THE EFFECTIVENESS OF COLCHICINE AND ORYZALIN ON POLYPLOIDY INDUCTION IN TEAK (*Tectona grandis* Linn. f.) IN VITRO

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THE EFFECTIVENESS OF COLCHICINE AND ORYZALIN ON POLYPLOIDY INDUCTION IN TEAK (*Tectona grandis* Linn. f.) IN VITRO. The Indonesian government has launched the rehabilitation of a community plantation forest program in the entire country that would be beneficial for remedying the shortage in domestic demand for teak wood every year. This program needs to be supported by the availability of quality seed resources and quality seedlings utilizing polyploid teak (*Tectona grandis*). Our study aimed to examine the effectiveness of colchicine and oryzalin to modify diploid into polyploid *T. grandis* based on growth response, morphological, anatomical, and cytological alteration, as well as the acclimatization ability of polyploid plantlets. The materials used were aseptic lateral shoots consisting of nodal segments immersed in antimitotic agents at the concentrations of 0, 15, and 30 µM for 5 days, then cultured on regeneration medium until the 8th week and followed by acclimatization. The results showed that colchicine at a concentration of 30 µM was the most effective to induce polyploidy of plantlets in the parameter of high growth rate, length of internodes, number of leaf plantlets, leaf surface area, and significant chlorophyll index content compared to the control. Anatomical analysis of polyploidy was characterized by increasing leaf thickness, stomata size, decreased stomatal density, and increased chloroplast content in guard cells. Based on the cytological examination of polyploidy plantlets, there was an increase in the number of chromosomes in the cell nucleus. The acclimatization of polyploid plantlets successfully induced rooting and 100% survival rate of grown plantlets. Polyploid seedlings were able to grow and well adapted to the environment condition of acclimatization.

Keywords: Acclimatization, chromosome number, polyploidy, Tectona grandis, tetraploidy

*EFEKTIVITAS KOLKISIN DAN ORYZALIN UNTUK INDUKSI POLIPLOIDI TANAMAN JATI (*Tectona grandis *Linn. f.) PADA KULTUR IN VITRO. Pemerintah Indonesia telah mencanangkan program rehabilitasi hutan tanaman rakyat di seluruh pelosok negeri yang dapat dimanfaatkan untuk pembangunan hutan berbasiskan kayu pertukangan sehingga dapat menambah suplai kayu jati atas permintaan tinggi industri setiap tahun. Program ini harus didukung tersedianya sumberdaya benih yang bermutu dan bibit yang berkualitas seperti jati (*Tectona grandis*) poliploid. Penelitian ini bertujuan untuk menguji efektivitas kolkisin dan oryzalin dalam mengubah jati diploid menjadi poliploid berdasarkan respon pertumbuhan, perubahan morfologi, anatomi dan sitologi serta kemampuannya dalam aklimatisasi. Bahan yang digunakan adalah pucuk lateral aseptik yang terdiri dari ruas nodul yang direndam dalam agen antimitotik pada konsentrasi 0, 15 dan 30 µM selama 5 hari, kemudian dikultur pada media regenerasi sampai berumur 8 minggu dan dilanjutkan untuk aklimatisasi. Hasil penelitian menunjukkan bahwa konsentrasi kolkisin 30 µM merupakan konsentrasi yang efektif untuk mengubah tingkat ploidi planlet jati berdasarkan parameter laju pertumbuhan tinggi, panjang ruas, jumlah planlet daun dan peningkatan luas permukaan daun serta jumlah klorofil yang signifikan dibandingkan dengan kontrol. Analisis anatomi poliploid ditandai dengan meningkatnya ketebalan daun, ukuran stomata, penurunan kerapatan stomata, dan peningkatan kandungan kloroplas pada sel penjaga. Berdasasarkan hasil uji sitologi planlet poliploid diperoleh*

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peningkatan jumlah kromosom pada sel inti. Aklimatisasi planlet jati poliploid berhasil menginduksi akar dan persentase hidup planlet yang tumbuh mencapai 100%. Bibit poliploid mampu tumbuh dan beradaptasi dengan baik pada kondisi lingkungan aklimatisasi.

Kata kunci: Aklimatisasi, jumlah kromosom, poliploidi, Tectona grandis, tetraploidi

I. INTRODUCTION

Teak (*Tectona grandis* Linn. f.) is one of the important traded woody plant commodities in Indonesia and the world. The teak wood industry has a wide market share, both in the home country and abroad. Yet, the world teak wood production is much lower compared to its demand. In 2019, teak wood still dominated the raw material for the national domestic industry, amounting to 500,000 m^3 (Kominfo Jatim, 2019). During the same period, the teak wood production of the state-owned forestry company, *Perum Perhutani*, only reached to 396,500 m^3 (BPS Statistics Indonesia, 2019).

In accordance with the above issues, the need for seeds to rehabilitate community forests, especially wood for woodworking, is also huge. According to the Directorate General of Watershed Management and Forest Reserve of the Ministry of Environment and Forestry of the Republic of Indonesia, the total area of community forests that have not been rehabilitated is 3.96 million hectares (Kementerian Lingkungan Hidup dan Kehutanan, 2020). One of the programs was on community plantation forests, which allow the community to have an access to forest resources, particularly for wood-working-based forest development. As one of the teak wood producing countries, this opportunity is a challenge for Indonesia to increase the total production of teak wood, bearing in mind that Indonesia has huge potential, both inland and human resources, which can be used as assets for forestry development. Forestry development, especially in the timber sector, must be supported by the availability of quality seeds and seedlings in adequate amounts and continuously.

