IN VITRO INDUCTION OF POLYPLOIDY IN OIL PALM *(Elaeis guineensis Jacq.)*

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Abstract

The effect of the three anti-mitotic agents: colchicine, oryzalin and amiprophos-methyl on the induction of polyploidization in oil palm were studied in vitro. Each anti-mitotic chemical was evaluated on 4 and 7-day old oil palm zygotic embryos and exposed for 7 and 10 days. Ploidy estimation was determined using the measurement of stomata size of guard cells in leaves and chromosome counting in root tips of the treated in vitro oil palm. Results revealed that percentage germination rate based on the LC_{50} estimation of oil palm zygotic embryo treated with colchicine and amiprophos-methyl was higher (53-80%) at 5-10 and 1-10 mg/L, respectively. Stomatal measurement of guard cells and chromosome counting in root tips were found to be a promising index in determining the ploidy level in oil palm. In terms of chromosome number, the three chemicals increased the chromosome number of the treated in vitro oil palm from 32 to 48 which also supports the results on stomata measurement of guard cells that ploidization in treated oil palm has occurred.

Keywords: *polyploidy, guard cells, stomata, oil palm, colchicine, oryzalin and amiprophos-methyl*

1.0 Introduction

Oil palm (*Elaeis guineensis* Jacq.), a perennial allogamous monocotyledonous tree is a very important commercial crop in the world which gives the highest yield of oil per hectare of any oil-producing crop (Gorret et al., 2004). During the 2005-2006 growing season, worldwide palm oil production was 39.8 million metric tons, of which 4.3 million tons was in the form of palm kernel oil. Thus, by far, palm oil is the most widely -produced tropical oil, which constitutes thirty percent of total edible oil production worldwide. Being the highest yielding oil-bearing crop, *in vitro* culture of oil palm has been applied to its genetic improvement using many methods (Chukwuemeka et al., 2005; Duval et al., 1993) to develop high- yielding collection and in order to remain competitive. Therefore, to remain competitive, oil palm growers and breeders need new and novel approaches for its genetic improvement (Abdullah et al., 2005).

The induction of polyploids has been found to be a valuable tool in the genetic improvement of many plants. In a narrow sense, polyploidization is an event in which the chromosome number is doubled. However, in a wider sense, it is an important process in nature and plant breeding (deWet, 1980). Polyploidization can result in larger and darker leaves, delays in flowering, larger inflorescences, prolongations of the flowering period, apomixes, larger fruits, and greater secondary metabolite production and yield (Dhawan and Lavania, 1996; Gao et al., 1996; Gu et al., 2005; Predieri, 2001; Roy *et al.*, 2001; Shao et al., 2003; Urwin et al., 2007). Hence, it is possible that polyploidy in oil palm may provide an improvement on agronomical traits. An

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efficient method for doubling chromosome number would be particularly useful for further production of tetraploid forms of *E. guineensis*.

Nowadays, breeding of oil palm is focused on hybridization. The breeder aims to combine the desirable traits from two different parental lines in the offspring through breeding programmes. It aims to select the offspring of plants which show the best combination of traits from both parent plants. Hybrids are considered superior because of their uniformity and diversity. However, there is a dearth of studies published for successful oil palm hybridization. To produce chromosome-doubled plants, colchicine treatment has mainly been used as an efficient strategy for various plant species. In fruit trees, colchicine has successfully been applied to produce polyploids in various crops such as grape (Notsuka et al., 2000), citrus (Gmitter and Ling, 1991), and loquat (Yahata et al., 2004). In blueberries, several successful results of chromosome doubling have been reported with colchicine treatment. Other microtubule depolymerizing compounds, including dinitroaniline: oryzalin, trifluralin and more recently phosphoric amide, amiprophos-methyl (APM), have also been used on many plants at various concentrations. All these chemicals act by binding to the tubulin dimmers preventing the formation of microtubules, and consequently, spindle fibers during cell division (Petersen et al., 2003).

This study aimed at developing an effective polyploidization system in the oil palm using treatment of the three anti-mitotic agents: colchicine, oryzalin and amiprophos-methyl of *in vitro* shoots and zygotic embryos. The effects of varying concentrations and the time of exposures to the three chemicals to increase ploidy in *E. guineensis* were analyzed. The basic method used for ploidy estimation is stomata size of guard cells measurement in the leaves and chromosome set counts from the root of oil palm.

