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Junshuo Qiu
Foshan University
Yuanyuan Wang
Foshan University
Jing Zhang
Foshan University
Tao Li
Foshan University
Yuanrong Xu
Foshan University
Tingting Wang
Foshan University
Tao Xie
xietao@fosu.edu.cn

Foshan University https://orcid.org/0000-0002-0373-7425

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Colchicine-Induced Polyploidy in pineapple (*Ananas comosus* L.) and Its Identification

Junshuo Qiu, Yuanyuan Wang*, Jing Zhang, Tao Li, Yuanrong Xu, Tingting Wang, Tao Xie*

Foshan University, Foshan 528231, China

*Corresponding author

***Corresponding author:**

Tao Xie
Foshan University, Foshan 528231, China
Tel number: +86-020-85501986
Email: xietao@fosu.edu.cn

Yuanyuan Wang
Foshan University, Foshan 528231, China
Tel number: +86-020-85501986
Email: 15626047153@163.com

Abstract

Enhancing the horticultural and commercial value of *Ananas comosus* through polyploidy has great potential, as does meeting market demands. This study used different colchicine treatments, durations, and concentrations to induce polyploidy in pineapple somatic embryo callus. Consequently, a maximum polyploid induction rate of 40.00% was achieved in somatic embryogenic callus exposed to 0.1% colchicine for 20 days. Flow cytometry revealed 202 tetraploids among the regenerated plants analyzed. Polyploid plants, in contrast to diploid ones, are morphologically characterized by having thicker, heavier, wider, and shorter leaves, a larger leaf area, a darker color, a shorter overall height, thicker stems, and fewer, shorter leaf spines that are more irregularly distributed and differently shaped. In polyploids, the stomata were both larger and less numerous than in diploid plants, and the stomatal guard cells and leaf epidermal cells were larger too. The study revealed that polyploid plants exhibited much higher levels of chlorophyll and PEPC enzyme activity than the diploid control plants. Compared to diploid control plants, polyploid plants displayed enhanced resistance to salt and heat following treatment. Overall, this research outlined an efficient strategy for inducing polyploidy in pineapples, paving the way for future advancements in pineapple germplasm resources.

Key words: pineapple, somatic embryogenesis, polyploid plant tolerance, tetraploidy

Abbreviations

MS: Murashige and Skoog, 2,4 – D: 2,4 -dichlorophenoxyacetic acid, 6-BA: 6-benzylaminopurine, IBA: Indole-3-butyric acid, NAA: 1-Naphthaleneacetic acid, PEPC: phosphoenolpyruvate carboxylase, PPDK: pyruvate phosphate dikinase, Rubisco: ribulose-1,5-bisphosphate carboxylase, REC: relative electrical conductivity, MDA: malondialdehyde, SOD: superoxide dismutase, CAT: catalase, Pro: proline, POD: peroxidase.

Introduction

Ananas comosus (*A. comosus*), is a perennial herbaceous plant from the Pineapple genus in the Pineappleaceae family, which contains around 8 species and 60 to 70 cultivated varieties (Hossain 2016; Bartholomew et al. 2018). With edible and economic benefits, the fruit is notable, and its leaves and flowers have a unique form. It also has potential for ornamental horticulture development (Bartholomew et al. 2018). Therefore, the research on polyploid induction of pineapple is of great significance for the creation of new germplasm and the improvement of variety characteristics. Nevertheless, China's pineapple industry relies on imported core germplasm resources due to a lack of domestically developed varieties. The existing varieties have issues like inadequate stress resistance and narrow adaptability, which seriously restrict industrial upgrading (Sun et al. 2019; Jing et al. 2022). Hence, the study of polyploid induction in pineapples is important for the creation of new germplasm and the advancement of variety characteristics.

Polyploidy, when it occurs naturally, is widespread in plants, and polyploidization is considered a vital mechanism for driving plant evolution and the creation of new species (Mizrachi et al. 2017). Polyploid plants tend to have advantages over diploid plants, including organ enlargement like flower and leaf thickening, enhanced stress resistance, and increased metabolite accumulation (Wu et al. 2025; Wang et al. 2025). Artificial polyploid induction is a significant method in plant breeding, utilized broadly in the breeding of crops (Zhang et al. 2024), fruit trees (Lu et al. 2024), vegetables (Xie et al. 2025), and flowers (Zheng et al. 2025) to capitalize on these advantages (Zhang et al.

2024; Lu et al. 2024; Xie et al. 2025). Preventing the formation of mitotic spindles with colchicine allows for the effective production of polyploid materials (Tapia-Campos et al. 2019). Multiple factors influence the success rate of polyploid induction by colchicine, including the species of plant, the parts that are treated, and differences in both colchicine concentration and treatment duration, leading to variations in induction effectiveness (Van Laere et al. 2011). In pineapple, 0.05% colchicine was applied to generate various genotypes, significantly impacting parameters like plant height, leaf number, stomatal length, leaf shape, and leaf color (Mursanto et al. 2021). The study indicated that treating pineapple callus with 0, 0.01%, 0.05% and 0.1% colchicine concentration enhance callus thickening, with this concentration being the most effective option (Istiqomah and Shofi 2018). Despite this, the research is still in its nascent exploratory stage, and a comprehensive induction system has yet to be developed. Meanwhile, there are evident shortcomings in the present research on the stress resistance mechanisms, photosynthetic physiology, and additional aspects of pineapple polyploids, demanding urgent and in-depth analysis.

Alongside an effective polyploid induction system, dependable identification techniques are vital for the successful acquisition of polyploids. As a quick and relatively precise technique, flow cytometry (FCM) is appropriate identifying artificially induced polyploids on a large scale (Liu et al. 2024). Counting chromosomes in the mitotic cells of root tips can effectively determine ploidy levels, it is a time-intensive process that demands expert skills. Thus, there have been initiatives to devise indirect methods for determining ploidy, such as employing stomatal area to tell apart diploid and tetraploid regenerated orchid plants (Chen et al. 2023). Distinguishing between diploid and tetraploid plants has also involved using the count of chloroplasts each guard cell pair (Wan et al. 1990) and variations in stomatal density (Wang et al. 2024).

The early-flowering mutant line '*Yulinglong*' of dwarf pineapple was utilized as the material in this study. The embryogenic callus culture system was used for polyploid induction experiments, involving a combination of soaking and co-cultivation methods, different durations, and colchicine concentrations (0, 0.01%, 0.05%, 0.1%). The ploidy of regenerated plants was determined by flow cytometry, and the differences between polyploid and diploid plants were systematically analyzed through morphological observations, chlorophyll content measurements, photosynthesis-related enzyme assessments, and salt and heat stress treatments. This research was conducted to develop an efficient polyploid induction technology for pineapples, supplying a theoretical basis and technical reference for breeding stress-resistant pineapples and innovating their germplasm.

Materials and methods

Plant materials, growth conditions and sample collections

The study focused on the novel cultivar '*Yu Linglong*' an early-flowering mutant strain that comes from the dwarf bromeliad 'himepine' (*A. comosus* cv. '*himepine*'). This cultivar was first introduced from Japan and then sourced from the experimental orchard of South China Agricultural University. Due to its unique early-flowering feature and compact growth habit, it is an ideal candidate for polyploid induction research.

The plant materials were maintained under strictly controlled environmental conditions. The callus tissues and test-tube seedlings of *A. comosus* cv. '*Yu Linglong*' were cultivated in a growth chamber with a 16-hour light and 8-hour dark photoperiod. The intensity of light during the photoperiod was adjusted to 50 - 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with the help of cool-white fluorescent lamps.

During the day, the temperature was kept at 24 ± 2°C, and at night, it was maintained at 16 ± 2°C, with the relative humidity held at 75 ± 5%. The nutrient solution for callus culture included Murashige and Skoog (MS) basal medium, 5.0 mg/L 2,4 -dichlorophenoxyacetic acid (2,4 - D), 0.5 mg/L 6-benzylaminopurine (6-BA), 30.0 g/L sucrose, and 7.0 g/L agar ($D_5B_{0.5}$) with the pH adjusted to 5.8 before being autoclaved at 121°C for 20 minutes. To promote shoot growth, the MS medium for the test-tube seedlings was modified to incorporate 2.0 mg/L Indole-3-butyric acid (IBA). The pineapple plants were grown in a greenhouse equipped with automated climate control systems. The environment inside the greenhouse was adjusted to replicate the conditions of a growth chamber, including similar photoperiod, temperature, and humidity. A sterilized mix of peat moss, perlite, and vermiculite in a 3:1:1 ratio was used to pot the plants, which were fertilized biweekly with a 20-20-20 NPK water-soluble fertilizer at 200 mg/L.

In the logarithmic growth phase, approximately 2 to 3 weeks after subculture, Ananas comosus callus was used for polyploid induction experiments. Samples from the test-tube seedlings were taken once they grew to a height of 5-6 cm, typically 4-5 weeks after being transferred to new medium. Leaves were individually collected from each seedling for characterization in terms of morphology, cytology, and physiology. To ensure accurate tracking and analysis throughout the study, all samples were labeled with detailed information, including the collection date, plant number, and treatment group.

Embryogenic callus induction

Pineapple explants were placed on a medium containing MS, 2.0 mg/L 6-BA, 2.5 mg/L 1-Naphthaleneacetic acid (NAA), 30 g/L sucrose, and 7.0 g/L agar ($B_2N_{2.5}$) to promote callus formation or seedling regeneration and subculturing. Once enough callus was subcultured, the pineapple callus was cut into 5 mm^3 pieces, the size of a soybean, and inoculated into $D_5B_{0.5}$ medium with a pH of 5.8 for embryogenic callus culture (Marchant et al. 2003). The setup of the experiment included 5 calluses per bottle, with 20 bottles (100 calluses) allocated for each treatment to comply with statistical sample size standards.

