

effect, S and s are genetic and environmental components of the competition effect, e is the residual, representing environmental variation between clones of the same genotype. Summations are over the four neighbours of the tree. The brackets group genetic and non-genetic terms of the equation. In Model I, where only a genetic and a residual effect are fitted, the genetic effect is equivalent to $(D + \Sigma S)$, where ΣS is the sum of neighbour genetic competition effects, whereas performance in monoculture is determined by the value of $D + 4S$, where S is the tree's own genetic competition component.

Equation (1) represents a random effects model. The only non-standard feature is the fact that each measurement is affected by more than one competition effect. To deal with the genetic competition term, for example, we 'overlay' the effects of four factors, each of which represents the genotype of one neighbour (e.g., north, east, south and west of the target tree). There is an analogy with the analysis of a diallel cross experiment, where the value of each cross is the sum of two line effects, one from the male and one from the female parent.

As already mentioned, we allow for covariances between D and S , and between d and s . Environmental covariances between d and s have been discussed above. The covariance between D and S (the direct and competition effects for the same tree) is σ_{SD} . This is also the covariance between S and D for two trees of the same genotype. Using standard results on resemblance between relatives, the covariance between D for a tree of one genotype and S for tree of a different genotype is $(1/2) \sigma_{SD}$ if the two genotypes belong to the same family (see page 4 for details of the family structure), and is otherwise zero. Similar results are obtained for covariances between values of D or S for two trees by replacing σ_{SD} by σ_D^2 or σ_S^2 .

These covariance structures are easily included in the mixed model, either by including additional random effects (family, genotype) in the model, or more directly by calculating a relationship matrix for the 48 genotypes and using the so-called 'animal' model.

A useful reference for the mixed and animal models is LYNCH and WALSH (1998).

Induction of 2n pollen by colchicine in *Populus × popularis* and its triploids breeding

By X. J. XI^{1),(2),(3),§}, X. B. JIANG^{1),(2),(3),§}, D. LI^{1),(2),(3)}, L. Q. GUO^{1),(2),(3)},
J. F. ZHANG^{1),(2),(3),*}, Z. Z. WEI^{1),(2),(3),(4)} and B. L. LI^{1),(2),(3),(5),*}

(Received 22th November 2010)

Abstract

Induction of 2n pollen is a required technique for cultivating polyploid via sexual polyploidy. Orthogonal design or Taguchi Design was applied to select the best treatment process of 2n pollen induction in *Populus × popularis* from different levels of the meiosis stage of

male flower buds, colchicine concentration, times of injection, and interval between injections. Flow cytometry and chromosome counting were used to identify the triploids from the offspring of *P. × euramericana*. (Dode) Guinier pollinated with induced pollen of *P. × popularis*. The results showed that high 2n pollen rate can be achieved by selecting the flower buds during diakinesis stage in meiosis, and then injecting 0.6% colchicine 4 times with 2 hours interval. The 2n pollen rate reached 62.10% by this process, and two triploids were obtained, which indicates that it is possible for cultivating triploids via 2n pollen induction by colchicine treatment in poplar. Results and protocol related to 2n pollen induction, polyploid identification and effect of 2n pollen in this study might be applicable in polyploidy breeding in section *Aigeiros* and *Tacamahaca* of poplar.

Key words: 2n pollen, poplar, polyploid breeding, colchicine, Orthogonal design.

Introduction

Polyploidy (having three or more complete sets of chromosomes) is considered to be a major pathway for plant evolution and can result in reproductive isolation and abrupt speciation (RAMSY and SCHEMSKE, 1998; SOLTIS et al., 2004; WENDEL, 2000). The effects of polyploidy on plant traits are also important to tree or plant

¹⁾ National Engineering Laboratory for Tree Breeding, Beijing Forestry University, 100083, Beijing, P. R. China.

²⁾ Key Laboratory of Genetics and Breeding in Forest Trees and Ornamental Plants, Ministry of Education, Beijing Forestry University, 100083, Beijing, P. R. China.

