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 growing in southern Spain, world's leading producer, with Fino de Jete as the main cultivar. In this cultivar, shoot regeneration from hypocotyl explants was investigated. Split explants (1 cm) and cross sections (0.3 cm), different culture media, and antioxidant treatments were evaluated. For split explants, a modified Murashige and Skoog medium supplemented with 0.3 mg L□□ 6-benzyladenine, resulting in shoot formation in 66% of explants with an average shoot length of 1.2 cm. In cross sections, using the same medium supplemented with 100 mg L□□ casein hydrolysate, 33% shoot regeneration and an average shoot length of 0.9 cm was achieved. This medium was also effective for hypocotyl cross section regeneration of Campas and Alboran cultivars. Additionally, the effect of carbon-dots nanoparticles (CDs; 0 to 0.8 mg L-1) and silver nanoparticles (AgNPs; 0 to 0.3 mg L-1), on shoot production using cross sections was evaluated. Treatment with CDs at 0.6 mg L□□ significantly reduced explant necrosis and promoted earlier shoot formation after 8 weeks of culture. Meanwhile, AgNPs markedly enhanced bud regeneration, shoot proliferation, and shoot elongation. These effects were most pronounced at 0.2 mg L□□ after 12 weeks of culture. Although rooting was achieved in control shoots (up to 40%), the efficiency of the rooting process in this material needs to be improved. Auxins and nanoparticles are currently being investigated to enhance this process. Completing a plant regeneration protocol from cherimoya hypocotyl explants would allow us to address the genetic transformation of this species in the future.

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 is 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. To develop new and improved genotypes of cherimoya with biotechnological tools, a plant regeneration protocol is necessary. In this sense, Encina 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; Padilla and Encina 2011). To address the current challenges in cultivation of cherimoya, regeneration and genetic transformation protocol for this species is needed. However, no protocol for shoot regeneration has so far been described. 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. Therefore, a primary objective of this work will be to develop a protocol for shoot formation from hypocotyl explants from seeds of the cherimoya "Fino de Jete" cv.

Despite these advances in micropropagation and germination in cherimoya, challenges remain in developing efficient regeneration protocols, particularly regarding explant necrosis and low and slow shoot development. Recently, nanotechnology has emerged as a powerful tool to overcome such limitations in plant biotechnology, demonstrating significant impacts on the growth and development of plants *in vitro* across a wide variety of species (Kim et al. 2017; Rohela et al. 2024; Balamurugan et

al. 2024). Because of their special qualities, 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). Thus, its positive effect is being demonstrated in micropropagation for the disinfection of explants (Abdolinejad et al. 2020; Khafri et al. 2022; Shaafi et al. 2022), in the multiplication and rooting phases of the shoots (Phong et al. 2022; Elsayh et al. 2022; Lai et al. 2022; Khattab et al. 2022; Tejada-Alvarado et al. 2022; Hegazi et al. 2021; Farrokhzad et al. 2022; Sarmast and Salehi 2022; Korpayev et al. 2021), as well as in the acclimatization of the obtained plants (Ung et al. 2022). Furthermore, other biotechnological processes including callogenesis and genetic transformation of tissues (Rajkumari et al. 2021; Malik et al. 2021) and protoplasts (Bansod et al. 2015) have also been enhanced with the use of AgNPs, giving good results even for inducing flowering *in vitro* (Rajput et al. 2024).

Likewise, carbon dots nanoparticles (CDs), a new type of carbon nanoparticles that show considerable advantages, such as easy synthesis, easy modification, excellent water solubility, strong fluorescence and very low toxicity, and show benefits in plant growth (Li et al. 2023). CDs can be easily absorbed by plants, so they seem to promote their growth and photosynthesis and activate their defense mechanisms (Kou et al. 2021; Qian et al. 2018; Wang et al. 2018; Zhang et al. 2012). Recent studies have demonstrated that CDs enhance photosynthetic efficiency through improved light conversion and nutrient delivery (Cheng et al. 2025) and promote coordinated regulation of nutrient uptake and photosynthesis (Hu et al. 2025).

CDs are also being used as a vehicle to introduce and disperse siRNA in plants for gene silencing and protection against fungi, viruses and insects (Schwartz et al. 2020; Wang et al. 2020; Zarrabi et al. 2024), and in *in vitro* culture for the genetic transformation of tissues (Campos et al. 2021) and of protoplasts (Lew et al. 2018). More recently, CDs have been successfully employed for DNA delivery into plant tissues, demonstrating their potential as carriers for genetic material without negatively affecting regeneration efficiency (Shivashakarappa et al. 2025). However, there are far fewer references on the use of CDs in *in vitro* tissue culture compared to metallic nanoparticles, with AgNPs being one of the most referenced (Balamurugan et al. 2024; Rohela et al. 2024). Therefore, an additional goal of this work is to study the effect of both AgNPs and CDs on the process of shoot regeneration in cherimoya.

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 (Figure 1).

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) consisted of WPM vitamins (Lloyd and McCown 1981; glycine- 2.00 mg L^{-1}, nicotinic acid-0.50 mg L^{-1}, pyridoxine HCl-0.50 mg L^{-1}, thiamine HCl -1.00 mg L^{-1}), 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose, and 3 mg L^{-1} 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 ℃ and 1.05 k·cm^{-2}. One intact seed was cultured per tube and placed in an incubator at 30 ± 1 °C in the dark. 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^{-1} 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 ℃ 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^{-1} N^6-benzyladenine (BA) and 100 mg L^{-1} casein hydrolysate (CH), unless otherwise indicated. The pH of media was adjusted to 5.74 and autoclaved for 20 min at 121 ℃ and 1.05 k·cm^{-2}. Twenty-five ml of medium was dispensed in 9 cm diameter Petri plates 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 dishes and subcultured to fresh medium in double-width Petri dishes 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 previous experiment, different salt formulations and cytokinins were tested using hypocotyl explants (data not shown). mMS medium was established as best medium and 0.3 mg L^{-1} BA as best cytokinin. Then, different regeneration experiments were carried out with LSP and CS hypocotyl explants. mMS medium supplemented with 0.3 mg L^{-1} BA and MS supplemented with 2 mg L^{-1} BA plus 0.5 mg L^{-1} NAA (Jordan 1988) were tested. Additionally, the effect of PVP (1 g L^{-1}) and CH (100 mg L^{-1}) on the regeneration of buds and shoots and necrosis of explants was studied. Three repetitions of each experiment were performed with twenty to thirty-explants per treatment and repetition. All cultures were incubated in the culture room at standard conditions, as previously indicated. Data on percentage regeneration, number of buds and regenerated shoots per explant, shoot length, callus and necrosis were collected after four, eight and twelve weeks.

