In Vitro Induction of Tetraploid Pummelo 'Nambangan' (*Citrus maxima* (Burm.) Merr.) By Colchicine Treatment Using Germinated Seed, Shoot Tip and Cotyledonary Node as Explants

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Abstract

Tetraploid citrus are important for interploidal hybridization to create triploid seedless citrus. Colchicine is the most commonly used as antimitotic agent to induce polyploid plants. Tetraploid induction by colchicine in Pummelo 'Nambangan' was conducted *in vitro* using different types of explants. The aim of this research was to induce tetraploid pummelo 'Nambangan' by colchicine treatment using germinated seed, shoot tip and cotyledonary node as explants. Tetraploid shoot induction was conducted by soaking germinated seeds, shoot tips and cotyledonary nodes in 0.1 % colchicine for 1, 3 and 5 hours. Regenerant shoots were grown on MS medium and their growth was observed after four weeks in culture. Ploidy level was determined using flow cytometry analysis. Stomata density, length and width of stomatal guard cell were also recorded. The results showed that shoot elongation was inhibited by colchicine treatment. Soaking of shoot tip explants in 0.1 % colchicine for 1 hour resulted in 66.66 % of putative tetraploid shoots. Compared to diploid shoots, tetraploids had lower stomata density but bigger in guard cell size.

Keywords: colchicine, tetraploid, pummelo (*Citrus maxima* (Burm.) Merr.), flow cytometry, stomata

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Introduction

The citrus gene pool is essentially diploid (2n=2×=18), therefore, ploidy manipulation is needed to support citrus breeding program. Tetraploid progenies are required to obtain interploidal crossing parents. Crossing tetraploid female parents with diploid pollen parents produces triploid seedless citrus (Jaskani *et al*., 2007, Ollitrault *et al*., 2008). Using monoembryonic citrus as tetraploid female parent was an effective way in interploidal hybridization to produce a single zygotic embryo (Aleza *et al*., 2009, Kainth & Grosser, 2010). Pummelo 'Nambangan' is one of monoembryonic citrus species which is commercially produced in Indonesia.

Several research had been done to create polyploid citrus. Stable autotetraploids in monoembryonic citrus cultivars have been produced by colchicine treatment of the axillary buds (Oiyama & Okudai, 1986), shoot tip (Aleza *et al*., 2009) and seeds (Kainth & Grooser, 2010). Oiyama & Okudai (1986) reported that autotetraploid plants was obtained through immersion of axillary buds of *Citrus clementina*, *Citrus hassaku*, and *Citrus tamurana* in 0.1% colchicine for 2-6 hours and these treated axillary buds and the untreated ones were grown to plants using trifoliate orange seedlings as rootstocks by micrografting technique. Tetraploid plants were identified by stomata size determination. Aleza *et al*. (2009) produced tetraploid of

'Clemenules' clementines using shoot tip immersed in 0.1 % colchicines. The tetraploid shoots were also micrografted for their propagation. Kainth & Grosser (2010) produced tetraploid pummelo (*Citrus grandis*) using pink or red-fleshed monoembryonic pummelo parents through 0.1 % colchicine treatment of meristematically active seeds *in vitro* for 12-24 hours and polyploid shoots were analyzed using flow cytometry.

The most commonly chemical used to increase citrus ploidy levels is colchicine. Colchicine is an alkaloid compound extracted from seeds and bulbs of the wild meadow saffron *Colchicum autumnale*. It is also usually used as antimitotic agent. Colchicine blocks the mechanism that regularly moves chromosomes to the respective poles by inhibited appearing spindle fiber (Eigsti & Dustin, 1955).

Explant type, colchicine concentration and its exposure time are important parameters which influence the efficiency of chromosome doubling. Low doses are not successfully produced polyploid plants, while excessively high doses are lethal (Dhooghe *et al*., 2011).

The aim of this research was to induce tetraploid pummelo 'Nambangan' by colchicine treatment using germinated seed, shoot tip and cotyledonary node as explants.

Materials and Methods

Explants preparation. Seeds were extracted from mature fruits of pummelo 'Nambangan' grown at the experimental garden Cikabayan, Bogor Agricultural University, Indonesia. The extracted seeds were surface sterilized by immersing them in 70 % ethanol for 1 hour, and then they were air-dried in a laminar airflow cabinet. All seed coats were removed then seeds were germinated on MS (Murashige & Skoog, 1962) medium without addition of plant growth regulators (MS0). After 1 week, germinated seeds with 0.5-0.8 cm radicula were used as explants (first type of explants). Shoot tip and cotyledonary node explants isolated from 4 weeks-old *in vitro* seedling were collected. Shoot tips at 3-4 cm length with 1-2 nodes without leaves were also used as explants (second type of explants). Hypocotyl segments containing cotyledonary nodes were also used as explants (third type of explants).

