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Seed vigor, photosynthesis and early growth of saplings of different triploid *Betula* families

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Abstract: Breeding scientists have given extensive attention to triploids in trees because of their importance to forestry. Consequently, creating and breeding triploids of good quality has become one of purposes of tree breeding. We chose two autotetraploids (*Betula platyphylla*, named Q10 and Q65) as female parents and eight hybrid diploids (*B. platyphylla* × *B. pendula*, named F1 – F8) as male parents to obtain progenies through controllable pollination, resulting in triploid progenies. Germination rate and germination energy of triploid seeds of Q65 were significantly higher ($P < 0.01$) than in triploid seeds of Q10. Triploid families with Q65 as female parent had a large quantity of saplings, whereas triploid families with Q10 as female parent had a small quantity of saplings. Triploid families with Q65 as female parent were generally superior in base diameter and height to base diameter ratio when compared to a diploid family. Q65×F3 was preliminarily recognized as the superior family. These results demonstrate that the female parent has a major influence on triploid progenies, although the male parent also has a small influence. The results provided a reference to build seed orchards of triploid birch trees, choose tetraploids as female parents and forecast triploid families of good quality.

Additional key words: birch, polyploidy, seeds, saplings

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Introduction

The natural European aspen (*Populus tremula*) triploid was discovered by Nilsson-Ehle (1936) and Müntzing (1936) in Sweden. Since then, plant-breeding scientists have given extensive attention to triploids in forest trees due to the huge growth of forestry. After Blackeslee and Avery discovered in 1937 that colchicine can loose the spindle fiber in cell division and induces chromosome doubling, a new methodology of plant breeding, known as polyploid breeding, was developed. Since then, this new technology has been extensively used in many fields of forestry breeding. Some researchers chose 2n pollen grains with the unreduced chromosome from a male flower of the diploid plant and obtained triploids through pollinating female flowers of normal diploid plants with the 2n pollen grains. They also chose 2n eggs from female flowers of the tetraploid plants and obtained triploids through fertilization of 2n eggs by n pollen grains of normal diploid plants. Triploid cultivars in *Populus* have been well-developed, in contrast to other species. Research reported that triploids in *P. tremula*, *P. tremuloides* and *P. tomentosa* bear such characteristics as greater growth and higher resistance against biotic stress and abiotic stress (Müntzing, 1936; Johnsson, 1942; Einspahr, 1963; Einspahr, 1984; Zhang et al. 2007; Zhang and Kang, 2010).

The members of the genus *Betula* form a particularly significant group of broadleaved trees in Eurasia and North America. Certain birch species, such as *B. platyphylla*, *B. pendula*, *B. pubescens* and *B. papyrifera*, are

valuable sources of wood and great importance is attached to breeding work aimed at their economic improvement (Eriksson and Jonsson, 1986). The natural triploid of the European birch (*B. verrucosa*) was discovered by Löve (1944), and showed "gigantism" in its morphological organs. Furthermore, Johnsson (1956) chose two tetraploids (*B. verrucosa* and *B. japonica* × *B. verrucosa*) as female parents and a diploid (*B. verrucosa*) as a male parent to obtain triploid progenies from open pollination. Since then, there were no reports about artificial triploid breeding in *Betula*, and triploid cultivars in *Betula* have had no effect in production. In fact, there were significant variations in triploid progenies from controllable pollination; however, the study mentioned above was only about triploid progenies from open pollination. In addition, Johnsson (1956) discovered that allotriploid progenies were superior in height to autotriploid progenies, which means that autotriploidy was not conducive to the growth in *Betula*. Researchers ignored the results of using tetraploids as female parents and diploids as male parents for distant hybridization from controllable pollination. Consequently, we obtained one hundred autotetraploids in *B. platyphylla,* which were derived from colchicine treatment in 2004. In 2009, we chose two autotetraploids (*B. platyphylla*) as female parents and eight hybrid diploids (*B. platyphylla* × *B. pendula*) as male parents, which were located in an intensive seed orchard to obtain the seeds of 13 triploid families. In this study, we analyzed seed vigor, photosynthesis and growth of saplings in the first vegetation period in these 13 triploid families, evaluated the influence of tetraploids as female parents and diploids as male parents to triploid progenies, and provided a reference to build a seed orchard of triploid birch.

Materials and Methods

Plant materials

Our investigation in April 2009 chose two autotetraploids (*B. platyphylla*, named Q10 and Q65), which had flowered at the age of six years, as female parents, and eight hybrid diploids (*B. platyphylla* × *B. pendula*, named F1, F2, F3, F4, F5, F6, F7 and F8), that had flowered at the age of 12 years, as male parents to obtain seeds from controllable pollination. We also chose seeds of diploids (*B. platyphylla*, named B10 and B65; B10 was the sister of Q10 and B65 was the sister of Q65) from open pollination as controls (Table 1). All parents were located in an intensive seed orchard in Harbin, China, and the male catkins on tetraploids had been removed before the flowering. Thus the seeds of 15 families had been obtained, which were sown in the greenhouse in March 2010, and the seedlings were transplanted into outdoor frames in May. The 15 families were planted at random in Maoershan National Forest Park, China.

