Environmental Pollution 233 (2018) 633–641

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Multiwall carbon nanotubes modulate paraquat toxicity in *Arabidopsis thaliana*☆

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ARTICLE INFO

Article history:
Received 26 July 2017
Received in revised form
5 October 2017
Accepted 28 October 2017

Keywords:
Arabidopsis thaliana
Multiwall carbon nanotubes
Paraquat
Oxidative stress
Proteomics
Photosynthesis

ABSTRACT

Carbon nanotubes can be either toxic or beneficial to plant growth and can also modulate toxicity of organic contaminants through surface sorption. The complex interacting toxic effects of carbon nanotubes and organic contaminants in plants have received little attention in the literature to date. In this study, the toxicity of multiwall carbon nanotubes (MWCNT, 50 mg/L) and paraquat (MV, 0.82 mg/L), separately or in combination, were evaluated at the physiological and the proteomic level in *Arabidopsis thaliana* for 7–14 days. The results revealed that the exposure to MWCNT had no inhibitory effect on the growth of shoots and leaves. Rather, MWCNT stimulated the relative electron transport rate and the effective photochemical quantum yield of PSII value as compared to the control by around 12% and lateral root production up to nearly 4-fold as compared to the control. The protective effect of MWCNT on MV toxicity on the root surface area could be quantitatively explained by the extent of MV adsorption on MWCNT and was related to stimulation of photosynthesis, antioxidant protection and number and area of lateral roots which in turn helped nutrient assimilation. The influence of MWCNT and MV on photosynthesis and oxidative stress at the physiological level was consistent with the proteomics analysis, with various over-expressed photosynthesis-related proteins (by more than 2 folds) and various under-expressed oxidative stress related proteins (by about 2–3 folds). This study brings new insights into the interactive effects of two xenobiotics (MWCNT and MV) on the physiology of a model plant.
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1. Introduction

Carbon nanotubes (CNT) are tubular structures of nanometer size with novel physical and chemical properties which have a wide range of applications in multiple sectors such as nanomedicines (Lacerda et al., 2006), electronics (Mink and Hussain, 2013), and energy harvesting (Abdulalmohsin et al., 2012). Within the last 20 years, the fate and effects of CNT in the environment have increasingly attracted research attention. CNT released in the environment are commonly of two types: single-wall carbon nanotubes (SWCNT), which consist of a single concentric graphite layer, and multiwall carbon nanotubes (MWCNT), which have several concentric cylinders (Harris, 2004). Although environmental concentrations of CNT are expected to be low and not to exert a threat to plants, negative environmental effects are possible in the future due to the increasing CNT production (Gottschalk et al., 2009). The effects of CNT on plants depend on the tested concentration of CNT, the target plant species, and the physical properties of the CNT such as charge and size (Cañas et al., 2008; Zhai et al., 2015). When present at concentrations in the low mg/L range (10–200), MWCNT can increase root growth of plant seedlings by promoting water uptake. MWCNT may hence have beneficial applications in biotechnology and in nanoagriculture (Khodakovskaya et al., 2013; Martínezballesta et al., 2016; Tiwari et al., 2014; Tripathi et al., 2011). However, at around 1000 mg/L, CNT can reduce zucchini growth (Stampoulis et al., 2009), up-regulate stress-related genes in tomatoes (Khodakovskaya et al.,

☆ This paper has been recommended for acceptance by Baoshan Xing.
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https://doi.org/10.1016/j.envpol.2017.10.116
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2011) and cause mesophyll cells necrosis and apoptosis in *Arabidopsis thaliana* (Yuan et al., 2011). CNT has also been proven to be toxic to microalgae. For example, SWCNT caused cell plasmolysis, decreased cell viability and inhibited cell division of the freshwater microalgae *Chlorella vulgaris* (Hu et al., 2015), while MWCNT inhibited the growth and photosynthesis of the euryhaline microalgae *Dunaliella tertiolecta* (Wei et al., 2010). These effects of both types of CNT on algae were observed in the mg/L level.

Since CNT are highly hydrophobic (Girifalco et al., 2000; Wang and Hobbie, 2003) and adsorb a range of contaminants (Hao et al., 2015; Miralles et al., 2012; Sanchís et al., 2015; Schwab et al., 2011; Zheng et al., 2016), they can on the one hand decrease bioavailable concentration of contaminants, whereas the adsorption of the contaminants in turn is likely to alter the properties of CNT (Ren et al., 2014; Yang et al., 2011). On the other hand, CNT can release contaminants when CNT-organic contaminant complexes are present close to the cell membranes of photosynthetic organisms, thus enhancing herbicide and polycyclic aromatic hydrocarbon toxicity (Glomstad et al., 2016; Schwab et al., 2013; Schwab et al., 2014). For example, MWCNT promoted the accumulation of organic contaminants in mustard, especially hexachlorobezene and 4, 4′-Dichlorodiphenyl trichloroethane (Chen et al., 2015).

