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PERTUMBUHAN DAN KARAKTERISASI FISIOLOGIS *Coleus amboinicus* Lour EX VITRO HASIL INDUKSI POLIPLOID

GROWTH AND PHYSIOLOYCAL CHARACTERIZATION OF Coleus amboinicus Lour EX VITRO RESULTS OF POLYPLOID INDUCTION

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ABSTRACT

Torbangun (Coleus amboinicus L.) is a local Indonesian medicinal plant traditionally used to promote breast milk (ASI) production. However, its propagation is limited because flowering is inhibited under unfavorable climatic conditions, and vegetative propagation through stem cuttings results in low genetic diversity. Increasing genetic variability through induced mutation and polyploidization offers a promising approach for crop improvement. To date, there has been no report on the morphological and physiological characterization of Torbangun polyploids induced by oryzalin. Therefore, this study aimed to evaluate the morphological and physiological characteristics of Torbangun clones derived from oryzalin-induced polyploidization. The plant materials consisted of six Torbangun clones (332Z, 332O, 333M, 333F, 513K, and 513W) obtained from the fifth subculture after exposure to 60 µM oryzalin for 72 hours. The experiment employed a completely randomized design (CRD) with one factor (clone) and eight replications, each represented by one plantlet per polybag. Growth parameters observed for 12 weeks included viability (%), plant height (cm), number of leaves, and number of axillary buds. Data were analyzed by ANOVA, followed by Duncan's test for mean separation. Ploidy levels were verified using ex vitro flow cytometry. The results showed that clone 513W exhibited superior growth performance with the highest number of leaves, axillary buds, and chlorophyll and carotenoid contents. Qualitatively, induced and control plants displayed similar morphological traits, but quantitative differences were observed in leaf thickness and area. These findings provide the first comprehensive characterization of oryzalin-induced Torbangun polyploids, offering insights for future breeding and conservation efforts.

ABSTRAK

Torbangun (*Coleus amboinicus* L.) merupakan salah satu jenis tanaman lokal Indonesia yang terbukti efektif memperlancar ASI. Tanaman Torbangun sulit untuk berkembang membentuk bunga karena iklim yang kurang mendukung. Perbanyakan tanaman ini dilakukan melalui stek batang, sehingga keragamannya sempit, untuk meningkatkan keragamannya salah satunya dengan menggunakan teknik mutasi. Keanekaragaman genetik Torbangun dapat ditingkatkan melalui pemuliaan tanaman dengan mutasi induksi poliploid menggunakan oryzalin. Sejauh ini belum ada laporan penelitian mengenai karakterisasi morfologi dan fisiologi Torbangun hasil induksi poliploidisasi menggunakan oryzalin, sehingga perlu dilakukan penelitian mengenai karakterisasi morfologi dan fisiologi Torbangun hasil induksi poliploidisasi menggunakan oryzalin. Bahan yang digunakan dalam penelitian ini adalah klon tanaman Torbangun 332Z, 332O, 333M, 333F, 513K dan 513W hasil dari lima subkultur mutasi induksi dengan konsentrasi oryzalin 60 µM, perendaman selama 72 jam. Percobaan menggunakan rancangan penelitian RAL (Rancangan Acak Lengkap) 1 faktorial yaitu klon dengan 8 ulangan/polibag per klon dan satuan pengamatan adalah 1 polibag berisi 1 planlet. Uji Duncan dilakukan apabila terdapat perbedaan nyata pada analisis ANOVA. Parameter pertumbuhan yang diamati adalah viabilitas (%); tinggi tanaman (cm); jumlah daun (untaian); jumlah tunas aksila. Pengamatan dilakukan setiap minggu selama 12 minggu. Analisis tingkat ploidi dengan flowcytometer Torbangun ex vitro. Hasil penelitian menunjukkan klon 513W mempunyai jumlah daun terbanyak, jumlah tunas terbanyak, klorofil tinggi, dan kandungan karotenoid tinggi. Karakter morfologi Torbangun kontrol dan induksi mempunyai karakteristik yang sama secara kualitatif. Perbedaan karakter morfologi terdapat pada karakter kuantitatif ketebalan daun dan luas daun.

