



In vitro tetraploid induction and plant regeneration of *Pinellia ternata*, a pearl-like herb

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Abstract

Pinellia ternata (Thunb.) is a widely used Chinese medicinal herb in many countries globally. However, asexual reproduction causes the germplasm of *P. ternata* to degrade, and there is limited research on genetic breeding. The objective of this study is to induce tetraploid ‘Pearl’ *P. ternata* plants by treating leaves and tubers with colchicine to acquire superior germplasm resources. The in vitro medium for autotetraploid plants was optimized through orthogonal experiments. Subsequently, the morphology, tissues, and cells of diploid and tetraploid plants were compared. The results indicated that the number of chromosomes in diploid plants was $2n=2x=66$, while the tetraploid plants were $2n=4x=132$ by somatic chromosome counting and flow cytometry analysis. The leaf was more suitable than the tuber for polyploid induction, and the maximum induction rate was 29.49% when leaves were treated with a 0.05% colchicine solution for 96 h. In MS medium supplemented with $0.1\text{ mg}\cdot\text{L}^{-1}$ 6-BA, $0.1\text{ mg}\cdot\text{L}^{-1}$ NAA, and $1.0\text{ mg}\cdot\text{L}^{-1}$ KT, tetraploid leaves successfully developed into plantlets with roots, achieving a 100% transplant survival rate. Tetraploid *P. ternata* exhibited larger and denser leaves, thicker petioles, and larger stomata but had shorter plants compared to diploid. Meanwhile, tetraploid leaves produced a greater number of tubers. This study induced tetraploids in *P. ternata* and established an artificial tetraploid rapid propagation system for *P. ternata*. It provided the theoretical foundation and experimental support for its genetic breeding and variety enhancement.

Key message

Tetraploid induction in vitro and autotetraploid regeneration were established for *P. ternata*. Compared to diploids, tetraploid plants had larger and denser leaves, thicker petioles, larger stomata, and produced more tubers.

Keywords *Pinellia ternata* (Thunb) · Polyploid induction · Rapid propagation system · Morphological characteristics

Introduction

Pinellia ternata (Thunb.) Breit is a perennial herb of the genus *Pinellia* and the family Araceae, primarily growing in eastern Asia. It has widespread distribution in China, especially in the Yangtze River basin, as well as in northeastern and northern China, with a small distribution in Tibet and Xinjiang (Flora of China Editorial Committee 1979). *P. ternata* tuber is one of the most widely used botanicals in China. Rich in alkaloids, volatile oils, and organic acids, it exhibits pharmacological effects such as cough suppression, phlegm resolution, and antitumor activity (Mao and He 2020). The distinctive pharmacological effects have heightened the market demand for *P. ternata*, leading to an expansion of its artificial planting area. However, its artificial propagation is primarily asexual, and the accumulation

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of viruses from year to year can result in a significant degeneration of its germplasm. Simultaneously, the variable natural environment, pests and diseases, and the phenomenon of falling seedlings (where the aboveground part withers in winter and regenerates in the next spring) also significantly constrain the yield of *P. ternata*. (Nagai et al. 2002). There is an urgent need for a new method to cultivate new *P. ternata* germplasm with ideal yield and resistance. At present, the breeding work of *P. ternata* still relies on mixed selection methods and systematic breeding methods. However, this traditional breeding method has limited ability to improve varieties, and the development and utilization of germplasm resources is relatively weak and time-consuming (Ding et al. 2021). Therefore, obtaining a substantial number of tetraploid resources of *P. ternata* through chromosome doubling is of great significance for artificial planting and the innovation of germplasm resources for *P. ternata*.

The inherent traits of polyploid plants may be fortified and enhanced due to the dose-effect, gene mutation, and nuclear-cytoplasmic ratio imbalance resulting from chromosome doubling (Niazian and Nalousi et al. 2020; Fernandes Gyorfy et al. 2021). Meanwhile, it will produce many new phenomena that the original diploid lacks, such as larger plants, higher yield, increased secondary metabolites, and enhanced adaptability (Pei et al. 2019; Wei et al. 2019). In *Arabidopsis thaliana* (L.) Heynh., the contents of lignin and cellulose in tetraploid and hexaploid plants were significantly lower than those in diploid plants (Corneille et al. 2019). Cell enlargement is the most famous effect of polyploids, but the final plant size and biomass of polyploids do not always increase because the increase in cell size and the decrease in cell number are an ecological adaptation strategy for plants (Iannicelli et al. 2020). In addition, polyploid plants are still prone to delayed flowering, low fruit set, seed sterility, and lengthy growth cycles (He et al. 2010). Despite these defects, these traits can be selected and may result in plants being domesticated as crops (Ding and Chen 2018; Niazian and Nalousi et al. 2020). At present, ploidy breeding has been successfully applied in some medicinal plants, such as *Cyclocodon lancifolius* (Roxb.) Kurzh, *Salix viminalis* E. L. Wolf, *Wedelia chinensis* (Osbeck.) Merr, *Cuminum cyminum* L., and so on (Dudits et al. 2016; Tsai et al. 2021; Xi et al. 2021; Sanaei-Hoveida et al. 2024). Since the rarity of polyploid plants in nature makes it difficult to provide the genetic diversity required for breeding, polyploids are typically induced artificially (Xi et al. 2021). Chemical mutagenesis is the most prevalent and effective method for inducing polyploidy; it effectively induces chromosome duplication in plant cells by inhibiting spindle filament and cell plate formation. Colchicine is currently the most widely used and effective chemical mutagen. Researchers have used colchicine to induce polyploid plants in *Raphanus*

sativus L., *Fagopyrum tataricum* (L.) Gaertn, *Echinacea purpurea* (L.), *Cyclocodon lancifolius* (Roxb.) Kurzh, *Lilium distichum* Nakai (Abdoli et al. 2013; Wang et al. 2017; Fu et al. 2019; Pei et al. 2019; Xi et al. 2021).

Chromosome doubling may change the expression pattern of duplicated genes (increase, silence), affect transcription and metabolism, and eventually lead to new phenotypic variation (Niazian and Nalousi et al. 2020). The most direct change is that the organ size of polyploid plants is larger than that of their diploid parents, known as the ‘gigas’ effect (Fu et al. 2019). The flowers, stems, and leaves in polyploids of *Dendrobium officinale* Kimura et Migo, *Lonicera japonica* Thunb. And *Lilium distichum* Nakai were larger than those in diploids. (Kong et al. 2017; Fu et al. 2019; Zhang and Gao 2020). In addition, allele doubling alters the gene dose and may lead to an increase in secondary metabolites (Niazian and Nalousi et al. 2020). These metabolites not only enhance the resistance and tolerance of plants, but also are of great significance for pharmacological research, especially for medicinal plants. The content of stevioside and rebaudioside A in tetraploid *Stevia rebaudiana* Bertoni. Was higher than that in diploid (Zhang et al. 2018); *Zingiber officinale* Roscoe tetraploid is not only greater than diploid in leaf length, leaf width, leaf diameter, and stem diameter but also higher in soluble sugar, soluble protein, and proline (Zhou et al. 2020); The main effective components of chlorogenic acid, luteoloside, hyperoside, and total flavonoids in *Lonicera japonica* Thunb. were higher than those in diploid (Kong et al. 2017). Therefore, ploidy breeding can not only provide a new breeding possibility for *P. ternata*, but also improve the yield, resistance, and secondary metabolite content. Although there are a large number of studies on the pharmacology and cultivation of *P. ternata*, no information on the induction of polyploidy by this plant has been reported so far. This is the first article on the induction of autotetraploidy by *P. ternata*. In this study, we utilized high-quality *P. ternata* ($2n=2x=66$) from the Zhaotong production region in Yunnan, China. Because the tubers of *P. ternata* grown in this region are spherical like pearls and rich in active constituents, they are known as ‘Pearl’ *P. ternate* (Hou et al. 2006). The autotetraploid of *P. ternata* was induced in vitro, and its regeneration system was established, providing an experimental basis for polyploid breeding of *P. ternata* variety enhancement.

