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Full Length Research Paper

In vitro induction of tetraploids from immature embryos through colchicine treatments in *Clivia miniata* Regel

Chong Wang and Jiajun Lei*

College of Horticulture, Shenyang Agricultural University, Shenyang 110866, People's Republic of China.

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The immature embryos of *Clivia miniata* Regel. cv. Ranchang were *in vitro* induced with 0.00, 0.01, 0.03 and 0.05% colchicine treatment for 10, 20 and 30 d, respectively on Murashige and Skoog (MS) medium supplemented with 2.0 mg L^{-1} a-Naphthaleneacetic acid (NAA) and 1.5 mg L^{-1} 6-Benzylaminopurine (BA). A total of 14 tetraploids were obtained with an average induction rate of 16.1%. The most effective treatment was 0.03% colchicine for 20 days with an induction rate of 30.0%. The stomata size (37.24 × 12.31 µm) of tetraploids was significantly larger than that of diploids (22.51 × 7.43 µm). The stomata density of tetraploids (13.80 mm^2) was significantly lower than that of diploids (30.78 mm^2). The chloroplast number of guard cells of tetraploids (41.53) was significantly more than that of diploids (28.00). Compared with diploids, tetraploids had thicker, wider, shorter, rougher and deeper-colorful leaves, fewer roots and slower growth. The leaf and stoma characteristics could be regarded as helpful indexes to identify colchicine-induced tetraploids in *C. miniata*.

Key words: *Clivia miniata* Regel., chromosome doubling, colchicine, tetraploid, stoma, chloroplast number.

INTRODUCTION

Clivia miniata Regel., an evergreen herbaceous flower belonging to the genus *Clivia* in the Amaryllidaceae, is native to Southern Africa (Ran et al., 2001a). *Clivia* is an important indoor potted flower due to its elegant leaf and colorful flower. The genus *Clivia* comprises seven recognized species in which *C. miniata* Regel. (2*n* = 2*x* = 22) is the most commonly cultivated species in the world (Ran and Simpson, 2005). Polyploidy has been significant in the evolutionary history of plants, which could broaden the diversity of gene pool (Adams and Wendel, 2005). It has been estimated that over 70% of land plants and 95% of ferns have some polyploidy in their evolutionary history (Otto and Whitton, 2000). Polyploidy are known to offer some advantages in many cases, such as large flowers, stronger stems and higher levels of resistance to disease, but their slower development and lower fertility were also evident (Sparnaaij, 1979). Colchicine combined with culture *in vitro* is an effective method to induce polyploidy, which could increase the efficiency of recovery of polyploid plants and reduce the incidence of chimeras (Compton et al., 1996; Chakraborti et al., 1998). With this method, a lot of ornamental polyploids have been obtained, such as *Alocasia* (Thao et al., 2003), *Cyclamen* (Takamura et al., 1996), *Gladiolus* (Suzuki et al., 2005), *Lilium* (Liu et al., 2009), *Phlox* (Zhang et al., 2008), *Rhododendron* (Väinölä, 2000) and *Rosa* (Roberts et al., 1990).

There are few reports on polyploid induction of *Clivia*. Niu et al. (1986) reported that tetraploids and hexaploids were obtained when seeds and seedlings were used as materials through the direct colchicine treatment. van Voorst (2006) reported that a few tetraploids and many chimeras were obtained from germinated seeds and mature embryos via colchicine induction *in vitro*. In our previous research, the 180-day immature embryos were the most effective explants for tetraploid induction (Wang et al., 2011), and as such, the present work aimed to seek for the effective combination of colchicine concentration and duration for inducing tetraploids from

*Corresponding author. E-mail: jiajunlei@yahoo.com.cn. Tel: +86-024-88487635. Fax: +86-024-88487175.

180-day embryos in *C. miniata* cv. Ranchang, and the leaf and stoma characteristics of tetraploids were compared with those of diploids.

