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In Vitro Initiation, Regeneration, and Characterization of Plants Derived from Mature Tetraploid Floral Explants of Date Palm (*Phoenix dactylifera* L.)

Ahmed Othmani [1,2], Amel Sellemi [1], Monia Jemni [3], Karim Kadri [4,5], Leen Leus [6] and Stefaan P. O. Werbrouck [7,*]

1 Laboratory for In Vitro Tissue Culture, Regional Centre for Research in Oasis Agriculture, Tozeur Km1, Degueche 2260, Tunisia; degletbey@yahoo.fr (A.O.); sallami.amel@gmail.com (A.S.)
2 LR21AGR03-Production and Protection for Sustainable Horticulture (2-PHD), Regional Research Centre on Horticulture and Organic Agriculture Chott Mariem, University of Sousse, Sousse 4042, Tunisia
3 Laboratory for Date Palm Technology, Regional Centre for Research in Oasis Agriculture, Tozeur Km1, Degueche 2260, Tunisia; monia.jemni@iresa.agrinet.tn
4 Biotechnology and Genetic Resources Laboratory, Regional Centre for Research in Oasis Agriculture, BO 62, Deguech 2260, Tunisia; kadrikarim2001@yahoo.fr
5 Laboratory of Biotechnology Applied to Agriculture, National Institute of Agronomic Research of Tunis, University of Carthage, 2049 Hedi Karray Street, Tunis 2036, Tunisia
6 Plant Sciences Unit, Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Caritasstraat 39, 9090 Melle, Belgium; leen.leus@ilvo.vlaanderen.be
7 Department of Plant & Crops, Faculty of Bioscience Engineering, Ghent University, Valentin Vaerwyckweg 1, 9000 Ghent, Belgium
* Correspondence: stefaan.werbrouck@ugent.be

Abstract: A stable tetraploid date palm mutant with the potential for increased fruit size was recovered from a sectorial di-tetraploid chimera via the in vitro culture of flower explants. Callus was induced using 2,4-D, followed by shoot regeneration on a medium containing NAA and BAP and rooting with IBA. Regenerated plantlets, confirmed as tetraploid via flow cytometry, were acclimatized and grown for six years. The leaves of tetraploids exhibited significantly wider petiole bases, thicker rachis and spines, broader leaflets, and a more intense green leaf color compared to diploids. However, leaf length, spine count, and overall leaf number were reduced. This is the first report of successful tetraploid recovery from a chimeric date palm, demonstrating the potential of this technique to generate novel germplasm and highlighting the phenotypic differences associated with tetraploidy in this species.

Keywords: chimera; flowers; flow cytometry; tetraploidy; field performance

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1. Introduction

The date palm (*Phoenix dactylifera* L.) is an important crop in arid and semi-arid regions, particularly in North Africa and the Middle East. Increasing demand has led to widespread cultivation of the 'Deglet Nour' cultivar, resulting in a monoculture that is vulnerable to disease outbreaks, pest infestations, and genetic erosion. However, this monoculture also provides opportunities for the discovery of novel mutations with the potential to increase the resilience and productivity of date palms. A prime example is the identification of a chimeric 'Deglet Nour' palm in the Tozeur Oasis (Tunisia), which exhibits both diploid and tetraploid sectors [1]. Tetraploid plants often exhibit valuable traits such as increased fruit size, enhanced stress tolerance, and altered growth habits, making them attractive targets for breeding programs. Therefore, the conservation and characterization of this rare tetraploid date palm genotype is critical for broadening the genetic base of cultivated date palms and developing improved cultivars with enhanced traits.

As highlighted in a recent review by Nimavat and Parikh [2], efficient micropropagation protocols are essential for the successful conservation and utilization of valuable date palm genotypes. They analyzed in detail the in vitro culture protocols for date palms and discussed the factors that influence the development of multiple shoots, roots, and organs. Details were given on the optimal choice of explants, media formulations, and combinations of growth regulators for successful propagation.

