

# Impact of zinc oxide nanoparticles and iron on *Stevia rebaudiana* Bertoni growth, nutrient uptake, and bioactive compounds under in vitro conditions

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## Research Article

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# Abstract

The experiment investigated the effects of different levels of zinc oxide nanoparticles (ZnONPs) (0, 10, 20, and 30 mg/L) and iron sulfate (13.9, 27.8, and 55.6 mg/L) on morphological and physiological responses of *Stevia rebaudiana* Bertoni plant under *in vitro* conditions. Results indicated that the combined application of ZnONPs at 10 mg and iron at 27.8 mg led to the highest increase in shoot number, height, and biomass, showing a respective rise of 17.37%, 39.66%, and 45.02% compared to control cultures. The highest pigment content and tissue antioxidant activity (83.48%) was observed with the combined presence of 10 mg/L ZnONPs and 27.8 mg/L iron. As ZnONP concentration increased in the culture medium, the combined effect on lipid peroxidation rate became more pronounced. The impact of ZnONPs on phenolic compound production varied depending on the specific substance. The iron content of shoots increased significantly by 41.11% under the influence of 27.8 mg/L iron and 10 mg/L ZnONP compared to control cultures. Interaction effects of treatments at various levels resulted in increased zinc content in shoots, peaking at 27.8 mg/L iron when ZnONP reached 20 mg/L, representing a 56.28% increment over control levels before slightly decreasing. The most increases in stevioside and rebaudioside were observed with the combination of 10 mg/L ZnONP and 27.8 mg/L iron, showing enhancements of 75.04% and 63.08%, respectively. These findings suggest that ZnONPs could stimulate the growth and enhance the bioactive components of stevia plants, making them a viable option as elicitors in *in vitro* batch cultures.

## Introduction

The stevia plant (*Stevia rebaudiana* Bertoni), produces non-absorbable sweet-tasting diterpene glycosides, including stevioside and rebaudioside. These glycosides are safe, non-toxic, and non-mutagenic, with sweetness levels over 300 times that of sucrose (Khiraoui et al. 2017; Ahmad et al. 2020a). Stevia is becoming increasingly popular as a sugar substitute, as it is calorie-free and has no known adverse health effects. This sugar substitute sweetens a wide range of products in the food and pharmaceutical industries, including soft drinks, beverages, gum, chocolates, cakes, jams, and even some medications (Naik and Poyil 2022).

Today, nanoparticles (NPs) are gaining importance in plant science due to their multifaceted impact on plant life. NPs, absorbed through roots or leaves, can positively, negatively, or neutrally influence plant growth and development, with mechanisms influenced by size, shape, and concentration (Azim et al. 2023).

ZnONPs can have significant impacts on cellular processes, structures, plant growth, and function, despite zinc being an essential element for many of these processes. They enhanced corn growth, leaf area, dry weight, and altered fatty acid profiles (Taheri et al. 2016), vegetative and yield parameters in corn (Tondey et al. 2021), root architecture in soybean (Yusefi-Tanha et al. 2022), amaranth biomass (Cai et al. 2022), photosynthesis in cucumber seedlings (Ghani et al. 2022) and seed germination and seedling length in onions (Abhilash et al. 2023). However, high concentrations of ZnONPs reduced photosynthesis in *Zea mays* and *Arabidopsis thaliana* (Zhao et al. 2015; Wang et al. 2018). Furthermore, ZnONPs affect the uptake of elements and heavy metals in plants. They reduced root cadmium and lead content; in the shoot, while copper and iron increased (Sharifan et al. 2020). Ahmed et al. (2023) found that applying ZnONPs to tomato plants increased zinc uptake and other nutrients, leading to improved plant growth and fruit production.

Studies reveal nanoparticles have the potential to alter the chemical profile of plant metabolites.

In various plants, ZnONPs increased the production of beneficial compounds: essential oil (Nekoukhou et al. 2022; Moghimipour et al. 2017), flavonoids and phenolics (Iziy et al. 2019), and polyphenols and antioxidants (Salachna

et al. 2021; Ruiz-Torres et al. 2021). On the other hand, some NPs, including copper hydroxide, cerium oxide, and copper oxide, actually lowered the amount of phenylpropanoids found in the plants studied (Huang et al. 2019; Zhang et al. 2019; Zhao et al. 2017). Interestingly, Kruszka et al. (2022) showed that certain NPs (silver, gold, copper, and palladium) increased the amount of xanthones, prenylated xanthones, and benzophenones in St. John's Wort (*Hypericum perforatum* L.) cells while decreasing the production of flavonoids and hydroxycinnamic acid derivatives. NPs' conflicting impacts on plant metabolism highlight their complexity and diversity. It emphasizes the need for further investigation into the underlying mechanisms and how specific factors influence the nature and direction of these changes.

In plant tissue culture, elicitation is a hopeful approach to improve the output of secondary metabolites, which is a powerful tool that allows for manipulating growth conditions and adding elicitors (Largia et al. 2023). Although some studies have investigated the effects of common elicitors such as salicylic acid and jasmonates on steviol glycoside biosynthesis in stevia, there is less information available on the impact of NPs on growth and steviol glycoside production in the plant. Studies showed a concentration-dependent effect of metal NPs on stevia. Low concentrations promoted plant growth, steviol glycoside production, and antioxidant activity. Conversely, higher concentrations exhibited phytotoxic effects, reducing biomass and bioactive compound production (Javed et al. 2017; Ahmad et al. 2020b). *In vitro* studies show the influence of NPs on plant growth and metabolite production can vary significantly depending on the specific NP used, its concentration, and the surrounding conditions in which the plants are grown (Humbal and Pathak 2023). Optimizing NP use for commercial Stevia production requires further research into their effects on steviol glycoside levels. Thus, this study investigates the potential effects of ZnONPs on growth parameters, metabolite production, and the uptake of zinc and iron by stevia plants grown in a tissue culture system.

## Materials and methods

### Sterilization and cultivation of explants

Stevia seeds were purchased from Pakan Seeds Company (Isfahan, Iran). Seeds were sterilized by immersion in a 2.5% sodium hypochlorite solution for 10 minutes, followed by four washes with sterile distilled water. They were then cultured in MS (Murashige and Skoog 1962) basal medium to obtain shoot apical explants. After the growth of seedlings, shoot apical explants were cut and cultured in MS medium comprising 30 g/L sucrose, 8 g/L agar with pH 5.7, and 6-benzylaminopurine growth regulator at a concentration of 2 mg/L to produce sufficient shoots for the experiments. The cultured explants were transferred to a growth chamber with a temperature of  $25 \pm 1^\circ\text{C}$ , humidity of 60%, and a light period of 16 hours per day for maintenance. Several subcultures were performed in the same medium to obtain new branches and sufficient samples for treatment. After six months of multiplication of stevia explants and getting sufficient plants under *in vitro* conditions, the shoots were prepared for cultivation in the following stages and treatment.

### Applying treatments

The treatments included ZnONPs (Sigma-Aldrich, Steinheim, Germany, Cas no. 1314-13-2) at (0, 10, 20, and 30) mg/L and iron sulfate at (9.13, 8.27, and 6.55) mg/L concentrations. The desired treatments were first sterilized using 0.2  $\mu\text{m}$  syringe filters. Before planting the shoots in the culture medium, the selected treatments were each added separately to the basal medium under sterile conditions. Distilled water was used as a control in the basal medium to study and compare the samples treated with the compounds mentioned above with untreated plants.

## Measurement of morphological traits

After one month and sufficient growth of the samples, traits such as the number of seedlings, fresh weight, height of seedlings, rooting, and observed color were recorded. Following the analysis of their morphological characteristics, the samples were frozen in liquid nitrogen and kept in a deep freezer (-80°C) for further studies.

## Measurement of photosynthetic pigments

For determining the chlorophyll content, 200 mg of freezing samples were extracted in dark conditions by ethanol (95%) at 4°C. After passing 72 h, samples were centrifuged at 15000×g for 10 min, and the supernatant was read by a spectrophotometer at wavelengths 664 and 648 nm (Baskar et al. 2018). The following formulas showed calculations of chlorophyll a, b, and carotenoids, respectively.

$$\text{Chlorophyll a} = V * (12.21 * A_{663} - 2.81 * A_{646}) / (1000 * W)$$

$$\text{Chlorophyll b} = V * (20.13 * A_{646} - 5.03 * A_{663}) / (1000 * W)$$

$$\text{Carotenoids} = (1000 * A_{470} - 1.82 * \text{Chla} - 85.02 * \text{Chlb}) / 198$$

The results were represented as mg/g FW.

## Measurement of phenolic compounds

To measure phenolic compounds, the Folin-Ciocalteu method was employed (Makkar et al. 2007). To prepare the plant extract, 200 mg of fresh tissue from each sample was first homogenized in an ethanolic acidic solution (ethanol: acetic acid, 99:1) and then centrifuged at 15000×g for 10 min. Subsequently, 100 µl of the supernatant of each sample was taken, and 100 µl of Folin-Ciocalteu reagent and 100 µl of 7% sodium carbonate were added to it. The amount of phenolic compounds was determined by measuring absorbance at a wavelength of 720 nm using gallic acid as the standard. Then, the total phenol content of each sample was calculated by plotting a standard curve and inserting the obtained values into the linear equation.

## Measurement of total flavonoid content

An aluminum chloride (AlCl<sub>3</sub>) reagent was used to measure the total flavonoid content (Stankovic 2011). To prepare the plant extract, 200 mg of fresh tissue from each sample was first homogenized in an ethanolic acidic solution (ethanol: acetic acid, 99:1) and then centrifuged at 15000×g for 10 min. Subsequently, 100 µl of the supernatant of each sample was taken and mixed with 200 µl of 2% aluminum chloride solution (AlCl<sub>3</sub>, 6H<sub>2</sub>O) (dissolved in 80% ethanol) for 30 minutes in darkness at room temperature. Then, the absorbance was measured at a wavelength of 430 nm. The total flavonoid content was calculated using the standard curve of apigenin as a reference.

