Distribution of Microtubular Cytoskeletons and Organelle Nucleoids During Microsporogenesis in a 2*n* Pollen Producer of Hybrid *Populus*

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Abstract

The distribution of microtubular cytoskeletons and organelle nucleoids during microsporogenesis in *Populus simonii* Carr. x *P. nigra* L. 'Tongliao' was studied by indirect immunofluorescence and 4',6-diamidino-2-phenylindole (DAPI) staining. Our purpose was to reveal the relationship between changes in microtubule and chromosome behavior and cytokinesis and to explain the mechanism of 2*n* pollen production. We observed stable frequencies of 2*n* pollen production, ranging from 1.56% to 2.29%, between 2006 and 2008. Moreover, conjoined pollen grains were also observed. Meiotic abnormalities, including univalents, lagging chromosomes and micronuclei, were observed during microsporogenesis. Triads and dyads were also detected in meiotic products. Parallel spindles in metaphase II were unable to fuse, owing to the existence of an organelle band. Regularly, in telophase II, primary phragmoplasts were organized between sister nuclei, and secondary phragmoplasts formed between non-sister nuclei. Cell plates were initiated by centrifugal expansion of phragmoplasts and cytoplasmic infurrowing started at the junctions between the microsporocyte wall and the expanded phragmoplasts. However, a secondary phragmoplast was absent in some microsporocytes. These observations suggest that the occurrence of 2*n* pollen may result from the partial failure of cytokinesis caused by the absence of secondary phragmoplasts in this hybrid.

Key words: microsporogenesis, microtubular cytoskeleton, organelle nucleoids, *Populus*, 2*n* pollen.

Introduction

Meiosis is essential for sexual reproduction and genetic recombination, and plays an important role in species evolution (KLECKNER, 1996). The chromosomes of sporophytes are doubled and apportioned equally into four microspores during the process of microsporogenesis. These fertile microspores serve to produce the next sporophyte generation via fertilization with eggs.

Microtubular cytoskeletons play an important role in nuclear division, cytokinesis, and apportionment of organelles during meiosis (SHELDON and DICKINSON, 1986; BROWN and LEMMON, 1988c, 1991a, b; TANAKA, 1991; SHAMINA et al., 2007). The arrangement of four daughter nuclei in telophase II is determined by the orientation of metaphase II spindles (BROWN and LEMMON, 1992). Abnormal orientation of spindles may cause meiotic nuclear restitution, resulting in the production of unreduced gametes (MOK and PELOQUIN, 1975; RAMANA, 1974, 1979; KANG, 2002).

Cytokinesis is mediated by the cytokinetic apparatus, including preprophase band of microtubules (PPB) and phragmoplast microtubules (GUNNING, 1982). In meiosis of higher plant, phragmoplast microtubules play a role in successive cytokinesis and in some instances of simultaneous cytokinesis (SHELDON and DICKINSON, 1986; BROWN and LEMMON, 1991a, b; SCHOPFER and HEPLER, 1991; SHAMINA et al., 2007). However, in another form of simultaneous cytokinesis, the formation of cell plates does not depend on phragmoplasts and cytokinesis is accomplished by the centrifugal expansion of cell plates (BROWN and LEMMON, 1988c, 1996). BROWN and LEMMON (1989, 1991a, b, 1996) consider that nuclear-based radial microtubule systems (RMSs) function in the organization and apportionment of cytoplasm, in the location of division planes, and in the definition of spore domains.

The genus *Populus* L. is widely distributed and cultivated throughout the world (RAE et al., 2007). As one of the most intensively studied forest tree species, the genus has become a model for forest tree biotechnology and genetic studies (JANSSON and DOUGLAS, 2007). The *Populus* genome is characterized by a high level of heterozygosity, which results in complicated chromosome behavior (SMITH, 1943; WANG et al., 2006). Although many cytological studies of microsporogenesis in *Populus* have been carried out using squashed preparation, the distribution and structure of microtubular cytoskeletons during meiosis continue to remain unclear.

P. simonii Carr. x *P. nigra* L. 'Tongliao' is a hybrid between two different sections (Tacamahaca and Aigeros) within the genus *Populus*; it has the properties of fast growth and high adaptability, and is easily propagated by cuttage. In this hybrid, meiosis of pollen mother cells exhibits a lack of complete homology between the genomes of the parental species. Some natural 2*n* pollen grains have been observed and the occurrence of this 2*n* pollen is assumed to be related to the existence of parallel spindles at metaphase II (WANG et al., 2006).

