

Full Length Research Paper

Preliminary study on the inducement effect of colchicine during microsporogenesis of *Ginkgo biloba* L.

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Accepted 2 May, 2011

This is the first report of colchicine treatment effect on microsporogenesis of *Ginkgo biloba* L. By high performance liquid chromatography (HPLC) analysis, colchicine was detected in microsporocyte inclusion from colchicine-treated microsporangium and microsporocyte of *G. biloba*. The change amount of colchicine was detected in mixture liquid after the protoplasm of *G. biloba* was co-cultured with colchicine by different conditions. Fixed with Carnoy's fluid and stained with improved phenol fuchsin, the development of colchicine-treated microsporogenesis could be examined with microscope. The results are as follows: The colchicine could intrude into treated microsporangium and microsporocyte, but intruded amount of colchicine was different when treatment conditions were different; the change of colchicine content in co-cultured mixtures liquid was few; and there were high frequencies of development tardiness in colchicine-induced microsporangium (more than 70%), but only a few dyads and triads were detected in meiotic products (less than 10%). These observations suggest that 2n pollen can be induced by colchicine but treatment conditions and slowing development of colchicine-treated microsporocyte may affect the inducement effect of colchicines.

Key words: *Ginkgo biloba* L., microsporogenesis, colchicine, 2n pollen, high performance liquid chromatography (HPLC).

INTRODUCTION

Ginkgo biloba L. is widely planted as an ornamental, the unusual shape of the crown, natural resistance to disease and yellow leaf color in fall make this a favorite street and park tree. It is a native single section species and being under state protection in China (Franklin, 1959; Chen, 2001). Various researches were conducted on its economic and ecological values, particularly in its value in pharmaceuticals. Researchers from China, Switzerland, France, Germany, Japan, the United States and several other countries have been working extensively on its

medical application (Du and Zhao, 2003; Andreas et al., 1992; Brunetti et al., 2006; DeKosky et al., 2008). Researches and application in this area have been popular since 1980s (Xing et al., 1997). Currently, *G. biloba* leaf extracts are short supplied in international pharmaceutical market and its plantation for other applications, such as landscaping, are also well demanded. It is highly desired to breed new cultivars with rapid growth, higher leaf yield and higher special secondary metabolite contents in leaves and fruits.

We urgently need to add the genetic improvement ways to increase the productivity of *G. biloba* for meeting the needs of the market. The methods used in genetic improvement of *G. biloba* is included: selective breeding (Li and Liu, 1999), crossing breeding (Huang and Dong, 2001), mutation breeding (Hu, 1998), clonal selection (Huang et al., 1997), genetic engineering and molecular marker (Wang et al., 2002). These measures can act on

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Abbreviations: HPLC, High performance liquid chromatography.

improving the productivity of the leaves and speed of growth to limited extent only (Tate et al., 2005). Triploid plants have fast growth, organs huge and high content of secondary metabolites, etc. The use of 2n pollen is a time-saving breeding of triploid efficient way, for which this experiment carried out relevant studies (Cai, 1988). Therefore, exploring the breeding of triploid *G. biloba* is most likely to make a breakthrough in the improving the thickness, size and quantity of leaves and increasing the account of secondary metabolites content as well as gaining the speed of growth.

Drawing on the successful experiences of ploidy breeding of other plants, one of the most rapid and the best method of breeding triploid trees is described as follows: Firstly, the doubled chromosome of male gamete to be induced and then put this doubled gamete on normal stigma, completing fertilization with normal female gamete and forming the 3n embryo (Einspahr, 1984; Kang, 2003; Gao et al., 2004). So, to achieve the purpose of breeding of triploid plants, we must select 2n pollen from nature or induce 2n pollen artificially. Some of 2n pollens can be found in some plants under natural conditions (Cai, 1988), but this phenomenon was never found in *G. biloba*, so we have to get them by artificial inducement.

Pre-meiosis, meiotic abnormalities and post-meiotic cytokinesis abnormalities, which are necessary to form 2n pollen (Kathleen et al., 1987), can be induced by colchicine treatment on flower buds. There are many plants which have obtained 2n gametes by using these methods. So, if we handle the microsporocyte of *G. biloba* with colchicine at proper time, the rate of mutation should be similar to the rate of other plants, such as in *Populus* (Kang et al., 1999; Zhang, 2006) and in *Eucommia* (Gao et al., 2004).

Our research group has studied on the 2n pollen induced by colchicine treatment for five years. From the experiment, we found that 2n pollen could be produced from the treated microsporocyte by colchicine, but the rate of mutation was still lower than that of other tree species (white *Populus* 88% (Kang et al., 1999), *Eucommia* 49.5% (Gao et al., 2004), black *Populus* 90.25% (Zhang, 2006), even the best result of our experiments was only 7% (Cheng et al., 2006). It is not enough to be used for the breeding of the triploid plants in *G. biloba*.

The main purpose of this study was to study the inducement effect of colchicine during microsporogenesis when the 2n pollen of *G. biloba* was induced by colchicine; to verify whether the colchicine residues in the *G. biloba* cell, whether the microsporangium, microsporocyte's wall and microsporocyte's protoplasm can interrupt or hinder the colchicine treating effects and to observe what the growth process of the microsporocyte is during microsporogenesis after the colchicine intrude into the microsporocyte of *G. biloba*. The results can help us to find the reason of the lower rate of 2n pollen production after microsporangium of *G. biloba* was treated by colchicine.

MATERIALS AND METHODS

Plant material culture

The male floral branches of *G. biloba* L. were collected in spring from the plantation (40.06787°N, 116.08134°E) of Beijing Forestry University, approximately 30 years old. The branches were cultured in greenhouse (20 to 30 °C) in order to induce meiosis.

The colchicine detection of untreated microsporangiums of *G. biloba*

About 50 g microsporangium of *G. biloba* were broken by grinding, and were made as the mixed sample solution by distilled water, after which the mixed sample solution was laid on the shaking table for about 4 h (26 °C, 200 rpm). After that it was centrifuged to separate the impurity for about 5 min by the high-speed refrigerated centrifuge (4 °C, 600 rpm). As a result, five replications per treatment were designed. In the end, the colchicine amount of the sample solution was tested by Agilent 1100 HPLC system. Each sample was tested three times, after which the peak area of the colchicine was recorded and the data was analyzed.

