Polyploid Induction in *Dendrobium secundum* (Bl.)Lindl. by *in vitro* Techniques

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Abstract

The effect of colchicine concentration on increasing the ploidy level of the orchid species *Dendrobium secundum* (Bl.) Lindl was studied. Chrolophyll content, stomatal characteristics, and flow cytometry were used for screening ploidy levels. The culture medium used to induce polyploidization was a VW_1 liquid medium containing 100 mg L^{-1} myo-inositol, 1 mg L^{-1} thiamine, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1} pyridoxine and 4 mg L^{-1} glycine, 15% coconut water and 20 g L^{-1} sucrose) supplemented with 0.05, 0.1, 0.15 and 0.2% concentration of colchicine incubated for 1, 2, 3, 4 and 5 days. High frequencies of polyploidy in regenerated plants were achieved in culture media supplemented with 0.05% colchicine, for 1 day.

Keywords: colchicine, protocorm, flow cytometry, orchid

Introduction

Orchid species in the genus *Dendrobium* are some of the most important types of commercial orchids used for cut flowers and potted plants. Flower sizes of some cultivars are small such as those seen for *D. secundum.* Chromosome doubling has been used in breeding programs for improving characteristics of orchid flowers (Silva et al., 2000). Colchicine treatment has become a common tool used for polyploid induction in many plants. Polyploid cells are larger than diploid cells. This greater cell volume frequently develops into thicker tissues, resulting in large-sized flowers (Chaicharoen, 1995)

Polyploids plant has been induced successfully from *in vitro* diploid plants by treating with cochicine in different concentrations. In pomegranate (*Punica granatum*), tetraploid plants were obtained by colchicine treatment of shoots propagated *in* vitro. Shoots cultured on MS medium supplemented with 10 mg L^{-1} colchicine, 1.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA for 30 days produced tetraploids at the high frequency of 20%. No tetraploids were detected by treating the shoots in 5000 mg L^{-1} colchicine for 114 h. Shoots treated by 5000 mg L^{-1} colchicine for 96 h produced three morphological mutants with narrow leaves, which were later confirmed as mixoploids that were separated into diploids and tetraploids after further subcultured. *In vitro* tetraploid plants had shorter roots, wider and shorter leaves than the diploid ones. Tetraploid pomegranate plants grew and flowered normally in pots, but possessed flowers with increased diameter and decreased length compared to diploids (Shao et al., 2003). In *Miscanthus sinensis*, different explant materials were treated with colchicine and oryzalin to induce chromosome doubling (Petersen et al., 2003). In *Alocasia*, shoot tips were treated in 1% dimethylsulfoxide solution with colchicine at concentrations of 0.01, 0.05, 0.1% and oryzalin at 0.005, 0.01, 0.05% at period of 24, 48 and 72 h, and ploidy levels could then be easily determined by flow cytometry. Variation in the morphological characteristic of leaf shape was found among the multiplied plants under the same growing condition. While the leaves of diploids had an elongated-heart shape, the leaves of tetraploids and chimeras tended

to be round (Thao et al., 2003). In *Dendrobium superbiens*, the number of flower in a large diploid inflorescence was much more than in the tetraploid. The tetraploid plants had lager flower than those in the diploids and the average width, length and thickness of sepals and petals of both diploids and tetraploid plants were significantly different (Chai-charoen, 1995). In *Cattleya intermedia*, cultured the protocorm-like bodies on NDM media with colchicine concentrations 0.05 and 0.1% appeared to be effective on the production of mixoploid and tetraploid (Silva et al., 2000)

Materials and Methods

Plant Material and *in vitro* Multipication

Protocorms of *D.secundum* were used as explant sources derived from culturs of *D. secundum* seed grown on modified VW medium (VW medium containing 100 mg L^{-1} myo-inositol, 1 mg L^{-1} thiamine, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1} pyridoxine and 4 mg L^{-1} glycine, 20 g L^{-1} sucrose, 15% coconut water and 0.8% agar at pH 5.4). The explants were cultured with a 16 hours photoperiod (light intensity 40 µ mole m^{-2} s^{-1}) at 25±2°C for 4 weeks. After 4 weeks of culturing the protocorms were formed.

For protocorm multiplication, protocorms were cultured on modified VW medium. The cultures were then maintained with a 16 hours photoperiod (light intensity 40 µmole $m^{-2}s^{-1}$) at 25±2°C for 4 weeks.

