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DOI:

[10.1021/acs.est.9b05794](https://doi.org/10.1021/acs.est.9b05794)

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Document Version

Peer reviewed version

Citation for published version (Harvard):

Zhang, P, Guo, Z, Luo, W, Monikh, FA, Xie, C, Valsami-Jones, E, Lynch, I & Zhang, Z 2020, 'Graphene oxide induced pH alteration, iron overload and subsequent oxidative damage in rice (*Oryza. sativa* L.): a new mechanism of nanomaterial phytotoxicity', *Environmental Science and Technology*, vol. 54, no. 6, pp. 3181-3190. <https://doi.org/10.1021/acs.est.9b05794>

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Contaminants in Aquatic and Terrestrial Environments

Graphene Oxide Induced pH Alteration, Iron Overload and Subsequent Oxidative Damage in Rice (*Oryza. sativa* L.): A New Mechanism of Nanomaterial Phytotoxicity

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Environ. Sci. Technol., **Just Accepted Manuscript** • DOI: 10.1021/acs.est.9b05794 • Publication Date (Web): 21 Feb 2020

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**Graphene Oxide Induced pH Alteration, Iron Overload and Subsequent
Oxidative Damage in Rice (*Oryza. sativa* L.): A New Mechanism of
Nanomaterial Phytotoxicity**

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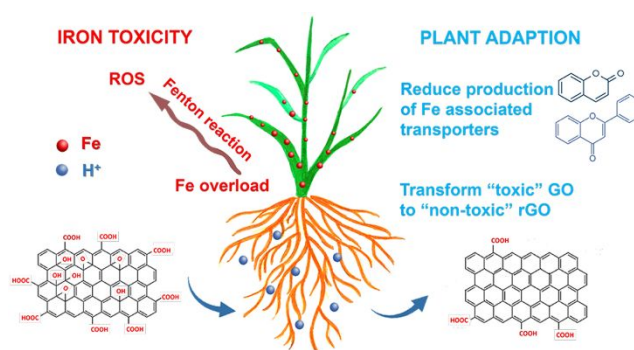
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ABSTRACT

The mechanism of graphene-based nanomaterial (GBM) induced phytotoxicity and its association with the GBM physicochemical properties are not yet fully understood. The present study compared the effects of graphene oxide (GO) and reduced GO (rGO) on rice seedling growth under hydroponic condition for 3 weeks. GO at 100 and 250 mg/L reduced shoot biomass (by 25% and 34%, respectively) and shoot elongation (by 17% and 43%, respectively), and caused oxidative damage, while rGO exhibited no overt effect except for the enhancement of the antioxidant enzyme activities, suggesting that surface oxygen content is a critical factor affecting the biological impacts of GBMs. GO treatments (100 mg/L and 250 mg/L) enhanced the iron (Fe) translocation and caused excessive Fe accumulation in shoots (2.2 and 3.6 times higher than control), which was found to be the main reason for the oxidative damage in shoots. GO-induced acidification of the nutrient solution was the main driver for the Fe overload in plants. In addition to the antioxidant regulators, the plants triggered other pathways to defend against the Fe toxicity, *via* downregulation of the Fe transport associated metabolites (mainly coumarins and flavonoids). Plant root exudates facilitated the reduction of toxic GO to non-toxic rGO, acting as another route for plant adaption to GO induced phytotoxicity. This study provides new insights into the mechanism of the phytotoxicity of GBMs. It also provides implications for agricultural application of GBM that the impacts of GBMs on the uptake of multiple nutrients in plants should be assessed simultaneously and reduced forms of GBMs are preferential to avoid toxicity.

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48 INTRODUCTION

49 Graphene is a two-dimensional carbon-based nanomaterial composed of a single layer of sp^2
50 hybridized carbon atoms.¹ It is considered one of the most promising engineered nanomaterials
51 (ENM) with the potential to be used in various sectors such as electronics, medical, energy and
52 environment,²⁻⁴ due to its unique electronic, thermal and mechanical properties. The increasing
53 production and use of graphene-based materials (GBMs) will inevitably increase the likelihood
54 of their release into the environment, and thus their potential adverse impacts on
55 environmental and human safety need to be fully assessed.⁵

56 There have been extensive studies regarding the toxicity of GBMs on cells and
57 microorganisms,^{6, 7} while knowledge of the potential impacts of GBMs on the growth of higher
58 plants is still lacking. There is concern that GBMs may affect plant growth and /or accumulate
59 in crops or vegetables,⁸ causing potential risks to human health. Recent studies showed that
60 GBMs have the potential to be used as a carrier for fertilizers to enable slow release of the
61 nutrients and thus enhance the nutrient use efficiency by plants;⁹⁻¹¹ however, such applications
62 in real agriculture are not currently pursued partially due to the concerns over the potential
63 adverse impacts of GBMs on the overall agricultural ecosystem, including soil functioning (e.g.,
64 bacterial community, enzyme activity),¹²⁻¹⁴ the potential trophic transfer of GBMs¹⁵ and
65 cumulative effects of GBMs after repeated application and over multiple growing seasons.

66 Overt toxicity of GBMs to plant such as inhibition of biomass production, shoot or root
67 elongation have been reported at high exposure concentrations.^{16, 17} However, a number of
68 studies also reported that GBMs induced physiological alterations (e.g., hormone levels,
69 nutrient uptake)^{17, 18} or oxidative stress (e.g., enhanced antioxidant enzymatic activities, lipid
70 peroxidation, or H_2O_2 over-accumulation)^{16, 19} even at environmentally relevant concentrations
71 (0.01~1mg/L),²⁰ suggesting that subtle physiological processes are more sensitive indices than
72 apparent toxicity indices (e.g., biomass, root/shoot length) for evaluating the phytotoxicity of
73 GBMs.²¹ In addition to the negative effects, positive effects resulting from exposure to GBMs on
74 plant growth are also reported. For example, hydrated graphene ribbons promoted the
75 germination of wheat seeds, upregulated carbohydrate, amino acids and fatty acids metabolism

during the germination and enhanced the tolerance of seeds to oxidative stress.²² Due to the hydrophilic nature of GO, it can act as a water transporter to promote the seed germination.²³

