



# Iron nano modulated growth and biosynthesis of steviol glycosides in *Stevia rebaudiana*

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## Abstract

Application of nanomaterials is becoming the most effective strategy of elicitation to produce a desirable level of plant biomass with complex medicinal compounds. This study was, designed to check the influence of commercial iron nanoparticles (FeNPs) on physical growth characteristics, antioxidant status and production of steviol glycosides of in vitro grown *Stevia rebaudiana*. Results indicated that lower concentrations of FeNPs (45 µg/L) had a positive influence on morphological growth parameters. At a higher dose (90, and 135 µg/L) FeNPs in culture media were found detrimental to growth characteristics and development. Furthermore, the stress caused by FeNPs at 135 µg/L in cultures produced higher levels of total phenolic content ( $3.2 \pm 0.042$  mg/g dry weight: DW), total flavonoid content ( $1.6 \pm 0.022$  mg/g DW) and antioxidant activity ( $73 \pm 4.6\%$ ). In addition, plants grown in the presence of FeNPs at 90 µg/L resulted in higher enzymatic antioxidant activities (SOD =  $3.2 \pm 0.042$  U/mg; POD =  $2.1 \pm 0.026$  U/mg; CAT =  $2.6 \pm 0.034$  U/mg and APx =  $3.3 \pm 0.043$  U/mg), respectively. Furthermore, exposure to a low dose of FeNPs (45 µg/L) exhibited the maximum amount of stevioside (stevioside:  $4.2 \pm 0.058$  mg/g (DW) and rebaudioside A:  $4.9 \pm 0.068$  mg/g DW) as compared to high doses. The current investigation confirms the effectiveness of FeNPs in growth media and offers a suitable prospect for commercially desirable production of *S. rebaudiana* biomass with higher sweet glycosides profiles in vitro.

## Key message

Lower concentrations of Iron nano particles (FeNPs) had a positive influence on morphological growth parameters, production of antioxidant secondary metabolites and natural calorie free steviol glycosides in *Stevia rebaudiana*.

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## Introduction

*Stevia rebaudiana* (commonly called Stevia) belongs to the family Asteraceae and acts as a natural sweetener with zero calories (Salehi et al. 2019). It is known as one of the finest substitutes of common sugar for food and pharmaceutical industries. Cultivation of Stevia has increased as a result of the interest of food industries due to the presence of steviol glycosides (SGs), particularly stevioside and rebaudioside A. These glycosides confer *S. rebaudiana* about 200–300 times higher sweetness than sucrose (Tavakoli et al. 2019, 2012). The low seed viability of *S. rebaudiana* has limited the large-scale production. Besides, the stem cutting method is time-consuming and requires high labor inputs which limits the stock of stem cutting for further commercial propagation (Kazmi et al. 2019a; Thiyagarajan and Venkatachalam 2012). This is why the current global production of Stevia is insufficient to meet the higher industrial demand, due to the lack of standardized protocols for the development of high-quality plants containing a higher amount of stevioside A and rebaudioside (Kazmi et al. 2019b; Yadav et al. 2011). To solve this problem, different research groups are focusing on biotechnology techniques to develop good quality stevia plants with sufficient size of the leaf for commercial purposes. In recent times, nanotechnology has revolutionized agriculture by promoting food and crop production (Khan et al. 2019b; Parisi et al. 2015). During the past decade, a variety of potent nanomaterials have been introduced to improve the efficiency, productivity and performance of several agricultural practices (Liu and Lal 2015).

Plant in vitro culture technology offers a pertinent platform for biomass production of commercially important medicinal and ornamental plants. It also provides a suitable platform to evaluate the impact of various elicitor compounds (biotic and abiotic factors) in culture media to improve morphological and physiological features of plants (Khan et al. 2019a). Plant micro propagation facilitates the proliferation of superior material at commercial scale, conservation of critically endangered species and production of the desirable level of precious secondary metabolites in plants (Sher Khan et al. 2019; Taj et al. 2019). Elicitors can serve as signaling molecules to activate a signal-transduction cascade which facilitates the gene expression related to plant growth and accelerate the biosynthesis of bioactive compounds (Saeed et al. 2017; Wang et al. 2015). Among various well-known abiotic elicitors, nanomaterials are emerging as a new class of elicitors with a promising approach for the production of plant biomass with maximum secondary metabolites in vitro (Khan et al. 2019b).

Previous research studies have documented the use of nanoparticles as elicitors in plant cell culture medium. For instance, zinc and iron oxides nanoparticles at an optimal level in growth media increased the production of hypericin and hyperforin in *Hypericum perforatum* in vitro cultures (Sharafi et al. 2013). According to Poborilova et al. (2013) the augmentation of Al-203 NPs (10–100 mg/mL) into the culture medium enhanced phenolic compounds in tobacco in vitro. In another study, it was suggested that AgNPs worked as good elicitors for the enhancement of secondary metabolites in *Cucumis anguria* root culture (Chung et al. 2018). To improve the leaf yield and steviol glycosides contents in Stevia, Iron nanoparticles (FeNPs) could be employed as nano elicitors. According to the reports of El-Temsah and Joner (2012) and Kim et al. (2015), FeNPs showed a hormetic effect on plants, that is, lower doses of FeNPs had a positive influence on the growth while higher levels of FeNPs resulted in detrimental effects on plants. Similarly, the efficiency of strawberries plant in vitro cultures was improved by application of FeNPs, thereby producing higher quantity and quality of strawberries (akbar Mozafari et al. 2018).

