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Induction of Polyploidy in *Dalbergia latifolia* Roxb. using Oryzalin

(Peninduksian Poliploidi dalam *Dalbergia latifolia* Roxb. menggunakan Oryzalin)

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ABSTRACT

The demand for rosewood wood keeps increasing, while the availability of large-diameter rosewood trees is becoming scarce. Rosewood is classified as vulnerable by the International Union for Conservation of Nature and Natural Resources (IUCN) and listed in Appendix II of Convention of International Trade of Endangered Species (CITES). The objective of this research was to develop a method to obtain fast-growing and more vigorous rosewood seedlings for quicker harvest through polyploidy induction using oryzalin. This experiment successfully produced tetraploid rosewood through polyploidy induction from seedlings. Polyploidy induction was carried out by soaking rosewood germinated seeds for 12 and 24 h in oryzalin solutions at concentrations of 0, 3.75, 7.5, 15, 30, 45, and 120 µM. The germinated seeds were then planted in a growth medium and maintained for 18 months. Ploidy level identification of those seedlings was conducted using flow cytometry and confirmed with stomata characterizations. Two accessions of tetraploid rosewood were obtained from the treatment of 15 µM oryzalin for 12 and 24 h soaking, while seven mixoploids were obtained by treatment of 12 h at various oryzalin concentrations. Induced tetraploid seedlings had longer stomata but lower stomata density and thicker leaves than their diploid counterparts. Both induced tetraploid and mixoploid rosewood seedlings did not exhibit greater vegetative vigor than their diploids up to 18 months of age. Therefore, oryzalin induced polyploidy, mixoploid and tetraploid, but it did not produce faster growing seedlings or better habitus of rosewood compared to their diploids, at least up to 18 months.

Keywords: Mixoploid; polyploidy; rosewood; seedling; tetraploid; vulnerable plant

ABSTRAK

Permintaan terhadap kayu *rosewood* terus meningkat, sementara ketersediaan pokok *rosewood* berdiameter besar semakin berkurangan. *Rosewood* dikelaskan sebagai rentan oleh Kesatuan Antarabangsa untuk Pemuliharaan Alam Semula Jadi dan Sumber Daya (IUCN) dan disenaraikan dalam Lampiran II Konvensyen Perdagangan Antarabangsa Spesies Terancam (CITES). Oleh itu, perdagangannya dihadkan oleh kuota. Justeru, campur tangan teknologi diperlukan untuk menghasilkan anak benih *rosewood* yang cepat tumbuh untuk penuaian yang lebih cepat. Objektif penyelidikan ini adalah untuk membangunkan dan mendapatkan anak benih *rosewood* yang cepat tumbuh dan lebih vigor melalui induksi poliploidi menggunakan oryzalin. Uji kaji ini berjaya menghasilkan tetraploid *rosewood* melalui induksi poliploidi daripada anak benih. Induksi poliploidi dilakukan dengan merendam anak benih *rosewood* selama 12 dan 24 jam dalam larutan oryzalin pada kepekatan 0, 3.75, 7.5, 15, 30, 45 dan 120 µM. Anak benih kemudian ditanam dalam medium pertumbuhan dan dipelihara selama 18 bulan. Pengenalpastian tahap ploidi anak benih dilakukan menggunakan sitometri aliran dan disahkan dengan pencirian stomata. Dua penerimaan tetraploid *rosewood* diperoleh daripada rawatan oryzalin 15 µM selama 12 dan 24 jam perendaman, manakala tujuh miksoploid diperoleh daripada rawatan 12 jam pada pelbagai kepekatan oryzalin. Anak benih tetraploid yang diinduksi mempunyai stomata yang lebih panjang tetapi kepadatan stomata yang lebih rendah dan daun yang lebih tebal berbanding anak benih diploid. Kedua-dua anak benih tetraploid dan miksoploid yang diinduksi tidak menunjukkan daya vegetatif yang lebih besar berbanding rakan diploid mereka sehingga umur 18 bulan. Oleh itu, poliploidi berhasil diinduksi oleh oryzalin, sama ada mixoploid dan

tetraploid, tetapi tidak menghasilkan anak benih *rosewood* yang tumbuh lebih cepat atau lebih baik berbanding rakan diploidnya, sekurang-kurangnya sehingga usia 18 bulan.

Kata kunci: Anak benih; miksoploid; poliploidi; *rosewood*; tetraploid; tumbuhan rentan

INTRODUCTION

Rosewood (*Dalbergia latifolia* Roxb.) belongs to the Fabaceae family. It is commonly found in tropical forests in India, Myanmar, Laos, and Thailand and scattered throughout Indonesia, primarily on the island of Java (Kertadikara & Prat 1995), and partly on other islands such as West Nusa Tenggara (Kusumadewi et al. 2022). Rosewood heartwood has a distinctive pattern, ranging from dark brown to blackish or purplish with white or black streaks. Rosewood wood also features longitudinal purple streaks clearly delineated by white sapwood, making it visually appealing. Therefore, the wood is widely used for quality furniture, ship interior, and aircraft components, decorative items with intricate carvings, such as jewelry boxes, wall decorations, and veneers (Arunkumar et al. 2022; Joker 2004; Prawirohatmodjo et al. 1994). Its strength and durability make it suitable for construction materials like frames, doors, windows, tools such as hoes, hammers, sharp weapons, and musical instruments like guitars. Additionally, rosewood is known for its resistance to termites and wood-decaying fungi (Prawirohatmodjo et al. 1994).

The beautiful and high-quality rosewood's timber has led to high demand. This phenomenon has resulted in excessive harvesting, threatening the sustainability of the rosewood population (Kementerian Kehutanan dan Lingkungan Hidup 2017). Since 1998, rosewood has been classified as vulnerable (VU) based on the IUCN Red List of Threatened Species (Lakhey, Pathak & Adhikari 2020). Moreover, since 2017, rosewood has been listed in Appendix II of CITES, meaning that the trade of rosewood wood is subject to quota limitations and strict licensing requirements (CITES 2017).

Cultivation of rosewood is needed as an alternative source of wood material to harvesting from nature. In nature, rosewood can propagate naturally through shoots that grow from roots (Vasudevan et al. 2020) and seeds (Arunkumar et al. 2022). However, propagation from seeds is reported to be less successful, as rosewood seeds have a low germination rate of 30-40% (Kumar et al. 2014; Prawirohatmodjo et al. 1994). Based on this problem, vegetative propagation becomes a choice. Vegetative propagation through root cuttings has been reported (Vasudevan et al. 2020). Vegetative propagation is beneficial for increasing superior seedlings having been identified, thus obtaining plantations with high production (Adinugraha et al. 2021). Identification of parent trees to obtain superior seedlings in rosewood has been conducted (Riastiwi et al. 2022). Additionally, the productivity of forest tree plantations can also be enhanced through conventional breeding to produce superior varieties (Kang 2020), as well as through somatic cell manipulation approaches, such as mutation or polyploidy induction (Abdolinejad, Shekafandeh & Jowkar 2021), aimed at obtaining tetraploid plants.

