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Induction of 2n pollen with colchicine during microsporogenesis in *Eucalyptus*

Jun Yang · Pengqiang Yao · Yun Li · Jiyou Mo · Jianzhong Wang · Xiangyang Kang

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Abstract Artificially inducing 2n gametes through chromosome doubling is an effective way to obtain polyploids. In this study, *Eucalyptus urophylla* microsporogenesis and flower development were investigated to guide 2n pollen induction. We also investigated suitable conditions for colchicine treatment. Our results showed that *E. urophylla* 2n pollen was spherical and had a large volume (mean diameter 28.57 ± 0.46 μm), while normal untreated pollen (mean diameter 19.68 ± 0.11 μm) was tetrahedron. The highest rate of 2n pollen production was 28.71 % when the flower buds, which ranged in size from 3.5 to 4.0 mm, underwent treatment with 0.5 % colchicine solution for 6 h. Further studies suggested that diplotene to diakinesis and metaphase I to telophase I were suitable meiotic stages for chromosomes doubling, due to asynchronous development of microsporogenesis between the anthers in a single flower bud. These data help to illuminate research in other areas, such as triploid eucalyptus production by chromosome doubling of female gametes.

Keywords 2n Pollen · Chromosome doubling · Colchicine · *Eucalyptus* · Microsporogenesis

Introduction

Eucalyptus tree species, which belonging to the family Myrtaceae (Ladiges et al. 2003), are one of the most important hardwoods planted worldwide (Doughty 2000). In the latest taxonomic revision, over 700 species, belonging to 13 main evolutionary lineages, were recognized (Brooker 2000). Although some varieties are widely planted around the world because of their rapid growth, straight form, valuable wood properties, wide adaptability to soils and climates, and ease of management through coppicing, these trees are still in the early stages of domestication compared to crop species (Eldridge et al. 1993; Potts and Dungey 2004; Potts and Jackson 1986; Potts and Reid 1988).

Polyploids, particularly triploids induced by sexual polyploidization, are of great value to tree breeders, as they combine the merits of both genome dosage effects and heterosis (Adiwilaga and Brown 1991; Barba-Gonzalez et al. 2006; Becerra and Orjeda 2002;

J. Yang · P. Yao · Y. Li · X. Kang
National Engineering Laboratory for Tree Breeding, Beijing Forestry University, Beijing 100083, People's Republic of China

J. Yang · P. Yao · Y. Li · X. Kang (✉)
Key Laboratory for Genetics and Breeding of Forest Trees and Ornamental Plants, Ministry of Education, Beijing Forestry University, Beijing 100083, People's Republic of China
e-mail: kangxy@bjfu.edu.cn

J. Mo · J. Wang
Guangxi Dongmen Forest Farm, Guangxi Zhuang Autonomous Region 532108, People's Republic of China

Dewitte et al. 2010). Some studies in *Populus* recently suggested that triploid trees had huge advantages in comparison with their diploid counterparts, such as faster growth rate, better timber quality, and higher stress resistance (Einspahr 1984; Weisgerber et al. 1980; Zhu et al. 1998). In general, the generation of 2n gametes through chromosome doubling is an effective way to obtain polyploids (Wang et al. 2010, 2012a, b; Lu et al. 2013, 2014). Since 2n gametes in a genus are rather exceptional, an efficient method of inducing 2n gametes is needed (Dewitte et al. 2009, 2010). While tetraploid *Eucalyptus* was artificially induced in vitro by colchicine treatment (Janaki-Ammal and Khosla 1969; Lin et al. 2010; Han et al. 2011), there are no reports of triploid *Eucalyptus* production induced by sexual polyploidzation (Myburg et al. 2007). Further, the production of 2n gametes by chromosome doubling during meiosis is conducive for genetic analysis and to accelerate the development of advanced germplasm in eucalyptus.

