



An efficient protocol for clonal propagation of avocado (*Persea americana* Mill.) rootstocks via etiolation-enhanced micropropagation

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Abstract

Avocado (*Persea americana* Mill.) represents a crop of strategic importance in Colombia, yet its cultivation faces significant challenges arising from high genetic heterogeneity in propagated plant material and unfavorable phytosanitary conditions. These constraints underscore the need for robust strategies to enable the clonal propagation of elite seedlings. In this study, we developed an optimized protocol for the in vitro micropropagation of Creole rootstocks and the cv. Hass. Apical and nodal explants derived from etiolated mother plants were successfully established under aseptic conditions. Etiolation of donor plants for seven weeks significantly enhanced shoot elongation and reduced tissue lignification, thereby improving explant competence and morphogenic responsiveness. Extended darkness preconditioning (15, 30, and 45 days) markedly reduced phenolic oxidation across genotypes, with “Creole 1” exhibiting the lowest phenolization rate (40%) following 45 days of dark treatment. An effective surface disinfection regime was achieved using 1% sodium hypochlorite (NaClO) for 5 min, supplemented with Benlate 50 WP[®] (1 g L⁻¹), and the antibiotics vancomycin (250 mg L⁻¹) and cefotaxime (500 mg L⁻¹). The hormonal combination of 6-benzylaminopurine (BAP, 1.5 mg L⁻¹), 1-naphthaleneacetic acid (NAA, 0.4 mg L⁻¹), and gibberellic acid (GA₃, 0.4 mg L⁻¹) resulted in the highest shoot induction frequency (62%) and elongation (1.35 cm). Continuous supplementation with activated charcoal (1 g L⁻¹) promoted spontaneous adventitious rooting during the multiplication phase (44–56%), thereby reducing dependence on exogenous auxin application. Overall, this integrated protocol effectively mitigates phenolic oxidation, enhances organogenic potential, and facilitates adventitious root formation, providing a reproducible framework for the clonal propagation of elite avocado rootstocks adapted to Colombian agroecological conditions.

Keywords Micrografting · Micropropagation · Etiolation · Phenolic oxidation · Adventitious rooting · Clonal propagation · Plant tissue culture

Abbreviations

WPM Woody Plant Medium
NaOH Sodium hydroxide
HCl Hydrochloric Acid

NaClO Sodium hypochlorite
HgCl₂ Mercuric chloride
AIA Indole-3acetic acid
BAP 6-Benzylaminopurine
GA₃ Gibberellic Acid
NAA 1-Naphthaleneacetic acid
CPPU N-(2-chloro-4-pyridyl)-N-phenylurea

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Introduction

Avocado (*Persea americana* Mill.) has experienced unprecedented global demand growth, driven by its exceptional nutritional profile—including high concentrations of monounsaturated fatty acids, proteins, minerals, and

vitamins—and demonstrated health benefits through cholesterol and triglyceride reduction via antioxidant mechanisms (Araújo et al. 2018; Stephen and Radhakrishnan 2022). This surge in consumption has positioned avocado as a crop of strategic economic importance, with applications spanning culinary, pharmaceutical, and cosmetic industries.

Commercial avocado production relies fundamentally on grafting technology; wherein vegetative scions of elite commercial cultivars are united with rootstock seedlings to optimize agronomic performance (Hernández et al. 2023). In Colombia, commercial plantations predominantly utilize cv. Hass scions grafted onto rootstocks derived from unselected Creole (landrace) seeds (Avocado National Council [ANC] 2013). This conventional propagation system, however, introduces critical constraints that significantly compromise productivity and orchard uniformity. Rootstock seeds are frequently sourced from local markets or production areas spanning contrasting agroecological zones, with no systematic selection criteria or provenance documentation (López-Galé et al. 2022). Consequently, the genetic identity and physiological quality of rootstock material remain unknown, a problem fundamentally rooted in the obligate cross-pollination reproductive biology of *P. americana*, which generates extensive inter-individual genetic heterogeneity within seed-derived populations (Lu et al. 2005; Fernández et al. 2019; Restrepo-Osorio et al. 2020; Cohen et al. 2023).

This genetic variability in rootstocks manifests as inconsistent field performance, including differential responses to edaphic stress, pathogen susceptibility, and scion compatibility—collectively undermining orchard productivity and economic returns. Furthermore, many nursery operations fail to maintain adequate phytosanitary standards and quality control protocols, resulting in the distribution of planting material with suboptimal health status and uncertain genetic characteristics, which further compromises graft establishment and long-term orchard success (Rabuma et al. 2020; Restrepo-Osorio et al. 2020). To address these systemic deficiencies, the Colombian avocado industry requires a transition toward clonal rootstock propagation systems that guarantee genetic uniformity, superior sanitary status, and predictable agronomic performance.

Current commercial clonal propagation technologies—including etiolation-based layering (Brokaw 1975) and double grafting (Ernst 1999) present substantial limitations: genotype-dependent variation in propagation success, intensive labor requirements and controlled-environment infrastructure, extended production cycles (24–36 months), and significant establishment losses (up to 50%) attributable to rootstock-scion incompatibility, graft-site phenolization, and pathogen transmission through infected propagation material (Habibi et al. 2022). In vitro micropropagation

theoretically offers superior alternatives through accelerated multiplication, year-round production, and pathogen elimination. However, commercial implementation remains elusive due to avocado's pronounced recalcitrance to tissue culture manipulations, characterized by: (i) high contamination rates during in vitro establishment (40–60% in most protocols), (ii) severe phenolic compound oxidation leading to tissue browning and necrosis (affecting up to 70% of explants), (iii) limited shoot multiplication coefficients (typically <2.0 shoots per explant per subculture), (iv) progressive decline in shoot elongation capacity across successive subcultures, and (v) extremely low adventitious rooting frequencies, particularly in mature-phase tissues (Castro et al. 1995; Barceló-Muñoz et al. 1999; De La Viña et al. 2001; Hiti-Bandaralage et al. 2022). Despite extensive research efforts globally over nine decades of plant tissue culture investigation (Negi et al. 2024), and recent advances in rendering recalcitrant woody plants more amenable to biotechnological manipulation (Ochatt et al. 2025), avocado remains among the most challenging species for commercial-scale micropropagation due to its pronounced physiological and biochemical barriers.

Phenolic oxidation represents the most critical bottleneck in avocado micropropagation. Upon explant wounding, polyphenol oxidase (PPO) and peroxidase (POD) enzymes catalyze the oxidation of phenolics into reactive quinones that polymerize into toxic brown compounds, causing tissue necrosis (Azofeifa 2009; Liu et al. 2024). Light exposure exacerbates this response by stimulating phenylpropanoid pathway activity. Although various mitigation strategies have been investigated—including antioxidants, activated charcoal incorporation, and darkness pretreatment (Solorzano Vega 1989; Castro et al. 1995; Barceló-Muñoz et al. 1999) no universally effective solution exists across genotypes.

Etiolation pretreatment has emerged as a promising approach for enhancing morphogenic competence of woody plant explants. Etiolated tissues exhibit reduced lignification, elevated endogenous auxins, and enhanced organogenic capacity (Bassuk and Maynard 1987; Sharma and Singh 2002; Massoumi et al. 2017). In avocado, maternal plant etiolation reduced explant oxidation in “West Indian” and “Colin V-33” rootstocks (Solorzano Vega 1989) and facilitated 20% rooting success in Duke-7 variety, although this remained substantially below rates achieved in less recalcitrant species (Wessels 1996). Similarly, activated charcoal incorporation has achieved up to 98% rooting in some studies through auxin stabilization and phenolic compound adsorption (Barceló-Muñoz et al. 1999; Premkumar et al. 2002), though results have not been consistently replicated across genotypes.

Despite extensive research, three critical knowledge gaps remain: (i) synergistic effects of maternal plant etiolation combined with extended in vitro explant darkness pretreatment have not been systematically evaluated for phenolic oxidation control, (ii) genotype-specific hormonal requirements for Colombian Creole germplasm remain undefined, and (iii) integrated protocols spanning establishment through rooting have not been optimized for Duke-7 and locally adapted Creole rootstocks that represent the genetic base of Colombian production systems.

Therefore, this study aimed to: (1) optimize maternal plant etiolation protocols to enhance explant morphogenic competence, (2) evaluate extended darkness pretreatment (15, 30, and 45 days) efficacy for phenolic oxidation mitigation across two Creole genotypes, Duke-7, and cv. Hass, (3) determine genotype-specific growth regulator combinations optimizing shoot induction and multiplication, and spontaneous adventitious rooting responses, and (4) evaluate supplemental auxin application for enhancing rooting in recalcitrant shoots.

