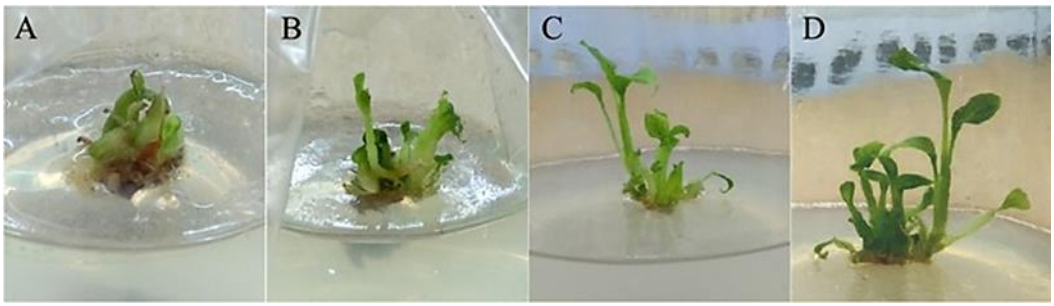

Figure 1. Establishment of aseptic system and initial proliferation culture. (A) Growth condition after 15 days of inoculation; (B) Growth condition after 30 days; (C) Growth condition after 45 days; (D) Growth condition after 60 days

Single factor experiment

The experimental results showed that the growth of buds of rhizome all were promoted on medium supplement with exogenous PGR alone, and the adventitious root occurrence was observed in all treatment group. Among cytokinin had a great influence on basal stem adventitious bud induction, high concentration 6-BA (5.5 mg·L^{-1} or more) had a significant effect to basal stem cluster bud induction (Figure 2A), followed by KT treatment (Figure 2B). The worst response was ZT treatment (Figure 2C). In NAA treatment group, a small number of adventitious buds occurrence from basal clusters were observed. Meanwhile, roots formation was observed in all NAA treatment, especially best response at 2.0 mg·L^{-1} NAA (Figure 2D). In contrast to the PGR group described above, the rhizome nodes expanded and callus formation only were observed in 2, 4-D group. In the 0.01 to 0.05 mg·L^{-1} concentration range of 2, 4-D, plants could grow normally without callus formation and proliferation (Figure 2E). Within the range of 0.5 to 1.5 mg·L^{-1} 2, 4-D, the rhizome expanded and elongated, and yellowish or white callus with loose texture generated. Meanwhile, adventitious buds did not differentiate and plant normal growth was inhibited (Figure 2F).

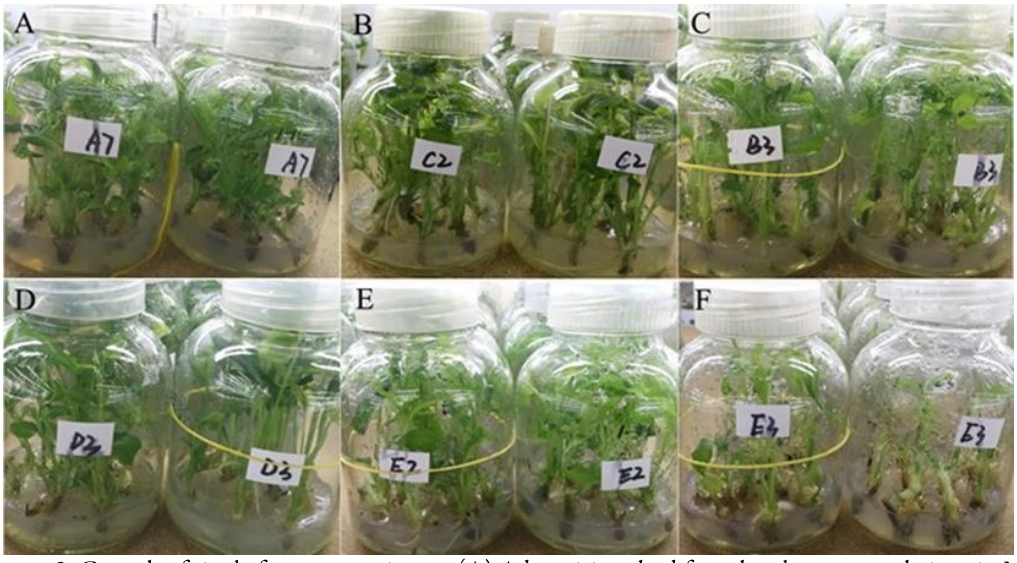

Figure 2. Growth of single factors experiment. (A) Adventitious bud from basal stem was obvious in MS medium with 7.5 mg·L^{-1} 6-BA; (B) Some of the materials grew adventitious buds from basal stems in MS medium with 7.5 mg·L^{-1} KT; (C) Adventitious bud from basal stem was occasionally observed in MS medium with 3.0 mg·L^{-1} ZT; (D) Adventitious bud from basal stem was occasionally observed in MS medium with 3.0 mg·L^{-1} NAA; (E) Plant growth was normal without proliferation in MS medium with 0.1 mg·L^{-1} 2,4-D; (F) In MS medium with 0.5 mg·L^{-1} 2, 4-D, the plants were dwarfed, the rhizomes were enlarged and elongated, and light yellow or white callus appeared at the base, without adventitious buds occurrence