Colchicine has been applied to zygotes, seedlings and tissue as a polyploidizing agent in many plants (Pereira et al. 2014; Takamura and Miyajima 1996; van Tuyl et al. 1992; Arisumi 1964; Kang et al. 2004; Wang et al. 2010, 2012a, b; Li et al. 2008; Kobayashi et al. 2008; Johnsson and Eklundh 1940). Xi et al. (2011) reported 2n pollen induction in Populus × popularis by arresting meiosis via colchicine treatment as well as triploid formation from the offspring of Populus × euramericana. (Dode) Guinier pollinated with induced pollen from Populus × popularis. Kang et al. (1999, 2000) successfully induced over 88 % 2n pollen with colchicine treatment at pachytene in *Populus,* suggested that the efficiency of 2n gamete induction depended on whether the treatment period was suitable for chromosome doubling during microsporogenesis. However, these conditions are poorly understood in Eucalyptus, which could be a potential obstacle to Eucalyptus polyploidizing induction.

In this study, *Eucalyptus urophylla* microsporogenesis and flower development were investigated to guide the induction of 2n pollen. Suitable conditions for colchicine treatment were also investigated as a basis for further research, such as triploid eucalyptus production by chromosome doubling of female gametes.

Materials and methods

Plant materials

Floral branches used in this study were selected from of an *Eucalyptus urophylla* clone ($2n = 2\times = 22$), which contains 10 individual trees. This clone was planted in a seed orchard at the Guangxi Dongmen Forest Farm (Guangxi Zhuang Autonomous Region, P R China), which was built in 1999.

Determination of the microsporogenesis development process

Eucalyptus flowers were observed in a cluster in an inflorescence, which was born in the axil of a leaf. The unit inflorescence is generally a simple umbel with 5–7 single flower buds (Doughty 2000). In *Eucalyptus urophylla*, flower development was asynchronous at different locations on a branch. The flower buds grew on the lower side of the branch showed greater increases in diameter. In addition, there is a relationship between microsporogenesis development and flower bud diameter growth (Yang and Kang 2015; Yang et al. 2016). In this study, when the microsporogenesis in the selected branches was observed for the first time, flower buds were sampled every 12 h before pollination in order to investigate the meiotic development of the pollen mother cell (PMC). All flower buds collected from the branch were fixed in Carnoy's fixative (ethanol:acetic acid, 7:3) for 24 h at 4 °C, and were then preserved in 70 % ethanol at 4 °C for long-term storage until cytological observation. Because flower buds differed in size at the same time on the same branch, flower buds were measured before being fixed for further analysis. Different sizes of flower buds at different stages of development were collected and measured from June to August 2013.

To analyze microsporogenesis, anthers containing cells undergoing meiosis were extracted from flower buds, squashed, and stained in 2 % acetocarmine for approximately 5 min. Slides containing cell spreads were observed under a microscope (BX51; Olympus, Tokyo, Japan). Photomicrographs were acquired using the Olympus DP70 camera system.

Colchicine treatment

After associating the development of microsporogenesis with flower morphological characteristics, a total of 2956 flower buds of suitable size from 8 branches were treated with 0.5 % colchicine solution for 3 or 6 h. In order to analyze their meiosis period, another 528 flower buds which were in the same state from nearby branches were measured and collected in the meantime for further cytological observation. Colchicine treatment was performed according to Kang et al. (2000), colchicine in a centrifuge tube was slowly injected to the appropriate location (an incision near the floral shoot) on a flowering branch. Untreated flower buds served as the control group. To determine the treatment efficiency, treated flower buds were collected around anthesis for further cytological analysis; treatment was considered to be successful when at least 1 % large pollen was present (Dewitte et al. 2010).

Statistical analysis

To assess for differences among and between treatment durations, 2n pollen production efficiency data, including the proportion of successfully treated flower buds which at least 1 % large pollen was present, were analyzed using an analysis of variance (ANOVA). When treatments were significantly different, a least significant difference (LSD) multiple comparison test was used for pairwise comparison. Prior to analysis, percent 2n pollen rate data were transformed by the arcsine of the square root of p/100. Pearson's correlation coefficient was calculated between the rate of 2n pollen production and the percentage of a certain male meiotic stage. All statistical analyses were performed using the SPSS software (version 19.0) or OriginPro software (version 8.5).

