

GROWTH AND MORPHOLOGICAL CHANGES AS AN EARLY INDICATION OF IN VITRO PLOIDIZATION OF TECTONA GRANDIS

*Respon Pertumbuhan dan Morfologi Planlet Jati (Tectona grandis) sebagai Deteksi Dini
poliploidi Pada Kultur In Vitro*

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Tanggal diterima: 3 November 2017; Tanggal direvisi: 19 Desember 2017;

Tanggal disetujui: 27 Desember 2017

ABSTRACT

Ploidization level estimation can be conducted in several methods, through morphological, growth response, anatomy, cytology, and molecular markers. The simplest and easiest methods are morphological marker and growth response. The study aimed to develop early detection method of polyploidy occurrence in in vitro *Tectona grandis* after treated by antimitotic agent colchicine. Nodal segments were immersed at 0, 15, and 30 μM colchicines for 5 days, then cultured for 8 weeks. Observations on plantlet height, number of leaves and morphology were performed at 2, 4, and 6 weeks after planting. Colchicine had high significant effect on the height and significant effect on leaves number. High concentration colchicine inhibited shoot elongation and leaves growth, however it increased morphological changes. The plantlets height of 0, 15, and 30 μM of colchicine treatment was 4.14; 3.82; 3.12 cm; while the number of leaves as much as 8.72; 8.4, and 7.5. Colchicine led to increase in morphological changes at the levels 0, 15, 30 μM were 26,60%; 46,66%; and 93,33%. Changes caused by polyploidy differ from media. Changes in polyploidy decreased the height, number of leaves, and induced morphological changes, whereas planting media resulted in vitrification. Response to colchicines in culture of *T. grandis* plantlet allows the growth and morphology to be a marker for early detection of polyploidization.

Key words: Colchicine, polyploidy detection, *tectona grandis*

ABSTRAK

*Penentuan tingkat ploidi dapat dilakukan dengan beberapa metode, antara lain: penanda morfologi, respon pertumbuhan, anatomi, sitologi, dan molekuler. Metode yang paling sederhana dan mudah dilakukan, terutama untuk deteksi dini yaitu dengan penanda morfologi dan respon pertumbuhan. Penelitian ini bertujuan untuk mengamati deteksi dini terjadinya poliploidisasi *Tectona grandis* akibat pemberian kolkisin pada kultur in vitro. Ruas nodus eksplan jati direndam dalam 3 konsentrasi kolkisin (0, 15, dan 30 μM) selama 5 hari, untuk selanjutnya dilakukan kultur in vitro selama 8 minggu. Pengamatan terhadap tinggi daun, jumlah daun dan morfologi daun dilakukan pada minggu ke-2, ke-4, dan ke-6 setelah tanam. Hasil penelitian menunjukkan pemberian kolkisin berpengaruh sangat nyata terhadap tinggi dan berpengaruh nyata terhadap jumlah daun. Pertumbuhan tinggi daun dan jumlah daun mengalami penurunan seiring dengan bertambahnya konsentrasi kolkisin. Tinggi planlet akibat perlakuan perendaman 0, 15, dan 30 μM kolkisin adalah 4,14; 3,82; dan 3,12 cm; sedangkan jumlah daun sebanyak 8,72; 8,4, dan 7,5. Peningkatan konsentrasi kolkisin menyebabkan peningkatan perubahan morfologi. Perubahan morfologi perlakuan kontrol, 15 dan 30 μM kolkisin sebesar 26,60%, 46,66%, dan 93,33%. Perubahan karena poliploidi berbeda dengan perubahan karena media. Perubahan akibat poliploidi menyebabkan perubahan pada tinggi planlet, jumlah daun, serta morfologi; sedangkan perubahan media tanam menyebabkan vitrifikasi. Adanya respon pemberian kolkisin pada kultur in vitro *T. grandis* memungkinkan pertumbuhan dan morfologi sebagai penanda untuk deteksi dini terjadinya poliploidi.*

Kata kunci: Deteksi poliploidi, kolkisin, *Tectona grandis*

I. INTRODUCTION

Teak (*Tectona grandis* L.f.) is a woody plant belonging to the Verbenaceae family that grows in tropical forests of India, Myanmar, Laos, Cambodia, Thailand, and Indonesia. In Indonesia teak grows well in Java, South Sulawesi, Southeast Sulawesi, Nusa Tenggara and Lampung (Martawijaya A, Kartasujana I, Kadir K, 2005).