We hypothesized that extended etiolation pretreatment (7 weeks) of maternal plants combined with prolonged darkness conditioning (45 days minimum) of in vitro explants would significantly reduce phenolic oxidation while enhancing shoot morphogenesis, that genotype-tailored cytokinin: auxin: gibberellin combinations would overcome recalcitrance barriers limiting multiplication efficiency, and that continuous activated charcoal supplementation would facilitate spontaneous adventitious rooting in Colombian avocado germplasm.

Materials and methods

Plant material and culture conditions

Assays were conducted at the AVOFRUIT avocado tree nursery in Supía (Caldas, Colombia) and the Plant Biotechnology Laboratory at EAFIT University in Medellín (Antioquia, Colombia). Plant material comprised four distinct genotype categories with specific roles in the experimental design: (1) ‘Creole 1’ and ‘Creole 2’ are landrace genotypes used exclusively as ROOTSTOCKS, derived from individual seedling selections within Colombian Creole germplasm populations exhibiting desirable agronomic characteristics (vigor, health, productivity); (2) ‘Duke-7’ is a commercially important ROOTSTOCK variety obtained from certified clonal sources; and (3) ‘Hass’ served dual roles—as SCION material for grafting experiments (sourced from young shoots of productive adult trees) and

as a rootstock genotype for comparative in vitro establishment assays. The physiological age of explant source material differed among genotypes: ‘Creole 1’, ‘Creole 2’, and ‘Duke-7’ explants were derived from 3-month-old grafted plants (cleft graft method) representing juvenile-phase material, maintained under nursery semi-controlled conditions. In contrast, ‘Hass’ explants for comparative in vitro assays were sourced from young shoots of productive adult trees, representing mature-phase material. This distinction is critical for interpreting morphogenic responses, as juvenile-phase tissues typically exhibit enhanced organogenic competence compared to mature-phase counterparts in recalcitrant woody species (Pijut et al. 2011; Wendling et al. 2014). All four genotypes (Creole 1, Creole 2, Duke-7, and Hass) were subjected to etiolation treatments and in vitro establishment protocols as described below, enabling direct comparison of morphogenic responses across diverse genetic backgrounds representative of Colombian avocado production systems. Conventional grafted plants (cleft graft method) at three months post-grafting maintained under avocado tree nursery semi-controlled conditions, served as the source of shoot segments for in vitro establishment. The basal culture medium used for in vitro culture was Woody Plant Medium (WPM) (Lloyd and McCown 1980). Media were supplemented with 25 g L⁻¹ sucrose, except for micrografting assay, which were supplemented with 20 g L⁻¹; 1.0 g L⁻¹ activated charcoal was added to media as an antioxidant agent, and pH was adjusted to 5.7±0.1 with (NaOH) 1 N and (HCl) 1 N before autoclaving. Sterilization was performed by autoclaving at 121 °C at 20 psi for 20 min.

The origin and genetic nature of plant material varied by genotype category. ‘Creole 1’ and ‘Creole 2’ genotypes were initially established from individual seedlings germinated from seeds collected from selected mother trees exhibiting desirable agronomic characteristics (vigor, health, productivity) at the AVOFRUIT nursery. Each ‘Creole’ designation therefore represents a distinct individual genotype selection that has been subsequently maintained clonally through grafting for experimental consistency. ‘Duke-7’ material was obtained from certified clonal sources with documented genetic identity. ‘Hass’ scion material was sourced from productive adult trees of verified cultivar identity. This origin from seed-derived selections implies inherent genetic individuality within each Creole genotype, consistent with the obligate outcrossing reproductive biology of *P. americana*, which generates extensive inter-individual heterozygosity (Lu et al. 2005; Fernández et al. 2019). Consequently, ‘Creole 1’ and ‘Creole 2’ should be understood as individual genotype selections rather than established clonal varieties.

Shoot induction for grafted mother plants on the avocado tree nursery

Shoots from young plants or active growth zones have better in vitro development than those from adult plants or resting buds (Nel and Kotzé 1984). The induction of new shoots is a better way. This procedure included cutting leaves from the apical meristem and tilting shoots horizontally tied with a rope (Fig. 1A, B, C). Each wound is sealed with a healing agent to prevent infection by pathogens or viruses.

In vitro establishment and shoot propagation

Shoots grafted from avocado mother plants were cut on nodal segments approximately 4 cm in length (Fig. 1B, D). After leaf removal, the explants were washed and immersed in an iodized soap solution for one (1) h. Different concentrations of disinfectants, such as sodium hypochlorite (NaClO) and mercuric chloride (HgCl₂), and exposure times, were evaluated. Also, 1 g L⁻¹ of benlate 50 WP[®] fungicide was used. Then, nodal segments were placed in the same exposure time and concentration of a solution of vancomycin hydrochloride[™] [250 mg L⁻¹] and [500 mg L⁻¹] cefotaxime[™] antibiotics.

Following disinfection, nodal segment explants from etiolated mother plants were established in vitro. To evaluate the effects of extended darkness exposure on phenolic oxidation control, in vitro cultures were maintained under complete darkness (photon flux density < 0.1 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 22 $\pm$ 2 °C for 15, 30, or 45 days prior to transfer to standard photoperiod conditions (12/12 h light/dark cycle, 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density). The incidence of phenolization was calculated as (number of phenolized explants / total explants) $\times$ 100. Fifteen explants per treatment were evaluated in two temporal replicates for the

“Creole 1”, Duke-7, and Hass varieties, while “Creole 2” had a single replicate due to limited explant availability.

Afterward, the explants were cultured on basal WPM medium without plant growth regulators to assess only the effect of light exposure on in vitro-established explants. Subsequently, nodal segments were transferred to basal WPM medium supplemented with different concentrations of plant growth regulators to evaluate their influence on shoot induction and development. 6-Benzylaminopurine (BAP) was tested at 0.5–2.5 mg L⁻¹, gibberellic acid (GA₃) at 0.1–0.6 mg L⁻¹, and α -naphthaleneacetic acid (NAA) at 0.1–0.6 mg L⁻¹, including a control treatment without regulators. The combinations were designed to progressively decrease hormone concentrations across treatments (T1–T5) to determine their effect on morphogenic response.

The initial explant to evaluate the multiplication coefficient was a 4 cm apical shoot, the presence of roots, lateral shoots, and all expanded leaves eliminated, leaving only the primordia leaf. Subcultures were made every six (6) weeks. Explants were carried through three (3) successive subculture cycles at 6-week intervals. After each subculture, newly developed shoots, greater than or equal to 4 cm in length were separated and transferred to fresh medium to maintain active physiological development. The number of shoots (multiplication rate) and root formation of the explants variables were monitored.

To determine the optimal growth regulator combination for shoot multiplication, a similar experiment combining BAP, NAA, and GA₃, was carried out (T1–T5), as described above.

Rooting assessment

Root formation was monitored visually throughout all multiplication subcultures. Shoots exhibiting spontaneous adventitious roots were quantified at each subculture interval.



Fig. 1 A. Shoot induction in the etiolation chamber. (B) Etiolated shoots exhibiting characteristic white coloration due to chlorophyll absence and small non-expanded leaves. (C) Elongated shoots resulting from etiolation. (D) Shoots with elongated internodes

Following the third subculture, shoots that remained recalcitrant to rooting were transferred to fresh WPM medium supplemented with indole-3-butyric acid (IBA) at 2.0 mg L⁻¹ for 6 additional weeks to assess auxin responsiveness. Rooting percentages were calculated for both spontaneous and IBA-supplemented treatments.

Statistical analysis

Data normality was assessed using the Shapiro-Wilk test ($\alpha=0.05$). For pairwise comparisons, Student's t-test was applied when data exhibited normal distribution; otherwise, the non-parametric Mann-Whitney U test was employed. Multiple group comparisons were conducted using the Kruskal-Wallis test or one-way ANOVA normality case followed by a Pairwise Wilcoxon Rank Sum or a Tukey HSD, respectively, when at least one different group was detected ($\alpha<0.05$). Additionally, "Bootstrapping" resampling with 10,000 permutations was implemented to validate statistical inferences, particularly for small sample sizes. Percentage data were arcsine-transformed prior to analysis to satisfy homoscedasticity assumptions. All statistical analyses were performed using R software version 3.1.2 (R Core Team 2024).