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 cvs., were germinated as previously described for Fino de Jete seeds (Padilla and Encina 2003). Two repetitions were performed and 30 to 40 explants per treatment and repetition were used. All cultures were incubated in the culture room at standard conditions, as previously indicated. Data on percentage

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-López et al. (2022) with different modifications; 10 mL of 25 mM $AgNO_3$ 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 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 15,000g 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^{-1}) and AgNPs (0, 0.1, 0.2, 0.3 mg L^{-1}). 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. Three repetitions of each experiment were performed, with 20-30 explant per treatment and repetition. The explants were maintained in RM with NPs for 12 weeks, with subcultures to fresh medium every 4 weeks. Control explants were maintained in RM without NPs. Data on percentage regeneration, number of buds, regenerated shoots per explant, shoot length, callus and necrosis were collected after four, eight and twelve weeks.

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

Photonic correlated spectroscopy techniques such as dynamic light scattering are the most used techniques to evaluate the size, distribution, surface charge and colloidal stability of different

nanomaterials (Mondejar et al., 2021). 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 1-2 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 (Figure 2A). 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. 2022) 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 (Figure 2B).

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

The first objective of this work was to develop a protocol to obtain shoot regeneration in cherimoya using Fino de Jete seed-derived hypocotyl explants. In previous experiments, we observed that buds always formed from 2 cm sections of hypocotyl, even in MS medium without growth regulators. In these explants, mMS medium plus 0.5 mg L^{-1} zeatin and, mMS medium plus 0.3 mg L^{-1} BA yielded the best results in terms of explants with shoots (data not shown), but we selected the medium with BA because of its lowest price. Subsequently, we tested the medium described by Jordan (1988), consisted of MS medium with 2 mg L^{-1} BA plus 0.5 mg L^{-1} NAA and a medium derived from the previous works mentioned consisted of mMS medium containing 0.3 mg L^{-1} BA in both LSP and CS explants. In addition, in both media and kind of explant we tested the antioxidants PVP (1 g L^{-1}) and CH (100 mg L^{-1}) (Jordan 1988). The results showed differences between types of explants and the media tested. Thus, 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, many buds grew from the epidermis (see figure 3A), and we 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, buds are seen to come mainly from the cut area (see figure 3C), as has also been described in cherimoya cv. `Concha Lisa´ by Jordan (1988) and in *A. muricata* (Bejoy and Hariharan 1992). Therefore, we observe a different origin of the bud in both explants which we believe to 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. With regards to the media tested, in LSP explants, best results were obtained with mMS medium plus 0.3 mg L^{-1} BA, with 100% regeneration, and 66% of explants having shoots with an average height of 1.2 cm (Table 1). Jordan´s (1988) medium produced similar results but shoots were significantly shorter. In the case of CS explants, significant differences were found, with better results obtained when mMS medium plus 0.3 mg L^{-1} BA and casein hydrolysate (100 mg L^{-1}) was used, with 70% regeneration and 34% of explants producing shoot with an average height of 0.9 cm (Table 1). In our study we did not observe a positive effect of PVP on

bud/shoot regeneration in any of the explants tested. Therefore, because of our regeneration experiments using hypocotyl explants of different sizes, we observed a high regenerative capacity in these explants, with rapid bud formation directly at certain positions of the epidermis, without intermediate callus formation, and not solely from cut surfaces. This appears to be a natural mechanism in this species, given that during germination, the cotyledons and epicotyl remain inside the seed, hanging (Figure 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) in his experiments, and good aspect of buds and shoots was observed (Figure 3A and 3C), which probably indicates genotype differences between cultivars. Jordan (1988) described callus proliferation at the base of CS explants and browning, which decreased when different antioxidants were added to the medium. In our case, we found a huge amount of very white and hard callus tissue in both explants tested when media were supplemented with NAA, that in the case of CS explants covered the whole explant (see figure 3B and 3D), preventing bud formation or proliferation (Table 1), despite the addition of antioxidant compounds (PVP or CH). However, in the mMS-0.3BA-100 CH medium, explants produced soft and cream-colored callus in most explants, with very few explants exhibiting hard callus.

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, we also observed variability in the *in vitro* regeneration capacity of the explant's regeneration using seeds with different ages, 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).

To our knowledge, this study represents the first report of shoot regeneration in cherimoya. A careful examination of Jordan's work (1988) suggests that the results described as shoot production (percentage of explants with shoots) at 4 weeks likely correspond to the formation of adventitious buds rather than fully developed shoots. In that study, neither the number of shoots per explant nor shoot length was reported, and no images documenting shoot regeneration were provided; instead, only a hypocotyl segment illustrating bud development was shown. In our experiments, after 4 weeks the regeneration frequency (percentage of explants with buds/shoots) ranged from 60 to 100%, whereas shoot formation was limited (0–2%). These results indicate that although bud formation occurs early, shoot development takes place at a later stage.

