



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

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## Abstract

Triploid *Atractylodes chinensis* (DC.) Koidz. 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<sup>-1</sup> 6-BA + 0.4 mg·L<sup>-1</sup> NAA medium, the regeneration rate was 61.33%. Strong roots occurred on 1/2 MS medium combined with 3% sucrose and 0.6 mg·L<sup>-1</sup> IBA. Rooting plantlets transplanted into a mixture of peat soil: perlite (1:1, v:v), the survival rate up to 90.00%. Histological analysis 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.

**Keywords** Triploid · *Atractylodes chinensis* · Micropropagation · Histology · Genetic stability

## Abbreviations

AR Atractylodis Rhizoma  
TCM Traditional Chinese medicine  
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

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## Introduction

*Atractylodes chinensis* (DC.) Koidz. is a perennial herb that belongs to Asteraceae family. This plant and *Atractylodes lancea* are the main sources of Atractylodis Rhizoma (AR), which is a traditional Chinese medicine (TCM) that is mainly distributed in China and widely used in East Asian

countries (Hiraoka 1993). According to the theory of TCM, AR is explained by its properties in eliminating dampness, invigorating spleen, and expelling wind for the treatment of rheumatic diseases, digestive disorders, night blindness, and influenza (Zhang et al. 2021; Xiao et al. 2002). Especially in China, *A. chinensis* 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). Up to now, more than 200 chemical compounds, notably sesquiterpenoids and alkynes have been isolated from AR, and many pharmacological activity studies strongly supported their remarkable clinical efficacy (Zhang et al. 2021; Kimura and Sumiyoshi 2012; Plengsuriyakarn et al. 2012). However, it has been demonstrated that the contents of the main components have big difference in ARs and mainly influenced by genetic factors (Tsusaka et al. 2019), therefore, the collection and reproduction of genetic resources of *A. chinensis* have significant practical significance to the development of AR industry.

*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 gasterodin in *Anoectochilus formosanus* Hayata (Chung et al. 2017), as well as an 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 simple sequence repetition (SSR) molecular markers and flow cytometry (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

The experiments were carried out with explants obtained from 2-year-old triploid *A. chinensis* plants (Fig. 1a), located by the scientific researchers during breeding and grown in the Medicinal Botanical Garden of Jilin Agricultural University, China. Fresh and tender stem explants with axillary or terminal buds were collected in early May.

### Sterilization of explants and initiation of in vitro culture

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 concentrations of sterilants for varying time periods [0.1% mercuric chloride (MC) for 5 min, 10 min, 15 min; 2% sodium hypochlorite (SH) and 0.05% chlorine dioxide (CD) for 20 min, 25 min, 30 min], and finally sterile water with 5 rinses. The surface water of explants was dried with sterile filter paper, and the explants with different buds (axillary or terminal) were cultured separately on Murashige and Skoog (1962) (MS) medium containing 3% sucrose, 0.55% agar and different concentrations of 6-benzyladenine (6-BA 1.0 mg·L<sup>-1</sup>, 1.5 mg·L<sup>-1</sup>, 2.0 mg·L<sup>-1</sup>) and 1-Naphthaleneacetic acid (NAA 0.2 mg·L<sup>-1</sup>, 0.4 mg·L<sup>-1</sup>, 0.6 mg·L<sup>-1</sup>). 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 ± 1°C under a 16 h photoperiod at a light intensity of 30 μmol m<sup>-2</sup> s<sup>-1</sup> provided by white fluorescent tubes. After 2 weeks, the sterilization effects were investigated. The adventitious 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. When the number of regenerated adventitious shoots reaches 8–10 per explant, and the height was 3–4 cm, they were harvested and prepared to proliferation, which takes about 5 weeks.

