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Production of Tetraploid Plants from Cotyledons of Diploid *Melia volkensii* Gürke

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Abstract: Polyploidy was induced in *Melia volkensii* (Mukau), a valuable native tree from the semi-arid regions of East Africa. Cotyledons of diploid *M. volkensii* (2n = 2x = 28) were treated with oryzalin for 0 (control), 1, 2, or 3 h with or without pretreatment with 1.1 µM thidiazuron. Cotyledons treated with 10 mg·L^{-1} oryzalin for three hours yielded 40% tetraploids. Pretreatment of cotyledons with thidiazuron for 18 days followed by treatment with oryzalin increased tetraploid plant production to 52.5%, but this also yielded more mixoploids. Compared to diploid *M. volkensii*, the tetraploid in vitro and young potted plants were compacter, with thicker stems, wider leaves, and a low density of longer and wider stomata. In the coming years, tetraploid *M. volkensii* plants will be observed in field trials and serve as a basis for further breeding efforts.

Keywords: in vitro chromosome doubling; *Melia volkensii*; oryzalin; polyploidy; regeneration

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1. Introduction

Traditional tree breeding methods necessitate a large space, and young trees require a significant amount of vegetative growth before flowering [1]. Furthermore, differences in reproductive cycles in trees [2], particularly in *Melia volkensii* (Mukau) species of the meliaceae family, which lacks seasonality in flowering [3], may make crosses difficult. Chromosome doubling is an essential technique for tree species breeding because it enriches germplasm banks and breeding programs with synthetic polyploids in a relatively short period of time [4,5].

The development of polyploid plants also offers opportunities to breed new genotypes with improved vigor and quality that are not categorized as genetically modified organisms. The process of chromosome doubling may make it possible to use sterile hybrids as a means to obtain fertile plants [6]. Polyploidization often leads to changes in the size of plant organs and improved tolerance to stress factors [7]. For example, under salt stress conditions, tetraploids of *Poncirus trifoliata* showed less leaf drop and chlorosis than diploid plants [8]. Autotetraploid plants of *Ziziphus jujuba* Mill. var. *spinosa* showed drought tolerance and improved photosynthetic system [9]. Tetraploid *Ficus carica* L. plants had superior morphological and physiological characteristics, higher phytohormones, phenolic compound content, total soluble sugars and proteins, and low antioxidants [10]. Fetouh et al. [11] reported that tetraploid *Ligustrum japonicum* showed significant changes in plant growth, leaf morphology, foliage, and alterations in rooting. Tetraploid plants can be distinguished from diploid plants by more chloroplasts and a darker green color [12,13]. The tetraploid *Populus* had significantly longer and wider stomata and lower stomatal density than the diploid [14–17]. In *Eucalyptus urophylla*, chromosome duplication resulted in slower-growing trees with a higher net

photosynthetic rate, more giant cells, thicker leaves, and a higher concentration of specific secondary metabolites [18]. The three-year-old tetraploid *Melia azedarach* developed faster than the diploid and had a bigger trunk and smaller, dark-green leaves [19]. Leaves of tetraploid *Mangifera indica* had larger chloroplasts, mesophyll cells, and stomatal guard cells, resulting in higher leaf elastic modulus and lower desiccation rates. The enlargement of the xylem and phloem cells resulted in lower hydraulic resistance [20].

In nature, polyploidization occurs through deviations in the sexual reproductive process [21]. Genomic duplications can also be artificially induced by treating explants in vitro or the meristems of plants in the field with antimitotic agents [22]. The most commonly used antimitotic agents are colchicine, oryzalin, and trifluralin [13,23,24]. During mitotic cell division, these antimitotic chemicals act by binding to an essential component for chromosome polar migration during mitosis called microtubules [25,26]. The effectiveness of these drugs in vivo or in vitro is influenced by propagation mechanisms, concentrations, exposure time, explant, and the presence of cells in mitosis [27]. Oryzalin is one of the most effective in promoting chromosome duplication in plants in vitro [13,28,29] and seed treatment in nurseries [30]. Oryzalin concentrations of 200 to 300 µM were effective in doubling chromosomes in pears [31]. The rate of tetraploid induction in *Platycladus orientalis*, *Thuja occidentalis*, and *T. plicata* treated with oryzalin was related to the duration of treatment [32].

Polyploid trees, *Melia volkensii* among others, may be of interest for forest plantations for providing better wood quality and performance under stress conditions compared to diploid trees [33,34]. Tetraploid *Acacia mangium* clones that were eight years old had pulp with longer and wider fibers, higher mass, porosity, and tear strength than diploid *Acacia mangium* clones that were the same age [35]. The polyploid *Betula platyphylla* resulted in significant increases in the wood's density as well as its characteristics, lignin content, and cellulose content in comparison to the diploid *Betula platyphylla* [36]. Tetraploid and triploid *Eucalyptus* spp. had wider stems and crowns, longer stems, and higher wood density than the diploid [37]. However, no cases of polyploid production have been reported in *M. volkensii*, which is a multi-purpose tree grown in semi-arid regions in Kenya. Therefore, it is essential to develop a method to induce polyploidy in *M. volkensii*. The first generation may have interesting morphology and physiology. Subsequent tetraploid or triploid generations may yield novel gene combinations that may provide physiological novelties. An experiment was set up to investigate the effects of oryzalin on the in vitro induction of tetraploid in *M. volkensii* trees and to compare the characteristics of diploid and tetraploid trees. We describe here a method to produce polyploid *M. volkensii* plants. To this end, we take advantage of the ease with which adventitious shoots can be induced on cotyledons in vitro [38]. Tetraploid plants were then morphologically evaluated for their potential added value for further breeding.