³⁾ The Tree and Ornamental Plant Breeding and Biotechnology Laboratory of State Forestry Administration, Beijing Forestry University, 100083, Beijing, P. R. China.

⁴⁾ Vegetable Research Center, Beijing Institute of Agriculture and Forestry, 100097, Beijing, P. R. China.

⁵⁾ Department of Forestry and Environmental Resources, North Carolina State University, Raleigh, 27695, North Carolina, USA.

^{*}) Corresponding authors:

JINFENG ZHANG, Beijing Forestry University, Campus Box 118, Beijing, 100083, P. R. China. Phone: 0086-10-62338415, fax: 0086-10-62338013. E-Mail: zjf@bjfu.edu.cn.

BAILIAN LI, North Carolina State University, 329 Daniels Hall, Campus Box 7112, Raleigh, NC, 27695-7112, USA. Phone 919-515-3201, fax 919-515-6835. E-Mail: Bailian_Li@ncsu.edu.

[§]) These authors contributed equally to this work.

breeders. Ploidy levels can influence plant vigor, gene expression, and crossability (NI et al., 2009; RANNEY, 2006). The induction of artificial polyploids has been utilized in the development of allopolyploids to restore fertility in sterile hybrids, enhance crossability and fertility of progeny, create seedless triploids, produce novel gene combinations, and increase the expression and diversity of secondary metabolites (CHEN and NI, 2006; CONTRERAS et al., 2007; OLSEN et al., 2006; WENDEL, 2000).

Triploid poplar clones, derived from 2n gametes, in section *Populus* and in section *Aigeiros* display faster growth over the normal diploids and are widely used in poplar plantation (ZHU et al., 1995; ZHANG et al., 2004; KANG et al., 2006). 2n pollen is a basic material for poplar sexual polyploidization. However, the natural occurrence rate of 2n pollen in poplar is very low, which is normally under 5%. Thus, artificial induction of 2n pollen is an essential technique for poplar polyploidy breeding. 2n pollen can be induced by physical factors such as temperature, radial ray, and ultrasonic. High temperature is the widest application factor among them. MASHKINA (1989) treated poplar and pine male flower buds at prophase I during meiosis with 38–40°C about 2h, gained 2n pollen 94.4% and 84.1%, respectively. 2n pollen also can be induced by chemical agents such as colchicine, oryzalin, and trifluralin (HUANG, 2002). Colchicine was first discovered as an effective cell division inhibitor in 1937 and has been utilized for a long time (EIGSTI and DUSTIN, 1955; HANCOCK, 1997; KANG et al., 2006). Most inductions of 2n pollen in poplar focus on the species in section *Populus* (HUANG et al., 2002) while few in other four sections in poplar genus. *P. × popularis* are the hybrid offspring of *P. simonii* × (*P. pyramidalis* + *Salix matsudana* mixed pollen) that were cultivated by the Chinese Academy of Forestry. *Salix* pollen was used as mentor pollen. They are widely planted in semiarid area in north China and display high resistance against cold and drought but slow growth. The objective of this paper was to explore the technique of 2n pollen induction and seek for the approach for polyploid cultivation of *P. × popularis* in order to enhance its growth for the future biomass production. This work may offer a start point for polyploidy breeding in section *Aigeiros* and *Tacamahaca* of poplar.

Materials and Methods

Plant materials

One male tree (P) of *P. × popularis* was used as paternal parent and 2n pollen induction in this study. One female trees (A) of *Populus × euramericana*. (Dode) Guinier was used as maternal parent. Controlled pollinations were performed on detached floral branches in greenhouse. Hybrids were produced using pollen radiation (2100 Rad, 339.7 Rad/min) (KANG et al., 2000a; ZHANG et al., 2009) in order to slow down the speed of 1n pollen germination and multiple pollinations. Young embryos of the hybrids were excised 25 to 35 d after the first pollination and cultured on the $\frac{1}{2}$ MS medium (half strength of MS macro nutrients, and other ingredients were no changed) with 30 g/l sucrose and 7 g/l agar. Two months later, hybrid plants were transplanted to the greenhouse and three weeks later they were transplanted to the field.

