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Effect of colchicine on survival rate and ploidy level of hydrid between *Dendrobium santana* x *D. friedericksianum* orchid

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Abstract The effect of colchicine on survival rate and ploidy level of hydrid between *Dendrobium santana* and *D. friedericksianum* orchid was studied. *In vitro* polyploid induction of this hydrid orchid was carried out by soaking nodal explant in different concentrations of colchicine for 24, 48 and 72 hours. The results showed that LD_{50} of colchicine for 24, 48 and 72 hours was 0. 052%, 0.041% and 0.011%, respectively. The treatment of colchicine at the above concentrations and durations increased DNA content from 33 to 50 % of the original DNA content as analyzed by flow cytometry (FCM) technique. This result was in accordance with physiological characteristics, which revealed the bigger size of guard cells but lower density and increase in number of chloroplasts in guard cell.

Keywords: *Dendrobium* hydrid, colchicine, survival rate

Introduction

Dendrobium is a popular orchid among all growers because it is easy to grow, bloom year round and various sizes of flowers and psuedobulbs. Apart from the benefits of using as an ornamental plant, some *Dendrobium* also be applied for medical purposes. *D. santana* is a hydrid between *D .moniliforme* (dwarf Nobile) and *D. friedericksianum* (Thai wild orchid), produces flowers many times in a year and form flower buds since small size at seedling stage. The mature orchids give numerous odor flowers of thick yellow petals. The hydrid of *D. santana* and *D. friedericksianum* should be heat tolerant orchid that is very easier to grow in tropical and beautifully marked in lip colours, which is suitable for growing as economic orchid in these areas. The hydrids can be used as a model for studying *in vitro* flowering that make value added produced as souvenirs.

So far, orchid is an economically important ornamental plant of Thailand. The export values of both flowers and seedlings of Thai orchids are over billion

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Baht per year. The orchid business has drastically increased in terms of quantity and value during the recent years. The competition among orchid production business is very intense, so a study on quality and quantity developments of orchids must be performed for commercial and conservative purposes. Moreover, plant breeding is very essential for creating bigger flowers by increasing chromosome using colchicine that can change plant characteristics to desirable traits.

The induction of polyploid or increase in chromosome set are plant breeding techniques for creating more biodiversity by increasing cell size affecting plants in having bigger size of vegetative parts and reproductive organs. These characteristics are different from polyploid plants that are developed from natural pollination, which can produce just a few polyploid plants.

The induction of polyploid plant can be performed by applying some chemicals inhibit the formation of spindle fibers. By this mechanism sister chromatid can not be pulled to the opposite pole of the cell during cell division at anaphase stage. Those chemicals are colchicine, orizaline, trifluralin, amiprophosemethyl and nitrous oxide. Among those chemicals colchicine is commonly used for ploidy induction purpose. Colchicine, a poisonous alkaloid extracted from the seeds and corms of *Colchicum autumnale*, is used in various times and concentrations. It is highly soluble in water, a desirable target in application is microtubule because it is matched with tubulin protein that is a part of microtubule, resulting in miss producing long fibers called spindle fibers during cell division. At anaphase, chromatic fiber cannot be attached at cell polar, so the chromosomes are increased from 2x to 4x, or polyploid (Starr and Taggart, 1995). Several attempts have been made to induce polyploidy orchids with colchicine. The first success of induced polyploid in orchid (*Laelia tranaei* var *alba*) was done by MacLeod (1947). The resulting plants were chimeras composed of predominantly diploid tissue and had low commercial value. The application of colchicine for polyploid induction in orchid development is the important method for plant development in order to increase sizes of flower, pseudobulb or other desired characteristics. The commonly used concentrations are 0.01–1.0%. The concentration of colchicine for polyploidy induction is species-specific (Silva *et al*., 2000; Kim *et al*., 2003; Vichiato *et al*., 2007; Atichart and Bunnag, 2007).