Results

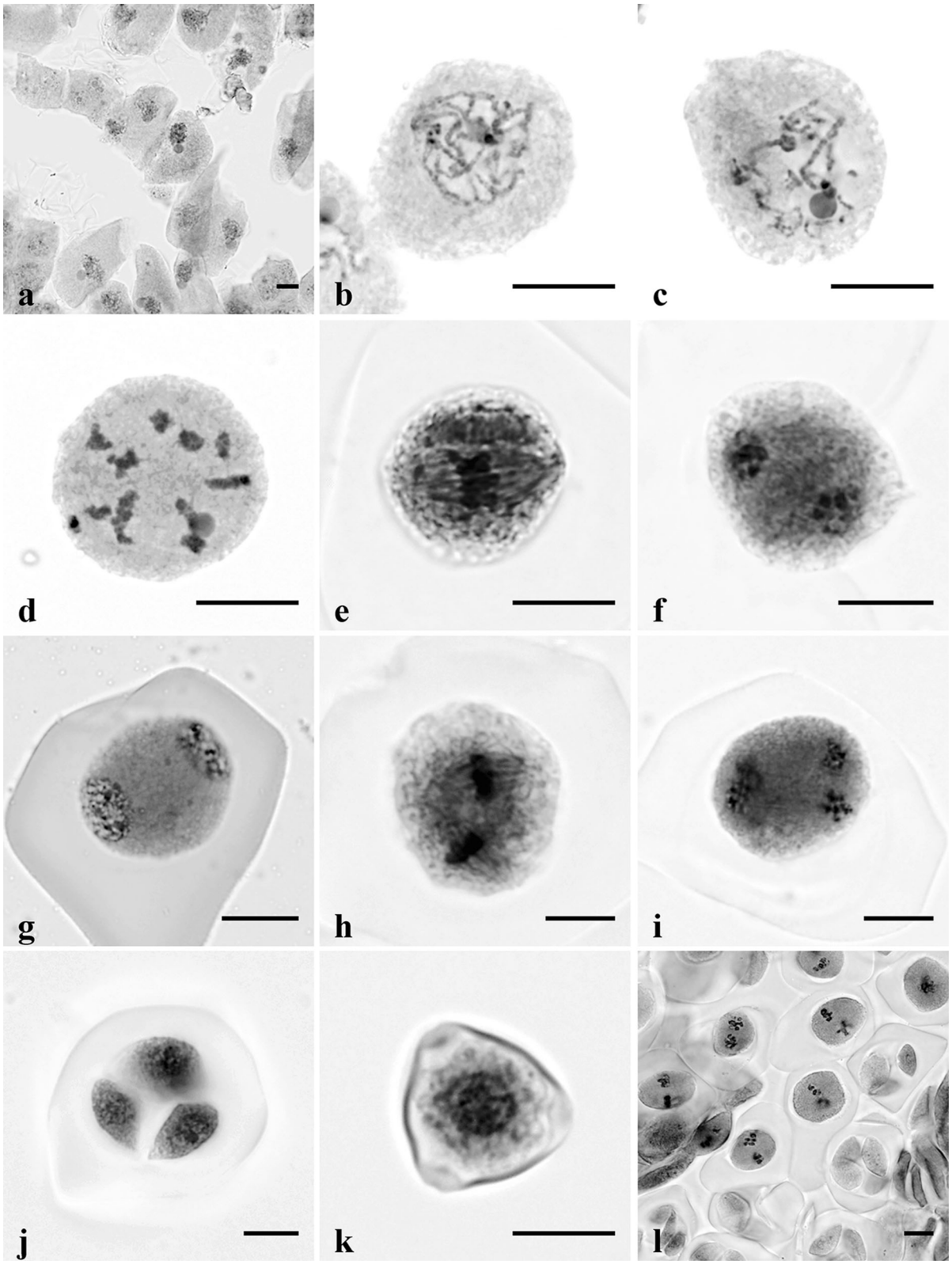
Flower bud size and relationship with microsporogenesis

