Polyploid Induction by Colchicine Treatments and Plant Regeneration of *Dendrobium chrysotoxum*

P. Atichart

Department of Biology, Faculty of Science, Mahasarakham University
Kantarawichai District, Mahasarakham 44150, Thailand

Corresponding author. Email: porntip_atichart@yahoo.com

Abstract

The purpose of this research was to investigate the effect of colchicines concentration and duration time to polyploid induction and plant regeneration of *Dendrobium chrysotoxum* L. The method was conducted by inclusion of colchicine into semi-solid VW medium. Protocorm like bodies (PLBs) of diploid *D. chrysotoxum* were treated with 0, 0.01, 0.02, 0.03, 0.04, and 0.05% colchicines (w/v) for 1, 2, 3, 4 and 5 days. The most effective treatment was 0.04% colchicine for 1 day which resulted in about 84% surviving PLBs and with 47 % of tetraploid orchids, as measured by flow cytometry. The treated PLBs were cultured on the same medium supplemented with 0, 0.5 and 1 mg L^{-1} NAA and 0, 0.5 and 1 mg L^{-1} BA for plant regeneration. Treated PLBs with 0.01% and 0.02% colchicines, the highest number of proliferated shoot (2.36 per explants and 2.44 per explant respectively) was obtained from 1 mg L^{-1} NAA and 0.5 mg L^{-1} BA. In the treatment with 0.03% and 0.04% colchicines, the highest number of proliferated shoot (3.40 per explants and 4.35 per explants respectively) was obtained from the culture media supplemented only with 0.5 mg L^{-1} BA and 1 mg L^{-1} BA .

Keywords: colchicine, protocorm, flow cytometry, orchid

Introduction

Dendrobium is one of the most important types of commercial orchids used for cut flowers and potted plants, flower sizes of some varieties are small such as *D. chrysotoxum*. It is a cluster of bright yellow, fifty cent size and honey scented. Chromosome doubling has been used in breeding program for improved new characteristics of orchid flower. Colchicine treatment has become a common tool use for polyploid induction in many plants. Economic crops such as wheat, oats, cotton, coffee, apples, roses, and bananas are polyploid (Luckett, 1989; Thao et al., 2003; Sundov et al., 2005). The process can occur naturally or through human manipulation. In general, polyploid plants exhibit superior phenotypes to those of diploids such as stronger stems, and thicker and larger leaves, flowers, fruits, and seeds. Polyploid induction can be used as a means to create and select new and better breeds for further use. In order to produce polyploidy plants, the chemical colchicine is widely used because of its effectiveness and availability but it is toxic to cells. Polyploids were induced successfully from in vitro plants of diploid by treating with cochicine in different concentrations in *Miscanthus sinensis* (Petersen et al., 2003), oil palm (Madon et al., 2005), sesame (Mensah et al., 2007), Ginger (Sakhanokho et al., 2009), Basil (Omidbaigi et al., 2010) and cocoyam (Oumar et al., 2011), different explant materials were treated with colchicine to induce chromosome doubling. Ploidy levels could be easily determined by flow cytometry (Costich et al., 1993; Allum et al., 2007; Sarathum et al., 2011; Oumar et al., 2011. The objectives for this experiment were to determine the effective concentrations of colchicine, the appropriate duration time in the polyploid induction

and plant regeneration from treated PLBs of *D. chrysotoxum*.

Materials and Methods

Plant Material

PLBs of *D. chrysotoxum* were used as explant sources derived from cultured of *D. chrysotoxum* seed on Vacin and Went (1949) medium (VW) 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 mg L^{-1} glycine, 20 g l mg L^{-1} sucrose, 15% coconut water and 0.8% agar at pH 5.4. The explants were cultured under 16 h photoperiod (light intensity 40 μmole m^{-2} s^{-1}) at 25±2°C for 4 weeks. After 4 weeks of cultured seeds were formed Protocorm like bodies (PLBs).

Colchicine Treatment

PLBs were treated with 0, 0.01, 0.02, 0.03, 0.04, and 0.05% colchicine for 1, 2, 3, 4 and 5 days. After the treatment, they were washed with steriled distilled water and then cultured on 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, 4 mg mg L^{-1} glycine, 15% coconut water, 20 g L^{-1} sucrose 8 g L^{-1} agar, pH 5.4. Each treatment was replicated 5 times and 50 protocorms were cultured per each replication. After culturing for 12 weeks, the survival plantlets was examined.