The intruding effect of colchicine on the microsporangium of *G. biloba*

The microsporangiums of *G. biloba* were instantly treated by colchicine solution when the microsporangiums of *G. biloba* were developing into pachytene. Three methods were taken, including the cotton soaking, the normal bottles soaking and the lucifugal bottle soaking (Figure 1). Two concentrations, 0.6 (6 mg/ml) and 0.8% (8 mg/ml) were used respectively for each treatment method (Cheng, 2006) and the control samples were instantly treated by distilled water only. Three replications per treatment were designed.

Two days later, the samples (they were treated by the 0.6 or 0.8% colchicine or the distilled water control solution) were picked up from the lively tree of *G. biloba*. All of the samples were rinsed quickly with ethanol (70%) three times and then rinsed with distilled water for several times till there was not any colchicine left in the wastewater. All of the treated samples were divided into several copies of 0.4 g for each tube and reserved in dark liquid nitrogen cryopreservation. The colchicine amounts of all treated samples were tested by HPLC.

The intruding effect of colchicine on microsporocyte of *G. biloba*

The microsporangium (50 g) of *G. biloba* was taken and the microsporocyte wall was broken by preliminary grinding, after which distilled water was added to them before mixing them evenly. The mixture was then filtered with the stainless steel mesh (100 mesh), and the filtrate was centrifuged for 10 min by the high-speed refrigerated centrifuge (4 °C, 600 rpm) (Wang et al., 2003). As a result, the microsporocyte precipitate was obtained. Similarly, 15 g was taken from the microsporocyte precipitate, and 50 ml distilled water was added to them, after which they were mixed evenly. The mixture was divided into 50 copies for each centrifuge tube. Then they were cultured with two concentrations of 0.6 and 0.8% colchicine under the period of 0.5, 1, 3, 6, 12 and 24 h, and the control samples were cultured with distilled water. Three replications per treatment were designed. After treatment, the treated samples were centrifuged 5 min by the high-speed refrigerated centrifuge (4 °C, 600 rpm), the precipitate which has been separated from the mixture was rinsed with distilled water for several times till the wastewater was without any colchicine. After that the rinsed precipitates were reserved into dark liquid nitrogen. The colchicine

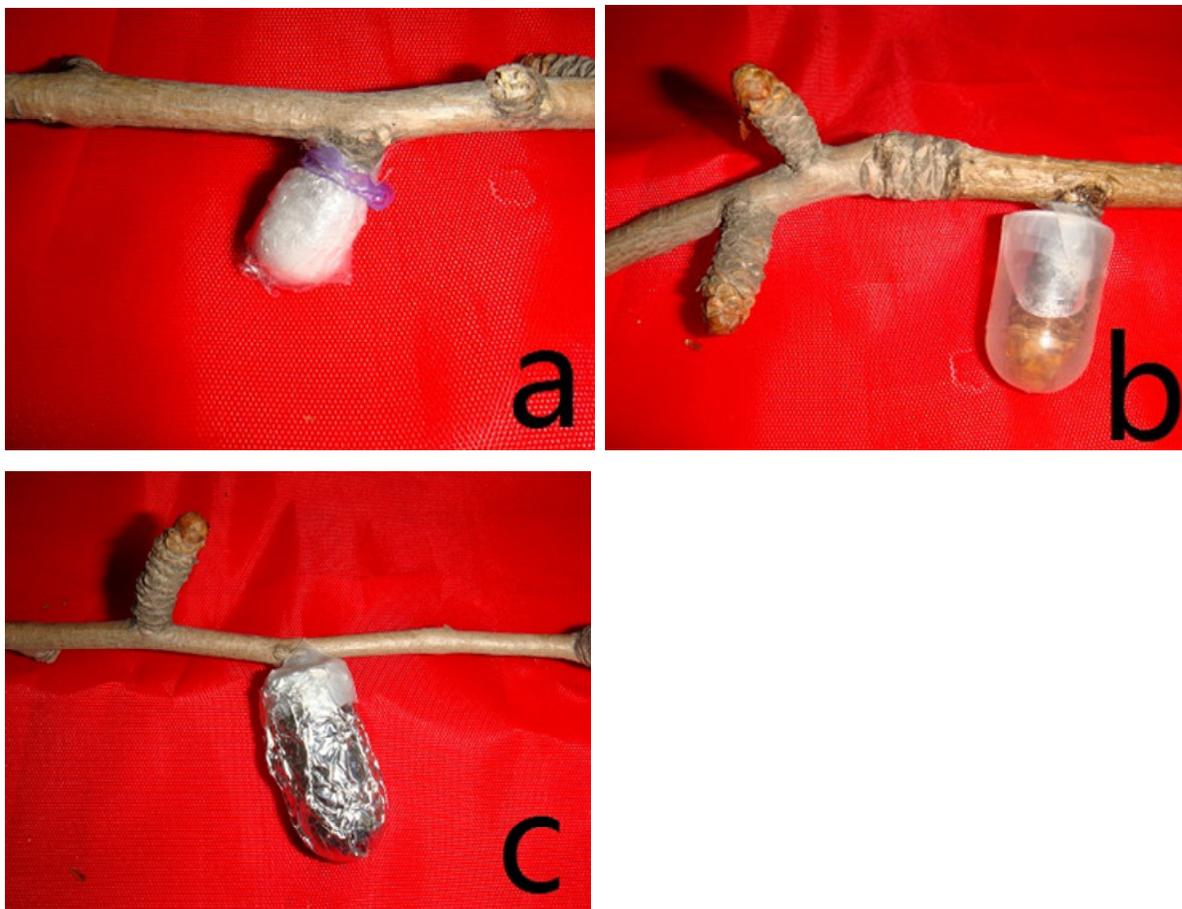


Figure 1. The Ginkgo's microsporangiums were treated with colchicine. A, the cotton soaking; B, the normal bottles soaking; C, the lucifugal bottle soaking.

amounts of all treated samples were tested by HPLC.