Materials and methods

Experimental materials and culture conditions

The experimental materials are leaves and tubers. The diploid tissue culture system has been successfully established

in the Yunnan Breeding and Cultivation Research and Development Centre of Endangered and Daodi Chinese Medicinal Materials (Long, 24°84'36" N, Lat, 102°82'10" E; Alt: 1878.3 m), data to be published. The study used sterile leaves and tubers derived from the same mother plant. On an ultra-clean bench, the leaves were cut into approximately 1.0 × 1.0 cm pieces with a surgical blade and inoculated for 15 days in our group's proven proliferation medium (MS + 0.05 mg·L⁻¹ 6-BA + 0.1 mg·L⁻¹ NAA + 0.5 mg·L⁻¹ KT) in diploid 'Pearl' *P. ternata*. The second material was the tubers produced after two generations of leaf culture (diameter between 1.0 and 1.5 cm).

The analytically pure plant growth regulators 1-naphthaleneacetic acid (NAA), kinetin (KT), N-(phenylmethyl)-9 H purin-6-amine (6-BA), sucrose, agar, MgCl₂·6H₂O, 3-(N-Morpholino)Propane Sulfonic Acid (MPOS), polyvinyl pyrrolidone (PVP), sodium citrate, Tritonx-100, Ethylenediaminetetraacetic acid disodium salt (Na₂EDTA) and propidium iodide (PI) for the experiment were purchased from Beijing Dingguo Changsheng Biotechnology Company (Beijing, China). All experiments utilized MS medium containing 3% sucrose and 0.47% agar; pH was adjusted to 5.6–5.8 with 1 N HCl and sterilized at 122 °C for 22 min in an autoclave. Different concentrations of colchicine solutions were sterilized under the same conditions as the medium; all hormone concentrations in this study are mass concentrations.

In culture conditions, the temperature in the culture room was maintained at 20 ± 2 °C, and the illumination was 10 h·d⁻¹ with a light intensity range of 1800–2500 lx.

Induction of autotetraploid 'Pearl' *P. Ternate*

The above leaves and tubers were agitated for 72, 84, 96, and 108 h in sterile colchicine solution at concentrations of 0.05% (w/v) and 0.1% (w/v); In the control group, colchicine solution was replaced by sterile water. At the conclusion of the treatment, and each material was rinsed 3 times with sterile water for not less than 3 min each time, and then these materials were inoculated in diploid *P. ternata* medium (MS + 0.05 mg·L⁻¹ 6-BA + 0.1 mg·L⁻¹ NAA + 0.5 mg·L⁻¹ KT). Each treatment group contained 5 bottles, and each bottle contained 10 materials. Number the obtained tetraploid plants of the first generation, maintaining the numbering of each tetraploid plant consistent with its progeny.

Chromosome counting and flow cytometry

When the roots of the plants in the bottles were between 0.5 and 1.0 cm in length, the root tips were immersed in a 0.05% (w/v) colchicine solution between 8:00 and 9:00 am and deposited in the dark at 4°C for 3 h. The treated root

tips were then washed three times with distilled water and transferred to Carnoy's fixative (70% ethanol: glacial acetic acid = 3:1, v/v) for 2 h at room temperature. The fixed root tips were washed twice with 70% ethanol and hydrolyzed in 1 M HCl for 15 min. After 5–6 washes with distilled water, the root tip was softened for 10 min in 45% acetic acid. The root tip was then milled on a glass slide and stained for 20 min with a dab of carbol fuchsin. Next, a coverslip was placed over the tissue and tapped with tweezers to disperse the tissue. Finally, the prepared temporary slide was viewed under an Olympus BH-2 microscope, and the number of chromosomes was determined. The autotetraploid plants of the first generation (the plants grown after colchicine treatment were the first generation) were numbered separately and cultivated in succession (the same number indicates propagation from the same mother plant). 10 plants were chosen at random for each generation, and their chromosome numbers were determined using chromosome counting, which was discontinued after 5 generations.

For flow cytometry analysis, the sample and the fresh leaf tissue (0.5 cm²) of the internal reference plant (*Zea mays* L.) were mixed at 1:1 and chopped. They were allowed to incubated in 1 ml nuclei isolation buffer (pH 7.0) containing 45 mM MgCl₂·6H₂O, 20 mM MOPS, 30 mM sodium citrate, 1% (w/v) PVP, 0.2% (v/v) Tritonx-100, 10 mM Na₂EDTA for 10 min, and then filtered through 30-μm nylon sieve to obtain nuclear suspension, then adding 500 μL of PI (50 mg·mL⁻¹) solution into the nuclear suspension, and placing it on ice to avoid light for 0.5–1 h. Finally, the nuclear suspension was analyzed by flow cytometry (BD FACScalibur), and the diploid of *P. ternata* was used as a control. 10,000 nuclei were collected for each test, and the coefficient of variation (CV) is controlled within 5%.

Development of an efficient autotetraploid propagation system

Material for orthogonal experiments were the leaves of autotetraploid plants, with 6-BA (0.05, 0.1, 0.5 mg·L⁻¹), KT (0.1, 0.5, 1.0 mg·L⁻¹), and NAA (0.05, 0.1, 0.5 mg·L⁻¹). The leaves were laid flat on the surface of the medium, and the proliferation coefficient was calculated after 45 days of incubation. The leaves, petioles, and tubers of *P. ternata* could be used as proliferation materials. The proliferation coefficient was calculated by summing the number of three materials (leaves, petioles, and tubers) produced by the leaves. Each treatment group was inoculated with 10 bottles containing 10 materials per bottle.

Acclimatization and transplantation of autotetraploid plants

When the tubers in the culture bottle grew to 1–2 cm in diameter, they were exposed to natural light for 6 days. The culture container's sealing film was then removed and exposed to light for 2 days. Next, remove the plantlets, wash the remaining agar solid from the root, and submerge them in 0.1% chlorothalonil for 5 min. Finally, the tubers (with leaves and roots removed) were transplanted into a bubble box (the inner diameter was 45.0×34.5×24.0 cm, the height of the humus was 6.0 cm) containing humus soil (sterilized with 0.2% potassium permanganate) and incubated (temperature is maintained between 23 and 25 °C and soil moisture content is maintained between 20 and 40%). Count the survival rate after 30 days of incubation.

Observations of the morphology and cytology of diploid and autotetraploid plants

Using calipers, observed and measured the tuber diameter, leaf thickness, leaf width, and petiole length of *P. ternata* and its autotetraploid plants (both of which were growing in the same environment). Using the method described by Fu et al. (2019) the number of stomata and stomatal density of the lower epidermis of the leaves were determined by observing 20 fields of view at 40x magnification. The leaves with the same growth time were selected, paraffin transverse sections of leaves with different ploidy levels were prepared, and the cell characteristics were examined under a light microscope. Repeat three times for each ploidy material, create 5 slides per repetition, and look at 5 fields of view on each slide.

Statistical index

The collected data were processed and analyzed using SPSS 26.0 (IBM Corp, Armonk, USA) and Excel (MC Corp, Redmond, USA) software. The induction rate and chimera rate were calculated by the number of regenerated plants in the first generation. If the tetraploid showed variation during 5 subcultures, it was regarded as a chimera.

Leaf (tuber) autotetraploid induction rate (%) = Number of tetraploid plantlets induced by leaves (tuber) / total number of leaf (tuber) regeneration plantlets × 100%.

Leaf (tuber) induction chimerism rate (%) = Number of chimeric plantlets induced by leaves (tuber) / total number of the leaf (tuber) regeneration plantlets × 100%.

Acclimatization survival rate (%) = Number of surviving plants / total number of transplanted plants × 100%.

Proliferation coefficient = Number of effective transfer materials (leaves, petioles, and tubers included) / total number of original inoculated materials.

