

Article

Auxin Treatment Enhances Adventitious Rooting While Genotype Modulates Root Elongation and Basal Callus Formation in *Coffea arabica* Cuttings

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Abstract

Adventitious rooting remains a major constraint for the clonal propagation of *Coffea arabica*, limiting the large-scale multiplication of elite genotypes. This study evaluated the effects of genotype, auxin treatment, and their interaction on adventitious rooting and basal callus formation in coffee cuttings under controlled nursery conditions, while morphophysiological traits were assessed as complementary indicators of cutting performance. A completely randomized 3×3 factorial design was used, including three hybrids (H3, Excelencia, and Milenio) and three plant growth regulator (PGR) treatments: control, indole-3-butyric acid (IBA), and a commercial auxin formulation (RH; NAA + IBA). Rooting probability and root number were significantly affected by PGR treatment, whereas the longest root length was influenced by hybrid, PGR treatment, and their interaction. Model-estimated rooting probability increased from 4.76% in the control to 17.05% under IBA and 35.73% under RH. Similarly, the estimated number of roots per cutting increased from 0.14 in the control to 0.99 under IBA and 1.85 under RH. Although the hybrid $\times$ PGR interaction was not significant for rooting probability, the highest observed rooting percentage was recorded in H3 under RH (52.38%), followed by Milenio under RH (33.33%). For the longest root length, the strongest responses were observed under RH, particularly in Milenio (71.15 mm) and H3 (70.08 mm). Callus formation varied among treatments, but its association with rooting performance was weak and inconsistent. Morphophysiological traits provided complementary information on cutting status but were not interpreted as direct mechanistic drivers of rooting. These findings indicate that adventitious rooting in *C. arabica* was more closely associated with genotype-dependent responsiveness to exogenous auxin than with the extent of callus formation. However, anatomical studies are needed to determine the developmental origin of root primordia and their possible relationship with callus tissue.

Keywords: *Coffea arabica*; adventitious rooting; clonal propagation; auxin responsiveness; indole-3-butyric acid; callogenesis; plant growth regulators; genotype-dependent response



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1. Introduction

Adventitious rooting is a critical step in the clonal propagation of woody crops, directly determining the efficiency and scalability of elite genotype multiplication [1]. In *Coffea arabica* L., a species of high economic importance, vegetative propagation through cuttings is increasingly used to maintain genetic uniformity and desirable agronomic traits. However, its large-scale application remains constrained by low and inconsistent rooting success under nursery conditions [2,3].

Adventitious root formation is a complex developmental process regulated by the interplay between endogenous physiological factors and external environmental conditions [4,5]. Among these, the physiological status of donor plants, nutrient availability, and environmental parameters such as temperature and humidity play key roles in determining rooting competence [6–8]. Importantly, genotype has been identified as a major source of variability, influencing both the magnitude and consistency of responses to exogenous auxin application.

Auxins are central regulators of adventitious rooting, controlling key processes such as cell dedifferentiation, division, and subsequent root primordia formation [9,10]. In coffee, auxin-based treatments have been reported to improve rooting responses in stem cuttings of *C. arabica*, including the use of IBA, NAA, and auxin-related combinations [2,11]. However, auxin-mediated responses are highly context-dependent and may lead to alternative developmental outcomes, including either adventitious root formation or undifferentiated callus proliferation, depending on tissue competence and hormonal balance [12,13].

Traditionally, callus formation has been considered an intermediate stage in the rooting process [14]. Nevertheless, visible callus formation does not always result in successful root emergence [15]. In several woody species, excessive callus proliferation has been associated with reduced rooting efficiency, indicating a potential trade-off in resource allocation between undifferentiated tissue growth and organized root development [16–18]. Despite this, the functional relationship between callus formation and rooting success remains poorly understood in *C. arabica*, where callus is frequently observed but not consistently associated with root emergence.

In addition to rooting traits, morphophysiological variables such as shoot development, gas exchange parameters, and chlorophyll content have been proposed as indicators of cutting performance [19,20]. However, their integrative relationship with rooting capacity and callus formation has not been fully elucidated in coffee, particularly under contrasting auxin treatments.

Given these limitations, it remains unclear how genotype modulates auxin responsiveness and whether callus formation is functionally linked to rooting success in *C. arabica* cuttings. Therefore, this study aimed to evaluate how genotype, auxin treatment, and their interaction influence adventitious rooting and basal callus formation in *C. arabica* cuttings under controlled nursery conditions. Morphophysiological traits were assessed as complementary indicators of cutting performance to support the interpretation of rooting responses, rather than as direct mechanistic determinants of root primordium induction.

Understanding genotype-dependent responsiveness to exogenous auxin is essential for optimizing propagation protocols, particularly when callus proliferation does not necessarily translate into successful root emergence. This framework allows rooting competence to be evaluated as the primary propagation outcome, while physiological traits are considered supportive indicators of cutting condition.

2. Materials and Methods

2.1. Plant Material and Study Conditions

Three *Coffea arabica* hybrids (H3, Milenio, and Excelencia) were used in this study (Table 1). Mother plants were pruned and maintained under nursery conditions (65% shade) for 90 days to promote the development of juvenile orthotropic shoots suitable for clonal propagation.

Table 1. Genetic origin of *Coffea arabica* hybrids.

Hybrid	Crossing *	Experimental Name **	Base Code of the Clone
F1	Caturra 7 × Etiope 531	Excelencia	LI_L05A26
F1	T5296 × Rume Sudan	Milenio	LI_L12A28
F1	Caturra × Ethiopian landrace accession “E531”	H3	LI_L13A22

* Hybrid group derived from Catimor or Timor lines crossed with Ethiopian parental material. ** Trade name according to the World Coffee Research coffee variety catalog.

All mother plants were managed under uniform agronomic conditions to minimize variability associated with nutritional status and physiological condition. During this period, plants were maintained following standard cultivation practices for *C. arabica* [21], including regular irrigation and routine phytosanitary management. Preventive disease control was applied using Phyton 27® (SERFI S.A., Peru; suspension concentrate; PQUA No. 3456-SENASA) (2 mL L^{−1}; 247 g L^{−1} CuSO₄·5H₂O).

Orthotropic shoots were collected from multiple mother plants within each hybrid and transferred to a polyethylene humidity chamber installed inside a microtunnel system (35% shade). For each hybrid, cuttings were obtained from five uniformly managed mother plants ($n = 5$). Prior to cutting collection, physiological, morphological, and nutritional traits of the donor plants were evaluated to verify comparable baseline conditions under nursery conditions. Mother plants were maintained under the same shade, irrigation, pruning, and phytosanitary management regime. Orthotropic shoots were collected from the identified mother plants and assigned to the corresponding PGR treatments according to the experimental design.

Environmental conditions within the microtunnel followed the natural photoperiod of the region (approximately 12 h light per day), with a mean temperature of 26 °C and relative humidity of 68%, continuously monitored using a data logger (ELITECH RC-4HC, Elitech Technology, Inc., San Jose, CA, USA).

All plant material used in the experiment was obtained from uniformly managed mother plants under identical environmental conditions. Therefore, subsequent analyses focused on the response of the propagation material under controlled nursery conditions.

2.2. Morphophysiological and Nutritional Evaluation of Mother Plants

Physiological measurements were performed 90 days after pruning using a portable photosynthesis system (LI-6800, LI-COR Biosciences, Lincoln, NE, USA) under controlled conditions of photosynthetically active radiation (PAR) and internal CO₂ concentration. The evaluated variables included intrinsic water-use efficiency (iWUE), net CO₂ assimilation rate (A), transpiration rate (E), and stomatal conductance (gs). Morphological variables included shoot length (cm) and basal stem diameter (mm).

For nutrient analysis, 0.2 g of lyophilized leaf tissue was digested using a nitric–hydrochloric acid digestion method following Borges et al. [22]. Macronutrients (P, K, Ca, and Mg) and micronutrients (Fe, Cu, Mn, and Zn) were quantified using microwave plasma atomic emission spectrometry (MP-AES 4100, Agilent Technologies, Santa Clara, CA, USA). Results were expressed as µg g^{−1} dry weight.