### Proliferation of shoots and rooting

The adventitious shoots were cut to a single or 2–3 shoots with a small clump after 5 weeks, transferred to proliferation medium (MS medium with 3% sucrose, 0.55% agar, 1.5 mg·L<sup>-1</sup> 6-BA and 0.4 mg·L<sup>-1</sup> NAA), and placed in a growth chamber with a 16-h photoperiod (50 μmol m<sup>-2</sup> s<sup>-1</sup> from cool white fluorescent tubes) at 22 ± 1°C. When the shoots grew to 3–5 cm, they were transferred to 1/2MS

rooting media containing different concentrations of sucrose (1%, 2% and 3%), NAA ( $0.5 \text{ mg}\cdot\text{L}^{-1}$ ,  $1.0 \text{ mg}\cdot\text{L}^{-1}$ ), and indole-3-butyric acid (IBA  $0.3 \text{ mg}\cdot\text{L}^{-1}$ ,  $0.6 \text{ mg}\cdot\text{L}^{-1}$ ) 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 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 with water. The plantlets were transplanted to a sterile mixture of culture substrates with different volume ratios [peat soil: perlite (2:1, v:v), peat soil: perlite (1:1, v:v), peat soil: perlite: vermiculite (2:1:1, v:v:v), peat soil: perlite: vermiculite (1:1:1, v:v:v)] 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 regenerated adventitious 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 Section (10  $\mu\text{m}$ , thickness) were obtained with a Fully Automated Rotary Microtome Leica RM2265. The samples were dyed using the Safranin-Fast Green stain (1% safranin O and 0.1% Fast Green FCF) method refers to Abdelbar (2017), 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  $0.5 \text{ cm}^2$  and immersed for 60 s in 200  $\mu\text{L}$  nuclei extraction buffer of the ploidy analysis kit (CyStain UV Precise P, Sysmex Corporation, Chuo-ku, Kobe, Hyogo, Japan) then chopped with a sharp razor blade. After incubating the obtained nuclear suspension for 3 min, the solution was filtered through 50  $\mu\text{m}$  CellTrics filters (Sysmex Corporation) into sample tubes. Staining buffer (800  $\mu\text{L}$ ) 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  $5 \times 10^3$ . 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  $-20^\circ\text{C}$  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  $\mu\text{L}$  reaction volumes, containing 1  $\mu\text{L}$  genomic DNA, 1  $\mu\text{L}$  of each primer (10 pmole), 10  $\mu\text{L}$  of 2 $\times$ PCR Master Mix (Shanghai Beyotime Biotechnology Co., Ltd., Shanghai, China), and 7  $\mu\text{L}$  ddH<sub>2</sub>O. The PCR procedure was performed at  $94^\circ\text{C}$  for 5 min, followed by 35 cycles each at  $94^\circ\text{C}$  for 45 s,  $60^\circ\text{C}$  for 45 s, and  $72^\circ\text{C}$  for 45 s, with a final extension at  $72^\circ\text{C}$  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% 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% 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% 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.

### Shoot induction

After 1 week of incubation, the stem segments enlarged and adventitious shoots were observed on the surface of some explants. After 3 weeks, an increasing number of adventitious 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 adventitious shoot induction rates of explants whether with terminal or axillary buds in the 5th group (6-BA 1.5 mg·L<sup>-1</sup>, NAA 0.4 mg·L<sup>-1</sup>) were significantly higher than those of the other groups, with 52.33% and 61.33%, respectively (Table 2). In general, when the concentration of 6-BA is in the range of 1.0–2.0 mg·L<sup>-1</sup>, the induction of adventitious shoots initially increased and then decreased, as the NAA concentration increased. Further study on the induction rates of different parts of explants suggested that the adventitious shoot induction rate of stem segments with axillary buds was higher than that with terminal buds for the majority of the treatments (Table 2). However, the callus induction rate of that with terminal buds was

higher than that with axillary buds, except for a few treatments in which no callus regeneration occurred (Table 2).

