

In vitro adventitious regeneration and plantlet transplantation of *Atractylodes chinensis* (DC.) Koidz., a valuable medicinal plant

Wenhao Jia (✉ jiawenhao32@163.com)

Jilin Agricultural University <https://orcid.org/0009-0007-0090-6282>

Junbo Rong

Jilin Agricultural University

Mengyang Zhang

Jilin Agricultural University

Wenyue Peng

Jilin Agricultural University

Xutong He

Jilin Agricultural University

Xiujuan Lei

Jilin Agricultural University <https://orcid.org/0000-0002-3188-2152>

Yingping Wang

Jilin Agricultural University

Research Article

Keywords: *Atractylodes chinensis*, Regeneration, Plantlet transplantation, Genetic stability

Posted Date: April 11th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2772597/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Version of Record: A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on July 18th, 2023. See the published version at <https://doi.org/10.1007/s11240-023-02573-9>.

Abstract

Triploid *Atractylodes chinensis* (DC.) Koidz. (*A. chinensis*) is a valuable genetic resource for medicinal plant breeding, and there is a demand to develop an efficient *in vitro* regeneration protocol to resolve triploid sterility in production. In this study, the effects of sterilization, bud type, 6-benzyladenine (6-BA) combined with 1-naphthaleneacetic acid (NAA) or indole-3-butyric acid (IBA) and transplanting materials on shoot regeneration, rooting and growth were explored. The genetic stability of regenerated plants was verified using flow cytometry (FCM) and simple sequence repetition (SSR) molecular markers. When axillary buds were sterilized with sodium hypochlorite for 30 min and cultured on Murashige and Skoog (MS) + 1.5 mg·L⁻¹ 6-BA + 0.4 mg·L⁻¹ NAA medium, the regeneration rate was 61.33%. Strong roots occurred on 1/2 MS combined with 3% sucrose and 0.6 mg·L⁻¹ IBA medium. Rooting plantlets transplanted into a mixture of peat soil: perlite (1:1, v/v), the survival rate up to 90.00%. Histological analyses revealed that shoot meristematic cells derived from cambium cells developed into the apical meristem and formed shoots. FCM analysis determined that the ploidy levels of 20 random regenerated plants were coincident with explants, the number and size of bands obtained by 6 pairs of SSR polymorphic primers were consistent. The results of this study show that our *in vitro* adventitious regeneration and plantlet transplantation protocol of triploid *A. chinensis* was efficient and produced strong genetic stability.

Key Message

In this study, an efficient and genetically stable adventitious regeneration of triploid *A. chinensis* was established for the first time.

Introduction

Atractylodes chinensis (DC.) Koidz. (*A. chinensis*) is a perennial herb that belongs to Asteraceae family. This plant is a main source of *Atractylodis Rhizoma* (Cangzhu), which is a traditional Chinese medicine (TCM) that is mainly distributed in the northeast of China and widely used in East Asian countries for the treatment of rheumatic diseases, digestive disorders, night blindness, and influenza (Chen et al. 2017). In China, *A. chinensis* was identified has a proven respiratory regulation effect and has played a critical role in the fight against COVID-19 as an important component of the Lung Cleansing and Detoxifying Decoction formula (Xu et al. 2021). Therefore, the demand and price of *A. chinensis* has increased significantly.

Atractylodes plants are usually diploid with 24 chromosomes (Hiraoka 1998), and a triploid mutant plant which has high yield was found when we did the fourth National Survey of TCM resources in China. Genome duplication of plants are always accompanied by quality improvement and yield increase compared to closely related diploids (Renny-Byfield and Wendel 2014).Chromosome doubling also affects the yield of secondary metabolites, *e.g.* increased content of terpene in *Cannabis sativa* L. (Parsons et al. 2019), of total flavonoid and gastrodin in *Anoectochilus formosanus* Hayata (Chung et al. 2017), as well as a increased content of organic acid, carotenoids in the *Citrus unshiu* Marcow (Sudo et al. 2021).Considering these, polyploid breeding has great advantages to the production of medicinal plants which are used as crude drugs. However, triploid sterility limited its application in *A. chinensis* breeding.

Micropropagation is a good method for the reproduction of clones and has been widely used with triploid Bermuda grass and triploid *Populus* (Lu et al. 2006; Zeng et al. 2019). To the best of our knowledge, there is no report on the micropropagation and genetic analysis of *A. chinensis*. In the present study, we use stem segments with buds to establish the triploid *A. chinensis* adventitious regeneration protocol and evaluate its genetic stability using SSR molecular markers and FCM, respectively. To explore the regeneration processes, the histological changes of the shoot during *in vitro* culture were observed using optical microscopy. This study can provide an effective method for the biotechnological breeding and genetic transformation of *A. chinensis*, and has great practical significance for the protection and utilization of the resources of this species.

Materials And Methods

Plant material

Donor triploid *A. chinensis* plants (Fig. 1a) were obtained from the Medicinal Botanical Garden of Jilin Agricultural University where they were located by the scientific researchers during breeding. Vigorous stem segments with axillary or terminal buds were used as explants.

Sterilization Of Explants And Induction Of Shoots

The explants were cut to a length of approximately 1 cm. A three-stage sterilization protocol was then applied to the explants: 75% alcohol solution for 30 s, followed by three different sterilants for varying time periods, and finally sterile water with 5 rinses. The explants were placed on sterile filter paper, the surface water was dried, and the samples were transferred to MS medium containing different concentrations of 6-benzyladenine (6-BA) and 1-Naphthaleneacetic acid (NAA). Three explants were placed in each bottle and ten bottles were used per experiment; each experiment was repeated 3 times. The cultures were incubated at $22 \pm 1^\circ\text{C}$ under a 16 h photoperiod at a light intensity of $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by white fluorescent tubes. After 2 weeks, the sterilization effects were investigated. The shoot and callus induction rates were calculated after 4 weeks. The survival rate was calculated as the number of viable explants/total number of explants. The regeneration rate was calculated as the number of induced explants/total number of explants.

Proliferation And Rooting Culture

The shoots were cut to a single shoot or multiple shoots with a small clump after 5 weeks, transferred to proliferation medium (MS with $1.5\text{mg}\cdot\text{L}^{-1}$ 6-BA and $0.4\text{mg}\cdot\text{L}^{-1}$ NAA), and placed in a growth chamber with a 16-h photoperiod ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$ from cool white fluorescent tubes) at $22 \pm 1^\circ\text{C}$. When the shoots grew to 3–5 cm, they were transferred to MS rooting media containing different concentrations of sucrose, NAA, and indole-3-butyric acid (IBA) and placed in a growth chamber with a 16-h photoperiod ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$ from cool white fluorescent tubes) at $22 \pm 1^\circ\text{C}$. The rooting time of each treatment was then recorded. After 4 weeks, the number of roots and root length were measured and the rooting rate was calculated.

Acclimatization And Transplanting

Plantlets with root lengths of approximately 3–5 cm, 5–7 cm, and 7–9 cm were selected and transferred to the greenhouse. Following 1 week of acclimatization after opening the bottle cap, the plantlets were extracted and the culture medium attached to the roots was removed by rinsing. The plantlets were transplanted to a sterile mixture of vermiculite, peat soil, and perlite with different volume ratios at room temperature for 4 weeks, during which time they were watered every 2 days to keep the leaf surfaces moist. The survival rate, root length, and growth situation were investigated after 8 weeks.