2.3. Rooting Treatments and Experimental Design

Orthotropic shoots (approximately 18 cm in length and 5 mm in diameter) were segmented into two-node cuttings (approximately 7 cm), with leaf area reduced to 25% to limit transpiration. Basal and apical cuts were standardized at 90° and 45°, respectively. Cuttings were surface-disinfected using Attack® (cymoxanil 80 g kg⁻¹ + mancozeb 640 g kg⁻¹; SERFI S.A., Lima, Peru; PQUA No. 4015-SENASA) (2 g L⁻¹) prior to treatment application.

Three plant growth regulator (PGR) treatments were evaluated: (i) indole-3-butyric acid (IBA; 0.2 g L⁻¹), (ii) a commercial auxin formulation [Root-Hor® (RH; Comercial Andina Industrial S.A.C., Lima, Peru)], and (iii) a control (no PGR). The IBA concentration used in this study (0.2 g L⁻¹) was selected based on previous reports indicating that exogenous auxin application improves adventitious rooting in *Coffea arabica* and other woody species [2,23–25]. Previous studies on coffee have shown that auxin-based treatments, including IBA, NAA, and combinations of auxins with boron, can improve rooting responses in stem cuttings of *C. arabica* cv. Mundo Novo and other coffee propagation systems [11]. In addition, preliminary nursery observations showed negligible rooting in the absence of auxin, supporting the need for exogenous auxin application under our propagation conditions.

The commercial auxin formulation Root-Hor® (RH; Comercial Andina Industrial S.A.C., Lima, Peru; PBUA No. 057-SENASA) was used as a soluble concentrate (SL) containing α -naphthaleneacetic acid (NAA; 0.40%) and indole-3-butyric acid (IBA; 0.10%). Based on the manufacturer's technical datasheet, RH was prepared at 5 mL L⁻¹, and the basal portion of each cutting was immersed for a standardized experimental period of 1 min. This product was selected as a commercially available auxin formulation for comparison with the single-auxin IBA treatment.

The experiment followed a completely randomized 3 × 3 factorial design (three hybrids × three PGR treatments), with 21 cuttings per hybrid × PGR combination ($n = 21$). Cuttings were randomly assigned to treatments and considered independent experimental units for statistical analysis.

Cuttings were established in PlugMix 8® substrate (Klasmann-Deilmann GmbH, Geeste, Germany; pH 5.5, 15% aeration, 80% water retention) and maintained under controlled nursery conditions as described above.

Cuttings were obtained from multiple mother plants within each hybrid to reduce potential bias associated with individual donor plants.

2.4. Evaluation of Morphological Development

Morphological development was monitored for 83 days after establishment. The number of leaves and shoot length were recorded periodically to assess vegetative growth dynamics.

Final evaluations were conducted at 90 days after establishment and included number of leaves, shoot length, and shoot biomass. Shoot biomass was determined as fresh weight and dry weight after oven-drying samples to constant weight at 60 °C in a forced-air oven.

Callus development was quantified by measuring callus area (CAB) and basal cutting area (BAC) using ImageJ version 1.54i (National Institutes of Health, Bethesda, MD, USA). Relative callus proportion was calculated as:

$$\text{PCF (\%)} = (\text{CAB}/\text{BAC}) \times 100 \quad (1)$$

Rooting performance was evaluated at 120 days after establishment and included rooting percentage (%), number of roots per cutting, and longest root length (mm).

2.5. Physiological Performance of Cuttings

Relative chlorophyll content was measured using a SPAD-502 meter (Konica Minolta, Osaka, Japan) at 15-day intervals. Final measurements were performed at 90 days after establishment on fully expanded leaves.

Gas exchange was measured at the end of each evaluation period on fully expanded leaves located in the middle third of the cuttings, between 10:00 and 13:00 h, using a portable photosynthesis system (LI-6800, LI-COR Biosciences, Lincoln, NE, USA). The evaluated parameters included net CO₂ assimilation rate (A , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), stomatal conductance (g_s , $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), transpiration rate (E , $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), and intrinsic water-use efficiency (iWUE, $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$), calculated as the ratio between A and g_s .

Chamber conditions were standardized at 600 $\mu\text{mol s}^{-1}$ airflow, 800 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ photosynthetic photon flux density (PPFD), 21 °C leaf temperature, 45% relative humidity, and a fan speed of 2.5 rpm, following the configuration described by López-Merino et al. [26]. All measurements were conducted under stable environmental conditions to ensure comparability among samples.

2.6. Statistical Analysis

Data were analyzed using R software [27] (version 4.5.2; R Core Team, Vienna, Austria). The experiment followed a completely randomized 3×3 factorial design with two fixed factors: hybrid (H3, Excelencia, and Milenio) and plant growth regulator (PGR) treatment (control, IBA, and RH). For each hybrid $\times$ PGR combination, 21 cuttings were evaluated ($n = 21$). Cuttings were obtained from five uniformly managed mother plants per hybrid and randomly assigned to PGR treatments to reduce potential donor-plant bias. Because individual cuttings were used as the experimental units and mother plants were used only as a source of propagation material under uniform nursery management, the statistical analyses focused on the fixed effects of hybrid, PGR treatment, and their interaction.

Rooting traits were analyzed using generalized linear models selected according to the distributional structure of each response variable. Rooting percentage was analyzed at the cutting level as a binary response variable (rooted vs. non-rooted) using a binomial generalized linear model (GLM) with a logit link. Number of roots was analyzed as count data. A Poisson GLM with a log link was initially fitted; however, because this model showed overdispersion, a negative binomial GLM with a log link was used instead. Longest root length was analyzed only for rooted cuttings using a Gamma GLM with a log link, as this variable was continuous and strictly positive.

For each rooting model, hybrid, PGR treatment, and their interaction were included as fixed effects. Type III likelihood-ratio tests were used to evaluate the significance of main effects and interactions. When the hybrid $\times$ PGR interaction was not significant, the main effect of PGR was interpreted and presented using estimated marginal means. When the interaction was significant, hybrid $\times$ PGR combinations were compared. Estimated marginal means or probabilities and their model-based 95% confidence intervals were obtained using the emmeans package. Pairwise comparisons were adjusted using Tukey's method, and significance letters were generated using compact letter display.

For GLM-based analyses, model adequacy was evaluated through inspection of residual diagnostics and assessment of overdispersion when appropriate. For variables analyzed using ANOVA, normality and homogeneity of variances were assessed using the Shapiro–Wilk and Levene's tests, respectively.

For vegetative development and basal callus traits that met parametric assumptions, data were analyzed using two-way analysis of variance (ANOVA) to test the main effects

of hybrid, PGR treatment, and their interaction. When significant effects were detected ($p \leq 0.05$), means were compared using Tukey's honestly significant difference (HSD) test.

For variables that did not meet parametric assumptions, data transformations were applied to improve normality and variance homogeneity. Specifically, relative callus proportion (PCF), shoot length, and dry weight were square-root transformed, whereas callus area (CAB) was log-transformed. Variables that satisfied assumptions after transformation were subsequently analyzed using the same ANOVA framework.

For physiological variables of cuttings, including intrinsic water-use efficiency (iWUE), transpiration rate (E), net CO₂ assimilation rate (A), stomatal conductance (gs), and relative chlorophyll content (SPAD), parametric assumptions were not satisfied even after transformation. Therefore, differences among hybrid $\times$ PGR combinations were evaluated using the Kruskal–Wallis test, followed by pairwise comparisons with Benjamini–Hochberg correction when significant differences were detected ($p \leq 0.05$). Results from these analyses were interpreted as combined treatment effects rather than as separate factorial effects.

Unless otherwise stated, descriptive results are presented as means $\pm$ standard deviations (SD). For rooting traits, model-estimated means or probabilities are presented with model-based 95% confidence intervals. Graphical analyses were performed using the ggplot2 package [28] and associated libraries in R.

Spearman correlation analysis was conducted to evaluate relationships among morphological, physiological, callus-related, and rooting variables. Correlation coefficients (ρ) and associated p -values were calculated using the rcorr function from the Hmisc package. Only significant correlations ($p < 0.05$) were considered for interpretation, and strong associations ($|\rho| \geq 0.60$) were emphasized in graphical representations.