2n pollen induction

Flower buds during prophase I in meiosis process were selected for 2n pollen induction experiment. During prophase I, there was almost no the phenotypical development of floral bud, so the amount of liquid is roughly 50 µl/time/floral bud and the bud was impregnated with colchicine liquid. Different concentration colchicine liquids were injected in the flower buds at varied times and intervals. The experiment design was Orthogonal Design or Taguchi Design $L_9(3^4)$, 4 factors were meiosis phase of flower buds, concentration of colchicine liquid, times of injection, and interval between two injections. Each factor was arranged 3 levels. For the first factor, 3 levels were leptotene, pachytene, and diakinesis. The 3 levels for the second factor were 0.2%, 0.4%, and 0.6%; for the third factor were 3, 4 and 5 times; for the final factor were 2h, 3h, and 4h. Untreated flower buds were used as control.

The 2n pollen rate was the number of large pollen grains ($d > 40 \mu\text{m}$)/400 observed pollen grain under the microscope, replicated 5 times. A total about of 2000 pollen grains were used for the calculation.

Pollen weight per catkin was the average pollen weight collected from 5 to 10 catkins for each of 9 treatments and the control.

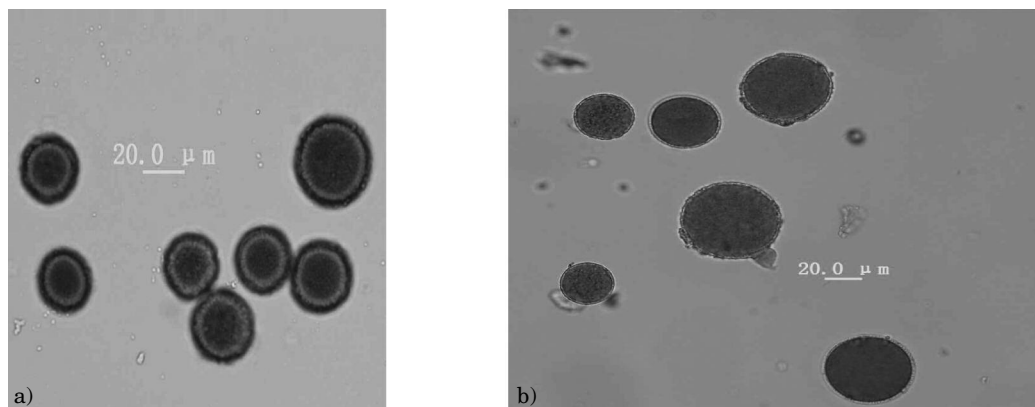


Figure 1. – Comparison of n and 2n pollen from *P. × popularis*. (a) Before induction; (b) After induction by colchicine.

Comparative pollen weight was pollen weight per catkin for each treatment/pollen weight per catkin for the control.

2n pollen rates were evaluated by analysis of variance with the statistical program Orthogonality Experiment Assistant II Version 3.1 and q test or S-N-K test according to *Forestry Experimental Design* (XU and HUANG, 1995; VUCHKOV and BOYADJEVA, 2001).

Flow cytometry analyses

A polyploid scan was made for all 16 hybrid offspring derived from A×P using flow cytometric analysis. Crude nuclei were prepared from about 500mg of fully expanded field-grown leaves by chopping each sample 30s with a sharp razor blade in 1 ml extraction buffer (DPOOLEŽEL et al., 1989). A 1.0 ml extraction buffer was added and gently blended for about 2 minute. The nuclei suspension was then filtered in a 50 µm nylon filter. The crude nuclei were sediment by a 5-min centrifugation of the filtrate at 800 rpm. The pellet was resuspended in 1 ml of 50 µg/ml propidium iodide (PI)/Triton X-100 stain-

ing solution with RNaseA (DARYNKIEWICZ and JUAN, 2001) for at least 30 min in the dark. The samples were then analyzed on a FACSCalibur Flow Cytometer (BD Biosciences, USA) with an argon laser emitting at 488 nm for excitation of propidium iodide. The mean nuclear DNA content of each plant sample was based on 10,000 scanned nuclei. A chromosome number and 2C DNA content known as diploid poplar tree 61# ($2\times=38$ and $2C\text{ DNA}=1.01\text{ pg}$) was used as an internal criterion. For each putative polyploid tree, the sample was independently characterized three times. Only measurements with coefficients of variation smaller than 8% were accepted. The polyploidy levels of the hybrid offspring were inferred by comparing the DNA content of the sample with the internal diploid.

