



# Effects of polyethylene terephthalate (PET) microplastics and acid rain on physiology and growth of *Lepidium sativum*<sup>☆</sup>

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## ABSTRACT

This study evaluated the chronic toxicity (30 days) of different sizes of polyethylene terephthalate (PET) microplastics (60–3000 µm) provided alone or in combination with acid rain, on garden cress (*Lepidium sativum*). Both biometrical and physiological traits have been evaluated: *i*) percentage inhibition of seed germination, plant height, leaf number and fresh biomass production; *ii*) oxidative stress responses (hydrogen peroxide; ascorbic acid and glutathione production); *iii*) impairment in photosynthetic machinery in term of pigments production; *iv*) aminolevulinic acid and proline production. Results highlighted that different sizes of PET, alone or in combination with acid rain, are able to negatively affect both biometrical and physiological plant traits. In particular, the lower size of microplastics is able to negatively affect growth and development, as well as to trigger the oxidative burst. Regarding the pigments production, PET coupled with acid rain, induced a higher production of Chl-b, and an inhibition of aminolevulinic acid.

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## 1. Introduction

Since the beginning of plastic large-scale production, about 70 years ago (Geyer et al., 2017), plastic has entered all anthropogenic activity, from work to daily life. Due to its useful features, such as waterproofness, insulation, flexibility, durability, lightness and low-cost production, it replaced the use of materials more environmentally sustainable, as well as wood or glass (Kouloumpis et al., 2020; Jiang et al., 2019). Among all types of plastics, polyethylene terephthalate (PET) is particularly used in food packaging for its peculiarities including heat, chemical and mechanical resistances (He et al., 2020). However, once in the environment, plastics can fragment into small plastic particles, known as microplastics (MPs). MPs are defined as plastic particles smaller than 5 mm (Klaine et al., 2012). Since when the problem of plastic pollution emerged, most of the attention has been paid on marine ecosystems, at the expense of terrestrial ones. This contradicts what has been pointed

out in other studies which evidenced that only 1% of MPs waste is released into marine ecosystems (Van Sebille et al., 2015). From this it can be inferred that the largest part of MPs is released in soil and freshwater (Li et al., 2020). At terrestrial level, the major supplier of microplastics is represented by the use of plastic mulching film and sewage sludge applied as fertilizer in agroecosystems. Sewage sludge, in fact, may carry huge number of MPs coming from cosmetic products or washing machine effluents. A recent study estimated that in European farms, about 43,000–63,000 tons of MPs are released every year (Lian et al., 2020; Nizzetto et al., 2016). Compared with aquatic ecosystems, terrestrial ones can potentially accumulate higher quantities of MPs because soil is not fluid, consequently, plastics are not able to move and may persist in soil over the time (Jiang et al., 2019).

Recent studies have already reported that the toxicological effects of MPs on soil organisms involve both growth and development inhibition in earthworm and wheat (Lwanga et al., 2017; Qi et al., 2018). Other research at plant level show that MPs are able to induce oxidative stress and genotoxicity on *L. sativum* and *V. faba*, respectively (Jiang et al., 2019; Pignattelli et al., 2020).

Acid rain is formed by the combination of sulphuric (H<sub>2</sub>SO<sub>4</sub>) and

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nitric acids (HNO<sub>3</sub>), both derived from sulphur dioxide (SO<sub>2</sub>) and nitrogen oxide (NO<sub>x</sub>), the major air pollutants emitted in the atmosphere from anthropogenic activities (Debnath et al., 2018). Normally, rainfall is slightly acid, but its pH value must be lower than 5.6 to be considered acid rain (Shu et al., 2019). It is well known that acidic precipitations are harmful for plants, in fact, they can damage the photosynthetic machinery, reduce the chlorophylls content and increase the production of reactive oxygen species; while at agroecosystem levels they are responsible for the crop yield losses (Debnath et al., 2018; Shu et al., 2019).

Like all sessile organisms, plants cannot escape from stressing factors, so they have evolved a complex structure to sense them and to defend themselves (Isah, 2019). The first line of defense consists in the production of trigger signal molecules, such as reactive oxygen species (ROS), which have the function, in turn, of activating the production of antioxidants substances. However, if the ratio ROS/antioxidant is unbalanced in favor of ROS production, an impairment of plant growth, photosynthesis, and biochemical processes can also occur (Choudury et al., 2013).

The species selected for the purpose of this study is the *Lepidium sativum*, also known as garden cress, an herbaceous species belonging to Brassicaceae family, widely used in many parts of the world as edible crop but also for phyto-therapeutic purposes (Smolinska and Leszczynska, 2017). Furthermore, its high fast-growing rate and sensitivity to toxic substances, make it suitable for phytotoxic experimentations (Smolinska, B., & Leszczynska J., 2017).

The present study is part of a bigger experimentation of which some results have already been published. In Pignattelli et al., 2020, different types of microplastics such as polypropylene (PP), polyethylene (PE), polyvinylchloride (PVC), and a commercial mixture (PE + PVC) have been tested in seeds of *Lepidium sativum*; in Pignattelli et al., (2021), three different sizes of PET microplastics have been tested on specimens of *Lepidium sativum* for six days. Based on the previous results, obtained from the short exposure of *Lepidium sativum* to different PET size provided alone or in combination with acid rain, the aim of this work is to investigate on the chronic toxicity (30 days), under controlled conditions. Two different sizes of plastics particles covering the range from 60 to 3000 µm were used. We evaluated at shoot level: i) biometric parameters (percentage inhibition of seed germination, plant height, leaf number and fresh biomass production), ii) oxidative stress (hydrogen peroxide, glutathione, ascorbic acid), iii) pigments production (Chlorophyll-a, -b and total carotenoids), iv) antioxidant production (aminolevulinic acid and proline). We speculate that different sizes of PET, alone or combined with acid rain, can interfere differently both at the growth and metabolic level.

