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# Induction and Identification of Colchicine-mediated Polyploidy in Three *Rosa roxburghii* Accessions

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***Abstract.*** **The induction of polyploidy in *Rosa roxburghii* has significant potential to enhance the horticultural and commercial value of this crop and meet specific market demands. In this study, seeds from three *R. roxburghii* germplasms were germinated and then treated with colchicine to induce polyploidy. The polyploids were identified using morphology, physiology, and cytology, and this study predicts prickleless traits in the polyploids through marker-assisted selection. The results indicate that the highest induction rate was reached after 12 hours of treatment with 1% colchicine. In total, 20 new mutants were successfully obtained, comprising two mutants from Wuci1 seed, three mutants from Wuci2 seed, and 15 mutants from Duanci1 seed. Aspects of morphology and physiology varied significantly between diploids and polyploids. The leaflet width and leaflet shape index of polyploid seedlings from three *R. roxburghii* germplasms showed a substantial increase, and the relative chlorophyll content was significantly higher than in diploid seedlings. Furthermore, there were differences in stomatal length, guard cell length, and stomatal density. Additionally, the main vein diameter, upper epidermal thickness, palisade tissue, and sponge tissue of polyploid leaves were greater than in those of diploid leaves. Furthermore, using insertion-deletion (InDel)-specific molecular markers, polymerase chain reaction (PCR) amplification was performed on 15 polyploids from Duanci1 seeds and predicted that three polyploids may carry the prickleless fruit trait. These polyploid mutants provide valuable materials for breeding large-fruited types of *R. roxburghii* without prickles and lay a theoretical foundation for future research.**

*Rosa roxburghii* Tratt., a perennial shrub belonging to the Rosaceae family, is mainly distributed in the southwest of China (Lu et al. 2021). Indeed, this plant is grown so widely that it is considered one of the 12 characteristic industries of the Guizhou Province. The cultivation area in this province alone currently exceeds 140,000 ha, with the main variety grown being Guinong5, due to its nutritional and medicinal value (Huang et al. 2023). However, the drawback of this variety is that it produces prickles on the surface of stems, petioles, sepals, and fruits. This presents challenges in plant management, fruit harvesting, transportation, processing, and consumption in its fresh form. Consequently, the overall development of the *R. roxburghii* industry is impeded and there is a need to develop new *R. roxburghii* germplasms that feature fruits without prickles.

*Rosa roxburghii* f. *eseiosa* Ku is a form of *R. roxburghii* that has smooth fruit skin without prickles (Ku and Robertson 2003). This characteristic makes it an excellent germplasm resource for breeding fresh-eating varieties, such as Wuci1 and Wuci2. However, the average weight of a single fruit is much smaller than those of Guinong5 (Jiang et al. 2022). Research has shown that polyploidy leads to the development of larger organs (Wang et al. 2023). Therefore, this study explores induced polyploidy of *R. roxburghii* by chemical mutagenesis. Colchicine is a mutagenic compound commonly used to induce polyploidization, and enlargement of plant organs, with prior use in apples (Zhang et al. 2015), pears (Sun et al. 2009), plums (Wang et al. 2023), kiwifruit (Xi et al. 2022), and jujube (Wang et al. 2023). From the results of these experiments and subsequent breeding, larger fruits have been obtained. Indeed, Wu et al. (2023) have recently performed colchicine treatment combined with tissue culture and rapid propagation technology to conduct doubling breeding of Wuci1 and Wuci2 to produce large-fruited cultivars of *R. roxburghii* without prickles. Apart from its application on vegetative organs, colchicine also can be used for seed treatment, and some studies have been conducted on colchicine mutagenesis of *R. roxburghii* seeds. Liao (2016) carried out such a study using Guinong5 seeds, immersing them in a 0.1% colchicine solution for either 24 or 48 h. However, they did not successfully induce polyploidy. On the other hand, Sha (2022) managed to obtain two mutants by immersing Guinong5 seeds in a 0.1% colchicine solution for 12 h. Additionally, Feng et al. (2016) induced *R. roxburghii* seeds (germplasm unknown) with colchicine and created two polyploids. The optimal induction condition was determined to be immersing the seeds in a 0.5% colchicine solution for 6 h. It is worth noting that the concentration and duration for colchicine induction differ among different *R. roxburghii* materials.

Studies have demonstrated that the inheritance of fruit prickle traits in *R. roxburghii* is governed by two pairs of genes and the absence of prickles in the fruit is marked by two pairs of recessive genes (Gao and Luo 1994). Although the fruits of Wuci1 and Wuci2 do not have prickles, their F1 progeny bear prickly fruit, such as Duanci1. By using Wuci1 and Wuci2 seeds, hybrid lines with large fruit with prickles were obtained, thereafter further breeding was required to obtain germplasm also without prickles. Trait separation can only occur in the F2 generation; therefore, plants grown from Duanci1seeds may have the potential to produce fruit without prickles. In fact, Jiang (2023) has discovered an insertion-deletion (InDel)-specific molecular marker that is significantly associated with the fruit without the prickles trait in Wuci2, which could be used for early selection of prickleless fruit traits in subsequent progenies. Molecular marker–assisted breeding technology has been successfully used in plants such as persimmon (Mitani et al. 2014), tomato (Kumar et al. 2021), onion (Sahoo et al. 2023), and soybean (Yerzhebayeva et al. 2023). It also has great potential in *R. roxburghii*.

Therefore, in this study, the seeds of these three *R. roxburghii* accessions were treated with colchicine to generate polyploid *R. roxburghii* seeds and understand the optimal duration and concentration for successfully obtaining polyploid lines. For the first time, mutation breeding of *R. roxburghi*i was combined with molecular marker–assisted breeding to directly acquire new germplasms with the

aim of growing large fruits without prickles. This has established the groundwork for breeding new polyploid varieties of *R. roxburghii* and developed methods that can be used to expedite breeding programs of *R. roxburghii* more widely.

## Materials and Methods

### Plant materials and mutagenic treatment

The experiment started in Nov 2022, at the laboratory of the College of Agriculture, Guizhou University, in Guiyang City, Guizhou Province. Before this, mature fruits of Wuci1, Wuci2, and Duanci1 *R. roxburghii* accessions were collected from the *R. roxburghii* germplasm resource nursery of Guizhou University (26°42.4080 N, 106°67.3530 E) on 21 Aug 2022 (Fig. 1). After removing the seeds from the fruit and cleaning them, they were mixed with damp sand (1- to 3-mm particle size) and stored in a refrigerator at 4 °C to stratify. The experimental process is shown in Fig. 1.

After the seeds germinated, seeds with well-developed embryonic roots (~5 mm in length) were exposed to a 0.1% (w/v) colchicine aqueous solution (Macklin, Shanghai, China) for 12 h and a 0.05% (w/v) colchicine solution for 24 h at 25 °C. The colchicine treatment was according to Sha's (2022) protocol with minor improvements, and was influenced by the optimal treatment plan for *R. roxburghii* seeds developed by Feng et al. (2016), where they were treated with a 0.5% (w/v) colchicine solution for 6 h. We soaked 150 seeds in each treatment and repeated three times. The remaining 900 Duanci1 seeds were then processed according to the optimal concentration. After processing, the seeds were rinsed with water and then sown in a tray filled with substrate containing perlite:peat at a 1:1 ratio. After growing three to five true leaves in the seed tray, the mutants were transplanted individually into pots and cultivated in a greenhouse.

$$\text{Survival rate \%} = (\text{number of surviving } \textit{R. roxburghii} \text{ seedlings/ number of germinated } \textit{R. roxburghii} \text{ seeds}) \times 100\%$$

