applied sciences

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The Interaction of Graphene Oxide with the Pollen—Stigma System: In Vivo Effects on the Sexual Reproduction of *Cucurbita pepo* L.

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Abstract: Applications involving graphene-related materials (GRMs) are becoming very common, raising concerns for their environmental impact. GRMs induce various effects on plants, but those on the sexual reproduction are still largely unknown. Here, the effects of graphene oxide (GO) and GO purified from production residues (PGO) were tested in vivo on the stigma of *Cucurbita pepo* L. ssp. *pepo* "*Greyzini*" (summer squash). Stigmas were exposed to GO or PGO for three hours and were then analyzed by environmental scanning electron microscopy to verify possible alterations to their surface. Stigmas were then hand-pollinated to verify the effects of the two GOs on pollen adhesion and germination on the stigma, and, subsequently, on the development of fruits and seeds. Severe damages to the stigma were not detected; nevertheless, both pollen adhesion and germination on the stigma decreased. Moreover, fruits developed defectively with signs of necrosis in the case of GO, whereas fruits did not ripen in the case of PGO and ovules did not develop seeds after both GOs treatments. These results highlight different mechanisms of interaction of the two materials with the pollen-stigma system, suggesting a possible negative impact of GO on the sexual reproduction of other seed plants.

Keywords: fruit development; flowers; nanomaterials; particulate matter; pollen germination; seed development; stigmatic surface

Citation: Zanelli, D.; Candotto Carniel, F.; Tretiach, M. The Interaction of Graphene Oxide with the Pollen—Stigma System: In Vivo Effects on the Sexual Reproduction of *Cucurbita pepo* L. *Appl. Sci.* **2021**, *11*, 6150. https://doi.org/10.3390/app11136150

Academic Editor: Ewa Kurczyńska

Received: 1 June 2021
Accepted: 29 June 2021
Published: 2 July 2021

1. Introduction

In 2004, the isolation of the first monolayer of graphene [1] kindled the research interest in graphene-related materials (GRMs) [2]. Their extraordinary chemical and physical properties boosted many fields of innovative technology, from opto-electronics and medicine to materials for automotive industry and constructions [3,4]. To date, GRM-enriched products, such as tires, asphalts, and sports equipment, are in an advanced stage of development and have already reached markets and civil society (for a list see www.graphene$-$info.com, accessed on 1 June 2021).

Despite their advantages, GRM-enabled products are subjected to degradation and breakage and will be disposed of at the end of their life cycle. This could lead to an unintended and undesirable release of GRM nanoparticles into the environment. Moreover, applications involving a direct voluntary release of GRMs into the environment are under development, such as GRM-composites used as pesticides [5,6], plant fertilizers [7–9], sand improvers for soil remediation [10], and drug enhancers [11]. GRM nanoparticles are extremely lightweight, thus, once dispersed in the atmosphere, they can be transported for very long distances, as described for carbon black in fine and ultrafine particulate matter (PM) [12]. These nanoparticles could settle on soil, water bodies, or vegetation with still unknown repercussions on organisms and functionality of ecosystems.

So far, literature reported both positive and negative effects of GRMs on seed plants, possibly owing to different experimental conditions (materials, concentrations, exposure

time, protocols, etc.), plant developmental stages (seed, seedling, adult plants, etc.), and/or species tested [13,14]. Notably, the aboveground organs of seed plants, such as leaves and flowers, act as natural traps for airborne PM [15,16], potentially including GRMs. Landing on flowers, airborne GRMs might affect one of the most important biological processes for life on Earth, i.e., the sexual reproduction of seed plants.

Recent in vitro studies demonstrated that few-layer graphene (FLG) and graphene oxide (GO) impair pollen tube emergence and, in the case of GO, pollen tube growth in *Corylus avellana* L. (common hazel) and *Nicotiana tabacum* L. (tobacco plant) [17,18]. It was hypothesized that FLG might mechanically impair/damage the germination pore and affect the intracellular redox homeostasis of the pollen. However, in a following in vivo study on *Cucurbita pepo* L. (summer squash) flowers [19], it was shown that FLG did not injure the structure of stigmas and pollen grains but affected pollen adhesion and germination over the stigmatic surface. Importantly, these effects were comparable to those of a naturally occurring 2D-nanomaterial (2D-NM), i.e., muscovite mica. Differently, the effects of GO on pollen were mainly related to the oxygen functional groups (e.g., carboxylic, epoxy oxydrilic) present on the GO lattice which makes GO acidic and able to bind/immobilize important cations, such as Ca^{2+}, for pollen tube germination and elongation [18].