## 2. Materials and method

### 2.1. Growth conditions, experimental set up and biometrical traits

Certified seeds of *Lepidium sativum* were obtained from ECOTOX LDS. A solution of acid rain, with a pH 4.5, was prepared as Liu et al. (2019), by using H<sub>2</sub>SO<sub>4</sub> and HNO<sub>3</sub>. The experimental conditions were those performed by Pignattelli et al. (2020). Plants were regularly monitored and watered twice a week with 8.5 mL of Milli-Q water, or acid rain solution, and finally sampled after 30 days of planting. Plants were grown in a climatic chamber under controlled environmental conditions (temperature ranging between 20 °C and 17 °C; relative air humidity ranging between 40% and 60%; photosynthetic photon flux density (PPFD) of 700 µmol m<sup>-2</sup> s<sup>-1</sup> for 14 h per day, from 06:00 to 20:00 local time).

PET micrometric flakes, with jagged edges and surface irregularity were obtained by double trituration of 1 mm industrial

pellets, the resulting gross-sized powder was sifted in order to obtain the desired particle-sizes: medium (61–499 µm; Ms), and large (500–1000 µm; Ls). A standardized methodology based on microscopy associated to Fourier Transform Infrared Spectroscopy technique (µFT-IR; Nicolet, iN10 MX; Thermo Fisher Scientific) was run for particle-size analysis and confirmed ranges theorized at the beginning of the experiment (all the details on PET-microplastic production and characterization are available on Piccardo et al., 2020). Two separate experimental set up have been prepared: the first one with plants exposed only to PET (indicated as PET-), and the second one composed by plants exposed to PET and acid rain (indicated as PET+). Two kind 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 times less than levels used by Rychter et al. (2010). Plant height measured by precision calliper, and leaf number were carried out once per week, from the start to the end of the experiment; while germination rate was measured after 6 days from the begin of the experiment; percentage inhibition of seed germination was carried out with following formula (ISO, 2016):

$$I\% = \frac{C_s - T_s}{C_s} \times 100$$

where C<sub>s</sub> are the germinated seeds of control group, and T<sub>s</sub> are the germinated seeds of each treatment.

In total 15 seeds per each treatment have been sown, the 10 specimens that germinated first were chosen, and the germinated seed numbers are obtained from the average of the 10 replicates used. The biomass was measured at the end of the experiment by weighing shoot fresh weight.

### 2.2. Hydrogen peroxide and antioxidants determination

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was measured spectrophotometrically after the reaction with potassium iodide (KI), according to the method proposed by Alexieva et al. (2001). The reaction was developed in trichloroacetic acid (TCA) and absorbance measured at 390 nm. The amount for H<sub>2</sub>O<sub>2</sub> was calculated using standard curve prepared with known concentrations of H<sub>2</sub>O<sub>2</sub>. The results were expressed as µg·g<sup>-1</sup> of fresh tissue weight (f. t. w.). Ascorbic acid (AsA) concentration was determined through the method proposed by Okamura (1980) and modified by Law et al. (1983). The assay was based on the reduction of Fe<sup>3+</sup> to Fe<sup>2+</sup> by ascorbate (As) in acidic solution. The absorbance at 525 nm was recorded. A standard curve of ascorbic acid (AsA) was used for calibration. Results were expressed as µg·g<sup>-1</sup> f.t.w. Glutathione (GSH) was determined using a modification of the Sedlak and Lindsay (1968) method. The determination was obtained through the extraction in TCA and by reaction with Ellman's reagent; the absorbance was read at 412 nm. A standard curve of GSH was used for calibration. The results were expressed as µg·g<sup>-1</sup> f.t.w. All spectrophotometric analyses were performed by UV/Vis spectrophotometry (ONDA, mod. UV-30 Scan).

### 2.3. Pigments determination

About 0.3 g of fresh leaf sample was homogenized with 6 mL of 80% acetone; then, sample mixture was centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatants were used to determine the chlorophylls and carotenoids content. Chlorophylls and carotenoids content were estimated by measuring the absorbance at 470, 645, and 663 nm. Thus, chlorophyll-a, chlorophyll-b, total chlorophylls and carotenoids were further calculated according to the formulae

described by literature (Bhushan et al., 2007). All spectrophotometric analyses were performed by UV/Vis spectrophotometry (ONDA, mod. UV-30 Scan).

## 2.4. Aminolevulinic acid and proline determination

The aminolevulinic acid (ALA) leaf content was measured according to Harel and Klein (1972). The determination was obtained through the extraction in TCA and by reaction with Ehrlich's reagent; the absorbance was read at 553 nm. A standard curve of ALA was used for calibration. The results were expressed as  $\mu\text{g}\cdot\text{g}^{-1}$  of fresh leaf weight (f.l.w.). Proline extraction and determination were performed according to Bates et al. (1973) with slight modifications. In brief, samples (0.2 g) were homogenized in liquid nitrogen and extracted with 70% ethanol (v/v). Extracts were held for 20 min at 95 °C, with 1 mL ninhydrin reagent [1% ninhydrin (w/v) in glacial acetic acid 60% (v/v), ethanol 20% (v/v)]. Proline content were measured by spectrophotometer at 520 nm; proline was used as external standard, and data were expressed in  $\mu\text{g}\cdot\text{g}^{-1}$  f.l.w. All spectrophotometric analyses were performed by UV/Vis spectrophotometry (ONDA, mod. UV-30 Scan).

## 2.5. 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 microplastics and acid rain effects on *Lepidium sativum* plants. A Holm-Sidak post-hoc test was applied to assess significantly 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 normalized biometrical and physiological responses to evaluate the significance of observed segregations according to the factors of the treatment (two levels, fixed; - MilliQ, + acid rain), and PET sizes (three levels, fixed; C, Ms, Ls).