Colchicine treatment and shoot regeneration

In the soaking procedure, a total of 12 experimental treatment groups were set up using four colchicine concentrations (0, 0.01%, 0.05%, and 0.1%) and three treatment durations (24, 48, and 72 hours). For each treatment, 100 pieces of embryonic callus were placed in a thermostatic shaker with an oscillation frequency of 50 times per minute to enhance colchicine penetration through mechanical agitation, and they were kept in the dark to prevent photodegradation. After soaking, thoroughly rinse three times using sterile water. Following induction, the callus was moved to a differentiation medium consisting of MS, 2.0 mg/L 6-BA, 1.0 mg/L NAA, 30 g/L sucrose, and 7.0 g/L agar (B_2N_1), where the induction process was monitored and documented. In roughly two months, the callus gave rise to adventitious buds, and the buds that regenerated from the callus were subjected to further analysis.

The mixed culture technique utilized $D_5B_{0.5}$ medium with colchicine concentrations of 0, 0.01%, 0.05%, and 0.1% for durations of 10, 20, and 30 days, respectively, creating a total of 12 experimental groups. By ensuring continuous exposure to colchicine, this method prolonged the action time. Each treatment used 100 pieces of embryonic callus, placed in a medium that contained colchicine. Selecting the culture cycle corresponded to various stages of cell division and differentiation. The 10-day treatment group focused on short-term induction, while the 30-day treatment group simulated the effects of long-term stress. After being cultured, the callus was moved

to B_2N_1 medium to resume growth. The mortality rate was monitored throughout the experiment. Adventitious buds were induced for additional analysis.

Flow cytometry for ploidy level estimation

Following two months in the regeneration medium, the adventitious buds that reached 1.5 cm were moved to a rooting medium (MS medium with 2 mg/L IBA, 30 g/L sucrose, and 9 g/L agar, pH 5.8) for a duration of one month. Flow cytometry was used to determine the ploidy level when the seedlings reached a height of approximately 3-5 cm. In this research, 209 mutant regenerated seedlings treated with colchicine were analyzed using flow cytometry. In particular, the fresh leaves from each sample were finely sliced with 500 μL of Partec HR-A nucleus extraction buffer (Sysmex Partec, Germany) using a razor blade in a petri dish. The mixture is passed through a Partec CellTrics 30 μm disposable filter (Sysmex Partec, Germany) to eliminate leaf tissue debris. Following this, the extracted nuclei were immersed in 1.5 mL of 4,6-diamidino-2-phenylindole (DAPI) staining buffer (Sysmex Partec, Germany) and allowed to sit in the dark for 5-10 minutes. The CyFlow Counter flow cytometer from Sysmex Partec, Germany, was employed to examine suspensions with stained nuclei. Using flow cytometry, the DNA content distribution curves of the induced regenerated plants and the control plants were assessed, with each sample tested no fewer than three times. First, the average peak value for each sample was computed using three repetitions, and then the average peak values for diploid, tetraploid, octoploid, and chimera were calculated individually. The ploidy level was assessed by comparing each sample's average peak to that of the diploid (Ochatt 2008). The induction rate was determined using this formula: induction rate (%) = (number of polyploid seedlings/total seedlings analyzed) × 100.

Leaf morphology measurement

Following flow cytometry analysis, when the regenerated plants attained a size of 3-5 cm, their morphological changes were systematically monitored and quantitatively evaluated, including growth potential (plant height, stem diameter, internode length), leaf phenotype (color, length, width, thickness), and overall plant type. Leaf color was determined with a hand-held colorimeter, accurate to ± 0.1. The measurement point was chosen in the vein-free section at the center of the leaf, and the three-color coordinate values of L^* (brightness, ranging from 0 to 100, where $L = 0$ is black and $L = 100$ is white), a* (red-green scale, positive red, negative green) and b^* (yellow-blue scale, positive yellow, negative blue) were documented. The leaf shape index is calculated by dividing the leaf length by the leaf width. Using a stereomicroscope, the shape, size, density, color, and opening angle of plant leaf spines were observed and measured. Three functional leaves, specifically the 2nd to 4th from the top, were measured for each plant, and each treatment group repeated this process with three plants to ensure statistical validity. For measuring morphological indexes, an electronic digital caliper with 0.1 mm accuracy was used to measure leaf length and width, while a micrometer with 0.01 mm accuracy was used for thickness.

Determination of leaf pigment and key photosynthetic enzyme contents

The chlorophyll content was measured using the leaf area method in this experiment. A total of 10 polyploid plants and 10 control plants that were growing well were chosen. Each plant was punched with a 0.2 cm^2 circular puncher, and five round leaves were arranged. After mixing, it was split into three sections and cut into pieces. The prepared 10 mL centrifuge tube contained the cut leaves and 1 mL of ethanol. The leaves were pulverized in a grinder for homogenization, then 9 mL of ethanol was added to the centrifuge tube, shaken thoroughly, and centrifuged at 10,000 rpm for 10 minutes. The supernatant was then extracted for testing. The ultraviolet spectrophotometer was

calibrated to zero using ethanol at wavelengths of 663, 646, and 470 nm, and then the absorbance of the supernatant was measured at these three wavelengths. The absorbance measurement results were used to calculate the concentrations of chlorophyll a, chlorophyll b, total chlorophyll, and pigment content individually (Babu et al. 2007). Three leaves were taken from each plant, sliced into pieces, and blended. The activities of photosynthesis-related enzymes, including phosphoenolpyruvate carboxylase (PEPC), pyruvate phosphate dikinase (PPDK), and ribulose-1,5-bisphosphate carboxylase (Rubisco), were measured using the samples, each with a weight of 0.5 grams. Three 0.5 g samples were weighed to measure the activities of photosynthesis-related enzymes, including PEPC, PPDK, Rubisco. Following the solarbio kit's guidelines, the determination method was conducted.

Determination of stomatal cell size and density

On the lower surface of the mature leaf midsections of both polyploid and control plants, transparent nail polish was spread. Once dried, the nail polish layer was gently peeled off to create epidermal imprints, which were mounted onto glass slides pre-treated with ultrapure water. The coating was leveled, and excess water was removed from the sides using absorbent paper (Zhang et al. 2023). The stomata characteristics were examined under a SZX16 (Nikon, Japan) microscope, with quantitative measurements of stomatal length, width, and density conducted to compare diploid and polyploid samples. Stomatal density was assessed by counting stomata in six evenly distributed microscopic fields, while the length, width, and area of 30 stomata were measured and calculated at each ploidy level.

Polyploid and diploid pineapple treated with high salt and high temperature

Stress treatments in this investigation were performed using a phytotron, with parameter settings guided by relevant literature on plant stress responses to ensure the scientific and rational nature of the experimental conditions. To eliminate the impact of environmental fluctuations on plants, polyploid and diploid control plants, which maintained consistent growth, were pre-acclimated for three days under salt stress or one day under heat stress, both in a standardized environment of 25 °C, 65% relative humidity, 4000 Lx light intensity, and a 16-hour photoperiod. During salt stress treatment, a 150 mM NaCl solution (Bahlaouan et al. 2025) was applied, with sampling divided into two distinct phases: the adaptation period from 0 to 7 days, and the continuous stress phase from 7 to 14 days. A heat-resistant treatment was applied at 40 °C, the critical temperature for pineapple heat damage (Hatfield and Prueger 2015), while soil moisture was maintained at 60 ± 5% of the field's water holding capacity through the weighing method for a duration of four days. Samples were collected on days 0, 2, and 4, with the initial stress period spanning days 0 to 2 and the continuous stress period covering days 2 to 4. Three biological replicates were established for each treatment group, and the top 3-5 functional leaves were gathered. Some samples were used to measure morphology, such as leaf thickness and chlorophyll content, while the rest were flash-frozen in liquid nitrogen and stored at -80 °C for subsequent physiological index analysis.

Morphological indices were assessed using standardized methods: leaf thickness was determined by folding the leaves several times and measuring with a vernier caliper accurate to 0.02 mm, calculated as leaf thickness = measured thickness/n. Fresh and dry weights were recorded using a 0.1 mg precision analytical balance (1/10,000 accuracy), with dry weights obtained by blanching at 105 °C for 30 min and then drying at 80°C until the mass was constant. The measurement of physiological parameters, including relative electrical conductivity (REC), protein (Pro) content,

soluble sugar content, malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, peroxidase (POD) activity, catalase (CAT) activity, and key photosynthetic enzyme activities, was conducted using the conductivity meter method, ninhydrin colorimetry, Coomassie Brilliant Blue G-250 staining, anthrone reagent method, thiobarbituric acid method, nitroblue tetrazolium (NBT) photoreduction, guaiacol oxidation, ultraviolet absorption method, respectively. Physiological parameters were assessed according to the solarbio kit guidelines. All measurements were done three times, and the averages were calculated to ensure the data's reliability.

Comprehensive evaluation of tolerance

The fuzzy membership function approach (Jain and Sharma 2020; Varshney and Goyal 2023) was used for a comprehensive evaluation of multiple indices, standardizing to remove dimension differences and fitting for multivariate stress response analysis. For the i-th index Xi, if it shows a positive correlation with stress tolerance (e.g., chlorophyll content, SOD activity), the membership function is F1(Xi) = (Xmax - Xmin) / (Xi - Xmin); if it shows a negative correlation (e.g., REC, MDA content), it uses F2(Xi) = 1 - (Xmax - Xmin) / (Xi - Xmin), where Xmin and Xmax represent the extreme values of the index across all treatment groups. To assess stress adaptability, the arithmetic mean of membership values across all indices was computed as the comprehensive tolerance index, with higher values reflecting stronger adaptability.

Data processing and visualization analysis

This study adopted a standardized data processing workflow, establishing a rigorous statistical analysis framework using Microsoft Excel 2010 and IBM SPSS Statistics 20.0 SPSS (Chicago, IL, USA). Raw data recording, organization, and basic statistics like mean and standard deviation were done using Excel 2010, whereas SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used for more advanced statistical analyses. A two-way ANOVA was conducted to examine the primary effects and interactions of different stress types (salt/heat) and ploidy levels (polyploid/diploid), with Duncan's multiple range test ($p<0.05$) employed to assess significant differences between groups. At the same time, Pearson correlation analysis ($|r|>0.7$ and $p<0.01$) was used to identify key indices significantly associated with stress tolerance, removing redundant variables to improve the scientific credibility of the evaluation system. Furthermore, SPSS 20.0 was used to perform One-way ANOVA and Duncan's test, with a significance level of $p<0.05$ to maintain the rigor of statistical inference.