Most male meiotic stages in *Eucalyptus urophylla* were observed during the cytological analysis (Fig. 1). The most remarkable cytological finding was that multiple microsporogenesis developmental stages were observed in one section of a bud (Fig. 1l). After individual observations of each anther, asynchronous microsporogenesis development was observed between anthers in a single flower bud (Table 1). In general, an *Eucalyptus urophylla* flower bud contains two types of stamens: a longer one (close to the style in a bud) and a shorter one (away from the style in a bud). Microsporogenesis of the longer stamens was always at a later developmental stage than that of the shorter stamens. Based on the regular rule of asynchronous meiotic development between different stamens, the meiotic developmental stage of short stamens was predictable when the data of longer stamens was known. To facilitate discrimination, the meiotic stage was determined based on the primary meiotic stage of the anther on the longer stamens (close to the style in a bud).

We studied the relationship between flower bud size and male meiotic stage to guide colchicine treatments (Table 2). In this study, male meiosis did not occur when the flower bud size was smaller than 3.0 mm. When the flower bud diameters ranged from 3.0 to 3.5 mm, some PMCs began to undergo male meiosis and some microsporocytes were in the PMC to diakinesis stages (Fig. 1d). When the flower bud diameters ranged from 3.5 to 4.0 mm, we observed that most meiotic stages were in the PMC to tetrad stages (Fig. 1j). Although several meiotic stages, including leptotene (Fig. 1a), pachytene (Fig. 1b), and diplotene (Fig. 1c) were observed, microspores (Fig. 1k) were first observed when the flower bud size increased to 4.5 mm. When the flower bud diameters ranged from 4.5 to 5.0 mm, meiotic stages, including metaphase I (Fig. 1e), anaphase I (Fig. 1f), telophase I (Fig. 1g), metaphase II (Fig. 1h), and anaphase II (Fig. 1i), were observed. When flower bud diameters were greater than 5.0 mm, the meiotic development stage was at least in prophase II.

2n pollen induction via colchicine treatment

According to the relationship between flower bud size and male meiotic stage, a total of 2956 flower buds were divided into six groups for colchicine treatment, based on their diameters (Table 3). Cytological observation of treated flower buds showed that 2n pollen produced by colchicine-induced microspore chromosome doubling was successful (Fig. 2). Cytokinesis was simultaneous in the control group; the observation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◀**Fig. 1** Microsporogenesis in *Eucalyptus urophylla*. Scale bar = 10 μm. **a** Late leptotene. **b** Pachytene. **c** Diplotene. **d** Diakinesis. **e** Metaphase I. **f** Anaphase I. **g** Telophase I. **h** Metaphase II. **i** Anaphase II. **j** Tetrad. **k** Microspore. **l** Multiple meiotic phases were observed in one section

of abundant dyads in the treated group indicated the validity of colchicine treatment for chromosome doubling during microsporogenesis (Fig. 2a). In the control group, untreated normal pollen was tetrahedron, and the pollen diameter ranged from 18.56 to 21.16 μm (19.68 ± 0.11 μm). Compared with the control group, giant pollen was spherical with a much greater volume (Fig. 2b); 2n pollen diameter ranged from 27.16 to 33.06 μm (28.57 ± 0.46 μm). The ANOVA revealed a significant difference between normal and 2n pollen ($F = 642.963$, $p = 0.010$).