Complete combination experiment

The results of three complete combination experiments could be seen from Table 3. In the combination experimental groups of KT and 2, 4-D, the basal of buds in the rhizome expended, formed pale yellow callus with loose texture, and hardly any adventitious bud occurrence, but the proliferation coefficient could reach 2.21-3.11 due to the phenomenon of propagation from nodes (Figure 3A). In the combination of 6-BA and 2, 4-D, the number of callus was higher than that of the group of KT and 2, 4-D, and had a significant phenomenon to propagation from nodes and accompanied with basal stem cluster buds occurrence. Meanwhile, the proliferation coefficient was higher than that of the group of KT and 2, 4-D, which could reach 4.31 (Figure 3B). As seen as from the above two complete combinations, the proliferation coefficient decreased significantly with the increase of 2,4-D concentration as the KT or 6-BA concentration was constant, which the enlargement of buds of rhizome basal became more obvious, but plant was stuntedness and growth was inhibited. In the combination of 6-BA and NAA, no callus formation and nodes propagation were observed, but on the basal of buds of rhizome had fasciculate adventitious bud appearance, and the best combination was 6.5 mg·L^{-1} 6-BA and 3.0 mg·L^{-1} NAA, which proliferation coefficient could reach 4.72 (Figure 3C).

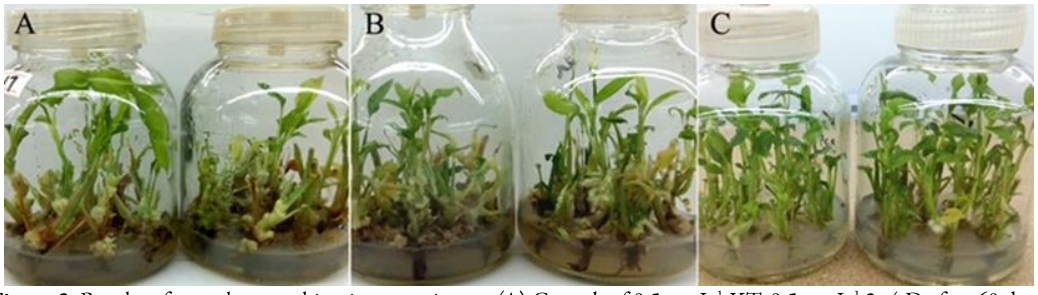

Figure 3. Results of complete combination experiment. (A) Growth of 0.5 mg·L^{-1} KT, 0.5 mg·L^{-1} 2, 4-D after 60 days; (B) Growth of 6.5 mg·L^{-1} 6-BA, 0.5 mg·L^{-1} 2, 4-D after 60 days; (C) Growth of 6.5 mg·L^{-1} 6-BA, 3.0 mg·L^{-1} NAA after 60 days

Table 3. Effects of different PGR concentration combinations on proliferation in *A. villosum*

No.	Plant growth regulator (mg·L^{-1})				Growth coefficient
	KT	6-BA	2,4-D	NAA	
CK	0	0	0	0	1.12±0.015 r
A1	0.5	0	0.5	0	3.11±0.029 i
A2	0.5	0	1.0	0	2.79±0.015 l
A3	0.5	0	1.5	0	2.34±0.017 p
A4	1.5	0	0.5	0	2.85±0.020 kl
A5	1.5	0	1.0	0	2.46±0.015 no
A6	1.5	0	1.5	0	2.38±0.020 p
A7	2.5	0	0.5	0	2.67±0.021 m
A8	2.5	0	1.0	0	2.40±0.015 op
A9	2.5	0	1.5	0	2.21±0.021 q
B1	0	6.5	0.5	0	4.31±0.020 bc
B2	0	6.5	1.0	0	3.34±0.015 h
B3	0	6.5	1.5	0	2.98±0.021 j
B4	0	7.5	0.5	0	3.46±0.021 g
B5	0	7.5	1.0	0	2.89±0.021 k
B6	0	7.5	1.5	0	2.48±0.021 n
B7	0	8.5	0.5	0	3.02±0.031 j
B8	0	8.5	1.0	0	2.78±0.021 l
B9	0	8.5	1.5	0	2.61±0.017 m
C1	0	6.5	0	3.0	4.72±0.015 a
C2	0	6.5	0	4.0	4.34±0.020 b
C3	0	6.5	0	5.0	4.01±0.021 d
C4	0	7.5	0	3.0	4.32±0.025 b
C5	0	7.5	0	4.0	4.06±0.036 d
C6	0	7.5	0	5.0	3.89±0.022 e
C7	0	8.5	0	3.0	4.24±0.029 c
C8	0	8.5	0	4.0	3.66±0.074 f
C9	0	8.5	0	5.0	3.53±0.015 g

Note: No.: number; CK: blank control group.

