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Full Length Research Paper

Polyploidy induction of clone of *Eucalyptus grandis* with colchicine

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The study was conducted to find the dependency of polyploid inductivity rate of axillary buds of *Eucalyptus grandis* clone Eg5 with colchicine on treatment conditions by applying Plackkett-Burman design and uniform design. According to regression analysis, the functional relation equation between polyploid inductivity rate and independent variables including colchicines concentration and soaking time was found with Adj R^2 as 0.9366 which is ideal. The maximum inductivity rate can reach 51% when the soaking time is 26.2 h and the colchicines concentration is 0.21%. The study result shows that inductivity rises at first and drops later with continuous increases of colchicine concentration and soaking time. The inductivity drops, because of the mass mortality of treated axillary buds. The difference of tree height between tetraploid plants and untreated plants was significant while the difference of ground diameter between them did not reach significant level.

Key words: Colchicine, polyploidy, eucalyptus, osmotic agent.

INTRODUCTION

Forestry ploidy breeding is a very important breeding method to acquire new variety. The vegetative organs of polyploid forest are usually relatively giant, so the technology of ploidy breeding can increase the yield and the quality of harvest, and can improve the resistance of breeding object (Janick and Moore, 1996; Bao and Louis, 1984), such as the triploid of Chinese white poplar, whose annual volume growth significantly increases.

Eucalyptus is a woody perennials which belongs to Myrtaceae (Eldridge, 1993). Nowadays, it is one of the fastest growing forest species in China because of its rapid growth, high level of adaptability and wide planting. So its area of artificial forest has reached 3,000,000 hectares in China and has ranked third in the world. Under serious wood shortage in China, if the new species of *Eucalyptus* can get higher yield, resistance and better quality, it can bring significant economic and social benefits. According to Leran's experience, the species which have cross pollination, perennial habit, vegetative propagation, fewer chromosomes and the nutritive organs needed by people are suitable to carry out the ploidy breeding (Kang, 2003; Hansen, 1998). The physiological characteristics of *Eucalyptus* are basically in line with the requirements earlier mentioned. *Eucalyptus* has the potential to become an improved diploid variety by doubling its chromosomes. However, preliminary studies concerning polyploid breeding of *Eucalyptus* have been done only on clone GL9 of *E. grandis* × *E. urophylla* (Hu et al., 2004), clone 12 ABL of *Eucalyptus* (Tan et al., 2005), and *Eucalyptus globulus* Labill (Lin et al., 2010; Notsuka, 2000). There are no ploidy breeding studies on the *E. grandis*.

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MATERIALS AND METHODS

This study used clone Eg5 of *E. grandis* as experimental material.

Establishment of main influencing factors

According to preliminary experiments and some references, the following eight factors were found to possibly affect inductivity rate of clone tissue of *E. grandis*; starting time, soaking concentration, soaking time, shake speed during soaking, concentration of penetrant, tenderness degree of treated stem segment and

Table 1. Factors and levels of Plackett-Burman design.

Factors	Factor levels and codes		Factors	Factor levels and codes	
	-1 level	1 level		-1 level	1 level
Starting time	8:00 am	12:00 am	Shake speed	80 r·min^{-1}	120 r·min^{-1}
Soaking concentration	0.15%	0.25%	Concentration	2%	3%
Soaking time	10 h	25 h	Tenderness degree of treated stem segment (d)	15	25
Soaking temperature	30°C	40°C	Colchicines concentration in medium	20 mg•L^{-1}	60 mg•L^{-1}

Table 2. Plackkett-Burman design of buds of polyploidy induction.

Test number	Factors of the experiment							
	Starting time (am)	Colchicines concentration* (%)	Soaking time (h)	Soaking temperature (°)	Shake speed (r·min^{-1})	Osmotic agent concentration (%)	Degree of tenderness (d)	Colchicine concentration in medium (mg·L^{-1})
1-1	12:00	0.15	25	30	80	2	25	60
1-2	12:00	0.25	10	40	80	2	15	60
1-3	8:00	0.25	25	30	120	2	15	20
1-4	12:00	0.15	25	40	80	3	15	20
1-5	12:00	0.25	10	40	120	2	25	20
1-6	12:00	0.25	25	30	120	3	15	60
1-7	8:00	0.25	25	40	80	3	25	20
1-8	8:00	0.15	25	40	120	2	25	60
1-9	8:00	0.15	10	40	120	3	15	60
1-10	12:00	0.15	10	30	120	3	25	20
1-11	8:00	0.25	10	30	80	3	25	60
1-12	8:00	0.15	10	30	80	2	15	20

