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ORIGINAL ARTICLE

Graphene effects on *Populus nigra*: assessment of sex-specific adaptive responses by in vitro culture

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Abstract

The application of graphene-related materials (GRMs) has increased considerably in various fields, posing a potential environmental risk. However, little is known about sex-related responses to GRMs in dioecious woody plants and in that regard, callus culture represents a reliable tool for toxicity and tolerance studies. In this work, the effects of different concentrations of graphene oxide (GO) and graphene nanoplatelets (GNP) on physiological traits of male and female clones of *Populus nigra* were investigated. After a 3-week treatment, at high concentrations, GO promoted in female calli, an increase in fresh weight and a reduction in protein content, accompanied by a remarkable enhancement of APX and CAT activity while no toxic effect was observed under GNP treatment. Instead, male cells displayed a greater sensitivity at lower GO concentration (25 mg/L), exhibiting a notable reduction in biomass, nutrient uptake and protein content, associated to an increase in APX and CAT activity. Similarly, at 25 mg/L, GNP caused a slight enhancement in lipid peroxidation (MDA) level and a significant decrease in protein content, accompanied by an increase in the production of flavonoids. These findings revealed sexually different responses to GO and GNP, with female clone exhibiting more tolerance compared to male one.

Key message

Populus nigra clones showed sex-specific physiological responses to graphene oxide and nanoplatelets *in vitro*. Females exhibited greater tolerance than males.

Keywords Callus culture · Dioecy · Graphene · Oxidative stress · *Populus* · Phytotoxicity

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Introduction

Since their discovery in 2004, graphene and its derivatives, collectively known as graphene-related materials (GRMs), have gained increasing attention due to their unique structure, large surface area and physicochemical properties, posing them for wide applications across several fields such as biomedicine (Singh et al. 2018), environmental protection as water treatment (Khaliha et al. 2022), engineering and nanoelectronics (Zhang et al. 2021a, b). GRMs include several materials such as graphene-oxide (GO), reduced graphene oxide (rGO), few-layer graphene (FLG) and graphene nanoplatelets (GNP), differing in oxidation level, lateral dimension, surface chemistry and layer number (Singh et al. 2018; Zhang et al. 2022). In recent years, increasing attention has been directed toward the potential applications of GRMs, particularly GO, in agriculture and forestry. Specifically, these materials can be used: as nanofertilizers to improve the controlled release of nutrients, thereby promoting plant growth and increasing crop yield; as antifungal and antibacterial agents, to improve stress tolerance; as nanosensors for plant monitoring and protection (Kamle et al. 2020; Abbas et al. 2022). However, the expanding use of GRMs in agro-forestry also raises important concerns regarding environmental safety and potential risks to human health.

While most toxicity research focuses on aquatic organisms, invertebrates, and cell lines, investigations into their effects on terrestrial plants are still underway (Malhotra et al. 2020; Xiao et al. 2022). As plants are primary producers, a comprehensive assessment of GRMs' phytotoxic effects is essential for their safe regulation and disposal, thereby mitigating environmental and human health risks (Wang et al. 2019; Yang et al. 2022). Hitherto, most studies on GRMs' biological effects have primarily focused on agricultural plants, reporting both stimulatory and inhibitory effects on plant growth and development, depending on concentration and duration of exposure, the specific experimental conditions and the plant species itself (Wang et al. 2019; Zhang et al. 2022). However, the knowledge about the impact of GRMs on woody plants remains largely unexplored. *Populus* species have become a model trees in woody plants studies due to their growth performance, small genome size, easy vegetative propagation and high biomass production (Iori et al. 2016). In addition, *Populus* species are dioecious with male and female reproductive organs on separate individuals. The sexual differences are not limited only to reproduction but also to their morphological, and physiological traits under different stress. Several studies have reported that male *Populus* plants tend to allocate more resources to vegetative growth and tolerance to environmental stress, such as salinity, cold, drought, nutrient deficiency and heavy metals, than female plants (Xu et al. 2008; Zhang et al. 2011; Liu et al. 2020). However, in other cases, no sex-related differences in adaptive strategies were detected (Juvany and Munné-Bosch 2015; Melnikova et al. 2017). Black poplar (*P. nigra* L.) is a common tree species in Europe, naturally growing in proximity to streams and rivers. This species is a valuable resource in breeding programs aimed at developing high-yielding hybrids for increased biomass production. Furthermore, previous studies have shown that two black poplar clones (the male clone Poli and the female clone 58–861) exhibit different adaptive responses to several abiotic stress (Regier et al. 2009; Iori et al. 2012a, 2023). However, little is known about the sex-specific responses to the long-term effects of GRMs. *In vitro* culture is considered an effective and efficient method to study stress responses in plants, especially in woody species characterised by long reproductive cycles (Confalonieri et al. 2003). In this context, in vitro screening can be used as a preliminary technique to provide useful information to improve the design for further field trials (Wijerathna-Yapa and Hiti-Bandaralage 2023). Hence, in this work, we employed callus culture of *Populus nigra* (clones Poli and 58–861) to investigate the effects of GO and GNP. Our aims were to determine if these GRMs inhibit poplar cell growth, induce oxidative stress in both genders, and to identify potential sex-specific differences in physiological responses. The availability of genetic linkage maps for these clones (Gaudet et al. 2008), enhances the utility of our findings in characterizing genetic determinants involved in adaptive mechanisms in dioecious species. Comprehending the sexual dimorphism in stress responses, at both the physiological and molecular levels, is crucial for informing breeding strategies but also for developing conservation and sustainable resource management strategies.

Materials and methods

Graphene-related materials

GO was purchased from LayerOne and used after an ultrapure water rinse to remove potential mobile metal residues (graphene oxide powder < 35 mesh, product code 1.8, XPS: O/C ratio 0.39 ± 0.01, C 70.1 ± 0.9%, O 27.2 ± 0.9%, N 0.2 ± 0.1%, S 1.0 ± 0.1%, Si 0.8 ± 0.1%, Cl 0.7 ± 0.1%, Mn below 0.1%) (Khaliha et al. 2022). GNP, produced from pyrolyzed waste tires by cycling and upcycling processes, was provided by NANOGRAFEN Co. (Gebze, Kocaeli, Turkey) and applied following an ultrapure water wash, as indicated for GO. The detailed characterization of the structural and morphological properties of GNP was reported in Okan et al. (2020).

