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Megaspore Chromosome Doubling in *Eucalyptus urophylla* S.T. Blake Induced by Colchicine Treatment to Produce Triploids

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Abstract: Triploids generally provide an advantage in vegetative growth in forest trees. However, the technique of triploid breeding is still an open field in the *Eucalyptus* tree species. This study aims to explore the colchicine treatment technique for megaspore chromosome doubling to establish triploids in this tree species. Cytological observation on microsporogenesis and megasporogenesis was carried out to guide megaspore chromosome doubling induced by colchicine treatment. Ploidy level in progenies was detected by flow cytometry and somatic chromosome counting. A relationship between microsporogenesis and megasporogenesis was established to guide the colchicine treatment. Seven triploids were obtained in the progenies, and the highest efficiency of triploid production was 6.25% when the flower buds underwent a 0.25% colchicine solution treatment for 6 h using an aspiration method seven days after the first observation of leptotene during microsporogenesis on the floral branch. Cytological analysis showed that the megasporocyte from leptotene to diakinesis may be the optimal period for megaspore chromosome doubling by colchicine treatment. Plant height, ground diameter, leaf area, and the photosynthetic parameter of triploid eucalypt were significantly higher than those of the diploid plant at 6 months old. Hybridization with 2n megaspores induced by colchicine treatment is an effective way for *Eucalyptus* triploid breeding. These results should accelerate the development of advanced germplasms in this tree species.

Keywords: polyploid breeding; 2n female gamete; microsporogenesis; megasporogenesis; stomata characteristics

1. Introduction

Eucalypts are one of the most important planted hardwoods worldwide [1]. The remarkable characteristics of this species, including their rapid growth, straight form, valuable wood properties, and wide adaptability, have driven them to be planted over 20 million hectares [2,3], having provided key renewable resources for the production of pulp, paper, biomaterials, and bioenergy in more than 100 countries across six continents [4–7]. In addition, they also have high concentrations of diverse essential oils for medicinal and industrial uses [8,9]. As a result of many years of study,

great achievements have been made on the genetic improvement of this species. However, there is still room for improvement, especially in regard to the creation of new germplasm in this tree species.

Polyploid plants are widely distributed in nature [10–12]. Previous research has shown that polyploid varieties with high productivity, high product quality, and strong resistance to stress play an important role in crop and horticultural plant production [13]. Similar observations have been made in the field of forestry, as polyploid breeding, particularly triploids induced by sexual polyploidization, has great potential in improving vegetative growth and economic traits. In recent years, triploid breeding has achieved great success in genetic improvement in *Populus*. Compared to their diploid counterparts, triploid *Populus* showed marked advantages, such as a faster growth rate, larger leaf organ, higher photosynthetic rate, better timber quality, and higher stress resistance [14–20]. For similar general purposes, triploid breeding plans should immediately become a part of the eucalypt genetic improvement program.

Triploids can be obtained through hybridization using diploid and natural polyploids [14] or by pollinating with natural [21] or artificial 2n pollen [22–24]; however, this method would be inefficient due to the weak development and competition of 2n pollen compared to normal pollen [25]. Recent developments in forestry breeding research demonstrated that female flower buds that underwent a colchicine treatment or were exposed to high temperature to induce megaspore or embryo sac chromosome doubling were a more effectively way to produce triploids, and the yield of triploids reached 16.67%–66.67% [26–33]. This study provides some guidelines for triploid breeding induced by gamete chromosome doubling in other similar tree species.

In *Eucalyptus*, there are no reports that indicate the existence of natural polyploids [34]. While tetraploid plants have been artificially induced in vitro via colchicine treatment [35–37], their development and application are still insufficient due to the difficulty of chimaera separation and the poor performance of the autotetraploid. We previously reported a method to obtain 2n pollen via colchicine treatment during microsporogenesis in *Eucalyptus urophylla* S.T. Blake with an expectation to produce triploids [38], but major problems, such as low yield and the purity of 2n pollen, limited its uses. Fortunately, these attempts uncovered some basic technical requirements, such as potential suitable periods for gamete chromosome doubling, which laid some foundation and gave some hope for further developments in eucalypt triploid breeding.

This study aims to explore a technique to produce triploids through female gamete chromosome doubling in *Eucalyptus*. Cytological observation on microsporogenesis and megasporogenesis were carried out to guide megaspore chromosome doubling to investigate the possibility of triploid induction by colchicine treatment using two different methods of application.

2. Materials and Methods

2.1. Plant Materials

Floral branches used in this study were selected from a *Eucalyptus urophylla* clone (2n = 2x = 22), which contains 10 individual trees. This clone was planted in a clonal test plantation at the Guangxi Dongmen Forest Farm (Guangxi Zhuang Autonomous Region, China), which was built in 2008.

