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Impact of the Leaf Cut Callus Development Stages of *Populus* on the Tetraploid Production Rate by Colchicine Treatment

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Abstract

This paper examined the impact of the leaf incision callus developmental status of *Populus* on the tetraploid production efficiency. Using diploid full-sib progeny [(*Populus pseudo-simonii* × *P. nigra* 'zheyin3#') × (*P.* × *beijingensis*)] as experimental material, leaf explants were gathered from seedlings of five genotypes selected randomly from full-sib hybrid progeny cultured in Murashige and Skoog basal medium with 0.4 mg/L 6-benzyladenine and 0.05 mg/L 1-naphthaleneacetic acid. The morphological and cytological characteristics of the incision callus (from the callus origin and adventitious bud development) were observed and divided into five stages based on the characteristics of the callus. The incision callus from each of the five stages was treated with 30 mg/L colchicine for 3 days. Then, the polyploidy level of the regenerated plants was confirmed by flow cytometry analysis and chromosome number counting. The results indicate that the rate of tetraploid production was significantly correlated with the callus development stage of *Populus* leaves; the most likely stage for chromosome doubling was Stage II, in which the calli initially formed around the cut end. To validate that callus developmental Stage II was the optimal callus developmental stage for chromosome doubling of diploid full-sib progeny, ten full-sib progeny genotypes were treated with 30 mg/L colchicine for 3 days at Stage II. All ten genotypes of the diploid progeny obtained tetraploid with no mixoploid production; the percentage of tetraploid induction was 7.9–13.2%. There were significant differences in the morphological characteristics of the leaves and roots of diploid and tetraploid plantlets.

Keywords *Populus* · Tetraploid induction · Callus development · Adventitious bud regeneration

Introduction

The genus *Populus* is an economically important model system for tree research and is widely distributing in the Northern Hemisphere (Bradshaw and others 2000). Due to the high growth performance, high-yield fiber production and resistance traits of triploid *Populus* (Kang and Zhu 2002; Yang and others 2006; Zhang and others 2012), triploid breeding programs have played an increasingly important role in *Populus* improvement. Therefore, as a parent for producing triploid trees, tetraploids have become an important breeding goal.

The major cytological mechanisms of polyploidy formation are the union of unreduced gametes, 2n pollen and somatic doubling. Of these, somatic chromosome doubling is the most common approach for obtaining tetraploids. The in vitro somatic chromosome doubling technique has been used to produce tetraploid plants artificially in forestry trees, including *Paulownia* (*Paulownia fortune*, Fan and others 2007), Betulaceae (*Betula*, Sarkilahti and Valanne 1990),

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and Poplar (*Populus pseudo-simonii* Kitag, Cai and Kang 2011). For the in vitro induction of somatic doubling, shoot tips (Thao and others 2003; Zhang and others 2008; Ewald and othersand others 2009), seeds, apical meristems (Liu and others 2007), nodal sections (Rose and others 2000), and petioles (Nilanthi and others 2009) can be treated. However, a higher frequency of chimeras (mixoploids) is associated with these induction methods. Leaves were ideal explant material for somatic doubling due to their high rate of shoot multiplication (Yadav and others 1996; Gu and Zhang 2005) and relatively low mixoploid rate following tetraploid induction (Nettancourt and others 1971; Kathal and others 1992).

Currently, most research on the technology of tetraploid induction is aimed at establishing an efficient procedure to induce tetraploids. These studies have shown that the rate of tetraploids is affected by the concentration of chemicals agents and exposure time using explants treated with antimitotic reagents (Dahanayake and Yang 2015; Abdoli and othersand others 2013; Acanda and others 2015; Widoretno 2016). However, the most suitable treatment conditions may change with the nuances of the explant culture environment (for example, culture temperature and medium composition) (Thorpe 1990; Saharan and others 2004; Lin and Zhang 2005) and explant genotype (Gandonou and others 2005; Xun and Zhao 2013), which may lead to a low rate of tetraploid production and unavoidably generate mixoploids. To solve this problem, it is necessary to find a more obvious, generally effective period for tetraploid induction in *Populus*.

In this study, we observed the shoot regeneration process of leaf explants of five genotype progeny [(*P. pseudo-simonii* × *P. nigra* 'zheyin3#') × (*Populus* × *beijingensis*)]. The shoot regeneration process was divided into five stages according to the developmental characteristics and cytological observations of the leaf incision callus. Then, we treated the incision callus at five stages with colchicine solution and analyzed the rate of tetraploid production. We successfully found the optimal chromosome doubling period and detailed the characteristics of the stage in which the calli initially formed around the cut end. To verify this, the leaves of ten diploid progeny were treated with colchicine as soon as the incision callus reached the optimal period for chromosome doubling.

