Effect of indole-3-butyric acid and gibberellic acid on rooting and growth of arracacha propagules

Efecto del ácido indol-3-butírico y ácido giberélico en el enraizamiento y crecimiento de propágulos de arracacha

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ABSTRACT

Ex situ conservation of Andean roots requires asexual propagation to maintain germplasm genetic integrity. This study evaluated the effects of phytohormones on adventitious root formation and growth in low-rooting arracacha (Arracacia xanthorrhiza) propagules (accession CUI15010083) from Colombia Germplasm Banks for Food and Agriculture. Indole-3-butyric acid (IBA; 7.5, 15, 1000 or 3000 mg L⁻¹) and gibberellic acid (GA; 50 or 100 mg L⁻¹) were applied at varying immersion times (1, 30 min for IBA; 5 min for GA), with a water-only control. Propagation was assessed across three phases: 1) water-based rooting, 2) acclimatization in soil, and 3) field transplant, measuring vigor, plant height, leaf length, and fresh/dry biomass. High IBA concentrations (1000-3000 mg L⁻¹; 1 min immersion) induced propagule mortality. Lower IBA doses ($\leq 15 \text{ mg L}^{-1}$; 30 min immersion) enhanced root quality, producing propagules with intermediate root category scores, though statistically comparable to controls. GA treatments (≤100 mg L⁻¹; 5 min immersion) showed no biomass differences relative to controls. Low-concentration IBA stimulated early rooting, improving adventitious root development, survival, and subsequent growth. GA primarily promoted cell elongation, confirming its role as a vegetative growth enhancer.

Key words: *Arracacia xanthorrhiza* Bancr., auxins, gibberellins, growth promoters, adventitious roots.

RESUMEN

La conservación ex situ de raíces andinas requiere de propagación asexual para preservar la integridad genética del germoplasma. Este estudio evaluó el efecto de fitohormonas en el enraizamiento y el crecimiento de propágulos de arracacha (Arracacia xanthorrhiza; accesión CUI15010083) con baja capacidad de formación de raíces adventicias, proveniente de los Bancos de Germoplasma para la Alimentación y la Agricultura de Colombia. Se aplicó ácido indol-3-butírico (AIB; 7,5, 15, 1000 o 3000 mg L⁻¹) y ácido giberélico (AG; 50 o 100 mg L⁻¹) en distintos tiempos de inmersión (1 y 30 min para AIB; 5 min para AG), junto con un testigo con agua. La propagación se analizó en tres etapas: 1) enraizamiento en agua, 2) aclimatación en suelo, y 3) trasplante a campo; evaluando vigor, altura de planta, longitud de hojas, y biomasa fresca/seca. Las dosis altas de AIB (1000-3000 mg L⁻¹; 1 min de exposición) causaron mortalidad en los propágulos. Dosis bajas de AIB (≤15 mg L⁻¹; 30 min) mejoraron la calidad del enraizamiento, con mayor cantidad de raíces en categorías intermedias, aunque sin diferencias estadísticas frente al testigo. Tratamientos con AG (≤100 mg L⁻¹; 5 min) no mostraron variaciones en biomasa respecto al testigo. El AIB en bajas concentraciones estimuló el enraizamiento temprano, facilitando la formación de raíces adventicias, la supervivencia y el crecimiento. El AG confirmó su rol como promotor del crecimiento vegetal, principalmente en la elongación celular.

Palabras Clave: *Arracacia xanthorrhiza* Bancr., auxinas, giberelinas, promotores del crecimiento, raíces adventicias.

Introduction

Arracacha (*Arracacia xanthorrhiza* Bancr., Apiaceae), also called "racacha" or "white carrot", is a socioeconomically important crop in Colombian and Andean agriculture due to its high local food value (Heywood, 2014). Colombian production rose from 49,587 to 105,408 t between 2009 and 2020, highlighting its significance for the country's

agriculture (MADR, 2022). The edible storage root, with varied colors of epidermis and pulp (Rincón Rueda *et al.*, 2021), is valued for its culinary versatility, easily digestible starches, and nutrient richness (Pacheco *et al.*, 2020).

