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Ploidy induction through secondary somatic embryo (SSE) of oil palm by colchicine treatment

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Polyploid oil palm plantlets (*Elaeis guineensis* Jacq.) were obtained by treating secondary somatic embryos (SSEs) with colchicine at different concentrations (0, 0.05, 0.10, 0.15, 0.20 %) for various durations of 12, 24, 48 h in MS liquid medium. The treatment was shaken on rotary shaker at 120 rpm in darkness at 25^0C. Flow cytometry analysis revealed that 0.20% (w/v) colchicine for 24 h gave the most effective result in producing tetraploid plantlets. Morphological and physiological evidence confirmed that tetraploid plantlets had thick and dark green leaves and flowers. Stomata size of tetraploid leaves was larger but lower in density than those of diploid plantlets. The nuclear DNA contents of tetraploid plantlets were two times higher than those of diploid plantlets as compare to standard DNA content of *Oryza sativa* cv. 'Nipponbare'.

Key words: oil palm (Elaeis quineensis Jacq.), colchicine, secondary somatic embryo

Introduction

Oil palm (*Elaeis guineensis* Jacq.), a member of Palmae family, is a valuable economically important source of vegetable oil, the most traded vegetable oil in the international market, and is increasingly used in the food industry. The majority of the commercial plantations of oil palm have been established by seed propagation. However, this method provides heterogeneity offspring due to heterozygosity of the two parents. At present, biotechnology-based techniques are relatively advanced in oil palm, including micro propagation through somatic embryogenesis, embryo rescue, genetic transformation and molecular marker assisted selection (Ashomore and Drew, 2006). Plant tissue culture is applied for plant breeding in order to overcome some limitations of the conventional breeding, and clonal propagation of oil palm

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through tissue culture is common (Rabechaultn *et al*., 1972; Teixeira *et al*., 1995; Aberlenc-Bertossi, 1999; Te-chato, 2002 ; Rajesh *et al*., 2003).

Tissue culture techniques have proven to be effective tools for breeding programs of several plant species. Desirable traits can be introduced from non commercial to commercial varieties via tissue culture techniques and maintained via asexual propagation methods (Vieira *et al*., 2005; Passos and Bernacci, 2005). The method for improving plant quality and quantity by inducing polyploid is of great important in many plants. Polyploidy often generates variants that may possess useful characteristics and also provide a wider germplasm base for breeding studies (Thao *et al*., 2003). Ploidy induction has been carried out for a variety of reasons. In case of oil palm, Madon *et al* (2005) reported the chromosome numbers of oil palm was 2n=2x=32 and induction of tetraploid should be 2n=4x=64. In citrus, tetraploid (2n=4x) parents were used to cross with 2x parents to produce seedless triploids (2n=3x) (Wu and Mooney, 2002). In the medicinal plants, *Scutellaria* (Gao *et al*., 2002) and *Artemisia* (De Jesus-Gonzalez and Weathers, 2003), tetraploid plant was aimed to increase the amounts of the secondary metabolites, baicalin and artemisinin. In azalea (De Schepper *et al*., 2004) and pomegranate (Shao *et al*., 2003) chromosome doubling has been used to obtain new ornamental characteristics.

Chromosome duplication using colchicine has long been used in plant breeding program. Colchicine, a compound that effectively arrests mitosis at the anaphase stage, has been found to have a significant effect on polyploid induction. In most plants, artificial polyploidy is often accompanied by increased cell size, leading to larger reproductive and vegetative organs (Adaniya and Shirai, 2001). In breeding programs, it is important to determine the ploidy level in a quick and simple way in various stages of plant development. In many plant species, there are correlation between ploidy level and physiological characteristics, such as chloroplast number in guard cell, size of stomata cell, stomata density and pollen grain diameter (Omidbaigi *et al*., 2010).

Flow cytometry (FCM) is a fast and accurate method for the estimation of the nuclear DNA content of different plant species (Thiem and Sliwinska, 2003; Bennett and Leith, 1995). The genomic DNA content of *E. guineensis* (*var* tenera) as reported by Rival *et al.* (1997) using flow cytometry was 3.786±0.125 pg. Srisawat *et al.* (2005) and Madon *et al.* (2008) reported the genome size for *E. guineensis* (*var.* tenera) was 3.76±0.02 pg and 3.83 ±0.31 pg, respectively. Flow cytometry was used in this study to estimate the nuclear DNA content of oil palm cultured *in vitro* and to study the stability of genome size during *in vitro* culture and to determine the ploidy level of treated plants.

The aims of this study were to induce tetraploid plantlets and analyse those plants by FMC first, followed by physiological and cytological markers for verification of diploids and tetraploids plantlets obtained from cholcicine-treated SSEs of oil palm.

Materials and methods

Plant Material and tissue culture

Secondary somatic embryos (SSEs) induced from single haustorium embryo (HEs) of *E. guineensis* Jacq. var *tenera* in SSEs induction medium or stress medium (Chehmalee and Te-chato, 2008) were used for treating with colchicine. To compare DNA content of colchicine-treated plants with normal plants standard DNA content of *Oryza sativa* cv. 'Nipponbare' leaves were applied. The DNA was kindly provided by Assoc. Prof. Dr. Ryo Akashi, Frontier Science Research Science Division of Bioresource Science. University of Miyazaki, Miyazaki, Japan.