Basal stem cluster bud occurrence, proliferation and culture of propagation from nodes

As seen as from $R_{6\text{-BA}} > R_{2,\,4\text{-D}} > R_{KT} > R_{NAA} > R_{Error}$ of basal stem cluster bud occurrence rate (Table 4 and 5), four factors were all reliability effects. It showed from analysis of variance (Table 6), 6-BA and 2, 4-D had an obvious effect ($P < 0.05$) and KT and NAA without significant effect ($P > 0.05$) about basal stem cluster bud occurrence. In regard to node reproduction occurrence rate, which showed that four factors also were all reliability effect from $R_{2,\,4\text{-D}} > R_{6\text{-BA}} > R_{NAA} > R_{KT} > R_{Error}$. It showed from variance analysis of Table 6, 2, 4-D had an extremely significant effect ($P < 0.01$) to nodes propagation, and 6-BA, KT and NAA without obvious effect ($P > 0.05$). On the final proliferation coefficient, which indicated that four factors contributed to cluster bud proliferation equally with reliable ability from $R_{2,\,4\text{-D}} > R_{6\text{-BA}} > R_{NAA} > R_{KT} > R_{Error}$. As could be seen from variance analysis (Table 6), 2, 4-D had an extremely significant effect ($P < 0.01$) and 6-BA, KT and NAA without obvious effect ($P > 0.05$). To sum up, the occurrence of basal stem cluster buds and node propagation of *A. villosum* was closely related with proliferation efficiency. Through the mean value analysis known, the highest frequency of proliferation cultivate was obtained on combination $A_3B_2C_1D_3$ with 7.5 mg·L^{-1} 6-BA, 5.0 mg·L^{-1} NAA, 1.5 mg·L^{-1} KT, 0.5 mg·L^{-1} 2, 4-D.

Table 4. The results of $L_{16}(4)^5$ orthogonal experiment for basal stem cluster buds, nodal propagation and proliferation

No.	Plant growth regulator (mg·L^{-1})					BSD (%)	NP (%)	GC
	A (6-BA)	B (KT)	C (2,4-D)	D (NAA)	E (Error)			
1	5.5	0.5	0.5	3.0	（1）	51.15±0.047	57.48±0.336	8.15±0.024
2	5.5	1.5	1.0	4.0	（2）	46.27±0.202	46.48±0.096	7.48±0.064
3	5.5	2.5	1.5	5.0	（3）	41.19±0.126	36.46±0.238	6.91±0.078
4	5.5	3.5	2.0	6.0	（4）	33.99±0.229	16.32±0.150	5.66±0.179
5	6.5	0.5	1.0	5.0	（4）	57.31±0.218	61.60±0.308	8.58±0.056
6	6.5	1.5	0.5	6.0	（3）	63.22±0.099	68.97±0.159	8.86±0.038
7	6.5	2.5	2.0	3.0	（2）	44.13±0.204	42.48±0.240	7.22±0.047
8	6.5	3.5	1.5	4.0	（1）	47.11±0.195	49.03±0.182	7.67±0.046
9	7.5	0.5	1.5	6.0	（2）	56.64±0.311	44.97±0.539	7.52±0.075
10	7.5	1.5	2.0	5.0	（1）	61.96±0.153	66.38±0.071	8.75±0.030
11	7.5	2.5	0.5	4.0	（4）	87.13±0.102	87.46±0.461	9.36±0.058
12	7.5	3.5	1.0	3.0	（3）	74.47±0.211	75.63±0.414	9.00±0.049
13	8.5	0.5	2.0	4.0	（3）	41.71±0.329	32.71±0.137	6.30±0.094
14	8.5	1.5	1.5	3.0	（4）	44.95±0.173	45.50±0305	7.47±0.074
15	8.5	2.5	1.0	6.0	（1）	58.69±0.358	60.29±0.261	8.48±0.067
16	8.5	3.5	0.5	5.0	（2）	73.40±0.180	74.04±0.417	8.87±0.047

Note: BSD (%): Incidence of cluster buds in basal stem; NP (%): Incidence of nodal propagation; GC: Growth coefficient. Same below.

