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ORIGINAL ARTICLE

CuO nanoparticles significantly influence in vitro culture, steviol glycosides, and antioxidant activities of *Stevia rebaudiana* Bertoni

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Abstract Copper oxide (CuO) nanoparticles (NPs) synthesized through co-precipitation method were employed in MS media during in vitro culture of *Stevia rebaudiana*. Physiological characteristics, production of steviol glycosides, and antioxidative parameters were investigated in regenerated plants. CuO NPs had crystalline monoclinic cubic cuprous oxides with average size 47 nm. The NPs were applied at 0, 0.1, 1.0, 10, 100 and 1000 mg/L in MS media for direct organogenesis of *S. rebaudiana* from nodal segments. Shoot organogenesis was found highest (88.5%) at 10 mg/L CuO and average shoot length, mean number of shoot per explant, and fresh weight were also found significantly higher at the same concentration. High performance liquid chromatography (HPLC) illustrated significant rise of bioactive major steviol glycosides (rebaudioside A and stevioside) at 10 mg/L CuO NPs in MS media. The oxidative stress produced by CuO nanoparticles on *S. rebaudiana* was affirmed by antioxidant activities i.e. total antioxidant activity (TAC), total reducing power (TRP) and 2,2-diphenyl-1-picryl hydrazyl (DPPH)-free radical scavenging activity. The oxidative stress generated by NPs involved production of antioxidative molecules total phenolic content (TPC), total flavonoid content (TFC) depending on NPs concentration. The study concludes that copper oxide nanoparticles functions as a stimulator of bioactive components productions, and can be employed in in vitro batch cultures.

Keywords Copper oxide nanoparticles · *Stevia rebaudiana* · Steviol glycosides · Oxidative stress · Phytotoxicity · Reactive oxygen species (ROS)

Introduction

Nanoparticles having 1–100 nm size possess large surface area as compared to their bulk counterparts. The physical and chemical nature of nanoparticles make them highly reactivity therefore are employed in industrial scale and also have biological properties (Yadav 2013; Kołodziejczak-Radzimska and Jesionowski 2014). Nanoparticles have positive as well as negative influence on biological systems (Boczkowski and Hoet 2010). In agriculture perspective, nanoparticles have been elucidated including biocide in plants, nano-fertilizer and nano-pesticide formulations, soil condition sensors, and targeted gene delivery in transformation (Aslani et al. 2014; Perreault et al. 2014).

Stevia rebaudiana belongs to family Asteraceae and is a perennial herb native to South America. The secondary metabolites such as steviol glycosides (rebaudioside A, stevioside, rebaudioside C, etc.) are well known as anti-diabetic, anti-bacterial and anti-cancerous (Dey et al. 2013)

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which produce in leaves at higher level. Different kinds of abiotic and biotic stresses have been employed to *S. rebaudiana* during in-vitro propagation to enhance the steviol glycosides production (Javed et al. 2017a).

Metal oxide nanoparticle stress elicitors such as zinc oxide (ZnO) and copper oxide (CuO) have gained enormous importance in recent years (Javed et al. 2017b). The reactivity and toxicity of metallic oxide NPs depend on their size, surface, structure, concentration, dissolution and exposure routes (Franklin et al. 2007; Jiang et al. 2009; Chang et al. 2012). CuO nanoparticles belong to the group of nanoparticles that are used for both household and industrial purposes. However, the CuO toxicity has largely been demonstrated in aquatic organisms such as algae and zebrafish (Aruoja et al. 2009). CuO intracellular oxidative stress involves the release of Cu ions (Cu^{+2}) causing toxicity after exceeding the maximum physiological tolerance range, hence disturb the balance between oxidation and anti-oxidation processes. CuO has largely been found phytotoxic because of generation of reactive oxygen species (ROS) and necrotic lesions, ultimately leading to the cell death (Chang et al. 2012).

The effect of CuO nanoparticles on the growth, photosynthesis and oxidative response has recently been studied in crop plant, *Oryza sativa, Brassica napus* (Da Costa and Sharma 2016; Zafar et al. 2016), *Lemna minor* (Duckweed), (Song et al. 2016; Perreault et al. 2014), *Landolti apunctata* (Shi et al. 2011), *Elodea nuttallii* (Regier et al. 2015). The production of steviol glycosides has been accomplished in the presence of abiotic stress i.e. metal (Pal et al. 2013; Jain et al. 2009), nutrient application (Allam et al. 2011; Utumi et al. 1999), osmotic stress (Vives et al. 2017); biotic stress i.e. endophytic fungi, genetic transformation (Pandey et al. 2016; Kilam et al. 2017).Copper is required for normal plant growth and development; however, it is toxic at higher levels. Due to its prominent role in development and stimulatory effect on secondary metabolites production, copper has received noticeable attention. However, despite of ions, to determine role of nanoparticles in secondary metabolite production, copper oxide nanoparticles were applied during in vitro propagation and for metabolite production of *S. rebaudiana*. Physiological characteristics of micropropagated shoots, steviol glycosides production, and non-enzymatic anti-oxidant activities in leaves of regenerated *S. rebaudiana* are evaluated.