Therefore, the objective of this study was to evaluate the effect of colchicine at different concentrations and durations on changes of chromosome of hydrid between *D. santana* and *D. friedericksianum* orchid and to investigate the changes in some physiological characters in order to fulfil market requirements. In addition, this study can be used as guideline for the

development of other Thai orchids in the future.

Materials and methods

Plant material

Six-month-old seedlings of hydrid between *D. santana* and *D. friedericksianum* induced from culturing seed on VW medium were used in this study. Subculture in same medium was rountinely carried out at two-month-intervals. The culture was maintained at 25±2℃ under 2,000 lux/m^2/sec of fluorescent lamp at 14 hours phototperiod.

Effect of cochicine on survival rate of hydrid between D. santana and D. friedericksianum and physiological characteristics

Survival rate

Nodal segment from *in vitro* seedling of hydrid between *D. santana* and *D. friedericksianum* was excised at size of 1.0 cm consisting of a stem with one node and soaked in 0.01%, 0.05% and 0.10 % colchicine, then placing on shaker (100 rpm) at 26±2^{o}C in the dark for 24, 48, and 72 hours. After that the explants were transferred to sterile paper for blotting dry and then transferred to culture on VW solidified medium without plant growth regulators. The survival rate and 50% lethal dose (LD_{50}) were recorded after one month of culture and statistically compared among those concetrations of colchicine and periods of soaking. In addition, physiological characters in terms of guard cell size, chloroplast number and stomatal density were evaluated and statistically compared among those treatment using 3-months-regenerated-shoots after colchicine treatment.

Analysis of DNA cotent by flow cytometry technique

The leaves from 10 explants of putative polyploid hydrid and 10 control explants were used for DNA content analysis. The leaf laminar was separated from midrib, sliced into 1-2 cm in size and placed in 6-cm diameter Petri-plate. Polyvinylpirrolidone (PVP) solution at volume of 700 µl was added to the plate and the leaves were chopped with sharp razor blade for 100 times. The mixture of chopping leaf laminar and PVP solution was filtered through 20 µm nylon mesh placed on funnel to centrifuge tubes. The filtrate was added with 20 µl propidium iodide (PI) solution, mixed by shaking the tubes gently for 2-3 times, then incubatedon ice 10 minutes. The sampling solutions were feeded into

Beckman Coulter Flow Cytometry. The DNA content was counted, recorded and compared between putative and control shoots in comparison with DNA of Japanese rice of Nipponbare [(*Oryza sativa*) cv. 'Nipponbare']. DNA content from both sources of shoots was calculated using the following equation:

$$\text{Sample (2C DNA)} = \frac{\text{G1 peak channel of sample}}{\text{G1 peak channel of } \textit{Oryza sativa}} \times 108 \text{ pg}$$

Statistical Analysis

The experiments were set up in completely rancomized design (CRD) with three treatments consisting of three replications per treatment. The test of significant differences among treatments were detected using Duncan's multiple range test (DMRT) at 5% confidence level.

Results

Effect of cochicine on survival rate and ploidy level

In vitro nodal explants of hydrid between *D. santana* and *D. friedericksianum* could survive at 100% in control treatment (without soaking in colchicine solution). Soaking the nodal explants in various concentrations of colchine at different period of times caused the decreament of survival rate. The explants treated with 0.01%, 0.05% and 0.10% colchicine for 24 hours had the survival rate of 60.00%, 60.00%, and 13.33%, respectively. The explants treated with the same concentration of colchicine for 48 hours had the survival rate of 76.67% 16.67% and 6.67 %, respectively. After the explants were treated with colchicine for 72 hours, the explants had lower survival rate of 13.33%, 6.67% and 16.67%, respectively (Table 1).