2.0 Materials and Methods

2.1 Preparation of Plant Materials

Bunches of African oil palm with mature fruits were collected from the oil palm plantation in Thailand. These were brought to the Energy Crop Laboratory for removal of fruits. Fruits were detached from the stalk, mesocarp was peeled and cut clearly until the embryo inside was seen, leaving a thin layer of the outer skin of the endosperm to protect the embryo upon sterilization. Seeds with embryos were cleaned with tap water for five (5) minutes and surface-sterilized by soaking in 70% ethanol for 30 seconds and in 20% bleach solution containing three drops of wetting agent, "Triton X-100" for 20 minutes inside the laminar hood. These were followed by three rinses in sterile distilled water for five minutes. The first rinse was mixed with 500 mg/L streptomycin as antibiotic.

2.2 Preparation of Culture Medium

The MS (Murashige and Skoog, 1962) medium in half strength concentration (½ MS) supplemented with 30 g/L sucrose and 2.5 g/L phytagel was used in the initiation of the growth of zygotic embryos. For 1 L medium, 10 ml of MS stock - I was measured using a pipette followed by 5 ml each of the MS stock II-MS stock V. The solutions were then stirred and sucrose was added into the solution. When the sucrose was totally dissolved, the solutions were filled up volume to 1000 ml by adding distilled water and adjusted the medium pH to 5.8. The agar was then poured and mixed

into the solution and put inside the microwave for 15 minutes to dissolve the gel. The medium was dispensed in a culture bottle containing 50 ml per bottle. These were sterilized in an autoclave at 121°C for 20 minutes and stored in the culture medium shelf.

2.3 Culture Initiation of Oil Palm Embryo

Sterilized seeds with embryos were cultured on the ½MS medium (Murashige and Skoog, 1962) with 30 g/L sucrose and 2.5 g/L phytagel with five embryos in each bottle. Embryo cultures were incubated at 28 ºC under light condition illuminated by cool-white fluorescent light and 60 µmol m^{-2} s^{-1} for 16 hours of photoperiod for 4 and 7 days. The germinated embryos were used for the experiment on the induction of polyploidization in the oil palm (*E. guineensis* Jacq.*)* using treatment of the three anti-mitotic chemicals such as colchicine, oryzalin and amiprophos-methyl of *in vitro* shoots and zygotic embryos.

2.4 Treatment with Anti-mitotic Agents

Germinated embryos from the ½MS media were transferred in a media added with the three anti-mitotic agents namely; colchicine, oryzalin and amiprophos-methyl at different concentrations. The anti-mitotic agents were incorporated into ½MS medium supplemented with 30 g/L sucrose and 2.5 g/L phytagel after autoclaving. The concentrations were as follows: colchicines at 5, 10 and 15 mg/L, oryzalin at 0.5, 1 and 5 mg/L and amiprophos-methyl at 1, 5 and 10 mg/L, respectively (Table 2). For the controls, embryos were cultured directly on ½ MS medium supplemented with 30 g/L sucrose and 2.5 g/L phytagel only. The effects of varying time of exposure to the three anti-mitotic chemicals and age of cultures were also determined.

Five embryos were planted for every control and treatment with three replicates. The cultures were incubated at 28 °C under light condition illuminated by cool-white fluorescent light and 60 µmol m^{-2} s^{-1} for 16 hours of photoperiod for weekly observation of morphological changes. The period or duration of treatment for each chemical was observed in 7 and 10 days.

2.5 Analysis of Polyploidy Plants

For the observations of morphological development, treated and untreated zygotic embryos of oil palm were described simultaneously based on the performance of their growth weekly. Percentage of germination was also calculated.

2.6 Chromosome Counting in Root tips

Chromosome counting was done using plantlets derived from both treated and untreated shoot tips. The root tips were cut from the plantlets and washed with tap water to separate the remnants of agar and fixed with 90% glacial acetic acid for 30 minutes. After 30 minutes, the glacial acetic acid was drained and rinsed with 70% ethanol for three times. Root tips were then hydrolyzed in 1N HCl in a water bath at 60°C for 45 minutes and rinsed with distilled water for three times. Root tips were squashed on a glass slide using the tip of the forceps to disperse the cells, stained using the 2% orcein solution for 30 minutes and covered a cover slip. Cells were spread by applying pressure (using thumb) on the cover slip layered with paper to absorb the excess staining solution. The edges of the cover slip were sealed with nail polish to prevent drying.