Tetraploid plants, which have four sets of chromosomes (Kurtz, Brand & Lubell-Brand 2020), exhibit larger stem diameters (Diallo et al. 2008; Longui et al. 2021), and in *Acacia mangium*, they produce pulp with longer fibers compared to their diploid counterparts (Griffin et al. 2014). Furthermore, compared to diploid plants, tetraploid plants are more adapted to environmental stresses, such as drought tolerance in teak (Ridwan et al. 2018) and in *Populus ussuriensis* (Xu et al. 2019), as well as salt tolerance in *Acacia senegal* (Diallo et al. 2022). Tetraploid plants are reportedly more resistant to biotic stresses such as pests and diseases (Li et al. 2019). On the other hand, changes resulting from tetraploid induction can have adverse effects. For example, tetraploid poplar plants experience a decrease in photosynthesis rate, carbohydrate synthesis, and decomposition abilities (Xu et al. 2020).

This study reports the successful induction of tetraploid rosewood using oryzalin for the first time. The tetraploidy of these seedlings was confirmed through flow cytometry, stomatal density and size assessment, and overall plant survival evaluation.

MATERIALS AND METHODS

MATERIALS

The rosewood seeds used were obtained from Ngawi Regency, East Java, and harvested as dry pods in August 2020, six months before their use in the experiment. These seeds were stored in an incubator cabinet (Incubate Box M-230FN, Taitec) at a temperature of 5 °C, following Riastiwi et al. (2023) for *Moringa oleifera*. The rosewood seeds were cleaned and washed using liquid soap. Subsequently, the seeds were soaked in a mixture of bactericidal solution (Agrept, containing active ingredient streptomycin sulfate 20%) and fungicide (Masalgin, containing active ingredient benomyl 50.4%) each at 2 gL^{-1} for 1 h. The seeds were then rinsed three times with sterile distillated water. Afterwards, the seeds were germinated on Petri dishes lined with kitchen paper towels and 10 mL of sterile distilled water. Germinated seeds with 2-4 cm roots (seedlings) of 8-10 days old were used for tetraploid induction with oryzalin.

POLYPLOIDY INDUCTION

The treatment consisted of two factors: oryzalin concentration (0, 3.75, 7.5, 15, 30, 45, and 120 µM) and soaking duration (12 and 24 h). A total of 100 germinated seeds, 8-10 days old having 2-4 cm roots length, were used for each treatment. The germinated seeds were soaked in 250 mL oryzalin solution in 1000 mL Erlenmeyer flasks according to the concentration and soaking duration treatment. The treated germinated seeds were left stationary and stored in a room at 20-25 °C with a relative humidity of 60-80% and diffuse lighting from room fluorescent lamps. After treatment, the rosewood germinated seeds were planted in plastic trays (37 × 30 × 12 cm) filled with planting medium to 10 cm from the base of the tray with a composition mixture of soil, charcoal rice husk, and manure (2:1:1). These plastic trays were placed in a greenhouse with 55% shade. The growing shoots were then sprayed with a 2 gL^{-1} Growmore solution (15N-15P-15K) using a sprayer. After 8 weeks, the seedlings were transferred to larger polybags (15 × 15 cm) and placed in the greenhouse with 55% shade for growth observation.

PLANT GROWTH OBSERVATION

Phenotypic observations on the plants resulting from ploidy induction included the percentage of surviving plants, plant height, number of nodes and compound leaves, ploidy level, stomatal density and size, and leaf thickness. The rate of surviving plants is calculated from the number of seedlings at 1 and 4 weeks of age divided by the initial number of seedlings in the treatment multiplied by 100%. Plant height and number of compound leaves were observed in all plants when the rosewood seedlings were 14 months old.

Eighteen months after polyploidy induction, their ploidy was determined. The determined mixoploid, tetraploid, and the control diploid were again observed for data recording. Data recording included several parameters: plant height (cm), number of nodes, number of leaves, leaflet area (cm^2), and stem diameter (cm). Plant height was measured from the base of the stem to the tip of the tallest leaf. The number of nodes included all nodes on the stem. The number of compound leaves included all fully opened leaves. Leaflet area measurement was calculated from the length and width of the leaflet. Stem diameter was measured on the plant stem at 5 cm from the base of the stem using calipers.

PLOIDY ANALYSIS WITH FLOW CYTOMETRY

The ploidy level resulting from induction was analyzed using the Attune NxT Flow Cytometer (Invitrogen, Waltham, MA, USA). Polyploid-induced plants that had been maintained for 18 months and showed different morphology indications were selected as samples for the analysis. As many as 327 plant seedlings were included in the ploidy determination. Additionally, only 30 plant seedlings from the untreated seeds served as controls.

Samples of young leaves measuring 1 cm^2 were placed in a 55 × 15 mm glass Petri dish, treated with 250 µL of 'CystainTM PI absolute P' reagent kit (Sysmex 05-05022), and finely chopped with a razor blade to release the nuclei from the cells and suspend them in the solution. The nucleus suspension was filtered from the leaf fragments using a cellTrics TM 30 µm filter and transferred to a cuvette tube. A 500 µL of staining solution Sysmex 05-05022 (2 mL staining buffer, 12 µL Propidium Iodide, and 6 RNase A) was added to the nucleus suspension. Subsequently, the cuvette containing the nucleus sample was placed in the flow cytometry sample port for analysis. Rosewood diploid leaf samples were set as references with the relative DNA peak histogram value set at channel 200. If the relative DNA peak histogram value was at channel 400, it indicated that the sample originated from a tetraploid plant. Meanwhile, a histogram displaying two peaks at channels 200 and 400 simultaneously indicated a mixoploid plant. The results of the flow cytometry analysis were presented in graphical form using Attune NxT software.

STOMATAL OBSERVATION

Stomatal observation was conducted on 18-month-old plants confirmed as tetraploid, mixoploid, and diploid using flow cytometry. The third leaf (consisting of 3 leaflets) from the shoot apex was selected as the sample. The observation was performed by applying clear nail polish (Revlon) to the lower surface of the leaf. Once the nail polish dried, transparent adhesive tape was attached to the leaf to remove the dried nail polish, having imprinted the lower leaf surface. The adhesive tape with the leaf surface imprint was mounted on a microscope slide. Stomatal imprints were observed at 400× magnification using an inverted Nikon Eclipse TS100 microscope (Nikon, Tokyo, Japan). Images of the leaf surface imprints were visualized on the LCD screen of an Indomicro Digital Camera model 2 MP (1920 × 1080) using IndomicroView N 23 software. Stomatal density was calculated in three observation fields on the LCD screen, measuring per mm^2 and randomly selected from each stomatal preparation. Subsequently, three stomata were randomly chosen from each field of view to measure their length.