### Shoots proliferation and rooting culture

Plantlets grew rapidly in the proliferation medium (Fig. 1d). After 2 weeks, new adventitious shoots emerged and grew to a cluster of shoots. 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 culture for 1 month, significant differences were observed in rooting time, rooting rate, root number, and root length of plantlets under different rooting culture media (Table 3). The effect of different concentrations of sucrose, NAA and IBA on rooting culture was shown in Table 3. When the concentration of IBA or NAA was constant in the rooting culture medium, with the increased sucrose concentrations, the rooting time advanced and the rooting rate decreased. Although both IBA and NAA could induce rooting, NAA has a more pronounced rooting response. When 3% sucrose and 1.0 mg·L<sup>-1</sup> 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<sup>-1</sup> NAA, the rooting rate increased to the 94.33%, which was significantly higher than other combinations. 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 + 0.6 mg·L<sup>-1</sup> 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<sup>-1</sup> IBA medium, the number of roots was high, and the roots were thin and long (Fig. 1f). In contrast, only a lower number of roots occurred in the 1/2MS + 1% sucrose + 0.6 mg·L<sup>-1</sup> IBA medium, and these roots were short and severely lignified. (Fig. 1g).

**Table 1** Effect of different sterilization protocols on explants

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

MC mercuric chloride; SH sodium hypochlorite; CD chlorine dioxide

\*Values represent mean ± SE. 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 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 7–9 cm and the transplanting mixture consisted of peat soil: perlite (1:1,



**Fig. 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 proliferation. **f–g**

Different status of roots after rooting culture 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

**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).

### Histological observations

The histological regeneration process of adventitious shoots was observed using the paraffin sections. During adventitious 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. 2a). As cells division become more active, meristematic cells undergo anticlinal

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

| Treatment | Plant hormone (mg·L <sup>-1</sup> ) |     | Stem segments with terminal bud |                            | Stem segments with 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 <sup>f</sup>         | 0 <sup>d</sup>             | 19.00±1.73 <sup>e</sup>         | 0 <sup>d</sup>             |
| 2         |                                     | 0.4 | 24.33±2.31 <sup>de</sup>        | 29.00±1.73 <sup>b</sup>    | 27.67±4.04 <sup>d</sup>         | 21.00±3.46 <sup>b</sup>    |
| 3         |                                     | 0.6 | 13.33±3.51 <sup>f</sup>         | 42.33±5.03 <sup>a</sup>    | 18.00±1.73 <sup>e</sup>         | 35.67±2.31 <sup>a</sup>    |
| 4         | 1.5                                 | 0.2 | 29.00±1.73 <sup>d</sup>         | 0 <sup>d</sup>             | 35.67±2.31 <sup>c</sup>         | 0 <sup>d</sup>             |
| 5         |                                     | 0.4 | 52.33±5.03 <sup>a</sup>         | 19.00±1.73 <sup>c</sup>    | 61.33±5.13 <sup>a</sup>         | 13.00±0.00 <sup>c</sup>    |
| 6         |                                     | 0.6 | 38.67±5.13 <sup>c</sup>         | 27.00±0.00 <sup>b</sup>    | 40.00±3.00 <sup>bc</sup>        | 19.00±1.73 <sup>b</sup>    |
| 7         | 2.0                                 | 0.2 | 22.00±1.73 <sup>e</sup>         | 0 <sup>d</sup>             | 28.00±1.73 <sup>d</sup>         | 0 <sup>d</sup>             |
| 8         |                                     | 0.4 | 43.00±0.00 <sup>bc</sup>        | 0 <sup>d</sup>             | 44.67±4.04 <sup>b</sup>         | 0 <sup>d</sup>             |
| 9         |                                     | 0.6 | 44.67±4.04 <sup>b</sup>         | 15.67±2.31 <sup>c</sup>    | 39.00±1.73 <sup>c</sup>         | 11.00±1.73 <sup>c</sup>    |

6-BA 6-Benzyladenine; NAA 1-Naphthaleneacetic acid

\*Values represent mean±SE. Different letters in the same column indicate significant differences according to the LSD test (P≤0.05) following ANOVA

**Table 3** Effect of different concentrations of sucrose, NAA and IBA on rooting culture

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

IBA Indole-3-butyric acid; NAA 1-Naphthaleneacetic acid

\*Values represent mean±SE. Different letters in the same column indicate significant differences according to the LSD test (P≤0.05) following ANOVA

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

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

\*Values represent mean±SE. Different letters in the same column indicate significant differences according to the LSD test (P≤0.05) following ANOVA

division to form adventitious bud primordia (Fig. 2b). 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. 2c). 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. 2d).