Induction of SSEs

Induction of SSEs was routinely carried out using single mature HE as the following procedure. Firstly, embryogenic calluses (ECs) were induced and maintained according to the method described by Te-chato (2002) on MS medium supplemented with 0.3 mg/l dicamba, 3% sucrose and 200 mg/l ascorbic acid. Secondly, ECs were transferred to MS medium supplemented with 0.1 mg/l dicamba for 1 month in order to induce HE. At this stage of culture pre-mature haustorium embryos developed at peripheral layer of the callus (Figure 1a). After subculture to the same medium component and culture for further 1 month well develop or mature HEs (Figure 1b) were obtained. Finally, single mature HEs were excised and transferred to culture on MS medium supplemented with 0.2 M sorbitol, 3% sucrose and 200 mg/l ascorbic acid, so called stress medium. The cultures were maintained at 26±4 ^{0}C under 14 h photoperiod at 1,300 lux illumination. After 3 months of culture cluster of SSEs (>10 SSEs) (Figure 1c) appeared at the basal part of HE. SSEs at this stage were used as plant material for colchicine treatment.

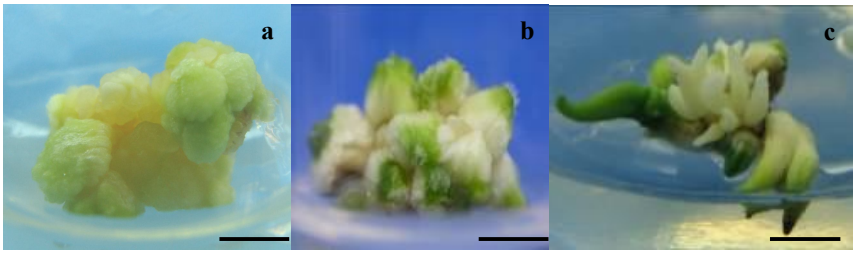

Fig.1. Development of pre-mature (a) and mature (b) HEs from peripheral layer of embryogenic callus and SSEs from single HE (c). (bar = 0.5 mm.)

Colchicine treatment

Ten clusters of SSEs for each treatment, with three replications, were immersed in 0, 0.05, 0.10, 0.15 and 0.20% colchicine (Sigma-Aldrich) solution and kept at room temperature on a shaker at 120 rpm for the periods of 12, 24, and 48 hours. Then treated SSEs were rinsed with distilled water and they were transferred to MS medium without plant growth regulators. The cultures were maintained under the same condition as induction of SSEs.

Ploidy level analysis

Diploid (control) and putative tetraploid plantlets were screened on the basis of flow cytometry. The putative tetraploid plantlets were examined six months later to validate ploidy stability and after 10 months of culture, those plantlets were classified according to their morphology (height, leaf shape and size, root length and shape) and physiology (stomata size, guard cell size and density and chloroplast number in guard cells).

Flow cytometric analysis

The nuclear DNA content was measured by flow cytometry according to the method described by Uozu *et al*. (1997). *Oryza sativa* cv. 'Nipponbare' (2C DNA content = 0.91pg) was used as an internal reference standard (Uozu *et al*.1997). Nuclei of oil palm and external reference standard were isolated from shoot apex and leaves from of each treatment. Leaf tissue at approximately 20-30 mg, were finely chopped with a razor blade in 1000 µl polyvinylpyrolidone (PVP, MW : 111.1/unit). Chopped tissues were filtered through 20 µm nylon mesh and collected in a polystyrene tube. The filtrate was stained with 50 µl of propidium iodide (PI) solution (1mg/ml; Sigma Chemical Company, USA). Samples were cultured at 4 ^{0}C in the dark for 15 minutes and examined using FCM.

At least 4,000 nuclei were analyzed in each sample. Analyses were performed using a FACSCalibur cytometer (Becton-Dickinson Japan) at the Frontier Science Research Science Division of Bioresource Science. University of Miyazaki, Japan. Nuclear DNA content (pg) was estimated by the following equation:

$$\text{Sample (2C DNA)} \frac{\text{G1 channel of sample}}{\text{G1peak channel of Oryza sativa}} \times 0.91 \text{ pg (Oryza sativa DNA content}$$

By the above procedure, polyploid plantlets of oil palm were screened and maintained on MS medium for further study.

Size and density of stomata and guard cell measurement

To estimate the suitability of the stomatal size and density as criteria for determination of ploidy level in oil palm, ten fully expanded leaves were sampled from diploid (control) and putative tetraploid plantlets. Leaf epidermis at abaxial surface were collected to prepare and examine under scanning electron microscopy (SEM). For SEM, leaves were fixed in 0.2 M Na_2HPO_4 – NaH_2PO_4 buffered solution (PB; pH 7.8) and immersed in 2.5% glutaraldehyde ($C_5H_8O_2$) in PB for 2 hours. After washing twice in PB, leaves were dehydrated through ethanol series to the critical-point drying, mounted on stubs, and sputter-coated with gold in an ion sputtering device (Spi module). Stomatal density, length, and width were determined from images obtained with a 1200 objective lens. Five fields (26.9 mm) were selected to determine stomatal density. For stomatal length and width, four fields were photographed, and waved randomly. Measurement and counting of stomata were carried out using using a SEM (Quanta 400, FEI) at an accelerating voltage of 1.0 kV (Hiroshi *et al*., 2008).

Chloroplast number in guard cells

For study of chloroplast number in the stomata guard cells of diploid and putative tetraploid plantlets, a few strips of epidermis were removed from the abaxial side of fully expanded leaves, and then mounted on microscope slide with a drop of distilled water and covered with cover slip to measure chloroplast number per guard cells (Sari *et al*., 1999).The chloroplast numbers was measured in the middle leaves from each of the tested branches from both sources of plantlets under light microscope at 1000X magnification with three replications.