Histological Observations

After the shoots were transferred to the proliferation medium, the regeneration sites were removed every 5 days and fixed with 50% FAA [formalin: glacial acetic acid: 50% ethanol = 5:5:90 (v/v/v)] for 48 h, and then embedded in paraffin. Paraffin sections (10 μm thick) were obtained with a Fully Automated Rotary Microtome Leica RM2265. The samples were dyed using the safranin fast green staining method, observed with a LEICA DFC7000 T light microscope after sealing, and photographs were retrieved using LAS X software. The histological changes of the shoot tissue cells were analyzed.

Flow Cytometry Analysis Of Ploidy Level

Fresh leaves collected from the transplants and diploid *A. chinensis* plants were washed with distilled water and wiped dry with filter paper. The leaves were cut to approximately 0.5 cm^2 and immersed for 60 s in 200 μL of the nuclei extraction buffer of the ploidy analysis kit (CyStain UV Precise P, Sysmex Corporation, Chuo-ku, Kobe, Hyogo, Japan). After incubating the obtained nuclear suspension for 3 min, the solution was filtered through 50 μm CellTrics filters (Sysmex Corporation) into sample tubes. Staining buffer (800 μL) from the ploidy analysis kit was added to the nuclear suspension, mixed well, and used for analysis after dyeing for 60 s. The filtrate was analyzed using a CytoFLEX flow cytometer with a detection wavelength of 355 nm, and the number of nuclei collected was 5×10^3 . The data were collected and analyzed using the CytExpert software of the flow cytometer.

Genetic Analysis With Ssr Molecular Markers

Fresh leaves (100 mg each) of 20 transplanted plants were randomly collected, and total DNA was extracted using the plant genome DNA extraction kit (DP350-03, Beijing Tiangen Biochemical Technology Co., Ltd., Beijing, China). DNA integrity was detected using 1% agarose gel electrophoresis, and the purity and concentration of DNA were determined using the Implen NanoPhotometer N50-Touch (Implen GmbH, Schatzbogen, München, Germany). DNA was diluted to a final concentration of 50 $\text{ng} \cdot \mu\text{L}^{-1}$ and stored at -20°C for future use. The primers used in the experiment were developed by Shakeel et al. (2016) based on *A. lancea* transcriptome sequencing. Thirty pairs of primers were randomly selected and synthesized by Shanghai Sangon Bioengineering Co., Ltd (Shanghai, China). PCR reactions were performed in 20 μL reaction volumes, containing 1 μL genomic DNA, 1 μL of each primer (10 pmole), 10 μL of 2 $\times$ PCR Master

Mix (Shanghai Beyotime Biotechnology Co., Ltd., Shanghai, China), and 7 μL ddH₂O. The PCR procedure was performed at 94°C for 5 min, followed by 35 cycles each at 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s, with a final extension at 72°C for 5 min. The PCR amplification products were subjected to electrophoresis with 6% polyacrylamide at 180 V for 3 h, dyed with silver nitrate, and photographed.

Data analysis

All experiments were repeated three times. The data were reported as means $\pm$ standard errors (SE). Means were analyzed by analysis of variance and significant differences between means were compared using the least significant difference (LSD) test with SPSS v.26.0. For all comparisons, statistical significance was considered at $P < 0.05$.

Results

Sterilization of explants

Surface sterilization of explants is the basis for successful cultures *in vitro*. In this study, sterilant type and sterilization time were analyzed to find a suitable sterilization protocol for the explants. The results showed that the survival and regeneration rates of explants treated with 2% sodium hypochlorite (SH) for 30 min were 64.33% and 56.67% respectively, which were higher than those of the other treatments (Table 1). Although the contamination rate of explants after 0.1% mercuric chloride (MC) treatment for 15 min was only 11.00%, the survival and regeneration rates were lower than those of the other treatments. Moreover, the regeneration effect of explants after 0.05% chlorine dioxide (CD) treatment was similar to that of the 2% SH treatment group, although contamination was considerable. After 30 min of sterilization, the contamination rate was still as high as 31%. The sterilization protocol we selected for the explants of *A. chinensis* was 75% alcohol treatment for 30 s, 2% SH for 30 min, and sterile water rinse with 5 replications.

Table 1
Effect of different sterilization protocols on explants

Disinfectant	sterilization time(min)	Contamination rate(%)	Survival rate(%)	Regeneration rate(%)
0.1%MC	5	24.33 ± 2.31 ^d	37.67 ± 4.04 ^d	35.33 ± 4.04 ^c
	10	15.67 ± 2.31 ^e	28.00 ± 1.73 ^e	25.67 ± 2.31 ^d
	15	11.00 ± 1.73 ^e	21.00 ± 1.73 ^f	17.00 ± 0.00 ^e
2%SH	20	44.33 ± 5.13 ^{ab}	49.00 ± 3.46 ^c	45.67 ± 2.31 ^b
	25	25.67 ± 2.31 ^d	54.33 ± 2.31 ^b	49.00 ± 1.73 ^b
	30	15.67 ± 2.31 ^e	64.33 ± 2.31 ^a	56.67 ± 3.51 ^a
0.05%CD	20	47.67 ± 4.04 ^a	49.00 ± 1.73 ^c	45.67 ± 2.31 ^b
	25	40.00 ± 3.00 ^b	52.00 ± 4.04 ^{bc}	48.00 ± 1.73 ^b
	30	31.00 ± 1.73 ^c	48.00 ± 1.73 ^c	44.67 ± 4.04 ^b

Different letters in the same column indicate significant differences according to the LSD test($P \leq 0.05$) following ANOVA

Shoot Induction

After 1 week of incubation, new shoots were observed in some of the explants. After 3 weeks, an increasing number of shoots had appeared and elongated (Fig. 1b), while after 5 weeks, cluster shoots and juvenile leaves were visible on the explants (Fig. 1c). At different concentrations of 6-BA and NAA, the shoot induction rates of terminal and axillary buds in the 5th group were higher than those of the other groups, with 52.33% and 61.33%, respectively (Table 2). In general, as the concentrations of 6-BA and NAA increased, the induction of shoots initially increased and then decreased. Further study on the induction rates of different explants suggested that the shoot induction rate of axillary buds was higher than that of terminal buds for the majority of the treatments (Fig. 2a). However, the callus induction rate for terminal buds was higher than that in axillary buds, except for a few treatments in which no callus regeneration occurred (Fig. 2b).

Table 2
Effects of different concentrations of 6-BA and NAA on shoot and callus induction

Treatment	Plant hormone (mg·L ⁻¹)		Terminal bud		Axillary bud	
	6-BA	NAA	Shoot induction rate(%)	Callus induction rate(%)	Shoot induction rate(%)	Callus induction rate(%)
1	1.0	0.2	15.67 ± 2.31 ^f	0 ^d	19.00 ± 1.73 ^e	0 ^d
2		0.4	24.33 ± 2.31 ^{de}	29.00 ± 1.73 ^b	27.67 ± 4.04 ^d	21.00 ± 3.46 ^b
3		0.6	13.33 ± 3.51 ^f	42.33 ± 5.03 ^a	18.00 ± 1.73 ^e	35.67 ± 2.31 ^a
4	1.5	0.2	29.00 ± 1.73 ^d	0 ^d	35.67 ± 2.31 ^c	0 ^d
5		0.4	52.33 ± 5.03 ^a	19.00 ± 1.73 ^c	61.33 ± 5.13 ^a	13.00 ± 0.00 ^c
6		0.6	38.67 ± 5.13 ^c	27.00 ± 0.00 ^b	40.00 ± 3.00 ^{bc}	19.00 ± 1.73 ^b
7	2.0	0.2	22.00 ± 1.73 ^e	0 ^d	28.00 ± 1.73 ^d	0 ^d
8		0.4	43.00 ± 0.00 ^{bc}	0 ^d	44.67 ± 4.04 ^b	0 ^d
9		0.6	44.67 ± 4.04 ^b	15.67 ± 2.31 ^c	39.00 ± 1.73 ^c	11.00 ± 1.73 ^c