Table 5. Results of average and analysis in BSD (%), NP (%) and GC

Factors		Plant growth regulator				
		A (6-BA)	B (KT)	C (2,4-D)	D (NAA)	E (Error)
BSD (%)	K_1	43.151	51.703	68.726	53.926	54.728
	K_2	52.942	54.351	59.186	55.554	55.111
	K_3	70.050	57.785	49.052	58.465	55.147
	K_4	54.940	57.244	44.119	53.138	56.098
	R_1	26.899	6.082	24.607	5.327	1.370
NP (%)	K_1	39.188	49.190	71.989	55.271	58.297
	K_2	55.521	56.834	61.001	53.921	51.993
	K_3	68.612	56.675	49.343	59.621	53.443
	K_4	53.133	53.754	34.121	47.641	52.721
	R_2	29.424	7.644	37.868	11.980	6.304
GC	K_1	7.051	7.638	8.811	7.962	8.262
	K_2	8.081	8.141	8.387	7.703	7.775
	K_3	8.658	7.993	7.699	8.277	7.769
	K_4	7.783	7.801	6.677	7.632	7.768
	R_3	1.608	0.503	2.134	0.645	0.494

Table 6. Results of ANOVA in BSD (%), NP (%) and GC

Factors	Sources	Type III sum of square	df	Mean square	F value	Significance
BSD (%)	A	1483.411	3	494.470	3.791	$P<0.05$
	B	81.292	3	27.097	0.129	$P>0.05$
	C	1399.277	3	466.426	3.570	$P<0.05$
	D	19.141	3	6.380	0.089	$P>0.05$
	E	10.838	3	3.613		
NP (%)	A	1743.695	3	581.232	2.126	$P > 0.05$
	B	46.243	3	15.414	0.126	$P > 0.05$
	C	2734.992	3	911.664	6.768	$P < 0.01$
	D	63.059	3	21.020	0.249	$P > 0.05$
	E	21.260	3	7.087		
GC	A	5.369	3	1.790	1.955	$P > 0.05$
	B	0.083	3	0.028	0.147	$P > 0.05$
	C	8.659	3	2.886	7.008	$P < 0.01$
	D	0.248	3	0.083	0.267	$P > 0.05$
	E	0.256	3	0.085		

Note: df: degree freedom.

Repeating the above combination experiment, basal stem started to appear the new buds and basal of bud was inflated with white after 15 days of materials inoculation (Figure 4A). After 30 days, basal stem cluster buds grown faster, petiole elongated, leaf blade stretched and white swelling of buds more obvious (Figure 4B). After 45 days of culture, the number of basal stem cluster buds increased further with the rhizome expands, the white swelling of buds gradually disappeared. In addition, adventitious roots were formed in the original white swelling, which had an upward trend (Figure 4C-D). After 60 days, rhizome apogeotropism growth, and node of everyone had adventitious buds occurrence. The phenomenon of budding at nodes was called nodes reproduction in this study (Figure 4E-F), the proliferation coefficient could reach about 9.5 right now.

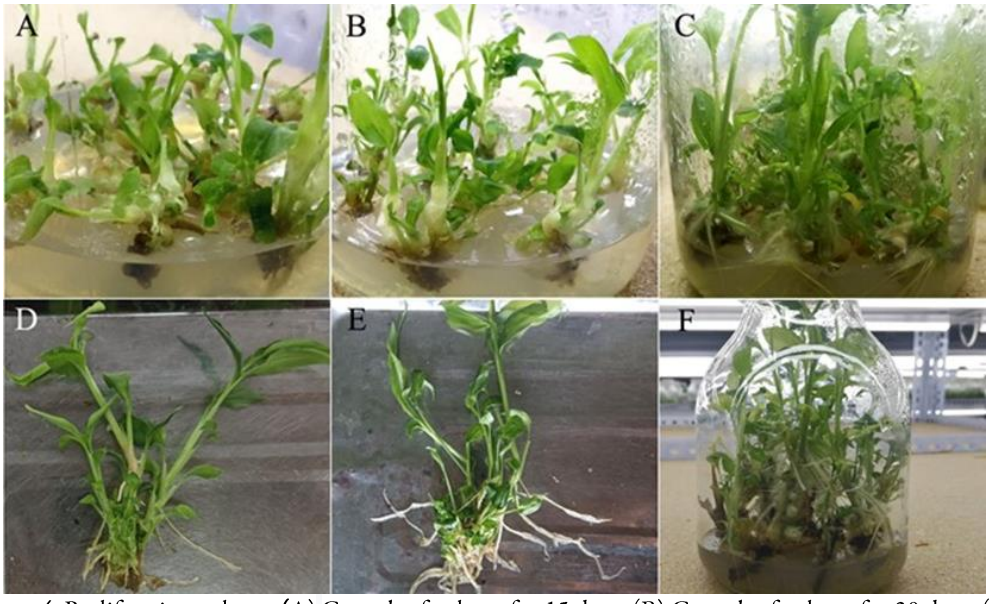

Figure 4. Proliferation culture. (A) Growth of culture for 15 days; (B) Growth of culture for 30 days; (C-D) Growth of culture for 45 days; (E-F) Growth of culture for 60 days