Colchicine Treatment

Protocorms were cultured on media with 0.05, 0.1, 0.15 and 0.2% colchicine for 1, 2, 3, 4 and 5 days. After the treatment, they were washed with steriled distilled water for 10 minutes and then cultured on modified VW medium. The experimental design was randomize complete block (RCB), each treatment was replicated 5 times and 50 protocorms were cultured per each replication. After culturing for 3 months, the surviving plantlets were examined.

Ploidy Level Determination

After 3 months, the proliferated shoots had their ploidy levels tested by flow cytometry. Young leaves of colchicine treated plants were used for flow cytometric measurement. Approximately 1 g of each leaf was chopped with a sharp razor blade in a 55-mm plastic dish, the hypotonic buffer Cy stain[R] UV ploidy were then add to the plant tissue (1 mL of one step DAPI staining solution) and then filtered through a 30 µm celltrics disposable filter. The samples were analyzed the flow cytometry with Partec PAII. The number and size of guard cells were examined after staining of the epidermis of leaves with safanine solution. Chrolophyll content was determined following the method of Arnon (1959).

Results and Discussion

Survival Rate

Protocorms that were not treated with colchicine multiplied rapidly during the two months of subculture. The survival of the explants after colchicine treatment depended on the concentration and on the duration of the treament. In general, higher concentrations and longer incubation durations reduced survival of explants. In particular colchicine at 0.2% was very toxic and all of explants died after 3 months of the culture at this concentration. Survival rate was significant differently (*p=0.005) in colchicine treatment at every concentration (Figures 1 and 2).

Chorophyll Content

After protocorms were treated with colchicine in concentration of 0.05, 0.1, 0.15 and 0.2% and culture for 2 months, chrophyll content decreased signifi-cantly different from the control (*p<0.005) (Table 2).

Determination of Polyploid

The gain in value was adjusted so that the peak of nuclei isolated from the control diploid plantlets (2C DNA) was set at about channel 200 showed peak 1 that had been determined by analyzing the

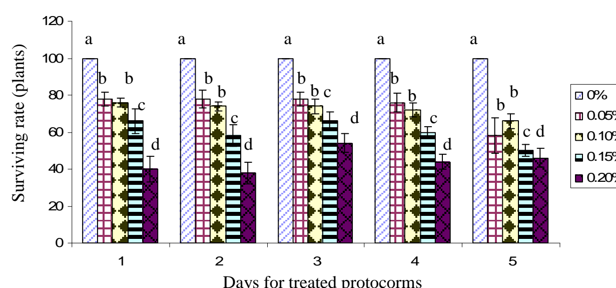

Figure 1. The effect of *in vitro* colchicine treatment on the survival rate of plantlets of *D. secundum.*