According to Monteuuis (2000), cconventional teak breeding using seed is still hindered by a number of difficulties, including: very limited seed production capability, asynchronous flowering time, low germination rate, high individual seed variability, not being economically feasible since it requires a large area for development, and a long period of time for preparation. In addition, seed qualification is difficult to be standardized due to compatibility issues during breeding. Moreover, the problem for teak plantation development with low productivity because the seeds and seedlings used by the farmer are generally not qualified.

New approach on teak quality improvement was initiated using mutation breeding strategy. Gamma-ray irradiation was used on Muna accession, which resulted in 10 putative mutants with superior characters (Zanzibar, Bramasto, Sianturi, Yuniarti, & Desmiati, 2015), and induction of 84% genetic diversity in in vitro plantlets (Parloangan, 2017). Another approach for stimulating mutation was polyploidy induction via in vitro culture. Polyploidy is generally the direction of the effect mutation, which increased in size (Rezende, Suzigan, Amorim, & Moraes, 2020), so it has the advantage of being more controlled than irradiation. Polyploidy can be induced by antimitotic agents on the organ, tissue or cells to obtain polyploid teak. So that it is expected to increase the character of teak to become superior, which can help improve the productivity and quality of teak wood as an industry related to bioresource material.

Various studies on the use of antimitotic agents for polyploidy induction have been done on many forestry plants. The purpose of those studies was to improve the quality and quantity

of plants and plant products, such as *Eucalyptus grandis* (Silva, Carvalho, & Clarindo, 2019), *E. dunnii* (Castillo, Lopez, Tavares, Santinaque, & Dalla Rizza, 2020), *Aquilaria malaccensis* (Siti-Suhaila et al., 2020), *Acacia mangium* (Griffin, 2014; Viet et al., 2020; Le et al., 2021), and *T. grandis* (Nugraha, 2012; Ridwan, Handayani, Riastiwi, & Witjaksono, 2018).

Polyploidy research conducted on the *A. mangium* tree showed positive responses. The tetraploid *A. mangium* tree has a gigantism effect and showed 21% coarser and thicker bark, 20% thicker polyads, 28% longer wood fiber, 17% thicker leaves, 12% wider leaves, and longer stomata (24.3 μm) compared to the diploid tree (Griffin, 2014). Likewise, the tetraploid tree produced a larger seed and a higher flowering capacity than the diploid tree (Le et al., 2021). Additionally, da Silva Souza et al. (2021) reported that polyploid Eucalyptus clones derived from the crosses of *E. grandis* × *E. urophylla* produced a longer and thicker cell wall of wood fibers than the diploids, thus increasing the fiber strength of the paper products.

In our study, antimitotic agents, i.e. colchicine and oryzalin, were induced into the in vitro culture of *T. grandis* using sterile apical meristems. Colchicine is a toxic chemical extracted from the seeds, tubers, and flowers of *Colchicum autumnale* L., which grows in Europe and the United Kingdom. The seeds and tubers contain 0.2–0.8% and 0.1–0.5% colchicine, respectively, and a small amount is found in the flowers (Lin et al., 2020). Oryzalin is a dinitroaniline herbicide that has the same performance as colchicine (Chen, Yu, Patterson, Sayer, & Powles, 2021) and is often used as an alternative to colchicine. Both antimitotic agents act as metaphase inhibitors. During metaphase, the microtubule spindle emerges from the microtubule organizing center (spindle). This spindle, composed of a- and b-tubulin dimers, is important for the migration of chromosome poles during anaphase. Therefore, inhibition of chromosome segregation results in a cell with a complete double chromosome, which is called polyploidy. Polyploidy has many consequences for plant growth and development and is applied in plant breeding (Iannicelli & Escandon, 2022).

The benefits of this research were to produce new mutants with high quality of performance, high yields, and adaptation to the environment to help increase the productivity of *T. grandis*. The purpose of our study was to examine the effectiveness level of antimitotic agent concentrations to achieve the precise dose for increasing the ploidy of *T. grandis* plantlets in in vitro culture and obtaining polyploid *T. grandis* seedlings after acclimatization.

II. MATERIAL AND METHOD

A. Place and Time of Experiment

The study was carried out at the Plant Micropropagation Laboratory, Laboratory for Biotechnology, BRIN, the Science and Technology Area of BJ Habibie, South Tangerang City, Banten, and the Micro Technical Laboratory, Department of Agronomy and Horticulture, Faculty of Agriculture, IPB University, Darmaga Campus, West Java. The study was conducted from January 2018 to June 2019.

B. Experimental Procedure

Source of explants and plant preparation

The source of the explants was obtained from the 2-year-old T. grandis mother plant of Muna accession planted in the mother plant collection park. The in vitro plants were initiated by culturing the axillary buds in the regeneration medium and multiplied until 8 weeks old with a height of 8 to 10 cm (Fauzan, Supriyanto, & Tajuddin, 2017a). Then, the shoots were cut to a size of 1 cm length for mutation induction. The shoots were planted on MS0 media and then immersed in antimitotic agents according to the treatment doses for 5 days. Subsequently, the shoot meristems were transferred into Murashige & Skoog regeneration media with the addition of BA (6-benzyladenin) and kinetin 0.5 mg/L each. The regeneration media used refers to Aguilar, Garita, Kim, Kim, and Moon (2019.