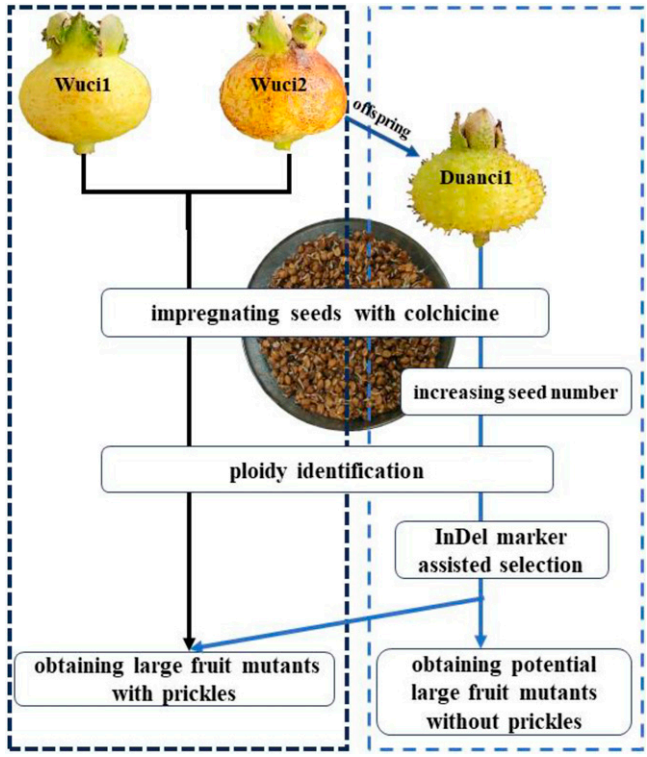


Fig. 1. The experimental process of polyploidy induction by treating the seeds of three different *Rosa roxburghii* accessions with colchicine.

$$\text{Variation rate \%} = (\text{number of mutants/ number of germinated } \textit{R. roxburghii} \text{ seeds}) \times 100\%$$

### Ploidy identification of mutants

*Observation of morphological characteristics.* The morphology of the seedlings was observed and compared with control seedlings of the same developmental stage to determine if the growth and external characteristics of seedlings treated with colchicine changed. Seedlings with morphological variation were selected as mutants. Here, we refer to the methods of Yan et al. (2022) and Feng et al. (2016) to measure the morphological indexes of plant height, basal diameter, leaflet length, leaflet width, and leaflet shape index of mutants. We selected leaves of mutant and control group plants with the same growth stage and environmental conditions and measured their leaflet length and leaflet width using a vernier scale. We also measured the chlorophyll content of leaflets from the mutant and control groups using a SPAD-502 chlorophyll meter (Minolta, Osaka, Japan). Mature leaflets were sampled at 5 months after germination, selecting from the tip of the leaf downward, leaflets 2 to 4. All experiments were performed in triplicate.

*Observation of guard cells and stomatal structure.* According to the method proposed by Yan et al. (2022), polyploid seedlings and control seedlings were selected from various *R. roxburghii* germplasm. Three leaves were taken from each plant for further analysis. Transparent nail polish was applied to both the adaxial and abaxial sides of the leaves, which were then left to dry for 3 to 4 min. Using tweezers, the nail polish formed on the back of the leaves was gently removed, placed onto a glass slide, covered with a cover slip, and observed under a microscope. Photographs were taken with the assistance of a Leica microscope (ICC50W, Wetzlar, Germany) equipped with a 40-fold objective lens. The dimensions (length and width) of diploid guard cells and stomata, as well as mutant guard cells and stomata, were measured. Furthermore, their respective densities were calculated to discern any differences between the two.

*Observation of leaflet anatomical structure.* Three mature leaflets (from top to bottom of the plant) were randomly selected from diploid and polyploid plants and placed in a centrifuge tube filled with a 50% FAA fixative solution for 24 h. Paraffin sectioning was then performed following the method of Zhang et al. (2018). Observations were made using Case Viewer (Version 2.4; 3DHISTECH, Hungary) and Adobe Photoshop CC 2019 (Adobe Inc., San Jose, CA, USA). The thickness of the upper and lower epidermis, palisade tissue, spongy tissue, and the diameter of the main veins were measured in 10 random fields for each leaflet.

*Ploidy analysis by flow cytometry.* Flow cytometry analysis was performed using a CyFlow Ploidy Analyzer (Sysmex-Partec, Goerlitz, Germany) and the CyStain® UV Precise P kit (Sysmex-Partec). The method developed by Wu et al. (2023) was used for this analysis. Fresh leaf tissue of ~0.5 $cm^2$ in area was taken from each treated seedling, added to 200 µL of extraction buffer (CyStain® UV Precise P, Sysmex-Partec) and cells ruptured with a sharp blade, and then filtered

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through a 30-μm mesh filter (CyStain® UV Precise P, Sysmex-Partec) to remove debris. Afterward, 800 μL of DAPI staining solution (CyStain® UV Precise P, Sysmex-Partec) was added, then the solution was measured using a laser wavelength of 365 nm.

*Root tip chromosome counting.* According to the methods described by Lin et al. (2015) and Wu et al. (2023), the polyploids identified by flow cytometry were re-identified using a chromosome compression method. Normal seedlings treated identically were used as the control. The root tips of seedlings, measuring ~0.5 cm and exhibiting a milky white appearance, were immersed in a 0.004-mol/L 8-hydroxyquinoline aqueous solution for 4 to 6 h. Afterward, they were rinsed two to three times with distilled water and placed in Carnoy's fixative at a temperature of 4 °C for more than an hour. If long-term storage was required, they were transferred to 70% ethanol. The fixed material was once again washed multiple times with distilled water and dissociated using a solution of ethanol and HCl (36% to 38%) in a ratio of 1:1 (v:v) for 6 min. Subsequently, they were soaked in distilled water for 10 to 20 min. Using tweezers, one root tip was transferred onto a clean slide, followed by the addition of two drops of carbol fuchsin staining solution for a staining duration of 15 to 20 min. The slide was then covered and carefully diffused using a pencil eraser. The prepared slide was observed under a Leica light microscope (ICC50W). Due to the small size of the chromosomes, using Leica optical microscopy imaging technology, the individual cells are first found using a low-power lens, and then switched to a ×100 oil lens for observation.

### InDel markers assistant selected breeding

According to the methodology outlined by Jiang (2023), InDel molecular markers were used to predict the prickleless fruit trait of polyploids from seeds of Duanci1. CD-21 primers (F: AACGTAAGAACTGGTTCGAAG, R: TCTCTGAGTTCATGAGCAACC) and CD-23 primers (F: GGTTAGGCAGATTAAGAGG, R: CACCTTGGATTCTTGGAGC) were used. First, the total DNA of the mutants from Duanci1 seeds and the leaves of Wuci2 were extracted using the cetyltrimethylammonium bromide (CTAB) method and BioTake Universal Plant DNA Extraction Kit (spin column type, model: DP1332). The PCR reaction system consisted of 20 μL, with 2 × Taq Master Mix 10 μL, 7 μL of $ddH_2O$, 0.5 μL of 10 μmol/L Primer-R, 0.5 μL of 10 μmol/L Primer-F, and 1 μL of 200 ng/μL total DNA template. The amplification program included pre-denaturation at 94 °C for 3 min, followed by 35 cycles of amplification. Each cycle comprised denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. The PCR product was then analyzed using a 1% agarose gel prepared with Gelred staining. Afterward, 7 μL of the PCR product was electrophoresed with a Bio-Rad (Hercules, CA, USA) electrophoresis device at 110 V for 25 min and detected on Bio-Rad gel imager.

### Statistics

Data were analyzed using GraphPad Prism 8 (La Jolla, CA, USA). The data were assessed using one-way analysis of variance (and nonparametric) with a Tukey test or Pearson correlation analysis. A significance level of $P < 0.05$ was considered statistically significant. Tables and charts were created using Microsoft Excel 2010 (Redmond, WA, USA) and Adobe Photoshop CC 2023. All experiments were repeated three times, and the results were the mean ± standard deviation.