Materials and Methods

Plant Materials

Floral branches of a female parent *Populus pseudo-simonii* × *P. nigra* 'zheyin3#' (2n = 2x = 38) were collected from a plantation in Tongliao, Inner Mongolia Autonomous Region, People's Republic of China. Floral branches of a male parent *Populus* × *beijingensis* (2n = 2x = 38) were collected at the campus of Beijing Forestry University. The branches were maintained in water-filled containers in the greenhouse (20/10 °C day/night). Three weeks after the female catkins were pollinated with pollen from the male parent, the seeds were collected from the female flower branches before the seed hairs began to feather out from the ovary to facilitate their disinfection. The seeds were immersed in 70% (v/v) ethanol for 30 s, and soaked in a solution of 1% (v/v) sodium hypochlorite for 4 min. After rinsing the seeds three times with sterile distilled water, they were placed horizontally on solid MS medium (Murashige and Skoog 1962) containing 3% (w/v) sucrose and 0.6% (w/v) agar. After several days, the seeds germinated. Twenty days later, the seedlings grew to about 10 cm in height. The leaves, apical buds and roots were cut off from the seedlings. Then the stems were cut into 1-cm segments with a leaf bud. Stem segments were inoculated vertically into shoot regeneration solid medium supplemented with 3% (w/v) sucrose, 0.6% (w/v) agar, 0.4 mg/L 6-benzyladenine (BA), and 0.05 mg/L NAA. After 30 days of culture, new adventitious shoots regenerated from the bud. Then, single shoots were excised and placed on half-strength MS medium supplemented with 0.1 mg/L indole butyric acid (IBA) for root induction. The established full-sib progeny included 200 genotypes. All media were adjusted to pH 5.8–6.2. The cultures were incubated at 25 °C under illumination at 30–40 µmol m^{-2}s^{-1} during a 14-h photoperiod.

In Vitro Callus Induction and Bud Regeneration

The leaves of five full-sib progeny genotypes (E1-G1, E1-G2, E1-G3, E1-G4, and E1-G5) randomly selected from the 200 progeny genotypes were used as explants and wounded by making two transverse cuts on the midrib without full separation, and then transferred to sterile plastic 9-cm-diameter Petri dishes containing 30 mL solid shoot regeneration medium supplemented with 3% (w/v) sucrose, 0.6% (w/v) agar, 0.4 mg/L BA, and 0.05 mg/L 1-naphthaleneacetic acid (NAA). A callus was induced from the leaf incision in the shoot regeneration medium; then, the callus differentiated into an adventitious bud.

Anatomical Observations of Callus Induction and Bud Formation at the Incision

Callus and adventitious bud development were studied to classify the developmental stages and identify the position of origin. First, the morphological characteristics of the callus leaves were recorded under a stereomicroscope (Olympus SZX12). Then, the development process was divided into five stages based on the characteristics of the callus at the leaf incision. Each callus stage was fixed in FAA fixative (5 mL 38% methyl aldehyde, 5 mL glacial

acetic acid, and 90 mL 50% alcohol) at 4 °C for 24 h, dehydrated in an ethanol series, and embedded in paraffin wax. Then, 8-μm sections were obtained and stained with iron hematoxylin. All samples were photographed under a microscope (Olympus BX51).

Induction of Tetraploids Using Callus Cells

Based on the cytological observations of callus induction and bud regeneration, leaves at each stage were selected as material for colchicine treatment. The leaves were pre-treated for 3 days in shoot regeneration medium, then treated with 30 mg/L colchicine for 3 days to produce tetraploids of the full-sib progeny [(*P. pseudo-simonii × P. nigra* 'zheyin3#') × (*Popupus × beijingensis*)] (Cai and Kang 2011). After treatment, the leaves were washed three times with sterile distilled water and transferred to shoot regeneration medium. The experiments were repeated three times with 15 explants per treatment.

Adventitious buds were regenerated from leaf callus. Single buds were excised and placed in half-strength MS root induction medium supplemented with 0.1 mg/L IBA. After 1 month, the plantlets were transferred to containers with a 1:1:2 mixture of peat, perlite, and sand to grow.

Ploidy Detection

Because flow cytometric analysis is a reliable method for determining ploidy level in *Populus* (Partec-PAS, Germany) (Galbraith and others 1983; Wang and others 2012), we determined the ploidy of plantlets using flow cytometry. Young leaves of the regeneration plants were chopped in a modified Galbraith's buffer (45 mM $MgCl_2 \cdot 6H_2O$, 20 mM MOPS, 30 mM sodium citrate, 0.1% Triton X-100, pH 7.0) using a sharp razor blade on ice. Subsequently, the nuclear suspension was filtered through a 30-μm plastic filter. Then nuclei were stained with 80 μL DAPI (5 mg/mL) for 30 s; samples were analyzed with Cyflow® Ploidy Analyzer (Partec). A known diploid plant was used as a control.

After flow cytometric analysis, ploidy levels of all putative regeneration tetraploid plants were confirmed by chromosome counting. Root tips were removed from the plantlets and pre-treated with 0.2% colchicine solution for 3 h at 25 °C. Subsequently, the materials were fixed in a fresh Carnoy's solution (acetic acid: ethanol, 1:3) for 24 h at 4 °C, and then hydrolyzed in 1N HCl at 60 °C for 10 min, rinsed with distilled water for 15 min. Root tip was transferred to a grass slide, stained with modified phenol solution on a slide glass, squashed with a cover slip and then observed at 100× oil lens using Olympus BX51 microscope.

Validation of the Optimal Callus Developmental Stage for Chromosome Doubling

To verify that the optimal callus developmental stage for chromosome doubling identified in the above experiment was effective for other diploid full-sib progeny of [(*P. pseudo-simonii × P. nigra* 'zheyin3#') × (*Populus × beijingensis*)], ten additional progeny genotypes (E2-G1, E2-G2, E2-G3, E2-G4, E2-G5, E2-G6, E2-G7, E2-G8, E2-G9, and E2-G10) were selected randomly from the 200 progeny genotypes. Then, leaves of the ten genotypes were cultured in shoot regeneration medium as in the above experiment. When the callus at leaf incisions developed to the optimal callus stage, it was treated with 30 mg/L colchicine for 3 days. The subsequent steps in the experimental procedure were the same as above.