Ploidy measurement

The DNA content in the leaves was evaluated by flow cytometry (PA-?, Partec, Germany), using the method of Zhang et al. (2006). Briefly, about 2 cm^2 of leaf was chopped with a razor blade in a plastic Petri dish containing 1.5 ml nuclei extraction buffer (10 mmol/l $MgSO_4 \cdot 7H_2O$, 50 mmol/l KCl, 5 mmol/l HEPES, 1% (*w/v*) PVP-40, 0.25% (*v/v*) Triton X-100, pH 8.0). The homogenate was filtered through a 30 μm Partec Celltrics™ nylon filter to remove cell debris, and 1 ml staining solution (Partec) was added. The analysis was performed automatically. The total DNA content of each ploidy was measured and the percentages of the cells that showed varied DNA content were processed by the DPAC software. Meanwhile, the DNA content of leaves in diploid was measured as a control group. Thirty plants were chosen at random from each family.

Mitotic chromosomes were isolated from young leaf buds, using the protoplast dropping method of Anamthawat-Jónsson (2003). Briefly, the buds were collected in iced water and treated for 24 h to arrest the metaphase before fixing them in a mixture of three parts of absolute ethanol and one part of glacial acetic acid. The fixed buds were digested in an enzyme mixture of 2.5% (*w/v*) cellulase Onozuka R10 (Merck) and 2.5% (*v/v*) pectinase (Sigma-Aldrich) for 5 h at room temperature. After hypotonic treatment with 75 mmol/l KCl for 5–10 min, and repeated fixation to clear cytoplasm, the protoplasts were dropped onto microscopic slides. The chromosomes were stained with carmine and visualized in a microscope (Axioimanger A1, ZEISS, Germany), using ×1000 magnification. Chromosomes were counted from 10–20 metaphases from each plant and 30 plants were chosen at random from each family.

Thousand seed weight and seed vigor measurements

One thousand seeds were sown in Petri dishes between layers of moist filter paper at 30°C in an incubator after being weighed. Germination was observed daily and a germination test was conducted with three replications. Germination rate is the percentage of germinating seeds seven days after planting relative to the total number of seeds tested and germination energy is the percentage of germinating seeds four days after planting relative to the total number of seeds tested (Farooq et al. 2005).

Table 1. Parents and the number of sapling in different families

Parents		Seed settings	The number of sapling	Parents		Seed settings	The number of sapling
Female parent	Male parent			Female parent	Male parent		
Q10	F1	×	0	Q65	F1	√	2160
	F2	√	6		F2	√	1656
	F3	√	8		F3	√	864
	F4	√	6		F4	√	348
	F5	×	0		F5	√	1440
	F6	√	8		F6	√	2244
	F7	√	28		F7	√	1368
	F8	√	16		F8	×	0
B10	F0	√	928	B65	F0	√	264

F0: open pollination: √: The family had seeds, ×: The family did not have any seeds.

Gas exchange and chlorophyll fluorescence measurements

The measurements of gas exchange and chlorophyll fluorescence were carried out on the same sapling during the first summer, and at least ten healthy, undamaged, and fully expanded leaves (usually the 3rd or 4th leaf from the apical meristem, each from different saplings) of different families were measured at random. An analysis of gas exchange was carried out by a portable photosynthesis system (LI-6400, LI-COR, USA), commencing at 8:00 am and usually lasting 3 hours. Leaf temperature was kept at 30æC via the temperature control device of the LI-6400. Irradiance was provided by a LI-6400-02B Red/Blue LED light source and was adjusted to 1000 μmol photons m^{-2} s^{-1}. The system was assembled using the LI-6400-01 CO_2 inject device that maintained the CO_2 concentration at 400 μmol CO_2 mol^{-1}. Water use efficiency was the ratio of net photosynthetic rate to transpiration rate according to Bierhuizen and Slatyer (1965).

Chlorophyll fluorescence was measured with a portable chlorophyll fluorometer (PAM-2500, Walz, Germany) by placing special fiberoptics (2010-F) in the leaf-clip holder (2030-B) at an angle of 60°, beginning at 23:00 am for 4 hours. Minimal fluorescence (F_o), maximal fluorescence (F_m), PS II photochemical efficiency (Φ_{PSII}), photochemical quenching (*qP*), and non-photochemical quenching (*NPQ*) were calculated automatically by the slow kinetics window of PAM-2500. Maximal photochemical efficiency of PS II in the dark (F_v/F_m) was calculated as: $F_v/F_m = (F_m - F_o) / F_m$ according to van Kooten and Snel (1990).

Growth measurements

The measurements of height, base diameter, height to base diameter ratio and apical bud diameter were undertaken after the end of vegetation period during the first year. At least sixty saplings were chosen at random in each family.

Statistical analysis

All data were analyzed by nested ANOVA, one-way ANOVA, Duncan test and Pearson's correlation coefficient. These tests were performed using SPSS 16.0 software (SPSS Inc.).

Results

Detection of birch triploids

The flow cytometry analysis of diploids, tetraploids and the progenies of tetraploids as female parents and diploids as male parents are shown in Figure 1. Figure 1a represents the DNA content of diploids, whose main peak was at channel 100. Figure 1b represented the DNA content of the progenies of tetraploid and diploid, whose main peak was at channel 152. Figure 1c represents the DNA content of tetraploids, whose main peak was at channel 198.

Chromosome counts of diploids, tetraploids and the progenies of tetraploids as female parents and diploids as male parents are shown in Figure 2. Figure 2a represents the chromosome of diploids, whose number was 28. Figure 2b shows the chromosome number of the progenies of tetraploid and diploid to be 42. Figure 2c displays the chromosome number of tetraploids at 56.

It is notable that the progenies of tetraploids and diploids had 1.5 times the DNA content and chromosome number of diploids, and had 0.75 times the DNA content and chromosome number of tetraploids. Consequently, the progenies of tetraploids as female parents and diploids as male parent were all triploid.