MATERIALS AND METHODS

Plant materials

C. miniata Regel. cv. Ranchang (2*n* = 2*x* = 22) was potted in greenhouse at Shenyang Agricultural University in China. The experiment was started on 20th March, 2008. The embryos were obtained from self-pollination. The embryo age referred to the days after pollination. Fruits of 180-day embryo were collected and washed with running water for 1 h, then disinfected with 70% (v/v) alcohol for 10 min, followed by rinsing thrice with sterile distilled water. Subsequently, the seeds were extracted from the fruits on a clean bench and immature embryos were excised from seeds by forceps.

Colchicine treatment

The inoculation and growth medium (IG medium) was MS basal medium supplemented with 2.0 mg L^{-1} NAA, 1.5 mg L^{-1} BA, 30 g L^{-1} sucrose and 5 g L^{-1} agar. The immature embryos were inoculated on IG medium containing 0.00, 0.01, 0.03 and 0.05% colchicine respectively, and treated for 10, 20 and 30 days respectively. Each treatment was replicated thrice with 20 explants per replication. The formula used for calculating the survival rate is given as:

Survival rate (%) = No. of survived embryos / No. of inoculated embryos × 100.

After ending the colchicine treatment, the explants were transferred to fresh IG medium at 30-days intervals for growing. When the shoots reached 3 cm in height, they were transferred to 1/2MS medium supplemented with 1.5 mg L^{-1} NAA and 2.0 g L^{-1} activated carbon for rooting. The formula used for calculating rooting rate is given as:

Rooting rate (%) = No. of rooted shoots / No. of inoculated shoots × 100.

The pH of the medium was adjusted to 5.8 with NaOH before autoclaving at 121℃ for 20 min. All cultures *in vitro* were maintained at 26 ± 2°C with a 16 h photoperiod using cool-white fluorescent lamps.

Chromosome count

The chromosome number of 87 colchicine-induced regenerated plantlets was identified. Root tips were excised from tube plantlets and pretreated with saturated 1,4-dichlorobenzene at 4°C for 24 h, then fixed in Carnoy's solution (95% ethanol: acetic acid = 3: 1, v/v) at 4°C for 2 h, followed by washing thrice with distilled water and hydrolyzed in 5 M hydrochloric acid for 10 to 15 min at room temperature. After soaked with distilled water for 20 min, the root-tip was squashed and stained by modified carbol fuchsin for 15 to 20 min (Wang et al., 2011). At least 10 clear division phases in metaphase per plantlet were observed under Olympus microscope.

Leaf and stoma characteristics

The leaf and stoma characteristics were observed from three colchicine-induced tetraploids. The leaf thickness, length, width and index were measured with three replications. Two fresh leaf disks (0.5 cm length × 0.5 cm width) from each tetraploid plantlet were cut and used for stoma observation. The leaf disks were decolored in Carnoy's solution for 48 h and rinsed with distilled water for 5 to 10 min, followed by staining with 1% KI-I_2 for 5 min and then observed under microscope (Wang et al., 2007). Five vision fields per leaf disk and five stomata per field were observed to determine the size of stomata and the number of chloroplasts. Ten vision fields per leaf disk were observed to determine stomata density (n/mm^2). The data were subjected to statistical analysis using Duncan's multiple range test (SPSS ver.16.0).

RESULTS

Effect of colchicine treatment on survival of immature embryos and shoot regeneration