## Measurement of anthocyanin content

To prepare the plant extract for the extraction and measurement of anthocyanins, initially, 200 mg of fresh tissue from each sample was homogenized in an ethanolic acidic solution (ethanol: acetic acid, 99:1) and then centrifuged at 15000×g for 10 min. Subsequently, the supernatant was separated and stored in darkness in the refrigerator overnight. Then, the absorbance was measured at a wavelength of 550 nm. The amount of anthocyanin in each sample was calculated using the extinction coefficient of 33000 M<sup>-1</sup> cm<sup>-1</sup> (Hara et al. 2003).

## Determination of antioxidant potential

To measure the antioxidant potential, 200 mg of fresh tissue from each sample was homogenized in methanol and then centrifuged at 15000×g for 10 min. Subsequently, for assessing the scavenging capacity of DPPH radicals (1,1-diphenyl-2-picrylhydrazyl), initially, 200 µl of the extract from each sample were mixed with 80 µl of DPPH solution (2.0 mM in methanol) as a source of free radicals. The samples were then placed in darkness at room temperature for 30 minutes. Afterward, the absorbance of each sample was measured at a wavelength of 517 nm (Baliyan et al. 2022). The blank solution consisted of 200 µl of ethanol + 80 µl of DPPH, while the control solution consisted of 200 µl of ethanol + 80 µl of the extract. Each sample's antioxidant potential percentage was calculated using the following formula.

$$(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100 = \text{Antioxidant potential (\%)}$$

## Measurement of membrane lipid peroxidation

To determine the extent of membrane damage by measuring the amount of malondialdehyde (MDA) as the end product of membrane lipid peroxidation, 200 mg of fresh tissue from each sample was homogenized with 10% trichloroacetic acid and then centrifuged at 15000×g for 10 min. Subsequently, 500 µl of the supernatant from each sample was mixed with 500 µl of 25% thiobarbituric acid and placed in a boiling water bath at 100 °C for 30 minutes. After cooling down the samples, the absorbance was measured at 600 nm and 532 nm wavelengths. The amount of MDA was calculated using the molar extinction coefficient (De Vos et al. 1991).

## Measurement of PAL enzyme activity

To extract and measure PAL enzyme activity, 200 mg of fresh tissue from each sample was homogenized with a Tris-HCl buffer (50 mM, pH 8.8) containing β-mercaptoethanol (15 mM) and then centrifuged at 15000×g for 10 min. The measurement of the PAL enzyme was based on the amount of cinnamic acid produced. This was done by initially taking 100 µl of the extracted sample from each and adding 500 µl of Tris buffer, 250 µl of 10 mM phenylalanine, and 200 µl of deionized water. The mixture was then incubated at 37°C in a water bath for one hour. The enzymatic reaction was stopped by adding 500 µl of 6 M HCl. Ethyl acetate was added to the samples for product extraction, followed by vortexing. After evaporating the ethyl acetate, the remaining solid was dissolved in 0.5 M NaOH solution. The absorbance of the samples was measured at a wavelength of 290 nm, and PAL enzyme activity was determined using cinnamic acid as a standard (Ochoa-Alejo and Gómez-Peralta 1993). One unit of PAL enzyme activity is the production of 1 µg of cinnamic acid per minute.

## Measurement of iron and zinc Content

To determine the levels of iron and zinc in the samples, an atomic absorption spectrometer (AA400, Perkin Elmer) was utilized. For this purpose, a dry digestion method was employed to prepare the extracts (Karpiuk et al. 2016). Initially, 200 mg of each sample was weighed and placed in labeled pouches. The samples were then dried for 48 hours at 72°C for desiccation. The dried samples were crushed in a mortar until they turned into powder. The powdered samples were placed in crucibles and transferred to a furnace for ash preparation. The samples were maintained at 800°C for 8 hours. After removing the samples from the furnace, 10 ml of 2N hydrochloric acid was added and placed on a hot plate at 80°C. The crucibles were left on the hot plate until just before boiling and the appearance of the first white vapor. Then, the contents inside the crucibles were poured into a 25 ml volumetric flask after passing through filter paper, and distilled water was added to reach the mark. After preparing the atomic absorption spectrometer, the extracts were injected into the device, and both iron and zinc levels were measured and calculated in the extracts.

# Extraction and measurement of rebaudioside and stevioside

To measure the levels of rebudioside and stevioside, the samples were homogenized in 70% ethanol and then centrifuged at 15000×g for 10 min. The separated solution was placed in a water bath at 70°C for 30 minutes. The samples were then evaporated, and 250 µl of HPLC-grade acetonitrile was added to the resulting residue and thoroughly mixed. The levels of stevioside and rebudioside were determined using High-Performance Liquid Chromatography (HPLC) (Knauer, Germany) and a C<sub>18</sub> column. The flow rate of the solvent was set to 1 ml/min, and the mobile phase was an isocratic mixture of water and acetonitrile in a ratio of 20:80, respectively. The detector used was UV-based, with a wavelength set at 210 nm. The amounts of stevioside and rebudioside were calculated by comparing them to related standard samples (Hearn and Subedi 2009).

## Statistical analysis

The experiment was executed following a factorial approach with a completely randomized design and included four replications. For statistical analysis, the SAS software (version 9.2) was utilized to perform both the analysis of variance and means comparison, employing Duncan's multiple range test.

## Results

### Shoot growth and proliferation

This study examined the effects of different concentrations of iron and ZnONPs on shoot growth. Tables 1 and 2 illustrate that shoot growth parameters underwent significant alterations at different iron and ZnONP concentrations. The highest number of shoots was observed at an iron concentration of 27.8 mg/L, with no significant difference in the other two concentrations. In the presence of ZnONPs, the parameter reached its maximum at 10 mg/L, after which it substantially declined. Both shoot height and fresh biomass exhibited a notable increase in response to iron and ZnONPs, up to 27.8 mg/L and 10 mg/L, respectively, before diminishing at higher concentrations of these factors. Moreover, the study revealed that the combined influence of iron and ZnONPs significantly impacted shoot growth compared to the control culture. As the level of ZnONPs increased to 10 mg/L, the effect of iron on shoot proliferation, height, and fresh biomass became notably potentiated. However, beyond that point, the interaction had no effect or adversely affected the parameters compared to the control culture. The most significant increase in shoot numbers, by 17.37%, was achieved when combining 27.8 mg/L of iron with 10 mg/L ZnONP, in contrast to the corresponding control. Regarding shoot height and fresh biomass, the most positive interaction was observed when using NPs at a concentration of 10 mg/L with varying iron concentrations, surpassing other combined treatments. The combined influence of NPs at 10 mg and 27.8 mg of iron resulted in the highest shoot height and biomass, showcasing a 39.66% and 45.02% increase compared to the control cultures. In contrast, the combined influence of all iron levels and ZnONPs at 20 mg/L showed no significant effect on the parameters, while at 30 mg/L, it significantly reduced them compared to the control cultures (Fig. 1).

Table 1

Comparison of the mean of traits measured in tissue culture of stevia plant under the effect of iron treatment.

| Fe<br>(mg/L) | Shoot<br>no./explant     | Height<br>(mm)               | FW<br>(g/sh)                | Chla<br>(mg/g<br>FW)        | Chlb<br>(mg/g<br>FW)        | Carotenoid<br>(mg/g FW)  | Phenolics<br>(mg/g<br>FW)   | Flavonoid<br>(mg/g<br>FW)   |
|--------------|--------------------------|------------------------------|-----------------------------|-----------------------------|-----------------------------|--------------------------|-----------------------------|-----------------------------|
| 13.9         | 7.15 ± 0.85 <sup>b</sup> | 16.21 ±<br>1.32 <sup>b</sup> | 3.25 ±<br>0.22 <sup>b</sup> | 0.85 ±<br>0.05 <sup>c</sup> | 0.49 ±<br>0.02 <sup>c</sup> | 1 ± 0.06 <sup>c</sup>    | 0.76 ±<br>0.03 <sup>c</sup> | 1.45 ±<br>0.07 <sup>c</sup> |
| 27.8         | 8.89 ± 0.97 <sup>a</sup> | 20.59 ±<br>2.37 <sup>a</sup> | 5.23 ±<br>0.63 <sup>a</sup> | 1.69 ±<br>0.11 <sup>a</sup> | 0.76 ±<br>0.05 <sup>a</sup> | 1.8 ± 0.11 <sup>a</sup>  | 0.88 ±<br>0.05 <sup>b</sup> | 1.65 ±<br>0.07 <sup>b</sup> |
| 55.6         | 7.42 ± 0.52 <sup>b</sup> | 15.99 ±<br>1.34 <sup>b</sup> | 3.67 ±<br>0.16 <sup>b</sup> | 1.35 ±<br>0.08 <sup>b</sup> | 0.54 ±<br>0.03 <sup>b</sup> | 1.28 ± 0.07 <sup>b</sup> | 1.14 ±<br>0.14 <sup>a</sup> | 1.71 ±<br>0.08 <sup>a</sup> |

The difference between the means that do not share letters is significant.