KATA KUNCI:
Aklimatisasi, *Coleus amboinicus*, ex vitro, oryzalin, torbangun

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1. INTRODUCTION

Torbangun (*Coleus amboinicus* Lour.) is a type of local Indonesian plant which has been proven to be effective in facilitating breast milk (ASI). In Indonesia, especially in North Sumatra, Torbangun leaves are consumed by mothers after giving birth and breastfeeding because Torbangun leaves are believed to increase 69% breast milk production volume (Damanik *et al*., 2001). According to Santosa (2001) and Damanik *et al*., (2006), the lactogogum effect produced by consuming Torbangun leaves is able to increase breast milk production more compared to consuming breast milk enhancing supplements such as molocco + B12, fenugreek seeds, and breast milk smoothing. Apart from quantity, the quality of breast milk, such as iron, zinc and potassium content, is superior to mothers who consume other supplements such as molocco + B12 and breast milk flows smoothly, thus affecting the baby's growth. Babies who drink breast milk from mothers who consume Torbangun leaves have better growth in weight, body length and head circumference when compared to mothers who consume other supplements (Santosa, 2001).

Research on Torbangun has mostly been carried out in the field of pharmacology (Hullatti & Bhattacharjee, 2011). Torbangun is still rarely cultivated commercially and is still often found on free land. The production of fresh biomass from Torbangun plants with compost fertilization was higher 125.21% than those without compost fertilization treatment. The growth of Torbangun plants in Indonesia is slow because of unfavourable climate so harvesting must also be done at a longer plant lifespan, which is 4-5 monts. Torbangun plants cannot be propagated by seed because the seeds from Torbangun plants are classified as sterile. People in Indonesia generally carry out vegetative propagation, namely through stem cuttings (Aziz, 2013). Cultivating Torbangun using stem cuttings makes it difficult to obtain new superior cultivars as a source of genetic diversity.

The genetic diversity of Torbangun can be increased by plant breeding. One of the goals of plant breeding is to obtain good quality plants, such as taste, color, size, biomass, bioactive content and so on. To increase the diversity of Torbangun, research has been carried out using induced mutations using gamma irradiation (Aisyah & Damanik, 2015), colchicine (Wibisono, 2019), oryzalin (Maulana et al., 2021) and ethyl methane sulfonate (Sari *et al.,* 2016). Conventional breeding techniques such as hybridization require quite a long time to obtain genetic diversity, so one technique to shorten the time is polyploidy induction.

Oryzalin can be used as an alternative compound for chromosome duplication and has less toxic properties than colchicine (Miguel & Leonhardt, 2011). oryzalin has a strong affinity for plant tubulin so it only requires a lower concentration (micromolar) to induce polyploid plants (Sattler *et al*., 2016). Changes in the number of chromosomes will change morphological, anatomical and physiological characteristics thereby increasing genetic diversity. Polyploidy in plants can occur naturally or artificially. One technique for artificial polyploid induction that is often used is by using chemicals such as oryzalin (Beranová *et al*., 2022). So far there has been no research report on the morphological characterization and secondary metabolites of torbangun resulting from polyploidization induction using oryzalin, so it is necessary to conduct research on the morphological and physiological characterization of torbangun resulting from polyploidization induction using oryzalin.

2. MATERIALS AND METHOD

2.1 Materials

The materials used in this research were Torbangun plant clones 332 Z, 332 O, 333 M, 333 F, 513 K and 513 W (Figure 1) resulting from the five subculture of induced mutations with oryzalin concentration of 60 μM, soaking for 72 hours, diploid control, MS media (Murashige and Skoog), sugar, aquabidest, NaOH, absolute ethanol, acetone,UV-Ploidy cystain buffer.