For asexual propagation to renew accessions in Agrosavia Germplasm Banks for Food and Agriculture (BGAA), variable rooting potential (RP) has been observed. Materials

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with low RP pose challenges for growth and survival and risk the conservation of genetic resources (Rosero Alpala *et al.*, 2023). To address this, propagules are pre-rooted to ensure nearly 100% field establishment (Santos & Carmo, 1998). Auxins are commonly used to stimulate high-quality root growth, enhancing plant uniformity and agricultural productivity (Pinto-Acero *et al.*, 2012).

Indole-3-butyric acid (IBA), a synthetic auxin insoluble in water, is highly effective in promoting rooting in plant species (Castrillón *et al.*, 2008). However, its low mobility and susceptibility to degradation by light or microbial enzymes limit its use (Othman & Leskovar, 2022). IBA has proven effective in inducing adventitious roots in tree seedlings; optimal doses range from 2000 to 3000 mg L⁻¹ (Román Clemente, 2014). Prolonged exposure to IBA can lead to excessive callus formation that hinders rooting, as in *Prunus* sp. rootstocks (Justamante *et al.*, 2022).

Gibberellins, like gibberellic acid (GA), are endogenous tetracyclic diterpenoid compounds that promote plant growth and development (Othman & Leskovar, 2022; Palma Soto et al., 2022; Peng et al., 2020). They enhance physiological processes such as gas exchange and source-sink relationships (Othman & Leskovar, 2022) and interact with environmental factors like light and temperature to stimulate seed germination and growth across phenological stages (Alcántara Cortés et al., 2019). GA induces stem elongation by stimulating apex cells, increasing cell wall plasticity, so that taller stems show higher gibberellin activity. However, excessive GA can lead to problems like hypertrophy, chlorosis, root rot, and inhibited secondary root formation, and these negatively impacting plant health and growth (Bashyal, 2018; Hernández Rodríguez et al., 2024).

Several factors, such as dosage, variety sensitivity, and plant conditions, are crucial when using rooting and growth promoters. This study aimed to evaluate the rooting and growth response of an arracacha accession from the BGAA

with low rooting potential, that was treated with auxins and gibberellins at varying concentrations and exposure times.

Materials and methods

The study was carried out during the years 2019 and 2020 at the La Selva Research Center in Rionegro, Antioquia, Colombia, located at 6°07'52.7" N, 75°24'51.9" W and an altitude of 2100 m a.s.l. The average temperature of the area is 16°C with a relative air humidity of 74.8%. The life zone is classified as low montane humid forest (bh-MB) (Holdridge, 1978).

Plant material

Propagules from the BGAA accession (CUI) BGVCOL 15010083 collected from 9-month-old mother plants were washed and cut to 1.5-2 cm lengths from the petiole insertion above the third cormel ring. They were disinfected by soaking in a chlorine dioxide (ClO₂) solution (2 ml L⁻¹) for 15 min. This accession exhibits low rhizogenic potential, marked by excessive callus formation and limited root development (Rosero Alpala *et al.*, 2023).

Experimental design and data analysis

The treatments were established from the two hormones used: indole-3-butyric acid with four application doses, gibberellic acid in two concentrations, and the control (water), for a total of seven treatments with four replicates, under a completely randomized experimental design. One experimental unit (EU) is a container with a volume of 354.9 ml containing a propagule, for a total of 28 EUs. The immersion time was adjusted according to the technical data sheet of each product (Tab. 1).

To evaluate the relationship between the ordinal qualitative variables, Kendall's Tau-b test was utilized for the number of equal response levels, and Kendall's Tau-c test for the number of different levels. The V-Cramer test was used to compare ordinal and dichotomous qualitative variables

TABLE 1. Description of treatments with indole-3-butyric acid (IBA) and gibberellic acid (GA) applied to arracacha propagules.