Colchicine treatment of *G. biloba* microsporocyte protoplasm

Microsporocyte protoplasm was obtained with enzyme treatment of live microsporocytes in solution that contained 2.0% cellulose (R-10, Yakult, Japan), 1.0% pectase (Y-23, Yakult, Japan), 5.0 mmol/l MES, 6.0 mmol/l CaCl_2 , 0.7 mmol/l K_2HPO_4 and 0.6 mol/l mannitol (pH 5.8) (Wang et al., 2003; Wu et al., 2009). The protoplasm-enzyme mixtures were stirred for 12 h at 60 rpm, then filtered with a stainless steel mesh (250 mesh) and rinsed with 22% sucrose solution. The filtrate was precipitated by centrifuging for 5 min at 4°C and 600 rpm, and then the precipitates were separated from the filtrate. The precipitates were suspended with 17% sucrose solution (5 times volume), for the protoplasm to float on top of the suspension. Then, the protoplasts were transferred into a new tube and the buffer [(5.0 mmol/l MES, 6.0 mmol/l CaCl_2 , 0.7 mmol/l K_2HPO_4 , 0.6 mol/l mannitol (pH5.8)) to 100 ml] was added to it (Frearson et al., 1973; Yan and Tian, 2005). Subsequently, the protoplasm mixture was transferred into 60 centrifuge tubes (1.5 ml in each tube) each tube was cultured with 0.6 or 0.8% colchicine for 0.5, 1, 3, 6, 12 and 24 h. Distilled water treatments were used to co-culture with the colchicine as control. Four replications per treatment were designed. After treatments, the mixtures were reserved in liquid nitrogen immediately. All samples were tested with HPLC in order to measure

the colchicine content.

The meiosis observation of *G. biloba* after colchicine treated

The microsporangiums of *G. biloba* were instantly treated by colchicine. When they were developing into pachytene, then the colchicine-treated microsporangium samples and untreated microsporangium samples (control) were then collected every 3 h, fixed for 2 to 24 h with Carnoy's fluid and stored in a vial at 4°C. The samples were squashed and stained with improved phenol fuchsin, while the meiotic stage was determined by the squash of untreated sample. However, the squash section was observed with optical microscope (Olympus BX-51), while the digital photograph was taken with Olympus DP70 photography system.

The HPLC determination of colchicine content

Preparation of standard

In preparing a standard, 10 mg colchicine are vacuum dry through P_2O_5 in a stable temperature (26°C) for 24 h and is dissolved in distilled water to 100 ml. Subsequently, 5 ml of it was taken and supplied with distilled water to 50 µg/ml (Yuan et al., 2007; Alberto and Stefano, 1998; Qiu et al., 2003).

Preparation of sample

The samples were taken out from liquid nitrogen and reserved in freezing temperature in darkness. Then, they were crushed up and dissolved in distilled water of 2 ml. At last, they were filtered with 0.45 μm microporous membrane.

The HPLC detection conditions were as follows: column: Agilent TC C18 (4.6 mm \times 150 mm \times 5 μm); mobile phase: $\text{CH}_3\text{OH}:\text{H}_2\text{O} = 44:56$; column temperature: 25°C; flow rate: 1 ml/min; detection wavelength: 246 nm; injection volume: 20 μl (Tai et al., 2006).

The linear relation analysis

In this analysis, 5, 10, 20, 30, 40 and 50 μl were taken accurately from the colchicine control solution and tested respectively, after which the regression equation in the colchicine solution was recorded and calculated by HPLC, before plotting the standard curve.

RESULTS

The linear relation analysis of colchicine

The regression equation was $Y = 2299.2X - 128.7$, in which X and Y stand for the amount (μg) and peak area of colchicine, respectively. The correlation coefficient was 0.99993 and the experiments showed that there was a linear relationship between the peak area and the amount of colchicine over the range of 0.25 to 2.5 μg .

Colchicine test results in the natural microsporangiums of *G. biloba*

It was not found that the colchicine in the natural microsporangium of *G. biloba* was not treated by colchicine. By HPLC analysis, there was a clear peak at a time period between 12.3 to 14 min in standard sample of colchicine, so the clear peak stood for the typical peak of colchicine itself; but there was no peak at same period in untreated microsporangium sample (Figure 2).

Infiltration effect of colchicine

The analysis results of HPLC indicated that colchicine remained in the microsporangiums after the microsporangiums of *G. biloba* were treated by 0.6 or 0.8% colchicine (Figure 3) with either, the cotton soaking, normal bottle soaking or lucifugal bottle soaking methods.

The HPLC analysis showed that the residue amount of colchicine was different in microsporangium of *G. biloba* when different treatment methods were used. Under the same detection concentration, there was highest residue amount of colchicine when the microsporangium was treated by the lucifugal bottle soaking (in 1 g microsporangium of *G. biloba*, there were 397.06 μg colchicine when the microsporangium was treated with 0.6% colchicine and there were 516.43 μg colchicine when the

microsporangium was treated with 0.8% colchicine) and there was the lowest residue amount of colchicine when the microsporangium was treated with the cotton soaking (in 1 g microsporangium of *G. biloba*, there were 183.80 μg colchicine when the microsporangium was treated with 0.6% colchicine and there were 265.96 μg colchicine when the microsporangium was treated with 0.8% colchicine). Under the same treatment method, the colchicine residue amount of 0.8% colchicine-treated microsporangium was a little higher than that of 0.6% colchicine-treated microsporangium (Table 1).

The same as the test result of colchicine-treated microsporangium, the HPLC analysis result of colchicine-treated microsporocyte showed that there were also colchicine remains in microsporocytes, when the microsporocytes were treated with 0.6 or 0.8% colchicine (Table 2). Also, tested at the same colchicine treatment period, the amount of colchicine in *Ginkgo's* microsporocytes which were treated with 0.8% colchicine was slightly more (about 1.5 times) than that in *Ginkgo's* microsporocytes treated with 0.6% colchicine.

In this study, it was found that the high-concentration of prolonged treatment (more than 0.8% colchicine) could not help add to the mutation rate of 2n pollen. Instead, it easily led microstrobilus of *G. biloba* to produce slow growth, deformity or death (Figure 4).

The test results of the microsporocyte's protoplasm co-cultured with the colchicine

The result of HPLC showed that the colchicine content of co-cultured sample group (the microsporocyte's protoplasm co-cultured with the colchicine.) was similar to the colchicine content of the control sample group (the distilled water co-cultured with the colchicine) (Table 3). And with the increase of processing time, from 0.5 to 24 h, the colchicine concentration was not reduced obviously in protoplasm-colchicine mixed solution along with the time increase during the process.