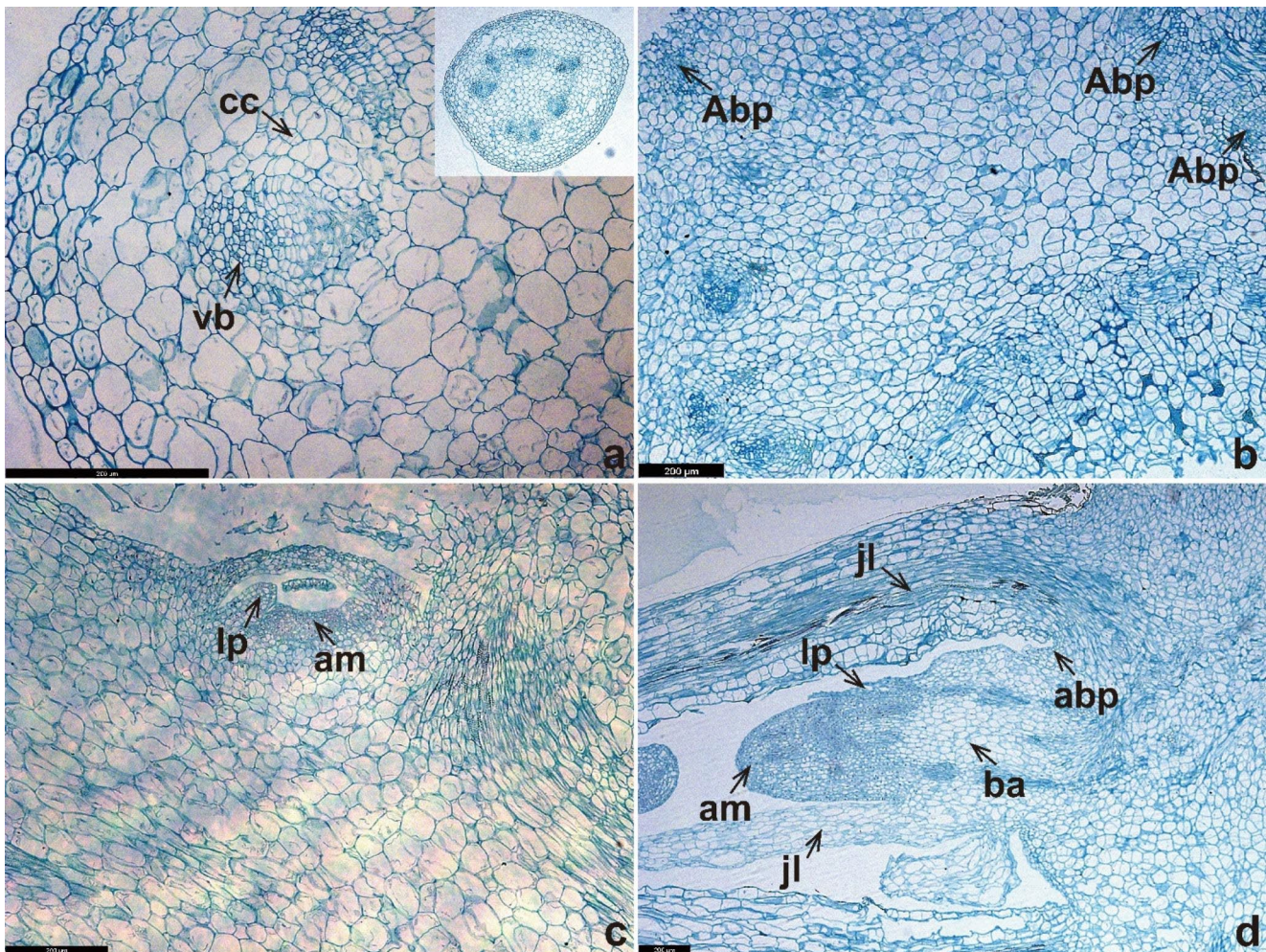
### Ploidy level determination

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  $1.8 \times 10^6$  (Fig. 3a, 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  $2.7 \times 10^6$  (Fig. 3c, d), and the ratio to the reference is 1.5, which indicates triploid status.