The aim of this study was to conserve this valuable tetraploid genotype by micropropagation and characterize the resulting plants. Flow cytometry confirmed the ploidy level of the regenerated plantlets, and their morphological traits were analyzed to assess their potential for future breeding applications. This is the first documented report of the successful regeneration and characterization of field-grown, in vitro-derived tetraploid date palm clones, providing valuable information for the development of ploidy-based breeding strategies in date palm cultivation.

2. Materials and Methods

2.1. Regeneration of Plants In Vitro

Mature female flowers were collected from the tetraploid sector of a chimeric 'Deglet Nour' date palm (Figure 1A,B). Diploid flowers were also collected from the same cultivar, serving as a reference control [1]. Flowers were collected in late March, after the first 20–25 cm of spathe had emerged from the base of the tetraploid leaves. The outer surfaces of the spathe and the cut surface were disinfected by immersion in ethanol (90%) for 20–60 s. The inflorescences were then sterilized with a 0.01% mercuric chloride solution for one hour and thoroughly rinsed three times with sterile distilled water. The inflorescences were cut into 1.5–2 cm segments, each containing 2–3 flowers, and placed horizontally on the culture medium to maximize contact. The initiation medium consisted of Murashige and Skoog (MS) salts and vitamins [3] supplemented with 30 g/L sucrose, 3 g/L activated charcoal (Sigma-Aldrich, St. Louis, MO, USA), 0.7% agar–agar (Chemi-Pharma, Cebalat Ben Ammar, Tunisia), and 0, 0.1, 0.2, 0.5, and 1 mg/L 2,4-D (MilliporeSigma, Burlington, MA, USA). For control purposes, the same medium was used for the diploid flowers, but only a concentration of 1 mg/L 2,4-D was used.

The culture medium was adjusted to a pH of 5.7 prior to autoclaving at 121.0 °C with 2.04 kg cm^{-2} pressure for 20 min. Thirty borosilicate test tubes (20 × 150 mm, Sigma-Aldrich) were prepared, each containing four explants. These cultures were incubated in darkness at a constant temperature of 28 ± 2 °C (LMS cooled incubator, Sevenoaks, UK). Subculturing was performed every four weeks for a total of nine months. When callus formation was observed, the organogenic calli were transferred to a multiplication medium (MM) consisting of MS salts and vitamins, 30 g/L sucrose, and 0.7% agar–agar supplemented with 1 mg/L NAA (MilliporeSigma, Bedford, MA, USA) and 1 mg/L BAP (MilliporeSigma, Bedford, MA, USA) to promote shoot regeneration and multiplication. The cultures were then maintained in a controlled environment at a temperature of 28 ± 2 °C with a photoperiod of 16/8 h light/dark and light intensity of 80 µmol m^{-2} s^{-1} E (PAR) provided by fluorescent lamps (Wellmax, Cheung Sha Wan, Hong Kong). The shoots were then transferred to an elongation medium (EM), identical to the previous medium but without plant growth regulators (PGRs). After four months on EM, well-developed shoots were transferred to a rooting medium (RM) containing the standard medium supplemented with 3 mg/L IBA (MilliporeSigma, Bedford, MA, USA) to induce root formation.

