Applied Biological Research 17 (1): 1-7; (2015)
DOI: 10.5958/0974-4517.2015.00001.4

PHYTOTOXICITY OF ZINC-NANOPARTICLES AND ITS INFLUENCE ON STEVIOSIDE PRODUCTION IN *Stevia rebaudiana* Bertoni

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(Received 16 July, 2014; accepted 19 November, 2014)

ABSTRACT

The present study was aimed to assess the effect of zinc-nanoparticles (Zn-NPs) on the growth and development of stevia (*Stevia rebaudiana* Bertoni) plant and to decipher the impact of Zn-NPs on secondary metabolite production. In present study, Zn-NP (< 100 nm) @ 50, 100, 200, 400 and 1000 mg L^{-1} concentration was tested. The nodal explants of stevia were cultured *in vitro* on MS medium where normal source of Zn was replaced with different concentrations of Zn-NPs. The Zn-NPs showed potential toxicity to stevia plant that was reflected from both morphological data as well as from the production of reactive oxygen species (ROS) scavenging enzymes under different treatment conditions. The 200 mg L^{-1} concentration of Zn-NP showed lower toxicity symptoms. The phytotoxicity severity increased at concentration of 400 mg L^{-1} and above with highest toxicity at 1000 mg L^{-1} concentration. The production of stevioside was significantly reduced when exposed to Zn-NP in a concentration depended manner. The study showed concentration dependent phytotoxic effect of Zn-NP on the physiology and stevioside production in stevia.

Key words: Phytotoxicity, ROS enzymes, stevia, Zn-nanoparticle, stevioside

INTRODUCTION

Nanoparticles (NPs) with dimension $\leq$100 nm fall in the transitional zone between individual atoms or molecules. The corresponding bulk materials in NP's can drastically modify the physiochemical properties of the materials and may generate adverse biological effects in organisms (Nel *et al*., 2006). The physiochemically-modified materials interact with living system in quite different manner; therefore, the information on these interactions is of paramount importance. The risks and benefits of many manufactured nano-materials into the environment have been documented (USEPA, 2007) which have aroused enormous attention of researchers in assessing their potential adverse impacts on ecosystems including human. The higher plants as a component of terrestrial environments are frequently exposed to NPs. There are many gaps in the current knowledge on their ecotoxicity and the challenges concerned to the biological effects of NPs (Monica and Cremonini, 2009).

M.Sc. dissertation report submitted at C.G. Bhakta Institute of Biotechnology, UKA Tarsadia University, Maliba Campus, Bardoli, Gujarat (India)

The unique properties of NPs such as high specific surface area, catalytic efficiency, surface energy, abundant reactive site and strong absorption enable NPs to significantly influence the living organisms. In most of NP studies related to their potential phytotoxicity, both positive and negative or inconsequential impacts have been reported (Menard *et al.*, 2011). Lin and Xing (2008) reported cell internalization and upward translocation of zinc oxide (ZnO) nanoparticles by *Lolium perenne*. Further, the presence of ZnO-NP significantly reduced ryegrass biomass including root tip and the root epidermal and cortical cells were highly vacuolated or collapsed. Lee *et al*. (2008) reported inhibition in growth rates of *Phaseolus radiatus* and *Triticum aestivum* on exposure to nanoparticles and depicted negative correlation of seedling length with the NP concentration. The phytotoxicity study of ZnO-NP in radish, rape, ryegrass, lettuce, corn and cucumber confirmed it to be one of the most toxic NP that could terminate root growth of test plants. Further, the ZnO-nano concentration of 400 mg L^{-1} inhibited seed germination (Lin and Xing, 2007).

The Zn phytotoxicity manifests as formation of smaller leaves and chlorosis of younger leaves, which may result in the reddening of younger leaves due to anthocyanin production (Masarovičová *et al.*, 2003). In severe cases plants may exhibit necrotic lesions on the leaves and eventually cause foliar death (Harmens *et al*., 1993). NPs may increase lipid membrane peroxidation upon contact to cell due to the increase in reactive oxygen (ROS) species (Nel *et al*., 2006). Plants elevate ROS scavenging enzyme production to overcome these toxic responses, therefore quantification of enzyme activities could be used as a measure in assessing phytotoxicity. The key ROS scavenging enzymes are peroxidase, catalase and superoxide dismutase. Metal contamination is reported to change the chemical composition of plant thus seriously impairing the quality, safety and efficacy of natural plant substances produced by medicinal plant species (Murch *et al*., 2003). The systematic study on the impact of NPs on secondary metabolites (SM) production has not so far been reported. The production of SM is influenced by imparted stress in plant and its play a vital role in stress tolerance. The Zn-NPs can efficiently penetrate the plant system as compared to their bulk counterparts, further the physiochemical properties of Zn-NPs are modified.