## 3. Results

### 3.1. Effects on plant growth

Biometrical traits of *L. sativum* showed significant differences between treatments ( $p < 0.05$ ), (Table 1). The percentage inhibition of seed germination (I %) is the only parameter that showed statistically significant interactions between treatments ( $p < 0.001$ ), acid rain ( $p < 0.001$ ), and treatments x acid rain ( $p < 0.001$ ). Control plants (C-) did not show any inhibition of germination, while

control plants under acid rain (C+) reported mean value of 10%. The same percentage characterized plants exposed to MPs and Milli-Q water. The treatment that most negatively affected the plants was the medium sizes (Ms+) PET added with acid rain. The shoot height (H) showed significant interaction only for acid rain ( $p < 0.001$ ); noteworthy is that plants treated with PET and acid rain together have showed shoots height values higher than plant treated only with microplastics. Similar to shoot height, the leaf number (#L) reported significant interaction only for acid rain ( $p < 0.001$ ). Control plants in Milli-Q water (C-) exhibited number of leaves higher than all the others. Furthermore, plants untreated with acid rain showed higher values than those treated with Milli-Q water. Concerning the fresh biomass, statistically significant interactions for treatments ( $p = 0.025$ ) and acid rain ( $p = 0.009$ ) were recorded. Higher values are showed for C- and Ls-, while plants treated with PET and acid rain together reported values lower than plants treated with PET only.

### 3.2. Reactive oxygen species and antioxidants

The hydrogen peroxide showed a significant accumulation in all plants (Fig. 1A): C+ produced more  $\text{H}_2\text{O}_2$  than C-, plants treated only with medium size of PET (Ms-) are characterized by the highest concentration of this reactive oxygen species (ROS), whilst the potential toxic effect induced by acid rain and plastic is totally buffered in Ls + plants which reported values similar to C-. Finally, hydrogen peroxide concentration has shown statistically significant interactions between treatments x acid rain ( $p = 0.012$ ), and treatments ( $p < 0.001$ ).

The concentrations of the antioxidant compound ascorbic acid (AsA) were significantly higher in all treatments with acid rain compare with those in MilliQ water, especially in C+ and Ms+ (Fig. 1B). Furthermore, AsA has recorded statistically significant interactions between treatments x acid rain ( $p < 0.001$ ), treatments ( $p < 0.001$ ), and acid Rain ( $p < 0.001$ ).

Glutathione concentrations between C- and C+ had similar values, and only Ls- was able to increase the mean glutathione production. Plants exposed to Ms+ and Ls + reported values near to zero. Globally, glutathione was statistically significant for the following interactions: treatments ( $p = 0.002$ ), acid rain ( $p < 0.001$ ), and treatments x acid rain ( $p < 0.001$ ).