Results and discussion

Shoot induction in grafted mother plants established in the avocado tree nursery

A total of 138 plants were evaluated in the etiolation chamber. New shoots formation on etiolated plants held under avocado tree nursery conditions began for the third-week trial (Fig. 1A). After the seventh week of darkness treatment, significant genotype-dependent variation in shoot production was observed ($p\leq 0.05$). "Creole 2" genotype exhibited the lowest shoot production (1.30 ± 0.61 shoots/

plant), while Duke-7 (2.28 ± 1.56 shoots/plant) and Hass (2.45 ± 1.59 shoots/plant) demonstrated significantly higher proliferation rates. Duke-7 additionally produced the greatest number of nodes per plant (19.43 ± 11.12), differing significantly from other genotypes (Table 1). These results exceed previously reported etiolation responses for Duke-7, where (Aguilera Palma 2007) and (Pullaz 2011) documented 1.2 and 1.4 shoots per plant, respectively, suggesting that the 7-week etiolation protocol optimized in this study enhances shoot induction efficiency.

Plants were kept in the dark conditions until the seventh-week allowing shoot development with white tissue coloration, due to a lack of chlorophyll and small unexpanded leaves (Fig. 1B). Moreover, elongated shoots with few internodes were separated from each other produced (Fig. 1C, D). Etiolation occurs because plants under dark conditions induce gene transcription responsible for elevated auxin biosynthesis, which increases shoot elongation. Cell expansion changes its orientation towards cell elongation in epidermal and cortical stem cells, just like the inhibition of cell proliferation that contributes to radial growth. There is a spatial redistribution in auxin transport, highly increased lateral stem transport, and a reduction in polar transport through the central cylinder, which causes vascular differentiation not to occur, and auxin concentration roots are reduced (Napier 2014). Recent molecular studies have elucidated the biphasic nature of auxin regulation during etiolation, where initial high auxin concentrations facilitate cellular dedifferentiation before transitioning to elongation-promoting levels that drive hypocotyl extension through differential gene expression patterns (Brumos et al. 2018; Du et al. 2022). This temporal auxin dynamics likely underlies the enhanced shoot development observed in etiolated avocado explants, where reduced lignification and elevated endogenous auxin create a metabolic state conducive to morphogenic competence (Bassuk and Maynard 1987; Massoumi et al. 2017). The genotype-specific variation in shoot production (Table 1) suggests differential capacity for auxin biosynthesis or transport regulation among the evaluated rootstocks (Mano and Nemoto 2012; Brumos et al. 2018), warranting future molecular characterization of auxin-responsive gene expression profiles. Etiolation has been associated with changes in phenolic substances, inhibiting AIA oxidase in etiolated shoots and producing a pronounced shoot elongation (Bassuk and Maynard 1987 a; Sharma and Singh 2002).

In vitro establishment and shoot propagation

In vitro establishment assays demonstrated that increasing disinfectant concentration and exposure time for both sodium hypochlorite (NaClO) and mercuric chloride (HgCl₂) reduced contamination rates while concomitantly

Table 1 Effect of darkness on "Creole 1 and Creole 2" genotypes and Duke-7 and Hass varieties, at seven weeks. Shoots number per plant and nodes number per plant were determined

Variety	Average number of shoots/ plant (N°)	Average number of nodes/plant (No.)
Creole 1	1.82 ± 1.36^{ab}	10.40 ± 5.70^{ab}
Creole 2	1.30 ± 0.61^b	8.90 ± 4.71^b
Duke-7	2.28 ± 1.56^b	19.43 ± 11.12^a
Hass	2.45 ± 1.59^a	9.55 ± 7.21^{ab}

Plants ($n=138$ total) were maintained in complete darkness for seven weeks in the etiolation chamber at the AVOFRUIT nursery. Values represent means $\pm$ standard deviation for the variables. Different letters indicate significant differences ($p\leq 0.05$) among genotypes (Kruskal-Wallis test followed by pairwise Wilcoxon rank sum test)

increasing explant mortality through phenolic oxidation (Fig. 2). The temporal dynamics of these four key establishment parameters across the five disinfection treatments revealed genotype-specific response patterns, with no significant differences ($p \geq 0.05$) among treatments within each genotype, except for the mortality variable in cv. Hass, which exhibited significantly lower values in T1 compared to T2 ($p \leq 0.05$). Notably, explants evaluated with HgCl_2 exhibited the lowest contamination percentage but also the highest phenolized explant percentage and bud mortality, significantly compromising *in vitro* establishment success.

Around avocado varieties, different NaClO concentrations (ranging between 0.5% and 4.0%) and exposure times (between 10 and 20 min) have been reported for apical buds and nodal segments disinfection, preventing contamination without causing phenolization (Solorzano Vega 1989; Castro et al. 1995; Wessels 1996; Barceló-Muñoz et al. 1999; De La Viña et al. 2001; Zulfiqar et al. 2009). However, optimization of disinfection protocols for woody species requires

balancing sterilization efficacy against explant viability—a particularly critical constraint in recalcitrant Lauraceae.

The trade-off between disinfection efficacy and explant viability represents a critical constraint in woody plant micropropagation. Although HgCl_2 treatments achieved lower contamination rates, the concomitant increase in phenolized explants and mortality across all genotypes (Fig. 2) rendered this approach counterproductive. The parallel visualization of these four interdependent variables reveals that treatments minimizing contamination (T5: 22–33% across genotypes) simultaneously compromised survival outcomes, with phenolization rates reaching 15–30% and mortality approaching 20–25%. The oxidative browning induced by mercuric chloride likely results from enhanced production of reactive oxygen species (ROS) during cellular stress responses, exacerbating endogenous phenolic oxidation beyond the capacity of the activated charcoal (1.0 g L^{-1}) incorporated in culture media to adsorb toxic quinone derivatives (Azofeifa 2009). In contrast, sodium hypochlorite at

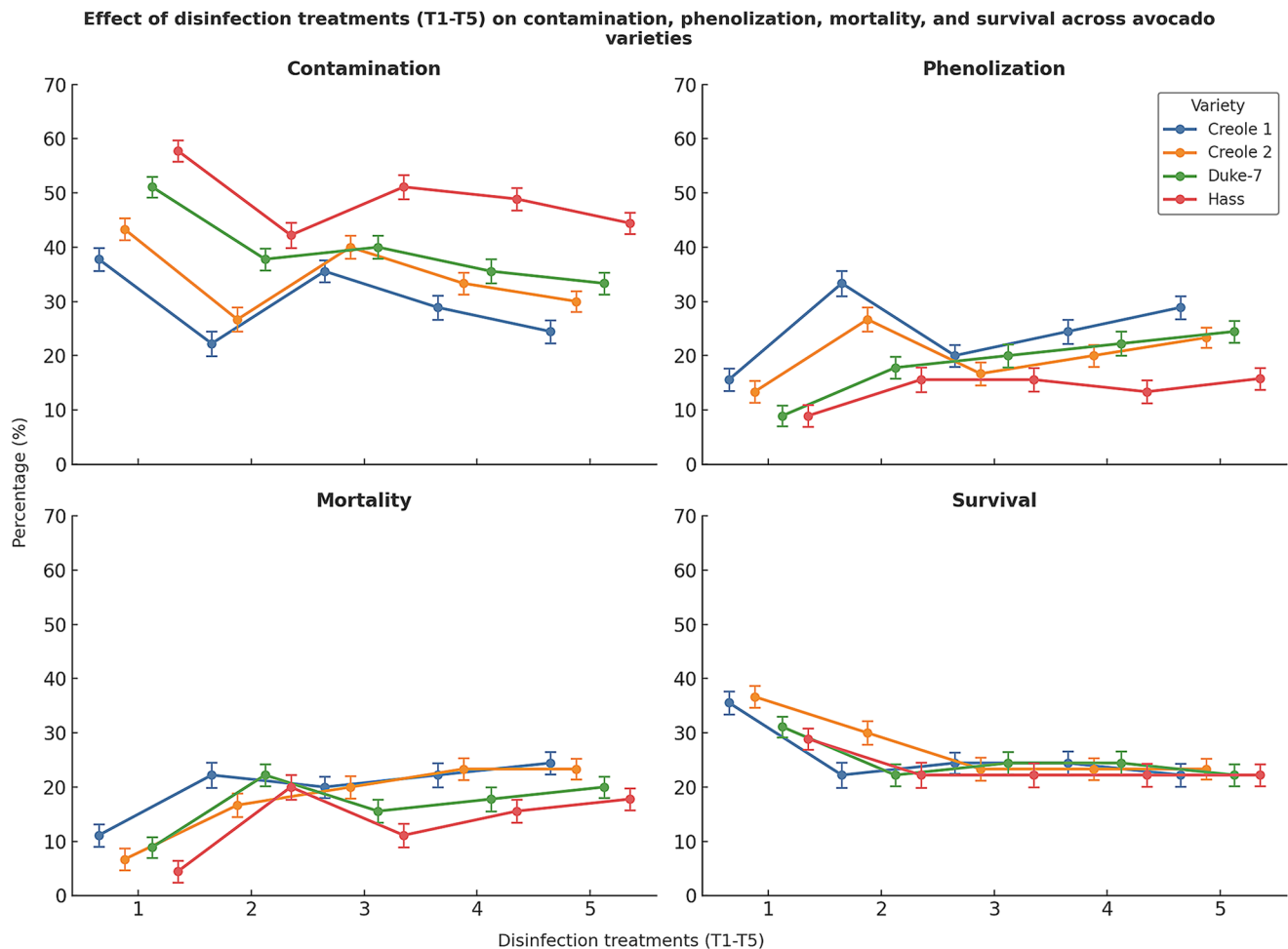


Fig. 2 Effect of surface disinfection treatments on contamination, phenolization, mortality, and survival across avocado genotypes. Treatments: T1 (NaClO 0.5% / 5 min); T2 (NaClO 1.0% / 10 min); T3 (HgCl_2 0.1% / 1 min); T4 (HgCl_2 0.1% / 2 min); T5 (HgCl_2 0.1% /