Different letters in the same column indicate significant differences according to the LSD test($P \leq 0.05$) following ANOVA

Rooting Culture

Plantlets grew rapidly in the proliferation medium. After 2 weeks, new shoots emerged and each single shoot grew to a cluster of shoots (Fig. 1d). In 4 weeks, an increased number of shoots occurred and juvenile leaves spread (Fig. 1e). The continuous extension of the shoots caused greater luxuriance in the plantlets. After rooting the culture for 1 month, significant differences were observed in rooting time, rooting rate, root number, and root length of plantlets under different rooting media (Table 3). In 1/2MS medium with increased sucrose concentrations, the rooting time advanced and the rooting rate decreased. When 3% sucrose and 1.0 mg·L⁻¹ NAA were applied, plantlets rooted earlier than those of other combinations, with an average of 7.7 days. After the addition of 1% sucrose and 1.0 mg·L⁻¹ NAA, the rooting rate increased to the 94.33%, which was the highest of all treatments. With the same sucrose concentration, higher concentrations of IBA and NAA correlated to a greater root number and length. The root number and root length of plantlets cultured in 1/2MS + 3% sucrose + 0.6mg·L⁻¹ IBA medium were higher than those of other combinations, averaging 7.7 roots and 9.2 cm, respectively. The root morphology of different rooting media was significantly different. In the 1/2MS + 3% sucrose + 0.6 mg·L⁻¹ IBA medium, the number of roots was

high, and the roots were thin and long (Fig. 1f). In contrast, the 1/2MS + 1% sucrose + 0.6 mg·L⁻¹ IBA medium produced a lower number of roots, and these roots were short and severely lignified. (Fig. 1g).

Table 3
Effect of different concentrations of sucrose, NAA and IBA on rooting culture MS media after culturing for 30 days

1/2MS +			Rooting time(day)	Rooting rate(%)	Roots no. (mean ± SE)	Root length(cm ± SE)
Sucrose(%)	IBA (mg·L ⁻¹)	NAA (mg·L ⁻¹)				
1	-	0.5	20.3 ± 1.0 ^a	80.00 ± 0.00 ^{bc}	2.6 ± 0.50 ^j	3.5 ± 0.15 ^j
	-	1.0	17.2 ± 0.9 ^b	94.33 ± 5.13 ^a	3.3 ± 0.46 ⁱ	4.0 ± 0.06 ⁱ
	0.3	-	19.6 ± 0.9 ^c	78.00 ± 1.73 ^c	3.8 ± 0.44 ^h	4.0 ± 0.12 ⁱ
	0.6	-	15.2 ± 1.0 ^e	83.33 ± 3.51 ^b	4.4 ± 0.50 ^f	5.2 ± 0.22 ^h
2	-	0.5	14.8 ± 1.1 ^e	70.00 ± 3.00 ^d	4.1 ± 0.31 ^g	5.5 ± 0.17 ^g
	-	1.0	13.3 ± 0.9 ^f	81.00 ± 3.46 ^{bc}	4.9 ± 0.39 ^e	5.7 ± 0.14 ^f
	0.3	-	16.0 ± 0.7 ^d	65.67 ± 2.31 ^d	5.4 ± 0.51 ^d	6.0 ± 0.12 ^e
	0.6	-	13.5 ± 0.8 ^f	78.00 ± 1.73 ^c	5.8 ± 0.37 ^c	7.1 ± 0.11 ^c
3	-	0.5	11.9 ± 0.9 ^g	54.33 ± 2.31 ^{ef}	5.6 ± 0.49 ^{cd}	6.2 ± 0.32 ^d
	-	1.0	7.7 ± 1.2 ^h	67.67 ± 4.04 ^d	6.7 ± 0.46 ^b	7.2 ± 0.23 ^b
	0.3	-	11.9 ± 0.7 ^g	50.00 ± 0.00 ^f	6.7 ± 0.42 ^b	8.0 ± 0.45 ^b
	0.6	-	8.3 ± 1.0 ^h	58.00 ± 1.73 ^e	7.7 ± 0.47 ^a	9.2 ± 0.39 ^a

Different letters in the same column indicate significant differences according to the LSD test ($P \leq 0.05$) following ANOVA

Acclimatization And Transplanting

Plantlets with a height of approximately 3–5 cm and a root length of 3–9 cm were acclimatized and transplanted (Fig. 1h). In the 2–3 weeks after transplanting, old leaves withered and juvenile leaves grew. After 4 weeks, the leaves expanded and thickened, their color deepened, and the outer edge spikes hardened (Fig. 1i). The effects of different cultivation mixtures on the survival rate of transplanted plantlets significantly differed (Table 4), and the effect on the root morphology of regenerated plantlets also varied. In the same mixture, plants with longer initial root lengths showed higher survival rates. When the initial root length was approximately 7–9 cm and the transplanting mixture consisted of peat soil: perlite (1:1, v:v), the transplanting survival rate was 90.00%, which was the highest observed rate. Eight weeks later, the mixture of peat soil: perlite (1:1, v:v) revealed a higher survival rate than those of other mixtures, with robust stems and leaves, deep green leaves, and thick roots (Fig. 1j). In contrast, the mixture of peat soil: perlite: vermiculite (1:1:1, v:v:v) showed relatively long and thin roots. The aboveground parts of the plantlets were soft and the leaves were light in color (Fig. 1k).

Table 4
Effect of different mixtures on plantlet transplanting of *A. chinensis*

Cultivation mixture(v:v)	Root length(cm)	Transplanting number	Transplanting survival number	Transplanting survival rate (%)	Root length(cm ± SE)	Growth situation
peat soil: perlite (2:1)	3 ~ 5	20	13	54.33 ± 2.31 ^{fg}	6.8 ± 0.33 ^h	Short taproot and thin lateral root
	5 ~ 7	20	14	61.00 ± 1.73 ^e	8.6 ± 0.40 ^g	
	7 ~ 9	30	22	64.33 ± 2.31 ^e	10.7 ± 0.22 ^d	
peat soil: perlite (1:1)	3 ~ 5	20	15	72.00 ± 1.73 ^d	9.1 ± 0.28 ^f	Strong taproot and a few lateral roots
	5 ~ 7	30	26	82.33 ± 4.04 ^b	10.0 ± 0.66 ^e	
	7 ~ 9	30	27	90.00 ± 3.00 ^a	14.1 ± 0.36 ^b	
peat soil: perlite: vermiculite (2:1:1)	3 ~ 5	30	22	71.00 ± 1.73 ^d	8.5 ± 0.21 ^g	Strong taproot and a few lateral roots
	5 ~ 7	20	16	77.00 ± 0.00 ^c	9.9 ± 0.24 ^e	
	7 ~ 9	30	26	81.00 ± 1.73 ^b	14.4 ± 0.40 ^b	
peat soil: perlite: vermiculite (1:1:1)	3 ~ 5	20	12	51.00 ± 1.73 ^g	8.6 ± 0.22 ^g	Long taproot and few lateral roots
	5 ~ 7	30	20	54.33 ± 2.31 ^{fg}	11.7 ± 0.58 ^c	
	7 ~ 9	20	14	57.00 ± 0.00 ^f	15.4 ± 0.37 ^a	

Different letters in the same column indicate significant differences according to the LSD test($P \leq 0.05$) following ANOVA

Histological Observations

The histological regeneration process of shoots was observed using the paraffin sections. During shoot proliferation, observations on the 5th day suggested that the cambium cells began to undergo periclinal division, after which they continued to divide to form smaller meristematic cells (Fig. 3a, b). On the 10th day, polar growth of the meristematic cells formed the apical meristem through cortex direction. The continuous division and proliferation of the apical meristem promoted the lateral protrusion of the leaf primordium, thereby enhancing this feature (Fig. 3c). Observations on the 15th day showed that differentiation occurred

from the apical meristem into shoots, and the leaf primordium into juvenile leaves. In addition, new undifferentiated leaf and axillary bud primordia developed which would further differentiate into shoots and juvenile leaves (Fig. 3d).