There are several reasons that may contribute to the inconsistent reports regarding the phytotoxicity of GBMs. Firstly, phytotoxicity of ENMs are species-dependent,²⁴ cross-species comparison is not always suitable. Secondly, using different culture media such as soil,²³ agar²⁵ and hydroponic solutions¹⁹ which have different compositions, can affect the behaviour, fate and toxicity of the GBMs. GBM in soil and agar media usually have low mobility and accessibility to plants, thereby lowering their impacts on plant growth found in such media when compared with those observed in hydroponic media. However, this might be not always true. For example, CeO₂ ENMs were reported to be more toxic to *Lactuca* plants in agar medium than in water, which is due to that *Lactuca* plants are more sensitive in agar than in water to the toxicity of Ce³⁺ ions released from CeO₂ ENMs.²⁶ Lastly, the physicochemical properties of the GBMs used in the previous studies are very diverse and are not fully described in many cases. In reviewing the studies on the effects of GBMs on higher plants (Table S1), more than half of the papers considered did not provide sufficient characterization data including lateral size, thickness and surface oxygen content, which are critical characteristics determining the biological effects of GBMs.⁵ Where provided, the given properties were very varied: the lateral size used in these studies ranged from 30 nm to 6.5 μ m, the layer thickness ranged from 0.3 nm to 3.5 nm, and the surface oxygen contents ranged from 3.51% to 38.8%. Clearly, more studies are required to provide sufficient data for cross comparison and elucidating the mechanism(s) of action of GBMs, and to determine the ranges of GBMs' properties that can be used safely to enhance plant growth and /or soil quality and nutrient cycling.

Common forms of graphene, including graphene (G), graphene oxide (GO) and reduced graphene oxide (rGO), are distinct in their surface oxygen contents. GO is the oxidized form of graphene, which contains abundant functional groups including carboxyl, hydroxyl, epoxy, and carbonyl groups.²⁷ These functional groups endow GO with high water dispersity and can be used for further functionalization of GO for different applications.³ Previous studies have suggested that GO and rGO have distinct antibacterial activities,²⁸ which is attributed to the

different modes of interaction of GO and rGO with cell membranes. We hypothesize that comparing the phytotoxicity of GO and rGO, which is yet to be studied, will allow acquisition of a mechanistic understanding of the actions of GBMs in plants. To do so, we investigated the impacts of GO and rGO on the growth of rice plants. Oxidative stress, perturbation of the uptake of macro- and micro- elements in plants, metabolic alteration and the transformation of GO and rGO in rice plants were compared to explore the mechanisms of the interaction of GO and rGO with plants and their consequences for plant health.

2. MATERIALS AND METHODS

2.1. Chemicals and Seeds

GO and rGO were purchased from Chengdu Organic Chemicals Co. Ltd (Chengdu, China). Morphology, lateral size, height, chemical structure, Zeta potential and hydrodynamic sizes of GO and rGO were characterized, details of which are described in the Supporting Information (Section 1). All other commercial chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Rice (*Oryza. sativa* L.) seeds were purchased from the Chinese Academy of Agricultural Sciences.

2.2. Plant Culture and Exposure

Rice seeds were germinated in the dark for 5 days after sterilization with 10% NaClO . Uniform seedlings were then selected and each seedling was anchored by a plastic foam with a hole and transferred into a 250 mL beaker containing 100 mL of modified 1/4 strength Hoagland solution. All the beakers were wrapped with black plastic bags to simulate the dark environment in soil. Six replicates were set for each treatment. The seedlings were allowed to grow in a growth chamber (PRX-450C, Saifu, China) with a day/night temperature of 28 °C /20 °C, day/night humidity of 50%/70% and a 14 h photoperiod for 10 days before treatment. GO and rGO were then added into freshly prepared nutrient solution to obtain suspensions with concentrations of 5, 50, 100 and 250 mg/L followed by ultrasonic pre-treatment for 10 min. The seedlings were then exposed to the GO and rGO suspensions and allowed to grow for three

131 weeks. The suspensions were replenished to 100 mL with fresh nutrient solution every two
132 days.

133 **2.3. Biomass Production, Seedling Length and Nutrient Content**

134 After three weeks of exposure to the GBMs, rice seedlings were gently lifted from the
135 suspensions, and the roots were rinsed with deionized water repeatedly. GO and rGO that were
136 attached to the roots were rinsed off with deionized water for further analysis. Residual GO and
137 rGO in the beakers were also collected for characterization. The roots and shoots were
138 separated and blotted with clean tissues, and the fresh weights were measured immediately.
139 The seedlings were then lyophilized, and the dry weights of the roots and shoots were
140 measured. To quantify the nutrient content (Fe, Cu, Mn, Zn, K, Ca, Mg, P) in plants, dried roots
141 and shoots were ground into fine powders and digested with a 3:1 mixture of HNO₃ and H₂O₂
142 on a heating plate (80°C for 1 h, 120 °C for 3 h, and 160 °C for 0.5 h). Elemental concentrations
143 in the digestion solution were then analyzed by inductively coupled plasma optical emission
144 spectroscopy (ICP-OES, Thermo, USA). Multi-element standard solutions (0.5~50 mg/L)
145 containing all the selected elements were used for external calibration. Blanks were analysed
146 between every six samples. Spiking recovery experiments and analysis of certified reference
147 material (GBW 07602 Bush Branches and Leaves) were performed for analytical method
148 validation. Recoveries and detection limits for all the elements are reported in Table S2.

149 **2.4. Stress Response of Rice to GO and rGO**

150 Fresh roots and shoots were excised, homogenized with cold PBS (50 mM, pH 7.8), and
151 centrifuged at 10,000 g and 4 °C for 10 min. The supernatants were collected for analyses of
152 superoxide dismutase (SOD) and peroxidase (POD) activities and malondialdehyde (MDA)
153 content using assay kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing,
154 China). Reactive oxygen species (ROS) accumulation in roots and leaves was examined by a
155 DCFH-DA staining method. Fresh leaves and roots were excised and incubated in DCFH-DA (10
156 mM in PBS) for 2 h followed by rinsing with PBS three times. ROS accumulation was imaged on
157 a fluorescence microscope (Olympus IX70) with an ex/em of 485nm/522nm. QA/QC for the
158 assays are described in Section 1, SI.