Plant material and experimental setup

Callus cultures of *P. nigra*, the male clone Poli and the female clone 58–861, were obtained by sub-culturing undifferentiated cell clusters from leaf tissue as reported by Iori et al. (2023). The experimental treatment was conducted in callus culture conditions. Before autoclaving, GO or GNP stock solutions were sonicated for 2 h and then, added to Murashige and Skoog (MS) medium (Murashige and Skoog 1962) at concentrations of 0, 25, 50 and 100 mg/L. Seven Petri dishes containing four calli each were used for each clone and treatment. After three weeks of exposure, for fresh weight (FW) and dry weight (DW) determination, each callus was collected, washed briefly with sterilized distilled water, dried on filter paper, and finally weighted. For biochemical analysis each callus was frozen in liquid N_2 and stored in a freezer at −80 °C.

Morphological observation by optical microscopy

Poplar calli cells were studied by optical microscopy to examine whether GO and GNP caused changes in cell morphology. At the end of the experiment, cell cultures were fixed overnight in ethanol/glacial acetic acid (3/1, v/v), placed on slides and observed under a light microscope (Zeiss Axioskop equipped with Axiocam MRc5).

Malondialdehyde (MDA) content and antioxidant enzymatic activities

The level of lipid peroxidation was determined by measuring malondialdehyde (MDA) content as reported by Iori et al. (2023). To determine antioxidant enzyme activities, frozen samples were homogenized in a pre-chilled mortar and pestle with two volumes of an ice-cold extraction buffer (50 mM potassium phosphate buffer, pH 7.0) containing 0.1% (w/v) ascorbic acid, 1% (w/v) PVPP, 1 mM Na_2-EDTA and 0.1% (v/v) Triton X-100. After centrifugation (15,000 g, 30 min at 4 °C), the supernatant fraction was set aside for assays of ascorbate peroxidase (APX) and catalase (CAT) activities. The total soluble protein content was quantified as described by Bradford (1976), using bovine serum albumin (BSA) as a standard.

Activity of ascorbate peroxidase (APX, EC 1.11.1.11) was determined by measuring the oxidation rate of ascorbate ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$) at 290 nm for 1 min (Nakano and Asada 1981). Assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 10 mM H_2O_2 and enzyme extract in a total volume of 1 mL. APX activity was expressed as µmol of ascorbate oxidized mg^{-1}protein min^{-1}. Catalase (CAT, EC 1.11.1.6) activity was measured by following the consumption of hydrogen peroxide ($\varepsilon = 36\ mM^{-1}\ cm^{-1}$) at 240 nm for 40 s as described by Aebi (1984) with minor modifications. The reaction mixture was composed of 50 mM potassium phosphate buffer (pH 7.0), 125 mM H_2O_2 and enzyme extract in a total volume of 1 mL. CAT activity was expressed as µmol of H_2O_2 mg^{-1} protein min^{-1}.

Determination of total phenolic content (TPC) and flavonoids (FC)

About 1 g of fresh material was extracted in ethanol (80%, v/v) at a final concentration of 10 mg mL^{-1}. The suspension was shaken overnight in the dark and then centrifuged at 3000 g for 20 min at 4 °C. The supernatant was processed or stored at–20 °C.

Total phenolic content was determined quantitatively using Folin-Ciocalteu (FC) reagent, with gallic acid as the standard by following the protocol published in Souid et al. (2023). The TPC was expressed as mg of gallic acid equivalents (GAE)/g of sample fresh weight (FW).

For total flavonoids quantification the protocol of Heimler et al. (2006) was applied with quercetin as the standard. Flavonoid content was expressed as mg quercetin equivalents (QE)/g of sample fresh weight (FW).

Nutrients analysis

Calli were kept in an oven at 60 °C until constant weight was obtained. Then, the oven-dried material was weighed and mineralised. Mineralisation was performed by treating 250 mg of dried samples with 4 mL of concentrated HNO_3, 3 mL of distilled water and 2 mL of H_2O_2 (30% v/v in water), followed by heating (EXCEL Microwave Chemistry Workstation, Preekem Scientific Instruments Co., Ltd., Shanghai, China) in a four-step procedure: 100 °C for 1 min at 250 psi, 140 °C for 1 min at 350 psi, 170 °C for 1 min at 450 psi and 200 °C for 12 min at 550 psi. Samples were then filtered and analysed. Determination of nutrient content was performed using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, 5800 Agilent Technologies, USA- LOD = 0.02 mg/L).

Statistical analysis

All results were represented as mean of three replicates ± standard deviations (SD). All data were checked for normality before analyses of variance. Statistical analysis was performed using R software with two-way analysis of variance (ANOVA), followed by Duncan's test. All statistical tests were considered significant at $p < 0.05$.

Results

GO and GNP effects on poplar calli growth

The effects of GO and GNP on the growth of poplar clones were analysed by evaluating biomass production (Fig. 1). Long-term GO exposure had different impact on the growth of the two poplar clones, as reported in Fig. 1a. Specifically, with increasing GO concentrations, the female clone 58–861 showed a significant enhancement (up to 54%) in fresh weight compared to the control, whereas the male clone Poli exhibited a marked reduction (up to 43%) with respect to the control, suggesting a detrimental effect of GO on Poli clone's biomass production. Moreover, GNP exposure revealed a significant interaction between sex and treatment for biomass production (Fig. 1b). Female clone displayed a concentration-dependent increase in fresh weight, reaching its highest value at 100 mg/L compared to the untreated control. Conversely, male clone exhibited a variable response to GNP concentration, resulting significantly higher at 25 and 100 mg/L compared to the control.