2.2. Determination of the Developmental Process of the Megasporogenesis

Eucalyptus flowers were observed in a cluster in an inflorescence born in the axil of a leaf, and the unit inflorescence was generally a simple umble with 5–7 single flower buds [2]. According to previous studies [39,40], the development of flower buds at different locations on a floral branch was generally asynchronous, and the relationship between microsporogenesis and flower development could be used to estimate the period of meiosis [38]. In this study, flower buds were sampled every 12 h when the flower bud grew to 3.0 mm in diameter in the selected floral branches until fertilization from June to August in 2016. Flower bud samples were fixed in FAA (formalin–acetic acid–alcohol) fixative (70% ethanol/acetic acid/40% formaldehyde, 90:5:5) at 4 °C for 24 h. The anthers containing cells undergoing

meiosis from each flower bud were extracted and stained with 2% acetocarmine for microsporogenesis observation. The ovaries from the same flower bud were embedded with paraffin, then sectioned at 8–10 μm and stained with iron hematoxylin for megasporogenesis observation. After cytological observations, the determination of the developmental process of the megasporogenesis was based on their relationship, which could be used to guide the colchicine treatment.

2.3. Colchicine Treatment

Floral branches, which were selected based on the determination of the developmental process of the megasporogenesis, were treated with 0.25% or 0.50% colchicine solution for 6 h from July to August in 2016. Colchicine treatments were performed in two different ways (Figure 1). In the first way, colchicine solution was slowly aspirated from a centrifuge tube (abbreviated as ACT, Figure 1a) into the appropriate location (a cut near the floral shoot) on a floral branch by capillary action, according to Yang et al. [38]. In the other way, colchicine solution was slowly injected using an infusion apparatus (abbreviated as IIA, Figure 1b) into the cut at the base of the floral branch. In addition, several untreated flower buds served as the control group. Considering the potential toxicity of colchicine, the operator should wear protective gloves and goggles while configuring the reagent or performing treatment using colchicine. When the treatment ends, the centrifuge tube or infusion apparatus with residual colchicine solution should be removed from the floral branches immediately and treated harmlessly after extensive dilution.

Figure 1. Colchicine treatment for *Eucalyptus urophylla* S.T. Blake flower buds during megasporogenesis. (**a**) Colchicine solution was slowly aspirated from a centrifuge tube into a cut near the floral shoot by capillary action (abbreviated as ACT, according to Yang et al. [38]). (**b**) Colchicine solution was injected using an infusion apparatus into a cut at the base of the floral branch (abbreviated as IIA).

Matured seeds both from the treated group and control group were collected and germinated in yellow soil in June, 2017. When the seedlings grew to approximately 5 cm in height, they were transplanted to containers with nutritious soil to promote growth for further ploidy level detection.

2.4. Detection of Ploidy Level in Progeny

When the seedlings grew to approximately 20 cm, both flow cytometry and somatic chromosome counting were used to detect the ploidy level of offspring. Flow cytometric analysis was conducted according to Pinto et al. [41]. In brief, nuclei were released from the cells by chopping approximately 0.5 g of young leaf material with a razor blade in Marie's isolation buffer [42] (50 mM glucose, 15 mM NaCl, 15 mM KCl, 5 mM EDTA Na_2, 50 mM sodium citrate, 0.5% Tween 20, 50 mM HEPES, pH 7.2). Subsequently, the nuclear suspension was filtered through a 50-μm nylon mesh to remove large debris, and then 10 μg mL^{-1} of 4',6-diamidino-2-phenylindole (DAPI) were added to stain the DNA for 5 min. Samples were analyzed by a flow cytometer (The CyFlow® Ploidy Analyzer, Sysmex Partec

GmbH, Görlitz, Germany). A known diploid plant of *E. urophylla* (2n = 2x = 22) was used as an external standard.

The ploidy level of all putative triploid plants was finally confirmed through somatic chromosome counting. Stem tips were removed from the seedlings and pretreated in a saturated solution of paradichlorobenzene for 4 h at room temperature, then washed once and fixed in fresh Carnoy's fixative (ethanol/acetic acid, 3:1) for at least 24 h at 4 °C. After that, samples were hydrolyzed in 38% HCl/ethanol (1:1) for 10 min at room temperature. After washing in distilled water three times for 15min, samples were squashed and stained with Carbol fuchsin, and then observed using a microscope (BX51, Olympus, Tokyo, Japan). Photomicrographs were acquired using the Olympus DP70 Camera System (DP70, Olympus, Tokyo, Japan).

2.5. Measurement of Phenotypic Traits of Triploid and Diploid Eucalypts

Seven diploid and seven triploid plants were used for stomata observation. Five leaves from each plant were selected for stomata observation. Mature leaves from each plant were peeled off using nail polish and then placed on a glass slide for observation, and photomicrography was performed using a microscope. The length and width of 20 stomata per leaf were measured randomly using the ImageJ software (version 1.51, NIH, Bethesda, MD, USA). Ten microscopic field areas per leaf were randomly selected to measure the stomata density. Significant differences were evaluated using *t* test at the 0.05 level of probability.

