Induction and characterization of polyploids from seeds of *Rhododendron fortunei* Lindl.

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Abstract

Most *Rhododendron* species are ornamental flowering species widely distributed in Asia, North America, and West Europe. *Rhododendron fortunei*, one of the endemic *Rhododendron* species in China, has beautiful flowers with bright colors and is being exploited to meet the needs of the flower market. Polyploid plants usually show superiority in growth, disease resistance, and adaption over their diploid relatives. Here, we report the first case of polyploid induction in *R. fortunei*. In order to induce polyploidy in *R. fortunei*, germinating seeds were treated with different concentrations of oryzalin for 16 h. By evaluating ploidy level with flow cytometry, a total of 34 polyploid *R. fortunei* lines, including 27 tetraploid lines and seven octoploid lines, were obtained. A comparison of treatments indicated that 7.5 mg L⁻¹ oryzalin was the optimal concentration for polyploid induction in seeds of *R. fortunei*. Compared with diploid plants, tetraploid and octoploid plants exhibited slower growth rates and had thicker and rounder curled leaves with more leaf epidermal hairs. Moreover, larger stomata at lower density were also observed in the leaves of polyploid plants. Chlorophyll contents were also significantly increased in polyploid plants, which leads to a darker green leaf color. Both small and large individuals exhibiting the same characteristics were observed among the obtained tetraploid plants. Overall, our study establishes a feasible method for polyploid induction in *R. fortunei*, thus providing a basis for breeding new *R. fortunei* varieties.

Keywords: *Rhododendron fortunei*, oryzalin, polyploid induction, flow cytometry

1. Introduction

China is very rich in *Rhododendron* resources and has a long history of cultivation. There are approximately 570 *Rhododendron* species in China, of which about 420 are precious and peculiar to China. Due to the underdeveloped industry and low utilization level of rhododendrons in China, these precious and endemic species are not widely used in variety development and breeding. *Rhododendron fortunei* is a small evergreen tree or shrub of the family Ericaceae and order Ericales, and is usually found in the
forest understory at altitudes as high as 620–2000 m (Wu et al. 2013). *Rhododendron fortunei* is native to the Shanxi, Hubei, Hunan, Henan, Anhui, Zhejiang, Jiangxi, Fujian, Guangdong, Guangxi, Sichuan, Guizhou and northeastern Yunnan of China (Wu et al. 2013). It has 6–12 flowers in a single terminal raceme umbellate, which has a light fragrance and red, pink, white, or purple coloring (Fig. 1-A). The flowering period lasts from April to May followed by the fruiting period that lasts from August to October (Bian and Jin 2006). The beautiful plant shape, green leaves, and lush flowers of *R. fortunei* make it a high value species in landscaping and flower markets. However, like many other *Rhododendron* species of China, *R. fortunei* cannot be effectively developed and applied to the market mainly due to its lack of strong resistances and desirable traits to adapt to the urban environment. Therefore, to develop a feasible method to improve its environmental adaption and horticultural traits is urgently necessary and important for selection and breeding of *R. fortunei*.

Polyploid means the presence of more than two sets of chromosomes above the diploid level in the nucleus of an organism, and polyploidization is a very common phenomenon in the plant kingdom, which is an important process in plant evolution and new species formation (Wood et al. 2009; Van de Peer et al. 2017). Polyploids often display significantly larger growth vitality than their diploid counterparts and exhibit bigger flowers and leaves, an intensification of colors, and thicker and more rigid foliage (Manzoor et al. 2019). Moreover, polyploidization increase the adaptability to different ecological niches. Most polyploids have the advantages of strong growth and adaptability, which allow survival under extremely unfavorable ecological conditions (Comai 2005; Sattler et al. 2016). The disease resistance, drought tolerance, and drought resistance of polyploids were also enhanced, which improve the quality and yield of products (Sattler et al. 2016). Therefore, the use of artificial methods to induce polyploids has become an important breeding method for crop and horticultural plants.