Morphological observations

Microscopic analysis revealed time- and dose-dependent increases in cell dimensions for the female clone 58–861 exposed to GO (Fig. 2). By day 21, female cells at higher GO concentrations (100 mg/L) were significantly larger (around 200 µm or more) compared to the control (less than 100 µm) and other tested concentrations. In contrast, the male clone Poli displayed a weaker response to GO, with no clear difference in cell size between the 50 mg/L and 100 mg/L treatments after 21 days. Similar trends were observed for both sexes under GNP exposure after 21 days, where cell size generally increased with longer exposure times and higher GNP doses. However, the male clone exhibited distinct behavior at the 25 mg/L concentration for both GO and GNP. At the lower concentration, GO promoted a general decrease in male cell size (50–100 µm), while GNP did not significantly alter cell size compared to the control. Interestingly, GNP exposure at 25 mg/L induced a change in male cell shape, with cells appearing rounder than in the control (Supplementary Fig. S1).

Oxidative damage determination by lipid peroxidation

MDA measurements revealed significant effects of both sexes and GO treatment, along with a crucial interaction between them (Fig. 3a). After three weeks of exposure, both clones exhibited a significant decrease in MDA content as the GO concentration increased, resulting more pronounced at 100 mg/L, especially in male clone. Compared to the respective controls, MDA content decreased by 20.11%, 33.02%, 36.94% in the female clone 58–861 and by 9.7%, 14.45%, 44.46% in the male clone Poli at GO concentrations ranging from 25 to 100 mg/L. These data suggest that the GO treatment did not cause membrane damage. About GNP, no significant interaction between sex and treatment was found (Fig. 3b). A downward trend in MDA content was observed in female clone as the GNP concentration increased, with a significant reduction observed at 100 mg/L GNP compared to control. In contrast, male clone exhibited no detectable increase in MDA levels at any GNP concentration.

Protein content

As reported in Fig. 4a, after three weeks, male and female clones showed sex-specific differences in total protein content after GO exposure. With increasing GO concentration, female callus cultures exhibited a significant decline in total protein content, with a decrease of 23.2% and 65% at 50 and 100 mg/L GO, respectively, compared with the control.

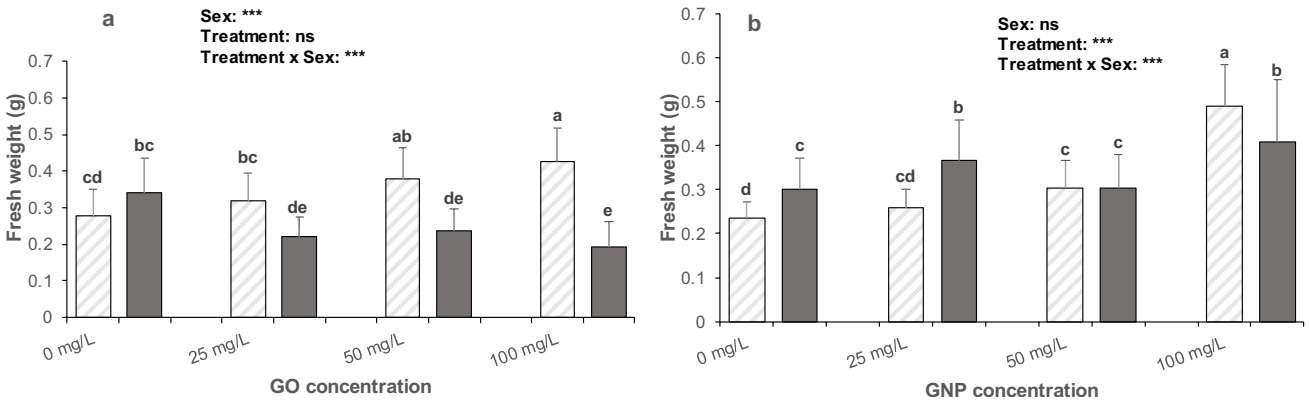

Fig. 1 Fresh biomass production in poplar calli (clone 58–861□; clone Poli■) exposed for three weeks to GO (**a**) and GNP (**b**) (±SD, n = 3). Different letters above bars indicate significant differences at $p < 0.05$ following Duncan's test