In the meantime, triploids and their diploid full-sib families were undergoing clonal propagation from the end of 2017. One triploid (genotype: T) and its diploid full-sib family (three genotypes: D1, D2, and D3) with 4 biological replications per genotype, which had been successfully propagated, were used for phenotypic traits measurement. In order to compare the differences between triploid and diploid plants, a total of ten phenotypic traits data were recorded when all the plants had grown up to 6 months old in the greenhouse. Height (H) and ground diameter (GD) were measured using the flexible rule. Mature leaves from the top branch of each plant were used to measure their photosynthetic parameters. The net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular carbon dioxide concentration (Ci), and transpiration rate (Tr) were measured using an LI-6400-02B portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA) at 8 to 10 o'clock on a sunny day in July 2018. All photosynthetic parameters were obtained under a photosynthetic photon flux density of 1400 $\mu mol^{-2} \cdot s^{-1}$, at 60% relative humidity, and with the CO_2 concentration held at 400 $\mu mol \cdot mol^{-1}$ using a CO_2 injecting system [43]. Then, the chlorophyll content index (CCI) and leaf area (LA) were recorded using a CCM-200 Plus chlorophyll content meter (OPTI-sciences Ins., Hudson, NH, USA) and CI-203 handheld laser leaf area meter (Li-Cor Inc., Lincoln, NE, USA) on the same day. The instantaneous water use efficiency (WUE_i) was calculated as Pn divided by Tr, and the photosynthetic efficiency of the whole leaf (PE_w) was calculated as Pn multiplied by LA [19].

2.6. Statistical Analysis

Comparisons of the phenotypic parameters were evaluated based on an ANOVA with Duncan's multiple range tests to determine whether there were any significant differences between triploid and diploid plants. Comparing the mean difference of stomata characteristics between diploid and triploid plants was calculated by paired-samples *t* test. All statistical analyses were performed using SPSS software (version 19.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Flower Bud Development and the Relationship between Male and Female Meiosis

Due to the regular rule of asynchronous meiotic development between different stamens, the meiotic stage of the flower bud was determined based on the primary leading meiotic stage [38]. We defined the first day as the statistical beginning (time maker) when leptotene was first observed

during microsporogenesis in the fastest growing flower bud on a selected floral branch, and then several flower buds from this branch were collected for cytological observations within the next 14 days. The results (Table 1) demonstrated that flower buds on the floral branch developed successively into meiotic phase over time. On the fifth day after the time maker, over 30% of flower buds were at the leptotene–pachytene stage and diplotene–diakinesis stage. Furthermore, on the seventh day after the time maker, 22.86% and 40% of flower buds were at metaphase I–metaphase II stage and meiosis II stage, respectively. On the ninth day after the time maker, the meiotic stages of most flower buds were primary at meiosis II; and on the fourteenth day after the time maker, over 60% of flower buds had developed to the microspore stage. These results indicated that the primary meiotic divisions of microspore mother cells of all the flower buds on selected branches could be estimated based on development days according to the time maker we set.

Table 1. Microsporogenesis stages of flower buds in selected floral branches on different days in *Eucalyptus urophylla*.

Date of Observation	Developmental Stage of Microsporogenesis						No. of Flower Buds Examined
	Stage I [a]	Stage II	Stage III	Stage IV	Stage V	Stage VI	
Day 1	76.67 [b]	23.33					60
Day 5	12.12	15.15	30.30	33.33	9.10		66
Day 7		11.43	14.28	22.86	40.00	11.43	70
Day 9		10.00	20.00	23.33	33.33	13.33	60
Day 14				13.33	23.33	63.33	60

[a] Stage I: Pollen mother cells (PMCs); Stage II: Leptotene to pachytene; Stage III: Diplotene to diakinesis; Stage IV: Metaphase I to telophase I; Stage V: Prophase II to tetrad; Stage VI: Microspore. [b] Percentages of different developmental stages observed.

Subsequently, a total of 296 flower buds on the selected floral branch were observed by paraffin sectioning to explore the relationship between microsporogenesis and megasporogenesis in a specific flower bud. The results (Table 2) showed that when the meiotic stage of the microspore mother cells were at metaphase I–telophase I, the primary meiotic stages of the megaspore mother cells were at leptotene–pachytene (75.51%). When the meiotic stages of the microspore mother cells were at meiosis II, the primary meiotic stages of the megaspore mother cells were at leptotene–pachytene (36.21%) and diplotene–diakinesis (58.62%). Finally, when the microspore mother cells were at tetrad, most of the megaspore mother cells had developed into functional megaspores (69.57%). This outcome suggested that the developmental stages of megaspore mother cells were always ahead of microspore mother cells in a flower bud, which contained a certain correspondence. The meiotic stages of megaspore mother cells could not be observed in time because they were wrapped in ovules, but they could be estimated based on the meiotic stages of microspore mother cells using the relationship between microsporogenesis and megasporogenesis, which could be easily observed using the traditional tableting technique to guide the megaspore chromosome doubling.

