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RESEARCH

Effects of nanoparticles on shoot formation of cherimoya (*Annona cherimola* Mill.) from hypocotyl explants

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Abstract

Cherimoya (*Annona cherimola* Mill.) is a subtropical fruit tree cultivated in southern Spain, the world's leading producer, where 'Fino de Jete' is the predominant cultivar. In this study, shoot regeneration from hypocotyl explants of this cultivar was investigated. Two explant types—1 cm-long longitudinally split pieces (LSP) and 0.3 cm cross sections (CS)—along with different culture media and antioxidant treatments, were evaluated. For LSP explants, a modified MS medium (mMS) supplemented with 0.3 mg/L 6-benzyladenine (BA) resulted in shoot formation in 66% of explants, with an average shoot length of 1.2 cm. CS explants cultured on mMS-0.3 BA medium supplemented with 100 mg/L casein hydrolysate showed an average shoot production rate of 34% and a mean shoot length of 0.9 cm. This medium was also effective for CS explants of the 'Campas' and 'Alborán' cultivars, achieving similar regeneration success to 'Fino de Jete'. Additionally, the effect of carbon-dots nanoparticles (CDs; 0 to 0.8 mg/L) and silver nanoparticles (AgNPs; 0 to 0.3 mg/L), on shoot production using CS explants was evaluated. CDs at 0.6 mg L^{-1} eliminated explant necrosis (0%) and promoted earlier shoot formation (76%) after 8 weeks. AgNPs at 0.2 mg L^{-1} significantly enhanced shoot regeneration, particularly at 12 weeks, increasing bud regeneration (91%), shoot proliferation (73.7%), and shoot elongation (2.5 cm). Rooting was achieved in control shoots (up to 40%), and acclimatization reached 100%; however, further optimization of the rooting process in regenerated material is required. The use of nanoparticles will be considered in future studies focusing on root induction and the development of genetic transformation protocols in cherimoya.

Key message

A protocol for cherimoya shoot regeneration from hypocotyl explants was developed applicable to different cultivars. Carbon dots and silver nanoparticles significantly enhanced shoot induction and development.

Keywords Cherimoya · Hypocotyl sections · Shoot formation · Carbon-dots nanoparticles · Silver nanoparticles

Introduction

Cherimoya, *Annona cherimola* Mill., is a subtropical fruit tree species of Andean origin well adapted to the subtropical conditions of southern Spain. In fact, Spain is the world's main producer of cherimoya fruit, with around 95% of the cultivated cherimoya based on the cultivar 'Fino de Jete' and 5% on the cultivar Campas. The consumption of cherimoya fruit and its export are limited mainly by the fast fruit ripening, the low resistance of fruit skin, and the high fruit seed number (Farré et al. 1999). Alboran, a new cultivar with low seed rate and high flower density, does not solve the main problems of this crop. In vitro culture is a biotechnological tool that could be of great interest to develop new and improved genotypes of cherimoya. In this sense, Encina

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et al. (1994) described the first procedure to propagate *A. cherimola* juvenile plants through in vitro culture. Later, a protocol for the in vitro seed germination was established (Padilla and Encina 2003) and selected adult genotypes of Fino de Jete and other cultivars were micropropagated (Padilla and Encina 2004, 2011). To address current challenges in cherimoya cultivation, an effective regeneration protocol for this species is needed. Jordan (1988) explored the formation of buds and callus from hypocotyl explants from seeds of the Chilean cultivar Concha Lisa but shoot production or shoot rooting was not achieved. More recently, Encina and Regalado (2026) used sections of 1.5 cm hypocotyls from Fino de Jete cv. to regenerate autotetraploid shoots following a colchicine treatment in order to address the high seed rate of cherimoya fruit. Successful rooting and greenhouse acclimatization of regenerated shoots was achieved. However, the process of shoot regeneration from hypocotyl explants in cherimoya is not well optimized, with low and slow shoot regeneration and, therefore, there is no suitable regeneration protocol available for the application of other biotechnological tools, such as gene transformation/editing. For all these reasons, in this work the effects of explant size, growth regulators and antioxidant compounds (e.g., PVP and casein hydrolysate) on shoot regeneration have been evaluated.

Nanoparticles (NPs), on the other hand, are significantly and favorably affecting in vitro plant culture. In this regard, silver nanoparticles (AgNPs) are among the most well-known nanomaterials and have been used in a variety of in vitro culture methods (Mahajan et al. 2022), with a demonstrated positive effect in micropropagation, e.g. explants disinfection (Abdolinejad et al. 2020; Khafri et al. 2022; Shaafi et al. 2022), shoot multiplication and rooting (Hegazi et al. 2021; Korpayev et al. 2021; Elsayh et al. 2022; Farrokhzad et al. 2022; Khattab et al. 2022; Lai et al. 2022; Phong et al. 2022; Sarmast and Salehi 2022; Tejada-Alvarado et al. 2022), and plantlet acclimatization (Ung et al. 2022). More precisely, several studies have documented the beneficial effects of AgNps on callus regeneration and shoot development, and the mechanism through which AgNps promote shoot regeneration has been determined (Saha and Dutta Gupta 2018; Jadczak et al. 2019; Malik et al. 2021; Phong et al. 2022; Baltazar Bernal et al. 2023). Carbon dots (CDs) are a type of nanoparticle notable for being low in toxicity, highly soluble in water, and easily absorbed by plants (Li et al. 2023). They can promote plants growth in vivo, enhance photosynthetic efficiency through improved light conversion and nutrient delivery, promote coordinated regulation of nutrient uptake and photosynthesis and activate plants defense mechanisms (Zhang et al. 2012; Qian et al. 2018; Wang et al. 2018; Kou et al. 2021; Cheng et al. 2025; Hu et al. 2025). Additionally, CDs have been widely used as gene carriers both in vivo (Schwartz et al. 2020; Wang et al. 2020; Zarrabi et al. 2024), and in vitro (Lew et al. 2018; Campos et al. 2021). Although CDs have been scarcely explored in plant tissue culture, and up to our knowledge, no reports have addressed their effects on shoot regeneration, a few studies have investigated other carbon-based nanoparticles, such as nanocarbon and carbon nanotubes, showing different effects on callus formation and shoot regeneration (Kokina et al. 2012; Chutipaijit and Sutjaritvorakul 2018). In order to facilitate future genetic modification and editing techniques, the primary goal of this work was to develop an optimized protocol for cherimoya shoot regeneration. Additionally, the potential of silver nanoparticles (AgNPs) and carbon dots (CDs) to enhance shoot formation in cherimoya hypocotyl explants was explored.

Materials and methods

Plant material and explant preparation

Annona cherimola seeds from cv. Fino de Jete, Campas and Alboran hand pollinated trees, were used as explant sources. Hypocotyl pieces from in vitro germinated healthy seedlings were selected for the shoot regeneration experiments. Two sizes of explants were used: (1) 1 cm long section split into two pieces (LSP) and placed with the cut surface on the medium, and (2) 0.3 cm cross sections (CS) with one of the cut surfaces on the medium (Fig. 1). To guarantee that every explant had the same dimensions, a paper millimeter ruler was positioned beneath the plate and used to cut the explants.

Culture media and culture conditions

Seeds were germinated in vitro following the protocol described by Padilla and Encina (2003) with modifications. Briefly, intact seeds were disinfected in a 2% sodium hypochlorite solution for 60 min, soaked for 24 h in sterile distilled water and disinfected again in a 1% sodium hypochlorite solution for 60 min, rinsing three times in distilled water, 5 min each. The seeds were incubated on paper bridge in liquid germination medium (Padilla and Encina 2003), which consisted of WPM vitamins (Lloyd and McCown 1981; glycine- 2.00 mg/L, nicotinic acid-0.50 mg/L, pyridoxine HCl-0.50 mg/L, thiamine HCl − 1.00 mg/L), 100 mg/L myo-inositol, 30 g L^{-1} sucrose, and 3 mg/L gibberellic acid (GA_3). The pH of medium was adjusted to 5.74 and 15 mL of medium was dispensed in 50 ml test tube covered with polypropylene caps and autoclaved for 15 min at 121 °C and 1.05 kg cm-2. One intact seed was cultured per tube and placed in an incubator at 30 ± 1 °C in the dark.