Materials and methods

Synthesis of CuO nanoparticles

CuO nanoparticles were synthesized by co-precipitation method as described by Javed et al. (2017a, b). It involved addition of 30 mL of 6 M aqueous sodium hydroxide (NaOH) solution to 600 mL of 0.2 M copper acetate monohydrate [Cu $(CH_3COO)_2 \cdot H_2O$; 98%, Sigma-Aldrich) solution and 2 mL of glacial acetic acid (CH_3COOH) solution in a drop-wise manner under continuous stirring at 100 °C. Blue color of solution changed to green and then brown. Finally, a black solution was obtained at pH 6–7 that was subsequently filtered, washed and then heated at 100 °C. Later on, the dried grinded powder was subjected to calcination at 500 °C for 4 h.

Characterization of CuO nanoparticles

Different analytical methods including X-ray diffraction (XRD), Fourier-transform infra-red (FTIR) spectra, scanning electron microscopy (SEM) and Energy dispersive X-ray (EDX) spectra were performed for characterization of CuO nanoparticles. The crystalline phase of the prepared sample was identified by X-ray diffraction (XRD) technique using a PANalytical Empyrean system. X-ray powder diffraction was performed at room temperature using *Cu Kα* radiation ($\lambda = 1.5406$ Å). The Fourier transform infrared (FTIR) spectra were recorded using FTIR spectrometer (Tensor 27 Bruker Germany) in the wave number ranging from 4000 to 500 cm^{-1}. Morphological studies of CuO NPs were done by field emission scanning electron microscope (FESEM, Nova NanoSEM 450) and operated at an accelerating voltage of 10 kV. Energy dispersive X-ray (EDX, Oxford Aztec) was utilized to determine the elemental composition (purity) of prepared CuO nanoparticles.

Preparation of medium having CuO nanoparticles

Murashige and Skoog (MS) (1962) medium was used as basal medium for shoot organogenesis. In MS media solution CuO nanoparticles were added at 0, 0.1, 1, 10, 100 or 1000 mg/L. Sucrose (30 g/L) was added and pH was adjusted to 5.7–5.8 using 0.1 N NaOH. The media was sonicated in water bath for 30 min for thorough dispersion of nanoparticles followed by addition of agar (8 g/L). The agar was dissolved by heating and media was dispensed as 30/100 mL conical flask while thorough mixing. The culture media was then autoclaved for 15 min at 121 °C and 1.06 kg/cm^2 pressure. The media was allowed to cool at room temperature before inoculation of explant.

Growth conditions of shoot organogenesis

The seeds of *S. rebaudiana* were purchased from POLISAN Tarim, Istanbul, Turkey. The seeds were cultured on plain MS medium after being disinfected with 0.1% (w/v) mercuric (II) chloride ($HgCl_2$) for 3 min. The axillary shoot nodes were excised from 4 weeks-old seedlings and incubated in media treatments having different concentrations of CuO nanoparticles. The cultured flasks were kept in growth room chamber having 16 h light/8 h dark photoperiod, provided by cool-white fluorescent light of 35 µmol/m/s irradiance and 24 ± 1 °C temperature at 55–60% rate of relative humidity. Each treatment had 15 nodal explants that were cultivated for 4 weeks. Finally, the physiological parameters involving percentage of shooting, mean length of shoots, mean number of leaves, and fresh weight of shoots produced by direct shoot organogenesis were revealed.

Extraction and analysis of steviol glycosides

Steviol glycosides were extracted from the leaves of in vitro regenerated shoots grown under CuO nanoparticles stress. The shoots propagated were carefully washed with sterile distilled water, and soaked on filter paper to remove excess water and dried in oven at 60 °C for 48 h.

The analysis of steviol glycosides was performed through high performance liquid chromatography (HPLC) according to method described by Yücesan et al. (2016). The dried plant material was grinded and 20 mg was suspended in 1 mL of 70% (v/v) methanol in a microcentrifuge tube. After incubation in an ultrasonic bath at 55 °C for 15 min, samples were centrifuged at 25 °C and 12,000 rpm for 10 min. The pellet was discarded and supernatant was filtered using 0.22 µm PTFE Millipore syringe filters.

Chromatography was performed using an autosampler (WPS-3000-SL Dionex Semi Prep Autosampler) injecting 10 µL of each sample, a binary pump (LPG 3400SD Dionex) solvent delivery system working at a flow rate of 0.8 mL/min, and a dual wavelength absorbance detector operating at 210 and 350 nm (MWD-3100 Dionex UV–VIS Detector). The column, Inertsil® ODS-3 (GL Sciences Inc., Japan) with 150 × 4.6 mm in length and 5 µm particle size, was kept warm at 40 °C in a column oven system (TCC-3000SD Dionex). At the end, isocratic flow was performed using acetonitrile and 1% (w/v) phosphoric acid buffer mixture (68:32) for 20 min.