Effect of cultivar on shoot regeneration

Once a regeneration protocol was established for hypocotyl explants of the Fino de Jete cultivar, mMS medium supplemented with 0.3 mg L□□ BA and 100 mg L□□ CH (hereafter referred to as regeneration medium (RM)) was identified as the most suitable for CS explants. This medium was subsequently evaluated using hypocotyl explants from the Campas and Alboran cultivars. In vitro, germination was higher in Fino de Jete seeds (79%) than in Campas (47%) and Alboran (40%); however, hypocotyls of comparable growth and quality were obtained across cultivars. The lower germination rates observed in Campas and other cultivars relative to Fino de Jete have been

previously reported (Padilla and Encina 2003), suggesting that the germination medium optimized for Fino de Jete may require adjustment when applied to other cultivars.

With respect to regeneration, CS explants from Campas and Alboran exhibited high regeneration frequencies, comparable to those obtained from Fino de Jete-derived CS explants (Table 2). These cultivars showed a higher number of buds per explant and lower levels of necrosis, although shoot length was shorter than in Fino de Jete. The percentage of explants forming shoots was also higher in Campas and Alboran, although differences were not statistically significant. Callus formation was similar between Fino de Jete and Campas explants and slightly lower in Alboran.

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. 1999), which may partly explain the comparable regeneration responses observed.

Effect of nanoparticles on shoot regeneration

During the shoot regeneration process from hypocotyl explants, particularly from small explants (CS), we observed that although numerous buds were formed and regeneration frequencies were high, the proportion of explants developing shoots remained relatively low. Moreover, when shoot formation occurred, the remaining buds on the same explant failed to develop further. Based on these observations, we investigated the effect of nanoparticles (NPs) on shoot formation.

Initially, low concentrations of carbon dots (CDs; 0.01, 0.1 and 0.2 mg L□□) 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□□) were tested. Increasing CDs concentration had a positive effect on some parameters, whereas others were not significantly affected (Figure 4). Regeneration frequency remained high across all treatments and was not increased by CDs supplementation (Figure 4A). In contrast, the number of buds per explant increased at some CDs concentrations, with the highest concentration tested (0.8 mg L□□) showing significantly higher mean values at 12 weeks (Figure 4B).

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 (Figure 4C). 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 (Figure 4D). 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 (Figure 4E). 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 (Figure 4F).

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 L□□ CDs. Although values remained higher than in the control, differences were no longer significant after 12 weeks (Figures 4G, 4H). 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⁻¹), 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 (Figure 5).

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. Their potential use as fungicides and bactericides has been explored, and several studies have reported positive effects on plant growth and enhanced tolerance to biotic and abiotic stresses (Kou et al. 2021; Li et al. 2023; Hu et al. 2025; Cheng et al. 2025). CDs have also been shown to enhance photosynthetic performance through multiple mechanisms, including improved light absorption and energy conversion (Cheng et al. 2025). However, reports on their application in in vitro plant tissue culture remain scarce.

Most available studies have focused on the use of CDs as elicitors to enhance the production of bioactive compounds in vitro, both in callus cultures (Ghorbanpour and Hadian 2015; Martinez-Chavez et al. 2024; Sigala-Aguilar 2024) and in cell suspension cultures (Heydari et al. 2020). 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 that high concentrations of multi-walled carbon nanotubes reduced callus formation in flax stem segment-derived cultures. Similarly, Chutipaijit and Sutjaritvorakul (2018) demonstrated 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 specifically for in vitro 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 (Figure 6). Data analysis indicated that 0.2 mg L⁻¹ 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 (Figures 7A, 7B). In addition, all tested AgNPs concentrations resulted in a higher number of shoots per explant and increased shoot length compared with the control (Figures 7C, 7D).

Callus formation was also influenced by AgNPs concentration: explants cultured with 0.1 and 0.2 mg L⁻¹ AgNPs produced significantly more callus than those treated with the highest concentration (0.3 mg L⁻¹), which showed values closer to the control (Figure 7E). Explant necrosis was not detected in either the control or the 0.2 mg L⁻¹ AgNPs treatment, whereas significantly higher necrosis levels were observed at 0.1 and 0.3 mg L⁻¹ (Figure 7F).

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⁻¹ (Figures 7G, 7H). Overall, AgNPs supplementation of the regeneration medium had a strong positive effect on hypocotyl CS explant regeneration, with 0.2 mg L⁻¹ representing the most effective concentration, as it consistently improved all evaluated parameters related to bud and shoot production (Figure 7).

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 in 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 (Elsay 2021; Ewais et al. 2015; 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. For example, in potato internode cultures, AgNPs supplementation increased both shoot number per explant and shoot length (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⁻¹ 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.

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, particularly at a concentration of 0.2 mg L⁻¹. The positive effects of AgNPs on in vitro regeneration have been attributed, at least in part, to their ability to modulate endogenous ethylene and abscisic acid levels in plant tissues (Manickavasagam et al. 2019; Sarmast and Salehi 2022).

On the other hand, Encina et al. (1994) described a method for the successful rooting of juvenile cherimoya material, achieving rooting rates of up to 95%. This protocol was applied to shoots that had been maintained in culture for more than one year and consists of three sequential steps using three different culture media. The first medium lacks growth regulators and contains activated carbon to remove residual exogenous cytokinin from the shoots; the second is an induction medium supplemented with a high auxin concentration (100 mg L^{-1} IBA); and the third is an elongation medium, also without growth regulators, to promote root development. This protocol was subsequently shown to be applicable to adult material that had undergone more than ten subcultures, although with a lower rooting percentage (approximately 50%) (Padilla and Encina 2004). However, when this protocol was applied to adventitious shoots regenerated in the present study and maintained for one to three subcultures, rooting frequencies did not exceed 40%. These results indicate that the rooting response of newly regenerated shoots differs from that of long-term cultured material and highlight the need for a more detailed analysis of the rooting process in this regenerated material. In this context, evaluating the potential effects of nanoparticles on root induction and development may represent a promising strategy to improve rooting efficiency. Therefore, we are currently investigating rooting in adventitious shoots, as well as the use of NPs, to enhance this process. The establishment of a reliable and reproducible regeneration protocol from cherimoya

hypocotyl explants would provide an essential foundation for future genetic transformation studies in this species.