Data collection and statistic analysis

SSEs of oil palm were initiated using 30 HEs. Each HE was stressed in 25x125 mm test tubes containing stress medium supplemented with colchicine for 3 months. The survival rate of HE or HE together with SSE was analyzed using the mean number of plantlets regenerated after SSEs were treated. Data were evaluated using the mean number of plantlets producing leaves and roots, average length and diameter of stomata and average size of guard cells and number of chloroplasts in guard cells. DNA contents of oil palm were statically analyzed by ANOVA (analysis of variance) and significant difference among the treatments was separated using Duncan's multiple range test (DMRT) at p=0.05.

Results and discussion

Effect of colchicine on survival rate

The effect of colchicine on the survival of HEs after immersing and culture for 3 months depended upon concentrations of colchicine and duration of treatment. Survival rate decreased with increase concentration of colchicine. Many plantlets survived in 12 and 24 h treatments with 0.05% to 0.20% colchicines. Survival rate obtained from 0.05%, 0.10% and 0.15% colchicine treatment for 48 h was 41%, 26.55% and 12.11%, respectively. Among those plantlets, they showed different ploidy characters as presented in Table 1. Colchicine at concentration of 0.10% for 12 h and 0.20% for 24 h appeared to be effective on the production of putative tetraploid plantlets (6.67% and 16.67% of origin treated plants). Morphological characters of those plantlets were quite different in both shape and size of leaves (Figure 2a, b and 2c). These characters could be further used in breeding program. In this present study, the most effective concentration and duration treatment for chromosome doubling in oil palm SSEs were 0.20% (w/v) for 24 h.

Table 1. Effects of concentrations and durations of colchicine treatment to SSEs on the survival rate and induction of putative polyploids (n = 30)

Concentration (%)	Duration	Survival rate	DNA C-value		Percentag of Tetraploid
	(h)	(%)	Mixoploid 2C-4C	Tetraploid 4C	(%)*
0	12	100	-	-	-
	24	100	-	-	-
	48	100	-	-	-
0.05	12	80	-	-	-
	24	70.11	-	-	-
	48	41	-	-	-
0.10	12	68.78	1	2	6.67
	24	57.33	-	-	-
	48	26.55	-	-	-
0.15	12	56.22	-	-	-
	24	48.66	-	-	-
	48	12.11	5	-	-
0.20	12	50.56	-	-	-
	24	42	-	5	16.67
	48	0	-	-	-

$$* \text{ \% of tetraploid plantlets } \frac{\text{Number of putative tetraploid plantlets}}{\text{Number of original plantlets (30 plantlets)}} \times X\ 100$$

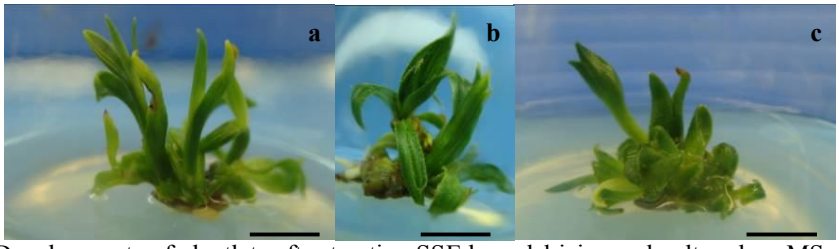

Fig.2. Developments of plantlets after treating SSE by colchicine and cultured on MS medium without plant growth regulator for 3 months. (a) normal plantlets (control) (bar = 0.5 mm), (b) plantlets obtained from treated SSEs with 0.10% colchicine for 12 h (bar = 0.5 mm), (c) plantlets obtained from treated SSEs with 0.20% colchicine for 24 h (bar = 0.8 mm)

Stability of ploidy level after treating with colchicines

Flow cytometry analysis (FCM)

Six months after treating SSEs with colchicine, the obtaining plantlets were classified into 3 types according to the ploidy level, diploid, mixoploid and tetraploid as shown in Figure 3. Flow cytometry showed a practical, reliable and rapid tool for screening of polyploidy plantlets after treating SSEs with colchicine. Similar results were also reported by Lee *et al*. (2002), Dolezel and Bartos (2005). In this present study, colchicine was proved to be a successful chemical in inducing chromosome doubling in oil palm. Peterson *et al*. (2003), Madon *et al*. (2005) and Omidbaigi *et al*. (2010) also found that colchicine effectively induced chromosome doubling in *Miscanthus sinensis*, oil palm and basil, respectively. According to Do *et al*. (1999) and Mene´ndez-Yuffa´ *et al*. (2000), ploidy level can be detected by conventional chromosome counting. However, the latter technique is laborious and lengthy, especially when the large number of samples is required (Roux *et al*., 2003).

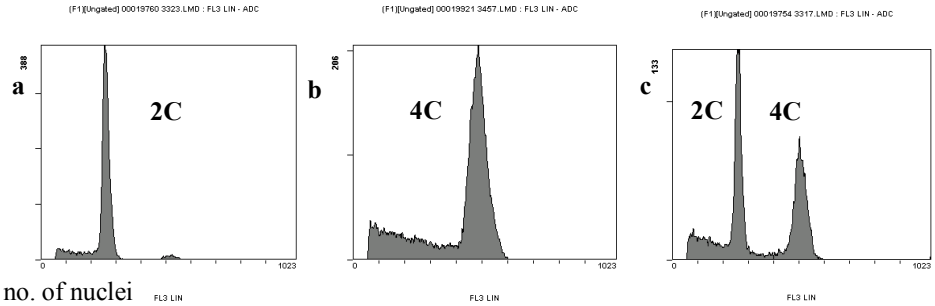

Fig. 3. Histogram of fluorescence intensity of nuclei isolated from oil palm plantlets obtained from colchicine treatment. (a) diploid (2C) (n=10) (b) tetraploid (4C) (n=7) and (c) mixoploid (2C-4C) (n=5).