Treatment	Hormone type - Active ingredient	Commercial presentation	Hormone concentration (mg L ⁻¹)	Immersion time (min)
IBA _ 7.5		Suspension	7.5	30
IBA _ 15.0	Auxin - IBA	3000 mg L ⁻¹	15.0	30
IBA _ 1000	AUXIII - IBA	Powder	1000	1
IBA _ 3000		99 %	3000	1
GA _ 50	Gibberellin - GA	Powder	50	5
GA _ 100	Gibbereiiii - GA	1 g 10 g ⁻¹	100	5
Control	Water	-	0	30

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or between dichotomous ones. A cluster analysis was performed at each stage, employing the most important variables for grouping the treatments. Qualitative variables were analyzed with cross tables to study their associations.

Descriptive analyses were conducted on quantitative variables to assess normality (Shapiro-Wilk), homoscedasticity (Levene), and residual independence (Durbin-Watson) at α =0.05. Variables meeting these assumptions were analyzed using parametric tests, with Duncan test for mean comparisons. Nonparametric methods, such as Kruskal-Wallis, were applied otherwise: T3-Dunnett for variables with normality but not homogeneity of variances, and Kruskal-Wallis with Bonferroni correction for those failing all assumptions. Analyses were performed using SAS v. 9.4.

Variables of rooting and growth

During the development of the experiment, three stages were considered in the vegetative propagation process: A) Rooting in water; B) Hardening in soil; and C) Plant growth in field, where the yield components and the harvest of storage roots were evaluated. The variables evaluated in the stages of the vegetative propagation process of

arracacha propagules treated with growth promoters are shown in Table 2.

Water turbidity was assessed through visual observation, based on the presence or absence of yellow coloration in the water. Root abundance was determined using the arracacha (Arracacia xanthorrhiza) rooting variable scale proposed by Rosero Alpala et al. (2023). Basal tissue texture was evaluated manually via tactile analysis, with softening levels classified into four categories: null (0), low (1), medium (2), and high (3). Adventitious root length was measured as the length of the longest root per propagule. Plant vigor was assessed based on leaf physical quality and developmental stage, categorized into three levels: low (1 = short stature, limited leaf development), medium (2 = intermediate stature, moderate development), and high (3 = tall stature, robust leaf development). Canopy diameter was calculated by averaging two perpendicular measurements taken in north-south and east-west orientations. Fresh and dry weights of the crown and storage root (Blas et al., 2008) were quantified using a Fenix-plus analytical balance (Lexus brand). Total yield was expressed in grams per square meter (g m⁻²).

TABLE 2. Variables evaluated in the vegetative propagation process stages of arracacha propagules treated with indole-3-butyric acid and gibberellic acid

Stage	Variable	Variable type	Scale/Unit	
	Survival			
A. Rooting in water (0-22 DAS)	Presence of leaves	Qualitative dichotomous	Yes (1), No (0)	
	Water turbidity			
	Root abundance	Qualitative ordinal	Null (0), low (1), medium (2), high (3)	
	Softening of basal tissues	Qualitative orullar		
	Survival	Qualitative dichotomous	Yes (1), no (0)	
	Number of leaves		Number	
B. Plant hardening in soil (23-49 DAS)	Plant height	Quantitative	cm	
	Root length		mm	
	Plant vigor	Qualitative ordinal	Low (1), medium (2), high (3)	
	Number of leaves		Number	
	Plant height		cm	
	Canopy diameter		cm	
	Leaf length	Quantitative	cm	
	Petiole length		cm	
C. Plant growth in field (50-365 DAS)	Colletotrichum incidence		%	
2. Flant growth in field (30-303 DAS)	Leaf fresh weight	Quannalive	g	
	"Crown" fresh weight (Blas et al., 2008)		g	
	Storage root fresh weight		g	
	Leaf dry matter		g	
	"Crown" dry matter		g	
	Storage root dry matter		g	