Previously, Yang et al. (2009) applied four concentrations of colchicine solution, specifically 0.5, 1.0, 2.0, and 3.0 mg mL\(^{-1}\) to induce polyploidy in *R. simsii*, *R. ovatum*, and *R. mariesii*. The results showed that the induced polyploids of three *Rhododendron* species exhibited obvious morphological changes in buds and flower buds, and 1.0 mg mL\(^{-1}\) colchicine was the most effective concentration for polyploidy induction (Yang et al. 2009). Eeckhaut et al. (2006) succeeded in inducing tetraploid *R. simsii* via the *in vitro* induction mode by using colchicine and oryzalin, thus rapidly achieving the possibility of crossbreeding *in vivo*, which is of particular interest for slow-growing plants. Besides, Schepper et al. (2004) carried out *in vitro* regeneration of tetraploid petal margin tissue, obtaining the first induced tetraploid ‘Belgian’ rhododendron, which displayed significantly improved flowering period and was easily propagated. Sakai et al. (2008) solved the problem that the seed or pollen vigor of sub-hybrids between evergreen and deciduous rhododendrons is much lower than that of their parents by doubling the chromosome number *in vitro* through polyploid induction with oryzalin. These studies indicated that artificial polyploidy induction method could be applied in breeding of rhododendrons and could potentially improve their horticultural traits.

Herbicides, such as colchicine, trifluralin and oryzalin, have been widely used for polyploid induction. A stable and feasible induction method by using these herbicides has been established for *R. simsii* (Eeckhout et al. 2001), which could provide a basis for *Rhododendron* hybrid breeding of new varieties. However, there are currently no reports on polyploid induction in *R. fortunei*. In this study, the seeds of *R. fortunei* were used as the materials for polyploid induction through oryzalin treatment. The ploidy level was estimated by flow cytometry and other characteristics of the resulting polyploids were also identified. The results establish a useful

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**Fig. 1** The flowers of *Rhododendron fortunei* (A) and effect of different concentration of oryzalin on seed germination (B). Error bars are SD.
method for polyploid induction in *R. fortunei*.

2. Materials and methods

2.1. Plant materials and oryzalin treatment

The seeds of *R. fortunei* were collected from the Huading Peak (29.252°N, 121.091°W) in Tiantai County, Zhejiang Province, China. The seeds were soaked in water at (25±2)°C for 24 h, then were surface-sterilized by immersion in 10% sodium hypochlorite for 10 min and rinsed with sterile distilled water for 6–8 times. Subsequently, these seeds were treated with 0 (CK), 5, 7.5 and 10 mg L\(^{-1}\) oryzalin for 16 h, respectively. Each treatment was repeated for three times with 200–300 seeds. Then, the oryzalin-treated seeds were thoroughly rinsed with sterile water for 4 times, scattering in a sterile 9-cm glass Petri dish with two sterilized moist filter papers. The seeds were germinated in a growth chamber with (25±2)°C temperature, 1500–2000 Lx light intensity and 12 h/day photoperiod. Then, the seedlings germinated from oryzalin-treated seeds were used for further analyses.

2.2. Determination of germination rate and radicle length

The germination rate of *R. fortunei* seeds was estimated after 15 d of incubation in glass dishes.

Induction rate=The number of polyploid seedlings/The number of seedlings treated with oryzalin×100%

Germination rate=The number of sprouted seeds/Total number of seeds×100%

The radicle length was measured under the stereoscope, and the statistics were carried out by using SPSS Software (SPSS, Inc., Chicago, IL, USA).

2.3. Flow cytometry for estimation of polyploid level and genome size

The germinated seeds of *R. fortunei* were transplanted to the nursery block (Jiffy, 38 mm) and grown for 3 mon. Then, the ploidy was estimated by flow cytometry according to a previous study (Eeckhaut et al. 2005). The leaves were sampled from the seedlings, and 40 seedlings of each concentration were detected. Briefly, 0.5 cm\(^2\) of the young leaves were quickly chopped with sharp blades and 0.4 mL CyStain UV Precise P (Partec) Nuclei extraction buffer was added to extract nuclei in the petri dish, then kept at room temperature and avoid light for 3–15 min. Subsequently, 1.6 mL CyStain UV Precise P (Partec) Staining dyeing solution was added into the Petri dish and mixed thoroughly and filtered into the sample tube and stand still avoiding light for 5–15 min. Finally, the ploidy detection was performed by using CyFlow Counter flow cytometry (Sysmex Partec, Germany). And, the voltage value, threshold value, average number, and CV value of each sample were recorded. The detection for each sample was performed with ten replicates. *Populus trichocarpa* (Nisqually-1) with a known genome size (480 Mbp) served as reference standards. The ploidy level and genome size were analyzed by comparing with the reference standards (Sliwinska 2018). The induction rate was calculated as follows:

Induction rate=(Number of polyploidy seedlings/Number of analyzed seedlings)×100%

2.4. Measurement of length, width, and area of leaves

After the flow cytometric analysis, identified diploid, tetraploid and octoploid seedlings were transferred to a greenhouse to grow for approximately 6 mon. Then, mature leaves from the middle part of plants were sampled for morphology measurements. Three mature leaves of middle parts were collected from 5 mon-old seedlings and scanned by plant leaf area measuring instrument (YMJ-G, Fangke Instrument, Weifang, China). The length, width, and areas of leaves were measured with Image J Software (Pandey and Singh 2011). At least ten plants of each ploidy level were included in leaf morphology measurements.

2.5. Determination of leaf pigment content

Mature leaves (0.1 g) were chopped into small pieces and soaked in 80% buffered acetone at 4°C for 48 h in the dark. Absorbance measurements of each extraction were made using a spectrophotometer at 665, 649, and 470 nm. According to the formula of the pigment concentration in the acetone extract, the contents of chlorophyll a and b, total chlorophyll and carotenoids were calculated, respectively (Manzoor et al. 2018).

2.6. Stomata measurements

For stomata measurements, the abaxial leaf surface of mature leaves about 0.1 cm\(^2\) was coated with transparent nail polish, then the lower epidermis was peeled off and put on a glass microscope slide for stomata analyses. The number of intact stomata, and stomatal apparatus length and width was measured by using the DXM 1200 microscope (Nikon, Japan). Four leaves were chosen from the same part of each of five diploid plants and polyploid plants. Stomatal length, width, and density were measured and compared between diploid and polyploid plants with 30 stomatal apparatus measured for each leaf. And stomatal density was determined by counting the number of stomata that were evenly distributed across six microscopic fields to
calculate the mean density (Li et al. 2018). The length and width of 30 stomata were measured, and stoma area was calculated using a previously described formula as follows:

Stoma area (mean) = 3.14 × Stoma length × Stoma width (mean)

2.7. Data processing

Data were analyzed by using SPSS version 15.0 (SPSS, Inc., Chicago, IL, USA). Significant differences between treatments were determined using a two-sample t-test at $P < 0.05$.

2.8. Transplant and tissue culture of polyploidy plants

To evaluate their stability, the polyploid plants were transplanted and grown in the greenhouse for another 2 years. After that, their ploidy levels were reconfirmed by flow cytometer as mentioned above. Furthermore, two tetraploid plants were chosen as explants of tissue culture to evaluate their stability. Tissue culture was performed as following: the stems with 1–2 axillary buds were cut from plant and soaked in 75% alcohol for 30 s, rinsed 3 times using sterile water, and then surface-sterilized by 0.1% HgCl₂ for 8 min, and finally rinsed by sterile water for 5 times. Then, the stems were cut into 2 cm in length and regenerated in the medium (WPM medium with 2 mg L⁻¹ ZT and 0.1 mg L⁻¹ NAA) for 2 mon (Wei et al. 2018). When regenerated plantlets grow up about 5 cm, the ploidy levels were then verified.

3. Results

3.1. Effect of oryzalin concentrations on the seed germination and radicle length

Oryzalin was generally toxic to seeds and affected their germination. To determine the optimal oryzalin concentration, three different concentrations were tested. Treatment with 5 mg L⁻¹ oryzalin resulted in a germination rate of 70.41%, which displayed no significant difference compared with the control (70.26%) (Fig. 1-B). The seed germination rate decreased to 68.92 and 66.80% when treated with 7.5 and 10 mg L⁻¹ oryzalin, respectively, indicating that high concentrations of oryzalin significantly inhibit R. fortunei seed germination.

After 15 days of cultivation, the radicle length of seedlings were observed and measured under a stereoscopic microscopy. As shown in Fig. 2, the hypocotyls of the oryzalin-treated seedlings were much thicker than that of the control, and the radicle lengths of oryzalin-treated seedlings were also significantly shorter than the diploid plants (Fig. 2-E). It means that oryzalin possibly inhibited hypocotyl's and radicle's growth. Moreover, the cotyledons of the oryzalin-treated seedlings showed darker green than control, suggesting that the increasement of the chlorophyll content.