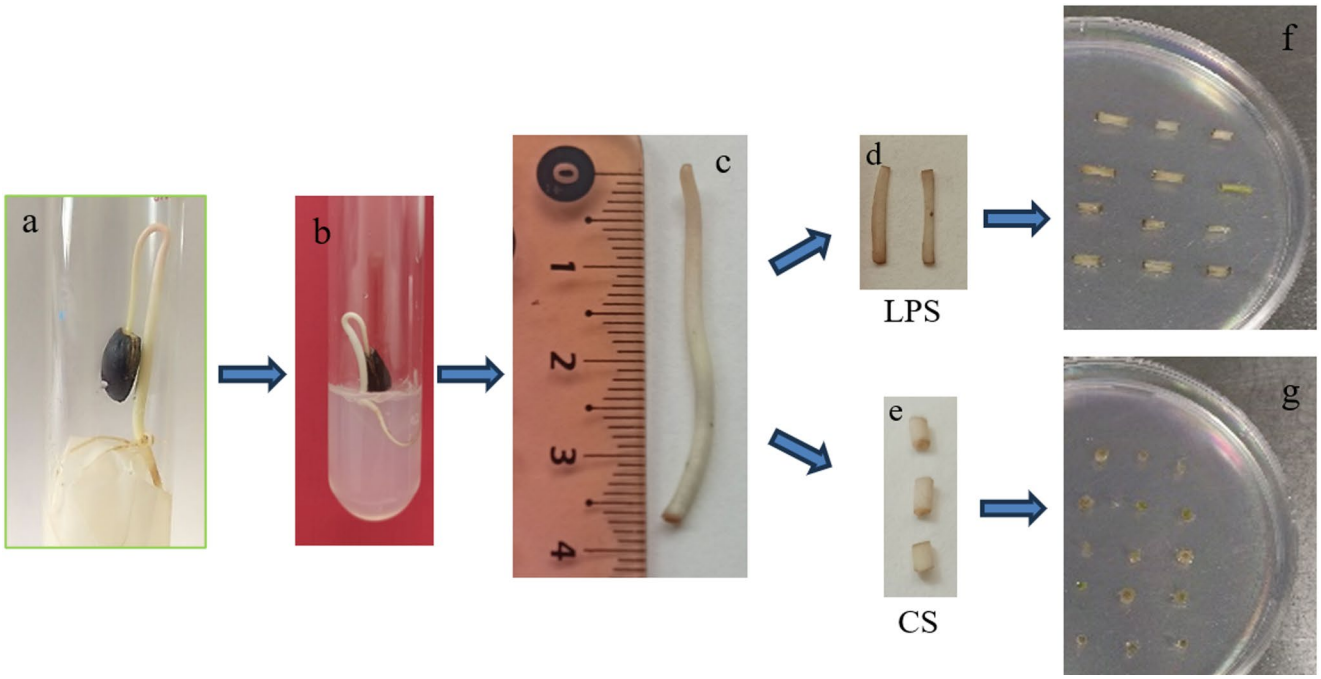


Fig. 1 Explant preparation. Germinated cherimoya seeds (**A**) were transferred to mMS* solid medium (**B**) to check for endogenous bacteria absence. The hypocotyl was separated from the seed (**C**) and cut into 2 types of explants: 1 cm long section split into two pieces (LSP: **D**) and placed with the cut surface on the medium (**F**), and 0.3 cm cross sections (CS; **e**) with one of the cut surfaces on the medium (**G**). *mMS: MS salt, WPM vitamins, 8764 mM (30 g/L) sucrose, and 0.6% of agar

Forty-two-day-old seedlings were transferred to modified MS (mMS) medium consisting of full-strength MS salt (Murashige and Skoog 1962), WPM vitamins, 30 g/L sucrose, and 0.6% of agar. The pH of medium was adjusted to 5.74 and 15 mL of medium was dispensed in 50 ml test tube covered with polypropylene caps and autoclaved for 15 min at 121 °C and 1.05 k/cm. One seedling was cultured per tube and placed in the culture room at 25 ± 1 °C in the dark to rule out endogenous contamination by bacteria in the seedlings.

In regeneration experiments, regeneration medium (RM) consists of mMS medium with 0.3 mg/L N^6-benzyladenine (BA) and 100 mg/L casein hydrolysate (CH), unless otherwise indicated. The pH of media was adjusted to 5.74 and autoclaved for 20 min at 121 °C and 1.05 kg cm^{-2}. Twenty-five ml of medium was dispensed in 9 cm diameter Petri dish with a sterile single-use pipette inside the flow hood. All cultures were incubated in the culture room at standard conditions, that is, at 25 ± 1 °C under a 16-h day photoperiod with a light intensity of 45 µmol m^{-2} s^{-1} (400–700 nm) photosynthetically active radiation (PAR). The explants were cultured in standard Petri dish and subcultured to fresh medium in double-width Petri dish every 4 weeks. In each regeneration experiment, explants from 4 to 7 different seeds were used, so that the explants from different seeds were distributed equally among the different treatments to compensate for the variability in regeneration among the seeds.

Effect of explant size, culture media and antioxidant compounds on shoot regeneration

In a preliminary experiment, different salt formulations and cytokinins were tested using Fino de Jete seed-derived 2 cm hypocotyl sections. Bud proliferation was observed even in MS medium without growth regulators. In these explants, mMS medium plus 0.5 mg/L zeatin or plus 0.3 mg/L BA yielded the best results, with similar regeneration rate (100% and 90%, respectively) and percentage of explants with shoots ≥ 0.5 (3.4 and 4.4 respectively), so BA was selected because of its lowest cost. Then, different regeneration experiments were carried out with LSP and CS Fino de Jete cv. seed-derived hypocotyl explants, and mMS medium with 0.3 mg/L BA, and the medium described by Jordan (1988), consisting in MS supplemented with 2 mg/L BA plus 0.5 mg/L NAA, were tested. Additionally, the effect of PVP (1 g L^{-1}) and CH (100 mg/L) (Jordan et al. 1988) on the regeneration of buds and shoots and necrosis of explants was studied. Each experiment was carried out three times using a total of sixty explants per treatment. All cultures were incubated in the growth room at standard conditions, as previously indicated. Data on percentage regeneration, number of buds and regenerated shoots per explant, shoot

length, and callus were collected after four, eight and twelve weeks. A stereomicroscope (Leica Microsystems, Germany) was used to visually the buds and evaluate the callus production in the explants. The callus production was categorized on a scale of 0 to 5, where 0 denotes the absence of callus and 5 denotes an explant covered with callus (Fig. 2). All of the experiments detailed in this work were conducted using this standard.

Effect of cultivar on shoot regeneration

In this set of experiments, we tested RM and hypocotyls CS explants from Campas and Alboran healthy seedlings, with Fino de Jete explants as control. Seeds from Campas and Alboran cv. trees were germinated as previously described for Fino de Jete seeds. Two repetitions, with a total of fifty explants per treatment, were carried out. All cultures were incubated in the culture room at standard conditions, as previously indicated. Data on regeneration, number of buds and regenerated shoots per explant, shoot length, callus and necrosis were collected after eight and twelve weeks.

Synthesis of nanoparticles (NPs)

Two kinds of NPs were tested in the shoot regeneration process, silver NPs (AgNPs) and carbon-dots NPs (CDs). Synthesis of AgNP was performed as previously described by Mondéjar et al. (2022) with different modifications; 10 mL of 25 mM AgNO3 was added dropwise at flow rate of 2 mL min-1 into 10 mL of *A. cherimola* aqueous extract under vigorous stirring. The suspension was kept by continuously stirring under white light, monitoring color change (whitish to dark brown solution) of the suspension over time. The AgNPs were collected after centrifugation at 15,000 g for 15 min at 4 °C and washed several times with MilliQ water and freeze dried at -40 °C. Carbon dots (CDs) were synthesized using a hydrothermal-pyrolysis method as described by Delgado-Martín et al. (2022). Briefly, a mixture of glucose (2 g) and branched polyethyleneimine 2000 MW (2 mL) in 20 mL of MilliQ water was subjected to hydrothermal pyrolysis at 180 °C for 6 h. Following pyrolysis, the crude product was purified through filtration using a 0.2 µm filter, followed by dialysis against deionized water using a membrane with a molecular weight cut-off (MWCO) of 1 kDa to remove unreacted precursors and smaller byproducts. The purified CD suspension was stored at room temperature. High resolution electron microscope images were obtained on a Jeol JEM 210 TEM microscope operating at 200 kV and equipped with an Oxford Link EDS detector. The resulting images were analyzed using Digital Micrograph™ software from Gatan.

