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Effects of graphene oxide nanoparticles on biochemical attributes in soybean (*Glycine max*) roots

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ABSTRACT

Graphene oxide (GO), as one of the most prominent members of the graphene family, has attracted ever-increasing interests because of many medical and industrial applications. The presence of nanomaterial (NMs) in the environment due to their wide applications and the potential for their uptake and transport by plants have raised many concerns about the adverse effects of these materials on the food chain. Therefore, it is necessary to investigate NM-plant interactions. In this work, due to the importance of soybean in the food industry, the effect of GO on some biochemical properties of soybean root was considered. Obtained results showed significant increase in POD and SOD enzyme activities, along with H_2O_2, phenolics and flavonoids contents, which were noticeable at the treatments of 800 and 1600 mg/L. The effect of GO on fresh weight, dry weight, length, and root volume of soybean were observed at different concentrations, with the highest effect at 400 mg/L.

Key words: Graphene oxide (GO), nanomaterial (NMs), Soybean, Reactive oxygen species (ROS).

INTRODUCTION

Toxicity of nanomaterials (NMs) is mainly related to their very small size, large surface area, and high reactivity (Magdolenova et al. 2014) Interaction of NMs with biological systems can be through chemical, mechanical, catalytic, and surface effects (Van Aken 2015). The decrease in photosynthetic rate, photosynthetic pigments, changes in the morphology and biomass, as well as degradation of proteins and nucleic acids are the toxic effects of NMs in plants (Yang et al. 2017). ROS generation has been regarded as one of the main responses of plants to NMs followed by oxidative damage and

cell death. The adverse effects of NM-induced ROS can be inhibited by the activation of the antioxidant defense systems in plants (Mahjouri et al. 2018). Antioxidantive enzymes for example superoxide dismutase (SOD), catalase (CAT), and peroxidases (POD), along with non-enzymatic antioxidants for instance ascorbate, glutathione, thiols, and phenolic compounds are the most important members of the defense system which protect plants against oxidative damage (Ma et al. 2010).

Graphene oxide (GO) is an oxidized derivative of graphene with oxygen-containing groups on the surface which provide many reactive sites for the binding of external functional groups such as small molecules, polymers, biological macromolecules, and inorganic nanoparticles (NPs) without the use of any reactants or additional surface mod (Huang et al. 2012). Unlike graphene, GO is capable to form stable solutions in water and some polar organic solvents. Besides, due to the exceptional electrochemical and catalytic properties, GO has promising applications in various biomedical and industrial fields (Jastrzębska et al. 2012). Before using in various applications, it is necessary to investigate environmental risk and health consequence of GO. Previous studies have been identified that GO at environmentally relevant concentrations (μg/L) can be easily absorbed by root hairs and accumulate in the cytosol of parenchyma cells in the root of *Arabidopsis* plants. Although, GO, at these concentrations, did not significantly influence development and basic physiological functions (Zhao et al. 2015). However, it has been reported that GO induced severe oxidative stress and membrane ion leakage under stress conditions (the mutual exposure to GO and polyethylene glycol 6000 (20%) or NaCl (200 mM)) in *Arabidopsis* seedlings (Wang et al. 2014). Considering the scarcity of studies, it is necessary to evaluate the effects of GO in plants.

Soybean (*Glycine max*) is one of the most important crops which plays a major role in the preparation of edible oil, plant protein, and chemical products. Soybean is also an alternative for biofuels (Pedrozo et al. 2018). Soybean has shown developmental and physiological responses to different NMs. The long-term exposure to 50 and 500 mg/kg of ZnO nanoparticles (NPs) adversely affected the growth and reproduction of soybean in a soil microcosm (Yoon et al. 2014). The presence of CeO_2 NPs in roots and its genotoxic effects on soybean plants have been reported (López-Moreno et al. 2010). Root exposure of Ag NPs showed significant reduction in soybean plant biomass, while increased the malondialdehyde and H_2O_2 contents of leaves. Uptake and translocation observations indicated the possibility of contamination of the edible parts of plants by Ag NPs (Li et al. 2017). In this study, the effect of GO on some biochemical aspects of soybean including hydrogen peroxide production, the activity of CAT, SOD, and POD, as well as total phenolics and flavonoid contents were assessed.