3.2. Detection and analysis of ploidy level

Forty plants of each treatment were used for ploidy level analysis through flow cytometry. The results indicated that the diploid plants showed a large peak at 25.87 (Fig. 3-A), whereas the tetraploid and octoploid plants showed a large peak shift at 50.81 and 103.48, respectively (Fig. 3-B and C), and the chimera showed two peaks at 25.53 and 51.56 (Fig. 3-D). According to the genome size of the diploid (758 Mb), the genome size of tetraploid and octoploid plants were estimated to be 1516 and 3032 Mb, respectively (Fig. 3-B and C). The efficiency of different oryzalin concentrations was also evaluated. As shown in Table 1, 7.5 mg L⁻¹ oryzalin was the most efficient treatment, which resulted in 33.3% tetraploids, 12.1% octoploids, and 21.2%...
chimera. Conversely, 10 mg L$^{-1}$ oryzalin treatment was associated with the lowest polyploidization efficiency, resulting in 24.4% tetraploids, 2.44% octoploids, and 43.9% chimera. In total, 27 tetraploid and 7 octoploid R. fortunei plants were obtained from 120 analyzed plants. Considering the germination rate, these combined results suggest that 7.5 mg L$^{-1}$ oryzalin is the optimal concentration to induce polyploidy in R. fortunei.

3.3. Stomatal observation of polyploid R. fortunei

Measurement of the size and density of stomata is another way to identify polyploid plants. As shown in Fig. 4 and Table 2, the stomata of tetraploid and octoploid R. fortunei were significantly larger than those of the diploid plants. Moreover, the stomatal density was significantly decreased in the tetraploid and octoploid plants (Table 2). All the observed characteristics of polyploid R. fortunei were consistent with those reported for other induced polyploid plants (Manzoor et al. 2018; Li et al. 2019; Rao et al. 2019).

3.4. Morphological differences of polyploid and diploid R. fortunei

As shown in Fig. 5, obvious morphological differences were observed between the polyploid and diploid plants. Compared with diploid R. fortunei, the polyploid plants exhibited generally rounder, harder, and thicker leaves, which sometimes were slightly twisted with a rough surface and darker coloring (Fig. 5). These differences were more obvious in the octoploid plants. Interestingly, there were two sizes of tetraploid plants, one of which had smaller leaves than diploid plants (Fig. 3-B), whereas the others exhibited...
significantly larger leaves than the diploid (Fig. 3-C). This phenomenon has also been reported during polyploid induction in Lagerstroemia indica (Ye et al. 2010) and Artemisia annua (Banyai et al. 2010).

Leaf characteristics of polyploid and diploid *R. fortunei* were measured. Table 3 and Fig. 5 contain data showing that the leaves of small tetraploid and octoploid plants appear much smaller in shape when compared with those of diploid plants. In diploid, small tetraploid, and octoploid plants, the average leaf lengths were 3.47, 1.65, and 1.15 cm, respectively, and the average leaf widths were 1.94, 1.10, and 0.99 cm, respectively. The average leaf areas of small tetraploid and octoploid plants were 1.37 and 0.89 cm², respectively, which were markedly smaller than that of diploid plants (5.16 cm²). By contrast, large tetraploid plants exhibited larger leaves compared with diploid plants. The average leaf length, width, and area of large tetraploid plants reached 3.60 cm, 2.43 cm, and 6.07 cm², respectively. However, the leaf indices of all polyploid plants were significantly smaller than diploid plants, which suggested that the leaves of polyploid *R. fortunei* were rounder.

### 3.5. Leaf pigment content of polyploid and diploid *R. fortunei*

Pigment contents in the leaves of polyploid and diploid *R. fortunei* were determined. As expected, the contents...
of chlorophyll a, chlorophyll b, and carotenoids were dramatically increased in the tetraploid and octoploid \textit{R. fortunei} compared with the diploid plants (Table 4). For example, the chlorophyll content of diploid plants was 0.643 mg g\(^{-1}\), whereas the chlorophyll contents in tetraploid and octoploid plants were 0.835 and 0.948 mg g\(^{-1}\), respectively (Table 4). These results explain why the leaves of polyploid \textit{R. fortunei} were darker green than those of diploid plants.