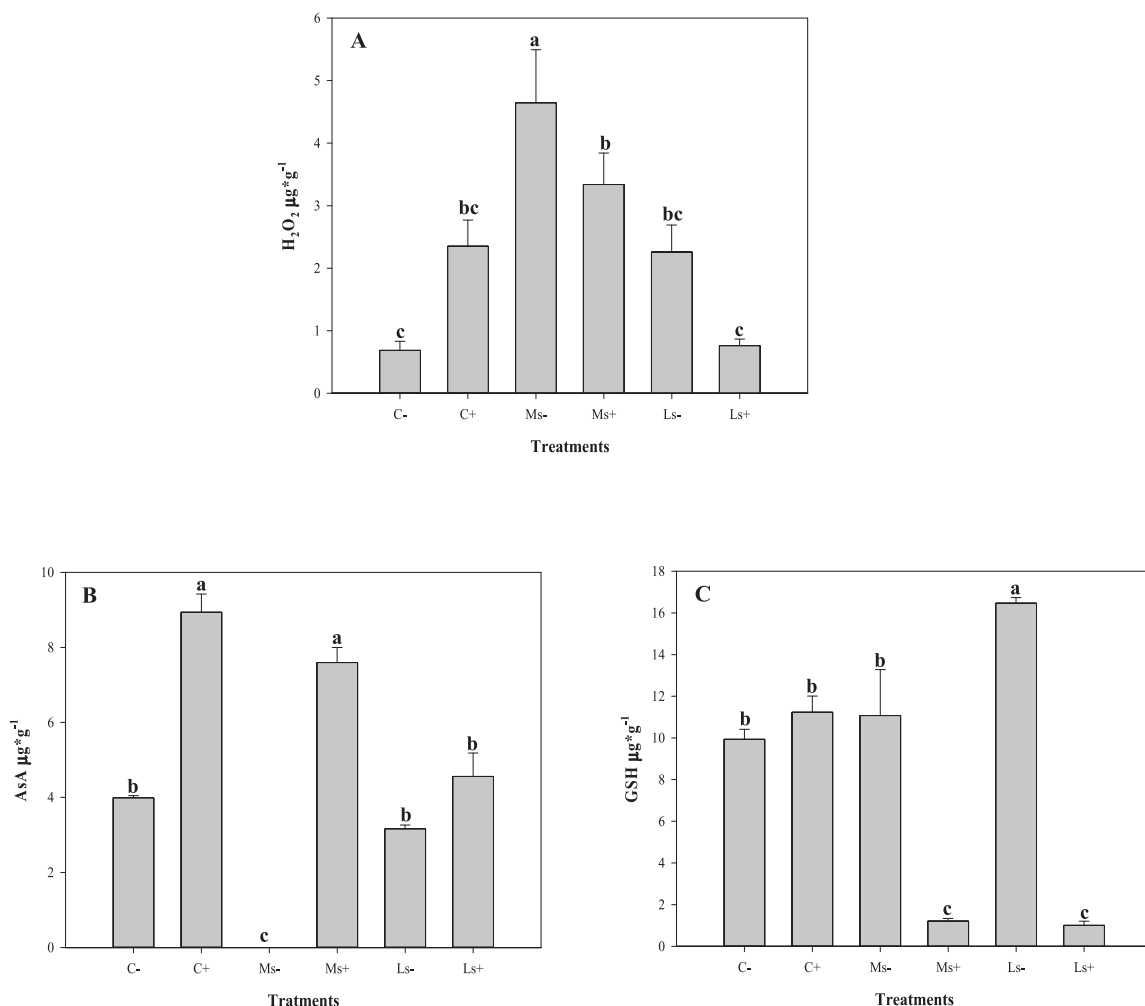
### 3.3. Pigments

Pigment concentrations are shown in Fig. 2A; each pigment has shown statistically significant differences. Regarding the chlorophyll-a (Chl-a), C+, and C- had similar values but the presence of plastic increased the production of the pigment: plants

**Table 1**

Biometrical parameters obtained in *Lepidium sativum* plants during the experiment (30 days). Percentage inhibition of seed germination (I%), shoots height (H, cm), leaf number (#L), and shoots biomass (B) exposed to different microplastic sizes (C = control, Ms = medium, Ls = large) are reported as mean values  $\pm$  standard error (SE;  $n = 10$ ). Two-way ANOVA was applied to determine significant differences between each treatment (p-level is given; \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ; ns = not significant). Different letters represent statistical differences between treatment for each chemical tested (Holm-Sidak method multiple comparison,  $p < 0.05$  level).

Treatments	I (%)		H (cm)		#L		B (g)	
	mean	se	mean	se	mean	se	mean	se
C-	0.0	<0.001 (c)	1.69	0.072 (b)	7.9	0.10 (a)	0.21	0.02 (a)
Ms-	9.9	0.100 (b)	1.34	0.175 (b)	6.3	0.71 (a)	0.12	0.02 (b)
Ls-	10.0	<0.001 (b)	1.37	0.163 (b)	7.0	0.78 (a)	0.21	0.05 (a)
C+	10.0	<0.001 (b)	2.46	0.369 (a)	4.9	0.56 (b)	0.07	0.01 (c)
Ms+	29.9	0.090 (a)	2.38	0.368 (a)	4.5	0.56 (b)	0.09	0.01 (c)
Ls+	10.0	<0.001 (b)	2.38	0.314 (a)	5.7	0.65 (b)	0.17	0.01 (b)
Treatments	***		n.s.		n.s.		*	
Acid Rain	***		***		***		**	
Treat x AR	***		n.s.		n.s.		n.s.	



**Fig. 1.** Biochemical responses to PET-induced stress obtained in *Lepidium sativum* plants exposed during the experiment (30 days). Measured levels of Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), ascorbic acid (AsA), and glutathione (GSH) in *L. sativum* leaves treated with different PET sizes (C = control, Ms = medium, Ls = large) are reported respectively in figure A, B, and C. Data are expressed as mean  $\pm$  standard error (SE,  $n = 3$ ). Two-way ANOVA was applied to determine significant differences between each treatment (p-level is given; \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ; ns = not significant). Different letters represent statistical differences between treatment for each chemical tested (Holm-Sidak method multiple comparison,  $p < 0.05$  level).

treated with Ms- and Ls- have shown values 1.5-fold and 2-fold higher than their counterparts treated with acid rain, respectively. Ms + treated plants did not differ from control, while the lowest concentration is showed by Ls + plants. This pigment has shown statistically significant interaction for each variation: treatments ( $p = 0.001$ ), acid rain ( $p < 0.001$ ), and treatments  $\times$  acid rain ( $p = 0.004$ ). The chlorophyll-b (Chl-b), conversely, showed highest values for the treatments that involved acid rain, particularly for Ms+ and C+. Plastic alone increased the values of Ms- and Ls- but no differences from C+ were reported by Ms+. As for Chl-a, Chl-b highlighted statistically significant interaction for the following variables: treatments ( $p < 0.001$ ), acid rain ( $p < 0.001$ ), and treatments  $\times$  acid rain ( $p < 0.001$ ).

Plants untreated with acid rain showed values of total carotenoids (Car) similar to Chl-b: in fact, Ms- and Ls-treated plants exhibited the highest values. On the contrary, plants treated with acid rain showed values from 2.0 to 2.5-folds lower than Chl-b. As for the other pigments, also for this latter has been recorded statistically significant interaction for each variable taken into account: treatments ( $p = 0.007$ ), acid rain ( $p = 0.003$ ), and treatments

$\times$  acid rain ( $p = 0.006$ ).