Teak wood has a high economic value and is classified in the first to second quality classes or grades in term of strength and the first class in term of durability, so it is suitable for industrial raw materials such as: construction, furniture, carving, and various other functions (Martawijaya, Kartasujana, & Kadir, 2005).

The teak industry has a large market share, both domestic and international (Adinugraha & Mahfudz, 2014). According to Kollert & Cherubini (2012) the total production of natural teak wood and world crop was estimated to reach \pm 2-2.5 million m³, while Indonesia's production until recently still reaches 455.995 m³ or about 20% of total world production (Perum Perhutani, 2015). As one of the teak producing countries, this information is a challenge for Indonesia to be able to increase the total teak production, considering Indonesia has significant potential, both land resources and human resources that can be used as forestry development investment especially in timber sector.

Forestry development in the timber sector should be supported by the availability of qualified seed resources or certified seedlings. One technological breakthrough that can be used is the doubling of chromosomes (polyploidy) through in vitro cultures. Polyploidy can be generated by colchicine induction to change the number of sets of diploid teak chromosomes into polyploid. So it is expected to change the teak properties into

better quality to help increase the teak productivity and quality as industrial raw materials.

Various studies on the use of mutagenic agents, such as colchicine, oryzalin, tryfluralin, and etc, for polyploidy have been done in order to improve the quality and quantity of forest crops such as *Eucalyptus grandis* (Han, C., Xu, J.M., Du, Z.H., Li, G.Y., Zeng, B.S., Wu, S.J., & Wang, 2011), *Eucalyptus globulus* (Lin et al., 2010), *Aquilaria malaccensis* (Suhaila et al., 2015), *Acacia crassicaarpa* (Lam, Harbard, & Koutoulis, 2014), and *Acacia mangium* (Griffin, 2014).

The results obtained by (Han, C., Xu, J.M., Du, Z.H., Li, G.Y., Zeng, B.S., Wu, S.J., & Wang, (2011); Lin et al., (2010); (Suhaila et al., 2015); Lam et al. (2014) demonstrated that the use of antimicrobial colchicine led to polyploidy development characterized by changes in growth rates and morphological changes. So it can be presumed that there is a correlation between morphological changes and growth response with ploidy levels.

Polyploidy development can be detected through alteration of growth and morphology at an early state. Early detection can be performed by observing the symptoms and signs or characters of specific physical changes that occurs in plants due to exposure to certain antimutagenic agent. Early detection is a crucial stage since it has advantages, among other things, that can help early screening or selection process of a large population into smaller samples. That way, it can save time, money and energy.

Ploidy character can be recognized directly or indirectly through several methods, such as: morphological marker, growth response, anatomy, cytology and molecular. The simplest and easiest method of early detection through indirect prediction, are morphological marker and growth response. This research aimed to develop the early detection method of

polyploidy occurrence in teak plantlets *in vitro* via growth response and morphological changes due to the influence of colchicine.

II. METHODOLOGY

A. Time and location of research

This research was conducted in Plant Micropropagation Laboratory-BPPT, Building 630 Puspiptek Area, Kota Tangerang Selatan, Banten Province from September 2016 to March 2017.