Plant Regeneration

Treated PLBs of *D. chrysotoxum* with colchicines were cultured on VW medium for 3 month they could not regenerated therefore treated PLBs would transfer to VW medium supplemented with 0, 0.5 and 1 mg L^{-1} α-naphthalene acetic acid (NAA) and 0, 0.5 and 1 mg L^{-1} Benzyl adenine (BA) for plant regeneration. The explants were cultured under 16 h photoperiod (light intensity 40 μmole m^{-2} s^{-1}) at 25±2°C. The observation was taken at regular intervals of one week up to the 20 weeks and the obtained result was recorded.

Ploidy Level Determination

After 20 weeks, the proliferated shoots were subjected to test their ploidy levels 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 Petri dish containing hypotonic buffer Cy stain ^{R}UV ploidy (one step DAPI staining solution) and then filtered through a 30 μm celltrics disposable filter. The samples were analyzed with Partec PAII.

Statistical Analysis

Mean values for each duration and concentration of colchicine treatment and plant regeneration were tested in five replications and subjected to factorial in Completely Randomized Design (CRD) analysis of variance and compared by Duncan's new multiple range test (DMRT) at $P<0.05$.

Results

Survival Rate

There are statistically significant differences ($p<0.05$) between the average survival rates over all duration and concentration of colchicines treatment, declining from 100% in the colchicines free control to 44% at the highest concentration of 0.05% treated for 3 days. The interaction between concentration and the duration of the colchicine treatments found that higher concentration and longer duration reduced survival of explants (Figure 1).

Plant Regeneration

Regeneration of treated PLBs after treated with colchicines on modified VW medium supplemented with 0, 0.5 and 1 mg L^{-1} NAA and 0, 0.5 and 1 mg L^{-1} BA. The mean number of proliferated shoot were evaluated after 20 weeks of cultures. The result showed significantly ($p<0.05$), treated PLBs with 0.01% and 0.02% colchicines, the highest number of proliferated shoot (2.36±0.4 per explants and 2.44±1.3 per explant respectively) was obtained from 1 mg L^{-1} NAA and 0.5 mg L^{-1} BA. But the treated PLBs with 0.03% and 0.04% colchicines, the highest number of proliferated shoot (3.40±1.26 per explants and 4.35±1.45 per explant respectively) was obtained from the culture media supplement only with 0.5 mg L^{-1} BA and 1 mg L^{-1} BA, respectively (Table 1).

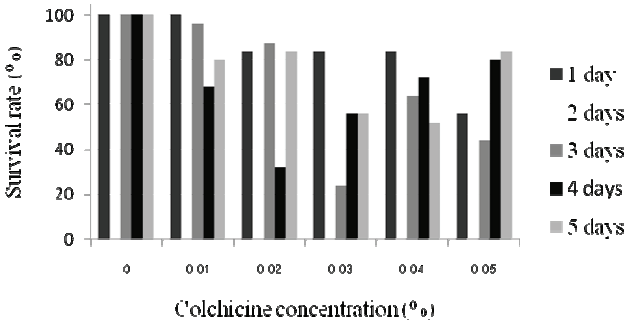

Figure 1 The effect of *in vitro* colchicine treatment on the survival rate of *D. chrysotoxum* PLBs.

Table 1 Effect of different concentration of α-naphthalene acetic acid (NAA) and Benzyl adenine (BA) on plant regeneration of *D. chrysotoxum* treated protocorm with colchicines.