CONCLUSION

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 proportion 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 regeneration, the use of these nanoparticles will be considered in future studies focusing on root induction and the development of genetic transformation protocols in cherimoya.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

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.

Ethic declaration

Not applicable.

Data availability statement

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

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Tables

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]	Regeneration (%)	Explants with shoots (%)	Shoot length (cm)
Cross Section	**mMS 0.3 BA-Control**	55 ab	16 bc	1.4 a
	mMS 0.3 BA-1PVP	18 b	29 ab	1 a
	mMS 0.3 BA-100CH	70 a	34 a	0.9 a
	MS 2 BA 0.5 NAA-Control	39 ab	9 c	0.8 a
	MS 2 BA 0.5 NAA-1PVP	21 b	5 c	2.0 -
	MS 2 BA 0.5 NAA -100CH	17 b	2 c	1.3 -
Long Split Peces	**mMS 0.3 BA-Control**	94 ab	66 ab	1.2 ab
	mMS 0.3 BA-1PVP	53 d	70 ab	0.9bc
	mMS 0.3 BA-100CH	84 cd	53 ab	1.1 abc
	MS 2 BA 0.5 NAA - Control	86 bc	72 ab	1.3 a
	MS 2 BA 0.5 NAA -1PVP	98 a	75 a	0.8 c
	MS 2 BA 0.5 NAA -100CH	74 c	47 b	1.1 abc

[a]mMS medium: full-strength MS salt (Murashige and Skoog, 1962), WPM vitamins (Lloyd and McCown, 1981), 30 g/l sucrose, and 0.6% of agar. MS: Murashige and Skoog (1962) medium. The numbers in front of the growth regulators BA and NAA are their concentrations expressed in mg L^{-1}. 1PVP: Polyvinylpyrrolidone 1 g L^{-1}; 100CH: casein hydrolysate 100 mg L^{-1}. 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).

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	Regeneration %	No. buds/explant	Explants with shoots %	No. shoots/explant	Shoot length (cm)	Callus (0 to 5)	Necrosis %
Fino de Jete	100 a	15.5 b	56 a	2.6 a	1.10 a	2.6 a	5.6 a
Campas	98 a	24.2 a	72 a	2.1 a	0.70 c	2.5 a	1.5 b
Alboran	100 a	23.8 a	65 a	3.3 a	0.97 b	2.1 b	0 b

[a]RM: full-strength MS salt (Murashige and Skoog, 1962), WPM vitamins (Lloyd and McCown, 1981), 30 g/l sucrose, and 0.6% of agar-0.3 mg L^{-1} BA-100 mg L^{-1} 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 and callus; $p<0.05$).

Figures

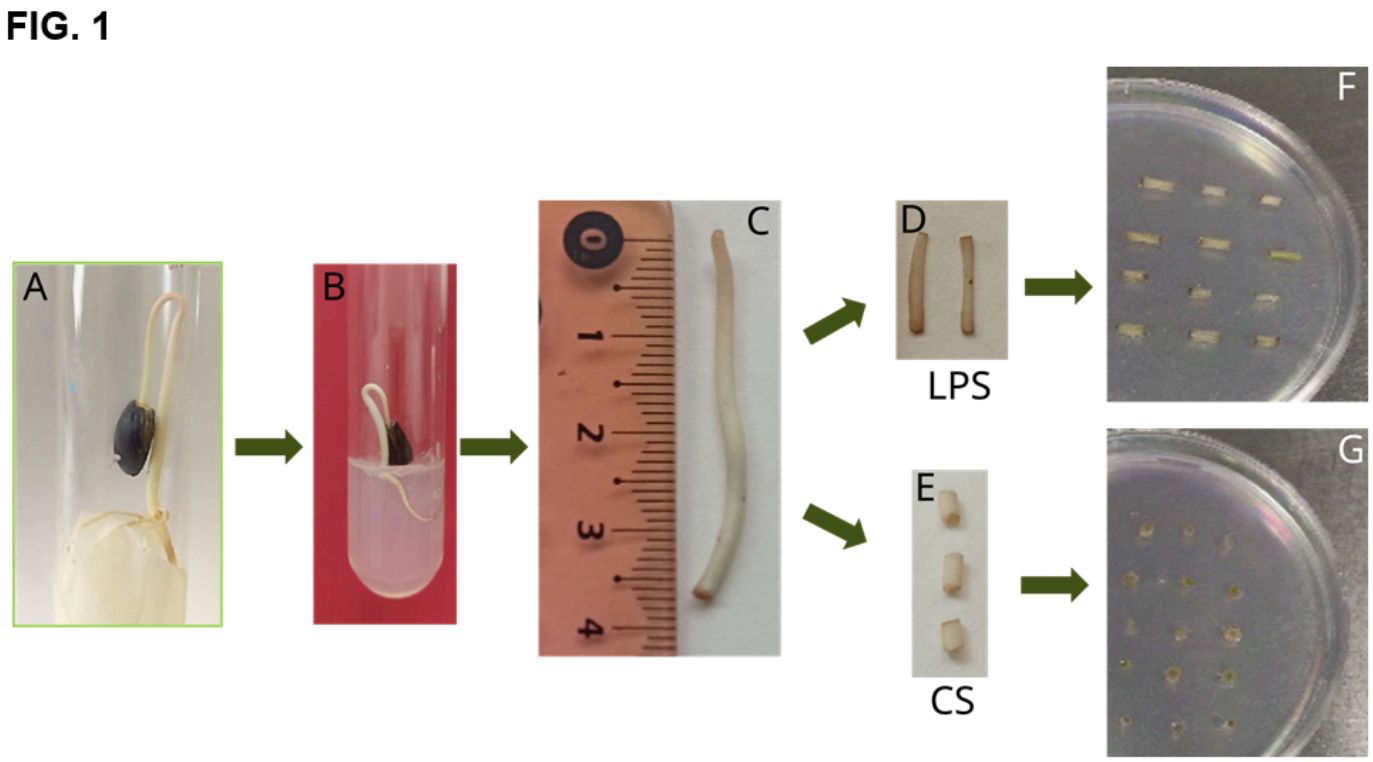

Figure 1

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 (Murashige and Skoog, 1962), WPM vitamins (Lloyd and McCown, 1981), 8764 mM (30 g/l) sucrose, and 0.6% of agar.