Ploidy Level Determination

The chromosome ploidy of transplanted plantlets of *A. chinensis* was identified by FCM. The position of the peak value of diploid *A. chinensis* in the abscissa is 1.8×10^6 (Fig. 4a, b), and this was used as the control during the chromosome ploidy analysis of the plantlets. According to the relative proportion of DNA content, the position of the peak value abscissa of transplanting plantlets is 2.7×10^6 (Fig. 4c, d), and the ratio to the reference is 1.5, which indicates triploid status.

SSR Analysis Of Genetic Stability

SSR primers (30 pairs) and the genomic DNA of *A. chinensis* from four regions were used to identify primer sets with polymorphic bands and a clear banding pattern, and 6 pairs of SSR primers were selected (Table 5). The genomic DNA of 20 regenerated plantlets was amplified by PCR with the chosen primers. The results revealed that 20 stable and clear bands were amplified by the 6 primer pairs, with an average of 3.83 amplified bands per primer. The size of amplified products ranged from 100 to 500 base pairs. However, further analysis of the electrophoretogram identified that the number and size of bands obtained by each primer were consistent in 20 materials, and that there were no polymorphisms in the bands (Fig. 2). No variation was detected with the 6 SSR primers in the regenerated plantlets of *A. chinensis* at the DNA level.

Table 5
sequences of the 6 pairs of SSR primer used in the study

Primer	Primer sequence F	Primer sequence R
CL2882	GATCTAACTCCCACCAGTTTCCT	GATCTAACTCCCACCAGTTTCCT
CL3515	TGAGCAACTAATTCAGGAGAAGG	TTGGGATGTTGTACACTCTGTTG
CL5771	TAATACCCGTGTTTCATCGTCTCT	GTCTGTTTGAGGAACATCGAATC
CL6819	ATGGAGAGGTCTGTATGGATCTG	GGCTGACTGTTACATCGTAGGTT
CL7052	TCCCCTCCAATAACTCCTAGAAC	GGTTGTTTTCTGTCTGATTAGG
CL7269	TGAAGAACTACACCCTCCTCAC	GACTTAGAACTTTGACCCCGTT

Discussion

Triploid can play an vital role in improving fruit traits, biomass, and abiotic stress tolerance in the future, leading to commercial benefits (Xu et al. 2022; Wang et al. 2022; Lourkisti et al. 2020). However, triploid sterility limits its application in many plants. Although grafting technology can solve triploid culture of partial species (Kaseb et al. 2023; Gakpetor et al. 2017; Trandel et al. 2021), it cannot be applied to all

plants. Consequently, establishing an *in vitro* regeneration protocol of *A. chinensis* is crucial. In the present study, stem segments with axillary or terminal buds were used as explants to rapidly cultivate triploid *A. chinensis* plantlets using micropropagation and plantlet regeneration technology, to provide conditions for the protection and utilization of genetic resources of this species.

Sterilization of explants is the basal in micropropagation. MC and SH are commonly used as explant sterilants (Huang et al. 2022; Rafiq et al. 2021; Chóez-Guaranda et al. 2021). While chlorine dioxide is a new sterilant with a wide range, commonly used in the sterilization of air, water, vegetables and fruits (Xu et al. 2022; Shimabukuro et al. 2020; Smith et al. 2015), it has also been reported to be used for explant sterilization (Maciel et al. 2022). Considering these, we analyzed the effects of the three sterilants at different time, and the results illustrated that although the overall sterilization effect of 0.1% MC was superior to that of 2% SH and 0.05% CD, the regeneration rate of the explants after inoculation was insufficient, indicating that 0.1% MC produced greater damage the explants than that of the other two compounds, with 0.05% CD causing little harm to the explants. If the CD concentration or sterilization time was properly adjusted, this effect could improve.

Shoot regeneration is affected by a variety of factors, of which the proportions of plant growth regulators (PGRs) and bud type are integral (Kirakosyan et al. 2022; Venkatachalam et al. 2015). In our research, it was found that under the same 6-BA and NAA concentration, the shoot regeneration and callus induction rate of different bud types were significantly different. The shoot regeneration rate of axillary buds was generally greater than that of terminal buds, while callus induction revealed the opposite. This is consistent with the research results of Vieitez et al. (2007) with chestnut, presumably due to the inhibitory effects of apical dominance on the development of shoots. With the increased concentrations of 6-BA and NAA, shoot regeneration first increased and then decreased, indicating that excessive PGRs concentrations inhibited the growth of shoots. In the medium with a low concentration of 6-BA and high concentration of NAA, the regeneration rate of the callus was high. The formation of the callus affects the differentiation of shoots and the rooting of plantlets.

IBA and NAA are the commonly used rooting hormones, and it is necessary to provide sucrose for plantlet growth (Amiri and Mohammadi 2021; Martin 2003). In our research, with the increase of IBA, NAA and sucrose concentrations, the rooting time was advanced and the rooting number and root length showed upward trends. However, higher the concentrations of sucrose resulted in lower rooting rates, indicating that high concentrations inhibited rooting (Nilanthi and Yang 2014). At equal sucrose concentrations, the rooting number and root length of the medium supplemented with IBA were higher than those of the medium supplemented with NAA, while the rooting rate showed the opposite effect, which may relate to adaptations of the plantlets to the low concentration of NAA during the growth period. Sreekisson et al. (2021) mentioned this possibility in the study of *Sceletium tortuosum* (L.) N. E. Br.

In the experiment related to plantlet acclimatization, *A. chinensis* plantlets showed an excellent root growth status after they were transplanted to any of two mixtures: peat soil: perlite (1:1) and peat soil: perlite: vermiculite (2:1:1, v:v:v). However, the plantlets transplanted into peat soil: perlite (2:1) and peat soil: perlite: vermiculite (1:1:1) showed a phenomenon of short or thin taproots and few lateral roots. This indicates that

in order to ensure a high survival rate and good growth status of the transplanted plants, the nutrition and air permeability of the transplant substrate are indispensable. The present result is in agreement with the previous findings observed in *Euryodendron excelsum* H. T. Chang (Chen et al. 2020) and *Argania spinosa* (L.) Skeels (Amghar et al. 2021).