B. Methods

1. Plant source and preparation of plantlets

Plant materials of nodal segment explants were obtained from 2-year-old *T. grandis* mother plant collection of Muna Island accession. The mother plant was generated from an ex vitro technique and maintained in the mother plant collection room of the Plant Micropropagation of Laboratory-BPPT. A one cm-long nodal segment was excised and sterilized before cultured in a regeneration medium. The regeneration medium used referred to (Srinivasan, R., Selvan, G.G., Karthikeyan, K., Chandran, C., Kulothungan, S., Govindasamy, 2012). After 4 weeks, 8 – 10 cm high shoots were harvested for studying the response of *T. grandis* L.f. to different colchicine concentrations.

2. Colchicine preparation and induction of polyploidy

Initially, colchicine solution was prepared by making a stock solution. The stock solution of 100 ppm concentration was prepared by weighing 2.5 mg colchicines powder. To enhance penetration of the colchicine solution into plant tissue, 2-3 drops of DMSO (Dimethyl Sulfide Oxide) was added into

the solution, then filter-sterilized using micro filter and added with sterile aquades up to 25 ml volume. The use of colchicine for the treatment of polyploidy induction was by firstly converting the colchicine stock from ppm concentration to μM unit in accordance with the treatment to be used, concentration 0, 15 and 30 μM .

Polyploidy treatments were performed by taking sterile buds produced by in vitro multiplication as planting material. Shoots used were composed of one nodal segment and 1 cm in length by removing all parts of the leaves and cultured on the regeneration media. Induction of mutation was done by immersing whole teak shoots in 0, 15, and 30 μM colchicine solution for 5 days. Subsequently, the culture was washed with sterile aquades and sub-cultured into the regeneration medium. The culture was then maintained for 8 weeks.

3. Experimental design

The experimental design used in this study was Completely Randomized Design with one factor, i.e the concentration of colchicine. Colchicine concentrations were: 0, 15 and 30 μM . Each treatment was repeated four times, whereas one replication consisted of five teak shoots planted in test tube that already contained the regeneration medium.

4. Observation and data analysis

Observation was carried out weekly for six weeks. Statistical analysis was performed using the analysis of variance (ANOVA) and Duncan Multiple Range Test at α level of 0.05 supported by the software SAS version 9.3. Parameters of observation in this study were plantlets height, number of leaves and type of morphological changes on the leaves and stems appearing during the observation. Treatment applied in the study can be seen in Table 1.

III. RESULTS AND DISCUSSION

A. Results

Based on ANOVA in general, the concentration of colchicines resulted in highly significant effect on the parameters of plantlets height and number of leaves at 2, 4 and 6 weeks after treatment (Table 2).

1. Height increase of plantlets

Figure 1 shows the result of colchicines treatments on height of plantlets. The colchicine significantly reduced the height of the *T. grandis* plantlets at 2, 4 and 6 weeks after treatment. At week 2 the growth of treatment K15 (15 µM) and K30 (30 µM) were significantly reduced compared to the control treatment. The height of the

control plantlet was 0.36 cm, whereas those of K15 and K30 were 0.23 cm and 0.14 cm, respectively.

At the 4th and 6th weeks all the treated plantlets experienced a significant decrease in growth. The treatments of K15 and K30 always experienced a lower growth rate than the control treatment. The height of plantlet on treatment of K15 increased in 4th week 4 to 6th from 1.71 cm to 3.82 cm, with an increase of 2.11 cm, while for treatment of K30 it happened from 1.29 to 3.12 cm, with an increase of 1.82 cm. The increase of plant height in the control treatment was from 2.32 to 4.44 cm, increased by 2.12 cm. So the K30 treatment experienced the lowest growth increase when compared with control treatment and K15. The height increase of the whole plantlets is shown in Figure 1.