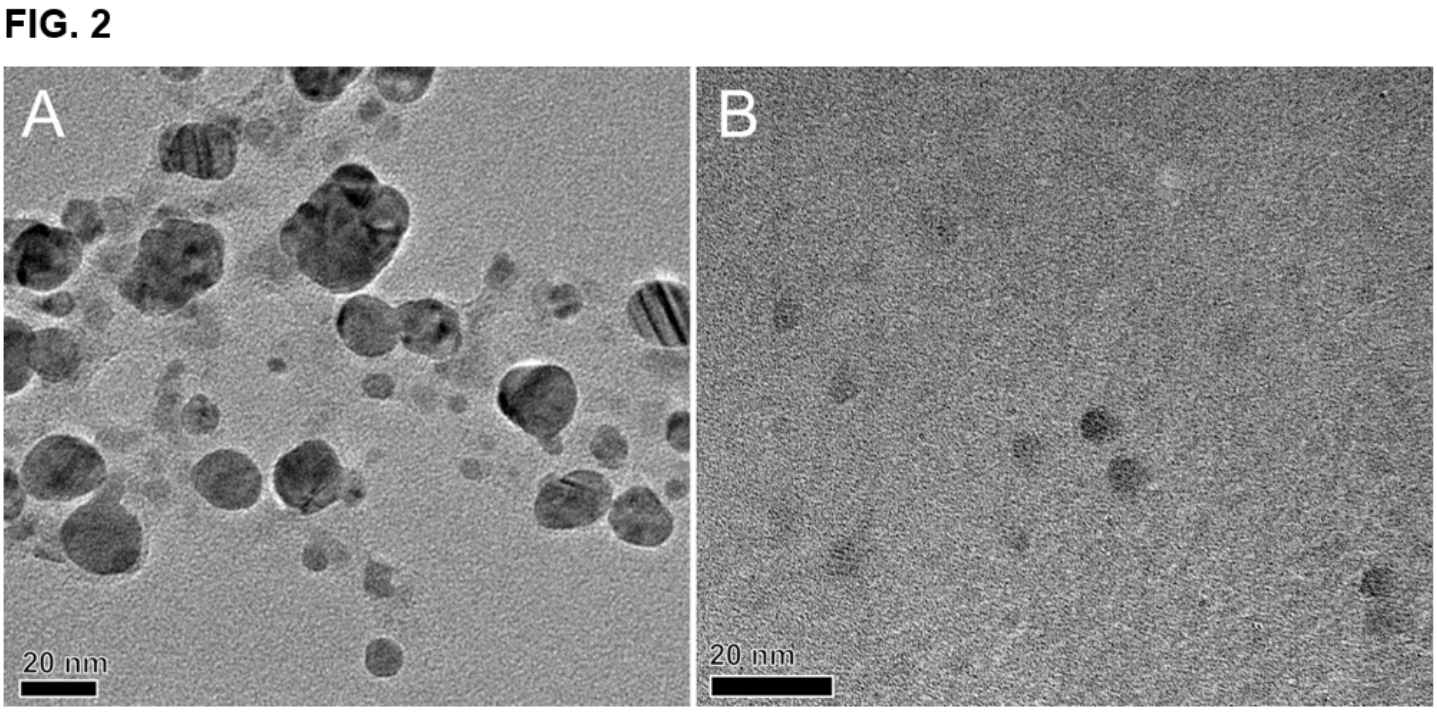

Figure 2

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

FIG. 3

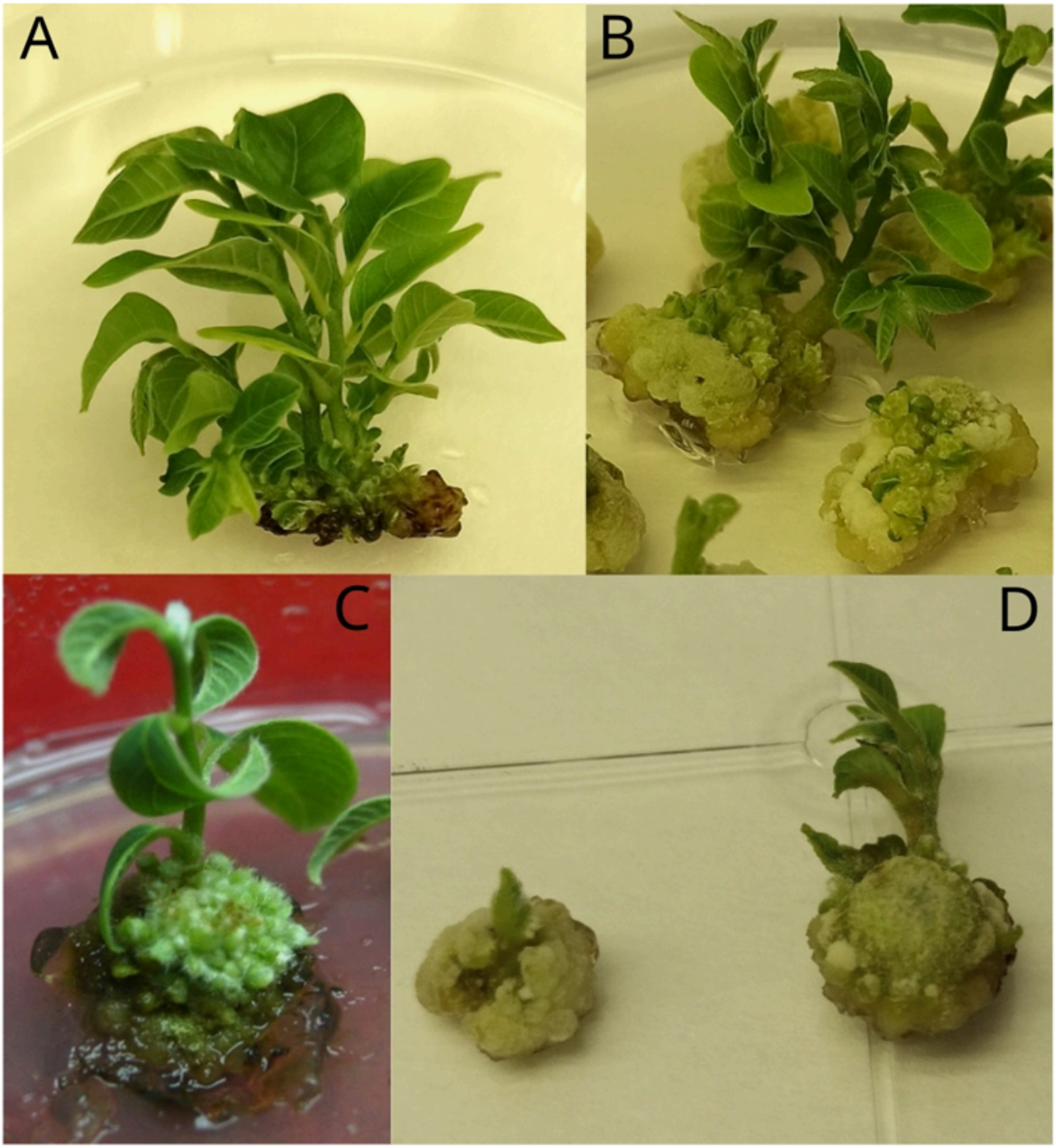

Figure 3

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 Jordan medium (Jordan, 1988). RM: full-strength MS salt (Murashige and Skoog, 1962), WPM vitamins (Lloyd and McCown, 1981), 30 g/l sucrose, and 0.6% of agar-0.3 mg L^{-1} BA-100 mg L^{-1} casein hydrolysate.

