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In vivo Induction of Tetraploid in Tangerine Citrus Plants (*Citrus reticulata* Blanco) with the Use of Colchicine

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ABSTRACT
This *in vivo* experiment was carried out at Sakhon Nakhon Rajabhat University, Sakhon Nakhon Province, Thailand during March-October 2013. The study aims to search for some possibilities in inducing a large number of tetraploid sets of chromosomes in tangerine citrus seedlings with the use of colchicine chemical. A Randomized Complete Block Design (RCBD) with four replications was used. Seeds of tangerine citrus were treated with colchicine solutions. The experiment consisted of seven treatments, i.e., T1 with 0.0% colchicine (control), T2 with 0.2% colchicine solution and submerged for 12 h, T3 with 0.2% colchicine solution and submerged for 24 h, T4 with 0.4% colchicine solution and submerged for 12 h, T5 with 0.4% colchicine solution and submerged for 24 h, T6 with 0.8% colchicine solution and submerged for 12 h, T7 with 0.8% colchicine solution and submerged for 24 h. The experiment was conducted for 91 days. The results showed that colchicine compound severely and significantly affected germination of tangerine citrus seeds. Colchicine of 0.2% in the solution with seeds submerged for 24 h gave the highest percentages of tetraploid chromosomes in seedlings of tangerine citrus (63.64%). Colchicine significantly affected seed germination, plant height, stomata density and leaf index of the tangerine citrus seeds and seedlings. Colchicine had no significant effect on poly-embryos, mono-embryos, leaf number, leaf area, leaf weight, leaf length and stomata length of the tangerine citrus seedlings.

Key words: *Citrus reticulata* Blanco, chromosomes, colchicine, tetraploid

INTRODUCTION

Tangerine citrus (*Citrus reticulata* Blanco) is an important kind of citrus plants being cultivated in many countries around the globe, particularly in the tropics. It is known as *Citrus nobilis* Andrew non Lou; *C. deliciosa* and *C. chrysocarpa*. It is popularly known amongst the English and American people as Mandarin and Tangerine, respectively (Verheij and Coronel, 1992). This citrus plant gave a large number of remarkable rounded fruits annually where the fruits provide juices of medicinal and nutritious values for human consumption. Its juices contained some considerable amount of Vitamin C where it gives sourness and sweetness tastes together. It is commonly known that in a 100 g of its juice, it could provide Vitamin C upto 42 mg and 82 μg of beta carotene. Its juice could contribute a lot in healing against thirsty and cold sickness and also bacterial infection in human beings. Apart from these, its dried peels can be used for burning in a fire place for some aromatic smoke that could possibly go against insect pests, particularly mosquitoes and able to purge the pests away. In Thailand, nowadays many growers gave less attention in cultivating this orchard crop due to many problems e.g., the problem on low prices of the imported fruits from China, the Philippines and other countries, hence growers could not compete with the low competitive prices. Furthermore, the problem on the attack of bacterial canker disease (*Xanthomonas axonopodis* pv. *citri*) due to a high degree of soil acidity where it tremendously

infect fruits, stems and leaves of the citrus plants. The plants may not be able to survive when seriously attacked (Suksri, 1999). Thus only a limited number of growers could be able to cultivate the citrus crop when they are able to produce a high quality of fruits with acceptable prices.

It seems more likely that orchard farms of citrus trees have declined in numbers, particularly in Thailand, thus it is of imperative value to search for some technology in improving the crop competency, in order to reduce investment cost, improve quality and production of fruits of the citrus plants. One way in improving the citrus plants is the application of colchicine to tangerine seeds as to induce more of tetraploid chromosomes. This is one way to attain mutated offspring of tetraploid chromosomes in plants which could be useful in producing seedless citrus fruits when breeding programme taken place between the citrus plants of tetraploid and diploid chromosomes. This method in improving breed of many crop plant species has been carried out successfully, e.g. Gmitter and Ling (1991) with citrus, Fassuliotis and Nelson (1992) with cantaloupe melon, Blanke *et al.* (1994) with apple and Van Duren *et al.* (1996) with banana. The colchicine alkaloid compound was firstly found by Agrawal (1998) and it was advocated that another study used this compound to increase homologous sets of chromosomes in seeds of the plants where it produced polyploidy chromosomes (Hill *et al.*, 1967). Therefore, the objective in carrying out this study focused on an *in vivo* induction of tetraploid in tangerine seeds with the use of colchicine as to produce mutated offspring of the citrus plants for better fruit quality and production.