In this investigation, in order to reveal the relationship between the change in microtubule and chromosome behavior and cytokinesis and to explain the mechanism of 2*n* pollen production, we analyzed the distribution of microtubules and organelle nucleoids during microsporogenesis in *P. simonii* x *P. nigra* 'Tongliao'.

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Material and Methods

Plant material

Male floral branches of *P. simonii* x *P. nigra* 'Tongliao' were collected in spring from a plantation, approximately 40 years old, located to the west of Tongliao City airport (Inner Mongolia Autonomous Region, P. R. China). The branches were cultured in a greenhouse (20–30 °C) in order to induce meiosis. The male flower buds were then collected every 3 h. The meiotic stage was determined by a 2% aceto-orcein squash of an anther. Anthers around the one used for the squash preparation were used for indirect immunofluorescence microscopy of microtubules and DNA staining.

Evaluation of 2n pollen

Pollen samples were collected from male catkins in the greenhouse and stored in a vial with allochroic silica gel at 4 °C. Pollen grains were removed from the vial using a needle, and mounted in a drop of 2% aceto-carmine on a microscopic slide. The frequency of 2*n* pollen was estimated based on pollen diameter.

Indirect immunofluorescence of microtubules and DNA staining

For indirect immunofluorescence of microtubules and DNA staining, our experimental material was fixed in 4% paraformaldehyde in a PEM buffer (50 mM PIPES, 5 mM EGTA, 2 mM $MgSO_4$, pH 6.9) for 45 min. After three 5-min rinses in the PEM buffer, the material was dipped in 10% dimethylsulfoxide (DMSO) for 15 min and then extracted with 1% Triton X-100 for 30 min. The material was then rinsed in the PEM buffer, followed by three 5-min rinses in a PBS buffer (137 mM NaCl, 2.7 mM KCl, 7 mM Na_2HPO_4, 1.5 mM KH_2PO_4,

Table 1. – Frequency of 2*n* pollen and conjoined one of *P. simonii* x *P. nigra* 'Tongliao' from 2006 to 2008.

Year	Number of analyzed pollen	Number of 2*n* pollen (%)	Number of conjoined pollen (%)
2006	1219	19 (1.56)	1 (0.08)
2007	1443	33 (2.29)	6 (0.42)
2008	1367	24 (1.76)	4 (0.29)

Table 2. – Meiotic abnormalities of *P. simonii* x *P. nigra* 'Tongliao' in 2007 and 2008.

Year	Phase	Number of analyzed cells	Abnormalities	Number of abnormal cells (%)
2007	Anaphase I	271	Lagging chromosomes	41 (15.13)
	Telophase I	237	Micronuclei	19 (8.02)
	Anaphase II	417	Micronuclei	21 (5.04)
			Parallel spindles	59 (14.15)
	Telophase II	355	Micronuclei	15 (4.23)
	Meiotic product	441	Triad	12 (2.72)
2008	Anaphase I	349	Lagging chromosomes	47 (13.47)
	Telophase I	263	Micronuclei	26 (9.89)
	Metaphase II	401	Micronuclei	15 (3.74)
	Anaphase II	223	Lagging chromosomes	7 (3.14)
			Micronuclei	8 (3.59)
			Parallel spindles	33 (14.80)
	Telophase II	469	Micronuclei	27 (5.76)
	Meiotic product	697	Triad	11 (1.58)
			Dyad	2 (0.29)

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pH 7.3). Thereafter, microsporocytes were squeezed out from the material and transferred to a slide coated with 0.1% poly-L-lysine (Sigma, P1274). The cells were incubated in a monoclonal anti-α-tubulin antibody (Sigma, T9026), diluted 1:100 with the PBS buffer, for 2 h at 37 °C in a moist chamber. Following further washing with the PBS buffer, the microsporocytes were incubated in a FITC-conjugated anti-mouse IgG (Sigma, F0257), diluted 1:50 with the PBS buffer, for 2 h at 37 °C in a dark chamber. After a final wash in the PBS buffer, the microsporocytes were mounted with a mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vectashield, H-1200). The preparations were observed and photographed using a Leica TCS-SP2 confocal laser scanning microscope.