DAS: days after sowing

A) Rooting in water

The propagules, in a mesh house, at an average ambient temperature of 18°C, were prepared and rooted in water (Rosero Alpala *et al.*, 2023). They were immersed in hormone solutions for the times recommended by the respective technical data sheet (Tab. 1). To promote the healing of the basal tissues, they were placed at rest and without water for 3 d in aluminum containers with a capacity of 750 ml. Every 3 d, the water was changed for 22 d. In the three stages, the variables presented in Table 2 were measured.

B) Plant hardening in soil

The propagules continued in mesh houses and planted in 236.6 ml plastic containers filled with loamy soil characterized with pH 5.6, 17.2% organic matter, 113.7 mg L⁻¹ phosphorus (P), 8.12 mg L⁻¹ sulfur (SO₄²⁻), 0.17 mg L⁻¹ boron (B), 13.13 cmol kg⁻¹ calcium (Ca), 2.54 cmol kg⁻¹ magnesium (Mg), 0.53 cmol kg⁻¹ potassium (K), and 16.3 cmol kg⁻¹ effective cation exchange capacity (ECEC). The soil was disinfected with chlorine dioxide (ClO₂) at 50 ml L⁻¹. To control soil phytopathogens, a biological fungicide containing *Bacillus* sp., *Trichoderma harzianum*, and *Trichoderma asperellum* was applied three times at 1 ml L⁻¹. Seedlings were watered twice weekly, and aphids were controlled 12 d after sowing using an abamectin-based insecticide at 0.5 ml per 500 ml of water.

C) Plant growth in field

At 50 days after sowing (DAS), propagules were transplanted into open field with 0.4 m spacing between plants and 1.0 m between rows. The soil was pre-treated and disinfected as described earlier. Agronomic management included preventive pest control, targeting beetle larvae ("chizas") with chlorpyrifos insecticide (0.5 ml L⁻¹). Fertilization was applied in three stages: i) at transplant, 2 g/plant of a compound fertilizer (10% N, 30% P₂O₅, 10% K₂O); ii) at 75 DAS, 15 g/plant of a mix containing 8 g of the same compound fertilizer, 3 g KCl, and 4 g of another fertilizer (8% N, 5% P₂O₅, 18% Ca, 6% Mg, 1.6% S, 0.14% Cu, 1% B, 2.5% Zn); and iii) at 225 DAS, 10 g/plant of a mix with 2 g of the compound fertilizer and 8 g KCl. Hilling was done at 90 DAS, and weeds were controlled every 30 d for the first six months. The entire plant was harvested at 365 d for storage roots yield evaluation.

Results and discussion

A) Rooting in water

At 22 d, root abundance, basal tissue softening, presence of leaves, and water turbidity were evaluated. Kendall's Tau-c showed no significant relationship between phytohormone

treatments and root abundance (0.041, P=0.835), basal tissue softening (0.395, P=0.002), or presence of leaves (V-Cramer=0.443, P=0.358). A significant medium relationship was found with water turbidity (V-Cramer=0.72A, P=0.012). Water turbidity showed significant medium associations with basal tissue softening (V-Cramer=0.7626, P=0.002) and root abundance (0.675, P=0.005). All treated propagules in all treatments survived this stage.

Cross-table analysis showed three groups: the first group with the following treatments IBA_7.5, IBA_15, GA_100; the control showed no turbidity or basal tissue softening, promoting root development. In contrast, a second group, those treated with IBA_3000 for 1 min exhibited high basal tissue softening (25%) and no root generation. Propagules treated with GA_50 for 5 min showed 25% with and 75% without basal tissue softening, resulting in low root generation. The third group was more marked in the stage of hardening in soil. Overall, increased basal tissue softening reduced rooting, and turbid water indicated propagule deterioration in hormone-treated groups (Fig. 1A, 1B and 1C).