Antimitotic preparations and polyploidy induction

The preparation of sterile antimitotic solutions, colchicine and oryzalin, was done by making a 100 ppm stock solution. Antimitotic agent treatment was accomplished with concentrations of 0 (as a control), 15, and 30 μM. Induction of polyploidy was performed on the axillary buds consisting of 1 cm nodes and planted on the regeneration medium, followed by soaking in the antimitotic agents for 5 days (Fauzan, Supriyanto, & Tajuddin, 2017b). The experimental design in completely randomized design with 20 replicates for each treatment was used (Table 1).

Table 1. The experimental design for colchicine and oryzalin treatments

No	Treatments	
	Antimitotic Agent	Concentration (μM)
1.	Control	0 (AM0)
2.	Oryzalin	15 (AM1)
3.	Oryzalin	30 (AM2)
4.	Colchicine	15 (AM3)
5.	Colchicine	30 (AM4)

Acclimatization of Polyploid Teak

Acclimatization was carried out in a greenhouse with an average daily temperature of 24.9 – 32.1°C, a humidity of 71.5 – 77.6% and a light intensity of 533.16 – 2664.58 lux. The acclimatization phase was initiated with the process of hardening off the *T. grandis* plantlets in the greenhouse condition for a week. The plantlets were washed in running tap water and soaked in a 2 g/L bactericide and fungicide mixture solution for 10 minutes, then dried at room temperature. Root induction was done by the ex vitro technique, referring to Badilla, Xavier, Murillo, and de Paiva (2017). Plantlets were then planted on fine husk charcoal media and incubated in a plastic hoop house under 75% screen net shade until the plantlets induced roots and grew well. Subsequently, the plants were transferred into a plastic hoop house under the shade of a 55% double-screen net for development and adaptation. For maintenance, watering was carried out using clean water every two days so that the soil did not dry out.

C. Observation and Data Analysis

Polyploidy Induction

Observations were performed once a week for six weeks. The parameters observed consisted of 3 categories, those were growth, anatomical, and cytological analysis. The growth parameters were measured on height, number of leaves, length of internodes, leaf area, and chlorophyll content index. The plant height, number of leaves, and length of internodes were measured directly using a ruler. The leaf area was measured using the Image-J program. The chlorophyll content index was measured using the chlorophyll meter type CCM-200 plus (Apogee).

The anatomical analysis included stomata density and the number of chloroplasts in the stomata cell. Stomata density and chloroplast anatomical tests were referred to the method of Baker, Yarkhunova, Vidal, Ewers, and Weinig (2017). Observation of stomata density was performed three times in randomly selected fields of view, each covering an area of 0.19625 mm2. The stomata were then selected randomly to measure their length and width using a microscope type BX 51 (Olympus) and a DP 25 type camera with the DP2-BSW software (Olympus). Observation and calculation of chloroplasts were done directly on the stomata by selecting three stomata samples randomly.

Data analysis was accomplished using the following formulas:

1. Growth

$$Height\ (cm) = \frac{\sum observed\ plant\ height\ accumulation}{\sum\ total\ plant\ observed} \quad (1)$$

$$Length\ of\ internode\ (cm) = \frac{\sum the\ observed\ internode\ length\ accumulation}{\sum\ total\ plant\ observed} \quad (2)$$

$$Number\ of\ leaves\ = \frac{\sum the\ observed\ accumulation\ of\ number\ of\ leaves}{\sum\ total\ plant\ observed} \quad (3)$$

$$The\ wide\ of\ leaf\ area\ (cm2) = \frac{\sum the\ observed\ leaf\ area\ accumulation}{\sum\ total\ plant\ observed} \quad (4)$$

2. Anatomy

$$\text{The thick of leaf } (\mu m) = \frac{\sum \text{the observed leaf thickness accumulation}}{\sum \text{total plant observed}} \quad (5)$$

$$\text{The length of stomata } (\mu m) = \frac{\sum \text{observed plant stomata length accumulation}}{\sum \text{total plant observed}} \quad (6)$$

$$\text{The width of stomata } (\mu m) = \frac{\sum \text{observed plant stomata width accumulation}}{\sum \text{total plant observed}} \quad (7)$$

$$\text{Stomatal density } (n/mm^2) = \frac{\sum \text{stomata}}{\text{Wide field of view}} \quad (8)$$

$$\text{Number of chloroplasts per stomata} = \frac{\sum \text{chloroplasts in stomata}}{\text{Number of stomata observed}} \quad (9)$$

3. The Effectiveness of Ploidy and Cytology

The ploidy levels were analysed based on indirect estimation by comparing the amount of diploid control chloroplast plants with the putative polyploid plants (Robinson et al., 2018). Further analysis to confirm the occurrence of polyploidy was implemented by cytological analysis. The method of cytological analysis refers to the modified method of Zeng, Liu, Du, and Kang (2019).

$$\text{The percentage of diploid } (\%) = \frac{\sum \text{diploid plant observed}}{\sum \text{total plant observed}} \times 100\% \quad (10)$$

$$\text{The percentage of polyploid } (\%) = \frac{\sum \text{polyploid plant observed}}{\sum \text{total plant observed}} \times 100\% \quad (11)$$

4. Acclimatization

$$\text{The percentage of rooting } (\%) = \frac{\sum \text{rooting plant observed}}{\sum \text{total plant observed}} \times 100\% \quad (12)$$

$$\text{Percentage of survival } (\%) = \frac{\sum \text{life plant observed}}{\sum \text{total plant observed}} x\ 100\% \quad (13)$$

$$\text{Height } (cm) = \frac{\sum \text{the observed plant height accumulation}}{\sum \text{total plant observed}} \quad (14)$$

$$\text{Diameter } (cm) = \frac{\sum \text{the observed diameter accumulation}}{\sum \text{total plant observed}} \quad (15)$$

$$\text{Number of leaves} = \frac{\sum \text{the observed plant leaf accumulation}}{\sum \text{total plant observed}} \quad (16)$$

The experiment was designed as a single factor, completely randomized experimental design, which was the concentration of antimitotic agents.