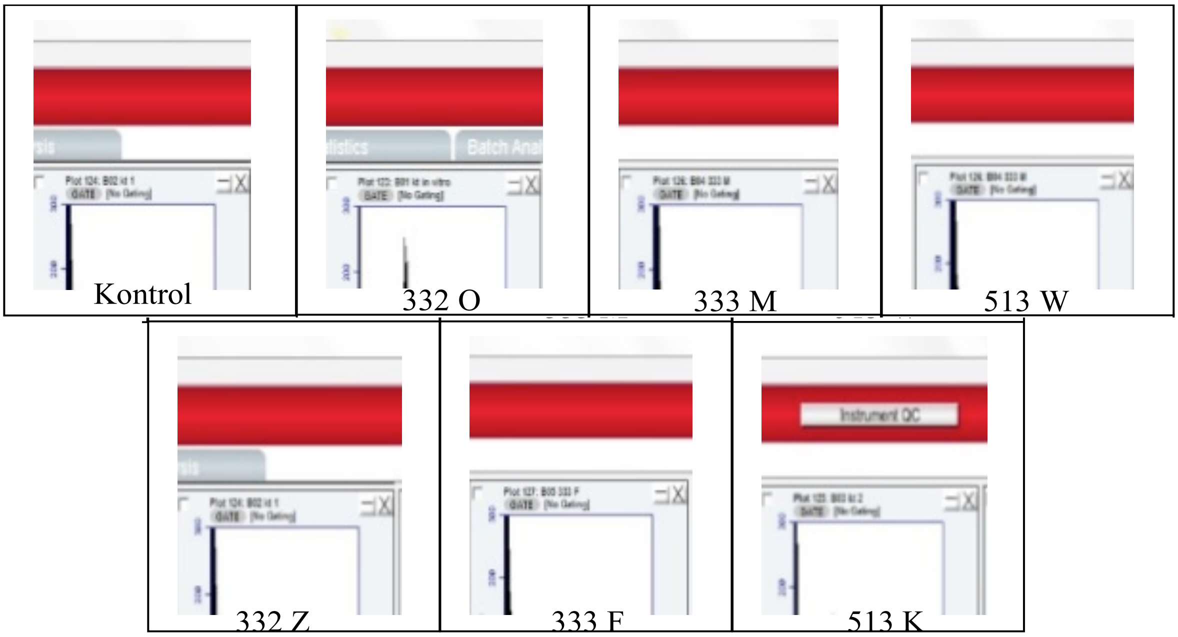

Figure 1. The materials used flowcytometer in this research were Torbangun plant clones 332 Z, 332 O, 333 M, 333 F, 513 K and 513 W.

The tools used are culture bottles, laminar air flow cabinets, bunsens, petri dishes, tweezers, scalpels, pH meters, autoclaves, analytical scales, hand sprayers, and UV/Vis spectrophotometers/ flowcytometers.

2.2 Method

Torbangun Growth Observation ex vitro. The Torbangun plantlets of clones 332 Z, 333 M, 513 K, 332 O, 333 F, 513 W (8 weeks after planting) and the rooted diploid control were removed from the bottle by adding a little water to the bottle, then shaking it slightly so that the plantlets would release themselves from the media. The plantlets that have been separated from the media are then removed from the bottle and washed thoroughly until there is no media left on the plantlet roots. The clean plantlets are then planted in acclimatization media. The media used for acclimatization are soil, cocopeat and husk charcoal in a ratio of 1:1:1. The acclimatization media was then put into a plastic polybag (20.5 cm x 10 cm). The polybag containing the plantlets is then covered using transparent plastic and placed in the shade for two weeks. After adapting for two weeks, the plastic cover is opened and the plants are placed in a place that gets sunlight. At 4 weeks of age, the planting medium is replaced with soil, manure and husk charcoal in a ratio of 1:1:1. The experiment used a 1 factorial RAL (completely randomized design) research design, namely clones with 8 replications/polybags per clone and the observation unit was 1 polybag containing 1 plantlet. The growth parameters observed were viability (%); plant height (cm); number of leaves (strands); number of axillary buds. Observations were carried out every week for 12 weeks.

Analysis of Chlorophyll and Carotenoid Content in Torbangun ex vitro. Leaf samples 332 Z, 333 M, 513 K, 332 O, 333 F, 513 W and 0.2 g diploid control were extracted with 10 ml of 80% acetone. The absorbance of the extract was measured at 645 and 663 nm for chlorophyll content and 480, 645 and 663 nm for carotenoid content (Sari & Hidayati, 2020). Observation of Torbangun morphology ex vitro. Torbangun morphological observations were carried out on plant type, stem character and leaf character. Observation of the type of plant, whether it is an upright type or a creeping type. Observation of stem characters in the form of stem shape, stem diameter (mm), branching, stem color, buds in the leaf axils (presence/absence) and hairs on the surface of the stem (presence/absence). Observation of leaf characters in the form of leaf shape, leaf edge, leaf tip, leaf vein color, upper leaf surface color, lower leaf surface color, leaf surface hairs, leaf thickness, leaf petiole color, leaf vein hairs, leaf position and leaf aroma (Nasution *et al*., 2017).