Therefore, owing to the important role of nano elicitors on enhancement of plant secondary metabolites, *S. rebaudiana* a medicinally important herb was subjected to the in vitro application of FeNPs to understand the interaction between FeNPs and *S. rebaudiana*. To the best of our knowledge, no research work is available to evaluate the impact of FeNPs on stevia plantlet development in vitro. This study aimed to assess the impact of various concentrations of FeNPs on all the morphological characteristics of *S. rebaudiana* in vitro and on the production of steviol glycoside.

## Materials and method

### Optimization of culture conditions for in vitro micropropagation

Three different concentrations of 6-Benzylaminopurine (BA) i.e. 1.5 mg/L, 3.0 mg/L and 4.5 mg/L were tested to optimize the suitable concentration for plant development in vitro. For this purpose, the growth medium was prepared by dissolving MS media supplemented with sucrose (3%), and appropriate concentrations of BA. The pH of the media was adjusted to 5.8. Then agar (7 g/L) was added to media before autoclaving at 121 °C for 20 min. Explants with nodal buds (1 cm) were collected from in vitro germinated plantlets and placed on MS media in aseptic conditions and incubated in the

growth chamber. The cultures were kept in the growth room having temperature ( $25 \pm 1$  °C), relative humidity (70%), light intensity ( $40\text{--}50$   $\mu\text{mol}/\text{m}^2/\text{s}$ ) and photoperiod of 16 h light and 8 h dark.

### Influence of FeNPs on physical growth characteristics of *S. rebaudiana*

The Iron nanoparticles (FeNPs) used in this study were purchased from (Sigma-Aldrich) and were of size ( $< 50$  nm). Suspended nanoparticle solution was prepared in distilled water and dispersed uniformly by following the protocol reported by Ali et al. (2019).

To evaluate the impact of FeNPs on physical growth parameters, plants established on MS medium with 3 mg/L BA were used as explant source and plants grown in this medium were selected as a control for this study. MS media with 30 g/L of sucrose, 3 mg/L of BA and 7 g/L of agar were prepared. The pH value was set at 5.8. After sterilization, the growth media was shifted to laminar airflow hood where the micro filter (0.22  $\mu$ ) sterilized FeNPs suspension at various rates of FeNPs (45, 90, 135  $\mu\text{g}/\text{L}$ ) were added. The medium was then distributed properly ( $7 \pm 1$  mL) in sterilized test tubes. Each test tube was stirred manually to prevent the agglomeration of FeNPs and was kept at 4 °C to solidify it quickly. The explants (nodal segments) were inoculated in media in culture tubes and incubated in growth room and morphological data (number of days to shoot and root initiation, number of roots, number of leaves, leaf diameter, fresh weight, dry weight and water content) were recorded. Data was collected from culture tubes after four weeks of period. For dry weight, the cultures of each treatment were placed in a dry oven at 50 °C for 2 days and then calculated the dry matter by using the following formula:

$$\text{Fresh weight/dry weight} \times 100$$

### Plant extract preparation

*Stevia rebaudiana* plants developed in vitro under the application of FeNPs and the control treatment were utilized for the phytochemical analysis. The procedure of plant extract preparation was carried out according to the protocol reported by Khan et al. (2013) with some minor modifications. Briefly, 300 mg dried powdered sample and 10 mL of 50% methanol (v/v) were mixed. These were mixed and shaken (24 rpm) at room temperature for 24 h. The mixture was then sonicated for 30 min followed by vortexing for 5 min and then again sonicated for 15 min. Later, the samples were centrifuged for 10 min

at 6,500 rpm and the supernatant was filtered by using syringe filter (0.02  $\mu\text{m}$ ). The extract was then diluted to 10 mg/L concentration and stored in the refrigerator at 4 °C until further use.

### Assessment of the effects of FeNPs on production of antioxidant secondary metabolites in *S. rebaudiana*

Total phenolic content (TPC) was assessed according to the protocol described by Ali et al. (2019). Briefly, 20  $\mu\text{L}$  of sample was taken from each extract (10 mg/mL) and added into the wells of a 96 well plate. Then, 90  $\mu\text{L}$  of the Folin–Ciocalteu reagent (10  $\times$  diluted) was added to the wells. The mixture was left to incubate at room temperature for 5 min and sodium carbonate (90  $\mu\text{L}$ ) was added that made a final volume (200  $\mu\text{L}$ ) of a mixture. A standard calibration curve was made wherein gallic acid (1.0–10 mg/mL) was used. Gallic acid at 1 mg/mL was considered as positive control and methanol (20  $\mu\text{L}$ ) was selected as a negative control. After 90 min of incubation of reaction mixture, the absorbance of samples was recorded at 630 nm with Biotek microplate reader (ELX 800, BIOTEK). The results were expressed as mg gallic acid equivalent (GAE) per g (mg GAE/g).