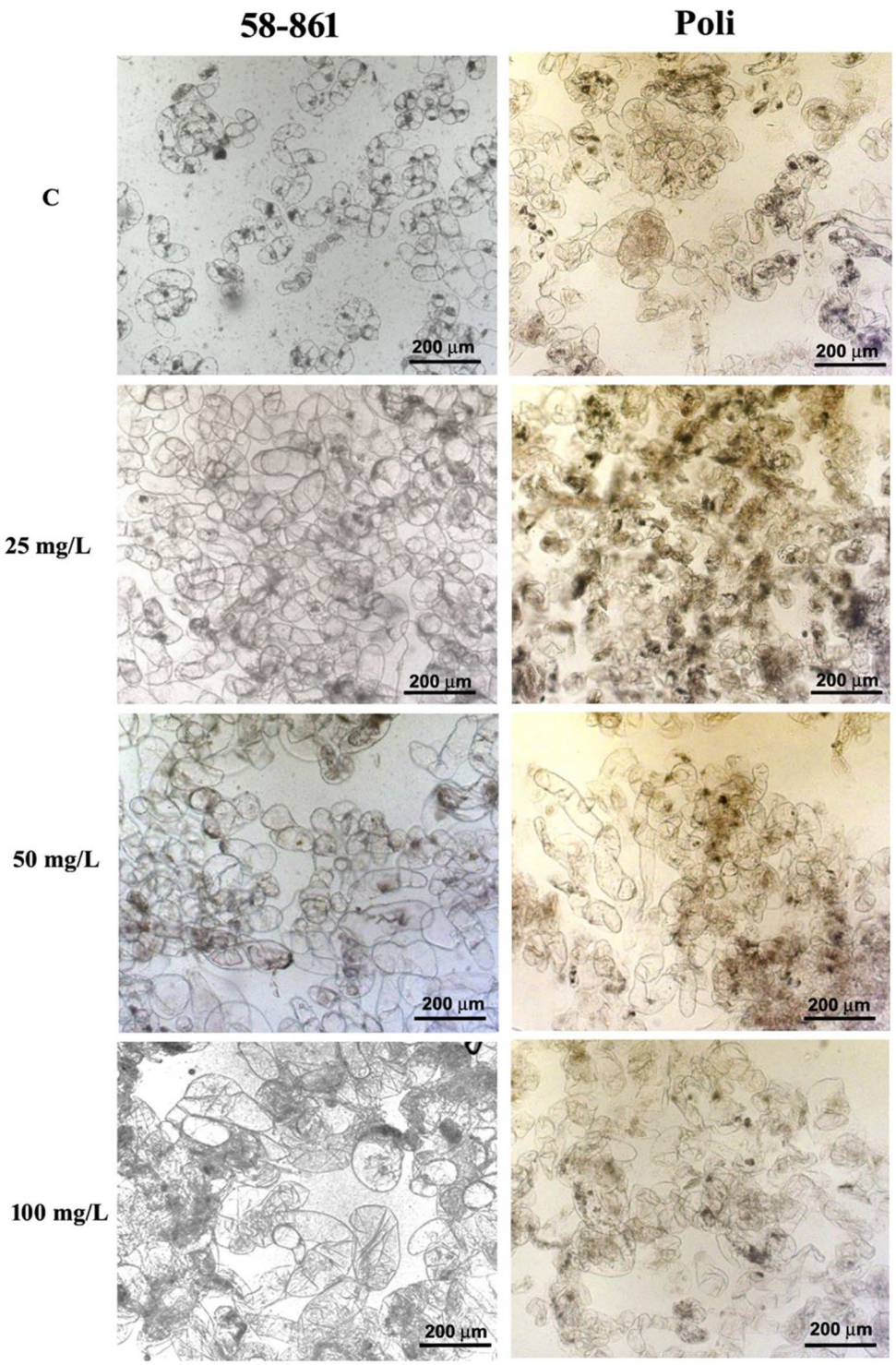

Fig. 2 Microscopic analysis of cell morphology in poplar clones, Poli and 58–861, exposed for three weeks to different GO concentrations

Conversely, total protein content decreased by 22.3% in the male clone Poli when exposed to the lowest GO concentration (25 mg/L), relative to the control. GNP treatment significantly affected total protein content in poplar calli (Fig. 4b). Callus cultures of 58–861 displayed a significant increase in protein content in a dose-dependent manner (up to 28%), with the most pronounced effect observed at 100 mg/L GNP compared to the control. Conversely, Poli cells exhibited a reduction in total protein content (14%) only at the lowest GNP concentration (25 mg/L). Notably, no significant effect was observed at higher concentrations (50 and 100 mg/L) compared to the control.

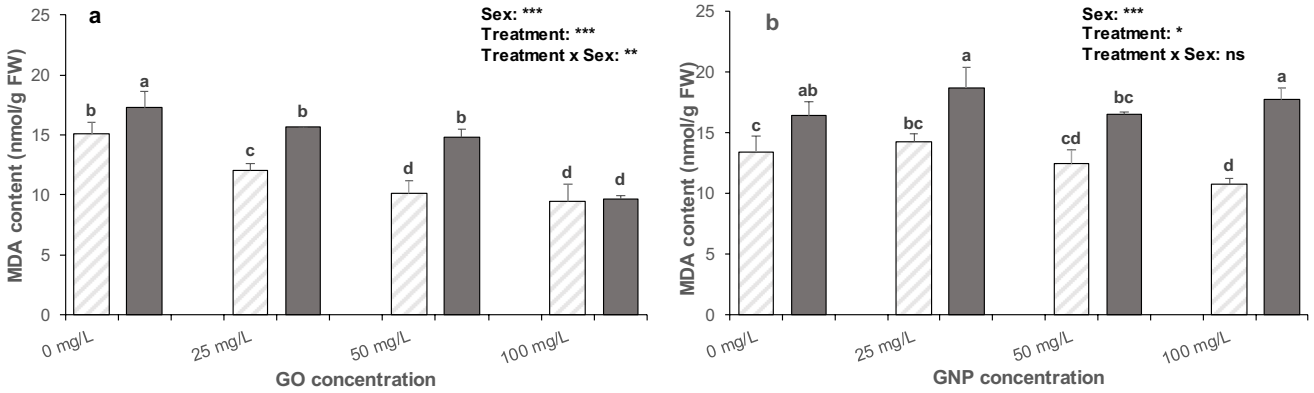

Fig. 3 Lipid peroxidation content (MDA) in poplar calli (clone 58–861 □; clone Poli ■) exposed for three weeks to GO (**a**) and GNP (**b**) (±SD, n = 3). Different letters above bars indicate significant differences at $p < 0.05$ following Duncan's test

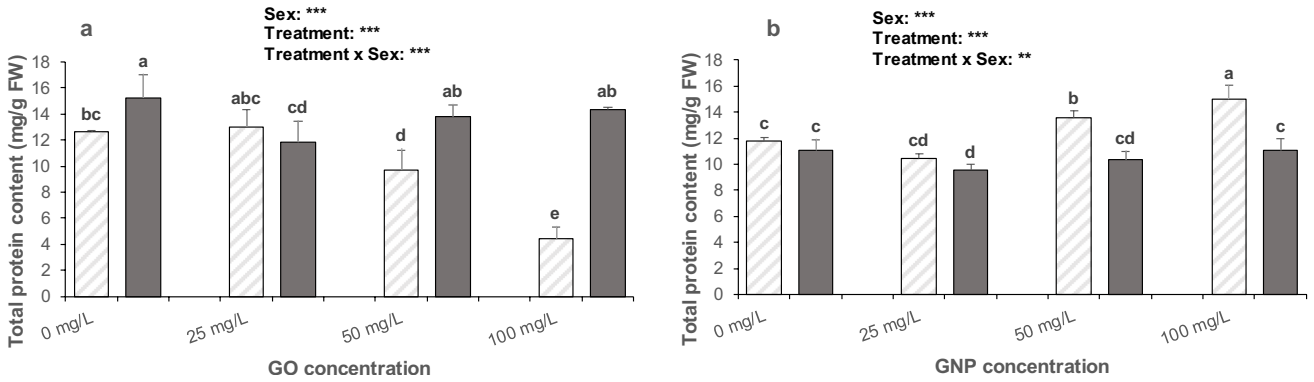

Fig. 4 Total protein content in poplar calli (clone 58–861 □; clone Poli ■) exposed for three weeks to GO (**a**) and GNP (**b**) (±SD, n = 3). Different letters above bars indicate significant differences at $p < 0.05$ following Duncan's test

Antioxidant enzymes activities

As reported in Fig. 5a, GO treatment significantly affected CAT activity, with the response varying between male and female *P. nigra*. In female clone, CAT activity showed no significant differences compared to control across all GO concentrations. In contrast, male clone displayed a significant increase in CAT activity at the lowest GO concentration (25 mg/L) while higher GO concentrations (50 and 100 mg/L) did not cause a further enhancement compared to the control. As shown in Fig. 5b, GNP treatment did not induce any significant effect on CAT activity, nor was there a significant interaction between clone and treatment. The male clone Poli consistently exhibited higher CAT activity in callus cultures compared to the opposite sex, irrespective of GNP treatment. Notably, GNP treatment significantly reduced CAT activity in the female clone 58–861, particularly at 100 mg/L compared to the control. In contrast, male clone exhibited no significant response to GNP treatment.