Table (Tabel) 1. The treatments of colchicine (*Perlakuan konsentrasi kolkisin*)

Antimitotic Agents (<i>Agen antimiotik</i>)	Treatments (<i>Perlakuan</i>)		Number of Repetition (<i>Jumlah pengulangan</i>)	Number of unit (<i>Jumlah satuan</i>)	Total
	Concentrations (<i>Konsentrasi</i>) (µM)	Soaking times days (<i>Waktu perendaman</i>)			
Control	0 (K0)	5	4	5	20
Colchicine	15 (K15)	5	4	5	20
Colchicine	30 (K30)	5	4	5	20
Total of unit (<i>Total satuan percobaan</i>)					60

Table (Tabel) 2. The varians analysis of colchicine influence for height and leaves number on 2, 4 and 6 week after treatments (*Hasil analisis ragam pengaruh kolkisin terhadap tinggi dan jumlah daun pada 2, 4 dan 6 MST*)

No	Parametre (<i>Parameter</i>)	Week (<i>Minggu ke-</i>)		
		2	4	6
1	Height (<i>Tinggi</i>)	0.0069**	0.0005**	0.0031**
2	Leaves number (<i>Jumlah daun</i>)	0.050*	0.050*	0.041*

Description (*Keterangan*): ** = Highly significant (*Sangat nyata*)

* = Significant (*Nyata*)

Tn = Not significant (*Tidak nyata*)

MST = Week after treatment (*Minggu setelah tanam*)

2. Amount of leaves

The colchicine induction treatment resulted in various responses to the amount of *T. grandis* leaves at 2, 4 and 6 weeks after planting. The antimitotic of colchicine at the concentrations of 15 and 30 μM significantly decreased the number of leaves (Figure 2). Concentrations of 15 and 30 μM caused a lower number of leaves growth responses although not always significantly different from the control treatment. Therefore, the addition

of the 15 and 30 μM colchicine may inhibit the growth of leaves.

Two week after treatment, the control, K15, and K30 treatments produced 2.75, 1.9, and 0.90 amount of leaves, respectively. The treatment of K15 had slightly lower amount of leaves than control. Conversely the treatment of K30 had the lowest leaf number and was significantly different when compared with other treatments. The 4th week had the same phenomena with the 2nd week.

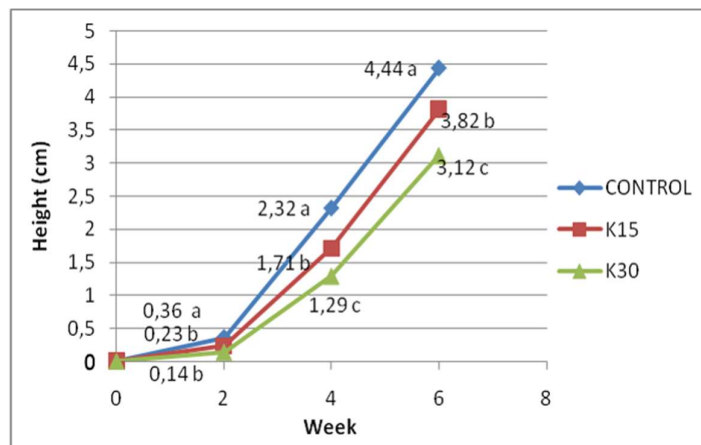


Figure (Gambar) 1. The average growth of *Tectona grandis* planlet at 2, 4 and 6 week after treatment (WAT) (*Rata-rata pertumbuhan T.grandis pada 2, 4 dan 6 minggu setelah perlakuan*). K15T5: Kolkisin 15 μM , T5: immersion time 5 days. K30T5: colchicine 30 μM , T5: immersion time 5 days.