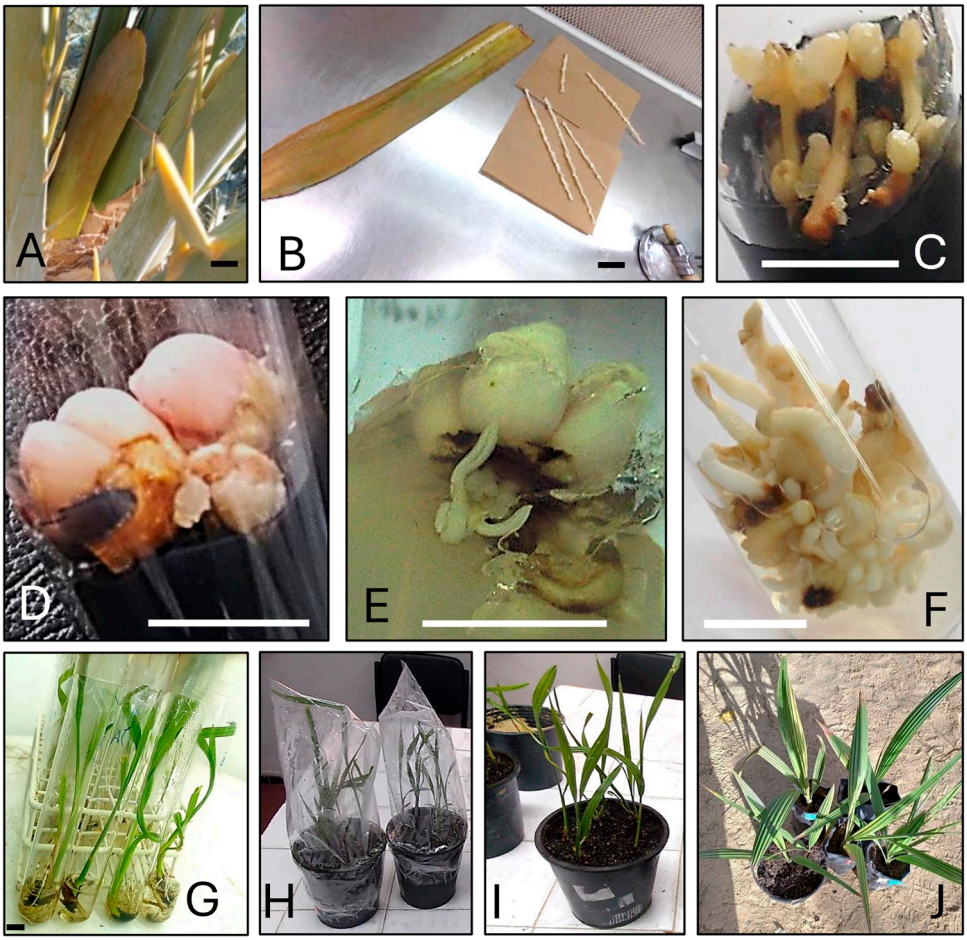

Figure 1. In vitro regeneration stages of plantlets derived from mature tetraploid inflorescences: (**A**) emerging flower spathe, (**B**) dissected flower spathe, (**C**) mature flower explants after one month on culture initiation medium (CIM), (**D**) callus induction after six months on CIM, (**E**) shoot organogenesis from callus after nine months on culture medium, (**F**) shoot proliferation, (**G**) vitro plants after three months on rooting medium (RM), (**H**) vitro plants potted in a 2:1 peat moss–sand mixture under a plastic cover, (**I**) vitro plants acclimatized for three months in a greenhouse, and (**J**) vitro plants acclimatized for six to eight months in a greenhouse. Scale bar = 1 cm.

2.2. Acclimatization

After regeneration, three-month-old plantlets with well-developed shoots and robust root systems were selected for acclimatization. Residual agar was carefully rinsed from the plantlets under running tap water. The plantlets were then individually potted in a 2:1 peat moss–sand mixture under a plastic cover and transferred to a greenhouse environment for acclimatization under natural sunlight with a controlled temperature of 28 ± 2 °C and 80–90% relative humidity. Plant survival, defined as the percentage of plants that successfully transitioned from the in vitro culture to ex vitro conditions, was evaluated after ten weeks.

2.3. Field Trial

Twenty acclimatized plants were planted in experimental plots at the CRRAO and CSFPAP research stations in Degache, Tunisia. These plants were grown alongside fifty in vitro-derived diploid 'Deglet Nour' plants as controls. To ensure adequate hydration, each plant was irrigated by drip irrigation, receiving 100 L of water per irrigation session. Irrigation schedules were adjusted seasonally: weekly in winter, bi-weekly in fall and spring, and three times per week in summer. Each 1 m × 1 m × 1 m planting pit was

fertilized annually with 5 kg of cow manure. Plants were grown under these conditions for six years.