On the basis of these premises, in this study we tested the hypothesis that GO might be potentially more hazardous than FLG to the sexual reproduction of seed plants as it could alter the conditions of the stigmatic environment, leading to unsuccessful pollen germination and, consequently, hindering the fertilization process. To verify this, stigmas of *Cucurbita pepo* L. ssp. *pepo* "*Greyzini*", a crop that is a model species for pollination studies [20], were exposed to GO and GO purified from production residues (PGO). At first, the GO effect on stigmatic surface integrity and on pollen adhesion and pollen germination was tested. Subsequently, the effect on fruit and seed production was assessed.

A better understanding of the possible negative effects of GO on the sexual reproduction of seed plants would help us to predict future scenarios and perhaps to adopt measures to engineer safely designed GRMs and derived applications.

2. Materials and Methods

2.1. GO and PGO Characterization

Graphene oxide (batch #GOB067) was kindly supplied by Graphenea (San Sebastián, Spain). A complete characterization of the material was reported by Fusco and co-authors [21] and a brief elemental analysis performed with a LECO CHNS−932 analyzer (LECO Corporation, St. Joseph, MI, USA) showed average values of 59.40 ± 0.10% C, 1.40 ± 0.10% H, 0.07 ± 0.02% N, < 36.6% O, and 2.50 ± 0.10% S. Raman spectroscopy performed with an inVia Raman Microscope (Renishaw, Wotton-under-Edge, UK) revealed the two characteristic D and G bands of GO, with their maxima located at ~1350 and ~1600 cm^{-1}, respectively. Lateral dimension was evaluated by laser diffraction in the GO slurry in the range from 6000 to 30,000 nm, with an average of 15,100 ± 400 nm. X-ray diffraction (XRD) analysis was performed on a GO dry film, revealing flakes with an average thickness of six layers. GO was firstly synthesized in acidic media (H_2SO_4) and then dispersed in water. To remove acidic residues due to the production process, purification was performed by repeated washings in distilled water, each followed by a centrifugation step at 4800× *g*. After purification, only around 2% of the starting S remained in the purified GO (PGO), mostly as SO_4^{2-} [22]. The acidity of the materials used in this study was verified with a HI5521 pH meter equipped with a HI1131B electrode (Hanna Instruments Italia s.r.l., Padua, Italy); data are reported in Table 1.

2.2. Plant Material and Exposure to GO

Seeds or young plants of *Cucurbita pepo* L. ssp. *pepo* "*Greyzini*" were purchased from Salto di Fondi-Società Cooperativa Agricola A.r.L. (Fondi, Italy) and were greenhouse-cultivated (18–22 °C) in the Botanical Garden of the University of Trieste from January to March and from February to April 2020. Plants were watered once a day with a ground-

based automatic irrigation system, fertilized (Concime universale Asso di Fiori, Cifo, S. Giorgio di Piano, Italy), and treated with antimycotics (Azupec 80WG, Ascenza Agro, Torres Vedra, Portugal and Jupiter WG, Isagro Spa, Adria, Italy) every two weeks.

Table 1. pH of dH_2O with graphene oxide (GO) and purified GO (PGO) at concentrations of 25, 50 and 100 μg mL^{-1}; values are reported as means ± s.d. (n = 3).

Materials	Concentration	pH
GO	25	4.62 ± 0.08
	50	3.86 ± 0.01
	100	3.53 ± 0.10
PGO	25	5.90 ± 0.38
	50	5.82 ± 0.17
	100	5.82 ± 0.32

Receptive stigmatic surfaces of *C. pepo* were exposed to GO and PGO applying all the needed efforts to assure workplace safety. For the experiments on stigma and pollen-stigma interaction, six pistils from six diverse individuals were excised, placed on a petri dish filled with 5 mL of agar gel, and kept at laboratory conditions (dim light and 21 °C). One stigma per each pistil was just brushed (control samples: CTRL; n = 6) or coated with 1 mg of either GO or PGO (treated samples; n = 6) using a paintbrush according to Zanelli and colleagues [19], and then kept at laboratory conditions before further processing. Preliminary tests indicated that brushing the stigmatic surface with a paintbrush did not affect stigmatic surface integrity, pollen-stigma interaction, and the subsequent fruit and seed production. For the experiments on fruit and seed production, three to five pistils, each from a different individual, were left on the mother plant. In this case, all three stigmas of each pistil were treated as described above.

