Induction, identification and characterization of tetraploidy in *Lycium ruthenicum*

Shupei Rao1,2), Xiangyang Kang1,2), Jian Li3) and Jinhuan Chen*1,2)

1) College of Biological Sciences and Technology, Beijing Forestry University, Beijing 100083, China  
2) National Engineering Laboratory for Tree Breeding, Beijing Forestry University, Beijing 100083, China  
3) State Key Laboratory of Seedling Bioengineering, Yinchuan 750004, China

*Lycium ruthenicum* of Solanaceae was widely used as healthy vegetables and natural medicine foods for containing numerous functional components in leaves, roots and fruits. In the present study, tetraploid plants of *L. ruthenicum* were obtained efficiently by treating their leaves with colchicine in vitro. The highest induction frequency of the tetraploids was 31.4%, which was obtained by preculturing the leaves for 10 days and then treating them with 100 mg/L of colchicine concentration for 48 h. The ploidy levels of the regenerated plants were determined by flow cytometry and chromosome counting methods. Cytological, morphological, and histological characterization validated the results of flow cytometry, revealing the differences between the two kinds of ploidy plants in their tissue culture stage and field production stages. Morphological indexes also provide a simple and intuitionistic method for distinguishing tetraploid from diploid plants. As the chromosome number increased, the stomatal size and number of the chloroplasts in the stomata also increased, but the stomatal density decreased. The results indicate that the chromosome number is correlated with the stomatal index. The generated tetraploid is a potentially useful cultivated variety and will be beneficial for producing triploid progeny in the future.

**Key Words:** Lycium, tetraploid, colchicine, flow cytometry.

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**Introduction**

*Lycium ruthenicum* is a defoliated shrub belonging to the family Solanaceae, and it is mainly distributed in the desert region of Northwest China, Central Asia, the Caucasus, Europe, Mongolia, and North African countries along the Mediterranean Sea (Guo et al. 2016, Han et al. 2014). *L. ruthenicum* can grow vigorously in saline-alkali soil; hence, it is an ideal plant for alleviating soil salinity and preventing soil desertification (Chen and Zhong 2014). In addition to its ecological values and afforestation potential, *L. ruthenicum* has been used directly as a raw ingredient in nutritional food products for animals and humans (Mocan et al. 2017). In Asian countries, *L. ruthenicum* has been used for at least 2000 years as a traditional food and medicine. As an important species of goji, *L. ruthenicum* remains in the wild state as most species from *Lycium* genus (Chen et al. 2018). In recent years, increasing researches have focused on the organizational components in black wolfberry. It is currently known that the leaves and ripe fruits of *L. ruthenicum* are rich in nutrients and functional components, such as anthocyanins, polysaccharides, and shikimate-mediated plant secondary metabolite, which are responsible for immune enhancement or antioxidant activity (Wang et al. 2018, Zeng et al. 2014). The fruits of *L. ruthenicum* are widely used as a traditional medicine for treating heart disease, menopause, and irregular menstruation (Chen et al. 2017, Zheng et al. 2011). The young soft leaves of *L. ruthenicum* have been widely used as functional tea, medicinal vegetables, and animal/poultry feed because of their particularly high nutrient content (Chen et al. 2018). However, the small size of fruits and leaves of *L. ruthenicum* cannot satisfy the market demand. Increasing its fruit size, leaf size and nutrient substances are great essential for the breeding and large-scale production of *L. ruthenicum*.

Polyploidy refers to the presence of extra chromosomes, which may originate from a single species or from different species, in addition to the complete homologous chromosomes (Gentric and Desdouets 2014). Polyploids are particularly common in angiosperms, and they act as key drivers of macro-evolutionary success (Mayrose et al. 2011, Pradhan et al. 2010). Since the first report on the polyploid evening primrose in 1907 (Lutz 1907), polyploidy has been extensively studied in the fields of genetic evolution and genomics (Borrell et al. 2015, Paterson et al. 2012, Soltis
et al. 2015, Song and Chen 2015). Most agricultural and ecological species are polyploids that display unique and novel hybrid phenotypes that are not shown by diploids (Washburn and Birchler 2014). Presently, considerable attention has been directed toward polyploids, particularly triploids, in commercial cultivation due to their various advantages and superior traits that are attributed to their altered polyploid DNA content; specifically, they often have large tissues, large number of fruits, decreased stomatal densities and seedless and capable of homeostasis in stressful environments (Beaulieu et al. 2008, Godfree et al. 2017, Hannweg et al. 2016, Kanchanapoom and Koarapatchaikul 2012, Wu et al. 2012). Chromosome manipulation has been recognized as an important approach for improving L. barbarum cultivars (Chen et al. 2018). The small leaves and numerous seeds of L. ruthenicum fruits limit their utilization. Therefore, polyploidization has substantial implications for the production of seedless and high-yield L. ruthenicum species.

