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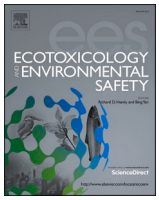

Research paper

Short-term physiological and biometrical responses of *Lepidium sativum* seedlings exposed to PET-made microplastics and acid rain

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ABSTRACT

Plastics enter in terrestrial natural system primarily by agricultural purposes, while acid rain is the result of anthropogenic activities. The synergistic effects of microplastics and acid rain on plant growth are not known. In this study, different sizes of polyethylene terephthalate (PET) and acid rain are tested on *Lepidium sativum*, in two separate experimental sets. In the first one we treated plants only with PET, in the second one we used PET and acid rain together. In both experimentations we analyzed: *i)* plant biometrical parameters (shoot height, leaf number, percentage inhibition of seed germination, fresh biomass), and *ii)* oxidative stress responses (hydrogen peroxide; ascorbic acid and glutathione). Results carried out from our experiments highlighted that different sizes of polyethylene terephthalate are able to affect plant growth and physiological responses, with or without acid rain supplied during acute toxicity (6 days).

Short description: This study showed that different sizes of PET microplastics affect physiological and biometrical responses of *Lepidum sativum* seedlings, with or without acid rain; roots and leaves responded differently.

1. Introduction

Thompson et al. (2009) defined our era as the "Plastic Age". Doubtless, plastic represents an essential component of our private and professional life. It was estimated that, in 2018, Europe produced 62 million tons of plastic and that 359 million tons were manufactured worldwide (Statista, 2018). The consequence of this large-scale production is that a large part of goods made of plastic is thrown away in waste dump and/or in the natural environment (Geyer et al., 2017).

Plastics released into the environment are generally classified, according to their size, in mega- (>100 mm), macro- (100–20 mm), meso- (20–5 mm), micro- (5000–0101 μm), and nanoplastics (<100 nm) following the classification criteria proposed by the literature (Barnes et al., 2009; Koelmans et al., 2015; Mattsson et al., 2015; Horton et al., 2017). Microplastics (MPs) are further divided according to the origin in primary and secondary. Primary MPs are particles produced deliberately in such dimensions by way of abrasive in cosmetic or industrial products (Chang, 2015; Napper et al., 2015). Nevertheless, the largest part of plastic present in the environment is of secondary origin, that is, originated from the fragmentation of larger plastic debris (Duis and Coors, 2016).

In terrestrial environment microplastics can enter as a consequence of agricultural practices such as the application of sewage sludge to fertilize the soils or by the fragmentation of other agrarian instruments. In fact, plastic made materials intended for agriculture, such as the mulching film used to control the temperature and weed growth, are exposed to destructive conditions such as soil tillage, sunlight, and high temperatures (Horton et al., 2017; Ng et al., 2018). Once released in soils, microplastic is not an immobile component: movements throughout the soil layers can be caused by ploughing and harvesting (Paustian et al., 1997), but also by other factors such as bio-pores created by soil biota. Soil cracking, in fact, represents the main responsible for the downward movements of microplastics (Majdalani et al., 2008; Rillig et al., 2017b). Moreover, the physical properties of microplastics such as shape, size, and hydrophobicity contribute to amplify their transport inside the soil (Wan and Wilson, 1994; Rillig

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et al., 2017a). Also sequestering processes like soil aggregation may influence the spatial microplastics distribution (Peng et al., 2017).

It is not attended that plants are able to uptake microplastic of larger size due to the physical barrier represented by root's cell wall that avoids penetration (Teuten et al., 2009). Nevertheless, both translocation of small particles and leaching of toxicants from larger ones could affect plants. Moreover, water-soluble additives and adsorbed chemicals can be leached out from plastics (Bejgarn et al., 2015), migrate into soils and be taken up by seedling roots (Zhang et al., 2017). Oxidative stress is one of the first signals indicative of phytotoxicity in plants. It involves the production of reactive oxygen species (ROS), such as the hydrogen peroxide, superoxide anion, and hydroxyl radical (Choudury et al., 2013), the accumulation of which can trigger the production of antioxidant molecules such as glutathione (GSH) or ascorbic acid (AsA). Nevertheless, if the ROS accumulation exceeds the antioxidant defenses, an impairment of plant growth, photosynthesis, and biochemical processes can also occur (Choudury et al., 2013).