The delay in root formation is probably due to the formation of a callus, in those treatments with prolonged exposure times, as observed in cuttings of Flordaguard peach *Prunus persica* L. (Justamante *et al.*, 2022). On the other hand, short exposures from 3 s to 2 min to concentrations higher than 500 mg L⁻¹ of IBA in woody species, such as peach, favored root development (Kaur, 2017; Natarajan *et al.*, 2023).

B) Plant hardening in soil

At 28 DAS, propagule survival showed a high and significant association with the treatments used (V-Cramer= 0.849, P=0.003). All propagules reached 100% survival, except those treated with IBA_3000, which had a survival rate of only 25% (Fig. 2).

Plant vigor lacked normality and homogeneity of variance (Shapiro-Wilk and Levene, P<0.05), with a significant treatment effect (Kendall's Tau-c=0.451, P=0.003). High vigor predominated in propagules treated with GA, IBA, and the control, except for those treated with 1000 mg L⁻¹ and 3000 mg L⁻¹ of IBA, which showed low to medium vigor, though differences were not statistically significant (Marginal homogeneity test, P=0.313). A low, inverse association was found between IBA concentrations and vigor (Kendall's Tau-c, r(17) = -0.457, P=0.002, Fig. 2).

Seedling height showed normality (Shapiro-Wilk, P=0.709) but not homoscedasticity (P=0.004) with no significant differences between treatments (P=0.765) and a coefficient of

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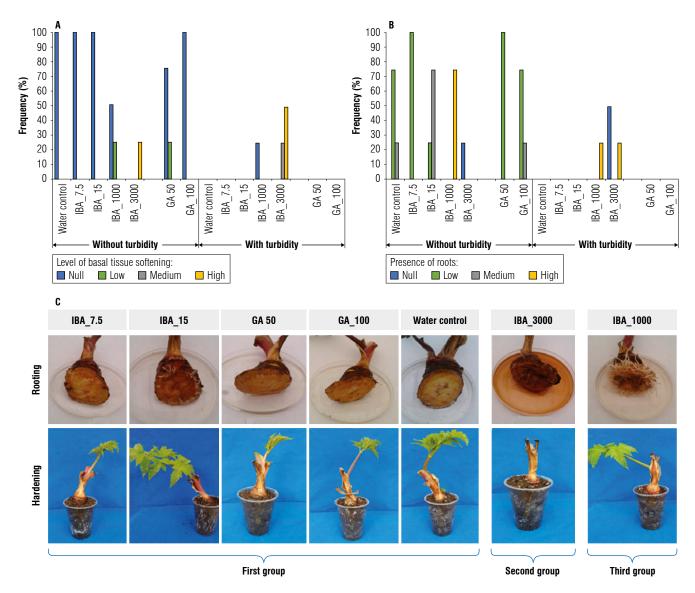


FIGURE 1. Frequencies of the level of basal tissue softening (A), presence of roots (B) and cluster characteristics for the rooting stage (C) in arracacha propagules at the rooting stage treated with indole butyric acid (IBA) and gibberellic acid (GA) in the presence and absence of water turbidity.

variation (CV) of 21.1%. Root length and leaf number did not meet assumptions (P<0.05). Trend curves indicated that height, root length, and leaf number decreased with IBA doses near 1000 mg L⁻¹, which also caused 69% chlorosis, and made up the third grouping of the treatments (Fig. 1B and 3). In contrast, *Passiflora nitida* cuttings treated with 1000 mg L⁻¹ IBA for 5 s showed optimal survival, rooting, and callus formation (Vale *et al.*, 2020).