D. Plant Growth Acclimatization Phase

Observation of growth parameters comprised of the number of life cultures, rooting, height of plant, number of leaves, and diameter of seedlings. The growth data were then statistically tested using the analysis of variance (ANOVA). Differences between treatments were determined by a Duncan multiple range test at the test level of $\alpha = 0.05$. Statistical analysis for observational data was carried out using the SAS program version 9.3.

III. RESULT AND DISCUSSION

A. Growth Response

The results showed that all explants survived and were regenerated. Figure 1 shows the process of mutation induction and plantlet regeneration *in vitro*.

Adding the antimitotic agents decreased the height and length of the internode of *T. grandis* plantlets and the number of leaves. On the contrary, these treatments increased the leaf surface area and chlorophyll content index of plantlets compared to control, as shown in Table 2. When the concentration of oryzalin increased from 15 to 30 µM, the height, internode length, and number of plantlets leaves decreased, from 3.37 to 2.92 cm, from 0.92 to 0.89 cm, and from 9.51 to 8.50 cm, respectively. However, these decreases were not significantly different. The treatment of oryzalin at 30 µM had the largest effect on the reduction of those parameters compared to the treatment of oryzalin at 15 µM and the control. The same phenomenon was seen in the effect of colchicine treatments on the plantlet height and the plantlet length of the internode. The higher the concentrations of colchicine, the shorter the height and internode length of plantlets (Table 2). The reduction in height and length might be associated with the polyploidy t stimulated by the antimitotic agents. According to Was et al. (2022), cell division was inhibited due to the doubled number of chromosomes. In some conditions, the chromosomes were multiplied or developed into polyploidy, increasing the level of complications in the chromosome pairing process (Syukur et al., 2019).

Comparing two treatments of colchicine and oryzalin at the same concentration of 15 and 30 µM, it showed that oryzalin has constantly lowered the average height and length of the

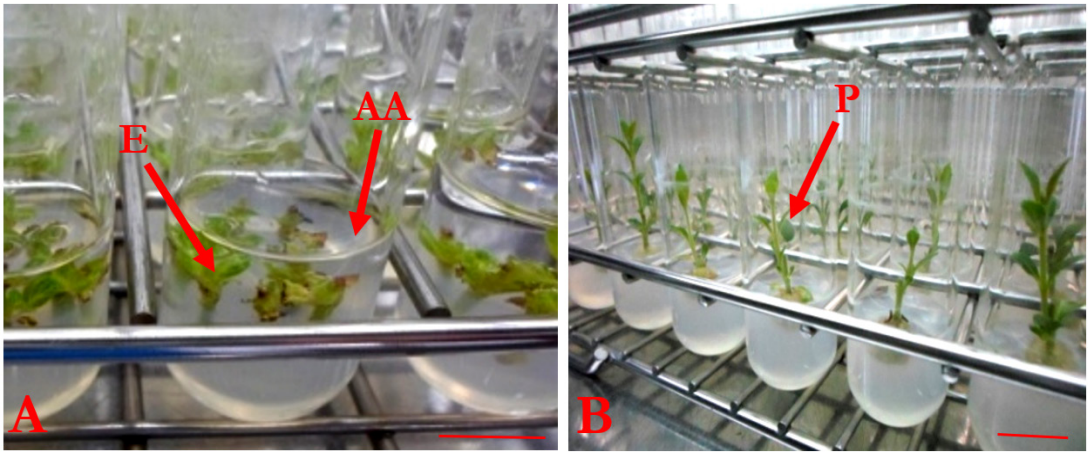

Figure 1. Explants on the in vitro media and immersing them in the antimitotic agents (A), regeneration of putative mutant shoots (B). AA: Antimitotic agent, E: Explants, P: Plantlets. Line Scale: 1 cm

Table 2. The growth response of plantlets *T. grandis* to various concentrations of antimitotic solution at 6 weeks after treatments

Treatment (µM)	Average Height (cm)	Average Length of Internode (cm)	Average Number of Leaves	Chlorophyll Content Index	Leaf Area (cm^2)
Control	5.06 a	1.17 a	10.58 a	7.37 c	1.03 b
Oryzalin 15	3.37 bc	0.92 b	9.51 ab	10.32 b	1.67 a
Oryzalin 30	2.92 c	0.89 b	8.50 b	10.74 ab	1.60 a
Colchicine 15	3.82 b	1.23 a	7.50 b	13.04 a	1.49 ab
Colchicine 30	3.13 bc	0.94 b	8.40 b	11.22 ab	1.79 a

Note: Numbers in the same column followed by the same letters are not significantly different based on the F-Test at a confidence level of 95%

internode. Nevertheless, both treatments were not significantly different on these parameters.