Total flavonoid content (TFC) was assayed as per the methodology reported by Chang et al. (2002). Briefly, 20  $\mu\text{L}$  of the 10 mg/mL sample was added into the microplate (96 well), followed by the addition of 10 mL aluminum chloride (10 g/L) and 10 mL of potassium acetate (98.15 g/L). In this assay, methanol (20 mL) and quercetin (1.0 mg/mL) were selected as negative and positive controls, respectively. The final volume was adjusted to 200 mL by adding 160 mL of distilled water and then incubated for 30 min. A standard calibration curve was made wherein rutin (1.0–10 mg/mL) was used for calibration. The absorbance was measured at 450 nm. The results were expressed as mg Quercetin equivalent (QE) per g (mg QE/g). Activities of antioxidative enzymes were analyzed in all the selected samples by following the protocol reported by Ali et al. (2018). Samples were homogenized using potassium phosphate buffer (2 mL), pH value (7.8), containing 1% polyvinylpyrrolidone (PVP) and 0.1 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C. The collected supernatant was used for estimation of superoxide dismutase (Giannopolitis and Ries 1977) and peroxidase (Abeles and Biles 1991), catalase (Arrigoni et al. 1992) and ascorbate peroxidase (Miyake et al. 2006) by using UV–Visible spectrophotometer. Moreover, DPPH assay (2,2-diphenyl-1-picrylhydrazyl) was performed as per the methodology reported by Abbasi et al. (2010).

## High performance liquid chromatography (HPLC) based quantification of Stevioside and Rebaudioside A

Stevioside and Rebaudioside A were determined and quantified in the in vitro raised plant samples through high-performance liquid chromatography (HPLC) by following the protocol described by Kazmi et al. (2019b). Briefly, for extract preparation dry powdered plant samples were added into a mixture of water–methanol (1:9 v/v) and were placed on a shaker at 30 °C overnight. Each sample was then carefully filtered through a general purpose filter paper (Whatman; 90 mm) followed by centrifugation at 4000 rpm for 30 min. Filtrate was concentrated further by using vacuum concentrator, and re-filtered using 0.45 µm filter paper and finally transferred to HPLC vials. Operating standards of Stevioside and Rebaudioside A were purchased from Sigma-Aldrich (USA). Vacuum pressure (− 40 °C;  $133 \times 10^{-3}$  bar) was employed for the lyophilization of the glycosides standards which were then mixed with HPLC grade water. Agilent 100 HPLC system was used for the analysis of Stevioside and Rebaudioside A in the selected plant samples. Injection volume was 20 µL for the plant samples and reference standard solutions. The column used for chromatographic analysis was Luna C18 (2) having the dimensions of (length: 250 mm; inner diameter: 4.6 mm, particle size: 5 µm). was taken for chromatographic analysis. Samples were separated in the column by using mobile phase A (acetonitrile/water (85:15 v/v) and mobile phase B (acetonitrile/water (75:25 v/v) having a flow rate of 1 mL/min.

Detection was carried out at 210 nm and additionally the spectrum was recorded in a range from 200 to 700 nm. The identification of Stevioside and Rebaudioside A was carried out based on the retention time of corresponding reference standards.

### Statistical analysis

Statistical analyses for all the selected parameters were conducted using SPSS ver. 16.0. One-way ANOVA was used to calculate the significant differences between each parameter

according to the post-hoc Tukey test ( $p \leq 0.05$ ). Graphs of all the parameters were plotted by using Graphpad Prism 5.

## Results and discussion

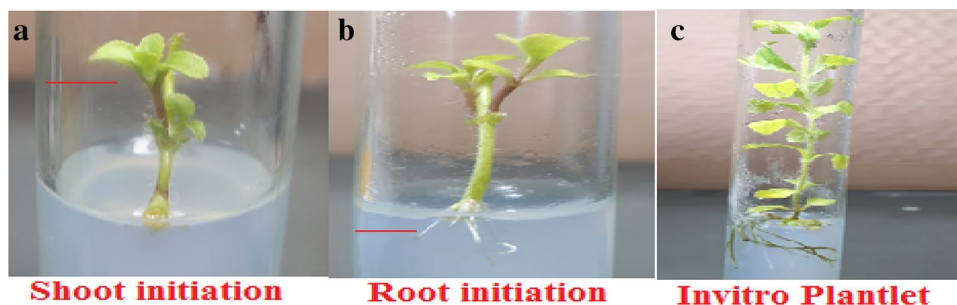
### Effects of FeNPs on in vitro growth and development of *Stevia rebaudiana*

During initial in vitro cultures optimization experiments, nodal explants derived from the in vitro germinated plantlets were exploited under the effects of varying levels of BA. Wherein, BA (3.0 mg/L) resulted in the early shoot initiation (6.2 days after inoculation), root initiation (10.1 days), highest percent response (80%) and a maximum number of roots (5.2) (Fig. 1).