Prepared samples were visualized by a light microscope and magnified by 20 x 10

and 40 x 10 and the chromosome counting and photographing were done with 100 x 10 objective. Number of chromosomes from control and treatment were compared.

2.7 Stomata Size of Guard Cells

The leaf samples from plantlets derived from treated and untreated (control) zygotic embryo or shoot tips were used to measure the length and width of stomata. A small area on the abaxial leaf surfaces was smeared with a nail polish and let it dried for few minutes. When dried, the nail polish impression was removed using a strip of transparent cellulose tape. The abaxial epidermis imprints that formed on the tape was placed on a glass slide after addition of one drop of tap water and covered with a cover slip. Under a light microscope, 10 samples of stomata per treatment including the control were examined and photographed for measurement.

Photographing was done with a 100 x 10 magnification. The length and diameter of ten stomata per leaf were measured using a Cell A Program at 100 x 10 magnification. The experiment was set-up in Completely Randomized Design (CRD) with 3 replications at 5 explants per replicate. The treatment means were compared using Duncan's Multiple Range Test (DMRT). Data was analyzed by SPSS version 14 software.

3.0 Results and Discussions

3.1 Effect of Anti-mitotic Agents on the Germination and Survival Rate of Oil Palm Zygotic Embryo

The effect of the three anti-mitotic agents: colchicines, oryzalin and amiprophos-methyl (APM) on the germination rate of oil palm embryo at different concentrations are shown in table 1.

Table 1. Percentage of germination of oil palm embryos treated with different anti-mitotic concentrations at varying time of exposure and age of culture.

Treatment	**Concentration (mg/L)**	**Duration of Treatment***			
		7 days		10 days	
		4-day old	7 day-old	4-day old	7-day old
Control (½ MS)	-	80.0a	93.3a	80.0a	80.0a
Colchicine	0.5	66.7ab	73.3abc	46.67ab	60.0b
	1.0	60.0ab	66.7abcd	40.0b	46.7bc
	5.0	53.0ab	46.7cd	33.3b	46.7b
Oryzalin	0.5	33.3b	40.0d	20.0c	0.0c
	1.0	47.7ab	0.0e	0.0c	0.0c
	5.0	0.0c	0.0e	0.0c	0.0c
Amiprosphos-methyl	1.0	66.7ab	80.0ab	33.3b	40.0bc
	5.0	66.7ab	73.3abc	26.7b	46.7bc
	10.0	80.0a	53.3bcd	26.7b	46.7bc

**Percentage of germinated embryo was investigated till 4 weeks after treated with anti-mitotic agents. Data are means from 3 replicates. Values in each group of embryos in each column followed by the same letter are not significantly different at the 0.05 level.*

There were significant differences found in the percentage of germination of oil palm embryos subjected to different anti-mitotic agents from 4 and 7 days after exposure, regardless of the age of culture (Table 1). Among the three anti-mitotic agents, percentage embryo germination rate was found to be higher in embryos treated with colchicines at different concentrations while oryzal in decreased significantly. Respective to the different concentrations of colchicine, germination rate was reduced at increasing concentration both in 4 and 7 day old culture. Furthermore, the result of the survival rate of the of oil palm zygotic embryos treated with different anti-mitotic concentrations based on LC_{50} at varying time of exposure and age of culture were presented in figure 1.

