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Effects of colchicine on survival rate, morphological, physiological and cytological characters of chang daeng orchid (*Rhynchostylis gigantean* var. *rubrum* Sagarik) *In Vitro*

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PLBs (Protocorm-like bodies) at stage of GI 5 of Chang Daeng Orchid *(Rhynchostylis gigantea* var. *rubrum* Sagarik) cultured in liquid NDM medium with 2% sucrose and 15% coconut water (CW) were used for treating with colchicines in order to doubling chromosome. The treatments consisted of five concentrations of colchicine (0, 0.05, 0.10, 0.15 and 0.20%) together with 3 duration of treatment 24, 48, and 72 hours. The results revealed that LD_{50} lethal dose affected to 50% of colchicine based on regression analysis was 0.14% for 72 hours. PLBs immersed in colchicines at concentrations 0.20% for 72 hours provided a suitable survival rate of 26% and induced the highest percentage of tetraploid (60%). The results provide the several different traits between tetraploid and diploid plants, especially height of the plant. Growth rate and height of tetraploid plantlets, including leaf shape and size were far lower than those of control. Anatomical characters of the tetraploid plants revealed the highest stomatal densities at 3.00 cells/mm^2 in comparison with control plantlets. The average number of chloroplasts obtained in tetraploid plantlets was 46 chloroplasts/guard cells, which lower than those of diploid plants. This evident indicated a negative correlation between chloroplast number and ploidy level. Morphological characters of tetraploid plantlets had higher average number of leaves than those of control. In case of root length, tetraploid plantlets was significantly lower than that of control. Cytological observation of root tip revealed that tetraploid plantlets had chromosome number (2n=4x=76) two times higher than those of control (2n=2x=38).

Key words: Colchicine, Chang Daeng Orchid, Rhynchostylis gigantean, Tetraploid

Introduction

Chang Daeng Orchid *(Rhynchostylis gigantea* var. *rubrum* Sagarik) belongs to Orchidaceae with the basic chromosome number of 2n=2x=38. The habitat of Chang Daeng Orchid are originally located at some country in south

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Asia, e.g., Thailand, Myanmar, and southern of China. In Thailand, there are naturally distributed in many parts such as Chiangmai and Nong khai. A size of Chang Dang canopy or flower is larger than another in their genus, characterized by their leaf, which represent as a large and thick leaf. The aerial root system found in most of them is large and green color. The flowers are compact and fragrant. Chang Daeng is highly popular because they are facilitating propagation on a basis of tissue culture and they can provide a number of fragrant flowers every year.

In Thailand, orchids are highly important because they have been exported as the first rank in comparison with other ornamental flower plants. Orchid business have been making the highly income of 1,200 million baht per year to Thailand (Supattra and Weenun, 2005). However, conventional propagations of the orchid are ineffective, especially by seed propagation because their seed components lack both of endosperm and cotyledon which are necessary for growth and development of a seeding (Sompong, 1995). In addition, seedlings derived from seed are high variation because their parents are a cross between different genetic materials of the orchid. Thereby, *in vitro* propagations from the thin cell layer organs are interested because it reduces the variation form the hybrid and also decrease time consuming in most of propagation process (Van Le *et al*., 1999). Colchicine application used for doubling chromosome in numerous experiment plants is a useful method, which usually contribute valuable traits, such as the larger flowers, the long time of flower period. However, using colchicine for double chromosome in Chang Daeng Orchid has not been published, therefore, the objective of this study are to study the effects of colchicine at various concentrations and various duration time of treatment in order to induce chromosome duplication.

Materials and methods

Plant Materials

Two-month-old PLBs at GI 5 stages induced from culturing seed on VW containing 15% CW were used in this study. PLBs were maintained in liquid NDM medium supplemented with 2% sucrose and 15% coconut water. The medium was adjusted to pH 5.2 and autoclaved at 121^{o} C for 15 min. The cultures were maintained at 26±4 ^{o}C under 75 µmol/m^2/sec of fluorescent lamp at 14 hour photoperiod. Subculture was routinely carried out at 10-14 days intervals for at least one year.

