Embryo sac chromosome doubling in *Populus alba* × *P. glandulosa* induced by high temperature exposure to produce triploids

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To determine the effects of the hours after pollination and the treatment durations on triploid production and reveal the effective stages of embryo sac chromosome doubling by high temperature exposure. At least three catkins were sampled, and 80 ovules were used for the determination of the embryo sac developmental process. Catkins (2–74 h after pollination) were treated to induce embryo sac chromosome doubling. Cytological observations revealed that the embryo sac development was a consecutive and asynchronous process. Fertilization occurred 50 h after pollination. In the offspring seedlings, 167 triploids were detected and the highest efficiency of triploid production was 87.0%. Among all the induced triploids, the most effective treatment period of inducing embryo sac chromosome doubling is from 26 to 50 h after pollination, and 121 triploids were obtained, representing 72.46% of the sum of all triploids. GLM-Univariate analysis indicated significant differences among the hours after pollination (F = 4.516, p = 0.045). However, the differences between the treatment durations (F = 0.077, p = 0.791) were not significant. Correlation analysis between the proportion of each embryo sac’s developmental stage and the percentage of triploid production indicated that the third mitotic division may be the most effective stage for 2n female gamete induction.

**Key Words:** triploid, 2n female gamete, embryo sac, high temperature exposure, *P. alba* × *P. glandulosa*.

**Introduction**

White *Populus* hybrid, *Populus alba* × *P. glandulosa*, is a timber species native to Korea. In 1984, the hybrid was introduced into China, and planted in the Guan Country nursery, Shandong province. Due to its high growth rate and good timber quality, it is used widely for landscape cultivation, ecological protection and the production of lumber in northern and northwestern China. In recent years, the forest industry has shown an increasing interest in planting *Populus* and its hybrids because of the shortage of wood supply. A breeding program for *P. alba* × *P. glandulosa* has been developed by Beijing Forestry University in cooperation with the Guan Country nursery.

Triploid *Populus* has many advantages, such as fast growth, superior timber quality and higher stress resistance over their diploid counterparts. Therefore, the triploid breeding plan has become an important part of *Populus* genetic improvement. Triploid hybrid *Populus* clones with better performance have been achieved in *P. tomentosa* (Zhu et al. 1995, Zhu 2006), white poplar hybrids (Kang et al. 2000b) and the section *Aigeiros* (Zhang et al. 2004). These triploid hybrid clones were successfully obtained by crossing 2n pollen with a normal female gamete. However, the rate of triploid production was low because 2n pollen germinated later on stigma and pollen tube grew slower than that of 1n pollen in *P. tomentosa* (Kang and Zhu 1997).

Recently, the utilization of 2n female gametes to increase the triploid production rate has been proven to be a more effective approach to produce triploid *Populus* (Lu et al. 2013, Wang et al. 2010, 2012) because there is no competition between 2n female gametes and 1n female gametes during fertilization (Li et al. 2008). Li et al. (2008) successfully induced 2n eggs by treating *P. alba* × *P. glandulosa* female buds with colchicine during macrosporogenesis, and produced 12 triploid hybrids. Wang et al. (2012) found that the relationship between flower bud morphological characteristics and female meiotic stages was established to guide high temperature treatments and reported the induction of 2n female gametes during macrosporogenesis of *P. pseudosimonii* Kitag. × *P. nigra* L. ‘Zheyin3#’ female catkins treated with high temperature exposure, obtaining 66.7% triploid production. Lu et al. (2013) also studied the relationship...
between flower bud morphological characteristics and female meiotic stages, and documented the induction of 2n female gametes during macrosporogenesis of *P. adenopoda* Maxim female catkins treated with high temperature exposure, obtaining 83.3% triploid production. Therefore, induction of 2n female gametes during macrosporogenesis is an appropriate method for effective triploid production.

