horticulturae

MDPI

Article

Optimizing In Vitro Establishment Protocols for 'Merensky 2' Avocado Rootstock (*Persea americana* Mill.)

Fernanda García-Cabrera [1], Mónica Castro [1,*], Ricardo Cautin [2], Carmen Estay [1], Leda Guzmán [3], María José Marchant [3] and Francesca Guerra [1]

[1] Laboratorio de Propagación, Escuela de Agronomía, Facultad de Ciencias Agronómicas y de los Alimentos, Pontificia Universidad Católica de Valparaíso, La Palma S/N, Quillota 2260000, Chile; fernanda.garcia.c@mail.pucv.cl (F.G.-C.); carmen.estay@pucv.cl (C.E.); francesca.guerra@pucv.cl (F.G.)

[2] Laboratorio de Especies Leñosas, Escuela de Agronomía, Facultad de Ciencias Agronómicas y de los Alimentos, Pontificia Universidad Católica de Valparaíso, La Palma S/N, Quillota 2260000, Chile; ricardo.cautin@pucv.cl

[3] Laboratorio de Biomedicina y Biocatálisis, Instituto de Química, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Avenida Universidad 330, Valparaíso 2340000, Chile; leda.guzman@pucv.cl (L.G.); maria.marchant.l@pucv.cl (M.J.M.)

* Correspondence: monica.castro@pucv.cl

Abstract

In vitro propagation of avocado faces several limitations. To optimize the establishment phase, we evaluated three plant material types: etiolated shoots, 30-day covered field shoots, and uncovered field shoots, collected at two time points. Biochemical and anatomical analyses were conducted to understand material performance during establishment. Across both collection times, etiolated shoots exhibited minimal oxidation, enhanced bud sprouting, reduced malondialdehyde (MDA) and reactive oxygen species (ROS) levels, increased peroxidase (POD) activity, and improved xylem development, consistently outperforming field-derived materials. Using etiolated shoots, we optimized disinfection and in vitro multiplication protocols. Pre-disinfection with 3 mL L^{-1} Phyton 27® and 2% sodium hypochlorite yielded the highest survival rates. In multiplication experiments, varying concentrations of 6-benzylaminopurine (BAP) and meta-topolin (MT), supplemented with gibberellic acid (GA_3), did not significantly affect growth variation. However, 8.88 µM BAP with 0.29 µM GA_3 resulted in the greatest number of sprouted buds.

Keywords: micropropagation; etiolation; anatomy; 6-benzylaminopurine (BAP); meta-topolin (MT); gibberellic acid (GA_3); antioxidant activity

Academic Editor: Sergey V. Dolgov

Received: 3 June 2025
Revised: 17 July 2025
Accepted: 18 July 2025
Published: 3 August 2025

Citation: García-Cabrera, F.; Castro, M.; Cautin, R.; Estay, C.; Guzmán, L.; Marchant, M.J.; Guerra, F. Optimizing In Vitro Establishment Protocols for 'Merensky 2' Avocado Rootstock (*Persea americana* Mill.). *Horticulturae* **2025**, *11*, 900. https://doi.org/10.3390/horticulturae11080900

1. Introduction

Avocado is a globally significant fruit crop, highly valued for its nutritional benefits and strong consumer demand [1]. This species, belonging to the Lauraceae family [2], is native to Central America and comprises three primary botanical races: Mexican, West Indian, and Guatemalan, which differ in their environmental adaptability and fruit quality profiles [3–5]. Among these, the 'Hass' cultivar has achieved global dominance due to its postharvest quality and market preference [6]. The optimal productivity of this cultivar in commercial orchards relies on selecting suitable rootstocks, such as 'Merensky 2', which demonstrates high tolerance to *Phytophthora cinnamomi* [7], salinity [8,9], and stress conditions commonly encountered in replanting scenarios [10].

Clonal propagation of elite avocado rootstocks is crucial for preserving genetic fidelity and ensuring uniform agronomic performance [11]. While propagation by seed

remains common, it is genetically heterogeneous and unsuitable for maintaining selected traits [11,12]. Traditional clonal methods, including air-layering as well as the Brokaw and Ernst etiolation techniques, have enabled propagation at a commercial scale [13–15]. However, these methods are laborious, time-consuming, and associated with high failure rates, ranging from 15% to 30%, particularly during grafting and rooting [16–18]. Additionally, they are limited by environmental dependency and genotype-specific responses [19–23]. Consequently, micropropagation is increasingly explored as a technically feasible alternative, offering the potential for year-round, scalable, and space-efficient production [17].

Another disadvantage that only the Brokaw technique has is that if the strangulation ring is poorly adjusted, it can allow the original root system to retain its functionality, which could compromise the development and dominance of the clonal rootstock root system. In vitro propagation offers a distinct alternative for the mass multiplication of commercially important fruit species [24]. Various treatments have been evaluated in avocado micropropagation, with varying results in terms of limitations and successes, depending on the rootstock used [17]. While these techniques (in vitro, Brokaw and Ernst) require specialized infrastructure and technology, micropropagation has the advantage that it can be performed throughout the year, requires less space [17], and allows for the production of a greater number of plants from a smaller amount of plant material.

During the establishment stage, factors such as pre-disinfection and disinfection of the plant material, the type and quality of the explant, the culture medium, and growth regulators are essential. Pre-disinfection and disinfection are critical steps to reduce losses caused by microorganisms, such as bacteria and fungi [25], which represent a major problem during this stage of in vitro culture [17]. For this purpose, fungicides and/or bactericides are used in pre-disinfection [25] and disinfectants, such as sodium hypochlorite, in disinfection, in order to reduce microbial contamination and achieve asepsis [26,27].

Various explant types have been utilized for avocado micropropagation, including mature embryos [28,29], immature embryos [28,30], pollen grains [31], cotyledon-derived callus tissue [32], leaf [33], meristematic tissues, and nodal segments [21]. In avocado in vitro propagation, nodal segments have demonstrated superior shoot development compared to meristem-based approaches, which exhibit notable limitations [17]. Similarly, explants containing axillary buds tend to outperform those with apical buds, showing enhanced morphogenetic responses [34,35]. Despite its advantages, micropropagation of avocado presents critical challenges during the establishment stage. Explant browning and necrosis due to oxidative stress are major limitations, often resulting from reactive oxygen species (ROS) accumulation triggered by wounding, high phenolic content, or suboptimal culture conditions [36–38]. Oxidative stress can lead to lipid peroxidation, typically measured by malondialdehyde (MDA) levels, which compromises explant viability [39,40]. Preconditioning treatments like etiolation in darkness can lower ROS levels by boosting antioxidant enzymes such as peroxidase (POD) and superoxide dismutase (SOD) [38,41,42]. These enzymatic systems help maintain redox homeostasis, improving in vitro performance and survival of the plant [43].

Various rejuvenation strategies have been employed to enhance explant physiological youth and improve in vitro responses. These include growth regulator application [44], exposure to darkness or etiolation treatments [41], grafting onto juvenile rootstocks [45], and seedling-derived explant use [46]. Otherwise, the explant's nutritional status is a critical determinant of in vitro shoot development, as initial nutrient reserves directly influence morphogenetic responses [47]. The accumulation of these reserves is closely linked to the plant's phenological stage [48]. It has been reported that stems contain higher soluble sugar and starch concentrations compared to other tissues, with peak total soluble sugar levels observed shortly after sprouting [49]. The anatomical characteristics of the propagation

material also play a pivotal role in determining its morphogenic capacity, which is strongly influenced by the explant's ontogenetic stage [50]. In avocado, etiolated shoots exposed to light for one to two weeks exhibit rapid anatomical changes in the phloem, transitioning from a thin, discontinuous line of phloem fibers to a thicker, continuous band [23,41]. Concurrently, an increase in xylem cell number and hemicellulose content is observed, indicating active tissue differentiation and vascular development [41].

In in vitro culture, the composition of the culture medium and the use of growth regulators are critical for optimizing the development and quality of the explant [51]. Most avocado micropropagation studies employ Murashige and Skoog (MS) medium as a basal formulation, often with modifications to mineral salts, vitamins, and plant growth regulators. Early research identified specific components of the standard MS medium as detrimental to avocado tissue culture [17]. Notably, ammonium nitrate induced tissue necrosis; a 33% reduction in its concentration significantly improved tissue health and shoot development [17]. Furthermore, avocado's sensitivity to chloride ions [52] suggests that reducing chloride concentrations in the medium could further enhance in vitro responses. Among growth regulators, BAP is the most used cytokinin in avocado micropropagation [17,42]. Other plant growth regulators applied include GA_3 [42], benzyladenine (BA) [16,53] and MT [36].

Thus, this study aimed to evaluate the in vitro establishment response of 'Merensky 2' avocado rootstock explants subjected to various conditioning treatments, assessed at two independent experimental time points. In addition, the study investigated the individual effects of disinfection protocols and growth regulator applications on the performance of etiolated material.

2. Materials and Methods

The study was performed at the Propagation Laboratory of the Pontificia Universidad Católica de Valparaíso (32°54′ S, 71°16′ W). Plant material was randomly collected from a seven-year-old 'Merensky 2' avocado rootstock mother plant orchard and subjected to annual pruning for rejuvenation. Shoots used in the experiment emerged following pruning in August 2023. In addition, etiolated 'Merensky 2' material obtained using the Brokaw technique from the laboratory's avocado clonal propagation unit was included. Both field-derived and etiolated materials were subjected to weekly disinfection for 30 days using Phyton-27® (3 mL L^{-1}); (Marketing ARM International, INC, Florida, USA). The experiments were conducted at the end of the first and second vegetative flushes in the study region, namely in late January and early April, respectively. Ambient temperatures were recorded for 30 days prior to each plant material collection to characterize the environmental conditions preceding each flush. The study site is located in a Mediterranean climate zone, typified by hot, dry summers and cool, partly cloudy winters, with an average annual precipitation of 264 mm [54]. Daily average incident shortwave solar radiation varies seasonally, reaching a maximum of 9.1 kWh m^{-2} in December and a minimum of 2.6 kWh m^{-2} in June [54].

2.1. In Vitro Establishment with Nodal Section Explants from First and Second Vegetative Flushes

Three types of plant material differentiated by conditioning treatment were used: T1–etiolated material (E1 and E2), T2–field material covered for 30 days (CF1 and CF2), and T3–uncovered field material (UF1 and UF2). The timing of treatment initiation and material collection (first and second vegetative flush) was based on the phenological stages described by Mena [55] for the Quillota province, complemented by weekly monitoring of the mother plants (Table 1).

Table 1. Characteristics of the conditioned plant material, according to treatment, used in the establishment 1 and 2 experiments of "Merensky 2" avocado rootstock.

Plant Material at the End of First Vegetative Flush (Sileptic Shoots)						
Treatments	**Material Type**	**Munsell Color Values**	**Phenological State (BBCH)**	**% Leaf Abscission**	**Average Temperature (°C)**	**Hours > 38 °C**
T1 (E1)	Etiolated material	Yellow 5Y (8/4 and 8/6)	Not applicable	0%	Not applicable	Not applicable
T2 (CF1)	Field material covered for 30 days	Green-yellow 5GY (5/6 and 5/8)	219/319	100%	21.6	71
T3 (UF1)	Uncovered field material	Green-yellow 2.5GY (5/6, 5/8 and 6/6)	219/319	0%	19.6	3
Plant material at the end of second vegetative flush (Proleptic shoots)						
T1 (E2)	Etiolated material	Yellow 5Y (8/4 and 8/6)	Not applicable	0%	Not applicable	Not applicable
T2 (CF2)	Field material covered for 30 days	Green-yellow 5GY (5/8 and 6/10)	223/328	40%	18.3	13
T3 (UF2)	Uncovered field material	Green-yellow 2.5GY (6/6) and 5GY (5/6)	223/328	0%	18.0	1

In the field, plants of the first and second flush vegetative growth were characterized according to Munsell color values [56] and BBCH (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie) [57]. From these plants, 20 branches measuring 4–5 mm in diameter were randomly selected. Half of the selected branches were covered for 30 days using 1.2 m-high, 17 cm-diameter Kraft paper cylinders (VIERLTDA, Santiago, Chile) with sealed upper ends (CF1 and CF2). Temperature was recorded using Elitech RC-4 dataloggers (Elitech, San Jose, CA, USA), with sensors placed at the midpoints of shoots in both CF1, CF2, UF1 and UF2 treatments (Table 1).