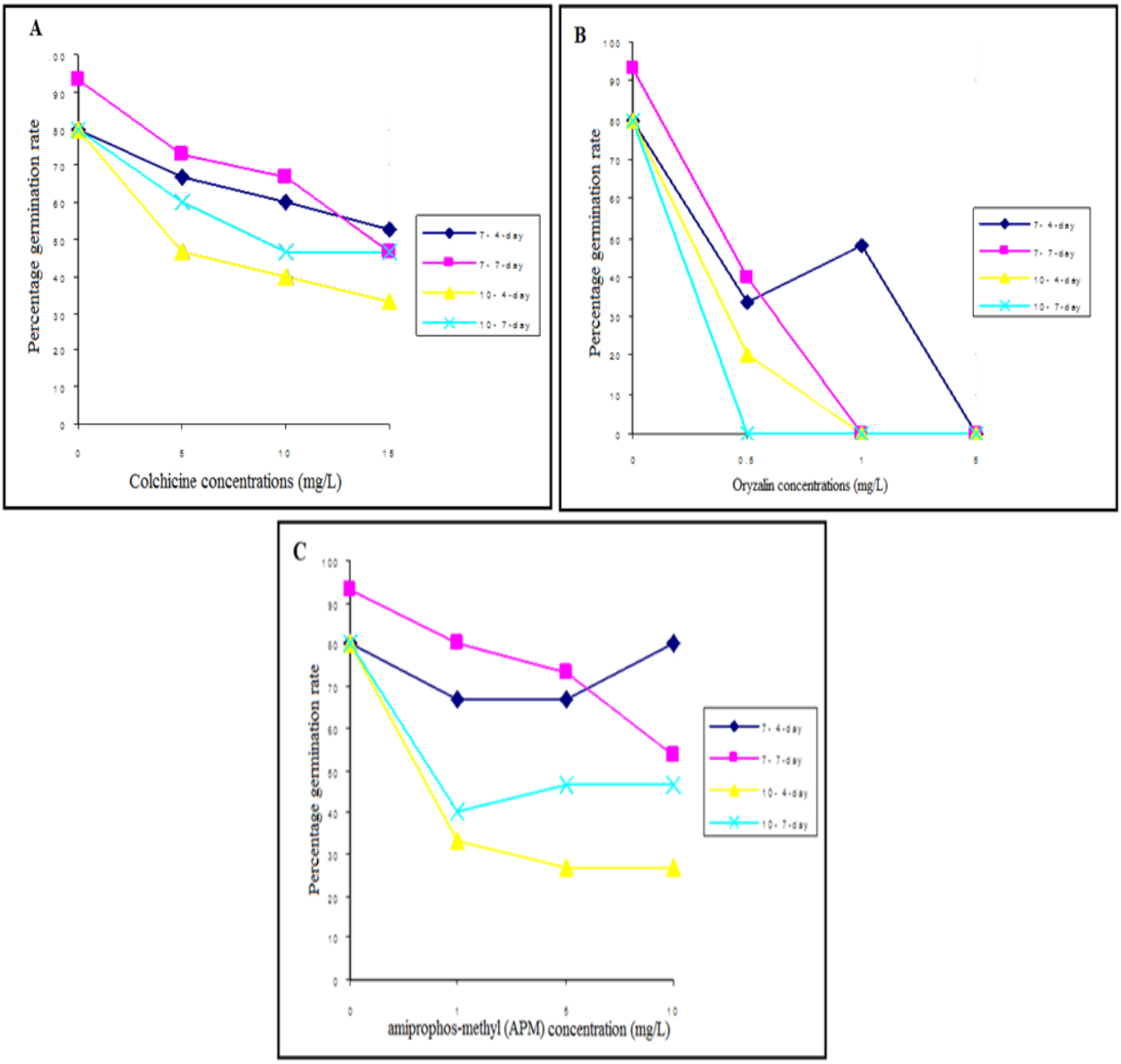

Figure 1. Percentage survival rate of oil palm zygotic embryos treated with different anti-mitotic concentrations: (A) colchicine (B) oryzalin, and (C) amiprophos-methyl (APM) based on LC_{50} at varying time of exposure and age of culture

Analyzing the relative toxicity of the three anti-mitotic chemicals based on the LC_{50}, it appears that oryzalin displayed somewhat greater overall toxicity relative to colchicine and APM. Oryzalin at different concentrations used in this experiment was found to be the most lethal for the growth and development of zygotic embryo of oil palm than colchicine and APM. This effect may be attributed to higher affinity of oryzalin for the plant tubulin, a protein that comprises the microtubules of the mitotic spindle apparatus (Hart and Sabnis, 1976; Okamura, 1980; van Harten, 1998). Oryzalin may also interfere with calcium ions involved in microtubule assembly (Weisenberg, 1972). For these reasons, lower concentrations of oryzalin, relative to colchicine and APM, should be typically employed for ploidy alteration in oil palm plants. These results show that the toxicity of these anti-mitotic chemicals is proportionate to the concentration and exposure duration and as a general rule, the lower the anti-mitotic concentration, the greater the survival rate. Too high concentration and longer duration of the treatment will lead to the formation of very few plants, as a result of toxicity (Hansen *et. al.*, 2000).