Effect of NPs in shoot regeneration

In these experiments, CS hypocotyls explants were used. Different concentrations of NPs were tested: CDs (0, 0.01, 0.1, 0.2, 0.4, 0.6 and 0.8 mg/L) and AgNPs (0, 0.1, 0.2, 0.3 mg/L). The NPs were added as an additional component to RM at the needed concentration prior to being autoclaved as previously indicated. All cultures were incubated in the culture room at standard conditions. Each experiment was carried out three times, using a total of 75 explants for each treatment. The explants

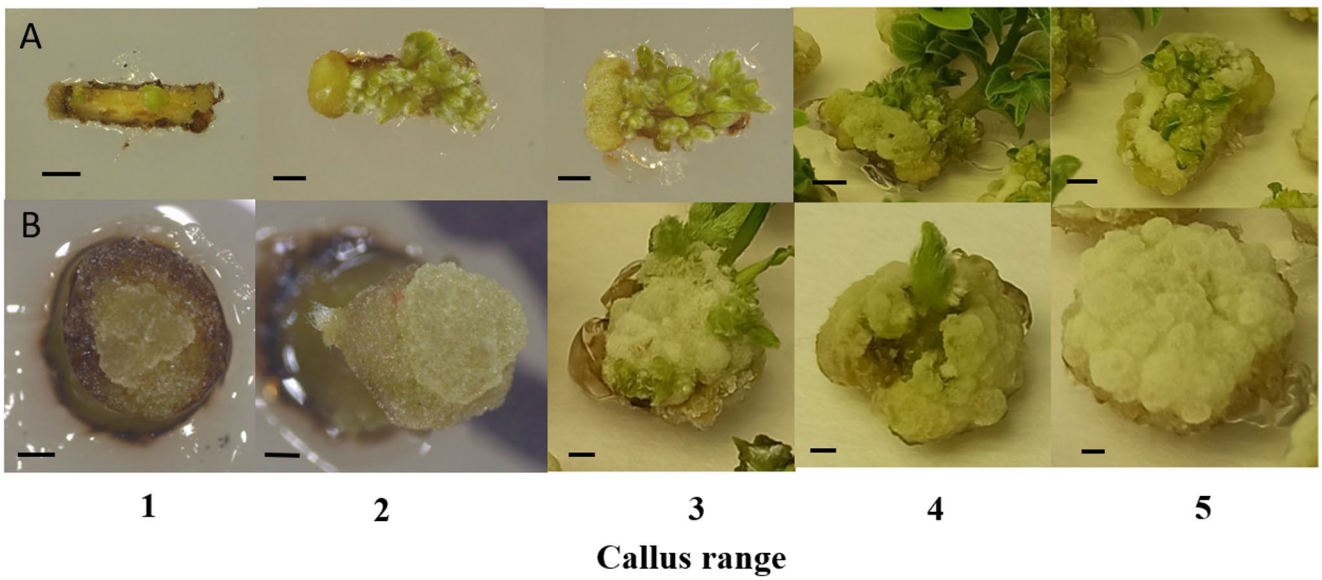


Fig. 2 Standardization of the callus present in LPS (**A**) and CS (**B**) hypocotyl explants. Scale bars represent 5 mm in LPS explants and 1 mm in CS explants

were maintained in RM with NPs for 12 weeks, with subcultures to fresh medium every 4 weeks. Control explants were maintained in RM medium without NPs. Data on percentage regeneration, number of buds, regenerated shoots per explant, shoot length, callus and necrosis were collected after eight and twelve weeks.

Rooting and acclimatization of regenerated shoots

Regenerated shoots in culture for 1 to 4 subcultures in mMS 0.15 mg/L BA medium were rooted according to the method described by Encina et al. (1994). Thus, shoots were first incubated for 3 days in light on mMS medium supplemented with 1 g/L activated charcoal and 20 g/L sucrose, then cultured for 10 days (7 dark / 3 light) on mMS medium containing 15 g/L sucrose, 200 mg/L citric acid and 100 mg/L IBA. Finally, shoots were transferred to root elongation medium, containing mMS with half-strength MS macroelements and 20 g/L sucrose. Rooted shoots were acclimatized under greenhouse conditions as follow: The shoots were removed from the tubes and washed with tap water to remove any remaining agar. Then, they were planted in 5 × 5 cm Jiffy pellets filled with a substrate consisting of peat, coconut fiber, perlite (9.5:1.5:1) and 0.83 mg/L of Osmocote Exact Mini (AICL Growing Solutions Iberia, Barcelona, Spain). The jiffy pellets and the substrate were pre-moistened and placed on moist perlite in a tray. The plants were maintained in a tunnel covered with plastic for 1 month, so that the humidity was above 90%. After that, the plastic of the tunnel was partially opened for one month, and fully opened later, allowing the plants to adapt to the ambient humidity. The plants were watered indirectly by adding water to the perlite in the tray so that the jiffies were always moist. Data on in vitro rooting and acclimatization survival rate was recorded after 4 weeks and for 3 months respectively.

Statistical analysis

SPSS/PC+ program (version 15.0, IBM Corp., Armonk, NY, USA) was used to do statistical analysis. Normally distributed variables were analyzed by analysis of variance (one-way ANOVA), and where significant differences were found, the values were compared according to Student-Newman-Keuls test. Variables expressed in percentages were analyzed by the χ^2 test.

Results and discussion

Characteristics of the nanoparticles used in this work

The AgNPs displayed a nanoparticle size of 77 nm and 0.35 of PDI confirming their uniform distribution and nano-scale size. These values were similar to those obtained in the synthesis of silver nanoparticles with extracts of other plant species or algae, which range from 1 to 2 nm to 95 nm depending on the plant part and species with which the NPs are synthesized (Dhaka et al. 2023). On the other hand, the biogenic nanoparticles displayed a negative surface charge with values close to -27 mV, confirming the well colloidal stability in aqueous suspensions (Jos et al. 2021). The TEM analysis confirms the spherical structure of biogenic nanoparticles with higher electro density corresponding to silver composition (Fig. 3A). The nanoparticles displayed a size distribution from 8 to 40 nm with rock-like shape, as observed in other biogenic silver nanoparticles using different plant extracts, such as *Iris tuberosa* (Mondéjar-López et al. 2021), wheat (Mondéjar-López et al. 2023), and other plant extracts (Bedlovicova et al. 2020). In contrast, the CDs exhibited a positive surface charge of approximately + 14.6 mV, 0.39 of PDI and a hydrodynamic diameter of approximately 6.5 nm, as determined by DLS analysis, while transmission electron microscopy (TEM) confirmed particle sizes around 5 nm (Fig. 3B).

Effect of explant size, culture media and antioxidant compounds on shoot regeneration

The main objective of this work was to develop a protocol to obtain shoot regeneration in cherimoya using Fino de Jete seed-derived hypocotyl explants, suitable for the development of improved varieties through genetic modification. Thus, the medium described by Jordan (1988), consisted of MS medium with 2 mg/L BA plus 0.5 mg/L NAA, and a medium derived from previous works, consisted of mMS medium containing 0.3 mg/L BA, in both LSP and CS explants were tested. In addition, antioxidants PVP (1 g/L) and CH (100 mg/L) (Jordan 1988), in both media and kind of explant were tested. The regeneration and shoot formation in LSP and CS hypocotyl explants from 4 to 12 weeks was examined based on published research on cherimoya hypocotyl regeneration (Jordan 1988). These authors claim to be able to find shoots at 4 weeks, but we were only able to see buds at this point and no shoots. Shoots appeared in our assays at 8 weeks, and there were some noticeable variations. It wasn't until 12 weeks that all of the variables under