Visual analysis was conducted using a combination of GraphPad Prism 8 and Adobe Photoshop CC 2017. Prism 8 facilitated the creation of standardized charts (such as bar graphs and line charts), automatically incorporating error bars (±SD) and statistical significance markers (*$p<0.05$, **$p<0.01$), with its ANOVA module verifying the statistical alignment between the data visualization and analysis results. Post-production optimization of charts was carried out with Photoshop CC 2017, focusing on resolution enhancement to at least 300 dpi, color mode unification to CMYK, and the standardization of legend annotations.

Results

Embryonic callus induction

Pineapple sucker segments were first inoculated on $B_2N_{2.5}$ medium to trigger callus formation. Afterward, the callus was transferred to MS medium with 3.0 mg/L 6-BA, 2.0 mg/L NAA, 30 g/L sucrose, and 7.0 g/L agar (B_3N_2), and cultured for an additional 30 days to obtain callus clusters with a diameter of 5-8 mm (Fig. 1A). The callus that had proliferated was introduced to $D_5B_{0.5}$ medium to initiate embryogenic callus induction. The surface of the induced embryogenic callus

displayed spherical granules that protruded, along with loose, friable, and non-compact granular tissue blocks (Fig. 1B, C).

Effect of colchicine treatment on callus induction

Using the immersion method to treat embryogenic callus, the mortality rate of calli rose as the treatment duration increased at the same colchicine concentration (Fig. 2A). With colchicine concentrations of 0.01% and 0.05%, the rate of phenotypic variation grew with the length of treatment, resulting in 18 and 28 phenotypic variation plants, respectively (Fig.2A, Table 1). With a colchicine concentration of 0.10%, extending the treatment duration led to a reduction in the phenotypic variation rate, resulting in 49 plants showing phenotypic differences. With a 24-hour treatment, the number of phenotypic variation plants reached its peak at 19 (Fig. 2A, Table 1). As the concentration of colchicine increased, both the mortality rate and phenotypic variation rate rose at the same treatment duration (Fig. 2A). A total of 19 plants exhibited phenotypic variations after being treated with 0.10% colchicine for 24 hours, achieving a variation rate of 19.00%, which was the best condition for the soaking method.

For the mixed culture approach, maintaining a constant treatment duration resulted in a marked increase in calli mortality rate as colchicine concentration went up (Fig. 2B, Table 1). Using colchicine at 0.01% and 0.05% concentrations for 10 days did not alter plant phenotypes, but a 0.1% concentration led to a 4.00% variation in phenotype. At the 20-day mark, the phenotypic variation rate rose with concentration, peaking at 40.00%. Nonetheless, at 30 days, the rate of phenotypic variation decreased with increased concentrations (Fig. 2B, Table 1). With the same colchicine concentration, mortality rates rose as treatment durations lengthened, whereas phenotypic variation rates first rose and then fell (Fig. 2B, Table 1). The most effective condition for the mixed culture method was a 20-day exposure to 0.10% colchicine, leading to the highest phenotypic variation rate of 40%.

There were notable differences in phenotypic variation rate and mortality between the two techniques. The mortality rate of pineapple embryogenic calli in the mixed culture method (35.00%–96.00%) was typically higher than in the immersion method (26.00%–46.00%). Compared to the soaking method, which had an 19.00% phenotypic variation rate, the mixed method achieved a significantly higher rate of 40.00% in regenerated plants. The immersion method's ideal condition was a 24-hour treatment with 0.1% colchicine, while the mixed culture method reached its highest induction efficiency with 0.1% colchicine treatment for 20 days. Taken together, these outcomes imply that using a mixed culture approach is more effective for pineapple polyploidy induction.

Polyploid identification by flow cytometry

The ploidy level of regenerated plants treated with different colchicines was evaluated by flow cytometry and FCS Express software. A total of 209 colchicine-treated phenotypically mutated plants were selected for ploidy analysis. As illustrated in Fig. 4 and Table S1, two distinct flow cytometry patterns were observed. The uninduced diploid plants exhibited a significant peak averaging 25.26 (Fig. 3A, Table S1), whereas some induced plants had their large peaks shift to an average of 48.92, signifying tetraploid (Fig. 3B, Table S1). A total of 202 tetraploids were successfully derived from 209 colchicine-treated mutant plants (Table 1, Table S1).

The comparison of polyploid induction efficiency under different colchicine treatments is shown in Table S1. Results indicated that the immersion method with 0.1% colchicine for 24 h achieved the highest induction rate of 18%, yielding 18 tetraploid plants (Table 1). In contrast, the mixed culture method with 0.1% colchicine for 20 days showed the highest induction rate of 40%,

generating 40 tetraploid plants. Other treatments showed notably lower induction rates. These findings confirm that the optimal condition for inducing polyploids in pineapple tissue-cultured seedlings is treating embryogenic callus with 0.1% colchicine via mixed culture for 20 days.

Morphological characteristics of *A. comosus* polyploids

Following the analysis of ploidy levels, notable variations in leaf width, thickness, and color were found between tetraploid (4X) and control (2X) plants. 4X plants, in particular, had wider, thicker, and darker leaves (Fig. 4). There were also significant differences in leaf color between tissue-cultured 4X and 2X. The handheld colorimeter data revealed that 2X plants had significantly greater L values (48.39±1.27), |a| values (8.30±0.56), and |b| values (29.51±1.84) than 4X plants, which had values of 38.41±1.05, 7.35±0.42, and 13.49±1.12 respectively, with $p<0.05$ (Table 2). The L value, reflecting lightness, indicated that 2X leaves were closer to white (L=100), while 4X leaves were darker. The a value, which is negative for green, and the b value, which is positive for yellow, indicated that 4X leaves exhibited less green saturation and yellowness compared to 2X, resulting in a dark green appearance (Fig. 4).

Plants with different ploidy were transplanted into the greenhouse and grown for another two months, after which their morphological characteristics were analyzed. Results showed that 4X plants exhibited typical polyploid traits in their leaves compared to 2X plants. As depicted in Table 2 and Fig. 5A, B, C, 4X plant leaves were thicker, heavier, and broader with larger leaf areas, while being shorter in length and having a reduced plant height (Fig. 5C, Table 3) compared to 2X plants. The 2X plants exhibited an average leaf thickness of 0.3575, with leaves measuring 9.64 cm in length, 0.66 cm in width, and covering an area of 5.67 cm² (Table 2). Differently, 4X plants had leaves with an average thickness of 0.5542, a length of 9.23 cm, a width of 0.98 cm, and an area of 6.57 cm² (Table 2). Also, the leaves of 2X plants were longer and narrower, with an average leaf shape index of 14.55, in contrast to 4X plants, which had an average index of 9.42 (Table 2). Analysis of leaf cross-sections confirmed that the leaves of 4X plants were considerably thicker, with both palisade and spongy tissues showing increased thickness, and they had a higher water content (Fig. 5A, Table 2, Table 3). With respect to overall plant morphology, 4X plants had a height of 10.05 cm, which is 8.8% less than 2X plants at 11.02 cm, but they showed an 85.0% increase in fresh leaf weight at 0.2377 g and a 41.8% increase in dry weight at 0.0173g, presenting a short and bulky phenotype.

The study of leaf spines indicated that both 2X and 4X plants had light green spines (Fig. 5C), although there were considerable differences in their distribution density and morphology. The leaves of 2X plants displayed spines uniformly distributed along the whole margin (left in Fig. 5D), with hook spines mostly in the central and lower sections, and straight spines at the tips angled upward at 40°. Conversely, in 4X plants, leaf spines were primarily located at the base of the leaf, nearly absent in the middle, and seldom found or missing at the tips, with a random mix of straight and hook spines, and significant differences in the bending angle of hook spines and the opening angle of straight spines. Measurement data showed that the average length of leaf spines in 2X plants was 0.62±0.04 mm, and the density was 7.94±0.58 spines/cm, which were significantly higher than 0.38±0.03 mm and 6.20±0.47 spines/cm in 4X plants ($p<0.05$) (Table 3). The statistical analysis revealed that the leaf spine length in 4X plants was reduced by 38.7%, and their density was 21.9% less compared to 2X plants, exhibiting the traits of fewer spines, shorter spines, and irregular distribution.

Stomatal characteristics of polyploid *A. comosus*

Typically, polyploids exhibit larger stomata at lower densities than diploids, which is a key feature of polyploids. This research involved microscopic examination of the lower epidermal stomata in 2X and 4X plants, focusing on detailed comparisons of stomatal density, shape, size, and the condition of epidermal cells using 100×, 40×, and 10× objective lenses (Fig. 6, Table 5). The comparative analysis showed notable differences in stomata between 4X and 2X plants. The stomata of 4X plants were long and elliptical with somewhat square ends, whereas those of 2X plants were round with slightly pointed ends (Fig. 6). In 4X plants, the stomatal count was notably less than in 2X plants, averaging 31.43 and 52.38 stomata per square millimeter (Table 5). Additionally, both stomata and epidermal cells in 4X plants were considerably larger than those in 2X plants. The lengths of guard cells and epidermal cells in 4X plants were 53.12 μm and 152.39 μm, respectively, surpassing the 33.91 μm and 93.86 μm lengths observed in 2X plants. With a guard cell width of 17.29 μm and an epidermal cell width of 35.46 μm, 4X plants exhibited larger dimensions than the 10.26 μm guard cell width and 26.42 μm epidermal cell width observed in 2X plants (Fig. 6, Table 4). The outcomes suggest that polyploid plants exhibit reduced stomatal density but possess larger stomata and epidermal cells, aligning with the usual traits of polyploid plants.