The analysis of LD_{50} of colchicine treatment at different concentrations and durations showed that the values of LD_{50} of colchicine treatment for 24, 48 and 72 hours were 0.052%, 0.041% and 0.011%, respectively (Figure 1). Multiple shoots developed from the colchicine-treated nodal explants of hydrid between *D. santana* and *D. friedericksianum* were illustrated in Figure 2.

Table 1. Effect of different concentrations and durations of colchicine treatment on survival rate of nodal explants and shoot formation after cultured on VW for 30 days

Colchicine (%)	Duration (hrs)	Explants	Survival rate (%)	Shoot formation (%)
0	-	30	100.00[a]	93.33
0.01	24	30	60.00[c]	33.33
	48	30	76.67[b]	39.29
	72	30	13.33[d]	50.00
0.05	24	30	60.00[c]	39.52
	48	30	16.67[d]	66.67
	72	30	6.67[d]	66.67
0.1	24	30	13.33[d]	83.33
	48	30	6.67[d]	66.67
	72	30	16.67[d]	100.00
F-test			*	ns
C.V(%) .			18.04	59.25

*=significantly different at $P \leq 0.05$
Ns = non significant difference
Mean values followed by the same letter(s) within a column are not significantly different. ($P \leq 0.05$)

Physiological characteristics

Colchicine at different concentrations and durations caused the alteration of some physiological charcteristics, especially guard cell size, density and number of chloroplasts in guard cell. For guard cell size the results showed that shoots obtained from colchicine-treated nodal explants at higher concentrations and longer durations had bigger size of guard cells. Whereas the density of guard cell decreased. The key parameter indicating increase in ploidy level is number of chloroplasts in guard cell. The result showed that the explants treated with 0.1% colchicine for 72 hours had the highest number of chloroplasts at 68 chloroplasts following by 0.01% colchicine for 72 hours had the number of chloroplast at 48.33 chloroplasts which was significant ($p \leq 0.05$) higher than that of control (without colchicine treatment, 32.56 chloroplasts) (Table 2, Figure 3).