To avoid overinterpretation of redundant associations, variables representing highly similar biological processes were evaluated before graphical presentation. When variables were strongly correlated ($|\rho| > 0.85$), representative traits were retained or emphasized based on biological relevance and interpretability. Rooting percentage, number of roots, and longest root length were retained in the correlation analysis because they represent complementary components of the rooting response.

3. Results

3.1. Physiological Response and Nutritional Status of Mother Plants

Physiological, morphological, and nutritional traits of mother plants were evaluated using a subset of representative individuals for each hybrid (Table 2). Intrinsic water-use efficiency (iWUE), net CO₂ assimilation rate (A), transpiration rate (E), stomatal conductance (gs), chlorophyll content, and shoot growth variables showed comparable values among genotypes.

Table 2. Physiological and morphological traits of *Coffea arabica* mother plants after pruning.

Hybrid ¹	iWUE ($\mu\text{mol mol}^{-1}$)	E (mmol $\text{m}^{-2} \text{s}^{-1}$)	A (μmol $\text{m}^{-2} \text{s}^{-1}$)	gs (mol $\text{m}^{-2} \text{s}^{-1}$)	Chlorophyll (SPAD)	Shoot Length (cm)	Stem Diameter (mm)
Excelencia	47.46 $\pm$ 6.99	2.62 $\pm$ 0.62	6.40 $\pm$ 0.37	0.13 $\pm$ 0.01	50.02 $\pm$ 1.47	17.99 $\pm$ 0.83	6.17 $\pm$ 0.28
Milenio	42.12 $\pm$ 3.24	1.95 $\pm$ 0.58	5.35 $\pm$ 0.82	0.13 $\pm$ 0.02	49.96 $\pm$ 1.86	17.15 $\pm$ 1.81	5.91 $\pm$ 0.35
H3	32.11 $\pm$ 4.90	2.85 $\pm$ 0.98	6.05 $\pm$ 0.31	0.19 $\pm$ 0.04	47.32 $\pm$ 0.80	19.44 $\pm$ 2.38	5.90 $\pm$ 0.18

¹ Values are presented as means $\pm$ standard deviations ($n = 5$ mother plants per hybrid). iWUE: intrinsic water-use efficiency; A: net CO₂ assimilation rate; E: transpiration rate; gs: stomatal conductance. Measurements were performed 90 days after pruning under controlled nursery conditions. These data were used to verify the homogeneity of the physiological status of donor plants prior to cutting collection.

Similarly, foliar nutrient concentrations did not differ substantially among hybrids, confirming a consistent nutritional status under the experimental conditions. Notably, relatively high concentrations of phosphorus ($2432.36 \mu\text{g g}^{-1}$) and potassium ($5684.26 \mu\text{g g}^{-1}$) were observed, both of which are associated with energy metabolism and osmotic regulation. This nutritional profile indicates that donor plants maintained adequate conditions to support subsequent cutting development.

Overall, no substantial differences were observed among hybrids in terms of physiological and nutritional status under the evaluated conditions, suggesting that donor plants exhibited homogeneous baseline conditions prior to cutting collection.

3.2. Vegetative Development of Cuttings

Vegetative development of cuttings at 90 days after establishment was significantly influenced by hybrid, plant growth regulator (PGR) treatment, and their interaction across all evaluated variables (two-way ANOVA, $p < 0.05$; Table 3).

Table 3. Effects of hybrid, plant growth regulator (PGR) treatment, and their interaction on vegetative development of *Coffea arabica* cuttings at 90 days after establishment.

Hybrid $\times$ PGR ¹	Number of Leaves	Shoot Length (cm)	Shoot Fresh Weight (mg)	Dry Weight (mg)
H3—Control	4.05 ± 0.92 ab	12.25 ± 3.07 a	195.56 ± 8.32 c	50.06 ± 2.73 c
H3—RH	4.62 ± 0.80 a	10.67 ± 2.34 a	296.52 ± 8.33 b	58.09 ± 3.37 b
H3—IBA	4.05 ± 0.92 ab	7.98 ± 1.87 bc	334.26 ± 11.72 a	61.93 ± 3.87 a
Excelencia—Control	3.52 ± 0.87 bc	6.60 ± 2.53 c	183.91 ± 7.98 d	34.27 ± 2.16 d
Excelencia—RH	3.10 ± 0.83 c	7.05 ± 2.08 c	172.45 ± 8.08 e	29.17 ± 1.48 e
Excelencia—IBA	3.95 ± 0.80 ab	7.74 ± 2.71 c	105.46 ± 6.27 h	24.30 ± 1.29 fg
Milenio—Control	3.48 ± 0.75 bc	7.45 ± 1.92 c	128.24 ± 6.88 g	23.43 ± 1.62 g
Milenio—RH	2.29 ± 0.72 d	10.19 ± 2.44 ab	147.28 ± 8.09 f	25.47 ± 1.76 f
Milenio—IBA	3.14 ± 0.79 c	7.43 ± 2.94 c	202.91 ± 9.28 c	29.40 ± 1.03 e
ANOVA (2 ways)				
Hybrid	*** ($p < 0.001$)	*** ($p < 0.001$)	*** ($p < 0.001$)	*** ($p < 0.001$)
PGRs	* ($p = 0.018$)	** ($p = 0.001$)	*** ($p < 0.001$)	*** ($p < 0.001$)
Hybrid $\times$ PGRs	*** ($p < 0.001$)	*** ($p < 0.001$)	*** ($p < 0.001$)	*** ($p < 0.001$)

¹ Values represent mean $\pm$ standard deviation ($n = 21$). Different lowercase letters within each column indicate significant differences among hybrid $\times$ PGR combinations according to Tukey's HSD test ($p < 0.05$). PGR: plant growth regulator; RH: commercial auxin formulation (Root-Hor[®]; NAA + IBA); IBA: indole-3-butyric acid. The asterisks indicate the levels of statistical significance. Their definitions have been added to the table footnote as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. The values in parentheses correspond to the exact p -values.

Leaf number varied among hybrid $\times$ PGR combinations, with the highest values observed in H3 under RH, whereas Milenio under RH showed the lowest values.

Shoot length exhibited a distinct response pattern. The greatest elongation was recorded in H3 under control conditions, while auxin treatments reduced shoot length in this hybrid. In contrast, shoot length in Excelencia and Milenio remained relatively stable across treatments.

Biomass accumulation showed a strong interaction effect (Table 3). The highest fresh and dry weights were observed in H3 under IBA, followed by H3 under RH, whereas the lowest values were recorded in Excelencia under IBA. Milenio exhibited intermediate biomass values that varied depending on the PGR treatment.

Overall, vegetative development showed significant variation among treatments among hybrid $\times$ PGR combinations, with H3 showing greater biomass accumulation under auxin treatments, while shoot elongation responses varied according to genotype and

treatment. These patterns were consistent with the visual differences observed among treatments (Figure 1).