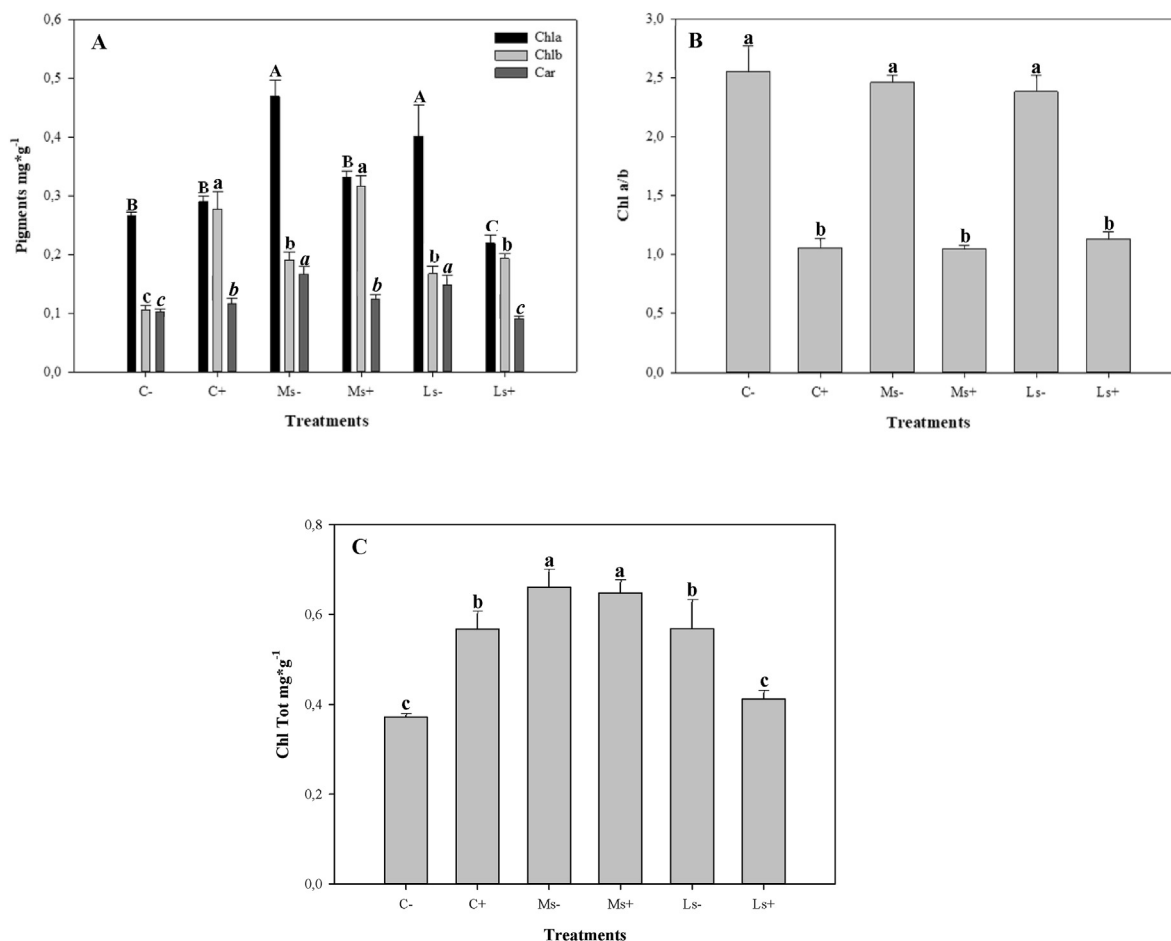
Chl-a/b ratios (Fig. 2B) of acid rain exposed plants were about 2.5-fold lower than the treatments without acid rain; the only significant interaction was shown from acid rain ( $p < 0.001$ ).

Finally, total chlorophylls (Fig. 2C) resulted statistically significant for treatments ( $p = 0.002$ ) and treatments  $\times$  acid rain ( $p = 0.005$ ). They showed a parabolic trend from C- to Ls + treated plants, with higher values recorded for Ms plants, both treated and untreated with acid rain.

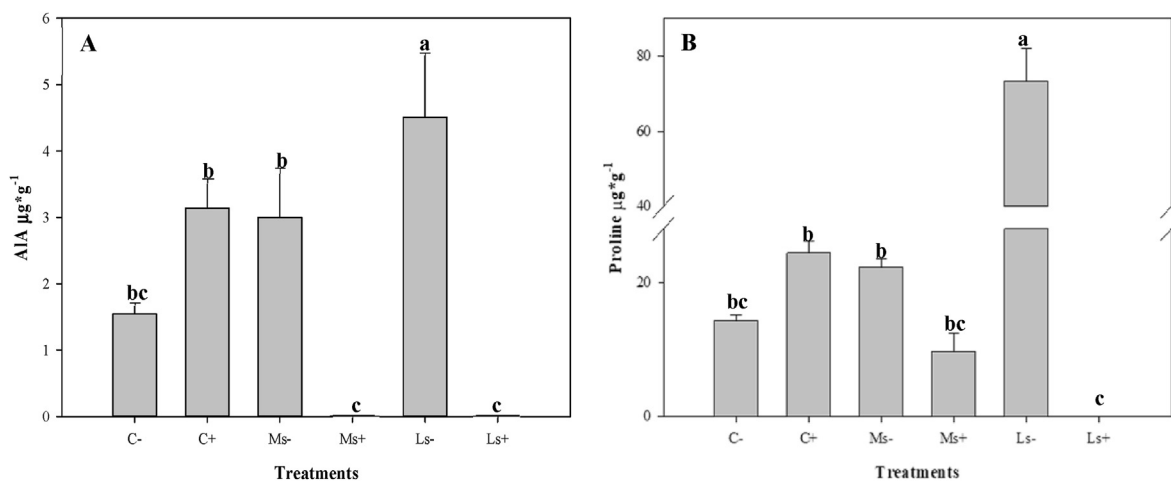
### 3.4. Aminolevulinic acid and proline

The aminolevulinic acid (ALA) production was greater in C+ than in C- (Fig. 3A). Highest values are recorded for Ls-, followed by Ms- and C+ treated plants; on the contrary, lowest values (near to zero) are shown by Ms+ and Ls+. Variables statistically significant are acid rain ( $p < 0.001$ ) and treatments  $\times$  acid rain ( $p < 0.001$ ).

Proline values (Fig. 3B) are characterized by a trend similar to ALA, showing highest concentrations for Ls-, followed by C+ and Ms-; while Ls + has recorded lowest value of all. From statistical



**Fig. 2.** Pigment concentrations, chlorophyll ratio and total chlorophylls concentration in *L. sativum* leaves exposed to PET-induced stress during the experiment (30 days). Pigments are represented in figure A, Chlorophylls ratio (Chl a/b) are represented in Figure B, while total chlorophyll concentrations in Figure C. The values are expressed as mean  $\pm$  standard error (SE,  $n = 3$ ). Two-way ANOVA was applied to determine significant differences between each treatment (p-level is given; \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\*;  $p < 0.001$ ; ns = not significant). Different letters represent statistical differences between treatment for each compound (Holm-Sidak method multiple comparison,  $p < 0.05$  level).



