

Are silver nanoparticles the “silver bullet” to promote diterpene production in *Stevia rebaudiana*?

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Abstract

S. rebaudiana is a sought after sweetener because of its low-calorie properties. However, the supply of suitable quantities of high quality propagation material is limited by inefficient propagation methods using conventional strategies. *In vitro* techniques combined with nanotechnology tools offer an attractive alternative not only for improved propagation but also for the stimulation of secondary metabolites which represent the targeted sweetener product for this crop. This short report provides an evaluation of silver nanoparticles applied in temporary immersion bioreactors for the abovementioned purpose. Different levels of AgNPs were supplied (0.0–37.5 mg/L) and after 21 d of growth, morphological and biochemical indicators were evaluated. Silver nanoparticles at 25 and 37.5 mg/L decreased shoot multiplication rate, shoot length, and the number of nodes and leaves per shoot compared with the control while no adverse effect was found at the lower tested concentration (12.5 mg/L). Shoot fresh and dry weights also showed statistically significant differences. Regarding the biochemical phenotypes, chlorophyll a, carotenoids and soluble phenolics were increased in plants supplied with 25 mg/L AgNPs, with the latter two indicators suggesting oxidative stress. Interestingly, endogenous levels of diterpenes were significantly increased with the application of 12.5 mg/L AgNPs. It is suggested that AgNPs show potential to act as elicitors to promote the production of diterpenes in stevia but that further work is required to understand the balance between oxidative damage and secondary metabolite production and that optimization of the protocol is required to improve the propagation potential of this strategy.

Introduction

Stevia rebaudiana Bertoni. (*Asteraceae*; Sweet Grass) is native to the subtropical region of Paraguay. It is a perennial shrub which has cast into the spotlight since the discovery that the leaves of this plant produce a sweet taste that is up to 300 times sweeter than sucrose. Due to the presence of low-calorie glucoside sweeteners which have beneficial effects on type II diabetes, *S. rebaudiana* is also regarded as a valuable medicinal plant (Brahmachari et al. 2011; Yüç et al. 2022; Dwivedi 2022). The plant also contains a range of natural antioxidants such as flavonoids, phenols, tannins and essential oils (Christaki et al. 2013; Chaiyana et al. 2021). Although stevia is cultivated outside of its natural range, challenges in production persist such as requirements for regular irrigation, warm temperatures, minimal frost, high light intensities, a naturally long growing season and the fact that it is a poor competitor with weeds. Self-incompatibility problems renders natural propagation difficult. Vegetative propagation is also restricted by limitations on the number of cuttings that may be obtained from a single plant and thereafter successfully established in soil (Carneiro et al. 1997). Therefore, there is a need for investigation of alternative strategies to generate propagation material, and the use of temporary immersion bioreactors represents one such feasible option (Vives et al. 2017).

Bionanotechnology is a rapidly evolving field where nanotechnology tools are increasingly being harnessed to address biological challenges. In a broad context, nanoparticles have been applied in the medical field (Chopra 2022; Hood 2022; Tortella et al. 2022), textile industry (Shah et al. 2022), electronics sector (Wang et al. 2022), the energy field (Kumar et al. 2022) and for environmental remediation (Rafeeq

et al. 2022). With regard to crop production, nanoparticles have been shown to promote seed germination and crop development (El-Batal et al. 2016; Kim et al. 2017). More specifically in plant tissue culture, nanoparticles have been used to stimulate *in vitro* propagation and improve the quality of plantlets such as *Prunus cerasifera* (Khafri et al. 2022), *Capsicum annum* (Asgari-Targhi et al. 2021), banana (Hasanin et al. 2021), chrysanthemum (Tung et al. 2021) and gray poplar (Vasyukova et al. 2021). Of greater relevance is the recent application of nanoparticles as novel elicitors to promote the accumulation of bioactive compounds in plants (Rivero-Montejo et al. 2021; Zhang et al. 2022). This short report provides an evaluation on the effect of silver nanoparticles (AgNPs) on *in vitro* growth stimulation and elicitation of secondary metabolite production in *Stevia* cultured in temporary immersion bioreactors. Different levels of silver nanoparticles were supplied in micropropagation medium for up to 21 d where after morphological and biochemical indicators were evaluated. This assessment included determination of endogenous levels of diterpenes, a chemical group that encompasses the natural sweeteners.