## Results

### Analysis of seedling survival rate and variation rate of different *R. roxburghii* accessions

As shown in Table 1, there were differences in seed survival rates among the three untreated *R. roxburghii* accessions, with the lowest survival rate recorded for Wuci2 seeds at 43.1%. However, after soaking the seeds with colchicine, the survival rates decreased. Following a 24-h treatment with 0.05% colchicine, the survival rate of the three seeds decreased by 42.4%, 50.7%, and 48.8% compared with the control, which is about the median lethal dose (LD50), but no mutants were obtained. Similarly, after a 6-h treatment with 0.5% colchicine, the germination rates of the three seeds sharply dropped to 11.0%, 14.0%, and 19.1%, respectively, and no mutants were obtained. On the other hand, soaking the seeds with 0.1% colchicine for 12 h resulted in survival rates of 34.4%, 38.3%, and 51.3% for the three accessions, respectively. Mutants were produced under these conditions, making it the most suitable condition for inducing polyploidy in the three seeds. Specifically, Wuci1 seeds obtained two mutants (C1, C2) with a mutation rate of 1.3%, Wuci2 seeds produced three mutants (Q1, Q2, Q3) with a mutation rate of 2.0%, and Duanci1 seeds obtained two mutants (D1, D2) with a mutation rate of 1.3%. Consequently, 900 seeds of Duanci1 were treated under the optimal induction conditions, resulting in the successful acquisition of 13 mutants (D3, D4, D5, D6, D7, D8, D9, D10, D11, D12, D13, D14, and D15).

### Ploidy identification of mutants

*Morphological identification.* After comparing morphologies of all the mutants and diploids from three *R. roxburghii* accessions, changes were found in the mutant plants. The results showed that the morphological characteristics of different mutants induced by the same seed did not differ significantly. A well-grown C1/Q1/D1 plant from Wuci1/Wuci2/Duanci1 seed mutants were taken for analysis. When compared with the control seedlings that were not treated, the mutants did not demonstrate significant differences in terms of plant height and stem diameter ($P < 0.05$). However, the leaflets of the mutants exhibited noticeable variations, such as obvious wrinkling, curling, widened width, and darker green color (Fig. 2A and B). The number of leaflets was reduced, with the mutant having fewer than seven and the diploid seedlings had more than nine. In addition, the leaflets of the mutant were significantly larger than that of the diploid (Fig. 2B). Furthermore, apical and lateral leaflets were significantly wider compared with the control, whereas no visible difference in leaflet length was observed (Fig. 2C and D). Significant changes were also observed in the apical and lateral leaflets of the mutants. Morphological data of all mutants were compared, and the results (Table 2) showed that both the width of the lateral and apical leaflets and the leaflet shape index showed a significant decrease ($P < 0.05$), whereas there was no

Table 1. Polyploidy induction of seeds from three different *Rosa roxburghii* accessions with colchicine.

| Genotype | Concn of colchicine (%) | Time (h) | No. of seed treatments | No. of survival | Seedling survival rate (%) | No. of variations | Variation rate (%) |
|---|---|---|---|---|---|---|---|
| Wuci1 | 0 | 12 | 150 | 92 | 61.3 ± 4.1 a | 0 | 0.0 |
| | 0.05 | 24 | 150 | 53 | 35.3 ± 3.3 d | 0 | 0.0 |
| | 0.1 | 12 | 150 | 52 | 34.7 ± 4.1 de | 2 | 1.3 |
| | 0.5 | 6 | 150 | 17 | 11.3 ± 2.5 h | 0 | 0.0 |
| Wuci2 | 0 | 12 | 150 | 65 | 43.3 ± 1.9 c | 0 | 0.0 |
| | 0.05 | 24 | 150 | 32 | 21.3 ± 0.9 f | 0 | 0.0 |
| | 0.1 | 12 | 150 | 58 | 38.7 ± 2.5 cd | 3 | 2.0 |
| | 0.5 | 6 | 150 | 21 | 14.0 ± 3.2 gh | 0 | 0.0 |
| Duanci1 | 0 | 12 | 150 | 86 | 57.3 ± 5.0 a | 0 | 0.0 |
| | 0.05 | 24 | 150 | 44 | 29.3 ± 2.5 e | 0 | 0.0 |
| | 0.1 | 12 | 150 | 77 | 51.3 ± 7.4 b | 2 | 1.3 |
| | 0.5 | 6 | 150 | 29 | 19.3 ± 4.1 fg | 0 | 0.0 |

Data are shown as mean ± standard deviation (n = 3). Different lowercase letters in the same column indicate significant differences ($P < 0.05$).

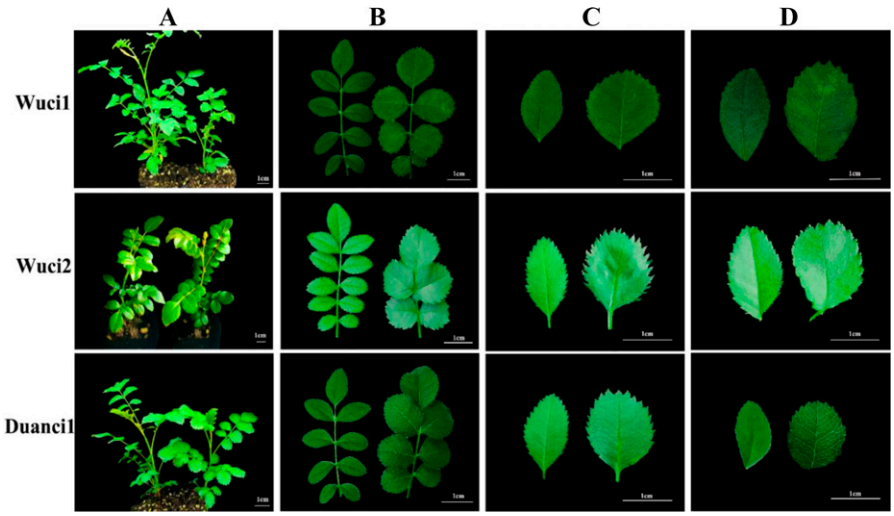


Fig. 2. The morphological observation of diploids and polyploids of *Rosa roxburghii*. (**A**) Plant; (**B**) leaf; (**C**) apical leaflet; (**D**) lateral leaflet, diploid (left), polyploid (right). Wuci1, Wuci2, and Duanci1 diploid and mutant materials C1, Q1, and D1 are shown here. Note: Wuci1 and Duanci1 seedlings were at 90 d post germination, and Wuci2 seedlings 110 d post germination.

significant difference in basal diameter and plant height. Therefore, the leaflet shape index can be considered the most reliable morphological parameter for distinguishing Wuci1/Wuci2/Duanci1 seed mutants from diploids.

*Comparison of chlorophyll content.* The leaves of polyploid plants exhibited a deeper color (Fig. 2) and all polyploid plants had a higher chlorophyll content than their diploid counterparts (Fig. 3). These results also show that there was no significant difference in the relative chlorophyll content between the three *R. roxburghii* accessions with the same ploidy. Specifically, the relative chlorophyll content of polyploid leaves from Wuci1, Wuci2, and Duanci1 seeds was at 120.22%, 126.89%, and 122.92% of the diploid controls, respectively.

*Cell stomatal identification.* The lengths and widths of guard cells and stomata, as well as stomatal density, were compared between diploid and polyploid plants of *R. roxburghii*. One of each Wuci1, Wuci2, and Duanci1 seed mutants is shown as an example (Fig. 4). The detailed data in Table 3 reveal significant differences in these parameters for polyploid plants compared with the diploid control. Specifically, the lower epidermis of polyploid seedlings exhibited increased lengths of stomata and guard cells. The stomatal length of the lower epidermis of mutant plants was 104.18%, 124.96%, and 127.86% of that of diploid plants, respectively. Similarly, the guard cell length of mutant plant seedlings was 108.58%, 119.19%, and 118.68% of diploid plants, respectively. Moreover, the guard cell width and stomatal width of mutants of Wuci2 seeds were significantly larger than those of diploid plants. Although not statistically significant, mutants of Wuci1 and Duanci1 seeds also showed on average larger guard cell and stomatal widths compared with the diploid plants. Furthermore, there was a significant difference in stomatal density between diploid and polyploid plants of the three *R. roxburghii* accessions, with polyploid plants showing a significantly lower stomatal density than diploid plants.