Chlorophyll content and key enzyme activity of photosynthesis in leaves of *A.comosus*

Morphological studies indicated that the green color of 4X leaves was noticeably darker compared to 2X leaves. The determination of pigment content indicated that chlorophyll levels in 4X plants were notably greater than in 2X plants, measuring 639.54 mg/m² compared to 501.15 mg/m² (Table 5). Specifically, 4X plants had a chlorophyll a content of 387.39 mg/m², a chlorophyll b content of 252.15 mg/m², and a chlorophyll a/b ratio of 1.54; while the chlorophyll a content of 2X plants was 286.36 mg/m², the chlorophyll b content was 214.80 mg/m², and the chlorophyll a/b ratio was 1.33. These results indicate that the chlorophyll content, chlorophyll a content, and chlorophyll a/b ratio of 4X plants were all significantly higher than those of 2X plants, while there was no obvious change in chlorophyll b content (Table 5).

This investigation evaluated the activities of PEPC, PPDK, and Rubisco. The data revealed that the activity of the PEPC enzyme in 4X plants was considerably higher compared to 2X plants, measuring 65.10 nM/min/g and 38.31 nM/min/g, respectively, representing a 70.0% increase (Table 5). There was no notable variation in PPDK enzyme activity between the two ploidy plants, which were 75.67 nM/min/g and 80.45 nM/min/g. Conversely, the activity of the Rubisco enzyme in 4X plants was notably reduced compared to 2X plants, measuring 0.31 nM/min/g and 0.39 nM/min/g, respectively, which represents a 20.5% reduction (Table 5). These outcomes demonstrate that tetraploid plants experienced a significant rise in PEPC enzyme activity, a considerable decline in Rubisco enzyme activity, and no significant alteration in PPDK enzyme activity. An increase in PEPC enzyme activity might significantly improve the carbon assimilation efficiency of 4X plants, especially enhancing their photosynthesis under conditions of low CO_2 concentration.

Comparison of salt and heat tolerance of plants of different ploidies

Under usual conditions, plants exposed to salt and heat stress often display yellowing leaves, diminished green hue, progressive water loss, wilting, and eventually perish. With greater tolerance, yellowing and chlorosis are less pronounced. This study involved observing and measuring the morphological and physiological indices of 2X and 4X plants under salt and heat stress conditions. Following 14 days of salt stress and 4 days of heat stress, the 4X plants exhibited considerably less morphological damage compared to the 2X plants. Following two phases of salt stress lasting 14 days, the leaves of 2X plants turned yellow and wilted, with yellowing noticeable at the tips and

bases, and significant yellowing of the upper leaves. In contrast, 4X plants exhibited yellowing only in the lower leaves, with little alteration in the upper leaves (Fig. 7A). After 4 days of heat stress, the leaves of 2X plants became noticeably yellow, particularly the lower ones, and some leaf bases were damaged and turned black. In contrast, 4X plants exhibited minimal changes, with only a few leaves tips yellowing (Fig. 7B). The data from the measurements (Table S2 and S3) indicated that neither salt stress nor heat stress significantly affected the leaf water content and thickness in plants of both ploidy types (Fig. 7C, D). The impact of the two stresses on leaf water content and thickness was relatively minor, yet they had a more pronounced effect on the morphology of control plants.

Exposure to salt and heat stress did not significantly affect the chlorophyll a content in plants of either ploidy (Fig. 7C, D). During the initial treatment phase, both salt stress over 7 days and heat stress over 2 days caused a significant decrease in total chlorophyll and chlorophyll b content (Fig. 7C, D), along with a significant increase in the chlorophyll a/b ratio. In 2X plants, the chlorophyll a/b ratio rose by 31.2% under salt stress and 27.6% under heat stress, whereas in 4X plants, the increases were 22.5% and 18.9%, respectively (Fig. 7C, D). During the second phase of treatment, which involved 14 days of salt stress followed by 4 days of heat stress, the total chlorophyll content, chlorophyll b content, and chlorophyll a/b ratio in plants of both ploidy levels showed a tendency to stabilize (Fig. 7C, D). It is noteworthy that under the same stress conditions, the increase in the chlorophyll a/b ratio in 2X plants was significantly greater than that in 4X plants, highlighting that diploid plants had a larger range of photosystem regulation during the early stress period. These results imply that in the initial phase of stress, there is a significant effect on the synthesis or degradation of chlorophyll b, causing a drop in total chlorophyll and a rise in the a/b ratio, whereas in the prolonged phase, plants adapt their physiological processes to stabilize chlorophyll metabolism. During the early stress period, polyploid plants experience a less significant drop in chlorophyll b and a smaller rise in the a/b ratio, potentially connected to their greater adaptability to stress.

Through multiple physiological indicators, the degree of plant cell damage and the activity of protective mechanisms under stress conditions can be quantitatively assessed. This research involved measuring 11 physiological indicators of plants subjected to salt stress (150 mM NaCl) and heat stress (40 °C). The data indicated that under salt stress, both ploidies of plants experienced similar changes in REC, MDA content, PEPC activity, PPDK activity, SOD activity, and CAT activity; while there were diverse trends in the changes of Rubisco activity, proline (Pro) content, soluble protein content, and POD activity (Fig. 8A, B). Plants with both ploidies exhibited the same changes in relative electrical conductivity, proline content, soluble protein content, and SOD and POD activities when subjected to heat stress; whereas the levels of malondialdehyde, PEPC activity, PPDK activity, Rubisco activity, soluble sugar content, and CAT activity showed varying patterns of change (Fig. 8C, D).

Overall, the early stress phase (7 days/2 days) saw 4X plants with significantly elevated PEPC, SOD, and CAT activities and Pro levels compared to 2X plants, and there were smaller increases in relative electrical conductivity and MDA, implying that 4X plants have more robust stress response capabilities and cell protection mechanisms.

To analyze the physiological indices of the two ploidy plants, the subordinate function method was applied, and the average membership degree was calculated (Table 6). The data revealed that under salt stress, the average membership degree of 4X plants initially increased and then decreased, peaking at 0.686 on the 7th day of treatment, which was 57.7% higher than the 0.435 of 2X plants;

the value decreased to 0.482 after 14 days of treatment, yet it was still higher than the 0.346 of 2X plants (Table 6). Salt tolerance was ranked as follows: 4X-7 d > 4X-14 d > 4X-0 d > 2X-7 d > 2X-0 d > 2X-14 d, which implies that 4X plants had the greatest overall tolerance at the midpoint of stress (7 days) and were more salt-tolerant than 2X plants. Under heat stress, the average membership degree of 4X plants consistently rose over time, reaching 0.557 after 4 days of treatment, which was 449.0% higher than the 0.374 observed in 2X plants; it also reached 0.548 after 2 days of treatment, significantly surpassing the 0.389 of 2X plants (Table 6). Heat tolerance was ranked as follows: 4X-4 days > 4X-2 days > 4X-0 days > 2X-2 days > 2X-4 days > 2X-0 days, demonstrating that polyploid plants had a more noticeable tolerance advantage after 4 days of heat stress. Generally, 4X exhibited significantly better salt and heat tolerance compared to 2X.

In heat stress treatment, the average membership degree of 4X plants increased continuously over time: it reached 0.557 at 4 days of treatment, which was 49.0% higher than that of 2X plants (0.374); it was 0.548 at 2 days of treatment, which was also significantly higher than 0.389 of 2X plants (Table 6). The heat tolerance ranking was: 4X-4 d > 4X-2 d > 4X-0 d > 2X-2 d > 2X-4 d > 2X-0 d, showing that the tolerance advantage of polyploid plants was more obvious under 4 days of heat stress. In general, the salt tolerance and heat tolerance of 4X plants were significantly better than those of 2X plants.

Discussion

Polyploid varieties are typically more valuable in farming and gardening because they possess better agronomic characteristics than diploids. The technique of artificially inducing polyploids is widely utilized in the breeding of crops (Zhang et al. 2024) and horticultural crops (Eng and Ho 2019; Lu et al. 2024;Xie et al. 2025; Zheng et al. 2025) by breeders. However, there are scarce reports on colchicine-induced polyploidy in pineapple. Enhancing polyploidy in this species is crucial for improving its horticultural characteristics and increasing its market value.

The development of plant tissue culture technology has recently spurred an increase in studies on polyploid induction using somatic embryos as explants. Acting as induction receptors, somatic embryos enable the efficient acquisition of autopolyploids, improve plant regeneration efficiency, and shorten the induction cycle (Eng and Ho 2019; Chu and Lyrene 2025). Colchicine, known as a common chemical mutagen, has been effectively employed in the induction of polyploidy in different plants. To induce polyploid, colchicine treatment combined with tissue culture primarily involves the soaking and co-cultivation methods (Suliman and Asander 2020; Yang et al. 2024). Nonetheless, various plants react differently to these two techniques, necessitating the optimization of reagent concentration and treatment time for each species. The study first evaluated the influence of colchicine concentration and treatment duration on mortality and the induction rate of polyploidy. The outcomes showed a notable rise in the mortality of pineapple callus as colchicine concentration and treatment duration rose (Table 1; Fig. 2), consistent with the toxic effects of high-dose colchicine documented in other polyploid induction studies (Ahmad et al. 2023). In opposition, the rate at which polyploids were induced did not continuously grow with concentration and duration (Table 1). Even though the mortality rate was significantly higher in the co-cultivation method, flow cytometry analysis revealed that it had a much higher induction rate compared to the immersion method (Table 1). A 20-day co-cultivation of embryogenic calli with 0.1% colchicine resulted in the highest polyploid induction rate of 40.00%, with no further increase from longer durations (Table 1).

Moreover, polyploids were obtained in this study by using colchicine on the embryogenic callus of the pineapple with no octoploids present. This absence highlights the stability of cell division within the embryogenic callus (Brassard et al. 1992). The synchronous division properties of these cells allow colchicine to predominantly induce a single chromosome doubling, from diploid to tetraploid, hindering further doubling to octoploids. In optimizing systems, this result highlights the advantages of using embryogenic callus as induction materials. The uniformity of their cells minimizes chimeras (Wu and Mooney 2002) and consistently produces tetraploids with stable genetic backgrounds, setting a standard for material selection in polyploid breeding of other crops and providing practical support for "precise induction of target ploidy". In pineapple research, consistently acquired tetraploids offer perfect materials for breeding stress-resistant varieties and conducting genetic analysis, removing the complications of higher ploidy on phenotypic assessment. This study has limitations. In subsequent studies, verifying the generality of the lack of octoploids and the ploidy tolerance threshold of pineapple cells can be achieved by increasing the sample size, improving detection techniques, or trying progressive induction methods. In-depth research on the genomic stability and breeding value of tetraploids will further expand the application scope of the results.