Table 1  
Continued.

| Fe<br>(mg/L) | Anthocyanin<br>(mg/g FW) | PAL<br>(µM<br>cinnamic<br>acid/min) | MDA<br>(nmol/g<br>FW)       | AP<br>(%)                    | Fe<br>content<br>(mg/kg<br>FW) | Zn<br>content<br>(mg/kg<br>FW) | Stevioside<br>content<br>(mg/g<br>FW) | Rebaudioside<br>content<br>(mg/g FW) |
|--------------|--------------------------|-------------------------------------|-----------------------------|------------------------------|--------------------------------|--------------------------------|---------------------------------------|--------------------------------------|
| 13.9         | 0.42 ± 0.04 <sup>c</sup> | 0.52 ±<br>0.03 <sup>c</sup>         | 0.32 ±<br>0.03 <sup>c</sup> | 51.35<br>± 3.15 <sup>c</sup> | 3.19 ±<br>0.12 <sup>c</sup>    | 1.81 ±<br>0.06 <sup>c</sup>    | 35.06 ±<br>3.06 <sup>b</sup>          | 18.34 ± 1.30 <sup>b</sup>            |
| 27.8         | 0.59 ± 0.07 <sup>b</sup> | 0.61 ±<br>0.04 <sup>b</sup>         | 0.38 ±<br>0.05 <sup>b</sup> | 56.18<br>± 4.63 <sup>b</sup> | 3.66 ±<br>0.10 <sup>b</sup>    | 1.83 ±<br>0.05 <sup>b</sup>    | 41.46 ±<br>3.31 <sup>ab</sup>         | 21.32 ± 1.10 <sup>ab</sup>           |
| 55.6         | 0.67 ± 0.03 <sup>a</sup> | 0.84 ±<br>0.09 <sup>a</sup>         | 0.47 ±<br>0.03 <sup>a</sup> | 61.37<br>± 2.85 <sup>a</sup> | 4.18 ±<br>0.13 <sup>a</sup>    | 1.80 ±<br>0.08 <sup>a</sup>    | 43.61 ±<br>2.42 <sup>a</sup>          | 22.3 ± 0.78 <sup>a</sup>             |

The difference between the means that do not share letters is significant.

Table 2  
Comparison of the mean of traits measured in the tissue culture of Stevia plant under the effect of ZnONPs treatment.

| ZnONPs (mg/L) | Shoot no./explant        | Height (mm)                | FW (g/p)                 | Chla (mg/g FW)           | Chlb (mg/g FW)           | Carotenoid (mg/g FW)     | Phenolics (mg/g FW)      | Flavonoid (mg/g FW)      |
|---------------|--------------------------|----------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| 0             | 7.24 ± 1.19 <sup>b</sup> | 19.6 ± 0.97 <sup>b</sup>   | 4.2 ± 0.15 <sup>b</sup>  | 1.25 ± 0.10 <sup>d</sup> | 0.56 ± 0.02 <sup>d</sup> | 1.02 ± 0.08 <sup>d</sup> | 0.83 ± 0.04 <sup>d</sup> | 0.98 ± 0.09 <sup>d</sup> |
| 10            | 8.44 ± 0.65 <sup>a</sup> | 25.02 ± 1.35 <sup>a</sup>  | 5.66 ± 0.16 <sup>a</sup> | 1.28 ± 0.22 <sup>c</sup> | 0.59 ± 0.01 <sup>c</sup> | 1.16 ± 0.25 <sup>c</sup> | 1.26 ± 0.03 <sup>b</sup> | 1.72 ± 0.13 <sup>a</sup> |
| 20            | 6.76 ± 0.59 <sup>b</sup> | 17.81 ± 1.05 <sup>bc</sup> | 3.53 ± 0.25 <sup>c</sup> | 1.34 ± 0.07 <sup>a</sup> | 0.62 ± 0.03 <sup>a</sup> | 1.38 ± 0.02 <sup>a</sup> | 1.55 ± 0.1 <sup>a</sup>  | 1.64 ± 0.05 <sup>b</sup> |
| 30            | 5.81 ± 1.12 <sup>c</sup> | 13.31 ± 2.38 <sup>c</sup>  | 3.14 ± 0.85 <sup>c</sup> | 1.32 ± 0.18 <sup>b</sup> | 0.61 ± 0.05 <sup>b</sup> | 1.27 ± 0.16 <sup>b</sup> | 1.16 ± 0.13 <sup>c</sup> | 1.26 ± 0.07 <sup>c</sup> |

The difference between the means that do not share letters is significant.

Table 2  
Continued.

| ZnONPs (mg/L) | Anthocyanin (mg/g FW)    | PAL (μM cinnamic acid/min) | MDA (nmol/g FW)          | AP (%)                    | Fe content (mg/kg FW)    | Zn content (mg/kg FW)    | Stevioside content (mg/g FW) | Rebaudioside content (mg/g FW) |
|---------------|--------------------------|----------------------------|--------------------------|---------------------------|--------------------------|--------------------------|------------------------------|--------------------------------|
| 0             | 0.54 ± 0.05 <sup>c</sup> | 0.45 ± 0.06 <sup>d</sup>   | 0.36 ± 0.06 <sup>d</sup> | 54.35 ± 3.51 <sup>d</sup> | 3.66 ± 0.12 <sup>c</sup> | 1.83 ± 0.05 <sup>c</sup> | 41.46 ± 3.01 <sup>d</sup>    | 21.32 ± 1.01 <sup>c</sup>      |
| 10            | 0.81 ± 0.04 <sup>a</sup> | 0.81 ± 0.08 <sup>a</sup>   | 0.54 ± 0.06 <sup>c</sup> | 82.52 ± 2.25 <sup>a</sup> | 5.07 ± 0.17 <sup>a</sup> | 2.81 ± 0.06 <sup>a</sup> | 72.61 ± 2.26 <sup>a</sup>    | 34.77 ± 0.98 <sup>a</sup>      |
| 20            | 0.69 ± 0.08 <sup>b</sup> | 0.65 ± 0.11 <sup>b</sup>   | 0.63 ± 0.04 <sup>b</sup> | 70.38 ± 1.93 <sup>b</sup> | 4.54 ± 0.15 <sup>b</sup> | 2.76 ± 0.05 <sup>a</sup> | 64.57 ± 1.01 <sup>b</sup>    | 33.46 ± 1.23 <sup>a</sup>      |
| 30            | 0.52 ± 0.07 <sup>c</sup> | 0.57 ± 0.05 <sup>c</sup>   | 0.74 ± 0.06 <sup>a</sup> | 65.42 ± 2.59 <sup>c</sup> | 3.96 ± 0.11 <sup>c</sup> | 2.48 ± 0.03 <sup>b</sup> | 52.89 ± 2.03 <sup>c</sup>    | 29.01 ± 1.04 <sup>b</sup>      |

The difference between the means that do not share letters is significant.

## Photosynthetic pigments

The study demonstrated that iron and ZnONP concentrations significantly affected photosynthetic pigment content in the shoot cultures. As shown in Table 1, iron presence significantly affected the shoot culture photosynthetic pigments (chlorophyll a, b, and carotenoid) content. The highest levels of these pigments were observed when the iron concentration was at 27.8 mg/L. Beyond this threshold, there was a decline in pigment content. Furthermore, the concentration of ZnONPs also yielded significant results. Increasing ZnONP

concentration up to 20 mg/L led to a notable increase in chlorophyll a, b, and carotenoid content compared to the control culture. However, there was a decrease in pigment content at the highest studied concentration of ZnONPs (Table 2). The interaction between ZnONPs and iron levels significantly influenced the content of the pigments. All iron levels positively influenced pigment content at a ZnONP concentration of 10 mg/L. The most significant pigment content was observed under the combined influence of 10 mg/L ZnONPs and 27.8 mg/L iron. Nevertheless, at higher levels of ZnONPs, this interaction became either insignificant or negatively impacted (Fig. 2).

## Phenolic compounds

The study findings revealed significant impacts of iron and ZnONPs in the culture medium on the biosynthesis of phenolic compounds in plant tissues. These effects vary depending on the concentrations of these substances and differ for total phenolics, flavonoids, and anthocyanins. Increased levels of iron in the culture medium showed a linear enhancement in the content of phenolics, flavonoids, and anthocyanins, as indicated in Table 1. The impact of ZnONP on the production of phenolic compounds varied based on the type of substance. The highest levels for flavonoids and anthocyanins were observed at 10 mg/L of this treatment, while for total phenols, the highest production occurred at 20 mg/L. Beyond these concentrations, higher treatment levels resulted in a decrease in the compounds content. Furthermore, the combined effect of iron and ZnONPs significantly influenced the production of these compounds. Increasing ZnONP concentrations notably intensified the effect of all iron levels on the biosynthesis of total phenolics and flavonoid compounds when compared to the control culture. As illustrated in Fig. 3A, raising the ZnONP level to 20 mg/L notably enhanced the iron impact on total phenolics production. The most synergistic effect and improvement in the biosynthesis of total phenolics, by 94.25%, were observed under the combined treatment of 27.8 mg/L of iron and 20 mg/L of ZnONP. For flavonoids and anthocyanins, the presence of ZnONP at 10 mg/L had the most positive interaction effect on the biosynthesis of these compounds at all iron levels. However, at higher ZnONP concentrations, this effect diminished. Specifically, the combined effect of 27.8 mg/L of iron and 10 mg/L of ZnONP led to increased biosynthesis of flavonoids and anthocyanins by 73.73% and 62.74% of the control culture, respectively (Figs. 3B and 3C).

## PAL enzyme activity

PAL is a regulatory enzyme in the phenylpropanoid pathway, which encompasses a wide range of secondary metabolites, including phenolic compounds. Our research findings indicate that PAL enzyme activity in stevia tissue culture was significantly influenced by the application of iron, ZnONPs, and their combined use. Iron demonstrated a favorable impact on PAL enzyme activity, with increasing iron concentrations resulting in a significant enhancement in enzyme activity (Table 1). Similarly, ZnONPs exhibited a positive effect on enzyme activity, significantly outperforming the control culture across all concentrations (Table 2). The highest enzyme activity was observed at 10 mg/L of ZnONPs, after which it declined, though it remained higher than that of the control culture. When these factors were applied together, their interaction significantly altered PAL enzyme activity, leading to distinct effects compared to their individual applications. The combination of 10 and 20 mg/L of ZnONPs intensified the impact of iron levels on enzyme activity. The combined effect of 27.8 mg/L iron and 10 mg/L ZnONP resulted in the highest enzyme activity, surpassing that of the control culture by 97.90% (Fig. 4).