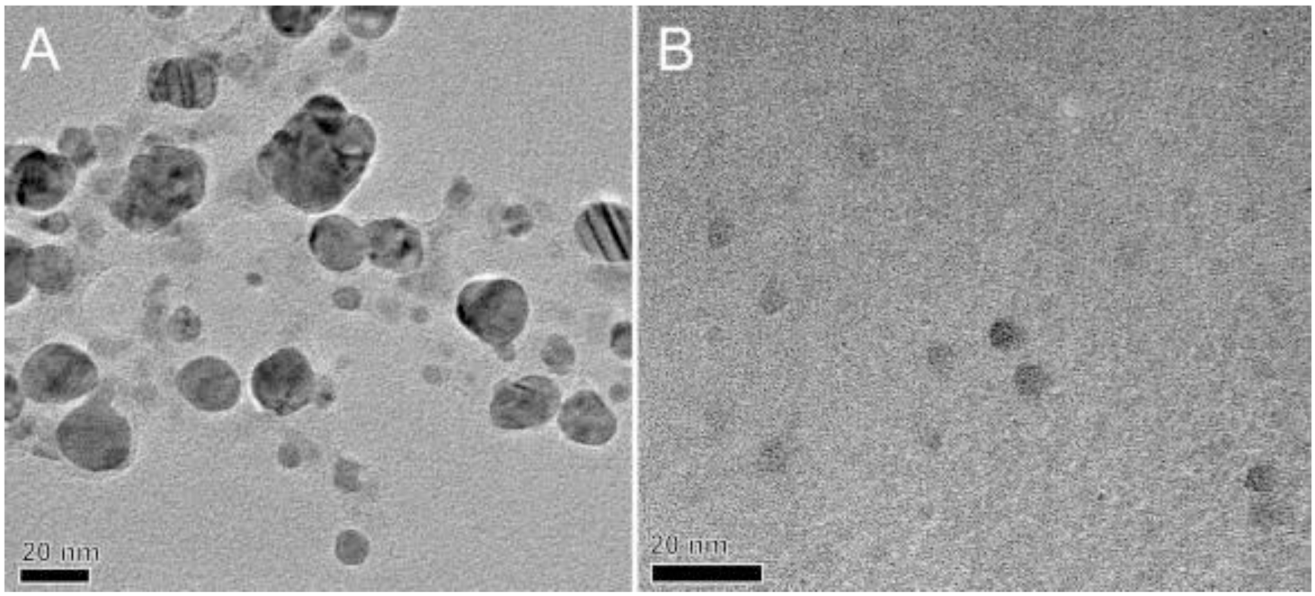


Fig. 3 TEM micrographs of biogenic AgNPs (**A**) and CDs (**B**)

Table 1 Effects of the type of explant, culture media and antioxidants on cherimoya hypocotyl explants shoot regeneration from Fino de Jete cv. seedlings after 12 weeks

Explant	Media-Treatment[a]	No. of explants	Regeneration (%)	Explants with shoots (%)	Shoot length (cm)
Cross Section	mMS 0.3 BA-Control	60	55 ± 4ab	16 ± 6 bc	1.4 ± 0.2 a
	mMS 0.3 BA-1PVP	60	18 ± 3b	29 ± 6 ab	1 ± 0.2 a
	mMS 0.3 BA-100CH	60	70 ± 1a	34 ± 3 a	0.9 ± 0.1a
	MS 2 BA 0.5 NAA-Control	60	39 ± 3ab	9 ± 8c	0.8 ± 0.1 a
	MS 2 BA 0.5 NAA-1PVP	60	21 ± 4b	5 ± 6c	2.0 ± 1.1 a
	MS 2 BA 0.5 NAA − 100CH	60	17 ± 4b	2 ± 4c	1.3 ± 0.3 a
Long Split Pieces	mMS 0.3 BA-Control	60	94 ± 1ab	66 ± 1ab	1.2 ± 0.1 ab
	mMS 0.3 BA-1PVP	60	53 ± 3 d	70 ± 1 ab	0.9 ± 0.6 bc
	mMS 0.3 BA-100CH	60	84 ± 1 cd	53 ± 1ab	1.1 ± 0.9 abc
	MS 2 BA 0.5 NAA -Control	60	86 ± 1 bc	72 ± 2 ab	1.3 ± 0.9 a
	MS 2 BA 0.5 NAA − 1PVP	60	98 ± 2 a	75 ± 2 a	0.8 ± 0.4 c
	MS 2 BA 0.5 NAA − 100CH	60	74 ± 2 c	47 ± 2 b	1.1 ± 0.1 abc

[a]mMS medium: full-strength MS salt, WPM vitamins, 30 g/l sucrose, and 0.6% of agar. The numbers in front of the growth regulators BA and NAA are their concentrations expressed in mg/L. 1PVP: Polyvinylpyrrolidone 1 g/L; 100CH: casein hydrolysate 100 mg/L. Values represent media ± SE. For each kind of explant values with different letters mean statistically significant according to the χ^2 test (regeneration and explants with shoots) or Student-Newman-Keuls ($p < 0.05$) (Shoot length)

investigation could be compared between the treatments. As a result, only 12 weeks' worth of data are shown.

Our findings revealed variations between the tested media and the different kinds of explants. Significantly higher percentages of regeneration were found when LSP explants were used compared to CS explants (Table 1). In the case of LSP, due to the explant being cut into two pieces and placed on medium, a callus was observed around the explant from which some buds emerged; however, most of the buds grew on the surface of the hypocotyls (see Fig. 4A), and it was observed in many explants that once a bud develops a shoot, this prevents the development of the others. Thus, LSP has a larger epidermis area for bud regeneration than CS. In fact, in cross sections, many buds are seen to come mainly from the cut area (see Fig. 4C), as has also been described in cherimoya cv. Concha Lisa by Jordan (1988) and in *A. muricata* (Bejoy and Hariharan 1992). Therefore, a different origin of buds in both explants was observed, which could be relevant when using one explant or another for *Agrobacterium*-mediated genetic transformation, since it is known that this bacterium only infects via wounds (Gelvin 2003), meaning the sections of 0.3 cm are probably a better choice for genetic transformation of cherimoya in the future.

Regarding the media tested, in LSP explants, best results were obtained with mMS-0.3BA (94% regeneration, and 66% of explants having shoots) and with medium

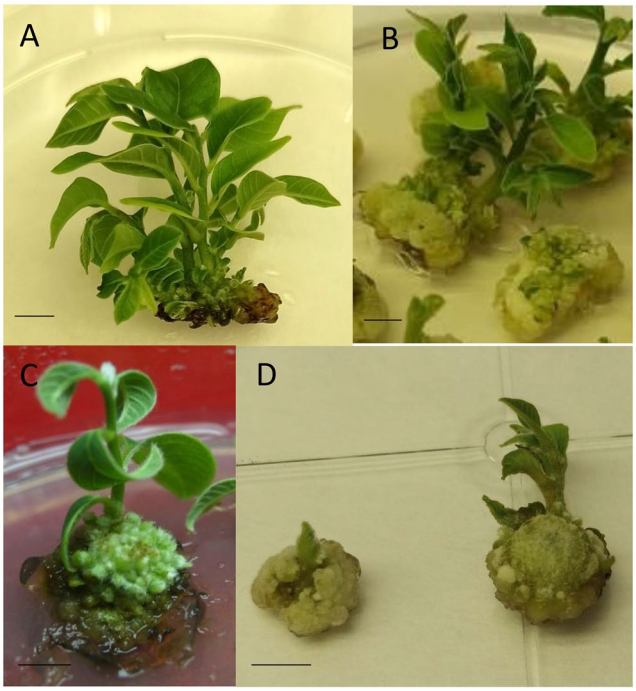


Fig. 4 Shoot regeneration from hypocotyl explants of cherimoya after 12 weeks in culture. Explants from Fino de Jete seeds. LPS (**A**) and CS (**B**) explants on RM. LSP (**C**) and CS (**D**) explants on MS-2BA-0.5NAA (Jordan 1988). RM: mMS-0.3BA-100 CH. Scale bar represents 5 mm in LPS explants and 1 mm in CS explants

MS-2BA-0.5NAA-1PVP (98% regeneration and 75% of explants having shoots), without significant differences between them. However, the average length of the shoots in the first medium was significantly higher (1.2 cm) than in the second (0.8 cm) (Table 1). In the case of CS explants, significant differences were found, with better results obtained when mMS-0.3BA-100CH was used, with 70% regeneration and 34% of explants producing shoot with an average height of 0.9 cm (Table 1). A positive effect of PVP was observed in LPS explants when medium was MS-2BA-0.5NAA, while in the case of CS explants, casein hydrolysate showed a better effect, mainly when the medium was mMS-0.3BA. In summary, a high regenerative capacity in both explants was observed, with rapid bud formation directly at certain positions of the epidermis, without intermediate callus formation. This high regenerative capacity in cherimoya hypocotyl explants could be a natural mechanism in this species, given that during germination, the cotyledons and epicotyl remain inside the seed, hanging (Fig. 1A), and can easily break, leaving the seed without an apical bud. On the other hand, no exudates and little browning on any kind of explant or medium tested was found, contrary to that described by Jordan (1988), and good aspect of buds and shoots was observed (Fig. 4A-C), which probably indicates genotype differences between cultivars.