In general, elevated ploidy levels bring about considerable morphological modifications, serving as dependable signs of polyploidy. Leaf morphology alterations are particularly noticeable and measurable. Unlike diploids, induced tetraploids generally exhibit larger leaves and significantly different leaf shapes (Suliman and Asander 2020;Wu et al. 2025; Wang et al. 2025). As earlier studies have shown, there are notable phenotypic differences in the leaves of diploid and polyploid pineapples: tetraploids have thicker leaves and larger leaf areas, yet their plant height and leaf length are reduced compared to diploid controls (Figs. 4 and 5; Tables 3 and 4). Polyploid *Lilium pumilum* features early blooming, broader leaves, a larger perianth, greater resistance to gray mold, and an increase in bulb fresh weight, which may lead to higher yields (Jitsuyama et al. 2023). Similarly, polyploids of *Passiflora edulis Sims* (Wang et al. 2024), *Populus alba* (Wang et al. 2025), *Clausena lansium* (Wu et al. 2025), and *Centella asiatica* (Sitthaphanit et al. 2024) all exhibit increased leaf area. Furthermore, observations on colchicine-induced polyploids demonstrated that tetraploids showed restricted growth and delayed development in plant height and leaf size during the early growth period, with an accelerated growth rate in the subsequent stage, resulting in significantly increased leaf size (Sitthaphanit et al. 2024). Based on these studies, polyploids may allocate more energy to the lateral growth (e.g., thickening and widening) of vegetative organs such as leaves to enhance photosynthetic area (enlarged leaf area), while reducing resource investment in vertical growth (plant height and leaf length). This strategy may be closely related to their environmental adaptation needs (Morton et al. 2021; Walczyk and Hersch-Green 2022).

Notably, polyploid pineapple plants displayed an uneven distribution, varied shapes, fewer numbers, and smaller leaf thorns (Fig. 5D; Table 4). In research on polyploid peonies, these plants had longer and more numerous trichomes, which is inconsistent with our findings (Zheng et al. 2025). This discrepancy might be explained by the inhibition of leaf thorn development in polyploid pineapples at the juvenile stage, whereas trichome growth in polyploid peonies accumulates advantages more readily at the mature stage due to polyploidization, leading to phenotypic differences. Observations of leaf cross-sections demonstrated that the palisade and spongy tissues in polyploid leaves were thicker than those in the control, with the palisade tissue having a more evident thickening trend (Fig. 5A). This trend causes a marked increase in leaf compactness and a

decrease in looseness, and such a compact leaf structure might improve the plant's resistance to stress (Ratnadewi et al. 2023; Cheng et al. 2024). Hence, the thickened palisade tissue in polyploid pineapple leaves could indicate an improvement in stress resistance.

Another typical indicator for assessing plant ploidy levels is the examination of stomatal characteristics (Yun et al. 2025). Induced polyploid pineapples, when compared to diploids, exhibited larger stomata and a significantly lower density of stomata (Fig. 6, Table 5), consistent with many past studies on the cytological characteristics of polyploid plants. For instance, tetraploid Passiflora edulis Sims had notably larger stomata than diploids, and there was a notable decrease in stomatal density with higher ploidy levels (Wang et al. 2024); research on tetraploid *Cinnamomum camphora* revealed that while stomatal width and length increased, their density decreased (Wang et al. 2024). Based on the research, stomata traits might serve as a more trustworthy indicator of polyploids.

A rise in chlorophyll levels is a typical occurrence during polyploid induction among different plant species (Horová et al. 2018). Compared to control plants, polyploid pineapple plants had a much higher chlorophyll leves in their leaves (Table 6), which accounts for their darker green appearance (Figs. 4 and 5). In a similar fashion, polyploid *Lilium pumilum* had more chlorophyll, enhanced photosynthesis, and greater yields than diploid varieties (Zhou et al. 2024); *Passiflora edulis Sims* tetraploids possessed a much higher chlorophyll level than diploids, which contributed to their superior photosynthetic capacity (Wang et al. 2024). The investigation revealed that chlorophyll was chiefly present in the spongy tissue of pineapples, with polyploid plants having a much thicker spongy tissue (Figure 5A). Studies conducted previously have demonstrated that the thickening of spongy tissue is associated with thylakoid thickening, thereby improving photosynthetic capacity in polyploids (Allen and Forsberg 2001; Li et al. 2012). Consequently, the increased chlorophyll content and enhanced ability to photosynthesize in polyploid pineapple plants may be connected to the thickening of leaves and the enlargement of spongy tissue.

PEPC, PPDK, and Rubisco enzyme activities are crucial physiological factors that control the efficiency of plant photosynthesis. The changes in their activity, in synergy with chlorophyll content, provide a complete reflection of the plant's photosynthetic ability. According to the study, PEPC activity was much greater in polyploids than in the control group, Rubisco activity was considerably reduced, and there was no significant change in PPDK activity (Table 6). The physiological mechanism behind this outcome can be further examined within the framework of photosynthetic metabolic pathways: in CAM plants, PEPC is the key enzyme that limits the rate of initial CO_2 fixation in photosynthesis, and enhanced PEPC activity is usually associated with a direct increase in the photosynthetic rate (Winter and Smith 2022; Xia et al. 2023). Rubisco, a key enzyme in the carbon assimilation phase, may have reduced activity due to changes in metabolic requirements in polyploids. The study shows a significant boost in total chlorophyll and chlorophyll a content in polyploids, implies that the light reaction stage can deliver more energy and reducing power (Papageorgiou et al. 2009), potentially diminishing the dependence on the efficiency of carbon fixation mediated by Rubisco (Croft et al. 2022). Crucially, the enhancement in PEPC activity greatly surpassed the decline in Rubisco activity. The 'compensatory effect' not only clarifies the increased photosynthetic ability of polyploids but also uncovers a possible new equilibrium strategy in regulating photosynthetic metabolism (Park et al. 2011), offering fresh perspectives on how polyploidization alters plant energy metabolism.

Pineapple leaves under salt and heat stress became yellow and wilted, with a significant decrease in chlorophyll content (Fig. 7C, D), while the chlorophyll a/b ratio increased more noticeably in control plants. This aligns with the results reported by Dong et al. (Hu et al. 2020) and Ibrahimova et al. (Zivcak et al. 2021). A stable chlorophyll a/b ratio is an important physiological indicator of a plant's tolerance (Paul 2017). Under high-temperature stress, plants with strong heat resistance can maintain a more stable ratio by minimizing the degradation of chlorophyll a (Bukovnik et al. 2007). This study observed that the chlorophyll a/b ratio in control plants, initially lower than in polyploids, eventually exceeded the polyploids with a significantly larger increase (Fig. 7C, D). This shifting discrepancy not only validates the trait of enhanced heat tolerance in polyploids but also uncovers the inherent mechanism behind their stress resistance superiority from the standpoint of photosynthetic machinery stability, thus offering physiological proof for clarifying the link between polyploid formation and environmental fitness.

To fully evaluate plant stress tolerance, it is necessary to integrate several physiological indicators. This investigation revealed that chlorophyll levels, along with Rubisco and POD activities, are significantly related to salt tolerance, while chlorophyll levels, soluble sugar content, and the activities of PEPC, PPDK, Rubisco, and CAT are more indicative of differences in heat tolerance (Fig. 8). Given that a single indicator cannot entirely capture plant stress resistance, the fuzzy membership function method, an effective tool for objectively assessing comprehensive resistance (Jain and Sharma 2020; Varshney and Goyal 2023), demonstrated its effectiveness in this instance. Noteworthy is that certain physiological indicators displayed significant changes only during the early (1-2 days) or middle (7 days) phases of stress (Figs. 7 and 8), consistent with the stage-specific nature of plant stress responses. Plants can quickly activate defense mechanisms, such as increasing enzyme activities, to maintain resistance under short-term stress, but prolonged stress might weaken resistance due to resource exhaustion (Bandurska 2022; Bogati and Walczak 2022). From the average value of membership degree, both polyploids and controls achieved maximum tolerance at 7 days under salt stress. However, under heat stress, controls peaked at 4 days, whereas polyploids kept improving (Table 6). The outcome not only lays the groundwork for pinpointing the critical time point for stress intervention but also implies that polyploid could have enhanced resistance potential under extended heat stress, which will inform future research on prolonged stress experiments to investigate the enduring mechanisms of polyploid stress resistance.

To summarize, polyploid pineapples can be effectively induced using colchicine on embryogenic callus. This research developed the most effective induction system by thoroughly evaluating different colchicine treatment techniques, concentrations, and durations. The efficiency of tetraploid induction, however, requires further enhancement. For important tropical horticultural crops like pineapples and Ananas, polyploid induction can generate new genetic resources for breeding. Further characterization is needed to understand the morphological changes induced by polyploidization. The polyploid plants from this study are preserved in a greenhouse and will be used for breeding work post-flowering.

Conclusions

This study achieved the successful creation of an effective polyploid induction system for pineapple. Through systematically comparing colchicine-based immersion and co-cultivation approaches, it was first clarified that the optimal protocol for inducing pineapple tetraploids is co-cultivation with 0.10% colchicine for 20 days, achieving an induction rate as high as 40.00%,

markedly outperforming the immersion method, which reached a maximum of only 18.00%. Using this technique, 121 pineapple tetraploid plants were effectively developed and confirmed through ploidy analysis. Compared to diploid controls, tetraploid plants show notable morphological and physiological benefits, including thicker, heavier, wider leaves with increased leaf area, more and longer epidermal hairs, dwarf plants with stouter stems; meanwhile, tetraploids possess larger stomatal apparatus, decreased stomatal density, and a notable increase in chlorophyll content. Significantly, the comprehensive evaluation of 18 physiological indicators via the fuzzy membership function method first clearly demonstrated that tetraploid pineapples possess superior salt and heat tolerance than diploids. Overall, this investigation supplies a solid technical pathway and high-quality germplasm resources for the breeding of polyploid pineapples.