2.3. ESEM Screening of Stigmatic Surfaces

Three CTRL and three treated stigmas were excised, attached to aluminium stubs, and observed using a Quanta250 SEM (FEI, OR, USA) which operated in an environmental mode (ESEM) to collect secondary electrons (accelerating voltage: 30 kV; chamber pressure: 90 Pa). The entire stigmatic surface of each sample was examined and 10−15 micrographs per sample were taken.

2.4. Pollen Adhesion and Germination over the Stigmatic Surface

CTRL and treated stigmas were hand-pollinated with 3 ± 0.5 mg of pollen (corresponding to 2407 ± 541 pollen grains) using a paintbrush. Pollen was harvested from *C. pepo* male flowers (n ≥ 7) ~2:30 h after flower blossoming (~7:30 a.m.) and its viability was assessed by the fluorescein diacetate (Sigma-Aldrich, Munich, Germany) fluorochromatic reaction [23], counting at least 200 pollens. Only pollen aliquots with viability higher than 80% were used. Pollen was gently and homogeneously brushed on a 4 × 4 mm spot using a greaseproof paper frame. Afterwards, pollen was let to germinate at laboratory conditions for 40 min. Pollen adhesion and germination onto the stigmatic surface were assessed following the protocol described in Zanelli and colleagues [19]. Pollen adhesion was estimated as follows: adhesion = number of pollen grains deposited minus number of pollen grains detached from the stigmatic surface (n = 6). The germination rate was expressed as % of germinated pollen still adhering to the stigma (n = 6) by counting, when possible, at least 200 pollen grains per stigma.

2.5. Fruit Biometrics and Seed Production

To verify the effect of GO on fruit and seed production, all three stigmas of one flower per plant (n = 3–5) were coated with GO or PGO or just brushed (CTRL). Hand-pollination was carried out, as explained above, ensuring an even distribution of pollen all over the three stigmatic lobes. Fruits that originated from flowers bearing treated stigmas (herein

referred to as CTRL, GO and PGO fruits) were harvested five weeks after pollination. Fresh mass, length, and maximum circumference of fruits were measured. Thereafter, fruits were dissected to visually inspect their internal structure for possible defects and to collect and count seeds. The latter were rinsed in dH_2O to remove residuals of the fruit pulp and left to dry out for 24 h before the analysis.

2.6. Statistical Analysis

Statistical analysis was performed using the software package Dplyr in R environment (R, version 3.6.3., 29 February 2020, The R foundation for statistical analysis) [24]. Differences in terms of pollen adhesion, and germination rates on the stigmatic surface, and fruit mass, length, circumference, and seed per fruit were assessed with generalized linear models (GLMs), assuming the treatments as categorical predictors. Differences among treatments were determined with the post-hoc pairwise *t*-test, applying the Bonferroni adjusted method.

3. Results and Discussion

The deposition of xenobiotics onto the stigmatic surface may have a negative effect on stigma-pollen interactions resulting in an impaired fruit and/or seed production [25–27]. For example, fungicides decrease pollen germination but do not alter fruit production in blueberry [25], apple [28] and grape, whereas they do affect seed production in grape [29]. Differently, pesticides lower pollen germination but not seed production in onion [27]. The GO tested in this study is a commercial product available in the market with dimensions in between those of PM 10 and of suspended powders with dimensions up to 30 μm but with a bi-dimensional geometry and lightweight that might lead to a longer time of permanence in the air. This might increase the chances of being intercepted by the aboveground organs of seed plants. Hence, in this study, the effect of a commercial GO and its purified version (PGO) was tested on stigma, pollen−stigma interaction, and fruit and seed production of *C. pepo*.

