Plant Cell, Tissue and Organ Culture (PCTOC) (2019) 138:571–581
https://doi.org/10.1007/s11240-019-01654-y

ORIGINAL ARTICLE

Oryzalin-induced chromosome doubling in triploid *Populus* and its effect on plant morphology and anatomy

Qingqing Zeng[1,2,3] · Zhao Liu[1,2,3] · Kang Du[1,2,3] · Xiangyang Kang[1,2,3]

Received: 3 September 2018 / Accepted: 14 July 2019 / Published online: 19 July 2019

Abstract

Populus is an important economical woody species due to its fast growth. In vitro induction of hexaploidy and investigation of morphological and anatomical characteristics in ((*Populus alba* × *P. glandulosa*) × *P. tomentosa*) were conducted in this study. Chromosome doubling was induced in vitro in a triploid clone ((*Populus alba* × *P. glandulosa*) × *P. tomentosa*) with oryzalin as a tubulin inhibitor. Nodal sections of 5 and 10 mm were exposed to 2.5 and 5.0 mg l^{-1} oryzalin for 24, 48 and 72 h. No significant differences in survival rates were observed between the different oryzalin dose, exposure time or nodal length; however, all rates were significantly lower than those in the no-oryzalin controls. The highest frequency of hexaploidy was 100% for the treatment of 5-mm nodes with 5 mg l^{-1} oryzalin for 72 h and the treatment of 10-mm nodes with 5 mg l^{-1} oryzalin for 24 h. The hexaploid plants were distinguishable from the triploid plants by morphological and anatomical characteristics. Chromosome doubling was accompanied by increases in the thickness and chlorophyll content of leaves. The stomata of hexaploids were larger and had a lower density than those of the original triploids. In particular, in triploid-to-hexaploid conversion, roots were less abundant, were shorter and had larger diameters. Root characteristics were determined to be suitable parameters for identifying putative hexaploids because they can be easily and quickly assessed.

Key message

In vitro induction of hexaploidy and investigation of morphological and anatomical characteristics of ((*Populus alba* × *P. glandulosa*) × *P. tomentosa*) at different ploidy levels were conducted in this study.

Keywords Chromosome doubling · Oryzalin · Nodal sections · Anatomical and morphological characteristics

Introduction

Polyploids are prevalent among plants, with estimates for angiosperms ranging from 30 to 70% (Masterson 1994), and have long been considered a prominent feature in the evolution of plants (Stebbins 1971). Many polyploid plants are widely used in agriculture (e.g., wheat, cotton, coffee, oat and canola), implying that polyploidy is often beneficial. The triploid *Populus* has been shown to have the characteristics of faster growth rates, better timber quality and stronger resistance to stress than their diploid counterparts (Zhu et al. 1995; Pu et al. 2002; Liao et al. 2018).The triploid *Populus* was selected from natural habitats (Nilsson-Ehle 1936; Peto 1938; Zhu et al. 1998) or obtained by crossing diploids with tetraploids or triploids (Johnsson 1945; Einspahr 1984). However, these methods show its inefficiency due to a lack of natural triploids and tetraploids. Another approach to produce triploids involves

Communicated by Henryk Flachowsky.

✉ Xiangyang Kang
1651029437@qq.com

[1] Beijing Advanced Innovation Center for Tree Breeding by Molecular Design, Beijing Forestry University, Beijing 100083, China

[2] National Engineering Laboratory for Tree Breeding, Beijing Forestry University, Beijing 100083, China

[3] Key Laboratory for Genetics and Breeding in Forest Trees and Ornamental Plants, Ministry of Education, College of Biological Sciences and Technology, Beijing Forestry University, Beijing 100083, China

utilization of spontaneous or artificial unreduced 2n pollen or induced 2n female gametes. Crossing 2n pollen with a normal female gamete has been widely used to obtain the triploid *Populus* (Zhu et al. 1995; Kang et al. 2000). However, the approach to obtain triploid *Populus* was replaced by utilization of 2n female gametes (Li et al. 2008; Lu et al. 2013) because 2n pollen exhibits less competitiveness in fertilizing female gametes in contrast to normal pollen, resulting in a low incidence of triploids (Kang and Zhu 1997). Moreover, triploid *Populus* arising from the union of a 2n female gamete with a normal reduced male gamete can be of greater value in breeding programs because which combine triploid advantages and maternal genetic dominance. However, the resulting triploids obtained by any method have low fertility due to irregular meiosis, thus limiting their application in *Populus* breeding programs. Chromosome doubling has been considered as a method for restoration of fertility (Lim et al. 2001; Dunn and Lindstrom 2007) and obtaining improved plant characteristics. The fiber strength of hexaploid cotton was stronger than that of the tetraploid parent (Muramoto 1969), hexaploid bread wheat exhibited greater salt tolerance than its tetraploid wheat progenitor (Yang et al. 2014), and hexaploid dandelion seedlings showed greater resistance than corresponding triploid seedlings (Zhong 2010). Chromosome doubling of the triploid *Populus* has not previously been achieved except for the hexaploid of *Populus alba* × *P. berolinensis* 'Yinzhong' (Liu et al. 2018). The triploid 'Yinzhong' was obtained by artificial hybridization, but the exact mechanism of formation was unknown, creating difficulties for further delicate research. However, although we were familiar with the formation mechanism of the triploid ((*Populus alba* × *P. glandulosa*) × *P. tomentosa*) arising from the fusion of unreduced female gametes (2n) and reduced pollen (n), the hexaploid ((*Populus alba* × *P. glandulosa*) × *P. tomentosa*) has not previously been achieved, which may be expected to produce fertile hexaploids and increase *Populus* germplasm with higher ploidy. Meanwhile, chromosome doubling of triploids in *Populus* was reported by Liu et al. (2018) by applying colchicine to leaves but with only low conversion frequencies. To achieve improved conversion frequencies, the type of spindle inhibitor used and the type of explants must be considered.

Colchicine has been widely used to increase plant ploidy levels since it was first reported to possess this ability in the 1930s (Blakeslee and Avery 1937). However, colchicine was gradually replaced by mitosis-inhibiting herbicides (oryzalin, trifluralin and amiprophos-methyl) in polyploidy breeding because of its low affinity for plant tubulin dimers compared to herbicides and because it caused side effects such as sterility, abnormal growth, and chromosome aberration. Previous studies have also shown that oryzalin was substantially more effective than colchicine for inducing chromosome doubling, such as *Rhododendron* (Väinölä 2000) and *Solanum* (Greplová et al. 2009).