## Antioxidant potential

The antioxidant system can potentially protect cells from oxidative stress and damage caused by reactive oxygen species (ROS). The findings revealed that both iron and ZnONPs individually enhanced tissue antioxidant potential.

As the concentration of iron increased, a dose-response relationship was observed, with a corresponding increase in antioxidant activity (Table 1). Similarly, increasing ZnONP concentrations in the culture medium also significantly augmented the scavenging activity of the tissues compared to the control cultures. Nevertheless, the antioxidant potential at 20 and 30 mg/L concentrations of ZnONPs to some extent decreased but still was significantly greater than the control culture (Table 2). The interaction between iron and ZnONPs was found to have a significant impact on tissue antioxidant potential. At all iron-tested concentrations, the presence of ZnONPs significantly potentiated their effect on this trait. The highest tissue antioxidant activity, reaching an impressive 83.48%, was achieved under the combined effect of 27.8 mg/L iron and 10 mg/L ZnONPs, which showed a 50.06% increase compared to the control culture (Fig. 5).

## **Lipid peroxidation rate**

MDA serves as a crucial indicator of cellular health, reflecting aspects such as lipid peroxidation, oxidative stress, and potential membrane damage. As illustrated in Table 1, under the influence of varying iron concentrations, MDA levels significantly increased, indicating heightened oxidative stress in the treated tissues. Similarly, applying ZnONPs led to a significant rise in MDA content, signifying an increase in cellular stress and lipid peroxidation in the treated stevia shoot tissues. Remarkably, the highest MDA content was observed at a ZnONP concentration of 30 mg/L, resulting in a 111.01% increase compared to the control culture (Table 2). The combined influence of these factors significantly affected the MDA content within the shoot tissues. Regardless of the levels of iron, the presence of ZnONPs intensified the rate of lipid peroxidation when compared to the control culture. As the concentration of ZnONPs in the culture medium increased, the combined effect on this characteristic became more evident, with the most notable synergistic effect observed at ZnONP concentrations of 20 and 30 mg/L, along with an iron concentration of 55.6 mg/L (Fig. 6).

## **Iron and zinc content**

The results showed that the iron content of the shoots significantly increased along with the enrichment of iron in the culture medium. However, in the case of zinc content, no significant difference was observed between the effects of iron levels on the cultures (Table 1). By increasing the ZnONPs level, the uptake of iron and zinc by shoots increased significantly, which was highest at 10 mg/L, and after that, although it was decreased, it was still higher than the control culture (Table 2). The combined effect of iron levels and ZnONP at 10 and 20 mg/L significantly increased the iron content of shoots compared to the control culture. The highest increase in iron content by 41.11% was observed under the effect of 27.8 mg/L iron and 10 mg/L of ZnONP compared to the control culture. However, the interaction at 30 mg/L ZnONP was positive only for 22.78 mg/L of iron, which was negative for other iron levels. The interaction effect of treatments at all levels led to increased zinc content of the shoot culture compared to the control culture. By increasing ZnONP to 20 mg/L, the zinc content peaked at 27.8 mg/L of iron, which was an increment of 56.28% more than the control, and then it decreased slightly (Fig. 7).

## **Stevia glycosides**

The results showed that the iron and ZnONP individually and jointly affected glycoside content and by increasing their levels the production of the metabolites in cultured shoots improved compared to the control culture. The highest content of the compounds was measured under the effect of 55.6 mg/L of iron and 10 mg/L of ZnONP in the culture medium (Tables 1 and 2). The combined effect of these factors also significantly affected and increased the biosynthesis of the glycosides compared to the control culture.

ZnONP intensified the effect of iron concentration, and this effect was most pronounced when it was at a level of 10 mg/L. However, as ZnONP levels increased beyond 10 mg/L, the interaction effect started to decrease, though it still remained higher than the control. The highest amount of stevioside (72.75 mg/L) was achieved when 10 mg/L of ZnONP and 27.8 mg/L of iron were combined. This resulted in a 75.04% increase in stevioside production compared to the control culture. For rebudioside, the highest increase (63.08%) compared to the control was also obtained when 10 mg/L of ZnONP was combined with 27.8 mg/L of iron. Even at higher ZnONP concentrations (20 and 30 mg/L), when combined with 27.8 mg/L of iron, there were still notable increases in glycoside content compared to the control, although the increase was lower than when ZnONP was at 10 mg/L. When 20 and 30 mg/L of ZnONP were added with 27.8 mg/L of iron, both stevioside and rebudioside contents significantly increased, with stevioside rising by 55.84% and 27.57% and rebudioside increasing by 56.94% and 36.07% compared to the control culture (Fig. 8).

## Discussion

NPs hold immense potential for revolutionizing various aspects of plant biotechnology, with the potential to influence various aspects of plant growth and development. The effects of NPs on plant tissue cultures can be diverse and complex, depending on various factors. The results of the study indicated that optimal concentrations of ZnONPs and iron enhanced shoot proliferation, height, and fresh biomass. In addition, synergistic effects were observed with specific concentration combinations, while higher concentrations had adverse effects. Our findings on the use of ZnONPs *in vitro* cultures align with previous research, demonstrating their positive effects on shoot growth and proliferation in plant tissue culture in a variety of plant species. Awad et al. (2020) investigated the percentage of shoot formation and the number of proliferated shoots in *Phoenix dactylifera* under the effect of ZnONPs. They observed a twofold increase in the multiplication rate of the proliferated shoot at 150 mg/L compared to the control treatment. ZnONPs have been used in tomato tissue cultures on an MS medium to induce callus production and plant regeneration (Alharby et al. 2016). Moreover, ZnONPs (1–20 mg/L) are also employed in MS medium to induce root formation of *Brassica nigra* plants (Zafar et al. 2016). A significant enhancement of shoot regeneration was observed when the concentration of ZnO NPs was increased to 10 mg/L. This trend aligns with the findings of Helaly et al. (2014), who demonstrated enhanced shoot regeneration in banana tissues treated with ZnONPs. Some research suggests that ZnONPs can act as catalytic cofactors for enzymes involved in key metabolic processes, such as nitrate reductase. By enhancing its activity, ZnO NPs can potentially increase plant growth (Alharby et al. 2016).

However, ZnONP also caused various detrimental effects in plants at high doses and durations that vary with different plants as well as with the size and shape of ZnONPs. For example, Wang et al. (2018) found that ZnONPs at concentrations of 400 and 800 mg/L significantly decreased the growth of the shoots and roots of tomato (*Lycopersicon esculentum* Mill.) plants. In addition, the ZnONPs adversely affect the growth of rape (Mousavi Kouhi et al. 2014), soybean (Yoon et al. 2014), and alfalfa (Bandyopadhyay et al. 2015) in a dose-dependent manner. Extensive research has been done to overcome the antagonist effect of ZnONPs, where low dose and duration of exposure are found to be beneficial in plants (Thounaojam et al. 2021).

It was revealed that the ZnONPs, iron, and their combination had a positive effect on pigment content, but only up to a certain point. Exceeding this threshold results in decreased pigment content. However, the positive interaction became negative at higher ZnONP levels. NPs can interact with plant photosystems and affect their photosynthesis and pigment production, either positively or negatively, depending on the type, concentration, duration, and mode of application of NPs (Ghorbanpour et al. 2021). Some NPs, such as mesoporous silica,

titanium dioxide, and carbon nanotubes, can enhance photosynthesis by increasing the chlorophyll content, the activity of the key enzyme Rubisco, the efficiency of photosystem II (PSII), and the CO<sub>2</sub> harvesting, as well as broadening the chloroplast photoabsorption spectrum (Mony et al. 2022). However, other NPs, such as iron oxide, silver, and ZnO, can inhibit photosynthesis by decreasing the chlorophyll content, the electron transport rate, the photosynthetic efficiency, and some other chlorophyll fluorescence parameters, as well as damaging the chloroplast components. For example, ZnONPs can reduce the photosynthesis regulating genes and cause oxidative stress in plants. Iron oxide NPs can impair the photosynthetic machinery and induce chlorosis (Ghorbanpour et al. 2021). The findings at lower ZnONP concentrations suggest a synergistic effect between ZnONPs and iron on pigments content. However, at higher ZnONP concentrations, this effect diminished or became antagonistic, highlighting the importance of considering the concentration-dependent nature of these interactions. Therefore, the effects of NPs on plant photosynthesis and pigments are complex and variable, and more research is needed to understand the underlying mechanisms and the optimal doses of NPs for plant cultivation.

Phenolic compound biosynthesis in stevia tissues was affected by treatments in the culture medium, varying with treatment type and concentrations. The biosynthesis of phenolic compounds in plant tissues can be influenced by various factors, such as environmental stress, genetic modification, and elicitation (Humbal and Pathak 2023). NPs as novel elicitors that can enhance the production of phenolic compounds in plant tissues by inducing stress responses, activating signaling pathways, and modulating gene expression (Selvakesavan et al. 2023). Different types of NPs have been reported to affect the biosynthesis of phenolic compounds in plant tissues, such as metallic, bimetallic, non-metallic, carbon-based, and composite NPs (Selvakesavan et al. 2023). For example, silver NPs increased the content of phenolic acids, carotenoids, and anthocyanins in basil leaves (Shahraki et al. 2024). Copper oxide NPs stimulated the production of gymnemic acid and phenolic compounds in cell suspension cultures of *Gymnema sylvestre* (Chung et al. 2019). Titanium dioxide NPs led to a massive increment in the production of valuable anticancer flavonoids such as xanthomicrol, cirsimaritin, and rosmarinic acid as polyphenols in hairy root cultures of *Dracocephalum kotschy* (Nourozi et al. 2021). In addition, carbon nanotubes improved the biosynthesis of phenolic compounds and flavonoids in callus cultures of *Fagonia indica* (Begum et al. 2023). Furthermore, some studies also showed that the composition of phenolics was affected by NPs. In lettuce seedlings, five phenolic compounds were decreased (3,4-diOH-benzaldehyde, ferulic acid, p-coumaric acid, salicylic acid, and vanillin) and two compounds (gallic acid and vanillic acid) were increased under the effect of NPs in comparison to control plants, while for sweet pepper an increase was observed for four compounds (chlorogenic acid, neochlorogenic acid, ferulic acid, and protocatechuic acid) (Kalisz et al. 2021).