The stigmatic surface of *C. pepo* is covered by clump-shaped structures made up of finger-like cells, i.e., the stigmatic papillae (Figure 1a,b). ESEM observations of CTRL samples revealed that the brushing of the stigmatic surface did not affect its integrity (Figure 1a). Papillae coated with GOs maintained their original shape even if they were slightly agglutinated and partially wilted at the apex. It is known that the direct interaction of GRMs with cells may cause physical damage, such as cuttings or piercings [30,31]. However, signs of severe damage as cytoplasmic leachates were not detected (Figure 1c,d), as already observed on the stigmatic surface of *C. pepo* coated with FLG and nanocrystals of the naturally occurring muscovite mica [19]. The absence of severe physical damage is in good agreement with previous works suggesting that the cell wall of plant cells is very difficult to damage and pierce through [18,32], even if the nanomaterials applied are very thin, have sharp edges, and their lateral dimensions are in the order of hundreds of nanometres. In fact, despite our GOs having lateral dimensions bigger than those of the NMs used in the previous study [19], it was as thin as FLG (6 vs. 3−4 layers, respectively) and thinner than muscovite mica (16 ± 13 layers).

The presence of GO over the stigmatic surface might alter the interaction between pollen and stigma [19,33,34], affecting both pollen adhesion and germination. In our case, the presence of GO caused the slight modification at the apex of the stigmatic papillae described above. This might be caused by the reactivity of GO, conferred by the oxygen functional groups bound to the graphene lattice. However, a decrease in pollen adhesion and germination might depend also on a physical interposition of GO flakes between single pollen grains and the stigmatic surface. In CTRL stigmas, pollen adhesion was 70 ± 11% (Figure 2a), whereas it decreased to 58 ± 18% and 51 ± 5%, in GO- and PGO-coated stigmas, respectively. This difference was statistically significant with respect to CTRL only in the case of PGO ($p < 0.05$, post hoc *t*-test for pairwise comparisons) (Table S1). PGO flakes in contact with the stigma appeared less folded than GO ones (Figure 1c,d), which might have decreased the surface of contact and the strength of the pollen adhesion to the

stigma. Differently, pollen germination significantly decreased from 65 ± 13% in CTRL stigmas (Figure 2b) to 17 ± 9% and 18 ± 19% in GO- and PGO-coated stigmas, respectively (Table S1). After three hours of germination, the pollen tubes generally penetrated deeper in the CTRL stigmatic tissues (Figure 3a) than in the GOs ones (Figure 3b,c).