Acid rains are considered together with global warming and ozone depletion as the biggest environmental disaster for the functioning of ecological systems (Xu et al., 2015). The formation of acid rain is mainly caused by anthropogenic emissions coming from fuel combustion, and traffic represents the principal source of fuel combustion emission in urban environments. The main compounds able to induce acid rains are sulphur dioxide (SO_2) and nitrogen oxides (NO_x) which react with the water molecules in the atmosphere to form acids (Shu et al., 2019). Rainfalls are defined as acid when their pH value is less than 5.6. It's well-known that acid rains affect seriously plants and environment around them (Debnath et al., 2018). At plant level, they are able to cause injuries in foliar tissue, induce the production of reactive oxygen species and reduce the growth and biomass production. At soil level, an increase in acidification affects nutrient availability for the plant's growth (Shu et al., 2019). If, on one side, scientific literature documented the harmful biological effects due to exposure to acid rain, to the best of our knowledge, any research focuses on the combined effect of microplastic particle-size and acid rains.

The polymer "PET" is a thermoplastic, so defined because when it is heated its chemical composition is not modified by the reaction, feature that makes it suitable for being recycled. For this reason, PET represents one of the major contributors to the total plastic amount on the planet (Gwada et al., 2019). Three different sizes of particles were tested, covering the dimensional range from small-sized to large-sized (5–3000 μm) microplastics, in order to evaluate if dimension can be considered a driving factor in PET toxicity on plants. In addition to the presence of microplastic, an acid rain scenario was created to evaluate the occurrence of synergic/antagonistic effects on plant toxicity. To evaluate plant response to plastic and acid rain, the vascular plant *Lepidium sativum* (garden cress) were chosen as model species. This is a fast-growing annual herbaceous plant species belonging to the Brassicaceae family, widespread worldwide (Nehdi et al., 2012). Its high sensitivity to phytotoxic substances makes it suitable for ecotoxicological assessments (Janecka and Fijalkowski, 2008; Adamcová et al., 2015; Sforzini et al., 2016; Smolinska and Leszczynska, 2017; Schiavo et al., 2018). Morphological and physiological responses taken into account were: i) biometric parameters (inhibition of seed germination, height of shoots, number of leaves, and fresh biomass production), and ii) compounds related to the occurrence of oxidative stress (hydrogen peroxide, glutathione, and ascorbic acid) in shoots and roots separately.

The aim of this work is to determine morphological and physiological effects recorded in shoots and roots of *Lepidium sativum* following the exposure to polyethylene terephthalate (PET) microplastics.

2. Materials and methods

2.1. Growth condition, experimental setup, and biometrical traits

Certified seeds of *Lepidium sativum* were obtained from ECOTOX$^{(R)}$ LDS; for acute toxicity we used Phytotoxkit® from MicroBioTest Inc slightly modified, following the conditions previously used (Pignattelli et al., 2020). In brief, transparent test plates (21 × 15.5 × 0.8 cm) composed of a bottom part separated by a middle ridge into two compartments, and a flat cover. Both parts have small rectangular cavities on their side for closing the plates tightly by a unique click system. One plate for each different size of microplastics tested, containing 5 seeds and 90 mL of unpolluted soil, without filter that separate plants from soil, was used. Before to sow the seeds, the capacity field of soil was tested and then the soil was soaked with 55 mL of Milli-Q water or simulated acid rain. A simulated acid rain of pH 4.5 was prepared adding H_2SO_4 and HNO_3, following the recipe proposed by Liu et al. (2019). The plants were grown in a climatic chamber under controlled environmental conditions (temperature range: 17–20 °C; relative air humidity range: 40–60%; photosynthetic photon flux density of 700 μmol m^{-2} s^{-1} for 14 h per day from 06:00–20:00 local time).

PET micrometric flakes, with jagged edges and surface irregularity, obtained by double trituration of 1 mm industrial pellets have been administered in three particle-sizes classes: small (5–60 μm; **G1**), medium (61–499 μm; **G2**), and large (500–3000 μm; **G3**). All the details on PET-microplastic production and characterization are available on Piccardo et al. (2020). As previously stated, the experimental setting included two different plant treatments: plants exposed only to PET (indicated as **PET-**), and the other was composed by plants exposed to PET and acid rain together (indicated as **PET+**). Two kinds of control treatments were used: control plants watered with Milli-Q water (**C-**), and control plants watered with acid rain (**C+**). For each treatment we used 0.02% (w/w microplastic/soil) content of microplastic, a concentration 5 time less than levels used by Rychter et al. (2010), corresponding to 0.092 g of PET in tested soils. Soil used for the experiment was collected in unpolluted natural site, its physical charatceristcs were as follows: pH value, measured in H_2O, 7.5, and conducibility 0.5 ds/m. Levels of total microplastic were determined before starting the experiments on six soil replicates following extraction and analytical methods reported by literature for microplastic in sediments (Renzi et al., 2020). Soil was considered acceptable to perform experiments if microplastic levels resulted under detection limits.