Ploidy level analysis with Torbangun ex vitro flowcytometer. Confirmation of the ploidy level was carried out on in vitro shoots in three subcultures using a flow cytometer (BD Accuri+, USA). Leaf pieces measuring approximately 0.5 × 0.5 cm were placed on a Petri dish then dripped with 1.5 mL of CyStain UV Ploidy buffer, then chopped until fine with a razor blade. The chopped leaves were filtered using a 30 µm millipore sieve. The filtrate is placed in a cuvette tube for analysis. Analysis was carried out at a wavelength of 400 nm and a speed of 1000 nuclei per second. The leaves of the diploid Torbangun plant were used as a standard. The average DNA content (mean) and coefficient of variation (CV) of each sample at each peak were observed and compared with control plants (diploid). Plant ploidy levels are determined according to multiples of the average amount of DNA content, based on a histogram (Maulana *et al*., 2021).

3. RESULT AND DISCUSSION

3.1 Axillary Shoots

The number of axillary shoots in the ex vitro treatment only appeared in the second week and the highest number of axillary shoots was clone 513 K with an average number of 8.62 and the lowest was clone 333 F with an average number of 2.00 (Table 1, Figure 2a). Clonal differences have a significant effect on the number of axillary shoots. Increasing the ploidy level has effect on the number of axillary shoots (Table 1). Differences in shoot growth in plants with higher ploidy levels are caused by reorganization and restructuring of the genome of polyploid plants, resulting in changes in gene expression patterns (Soltis, 2015). The reason the control explants had a greater number of shoots was due to the presence of endogenous hormones. Bella *et al*., (2016) stated that if the concentration of cytokinin is greater than auxin, it will stimulate the growth of shoots and leaves.

3.2 Shoots Height

Observation of shoot height showed that in each clone there was an increase in shoot height from the second week to the twelfth week (Figure 2b). Clone differences have a significant effect on shoot height. Increasing the ploidy level has no effect on shoot height. Clone 332 O showed a higher average value of 49.12 cm at the twelfth week and the lowest shoot height was found in clone 333 F at 32.00 cm (Table 1). Plants treated with oryzalin at certain concentrations will give different responses. A balanced concentration of oryzalin can increase plant growth.

According to Defiani & Ni Wayan (2014), plant growth constraints are caused by disrupted cell division at concentrations that exceed plant needs. Plants that are induced using antimitotic compounds will show a different response because the cells will adapt first before continuing growth. The length of exposure time and concentration of oryzalin influence plant adaptation patterns. The adaptations made by plants vary. When exposed to antimitotic substances, plants will show a response with slow growth. However, at the right time plants will increase their regenerative capacity towards the best growth if the concentration given is appropriate (Carvalho *et al*., 2016).

3.3 Number of Leaves

The number of leaves (*ex vitro*) began to grow in the second week and continued to increase until the twelfth week (Figure 2c). Clone differences have a significant effect on the number of leaves. Increasing the ploidy level has no effect on number of leaves. Clone 513 W produced the highest number of leaves (88.50) compared to the control and other polyploid clones (Table 1). The ploidy level did not affect the number of leaves, this was thought to be because the administration of oryzalin was not able to affect all plant cells but only some cells. Avery *et al*., (1947) revealed that the appropriate use of mutagens can result in variations in morphological characters. Furthermore, Barnabas *et al*., (1999) explained that the colchicine treatment given was not appropriate so that it did not show any changes in morphological characters, this was because colchicine activity only affected some cells, so that mutations did not occur in all parts of the plant, oryzalin is a mutagen like colchicine. The administration of the oryzalin compound must be precise because the higher the concentration obtained by the plant, the more it will damage the cells and fail to divide (Handayani *et al*., 2017). These results are comparable to research by Ermayanti *et al*., (2014) who stated that the number of leaves of Artemisia annua L. induced by 15 and 30 μM oryzalin had a greater number of leaves than control plants. The control mean had a number of 21.75, while the result of 15 μM oryzalin induction had a value of 28.33, and 30 μM oryzalin induction had a value of 24.92 strands. The large number of leaves is also directly proportional with the large number of axillary shoots formed. The more axillary buds are formed, the more leaves will be produced on the plant.