Preparation and Analysis of Mesophyll Cell Suspensions to Count Chloroplasts

Leaves located third from the shoot tip were cut into 0.5–1 mm² pieces and transferred to 10 mL of modified enzyme solution [0.6 M mannitol, cellulase (Onozuka R-10) (3% w/v), Macerozme R-10 (0.2% w/v), and Pectolyase Y-23 (0.05% w/v)] at 25 °C for 3 h (Cai 2011). The number of chloroplasts was counted under a 40× lens using ultraviolet or bright field illumination (Olympus BX51 microscope).

Comparison of the Root Properties and Plant Height of Diploid and Tetraploid Plants

The tetraploid and diploid counterparts of the five genotypes from the first experiment were used for the analysis of root properties and plant height. After the diploid and tetraploid plants had grown in the solid root medium for 1 month, ten plantlets were selected randomly from each genotype. The numbers of primary and lateral roots and the plant height of the diploids and tetraploids were reported; primary root length was measured using vernier calipers; and root diameter was measured under an Olympus BX51 microscope. After the diploid and tetraploid plants had been transplanted and grown in a greenhouse for 3 months, the plant height of the diploids and tetraploids was compared again. These measurements were repeated three times, and each replicate consisted of 30 plantlets for each ploidy. Data presented are the mean ± standard error (SE).

Statistical Analysis

The statistical analyses were performed using SPSS ver. 19.0. (SPSS, Chicago, IL, USA). An analysis of variance was performed and Duncan's multiple range test was used to assess differences between treatments. A *p* value < 0.05 was

considered significant, and a *p* value < 0.01 was considered highly significant.

Results

Leaf Callus and Adventitious Bud Development

The entire process of incision callus induction and shoot regeneration was classified into five stages, based on developmental characteristics (Table 1; Fig. 1). (I) No callus formed on the leaf incision (Fig. 1a). (II) Callus initiation stage at the leaf incision (Fig. 1b). The callus at the cut end had a crystalline surface, and small calli were visible. Callus width less than 1 mm. (III) Fast-growing callus stage at the leaf incision (Fig. 1c). After induction, the callus

proliferated quickly. The incision callus was soft, watery, and white or light cream and translucent. The width of the callus increased to 1–2 mm. (IV) Slow-growing callus stage at the leaf incision (Fig. 1d). The callus turned yellow-cream colored with a granular primordium meristem. The width of the callus increased to 2–4 mm. (V) Bud initiation stage at the leaf incision. The callus became gray-cream colored, and bud morphogenesis was observed (Fig. 1e). Interestingly, the developmental stages of the incision callus of different genotypes were asynchronous. The elapsed times for the five genotypes are shown in Table 2.

The relationships between the morphological observations and cytological characteristics of the five developmental stages were established (Table 1) and were used as a tetraploid induction guide. Five stages corresponding to the cytological characteristics of callus development were

Table 1 Developmental characteristics of cut leaf calli and corresponding cytological observations

| Stage	Typical characteristics of the leaf cut	Cytological observations
I	No callus forms	Mesophyll cells near the cut edges enlarged; palisade cells begin to divide
II	Calli initially develop around the cut end	Cell divisions began to form callus at the cut edge; array structure was loose
III	Rapid callus growth	Meristematic cellular masses form in the callus subsurface
IV	Callus growth decelerates, small particles emerge	Initiation of the bud primordium from superficial meristematic tissue
V	The callus turns a gray-cream color, adventitious buds form	Adventitious bud morphology and structure are clearly visible
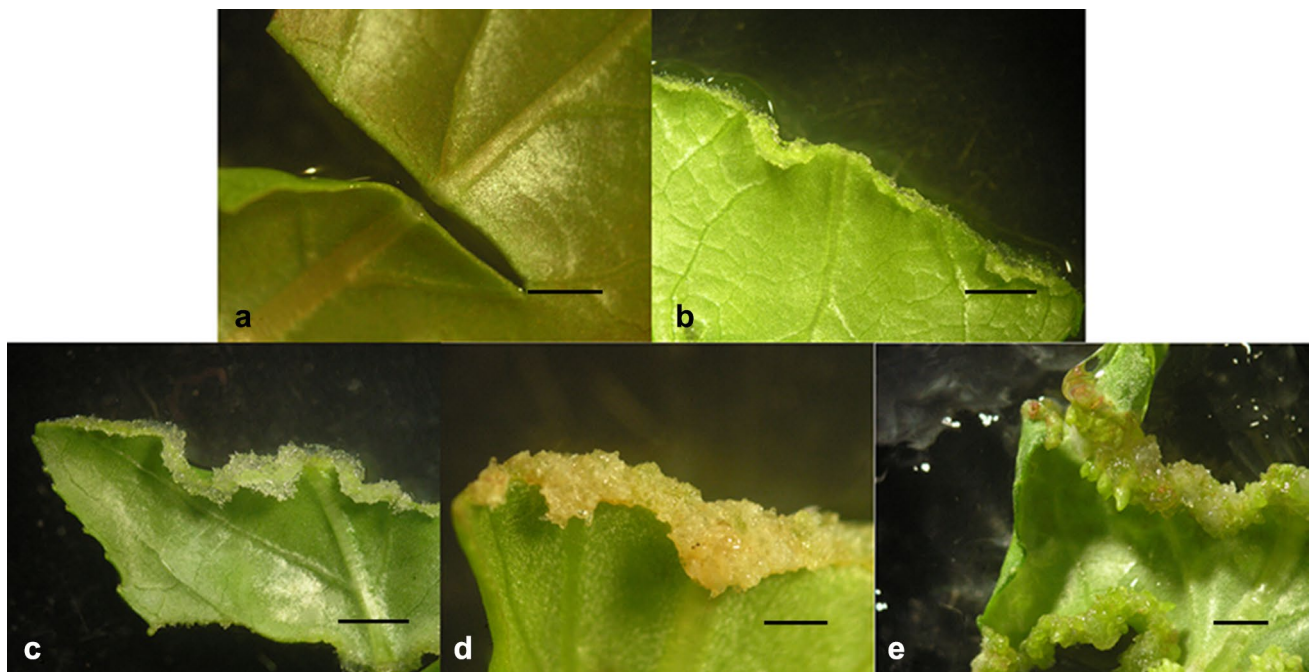
Fig. 1 Callus induction from (*P. pseudo-simonii* × *P. nigra* 'zheyin3#') × (*Populus* × *beijingensis*). **a** Stage I, cut leaves with no callus. **b** Stage II, the initiation of callus around the cut leaves; the callus is < 1 mm wide. **c** Stage III, characterized by fast growing cov-

