Introduction

Microsporogenesis is an important process in plant reproduction, which includes several series of developmental stages from sporogenous cells to microspores. Any unpredictable disturbance during the meiotic process reduces gamete viability. Recent efforts have been made to describe microsporogenesis and pollen formation from cytological and genetic perspectives in model plant species (Blackmore et al. 2007, Chen et al. 2005, McCormick 2004, Scott et al. 2004).

The relationship between microsporogenesis and flower development has been examined in some trees (Lu et al. 2013, Wang, C. et al. 2010). Generally, microsporogenesis and pollen formation are precisely timed and choreographed, and meiosis occurs in a precise chronological order that correlates with the flower bud size (Koltunow et al. 1990, Scott et al. 1991). Mao et al. (2013) reported a significant correlation between the male meiotic stage and mean diameter of male buds in *Eucommia ulmoides*. Xu et al. (2013) compared microsporogenesis and anther development in some species from Magnoliaceae and found that the middle layer cells from anthers started to degenerate gradually during the microspore interphase stage or meiosis stage.

Eucalyptus tree species, commonly referred to as eucalypts, are one of the most important planted hardwoods in the world (Doughty 2000). They are generally long-lived evergreen species belonging to the family Myrtaceae (Ladiges et al. 2003). Eucalypts are still in the early stages of domestication compared to crop species, but are becoming the most advanced genetic forest tree material because of their rapid growth, straight form, valuable wood properties, wide adaptability to soils and climates, and ease of management through coppicing (Eldridge et al. 1993, Potts 2004). Over 700 species belonging to 13 main evolutionary lineages are recognized in the latest taxonomic revision (Brooker 2000). Interspecific hybridization often occurs in natural populations, and some of their offspring become the most important planting varieties (Potts and Jackson 1986, Potts and Reid 1988). Davis (1968) reported that the process of microsporogenesis in *Eucalyptus melliodora* A. Cunn. was normal, archesporial cells developed into sporogenous cells, the ensuing meiotic stages were regular, and cytokinesis was simultaneous. However, our understandings of hybrid species such as *Eucalyptus urophylla × E. tereticornis*, especially the relationship between microsporogenesis and flower development, still remains incomplete.

In addition, in other trees such as *Populus*, triploid plants showed marked 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 recent years, the generation of 2n gametes through chromosome doubling has been shown to be an effective method to obtain polyploids (Lu et al. 2013, Wang, J. et al. 2010, 2012). While tetraploid Eucalyptus has been artificially induced (Han et al. 2011, Janaki-Ammal and Khosla 1969, Lin et al. 2010), there are no reports of polyploid Eucalyptus in nature (Myburg et al. 2007), and 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. Furthermore, previous research on Populus (Kang et al. 1999, 2000) showed that the efficiency of induction of 2n gametes depended on whether the treatment period during microsporogenesis for chromosome doubling was suitable. In other trees such as Populus, over 88% 2n pollen were induced with colchicine treatment at pachytene by Kang (1999, 2000). However, these processes are poorly understood in Eucalyptus. Thus, it is important to increase our understanding of eucalyptus.

In this study, microsporogenesis and flower development in E. urophylla × E. tereticornis was investigated using chromosome tableting to distinguish between the developmental stages of microsporogenesis for further breeding research such as induction of 2n pollen by chromosome doubling.

Materials and Methods

Plant material

Eucalyptus urophylla × E. tereticornis used in this study was planted in a seed orchard at the Guangxi Dongmen Forest Farm built in 1999.

Flower collection and data recording

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. Different sizes of flower buds at different stages of development were measured or collected from July to August 2012.

In the selected flower branch, each umbel was numbered with an increasing Arabic serial number from the bottom to the top according to their locations on the branch (Fig. 1). These small branches constituted a larger branch and were also numbered with lowercase letters from bottom to top according to their different locations. These data (including the location of serial numbers) were recorded for further analysis. In the meantime, flower bud samples were collected every 2 days for subsequent cytological observation.