Materials And Methods

The establishment of aseptic cultures of the Morita II cultivar was achieved following the method of Espinal de Rueda et al. (2006). In this regard, nodal segments were excised from mother plants maintained in a greenhouse and were disinfected with sodium hypochlorite (1% v:v) for 5 min, washed three times with sterile distilled water and placed on semi-solid Murashige and Skoog (1962) medium supplemented with 30 g/L sucrose, 100 mg/L myo-inositol, 1 mg/L thiamine and 6 g/l agar for 21 d. Following bud break, shoot multiplication was performed using the same medium formulation with the addition of 0.25 mg/L benzyl amino purine (BAP). After seven cycles of multiplication (with each cycle lasting for 21 d), uniform nodal segments were selected to initiate experiments in bioreactors. Nodal segments (1 cm) containing two axillary buds were grown in temporary immersion bioreactors as recommended by Vives et al. (2017). The culture medium was supplemented with four concentrations of silver nanoparticles, i.e. 0; 12.5; 25.0 and 37.5 mg/L.

Argovit™ silver nanoparticles were provided by the Scientific-Production Centre Vector-Vita Ltd (Novosibirsk, Russia, <http://vector-vita.com/>) as a commercially manufactured product. The Argovit™ formulation is a highly dispersed AgNP suspension at an overall concentration of 200 mg/mL, stabilized with 18.8% polyvinylpyrrolidone (PVP) and containing 1.2% of metallic silver (Bello-Bello et al. 2018). Characterization of Argovit™ AgNPs have been previously reported (Valenzuela-Salas et al. 2019) and is therefore not presented in the current study. The Argovit™ AgNP suspension was diluted with sterile distilled water to reach the targeted concentrations.

For each treatment there were three bioreactors with 20 nodal segments in each. Bioreactors (1000 mL glass flasks) contained 250 mL medium (equivalent to 12.5 mL/explant). All cultures were maintained under cool fluorescent light ($80 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 16 h photoperiod) at $28 \pm 2^\circ\text{C}$. After 21 d of growth, plants were removed from vessels and the following indicators were measured: shoot multiplication rate, length of the primary shoot, number of nodes and leaves per shoot, biomass (shoot fresh and dry weight); and

levels of chlorophyll a, chlorophyll b, carotenoids, soluble phenolics, cell wall-linked phenolics, lignin and diterpenes.

The extraction of chlorophyll pigments were carried out in 5.0 ml of acetone (80%, v:v). Samples were centrifuged (14086.8 xg, 4°C, 15 min), supernatants were collected and the absorbance read at 646.6 and 663.6 nm using a RAYLEIGH, VIS-723G spectrophotometer (Porra 2002). To determine carotenoid levels, absorbance of the same extract was assessed at 470 nm (Lichtenthaler 1987). Phenolic compounds were extracted in 0.5 ml methanol and quantified using a colorimetric assay based on reaction with Folin Ciocalteu reagent (mg gallic acid equivalents per g fresh weight) (Gurr et al. 1992). A spectrophotometric method was also used to measure levels of lignin. Samples were extracted in NaOH solution and the pH was adjusted to 7.0 and 12.3. The amount of lignin was calculated by the difference between A245 (pH 7.0) and A350 (pH 12.3) (Stafford 1960).

Levels of diterpenes were assessed using the method outlined by Vázquez-Baxcajay et al. (2014). Shoots were oven-dried (HS 62A) at 65°C for 72 h and then milled in a Micro-Feinmuhle-Culatti mill at 500 rpm to a particle diameter of 0.7 mm. For extraction, 90% ethanol (2 mL per 0.2 g of dry plant material) was used. The extraction mixture was shaken for 2 hours at room temperature. The resulting homogenate was centrifuged at 8000 × g for 10 min and the supernatant was collected for diterpene measurement. To quantify diterpenes, the absorbance at 210 nm was recorded. Diterpene content was evaluated using a calibration curve (1 mg/mL) (Kolb et al. 2001).