Results

Occurrence of 2n and conjoined pollen

In *P. simonii* x *P. nigra* 'Tongliao', a low frequency of 2*n* pollen production (1.56% to 2.29%; *Fig. 1a*) was recorded in each year from 2006 to 2008, and conjoined pollen (*Fig. 1b*) was also observed. Compared with the years 2006 and 2008, there were relatively high frequencies of 2*n* and conjoined pollen grains in 2007 (*Table 1*).

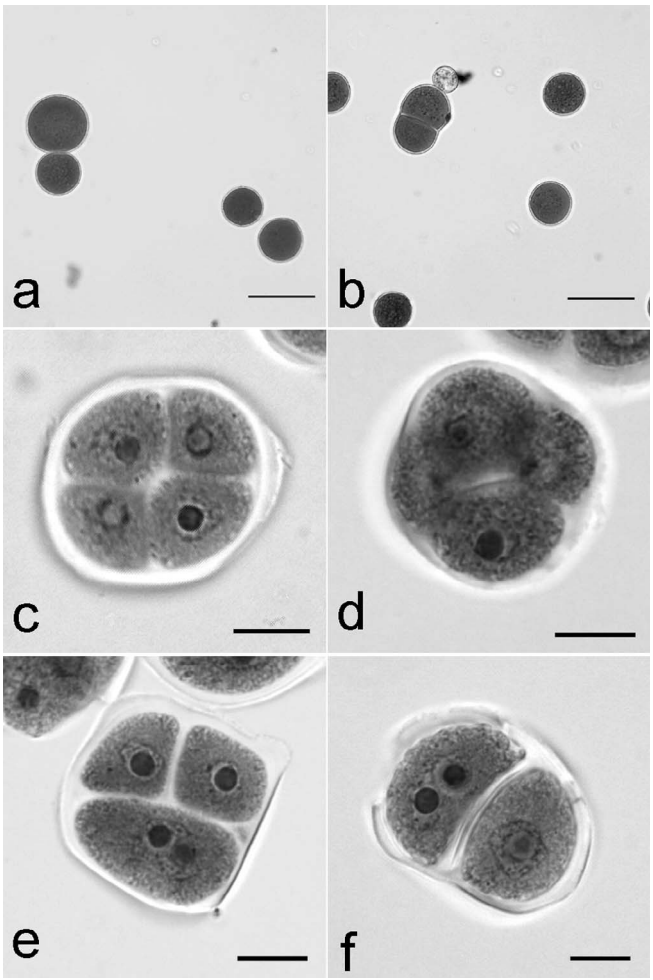

Figure 1. – Variation of pollen and different sporad patterns in *P. simonii* x *P. nigra* 'Tongliao'. (a) 2n and normal pollen; (b) Conjoined and normal pollen; (c) Tetragonal tetrad developed from metaphase II parallel spindles; (d) Tetrahedral tetrad formed from perpendicular spindles; (e) Triad formed by partial absence of cytokinesis, fusion of two nuclei in undivided spore; (f) Dyad. Bars in a and b are equal to 50 μm; bars in c to f are equal to 10 μm.

Abnormality of meiosis

It was found that meiosis in *P. simonii* x *P. nigra* 'Tongliao' proceeded to completion with a typically simultaneous cytokinesis. Abnormalities were, however, observed during microsporogenesis. There were steady frequencies of abnormalities between 2007 and 2008 (*Table 2*). In 2007, although more than 18% cells were observed with lagging chromosomes in anaphase I, only approximately half of these developed into cells with micronuclei in telophase I. After the second meiosis, the frequency of telophase II cells with micronuclei declined to less than 5%. Parallel spindles in anaphase II were observed at a stable frequency of approximately 15%. In the meiotic products, apart from tetrads with tetragonal and tetrahedral arrangements (*Figs. 1c* and *d*), triads were observed in both 2007 and 2008, and a few dyads were detected in 2008. *Figure 1e* shows an undivided spore of a triad, in which two nuclei are observed in the process of fusion. *Figure 1f* shows that in one of the undivided spores of a dyad, two nuclei are about to fuse, and that in the other spore, one fused nucleus has already formed.

Changes of microtubule and organelle nucleoids

During the microsporogenesis of *P. simonii* x *P. nigra* 'Tongliao', the distribution of microtubular cytoskeletons and organelle nucleoids (plastid and mitochondrial) changed regularly. However, some abnormalities with abnormal changes of microtubule and organelle nucleioids were observed.