3.2 Effect of Anti-mitotic Agents on the Growth and Morphological Development of Oil Palm Zygotic Embryo

Morphological changes such as the characteristics of leaves and roots of the treated plantlets were completely different from the control. Roots of the treated oil palm plants were shorter than those of the control. Colchicine treated oil palm have a broader and greener leaf than those from the other anti-mitotic agents. These phenomena could be explained by assuming that colchicine could have a similar effect as cytokinins. According to Ruiz & Vasquez (1992), the colchicine added to the media could, to a certain extent, modify the auxin/ cytokinin relation and, therefore change the growth of the cell population in culture. In the case of oil palm treated with oryzalin and APM, it showed a typical darker green leaves, often narrower and shorter which was similar to characteristics of tetraploid in Citrus *spp.*, as described by Wu and Mooney (2002). High concentrations of oryzalin and APM coupled with a long exposure time were detrimental to subsequent seedling growth and produced tetraploids with undesirable characteristics such as stunted growth (Fig. 2).

3.3 Effect of Anti-mitotic Agents on the Stomatal Size of Guard Cells of Oil Palm Zygotic Embryo

As observed in the study, the leaf abaxial epidermis under the light microscope confirmed the differences in the number of stomata and length of guard cells between the diploid oil palm plants (control shoot) and the polyploid oil palm plants (treated shoot) as shown in table 2 & 3 and visualized in figs. 3 & 4.

Results revealed that treated leaf and shoot tip-derived samples of oil palm plants showed largest size of stomata respective to width and length and were found to be significantly different. As observed the length in stomata size of guard cells reached 32.2 µm in zygotic embryo-derived shoots and 33.7 µm in shoot tips in both colchicine and oryzalin at 0.5 mg/L and colchicine at 100 mg/L concentration, respectively (Table 2 and 3). Respective to stomata width of guard cell, the differences between diploid and polyploid plants were highly significant. The average stomatal width of guard cell of the polyploidy plants was about 21.5 µm in