MATERIALS AND METHODS

***In vivo* colchicine treatments in seeds:** Sixty marketable ripe fruits of a similar size of tangerine citrus obtained from the local market at Sakhon Nakhon Province were used. The tangerine citrus fruits were washed with tap water twice allowed to dry off then peeled out the skins of fruits for juices and the fully developed seeds. Eight hundred and forty fully matured seeds were carefully chosen and washed with trap water twice then placed under 10% Clorox chemical for 10 min to avoid contamination of some pathogenic organisms and finally washed with trap water for three times. The experiment consisted of seven treatments and each treatment was replicated four times. A Randomized Complete Block Design (RCBD) was used. Four rates of colchicine were used i.e., 0.0, 0.2, 0.4 and 0.8%. Two submerging periods of seeds in the colchicine solutions were used (12 and 24 h). The seven treatments consisted of: T1 without colchicine (control), T2 with a colchicine solution at a rate of 0.2%, submerged for 12 h, T3 with a colchicine solution at a rate of 0.2%, submerged for 24 h, T4 with a colchicine solution at a rate of 0.4%, submerged for 12 h, T5 with a colchicine solution at a rate of 0.4%, submerged for 24 h, T6 with a colchicine solution at a rate of 0.8%, submerged for 12 h and T7 with a colchicine solution at a rate of 0.8%, submerged for 24 h. All of the treated seeds were carefully washed with ionized water for three times then sown the seeds into plastic germination trays containing fine sand plus autoclaved fluff coir (1:1 by volume). Each tray has a dimension of 30×40×10 cm in width, length and height, respectively. Thirty fully developed seeds were used for each tray. Each germination tray contained a full amount of compost materials (approximately 95%) for germination and growth and each germination tray was used as a replication. The treated and untreated seeds were individually allocated into their respective treatments to the depth of approximately 2 cm then daily water was taken place once in the morning for thirteen weeks (91 days) of the experimental period. At the end of the experimental period, the plant samples were measured where they were categorized into three groups, i.e., the first group included germination percentage, tetraploidy percentage, poly-embryos percentage and mono-embryos percentage, the second group included the measurements on height of individual seedlings (cm), leaf fresh weight (g), number of leaves, length of leaves (cm), width of leaves (cm) and leaf index (length of leaf/width of leaf (Daughtry, 990) and the third group included length of stomata of third and forth leaves and also number of stomata mm^{-2}. For cytometric analysis, a flow cytometric analysis on poly-ploid percentages was carried out with the Flow Cytometer PA II with the application of the Cystain UV Precise P: high resolution DNA staining kit followed the method described by Petersen *et al.* (2003). The collected data were statistically analyzed using a computer program (SAS., 1998).

Statistical analysis: The Duncan's Multiple Range Test (DMRT) was used for least significant differences among the seven treatments used.