Table 3 shows the effect of colchicine treatment on 2n pollen formation in different *Eucalyptus urophylla* flower bud sizes. Although only a few flower buds did not survive during the treatment because of the chemical or mechanical damage, our results showed that the highest rate of successful treatment was 28.71 % when the group that ranged in size from 3.5 to 4.0 mm underwent treatment with a 0.5 % colchicine solution for 6 h. Treatments were not valid in groups that were less than 3.0 mm or greater than 5.0 mm in size. According to the 2n pollen production efficiency data, the ANOVA revealed significant differences among groups of different flower bud sizes ($F = 8.684$, $p = 0.010$). The effects of treatment duration did not significantly differ ($F = 0.645$, $p = 0.441$). Further, the LSD multiple comparison tests showed that 2n pollen production efficiency was not significantly different between the groups that ranged from 3.5 to 4.0 and 4.0 to 4.5 mm; however, the 2n pollen production efficiency of these two groups was significantly better than that in groups with diameters ranging from 3.0 to 3.5 mm ($\alpha = 0.05$). Therefore, our data suggest that flower bud diameters between 3.5 to 4.5 mm were suitable for microspore chromosome doubling in this species of eucalypt.

Stages in which colchicine treatment effectively induced pollen chromosome doubling

To determine the meiotic stages in which treatment was effective at microspore chromosome doubling, six groups comprised of 528 total flower buds of different sizes were analyzed by cytological observation (Table 4). Although each group of different-sized flower buds had asynchronous meiotic development, some meiotic stages were predominant. For example, in the group of flower buds that ranged in size from 3.5 to 4.0 mm, 29.08 and 31.21 % were in the leptotene to pachytene and diplotene to diakinesis meiotic stages, respectively, which was higher than other stages observed in the same group.

We used Pearson's correlation analyses to determine the correlation between the 2n pollen rate and male meiotic stage percentage following treatment with 0.5 % colchicine solution for 6 h (i.e., one of the most suitable treatment conditions for microspore chromosome doubling). On the vertical coordinates in each graph (Fig. 3), meiotic stages proportion of treated flower buds in each size group were determined based on the relationship between microsporogenesis development and flower bud diameter growth (Table 4). The percentage of both meiotic stages (i.e., diplotene to diakinesis and metaphase I to telophase I) was significantly positively correlated with 2n pollen production in different flower bud size groups ($r = 0.8955$, $p = 0.0558$ and $r = 0.8424$, $p = 0.00353$, Fig. 3). However, the percentage value of the group diplotene to diakinesis cannot match the 2n pollen efficiency, neither did the group metaphase I to telophase I. Then, the sum of the percentage of these two groups was also significant positively correlated with 2n pollen production ($r = 0.9704$, $p = 0.0052$), suggesting that both of these two meiotic stage groups may be suitable treatment periods for chromosome doubling. In addition, the rate of flower buds successfully treated was gradually decreased with increasing the ratio of microspore at the end of the microsporogenesis ($r = -0.3889$, $p = 0.4460$).

Discussion

Previous research showed that the pollen ploidy level was related to their size (Adiwilaga and Brown 1991; Akutsu et al. 2007; Crespel et al. 2006; Dewitte et al. 2009, 2010; Okazaki et al. 2005): the diameter of 2n pollen was about 28–50 % larger than normal pollen depending on the species (Dewitte et al. 2009, 2010; Akutsu et al. 2007; Mao et al. 2013). Our results demonstrated that colchicine treatment could be an effective way to increase pollen size in *Eucalyptus*

Table 1 Asynchronous meiosis among *Eucalyptus urophylla* stamens

		Frequency of different developmental stage of microsporogenesis (%)						No. of anthers examined
		PMC	Leptotene to pachytene	Diplotene to diakinesis	Metaphase I to telophase I	Prophase II to tetrad	Microspore	
Umbel 1	Short stamens	10.33	69.07	20.62				97
	Long stamens				15.04	76.11	8.85	113
Umbel 2	Short stamens		27.17	68.48	4.35			92
	Long stamens					42.16	57.84	102
Umbel 3	Short stamens				14.67	85.33		75
	Long stamens						100.00	60

PMC pollen mother cell

Table 2 Relationship between flower bud sizes and microsporogenesis developmental stages in *Eucalyptus urophylla*

Diameter (mm)	Representative male meiotic stages
$d < 3.0$	PMC
$3.0 \leq d < 3.5$	PMC to diakinesis
$3.5 \leq d < 4.0$	PMC to tetrad
$4.0 \leq d < 4.5$	Leptotene to microspore
$4.5 \leq d < 5.0$	Metaphase I to microspore
$d \geq 5.0$	Prophase II to microspore