DNA content of oil palm

DNA content of oil palm was quantitative analyzed using *Oryza sativa* cv. 'Nipponbare' (2C = 0.91; Uozu *et al*.,1997) as an external reference plant. Analysis of DNA content of oil palm after chromosome doubling with colchicine treatment revealed that the tetraploid plantlets gave the highest DNA content (4C = 7.45 pg), while diploid plantlets have two times lower than that of tetraploid plantlets (2C = 3.75 pg). On the other hand the number of base pairs of haploid genome for diploid and tetraploid plantlets was 1.81 and 2.61 x

10^9 bps, respectively (Table 2, Figure 4). From this result it is clearly demonstrated that DNA content has close correlated with the ploidy level So, the absolute nuclear DNA content can be used as an indicator for detection of ploidy level in oil palm.

In conclusion, it is possible to verify DNA content of oil palm by FACScalibur flow cytometer using *Oryza sativa* cv. 'Nipponbare' as an external plant. In addition, *Petunia hybrida* (2C=2.85 pg) was also popularly used as a reference in flow cytometric analysis, such as that of date palm (Siljak-Yakovlev *et al*., 1996), oil palm (Rival *et al*., 1997) and coconut (Sandoval *et al*., 2003), however, DNA content of petunia was not available in this investigation. Srisawat *et al*. (2005) reported that the highest DNA content of oil palm (4.72 pg; 2C) was found when standard DNA of corn was used as reference. The lowest DNA content (3.77 pg; 2C) was obtained when standard DNA of soy bean was used as reference. Similar result was also reported by Rival *et al*. (1997) who found a significantly lower DNA content in different plant tissue. They explaination to this situation might be due to different chromatin condensation in the nuclei of plant material, which caused different fluorochrome accessibility.

Based on the present results, we can conclude that flow cytometry is a good indicator for cell division activity in the cell cycle and potentially use in tissue culture of many plant species (Winkelman *et al*., 1998; Sandoval *et al*., 2003; Srisawat *et al*., 2005). Thus, the difference in DNA content of diploid and tetraploid plantlets resulted from the changes in chromosome number. It is more probable that euploidy or aneuploidy occurred in the cell of leaves that changes in chromosome number and/or rearrangement of their structure often occur (Karp *et al*., 1987; Bajaj, 1990; Kevers *et al*., 1999; Makowczy´nska *et al*., 2008)

Table 2. Genome size (2C nuclear DNA content in pg) of diploid and tetraploid plant of *E. guineensis* Jacq.(cv.Tenera) using *Oryza sativa* cv.'Nipponbare' as an external reference standard. The means are based on 3 replicated of experiment

Plant	No. of plants	Chromosome no.	DNA content (pg)	Base pairs (Mpb)
E. guineensis Jacq. cv. 'Tenera'	3	32	3.75 ± 0.26^a	$1.81 \times 10^9 \pm 0.05$
E. guineensis Jacq. cv. 'Tenera'	3	64	7.45 ± 0.25^b	$2.61 \times 10^9 \pm 0.06$
Oryza sativa cv. 'Nipponbare'	3	24	0.91	438

Data represents mean ±SD of three replicates per treatment.
Means within columns separated by Duncan's Multiple Range Test at P=0.05.
Different letters indicate significant difference at $p< 0.05$ (DMRT)

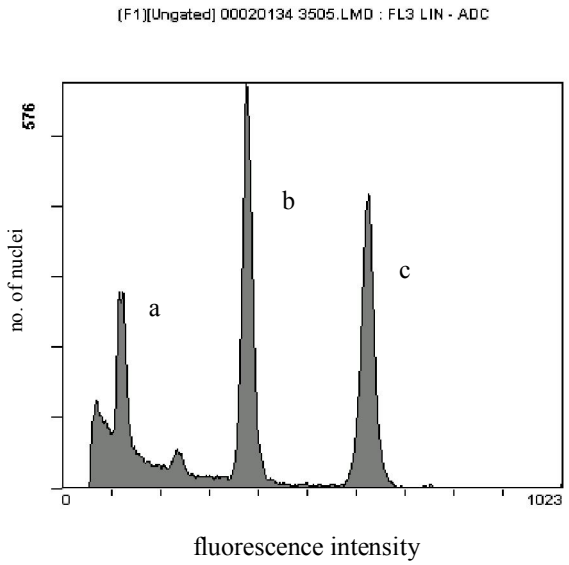

Fig. 4. Comparison of fluorescence intensity histogram among *E. guineensis* Jacq. cv. 'Tenera' control (b) and treated with 0.20% colchicine for 24 hours, (c) using *Oryza sativa* cv. 'Nipponbare' (a) as an external reference plant (n=3)