A number of tissues can be involved in the formation of shoots, such as the pericycle, subepidermal cells, and epidermal cells, depending on the plant (Atta et al. 2009; Wang et al. 2015). This study suggested that the shoots of *A. chinensis* originated from cambium cells and that the shoots had obvious morphological characteristics at different development stages. The cambium cells first divided and proliferated into meristematic cells that continued to differentiate into apical meristems and leaf primordia, which finally grew into shoots and juvenile leaves, with new leaf and axillary bud primordia appearing. There has been contention regarding the formation sequence of bud and leaf primordia (Bowman and Eshed 2000; Barton and Poethig 1993). However, the results of this experiment show that these features are formed simultaneously in *A. chinensis*.

The rapid propagation of tissue cultures has common chromosome and gene level variations (D'Amato and Bayliss 1985). A number of factors can affect the genetic stability of regenerated plants, including explant type used, *in vitro* culture duration, and plant genotype (Debnath and Ghosh 2022). FCM and DNA molecular markers are effective methods for quickly identifying the existence of these variations (Escobedo-Gracia-Medrano et al. 2018; Sultana et al. 2022). In the present study, the 20 randomly selected regenerated plants of *A. chinensis* was identified using FCM and SSR molecular markers. The results revealed that, similar to the explant materials, all 20 plants were triploid with no chromosomal variation and the number and size of bands obtained by each primer were consistent, no polymorphism was present. This is consistent with several cases of stable genetics have been reported, including axillary bud regeneration of *Hedysarum theinum* Krasnob. plants (Erst et al. 2015), *in vitro* propagation of different strawberry cultivars (Naing et al. 2019), *in vitro* propagation of *Terminalia arjuna* (Gupta et al. 2014), and axillary bud proliferation of chestnut rootstock hybrids (Carvalho et al. 2004).

Conclusion

In conclusion, the current study established an effective *in vitro* regeneration protocol of *A. chinensis*. The findings indicate that it is viable to overcome triploid sterility by micropropagation. Finally, this study showed that the regenerated plants obtained by this method had good genetic stability identified by FCM and SSR molecular markers.

Abbreviations

6-BA	6-Benzyladenine
CD	Chlorine dioxide
FCM	Flow cytometry
MC	Mercuric chloride
IBA	Indole-3-butyric acid
MS	Murashige and Skoog
SH	Sodium hypochlorite
NAA	1-Naphthaleneacetic acid
PGRs	Plant growth regulators
SSR	Simple sequence repetition

Declarations

Author contributions Wenhao Jia, Xiujuan Lei and Yingping Wang contributed to the study conception and design. Material preparation, data collection and analysis were performed by Junbo Rong, Mengyang Zhang, Wenyue Peng and Xutong He. The first draft of the manuscript was written by Wenhao Jia and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This work was supported by the Key Research and Development Project Fund of Jilin Province (20200404005YY).

Conflict of interest The authors declare that they have no conflict of interest.

Consent to participate Informed consent was obtained from all individual participants included in the study

References

1. Amghar I, Ibriz M, Ibrahimi M, et al (2021) In Vitro Root Induction from Argan (*Argania spinosa* (L.) Skeels) Adventitious Shoots: Influence of Ammonium Nitrate, Auxins, Silver Nitrate and Putrescine, and Evaluation of Plantlet Acclimatization. *Plants* (Basel) 10(6):1062. <https://doi.org/10.3390/plants10061062>
2. Atta R, Laurens L, Boucheron-Dubuisson E, et al (2009) Pluripotency of Arabidopsis xylem pericycle underlies shoot regeneration from root and hypocotyl explants grown *in vitro*. *The Plant Journal* 57(4): 626-644. <https://doi.org/10.1111/j.1365-313X.2008.03715.x>
3. Amiri S, Mohammadi R (2021) Establishment of an efficient *in vitro* propagation protocol for Sumac (*Rhus coriaria* L.) and confirmation of the genetic homogeneity. *Sci Rep* 11(1):173. doi: 10.1038/s41598-020-80550-4

4. Bowman JL, Eshed Y (2000) Formation and maintenance of the shoot apical meristem. Trends in plant science 5(3): 110-115. [https://doi.org/10.1016/S1360-1385\(00\)01569-7](https://doi.org/10.1016/S1360-1385(00)01569-7)
5. Barton MK, Poethig RS (1993) Formation of the shoot apical meristem in *Arabidopsis thaliana*: an analysis of development in the wild type and in the shoot meristemless mutant. Development 119(3): 823-831. <https://doi.org/10.1242/dev.119.3.823>
6. Carvalho LC, Goulão L, Oliveira C, et al (2004) RAPD assessment for identification of clonal identity and genetic stability of *in vitro* propagated chestnut hybrids. Plant Cell, Tissue and Organ Culture 77: 23-27. <https://doi.org/10.1023/B:TICU.0000016482.54896.54>
7. Chóez-Guaranda I, García J, Sánchez C, et al (2021) Identification of lupeol produced by *Vernonanthura patens* (Kunth) H. Rob. leaf callus culture. Nat Prod Res 35(3):503-507. <https://doi.org/10.1080/14786419.2019.1636239>
8. Chung HH, Shi SK, Huang B, et al (2017) Enhanced agronomic traits and medicinal constituents of autotetraploids in *Anoectochilus formosanus* Hayata, a top-grade medicinal orchid. Molecules 22(11): 1907. <https://doi.org/10.3390/molecules22111907>
9. Chen F, Wei YX, Zhang JM, et al (2017) Transcriptomics analysis investigates sesquiterpenoids accumulation pattern in different tissues of *Atractylodes lancea* (Thunb.) DC. Plantlet. Plant Cell, Tissue and Organ Culture 130: 73-90. <https://doi.org/10.1007/s11240-017-1205-8>
10. Chen S, Xiong Y, Wu T, et al (2020) Axillary shoot proliferation and plant regeneration in *Euryodendron excelsum* HT Chang, a critically endangered species endemic to China. Scientific Reports 10(1): 14402. <https://doi.org/10.1038/s41598-020-71360-9>
11. D'Amato F, Bayliss MW (1985) Cytogenetics of plant cell and tissue cultures and their regenerates. Critical Reviews in Plant Sciences 3(1): 73-112. <https://doi.org/10.1080/07352688509382204>
12. Debnath SC, Ghosh A (2022) Phenotypic variation and epigenetic insight into tissue culture berry crops. Front Plant Sci 13:1042726. doi: 10.3389/fpls.2022.1042726
13. Escobedo-Gracia-Medrano RM, Burgos-Tan MJ, Ku-Cauich JR, et al (2018) Using flow cytometry analysis in plant tissue culture derived plants. In: Plant Cell Culture Protocols. Humana, New York, pp 317-332.
14. Erst AA, Zvyagina NS, Novikova TI, et al (2015) Clonal micropropagation of a rare species *Hedysarum theinum* Krasnob.(Fabaceae) and assessment of the genetic stability of regenerated plants using ISSR markers. Russian Journal of Genetics 51: 158-162. <https://doi.org/10.1134/s1022795415020076>
15. Gakpetor PM, Mohammed H, Moreti D, et al (2017) Periclinal chimera technique: new plant breeding approach. Genet Mol Res 16(3). doi: 10.4238/gmr16039790
16. Gupta AK, Rai MK, Phulwaria M, et al (2014) In vitro propagation, encapsulation, and genetic fidelity analysis of *Terminalia arjuna*: a cardioprotective medicinal tree. Applied biochemistry and biotechnology 173: 1481-1494. <https://doi.org/10.1007/s12010-014-0920-4>
17. Hiraoka N (1998) *Atractylodes lancea* autotetraploids induced by colchicine treatment of shoot cultures. Biol Pharm Bull 21(5):479-83. <https://doi.org/10.1248/bpb.21.479>
18. Huang T, Zhang H, Zhao R, et al (2022) Establishing an Efficient Regeneration System for Tissue Culture in *Bougainvillea buttiana* 'Miss Manila'. Plants (Basel) 11(18):2372.