The *in vitro* culture experiment, in a completely randomized design, was repeated three times and representative data are summarized here, while the diterpene quantification was repeated nine times. The statistical programme SPSS (version 20.0) was used to perform One-Way ANOVA and Tukey post-hoc tests ($\alpha = 0.05$). Normal distribution and homogeneity of variances were also demonstrated according to Kolmogorov-Smirnov ($\alpha = 0.05$) and Levene ($\alpha = 0.05$) tests, respectively. For the statistical analysis only, discrete variables (leaves and number of nodes) were transformed according to $y' = y^{0.5}$.

In addition, the overall coefficient of variation (OCV) was calculated as follows: (standard deviation/average) * 100. In this formula, we considered the average values of the four treatments to calculate the standard deviation and average. For this comparison, the larger the difference between the four treatments compared, the higher the OCV (Lorenzo et al. 2015). The OCVs were classified as *Low* = 8.77 to 19.58%, *Medium* = 19.58 to 30.38% and *High* = 30.38 to 41.19%.

Results

Shoots of *S. rebaudiana* were exposed to varying concentrations of AgNPs for 21 d in temporary immersion bioreactors. Evaluation of morphological characteristics showed that concentrations of 25 and 37.5 mg/L of AgNPs resulted in a reduction in shoot multiplication (Fig. 1A), shoot length (Fig. 1B), the number of nodes (Fig. 1C) and leaves per shoot (Fig. 1D) relative to the control. A similar trend was

observed for shoot fresh and dry weights (Fig. 1E and F). However, it is highlighted that these phenotypic indicators (Fig. 1) presented *Low* OCVs which indicate a minor effect of silver nanoparticles.

In terms of plant biochemical response (Fig. 2), *Low* OCVs were also observed for the levels of chlorophyll b (Fig. 2B) and lignin (Fig. 2F). In contrast, a pattern was evident whereby levels of chlorophyll a (Fig. 2A), carotenoids (Fig. 2C) and soluble phenolics were all increased following exposure to 25 mg/L AgNPs and with all mentioned indicators displaying *High* OCVs. Although a decreasing trend in cell wall-linked phenolics was observed as AgNP concentration was increased (Fig. 2E), this indicator also presented a *High* OCV. Interestingly, endogenous levels of diterpenes were significantly increased with 12.5 mg/L silver nanoparticles (Fig. 2G) with a *Medium* OCV.

Discussion

The AgNPs are nanoparticles of silver that measure between 1 and 100 nm in size. Nanoparticles can be generated in a variety of shapes depending on the required application. In most cases, AgNPs are spherical in form, however, diamond, octagonal, and thin sheets are also common (Graf et al. 2003). One of the benefits of nanoparticles is that their large surface area:volume ratio renders them amenable to the coordination of a vast number of ligands. Considering the varying applications of nanoparticles within the nanobiotechnology sphere, studies are in progress to assess the efficacy, biosafety and biodistribution of AgNPs in plants, animals and humans (Cassano et al. 2019).

While nanotechnology applications in other sectors are well advanced, there has been substantially less work undertaken in the plant biotechnology field. The development of efficient micropropagation protocols are dependent on the optimization of a range of factors including culture medium components, plant growth regulators, etc. (Sehgal and Joshi 2022). The inclusion of nanomaterials in biotechnology offers a novel alternative to optimize various stages such as explant disinfection and pathogen removal, somatic embryogenesis, shoot and root proliferation, secondary metabolite production, etc. (Kim et al. 2017). Based on superior physiological characteristics, AgNPs have been at the forefront of nanomaterials research. As highlighted above, nanoparticles can be synthesized in the laboratory by end-users or commercially prepared formulations can be used. Argovit™ is a commercially available solution of AgNPs that has attained regulatory approval for use in veterinary and human applications in Russia and other countries (Borrego et al. 2016) and was therefore used in the present study.