Triploid seeds vigor

One-way ANOVA showed a highly significant ($P < 0.01$) variation in different families from the same female parent, with regard to measures of germination rate and germination energy in different families (Ta-

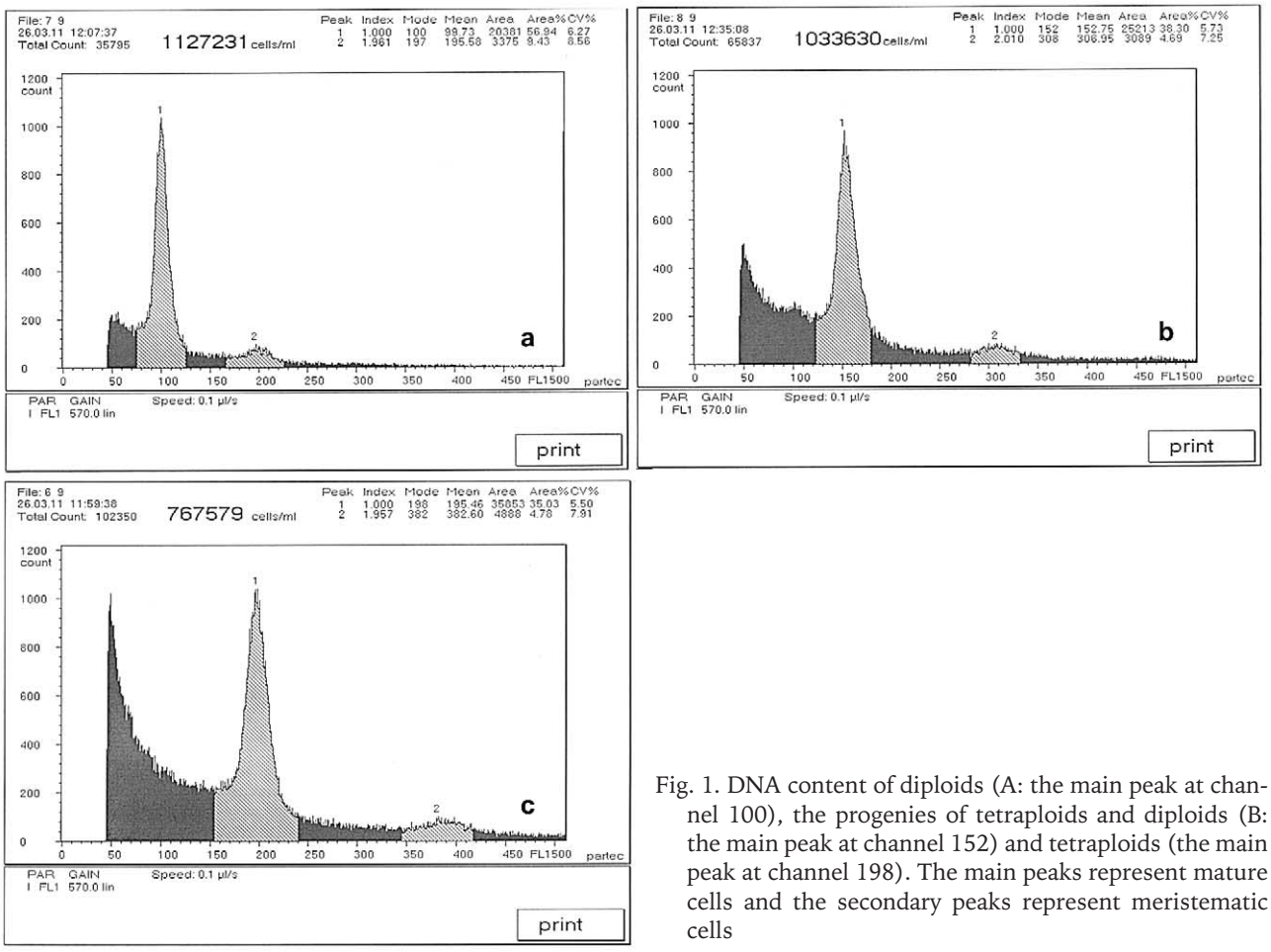

Fig. 1. DNA content of diploids (A: the main peak at channel 100), the progenies of tetraploids and diploids (B: the main peak at channel 152) and tetraploids (the main peak at channel 198). The main peaks represent mature cells and the secondary peaks represent meristematic cells

ble 2). Germination rate and germination energy of triploid seeds from Q10 was significantly ($P < 0.05$) lower than those of diploid seeds from B10; germination rate and germination energy of triploid seeds from Q65 was also significantly lower than those of diploid seeds from B65, except Q65×F3 and Q65×F2 (Fig. 3). In addition, the germination rates of triploid seeds from Q65 and Q10 were 40.00% and 11.61% respectively, and the germination energy of triploid seeds from Q65 and Q10 were 37.05% and 5.11%, respectively (Table 3). Germination rate and germination energy of triploid seeds from Q65 was significantly higher than those of triploid seeds from Q10, as shown by nested ANOVA (Table 4). These results suggest that triploid seed vigor was inferior to diploid seed vigor, but the vigor of triploid seeds was decided by the female parents in the case of an identical male parent.