Colchicine treatments and plantlet regeneration. Colchicine stock solution was prepared by dissolving colchicine in a few drops of dimethylsulfoxide (DMSO) followed by the addition of sterile water to bring the final concentration to 0.5 %. This solution was filter sterilized. Working solution was prepared with diluting stock solution with Murashige & Tucker (MT) liquid medium containing 50 g/L sucrose (Murashige & Tucker, 1969) until final concentration of 0.1 %. Ten replicates of each explant were immersed in 0.1 % colchicine for 1, 3 and 5 hours and controls were immersed in liquid MT medium for 1 hour. Explants were submerged in flasks and flasks were incubated on a rotary shaker with 30 rpm in a room temperature (Kainth & Grosser, 2010).

After colchicine treatment, explants were taken out and placed on solid MS0 medium (solidified with gellan gum at 3 g/L) then incubated under continuous light conditions provided by cool white fluorescent tube with 1000-1400 lux light intensity at a 25-27 ºC culture room. Percentages of survived explants were observed after 4 weeks in culture. Five regenerant shoots from each explant types and duration colchicine treatment were transfer to Grosser & Gmitter rooting medium (Grosser & Gmitter, 1990). Shoot number and height, node number, leaves number and length, and root number from explants were observed at 8 weeks after the colchicine treatment.

Ploidy analysis and stomata measurement. Ploidy level was analyzed using flow cytometry (CyFlow ® Space. Partec, Germany). Leaves from *in vitro* germinated seeds were used as control diploid. Leaves of regenerants treated with colchicine were analyzed according to the protocol developed for bananas (Dolezel *et al*., 2004). Approximately 0.4 cm^2 of one leaf blade was chopped in the extraction buffer, then they were passed through a 30 µm nylon mesh screen and stained with Fluorescent dye Propidium Iodide (Partec, Germany). Position

of G0/G1 peak on channel 200 was used to determine the position of diploid peak on the histogram presentation from the analyzer. Tetraploid shoots were identified by the presence of peak on channel 400. Result of ploidy level analysis from each regenerant was assessed by calculating Tetraploid Induction Efficiency (TIE) by the formula given in Kainth & Grosser (2010).

Stomata density and size of guard cell from lower epidermis cells of the leaves were also determined. Epidermis of the leaves was covered with a thin layer of clear nail polish and left to dry for few minutes to make epidermal impression. Dry layer of epidermis cell cover with nail polish were peeled off by using clear tape then they were sticked onto a microscope slide. Each slide was examined under inverted light microscopy (Leica DMIL LED) with a magnification of 400 times. Stomata number, length and width of guard cell were recorded. Measurement was conducted using software Leica Application Suite v3.8. Stomata density, length and width of guard cells were recorded from 5 fields of views taken from 3 leaves, each leaf isolated from different shoot. Data were analyzed by variance analysis (ANOVA), followed by Duncan's Multiple Range Test (DMRT) at 5 % level of probability from mean comparison.

Results

Effect of colchicine on explants survival rate and growth of regenerant.

Duration of colchicine treatment affected the survival rate of different explant types. Figure 1 demonstrates that different explants had different survival percentage. Germinated seeds had high survival rates (90-100 %), whereas cotyledonary node explants had low survival rate (30-40 %). Shoot tips had the highest survival rate compared to germinated seed and cotyledonary node explants. The survival rates of explants were declined in relation to the treatments of colchicine soaking duration. The lowest survival rate was observed at 5 hours treatment which was about 10 %.