2.4. Ploidy Analysis

Ploidy analysis was performed using a CyFlow Space flow cytometer equipped with a UV LED and Flomax 2.9 software to determine histogram peak positions [4]. Leaf tissue was prepared by chopping according to Galbraith et al. [5] and stained with 4′, 6-diamidino-2-phenylindole (Sigma-Aldrich, Overijse, Belgium) according to Otto [6]. Leaf material from diploid 'Deglet Nour' plants grown in vitro was used as a reference. Three samples per plant tissue were examined, for a total of six plants.

2.5. Phenotypic Characterization of Leaves

A quantitative assessment of leaf characteristics was performed according to the date palm descriptors established by IPGRI [7]. The parameters measured included number of leaves (NL), leaf length (LL, cm), leaf width at the base of the petiole (WbP, cm), maximum leaf thickness (MT, cm), maximum leaf length (MlL, cm), length of the apical leaf (LaL, cm), apical leaf width (WaL, cm), petiole thickness (TP, cm), average number of spines (AnS), maximum spine thickness (MtS, cm), and maximum spine length (MlS, cm). Three leaves were sampled from each plant, representing the lower, middle, and upper parts of the crown. Six in vitro-derived plants were included in this study: three diploids (used as a standard) and three tetraploids.

2.6. Color of Leaflets

A colorimeter (Minolta CR 300, Ramsey, NJ, USA) was used to monitor leaf color (C standard C.I.E. illumination, 0° viewing) and the results were expressed in the CIELAB (L*a*b*) color space. These measurements were made for both large and normal leaflets at 10 different locations on the leaf surface. For each leaflet (large and normal), the mean values of three key parameters (L*, a*, and b*) were calculated across ten different measurement locations on the leaflet surface, following the guidelines of the Commission Internationale de l'éclairage (CIE) [8]. These parameters included lightness (L*), red/green coordinate (a*), and blue/yellow coordinate (b*).

2.7. Observation of Stomata

Leaf samples (n = 60) were collected from 6-year-old experimental plants grown in the CRRAO plot. These included both diploid (standard) and tetraploid in vitro-derived date palms, with ten leaves sampled per plant. To determine mean stomatal size (length and width) and density (stomata per mm^2), the upper leaf surface was imprinted following the established method of Hamill et al. [9]. Clear nail polish was applied, allowed to dry, and then peeled to create a leaf impression. This impression was mounted on a microscope slide with a drop of water for observation. Stomata were visualized using a Leica DM1000 LED compound biological microscope (Wetzlar, Germany), and ten images were captured per imprint for subsequent analysis.

2.8. Data Analysis

Descriptive statistics were used to analyze leaf and stomatal characteristics. Data were collected in triplicate for each parameter. Mean, maximum, and minimum values were calculated. To compare the data sets, either independent sample *t*-tests (5% significance level) or one-way analysis of variance (ANOVA) was used, followed by Newman and Keuls' test for post hoc comparisons at a significance level of $p = 0.05$.

3. Results

3.1. Regeneration of Vitro Plants

The regeneration process of tetraploid vitro plants is illustrated in Figure 1. While most explants showed significant growth in size, they lacked any further morphogenetic re-

sponse for a period of 8–9 months. Root formation and necrosis were rare observations. On only initiation medium (IM) containing the highest dose of 1 mg/L 2,4-D, a limited proportion of flowers (6.22%) developed callus at the basal zone (Figure 1C,D). This callus tissue, composed of meristematic cells, underwent differentiation into a string of buds. After 3–5 months on elongation medium (EM), these buds matured into recognizable adventitious shoots (Figure 1E,F). Transfer of isolated shoots to rooting medium (RM) significantly enhanced root development (Figure 1G), resulting in the formation of vigorous plantlets within 2–4 months (Figure 1H,I). Twenty of these plantlets were successfully transferred to a greenhouse environment and all survived and continued to grow (Figure 1J).