Cytological observation

All flower buds collected from the branch were fixed in Carnoy’s fixative (ethanol : acetic acid, 7 : 3) at 4°C for 24 h and were then preserved in 70% ethanol at 4°C for long-term storage before observation. To analyze microsporogenesis, anthers containing cells during meiotic courses were extracted from flower buds, squashed, and stained in 2% aceto carmine for about 5 min. The slides containing cell spreads were obtained using a microscope (BX51; Olympus, Tokyo, Japan). Microphotographs were taken using the Olympus DP70 camera system.

Results

Microsporogenesis

At the beginning of meiosis, pollen mother cells (PMCs) with thick cytoplasm huddled together in the anther and were difficult to separate. In the early leptotene stage (Fig. 2A), filiform chromosomes were irregularly distributed around the nucleolus where the cytoplasm was still thick. Chromosomes gradually gathered at one side of the nucleolus during the late leptotene stage (Fig. 2B), which presented as a truss. At this stage, thick and translucent callose surrounded the PMC, which separated the cells and allowed for better observation. The chromosomes continued to coarsen during the pachytene stage (Fig. 2C) and chromosomes remained entangled. During the diplotene stage (Fig. 2D), homologous chromosomes began to repel each other, although chromosome chiasmate was clearly observed. During the diakinesis stages (Fig. 2E), chromosomes were highly coagulated and 11 homologous bivalents separated equally in the cell, which facilitated chromosome counting. During metaphase I (Fig. 2F), chromosomes were neatly established along the equatorial plate. Subsequently, during anaphase I (Fig. 2G), chromosomes of homologous pairs were pulled to two ends of the cell. During telophase I (Fig. 2H), uncoiling chromosomes and some small nucleolus were observed. Prophase II (Fig. 2I) was making an appearance at

Fig. 1. Location map of umbels on the branches. Each umbel was numbered with an increasing Arabic serial number from the bottom to the top according to their locations on the branch. These small branches constituted a larger branch and were also numbered with lowercase letters from bottom to top according to their different locations.
Flower development

When the microsporogenesis was observed for the first time (Day 1), the mean diameter and the location serial number of each umbel from a large selected flower branch was recorded over 21 days, and data from days 1, 5, 7, 9, 14, and 21 were used to analyze flower growth and development. The results showed that umbels according to their locations were at different development stages of growth at the same time (Fig. 3). The majority of umbels (with Arabic serial numbers) on the lower locations of a small branch grew larger than the upper locations. The maximum interpolation of mean diameter in the same small branch was greater than 1.00 mm (umbels b1 and b4 on day 1). However, the umbel sizes between small branches (with lowercase serial letters) from a selected large branch differed. In addition, they did not show the same role as the umbels growing on the same small branch. The umbels from branch c grew bigger than those on branch a, but grew lower than those on branch a (on day 1, 5, 7, 9, 14, and 21).

All umbels from the selected large branch were grown daily (Fig. 4). Over 21 days of observation, the average increased diameter of all umbels was 0.84 mm. The mean diameters of 19.51% of umbels increased by more than 1.00 mm. However, the flowers growth rates differed every day. The results showed that the umbels grew rapidly from day 1 to 9 and slowly on the remaining days.

Relationship between microsporogenesis and flower development

The relationship between the size of flower buds and the stages of microsporogenesis during each day was studied (Table 2). In total, 1,201 flower buds on a selected large branch were cytologically examined. On each day, flower bud samples were divided into several groups based on their diameters. As already stated in a previous report regarding asynchronous meiosis between stamens in a flower bud (Table 1), 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).