Different explant types have been used in chromosome doubling of *Populus*, including leaves (Cai and Kang 2001; Xu et al. 2016; Liu et al. 2018), seeds (Mattila 1961; Qi et al. 2010) and zygotic embryos (Wang et al. 2013; Guo et al. 2017). However, reports on nodal segments used as explants in chromosome doubling in *Populus* are rare (Ewald et al. 2009). In other species, nodal segments have been proven to be efficient in polyploid induction (Rose et al. 2000; Kermani et al. 2003; Allum et al. 2007; Dunn and Lindstrom 2007; Viehmannová et al. 2012). To explore a more efficient methodology for obtaining artificially induced polyploids in *Populus*, the present experiment was conducted by applying oryzalin to nodal segments.

The present investigation aimed to induce hexaploids in ((*Populus alba* × *P. glandulosa*) × *P. tomentosa*) for fertility restoration, enabling continued introgression of desired genes into the cultivated gene pool and accelerating the development of novel, improved selections of *Populus* to meet human needs.

In this study, an appropriate protocol for in vitro hexaploid induction was established by investigating the effects of different drug concentrations, exposure times and explant lengths. Furthermore, the morphological and anatomical characteristics of ((*Populus alba* × *P. glandulosa*) × *P. tomentosa*) at different ploidy levels were assessed. The cultivar '((*Populus alba* × *P. glandulosa*) × *P. tomentosa*)' was selected for chromosome doubling because hybridization with unreduced female gametes can theoretically combine heterosis, triploid advantages and superior maternal traits, and Li and Kang (2007) found which surpassed the diploid counterpart in height and diameter by 33% and 38%, respectively, in 2 years.

Materials and methods

Plant material

The National Engineering Laboratory for Tree Breeding, Beijing Forestry University, China supplied tissue culture plantlets of the triploid ((*Populus alba* × *P. glandulosa*) × *P. tomentosa*) hybrid.

Tissue culture methods

Nodal explants were introduced into solid shoot multiplication medium containing MS (Murashige and Skoog 1962) medium, benzyladenine (BA, 0.1 mg l^{-1}) and naphthaleneacetic acid (NAA, 0.1 mg l^{-1}). The regenerated shoots excised from the nodal explants were transferred to the

rooting medium for 4 weeks, with contained half-strength MS medium and indole-3-butyric acid (IBA, 0.5 mg l^{-1}) to induce shoot elongation. The rooting shoots were used as the source of explants for the induction of polyploids. The pH of the media were adjusted to 5.8 with 0.1 N NaOH before autoclaving for 20 min at 121 °C. Oryzalin from a stock solution in dimethyl sulfoxide (DMSO) was filter-sterilized before its addition to the autoclaved liquid multiplication medium. Plant agar (6 g l^{-1}) was added when solidified media were required. Nodal sections in liquid medium containing oryzalin were kept in darkness, but all other cultures were illuminated by fluorescent tubes with a 16-h photoperiod of 40 µmol m^{-2} s^{-1} in a growth chamber at 25 ± 1 °C.

Polyploid induction

Nodal sections excised from the rooting plantlets were used for polyploid induction. The experiment was carried out by using two different concentrations of oryzalin (2.5 and 5.0 mg l^{-1}) and three different exposure times (24, 48, 72 h) plus two types of nodal sections (5 mm and 10 mm), with 60 nodal sections per treatment. For each treatment, the nodal segments were immersed in liquid multiplication medium containing oryzalin. One set of controls (0 h exposure) consisting of 60 nodes was prepared for 5-mm nodes and another set of 60 nodes was prepared for 10-mm nodes. After exposure to oryzalin or oryzalin-free medium (controls) in liquid shoot multiplication medium, the explants were washed three times with sterilized water and transferred to fresh solid shoot multiplication medium without oryzalin and subcultured every 4 weeks. Survival rates were recorded and the ploidy level of the regenerated shoots was analyzed.

Flow cytometric analysis

The ploidy level of the regenerated shoots arising from oryzalin-treated explants was estimated preliminarily by flow cytometry due to its rapid operation. The machine demonstrated the ploidy of the examined samples by detecting the amount of fluorescence emitted by stained DNA, which was illuminated by a light source. Approximately 20 mg of young leaves from the regenerated shoots was chopped on a petri dish with a blade razor in 1 ml of modified Galbraith's buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, 0.1% (v/v) Triton X-100, pH 7.0 (Galbraith et al. 1983)), and then the suspension of the released nuclei was filtered by a nylon filter with a pore size of 37.4 µm into a 2-ml centrifuge tube. Then, 200 µl of DAPI (1 mg l^{-1}) as a staining solution was added to the filtered suspension and the ploidy level was analyzed with a flow cytometer (Partec-PAS, Germany). Leaves from untreated triploids ((*Populus alba* × *P. glandulosa*) × *P. tomentosa*) were selected as the calibration standard.

Chromosome counting

The ploidy level was further confirmed by chromosome counting to precisely determine the number of chromosomes. Shoot tips were collected and immersed in saturated 1,4-dichlorobenzene solution for 4 h as pretreatment. Then, they were fixed in Carnoy's solution (ethanol:glacial acetic acid = 3:1) at 4 °C for 24 h to kill the cells and maintain their structure. Next, these samples were digested by 3% Cellulase RS, 0.5% Macerozyme R-10 and 0.1% Pectinase Y-23 for 2 h to break the linkages among the cell walls and remove them. After three rinses with water, the digested samples were squashed in carbol fuchsin solution and observed under an Olympus BX51 microscope. The plants with a chromosome number of 57 were classified as triploid plants (2n = 3x = 57), and the plants with a chromosome number of 114 were classified as hexaploids (2n = 6x = 114). Subsequently, hexaploid shoots and their triploid counterparts were cultured in rooting medium for 4 weeks and the rooting shoots were used to assess various characteristics.

Chlorophyll content determination

The fully expanded leaves of three selected individuals for each ploid were cut to 1 × 1 cm^2 and immersed in 5 ml of aqueous acetone (80%) for 48 h in darkness to extract chlorophyll. Chlorophyll concentrations were estimated spectrophotometrically using the equations by Arnon (1949).

Morphological characteristics and stomatal analysis

The height of plantlets and the number and length of roots of three selected individuals for each ploid were recorded. Stomata were obtained by removing a few pieces of epidermal layers from the abaxial side of the expanded leaves of triploid and hexaploid plants using transparent tape techniques. Stomatal characteristics were observed under an Olympus BX51 microscope and images were taken with an Olympus DP73 camera system.

Anatomical characteristics

For anatomical studies, we collected fragments of fully expanded leaves and roots of three selected individuals for each ploid. Samples were fixed in 2.5% glutaraldehyde for 1 h and then washed with 0.1 M potassium phosphate buffer. Subsequently, the fixed samples were postfixed in 1% osmium tetroxide for 1.5 h at 4 °C, dehydrated through an ethanol series, and embedded in Spurr's resin (Spurr 1969). Transverse sections of the leaves and roots were cut on an

ultramicrotome (Leica UC6), mounted on glass slides and stained with 0.5% toluidine blue. Finally, the micrographs of the sections were photographed and the images were used for measurements.