2. Materials and Methods

2.1. Plant Material and Initiation

Melia volkensii seeds were obtained from Better Globe Forestry Ltd. (BGF), Kenya. The seeds were rinsed with 70% ethanol for one minute, sterilized externally with 15% bleach (5% sodium hypochlorite) plus 0.005% detergent (Teepol) for 15 min and then rinsed three times with sterile distilled water. The seed coats were removed with forceps and a scalpel, and seeds were then transferred into a tube containing 10 mL of basic medium consisting of Murashige and Skoog's (MS) macro- and micro-salts and vitamins [39] supplemented with 30 $g \cdot L^{-1}$ sucrose and 7 $g \cdot L^{-1}$ plant agar (Duchefa, 2003 RV Haarlem, The Netherlands). The pH of the medium was adjusted to 5.8 with 1 M KOH and 1 M HCL before autoclaving at 121 °C for 20 min. The cultures were kept in a growth chamber at 22 ± 1 °C and a photoperiod of 16/8 h light/dark. After two weeks, the cotyledons were collected and subsequent subculturing of the germinated shoots occurred every four weeks.

2.2. Chromosome Counting

Chromosome numbers of some *Melia* species have already been reported, but not for *Melia volkensii*. This led to the necessity of performing chromosome counting. The root tips of diploid plants were harvested from acclimatized *M. volkensii* plants in vitro. The root tips were pretreated for one hour at 4 °C in 0.1% colchicine plus 20 µL of 500× 8-hydroxyquinoline [40]. Then the root tips were fixed 3:1 ethanol:acetic acid for 60 min. Cell suspension and slide preparation were made according to the "SteamDrop" procedure of Kirov et al. [41]. Root tips were incubated at 37 °C for 60 min in a 0.6% enzyme mixture (0.6% cellulase and 0.6% pectolyase in 0.1 M citrate buffer (pH = 4.6). As fixatives, 1:1 and 2:1 ethanol:acetic acid were used to prepare the slides. The slides were stained with 4′,6-diamidino-2-phenylindole (DAPI) (100 µg/mL) and analyzed using a fluorescence microscope (AxioImager M2, Carl Zeiss MicroImaging, Belgium; 1000× magnification) equipped with an Axiocam MRm camera and ZEN software (Carl Zeiss MicroImaging, Zaventem, Belgium). Chromosome counting was performed on nine metaphases using ImageJ (imagej.net).

2.3. Polyploidization

In the first experiment, cotyledons from two-week-old in vitro seedlings (Figure 1a) were treated with 10 mg·L^{-1} oryzalin (dissolved in dimethyl sulfoxide (DMSO) for 1, 2, and 3 h. The concentration of oryzalin was selected based on the internal protocol of the Applied In Vitro Plant Biotechnology Laboratory, Bioscience Engineering, Ghent University. After this treatment, the cotyledons were transferred to MS with 1.1 µM TDZ for 18 days and then transferred to basal MS medium with 1 µM indole-3-acetic acid (IAA) and 2 µM 6-Benzylaminopurine (BAP) or 5 µM meta-Topolin Riboside (mTR). All cultures were incubated in a growth chamber with 16 h/8 h day/night provided with PURPL® LED PANEL LIGHT (LED type: 4014, model: PAN-3000K-30X120). Twenty-one days after transfer, cultures were transferred to a medium containing 2 µM BAP or 5 mM mTR but without IAA.

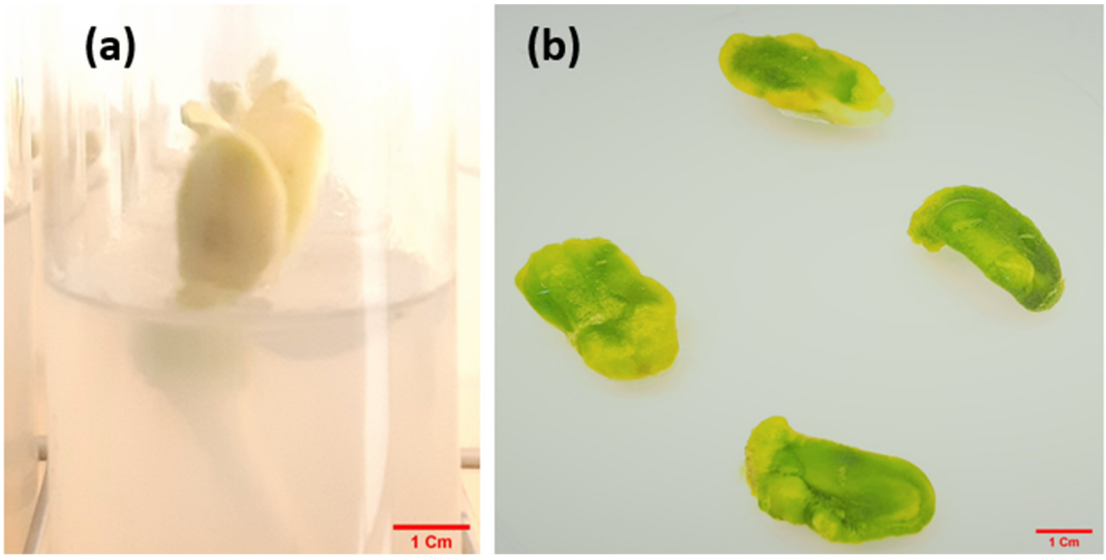

Figure 1. *Melia volkensii* cotyledons. (**a**) Two-week-old cotyledons under basal MS medium during initiation. Scale bar = 1 cm. (**b**) Cotyledons change color after 18 days in medium containing 1.1 µM TDZ.