The immature embryos inoculated on IG medium without colchicine turned from light yellow to yellow and turned to green after culturing for about 5 days; the plantules swelled and coleoptiles emerged after culturing for 15 days (Figure 1a), followed by germination of the shoots 30 days later (Figure 1b); the regenerated shoots from immature embryos grew to above 3 cm in height after 90 days (Figure 1c). But, the immature embryos inoculated on medium supplemented with colchicine germinated later about 20 days than without colchicine. The colchicine treatment usually resulted in partly browning or completely browning of embryos. Some browning embryos cultured on medium supplemented with 0.03% colchicines for 30 days could regenerate shoots after culturing for about 40 days on colchicines-free medium (Figure 1d). Only few completely browning embryos cultured on medium supplemented with 0.05% colchicines for 20 days could survive and re-germinate after being transferred to IG medium for about 60 days (Figure 1e). The survival rate of embryos decreased with the increases of colchicine concentration and treatment duration. The survival rate decreased from 56.7 to 23.3% when the duration was prolonged from 10 to 30 days at 0.03% colchicine, and decreased from 65.0 to 25.0% when the colchicine concentration increased from 0.01 to 0.05% at the treatment for 20 days (Table 1). In the control, the rooting rate of shoots and the root number per shoot were 45.4% and 2.7 in average respectively. But for the colchicine-induced shoots, were 29.7% and 1.5 in average respectively, and the rooting rate and the root number per shoot decreased with the increase of colchicine concentration (Table 2).

Ploidy determination

The chromosome number of 87 plantlets selected randomly from all colchicine-induced shoots was identified, 14 tetraploids (16.1%), 33 mixoploids (37.9%) and 40 diploids (46.0%) were obtained (Figure 2a and b). There was higher survival rate and induction rate when

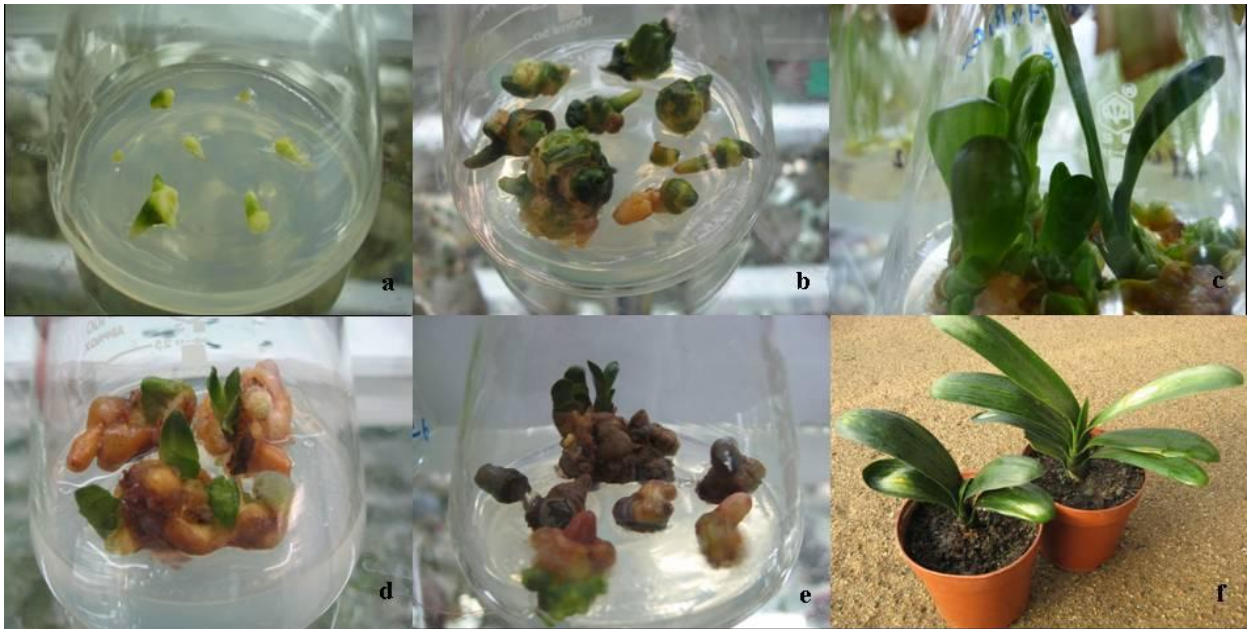

Figure 1. Shoot regeneration on colchicine-free medium and tetraploid induction from immature embryos *in vitro* treated with colchicine in *C. miniata* cv. Ranchang; (a) Plantules swelled and emergence of coleoptiles after culturing for 15 d on colchicines-free medium; (b) Shoots germinated after culturing for 30 d on colchicines-free medium; (c) Regenerated shoots from immature embryos grew to above 3 cm in height after culturing for 90 d on colchicine-free medium; (d) Partly browning embryos cultured on medium supplemented with 0.03% colchicine for 30 d regenerated shoots after transferring on colchicine-free medium for 40 d; (e) Completely browning embryos cultured on medium supplemented with 0.05% colchicine for 20 d re-germinated shoots after transferring on colchicine-free medium for 60 d; (f) Colchicine-induced tetraploid (left) and diploid (right) plants in pots.