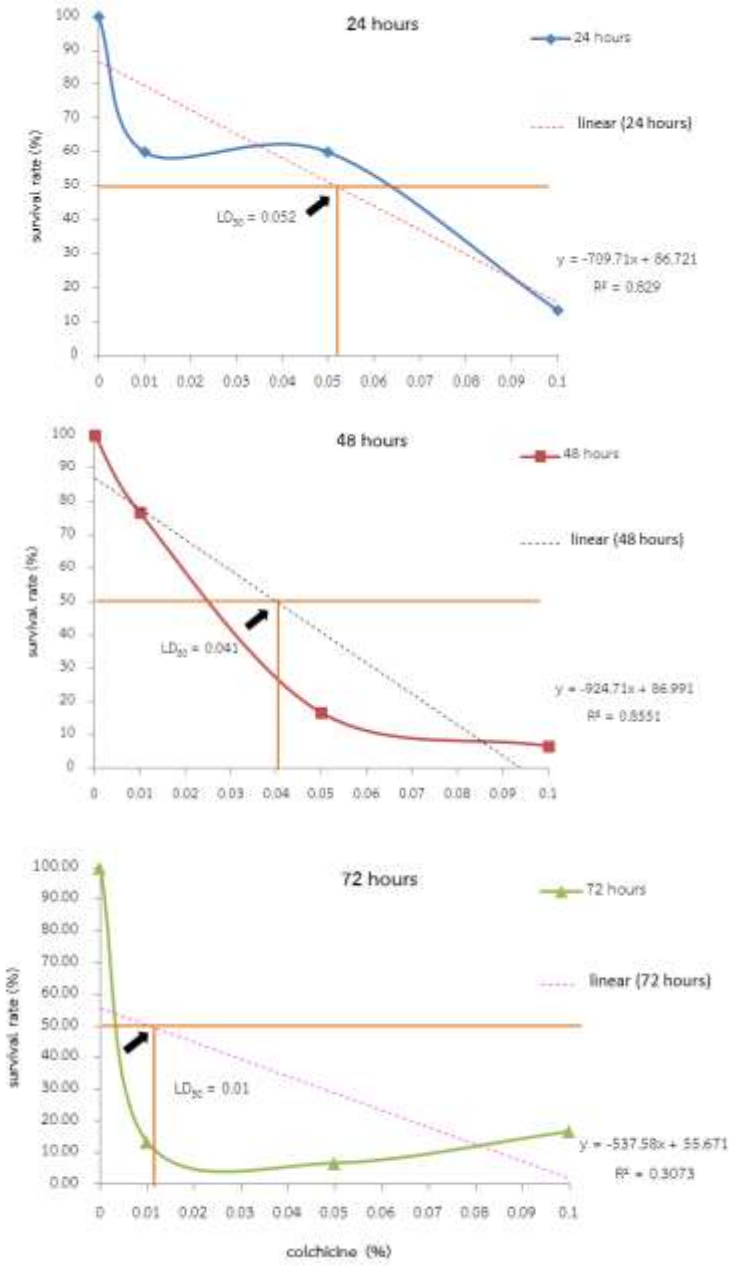

Figure 1. Survival rate at 50 % (LD_{50}) of hydrid between *D. santana* and *D. friedericksianum* after soaking nodal explants in different concentrations and durations of colchicine subsequent to culture on solidified VW medium for 30 days

Figure 2. Multiple shoots developed from nodal explants of hydrid between *D. santana* and *D. friedericksianum* treated with 0.1% colchicine for 48 hours subsequent to culture on solidified VW medium for 60 days (bar = 1 cm)

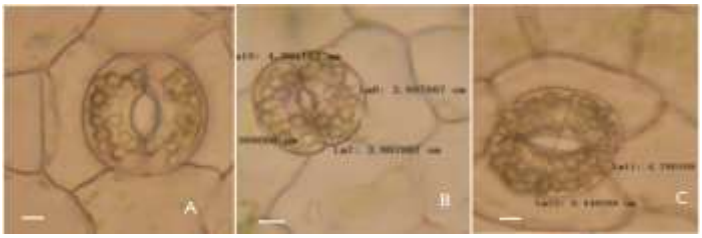

Figure 3. Chloroplasts in guard cell of hydrid between *D. santana* and *D. friedericksianum after* 8 months culture on VW medium (bar = 1 μm); (A) Control; (B) Hydrid obtained after treatment with 0.05% colchicine for 72 h.; (C) Hydrid obtained after treatment with 0.1% colchicine for 72 h.

Table 2. Effect of different concentrations and durations of colchicine treatment on some physiological characteristics of hydrid between *D. santana* and *D. friedericksianum*

colchicine		**Guard cell size (μm)**		**Stomatal density (mm^2)**	**Chloroplast number**
Concentration (%)	**Duration** (**hr**)	**lenght**	**width**		
0	24	3.81^{c}	3.75^{de}	19.67^{a}	32.56^{c}
0.01	24	3.58^{d}	4.14^{c}	14.33^{c}	30.33^{cd}
	48	3.67^{d}	4.69^{b}	17.25^{c}	21.67^{e}
	72	4.94^{a}	4.73^{b}	9.00^{f}	48.33^{b}
0.05	24	3.66^{d}	3.28^{f}	20.67^{a}	21.00^{e}
	48	4.30^{b}	3.86^{de}	13.00^{c}	33.00^{c}
	72	3.94^{c}	3.93^{d}	8.67^{e}	35.67^{c}
0.10	24	4.30^{b}	3.38^{f}	14.33^{c}	26.00^{de}
	48	3.61^{d}	3.72^{e}	21.00^{a}	24.67^{e}
	72	5.04^{a}	5.22^{a}	11.00^{d}	68.68^{a}
F-test		*	*	*	*
C.V.(%)		3.41	2.76	7.65	8.92

*=significant different at $P \leq 0.05$

Mean values followed by the same letter(s) within a column are not significantly different ($P \leq 0.05$).