PMC pollen mother cell

urophylla (Fig. 2); the mean diameter of treated pollen increased by 40 % compared with the control group. The pollen morphology was also different between the treated and control groups, which indicated that the increased DNA amount affected the shape of the pollen wall (Blackmore et al. 2007). These changes could be a simple way to distinguish giant pollen from normal pollen. Furthermore, cytokinesis during microsporogenesis in Eucalyptus was simultaneous (Davis 1968, 1969). Observation of dyads in our study indicated the validity of colchicine treatment for inducing chromosome doubling, which could be another way to identify the occurrence of 2n pollen.

It was known that flower bud size could be correlated with the pollen development stage during microsporogenesis in many plants (Wang et al. 2010, 2012a, b; Lu et al. 2013, 2014; Kang et al. 1999, 2000; Mao et al. 2013; Gao et al. 2004). Based on our study in *Eucalyptus urophylla*, pollen developmental stages among different flower buds were not synchronous. However six flower bud size groups, which were categorized by diameter, could be correlated to developmental stages ranging over several adjacent meiotic stages. According to previous research, the efficiency of 2n gametes induction depended on whether the treatment period was suitable for chromosome doubling during microsporogenesis (Kang et al. 1999, 2000). Gao et al. (2004) directly injected colchicine solution into flower buds at pachytene, and successfully induced 2n pollen in *Eucommia ulmoides;* the highest proportion was 49.5 %. Similar to *Populus*, over 80 % of 2n pollen was induced in the late laptotene to pachytene following colchicine treatment (Kang et al. 1999, 2000; Li et al. 2014). In this study, the highest rate of 2n pollen production was 28.71 %, when the diameter of flower buds ranged from 3.5 to 4.0 mm. Further research showed that the main meiotic stages were comprised of leptotene to diakinesis in this treatment group. In addition, it was notable that 13.13 % of 2n pollen was induced when the diameter of flower buds ranged from 4.5 to 5.0 mm, while the main meiotic stages were comprised of prophase II to microspore in this treatment group. This difference could be due to the asynchronous development of microsporogenesis between anthers in each eucalyptus flower bud. Davis (1968, 1969) reported the existence of two different length stamens in eucalyptus flower buds: the first-formed stamens (which were longer and closer to the style in a bud), and the shorter stamen away from the style. According to our study, the meiotic stages in the longer stamens were more advanced than that of the shorter stamens. To facilitate discrimination, the meiotic stage was determined based on the primary meiotic stage of the anther on the longer stamens (close to the style in a bud). In a single flower bud, some of the stamens were still in the early meiotic stages, which may be suitable for chromosome

Table 3 Effect of colchicine treatment on 2n pollen formation in different *Eucalyptus urophylla* flower bud sizes

Diameter (mm)	Treatment duration (h)	No. of flower buds treated	No. of flower buds survived	No. of flower buds successfully treated	Rate of flower buds successfully treated (%)
$d < 3.0$	3	17	5	0	0.00
	6	36	9	0	0.00
$3.0 \leq d < 3.5$	3	153	75	1	1.33
	6	212	116	5	4.31
$3.5 \leq d < 4.0$	3	295	236	37	15.68
	6	577	404	116	28.71
$4.0 \leq d < 4.5$	3	304	262	35	13.36
	6	685	521	95	18.23
$4.5 \leq d < 5.0$	3	134	105	8	7.62
	6	341	297	39	13.13
$d \geq 5.0$	3	70	55	0	0.00
	6	132	103	0	0.00

