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Effect of Ag Nanoparticles on Morphological and Physio-biochemical Traits of the Medicinal Plant *Stevia Rebaudiana*

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Abstract. Nowadays, overproduction of secondary metabolites in remedial herbs through giving biotic/abiotic stresses is an interesting area of research. In the current study, the influences of various concentrations of silver nanoparticles (Ag NPs) were evaluated on several morphological and physio-biochemical traits, such as the steviol glycosides level in *Stevia*. The findings showed that the herbs incubated with 400-ppm Ag NPs own the highest dry and fresh weight of shoot, while those incubated with 80- up to 200- ppm Ag NPs own the highest steviol glycosides content. As a result, we successfully improve the content of stevioside glycoside up to 1.75-fold by applying the 80- up to 200-ppm Ag NPs in *Stevia* medicinal plant. Moreover, our findings revealed that low concentrations the Ag NPs lead to an increase of glutathione content and total antioxidant capacity, and a decrease of MDA, whereas treatments at higher concentrations induced adverse effects for the plant. As a result, the treatment with Ag NPs low concentrations had a favorable efficacy on physio-biochemical and morphological characteristics of *Stevia*. These achievements are very promising, because they revealed a considerable capability for the Ag NPs application in enhancing the secondary metabolites in *Stevia* remedial herb. The present study is the first case assessing the desirable influences of Ag NPs on the Stevia, in regard with shifting of biosynthetic pathway of steviol glycosides in a concentration-dependent manner.

Keywords: Ag Nanoparticles, Medicinal Plant, *Stevia*, Steviol Glycosides.

INTRODUCTION

Stevia (*Stevia Rebaudiana* Bertoni) is a medicinal perennial herb sweet in taste. This herb is a member of Asteraceae family and native to Paraguay as well as Brazil (Shivanna *et al.* 2012). *Stevia* gives rise to steviosides and rebaudiosides as zerocalorie diterpene glycosides, and naturally keeps safe from obesity, hypertension, and diabetes mellitus (Thiyagarajan and Venkatachalam 2012; Goyal *et al.* 2010; Geuns 2003). *Stevia* can be propagated through tissue culture techniques for producing elite varieties (Yucesan et al. 2016). The less efficiency of stem cutting and poor seeds germination actually led to difficulties for *in vitro* large-scale propagation of this herb (Hendaw-

ey *et al.* 2015). Up to now a number of approaches have been established to achieve an increased content of secondary metabolites from the leaf tissues of *Stevia* (Javed *et al.* 2017a; sı *et al.* 2020; Liu *et al.* 2021).

Biotic and abiotic elicitors own the potential of bringing about the larger level of secondary metabolites as well as sweetening compounds through modification of metabolic cycles (Sabzehzari and Naghavi 2018, 2019; Sabzehzari *et al.* 2020, 2019; Gupta *et al.* 2015; Peng *et al.* 2021; Ma *et al.* 2021). The elicitors, however, are beneficial up to particular threshold levels because being cytotoxic at much higher concentration (Javed *et al.* 2017a; Hendawey *et al.* 2015; Chen *et al.* 2021; Bi *et al.* 2021). The cytotoxic influences of various nanoparticles as abiotic elicitors have been recorded in a variety of crops/plants (Javed *et al.* 2017b, c; Shaw and Hossain 2013; Lin and Xing 2008; Lee *et al.* 2008, 2010; Spanò *et al.* 2020).

In terms of Stevia, only a few types of nanoparticles have been evaluated. For instance, Rezaizad *et al.* (2019) observed that the plants incubated with 200 ppm TiO_2 NPs had the lowest MDA extent and the highest steviol glycosides content, while those incubated with 400 ppm TiO_2 NPs had the highest fresh and dry weights of shoot. Accordingly, the authors suggested that the treatment with TiO_2 NPs leads to a positive influence on phytochemical and morphological attributes in Stevia herb. Javed *et al.* (2018) observed that total reducing power (TRP), total antioxidant capacity (TAC), scavenging activity of free radical (DPPH), and total phenolic content (TPC) were highest at 10 ppm of CuO NPs, while the highest level of total flavonoid content (TFC), DPPH, and TPC were registered at 100 ppm concentration of ZnO NPs. Their results clearly showed that CuO NPs are more cytotoxic to Stevia plant when compared to ZnO NPs. Thus, the authors proposed a promising way for future research using CuO or ZnO NPs for increasing commercially significant secondary metabolites in various medicinal herbs.