Stomatal density = Number of stomata within the field of view / area of the field of view.

Result

Autotetraploid induction and identification results

Table 1 displays the results of tetraploid induction from leaves and tubers treated with colchicine. As colchicine concentration and treatment time increased, the number of tubers produced per leaf in the leaf treatment group decreased substantially, and the growth rate was also inhibited. The number of sprouted tubers per bottle of leaves decreased by 30% when treated with 0.05% colchicine for 72 h. When leaves were treated with 0.1% colchicine for 108 h, only half as many tubers sprouted per bottle as in the blank group. When the tubers were stimulated by external stimuli (trauma or colchicine solution), more regenerated plantlets would be differentiated. In the tuber treatment group, treated tubers began to expand and deform after approximately 10 days of incubation (Fig. 1A), transformed to a pseudo-dead state after 30 days, and after 3 months, new plants emerged from the brown tubers (Fig. 1B). The number of chromosomes in diploid plants was determined to be $2n = 2x = 66$ by observing temporary slides of root tip chromosomes from the blank group (Fig. 1C). The chromosome number of tetraploid plants in each treatment group was determined to be $2n = 4x = 132$ (Fig. 1D). Meanwhile, the tetraploid plant leaves were identified by flow cytometry (Fig. 2). In the figure, the abscissa value represents the fluorescence channel value, and the ordinate value represents the relative value of the number of cells measured; the left peak represents the reference plant (*Zea mays* L.), and the right peak represents the sample plant. The ploidy of the test materials can be seen from the peak abscissa; the peak abscissa of diploid plants was around 200 (Fig. 2A), while the peak abscissa of autotetraploid plants was around 400 (Fig. 2B), and the value of autotetraploid plants was about twice that of diploid plants.

In addition, under the same treatment conditions, the tetraploid induction rate of leaves was significantly higher than that of tubers; the highest tetraploid induction rate of 29.49% was achieved in leaves at 0.05% colchicine treatment for 96 h, whereas the highest tetraploid induction rate of 21.56% was achieved in tubers at 0.1% colchicine treatment for 108 h. However, chimeras were found in both materials across all treatment groups. The chimera rate increased with the increase of treatment time and colchicine

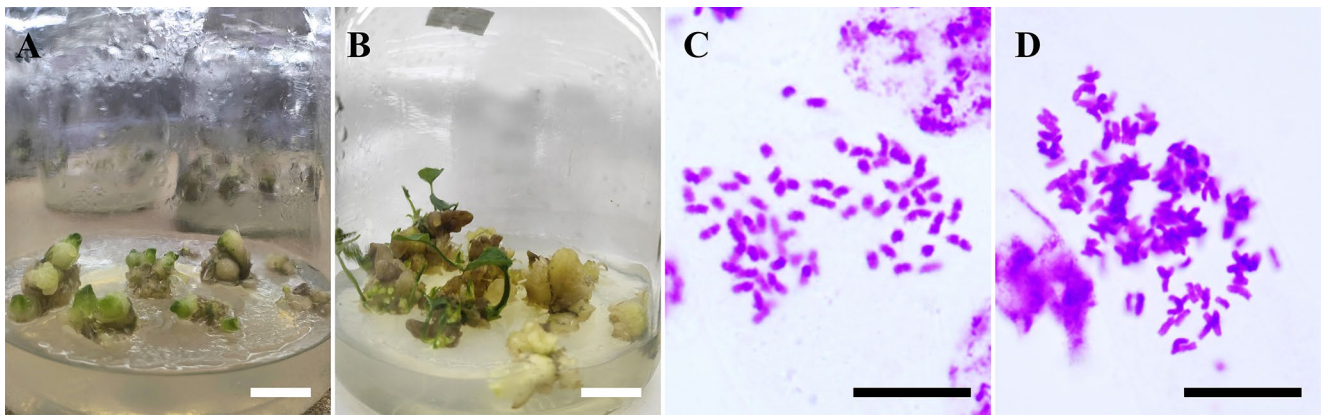


Fig. 1 Phenomenon of pseudo-death in tubers and chromosomes of root tips with different ploidy. **A** Tuber growth after treatment with 0.05% colchicine and incubation in medium for 10 days; **B** Tuber

growth after treatment with 0.05% colchicine and incubation in medium for 80 days; **C** $2n=2x=66$; **D** $2n=4x=132$. Scale bars: **A**, **B**=2 cm; **C**, **D**=10 mm

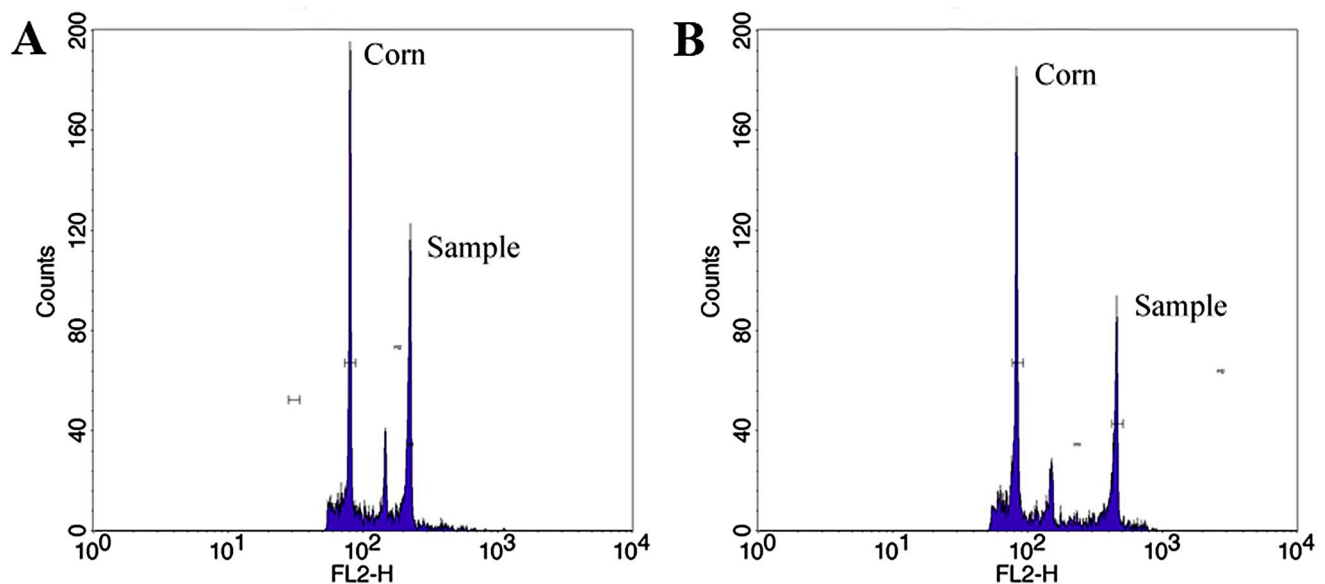


Fig. 2 The results of flow cytometry analysis. **A** control diploid plant; **B** tetraploid plant. The left peak is the internal reference plant, and the right peak is the test sample

solution concentration, but long-term colchicine soaking would reduce the number of regenerated plants, resulting in a decrease in the chimera rate. Microscopic observation and flow cytometry analysis showed that all chimeras contained only diploid and tetraploid cells, and no other ploidy cells (octoploid) appeared (Table 1). Comparing the two materials, the leaves were more suitable for low concentration and prolonged colchicine treatment to induction tetraploid, whereas the tubers- induced tetraploid is more effective under high concentration and prolonged treatment. The interval between treatment and identification of tubers was nearly double that of leaves, and the rate of tetraploid induction in tubers was lower than that of leaves. The optimal treatment for inducing autotetraploidy in ‘Pearl’ *P. ternata*

was the 96-hour treatment of leaves with a colchicine solution containing 0.05% colchicine.