FIG. 4

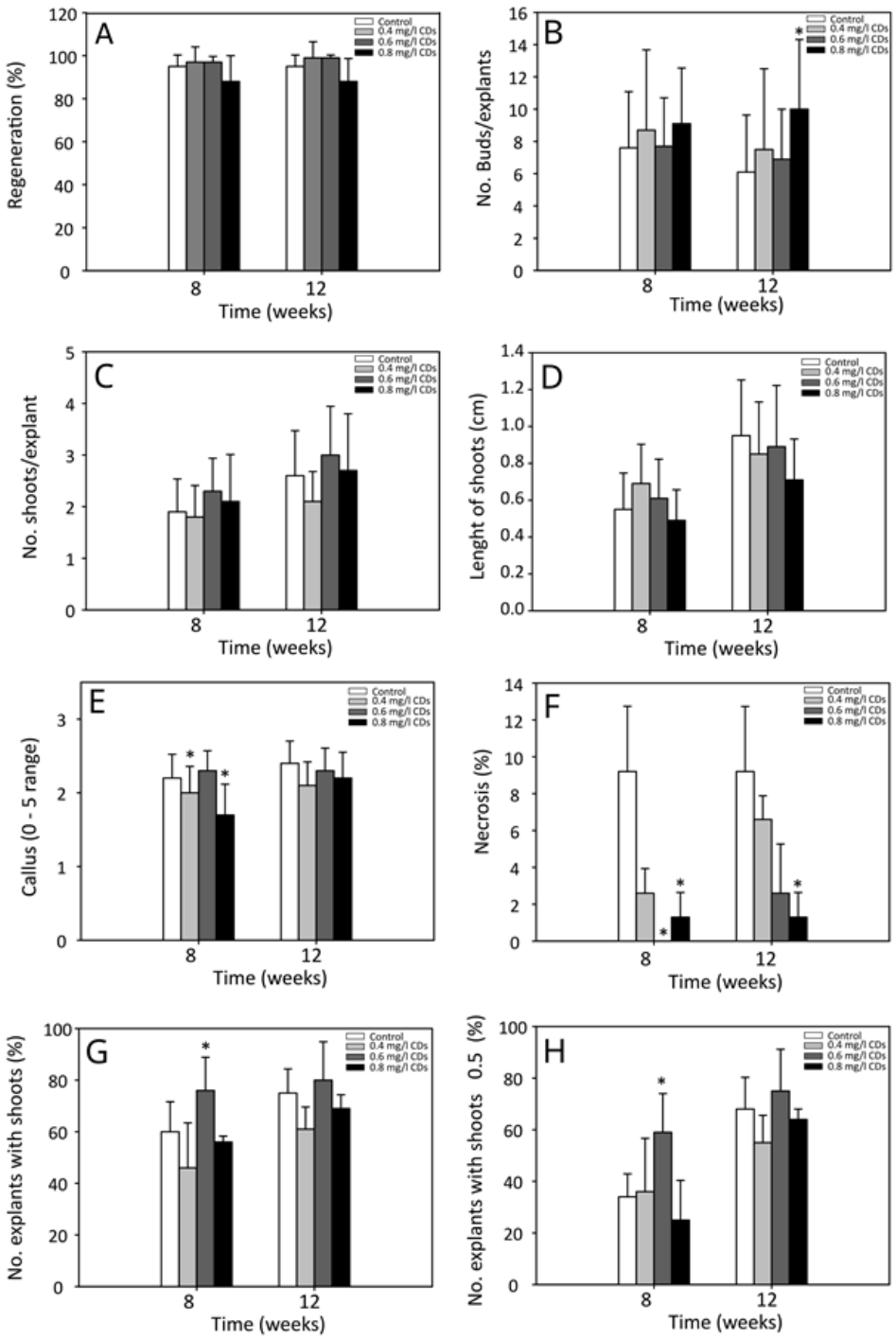

Figure 4

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-1). 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 (Murashige and Skoog, 1962), WPM vitamins (Lloyd and McCown, 1981), 30 g/l sucrose, and 0.6% of agar-0.3 mg L^{-1} BA-100 mg L^{-1} casein hydrolysate.