One way of obtaining triploids is to cross diploids with tetraploids. Thus, an increasing number of tetraploids have been generated from various plant species by using antimitotic chemicals (such as oryzalin, trifluralin, and colchicine) in vitro (Cheng et al. 2012, de Carvalho et al. 2016, Dhooghe et al. 2011, Xie et al. 2015). In view of the success of the technology, many vegetatively propagated flowers, fruits, and agricultural crops with major economic values have been artificially induced with polyploids in vitro (Dhooghe et al. 2011), including Rosa rugosa (Allum et al. 2007), kiwi (Wu et al. 2012), grape (Chung et al. 2014), orchid (Chung et al. 2014), pear (Kadota and Niimi 2002), Solanum lycopersicum (Cola et al. 2014), and Ziziphus jujuba (Cui et al. 2017, Gu et al. 2005). In polyploid studies, researchers usually retain isotype diploid controls by using leaves rather than seeds, thereby facilitating comparative studies between tetraploid and its original diploid. However, to the best of our knowledge, the production of L. ruthenicum tetraploids by treating L. ruthenicum leaves with colchicine in vitro is poorly studied. In our study, we used colchicine directly to soak young diploid (2n = 2x = 24) leaves from tissue culture seedlings to regenerate adventitious buds for polyploid induction and established an efficient method to obtain tetraploids from the leaves of L. ruthenicum. Colchicine concentration and standardization of optimum treatment durations were discussed. We further analyzed the morphological characteristics of the resultant tetraploid plants grown in a sterile room for 30 days and in the field for 8 months and we applied a potential method to distinguish tetraploid from diploid types. The established method greatly improved the mutagenic efficiency, reduced the chimera ratio, and provided valuable parental germplasm for further triploid breeding study of Lycium species.

Materials and Methods

Plant materials

The regenerated shoots of L. ruthenicum were derived from full seeds collected in Xinjiang Province. The seeds were disinfected with 75% (v/v) ethanol and 70% (v/v) sodium hypochlorite disinfectant (Beijing BioDee Biotechnology Co. Ltd). Then, such seeds were inoculated into Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 3% (w/v) sucrose (Beijing BioDee Biotechnology Co. Ltd) and 0.7% (w/v) agar (Beijing BioDee Biotechnology Co. Ltd.) until seed germination. Each seedling was clonally propagated as described below to establish a genetically uniform plant line. When the seedlings grew to 5–6 cm, the shoots were sliced into 1 cm sections with at least one axillary bud and transferred to the MS medium containing 0.49 μM indole-3-butyric acid (IBA, Beijing BioDee Biotechnology Co. Ltd.) at pH 6.0. The cultures were maintained at 24 ± 1°C under a 16 h photoperiod using cool-white fluorescent light with 3klx intensity.

Shoot regeneration and colchicine treatment

Fully expanded leaves were collected from a single plant line 30 days after subculture and used for shoot regeneration. The experiment consisted of 24 treatments using colchicine and 6 treatments without using colchicine. Each of treatment processed 27 explants and was repeated three times. The explants were transected into a 0.5 cm × 0.5 cm square and precultured in MS medium, which contained 0.44 μM 6-benzyladenine (6-BA), 0.53 μM 1-naphthlcetic acid (NAA), and 2.32 μM kinetin (KT), for 10 or 20 days (Fig. 1a). When the callus appeared in the edge of leaves, they were transferred to a liquid medium that contained 1% (v/v) dimethyl sulfoxide to increase the efficiency and different concentrations of colchicine (0, 50, 100, 150, or 200 mg/L) for 24, 48, or 72 h in the dark. After the colchicine induction treatment, the explants were washed three times with sterile water and transferred to MS medium, which contained 0.44 μM BA, 0.53 μM NAA, and 2.32 μM KT, without colchicine for 30 days to obtain adventitious buds (Figs. 1c, 1d). When the adventitious shoots grew to 1.5 cm, they were transferred to half-strength MS rooting medium containing 0.49 μM IBA to promote root growth (Fig. 1e). All chemicals mentioned in this section were purchased from the Beijing BioDee Biotechnology Co. Ltd.