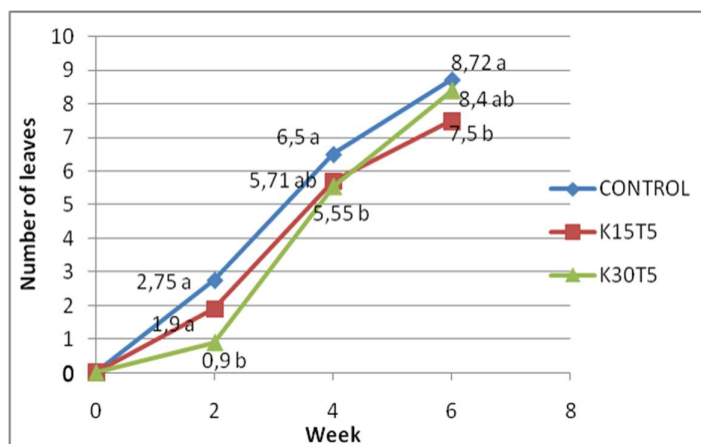


Figure (Gambar) 2. The average decrease of number of leaves of *T. grandis* at 2nd, 4th and 6th week after treatment (WAT) (*Rata-rata penurunan jumlah daun T. grandis pada 2, 4 dan 6 minggu setelah perlakuan*). K15T5: Kolkisin 15 μM , T5: immersion time 5 days. K30T5: colchicine 30 μM , T5: immersion time 5 days.

At the 6th week, treatment of K15 resulted in the lowest amount of leaves when compared with control and K30 treatment. There was a significant decrease in amount of leaves on the K15 treatment. The average number of leaves on treatment of K30 was increased significantly from 4th to 6th week, which was from 5.55 to 8.40. It seems that at a concentration of 30 μ M some plantlets mutated and changed the leaf number from 2 sets to 3 sets or more (as shown in Figure 5D). The average number of leaves of all treatments is displayed in Figure 2.

The result of this study confirmed that colchicine could inhibit the height and amount of leaves of *Tectona grandis* plantlets. The higher the concentration of colchicine, the lower the height and number of leaves (Figure 3). The same results were obtained by Hui, Y., Li, Z., Shuhui, (2012) that colchicine treatment inhibited the regeneration of *Pinus bungeana* shoots, and the growth of shoot increased with the decrease of colchicine concentration. These results were also in agreement with the experiment recorded by Lam et al., (2014) that increasing the concentrations of colchicine would decrease the average height of *Acacia crassicarpa* A. Cunn. Ex Benth. Likewise, the seedlings produced were shorter than that of control diploid.

3. Change of plantlets morphology

Morphological changes of teak plantlets in response to colchicine induction treatments can be seen in Table 3 below.

B. Discussion

1. Growth response

The plant growth and number of leaves inhibition is presumably due to effect of mitotic agents which can cause cellular and meristematic tissue damage. According to Suryo, (2007) cell division may be inhibited, which is triggered by the number of chromosomes doubling due to mitotic agents. Chromosomes doubling or

polyploidy increased the complication during pairing process (Syukur, M., & Sastrosumarjo, 2013). For diploid cell during the mitosis and meiosis, the process involves the pairing of homologous chromosome prior to cytokinesis. In polyploidy cell the pairing processes takes longer time than diploid chromosomes, because there are more than two chromosomes for one set. Furthermore, Sastrodumarjo et al. (2013) stated that there is a mechanism that coordinates the pairing along the whole chromosome through similarity of DNA sequences across chromosomal, so, its impact of slowing the pairing process can directly decrease of plant tissue growth.

2. Change of plantlets morphology

Colchicine significantly influenced morphological changes of *T. grandis* plantlet at the 2nd, 4th and 6th week after treatment. Increasing concentration of colchicine resulted in raise of the morphological changes of plantlet. The morphological changes appeared at the 2nd week after treatments observed in the K15 treatment 13.33% of the plantlets had incomplete dissection characteristic with two sets of leaves attached together (Figure 4B). Significant morphological changes was observed at the 4th week after treatment on treatment K15 and K30 which the proportion was 46.6 and 73.33%, respectively. Furthermore at the 6th week, the treatment of K30 had very intensive morphological change i.e. up to 93.33%. Morphological changes observed in the treatment of K15 and K30 were modification of stems, leaves and growth types. The change in the shape of the stems was caused by the emergence of new branches that bends the stems, whereas on control plantlets no branches were found. The leaves were observed on the change of shape, edge, position, thickness, color, and size. Whereas the growth type changed was the dwarf plantlets.