FIG. 5

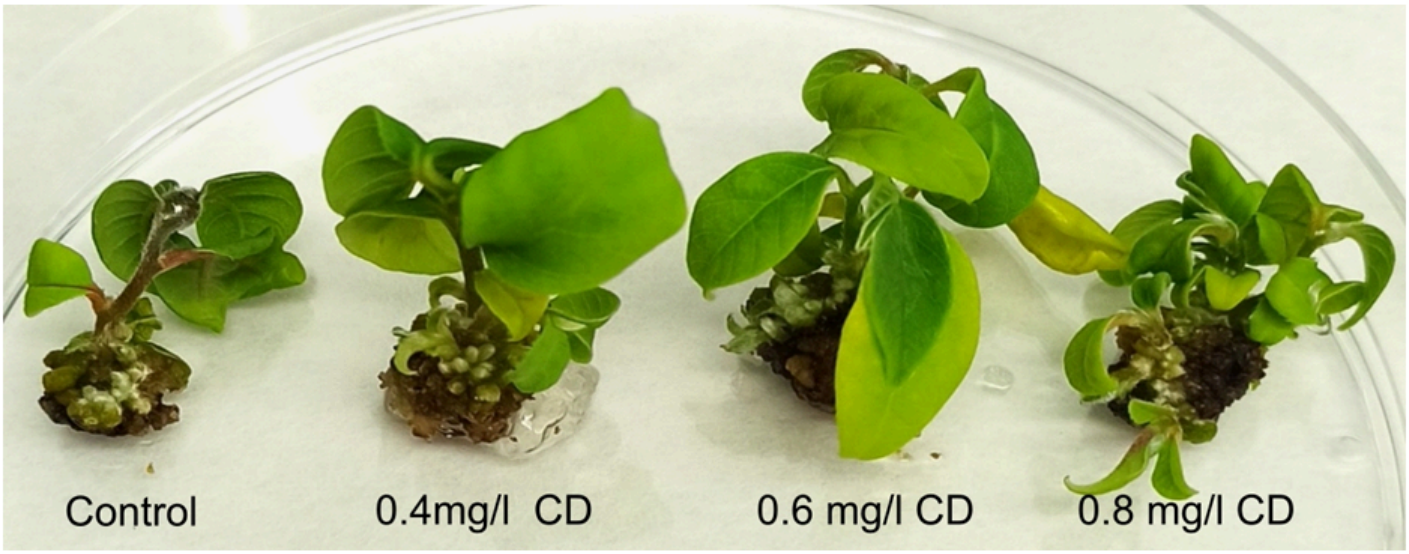

Figure 5

Aspect of the 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 (Murashige and Skoog, 1962), WPM vitamins (Lloyd and McCown, 1981), 30 g/l sucrose, and 0.6% of agar-0.3 mg L^{-1} BA-100 mg L^{-1} casein hydrolysate).