<https://doi.org/10.3390/plants11182372>

19. Kirakosyan RN, Kalashnikova EA, Abubakarov HG, et al (2022) Influence of Mineral Treatment, Plant Growth Regulators and Artificial Light on the Growth of Jewel Sweet Potato (*Ipomoea batatas* Lam. cv. Jewel) *In Vitro. Life* (Basel) 13(1):52. <https://doi.org/10.3390/life13010052>
20. Kaseb MO, Umer MJ, Lu X, et al (2023) Comparative physiological and biochemical mechanisms in diploid, triploid, and tetraploid watermelon (*Citrullus lanatus* L.) grafted by branches. *Sci Rep* 13(1):4993. <https://doi.org/10.1038/s41598-023-32225-z>
21. Lourkisti R, Froelicher Y, Herbette S, et al (2020) Triploid citrus genotypes have a better tolerance to natural chilling conditions of photosynthetic capacities and specific leaf volatile organic compounds. *Frontiers in plant science* 11: 330. <https://doi.org/10.3389/fpls.2020.00330>
22. Lu S, Wang Z, Peng X, et al (2006) An efficient callus suspension culture system for triploid bermudagrass (*Cynodon transvaalensis* × *C. dactylon*) and somaclonal variations. *Plant Cell, Tissue and Organ Culture* 87: 77-84. <https://doi.org/10.1007/s11240-006-9138-7>
23. Martin KP (2003) Rapid in vitro multiplication and ex vitro rooting of *Rotula aquatica* Lour., a rare rhoeophytic woody medicinal plant. *Plant Cell Rep* 21(5):415-20. <https://doi.org/10.1007/s00299-002-0547-8>
24. Maciel G, Lopes AA, Cantrell CL, et al (2022) Jasmonates promote enhanced production of bioactive caffeoylquinic acid derivative in *Eclipta prostrata* (L.) L. hairy roots. *Plant Cell, Tissue and Organ Culture* 149, 363–369. <https://doi.org/10.1007/s11240-021-02201-4>
25. Naing AH, Kim SH, Chung MY, et al (2019) In vitro propagation method for production of morphologically and genetically stable plants of different strawberry cultivars. *Plant methods* 15: 1-10. <https://doi.org/10.1186/s13007-019-0421-0>
26. Nilanthi D, Yang YS (2014) Effects of sucrose and other additives on in vitro growth and development of purple coneflower (*Echinacea purpurea* L.). *Advances in Biology* 2014, 2014(1):1-4.
27. Parsons JL, Martin SL, James T, et al (2019) Polyploidization for the genetic improvement of *Cannabis sativa*. *Frontiers in Plant Science* 476. <https://doi.org/10.3389/fpls.2019.00476>
28. Rafiq S, Rather ZA, Bhat RA, et al (2021) Standardization of *in vitro* micropropagation procedure of Oriental *Lilium* Hybrid Cv. 'Ravenna'. *Saudi J Biol Sci* 28(12):7581-7587. <https://doi.org/10.1016/j.sjbs.2021.09.064>
29. Renny-Byfield S, Wendel JF (2014) Doubling down on genomes: polyploidy and crop plants. *American journal of botany* 101(10): 1711-1725. <https://doi.org/10.3732/ajb.1400119>
30. Shimabukuro PMS, Duarte ML, Imoto AM, et al (2020) Environmental cleaning to prevent COVID-19 infection. A rapid systematic review. *Sao Paulo Med J.* 138(6):505-514. <https://doi.org/10.1590/1516-3180.2020.0417.09092020>
31. Sultana KW, Das S, Chandra I, et al (2022) Efficient micropropagation of *Thunbergia coccinea* Wall. and genetic homogeneity assessment through RAPD and ISSR markers. *Sci Rep* 12(1):1683. <https://doi.org/10.1038/s41598-022-05787-7>
32. Smith DJ, Ernst W, Herges GR. (2015) Chloroxyanion Residues in Cantaloupe and Tomatoes after Chlorine Dioxide Gas Sanitation. *J Agric Food Chem* 63(43):9640-9.

<https://doi.org/10.1021/acs.jafc.5b04153>

33. Sreekissoon A, Plačková L, Doležal K, et al (2021) In vitro and ex vivo vegetative propagation and cytokinin profiles of *Sceletium tortuosum* (L.) NE Br.: A South African medicinal plant. *Plant Cell, Tissue and Organ Culture* 145: 191-202. <https://doi.org/10.1007/s11240-020-02001-2>
34. Sudo M, Yasuda K, Yahata M et al (2021) Morphological Characteristics, Fruit Qualities and Evaluation of Reproductive Functions in Autotetraploid Satsuma Mandarin (*Citrus unshiu* Marcow.) *Agronomy* 11(12): 2441. <https://doi.org/10.3390/agronomy11122441>
35. Shakeel A, Zhan C, Yang Y, et al 2016 The Transcript Profile of a Traditional Chinese Medicine, *Atractylodes lancea*, Revealing Its Sesquiterpenoid Biosynthesis of the Major Active Components. *Plos One* 11(3):e0151975. <https://doi.org/10.1371/journal.pone.0151975>
36. Trandel MA, Johanningsmeier S, Schultheis J, et al (2021) Cell Wall Polysaccharide Composition of Grafted 'Liberty' Watermelon With Reduced Incidence of Hollow Heart Defect. *Front Plant Sci* 12:623723. <https://doi.org/10.3389/fpls.2021.623723>
37. Venkatachalam P, Kalaiarasi K, Sreeramanan S. (2015) Influence of plant growth regulators (PGRs) and various additives on *in vitro* plant propagation of *Bambusa arundinacea* (Retz.) Wild: A recalcitrant bamboo species. *J Genet Eng Biotechnol* 13(2):193-200. <https://doi.org/10.1016/j.jgeb.2015.09.006>
38. Vieitez AM, Sánchez MC, García-Nimo ML, et al (2007) Protocol for micropropagation of *Castanea sativa*. In: *Protocols for micropropagation of woody trees and fruits*. Springer, Dordrecht, pp 299-312
39. Wang H, Li M, Yang Y, et al (2015) Histological and endogenous plant growth regulators changes associated with adventitious shoot regeneration from in vitro leaf explants of strawberry (*Fragaria × ananassa* cv.'Honeoye'). *Plant Cell, Tissue and Organ Culture* 123: 479-488. <https://doi.org/10.1007/s11240-015-0851-y>
40. Wang L, Tu M, Li J, et al (2022) Photosynthetic Efficiency and Glyco-Metabolism Changes in Artificial Triploid Loquats Contribute to Heterosis Manifestation. *Int. J. Mol. Sci* 23, 11337. <https://doi.org/10.3390/ijms231911337>
41. Xu F, Hou T, Shen A, et al (2021) Mechanism deconvolution of Qing Fei Pai Du decoction for treatment of Coronavirus Disease 2019 (COVID-19) by label-free integrative pharmacology assays. *Journal of ethnopharmacology* 280: 114488. <https://doi.org/10.1016/j.jep.2021.114488>
42. Xu MY, Lin YL, Zhang TY, et al (2022) Chlorine dioxide-based oxidation processes for water purification A review. *J Hazard Mater* 436:129195. <https://doi.org/10.1016/j.jhazmat.2022.129195>
43. Xu T, Zhang S, Du K, et al (2022) Insights into the Molecular Regulation of Lignin Content in Triploid Poplar Leaves. *Int. J. Mol. Sci* 23, 4603. <https://doi.org/10.3390/ijms23094603>
44. Zeng Q, Han Z, Kang X (2019) Adventitious shoot regeneration from leaf, petiole and root explants in triploid (*Populus alba* × *P. glandulosa*) × *P. tomentosa*. *Plant Cell, Tissue and Organ Culture* 138: 121-130. <https://doi.org/10.1007/s11240-019-01608-4>