colchicines concentration in medium. The penetrant used is Azone. The days from the last time of transferring untreated proliferation seedlings is defined as the tenderness degree of treated stem segment. “Colchicines concentration in medium" refers to the concentration of colchicines added to modified Murashige and Skoog medium (MS medium) used in culturing treated stem segment after treatment (Aneja, 1969). The mass of colchicines contained in one liter of medium after sterilization at 100°C is defined as the concentration value. Plackkett-Burman design is used to find the significant factors on the inductivity rate of polyploid. Plackkett-Burman design is shown in Tables 1 and 2.

The use of uniform design

The two significant factors, "soaking concentration" and "soaking time", are used in uniform design of polyploid induction of buds as shown in Table 3 (Fang et al., 1994).

Implementation of the experiment

The tissue culture seedlings to be treated were cut into segments of 1 cm on a clean bench. The leaves were cut off and segments with auxiliary buds were selected for polyploidization. About 100 ml of sterile colchicine solution of different concentrations was poured into a 250 ml bottle. Auxiliary buds of a specific amount to be treated were put into the bottle. The cap was twisted on tight, and the bottle was then put on a shaker and treated at a specified speed and for a certain time. After treatment, segments were rinsed with sterile water to remove residual colchicine solution. Then, the segments were transplanted in MS medium. The culture temperature was set at 25°C. After 15 to 20 days, they were transferred once. During the

Table 3. Uniform design of polyploidy induction of buds.

Test number	Colchicine concentration (%)	Soaking time (h)
2-1	0.10	28
2-2	0.14	22
2-3	0.18	32
2-4	0.22	26
2-5	0.26	20
2-6	0.30	30
2-7	0.34	24

Table 4. Inductivity rate of Plackkett-Burman experiment.

Test number	1-1	1-2	1-3	1-4	1-5	1-6	1-7	1-8	1-9	1-10	1-11	1-12
Inductivity (%)	43	21	47	41	22	49	50	42	18	16	24	15

operation, the sterile tissue culture seedlings were being contaminated. After the seg-ments are transferred three times, flow cytometry is used to test the leaves for the ploidy of treated plants. Root ends of proliferated induced plantlets of both polyploidized and pre-polyploidized are obtained. Then they are compressed and stained, and chromosomes are counted.

Analysis of experimental data

SAS software is used for the analysis of Plackkett-Burman experimental design. Two softwares, 1stOpt and Matlab, were used for the fitting of regression equation. The Origin Software is used for making a 3D diagram and contour mapping.

RESULTS

Detection of inductivity rate

Before treatment, the ploidy of tissue culture seedlings was 2x, and after treatment, plantlets of 4x ploidy was obtained. Inductivity rates of each treatment in Plack-kett-Burman design are shown in Table 4. Polyploidized tetraploid tissue culture seedlings and untreated diploid tissue culture seedlings were compared in terms of body appearance. The differences are shown in Figure 1.

SAS software was used to analyze the data obtained. The results are shown in Table 5. Table 5 shows that the two independent variables, "colchicine concen-tration" and "soaking time", reach significant level. So in the uniform design in the next step, only these two independent variables were retained.

Analysis of uniform design

Flow cytometry is used to test treated buds according to the uniform design to determine ploidy. The measured inductivity rates are shown in Table 6.

The concentration of colchicines was set as x_1, the soaking time as x_2, and tetraploid inductivity rate as y_1. Regression analyses were made according to the experiment design and the data was obtained. Linear regression was analyzed, and the fitting effect is shown in Table 7.