In the second experiment, two weeks after seeds were sown in vitro, cotyledons were detached from the seedlings and transferred to Petri dishes containing basal medium supplemented with 1.1 µM Thidiazuron (TDZ). The cotyledons began to respond to TDZ after 18 days by turning green (Figure 1b). Then, cotyledons were treated with 10 mg·L^{-1} oryzalin for 1, 2, and 3 h, washed twice in sterile distilled water, and transferred into Petri dishes containing MS medium containing 1.1 µM TDZ for 21 days. For each treatment, a total of fourteen cotyledons were used, and the experiment was repeated three times.

Shoot meristem formation was observed after 21 days, and at that time the cotyledons were transferred to MS supplemented with 1 µM Indole-3-acetic acid (IAA) and 2 µM 6-benzylaminopurine (BAP), according to Mulanda et al. [38] or with 5 µM meta-Topolin Riboside (mTR). Shoots from cotyledons that had not received oryzalin treatment were used as controls. Conditions in the growth chamber were similar to those of the first experiment. Twenty-one days after transfer, regenerated shoots were propagated on a medium containing 2 µM BAP or 5 mM mTR without IAA. The regenerated microshoots were grown in 10 mL tubes of MS medium containing 1 µM mTR.

2.4. Ploidy Determination

Ploidy content of regenerated shoots with three leaves was determined by flow cytometry. For each treatment shoots resulting from eight cotyledons and five shoots per cotyledon were analyzed, as well as twenty control plants measured as external control. Sample preparation and staining were performed with Cystain™ PI absolute P (REF: 05-5022) (Sysmex Partec GmbH, Görlitz, Germany) kit. Approximately 0.5 cm^2 of the leaf was placed in a Petri dish to which extraction buffer was added. The leaf tissue was chopped with a sharp razor blade. The sample was filtered through a 50 µM CellTrics™ filter. Then, 1 mL of staining solution was added with 20 mL of buffer, 120 µL of propidium iodide, and 60 µL of RNase A stock solution. Before analysis, the sample was incubated for 60 min in the dark at room temperature. The amplification of the instrument (CyTecs, GmbH, Amflugplatz 13, D-02828 Görlitz) was tuned to a scale of 362 channels, with channel 100 roughly corresponding to the peak. Samples were analyzed using the same instrument settings to obtain comparable relative fluorescence intensity values. The reference fluorescence of stained nuclei was set by scanning untreated *M. volkensii* (2n = 2x = 28) plants and a known diploid *Paulownia tomentosa* (2n = 2x = 40) prior to scanning any samples.

2.5. In Vitro Propagation of the Tetraploid Melia volkensii Plants

The identified tetraploid shoots were micropropagated using the basal medium supplemented with 5 µM mTR as described by Dushimimana et al. [42] with minor modifications. Six shoots with one node and two leaves per shoot were grown in 720 mL plastic vessels (Eco2Box with XXL green filter, Duchefa, The Netherlands) filled with 150 mL basal medium with 5 µM mTR. Diploid shoots were grown as controls on the same medium.

2.6. Morphological Characteristics of the Tetraploid Melia volkensii in Comparison of Diploids

After four weeks in the culture medium, morphological analysis was performed on 15 clusters of one diploid and four tetraploid genotypes (T192-1, T482-2, T432-1, and T841-1). Therefore, the number of shoots per explant, shoot length (cm), shoot diameter (mm), number of leaves per shoot, shoot internode length (mm), and number of leaflets per leaf of the most elongated shoots were measured and compared between diploids and tetraploids. In addition, leaf morphology of tetraploid plants was compared with that of diploid plants using three leaves from each tetraploid and diploid plantlet. Leaf length and width (cm) and leaf area (cm^2) were assessed on four-week-old shoots. Leaf epidermis was collected with clear nail polish, which was taped with tape after drying [43]. Observations of the stomata were performed with a light microscope (VisiScope, TL384P, VWR, Avantor, PA, USA) and software (Olympus EPview™ v2.9.6_20201224: http://www.olympus-sis.com/ (accessed on 29 August 2022)). Stomatal length and width (µm) were assessed using 75 stomata from diploid or tetraploid plantlets. Stomatal density was analyzed by counting stomata in five microscopic fields at 400× magnification, as described by Li et al. [44]. The leaf area was determined after four weeks in the culture medium. Photographs of three fully expanded leaves per plant were used to determine leaf area using image analysis (ImageJ, Downloads (imagej.net)). For acclimatized polyploid *M. volkensii*, the leaf morphology of three-month-old plants was assessed from three leaves of two acclimatized tetraploid or diploid plants. Stomatal length and width were evaluated as described above.