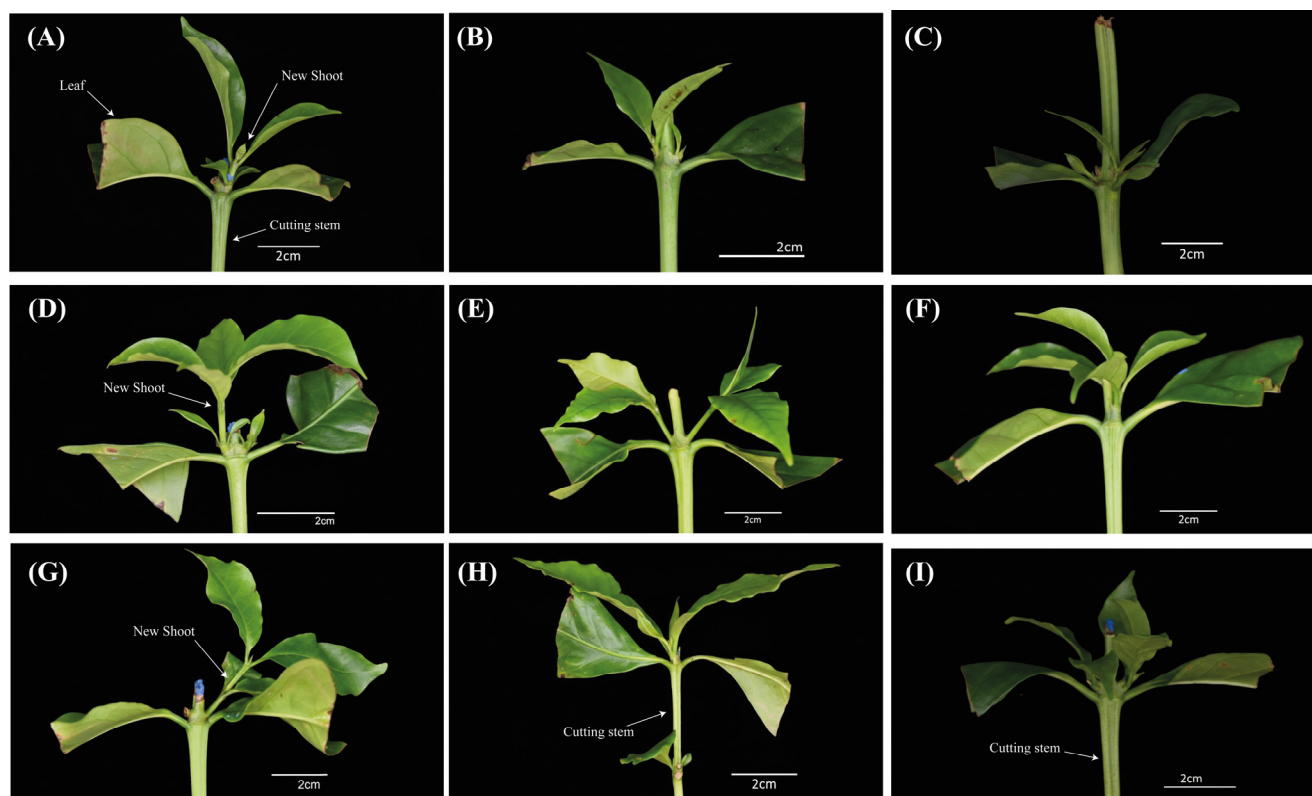


Figure 1. Representative vegetative development of *Coffea arabica* cuttings from three hybrids under different plant growth regulator (PGR) treatments at 90 days after establishment. (A–C) H3 hybrid under control, RH, and IBA treatments, respectively; (D–F) Excelencia hybrid under control, RH, and IBA; (G–I) Milenio hybrid under control, RH, and IBA. Relevant vegetative structures, including leaves, new shoots, and the cutting stem, are labeled in representative panels. PGR: plant growth regulator; RH: commercial auxin formulation (Root-Hor[®]; NAA + IBA); IBA: indole-3-butyric acid.

3.3. Rooting Response

For rooting percentage, the Type III analysis showed a significant effect of PGR treatment, whereas hybrid and the hybrid $\times$ PGR interaction were not significant (Table 4). Accordingly, rooting percentage is presented as the main PGR effect in Figure 2A. Model-estimated rooting probability increased from 4.76% in the control to 17.05% under IBA and 35.73% under RH.

Table 4. Type III likelihood-ratio tests from generalized linear models for rooting traits of *Coffea arabica* cuttings.

Factor ¹	Rooting (%)	Number of Roots	Longest Root (mm)
Hybrid	ns ($p = 0.571$)	ns ($p = 0.202$)	*** ($p < 0.001$)
PGRs	*** ($p < 0.001$)	*** ($p < 0.001$)	*** ($p < 0.001$)
Hybrid $\times$ PGRs	ns ($p = 0.933$)	ns ($p = 0.631$)	*** ($p < 0.001$)

¹ Rooting percentage was analyzed using a binomial GLM with logit link. Number of roots was analyzed using a negative binomial GLM with log link because the initial Poisson model showed overdispersion. Longest root length was analyzed using a Gamma GLM with log link considering only rooted cuttings. Significance was assessed using Type III likelihood-ratio tests. ns: not significant; *** $p < 0.001$. PGR: plant growth regulator; RH: commercial auxin formulation; IBA: indole-3-butyric acid.

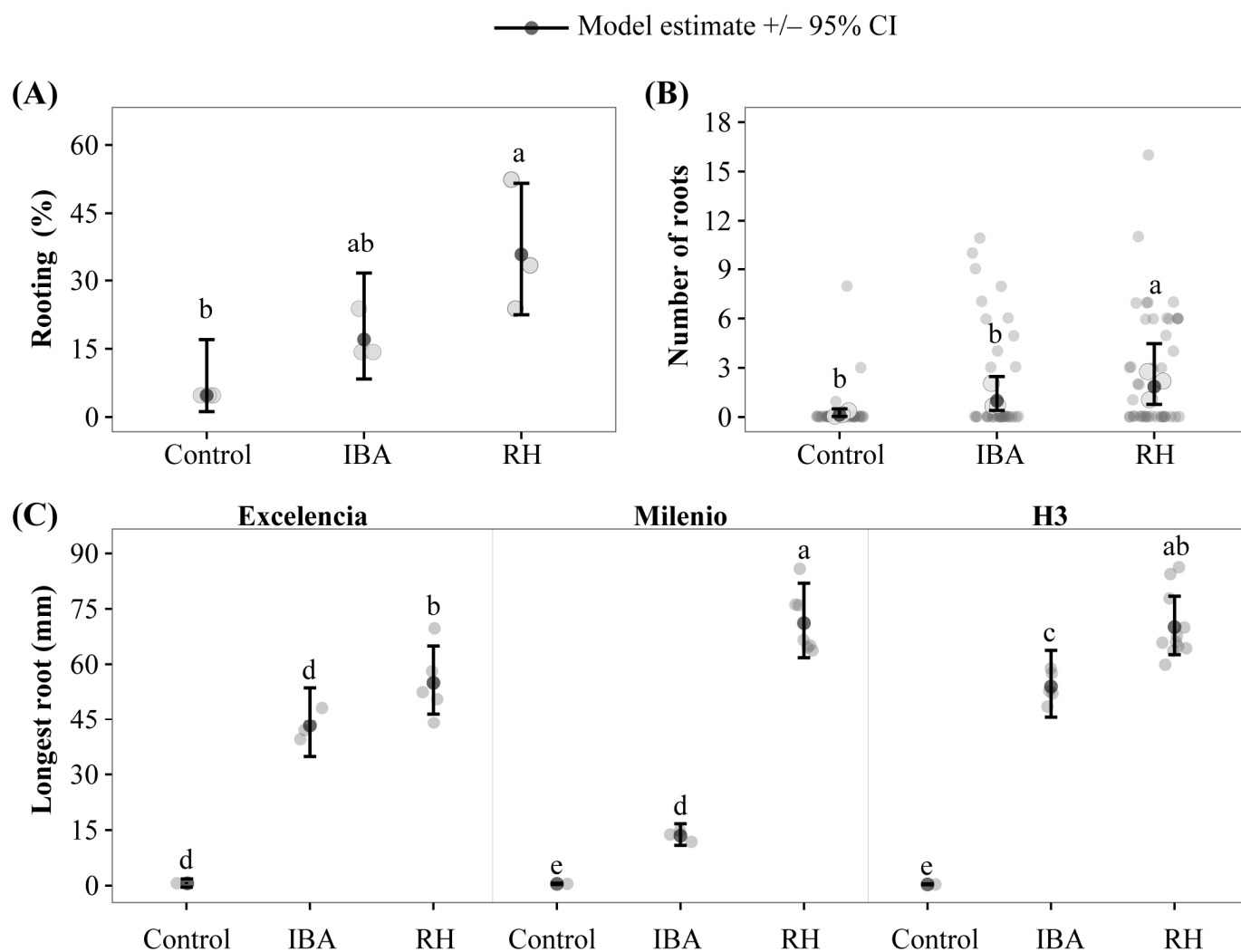


Figure 2. Model-estimated rooting responses of *Coffea arabica* cuttings under different plant growth regulator (PGR) treatments. (A) Rooting percentage analyzed using a binomial generalized linear model with logit link and presented as model-estimated rooting probability. (B) Number of roots per cutting analyzed using a negative binomial generalized linear model with log link. (C) Longest root length analyzed using a Gamma generalized linear model with log link, considering only rooted cuttings. Large colored points represent model-estimated means or probabilities, and error bars indicate model-based 95% confidence intervals. Small grey points represent observed data. Different lowercase letters indicate significant differences according to Tukey-adjusted comparisons of estimated marginal means ($p \leq 0.05$). PGR: plant growth regulator; IBA: indole-3-butyric acid; RH: commercial auxin formulation containing NAA + IBA. The raw dataset and complete statistical output used to generate Figure 2 are provided in Supplementary File S1.