It can be seen from Table 7 that the relationship between y_1 and x_1, x_2, is not a simple linear regression. Non-linear regression is needed for curve fitting. The softwares MATLAB and 1stOpt were used for modeling. The coefficients of curve equations were determined. The regression equation was obtained as follows:

$$y_1 = -1.15410 + 300.39062x_1 - 86914x_1^2 + 0.10258x_2 - 0.00207x_2^2 - 2.72438x_1x_2$$

Regression effect is satisfactory because Adj R^2 is 0.9366. The Lingo Software was used to obtain the maximum value of y_1 which was 0.51. Then, x_1 and x_2 were set as 0.0021 (0.21%) and 26.2, respectively. This shows that when the colchicine concentration was 0.21% and the soaking time was 26.2 h, the inductivity rate reached the maximum (51%). This result is con-sistent with the one obtained in experimental conditions number 2 to 4 in the uniform design.

Origin Software was used to make the 3D diagram

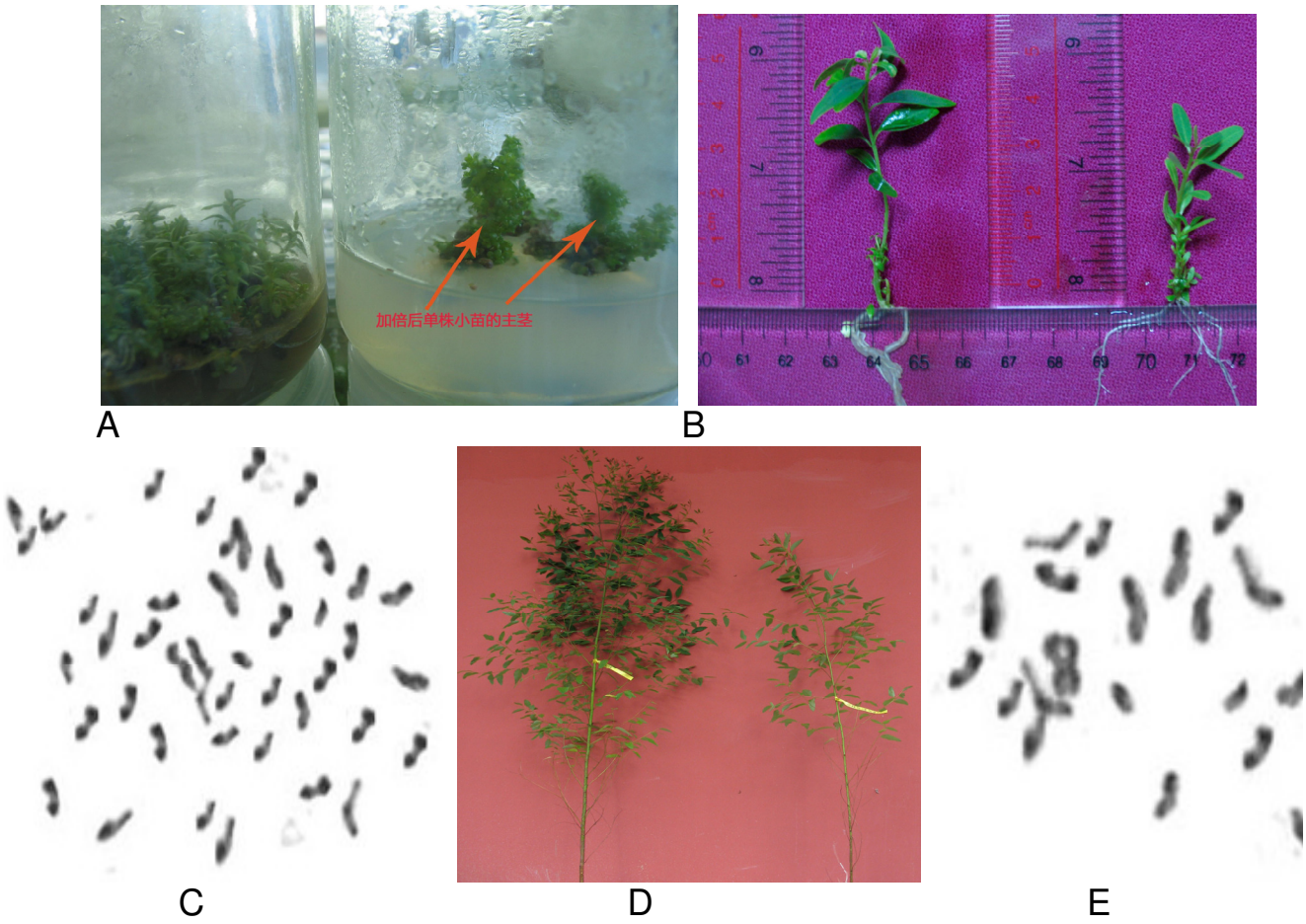

Figure 1. The comparison between the plantlet without treatment and the plantlet with treatment. a: The tissue culture seedlings without treatment are in the left bottle and those with treatment are in the right bottle; b and d: The tissue culture seedling with treatment is on the left while the one without treatment is on the right; c: Chromosome map of tetraploid plantlet of Eg5 clone of *E. grandis*; e: Chromosome map of diploid planlet of Eg5 clone of *E. grandis*.