Establishment of an autotetraploid plant regeneration system

The results of the orthogonal experiments are shown in Table 2, and the extreme difference in proliferation coefficients showed that $R_{6-BA} > R_{KT} > R_{NAA} > R_{Error}$, indicating that all three factors were effective for the proliferation culture of autotetraploid *P. ternata*. It was evident from the ANOVA results (Table 3) that 6-BA had a significant effect on the leaf proliferation coefficient ($P < 0.05$), whereas KT and NAA did not. Duncan’s test for the three levels of 6-BA (Table 4) showed that the most suitable concentration for

Table 1 Autotetraploid induction results of *P. ternata*

Material	Concentration	Time	NRP	Tetraploid		Chimera	
				Number	Rate (%)	Number	Rate (%)
Leaf	0.05	72	100.00±0.88a	10.80±0.416c	10.81±0.43d	12.00±0.87c	12.05±0.24d
		84	90.60±0.78b	14.20±0.277b	15.58±0.33c	14.00±0.61b	15.46±0.35c
		96	78.00±0.84c	23.00±0.650a	29.49±0.79a	15.20±0.51a	19.60±0.52a
		108	69.60±0.87d	14.40±0.300b	20.60±0.47b	11.00±0.34b	17.13±0.50b
	0.1	72	83.80±0.61a	9.20±0.41c	10.85±0.47c	13.00±0.41b	15.53±0.75c
		84	75.80±0.53b	12.80±0.38a	17.03±0.51b	17.20±0.90a	22.72±0.37a
		96	67.80±0.74c	14.00±0.58a	20.70±0.95a	13.60±0.75b	20.06±0.41b
		108	54.20±0.90d	11.00±0.52b	20.40±1.05a	7.40±0.66c	13.81±0.51d
Tuber	0.05	72	25.80±0.53a	1.00±0.21b	3.84±0.80c	2.80±0.46b	10.57±0.62d
		84	20.20±0.66b	1.80±0.33ab	8.81±1.63b	3.40±0.53ab	14.94±1.19c
		96	17.60±0.40c	2.40±0.34a	14.10±1.85a	4.00±0.33a	20.61±2.14a
		108	16.00±0.31d	1.60±0.21ab	10.70±1.50ab	2.60±0.43b	19.11±1.39b
	0.1	72	19.20±0.41a	1.00±0.23b	5.81±1.29b	2.40±0.55b	12.55±1.33d
		84	16.20±0.49b	1.40±0.22ab	9.35±1.45b	3.20±0.56a	17.65±0.96c
		96	13.20±0.44c	2.20±0.29a	10.75±2.36b	3.40±0.36a	23.90±2.05a
		108	10.40±0.371d	1.40±0.31ab	21.56±3.21a	2.00±0.47b	20.57±1.61b

Note NRP: Number of regenerated plants; Different letters in the table indicate significant differences in the 5% level; Mean ± Standard error

Table 2 The $L_9 (3^4)$ orthogonal test results of autotetraploid *P. ternata* one-step seedling formation

No.	Factors ($\text{mg}\cdot\text{L}^{-1}$)				Proliferation coefficient
	A (6-BA)	B (NAA)	C (KT)	Error	
1	1	1	1	1	65.20±0.37
2	1	2	2	2	69.80±0.37
3	1	3	3	3	73.40±0.25
4	2	1	2	3	84.00±0.71
5	2	2	3	1	99.00±0.32
6	2	3	1	2	89.80±0.37
7	3	1	3	2	78.00±0.32
8	3	2	1	3	76.80±0.37
9	3	3	2	1	80.40±0.25
Proliferation coefficient	<i>K1</i>	69.47	75.73	77.27	81.53
	<i>K2</i>	90.93	81.87	78.07	79.20
	<i>K3</i>	78.40	81.20	83.47	78.07
	<i>R</i>	21.46	6.14	6.2	3.46

Note *K*: mean; *R*: range ($R = K_{\max} - K_{\min}$); Mean ± Standard error

Table 3 Analysis of variance for proliferation coefficient

Factors	Source	Type III sum of squares	DOF	Mean square	F-value	Significance
Proliferation coefficient	A (6-BA)	697.707	2.00	348.853	37.218	$P < 0.05$
	B (NAA)	67.947	2.00	33.973	3.624	$P > 0.05$
	C (KT)	68.240	2.00	34.120	3.62	$P > 0.05$
	Error	18.747	2.00	9.373		

Table 4 Duncan's test in three levels of 6-BA

Factor	Levels	Mean	0.05 Level
6-BA	2	90.93	a
	3	78.40	b
	1	69.47	b

Note Different letters in the table indicate significant differences in the 5% level

the proliferation of *P. ternata* was level 2 (0.1 $\text{mg}\cdot\text{L}^{-1}$), followed by level 3 (0.5 $\text{mg}\cdot\text{L}^{-1}$) and level 1 (0.05 $\text{mg}\cdot\text{L}^{-1}$). The mean analysis showed that the best hormone combination for the simultaneous culture of proliferation and rooting of 'Pearl' *P. ternata* was MS+0.1 $\text{mg}\cdot\text{L}^{-1}$ 6-BA+0.1 $\text{mg}\cdot\text{L}^{-1}$ NAA+1.0 $\text{mg}\cdot\text{L}^{-1}$ KT.

The leaves of 'Pearl' *P. ternata* plants were inoculated with the optimal medium described above. After 15 days,

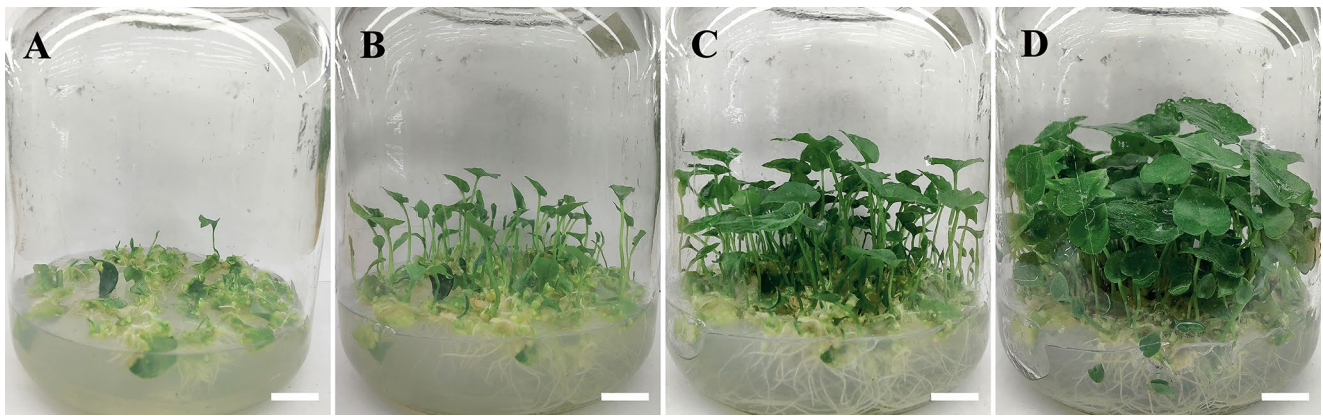


Fig. 3 Leaf proliferation in autotetraploid plants of ‘Pearl’ *P. ternate*. **A** Culture for 15 days; **B** Culture for 25 days; **C** Culture for 35 days; **D** Culture for 45 days. Scale bars=2 cm