Shoots from CF1, CF2, UF1, and UF2 were collected using pruning shears, while E1 and E2 shoots were harvested with a scalpel. In the Propagation Laboratory, leaves were removed, and stems were sectioned into 1.5 cm segments, each containing at least one bud. These segments were first washed with commercial detergent (Quix, Unilever Chile, Laboratorio Dukay S.A., Santiago, Chile) and rinsed under tap water for 15 min. Following this, they were disinfected by immersion in a 1.5% sodium hypochlorite (NaClO) solution (Comercial Vimaroni S.A., Quilpué, Chile), supplemented with 2838.97 µM ascorbic acid (AA) (Merck KGaA, Darmstadt, Germany) and 2602.49 µM citric acid (CA) (Comercial Vimaroni S.A., Quilpué, Chile), along with two drops of Tween 20 (Loba Chemie, Laboratory Reagents & Fine Chemicals, Mumbai, India). This solution was kept under constant agitation for 15 min. After disinfection, the segments were transferred to a laminar flow hood, where they were triple-rinsed with sterile distilled water containing 2838.97 µM AA and 2602.49 µM CA, then dried with absorbent paper.

One-centimeter nodal section explants, each with a single bud, were placed in culture flasks at a 40–50° angle relative to the medium surface. The culture medium was a modified Murashige and Skoog (1962) (MS) [58] formulation (Sigma-Aldrich, St. Louis, MO, USA), with the concentrations of ammonium nitrate and calcium chloride reduced to 33% of their original values and complemented with 30 g L^{-1} sucrose. Thirty-five mL flasks, each containing 8 mL of medium and one explant, were used. The pH was adjusted to 5.7 ± 0.1, and the medium was solidified with 6.5 g L^{-1} agar (Algas Marinas S.A., Santiago, Chile). Cultures were incubated in a growth chamber at 25 ± 1 °C with a 16-h light photoperiod (3000 Lux). To minimize oxidation, cultures were kept in darkness for 15 days. After this period, cultures were transferred to indirect light (500 Lux) and subsequently to direct light (3000 Lux). For each treatment, twelve explants were randomly selected and evaluated in three replicates. After 45 days, the percentage of explants exhibiting fungal or bacterial contamination, oxidation, survival, and sprouting (defined as shoots ≥ 4 mm) was recorded.

2.2. Biochemical Assays for Plant Material from First and Second Vegetative Flushes

ROS, SOD, POD, MDA and, Protein and Sugar Content

To evaluate ROS levels, 200 mg of vegetal material was ground and homogenized in 1 mL of 10 mM Tris-HCl buffer (pH 7.2) (Sigma-Aldrich, St. Louis, MO, USA). The collected supernatant was diluted in extraction buffer for fluorescence measurement, both with and without 1 mM H_2DCFDA-DCF probe. ROS formation was determined by measuring the fluorescence intensity of the probe's oxidation product (λexc/λem: 490/525 nm). Kinetic measurements were performed using a Skanit® Appliskan multiplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 30 min, with light exclusion [59].

To evaluate antioxidant enzyme activity on ROS generation, 200 mg vegetal material was used. Samples were centrifuged at 15,000× *g* for 20 min at 4 °C and then supernatant and pellet were resuspended in 0.8 mL of the same buffer. The SOD and POD enzyme activities were measured [60].

To determinate SOD activity the assay was based on the inhibition of nitro blue tetrazolium (NBT) reduction by superoxide radicals. A 2 mL reaction mix included 50 mM phosphate buffer (pH 7.8), 2 mM EDTA (Sigma-Aldrich, St. Louis, MO, USA), 9.9 mM L-methionine, 55 µM NBT, 0.025% Triton X-100, and 40 µL sample extract. To start the reaction, 20 µL of 1 mM riboflavin was added. The mix was exposed to a 15 W fluorescent light at 12 cm for 10 min while shaking. The blank was kept in darkness. Absorbance was recorded at 560 nm and quantified with a standard curve from pure SOD [60].

To determinate POD activity the assay mixture included 50 mM potassium phosphate buffer (pH 7.0) (Sigma-Aldrich, St. Louis, MO, USA), 0.5 mM guaiacol, 0.5 mM H_2O_2, and 0.01 mL sample extract. The reaction was initiated by adding H_2O_2, and absorbance was measured for 3 min. POD activity was determined by measuring the increase in absorbance at 470 nm, which corresponds to guaiacol oxidation, using an extinction coefficient of 26.6 mM cm^{-1} [60].

Lipid peroxidation was evaluated using the thiobarbituric acid (TBA) assay, specifically measuring malondialdehyde (MDA) as described in Zeb and Ullah [61]. Briefly, the supernatant absorbance was measured at 532 nm using an EPOCH microplate reader (ELx800, BioTek®, VT, USA). Non-specific absorbance at 600 nm was subtracted. The MDA-TBA complex concentration was calculated [61].

Total protein in the previously obtained extracts used for evaluated SOD, POD and MDA was quantified using the BCA method, following the manufacturer's instructions. Absorbance was measured at 562 nm, and protein concentration was determined by interpolation from a standard curve prepared with bovine serum albumin. Samples were analyzed in biological and technical triplicates [62]. In addition, we evaluated the effect of different growth conditions on the content of total soluble sugars and reducing sugars in 100 mg of collected plant material. Standard sugar samples (glucose, fructose, and sucrose) were prepared at 1 mg mL^{-1} in water and used as reference. For this, the µg reducing sugars per mg fresh tissue was determined using the DNS method [63].

Total sugar content was determined using the phenol–sulfuric acid method described by Doran and De Souza [64]. Briefly, the reaction mixture was incubated at 37 °C for 10 min, and absorbance was measured at 490 nm using an EPOCH microplate reader (BioTek). Data were interpolated into a glucose calibration curve, and results were expressed as µg total sugars per mg fresh tissue.

Both standard solutions and samples were repeatedly spotted (1 µL × 4) onto silica gel GF_{254} TLC plates (Merck Millipore, Darmstadt, Germany) at predefined positions. The TLC plates were developed using a chloroform–acetic-acid–water mobile phase (3:3.5:0.5 *v*/*v*) and dried at room temperature. Plates were then sprayed with ethanol–anisaldehyde–sulfuric acid (18:1:1) (Sigma-Aldrich, St. Louis, MO, USA) and visualized at 120 °C.

2.3. Anatomical Study

For this study, explants from treatments E1, CF1, UF1, E2, CF2, and UF2 were collected and processed according to the procedures described in Section 2.1. Each nodal section explant per treatment was washed with Tween 20, dried, and fixed in FAA solution (40% formalin (Agromaipo, Santiago, Chile), ethanol (MERCK®, Darmstadt, Germany), and acetic acid (MERCK®, Darmstadt, Germany)) for 72 h. Samples were then dehydrated using a graded ethanol series. After dehydration, samples were cleared in xylene (MERCK®, Darmstadt, Germany) for two 1-h cycles. Finally, they were infiltrated with Histosec (MERCK®, Darmstadt, Germany) through three changes to form paraffin blocks. Transverse sections of avocado rootstock stems, taken just below the bud, were cut to a thickness of 10 µm using a LEICA RM 2235 rotary microtome. Tissue staining was performed with alcoholic safranin (ARQUIMED, Santiago, Chile) and light green (HiMedia, Mumbai, India). Stained sections were examined and photographed using a LEICA ICC50 W camera mounted on a microscope at 4× and 10× magnification. Tissue visualization, cell counting, and measurements were conducted using ImageJ version 1.54 (Wayne Rasband and contributors, National Institutes of Health, Bethesda, MD, USA). Transverse stem sections revealed distinct structural developments across the tissue [23]. Measurements were taken by treatment and date in two defined zones of the avocado stem: zone 1 (adjacent to the bud, up to 1 mm away) and zone 2 (more than 2 mm from the bud). Within each zone, the following parameters were quantified using ImageJ software: distance from the epidermis to the cambial zone (inclusive), xylem zone length, number of phloem fibers and xylem vessels, fiber and xylem vessel wall thickness, and xylem vessel lumen area. Photomicrographs were calibrated using a reference scale, establishing a pixel-to-micron ratio of 1 pixel = 1.693 µm.

2.4. In Vitro Establishment of Etiolated Material

Disinfection Experiment with Etiolated Material

The culture medium used was modified MS. It included $Ca(NO_3)_2$ (4.5 meq), KNO_3 (10.9 meq), and NH_4NO_3 (7.5 meq). This experiment utilized etiolated (E) material sourced from the Cloning Unit of the Propagation Laboratory. A completely randomized design was implemented, resulting in four disinfection treatments. These treatments combined two NaClO concentrations with two pre-disinfection applications of Phyton-27®, applied 4 and 2 days prior to in vitro establishment. The treatments were as follows: T1: 1.5% NaClO + 1.5 mL L^{-1} Phyton-27®; T2: 2% NaClO + 1.5 mL L^{-1} Phyton-27®; T3: 1.5% NaClO + 3 mL L^{-1} Phyton-27®; and T4: 2% NaClO + 3 mL L^{-1} Phyton-27®. Nine explants per treatment were used, with three replicates per treatment. After 30 days, the percentage of explants showing infection (fungal or bacterial), oxidation, and survival was recorded.

2.5. Multiplication Experiments of Etiolated Explants

In the multiplication experiments, the same basal medium was used as in the establishment stage.

2.5.1. Explants Multiplication Using BAP and GA_3

This experiment utilized sprouted nodal section explants obtained from the prior in vitro establishment of etiolated material. A completely randomized design was applied, comprising eight treatments with five replicates each (one explant per replicate). To evaluate the individual and combined effects of growth regulators, three concentrations of BAP (Sigma-Aldrich, Steinheim, Germany) and one concentration of GA_3 (Merck, Darmstadt, Germany) were tested. The treatments were as follows: T0: Control (no growth regulators); T1: 4.44 µM BAP; T2: 8.88 µM BAP; T3: 13.32 µM BAP;

T4: 0.29 µM GA_3; T5: 4.44 µM BAP + 0.29 µM GA_3; T6: 8.88 µM BAP + 0.29 µM GA_3; T7: 13.32 µM BAP + 0.29 µM GA_3. Shoot length was measured at the beginning of the experiment and again after 30 days to assess growth variation.

2.5.2. Explants Multiplication Using MT and GA_3

These experiments employed sprouted nodal section explants derived from the previous in vitro establishment of etiolated material. A completely randomized design was implemented, consisting of six treatments with five replicates each (one explant per replicate). To evaluate the individual and combined effects of growth regulators, two concentrations of MT (MedChemExpress, Monmouth Junction, NJ, USA) and one concentration of GA_3 (Merck, Darmstadt, Germany) were tested. The treatments were as follows: T0: Control (no growth regulators); T1: 0.41 µM MT; T2: 0.83 µM MT; T3: 0.29 µM GA_3; T4: 0.41 µM MT + 0.29 µM GA_3; and T5: 0.83 µM MT + 0.29 µM GA_3. Shoot length was recorded at the start and after 30 days to assess growth variation.