Biometrical measurements were performed after six days from the beginning of the experiment on 15 plants for treatment (5 plants x 3 replicates). In detail, the height of the shoot (measured using a precision caliper), the number of leaves and the germination rate of seeds were determined. The percentage of inhibition of seed germination was carried out following the formula: ((GsC–GsT)/GsC)*100; where GsC is seeds that germinated in the control group, and GsT is seeds that germinated in the treated group. The number of germinated seeds was calculated as an average of germination among the experimental replicates tested. Plants' biomass was measured at the end of the experiment by weighing fresh overall plant, with four decimal places scale.

2.2. Hydrogen peroxide, and antioxidants determinations

Before starting extraction, survived plants for treatment (root and shoot separately) were grinded with liquid nitrogen and then stored at −20 °C. In this research, levels of hydrogen peroxide (H_2O_2) was determined by spectrophotometry (390 nm) after reaction with potassium iodide and the development of trichloroacetic acid (TCA) comparing to a standard curve of known H_2O_2 levels as proposed by Alexieva et al. (2001). Concentrations of ascorbic acid (AsA) were measured based on the reduction of Fe^{3+} to Fe^{2+} by ascorbate in acidic solution at 525 nm comparing to a standard curve of AsA and following Okamura (1980) modified by Law et al. (1983) methods. Glutathione (GSH) was determined at 412 nm comparing samples, previously extracted in TCA and reacted with Ellman's reagent, with a standard curve of GSH (Sedlak and Lindsay, 1968).

All the obtained results were expressed as μg*g^{-1} f.w. All spectrophotometric analyses were performed by UV/Vis spectrophotometry

(ONDA, mod. UV-30 Scan).

2.3. Statistical analysis

Descriptive statistics (means, standard errors) were performed for all measured parameters using SigmaPlot 12.5 (SPSS Inc., Chicago, IL) scientific data analysis and graphing software. Analysis of Variance, Two-way ANOVA, was applied to test the different PET sizes effects on *Lepidium sativum* plants. A Fisher-LSD post-hoc test was applied to assess significant differences among treatments ($p < 0.05$ level). Multivariate statistics were performed by Primer v7.0 (Primer-E Ltd., Plymouth Marine Laboratory, UK) on Euclidean matrices of distance calculated on standardized (square root) and normalized biometrical and biochemical responses to evaluate the significance of observed segregations according to the factors of the treatment (two levels, fixed; - MilliQ, + acid rain), and PET grain-sizes (three levels, fixed; G1, G2, G3). Plants' tissues were indicated as L (leaves) and Ro (roots) following the abbreviation of the biochemical response measured (as for example AsA Ro = ascorbic acid levels in roots).

3. Results

3.1. Effects on plant growth

Biometrical traits of L. *sativum* treated with PET (-) and PET supplied with acid rain (+) are shown in Table 1. The inhibition percentage of seed germination (I%), is the only biometrical traits, that showed statistically significant interactions between treatments ($p < 0.001$), acid rain ($p < 0.001$), and treatments x acid rain ($p < 0.001$). Different particle-sizes of PET differently affected the germination rates, although all treatments had negative impacts (except in control plants and G3+). Interestingly, plants treated only with PET are most affected than plants treated also with acid rain, infact the largest size of PET (i.e. G3-) severely affected the germination, followed by G1- treated plants, while the G2- treated plants reported low consequences. Conversely, between the plants treated with microplastics and acid rain together, G2+, followed to G1+, were those most negatively affected. The shoot height showed significant interaction among acid rain ($p = 0.003$) and treatments x acid rain ($p < 0.001$), it was consistent with the results found for germination, as well as for the leaves number that resulted in the same significant interaction but with different p values: $p = 0.032$ and $p = 0.005$ detected for acid rain and treatments x acid rain respectively. The biomass produced, albeit not statistically significant, is resulted in agreement with the other biometrical traits detected.