RESULTS

Germination, tetraploid, poly-embryos and mono-embryos percentage: The results showed that the germination percentages were highest with T1 (control) and lowest with T6 with mean values of 96.68 and 24.17, respectively (Table 1). The differences were large and highly significant. The percentages of tetraploid were highest with T3 and lowest with T7 with mean values of 63.64 and 14.00%, respectively. In most cases, the higher the amount of colchicines content in the solution the lower the percentages of tetraploid. With the

Table 1: Germination, tetraploid, poly-embryos and mono-embryos (%) of citrus seedlings, grown at Sakhon Nakhon Rajabhat University, Northeast Thailand

Treatments	Germination	Tetraploid	Poly-embryos	Mono-embryos
	----------(%)----------			
T1 (0, 0)	96.668^{a}	0.00	32.86	67.15
T2 (0.2, 12)	64.168^{b}	28.57	41.65	58.35
T3 (0.2, 24)	46.668^{c}	63.64	39.28	60.72
T4 (0.4, 12)	34.165^{cd}	50.00	48.61	51.39
T5 (0.4, 24)	30.835^{cd}	50.00	40.85	59.41
T6 (0.8, 12)	24.165^{d}	33.33	56.94	43.06
T7 (0.8, 24)	39.165^{cd}	14.00	51.12	48.88
F-test	***	-	ns	ns
C.V. (%)	21.49	-	39.17	31.38

Letter's in each column indicated least significant differences of Multiple Range Test (MRT) at probability (p) of ***0.01, ns: Non significant

Table 2: Plant height, leaf fresh weights, numbers of leaves, leaf length, leaf width and leaf index of citrus seedlings, grown at Sakhon Nakhon Rajabhat University, Northeast Thailand

Treatments	Plant heights (cm)	Leaf weights (g)	No. of leaves	Leaf length (cm)	Leaf width (cm)	Leaf index (cm)
T1 (0, 0)	4.27^{a}	0.02690	4.25	2.1750	1.13	0.51950^{b}
T2 (0.2, 12)	3.31^{b}	0.02635	4.02	2.1000	1.15	0.55550^{b}
T3 (0.2, 24)	2.81^{b}	0.02048	4.00	1.8125	0.99	0.54400^{b}
T4 (0.4, 12)	3.53^{ab}	0.02528	4.13	1.9525	1.09	0.54750^{b}
T5 (0.4, 24)	3.10^{b}	0.07695	4.41	2.0225	1.02	0.50550^{b}
T6 (0.8, 12)	3.03^{b}	0.02643	3.75	1.4600	1.05	0.76550^{a}
T7 (0.8, 24)	3.49^{ab}	0.02770	3.66	1.8650	1.11	0.61675^{b}
F-test	*	ns	ns	ns	ns	*
c.v. (%)	15.26	117.29	15.93	17.56	8.47	16.75

Letter's in each column indicated least significant differences of Multiple Range Test (MRT) at probability (p) of *0.05, ns: Non significant

percentages of poly-embryos, the highest percentage was found with T6 with a value of 56.94 and the lowest was found with T1 (32.86%). There was no consistent trend due to treatments and the differences were not statistically significant. For mono-embryo percentages, the highest value was found with T1 (67.15%) and lowest with T6 (43.06%). There was no trend due to treatments found.

Plant height, number of leaves, length of leaf, width of leaf and leaf index: The results revealed that the highest mean value on plant height was found with T1 (4.27 cm) and lowest with T3 (2.81 cm). The difference was large and statistically significant (Table 2). Nevertheless, plant heights were similar amongst the treated plants (T2-T7). The seedlings of the colchicine treated seeds gave lower values of plant heights than that of the control treatment (T1). With leaf numbers, it showed that leaf numbers were highest with T5 and lowest with T7 with mean values of 4.41 and 3.66, respectively. There was no statistical difference found among the treatments used yet the highest level of colchicine (0.8%) gave the lowest leaf numbers whilst the rest were slightly higher. For length of leaves, a similar trend as that of leaf numbers was found, i.e., the seedlings of the colchicine treated seeds gave lower values than that of the control treatment. There was no statistical difference found among the treatments used. The results on leaf width showed that leaf width was highest with T2 (1.15 cm) and lowest with T5 yet there was no statistical difference found among the treatments used. Nevertheless, leaf index value was highest with T6 (0.766) and lowest with T5 (0.506) where T6 was significantly higher than the rest including the control treatment. When observed with naked eyes, it was found that those plants derived from colchicines treated seeds gave much larger leaf width (oval shape) than the control treatment.