On the other hand, the variables increased with IBA concentrations lower than 15 mg L⁻¹ and imbibition for 1 min and higher than 2000 mg L⁻¹ with exposure for 30 min. In the treatments with GA, the height increased with concentrations up to 100 mg L⁻¹, imbibed for 5 min

without presenting significant differences with the control (*P*>0.05), while the root length and the number of leaves decreased (Fig. 3). These results could be explained by the effect found in the excessive formation of calluses caused by IBA concentrations that are much lower than those applied in this work. In a work where *Prunus* cuttings were treated with 0.9 mg L⁻¹ with constant incubations of 24 h, the prolonged exposure caused the excessive formation of calluses that limited root development. This work concluded that the short incubation with IBA is sufficient to trigger root initiation (Justamante *et al.*, 2022).

In *Arracacia* sp., 7.5 ml L⁻¹ of IBA and GA applied for 30 min increased root number and length, confirming their

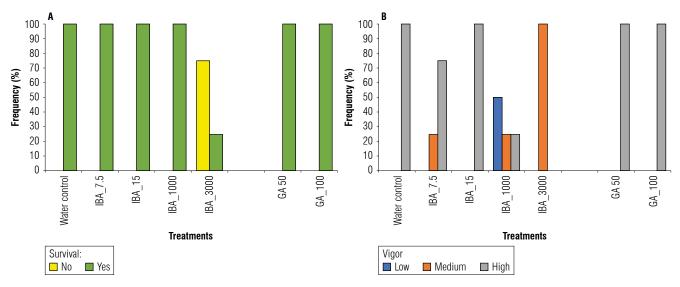


FIGURE 2. Frequencies of survival (A) and vigor (B) of arracacha propagules at the hardening stage treated with indole-3-butyric acid (IBA) and gibberellic acid (GA).

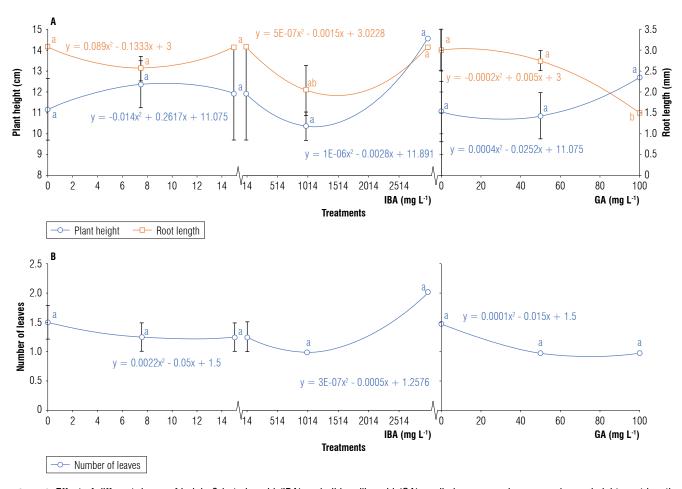


FIGURE 3. Effect of different doses of indole-3-butyric acid (IBA) and gibberellic acid (GA) applied on arracacha propagules on height, root length (A), and number of leaves (B) during hardening in soil. Averages of a series with the same lowercase letter do not show significant differences (For height: T3-Dunnett, for root length and leaf number: Kruskal-Wallis with homogeneous subsets based on asymptotic and Bonferroni significances, Wallis with homogeneous subsets based on asymptotic and Bonferroni significances, >0.05). Mean with standard error (SE) bars with n=4 for treatments.

effectiveness as rooting stimulators (Reghin *et al.*, 2000). Higher doses of 25 and 50 mg L⁻¹ of IBA in *Gossypium barbadense* L. cuttings showed moderate rooting (Rojas-Idrogo *et al.*, 2013), but lower doses in potato (*Solanum tuberosum* L.) shoots treated with 1.0 mg L⁻¹ of IBA registered efficient root production (Sharde *et al.*, 2024). Moreover, rooting was obtained in *Viola odorata* L. using leaves as propagules and treated with 1.5 mg L⁻¹ of IBA (Vilas Haralkar & Raosaheb Biradar, 2020). These publications demonstrate the significant effect of a range of IBA concentrations used in different species in short exposure times.