Table 1. Number of axillary shoots, shoot height and number of leaves of the Torbangun clone 12 weeks after planting.

Clone	Number of Axillary Shoots	Shoot Height (cm)	Number of Leaves
Control (Diploid)	5,25 c	44,38 d	46,75 b
513 K (Triploid)	8,62 g	41,38 c	78,00 e
513 W (Tetraploid)	7,62 f	44,25 d	88,50 f
333 M (Triploid)	3,62 b	42,38 c	36,75 a
333 F (Tetraploid)	2,00 a	32,00 a	35,75 a
332 Z (Triploid)	7,12 e	47,00 e	69,25 d
332 O (Tetraploid)	6,00 d	49,12 e	55,25 c

Note: Numbers followed by different letters in the same column indicate significantly different results based on the DMRT multiple interval test with a level of 5%.

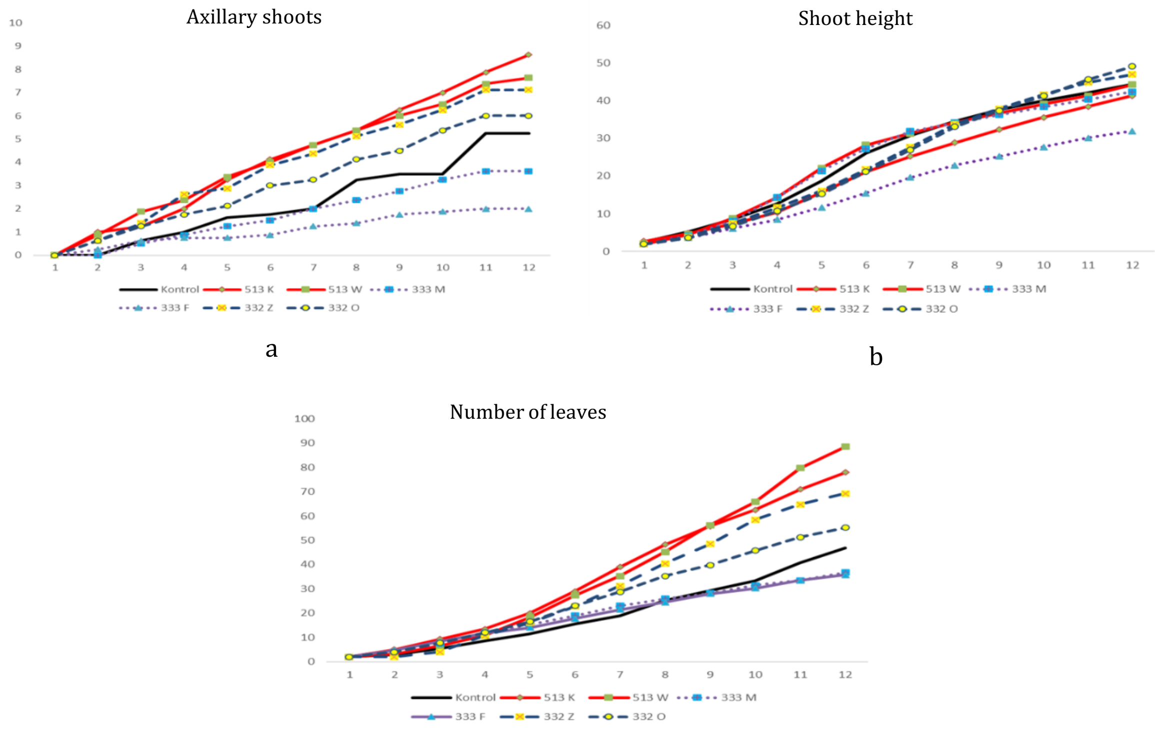

Figure 2. (a) Number of axillary shoots, (b) Shoot height (cm), (c) Number of leaves of control Torbangun plants and results of oryzalin induction clones 513 K, 513 W, 333 M, 332 Z, 333 F, 332 O aged 1-12 weeks after planting (*ex vitro*).