Flow cytometry for ploidy analysis

Apical leaves located in the fourth to fifth piece from 25- to 30-day-old seedlings obtained after colchicine treatments were examined for polyploid content by flow cytometry (Partec-PAS, Germany). Isogenic diploids were used as the controls. The selected leaves were quickly transected into individual cubes by using a single-edged sharp blade in a plastic petri dish (6 cm in diameter), to which 1 mL of lysate (pH 7.0), consisting of 45 mM magnesium chloride hexahydrate, 20 mM 3-(N-morpholino) propanesulfonic
acid, 30 mM sodium citrate, 0.5% (v/v) Triton X-100, and 1% (w/v) polyvinylpyrrolidone was added (Galbraith et al. 1983). After being chilled on ice for 1 min, a 37 μm cell sieve was used to filter the solution into a flow tube. Then, 200 μL of 4′,6-diamidino-2-phenylindole (DAPI, 10 μg/mL) fluorescence staining solution was added for 1–2 min in the dark and subsequently detected for polyploidy by using the Cyflow® Ploidy Analyzer (Partec). The standard peak of the diploid control was adjusted to channel 50. In general, 8,000–10,000 nuclei were measured per sample.

Chromosome counting
When the roots of the colchicine-treated plants grew up to a length of 1–1.5 cm, they were cut into lengths of 0.5 cm at the time of exuberant apical cell division, placed in distilled water for 24 h at 4°C, and transferred to Carnoy’s fluid (ethanol:acetic acid ratio = 3:1, by vol.) at 4°C for 24 h. After rinsing with distilled water for 3–4 times, 1 M HCl was added to dissociate the material in a constant-temperature water bath at 60°C for 15 min, and the samples were stained with a modified phenol fuchsine solution for 5 min on a glass slide. The chromosome number was observed using the oil lens of an OLYMPUS CX23 microscope after pressed-disk technique with a cover slip.

Growth parameters
Plant height, weight and stem diameter are indicators of plant growth. The height and stem diameter of diploids and tetraploids grown for 30 days were measured. The fresh weight (FW) and Dry weight (DW) of whole plant, 10 leaves and per centimeter of stem were measured. Paper-cut weighing method was used to measure the leaf area of the upper, middle, and lower leaves of the diploids and tetraploids. The width and thickness of both ploidy plants were observed by scanning electron microscopy (SEM). The fourth leaf was sliced from the upper part of the plant and immediately transferred to 2.5% glutaraldehyde at room temperature for 2 h. The samples were washed three times with distilled water and dehydrated with a series of ethanol solutions (50%, 70%, 80%, 90%, 95% and 100%). They were placed in tert-butanol for 1–2 h and then immediately freeze-dried. SEM (Hitachi S-3400N) was used to observe the transverse section of the leaf. The soil and plant analyzer development (SPAD) value of the diploid and tetraploid leaves, which were grown for 30 and 90 days, were measured using the Minolta chlorophyll meter (SPAD-502, Konica Minolta, Osaka, Japan) in vivo. A total of four random leaves of the upper, middle, and lower parts of each plant were selected in the same position, and each leaf was measured three times.

Stomatal analysis and chloroplast count
The diploid (control) and tetraploid plants were subjected to stomatal analysis. The abaxial epidermis of the leaf, which was positioned on the fourth from the shoot tip of the plant, was peeled off and placed in the center of a glass slide with distilled water. We observed 10 random fields of view per leaf to calculate the stomatal density and randomly selected 30 stomata for stomatal size measurement. We observed the number of chloroplasts between two guard cells in each stoma by laser confocal microscope (Leica TCS SP8 CARS) and randomly selected 10 stomata for each leaf to count.

Statistical analysis
Analysis of variance and Duncan’s multiple analysis were performed using SPSS 20.0 (International Business Machines Corporation, Armonk, NY, USA). Differences were considered statistically significant when $p < 0.05$. 