For number of roots, the initial Poisson model showed overdispersion; therefore, a negative binomial GLM was used. The Type III analysis showed a significant effect of PGR treatment, while hybrid and the hybrid $\times$ PGR interaction were not significant (Table 4). Accordingly, number of roots is presented as the main PGR effect in Figure 2B. Model-estimated root number increased from 0.14 roots per cutting in the control to 0.99 under IBA and 1.85 under RH.

For longest root length, the Gamma GLM showed significant effects of hybrid, PGR treatment, and their interaction (Table 4). Therefore, this trait is presented by hybrid $\times$ PGR combinations in Figure 2C. The longest roots were estimated under RH, particularly in Milenio (71.15 mm) and H3 (70.08 mm), followed by Excelencia under RH (54.98 mm) and

H3 under IBA (53.99 mm), whereas control treatments showed negligible root elongation (<1 mm).

Representative root phenotypes were included to complement the model-based estimates and to visually illustrate the range of adventitious root development observed among cuttings at 120 days after establishment (Figure 3).

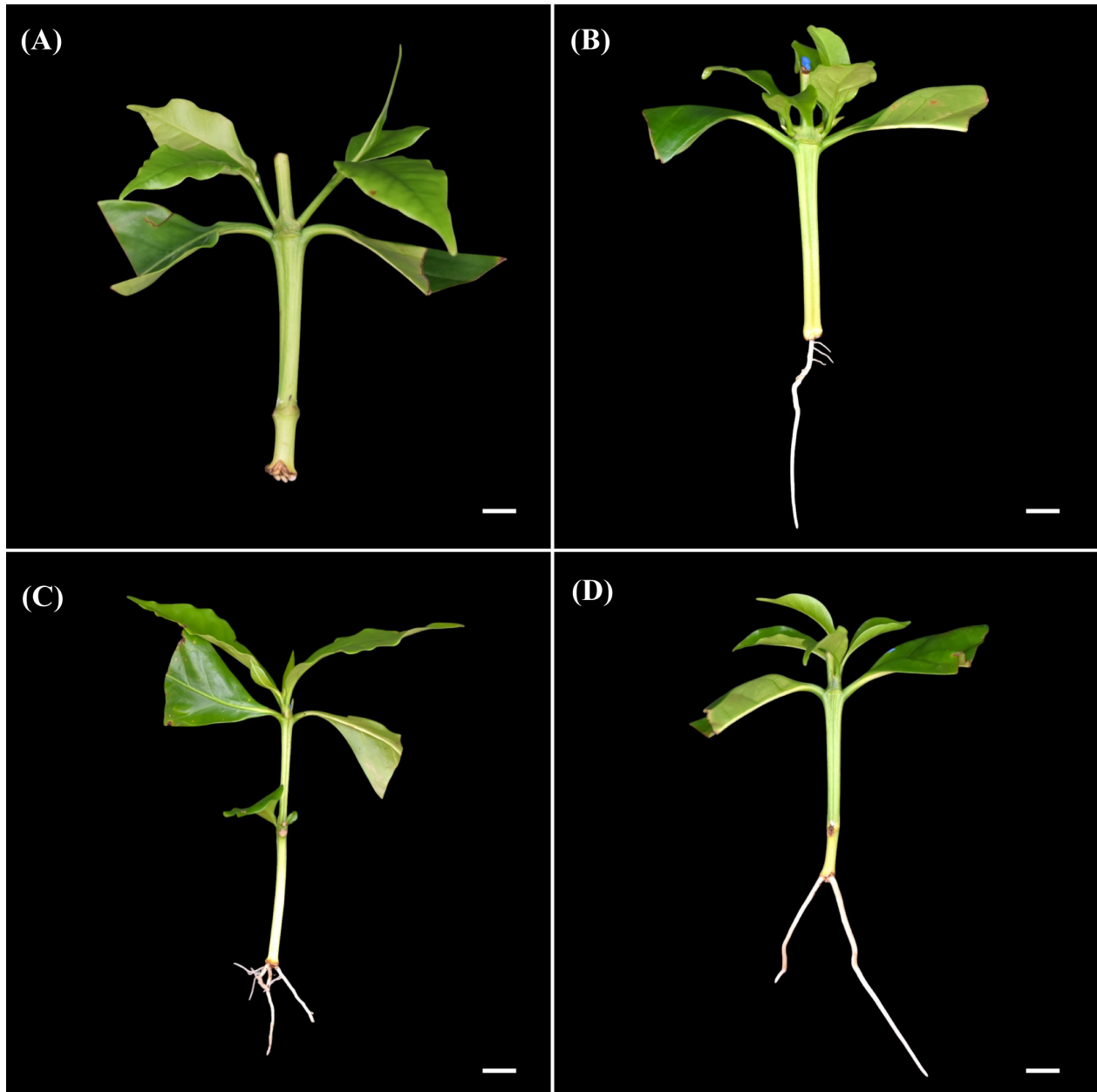


Figure 3. Representative root phenotypes of *Coffea arabica* cuttings at 120 days after establishment. Panels illustrate the phenotypic range observed across the experiment, from cuttings without visible adventitious roots (A), to cuttings with incipient root emergence (B), multiple short adventitious roots (C), and elongated adventitious roots (D). This visual pattern is consistent with the quantitative rooting responses, in which control treatments showed very low rooting percentages, IBA produced intermediate responses, and RH promoted greater root emergence and elongation, particularly in H3 and Milenio. Images were included to complement the model-based estimates shown in Figure 2. Root systems were gently cleaned before imaging. Scale bar = 10 mm.

3.4. Basal Callus Formation and Morphology

Basal callus formation was significantly affected by hybrid, plant growth regulator (PGR) treatment, and their interaction for all evaluated variables (two-way ANOVA, $p < 0.05$; Table 5).

Table 5. Interaction effects of hybrid and plant growth regulator (PGR) treatments on basal callus formation in *Coffea arabica* cuttings.

Hybrid × PGR ¹	PCF	CAB	CDW
H3—Control	61.08 ± 8.12 cd	27.6 ± 5.62 b	3.10 ± 0.55 c
H3—RH	62.56 ± 8.56 c	22.9 ± 5.50 c	2.04 ± 0.28 e
H3—IBA	72.82 ± 3.5 b	21.93 ± 4.54 cd	2.34 ± 0.34 de
Excelencia—Control	38.64 ± 9.1 e	8.45 ± 1.09 g	2.72 ± 0.45 cd
Excelencia—RH	59.72 ± 5.87 cd	18.76 ± 3.32 cd	2.69 ± 0.47 cd
Excelencia—IBA	58.39 ± 8.12 cd	16.79 ± 3.12 ef	2.63 ± 0.66 d
Milenio—Control	54.48 ± 7.04 d	14.53 ± 0.36 f	3.6 ± 0.30 b
Milenio—RH	84.36 ± 8.67 a	42.92 ± 8.46 a	5.66 ± 0.47 a
Milenio—IBA	77.89 ± 8.64 ab	28.62 ± 6.59 b	2.06 ± 0.10 e
ANOVA (2 ways)			
Hybrid	*** (<0.001)	*** (<0.001)	*** (<0.001)
PGRs	*** (<0.001)	** (0.001)	*** (<0.001)
Hybrid × PGRs	*** (<0.001)	*** (<0.001)	*** (<0.001)

¹ Values represent mean ± standard deviation ($n = 21$). Different lowercase letters within each column indicate significant differences among hybrid × PGR combinations according to Tukey's HSD test ($p < 0.05$). PCF: relative callus proportion (%); CAB: callus area (mm²); CDW: dry weight of callus (mg); RH: commercial auxin formulation (NAA 0.40% + IBA 0.10%); IBA: indole-3-butyric acid. The asterisks indicate the levels of statistical significance. Their definitions have been added to the table footnote as follows: ** $p < 0.01$ and *** $p < 0.001$. The values in parentheses correspond to the exact p -values.