However, the effects of NPs on the biosynthesis of phenolic compounds in plant tissues are not always positive. Some NPs may also have negative or toxic effects on plant growth, development, and metabolism, depending on the concentration, duration, plant species, and mode of application of NPs (Hu et al. 2022). Therefore, the effects of NPs on the biosynthesis of phenolic compounds in plant tissues are complex and variable, and more research is needed to understand the underlying mechanisms and the optimal doses of NPs for plant cultivation and improvement.

This research demonstrated a significant influence of ZnONPs and their combined treatment on PAL enzyme activity within stevia tissues. One of the ways that NPs affect plant growth and development is by influencing the activity of key enzymes. PAL is a key enzyme in the phenylpropanoid pathway, which is responsible for the biosynthesis of a variety of secondary metabolites, including flavonoids, phenolics, and lignans. PAL activity is often upregulated in response to stress, such as pathogen infection or wounding. NPs have been shown to affect

PAL activity in plant tissue culture. In this respect, Ghalamboran et al. (2023), showed that the total protein and phenylalanine level in rice kernel decreased under all concentrations of chitosan NPs compared to the control, while the activity of phenylalanine ammonia-lyase was higher than that of the control. Phytochemical analysis of the callus cultures showed higher production of phenolics, flavonoids phenylalanine ammonia-lyase activity, and antioxidant activity, respectively, in the callus cultures of *Caralluma tuberculata* in the presence of AgNPs (Ali et al. 2019). The application of SeNPs increased the synthesis of secondary metabolites through increases in the expression of biosynthesis pathway-related genes: PAL and 4-coumaroyl CoA ligase, in bitter melon (Rajaei Behbahani et al. 2020), and pepper plant (Li et al. 2020). In addition, using SeNPs increased the activities of the PAL enzyme, which is involved in the synthesis of secondary metabolites in plants through the phenylpropanoid biosynthetic pathway (Abedi et al. 2021). Another study by Karimzadeh et al. (2019) found that the highest activity of PAL was observed in nano-ZnO treatment, whereas the effect of nano-TiO<sub>2</sub> on PAL enzyme activity was not statistically significant. The exact mechanisms by which NPs affect PAL activity are not fully understood. However, it is thought that NPs may interact with plant cell membranes and signaling pathways to trigger the upregulation of PAL gene expression.

The study found that ZnONP led to an increase in the antioxidant activity of tissues. Some studies evidenced that NPs can enhance antioxidant activity in plant tissues. For example, Ishtiaq et al. (2023) found that seed priming with selenium NPs (SeNPs) increased the activity of the antioxidant enzymes, the content of the antioxidant vitamins C and E as well as reduced glutathione and oxidized glutathione content in tomatoes. In addition, it was reported that applying SeNPs upregulated the antioxidant defense enzymes in plants and the scavenging capacity of free radicals in *Mangifera indica*, *Sorghum bicolor*, and citrus (Garza-García et al. 2021; Shahbaz et al. 2023; Djanaguiraman et al. 2018; Alvi et al. 2021). ZnONPs were found to play an important role in controlling reactive oxygen species (ROS) and protecting plant cells from oxidative stress (Alharby et al. 2016). However, NPs also have negative effects on plants, including affection of antioxidant enzyme activity, oxidative stress, and increased chromosomal and micronucleus abnormalities, which may affect plant root growth, and seed germination. Disruption of ROS antioxidant mechanisms in *Allium cepa* and *Lathyrus sativus* by NPs causes cell cycle arrest, DNA damage, and cell death, resulting in cytotoxicity (Panda et al. 2017; Sun et al. 2019). Lower doses of ZnONPs were expected to have beneficial effects, but higher doses may reduce plant growth and induce stress due to increased zinc accumulation (ur Rehman et al. 2023).

The treatments including ZnONPs administered in this study had a significant impact on MDA content or lipid peroxidation within the stevia shoot tissues. Studies have reported that NPs can increase or decrease MDA levels, depending on the type of NP, its size, concentration, surface properties, and plant species. One of the proposed mechanisms by which NPs affect MDA levels is by inducing oxidative stress in plant cells. This triggers the production of reactive oxygen species (ROS), which can damage lipids and other cellular components (Zia-ur-Rehman et al. 2023). It was shown that all ZnONP treatments increased antioxidant capacity and oxidative stress, along with increased MDA content in *Chenopodium murale* (Zoufan et al. 2020). In eggplant (*Solanum melongena* L.), the NPs (NiO, CuO, and ZnO) induced a high amount of ROS, which led to a higher amount of MDA as a lipid oxidation marker (Baskar et al. 2018). However, AgNPs decreased hydrogen peroxide, ROS, and lipid peroxidation levels and thus improved the growth of rice seedlings (Gupta et al. 2018). In addition, increased activities of antioxidant enzymes and reduced levels of ROS and MDA were observed in *Daucus carota* L. under the effect of AgNPs (Faiz et al. 2022).

Adding ZnONPs to the culture medium enhanced the uptake of both iron and zinc by the shoots. However, the increase in uptake wasn't proportional to the increase in ZnONP concentration. It was revealed that NPs can affect

mineral accumulation in plants by influencing their uptake, translocation, and distribution within plant tissues. Some NPs can enhance mineral absorption and transport by plants, while others can interfere with or inhibit these processes (Yang et al. 2020). The effect of NPs on mineral accumulation in plants depends on several factors, such as the type, size, shape, concentration, and surface coating of the NPs, as well as the plant species, growth stage, and environmental conditions (Mishra et al. 2014). According to some studies, NPs can have positive effects on mineral accumulation in plants. For example, the ZnONPs addition was found to increase the zinc accumulation in tomato callus tissue. Meanwhile, no significant differences were found between the ZnONPs and control treatment for K, N, and P content (Alharby et al. 2016). Soybean (*Glycine max* L.) exposed to amendments of Fe<sub>2</sub>O<sub>3</sub> NPs during an eight-week growing period enhanced potassium, zinc, iron, and nitrogen content in the plant (Yang et al. 2020). The higher N, P, Zn, and Cu concentrations were recorded under TiO<sub>2</sub>NPs treatment in the wheat (*Triticum vulgare* L.) plant (Dağhan et al. 2020). Treatment with Fe<sub>3</sub>O<sub>4</sub> NPs led to noticeable increases in the leaf Fe, P, and K content in wheat (*Triticum aestivum*) plants (Feng et al. 2022). The work of Sundaria et al. (2019) demonstrated that seed priming by iron oxide Fe<sub>2</sub>O<sub>3</sub> NPs in two contrasting wheat genotypes induced germination, improved growth parameters, and enhanced accumulation of Fe in the grain. Different concentrations of Fe<sub>3</sub>O<sub>4</sub>NPs increased significantly some nutrient contents of moringa (*Moringa oleifera*) leaves (N, P, K, and K/Na) compared with untreated control plants, meanwhile decreasing Na contents (Tawfik et al. 2021). The Zn content in rice leaf and seed was higher in ZnO nano-treated plant samples compared to ZnSO<sub>4</sub> treatment (Rameshraddy et al. 2017). The exposure to ZnONPs increased leaf fresh and dry weight and leaf Zn content in red perilla as compared with untreated control (Salachna et al. 2020). Similar outcomes were achieved in earlier studies in beans and tomatoes grown in the soil enriched with 3, 20, or 225 mg ZnONPs kg<sup>-1</sup> (García-Gómez et al. 2020).

Meanwhile, nano-Si application significantly increased concentrations of K, Mg, and Fe in rice grains and rachises, but had no significant effect on concentrations of Ca, Zn, and Mn in them (Chen et al. 2018). Application of a foliar spray of SiO<sub>2</sub> NPs to rice seedlings in hydroponics decreased Ca and enhanced Mg, Fe, and Zn in shoots and roots. However, this decrease or increase in uptake of Fe, Cu, and Mn by plants depended on plant organ and nutrient type (Wang et al. 2015a; Wang et al. 2015b). Therefore, the effect of NPs on mineral accumulation in plants is complex and variable, and it requires further research and evaluation to understand the underlying mechanisms and potential applications or risks of nanotechnology in agriculture.

The results showed that ZnONPs and iron individually and in combination influenced the production of glycosides in stevia-cultured shoots. Several studies have shown that NPs can increase the production of secondary metabolites including glycosides in tissue cultures of stevia and other plants. Javed et al. (2017) found that under 1 mg L<sup>-1</sup> of ZnONPs increased the glycoside content in *Stevia rebaudiana* shoot cultures by up to 100%. However, the formation of other secondary metabolites and the physiological parameters showed a sudden decline after crossing a threshold of 1 mg L<sup>-1</sup> concentration of ZnONPs and falling to a minimum at 1000 mg L<sup>-1</sup>, elucidating the maximum phytotoxic effect of ZnONPs at this concentration. The selenium and titanium dioxide NPs increased the concentration of stevioside and rebaudioside A in stevia plants (Sheikhalipour et al. 2021). Although zinc oxide and copper oxide NPs maximized levels of total phenolic content, total flavonoid content, and total antioxidant capacity in stevia callus cultures, surprisingly, none of the cultures produced steviol glycosides (Javed et al. 2018).