When the plantlets were subjected to 15 and 30 µM concentrations of oryzalin and colchicine, the results improved the chlorophyll content index and leaf area. The addition of oryzalin from the concentration of 15 to 30 µM showed an increase in chlorophyll content index; however, this did not occur in colchicine. Doubling the colchicine concentration from 15 to 30 µM increased the leaf surface area, yet decreased the chlorophyll content index, although not significantly different. It seems that an increase in the concentration of colchicine up to 30 µM damaged the chloroplast organelles, causing them to reduce the chlorophyll content (Cunha Neto et al., 2020; Kim et al., 2021). Adding colchicine at the concentrations of 15 and 30 µM, resulted a higher average of chlorophyll content index compared to oryzalin, though not widely different. Based on the parameters of growth responses in our study, it is assumed that oryzalin has a greater impact on the reduction of plantlet height, length of internode, and number of leaves than colchicine.

Table 2 also displays that increasing the antimitotic concentration was the cause of increasing the leaf surface area and the chlorophyll content of *T. grandis*. The use of colchicine and oryzalin at concentrations of 15 and 30 µM, resulted in a wider leaf surface (Figure 2) and higher chlorophyll content compared to the control. It is presumed that an increase in leaf surface area would increase the number of chloroplast organelles in the leaf cells. The more chloroplast organelles, the ability of the chloroplasts to produce chlorophyll pigments

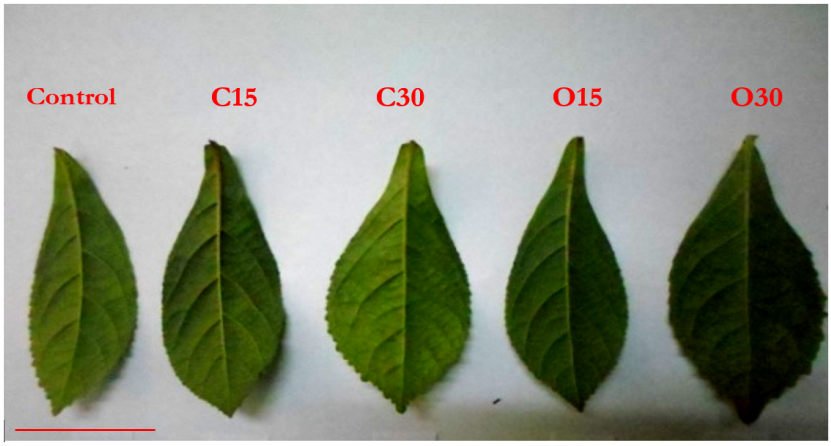

Figure 2. The leaf surface area at various treatments of control, colchicine (C15 and C30), oryzalin (O15 and O30). The leaves were obtained from the 7 days-old in vitro plantlets. Line Scale: 0.5 cm

Table 3. The anatomical response of *T. grandis* leaves at various concentrations of colchicine and oryzalin

Treatment (µM)	Leaf Thickness (µm)	Length of Stomata (µm)	Width of Stomata (µm)	Index (L/W Stomata)	Stomata density (per mm^2)	Number of Chloroplasts (per stomata)
Control	87.83 a	21.59 c	16.44 c	1.31 a	318.62 a	9.77 c
Oryzalin 15	93.43 a	23.97 bc	18.16 b	1.32 a	262.05 ab	11.37 bc
Oryzalin 30	102.81 a	27.82 b	19.62 b	1.42 a	202.03 cd	12.67 ab
Colchicine 15	99.77 a	24.85 bc	19.29 b	1.28 a	250.51 bc	10.88 bc
Colchicine 30	119.45 a	32.63 a	21.15 a	1.55 a	188.16 d	14.07 a

Note: Numbers in the same column followed by the same letters are not significantly different based on the F-Test at a confidence level of 95%

increased during the photosynthetic process, indirectly resulting in changes in leaf color that look darker green than the control. The result of our study coincided with that of Denaeghel et al. (2018). In their study on Escallonia illinita and *E. rubra*, they observed that one of the indicators of polyploidy was the increase in leaf area. Yan, Zhang and Zhang (2021) claimed that polyploid poplar (Populus euroamericana) had characteristics of increasing in leaf, thicker and rounder leaves, with a larger size of stomata and more of chloroplasts, but a lower stomatal index on abaxial leaf surfaces.

B. Anatomical and Cytological Responses

Anatomical and cytological analysis performed additional indirect and direct analysis of polyploid. The assessment was executed through observation of chloroplasts, stomata, and chromosome numbers. According to Manzoor, Ahmad, Bashir, Hafiz, and Silvestri, (2019), calculating the chloroplast content in stomata guard cells is an indirect method that has the advantage of being simple and fast for identifying ploidy levels. Furthermore, Tammu, Nuringtyas, and Daryono (2021) stated that the analysis has an accuracy rate of 90%. The results of Duncan's test on the anatomical response of leaves at various antimitotic concentrations are shown in Table 3.

It reveals that oryzalin and colchicine at concentrations of 15 and 30 µM increased the leaf thickness (Figure 3), length, and width of the stomata (Figure 4), as well as the number of chloroplasts in stomata guard cells (Table 3). Increasing the concentration of oryzalin and