The culture of rooting

The results of rooting culture were seen on Table 7. The rooting rate of blank control in experimental group A was 54.17 %, rooting rate of blank control in experimental group B was 41.67 % Rooting rate of all experimental group were 100 % except blank control, which indicate that exogenous PGRs had an obvious effect to adventitious roots induction. NAA was added separately to induce adventitious roots was better than the combination of NAA and 6-BA, and the former without adventitious cluster buds formation. After 60 days of culture, the plant grew well and had thick roots, it was very conducive to the next domestication and transplanting works. The experiment also found that the growth of rooting plantlet in experimental group B was weaker than experimental group A when PGR concentration and growth environment remain unchanged. Meanwhile, plants in B group were thin (Figure 5A-B), and fibrous roots were short and numerous, which were easily broken during cleaning (Figure 5C), so not conducive to cultivation. In conclusion, the best medium of rooting in this study was R 01 with 1/2 MS containing 2.0 mg·L^{-1} NAA (Figure 5D-L).

Table 7. Effect of different NAA/6-BA/AC concentration on rooting of plantlets

No.	Plant growth regulator (mg·L^{-1})		Inoculation bottle	Average number of adventitious roots per plant
	NAA (mg·L^{-1})	6-BA (mg·L^{-1})		
CK-1	0.00	0.00	10	0.65±0.150 f
R 01	2.00	0.00	10	9.35±0.488 a
R 02	2.50	0.00	10	8.10±0.362 b
R 03	3.00	0.00	10	6.20±0.367 c
R 04	2.00	0.05	10	7.75±0.331 b
R 05	2.00	0.10	10	6.75±0.239 c
R 06	2.00	0.50	10	5.55±0.294 c
With activated charcoal (1.0 g L^{-1})				
CK-2	0.00	0.00	20	0.40±0.112 f
R 07	2.00	0.00	20	2.35±0.182 e
R 08	2.50	0.00	20	2.55±0.294 e
R 09	3.00	0.00	20	2.95±0.266 e
R 10	2.00	0.05	20	4.45±0.285 d
R 11	2.00	0.10	20	2.85±0.244 e
R 12	2.00	0.50	20	2.85±0.233 e