Table 5. Results and analysis of Plackkett-Burman experiment.

Factors	t	Pr > |t|	Statistic significance
Starting time	-1.04447	0.3730	
Soaking concentration of colchicine	9.922426	0.0022	**
Soaking time	40.73417	0.0001	**
Soaking temperation	$7.25*10^{-16}$	1.0000	
Shaking speed	$-2.17*10^{-15}$	1.0000	
Osmotic agent concentration	2.088932	0.1279	
Degree of tenderness of treated stems	1.566699	0.2152	
Concentration of colchicines in medium	1.578962	0.2056	

and contour map which reflect response variables and independent variables. They are shown in Figure 2.

Through the expression of function, it is observed that when the inductivity rate is obtained and x_2 is fixed, y_1 increases along with x_1, and then decreases. In other words, the inductivity rate increases with the increase of concentration, and then decreases afterward. When x_1 is fixed, y_1 increases with x_2 and then decreases also. However, the inductivity rate increases and then decreases with the treatment time which was continuously extended.

In the experiment, it was found that when colchicine concentration was higher than 0.21% and the soaking time was greater than 26.2 h, the inductivity rate rapidly

Table 6. Results of uniform design.

Test number	Soaking concentration. of colchicines (x_1, %)	Soaking time (x_2 , h)	Inductivity rate (y_1, %)
2-1	0.10	28	0.39
2-2	0.14	22	0.43
2-3	0.18	32	0.42
2-4	0.22	26	0.51
2-5	0.26	20	0.41
2-6	0.30	30	0.43
2-7	0.34	24	0.35

