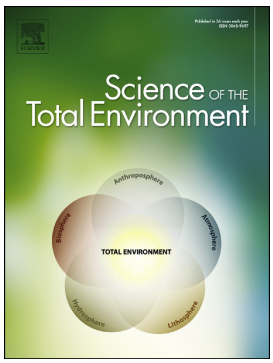

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PHYSIOLOGICAL RESPONSES OF GARDEN CRESS (*L. sativum*) TO DIFFERENT TYPES OF MICROPLASTICS

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ABSTRACT

In this study, for the first time, acute and chronic toxicity caused by four different kinds of microplastics: polypropylene (PP), polyethylene (PE), polyvinylchloride (PVC), and a commercial mixture (PE+PVC) on *Lepidium sativum* were evaluated. Parameters considered were: *i)* biometric parameters (e.g. percentage inhibition of seed germination, plant height, leaf number and fresh biomass productions); and *ii)* oxidative stress (e.g. levels of hydrogen peroxide, glutathione, and ascorbic acid). On plants exposed to chronic stress chlorophylls, carotenoids, aminolaevulinic acid, and proline productions were, also, evaluated. PVC resulted the most toxic than other plastic materials tested. This study represents the first paper highlighting microplastics are able to produce oxidative burst in tested plants and could represent an important starting point for future researches on biochemical effects of microplastic in terrestrial environments such as agroecosystems.

Keywords: Polypropylene; polyethylene; polyvinylchloride; plastic packaging; microplastics; plant exposure.

1. INTRODUCTION

The “plastic problem” originated around 70 years ago (1950) with the start of its large-scale production (Geyer et al., 2017). Since then, in 2018, the plastic produced in Europe is close to 62 million tons, while in the same year 359 million tons were produced worldwide (Statista, 2018). Due to their resistance to biodegradability, plastics accumulate rather than decompose when disposed in landfills or released in the environment (Geyer et al., 2017).

The term “microplastics” includes all plastic particles with dimensions less than 5 mm. They are found in the environment as primary microplastics, deliberately manufactured in micro-sizes or as secondary microplastics generated by the fragmentation of larger plastic litter (Duis and Coors, 2016). Due to the increasingly widespread presence of microplastics in different ecosystems, these materials attracted the global attention as emerging "new generation" contaminants (Karthik et al., 2018; Tang et al., 2018; Zhao et al., 2018; Zhu et al., 2018; Di and Wang, 2018; Lin et al., 2018; Rodrigues et al., 2018; Sighicelli et al., 2018; Horton et al., 2017; Zhang and Liu, 2018).

Agro-ecosystems are considered the main source of microplastic pollution in terrestrial environments (Ng et al., 2018). In fact, in these ecosystems, primary microplastics come from the application of sewage sludge used to fertilise the soil; while, secondary microplastics can originate from plastic mulch films or from plastic materials used to cover greenhouses. The process of plastic fragmentation producing secondary microplastics can originate not only from agricultural practices, but also from the environmental exposure itself due to the destructive action of exposure to sunlight and temperatures (Horton et al., 2017; Lv et al., 2019). The deposition and movement of microplastics in soil are influenced by both biota activities and soil tillage. Main responsible for the downward movement are biopores created by soil biota or soil cracking (Rilling et al., 2017; Majadlani et al., 2008); while the horizontal movement of microplastics is caused by harvesting and ploughing (Paustian et al., 1997). The physical properties of microplastics (i.e. size, shape and hydrophobicity) can influence their transport within the soil (Rilling et al., 2017; Wan and Wilson,