3.2. Ploidy Analysis

Flow cytometry analysis (Figure 2) revealed the ploidy of the regenerated plants. The histogram shows distinct peaks corresponding to the diploid control (approximately 110 fluorescence channels) and the tetraploid plants (approximately 210 RFU), confirming the maintenance of the tetraploid state in the regenerated plants.

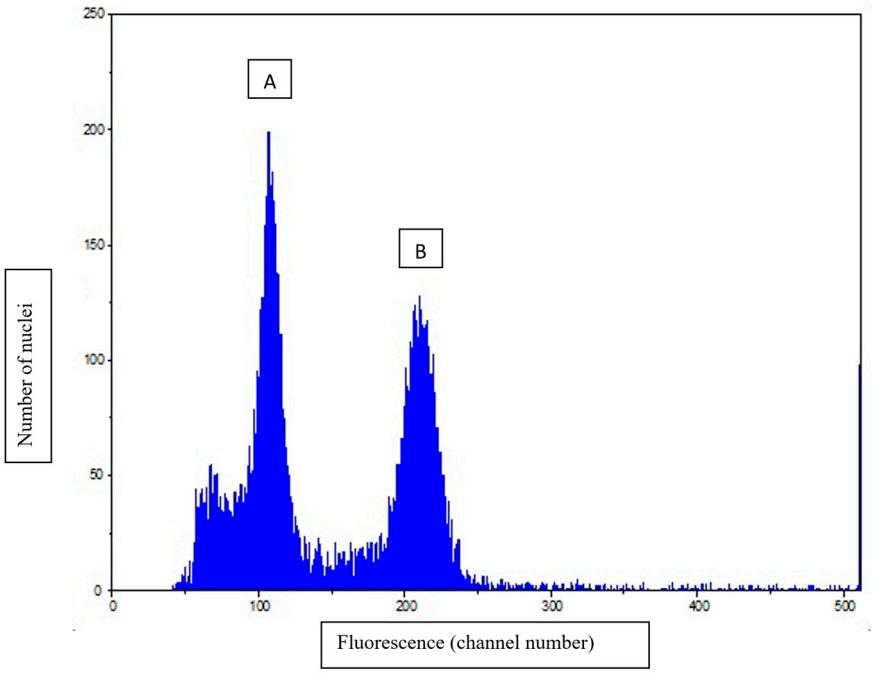

Figure 2. Flow cytometry histograms of ploidy levels in 'Deglet Nour' date palm. (A) Diploid control with a peak fluorescence at channel ~110. (B) Tetraploid vitro plants with a peak fluorescence at channel ~210, confirming tetraploidy.

3.3. Phenotypic Characterization of Leaves

Significant morphological differences between diploid and tetraploid genotypes were observed in all eleven leaf parameters analyzed (Table 1, Figures 3–5). Compared to their diploid counterparts, tetraploid leaves showed an increased width at the petiole base (WbP), as well as thicker rachis and spines (MT and MtS). Leaflets located in the central part of the leaf showed greater width (MlL) in tetraploids, and the apical pinnae of the apical leaflet were both longer (LaL) and wider (WaL). Conversely, tetraploid plants showed a reduction in total leaf length (LL), average spine number (AnS), maximum spine length (MlS), and total leaf number (NL). In addition, tetraploid leaves had a more intense green coloration compared to diploids (Table 2).

Table 1. Leaf morphology parameters, demonstrating significant differences between six-year-old, in vitro-derived diploid and tetraploid date palms grown under identical field conditions.