ering the cut end. The callus is 1–2 mm wide. **d** Stage IV, the callus is 2–4 mm wide. **e** Stage V, calli grew gradually into new green shoots. Bars is 2 mm in **a**, bars are equal 3 mm in **b, c, d** and 5 mm in **e**

Table 2 Number of culture days to arrive at the different stages in the five genotypes

Genotype	Arrive at stage I (days)	Arrive at stage II (days)	Arrive at stage III (days)	Arrive at stage IV (days)	Arrive at stage V (days)
E1-G1	0–2	3–3.5	4.5–6	8–10	13.5–15
E1-G2	0–4	5–5.5	6–7	10.5–12	16–19
E1-G3	0–5	5.5–6.5	6.5–8	9–11	20–23
E1-G4	0–7	8–9	9–10.5	13–15.5	26–28
E1-G5	0–3	3.5–4	5.5–7	8–9	15–18

observed. In stage I, mesophyll cells near the cut edges began to enlarge and palisade cells began to divide (Fig. 2a, b). In stage II, cells began dividing, and callus cells formed on the cut edge of the leaf. A loose structure formed and nuclei were clearly visible (Fig. 2c, d). In stage III, meristematic cell masses were produced on the subsurface of calli; the mass was formed from small cells and had a dense structure; many starch grains appeared in cells (Fig. 2e, f).
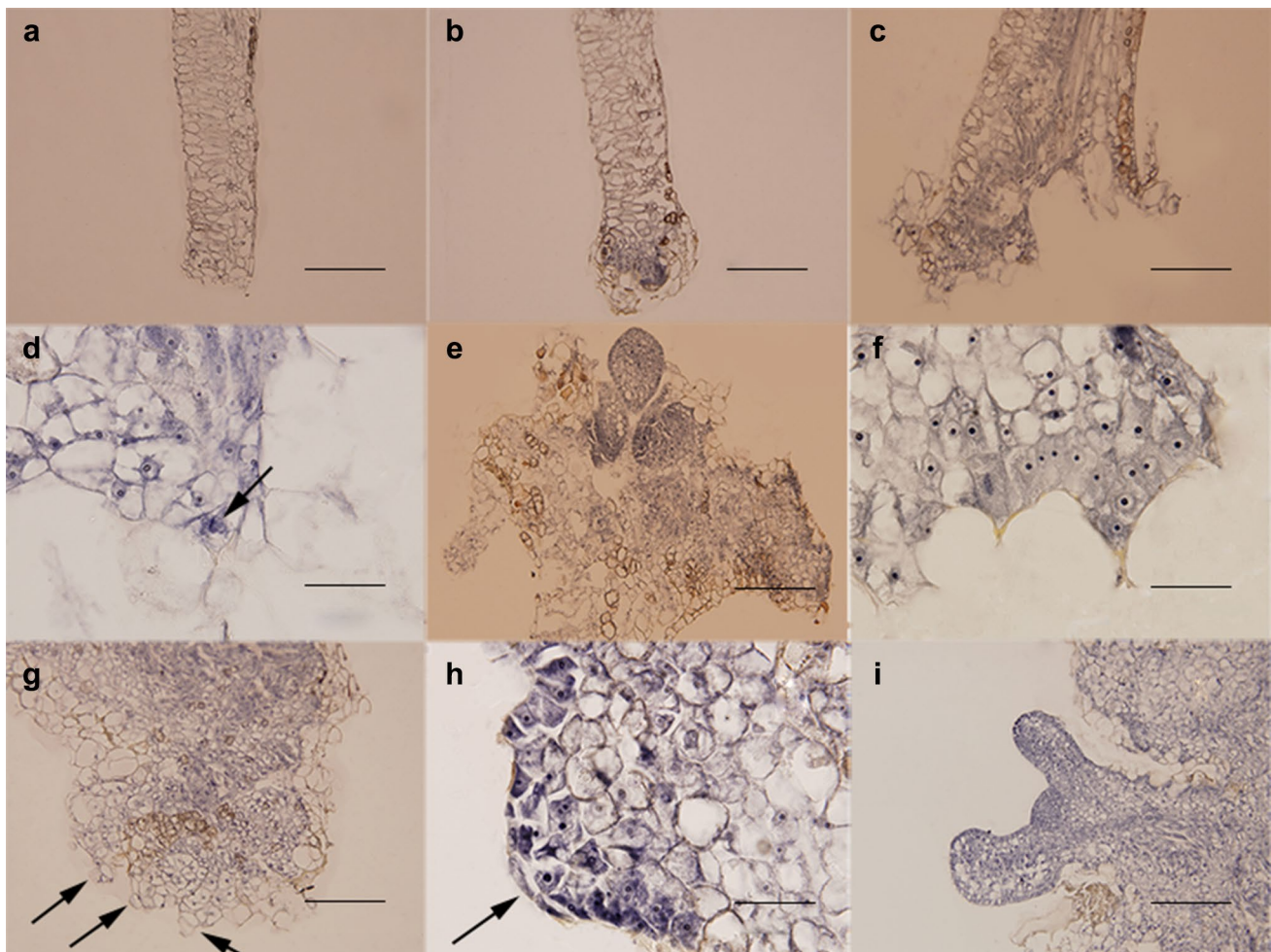