**Fig. 3.** Aminolevulinic acid and proline concentrations in *L. sativum* leaves exposed to PET-induced stress during the experiment (30 days). Aminolevulinic acid (AIA) is represented in figure A, proline in Figure B. Values are expressed as mean  $\pm$  standard error (SE,  $n = 3$ ). Two-way ANOVA was applied to determine significant differences between each treatment (p-level is given; \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\*;  $p < 0.001$ ; ns = not significant). Different letters represent statistical differences between treatment for each compound (Holm-Sidak method multiple comparison,  $p < 0.05$  level).



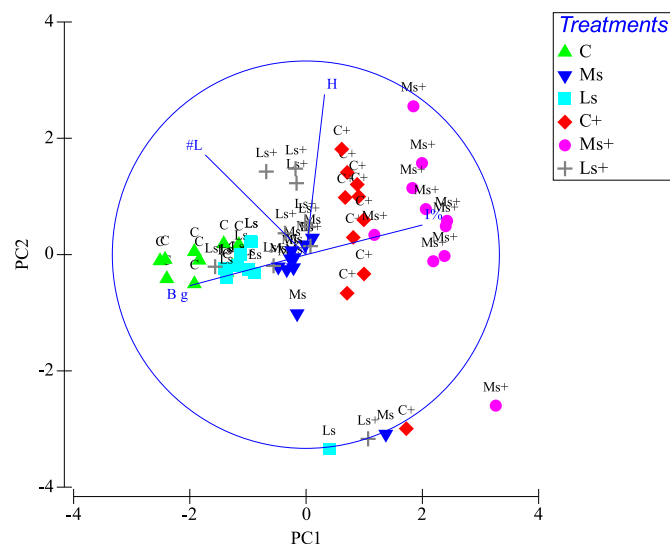
analyses, it resulted significant for each variable: treatments ( $p = 0.003$ ), acid rain ( $p < 0.001$ ), and treatments x acid rain ( $p < 0.001$ ).

### 3.5. An overview on multivariate statistics

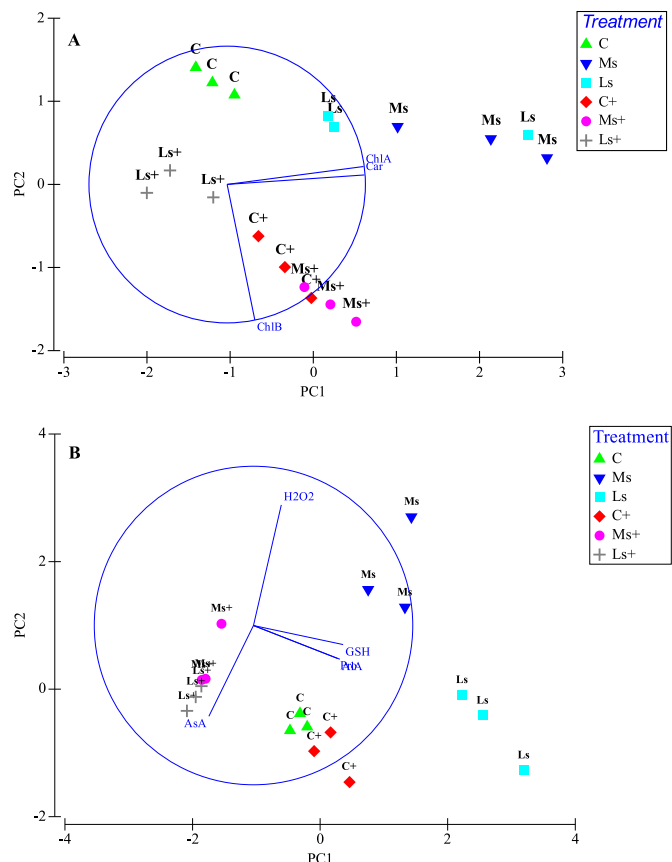
The Principal Component Analyses (PCA), performed on biometrical traits (percentage inhibition of germinability, shoot height, leaf number and biomass produced) (Fig. 4), explained 92.6% of the total variance (47.8, 32.5, 12.3% respectively). The eigenvectors related to PC1 have recorded a positive correlation for  $I\%$  (0.602) and  $H$  (0.096), while negative were showed for  $\#L$  and  $B$  ( $-0.520$ ,  $-0.599$ ). Regarding as PC2, it was strongly positively correlated with  $H$  (0.828), and in minor way for  $\#L$  (0.515). The only negative correlation for this axis was biomass production ( $-0.160$ ). The PERMANOVA analyses based on the Euclidian distance, performed on treatments versus acid rain, has noticed 9.417 and 0.001 values for Pseudo-F and P respectively. PCA performed on pigments (Chl-a, Chl-b and Car), (Fig. 5A), showed the 99.9% of the total variance (66.6, 32.7, 0.6% respectively). The related eigenvectors showing higher positive correlations for PC1 were: Chl-a (0.698), and Car (0.702); while for PC2, Chl-b was the only negatively and strongly correlated ( $-0.989$ ), compared with other two pigments, (0.131) Chl-a and Car (0.069), respectively. PERMANOVA analyses recorded 10.968 and 0.001 values for Pseudo-F and P. The last PCA performed on biochemical compounds (Fig. 5B) has recorded 92.1% of the total variance, so divided: 55.6, 22.1, and 14.3% for each axis. The eigenvectors related with PC1 were mostly positive, except for AsA ( $-0.280$ ); conversely, for PC2 the only positive correlation was showed from  $H_2O_2$ . The PERMANOVA analyses, performed on treatments versus acid rain, has noticed 17.502 and 0.001 values for Pseudo-F and P, respectively.