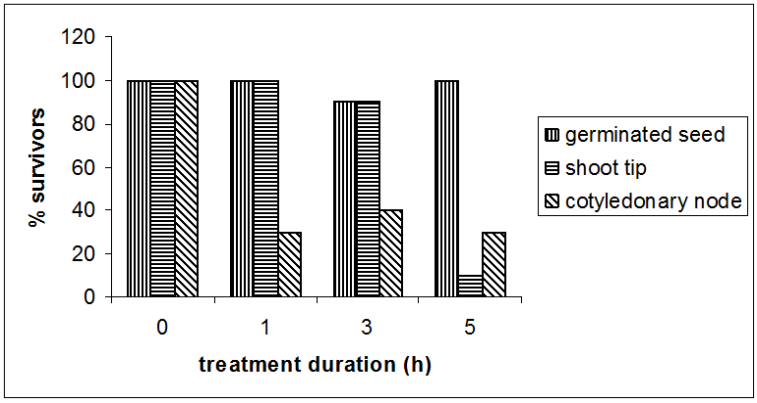

Figure 1. Effect of different explants and treatment duration of colchicine on explants survival percentage

Table 1 shows growth of regenerant shoots from different explants 8 weeks after colchicine treatment. Analysis of variance showed that different explants and duration of treatments affected all growth parameters significantly. All explants showed decrease in shoot number, and height, node number, leaf number and length and also root number along with increasing treatment of duration soaking time (Table 1, Figures 2, 3, and 4). Shoot height of germinated seed explants were significantly different compared to the control treatment. Leaf length of shoot tip explants was also lower significantly different with that of the control treatment. Shoot height and root number of cotyledonary node was much shorter significantly different compared to the control treatment (Table 1).

Table 1. Effect of different duration treatment at different explants on regenerant shoots growth 8 weeks after colchicine treatment

Treatments		Shoot number	Shoot height (cm)	Node number	Leaf number	Leaf length (cm)	Root number
Explants	duration (hour)						
Germina-	Control	1.0±0.0c	8.0±0.7a	2.6±0.5abcd	5.2±0.8ab	2.4±0.2ab	1.0±0.0ab
ted seed	1	2.0±0.0a	6.7±1.1b	4.0±0.7a	6.6±2.1a	2.8±0.8a	1.0±0.0ab
	3	1.0±0.0c	3.9±0.8c	3.4±1.1abc	5.2±2.9ab	2.7±0.3a	1.0±0.0ab
	5	1.0±0.0c	3.0±0.6de	3.4±0.9abc	3.6±1.1b	2.3±0.4ab	1.0±0.0ab
Shoot tip	Control	1.2±0.4bc	3.6±0.4cd	2.8±1.9ab	3.4±0.5b	1.5±0.4cde	0.8±0.8b
	1	1.8±0.4a	3.9±0.4c	2.6±1.2cd	3.4±0.9b	1.0±0.0de	0.8±0.4b
	3	1.0±0.0c	3.0±0.0de	2.2±1.1abcd	3.2±0.4b	0.9±0.2ef	0.0±0.0c
	5	0.2±0.4d	2.7±0.3e	1.2±1.7abc	0.2±0.4c	0.2±0.4f	0.0±0.0c
Cotyledo	Control	1.6±0.5bc	3.7±0.9cd	3.6±1.9abcd	5.4±2.5ab	2.4±0.4bc	1.4±0.5a
-nary	1	1.2±0.4c	0.9±0.5f	2.0±1.2abcd	5.2±1.7ab	1.6±1.0cd	0.2±0.4c
node	3	1.0±0.0c	0.7±0.3f	2.6±1.1bcd	4.2±1.3ab	1.4±0.5cde	0.2±0.4c
	5	1.0±0.0c	0.7±0.4f	1.2±1.7d	5.4±2.6ab	1.2±0.3cde	0.6±0.5bc

For each column, Mean± SD followed by letter(s) are significantly different (P=0.05) according to ANOVA.