MATERIALS AND METHODS

Plant cultures and treatment

Soybean seeds (Kowsar cultivar) were surface sterilized using 10% sodium hypochlorite solution (2 min) and then washed with sterile distilled water for several times. After being germinated on petri dishes with filter papers for 1 week, seedlings were transferred to the pots containing perlite and kept at the growth chamber under the daily photoperiod of 16 h light and 8h dark at 24 ± 1 ˚C. Plants were nourished with Hoagland solution (pH 6.3-6.5) (Hoagland & Arnon 1950). Experiments were conducted in the two-leaf stage of growth after 2 weeks of culture. 3 plants were used for each pot with 3 independent replicates. Plants were treated with different concentrations of GO (99%, 3.4-7 nm, 6-10 layers) solution (0, 100, 200, 400, 800 and 1600 mg/L) for 3 weeks (3 times a week). The root samples immersed in liquid nitrogen and stored in a -80 °C freezer until using in biochemical analysis.

Measurement of growth parameters

The harvested plant roots were washed with distilled water and the following parameters were recorded: root volume (cm^3, determined by water displacement), longest root length (mm), and root fresh and dry weight (dried at 70 °C for 72 h).

Measurement of H_2O_2 content

For calculating of the H_2O_2 accumulation, 0.1 g of the root samples was ground in a 0.1% trichloroacetic acid (TCA) solution at 4 ºC. After centrifuging (12,000 *g* for 15 min at 4 °C), 0.5 mL of resulted clear extract was mixed with 0.5 mL of 10 mM potassium phosphate buffer (pH 7) and 1 mL of 1 M potassium iodide reagent. The reaction mixture was kept at 25 °C for 15 min and finally its absorbance was identified at 390 nm. The amount of H_2O_2 was calculated based on a standard curve (Velikova et al. 2000).

Antioxidant enzyme activity

A quantity of 0.1 g of the untreated and treated soybean roots was ground with 1 mL of 50 mM potassium phosphate buffer (pH 7) at 4 °C. After centrifugation (10,000 g at 4 °C for 20 min), the obtained supernatant was used at the antioxidant enzyme activity experiments. Total SOD activity was determined by Winterbourn et al. (1976). One unit of the enzyme activity was considered as the amount of SOD enzyme needed for the inhibition of 50% of nitro blue tetrazolium chloride reduction. Total POD activity was measured by Chance and Maehly method (Chance & Maehly 1955). POD activity was determined as the enzyme quantity which converts 1 μmol guaiacol to tetraguaiacol per minute per ml. The Bradford reagent and bovine

serum albumin (BSA) were used as standard for calculating total protein content (Bradford 1976).

Total phenolic and flavonoid contents

Phenolic compounds content was measured using the Folin-Ciocalteu method (Singleton et al. 1999). Accordingly, 100 µl of methanol extract (0.1 g root extracted using 1 mL of methanol) and 100 µl of Folin reagent were dissolved in 2.5 ml of distilled water. After 6 min, 150 µl of 20% sodium bicarbonate was added to the reaction mixture and stored at dark condition for 30 min. Finally, the absorbance of the mixture was read at 765 nm. Total phenol content was determined as µg/g fresh weight according to obtained standard curve using gallic acid.

Flavonoid content of the methanolic extracts was quantified by colorimetric method using aluminum chloride (Quettier-Deleu et al. 2000). An amount of 0.5 ml of methanol extract was mixed with 0.5 ml of 2% aluminum chloride dissolved in methanol and kept at 25 ºC for 60 minutes in the dark. Then, the absorbance of the solutions was recorded at 415 nm by spectrophotometer and total flavonoid content was expressed as µg/g fresh weight based on quercetin standard curve.