Morphological Parameters of Leaves	Diploid Vitro Plants	Tetraploid Vitro Plants
Length of the leaf (m)	2.57 ± 0.18 a	1.86 ± 0.13 b
Thickness of the rachis (cm)	2.69 ± 0.20 b	3.25 ± 0.29 a
Width of the leaf at the base of the petiole (cm)	9.97 ± 0.43 b	13.44 ± 1.00 a
Average number of spines	33.88 ± 3.27 a	15.66 ± 1.58 b
Max. thickness of spines (cm)	0.75 ± 0.03 b	0.95 ± 0.06 a
Max. length of spines (cm)	20.96 ± 1.51 a	16.97 ± 1.34 b
Number of leaflets (pinnae)	93.44 ± 7.91 a	81.88 ± 1.00 b
Max. width of leaflets in the middle of the leaf (cm)	2.52 ± 0.05 b	3.52 ± 0.18 a
Max. length of the leaflets in the middle of the leaf (cm)	40.81 ± 0.75 b	48.85 ± 2.05 a
Length of the apical pinnae (cm)	26.78 ± 1.04 b	38.92 ± 1.08 a
Width of the apical leaflet (cm)	2.04 ± 0.04 b	3.17 ± 0.51 a

Three leaves were used for each vitroplant (3 diploids and 3 tetraploids). Values followed by the same letters are not significantly different ($p < 0.05$).

Figure 3. Six-year-old 'Deglet Nour' date palms after in vitro regeneration. **Left**: Diploid. **Right**: Tetraploid. Scale bar = 20 cm.

Figure 4. Comparison of leaves from six-year-old, in vitro-derived date palms. **Top**: Diploid. **Bottom**: Tetraploid. Scale bar = 10 cm.

Figure 5. Comparison of leaflets from six-year-old diploid (**top**) and tetraploid (**bottom**) date palms. Note the increased width and thicker spines in the tetraploid leaflet. Scale bar = 2 cm.

Table 2. Leaflet color parameters in diploid and tetraploid six-year-old 'Deglet Nour' date palms observed after in vitro regeneration (CIELAB: L: represents lightness. It ranges from 0 (black) to 100 (white). a: represents the position between red and green. Positive values indicate red, while negative values indicate green. b: represents the position between yellow and blue. Positive values indicate yellow, while negative values indicate blue). Values are means (n = 3) ± SD. Different letters indicate significant differences ($p \leq 0.05$) by Tukey's test.

Leaflet Origin	L	a	b
diploid	46.61 ± 1.73 a	−11.28 ± 0.65 a	14.83 ± 1.25 a
tetraploid	47.92 ± 1.72 a	−10.35 ± 1.12 b	18.15 ± 1.45 b

3.4. Characteristics of Stomata

Table 3 and Figure 6 illustrate significant differences in stomatal characteristics between diploid and tetraploid in vitro-derived plants, particularly with respect to stomatal size and density. Tetraploid plantlets exhibited significantly larger mean stomatal length (74.4 µm) and width (33.6 µm) compared to their diploid counterparts (61.2 µm and 28.4 µm, respectively). Conversely, tetraploid plants exhibited a significantly reduced stomatal density, with a 40.49% decrease in the mean number of stomata per mm^2.

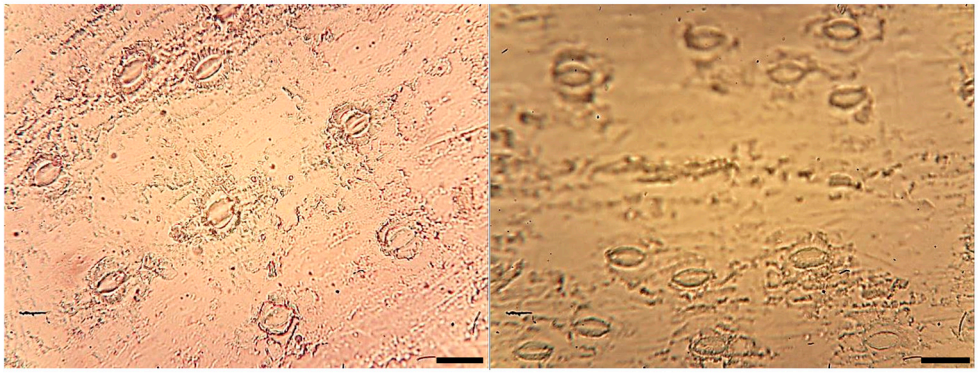

Figure 6. Comparison of stomata on the upper leaf surface of field-grown diploid (**right**) and tetraploid (**left**) date palms. Scale bar = 30 µm.