Chromosome counting

The fresh root apical meristem tissues from water cultured cuttings for each of the 2 putative polyploidy trees were collected for somatic chromosome counts. Conventional squashes of root tip somatic cells were used under

Table 1. – The 2n pollen ratio of *P. × popularis* by colchicine induction.

Treatment No.	Meiosis stage	Colchicine concentration (%)	Injection times	Interval (h)	2n pollen rate* (%)	Arcsine conversion of 2n pollen rate	Pollen weight per catkin*	Comparative pollen weight
1	leptotene	0.2	3	2	2.95	9.97	4.60	1.15
2	leptotene	0.4	4	3	13.55	21.64	0.23	0.06
3	leptotene	0.6	5	4	41.28	39.99	0.73	0.18
4	pachytene	0.2	4	4	12.15	20.44	3.16	0.79
5	pachytene	0.4	5	2	32.25	34.63	1.26	0.32
6	pachytene	0.6	3	3	39.55	39.00	2.38	0.60
7	diakinesis	0.2	5	3	4.25	11.97	1.96	0.49
8	diakinesis	0.4	3	4	26.25	30.85	5.57	1.39
9	diakinesis	0.6	4	2	62.10	52.00	3.42	0.86
Mean1	23.867	14.127 ^a	26.607	32.200				
Mean 2	31.357	29.040 ^a	31.360	24.203				
Mean 3	31.607	43.663 ^b	28.863	30.427				
Range	7.740	29.536	4.753	7.997				

* Note: 2n pollen rate of untreated buds was 1.25%; Pollen weight per catkin of untreated control was 40 mg.

^{a,b} following the figures in the same columns means significantly differences at $P<0.05$ by q test or S-N-K test.

Table 2. – Variance analysis of 2n pollen ratio of *P. × popularis* by colchicine induction.

Factor	SS	df	F	F $\alpha=0.05$
Meiosis Stage	116.707	2	3.422	19.000
Colchicine	1308.664	2	38.581*	19.000
interval	105.821	2	3.120	19.000
Injection Times (Random Error)	33.920	2		

* Note: significantly differences at $P<0.05$.