In higher plants, the megasporocyte undergoes meiosis and develops into a functional megaspore. Subsequently, the functional megaspore undergoes three successive mitotic divisions and forms into a mature embryo sac. Theoretically, the three successive mitotic divisions provide a high chance for the induction of embryo sac chromosome doubling. Embryo sac chromosome doubling was successfully induced by colchicine in *P. pseudo-simonii* Kitag. × *P. nigra* L. ‘Zheyin3#’ (Wang *et al.* 2010) between 18 and 96 hours after pollination, and 23 triploid individuals were obtained. Lu *et al.* (2013) was also recorded the induction of embryo sac chromosome doubling of *P. adenopoda* Maxim female catkins treated with high temperature exposure between 6 and 72 hours after pollination, obtaining 51 triploid plants. However, the effects of the hours after pollination and the treatment durations on triploid production were not revealed.

High temperature exposure, as a physical mutagenic agent, is often used to induce polyploidy in plants due to its operational advantages and uniformity of treatment (Kang *et al.* 2000a, Mashkina *et al.* 1989, Randolph 1932, Wang *et al.* 2012, Zhang *et al.* 2002). On the one hand, 2n gametes were successfully induced in *Populus* ‘Zheyin3#’ (Wang *et al.* 2012), *P. adenopoda* (Lu *et al.* 2013), and *Populus* hybrids (Kang *et al.* 2000a), by high temperature exposure. For example, Kang *et al.* (2000a) induced more than 80% 2n male gametes with high temperature exposure in *P. tomentosa* × *P. Bolleena*. On the other hand, tetraploidy in *Populus* has also been successfully induced by high temperature exposure during the first zygote division. Lu *et al.* (2014) reported that 32 tetraploids were confirmed by both flow cytometric analysis and chromosome number counting, and the highest rate of tetraploid production was 14.12% in *P. adenopoda*, and 25 tetraploids were determined in *Populus* ‘Zheyin3#’ (Wang *et al.* 2013).

Embryo sac chromosome doubling by high temperature exposure to create hybrid triploids in *P. tomentosa* has been attempted by Kang *et al.* (2015), and no significant differences in triploid production for both different temperature and treatment durations were observed. Due to serious female gamete abortion (Zhu 2006), more female catkins are needed to get the same number of triploids, and there is no information about the effective stages of embryo sac chromosome doubling induced by high temperature exposure. Compared with *P. tomentosa*, the hybrid, *Populus alba* × *P. glandulosa*, is much better in fertility and often used as a female parent in white *Populus* improvement. However, information on the development process of embryo sac, the effects of the hours after pollination and the treatment durations on triploid production, and the effective stages of embryo sac chromosome doubling by high temperature exposure have been unknown.

The objective of this study is, based on the cytological observation of embryo sac development, to develop an embryo sac chromosome doubling technique and effectively increase triploid production rate by high temperature exposure in *P. alba* × *P. glandulosa*. These findings will help provide more specific information on embryo sac chromosome doubling by high temperature exposure in *Populus*.

### Materials and Methods

#### Plant materials

The mother branches of *P. alba* × *P. glandulosa* (2n = 2x = 38), a hybrid with good fertility under normal conditions, were collected from the Guan Country nursery of Shandong province. Some male floral branches (2n = 2x = 38) were sampled from *P. tomentosa* ‘Baotoubai’ growing at the Guan Country nursery. All sampled branches were trimmed and cultured in tape water in a greenhouse (10–20°C) at Beijing Forestry University to force floral development. No nutritional composition was added into tape water.

#### Determination of the developmental process of the embryo sac

For observation of the development of the embryo sacs, female catkins were sampled every 12 h from 2 h after pollination until seed maturity. The sampled catkins were fixed in FAA (70% ethanol/acetacid/40% formaldehyde, 90:5:5) at 4°C for 24 h.

Ovaries from each fixed catkin were randomly removed, dehydrated in alcohol, embedded with paraffin and sectioned (between 8–10 μm). The sections were stained with iron hematoxylin and photographed under an Olympus BX61 microscope. At least three catkins were sampled and 80 ovules were used for statistical analysis.