Leaf area, length of stomata and density of stomata: With leaf area, leaf area was highest with the control treatment (T1) with a value of 2.41 cm^2 and lowest with T5 with a value of 1.53 cm^2 (Table 3). There was no statistically significant difference found among the treatments used yet, it revealed that leaf areas of the seedlings with colchicine treated seeds were lower than the control treatment. For length of stomata, the results showed that T6 gave the highest length of stomata (26.72 μm) and lowest with T1 (control treatment) with a mean value of 22.13 μm. There was no statistical difference

Table 3: Leaf area/plant, stomata length and stomata density of citrus seedlings, grown at Sakhon Nakhon Rajabhat University, Northeast Thailand

Treatments	Leaf area (cm^2)	Stomata length (μm)	Stomata density (stomata mm^{-2})
T1 (0, 0)	2.4058	22.133	79.00^{bc}
T2 (0.2, 12)	1.8418	25.375	98.50^{a}
T3 (0.2, 24)	1.6328	26.490	84.25^{ab}
T4 (0.4, 12)	1.7578	25.943	78.50^{bc}
T5 (0.4, 24)	1.5255	25.375	70.50^{bc}
T6 (0.8, 12)	1.5998	26.720	65.25^{c}
T7 (0.8, 24)	1.8010	24.415	77.50^{bc}
F-test	ns	ns	*
c.v. (%)	15.63	12.41	13.24

Letter's) in each column indicated least significant differences of Multiple Range Test (DMRT) at probability (p) of *0.05, ns: Non significant

found among the treatments used yet stomatal lengths, in all cases, were much longer than that of the control treatment. With density of stomata, it was found that density of stomata mm^{-2} was highest with T2 (98.50 stomata mm^{-2}) and the lowest was found with T6 with a value of 65.25 stomata mm^{-2}. The effect due to colchicine compound was large and statistically significant.

Observations on morphological appearance of tangerine seedlings: It appears that all seedlings derived from the colchicine treated seeds possessed a kind of dwarf plants, with short thick stems and dark green oval or rounded shape of leaves, whilst that of the control treatment, the seedlings were taller with elongated leaves and a slightly yellowish appearance. This type of appearance may provide some advantage for the harvest of fruits, i.e., when the trees bearing fruits, the plants may not as tall as that of the untreated plants.

DISCUSSION

With the results on germination of the citrus seeds, the results showed that the germination percentage of seeds of T1 (control or untreated seeds) was highest whilst those treated seeds with the use of colchicine solutions gave much lesser percentages than that of the control treatment. The differences were large and highly significant. The results indicated that colchicine inhibited germination of tangerine seeds although the decrease due to an increase in colchicine concentration in the solutions was not consistently found. The results clearly showed that colchicine in the solutions inhibited germination percentage of the tangerine seeds. An increase in colchicine

level in the solutions greater than 0.2% gave a similar germination percentage. The results indicated that colchicine of 0.2% could be an optimum level for use in seed treatment for tangerine citrus plants. The decrease in germination percentage confirm the work carried out by Wongpiyasatid *et al.* (2003) with *Gossypium arboretum* PM_2 (light brown cotton species) and PM_3 (white cotton species). They showed that germination percentage of the PM_2 seeds decreased from 95-78% whilst the PM_3 decreased from 100-72%. Similarly, Dhamayanthi and Gotmare (2010) also with cotton seeds of two species (*Gossypium armourianum* and *G. aridum*) where 0.5, 0.7, 0.9, 1.0 1.5, 1.7 and 1.9% of colchicine solutions were used. They also concluded that colchicine significantly inhibited germination of the cotton seeds. In other study, Pirkoohi *et al.* (2011) with mint crop reported that mint seeds treated with 0.05, 0.1 and 0.4% colchicine solutions gave germination of 80.30, 74.50 and 57.00%, respectively. With this current study it was found that the concentration of colchicine solution of 0.2% gave highly significant germination percentage than any other higher levels of colchicine where further increases gave no significant effect on germination percentages. Therefore, a concentration of 0.2% in the solution of T3, i.e., when the tangerine seeds were submerged in the solution for 24 h, it gave the highest tetraploid up to 63.64%. The establishment of tetraploid percentage increased from T2 to T3 and then a decline whilst T1 (control) did not provide any tetraploid percentage. The results indicated that colchicine has its significant effect in inducing tetraploid chromosomes production in the citrus seedlings and the highest percentage reached a value of 63.64%. There were no significant effects due to colchicine solutions on both poly-embryos and mono-embryos, yet all colchicine treated seedlings gave much higher poly-embryos than the control treatment, except those of the mono-embryos where all treated seedlings gave slightly lower percentages than the control treatment yet no significant difference was found.

