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Identification of tetraploid mutants of *Platycodon grandiflorus* by colchicine induction

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Abstract — *Platycodon grandiflorus* is an important medicinal plant in China and its root has been used as medicine or food for centuries. Polyploidy may increase the amounts of functional compounds in vegetative organs for medicinal plants. So polyploid manipulation for medicinal plants is an effective approach of germplasm development. This research focused on tetraploid induction of *P. grandiflorus* by modified colchicine method. The diploid seedlings were induced under three treatments (24h, 48h and 72h) to test the best treatment time. Morphology and cytology identifications between obtained mutants and diploid controls were also conducted. The seedling growth and development of all mutants was more stunted than controls. According to preliminary morphological characteristics, mutant rates in different treatment times were statistically estimated and the highest mutant rate was 50% under the treatment of 72h. The chromosome number of most mutants was 36 (2n=4x), while the chromosome number of diploid controls was 18 (2n=2x) by cytology observation of root tip cells. Chimeras and octoploids were also identified from obtained mutants. By microscope observation of low leaf epidermis, there were significant differences for stoma area between tetraploid mutants and diploid controls. As a result, tetraploid mutants of *P. grandiflorus* were successfully obtained by modified colchicine method and their desirable traits would be further evaluated to incorporate into next breeding and pharmacy production program.

Key words: colchicine; identification; mitosis; *Platycodon grandiflorus*; tetraploid induction.

INTRODUCTION

Recently, herbs have begun to attract attention as health-beneficial foods (physiologically functional foods) and raw materials for the development of drugs. Herbal medicines derived from plants are being increasingly utilized to treat a wide variety of clinical diseases, even though relatively little is known about their modes of action (LEE *et al.* 2004).

Platycodon grandiflorus is a species of perennial flowering plant of the family Campanulaceae. It is native to East Asia (such as China, Korea, Japan, and East Siberia) and it has been used as medicine or food for centuries in China. In Europe, it is cultivated mainly as an ornamental plant (LEE *et al.* 2002). However it is presently a hot research topic in Asia because of its potential health care uses. The root of this species (*radix platycodi*) is used extensively as an anti-inflammatory in the treatment of coughs and colds (LEE 1973). This plant cultivated for more than twenty years, prevented hypercholesterolemia and hyperlipidemia (KIM *et al.* 1995) and enhanced the functions of macrophages (CHOI *et al.* 2001). Recent studies show that platycodins are one of the most essential functional components in *Platycodi Radix* in terms of the inhibition of pancreatic lipase (ZHAO and KIM 2004), cholesterol lowering, and antiobesity effects (ZHAO *et al.* 2006).

Polyploidy is the condition of having three or more complete sets of chromosomes and is relatively common in plants (JONES *et al.* 2007). Polyploids, although frequently encounter low

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seed-setting rates or complete sterility (LEWIS 1980), usually show larger organ size and superior cold tolerance (KATO and BIRCHLER 2006). In some cases, polyploids may also have enhanced ornamental characteristics (KEHR 1996) which flowers are usually larger in size, with rounder conformation, and greater substance than the diploids (WIMBER and WIMBER 1967). For this reason, the polyploidy procedure is used for root crops, fruit trees and ornamental flowers (KATO and BIRCHLER 2006). For medicinal plants, polyploidy may increase the amounts of secondary metabolites (THAO *et al.* 2003) which functional compounds are accumulated in the vegetative parts such as purple coneflower (GAO *et al.* 1996; NILANTHI *et al.* 2009). So polyploid breeding is an effective approach of germplasm development for medicinal plants.

In the late 1930s, it was discovered that colchicine inhibited the formation of spindle fibers and temporarily arrests mitosis at the anaphase stage. At this point, the chromosomes have replicated, but cell division has not yet taken place resulting in polyploid cells (BLAKESLEE and AVERY 1937).

Methods using colchicine for polyploidy induction are common for a range of plant species (LUCKETT 1989; ISHIZAKA and UEMATSU 1994; PINHEIRO *et al.* 2000; PETERSEN *et al.* 2003; LIU *et al.* 2007). Some reports of *P. grandiflorus* by colchicine induction have also been available (WANG *et al.* 2006). The objectives of our study were to explore more simple method and an effective concentration of colchicine for tetraploid mutagenesis of *P. grandiflorus*, to further identify tetraploid mutants by chromosome counting and stoma measuring.