## 4. Discussion

Since that MPs enter the environment, they can accumulate at soil level and persist over the time affecting growth and development of plants. Some biometrical parameters are useful tools to evaluate, preliminarily, the MPs toxicity (Lian et al., 2020). The present research has shown that different sizes of PET



**Fig. 4.** Principal component analyses performed on biometrical data. PCA performed on biometrical traits ( $I\%$ ,  $H$ ,  $\#L$  and  $B$ ) of plants exposed to different PET sizes under natural and acid rain (+) conditions.



**Fig. 5.** Principal component analyses performed on physiological data. PCA performed on (A) pigments, (B) oxidant ( $H_2O_2$ ) and antioxidants (GSH, AsA, AIA, Pro) compounds of plants exposed to different PET sizes under natural and acid rain (+) conditions.

microplastics, added or not with acid rain, differently affect biometrical traits and physiological response of garden cress. Overall, we observed that germinability inhibition is affected by both different size of PET and the combination of plastic and acid rain, with special attention on plants treated with Ms and acid rain together ( $Ms+$ ). These results are in agreement with previous studies on *L. sativum*, performed by using polycarbonate, polyethylene and polypropylene microplastics (Pflugmacher et al., 2020; Pignattelli et al., 2020). Regarding the shoot length, is clear that acid rain improves its elongation; this could be explained by the nitrogen supplied through acid rain (Liu et al., 2019). A completely opposite scenario, instead, is showed by number of leaf and biomass produced; in these cases, the plants more negatively affected were those treated with acid rain. Considering the two distinct experimental set-ups (MPs-alone, and MPs + acid rain), in MPs-alone set, plants more affected were  $Ms-$ ; instead, in the acid rain set,  $Ms+$  and  $C+$  were more affected for leaf number and biomass produced, respectively. These results suggest that size of microplastics plays a key role in growth and development of plant, regardless of acid rain. The multivariate analysis based on Euclidian distance, was performed to examine the separation between PET size in natural and acidified conditions. The first principal coordinate axis showed that simulated environmental conditions (acid rain), have an effect on different PET size supplied. Noteworthy, is that plants treated only with PET were clustering along the PC1. On the other hand, only PET supplied with acid rain were separated along the second principal coordinate axis, particularly  $Ms+$  and  $C+$ ; in minor way  $Ls+$ . These latter confirm the results mentioned above, where acid rain is proposed like an enhancer stress factor.

Being sessile organisms, plants cannot escape from stressing environmental conditions, so when they are subjected to these, an increase of reactive oxygen species (ROS) production occur (Ahmad et al., 2018). ROS are considered a waste product of plant aerobic metabolism. Their harmful production is due to the excess excitation energy, that which in turn happens when the light-utilization capacity of a plant is unbalanced compared with its light harvesting capacity. Plants produce different kind of ROS, such as superoxide anion ( $O_2^-$ ), hydroxyl radical ( $OH^\cdot$ ) and hydrogen peroxide ( $H_2O_2$ ); between these,  $H_2O_2$  is the main ROS produced by plants (Li et al., 2020).  $H_2O_2$  can be formed both through reduction of oxygen ( $O_2$ ), and by dismutation of  $O_2^-$  operated by superoxide dismutase (SOD) (Noctor et al., 2018). Overall, in the present study,  $H_2O_2$  increases in both control treatments, when lower size of plastics is added. Noticeable is that higher values of  $H_2O_2$  are recorded in Ms treated plants without acid rain supplied. This indicate that ROS metabolism is perturbed mainly by the microplastics size rather than acid rain. Furthermore, results obtained on Ms (- and +) treatments were confirmed, also, by biometrical results, because these latter two were either more negatively affected for each biometrical trait.

When plants are subjected to oxidative stress, they activate an antioxidant compounds production operated by enzymatic systems. The enzymes mostly involved in ROS scavenging and detoxification are superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and catalase (CAT). SOD is involved in the first conversion of superoxide anion ( $O_2^-$ ) in hydrogen peroxide ( $H_2O_2$ ). Subsequently, APX, GPX and CAT work in concert to convert  $H_2O_2$  in  $H_2O$  in a detoxification process. The conversion operated by APX to obtain  $H_2O$  from  $H_2O_2$  needs ascorbate and reduced glutathione (GSH) through the ascorbate-glutathione cycle. More precisely,  $H_2O_2$  is transformed in  $H_2O$  through the oxidation reaction of ascorbate to mono dehydroascorbate (MDA), to produce dehydroascorbate by dismutation. GPX, such as APX, uses GSH to produce  $H_2O$  from  $H_2O_2$ . Furthermore, CAT is involved in conversion and scavenging from  $H_2O_2$  produced during the mitochondrial electron transport,  $\beta$ -oxidation of fatty acids and in photorespiratory oxidation, to  $O_2$  through the production of  $H_2O$  (Choudhury et al., 2013; Quant et al., 2008). In ascorbate-glutathione cycle, AsA and GSH are used as buffers by peroxidases to produce  $H_2O$  from  $H_2O_2$  (Gao et al., 2018). In plant tissue ascorbic acid (AsA) covers three main biological roles: it is an enzyme cofactor, a radical scavenger and a donor/acceptor in electron transport mechanism. First, AsA is able to modulate enzymatic reaction, its function is to maintain the transition metal ion centres of these enzymes in a reduced form, representing a potent enhancer of enzyme activity. Second, AsA interacts either enzymatically or non-enzymatically with reactive oxygen species. Its ability to interact with ROS makes AsA an active player in processes modulation such as lignification, cell division and hypersensitive response. The biological importance of the ascorbic acid in antioxidant functions, is due to its ability to finish the radical chain reactions through the production of non-toxic compounds such as DHA. Another relevant peculiarity of the AsA non-enzymatic antioxidant activity, is its involvement in the regeneration of the lipophilic, membrane-associated  $\alpha$ -tocopherol, radical. In the frame of photosynthetic machinery, AsA is important in the chloroplast activity by its use of substrate for APX, to scavenge peroxide generated in thylakoids. Third, due to its monovalent cation nature at physiological pH, ascorbic acid is unable to permeate the membranes. Thus, the process that involves the crossing of the membrane by AsA is mediated by carriers. The subcellular compartments where it was found are chloroplast, apoplast, and vacuole (Davey et al., 2000).