Callus-related traits showed significant variation among hybrid × PGR combinations (Table 5). The highest relative callus proportion (PCF), callus area (CAB), and callus dry weight (CDW) were consistently observed in Milenio under RH, whereas the lowest values were generally recorded in Excelencia under control conditions.

Intermediate responses were observed in H3 and Milenio under IBA, while H3 under control exhibited relatively high callus area compared to other treatments. Across variables, RH treatment was associated with greater callus development, particularly in Milenio, whereas Excelencia showed comparatively lower callus formation across treatments.

Callus development exhibited a strong genotype-dependent response to PGR treatments, with Milenio showing the most pronounced callogenic response and Excelencia the lowest.

Notably, the treatments that promoted the highest callus formation did not necessarily correspond to those with the highest rooting performance.

These quantitative patterns were consistent with the morphological differences observed in basal callus structure (Figure 4).

3.5. Complementary Physiological Status of Cuttings

Gas exchange parameters and chlorophyll-related traits were significantly affected by the interaction between hybrid and plant growth regulator (PGR) treatments (Kruskal–Wallis, $p < 0.001$ for all variables; Table 6).

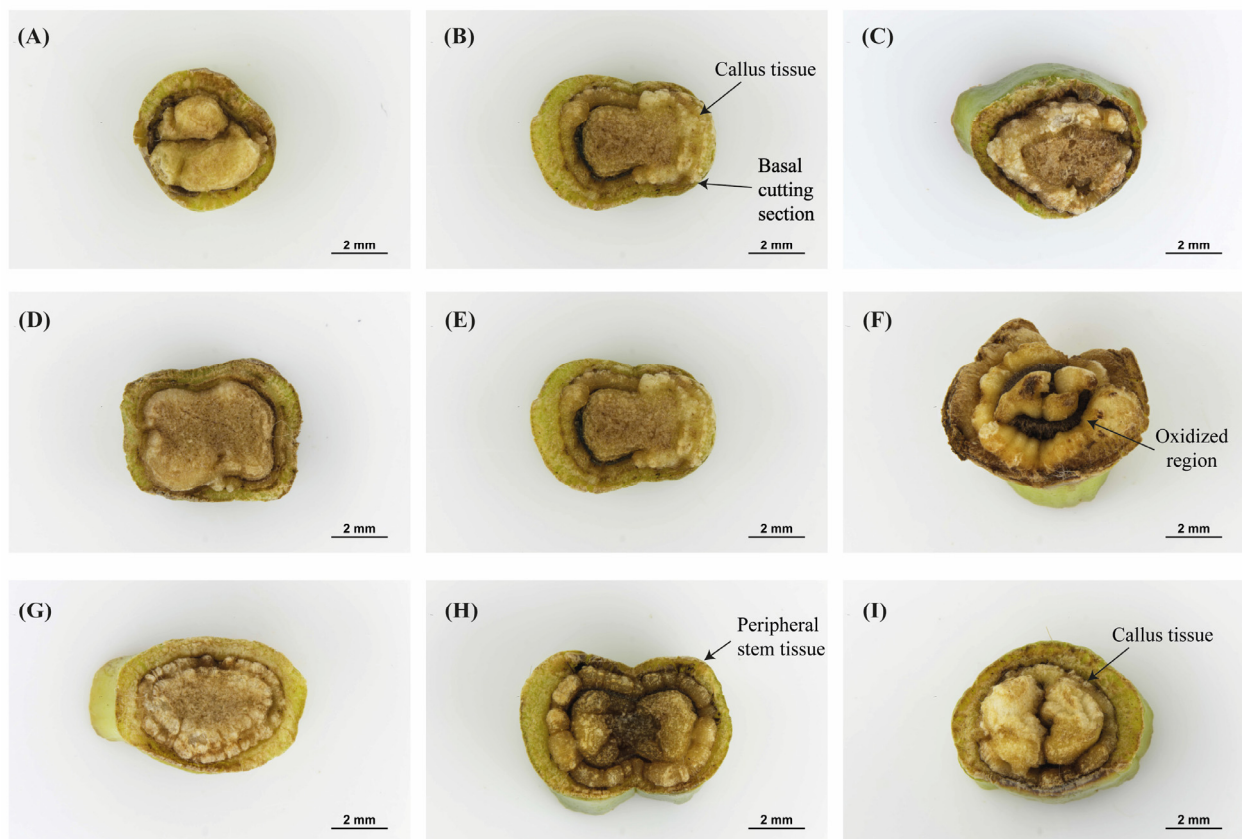


Figure 4. Representative basal callus morphology in *Coffea arabica* cuttings from three hybrids under different plant growth regulator (PGR) treatments at 120 days after establishment. (A–C) H3 under control, RH, and IBA treatments, respectively; (D–F) Excelencia under control, RH, and IBA treatments, respectively; and (G–I) Milenio under control, RH, and IBA treatments, respectively. Relevant visible structures, including callus tissue, the basal cutting section, peripheral stem tissue, and visually oxidized regions, are labeled in representative panels. PGR: plant growth regulator; RH: commercial auxin formulation (Root-Hor[®]; NAA 0.40% + IBA 0.10%); IBA: indole-3-butyric acid. Scale bars = 2 mm.

Physiological variables showed significant variation among treatments among hybrid $\times$ PGR combinations (Table 6). The highest net CO₂ assimilation rate (A) was observed in H3 under RH, followed by H3 under IBA and Milenio under RH, whereas the lowest values were recorded in Excelencia under control conditions.

A similar pattern was observed for transpiration rate (E), with the highest value recorded in Milenio under RH and the lowest in Excelencia under control conditions.

Stomatal conductance (gs) also varied among treatments, with the highest values observed in Milenio under RH and H3 under IBA, while lower values were recorded in Excelencia under control and Milenio under IBA.

In contrast, intrinsic water-use efficiency (iWUE), calculated as the ratio between net CO₂ assimilation and stomatal conductance (A/gs), showed a distinct distribution across treatments. The highest values were observed in H3 under RH and Excelencia under control conditions, whereas the lowest values were recorded in Milenio under RH.

Chlorophyll content (SPAD) varied within a narrower range compared to gas exchange variables, with the highest values observed in H3 under IBA and lower values in Excelencia and Milenio under RH treatment.

Table 6. Interaction effects of hybrid and auxin-based plant growth regulators on gas exchange and chlorophyll-related physiological parameters in *Coffea arabica* cuttings.

Hybrid × PGR	A ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	E ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$)	gs ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$)	iWUE ($\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$)	SPAD
H3—Control	1.27 ± 0.06 cd	0.21 ± 0.03 h	0.03 ± 0.00 cd	45.73 ± 4.85 e	38.80 ± 1.03 b
H3—RH	2.53 ± 0.18 a	0.31 ± 0.03 e	0.02 ± 0.00 a	153.27 ± 8.14 a	38.63 ± 2.21 b
H3—IBA	2.07 ± 0.09 b	0.45 ± 0.03 b	0.07 ± 0.00 b	29.66 ± 2.19 g	40.92 ± 1.94 a
Excelencia—Control	0.61 ± 0.02 g	0.11 ± 0.01 i	0.01 ± 0.00 g	141.69 ± 7.56 b	36.43 ± 1.03 c
Excelencia—RH	1.25 ± 0.07 d	0.33 ± 0.01 d	0.02 ± 0.00 d	74.68 ± 9.25 d	30.62 ± 2.21 d
Excelencia—IBA	0.70 ± 0.04 f	0.37 ± 0.01 c	0.02 ± 0.00 f	34.06 ± 2.70 f	38.09 ± 1.98 b
Milenio—Control	1.29 ± 0.04 c	0.22 ± 0.01 g	0.02 ± 0.00 c	70.84 ± 7.67 d	38.75 ± 2.54 b
Milenio—RH	2.06 ± 0.07 b	1.22 ± 0.07 a	0.09 ± 0.00 b	25.36 ± 1.80 h	32.17 ± 2.44 d
Milenio—IBA	1.09 ± 0.05 e	0.26 ± 0.04 f	0.01 ± 0.00 e	95.97 ± 3.39 c	30.99 ± 3.02 d
Kruskal–Wallis test (Hybrid × PGR)					
Hybrid × PGR	*** ($p < 0.001$)	*** ($p < 0.001$)	*** ($p < 0.001$)	*** ($p < 0.001$)	*** ($p < 0.001$)

Values represent mean ± standard deviation ($n = 21$). Different lowercase letters within each column indicate significant differences among hybrid × PGR combinations according to the Kruskal–Wallis test followed by pairwise comparisons with Benjamini–Hochberg adjustment ($p \leq 0.05$). A: net CO_2 assimilation rate; E: transpiration rate; gs: stomatal conductance; iWUE: intrinsic water-use efficiency (A/gs); SPAD: relative chlorophyll content. RH: commercial auxin formulation (NAA 0.40% + IBA 0.10%); IBA: indole-3-butyric acid. *** $p < 0.001$.