159 2.5. Characterization of GO and rGO After Interaction with Plants

160 After harvesting of the plants, GO and rGO that were attached to the root surface were washed
161 off from the roots using ddH₂O (named "GO-W and rGO-W") and collected by centrifugation
162 (10,000 g, 30 min). The pellets were then rinsed with hydrochloric acid and ethanol repeatedly
163 to remove salts and organic components.²⁹ The obtained pellets were then freeze-dried for
164 analysis. The residual solutions in the beaker after removal of the plants were also collected
165 and rinsed by the same procedure described above. GO and rGO incubated in nutrient solution
166 for three weeks without the presence of plants were also collected for comparison. All the
167 materials described above (washed and residual) were analyzed by Raman (Horiba Scientific,
168 Japan), FTIR (Bruker Tensor 27 spectrometer, Germany), UV-vis spectroscopy (Purkinje
169 General, Beijing), and XPS (ESCALAB 250Xi, Thermo Scientific, USA). To analyze the GO and rGO
170 on the root surface *in situ*, fresh root apexes were freeze-dried and analysed on a Raman
171 spectrometer (Horiba Scientific, Japan). Fresh root apexes were also excised, fixed and
172 sectioned for TEM observation (see details in the Supporting Information).

173 2.6. Xylem Sap Collection and Fe concentration Analysis

174 Rice seedlings were exposed to GO and rGO for 3 weeks as per the exposure procedure in
175 section 2.2 and then cut off at 2 cm above the root-shoot interface. The cut surface of the shoot
176 was cleaned with DI water and then a silicon tube was fit to the stump (Fig. S1). The xylem sap
177 was collected after 24 h with a pipette and digested in HNO₃ (70%). Iron concentrations in the
178 xylem saps were analysed by ICP-OES (Thermo, USA).

179 2.7. Metabolomics analysis of rice leaves

180 The fresh rice leaves were thoroughly rinsed with ddH₂O after harvest and ground into powder
181 in liquid nitrogen. For each sample, 100 mg powder was transferred to a 1.5 mL Eppendorf tube
182 and mixed with 2 mL methanol by vortexing vigorously. Samples were ultrasonicated for 1 hour
183 min at 4°C and dried under a stream of N₂. Then, 500 µL of cold methanol was added to each
184 sample. The samples were mixed by vigorous vortexing followed by centrifugation at 12,000
185 rpm for 10 min at 4°C. A 300 µL aliquot of supernatant was then transferred into a glass

sampling vial for analysis. Samples were then analysed by liquid chromatography-tandem MS (LC-MS/MS). Details of the measurement and data analysis are described in the Supporting Information.

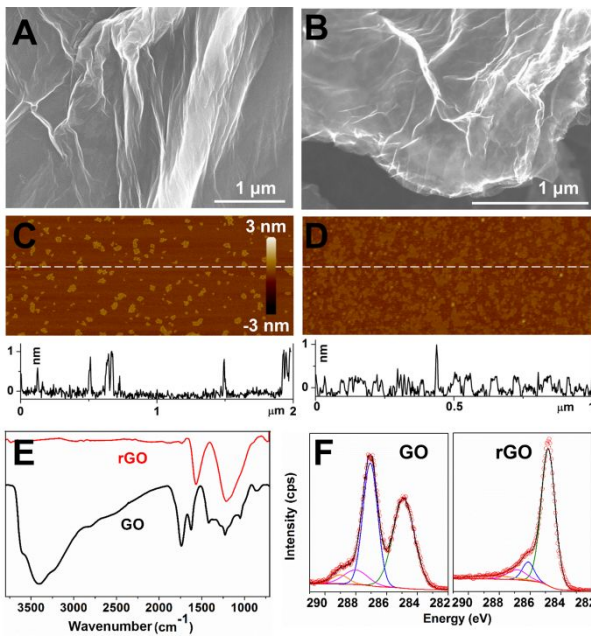
2.8. Data processing

All statistical data were presented as means \pm standard deviation. Statistical analysis was performed using IBM SPSS Statistics 19. One way ANOVA with a Tukey's test was applied after testing the data for normality and homoscedasticity, to analyse whether there were significant differences for the data of biomass, root length, stress response, and elemental concentrations between exposure conditions. $P < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Characterization of GO and rGO

SEM images show the morphology of GO and rGO sheets (Fig. 1A and 1B). The average sizes of GO ($0.089 \pm 0.023 \mu\text{m}$) and rGO ($0.078 \pm 0.034 \mu\text{m}$) are not significantly different (Fig. 1C and 1D). The size distributions are shown in Fig. S2. AFM height profiles show that GO and rGO sheets have a thickness of 0.78 ± 0.26 and 0.44 ± 0.23 nm, respectively. FTIR spectra confirm that GO has a significantly higher amount of oxygen-containing groups (O-H group at 3400 cm^{-1} , C=O group at 1726 cm^{-1} , C-O group at 1416 and 1052 cm^{-1}) than rGO. XPS survey analysis shows 34.6% and 7.8% of atomic oxygen in GO and rGO, respectively (Fig. S3). Peak fitting analysis of high-resolution XPS spectra suggests that the amount of oxygen containing groups in GO and rGO are 61% and 23%, respectively. DLS analysis suggests that rGO shows a positive charge in both water and nutrient solution, while GO is negatively charged (Table S3). The hydrodynamic diameters of GO and rGO were larger in nutrient solution (1398 ± 347 nm and 1599 ± 368 nm) than in deionized water (1194 ± 123 nm and 865 ± 98 nm), indicating agglomeration of GO and rGO in nutrient solution, and the sizes of GO and rGO in nutrient solution were similar. The high salinity of the nutrient solution contributed to the compression of the double electric layer on the surface of nanomaterials and subsequent aggregation.³⁰



213

214 **Fig. 1.** Characterization of GO and rGO. SEM images of GO (A) and rGO (B); AFM images and
215 height profiles of GO (C) and rGO (D); FTIR spectra of GO and rGO (E); XPS spectra of GO and
216 rGO (F).