Colchicine treatment

PLBs at GI 5 stages were treated with 0.00 (control), 0.05, 0.10, 0.15 and 0.20 % colchicines and incubated on a rotary shaker for 24, 48 and 72 h. After the end period of treatment, they were washed three times with sterile distilled water, then transferred to NDM medium supplemented with 2% sucrose, 15% coconut water and 0.75 % agar and subcultured monthly intervals. The survival rate, morphological, physiological and cytological characters were recorded at the fifth months after the culture and compared statistically. Morphological, anatomical and physiological analysis.

Evaluation of stomatal density, chloroplast number and morphology

Leaf samples of Chang Daeng Orchid were excised from putative tetraploid and diploid plantlet. The lower epidermis layer in the central part of the leaves were gently removed with pointed tweezers and placed on glass slide. The five sample leaves were used in each treatment, at least 10 randomly selected fields or view were observed and photographed using compound microscope. After taking photograph, the stomatal size and density were recorded and compared statistically.

During the growing period of five months, the morphological characteristics were compared between putative tetraploid and diploid plantlets. The number and length of leave and root were statistically compared from five randomly selected leaves and roots from each treatment.

Cytological analysis

Chromosome preparation and staining were performed according to the method described by Sharma and Sharma (1980) with some modifications. Actively growing root tips about 1 cm. in length from five plants in each treatment were excised and pre-treated with α-bromonaphthalene kept at 16 ^{o} C for 24 h, washed and transferred to fixative solution consisted of 95% ethanol : acetic acid (3:1, v/v) for at least 24 h. The fixed root tips were then hydrolyzed in 1 *N* HCl for 5 min at 60 ^{o} C. After hydrolysis, root tips were rinsed several time with distilled water and stained by carbol Fuchsin solution at room temperature for about 3-4 h. The stained region of root were cut and placed on a glass slide and squashed in 1-2 drops of carbol Fuchsin solution. These root tissues were observed under the compound microscope at magnification of 40x.

Statistical analysis

Mean data were analyzed using a one-way analysis of variance (ANOVA). Significant differences among treatments were detected using Duncan's multiple range tests (DMRT) at the 0.01 or 0.05 level of probability.

Results and discussion

Survival rate and plant regeneration

Some of PLBs gradually turned brown and died after incubation on NDM medium supplemented with 2% sucrose and 15% CW for 1 month. Survival rate of them was gradually reduced when treated with a high concentration of colchicine and with along period of immersion (Table 1). Survival rate of PLBs treated with colchicines at concentration of 0.05-0.10 % was nearly of 50 % while regression analysis represented that 50% survival rate of PLBs was obtained with 0.14% colchicine at 72 h (Fig 1). In contrast, samples which treated with same a concentration at 48 h gave the highest percentage of survival rate. However, high concentration of colchicines and longer duration of treatment or immersion provided the reduction of survival rate. Similar results were also reported by Nguyen *et al*. (2003) and Pincheiro *et al*. (2000). Fluctuation in survival rate obtained in the present study may be due to a small numbers of replications and different performance of PLBs (including, physiological stages of development, vigor etc.). Non-uniform PLBs give different response at the same concentration of colchicines. Generally, vigor PLBs can tolerate to high concentration of chemical mutagens better than weak PLBs. However, this effect had been consistented only for a short periods, for long time period, the survival rates were reduced to be zero because PLBs accumulate toxicity form colchicine, which causing to cell destruction (Rungrueng, 2537).

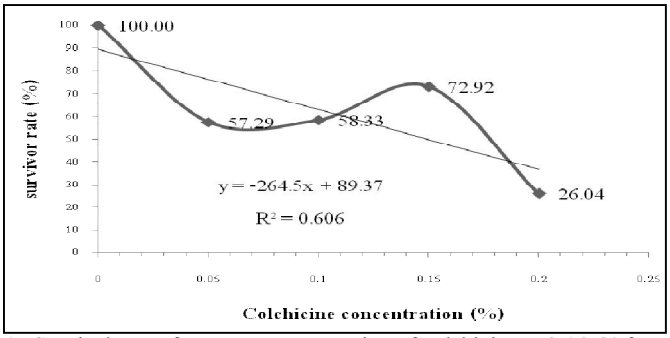

Fig. 1. Survival rate of PLBs at concentration of colchicine at 0.14 % for 72 h.