In terms of Ag NPs, Kaveh et al. (2013) observed that exposure to higher concentration of these nanoparticles (up to 20 ppm) led to a decrement of the biomass in Arabidopsis. Similarly, Dimkpa *et al.* (2013) recorded that Ag NPs treatment decreased the roots and shoots length in a dose-dependent way in wheat. Nair and Chung (2014a) also recorded that Ag NPs treatment decreased root and shoot weight and root elongation in rice. Al-Huqail *et al.* (2018) registered a decrease in the total protein content, total chlorophyll content, fresh weight, and root and shoot elongation after exposure to Ag NPs in *Lupinus termis*. Patlolla *et al.* (2012) reported that Ag NPs treatment enhanced the micronuclei and chromosomal aberrations and declined the mitotic index in root tips of broad bean, proposing that mitosis and cell cycle in root tips was disrupted by silver nanoparticles. However, there are several studies that documented the positive effect of silver nanoparticles on plant growth and development in a plant-dependent manner (Reviewed in Yan and Chen, 2019). However, there is no report on the effect of silver nanoparticles on Stevia plant. Based on what has been mentioned about the value of Stevia secondary metabolites and the effect of silver nanoparticles, the present study was focused on the evaluation of the photocatalytic efficacy of Ag NPs on the phytochemical as well as morphological characteristics of Stevia plant under controlled conditions.

MATERIAL AND METHODS

Plant material and growth conditions

The seeds of Stevia were provided by the College of Agriculture and Natural Resources, University of Tehran, Iran. The current research was carried out through a completely randomized design with three replications. After germination, the seedlings were moved to pot comprising pittmoss and perlite (1:1) (each pot including three samples), and then put in growth chambers for 10 days under 18-h light at 25°C to grow and take root. The seedlings were moderately irrigated on the first day, and then watered every two days through 50% Hoagland solutions. The treatments in this study were performed at increasing concentrations (0, 20, 40, 60, 80, 100, 200, 400 and 800) of Ag NPs. After being developed, the leaves of all plants were sprayed by Ag NPs on the 11th day. The subsequent spraying operations were performed one week later, followed by the last spraying operations two weeks later. At the end of the treatments some morphological and physio-biochemical traits like the dry and fresh weights of shoot, MDA level as a measure of membrane lipid peroxidation, glutathione content, total antioxidant capacity and the steviol glycosides content were assayed in the *Stevia* leaves.

Morphological evaluation

To estimate the fresh weights, the shoots (leaves as well as stems) were washed, cut into pieces, and eventually the shoot fresh weights were registered in g. To measure the dry weights, the samples were dried at 60°C by uing an oven, and ultimately the weights were registered in g.

Physio-biochemical analysis

The estimation of the level of malondialdehyde (MDA), as output derived from lipid peroxidation, has been performed by the method described by Cakmak and Horst (1991). The absorbance at 532 and 600 nm of cell extracts was recorded and the average of the readings in triplicate was utilized for estimating the level of MDA by 155 $mM^{-1}cm^{-1}$ extinction coefficient as follows: malondialdehyde (nM)= $\Delta A_{(532-600)}/1.56*10^5$. The glutathione content (GSH) was calculated through the procedure as elucidated by Moron *et al.* (1979). For evaluation of total antioxidant capacity, 100 µL stock solution of each specimen (5 mg/mL in dimethyl sulfoxide) was combined with 1000 µL reagent solution, including 0.7 M of sulfuric acid, 5 mM of ammonium molybdate, and 30 mM of sodium phosphate. The reaction admixture was maintained for an hour and a half at 95°C, followed by cooling at 25°C. The absorbance of specimens was recorded at 695 nm through micro plate reader in triplicates. Vitamin C was utilized as standard. The data was represented as µg AA/mg (i.e., mg ascorbic acid equivalent) (Ali et al. 2015).

Measurement of steviol glycosides

To calculate the steviol glycosides content in leaves, 100mg of dry leaves was incubated in 10 ml methanol for 15 min. The residual solvent was removed and the solid residue was solubilized in 5ml water/acetonitrile mix (20:80). The extract (20µL/ml) was injected into the HPLC column (Cosmosoil 5 NH2-MS with particle size of 5 µm, 4.5 mm in diameter, and 15 cm in length) linked to the HPLC (Rezaizad et al. 2019).The moving phase of 80% of acetonitrile as well as 20% distilled water was set at a rate of 1 ml/min.