3 min). All treatments included Benlate 50 WP[®] (1 g L^{-1}) and antibiotics (vancomycin 250 mg L^{-1} + cefotaxime 500 mg L^{-1}). Bars represent means $\pm$ standard deviation ($n=15$ explants per treatment, two temporal replicates)

optimized concentrations (1% for 5 min) combined with systemic antibiotics (vancomycin 250 mg L⁻¹ + cefotaxime 500 mg L⁻¹) achieved acceptable contamination control (22–35% across genotypes) while maintaining explant viability > 60%, representing a more balanced approach for recalcitrant Lauraceae species.

Effect of light and hormonal regulators on shoot induction and development in vitro propagation shoots

In in vitro avocado culture, it is common for tissues to release phenolic substances when tissue is wounded, producing a deep brown color in a culture medium that can become toxic to explants. Light condition plays a critical role in the overproduction of these compounds (Azofeifa 2009; Liu et al. 2024). In vitro avocado buds were maintained for fifteen, thirty or even forty-five days in dark conditions before transferring to photoperiod conditions to assess the effect of extended darkness pretreatment on explant phenolization.

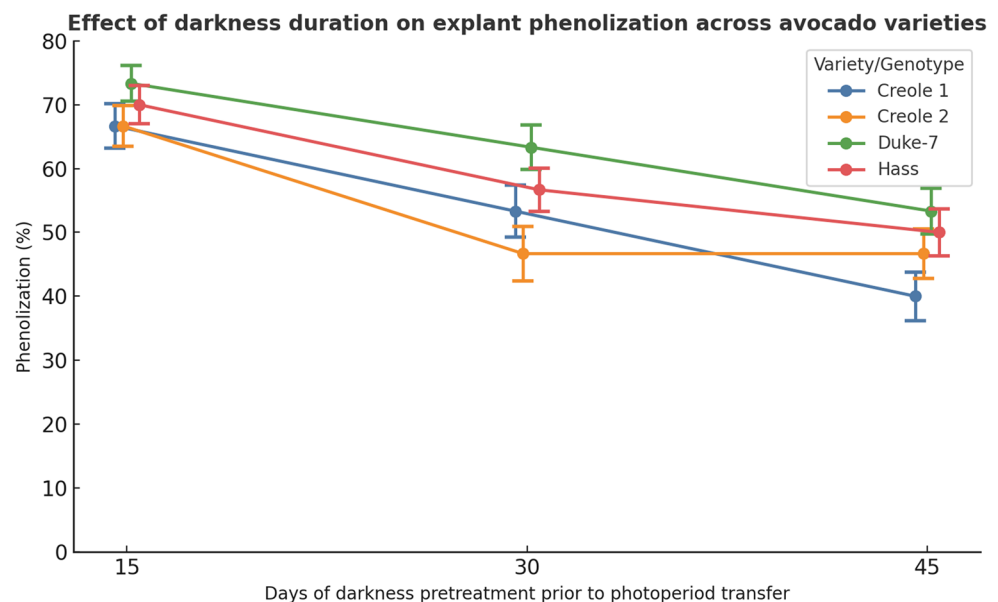
When explants remained for only fifteen days in dark conditions before transferring to photoperiod, all varieties exhibited elevated phenolization rates (66–73%; Fig. 3), with Duke-7 and cv. Hass demonstrating the highest incidence (73% and 70%, respectively). Conversely, explants preconditioned for 45 days of darkness prior to photoperiod transfer displayed a pronounced dose-dependent reduction in phenolization across all evaluated genotypes (Fig. 3). The temporal trajectory of phenolic oxidation declines revealed genotype-specific kinetics, with “Creole 1” exhibiting the steepest reduction gradient (from 67% to 40%; 40.3% relative decrease) and attaining the lowest terminal phenolization rate at 45 days—significantly different from all other genotypes ($p \leq 0.05$). Notably, Duke-7 maintained

the highest residual phenolization (53%) despite extended darkness pretreatment, suggesting this commercially important rootstock harbors constitutive phenolic metabolism that is only partially amenable to darkness-mediated downregulation.

The progressive, non-linear reduction in phenolization with extended darkness pretreatment (Fig. 3) reveals the temporal dynamics of phenolic metabolism during the ex vitro-to-in vitro transition. The response curves demonstrate that the most substantial phenolization decline occurs during the 15-to-30-day interval (mean reduction: 11.7% points across genotypes), with diminishing marginal returns between 30 and 45 days (mean reduction: 6.0% points), suggesting an asymptotic approach to genotype-specific phenolic homeostasis thresholds. Phenylalanine ammonia lyase (PAL), the first committed enzyme in phenylpropanoid biosynthesis, is known to exhibit reduced activity under prolonged darkness in various plant species (Zhang and Liu 2015), which would be expected to limit substrate availability for downstream phenolic production. We hypothesize that the 45-day darkness protocol likely enables metabolic adjustment wherein accumulated phenolic compounds may be enzymatically degraded, conjugated to less reactive forms through glycosylation, or sequestered in vacuoles, thereby potentially reducing their cytotoxic potential upon subsequent light exposure.

The superior response of “Creole 1” (40% phenolization at 45 days, $p \leq 0.05$) compared to other genotypes suggests cultivar-specific variation in phenolic homeostasis mechanisms, possibly involving differential PAL regulation, enhanced polyphenol oxidase (PPO) inhibitor production, or more efficient vacuolar compartmentalization systems (Nurzyńska-Wierdak 2023). Furthermore, residual phenolization persisting even after 45 days of darkness (40–53%

Fig. 3 Effect of extended darkness duration on phenolic oxidation in in vitro-established avocado explants. Phenolization incidence was calculated as (phenolized explants / total explants) $\times$ 100. Bars represent means $\pm$ standard deviation ($n=15$ explants per treatment, two temporal replicates, except ‘Creole 2’ with one replicate)



across all genotypes) suggests that darkness pretreatment alone may not be sufficient to fully overcome avocado's recalcitrance to in vitro conditions, presumably reflecting constitutive phenolic production associated with lignification and secondary cell wall formation characteristic of woody perennials.

These findings align with previous reports in “West Indian” and “Colin V-33” rootstocks (Solorzano Vega 1989), where maternal plant etiolation reduced explant oxidation in in vitro seedlings. Similarly, “Velvick” and “Lula” rootstocks exhibited 45% phenolization when darkness pretreatment was limited to 8 days (Castro et al. 1995), corroborating our observation that brief darkness exposure is insufficient for effective phenolic oxidation control. However, our study extends previous understanding by systematically demonstrating dose-dependent phenolization reduction with darkness duration—a relationship not previously quantified for Colombian Creole germplasm. The persistence of phenolization in Duke-7 and Hass (50–53% at 45 days) despite extended darkness pretreatment suggests these commercially important genotypes require additional interventions beyond the darkness-mediated PAL downregulation achieved in this study. Alternative strategies for phenolization control merit systematic investigation, including: (i) direct PAL inhibitors (e.g., 2-aminoindane-2-phosphonic acid, AIP) to pharmacologically suppress phenylpropanoid flux at its committed step; (ii) enhanced antioxidant supplementation (e.g., ascorbic acid, polyvinylpyrrolidone) to scavenge reactive quinones before polymerization; and (iii) refined auxin pulse treatments that optimize the balance between root-promoting concentrations and phenolic-exacerbating oxidative stress.