LEAF THICKNESS OBSERVATION

Leaf thickness was measured on the 18-month-old plants and confirmed as tetraploid, mixoploid, and diploid. The third leaf (consisting of 3 leaflets) from the shoot apex was selected. The sample leaf was cut transversely and then placed on a microscope slide. Leaf thickness was observed using a Nikon SMZ 1000 stereo microscope (Nikon, Tokyo, Japan) and visualized on the LCD screen of

an Indomicro Digital Camera model 2 MP (1920 × 1080) using IndomicroView N 23 software.

Data Analysis

The data were presented as means and standard errors and visually represented in histograms. Histograms were created using SigmaPlot 15.0, and the data were analyzed using two-way ANOVA in IBM SPSS Statistics 21 at a confidence level of 95% and differences among means were detected with DMRT (Duncan Multiple Range Test) at the same confidence level.

RESULTS

SURVIVAL AND GROWTH OF THE ORYZALIN TREATED PLANTS

Oryzalin treatment affected the survival of rosewood seedlings (Figure 1), but the treatment did not decrease the survival rate at the tested concentrations. In the control treatment, soaking the seedlings increased the survival percentage with increasing soaking duration, but the opposite occurred with oryzalin concentrations. Generally, the seedling survival rate decreased with increasing soaking duration from 12 to 24 h. Soaking the seedlings in oryzalin affected survival and seedling growth up to 4 weeks after treatment, with the overall percentage of seedling growth reduced in the 4th week compared to the 1st week.

The treatment combination of soaking duration and oryzalin concentration did not show any interaction in all growth variables, namely plant height (P = 0.266), number of nodes (P = 0.207), and number of leaves (P = 0.299). However, the oryzalin concentration treatment as a single factor significantly influenced plant height (P = 0.003) (Table 1), with a response that tends to be parabolic with the optimum concentration 7.5 µM. Meanwhile, soaking duration did not significantly affect plant height (P = 0.194) but significantly affected the number of nodes (P = 0.027) and number of leaves (P = 0.05).

PLOIDY ANALYSIS BASED ON FLOW CYTOMETRY

Flow cytometry analysis of leaf samples from oryzalin-treated plants compared to untreated controls is presented in Figure 2. In the diploid control, the graph's peak indicates a relative DNA content of 198.48 (Figure 2(A)). Meanwhile, the graph's peak in the tetraploid sample is 402.68, with a covariance (CV) value of 4.49% (Figure 2(B)). The peak of the mixoploid sample is slightly higher than the diploid and tetraploid samples, with relative DNA content peaking at 202.91 and 407.49, respectively, with CV values of 8.62% and 6.22%, respectively (Figure 2(C)).

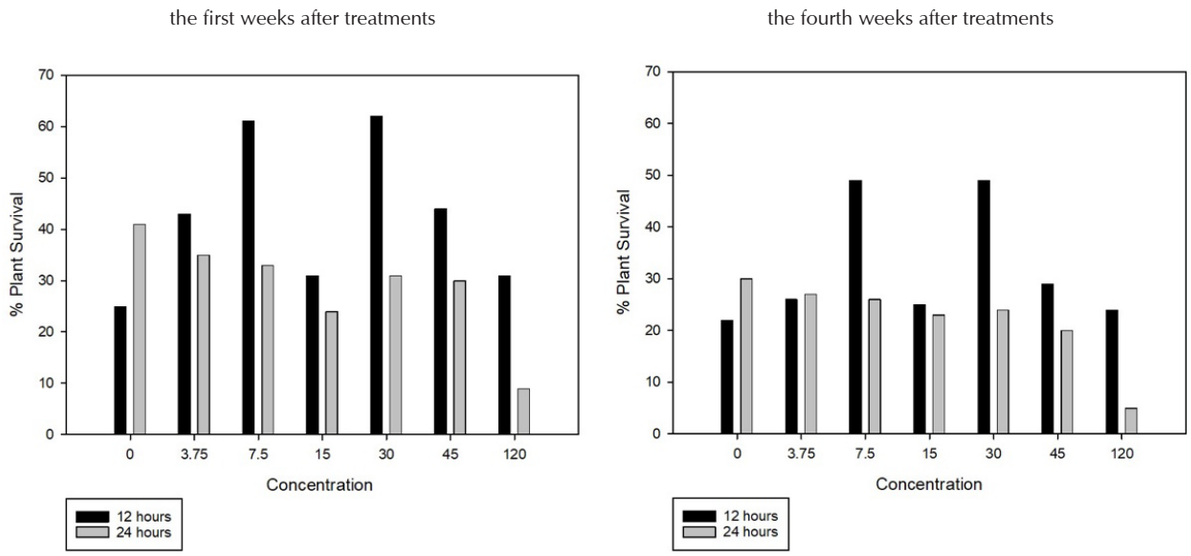

FIGURE 1. The effect of oryzalin concentrations of 0, 3.75, 7.5, 15, 30, 45, and 120 µM and soaking duration of 12 and 24 h on the percentage of plant survival at the first and the fourth weeks after treatments. There were 100 germinated seeds per treatment

TABLE 1. The effect of concentration and soaking duration of rosewood seedlings in oryzalin solution on the average growth of 14-month-old rosewood plants

Oryzalin concentration (µM)	Plant height (cm)	Number of nodes	Number of leaves
0	23.91 ± 1.41 ab	11.44 ± 0.64	10.31 ± 1.01
3.75	19.72 ± 1.23 bc	10.38 ± 0.72	10.31 ± 0.82
7.5	25.22 ± 1.54 a	11.75 ± 0.71	11.19 ± 0.67
15	17.66 ± 1.77 c	8.94 ± 1.17	8.06 ± 0.99
30	18.63 ± 1.57 c	11.00 ± 0.95	11.44 ± 1.02
45	22.06 ± 1.33 abc	10.94 ± 0.64	10.94 ± 0.57
120	18.09 ± 3.12 c	9.38 ± 1.67	9.63 ± 1.58
Soaking 12 h	19.97 ± 0.37	9.80 ± 0.19b	9.57 ± 0.24b
Soaking 24 h	21.54 ± 0.60	11.29 ± 0.24a	10.96 ± 0.20a

Data presented as mean ± standard error. Different letters in the same column indicate significant differences at p≤0.05 according to DMRT