Significant callus proliferation was observed during the regeneration process in both explants when the medium was MS-2BA-0.5NAA medium, with no differential effect of the antioxidants on the callogenesis. In fact, callus proliferation of ranks 4 and 5 was only seen in media that contained 2BA-0.5 NAA. Thus, in MS-0.3BA media and in both types of explants, 62% of the explants showed callus range 2. However, in CS explants cultures on MS-2BA-0.5NAA medium, 55% of explants showed callus 4, while in LPS explants most of the explant showed callus 3 (35%) and callus 4 (40%), and even 25% of them showed callus 5. Jordan (1988) described callus proliferation at the base of CS explants, which decreased when different antioxidants were added to the medium. In our case, a huge amount of very white and hard callus tissue in both explants tested was found when the medium was MS-2BA-0.5NAA, which, in the case of CS explants, covered the whole explant (see Figs. 3D and 4B), preventing bud formation and proliferation, despite the addition of PVP or CH (Table 1).

In *Annona cherimola* x *A. squamosa* (atemoya) cv. African Pride (Rasai et al. 1994) and *A. squamosa* (Lemos and Blake 1996b), no NAA was needed for bud and shoot formation, while in *A. muricata* NAA was essential (Bejoy and Hariharan 1992; Lemos and Blake 1996a), indicating differences between both species and cultivars. Regardless of these differences in regeneration capacity between cultivars and species, widely described in the literature, variability in the in vitro regeneration capacity of the explant's regeneration using seeds with different ages was also observed, thus, explants from freshly harvested seeds, showed higher regeneration rate. This could be related to the germination capacity of the seeds, where we observed that seed germination rate fluctuated from 60 to 90%, with an effect of seed storage time (Padilla and Encina 2003).

The findings from this series of studies have improved our knowledge of the cherimoya explant shoot regeneration process. A high regenerative capacity of cherimoya hypocotyls was confirmed, with bud development occurring predominantly in the epidermal region, mainly in large explants, in agreement with previous studies (Jordan 1988; Encina and Regalado 2026). In small explants, like CS explants, many buds seen to come from the cut area (Fig. 4C), as previously reported (Jordan 1988; Bejoy and Hariharan 1992). This regenerative response was more pronounced in larger explants (Table 1).

Regeneration was characterized by a high percentage of explants forming buds; however, this did not result in substantial shoot proliferation. In addition, shoot development was slow, being almost negligible after 4 weeks, in contrast to the findings reported by Jordan (1988). For LPS explants, mMS-0.3BA and MS-2 mg/L BA-0.5 mg/L NAA-1PVP media could be recommended for shoot induction; however,

longer explants (≥ 1 cm) display high regenerative capacity in alternative media, as reported by Encina and Regalado (2026). In the case of CS explants, the most suitable medium was mMS-0.3 mg/L BA-100 mg/L CH.

Effect of cultivar on shoot regeneration

Once a regeneration protocol was established for hypocotyl explants of the Fino de Jete cultivar, mMS 0.3BA-100 CH was identified as the most suitable for CS explants (hereafter referred to as regeneration medium (RM). This medium was subsequently evaluated using hypocotyl explants from the Campas and Alboran cultivars. In these studies, when Campas and Alborán seeds were germinated to obtain the hypocotyl explants, we corroborated previous findings, confirming lower germination rates in Campas relative to Fino de Jete (Padilla and Encina 2003), and a similar to Campas germination rate in Alboran seeds, suggesting that the germination medium optimized for Fino de Jete may require adjustment when applied to other cultivars.

The three cultivars' CS explants showed a comparable rate of regeneration (Table 2). Campas and Alboran-derived CS explants showed a higher number of buds per explant and lower levels of necrosis, although no significant differences were observed in the number of shoots per explant, and shoot length was shorter in both cv. compared to Fino de Jete (Table 2).

Callogenesis was similar among treatments. Thus, the largest percentage of explants presented callus 2 (Fino de Jete 60%; Campas 51%; Alboran 56%), and to a lesser extent callus 3 (Fino de Jete 30%; Campas 38%; Alboran 29%). On the other hand, Jordan (1988) describes extensive necrosis in hypocotyl explants from the Concha Lisa cultivar. However, very little necrosis in explants from Fino de Jete seeds was observed in the experiments described in the previous section. Explant necrosis was measured in this set of experiments to determine whether the cultivar had an impact; however, very low percentages of necrosis were noted in the explants from all three cultivars (Table 2).

The favorable regeneration response observed in the Spanish cultivars 'Campas' and 'Alboran' may be related to their greater genetic similarity compared with the Chilean cultivar Concha Lisa. Previous studies based on molecular markers have reported higher genetic similarity between Campas and Fino de Jete than between these cultivars and Concha Lisa (Perfectti and Pascual 1998; Escribano et al. 2007), which may partly explain the comparable regeneration responses observed.

Effect of nanoparticles on shoot regeneration

During the shoot regeneration process from hypocotyl explants, as previously mentioned, particularly from small explants (CS), a high percentage of explants with buds was observed, but the number of explants with shoots was considerably lower. Moreover, when shoot formation occurred, the remaining buds on the same explant failed to develop further. Based on these observations, the effect of nanoparticles (NPs) on shoot formation was investigated.

Initially, low concentrations of carbon dots (CDs; 0.01, 0.1 and 0.2 mg L^{-1}) in RM were evaluated; however, no differences were detected in any of the analyzed parameters compared with the control treatment (data not shown). In subsequent experiments, higher concentrations of CDs (0.4, 0.6 and 0.8 mg L^{-1}) were tested. Increasing CDs concentration had a positive effect on some parameters, whereas others were not significantly affected (Fig. 5). Regeneration frequency remained high across all treatments and was not increased by CDs supplementation (Fig. 5A). In contrast, the number of buds per explant increased at some CDs concentrations, with the highest concentration tested (0.8 mg L^{-1}) showing significantly higher mean values at 12 weeks (Fig. 5B).

The number of shoots per explant was higher than in the control at the two highest CDs concentrations, although differences were not statistically significant at either 8 or 12 weeks (Fig. 5C). Similarly, shoot length was not significantly improved by CDs treatment, although mean values were higher at 0.4 and 0.6 mg/L after 8 weeks (Fig. 5D). Callus formation was significantly reduced at 0.4 and 0.8 mg/L after 8 weeks; however, this effect was no longer observed at 12 weeks (Fig. 5E). Explant necrosis was consistently reduced by CDs application, with significant reductions observed at 0.6 and 0.8 mg/L after 8 weeks, and at the highest concentration at both 8 and 12 weeks (Fig. 5F).