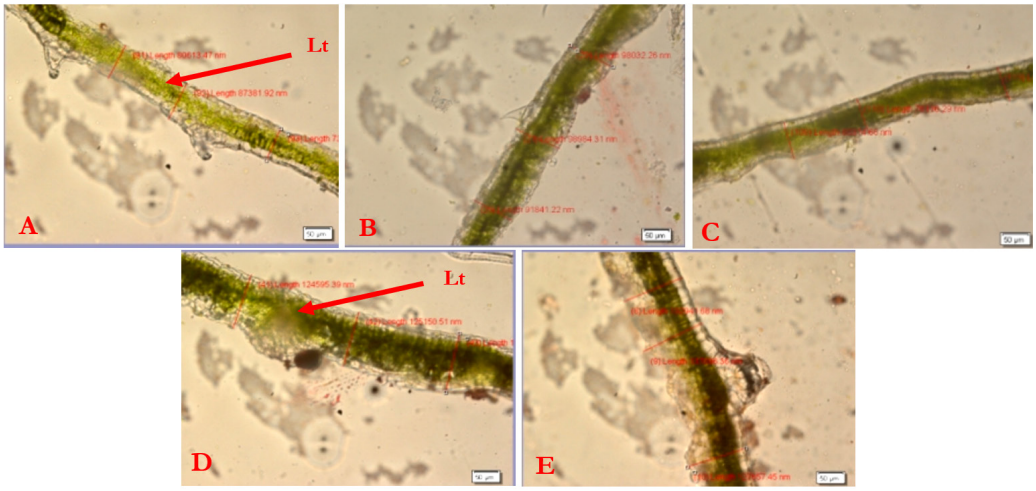

Figure 3. Anatomical leaf thickness of diploid and polyploid *T. grandis*. (A) Control, (B) Colchicine 15 µM, (C) Oryzalin 15 µM, (D) Colchicine 30 µM, (E) Oryzalin 30 µM.

Note: Lt=Leaf thickness. Line Scale: 50 µm

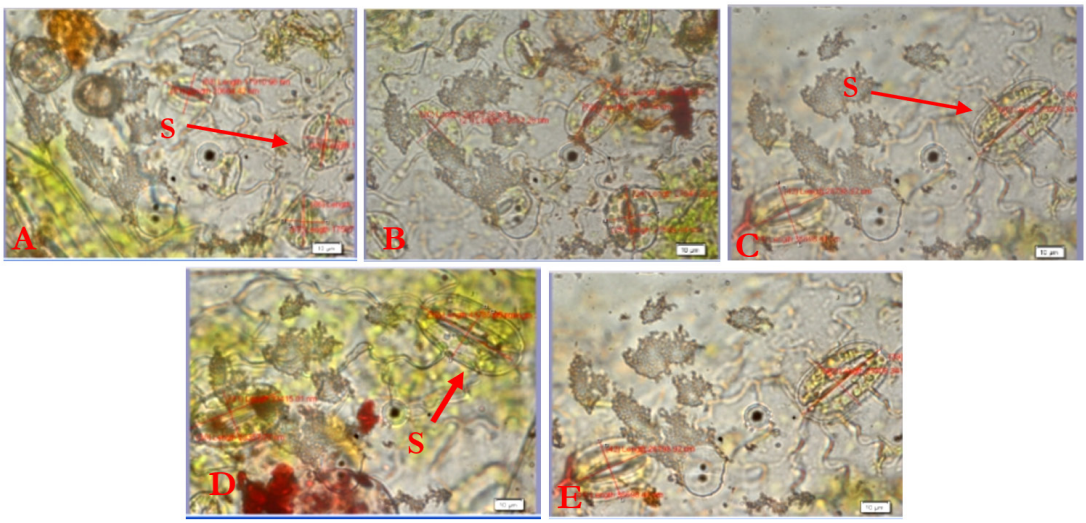

Figure 4. Leaf anatomical size of stomata of diploid and polyploid *T. grandis*. (A) Control, (B) Colchicine 15 µM, (C) Oryzalin 15 µM, (D) Colchicine 30 µM, (E) Oryzalin 30 µM.

Note: S=Stomata. Line Scale: 10 µm

colchicine from 15 to 30 µM tends to increase length and width of stomata considerably. Moreover, increasing the concentration from 15 to 30 µM also multiplied the significant number of chloroplasts, from 9.77 (control) up to 12.67 and 14.07, respectively. The concentration of oryzalin and colchicine at 30 µM influenced the alteration of the ploidy level from diploid to polyploid. These findings correspond to Oberprieler, Talianova, and Griesenbeck (2019) results on the Leucanthemum. In their experiment, the result showed that polyploid plants had more chloroplasts than diploids. The size of stomata is associated with the number of chloroplasts in stomatal guard cells. Increasing the number of chloroplasts per guard cell resulted in changes in the length and width of the stomata (Manzoor et al., 2019). Despite that, oryzalin and colchicine significantly reduced the stomata density per millimeter square (mm2) cell (Table 3). The stomatal L/W index shows the shape of the stomata, so the higher

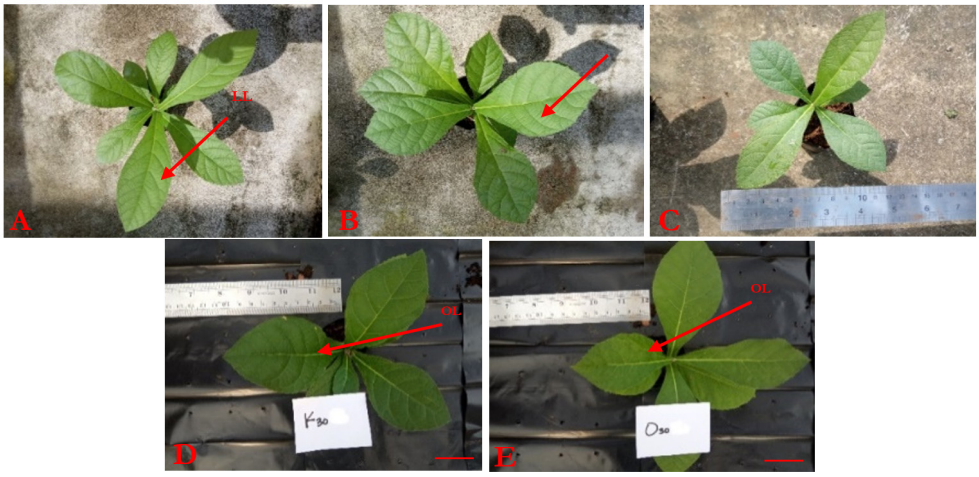

Figure 5. Leaf morphological performance of diploid and polyploid *T. grandis*. The growth of 3 months-old plants after acclimatization. (A) Control, (B) Colchicine 15 µM, (C) Oryzalin 15 µM, (D) Colchicine 30 µM, (E) Oryzalin 30 µM.