Supplementary information Table S1. Physiological indicators of salt tolerance in plants with varying ploidy levels; Table S2. Physiological indicators of heat tolerance in plants with varying ploidy levels.

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Author Contributions JSQ: Conceptualization, Formal Analysis, Data Curation, Writing – Original Draft Preparation; TX: Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision, Funding Acquisition; YYW: Conceptualization, Methodology, Resources, Data Curation, Writing – Review & Editing, Visualization, Project Administration; JZ: Validation, Formal Analysis; YRX: Investigation, Investigation; TL: Validation, Data Curation; TTW: Validation. All authors read and approved the final manuscript.

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Data Availability All data are included in the main text.

Conflicts of Interest The authors declare no conflict of interest.

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Figure legends

Fig. 1 Comparison of non-embryogenic and embryogenic callus. (A) Non-embryogenic callus. (B) and (C) Induced embryogenic callus. Scale bar = 500 μm in the smaller circle. Scale bar = 2mm in the larger circle.

Fig. 2 Effects of colchicine treatment on induction of pineapple callus. (A) Induction results of immersion method. (B) Induction results of mixed culture method.

Fig. 3 Analysis of ploidy level for induced plants by flow cytometry. (A) The distribution curve of leaf DNA content of control plants (diploid plants). (B) The distribution curve of DNA content in the leaves of mutant plants.

Fig. 4 Preliminary screening of regenerated plants. (A) Regenerated plants of control treatment. (B) Regenerated plants treated with colchicine.

Fig. 5 Analysis of leaf-associated characteristics in plants regenerated and transplanted for 60 days. (A) The 2X plant's leaves are shown in longitudinal section on the left, and the 4X plant's leaves are shown on the right. Scale bar = 200 μm. (B) and (C) The 2X plants are on the left, and the 4X plants are on the right. (D) Analysis and comparison of leaf thorns. From top to bottom are the tip, middle, and base of the leaf; from left to right are leaves from the 2X plant and the 4X plant.

Fig. 6 Stomatal observation. The figure displays observation results using 100×, 40×, and 10× objective lenses from left to right. The top row shows the leaf stomata of the 2X plant, while the bottom row shows those of the 4X plant. Scale bar = 20 μm.

Fig. 7 Changes in morphology of plants with different ploidy levels under stress and the corresponding physiological index variations in their leaves. (A) Changes in plants under salt stress. (B) Changes in plants under heat stress. (C) Histogram depicting physiological metrics for leaves treated with salt. (D) Histogram illustrating physiological metrics for leaves exposed to heat. The control plant is labeled as 2X, and the variation plant is labeled as 4X. The data are presented as average values. All values are averages, and significant differences within the same group are indicated by different lowercase English letters ($p<0.05$).

Fig. 8 Changes of other relevant physiological indicators of different ploidy plants under stress. (A) and (B) show histograms related to salt treatment. (C) and (D) display histograms for heat treatment. The control plant is labeled as 2X, and the variation plant is labeled as 4X. The data are presented as average values. All data are presented as averages, with notable differences among different lowercase English letters within the same data group ($p<0.05$).

Figures

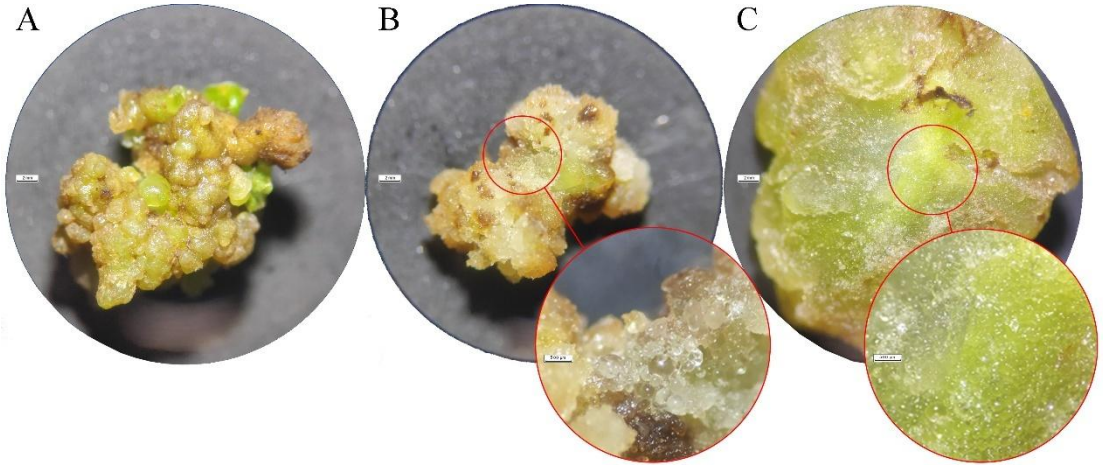
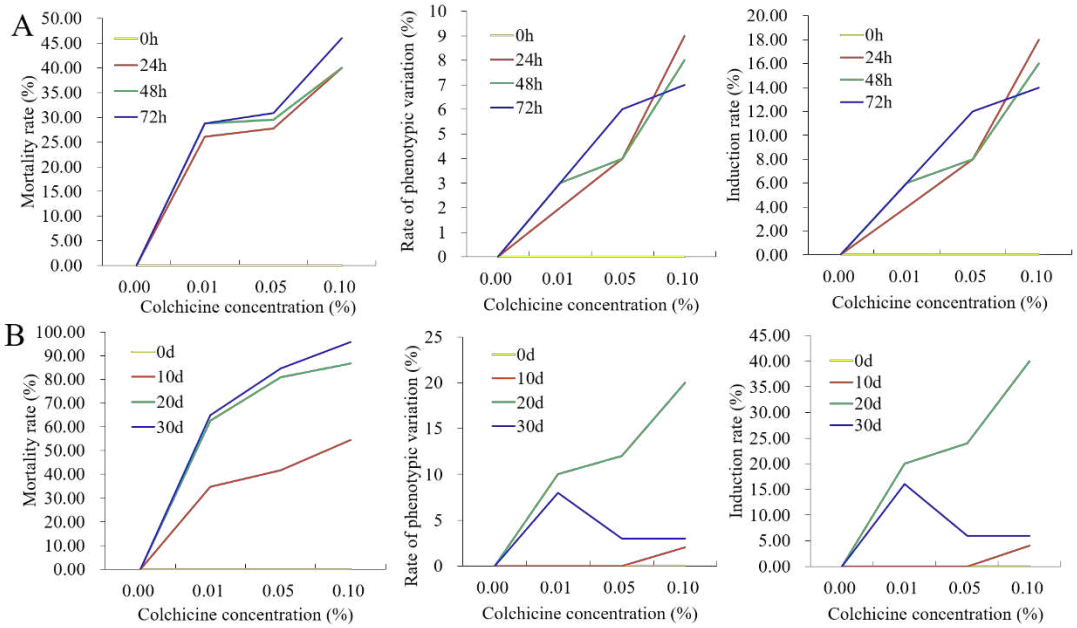


Fig. 1



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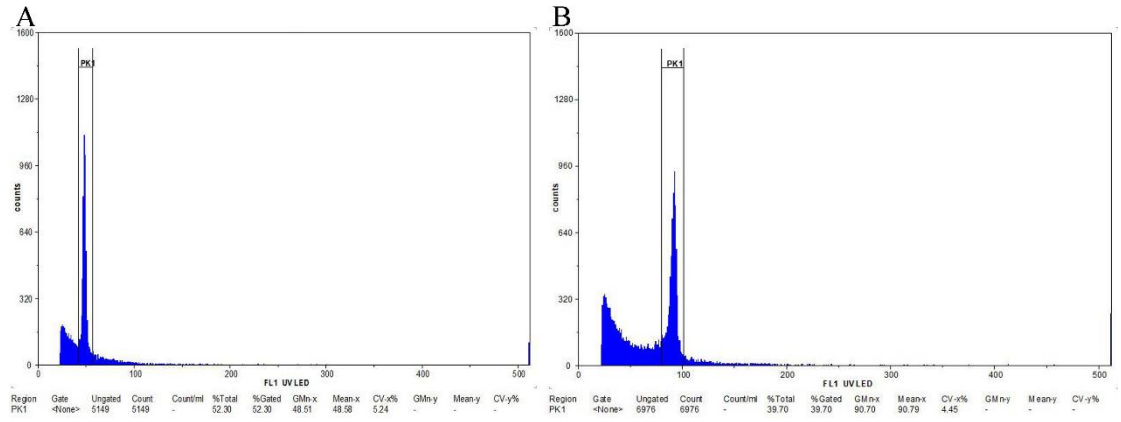

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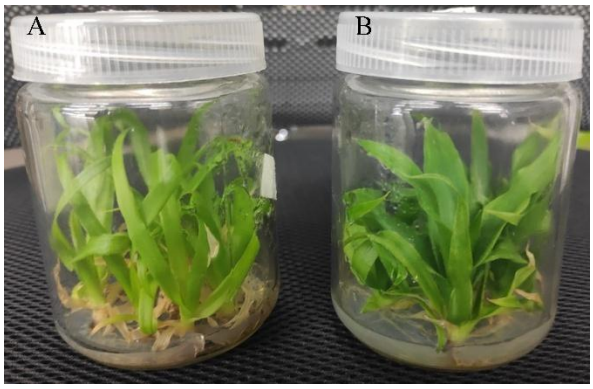
A
B