Fig. 2 Anatomic observations of callus formation and organogenesis in [(*P. pseudo-simonii* × *P. nigra* 'zheyin3#') × (*Populus* × *beijingensis*)] leaves cultured in vitro. Stage I: **a** Transverse section of leaf explant. **b** Enlarged mesophyll cells near the cut edge and a cell mass is induced from mesophyll cells near the palisade layer. Stage II: **c** Active cell division and callus forming on the cut edge of the leaf explant. **d** Initial callus cell forming with clearly visible nucleus. Stage III: **e** Rapidly proliferating callus. **f** Cellular masses forming on the callus subsurface, with small tightly packed cuticular cells emerging. Nuclei became more apparent, and starch accumulated in the cells. Stage IV: **g** Primordium meristem forming from the callus surface. **h** Bud close-up Stage V: **i** Adventitious bud derived from the callus surface. Bars are equal 200 μm in **a, b, c, e, g, i** and 20 μm in **d, f, h**

In stage IV, primordial buds formed near the surface of calli (Fig. 2g, h). In stage V, adventitious buds regenerated from the callus and were of external origin (Fig. 2i).

Tetraploid Production

The percentages of tetraploid production of the diploid progeny of the five genotypes induced at different callus developmental stages are presented in Table 3. Treatment callus stages that did not produce tetraploids are not listed. All regenerated plantlets were examined using flow cytometry counting, which showed that all these putative tetraploids were real tetraploids ($2n = 4x = 76$; Fig. 3). Using the tetraploid induction frequency data (Table 3), GLM-univariate analysis indicated highly significant differences among the callus development stages ($F = 44.7$, $p = 0.000$), showing that selecting the suitable stage is important for tetraploid production. The induction results at Stage II and III in Table 3 clearly show that those treated with colchicine at stage II all produced tetraploids; the percentage of tetraploid induction was 5.4–10.3%, with no production of mixoploids. In comparison, when the progeny of the five genotypes were treated with colchicine as soon as the leaf incision callus reached stage III, only one genotype produced a tetraploid and the tetraploid induction frequency was only 2.9%; the remaining genotypes produced mixoploids only, and the highest mixoploid production frequency was 11.1%. No tetraploids or mixoploids of the five genotypes were obtained for leaves treated at Stage I, IV, or V. Therefore, Stage II was the effective period for chromosome doubling.

Validation of the Optimal Callus Developmental Stage for Chromosome Doubling

Table 4 presents the tetraploids production results for the additional ten progeny that were treated at callus development Stage II. All produced tetraploids, and the percentage of tetraploid induction was 7.9–13.2%, and never mixoploid was monitored in the regeneration plantlets. These results verify that Stage II is the appropriate period for chromosome doubling of diploid full-sib progeny of [(*P. pseudo-simonii* × *P. nigra* 'zheyin3#') × (*Populus* × *beijingensis*)].

Mean Chloroplast Number per Mesophyll Cell in Diploids and Tetraploids

The ranges of variation in chloroplast numbers per mesophyll cell were 20–23 and 13–15, respectively, for tetraploid and diploid. The average chloroplast numbers of 30 tetraploid plantlets were approximately twice that of diploids (21.2 ± 0.7 vs. 14.0 ± 0.9, respectively) (Table 5). Comparison of chloroplast number per mesophyll cell between tetraploids and diploids revealed significant variation (Fig. 4; Table 5).

Leaf, Root, and Plant Height Characteristics of Diploids and Tetraploids

Comparing the leaves and roots of diploids and tetraploids, the leaf margins of tetraploids were strongly toothed (Fig. 5a), and the tetraploid plantlets had dark green leaves, thicker roots, and stunted growth (Fig. 5b). Figure 6 shows the analysis of root and plant height of diploids and tetraploids, comparing the primary root length, primary root diameter, primary root number, lateral root number, and plant height. The primary root length, primary root diameter, primary root number, lateral root number and plant height (3 months) of tetraploids and diploids differed significantly $p < 0.01$), and the plant height (1 month) of tetraploids and diploids differed significantly ($p < 0.05$). Therefore, the leaf, root, and plant height characteristics are useful markers for tetraploid screening in [(*P. pseudo-simonii* × *P. nigra* 'zheyin3#') × (*Populus* × *beijingensis*)].