2.6. Statistical Analysis

For all in vitro propagation tests and the anatomical study, a Generalized Linear Model (GLM) and a variance components analysis were used, evaluating the differences between treatments using the Tukey test ($p \leq 0.05$) in the Minitab 19 statistical software (Minitab Inc., State College, PA, USA). For biochemical assays, a one-way analysis of variance (ANOVA) was performed, followed by Tukey's multiple comparisons test, with a 95% confidence interval. All analyses were performed using GraphPad Prism 10.4 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. In Vitro Establishment with Nodal Section Explants from First and Second Vegetative Flushes

3.1.1. Contamination, Oxidation, Survival, and Sprouting in Nodal Section from the End of the First Vegetative Flush

During the in vitro establishment (Table 2), significant differences ($p \leq 0.05$) were observed among treatments in both explant loss due to oxidation and in the sprouting percentage. The UF1 treatment exhibited the highest percentage of explant loss attributable to oxidation, whereas E1 showed no oxidation-related losses. In terms of sprouting, E1 demonstrated a significantly higher sprouting percentage compared to UF1, which had the lowest sprouting performance among the treatments.

Table 2. In vitro establishment response of 'Merensky 2' avocado rootstock nodal section explants collected at the end of the first vegetative flush.

Treatment	Contamination				
	Bacterial (%)	**Fungal (%)**	**Oxidation (%)**	**Survival (%)**	**Sprouting ≥ 4 mm (%)**
E1	33.3 ± 5.6	8.3 ± 1.3	0.0 ± 0.0 c	58.3 ± 4.4	44.0 ± 4.8 a
CF1	19.4 ± 4.6	24.8 ± 3.0	19.5 ± 4.7 b	36.1 ± 3.3	19.4 ± 3.7 ab
UF1	22.0 ± 3.7	13.9 ± 2.8	36.1 ± 3.6 a *	25.0 ± 2.3	5.5 ± 1.7 b
p	n.s.	n.s.	0.001	n.s.	0.012

E1: Etiolated material; CF1: Field material covered for 30 days; UF1: Uncovered field material. Values are presented as mean ± SD. * Within each column, values with different letters indicate statistically significant differences (Tukey's test, $p \leq 0.05$). n.s.: $p > 0.05$.

3.1.2. Contamination, Oxidation, Survival, and Sprouting in Nodal Section from the End of the Second Vegetative Flush

In vitro establishment response for this time point was analyzed (Table 3), revealing that the percentages of oxidation-related loss, survival, and sprouting were significantly

different ($p \leq 0.05$). Specifically, E2 exhibited a lower percentage of oxidation and higher survival and sprouting compared to UF2.

Table 3. In vitro establishment response of 'Merensky 2' avocado *rootstock* nodal section explants collected at the end of the second vegetative flush.

Treatment	Contamination				
	Bacterial (%)	Fungal (%)	Oxidation (%)	Survival (%)	Sprouting ≥ 4 mm (%)
E2	30.6 ± 3.3	16.7 ± 4.3	0.0 ± 0.0 b	52.8 ± 2.7 a	41.7 ± 6.6 a
CF2	27.8 ± 2.0	33.3 ± 3.3	5.6 ± 0.6 ab	33.3 ± 3.3 ab	19.4 ± 2.7 ab
UF2	41.7 ± 3.6	13.9 ± 2.7	19.5 ± 1.7 a *	25 ± 2.3 b	5.5 ± 1.7 b
p	n.s.	n.s.	0.021	0.035	0.032

E2: Etiolated material; CF2: Field material covered for 30 days; UF2: Uncovered field material. Values are presented as mean ± SD. * Within each column, values with different letters indicate statistically significant differences (Tukey's test, $p \leq 0.05$). n.s.: $p > 0.05$.

3.2. Biochemical Assays for Plant Material from First and Second Vegetative Flushes

ROS, SOD, POD, MDA and, Protein and Sugar Content

In the first vegetative flush, biochemical assays revealed significant differences ($p \leq 0.05$) in protein content, ROS levels, antioxidant enzyme activity, and MDA content among treatments. Protein content (Figure 1a) was highest in UF1, followed by CF1, and lowest in E1. MDA content (Figure 1b), a marker of oxidative stress, was greatest in CF1, intermediate in UF1, and lowest in E1. POD activity (Figure 1c) was significantly higher in E1 compared to CF1 and UF1, while SOD activity (Figure 1d) was highest in CF1, followed by UF1, and lowest in E1. ROS levels (Figure 1e) were highest in CF1, followed by E1, and lowest in UF1. On the other hand, significant differences ($p \leq 0.05$) were observed in total sugar content (Figure 1f) and reducing sugar content (Figure 1g). UF1 showed the highest levels of both total and reducing sugars, followed by E1, with CF1 exhibiting the lowest values. The ratio of reducing-to-total sugar content (Figure 1h) was greatest in UF1, while E1 and CF1 displayed similar, lower ratios.

For the second vegetative flush, significant differences ($p \leq 0.05$) were found in ROS levels, antioxidant activity, and MDA content. Protein content (Figure 2a) was highest in UF2, followed by CF2, and lowest in E2. MDA content (Figure 2b), an indicator of oxidative stress, was also highest in UF2, intermediate in CF2, and lowest in E2. POD activity (Figure 2c) was greatest in E2 and lower in both CF2 and UF2. SOD activity (Figure 2d) peaked in UF2, followed by CF2, and was lowest in E2. ROS levels (Figure 2e) were highest in CF2, moderate in UF2, and lowest in E2. In addition, analysis of reducing sugar content (Figure 2f) and total sugar content (Figure 2g) showed that UF2 exhibited the highest total sugar levels, followed by CF2, with E2 showing the lowest values. Reducing sugar content was highest in UF1, with lower values in CF2 and E2. The ratio of reducing-to-total sugar content (Figure 2h) was greatest in UF2, while E2 and CF2 displayed similar lower ratios.

For the first vegetative flush, thin-layer chromatography (TLC) analysis of sugars (Figure 3a) yielded qualitative results, with stain intensity reflecting the relative abundance of each compound. Fructose content was higher in E1 compared to CF1 and UF1, whereas glucose was more abundant in CF1 and UF1 than in E1. Sucrose was not detected in any of the treatments.

On the other hand, for the second vegetative flush, the TLC analysis of sugars (Figure 3b) showed higher fructose levels in E2 compared to CF2 and UF2, while glucose levels were higher in CF2 and UF2 relative to E2. Sucrose was not detected in any of the treatments.

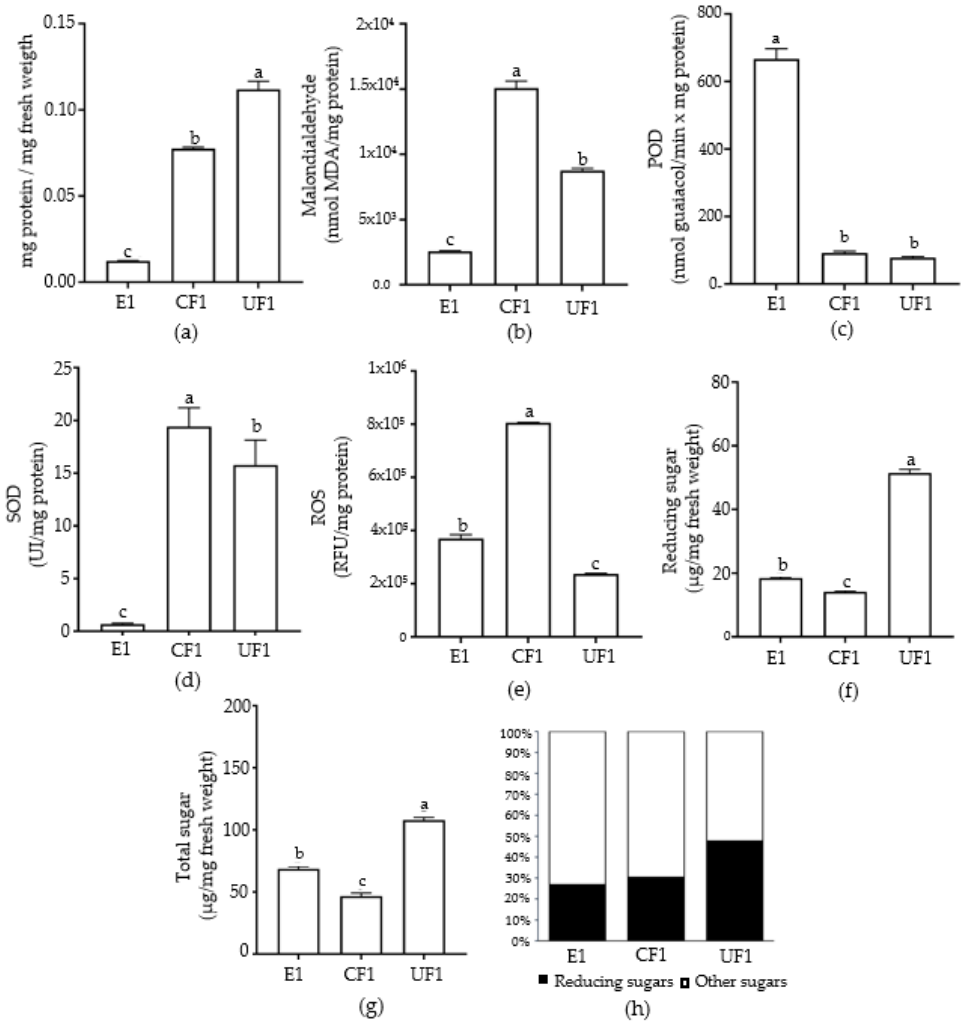

Figure 1. Biochemical analysis of 'Merensky 2' avocado rootstock stem sections collected at the end of the first vegetative flush. Parameters assessed include (**a**) Protein content (relative to fresh weight), (**b**) Malondialdehyde (MDA) content, (**c**) Peroxidase (POD) activity, (**d**) Superoxide dismutase (SOD) activity, (**e**) Reactive oxygen species (ROS) levels, (**f**) Reducing sugar, (**g**) Total sugar and (**h**) Ratio of reducing sugar-to-total sugars. Treatments: E1—etiolated material; CF1—field material covered for 30 days; UF1—uncovered field material. Bars with different letters denote statistically significant differences (ANOVA, Tukey's test, $p \leq 0.05$).

3.3. Anatomical Study

At the anatomical level, significant differences ($p \leq 0.05$) were observed among treatments at the end of the first vegetative flush. In zone 1 (Table 4), statistically significant differences were detected in the distance from the epidermis to the cambial zone (E-Cam), xylem zone length (Xi), phloem fiber count (NFPh), xylem vessel count (NVXi), xylem vessel wall thickness (GPVXi), and xylem vessel lumen area (ALVXi). E-Cam was greater in CF1 and UF1; Xi was significantly greater in UF1; NFPh was highest in E1; NVXi differed significantly between E1 and UF1; GPVXi was greater in CF1; and ALVXi showed significant variation between CF1 and UF1.

In zone 2 (Table 4), E-Cam was again greater in CF1 and UF1; Xi and NFPh were greater in E1; NVXi was higher in both E1 and CF1; and GPVXi was greater in CF1 and E1. No significant differences ($p > 0.05$) were observed in phloem fiber wall thickness (GPFPh).