Statistical analysis

The experimental results were subjected to analysis of variance (ANOVA) with IBM SPSS 20.0 (IBM Inc., New York, NY, USA). Percentages were subjected to arcsine transformation before statistical analysis (Snedecor and Cochran 1967). Image J (http://rsb.info.nih.gov/ij/) was used to analyze the images of stomata properties and anatomical characteristics of leaves and roots.

Results and discussion

Polyploid induction

The effects of different concentrations of oryzalin, explant length, and the duration of treatment on polyploid induction were investigated in this study. According to survival rate and hexaploid induction frequency data of 12 treatments (Table 1), GLM-univariate analysis (Table 2) indicated that significant differences in survival rates were not observed between the different oryzalin dose, exposure time or nodal length. Whereas, survival rates decreases with increased drug concentration, longer exposure time, and decreased nodal length. The controls showed survival rates of 90% and 91.67% (data not shown), the rates under different oryzalin treatments ranged from 0 to 16.67% and from 1.67 to 11.67% for the 5-mm and 10-mm nodal segments, respectively (Table 1). The survival rate of explants treated with oryzalin was significantly lower than that in the no-oryzalin control group, which is consistent with polyploidization in other species (Kermani et al. 2003; Dunn and Lindstrom 2007). This lower rate may be associated with the toxic effect of this antimitotic agent, which binds to plant tubulin and inhibits polymerization of microtubules (Morejohn et al. 1987), which are basic components of the cytoskeleton and are involved in many fundamental cellular processes (Nogales 2000), thus causing dramatic consequences for the viability of plant cells.

The ploidy level of the surviving plants was determined by flow cytometry and further confirmed by chromosome counting. The peak of triploid control plants (3x) was set at channel 50 (Fig. 1a), and the peak of hexaploid plants arising from oryzalin-treated shoots was set at channel 100 (Fig. 1b). The chromosomes of shoot tips of selected plants by flow cytometry further confirmed that triploids had 57 chromosomes and hexaploids had 114 chromosomes (Fig. 2).

Table 2 Effects of the three main factors: oryzalin concentration, exposure time and nodal length on polyploidization in ((*Populus. alba*×*P.glandulosa*)×*P. tomentosa*)

Main factors	Survival rate (%)	Polyploidization efficiency (%)
Oryzalin concentration		
2.5	6.39	10.71
5.0	2.22	47.22
Exposure time		
24	6.25	25.00
48	4.58	11.90
72	2.08	50.00
Nodal length		
5	3.33	16.67
10	5.28	41.27

Table 1 The effect of oryzalin treatments on plant survival and polyplodization in ((*Populus alba*×*P.glandulosa*)×*P. tomentosa*)

Length of stem (mm)	Oryzalin concentration (mg l^{-1})	Exposure time (h)	No. of treated explants (%)	No. of survival treated explants (%)	No. of polyploids (%)
5	2.5	24	60	10 (16.67)	0 (0)
5	2.5	48	60	0 (0)	0 (0)
5	2.5	72	60	0 (0)	0 (0)
5	5.0	24	60	0 (0)	0 (0)
5	5.0	48	60	1 (1.67)	0 (0)
5	5.0	72	60	1 (1.67)	1 (100)
10	2.5	24	60	4 (6.67)	0 (0)
10	2.5	48	60	7 (11.67)	1 (14.29)
10	2.5	72	60	2 (3.33)	1 (50.00)
10	5.0	24	60	1 (1.67)	1 (100.00)
10	5.0	48	60	3 (5.00)	1 (33.33)
10	5.0	72	60	2 (3.33)	1 (50.00)

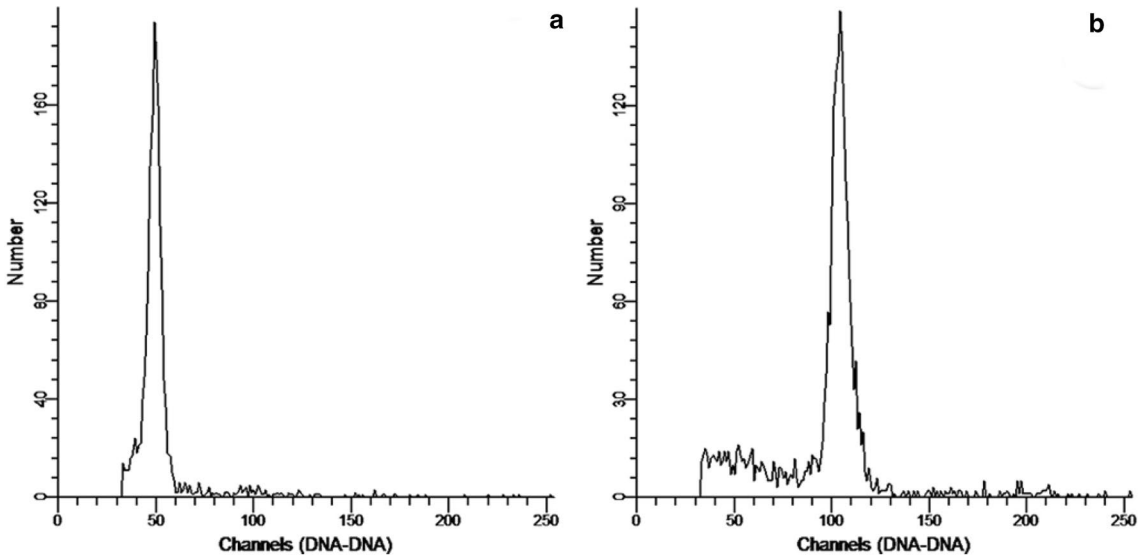

Fig. 1 Histograms of flow cytometric analysis of ((*Populus.alba*×*P.glandulosa*)×*P.tomeantosa*.) **a** triploid control plant (3x = 57), **b** induced hexaploid plant (6x = 114)