A positive correlation was found between weight and seed vigor of all 1000 seeds. The correlation be-

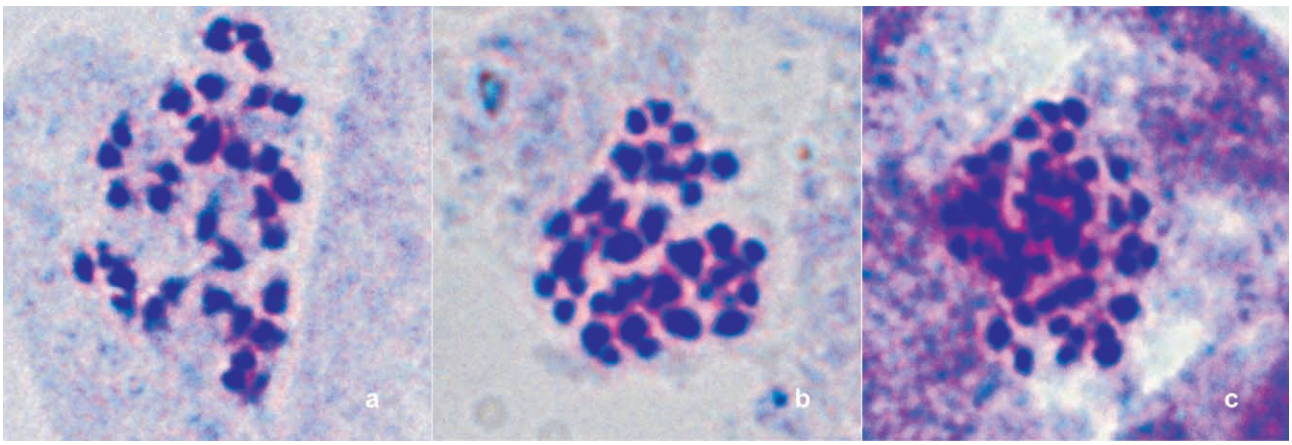

Fig. 2. Chromosome number of diploids (A: 2n=2x=28), the progenies of tetraploids and diploids (B: 2n=3x=42) and tetraploids (2n=4x=56)

Table 2. One-way ANOVA test of different families from the same female parent on germination rate, germination energy and thousand seed weight

Female parent	Dependent variable	*SS*	*df*	*MS*	*F*	*P*
Q10 and B10	Germination rate	0.271	6	0.045	11.394	0.000
	Germination energy	0.469	6	0.078	26.950	0.000
	Thousand seed weight	0.022	6	0.004	29.530	0.000
Q65 and B65	Germination rate	0.149	7	0.021	13.383	0.000
	Germination energy	0.139	7	0.020	9.128	0.000
	Thousand seed weight	0.237	7	0.034	80.072	0.000