Exposure to GO significantly affected APX activity, with the response varying between males and females at different GO concentrations (Fig. 5c). Clone 58–861 displayed a significant increase in APX activity at 100 mg/L GO compared to the control, irrespective of treatment. Conversely, clone Poli exhibited an upregulation of APX activity at the lowest concentration (25 mg/L), mirroring the pattern observed for CAT, but followed by a significant decrease at the highest GO concentration, not statistically different from the control. Similar to GO treatment, GNP treatment significantly impacted APX activity, with a crucial interaction observed between clone and treatment (Fig. 5d). Interestingly, female clone exhibited no significant change in APX activity following GNP exposure. In contrast, male clone displayed a marked decrease in APX activity at higher GNP concentrations, particularly at 100 mg/L compared to the control.

Total phenolic and flavonoid contents

GO exposure affected total phenolic content (TPC) differently between male and female *P. nigra* (Fig. 6a). Overall, under control conditions, the female clone 58–861 exhibited higher TPC compared to the opposite gender. Particularly in female callus cultures, GO treatment caused a significant increase in TPC at 25 mg/L, followed by a marked decrease at higher concentrations (50 and 100 mg/L), compared to the control. In contrast, the male clone Poli displayed a

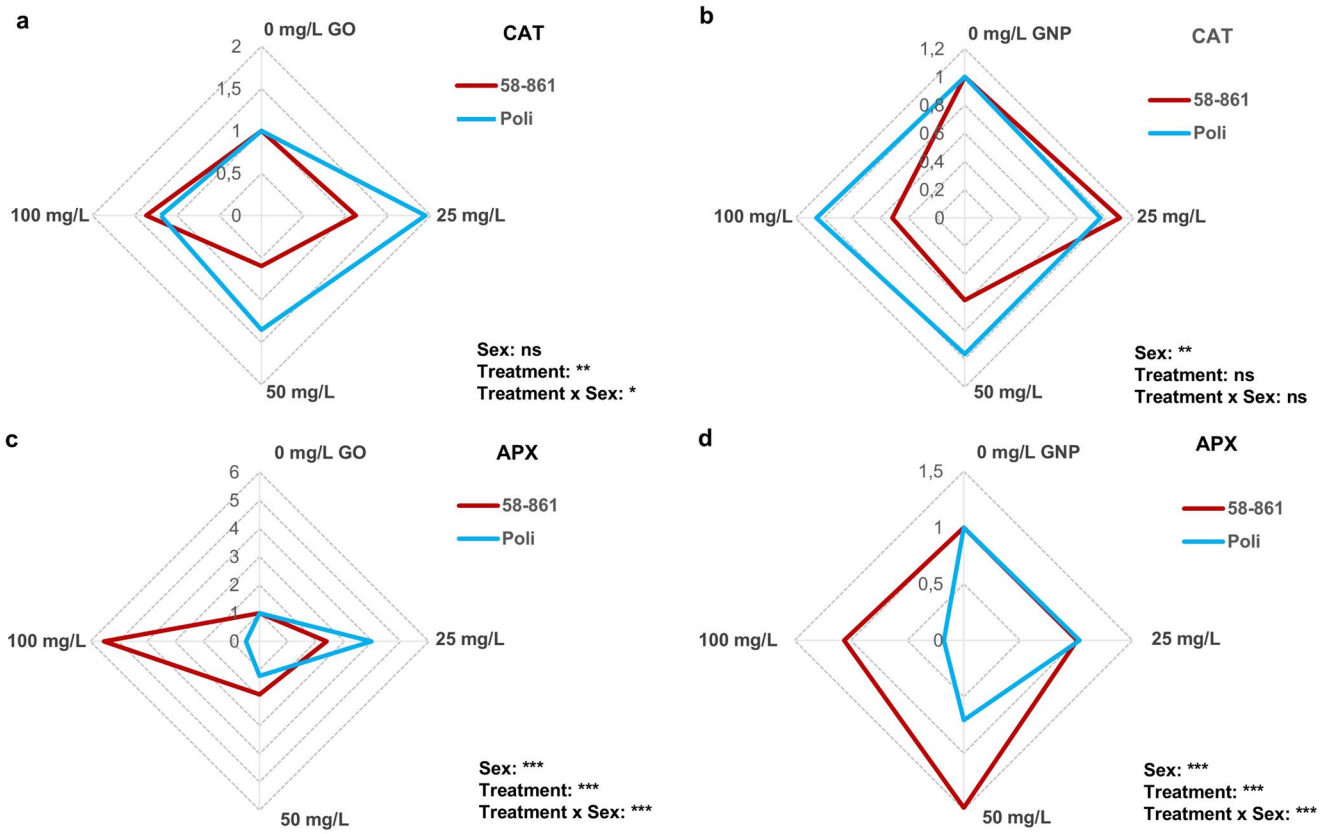

Fig. 5 Radar plot representation of catalase (CAT) and ascorbate peroxidase (APX) activities in poplar calli (clones 58–861 and Poli) exposed for three weeks to GO (**a–c**) and GNP (**b–d**). The data are the average of three biological replicates and report the values with respect to calli grown in the control condition (0 mg/L = control = 1). A detailed Duncan's test ($p < 0.05$) was done before normalization and it is provided in Supplementary Table S1 and S2, respectively