Golinejad et al. (2023) found that cells treated with gold NPs had the highest levels of phenols after 8 hours. The highest amount of taxanes, both inside and outside the cells, was found in cells treated with a lower dose of NPs after 24 hours. *Hyoscyamus reticulatus* transformed roots treated with SiO<sub>2</sub> NPs offer a promising approach to significantly enhance the production of hyoscyamine and scopolamine, while the same method showed less

potential in *Hyoscyamus pusillus* (Hedayati et al. 2020). Shahhoseini et al. (2020) showed that ZnONP treatment significantly increased essential oil content and zinc absorption in the Feverfew (*Tanacetum parthenium* (L.) Schultz Bip.) plant while decreasing parthenolide levels. This suggests a potential trade-off between maximizing essential oil production and maintaining the plant's natural chemical profile.

## Conclusions

This research demonstrates that ZnONPs and iron can have both positive and negative effects on the growth and proliferation of shoot, nutrient uptake, and biochemical content in stevia tissue culture, depending on the concentration and combination used. Synergistic effects were observed with specific combinations, while higher concentrations had adverse effects. Optimizing these factors is crucial for harnessing the potential benefits of NPs while minimizing potential risks. Further research is needed to fully understand the mechanisms behind these effects and to develop safe and effective methods for using NPs in plant biotechnology.

## Declarations

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**Data availability:** The data used in this study will be made available on request.

**Conflict of interest:** The authors state that they have no competing financial interests related to the publication of this work.

**Author contributions** Seyed Mohammad Javad Lankarani: Methodology, Software, Data acquisition, and Writing the original draft. Jaber Karimi: Supervision, Conceptualization, Validation, and Revising the manuscript. Ayatollah Rezaei: Supervision, Conceptualization, Validation, and Revising the manuscript.

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## Figures

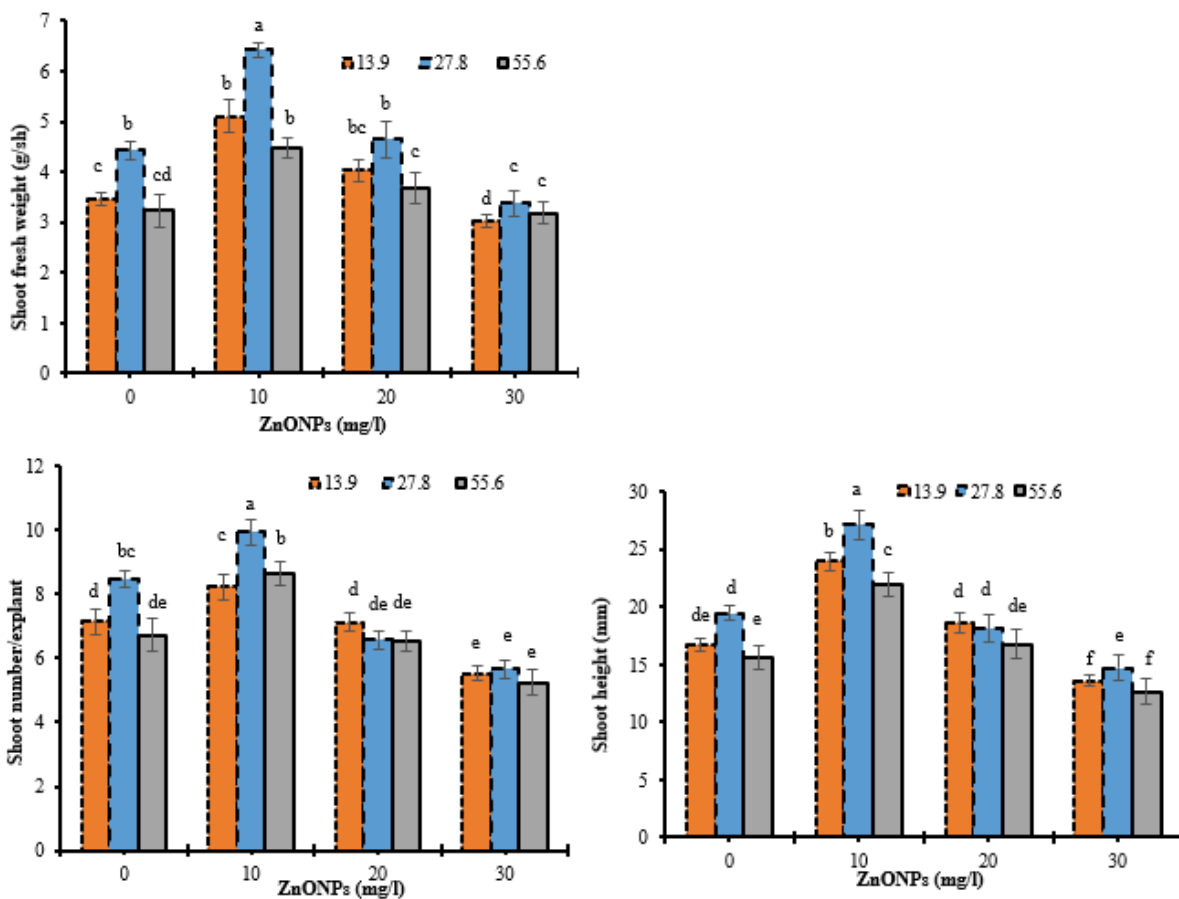


**Figure 1**

Change in the shoot number, height, and fresh weight in tissue culture of stevia plant under the interaction effect of iron and ZnONPs. The data represents the mean values of four replicates. The means comparison was performed by Duncan's method ( $\alpha = 0.05$ ). The difference between means that do not have common letters is significant.

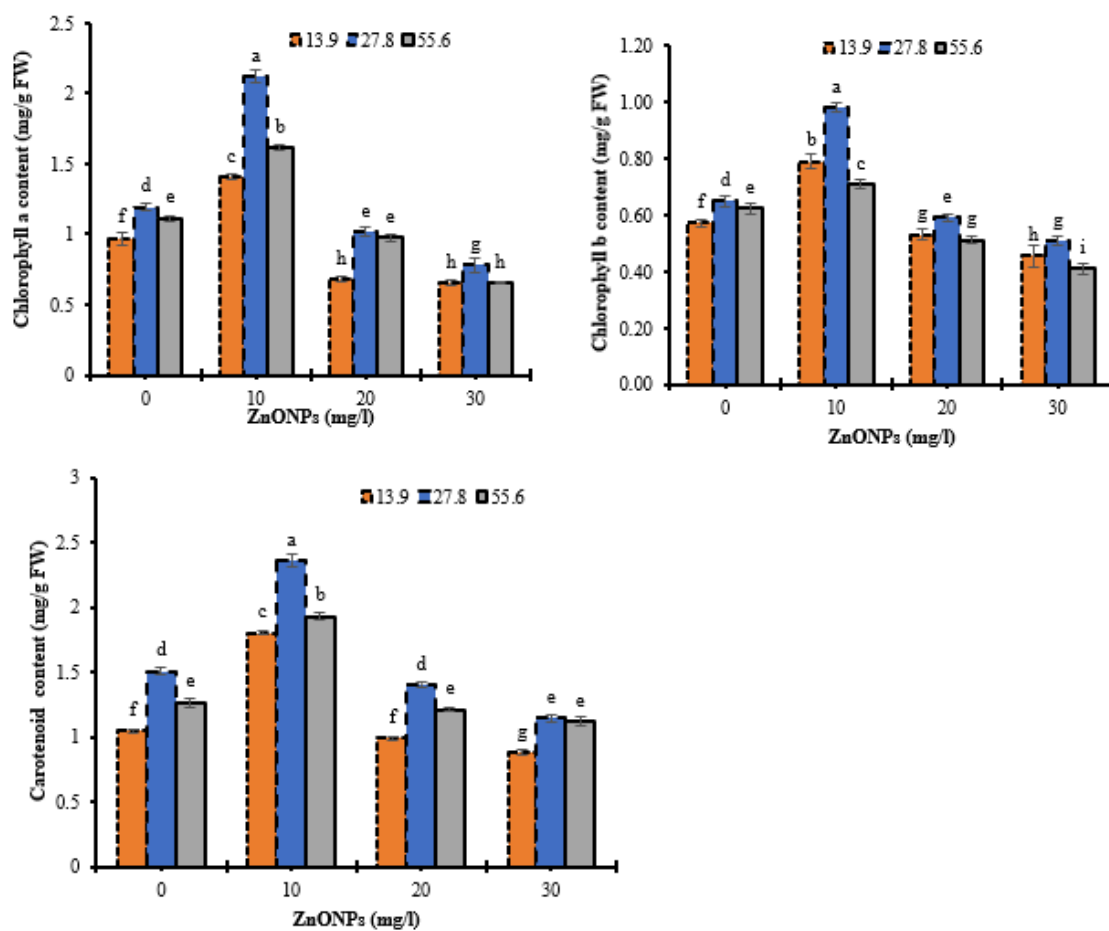


**Figure 2**

Change in the chlorophyll a, b, and carotenoid content in tissue culture of stevia plant under the interaction effect of iron and ZnONPs. The data represents the mean values of four replicates. The means comparison was performed by Duncan's method ( $\alpha = 0.05$ ). The difference between means that do not have common letters is significant.