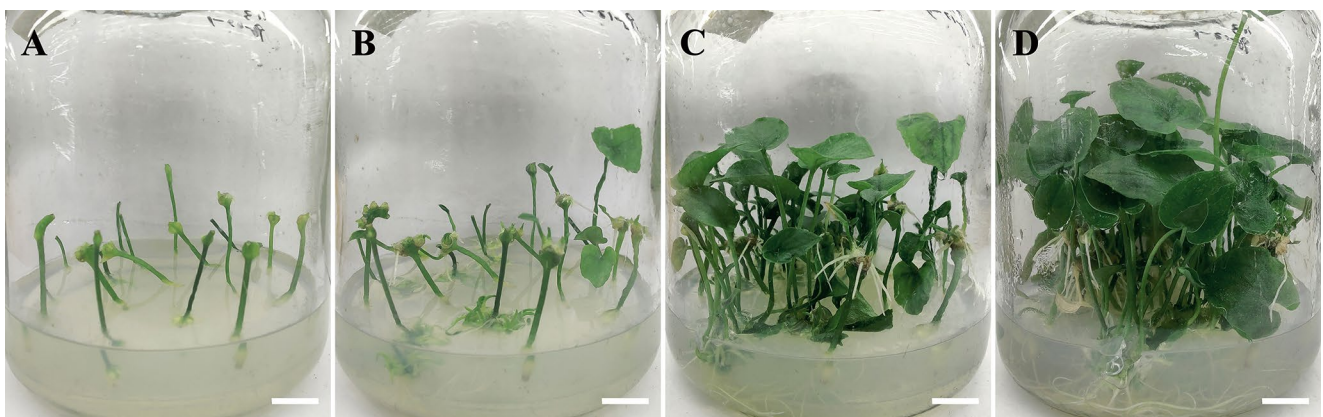


Fig. 4 Petiole proliferation in autotetraploid plants of ‘Pearl’ *P. ternate*. **A** Culture for 15 days; **B** Culture for 25 days; **C** Culture for 35 days; **D** Culture for 45 days. Scale bars=2 cm

small yellow-green tubers formed on the leaf surface, and leaf buds and adventitious roots emerged on the tubers (Fig. 3A). After 25 days of cultivation, the leaves were almost entirely covered by small tubers, new leaves were emerging on all tubers, and adventitious roots were evident (Fig. 3B). After 35 days, the small tubers grew in size, and the leaves became dark green (Fig. 3C). After 45 days, the regenerated plants flourished in growth, and adventitious roots covered the entire substrate (Fig. 3D). 15 days after inoculation, petioles exhibit apical expansion (Fig. 4A). After 25 days in culture, small tubers formed at the base of the petiole, and pearl buds with adventitious roots and leaves formed at the apical expansion (Fig. 4B). After 35 days, all the pearl buds on the petiole’s apex produced new leaves (Fig. 4C). Both the pearl buds at the apex of the petiole and the small tubers at the base of the petiole regenerated into vigorously growing plants after 45 days (Fig. 4D). Tubers can only produce leaves and petioles without stimulation. 10 days after inoculation, tubers developed a few leaf buds (Fig. 5A). After 20 days, the number of leaf buds on the tubers increased, and adventitious roots became visible

(Fig. 5B). After 30 days, the leaves expanded swiftly, the petioles lengthened, and the adventitious roots multiplied (Fig. 5C). After 40 days, the petioles and leaves grew vigorously, the tubers enlarged, and the adventitious roots were robust (Fig. 5D). At this time, the proliferation coefficient was calculated by dividing the three materials of leaves, petioles, and tubers, and it could reach 99.0.

The result of acclimatization and transplanting

After acclimatization in the bottle, the petioles, leaves, and roots of the plants were removed, leaving only the tubers (Fig. 6A). The tubers were transferred to a foam box to keep warm and moist, and after 10 days, some of the tubers produced new leaves with curled leaves and short petioles (Fig. 6B). After 20 days, all tubers had successfully sprouted with spreading leaves and elongated petioles (Fig. 6C). After 30 days, the leaves continued to increase, and the plants grew better (Fig. 6D). ‘Pearl’ *P. ternata* tetraploid plants all grew normally after transplanting and were more likely to

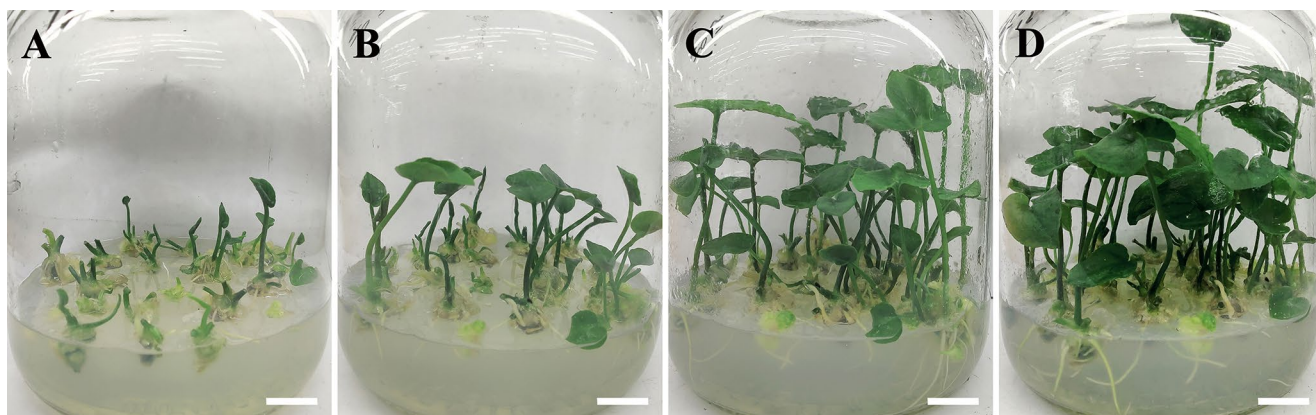


Fig. 5 Tuber proliferation in autotetraploid plants of ‘Pearl’ *P. ternate*. **A** Culture for 10 days; **B** Culture for 20 days; **C** Culture for 30 days; **D** Culture for 40 days. Scale bars = 2 cm

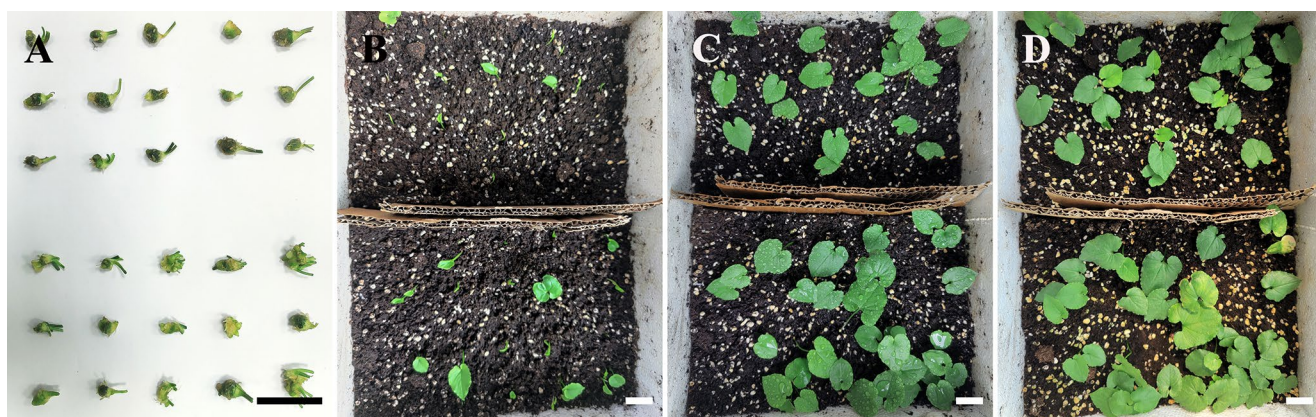


Fig. 6 Comparison of transplants of diploid and autotetraploid plants of *P. ternate*. **A** Tubers with leaves and roots cut off; **B** Growth after 10 days of planting; **C** Growth after 20 days of planting; **D** Growth after 30 days of planting. Top: diploid; bottom: tetraploid. Scale bars = 2 cm

survive and grow more robustly than diploid plants, with an acclimatization survival rate of 100%.