Table (Tabel) 3. Comparison the morphological changes of *T. grandis* plantlets on 2nd, 4th, and 6th weeks after treatments (Perbandingan perubahan morfologi planlet *T. grandis* pada 2, 4 dan 6 minggu setelah perlakuan)

No	Treatments (Perlakuan)	(%) Morphological changes 2 th Week (Perubahan morfologi pada minggu ke-2)	(%) Morphological changes 4 th Week (Perubahan morfologi pada minggu ke-4)	(%) Morphological changes 6 th Week (Perubahan morfologi pada minggu ke-6)	Descriptions (Keterangan)
1	Control	0.00 (0/15)	20.00 (3/15)	26.60 (4/15)	Week 2, buds have not developed and no leaves Week 4, intermittent leaves Week 6, intermittent with 2 sets of leaves
2	K15	13.33 (2/15)	46.66 (7/15)	46.66 (7/15)	Week 2, two sets of fused leaves Week 4, round leaves, large jagged edges, intermittent leaves, 2 sets of fused leaves. Week 6, round leaves, jagged edges, 3 sets of opposite leaves, intermittent leaves, 2 sets of fused leaves
3	K30	0.00 (0/15)	73.33 (11/15)	93.33 (14/15)	Week 2, buds have not growth, no leaves Week 4, round leaves, intermittent leaves, leafy edges, large jagged leaves, split leaf tips, 4 sets of opposite leaves, chimera Week 6, round leaves, intermittent leaf, leafy edges, large jagged edges, split leaves tips, 4 sets of opposite leaves, chimera

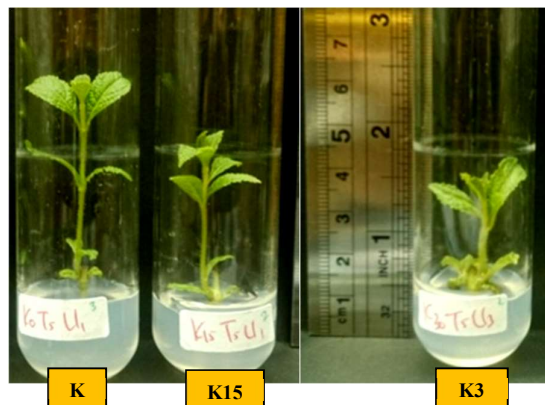


Figure (Gambar) 3. Colchicine influence on height of *T. grandis* plantlets on 4th WAT (Pengaruh kolkisin terhadap tinggi planlet *T. grandis* pada 4 minggu setelah perlakuan)

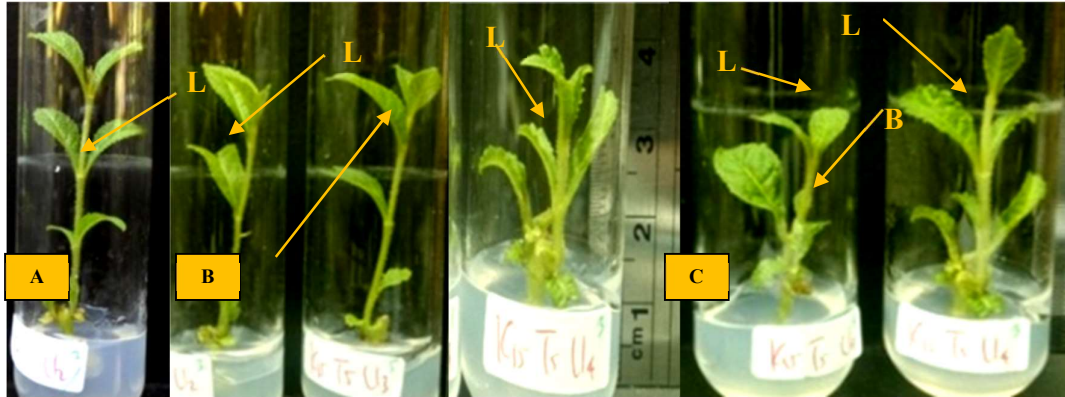


Figure (Gambar) 4. Normal leaves shape and position (A), intermittent leaves position and split end (B), branched stem, thick and jagged leaves edge (C). L: Leaves, B: Branches (*Bentuk dan posisi daun normal (A), posisi daun berselang dan ujung daun terbelah (B), batang bercabang tepi daun beringgit besar (C). L: Daun, B: cabang*)