Statistical analysis

The data were analyzed with one-way analysis of variance (ANOVA; IBM–SPSS ver. 22) followed by Duncan's multiple range test ($P \leq 0.05$). All data are expressed as average values from the three independent experiments ± standard error of the means (SE), (Table1).

RESULTS AND DISCUSSION

Analysis of variance

Analysis of variance (ANOVA) revealed significant differences among distinct concentration of GO in all evaluated traits in soybean (Table 1).

Table1. Analysis of variance of the effects of different concentrations of GO on the studied traits in soybean.

		Mean squares								
Source of variation	**df**	**Fresh weight**	**Dry weight**	**Plant height**	**Root volume**	**POD**	**SOD**	**Flavonoid**	**Phenol**	**H_2O_2**
GO concentration	5	0.655**	0.002*	24.91**	18.00**	5.90*	6.8**	193.64**	0.15*	0.40**
Error	12	0.13	0.001	5.317	0.01	0.51	0.79	3.99	0.004	0.003

"*" and "**" are showing significance at 5% and 1% probability level, respectively.

Effect of GO on the growth

Figures 1A-D show the effect of different concentrations of GO on the fresh weight, dry weight, length, and volume of soybean root. There was a significant increase ($p < 0.05$) in fresh weight at 200 and 400 mg/L of GO as well as dry weight at 400 mg/L of GO compared to the control. Treatment of 400 mg/L of GO considerably augmented root length compared to control plants, while other concentrations had no significant effect on root length. Increase in GO concentration caused a significant decrease in root volume of treated soybean plants in comparison to the control especially at 1600 mg/L of GO. Decrease in growth parameters of soybean root at higher concentration can be attributed to the toxicity of GO. Begum et al. (2011) reported that different concentrations of graphene inhibited root, shoot, and biomass growth in cabbage, tomatoes, and red spinach, which was clearly evident at the concentration of 2000 mg/L. Similar results were observed in *Phaseolus mungo* and *Brassica juncea* treated with carbon nanotubes (Stampoulis et al. 2009). It has been suggested that the difference in the toxicity of NMs on the plant is most likely due to differences in root anatomy because the xylem structures determine the rate of water transfer and this difference may result in different absorption rates of the NMs (Lee et al. 2010).

Effect of GO on H_2O_2 production

At the high concentrations of GO (800 and 1600 mg/L) an increase in H_2O_2 level were observed (Fig. 2). It has been identified that the overexpression of ROS induced by various stresses can result in lipid peroxidation, protein oxidation, DNA damage, and activation of the programmed cell death pathway. Some reports are in accordance with our results. For example, a time-dependent increase in ROS content was observed in rice suspension cultures treated with 20 mg/L of multi-walled carbon nanotubes which was 3.5 times higher than the control (Tan et al. 2009). Increased ROS production and membrane damage were detected in tobacco BY-2 cells under 0.01 mg/ml of water-soluble carboxy-fullerene for 3 days (Liu et al. 2010). Increase in ROS levels and lipid peroxidation were attributed to the accumulation of multi-walled carbon nanotubes and carboxy fullerenes in the cell wall of rice and BY-2 tobacco.

Effect of GO on the activity of antioxidant enzymes

In order to evaluate the toxicity of GO, activity of SOD and POD, as important enzymes of plant antioxidant defense system for ROS scavenging, was investigated. According to the results, different concentrations of GO showed different effects on the activity of antioxidant enzymes. Higher SOD activity was recorded in plants exposed to 800 and

1600 mg/L of GO when compared with the plants under 0, 100, 200, and 400 mg/L treatments (Fig. 3A). The highest amount of POD activity was observed in soybean roots after treatment with 800 and 1600 mg/L of GO (Fig. 3B). It has been reported that generation of ROS is one of the important mechanisms of NM-induced toxicity which cause oxidative damage and cell death in plants. AS a response, plants increase the levels of antioxidant enzymes to overcome the stress. Therefore, measuring the antioxidant enzymes activity for example SOD and POD can be considered as an important biomarker in NMs toxicity assessments (Rico et al. 2015). Response of enzymatic antioxidant system to NMs can vary greatly with several factors such as plant species, plant growth stage, composition and concentration of the applied NMs (Rizwan et al. 2017). Anjum et al. (2014) reported that a significant increase in the APX and CAT activity with 800 and 400 mg/L of GO in *Vicia faba* helped root tissues to maintain H_2O_2 accumulation in an optimum level which can activate plant response against the stress.