In the present report, AgNPs did not promote plantlet growth and development relative to the control (Fig. 1A-F, *Low* OCVs). For most of the measured parameters, the phenotypic response of stevia plants were similar for the control and 12.5 mg/L AgNPs, with a subsequent decline in growth at the higher tested concentrations. Similar results were reported by Timoteo et al. (2019) with *Campomanesia rufa* and Spinoso-Castillo et al. (2017) with *Vanilla planifolia*. However, in contrast to the present work, the latter study reported improved shoot proliferation at the lower tested concentrations.

Evaluation of photosynthetic pigments was undertaken to provide an indication of photosynthetic activity (Al-Ramamneh et al. 2022). The Argovit™ AgNPs are reported to play an important role in the synthesis of

photosynthetic pigments (Spinoso-Castillo et al. 2017), however inconclusive results were found in the present study. In this regard, an enhancement of chlorophyll a (*High* OCV) was found in plants exposed to 25 mg/L AgNPs but there was no improvement in levels of chlorophyll b. Accumulation of carotenoids is perceived as an indicator of photodamage (Prasad and Chattopadhyay 2016) via oxidative stress (Al-Sammarraie et al. 2020) and in the current study, plant carotenoid content was highest at 25 mg/L AgNP which also corresponded with elevated levels of soluble phenolics (also associated with oxidative stress-discussed below).

A delicate balance exists between the use of nanoparticles as elicitors to promote the accumulation of secondary metabolites and toxicity to plants. At low levels (< 100 mg/L), AgNPs stimulate secondary metabolite synthesis but at higher levels (e.g. 100–250 mg/L), this is inhibited. The central component to the proposed toxicity and/or stimulatory effect of nanoparticles is the production and quenching of ROS. Silver nanoparticles are heavy metals which induce ROS formation in subcellular compartments upon uptake (Rani et al. 2016) and plants attempt to counteract this effect by increasing the production of phenolic compounds with antioxidant capacity (Yasur and Rani 2013). Therefore at low levels, the ROS generated in response to nanoparticles can be quenched with antioxidant defense systems, ultimately leading to improved plant growth via efficient nutrient uptake and metabolism. At high concentrations, nanoparticles result in the excess production of ROS which destabilize antioxidant systems resulting in lipid peroxidation, subcellular damage and a concomitant reduction in transpiration and plant growth (Zhang et al. 2022). In *S. rebaudiana*, it was shown that increased levels of soluble phenolics were produced when plants were exposed to 25 mg/L AgNP with a decline in levels observed at 37.5 mg/L. Spinoso-Castillo et al. (2017) contended that at high Argovit™ concentrations, phenolic compound contents (and consequent antioxidant capacity) was reduced as a consequence of ROS production leading to a decrease in shoot number and length, the latter finding was also observed in the present study.

In the present study, the levels of lignin were marginally increased in response to elevated concentrations of AgNPs as similarly reported by Bernard et al. (2015) working on *Thymus daenensis*. As highlighted by Mishra et al. (2017) the effect of AgNPs on lignification of cell walls remains largely unstudied. Bernard et al. (2015) have suggested a possible correlation between lignification and availability of polyamines and some work has been done on lignification in response to pathogen attack which can be ameliorated by AgNPs (Mishra et al. 2017) where it was proposed that lignification was more pronounced under stress conditions.