Meiotic abnormalities observe in the colchicine-induced microsporocyte

Some abnormalities were observed during microsporogenesis in the colchicine-treated *G. biloba*. There were steady and high frequencies of growth tardiness in the colchicine-induced microsporangium from 2007 to 2010 (Table 4). The results showed that the colchicine-treated microsporocytes grew very slowly from pachytene to anaphase I in the meiosis (Figure 5b, e and h). Even they stopped growing when the microsporangium of *G. biloba* was treated with colchicine (Figure 5c, f and i). It was found that there were some abnormalities of chromosome segregation from metaphase II to anaphase II during microsporogenesis (Figure 6). And a few dyads

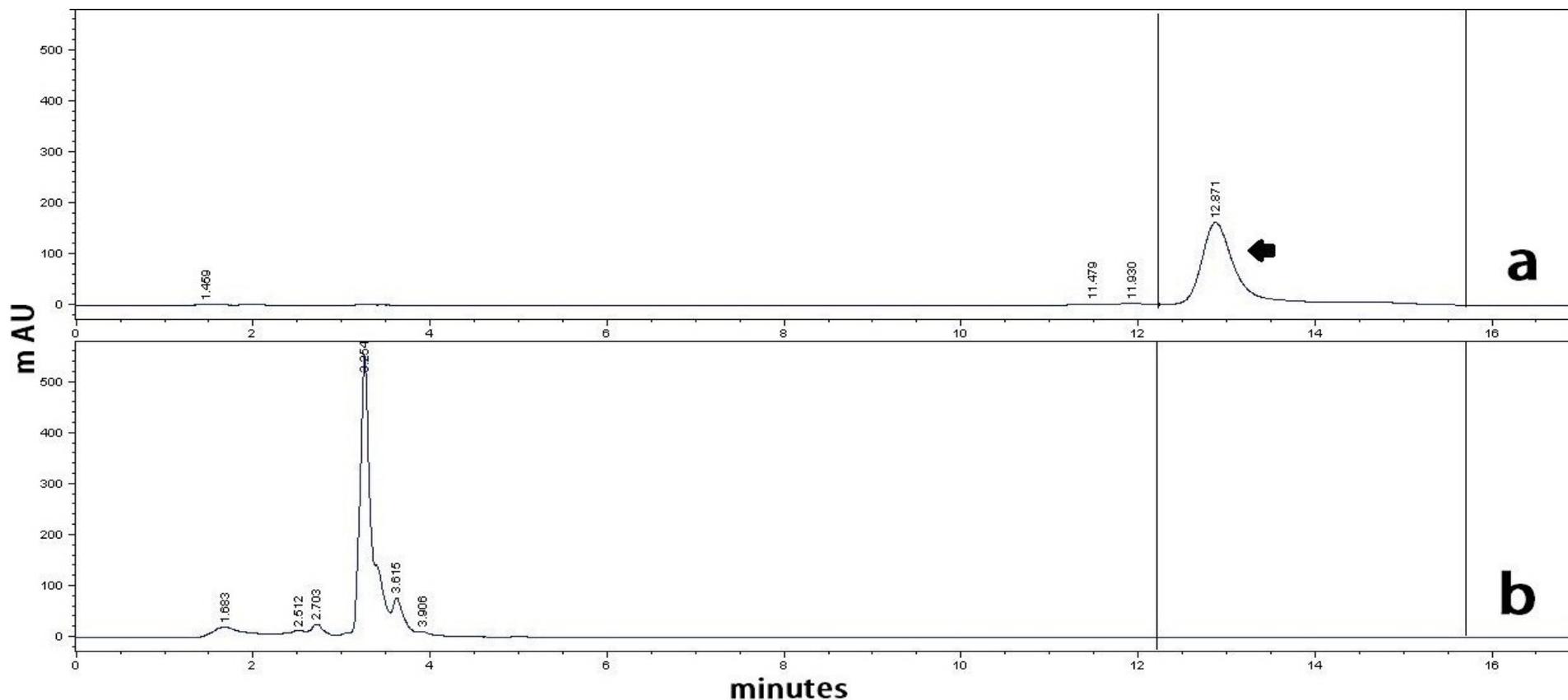


Figure 2. The colchicine content induction. The detection wavelength was 246 nm; the top is the colchicine sample from STD of colchicine, the bottom is the microsporangium sample from the normal microsporangiums of *G. biloba*. The peak between two vertical lines is the typical peak of colchicine (arrow).

and triads were detected in the meiotic products (Table 5). However, these abnormalities were not found in the untreated microsporangium of *G. biloba*.

DISCUSSION

There was a dispute about the presence of colchicine in *G. biloba* extract. Howard et al. (2001)

assumed the presence of colchicine in *G. biloba* extract; however, other researchers confirmed the absence of colchicine in *G. biloba* products (Li et al., 2002a, b). In this study, we also noticed the absence of colchicine in untreated microsporangium of *G. biloba*. It indicates that the cell tissue cannot naturally produce colchicines, which could disturb the process of artificial induced 2n pollen in *G. biloba*.

The microsporocytes of *G. biloba* are packaged in its microsporangium, due to the fact that the microsporocyte wall controls the internal and external substances circulations (Takahisa, 2006). Various amounts of colchicine residue content were observed in *G. biloba* with different treatment conditions (Figure 7), indicating that colchicine can penetrate through the microsporangium wall and microsporocyte wall in *G. biloba* (Figures 7 and 8).