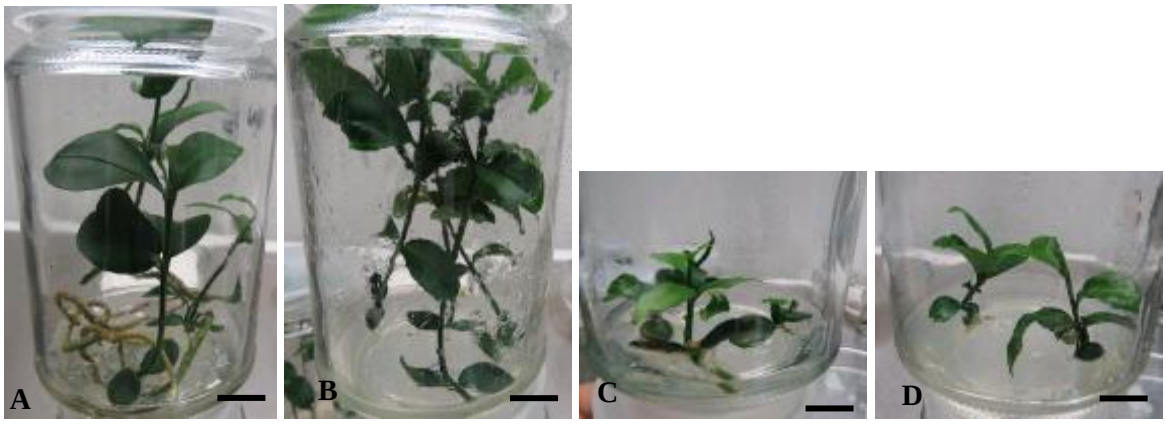

Figure 2. Growth of shoots from germinated seeds regeneration, 8 weeks after immersed in 0.1 % colchicine and control: A. Control, B. for 1 hour, C. for 3 hours and D. for 5 hours. (bar=1cm).

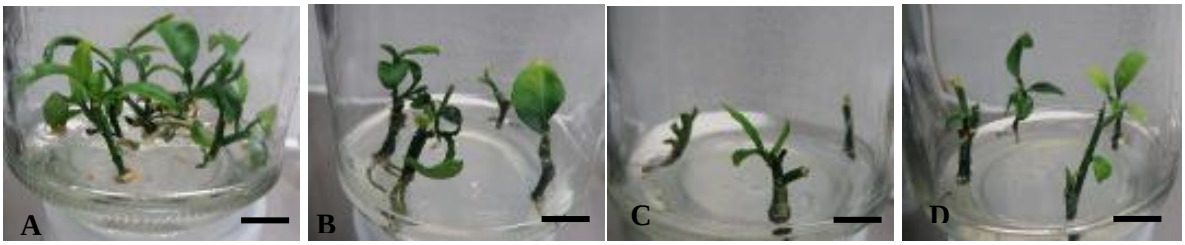

Figure 3. Growth of shoots from shoot tip regeneration, 8 weeks after immersed in 0.1 % colchicine and control: A. Control, B. for 1 hour, C. for 3 hours and D. for 5 hours.

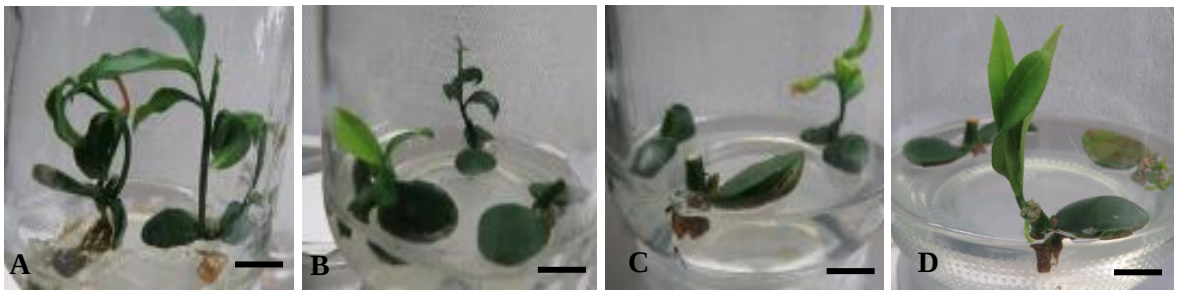

Figure 4. Growth of shoots from cotyledonary node regeneration, 8 weeks after immersed in 0.1 % colchicine and control: A. Control, B. for 1 hour, C. for 3 hours and D. for 5 hours.

Ploidy analysis and stomata measurement.

Ploidy level of all regenerant shoots is shown on Table 2. Diploid, mixoploid and tetraploid shoots were regenerated from survival shoots with colchicine treatment. Control treatment of all explant types was produced 100 % of diploid shoots. The highest of Tetraploid Induction Efficiency was achieved from shoot tip explant treated with cholchicine for 1 hour and the lowest was achieved from cotyledonary node explant soaked for 1 hour in cholchicine solution. Figure 5 shows histograms of diploid, mixoploid and tetraploid plantlets. Figure 5A shows diploid profile of a control (untreated explants). Figure 5B shows mixoploid profile as chimeric regenerant shoot having diploid and tetraploid nuclei, meanwhile Figure 5C indicates non chimeric tetraploid profile.

All upper epidermis cells had no stomata. Microscopic observation of leaf surface imprints confirmed that the presence of stomata were only on the lower epidermis. Stomata observed on lower epidermis of pummelo 'Nambangan' leaves are shown on Figure 6. Analysis of variance on stomata density, guard cell width and length showed significantly different for diploid, mixoploid and tetraploid shoots. Stomata density was lower and guard cells size was greater when ploidy level was higher (Table 3).