Overall, the present study, showed that PET-MPs, added or not with acid rain, are able to induce oxidative stress and consequently

trigger the antioxidant compounds production of AsA and GSH. Furthermore, results recorded from these latter two antioxidants have highlighted an opposite behaviour: higher AsA values are showed by plants treated with acid rain; conversely, higher GSH production is resulted, mainly, by plants treated only with PET. Lower values of AsA in Ms- and Ls-treated plants, associated with high concentrations of  $H_2O_2$  and GSH for the same treatments, can have a dual explanation: ascorbic acid is directly involved in counteracting ROS but also in GSH regeneration from Haliwell-Asada cycle; as already demonstrated in our previous work (Pignattelli et al., 2020). On the other hand, for plants treated with acid rain, seems that GSH is directly involved in ROS removal by its depletion (Polle, 2001; Tarrago et al., 2009). Concerning the multivariate analyses, the effect of plastic size, coupled or not with acid rain, is clearly separated along the PC2 indicating that these treatments exert a different influence at plant level.

In plants, chlorophylls play a key role in light energy transformation through its absorption, transmission and distribution which, in turn, results in a synthesis of organic products (Li et al., 2015). In our work we found that Chl-a, for plants treated only with PET, was always higher than control, as already reported in tomato plants stressed with salt (Li et al., 2015; Romero-Aranda et al., 2001). The increase of Chl content can be due to two different processes: to the decrease in Chl degradation, or conversely, to the increased Chl synthesis (Li et al., 2015). Noteworthy, is that Chl-b, in plants treated with acid rain, is always higher than plants treated only with PET; this higher production can be explained by the fact that Chl-b is formed from the Chl-a. Normally, Chl-a concentration is higher than Chl-b because it is present both in photosynthetic reaction centres and in light-harvesting antennae (Fang et al., 2020). Our data suggest that acid rain could probably cause an impairment in Chl-a production, that which in turn results in a lower production of this latter pigment, or that the demand of Chl-b is higher respect to the Chl-a production, rather than pheophytins production.

In fact, at macroscopical level, pheophytin production is visible through the degreasing of leaves, that is due to the Mg-removal; this supposition is supported by the fact that, in our study, no browning colours is recorded in these leaves (Schelbert et al., 2009). Furthermore, our data showed that Chl-a/b ratio was unbalanced in favour of plants treated with PET and acid rain confirming the values found for Chl concentration; in fact, an unbalanced ratio of this pigments can decrease the photosynthetic system capacity by reducing its efficiency in photosynthetic reaction centres (Fang et al., 2020). Concerning the multivariate analyses related to chlorophyll production, plants treated only with PET are clearly separated along the PC1, while PET plus acid rain treated plants are mainly separated along the PC2; these results indicate that simulated environmental condition exerts an effect on different PET size supplied at plant level.

Aminolevulinic acid (ALA) is a compound belonging to the tetrapyrroles pathway, it is a precursor of chlorophyll biosynthesis and not only, because it is involved in stress tolerance by inducing the production of antioxidant compounds such as ascorbic acid and glutathione (Wu et al., 2019). Noticeable is that ALA production was completely inhibited for Ms+ and Ls + if compared with C+, it could be due to the antagonistic effect supplied by PET and acid rain. This result is in agreement with records on pigments, in fact, plants treated with PET and acid rain showed lower pigments production, particularly, for LS+. The higher ALA production recorded for Ls- and Ms- is in agreement with pigments production but another explanation is also plausible. As mentioned above, ALA is involved in counteracting oxidative stress, its higher production corresponds to higher GSH production. The proline function as osmo-protectant and antioxidant against abiotic stress is well

known (Szabados and Savoure, 2010). Data collected in this study showed that proline and AIA follow almost the same trend, in fact, at higher proline values corresponded higher AIA values suggesting that in plants treated with MPs there is no competition in favour of proline production, as reported for salt stress by other authors (Averina et al., 2010; Xiong et al., 2018). Concluding, this study confirmed the results found in our previous work (Pignattelli et al., 2020).

## 5. Conclusion

Our research highlighted that different size of PET, supplied or not with acid rain, negatively affect biometrical and physiological traits of garden cress after long exposure. At biometrical level plants most negatively affected were those treated with lower size of PET, and the addition of acid rain inhibited or delayed the plant growth and development except for shoot height. Both treatments were able to induce oxidative stress in term of  $H_2O_2$  production, especially for Ms treated plants; while antioxidants production have showed an opposite trend: ascorbic acid was mainly produced by plant treated with acid rain, and glutathione by plants treated only with PET. Furthermore, the addition of acid rain has evidenced a higher production of Chl-b at expense of Chl-a, which in turn results in a lower production of this latter pigment. Finally, AIA production was totally inhibited by PET + acid rain treatment and AIA and proline productions followed the same trend: higher values of AIA corresponded to higher values of proline.

## Authors' contribution

S.P. = performed experiment development, statistical analyses, and the first, draft of the paper; A.B. = performed laboratory analyses; M. P. = performed the microplastics pre-treatment and the revision of the first and final draft of the paper; A.T. = performed the microparticle production and a critical revision of the dataset and the revision of the final draft; M.R. = perform found recruitment, experimental project plan, revision of the final draft for the production of the submitted version.

## 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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