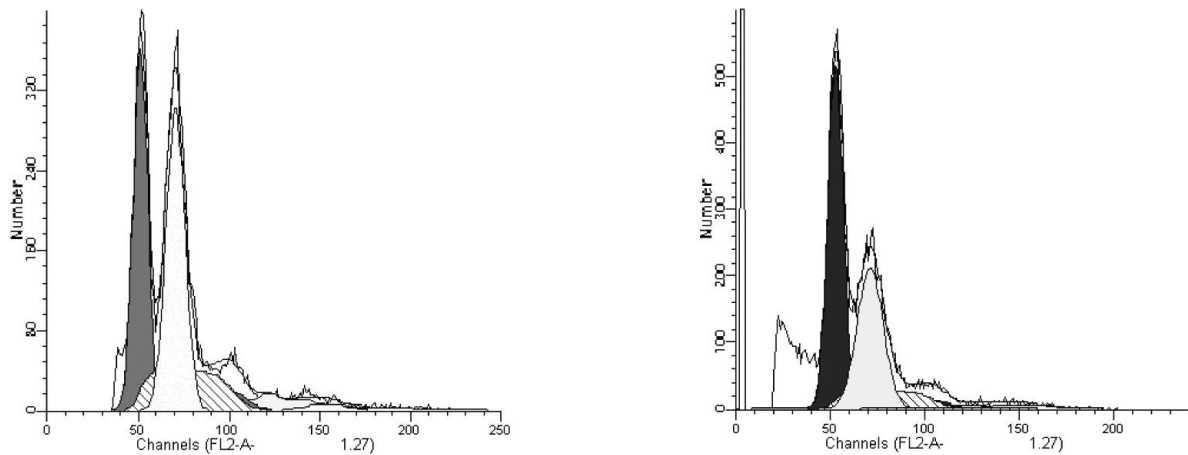


Figure 2. – The DNA content of triploids pollinated with $2n$ pollen of *P. × popularis*. (a) Flow cytometry of nuclear DNA content of triploid hybrid 65# and diploid control [left gray peak for control ($2\times$), right white peak for 65# ($3\times$)]; (b) Flow cytometry of nuclear DNA content of triploid hybrid 73# and diploid control [left black peak for control ($2\times$), right gray peak for 73# ($3\times$)].

a light microscope equipped with a digital camera system. The somatic cell squashes were prepared from root apical meristem tissues as described by LI (1996) with minor modifications. Briefly, actively growing root tips were cut and placed into a saturated solution of 1,4-dichlorobenzene for 3 h at room temperature to shorten chromosomes. Then the pretreated root tips were fixed in 3:1 ethanol-acetic acid solution for 24 h at room temperature. The fixed root tips were washed in flowing deionized water for 10 min, then digested in cytolase (95% ethanol:hydrochloric acid=1:1) solution at 30°C for 5 to 10 min. Chromosomes were stained with Carbor fuchsin. Thirty cell nuclei per individual were analyzed for chromosome counting.

Results

2n pollen induction

$2n$ pollen was obviously increased in treated buds comparison to untreated control buds (Figure 1) although the control had 1.25% $2n$ pollen and varied in size. Flower buds with 9 varied treatments showed different $2n$ pollen rate (Table 1). The result of variance analysis (Table 2) for every factor showed that only the difference among colchicine concentration levels was significant. Further q test showed that only 0.6% colchicine had significant difference from 0.2%. The best inducing factors combination selected by this Orthogonal Design experiment was diakinesis, 0.6% colchicine, 4 times of injection and 2 hours interval. This means that high $2n$ pollen rate can be achieved by selecting the flower buds during diakinesis stage in meiosis, then injecting 0.6% colchicine 4 times with 2 hours interval.

Triploid detection

Two triploids 65# ($3\times$) and 73# ($3\times$) were gained in the total 16 offspring of $A\times P$ by flow cytometer scan (Figure 2a, b) and further proved by chromosome counting (Figure 3b, c). The DNA content values of 65# was the $2C$ value = 1.37 ± 0.02 pg (the second value is the standard deviation), 73# $2C$ value = 1.59 ± 0.19 pg and

the internal criterion 61# $2C$ value = 1.01 pg. It is hard to obtain an unambiguous picture of all the chromosomes of a poplar. The chromosome counting results of average chromosome number from 30 nuclei per individual were for 65#, $2n=55$, for 73#, $2n=56$, and for the

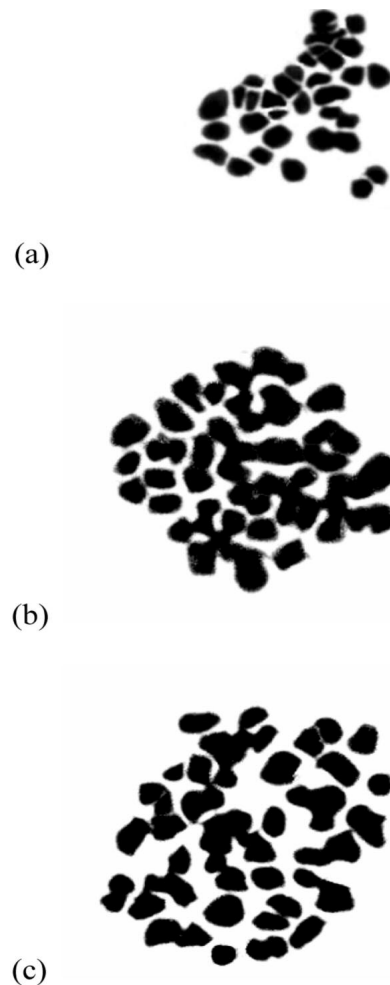


Figure 3. – The chromosomes of triploid hybrids poplar and their diploid control. (a) Diploid 61# ($2\times$); (b) Triploid 65# ($3\times$); (c) Triploid 73# ($3\times$).