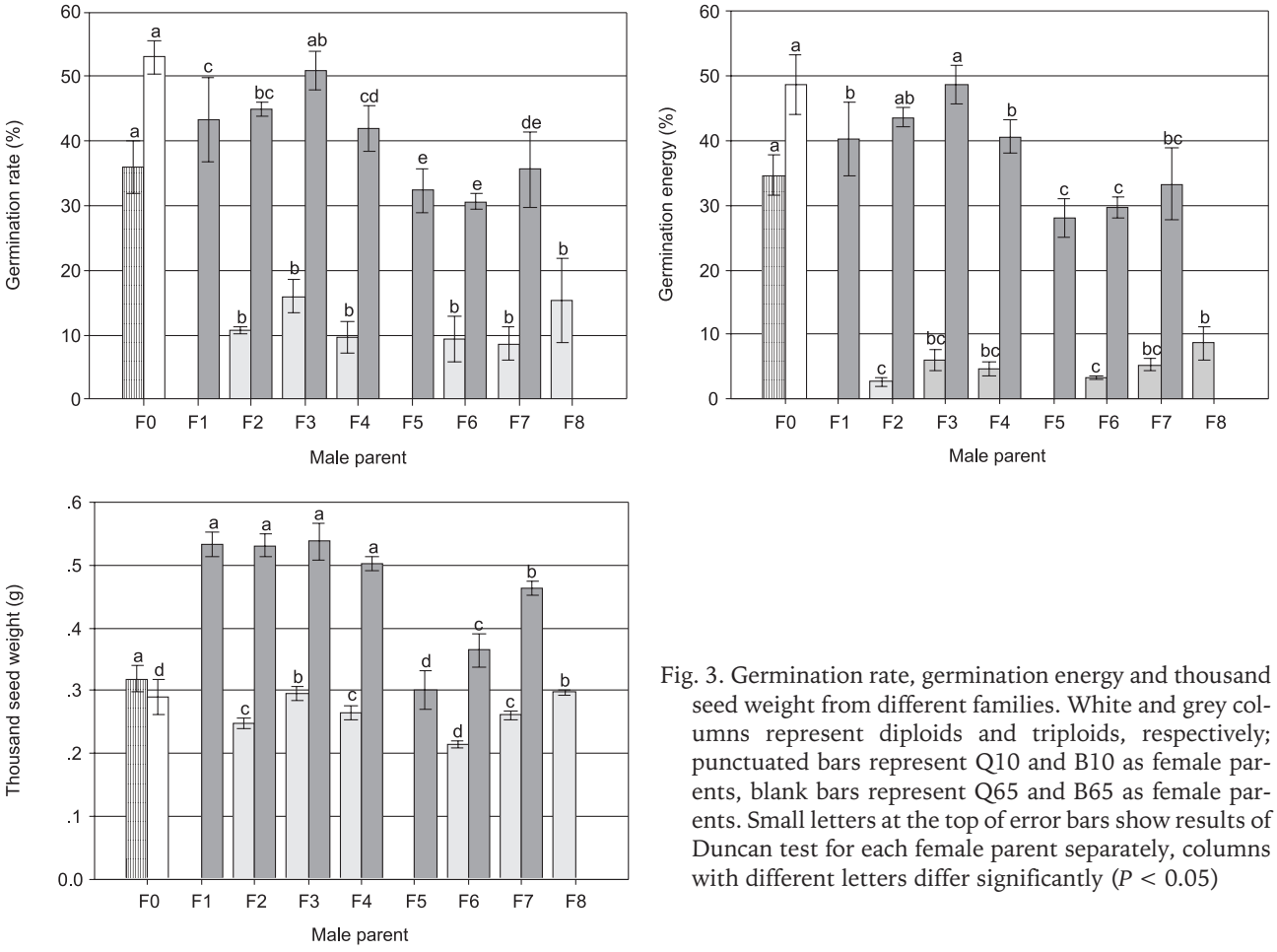

Fig. 3. Germination rate, germination energy and thousand seed weight from different families. White and grey columns represent diploids and triploids, respectively; punctuated bars represent Q10 and B10 as female parents, blank bars represent Q65 and B65 as female parents. Small letters at the top of error bars show results of Duncan test for each female parent separately, columns with different letters differ significantly ($P < 0.05$)

tween seed weight and germination rate was statistically significant ($r = 0.729$; $P < 0.01$; n = 45) in different families; a similar condition was also found between seed weight and germination energy ($r = 0.740$; $P < 0.01$; n = 45). The results indicate that empty seeds can be characterized by any vigor.

Table 3. Differentiation of seeds from Q10 and Q65 by germination rate, germination energy and thousand seed weight

Female parent	Germination rate (%)	Germination energy (%)	Thousand seed weight (g)
Q10	11.61 ± 4.57	5.11 ± 2.91	0.263 ± 0.030
Q65	40.00 ± 7.75	37.05 ± 7.68	0.462 ± 0.091

Table 4. Nested ANOVA test of female parents and families on germination rate, germination energy and thousand seed weight

Source	Dependent variable	*SS*	*df*	*MS*	*F*	*P*
Between female parents	Germination rate	1.132	1	1.132	386.714	0.000
	Germination energy	1.821	1	1.821	733.235	0.000
	Thousand seed weight	0.381	1	0.381	1636.132	0.000
Among families within female parent	Germination rate	0.141	11	0.013	4.374	0.001
	Germination energy	0.139	11	0.013	5.087	0.000
	Thousand seed weight	0.173	11	0.016	67.470	0.000

Photosynthesis of triploid birch saplings

The seeds from Q10 were exiguous and showed low germination so that the number of progeny saplings of Q10 did not meet the requirements for statistical analysis. Consequently, we only measured gas exchange, chlorophyll fluorescence and growth of progeny saplings of Q65 and B65. Measurements of net photosynthetic rate, transpiration rate and water use efficiency in different families showed that there were highly significant variations in different families from Q65 and B65 (Table 5), whereas there was a similar tendency between those of different triploid families and the diploid family. The net photosynthetic rate of each triploid family was significantly inferior to that of the diploid family; water use efficiency of each triploid family was also significantly lower than that of the diploid family except Q65×F4 (Fig. 4).

Measurements of F_v/F_m, Φ_{PSII}, *qP* and *NPQ* in different families showed highly significant variations of Φ_{PSII} and *qP* between different families from Q65 and B65 (Table 6), whereas a similar tendency between different triploid families and the diploid family was seen in F_v/F_m, Φ_{PSII}, *qP* and *NPQ*. F_v/F_m and *NPQ* of each triploid family were not significantly different from those of the diploid family; Φ_{PSII} and *qP* of each triploid family were also not significantly different from those of the diploid family, except Q65×F1 and Q65×F5 (Fig. 5). These results suggest that the net photosynthetic rate and water use efficiency of triploid saplings were inferior to those of diploid saplings, but there was no significant variation in chlorophyll fluorescence between different triploid families and diploid family.

Table 5. One-way ANOVA test of different families from Q65 and B65 on net photosynthetic rate, transpiration rate and water use efficiency

Dependent variable	*SS*	*df*	*MS*	*F*	*P*
Net photosynthetic rate	136.316	7	19.474	12.345	0.000
Transpiration rate	26.500	7	3.786	7.783	0.000
Water use efficiency	14.910	7	2.130	9.956	0.000

Table 6. One-way ANOVA test of different families from Q65 and B65 on F_v/F_m, $\Phi_{PS?}$, *qP* and *NPQ*

Dependent variable	*SS*	*df*	*MS*	*F*	*P*
F_v/F_m	0.009	7	0.001	1.854	0.090
$\Phi_{PS?}$	0.276	7	0.039	6.484	0.000
qP	0.621	7	0.089	5.728	0.000
NPQ	0.522	7	0.075	2.125	0.052

