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Single-bilayer graphene oxide sheet impacts and underlying potential mechanism assessment in germinating faba bean (*Vicia faba* L.)

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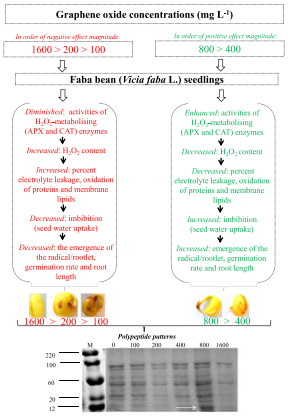
HIGHLIGHTS

- Graphene oxide (GO) impacted *Vicia faba* both positively and negatively.
- GO (1600 > 200 > 100 mg GO L^{-1}) elevated oxidative stress but lowered its metabolism.
- GO (800 > 400 mg GO L^{-1}) enhanced H_2O_2 scavenging and improved *V. faba* health status.
- *V. faba*-root-polypeptide patterns substantiated GO-positive and -negative impacts.
- Results imply 800 > 400 mg GO L^{-1}-safe nature and also warrant further studies.

GRAPHICAL ABSTRACT



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ABSTRACT

This study investigates the impact of different single-bilayer graphene oxide sheet (hereafter 'graphene oxide', GO; size: 0.5–5 μm) concentrations (0, 100, 200, 400, 800 and 1600 mg L^{-1}) and underlying potential mechanisms in germinating faba bean (*Vicia faba* L.) seedlings. The study revealed both positive and negative concentration-dependent GO-effects on *V. faba*. Significant negative impacts of GO concentrations (ordered by magnitude of effect: 1600 > 200 > 100 mg GO L^{-1}) were indicated by decreases in growth parameters and the activity of H_2O_2-decomposing enzymes (ascorbate peroxidase, APX; catalase, CAT), and by increases in the levels of electrolyte leakage (EL), H_2O_2, and lipid and protein oxidation. The positive impacts of 400 and 800 mg GO L^{-1} included significant improvements in *V. faba* health status indicated by decreased levels of EL, H_2O_2, and lipid and protein oxidation, and by enhanced H_2O_2-decomposing APX and CAT activity, and increased proline and seed-relative water content. *V. faba* seedlings-polypeptide patterns strongly substantiated these GO-concentration effects. Overall, the positive effects of these two GO concentrations (800 > 400 mg L^{-1}) on *V. faba* seedlings indicate their safe nature and allow to suggest further studies.

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1. Introduction

Single-bilayer graphene oxide sheet (hereafter termed 'graphene oxide', GO; size: 0.5–5 μm) is a water-soluble derivative of graphene (a novel one-atom-thick two-dimensional nanoparticle composed of a single layer of sp2-bonded carbon atoms) and has been widely used as a precursor of graphene-based nanoparticle composites (Geim, 2009; Xu and Wang, 2012). Extensive anchoring of engineered GO onto the surface of river sand is used to produce effective adsorbents for the removal of heavy metal ions, pesticides and natural dyes (Sreeprasad et al., 2011; Gupta et al., 2012). Owing to the wide use of GO-based adsorbents in environmental cleanup, it is clear that organisms are widely exposed to GO and GO can thus be transferred to the human/animal food chain. Therefore, the ultimate effects of GO-based materials on both the environment and biota (including plants) are of prime concern. Plants are the essential base component of all ecosystems and they are vital for devising and implementing sustainable mitigation or control measures against pollution by engineered nanoparticles (Navarro et al., 2008; Ma et al., 2010). However, to date, studies on interaction of crop plants and engineered GO are scarce (Begum et al., 2011; Anjum et al., 2013a), and GO phytotoxicity studies have yielded only speculative and unsubstantiated results reporting only minor or no effects on higher plants (Begum et al., 2011). Two recent reports reflect a partial mechanism underlying GO impact on plants in which GO-induced reactive oxygen species (ROS) generation (Begum et al., 2011) and glutathione (GSH) redox system impairments (Anjum et al., 2013a) were found to be the major factors controlling plant responses to GO.

Being sedentary in nature, plants have limited mechanisms to avoid exposure to environmental contaminants. GO impacts on plants have been reported to be dependent on plant species/genotypes and/or time/duration of exposure (Begum et al., 2011). Nanoparticles have been extensively reported to impair plant growth, photosynthesis and its related variables, and also to interfere with plant–water–nutrient pathways in different plant models (Lin and Xing, 2008; Asli and Neumann, 2009; Dimkpa et al., 2012; Zhao et al., 2012). High levels of ROS have also been found in different plant species exposed to a number of nanoparticles including ZnO (Lin and Xing, 2008), TiO_2 (Asli and Neumann, 2009), CuO and ZnO (Dimkpa et al., 2012), CeO_2 (Zhao et al., 2012; Rico et al., 2013) and GO (Begum et al., 2011).