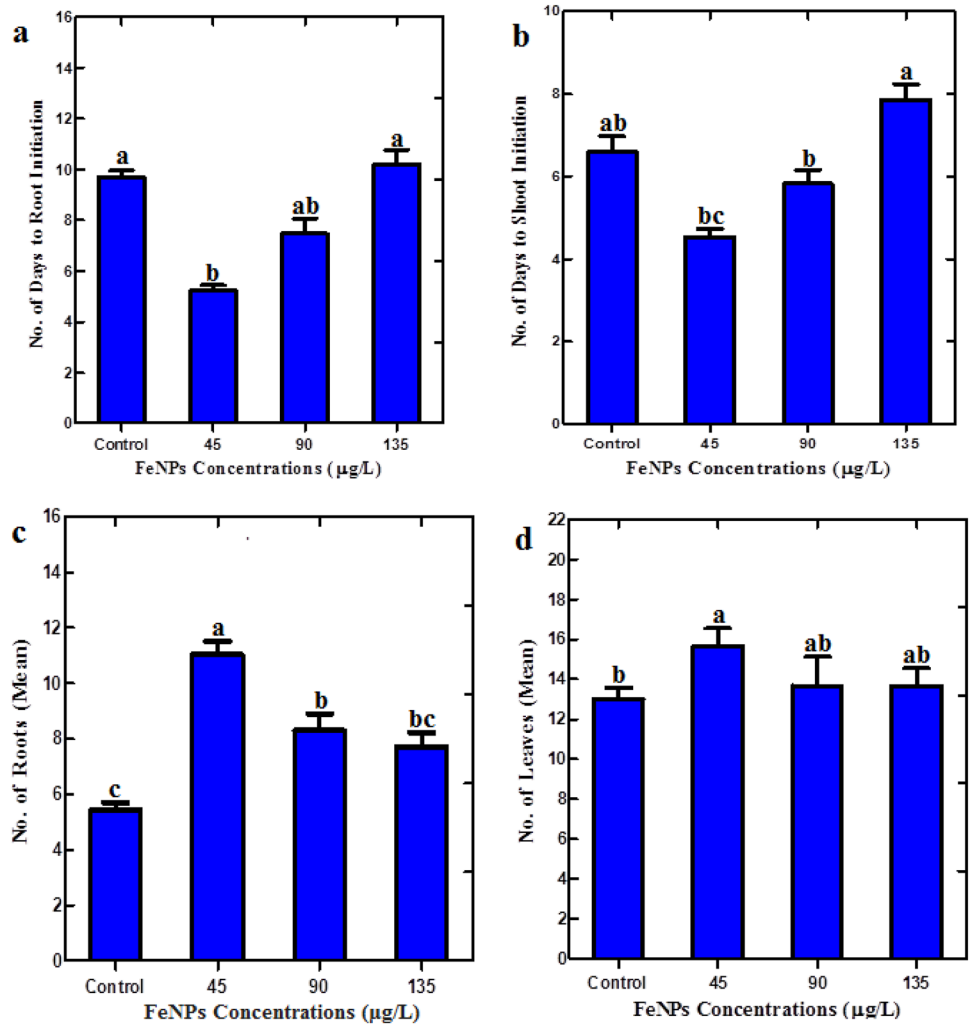
In the subsequent experiments on the application of FeNPs on in vitro plantlet growth and development, it was determined that the effects of FeNPs on *S. rebaudiana* were dependent on the additive concentrations of the nanoparticles. The MS media supplemented with a lower level of FeNPs (45 µg/L) induced early shoot initiation (4.5 days), root initiation (5.2 days) and a maximum number of roots ( $n = 10.5$ ). However, higher doses of FeNPs (90 and 135 µg/L) in the culture media resulted late shoot and root initiation (7.2 and 9.1 days, respectively), and a minimum number of the root ( $n = 6.7$ ) (Fig. 2 a-d). This might be due to the toxicity developed by applying higher levels of NPs which exhibit a negative impact on plant growth and development (Khan et al. 2019b).

Overall, lower concentrations of FeNPs had a positive effect on plant growth, whereas, high concentrations of FeNPs decreased the various growth parameters significantly (Figs. 2a–d, 3a, b, 4a–d). Our results are in accordance with the findings of Jamzad Fard et al. (2013) wherein they reported enhanced growth of in vitro *Rosa chinensis* by applying the optimal level of FeNPs in culture media. Similarly, the reports of Sheykhbaglou et al. (2010) and Rui et al. (2016) also confirmed that iron oxide nanoparticles at low concentrations significantly increase the biomass of soybean and peanut plant, respectively. In corn, the low dose of FeNPs was beneficial for various growth parameters while

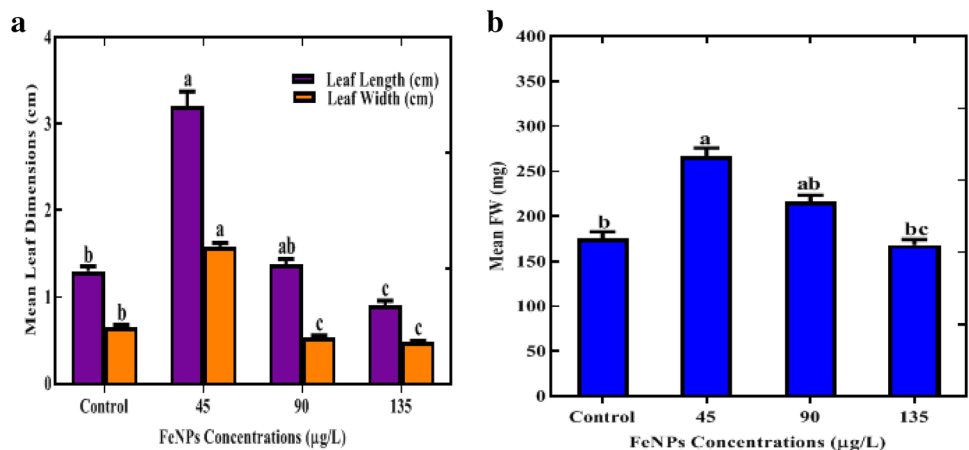
**Fig. 1** Optimization of plant growth regulators for in vitro plantlet development. Shoot initiation on MS media augmented with BA (3 mg/L) (a), root initiation on MS media augmented with BA (3 mg/L) (b), in vitro plantlet development on MS media augmented with BA (3 mg/L) (c)