Statistical analysis

The q data was analyzed through SPSS Ver. 20. In variance analysis, p=0.05 was taken into consideration as significant level.

RESULTS AND DISCUSSION

Influence of Ag NPs on the dry and fresh weights of shoot, membrane lipid peroxidation, glutathione content, total antioxidant capacity and the steviol glycosides content was assayed in the Stevia plants. The findings revealed that Ag NPs own a considerable positive efficacy on the analyzed traits.

Influence of Ag NPs on the dry and fresh weights of shoot

A comparison of mean dry and fresh weight of shoot in Ag NPs-treated Stevia plants indicated that the 0 ppm Ag NPs (control) sample has the lowest shoot weight, whereas the 400-ppm concentration of Ag NPs has the highest (Figures 1, 2). The fresh and dry weights were increased up to 5- and 3-fold in the 400-ppm Ag NPs-treated Stevia plants when compared to control, respectively. In order to explain our observations, we can suppose that Ag NPs can stimulate root strength and enhance the capability of the root to uptake nutrients and water, finally leading to an increment in the fresh and dry weights of the shoot, as reported by Vannini et al. (2013) in *Eruca sativa*. Moreover, it was found that the using of Ag NPs in the early developmental stages has ability to increases the carbon fixation efficiency and photosynthesis rate in the plants, resulting in enhancing of the yield of dry matter (Rezaizad et al., 2019). However, the 800-ppm Ag NPs treatment led to a considerable decrease in dry and fresh weight of shoot in treated Stevia plants. Similarly, in *Sorghum bicolor*,

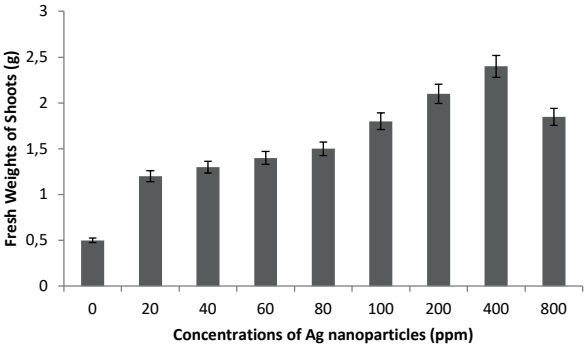

Figure 1. Efficacy of various concentrations of Ag nanoparticles on fresh weight of shoots in Stevia plant.

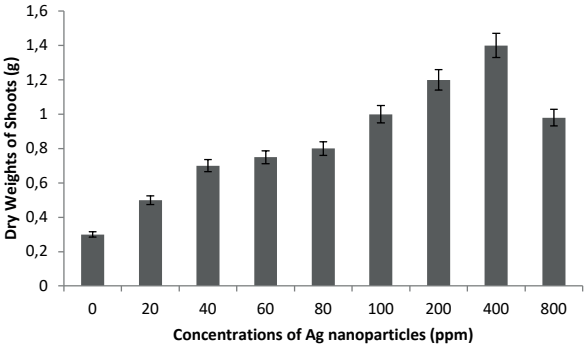

Figure 2. Efficacy of various concentrations of Ag nanoparticles on dry weight of shoots in Stevia plant.

Krishnaraj *et al*. (2012) observed at low concentrations of Ag NPs, an increase in the shoot and root length and weight, while at high concentrations a decline in the length and weight.

Influence of Ag NPs on the MDA

The production rate of free oxygen radicals has a close relationship with the strength of the membrane (Shivanna *et al*. 2012). Malondialdehyde, as a reactive oxygen species leads to peroxidation of membrane lipid, enhancing membranes permeability, whereas declining membranes strength. Thus, the content of MDA serves as a measure of the lipid peroxidation, offering an image of cell damages (Yan and Chen, 2019).