3.6. Spearman Correlations Among Rooting, Callus, Morphological, and Physiological Traits

Correlation analysis revealed a structured pattern of associations among morphological, physiological, callus-related, and rooting traits (Figure 5). Strong correlations ($|r| \geq 0.60$, $p < 0.05$) highlighted coordinated responses primarily among gas exchange and rooting variables.

Rooting traits exhibited the strongest internal associations. Rooting percentage was highly correlated with number of roots ($\rho = 0.89$) and root length ($\rho = 0.87$), while number of roots and root length were also strongly correlated ($\rho = 0.86$), indicating a consistent and integrated rooting response across treatments. Rooting percentage was positively associated with net photosynthetic rate (A; $\rho = 0.70$) and transpiration rate (E; $\rho = 0.72$).

Gas exchange variables showed strong interrelationships. Transpiration rate (E) was positively correlated with stomatal conductance (gs; $\rho = 0.66$) and with rooting traits, including number of roots ($\rho = 0.81$) and root length ($\rho = 0.77$). In contrast, intrinsic water-use efficiency (iWUE) was negatively correlated with gs ($\rho = -0.86$) and E ($\rho = -0.67$), reflecting an inverse relationship between water-use efficiency and gas exchange activity.

Callus-related variables showed comparatively weaker associations with physiological and rooting traits. Relative callus proportion (PCF) was moderately correlated with E ($\rho = 0.57$) and rooting percentage ($\rho = 0.51$). This pattern provides quantitative support for the statement that callus formation was weakly and inconsistently associated with rooting performance, particularly when compared with the stronger correlations observed among rooting traits themselves. However, these associations were weaker than those observed among rooting traits and between rooting traits and gas exchange variables, supporting the interpretation that callus formation alone was not a reliable predictor of rooting success.

In contrast, morphological traits showed weaker and less consistent associations. Shoot length was moderately correlated with A ($\rho = 0.46$) and rooting traits ($\rho = 0.33$ – 0.37), whereas number of leaves exhibited generally weak correlations across variables.

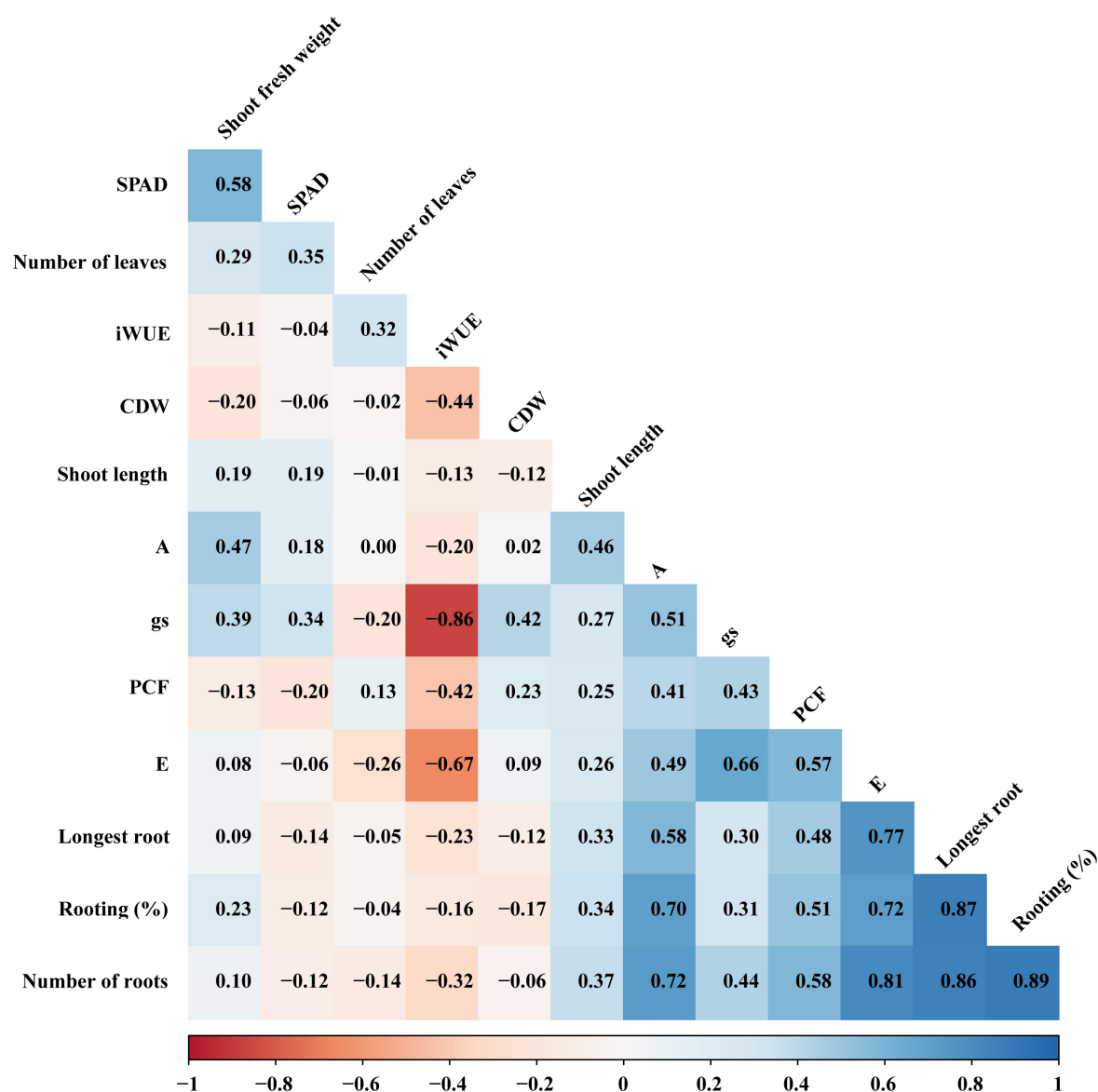


Figure 5. Correlation matrix of morphological, physiological, callus-related, and rooting traits in *Coffea arabica* cuttings. Spearman correlation coefficients (ρ) among morphological traits, physiological traits, callus-related variables, and rooting variables are presented as a heatmap. Color intensity represents the strength and direction of correlations, ranging from -1 (red, negative correlation) to $+1$ (blue, positive correlation). Only one triangle of the matrix is shown to improve visualization. Strong correlations ($|\rho| \geq 0.60$) indicate coordinated associations among physiological activity and rooting performance.

4. Discussion

The physiological and nutritional status of mother plants constitutes a critical baseline for successful clonal propagation. Nutrient availability, particularly macronutrients such as N, P, K, Ca, and Mg, directly influences reserve accumulation in cuttings and their subsequent growth performance [29–31]. In the present study, the relatively high concentrations of phosphorus and potassium indicate a favorable nutritional status. However, physiological parameters such as net photosynthesis, transpiration, and chlorophyll content were lower than those reported under optimal conditions, likely reflecting transient reductions in photosynthetic capacity following pruning and associated shifts in carbon allocation dynamics [32–34].