2.7. Statistical Analysis of Data

Data were analyzed using IBM® SPSS® statistics (version 28), (Armonk, New York 10504-1722, NY, USA). One-way analysis of variance (ANOVA) was used to test for significant differences between treatments, and a post hoc Duncan's multiple range test at 5% was used to separate means.

3. Results

3.1. Chromosome Counting

Chromosome counting of diploid *M. volkensii* plants was performed before oryzalin treatment. This was done because the total number of chromosomes in these plants was unknown. Well-spread chromosomes in metaphase (Figure 2) with clear centromeres were observed in cells from the root tips of acclimatized plants, showing a chromosome number of *M. volkensii* of 2n = 2x = 28 (Figure 2).

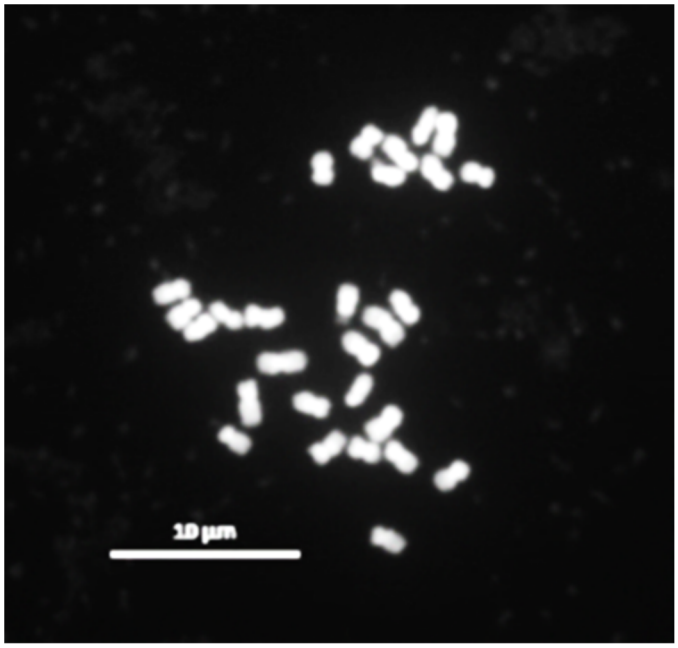

Figure 2. Chromosome number of *Melia volkensii*. Well spread metaphase (2x = 28).

3.2. Induction of Tetraploidy

Shoot growth was observed in both control cotyledons and cotyledons treated with 10 mg·L^{-1} oryzalin. After 21 days in the growth medium, shoots from oryzalin-treated cotyledons were less vigorous than those from untreated cotyledons (Figure 3).

No tetraploids or mixoploids were detected in the control treatments. When oryzalin was applied following TDZ pretreatment for 18 days, incubation for three hours in 10 mg·L^{-1} oryzalin was the most favorable. In the trial where TDZ was not used as a pretreatment, forty percent of the induced shoots were tetraploid (Table 1), whereas only 4.8% were mixoploid plants. Cotyledons treated with TDZ for 18 days followed by 1, 2, and 3 h of treatment with 10 mg·L^{-1} oryzalin resulted in 32.5%, 40%, and 52.5% polyploid plants, respectively (Table 1). A lot of mixoploidy (20%) was also observed. Flow cytometric analysis of nuclei from leaves of diploid *M. volkensii* plants showed a large peak (2x) of diploid nuclei, while the tetraploid peak (4x) was shifted twice (Figure 4) compared to the diploid peak in logarithmic scale of three, FL3 (log3). The mixoploid plants showed both large diploid and tetraploid peaks.