3.2. Reactive oxygen species and antioxidants

Plants treated with PET (-) and PET supplied with acid rain (+) show a significant production of hydrogen peroxide (H_2O_2), glutathione (GSH) and ascorbic acid (AsA) at foliar level compared to controls (Fig. 1A, C, and E). C+, G3- treated plants followed by G1- produced the highest concentration of H_2O_2. Finally, the concentration of this compound has shown statistically significant interactions between treatments x acid rain ($p = 0.012$), and treatments ($p < 0.001$). The higher GSH production is recorded for acid rain treated plants if compared with those untreated, particularly for C+ and G3+ treated plants; such as for H_2O_2, for PET treated plants, higher concentration is shown by the G3- plants. Furthermore this latter antioxidant recorded significant interaction for each factors considered: treatments ($p = 0.034$), acid rain ($p = 0.002$) and treatments x acid rain ($p = 0.013$). The AsA concentration was significantly higher in C+, followed to G1- and G2- treated plants, it has shown significant interaction only for treatments x acid rain ($p = 0.011$).

At root level, both oxidant than antioxidants concentrations are statistically significant (Fig. 1B, D, F). Overall, H_2O_2 concentration is higher in roots than is shoots; in this case, higher concentrations are found in G3- and G1+ treated plants; the only statistically significant interaction is treatments x acid rain ($p = 0.009$). On the contrary, at root level lower antioxidant concentrations than at shoot levels are always detected. Higher GSH production is recorded for G1+ followed to G1- and G3- treated plants; furthermore, it showed all the interactions statistically significant with the same value ($p < 0.001$). AsA concentration has resulted in higher concentration for C+ and G3- exposed plants, while C-, G1+ and G2+ reported values near to zero. Globally, ascorbic acid was significant for the following interactions: treatments ($p = 0.004$) and treatments x acid rain ($p < 0.001$).

3.3. Overview on multivariate statistics

The first three axes of Principal Component Analyses performed on biometrical data (inhibition of germination %, biomass, leaves height, and number of leaves) explained 99.6% of the total variance (64.8, 25.3, 9.5% respectively). Eigenvectors related to the two axes represented in Fig. 2(A) showed a positive correlation to inhibition of seeds germination (0.486) and negative correlation with height of shoots and number of leaves (respectively −0.568 and −0.556) for PC1. On the contrary, the second axe (PC2) was strongly positively related to biomass (0.719) and negatively related to number of leaves (−0.421). ANOSIM test two-ways performed on factors treatment versus particle sizes highlighted significant responses for both of them (significant level of sample statistic of 0.01%). The first three axes of Principal Component Analyses performed on biochemical data (H_2O_2, GSH, and AsA in both leaves and roots)

Table 1
Biometrical parameters obtained in *Lepidium sativum* plants treated with PET(-) and PET additioned with acid rain (+). Percentage inhibition of germination (I%), shoots height (H), leaf number (#L), and total biomass (B) exposed to different PET sizes are reported as mean values ±standard error (SE; n=10). Two-way ANOVA was applied to determine significant differences between treatments (G1=6–60 μm; G2=61–499 μm; G3=500–3000 μm) and controls (C). p-level is given; *= $p < 0.05$; **=$p < 0.01$; ***; $p < 0.001$; ns=not significant.

Treatements	I (%)		H (cm)		#L		B (g)	
	mean	se	mean	se	mean	se	mean	se
C-	0.00	<0.001(e)	0.38	±0.051(b)	1.8	±0.20(b)	0.051	0.013
G1-	44.44	<0.001(b)	0.17	±0.058(c)	1.0	±0.33(d)	0.028	0.002
G2-	11.11	<0.001(d)	0.35	±0.062(b)	1.6	±0.26(c)	0.029	0.005
G3-	66.66	<0.001(a)	0.08	±0.041(d)	0.6	±0.30(d)	0.015	0.002
C+	0.00	<0.001(e)	0.40	±0.045(a)	1.8	±0.20(b)	0.041	0.010
G1+	11.11	<0.001(d)	0.34	±0.061(b)	1.6	±0.26(c)	0.030	0.004
G2+	33.33	±0.001(c)	0.26	±0.074(c)	1.2	±0.32(c)	0.042	0.004
G3+	0.00	<0.001(e)	0.44	±0.017(a)	2.0	±0.00(a)	0.048	0.005
Treatments	***		n.s.		n.s.		n.s.	
Acid rain	***		**		*		n.s.	
Treat x AR	***		***		**		n.s.	