In the prophase of the first meiotic division, organelle nucleoids were distributed uniformly in the cytoplasm (*Fig. 2a*), and microtubules formed random networks in the cytoplasm (*Fig. 2b*). During metaphase I and anaphase I, the organelle nucleoids tended to concentrate around the spindle (*Figs. 2c* and *e*). In metaphase I, a dipolar spindle with kinetochore microtubules and pole-to-pole non-kinetochore microtubules formed (*Fig. 2d*). Chromosomes were arrayed on the equatorial plane. In some microsporocytes, two univalents, without kinetochore microtubules, roamed about in the spindle region and the other two were pulled by kinetochore microtubules to one pole precociously (*Figs. 2c* and *d*). In anaphase I, the kinetochore microtubules shortened and interzonal microtubules became conspicuous (*Fig. 2f*). Homologous chromosomes were separated and pulled to two poles and the univalents started to lag behind due to a defect in the kinetochore microtubules (*Figs. 2e* and *f*).

Organelle nucleoids migrated from the periphery of the spindle to the equatorial region during telophase I (*Fig. 2g*). As the nuclear envelopes re-formed, nuclear-based microtubules decreased, and a typical phragmoplast, divided into two halves by a dark zone, was initiated in the interzone between daughter nuclei (*Fig. 2h*). Subsequently, as the phragmoplast expanded

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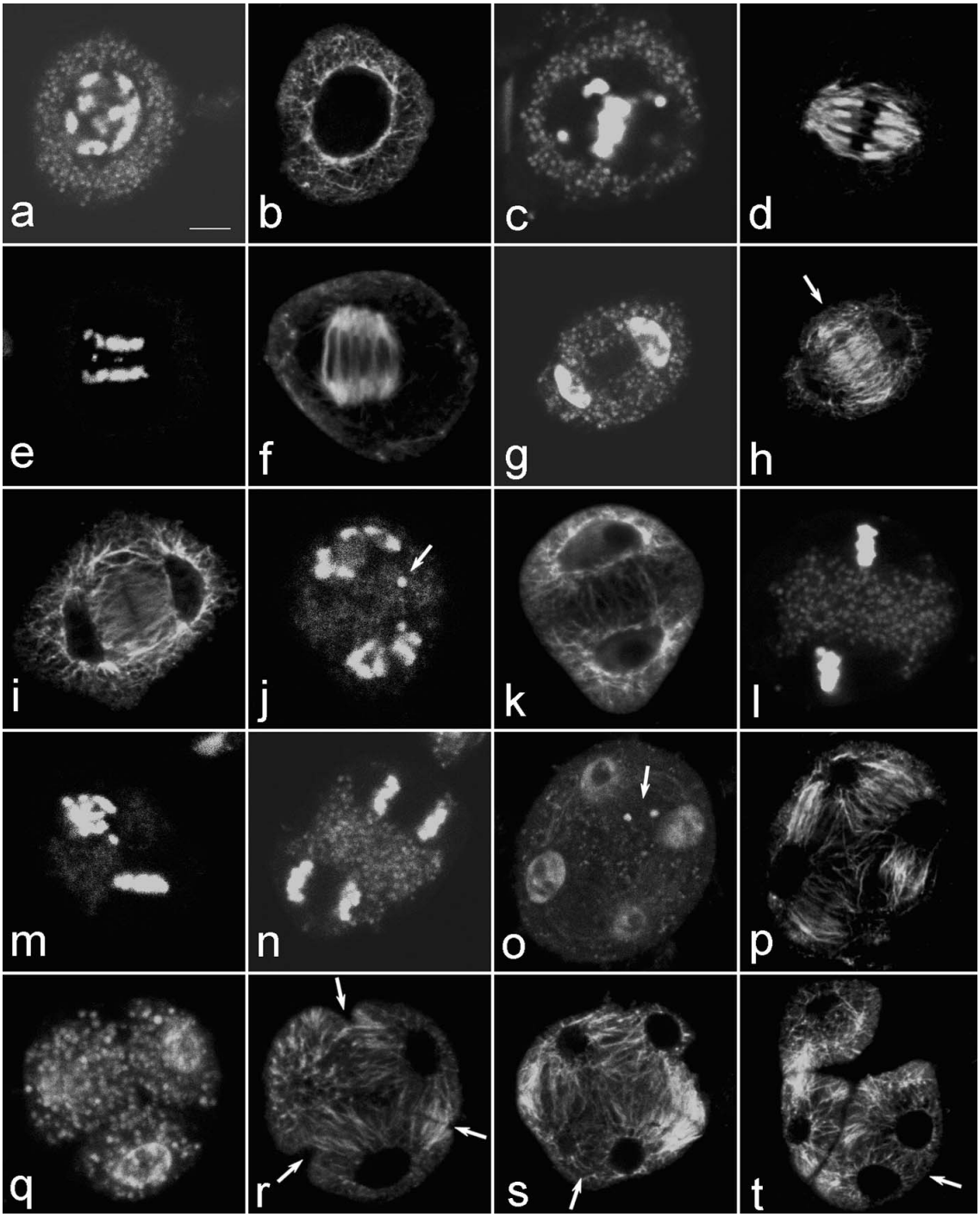