Figures



Figure 1

In vitro regeneration and plantlet transplantation of triploid *A. chinensis*. **a** Plant morphology of triploid *A. chinensis* explants. **b** Shoots morphology induced by explants for 3 weeks. **c** Shoots morphology induced by explants for 5 weeks. **d** Morphology of shoots before proliferation. **e** Morphology of shoots after multiplication. **f-g** Different status of roots after rooting for 30 days **h** Overall morphology of plantlet before transplantation. **i** Plant morphology after 4 weeks of transplanting. **j-k** Different status of roots after 8 weeks of transplanting

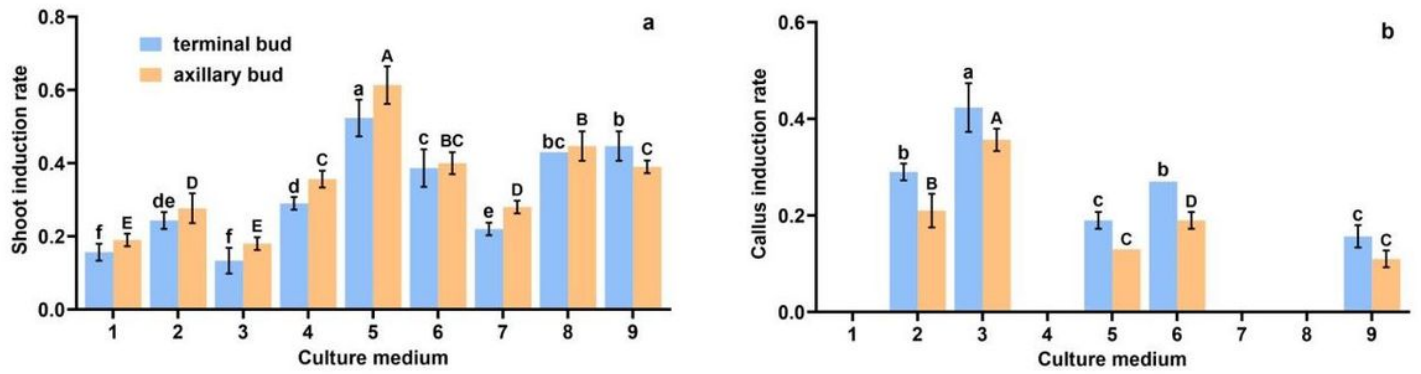


Figure 2

Effects of different concentrations of 6-BA and NAA on different explants. **a** Shoot induction rate. **b** Callus induction rate. Different letters in the same color indicate significant differences according to the LSD test ($P \leq 0.05$) following ANOVA

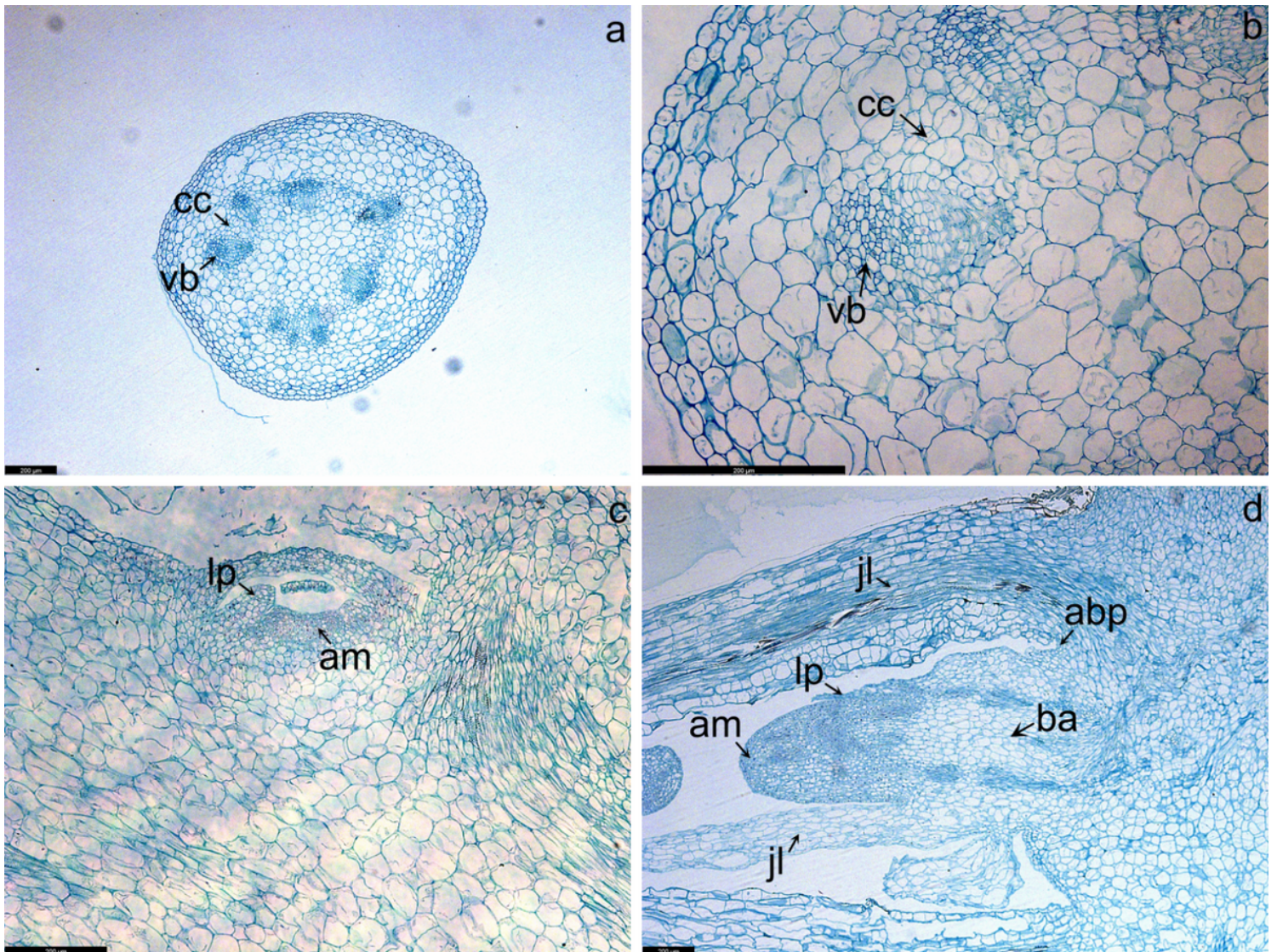
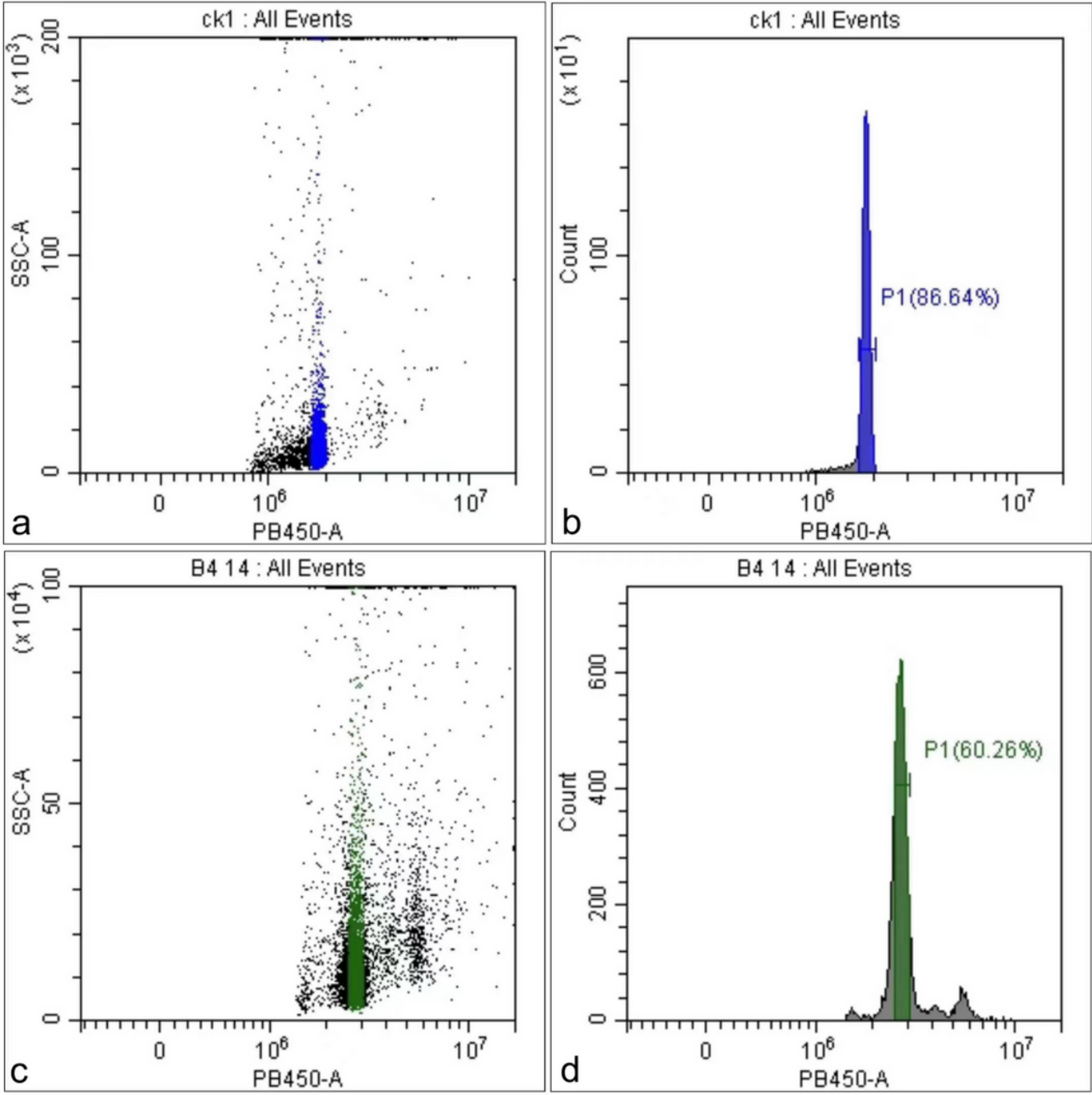