### SSR analysis of genetic stability

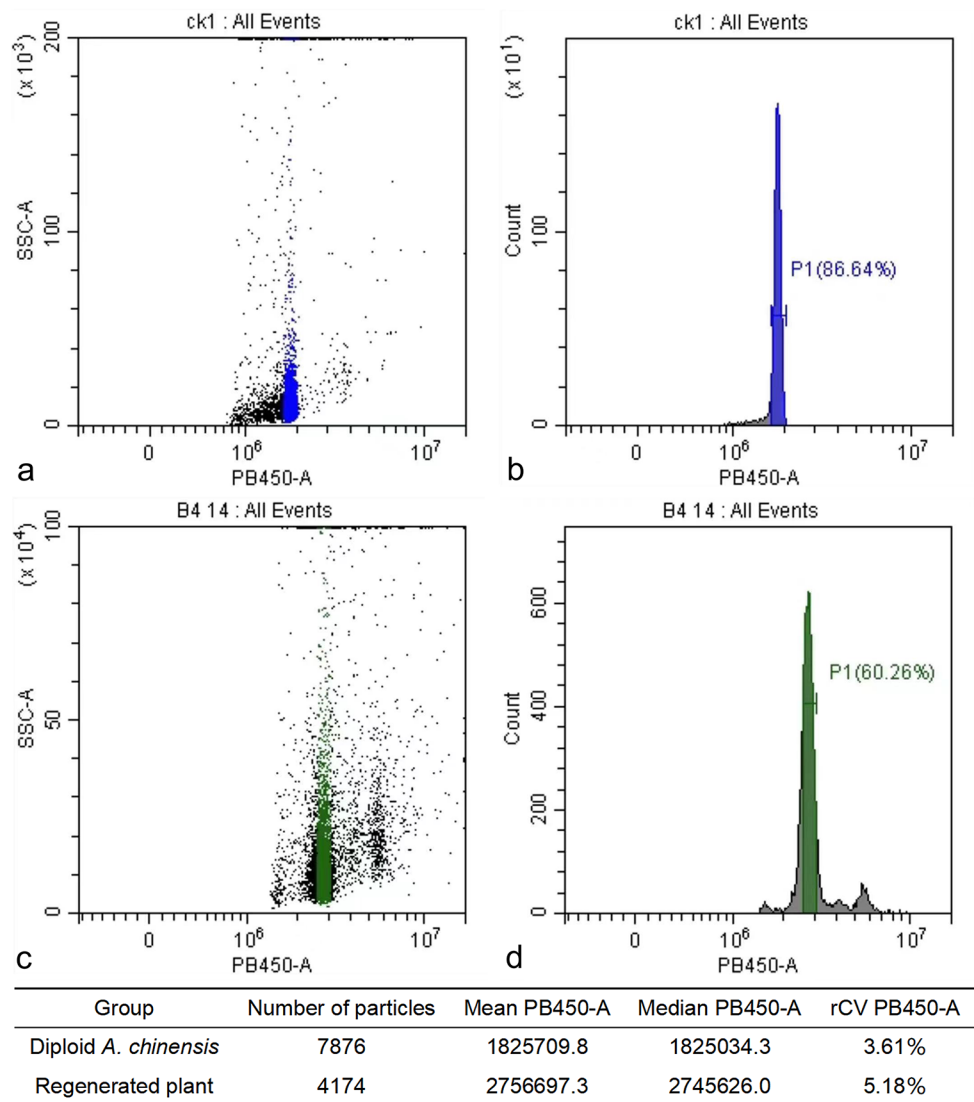
SSR primers (30 pairs) and the genomic DNA of *A. chinensis* from four regions were used to identify primer sets with polymorphic bands and a clear banding pattern, and 6 pairs of SSR primers were selected (Table 5). The genomic DNA of 20 regenerated plantlets was amplified by PCR with the chosen primers. 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