217

218 **3.2. GO and rGO showed distinct effects on rice seedling growth**

219 As shown in Fig. 2, GO and rGO showed distinct impacts on the seedling growth of rice plants.
220 GO showed no effect on root elongation after three weeks of dosing, but significantly reduced
221 the shoot length by 11% at 100 mg/L and by 40% at 250 mg/L (Fig. 2A). GO at 100 mg/L and
222 250 mg/L also reduced the fresh biomass of both roots and shoots (Fig. 2C), and the dry weight
223 of shoots at 100 mg/L and 250 mg/L (Fig. 2E). In contrast, rGO showed no effect on seedling
224 elongation and biomass production at all concentrations (Fig. 2B, 2D and 2F).

225 Since the lateral size and hydrodynamic size of GO and rGO are not significantly different,
226 they are not related to the different toxicity between GO and rGO. The thicknesses of GO and
227 rGO are slightly different. It has been reported that increasing the thickness would decrease the
228 sharpness of the edge thus weakening the “nanoknife” effect,³¹ that is, GO with a bigger
229 thickness should show lower effects on plant growths than rGO. However, our result is the

opposite, suggesting that thickness is also not a determining factor. These indicate that phytotoxicity of GBMs is mainly dependent on their surface oxygen content.

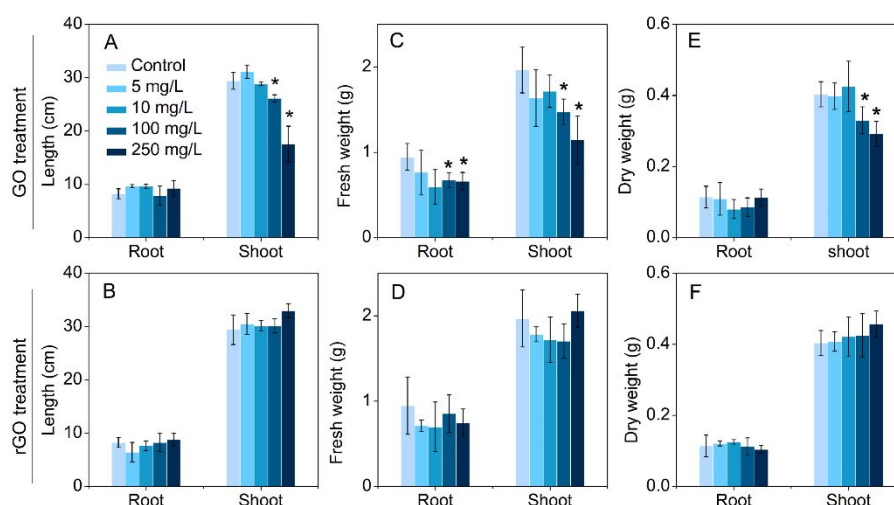
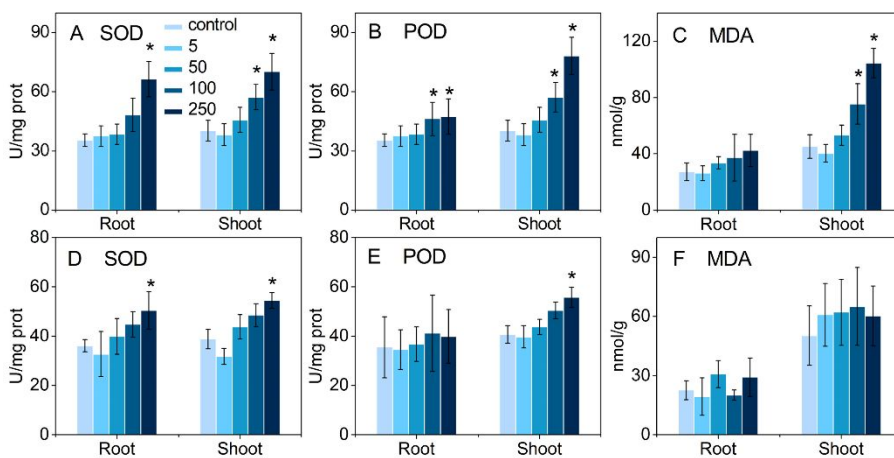


Fig. 2. Seedling lengths (A and B), fresh weights (C and D), and dry weights (E and F) of rice seedlings after exposure to different concentrations of GO and rGO for 3 weeks. Top row shows GO treatments, bottom row shows rGO treatments. * indicates a significant difference compared with control at $P < 0.05$.

3.3. Oxidative stress responses induced by GO and rGO

To further explore the underlying mechanisms of the different responses of rice seedlings and plants to GO and rGO, we examined the oxidative stress responses of rice seedlings to GO and rGO exposure (Fig. 3). The activities of antioxidant enzymes including SOD (Fig. 3A) and POD (Fig. 3B) in shoots following GO treatment were significantly enhanced at 100 mg/L and 250 mg/L whilst the MDA contents in shoots increased by 37% and 70% (Fig. 3C), respectively. No obvious changes of SOD, POD and MDA were observed in roots. In rGO treatments, SOD and POD activity only increased at the highest exposure concentration (250 mg/L) (Fig. 3D and 3E), and there was no alteration of MDA content in either roots or shoots (Fig. 3F). Significant overproduction of ROS was found in shoots with GO treatment while no obvious change was found with rGO treatment (Fig. S4). The enhanced activities of antioxidant enzymes represents a defence mechanism of plants against ambient stress. Both GO and rGO triggered the stress

250 response of plants, with the enzymatic antioxidant system failing to protect the plants against
251 the GO exposure, with the evidence showing that ROS and MDA over accumulated in GO-
252 exposed plants. Notably, the most overt over accumulation of MDA and ROS in response to GO
253 treatment was found in shoots rather than roots. A similar phenomenon was also reported with
254 maize plants where leaves were more sensitive than roots to the oxidative stress induced by
255 sulfonated graphene;³² the MDA content in roots was increased only by the highest GO
256 concentration (500 mg/L) while that in leaves was enhanced by GO concentrations ranging
257 from 100 ~ 500 mg/L. The underlying mechanism was explored in the following studies.