Table 3 Effects of the different incision callus developmental stages on tetraploid induction in the diploid progeny of five genotypes of (*P. pseudo-simonii* × *P. nigra* 'zheyin3#') × (*P.* × *beijingensis*)

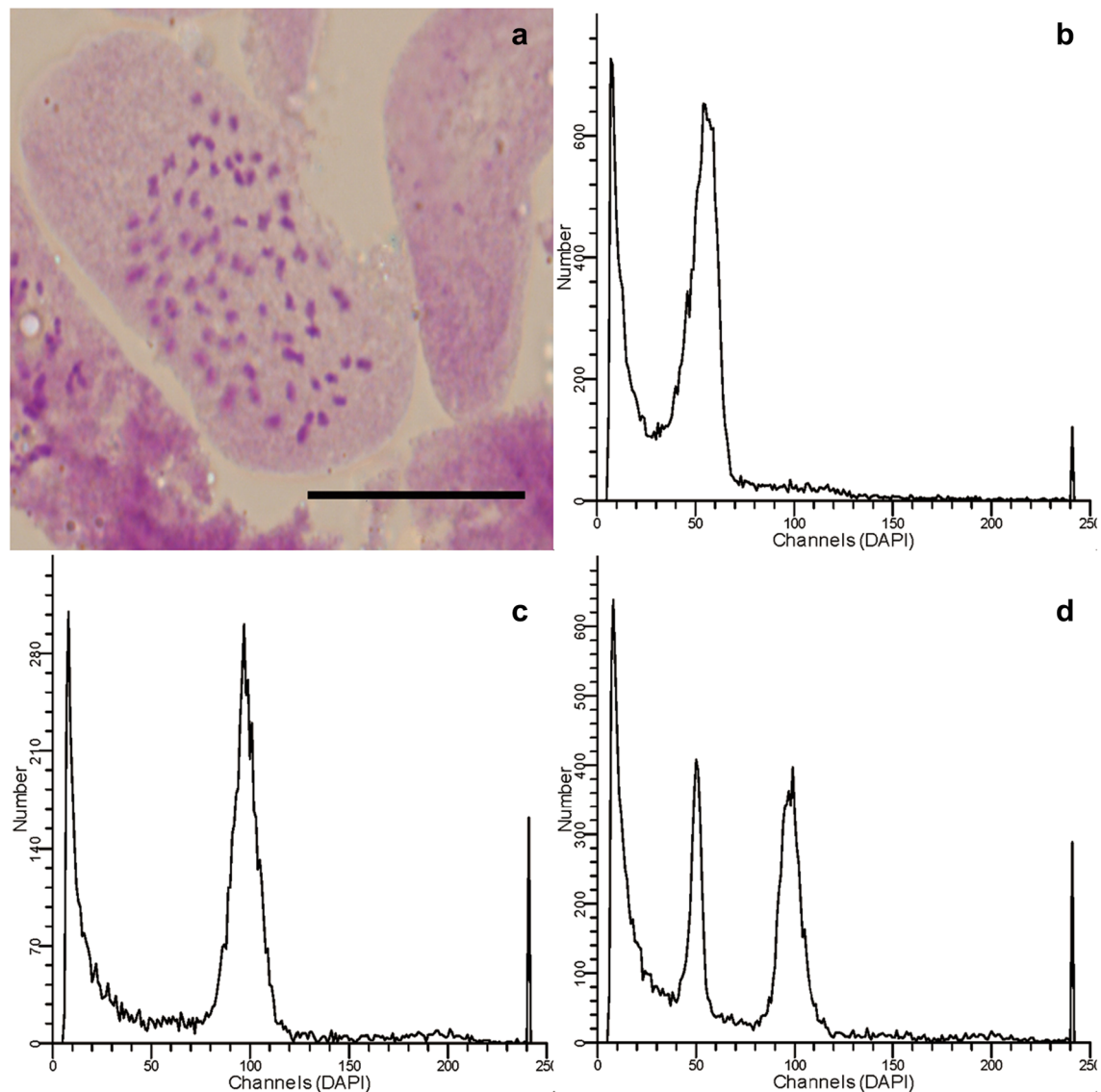| Genotype	Callus stage of leaf incision	No. 4x	No. mixo	No. plants examined	Mixo (%)	4x (%)
E1-G1	II	2	0	37	0	5.4
	III	1	1	34	2.9	2.9
E1-G2	II	2	0	25	0	8
	III	0	0	24	0	0
E1-G3	II	3	0	31	0	9.7
	III	0	2	18	11.1	0
E1-G4	II	2	0	31	0	6.4
	III	0	2	25	8	0
E1-G5	II	3	0	29	0	10.3
	III	0	2	28	7.1	0

Fig. 3 Chromosome counts of seedlings derived from colchicine doubling. **a** Somatic chromosome number in a tetraploid (2n = 4x = 76). **b–d** Flow cytometric analyses of a diploid, tetraploid, and mixoploid, respectively

Discussion

This study demonstrated that the tetraploid induction frequency was highly dependent on the treatment stage. An analysis of the tetraploid induction frequency of the five callus developmental stages confirmed that the optimal chromosome doubling stage was Stage II. By treating the leaf callus at Stage II with colchicine, tetraploids were successfully obtained in the progeny of five genotypes, with no production of mixoploids. Subsequently, the leaves of an additional ten progeny were treated with colchicine as soon as the incision callus reached Stage II and all ten genotypes produced tetraploid plantlets, verifying that Stage II is the effective period for chromosome doubling

of diploid full-sib progeny of [(*P. pseudo-simonii* × *P. nigra* 'zheyin3#') × (*Populus* × *beijingensis*)].