diploid control 61#, $2n=36$; The maximal chromosome number for those three individuals were: for 65#, $2n=57$, for 73#, $2n=57$, and for the diploid control 61#, $2n=38$.

As shown in *Fig. 2a, b*, 65# ($3\times$) and 73# ($3\times$) had about 1.5 times more DNA content than 61# ($2\times$), respectively. They also had about 1.5 times chromosomes contrast to 61# ($2\times$) (*Figure 3a, b, c*).

Discussion

The technique of $2n$ pollen induction

The technique of $2n$ pollen induction is a systematic process involved in several key points. The first factor is the concentration of colchicine. It has significant influence in the $2n$ pollen induction, as showed in *Table 1*, at high level (0.4% and 0.6%) over the low level (0.2%). The second is meiosis stage of flower buds. Prophase I is an important stage for induction. We got little $2n$ pollen rate and almost as the same as control but the released pollen weight was dramatically dropped in comparison to the control when the flower buds were induced in other stage (data not shown). So, the observation of flower buds development is pivotal work. The judgment of prophase I of bud can either directly be observed by microscope or indirectly be judged by the colour of anther or both. The whole meiosis process developed in 3 days in green house, Prophase I normally keep two days. According to our experience, apical part of anther developed slowly than middle and basal parts, middle and basal parts almost developed as the same speed. So, we observed the middle anthers of bud by microscope only to judge the leptotene, pachytene, and diakinesis stage. The apical part was scissored out with bract together. This was not only good for keeping buds in the right stage but also good for injection to escape the bract glue to stick the needle. anther from green to yellow is at the prophase I stage. If it turns to light red or even deep red, it develops in to metaphase I or anaphase I. This is different from *P. tomentosa* (KANG et al., 2000b). Its catkin grows out of bract and anther turns light red at prophase I stage while the catkin of *P. × popularis* is wrapped in the bract at this stage. This is similar to *P. ussuriensis* (QI et al., 2009). Its catkin also is wrapped in the bract but the anther color is green at prophase I stage. Within prophase I, the efficiency of induction is obviously high in pachytene and diakinesis over in leptotene although the difference does not reach at the significant level in statistics. One of the advantages of Orthogonal design is to select a best combination of different level of every factor. In our experiment, the best inducing combination is diakinesis, 0.6% colchicine, 4 times of injection with 2h interval. Following this process, we induced *P. × popularis* in 2004, 2005 and 2006 and got 59.37%, 42.9%, and 73.95% $2n$ pollen, respectively. The efficiency of the process is stable and high.

Comparison of the methods for polyploid identification in poplar

Identification of polyploid is an important hinge for polyploidy breeding. Short or long breeding cycle mostly

depends on if polyploid can be identified quickly and accurately or not. So, the selection of identification methods is worth to carefully think about. All the methods can be divided into four types: morphological markers, chromosome counting, DNA content flow cytometer, and codominant DNA molecular markers. Morphological markers include leaf color (BRADSHAW and STETTLER, 1993), leaf or whole tree size (NILSSON-EHLE, 1936; MÜNTZING, 1936), and chloroplast number in the guard cell of stomata (EWALD et al., 2009). Chromosome counting is the direct evidence for polyploid identification but it is a time consuming and labour intensive work. Flow cytometric analysis is quick and accurate but it requires a special equipment and cost. Codominant DNA molecular markers can only distinguish part of the polyploid (ZHANG et al., 2009). Concerning the use of the morphological markers which were mentioned it has to be added that not all markers are of comparable importance. Leaf colour, size and shape are no reliable markers especially as it concerns hybrids of wide origin. It is the same with growth: among many fast growing plant there are triploids, but not all triploids are fast growing. Of course breeders are only interested in the fast growing ones. Flow cytometry is relatively cheap concerning the running costs, but it has some critical points. Nevertheless with internal standards, as it was shown here, it allows a fast and reliable determination of ploidy levels. Our results indicate that a good process of polyploid confirmation is using morphological markers such as chloroplast number first for a large scale pre-screening, then doing the further confirm test by flow cytometry or chromosome counting in a narrow scope.