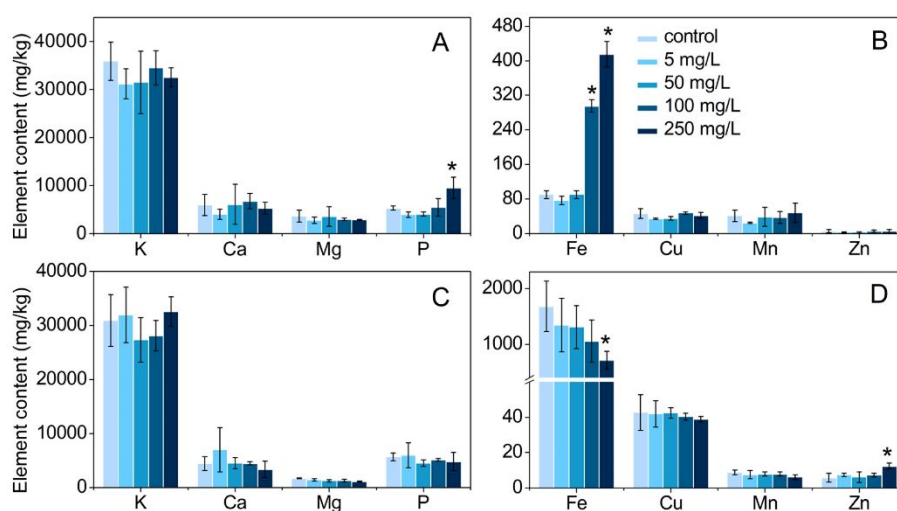


259 **Fig. 3.** SOD (A and D) and POD (B and E) activities, and MDA (C and F) contents in rice after
260 exposure to GO and rGO for 3 weeks. Top row: GO treatments, bottom row: rGO treatments. *
261 indicates significant difference compared with control at P < 0.05.

263 3.4. Alteration of the uptake of macro- and micro- elements in plants

264 To further examine the impact of GO and rGO on plant growth, we measured the uptake of
265 several key nutrients that are essential for plant growth. rGO decreased the Cu level in plant
266 tissues at 50 mg/L but showed no effects on the level of other elements (Fig. S5); however, GO
267 induced alteration of the levels of several elements including P and Fe in shoots and Fe and Zn
268 in roots (Fig. 4). Surprisingly, the Fe level in shoot (415 mg/kg) treated with 250 mg/L of GO
269 was enhanced by 3.6 times as compared with that in the control plants (90 mg/kg). Rice plants
270 usually maintain 60~300 mg/kg of Fe; when the Fe content exceeds 400 mg/kg the plant will

271 experience toxicity due to Fe overload.³³ The excessive Fe is translocated upwards and
 272 accumulated in leaves, impairing the physiological processes of plants by generating ROS via
 273 the Haber-Weiss or Fenton reactions.³⁴ In our study, the total Fe content in the GO-exposed
 274 plants was not significantly changed (Fig. S6A); however, the translocation of Fe from root to
 275 shoot was greatly enhanced (Fig. S6B). The Fe level in shoots was increased up to 415 mg/kg
 276 by the 250 mg/L GO treatment, which is correlated with the over accumulation of ROS and
 277 altered antioxidant enzymatic activities in plant leaves. These results suggested that GO
 278 induced Fe overload and consequent oxidative stress in leaves is one possible mechanism
 279 causing the phytotoxicity observed. The increased P level (Fig. 4A) was unlikely to be the driver
 280 of the toxicity because the highest P level in the shoot (9.5 mg/g) was still below the
 281 concentration (>13 mg/g) at which P may become toxic to gramineous plants.³⁵ Additionally,
 282 the toxicity in shoots occurred at 100 mg/L when there is no change of P levels, suggesting that
 283 P was not necessary for the occurrence of the toxicity.



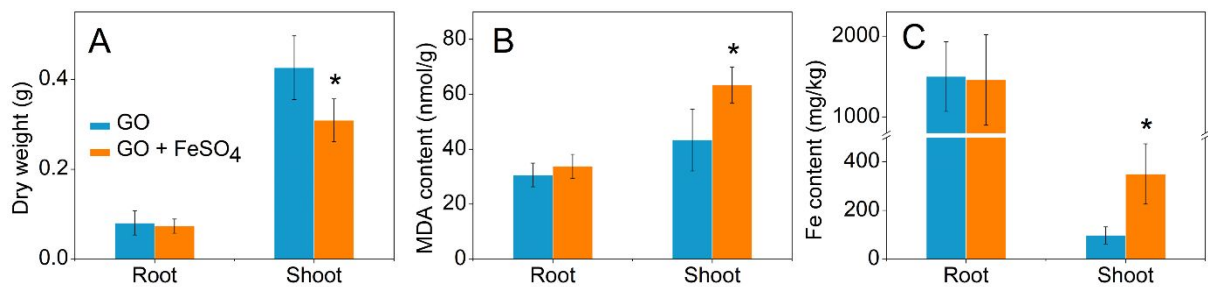
284
 285 **Fig. 4.** Macronutrient and micronutrient contents in shoots (A and B) and roots (C and D) of
 286 plants after exposure to different concentrations of GO for 3 weeks. * indicates significant
 287 difference compared with control at P < 0.05.

288

289 3.5. Iron overload contributes more than GO *per se* to the GO induced phytotoxicity

290 Since both GO *per se* and Fe overload may induce oxidative stress in plants, a follow-up question
291 is to understand the contributions of GO and Fe overload to the induced oxidative stress and
292 phytotoxicity. In our study, the oxidative stress, lipid peroxidation and overt phytotoxicity
293 (reduction of biomass and seedling length) were found for leaves rather than roots; this pattern
294 is similar with that found in Fe overloaded plants rather than graphene or other ENM treated
295 plants. For example, it was reported that excessive FeSO₄ treatment enhanced the MDA content
296 in rice leaves by 134% while having no effect on the MDA levels in roots.³⁶ While for ENMs, the
297 roots are usually more sensitive than the leaves to ENM-induced toxicity, which might be due
298 to the fact that most of the ENMs are adsorbed onto the root surface while the upward
299 translocation of ENMs is limited.³⁷

300 Therefore, we deduced that excessive Fe uptake might be the main contributor to the toxicity
301 found in this study rather than GO *per se*. To prove this hypothesis, we added an excessive
302 amount of Fe (4 mM FeSO₄) to the 50 mg/L GO suspension and examined the rice seedlings
303 growth. As compared with GO treatment alone, the dry weight of leaves was reduced by 27%
304 (Fig. 5A) and the MDA content in leaves was upregulated by 46% (Fig. 5B) after exposure to
305 GO+FeSO₄, which are correlated with a significantly enhanced Fe level in leaves (Fig. 5C). These
306 results suggest that Fe overload contributed more than GO *per se* to the oxidative stress and
307 subsequent toxicity in rice plants, although the effects of GO and rGO cannot be simply ignored
308 since GO and rGO *per se* may generate ROS.³⁸



310 **Fig. 5.** Dry weight (A), MDA content (B) and Fe content (C) in rice plants after exposure to GO
311 (50 mg/L) and a mixture of GO (50 mg/L) and FeSO₄ (4 mM) for 3 weeks. * indicates significant
312 difference compared with GO treatment at P < 0.05.