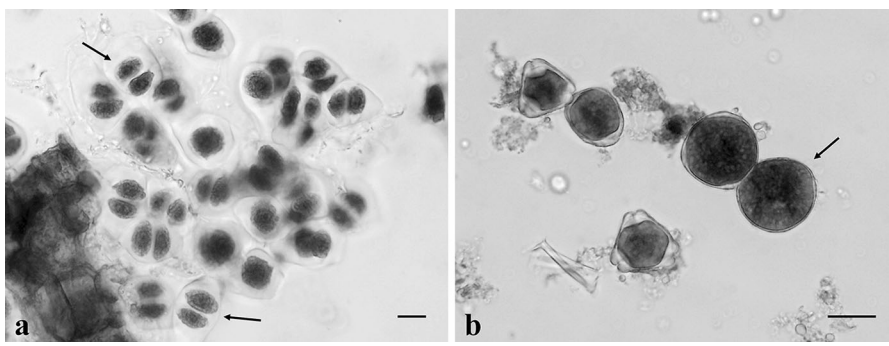

Fig. 2 Effect of colchicine treatment on pollen mother cells (PMCs) in *Eucalyptus urophylla*. Scale bar = 10 μm. **a** Observation of dyad (*arrows*) indicated the validity of colchicine treatment for chromosome doubling during microsporogenesis. **b** Compared with normal tetrahedral pollen, 2n pollen (*arrow*) was spherical and had a much larger volume

Table 4 Detailed presentation of micorporogenesis in different flower bud sizes for treatments

Diameter (mm)	Frequency of different developmental stages of microsporogenesis (%)						No. of flower buds examined
	PMC	Leptotene to pachytene	Diplotene to diakinesis	Metaphase I to telophase I	Prophase II to tetrad	Microspore	
$d < 3.0$	100.00						38
$3.0 \leq d < 3.5$	76.47	18.63	4.9				102
$3.5 \leq d < 4.0$	11.35	29.08	31.21	22.70	5.66		141
$4.0 \leq d < 4.5$		8.20	21.31	33.61	28.69	8.19	122
$4.5 \leq d < 5.0$				20.25	36.71	43.04	79
$d \geq 5.0$					4.35	95.65	46

PMC pollen mother cell

doubling, while later meiotic stages (e.g., tetrad) were observed in the bigger-size group of treated flower buds. This phenomenon may make it difficult for breeders to judge the optimal time for treatment, but also make the potential treatment period longer than expected. Therefore, determining the optimal