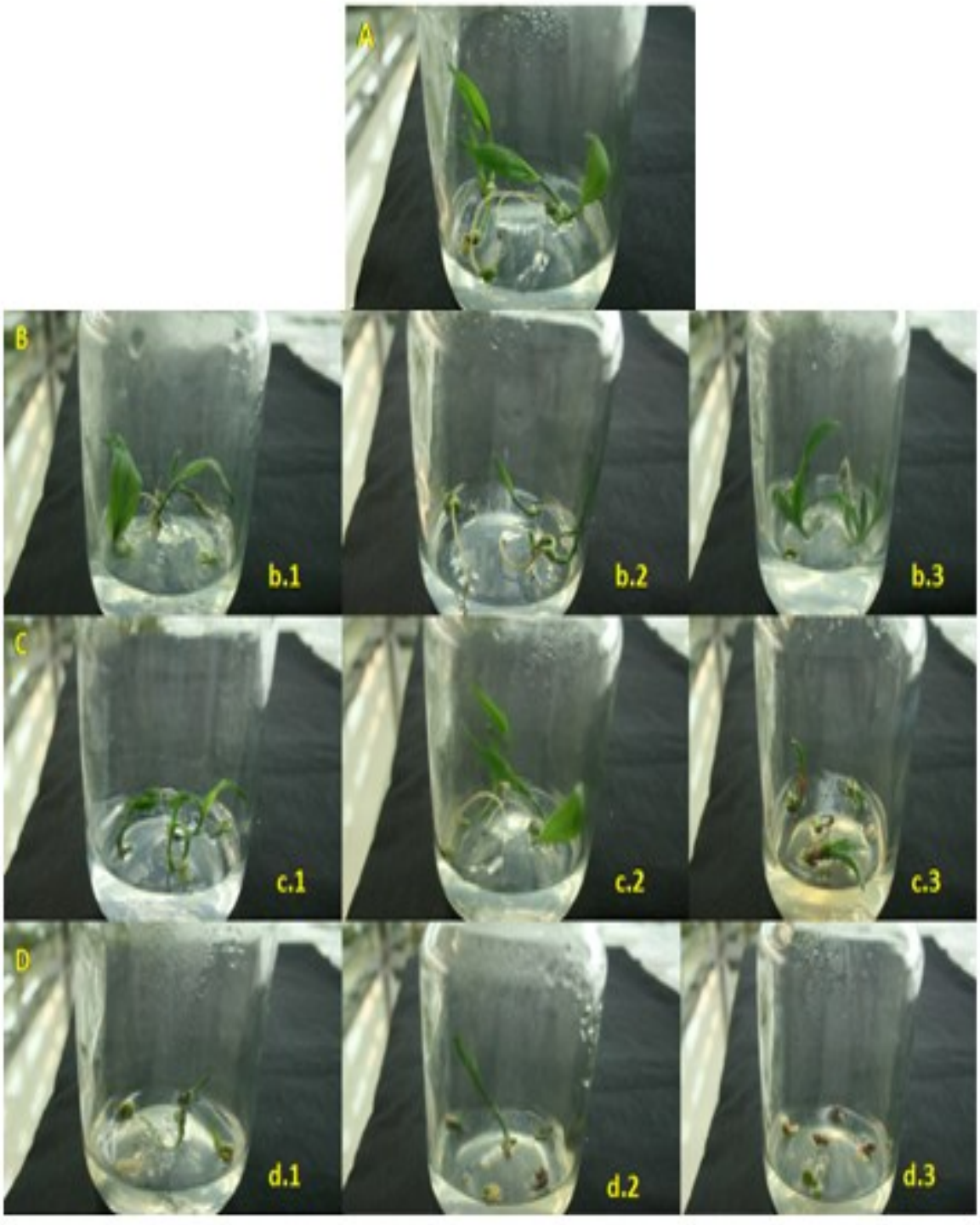

Figure 2. Development of a 7-day old zygotic embryos at 4 weeks after treated with anti-mitotic agent. (*A. Control ½MS; B. Colchicine (b. 1) 5.0 mg/L (b. 2) 10 mg/L (b.3) 15 mg/L; C. Oryzalin (c.1) 0.5 mg/L (c.2) 1.0 mg/L (c.3) 5.0 mg/L ; D. Amiprophos-methyl (d.1) 1.0 mg/L (d.2) 5.0 mg/L (d.3) 10 mg/L.*

Table 2. Stomatal length and width of guard cell of leaf samples from a diploid (control) and polyploid (derived after treatment with anti-mitotic agent) zygotic embryo-derived oil palm plants.

Treatment	Concentration (mg/L)	Size of Stomata (µm)*	
		Length	Width
Control (½ MS)	-	24.6 bc	14.8 d
Colchicine	0.5	27.1 bc	17.6 bc
	1.0	32.0 a	21.5 a
	5.0	28.7 b	16.0 bc
Oryzalin	0.1	28.7 b	15.0 cd
	0.5	32.2 a	14.6 d
	1.0	27.5 bc	16.1 bcd
Amiprosphos-methyl	5.0	28.1 b	17.99 b

**An average of 10 stomata per treatment. Values in each column followed by the same letter are not significantly different at the 0.05 level.*

Table 3. Stomatal length and width of guard cell of a diploid (control) and polyploid shoot tip-derived oil palm plants

Treatment	Concentration (mg/L)	Size of Stomata (µm)*	
		Length	Width
Control (½ MS)	-	21.3 d	14.8 c
Colchicine	100	33.7 a	17.6 b
Oryzalin	0.1	29.3 b	19.8 a
	0.2	25.5 c	16.9 b
	0.5	27.7 b	16.4 c
Amiprosphos-methyl	5.0	23.9 c	20.8 a

**An average of 10 stomata per treatment. Values in each column followed by the same letter are not significantly different at the 0.05 level.*

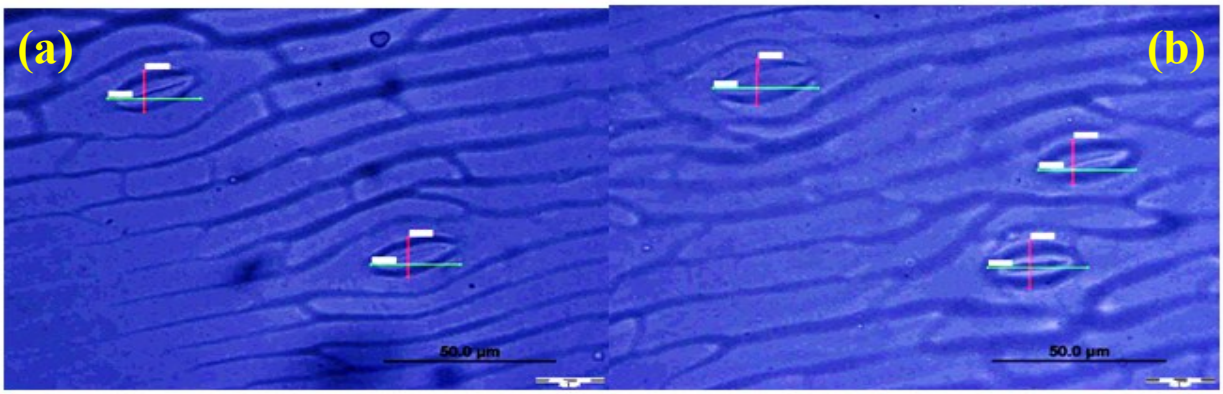

Figure 3. Length and width from leaf sample of diploid oil palm plants from (a) zygotic embryo-derived shoot and (b) shoot-tip culture.