C) Plant growth in field

At this stage, no significant differences (*P*>0.05) were found between treatments and the evaluated variables. Plant height, canopy, leaf, and petiole lengths met statistical assumptions. Seedlings treated with IBA_1000 and IBA_3000 for 1 min did not survive. *Colletotrichum* incidence was low, averaging 5% in GA_100-treated seedlings soaked for 5 min. Similar results were observed

in arracacha propagules treated with 500 mg L⁻¹ IBA for 20 s, achieving 95% rooting, though no differences from the control (hormone-free) were noted, and higher doses proved phytotoxic (Câmara, 1992).

At 365 DAS, variables such as fresh weight and dry matter of leaves, crown, and storage roots did not meet normality, and crown dry matter lacked homoscedasticity (*P*<0.05). Treatment effects showed no significant differences based on ANOVA and Kruskal-Wallis tests (*P*>0.05). Height, canopy diameter, and lengths of leaves, and petioles varied with hormone type: IBA-treated plants showed a slight increase at 7.5 mg L⁻¹, declining at lower or higher doses, while GA-treated plants exhibited reduced height and length with increasing doses, though not statistically different from the control (Fig. 4).

The results coincide with those found in potato, where treatments were applied with GA doses of 20 mg L⁻¹, lower than those used in the present trial, obtaining the highest

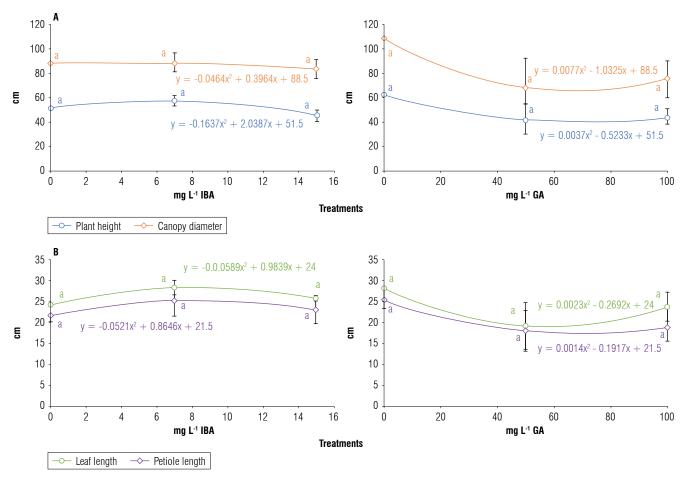


FIGURE 4. Behavior of plant height, canopy diameter (A), and leaf and petiole length (B) during the transplant to the field stage of propagules treated with different doses and exposure times of indole-3-butyric acid (IBA) and gibberellic acid (GA). Averages of a series with the same letter do not show significant statistical differences (Duncan, P > 0.05). Mean standard error (SE) bars with n = 4 for treatments.

root length and leaf area values, while concentrations higher than 300 mg L⁻¹ inhibited their development (Almanza Merchán *et al.*, 2015). It is possible that, given the primary function of GA, which acts on stems elongation, its action on cell growth is not as strong (Almanza Merchán *et al.*, 2015). The responses of different doses to stimulate root development were not significant concerning the control treatment with only water.

The effect on stem elongation was evident in tomato seeds evaluated with gibberellins concentrations that inhibited root growth to a certain degree and where the growth response was produced by low concentrations of gibberellin, together with a longer imbibition time (Balaguera-López *et al.*, 2009). Plants from seeds treated with 300 mg L⁻¹ for 36 h showed the highest increases in leaf area, fresh leaf, rootstock mass, root length, and vigor. The lowest response occurred with the 18 h imbibition treatment and 600 mg L⁻¹ of GA. They also found that the GA concentrations

evaluated (300, 600, and 900 mg L⁻¹) inhibited root growth to a certain degree and that the growth response was due to low GA concentrations and a longer imbibition time (36 h) (Balaguera-López *et al.*, 2009).