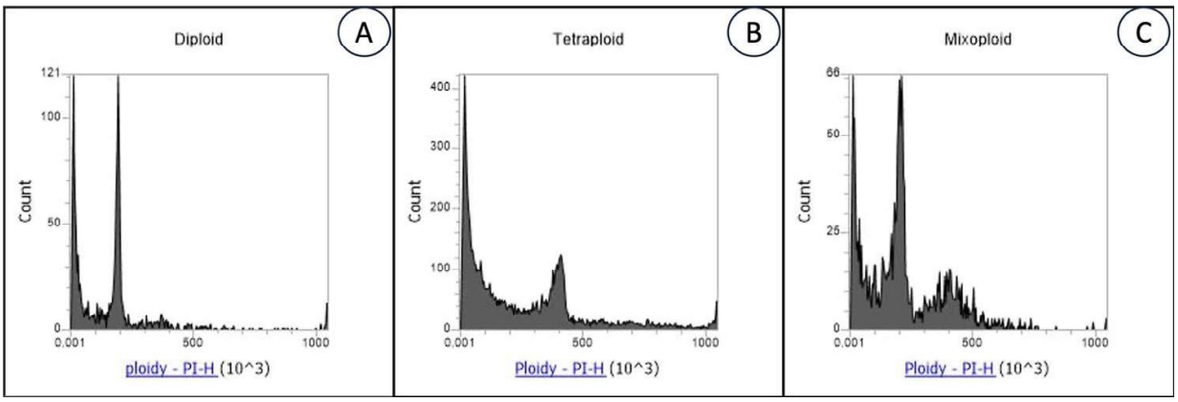

FIGURE 2. Flow cytometer analysis of selected plants from oryzalin-treated and untreated control: (A) diploid clone in untreated control peaks at 198.48 with a CV of 5.80%.(B) tetraploid clone peaks at channel 402.68with a CV of 4.49%. (C) mixoploid clone produces two peaks at 202.91 and 407.49 with CV values of 8.62% and 6.22%, respectively

POLYPLOIDY STABILITY

After the soaking treatment with oryzalin, most plants did not survive, particularly during the acclimatization and maintenance in the nursery. Only 379 plants survived out of the initial treated 1400 g seedlings. Among these, 52 plants were from the control group, and the remaining 327 plants were from the oryzalin treatment. The ploidy levels of 327 plants were analyzed using a flow cytometer as described above. The flow cytometer analysis successfully identified 2 tetraploid plants and 7 mixoploid plants (Table 2). The two tetraploid plants were obtained from the treatment of 15 µM oryzalin and soaking for 12 and 24 h. Meanwhile, the 7 mixoploid plants were obtained from the oryzalin treatment with concentrations ranging from 7.5 to 120 µM and 12 h-soaking times in 5 plants, and only 2 mixoploid plants were obtained from 24 h-soaking times (Table 2).

STOMATA SIZE AND DENSITY IN DIPLOID, TETRAPLOID, AND MIXOPLOID ROSEWOOD PLANTS

The average length of stomata in tetraploid plants was 12.92 ± 0.17 µm, which was longer than mixoploid plants (10.73 ± 0.27 µm) and significantly longer than diploid controls (10.59 ± 0.29 µm) with (P = 0.00) (Table 3). Tetraploid plants have stomata that are approximately 20% longer than those of diploid plants. The stomata density of tetraploid plants (120.87 ± 6.53 per mm^2) was significantly lower (Pr>F=0.00) than that of the diploid plants (180.13 ± 2.67 cells per mm^2).

LEAF THICKNESS IN DIPLOID, TETRAPLOID, AND MIXOPLOID ROSEWOOD PLANTS

The leaf thickness among diploid, tetraploid, and mixoploid plants significantly differs (P = 0.00). The tetraploid rosewood leaf is thicker than both diploid and mixoploid counterparts (Figure 3). Compared to the diploid leaves, the tetraploid leaves had an average about 46% thicker, while the mixoploid leaves was just 1.5% thinner (Table 4).

MORPHOLOGY OF DIPLOID CONTROL, TETRAPLOID, AND MIXOPLOID PLANTS IN THE NURSERY

Observations on the morphology of tetraploid plants in the nursery showed no growth improvement over the diploid counterparts. They were significantly shorter and had fewer nodes, fewer leaf numbers and leaf areas, and fewer stem diameters (Table 5, Figure 4). Similarly, the mixoploid plants tend to have smaller growth variables than the diploid counterparts, particularly in the number of nodes and leaves (Table 5, Figure 4).

TABLE 2. Flow cytometer observation results from 357 leaf samples

Oryzalin concentration (μM)	Soaking duration (h)	Number of Samples	Number of polyploids		
			Diploid	Tetraploid	Mixoploid
0 (control)	12	15	15	0	0
3.75	12	26	26	0	0
7.5	12	49	48	0	1
15	12	25	24	1	0
30	12	49	47	0	2
45	12	29	28	0	1
120	12	24	23	0	1
0 (control)	24	15	15	0	0
3,75	24	27	27	0	0
7,5	24	26	26	0	0
15	24	23	22	1	0
30	24	24	24	0	0
45	24	20	18	0	2
120	24	5	5	0	0
Total		357	348 (97.48%)	2 (0.56%)	7 (1.96%)

TABLE 3. Stomata size and density in diploid control, tetraploid, and mixoploid plants

Ploidy level	Stomata length (μm)	Stomata density/mm^2
Diploid	10.59 ± 0.29b	180.13 ± 2.67a
Tetraploid	12.92 ± 0.17a	120.87 ± 6.53b
Mixoploid	10.73 ± 0.27b	105.67 ±2.40c

Stomatal density = number of stomata per mm^2. Data are presented as mean ± standard error. Different letters in the same column indicate significant differences at p≤0.05 DMRT

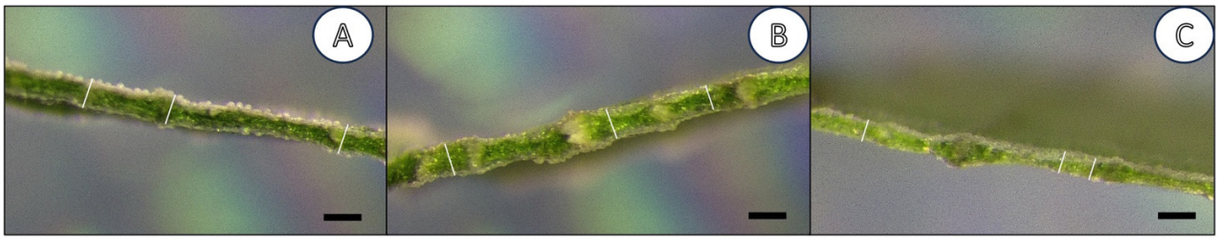

FIGURE 3. Leaf thickness of rosewood plants: (A) diploid plant, (B) tetraploid plant, (C) mixoploid plant. Bar=100 mm