Table 1. Effect of concentration and duration of colchicine on the survival rate, shoot regeneration and polyploidy production of immature embryos in *C. miniata* cv. 'Ranchang'.

Colchicine concentration (%)	Duration (d)	No. of inoculated embryos	No. of survival embryos	Survival rate of embryos [a] (%)	No. of shoots identified	No. of shoots with different ploidy (percentage)		
						Diploid	Tetraploid	Mixoploid
0.00	10	60	60	100.0	5	5 (100.0)	0	0
	20	60	57	95.0	5	5 (100.0)	0	0
	30	60	55	91.7	5	5 (100.0)	0	0
Total		180	172	95.6	15	15 (100.0)	0	0
0.01	10	60	49	81.7	12	10 (83.3)	0	2 (16.7)
	20	60	39	65.0	12	9 (75.0)	2 (16.7)	1 (8.3)
	30	60	32	53.3	12	5 (41.7)	3 (25.0)	4 (33.3)
Total		180	120	66.7	36	24 (66.7)	5 (13.9)	7 (19.4)

Table 1. Contd.

	10	60	34	56.7	12	8 (66.7)	2 (16.7)	2 (16.7)
0.03	20	60	28	46.7	10	2 (20.0)	3 (30.0)	5 (50.0)
	30	60	14	23.3	7	2 (28.6)	1 (14.3)	4 (57.1)
Total		180	76	42.2	29	12 (41.4)	6 (20.7)	11 (37.9)
	10	60	23	38.3	10	2 (20.0)	2 (20.0)	6 (60.0)
0.05	20	60	15	25.0	8	2 (25.0)	1 (12.5)	5 (62.5)
	30	60	7	11.7	4	0	0	4 (100.0)
Total		180	45	25.0	22	4 (18.2)	3 (13.6)	15 (68.2)

[a] The survival rate was assessed after the embryos had been cultured for 60 days.

Table 2. The rooting rate and root number per shoot derived from different combinations of colchicine concentration and duration in *C. miniata* cv. 'Ranchang'.

Colchicine concentration (%)	Duration (d)	No. of inoculated shoot	No. of rooted shoot	Rooting rate [a] (%)	No. of root per shoot
	10	20	9	45.0	2.7
0.00	20	17	7	41.2	2.8
	30	16	8	50.0	2.6
	10	15	5	33.3	2.0
0.01	20	24	7	29.2	2.3
	30	13	4	30.8	1.0
	10	17	6	35.3	1.2
0.03	20	14	4	28.6	2.0
	30	8	2	25.0	1.0
	10	20	6	30.0	1.7
0.05	20	15	4	26.7	1.3
	30	7	2	28.6	0.6

[a], The rooting rate and root number per shoot was assessed after cultured for 30 days.

treated with higher concentration plus shorter duration. The induction rate of tetraploid was 13.9% when treated with 0.01% colchicine, and 20.7% when treated with 0.03% colchicine, but decreased to 13.6% when treated with 0.05% colchicine. When the duration prolonged from 10 to 20 d at 0.05% colchicine, the induction rate was reduced by 37.5% due to more mixoploids were obtained, and no tetraploids could be induced