Table 2. Ploidy analysis of regenerants from different explants and treatment duration, 4 weeks after treatment

Explant	Treatment duration (hour)	Ploidy analysis/total regenerant (%)			% TIE
		Diploid	Mixoploid (diploid and tetraploid)	Tetraploid	
Germinated seed	control	100.0	0.0	0.0	0.0
	1	90.0	10.0	0.0	0.0
	3	55.5	0.0	44.4	39.9
	5	63.6	9.1	27.3	27.3
Shoot tip	control	100.0	0.0	0.0	0.0
	1	6.6	26.6	66.6	66.6
	3	0.0	63.6	36.3	32.7
	5	100.0	0.0	0.0	0.0
Cotyledonary node	control	100.0	0.0	0.0	0.0
	1	60.0	0.0	40.0	12.0
	3	83.3	16.6	0.0	0.0
	5	100.0	0.0	0.0	0.0

%TIE (Tetraploid Induction Eficiency) = (% explant survival × % teraploid regenerant)/100

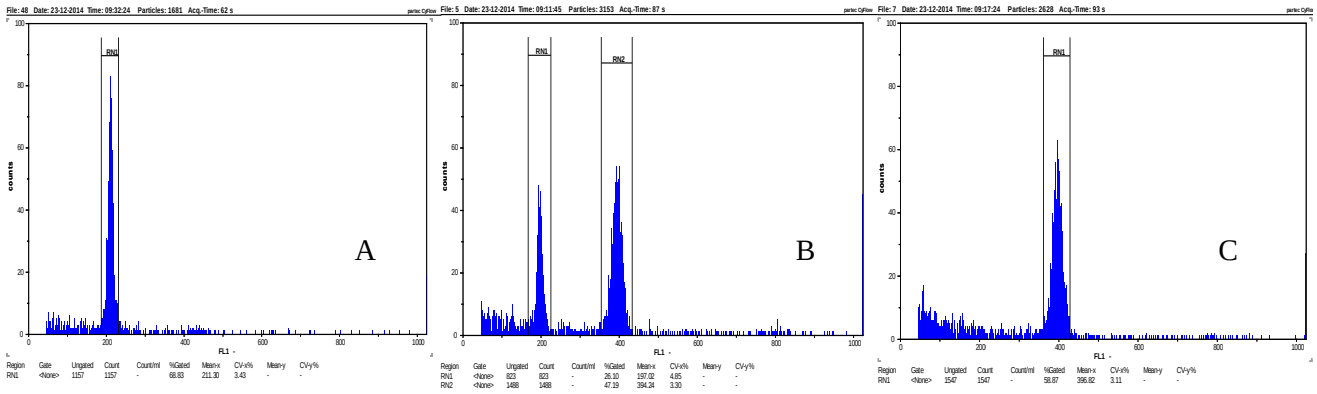

Figure 5. Flow cytometry histograms of pummelo 'Nambangan' shoots from *in vitro* colchicine treatment and control: A. diploid (control), B. mixoploid and C. tetraploid (X axis showed DNA content and Y axis showed number of nuclei).

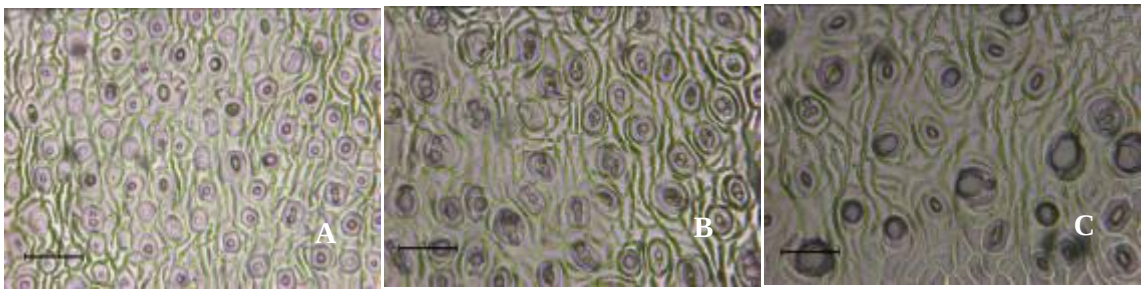

Figure 6. Stomata on lower leaf epidermis of pummelo 'Nambangan' shoots from *in vitro* colchicine treatment and control: A. Diploid (control), B. Mixoploid, and C. Tetraploid (bar = 50 µm).