However, the 200- up to 800-ppm Ag NPs treatments resulted in an increase in MDA level in treated Stevia plants (Figures 3). Similarly, Thiruvengadam *et al*. (2015) evaluated the effect of Ag NPs exposure in turnip seedlings, observing that a higher concentration of Ag NPs led to overproduction of superoxide radicals and increased the lipid peroxidation; hydrogen peroxide production was also enhanced after exposure to silver nanoparticles. Nair and Chung (2014b) recorded that exposure to Ag NPs leads to an increment in lipid peroxidation and hydrogen peroxide production in rice root and shoot in a dose-dependent way. Nair and Chung (2014a) documented that lipid peroxidation increases after exposure to silver nanoparticles in Arabidopsis. De La Torre-Roche *et al*. (2013) also documented that Ag NPs exposure with concentration at 500 up to 2000 ppm caused a considerable increase in MDA level in soybean. Overall, our results revealed that the 100-ppm Ag NPs treatment can decline the level of MDA, whereas Ag NPs treatment with a concentration above 100-ppm represents an adverse effect likely because of damaging to thylakoid membrane structure (Nair and Chung 2014a; Shivanna *et al*. 2012).

Influence of Ag NPs on the glutathione content

In light of our findings, glutathione content was enhanced in *Stevia* plants after 100-ppm Ag NPs treatments when compared to the 0 ppm (control) concentration. However, the 200- up to 800-ppm Ag NPs treatments led into a decrease in glutathione content in treated *Stevia* plants. The glutathione extent was increased up to 2-fold in the 100-ppm Ag NPs-treated herbs when compared to control conditions (Figures 4). Similarly, Nair and Chung, (2014b) reported an increased glutathione content after exposure *Arabidopsis thaliana* seedlings to the high concentration of Ag NPs. The enhanced level of glutathione may be resulted from the increased glutathione biosynthesis, expression of glutathione S-transferase and glutathione reductase genes, and sulfur assimilation after exposure to Ag NPs (Nair and Chung, 2014b). After silver NPs exposure, a considerable increase in shoots glutathione content was observed, proposing that plant employs glutathione to decrease the effect of ROS derived from the high concentration of silver nanoparticles (Mirzajani *et al*. 2013). These outputs are in coincidence with Jiang *et al*. (2014), who showed that silver own an important function in the increase of antioxidant potentials like glutathione content in *Spirodela polyrhiza* plant. In fact, antioxidants such as glutathione present a key role in detoxification of toxic metal ions (Pompella *et al*. 2003). Indeed glutathione is a key antioxidant in crops/plants, and preserves significant cellular components from reactive oxygen species (Singh and Sinha, 2005).

Influence of Ag NPs on the total antioxidant capacity

We found that total antioxidant capacity enhanced in *Stevia* plants after incubation with the 20- up to 200-ppm Ag NPs concentrations in contrast to the con-

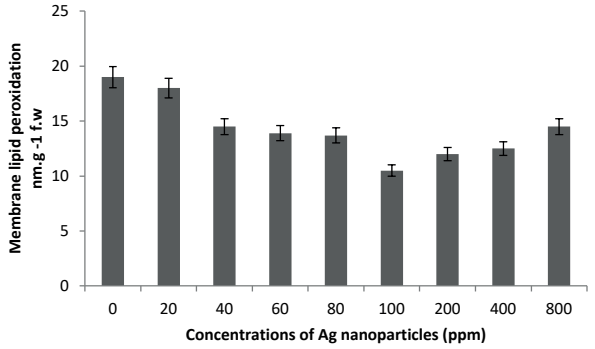

Figure 3. Efficacy of various concentrations of Ag nanoparticles on the MDA (membrane lipid peroxidation) in Stevia plant.

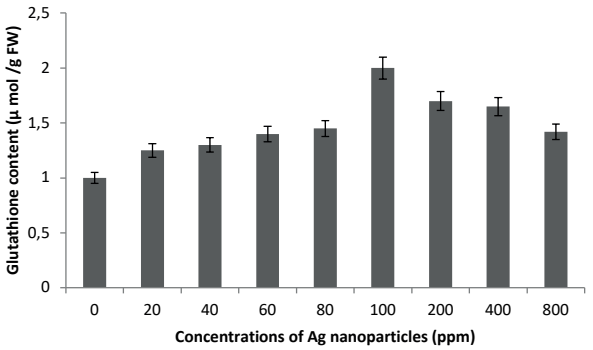

Figure 4. Efficacy of various concentrations of Ag nanoparticles on glutathione content in Stevia plant.

trol conditions. However, the 400- and 800-ppm Ag NPs treatment resulted in a decrease in total antioxidant capacity in the *Stevia* herbs. The total antioxidant capacity was increased up to 1.65-fold in the 100- and 200-ppm Ag NPs-treated *Stevia* plants when compared to control (Figures 5). In line with our findings, Jiang *et al*. (2014) reported Ag NPs can induce accumulation of ROS, and alter antioxidant system in the *Spirodela polyrhiza* aquatic plant. Qian et al. also observed that Ag NPs accumulated in the leaves of Arabidopsis and changed the transcription of aquaporin and antioxidant genes, proposing that Ag nanoparticles can alter the balance between antioxidant as well as oxidant systems (Qian *et al.* 2013).