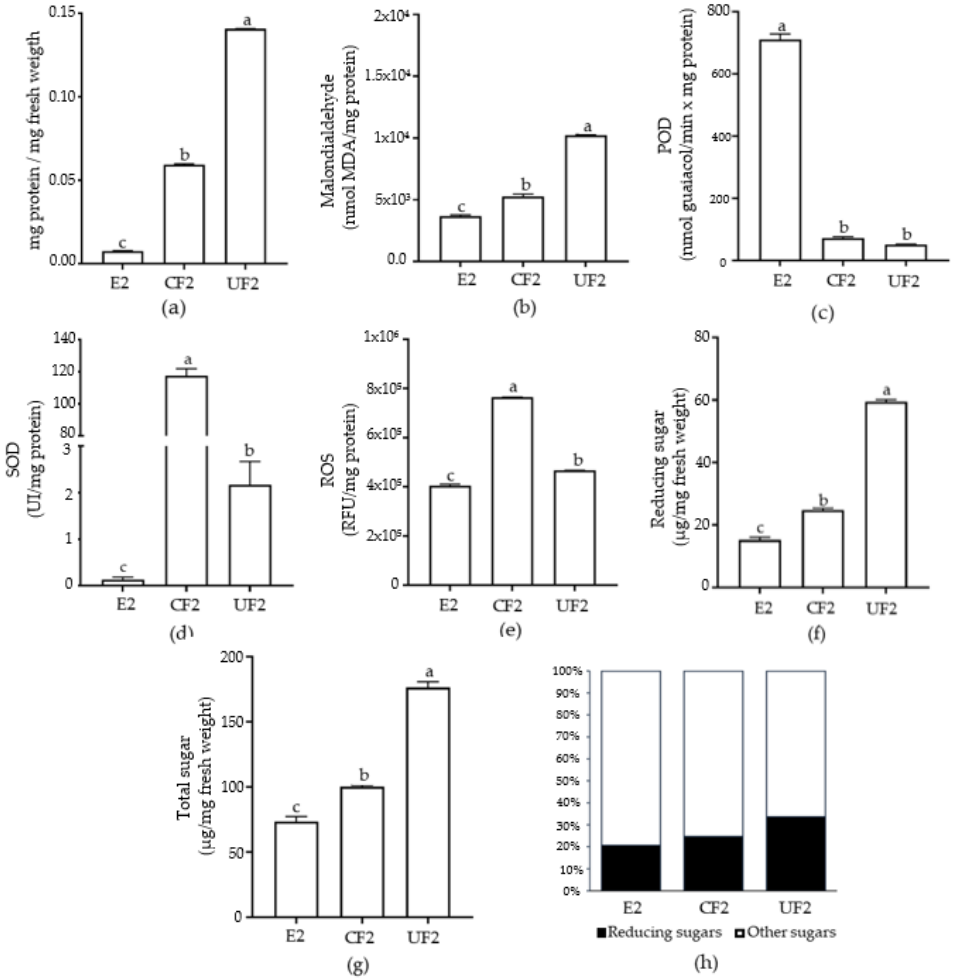

Figure 2. Biochemical analysis of 'Merensky 2' avocado rootstock stem sections collected at the end of the second vegetative flush. Parameters assessed include (**a**) Protein content (relative to fresh weight), (**b**) Malondialdehyde (MDA) content, (**c**) Peroxidase (POD) activity, (**d**) Superoxide dismutase (SOD) activity, (**e**) Reactive oxygen species (ROS) levels, (**f**) Reducing sugar, (**g**) Total sugar and (**h**) Ratio of reducing sugar-to-total sugars. Treatments: E2—etiolated material; CF2—field material covered for 30 days; UF2—uncovered field material. Bars with different letters denote statistically significant differences (ANOVA, Tukey's test, $p \leq 0.05$).

Notably, a continuous xylem zone encircling the stem was observed in E1 (Figure 4a), in contrast to the discontinuous pattern seen in CF1 (Figure 4b) and UF1 (Figure 4c). In CF1 and UF1, phloem fibers appeared agglomerated and poorly lignified (Figure 4e,f,h,i). In E1, cell walls appeared weaker with the formation of intercellular spaces (Figure 4d,g).

Measurements from zone 1 (Table 5) revealed significant differences in several anatomical parameters at the end of the second vegetative flush: distance from the epidermis to the cambial zone (E-Cam), xylem zone length (Xi), phloem fiber count (NFPh), phloem fiber wall thickness (GPFPh), xylem vessel wall thickness (GPVXi), and xylem vessel lumen area (ALVXi). E-Cam was greater in CF2 and UF2, while Xi and NFPh were greater in E2. GPFPh was higher in CF2 and UF2, GPVXi differed significantly between E2 and UF2 (greater in UF2) and ALVXi was highest in E2.

In zone 2 (Table 5), significant differences were observed in the following parameters: E-Cam, Xi, NFPh, GPVXi, and ALVXi. E-Cam was greater in UF2, while Xi and NFPh were greater in E2. GPVXi differed significantly between UF2 and CF2, with greater thickness in CF2. ALVXi was highest in E2. Notably, E2 exhibited a continuous xylem ring with

thickened vessel walls, particularly in zone 1, compared to CF2 and UF2. Phloem fibers appeared agglomerated and poorly lignified in CF2 and UF2 (Figure 5).

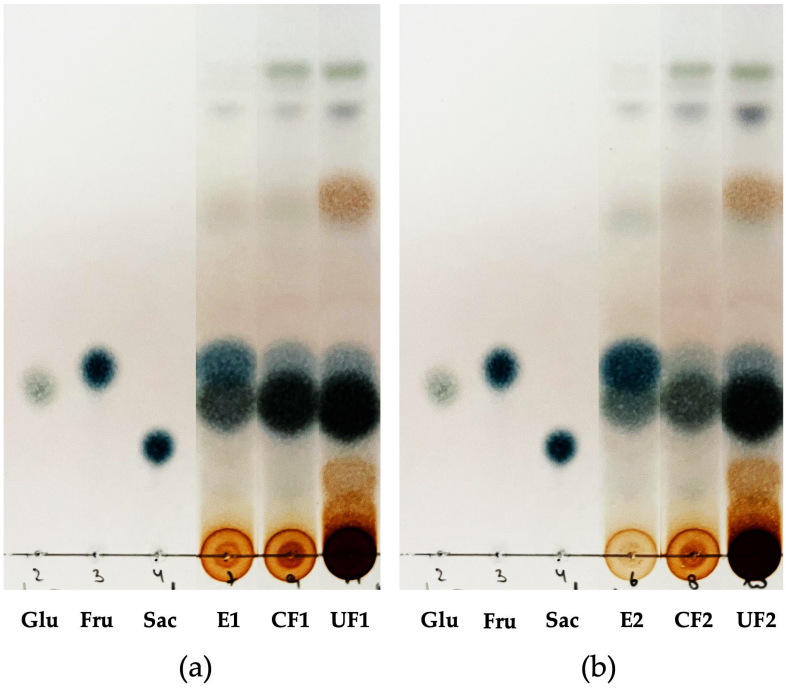

Figure 3. Thin-layer chromatogram (TLC) showing glucose (Glu), fructose (Fru), and sucrose (Suc) standards alongside ethanolic extracts from 'Merensky 2' avocado rootstock stems. (**a**) First vegetative flush, (**b**) Second vegetative flush. Etiolated material (E1 and E2), field material covered for 30 days (CF1 and CF2), and uncovered field material (UF1 and UF2).

Table 4. Anatomical evaluation of zone 1 and 2 in 'Merensky 2' avocado rootstock stems at the end of the first vegetative flush.

Zone 1							
Treatment	**E-Cam (µm)**	**Xi (µm)**	**NFPh**	**GPFPh (µm)**	**NVXi**	**GPVXi (µm)**	**ALVXi (µm^2)**
E1	287.8 ± 17.9 b	131.9 ± 12.9 b	31.8 ± 7.8 a	3.2 ± 0.5	24.8 ± 2.1 a	1.6 ± 0.2 c	284.1 ± 11.3 ab
CF1	620.8 ± 25.4 a	151 ± 12.4 b	14.7 ± 3.5 b	2.5 ± 0.8	21.5 ± 3.5 ab	3.2 ± 0.3 a	251.8 ± 13.2 b
UF1	619.2 ± 21.1 a *	184.6 ± 10.7 a	16.6 ± 5.5 b	2.3 ± 0.8	16.7 ± 3.8 b	2.6 ± 0.2 b	353.2 ± 14.2 a
p	0.000	0.000	0.000	n.s.	0.020	0.000	0.002
Zone 2							
Treatment	**E-Cam (µm)**	**Xi (µm)**	**NFPh**	**GPFPh (µm)**	**NVXi**	**GPXi (µm)**	**ALVXi (µm^2)**
E1	294.8 ± 11.7 b	105.7 ± 15.0 a	21.2 ± 3.8 a	2.7 ± 0.2	17.7 ± 2.6 a	1.7 ± 0.1 b	253.9 ± 11.7
CF1	437.8 ± 17.6 a	57.7 ± 13.4 b	4.5 ± 1.3 b	2.7 ± 0.1	12.2 ± 1.5 a	2.8 ± 0.4 a	250.8 ± 10.0
UF1	439.0 ± 13.9 a *	67.8 ± 19.3 b	3.5 ± 1.8 b	2.1 ± 0.3	5.7 ± 0.7 b	2.7 ± 0.1 a	237.9 ± 14.7
p	0.000	0.000	0.000	n.s.	0.000	0.000	n.s.

E1: Etiolated material; CF1: Field material covered for 30 days; UF1: Uncovered field material. E-Cam: Distance from epidermis to cambial zone; Xi: Xylem zone length; GPFPh: Phloem fiber wall thickness; NVXi: Xylem vessel count; GPVXi: Xylem vessel wall thickness; ALVXi: Xylem vessel lumen area. Values are presented as mean ± SD. * Within each column, values with different letters indicate statistically significant differences (Tukey's test, $p \leq 0.05$). n.s.: $p > 0.05$.

3.4. In Vitro Establishment of Etiolated Material

Disinfection Experiment with Etiolated Material

Treatment 4, which combined the highest concentration of Phyton-27® (3 mL L^{-1}) with the highest concentration of NaClO (2%), resulted in the highest survival rate and the

lowest contamination-related losses (Table 6). Notably, none of the treatments exhibited losses due to oxidation.

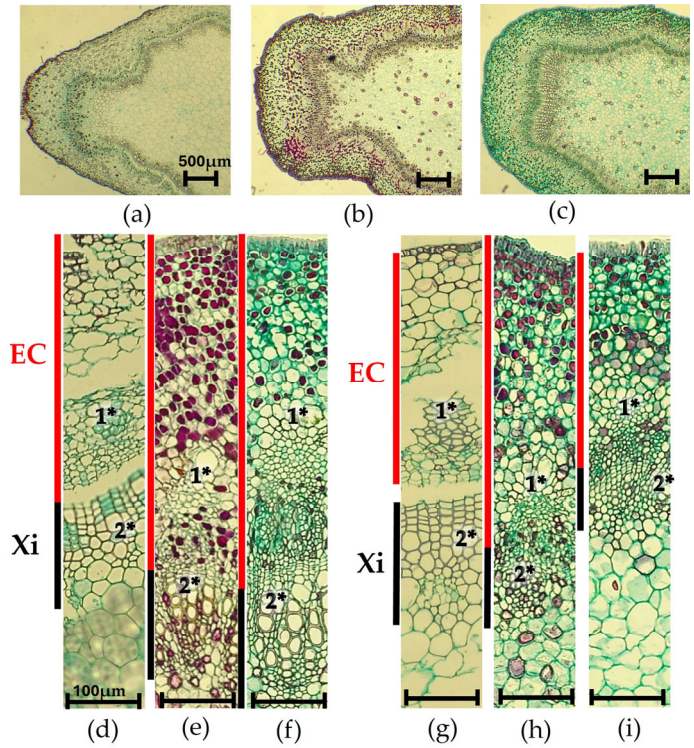

Figure 4. Transverse sections of 'Merensky 2' avocado rootstock stems collected and fixed in FAA at the end of the first vegetative flush: (**a**) etiolated material, (**b**) covered field material for 30 days, (**c**) uncovered field material, (**d**) etiolated material (zone 1), (**e**) covered field material for 30 days (zone 1), (**f**) uncovered field material (zone 1), (**g**) etiolated material (zone 2), (**h**) covered field material for 30 days (zone 2), (**i**) uncovered field material (zone 2). Key: EC = distance from epidermis to cambial zone, Xi = xylem zone length, 1* = phloem fibers, 2* = xylem vessels. Scale bars: (**a**–**c**) 500 µm; (**d**–**i**) 100 µm.

Table 5. Anatomical evaluation of zone 1 and 2 in 'Merensky 2' avocado rootstock stems at the end of the second vegetative flush.