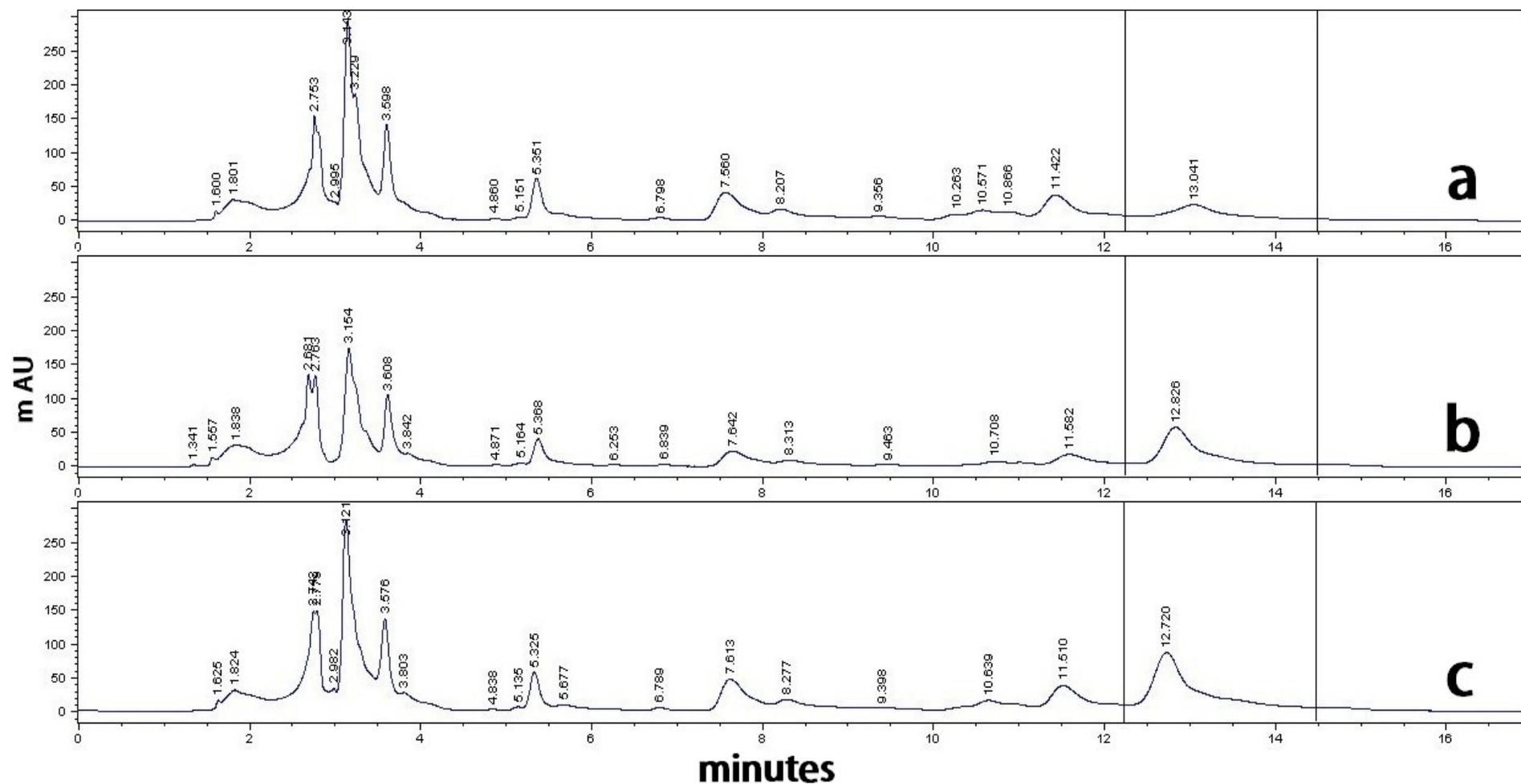


Figure 3. The colchicine content in the microsporangium of *G. biloba* was induced by 0.6% colchicine induction. The detection wavelength was 246 nm; A, the cotton soaking; B, the normal bottle soaking; C, the lucifugal bottle soaking. The peak between two vertical lines is the typical peak of colchicine.

Table 1. The average colchicine content in 1 g microsporangium of *G. biloba* were induced with different colchicine treatment method and concentration.

Concentration (mg/ml)	Cotton soaking (μg)	Normal bottle soaking (μg)	Lucifugal bottle soaking (μg)
6	183.80 \pm 10.03	329.77 \pm 8.713	397.06 \pm 14.59
8	265.96 \pm 9.19	366.92 \pm 10.28	516.43 \pm 16.09

Table 2. The colchicine's content in 1 gram microsporocytes of *G. biloba* treated with colchicine.

Treatment time (h)	0.6%				0.8%			
	Control (μg)	1 (μg)	2 (μg)	3 (μg)	Control (μg)	1 (μg)	2 (μg)	3 (μg)
0.5	0	298.60 \pm 10.44	299.18 \pm 11.21	286.74 \pm 7.59	0	332.61 \pm 15.29	345.89 \pm 10.47	331.25 \pm 9.32
1	0	275.55 \pm 7.20	279.57 \pm 9.10	308.60 \pm 7.86	0	352.62 \pm 8.95	317.41 \pm 7.13	347.72 \pm 10.54
3	0	255.71 \pm 9.05	248.70 \pm 11.03	274.24 \pm 11.26	0	313.24 \pm 6.50	307.44 \pm 9.43	331.77 \pm 10.60
6	0	258.55 \pm 7.54	250.55 \pm 9.79	240.20 \pm 11.11	0	331.65 \pm 10.44	319.71 \pm 6.97	324.20 \pm 10.90
12	0	254.78 \pm 10.52	252.52 \pm 11.02	229.04 \pm 7.31	0	301.47 \pm 8.14	314.34 \pm 9.72	277.14 \pm 12.27
24	0	223.12 \pm 5.87	246.90 \pm 8.04	222.16 \pm 6.71	0	280.18 \pm 8.11	300.12 \pm 5.78	313.27 \pm 7.30

**Figure 4.** Growth of Ginkgo's microstrobilus, treated with more than 0.8% colchicine by the lucifugal bottle soaking. A: normal growth without colchicine treatment; B: slow growth; C: deformity growth; D: death of microstrobilus.

Table 3. The colchicine concentration of the microsporocyte's protoplasm-colchicine mixture in *G. biloba*.

Treatment time (h)	0.6%					0.8%				
	Water-colchicine control ($\mu\text{g/ml}$)	1 ($\mu\text{g/ml}$)	2 ($\mu\text{g/ml}$)	3 ($\mu\text{g/ml}$)	4 ($\mu\text{g/ml}$)	Water-colchicine control ($\mu\text{g/ml}$)	1 ($\mu\text{g/ml}$)	2 ($\mu\text{g/ml}$)	3 ($\mu\text{g/ml}$)	4 ($\mu\text{g/ml}$)
0.5	29.65±0.07	29.37±0.03	28.75±0.11	29.06±0.04	28.99±0.07	38.16±0.08	38.93±0.08	38±0.0860	36.67±0.10	37.55±0.08
1	29.16±0.06	29.6±0.08	28.86±0.04	28.74±0.08	29.27±0.06	38.24±0.11	39.95±0.06	38.32±0.05	39.15±0.06	38.35±0.08
3	30.73±1.61	29.60±0.11	28.69±0.08	29.18±0.08	29.01±0.08	37.74±0.05	36.85±0.07	39.44±0.10	38.00±0.10	39.21±0.03
6	29.60±0.19	30.73±0.97	29.37±0.08	29.62±0.12	30.29±0.09	39.96±0.11	36.38±0.08	38.20±0.05	38.62±0.05	37.71±0.04
12	29.12±0.10	28.50±0.08	26.85±0.10	29.36±0.04	29.01±0.10	40.30±0.04	38.59±0.03	38.46±0.11	40.30±0.10	38.59±0.05
24	29.19±0.03	29.27±0.05	28.17±0.10	29.22±0.06	29.38±0.09	39.64±0.09	39.50±0.09	39.40±0.04	39.33±0.06	39.69 ±0.06

Table 4. The observation statistics of microsporocyte development tardiness in the colchicine-induced microsporangiums of *G. biloba* from 2007 to 2010.