Figure 2. – Distribution of organelle nucleoids and microtubules during microsporogenesis. Bar is equal to 5 μm. (a) Condensed chromosomes and organelle nucleoids in prophase I; (b) Microtubule network in the same microsporocyte as shown in a; (c) Univalents and the organelle nucleoids around the spindle in metaphase I; (d) A dipolar spindle with kinetochore microtubules and pole-to-pole non-kinetochore microtubules in the same microsporocyte as shown in c; (e) Lagging chromosomes in the equatorial region at anaphase I; (f) The shortened kinetochore microtubules and conspicuous interzonal microtubules in the same cell as shown in e; (g) Return of organelle nucleoids to the equatorial region during telophase I; (h) Phragmoplast with a dark zone (arrow) in telophase I; (i) Centrifugal expansion of a phragmoplast; (j) Formation of an organelle band and a micronucleus (arrow) in prophase II; (k) Nuclear-based radial microtubules in the same cell as shown in j; (l) Metaphase II spindles with parallel orientation; (m) Metaphase II spindles with perpendicular orientation; (n) A conspicuous organelle band in anaphase II; (o) Telophase II with tetragonal arrangement, showing that two micronuclei (arrow) remained in the cytoplasm; (p) The microtubules in the same cell as shown in o, showing broad primary phragmoplasts between sister nuclei and narrow secondary phragmoplasts between non-sister nuclei; (q) Three visible nuclei in late telophase II, another one is out of focus; (r) Infurrowing of cytoplasm at tangential sites between the periphery of the microsporocyte and expanded phragmoplasts (arrows); (s) Atypical telophase II, showing the secondary phragmoplast formed between one pair of non-sister nuclei, and the lack of development between the other pair (arrow); (t) Microtubular arrays in a triad, the arrow indicates the lack of a cell plate.

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centrifugally, the nuclear-based microtubules proliferated, although the dark zone was still visible in the equatorial region (*Fig. 2i*). After the first division, no cell plate formed in the equatorial region, which was occupied by organelles.

By prophase II, the organelle nucleiods were localized in the equatorial region and formed a band of organelles that separated the cytoplasm into dyad domains (*Fig. 2j*). Nuclear-based radial microtubules replaced the phragmoplast and defined the nuclear cytoplasmic domains (*Fig. 2k*). In some microsporocytes, micronuclei, which wandered in the interzonal region because of a lack of surrounding microtubules, were observed (*Figs. 2j* and *k*).

Metaphase II spindles were oriented as either parallel or perpendicular alignments (*Figs. 2l* and *m*). The axes of the spindles aligned parallel to the band of organelles. This band remained in the equatorial region until anaphase II (*Fig. 2n*). As the nuclear envelopes reformed, the organelle nucleoids returned to the cytoplasmic domains of the microsporocyte (*Fig. 2o*). Tetragonal and tetrahedral arrangements of four daughter nuclei formed in the microsporocytes during telophase II (*Figs. 2o* and *2q*) due to the different orientation of the metaphase II spindles. Primary phragmoplasts were developed from the interzonal microtubules between sister nuclei. Subsequently, two narrow secondary phragmoplasts were initiated simultaneously between non-sister nuclei (*Fig. 2p*). Micronuclei lacking surrounding microtubules were observed in some telophase II cells (*Figs. 2o* and *p*). Cell plates formed in association with a centrifugal expansion of phragmoplasts after the second meiosis (*Fig. 2r*). Organelle nucleoids, distributed around the daughter nuclei, were apportioned equally into four microspores (*Fig. 2q*). Infurrowing of cytoplasm began at the junctions between the microsporocyte wall and the expanded phragmoplasts (*Fig. 2r*).