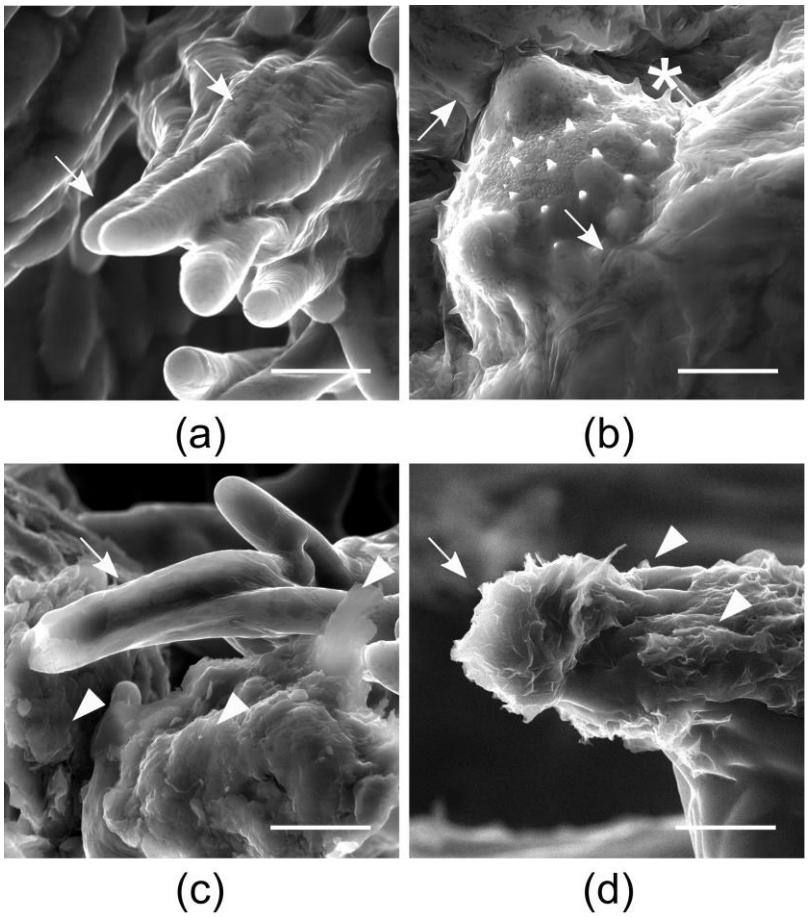

Figure 1. SEM micrographs of stigmas of *Cucurbita pepo* L. coated with 0 (CTRL) (**a**,**b**) or 1 mg of graphene oxide (GO) (**c**) and purified GO (PGO) (**d**), and pollinated after three hours (for more details, see Sections 2.2 and 2.3). Stigmatic papillae, pollen grains and GO flakes/nanoparticles are indicated with arrows, asterisks, and arrowheads, respectively. Bars = 50 µm.

In vitro experiments reported that GO had an inhibitory effect on pollen tube emergence and elongation in *C. avellana* and *N. tabacum*, which was mainly caused by GO acidic properties [17]. However, considering that PGO had a sub-neutral pH (Table 1) and that the pH-neutral FLG and muscovite mica caused comparable results [19] to those of GO, the aforementioned effects might derive from a different interaction mechanism. Coating flowers of *Pistacia vera* L. with dust before pollination [35] decreased the production of fruits. The authors hypothesized that the dust at the interface between pollen and stigma impaired the chemical signaling occurring between them, thus lowering the number of pollen grains able to germinate and to complete fertilization. This suggests that fewer pollen grains in contact with the stigma and a decreased pollen germination rate due to low pollen density over the stigmatic surface, i.e., a "pollen population effect" [36], are the cause of a reduced fertilization success. Accordingly, experiments on pollen loading and dilution of pollen [37] showed that lowering the density of pollen grains over the stigma decreased the percentage of germinated pollen grains and, consequently, the development of fruits and seeds [38].

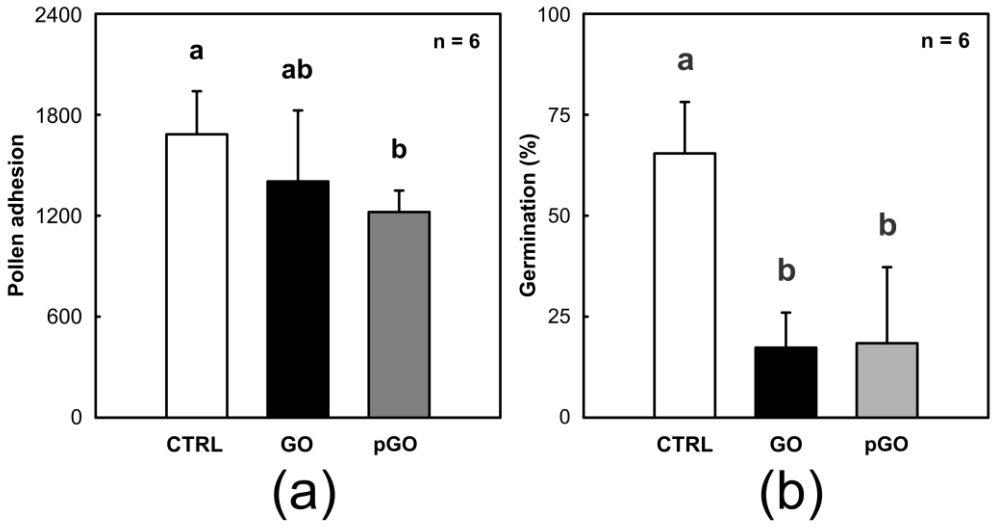

Figure 2. Pollen adhesion (**a**) and pollen germination (**b**) observed on *Cucurbita pepo* L. stigmas coated with 0 (CTRL) or 1 mg of graphene oxide (GO) and purified GO (PGO), and pollinated after three hours (for more details, see Sections 2.2 and 2.4). Values are means ± s.d. (n = 6 stigmas). Statistically different groups are marked with different letters (GLM analysis, followed by Bonferroni *t*-test for more details, see Table S1).