Morphological characteristics of the putative tetraploid plant

The morphological of diploid and putative tetraploid plantlets were distinguished after 10 months of culture. The results showed the difference between controlled diploid and putative tetraploid plantlets. Leaves of putative teraploid plantlets were actually shorter and thicker than those of the control. The color of the leaves was also darker and the shape of leaves changes from lanceolate to ovate (Figure 5a, b). In case of growth rate, the putative tetraploid plantlets were less than control (Figure 5c). Moreover, the root of putative tetraploid plantlets were bigger and shorter than diploid plantlets (Figure 5d, e, f and Table 3) while leaf characteristics and growth rate of mixoploid plantlets were the same with control, however, root was bigger. After 1 year of maintenance, *in vitro* conditions in flowering occurred in some putative tetraploid plantlets(Figure 5g, h, i, j).In most plantlets, tetraploid plantlets had bigger organs than those of diploid plantlets like those reported in basil (Omidbaigi *et al*., 2010). However, some organs of tetraploid plantlets of oil palm were smaller than diploid plantlets. Similar results to this present study were also reported in oil palm by Madon *et al.* (2005). Clarindo *et a*l. (2008) reported that colchicine could induce polyploidization which is the most common change in chromosome number.

This present results also confirmed that the morphological of oil palm has been correlated with a change in ploidy of the cells. In accordance with Wyman *et al.* (1992) plants with aberrant chromosome numbers may exhibit normal phenotypes, however, in this result the morphological changes were not reliable and accurate indicators for identification of tetraploid plantlets.

Table 3. Morphological characters of ploidy plantlets obtained from treating SSEs with colchicine at concentration of 0.10% colchicine for 12 h, 0.20% colchicine for 24 h and maintained on MS medium without plant growth regulators for 10 months

Ploidy level	Size of leaf from the top		Size of root	
	Length (cm)	Width (cm)	Length (cm)	Width (cm)
Diploid (2x)	9.27 ± 1.62^{a}	0.92 ± 0.13^{a}	1.30 ± 0.07^{a}	0.12 ± 0.02^{a}
Mixoploid (2x-4x)	8.36 ± 1.24^{a}	0.86 ± 0.15^{a}	1.21 ± 0.03^{b}	0.20 ± 0.01^{b}
Tetraploid (4x)	3.59 ± 0.92^{b}	0.57 ± 0.13^{b}	0.64 ± 0.06^{c}	0.24 ± 0.02^{c}

Data represents mean ±SD of three replicates per treatment in ten repeated experiments.
Means within columns separated by Duncan's Multiple Range Test at P=0.05.
Different letters indicate significant difference at $p< 0.05$ (DMRT)

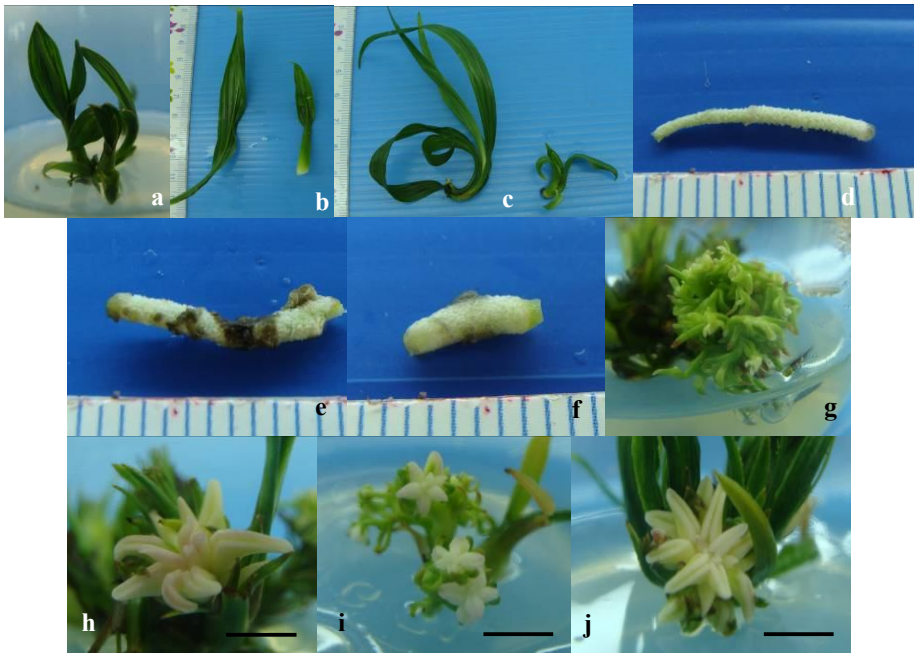

Fig. 5. Morphological characteristics of oil palm plantlets obtained from colchicine treatment.
a, b: leaf size and shape of tetraploid plantlets
c: growth of putative tetraploid plantlets (left) and tetraploid plantlets (right)
d, e, f: roots of diploid plants, mixoploid plants and tetraploid plantlets, respectively
g, h, i, j: flowering of putative tetraploid plantlets (bar = 0.5 mm.)

Size and density of stomata and guard cells

The results of size and density of stomata were different between diploid and putative tetraploid plantlets (Table 4 and Figure 6a, b, c). Putative tetraploid plantlets showed larger size of stomata but two times lower in density. From this point stomata characteristics (stomata size and stomata density) can be a simple and efficient parameters for identification the difference between diploid and tetraploid plantlets of oil palm treated with colchicines *in vitro* like those reports by Yudanova *et al*. (2002) and Gu *et al*. (2005). Moreover, the guard cells of putative tetraploid plantlets were observed to be abnormal and succulent. For mixoploid plants, the size of stomata nearly the same as control plantlets but the density of stomata were lower than diploid plantlets with some of guard cells was broken. However, Madon *et al*. (2005) reported that there was no significant difference in the stomata density between the diploid and polyploidy plant of oil palm.

