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Full Length Research Paper

Production of polyploids from cultured shoot tips of *Eucalyptus globulus* Labill by treatment with colchicine

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Polyploids from cultured shoot tips of *Eucalyptus globulus* were produced by treatment with colchicine. Results showed that the combination of 0.5% colchicine and treating multiple shoot clumps for 4 days was the most appropriate conditions for *E. globulus* polyploidy induction and the effect of the use of multiple shoot clumps for colchicine polyploidy-induced was better than single buds. By comparing the polyploidy plants with normal diploid ones in morphology, leaves of polyploid plants were thicker, larger, and darker green. The chromosome number of polyploidy plants that had been identified in morphology was 2n = 4x = 44, while that of diploids was 2n = 2x = 22.

Key words: *Eucalyptus globulus*, polyploids, cultured shoot, colchicine.

INTRODUCTION

Eucalyptus globulus, a bluegum plant of the family *Myrtaceae* (Liu et al., 2004), is one of the most widely planted eucalypt in the world. It is widely distributed in Guangdong, Guangxi, Yunnan and Sichuan Provinces of China (Liu et al., 2003) and is widely grown in Australia, Portugal, Spain, Chile, China, Colombia, Ethiopia, Peru, USA (California) and several other countries (Li et al., 2000). The leaves, root barks and fruits have been used as folk medicine for anti-inflammation, antimicrobial, anticancer, treatment of injures and so on (Chen et al., 2002). Now tissue culture of *E. globulus* was successful and the seedlings from tissue culture were used in forestations widely (Mo et al., 2009). Currently it is the subject of many genetic improvement programs (Eldridge et al., 1993; Byrne et al., 1997; Gion et al., 2000; Moran et al., 2002).

Colchicine is used for inducing polyploidy in plant cells during cellular division by inhibiting chromosome segregation during meiosis. In plant cells it is not only usually well tolerated, but results in plants which are higher contain chemical constituents, larger, hardier, faster growing and in general more desirable than the normally diploid parents; for this reason, this type of genetic manipulation is frequently used in breeding plants commercially. But there is still no report about the inducing polyploidy of *E. globulus*. The aim of the present study was to induce the polyploidy and identify it by detecting the number of chromosome in regenerated plants of *E. globulus* treated by colchicine.

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Abbreviations: IAA, Indole-3-acetic acid; **DMSO,** dimethyl sulphoxide; **MS,** Murashige and Skoog; **NAA,** α-naphthalene acetic acid; **IBA,** indole-3-butyric acid; **BA,** 6-benzylaminopurine.

MATERIALS AND METHODS

Explants source, surface sterilization and initial culture

Shoot tips were excised from the trees of *E. globulus* Labill at Southwest Forestry University. All the explants were rinsed for 30 min under tap water. Before use, the explants were immersed in 70% ethanol for 8 - 10 s and then in 0.1% $HgCl_2$ for 5 - 8 min (depending on degree of tenderness). Finally, the explants were washed with 4 or 5 rinses of sterile water to eliminate the sterilization agent. The sterilized explants were cut into segments with one to two axillary buds and inoculated in Murashige and Skoog's medium supplemented with 0.2 mg/l indoleacetic acid (IAA) to select optimal medium for initial culture.

Tissue culture and treatment of colchicine

Tissue culture was performed as described by Mo et al. (2009). Calli induction was carried on the modified MS medium described as Li et

Table 1. Polyploid induction effect of colchicine gradient and the processing time for multiple shoot clumps.

Colchicine concentration (%)	Processing time (days)	Number of multiple shoot clumps	Number of mortality	Mortality rate (%)	Number of variation	Rate of variation (%)
0.2	2	30	3	10	2	6.7
	3	30	4	13.3	3	10
	4	30	9	30	7	23.3
	5	30	10	33.3	6	20
0.3	2	30	3	10	3	10
	3	30	5	16.7	4	13.3
	4	30	10	33.3	8	26.7
	5	30	12	40	9	30
0.4	2	30	5	16.7	2	6.7
	3	30	7	23.3	4	13.3
	4	30	12	40	8	26.7
	5	30	17	56.6	7	23.3
0.5	2	30	6	20	3	10
	3	30	10	33.3	5	16.7
	4	30	13	43.3	11	36.7
	5	30	18	60	9	30