3.2. Triploid Production via Megaspore Chromosome Doubling by Colchicine Treatment

From the fifth to tenth day after the time maker was determined, flower buds on the selected branch were treated everyday using colchicine solution. After treatment by two different methods, a total of 1833 progenies were bred in different treatment groups, and a total of 638 progenies was bred in the control group. The ploidy levels in all the progenies were detected by flow cytometry and somatic chromosome counting, and a total of 7 triploids (2n = 3x = 33) were obtained (Figure 2). The results (Table 3) showed that 5 triploids were obtained in the progenies when the flower buds underwent a 0.25% colchicine solution treatment using the ACT treatment method seven days after the time maker was determined, which equated to a triploid yield of 6.25%. Only 1 triploid was obtained when the flower buds underwent a 0.50% colchicine solution treatment using the same treatment method six days after the time maker, and only 1 triploid was obtained when the flower buds underwent a 0.25%

colchicine solution treatment using the IIA treatment method. All the progenies in the control group were diploid (2n = 2x = 22).

Table 2. Relationship between microsporogenesis and megasporogenesis in *Eucalyptus urophylla*.

Developmental Stage of Megasporogenesis (%)	Developmental stage of Microsporogenesis				
	PMC to Diakinesis	Metaphase I to Telophase I	Prophase II to Telophase II	Tetrad	Microspore
MMC	100.00 [a] (70 [b])				
Leptotene to pachytene		75.51 (185)	36.21 (84)		
Diplotene to diakinesis		24.49 (60)	58.62 (136)	23.45 (57)	
Metaphase I to telophase II			5.17 (12)	25.93 (63)	
Tetrad				50.62 (123)	30.43 (42)
Functional megaspore					69.57 (96)
No. of flower buds examined	35	49	58	81	73

[a] Percentages of different developmental stages observed. [b] Numbers of ovaries examined.

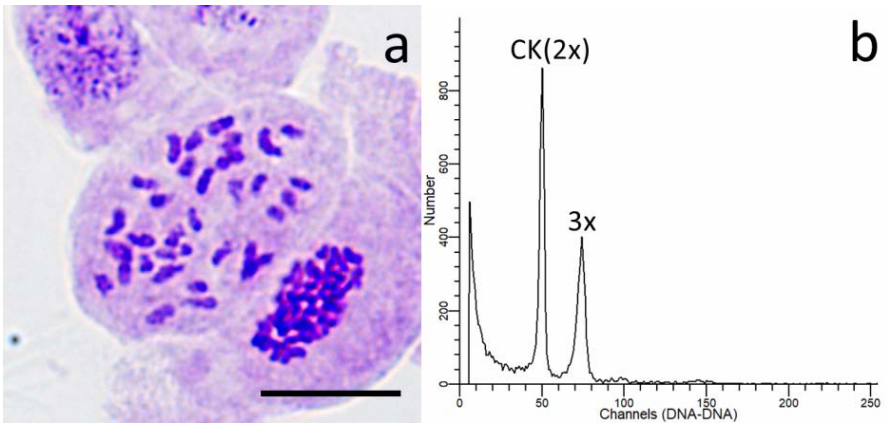

Figure 2. Ploidy level detection of offspring derived from megaspore chromosome doubling by colchicine treatment in *Eucalyptus urophylla*. (**a**) Somatic chromosome counting of triploid plant (2n = 3x = 33, scale bar = 10 μm). (**b**) Histogram presenting the flow cytometric analysis results.

3.3. The Effective Meiotic Stages for Megaspore Chromosome Doubling by Colchicine Treatment

During colchicine treatment, a total of 172 flower buds whose meiotic stage of the megaspore mother cells were the same as the treatment groups were observed to explore the optimal period for induction of megaspore chromosome doubling by colchicine treatment. The results (Table 4) showed that in the treatment groups in which triploids were successfully obtained, although the meiotic stage of the megaspore mother cells of flower buds on the selected branch was inconsistent, they were mostly at the leptotene–pachytene stage and the diplotene–diakinesis stage. For instance, the meiotic stage of the megaspore mother cells of flower buds in the treatment group was primarily at the leptotene–pachytene stage (37.93%) and the diplotene–diakinesis stage (51.72%) on the seventh day after the time maker was determined, while most of the triploids (5 out of 7) were successfully obtained by the treatment of the day. This result indicated that the optimal period for megaspore chromosome doubling by colchicine treatment might be when the megasporocyte progresses from the leptotene to diakinesis stage.

Table 3. Triploid production via megaspore chromosome doubling by colchicine treatment in *Eucalyptus urophylla*.