Morphological and anatomical comparison results

The growth of autotetraploid plantlets of *P. ternata* differed significantly from that of diploid plantlets. After 15 days of leaf culture, most of the tetraploid leaves had formed small tubers with adventitious roots, while the diploid leaves formed only a few small tubers on the surface (Fig. 7A and B; top right). Observations revealed that the tetraploid plantlets grew quicker and that the tetraploid leaves produced a greater number of small tubers than the diploid leaves. However, after 30 days of incubation, the development of the tetraploid plantlets was inhibited, and the height of the plants at 45 days of growth was not significantly different from the height at 30 days. At this time, the diploid plants grew faster than the tetraploid plants, and their height exceeded that of the tetraploid plants (Fig. 7A and B). There was no difference in the time required for tetraploid and diploid petioles to produce pearl buds. However, pearl buds on

tetraploid petioles produced leaves more quickly than those on diploid petioles, and tetraploid pearl buds developed into more robust plantlets (Fig. 7C and D). The tubers of both tetraploid and diploid plants produced leaf buds simultaneously, but the diploid tubers produced more leaf buds and grew more quickly; after 40 days of growth, the diploid plants were taller and had more leaves than the tetraploid plants (Fig. 7E and F).

The *P. ternate* autotetraploid differed significantly from the diploids in morphology and cytoarchitecture, as shown in Table 5. The tetraploid plantlets measured approximately 8.5 cm in height, while the diploid plantlets measured approximately 11 cm (Fig. 8A). Compared to diploids, autotetraploid had wider leaves, darker leaf coloration, and a more rounded leaf shape (Fig. 8B). The petioles of tetraploid plants were stronger but shorter than those of diploid plants (Fig. 8C). There was no significant difference in the size and number of roots between tetraploid and diploid tubers, but the tetraploid tubers produced roots that were thicker and shorter (Fig. 8D). It was discovered that the cell and tissue thickness of tetraploid leaves was greater than

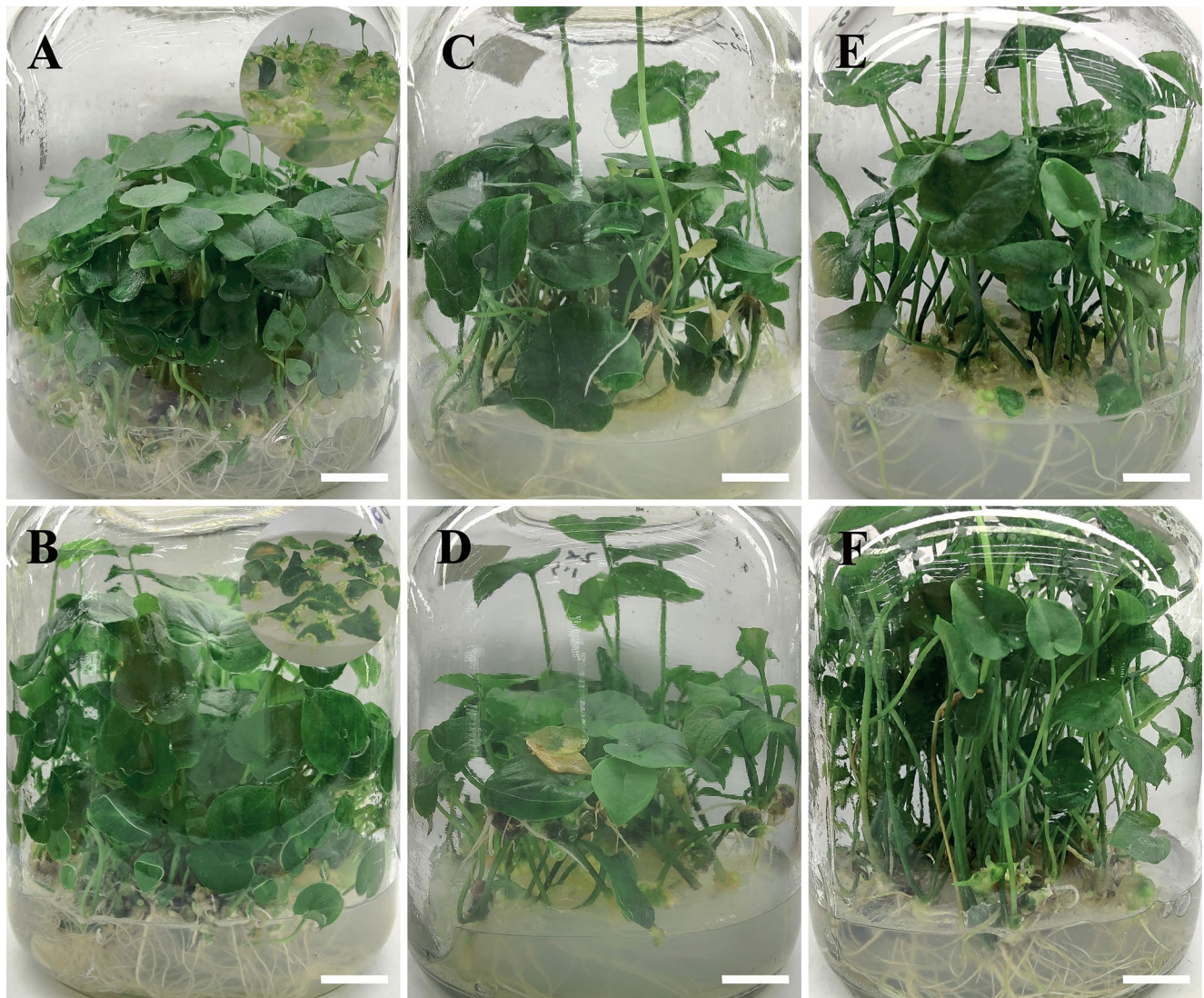


Fig. 7 In-bottle comparison of autotetraploid and diploid *P. ternate*. **A**, **C**, **E** Tetraploid; **B**, **D**, **F** Diploid; **A**, **B** Plantlets formed after 50 days of leaf culture, the upper right corner shows the small tubers produced

after 15 days of leaf culture; **C**, **D** Plantlets formed after 60 days of petiole incubation; **E**, **F** Plantlets formed after 40 days of tuber culture. Scale bars = 2 cm

that of diploid leaves (Fig. 9A and B). Under a microscope, the stomata of the lower epidermis of tetraploid and diploid leaves were observed. The results demonstrated that the stomata of tetraploid leaves were larger than those of diploid leaves, but the density of stomata was substantially lower than that of diploid leaves, which was only half that of diploid leaves (Fig. 9C, D, and E). In terms of morphology and cellular tissue structure, the *P. ternate* tetraploid showed typical polyploid characteristics and differed significantly from the diploid.

Discussion

Effect of material choice on chromosome doubling

Importantly influencing the polyploid induction process was the type of plant material, and tissues or organs with vigorous cell division were generally regarded as the most suitable induction material (Li et al. 2018). Seeds are less functionally differentiated, most cells divide at a more uniform rate, and their cells are subject to similar effects when treated with growth inhibitors, so chimera formation is reduced or prevented (Xi et al. 2021). However, some species were not suitable for doubling using seeds, and chromosome doubling was not successfully achieved in *C. lancifolius* (Roxb.) Kurz and *Lepidium sativum* L.