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Fig. 1. Plant regeneration from leaves of *L. ruthenicum*. a: 0.5 cm × 0.5 cm leaf inoculation into MS differentiation medium. b: The blade has grown more than 1 mm of callus. c: Growing buds from callus. d: Leaves treated with colchicine successfully differentiated into adventitious buds. e: Adventitious buds transferred to half-strength MS medium. f: Adventitious buds have already taken root.
Results

Ploidy level identification
A dominant peak in the flow cytometry analysis of the DAPI-stained nuclei histogram demonstrated the ploidy level of the plants (Fig. 2). The G1 peak of the diploid control appeared in the vicinity of channel 50, whereas the G1 peak appeared at channel 100 in the tetraploids. Both chromosome counting and flow cytometry analysis are standard methods for verifying polyploidy. Moreover, chromosome counting can accurately confirm the plants’ ploidy level, which was analyzed by flow cytometry. Cytological analysis showed that the metaphase chromosome number of the diploid plant was \(2n = 2 \times = 24\) (Fig. 3a), and that of the tetraploid plant was \(2n = 4 \times = 48\) (Fig. 3b), which were consistent with the results of flow cytometry analysis.

Tetraploid induction frequencies
A total of 30 treatment conditions involving various preculture durations, exposure time, and colchicine concentrations were set for tetraploid induction frequency analyses (Table 1). As shown, different treatment conditions led to substantial differences in the frequency of tetraploid induction. All four colchicine concentrations could successfully induce the tetraploid of \(L.\) ruthenicum. The highest frequency of polyploid induction was 31.4% for the eighth treatment

| Treatment number | Colchicine concentration (mg/L) | Preculture duration (days) | Exposure time (h) | Survival rate (%)a | No. of regenerated shoots | No. of diploid | No. of mixoploid | No. of tetraploid | Tetraploid induction rate (%) |
|------------------|---------------------------------|-----------------------------|-------------------|--------------------|--------------------------|----------------|-----------------|-----------------|-----------------------------|
| 1                | 0                               | 10                          | 72                | 92.6 ± 6.41        | 48                       | 48             | 0               | 0               | 0              |
| 2                | 48                              | 48                          | 100 ± 0.00        | 46.2 ± 6.41        | 46                       | 46             | 0               | 0               | 0              |
| 3                | 24                              | 24                          | 100 ± 0.00        | 96.2 ± 6.41        | 52                       | 52             | 0               | 0               | 0              |
| 4                | 20                              | 72                          | 100 ± 0.00        | 96.2 ± 6.41        | 53                       | 53             | 0               | 0               | 0              |
| 5                | 24                              | 48                          | 100 ± 0.00        | 100 ± 0.00         | 57                       | 57             | 0               | 0               | 0              |
| 6                | 24                              | 24                          | 100 ± 0.00        | 100 ± 0.00         | 55                       | 55             | 0               | 0               | 0              |
| 7                | 24                              | 50                          | 100 ± 0.00        | 88.9 ± 6.41        | 36                       | 33             | 0               | 3               | 8.3            |
| 8                | 24                              | 48                          | 100 ± 0.00        | 92.6 ± 6.41        | 39                       | 36             | 0               | 3               | 7.7            |
| 9                | 24                              | 24                          | 100 ± 0.00        | 100 ± 0.00         | 46                       | 43             | 1               | 2               | 4.3            |
| 10               | 24                              | 20                          | 100 ± 0.00        | 92.6 ± 6.41        | 42                       | 33             | 0               | 9               | 21.4           |
| 11               | 24                              | 48                          | 100 ± 0.00        | 100 ± 0.00         | 52                       | 39             | 1               | 12              | 23.1           |
| 12               | 24                              | 100                         | 100 ± 0.00        | 92.6 ± 6.41        | 49                       | 44             | 1               | 4               | 8.2            |
| 13               | 100                             | 10                          | 100 ± 0.00        | 92.6 ± 6.41        | 40                       | 30             | 0               | 10              | 25             |
| 14               | 48                              | 48                          | 100 ± 0.00        | 100 ± 0.00         | 51                       | 35             | 0               | 16              | 31.4           |
| 15               | 24                              | 24                          | 100 ± 0.00        | 96.3 ± 6.41        | 49                       | 42             | 0               | 7               | 14.3           |
| 16               | 24                              | 20                          | 100 ± 0.00        | 85.2 ± 12.82       | 35                       | 32             | 0               | 3               | 8.6            |
| 17               | 24                              | 48                          | 100 ± 0.00        | 88.9 ± 11.1        | 40                       | 36             | 0               | 4               | 10.0           |
| 18               | 24                              | 48                          | 100 ± 0.00        | 96.3 ± 6.41        | 47                       | 45             | 0               | 2               | 4.3            |
| 19               | 150                             | 10                          | 100 ± 0.00        | 70.4 ± 6.41        | 27                       | 24             | 0               | 3               | 11.1           |
| 20               | 150                             | 48                          | 100 ± 0.00        | 74.1 ± 6.41        | 29                       | 25             | 0               | 4               | 13.8           |
| 21               | 150                             | 24                          | 100 ± 0.00        | 81.5 ± 6.41        | 34                       | 32             | 0               | 2               | 5.9            |
| 22               | 150                             | 24                          | 100 ± 0.00        | 74.1 ± 6.41        | 31                       | 27             | 0               | 4               | 12.9           |
| 23               | 150                             | 48                          | 100 ± 0.00        | 88.9 ± 11.10       | 41                       | 37             | 0               | 4               | 9.8            |
| 24               | 150                             | 24                          | 100 ± 0.00        | 85.2 ± 6.41        | 38                       | 35             | 0               | 3               | 7.9            |
| 25               | 150                             | 20                          | 100 ± 0.00        | 96.3 ± 6.41        | 47                       | 45             | 0               | 2               | 4.3            |
| 26               | 200                             | 72                          | 100 ± 0.00        | 0.0 ± 0.00         | 0                        | 0               | 0               | 0               | 0              |
| 27               | 200                             | 48                          | 11.1 ± 0.00       | 7.4 ± 6.41         | 4                        | 4               | 0               | 0               | 0              |
| 28               | 200                             | 24                          | 0.0 ± 0.00        | 7.4 ± 6.41         | 4                        | 4               | 0               | 0               | 0              |
| 29               | 200                             | 48                          | 0.0 ± 0.00        | 18.5 ± 6.41        | 13                       | 12             | 0               | 1               | 7.7            |
| 30               | 200                             | 24                          | 0.0 ± 0.00        | 3.7 ± 6.41         | 3                        | 3               | 0               | 0               | 0              |

