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Potential toxicity of nano-graphene oxide on callus cell of *Plantago major* L. under polyethylene glycol-induced dehydration

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ABSTRACT

Graphene-based nanomaterials have shown a great potential towards improving plant performance in various contexts. However, their increased application over the last few years has raised concerns about their potential biological and environmental risks, warranting optimization and safety assessment considerations. The current study was performed to explore the potential impacts of nano-garphene oxide (NGO) at various concentrations (100–800 μg mL^{-1}) on morphological, physiological and biochemical responses of *Plantago major* L. calli cultures under normal and polyethylene glycol-induced drought stress conditions. Leaf-derived calli on the ½ MS-*treated medium with* polyethylene glycol showed a decrease in relative growth rate (78.5%), osmotic potential value (48.2%) and an increase in dry matter (35.1%) and H_2O_2 (54.2%) contents at the highest employed NGO concentration compared with control ($p < 0.05$). The engineered NGO affected secondary metabolites and amino acid contents under normal water availability as well: at 800 μg mL^{-1}, NGO significantly increased total phenolic (40.9%) and flavonoid (35.3%) contents, but significantly reduced proline (26.9%) content compared to the respective control. The integrated biological marker (IBR/*n*) index for antioxidant enzymes (SOD, CAT, POD, and APX) activities was differentially influenced by the experimental treatments. Overall, the results demonstrated that NGO can positively affect the performance of *P. major* L. calli cells when applied at specific concentrations, and provide useful inputs into the further studies on phytotoxicity assessment of NGO.

1. Introduction

The impressive properties of graphene-based nanomaterials including two-dimensional (2D) structure, mechanical flexibility, unique electronic and optical attributes, *high* electrical conductivity and chemical stability have been greatly broaden their applications in various fields such as medicine, chemistry, biology, sensors, exploration and their related interdisciplinarities over the past few years (Shehzad et al., 2016; Shen et al., 2016; Dreyer et al., 2010).

The interaction of nanomaterials with biological molecules such as nucleic acids and proteins as well as its uptake, cytotoxicity, pharmacokinetics and hematopathology have extensively been investigated in beneficial microorganisms (Gurunathan, 2015), animal and human cells (Lanphere et al., 2014; Zhang et al., 2010; Wu et al., 2015), while its phytotoxicity (at genetic levels) remains mainly unexplored particularly under environmental perturbations (Singh et al., 2017), though there is available literature on the impacts of NGO and graphene-based materials on germination and growth of certain plant species. According to Begum et al. (2011), exposure of cabbage, tomato and red spinach to various graphene concentrations reduced leaf growth of the treated plants. They found a graphene dose-dependent increase in H_2O_2 generation, electrolyte leakage and cell death in treated plants. Other reports also reflect a partial mechanism underlying graphene oxide impacts on growth and development of *Vicia faba* L. in which graphene oxide-induced reactive oxygen species (ROS) production, antioxidant enzyme activities, lipid and protein oxidation, and glutathione redox system impairments were found to be the primary controlling factors in plant responses to graphene oxide (Anjum et al., 2013, 2014).

Nevertheless, the information concerning graphene oxide effects on physiological process of plants appears to be controversial. For example, it has been reported that exposure of *Arabidopsis thaliana* to different graphene oxide levels did not obviously influence germination and early growth parameters, and could not increase H_2O_2 production, oxidative stress induction, malondialdehyde content, and antioxidant enzymes activities, implying physiological basis for safety property of graphene oxide at the examined concentrations in *Arabidopsis thaliana*

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(Zhao et al., 2015). Most recently, however, Dasgupta et al. (2017) investigated the degree of biosafety of the reduced garphene oxide, and found that it to be safe under 100 μg mL^{-1}. Conversely, the hydrophilic nature and water-transporting characteristics of garphene oxide were exploited to enhance germination, growth and yield of spinach and chive at lower doses (Yijia et al., 2017).

Despite raising the number of researches over the last few years regarding interaction of nanomaterials with plants (Tripathi et al., 2017) little studies have been performed about the role of *carbonaceous* nanomaterials on plant cellular phenomenon under abiotic stresses. However, Wang et al. (2014) reported that graphene oxide exposure combined with drought or salt stress induced severe alterations in expression patterns of the genes required for root development and abiotic stress in Arabidopsis plants, resulting in more severe loss in morphology along with increase in ROS generation or membrane ion leakage and decrease in activity of specific antioxidant enzymes (Wang et al., 2014). Recently, Hatami et al. (2017) reported that exposure to low concentrations of single-walled carbon nanotubes ameliorated the polyethylene glycol-induced drought stress (up to moderate levels only) in *Hyoscyamus niger* L. seedlings through activation of various antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX), and also biosynthesis of metabolites such as proteins, phenols and proline. These findings present the complex interactions of carbon-based nanomaterials with plant species and emphasize the need for further investigation.

Drought stress is known as one of the most important factors inhibiting plant growth and productivity, especially in the arid and semiarid regions (Baiazidi-Aghdam et al., 2016). This kind of abiotic stress may interrupt the regular cell metabolism and trigger oxidative stress in plant tissues, leading to decrease in photosynthetic electron chain and increase in ROS production (Fu and Huang, 2001). However, expression of antioxidant molecules and biosynthesis of specific metabolites can improve plant performance in such regions.