Recent advances in recalcitrant woody species provide compelling evidence for integrated approaches; Martins and collaborators (Martins et al. 2024) demonstrated that in *Quercus robur*, activated charcoal combined with optimized auxin regimes achieved superior morphophysiological outcomes compared to either treatment alone, with enhanced hydraulic conductivity and photosynthetic apparatus performance in regenerated plantlets. Critically, their findings suggest that activated charcoal efficacy extends beyond simple phenolic adsorption to encompass auxin stabilization and ethylene removal—mechanisms likely operative in our avocado protocol. Furthermore, the auxin–ethylene balance emerges as a pivotal regulatory axis for adventitious root formation in avocado. Duman and collaborators (Duman et al. 2020) demonstrated that fine-tuning this hormonal interplay through short de-etiolation periods significantly enhanced rooting competence in the recalcitrant VC801 rootstock, with transcriptomic analyses revealing enrichment of ethylene-responsive transcripts in etiolated branches and pectin-modifying enzymes in de-etiolated tissues. These findings

suggest that our extended etiolation protocol, while effective for phenolic reduction, may benefit from a subsequent brief light exposure period to optimize cell-wall properties and hormonal homeostasis prior to root induction—a refinement that could potentially improve the 44–56% rooting rates achieved herein. To achieve phenolization rates compatible with commercial-scale micropropagation (typically <20%), future protocols should systematically evaluate these complementary interventions, either individually or in factorial combinations, across the genotypic diversity represented in Colombian avocado production systems.

External factors, like light conditions, induce organogenic responses in the avocado in vitro culture (Schroeder 1976; Solorzano Vega 1989). An etiolated tissue is less lignified and has a higher content of auxins and AIA oxidase inhibitors. Moreover, it develops undifferentiated tissue that could increase root primordia initiation, which helps to make rhizogenesis easier (Bassuk and Maynard 1987; Massoumi et al. 2017). However, this assesses a low root induction percentage of all varieties at this stage (Fig. 4A, D) (Table 2). It was reported for the Duke-7 variety that etiolation was necessary for plant rooting in 20% of assays (Wessels 1996). However, as reported by (González Rosas and Salazar García 1984), better rooting (70%) was obtained with partial light (6.76 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for the “*Persea americana* var. *americana*” West Indian race. These results indicate that root induction response also depends on the genotype.

Statistical analysis showed shoot induction (Fig. 5A, B, C, D) was influenced by growth regulators combination ($p \leq 0.05$) for varieties and genotypes evaluated, except for “Creole 2”. “Creole 1”, Duke-7, and Hass variety, the highest shoot induction percentage, was for treatment three, with a hormonal combination [1.5 mg L⁻¹] BAP, [0.4 mg L⁻¹] GA₃, and [0.4 mg L⁻¹] NAA. “Creole 2”, there were no differences between hormonal treatments. Therefore, for this stage, any evaluated treatments can be chosen for shoot induction (Table 3).

Nevertheless, multiple shoot formations were observed in all varieties evaluated at the same bud (Fig. 6A, B), which coincides with (González Rosas and Salazar García 1984) in the “*Persea americana* var. *americana*” Antillean race. However, very few explants showed this characteristic.

The differential hormonal responsiveness observed among genotypes (Table 3) underscores the genotype-dependency of avocado micropropagation protocols. Treatment 3 (BAP 1.5 mg L⁻¹ + NAA 0.4 mg L⁻¹ + GA₃ 0.4 mg L⁻¹) optimized both shoot induction percentage (62% in “Creole 1” and Hass) and elongation (1.17–1.35 cm) in three of four genotypes, suggesting this balanced cytokinin: auxin: gibberellin ratio (3.75:1:1) effectively promotes meristematic activity while maintaining sufficient auxin for cell expansion. The notable exception “Creole 2” showing

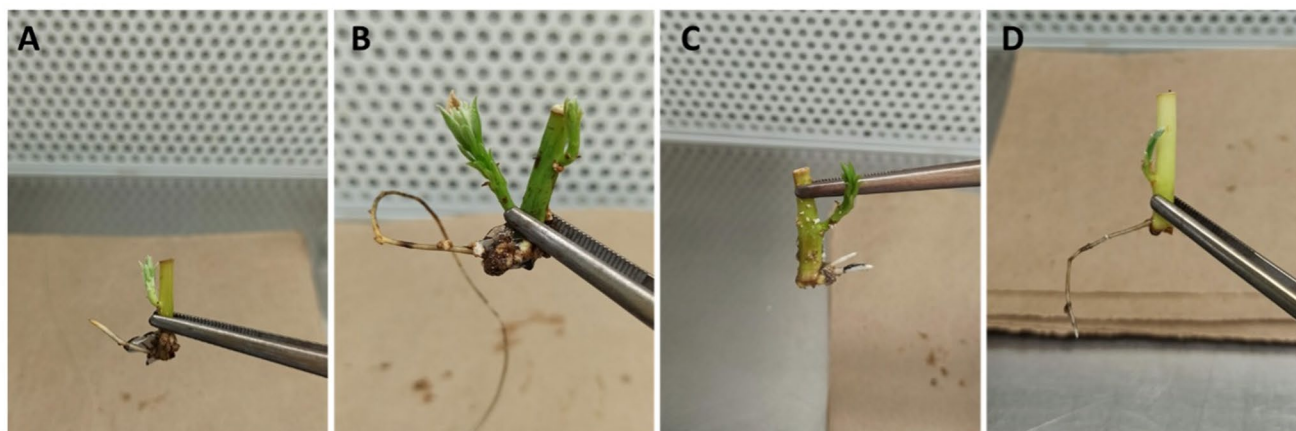


Fig. 4 Root induction in in vitro-cultured avocado genotypes and varieties evaluated. (A) “Creole 1”. (B) “Creole 2”. (C) Duke-7. (D) Hass. Explants were cultured on WPM medium supplemented with T3

growth regulator combination (BAP 1.5 mg L⁻¹ + NAA 0.4 mg L⁻¹ + GA₃ 0.4 mg L⁻¹) and 1.0 g L⁻¹ activated charcoal

Table 2 Percentage of shoots in vitro established with roots and callus formation from “Creole 1 and 2” genotypes, and Duke-7 and Hass varieties

Variety	% roots	% callus
Creole 1	11.11 ± 0.44 ^a	75.56 ± 0.32 ^a
Creole 2	13.33 ± 0.45 ^a	73.33 ± 0.34 ^a
Duke-7	15.56 ± 0.46 ^a	71.11 ± 0.47 ^a
Hass	11.11 ± 0.47 ^a	68.89 ± 0.32 ^a

Explants were cultured on WPM basal medium supplemented with 1.0 g L⁻¹ activated charcoal under 12/12 h photoperiod following 45 days of darkness pretreatment. Values represent means ± standard deviation ($n=45$ explants per genotype). Different letters within columns indicate significant differences among genotypes (Kruskal-Wallis test followed by pairwise Wilcoxon rank sum test, $\alpha=0.05$)

no significant differences among treatments indicates this genotype may possess altered endogenous hormone homeostasis or receptor sensitivity, requiring alternative optimization strategies.

The superior elongation achieved with intermediate BAP concentrations (1.5 mg L⁻¹) compared to higher doses (2.0 mg L⁻¹) aligns with classical cytokinin dose-response curves, where excessive cytokinin concentrations inhibit elongation while promoting lateral bud proliferation—a phenomenon attributed to altered auxin: cytokinin ratios favoring shoot initiation over cell expansion. Gibberellin supplementation (0.3–0.5 mg L⁻¹ GA₃) likely mitigated this cytokinin-mediated elongation inhibition by promoting cell elongation through independent signaling pathways involving DELLA protein degradation and cell wall loosening (Ito et al. 2018).