Year	Treated time (h)	Number of analyzed microsporocyte	Number of abnormal microsporocyte (%)
2007	12	437	329 (75.3)
	24	398	305 (76.3)
	48	452	366 (81.0)
2008	12	381	273 (71.7)
	24	353	257 (72.8)
	48	410	324 (79.0)
2009	12	464	390 (84.1)
	24	563	502 (89.2)
	48	519	468 (90.1)
2010	12	521	431 (82.7)
	24	475	400 (84.2)
	48	526	463 (88.0)

We believe that the microsporangium and microsporocyte wall have limited impact on the process which 2n pollens were induced by colchicines in *G. biloba*. Because the amount of colchicine inside treated microsporocyte was more

when the microsporocyte was treated with higher concentration of colchicine, combined with the earlier mentioned analysis, it indicated that the microsporocyte wall cannot control the intruding of colchicine and keep the amount of colchicine on a

steady level in the microsporocyte of *G. biloba*.

The intruded amount of colchicine was affected by different medium and light intensity in the different treatment conditions. The difference between the three treatment methods were actually different

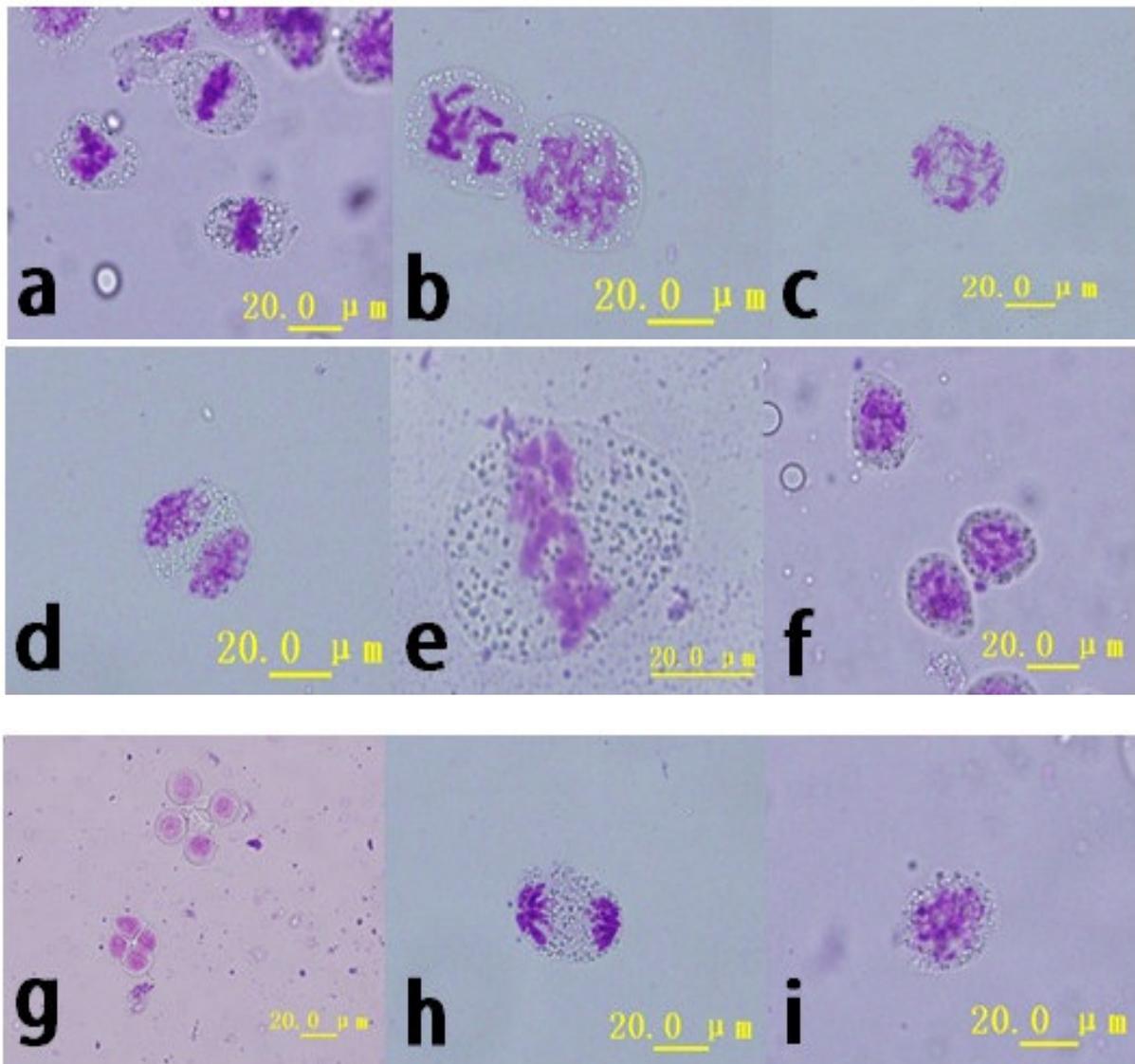


Figure 5. The microsporogenesis condition of microsporangium had treated from 12 to 48 h by colchicine in *G. biloba*. The microsporogenesis condition after 12 h: A, B and C; the microsporogenesis condition after 24 h: D, E and F; the microsporogenesis condition after 48 h: G, H and I. The normal growth condition of normal microsporocyte: A, D and G; the growth tardiness condition of colchicine-induced microsporocyte: B, E and H; the stopping growth condition of colchicine-induced microsporocyte: C, F and I.

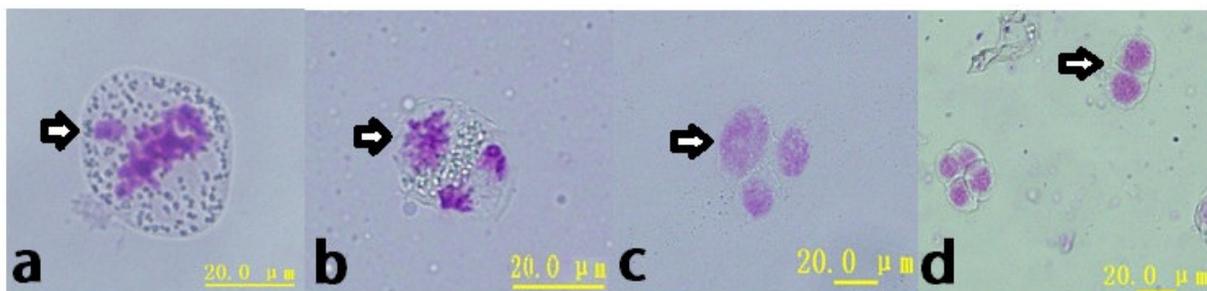


Figure 6. Some abnormalities of chromosome segregation after the microsporangiums of Gink *G. biloba* were induced by colchicine. A: the univalent (arrow) in metaphase I; B: the slow segregation of single side chromosome (arrow) in anaphase II; C: the big cell (arrow) in triad; D: the dyad (arrow).