In this study, treating the leaves at Stage I with colchicine, produced no tetraploid plantlets. This indicates that tetraploids do not originate from mesophyll cell chromosome doubling directly, but from the subsequent callus developmental stages. Moreover, tetraploid plantlets were easily obtained with almost no production of mixoploids by treating the leaf callus at Stage II with colchicine. This is because the bud primordia originate from a single cell (Broertjes and Keen 1980; Broertjes and Harten 1985; Yang and Schmidt 1994), and the callus cells at Stage II were newly formed and had not further divided into the meristematic multicellular stage. By treating the leaves during this period, the mitotic

Table 4 Tetraploid induction in the progeny of ten genotypes of (*P. pseudo-simonii* × *P. nigra* 'zheyin3#') × (*Populus* × *beijingensis*) induced in the callus at developmental Stage II

Genotype	Pre-culture duration of arrival at Stage II (days)	No. plants examined	4x (%)
E2-G1	2	39.3 ± 3.2	12.6 ± 1.6
E2-G2	4	24.7 ± 2.5	8.2 ± 0.9
E2-G3	5	37.3 ± 4.2	9.9 ± 1.2
E2-G4	5.5	38.7 ± 3.5	9.5 ± 1.9
E2-G5	5.5	51.7 ± 9.5	9.9 ± 1.9
E2-G6	5.5	32.7 ± 3.1	8.3 ± 2.4
E2-G7	4.5	38.7 ± 5.7	10.0 ± 5.2
E2-G8	7	26.3 ± 4.9	6.7 ± 3.1
E2-G9	3.5	38.0 ± 2.6	12.3 ± 1.5
E2-G10	5	41.0 ± 4.4	5.9 ± 3.2

metaphase cells had doubled into polyploid cells, which further divided into the meristematic tissue of the polyploid. The apex of the tetraploid adventitious shoot was formed from meristematic tissue on the outer surface of the callus.

When the callus of leaves that had developed to Stage III was treated with colchicine, many mixoploids and a few tetraploids were produced. Most of the callus cells in this stage have formed multicellular meristematic masses in the leaf incision subsurface. Some cells in the meristematic masses became polyploid, whereas many others were unaffected and remained diploid when treated with colchicine. Consequently, the adjacent normal and polyploid cells on the outer surface developed into mixoploid buds. On treating the callus of leaves that had developed to Stage IV or V with colchicine, no tetraploids were produced in any of five genotypes. This is because the bud primordium had formed from the superficial meristematic tissue in Stage IV or V. A

Table 5 Comparison of the number of chloroplasts between tetraploid and diploid plants

Characters	Tetraploids		Diploids		*t* value	*p* value
	Range	Mean ± SE	Range	Mean ± SE		
Chloroplasts number (no./mesophyll cell)	20–23	21.2 ± 0.7	13–15	14 ± 0.9	18.5**	<0.001

**Significant differences between tetraploids and diploids at α = 0.01 in the *t* test

Fig. 4 Fluorescence microscope images of the mesophyll chloroplasts in leaf samples from diploid and tetraploid plants. **a** Fluorescence images of diploid mesophyll chloroplasts. **b** Fluorescence images of tetraploid mesophyll chloroplasts
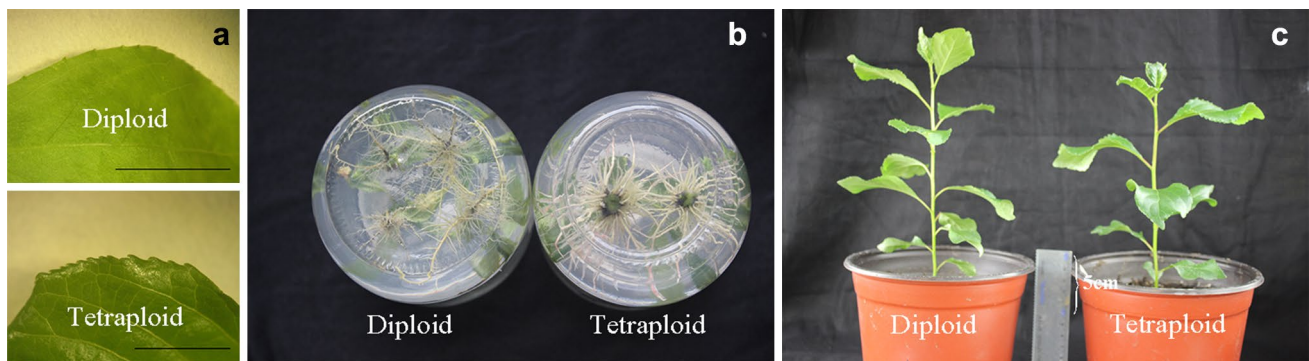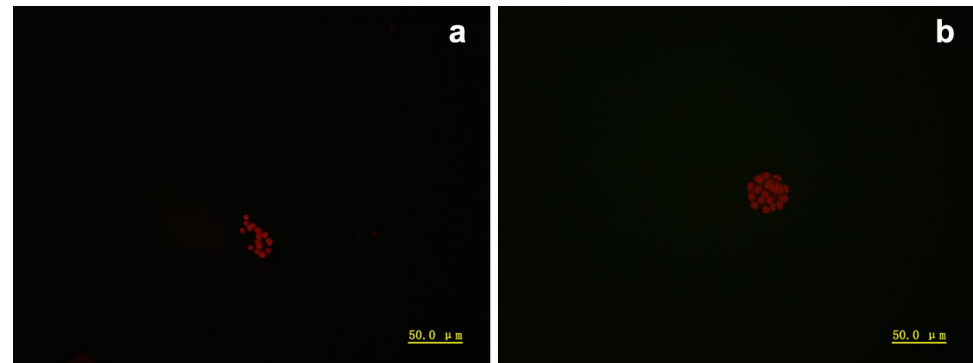