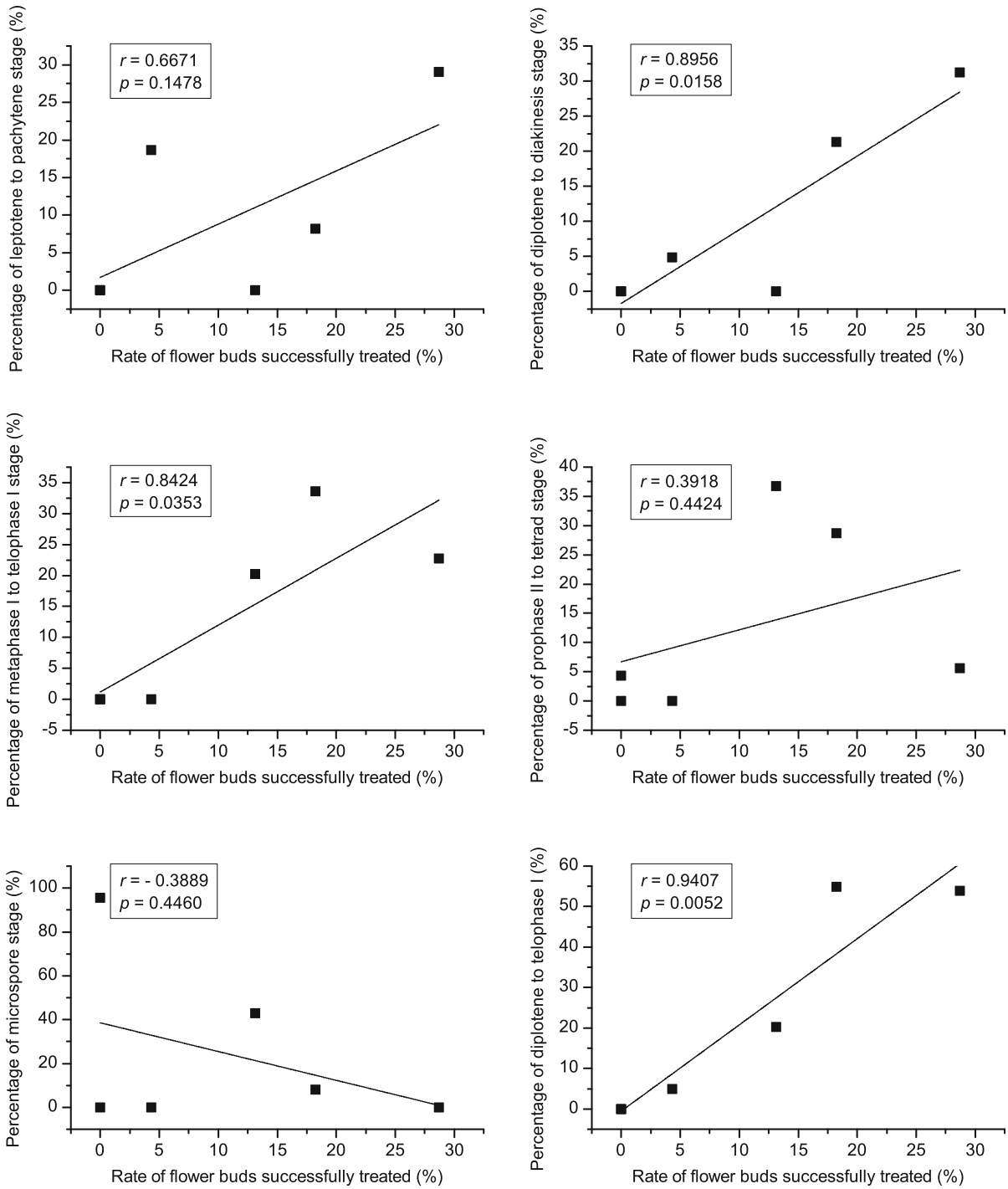

Fig. 3 Pearson's correlation analyses of the 2n pollen production rate and male meiotic stages following treatment with 0.5 % colchicines solution for 6 h. Each point plotted by 2n pollen production rates and the proportion of meiotic stages. On the vertical coordinates in each graph, meiotic stage proportion of treated flower buds were determined based on the relationship between microsporogenesis development and flower bud diameter growth

treatment time should mainly based on flower bud size in order to increase the effective rate of treatment (Yang and Kang 2015; Yang et al. 2016). Further, a treatment duration of 6 h was better than 3 h to induce 2n pollen production. The most likely reason was that the colchicine solution was slowly injected into the appropriate location on flowering branch base, and it may take some time to be delivered to the anthers. Compared to previous work by other breeders (Kang et al. 1999, 2000; Li et al. 2014; Mao et al. 2013; Gao et al. 2004, Dover 1972; Wang et al. 2010; Xi et al. 2014), it could effectively reduce mechanical damage to the flower buds and improve the survival rates of treated flower buds.

2n gametes are an important source for innovation in plant breeding (Dewitte et al. 2010). Benefited from the successful induction of 2n pollen, triploids were obtained from offspring crossed with a diploid mother plant (Adiwilaga and Brown 1991; Dewitte et al. 2010; Okazaki et al. 2005; Hahn et al. 1990). In recently years, triploid breeding via 2n egg induction has become one of the most powerful approaches for the improving in *Populus* (Wang et al. 2010, 2012a, b; Lu et al. 2013, 2014; Li et al. 2008). Compared with microspore chromosome doubling, megasporogenesis and megagametogenesis chromosome doubling had some advantages in yield efficiency to produce triploids in *Populus*, because these could directly generate triploids after control or open pollination without distinguishing or removing normal pollen from 2n pollen. Our study on 2n pollen induction, particularly the determination of suitable treatment conditions for chromosome doubling, could provide illumination for further polyploidy breeding in *Eucalyptus*.

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