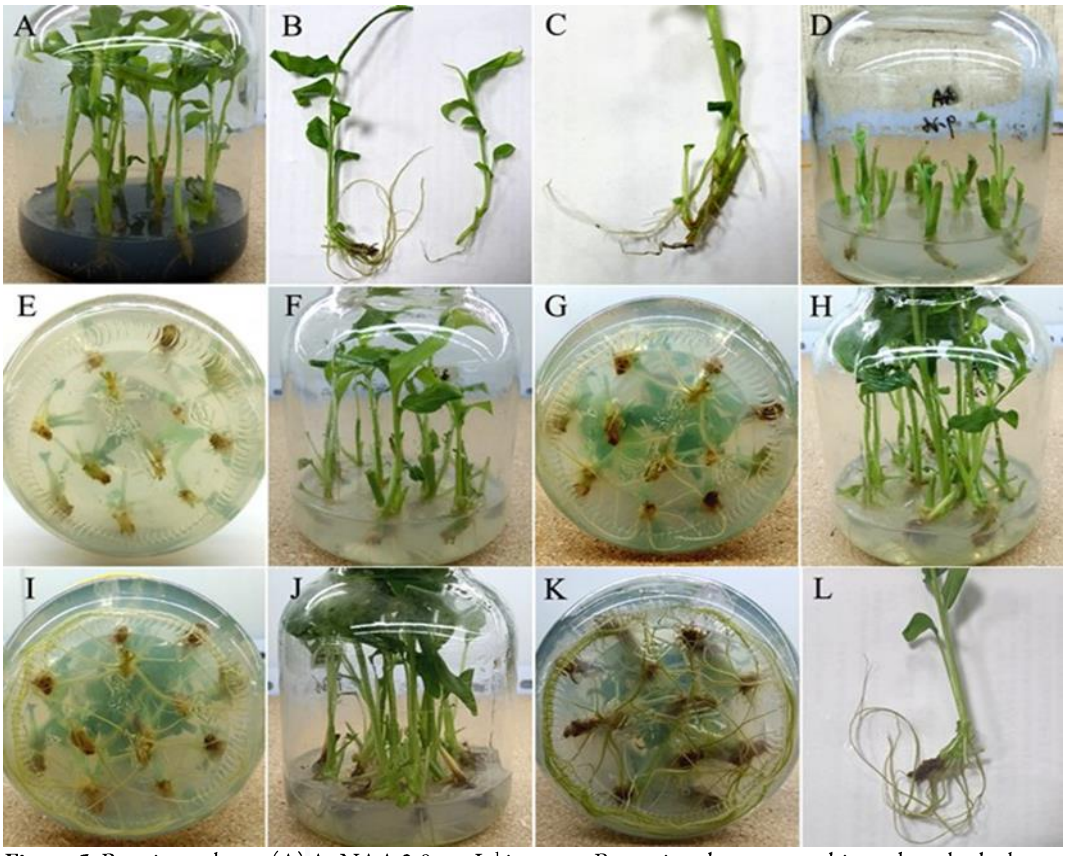

Figure 5. Rooting culture. (A) At NAA 2.0 mg·L^{-1} in group B, rooting shoots were thin and weak, the leaves could not expand normally; (B) Left is group A rooting seedling, right is group B rooting plantlet; (C) In group B, plantlets were not developed, the number of taproots was small, and the fibrous roots were weak; (D-E) Adventitious roots appeared and leaf buds began to germinate after 15 days; (F-G) New adventitious roots appeared continuously, petiole elongated and leaves gradually expanded after 30 days; (H-I) The adventitious roots became thicker and further elongated, and the plants grow rapidly after 45 days. (J-K) Plantlets were strong with thick roots, and the plants were dark green after 60 days; (L) Indeterminate roots of test-tube plantlets

Domesticated transplanting and others

The survival rate of transplanted *in vitro* after 60 days was 100% by domestication (Figure 6A). After 120 days, petiole elongated, leave gradually spread and new leaves grew rapidly (Figure 6B-C). The paddy field grew well after 90 days of transplanting (Figure 6D).

It was noteworthy from this study, the swollen rhizome induced by single factor 2, 4-D group was used as explants that transplanted into the optimal proliferation medium obtained by complete combination and orthogonal experiment, which had no adventitious bud formation (Figure 6E-F). Developing for 60-80 days, the basal of plant further expanded and a few calli were produced, but plant had no obvious growth, and gradually browned and died. Basal cluster bud was observed by microscope, known that all adventitious buds on nodes of basal stem were found and without callus occurrence (Figure 6G-H).

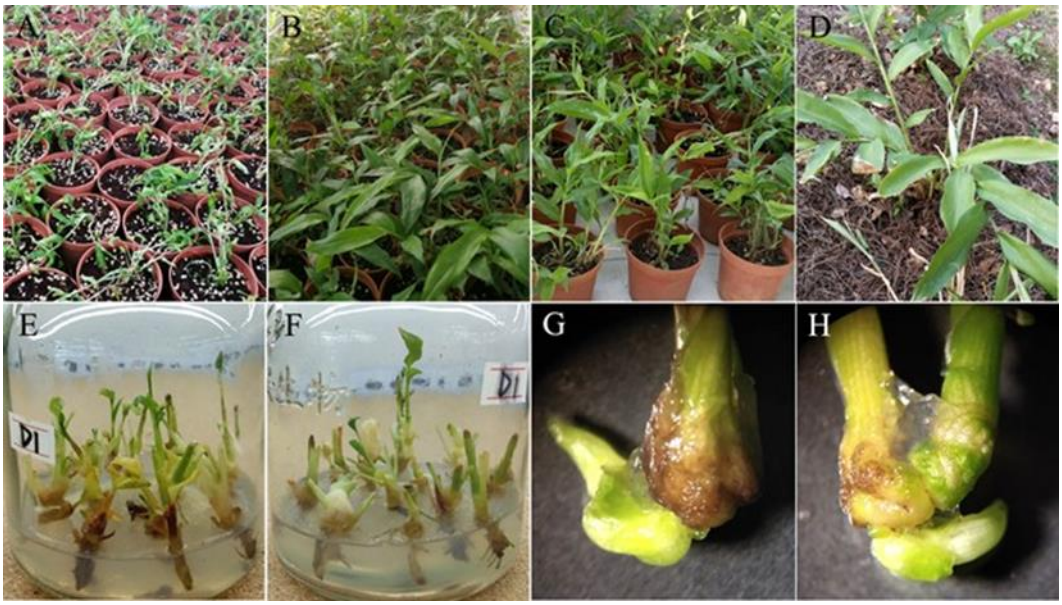

Figure 6. Domesticated transplanting and others. (A) Transplanted in vitro after 60 days; (B-C) Transplanted in vitro after 120 days; (D) Growth of paddy field after 90 days of transplanting; (E) Materials in completely combine t experiments; (F) Materials in orthogonal combination experiments; (G-H) Microscopic view of basal stem cluster buds occur. 40X