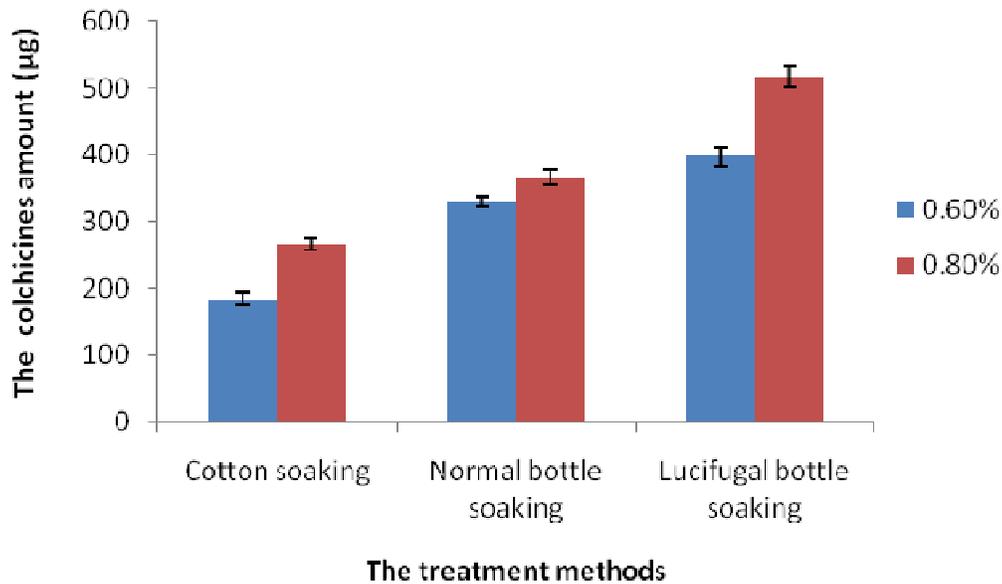


Figure 7. The average colchicine's content in 1 g *G. biloba* microsporocyte with different colchicine treatment. Unit: (µg).

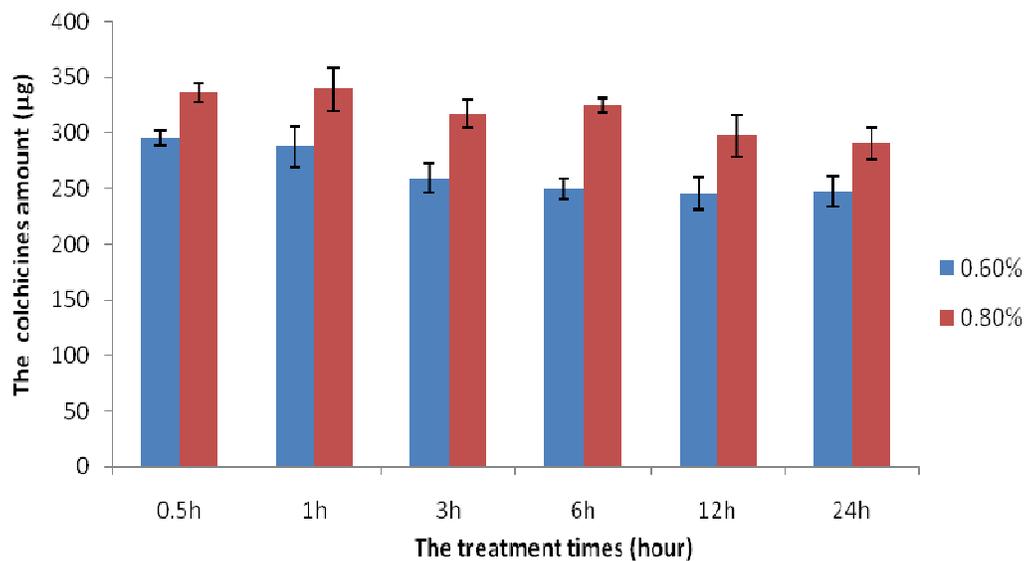


Figure 8. The practical colchicine's effect content in 1 g microsporocytes of *G. biloba* were induced by colchicine induction. Unit: (µg).

medium and light intensive. Compared with the other two treatments methods, there was only one different medium (the cotton) in the cotton soaking treatment method; the cotton has adsorption (Fras-Zemljic and Stenius, 2006), the colchicine liquid may be absorbed by the cotton and only a small part of colchicine acting on microspore and the test results also demonstrated that the colchicine residue amount was less than the other two treatments methods in the microspore of *G. biloba*. The amount of colchicine in the normal bottle soaking was less than that

in the lucifugal bottle soaking; although, the difference between the two treatment methods was only the light intensity.

The excessive colchicine was not good for the addition of the mutation rate of 2n pollen, when the 2n pollen of *G. biloba* was induced by colchicine. The colchicine is very toxic to the cell, in that the high concentration of colchicine treatment can kill the plant cells (Kubler, 2000). On the induced process of 2n pollen, if the concentration of colchicine was added excessively to the treatment, more