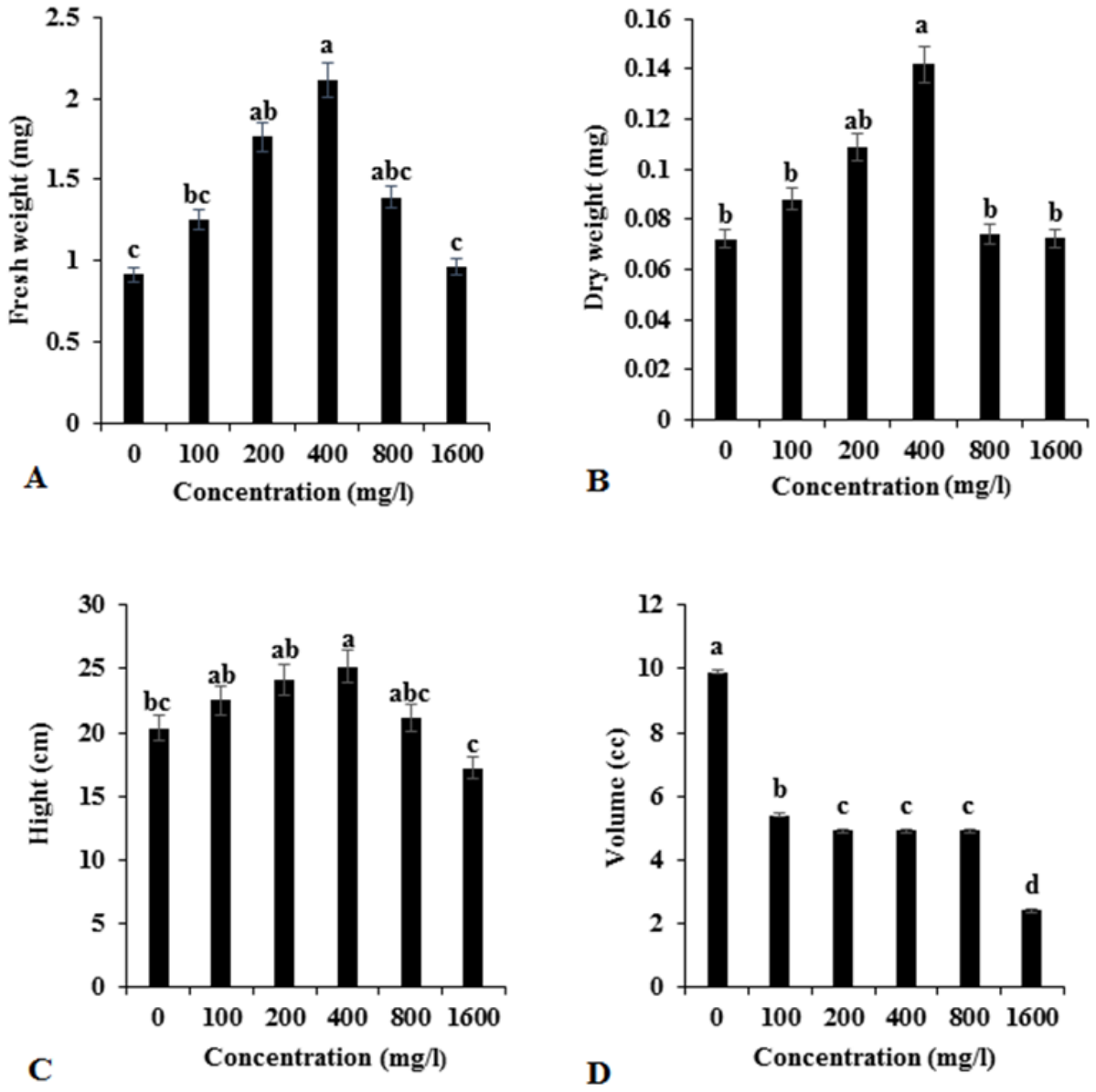

Fig.1. Comparison of the effect of different concentrations of GO on fresh weight (A), dry weight (B), height (C) and root volume (D) in soybean.