**Fig. 2** Changes in morphological parameters in response to different concentrations of FeNPs as compared to the control plant in *S. rebaudiana*. Number of days to root initiation, number of days to shoot initiation (a, b), Number of root development, number of leaves development (c, d). Columns data with different letter/s differ significantly ( $p < 0.05$ )



**Fig. 3** Changes in morphological parameters in response to different concentrations of FeNPs as compared to control plant in *S. rebaudiana* leaf dimensions, fresh weight (a, b). Columns data with different letter/s differ significantly ( $p < 0.05$ )

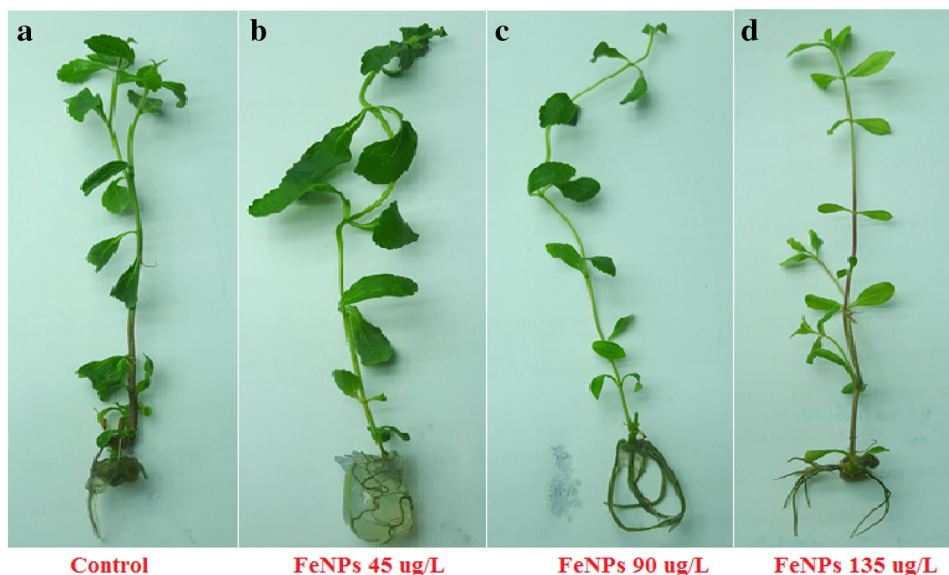


high dose drastically reduced the plant growth (Li et al. 2016). Similarly, many previous scientific reports about the impact of various nanoparticles on plants have also shown beneficial as well as a detrimental role in the form of enhancing or reducing the plant growth and their productivity (Ali

et al. 2019; Khan et al. 2019b; Ma et al. 2016; Mohammad et al. 2019; Sarmast et al. 2015).

It was also noted in the current investigations that FeNPs positively influenced some of the essential growth parameters i.e. leaf diameter, number of leaves, fresh weight and dry

**Fig. 4** Influence of different concentration of FeNPs on the growth and development of *S. rebaudiana* as compared to control plant. **a** Developed on MS media supplemented with BA (3 mg/L) developed on MS media supplemented with BA (3 mg/L) + FeNPs 45  $\mu$ g/L (**b**), developed on MS media supplemented with BA (3 mg/L) + FeNPs 90  $\mu$ g/L (**c**), developed on MS media supplemented with BA (3 mg/L) + FeNPs 135  $\mu$ g/L (**d**)



weight in *S. rebaudiana*. Among the different concentrations tested, 45  $\mu$ g/L FeNPs was found most effective in enhancing the leaf width (1.5 cm) and length (2.9 cm), a number of leaves ( $n = 16$ ), fresh weight (260 mg), dry weight (19.24 mg) which were higher as compared with control and other applied treatments (Figs. 3a, b, 5a–d). Similarly, Desai Heta et al. (2017) also assessed the influence of magnesium nanoparticles (MgNPs) on the growth and development of *S. rebaudiana*. They revealed that MgNPs helped improve leaf length, leaf width, shoot length and number of shoot formation. Similarly, in the present study, optimal levels of FeNPs are very crucial in MS media for the optimal growth of plantlets.