Table 7. The fitting effect of linear regression

Root MSE	0.05809	R^2	0.0493
Dependent Mean	0.42000	Adj R^2	-0.4261

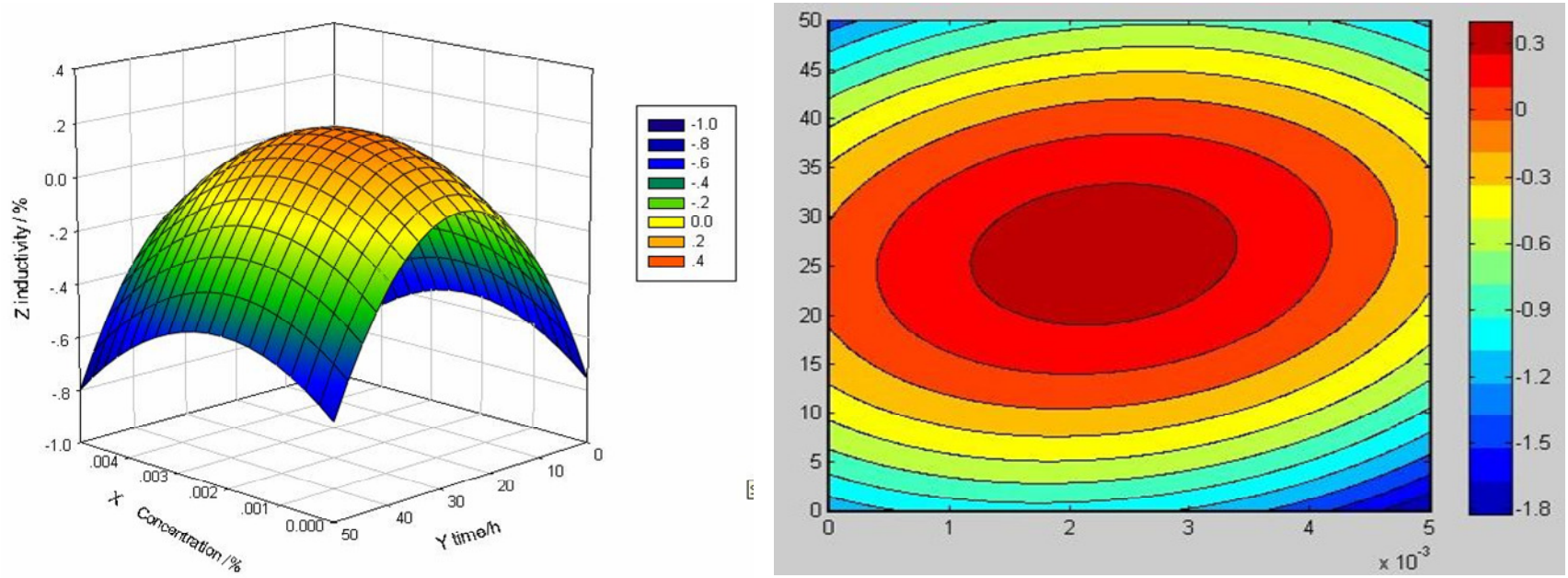

Figure 2. Trend surface and contour graph of the relationship between inductivity and independent variables including colchicine concentration and soaking time.

Table 8. Variance Analysis of tree height between plants with and without treatment

Parameter	Sum of Squares	df	Mean Square	F	Significance
Between Groups	0.123	1	0.123	9.281	0.016
Within Groups	0.106	8	0.013		
Total	0.229	9			

Table 9. Variance Analysis of ground diameter between plants with and without treatment

Parameter	Sum of Squares	df	Mean Square	F	Significance
Between Groups	0.014	1	0.014	4.068	0.078
Within Groups	0.028	8	0.004		
Total	0.043	9			

declined because mortality rate of treated buds continuously increased and eventually reached 100%. It was shown that when the solution of colchicine doubles buds' chromosomes, at the same time, under conditions of larger concentration and longer time, buds were also seriously poisoned and harmed to death.

Table 8 shows that the tree height differences bet-ween treated and untreated plantlets are significant. Table 9 shows that the ground diameter differences between treated and untreated plantlets are significant.

DISCUSSION

Compared with the orthogonal design, the number of experiments of uniform design will not greatly increase even when experimental treatments are many (Liu et al., 2005). This significantly reduces the test volume and saves test cost. In this study, both flow cytometry and chromosome microscopic examination were used to test ploidy. The two methods were compared, and it was found that flow cytometry testing was fast and labor-saving, especially in case of a large number of samples.

In this study, obtained tetraploid tissues still have the possibility of becoming chimerism. They can be purified through the following two methods to become a com-plete tetraploid plant:

(1) Plants are tested to determine whether or not they are really tetraploid plants and then explants are taken for tissue culture (or cuttage). New plants are obtained and tested again. If chimerism is found, then this process is repeated until tetraploid plants are found.
(2) If the treated plant gets normal reproductive development and can produce flowers and seeds, and if the ploidy of hermaphroditic zygote is 2x, self-pollination is used to obtain homozygous tetraploid.

In Tan et al. (2005) polyploid induction study of *E.* 12ABL, the highest inductivity rate reached 40% lower than the maximum inductivity rate of tetraploid *E. grandis* in this study. In Tan's experiment, under both kinds of conditions (10 h, 0.75% and 0.25%, 22 h), the maximum inductivity rate was obtained. In this study, only under colchicine concentration of 0.21% and soak-ing time of 26.2 h was the maximum inductivity rate obtained. After induction, Hu (Hu et al., 2004) obtained treated plants of clone GL9 of *E.* by adding 3 to 5 chromosomes on basis of 22 chromosomes. This is not consistent with the fact that tetraploid plants should have 44 chromosomes (Figure 1c).

It is necessary to continue to track growth comparison between the polyploidized and un-polyploidized clone Eg5 of *E. grandis*. Then, it can be determined whether the tetraploid plant has better growth and whether the plant has potential for farming production. In other species, polyploids of different ploidies were compared, and it was found that triploids have more feasibility of excellent traits. Through sexual reproduction, tetraploid clone Eg5 obtained in this study can produce triploid clone Eg5 of *E. grandis* and triploid of interspecific hybridization. Therefore, it can provide new superior variety for the subsequent breeding of triploid *Eucalyptus*.

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