#### High temperature treatment

When the stigmas of the catkins were at the receptive stage, pollination was conducted using fresh pollen of the *P. tomentosa* clone ‘Baotoubai’. Then, 2h after pollination, pollinated inflorescences were sampled every 12 h after exposure to 42°C for 2 and 4 h. Untreated catkins served as the control group.

The female floral branches were further hydroponically cultured. Seeds were harvested after approximately 4 weeks. They were sown in clay pots in a greenhouse and seedlings were planted into nutrition pots which were made of black plastic when a height of 4–5 cm was reached to promote growth. The size of nutrition pot is 8.0 cm × 10.0 cm.

#### Ploidy analysis by flow cytometry

Flow cytometry measurements were conducted using a flow cytometer (BD FACSCalibur USA). Approximately
0.5 g of young leaves were chopped with a sharp blade into a 55-mm Petri dish containing nuclei extraction solution (Galbraith et al. 1983) (0.2 mM Tris-HCl, 45 mM MgCl₂, 30 mM sodium citrate, 20 mM 4-morpholinepropane sulfonate, 1% (v/v) Triton X-100, pH 7.0) and then filtered through a 40-μm nylon mesh. The suspension of released nuclei was stained with 50 μl of 4',6-diamidino-2-phenylindole (DAPI, 10 mg/ml) for 5 min. At least 2000 nuclei of each sample were detected. Three samples were collected per plant. The leaf sample from a known diploid plant of *P. tomentosa* (2n = 2x = 38) was used as an external standard. The plant DNA C-value of *Populus* is 0.46 pg (Kew C-value database). The standard peak was managed to appear at about channel 50 of relative fluorescent intensity.

**Chromosome counting**

The ploidy level of each offspring was finally confirmed by chromosome counting. Stem tips were excised from the seedlings and pretreated in a saturated solution of para-chlorobenzene for 4 h, then washed once and fixed in fresh Carnoy’s fluid (ethanol/acetic acid, 3:1) for at least 24 h at 4°C. Fixed stem tips were hydrolyzed in 38% HCl/ethanol (1:1) for 20 min at room temperature and then rinsed three times (each for 10 min) with distilled water. The hydrolyzed samples were stained with Carbol fuchsin, squashed with a cover slip and then observed at 100× oil lens magnification using an Olympus BX61 microscope.

**Stomata observations**

For the stomata observations, leaves from ten diploid and triploid plants (5 each) were selected. The lower epidermis of mature leaves (leaf No. 5 from the top of the plant) was peeled off using nail polish, then placed on glass slides, and observed under an Olympus BX61 microscope. To collect the length and width data of the stomata, 20 stomata per leaf were measured randomly using an ocular scale. To record the stomatal density, 10 microscopic field areas per leaf were randomly selected. Significant differences were evaluated using a t test at the 0.05 level of probability.

**Statistical analysis**

The triploid production data (induced by embryo sac chromosome doubling) were analyzed using GLM-Univariate to reveal the differences between the hours after pollination and the treatment durations. Pearson’s correlation coefficient was calculated for the rate of total triploid production at different hours after pollination and the percentage developmental stage of the embryo sac. All statistical analyses were performed using SPSS software (SPSS for Windows, version 13, SPSS, Chicago, IL).

**Results**

**Process of embryo sac development in *P. alba × P. glandulosa***

Embryo sac development of *P. alba × P. glandulosa* belonged to the typical *polygonum*-type. Megaspore mother cells formed a functional megaspore via meiosis. After three sequential mitotic divisions, functional megaspores developed into a 7-celled mature embryo sac (Fig. 1). Three antipodal cells, a central cell, an egg cell and two synergid cells were observed in the mature embryo sac (Fig. 1E).