Preparation of extract and anti-oxidant assays

The leaf extracts of *S. rebaudiana* were prepared by drying the leaves, and then taking 0.1 g of their fine powder obtained under different CuO concentrations. 500 µL of methanol was used for the dissolution of powder. It was vortexed for 5 min and then sonicated for 30 min followed by 15 min centrifugation at 10,000 rpm. The pellet was discarded and supernatant was stored to perform different antioxidant activities.

Determination of total phenolic content

Method of Ali et al. (2017) was performed after slight modifications, to estimate the total phenolic content in leaf extracts of *Stevia*. The process involved transfer of an aliquot of 20 µL (4 mg/mL) dimethyl sulfoxide (DMSO) stock solution of each sample to the respective well of 96 well plate, and then the addition of 90 µL of Folin–Ciocalteu reagent in it. The plate was kept for 5 min and later on, 90 µL of sodium carbonate was added to the reaction mixture. All samples were run in triplicate and their absorbance was obtained at 630 nm using microplate reader. Gallic acid was used as standard and the results were expressed as mg Gallic acid equivalent (µg GAE/mg).

Determination of total flavonoid content

Aluminum chloride colorimetric method of Zafar et al. (2017) was performed after slight modification, to determine total flavonoid content of different leaf extracts of *Stevia*. 10 µL of 10% aluminum chloride, 10 µL of 1.0 M potassium acetate and 160 µL of distilled water were added to the aliquot of 20 µL (4 mg/mL) DMSO stock solution of each sample contained in the respective well of 96 well plate. It was kept at room temperature for 30 min. The samples were run in triplicate and their absorbance was measured at 630 nm using microplate reader. Quercetin was used as standard and the results were expressed as mg quercetin equivalent (µg QE/mg).

Determination of total antioxidant capacity

Total antioxidant capacity was evaluated by the procedure of Ali et al. (2015) after slight modification. An aliquot of 100 µL from stock solution of each sample (4 mg/mL in DMSO) was mixed with 900 µL reagent solutions containing 0.6 M sulfuric acid, 4 mM ammonium molybdate and 28 mM sodium phosphate. The reaction mixture was incubated at 95 °C for 90 min, and later on cooled at room temperature. All samples were run in triplicate and their absorbance was measured at 695 nm using microplate reader. Ascorbic acid was used as standard and the results were expressed as mg ascorbic acid equivalent (µg AA/mg).

Determination of total reducing power

Total reducing power of samples was calculated according to the procedure of Rehman et al. (2014) after slight modifications. 100 µL of each sample (4 mg/mL in DMSO) was mixed with 200 µL of phosphate buffer (0.2 M, pH 6.6) and 250 µL of 1% w/v potassium ferricyanide. The resulting mixture was incubated at 50 °C for 20 min. Thereafter, the reaction was acidified with 200 µL of 10% w/v trichloroacetic acid and centrifugation was performed at 3000 rpm for 10 min. The pellet was discarded and the supernatant (150 µL) obtained was mixed with 50 µL of 0.1% w/v ferric chloride solution. All samples were run in triplicate and their absorbance was measured at 630 nm. Ascorbic acid was used as standard and the results were expressed as mg ascorbic acid equivalent (µg AA/mg).

Determination of DPPH free radical scavenging activity

Since the over-production and accumulation of free radicals is damaging to plant cells, the ability of antioxidants produced to prevent oxidative damage was elucidated by 2,2-diphenyl-1-picryl hydrazyl (DPPH) reagent. This assay was performed according to the protocol of Haq et al. (2012) after slight modifications. 10 µL (4 mg/mL) of *Stevia* leaf extracts was mixed with 190 µL of DPPH (0.004% w/v in methanol). The resulting reaction mixture was incubated in darkness for a period of 1 h. All samples were run in triplicate and their absorbance was measured at 515 nm of wavelength using microplate reader. Ascorbic acid was used as positive control while DMSO as negative control.

$$\begin{aligned} \%\ \textit{inhibition of test sample} &= \%\text{scavenging activity} \\ &= (1 - \mathrm{A_{bs}}/\mathrm{A_{bc}}) \times 100 \end{aligned} \tag{1}$$

where $\mathrm{A_{bs}}$ indicates the absorbance of DPPH solution with sample, and $\mathrm{A_{bc}}$ is the absorbance of only DPPH solution. The IC_{50} was calculated by using Table curve software 2D Ver. 4.

Statistical analysis

The design of experiments was randomized and the statistical analysis of data was performed using SPSS, Version 17.0 (SPSS Inc., Chicago, IL, USA). Statistical difference was determined using ANOVA, and the significance of difference between means ± SE (standard error) values was obtained using Duncan's multiple range tests performed at $p < 0.05$.