3.4 Morphology of Torbangun

The morphology of Torbangun was observed on plant type, stem character and leaf character (Table 2, Figure 3). Torbangun plants, diploid clones and clones 333 F, 333 M, 513 K, 513 W, 332 O and 332 Z are upright plant types. The morphology of the stem characters of Torbangun clones, diploid clones and clones 333 F, 333 M, 513 K, 513 W, 332 O and 332 Z have similarities, namely square stem shape, upward curved branches, purple green stem color, buds in the leaf axils, and there are hairs on the surface of the stem. The diploid stem diameter is smaller than the triploid and tetraploid clones. The leaf characteristics of Torbangun clones, diploid clones and clones 333 F, 333 M, 513 K, 513 W, 332 O and 332 Z have similarities, namely oval leaf shape, serrated leaf edges, blunt leaf tips, light green leaf vein color, upper surface leaf color, dark green, the color of the lower surface of the leaves is light green, there are hairs on the surface of the leaves, the color of the leaf stalks is purple green, the position of the leaves is facing each other and the aroma of the leaves is strong. The existence of phenotypic variations between one individual and another in the treatment can be different, not all individual plants that are treated experience phenotypic variations. Phenotypic variations can occur in almost all parts of the plant but can also occur in only a few parts of the organ. The variations that occur indicate that cell division activity between individual plants is not the same.

In individual plants that have more cells that are actively dividing when oryzalin is given, this will result in a higher percentage of mutated cells. On the other hand, individual plants that have fewer cells that are actively dividing when oryzalin is given will result in only a few cells being mutated or even not affecting the phenotypic changes at all (Normasiwi S & Nurlaeni Y, 2014). Oryzalin given to each individual plant does not affect all plant cells, but only some cells.

Figure 3. Performance of *ex vitro* tetraploids Torbangun after twelve weeks

Table 2. Observation results of diploid Torbangun morphology and induction results (513 K, 513 W, 333 M. 332 Z, 333 F and 332 O)on twelve weeks after planting.

Morphology	Clone						
	Control/ Diploid	**513 K**	**513 W**	**333 M**	**333 F**	**332 Z**	**332 O**
A. Plant Type	Upright	Upright	Upright	Upright	Upright	Upright	Upright
B. Stem Character							
Stem Shape	Rectangle	Rectangle	Rectangle	Rectangle	Rectangle	Rectangle	Rectangle
Stem Diameter (cm)	6,9	7,9	9,9	7,5	7,7	8,1	8,2
Stem Branch	Upper Arch	Upper Arch	Upper Arch	Upper Arch	Upper Arch	Upper Arch	Upper Arch
Stem Colour	Green	Purple Green	Purple Green	Purple Green	Purple Green	Purple Green	Purple Green
Buds in Leaf Axis	yes	yes	yes	yes	yes	yes	yes
Hairs on the Surface of the Stem	yes	yes	yes	yes	yes	yes	yes
C. Leaf Character							
Leaf Shape	Oval	Oval	Oval	Oval	Oval	Oval	Oval
Leaf Edge	Serration	Serration	Serration	Serration	Serration	Serration	Serration
Leaf Tip	Blunt	Blunt	Blunt	Blunt	Blunt	Blunt	Blunt
Leaf Vain Color	Light Green	Light Green	Light Green	Light Green	Light Green	Light Green	Light Green
Upper Surface Leaf Color	Green	Green	Green	Green	Green	Green	Green
Lower Surface Leaf Color	Light Green	Light Green	Light Green	Light Green	Light Green	Light Green	Light Green
Hairs on Leaf Surface	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Leaf Thickness	38,21	74,01	98,36	87,11	61,31	74,82	84,14
Leaf Petiole Color	Green	Green purple	Green purple	Green purple	Green purple	Green purple	Green purple
Leaf Spines	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Leaf Position	Pair	Pair	Pair	Pair	Pair	Pair	Pair
Leaf Aroma	Strong	Strong	Strong	Strong	Strong	Strong	Strong
Leaf Area (cm^2)	7,772	13,120	13,359	9,962	10,539	13,605	18,813
Bone Leaves	Finger	Finger	Finger	Finger	Finger	Finger	Finger