3.6. Stability of the polyploid \textit{R. fortunei} plants

All the obtained polyploid \textit{R. fortunei} plants were transplanted and cultivated in a greenhouse for 2 years. Currently, 24 of 27 tetraploids and 4 of 7 octoploids still grow well in the greenhouse. Further investigation on morphological traits showed that the newly formed leaves of polyploid plants still exhibited similar leaf characteristics (Fig. 6, Table 5). The leaves of tetraploid plants maintained their thicker, rounder, and dark-green appearance (Fig. 6-B and D). As shown in Table 5, the average leaf width and area of tetraploid plants were 6.46 cm and 65.73 cm\(^2\), which were 1.38 and 1.34 times larger than those of diploid plants, respectively. In addition, the plant height of tetraploids (22.13 cm) was slightly shorter than that of diploids (25.27 cm), while their stem diameters were dramatically increased to 1.10 cm.

### Table 3 Leaf characteristics of the polyploid and diploid plants

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Leaf length (cm)</th>
<th>Leaf width (cm)</th>
<th>Leaf index</th>
<th>Area (cm(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td>3.47±0.24 a</td>
<td>1.94±0.15 b</td>
<td>1.79±0.09 a</td>
<td>5.16±0.72 a</td>
</tr>
<tr>
<td>Tetraploid (small)</td>
<td>1.65±0.07 b</td>
<td>1.10±0.05 c</td>
<td>1.50±0.08 b</td>
<td>1.37±0.09 b</td>
</tr>
<tr>
<td>Tetraploid (big)</td>
<td>3.60±0.21 c</td>
<td>2.43±0.09 a</td>
<td>1.48±0.06 c</td>
<td>6.07±0.62 c</td>
</tr>
<tr>
<td>Octoploid</td>
<td>1.15±0.05 d</td>
<td>0.99±0.04 d</td>
<td>1.16±0.06 d</td>
<td>0.89±0.06 d</td>
</tr>
</tbody>
</table>

Data are mean±SD. Values within the same column followed by different lowercase letters are significantly different according to two-sample t-test (\(P<0.05\)).

### Table 4 Content of leaf pigment in diploidy, tetraploidy, and octoploidy \textit{Rhododendron fortunei}

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Chlorophyll a+b (mg g(^{-1}))</th>
<th>Chlorophyll a (mg g(^{-1}))</th>
<th>Chlorophyll b (mg g(^{-1}))</th>
<th>Carotenoid (mg g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td>0.643±0.042 c</td>
<td>0.484±0.028 c</td>
<td>0.155±0.006 c</td>
<td>0.132±0.004 c</td>
</tr>
<tr>
<td>Tetraploid</td>
<td>0.835±0.031 b</td>
<td>0.626±0.039 b</td>
<td>0.212±0.022 b</td>
<td>0.166±0.007 b</td>
</tr>
<tr>
<td>Octoploid</td>
<td>0.948±0.031 a</td>
<td>0.721±0.049 a</td>
<td>0.237±0.021 a</td>
<td>0.191±0.003 a</td>
</tr>
</tbody>
</table>

Data are mean±SD. Values within the same column followed by different lowercase letters are significantly different according to two-sample t-test (\(P<0.05\)).

![Fig. 6](image-url) Stability analysis of the polyploid \textit{Rhododendron fortunei} plants. A and B, diploid and tetraploid plants cultivated for 2 years. C and D, leaves of diploid and tetraploid plants, respectively. E, octoploid plant cultivated for 2 years. F–H, ploidy level of diploid, tetraploid and octoploid plants, respectively.
Remarkably, the growth and development of octoploid plants are highly aberrant with extreme slow growth rate and dwarfing plant height (Fig. 6-E, Table 5). Compared with diploid and tetraploid plants, the leaves of octoploid plants became highly deformed with very smaller size in leaf length, width and area (Table 5). This means that those octoploid plants are not suitable for utilization as breeding resource. The ploidy levels of these plants were reconfirmed by flow cytometry, which revealed no change in ploidy levels (Fig. 6-F–H).

Furthermore, two tetraploid plants were selected out to be propagated by tissue culture. As shown in Fig. 7, tissue-cultured plantlets of these two tetraploids also have rounder and curled leaves with darker green compared with diploid plants (Fig. 7-A–E). And their ploidy levels were also verified by flow cytometry after propagated for three generations. The result showed similar ploidy level as their explant sources (Fig. 7-F and G). These results demonstrate that the polyplloid R. fortunei plants induced by our method are genetically stable.