The leaves of stevia (*Stevia rebaudiana* Bertoni) are used as a sweetener and sugar substitute. Its steviol glycoside extracts (stevioside) are 300 times sweeter than normal sugar (Abdullateef and Osman, 2012). Stevia has attracted attention due to rise in demand for low-carbohydrate and low-sugar sweeteners. Since stevia has negligible effect on blood glucose, it is attractive to the people on carbohydrate-controlled diets especially diabetic people. The present study was aimed to improve our understanding about the effect of Zn-NP on the growth and development of stevia plant and stevioside production, a pharmaceutically and economically important SM production of stevia.

MATERIALS AND METHODS

This study was conducted at Department of Plant Molecular Biology and Biotechnology, ASPEE College of Horticulture and Forestry, Navsari Agricultural University, Navsari, Gujarat, India, in the year 2012-2013. The stevia (*Stevia rebaudiana* Bertoni) was grown *in vitro* on MS basal media (Modi *et al*., 2012) up to three subcultures of 21 days. The nodal segments from these *in vitro* grown plants were taken as explants and cultured on MS basal medium (positive control), MS medium devoid of normal Zn source *i.e.* $ZnSO_4.7H_2O$ (negative control) and MS medium in which normal source of Zn was replaced with different concentrations *viz.*, 50, 100, 200, 400 and 1000 mg $L^{-1}$of Zn-NP (<100 nm; Sigma) as earlier described by Lin and Xing (2007). The experiment was conducted in a completely randomized design where five explants per bottle and ten bottles per treatment were inoculated.

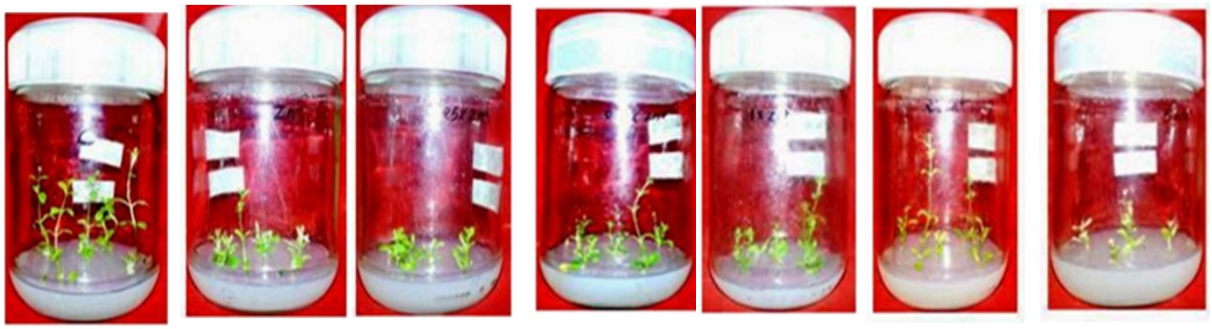
Different morphological observations *viz.*, leaf length, leaf width, shoot length, number of node culture^{-1}, internodal space and number of shoot culture^{-1} were taken at 21 days after incubation (DAI) on treatment media. These observations were recorded for each repetition of treatment and expressed in terms of mean. The standard statistical analysis such as standard error of mean, critical difference and coefficient of variation was performed to decipher the significance of treatment, if any, as per Panse and Sukhatme (1978).

To determine the phytotoxic effect of Zn-NP on stevia the ROS scavenging enzyme activity was studied from leaf tissues at 21 DAI. The superoxide dismutase (SOD; EC 1.15.1.1), guaiacol peroxidase (GPOX; EC 1.11.1.7) and catalase (CAT; EC 1.11.1.6) were estimated as per the standard procedures (Sadasivam and Manickam, 1992).