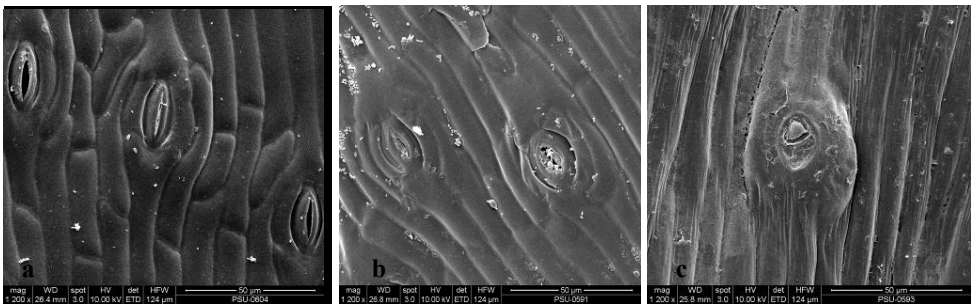

Fig. 6. Size and density of stomata from the plantlets obtained from treating SSEs with colchicine. (a) control, (b) mixoploid plantlet (treated with 0.10% colchicine for 12 h) and (c) tetraploid plantlet (treated with 0.20% colchicine for 24 h)

Chloroplast number in guard cells

It was clearly observed that chloroplast numbers in guard cells of diploid plantlet was two times lower than those in putative tetraploid plantlets (treated with 0.2% colchicine for 24 h). In one guard cell of diploid plantlets, 14.4 chloroplasts were found whereas 28 chloroplasts were found in tetraploid plantlets (Table 4 and Figure 7a, 7c). This experiment was similar to the research of basil reported by Omidbaigi *et al.* (2010). Moreover, chloroplast number in one guard cell of mixoploid plantlets (Figure 7b) was nearly the same as control (13.5 chloroplasts). However, the size of chloroplast in a guard cell decreased with the increase in ploidy level. Putative tetraploid plantlets and mixoploid plantlets had size of chloroplasts in a guard cell at 2.0 µm whereas the size of those in diploid was 4.0 µm. From this present results chloroplasts

number and the size of chloroplast is genetically correlated with the ploidy level of a plant. Similar result was also reported by Yudanova *et al*. (2002) and Aryavand *et al*. (2003). Accordingly, chloroplasts number and their size might be efficiently used as parameters for distinguishing ploidy level of oil palm.

Table 4. Size and density of stomata, size of chloroplast and chloroplast number in guard cells of the plantlets obtained from treating SSEs with colchicine at concentration of 0.10% colchicine for 12 h, 0.20% colchicine for 24 h and maintained on MS medium without plant growth regulators for 10 months

Characteristic	Diploid	Mixoploid	Tetraploid
stomata length (µm)	25.82 ± 2.58^{a}	26.61 ± 3.31^{a}	29.23 ± 5.70^{a}
stomata diameter (µm)	9.75 ± 1.21^{c}	14.39 ± 2.69^{b}	19.11 ± 3.67^{a}
stomata density $(mm)^2$	2.90 ± 0.32^{a}	2.00 ± 0.47^{b}	1.10 ± 0.32^{c}
No. of chloroplast/guard cell	14.40 ± 0.70^{b}	13.50 ± 0.53^{c}	28.00 ± 1.15^{a}
size of chloroplast (µm)	4.00 ± 0.00^{b}	2.00 ± 0.00^{a}	2.00 ± 0.00^{a}

Data represents mean ±SD of three replicates per treatment in ten repeated experiments.
Means within columns separated by Duncan's Multiple Range Test at P=0.05.
Different letters indicate significant difference at $p < 0.05$ (DMRT)

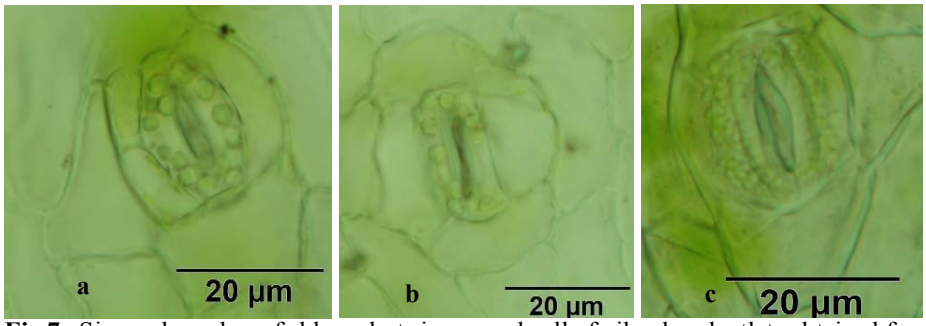

Fig.7. Size and number of chloroplasts in a guard cell of oil palm plantlets obtained from colchicine treatment (a) control, (b) mixoploid plantlet and (c) tetraploid plantlet

Conclusion

The results revealed that colchicine can increase the ploidy level of oil palm. In this present experiment, the analysis of diploid and tetraploid plantlets were based on DNA content, plant morphology, size and density of stomata, chloroplast number in guard cells and size of chloroplast. The most efficient method to identify the ploidy level was flow cytometry. This method is suitable, quick and easy for identifying the ploidy level of oil palm. Morphological and physiological characters are an effective method in preliminary screening of

tetraploid plantlets in oil palm. This method can generate useful genetic variability for oil palm improvement.