Occasionally, in some microsporocyts at telophase II, one normal secondary phragmoplast was initiated between one pair of non-sister nuclei; however, the other secondary phragmoplast failed to develop between the other pair (*Fig. 2s*). This resulted in cytokinetic failure in the tetrad, with two nuclei remaining in one microspore (*Fig. 2t*).

Discussion

Meiotic chromosome behavior is related to changes in microtubules (Brown and Lemmon, 1989; Shamina et al., 2007). Separation of chromosomes is caused by the shortening of kinetochore microtubules, whereas formation of lagging chromosomes results from a lack of kinetochore microtubules. Micronuclei enclosed by microtubular arrays can form minispindles during the second division and develop into small supernumerary spores (Brown and Lemmon, 1989, 1992). In our study, no microtubules were formed around the micronucleus and no supernumerary spores were observed. This suggests that the micronucleus may be assimilated in the cytoplasm or incorporated into daughter nuclei during the second meiosis and that microtubular arrays are responsible for the fate of micronuclei.

The site of cell division is marked by a girdling PPB in advance of mitosis (Gunning et al., 1978; Brown and Lemmon, 1988b). However, the PPB has never been reported in plant meiosis. In some lower plants, meiotic division planes are determined by cytoplasmic lobing in prophase I (Brown and Lemmon, 1988a). In flowering plants, it is not possible to confirm the sites of division planes prior to meiosis. RMSs are, nevertheless, considered to play a primary role in the organization and apportionment of cytoplasm, the location of division planes, and the definition of nuclear cytoplasmic domains (Brown and Lemmon, 1989, 1991a, b, 1996). In this hybrid, the orientation of metaphase II spindles defined the arrangement of the four daughter nuclei of telophase II, which was accompanied by different patterns of tetrads. This indicates that the alignment of metaphase II spindles is responsible for determining the configuration of tetrads (Brown and Lemmon, 1992).

In the mitosis of vegetative cells, the cytokinetic apparatus consists of PPB and phragmoplast (Gunning, 1982). In the meiotic cytokinesis, however, no PPB forms and phragmoplasts play an important role. In the successive cytokinesis of microsporocytes, phragmoplasts develop in the interzonal region between daughter nuclei. The formation of cell plates is associated with centrifugal expansion of the phragmoplasts and cytokinesis is completed by cytoplasmic infurrowing (Sheldon and Dickinson, 1986; Shamina et al., 2007). In the simultaneous type of microsporogenesis, the phragmoplasts play different roles. In the genus *Phalaenopsis*, cell plates develop in association with the centrifugal expansion of phragmoplasts and cytokinesis is completed by expansion and fusion of each of the cell plates (Brown and Lemmon, 1991b). However, in some other forms of simultaneous cytokinesis, the formation of cell plates does not depend on phragmoplasts, and cytokinesis is associated with a centrifugal expansion of cell plates (Brown and Lemmon, 1988c, 1996). In this study, primary phragmoplasts were formed between sister nuclei and secondary phragmoplasts developed between non-sister nuclei. Cell plates were initiated by centrifugal expansion of phragmoplasts and simultaneous cytokinesis was accomplished by cytoplasmic infurrowing. These abundant variations of cytokinetic pattern may be related to the evolutionary scale in different species (Cai, 1999).

The distribution of organelle nucleoids can be distinguished by DAPI staining during microsporogenesis (Tanaka, 1991). A large number of studies, conducted using light and electron microscopy, have demonstrated that organelles are distributed regularly during meiosis (Dickinson, 1981; Tanaka, 1991; Brown and Lemmon, 1988c, 1991a, b, 1996). In the hybrid described here, organelle nucleoids were concentrated around the spindle in metaphase I. After the first meiosis, the organelle nucleoids migrated to the equatorial region and formed a band that separates the cytoplasm into dyad domains (Brown and Lemmon, 1991b, 1996). The organelles were apportioned equally to four microspores with simultaneous cytokinesis. The movements and distributions of organelle nucleoids are attributed to radial microtubules emanating from cell nuclei (Tanaka, 1991).