Figure 3

Histological observation of shoot regeneration of *A. chinensis*. **a-b** Meristematic cells formation stage (Cross section, 50×; Cross section, 200×). **c** Apical meristem and leaf primordium formation stage (Longitudinal section, 100×). **d** Shoot differentiation stage (Longitudinal section, 50×) **cc** Cambium cell **vb** Vascular bundle **lp** Leaf primordium **am** Apical meristem **jl** Juvenile leaf **abp** Axillary bud primordium **ba** Bud axis



Group	Number of particles	Mean PB450-A	Median PB450-A	rCV PB450-A
Diploid <i>A. chinensis</i>	7876	1825709.8	1825034.3	3.61%
Regenerated plant	4174	2756697.3	2745626.0	5.18%

Figure 4

Scatter diagrams and histograms of FCM and the accompanying table of DNA content. **a-b** Diploid *A. chinensis*. **c-d** Regenerated plantlets

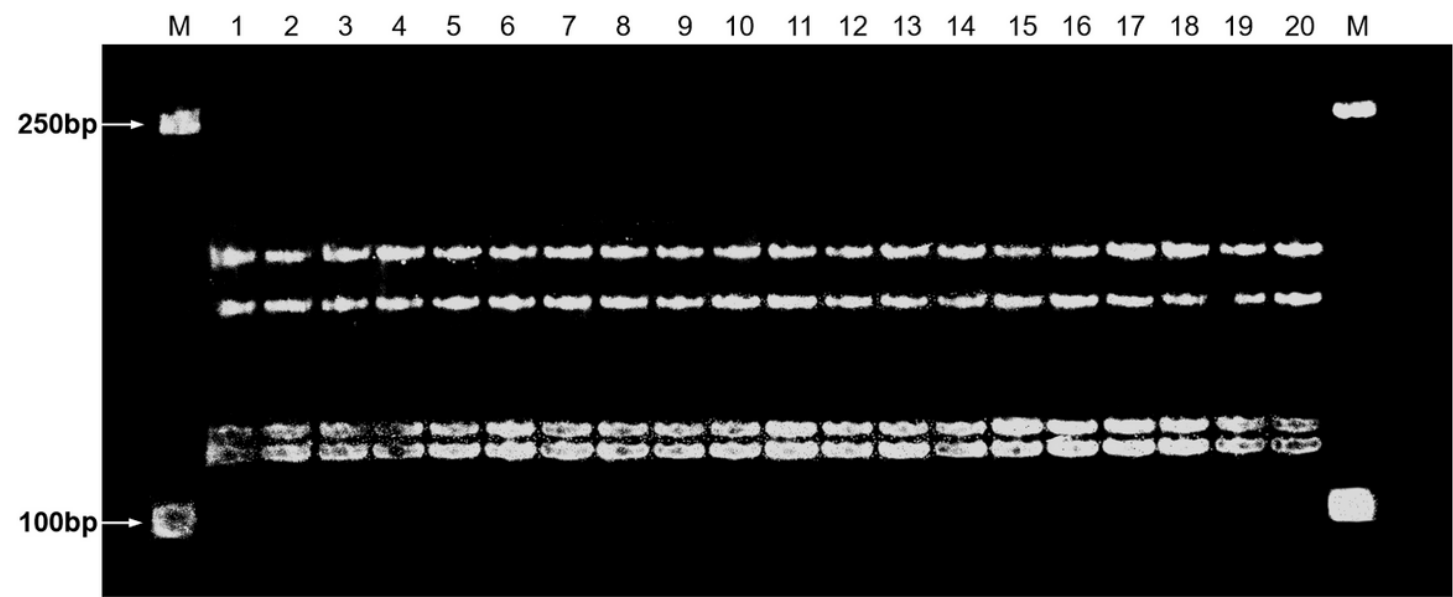


Figure 5

Amplified bands of 20 regenerated plantlets of *A. chinensis* by primer CL7269. M: DL 2000 DNA Marker; 1~20: 20 regenerated plants of triploid *A. chinensis*