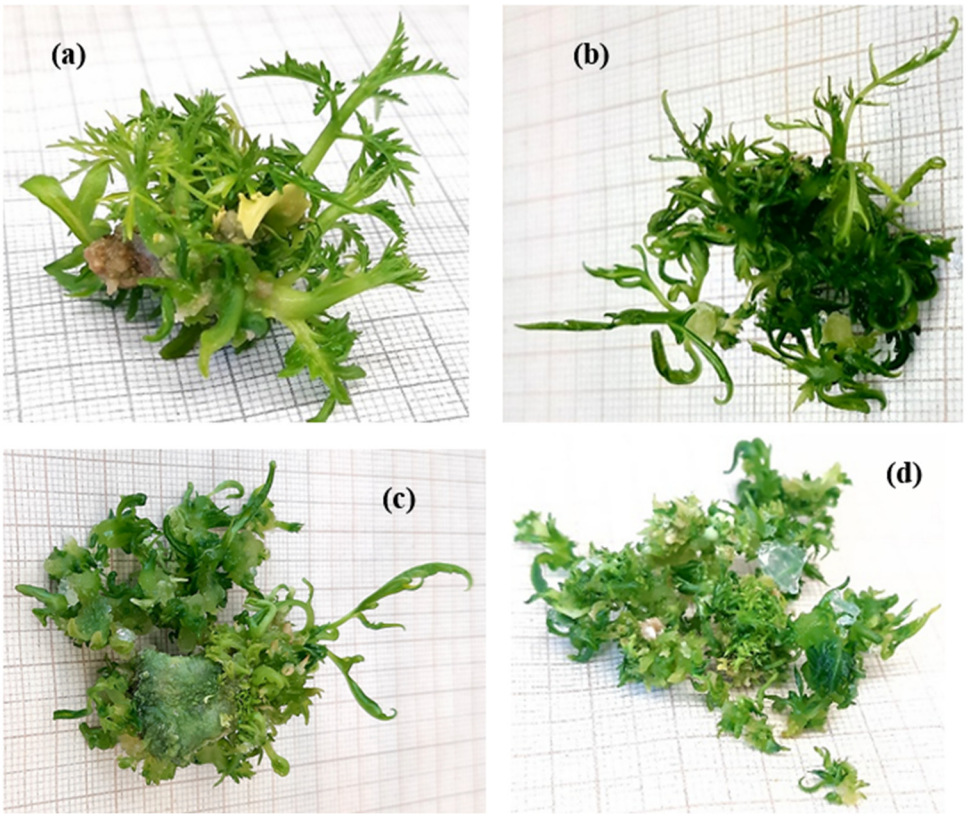

Figure 3. Multiple shoot formation on detached cotyledons after 21 days treatment with 10 mg·L^{-1} oryzalin. (**a**) Induced shoots on untreated cotyledons. (**b**) Induced shoots on oryzalin-treated cotyledons for one hour. (**c**) Induced shoots on oryzalin-treated cotyledons for two hours. (**d**) Induced shoots on oryzalin-treated cotyledons for three hours.

Table 1. Effect of oryzalin on polyploid induction during incubation of fresh and pretreated (TDZ) *Melia volkensii* cotyledons at different time intervals.

TDZ Concentration *	Oryzalin Treatment (Hours) Duration	Analyzedplants	Tetraploid Percentage (%)	Mixoploid Percentage (%)
0 µM	0	20	0.0	0.0
	1	40	12.5	0.0
	2	40	2.5	0.0
	3	40	40.0	4.8
1.1 µM	1	40	32.5	20.0
	2	40	40.0	5.0
	3	40	52.5	20.0

* The concentrations of 0 µM TDZ represents the first experiment in which no TDZ pretreatment was performed, while 1.1 µM TDZ represents the second experiment in which 18 days of TDZ pretreatment were performed prior to oryzalin treatment.

3.3. Morphological Characteristics of In Vitro Tetraploid Melia volkensii

The number of regenerated shoots per explant, leaves per leaf, and shoot internode length did not differ significantly between diploid and tetraploid in vitro plants. During the in vitro phase (Figure 5), polyploidization significantly affected shoot length, shoot diameter, shoot internode length (Table 2), leaf area, and stomatal size (Figure 6). Tetraploid genotypes had significantly ($p < 0.001$) shorter shoots with larger shoot diameters and fewer leaves with much larger leaf area (4.49 cm^2) than diploids (2.96 cm^2). The leaf length of diploid and tetraploid plants was not significantly different. Tetraploid *M. volkensii* had

longer and wider stomata compared to diploid *M. volkensii* (Figure 6B), which were much larger (Figure 7), with an average length of 389.7 µm and a width of 289.1 µm versus a length of 219.9 µm and a width of 188.7 µm in diploids.