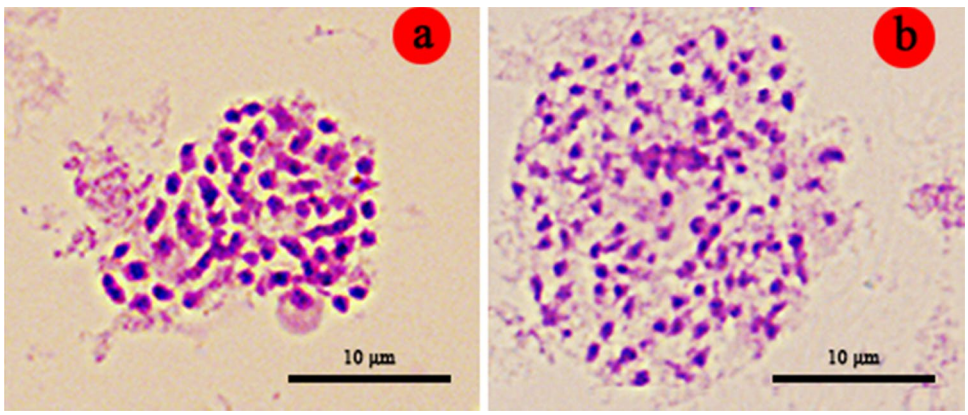

Fig. 2 chromosomes of shoot tip cell of ((*Populus.alba*×*P. glandulosa*)×*P.tomeantosa*). **a** triploid control plant (3x = 57), **b** induced hexaploid plant (6x = 114) (Bars = 10 µm)

In our experiment, significant differences in polyploid induction efficiency were not observed between the different durations of exposure, oryzalin concentrations, or nodal lengths (Table 2). We detected 6 hexaploids from 31 surviving plants arising from oryzalin-treated nodal segments. The hexaploid percentage was 19.35% in our study, which is higher than 5.80% obtained by Liu et al. (2018). Meanwhile, the highest frequency of hexaploidy was 100% for the treatment of 5-mm nodes with 5 mg l^{-1} oryzalin for 72 h and the treatment of 10-mm nodes with 5 mg l^{-1} oryzalin for 24 h. These results indicated that applying oryzalin to nodal segments was an efficient method for chromosome doubling in *Populus*.

Chlorophyll

An overall increase in the chlorophyll content of 6× leaves was observed when calculated per unit leaf area (Table 3). The results are consistent with the findings of other studies showing that plants with a higher ploidy level had leaves with significantly greater chlorophyll content and a darker green color than plants with a lower ploidy level (Ntuli and Zobolo 2008; Murti et al. 2012).

Leaf anatomical characteristics

The anatomical structures of the leaves of ((*Populus alba*×*P. glandulosa*)×*P. tomentosa*) plants at different ploidy levels are shown in Fig. 3. The anatomical structures of the leaves of triploids and hexaploids indicated that both leaves were bifacial and differentiated to adaxial epidermal cells, mesophyll cells and abaxial epidermal cells. No differentiation of palisade and spongy tissue cells was apparent in mesophyll layers in our study for the triploids and hexaploids, which is consistent with the results obtained by Xin et al. (2017). However, other studies have reported

Table 3 Effect of ploidy level on characteristics of [(*Populus.alba* × *P. glandulosa*) × *P. tomentosa*]

Characteristics	Triploid	Hexaploid
Total chlorophyll (mg dm^{-2})	3.56 ± 0.12b	4.84 ± 0.03a
Stomata length (μm)	19.53 ± 0.40b	31.16 ± 1.77a
Stomatal width (μm)	9.52 ± 0.20	12.11 ± 1.76
Stomatal density (No. μm^{-2})	155.42 ± 3.35a	69.13 ± 1.16b
Height (cm)	6.10 ± 0.21a	3.93 ± 0.07b
Root length (cm)	6.37 ± 0.41a	2.06 ± 0.14b
No. of the root	4.56 ± 0.11a	2.33 ± 0.33b
Root diameter (μm)	768.01 ± 17.42b	1063.79. ± 27.79a
Vascular cylinder diameter (μm)	187.76 ± 4.49b	272.15 ± 2.81a
Root cortex diameter (μm)	580.26 ± 12.93b	791.64 ± 25.07a
Arenchyma/cortex (%)	33.16 ± 1.13a	12.67 ± 0.15b
Leaf thickness (μm)	84.73 ± 1.13b	124.18 ± 1.28a
The leaf adaxial epidermis (μm)	21.66 ± 0.28b	24.75 ± 0.75a
The leaf abaxial epidermis (μm)	12.35 ± 0.73	13.97 ± 0.29
Mesophyll thickness (μm)	50.72 ± 0.93b	85.46 ± 1.04a

Means with each row with different letters are significantly different at P = 0.05

A values for all parameters correspond to mean ± SE

that mesophyll cells were distinctly arranged into palisade and spongy tissue cells (Romero-Aranda et al. 1997; Vyas et al. 2007; Zhang et al. 2012). This discrepancy may be due to variations in mature leaves and the use of different species. Table 3 shows a significant difference in leaf thickness between the hexaploids (124.18 μm) and triploids (84.73 μm). Increasing leaf thickness caused by an increasing ploidy level has been observed in *Phlox drummondii* (Vyas et al. 2007), *Pennisetum americanum* (Warner and Edwards 1988) and citrus (Romero-Aranda et al. 1997). In this study, the increased thickness was mainly caused by the thicker mesophyll layers, which accounted for more than half of the total leaf thickness, resulting in 68% greater thickness than that of the triploid leaves.

Stomatal characteristics

Generally, stomatal characteristics have been used for rapid and efficient early identification of polyploids, especially when a number of putative polyploids need to be identified (Cohen and Yao 1996; Gu et al. 2005; Tang et al. 2010). In the induced polyploid plants, significant increases in stomatal length and stomatal width were reported by Tavan et al. (2015) in *Thymus persicus*, in *Paulownia tomentosa* by Tang et al. (2010). However, Table 3 shows a significant increase in stomatal length but not stomatal width in the induced hexaploids, which is consistent with the results obtained by Kaensaksiri et al. (2011). The hexaploid plants displayed significantly decreased stomatal density compared with the triploids (Fig. 4), as reported in *Lagerstroemia indica* L. (Zhang et al. 2010) and *Vitis vinifera* L. (Yang et al. 2006), which may be due to larger epidermal cell size and less stomatal differentiation at the higher ploidy level (Mishra 1997).