On day 1, approximately 63.00% of flower buds had diameters between 3.0 and 3.5 mm. This rate consistently decreased to 4.85% on day 21 due to growth of the flower bud. From day 7 to 14, the majority of flower buds showed diameters between 3.5 and 4.0 mm (73% on day 7, 75.62% on

Table 1. Asynchronous meiosis between stamens in *Eucalyptus urophylla × E. tereticornis*

| Umbel 1 | Short Stamens | Long Stamens | PMCs | Leptotene to Pachytene | Diplotene to Diakinesis | Metaphase I to Telophase I | Prophase II to Tetrad | Microspore | Total number of anthers examined |
|---------|---------------|--------------|------|------------------------|------------------------|--------------------------|-------------------------|------------|--------------------------------|
|         | 8.96*        | 65.17       | 25.87 |                        |                        | 5.62                     | 81.46                   | 12.92      | 64                             |
| Umbel 2 | Short Stamens | 34.33       | 62.19 |                        |                        | 3.48                     | 48.06                   | 51.94      | 75                             |
| Umbel 3 | Short Stamens | 19.47       | 80.53 |                        |                        |                          |                         | 100        | 60                             |

* The percentage of meiotic stages in each type of stamen.
day 9, and 70.00% on day 14). At the end of the experimental period, half of flower buds had diameters over 4.0 mm.

Cytological observation showed that the meiotic stages differed between groups each day. The leptotene stage was first observed on day 1, and other meiotic divisions were observed 5 days later. The majority of meiotic stages (from leptotene to tetrad) were observed from day 5 to 9, suggesting that microsporogenesis in a branch could last 5 days. The leptotene and pachytene were not observed after day 14. The results also showed that the majority of microsporogenesis occurred when the flower bud diameters ranged from 3.0 to 4.0 mm. Diplotene to telophase I stages were more commonly observed in groups with diameters ranging 3.0–4.0 mm from day 5 to 9.

**Discussion**

Natural and artificial hybridization between different subgenera does not occur (Pryor and Johnson 1971), although hybridization within subgenera is relatively common (Griffin et al. 1988). In this study, both the parents of *E. urophylla × E. tereticornis* belong to the subgenus *Symphymyrtus*, which means they could crossbreed freely. Similarly, as shown previously in *E. Melliodora* (Davis 1968), the process of meiosis in *E. urophylla × E. tereticornis* was quite normal, and 11 homologous chromosomes paired up around the metaphase plate at metaphase I. In addition, no lagging chromosome was observed. At the end of telophase II, callose began to dissociate and cytokinesis occurred without any abnormalities, indicating that the parents of *E. urophylla × E. tereticornis* were genetically closely related. The fertility of the hybrid was not affected by heterozygosity; therefore, *E. urophylla × E. tereticornis* may be the appropriate parent for further breeding studies.

Davis (1968, 1969) reported two different length stamens in the eucalyptus flower buds; the first-formed stamens (which were longer and closer to the style in a bud) and the shorter stamens away from the style. These results are consistent with our findings. After anthesis, the longer stamens formed the outermost staminal whorl. Based on massive cells from one flower bud examined under the microscope, strongly asynchronous processes of meiosis were observed. In a single flower, the meiotic stages of PMCs in longer stamens were always ahead of the shorter stamens. For example, some PMCs originated from shorter stamens in the leptotene stage, while others developed into the microspore stage (Table 1). These phenomena persisted until all of the cells developed to mononuclear pollen before anthesis. This form of asynchronism is not rare in other trees and could be a mechanism of environmental adaptation (Wyatt 1982).

In this study, the size of umbels differed according to their location on the branch, which is common in other tree species. The majority of eucalyptus are perennial
Table 2. Microsporogenesis stages in *Eucalyptus urophylla* × *E. tereticornis* in different-sized groups of diameters on different days