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It has been demonstrated that the production of unreduced pollen in hybrids is considerably greater than that in non-hybrids (RAMSEY and SCHEMSKE, 1998), due to poor chromosome pairing and non-disjunction in interspecific hybrid. Large pollen, with a diameter upward of 37 µm, is considered as 2*n* pollen in the genus *Populus* (MASHKINA et al., 1989). In *P. simonii* x *P. nigra* 'Tongliao', as a hybrid between two different sections, genomic heterozygosity was demonstrated, including lagging chromosomes and micronuclei. However, in our observations, we found that no more than two chromosomes lagged. It is not strong enough to produce 2*n* pollen. Moreover, in this investigation, the frequency of chromosome-lagging and the presence of micronuclei were evidently higher than that of 2*n* pollen production. This suggests that genomic heterozygosity is not a crucial factor for 2*n* pollen production.

The abnormal orientation of metaphase II spindles, such as parallel, fused, and tripolar spindles, is responsible for unreduced pollen production (MOK and PELOQUIN, 1975; RAMANNA, 1974, 1979; KANG, 2002). In *P. simonii* x *P. nigra* 'Tongliao', formation of large pollen is presumed to be attributed to the fusion of parallel spindles (WANG et al., 2006). In the present investigation, although parallel spindles were observed, their frequency was evidently higher than 2*n* pollen production. Furthermore, the parallel spindles remained at different sides of the organelle band and resulted in a tetragonal arrangement of four daughter nuclei in telophase II, which developed into a tetragonal tetrad. This suggests that the organelle band may serve to prevent parallel spindles from fusing and that the parallel spindles are not the major mechanism of 2*n* pollen production in this hybrid *Populus*.

Lack of meiotic cytokinesis is also considered as one of the reasons for unreduced gamete formation (PFEIFFER and BINGHAM, 1983; RAY and TOKACH, 1992). In potatoes, analysis of microtubule cytoskeletons has revealed that meiotic nuclear restitution occurs by the fusion of non-sister nuclei, which depends on the absence of secondary interzonal microtubules and on the distance between nuclei (CONICELLA et al., 2003). In the present investigation, triads and dyads were observed, which indicates the occurrence of abnormal cytokinesis. In some microsporocytes of this hybrid, no secondary phragmoplast was established between non-sister nuclei in telophase II. We speculate that partial cytokinetic defects, caused by the absence of a secondary phragmoplast, may be a reason for 2*n* pollen production in *P. simonii* x *P. nigra* 'Tongliao'.

The production of conjoined pollen is an interesting event. After sporad formation, the degradation of the callosic wall surrounding each microspore is responsible for microspore separation (RHEE and SOMERVILLE, 1998). In the conjoined pollen, a cell wall was established; however, the separation of microspores was aborted, suggesting that the degradation of the callosic wall had failed. After cytokinesis, cell wall deposition follows the formation of a cell plate, which is associated with the centrifugal expansion of phragmoplasts (SHELDON and DICKINSON, 1986; SHAMINA et al., 2007). Therefore, the formation of the conjoined pollen may be related to the abnormal development of phragmoplasts in this hybrid.

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A Novel Gel-based Method for Isolation of Stigmas During Controlled Pollination Experiments

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Abstract

In forestry, controlled pollination (CP) allows the combining of genetic material of selected elite trees to produce high quality, and consequently high value, seed. The aim of the present study was to develop a novel isolation method that would allow the technique to be conducted without expensive and time-consuming bagging, making CPs on small-flowered eucalypts commercially viable. We compared the current method of isolating inflorescences using exclusion bags to a novel method which uses sodium alginate gel. Sodium alginate was effective in keeping external pollen away from the stigma, since no seed was produced in those treatments that were not manually pollinated but isolated in this way. In addition, flowers hand-pollinated and isolated with sodium alginate produced progeny that were 100% outcrossed with the applied pollen. The exclusion bags, on the other hand, were not as effective in protecting the stigma as seed was produced in those treatments that were isolated with an exclusion bag without being hand-pollinated. Sodium alginate isolation also increased the efficiency of control-pollinations as the gel was naturally shed, removing the need for operators to return to the tree to remove the isolation material.

Key words: control-pollination, bud isolation, sodium alginate, exclusion bag, *Eucalyptus*, Artificially Induced Protogyny.

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