313

3.6. Mechanisms involved in the overload of Fe in leaves

Under anaerobic conditions, e.g. in paddy fields, Fe usually exists in the form of Fe^{2+} which is bioavailable for plant uptake. To avoid over accumulation of Fe, rice roots can release oxygen and oxidase to oxidize the Fe^{2+} to Fe^{3+} , which precipitates to form a coating on the roots named “Fe plaque”.³³ The Fe plaque can prevent not only the uptake of excessive Fe^{2+} but also the entry of toxic heavy metals into plants. However, a decrease of pH can significantly promote the reduction of insoluble Fe^{3+} to Fe^{2+} that eventually leads to Fe toxicity, which affects a significant proportion of rice fields in many developing countries.³³

The pH mediated Fe uptake not only applies for plants cultured in soil but also applies for hydroponic culture. It was also reported that low pH can significantly promote Fe^{2+} uptake by plants.³⁹ We found that GO acidified the nutrient solution while rGO didn't change the pH significantly (Fig. 6A). The pH of the GO suspensions decreased with increasing GO concentration. The pH values for 100 mg/L and 250 mg/L of GO in NS are 3.95 and 3.32, respectively, which are much lower than that of the normal NS (pH 5.5). The low pH itself was unlikely the main reason for the toxicity based on two reasons: 1) Rice is relatively tolerant of acidic conditions. Previous studies showed that rice can grow normally at pHs as low as 3.4, but the growth can be greatly impaired if Fe contents in the soil increased.⁴⁰ 2) Impairment of the root growth should be also observed if pH was the reason for the toxicity. However in our study only the growth of shoots was impaired. Therefore, the low pH itself is not the main driver of the toxicity. Instead, the GO induced decrease of pH can increase Fe mobilization and cause the observed Fe overload in the shoots.³⁹ Fig. 6B further showed that the Fe content flux in the xylem sap was significantly enhanced by GO treatment.

Considering the high capacity for absorption of GBMs which results from their high surface area, we further examined another possibility, which is that GO may enrich Fe on their surface by adsorption and then translocate to the leaves which may enhance the Fe uptake. To do so, we compared the adsorption of Fe on GO and rGO (see method in Section 1, SI). The amount of Fe adsorbed onto GO and rGO was 1.22 mg/L and 1.31 mg/L (Fig. 6C), respectively, which was nearly half of the Fe present in the nutrient solution (2.9 mg/L). We then estimated the amount

of Fe that can be translocated with the GO and rGO to the shoots (see method for estimation of uptake in Section 1, SI). Only 0.098 mg/kg of Fe can be attributed to transport into the plants *via* adsorption onto the GO or rGO. This is negligible compared with the total amount of Fe that was accumulated in the leaves (325 mg/kg), suggesting that the contribution of this mechanism to Fe overload is negligible.

When the pH of the GO suspension was adjusted back to normal pH (5.5) (see details of methods in SI), the phytotoxicity was eliminated (Fig. 6D and 6E) and the Fe content in shoots was normal (Fig. 6F). These results provide solid evidence that Fe overload is the main cause of the GO induced toxicity.

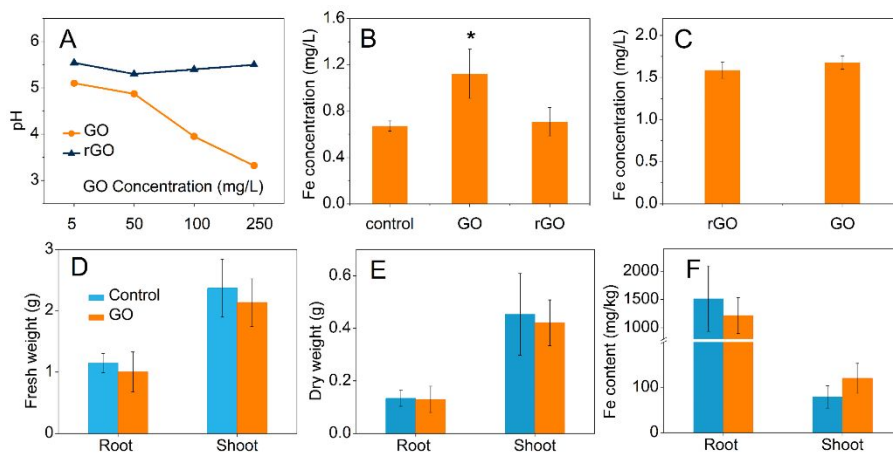


Fig. 6. pH values of GO and rGO suspensions in nutrient solution (A), the free Fe concentration in collected xylem saps (B), and the free Fe concentration in nutrient solution after incubation with GO and rGO (C) from a starting concentration of 2.9 mg/L. Fresh weight (D) and dry weight (E) of plant and Fe content (F) in plant after exposure to pH adjusted GO suspension (250 mg/L, pH 5.5) for 3 weeks. * indicates significant difference compared with control at $P < 0.05$.