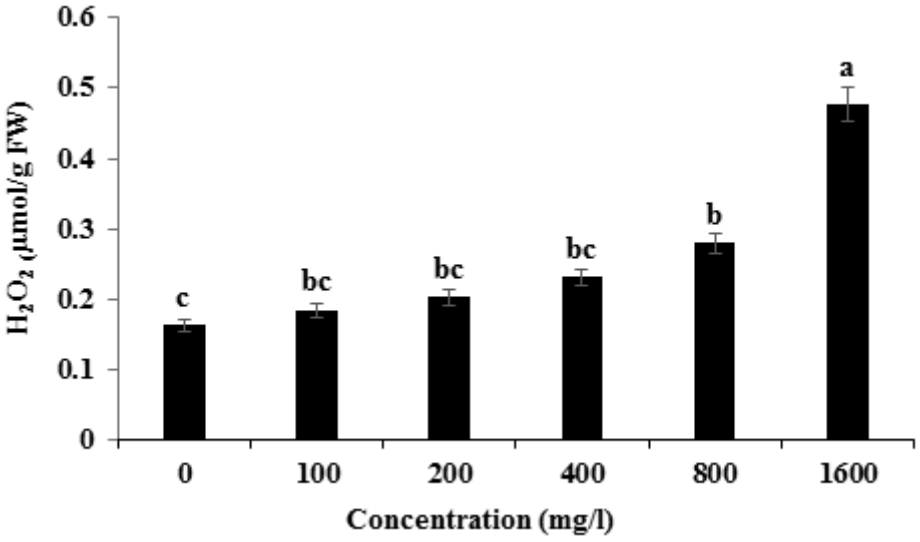

Fig.2. The effect of different concentrations of GO on H_2O_2 content in soybean root

Effect of GO on the total phenol and flavonoid content

According to the results, the highest amount of total phenol was observed in soybean roots exposed to 800 and 1600 mg/L of GO (Fig. 4A). The highest total flavonoid content also was measured in the roots treated with 1600 mg/L of GO (42.9 µg/g FW) (Fig. 4B). Because the amount of H_2O_2 at the concentration of 1600 mg/L of GO was also significantly higher than those of the other concentrations, it can be concluded that increasing the content of antioxidant compounds can contribute to stress tolerance induced by NMs through enhancing the capacity of protective mechanisms against oxidative damage. Phenolic compounds have been reported to exert their effects via scavenging of ROS. It has been reported that NMs can cause increase in the production of phenolic and flavonoid compounds in plants (Ghorbanpour et al. 2018; Mahjouri et al. 2019).