Results and discussion

XRD results

The powder patterns were recorded with the use of Empyrean PANalytical X-ray diffractometer with Bragg–Brentano geometry using Cu Kα radiation (λ = 1.54 Å). The step-scan covered the angular range 20–80° with the step of 0.02°. Figure 1a shows the XRD pattern of CuO nanoparticle. The diffraction data revealed that the material was

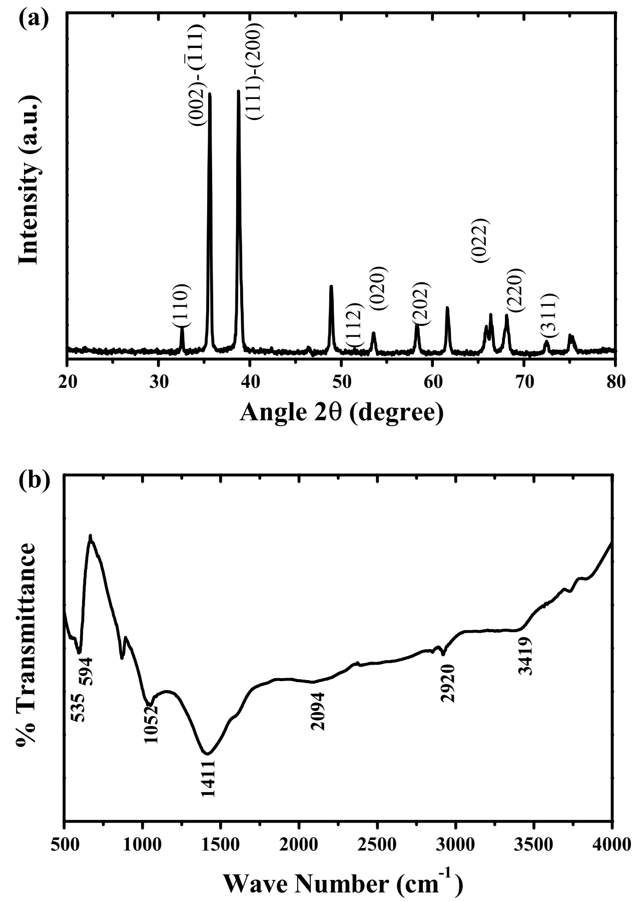

Fig. 1 **a** X-ray diffraction pattern, **b** FTIR spectrum of the CuO nanoparticles

composed of crystalline monoclinic cubic cuprous oxides (Fig. 1a). The peak positions are in good agreement with the PCPDFWIN data card 895899. The crystallite size determination was carried out using the Scherrer equation (Eq. 2) (Cullity 1978).

$$D = \frac{k\lambda}{\beta \cos \theta_B} \tag{2}$$

where D is the crystallite size, k is a constant (~0.94 assuming that the particles are spherical), λ is the wavelength of the X-ray radiation, β is the line width at half maximum intensity of the peak and θ_B is the angle of diffraction. The particles size obtained from the XRD data for CuO, is 47 nm. The lattice parameter of CuO monoclinic structure and the plane spacing d is related to the lattice constant and the Miller indices *(hkl)* by Eq. 3 (Cullity 1978)

$$\frac{1}{d_{hkl}^2} = \frac{1}{\sin^2\beta}\left(\frac{h^2}{a^2} + \frac{k^2\sin^2\beta}{b^2} + \frac{l^2}{c^2} - \frac{2hl\cos\beta}{ac}\right) \tag{3}$$

Using above equation along with the Bragg's law ($2d_{hkl}sin\theta = n\lambda$), we calculate the values of lattice parameters. Monoclinic CuO crystal have the lattice constants $a = 4.69$ Å, $b = 3.418$ Å and $c = 5.122$ Å and angle $\beta = 99.57°$.

FTIR results

Figure 1b shows the FTIR spectrum of CuO nanoparticles. Various well-defined peaks were observed at 535, 594, 869, 1052, 1411, 2094, 2847, 2920 and 3419 cm^{-1}. The appearance of the peaks at 535, 594 corresponds to the characteristic stretching vibrations of Cu–O bonds in the monoclinic crystal structure of CuO (Zheng and Liu 2007; Ethiraj and Kang 2012) and 869 cm^{-1} corresponds well to Cu–O–H vibration (Yu et al. 2012; Park and Kim 2004). The bond at 1052 cm^{-1} is due to the C–O stretching vibration (Zhao et al. 2012). Origin of two well-defined absorption bands at 1411 cm^{-1} is due to the CH_3 group and CH_3 asymmetrical stretching mode present on the surface of CuO nanostructures. The bands at 2847 and 2920 cm^{-1} are assigned to $-CH_2$ and C–H stretching mode. The absorption peak around 2094 cm^{-1} is due to the existence of CO_2 molecules in air, and the peak around 3419 cm^{-1} is assigned to the O–H stretching vibration.