3.7. Plant defence against Fe overload via reduced production of Fe transport associated metabolites

In our study, we found depressed production or excretion of iron-mobilizing coumarin-type compounds and iron-chelating flavonoid-type compounds (Fig. 7) in the leaves of GO treated plants, which may act as an important component of the iron depletion strategy in response to

363 Fe overload. Coumarin-derived phenolics or their corresponding glycosides were all
364 dramatically depressed in the GO treated plants. For example, psoralen, a linear
365 furanocoumarin, was decreased by 36% (Fig. 7A). The coumaric acid ester, 3-hydroxycinnamic
366 acid, and cinnamoylcholine (a cinnamic acid ester), which are intermediates in the coumarin
367 biosynthetic pathway, were significantly decreased by 78%, 62%, and 60%, respectively. The
368 glycosides, such as coumarin glycoside (4-methylumbelliferyl glucuronide) and cinnamoyl
369 glycoside, were also significantly decreased. In addition, an array of flavonoid-derived
370 compounds, including flavonoids (e.g. flavanones, flavones, flavonols, anthocyanidin), the
371 methylated derivatives, and their glycosides were all downregulated in the GO exposed plants
372 (Fig. 7B). For example, the concentrations of cyanidin (a type of anthocyanidin), kaempferol (a
373 natural flavonol), naringenin (a flavanone), 6-hydroxyflavone (a flavone), and corymbosin (a
374 flavone), were depressed by 70%, 66%, 48%, 56% and 44%, respectively. The decrement for
375 other flavonoid derivatives ranged from 41% to 65%. These data suggest that regulatory
376 mechanisms at the metabolic level were evoked in leaves in order to sustain the iron
377 homeostasis in response to GO-induced Fe overload.

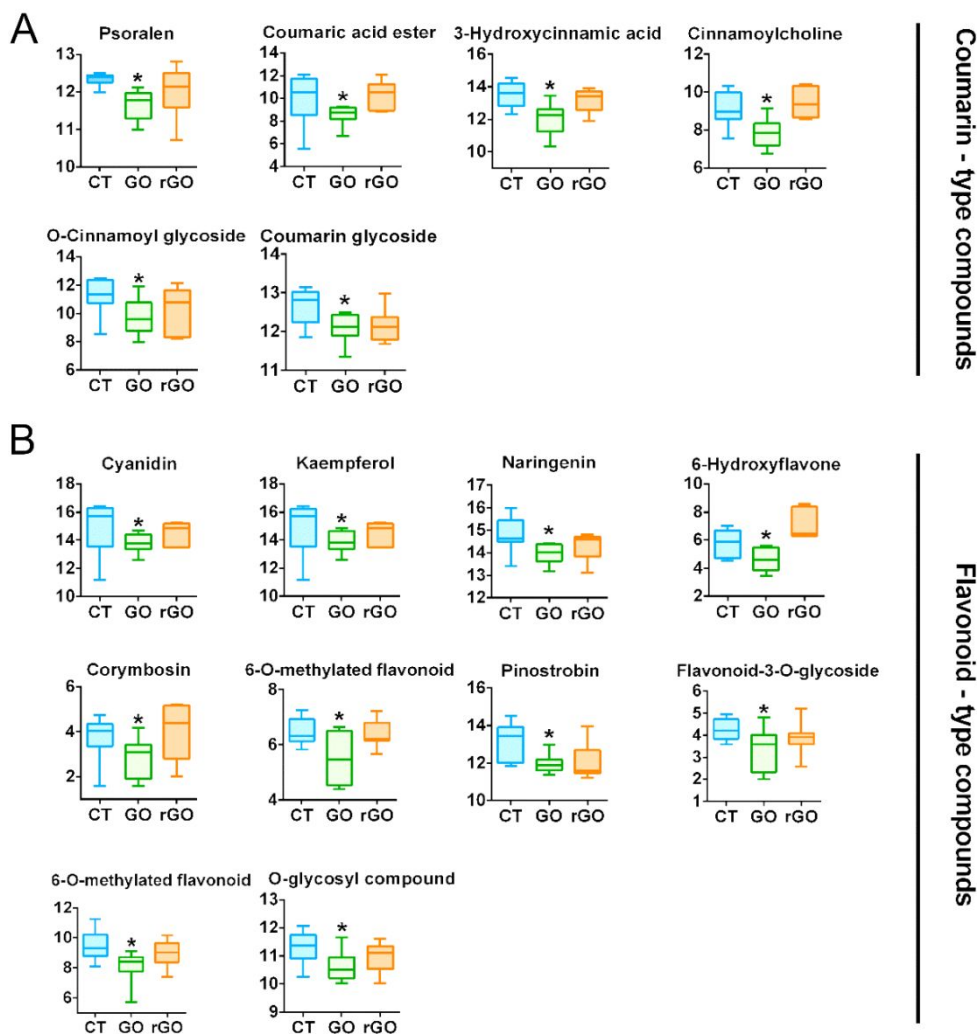


Fig. 7. Box plots of relative abundance of coumarin-type compounds (A) and flavonoid-type compounds (B) in the leaves of rice treated with 250 mg/L GO and rGO compared to the untreated controls (n=8). * indicates significant difference compared with control.