These findings corroborate previous reports where BA (alone or combined with GA₃) optimized avocado shoot induction (Dalsaso Eric Guevara 1988; Rodríguez et al. 1999) but extend the understanding by systematically evaluating three-hormone combinations across multiple Colombian genotypes. The genotype-specific optimal

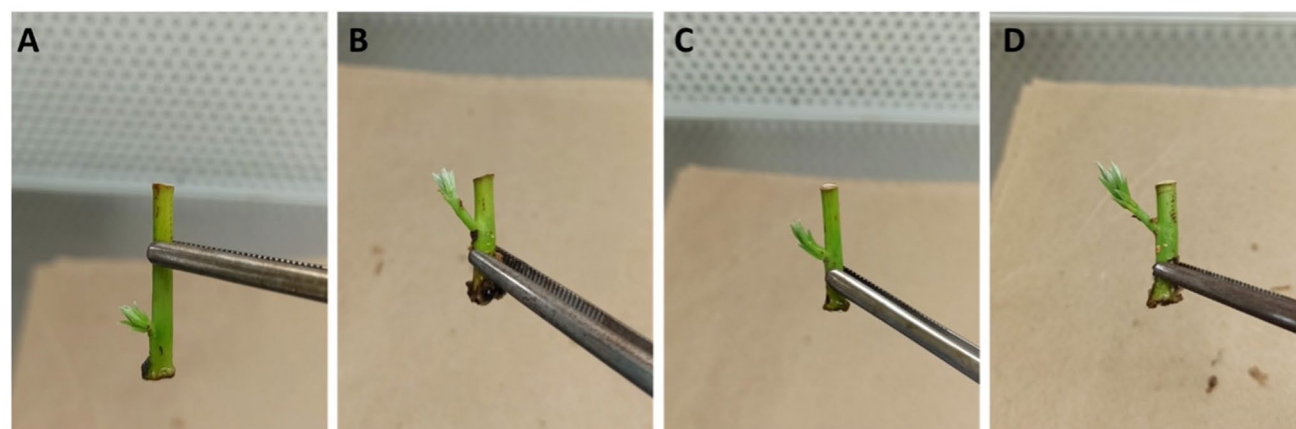


Fig. 5 Shoot induction response in in vitro-established avocado explants under optimal growth regulator treatment. (A) “Creole 1”. (B) “Creole 2”. (C) Duke-7. (D) Hass. Nodal segment explants cultured

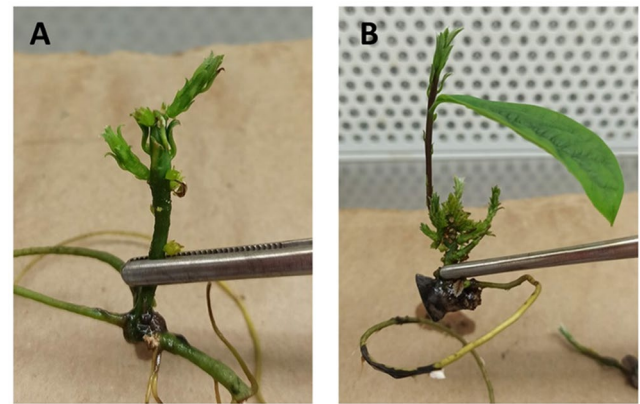
on WPM medium supplemented with T3 treatment (BAP 1.5 mg L⁻¹ + NAA 0.4 mg L⁻¹ + GA₃ 0.4 mg L⁻¹) and 1.0 g L⁻¹ activated charcoal

Table 3 Effect of growth regulators combinations on shoot induction and elongation of in vitro-established avocado explants from “Creole 1 and 2” genotypes, and Duke-7 and Hass varieties

Variety	Plant regulator	% average shoot induction	Average shoot height/explant
Creole 1	T ₁	46.67±0.51 ^{ab}	0.73±0.28 ^b
	T ₂	42.22±0.52 ^{ab}	0.72±0.35 ^b
	T ₃	62.22±0.49 ^a	1.17±0.28 ^a
	T ₄	51.11±0.51 ^{ab}	0.83±0.31 ^b
	T ₅	53.33±0.61 ^{ab}	1.15±0.39 ^a
	T ₆	35.56±0.48 ^b	0.58±0.26 ^c
Creole 2	T ₁	53.33±0.52 ^a	0.75±0.26 ^b
	T ₂	53.33±0.48 ^a	1.24±0.35 ^a
	T ₃	53.33±0.62 ^a	1.05±0.42 ^a
	T ₄	53.33±0.51 ^a	0.77±0.34 ^b
	T ₅	46.67±0.52 ^a	0.61±0.30 ^{bc}
	T ₆	33.33±0.49 ^a	0.47±0.61 ^c
Duke-7	T ₁	57.78±0.48 ^a	0.80±0.20 ^c
	T ₂	48.89±0.51 ^{ab}	0.72±0.24 ^{cd}
	T ₃	60.00±0.50 ^a	1.35±0.42 ^a
	T ₄	57.78±0.62 ^{ab}	1.15±0.35 ^b
	T ₅	57.78±0.50 ^{ab}	0.68±0.24 ^{cd}
	T ₆	37.78±0.49 ^b	0.61±0.65 ^d
Hass	T ₁	48.89±0.51 ^{ab}	0.74±0.24 ^b
	T ₂	53.33±0.68 ^{ab}	0.75±0.23 ^b
	T ₃	62.22±0.49 ^a	1.21±0.35 ^a
	T ₄	57.78±0.75 ^{ab}	1.28±0.39 ^a
	T ₅	51.11±0.50 ^{ab}	0.60±0.24 ^c
	T ₆	40.00±0.69 ^b	0.49±0.15 ^c

Treatments: T₁ (BAP 2.5 mg L⁻¹ + NAA 0.6 mg L⁻¹ + GA₃ 0.6 mg L⁻¹); T₂ (BAP 2.0 mg L⁻¹ + NAA 0.5 mg L⁻¹ + GA₃ 0.5 mg L⁻¹); T₃ (BAP 1.5 mg L⁻¹ + NAA 0.4 mg L⁻¹ + GA₃ 0.4 mg L⁻¹); T₄ (BAP 1.0 mg L⁻¹ + NAA 0.3 mg L⁻¹ + GA₃ 0.3 mg L⁻¹); T₅ (BAP 0.5 mg L⁻¹ + NAA 0.1 mg L⁻¹ + GA₃ 0.1 mg L⁻¹); T₆ (Control, no growth regulators). Values represent means±standard deviation (n=15 explants per treatment, two temporal replicates, except ‘Creole 2’ with one replicate due to limited explant availability). Different letters within columns for each genotype indicate significant differences among treatments (Kruskal-Wallis test followed by pairwise Wilcoxon rank sum test, $\alpha=0.05$)

combinations identified here (Table 3) provide actionable protocols for targeted rootstock propagation programs, though the modest elongation achieved (maximum 1.35 cm in Duke-7 after 90 days) highlights persistent recalcitrance requiring further optimization. The present protocol was validated across four genotypes representing Colombian Creole germplasm and commercially important rootstocks. However, the genotype-specific responses observed—particularly the differential phenolization rates (40–53% at 45 days of darkness) and hormonal sensitivities (with “Creole 2” showing no significant differences among treatments)—underscore the need for validation across additional germplasm to establish broader applicability. This limitation reflects the well-documented genotype-dependency characteristic of woody plant micropropagation, wherein optimal

**Fig. 6** Shoot multiplication and elongation during subculture of in vitro-propagated avocado. (A) Multiple shoot formation from a single nodal explant during Multiplication phase. (B) Individual elongated shoot ready for separation and transfer to fresh medium

conditions for one genotype may prove suboptimal or even deleterious for another (Abdalla et al. 2022). Future studies should therefore expand genotype testing to include additional Creole lines from diverse Colombian agroecological zones, as well as other widely used commercial rootstocks to determine the extent to which the present protocol can be generalized across the genetic diversity of *P. americana*.

Effect of light and hormonal regulators on in vitro shoot multiplication and development

New shoot response was induced during subcultures, as noted by (Castro et al. 1995), ranging between zero and five shoots (Fig. 1). Likewise, few induced shoots reached 4 cm of elongation but (Fig. 1) taking 60 d (in average) affecting multiplication time, which usually takes 45 d. Although only three subcultures were assessed, the following subcultures were monitored. Height growth of new induced shoots was lower with increasing subcultures. This recalcitrant response reported for the ‘Hass’ variety (Rohim et al. 2013), in which growth shoots decrease (from 3.8 cm to 1.47 cm), was only found until the sixth subculture. This response is found in other Lauraceae species, such as *Persea indica*. This species was only found from the seventh subculture onwards (Nel and Kotzé 1984). The progressive decline in shoot elongation during successive subcultures is characteristic of in vitro recalcitrance in avocado micropropagation, with only 18–24% of shoots reaching the 4 cm threshold needed for autonomous growth. We explicitly acknowledge that this fundamental constraint represents the principal bottleneck limiting commercial-scale avocado micropropagation, and the present protocol—while achieving meaningful improvements in establishment efficiency and spontaneous rooting—does not fully overcome this limitation. The multiplication rates achieved in the present study (1.03–1.37