Table 2 Effect of the cherimoya cultivar on the regeneration of hypocotyl explants (0.3 cm cross sections) in RM[a] after 12 weeks

Cultivar	No. of explants	Regeneration %	No. buds/explant	Explants with shoots %	No. shoots/explant	Shoot length (cm)	Necrosis %
Fino de Jete	50	100 ± 0 a	15.5 ± 0.6 b	56 ± 8 a	2.6 ± 0.5 a	1.10 ± 0.14 a	6 ± 1 a
Campas	50	98 ± 1 a	24.2 ± 4.4 a	72 ± 10 a	2.1 ± 0.3 a	0.70 ± 0 c	2 ± 2 b
Alboran	50	100 ± 0 a	23.8 ± 5.4 a	65 ± 11 a	3.3 ± 0.6 a	0.97 ± 0.3 b	0 ± 0 b

[a]RM: full-strength MS salt, WPM vitamins, 30 g/L sucrose, and 0.6% of agar-0.3 mg/L BA-100 mg/L casein hydrolysate. Mean values with different letters mean statistically significant according to the χ^2 test (regeneration, explants with shoots and necrosis) or Student-Newman-Keuls (No. bud/explant, No. shoots/explant, shoot length $p < 0.05$)

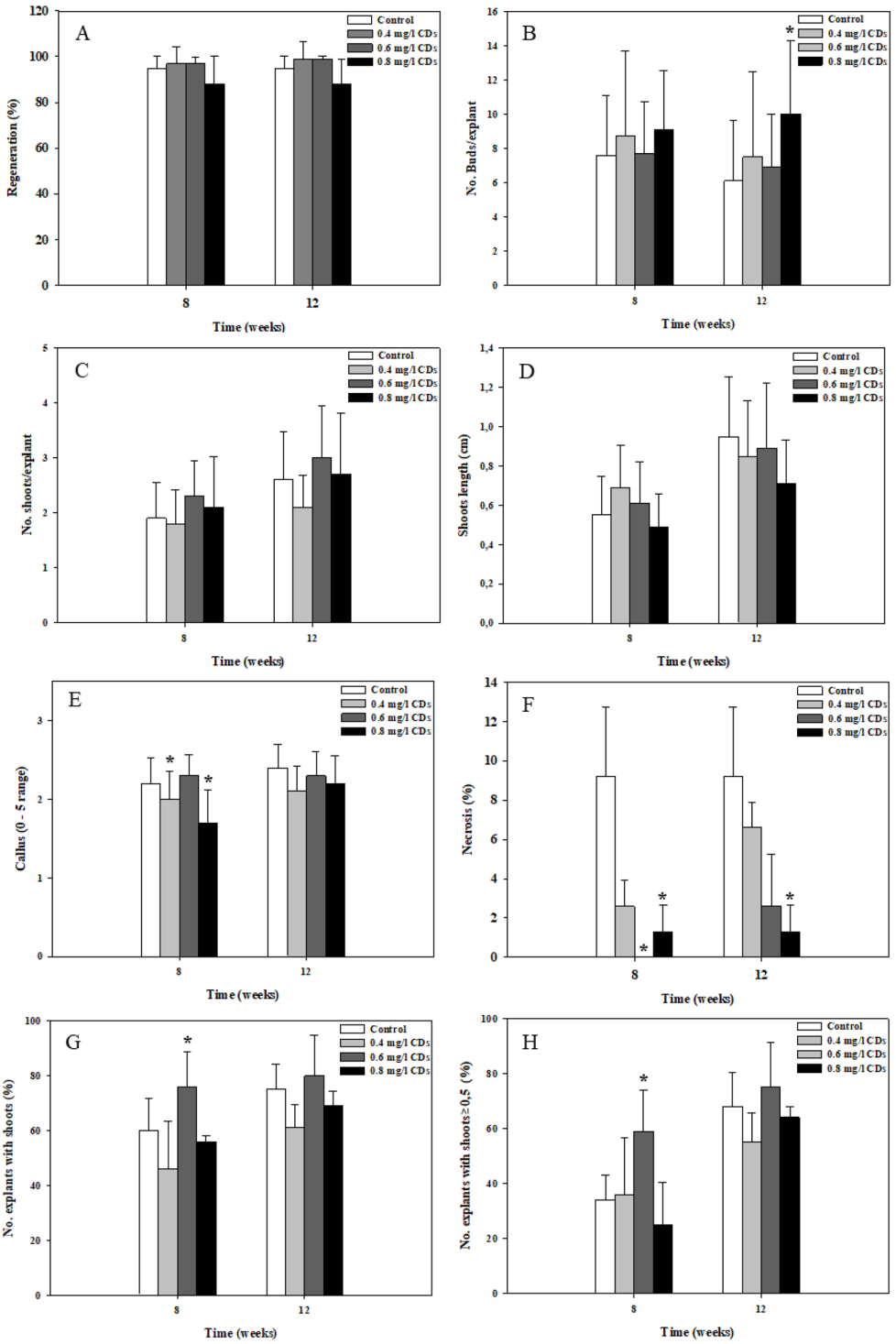


Fig. 5 Effect of CDs on cherimoya hypocotyl CS explants regeneration on RM supplemented with different CDs concentrations (0, 0.4, 0.6 and 0.8 mg/L). (**A**) regeneration percentage, (**B**) number of buds per explant, (**C**) number of shoots per explant, (**D**) shoot length, (**E**) callus proliferation on explants (range 0–5), (**F**) percentage of explant with necrosis, (**G**) percentage of explants with shoots, (**H**) percentage of explants with shoots higher or equal to 0.5 cm. Columns represent mean values ± SE. An asterisk (*) indicates significant differences between treatments according to Student–Newman–Keul's test ($p < 0.05$) for normal variables and according to χ^2 test for binomial variables. Data after 8 and 12 weeks. RM: full-strength MS salt, WPM vitamins, 30 g/L sucrose, and 0.6% of agar-0.3 mg/L BA-100 mg/L casein hydrolysate

Analysis of the percentage of explants producing shoots, as well as those producing shoots ≥ 0.5 cm in length, revealed a significant increase at 0.6 mg/LCDs. Although values remained higher than in the control, differences were no longer significant after 12 weeks (Fig. 5G-H). Overall, CDs supplementation reduced explant necrosis, particularly at higher concentrations, and promoted earlier shoot formation at 0.6 mg/L. In contrast, in control explants the transition from buds to shoots occurred later, between 8 and 12 weeks. At the highest concentration tested (0.8 mg/L), a higher number of buds and lower necrosis were observed, but shoot formation was reduced, suggesting a possible concentration-dependent inhibitory or toxic effect. Shoots

regenerated in media containing CDs showed a morphology comparable to that of control shoots (Fig. 6).

In recent years, CDs, also referred to as carbon quantum dots, have attracted increasing attention in agriculture due to their low toxicity, low production cost, high stability and limited environmental impact. Studies conducted on CDs effects on plants in vivo have not only revealed its positive effects on plant growth but also its mode of functioning (Kou et al. 2021; Li et al. 2023; Hu et al. 2025; Cheng et al. 2025). Hence, significant enhanced macro- and micronutrient absorption in hydroponically grown plants has been reported, leading to improved root architecture and shoot development (Kou et al. 2021; Hu et al. 2025).

CDs have also been shown to enhance photosynthetic performance through multiple mechanisms, including improved light absorption and energy conversion (Guo et al. 2022; Cheng et al. 2025). In this sense, it has been suggested that the small size of the CDs used (mostly < 10 nm) likely facilitates efficient root uptake, intracellular transport, and penetration into photosynthetic tissues, thereby enhancing photochemical electron transport and CO2 assimilation (Zheng et al. 2017; Tan et al. 2021; Hu et al. 2025). Additionally, an activation of antioxidant defense of the plant has been reported, showing that antioxidant enzyme activities rise at intermediate nanoparticle concentrations (Jadczak et al. 2020; Singh et al. 2021). Taking all these studies together, it appears that the effect of CDs is due to their size and surface characteristics, which allow them to easily penetrate the plants tissues and act as a carrier, improving plant growth in a dose-dependent manner. However, reports on their application in in vitro plant tissue culture remain scarce and most available studies have focused on the use of CDs as elicitors to enhance the production of bioactive compounds in vitro (Ghorbanpour and Hadian 2015; Heydari et al. 2020; Martinez-Chavez et al. 2024; Sigala-Aguilar et al. 2024). Their application in regeneration systems is even more limited. To date, only a few studies have examined the effects of other carbon-based nanoparticles, such as nanocarbon (carbon black) or carbon nanotubes, on callus formation and shoot regeneration. Kokina et al. (2012) reported a negative effect of multi-walled carbon nanotubes on flax somatic embryogenesis, with reduced callus formation in stem segment-derived cultures and an increased DNA methylation in a dose-dependent manner. However, Chutipaijit and Sutjaritvorakul (2018) reported that nanocarbon improved both callus production and shoot regeneration from rice seed-derived calluses compared with activated carbon, with optimal concentrations of 5 and 20 mg L^{-1}.