Fig. 4

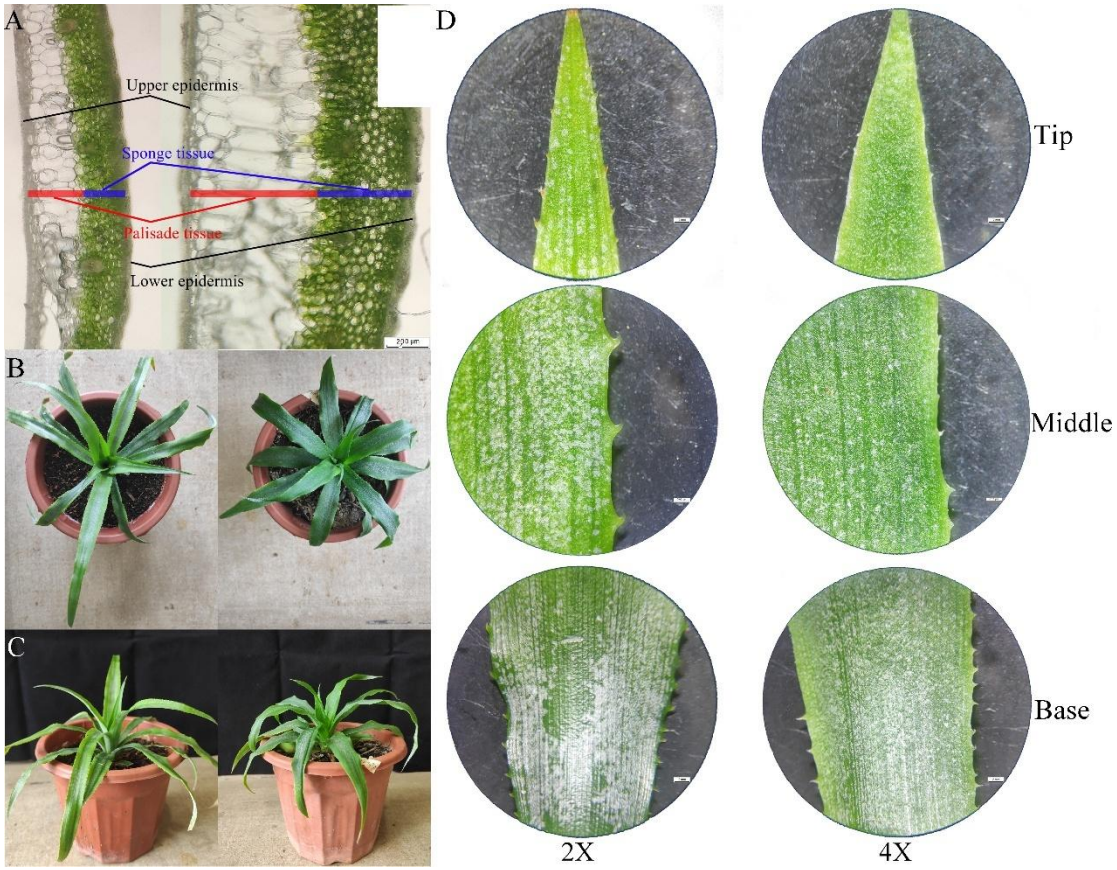
A
Upper epidermis
Sponge tissue
Palisade tissue
Lower epidermis
B
C
D
Tip
Middle
Base
2X
4X

Fig. 5

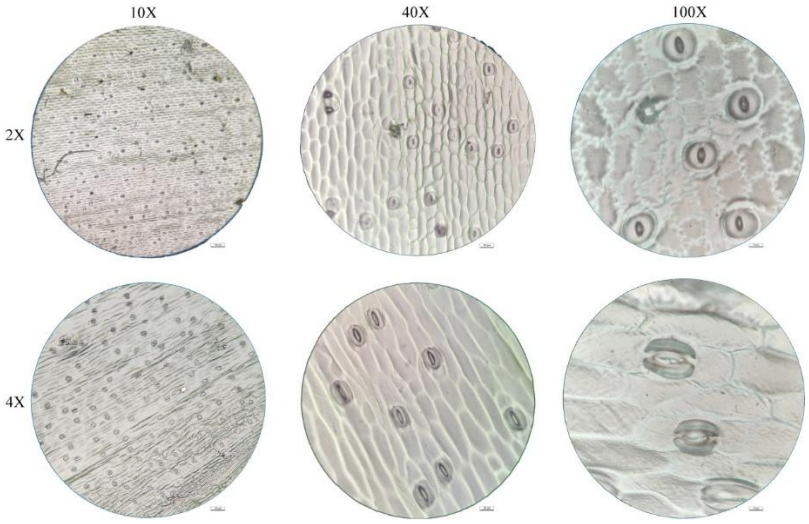
10X
40X
100X
2X
4X

Fig. 6

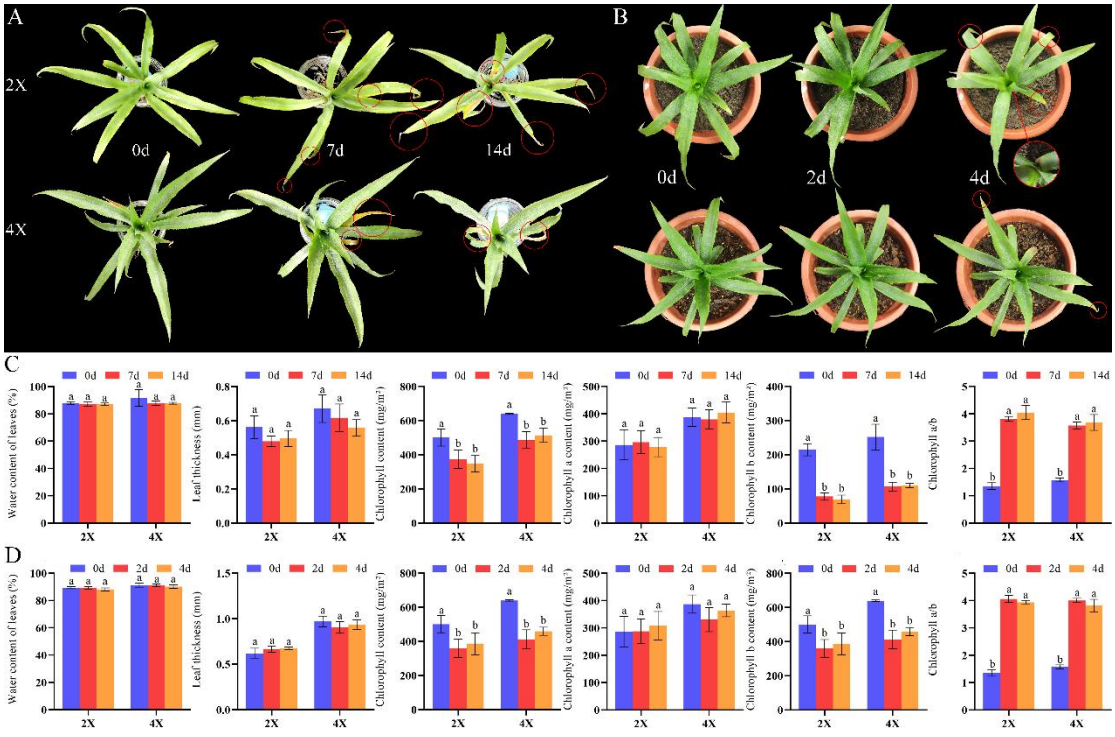
A
2X
4X
0d
7d
14d
B
0d
2d
4d
C
D
Water content of leaves (%)
Leaf thickness (mm)
Chlorophyll content (mg/m²)
Chlorophyll a content (mg/m²)
Chlorophyll b content (mg/m²)
Chlorophyll a/b
2X
4X

Fig. 7

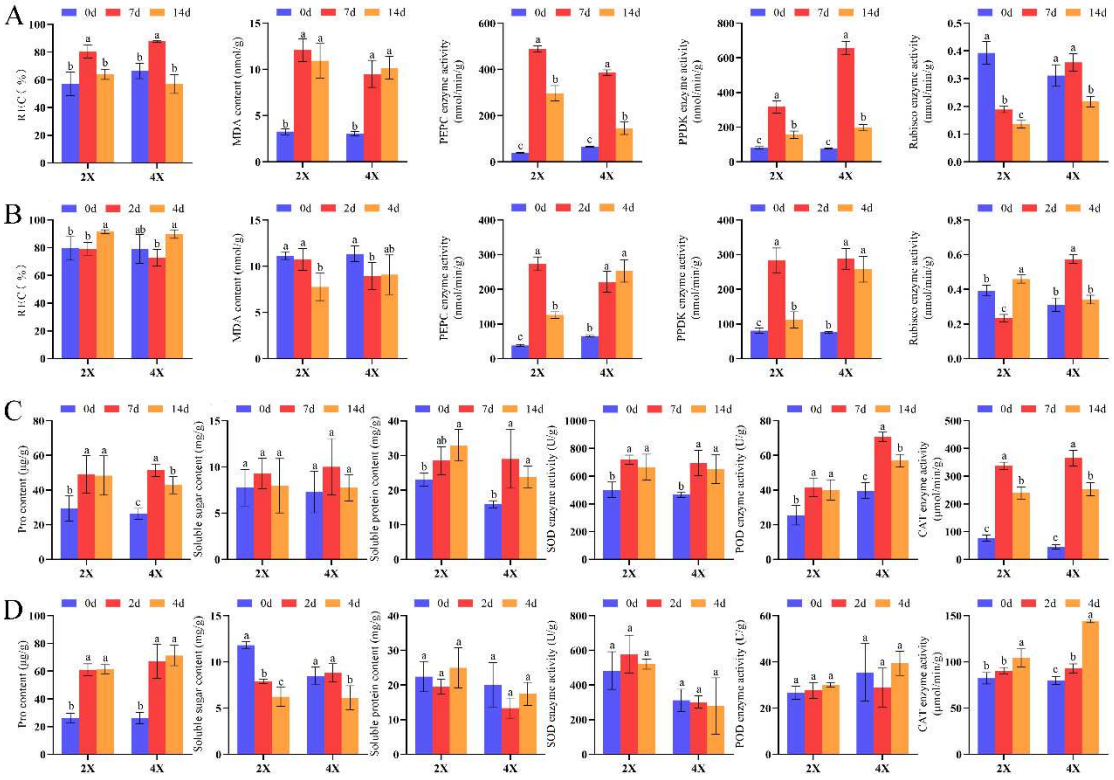
A
B
C
D
0d
2d
4d
7d
14d
RWC (%)
MDA content (nmol/g)
PEPC enzyme activity (nmol/min/g)
PPDK enzyme activity (nmol/min/g)
Rubisco enzyme activity (nmol/min/g)
Pro content (μg/g)
Soluble sugar content (mg/g)
Soluble protein content (mg/g)
SOD enzyme activity (U/g)
POD enzyme activity (U/g)
CAT enzyme activity (μmol/min/g)
2X
4X

Fig. 8

842 **Table 1** Effect of different colchicine on polyploid induction of *A. comosus*.