shoots/explant) align with the inherent recalcitrance of *Persea americana* and remain consistent with values reported for other recalcitrant woody species, where coefficients rarely exceed 2.0 shoots per explant per subculture (Abdalla et al. 2022). This progressive decline likely involves multiple physiological constraints operating simultaneously. Notably, while “Creole 1” exhibited enhanced multiplication in the third subculture with optimal hormonal treatments (T2–T3; Fig. 7, left panel), both Duke-7 and cv. Hass maintained relatively stable proliferation rates across subcultures (Fig. 7, center and right panels), suggesting that cytokinin signaling remains effective for shoot initiation even as elongation pathways progressively deteriorate. The contrasting trajectories between genotypes—with “Creole 1” showing enhancement (T3: 0.93→1.37 shoots/explant) and cv. Hass displaying gradual attenuation (T5: 0.70→0.75 shoots/explant)—underscore the critical importance of genotype × hormone × subculture interactions in determining long-term multiplication sustainability. Extended culture may also induce genetic and epigenetic changes, impairing regeneration, as chromatin accessibility for auxin biosynthesis genes becomes limited (Duta-Cornescu et al. 2023). Additionally, gradual depletion of micronutrients, especially iron and zinc, or accumulation of inhibitory metabolites such as ethylene and phenolic oxidation products may restrict growth (Ahmed et al. 2024; Liu et al. 2024). These mechanistic constraints—epigenetic modifications, nutrient exhaustion, and hormonal imbalances—represent fundamental barriers that have hindered commercial-scale avocado micropropagation globally, and their persistence in the present protocol, despite the integrated etiolation and activated charcoal approach, highlights the need for novel intervention strategies targeting these specific bottlenecks. Genotype-specific multiplication rates highlight the need for cultivar-specific protocols; Duke-7 and “Creole 1” maintained higher

multiplication (1.03–1.37 shoots/explant) than “Creole 2” and Hass, possibly due to differences in hormone balance, antioxidant capacity, or structural traits affecting nutrient and hormone uptake (López-Galé et al. 2022).

Significant differences ($p \leq 0.05$) were detected among growth regulator treatments for multiplication efficiency. The temporal evolution of shoot proliferation across three consecutive subcultures (Fig. 7) revealed genotype-specific hormonal response patterns and subculture-dependent multiplication kinetics. “Creole 1” attained maximum multiplication rates with T3 (BAP 1.5 mg L⁻¹ + NAA 0.4 mg L⁻¹ + GA₃ 0.4 mg L⁻¹), reaching 1.37 shoots per explant by the third subculture—a 43% increase relative to the initial subculture. Duke-7 exhibited optimal proliferation with T2 (BAP 1.0 mg L⁻¹ + NAA 0.3 mg L⁻¹ + GA₃ 0.3 mg L⁻¹), achieving stable multiplication rates (1.03–1.07 shoots/explant) across all subcultures. Cv. Hass demonstrated comparable performance across T2, T3, and T4 treatments (1.00–1.13 shoots/explant), suggesting broader hormonal plasticity in this commercial cultivar. These results coincide with those reported by (Rodríguez et al. 1999), who obtained the best results on the number of induced shoots, where treatment contained BAP and GA₃. However, for “Velvick” and “Lula” rootstocks (Castro et al. 1995), a similar hormonal combination was not adequate for new shoot induction, demonstrating this response as a dependent genotype. Appropriate selection of growth regulator and concentration is critical; an optimal concentration increases the protein synthesis that promotes the development of pre-existing meristematic points.

Different responses in multiplication rate have been obtained depending on the avocado variety. For ‘Velvick’ and ‘Lula’ rootstocks, authors reported 1.5 shoots per explant using 0.1 mg L⁻¹ of CPPU [N-(2-chloro-4-pyridyl)-N-phenylurea] concentration (Castro et al. 1995). On the

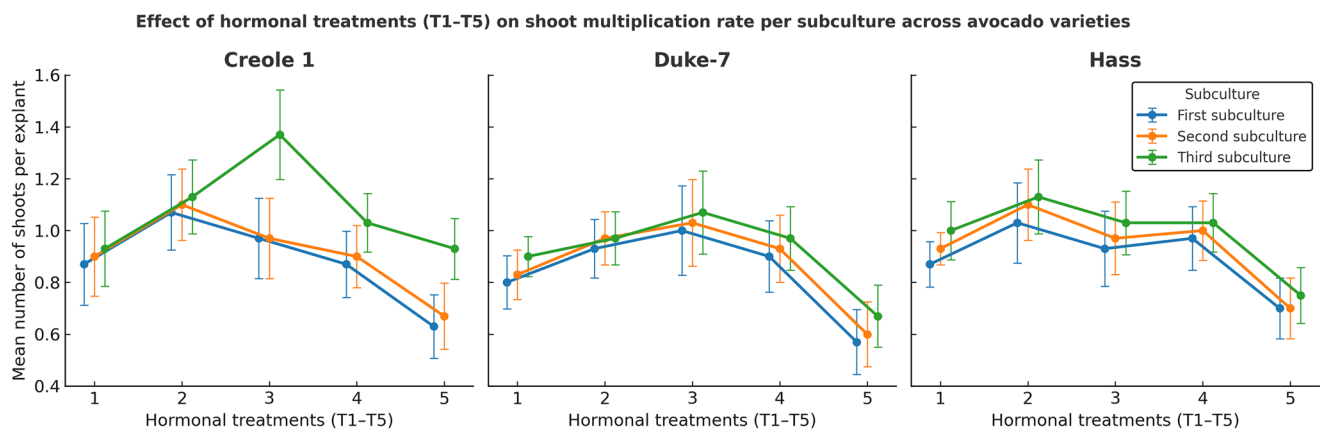


Fig. 7 Effect of hormonal treatments (T1–T5) on shoot multiplication rate across three consecutive subcultures in avocado genotypes. Treatments: T1 (BAP 2.5 mg L⁻¹ + NAA 0.6 mg L⁻¹ + GA₃ 0.6 mg L⁻¹); T2 (BAP 2.0 mg L⁻¹ + NAA 0.5 mg L⁻¹ + GA₃ 0.5 mg L⁻¹); T3 (BAP 1.5

mg L⁻¹ + NAA 0.4 mg L⁻¹ + GA₃ 0.4 mg L⁻¹); T4 (BAP 1.0 mg L⁻¹ + NAA 0.3 mg L⁻¹ + GA₃ 0.3 mg L⁻¹); T5 (BAP 0.5 mg L⁻¹ + NAA 0.1 mg L⁻¹ + GA₃ 0.1 mg L⁻¹); T6 (Control, no growth regulators). Bars represent means ± standard deviation

Table 4 Percentage of in vitro rooted shoots and callus formation from “Creole 1 and 2” genotypes, Duke-7 and Hass varieties, under different growth regulators and light conditions

Variety	% roots
Creole 1	55.56
Duke-7	44.44
Hass	48.89

other hand, for “West Indian” and Colin V-33” rootstocks, three shoots per explant were reached using a combination of GA3 and BA (Solorzano Vega 1989). Also, one (1) shoot per explant was obtained for “*Persea americana* Mill” using 2% peptone (Nhut et al. 2008).

The rooting stage is reported as the most critical and rate-limiting step of avocado tissue in vitro micropropagation (Hiti-Bandaralage et al. 2022). Our results showed shoots rooting on multiplying phases through subcultures. First subculturing, rooting reached 55.56% in the “Creole 1”, 44.44% in Duke-7, and 48.89% in the Hass variety (Table 4). Here we used activated charcoal for the in vitro culture phases, which could influence root formation, shoot development, and growth.

The spontaneous rooting rates achieved during multiplication (44–56% across genotypes; Table 4) represent a substantial advancement over conventional avocado micropropagation protocols, which typically report <30% rooting success (Hiti-Bandaralage et al. 2022). This improvement is attributed primarily to continuous activated charcoal supplementation (1.0 g L⁻¹) throughout all culture phases—a protocol refinement yielding multiple synergistic benefits: Phenolic adsorption, activated charcoal continuously binds oxidized phenolic compounds and quinone derivatives, preventing accumulation to phytotoxic concentrations that inhibit root primordium differentiation. Similar beneficial effects of activated charcoal on adventitious rooting have been recently documented in recalcitrant woody species such as *Quercus robur*, where activated charcoal promoted superior root development compared to exogenous auxin treatments through enhanced morphophysiological status and improved hydraulic conductivity (Martins et al. 2024). This is particularly critical in avocado, where constitutive phenolic production remains elevated even under optimized culture conditions. Auxin stabilization, charcoal may protect endogenous indole-3-acetic acid (IAA) from oxidative degradation by phenolic-derived reactive oxygen species, maintaining auxin concentrations favorable for adventitious root initiation without requiring exogenous auxin supplementation during multiplication. Ethylene removal, by adsorbing ethylene produced during tissue stress responses, activated charcoal eliminates this gaseous hormone’s well-documented inhibitory effects on rooting in recalcitrant woody species.