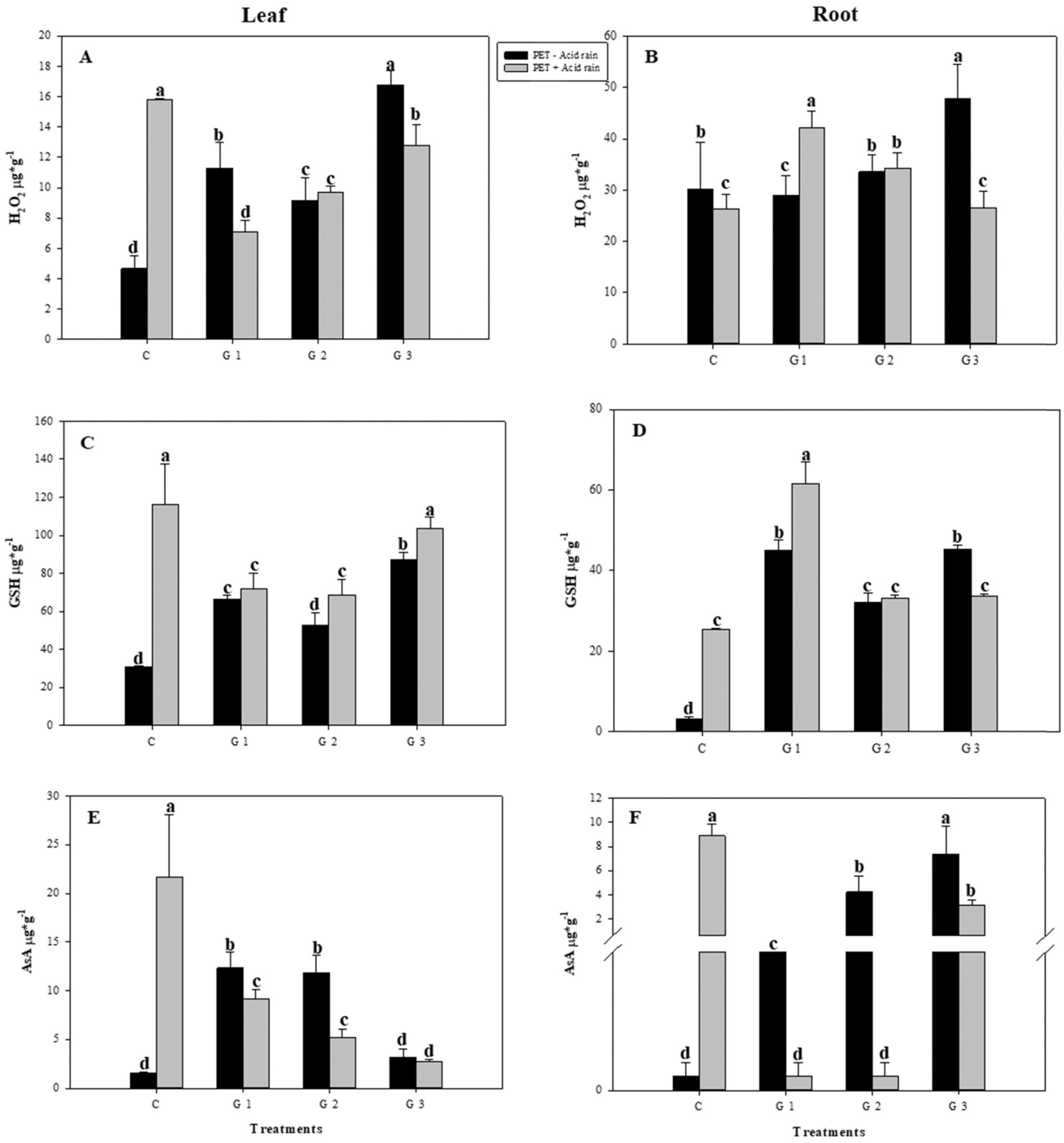

Fig. 1. Biochemical responses to PET (-) and PET plus acid rain (+) in *L. sativum* leaves and roots. Measured concentrations of hydrogen peroxide (H_2O_2), ascorbic acid (AsA) and glutathione (GSH). Two-way ANOVA was applied to determine significant differences between treatments (G1 = 6–60 µm; G2 = 61–499 µm; G3 = 500–3000 µm) and controls (C). Data are expressed as mean ±standard error (SE, n=3). Different letters represent statistical differences between treatments for each tested chemical (Fisher-LSD multiple comparison, $p < 0.01$ level).