A number of reports indicate that ROS (such as $O_2^{\bullet-}$, $OH^{\bullet}$, and H_2O_2) induce oxidative damage in bio-molecules including nucleic acids, proteins and membrane lipids leading to weakening of membrane integrity, elevated electrolyte leakage (EL), and eventually to cell and plant death (Gill and Tuteja, 2010; Anjum et al., 2012, 2013b). Thus, the tissue levels of H_2O_2 (a predominant toxic intermediate produced during the oxidative burst), lipid peroxidation products (thiobarbituric acid reactive substances, TBARS) and the EL have been extensively reported as indicators of oxidative injury status in stressed plants (Gill and Tuteja, 2010; Anjum et al., 2012). Although the significance of ascorbate peroxidases (APX, EC 1.11.1.11) and catalase (CAT, EC 1.11.1.6), important enzymes for the scavenging of H_2O_2, has been demonstrated in different plant species exposed to non-nano-sized materials (reviewed by Gill and Tuteja, 2010; Anjum et al., 2012) and CeO_2 nanoparticles (Rico et al., 2013), the literature contains no report on this mechanism in GO-exposed plants.

The accumulation of osmolytes (such as excess free proline) has been extensively reported in different plant species where these compounds help plants to survive under various stress conditions by controlling various physiological functions viz., osmotic adjustment, sub-cellular structure stabilization, and free radical scavenging (Sharma and Dietz, 2006). Additionally, plant polypeptide patterns have been reported to be modulated under biotic and abiotic stress exposure which can be easily visualized using sodium dodecyl sulfate–polyacrylamide-gel electrophoresis (SDS-PAGE) (Sobkowiak and Deckert, 2006; Ahsan et al., 2007). As a fundamental step toward proteomic studies the SDS-PAGE of plant proteins has also been shown to yield important information for understanding the molecular mechanisms of stress responses and developing transgenic plants with enhanced tolerance to stress (Ahsan et al., 2007). In the light of views thus far available on the GO phytotoxicity and tolerance mechanisms (Begum et al., 2011) and the known differential impairments in cellular GSH redox system components in higher plants under GO exposure (Anjum et al., 2013a), it was hypothesized that cross-talk among oxidative stress parameters and their metabolizing enzymes, and the modulation of seedlings polypeptide patterns may illuminate the basic mechanisms underlying sensitivity to GO concentrations in the food crop — faba bean (*Vicia faba* L.). To test this hypothesis, the present study aimed: (i) to investigate the impact of GO on the growth traits (germination rate, root length) and water relations (measured as seed-relative water content); (ii) to assess cell membrane permeability (measured as EL), oxidative stress and its metabolism (measured as lipid and protein oxidation, H_2O_2 content and the activity of its decomposing enzymes — APX and CAT), and osmolyte level (measured as proline content); and (iii) to explore the potential relationship between GO-mediated anomalies with the correlative modulation of seedlings polypeptide patterns.

V. faba was chosen as a model plant system and the germinating seedling as a model test plant stage for this study because *V. faba* is among the most common food crops and a primary dietary legume for humans and animals. It contributes about 33% of the dietary protein nitrogen needs of humans (Popelka et al., 2004). Germination and/or seedling — an early stage of growth and a complex physiological process in plants are widely used for evaluation of environmental-contaminants-phytotoxicity (Markwiese et al., 2001; Ahsan et al., 2007; Anjum et al., 2013a).

2. Materials and methods

2.1. Graphene oxide test solution preparation

Having characterized the as-synthesized GO (Anjum et al., 2013a), the test solution was prepared following the procedure described elsewhere (Anjum et al., 2013a). In brief, GO test concentrations (0, 100, 200, 400, 800 and 1600 mg GO L^{-1}) were prepared from a stock graphene aqueous suspension using freshly prepared deionized water and subsequently vortexed for 20 s and sonicated for 2 × 20 s with a 20 s interval. GO test solutions were neutralized using an aqueous 0.1 mol L^{-1} NaOH solution to achieve the pH values (6.3–6.5) favorable to plant growth (Begum et al., 2011).

2.2. Seed culture conditions and treatments

V. faba seeds were cultured and treated following the procedure described elsewhere (Anjum et al., 2013a). In brief, healthy and equal-sized *V. faba* seeds were surface sterilized by immersion in 10% NaClO solution for 10 min (USEPA, 1996) and subsequent vigorous rinsing with sterilized double-distilled water. *V. faba* seeds were sown (10 seeds for each treatment) in five replicates on Petri dishes with 90-mm filter paper (Whatman No. 1) round strips, soaked in 4 ml solution with GO concentrations (0, 100, 200, 400, 800 and 1600 mg L^{-1}), covered with a lid and incubated in the dark at 23 ± 2 °C until germination (Ahsan et al., 2007). The seeds were considered germinated when the root measured at least 20 mm in length for controls (USEPA, 1996). Roots were excised with sharp sterile blades, and estimations for both germination and growth trait were performed. Roots were either used fresh for biochemical estimations or dipped into liquid nitrogen and stored at −80 °C for further assays.

2.3. Seedlings growth and relative water content

The number of germinated seeds at each GO concentration was counted to estimate percent germination. Root length was measured using a meter scale. The relative water content (RWC) of the seeds