| Diameter (mm) | Stage of Microsporogenesis | Number of flowers examined |
|---------------|-----------------------------|---------------------------|
|               | PMCs | Leptotene to Pachytene | Diploptene to Diakinesis | Metaphase I to Telophase I | Prophase II to Tetrad | Microspore |
| Day 1         |      |                      |                          |                            |                         |             |
| d < 3.0       | 100.00* | 67.46 | 32.54 |                          |                            |                          | 54          |
| 3.0 ≤ d < 3.5 | 40.00 | 60.00 | 40.00 | 30.00 | 10.00 | 10.00 | 126         |
| 3.5 ≤ d < 4.0 | 75.00 | 25.00 | 75.00 | 25.00 | 25.00 | 25.00 | 20          |
| Day 5         |      |                      |                          |                            |                         |             |
| d < 3.0       | 4.88  | 5.57  | 4.88  | 5.57  | 4.88  | 5.57  | 4           |
| 3.0 ≤ d < 3.5 | 38.60 | 38.60 | 38.60 | 38.60 | 38.60 | 38.60 | 114         |
| 3.5 ≤ d < 4.0 | 34.15 | 34.15 | 34.15 | 34.15 | 34.15 | 34.15 | 82          |
| Day 7         |      |                      |                          |                            |                         |             |
| 3.0 ≤ d < 3.5 | 24.07 | 24.07 | 24.07 | 24.07 | 24.07 | 24.07 | 146         |
| 3.5 ≤ d < 4.0 | 7.53  | 7.53  | 7.53  | 7.53  | 7.53  | 7.53  | 146         |
| Day 9         |      |                      |                          |                            |                         |             |
| 3.0 ≤ d < 3.5 | 14.82 | 14.82 | 14.82 | 14.82 | 14.82 | 14.82 | 54          |
| 3.5 ≤ d < 4.0 | 34.93 | 34.93 | 34.93 | 34.93 | 34.93 | 34.93 | 146         |
| Day 14        |      |                      |                          |                            |                         |             |
| 3.0 ≤ d < 3.5 | 20.00 | 20.00 | 20.00 | 20.00 | 20.00 | 20.00 | 20          |
| 3.5 ≤ d < 4.0 | 11.54 | 11.54 | 11.54 | 11.54 | 11.54 | 11.54 | 146         |
| 4.0 ≤ d < 4.5 | 61.11 | 61.11 | 61.11 | 61.11 | 61.11 | 61.11 | 90          |
| Day 21        |      |                      |                          |                            |                         |             |
| 3.0 ≤ d < 3.5 | 37.00 | 37.00 | 37.00 | 37.00 | 37.00 | 37.00 | 100         |
| 3.5 ≤ d < 4.0 | 63.00 | 63.00 | 63.00 | 63.00 | 63.00 | 63.00 | 100         |
| 4.0 ≤ d < 4.5 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 6           |

*The percentage of meiosis stage in each group divided by diameters.*
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megaphanerophytes, and predicting flower growth development of the whole tree is difficult. However, assessing flower growth development in a selected branch is possible. At the same time, the umbels contained several single flowers growing on the lower location of a small branch and were larger than the upper umbel. However, this rule does not apply when comparing different small branches. Several studies comparing microsporogenesis development and the diameters of flower buds in other plants have been reported (Custódio *et al.* 2005, Wang, *C.* *et al.* 2010). In this study, the sizes of flower buds are known to increase over time, and microsporogenesis in *E. urophylla × E. tereticornis* tended to occur when the diameters of flower buds were between 3.0 and 4.0 mm. The specific meiotic stages in the similar-sized flower buds differed at different times. At later development days, later meiotic stages appeared more often.

Studies on other species of trees demonstrated that the suitable period of high-temperature treatment to induce 2n gametes was between diakinesis and metaphase I (Kang *et al.* 2000, Lu *et al.* 2013, Mao *et al.* 2013). In *E. urophylla × E. tereticornis*, the percentage of each meiotic stage in a branch containing multiple sizes of flowers buds were different on different days (**Fig. 5**). The leptotene stage was first observed in the smallest flower bud as a starting point, and meiosis in this whole branch likely occurred over the next 5–9 days. Diplotene to diakinesis stages in the whole branch accounted for 36.00%, 26.00%, and 25.91% of developmental stages on day 5, 7, and 9, respectively. That may be the most suitable period for treatment. Due to asynchronous meiosis between stamens in a flower, this period may be longer than expected.

In summary, this study has shown that microsporogenesis in *E. urophylla × E. tereticornis* is normal but asynchronous. Meiotic development differed between different lengths of stamens. Flower bud growth development was also asynchronous in the whole branch. The relationship between microsporogenesis and flower development could be used to predict and characterize microsporogenesis in a whole flower branch, which provides a convenient method for further studies in this tree species.

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**Fig. 5.** The ratio of each meiosis period on different days.
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