The results obtained in this study are in agreement with those reported previously by Lin et al. (2010), that increasing the colchicine concentration and soaking time increased the average number of mutants of *E. globulus* Labill *in vitro*. Similar findings were obtained by Lam et al. (2014) which generated high frequency of abnormalities seedling of *A. crassicarpa* A. Cunn. Ex Benth as much as 55% and 57%, resulted from the immersion of colchicine and oryzalin, respectively. These treatments resulted in abnormalities on leaves and filodia (false leaves).

One of the characteristic of morphological changes on leaves was its shape. The leaves shape was changed from lancet to rounded leaves, with a low ratio of leaves width to leaves length. Additionally, the leaves were thicker, rougher, darker in color, and with thicker stem (Figure 4E). Our results were in accordance with results described by (Griffin, 2014) that the polyploids *Acacia* plants have wider leaves surface compared to diploid *Acacia*. The same results were reported by Lin et al. (2010) on *in vitro* polyploids *E. globulus* plants which had thicker, wider and darker green

leaves. Similarly, the polyploid plants of rose showed thicker and darker green leaves than the control diploid plants (Kermani Sarasan, Roberts, Yokoya, Wentworth Sieber., 2003). Similarly of Griffin (2014), tetraploids *A. mangium* produced gigantism effect. The results showed that treated plants had 21% rougher and thicker skin, 20% thicker polyads, 28% longer wood fibers, 17% thicker leaves, 12% wider leaves, 24.3 μ m longer stomata than that of diploid *A. mangium*. Further results by Suhaila et al. (2015) on *A. malaccensis* plant showed that tetraploid plants underwent some increase in the essential oil content such as β -patchoulene, β -elemente, longifolene, β -cedrene, Didehydro-cycloisolongifolene which was higher than that of its diploid counterpart.

According to Zulkarnain, (2009) clonal propagation through *in vitro* culture can cause temporary mutation. One of the indications of somaclonal changes that occur is the morphological changes due to the interaction of physiological components (cells and tissues) with the culture environment during the process of *in vitro* culture. These changes are results of long-term exposure to chemicals and

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growth regulators contained in culture media. These phenomena were observed on the control plantlets that resulted in 26.66% morphological change, for example the position of sets of intermittent leaves. Such morphological changes were not permanent. As the plantlets grew, the characters exhibiting abnormalities turned back to normal after several weeks, indicated by the appearance of a new leaves set.

The use of antimetabolic agents (colchicine) is presumed as a factor causing permanent morphological changes on plantlets. Colchicine as a mutagen is toxic and highly destructive (Lin et al., 2010b). This mutagen is physically and chemically penetrating into the cell nucleus which affects and inhibit microtubule organizing center (MTOC). This inhibition presumably resulted in abnormal changes in plantlets morphology (Figures 4, 5, 6 & 7).