Table 5 Comparison of morphology and stomata of diploid and autotetraploid plants of *P. ternate*

Ploidy	NLBT	Plant height (cm)	Petiole diameter (mm)	Leaf length (cm)	Leaf wide (cm)	Stomatal length (μm)	Stomatal frequency
2X	4.21 \pm 0.88a	11.19 \pm 0.24a	1.53 \pm 0.59b	3.14 \pm 0.78b	2.14 \pm 0.46b	30.15 \pm 0.58b	5.28 \pm 0.45a
4X	1.22 \pm 0.78b	8.56 \pm 0.56b	2.80 \pm 0.98a	4.18 \pm 0.37a	3.45 \pm 0.56a	50.13 \pm 0.98a	3.18 \pm 0.46b

Note NLBT: Number of leaf buds on the tuber

even when the seed coat was removed and germinated seeds were used; this phenomenon may occur because the seeds of these plants were insensitive to colchicine, or it may be that seeds were stimulated by colchicine and triggered their dormancy mechanism (Aqafarini et al. 2019; Xi et al. 2021). Simultaneously, the seed induction polyploid induction rate was low, the number of seeds required was large, and survival and propagation after successful seed doubling were more challenging, so successful use of seed induction autotetraploid was reported less frequently (Abdoli et al. 2013; Pei et al. 2019). For these reasons, researchers mostly conduct in vitro polyploid induction, which was established based on the target plant regeneration system. However, this method has high laboratory setup and tissue culture technology requirements (Khalili et al. 2020). However, in vitro induction of polyploids led to the formation of more chimeras, making screening polyploid plants more challenging (Aqafarini et al. 2019; Khalili et al. 2020). When inducing polyploids in vitro, stem segments, stem tips, scales, and somatic embryos were typically used, and the potential for doubled chromosomes varied between explants. The stem tip of *C. lancifolius* was more suitable to polyploid induction than the seeds (Xi et al. 2021); the somatic embryos of *L. distichum* Nakai and *L. cernuum* Komar were more suitable to polyploid induction than the scales and no chimeras were produced (Fu et al. 2019). In addition, the developmental stage of the plant receptor (age of the explants) was a crucial factor in the successful induction of polyploids. The cotyledon stage and true leaf stage (when there were two true leaves) of *L. sativum* were treated with colchicine, and it was discovered that polyploid plants were easier to obtain in the true leaf stage (Aqafarini et al. 2019). In this study, *P. ternate* leaves served as the first source of induction material; following colchicine treatment, the number of regenerated plants decreased relative to the control group, whereas the tetraploid induction rate was at its maximum. The tubers were the second material utilized for this study. Under either treatment, the tubers expanded, and the leaf buds on the surface died, indicating that colchicine has a greater toxic effect on *P. ternate* tubers. The leaf buds within the tubers began to develop into new plants after a period of cultivation, but their induction rate was lower than that of the leaves. The use of explants containing cells at the same developmental stage might decrease chimera formation. Although both the leaves and tubers of *P. ternate* were capable of inducing tetraploids, leaves with a single cell source might have higher polyploid induction rates and a lower incidence of chimerism than tubers with multiple layers of differentiated cells. This phenomenon has been previously reported in *Papriopedilum villosum* var. *boxallii* Veitch (Huy et al. 2019). Chimeras appeared in all treatment groups in this study, and chimeras were common in polyploid induction

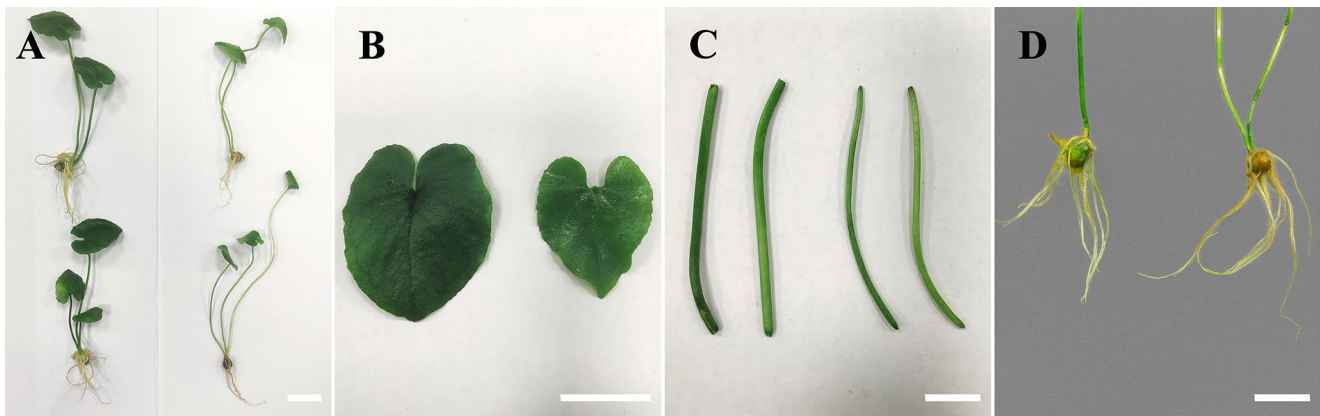


Fig. 8 Comparison of plant morphology between autotetraploid and diploids of *P. ternate*. **A** Comparison of intact plants; **B** Comparison of leaves; **C** Comparison of petioles; **D** Comparison of tubers and root systems. Left: tetraploid; right: diploid. Scale bars = 2 cm

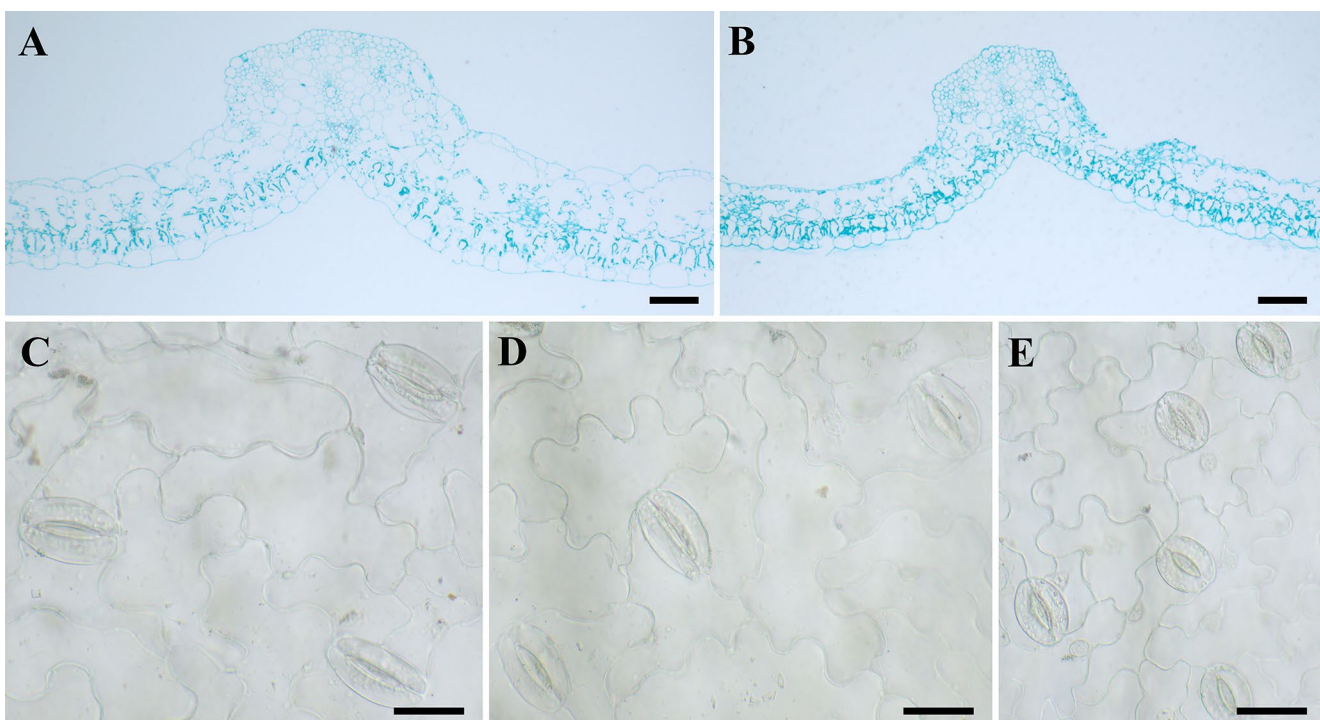


Fig. 9 Comparison of cell and tissue structure between the autotetraploid and diploids of *P. ternate*. **A** Longitudinal micrograph of the tetraploid leaf; **B** Longitudinal micrograph of the diploid leaf; **C, D**

Micrograph of subepidermal stomata of the tetraploid leaf; **E** Micrograph of subepidermal stomata of the diploid leaf. Scale bars: A, B = 200 mm; C, D, E = 50 mm

(Fu et al. 2019; Xi et al. 2021). The uneven penetration of multicellular tissue and colchicine into meristem is the main reason for the formation of chimera. In the apical bud sections of *Manihot esculenta* Crantz diploid and chimera, it was found that the cells of the L1 layer (epidermal cells) and L2 layer (subepidermal cells) of the chimera were significantly larger than those of diploid (Hashimoto-Freitas and Nassar 2013). Chimeras are usually considered to be failures or undesirable by-products of polyploidization studies, and they cannot produce stable polyploids (Eng et al. 2021). However, chimeras still have some value in plant

development research, and the new genetic types produced by chimeras may be beneficial to breeding research (Yan et al. 2014).