To our knowledge, the present study is the first to evaluate the use of CDs in *Annona* shoot regeneration. The observed reduction in explant necrosis and the promotion of earlier shoot formation may represent a valuable advantage for the development of genetic transformation protocols in cherimoya, particularly considering that the use of CDs in plant genetic transformation has already been reported in other species (Othman et al. 2024; Shivashakarappa et al. 2025).

With respect to the use of silver nanoparticles (AgNPs) in the bud and shoot regeneration process from cherimoya hypocotyl explants, AgNPs supplementation in RM produced a markedly stronger effect on regeneration than CDs. This effect was readily visible on the culture plates (Fig. 7).

Data analysis indicated that 0.2 mg/L AgNPs was the most effective concentration. At this level, both regeneration frequency and the number of buds per explant were significantly higher than in the control treatment (Fig. 8A-B). In addition, all tested AgNPs concentrations resulted in a higher number of shoots per explant and increased shoot length compared with the control (Fig. 8C-D). Callus formation was also influenced by AgNPs concentration: explants cultured with 0.1 and 0.2 mg/L AgNPs produced significantly more callus than those treated with the highest concentration (0.3 mg L^{-1}), which showed values closer to the control (Fig. 8E). Explant necrosis was not detected in either the control or the 0.2 mg/L AgNPs treatment, whereas significantly higher necrosis levels were observed at 0.1 and 0.3 mg/L

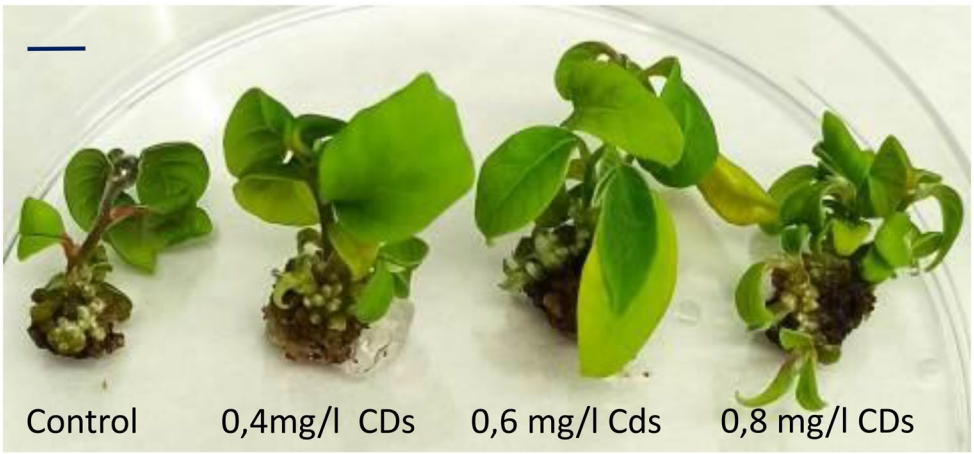


Fig. 6 Aspect of the shoots after three different treatments with CDs on cherimoya hypocotyl CS explants. Control explants lack CDs. Explants are shown at 12 weeks since cultivation on RM (full-strength MS salt, WPM vitamins, 30 g/L sucrose, and 0.6% of agar-0.3 mg/L BA-100 mg/L casein hydrolysate). Scale bar represents 5 mm

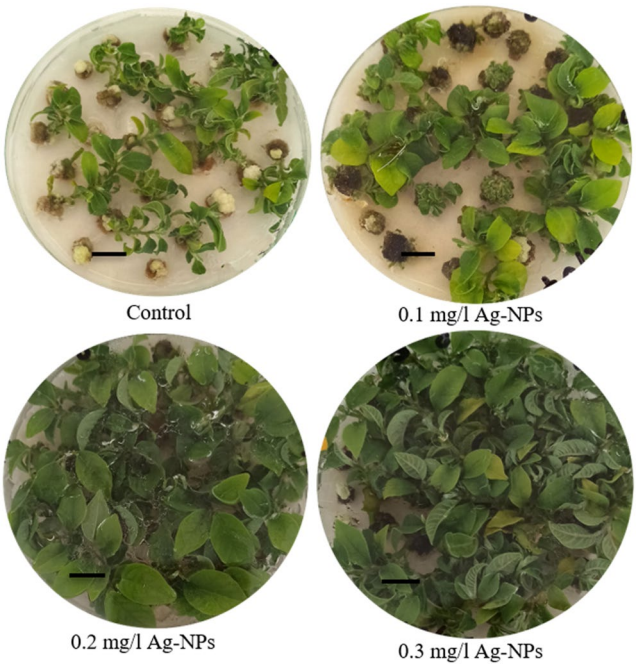


Fig. 7 Aspect of the of the shoots after three different treatments with AgNPs on cherimoya hypocotyl CS explants on RM (full-strength MS salt, WPM vitamins, 30 g/l sucrose, and 0.6% of agar-0.3 mg/L BA-100 mg/L casein hydrolysate). Explants are shown at 12 weeks since cultivation. Scale bar represents 1 cm

(Fig. 8F). Analysis of the proportion of explants producing shoots, as well as explants with shoots ≥ 0.5 cm in length, showed that all AgNPs treatments increased these parameters relative to the control after 12 weeks; however, differences were statistically significant only at 0.2 mg/L (Fig. 8G-H). Overall, AgNPs supplementation of the regeneration medium had a strong positive effect on hypocotyl CS explant regeneration, with 0.2 mg/L representing the most effective concentration, as it consistently improved all evaluated parameters related to bud and shoot production (Fig. 8).

The beneficial effects of AgNps on in vitro shoot growth and adventitious shoot regeneration have been widely reported (Balamurugan et al. 2024; Guha et al. 2024). Significant improvements on shoot proliferation, shoot number per explant and shoot length have been described in several species, including olive (Hasanin et al. 2024) and apricot (Pérez-Caselles et al. 2023). AgNPs have also been shown to enhance embryogenic callus formation and to promote somatic embryo proliferation and maturation (Ewais et al. 2015; Elsayh 2021; Phong et al. 2023).

Within organogenic regeneration systems, AgNPs-induced improvements in callus induction and adventitious shoot formation have been reported in different explants and species. Thus, in potato internode cultures, AgNPs supplementation increased both shoot number per explant and shoot length (Baltazar Bernal et al. 2023). In rice, AgNPs promoted callus induction and callus regeneration at concentrations of 10 and 5 mg L^{-1}, respectively (Manickavasagam et al. 2019). Similarly, in passion fruit, supplementation of the regeneration medium with 1 mg/L AgNPs increased both the percentage of explants producing shoots and the number of shoots per explant (Phong et al. 2023). Mustafa et al. (2017) also reported enhanced callus induction, as well as increased fresh and dry biomass, in hypocotyl explants derived from seeds treated with AgNPs.

To better understand how nanoparticles interact with the tissues in vitro, their effects have been investigated genetically, molecularly, and physiologically in in vitro culture systems. Castro-González et al. (2019) found that AgNPs in stevia shoots were transported from the culture medium and accumulated within plant tissues. Several studies on callus organogenesis have described the influence of silver AgNPs on the gene expression of genes sensitive to cytokinins, auxins, ethylene, and ABA (Manickavasagam et al. 2019; Phong et al. 2022; Sarmast and Salehi 2022; Baltazar Bernal et al. 2023). This effect has been described as dose-dependent, such that optimal AgNP concentrations resulted in a decrease in the expression of genes sensitive to ethylene and ABA, while maintaining the cytokinin/auxin ratio, whereas this ratio was altered at suboptimal doses (Baltazar Bernal et al. 2023). In rice callus regeneration, a dose-dependent effect of AgNPs on the DMA, proline, and H_2O_2 values, normally produced by plants in response to stress, has also been described (Manickavasagam et al. 2019). The results obtained in the present study are consistent with these reports and confirm the effectiveness of AgNPs in promoting bud, shoot and callus formation in cherimoya hypocotyl explants in a dose-dependent manner, with 0.2 mg/L AgNps as optimal dose.