When comparing the transverse anatomical structure of the leaves of polyploid and diploid plants, a number of significant differences were observed. In Fig. 5, the leaflet anatomical structure of Wuci1, Wuci2, and Duanci1 seed mutants is shown. The upper epidermal cells of diploid plants are closely arranged and mostly have a flat quadrilateral shape. In contrast, the upper epidermal cells of polyploid plants are mainly oval or quadrilateral. Furthermore, the leaflet vein tissue in polyploid leaves is more developed than in diploids (Fig. 5A and B). The palisade tissue consists of two layers of cells, with the columnar cells of diploid plants being shorter than those of polyploid plants (Fig. 5C and D). The anatomical data of all mutants of the three *R. roxburghii* cultivars were further explored in Table 4, showing an increase in the main vein diameter, leaflet thickness, upper epidermis thickness, palisade tissue thickness, and spongy tissue thickness of the leaves in the polyploid plants of the three *R. roxburghii* accessions. There was no significant difference in lamina thickness between polyploidy and diploid plants obtained from seeds of Wuci1, but the lamina thickness of polyploid plants obtained from seeds of Wuci2 and Duanci1 was significantly greater than diploid. The main vein diameter, upper epidermis thickness, palisade tissue thickness, and sponge tissue thickness of the three polyploid lines were all significantly greater than the diploid.

*Ploidy analysis by flow cytometry.* According to various processing times and growth conditions, flow cytometry was conducted on the young leaves of 20 mutants of three *R. roxburghii* accessions in batches. The results revealed three distinct main peak modes. In Fig. 6, only the results of Duanci1 diploid and mutant materials D1 and D8 were taken as representative pictures. The

Table 2. The morphometry of diploids and polyploids of *Rosa roxburghii*.

| Genotype | Ploidy | Plant ht (mm) | Base diam (mm) | Lateral leaflet length (mm) | Apical leaflet length (mm) | Lateral leaflet width (mm) | Apical leaflet width (mm) | Apical leaflet shape index (length/width) | Lateral leaflet shape index (length/width) |
|---|---|---|---|---|---|---|---|---|---|
| Wuci1 | Diploid | 81.83 ± 4.58 bc | 1.82 ± 0.04 a | 17.45 ± 0.16 a | 17.84 ± 0.12 a | 9.53 ± 0.14 b | 11.97 ± 0.09 c | 1.49 ± 0.02 b | 1.83 ± 0.03 a |
| | Polyploid | 69.93 ± 14.47 c | 1.89 ± 0.11 a | 15.69 ± 0.44 b | 17.72 ± 0.08 a | 11.37 ± 0.12 a | 13.64 ± 0.15 b | 1.30 ± 0.02 c | 1.38 ± 0.03 b |
| Wuci2 | Diploid | 134.81 ± 15.10 a | 1.92 ± 0.14 a | 11.89 ± 0.25 d | 13.79 ± 0.22 d | 6.40 ± 0.21 c | 8.07 ± 0.09 d | 1.71 ± 0.04 a | 1.86 ± 0.10 a |
| | Polyploid | 120.80 ± 11.19 a | 2.39 ± 0.07 a | 11.66 ± 0.47 d | 15.28 ± 0.12 bc | 9.23 ± 0.73 b | 12.04 ± 0.19 c | 1.27 ± 0.02 c | 1.23 ± 0.05 b |
| Duanci1 | Diploid | 67.59 ± 1.59 c | 1.78 ± 0.16 a | 13.86 ± 0.01 c | 14.83 ± 0.72 cd | 7.59 ± 0.27 c | 8.47 ± 0.45 d | 1.75 ± 0.04 a | 1.83 ± 0.06 a |
| | Polyploid | 76.09 ± 7.97 bc | 1.91 ± 0.20 a | 13.77 ± 0.05 c | 16.23 ± 0.46 b | 10.75 ± 0.48 ab | 14.92 ± 0.11 a | 1.09 ± 0.02 d | 1.28 ± 0.06 b |

The data are shown as mean ± standard deviation (n = 3) from three *R. roxburghii* accessions, with different lowercase letters in the same column indicating significant differences ($P < 0.05$).

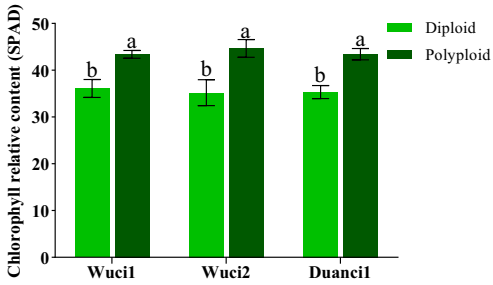


Fig. 3. The relative chlorophyll content in the leaves of diploids and polyploids of *Rosa roxburghii*. The data are shown as mean ± standard deviation (n = 3) from three *R. roxburghii* accessions. Different lowercase letters indicate significant differences ($P < 0.05$).

diploid control plant exhibited a main peak near a fluorescence intensity of 20,480.19 (Fig. 6A), with a coefficient of variation of 5.65. The mutant (Fig. 6B) displayed a maximum fluorescence intensity of 39,647.24 and a coefficient of variation of 4.99, indicating its identification as a polyploid. In the seedlings with two main peaks (Fig. 6C), One has a fluorescence intensity of 20,531.62 with a coefficient of variation of 4.05, and the other has a fluorescence density of 39,857.32 with a coefficient of variation of 3.67, classified as a mixoploid. Based on these findings, a total of one, two, and six polyploids of mutants obtained from Wuci1, Wuci2, and Duanci1 seeds were identified, respectively. Mutants obtained from one Wuci1, one Wuci2, and nine Duanci1 seeds were identified as mixoploids.

*Root tip chromosome counting.* The mutants obtained were analyzed using flow cytometry, and their chromosome number was further verified through root tip staining. The results revealed that the diploid root tip had a chromosome number of 2n = 2X = 14 (Fig. 6D), whereas the polyploid root tip had a chromosome number of 2n = 4X = 28 (Fig. 6E). The mixoploids contained either 14 or 28 chromosomes per nucleus. The mixoploids showed stable mixed ploidy levels over the duration of this experiment. These findings are consistent with the results of the flow cytometry analysis. Based on these results, it can be concluded that among the Wuci1, Wuci2, and Duanci1 seed mutants, one, two, and six were identified as polyploids, respectively.

### Selection and characterization of mutants with the trait of fruit without prickles

*InDel marker assistant selected mutants with the trait of fruit without prickles.* The DNA of Wuci2 and 15 mutants from Duanci1 seeds was amplified using a specific InDel marker that is significantly associated with the trait of fruit without prickles. A specific band type, labeled as ABC (Fig. 7), indicated the absence of prickles in the fruit of Wuci2. Mutants D3, D4, D6, D8, D10, and D11 also showed the same band type. Based on this observation, out of the 15 mutants from Duanci1 seeds, six promising mutants were predicted to have large fruit without prickles.

*Morphological, anatomical, and cytological characterization of mutants without fruit prickles.* As shown in Fig. 8, the morphology, anatomical structure, flow cytometry, and chromosome number of the six promising mutants were significantly different from those of the diploids. The seedlings of all six mutants showed larger and deeper green leaves. The number of stomata in leaves decreased significantly and the stomata were larger. The anatomical results showed that the leaflet thickness, palisade tissue, and spongy tissue structure of the mutant increased. Flow cytometry and chromosome data showed that the six mutants showed obvious polyploid characteristics. The peak fluorescence intensity of the diploid control was 22,684.9, and the peak fluorescence intensity of the mutant was ∼48,000. It showed that D3, D4, and D6 were polyploids and therefore, three of the six promising mutants were likely to be polyploid plants without prickles.