3.8. Biotransformation of GO as a pathway to alleviate GO-induced phytotoxicity

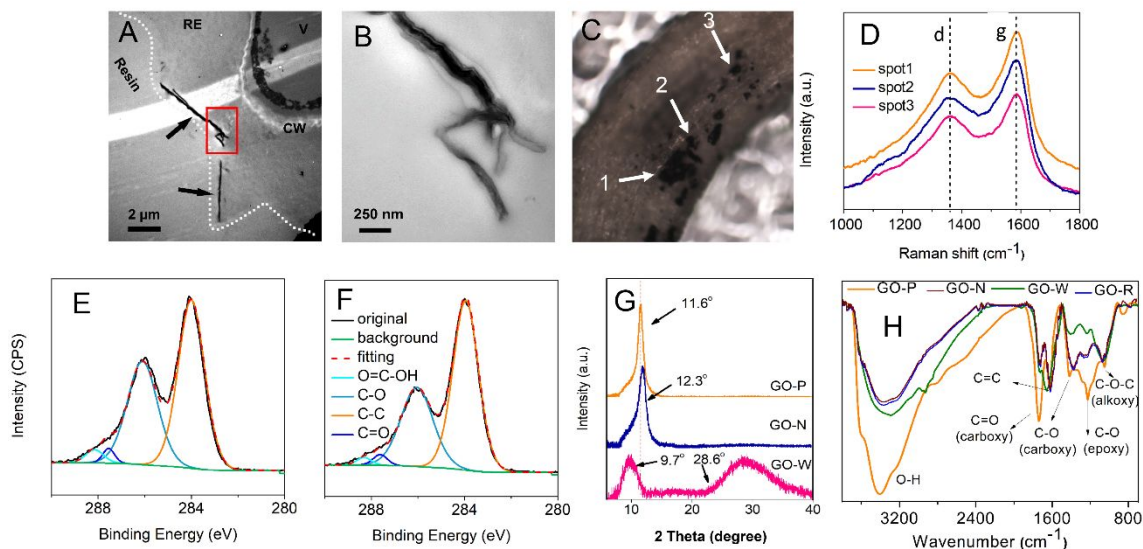
ENMs may transform by interaction with plants, the process of which may determine their subsequent behaviour, fate and toxicity in plants. CeO₂ ENMs are reported to be transformed in many plant species,^{41, 42} being reduced and releasing Ce³⁺, which is found to be responsible for the toxicity of CeO₂ ENMs to *Lactuca* plants.⁴² The released Ce³⁺ can bind with phosphates, which could be a detoxification process.^{43, 44} Transformation of graphene materials has been reported in bacteria,²⁸ plants⁴⁵ and in water under sunlight.⁴⁶ Free radicals (OH•) were

reported to be involved in the transformation of graphene into CO₂ in plant leaves, the process of which contributed to the elimination of graphene from plants following uptake and may thus reduce graphene induced phytotoxicity.⁴⁵ Immobilization of root exudates onto GO and formation of ligand-GO complexes were also reported, which decreased the surface charge and increased the unpaired electrons and the toxicity of the GO to zebrafish.⁴⁷

We found that GO adsorbed onto the root surface, with a significant change of morphology from sheet to a folded shape (Fig. 8A and 8B). A root exudate (RE) layer between the GO and the root epidermis cells can be observed, which might act as a barrier to prevent the GO from entering the roots. Adsorption of GO onto the root surface was also clearly visible under the Raman microscope (Fig. 8C). The Id/Ig ratios (0.715-0.783) of the Raman spectra, collected from three spots on the roots (Figure 8D), were significantly lower than that of pristine GO (0.98, Fig. S7, Table S4), suggesting that roots enhanced the disorder in the structure of GO.⁴⁷ The O/C ratios of GO decreased significantly after interaction with plants (Fig. 8E and 8F, Table S4), suggesting the partial transformation of GO into rGO. GO-W (which were washed from the root surface) showed a higher reduction degree (O/C, 0.31) than GO-R (O/C, 0.4), suggesting that direct contact with the plant roots accelerated the reduction of GO. XRD analysis further confirmed the transformation of GO into rGO (Fig. 8G). Incubation in nutrient solution (GO-N) only induced a slight shift of the (002) peak of GO from 11.6° to 12.3°, suggesting no alteration of the crystal structure but a decrease of the lattice spacing. However, interaction of GO with roots (GO-W) not only induced a shift of the (002) peak to 9.7° but also led to the formation of a new peak at 28.6° which is attributed to the (002) peak of rGO, suggesting the reduction of GO.⁴⁸ In agreement with the XPS and XRD results, FTIR showed that GO-W was reduced to a higher degree than GO-R, suggesting that contact of GO with plant roots facilitated the transformation of GO to rGO (Fig. 8H). The transformation of “toxic” GO into a relatively low-toxic “rGO” might act as a pathway to alleviate the toxicity of GO. The potential role of root-associated microbes in the transformation process remains to be explored.

Adsorption of rGO onto the root surface was also observed by TEM (Fig. S9). FTIR spectra (Fig. S10) showed increased intensity of surface oxygen content after interaction with plant

418 roots (rGO-W), suggesting partial oxidation of rGO. However, XRD analysis showed that the
419 main peak (002) of rGO has not shifted (Fig. S11), suggesting no changes to the crystal structure.
420 The increased surface oxygen content observed by FTIR might be due to the adsorption of
421 organic compounds from root exudates.



422
423 **Fig. 8.** Characterization of GO after interaction with plants for 3 weeks. (A and B) TEM images
424 of root sections; B is the magnified image of the rectangle area shown in A. RE indicates the root
425 exudate, CW indicates the cell wall. (C) Optical image of roots. (D) Raman spectra collected at
426 the three spots shown in C; d and g indicate the d band at 1363 cm^{-1} and g band at 1593 cm^{-1} ,
427 respectively. The intensity ratios of d to g, i.e. I_d/I_g ratios, were 0.715 (spot 1), 0.723 (spot 2)
428 and 0.783 (spot 3), respectively. (E) XPS spectra of GO-R (GO in residual NS after removal of
429 plants). (F) Raman spectra of GO-W (GO washed off from roots). (G) XRD spectra of GO-P
430 (pristine GO), GO-N (GO incubated in nutrient solution for 3 weeks) and GO-W. (H) FTIR spectra
431 of GO-P, GO-N, GO-W and GO-R.

432
433 The present study reports for the first time a new mechanism of ENM induced phytotoxicity,
434 i.e. GO induced pH alteration of nutrient solution and subsequent Fe over accumulation and
435 oxidative damage in plant leaves. Some previous studies have suggested that ENMs can disturb
436 the macro and micro element distribution in plants, however, a clear interpretation of these
437 findings are lacking. The present study indicates that ENMs may cause toxicity to plants

indirectly by altering the micronutrient uptake. The apparently different impact of GO and rGO on plant growth suggests that the phytotoxicity of GBMs is highly related to their surface oxygen content. The inconsistent use of GO or rGO with different surface oxygen densities might be one of the reasons that explain the inconsistency in current literature. It should be noted that this is a short term study carried out in hydroponic condition. Effects of GBMs on plant in realistic soil environment over longer exposure time might be different and the mechanisms involved will be complicated by the soil components, which requires further studies.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website. It includes additional experimental details and results.

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ACKNOWLEDGEMENT

This work was supported by Marie Skłodowska-Curie Individual Fellowships (NanoLabels Grant Agreement No. 750455 to PZ; NanoBBB Grant Agreement No. 798505 to ZG) under the European Union's Horizon 2020 research program.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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