The leaf tissues of 21 DAI plants were also analyzed for stevioside content using the method of Bovanová *et al.* (1998). Prominence UFLC, high performance liquid chromatogram (HPLC) equipped with PDA detector 2800 (Shimadzu; Japan) and C-18 column were used in this analysis. The mobile phase was 70% methanol (Merck, Germany) with flow rate of 0.9 mL min^{-1}. The stevioside were detected at 210 nm and identified by retention time and co-chromatographed by spiking the sample with the authentic standard i.e. Stevioside (50956-10 MG, Sigma, USA).

RESULTS AND DISCUSSION

Normal growth was observed in *Stevia rebaudiana* plants grown on MS basal media supplemented with Zn in the form of $ZnSO_4.7H_2O$ (Fig. 1a). The typical symptoms of Zn deficiency, *viz.*, whitish leaves due to the decrease in chlorophyll content, reduced narrow leaves, reddening of stem and axillary bud due to anthocyanin production, were observed in stevia plant raised on MS medium devoid of Zn (Fig. 1b). The Zn deficient plant showed dwarfing reflected with reduction in number of node culture^{-1} and shoot culture^{-1}. The plant cultured on MS medium supplemented with Zn-NPs (< 100 nm) showed phytotoxicity in a concentration dependent manner (Fig. 1c-g). The Zn nanoparticle up to 200 mg L^{-1} concentration showed increase in leaves length and width but higher concentration caused decrease in leaf length and width along with manifestation of phytotoxicity symptoms (yellowing of leaves). The shoot length, number of shoot culture^{-1} and number of node culture^{-1} were lower in Zn-NP treated plants upto 400 mgL^{-1} concentration as compared to the control but were at par with negative controlled plant. The 1000 mg L^{-1} concentration of Zn-NP drastically reduced these parameters and maximized the phytotoxicity (Table 1).



A B C D E F G

Fig. 1: Effects of various concentrations of zinc-nanoparticles on the physiology of *Stevia rebaudiana* plants at 21 DAI. A) MS basal medium (positive control); B) Zn-free MS medium (negative control); Zn-free MS medium supplemented with Zn-NP at the concentration of 50 mg L^{-1} (C), 100 mg L^{-1} (D), 200 mg L^{-1} (E), 400 mg L^{-1} (F) and 1000 mg L^{-1} (G)

Table 1: Effect of different concentration of zinc-nanoparticles (< 100 nm) replacement in MS medium on physiological characteristics of *Stevia rebaudiana*

Treatments	Leaf length (cm)	Leaf width (cm)	Shoot length (cm)	Nodes culture^{-1}	Internodal space (cm)	Shoots culture^{-1}
Zn (positive control)	0.47	0.27	6.67	4.00	1.07	6.00
Zn (negative control)	0.38	0.23	3.25	2.50	1.15	4.50
Zn-NP @ 50 mg L^{-1}	0.88	0.45	3.13	2.50	1.18	2.75
Zn-NP @ 100 mg L^{-1}	0.78	0.48	2.38	2.25	1.05	2.75
Zn-NP @ 200 mg L^{-1}	0.75	0.38	3.75	2.75	1.10	4.50
Zn-NP @ 400 mg L^{-1}	0.50	0.25	3.50	2.67	1.17	4.00
Zn-NP @ 1000 mg L^{-1}	0.45	0.30	1.50	2.00	0.60	2.00
Mean	0.60	0.33	3.02	2.67	1.04	3.79
SEM±	0.02	0.02	0.09	0.18	0.05	0.18
$CD_{0.05}$	0.05	0.05	0.27	0.54	0.14	0.54
CV (%)	5.06	5.06	4.92	6.55	6.66	6.11

Exposure of plant to abiotic stress leads to the production of free radicals that alter normal physiological activities of plant. The plant produces various enzymes that help to scavenge the ROS produced in their cells. In present study, lower activity of ROS scavenging enzymes, *viz.*, catalase, guaiacol peroxidase and superoxide dismutase was observed in the leaves of stevia plant under controlled condition (Table 2). The Zn deficiency increased these enzymes activities, whereas exposure of stevia plants to Zn-NP drastically increased the guaiacol peroxidase activity up to 100 mg L^{-1} Zn-NP and reduced thereafter. The maximum guaiacol peroxidase activity (98.46 µM min^{-1} g^{-1} protein) was observed at 100 mg L^{-1} Zn-NP. The use of 1000 mg L^{-1} Zn-NP gave highest catalase activity (68.43 µM^{-1} min^{-1} g^{-1} protein). Other concentrations of Zn-NP also showed significantly higher catalase activity over control. Superoxide dismutase activity increased slightly under different Zn-NP treatment conditions with maximum activity observed at 1000 mg L^{-1} Zn-NP (7.15 unit g^{-1} protein) that is at par with negative control (6.94 unit g^{-1} protein). The productions of ROS scavenging enzymes are general measures to protect plant against stress. Increase in ROS activity can directly correlated with imparted stress to plant.