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References

Aberlenc-Bertossi F., Noirot, M. and Duval, Y. (1999). BA enhances the germination of oil palm somatic embryos derived from embryogenic suspension cultures. Plant Cell, Tissue and Organ Culture 66: 53-57.

Adaniya, S. and Shirai, D. (2001). *In vitro* induction of tetraploid ginger (*Zingiber officinali* Roscoe) and its pollen fertility and germinability. Scientia Horticulturae 88: 277-287.

Ashomore, S.E. and Drew, R.A. (2006). The application of biotechnology in an integrated project of conservation and utilization of papaya and its wild relatives. Acta Horticul- turae 725: 89-94.

Aryavand, A., Ehdaie, B., Tran, B. and Waines, J.G. (2003). Stomata frequency and size Differenttiate ploidy levels in *Aegilops neglecta*. Genetic Resources and Crop Evolution 50:175-182.

Bajaj, Y.P.S. (1990). Somaclonal variation origin, induction, cryopreservation, and implications in plant breeding. In: Bajaj,YPS (ed.) Biotechnology in agriculture and forestry. Somaclonal variation in crop improvement, vol 11, Springer, Berlin Heidelberg New- York Tokyo, pp 3-35.

Bennett, M.D. and Leitch, I.J. (1995). Nuclear DNA amounts in angiosperms. Annal of Botany. 76: 113-176.

Clarindo, W.R., de Carvalho, C.R., Santos Arau´jo, F., de Abreu, I.S. and Otoni, W.C. (2008). Recovering polyploid papaya *in vitro* regenerants as screened by flow cytometry. Plant Cell, Tissue and Organ Culture 92: 207-214.

Chehmalee, S. and Te-chato, S. (2008). Induction of somatic embryogenesis and plantlet rege-neration from cultured zygotic embryo of oil palm. Journal of Agricultural Technology 4: 137-146.

De Schepper, S., Leus, L., Eeckhaut, T., Van Bockstaele, E., Debergh, P. and Deloose, M. (2004). Somatic polyploid petals: regeneration offers new roads for breeding Belgian potazaleas. Plant Cell, Tissue and Organ Culture 76: 183-188.

De Jesus-Gonzalez, L. and Weathers, P.J. (2003). Tetraploid *Artemisia* annual hairy roots produce more artemisinin than diploids. Plant Cell Reports 21: 809-813.

Do, G., Seo, B. and Ko, J. (1999). Analysis of somaclonal variation through tissue culture and chromosomal localization of rDNA sites by fluorescent in situ hybridization in wild *Allium tuberosum* and a regenerated variant. Plant Cell, Tissue and Organ Culture 57: 113-119

Dolezel, J. and Bartos, J. (2005). Plant DNA flow cytometry and estimation of nuclear genome size. Annals of Botany 95: 99-110.

Gao, S.L., Chen, B.J. and Zhu, D.N. (2002). *In vitro* production and identification of autote-traploids of *Scutellaria baicalensis*. Plant Cell, Tissue and Organ Culture 70: 289-293.

Gu, X.F., Yang, A.F., Meng, H. and Zhang, J.R. (2005). *In vitro* induction of tetraploid *Zizyphus jujuba* Mill. cv. Zhanhua. Plant Cell Reports 24: 671-676.

Hiroshi, S., Shuhei, T., Yumi, U., Harumi, T., Mayumi, Y., Kazuo, S., Akiteru, A. and Hideo, H. (2008). The stomal morphology of parasitic females of *Strongyloides* pp.by scanning electron microscopy. Parasitology Research 02: 541-546.

Karp, A., Wu, Q.S., Steele S.H. and Jones, M.G.K. (1987). Chromosome variation in dividing protoplasts and cell suspensions of wheat. Theoretical and Applied Genetics 74: 140-146.

Kevers, C., Greimers, R., Franck, T., Dommers, J. and Gaspar, I. (1999). Flow cytometry esti- mation of nuclear size and ploidy level of habituated calli of sugar beet. Biology of Plants 42: 321-332.

Lee, J.H., Arumuganathan, K. and Kaeppler, S.M. (2002). Variability of chromosomal DNA contents in maize (*Zea mays* L.) inbred and hybrid lines. Planta 215: 666-671.

Luckett, D. (1989). Colchicine mutagenesis is associated with substantial heritable variation in cotton. Euphytic 42: 177-182.

Madon, M., Clyde, M.M., Hashim, H., Mohd, Y.Y., Mat, H. and Saratha, S. (2005). Polyploidy induction of oil palm through colchicine and oryzaline treatments. Jounal of Oil Palm Research 17: 110-123.

Madon, M., Phoon, L.Q., Clyde, M.M. and Mohd Din, A. (2008). Application of flow cyto-metry for estimation of nuclear DNA content in Elaeis . Jounal of Oil Palm Research 20: 447-452.

Makowczy´nska, J., Andrzejewska-Golec, E. and Sliwinska, E. (2008). Nuclear DNA content in different plant materials of *Plantago asiatica* L. cultured *in vitro*. Plant Cell, Tissue and Organ Culture 94: 65-71.

Mene´ndez-Yuffa´, A., Silva, R.F. and Rios, L. (2000). Mitotic aberrations in coffee (*Coffea arabica* cv. 'Catimor') leaf explants and their derived embryogenic calli. Electronic Journal of Biotechnology 3:161-166.

Omidbaigi, R., Mirzaee, M., Hassani, M.E. and Moghadam, M.S. (2010). Induction and Identi-fication of polyploidy in basil (*Ocimum basilicum* L.) medicinal plant by colchicine treatment. International Journal of Plant Production. 4: 87-98.