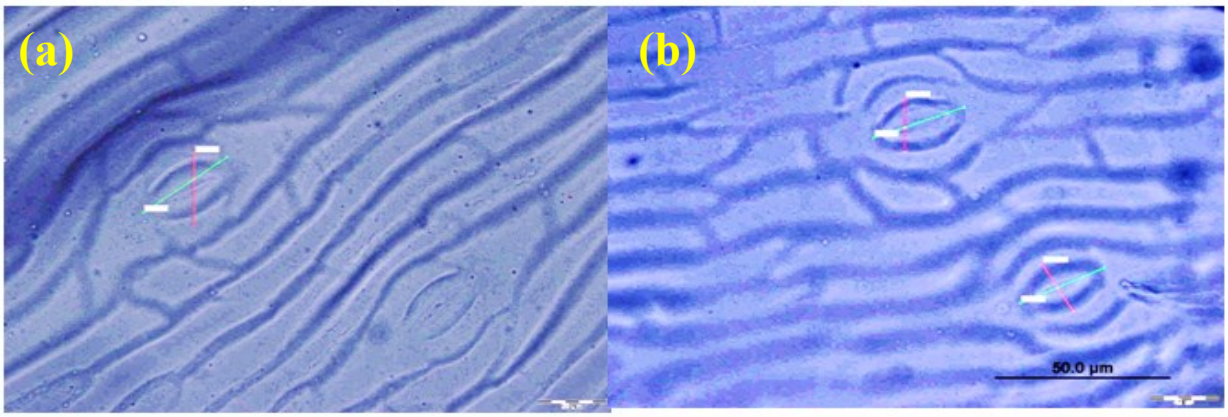

Figure 4. Length and width from leaf sample of polyploid oil palm plants from (a) treated zygotic embryo-derived shoot and (b) treated shoot tip.

zygotic embryo-derived shoots and 20.8 µm in shoot tip-derived from the treatment of colchicine at 1 mg/L and amiprophos-methyl at 5 mg/L concentrations, respectively. The length and width of stomata of guard cell of the treated zygotic embryo-derived shoots was 7.4 and 6.7 µm respectively, higher than those of the control. In this results, leaves of shoot derived from colchicine treatment consistently has longer stomatal size of guard cells with the concentration of 1 mg/L and 100 mg/l in zygotic derived-shoots and shoot tips, respectively, compared to other treatments and control. The above result is visually seen in figures 3 and 4 which profoundly showed an increased in stomatal size in those treated oil palm plants.

The result above coincides with the previous reported studies of Cohen and Yao (1996); Sajjad et al., (2013) and Samala & Te-chato (2010). Polyploidy could be due to lack of normal chromatid separation since the agents employed inhibits spindle assembly. Chromatids fail to move to the poles and eventually become enclosed in a new nuclear membrane and proceed into interphase as a doubled number of chromosomes (Hague and Jones 1987). On the other hand, the larger stomatal size observed among treated plants is similar to the study of Chen & Gao (2007) which revealed occurrence of polyploidy. Earlier studies of induced and spontaneous polyploid plants have frequently observed that the size and number of stomata and the number of chloroplasts within the guard cells change significantly in the event of chromosome doubling, compared with the diploid state (Nigel et al., 2007)

3.4 Chromosome Counting in Root tips

The results of the chromosome scoring of plantlets examined from untreated oil palm zygotic embryo showed two sets of chromosome (n = 32) while the same data in the treated zygotic embryo-derived shoot and shoot tips was polyploid (n= 42 - 48) (Fig. 5). Root tip samples examined from the colchicine treated oil palm plant has a higher increase in number of chromosomes in both the zygotic-derived shoots and shoot tips (Table 5). It is clearly shows that all these three anti-mitotic agents are potential to induce polyploids in oil palm.