al. (2000). For initial multiple shoot induction, the explants were cultured on Murashige and Skoog's medium supplemented with 2.0 mg/l BA + 0.5 mg/l IBA + 0.1 mg/l NAA + 20 g/l sugar. Small micro shoots grown on subculture medium were transferred to MS media supplemented with 2.0 - 2.5 mg/l BA + 0.5 mg/l IBA + 0.1 mg/l NAA + 20 g/l sugar for elongation. The elongated shoots were placed into 100 ml solution containing 750 mg/l colchicine and 20 ml/l of dimethyl sulphoxide (DMSO) and the tops were immersed 3 cm under the surface of the solution. Enough light was supplied and the plants were treated under a temperature of 23 to 24℃ for 5 h. At the end of the treatment the shoots were transferred to elongation culture medium. After 20 days the tops were harvested and transferred to subculture medium for elongation again. The elongated shoots of 3 - 4 cm length were subsequently transferred to half strength liquid MS medium supplemented with 0.5 mg/l IBA + 0.05 mg/l NAA + 15 g/l sugar for rooting. After rooting, the bottoms of plants were washed thoroughly with running tap water and then planted into pots and kept at a temperature of about 20℃ for two weeks. And they were transplanted to the field.

Chromosome observation

Root tip meristematic cells from plants were used as a source of mitoses. Excised roots were treated with 2 mM 8 -hydroxyquinoline for 1-4 h at room temperature, fixed in a 3:1 (v/v) mixture of methanol and glacial acetic acid and stored at -20℃ until required. Excised roots were washed in 0.01 M citric acid-sodium citrate buffer (pH ~ 4.8) for 20 min and digested enzymatically for 1-1.5 h at 37℃ in a mixture of 1% (w/v) cellulase (Sangon), 1% (w/v) Onozuka R-10 cellulase (Sangon) and 20% (v/v) pectinase (Sigma). After separation from the non-meristematic parts, root tips were squashed in a drop of 45% acetic acid and the preparations were frozen. Cover slips were removed and the preparations were postfixed in chilled 3:1 (v/v) ethanol: glacial acetic acid, followed by dehydration in absolute ethanol and air-drying. All images were acquired using an Olympus Camedia C-4040Z digital camera attached to a Leica DMRB microscope. Image processing was done using Micrografx (Corel) Picture Publisher software.

RESULTS AND DISCUSSION

The tight calli with shoot organic potentiality could be obtained on the modified MS medium described as Li et al. (2000). In contrast, we founded that only loose calli without shoot organic potentiality were obtained on MS medium supplemented with 0.2 mg/l indoleacetic acid (IAA). And we also found that coconut milk had no effect for inducing callus. The calli began to form multiple shoot clumps after transferred to multiple shoot induction medium 15 days. And the callus would also become bigger after transferred to subculture medium. In the optimal medium for multiplication, the propagation coefficient was 4.7. In the rooting medium, the rooting rate was 100% and the mean root number of 3.9.

The impacts of processing time and colchicine concentration on eucalyptus buds polyploidy-induced were remarkable. With colchicine processing time extension, induction rate would increase. As a result of that, colchicine had a strong toxic effect on cells, after dealing with up to a certain length of time, the induction rate of increase with the time was not so obvious, but would increase the bud mortality. When the processing time up to the 4d or 5d, the mortality rate were highest and both of them were more than 30% (Table 1). While the induction rate was on the same level, the mortality rate of 5d was 10% higher than that of 4d (Table 1). Hence, processing 4d was more appropriate. The polyploidy induction rate was increased with the concentration of colchicine and 0.5%

Table 2. The Comparison of effect for processing single shoots and multiple shoot clumps with 0.5% colchicine.