1994). Furthermore, the distribution of microplastics is influenced by sequestering processes such as soil aggregation (Peng et al., 2017). Plants are not expected to absorb or translocate microplastics. In fact, the high molecular weight of microplastics and/or their large size (Teuten et al., 2009) prevent penetration through cell wall at the root level. On the other hand, there is no experimental evidence that confirms the toxicity of nano- and microplastics on plants (Ng et al., 2018). The researches performed from Lin et al (2009) and Liu et al (2009), respectively on rice and *Nicotiana tabacum*, pointed out the capacity of plant cells to uptake carbon-based nanotubes and fullerenes. Subsequently, other studies were conducted on the effects of nanoparticles on edible crops; highlighting a wide range of positive, negative and neutral interactions (Husen and Siddiqi, 2014; Ma et al., 2010; Rico et al., 2011; Wang et al., 2016). Plants are organisms capable of metabolizing a wide range of pollutants, such as polychlorinated compounds and polycyclic aromatic hydrocarbons (Sanderman Jr., 1992). Others chemical pollutants released by plastic (Fossi et al., 2016) could be absorbed by plants and could cause oxidative stress during the activation of the metabolic patterns of the plant (Choudury et al., 2013).

To evaluate the response of plants to different microplastics, this study used *Lepidium sativum* L., also known as garden cress, a fast-growing annual herbaceous plant belonging to the *Brassicaceae* family, as model species. It is a species native from Egypt and Asia but now it is widespread at world level for culinary and phytotherapeutically purposes (Nehdi et al., 2012). Its high sensitivity to phytotoxic substances makes it suitable for biological tests (Janecka and Fijalkowski, 2008; Smolinska and Leszczynska, 2017). The physiological and biochemical mechanisms involved in stress, caused by the exposure to microplastics, are not completely understood and clarified. Normally, phytotoxicity is related to the production of reactive oxygen species (ROS), such as hydrogen peroxide, superoxide anion, and hydroxyl radical (Choudury et al., 2013). The ROS accumulation in plant cell leads to an impairment of plant growth, photosynthesis, and biochemical processes (Choudury et al., 2013). Several studies indicate that the exposure of the plant to abiotic stresses, such as heavy metals, affect the production of chlorophylls and carotenoids by interfering

with the synthesis of chlorophyll by the inhibition of enzymes involved in this process (Van Assche and Clijster, 1990; Kastori et al., 1998; Lenti et al., 2002). To counteract oxidative stress, plants have developed an antioxidant system that involves a lot of products such as glutathione (GSH), and ascorbic acid (AA) (Choudury et al., 2013).

The aim of this paper is to better understand the mechanisms caused and connected to phytotoxicity of microplastics. In the present study, we tested under controlled conditions, different times of exposure related to acute and chronic toxicity caused by four different kinds of microplastics supplied to *Lepidium sativum*: polypropylene (PP), polyethylene (PE), polyvinylchloride (PVC), and a commercial material used in agriculture (mixture of PE+PVC). Effects accounted were biometric parameters such as percentage inhibition of seed germination, plant height, leaf number and fresh biomass production. Oxidative stress (hydrogen peroxide, glutathione, ascorbic acid) was also evaluated as biochemical effects following the exposure to microplastics. On plants exposed to chronic stress chlorophylls, carotenoids, aminolaevulinic acid, and proline production were also evaluated. We hypothesized that *L. sativum* treated with several types of microplastic under different exposure temporal length (short and long), will respond differently about its growth and physiological mechanisms following the activation of metabolic response to toxicity.

2. MATERIALS AND METHODS

2.1. Growth condition, experimental set up and biometrical traits

Certified seeds of *Lepidium sativum* were obtained from ECOTOX LDS. The PhytoToxKit from MicroBioTest Inc were used for acute toxicity test. One plate supplied from PhytoToxKit, for each kind of microplastic tested, containing 10 seeds and filled with 90 mL of natural soil was used. Before the seeds sowing, the field capacity was tested. Soil, previously dried in oven, was then soaked with 55 mL of Milli-Q water. After that microplastics were added. Acute stress was tested after 6 days from seeding.