Morphological analysis

Morphological observation revealed that both size and shape of leaves of putative tetraploid were far lower than those of control plantlets. The leaves of putative tetraploid plantlets were short and narrow leading to a short stem (dwarf type). Moreover, the number of leaf per plant was reduced into half of control plantlets (Fig 2). This phenomenon was also observed in oil palm (Samala and Te-chato, 2012) which obtained from treating secondary somatic embryo (SSE) with 0.2% colchicine for 12 hours. Eventhough colchicine was reported to increase size of plant organ, leaf, flower and fruit, it had an adverse effect in Chang Daeng Orchid in this study and oil palm (Samala and Te-chato, 2012). For morphological characters, number and length of leaves were different between control and putative tetraploid plantlets (plantlets derived from treating PLBs with 0.20 % concentration for 72 h). Leave number of putative tetraploid plantlets were 3.50 leaves/plant while those of control diploid plantlets were 2.80 leaves.

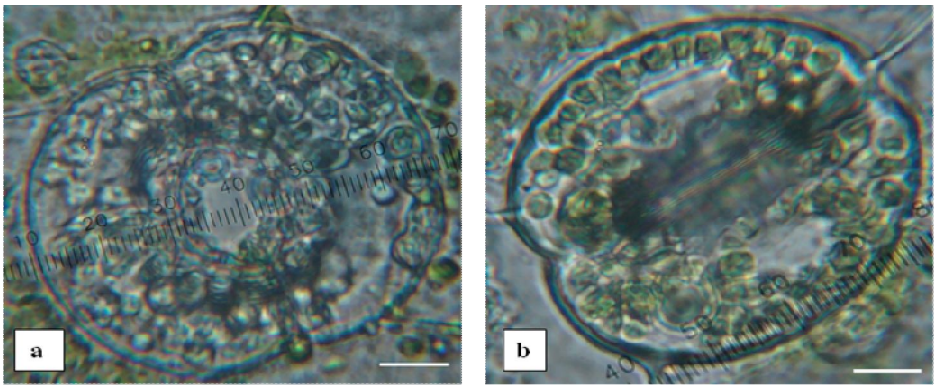

Fig. 2. Chloroplast number of Chang Daeng Orchid after treating with 0 % colchicine (a) and 0.15 % colchicine for 72 h (b).

Chromosome observation

The chromosome number of diploid plant was 38. Colchicine at 0.20 % for 72 h showed the best result with 3 tetraploid out of 5 explants examined (60%) (Table 4) The chromosome counting technique used to confirm a double of chromosome was the directly conventional method, which needed of time consuming and labors. The flow cytrometric technique was an alternative method, which was more preferable in various experiments because it was fast and accurate.

Anatomical and physiological analysis

The width and length of guard cell, stomatal density, chloroplast number and morphology

The width and length of guard cell of tetraploid plant was 53.33 and 45.83 μm, respectively longer than that of diploid plantlets. Similar result was observe in stomatal density which showed that tetraploid plantlets had higher than that of the diploid (Table 2). In general, PLBs immersed in high colchicine concentration and duration time showed the larger size of guard cell than PLBs treated with low and no colchicine conditions. These results were in accordance with the colchicine treatment in banana tissue (Saradhuldhat, 2540).

Furthermore, the length of guard cell was associated with different ploidy levels, high ploidy levels revealed high lengths of guard cell. Similar result was also found in various orchid studies (Atichart and Bunnage, 2007; Fan *et al*., 2003; Silva *et al*, 2000). Nevertheless, Kim *et al*. (2003) observed a non different of guard cell sizes in diploid and tetraploid *Cymbidium,* indicating that changing of guard cell size was varied in different types of plant species. For stomatal density, in general, it is unlike a size of guard cell, high ploidy levels caused to low stomata density, which were found in numerous plant e.g., *Cattleya intermedia* Lindl. (Silva *et al*., 2003), banana (Saradhulhat and Salayai, 2001). However, those results were contrasted from this experiment that high stomatal density were observed in high ploidy level, in agreement with a report of Kim *et al*. (2003) who studied in *Cymbidium*.