Influence of Ag NPs on the stevioside glycoside content

Analysis of the stevioside glycoside content revealed that 80- up to 200-ppm Ag NPs concentrations led into the highest stevioside glycoside content, whereas spraying the Ag NPs at 400- and 800-ppm concentrations lead to a decrease in percentage weight of this compound (Figures 6). As a result, we successfully improve the content of stevioside glycoside up to 1.75-fold by applying the 80- up to 200-ppm Ag NPs in *Stevia* medicinal plant. To the best of our knowledge, no research has ever been documented the Ag NPs application on *Stevia* medicinal herb so far. However, previous findings applying copper, silicon, iron, as well as TiO_2 NPs on this herb showed that the higher concentrations of copper and iron nanoparticles lead to the higher levels of stevioside when compared to control (Rezaizad *et al.* 2019). For silicon NPs, the findings indicated that the highest stevioside content is generated at low concentrations (0.20 up to 2 mg/L) and the least extent of stevioside glycoside is produced at the 4 up to 8 mg/L concentrations of silicon NPs (Hendawey *et al.* 2015).

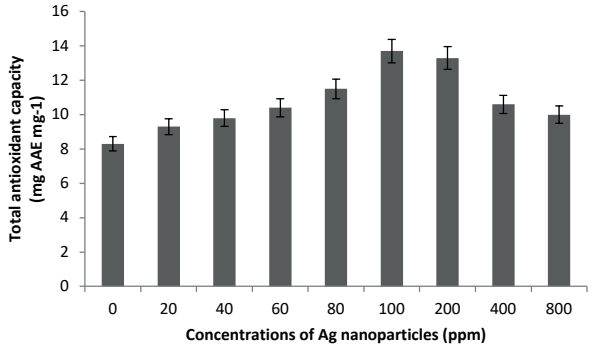

Figure 5. Efficacy of various concentrations of Ag nanoparticles on total antioxidant capacity in Stevia plant.

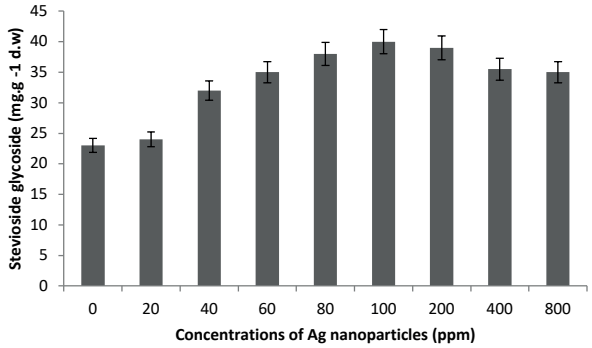

Figure 6. Efficacy of various concentrations of Ag nanoparticles on stevioside glycoside content in Stevia plant

CONCLUSION

Many studies showed the adverse effect of silver nanoparticles on various crops/plants at molecular, cellular, physiological, and morphological level (Reviewed in Yan and Chen 2019; Wang *et al.* 2021; Yin *et al.* 2021; Zhao *et al.* 2021). However, a number of researches recorded the positive effect of Ag NPs on plants growth and development, and on physio-biochemical parameters (Vannini *et al.* 2013; Krishnaraj *et al.* 2012; Jia *et al.* 2020; Shi *et al.* 2021; Zheng *et al.* 2021; Zhu *et al.* 2021). These contradictory findings reveal the complexity of the response of crops/plants to silver nanoparticles, which are not only determined through the features of Ag NPs (Ag chemical form, surface coating, shape, concentration, size, etc.), but are also dependent on the plant systems utilized (developmental stage, organ, tissue, species, etc.) and experimental methodologies (exposure time, exposure procedure, medium, etc.). We firstly reported the Ag NPs, at low concentrations, had a favorable efficacy on physio-biochemical and morphological characteristics of *Stevia* herb, while the high concentrations had an adverse effect.

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