Effect of $2n$ pollen in polyploidy breeding

$2n$ pollen as an essential material for sexual polyploidy can produce polyploidy without cytochimera like somatic induction and unstable performance. Triploids *P. tomentosa* derived from $2n$ pollen have stable fast growing character (ZHU et al., 1995) and are 2 to 3 times volume as the diploid control at 8 years old. $2n$ pollen can be used to overcome the hybrid barrier between different ploidy levels plant species. Cultivated potato ($4x$) crossed the $2n$ pollen of wild potato ($2x$) to increase the resistance (WATANABE et al., 1992). The basic effect of $2n$ pollen is to cultivate polyploid and this has a significant impact in the recently enhanced activities aimed at biomass production because of the characters of polyploid such as fast growth, high resistance, sterile of odd ploidy plant, more metabolic production and so on. So, induction of $2n$ pollen to cultivate polyploid in poplar could apply polyploidy and hybrid vigor simultaneously in one breeding program and has a prospective future.

In conclusion, it is possible for cultivating triploids via $2n$ pollen induction by colchicine treatment in poplar. The key points for $2n$ pollen induction in *P. × popularis* are colchicine concentration and meiosis stage of flower buds. Stable and high efficiency of $2n$ pollen inducing technique may accelerate the polyploidy breeding of poplar. The performance of triploids need further field test to select the applied clones for biomass production.

Acknowledgements

This research was supported by the Project of National Natural Science Foundation of China (No. 31070598), Chinese Chang-Jiang Scholar funds and the key Project of Chinese Ministry of Education (No. 109022). We thank Professor J. G. ZHANG and D. S. HUANG for offering the material of *P. × popularis*.