3.5 Physiology of Torbangun

The chlorophyll content of ex vitro plants is higher than in vitro (Table 3). According to Lan *et al.*, (2020), oryzalin induction changes the ploidy level to a higher level, stomata become larger in polyploid plants, absorb light and carbon dioxide at a higher rate and the chlorophyll and carotenoid content of polyploids is higher.

According to Suzuki *et al.*, (1997) chlorophyll biosynthesis is carried out by certain genes in the chromosomes. These genes encode enzymes that will play a role in the tetrapyrrole biosynthesis pathway as the structural center of chlorophyll. The chlorophyll content in the leaves will affect the photosynthesis reaction. Low chlorophyll levels certainly will not maximize photosynthesis reactions. According to Nurcahyani *et al.*, (2020), when the photosynthesis reaction is not optimal, the carbohydrate compounds produced also cannot be optimal. Sunlight and adequate environmental conditions not only support the photosynthesis process, but also the formation of chlorophyll. Ex vitro plants absorb more carbon dioxide. If CO_2 levels in cells are low then photosynthesis will decrease. The conditions in the culture container in conventional propagation have a low CO_2 concentration during photoperiodicity so that the photosynthesis process almost does not occur (Kubota, 2002).

Table 3. Results of chlorophyll analysis of diploid Torbangun leaves and induction results (513 K, 513 W, 333 M, 332 Z, 333 F and 332 O) *ex vitro*.

Treatment	Chlorophyll A	Chlorophyll B	Total Chlorophyll (mg/L)	Total Carotenoid (mg/L)
Control/Diploid	12,50	9,41	21,91	0,68
513 K Triploid	14,24	14,36	28,60	0,81
513 W Tetraploid	14,80	14,74	29,54	0,85
333 M Triploid	15,08	14,66	29,74	0,78
333 F Tetraploid	15,33	15,54	30,87	0,81
332 Z Triploid	14,22	11,94	26,16	0,77
332 O Tetraploid	15,60	12,63	28,23	0,85

Carotenoids are terpenoid compounds which are photosynthetic pigments with color effects ranging between red and yellow. The chlorophyll content will decrease in the aging phase. Carotenoids in leaves, as photosynthetic accessory pigments, generally increase when chlorophyll content decreases (Yang *et al*., 2014). Carotenoid content is a parameter that influences plant metabolic processes through photosynthesis. Carotenoids in plants function as photoprotectors which work to prevent damage due to photooxidation because chlorophyll will undergo photooxidation when exposed to light.

Carotenoids can prevent the formation of triplet oxygen (chlorophyll bonds with oxygen) so that it cannot produce single oxygen because single oxygen is a strong oxidant that will oxidize chlorophyll. The photosynthesis system in plants then inspired the formation of dye-sensitized solar cells for the renewable energy sector (Kusumaningrum & Zainuri, 2013). Llorente *et al*., (2016) suggested that Light is an important environmental factor involved in regulating the carotenoid biosynthesis. Carotenoids play essential functions in photosynthesis for photosystem assembly, light-harvesting, and photoprotection (Domonkos *et al,* 2013). Research by Marin *et al*., (2017) also obtained data that the chlorophyll content in Fabaceae sp. positively correlated with the carotenoid content. Carotenoids can function as precursors of vitamin A, as antioxidants, as anticancer and antiobesity agents (Syukri, 2021). The superior Torbangun clone that can be selected for in vitro propagation is clone 513 W with the highest number of axillary shoots and leaves.

4. CONCLUSIONS

Clone 513 K is the best clone with the highest number of shoots. Clone 332 O is the best clone with the highest shoot height. Clone 513 W is the best clone with the highest number of leaves and total carotenoid. Clone 333 F is the best clone with the highest total chlorophyll. The morphological characters of the control and induced Torbangun have qualitatively the same characteristics. Differences in morphological characters are found in the quantitative characters of leaf thickness and leaf area.

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