Chloroplast number of tetraploid plants in this study was 46.67 chloroplasts that lower than diploid plants (Fig. 2, Table 2). A number of chloroplast cloud be considered as another indicator for chromosome doubling. Naturally, high ploidy level provided a high number of chloroplast, however it may be depended on kinds of plant tissues (Liu *et al*., 2007). Contrary results were obtained in this study, Putative tetraploid plantlets of Charng dang orchids gave the lower of chloroplast number than diploids, which may be occurred from working of abnormal cell structures. In case of root, putative tetraploid plantlets had number of roots and root length significant lower than those of the diploid (Fig 4, Table 3). Polyploid plants usually had larger organ than diploids that found in numerous plant types (Gu *et al*., 2005). Morphological of leaf and root characters obtained in putative tetraploid Chang Daeng Orchid in the present study was lower than the diploids, similar to *Phalaenopsis* orchid experiment which was reported by Griesbach (1981).

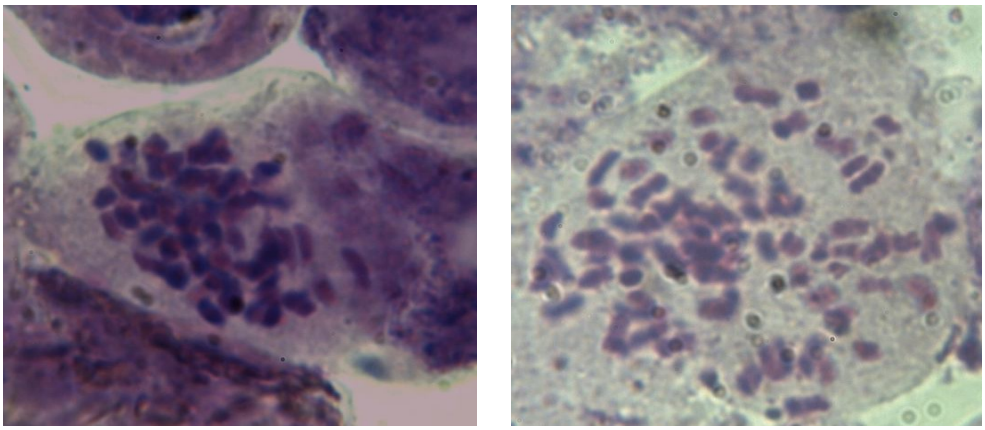

Fig. 3. Chromosome of regenerated Chang Daeng Orchid after treating with different concentrations of colchicine; 2n=2x=38 (a) and 2n=4x=76 (b) (Bars 5 µm.)