Zone 1							
Treatment	**E-Cam (µm)**	**Xi (µm)**	**NFPh**	**GPFPh (µm)**	**NVXi**	**GPVXi (µm)**	**ALVXi (µm²)**
E2	278.6 ± 14.6 b	164.8 ± 14.1 a	16.5 ± 2.6 a	3.2 ± 0.8 a	21.5 ± 3.6	1.8 ± 0.3 b	394.5 ± 16.3 a
CF2	529.4 ± 16.9 a *	95.1 ± 12.2 b	5.0 ± 1.0 b	1.7 ± 0.5 b	16.6 ± 2.6	2.1 ± 0.3 ab	157.2 ± 10.6 c
UF2	510.9 ± 10.5 a	110.4 ± 16.8 b	4.0 ± 1.4 b	2.6 ± 0.3 a	16.3 ± 1.7	2.4 ± 0.5 a	258.4 ± 12.1 b
p	0.000	0.000	0.000	0.000	n.s.	0.002	0.000
Zone 2							
Treatment	**E-Cam (µm)**	**Xi (µm)**	**NFPh**	**GPFPh (µm)**	**NVXi**	**GPXi (µm)**	**ALVXi (µm²)**
E2	271 ± 11.9 c	141.9 ± 10.4 a *	21.2 ± 2.4 a	3.1 ± 0.2 ab	19.3 ± 3.8	1.6 ± 0.3 b	422.4 ± 11.7 a
CF2	429.7 ± 12.9 b	59.6 ± 8.3 b	2.5 ± 0.3 b	3.7 ± 0.3 a	16.2 ± 2.1	2.8 ± 0.4 a	195.9 ± 10.0 b
UF2	505.2 ± 11.9 a	65.9 ± 7.1 b	6.3 ± 1.3 b	2.3 ± 0.1 b	14.7 ± 2.3	1.9 ± 0.2 b	278.5 ± 10.3 b
p	0.000	0.000	0.000	0.004	n.s.	0.000	0.000

E2: Etiolated material; CF2: Field material covered for 30 days; UF2: Uncovered field material. E-Cam: Distance from epidermis to cambial zone; Xi: Xylem zone length; GPFPh: Phloem fiber wall thickness; NVXi: Xylem vessel count; GPVXi: Xylem vessel wall thickness; ALVXi: Xylem vessel lumen area. Values are presented as mean ± SD. * Within each column, values with different letters indicate statistically significant differences (Tukey's test, $p \leq 0.05$). n.s.: $p > 0.05$.

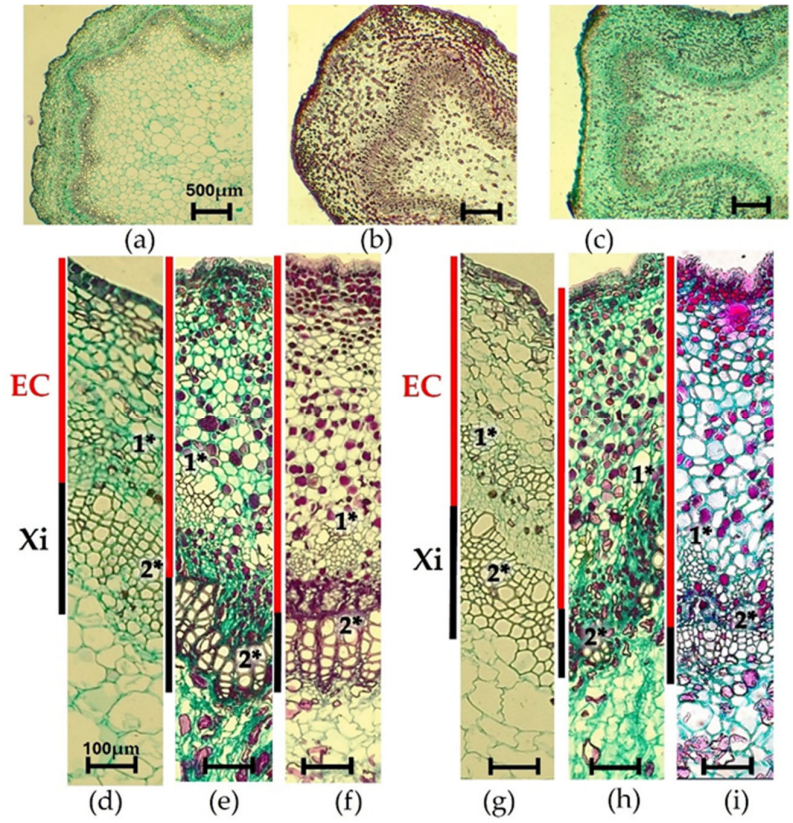

Figure 5. Transverse sections of '*Merensky 2*' avocado rootstock stems collected and fixed in FAA at the end of the second vegetative flush: (**a**) etiolated material, (**b**) covered field material for 30 days, (**c**) uncovered field material, (**d**) etiolated material (zone 1), (**e**) covered field material for 30 days (zone 1), (**f**) uncovered field material (zone 1), (**g**) etiolated material (zone 2), (**h**) covered field material for 30 days (zone 2), (**i**) uncovered field material (zone 2). Key: EC = distance from epidermis to cambial zone, Xi = xylem zone length, 1* = phloem fibers, 2* = xylem vessels. Scale bars: (**a**–**c**) 500 µm; (**d**–**i**) 100 µm.

Table 6. Percentage of contamination and survival of 'Merensky 2' avocado rootstock explants in vitro.

Treatments	Contamination		
	Bacterial (%)	**Fungal (%)**	**Survival (%)**
T1	59.3 ± 6.4 a *	40.7 ± 4.4	3.7 ± 1.4 b
T2	30 ± 3.1 bc	60 ± 4.1	11.1 ± 0.0 b
T3	40.7 ± 3.2 b	37.4 ± 3.6	11.1 ± 2.4 b
T4	18.5 ± 2.5 c	37 ± 3.4	44.4 ± 0.0 a
p	0.000	n.s.	0.000

T1: 1.5% NaClO + 1.5 mL L^{-1} Phyton-27®; T2: 2% NaClO + 1.5 mL L^{-1} Phyton-27®; T3: 1.5% NaClO + 3 mL L^{-1} Phyton-27®; T4: 2% NaClO + 3 mL L^{-1} Phyton-27®. Values are presented as mean ± SD. * Within each column, values with different letters indicate statistically significant differences (Tukey's test, $p \leq 0.05$). n.s.: $p > 0.05$.

3.5. Multiplication Experiments of Etiolated Explants

3.5.1. Explants Multiplication Using BAP and GA_3

Shoot growth did not vary significantly across the different hormone concentrations and combinations ($p > 0.05$) (Table 7) (Figure 6). However, lateral bud formation was significantly influenced ($p \leq 0.05$), with treatments 3, 5, and 6 showing the highest number of lateral buds. Notably, chlorosis and leaf abscission were observed exclusively in plants from treatment 4 (0.29 µM GA_3) (Figure 7).

Table 7. Shoot growth and lateral bud formation in 'Merensky 2' avocado rootstock explants treated with BAP and GA_3.

Treatment	Growth Variation (mm)	N° of Lateral Buds
Control	1.0 ± 0.3	0 ± 0.0 b
T1	1.3 ± 0.2	0 ± 0.0 b
T2	1.6 ± 0.1	0.2 ± 0.1 b
T3	1.9 ± 0.4	1.2 ± 0.2 ab
T4	0.9 ± 0.3	0 ± 0.0 b
T5	2.0 ± 0.5	1.0 ± 0.2 ab
T6	2.5 ± 0.6	1.8 ± 0.2 a *
T7	2.1 ± 0.5	0.6 ± 0.2 ab
p	n.s.	0.002

Control: No growth regulators; T1: 4.44 µM BAP; T2: 8.88 µM BAP; T3: 13.32 µM BAP; T4: 0.29 µM GA_3; T5: 4.44 µM BAP + 0.29 µM GA_3; T6: 8.88 µM BAP + 0.29 µM GA_3; T7: 13.32 µM BAP + 0.29 µM GA_3. Values are presented as mean ± SD. * Within each column, values with different letters indicate statistically significant differences (Tukey's test, $p \leq 0.05$). n.s.: $p > 0.05$.

Figure 6. In vitro multiplication of 'Merensky 2' avocado rootstock material. Scale bar: 1 cm.

3.5.2. Explants Multiplication Using MT and GA_3

The in vitro shoot growth response of nodal sections to varying concentrations of MT and its combination with 0.29 µM GA_3 (Table 8) showed no significant differences in shoot elongation. In addition, lateral bud formation was absent across all treatments. However, treatments containing MT demonstrated enhanced leaf development compared to the control and T3 (Figure 7).

Table 8. Shoot growth response of 'Merensky 2' avocado rootstock explants treated with MT and GA_3.

Treatment	Growth Variation (mm)
Control	1.0 ± 0.2
T1	2.6 ± 0.4
T2	2.2 ± 0.3
T3	0.9 ± 0.2
T4	2.3 ± 0.4
T5	2 ± 0.2
p	n.s.

Control: No growth regulators; T1: 0.41 µM MT; T2: 0.83 µM MT; T3: 0.29 µM GA_3; T4: 0.41 µM MT + 0.29 µM GA_3; T5: 0.83 µM MT + 0.29 µM GA_3. Values are presented as mean ± SD. n.s.: $p > 0.05$.

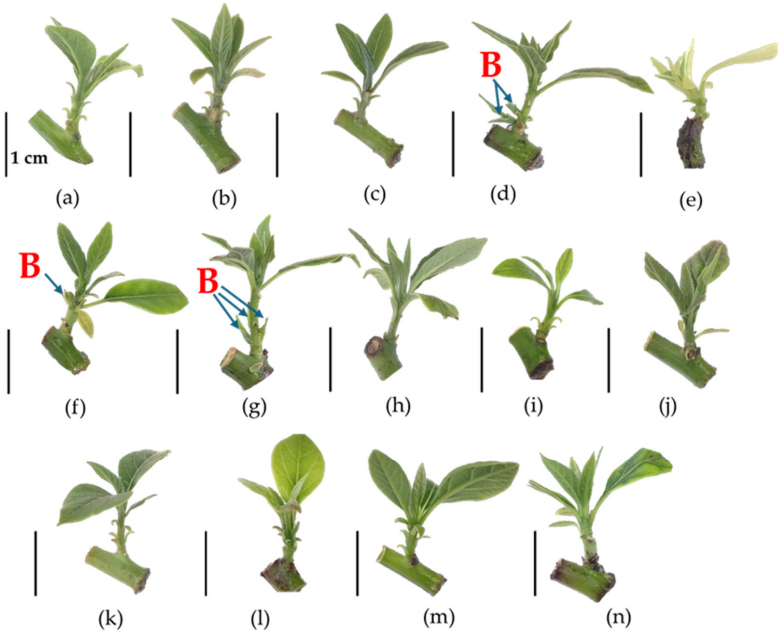

Figure 7. Shoot growth of 'Merensky 2' avocado rootstock explants in vitro, 30 days after initiation of hormonal treatments. (**a**–**h**): BAP and GA_3. (**i**–**n**): MT and GA_3. (**a**) Control (no hormones), (**b**) 0.29 µM GA_3, (**c**) 4.44 µM BAP, (**d**) 8.88 µM BAP, (**e**) 13.32 µM BAP, (**f**) 4.44 µM + 0.29 µM GA_3, (**g**) 8.88 µM BAP + 0.29 µM GA_3, (**h**) 13.32 µM BAP + 0.29 µM GA_3. (**i**) Control; (**j**)T1: 0.41 µM MT; (**k**)T2: 0.83 µM MT; (**l**)T3: 0.29 µM GA_3; (**m**)T4: 0.41 µM MT + 0.29 µM GA_3; (**n**)T5: 0.83 µM MT + 0.29 µM GA_3. B = axial bud sprouting. Scale bar: 1 cm.