The fresh weight of the leaf, crown, and storage root showed the same tendency when increasing the dose of the GA and IBA promoters; the correlations were positive and higher than Pearson r=0.89. Leaf and crown height tended to decrease when increasing the dose, while in IBA, the weight of the storage root increased in plants treated with IBA_7.5 exposed for 30 min but decreased in IBA_15 with the same imbibition time without presenting statistical differences (*P*>0.05, Fig. 5A). In cuttings of herbaceous species such as *Mentha spicata* L. immersed for 0, 10, and 20 min in a solution of 500 mg L⁻¹ of IBA, the number of roots increased by prolonging the immersion time to 20 min (Ferraz *et al.*, 2018).

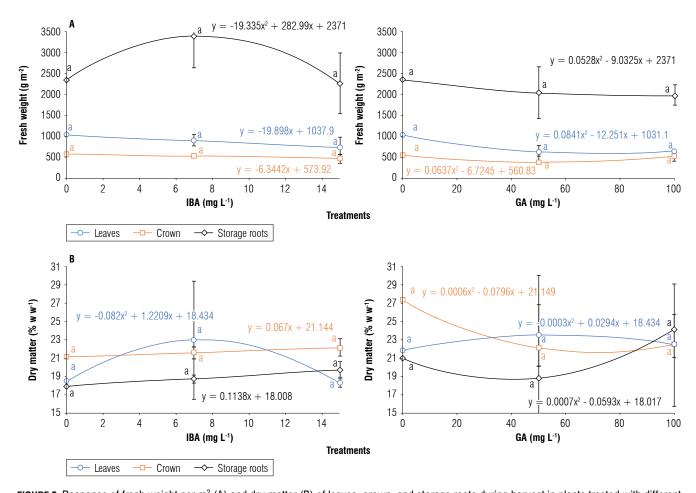


FIGURE 5. Response of fresh weight per m^2 (A) and dry matter (B) of leaves, crown, and storage roots during harvest in plants treated with different doses of indole-3-butyric acid (IBA) and gibberellic acid (GA). Averages with the same letter do not present significant statistical differences (Duncan, P > 0.05 for fresh weight and Kruskal-Wallis with homogeneous subsets based on asymptotic significances and Bonferroni, P > 0.05 for dry matter). The bars of the averages represent the standard error (SE) with n = 4 for treatments.

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Dry matter changed according to the hormone used, although there were no significant differences (P>0.05). With the GA treatments, irregular trends were registered in the storage root. The dry matter of leaves and storage roots decreased in plants treated with GA_50 and increased slightly with doses of GA_100. In plants treated with IBA, the dry matter of crown and storage roots had a slight increase with increasing doses, but in leaves it increased up to IBA_7.5 and decreased with a dose of IBA_15 without presenting statistical differences (P>0.05, Fig. 5B).

Conclusions

Treatment with indole-3-butyric acid (IBA) at low concentrations and varying exposure times stimulated early rooting of arracacha propagules; this facilitated adventitious root formation, survival and growth.

The primary role of gibberellic acid as a plant growth promoter was confirmed, particularly its involvement in cell elongation. However, since its most pronounced effects are observed through exogenous applications, such as foliar spraying, no significant differences in biomass production were detected between the treated plants and the control group.

It is important to continue researching the sensitivity of the concentrations of the IBA and GA promoters and the exposure times in non-woody species, such as arracacha, to improve the response in root and biomass development.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

MGRA contributed to the study of conception and design. MGRA and CEVA performed the material preparation and data collection. JLF performed the analysis. MGRA, CEVA, JLF and JPGM wrote the first draft of the manuscript, and all the authors commented on the previous versions of the manuscript. All authors have read and approved the final version of the manuscript.

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