Treatment	Colchicine concentration (%)	Processing time	Mortality rate (%)	Number of phenotypic variations	variant rate (%)	Number of polyploids	Polyploids rate (%)
CK	0	0	0	0	0	0	0
immersion method	0.01	24 h	26.00	5	5.00	4	4.00
		48 h	29.00	6	6.00	6	6.00
		72 h	29.00	7	7.00	6	6.00
	0.05	24 h	28.00	8	8.00	8	8.00
		48 h	30.00	8	8.00	8	8.00
		72 h	31.00	12	12.00	11	11.00
	0.10	24 h	40.00	19	19.00	18	18.00
		48 h	40.00	16	16.00	16	16.00
		72 h	46.00	14	14.00	13	13.00
Mixed method	0.01	10 d	35.00	0	0.00	0	0.00
		20 d	63.00	20	20.00	19	19.00
		30 d	65.00	16	16.00	16	16.00
	0.05	10 d	42.00	0	0.00	0	0.00
		20 d	81.00	23	23.00	22	22.00
		30 d	85.00	6	6.00	6	6.00
	0.10	10 d	55.00	4	4.00	4	4.00
		20 d	87.00	40	40.00	40	40.00
		30 d	96.00	5	5.00	5	5.00

843

844 **Table 2 Leaf Indexes.**

Material	*L*	*a*	*b*	Leaf thickness (mm)	Leaf length (cm)	Leaf diameter (cm)	Leaf area (cm^2)	Leaf shape index
2X	48.39*	-8.30*	29.51*	0.3575	9.60*	0.66	5.67	14.55*
4X	38.41	-7.35	13.49	0.5542*	9.23	0.98*	6.57*	9.42

845 *L* indicates brightness, where *L*=0 is black and *L*=100 is white. The '*a*' value signifies red and green, with a
846 positive 'a' indicating red and a negative '*a*' indicating green. The '*b*' value denotes yellow and blue, with a positive
847 '*b*' indicating yellow and a negative '*b*' indicating blue. The control plant is labeled as 2X, and the tetraploid plant is
848 labeled as 4X. The data shown are average values. An asterisk (*) next to the same column of data indicates
849 significant differences ($p<0.05$).

850

851 **Table 3 Plant and leaf thorn Indexes.**

Material	Plant height (cm)	Fresh weight of leaves (g)	Dry weight of leaves (g)	Water content of leaves (%)	Leaf thorn length (mm)	Leaf thorn density (pcs/cm)
2X	11.02*	0.1285	0.0122	90.53	0.62*	7.94*
4X	10.05	0.2377*	0.0173*	92.73*	0.38	6.20

852 The control plant is labeled as 2X, and the tetraploid plant is labeled as 4X. The data shown are average values.

An asterisk (*) next to the same column of data indicates significant differences (p<0.05).

Table 4 Stomatal characteristics.

Material	Stomatal density (/mm^2)	Guard cell length (μm)	Guard cell diameter (μm)	Epidermal cell length (μm)	Epidermal cell diameter (μm)
2X	52.38*	33.91	10.26	93.86	26.42
4X	31.43	53.12*	17.29*	152.39*	35.46*

The control plant is labeled as 2X, and the variation plant is labeled as 4X. The data are presented as average values. An asterisk (*) after the same column of data indicates significant differences (p<0.05).

Table 5 Chlorophyll concent and crucial photosynthetic enzyme activities in plants of different ploidy levels.

Measurement index	2X	4X
the chlorophyll content （mg/m²）	501.15	639.54*
chlorophyll a content （mg/m²）	286.36	387.39*
chlorophyll b content （mg/m²）	214.80	252.15
chlorophyll a/b ratio	1.33	1.54*
PEPC enzyme activity （nM/min/g）	38.31	65.10*
PPDK enzyme activity （nM/min/g）	80.45	75.67
Rubisco enzyme activity （nM/min/g）	0.39*	0.31

The control plant is labeled as 2X, and the variation plant is labeled as 4X. The data are presented as average values. An asterisk (*) after the same column of data indicates significant differences (p<0.05).

Table 6 Analysis of tolerance membership function of different ploidy plants.

Treatment	Plant ploidy	Processing time (d)	Value of membership function	Endurance ranking
Salt	2X	0	0.384	5
		7	0.435	4
		14	0.346	6
	4X	0	0.476	3
		7	0.686	1
		14	0.482	2
Heat	2X	0	0.337	6
		2	0.389	4
		4	0.374	5
	4X	0	0.487	3
		2	0.548	2
		4	0.557	1

The control plant is designated as 2X, while the polyploid plant is designated as 4X. The membership function value represents the mean of all the indicators' membership function values.

870 **Table S1** Ploidy level analysis for induced polyploids of *A. comosus.*

Ploidy Level	No. of Evaluated Plants	Peak Value
Diploid	35	25.26 ± 0.96
Tetraploid	209	97.90 ± 1.74

871

Table S2 Physiological indicators of salt tolerance in plants with varying ploidy levels.

Measurement index	Ploidy level	0 d	7 d	14 d
Water content of leaves (%)	2X	87.83a	87.16a	86.99a
	4X	91.72a	87.85a	87.78a
Leaf thickness (mm)	2X	0.5625a	0.4800a	0.4958a
	4X	0.6708a	0.6175a	0.5575a
the chlorophyll content (mg/m²)	2X	501.15a	373.89b	347.55b
	4X	639.54a	485.61b	514.51b
chlorophyll a content (mg/m²)	2X	286.36a	296.21a	278.27a
	4X	387.39a	379.21a	404.68a
chlorophyll b content (mg/m²)	2X	214.80a	77.69b	69.28b
	4X	252.15a	106.40b	109.83b
chlorophyll a/b ratio	2X	1.33b	3.81a	4.02a
	4X	1.54b	3.56a	3.68a
PEPC enzyme activity (nM/min/g)	2X	38.31c	488.63a	296.87b
	4X	65.10c	386.06a	145.30b
PPDK enzyme activity (nM/min/g)	2X	80.45c	317.67a	156.11b
	4X	75.67c	658.34a	198.83b
Rubisco enzyme activity (nM/min/g)	2X	0.39a	0.19b	0.14c
	4X	0.31a	0.36a	0.22b
REC (%)	2X	57.06b	80.54a	63.97b
	4X	66.44b	87.83a	56.89b
MDA content (nmol/g)	2X	3.27b	12.08a	10.94a
	4X	3.06b	9.52a	10.17a
Pro content (μg/g)	2X	29.43b	49.11a	48.40a
	4X	26.34c	51.47a	42.88b
soluble sugar content (mg/g)	2X	7.77a	9.31a	7.97a
	4X	7.30a	10.02a	7.75a
MDA content (mg/g)	2X	23.04b	28.50ab	32.96a
	4X	15.91b	29.01a	23.81a
SOD activity (U/g)	2X	501.20b	719.35a	665.74a
	4X	466.60b	696.55a	651.94a
POD activity (U/g)	2X	25.42b	41.50a	40.05a
	4X	39.63c	70.73a	56.91b
CAT activity (μM/min/g)	2X	76.68c	336.85a	239.06b
	4X	44.98c	365.23a	253.07b

All the data presented are average values. Significant differences (p<0.05) are indicated by different lowercase English letters following the same row of data.

Table S3 Physiological indicators of heat tolerance in plants with varying ploidy levels.

Measurement index	Ploidy level	0 d	2 d	4 d
Water content of leaves (%)	2X	89.37a	89.38a	88.15a
	4X	91.19a	91.10a	90.21a
Leaf thickness (mm)	2X	0.6167a	0.6625a	0.6750a
	4X	0.9681a	0.9033a	0.9333a
the chlorophyll content (mg/m²)	2X	501.15a	359.08b	386.22b
	4X	639.54a	413.14b	459.25b
chlorophyll a content (mg/m²)	2X	286.36a	288.32a	308.06a
	4X	387.39a	330.62a	363.98a
chlorophyll b content (mg/m²)	2X	214.80a	70.76b	78.16b
	4X	252.15a	82.52b	95.27b
chlorophyll a/b ratio	2X	1.33b	4.07a	3.94a
	4X	1.54b	4.01a	3.82a
PEPC enzyme activity (nM/min/g)	2X	38.31c	274.57a	125.68b
	4X	65.10b	221.77a	235.21a
PPDK enzyme activity (nM/min/g)	2X	80.45c	283.51a	111.72b
	4X	75.67b	288.04a	258.22a
Rubisco enzyme activity (nmol/min/g)	2X	0.39b	0.24c	0.46a
	4X	0.31b	0.57a	0.35b
REC (%)	2X	79.45b	79.24b	91.45a
	4X	79.09ab	72.76b	89.79a
MDA content (nmol/g)	2X	11.15a	10.74a	7.76b
	4X	11.35a	8.95b	9.08ab
Pro content (μg/g)	2X	26.27b	61.06a	61.45a
	4X	26.05b	67.24a	71.27a
soluble sugar content (mg/g)	2X	11.81a	7.92b	6.24c
	4X	8.49a	8.82a	6.11b
MDA content (mg/g)	2X	22.44a	19.48a	24.94a
	4X	20.09a	13.33a	17.48a
SOD activity (U/g)	2X	483.82a	578.40a	520.49a
	4X	312.14a	301.47a	278.51a
POD activity (U/g)	2X	26.68a	27.71a	30.15a
	4X	35.49a	28.82a	39.44a
CAT activity (μM/min/g)	2X	82.26b	90.55b	104.54a
	4X	79.92c	92.91b	144.09a

All the data presented are average values. Significant differences ($p<0.05$) are indicated by different lowercase English letters following the same row of data.

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