To produce chromosome-doubled plants, colchicine treatment has mainly been used as an efficient strategy for various plant species. In fruit trees, colchicine has successfully been applied to produce polyploids in various crops such as grape

(Notsuka *et al.*, 2000), citrus (Gmitter and Ling, 1991), and loquat (Yahata *et al.*, 2004). In blueberries, several successful results of chromosome doubling have been reported with colchicine treatment. Other microtubule depolymerizing compounds, including dinitroaniline: oryzalin, trifluralin and more recently phosphoric amide, amiprophos-methyl (APM), have also been used on many plants at various concentrations. All these chemicals act by binding to the tubulin dimmers preventing the formation of microtubules, and consequently, spindle fibers during cell division (Petersen *et al.*, 2003).

Table 5 . The effect of anti-mitotic agents on polyploidy as showed in the number of chromosome analyzed from root samples of oil palm plants derived after treatment with anti-mitotic agents.

Treatment	**Number of Chromosomes**	
	Zygotic-derived shoots	**Shoot tip**
Control (½ MS)	32 b	32 b
Colchicine	45 a	48 a
Oryzalin	42 a	45 a
Amiprosphos-methyl	45 a	44 a

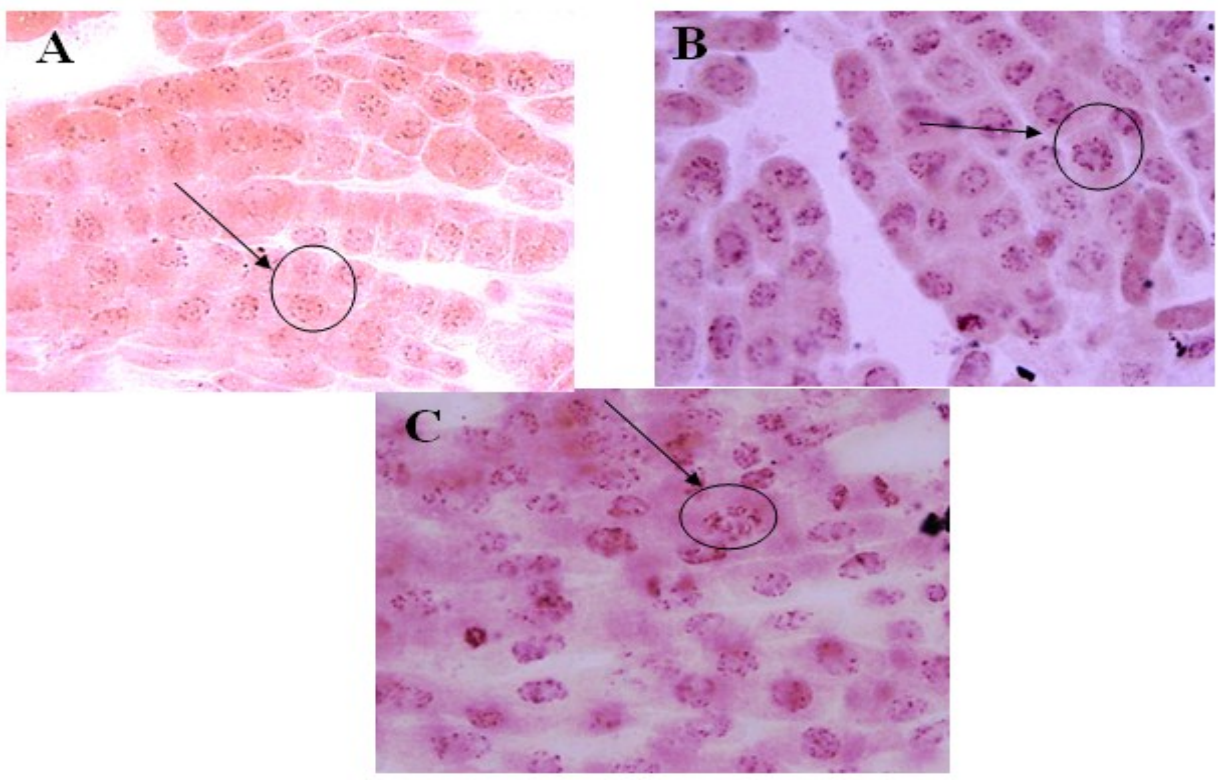

Figure 5. Number of chromosome from root samples of (A) untreated (B) treated shoot tip and (C) treated zygotic-derived shoot of oil palm plants.

4.0 Conclusion

The study revealed that colchicine, oryzalin and amiprophos-methyl are promising anti-mitotic chemicals that induced polyploidy in zygotic embryo of oil palm. Considerable morphological variation can be traced in physiological characteristics such as the size of stomata cell & chromosomal number.

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