References

- BRADSHAW, H. D. and R. F. STETTLER (1993): Molecular genetics of growth and development in *Populus*. I. Triploidy in hybrid poplars. Theoretical and Applied Genetics **86**: 301–307.
- CHEN, Z. J. and Z. F. NI (2006): Mechanisms of genomic rearrangements and gene expression changes in plant polyploids. BioEssays **28**(3): 240–252.
- CONTRERAS, R. N., T. G. RANNEY and S. P. TALLURY (2007): Reproductive behavior of diploid and allotetraploid Rhododendron L. 'Fragrant Affinity'. HortScience **42**: 31–34.
- DARZYŃKIEWICZ, Z. and G. JUAN (2001): DNA Content Measurement for DNA Ploidy and Cell Cycle Analysis, pp. 1–24. In: Current Protocols in Cytometry, edited by J. P. ROBINSON, John Wiley & Sons Inc, New York.
- DOLEŽEL, J., P. BINAROVÁ and S. LUCRETTI (1989): Analysis of nuclear DNA content in plant cells by flow cytometry. Biologia Plantarum **31**(2): 113–120.
- EIGSTI, O. J. and P. DUSTIN (1955): Colchicine in agriculture, medicine, biology, chemistry. Iowa State College Press, Iowa.
- EWALD, D., K. ULRICH, G. NAUJOKS and M. B. SCHRÖDER (2009): Induction of tetraploid poplar and black locust plants using colchicine: chloroplast number as an early marker for selecting polyploids *in vitro*. Plant Cell, Tissue and Organ Culture **99**(3): 353–357.
- HANCOCK, J. F. (1997): The colchicine story. HortScience **32**: 1011–1012.
- HUANG, Q. J., Z. Y. ZHANG and X. Y. KANG (2002): 2n pollen of *P. tomentosa* × *P. bolleana* induced by four antimicrotubule agents. Journal of Beijing Forestry University **24**: 12–15.
- KANG, X. Y., J. ZHANG, P. D. ZHANG and Y. H. LI (2006): Induction of triploids in white poplar, pp. 71 in International Poplar Symposium IV, Nanjing.
- KANG, X. Y., Z. T. ZHU and H. B. LIN (2000a): Radiosensitivity of different ploidy pollen in white poplar and its application. Acta Genetica Sinica **27**: 78–82.
- KANG, X. Y., Z. T. ZHU and Z. Y. ZHANG (2000b): Meiosis and its stages of pollen mother cells in Chinese white poplar. Journal of Beijing Forestry University **22**: 5–7.
- LI, M. and Z. ZHANG (1996): Crop chromosome and its research technique. China Agricultural Press, Beijing.
- MASHKINA, O. S., L. M. BURDAEVA and M. M. BELOZEROVA (1989): Method of obtaining diploid pollen of woody species. Lesovedenie **1**: 19–25.
- MÜNTZING, A. (1936): The chromosome of a giant *Populus tremula*. Hereditas **21**: 383–393.
- NI, Z. F., E. D. KIM, M. HA, E. LACKEY, J. X. LIU and Y. R. ZHANG et al. (2009): Altered circadian rhythms regulate growth vigor in hybrids and allopolyploids. Nature **457**(7227): 327–331.
- NILSSON-EHLE, H. (1936): Über eine in der Natur gefundene Gigasform von *Populus tremula*. Hereditas **21**: 379–382.
- OLSEN, R. T., T. G. RANNEY and Z. VILORIA (2006): Reproductive behavior of induced allotetraploid × Chitalpa and *in vitro* embryo culture of polyploid progeny. Journal of the American Society for Horticultural Science **131**(6): 716–724.
- QI, C. L., K. L. LI, J. XIAO, Z. X. LI, C. L. JIN and W. H. TENG (2009): Meiosis of Pollen Mother Cells and Pollen Development of *Populus ussuriensis*. Journal of Northeast Forestry University **37**: 72–75.
- RAMSEY, J. and D. W. SCHEMSKE (1998): Pathways, mechanisms, and rates of polyploidy formation in flowering plants. Annual Review of Ecology and Systematics **29**: 467–501.
- RANNEY, T. G. (2006): Polyploidy: From evolution to new plant development. Combined Proceedings, International Plant Propagators' Society **56**: 137–142.
- SOLTIS, D. E., P. S. SOLTIS and J. A. TATE (2004): Advances in the study of polyploidy since Plant Speciation. New Phytologist **161**(1): 173–191.
- VOUCHKOV, I. N. and L. N. BOYADJIEVA (2001): Quality Improvement with Design of Experiments: A Response Surface Approach. Springer-Verlag, Berlin.
- WATANABE, K., H. M. EI-NASHAAR and M. IWANAGA (1992): Transmission of bacterial wilt resistance by first division restitution (FDR) 2n pollen via 4x × 2x crosses in potatoes. Euphytica **60**(1): 21–2.
- WENDEL, J. F. (2000): Genome evolution in polyploids. Plant Molecular Biology **42**(1): 225–249.
- XU, J. R. and Z. H. HUANG (1999): Forestry Experimental Design. Chinese Forestry Publishing House, Beijing.
- ZHANG, J. F., Z. Z. WEI, D. LI and B. L. LI (2009): Using SSR markers to study the mechanism of 2n pollen formation in *Populus × euramericana* (Dode) guinier and *P. × popularis*. Annals of Forest Science **66**(5): 53–62.
- ZHANG, S., L. QI, C. CHEN, X. LI, W. SONG and R. CHEN et al. (2004): A report of triploid *Populus* of the section *Aigeiros*. Silvae Genetica **53**(2): 69–75.
- ZHU, Z. T., H. B. LIN and X. Y. KANG (1995): Studies on allotriploid breeding of *Populus tomentosa* B301 clones. Scientia Silvae Sinicae **31**(6): 499–505.