Treatment concentration (%)	Growth regulator (mg L^{-1})		Mean number of regenerated shoots (mean±S.E)
	NAA	BA	
0.01	0	0	1.72 ± 0.2bc
	0	0.5	1.20 ± 0.4abc
	0	1	0.40 ± 0.5 a
	0.5	0	0.00 ± 0.3 a
	0.5	0.5	0.60 ± 0.1ab
	0.5	1	0.76 ± 0.3 ab
	1	0	2.36 ± 0.7
	1	0.5	2.36 ± 0.4 c
	1	1	0.20 ± 0.2 a
0.02	0	0	2.30 ± 1.35bc
	0	0.5	0.70 ± 0.4ab
	0	1	3.13 ± 1.267c
	0.5	0	0.40 ± 0.4a
	0.5	0.5	0.80 ± 0.6ab
	0.5	1	1.30 ± 0.3ab
	1	0	1.46 ± 1.23 abc
	1	0.5	2.44 ± 1.35bc
	1	1	0.00 ± 0.23 ab
0.03	0	0	2.26 ± 1.23bc
	0	0.5	3.40 ± 1.26 ab
	0	1	0.50 ± 0.4a
	0.5	0	1.52 ± 0.6ab
	0.5	0.5	1.52 ± 0.6 ab
	0.5	1	2.10 ± 1.34 bc
	1	0	0.80 ± 0.5 ab
	1	0.5	0.90 ± 0.4 ab
	1	1	1.44 ± 1.15 ab
0.04	0	0	0.86 ± 0.5 a
	0	0.5	3.56 ± 1.52b
	0	1	4.35 ± 1.45 b
	0.5	0	1.70 ± 1.23a
	0.5	0.5	0.80 ± 0.5 a
	0.5	1	0.86 ± 0.5a
	1	0	1.40 ± 1.25a
	1	0.5	1.00 ± 0.9 a
	1	1	0.40 ± 0.3 a

Determination of Ploidy Level by Flow Cytometric Analysis

In diploid control plants, used as an external standard, 100% of the nuclei were in G1 phase of the cell cycle, peaking at a relative nuclear DNA content of 200 (chanel number) indicated that the diploid (Figure 3A), whereas the tetraploid remaining nuclei appeared at a relative nuclear DNA content of 400 (chanel number), in late S or G2 phase of the cell cycle (Figure 3B), and mixoploid remaining nuclei appeared at a relative nuclear DNA content of 200 and 400 (chanel number) (Figure 3C)The ploidy level of the treated plantlets was affected by the concentration of colchicine and the duration of treatment. When plantlets were treated with 0.04% colchicine, plantlets exhibit increases in ploidy level. At the same concentration of colchicines (0.04%) but when the time of exposure was increased to 2 days, 30% tetraploids and 46% mixoploids (2x+4x) were found among tested plantlets (Table 2).

Discussion

In vitro induction of polyploid in *D. chrysotoxum* proved successful when treated PLBs with colchicines concentration at 0.04% for 1 and 2 days, Tetraploid and mixoploid were found. Several reports used colchicine as antimitotic substances. It binds to cell protein tubulin and arrests mitosis in metaphase due to failure of spindle formation. It causes depolymerisation and disappearance of the fibrillar microtubules in granulocytes and other motile cells, inhibiting their migration as well as metabolic and phagocytic activity (Sundov et al., 2005) In many plant species colchicine causes side effects such as sterility, abnormal growth and morphology, chromosome losses or rearrangements and gene mutation (Luckett, 1989). Using high colchicines concentration at 0.1, 0.15 and 0.2% for longer than 24 h, chrolophyll contents were decreased and plantets were died after treated. The survival of the explants after colchicine treatments depend on the concentration and duration of the treatment. In general, higher coccentration and longer duration reduced survival of plants (Thao et al., 2003; Atichart and Bunnag, 2007).

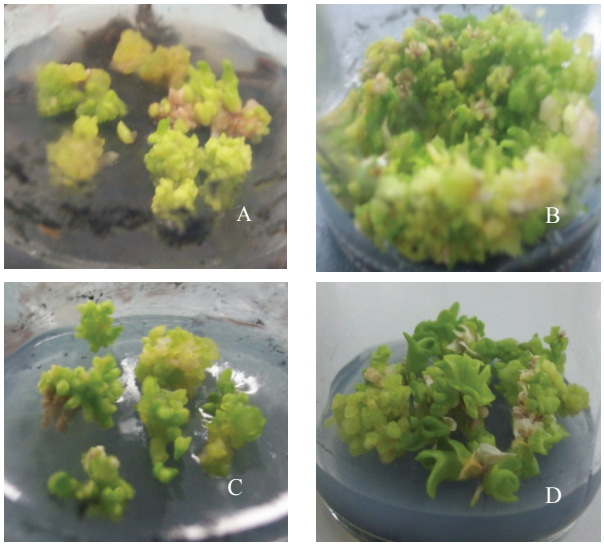

Figure 2 Plant regeneration of *D. chrysotoxum* treated protocorm with co% cultured on 0.03% cultured on VW lchicines (A), 0.01% cultured on VW supplement with 1 mg L^{-1} NAA and 0.5 mg L^{-1} BA (B), VW supplement with 1 mg L^{-1} NAA and 0.5 mg L^{-1} BA (C), and 0.02 supplement with 0.5 mg L^{-1} BA 0.04% cultured on VW supplement with 1 mg L^{-1} BA (D).