FIG. 6

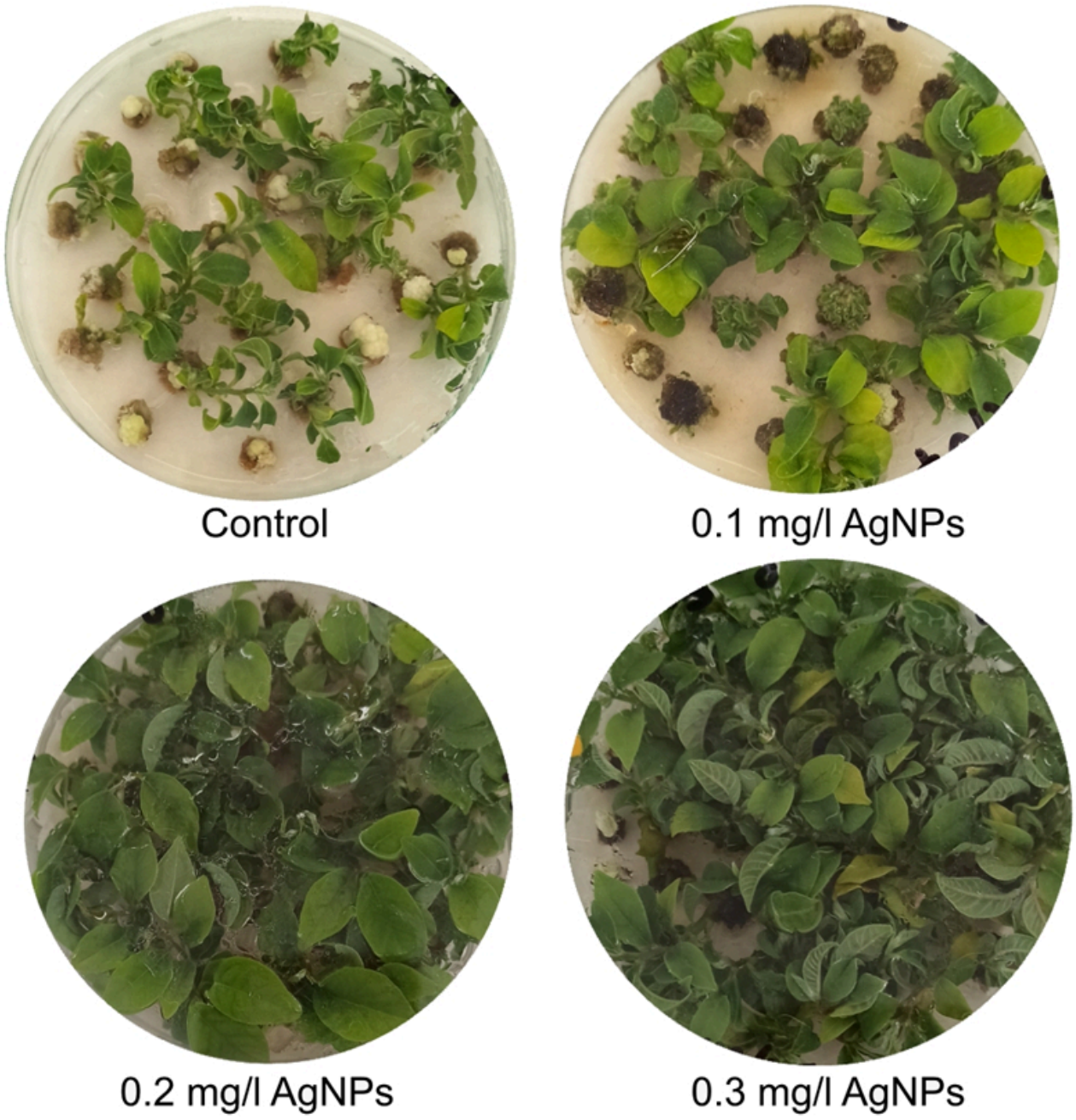

Figure 6

Aspect of the of the shoots after three different treatments with AgNPs on cherimoya hypocotyl CS explants on RM (full-strength MS salt (Murashige and Skoog, 1962), WPM vitamins (Lloyd and McCown, 1981), 30 g/l sucrose, and 0.6% of agar-0.3 mg L^{-1} BA-100 mg L^{-1} casein hydrolysate). Explants are shown at 12 weeks since cultivation.

FIG. 7

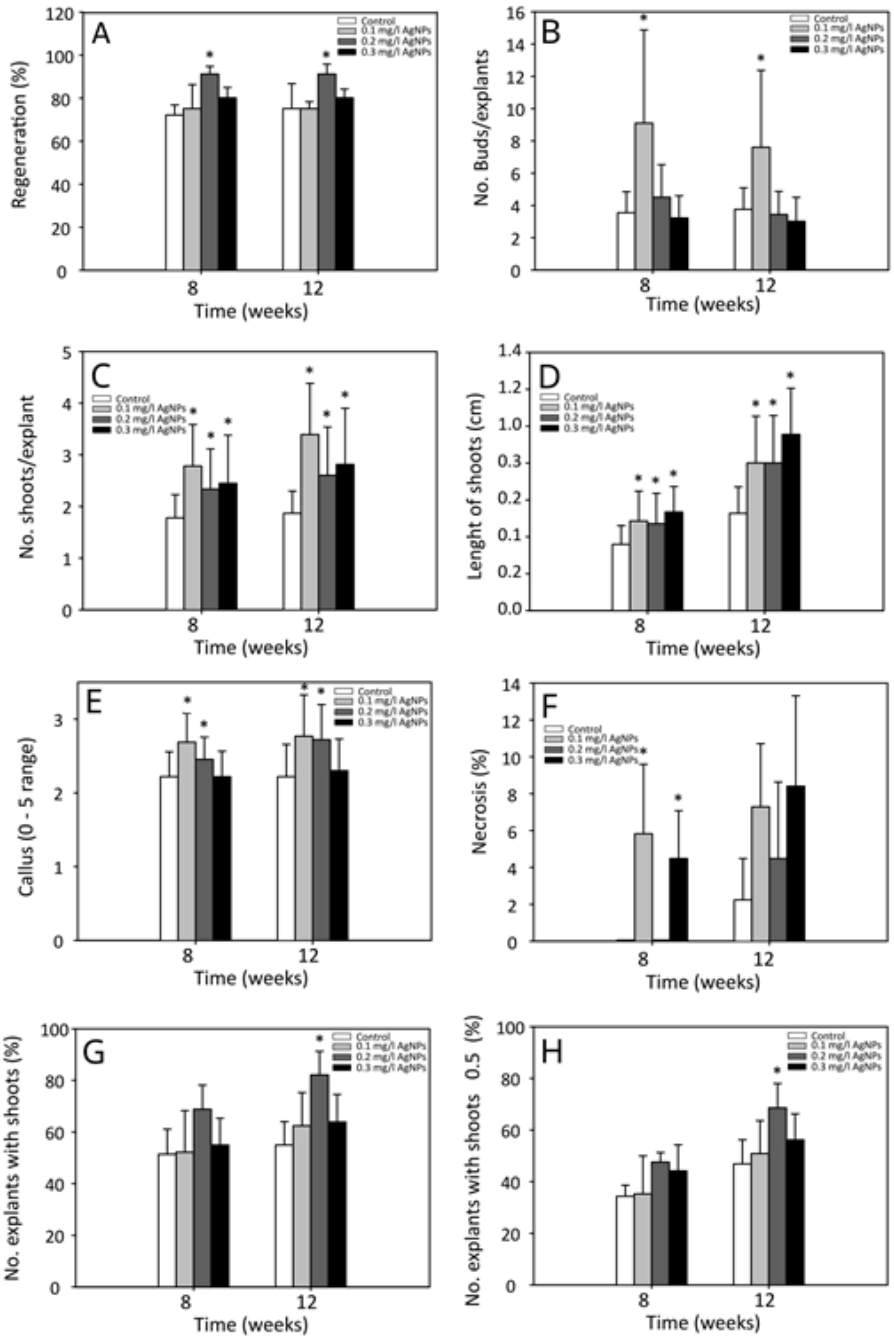

Figure 7

Effects of AgNPs concentration (0.1, 0.2, 0.3 mg L^{-1}) on cherimoya hypocotyl CS explants shoot regeneration in RM (full-strength MS salt (Murashige and Skoog, 1962), WPM vitamins (Lloyd and McCown, 1981), 30 g/l sucrose, and 0.6% of agar-0.3 mg L^{-1} BA-100 mg L^{-1} casein hydrolysate). 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.