4. Discussion

This study demonstrated that explant oxidation represents a major barrier to successful avocado micropropagation, primarily associated with elevated reactive oxygen species (ROS) accumulation. Etiolated explants (treatments E1 and E2) showed higher sprouting rates and no losses due to oxidation, in contrast to uncovered field explants (UF1 and UF2), which exhibited greater oxidation and reduced sprouting. Similar benefits of etiolation preconditioning have been reported in other avocado rootstocks [65], and species such as mango, where dark pre-treatments minimize browning and enhance in vitro sprouting [66,67]. Physiologically, this can be attributed to antioxidant capacity; etiolated material showed elevated POD levels [68], a key enzyme in ROS degradation. The elevated POD content in etiolated tissues promotes the elimination of peroxides and free radicals (ROS), preventing them from reaching damaging levels. In plants, POD, in conjunction with SOD, forms the first line of defense against oxidative stress [43]. When antioxidant enzyme levels are insufficient, ROS accumulate, leading to cell damage and death [43,66,69]. Etiolated explants (E1 and E2) exhibited lower ROS and MDA content, and higher POD activity compared to both covered (CF1, CF2) and uncovered (UF1, UF2) field treatments. The reduced MDA content in etiolated material indicates less lipid membrane damage due to oxidative stress, reflecting an improved physiological state. Conversely, covered (CF1 and CF2) and uncovered (UF1 and UF2) field explants exhibited the highest ROS and MDA accumulation, coupled with relatively low POD and SOD activity, indicating an antioxidant imbalance. Under abiotic stress (e.g., wounding, heat), elevated ROS levels activate PPO and other oxidases, leading to tissue browning and death [66,68]. Therefore, abiotic stresses, such as the high summer temperatures to which CF1 and UF1 explants were exposed at the end of the first vegetative flush, likely explain the increased oxidative losses compared to CF2 and UF2 explants from the end of the second vegetative flush. This is supported by the higher MDA content in CF1 compared to UF1, suggesting that the

covered material, due to prolonged exposure to temperatures exceeding 40 °C, activated a more robust defense against stress. Specifically, the increased ROS levels in CF1 likely triggered an upregulation of antioxidant molecules to regulate ROS content [68], resulting in lower oxidation losses at that time. It has been observed in several plant species that when subjected to moderate-to-severe combined stress, they respond better to future stress [70]. Considering this background, temperature control of field material prior to collection could have allowed better results in the in vitro establishment of avocado. In *Saccharum* spp. callus grown in vitro on NaCl medium when transferred to a salt-free medium, regenerated shoots showed significantly higher levels of antioxidants compared to the control treatment, suggesting a higher responsiveness to possible oxidative damage [71].

In future studies, it would be relevant to evaluate the prior application of a compound that increases antioxidant activity, with the objective of reducing oxidative losses in the establishment. In this context, the use of brassinosteroids would be a good alternative to evaluate in order to reduce oxidative loss in field material during in vitro establishment [72].

Dark treatments mitigate ROS peaks, thereby reducing explant oxidation [66,67]. In this study, pre-etiolation of shoots prior to in vitro establishment nearly eliminated oxidative losses, while partial covering of field branches significantly reduced browning compared to fully exposed material. This is evidenced by ~19.5% and ~5.6% oxidized explants in CF1 and CF2, respectively, versus ~36.1% and ~19.5% oxidized explants in UF1 and UF2, respectively. This reduction could be attributed to higher relative SOD activity, although it was insufficient to fully neutralize the generated ROS. In essence, etiolated material entered the in vitro system with a low oxidative load and robust antioxidant defenses, preventing browning and enhancing survival and sprouting. This starkly contrasts with explants from covered or uncovered fields, where high MDA and low antioxidant activity compromised tissue viability.

Initial carbohydrate profiles varied among the materials. Specifically, etiolated material (E1 and E2) exhibited higher fructose levels, while field explants displayed higher glucose levels. Sucrose was undetectable in all treatments, suggesting rapid utilization within the sampled tissue. Uncovered field material (UF1 and UF2) contained the highest total and reducing sugar content, exceeding that of covered and etiolated materials. This indicates that light-exposed material (UF1 and UF2) possessed greater carbohydrate reserves at the time of in vitro establishment, attributable to photosynthetic accumulation. Conversely, sugars in etiolated stems (E1 and E2) likely originated from the mobilization of internal reserves [73]. Shading has been shown to reduce photosynthetic activity in plants, leading to decreased sugar content and ATP formation [74]. This could partially explain the weaker anatomical structure of etiolated material (E1 and E2) compared to field material (CF1, CF2, UF1, and UF2), and the lack of significant differences between covered and uncovered field material. The latter may be attributed to the short covering period, given that field branches were initially photosynthetically active [23]. A lower total sugar content was observed in CF1, likely due to shading and heat-induced leaf loss, which directly reduces the photosynthetically active area [73]. Conversely, CF2, which experienced minimal leaf loss, exhibited higher sugar content.

Xylem characteristics are also relevant. A study in cucumber (*Cucumis sativus* L.) demonstrated that increased xylem vessel area in the stem and vascular bundle area in the root resulted in higher yields [75]. In this study, only nodal sections, directly contacting the culture medium, were used; therefore, rooted material was not examined. The xylem zone length, xylem vessel count, and xylem vessel lumen area could potentially correlate with higher sprouting responses. This characteristic was observed in etiolated material [76]. Although the xylem zone length in etiolated material was not greater than that in covered or uncovered field material, the etiolated material exhibited a continuous xylem zone in

the stem cross-section. In contrast, covered and uncovered field material showed a similar xylem zone length only in zone 1 and a reduced xylem zone length and fewer xylem vessels in zone 2. Xylem characteristics may be a contributing factor, alongside other variables, such as hydraulic conductivity, which affects water and nutrient transport [77]. It would be expected that field material covered for 30 days at the end of the first vegetative flush (CF1), during the hottest month [78] with temperatures exceeding 40 °C, would exhibit increased xylem wall thickness [79]. However, this was not observed, likely due to the near-complete loss of leaves, resulting from high-temperature damage to the photosynthetic and foliar apparatus [80]. This leaf loss reduced evapotranspiration and, consequently, plant adaptation [81], leading to a smaller xylem cell lumen area in CF1 compared to uncovered branches.

Given the availability of etiolated plant material and its favorable response, particularly the absence of oxidation-related losses, subsequent experiments focused on optimizing survival with this material. Preliminary experiments without pre-disinfection resulted in 100% contamination due to bacterial presence (unpublished data). Utilizing Phyton-27®, a bactericide–fungicide product with a cupric component, has demonstrated effective control of bacterial diseases [82]. The highest tested dose of Phyton-27®, in combination with the highest NaClO concentration, yielded the highest survival rates without oxidative losses. Therefore, future studies could explore higher combination doses of these products to further enhance survival while minimizing chemical stress-induced oxidative damage.

In in vitro multiplication studies, it has been shown that BAP concentrations of 2.22 µM and 13.32 µM induce a greater number of shoots in avocado [35], which is in agreement with our observations, where an increase in bud formation and sprouting was observed with the use of 13.32 µM of BAP. Moreover, it has been reported in in vitro studies that gibberellins improve bud sprouting and stem elongation [83]. However, in our study, applying GA_3 as the only growth regulator did not promote shoot formation and growth. On the contrary, negative effects such as leaf abscission and chlorosis were observed. This is in agreement with other studies, where GA_3-supplemented culture medium showed physiological disorders, e.g., apex and leaf necrosis [84–86], possibly due to hormonal imbalance [87]. Meanwhile, its application in conjunction with BAP did not show a detrimental effect and was associated with increased bud formation and sprouting. This response has been observed in other species, where the combination with a species-specific concentration of cytokinin and gibberellin has a positive impact on growth and multiplication [85,88–90], which is possibly due to the balance in biosynthesis and signal transduction levels achieved [87,90]. On the other hand, the use of culture media supplemented with MT or MT in combination with GA_3 did not affect shoot growth, unlike the response obtained in another in vitro trial using the avocado rootstock 'Velvick' with the same concentrations [36]. This difference may be due to the characteristics of the plant, since, depending on the species and genotype, the response and sensitivity to the growth regulator will be different [86,87,91], which is related to the endogenous hormone content of the plant [87,90]. Therefore, future experiments using growth regulators and different concentrations, either individually or in combination, will make it possible to identify the treatments that obtain better and faster development of the 'Merensky 2' rootstock under in vitro conditions, which will make it possible to optimize stages of the process, such as rooting. Of the above, it has been shown in another species that a vigorous shoot obtained at the multiplication stage achieves a better rooting response [91]. It is possible that the presence of the nodal section limited the efficacy of the growth regulators; therefore, evaluating these regulators using only shoot explants may yield improved results.

5. Conclusions

Etiolated 'Merensky 2' avocado rootstock material exhibited a superior in vitro establishment response due to a combination of biochemical, anatomical, and environmental factors. These explants exhibited reduced oxidative stress at the initial stages, which contributed to increased survival rates in culture medium. Disinfection of etiolated material with a combination of 3 mL L^{-1} of Phyton-27® and 2% NaClO produced the lowest contamination rates. Furthermore, bud induction was enhanced by treatments with BAP alone or combination with GA_3.

Author Contributions: Conceptualization, F.G.-C. and M.C.; methodology, F.G.-C., M.C. and M.J.M.; formal analysis, F.G.-C. and M.J.M.; investigation, F.G.-C. and M.C.; resources, M.C.; writing—original draft preparation, F.G.-C., M.C.; R.C., C.E., M.J.M., L.G. and F.G.; writing—review and editing, F.G.-C., M.C.; R.C., C.E., M.J.M., L.G. and F.G.; funding acquisition, M.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Propagation Laboratory, Pontificia Universidad Católica de Valparaíso.

Data Availability Statement: Data is contained within the article.

Acknowledgments: The authors would like to express their gratitude to the Propagation Laboratory for the unconditional support given to this research. We gratefully acknowledge the Morphohistology Laboratory, Universidad de Viña del Mar, for their collaboration and willingness to assist with the anatomical study, and the Biomedicine and Biocatalysis Laboratory, PUCV, for their contribution to the biochemical study.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Fonseca, P.; Alves, M.; Dellinghausen, C.; Barboza, C.R. Avocado: Characteristics, health benefits and uses. *Cienc. Rural* **2016**, *46*, 747–754.
2. Lorenzo-Manzanarez, J.L.; Enríquez-Valencia, A.J.; Olivares-García, C.A.; Ibarra-Laclette, E.; Velázquez-López, O.; Ruiz-May, E.; Loyola-Vargas, V.M.; Kú-González, A.F.; Arteaga-Vázquez, M.A.; Mata-Rosas, M. Genome-wide analysis of ARF gene family and miR160 in avocado (*Persea americana* Mill.) and their roles in somatic embryogenesis from zygotic embryos. *Planta* **2025**, *261*, 61. [CrossRef] [PubMed]
3. Hormaza, I.; Alcaraz, L. Fisiología de la floración y cuajado. In *Cultivo, Poscosecha y Procesado del Aguacate*, 1st ed.; Namesny, A., Conesa, C., Hormaza, I., Lobo, G., Eds.; SPE3: Valencia, España, 2020; pp. 47–60.
4. Li, J.; Eltaher, S.; Freeman, B.; Singh, S.; Ali, G.S. Comprehensive genetic diversity and genome-wide association studies revealed the genetic basis of avocado fruit quality traits. *Front. Plant Sci.* **2024**, *15*, 1433436. [CrossRef]
5. Wolstenholme, B.N. Ecología: Clima y Suelo. In *El Aguacate: Botánica, Producción y Usos*, 2nd ed.; Schaffer, B., Wolstenholme, B.N., Whiley, A., Eds.; Ediciones Universitarias de Valparaíso: Valparaíso, Chile, 2015; pp. 113–150.
6. Peña, L.S.; Rebollar, S.; Callejas, N.; Hernández, J.; Gómez, G. Análisis de viabilidad económica para la producción comercial de aguacate Hass. *Rev. Mex. Agroneg.* **2015**, *36*, 1325–1338.
7. Fick, A.; Swart, V.; Backer, R.; Bombarely, A.; Engelbrecht, J.; Van den Berg, N. Partially resistant avocado rootstock Dusa® shows prolonged upregulation of nucleotide binding-leucine rich repeat genes in response to Phytophthora cinnamomi infection. *Front. Plant Sci.* **2022**, *13*, 793644. [CrossRef]
8. Crane, J.H.; Douhan, G.; Faber, B.A.; Arpaia, M.L.; Bender, C.F.; Balerdi, C.F.; Barrientos-Priego, A.F. Cultivares y portainjertos. In *El Aguacate: Botánica, producción y usos*, 2nd ed.; Schaffer, B., Wolstenholme, B.N., Whiley, A., Eds.; Ediciones Universitarias de Valparaíso: Valparaíso, Chile, 2015; pp. 243–281.
9. Celis, N.; Suarez, D.L.; Wu, L.; Li, R.; Arpaia, M.L.; Mauk, P. Salt Tolerance and Growth of 13 Avocado Rootstocks Related Best to Chloride Uptake. *HortSci.* **2018**, *53*, 1737–1745. [CrossRef]
10. Lemus, G.; Ferreyra, R.; Gil, P.; Sepúlveda, P.; Maldonado, P.; Toledo, C.; Barrera, C.; Celedón de, A.J. *El Cultivo del Palto*, 3rd ed.; Instituto de Investigaciones Agropecuarias: Santiago, Chile, 2010; p. 80.
11. Sánchez-González, E.I.; Gutiérrez-Díez, A.; Mayek-Pérez, N. Outcrossing Rate and Genetic Variability in Mexican Race Avocado. *J. Am. Soc. Hortic. Sci.* **2020**, *145*, 53–59. [CrossRef]