In brief, our finding demonstrated that compared to triploids, hexaploids had a larger stomatal size and a lower stomatal density, Which were also observed by Liu et al. (2018) based on the in vitro application of colchicine to another triploid *Populus*, 'Yinzhong'. Analysis of the results obtained by Liu et al. (2018) and our study showed that stomatal characteristics had the same varying tendency in triploid to hexaploid conversions, regardless of oryzalin or

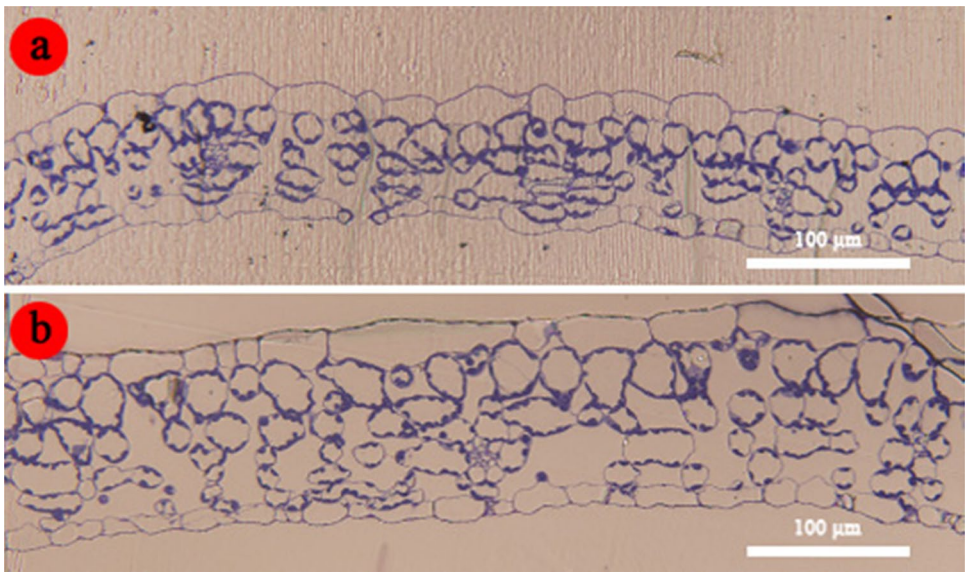

Fig. 3 Cross sections of leaves of ((*Populus.alba* × *P.glandulosa*) × *P.tomeantosa*) **a** triploid control plant, **b** induced hexaploid plant (Bars = 100 μm)

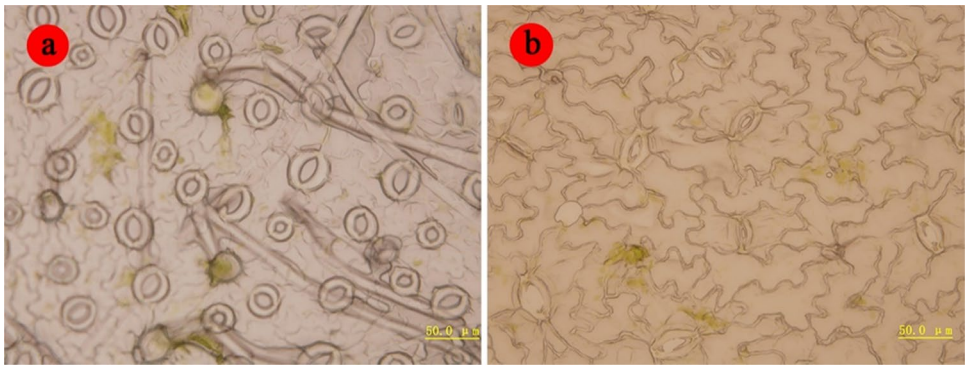

Fig. 4 Stomata characteristics of ((*Populus.alba* × *P.glandulosa*) × *P.tomeantosa*) **a** triploid control plant, **b** induced hexaploid plant (Bars = 50 µm)

colchicine used as antimitotic agents. These findings indicated that morphological changes may be independent of the type of antimitotic substances in polyploidization, which further suggested that the ploidy level may be considered to be a major factor affecting the variation in morphological characteristics.

Stomata have the most prolific distribution in higher plants and locate in the epidermal layers of most aerial parts (Willmer and Fricker 1996). Plant physiologists consider that stomata play a crucial role in regulating the transpiration of vegetation by adapting their mobility and structure. Many studies have shown that stomatal structure is especially plastic to water deficiency, exhibiting increases in stomatal density (Yang and Wang 2001; Zhang et al. 2006) and decreases in stomatal size (Spence et al. 1986). Meanwhile, Carpenter and Smith (1975) found that trees inhabiting dry sites tended to have higher stomatal density and smaller stomatal size than mesic species. These findings indicate that stomata with a small size and high density may enhance the adaptation of plants to drought; therefore, triploids may have greater tolerance under drought conditions than hexaploids.

Root characteristics

Root systems have two major functions. The first is essentially physiological, including the absorption of water and nutrients, and the second is mainly mechanical, maintaining the stability of plants in soil. Quantifying the root system is important because root growth reflects plant productivity and adaptability to the environment.

In this study, the lengths of the roots of chromosome-doubled plants were shorter than those of the original plants (Table 3), as reported in induced tetraploids of *Thymus persicus* (Tavan et al. 2015) and *Punica granatum* (Shao et al. 2003). Hexaploid plants presented fewer root numbers per plant compared to triploid plants (Table 3), while Shao et al. (2003) found that the number of roots per plant was similar between original plants and chromosome-doubled plants. These differences are probably due to the different species used in the studies. Shorter and fewer roots may be the reason that the hexaploids exhibited slow growth and dwarfism (Fig. 5) because root length is assumed to be proportional to resource acquisition (Eissenstat and Yanai 1997), and an underdeveloped underground portion (root) may not support a high aerial part in terms of structural physics.

Anatomical differences in roots are usually closely connected with many physiological functions of the root system. Thus, ploidy level effects on root anatomy can be examined to determine polyploids' adaption to various environments. However, studies on ploidy level effects on root anatomy are rare (Wang 2013). Hexaploids of *Populus* are novel germplasms whose root anatomical characteristics have not been studied. The anatomical structures of the roots of ((*Populus alba* × *P. glandulosa*) × *P. tomentosa*) plants at different ploidy levels are shown in Fig. 6.

We biometrically described the root cortex, vascular cylinder and root diameters of ((*Populus alba* × *P. glandulosa*) × *P. tomentosa*) plants at different ploidy levels. Significant differences in the cortex and vascular cylinder diameters were observed between triploids (580.26 µm and 187.76 µm, respectively) and hexaploids (791.64 µm and 272.15 µm, respectively) (Table 3), leading to a significant increase in the overall root diameter in the hexaploids compared with the triploids. Table 3 shows that the hexaploids displayed a lower proportion of aerenchyma in the cortex compared to the triploids.

In a previous study, hydraulic conductivity was inversely related to root diameter, suggesting that thinner roots usually had greater hydraulic conductivity (Rieger and Litvin 1999). A thinner root cortical diameter was observed in drought-tolerant cultivars versus drought-intolerant cultivars (Maciel et al. 2015). Therefore, drought-tolerant plants can be assumed to have a reduced cortical diameter and a thinner root diameter than drought-intolerant plants, which are conducive to absorbing surrounding water within soil.