Table 3. Analysis of stomata density, length and width of guard cells on diploid, mixoploid and tetraploid regenerant

Ploidy	Stomata density (stomata/mm^2)	Guard cell length (µm)	Guard cell width (µm)
Diploid	888.6±0.1a	21.1 ±1.4b	17.2± 0.8b
Mixoploid (diploid-tetraploid)	439.5±0.0b	29.7 ±1.3a	25.1± 1.6a
Tetraploid	318.4±0.0b	30.8 ±1.5a	24.5± 1.1a

For each column, Mean ± s.e. followed by letter(s) are significantly different (*P*=0.05) according to ANOVA.

Discussion

Effect of colchicine treatment on shoot tip explant survival rate clearly indicates that shoot tip was the best explants for polyploid induction in pummelo 'Nambangan' compared to germinated seeds and cotyledonary nodes. Explant survival rate declined simultaneously with the increase in colchicine treatments duration. Different effect of colchicine treatment on different type of explants was reasonable, because explant survival rate was affected by the permeability of the explants tissue and transport capability of the antimitotic agent to the meristematic tissue (Allum *et al*., 2007).

Shoots regenerated from colchicine treatments gave significantly lower growth compared with control (Table 1). In this study explants immersed in longer colchicine treatment gave more stunted shoot regeneration. Levels of growth obstacle was affected by sensitivity of explants tissue, shoot tip was the most sensitive explants compared to germinated seed and cotyledonary node. Growth inhibition after colchicine treatment in citrus was also confirmed by Oiyama & Okudai (1986) in monoembryonic citrus cultivar, Gmitter & Ling (1991) in sweet orange and tangelos, Wu & Mooney (2002) in tangor, Kainth & Grosser (2010) in pummelo. This growth inhibition indicates that colchicine was toxic to explants (Dhooghe *et al*., 2011).

Ploidy analysis of regenerant shoots showed that genetic variability of pummelo 'Nambangan' was improved (Table 2). Tetraploid regenerants were produced from all type of explants. In this research, Tetraploid Induction Efficiency (TIE) of all explants was higher than that of 'Clementine', 'Hassaku' and 'Hyuganatsu' (Oiyama & Okudai, 1986), 'Clemenules' clementines, 'Fina' clementines, 'Marisol' clementines and 'Moncada' mandarin (Aleza *et al*., 2009) and 'Hirado Buntan' pink pummelo (Kainth & Grosser, 2010) with axillary tip, shoot tip and germinated seed explants with 0.1 % colchicine concentration. Oiyama & Okudai (1986) immersed axillary bud in 0.1 % colchicine for 2 and 6 hours resulted one tetraploid grafted plant from 44 treated buds in the three cultivars. Aleza *et al*. (2009) reported that immersed shoot tip in 0.1 % colchicine for 3 and 24 hours resulted in 2× and 4× cytochimeras. Kainth & Grosser (2010) meanwhile immersed germinated seeds explant for 12 and 24 hours, resulted in 1-2 tetraploid plant.

This study showed that shoot tip was the best explant for tetraploid induction of pummelo 'Nambangan' and highest efficient

treatment was 0.1 % colchicine for 1 hour immersion. This treatment gave 66.66 % tetraploid shoot and 26.66 % mixoploids.

Stomata density of tetraploid regenerants were lower, stomata guard cell length and width were bigger than diploid regenerants (Figure 6 and Table 3). Tetraploid plant leaf characteristic were lower in stomata distribution but bigger size of stomata guard cell (Oiyama & Okudai, 1986; Gu *et al*., 2005; and Yang *et al*., 2006). Commonly, polyploid plants have increase in their stomata size. When the stomata cells are bigger than the normal size, the plant would make fewer in sufficient CO_2 uptake and have lower transpiration rate. This will be beneficial in supporting plant growth (Li *et al*., 1996; Maherali *et al*., 2009).

Conclusion

Tetraploid pummelo 'Nambangan' was obtained by immersing germinated seed, shoot tip and cotyledonary node explant in 0.1 % colchicine for 1, 3, and 5 hours. Treatment duration significantly inhibited regeneration of explants into shoots. Shoot tip explant was the most sensitive explant when immersed in 0.1 % colchicine, and produced 66.66 % putative tetraploid when immersed for 1 hour. Stomata density of tetraploid shoots was lower, however, stomata guard cell length and width was bigger.

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