12. Li, W.; Ma, X.; Wang, S.; Huang, W.; Jiang, M. The Leafy-Stem-Buried Etiolation Contributed to the High Efficiency of Rootstock Vegetative Propagation in Avocado (*Persea americana*). *Horticulturae* **2024**, *10*, 770. [CrossRef]
13. Ernst, A.A.; Whiley, A.W.; Bender, G.S. Propagación. In *El Aguacate: Botánica, Producción y Usos*, 2nd ed.; Schaffer, B., Wolstenholme, B.N., Whiley, A., Eds.; Ediciones Universitarias de Valparaíso: Valparaíso, Chile, 2015; pp. 283–320.
14. Alberti, M.; Brogio, B.; Silva, S.; Cantuarias-Avilés, T.; Fassio, C. Avances en la propagación del aguacate. *Rev. Bras. Frutic.* **2018**, *40*, e-782. [CrossRef]
15. Ernst, A. Micro cloning: A multiple cloning technique for avocados using micro containers. *Rev. Chapingo Ser. Hortic.* **1999**, *5*, 217–220.
16. Cortés-Rodríguez, M.A.; López-Gómez, R.; Martínez-Pacheco, M.M.; Suárez-Rodríguez, L.M.; Hernández-García, A.; Salgado-Garciglia, R.; Vidales Fernández, I.; Ángel Palomares, M.E.A. In Vitro Propagation of Mexican Race Avocado (*Persea americana* Mill. var. drymifolia). *Acta Hortic.* **2011**, *923*, 47–52. [CrossRef]
17. Hiti-Bandaralage, J.C.; Hayward, A.; Mitter, N. Micropropagation of Avocado (*Persea americana* Mill.). *Am. J. Plant Sci.* **2017**, *8*, 2898–2921. [CrossRef]
18. Tripathi, P.; Karunakaran, G. Standardization of propagation method in avocado (*Persea americana*). *J. Appl. Hortic.* **2019**, *21*, 67–69. [CrossRef]
19. De Morais, I.; Cavalcante, I.; Franco, D.; Martins, A. Cloning of avocado cultivar "Duke 7" (*Persea americana* Mill.) using air-layering technique. *Rev. Bras. Frutic.* **2008**, *30*, 759–763.
20. Mitra, S.K.; Pathak, P.K. Recent development in the propagation of tropical and subtropical fruit crops by cutting. *Acta Hortic.* **2018**, *1205*, 721–726. [CrossRef]
21. Hiti-Bandaralage, J.C.A.; Hayward, A.; O'Brien, C.; Gleeson, M.; Nak, W.; Mitter, N. Advances in avocado propagation for the sustainable supply of planting materials. In *Achieving Sustainable Cultivation of Tropical Fruit*; Burleigh Dodds Science Publishing: Cambridge, UK, 2020; pp. 215–238. Available online: https://shop.bdspublishing.com/store/bds/detail/product/3-190-9781786765369 (accessed on 10 January 2025).
22. Velho da Silveira, S.; Vítor Dutra de Souza, P.; Koller, O. Vegetative propagation of avocado, by stem cuttings. *Rev. Bras. Frutic.* **2004**, *26*, 191–192.
23. Duman, Z.; Hadas-Brandwein, G.; Eliyahu, A.; Belausov, E.; Abu-Abied, M.; Yeselson, Y.; Faigenboim, A.; Lichter, A.; Irihimovitch, V.; Sadot, E. Short De-Etiolation Increases the Rooting of VC801 Avocado Rootstock. *Plants* **2020**, *9*, 1481. [CrossRef]
24. Godoy, S.; Tapia, E.; Seit, P.; Andrade, D.; Sánchez, E.; Andrade, P.; Almeida, A.M.; Prieto, H. Temporary immersion systems for the mass propagation of sweet cherry cultivars and cherry rootstocks: Development of a micropropagation procedure and effect of culture conditions on plant quality. *In Vitro Cell. Dev. Biol. Plant* **2017**, *53*, 494–504. [CrossRef]
25. Joya-Dávila, J.G.; Ruíz-Sesma, B.; Lecona-Guzmán, C.A.; Ruiz-Lau, N.; Ruíz-Valdiviezo, V.M.; Rojas-Martínez, R.I.; Gutiérrez Miceli, F.A. Field phytoprotection of *Coffea arabica* mother plants, disinfection and callogenesis induction. *Agrociencia* **2023**, *57*, 31–40. [CrossRef]
26. Tariq, R.; Khurshid, Z.; Ahmed, W.; Adanir, N. Anti-bacterial efficacy of Aloe vera against *E. Faecalis* in comparison to other intracanal medicaments: A systematic review and meta-analysis. *Saudi Dent. J.* **2023**, *35*, 451–467.
27. Sandhu, M.; Wani, S.H.; Jiménez, V.M. In vitro propagation of bamboo species through axillary shoot proliferation: A review. *Plant Cell Tissue Organ Cult.* **2018**, *132*, 27–53. [CrossRef]
28. Rodríguez, N.; Rodríguez, V.; Alvarez, M. Cultivo in vitro de embriones maduros e inmaduros de aguacatero (*Persea americana* Mill.). *Agric. Tec.* **1997**, *57*, 154–158.
29. Suarez, I.E.; Schnell, R.A.; Kuhn, D.N.; Litz, R. *Microinjerto de plantas de aguacate* (*Persea americana*) infectadas con ASBVd. *Plant Cell Tissue Organ Cult.* **2005**, *80*, 179–185. [CrossRef]
30. Márquez-Martín, B.; Pliego-Alfaro, F.; Sánchez-Romero, C. Improvement of plant recovery from avocado zygotic embryos by desiccation under high relative humidity conditions. *Sci. Hortic.* **2017**, *222*, 169–174. [CrossRef]
31. Alcaraz, M.L.; Montserrat, M.; Hormaza, J.I. In vitro pollen germination in avocado *(Persea americana Mill.)*: Optimization of the method and effect of temperature. *Sci. Hortic.* **2011**, *130*, 152–156. [CrossRef]
32. Blumenfeld, A.; Gazit, S. Growth of Avocado Fruit Callus and Its Relation to Exogenous and Endogenous Cytokinins. *Physiol. Plant.* **1971**, *25*, 369–371. [CrossRef]
33. Oyerinde, R.O.; Mycock, D.J. Callogenesis from *Persea americana* (Mill.) leaf tissues: A histological comparison of materials grown in greenhouse and in vitro. *Pak. J. Bot.* **2024**, *56*, 1497–1506. [CrossRef]
34. Barceló-Muñoz, A.; Pliego-Alfaro, F. Micropropagation of Avocado (*Persea americana* Mill.). In *Micropropagation of Woody Trees and Fruits*; Jain, S.M., Ishii, K., Eds.; Forestry Sciences: Tokyo, Japan, 2003; Volume 75, pp. 519–542.
35. Zulfiqar, F.; Abbasi, N.; Ahmad, T.; Ahmed, I. Effect of explant sources and different concentrations of plant growth regulators on In vitro shoot proliferation and rooting of avocado (*Persea americana Mill.*) cv. "Fuerte". *Pak. J. Bot.* **2009**, *41*, 2333–2346.