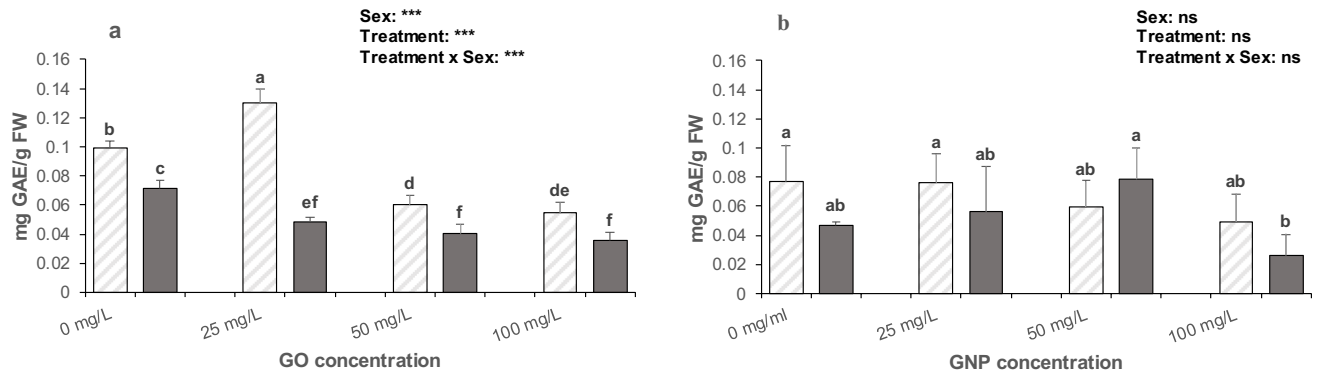

Fig. 6 Total phenolic content in poplar calli (clone 58–861 □; clone Poli ■) exposed for three weeks to GO (**a**) and GNP (**b**) (± SD, n = 3). Different letters above bars indicate significant differences at $p < 0.05$ following Duncan's test

significant decrease in TPC upon GO exposure compared to the control but no statistical differences among treated calli were observed. Unlike GO treatment, GNP exposure had no effect on total phenolic content in both genders (Fig. 6b).

Consistent with the findings for total phenolic content, female clone exhibited higher flavonoid content compared to the opposite sex in control conditions (Fig. 7). Specifically, GO exposure reduced flavonoid content in clone 58–861 callus cultures at 50 and 100 mg/L, with no significant effect observed at 25 mg/L compared to the control. In contrast to female clone, GO exposure caused a slight increase in flavonoid content in male clone compared to the control. However, no significant differences in content were observed among GO-treated Poli calli (Fig. 7a).

Under GNP exposure, calli of the two poplar clones revealed a significant difference in the content of flavonoids

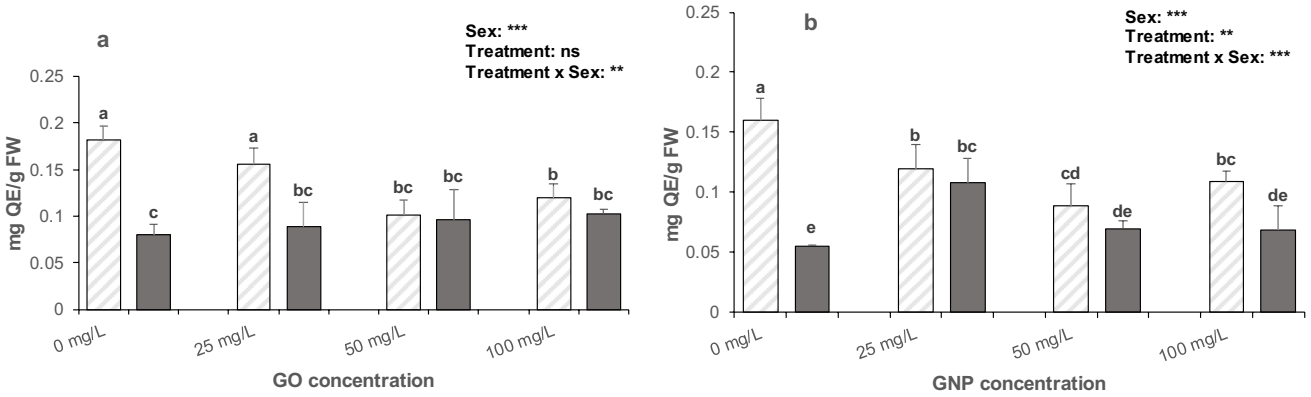

Fig. 7 Total flavonoid content in poplar calli (clone 58–861 □; clone Poli ■) exposed for three weeks to GO (a) and GNP (b) (±SD, n = 3). Different letters above bars indicate significant differences at $p < 0.05$ following Duncan's test

as shown in Fig. 7b. GNP treatment caused a significant decrease in flavonoid content in female cells, with the effect most pronounced at 50 mg/L compared to the control. Moreover, the male clone Poli exhibited a significant increase in flavonoid content only at the lowest GNP concentration (25 mg/L) compared to the control while no significant differences were observed at higher concentrations.

Effects of GO on nutrient uptake

According to the data obtained on biomass production, nutrient content in calli exposed to GO was quantified. Pearson's correlation coefficient analysis revealed significant negative and positive correlations between uptake of micro- and macronutrients and exposure to different GO concentrations, particularly in the male clone Poli (SupplementaryTable S3). Specifically, a marked reduction in the uptake of B, Ca, Cu, Fe, K, Na and S was detected as the GO level increased. Interestingly, in the female clone 58–861, GO treatment had no significant impact on the mineral content (Supplementary Table S4).

Discussion

The development of stress tolerance is a complex process driven by adaptive changes in genetic, biochemical, and morpho-physiological traits. This process often differs between male and female dioecious plants, reflecting sexual dimorphism in abiotic stress tolerance. It's generally hypothesized that these sex-related differences under adverse conditions, arise from differing adaptive strategies. Females typically invest more resources in preserving their reproductive organs, while males tend to maintain their vegetative growth (Juvany and Munnè-Bosch, 2015). Understanding the complex interplay between abiotic stress and sexual differences in tolerance is crucial for the conservation and management of dioecious plant species.