TABLE 4. Leaf thickness among diploid control, tetraploid, and mixoploid plants

Ploidy level	Average leaf thickness (μm)	Difference compared to diploid control (%)
Diploid	67 ± 5 b	0
Tetraploid	98 ± 3 a	46
Mixoploid	66 ± 3 b	-1.5

The smallest leaf thickness scale is in millimeters. Different letters in the same column indicate a significant difference at $p \leq 0.05$ according to DMRT

TABLE 5. Growth of diploid, tetraploid, and mixoploid rosewood plants at 18 months of age

Growth variables	Ploidy level		
	Diploid (n=5)[1]	Tetraploid (n=2)	Mixoploid (n=7)
Plant height (cm)	14.3 ± 1.2 [2]	12.0 ± 0.4	12.1 ± 1.1
Number of node	9.8 ± 0.6	8.0 ± 0.0	8.4 ± 0.4
Number of leaves	5.4 ± 0.6	4.5 ± 0.5	3.1 ± 0.5
Leaf area (cm^2)	2.7 ± 0.5	1.6 ± 0.8	2.2 ± 0.5
Stem diameter (cm)	0.20 ± 0.0	0.18 ± 0.0	0.20 ± 0.0

[1]n = number of samples. Five diploid plant samples were randomly selected from the control treatment of water immersion

[2]Presented as mean values and standard errors

FIGURE 4. Morphology of rosewood plants: (A) diploid plant, (B) tetraploid plant, (C) mixoploid plant. Bar=10 cm

DISCUSSION

As an anti-mitotic agent, Oryzalin has long been used to induce tetraploid plants. Oryzalin has been reported to be effective in inducing tetraploidy in several woody plants, such as *Eucalyptus urophylla* (de Moura et al. 2020), *Moringa oleifera* (Ridwan & Witjaksono 2020), and herbaceous plants like bananas (Poerba et al. 2019a, 2019b, 2014; Van Duren et al. 1996) and taro (Handayani et al. 2023). We have successfully obtained tetraploid and mixoploid rosewood seedlings using a similar method, which involves soaking seedlings in an oryzalin solution for a specific period. Of 327 surviving seedlings, 2 were tetraploid, and 7 were mixoploid. This result represents the first report of successful tetraploid induction in rosewood seedlings, a perennial tree, as confirmed by flow cytometry analysis, stomatal size, and density.

Tetraploid induction in rosewood has been attempted repeatedly (unpublished data). Some of these treatments included oryzalin at concentrations of 0, 30, 60, 120, 240, and 480 µM with a soaking duration of 1 day, resulting in no surviving seedlings beyond 120 µM concentration. Subsequent treatments at concentrations of 0, 3.75, 7.5, 15, 30, 60, and 120 µM with soaking durations of 1 day, 3 days, and 5 days resulted in no seedlings surviving beyond 1 day of soaking. Further treatments at concentrations of 0, 3.75, 7.5, 15, 30, 45, and 120 µM with a soaking duration of 1 day yielded 1 mixoploid rosewood plant. However, this mixoploid rosewood plant eventually died due to its weak condition. Based on these preliminary studies, we conducted oryzalin treatments with soaking durations of 12 and 24 h.

However, the oryzalin induction treatment resulted in a decrease in the survival rate of rosewood seedlings. The percentage of seedling survival also decreased with longer soaking durations from 12 to 24 h. The effect of this decrease in growth percentage persisted for up to 4 weeks after treatment. The soaking duration and the effective concentration of oryzalin for inducing tetraploidy varies in different plants. For example, in *Rhododendron fortune*, the most effective soaking time with oryzalin was found to be 16 h at a concentration of 2.60 µM (Lan et al. 2020), while tetraploid induction in *Lilium rosthornii* was achieved with a 1-day soaking at a concentration of 34.6 µM (Wang et al. 2020). In *Dendrobium officinale*, a 1-day soaking with a concentration of 14.4 µM was effective in obtaining tetraploid plants (Zhang & Gao 2020). Meanwhile, for *Mentha spicata*, a soaking duration of two days with a concentration of 40 µM was influential in obtaining polyploid plants (Bharati et al. 2023). In *Populus* sp., a 3-day soaking with a concentration of 50 mg L^{-1} was declared effective for polyploidy induction (Ren, Jing & Kang 2021). The most effective soaking duration for inducing tetraploidy in rosewood seedlings was 12 and 24 h at an oryzalin concentration of 15 µM. An effective soaking duration for tetraploidy ranging from 12-24 h has also been reported in *Agastache foeniculum* (Talebi et al. 2017), *Cannabis sativa* (Parson et al. 2019), *Thymus vulgaris* (Navrátilová et al. 2021), and *Acacia crassicarpa* (Lam, Harbard & Koutoulis 2014).

The effective concentration of oryzalin for inducing rosewood tetraploidy of 15 µM is relatively low compared to the concentrations used for other plants. For instance, the concentration of oryzalin used to induce polyploidy in hebe 'Oratia Plant Beauty' is relatively high at 289 µM (Gallone, Hunter & Douglas 2014). However, in the case of *Hylocereus megalanthus* (Tel-Zur et al. 2011), only a concentration of 0.7 µM is needed to induce plant polyploidy.

The low percentage of plant survival after soaking in oryzalin treatments indicates that oryzalin is toxic to rosewood seedlings. In addition to oryzalin, colchicine is also commonly used to induce plant polyploidy (Petersen, Hagberg & Kristiansen 2003; Talebi et al. 2017). However, oryzalin is preferred over colchicine for tetraploidy induction because it is effective at lower concentrations (Ganga & Chezhiyan 2002; Kanchanapoom & Koarapatchaikul 2012; Van Duren et al. 1996) and has lower toxicity levels (Dhooghe et al. 2009; Ramulu, Verhoeven & Dijkhuis 1991). In the case of *Ranunculus asiaticus*, oryzalin demonstrated more significant toxicity than trifluralin and colchicine (Dhooghe et al. 2009). In *Cnidium officinale*, a concentration of 4 mgL^{-1} oryzalin can induce tetraploidy. However, concentrations surpassing 4 mgL^{-1} show toxic effects (Kim et al. 2021).

Compared to diploid seedlings, tetraploid rosewood showed morphological differences at the microscopic level, namely lower stomatal density, larger stomata size, and thicker leaves. The characteristic increase in stomatal size and decrease in density in tetraploid plants compared to their diploid counterparts was also evident in induced tetraploid water spinach (kangkong) plants (Rahmi, Witjaksono & Ratnadewi 2019), induced tetraploid teak (Ridwan et al. 2018), several induced tetraploid banana varieties (Poerba et al. 2019a, 2019b), induced tetraploid *Melia volkensii* (Dushimimana et al. 2023), and induced tetraploid *Robinia pseudoacacia* (Li et al. 2021). The thicker leaf characteristic in tetraploid plants was also found in poplar 84 K (*P. alba* × *P. glandulosa*) (Ren, Jing & Kang 2021) and induced tetraploid *Averrhoa carambola* L. (Hu et al. 2021).