The results on plant height showed that plant heights of the seedlings derived from the treated seeds were significantly shorter than the control treatment, particularly with T3 where the mean value of the seedlings was much shorter than the rest. Thus colchicine severely affected plant height. It was found with naked eyes that the seedlings derived from the colchicine treated seeds performed healthier and firmly established better than the control. The results agree with the work reported by Wongpiyasatid *et al.* (2003) with cotton. Therefore, colchicine had its significant effect on plant height of the tangerine seedlings. Colchicine did not affect leaf fresh weights, numbers of leaves, leaf length and leaf width but did with Leaf Index (LI) where the LI value of T6 was significantly greater than the rest. The difference may be due to the greater expansion of leaf area where most of the colchicine treated plants possessed larger leaf area than the control. The results confirm the work reported by Grouh *et al.* (2011) with *Salvia hians* plants of mint family. They reported that seedlings with greater amount of tetraploid sets of chromosomes gave greater LI than those the diploid ones.

There were no statistical differences found on leaf area of the tangerine seedling, yet stomatal lengths in leaves in all treated seedlings were significantly greater than that of the control treatment. The results indicated that colchicine significantly increased growth of stomatal lengths of leaves of the tangerine seedlings. The results agree with the studies carried out by Chinachit and Sreemaung (2008) on *Eulophia andamanensis* Reichb.f, Majdi *et al.* (2010) on *Salvia hains*, Grouh *et al.* (2011) on mint of *Salvia hians*, Kerdsuwan and Te-chato (2012) on Chang Daeng orchid plants. For the results on density of stomata, it revealed that the highest value of stomata was found with T2 then a slightly decline with T3 whilst the rest were somewhat similar. The difference was large and statistically significant. The results evidently showed that the required submerged time and level of colchicine for tangerine seeds to reach an optimum density of stomata in leaves of the citrus seedlings were 12 h with a concentration of a colchicine solution of 0.2% only. Higher levels of colchicine gave no significant effect hence optimum colchicine for use in treating the tangerine seeds as to attain optimum tetraploid sets of chromosomes could be only up to 0.2% with 24 h (T3) submerged in the solution.

The results derived from this investigation indicated that experiments carried out with the use of tangerine citrus under *in vivo* condition were successfully achieved. A large number of tetraploid chromosomes of the tangerine citrus were induced hence some other *in vitro* experiments may not be necessary since its investment could be much higher for the *in vitro* conditions. Therefore, this finding may be of significant value for further breeding programme for the improvement of tangerine citrus plants.

CONCLUSION

It was found that colchicine compound severely and significantly affected germination of tangerine citrus seeds. A level of colchicine of 0.2% in the solution with the seeds being submerged for 24 h (T3) gave the highest value of tetraploid chromosomes in seedlings of the tangerine citrus plants (63.64%), hence T3 was considered to be the best treatment to be recommended. Colchicine significantly affected seed germination, plant height, stomata density and leaf index of the tangerine citrus seedlings but not with other measured parameters such as poly-embryos, mono-embryos and etc.

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REFERENCES

Agrawal, R.L., 1998. Fundamentals of Plant Breeding and Hybrid Seed Production. Science Publishers, New Hampshire, ISBN-13: 978-1578080298, Pages: 394.