This work revealed significant sexual dimorphism in the physiological and biochemical responses to GO and GNP in *P. nigra* males and females. Although the predicted environmental concentration of GRMs may be in the range of ng/L or µg/L (Zhao et al. 2015), in this study higher GO and GNP concentrations as well as long-term exposure were chosen in order to elicit a detectable response in plant cells and to evaluate the potential GRMs environmental risk.

For phytotoxicity assessment, plant growth is frequently employed as a physiological parameter. Hitherto, most of the published literature on GRMs' impact on plants, has reported a range of responses, including stimulation, inhibition or no observed effects on plant morphology and physiology. This variability is likely due to several factors, such as the selected plant species, the GRMs' physicochemical properties, the experimental conditions and the duration of exposure (Yang et al. 2022; Zhang et al. 2022). Therefore, this high variability hinders comparisons between toxicity tests and prevents a definitive assessment of GRMs'overall impact on plants (Wang et al. 2019). In the current study, the male clone Poli and the female clone of *P. nigra* exhibited a divergent sensitivity to GO after 21 days of exposure. Specifically, clone 58–861 showed a significant dose-dependent enhancement in fresh weight, whereas all tested GO concentrations inhibited growth in clone Poli. Our results suggested that under GO exposure, female clone tend to allocate more resources to growth compared to the male clone. Consistent with observations in clone 58–861, an increase in biomass production was detected in mature tomato plants after one month of exposure to 50–100 mg/L GO (Guo et al. 2021). Similarly, Ghorbanpour et al. (2018) observed enhanced cell growth rate in *Plantago major* L. exposed for 49 days to 100 and 200 µg/mL GO. Likewise, Zhang et al. (2021a, b) reported an increase in *Aloe vera* L. growth following long-term GO exposure (0–100 mg/L). Conversely,

several studies reported decreased biomass production upon GO exposure, consistent with findings for clone Poli. For instance, Shen et al. (2019) observed this effect in five rice species at 50 mg/L, and Xiao et al. (2022) found similar results in *Brassica napus* L. treated with 1000 mg/L GO for 15 days. Furthermore, Begum et al. (2011) observed a negative effect on various vegetables (cabbage, tomato, lettuce, red spinach) exposed to graphene (500–2000 mg/L) for 20 days.

The observed dose-dependent increase in biomass of clone 58–861 following GO treatment might be associated with its larger cell size. This enlargement at higher GO doses could potentially be due to changes in protein content, osmotic regulators, and membrane permeability, leading to enhanced water uptake and subsequent cell expansion. While the exact mechanism by which nanomaterials, like GO, affect plant growth remains unclear, their physicochemical properties (size, surface chemistry and shape) are believed to play a key role (Ghorbanpour et al. 2018; Wang et al. 2019). As reported by Zhao et al. (2023), GO may adhere to the cell wall through the covalently bonded groups or even enter plant cells, affecting metabolic processes and leading to either growth enhancement or inhibition. Notably, increased cell growth may be related to the activation of aquaporins, water channel proteins crucial for plant-water relations. Khodakovskaya et al. (2012) observed such an activation in tobacco cells exposed to carbon nanotubes, highlighting a potential mechanism for GO's effect.

Moreover, changes in plant growth and development are well-documented to be related to the uptake of macro- and micronutrients essential for various metabolic pathways (Zhao et al. 2022). In our study, male clone exhibited a decline in K, B, Ca, Cu, Fe, Na and S content with the increasing GO concentration, suggesting an imbalance of nutrient homeostasis within callus cells, potentially contributing to the observed growth inhibition. A reduction in the levels of K, Fe and B was also observed in white clover (*Trifolium repens* L.) (Zhao et al. 2022) and alfalfa (*Medicago sativa* L.) (Zhao et al. 2023) exposed to different GO concentrations for 50 and 100 days.

About GNP, both sexes showed an upward trend in the biomass production with increasing concentration in the culture medium. Interestingly, in the male clone Poli treated with 25 mg/L GNP, cell size remained similar to the control, yet a decrease in protein content was observed alongside an enhancement in biomass production, suggesting a higher proliferation rate of callus, rather than cell expansion driven by osmotic processes, consistent with the findings by Khodakovskaya et al. (2012). These results highlighted that different nanomaterials can elicit distinct responses within the same clone. Specifically, in contrast to observations with GO, the male clone maintained its growth capacity similarly to the female clone.

Previous investigations have pointed out that graphene nanomaterials can induce the production of reactive oxygen species (ROS) in plant cells. This oxidative stress can damage cellular components like lipids, DNA and proteins, ultimately leading to decreased biomass production (Zhang et al. 2016; Zhao et al. 2023). However, ROS play a complex role in plants. At low concentration, they act as signaling molecules, regulating several physiological processes such as growth, development and stress responses (Hasanuzzaman et al. 2020; Huang et al. 2019). Therefore, a delicate balance between ROS production and the scavenging mechanisms determines whether ROS act as damaging agents or signaling molecules.

Under oxidative stress, ROS generation can cause cell membrane damage through lipid peroxidation, affecting membrane fluidity and permeability. Malondialdehyde (MDA) is a major lipid peroxidation product, and its measurement has been used as a biomarker of oxidative stress (Iori et al. 2023; Zhao et al. 2022). Our findings showed that in both sexes, GO treatment induced a decrease in MDA level in a dose-dependent manner, resulting more evident at 100 mg/L, especially in male clone. On the contrary, following GNP exposure, in female clone a significant reduction in MDA content was observed at 100 mg/L whereas in the male clone Poli no lipid peroxidation was induced. Our findings align with Xiao et al. (2022) who reported decreased MDA levels in *Brassica napus* L. exposed to reduced GO for 15 days. Instead, no significant effect on MDA content was observed in Arabidopsis plants exposed to 10–1000 mg/L GO for 32 days (Zhao et al. 2015), in *Brassica napus* L. treated with 5–100 mg/L GO for 15 days (Cheng et al. 2016), and in five rice genotypes grown for 16 days in the presence of 5–150 mg/L GO (He et al. 2021). Therefore, our findings suggested that changes in fresh weight of both sexes could not be ascribed to lipid peroxidation and that GO and GNP did not cause severe H_2O_2 production.