36. Hiti-Bandaralage, J.; Hayward, A.; O'Brien, C.; Mitter, N. Gibberellin and cytokinin in synergy for a rapid nodal multiplication system of avocado. In Proceedings of the Actas VIII Congreso Mundial del Aguacate 2015, Lima, Perú, 13–18 September 2015; pp. 95–98.
37. Akter, S.; Huang, J.; Waszczak, C.; Jacques, S.; Gevaert, K.; Van Breusegem, F.; Messens, J. Cysteines under ROS attack in plants: A proteomics view. *J. Exp. Bot.* **2015**, *66*, 2935–2944. [CrossRef]
38. Yin, M.; Vargas, A.; Fuentealba, C.; Shahid, M.; Bassil, E.; Schaffer, B. Differences in physiological and biochemical responses to short-term flooding among the three avocado (*Persea americana* Mill.) races. *Plant Physiol. Biochem.* **2023**, *196*, 925–939. [CrossRef]
39. Sun, Y.; Zhang, J.; Li, X.; Guo, J. Mechanisms of ROS regulation under abiotic stress in plants: From signaling to tolerance. *Physiol. Plant.* **2021**, *173*, 574–587.
40. Xu, L.; Wang, X.; Zhang, Y.; Tang, Z. Lipid peroxidation and ROS production modulated by temperature in in vitro plant cultures. *Plant Cell Tissue Organ Cult.* **2020**, *141*, 543–556.
41. Hiti-Bandaralage, J.; Hayward, A.; Mitter, N. Structural Disparity of Avocado Rootstocks In Vitro for Rooting and Acclimation Success. *Int. J. Plant Biol.* **2022**, *13*, 426–442. [CrossRef]
42. Güler, G.; Gübbük, H.; Arslan, M. The effect of antioxidants on micropropagation of avocado by nodal segments. *Hortic. Stud.* **2021**, *38*, 50–55. [CrossRef]
43. Azofeifa, A. Problemas de oxidación y oscurecimiento de explantes cultivados in vitro. *Agron. Mesoam.* **2009**, *20*, 153–175. [CrossRef]
44. Bon, M.C.; Riccardi, F.; Monteuuis, O. Influence of phase change within a 90-year-old *Sequoia sempervirens* on its in vitro organogenic capacity and protein patterns. *Trees* **1994**, *8*, 283–287. [CrossRef]
45. Singh, P.; Singh, R.K.; Kumar, A. Serial grafting as a rejuvenation strategy in mature woody plants. *Hortic. Plant J.* **2020**, *6*, 249–258.
46. Thorne, H.B.; Axtens, J.; Best, T. Perceptual Factors Influencing the Adoption of Innovative Tissue Culture Technology by the Australian Avocado Industry. *Agriculture* **2022**, *12*, 1288. [CrossRef]
47. Martínez-Alonso, C.; Kidelman, A.; Feito, I.; Velasco, T.; Alía, R.; Gaspar, M.J.; Majada, J. Optimization of seasonality and mother plant nutrition for vegetative propagation of *Pinus pinaster* Ait. *New For.* **2012**, *43*, 651–663. [CrossRef]
48. Wolstenholme, B.N.; Whiley, A.W. Carbohydrate and phenological cycling as management tools for avocado orchards. *SAAGA Yearb.* **1989**, *12*, 33–37.
49. Liu, X.; Robinson, P.W.; Madore, M.A.; Witney, G.W.; Arpaia, M.L. 'Hass' Avocado Carbohydrate Fluctuations. I. Growth and Phenology. *J. Am. Soc. Hortic. Sci.* **1999**, *124*, 671–675. [CrossRef]
50. Vidal Cob Uicab, J.; Ríos Leal, D.; Sabja, A.M.; Cartes Riquelme, P.; Sánchez Olate, M. Organogénesis directa para la propagación in vitro de *Quillaja saponaria* Molina en Sudamérica Austral. *Rev. Mex. Cienc. For.* **2016**, *7*, 57–68.
51. Benson, E. In vitro plant recalcitrance: An introduction. *In Vitro Cell. Dev. Biol.–Plant* **2000**, *36*, 141–148. [CrossRef]
52. Lazare, S.; Cohen, Y.; Goldshtein, E.; Yermiyahu, U.; Ben-Gal, A.; Dag, A. Rootstock-Dependent Response of Hass Avocado to Salt Stress. *Plants* **2021**, *10*, 1672. [CrossRef]
53. Mohamed-yesseem, Y. In Vitro Propagation of Avocado (*Persea americana* Mill.). In *California Avocado Society 1993 Yearbook*; California Avocado Society: Ventura, CA, USA, 1993; Volume 77, pp. 107–111.
54. Datos Históricos Meteorológicos de 2024 en Quillota Chile. Available online: https://es.weatherspark.com/h/y/25817/2024/Datos-hist%C3%B3ricos-meteorol%C3%B3gicos-de-2024-en-Quillota-Chile#google_vignette (accessed on 2 January 2025).
55. Mena, F. Fenología del palto, su uso como base del manejo productivo. In Proceedings of the 2nd Seminario Internacional de Paltos, Quillota, Chile, 29 September–1 October 2004; pp. 1–8.
56. Munsell Color. *Munsell Color Chart for Plant Tissues with Genuine Munsell Color Chips*; Munsell Color Company: Grand Rapids, MI, USA, 2012; p. 9.
57. Alcaraz, M.L.; Thorp, T.G.; Hormaza, J.I. Phenological growth stages of avocado (*Persea americana*) according to the BBCH scale. *Sci. Hortic.* **2013**, *164*, 434–439. [CrossRef]
58. Murashige, T.; Skoog, F.A. Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Plant Physiol.* **1962**, *15*, 473–497. [CrossRef]
59. Giannopolitis, C.N.; Ries, S.K. Superoxide dismutases. *Plant Physiol.* **1977**, *59*, 309–314. [CrossRef] [PubMed]
60. Gohari, G.; Panahirad, S.; Sadeghi, M.; Akbari, A.; Zareei, E.; Zahedi, S.M.; Bahrami, M.K.; Fotopoulos, V. Putrescine-functionalized carbon quantum dot (put-CQD) nanoparticles effectively prime grapevine (*Vitis vinifera* cv. 'Sultana') against salt stress. *BMC Plant Biol.* **2021**, *21*, 120. [CrossRef]
61. Zeb, A.; Ullah, F. A Simple Spectrophotometric Method for the Determination of Thiobarbituric Acid Reactive Substances in Fried Fast Foods. *J. Anal. Methods Chem.* **2016**, *2016*, 9412767. [CrossRef]
62. BCA Protein Assay Kit: Protocol for Total Protein Determination. (Cat. No. SC-202389) [Hoja de Datos]. Available online: https://datasheets.scbt.com/sc-202389.pdf (accessed on 14 November 2023).
63. Gusakov, A.V.; Kondratyeva, E.G.; Sinitsyn, A.P. Comparison of two methods for assaying reducing sugars in the determination of carbohydrase activities. *Int. J. Anal. Chem.* **2011**, *2011*, 283658. [CrossRef]

64. Total Soluble Sugar Quantification from Ethanolic Plant Extracts. Available online: https://www.protocols.io/view/total-soluble-sugar-quantification-from-ethanolic-261genp7og47/v1 (accessed on 14 November 2023).
65. Zirari, A.; Lionakis, S.M. Effect of cultivar, explant type, etiolation pretreatment and the age of plant material on the in vitro regeneration ability of avocado (*Persea americana*). *Acta Hortic.* **1994**, *365*, 69–76. [CrossRef]
66. Krishna, H.; Singh, S. Biotechnological advances in mango (*Mangifera indica* L.) and their future implication in crop improvement—A review. *Biotechnol. Adv.* **2007**, *25*, 223–243. [CrossRef]
67. Krishna, H.; Sairam, R.K.; Singh, S.K.; Patel, V.B.; Sharma, R.R.; Grover, M.; Nain, L.; Sachdev, A. Mango explant browning: Effect of ontogenic age, mycorrhization and pre-treatments. *Sci. Hortic.* **2008**, *118*, 132–138. [CrossRef]
68. Lu, N.; Dai, L.; Luo, Z.; Wang, S.; Wen, Y.; Duan, H.; Hou, R.; Sun, Y.; Li, Y. Characterization of the Transcriptome and Gene Expression of Tetraploid Black Locust Cuttings in Response to Etiolation. *Genes* **2017**, *8*, 345. [CrossRef] [PubMed]
69. Kahn, V. Effect of some phenolic compounds on the oxidation of 4-methyl catechol catalyzed by avocado polyphenoloxidase. *J. Food Sci.* **1976**, *41*, 1011–1012. [CrossRef]
70. Kambona, C.M.; Koua, P.A.; Léon, J.; Ballovora, A. Stress memory and its regulation in plants experiencing recurrent drought conditions. *Theor. Appl. Genet.* **2023**, *136*, 26. [CrossRef]
71. Munir, N.; Aftab, F. Changes in activities of antioxidant enzymes in response to NaCl stress in callus cultures and regenerated plants of sugarcane. *J. Anim. Plant Sci.* **2013**, *23*, 203–209.
72. Khalid, A.; Aftab, F. Effect of exogenous application of 24-epibrassinolide on growth, protein contents, and antioxidant enzyme activities of in vitro-grown *Solanum tuberosum* L. under salt stress. *In Vitro Cell. Dev. Biol. Plant* **2016**, *52*, 81–91. [CrossRef]
73. Tesfay, S.Z.; Bertling, I.; Bower, J.P.; Lovatt, C. The quest for the function of 'Hass' avocado carbohydrates: Clues from fruit and seed development as well as seed germination. *Aust. J. Bot.* **2012**, *60*, 79–86. [CrossRef]
74. Raza, M.A.; Feng, L.Y.; Iqbal, N.; Khan, I.; Meraj, T.A.; Xi, Z.J.; Naeem, M.; Ahmed, S.; Sattar, M.T.; Chen, Y.K.; et al. Effects of contrasting shade treatments on the carbon production and antioxidant activities of soybean plants. *Funct. Plant Biol.* **2020**, *47*, 342. [CrossRef]
75. Robledo-Torres, V.; González-Cortés, A.; Luna-García, L.R.; Mendoza-Villarreal, R.; Pérez-Rodríguez, M.Á.; Camposeco-Montejo, N. Histological Variations in Cucumber Grafted Plants and Their Effect on Yield. *Agronomy* **2024**, *14*, 1377. [CrossRef]
76. Wang, B.; Zhang, Y.; Dong, N.; Chen, Y.; Zhang, Y.; Hao, Y.; Qi, J. Comparative transcriptome analyses provide novel insights into etiolated shoot development of walnut (*Juglans regia* L.). *Plant* **2020**, *252*, 74. [CrossRef] [PubMed]
77. Albornoz, F.; Pérez-Donoso, A.G.; Leigh Urbina, J.; Monasterio, M.; Gómez, M.; Steinfort, Ú. Nitrate Transport Rate in the Xylem of Tomato Plants Grafted onto a Vigorous Rootstock. *Agronomy* **2020**, *10*, 182. [CrossRef]
78. Clima y Vegetación Región Metropolitana de Santiago. Available online: https://www.bcn.cl/siit/nuestropais/region13/clima.htm#:~:text=Chile%20Nuestro%20Pa%C3%ADs&text=La%20temperatura%20mdia%20anual%20es,con%207%2C7%C2%B0C (accessed on 16 October 2024).
79. Fonti, F.; Bryukhanova, M.V.; Myglan, A.S.; Kirdyanov, A.V.; Naumova, O.V.; Vaganov, A. Temperature-induced responses of xylem structure of *Larix sibirica* (Pinaceae) from the Russian Altay. *Am. J. Bot.* **2013**, *100*, 1332–1343. [CrossRef]
80. Ruehr, N.; Grote, R.; Mayr, S.; Arneth, A. Beyond the extreme: Recovery of carbon and water relations in woody plants following heat and drought stress. *Tree Physiol.* **2019**, *39*, 1285–1299. [CrossRef]
81. Wang, A.; Li, S.; Cui, H.; Liu, Y.; Lu, Y.; Hao, G. Divergence in leaf and cambium phenologies among three temperate tree species of different wood types with special reference to xylem hydraulics. *Front. Plant Sci.* **2025**, *16*, 1562873. [CrossRef]
82. Aldwinckle, H.S.; Bhaskara, M.V.; Norelli, J.L. Evaluation of control of fire blight infection of apple blossoms and shoots with sar inducers, biological agents, a growth regulator, copper compounds, and other materials. *Acta Hortic.* **2002**, *590*, 325–331. [CrossRef]
83. Hansen, E.; Olsen, J.; Junttila, O. Gibberellins and Subapical Cell Divisions in Relation to Bud Set and Bud Break in *Salix pentandra*. *J. Plant Growth Regul.* **1999**, *18*, 167–170. [CrossRef]
84. Bouza, L.; Jacques, M.; Miginiac, E. In vitro propagation of *Paeonia suffruticosa* Andr. cv. 'Mme de Vatry': Developmental effects of exogenous hormones during the multiplication phase. *Sci. Hortic.* **1994**, *57*, 241–251.
85. Fráguas, C.B.; Pasqual, M.; Dutra, L.F. Micropropagation of fig (*Ficus carica* L.) 'Roxo de Valinhos' plants. *In Vitro Cell. Dev. Biol. Plant* **2004**, *40*, 471–474. [CrossRef]
86. Žiauka, J.; Kuusienė, S. Plant Hormone Gibberellin Induces Decline of Viability in Isolated Larch Shoot Buds. *Baltic For.* **2009**, *15*, 13–22.
87. Nordström, A.; Tarkowski, P.; Tarkowska, D.; Norbaek, R.; Astot, C.; Dolezal, K.; Sandberg, G. Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: A factor of potential importance for auxin-cytokinin-regulated development. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 8039–8044. [CrossRef]
88. Oliveira, J.P.; Koblitz, M.G.B.; Ferreira, M.S.; Cameron, L.C.; Macedo, A.F. Comparative metabolomic responses to gibberellic acid and 6-benzylaminopurine in *Cunila menthoides* Benth. (*Lamiaceae*): A contribution to understand the metabolic pathways. *Plant Cell Rep.* **2018**, *37*, 1173–1185. [CrossRef]

89. Ahmad, A.; Ahmad, N.; Anis, M.; Alatar, A.; Abdel Salam, E.; Qahtan, A. Gibberellic acid and thidiazuron promote micropropagation of an endangered woody tree (*Pterocarpus marsupium* Roxb.) using in vitro seedlings. *Plant Cell Tissue Organ Cult.* **2021**, *144*, 449–462. [CrossRef]
90. Pasternak, T.P.; Steinmacher, D. Plant Growth Regulation in Cell and Tissue Culture In Vitro. *Plants* **2024**, *13*, 327. [CrossRef]
91. Silva de Oliveira, L.; Brondani, G.; Batagin-Piotto, K.; Calsavara, R.; Gonçalves, A.; De Almeida, M. Micropropagation of *Eucalyptus cloeziana* mature trees. *Aust. For.* **2015**, *78*, 219–231. [CrossRef]