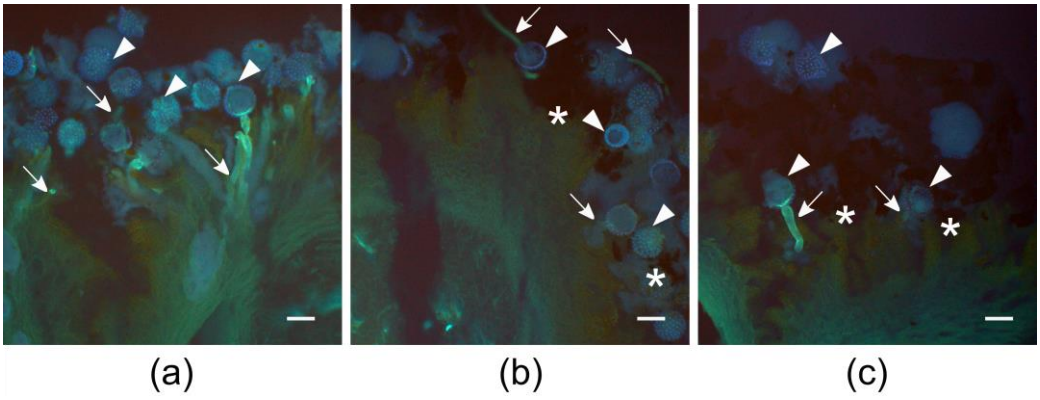

Figure 3. Fluorescence micrographs of stigmas of *Cucurbita pepo* L. coated with 0 (CTRL) (**a**) or 1 mg of graphene oxide (GO) (**b**) and purified GO (PGO) (**c**), and pollinated after three hours (for more details, see Sections 2.2 and 2.4). Pollen tubes, pollen grains and GO flakes/nanoparticles are indicated with arrows, arrowheads, and asterisks, respectively. Bars = 100 µm.

The presence of reactive substances on the stigmatic surface can impair not only the quantity but also the quality of fruits. For *P. vera*, it was shown that the coating of flowers with dust reduces fruit size and increases parthenocarpy (i.e., production of fruits without fertilization of ovules) [35]. In this study, CTRL fruits had a mass of 437.1 ± 168.8 g, a length of 21.3 ± 3.1 cm, a circumference of 26.1 ± 5.7 cm, and bore 37 ± 25 seeds per fruit. Differently, GO fruits developed defectively (Figure 4c,d), with significantly lower fresh mass, length, and maximum circumference than CTRL fruits (Table S2). Furthermore, ovules that did not develop seeds, dead tissues, and cavities in the upper part of the ovary were also observed (Figure 4c,d, asterisks). This could be caused by the highly reactive residues (such as H_2SO_4, H_2O_2 or $KMnO_4$) of the GO production process. Once in contact with the stigmatic surface, they might dissolve in the intercellular spaces and injure the underneath pericarp tissues. A similar effect was observed as a consequence of the depositions of active molecules and materials, such as herbicides [35], fungicides [39], pesticides [26], or cement-kiln dusts [40], which can dissolve and release active compounds causing necrosis and blocking seed and, subsequently, fruit development. In the case of PGO, fruits never reached maturation (Figure 4e,f), because ovule fecundation did not take place. This is in accordance with the hypothesis that a physical interposition of planar NMs

between pollen and stigma increased the "pollen population effect" and caused a reduced pollen load and a deteriorated pollen-stigma signaling, altering the fertilization process and, subsequently, fruit and seed production.