Increasing evidence indicates that the root cortical aerenchyma is associated with adaptation to stress (Jackson and Armstrong 1999; Rieger and Litvin 1999; Fan et al. 2003). The root cortical aerenchyma increases drought tolerance by reducing root metabolic costs by converting living cortical

Fig. 5 Morphological characteristics of ((*Populus.alba* × *P. glandulosa*) × *P.tomeantosa*) **a** triploid control plant, **b** induced hexaploid plant (Bars = 1 cm)

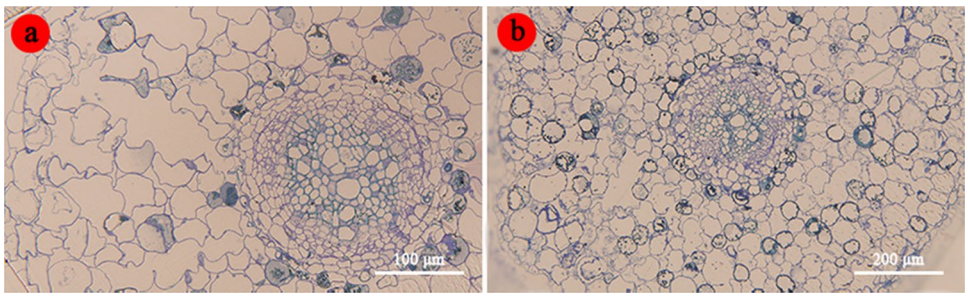

Fig. 6 Cross sections of roots of (*Populus.alba* × *P.glandulosa*) × *P.tomeantosa*. **a** triploid control plant (Bars = 100 μm), **b** induced hexaploid plant (Bars = 200 μm)

tissue to air space, thus allowing roots have more carbon and energy available for root growth and extension to deeper soils under water stress (Zhu et al. 2010). Taken together, we surmise that triploids may have greater tolerance under conditions of drought than hexaploids because triploids have a thinner root diameter, a thinner root cortex diameter, and a higher proportion of aerenchyma in the cortex compared to hexaploids.

Several studies have discriminated plants of different ploidy levels based on stomatal parameters (Kadota and Niimi 2002; Portela de Carvalho et al. 2005). Others have reported that ploidy levels were evaluated by counting the number of chloroplasts in the guard cells of stomata from leaves of regenerated plants (Compton et al. 1996; Zhang et al. 2010). Morphological characteristics such as thicker stems (Viehmannová et al. 2012), increased floral organs (Tang et al. 2010), or an increased leaf width by length ratio (Shao et al. 2003) have been reported to be reliable indicators for determining putative polyploids by macrography, decreasing the work load involved in cytological analysis. In vitro comparison of roots between triploids and hexaploids showed that the roots of hexaploids were shorter and thicker than those of triploids. Shorter and thicker roots were also noted in induced polyploids of *Echinacea purpurea* L. (Dahanayake and Yue-Sheng 2013) and *Punica granatum* (Shao et al. 2003). Therefore, root morphological parameters can be used as primary selection criteria for polyploids because determination of root morphology is simple, rapid and does not require expensive instruments. In addition to morphological markers, flow cytometry and chromosome counting can also be used to precisely confirm ploidy levels.

Conclusions

In conclusion, we found that in vitro application of oryzalin to nodal tissue can efficiently induce chromosome doubling in *Populus*.

Some vegetative characteristic changes were associated with chromosome doubling, including reduced height, thicker and darker green leaves, and an increased root diameter with a correspondingly decreased root number and root length in ((*Populus alba* × *P. glandulosa*) × *P. tomentosa*), suggesting that chromosome doubling can be used to modify plant phenotypes.

According to the variations in plant phenotypic characteristics, we surmise that the hexaploids produced in this work may have lower drought susceptibility. However, this disadvantage may be offset by the fertility of the hexaploids, which facilitates introgression of genes among *Populus*. Additionally, the hexaploid level of ((*Populus alba* × *P. glandulosa*) × *P. tomentosa*), in which three genomes are combined, may provide interesting opportunities for *Populus* breeding.

Acknowledgements This research was supported by National Natural Science Foundation of China for Molecular basis of the vegetative growth advantage in allotriploid poplar (31530012).

Author contributions KXY and ZQQ conceived and designed the experiments; ZQQ and LZ performed the experiments; ZQQ analyzed the data and wrote the manuscript; DK processed pictures.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Allum JF, Bringloe DH, Roberts AV (2007) Chromosome doubling in a *Rosa rugosa* Thunb. hybrid by exposure of in vitro nodes to oryzalin: the effects of node length, oryzalin concentration and exposure time. Plant Cell Rep 26:1977–1984. https://doi.org/10.1007/s00299-007-0411-y

Arnon DJ (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24:1–15. https://doi.org/10.1104/pp.24.1.1

Blakeslee AF, Avery AG (1937) Methods of inducing doubling of chromosomes in plants by treatment with colchicine. J Hered 28:393–411. https://doi.org/10.1093/oxfordjournals.jhered.a104294

Cai X, Kang XY (2011) In vitro tetraploid induction from leaf explants of *Populus pseudo-simonii* Kitag. Plant Cell Rep 30:1771–1778. https://doi.org/10.1007/s00299-011-1085-z

Carpenter SB, Smith ND (1975) Stomatal distribution and size in southern Appalachian hardwoods. Can J Bot 53:1153–1156. https://doi.org/10.1139/b75-137

Cohen D, Yao JL (1996) In vitro chromosome doubling of nine Zantedeschia cultivars. Plant Cell Tissue Organ Cult 47:43–49. https://doi.org/10.1007/BF02318964

Compton ME, Gray DJ, Elmstrom GW (1996) Identification of tetraploid regenerants from cotyledons of diploid watermelon cultured in vitro. Euphytica 87:165–172. https://doi.org/10.1007/bf00023744

Dahanayake N, Yue-Sheng Y (2013) Identification of ploidy levels of in vitro grown purple coneflower (*Echinacea purpurea* L.). J Univ Ruhuna 1:17–21. https://doi.org/10.4038/jur.v1i1.6156

Dunn BL, Lindstrom JT (2007) Oryzalin-induced chromosome doubling in Buddleja to facilitate interspecific hybridization. HortScience 42:1326–1328. https://doi.org/10.1038/194384a0

Einspahr DW (1984) Production and utilization of triploid hybrid aspen. Iowa State J Res 58:401–409

Eissenstat DM, Yanai RD (1997) The ecology of root life span. Adv Ecol Res 27:1–60

Ewald D, Ulrich K, Naujoks G, Schröder MB (2009) Induction of tetraploid poplar and black locust plants using colchicine: chloroplast number as an early marker for selecting polyploids in vitro. Plant Cell Tissue Organ Cult 99:353–357. https://doi.org/10.1007/s11240-009-9601-3

Fan MS, Zhu JM, Richards C, Brown KM, Lynch JP (2003) Physiological roles for aerenchyma in phosphorus-stressed roots. Funct Plant Biol 30:493–506. https://doi.org/10.1071/FP03046

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220:1049–1051. https://doi.org/10.1126/science.220.4601.1049