SEM and EDX analysis

Morphology of the sample was investigated using field emission scanning electron microscope (FESEM). Specimens were prepared by sticking CuO nanoparticles to the carbon tape, and blow away the excess of powder with compressed air. This specimen was sputter coated with a thin Au–Pd layer of about 3 nm thick in vacuum to avoid the charging. Typical SEM micrograph for prepared CuO nanoparticles is shown in Fig. 2. The SEM micrograph clearly showed irregular shaped morphologies of CuO nanoparticles. The SEM observation showed the presence of agglomerated nanoparticles with an average size of 40–100 nm.

The EDX spectrum of CuO nanoparticles is given in Fig. 3. The EDX results show that there are no other elemental impurities present in the prepared CuO nanoparticles.

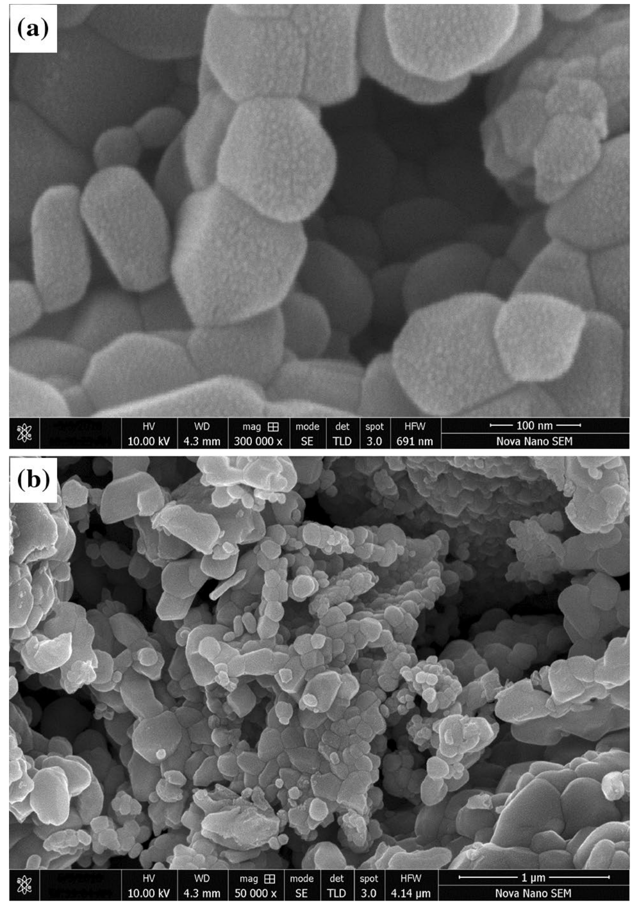

Fig. 2 FESEM image of CuO nanoparticles **a** high resolution image, **b** low resolution image

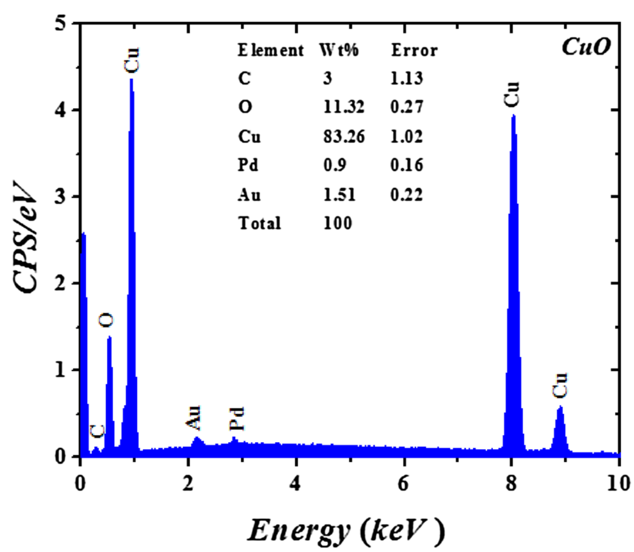

Fig. 3 Energy dispersive X-ray profile of CuO nanoparticles

Estimation of physiological parameters and steviol glycosides

The results (Table 1 and Fig. 4a, b) show that the growth of *S. rebaudiana* shoots reached to maximum at 10 mg/L of CuO nanoparticles. However, after attaining this threshold level, further increase in NPs concentration resulted in toxicity to the plant. Table 1 indicates that the highest amount of shooting (~90%) occurred in MS medium supplemented with 10 mg/L of CuO nanoparticles. In contrary, the least

Table 1 Comparison of physiological parameters in 4 weeks old shoots produced from nodal explants on MS medium supplemented with different concentrations of CuO nanoparticles