MATERIAL AND METHODS

Plant material - *P. grandiflorus* came from Natural Medicinal Plant Garden in Shanxi province. Field experiments were finished in this Garden and inside experiments were carried out in herbal breeding lab of Shanxi Agricultural University.

Polyploidy inducing - Approximately 5 seeds were planted in every separate pots with sunshine potting soil. When seedlings were at the cotyledon stage, 96 pots were chosen for treatments and 15 pots were left as controls. Treatment pots were divided into three groups: 32 pots for 24h (one applicaton), 32 pots for 48h (two applicatons) and 32 pots for 72h (three applicatons). The suitable plant (subsample) in each pot was treated by modified method of 1% agar with 0.05% colchicine semi-solid. A single drop (2-4 μL) of the warm (~50°C) semi-solid was painted between cotyledons of each seedling to cover the apical bud. That was one applicaton and two or three applications were conducted in every 24h interval time respectively. The treated pots were then covered by a plastic cup under a high humidity growth chamber at 25°C. After each treatment, plastic cup was uncovered and the residue was carefully removed with tweezer. Sprinkling water and nutritive solution (0.3g CON_2H_4 and 0.5g KH_2PO_4 in 1L distilled water) were applied in one separate day to help recover the seedling growth for two weeks and then seedlings were transplanted in the field.

Morphological examination - Morphological characteristics of three treatments were examined during the seedling growth period. Retardant growth, crimpled and dark green leaf were measured as mutant preliminary indices and mutant rates of different treatments were statistically estimated.

Cytological identification - The ploidy level of *P. grandiflorus* was estimated by chromosome counting of root tips from obtained mutants earlier determining by morphological characteristics. Chromosome spreads were made using the modified method of LI and ZHANG (1998). Seeds were soaked in 60°C water for 24h, and then germinated at 27°C. When roots were about 0.5-1cm long, root tips were sampled and pretreated with a saturated solution of p-Dichlorobenzene and aqueous α-bromonaphthalene for 2.5 h at 20ºC, then washed in distilled water for several times and subsequently fixed in freshly prepared absolute ethanol-glacial acetic acid (3:1) at 4ºC for 24h, finally stored in 70% ethanol at 4ºC for use. Hydrolysis was performed in 1N HCl for 2 minutes at room temperature (~22°C), then washed in distilled water for several times. The softened meristem was placed in a drop of Carbol fuchsin solution on a clean microscope slide and squashed between slide and cover slip. Microscope slides were examined with a OLYMPUS BX51 using a 40x objective. The chromosome number of root tips of all treatments was counted in at least ten cells herein. A plant with all root tip cells showing 18 chromosomes was classified as diploid, and with all cells showing 36 chromosomes was determined as tetraploid. Chimera was confirmed as the condition of 18 chromosomes in some cells and 36 chromosomes in other cells of the same root tip.

Stoma measurement - The ploidy level of *P. grandiflorus* was estimated either by stoma measurement. Low leaf epidermis was torn from obtained tetraploids and diploid controls in the same internode (the ninth) and mounted on a clean slide with a drop of distilled water respectively. Another drop of 1% I-KI solution was added to stain the stoma and covered with cover slip. Slides were examined with a OLYMPUS BX51 using a 40x objective. The density and size of stoma under the same magnified scope were measured. At least 10 scopes were chosen and mean numbers were statistically calculated.

Data analysis - The experimental design was completely randomized and data analyses were performed using SAS software (SAS Institute, Cary, NC, 1995).