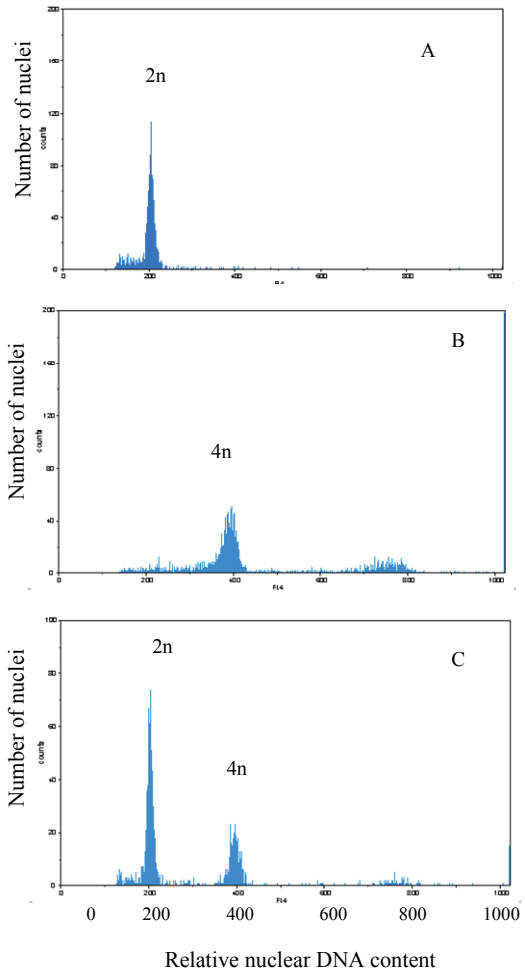

Figure 3 Histograms showing different ploidy levels for the standard diploid (A) the induced tetraploid and (C) mixoploid.

Treated PLBs of *D. chrysotoxum* with colchicines in various concentration and duration could not regenerated to plantlet. Used of growth regulators may help them to shoot proliferated. The type and concentration of growth regulators are an initial consideration for micropropagation of orchid species (Genkov and Ivanova, 1995). Addition of cytokinin to the medium increase the number of shoots which demonstrates the significance of exogenous cytokinin to enhance the multiple shoots. BA influences shoot proliferation by stimulating quick cell divisions to induce large number of multiple shoots (Yakimova et al., 2000; Roy and Banerjee, 2002; Ronzhina, 2003; Hameed et al., 2006). Results are also according to Asghar et al. (20011) who reported that axillary buds of orchid *Dendrobium nobile* var. Emma white were proliferated by using phytotechnology medium (O753) supplemented with benzylaminopurine (BAP) and kinetin (Kin) as well as coconut water (CW) and Roy and Banerjee (2002) who reported that BAP enhances the shoot multiplication more actively than Kin. BAP provided smaller lengths of proliferated shoots in contrast to shoots number. Being a strong cytokinin, it depresses shoot length by an increase in number of axillary buds (Hameed et al., 2006).

The following conclusions regarding the efficiency of polyploidy induction, base on the in vitro application of colchicines to *D. chrysotoxum*, plantlets of this study will multiply and observation about plant growth, plant morphology such as the number of leaves and flower per plant, plant size and flower size and determine chlorophyll content for the next experiment.

Acknowledgments

This research was supported by Mahasarakham University and 2553 research fund form National Research Council of Thailand.

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Table 2 Ploidy level was analyzed two months after colchicines treatment.

Treatment concentration (%)	Duration (days)	Ploidy level (%)		
		2X	4X	2x + 4X
Control				
0.04 colchicine	0	100	0	0
	1	53	47	0
	2	24	30	46

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Manuscript received 3 March 2013, accepted 22 May 2013