Table 3. Comparison of stomata characteristics (size and number per mm^2) in the upper surface of leaves sampled from diploid and tetraploid date palm vitro plants.

Leaves	Stomata		
	Length (µm)	Width (µm)	Number (mm^2)
Diploid vitroplant	61.11 ± 0.13 b	28.40 ± 0.13 b	154.6 ± 6.13 a
Tetraploid vitroplant	74.40 ± 0.16 a	33.60 ± 0.17 a	119.00 ± 1.16 b

Each value represents the average of ten replicates. Values followed by different letters are significantly different ($p < 0.0001$).

4. Discussion

Initiating cultures directly from the apical meristem of the tetraploid offshoot was considered a high-risk strategy due to the potential for contamination, which could result in the irretrievable loss of this unique genetic resource. Therefore, we decided to use mature female flowers as the explant source. This approach is consistent with established practices, as female flowers can be harvested annually without compromising the health of the mother plant [10].

Successful in vitro regeneration from mature female date palm inflorescences was achieved. The efficiency of conversion of floral buds into vegetative shoots is known to be influenced by several factors, including the developmental stage of the flower at the time of explant collection [11,12], the type and concentration of plant growth regulators used in the culture medium [13], and the specific physicochemical conditions used during subculturing [10]. Building on previous research by Kriaâ et al. [10] and Zayed et al. [12], which demonstrated the in vitro regenerative potential of female date palm flowers harvested at their final stage of development, this work highlights the presence of residual meristematic zones within the floral bracts (sepals and petals) that contribute to this regenerative capacity.

Our results indicate that callus formation was induced exclusively in the basal region of mature tetraploid female flowers (6.22%) when cultured on MS medium supplemented with the highest dose of 1 mg/L 2,4-D. This observation is consistent with the work of Kriaâ et al. [10], who attributed this preferential callus formation to the presence of meristematic cells within the basal region that showed an increased responsiveness to plant growth regulators. While they reported occasional callus proliferation on other floral organs (sepals, petals, carpels), this was not observed in our experimental setup. Similarly, the observations of Zayed et al. [12] with callus initiation on perianth segments, carpels, and receptacles were not replicated in our study.

Research on direct shoot organogenesis from date palm inflorescences is limited. Loutfi and Chlyah [14] successfully produced shoot primordia using [15] supplemented with 0.5 mg/L NAA, 2 mg/L BAP, and 1 mg/L 2ip. Khierallah et al. [16] found that a combination of 2 mg/L NAA and 4 mg/L BAP induced bud formation on inflorescence explants. Mazri and Meziani [17] reported that 2,4-D was the most effective auxin for the induction of embryogenic callus in date palm plants from either shoot tips or inflorescences. Kriaâ et al. [18] showed that 10 mg/L 2,4-D was more effective than lower concentrations for inducing somatic and organogenic callogenesis from mature flowers of 'Deglet Nour' date palm. Abdelghaffar et al. [18] obtained the highest percentage of callus induction in three cultivars on MS medium containing 9 µM 2,4-D + 5.7 µM IAA + 10 µM NAA and the best callus growth on MS medium containing 4.5 µM 2,4-D + 9.8 µM 2-iP + 1.5 AC. MS medium with 4.4 µM BA + 9.8 µM 2-iP produced the highest number of somatic embryos and shoots. However, we chose to use lower concentrations of growth regulators to minimize the risk of somaclonal variation, as documented by Loschiavo et al. [19] and Joshi and Dhawan [20]. Using 1 mg/L 2,4-D on tetraploid 'Deglet Nour' inflorescences, we successfully induced callus formation from mature female flowers. The achieved callus induction rate (6.22%) was relatively low, possibly reflecting a negative influence of tetraploidy on regeneration from mature flowers. Whole genome duplication events can cause significant

genetic and epigenetic changes, including altered gene expression and DNA methylation. These changes can affect genes involved in organogenesis and regeneration, affecting the in vitro response of polyploid plants [21]. However, the effects of polyploidy on in vitro regeneration vary between species. Some polyploids show reduced regenerative capacity, while others exhibit enhanced regeneration in certain tissues or culture conditions [22]. This variability is likely due to the complex interplay between ploidy level, gene expression, and hormonal balance. Polyploidization can alter endogenous hormone levels and sensitivity to exogenous plant growth regulators, potentially disrupting the delicate balance required for successful in vitro organogenesis and reducing the regenerative capacity of some polyploids [23].