The induced tetraploid rosewood seedlings did not exhibit overall morphological characteristics typical of tetraploid plants, such as increased leaf width in *Eucalyptus polybractea* (Fernando et al. 2019), date palm (Othmani et al. 2020), *Betula pendula* (Zhang et al. 2022), and *Sorbus pohuashanensis* (Hance) Hedl (Zhang et al. 2023), as well as faster growth rates and more giant cells and organs than diploid plants in woody trees (Zhang et al. 2023). The increase in leaf area might be due to the doubling of chromosomes, leading to increased cell size and overall leaf size. However, unlike other trees, rosewood seedlings with

induced polyploidy had smaller leaf area compared to their diploid counterparts, at 18 months of age.

Tetraploid induction does not always result in the characteristics mentioned earlier. Tetraploid induction has been reported to render plants intolerant to diseases (Kunwar et al. 2023), slower in growth (Liu et al. 2022; Wu et al. 2023), with smaller stem diameters, fewer branches, and shorter shoots (Podwyszyńska et al. 2021). In banana plants, tetraploid individuals were weaker than their diploid counterparts (Bakry et al. 2007) for the former were prone to more leaf breakage to wind than that of the latter. Additionally, tetraploid induction can lead to dwarfism (Chen et al. 2022; Dong et al. 2023). This dwarfism is caused by changes in the expression of several genes in the plant hormone pathway, reducing the content of IAA and GA while increasing the content of ABA and JA (Ren et al. 2022).

The tetraploid and mixoploid plants obtained also did not exhibit superior traits like typical tetraploid plants. Nevertheless, obtaining these tetraploid rosewood plants could be of used for further genetic improvement. Planting tetraploid and diploid rosewood trees together or nearby to each other opens up the possibility of crossing, which may result in triploid rosewood plants. Triploid plants typically have seedless fruits, potentially leading to more significant biomass accumulation (Wang et al. 2016).

CONCLUSIONS

Polyploid induction in rosewood seedlings was achieved by soaking rosewood seedlings in oryzalin solution with concentrations ranging from 7.5-120 µM for 12 and 24 h. A total of 2 tetraploid seedlings were obtained with soaking durations of 12 and 24 h in 15 µM oryzalin. Meanwhile, seven accessions of mixoploid seedlings were obtained with a 12-h soaking duration at various oryzalin concentrations. Tetraploid plants were identified using flow cytometry and confirmed by their larger stomata size and lower stomata density than diploid seedlings. Mixoploid seedlings exhibited intermediate stomata size between the two ploidies and the lowest stomata density. The tetraploid and mixoploid seedlings did not show superior vegetative growth compared to their diploid counterparts, up to 18 months of age.

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REFERENCES

Abdolinejad, R., Shekafandeh, A. & Jowkar, A. 2021. *In vitro* tetraploidy induction creates enhancements in morphological, physiological and phytochemical characteristics in the fig tree (*Ficus carica* L.). *Plant Physiology and Biochemistry* 166: 191-202.

Adinugraha, H.A., Windyarini, E., Hasnah, T.M., Priyanto, A., Firdaus, H. & Leksono, B. 2021. The growth of *Calophyllum inophyllum* scions taken from provenance seed stand in Wonogiri, Central Java. *Jurnal WASIAN* 8(1): 1-9.

Arunkumar, A.N., Warrier, R.R., Kher, M.M. & Teixeira da Silva, J.A. 2022. Indian rosewood (*Dalbergia latifolia* Roxb.): Biology, utilisation, and conservation practices. *Trees* 36(3): 883-898.

Bakry, F., De La Reberdiere, N.P., Pichot, S. & Jenny, C. 2007. In liquid medium colchicine treatment induces non chimerical doubled-diploids in a wide range of mono-and interspecific diploid banana clones. *Fruits* 62(1): 3-12.

Bharati, R., Fernández-Cusimamani, E., Gupta, A., Novy, P., Moses, O., Severová, L. & Šrédl, K. 2023. Oryzalin induces polyploidy with superior morphology and increased levels of essential oil production in *Mentha spicata* L. *Industrial Crops and Products* 198: 116683.

Chen, S., Zhang, Y., Zhang, T., Zhan, D., Pang, Z., Zhao, J. & Zhang, J. 2022. Comparative transcriptomic, anatomical and phytohormone analyses provide new insights into hormone-mediated tetraploid dwarfing in hybrid sweetgum (*Liquidambar styraciflua* × *L. formosana*). *Front. Plant Science* 13: 924044.

CITES. 2017. *Notification to the Parties*. https://cites.org/sites/default/files/notif/E-Notif-2017-010.pdf. Accessed on 3 February 2024.

de Moura, L.C., Xavier, A., Viccini, L.F., Batista, D.S., de Matos, E.M., Gallo, R. & Otoni, W.C. 2020. Induction and evaluation of tetraploid plants of *'Eucalyptus urophylla'* clones. *Australian Journal of Crop Science* 14(11): 1786-1793.

Dhooghe, E., Denis, S., Eeckhaut, T., Reheul, D. & Van Labeke, M-C. 2009. *In vitro* induction of tetraploids in ornamental *Ranunculus*. *Euphytica* 168: 33-40.

Diallo, A.M., Kjaer, E.D., Nielsen, I.R. & Ræbild, A. 2022. Response of di- and tetraploid *Acacia senegal* (L.) Willd. seedlings to salt stress. https://dx.doi.org/10.2139/ssrn.4272536

Diallo, A., Gbeassor, M., Vovor, A., Eklu-Gadegbeku, K., Aklikokou, K., Agbonon, A., Abena, A.A., de Souza, C. & Akpagana, K. 2008. Effect of *Tectona grandis* on phenylhydrazine-induced anaemia in rats. *Fitoterapia* 79(5): 332-336.

Dong, R., Pei, Y., Hong, L., Nie, J., Chen, Y., Yan, H. & Liu, Y. 2023. Tetraploid induction identification and transcriptome preliminary analysis of black currant (*Ribes rubrum* L.). *Plant Cell, Tissue and Organ Culture* (PCTOC) 155(3): 861-872.

Dushimimana, C., Van Laere, K., Magomere, T., Smagghe, G. & Werbrouck, S.P. 2023. Production of tetraploid plants from cotyledons of diploid *Melia volkensii* Gürke. *Horticulturae* 9(7): 791.

Fernando, S.C., Goodger, J.Q., Chew, B.L., Cohen, T.J. & Woodrow, I.E. 2019. Induction and characterisation of tetraploidy in *Eucalyptus polybractea* RT Baker. *Industrial Crops and Products* 140: 111633.