Paraquat (also known as methyl viologen, MV) is a broad spectrum contact herbicide, widely used in agricultural crops for weed control (Aksakal, 2013). Paraquat residues in soils where paraquat was applied for agricultural use were about a few to several hundred mg/kg (Roberts et al., 2002). These concentrations are potentially harmful to soil organisms. Although CNT can adsorb several organic contaminants and thus affect their bioavailability and toxicity, little is known about the interacting effects of CNT and MV mixtures on plant development. Such knowledge is necessary to better evaluate the environmental risk associated with both compounds and will help to better understand the mechanisms of interactions between these two widespread xenobiotics and their combined effects on plant growth. In the present study, we set out to evaluate the effects of MV and MWCNT on the physiology, morphology and proteome of the model plant *A. thaliana*. Our integrative approach reveals the intricate molecular interactions at the basis of MV toxicity in *A. thaliana* and the extent of interactions between MWCNT and MV. This study may help to develop strategies to reduce the toxicity of contaminants in agriculture fields using MWCNT.

2. Materials and methods

2.1. Preparation of MWCNT dispersions and MV dissolution

Multiwall carbon nanotubes (purity >95%) and MV (purity = 98%) were purchased from Aladdin (Shanghai, China). MWCNT were suspended in ultrapure water containing 0.25% (w/v) gum Arabic, which has been proven to be biocompatible and to favor the dispersion of MWCNT in the aqueous phase (Larue et al., 2012). The suspensions were sonicated during 3 × 30 min in an ultrasonication bath for optimal dispersion. MV powder was dissolved in ultrapure water and stored at 4 °C, and used for the experiment within a one-month timeframe after preparation.

2.2. Microscopic and physico-chemical analysis of MWCNT dispersions

A dispersion of 50 mg/L MWCNT was sonicated prior to transmission electron microscopy (TEM) analysis (Hitachi H-7650, Japan) to observe its micromorphology. The hydrodynamic size of the MWCNT was measured using a dynamic light scattering instrument (Brookhaven BI-200SM, USA). The zeta potential of MWCNT dispersions with or without MV was measured using a Zetasizer Nano ZS instrument (Malvern, UK).

2.3. Measurements of physiological parameters

A. thaliana seedlings were exposed to MWCNT and MV separately or in combination for 7 and 14 d prior to measurement of the physiological parameters. The details of the preparation of the plant material and the exposure assays are provided in the Supporting Information (SI), Section S1. After exposure, the measurements of chlorophyll fluorescence, fresh weight, chlorophyll and anthocyanin content, as well as root morphology were performed for evaluation of plant growth (see details in SI, Section S2). All analyses related to oxidative stress were performed on plant leaves, the presence of superoxide radicals (O_2^-) in leaves was measured using nitroblue tetrazolium (NBT) (Rao and Davis, 1999) (see details in SI, Section S3). The measurements of malondialdehyde (MDA), catalase (CAT), glutathione (GSH) and superoxide dismutase (SOD) were performed with commercial kits (Nanjing Jiancheng, Nanjing, China).

2.4. Measurements of MV and nutrients in the culture medium

The content of MV in the culture medium was measured by means of high performance liquid chromatography (HPLC). The NO_3^- and PO_4^{3-} concentrations in the culture medium were measured using spectrophotometry (Frankovich and Jones, 1998; Sun et al., 2016) (the details for the measurements of MV, NO_3^- and PO_4^{3-} are provided in the SI, section S4).

2.5. Total protein extraction and label-free quantification

Approximately 0.5 g of *A. thaliana* shoots and leaves were collected for total protein extraction. Crude protein extraction was performed by means of acetone precipitation as detailed in Qian et al. (2015). The crude protein was then lysed according to the instructions provided in the Bradford Assay protein quantitation kit (Sangon Biotech, Shanghai, China) before quantification. An identical quantity of proteins from different groups was reduced and alkylated, then digested with trypsin (Promega, Beijing, China) for 12 h at 37 °C. After desalination, the peptides were finally dried by vacuum centrifugation. One microgram of peptide was separated using a reversed-phase analytical column (Acclaim PepMap RSLC, Thermo Scientific) and analyzed using a Q Exactive plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo FisherScientific Waltham, MA) afterwards. Proteomic data were retrieved with the MaxQuant software (v1.0.13.13).

2.6. Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). One way analysis of variance (ANOVA) was used to evaluate significant statistical differences ($p < 0.05$) using the StatView 5.0 program. The preliminary conditions of ANOVAs (normality and homogeneity of variances of residuals) were tested and if the conditions were violated, the data were transformed prior to ANOVAs. For proteomic results analysis, a threshold of 1.8-fold over- or under-regulation was chosen to define differentially expressed proteins, (e.g. for a given protein in the control and MWCNT groups, if the ratio of the protein in MWCNT to control was over 1.8, we identified the protein as an over-expressed protein in the MWCNT group, if the ratio was below 0.56, we identified it as an under-expressed protein, with a statistically significant difference, $p < 0.05$). The database for annotation, visualization and integrated

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