RESULTS AND DISCUSSION

Estimation of mutant rates - Morphology characters are also earlier evidence to estimate ploidy level. By colchicine treatment, the seedling growth and development of mutants was considerably stunted and mutants were morphological-

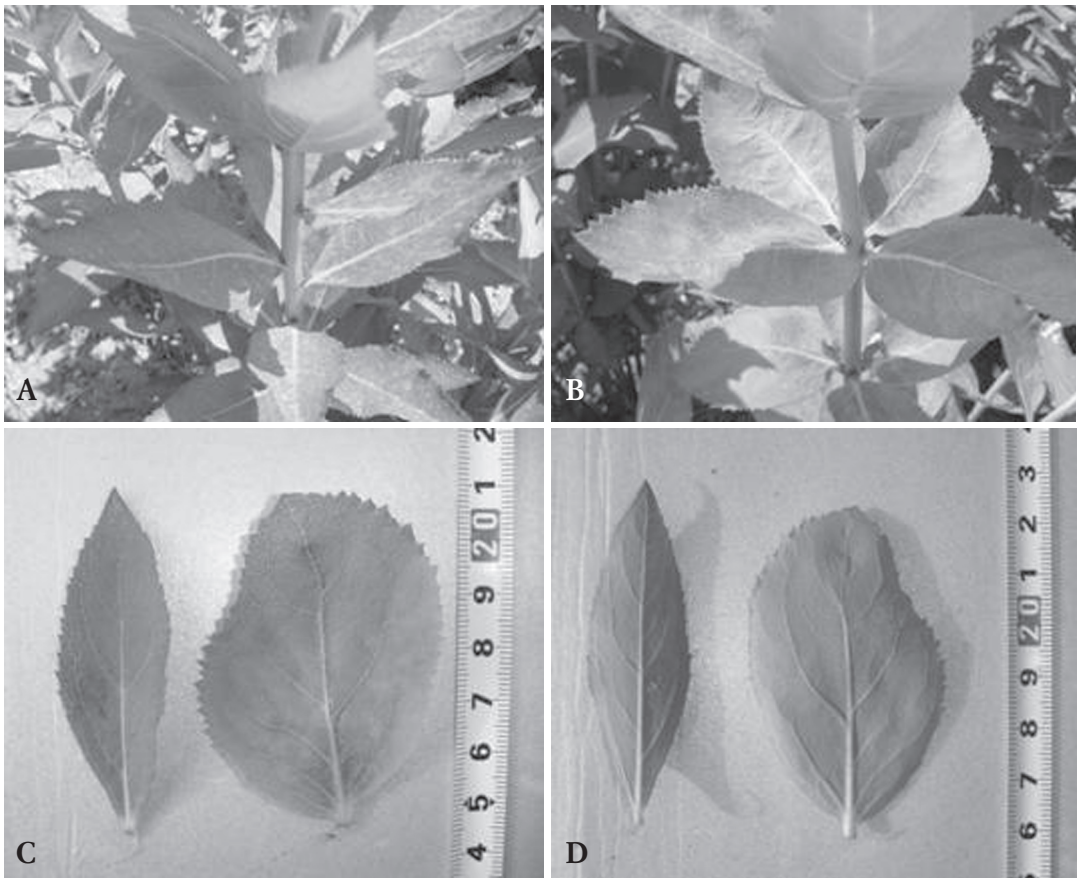

Fig. 1— Comparision of leaf (A) alternate leaf arrangement of control; (B) whorled leaf arrangement of mutant; (C, D) control leaf-left and mutant leaf-right.

TABLE 1 — Colchicine effect on *P. grandiflorus* seedlings in different treatment.

Treatment duration	Number of treatment	Number of death	Number of mutant	Mortality rate	Mutant rate
72h	32	8	16	25%	50%
48h	32	7	11	21.88%	34.38%
24h	32	9	5	28.13%	15.63%

ly distinguished by luxuriant vegetative growth showing broader and thicker leaf, whorled leaf arrangement comparing with alternate leaf arrangement of controls (Fig. 1). The mutant rates of 24h, 48h and 72h were 15.63%, 34.38% and 50% respectively (Table 1). This study gave the suggestion that colchicine treatment of 72h was the most effective for mutagenesis, yielding 50% of mutants. At the same time the increasing number of application did not increase the number of death over other applications. From X^2-test in table 1, we can see these three kind of treatments have little different influence on mortality rate and mutant rate respectively. WATROUS and WIMBER (1988) obtained more than 50% of tetraploids by treating protocorms of *Paphiopedilum* with 0.05% of colchicine treatment less than 10 days. GRIESBACH (1981) also produced 50% of *Phalaenopsis* tetraploids using 0.05% of colchicine with a long treatment (10-14 days).