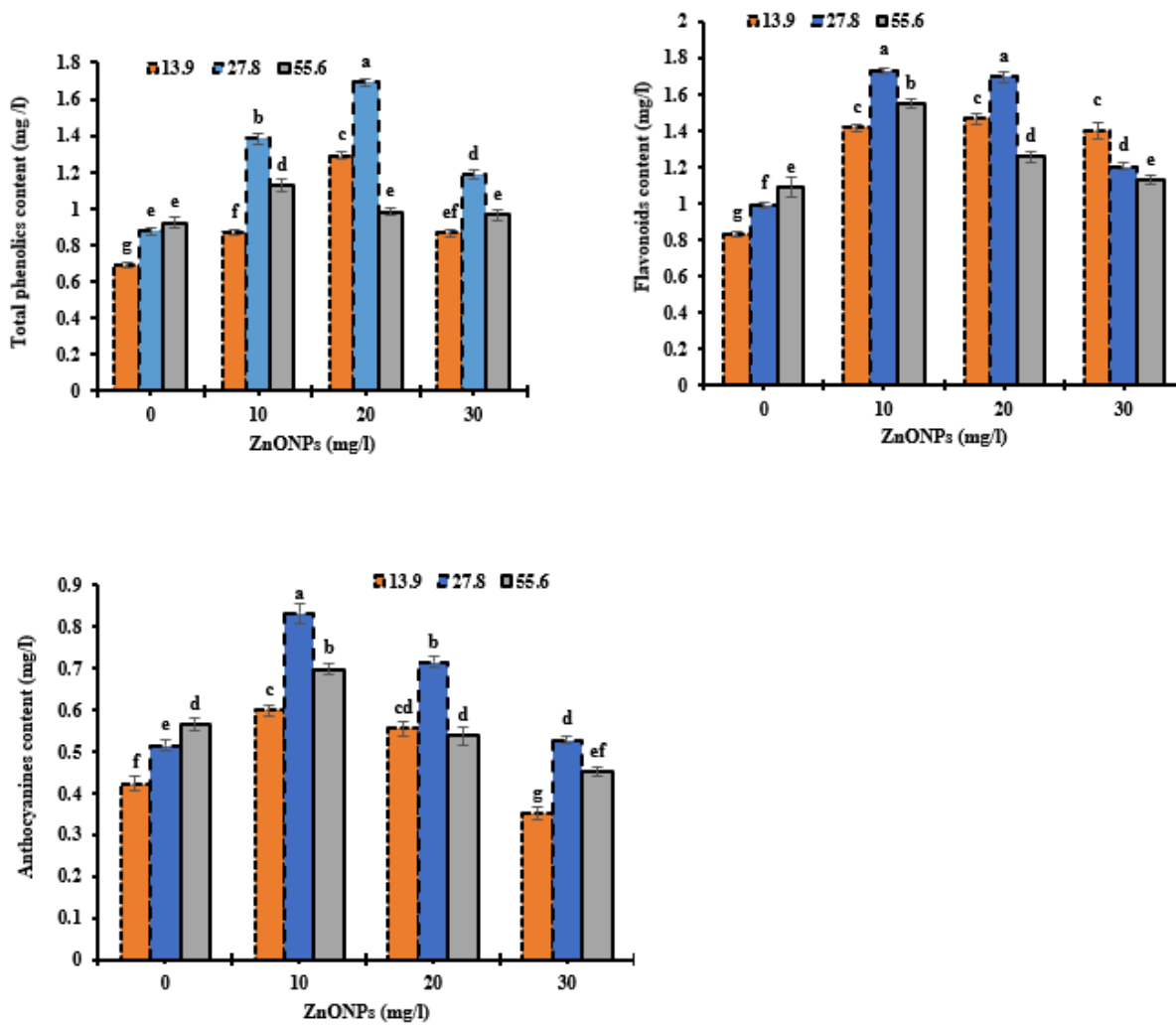


**Figure 3**

Change in the total phenolics, flavonoids, and anthocyanin content in tissue culture of stevia plant under the interaction effect of iron and ZnONPs. The data represents the mean values of four replicates. The means comparison was performed by Duncan's method ( $\alpha = 0.05$ ). The difference between means that do not have common letters is significant.

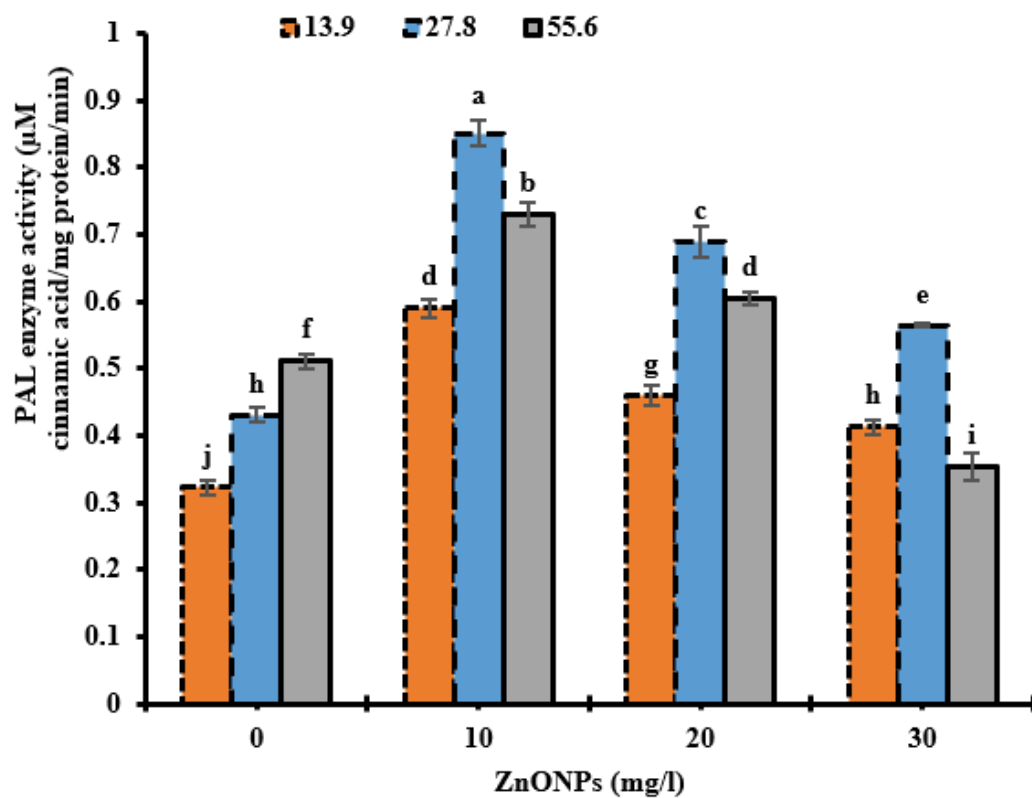


**Figure 4**

Change in the PAL enzyme activity in tissue culture of stevia plant under the interaction effect of iron and ZnONPs. The data represents the mean values of four replicates. The means comparison was performed by Duncan's method ( $\alpha = 0.05$ ). The difference between means that do not have common letters is significant.

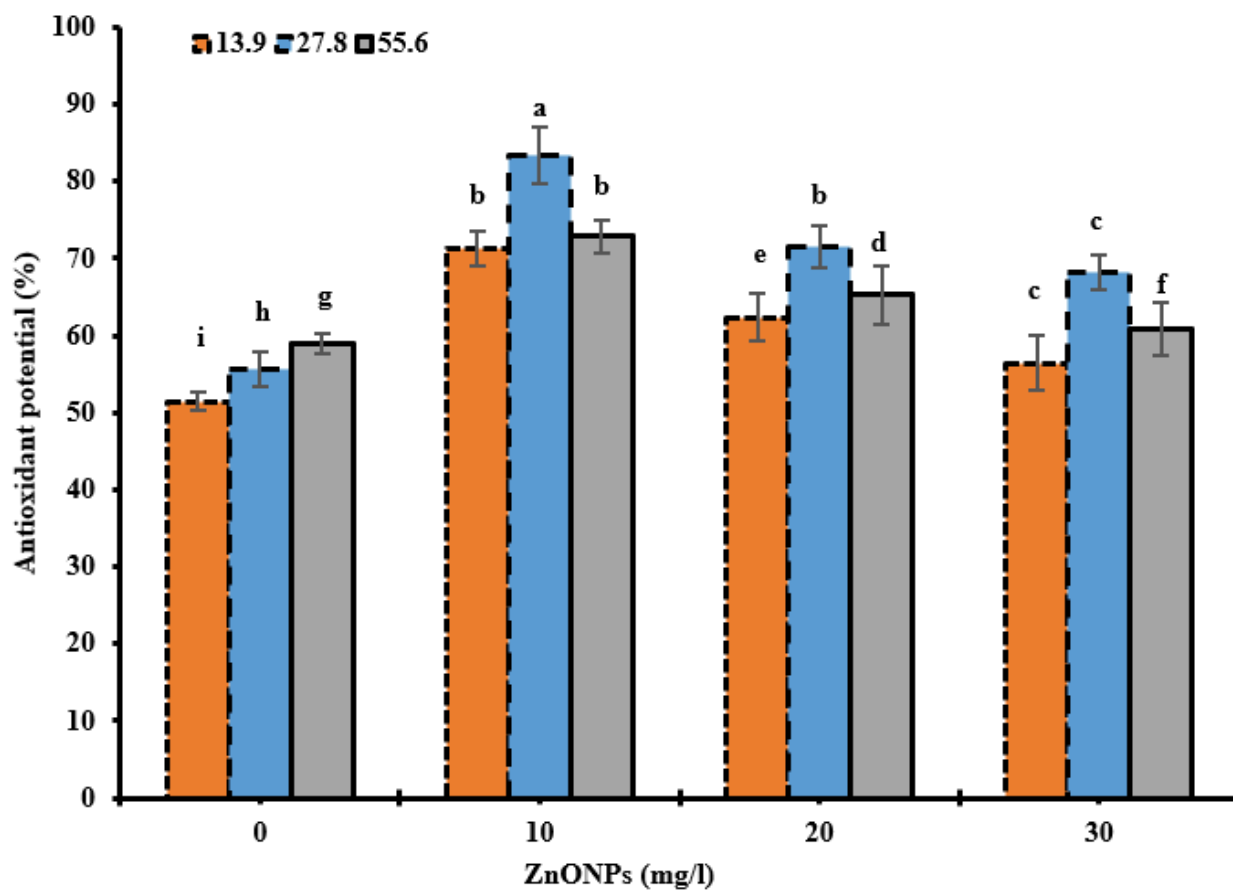


**Figure 5**

Change in the antioxidant potential in tissue culture of stevia plant under the interaction effect of iron and ZnONPs. The data represents the mean values of four replicates. The means comparison was performed by Duncan's method ( $\alpha = 0.05$ ). The difference between means that do not have common letters is significant.