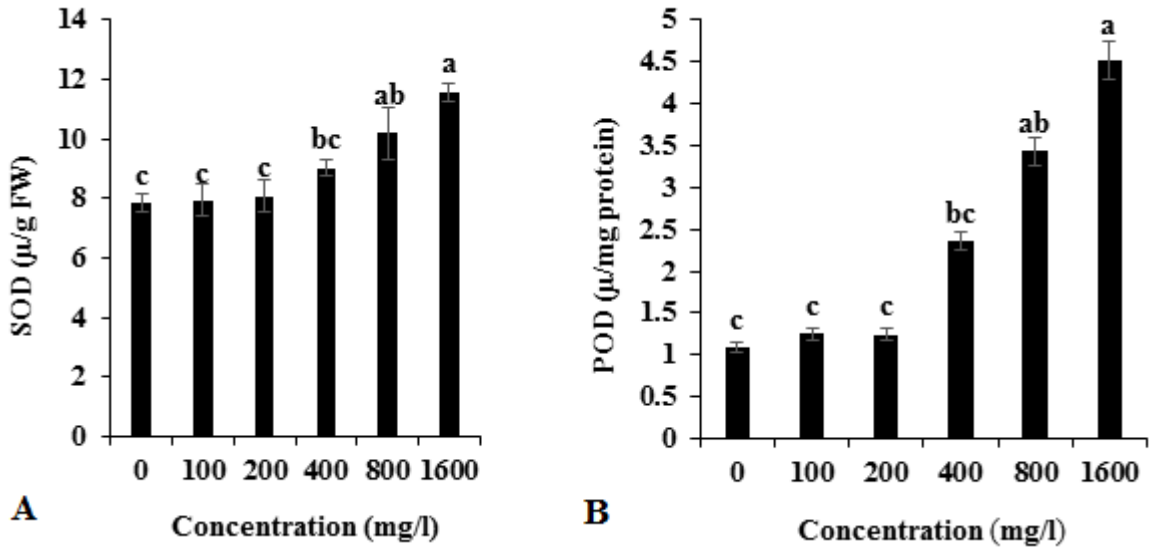

Fig.3. The effects of different concentrations of GO on SOD (A) and POD (B).

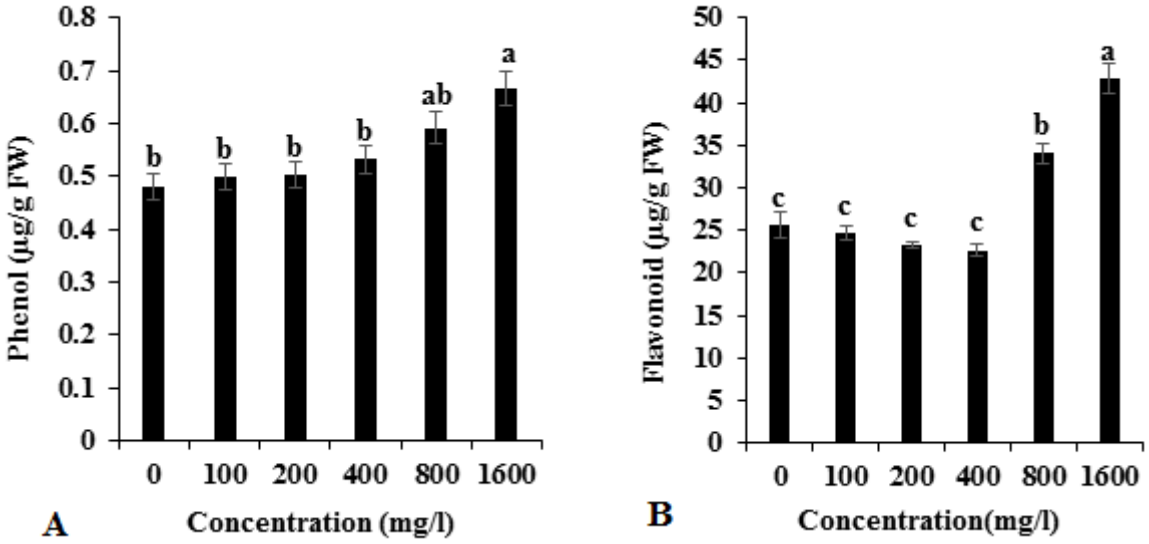

Fig.4. The effects of different concentrations of GO on the amount of phenol (A) and flavonoid (B).

Our results revealed the toxic impacts of GO on soybean. Totally, increase in the concentration of GO in culture medium led to the reduction in growth parameters of soybean root. Besides, augmented antioxidant enzyme activity and accumulation of non-enzymatic compounds showed that higher concentrations of GO induced oxidative stress in soybean root. In spite of the importance of GO in many industrial and biochemical applications, our results give a warning of adverse effects of GO on food chain.

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