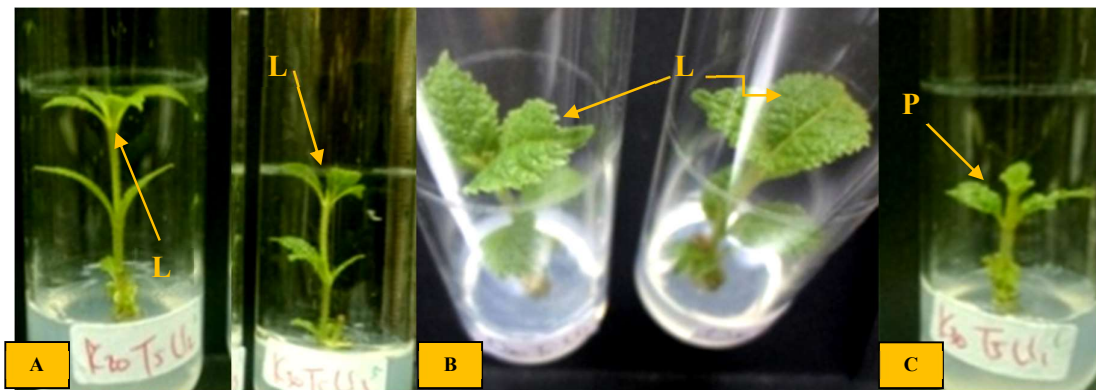


Figure (Gambar) 5. The 3rd and 4th position set of leaves facing each other (A), rounded and wavy leaves end with stiff and rough surface (B), dwarf plantlets (C). L: Leaves, P: Planlet (3 – 4 posisi daun menyatu (A), tepi daun berombak (B), planlet tumbuh kerdil. L: Daun, P: Planlet)



Figure (Gambar) 6 Several changes in leaves morphology from normal (K0) to abnormal (K15 & K30) (read: from left above to right below). N: Normal (Beberapa perubahan morfologi daun dari normal menjadi tidak normal (K15 & K30) (baca: dari kiri ke kanan). N: Normal)



Figure (Gambar) 7 Change of leaves position and two sets of leaves merged into one set (A), change of branching shape (B). L: Leaves, B: Branches (*Perubahan posisi daun dan 2 set bergabung menjadi (A), Batang bercabang (B). L: Daun, B: Cabang*)

3. Early indication of polyploidy

The treatment of K30 (30 μM) resulted in the most significant growth and morphological alterations of *T. grandis* plantlets. These alterations were thought to be associated with changes in the ploidy level of plantlets. This finding was supported by the results of several previous studies regarding the effects of polyploidy induction in plants. Viehmannová, Trávníčková, Špatenková, Černá, Trávníček, (2012) described that morphologically the growth of polyploid *U. tuberosus* plants was slower and shorter than diploid plants. In addition the morphology of leaves had smaller surface area. Dunn, (2007) did an experiment on Buddleja plant and reported the same response, which changed in leaves size, leaves color, stem thickness and increase in pollen production as an early indicator of polyploidization. Moreover, tetraploid and octaploid *A. crassicaarpa* were shorter than their diploid counterparts (Lam et al., 2014). Other characteristics phenomena occurring in polyploid plants were large nuclei and cell contents, increase in the

size of leaves and flowers, and an increase and difference in chemical constituents including carbohydrates, proteins, vitamins and alkaloids (Poespodarsono S, 1988).

From the polyploid characteristics described above, we assumed that the *T. grandis* plantlets resulted from our study has changed from diploid to polyploid. These changes were indicated by decrease of the height of plantlets, reduced the amount of leaves and the occurrence of morphological changes on plantlets. To prove the occurrence of polyploidization we need further verification by using the methods of anatomical, cytological, or molecular markers. This method can be used for an early detection of polyploidy in a quick, easy, and practical way to screen for large numbers of plantlets.

IV. CONCLUSION AND SUGGESTION

A. Conclusion

Raising the concentration of colchicine resulted in the decreasing of the

height of plantlets, smaller number of leaves and the increase of morphological changes on plantlets. As for plantlets morphology, the accumulation changes in control were 26.60%, and then increased in K15 treatment for 46.66% and K30 for 93.33%. Based on the changes observed in plantlets growth and morphology, we may identify the polyploidization of *T. grandis*. The growth and morphological alteration data can be used for early detection of polyploidy in *T. grandis*.

B. Suggestion

Further testing was required using anatomical and cytologic markers to reinforce the direct prediction of (*T. grandis*) polyploidy.

KNOWLEDGEMENT

Thanks to the Agency for Assessment and Application of Technology who has facilitated and funded this research.

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