Flow cytometry analysis revealed that DNA content of hydrid between *D. santana* and *D. friedericksianum* were mixoploid. DNA content from leaves of shoots derived from non-treated nodal explants was diploid at quantity of 3.07±0.11 picogram. The base pair size was at $1.48x10^9$ bp. While the DNA content of the hydrid treated with colchicine at every concentration and duration was between 2.98±0.04 to 3.64±0.09 picogram. The base pair size was between $1.44x10^9$ bp to $1.76x10^9$ bp. (Table 3, Figure 4).

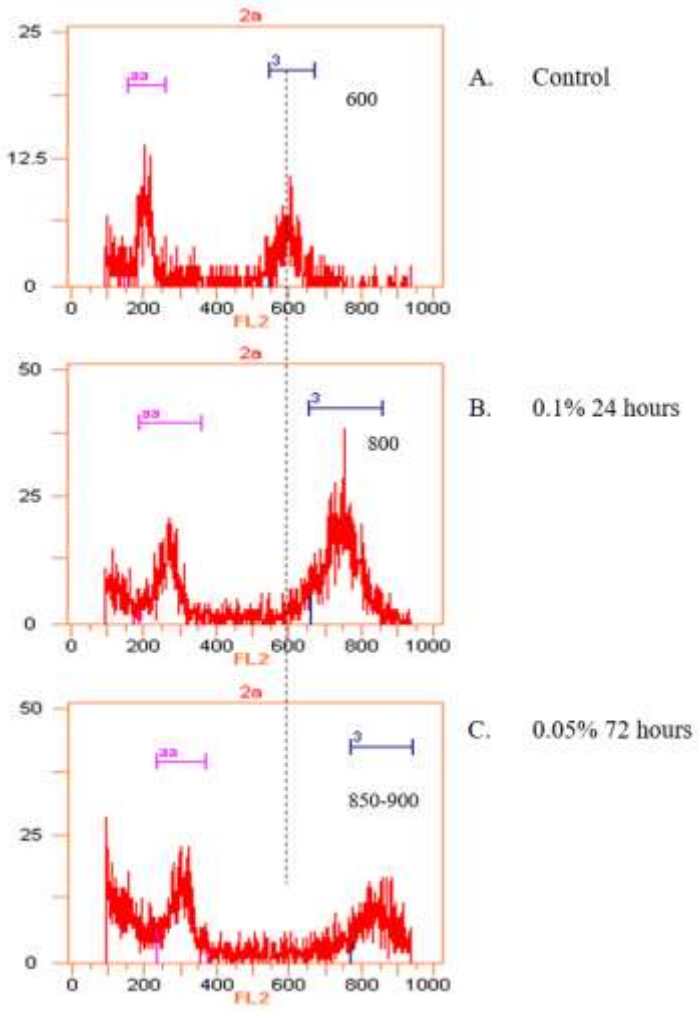

Figure 4. Histograms showing DNA content of hydrid between *D. santana* and *D. friedericksianum* treated with different concentrations and durations of colchicine in comparison with DNA content of Nipponbare rice

Table 3. Mean values of DNA content of hydrid between *D. santana* and *D. friedericksianum* treated with different concentrations and durations of colchicine

Colchicine		Treatment number	DNA content ±SE (pg $2C^{-1}$)	Mbp*
Concentration (%)	Duration (h)			
0	24	3	3.07±0.11	1.48
0.01	24	3	3.18±0.03	1.53
	48	3	3.64±0.09	1.76
	72	3	3.19±0.12	1.54
0.05	24	3	3.61±0.27	1.74
	48	3	3.09±0.07	1.49
	72	3	3.01±0.07	1.45
0.10	24	3	3.31±0.19	1.60
	48	3	2.98±0.04	1.44
	72	3	3.16±0.21	1.52