explained 84.5% of the total variance (45.3, 23.2, 16.0% respectively). Eigenvectors related to the two axes represented in Fig. 2(b) showed a negative correlation to H_2O_2, GSH in leaves and AsA in roots (respectively −0.533, −0.529, −0.461) for PC1. On the contrary, the second axe (PC2) was strongly positively related to H_2O_2, GSH in roots (respectively 0.733, and 0.565). ANOSIM test two-ways performed on factors treatment versus particle sizes highlighted significant responses for both of them (significant level of sample statistic respectively of 0.01% and 1.20%). A significant difference in biomarkers expression in roots and leaves was highlighted following PET exposure.

4. Discussion

Different sizes of polyethylene terephthalate (PET) are able to differently affect plant's biometric traits, either with the addition or not of simulated acid rain. After 6 days of exposure, the plants most affected

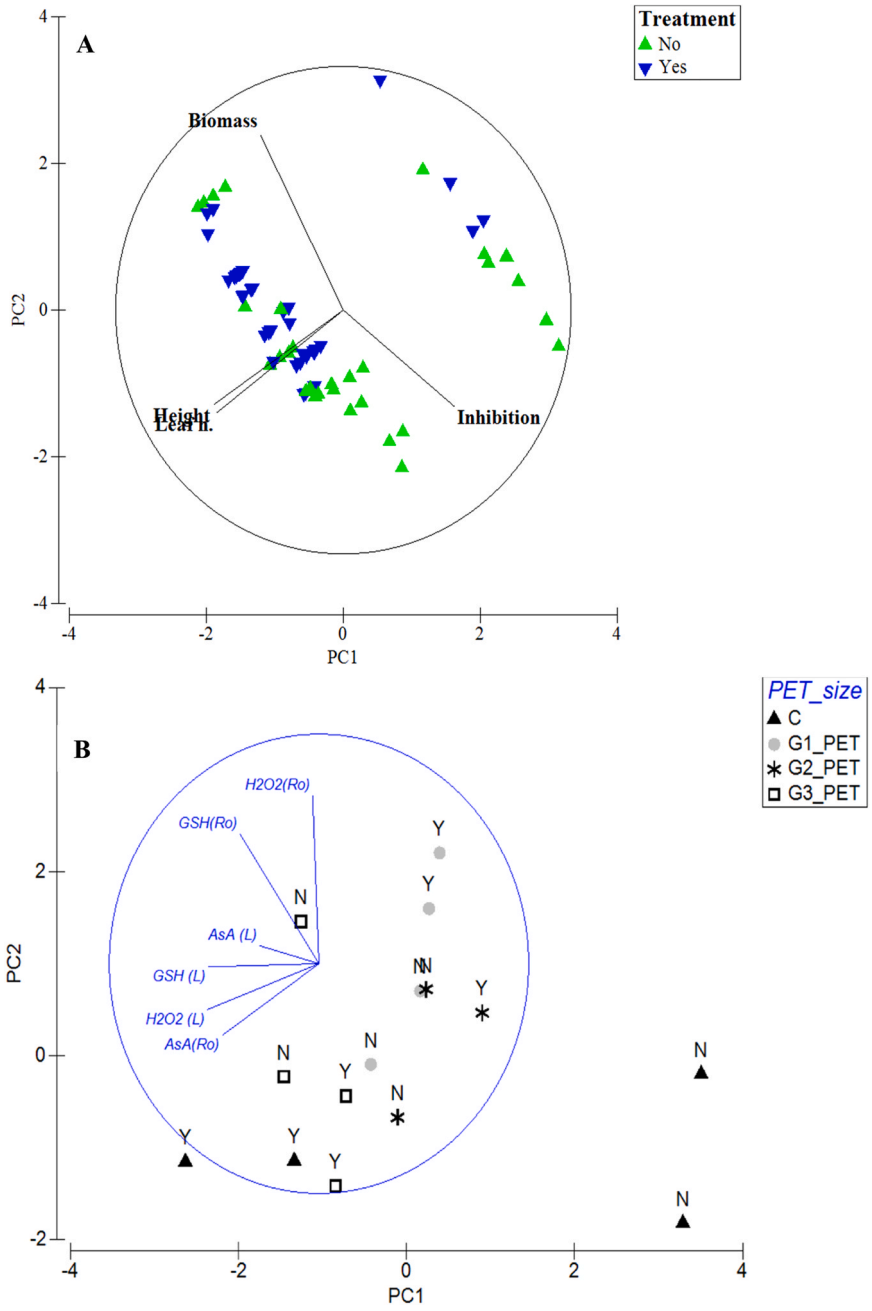

Fig. 2. Principal component analyses performed on biometrical and biochemical data**.** Fig. 2(A) represents PCA performed on biometrical data of plants exposed to different grain-size of PET under natural and acid rains conditions. Fig. 2(B) represents PCA performed on biochemical data of plants exposed to different grain-size of PET under natural and acid rains conditions. Experimental controls are also reported. Notes: L= leaves; Ro=roots.

by PET (-) treatments were those exposed to the bigger size of PET (i. e., G3), followed by G1 and G2. Plants exposed to G1 an G2 showed comparable biomass production, although growth values were almost 2-fold higher in plants exposed to G2 than G1. Plants treated with plastics and acid rain together (PET+) showed that G2 mostly affected each trait considered, except for biomass that showed almost the same value of the control plants. Comparing experiments, plants treated with PET (+) responded in different manner. In fact, overall PET (+) plants showed higher values than those supplied only with PET. From a biometrical point of view, it seemed that in the short term, PET toxicity was perceived earlier respect plants treated with PET and acid rain (PET+).