Despite these reductions, intrinsic water-use efficiency (iWUE) remained within the optimal range reported for *Coffea arabica* [26,35–38], indicating that donor plants maintained sufficient physiological functionality to support vegetative propagation. This balance between carbon assimilation and water loss is essential for sustaining metabolic activity and ensuring the availability of photoassimilates required for root initiation [39,40]. The homogeneity observed among mother plants supports the interpretation that differences in rooting performance were primarily driven by genotype and hormonal treatments rather than initial plant condition.

Rooting performance was strongly influenced by exogenous auxin application, although the statistical pattern differed among rooting traits. Rooting percentage and number of roots were mainly affected by PGR treatment, whereas the longest root length showed a significant hybrid $\times$ PGR interaction. The commercial formulation RH (NAA + IBA) produced the highest model-estimated rooting probability and root number, while also promoting longer roots in Milenio and H3. This enhanced response may be related to the complementary properties of auxin types, including differences in stability, transport, and cellular uptake, which can influence adventitious rooting competence in woody cuttings [23,41,42].

Genotypic differences were clearly evident, with H3 exhibiting superior rooting performance and biomass accumulation under auxin treatments. This response suggests higher intrinsic developmental competence for root organogenesis, a key determinant of adventitious rooting capacity in woody species [10,15,43]. The superior rooting response observed in H3 may reflect greater phenotypic responsiveness to exogenous auxin under the evaluated nursery conditions. However, because endogenous hormone levels, gene expression, and transcriptomic profiles were not assessed, this interpretation should be considered functional and phenotypic rather than mechanistic. Therefore, the genotype-dependent responses observed in this study should not be interpreted as direct evidence of differential auxin signaling, hormonal regulation, or molecular control. Instead, they indicate that the evaluated hybrids differed in their rooting performance when exposed to exogenous auxin treatments, consistent with the recognized role of auxins in adventitious rooting of woody species [23,24,42,43]. Future studies integrating endogenous hormone quantification, histological analysis, and transcriptomic approaches would be necessary to identify the mechanisms underlying these contrasting responses.

Morphophysiological traits were interpreted as complementary indicators of cutting status rather than direct mechanistic determinants of adventitious rooting. H3 exhibited higher photosynthetic activity and intrinsic water-use efficiency under propagation conditions, which coincided with its superior rooting performance, particularly under RH treatment (Table 6; Figure 2). In contrast, Milenio showed increased transpiration and stomatal conductance under auxin treatments; however, this did not translate into the highest rooting percentage. Therefore, gas exchange responses alone were not sufficient to explain adventitious rooting performance across hybrids.

Callus formation exhibited a strong genotype $\times$ PGR interaction, with Milenio showing the highest callogenic response, particularly under RH treatment. However, increased callus development was not associated with improved rooting performance. Correlation analysis revealed weak and inconsistent relationships between callus-related traits and rooting variables, whereas strong associations were observed among rooting traits and gas exchange parameters. Specifically, rooting percentage was strongly correlated with number of roots ($\rho = 0.89$) and the longest root length ($\rho = 0.87$), whereas relative callus proportion showed only a moderate association with rooting percentage ($\rho = 0.51$), reinforcing that visible callus formation was not a reliable predictor of rooting success. These results suggest

that callus formation had limited predictive value for rooting success under the conditions of this study.

An important finding of this study was the occurrence of abundant basal callus formation without a proportional increase in root emergence. This response was particularly evident in treatments where callus-related traits were high but rooting performance remained moderate or low. For instance, Milenio under RH showed the highest relative callus proportion, callus area, and callus dry weight, but this did not correspond to the highest rooting percentage. In contrast, H3 under RH exhibited the highest rooting percentage, despite showing lower callus development than Milenio under the same auxin treatment. These contrasting patterns suggest that, under the evaluated conditions, callus proliferation was not a reliable phenotypic predictor of adventitious rooting success in *Coffea arabica* cuttings.

From a physiological perspective, abundant callus proliferation could be interpreted as a possible alternative developmental sink for carbohydrates, nutrients, and hormonal signals; however, this hypothesis remains speculative because carbon allocation, carbohydrate reserves, endogenous hormone levels, and anatomical development were not directly assessed in the present study. This interpretation is consistent with previous evidence indicating that callus formation and adventitious root formation may involve distinct, although partially overlapping, developmental processes, and that successful rooting requires not only cellular dedifferentiation but also organized redifferentiation into root primordia [14,15,24,25,44]. However, because anatomical sections were not performed in the present study, it was not possible to determine whether roots originated directly from vascular tissues, from callus-associated tissues, or from both. Therefore, the observed callus-without-rooting response should be interpreted as phenotypic evidence of a weak association between callus proliferation and root emergence, rather than as anatomical proof of independent developmental pathways.

Overall, the present study indicates that adventitious rooting in *C. arabica* cuttings was more closely associated with genotype-dependent responsiveness to exogenous auxin than with the extent of callus formation. The superior rooting performance of H3, particularly under RH, supports the need for genotype-specific propagation protocols. Morphophysiological traits provided useful complementary information on cutting status, but they were not considered direct mechanistic evidence of adventitious rooting. These findings highlight the importance of prioritizing rooting performance and callus–root relationships when optimizing auxin-based clonal propagation protocols in coffee.

From an applied perspective, these results provide a framework for improving propagation protocols in coffee through the integration of genotype selection and targeted auxin management. Successful adventitious rooting also supported subsequent plant establishment, as illustrated by a representative rooted cutting maintained up to 240 days after establishment (Figure S1). However, this image is presented only as complementary visual evidence of post-rooting development and was not included in the statistical analyses of rooting traits, which were conducted 120 days after establishment. More broadly, these results suggest that phenotypic rooting competence, rather than visible callus formation alone, should be considered the central criterion for evaluating auxin-based propagation success in woody cuttings [24,25,44].

5. Conclusions

This study shows that adventitious rooting in *Coffea arabica* cuttings is strongly influenced by exogenous auxin application, with the magnitude of the response depending on the evaluated rooting trait. Rooting percentage and number of roots were mainly affected by PGR treatment, whereas the longest root length showed a significant hybrid ×

PGR interaction. Among the evaluated treatments, the combined auxin formulation RH (NAA + IBA) provided the most consistent improvement in rooting responses, particularly for rooting probability and root production.

Importantly, callus formation was not a reliable predictor of rooting success under the evaluated conditions. The weak association between callus-related traits and rooting variables, together with the stronger relationships observed between rooting and gas exchange parameters, suggests a partial phenotypic decoupling between callus proliferation and root emergence. However, anatomical and histological analyses would be required to determine whether callogenesis and root organogenesis are developmentally independent processes in *Coffea arabica* cuttings.

From a practical perspective, these findings support the use of targeted auxin management to improve clonal propagation efficiency in coffee. They also suggest that visible callus formation alone should not be used as a reliable indicator of rooting success and that rooting competence should be evaluated directly through root emergence and root development traits.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/crops6040063/s1>. Figure S1: Representative post-rooting establishment of a rooted *Coffea arabica* cutting at 240 days after establishment; Table S1: Raw observations for rooting status, number of roots, and longest root length of *Coffea arabica* cuttings under hybrid $\times$ PGR combinations; Table S2: Binomial GLM output for rooting percentage; Table S3: Negative binomial GLM output for number of roots; Table S4: Gamma GLM output for longest root length considering only rooted cuttings; Table S5: Summary of Type III likelihood-ratio tests for rooting traits; Table S6: Observed raw summaries of rooting percentage, root number, and longest root length by hybrid $\times$ PGR combination.

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Data Availability Statement: The data supporting the findings of this study are available from the corresponding author upon reasonable request. The dataset used to generate Figure 2, together with the complete statistical output including model summaries, Type III tests, estimated marginal means, pairwise comparisons, and compact letter displays, is provided as Supplementary Materials.

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Conflicts of Interest: The authors declare no conflicts of interest. The commercial rooting formulation Root-Hor[®] was used solely as an experimental treatment. The authors have no financial or non-financial relationship with the manufacturer, distributor, or supplier of this product, and no commercial entity was involved in the study design, data collection, analysis, interpretation, or manuscript preparation.

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