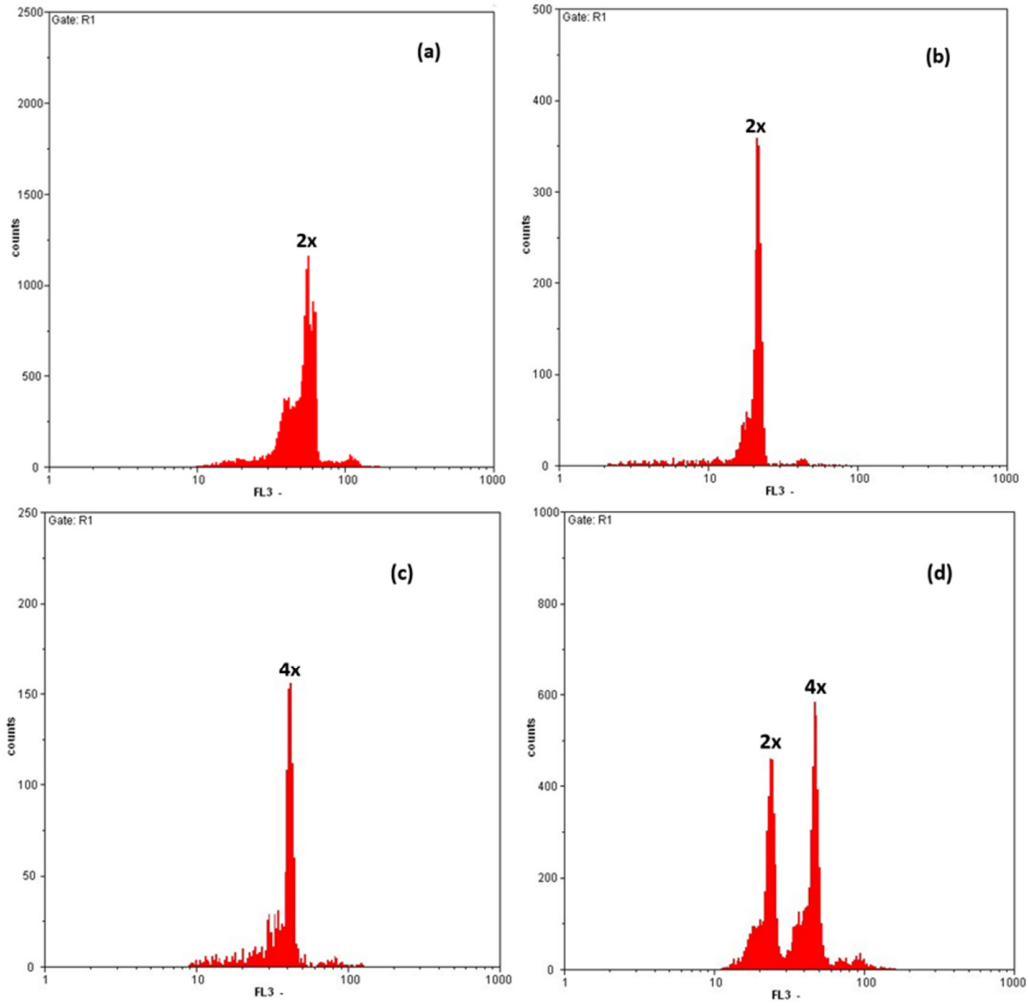

Figure 4. Flow cytometric analysis of nuclei from references and tetraploid *M. volkensii*. (**a**) diploid (2x) *Paulownia tomentosa*. (**b**) Diploid (2x) *Melia volkensii*. (**c**) Tetraploid (4x) *Melia volkensii*. (**d**) Mixoploid *Melia volkensii*.

Table 2. Morphological comparisons of tetraploid and diploid *Melia volkensii* during propagation after 4 weeks in culture medium.

Genotypes	Number of Shoots	Shoot Length (cm)	Shoot Diameter (mm)	Number of Leaves Per Shoot	Number of Leaflets Per Leaflet	Shoot Internode Length (mm)
Diploid	4.73 ± 0.30	4.41 ± 0.27 b	2.36 ± 0.08 a	7.60 ± 0.27 c	7.67 ± 0.25	5.4153 ± 0.24
T192-1	4.73 ± 0.33	3.37 ± 0.19 a	3.14 ± 0.11 b	5.80 ± 0.30 b	7.27 ± 0.27	4.8360 ± 0.23
T482-2	5.07 ± 0.38	3.69 ± 0.16 a	3.10 ± 0.11 b	5.40 ± 0.21 ab	7.60 ± 0.23	4.6500 ± 0.27
T432-1	5.73 ± 0.54	4.33 ± 0.28 b	3.32 ± 0.15 bc	6.07 ± 0.25 b	7.27 ± 0.18	4.2147 ± 0.43
T841-1	5.33 ± 0.48	3.26 ± 0.15 a	3.58 ± 0.19 c	4.87 ± 0.29 a	7.00 ± 0.28	4.5987 ± 0.39
p-value	0.388	<0.001	<0.001	<0.001	0.299	0.129

Note: Means followed by the same letters within a column are not significantly different at $p \leq 0.05$ (Duncan's multiple range test at 5%).

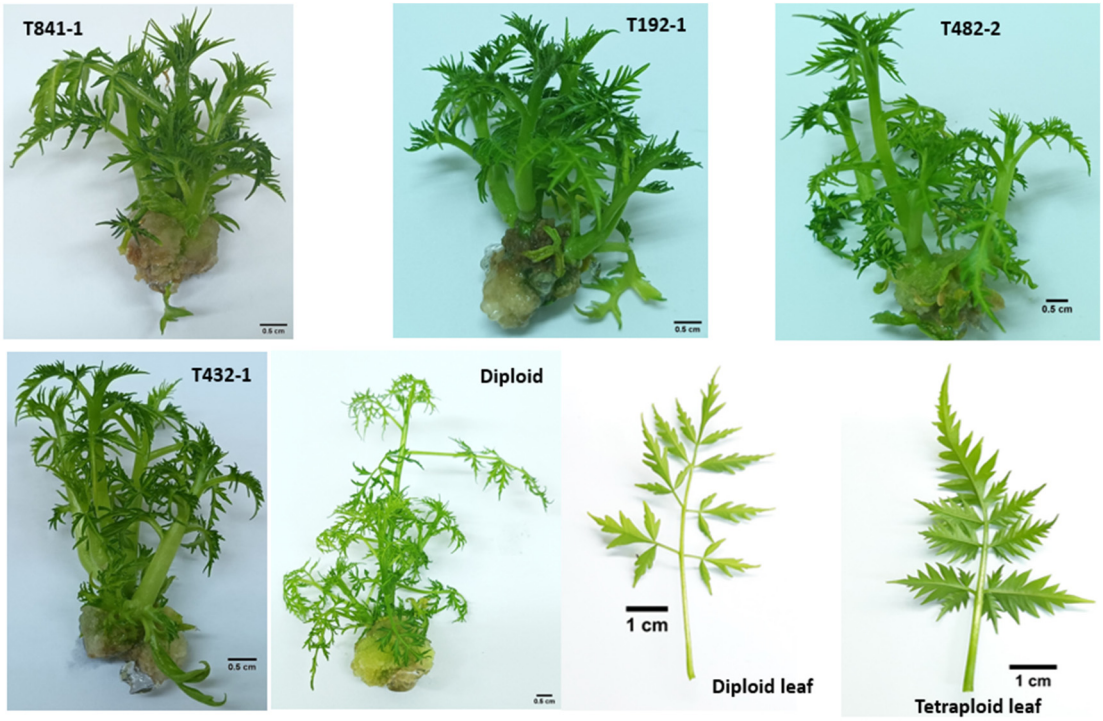

Figure 5. Morphological observation of tetraploid and diploid *Melia volkensii* after 4 weeks in culture medium. T841-1, T192-1, T482-1, and T432-1 are tetraploid genotypes.