Date of Treatment	Treatment Type	Treatment Concentration (%)	No. of Seedlings	No. of Triploids	Triploid Production Rate (%)
Day 5	IIA [a]	0.25	177	0	0
		0.50	120	0	0
	ACT [b]	0.25	72	0	0
		0.50	27	0	0
Day 6	IIA	0.25	194	0	0
		0.50	144	0	0
	ACT	0.25	93	0	0
		0.50	31	1	3.23
Day 7	IIA	0.25	139	1	0.72
		0.50	101	0	0
	ACT	0.25	80	5	6.25
		0.50	19	0	0
Day 8	IIA	0.25	117	0	0
		0.50	96	0	0
	ACT	0.25	51	0	0
		0.50	8	0	0
Day 9	IIA	0.25	93	0	0
		0.50	82	0	0
	ACT	0.25	44	0	0
		0.50	3	0	0
Day 10	IIA	0.25	66	0	0
		0.50	42	0	0
	ACT	0.25	27	0	0
		0.50	7	0	0
Treatment			1833	7	0.38
Control			638	0	0
Total			2471	7	

[a] Inject using an infusion apparatus into a cut at the base of the floral branch (abbreviated as IIA). [b] Aspirate from a centrifuge tube into a cut near the floral shoot (abbreviated as ACT).

Table 4. Megasporogenesis stages on different days for colchicine treatments in *Eucalyptus urophylla*.

Date of Treatment	Developmental Stage of Megasporogenesis					No. of Flower Buds Examined
	Leptotene to Pachytene	Diplotene to Diakinesis	Metaphase I to Telophase II	Tetrad	Functional Megaspore	
Day 5	77.42 [a] (120 [b])	22.58 (35)				31
Day 6	54.60 (83)	42.11 (64)	3.29 (5)			30
Day 7	37.93 (44)	51.72 (60)	10.34 (12)			29
Day 8	11.95 (19)	33.33 (53)	41.51 (66)	13.20 (21)		27
Day 9		29.41 (40)	44.12 (60)	26.47 (36)		34
Day 10			15.79 (21)	63.91 (85)	20.30 (27)	21

[a] Percentages of developmental stages observed. [b] Numbers of ovaries examined.

3.4. Phenotypic Traits of Triploid and Diploid Plants

Stomata characteristics of triploid and diploid plants were observed when the height of the plant reached over 30 cm. The results (Table 5) demonstrated that the stomata length and width of the triploid plants (16.200 ± 1.558 and 9.820 ± 1.256 μm, respectively) were significantly higher than those of the diploid plants (12.306 ± 0.740 and 6.567 ± 0.757 μm, respectively), but the diploid stomata density was approximately 1.83 times higher than that of the triploid (Figure 3).

Table 5. Stomatal characteristics of diploid and triploid plants in *Eucalyptus urophylla*.

Ploidy Level	Stomata Length (μm)	Stomata Width (μm)	Stomata Density (no./mm^2)
Diploid	12.306 ± 0.740	6.567 ± 0.757	468.32 ± 40.16
Triploid	16.200 ± 1.558	9.820 ± 1.256	256.20 ± 57.08
Significance [a]	*	*	*

[a] Asterisk represents a significant difference based on a two-sample *t* test ($p < 0.05$).

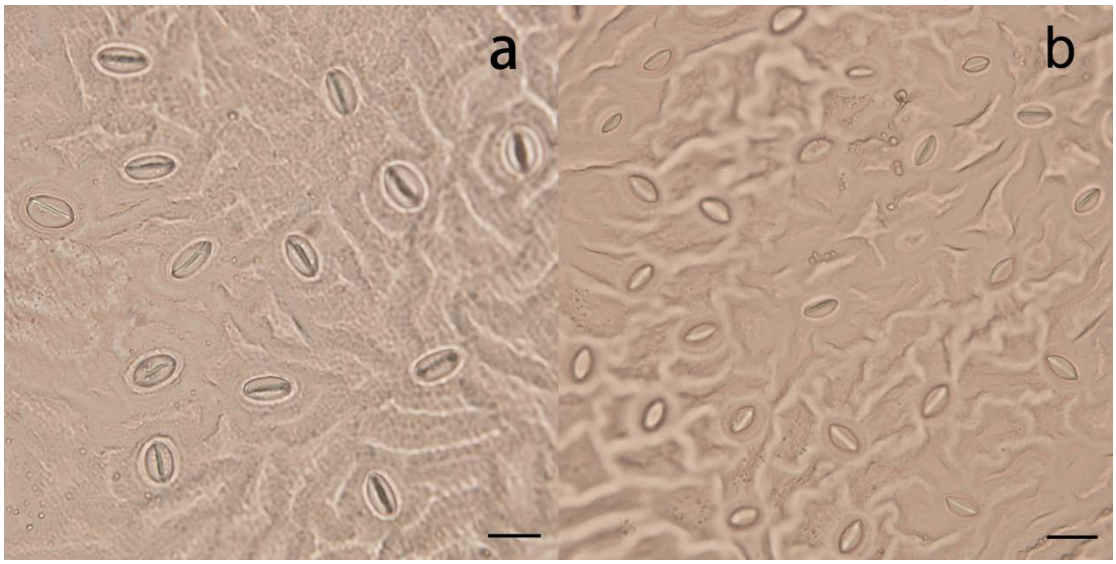

Figure 3. Stomata size and density in *Eucalyptus urophylla* (scale bar = 20 μm). (**a**) Triploid plant. (**b**) Diploid plant.