Conc. of CuO nanoparticles (ppm)	% of nodal explants shooting	Mean shoot length (cm)	Mean no. of nodes per explant	Mean no. of leaves per regenerated shoot	FW of shoots per explant (g)
0	84.7	4.1 ± 0.1^{d}	4.6 ± 0.1^{a}	13.1 ± 0.3^{c}	0.16 ± 0.0^{d}
0.1	85.4	4.3 ± 0.1^{bc}	4.7 ± 0.1^{a}	13.5 ± 0.3^{c}	0.30 ± 0.0^{c}
1.0	86.7	4.5 ± 0.1^{b}	$4.8 \pm 0.^{1a}$	14.3 ± 0.3^{b}	0.39 ± 0.0^{b}
10	88.5	4.9 ± 0.2^{a}	4.9 ± 0.1^{a}	16.1 ± 0.4^{a}	0.44 ± 0.0^{a}
100	68.5	3.8 ± 0.0^{d}	2.3 ± 0.0^{b}	9.88 ± 0.2^{d}	0.14 ± 0.0^{d}
1000	40.8	1.0 ± 0.0^{e}	0.3 ± 0.0^{c}	4.66 ± 0.0^{e}	0.01 ± 0.0^{e}

±: standard error, the means with the same letter within the columns are not significantly different according to Duncan's multiple range test at confidence level of 95%

shooting frequency (~41%) was obtained in MS medium containing 1000 mg/L of CuO nanoparticles. Maximum shoot length (4.9 cm) was achieved with MS medium augmented with 10 mg/L of CuO nanoparticles, followed by MS containing 1 mg/L (4.5 cm). Similarly, highest number of nodes (4.9) and leaves (16.1) were also recorded by 10 mg/L CuO nanoparticles treatment. Fresh weight of shoots was also measured to be highest in MS medium fortified with 10 mg/L of CuO nanoparticles (0.44 g).Direct shoot organogenesis is a method used to perform in vitro shoot growth of *S. rebaudiana* and presence of nanoparticles stimulates metabolic pathways for production of active constituents (Dimkpa et al. 2013). Mineral nutrients are the basic components of tissue culture media and the explant responds based on type and concentration of nutrient. Various Cu-containing enzymes involved in electron transport, protein and carbohydrate biosynthesis also play a role in plant regeneration (Niedz and Evens 2007). However, the threshold level of each component is critical. Copper has been investigated as stimulatory component for induction of in vitro culture of many plant species i.e. Eleusine, Cucumis, Sorghum, Lepidium, Capsicum, Triticale, barley, wheat, rice, Tinospora (Garcia-Sago et al. 1991; Purnhauser and Gyulai 1993; Dahleen 1995; Sahrawat and Chand 1999; Pande et al. 2000; Kumar et al. 2003; Nirwan and Kothari 2003; Kothariet al. 2004; Tahiliani and Kothari 2004). Based on these findings Cu nanoparticles were used in this study that reflects that CuO NPs also has stimulatory effect on in vitro shoot regeneration and production of secondary metabolites.

Figure 5 shows the production of steviol glycosides (rebaudioside A and stevioside) in *S. rebaudiana* in the presence of up to 10 mg/L of CuO nanoparticles, thereafter, it causes phytotoxicity. The amount of rebaudioside A enhanced from 2.07% in control MS treatment to 4.17% in MS treatment supplemented with 10 mg/L of CuO nanoparticles. On gradual increasing the CuO nanoparticles concentration, decrease in rebaudioside A amount was observed. Similarly, HPLC spectra for quantity of stevioside reveales that the amount of stevioside was 0.73% obtained in control group that increased up to 1.19–2.06% in treatments having 1 and 10 mg/L of CuO nanoparticles, respectively. CuO nanoparticles have been considered as abiotic stress elicitors that positively effect the growth parameters, and enhance the quantity of steviol glycosides as well as non-enzymatic antioxidant activities found specifically in the *Stevia* leaves. This positive influence had been observed in the start, but after crossing a threshold barrior of 10 mg/L of CuO nanoparticles, a sudden decline was investigated as a result of continued addition of CuO nanoparticles to the growth medium. Zafar et al. (2017) have reported that at some level CuO NPs have beneficial role and it is mainly due to release of Cu ions from the nanoparticles that are taken up by the cells and play a pivotal role in plant biochemistry. Therefore, significant variation in plant biomass, steviol glycoside and antioxidants are observed. Allam et al. (2001) showed a significant effect of nitrogen to enhance the concentration of stevioside in the stevia leaf while deficiency of some nutrients like potassium and calcium decreased the concentration of stevioside on a dry weight basis (Utumi et al. 1999). The accumulation of steviol glycoside in cells of *S. rebaudiana* relates to the extent of the development of the membrane system of chloroplast and the content of photosynthetic pigments (Ladygin et al. 2008).