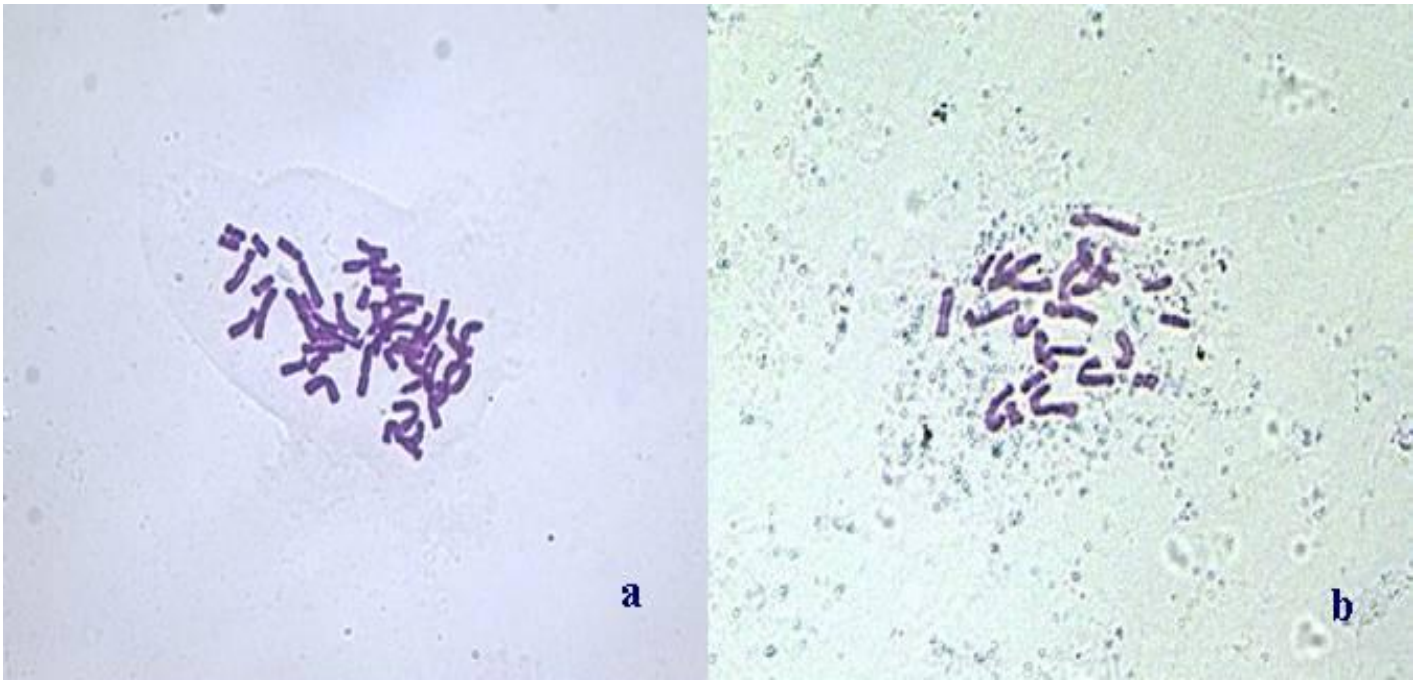

Figure 2. Chromosome number of root tips of colchicine-induced tetraploid plant derived from immature embryos *in vitro* treated with colchicine and control diploid plant in *C. miniata* cv. Ranchang.(a) Tetraploid (2*n* = 4*x*= 44); (b) Diploid (2*n* = 2*x* = 22).

Table 3. Leaf characteristics of tetraploid and diploid plants in *C. miniata* cv. 'Ranchang'.

Ploidy	Leaf thickness (mm)	Leaf length (cm)	Leaf width (cm)	Leaf index
Tetraploid 1	1.43±0.13^{a}	6.54±1.2^{b}	3.96±1.16^{a}	1.73±0.38^{b}
Tetraploid 2	1.59±0.33^{a}	6.12±0.91^{b}	2.90±0.52^{b}	2.36±0.67^{b}
Tetraploid 3	1.42±0.18^{a}	7.68±1.16^{b}	3.14±0.38^{ab}	2.68±0.57^{b}
Diploid	1.02±0.1^{b}	10.38±2.23^{a}	1.86±0.35^{c}	5.75±1.50^{a}

Each value represents the mean ± SD of three replicates. Data within columns followed by the same letter are not significantly different at the 0.05 level by Duncan's multiple range test.

when treated with 0.05% colchicine for 30 days. Therefore, the most effective treatment was 0.03% colchicine for 20 days, with a higher induction rate of 30.0% and survival rate of 46.7% (Table 1).