One of the triploid eucalypts (genotype: T) and its diploid full-sib family (three genotypes: D1, D2, and D3) were used for phenotypic traits measurement at 6 months old. The results (Table 6 and Table S1) demonstrated that triploid eucalypt had a visible advantage in vegetative growth. In terms of plant height (H), ground diameter (GD) and leaf area (LA), triploid eucalypt (117.23 ± 9.34 cm, 14.00 ± 1.40 mm and 42.75 ± 12.64 cm^2, respectively) is significantly higher ($p < 0.01$) than the diploid group (88.62 ± 12.06 cm, 10.86 ± 1.12 mm and 23.09 ± 5.81 cm^2, respectively) (Figures 4 and 5). In addition, the results of photosynthetic parameter determination indicated that triploid eucalypt also has higher photosynthetic efficiency. For example, the net photosynthetic rate (Pn), chlorophyll content index (CCI), and photosynthetic efficiency of whole leaf (PE_w) showed highly significant differences ($p < 0.01$) between the triploid plant (15.46 ± 1.57 μmol·m^{-2}·s^{-1}, 18.06 ± 1.14 and 0.0650 ± 0.0165 μmol·s^{-1}, respectively) and diploid group (9.37 ± 3.05 μmol·m^{-2}·s^{-1}, 11.52 ± 2.81 and 0.0217 ± 0.0097 μmol·s^{-1}, respectively) based on one-way ANOVA F tests, and the triploid always has the maximum value of the data (Table 6 and Table S1).

Table 6. Descriptive statistics (mean values ± SD) of ten quantitative traits measured from the top mature leaf of *Eucalyptus urophylla* at 6 months old.

Genotype	H (cm)	GD (mm)	Pn ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	Gs ($mol \cdot m^{-2} \cdot s^{-1}$)	Ci ($\mu mol \cdot m^{-1}$)
D1	92.88 ± 5.17 b	10.36 ± 0.82 c	10.91 ± 2.32 b	0.203 ± 0.073 a	276.70 ± 13.64 a
D2	99.28 ± 5.54 b	10.20 ± 0.60 c	8.28 ± 3.70 b	0.109 ± 0.080 b	232.07 ± 20.91 b
D3	73.70 ± 4.35 c	12.01 ± 0.89 b	8.92 ± 2.52 b	0.195 ± 0.084 a	274.56 ± 35.47 a
T	117.23 ± 9.34 a	14.00 ± 1.40 a	15.46 ± 1.57 a	0.214 ± 0.061 a	248.60 ± 26.56 ab
Mean	95.77 ± 16.89	11.65 ± 1.82	10.89 ± 3.82	0.180 ± 0.084	257.98 ± 30.97
F	94.760 **	39.793 **	18.090 **	4.953 **	8.564 **
Genotype	**Tr ($mmol \cdot m^{-2} \cdot s^{-1}$)**	**WUE_i**	**CCI**	**LA (cm^2)**	**PE_w ($\mu mol \cdot s^{-1}$)**
D1	4.04 ± 0.95 a	2.73 ± 0.32 b	11.07 ± 1.39 b	22.06 ± 5.62 bc	0.0242 ± 0.0087 b
D2	2.39 ± 1.39 b	3.75 ± 0.80 a	10.52 ± 2.48 b	28.53 ± 3.51 b	0.0241 ± 0.0124 b
D3	4.21 ± 1.64 a	2.32 ± 0.62 b	12.97 ± 3.66 b	18.69 ± 2.97 c	0.0168 ± 0.0058 b
T	4.69 ± 1.66 a	3.71 ± 1.33 a	18.06 ± 1.15 a	42.75 ± 12.64 a	0.0650 ± 0.0165 a
Mean	3.83 ± 1.64	3.13 ± 1.03	13.15 ± 3.79	28.00 ± 11.68	0.0325 ± 0.0222
F	5.806 **	8.452 **	24.854 **	25.568 **	43.013 **

The values of each column followed by the different letters show statistically significant differences at $p < 0.05$; Asterisks represent significant differences among different genotypes as: * $p < 0.05$, ** $p < 0.01$; H: Height; GD: Ground diameter; Pn: Net photosynthetic rate; Gs: Stomatal conductance; Ci: Intercellular carbon dioxide concentration; Tr: Transpiration rate; WUE_i: Instantaneous water use efficiency; CCI: Chlorophyll content index; LA: Leaf area; PE_w: Photosynthetic efficiency of whole leaf.