Greplová M, Polzerová H, Domkářová J (2009) Intra- and inter-specific crosses of Solanum materials after mitotic polyploidization in vitro. Plant Breed 128:651–657. https://doi.org/10.1111/j.1439-0523.2009.01632.x

Gu XF, Yang AF, Meng H, Zhang JR (2005) In vitro induction of tetraploid plants from diploid *Zizyphus jujuba* Mill. cv. Zhanhua. Plant Cell Rep 24:671–676. https://doi.org/10.1007/s00299-005-0017-1

Guo LQ, Xu WT, Zhang Y, Zhang JF, Wei ZZ (2017) Inducing triploids and tetraploids with high temperatures in *Populus* sect. Tacamahaca. Plant Cell Rep 36:313–326. https://doi.org/10.1007/s00299-016-2081-0

Jackson MB, Armstrong W (1999) Formation of aerenchyma and the processes of plant ventilation in relation to soil flooding and submergence. Plant Biol 1:274–287. https://doi.org/10.1111/j.1438-8677.1999.tb00253.x

Johnsson H (1945) The triploid progeny of the cross diploid × tetraploid *Populus tremula* Hereditas 31:411–440. https://doi.org/10.1111/j.1601-5223.1945.tb02761.x

Kadota M, Niimi Y (2002) In vitro induction of tetraploid plants from a diploid Japanese pear cultivar (*Pyrus pyrifolia* N. cv. Hosui). Plant Cell Rep 21:282–286. https://doi.org/10.1007/s00299-002-0509-1

Kaensaksiri T, Soontornchainaksaeng P, Soonthornchareonnon N, Prathanturarug S (2011) In vitro induction of polyploidy in *Centella asiatica* (L.) Urban. Plant Cell Tissue Organ Cult 107:187–194. https://doi.org/10.1007/s11240-011-9969-8

Kang XY, Zhu ZT (1997) A study on the 2n pollen vitality and germinant characteristics of white populars. Acta Bot Yunnanica 19:402–406

Kang XY, Zhu ZT, Zhang ZY (2000) Breeding of triploids by the reciprocal crossing of *Populus alba* × *P. glandulosa* and *P. tomentosa* × *P. bolleana*. J Beijing For Univ 22:8–11. https://doi.org/10.1332/j.1000-1522.2000.06.003

Kermani MJ, Sarasan V, Roberts AV, Yokoya K, Wentworth J, Sieber VK (2003) Oryzalin-induced chromosome doubling in *Rosa* and its effect on plant morphology and pollen viability. Theor Appl Genet 107:1195–1200. https://doi.org/10.1007/s00122-003-1374-1

Li YH, Kang XY (2007) Triploid induction in white poplar by chromosome doubling of megaspore. J Beijing For Univ 29:22–25. https://doi.org/10.13332/j.1000-1522.2007.05.017

Li YH, Kang XY, Wang SD, Zhang ZH, Chen HW (2008) Triploid induction in *Populus alba* × *P. glandulosa* by chromosome doubling of female gametes. Silvae Genet 57:37–40

Liao T, Wang Y, Xu CP, Li Y, Kang XY (2018) Adaptive photosynthetic and physiological responses to drought and rewatering in triploid *Populus* populations. Photosynthetica 56:578–590. https://doi.org/10.1007/s11099-017-0704-5

Lim KB, Ramanna MS, Tuyl JMV (2001) Comparison of homeologous recombination frequency betweeen mitotic and meiotic polyploidization in BC1 progeny of interspecific lily hybrids. Acta Hort 552:65–72

Liu WT, Zheng YF, Song SY, Huo BB, Li DL, Wang J (2018) In vitro induction of allohexaploid and resulting phenotypic variation in *Populus*. Plant Cell Tiss Organ Cult 134:183–192. https://doi.org/10.1007/s11240-018-1411-z

Lu M, Zhang PD, Kang XY (2013) Induction of 2n female gametes in *Populus adenopoda* Maxim by high temperature exposure during female gametophyte development. Breed Sci 63:96–103. https://doi.org/10.1270/jsbbs.63.96

Maciel JRDC, Oliveira DD, Fadin DA, Sajo MDG, Pedroso-De-Moraes C (2015) Morpho-anatomical characteristics conferring drought tolerance in roots of sugar cane genotypes (*Saccharum* L., Poaceae). Braz J Bot 38:951–960. https://doi.org/10.1007/s40415-015-0191-5

Masterson J (1994) Stomatal size in fossil plants: evidence for polyploidy in majority of angiosperms. Science 264:421–424. https://doi.org/10.1126/science.264.5157.421

Mattila RE (1961) On the production of the tetraploid hybrid aspen by colchicine treatment. Hereditas 47:631–640. https://doi.org/10.1111/j.1601-5223.1961.tb01792.x

Mishra MK (1997) Stomatal characteristics at different ploidy levels in *Coffea* L. Ann Bot 80:689–692. https://doi.org/10.1006/anbo.1997.0491

Morejohn LC, Bureau TE, Molè-Bajer J, Bajer AS, Fosket DE (1987) Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization in vitro. Planta 172:252–264. https://doi.org/10.2307/23379101

Muramoto H (1969) Hexaploid cotton: some plant and fiber properties. Crop Sci 9:27–29. https://doi.org/10.2135/cropsci1969.0011183X000900010009x

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tabacco tissue cultures. Physiol Plant 15:473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Murti RH, Kim HY, Yeoung YR (2012) Morphological and anatomical characters of ploidy mutants of strawberry. Int J Agric Biol 14:204–210

Nilsson-Ehle H (1936) Note regarding the gigas form of *Populus tremula* found in nature. Hereditas 21:372–382

Nogales E (2000) Structural insights into microtubule function. Annu Rev Biochem 69:277–302. https://doi.org/10.1146/annurev.biochem.69.1.277

Ntuli NR, Zobolo AM (2008) Effect of water stress on growth of colchicine induced polyploid *Coccinia palmata* and *Lagenaria sphaerica* plants. Afr J Biotechnol 7:3648–3652. https://doi.org/10.5897/ajb08.688

Peto FH (1938) Cytology of poplar species and natural hybrids. Can J For Res 16:445–455. https://doi.org/10.1139/cjr38c-041

Portela de Carvalho JFR, de Carvalho CR, Otoni WC (2005) In vitro induction of polyploidy in annatto (*Bixa orellana*). Plant Cell Tissue Organ Cult 80:69–75. https://doi.org/10.1007/s11240-004-8833-5

Pu JW, Song JL, Xie RJ (2002) Characteristics of lignin structure of triploid clones of *Populus tomentosa* Carr. J Beijing For Univ 24:211–215. https://doi.org/10.13332/j.1000-1522.2002.z1.042