Materials	Processing time (days)	Number of shoots (or clumps)	Number of mortality	Mortality rate (%)	Number of mutation	Rate of mutation (%)
Single shoots	2	30	7	23.3	1	3.3
	3	30	13	43.3	3	10
	4	30	17	56.7	9	30
	5	30	21	70	6	20
Multiple shoot clumps	2	30	6	20	3	10
	3	30	11	36.7	5	16.7
	4	30	15	50	11	36.7
	5	30	19	63.3	7	23.2

Figure 1. *Eucalyptus globulus* plants and the ones treated by colchicine. Note: Plants in the left bottle were *Eucalyptus globulus* and plants in right one were the *Eucalyptus globulus* polypoid.

of colchicine had the greatest effect of the stimulation and the rate of mutation was highest. Because of the highest mortality rate (60%) at the concentration of 0.5%, taking into account the induced efficiency, higher concentrations were not been conducted in the experiment. Hence, a combination of 0.5% colchicine and treating multiple shoot clumps for 4 days was the most appropriate condition for *E. globulus* polyploidy induction.

Treated with 0.5% colchicine for 2 ~ 5d, respectively, to deal with multiple shoot clumps and single bud, the results showed that the rate of colchicine-induced mutations among single-bud slightly was a little bit higher (Table 2). However, mortality rate among the single-bud was also high and the highest reached 70% (Table 2). The recovery time of single buds were much longer than that of the multiple shoot clumps and the growth of former one was much slower than the later one. Therefore the effect of the use of multiple shoot clumps for colchicine polyploid-induced was better than single buds. Increase of colchicine processing time and concentration, the stimulating effect to the buds would also enhance and the mutation rates would also increase, and as a result of that colchicine were poisoned to multiple shoot clumps, the mortality would also increased (Table 2).

By comparing the polyploidy plants with normal diploid ones in morphology, leaves of polyploidy plants were

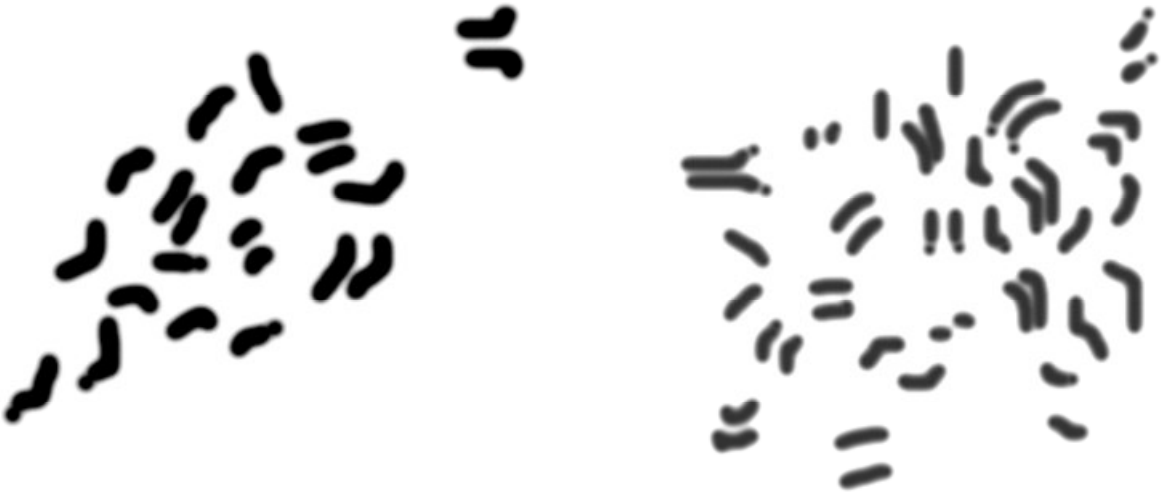

Figure 2. Chromosome number of *Eucalyptus globulus* plant and the one of the micropropagated plants treated by colchicine. Note: The left picture was the normal cell of *Eucalyptus globulus* and the chromosome number was 22; the right one was the polyploidy cell and the chromosome number was 44.

thicker, larger, and darker green (Figure 1). The figure 1 showed that some of the plants had become polyploidy and some of them had not, even they were provided with the same conditions (in the same bottle). The chromosome number of polyploidy plants that had been identified in morphology was 2n = 4x = 44, while that of diploid was 2n = 2x = 22 (Figure 2). Results of chromosome observation showed that the manufacture of chromosome doubling was successful and the chromosome numbers of some the plants treated had been doubled.

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