In stevia, the diterpene steviol glycoside provides the characteristic sweet taste attributed to this plant. Given the increasing demand for steviol as a zero calorie sweetener, the present study considered the role of AgNPs as elicitors to promote the production of these bioactive compounds (assessed as total diterpene content). The results showed that AgNPs applied at 12.5 and 25 mg/L yielded a higher concentration of diterpenes than the control. Similarly, Ramezani et al. (2019) reported that AgNPs stimulated the production of various glycosides (particularly stevioside) in *S. rebaudiana*. However, that study did not report on plant growth in response to AgNP application. Based on our initial study, we

propose that at low concentrations (12.5 mg/L), AgNPs could act as a positive stimulus for the production of diterpenes in stevia with neutral effects on plant growth. At higher concentrations (25 mg/L), AgNPs yield marginally more diterpenes than untreated plants, but this is accompanied by putative free radical-mediated cellular damage (exhibited by elevated carotenoid and soluble phenolics production). The role of nanoparticles in eliciting stimulation of secondary metabolites is complex involving cross talk between multiple pathways with calcium and ROS reportedly acting as secondary messengers and upregulation of MAPK networks ultimately leading to transcriptional regulation of secondary metabolism (Ramezani et al. 2019). Further studies are required to elucidate the exact role attributed to ROS in this process (including the associated antioxidant response) and where modifications can be made to promote diterpene production without adverse effects.

Declarations

Author contribution

IA, MG, JCGR, EH, NB, AP, OC, JCL and ME designed the research; IA and MG conducted the experiment; IA, JCGR, EH, NB, AP, OC, JCL and ME analyzed the data and wrote the paper; and EH, JCL and ME had primary responsibility for the final content. All authors have read and approved the final manuscript.

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Conflict of interest Authors do not have any conflict of interests.

Data availability statement The data that support the findings of this study are available from the corresponding author (ME) upon reasonable request.

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Figures

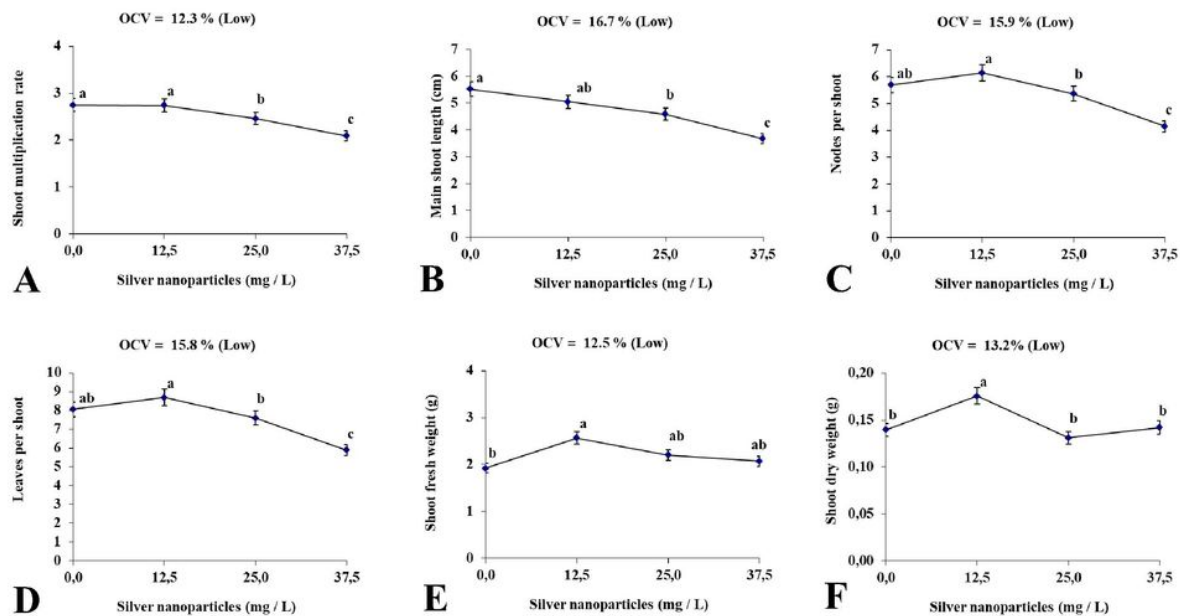


Figure 1

Visual phenotypes of stevia cultured in temporary immersion bioreactors with silver nanoparticles. Results with the same *letter* are not statistically different (One-Way ANOVA, Tukey, $p > 0.05$). OCV were classified as *Low* when varied from 8.77 to 19.58%, *Medium* from 19.58 to 30.38%, and *High* from 30.38 to 41.19%. Vertical bars represent SE.