*a Survival rate refers to the percentage of differentiated successfully explants to the total number of explants. All data represent the mean ± SE of three replicates.

Growth of colchicine-treated leaves
The condensed regeneration rate of the explant is the remarkable outcome of colchicine treatment because of its adverse effect on cell division. The survival rate, which was influenced by preculture duration, exposure time and colchicine concentration, was measured by the number of surviving leaves after the colchicine-treated leaves were grown for 30 days (Table 1). The survival rates of all explants were below 20%, regardless of exposure time and preculture duration at high colchicine concentrations (200 mg/L). At 200 mg/L for 72 h, all explants died. At the same concentration, the survival rate for the 10-day preculture duration was lower than that for the preculture for 20 days. The toxic effects on the leaves were remarkably reduced as the concentration of colchicine was reduced, and the differentiation rate increased to 70%–100% (Fig. 1a–1d). The surviving explants grew healthy adventitious buds (Fig. 1c, 1d). Regenerated shoots were transferred to half-strength MS medium containing 0.49 μM IBA for rooting (Fig. 1e). After 4 weeks, the developed roots of tetraploids grew completely (Fig. 1f).
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**Morphological features**

Leaf area, width, thickness and weight are morphologic traits that are closely related to the light energy utilization efficiency of plants. As shown in Figs. 5–7, the tetraploids obtained from colchicine treatment grew slower and lesser in height than the untreated plants grown in a sterile room for 30 days and in the field for 8 months, respectively, and they displayed morphological traits such as strong stems, wider blade, increased leaf area, and irregular edges (Figs. 5–7). The differences in the width and thickness between the diploid and tetraploid leaves were observed by using the objective lens of a scanning electron microscope at 65× magnification. The tetraploid clearly had wider and thicker leaves than the diploid (Fig. 5c, 5d). The diploid has obviously higher weight than the tetraploid at the whole plant level, which may be related to its faster growth rate. However, the biomass of tetraploid is significantly higher than that of diploid after we fixed the length or numbers of specific tissues (Fig. 6d–6f).