*1 picogram DNA =965 Mbp

Discussion

The nodal explant of hydrid orchid between *D. santana* and *D. friedericksianum* soaked in colchicine with different concentrations and durations for 30 days showed different response in survival rate. High concentration of colchicine with longer duration of application yielded low survival rate of cultured explant subsequent to shoot development (multiple shoot formation). The result was in accordant with *D. scabrilingue* Lindl. (Sarathum *et al*., 2008) and *D. formosum* (Petchang, 2010). It is assumed that high concentration of colchicine would be toxic to plant cell, caused imbalance of cell and affected to internal process of cell causing plants died eventually. So far, increase in ploidy of *Dendrobium* from protocorm and protocorm-like bodies (PLBs) had been reported. For example, Sanguthai *et al.* (1973) could induce numerous hexaploid and mixoploid *Dendrobium* by soaking PLBs in 0.1% colchicine solution. While, *D. scabrilligue* gave a good results in survival rate of protocorm at 36.8% from soaking PLBs in 0.075% colchicine for 14 days. After treating with colchicine ploidy level or DNA content was investigated with flow cytometry technique. The results showed that the polyploid could be induced at 43.1% (Sarathum *et al.,* 2010). Watrous and Wimber (1988) could induce tetraploid of *Paphiopedilum* for more than 50% when soaking protocorm in 0.05% colchicine for 3-10 days. For other orchids Silva *et al*. (2000) reported that soaking of PLBs of *Cattleya* in 0.05-0.1% colchicine for 4 days was the optimum treatment for the increment of ploidy level, but longer duration at 10-14 days was required for *Phalaenopsis*

(Griesbach, 1981). However, polyploid induction from nodal explant of *Dendrobium* had never been reported. In this present study, soaking of nodal segment of hydrid between *D. santana* and *D. friedericksianum* in 0.05-0.1% colchicine for 24-72 hours could promote the increment of DNA content at 33-50% of the original content as revealed by flow cytometry techniques. This could be assumed that hydrid plantlets obtained from treating nodal explants with colchicine are mixoploid, and some plants would be triploid because the DNA content was increased more than 50% from the control plants.

After comparing stomatal size and density including chloroplast number of hydrid between *D. santana* and *D. friedericksianum* orchid treated with different concentrations of colchicine and durations, the results showed that the explants treated with higher concentration and longer duration of colchicine had higher guard cell size and chloroplast numbers. This result was in accordant with guard cell size of *Doritis* treated with colchicine. Octaploid plants of *Doritis* had the longest guard cell followed by tetraploid plants. The diploid had the shortest guard cell (Jiwanit, 2009). The results were in accordant with *D. secuntum* (Atichart and Bunnag, 2007), *Cattleya intermedia* (Silva *et al*., 2000), and *Phalaenopsis* (Chen *et al*., 2009). However, in *Cymbidium*, the results showed that guard cells of diploid, triploid, and tetraploid did not have different sizes. (Kim *et al.*, 2003) The comparison of stomatal density of hydrid between *D. santana* and *D. friedericksianum* orchid in this study showed that the treatment with higher concentration of colchicine with longer duration affecting the decrement in stomatal density. Similar result was also reported in *Doritis*. Stomatal density was decreased when ploidy level was increased. (Jiwanit, 2009). However, in Charng Daeng orchid, stomatal density was increased with the higher concentration of colchicine. (Kerdsuwan and Te-chato, 2012). However, the key parameter that indicates the duplication of chromosome is number of chloroplast in guard cell. Generally, increase in number of chromosome have a close relation with the increase in number of chloroplasts in guard cell. The result obtained in this present study showed that chloroplast number of mixoploid plants was increased slightly higer than 2 times when compare with diploid plants. DNA analyse also showed the increament of DNA content or chromosome but not two times like the number of choloroplasts. Next investigation will be carried out by root tip chromosome counting to make sure about the duplication of chromosome.

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