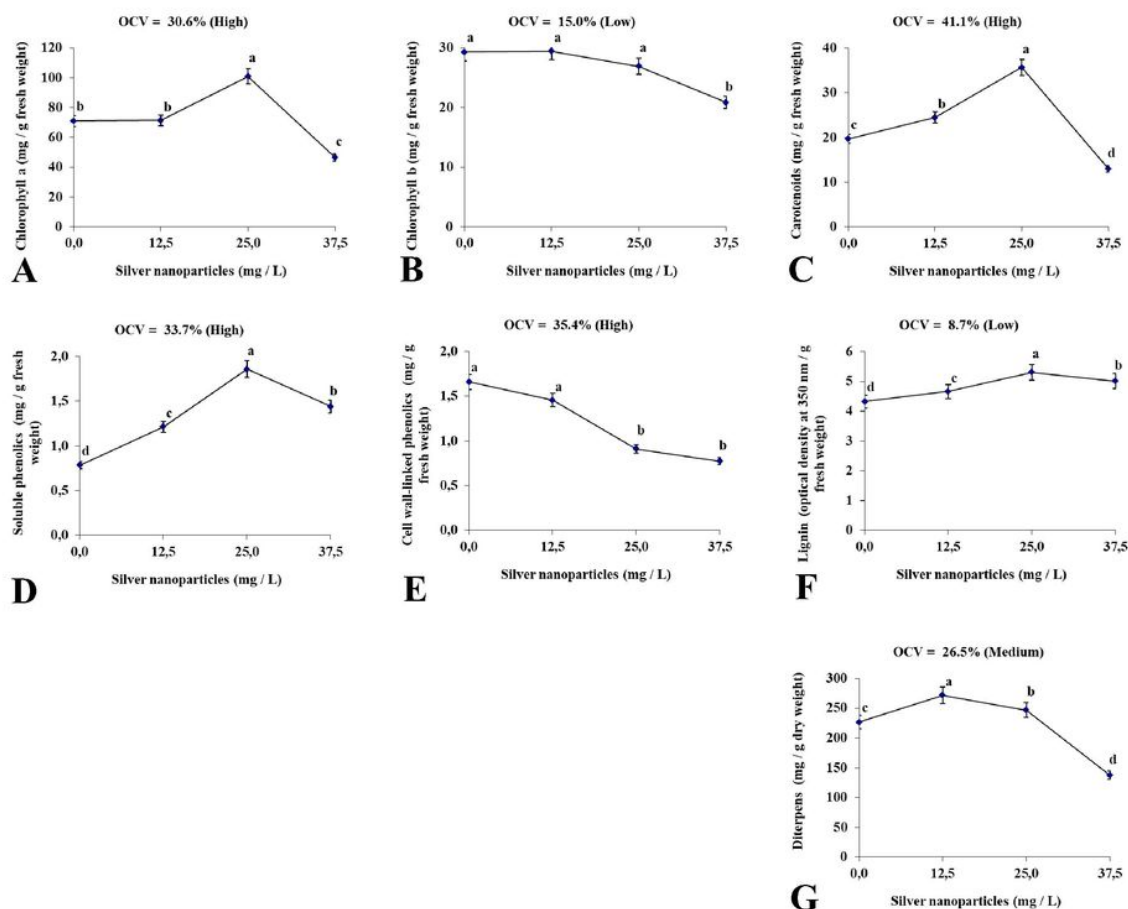


Figure 2

Biochemical phenotypes of stevia cultured in temporary immersion bioreactors with silver nanoparticles. Results with the same *letter* are not statistically different (One-Way ANOVA, Tukey, $p > 0.05$). OCV were classified as *Low* when varied from 8.77 to 19.58%, *Medium* from 19.58 to 30.38%, and *High* from 30.38 to 41.19%. Vertical bars represent SE.