Sweet tasting stevioside (STS) is a diterpene glycoside 300 times sweeter than sucrose produced in stevia plant (Abdullateef and Osman, 2012). The stevioside concentration was measured by HPLC and standard stevioside peak observed at 3.13 min retention time (RT) which was used for the identification of stevioside from sample. The highest stevioside content 0.80% was

Table 2: Effect of different concentration of zinc-nanoparticles (< 100 nm) replacement in MS medium on stevia ROS activity

Treatment	Catalase (µM min^{-1} g^{-1} protein)	Guaiacol peroxidase (µM min^{-1} g^{-1} protein)	Superoxide dismutase (Unit g^{-1} protein)
Zn (positive control)	7.11	35.00	2.50
Zn (negative control)	10.06	50.21	6.94
Zn-NP @ 50 mg L^{-1}	56.75	91.56	2.63
Zn-NP @ 100 mg L^{-1}	52.69	98.46	4.45
Zn-NP @ 200 mg L^{-1}	64.33	56.62	4.11
Zn-NP @ 400 mg L^{-1}	60.65	48.66	4.17
Zn-NP @ 1000 mg L^{-1}	68.43	60.60	7.15
Mean	45.72	63.01	4.56
SEM±	0.24	0.27	0.07
$CD_{0.05}$	0.69	0.78	0.21
CV (%)	0.89	0.73	2.73