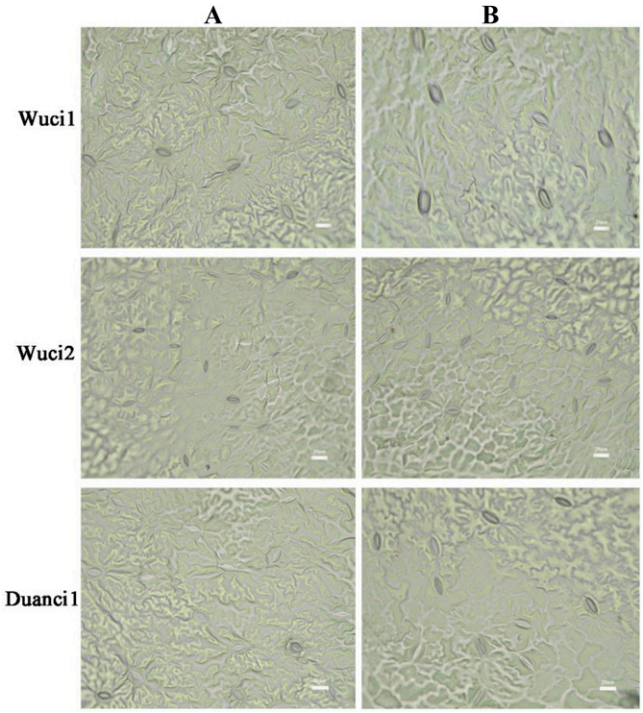


Fig. 4. The leaflet stomata observation of diploids and polyploids of *Rosa roxburghii*. (**A**) Diploid; (**B**) polyploid. The results of Wuci1, Wuci2, and Duanci1 diploid CK and mutant materials C1, Q1, and D1 are shown.

## Discussion

### Mutagenic effect of different *R. roxburghii* accessions

In this study, the optimal condition for inducing polyploidy is to treat germinating *R. roxburghii* seeds with 0.1% colchicine for 12 h. This resulted in the acquisition of 20 new mutant materials, including one polyploid from Wuci1 seeds, two polyploids from Wuci2 seeds, and six polyploids from Duanci1 seeds. This is the highest number reported in *R. roxburghii*. Various methods can be used to achieve polyploidy with colchicine, but soaking seeds is widely recognized as the most convenient and effective approach (Li et al. 2022). This method has also been successfully applied to other plants, such as *Hemerocallis fulva* (Zhang et al. 2022) and *Loropetalum chinense* var. *rubrum* (Deng et al. 2022). Previous studies on polyploidy in *R. roxburghii* by Sha (2022) and Feng et al. (2016) each obtained two polyploids using the soaking method to treat germinated seeds. They found that the optimal treatment was 12 h of 0.1% colchicine (Sha 2022) and 6 h of 0.5% colchicine (Feng et al. 2016). This study used both treatments and obtained the same results as Sha (2022). Unfortunately, we did not produce any mutants using the treatment method recommended by Feng et al. (2016), whereby here polyploidy was not achieved after 12-h treatment with 0.1% colchicine. This may be due to the different accessions used, as the germplasm used by Feng et al. (2016) was not disclosed in the article. In our experiment, the three *R. roxburghii* accessions did not yield mutant plants after treatment with 0.05% colchicine for 24 h; however, in our preliminary experiments, this

Table 3. The leaflet stomata measurement of diploids and polyploids of *Rosa roxburghii*.

| Genotype | Ploidy | Length of guard cell (μm) | Width of guard cell (μm) | Stoma length (μm) | Stoma width (μm) | No. of stoma (10 × 40) |
|---|---|---|---|---|---|---|
| Wuci1 | Polyploid | 25.30 ± 1.68 a | 17.32 ± 0.86 a | 17.19 ± 0.90 a | 7.42 ± 0.85 a | 7.20 ± 0.90 e |
| | Diploid | 23.30 ± 1.81 ab | 15.53 ± 1.68 ab | 16.50 ± 1.45 ab | 7.79 ± 1.01 a | 11.70 ± 1.35 cd |
| Wuci2 | Polyploid | 21.86 ± 1.50 b | 14.26 ± 1.82 b | 14.42 ± 1.35 b | 6.73 ± 1.01 a | 19.90 ± 2.39 b |
| | Diploid | 18.34 ± 1.46 c | 11.93 ± 0.79 c | 11.54 ± 1.44 d | 5.19 ± 1.09 b | 38.30 ± 3.44 a |
| Duanci1 | Polyploid | 25.92 ± 1.75 a | 15.92 ± 1.95 ab | 18.08 ± 1.78 a | 6.71 ± 1.43 a | 9.60 ± 0.80 de |
| | Diploid | 21.84 ± 1.06 b | 14.17 ± 1.38 b | 14.14 ± 1.63 c | 6.42 ± 1.03 ab | 14.30 ± 2.05 c |

The data are shown as mean ± standard deviation (n = 3) from three *R. roxburghii* accessions. Different lowercase letters in the same column indicate significant differences ($P < 0.05$).

treatment induced polyploidy in limited numbers of 'Guinong5' seeds. Therefore, increasing the number of treated seeds may be key in producing polyploid plants due to the low success rates. Similarly, increasing the number of treated seeds may possibly result in polyploids after 6 h of 0.5% colchicine treatment. However, this combination of high concentration and short duration was found to be unsuitable for the seeds of Wuci1, Wuci2, and Duanci1 materials grown in Guizhou Province.

The survival rate of different *R. roxburghii* germplasm resource seeds treated with colchicine varied (Table 1). This indicates that there may be variations in the mutagenic effects or toxicity to different germplasms. One possible reason is that there are differences in germination times among the three cultivars. This is due to variations in chilling requirements and seed vitality, resulting in inconsistent germination times. Even seeds of the same material germinated in distinct batches. The first batch usually germinates in October–November of the same year and is relatively uniform. The second batch will not germinate until January of the following year, and it will be sporadic. As a result, it becomes challenging to handle all the seeds in a timely and simultaneous manner, leading to delayed treatment and lower survival rates in some cases. For example, the treatment of Wuci2 (0.05% colchicine, 24 h) showed a low survival rate (21.3%). Second, the embryonic root growth rate of the three *R. roxburghii* accessions differs. An excessively long embryonic root is more susceptible to mechanical damage, thereby reducing the overall survival rate. Comparing the three accessions in the control treatment, Wuci2 seeds exhibited the longest embryonic root during treatment, making it more susceptible to mechanical damage and resulting in the lowest survival rate (43.1%). Therefore, this study can conclude that using fresh *R. roxburghii* seeds in the same year they developed, while ensuring that embryonic roots do not grow excessively, can achieve the best results.