Shoots failing to root spontaneously were transferred to IBA-supplemented medium (2.0 mg L⁻¹) for 6 weeks yet

achieved only modest additional rooting (13.33–15.56%). While the 44–56% spontaneous rooting rates achieved represent a substantial advancement over conventional protocols reporting <30% success (Hiti-Bandaralage et al. 2022), the residual phenolization (40–53%) and limited IBA responsiveness (13–15% additional rooting) highlight persistent barriers requiring further investigation. The limited IBA responsiveness observed in the present study contrasts with protocols reported for other woody species, where auxin pulse treatments followed by transfer to activated charcoal-supplemented medium have achieved substantially higher rooting rates (Barceló-Muñoz et al. 1999; Martins et al. 2024). Several factors may explain this discrepancy: (i) the continuous activated charcoal supplementation throughout our protocol, while beneficial for phenolic adsorption, may have concurrently adsorbed a significant proportion of both exogenous IBA and endogenous auxins, potentially reducing available auxin concentrations below optimal thresholds for root induction; (ii) genotype-specific anatomical barriers, including cortical sclerification patterns and cambial architecture unique to Colombian Creole germplasm, may physically impede root primordium initiation regardless of auxin availability (Hiti-Bandaralage et al. 2022); and (iii) the Colombian Creole genotypes evaluated herein may possess distinct auxin sensitivity profiles or transport dynamics compared to internationally characterized rootstocks such as Duke-7.

Recent literature supports that avocado’s recalcitrance involves complex auxin–ethylene interactions and cell-wall properties; (Duman et al. 2020) demonstrated that fine-tuning the auxin–ethylene balance through short de-etiolation periods significantly enhanced rooting competence in the recalcitrant VC801 rootstock, suggesting that our extended etiolation protocol may benefit from subsequent brief light exposure to optimize hormonal homeostasis prior to root induction. This limited IBA responsiveness suggests that beyond hormonal supplementation, intrinsic anatomical or physiological barriers prevent root formation in a subset of microshoots. Histological studies in avocado have demonstrated that rooting capacity correlates with specific vascular tissue architecture—particularly prominent fascicular cambium and reduced cortical sclerification (Hiti-Bandaralage et al. 2022). Shoots lacking these favorable anatomical features likely represent the recalcitrant cohort observed in our assays. Future investigations should evaluate modified auxin application strategies to optimize rooting responses in these genotypes, including: (i) higher IBA concentrations or alternative auxins such as indole-3-acetic acid (IAA) or phenylacetic acid (PAA); (ii) shorter auxin pulse durations (24–72 h) followed by transfer to auxin-free medium with activated charcoal, rather than continuous IBA exposure; (iii) sequential auxin–charcoal transfer protocols

that temporally separate the auxin induction phase from the phenolic adsorption phase; and (iv) comparative assessment of rooting competence across a broader range of Colombian Creole genotypes to identify superior responders for commercial propagation programs.

Commercial implications and protocol benchmarking

From a commercial perspective, the integrated protocol developed herein offers potential advantages over conventional etiolation-based layering methods, which typically require 24–36 months from initial propagation to field-ready plants and experience 40–50% establishment losses attributable to rootstock–scion incompatibility, graft-site phenolization, and pathogen transmission (Habibi et al. 2022). The *in vitro* approach substantially reduces production cycle time—with rooted plantlets achievable within 4–5 months from explant establishment—and enables year-round multiplication independent of seasonal constraints that limit conventional nursery operations. Furthermore, the aseptic conditions inherent to micropropagation eliminate the risk of systemic pathogen transmission that compromises a significant proportion of conventionally propagated material.

However, realistic assessment of commercial viability requires acknowledgment of the infrastructure and expertise barriers associated with *in vitro* systems. Tissue culture laboratories demand significant capital investment in laminar flow cabinets, autoclaves, controlled-environment growth chambers, and trained personnel, costs that may be prohibitive for small-scale nursery operations. Additionally, the acclimatization phase—transferring *in vitro*-rooted plantlets to *ex vitro* conditions—represents a critical bottleneck where survival rates can decline substantially if environmental transitions are not carefully managed (Hiti-Bandaralage et al. 2022). Detailed cost-benefit analyses comparing infrastructure requirements, labor intensity, consumable expenses, and per-plantlet production costs between conventional and micropropagation systems remain essential for informed commercial adoption decisions. Such analyses should incorporate the long-term economic benefits of genetic uniformity and pathogen-free status, which may offset higher initial production costs through improved orchard establishment rates and reduced replanting requirements.

Conclusions

This study presents an integrated micropropagation protocol for avocado (*Persea americana* Mill.) rootstocks that addresses multiple recalcitrance barriers through a systematic approach combining maternal plant conditioning,

explant pretreatment, and culture medium optimization. Rapid shoot induction and development at the *in vitro* establishment stage were achieved by subjecting mother plants in the tree nursery to etiolation for seven weeks before *in vitro* establishment. An effective, a methodology was developed for nodal segment disinfection, on “Creole 1” and “Creole 2” genotypes, and Duke-7 and Hass varieties, enabling successful *in vitro* establishment. This methodology comprises: (1) Benomyl fungicide (1 mg L^{-1}) for 1 h; (2) sodium hypochlorite (1%) for 5 min; and (3) the antibiotics vancomycin (250 mg L^{-1}) and cefotaxime (500 mg L^{-1}), achieving a contamination rate below 40%. To reduce *in vitro* explants phenolization in “Creole 1”, “Creole 2” genotypes and Duke-7, Hass varieties, extended darkness pretreatment of at least 15 days is required before transfer to photoperiod, with 45 days yielding optimal results for “Creole 1”. The hormonal combination of BAP (1.5 mg L^{-1}), NAA (0.4 mg L^{-1}), and GA₃ (0.4 mg L^{-1}) achieved the highest shoot induction frequency (62%) and elongation (1.35 cm). Moreover, “Creole 1” and “Creole 2” genotypes and Duke-7 and Hass varieties nodal segments achieved a significant induction of new shoots; nevertheless, only 18–24% reached the minimum length (4 cm) required to guarantee their survival through subcultures. Notably, continuous activated charcoal supplementation (1 g L^{-1}) promoted spontaneous adventitious rooting during multiplication (44–56%), substantially reducing dependence on exogenous auxin application and representing a significant advancement over conventional protocols reporting <30% success.

Future perspectives

Despite the advances achieved, several critical areas warrant further investigation to enable commercial-scale implementation of this protocol. *First*, genotype validation should be expanded to include additional Creole lines from diverse Colombian agroecological zones, as well as internationally important rootstocks to establish protocol transferability and broader commercial applicability across the genetic diversity of *P. americana*. *Second*, assessment of clonal fidelity through molecular markers represents a critical requirement for commercial deployment. Recent studies demonstrate that SSR and ISSR markers provide reliable detection of somaclonal variation in micropropagated woody plants, with true-to-type confirmation rates exceeding 86% when appropriate protocols are employed (Gupta et al. 2009; Debnath 2017). Given that extended *in vitro* culture may induce epigenetic modifications affecting regeneration capacity (Duta-Cornescu et al. 2023), future studies should incorporate molecular fidelity assessment to ensure genetic stability of micropropagated avocado rootstocks throughout successive subcultures. *Third*, promising avenues for protocol enhancement include: (i)

integration with micrografting techniques to combine optimized rootstock multiplication with scion establishment in a unified propagation system, potentially reducing the time from in vitro establishment to field-ready plants; (ii) adaptation to temporary immersion bioreactor systems (TIBs) to achieve automation, improved gas exchange, and scalability for commercial production volumes; and (iii) development of cryopreservation protocols for long-term germplasm conservation of elite Colombian avocado genotypes, ensuring preservation of superior rootstock selections identified through field performance evaluation. *Finally*, elucidation of the molecular mechanisms underlying the persistent recalcitrance observed—particularly the progressive decline in shoot elongation across subcultures and the limited IBA responsiveness in non-rooting cohorts—may reveal novel intervention targets. Transcriptomic and metabolomic profiling of responsive versus recalcitrant explants could identify candidate genes and metabolic pathways for targeted manipulation through either cultural modifications or biotechnological approaches.

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Author contributions DV-M designed and conducted the experiments. CR-O, LC-O, and AGC conducted avocado in vitro culture experiments. SS-P directed and conducted experiments with avocado tree nursery plants. CR-O and AGC analyzed the data and authored the article. DV-M and CR-O provided substantial contributions to data analysis and manuscript revision. LC-G contributed to managing access to biological resources, generating agreements between the executing entities, and managing Material Transfer Agreements (MTA). All authors read and approved the final manuscript.

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Data availability All data generated and analyzed during this study are included in this document. No additional datasets were generated or analyzed that require deposition in public repositories.

Declarations

Ethical approval Not applicable.

Competing interests All authors declare that they have no competing interests.

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