**Chlorophyll SPAD value determination**

Chlorophyll is one of the most important parameters that is often used as a physiological index to investigate plant

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**Fig. 2.** Flow cytometry analysis of *L. ruthenicum*. a: Diploid (control). b: Tetraploid.

**Fig. 3.** Number of chromosomes in root tips of regenerated plants of *L. ruthenicum*. a: Chromosome number of diploid plant (2n = 2x = 24) (bars: 10 μm). b: Chromosome number of tetraploid plant (2n = 4x = 48) (bars: 10 μm).

**Table 2.** Comparison of stomatal traits between diploids and tetraploids

| Ploidy level | Stomatal length (μm) | Stomatal width (μm) | Stomatal density (mm²) |
|--------------|----------------------|---------------------|-----------------------|
| Diploid      | 23.0 ± 2.91b         | 21.3 ± 2.15b        | 189.6 ± 19.20a        |
| Tetraploid   | 37.2 ± 2.30a         | 38.4 ± 1.99a        | 94.3 ± 10.54b         |

Lower case letters represent a significant difference in Duncan’s multiple range test at the 0.05 significance level.
typic variation, adaptability, and genetic diversity in plant evolution. Polyploids are commonly found in angiosperms, and act as key drivers of macroevolutionary success (Mayrose et al. 2011, Pradhan et al. 2010). Polyploidy can be induced through many different ways. Callus is typically used as the material for polyploidy induction; the mitosis-inducing polyploidy pathway seemed to produce chimeras more easily when callus-derived shoots were used. As for seed-derived polyploid induction, we cannot confirm the polyploidy of the original seeds and retain the isotype diploid controls. In this study, we proposed to use the leaves in vitro by colchicine treatment method to induce tetraploid. Although mixoploids also appeared, their frequency was considerably lower than that of the tetraploids. Thus, we provided an efficient colchicine mediated method for in vitro tetraploidization from leaf explants and established its confirmation method through a series of experiments. The optimal colchicine treatment was determined experimentally according to the various materials because of their different sensitivities to colchicine (Cui et al. 2017, Zhang et al. 2008). According to previous reports, Ziziphus jujuba Mill. cv. Zhanhua and Ziziphus jujuba Mill. var. spinosa have different colchicine concentration and duration, indicating that different species have different colchicine tolerance (Cui et al. 2017, Gu et al. 2005). The critical effects of preculture duration, colchicine concentrations, and exposure time on the induction of tetraploids were explored. Our results showed that a low concentration of colchicine or a short exposure time could increase the survival rate of explants, whereas the induction rate of tetraploids could be seriously reduced. An excessively high colchicine concentration or a long exposure time increases the mortality of the explants due to its toxicity, indicating that efficient tetraploid acquisition must be based on the tolerance of the species to growth and development. SPAD values reflect the relative amount of chlorophyll. The chlorophyll SPAD values of the upper, middle, and lower parts of leaves from 30- and 90-day-old diploid and tetraploid seedlings were measured in vivo. At 30 days, the chlorophyll SPAD value of both the upper new leaf and lower old leaf of the tetraploid was higher than that of the diploid. At 90 days, the SPAD value in the lower part of the tetraploid plant was less than that of the diploid, although the upper new leaf still had a higher SPAD value than that of the diploid (Fig. 6g). In general, the chlorophyll SPAD value of the tetraploid was significantly higher ($p < 0.05$) than that of the diploid, indicating that the tetraploid has a higher photosynthetic capacity and photosynthetic activity, and that it can convert more photosynthetic products. In a short period of time, the chlorophyll SPAD value of the tetraploid was higher than that of the diploid. Over time, the photosynthetic capacity of the lower old leaves of the tetraploid became slightly weaker than that of the diploid, but the photosynthetic capacity of the new upper leaves was still stronger than that of the diploid.