Fig. 5 Leaf and root characteristics of diploids and tetraploids. **a** Leaf margins of regenerated diploids (top) and tetraploids (bottom) Bars = 1 cm. **b** Root variation in regenerated diploids (left) and tetra-ploids (right) after 1 month. **c** Transplants of regenerated diploids (left) and tetraploids (right)
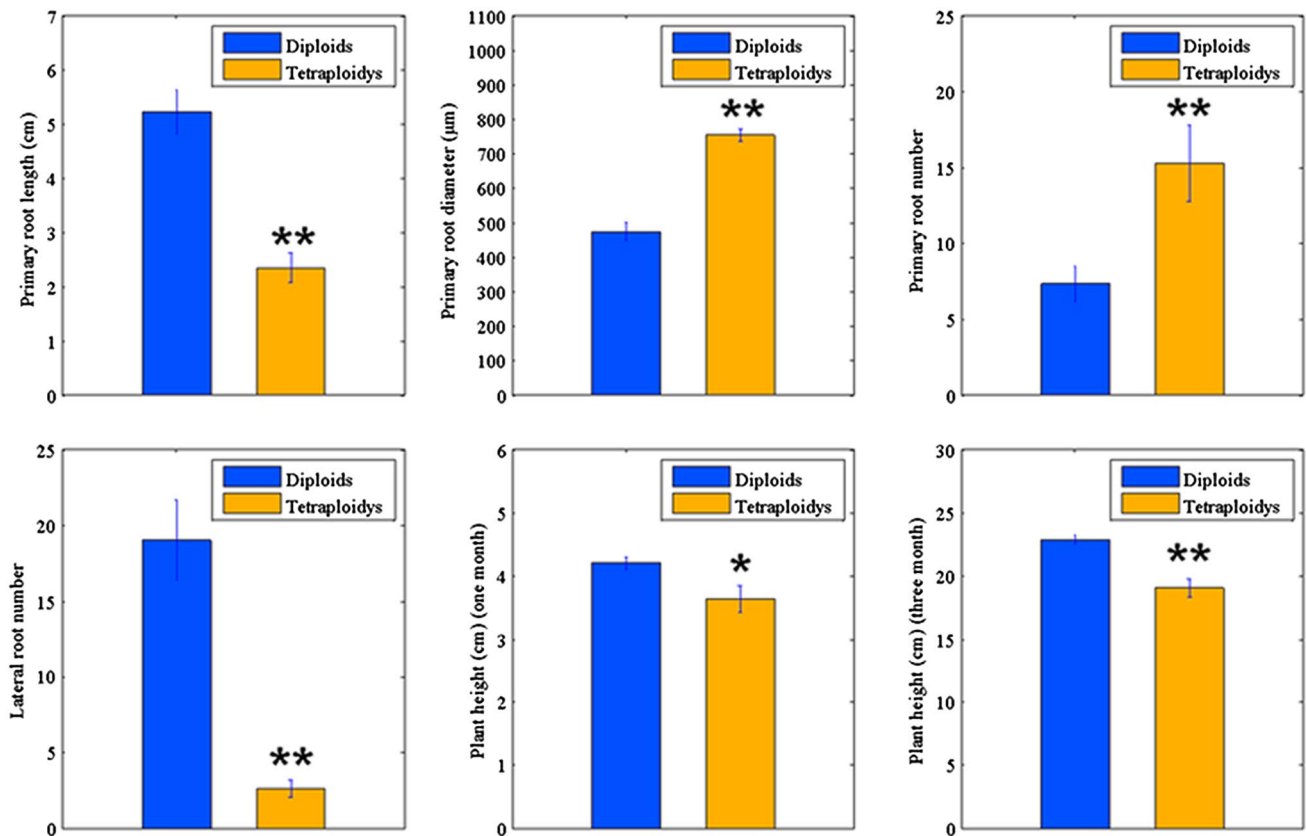
Fig. 6 Comparison of the primary and lateral root and plant height characteristics of diploids and tetraploids. (*$p < 0.05$; **$p < 0.01$)

few mitotic metaphase cells of the bud primordium doubled into tetraploid cells; however, because the bud primordium consisted of countless cells, the limited quantity of doubled cells would not change the ploidy level of the bud.

Previous studies have reported indirect methods to determine ploidy level, such as morphological traits analysis, particularly stomata. Stomata of tetraploids are usually larger and occur at a lower density, but have more chloroplasts per guard cell compared with those of diploids (Tang and others 2010; Widoretno 2016). In this study, the mean chloroplast number per mesophyll cell and leaf and root characteristics differed significantly between diploids and tetraploids and could be used to distinguish tetraploids from diploids, which may explain why the leaves of tetraploids were thicker and had higher chlorophyll content than those of diploids (Mathura and others 2006; Allario and others 2011).

It is generally believed that tetraploids display a larger morphology or higher than diploid, such as *Arabidopsis* (Ni and others 2009), *Morus* (Dai and others 2015). However, our study found that plant height of tetraploids was lower than diploids; this was consistent with the research on *Citrus* (Allario and others 2011) and *Birch* (Mu and others 2012). The phenotypes of different species were variant after tetraploidization, and it was probably because

the mechanism of growth regulation was different. To reveal the mechanism of phenotype variation in *populus* tetraploid, further studies were necessary.

The tetraploid production efficiency was significantly correlated with the leaf incision callus developmental stage. Treating the callus at Stage II with colchicine led to chromosome doubling in diploid full-sib progeny of [(*P. pseudo-simonii* × *P. nigra* 'zheyin3#') × (*Populus* × *bei-jingensis*)]. This novel method overcomes the problem of mixoploidy. In addition, our method is much simpler, faster, and more convenient than traditional in vitro tetraploid induction methods.

Compliance with Ethical Standards

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

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