Multivariate analyses supported that factors tested (treatment: - versus +; particle size of PET G1, G2, G3) produced significant effects on biometric and biochemical variables measured in L. *sativum*. The field of plant-microplastic interaction is still quite unexplored, even more so the acute toxicity caused by microplastics and by microplastics associated with acid rain. Only one research took into consideration the microplastics toxicity from a purely agronomic point of view and considered the effects in terms of yield or biomass production in medium-long exposure. A previous study performed on *Triticum sativum*, found that starch-based biodegradable plastic is able to negatively affect the biomass production and the number of emitted leaves more than low density polyethylene (LDPE), polymers that are both commonly used in agriculture as mulching film (Qi et al., 2018). Other research carried out on horse bean and corn (Tao et al., 2012; Zhang et al., 2015) treated with LDPE powder reported that this plastic improves soil fertility. Research performed on two varieties of cotton, instead, pointed out that both yield and biomass productions at boll, and boll opening levels decreased when plastic concentration increased (Dong et al., 2015). Moreover, experimentation conducted on turnip, radish, cress, and

monocotyledonous oat using the poly-(butylene adipate-co-terephthalate), a plastic mulching film known as PBAT, highlighted no effect on the growth of tested plants indicating an absence of plastic toxicity (Rychter et al., 2010; Muroi et al., 2016). As mentioned above, there is nothing, in literature, about acid rain and microplastics interaction. At biometrical level there are discordant results on the effect of acid rain, maybe due to different pH values supplied to the plant. Debnath et al. (2018), working on tomato, found that acid rain negatively affects plant growth parameters; on the other hand, researches carried out on *Elaeocarpus gravipetalus* and *Jatropha curcas* highlighted a positive effect on biometrical traits (Liu et al., 2019; Shu et al., 2019). Results obtained by this research are mostly in agreement with those found by Liu and Shu et al. (2019, 2019). Growth parameters measured in plants exposed to acid rain treatments, in fact, underline higher values than plants treated only with microplastics; this could be explained by the nitrogen added through acid rain application that could have improved the performance of plants (Liu et al., 2019).