### 4. Discussion

As an endemic species of Rhododendron, R. fortunei is a small tree of high ornamental value and great commercial potential (Bian and Jin 2006). It is important to make more R. fortunei varieties for breeding of this species. However, tree breeding is usually labor-intensive and time-consuming.

#### Table 5 Morphological traits of 2 years-old polyploid and diploid Rhododendron fortunei plants

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Plant height (cm)</th>
<th>Stem diameter (cm)</th>
<th>Petiole length (cm)</th>
<th>Leaf length (cm)</th>
<th>Leaf width (cm)</th>
<th>Leaf index</th>
<th>Area (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td>25.27±2.19 a</td>
<td>0.83±0.15 b</td>
<td>2.40±0.26 a</td>
<td>14.24±2.05 a</td>
<td>4.69±1.09 b</td>
<td>3.11±0.45 a</td>
<td>48.98±7.43 b</td>
</tr>
<tr>
<td>Tetraploid</td>
<td>22.13±3.84 a</td>
<td>1.1±0.14 a</td>
<td>2.55±0.34 a</td>
<td>12.67±2.28 b</td>
<td>6.46±0.70 a</td>
<td>1.97±0.37 b</td>
<td>65.73±9.10 a</td>
</tr>
<tr>
<td>Octoploid</td>
<td>6.50±1.22 b</td>
<td>0.42±0.07 c</td>
<td>0.44±0.11 b</td>
<td>2.56±0.34 c</td>
<td>1.87±0.24 c</td>
<td>1.37±0.10 c</td>
<td>4.01±0.73 c</td>
</tr>
</tbody>
</table>

Data are mean±SD. Values within the same column followed by different lowercase letters are significantly different (P<0.05).

![Fig. 7 Stability analysis of tissue-cultured plantlets of tetraploid Rhododendron fortunei plants. A, diploid plantlet. B and C, tetraploid plantlets. D and E, leaves of diploid and tetraploid plantlets, respectively. F and G, ploidy levels of diploid and tetraploid plantlets, respectively. In A–C, bar=1 cm; in D and E, bar=1 mm.](image-url)
(Wilde et al. 2015). Polyploid induction through chemical treatment has been proven to be an effective method to increase the mutagenesis rate and obtain polyploids in ornamental plants (Sattler et al. 2016; Manzoor et al. 2019). The theory of polyploid induction suggests that cells with strong division ability may be easier to induce polyploidy. Therefore, it is necessary to select tissues or organs that have strong cell division and strong proliferative ability as the inducing materials. In previous studies, seeds, adventitious buds, and shoot tips have been typically used as materials for polyploid induction (He et al. 2016; Eng and Ho 2019). Similarly, seeds of R. fortunei were successfully used to create polyploid plants in this study.

Colchicine is a widely-used chemical for inducing polyploidy. However, it does not result in stable polyploidization, and it is highly toxic and easily causes chromosome deletion, resulting in the abnormal growth of regenerated plants (Manzoor et al. 2019). The herbicide oryzalin has low toxicity for animals and results in good induction of polyploidy in plants (Ascough et al. 2008; Gallone et al. 2014). For example, following the application of oryzalin to induce polyploidy in ornamental Alocasia, the tetraploid Alocasia plants exhibited slower growth, thicker leaves, stems and roots, larger stomatal apparatus and increased number of chloroplasts (Thao et al. 2003). In oryzalin-induced tetraploid Rosa, there is a significant increase in pollen viability and petal number (Kermani et al. 2003). In Rhododendron species, the tetraploid R. simsii was also obtained by oryzalin treatment of seedlings (Eeckhaut et al. 2006). In our study, through oryzalin treatment of seeds of R. fortunei, tetraploid and octoploid plants were obtained for the first time, which exhibited obvious morphological changes (Fig. 5).

Treatment time and concentration of chemical inducer are critical for polyploid induction. The most efficient condition for polyploid induction in Stevia rebaudiana is soaking for 24 h in 0.1% colchicine, which resulted in 6% of tetraploid and octoploid R. fortunei was undoubtedly 7.5 mg L⁻¹ oryzalin (Table 1). Therefore, an appropriate concentration of chemical inducer is a key factor for the successful induction of polyploids.