The embryo sac development was a consecutive and asynchronous process (Table 1). Generally, it started before stigma receptivity of *P. alba × P. glandulosa* was initiated. Three micropylar megaspores in the same tetrad began to degenerate and a uni-nucleate embryo sac derived from the enlarged functional megaspore at the chalazal end was formed (Fig. 1A). Two hours after pollination, the percentage of the uni-nucleate embryo sac was the highest (48.75%). Other stages, such as the functional megaspore, the two-nucleate embryo sac (Fig. 1B), the four-nucleate sac (Fig. 1C) and even the eight-nucleate sac (Fig. 1D), were also observed. At 26 h after pollination, the occurrence of the two-nucleate embryo sac became predominant (33.75%). As the mitosis of the embryo sacs proceeded, the percentage of the four-nucleate embryo sacs gradually increased, exhibiting higher proportions than the other stages 38 h after pollination. Subsequently, all cells developed into eight-nucleate and mature embryo sacs in succession. Between 50 h and 74 h after pollination, fertilization was occurred (Fig. 1G).

**Embryo sac chromosome doubling induced by high temperature exposure**

After the pollinated catkins of *P. alba × P. glandulosa* were treated with high temperature, fertilization was hampered because the stigmas became dry and brown. Some catkins treated with high temperature died, resulting in limited seed collection for some treatments.
A total of 966 seeds were collected from the surviving treated and control catkins (Table 2). After seed sowing and the transfer all of young seedlings, only 305 seedlings remained in the field. All triploid offspring were determined by flow cytometry, and 167 putative triploids were detected by treating pollinated catkins of *P. alba* × *P. glandulosa* with high temperature exposure to induce embryo sac chromosome doubling. Further somatic chromosome counting indicated that all these putative triploids were real triploids (2n = 3x = 57, Fig. 2A, 2B). All triploids were from the treated groups. No triploids were observed in the control group, suggesting that 2n female gamete formation and fertilization between normal female gametes and 2n pollen occur rarely.

The number and efficiency of triploid production in the different treatments are presented in Table 2. No triploids were produced in some treatments, due to the absence of surviving seedlings. A total of 121 triploids were obtained from the treatments 26–50 h after pollination, representing 72.46% of the sum of all triploids. Among all treatments, the highest number of triploids produced was 85, which was observed 26 h after pollination. GLM-Univariate analysis indicated that there were significant differences in triploid production among the hours after pollination (F = 4.845, p = 0.038). However, the differences between the treatment durations (F = 0.100, p = 0.763) were not significant.

The effective stages of embryo sac chromosome doubling induced by high temperature exposure

To determine the effective stages of embryo sac chromosome doubling, Pearson’s correlation analyses were conducted between the percentage of each developmental stage of the embryo sacs (Table 1) and the rate of triploid production (Table 2). A moderately positive correlation was observed between the percentage of four-nucleate embryo sac and fertilization (r = 0.680, p = 0.092). A weak positive correlation was also observed between the percentage of two- and eight-nucleate embryo sacs and the rate of triploid production (r = 0.180, p = 0.700 and r = 0.068, p = 0.885, respectively). However, a weak negative correlation was found between the percentage of uni-nucleate embryo sacs and the rate of triploid production (r = –0.024, p = 0.960).

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**Table 1.** Process of embryo sac development in *P. alba* × *P. glandulosa*

| Hours after pollination | Functional megaspore | Uni-nucleate embryo sac | Two-nucleate embryo sac | Four-nucleate embryo sac | Eight-nucleate embryo sac and mature embryo sac | Fertilization and post |
|------------------------|----------------------|-------------------------|------------------------|--------------------------|-----------------------------------------------|----------------------|
| 2                      | 28.8⁹ (23³)          | 48.8 (39)               | 17.5 (14)              | 3.75 (3)                 | 1.25 (1)                                       | 32.0                 |
| 14                     | 6.25 (5)             | 42.5 (34)               | 35.0 (28)              | 12.5 (10)                | 3.75 (3)                                       | 81.5                 |
| 26                     | 26.3 (21)            | 33.8 (27)               | 28.8 (23)              | 11.3 (9)                 |                                               | 81.5                 |
| 38                     | 15.0 (12)            | 23.8 (19)               | 40.0 (32)              | 21.3 (17)                |                                               | 81.5                 |
| 50                     | 7.50 (6)             | 13.8 (11)               | 32.5 (26)              | 41.3 (33)                | 5.00 (4)                                       | 81.5                 |
| 62                     | 2.50 (2)             | 8.75 (7)                | 12.5 (10)              | 30.0 (24)                | 46.3 (37)                                      | 81.5                 |
| 74                     | 1.25 (1)             | 11.3 (9)                | 21.3 (17)              | 66.3 (53)                |                                               | 81.5                 |

⁹ Figures are frequencies (in %) of ovules showing different developmental stages at different times after pollination. Number of ovaries examined at each time point = 80.