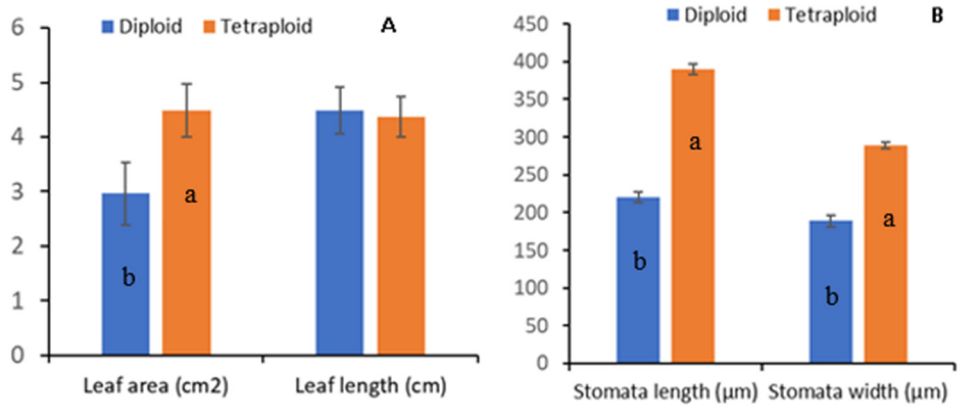

Figure 6. A comparison of tetraploid and diploid leaf and stomata of *Melia volkensii* after four weeks of in vitro propagation. (**A**) Tetraploid and diploid *Melia volkensii* leaf area and length. (**B**) Tetraploid and diploid *Melia volkensii* stomata length and width. Data are reported as mean ± standard error. Note: In the figure, different letters within variables indicate significant differences at 5%.

3.4. Leaf and Stomatal Morphology of Tetraploid and Diploid Melia volkensii under Greenhouse Conditions

The greenhouse-acclimated tetraploid potted plants showed promising growth. Compared to the diploid plant, the tetraploid plant showed distinct leaf and stomatal characteristics (Table 3). The tetraploid plant was short and thick, with broad leaves of 75.74 cm^2, 19.96 cm of leaf length, and a short petiole of 6.13 cm compared to a leaf area of 57.54 cm^2, leaf length of 22.35 cm and petiole of 8.58 cm for diploid *M. volkensii* (Figure 8). Diploid *M. volkensii* developed significantly ($p < 0.05$) longer leaves with elongated petioles than tetraploid *M. volkensii*. Image analysis of stomata showed that the length and width of tetraploid stomata were significantly different from those of diploid *M. volkensii*. On average, stomatal density was lower in tetraploid leaves than in diploid leaves.

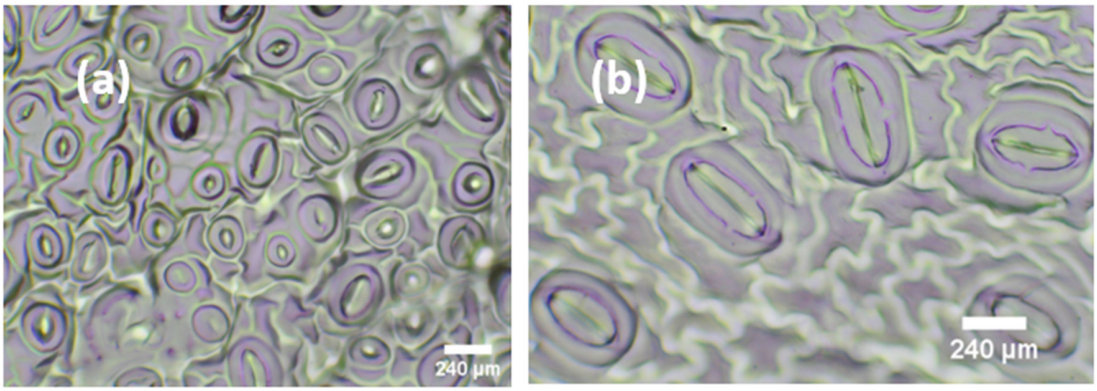

Figure 7. *Melia volkensii* stomata during in vitro propagation. (**a**) diploid; (**b**) tetraploid. Photographs were taken under a microscope at 400× magnification.

Table 3. Leaf and stomatal morphological characteristics of acclimated tetraploid and diploid *Melia volkensii* plants.

Morphological Characteristics	Diploid	Tetraploid
Leaf area (cm^2)	57.54 ± 4.32	75.74 ± 5.09 *
Leaf length (cm)	22.35 ± 0.50	19.96 ± 0.86 *
Petiole length (cm)	8.58 ± 0.27	6.13 ± 0.34 ***
Stomata length (µm)	1024.70 ± 14.12	1360 ± 14.56 ***
Stomata Width (µm)	735.65 ± 10.58	913.12 ± 10.18 ***
Stomata density (per unit area)	22.80 ± 2.52	11.60 ± 2.25 *

* Mean values are significant at $p < 0.05$, *** Mean values are significant at $p < 0.001$.

Figure 8. Leaf morphology of tetraploid and diploid *M. volkensii* plantlets after three months of growth under greenhouse conditions. The leaf image was taken from the third leaf of a tetraploid or diploid plant.