Note: LL=Lancet Leaf, OL=Obovate Leaf.

Table 4. The percentages of polyploidy rate in *T. grandis* culture *in vitro*

Treatment		Life Culture	(%) Ploidy	
Antimitotic agents	Concentration	(%)	Diploid	Polyploid
Control	0 µM	100	100 (9/9)	0 (0/9)
Oryzalin 15	15 µM	100	66.67 (6/9)	33.33 (3/9)
Oryzalin 30	30 µM	100	22.22 (2/9)	77.78 (7/9)
Colchicine 15	15 µM	100	55.56 (5/9)	44.44 (4/9)
Colchicine 30	30 µM	100	11.11 (1/9)	88.89 (8/9)

the stomata index, the more oval and larger the stomata shape. It is proven that increasing the concentration of oryzalin and colchicine can change the shape of the stomata from round to oval (Figure 4). Correspondingly, the shape of the leaves was also affected by the antimitotic treatments. The 30 µM concentration oryzalin and colchicine-treated *T. grandis* plants produced oval to obovate leaves, while the control plant had lancet-shaped leaves (Figure 5). The same phenomena were noticed on induced polyploidy plants, in which leaves were changed by the ploidy level (Manzoor et al., 2019; Wilson, Fradera-Soler, Summers, Sturrock, & Fleming, 2021).

The survival percentage and ploidy of T. grandis plantlets after antimitotic induction is presented in Table 4. The results showed that the immersion method at various concentrations of antimitotic agents combined with culturing on regeneration medium gave rise to a plant survival rate of 100% and a polyploidy rate ranging from 33.33 to 88.89%. It reveals that the higher the concentration of antimitotic added, the higher the percentage of polyploidy obtained. The largest polyploidy percentage was obtained at a colchicine concentration of 30 µM. At the same concentration, colchicine always produced a higher polyploidy rate compared to oryzalin. The results of our study showed that the polyploid *T. grandi*s obtained was higher than that of Nugraha (2012), who combined the method of immersion and planting of *T. grandis* shoots in the antimitotic agent oryzalin at 5 µM. He generated an explant survival rate of 50-70% and a polyploidy success rate of 61.9%.

Comparing our results with Nugraha (2012), it was assumed that the highest percentage of polyploidy plantlets was obtained at a colchicine concentration of 30 µM. It showed that colchicine was more effective compared to oryzalin in generating polyploid *T. grandis*. It is believed that the method of culturing on the regeneration medium combined with immersion in the antimitotic solution directly impacts the antimitotic agents entering plant cells through the mechanism of absorption in the mitotic phase of the cell cycle. Syukur et al. (2019) verified that the application of antimitotic agents is more effective at the metaphase stage of mitotic cells, which affects the polyploidy in plants.

Further analysis to ensure alteration in the ploidy level was accomplished directly by looking at differences in the number of chromosomes in the cell nucleus. This direct observation and calculation are the most precise and accurate method. The number of chromosomes is easily calculated when the cell is in a late prophase before metaphase (Doyle & Coate, 2019). In this phase, the chromosomes are perfectly condensed, so it is easy to observe their morphology and quantity (Syukur et al., 2019).

The results demonstrated that the number of polyploid chromosomes was higher than the control diploid. Based on cytological examination, there was an increase in the ploidy level of *T. grandis*, from diploid to polyploid, resulting from the treatment of colchicine and oryzalin. The alteration in the number of *T. grandis* chromosomes from diploid (2n = 2x = 36) into polyploid was identified as follows: 1) tetraploid [2n = 4x = 72] after treatment of colchicine 30 µM; 2) polyploidy-1 [2n = 5x = 90] resulted from treatment of Oryzalin 15 µM; and 3) polyploidy-2 [2n = 9x = 162] resulted from treatment of Oryzalin 30 µM (Figure 3). Unfortunately, the chromosome numbers from colchicine 15 µM treatment specimens could not be identified, even after several attempts (Figure 6B). The reason is because the metaphase phase of mitosis in T. grandis cells was too short a time, so it was difficult to observe.