Gallone, A., Hunter, A. & Douglas, G.C. 2014. Polyploid induction in vitro using colchicine and oryzalin on Hebe 'Oratia Beauty': Production and characterization of the vegetative traits. *Scientia Horticulturae* 179: 59-66.

Ganga, M. & Chezhiyan, N. 2002. Influence of the antimitotic agents colchicine and oryzalin on in vitro regeneration and chromosome doubling of diploid bananas (*Musa* spp.). *The Journal of Horticultural Science and Biotechnology* 77(5): 572-575.

Griffin, A.R., Twayi, H., Braunstein, R., Downes, G.M., Son, D.H. & Harwood, C.E. 2014. A comparison of fibre and pulp properties of diploid and Tetraploid *Acacia Mangium* grown in Vietnam. *Appita* 67(1): 43-49.

Handayani, T., Prawestri, A.D., Rahayu, R.S. & Leksonowati, A. 2023. Oryzalin-induced taro (*Colocasia esculenta* L.) tetraploid and diploid assessment for growth and agronomic traits. *SABRAO Journal of Breeding and Genetics* 55(1): 163-174.

Hu, Y., Sun, D., Hu, H., Zuo, X., Xia, T. & Xie, J. 2021. A comparative study on morphological and fruit quality traits of diploid and polyploid carambola (*Averrhoa carambola* L.) genotypes. *Scientia Horticulturae* 277: 109843.

Joker, D. 2004. Seed leaflet: *Dalbergia latifolia* Roxb. Forest & Landscape Denmark. http://dfsc.dk/pdf/Seedleaflets/Dalbergia_latifolia_90.pdf. Accessed on 9 February 2024.

Kanchanapoom, K. & Koarapatchaikul, K. 2012. *In vitro* induction of tetraploid plants from callus cultures of diploid bananas (*Musa acuminata*, AA group) 'Kluai Leb Mu Nang' and 'Kluai Sa.' *Euphytica* 183(1): 111-117.

Kang, X. 2020. Research progress of forest genetics and tree breeding. *Journal of Nanjing Forestry University* 44(3): 1-10.

Kementerian Kehutanan dan Lingkungan Hidup. 2017. Peraturan terbaru peredaran kayu sonokeling (*Dalbergia latifolia*) dalam negeri dan luar negeri. http://ksdae.menlhk.go.id/. Accessed on 9 February 2024.

Kertadikara, A. & Prat, D. 1995. Genetic structure and mating system in teak (*Tectona grandis* L.f.) provenances. *Silvae Genetica* 44: 104-110.

Kim, H.E., Han, J.E., Lee, H., Kim, J.H., Kim, H.H., Lee, K.Y., Shin, J.H., Kim, H.K. & Park, S.Y. 2021. Tetraploidization increases the contents of functional metabolites in *Cnidium officinale. Agronomy* 11(8): 1561.

Kumar, V., Kumar, S., Jha, S. & Jijeesh, X. 2014. Infuence of de-oiled seed cakes on seedling performance of East Indian rosewood (*Dalbergia latifoila* Roxb.). *Soil and Environment* 33(2): 169-174.

Kunwar, S., Meyering, B., Grosser, J., Gmitter Jr., F.G., Castle, W.S. & Albrecht, U. 2023. Field performance of 'Valencia' orange trees on diploid and tetraploid rootstocks in different huanglongbing-endemic growing environments. *Scientia Horticulturae* 309: 111635.

Kurtz, L.E., Brand, M.H. & Lubell-Brand, J.D. 2020. Production of tetraploid and triploid hemp. *HortScience* 55(10): 1703-1707.

Kusumadewi, Y., Susila, Rachmat, H.H., Dwiyanti, F.G., Atikah, T.D., Subiakto, A., Pratama, B., Setyawati, T., Wardani, W., Fambayun, R.A., Arrofaha, N. & Kamal, I. 2022. Population genetic of the Indonesian rosewood (*Dalbergia latifolia*) from Java and West Nusa Tenggara revealed using sequence-related amplified polymorphism. *Forest Science and Technology* 18(4): 172-181.

Lakhey, P., Pathak, J. & Adhikari, B. 2020. *Dalbergia latifolia*. The IUCN Red List of Threatened Species.

Lam, H.K., Harbard, J. & Koutoulis, A. 2014. Tetraploid induction of *Acacia crassicarpa* using colchicine and oryzalin. *Journal of Tropical Forest Science* 26(3): 347-354.

Lan, M.O., Chen, J.H., Fei, C., Xu, Q.W., Tong, Z.K., Huang, H.H., Dong, R.H., Lou, X.Z. & Lin, E.P. 2020. Induction and characterization of polyploids from seeds of *Rhododendron fortunei* Lindl. *Journal of Integrative Agriculture* 19(8): 2016-2026.

Li, W., Zhang, Q., Wang, S., Langham, M.A., Singh, D., Bowden, R.L. & Xu, S.S. 2019. Development and characterization of wheat–sea wheatgrass (*Thinopyrum junceiforme*) amphiploids for biotic stress resistance and abiotic stress tolerance. *Theoretical and Applied Genetics* 132(1): 163-175.

Li, X., Zhang, Z., Ren, Y., Feng, Y., Guo, Q., Dong, L., Sun, Y. & Li, Y. 2021. Induction and early identification of tetraploid black locust by hypocotyl *in vitro*. *In Vitro Cellular & Developmental Biology-Plant* 57: 372-379.

Liu, Z., Wang, J., Qiu, B., Ma, Z., Lu, T., Kang, X. & Yang, J. 2022. Induction and characterization of tetraploid through zygotic chromosome doubling in *Eucalyptus urophylla*. *Frontiers in Plant Science* 13: 870698.

Longui, E.L., Custódio, G.H., Amorim, E.P., da Silva Jr., F.G., Oda, S. & Souza, I.C.G. 2021. Differences in wood properties among *Eucalyptus grandis* and *Eucalyptus grandis* × *Eucalyptus urophylla* with different degrees of ploidy. *Research, Society and Development* 10(16): e395101624035.

Navrátilová, B., Švécarová, M., Bednář, J. & Ondřej, V. 2021. *In vitro* polyploidization of *Thymus vulgaris* L. and its effect on composition of essential oils. *Agronomy* 11(3): 596-607.

Othmani, A., Jemni, M., Leus, L., Sellemi, A., Artés, F. & Werbrouck, S. 2020. A ploidy chimera reveals the effects of tetraploidy in date palm. *European Journal of Horticultural Science* 85(3): 137-144.

Parson, J.L., Martin, S.L., Golenia, G. & James, T. 2019. Polyploidization for the genetic improvement of *Cannabis sativa*. *Frontier Plant Science* 10: 476.