Our combined analysis of leaf morphology, stomatal characteristics, and flow cytometry data conclusively demonstrates that all in vitro-derived plants regenerated from the tetraploid sector were indeed tetraploid. Furthermore, this study reveals that polyploidy significantly alters leaf morphology, a phenomenon likely attributable to the increased cell size characteristic of polyploids [24]. Tetraploid trees often exhibit enhanced drought tolerance but may have slower growth [25]. Physiological adaptations include larger leaves and more efficient antioxidant systems. This adaptive response is relevant for forestry and environmental management, particularly in drought-prone areas and ecological restoration [26,27].

As shown in Table 2, the tetraploid date palms exhibited a darker green color. This phenomenon is often observed in tetraploid plants and is attributed to various factors. Increased chlorophyll content and thicker leaves with more chloroplasts [28] and larger cells [29] contribute to this darker coloration. These changes are linked to increased gene dosage, altered leaf anatomy, and enhanced stress tolerance in tetraploids [30].

Figure 2 highlights the visually distinctive characteristics of the tetraploid date palm, including its appealing coloration and silhouette, which enhance its ornamental value. The increased leaf size is also well suited for traditional applications such as basket weaving and fiber extraction.

In general, polyploidy may confer increased resilience to both biotic and abiotic stressors due to a more robust structure, particularly in terms of stomatal characteristics [31,32]. Reduced stomatal density has been associated with improved drought tolerance in a number of plant species, including arabidopsis [33], barley [34], rice [35], wheat [36], and ziziphus [37]. Although preliminary, these results suggest that similar advantages may exist for the tetraploid date palm. The reduced leaf length observed in tetraploid plants allows for closer spacing within a date palm orchard, potentially increasing overall yield per hectare. Polyploid plants are often associated with improvements in fruit characteristics, as documented in apple [38], pear [39], and ziziphus [37]. However, the original tetraploid sector on the diploid date palm produced primarily inedible, seedless fruits. This was probably due to pollination with monoploid pollen from diploid cultivars [1]. To overcome this problem, research efforts are currently focused on developing other tetraploid genotypes and crossing them with tetraploid female plants. The goal is to produce larger, edible fruits containing tetraploid embryos.

5. Conclusions

This study successfully regenerated a unique tetraploid date palm genotype from mature female flowers, preserving a valuable and unique genetic resource. Flow cytometry confirmed the stable tetraploid state of the regenerated plants. These tetraploids exhibited distinct morphological and anatomical features compared to their diploid counterparts, including broader leaves, thicker spines, shorter stature, and altered stomata characteristics. These findings highlight the significant impact of polyploidization on date palm phenotype and potential adaptability. Future research should explore the physiological and agronomic consequences of these traits, particularly in relation to fruit production, stress tolerance, and ornamental applications. Furthermore, creating a tetraploid breeding population

through the induction of tetraploidy in elite pollinators holds promise for enhancing genetic diversity and improving date palm cultivation worldwide.

Author Contributions: A.O. and S.P.O.W. conceived and designed the experiment; A.O. collected the inflorescences; A.O. and A.S. were responsible for the in vitro tissue culture of tetraploid inflorescences from decontamination to obtaining the vitro plants; A.O. and K.K. participated in the microscopic observation of the stomata; A.O. and M.J. participated in the statistical analysis; L.L. and S.P.O.W. performed the ploidy analysis of the vitro plants using flow cytometry; and A.O. and S.P.O.W. conceived and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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