Evaluation of antioxidant activities

Table 2 illustrates that a premier quantity of total phenolic content (6.22 µg GAE/mg), total flavonoid content (7.49 µg QE/mg), total anti-oxidant capacity (11.9 µgAAE/mg), total reducing power (11.5 µg AAE/mg) and % DPPH inhibition (74.8%) was assessed from *Stevia* leaves obtained from MS medium containing 10 mg/L of CuO nanoparticles. However, the lowest total phenolic content (3.99 µg GAE/mg), total flavonoid content (2.11 µg QE/mg), total anti-oxidant capacity (9.16 µgAAE/mg), total reducing power (10.3 µg AAE/mg) and % DPPH inhibition (58.5%) were found out from extracts containing 1000 mg/L of CuO nanoparticles employed in MS medium.The enzymatic and non-enzymatic antioxidants naturally present in plants actually help them cope with

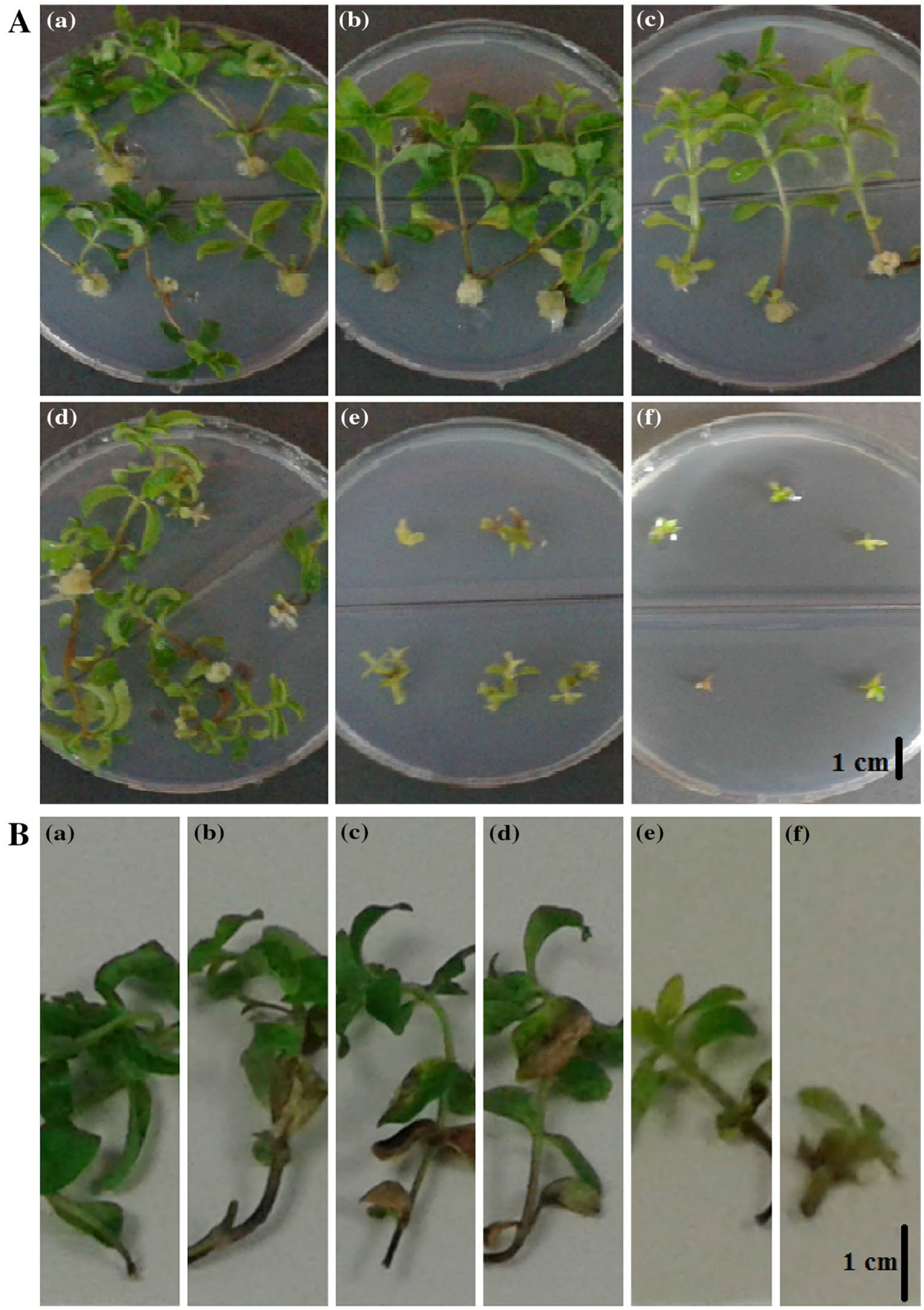

Fig. 4 **A** Shoot organogenesis of *Stevia* in MS basal medium containing **a** no CuO, **b** 0.1 mg/L CuO, **c** 1 mg/L CuO, **d** 10 mg/L CuO, **e** 100 mg/L CuO, **f** 1000 mg/L CuO nanoparticles. **B** Comparison of different-sized shoots of *Stevia* obtained in MS medium having **a** no CuO, **b** 0.1 mg/L CuO, **c** 1 mg/L CuO, **d** 10 mg/L CuO, **e** 100 mg/L CuO, **f** 1000 mg/L CuO nanoparticles

an oxidative stress of metal ions or free radicals (Klaper et al. 2009). Hence, the imbalance of endogeneous plant growth regulators and an accumulation of reactive oxygen species (ROS) causes depletion of plant cells and their activities (Choi and Hu 2008). Based on our study, CuO nanoparticles implicated an intracellular oxidative stress by the release of metal ions (Cu^{+2}) or free radicals into MS culture medium (Gajewska and Skłodowska 2007; Dimkpa et al. 2014), and as a consequence of oxidative damage at 1000 mg/L, an impaired growth, reduced steviol glycoside quantity, and mitigation of antioxidant activities was observed.