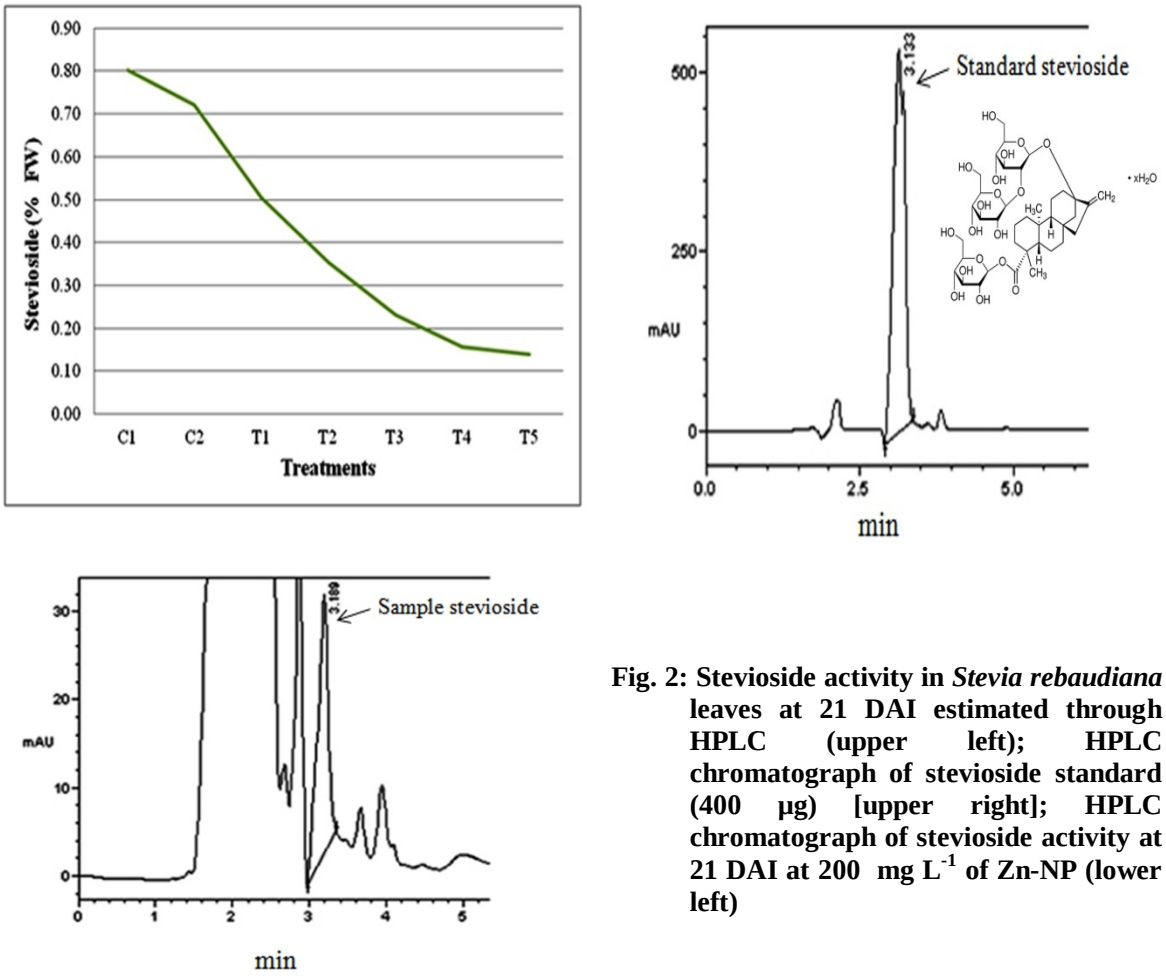


Fig. 2: Stevioside activity in *Stevia rebaudiana* leaves at 21 DAI estimated through HPLC (upper left); HPLC chromatograph of stevioside standard (400 µg) [upper right]; HPLC chromatograph of stevioside activity at 21 DAI at 200 mg L^{-1} of Zn-NP (lower left)

observed in leaves raised on basal MS medium alone. Zn deficiency leads to reduction in stevioside content, whereas the Zn metal NP treatment drastically reduced the same in the concentration dependant manner. The inverse relation between the stevioside content and Zn-NPs concentration in culture media was observed (Fig. 2).

Compared to other contaminant NP size plays important role in the physio-chemical behaviour, reactivity and its toxicity (Monica and Cremonini, 2009). In present study the concentration dependent phytotoxicity of Zn-NP was observed. The increased concentrations of NPs resulted in severe symptoms of toxicity. The 200 mg L^{-1} concentration reported lower toxicity symptoms, the phytotoxicity severity increased above the 400 mg L^{-1} concentration. The 1000 mg L^{-1} concentration showed highest toxicity. Lee *et al*. (2010) reported that ZnO-NP at 400 mg L^{-1} concentration inhibited the germination in *Arabidopsis thaliana*. In present study 1000 mg L^{-1} Zn-NP concentration showed severe phytotoxicity to setvia plant. The ROS scavenging enzymes showed its potentiality as marker for study of phytotoxicity under *in vitro* condition. The catalase, guaiacol peroxidase and superoxide dismutase activity drastically increased upon exposure to Zn-NP. The catalase activity in buckwheat seemed to be increased upon ZnO-NP exposure and showed saturation pattern at the high dose i.e., 1,000 and 2,000 mg L^{-1} (Lee *et al*., 2013). This phenomenon resulted from high dose of NPs that produced too much ROS, which exceeded the scavenging capacity of catalase, inhibited its activity and produced oxidative damage (Xia *et al.* 2006; Kim *et al*. 2009). Nel *et al.* (2006) reported that NPs may increase lipid membrane peroxidation upon contact to cell due to the increase in ROS. The increased ROS enzymes can be exploited as measure of phytotoxicity. The short term exposure of tomato seed to NPs has been reported to significantly

reduce the root growth and causes oxidative imbalance marked with enhanced level of antioxidant marker enzymes, as a manifestation of phytotoxicity (Faisal *et al*., 2013). Murch *et al*., (2003) reported that metal contamination change the chemical composition of plant thereby, seriously impacting the quality, safety and efficacy of natural plant substances produced by medicinal plant species. The toxicity to plant leads to change in metabolic flux and there by the production of SM is altered. Further if the SM is engaged to combat stress its concentration changes drastically. The Zn-NP significantly altered the stevioside production, and the alteration was NP concentration dependant. The concentrations > 200 mg L^{-1} significantly reduced the stevioside production in stevia which further reduced with increasing concentration of Zn-NP.

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