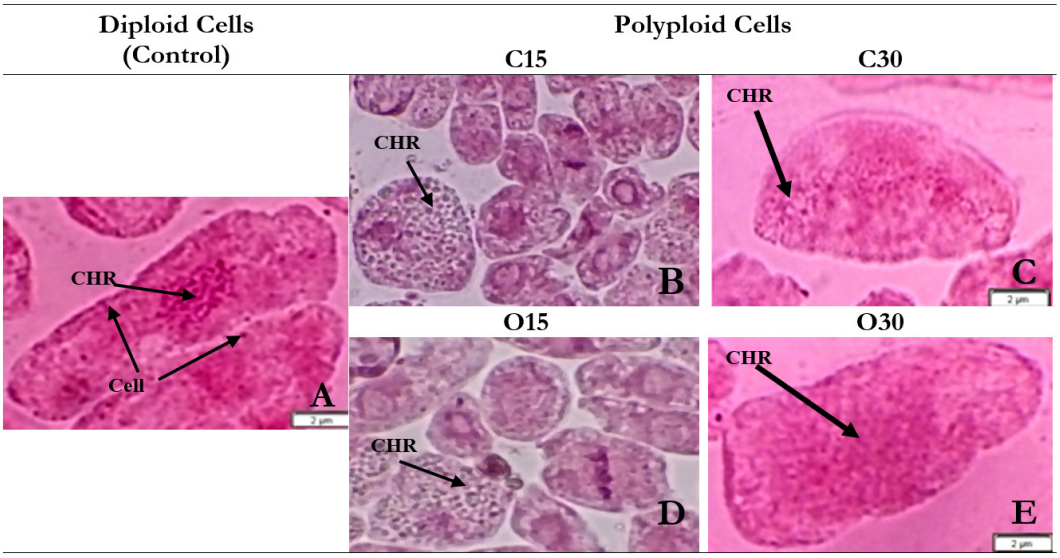

Figure 6. Image of polyploid cell of *T. grandis* under microscope resulted from various treatments of control, colchicine (C15 and C30), oryzalin (O15 and O30). A) Diploid cells [2n = 2x = 36]; B) Unidentified; C) Tetraploid cells [2n = 4x = 72]; D) Polyploid-1 cells [2n = 5x = 90]; E) Polyploid-2 cells [2n = 9x = 162]. Note: CHR=chromosomes. Line Scale: 2 µm.

Table 5. The growth of plantlets on germination medium at 6 weeks after acclimatization

Antimitotic Treatment (μM)	Rooting (%)	Survival (%)	Height (cm)	Stem Diameter (cm)	Number of leaves
Control	100 a	100 a	12.04 ab	0.151 a	11.11 ab
Oryzalin 15	100 a	100 a	9.45 c	0.141 a	9.33 b
Oryzalin 30	100 a	100 a	12.68 a	0.149 a	11.22 ab
Colchicine 15	100 a	100 a	10.65 bc	0.147 a	12.17 a
Colchicine 30	100 a	100 a	12.83 a	0.169 a	10.61 b

Note: Numbers in the same column followed by the same letters are not significantly different based on the F-Test at a confidence level of 95%

C. Acclimatization of Polyploid *T. grandis*

The results of the acclimatization and growth of polyploid *T. grandis* plantlets are revealed in Table 5.

Six weeks after acclimatization, we evaluated all plantlets resulting from antimitotic agent treatments. All the plantlets were developed successfully and were well-rooted. The average number of primary roots produced ranged from 3-5 roots per plantlet. Table 5 shows that the colchicine 30 (M) treatment had the best average plantlets height and stem diameter compared to other treatments. Nevertheless, it was not considerably different from the treatment with oryzalin (30 μM) and the control. Likewise, the number of leaves demonstrated the same phenomenon. Based on the evaluation of these parameters, it has been proven that polyploidy occurs in *T. grandis* seedlings, which is indicated by growing faster and bigger (Figure 7). Kardiman and Raebild (2018) argued that increasing the size of stomata leads to high rates of biomass accumulation, and consequently increasing plant growth.

Based on leaf morphology, particularly the edge of the *T. grandis* leaf was changed due to the ploidy level (shown in the Figure 7 by arrows). The usual diploid *T. grandis* leaf has entire to denticulate types of leaf margins, which are smooth to small-toothed edges. Increasing the ploidy level of *T. grandis* from diploid to polyploid resulted in change at the edge of the leaf. Polyploid *T. grandis* leaf has serrate to dentate types of leaf margin, which have a notched like a saw with teeth margin. The same occurrences were reported by other researchers on their polyploid plants (Baker et al., 2017; Corneillie et al., 2019; Mo et al., 2020).

Based on the data analysis in our study, it's believed that colchicine and oryzalin change the morphology and anatomy of teak seedlings from diploid to polyploid. The growth characteristics and wood performance could be recognized after stability tests and clonal tests at multiple locations in order to obtain more comprehensive data and information regarding changes in the genetic and physiological performance of teak seedlings from diploid to polyploid.

IV. CONCLUSION

Antimitotic agents affected the reduction of height, internode length and the number of leaves in *T. grandis* plantlets. They also increased the leaf surface area and chlorophyll content index. Additionally, oryzalin and colchicine increased the leaf thickness, length and width, and the number of chloroplasts in stomata, however, reduced the stomata density significantly. Among four treatments of antimitotic agents (oryzalin 15 μM, oryzalin 30 μM, colchicine 15 μM, and colchicine 30 μM), on the plantlets of *T. grandi*s the treatment of colchicine 30 μM, was the most effective to generate polyploidy plantlets, with a rate of effectiveness of 88.89%. The acclimatization of the *T. grandis* plantlet resulted in a high percentage of survival and rooting, as well as bigger and faster-growing plantlets. The polyploid *T. grandis* plantlets showed a fast

Figure 7. The growth of polyploid *T. grandis* plantlet during acclimatization after 15, 35, and 75 days at various treatments of control (A), colchicine 15 µM (B), colchicine 30 µM (C), oryzalin 15 µM (D) and oryzalin 30 µM (E). Line Scale: 1 cm.

growth and had more leaves compared to control. From this study we obtained the polyploid *T. grandis* as tetraploid [2n = 4x = 72], polyploid-1 [2n = 5x = 90], and polyploid-2 [2n = 9x = 162].

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