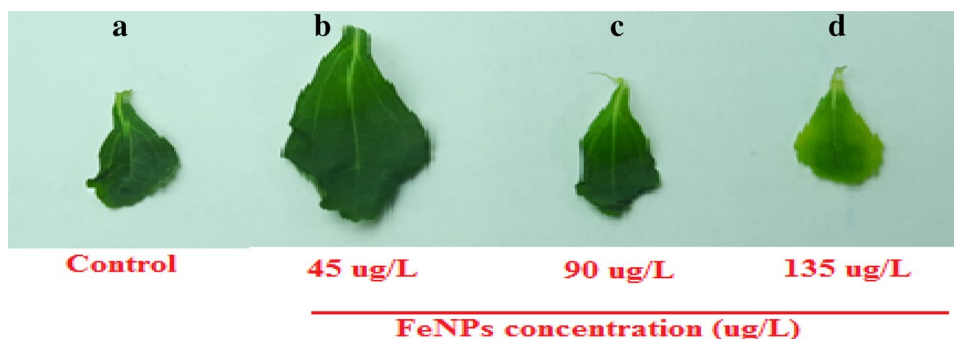
Interestingly, the high level of FeNP (135  $\mu$ g/L) in culture media considerably reduced the leaf length (1.0 cm), leaf width (0.5 cm), number of leaves developed ( $n = 14$ ), fresh weight (160 mg) and dry weight (18.72 mg) as compared to the results obtained with 45  $\mu$ g/L FeNPs (Fig. 3 a-b). The detrimental effects of higher concentrations of nanoparticles on plant growth are more obvious and are reported by many studies (Keller and Lazareva 2014; Pokhrel and Dubey 2013; Zuverza-Mena et al. 2016). Higher concentrations of NPs

have been reported to start the production of ethylene that increases the activity of chlorophyllase enzyme which might have destroyed the internal chloroplast membrane and thus hampered growth parameters (Khan et al. 2019b). This may be linked with the pale-yellow appearance of leaves with lesser diameters in plantlets of *S. rebaudiana* (Fig. 5).

It was also observed in the present study that the treatments at which the plants were found to accumulate higher biomass contained a higher percentage of water content. The moisture content varied from 88 to 92% in all the applied treatments. Maximum water content was observed when the plants were developed on MS media containing a lower amount (45  $\mu$ g/L) of NPs. On the contrary, increasing the level of FeNPs in growth media substantially reduced the percentage of water content. However, the results were not significantly different (data not shown).

Sufficient water uptake from the culture media is essential for plant cellular metabolism and growth (Dilshad et al. 2020; Yousaf et al. 2019). Earlier, Qian et al. (2013) and Maurel et al. (2015) suggested that nanomaterials activate the water channels i.e. aquaporin in plants which are transmembrane proteins. Aquaporins not only facilitate the

**Fig. 5** Influence of different concentration of FeNPs on leaf size of *S. rebaudiana* on MS media supplemented with BA (3 mg/L) (a), BA (3 mg/L) + FeNPs 45  $\mu$ g/L (b), BA (3 mg/L) + FeNPs 90  $\mu$ g/L (c), BA (3 mg/L) + FeNPs 135  $\mu$ g/L (d)



diffusion of water across biological membranes and regulate water homeostasis, but also facilitate uptake of nutrients, and gases such as CO<sub>2</sub>. Previous studies also indicated that certain concentrations of silver nanoparticles and carbon nanotubes activated the expression of aquaporin genes in tomato roots and Arabidopsis seedlings, respectively (Qian et al. 2013; Villagarcia et al. 2012). In our results, the application of FeNPs at a certain level in culture media may have activated the expression of aquaporin genes which enhanced the uptake of water content and thus improved the percentage of moisture content in stevia plant grown in vitro.

### Effects of FeNPs supplementation on the accumulation of secondary metabolites and antioxidant activity

Our results demonstrated that *S. rebaudiana* plants grown under the presence of FeNPs in culture media have shown higher levels of phenols and flavonoids than the plants grown under control treatment (Fig. 6). Maximum TPC, TFC (3.2 ± 0.042 mg GAE/g and 1.6 ± 0.022 mg QAE/g, respectively) and DPPH free radical scavenging activity (73 ± 4.6%) were recorded in plants, micro-propagated under higher concentrations of FeNPs (135 µg/L). Plants grown in stress free conditions (i.e. control) produced comparatively less TPC (1.6 ± 0.022 mg GAE/g), TFC (0.4 ± 0.009 mg QAE/g) and DPPH free radical scavenging activity (65 ± 4.3%) (Fig. 6). However, lower concentrations of FeNPs (45, and 90 µg/L) moderately enhanced the levels of TPC, TFC and DPPH free radical scavenging activity. Similar earlier studies with other nanoparticles on in vitro cultures of *S. rebaudiana* have significantly enhanced phenols and flavonoid contents (Ghazal et al. 2018; Golkar et al. 2019; Javed et al. 2017a). Iron oxide magnetic nanoparticles are reported for their role of release of iron in plant cells and