DNA formation and chromosome doubling need a lot of energy which slows the split rate of polyploidy cells, so the growth of polyploid mutants was retardant. The longer the theatment time is, the slower the growth speed is. The deformity of mutants was mainly caused by the destroy of spindle from colchicine poison and the confusion between animal poles and plant poles, which leads to single, three and four poles. This phenomena could be explained by assuming that colchicine could have an effect similar to the cytokinins' effect (WEBSTER and DAVIDSN 1969) and the ratio and absolute quantity of auxin in polyploid also increased its deformity. RUIZ and VÁZQUEZ (1982) found that colchicine added to the media could modify the auxin/cytokinin relation to a certain extent and therefore change the growth of cell population in culture. Colchicine mainly concentrated on the metaphase of cell mitosis, however not all metaphase cells became polyploids. The growth rate and cell volume of diploids and polyploids are different, so the combination of diploid and polyploid cells caused crimpled leaf.

Chromosome counting - The term "ploidy" or "ploidy level" refers to the number of complete sets of chromosomes and is notated by an "x". An individual with two sets of chromosomes is referred to as a diploid (2x), three sets would be a triploid (3x), and so on with tetraploid (4x),

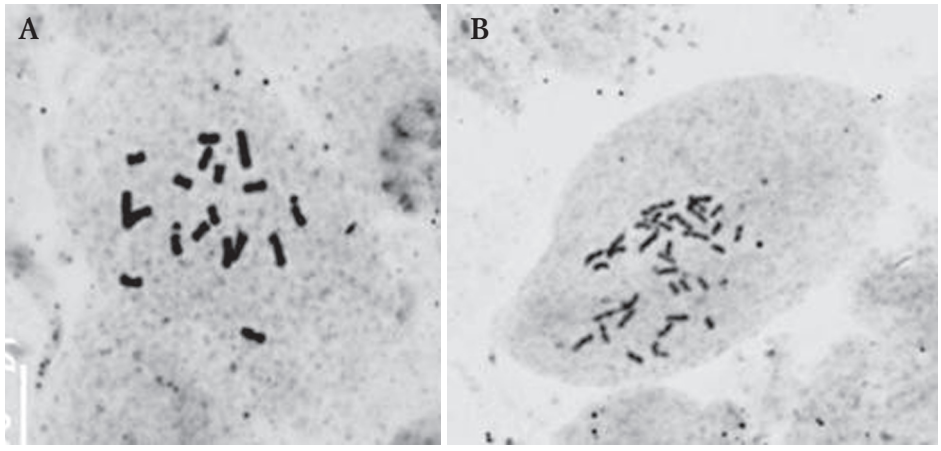

Fig. 2 — Metaphase chromosomes of mitosis in root tips (A) diploid control; (B) obtained tetraploid (10X40).

TABLE 2 — Different ploidy level of obtained plants under three treatments.

Treatment durations	Number of treatments	Number of diploids	Number of Obtained mutants	Different ploidy number of obtained mutants		
				Tetraploid	Octoploid	Chimera
72h	32	8	16	12	1	3
48h	32	14	11	8	1	2
24h	32	18	5	4	0	1

pentaploid (5x), hexaploid (6x), etc (THOMAS and JEFF 2008).

Chromosome counting in root-tip cells is the direct evidence to test ploidy level in plants. All obtained mutants were examined by chromosome counting and most of them were confirmed as tetraploids in this study. The chromosome number of obtained tetraploids was 36, but the chromosome number of diploid controls was 18 by microscope observation of metaphase plates in mitosis from root tips (Fig. 2). Different treatments of *P. grandiflorus* actually resulted in a range of different ploidy level including diploids, tetraploids, also some octoploids and chimeras (Table 2). From X^2-test in table 2, three different treatments have distinct effect on the number of obtained tetraploid, octoploid and chimera. High-level polyploids can be stunted and malformed, possibly resulting from the extreme genetic redundancy and somatic instability that leads to chimeral tissue. Chimera is refered to two or more kind genotype tissue and plant. Due to insynchronization of meristem, not all cells of colchicine treatments are polyploidy.