Discussion

Polyploidization is highly important for increasing pheno-
Polyploid breeding in *Lycium ruthenicum* BS

We used apical leaf to conduct tetraploid induction according to the method of Li *et al.* (2016). Consequently, all the leaves had a lethal performance on all provenances we used, indicating that the method is not suitable for all genotypes of *L. ruthenicum* we tested. Then, we established a soaking method with five concentrations including 0, 50, 100, 150, and 200 mg/L of colchicine to treat the leaves, of which 0 mg/L colchicine was set to eliminate the influence caused by external factors of the experiment. All four concentrations of colchicine successfully induced the tetraploid of *L. ruthenicum* during our test, whereas the frequency varied greatly. The highest frequency of tetraploid induction was achieved by the preculture for 10 days with 100 mg/L of colchicine concentration for 48 h, reaching a frequency of 31.4%. Hence, low concentration of colchicine facilitated the acquisition of tetraploidy for *L. ruthenicum*. We also performed doubling experiments on four other genotypes and found the optimal induction condition for these genotypes was similar. Although two genotypes have an overall lower induction frequency, the best condition to induce tetraploid was occurred when using

**Fig. 6.** Comparison of morphological difference between diploids and tetraploids of early stage. a: Plant height for 30-day-old seedlings. b: Stem diameter for 30-day-old seedlings. c: Leaf area for 30-day-old seedlings. d: Fresh weight or dry weight for whole plant of diploid and tetraploid. e: Fresh weight or dry weight for 10 leaves of diploid and tetraploid. f: Fresh weight or dry weight for per cm stem of diploid and tetraploid. g: Chlorophyll content of diploid and tetraploid leaves grown for 30-day-old seedlings and 90-day-old seedlings.

**Fig. 7.** Comparison of morphological difference between diploids and tetraploids field seedlings growing for 8 months. a: Morphology of diploid plants. b: Morphology of tetraploid plants.
100 mg/L colchicine concentration for 48 h. The results indicate that the preculture duration, colchicine concentration, and exposure time but not genotype had significant impacts on tetraploid induction rate.

As a simple, convenient, and rapid technique, flow cytometry is widely used for DNA content analysis of different plant species and for the preliminary identification of chromosome ploidy (Han et al. 2018, Podwyszynska et al. 2017). In our study, the flow cytometry results were consistent with the chromosomal counting method, which is considered a completely accurate method for ploidy identification. Stomatal analysis and chloroplast number are also effective methods for identifying ploidy (Tang et al. 2010). An increase in the number of chromosomes reduced the stomatal density and increased the stomatal size. The number of chloroplasts in tetraploids of stomatal guard cells was approximately twice that in diploids. Meanwhile, the size of stomata and chloroplast frequencies in stomata were inversely proportional in relation to the ploidy difference. Thus, the stomatal density and size could serve as indexes for distinguishing the tetraploids of L. ruthenicum from the diploids.

Chromosome doubling often produces apparent morphological changes in stem diameters, leaf area, and shape of leaf edges in some species (Cai et al. 2015, Han et al. 2018, Luo et al. 2018). According to the morphological measurement of the seedlings, the height of the tetraploids of L. ruthenicum in a tissue culture room, as well as in the field, grew more slowly than that of diploids. The whole plant weight of tetraploids was obviously lower than diploid, which may result from lower growth rate of tetraploid. The leaf area and stem diameter were greatly increased in tetraploids. Accordingly, the biomass of tetraploid is significantly higher than that of diploid when measuring the fixing unit of respective tissues. Although a minimum of 3 years is required to obtain consistent data of the fruit characteristics of tetraploid or acquire triploid plants, the larger leaves presented herein imply that the tetraploid is a potentially useful cultivated variety. Furthermore, the leaf margins were irregular, the thickness of the leaves increased, and the color of leaves was darkened in tetraploids. Chlorophyll is the basic substance of plant photosynthesis, and it reflects a plant’s growth and development status, physiological metabolism, and nutritional status. The SPAD value is positively correlates with the chlorophyll content (Ling et al. 2011, Neufeld et al. 2006). To observe whether color deepening is related to chlorophyll content, we found that the tetraploid chlorophyll SPAD value was remarkably higher than that of the diploid. Based on the slower growth, stronger stem, and larger leaves, we could easily distinguish tetraploid seedlings from diploids through macroscopic observation. We may find the natural tetraploid of L. ruthenicum according to these phenotypic characteristics.

In summary, we explored a highly efficient method for inducing the tetraploids of L. ruthenicum. We determined the suitable colchicine treatment concentration and exposure time for inducing the tetraploids of L. ruthenicum, thereby greatly improving the efficiency of mutagenesis and reducing the chimera ratio. The generated tetraploid provided the foundation for the triploid breeding of L. ruthenicum, which may improve the industrialized production of L. ruthenicum. Future studies should focus on the traits and yields of tetraploid fruit, the molecular changes that occur internally, and the triploid breeding of L. ruthenicum.

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