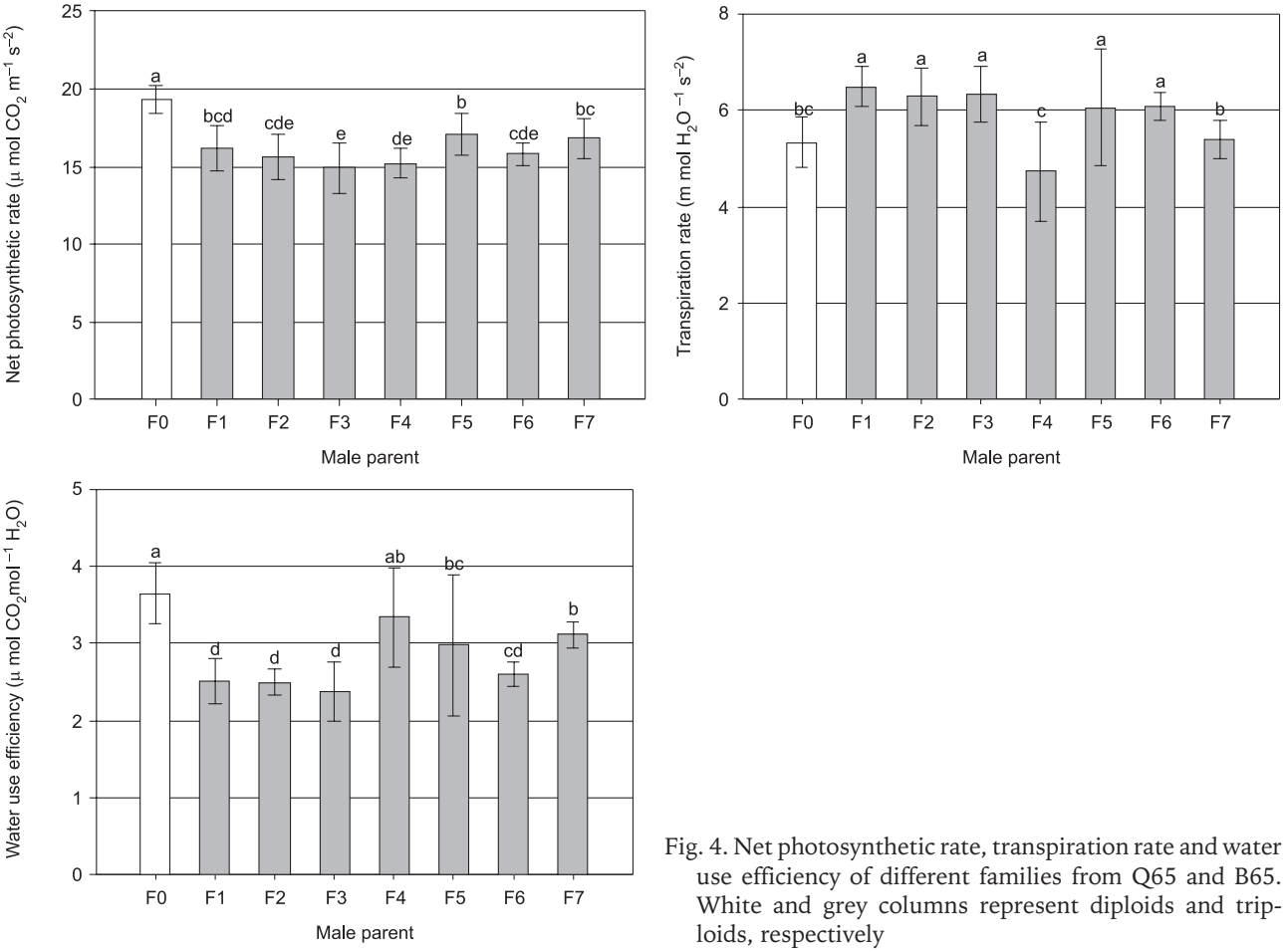

Fig. 4. Net photosynthetic rate, transpiration rate and water use efficiency of different families from Q65 and B65. White and grey columns represent diploids and triploids, respectively