Because of their sessile lifestyle, plants cannot escape from environmental stressors. For this reason, plants developed a series of metabolic mechanisms to counteract stresses (Isah, 2019). The reactive oxygen species in general, and hydrogen peroxide (H_2O_2) in particular, play a key role in the signal transduction cascade, that in turn trigger the signal molecules involved in the stress factors sensing. The oxygen (O_2) is the final electron acceptor during respiration, and ROS production can begin from O_2 consumed by plant during this process. The H_2O_2 production, in plants, at low concentration acts as a signal molecule, while at high concentrations the redox homeostasis is unbalanced and antioxidant system cannot act as scavenging system, so programmed cell death occurs (Quan et al., 2008). Our results, at leaf level, show a different H_2O_2 production related both to the different sizes of PET and acid rain supplied alone or together respectively. In the treatment that involved only microplastics, G3 treated plants resulted most affected, indicating that plastic size, could have a strong influence on phytotoxicity. In the double treatment, instead, toxicity is more strongly related to acid rain rather than to PET supplied: in fact, control plants (C+) showed not only a higher H_2O_2 production than other treatments, but also a higher concentration than control plants grown on MilliQ water (C-). These results are in agreement with data obtained on tomato plants (Debnath et al., 2018) and *Arabidopsis* (Qiao et al., 2018), but are in disagreement with those found on rice (Ren et al., 2018) in which no difference in H_2O_2 content between control and treated plants were recorded. This could be explained by the different sensitivity of plants to the stress factor even when supplied in the same intensity. Not considering the C+ results, PETs size is related to H_2O_2 production: an increase in H_2O_2 production followed an increase in particle dimensions. Roots of both treatments showed higher H_2O_2 production than leaves. In addition, results carried out from root plants treated with PET only, are coherent with those found on leaf exposed to PET and acid rain: in fact, plants most affected were those treated with G3. Conversely, roots of double treated plants showed an interesting different H_2O_2 production: control plants were unaffected from acid rain, while a decreasing trend is showed from smaller to bigger size of PET indicating that smaller plastic size, mixed with an acid environment, has a strong toxic impact effect at root level.

In order to counteract oxidative burst, plants have developed an antioxidant system that works to avoid an excessive and harmful accumulation of reactive oxygen species (ROS) that are formed following environmental stresses (Liu et al., 2019). The antioxidants molecules, of non-enzymatic origin, mainly involved in ROS detoxification are glutathione and ascorbic acid for a dualistic reason: the peroxidases make these two molecules able to react quickly with H_2O_2; subsequently, the reductases regenerate their oxidized forms just as quickly (Noctor et al., 2018). Glutathione is a thiol-type, low molecular weight antioxidant, involved in the first line of defense against ROS (Liu et al., 2019), its function may be to reduce superoxide and also be used as an enzymatic substrate (Noctor et al., 2018). In the present study, in both treatments, GSH concentration at foliar level followed the same H_2O_2 trend. At root level we had almost the same H_2O_2 situation. Overall, at leaf level we have recorded higher GSH content than at root level; and double treated plants (acid rains + microplastics) showed always a higher glutathione concentration than plants treated only with PET. This latter feature can be explained by the fact that sulfate ion (SO_4^{2-}), supplied indirectly by acid rain, could be assimilated and used by plants to produce GSH (Qiao et al., 2018).

Ascorbic acid (AsA), in plants, is important for its involvement in ROS chemical removal and also because it works like cofactor for peroxidases (Noctor et al., 2018). Globally, at foliar level, plant treated with PET- have shown higher AsA values than double treated plants due to the particular contribution of G1 and G2. In addition, a size dependent response characterized by the decrease of acid production as the PET size increases, describes the AsA trend in PET and acid rain treated plants. At root level, PET treated plants showed an increasing trend of AsA from both to G3. On the whole, our results on acid ascorbic production show that its concentration is always lower than GSH, both at foliar than root levels. A possible explanation is that GSH is involved not only to counteract H_2O_2 but also in the ascorbate restoration from dehydroascorbate (DHA), both chemically and enzymatically (Noctor et al., 2018).

5. Conclusion

Our research highlighted, for the first time, that different size of microplastic of polyethylene terephthalate (PET) differently affect the growth and development of garden cress (*Lepidium sativum*), with or without acid rain supplied. Both in leaves and roots, PET (-) toxicity impacted plants almost in the same way concerning the production of ROS and antioxidant molecules. In PET (+) experimental set up (acid rain + microplastics), instead, plants responded differently: shoots were mostly affected by acid rain while roots are mostly affected by the size of PET.

6. Future research perspectives

Based on the data obtained from this work, the next step will be evaluate plants chronic toxicity on the same treatements provide here. Particularly will be useful assess if plants will be able to adapt in long exposure, and in which biometric parameters and metabolic activities will be damaged from this exposure. After that, will be interesting also evaluate this toxicity at field level.

CRediT authorship contribution statement

S.P.: Conceptualization, Laboratory activities, Laboratory analyses, Statistics. **A.B.:** Laboratory analyses, First draft of manuscript. **M.P.:** Revised first draft of the manuscript. **S.F.:** Microplastic used in experiments, Formatted ms for the submission. **A.T.:** Revised final draft of the manuscript. **M.R.:** Conceptualization of experiments, Laboratory activities management, Revisions to first and final drafts, Funds recruitment, and Project rendicontation.

Declaration of Competing Interest

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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