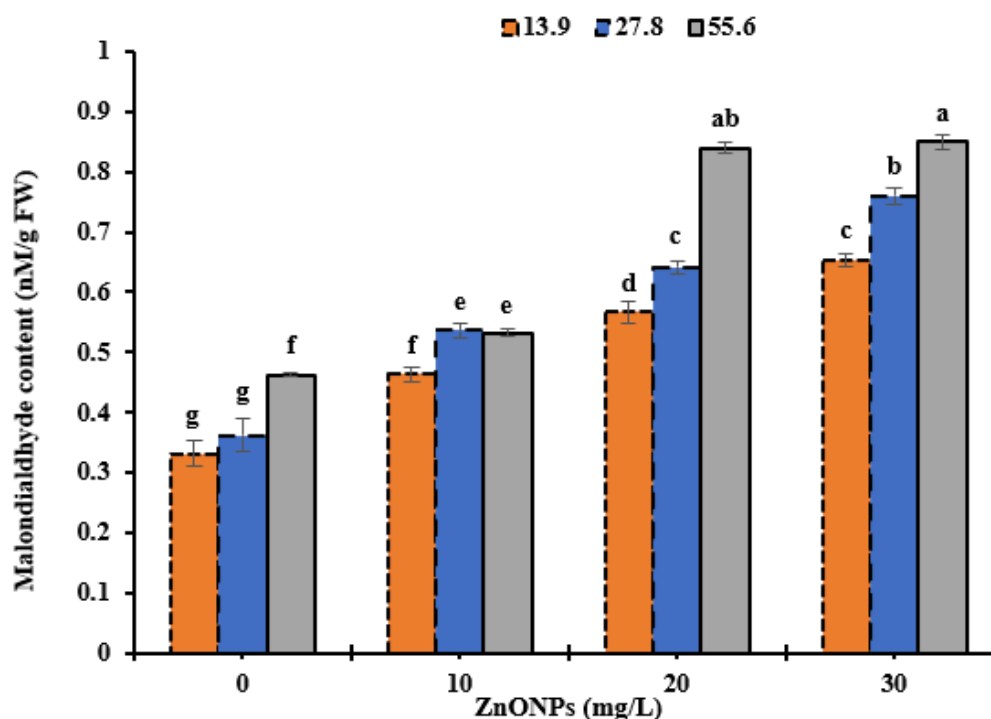


**Figure 6**

Change in the malondialdehyde content in tissue culture of stevia plant under the interaction effect of iron and ZnONPs. The data represents the mean values of four replicates. The means comparison was performed by Duncan's method ( $\alpha = 0.05$ ). The difference between means that do not have common letters is significant.

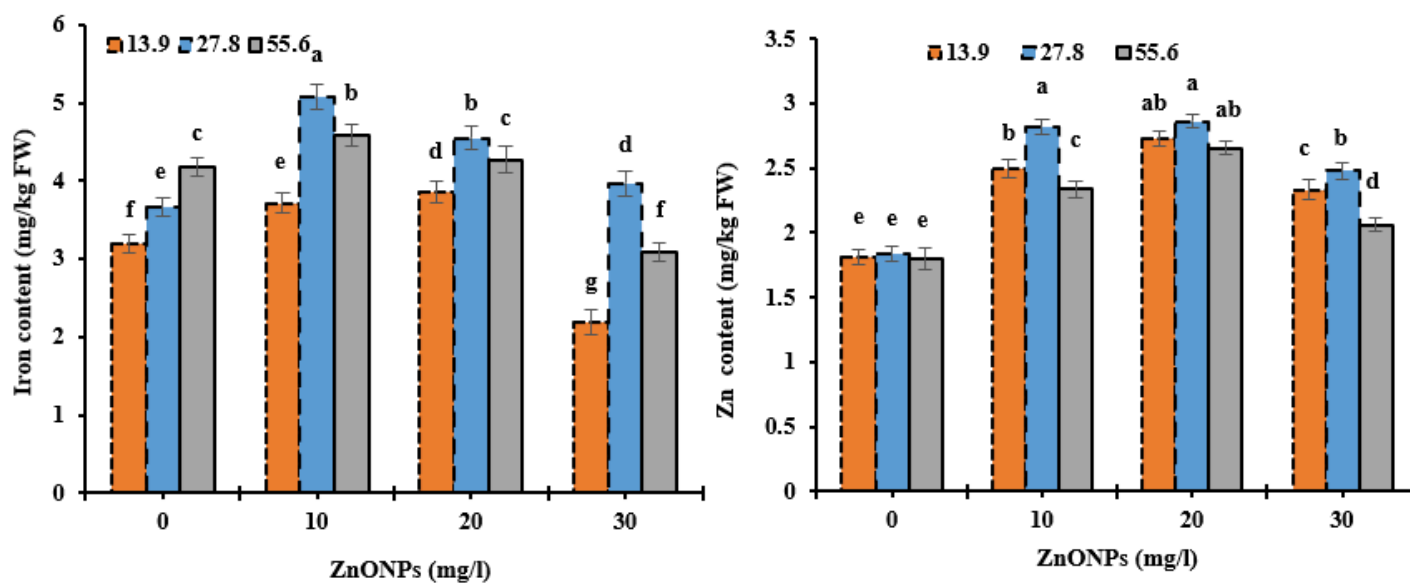


**Figure 7**

Change in the iron and zinc content in tissue culture of stevia plant under the interaction effect of iron and ZnONPs. The data represents the mean values of four replicates. The means comparison was performed by

Duncan's method ( $\alpha = 0.05$ ). The difference between means that do not have common letters is significant.

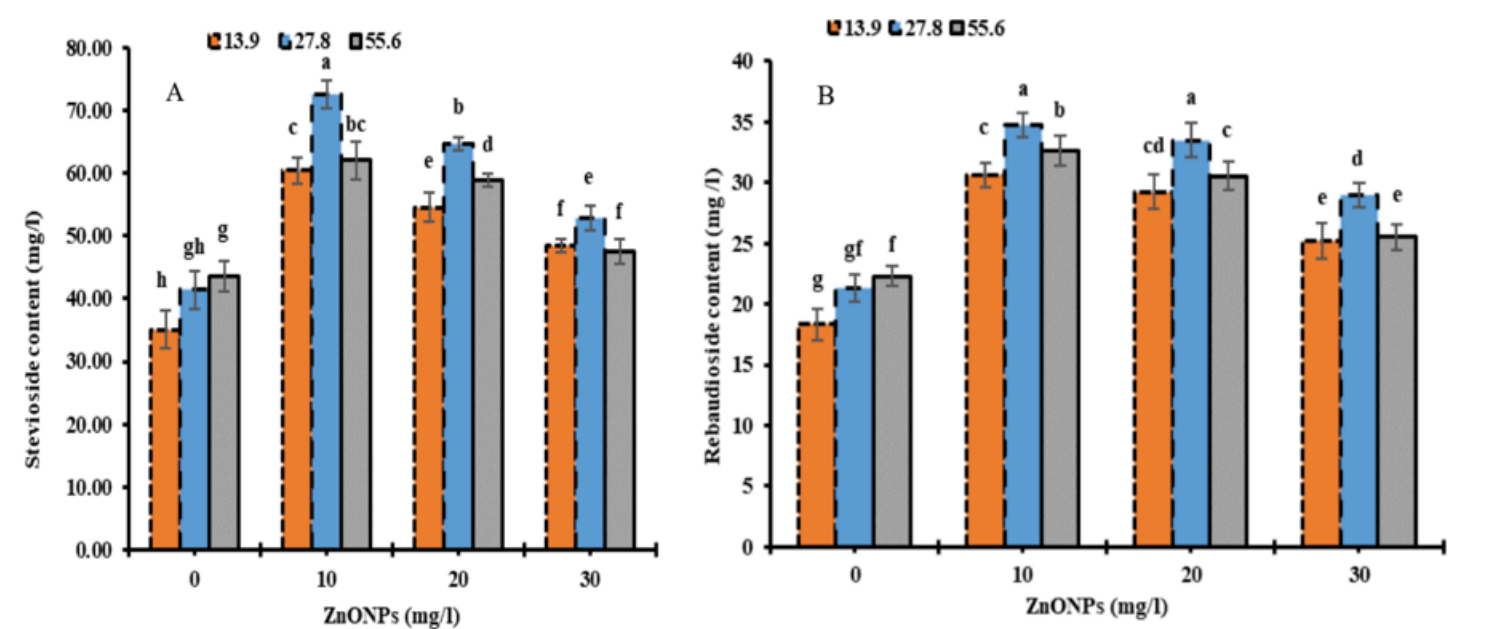


**Figure 8**

Change in the (A) stevioside and (B) rebaudioside content in tissue culture of stevia plant under the interaction effect of iron and ZnONPs. The data represents the mean values of four replicates. The means comparison was performed by Duncan's method ( $\alpha = 0.05$ ). The difference between means that do not have common letters is significant.