Discussion

The proliferation mode and nodal propagation phenomenon of A. villosum

According to report, the culture method of rhizome or tuber is direct organ and indirect organ occurrence (Nayak, 2000; Zhao *et al.*, 2003; Diao *et al.*, 2011; Bisht *et al.*, 2012; Li *et al.*, 2017; Behera *et al.*, 2018). Rhizome of *A. villosum* could form cluster adventitious buds during the process of proliferation, as called basal stem cluster buds. Views on the origin of basal stem cluster bud were different in different researcher. About *Codonopsis bulleyana* Forest ex Diels (Wang *et al.*, 2018), the researcher argues that the basal stem adventitious buds were obtained by indirect organogenesis method with callus to adventitious bud. In *Lycium ruthenicum* Murr. (Li *et al.*, 2020) and *Halocnemum strobilaceum* (Pall.) Bieb. (Ti *et al.*, 2016), researchers contend that basal stem cluster buds actually were clump axillary bud, namely direct organ occurrence. In the complete combination and orthogonal experiment of *A. villosum* added with 2, 4-D, a partial cluster buds were very similar to adventitious buds of callus redifferentiation in morphology. Through microscopic observation and comparison, found that the clustered buds all occurred at nodes. As a general rule bud that grows on a branch with a fixed position was called definite bud, while buds without a certain position from internode of stem, root or leaf were called adventitious buds. Basal stem of *A. villosum* extremely shortened to like root with abundant nodes, which formed multiple buds in a certain place and the buds were so many that vision was confused. On the other hand, in the culture only enlargement of basal nodes was observed, but the callus with differentiation ability did not appear. Meanwhile, have a distinct connection by the vascular bundle in between cluster bud and plantlet, so it could be judged as fixed bud. Therefore, this study believed that the proliferation method of basal stem cluster bud in *A. villosum* was essentially axillary bud sprout, namely direct organ occurrence. In the process culture, effect of enlarging the contact area between material and medium was achieved by loose callus, which helped the material to get nutrients more efficiently from medium.

In addition, in the complete combination experiment with the addition of 2, 4-D and especially in the orthogonal experiment, the base of basal stem cluster bud appeared white expansion in the early stage of culture.

With the extension of culture time, the white expansion decreased and even disappear, internode of basal stem cluster bud stretched, adventitious roots were generated from node and without lateral bud occurrence. A completely new material (each material with 1-2 nodes) was obtained by cutting the basal stem cluster bud. This phenomenon was called node propagation in this study. About description of the morphology and structure of *A. villosum* in Flora of China, the stem was an abnormal stem that shape like root, growth is scattered and prostrate on the ground (Flora of China Editorial Committee and Chinese Academy of Sciences, 1982). Propagation from nodes in *A. villosum* similar to stolon, which can induction of leaf and adventitious root per node, and can grow into a new individual after leaving a female parent. Stolon grows in a horizontal direction and only internode of branches adjacent to base is longer. Shoots produced by nodes propagation are upward growing and internodes elongate continuously with plant growth. To conjecture under effect of exogenous PGRs and especially 2, 4-D in the early stage, promoting nutrients accumulate at the base of rhizome bud which showed a white swelling shape. Subsequently in the later made the new buds formed the overground branches that grow behind the ground, the internodes became longer at the same time, and adventitious roots appeared on the nodes. However, no lateral bud formed on the nodes, and the nodes grew upward one by one, internodes were larger or smaller. This moment, the white swelling reduced or disappeared. The proliferation coefficient of *A. villosum* bottle plantlet greatly increased by propagation nodes and basal stem cluster buds. Proliferation coefficient in this study far more than the existing reports of Zingiberaceae plants, can not only meet the need of large-scale production, but also maintain the excellent character of the female parent, and is the most effective proliferation mode for artificial rapid propagation.