Leaf characteristics

There were significant differences ($P<0.05$) in leaf thickness, length and width, and leaf index between tetraploids and diploids (Table 3). Compared with the diploids, tetraploids had a thicker, shorter and broader leaf, and lower leaf index (Figure 1f). In addition, the slower growth, rougher leaf surface, deeper leaf color and fewer roots were also observed in tetraploids. Therefore, the leaf characteristics were helpful indexes for early screening of tetraploids.

Stoma characteristics

The stomata of *C. miniata* were located in the lower epidermis. There were significant differences ($p<0.05$) in sizes of stomata and guard cells, and chloroplast number in guard cells between tetraploids and diploids (Table 4). Compared with diploids, the stoma length of tetraploids was 37.24 μm, significantly increased by 65.44%. The length and width of guard cells of tetraploids were 59.01 and 22.15 μm respectively, significantly increased by 38.88 and 36.81% respectively. The average number of chloroplast in guard cells was significantly increased by 48.32% from diploids (28.00) to tetraploids (41.53) (Figure 3a and b). The stoma density of tetraploids was significantly reduced by 55.17% (Figure 3c and d). Therefore, the stoma characteristics were also used as helpful indexes for early screening of tetraploids.

Table 4. Characteristics of stoma and guard cells of tetraploid and diploid plants in *C. miniata* cv. 'Ranchang'.

Ploidy	Stoma length (µm)	Stoma width (µm)	Stoma destiny (n/mm^2)	Guard cell length (µm)	Guard cell width (µm)	Chloroplast number in guard cells
Tetraploid 1	32.65±2.83^{b}	11.63±3.31^{b}	13.12±2.64^{b}	55.06±1.55^{b}	20.89±1.45^{a}	42.40±7.30^{a}
Tetraploid 2	37.32±5.99^{ab}	11.06±1.25^{ab}	17.10±7.49^{b}	56.46±2.97^{b}	22.85±1.01^{a}	41.80±4.92^{a}
Tetraploid 3	41.75±6.69^{a}	14.23±4.14^{a}	11.18±4.17^{b}	65.50±2.95^{a}	22.71±1.92^{a}	40.40±4.16^{a}
Diploid	22.51±2.9^{c}	7.43±2.70^{ab}	30.78±9.17^{a}	42.49±7.78^{c}	16.19±2.25^{b}	28.00±3.16^{b}

Each value represents the mean ± SD of three replicates. Data within columns followed by the same letter are not significantly different at the 0.05 level by Duncan's multiple range test.