Figure 4. Eucalypts grown in greenhouse at 6 months old. (**a**) Triploid plant. (**b**) Diploid plant.

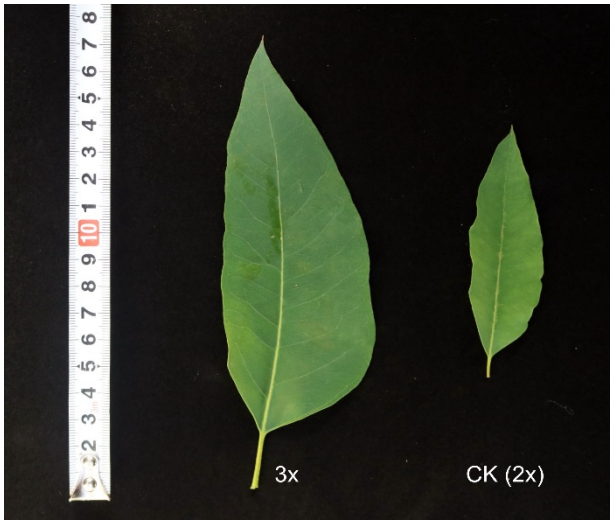

Figure 5. Triploid and diploid mature leaves in *Eucalyptus urophylla*.

4. Discussion

The discovery and utilization of polyploid in forestry breeding were very early. It was 1936 when the discovery of natural triploid poplars exhibiting excellent properties was first reported [44,45]. Afterward, many studies looking at the artificial induction of polyploid began [14,21,46], and many excellent results were obtained in the last 10 years [27–33]. Previous research has shown that polyploid breeding is more successful upon utilization of heterosis [47–49]. To realize the comprehensive utilization of the dosage effect and heterosis in *Eucalyptus*, a more reasonable process could be to obtain triploids by sexual polyploidization. The first study on artificial induction of 2n pollen in *Eucalyptus* was performed by Yang et al. [38]. However, compared with an anemophilous flower, such as *Populus*, eucalypt produced little pollen, which could not be used to produce triploids due to the low yield and purity of the 2n pollen. In this study, we successfully obtained 7 triploid progenies with heterozygosity via megaspore chromosome doubling for the first time. The stomata characteristics of these triploids were consistent with other studies [50–53]. The increased cell size as exemplified by enlarged stomata size in triploid eucalypt may play an important role in increasing the size of the leaves, which was also observed in this study [54]. In addition, the higher photosynthetic efficiency of these triploid eucalypts may be able to explain their significant faster growth in plant height and ground diameter [19]. Considering the outstanding growth advantage exhibited by triploid in this study, our study provides a new perspective and method for germplasm creation in *Eucalyptus*, which may have a significant impact on further varietal improvement and genetic research in this tree species.

In general, the primary means of colchicine treatment includes immersing, injection, coating, etc., and the treatment method employed should be specifically designed according to the characteristics of the plant material. However, the eucalypt flower buds were coated with wax [55,56], which prevented the colchicine from entering the ovary via immersing or coating. The internal partition of the flower buds was compact as well, any damage to the flower bud structures could lead to death, which made it impossible to inject the colchicine solution directly into the flower bud. To alleviate this challenge, we injected the colchicine solution using the IIA method to transport the solution into the ovaries through the transfusion tissue, which differs from the directly injected method in *Populus* [26,29,33]. This method could treat more flower buds on the branches all at once with less mechanical damage, but the effective rate of this treatment method was low in this study because it took more time on transporting. This outcome made measuring the specific time when the colchicine arrived in the ovaries and the final concentration of the treatment inconclusive. Previous research also provided an ACT mothed to induce 2n pollen in *Eucalyptus urophylla* [38]. We also used this method to induce 2n female gametes in this study, and successfully obtained 6 triploid progenies in total. This result

indicated that the ACT method was more direct and instantaneous because the colchicine was rapidly transported into the ovaries with less dissipation and dilution. It should also be noted that after sowing and seeding, the quantity of seedlings that underwent the ACT treatment group was less than that in the IIA group, and the quantity of seedlings that underwent a higher colchicine concentration treatment was also less than the quantity of seedlings that were from the lower group. These results indicated that the toxicity of colchicine on plant materials became high when treatment intensity was increased, consistent with previous studies [29,33,38]. The high yield of triploid induction is not always realized through increased colchicine treatment intensity, but instead could cause shedding of flower buds, and the optimal treatment intensity should be set based on the tolerance of the plant materials in the future studies.