4. Discussion

Before performing the *M. volkensii* chromosomal doubling assay, the chromosome number was counted to ensure that the correct number for diploid was known and to establish a baseline for tracking changes after the assay. In accordance with the "SteamDrop" method [41], our chromosome counts indicate that diploid *M. volkensii* plants have 2n = 2x = 28 chromosomes. To our knowledge, this has never been reported before in the *M. volkensii* species. This number of chromosomes of 2n = 28 is consistent with those of other Meliaceae, such as *Melia azedarach, Trichilia connaroides*, *Walsura trifolia* [45], *Walsura piscida* [46], *Azadirachta indica* [45–47]. In contrast, *Swietenia macrophylla* (2n = 54), *S. mahogani* (2n = 54), *Toona ciliata* (2n = 56), and *Aglaia adorate* (2n = 168), also belonging to the Meliaceae, are probably autopolyploids [46]. Based on the number of chromosomes found in diploid *M. volkensii*, it was hypothesized that the tetraploid plants resulting from polyploidization of this species would have 2n = 4x = 56 chromosomes.

With references to diploid *M. volkensii* and *P. tomentosa*, flow cytometry results showed that tetraploid *M. volkensii* was successfully generated from cotyledons treated with oryzalin. For the induction of polyploidy, cells in mitosis are essential [48] and the type of mitosis inhibitor also plays a role. Jambhale et al. [19] could obtain polyploid *M. azedarach* with 0.5% colchicine. However, treatment with oryzalin resulted in more polyploid plants than with colchicine [23,26,49]. The number of polyploid *M. volkensii* plants obtained varied with the duration of oryzalin treatment. Similar findings were reported by Miguel and Leonhardt [50] who showed that the duration of oryzalin treatment increased the number of polyploid plants produced in orchids. The highest polyploid induction of 52.5% was recorded in cotyledons of *M. volkensii* that were pretreated with TDZ for 18 days and then treated with oryzalin for three hours. However, the disadvantage was that more ploidy chimeras were formed. The increased production of tetraploids and mixoploids may be a result of oryzalin hitting the dividing cells of the developing adventitious bud in cotyledons treated with TDZ. Thidiazuron has been reported to increase the induction of adventitious bud during in vitro propagation [38,51–53]. The mixoploids obtained were not considered for propagation because creating of homogeneous tetraploids out of these mixoploids would require another adventitious shoot regeneration step [16]. The findings indicate that treating a responding cell with oryzalin increases the number of polyploid plants produced.

The tetraploid *M. volkensii* had remarkably shorter leaves, larger shoot diameter, and wider leaves than the diploid specimens. This is consistent with previous reports in *Juncus effusus* [54] and *Gerbera jamesonii* Bolus cv. Sciella [55] and *Ligustrum japonicum* [11]. In contrast, Jambhale et al. [19] stated that the polyploid *M. azedarach* had smaller leaves than the diploid variety. There was no significant difference in leaf length between tetraploid and diploid plants. Li et al. [44] reported similar findings for the leaf length of *Bletilla striata* in vitro culture. In contrast, there was a slightly significant difference in leaf length between tetraploid and diploid *M. volkensii* plants in pots. This variation in leaf length could be due to species and growing conditions. Similar findings regarding the leaf length of diploid and tetraploid *Escallonia* species were reported by Denaeghel et al. [29]. Stomata of the tetraploid *M. volkensii* were significantly larger with greater length and width than those of the diploid. Similar results of larger stomata in tetraploids than in diploids have been reported in *Paulownia tomentosa* [56] and *Gerbera jamesonii* Bolus cv. Sciella [55], *Eucommia ulmoides* [57] and apple [28]. Similar findings were reported by Tang et al. [56], who reported that tetraploid *P. tomentosa* plants could be distinguished from diploid ones by virtue of their longer stomata and reduced stomata frequency.

Tetraploid Mukau trees probably will perform better in a variety of environmental conditions than their relative diploid. In addition, polyploid Mukau could produce timber with superior wood quality and properties. In an 18-year field trial and growth chamber experiment, it was observed that polyploid *Acacia senegal* (L.) showed not only large leaf and stomata but also an adaptive advantage in drought tolerance with greater height and trunk diameter [33]. Drought tolerance in an autotetraploid apple clone (Redchief) was reported with only minimal variation in growth, and it also showed superior resistance to fire blight [58]. Tetraploid *Robinia pseudoacacia* L. clones adapted to drought by reducing

intercellular CO_2, net photosynthetic rate, and stomatal conductance more than diploid clones [59]. Tetraploid *F. carica* clones showed higher tolerance to water stress by maintaining relative water content while producing high levels of stress hormones, enhancing the enzymatic defense system, and adjusting osmotic pressure [60]. Polyploid eucalyptus hybrids are larger in size, have more fibers, and have thicker walls compared to diploid relatives [61]. It is also possible that tetraploid *M. volkensii* plants possess higher quantities of medicinal and biopesticides chemicals than diploid plants. Navrátilová et al. [62] discovered that tetraploid *Ajuga reptans* plants have a higher concentration of phytochemicals than diploid ones.

5. Conclusions

The altered morphology of these tetraploid trees could be very interesting and will be followed under field conditions for many years. The future will show to what extent their wood quality will change and if their stress resistance will be affected. We expect that the plants produced in this study will form the basis for new breeding programs and produce trees with a new phenotype.

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