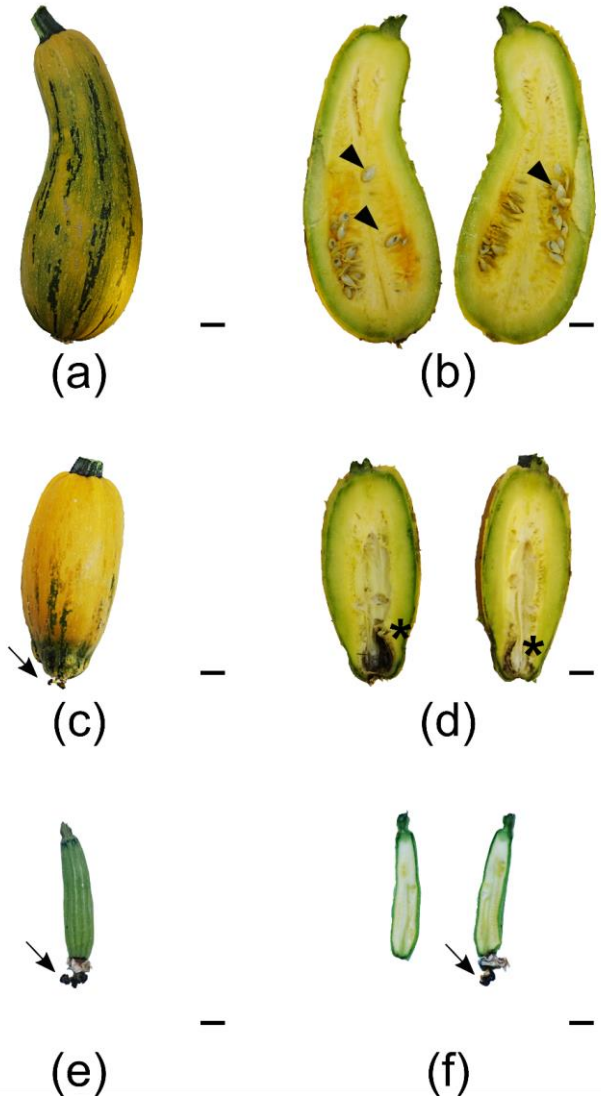

Figure 4. Photographs of 5-week-old fruits of *Cucurbita pepo* L., entire (left column) or dissected (right column), originated from stigmas coated with 0 (CTRL) (**a**,**b**) or 1 mg of graphene oxide (GO) (**c**,**d**) and purified GO (PGO) (**e**,**f**), and pollinated after three hours (for more details, see Sections 2.2 and 2.5). GO flakes/nanoparticles, seeds and dead tissues are indicated with arrows, arrowheads and asterisks, respectively. Bars = 2 cm.

4. Conclusions

In this study, the effect of GO interaction with the pollen−stigma system was verified on the entire reproduction process of the model plant, *C. pepo*, which is also an economically important vegetable. The stigmatic surface integrity was not compromised by GO; still, pollen adhesion and germination over the stigma decreased, fruit development was altered, and seed production was completely suppressed. The similar effect of GO and PGO and the comparable effect of further 2D-NMs (i.e., FLG and muscovite mica) support the hypothesis that the physical interposition of planar nanoparticles between pollen and stigma compromises the reproduction process by lowering the pollen load and affecting pollen-stigma signaling. However, the necrotic effect of GO on fruits also suggests a chemical interaction of this material (and its potential contaminants) with the plant tissues,

similar to other reactive substances. Our samples were exposed to high amounts of GO which were unlikely to occur in the environment in a plant whose flowers remain open for a few hours only. Nonetheless, this allowed us to highlight possible effects due to GO depositions on stigmas caused by very localized and abundant releases of these materials, such as sprays of GO-based fungicides or pesticides. Future research should test quantities more similar to what is expected to be found in the environment and with techniques mimicking atmospheric dry depositions. Special attention should be focused on anemophilous plants, whose flowers remain exposed to airborne particulate for days or even weeks.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11136150/s1, Table S1: GLM results of pollen adhesion % in in vivo germination, Table S2: GLM results of fresh mass, length, maximum circumference of fruits and number of seeds produced per fruit.

Author Contributions: Conceptualization, D.Z., F.C.C. and M.T.; methodology, D.Z., F.C.C. and M.T.; validation, D.Z. and F.C.C.; formal analysis, D.Z.; investigation, D.Z. and F.C.C.; resources, D.Z. and F.C.C.; writing—original draft preparation, D.Z. and F.C.C.; writing—review and editing, D.Z., F.C.C. and M.T.; visualization, D.Z., F.C.C. and M.T.; supervision, F.C.C. and M.T.; project administration, F.C.C. and M.T.; funding acquisition, M.T. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Graphene Flagship Core 2 and Core 3 grant agreement (no. 785219 and 881603, respectively), the University of Trieste DOTTAMBIENTEVITA34-18.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors would like to thank Andrea Pannozzo (Fondi) for the advice and discussion on plants growth, Davide Porrelli and Gianluca Turco (University of Trieste), for the help with the ESEM analysis.

Conflicts of Interest: The authors declare no conflict of interest.

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