It is well established that, under abiotic stress, ROS initially target proteins and induce modifications, directly or indirectly, increasing the susceptibility of proteins to proteolysis and, consequently, reducing protein content (Choudhary et al. 2020). In the present study, in the male clone Poli, both GO and GNP induced a significant reduction in protein level only at 25 mg/L, whereas in the female clone 58–861 total protein content markedly decreased mostly at the higher GO concentration (100 mg/L) and, on the contrary, increased at the corresponding GNP dose. In general, a decline in protein content in dose-dependent manner was also detected in Changbai larch (*Larix olgensis* A. Henry) (Song et al. 2020) and alfalfa (*Medicago sativa* L.) (Zhao et al. 2023) exposed to GO, suggesting that a key mechanism

involved in toxic effect of this nanomaterial could be protein oxidation. Taking into account that in both sexes no lipid peroxidation was observed, it is conceivable that under GO and GNP treatment, synthesis of stress-related proteins, such as osmotic regulators, was not affected and poplar cells maintained osmoregulation ability, preserving membranes (Jan et al. 2022; Zhao et al. 2023).

To maintain cellular redox homeostasis and physiological activities during oxidative stress, plant cells activate a defence mechanism involving several antioxidative enzymes (e.g., CAT and APX) and metabolites (e.g. phenols, flavonoids and anthocyanins) (Ren et al. 2020). However, as highlighted by Hasanuzzaman et al. (2020), antioxidative defence approaches differs among plant species and genotypes, as well as stress types and duration. Among antioxidant enzymes, CAT is crucial for maintaining the redox balance during oxidative stress while APX plays an essential role in the control of intracellular amount of H_2O_2 (Nadarajah 2020). In this study, calli of the two poplar clones revealed differences in CAT and APX activities. In female clone, GO exposure did not affect antioxidant activity of CAT whereas at 100 mg/L it improved APX activity, suggesting that APX is more active in ensuring protection against oxidative stress, promoting cell growth. In male clone, both CAT and APX was up-regulated at the lowest GO concentration (25 mg/L). Similar results on the induction of APX as well as CAT activity in the male clone Poli has been previously detected upon exposure to cadmium (Iori et al. 2012a), ibuprofen (Iori et al. 2012b) and AgNPs-Cit-L-Cys (Iori et al. 2023) and it could be ascribed to the phenomenon of hormesis, where low doses of a stressor can elicit a stimulatory response (Zhang et al. 2016). In that regard, in the current study, it is conceivable that in clone Poli, GO could stimulate adaptive responses at the lowest dose (25 mg/L), at which a marked reduction in total protein content occurred, highlighting a very sensitive callus culture response to counteract GO effects.

About GNP, in the female clone 58–861 the specific activity of CAT showed a significant decline mostly at 100 mg/L whereas APX activity was unaffected by GNP exposure. In male clone, GNP treatment caused a significant reduction in APX activity at 100 mg/L whereas it did not induce any effect on CAT activity. The decrease in CAT activity detected in clone 58–861 at the highest GNP concentration is consistent with studies by Ghorbanpour et al. (2018) and Xiao et al. (2022) in which the specific activity of CAT in *Plantago major* L. and *Brassica napus* L., respectively, was down-regulated at high GO dose.

Beyond antioxidant enzymes, the ROS-scavenging system includes secondary metabolites, such as phenols and flavonoids. These metabolites are crucial for regulating plant growth, development, and defence by maintaining ROS homeostasis (Berni et al. 2019; Daryanavard et al. 2023). In the female clone 58–861, GO exposure caused a rise in phenolic content at the lowest concentration (25 mg/L), coinciding with increased APX activity. However, at higher concentrations, both total phenolic and flavonoid contents decreased, while APX and CAT were up-regulated. In the male clone Poli, a decrease in phenolic level was observed in the presence of GO whereas flavonoids were not affected. In the presence of GNP, no effect on total phenolic content was observed in both sexes whereas differences in distribution of flavonoids were detected. In particular, GNP treatment induced a significant decrease in total flavonoid content in female clone, which corresponded CAT inhibition and enhancement in total protein content, whereas in male clone it caused a marked enhancement only at 25 mg/L consistent with a reduction in total protein level and induction of hormetic dose response.

A reduction in secondary metabolites at higher doses of GRMs was observed also by Samadi et al. (2023) in *Thymus daenensis* celak. seedlings exposed to various concentrations of single-walled carbon nanotubes (SWCNTs). Such a trend in total phenolic and flavonoid contents was likewise detected by Dutta Gupta et al. (2018) in rice seedlings exposed to AgNPs, suggesting a mild or non-toxic impact of nanomaterials.

Conclusion

The comparative analysis of female and male clones of *P. nigra* revealed sexual differences in responses to GO and GNP, highlighting their differential sensitivities. In particular, long-term exposure to high GO concentrations positively affected health status of the female clone 58–861, as evidenced by enhanced biomass production as well as activity of antioxidant enzymes (APX and CAT) and lower MDA content, indicative of considerable tolerance. In contrast, the male clone Poli showed a greater sensitivity to the lowest GO concentration (25 mg/L), as highlighted by reduced cell growth and nutrient uptake, alongside a decrease in MDA content and an enhancement in APX and CAT activity. The absence of detectable lipid peroxidation suggests that GO, in both genders, may act as a mild stress signal, able to alter ROS levels, without reaching cytotoxicity thresholds, and to exert some detrimental effects on protein content, counteracted by activity of antioxidant defence enzymes. About GNP, our findings suggested a greater tolerance in female clone, as evidenced by increased fresh weight and protein content at higher GNP concentrations. As under GO treatment, male clone, displayed a noticeable sensivity to the lowest GNP concentration (25 mg/L) consistent with a slight enhancement in MDA levels, a significant reduction

in protein content, and a rise in the production of flavonoids, secondary metabolites with antioxidative properties, known to scavenge and repair ROS-induced damage. It is worth noting that these results contrast with most literature, which generally report greater male tolerance compared to females. Since genetic map for this plant species is available, the large variability observed between male and female clones of *P. nigra* could be further exploited with a genetic approach to identify key genes or mechanisms involved in the adaptive response in dioecious species.

However, it is worth noting that these results should be interpreted carefully, as this study was conducted under *in vitro* conditions using callus cultures. Further field experiments are necessary to validate these findings in whole plants.

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Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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