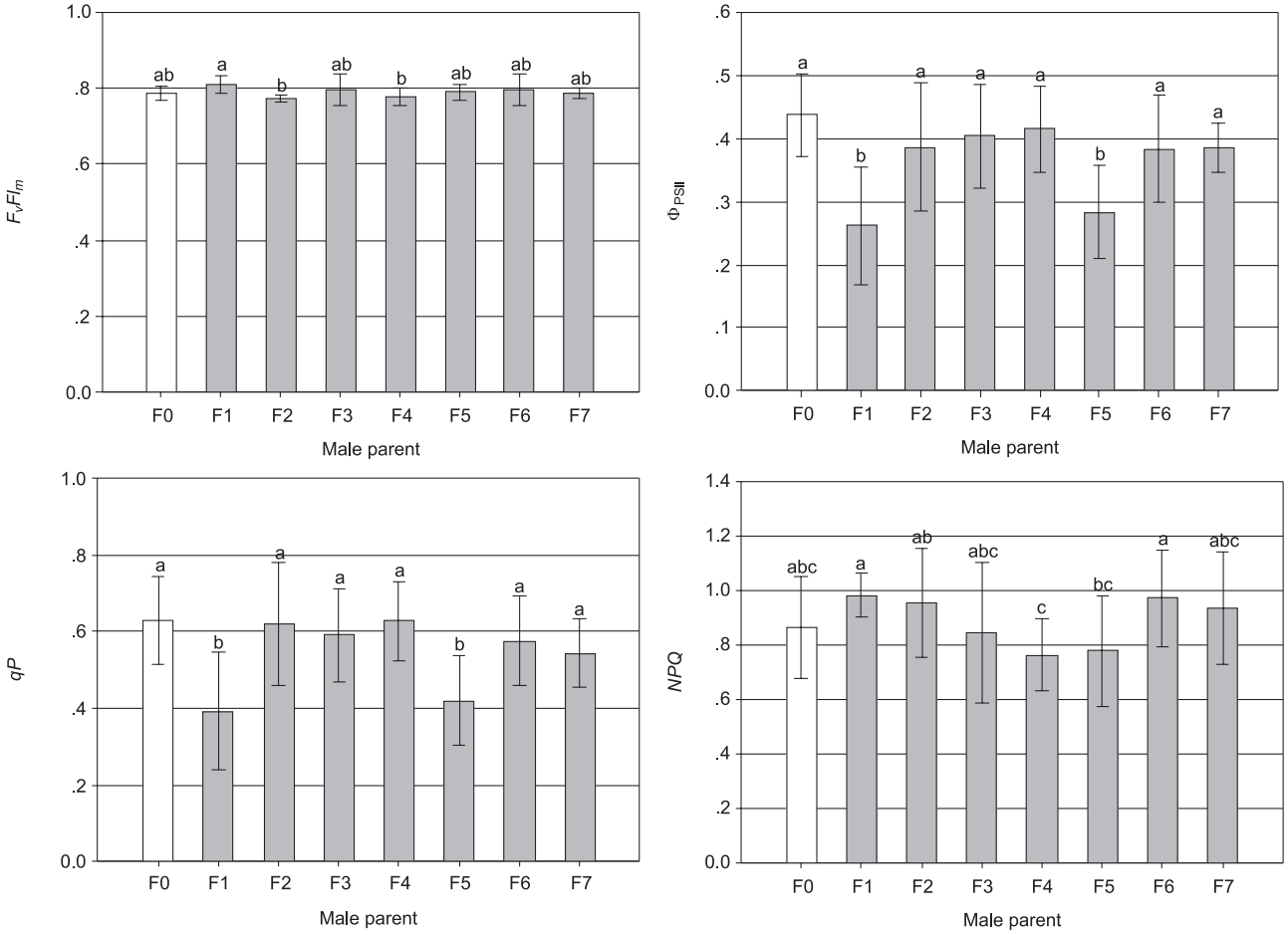

Fig. 5. F_v/F_m, Φ_{PS}, *qP* and *NPQ* of different families from Q65 and B65

Growth of triploid birch saplings

Measurement of sapling height, base diameter, height to base diameter ratio and apical bud diameter in different families showed that there were highly significant variations in different families from Q65 and B65 on height, base diameter, and height to base diameter ratio (Table 7), whereas there was a similar tendency between different triploid families and diploid family on height, base diameter, height to base diameter ratio and apical bud diameter. The base diameter of each triploid family was significantly superior to that of the diploid family; height to base diameter ratio of each triploid family was significantly inferior to that of diploid family; in apical bud diameter of each triploid family there was no significant variation to that of diploid family; the height of each triploid family was also not significantly different from that of the diploid family, except Q65×F4 (Fig. 6). These results indicate that the growth in height of triploids was equal to that of diploids but that the growth in base diameter of triploids had been bigger than that of diploids. Consequently, the triploid yearlings were stouter and the diploid ones were more slender. Meanwhile, the growth of the Q65×F3 triploid family was stouter than that of the diploid family, and the germination rate, germination energy, height and apical bud diameter of Q65×F3 triploid family was not inferior to those of the diploid family. For these reasons, it was preliminarily recognized that F3 was the superior male parent and Q65×F3 was the superior family.

Table 7. One-way ANOVA test of different families from Q65 and B65 on height, base diameter, height to base diameter ratio and apical bud diameter

Dependent variable	*SS*	*df*	*MS*	*F*	*P*
Height	4404.829	7	629.261	4.655	0.000
Base diameter	97.443	7	13.920	13.241	0.000
Height to base diameter ratio	105606.069	7	15086.581	16.295	0.000
Apical bud diameter	0.831	7	0.119	0.488	0.844

Discussion

Triploid breeding has great potential for fast growing trees of good quality. The growth rate of the triploid poplar (*P. tomentosa*) was approximately