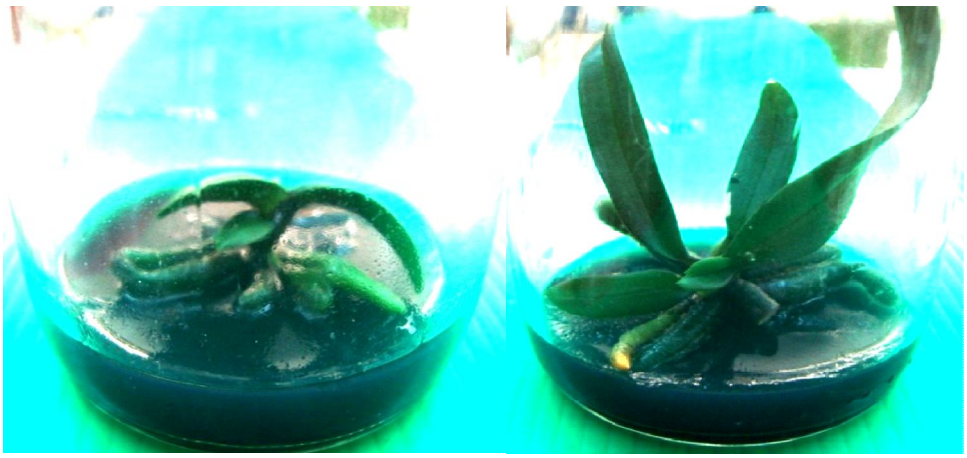

Fig. 4. Different morphological characters of Chang Daeng orchid after treating with different concentrations of colchicine; diploid (a) and tetraploid (b). (bars 10 cm)

Table 2. Effect of colchicine concentration and duration time on physiological characters

Colchicine (%) Duration time		Guard cell width (µm)	Guard cell length (µm)	Density of guard cell (cell/mm^2)	Chloroplast number
0 (control)		44.16	40.00	1.00	68.33
0.05	24	48.33	47.50	1.00	62.67
	48	58.33	50.00	1.33	63.33
	72	50.00	50.00	1.33	69.00
0.10	24	50.00	41.67	1.33	56.00
	48	50.00	43.33	1.33	58.67
	72	50.00	47.50	1.00	52.67
0.15	24	54.17	37.50	2.33	48.33
	48	52.50	50.83	2.00	40.67
	72	53.33	50.00	2.00	45.67
0.20	24	50.00	47.50	2.33	44.33
	48	61.67	54.17	2.67	49.67
	72	53.33	45.83	3.00	46.67
F-test Duration time		ns	ns	ns	ns
Concentration		**	ns	**	**
C.V. (%)		6.73	8.27	14.76	7.12

Table 3. Effect of colchicine concentrations and duration times on morphological characters of Chang Daeng orchid

Colchicine (%)	Duration time	Leave no.	Leave length	Root no.	Root length
0 (control)		2.80	2.52	5.40	2.86
0.05	24	3.00	3.02	3.40	1.76
	48	4.00	2.20	3.40	0.78
	72	3.40	2.36	3.40	1.54
0.10	24	3.60	2.04	3.20	1.38
	48	4.20	1.50	2.60	1.52
	72	3.00	1.70	3.20	2.82
0.15	24	3.20	2.44	3.20	2.20
	48	2.40	2.00	4.60	3.06
	72	2.20	2.54	3.40	3.50
0.20	24	3.00	2.28	4.20	2.20
	48	3.80	2.70	2.80	2.50
	72	3.50	2.34	3.20	2.48
F-test		**	**	*	**
C.V. (%)		26.60	31.41	34.43	41.13
$LSD_{0.01}$		1.45	1.21	2.06	1.53

Table 4. Effect of colchicine concentration and duration time on ploidy level in Chang Daeng orchid

Colchicine (%)	Duration time (cm)	No.of observed plants	Ploidy level (%)		
			diploid	mixoploid	tatraploid
0 (control)		5	5(100)	0	0
0.05	24	5	5 (100)	0	0
	48	5	5 (100)	0	0
	72	5	3 (60)	1 (10)	1 (10)
0.10	24	5	4 (80)	1 (20)	0
	48	5	3 (60)	1 (10)	1 (10)
	72	0	-	-	-
0.15	24	5	4 (80)	1 (20)	0
	48	5	2 (40)	3 (60)	0
	72	5	4 (80)	0	1 (20)
0.20	24	0	-	-	-
	48	5	2 (40)	1 (20)	2 (40)
	72	5	1 (20)	1 (20)	3(60)
total		55	38 (69.09)	9(16.36)	8(14.55)

Conclusion

PLBs treated with colchicine at 0.20% concentration for 72 h gave the survival rate at 26% and the highest percentage of tetraploid plantlets at 60 from 5 plantlets. The tetraploid plantlets had the highest density of guard cell at 3.00 cell/mm^2 when compared to those from diploid ones. The chloroplast number and root length of tetraploid plantlets were lower than that of control treatment (diploid plantlets). Cytological observation from root tip showed tetraploid chromosome 2n=4X=76.

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