The use of in vitro culture model to investigate stress responses is based on the fact that in vitro cultured cells behave similarly to cells of whole plants exposed to abiotic stress conditions such as drought and salinity (Attree et al., 1991). Moreover, plant cell and tissue culture techniques minimize environmental variations due to the use of specific nutrient media, controlled conditions, homogeneity of nutrient availability and stress severity (Sakthivelu et al., 2008). To the best of our knowledge, no previous study has been performed regarding the effects of NGO exposure and drought stress on medicinal plants under in vitro culture conditions, and the possible mechanisms underlying positive or negative responses have yet to be fully clarified.

Common plantain (*Plantago major* L.), one of the most abundant and widely distributed medicinal plants in the world, has been used for different pharmaceutical uses (Haddadian et al., 2014). Among the plant genotypes, *P. major* L. is known as a high responsive species for in vitro callus induction and regeneration (Ghorbanpour and Khadivi-Khub, 2015), being therefore selected for this study.

This study aimed to identify the effects of NGO at different concentrations on morpho-physiological and biochemical traits in calli derived from leaf tissues of *P. major* L. under polyethylene glycol (PEG)-induced dehydration in vitro.

2. Materials and methods

2.1. Characterization and preparation of NGO test solution

NGO used in this study was obtained from NANOSANY (Iranian Nanomaterials Pioneers Co. Ltd., Mashhad, Iran). The NGO powder was dispersed in DI-water without adding surfactants using a sonicator water bath with high operating frequencies (~ 40 kHz, 100 W) for 30 min (Zhao et al., 2015). After sonication, the microscopic morphology and other properties of the samples were characterized using scanning electron microscopy (SEM, Hitachi S-4160, Tokyo, Japan), transmission electron microscopy (TEM, Hitachi, Tokyo), and X-ray diffraction (XRD, Philips-X'Pert MPD X-ray diffractometer) techniques. Elemental contents of NGO sheet were determined using an Agilent apparatus (model 7700 × ICP-MS, Tokyo, Japan). The Brunauer-Emmett-Teller (*BET*) *surface area* of samples was carried out through nitrogen adsorption on a Quantachrome NOVA 2000 surface analyzer.

2.2. Explant preparation, calli cultures and treatments

Leaf explants were excised from in vitro seed-derived plantlets (20 day old) of *P. major* L. To develop calli cultures, approximately 4–5-mm^2 leaf pieces were placed on a *half-strength* Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with different concentrations of NGO and polyethylene glycol (PEG 6000, Merck) as follows:

The exposure solution for biological study was prepared following the method described by Anjum et al. (2013). Briefly, NGO test concentrations (0, 100, 200, 400, and 800 μg mL^{-1}) were prepared from a stock NGO aqueous suspension using freshly prepared DI-water by vortexing for 20 s, followed by sonication for 2 × 20 s with a 20 s interval. GO test solutions were neutralized using an aqueous solution of NaOH (0.1 mol L^{-1}) to attain the pH values (6.3–6.5) suitable for plant growth (Begum et al., 2011).

To induce drought stress conditions, polyethylene glycol-infused agar plates were prepared, according to a modified version of the method described by Verslues et al. (2006). Polyethylene glycol-infused agar plates were made by dissolving solid polyethylene glycol in a sterilized solution of ½ MS basal medium with 2 mM *2*-(N-morpholino) ethanesulfonic acid (MES) buffer (pH 5.7). This polyethylene glycol solution was then overlaid on agar-solidified (15 g L^{-1} Phyto agar) ½ MS medium plates (3:2; v/v). Then, the agar medium and polyethylene glycol solution were allowed to equilibrate for at least 12 h prior to the excess polyethylene glycol solution removal. Drought stress intensity was expressed as the concentration of the overlayed polyethylene glycol solution: 0% (unstressed control) or 35% (drought stress), abbreviated as -PEG (without polyethylene glycol) and +PEG (with polyethylene glycol), respectively (Verslues et al., 2006). All the cultures were incubated in a growth room at 24 ± 1 °C and 16/8 h illumination (40 μmol m^{-2} s^{-1}), provided by fluorescent tube lights (40 W). *Subcultures were* being performed every *15 days* in fresh ½ MS medium supplemented with the aforesaid concentrations of NGO and polyethylene glycol.

2.3. SEM observations and Raman spectroscopy detection

The calli segments were fixed by immersion in a fixing solution containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), and stored at 4 °C overnight (Wang et al., 2002). The samples were rinsed three times with 0.1 M cacodylate, post-fixed with 1% osmium tetraoxide in the same buffer, and subsequently dehydrated in a graded ethanol series (30%, 50%, 70%, 85%, 95%, and 100% v/v), and embedded in Epon 812 resin. The surface morphology of calli cells was investigated using SEM (Hitachi S-4160, Tokyo, Japan). Raman scattering measurements were performed on treated callus to confirm the presence of NGO inside the cells using a Raman spectrometer (Takram P50CoR10, Teksan, Iran) (Holt et al., 2012; Drescher and Kneipp, 2012).

2.4. Callus morphology and physiology assay

Morphological characteristics including calli color and texture as well as physiological traits were investigated as follows:

2.4.1. Relative growth rate

Calli from all treatments were collected after 49 days of initial culture and the final fresh weight (FW) was measured immediately. For