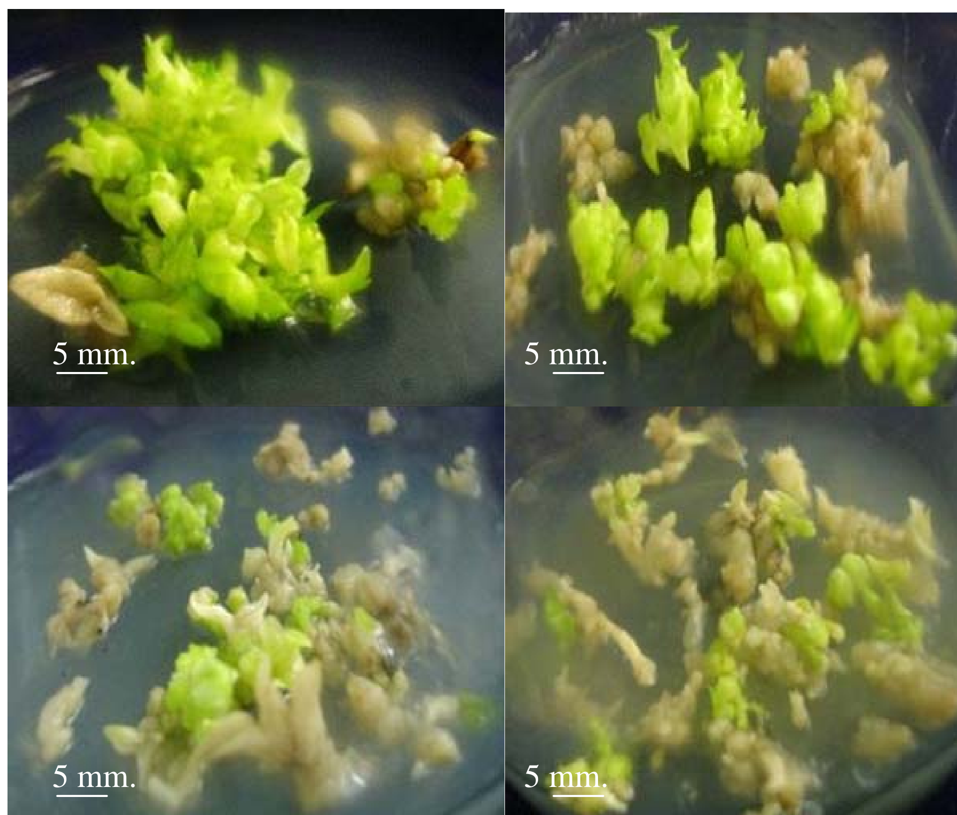

Figure 2 The survival of *D. secundum* plantlets after treated with colchicine 0.05% (A) 48 h (B) 72 h (C) 96 h and (D)120 h. Examined after culture on VW medium without colchicines for 3 months.

Table 1 The average length and number of stomata of diploid and polyploidy *D. secundum*.

Ploidy level	No. of Plant examined	Length of guard cell (µm±SE)	No. of stomata (mm^{-2} ±SE)
Diploid	20	30±0.47	118 ±2.94
Polyploid	20	38±1.09	132±2.94

Table 2 The effect of *in vitro* colchicine treatment on chrolophyll contents of plantlets of *D. secundum.*

Day	Chrolophyll content (mg g^{-1}fresh weigh)±SE				
	Colchicine concentration (%)				
	0	0.05	0.1	0.15	0.2
1	0.263±0.16[a]	0.078±0.01[b]	0.096±0.01[b]	0.174±0.01[c]	0.167±0.01[c]
2	0.254±0.16[a]	0.070±0.01[b]	0.099±0.01[b]	0.116±0.01[c]	0.118±0.01[c]
3	0.244±0.16[a]	0.146±0.01[b]	0.080±0.01[b]	0.128±0.01[c]	0.169±0.01[c]
4	0.264±0.16[a]	0.114±0.02[b]	0.152±0.01[b]	0.131±0.01[c]	0.149±0.01[c]
5	0.265±0.16[a]	0.152±0.02[b]	0.106±0.01[b]	0.127±0.01[c]	0.115±0.01[c]

standard with known ploidy (Figure 3A). Tetraploids with 4C DNA showed a histogram with a peak 2 at channel 400 (Figure 3B). Hexaploids and octaploids were showed histogram with peaks 3 and 4 at channel 600 and 800, respectively (Figure 3C and 3D).

Characteristics of Diploids and Polyploids

In vitro comparison of roots, leaves and stem among diploids and polyploids showed that the roots of polyploid plantlets were shorter and thicker than those of diploids. The root length of the polyploid were about one-third of the root length of the diploids. The stems and leaves of polyploid plantlets were thicker than those of the diploids. (Figure 4) Flower size of polyploid plantlets were larger than those for the diploid plantlets (Figure 5).

Guard cells of multiplied plantlets were obserbed and their size were measured. The averaged length of diploid guard cells was 30±0.47 µm and the averaged length of polyploid guard cells was 38