The polyploid *R. roxburghii* exhibits larger organs and more pronounced biological traits compared with the diploid variety. The leaflet width of the three polyploid *R. roxburghii* resources significantly increased, whereas the leaflet shape index decreased significantly (Fig. 2 and Table 2). Polyploidization plays a crucial role in breeding varieties and diversifying plants (Heslop-Harrison et al. 2022; Zhang et al. 2020). Changes in chromosome ploidy led to alterations in the morphological, physiological, and cytological characteristics of plants (Haist et al. 2023; Moetamedipoor et al. 2022). These changes are typically characterized by thicker, larger, and darker green leaves, as well as bigger flowers and fruits (Wang et al. 2020, 2023; Yan et al. 2022). However, the effects of polyploidization on the same species can vary depending on the genotype of the individual (Podwyszyńska et al. 2018). Colchicine-induced seeds of *Buddleja lindleyana* exhibit different polyploid morphologies (Yan et al. 2022). Among plants with the same ploidy, the blade thickness of polyploids obtained from Wuci1 seeds is significantly different from that obtained from Wuci2 and Duanci1 seeds, but not significantly from that of the diploid plants. Polyploid plants tend to have larger organs, increased leaflet area, higher amounts of photosynthetic pigments, thicker epidermis and sponge tissue, as well as more and thicker thylakoid lamellae (Cao et al. 2018; Xue et al. 2017). Consequently, polyploid plants often exhibit higher photosynthetic capacity compared with their original diploid counterparts, further improving the crop. In this study, the leaves of polyploid *R. roxburghii* plants from three different accessions were larger than those of the diploid control, and the chlorophyll content was significantly higher. The size of stomata and guard cells in the leaves of polyploids from Wuci1 seeds were larger than those of diploid plants, although the difference was not significant. However, the stomata and guard cells of polyploid obtained from Wuci2 and Duanci1 seeds were significantly larger than those of diploids (Table 3),

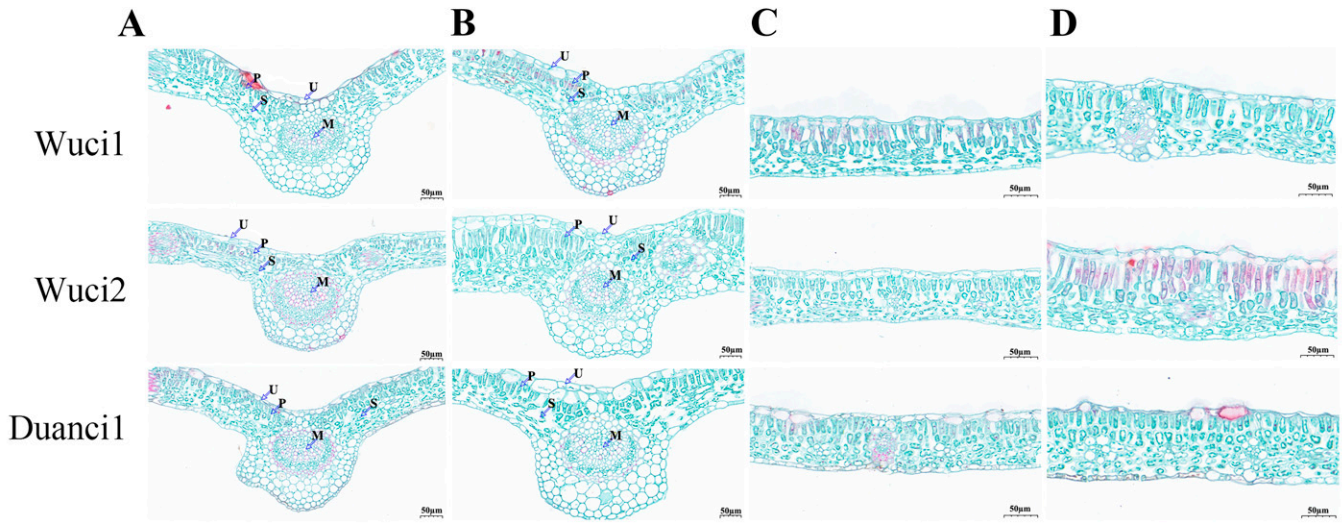


Fig. 5. The leaflet anatomical structure observation of diploids and polyploids of *Rosa roxburghii*. (**A**, **C**) Diploid; (**B**, **D**) polyploid. U = upper epidermis; P = palisade tissue; M = main vein; S = sponge tissue. The results of Wuci1, Wuci2, and Duanci1 diploid CK and mutant materials C1, Q1, and D1 are shown.

Table 4. The leaflet anatomical structure measurement of diploids and polyploids of *R. roxburghii*.

| Genotype | Ploidy | Blade thickness (μm) | Main vein diam (μm) | Upper epidermis thickness (μm) | Palisade tissue thickness (μm) | Sponge tissue thickness (μm) |
|---|---|---|---|---|---|---|
| Wuci1 | Polyploid | 101.80 ± 4.66 c | 193.27 ± 1.17 ab | 22.67 ± 0.66 a | 45.10 ± 1.08 b | 38.63 ± 0.95 a |
| | Diploid | 94.13 ± 2.50 cd | 155.80 ± 1.61 c | 13.53 ± 0.82 d | 40.00 ± 0.70 d | 28.07 ± 2.32 b |
| Wuci2 | Polyploid | 164.73 ± 3.77 a | 180.93 ± 1.48 a | 17.63 ± 0.58 a | 62.17 ± 0.73 a | 41.77 ± 1.99 a |
| | Diploid | 84.30 ± 1.43 d | 157.37 ± 0.12 c | 11.03 ± 0.71 dc | 33.43 ± 1.02 c | 28.77 ± 1.96 b |
| Duanci1 | Polyploid | 115.57 ± 1.34 b | 188.70 ± 4.46 b | 21.87 ± 1.51 b | 47.77 ± 1.45 b | 40.60 ± 1.69 a |
| | Diploid | 86.83 ± 0.74 d | 152.37 ± 2.68 c | 11.13 ± 0.61 c | 34.43 ± 2.09 c | 30.67 ± 1.63 b |

The data are shown as mean ± standard deviation (n = 3) from three *R. roxburghii* accessions. Different lowercase letters in the same column indicate significant differences ($P < 0.05$).

and the stomatal density in all three polyploid plants was significantly lower compared with diploids. In addition, the main vein diameter, upper epidermis thickness, palisade tissue, and spongy tissue of the leaves in the three polyploid *R. roxburghii* plants showed significant increases (Table 4). Therefore, the enlargement of organs in polyploid plants may be attributed to tissue thickening (Lei et al. 2023; Lin et al. 2023). Compared with diploids, polyploids lead to larger guard cells and a greater number of chloroplasts (Bhuvaneswari et al. 2019; Fakhrzad et al. 2022). With an increased number of chloroplasts in stomatal guard cells, the chlorophyll content is likely to also increase (Haist et al. 2023). As a result, most polyploid leaves exhibit darker colors and have a stronger photosynthetic capacity (Bharati et al. 2023; Du et al. 2021). This finding aligns with previous studies on apple (Xue et al. 2017). In addition, in this study, there were no significant differences in morphology, physiology, and anatomy of 20 polyploids, but nine polyploid seedlings were obtained by flow cytometry and chromosome number identification. Thus, leaflet width, leaflet shape index, stomatal density, thickness of the leaflet upper epidermis, palisade tissue, sponge tissue, and chlorophyll content can be used as preliminary identification indices for polyploidy. Flow cytometry and chromosome count were needed to further confirm the ploidy. These parameters can provide an effective foundation for establishing new polyploid germplasm of *R. roxburghii* accessions.