Conclusion

In conclusion, it has been demonstrated from these findings that CuO nanoparticles confer positive effect for in-vitro *Stevia* growth dynamics and steviol glycoside production. In this regard, a significantly enhanced amount

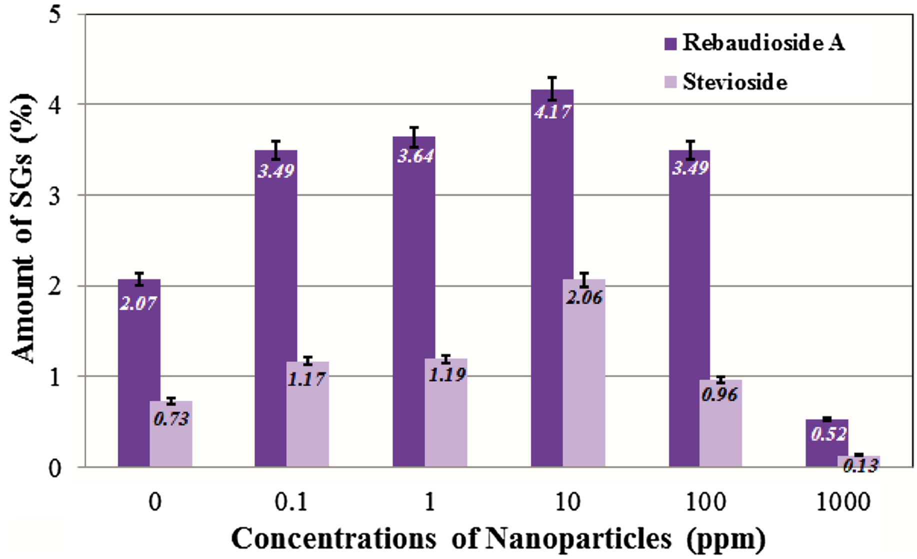

Fig. 5 Effect of CuO nanoparticles at different concentrations ranging between 0 and 1000 ppm on rebaudioside A content represented with dark purple bars and stevioside content with light purple bars. Error bars have been shown as standard deviation on each bar. (Color figure online)

Table 2 Comparison of phytochemical assays in 4 weeks old shoots produced from nodal explants on MS medium supplemented with different concentrations of CuO nanoparticles

Conc. of CuO nanoparticles (ppm)	TPC (μg GAE/mg)	TFC (μg QE/mg)	TAC (μg AAE/mg)	TRP(μg AAE/mg)	% DPPH inhibition
0	4.5 ± 0.0^{c}	3.8 ± 0.0^{d}	10.4 ± 0.0^{b}	10.5 ± 0.0^{c}	60.6
0.1	4.6 ± 0.0^{c}	6.3 ± 0.0^{b}	10.9 ± 0.0^{b}	10.7 ± 0.0^{b}	66.7
1.0	5.6 ± 0.0^{b}	6.3 ± 0.0^{b}	11.5 ± 0.0^{a}	10.8 ± 0.0^{b}	68.6
10	6.2 ± 0.0^{a}	7.5 ± 0.0^{a}	11.9 ± 0.0^{a}	11.5 ± 0.0^{a}	74.8
100	4.1 ± 0.0^{d}	5.7 ± 0.0^{c}	9.52 ± 0.0^{c}	10.6 ± 0.0^{bc}	64.2
1000	3.9 ± 0.0^{e}	2.1 ± 0.0^{e}	9.16 ± 0.0^{d}	10.3 ± 0.0^{d}	58.5

±: standard error, the means with the same letter within the columns are not significantly different according to Duncan's multiple range test at confidence level of 95%

TPC total phenolic content, *GAE* gallic acid equivalent, *TFC* total flavonoid content, *QE* quercitine equivalent, *TAC* total antioxidant capacity, *TRP* total reducing power, *AAE* ascorbic acid equivalent, *DPPH* 2,2-diphenyl-1-picryl hydrazyl

of secondary metabolites and antioxidant activities have been obtained at 10 mg/L of CuO nanoparticles concentration employed in MS basal medium. In the meanwhile, phytotoxic effects of CuO nanoparticles have also been observed, and the highest level of phtotoxicity has been achieved by CuO nanoparticles at 1000 mg/L concentration. Our research opens up new avenues for the study of metabolic pathways in context of an interaction between different concentrations of nanoparticles and in vitro grown medicinal plants.

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Author contributions RJ designed the study and wrote manuscript. AM and RJ performed experimental work. BY and RJ analyzed the data. MZ and EG critically reviewed the manuscript and added to its technical part. All authors have contributed, seen and approved the manuscript.

Compliance with ethical standards

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

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