Effect of hormones on different ploidy levels in plants

There are three primary types of meristematic tissues in plants: apical meristematic tissues in stems and roots and the procambium layer in vascular tissues (Ye and Zhong 2015). Materials containing meristematic tissues are

capable of producing new plants in plant regeneration in vitro more rapidly. The leaf achieved rapid in vitro reproduction mainly via the vascular bundle formation layer contained in the leaf veins, and the formation layer's activity was regulated by complex hormones, primarily cytokinin, and auxin regulation (Nieminen et al. 2015). Important factors influencing plant regeneration in vitro were plant hormones, whereas endogenous hormones were thought to undergo drastic changes after double chromosomes. In tetraploid leaves of *Salix viminalis* E. L. Wolf, the levels of active gibberellins, cytokinin, salicylic acid, and jasmonic acid were significantly higher than in diploid leaves, and elevated levels of indoleacetic acid, active cytokinin, active gibberellins, and salicylic acid were detected in their root tips (Dudits et al. 2016). In the *Liquidambar styraciflua* × *L. formosana* tetraploid, genes that produce phytohormones in the root and stem were generally turned down, and auxin, gibberellin, and oleuropein lactone contents were significantly lower than in the diploid (Chen et al. 2022). The tetraploids of *Populus alba* × *P. glandulosa* had increased indole acetic acid and gibberellin content and decreased jasmonic acid and abscisic acid content compared to the diploids (Ren et al. 2022). The medium for diploid plants is frequently unsuitable for the growth of their autotetraploid counterparts because the conditions for in vitro regeneration changed after chromosome duplication due to changes in endogenous hormone content. In this study, the autotetraploid of *P. ternate* had three induction materials: leaf blade, petiole, and tuber. The tuber was able to grow normally in the medium of diploid plants, whereas the leaf and petiole did not achieve complete regeneration, and the without re-differentiation leaves and petioles progressively yellowed and died as the culture time was prolonged. Chromosome doubling has a profound effect on hormone homeostasis and tissue development in plants. Changes in endogenous hormone concentrations are likely to inhibit or prevent the formation of microscopic tubers in the procambium layer of leaf vascular bundles (Swarup et al. 2008; Klimešová et al. 2009). In *C. lancifolius*, the level of cytokinin in the medium of tetraploid plants was 10 times lower than in the medium of diploid plants, and tetraploid plants were able to regenerate without passing through a callus and achieved simultaneous proliferation and rooting (Xi et al. 2020, 2021). We modified the medium optimal for the culture of diploid and reformulated the exogenous hormone concentration for orthogonal proliferation experiments to obtain a new medium suitable for the growth and development of autotetraploid *P. ternate*. Compared to the medium optimal for the culture of diploid (MS + 0.05 mg·L⁻¹ 6-BA + 0.1 mg·L⁻¹ NAA + 0.5 mg·L⁻¹ KT), the medium optimal for the culture of tetraploid (MS + 0.1 mg·L⁻¹ 6-BA + 0.1 mg·L⁻¹ NAA + 1.0 mg·L⁻¹ KT) contained higher concentrations

of 6-BA and KT, while the NAA concentration remained unchanged. Cytokinin was directly related to the production of small tubers in the leaves of *P. ternate*, and increasing the concentration of exogenous cytokinin in the medium indirectly indicates that the expression of genes involved in the synthesis of endogenous cytokinin was suppressed in *P. ternate* after double chromosomes. Even though the proliferation coefficient of autotetraploid leaves decreased in a tetraploid medium, they still retained the advantages of direct organ regeneration and simultaneous proliferation and rooting.

Effect of chromosome double on plant reproduction potential

Polyploidy (whole genome duplication, or WGD) was a crucial mechanism for species formation, genome evolution, and biodiversity maintenance, as well as a traditional strategy for the initial domestication of crops (Soltis and Soltis 2016). However, it is unknown how polyploidy persists and achieves evolutionary success in nature. Sexual reproduction in nascent polyploids frequently occurs in error due to irregular chromosome segregation, resulting in the incomplete formation of reproductive structures or the production of malformed embryos (Sattler et al. 2016). In the case of reduced sexual reproduction, nascent tetraploids may reallocate their internal resources to asexual reproduction (rhizome, stolon, bead bud, etc.) (Vallejo-Marín et al., 2010). It has also been reported that chromosome doubling can immediately alter the phenotype and reproductive pattern of plants, that the asexual reproduction of nascent polyploids may differ from diploids in number and size due to gene dosage effects or gene interactions, and that whole genome duplication increases the rate of asexual reproduction in nascent polyploids (Cifuentes et al. 2010; Ramsey and Schemske 2002). *Chamerion angustifolium* (Linnaeus) Holub tetraploids produce more and smaller root buds than diploids under greenhouse conditions (Van Drunen and Husband 2018). Tetraploid *Solidago gigantea* Aiton plants have more root buds than diploid plants under natural conditions (Schlaepfer et al. 2010). In an in vitro culture of *Gentiana decumbens* L.f., cells from tetraploid plants entered a new cell cycle more readily than cells from diploid plants, indicating that tetraploid plants could form regenerating plants faster than diploid plants (Tomiczak et al. 2015). In this study on the in vitro regeneration of diploid and autotetraploid leaves of 'Pearl' *P. ternata*, it was discovered that tetraploid leaves formed small tubers much more quickly and in greater quantity than diploid leaves. Although it was unclear how whole genome duplication directly affects asexual reproduction, polyploids have influenced gene expression of meristematic tissue growth and regulatory

hormones (Dai et al. 2015). *P. ternata* tuber originates from the procambium layer in the vascular tissue in the leaf veins, and whole genome duplication results in an increase in the number of leaf vein tissues as well as the transfer of energy from sexual reproduction to asexual reproduction patterns, which most likely leads to an increase in the rate and number of bud regeneration in tetraploids (Klimešová et al. 2009). Under natural conditions, asexual propagation of *P. ternata* involves the formation of new plants from pearl buds and leaf buds (produced by tubers), and if the above reasons are followed, the number of leaf buds on the tubers of tetraploid plants will be greater than that of diploids, but the number of leaf buds produced by diploid tubers during in vitro regeneration is greater than that of autotetraploid. Tubers grow to a certain age before leaf buds are formed, and autotetraploid plants may have slow growth due to chromosome doubling. Therefore, our group hypothesized that the number of leaf buds produced by autotetraploid plants of ‘Pearl’ *P. ternata* was less than that of diploid, primarily due to the slow growth of the plants; after cultivation in the soil, the number of leaf buds produced by autotetraploid tubers would exceed that of diploid.

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Author contributions The authors confirm their contribution to the paper as follows: methodology, performing experiments, and drafting: YR; work design and performing experiments: JG; investigation and data analysis: XD; conceptualization, work design, and methodology: HY; methodology and revising the manuscript: HH. All authors reviewed the results and approved the final version of the manuscript.

Data availability All the data of this study are available within the paper.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest to report regarding the present study.

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