**Fig. 2** Histological observation of shoot regeneration of *A. chinensis*. **a** Meristematic cells formation stage (Cross section, 200 $\times$ ). **b** The differentiation of meristem cells into adventitious bud primordia stage (Cross section, 50 $\times$ ). **c** Apical meristem and leaf primordium forma-

tion stage (Longitudinal section, 100 $\times$ ). **d** Shoot differentiation stage (Longitudinal section, 50 $\times$ ) **cc** Cambium cell **vb** Vascular bundle **Abp** Adventitious bud primordium **lp** Leaf primordium **am** Apical meristem **jl** Juvenile leaf **abp** Axillary bud primordium **ba** Bud axis

**Fig. 3** Scatter diagrams and histograms of FCM and the accompanying table of DNA content. **a–b** Diploid *A. chinensis*. **c–d** Regenerated plantlets



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. 4). No variation was detected with the 6 SSR primers in the regenerated plantlets of *A. chinensis* at the DNA level.

## Discussion

Triploid can play a vital role in improving fruit traits, biomass, and abiotic stress tolerance in the future, leading to commercial benefits (Xu et al. 2022b; 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

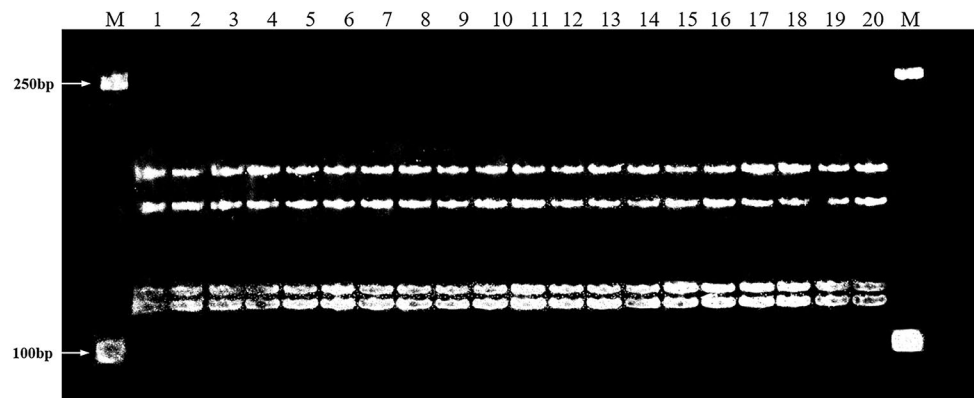
present study, stem segments with axillary or terminal buds were used as explants to rapidly cultivate triploid *A. chinensis* plantlets using micropropagation and plantlet regeneration technology, to provide conditions for the protection and utilization of genetic resources of this species.

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. 2022a; 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



**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  | TTGGGATGTTGTACTACTCTGTTG  |
| CL5771 | TAATACCCGTGTTTCATCGTCTCT | GTCTGTTTGGAGAACATCGAATC   |
| CL6819 | ATGGAGAGGTCTGTATGGATCTG  | GGCTGACTGTTACATCGTAGGTT   |
| CL7052 | TCCCCTCCAATAACTCCTAGAAC  | GGTTGTTTTCCTGTCTGATTAGG   |
| CL7269 | TGAAGAACTACACCCTCCTCAC   | GACTTAGAAAACCTTTGACCCCGTT |

**Fig. 4** 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*

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. Sreekissoon 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). 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. This investigation is of great significance for enriching the genetic resources of *A. chinensis* and can provide material basis for the study of triploid abortion mechanism.

**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.

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**Data Availability** All relevant data are within the paper.

## Declarations

**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.

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