Passos, I.R.S. and Bernacci, L.C. (2005). Tissue culture applied to in vitro germplasm conserva-tion and breeding of passion fruit (Passi (ora spp.).In: Faleiro, F.G., Junqueira. N.T.V., Braga, M.F.(eds) Passion fruit:germplasm and breeding. Embrapa Cerrados, Planaltina, DF, Brazil, pp 360-384 (in Portuguese).

Petersen, K.K., Hagberg, P. and Kristiansek, K. (2003). Colchicine and oryzalin mediated chromosome doubling in different genotypes of *Miscanthus sinensis*. Plant Cell, Tissue and Organ Culture 73: 137-146.

Rabechault, H., Martin, J.P. and Gas, S. (1972). Researches surla culture des rissue de palmier a huile (*Elaeis guineensis* Jacq). Oleagineux 27: 531-534.

Rajesh, M.K., Radha, E., Karun, A. and Parthasarathy, V.A. (2003). Plant regeneration from embryo-derived callus of oil palm - the effect of exogenous polyamines. Plant Cell, Tissue and organ Culture 75: 41-47.

Rival, A., Beule, T., Barre, P., Hamon, S., Duval, Y. and Noirot, M. (1997). Comparative flow cytometric estimation of nuclear DNA oil palm content in (*Elaeis guineensis* Jacq.) tissue cultures and seed-derived plants. Plant Cell Reports 16: 884-887.

Roux, N., Toloza, A. and Radecki, Z. (2003). Rapid detection of aneuploidy using flow cyto- metry. Plant Cell Reports 21: 483-490.

Sandoval, A.., Hocher, V. and Verdeil, J.L. (2003). Flow cytometric analysis of the cycle in different coconut palm (*Cocos nucifera* L.) tissues cultured *in vitro*. Plant Cell Reports 22: 25-31.

Sari, N., Abak, K. and Pitrat, M. (1999). Comparison of ploidy level screening methods in water-melon: *Citrullus lanatus* (Thunb.) Matusum. And Nakai. Scientia Horticulturae 82: 265-277.

Shao, J., Chen, C. and Deng, X. (2003). *In vitro* induction of tetraploid in pomegranate (*Punica granatum*). Plant Cell, Tissue and Organ Culture 75: 241-246.

Siljak-Yakovlev, S., Benmalek, S., Cerbah, M., Coba de la Pena, T., Bounaga, N., Brown, S.C. and Sarr, A. (1996). Chromosomal sex determination and heterochromatin structure in date palm. Sexual Plant Reproduction 9: 127-132.

Srisawat, T., Kanchanapoom, K., Pattanapanyasat, K., Srikul, S. and Chuthammathat, W. (2005). Flow cytometric analysis of oil palm: a preliminary analysis for cultivars and genomic DNA alteration. Songklanakarin Journal Science Technology 27: 645-652.

Te-chato, S. (2002). Improve callus induction and embryogenic callus formation from cultured young leaves of oil palm seedling. Thai Journal of Agricultural Science 35: 407-413.
Teixeira, J.B., Sondahl, M.R., Nakamura, T. and Kirby, E.G. (1995). Establishment of oil palm cell suspension and plant regeneration. Plant Cell,Tissue and organ Culture 40: 105-111.
Thao, N.T.O., Ureshino, K., Miyajima, I., Ozaki, Y. and Okubo, H. (2003). Induction of tetra- ploids in ornamental *Alocasia* through colchicine and oryzalin treatments. Plant Cell, Tissue and Organ Culture 72: 19-25.
Thiem, B. and Sliwinska, E. (2003). Flow cytometric analysis of nuclear DNA content in cloudberry (*Rubus chamaemorus* L.) *in vitro* cultures. Plant Science 164: 129-134.
Uozu, S., Ikehashi, H., Ohmido, N. and Ohtsubo, H. (1997). Repetitive sequences:cause for variation in genome size and chromosome morphology in the genus *Oryza*. Plant Molecular and Biology 35: 791-799.
Vieira, M.L.C., Oliveira, E.J., Matta, F.P., Padua, J.G. and Monteiro, M. (2005). Biotechnological methods applied to passion fruit breeding.In: Faleiro, F.G., Junqueira, N.T.V., Braga,M.F.(eds) Passion fruit: germplasm and breeding.Embrapa Cerrados,Planaltina, DF, Brazil, pp 41-453 (in Portuguese).
Winkelmann, T., Sangwan, R.S. and Schwenkle, H.G. (1998). Flow cytometric analyses in embryogenic and non-embryogenic callus lines of *Cyclamen persicum* Mill.: relation between ploidy level and competence for somatic embryogenesis. Plant Cell Reports 17: 400-404.
Wu, J. and Mooney, P. (2002). Autotetraploid tangor plant regeneration from *in vitro citrus* somatic embryogenic callus treated with colchicine. Plant Cell, Tissue and Organ Culture 70: 99-104.
Wyman, J., Brassard, N. and Flipo, D. (1992). Ploidy level stability of callus tissue, axillary and adventitious shoots of *Larix* X *eurolepis* Henry regenerated *in vitro*. Plant Science 85: 189-196.
Yudanova, S.S., Maletskaya, E.I. and Maletskii, S.I. (2002). Variability of chloroplast number in populations of stomatal guard cells in sugar beet *Beta vulgaris* L. Russian Journal of Genetics 38:72–78.

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