### Molecular marker–assisted selection breeding

This experiment used specific molecular markers to assist in selecting new varieties of *R. roxburghii* without prickles. After identification, six polyploids of *R. roxburghii* without prickles were obtained, displaying the same three-band spineless trait as Wuci2. Among these, three were identified as polyploidy while the remaining three were found to be mixoploid (Fig. 8). These materials have established a strong foundation for the cultivation of large-fruited *R. roxburghii* without prickles for fresh-food cultivars. Compared with other mutants in Duanci1, the six promising mutants showed no significant differences in morphological characteristics, stomatal number, and anatomical structure (Fig. 8). It was difficult to distinguish whether the fruit has prickles or not. As early as 1994, marker-assisted selection has been applied for predicting the early fruit color of apple seedlings (Cheng et al. 1994) and for screening apple scab resistance (Minou et al. 1998). Over the past 20 years, marker-assisted selection (MAS) has been extensively studied in fruit tree breeding, leading to the development of various marker-assisted selection breeding techniques (Mori and Cipriani 2023). In the case of *R. roxburghii*, molecular markers have been employed since 2003 to explore the genetic relationship between *R. roxburghii* and some related species (Wen et al. 2003) and to identify major genotypes (Wen et al. 2003). Subsequently, these markers have been used for genotype classification, pedigree analysis, genetic map construction, and the identification of genotype

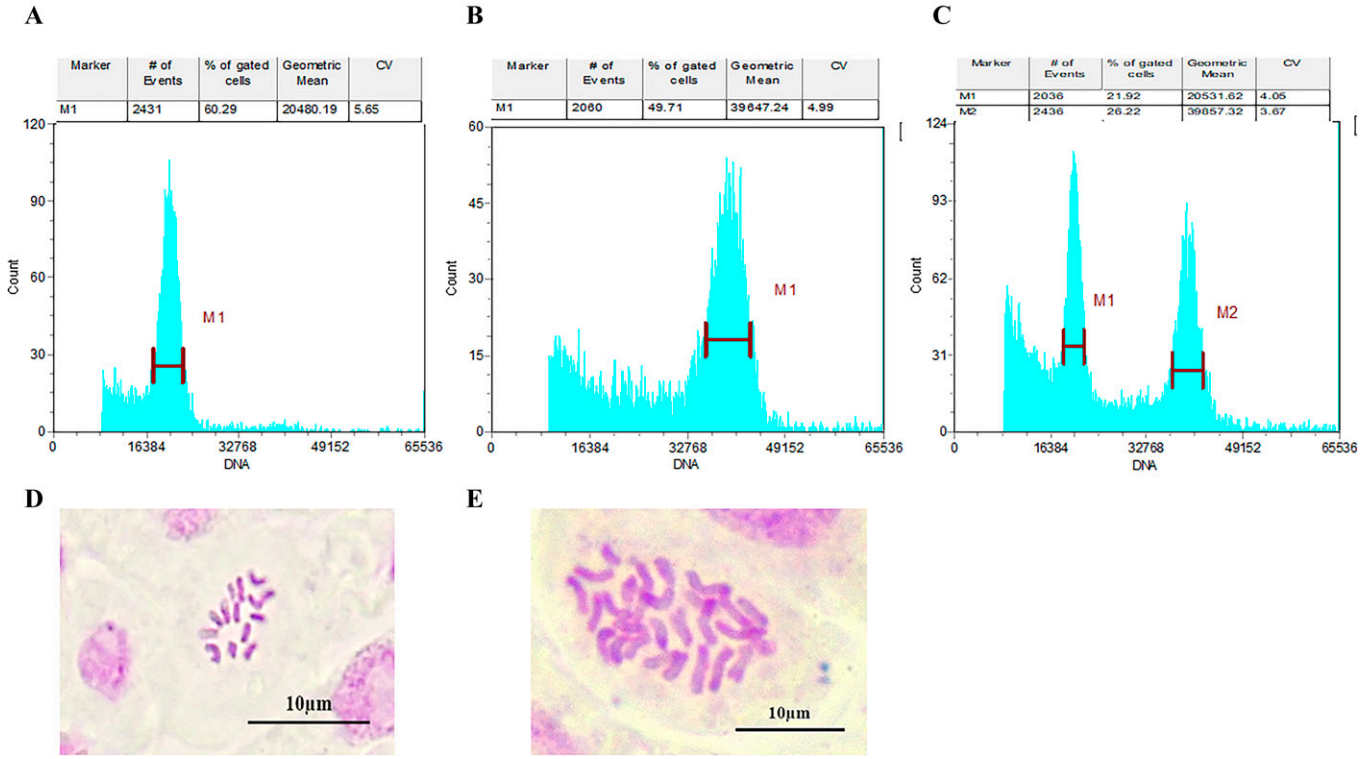


Fig. 6. The ploidy identification of *Rosa roxburghii* by flow cytometry and chromosome counting. (**A**) Diploid; (**B**) polyploid; (**C**) mixoploid; (**D**) diploid (2n = 2X = 14); (**E**) polyploid (2n = 4X = 28), the diploid is the reference standard. All mutants were identified by flow cytometry and chromosome number. Only the results of Duanci1 diploid and mutant materials D1 and D8 are shown here.

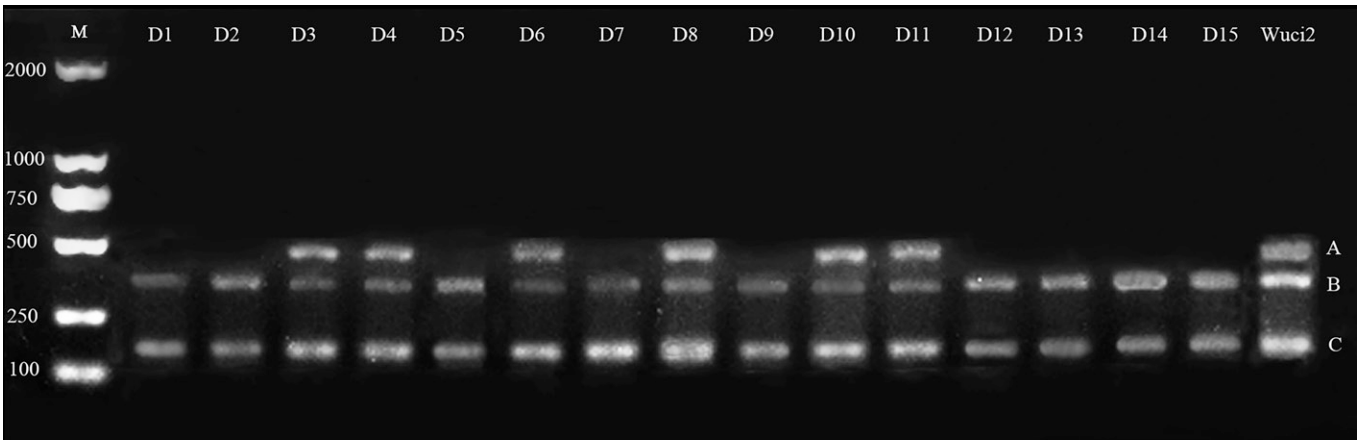


Fig. 7. Agarose gel electrophoresis map of InDel marker assistant selected mutants with the trait of fruit without prickles. M = DNA Marker (D2000); D1–D15 represent the polymerase chain reaction (PCR) products of 15 different genotypes of mutants from Duanci1 seeds with InDel marker, ABC represents a specific band type of Wuci2 plant PCR products.

markers (Chen et al. 2017; Lu et al. 2020; Sha 2022). However, there is a lack of research on molecular marker–assisted selection breeding in *R roxburghii*. Jiang (2023) used InDel-specific molecular markers to distinguish Wuci2, and this study for the first time used them to identify early seedlings with prickles or not. In addition, the author did not find any significant difference in morphology and physiology between the polyploid and mixoploid seedlings this study screened. The exact reason for this is still unclear, but mutations in the genes that control its morphology and physiology may occur. Therefore, MAS can be used to obtain additional information on these related issues in the future Moreover, MAS also can be used to further investigate and develop resistance to powdery mildew, top rot, and other diseases (Han et al. 2023; Sahoo et al. 2023), as well as phenological traits associated with fruit production (Mori and Cipriani 2023), such as early or late maturity and flesh texture (Yerzhebayeva et al. 2023), along with other relevant markers.