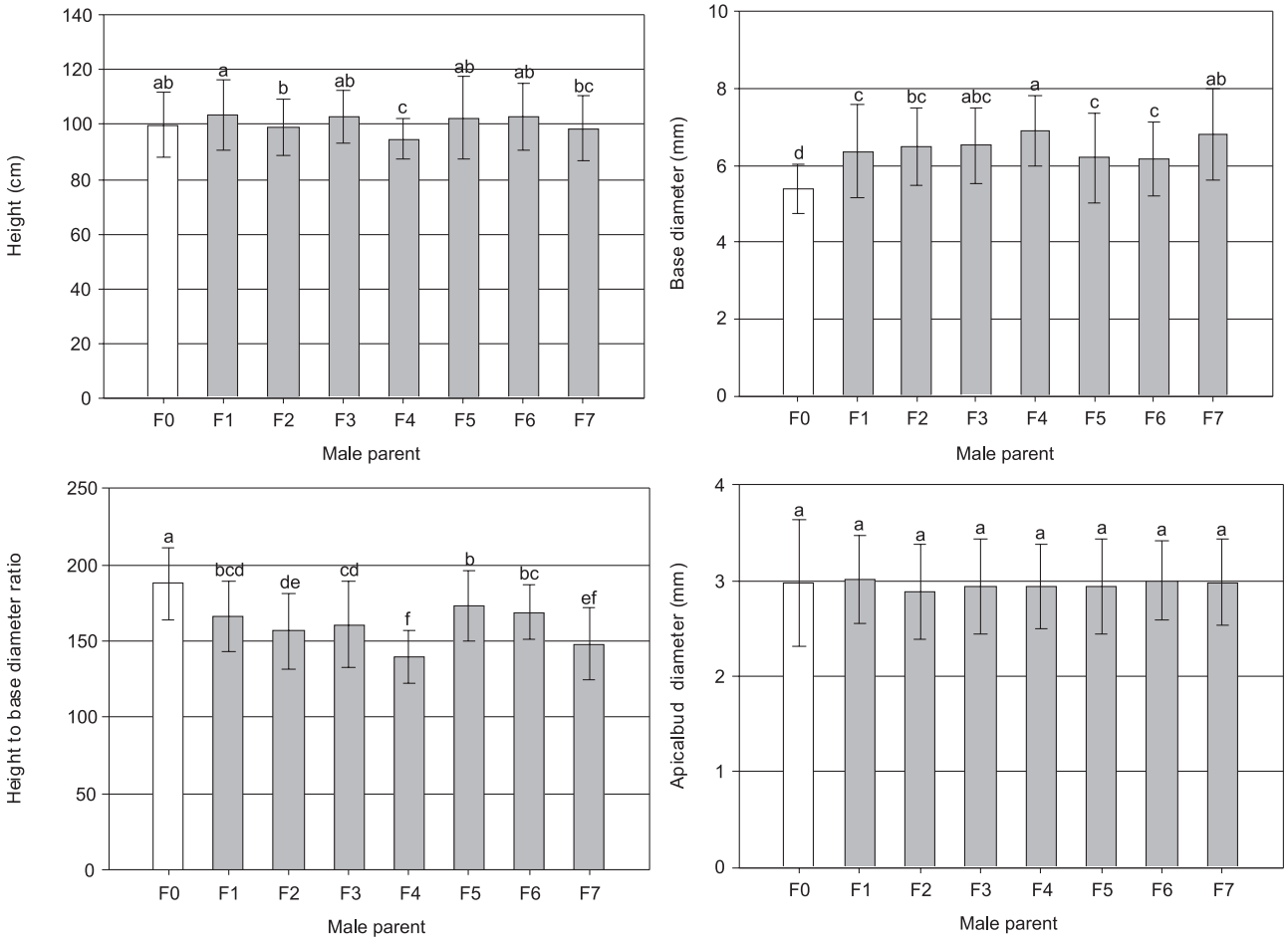

Fig. 6. Height, base diameter, height to base diameter ratio and apical bud diameter of different families from Q65 and B65

double that of a diploid plant, and the trees showed a higher wood density, longer fibers and improved pulp properties (Zhu et al. 1995; Xing and Zhang, 2000; Xing et al. 2004). Triploid mulberry (*Morus alba*) had a vigorous vegetative growth, which was characterized by long, straight and thick branches, large and thick leaves, and the yield of leaves was higher than in diploids (Yu et al. 2004). Black pine (*Pinus thunbergii*) with triploid chromosomes showed higher SOD levels than diploid trees and markedly inhibited lipid peroxide formation, since the SOD gene resides on a chromosome (Niwa and Sasaki, 2003). Interspecific hybrids of individuals with different ploidy is the most simple and effective method to obtain new polyploids. By using this method, artificially induced triploids have been produced in *Populus* (Einspahr, 1984), *B. verrucosa* (Johnsson, 1956), *Alnus glusinosa* (Johnsson, 1950) and *M. alba* (Dwivedi et al. 1989). Of these triploids, allotriploid aspen (*P. tremula* × *P. tremuloides*) made great contributions to the breeding of aspen that improved pulp properties (Einspahr, 1984). In some regions of North Europe and America, researchers even obtained seeds of tetraploid female aspen from open pollination and then used the seeds to forestation. This study obtained the progeny saplings of autotetraploids (*B. platyphylla*) derived from colchicine treatment as female parents and hybrid diploids (*B. platyphylla* × *B. pendula*) as male parents. All plants were triploid, as detected by flow cytometry and chromosome count. This result provides a simple and effective method to obtain triploid birch.

Germination rate and germination energy of triploid seeds from Q65 was highly significantly superior to those of triploid seeds from Q10, indicating that triploid seed vigor was decided by the female parents in case of a similar male parent. Abnormal meiosis of Q10 resulted in seeds showing considerable sterility. The fertility of seeds from Q65 was significantly higher than that of diploid seeds from B10. These results suggest that triploid seed vigor is heritable and choosing tetraploids whose gametes have a higher fertility could obtain triploid seeds with higher germination rates and germination energy. In addition, triploid progeny saplings of Q65 were abundant and normal whereas those of Q10 were exiguous and abnormal; this phenomenon demonstrated that the female parents had a major influence on their triploid progenies.

According to Li et al. (2009), the survival ratio of trees in forestations is a positive correlation between base diameter and the height to base diameter ratio of

saplings if the height of saplings is normal. In addition, the stem erecting is in positive correlation to the apical bud diameter, according to birch silviculture experiments conducted by the author over several decades. The growth difference in different families of Q65 and B65 indicated significant variation between different families. Nevertheless, there was a similar tendency in the growth of triploid saplings in different families compared to that of diploid saplings characterized by a higher base diameter, a lower height to base diameter ratio, an equal height and equal apical bud diameter. This result suggests that triploid birch were not only equaled to diploids in growth and stem erecting, but also superior to diploids in survival ratio of a forestation because of lower height to base diameter ratio. On this basis, it is also preliminary superior to plants with tetraploids as female parent and diploids as male parent with regard to seed vigor and sapling growth of triploid progenies. In the further research of this study, the seed vigor and sapling growth of diploid progenies from B65 were both superior to those of diploid progenies from B10 at the condition of open pollination, which suggests that choosing the seeds of plus birch trees to be treated with colchicine could select tetraploid trees as superior female parents.

It is feasible to build a seed orchard of triploid birch trees on the basis of selecting fertile tetraploids as female parents and fertile diploids as male parents, similar to triploid aspen, and then to obtain a large number of triploid birch since it is difficult to obtain birch clones. This study provides a reference to build a seed orchard of triploid birch, choose tetraploids as female parents and expect triploid families of good quality.

It is currently impossible to draw any conclusions about the future development of the triploid families at this early stage. Consequently, it is advisable to make further measurements on growth, wood properties, gene expression and heteromorphosis stability of triploid birch, and regard polyploid breeding as a new approach for genetic improvement of forestry trees.

Acknowledgements

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