Synergistic effect of exogenous PGRs during proliferation

Exogenous PGRs play an important role in regulating plant regeneration *in vitro*. Different kinds of PGRs and mass concentration have extremely important effects on regulating cell dedifferentiation, proliferation, growth, morphological structure and other aspects (Fu *et al.*, 2018; Kumari *et al.*, 2018). Available with proliferation coefficient as standard, in artificial propagation of *A. villosum*, orthogonal experiment > complete combination experiment > single factor experiment. It proves that the synergistic effect of multiple factors is more conducive to proliferation. With the rapid development of tissue culture technology in 1970s to 1980s, many researchers have pointed out that cytokinin exists in free form in plants, and its main synthesis site is root tip, which can stimulate RNA synthesis and regulate cell cycle (Van-Staden and Davey, 1979; Chen *et al.*, 1985; Sundberg *et al.*, 1991). Auxin not only promotes cell elongation, but also stimulates vascular bundle differentiation and participates in bud and root differentiation. For example, the main reason plants grow fastest in the spring is that the tender buds produce auxin to stimulate cell division of cambium (Borthakur *et al.*, 1998; Aloni, 2010; Xi *et al.*, 2020). Therefore, in plant tissue culture, cytokinin and auxin need to be added simultaneously in proliferation culture stage, and only appropriate auxin should be added in rooting culture. In this study, based on the unsatisfactory proliferation effect of three complete combination experiments. Four PGRs with 6-BA, NAA, 2, 4-D and 6-BA, KT, 2, 4-D were used as factor respectively to perform two groups of $L_9(3^4)$ orthogonal experiments, which found that there was no significant difference between the result and the optimal multiplication coefficient of complete combination. Meanwhile, the phenomenon of node reproduction was not obvious, so it was not included in the text writing. Then the results of $L_{16}(4^5)$ orthogonal experiment showed that satisfactory proliferation effect was obtained in basal stem cluster bud occurrence or propagation from nodes, which indicated that the synergistic effect of many factors among 6-BA, KT, 2, 4-D and NAA was particularly significant in the proliferation culture of *A. villosum*. Because the simultaneous use of two kinds of cytokinin and two kinds of archusia requires huge workload, such examples are rare. According to the results of this study, it is speculated that the reason why *A. villosum* is insensitive to one or two or even three exogenous PGRs is that it is born in the shade and humidity of low-altitude mountain areas. Its physiological activity is vigorous all the year round, a large number of endogenous cytokinin and auxin

are accumulated in plants especially underground rhizomes. Meanwhile, it explants also the reason why the four exogenous PGRs used all high concentration in the multiplication culture of *A. villosum*.

It is particularly noteworthy that the role of 2, 4-D in the proliferation of *A. villosum*. On the whole, although 2, 4-D is usually used to induce callus and promote root growth, it also strongly inhibits bud formation and affects organ development. In the process of proliferation of *A. villosum*, although 2, 4-D (0.5 to 1.5 $mg{\cdot}L^{-1}$) alone caused irreversible damage in the culture, the combination of 2, 4-D with other PGRs directly lead to the phenomenon of nodes propagation. It is speculated that in the early stage of culture, the white swelling was induced at the base of rhizome bud by 2, 4-D alone, and the white swelling not disappeared, but a large number of calli without redifferentiation ability were produced after partial ruptured. On the other hand, under the combined action of 2, 4-D and other PGRs, the white swelling was transformed into the material basis of the back-ground growth, thus resulting in the unique phenomenon of nodes propagation.

The culture of rooting

Adventitious roots all occur in the single factor, complete combination and orthogonal experiment of *A. villosum*. The phenomenon that root and bud of Zingiberaceae occur at the same time had been reported in *Alpinia officinarum* Hance (Borthakur *et al.*, 1998), *Kaempferia galanga* Linn. (Shirin *et al.*, 2000), *Kaempferia galanga* Linn. (Behera *et al.*, 2018), *A. calcarata* Rosc. (Bhowmik *et al.*, 2016). Similar to *A. calcarata*, *A. villosum* can also proliferate and take root simultaneously. However, plantlets obtained were weak, had slender roots, and the survival rate of domestication and transplantation was very low, so culture of rooting was conducted separately. In this study, although the addition of AC provided a dark condition for the rooting of *in vitro* plantlets, due to the non-selective adsorption of AC, the concentration of organic compounds and exogenous PGRs may be reduced in the culture medium, resulting in weak plantlet of rooting, more fibrous roots, easy breakage and significantly reduced number of adventitious roots, which was not conducive to transplanting. Therefore, AC should not be used in the rooting process of artificial rapid propagation of *A. villosum*.

Conclusions

In this study, the rhizome of *A. villosum* was used as explant to establish a complete and efficient rapid propagation system. Compared with similar studies, the proliferation rate of plantlets and survival rate of transplantation have been substantially improved. In the proliferation process of basal stem cluster bud, the unique phenomenon of node propagation made the proliferation coefficient greatly increased. The phenomenon of node propagation was first found in Zingiberaceae plants, which provided a feasible idea for the propagation of other plants in this family *in vitro*. The research results laid an experimental foundation for large-scale production, and the genetically stable and high-quality plantlets were obtained in a short time to improve artificial planting efficiency and solve the market demand and quality problems. What's more, 2, 4-D played a key role in node propagation, and its mechanism was worthy of further study, especially in molecular aspects.

Authors' Contributions

Writing, editing and experimental strategy: JR-G; Data curation: YR, JR-G; Investigation and Inoculation: XM, YR, JR-G; Funding acquisition and review: FR-X; Supervision, review and guide: HY-H. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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