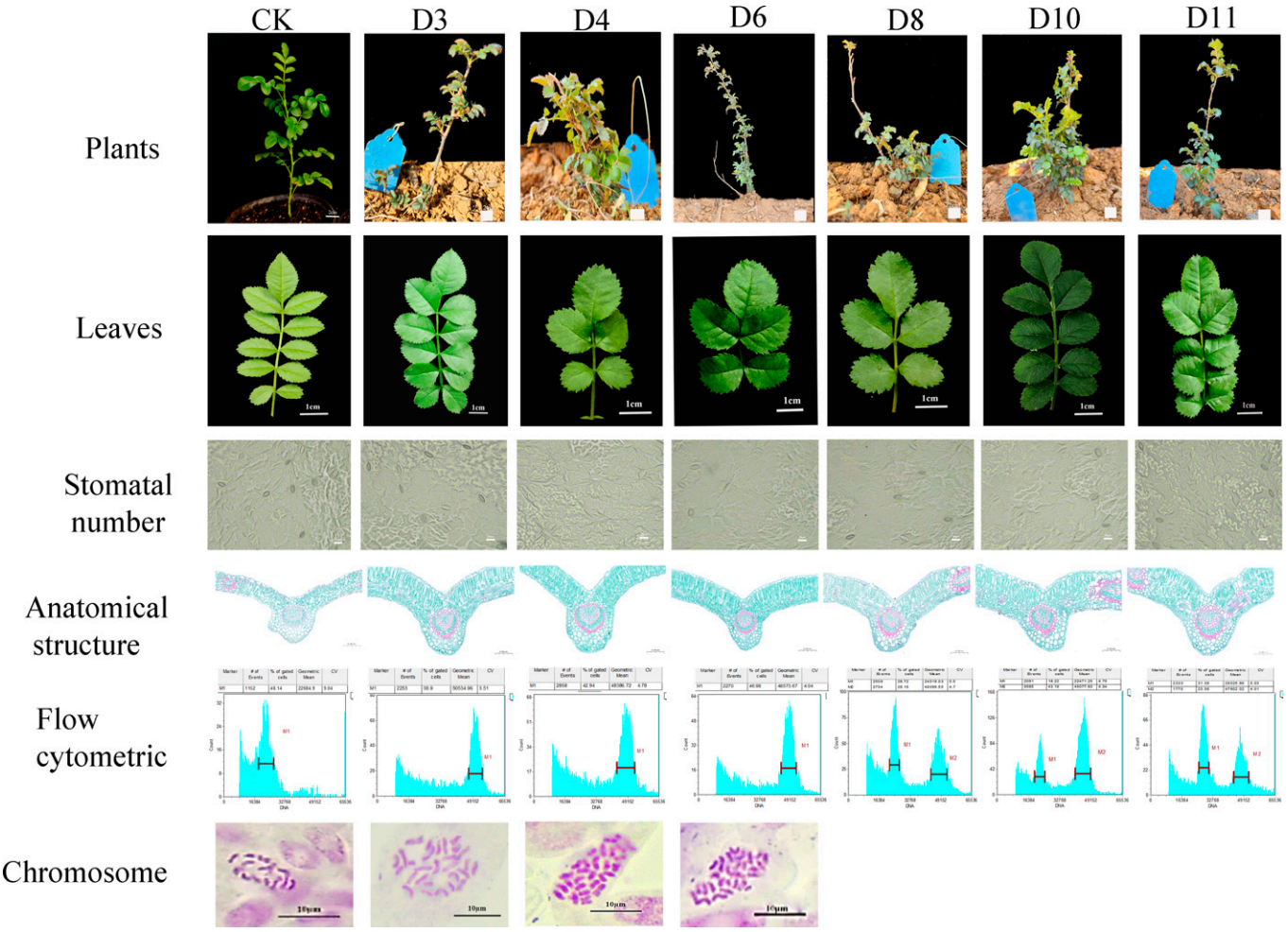


Fig. 8. Morphology, anatomy, and cell characterization of six mutants with the trait of fruit without prickles. CK = diploid control; D3, D4, D6, D8, D10, and D11 represent six promising mutants from the Duanci1 seeds.

These markers will play an important role in *R. roxburghii* breeding programs.

### Perspectives on breeding and further applications of polyploidy in *R. roxburghii*

Chromosome doubling is an important method for artificially obtaining polyploidy, which facilitates the transfer of advantageous genes from accessions to cultivated varieties. This technique has been effectively applied in the breeding of *R. roxburghii* (Feng et al. 2016; Wu et al. 2023). In this experiment, the author successfully performed doubling treatments on three *R. roxburghii* germplasm resource seeds using colchicine induction, resulting in nine polyploid plants that were induced from seeds of Wuci1, Wuci2, and Duanci1.

The mixoploid traits are unstable and tend to revert to diploid, but some later polyploidized. The most commonly used method for separating mixoploids is to obtain homozygous polyploid plants through plant regeneration technology and the tissue continuous cutting separation method (Liu et al. 2020; Zhou et al. 2021). Once the ploidy traits are stable, the resulting polyploid plants can be used in *R. roxburghii* breeding efforts. Preliminary experimental results indicate that, compared with diploids, the leaflet size of polyploids of three *R. roxburghii* accessions increased. This suggests that polyploids have the potential to produce larger biomass per unit of land area, resulting in a significant improvement in total yield. The study by Yang et al. (2022) showed that phenolic compounds present in *R. roxburghii* leaves are an effective source of phenolic ingredients in functional beverages and nutritional products. Zhou et al. (2019) demonstrated that mature leaves of *R. roxburghii* contain the highest amounts of total phenols, total flavonoids, and vitamin C, and exhibit the strongest in vitro antioxidant activity. Leaves at longer developmental stages accumulate higher levels of triterpenoids. Therefore, as polyploidy can produce more and larger leaves in *R. roxburghii*, it may also have potential for improving its antioxidant capacity and accumulation of bioactive substances. Flavonoids, phenolic compounds, and antioxidant capacity in *R. roxburghii* fruits are significantly correlated (Jiang et al. 2022; Li et al. 2022), playing an extremely important role in the development of functional foods, nutritional products, drugs, or high-value-added products. Jiang et al. (2022) found that Wuci1 contains abundant phenolic substances and screened out 23 characteristic metabolites, mainly including phenolic acids and triterpenoids. Due to the gigantism of polyploidy organs, it is speculated that polyploid *R. roxburghii* plants yield larger fruits. Thus, the three polyploid *R. roxburghii* plants are of significant importance for commercial cultivation. Because both Wuci1 and Wuci2 polyploid plants were induced from seeds, and the inheritance of the fruit prickle trait in *R. roxburghii* is controlled by two pairs of recessive genes, the obtained polyploids from Wuci1 seeds and Wuci2 seeds can serve as hybrid parents for breeding large-fruited *R. roxburghii* without prickles. Among the six polyploid plants obtained from the seeds of Duanci1, three lines were predicted to be prickleless through molecular marker–assisted selection. Duanci1 offers other agronomic advantages such as being resistant to powdery mildew, and so the polyploid seedlings would be of great value if they are confirmed to yield large fruits without prickles.

However, due to the long juvenile period and slower growth rate of polyploid plants compared with diploid plants, the mutant materials obtained in this study are still in the juvenile stage, and only the leaflet and stem morphological characteristics and basic physiological indicators of the seed mutant polyploids. For the biological characteristics of polyploidy, including growth and fruiting habits, quality, resistance, and phenology, and the molecular biological characteristics of polyploids, including metabolites and gene expression, no further research was conducted. Therefore, subsequent research shall focus on the biological and molecular biological characteristics of polyploid plants and fruits, and compare the differences in metabolites, gene expression, and fruit quality between polyploid and diploid lines.

## Conclusion

Polyploid plants were successfully obtained by treating the seeds of three *R. roxburghii* accessions with 0.1% colchicine for 12 h. In the breeding of *R. roxburghii* for the first time, using InDel-specific molecular markers, three polyploid plants without prickle traits were identified, providing a foundation for the cultivation of large-fruited *R. roxburghii* without prickles. This study provides valuable insights for the induction and identification of polyploid *R. roxburghii* seedlings and opens up avenues for further research on the practical application of polyploid *R. roxburghii* in cultivation. The limitation lies in the longer breeding cycle of woody fruit trees, which requires 3 years or even longer to validate the authenticity of the predicted results.

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