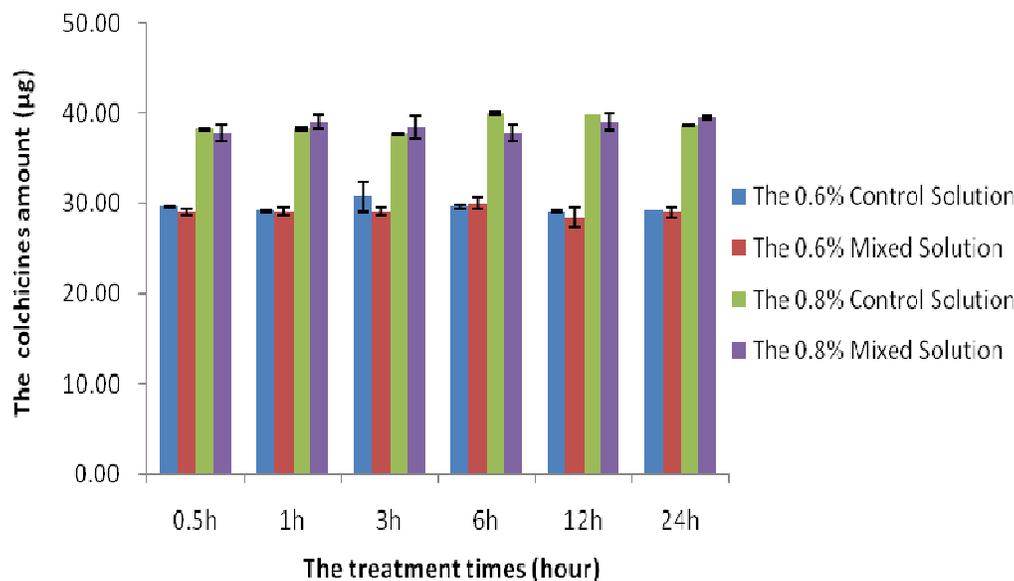


Figure 9. After colchicine was co-cultured with the microsporocyte's protoplasm in *G. biloba*, the concentration comparison diagram of the colchicine between the microsporocyte's protoplasm-colchicine solution and the control solution. Unit: (µg).

mutation rate of 2n pollen was not always obtained; instead the plant could produce the mass mortality (Kang et al., 1999; Gao et al., 2004). In our study, we also found this phenomenon. So we think the best concentration of colchicine was not more than 0.8% when 2n pollen of *G. biloba* was induced by colchicine. Under this concentration, the Ginkgo's microstrobilus could grow naturally.

The chemical constitution of colchicine was not destructed except for degradation itself, when microsporocyte was intruded into and treated by colchicine. The test results of co-cultured samples (the microsporocyte's protoplasm was co-cultured with the colchicine) indicated that the degradation of colchicine was few and the practical amount of colchicine was not affected by the protoplasm of *G. biloba* along with total culture time among the period of 0.5 to 24 h (Figure 9). Protoplasm is composed of a mixture of small molecules; there are almost all chemical substances of cell in protoplasm (Arthur and John, 2010). This research showed that the pachytene was the best induced period of 2n pollen and we found that the duration of pachytene was less than 12 h in *G. biloba*. So the experimentation (the microsporocyte's protoplasm was co-cultured with the colchicine.) can veritably reflect whether the chemical constitution of colchicine was affected by the inclusion of microsporocyte when the 2n pollen of *G. biloba* was induced by colchicine.

The 2n pollen production of plant is affected deeply by heredity and environmental change (Veilleux and Lauer, 1981; Bretagnolle and Lumaret, 1995). But it was never found that there was naturally 2n pollen of *G. biloba*, so it was very difficult to produce 2n pollen of the *G. biloba* like that produced by *Populus* (Wang and Kang, 2009). The

research on other plants shows that the treatment of colchicine can induce dyads and triads to produce the 2n pollen production (Johnsson and Eklundh, 1940; Gulyaeva and Sviridova, 1979; Kang et al., 1999) and we found that there were a few dyads (Figure 6d), triads (Figure 6c) and other meiotic abnormalities (Figure 6 a, b) in the meiotic products of colchicine-induced microsporangium after the microsporangium *G. biloba* was treated by colchicine in our study and these have never been found in untreated microsporangium of *G. biloba*. Combined with the result of HPLC analysis, we know that the colchicine liquid can intrude into the cell of microsporocyte and act on the microsporocyte's protoplasm. But, we also detected that there were only a few dyads and triads in the meiotic products (less than 10%). Combined with the research result of Cheng et al. (2006), we affirm that the 2n pollen of *G. biloba* (Cheng et al., 2006) can be induced by colchicine, but the rate of mutation was low because the microsporocytes of *G. biloba* could be induced to produce only a few dyads and triads by colchicine.

The slow development of colchicine-treated microsporocyte may lead to lower mutation rate of 2n pollen in *G. biloba*. Through the observation of the process of microsporogenesis when *G. biloba* was treated by colchicine, it was found that the development of many microsporocytes became slower in the colchicine-treated microsporangium (more than 70%), some microsporocytes even stopped developing. This phenomenon was not the same as that the poison treatment of high concentrations colchicine leads to abnormality (Figure 4), but it was similar to the reaction of hypoxia in plant (Fukao and Bailey-Serres, 2004; Klaring and Zude, 2009). So we conjecture that this phenomenon is a hypoxia response in

microsporocyte and it is led by colchicine liquid soaking when the colchicine intrude into the cell of microsporocyte and act on the microsporocyte's protoplasm, it can make development of microsporocyte to change slow and lead the colchicine treatment to miss the best treatment period and finally, disturb the mutation rate of $2n$ pollen.

Also, the other research shows that the cell can produce one kind of metallothionein (MT) when the colchicine intrudes and damages the cell. The metallothionein will help to preserve cell tissue and prevent colchicine from the damage (Itoh et al., 1997; Qiu et al., 2005). Although, the concentration of colchicine did not change, as liquid chromatography can only detect the total of colchicine in the microsporocyte of *G. biloba* and it is difficult to distinguish that the colchicine is free or binding states. Therefore, we cannot determine the state of colchicine in the treated cells of *G. biloba* and cannot know how much colchicine is acted on and combined with the tubulin. What is certain is that the microsporocyte's protoplasm does not impact on the structure of colchicine. But we cannot rule out that the possible existence of other materials in protoplasm interfered the process which the colchicine combine with the tubulin. There may be metallothionein or other proteins similar to metallothionein in *G. biloba*, which cannot destroy the chemical structure of colchicines, but can interfere with the process in which the tubulin combines with colchicine. Of course, this also requires the relevant follow-up tests for further verification.

Base on the earlier mentioned analysis, we think that the best induced method is the lucifugal bottle soaking method with 0.8% colchicine and the treated conditions and slowing of microsporocyte of *G. biloba* may affect the inducement effect when $2n$ pollen was induced by colchicine. So, if we can find right conditions to reduce the frequencies of growing tardiness in the colchicine-induced microsporangium, maybe it can produce more mutation rate of $2n$ pollen after microsporangium of *G. biloba* is treated with colchicine. The result of our study can provide basis for further exploration of how to increase the mutation effects of the $2n$ pollen of *G. biloba* in future.

ACKNOWLEDGEMENT

The authors greatly appreciate the financial support from the National Science Foundation of China (30771747).

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