³ The statistical number of ovaries examined.

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**Table 2.** Triploid induction of embryo sac chromosome doubling by high-temperature in *P. alba* × *P. glandulosa*

| Hours after pollination | Treatment duration (h) | Seeds number | Total No. of seedlings | No. of triploid seedlings | Triploid production rate (%) |
|------------------------|------------------------|--------------|------------------------|--------------------------|------------------------------|
| 2                      | 2                      | 52           | 25                     | 8                        | 32.0                         |
| 14                     | 4                      | 71           | 27                     | 22                       | 81.5                         |
| 26                     | 2                      | 163          | 41                     | 35                       | 85.4                         |
| 38                     | 4                      | 202          | 61                     | 50                       | 82.0                         |
| 50                     | 2                      | 45           | 16                     | 13                       | 81.3                         |
| 62                     | 2                      | 72           | 23                     | 20                       | 87.0                         |
| 74                     | 4                      | 5            | 0                      | 0                        | 0                            |
| Control                | 209                    | 67           | 0                      | 0                        | 0                            |
| Total                  | 966                    | 305          | 167                    |                           |                              |

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**Fig. 2.** Ploidy level detection of offspring derived from embryo sac chromosome doubling in *P. alba* × *P. glandulosa* subjected to high temperature (Scale bar = 20 μm). (A) Flow cytometric detection of a nuclei mixture from young leaves from diploid and triploid seedlings. (B) Somatic chromosome number of triploids (2n = 3x = 57).
Embryo sac chromosome doubling by high temperature exposure

Table 3. Stomatal characteristics of diploid and triploid plants of P. alba × P. glandulosa

| Ploidy level | Stomata length (μm) | Stomata width (μm) | Stomata density (no./microscopic field) |
|--------------|----------------------|--------------------|----------------------------------------|
| Diploid      | 17.98 ± 2.38         | 12.72 ± 1.79       | 27.31 ± 0.91                           |
| Triploid     | 27.87 ± 2.25         | 19.69 ± 2.90       | 16.27 ± 0.68                           |

Data represent the mean ± SD.
* Represents a significant difference at the p = 0.05 level according to a two-sample t-test.

Stomatal characteristics of triploids and diploids

The stomatal density and size (length and width) of the triploids and diploids are presented in Table 3. The stomatal length and width of the triploid plants (27.87 ± 2.25 and 19.69 ± 2.90 μm, respectively) were significantly higher than those of the diploid plants (17.98 ± 2.38 and 12.72 ± 1.79 μm, respectively) (t test). In general, an increase in stomatal size results in a decrease in stomatal density. Therefore, compared with diploids, a dramatic decrease in the stomatal density of the triploids was observed (Fig. 3A, 3B). The average density of stomata per microscopic field of the diploids was 1.7-fold higher than that of the triploids.

Discussion

In previous studies, high temperature exposure was usually used to induce 2n pollen in plants (Kang et al. 2000a, Mashkina et al. 1989, Randolph 1932, Zhang et al. 2002). Mashkina et al. (1989) and Kang and Zhu (1997) induced more than 80% 2n pollen in Populus using high temperature. However, the efficiency of triploid production via pollination with induced 2n pollen was low (12.9% at most, published in Kang et al. 2000b) because of weak competition from normal pollen (Kang and Zhu 1997). Recently, the induction of 2n female gametes during macросporogenesis of P. pseudo-simonii Kitag. × P. nigra L. ‘Zheyn3#’ and P. adenopoda Maxim with high temperature exposure was reported by Lu et al. (2013) and Wang et al. (2012). The highest frequency of triploid production ranged from 66.7 to 83.3%, which was much higher than that of previous studies (Kang et al. 2000b), suggesting megaspore chromosome doubling induced by high temperature exposure is a more effective approach for triploid production in Populus.