Previous research has shown that the efficiency of 2n gamete induction depended on whether the treatment period was suitable for chromosome doubling. Kang et al. [22,57] determined that the most suitable period for 2n pollen induction in *Populus* via colchicine treatment was at pachytene during microsporogenesis, and the most suitable period for megaspore chromosome doubling was from leptotene to pachytene [26]. Therefore, we needed to determine the accurate meiosis stage immediately. Derived from the relationship between microsporogenesis and megasporogenesis in *E. urophylla*, on the fifth day after the time maker was determined, most of the flower buds' meiotic division of the microsporocytes developed to metaphase I–telophase I, while the meiotic division of the megasporocytes developed to leptotene–pachytene. On the seventh to ninth day after the time maker was determined, most of the flower buds' microsporocytes developed to the prophase II–tetrad phase of meiotic division, while the meiotic division of the megasporocytes developed to diplotene–diakinesis. These results indicated that the fifth day and the following few days would be the optimal treatment period for chromosome doubling using colchicine solution.

Results in this study also indicated that the meiotic stages at leptotene–diakinesis during megasporogenesis in the flower buds might be the optimal meiosis period for megaspore chromosome doubling by colchicine treatment, which was consistent with previous research on 2n pollen induction in *Eucalyptus urophylla* [38]. However, there were some differences with previous research on *Populus*, which had found the optimal periods for megaspore chromosome doubling via colchicine was when the megasporocytes were at leptotene–pachytene [26,33]. The most likely reason for this difference is that the times for megasporogenesis are different across different plant species. As a result, in this study, colchicine treatment proceeded when the megasporocytes were at diplotene–diakinesis and could gain the greatest number of triploids, but the most suitable period for megaspore chromosome doubling is a topic for further research.

Colchicine is widely used for megaspore chromosome doubling. In this study, the triploid yield was lower than in previous research on *Populus* [26,33]. Similarly, previous studies on 2n pollen induction via colchicine treatment demonstrated that the highest yield of 2n pollen in *Eucalyptus* was 28.71% [38], while the yields were 88% [57] and 82.83% [58] in *Populus*. The most likely reason for this discrepancy is that the flower bud growth and megasporogenesis on the whole floral branch in *Eucalyptus* is asynchronous [39,40]. While poplars can be cultivated in a hydroponic greenhouse to maintain relative consistency in the process of flower bud development, it is difficult to control the flower bud growth and megasporogenesis of eucalypt in a wild environment due to the longstanding flower bud developmental phase and fruit-setting period, which exacerbates the asynchronism because of the environmental effect. The relationship between flower bud growth and microsporogenesis among different flower buds on different locations in the floral branch has been studied [39,40], and the asynchronism among different flower buds may result in a lower yield of 2n pollen induction [38]. The unique features of meiosis in microspore mother cells are probably similar to those of megaspore mother cells, which could also be influenced by the asynchronism among different flower buds on different locations in the floral branch. Therefore, it would be difficult to ensure that all flower buds within a treatment group were at the same developmental stages during megasporogenesis, thereby further reducing the triploid yields. A possible solution might be to remove flower buds in

treatment groups that are significantly different in their developmental stage based on morphologic and cytological observations to make sure the rest of the flower buds developed more uniformly, which might increase the efficiency of colchicine treatment for a better yield of triploid induction in future studies.

From the present research that demonstrates high yield of triploid induction in *Populus*, there is still room for improvement in triploid breeding research in *Eucalyptus* through the optimization of techniques, including the timing and method of treatment. In addition, it has been reported that female flower buds from *Populus* that were treated with colchicine solution after pollination by embryo sac chromosome doubling could also produce triploids [29,59]; this outcome presents a possible new way of triploid germplasm creation in *Eucalyptus*. In recent years, high temperature treatment was widely used for triploid breeding with a higher efficiency compared to that of colchicine treatment due to a more direct effect on the 2n gamete production process [27,28,30–32]. Compared with colchicine treatment, the temperature could increase quickly to save the time the colchicine transportation needed, which would enable us to distinguish the meiotic stages for chromosome doubling more accurately to increase the efficiency of triploid production. High temperature treatment could also avoid the toxicity of colchicine to the plant materials, thereby further raising the yield of triploid induction. This outcome suggests that both the megaspore and the embryo sac chromosome doubling induced by high temperature treatment is a potentially new direction for research for eucalypt breeders, as it would be a more efficient way for triploid germplasm creation in this tree species.

5. Conclusions

The results from this study indicate that the meiotic stages of megasporogenesis could be deduced from the meiotic stages of microsporogenesis, an easy observation based on their relationship of guided megaspore chromosome doubling induced by colchicine treatment. Both ACT and IIA are effective methods for triploid induction by colchicine treatment during megasporogenesis. Hybridization with 2n megaspores induced by colchicine treatment is an effective way for *Eucalyptus* triploid breeding, which could accelerate the development of advanced germplasms in this tree species.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/9/11/728/s1, Table S1: Detailed record data of ten quantitative traits measured from the top mature leaf of *Eucalyptus urophylla* S.T. Blake at 6 months old.

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