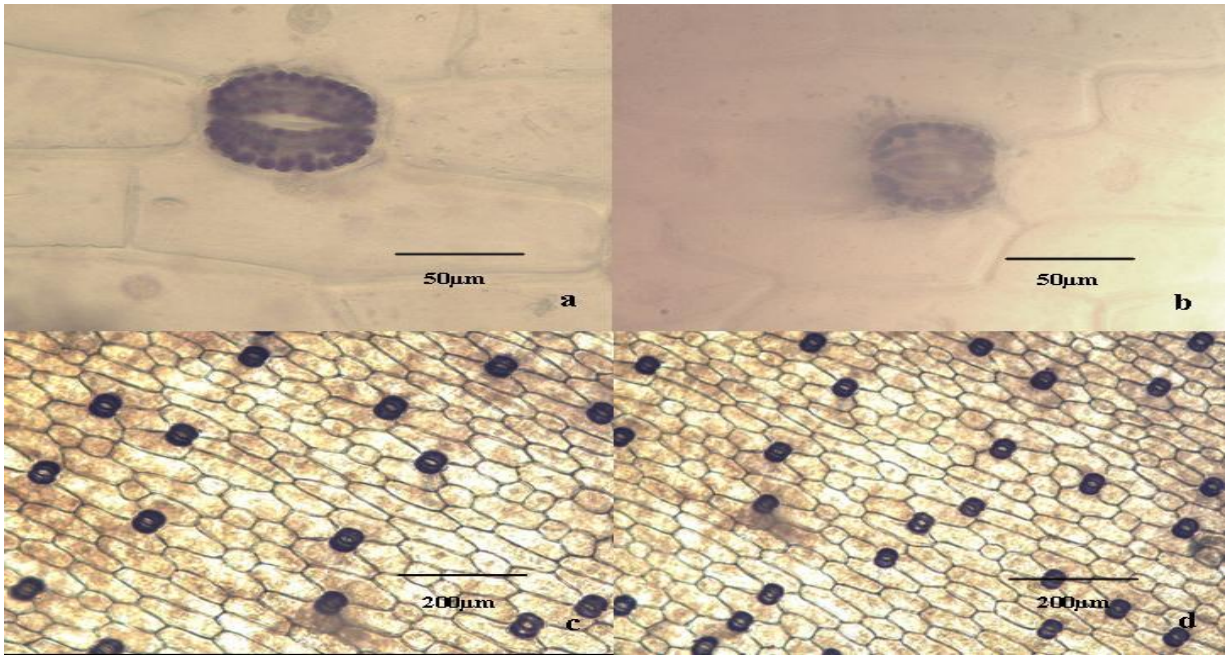

Figure 3. The size and density of stomata and chloroplast number in guard cells of colchicine-induced tetraploid and control diploid plants in *C. miniata* cv. Ranchang; (a, b) Stoma size and chloroplast number in guard cells of tetraploid (a) and diploid (b) plants; (c, d) Stoma density of tetraploid (c) and diploid (d) plants.

DISCUSSION

The explants, colchicine concentration and treatment duration were critical factors for polyploidy induction (Chakraborti et al., 1998; Quesenberry et al., 2010). Finnie and van Staden (1999) reported many tissues including meristems, seeds, embryos, ovaries, petals and pedicels could be used as explants, although, plantlet regeneration was slow in *C. miniata*. The immature embryo was an effective explant for polyploidy induction *in vitro* in *Clivia* because of more available plantlet regeneration and relative shorter induction cycle. Usually, lower colchicine concentration combined with longer duration was suitable for polyploidy induction *in vitro* in ornamental plants (Lei and Wang, 2012). In our experiment, the induction rate of tetraploids could reach 25.0 and 30.0% respectively when treated with 0.01% colchicine for 30 days and 0.03% colchicine for 20 days in *C. miniata*. Zheng et al. (2003) reported that the induction rate of tetraploids could reach 26% when using 0.1 to 0.2% colchicine for 12 to 36 h in *Viola tricolor*.

Chromosome count was the most direct and accurate method to identify polyploidy, but an early and preliminary screening could greatly reduce workload, (Ye and Tong, 2010) especially, for plants with a longer induction and growth cycle, such as *Clivia*. Usually, the tetraploid plants had broader and thicker leaves and deeper leaf color (Liu et al., 2007). Cui et al. (2009) reported that the tetraploid of *Phalaenopsis* had rougher leaf surface and darker green leaf color.

Simultaneously, the size and destiny of stomata could be used as useful parameters for distinguishing ploidy level in the early period of seedlings due to larger stoma and lower stoma destiny in tetraploids (Yang et al., 2006;

Zhang et al., 2010). The chloroplast number in guard cells of stomata was positively correlated with ploidy levels in some plants (Chaudari et al., 1975; Yuan et al., 2009; Zhang et al., 2005). In our experiment, leaf characteristics, the slower growth and fewer roots of colchicines-induced tetraploids were helpful for preliminary identification of polyploids in *C. miniata*, and there were significant differences in stoma length and density, the size of guard cells, and chloroplast number in guard cells between tetraploids and diploids.

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