Qi C, Jin C, Li K, Li Z, Zhao H (2010) Comparison of photosynthetic characteristics and leaf anatomy structure of different ploidy *Populus ussuriensis* Kom. Plant Physiol Commun 46(9):917–922. https://doi.org/10.13592/j.cnki.ppj.2010.09.022

Rieger M, Litvin P (1999) Root system hydraulic conductivity in species with contrasting root anatomy. J Exp Bot 50:201–209. https://doi.org/10.1093/jxb/50.331.201

Romero-Aranda R, Bondada BR, Syvertsen JP, Grosser JW (1997) Leaf characteristics and net gas exchange of diploid and autotetraploid citrus. Ann Bot 79:153–160. https://doi.org/10.1006/anbo.1996.0326

Rose JB, Kubba J, Tobutt KR (2000) Chromosome doubling in sterile *Syringa vulgaris* × *S. pinnatifolia* hybrids by in vitro culture of nodal explants. Plant Cell Tissue Organ Cult 63:127–132. https://doi.org/10.1023/A:1006472101185

Shao JZ, Chen C, Deng XX (2003) In vitro induction of tetraploid in pomegranate (*Punica granatum*). Plant Cell Tissue Organ Cult 75:241–246. https://doi.org/10.1023/A:1025871810813

Snedecor WG, Cochran GW (1967) Statistical methods. The Iowa State Univesity Press, Iowa

Spence RD, Wu H, Sharpe PJH, Clark KG (1986) Water stress effects on guard cell anatomy and the mechanical advantage of the epidermal cells. Plant Cell Environ 9:197–202. https://doi.org/10.1111/1365-3040.ep11611639

Spurr AR (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26:31–43. https://doi.org/10.1016/S0022-5320(69)90033-1

Stebbins GL (1971) Chromosomal evolution in higher plants. Addison-Wesley, London

Tang ZQ, Chen DL, Song ZJ, He YC, Cai DT (2010) In vitro induction and identification of tetraploid plants of *Paulownia tomentosa*. Plant Cell Tissue Organ Cult 102:213–220. https://doi.org/10.1007/s11240-010-9724-6

Tavan M, Mirjalili MH, Karimzadeh G (2015) In vitro polyploidy induction: changes in morphological, anatomical and phytochemical characteristics of *Thymus persicus* (Lamiaceae). Plant Cell Tissue Organ Cult 122:573–583. https://doi.org/10.1007/s11240-015-0789-0

Väinölä A (2000) Polyploidization and early screening of Rhododendron hybrids. Euphytica 112:239–244. https://doi.org/10.1023/A:1003994800440

Viehmannová I, Trávníčková M, Špatenková E, Černá M, Trávníček P (2012) Induced polyploidization and its influence on yield, morphological, and qualitative characteristics of microtubers in *Ullucus tuberosus*. Plant Cell Tissue Organ Cult 109:83–90. https://doi.org/10.1007/s11240-011-0076-7

Vyas P, Bisht MS, Miyazawa S, Yano S, Noguchi K, Terashima I, Funayama-Noguchi S (2007) Effect of polyploidy on photosynthetic properties and anatomy in leaves of *Phlox drummondii*. Funct Plant Biol 34:673–682. https://doi.org/10.1071/FP07020

Wang WF (2013) The ecophysiological mechanisms of maintaining whole-plant water balance. Dissertation, University of Chinese Academy of Sciences

Wang J, Shi L, Song SY, Tian J, Kang XY (2013) Tetraploid production through zygotic chromosome doubling in *Populus*. Silva Fenn 47:1–12

Warner DA, Edwards GE (1988) C4 photosynthesis and leaf anatomy in diploid and autotetraploid *Pennisetum americanum* (pearl millet). Plant Sci 56:85–92. https://doi.org/10.1016/0168-9452(88)90190-2

Willmer C, Fricker M (1996) The distribution of stomata. In: Black M, Charlwood M (eds) Stomata, 2nd edn. Chapman and Hall, London, pp 12–35

Xin YL, Kuang F, Tang JR, Qiu F, Li B, Xin PY (2017) Difference analysis on stem and leaf anatomical structure of different ploidy *Psammosilene tunicoides*. North Horticult 8:160–164. https://doi.org/10.11937/bfyy.201708037

Xu CP, Huang Z, Liao T, Li Y, Kang XY (2016) In vitro tetraploid plants regeneration from leaf explants of multiple genotypes in *Populus*. Plant Cell Tissue Organ Cult 125:1–9. https://doi.org/10.1007/s11240-015-0922-0

Yang HM, Wang GX (2001) Leaf stomatal densities and distribution in *Triticum aestivum* under drought and CO_2 enrichment. Acta Phytoecol Sinica 25:312–316

Yang XM, Cao ZY, An LZ, Wang YM, Fang XW (2006) In vitro tetraploid induction via colchicine treatment from diploid somatic embryos in grapevine (*Vitis vinifera* L.). Euphytica 152:217–224. https://doi.org/10.1007/s10681-006-9203-7

Yang CW, Zhao L, Zhang HK, Yang ZZ, Wang H, Wen SS, Zhang CY, Rustgi S, von Wettstein D, Liu B (2014) Evolution of physiological responses to salt stress in hexaploid wheat. Proc Natl Acad Sci USA 111:11882–11887. https://doi.org/10.1073/pnas.1412839111

Zhang YP, Wang ZM, Wu YC, Zhang X (2006) Stomatal characteristics of different green organs in wheat under different irrigation regimes. Acta Agron Sinica 32:70–75

Zhang QY, Luo FX, Liu L, Guo FC (2010) In vitro induction of tetraploids in crape myrtle (*Lagerstroemia indica* L.). Plant Cell Tissue Organ Cult 101:41–47. https://doi.org/10.1007/s11240-009-9660-5

Zhang XS, Zhai XQ, Fan GQ, Deng MJ, Zhao ZL (2012) Observation on microstructure of leaves and stress tolerance analysis of different tetraploid Paulownia. J Henan Agric Univ 46:646–650. https://doi.org/10.16445/j.cnki.1000-2340.2012.06.023

Zhong SM (2010) Study on artificial doubling and application value of dandelion (*Taraxacum mongolicum* Hand.-Mazz.). Dissertation, Huazhong Agricultural University

Zhu ZT, Lin HB, Kang XY (1995) Studies on allotriploid breeding of *Populus tomentosa* B301 clones. Sci Silvae Sin 31:499–505

Zhu ZT, Kang XY, Zhang ZY (1998) Studies on selection of natural triploids of *Populus tomentosa*. Sci Silvae Sin 34:22–31

Zhu JM, Brown KM, Lynch JP (2010) Root cortical aerenchyma improves the drought tolerance of maize (*Zea mays* L.). Plant Cell Environ 33:740–749. https://doi.org/10.1111/j.1365-3040.2009.02099.x