Petersen, K.K., Hagberg, P. & Kristiansen, K. 2003. Colchicine and oryzalin mediated chromosome doubling in different genotypes of *Miscanthus sinensis*. *Plant Cell, Tissue and Organ Culture* 73: 137-146.

Podwyszyńska, M., Markiewicz, M., Broniarek-Niemiec, A., Matysiak, B. & Marasek-Ciolakowska, A. 2021. Apple autotetraploids with enhanced resistance to apple scab (*Venturia inaequalis*) due to genome duplication-phenotypic and genetic evaluation. *International Journal of Molecular Sciences* 22(2): 527.

Poerba, Y., Martanti, D., Handayani, T. & Witjaksono. 2019a. Morphology and reproductive function of induced autotetraploi banana by chromosome doubling. *SABRAO Journal of Breeding and Genetics* 51(2): 175-190.

Poerba, Y.S., Martanti, D., Handayani, T. & Witjaksono. 2019b. Induction of banana autotetraploids "Klutuk Sukun" and their reproductive function for producing triploid hybrids. *Asian Journal of Plant Sciences* 18(2): 91-100.

Poerba, Y.S., Ahmad, F., Witjaksono & Handayani, T. 2014. Induction and characterization of tetraploid pisang mas lumut. *Journal Biologi Indonesia* 10(2): 191-200.

Prawirohatmodjo, S., Suranto, J., Martawijaya, A., den Outer, R.W. & Sosef, M.S.M. 1994. *Plant Resources of South-East Asia. Timber Trees: Major Commercial Timbers*. Bogor: PROSEA.

Rahmi, P., Witjaksono, & Ratnadewi, D. 2019. Induksi poliploidi tanaman kangkung (*Ipomoea aquatica* Forssk.) kultivar Salina *in vitro* dengan oryzalin. *Jurnal Biologi Indonesia* 15(1): 1-8.

Ramulu, K., Verhoeven, H.A. & Dijkhuis, P. 1991. Mitotic blocking, micronucleation, and chromosome doubling by oryzalin, amiprophos-methyl, and colchicine in potato. *Protoplasma* 160: 65-71.

Ren, Y., Jing, Y. & Kang, X. 2021. *In vitro* induction of tetraploid and resulting trait variation in *Populus alba*× *Populus glandulosa* clone 84 K. *Plant Cell, Tissue and Organ* 146: 285-296.

Ren, Y., Zhang, S., Xu, T. & Kang, X. 2022. Morphological, transcriptome, and hormone analysis of dwarfism in tetraploids of *Populus alba* × *P. glandulosa*. *International Journal of Molecular Sciences* 23(17): 9762.

Riastiwi, I., Prawestri, A.D., Ridwan & Damayanto, I.P.G.P. 2023. Viability of *Moringa oleifera* seeds stored at different temperatures and recent status of *Moringa oleifera* collections in seed banks worldwide. *Journal of Applied Botany and Food Quality* 96: 67-74.

Riastiwi, I., Witjaksono, Ratnadewi, D. & Siregar, U.J. 2022. Genetic diversity of rosewood (*Dalbergia latifolia*) in Yogyakarta, Indonesia for plus trees selection. *Biodiversitas Journal of Biological Diversity* 23(5): 2630-2639.

Ridwan & Witjaksono. 2020. Induction of autotetraploid moringa plant (*Moringa oleifera*) using oryzalin. *Biodiversitas Journal of Biological Diversity* 21(9): 4086-4093.

Ridwan, Handayani, T., Riastiwi, I. & Witjaksono. 2018. Tetraploid teak seedling was more tolerant to drought stress than its diploid seedling. *Jurnal Penelitian Kehutanan Wallacea* 7(1): 1-11.

Talebi, S.F., Saharkhiz, M.J., Kermani, M.J., Sharafi, Y. & Fard, F.R. 2017. Effect of different antimitotic agents on polyploidy induction of anise hyssop (*Agastache foeniculum* L.). *Caryologia* 70(2): 184-193.

Tel-Zur, N., Mizrahi, Y., Cisneros, A., Mouyal, J., Schneider, B. & Doyle, J.J. 2011. Phenotypic and genomic characterization of vine cactus collection (Cactaceae). *Genetic Resources and Crop Evolution* 58: 1075-1085.

Van Duren, M., Morpurgo, R., Dolezel, J. & Afza, R. 1996. Induction and verification of autotetraploids in diploid banana (*Musa acuminata*) by *in vitro* techniques. *Euphytica* 88(1): 25-34.

Vasudevan, R., Sasidharan, K.R., Venkataramanan, K.S., Thangamani, D. & Sivanantham, S. 2020. Standardization of macropropagation technique for *Dalbergia latifolia* Roxb. through root cuttings, its application in production of quality planting stock and conservation of genetic resources. *International Journal of Advanced Research and Review* 5(11): 9-19.

Wang, L.J., Zhang, Q., Cao, Q. Z., Gao, X. & Jia, G.X. 2020. An efficient method for inducing multiple genotypes of tetraploids *Lilium rosthornii* Diels. *Plant Cell, Tissue and Organ Culture* 141: 499-510.

Wang, X., Cheng, Z.M., Zhi, S. & Xu, F. 2016. Breeding triploid plants: A review. *Czech Journal of Genetics and Plant Breeding* 52(2): 41-54.

Wu, J., Zhou, Q., Sang, Y., Zhao, Y., Kong, B., Li, L., Du, J., Ma, L., Lu, M. & Zhang, P. 2023. *In vitro* induction of tetraploidy and its effects on phenotypic variations in *Populus hopeiensis*. *BMC Plant Biology* 23: 557.

Xu, C., Zhang, Y., Han, Q. & Kang, X. 2020. Molecular mechanism of slow vegetative growth in populus tetraploid. *Genes* 11(12): 1417.

Xu, J., Jin, J., Zhao, H. & Li, K. 2019. Drought stress tolerance analysis of *Populus ussuriensis* clones with different ploidies. *Journal of Forestry Research* 30(4): 1267-1275.

Zhang, X. & Gao, J. 2020. *In vitro* tetraploid induction from multigenotype protocorms and tetraploid regeneration in *Dendrobium officinale*. *Plant Cell, Tissue and Organ Culture* 141: 289-298.

Zhang, X., Chen, K., Wang, W., Liu, G., Yang, C. & Jiang, J. 2022. Differences in leaf morphology and related gene expression between diploid and tetraploid birch (*Betula pendula*). *International Journal of Molecular Sciences* 23(21): 12966.

Zhang, Z., Zhang, Y., Di, Z., Zhang, R., Mu, Y., Sun, T., Tian, Z., Lu, Y. & Zheng, J. 2023. Tetraploid induction with leaf morphology and sunburn variation in *Sorbus pohuashanensis* (Hance) Hedl. *Forests* 14(8): 1589.

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