Rooting and acclimatization of regenerated shoots

Adventitious shoots regenerated in MR medium were subcultured in MS-0.15BA medium for 1 to 3 subculture and, subsequently, rooting was induced following the method described by Encina et al. (1994). Although rooting was achieved (Fig. 9), rooting did not exceed 40%. This value is low for juvenile material and highlight the need for a more detailed analysis of the rooting process in this regenerated material. In previous published works using the same rooting method, higher percentages were reported. Thus, in the 1994 work by Encina et al., up to 95% rooting was achieved, and more recently, Encina and Regalado (2026) rooted regenerated shoots from hypocotyl explants treated with colchicine with an 80% rooting success rate. In both

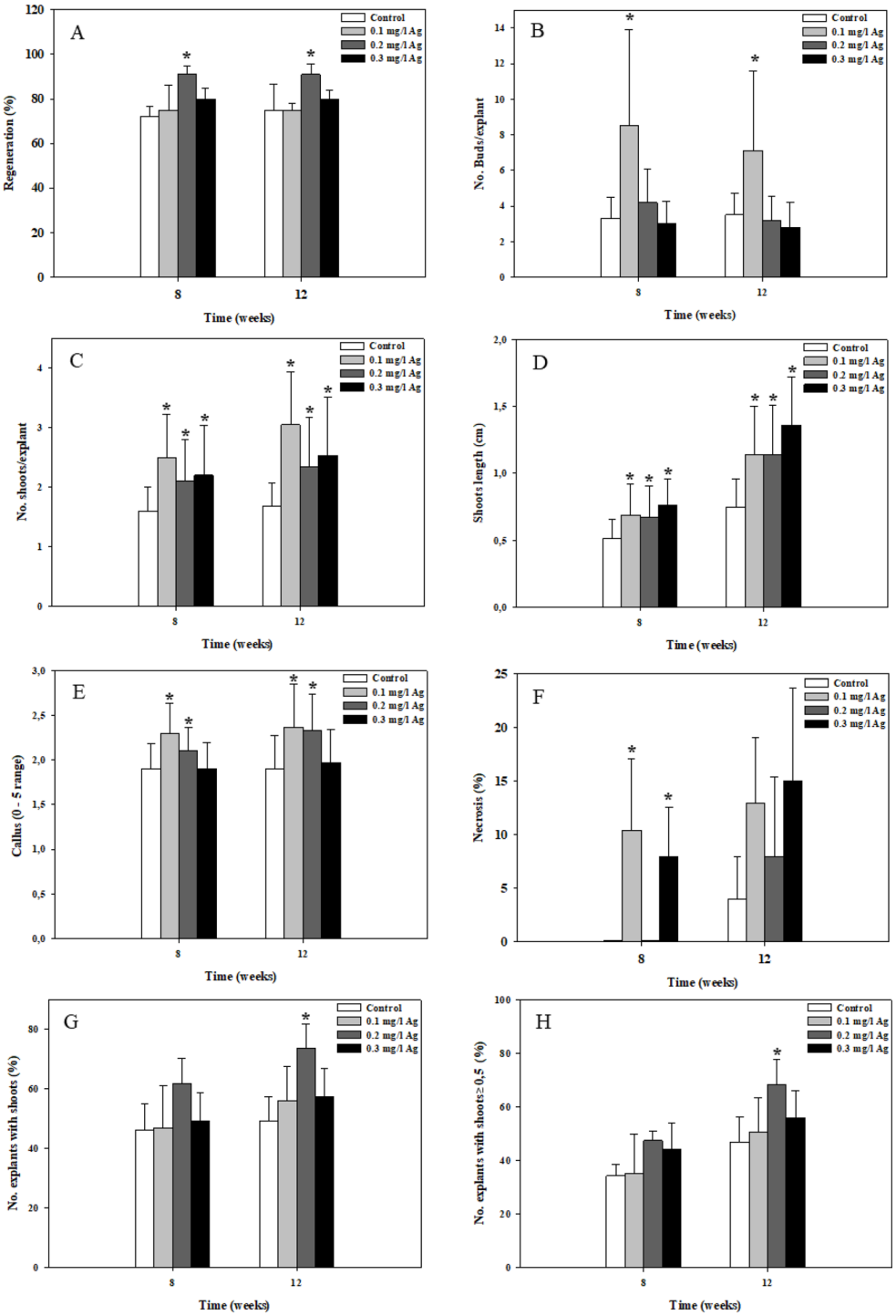


Fig. 8 Effects of AgNPs concentration (0.1, 0.2, 0.3 mg/L) on cherimoya hypocotyl CS explants shoot regeneration in RM (full-strength MS salt, WPM vitamins, 30 g/L sucrose, and 0.6% of agar-0.3 mg/L BA-100 mg/L casein hydrolysate). (**A**) regeneration percentage, (**B**) number of buds per explant, (**C**) number of shoots per explant, (**D**) shoot length, (**E**) callus proliferation on explants (range 0–5), (**F**) percentage of explant with necrosis, (**G**) percentage of explants with shoots, (**H**) percentage of explants with shoots higher or equal to 0.5 cm. Columns represent mean values ± SE. An asterisk (*) indicates significant differences between treatments according to Student–Newman–Keuls test ($p < 0.05$) for normal variables, and according to χ^2 test for binomial variables. Data after 8 and 12 weeks

studies, shoots were in cultivation for more than 1 year. So, these results could indicate that the physiological state of 1 to 3 subculture-regenerated shoots from the present study differs from those obtained in previous works and/or that a higher number of subcultures could produce a state of shoot rejuvenation, improving rooting process. In this context and considering that Nps have been used successfully in the rooting of different species (Hegazi et al. 2021; Korpayev et al. 2021; Elsayh et al. 2022), evaluating the potential effects of nanoparticles on root induction and development may represent a promising strategy to improve rooting efficiency of this type of regenerated material. Plantlet acclimatization was not a limiting phase in the micropropagation of plantlets and 100% of the rooted shoots were successfully acclimatized to the greenhouse (Fig. 9).

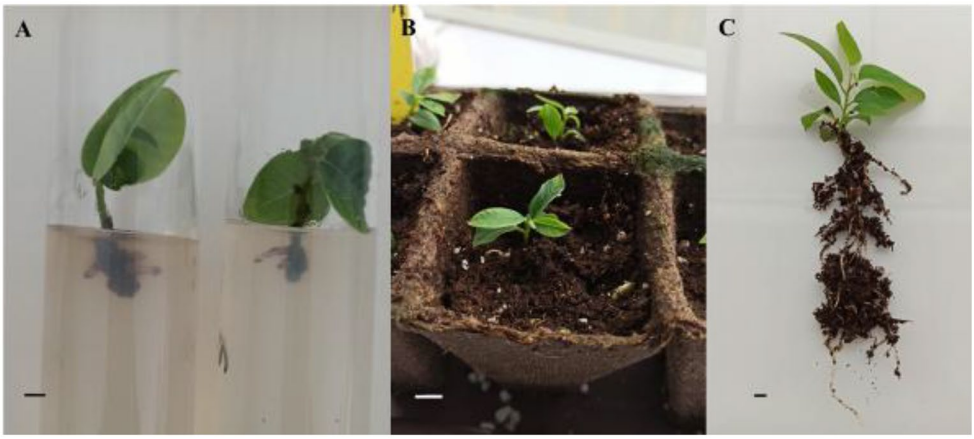


Fig. 9 Rooting and acclimatization of regenerated shoots. (**A**) Regenerated shoots with roots after 4 weeks. (**B**) Rooted shoots after 1 month of acclimatization. (**C**) Root development in acclimatizes plantlet after 12 weeks. Scale bars represent 5 mm

Conclusions

A regeneration medium for bud induction and shoot development from cherimoya hypocotyl explants was successfully established. The application of nanoparticles to the regeneration medium significantly enhanced adventitious shoot regeneration from cherimoya hypocotyl CS explants. CDs reduced explant necrosis and promoted earlier shoot formation, whereas AgNPs increased both the rate of explants producing shoots and shoot length. Further optimisation of the rooting process in regenerated material is required to achieve higher rooting efficiencies. Based on the positive effects observed during shoot formation, the use of these nanoparticles will be considered in future studies focusing on root induction and the development of genetic transformation protocols in cherimoya.

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Author contributions Regeneration experiments, data collection and analysis were performed by Isabel María González Padilla. Carlos Lopez Encina contributed to the study conception and regeneration experiments design. Carbon-dots synthesis and experiment design were performed by Leonardo Velasco. Silver nanoparticles synthesis and experiment design were performed by Enrique Niza. The first draft of the manuscript was written by Isabel María González Padilla and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The authors confirm that the data will be available on request.

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

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