Ovary was always thought as suitable inducing treatment position but chemical is not easy to get into and it is difficult to control the treatment time. Seed treatment was also not successful because of the drastic effect of the drug on roots, which failed to produce lateral roots or to show any appreciable development after treatment (KUMAR and ABRAHAM 1942). Shoots on older plants can be treated, but it is often less successful and results in a greater percentage of cytochimeras. Axillary or sub-axillary meristems are usually induced for orchid and chemical solutions can be applied to buds using cotton, agar, or lanolin or by dipping branch tips into a solution for a few hours or days (THOMAS and JEFF 2008), but chimeras can be obtained and polyploidy cells were easy to be substituted by diploid cells. One of the easiest and most effective method is to work with a large number of seedlings with small, actively growing meristems. Seedlings can be soaked or the apical meristems can be submerged with different concentrations, durations, or frequencies of a given doubling agent. In our study, improved colchicine method was used to apical buds and chemical can be transported to other parts of plant by fascicular. If those parts include hypo-meristem, induction can be produced twice again and cause far effect. Direct part of colchicine treatment can cause original effect. Besides, near effect can

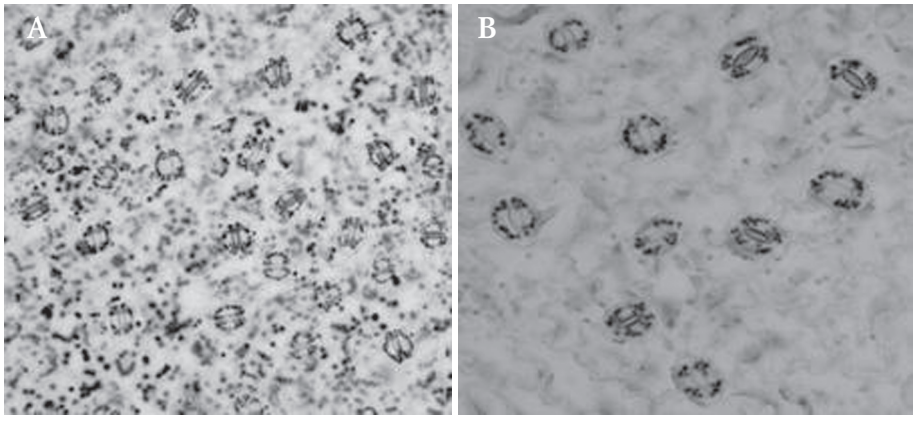

Fig. 3 — Stoma comparison of low leaf epidermis (A) diploid control; (B) obtained tetraploid (10X40).

TABLE 3— Comparision of stoma between tetraploids and diploid controls.

Treatment	The length of stoma/um	The width of stoma/um	Stoma Density/10 um²
Control	8.3±0.9 a A	6.8±0.7 a A	10.3±1.4 a A
Tetraploid	11.2±1.2 b B	9.6±1.3 b B	8.5±0.8 b B
Change rate (%)	34.94	41.18	-17.48

Note: Small letters stand for significance at 0.05 level and capital letters stand for significance at 0.01 level.

be produced by such plasmodesmata, pinocytosis and osmotic pressure. All these three effects are the main reason for our successful tetraploid mutagenesis.

Stoma measurement - Chromosome counting in root-tip cells is an accurate procedure to determine the ploidy level, but it is time-consuming and requires much experience, so stoma studies can help in screening the germplasm. In this study, two epidermis from strictly comparable leaves in the same internode (the ninth) showed an increase in the size of stoma between obtained tetraploids and diploid controls in table 3 and Fig. 3. By microscope measurement, the length and width of stoma ranged from 8.3um, 6.8um of controls to 11.2 um, 9.6um of tetraploids increased by 34.94%, 41.18% respectively. But it was decreased by 17.48% from controls (10.3/10um^2) to tetraploids (8.5/10um^2) for the stoma density (Table 3). There were significant differences between tetraploids and controls at 0.01 or 0.05 level. This study suggested that polyploid mutants could be identified by stoma measurement and it is an important cytology phenomena for stoma distinction in different ploidy plants.

It would be our current study for tetraploid characterization and further study to compare functional components between obtained tetraploids and diploid controls for this medicinal plant. The desirable traits of this tetraploid plants would be evaluated to incorporate into a further breeding and pharmacy production program.

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