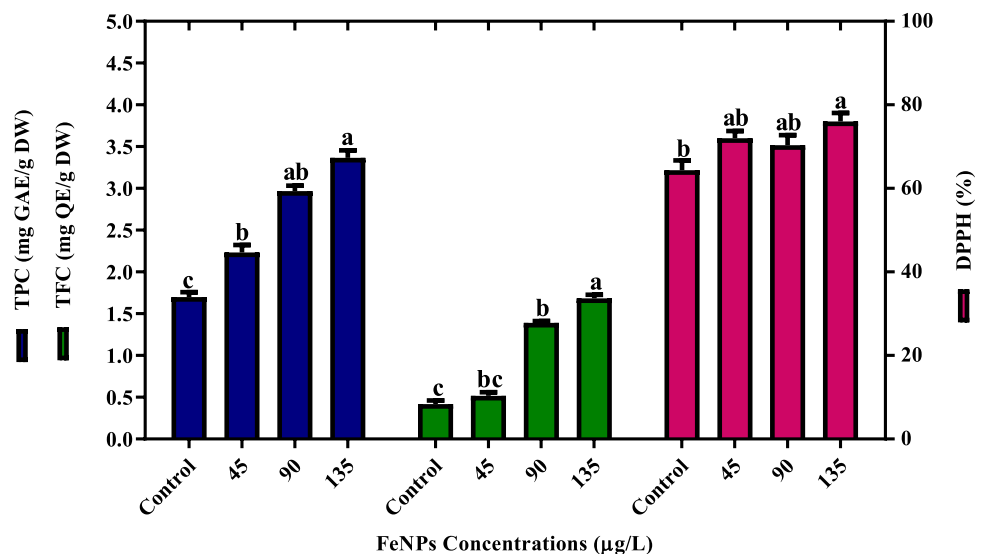
stimulation of Fenton reaction which eventually result in generation of reactive free radicals (Taghizadeh et al. 2019).

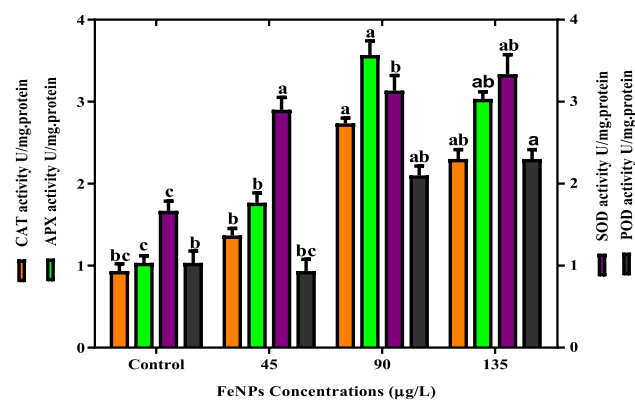
To date, the underlying mechanism of the effects of nanomaterials on secondary metabolites in plants has not yet been well documented. One of the probable mechanisms to explain this scenario is that the nanoparticles induce oxidative stress to plant and cause the plant to cope with the stress through a variation in physiological, and biochemical processes in plants (Ali et al. 2019; Khan et al. 2019b). In such a stressful environment, the imbalanced equilibrium between reactive oxygen species (ROS) generation and scavengers drastically damages macromolecules (nucleic acids, proteins, carbohydrates, and lipids) which ultimately lead to cell death (Jhinjer et al. 2016; Mohammad et al. 2019). Besides, it is also suggested that ROS induce harmful effects on chlorophyll content in plants thus causing damage to the cell which might trigger secondary metabolism to cope with the damage (Asada 2006). It has been shown through various studies that the application of different nanoparticles of metals such as Cerium, and Titanium accelerate the ROS production that triggers secondary metabolism to cope with the harmful impact on plant growth and development (Ali et al. 2019; Ghazal et al. 2018; Golkar et al. 2019; Javed et al. 2017a; Khan et al. 2019b).

Our results indicated that the exposure of *S. rebaudiana* to FeNPs improved the production of polyphenols and DPPH free radical scavenging activity compared to the control treatment.

Plants fight ROS through a strong enzymatic antioxidant system. In this study, application of FeNPs to in vitro cultures of *S. rebaudiana* significantly stimulated the antioxidant enzyme activities (SOD, POD, CAT, APx). Maximum SOD (1.6 ± 0.022 U/mg), POD (0.8 ± 0.015 U/mg), CAT (0.9 ± 0.016 U/mg protein) and APx activity (3.3 ± 0.043 U/mg) were detected in plants treated with FeNPs (90 µg/L).

**Fig. 6** Influence of different concentrations of FeNPs on total phenolic content (TPC), total flavonoid content (TFC) and DPPH free radical scavenging activity. Columns data with different letter/s differ significantly at  $p < 0.05$





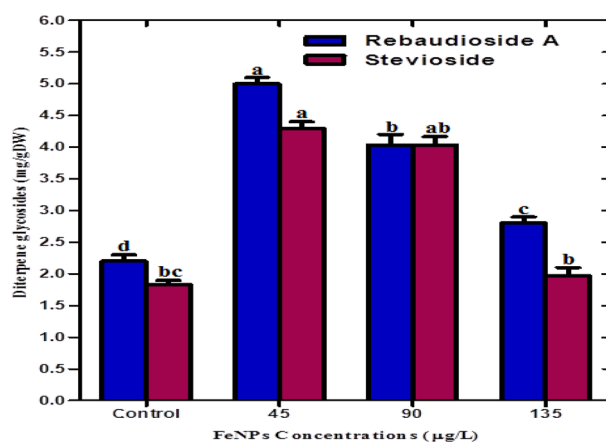
**Fig. 7** Influence of different concentration of FeNPs on *SOD* superoxide dismutase, *POD* peroxidase, *CAT* catalase, *APx* ascorbate peroxidase columns data with different letter/s differ significantly at  $p < 0.05$

However, higher concentrations (135 µg/L) of FeNPs only slightly improved the activities of *SOD*, *POD*, *CAT*, *APx*. As compared to NPs-treated plants, a minimal enzymatic activity ( $SOD = 1.6 \pm 0.022$  U/mg;  $POD = 3.3 \pm 0.043$  U/mg;  $CAT = 3.3 \pm 0.043$  U/mg;  $APx = 1.2 \pm 0.026$  U/mg) were observed in control (Fig. 7).