Blanke, M.M., M. Hofer and R.J. Pring, 1994. Stomata and structure tetraploid apple leaves cultured *in-vitro*. Ann. Bot., 73: 651-654.

Chinachit, W. and S. Sreemaung, 2008. [Colchicine affecting the alteration of ploidy level in plantlets of *Eulophia andamanensis* Reichb.f]. Agric. Sci. J., 39: 275-277, (In Thai).

Daughtry, C.S.T., 1990. Direct measurements of canopy structure. Remote Sens. Rev., 5: 45-60.

Dhamayanthi, K.P.M. and V. Gotmare, 2010. Induction of polyploidy in two diploid wild cotton (*G. armourianum* and *G. aridum*) species by colchicine treatment. Electron. J. Plant Breed., 1 : 966-972.

Fassuliotis, G. and B.V. Nelson, 1992. Regeneration of tetraploid muskmelons from cotyledons and their morphological differences from two diploid muskmelon genotypes. J. Am. Soc. Horticult. Sci., 117: 863-866.

Gmitter, Jr. F.G. and X. Ling, 1991. Embryogenesis *in vitro* and nonchimeric tetraploid plant recovery from undeveloped citrus ovules treated with colchicine. J. Am. Soc. Horticult. Sci., 116: 317-321.

Grouh, M.S.H., H. Meftahizade, N. Lotfi, V. Rahimi and B. Baniasadi, 2011. Doubling the chromosome number of *Salvia hains* using colchicine: Evaluation of morphological traits of recovered plants. J. Med. Plants Res., 5: 4892-4898.

Hill, J.B., H.W. Poop and A.R. Grove Jr., 1967. Botany: A Textbook for Colleges. 4th Edn., McGraw-Hill, Toronto, ON., Pages: 614.

Kerdsuwan, N. and S. Te-chato, 2012. Effects of colchicine on survival rate, morphological, physiological and cytological characters of chang daeng orchid (*Rhynchostylis gigantean* var. *rubrum* Sagarik) *in vitro*. Int. J. Agric. Technol., 8: 1451-1460.

Majdi, M., G. Karimzadeh, M.A. Malboobi, R. Omidbaigi and G. Mirzaghaderi, 2010. Induction of tetraploidy to feverfew (*Tanacetum parthenium* Schulz-Bip.): Morphological, physiological, cytological and phytochemical changes. HortScience, 45: 16-21.

Petersen, K.K., P. Hagberg and K. Kristiansen, 2003. Colchicine and oryzalin mediated chromosome doubling in different genotypes of *Miscanthus sinensis*. Plant Cell Tissue Org. Cult., 73 : 137-146.

Pirkoohi, M.H., M. Keyvanloo and M. Hasanpoor, 2011. Colchicine-induced ploidy in mint by seed treatment. Int. J. Agric. Crop Sci., 3-4: 102-104.

SAS., 1998. SAS/STAT User's Guide. Version 6, SAS Institute Inc., Cary, North Carolina, USA.

Suksri, A., 1999. Some Agronomic and Physiological Aspects in Growing Crops in Northeast Thailand. 1st Edn., Khon Kaen University Press, Khon Kaen, Thailand, pp: 212.

Van Duren, M., R. Morpurgon, J. Dolezel and R. Afza, 1996. Induction and verification of autotetraploids in diploid banana (*Musa acuminata*) by *in vitro* techniques. Euphytica, 88: 25-34.

Verheij, E.W.M. and R.E. Coronel, 1992. Plant Resources of South-East Asia No. 2: Edible Fruits and Nuts. PROSEA, Bogor, Indonesia.

Wongpiyasatid, A., P. Hormchan and N. Rattanadilok, 2003. Preliminary test of polyploidy induction in cotton (*Gossypium arboretum*) using colchicine treatment. Kasetsart J. Nat. Sci., 37: 27-32.