In terms of the detection of ploidy level, chromosome counting is one of the most reliable methods to determine ploidy. However, due to the extremely thin roots and small chromosomes of Rhododendron species, it was difficult to use root tips, shoot tips, or buds as materials for chromosome counting. Alternatively, flow cytometry has the advantages of convenience, rapidity, and result repeatability, and its reliability of identifying genome size and ploidy level has been confirmed in many cases of Lilium regale, Lycium ruthenicum, Actinidia chinesis and Rhododendron (Eeckhaut et al. 2005; Jones et al. 2007; Izadi et al. 2019; Li et al. 2019; Rao et al. 2019). In our study, tetraploid, octoploid, and chimera plants were all successfully identified by flow cytometry. Moreover, these polyploids were further validated by their morphological characteristics. Thickened and rounder leaves, larger stomata at lower density, and higher pigment content, which are typical traits of polyploid plants, were observed in the identified polyploid R. fortunei. These results indicate that flow cytometry is a reliable method for ploidy level identification.

As revealed by abundant studies, the most distinctive feature of polyploidy in plants is the increase in cell size due to the increase in nuclear content, because of which polyploid individuals may generate larger organs than their diploid counterparts (Manzoor et al. 2019). Larger organs, such as leaves, flowers and seeds, are valuable traits for breeding of most ornamental plants. Therefore, to induce polyploidy has been one of widely used tools in breeding of ornamental plants. For instance, colchicine treatment produced larger flower height with increased lip width in wishbone flower (Torenia fournieri) (Boonbongkarn et al. 2013), and increased flower diameter up to 1.2–1.3 folds in tetraploid plants of matted sea-lavender (Limonium bellidifolium) as compared to its diploid plants (Mori et al. 2016). Induced tetraploid also increased leaf area and changed leaf shape in Arabidopsis (Arabidopsis thaliana) (Comellie et al. 2019), marigold (Tagates erecta) (SadhuKhann et al. 2014), Bletilla striata (Li et al. 2018) and Lycium ruthenicum (Rao et al. 2019). Similarly, obvious morphological changes in leaves were also observed in our study. Some of induced tetraploid R. fortunei plants exhibited larger, rounder and thicker leaves with more epidermal hairs (Figs. 5–7). Interestingly, we also noticed that some of the tetraploid R. fortunei plants were much smaller in size than diploids (Fig. 5). As observed in Lagerstroemia indica and Artemisia annua (Banyai et al. 2010; Ye et al. 2010), this may cause by reduction in the number of cell divisions in polyploids.

In addition, polyploidization also brings a significant impact
on the number of plant physiological processes (Sattler et al. 2016; Manzoor et al. 2019). For instance, leaf primary metabolite, such as sucrose, proline and γ-aminobutyric acid (GABA), was remarkably upregulated in citrus polyploids. Compared with citrus diploids, tricarboxylic acid cycle intermediates — citric acid, malic acid, fumaric acid and succinic acid were also increased in citrus tetraploids (Tan et al. 2015). On the contrary, the levels of some secondary metabolites, including phenylpropanoids and terpenoids, tended to decrease, which may alleviate genomic stress to promote vitality and growth in the early stages of genomic doubling (Tan et al. 2017). In our study, the pigment contents were dramatically increased in polyploid R. fortunei plants (Table 4). Increase in chlorophyll content may promote photosynthesis to affect carbohydrate metabolism, which might change flowering behaviors in polyploids (Meyers and Levin 2006). Besides, larger stomata with low density have also been observed in the induced polyploid R. fortunei plants (Fig. 4, Table 2). It is believed that these changes could reduce the transpiration rate in polyploid plants. Additionally, thicker leaves also possibly retain higher water content, which may improve their tolerance to drought for those polyploid R. fortunei plants.

5. Conclusion

In this study, a novel effective method was established for polyploid induction of R. fortunei. The data indicates that a 16-h treatment with 7.5 mg L⁻¹ oryzalin is the optimal condition to obtain polyploid plants. Seeds treated with these conditions maintained a germination rate of 68.92% and exhibited a tetraploid induction rate of up to 33.3%. Compared with diploid plants, the polyploids exhibited obvious morphological changes including thicker and rounder leaves with larger stomata and higher pigment contents. These morphological characteristics and the ploidy level were relatively stable during prolonged cultivation and tissue culture. Overall, the results of this study provide an important technical foundation for polyploid breeding of R. fortunei, and significantly contribute towards solving practical problems in the breeding of new varieties of Rhododendron hybrids.

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References