During embryo sac development in higher plants, the functional megaspore undergoes three successive mitotic divisions and forms a mature embryo sac. Therefore, diploid (2n) eggs could be created when applying a mutagenic agent, such as colchicine, to dividing functional megaspores. In the polyploid breeding program of white Populus, Kang et al. (2004) obtained 21 triploids (51.7% production efficiency) through embryo sac chromosome doubling with a colchicine solution. Wang et al. (2010) achieved 23 triploids (66.7% production efficiency) through embryo sac chromosome doubling by applying a colchicine solution to P. pseudo-simonii Kitag. × P. nigra L. ‘Zheyn3#’. In our study, high temperature exposure yielded a better outcome in embryo sac chromosome doubling (167 triploids and 87.0% production efficiency), demonstrating that high temperature exposure is more suitable for diploid egg induction of Populus than colchicine. However, since the response of the female gametophyte to high temperatures can vary according to genotype (Wahid et al. 2007), the temperature range and duration should be adjusted with different genotypes of Populus.

Hours after pollination responded developmental stage of embryo sac (Table 1). Therefore, it is vital to determine the right time for inducing embryo sac chromosome doubling by a mutagenic agent. From 24 to 48 h after pollination, the triploid production rate induced by embryo sac chromosome doubling in P. tomentosa × P. bolleana (Kang et al. 2004) and P. pseudo-simonii × P. nigra ‘Zheyn3#’ (Wang et al. 2010) was more than 50%. Lu et al. (2013) reported that a total of 34 triploids were obtained from the treatments 12–24 h after pollination, representing 66.7% of the sum of all triploids in P. adenopoda. In the present study, the most effective period was from 26 to 50 h after pollination and 121 triploids were produced, representing 72.46% of the sum of all triploids. The efficiency of triploid production was slightly higher than that in the previously mentioned study.

Applying a mutagenic agent to cells at a suitable stage is important for the induction of chromosome doubling. Lu et al. (2013) documented that two-nucleate embryo sacs may be the most effective stage to induce 2n female gametes in P. adenopoda. However, a moderately positive correlation between the percentage of the four-nucleate embryo sacs and the rate of triploid production (r = 0.680, p = 0.092) was observed, suggesting that the third mitotic division during embryo sac development may be effective for 2n female gamete induction of P. alba × P. glandulosa. This is in agreement with the findings presented by Wang et al. (2010) for P. pseudo-simonii × P. nigra ‘Zheyn3#’.

Chromosome doubling always results in anatomical and morphological changes within plants, such as larger stomata, thicker leaves and stronger stems (Allum et al. 2007, Chakraborti et al. 1998, Ewald et al. 2009, Liu et al. 2007, Lu et al. 2014, Sun et al. 2009, Yang et al. 2006). Although

Fig. 3. Stomatal size and density of a P. alba × P. glandulosa leaf. Bars: 50 μm. (A) Stomata of a diploid plant leaf. (B) Stomata of a tetraploid plant leaf.
polyploids derived from somatic chromosome doubling do not produce new genetic material, the changes in them might be due to duplication of existing chromosomes and their functional alterations (Dhooghe et al. 2010, Parisod et al. 2010, Ranney 2006). In the present study, the mean length of the stomata was longer in triploid than in diploid plants, and the stomatal density decreased with an increase in the ploidy level. The average density of stomata per microscopic field was 1.7-fold higher for the diploids compared with the triploids.

Triploid breeding programs for woody plants have, in general, emphasized improvements in trunk biomass accumulation, adaptability, and disease resistance (Zhu 2006). Our study reports the first step in such a breeding program, i.e., the production of triploids for inter-breeding with diploids. Further work should be focused on evaluating the growth and wood properties of triploids.

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