Furthermore, lower *SOD* and *POD* activities were observed when cultures were supplemented with lower doses of FeNPs (45 µg/L), this means lower availability of iron, which can be linked with lower availability of iron as described by El-Temsah and Joner (2012).

### Production of Steviol glycosides in *S. rebaudiana* in response to FeNPs

Through these results, we have shown that plants propagated in the presence of FeNPs (45 µg/L) produced higher quantities of Rebaudioside A and Stevioside ( $4.9 \pm 0.068$  mg/g DW and  $4.2 \pm 0.058$  mg/g DW, respectively). As indicated in Fig. 8, exposure to higher concentrations of FeNPs (135 µg/L), showed reduction in Rebaudioside A and Stevioside content ( $4.2 \pm 0.058$  mg/g DW and  $3.9 \pm 0.048$  mg/g DW, respectively). The higher production of these steviol glycosides can be linked with the healthy leaves and higher chlorophyll content. This can be explained by the fact that the synthesis of steviol glycosides begins in chloroplast and the production of these compounds depend on the integrity of chloroplast membrane structure (Ladygin et al. 2008). Thus, healthy leaves with well-developed chlorophyll systems can produce a desirable amount of steviol glycosides. These results can be correlated with our results where optimal concentrations of FeNPs produced healthy plantlets with higher leaf diameter and greenish in appearance (Fig. 5). It has been proposed that the enhancement of steviol glycosides due to some foreign stress inducer compounds can regulate glycosyltransferase



**Fig. 8** Influence of different concentrations of FeNPs on the production of Steviol glycosides (Stevioside and Rebaudioside A). Columns data represent the mean values differ significantly at  $p < 0.05$

enzymes. The activation of a diverse group of these glycosyltransferase enzymes can shift a sugar residue from an activated donor to an acceptor molecule and consequently, the production of steviol glycosides in *S. rebaudiana* (Vazquez-Hernandez et al. 2019). Furthermore, for well develop chloroplast structure and thus for sufficient growth, the plant needs optimal quantities of iron, because both their deficiency as well as the excessive amount may disrupt the metabolic process. Brittenham (1994) also suggested that the presence of iron with adequate quantity significantly activates more than 100 enzymes which are important for biochemical reactions. There are studies wherein, different elicitors could significantly enhance the content of stevioid and rebaudioid in vitro cultures of Stevia, by upregulation of enzymes involved in secondary metabolism (Kazmi et al. 2019a). For instance, Golkar et al. (2019) reported that AgNPs (45 mg/L) enhanced Rebaudioside A and Stevioside content in callus culture of *S. rebaudiana*. Javed et al. (2017b) evaluated the effects of ZnO NPs on shoot regeneration and induction of steviol glycosides in *S. rebaudiana*. They concluded that application of ZnO nanoparticles at 1 mg/L resulted in the optimal shoot formation and enhanced production of rebaudioside A and stevioside in the in vitro raised shoot cultures. However, at higher doses ZnO NPs showed detrimental effects on growth and biosynthesis of glycosides. In another study, Magangana et al. (2018) observed differential effects of nitrogen and phosphate tested in vitro on growth and biosynthesis of steviosides in *S. rebaudiana*.

Our study showed the production of Rebaudioside A and Stevioside only responded well to the lower concentration of FeNPs (45 µg/L). This concentration not only reduced the toxicity of FeNPs but also enhanced the content of steviol glycosides. It was observed that after providing the media with higher concentrations of FeNPs, a decline in the production of the steviol glycosides was observed. This might be



the result of the induction of nanotoxicity in cultures which alter the enzymatic processes and cause negative effects. In another study, Castro-González et al. (2019) showed that AgNPs caused toxic effects above from the optimal level and negatively influence the physio-biochemical activity.

## Conclusions

The current research established the in vitro effects of FeNPs on plant growth, development and antioxidant profile of *S. rebaudiana*. Our results demonstrated that employing nano-elicitors (FeNPs) to *S. rebaudiana* accelerated the plant growth and production of bioactive compounds. While the exposure of the cultures to higher concentrations of FeNPs inhibits their growth and development, lower concentrations of FeNPs considerably stimulated the plant's physical characteristics especially improving the leaf diameter and morphology. The application of FeNPs was also found to increase the production of polyphenols associated with essential enzymatic antioxidant activities in cultures as compared to control. Similarly, cultures developed under low concentrations of FeNPs significantly enhanced the Steviol glycosides in comparison to treatments with higher levels of FeNPs. The current protocol can serve as an initiative of nano-elicitors for application in large scale commercial production of in vitro *S. rebaudiana* with a sufficient amount of Steviosides.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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