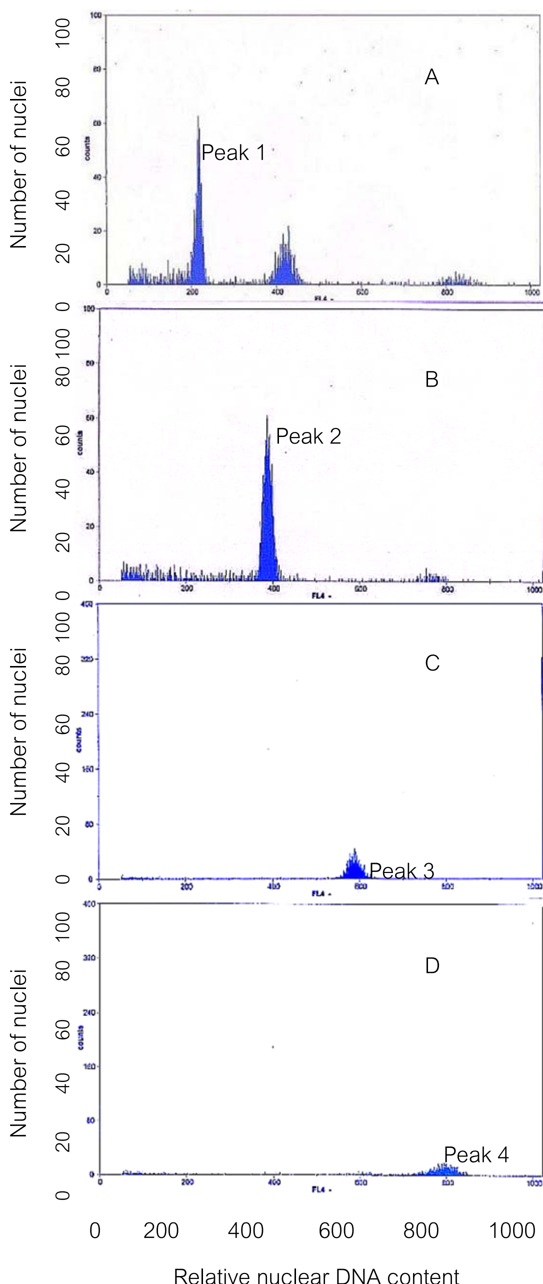

Figure 3 Flow cytometric histograms of (A) 2x, (B) 4x, (C) 6x and (D) 8x plantlets.

Figure 4 Variation in leaves, roots and stems of *D. secundum* diploid plantlets (A and C) and polyploid plantlets (B and D).

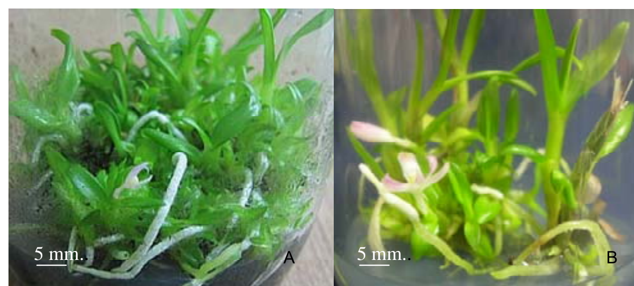

Figure 5 Comparison of flower size between diploid plantles (A) and polyploidy plantlets (B).

±1.09 µm. The number of stomata that were diploid was 132±2.94 mm^{-2} and the number of polyploids was 118±2.94 mm^{-2} (Table 1 and Figure 6)

Conclusions

In vitro induction of polyploidy in *D. secundum* was proved successful when protocorms and plantlets were treated with colchicines at a concentration of 0.05 % for 1 day. Morphological characteristics such as leaf stem root and flower size were larger and thicker in the polyploids when compared to the diploids. Several reports used colchicine as antimitotic substance. It binds to the cell protein tubulin and arrests mitosis in metaphase due to the inhibition of spindle formation. It causes depolymerisation and the disappearance of the fibrillar microtubules in granulocytes and other motile cells, inhibiting their migration as well as their metabolic and phagocytic activity (Sundov et al., 2005). In *C. intermedia* used colchicine concentration between 0.05 and 0.1% for 4 days treatment to be effective on the production of mixoploid and tetraploid (Silva et al., 2000). Griesbach (1981) obtained 50% of tetraploid *Phalaenopsis* using 0.05% of colchicines, however using a more prolonged treatment (10-14 days).

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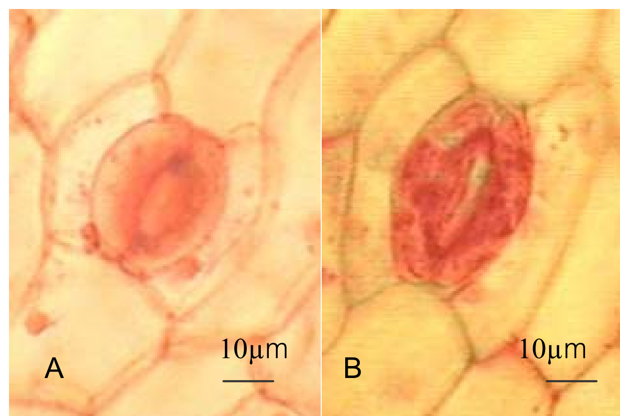

Figure 6 Guard cells of *D. secundum* diploid plantlets (A) and polyploidy plantlets (B).

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