In chronic toxicity experiment, seeds were sown in pot (5.5 cm diameter x 6 cm depth) containing 50 mL of natural soil already dried, with the field capacity assessed, and microplastics added. One seed per pot and 10 seeds per treatment were sown; plants were regularly monitored and watered, twice a week with 8.5 mL of Milli-Q water per time and they were sampled after 21 days from sowing. In both toxicity tests, plants were grown in a climatic chamber under controlled environmental conditions (temperature ranging between 17-20 °C; relative air humidity ranging between 40-60 %; photosynthetic photon flux density (PPFD) of 700 $\mu molm^{-2}s^{-1}$ for 14 h per day (from 06:00 to 20:00 local time). All the experiment was carried out by applying five different treatment conditions: *i)* control (C), plants were exposed only to natural soil; *ii)* soil added with polyethylene (PE); *iii)* soil added with polyvinylchloride (PVC); *iv)* soil added with polypropylene (PP); *v)* soil added with a commercial material used in agriculture (mixture of PE+PVC). In this study we added 0.02% (w/w) of microplastic to soil. Tested concentration was 5 time less than exposure levels used by Rychter et al. (2010). About 0.092 g (±0.002 g) of microplastic were added to 500 mL of natural soil (500 g of soaked soil; 184±4 mg/kg). Soil used to set experiments was collected by a local operator from a natural unpolluted protected area (Regional Natural Park). To evaluate levels of microplastics in natural soil used to perform experiments, levels of microplastics were determined on three replicates (n=3) following extraction and chemical quantification methods reported by literature for sediments (Renzi et al., 2020). Results highlighted microplastic pollution in natural soil was <0.5 mg/kg.

Microplastics fragments were obtained by grinding, with liquid nitrogen, of larger pieces. Obtained powder was then sieved by 0.125 mm ASTM sieve to remove larger plastic fragments. The powder passed the sieve was collected and rapidly washed with ethanol 75% and subsequently dried in oven at 40°C in a large glass-made Petri capsule. Plant height and leaf number were measured once per week. Germination rate were measured after 6 days from the begin of the experiment; percentage inhibition of seed germination (I%) was calculated using the following formula (ISO, 2016):

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

Where C_s are the germinated seeds of control group, and T_s are the germinated seeds of each treatment. The germinated seed numbers are obtained from the average of the replicates used. The biomass was measured at the end of the experiment, during the sampling, by weighing shoot (f.w., fresh weight).

2.2. Hydrogen peroxide, antioxidants, and proline determination

Hydrogen peroxide (H_2O_2) was measured spectrophotometrically after reaction with potassium iodide (KI), according to a method proposed by Alexieva et al. (2001). The reaction was developed in trichloroacetic acid (TCA) and absorbance was measured at 390 nm. The amount for H_2O_2 was calculated using standard curve prepared with known concentrations of H_2O_2. The results were expressed as $\mu g*g^{-1}$ fresh leaf weight (f.l.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^{3+} to Fe^{2+} 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 $\mu g*g^{-1}$ f.l.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 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 $\mu g*g^{-1}$ 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 $\mu g*g^{-1}$ f.l.w. All spectrophotometric analyses were performed by UV/Vis spectrophotometry (ONDA, mod. UV-30 Scan).

2.3. Pigments and aminolaevulinic acid 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. Then, chlorophyll-a, chlorophyll-b, total chlorophylls and carotenoids were further calculated according to formulae described by the literature (Bhushan et al., 2007). The aminolaevulinic acid (AlA) leaf content was measured according to Haren and Klein (1972). The determination was obtained through the extraction in TCA and 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 $\mu g*g^{-1}$ fresh leaf weight. All spectrophotometric analyses were performed by UV/Vis spectrophotometry (ONDA, mod. UV-30 Scan).

2.4. 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, one-way ANOVA, was applied to test the different microplastics effects on *Lepidium sativum* plants. A Fisher-LSD post-hoc test was applied to asses significantly differences among treatments ($p < 0.05$ level).

3. RESULTS

3.1. Effects on the growth of *Lepidium sativum*

Acute toxicity experiments (6 days). Biometrical parameters recorded in exposed plants and controls are summarized in **Table 1** (I%, percentage of inhibition of germination; H, shoots height;

#L, leaf number; B, shoots biomass). All tested parameters are statistically significant, except the leaf number. ANOVA analyses show a significant interaction effect between treatments at the following significance levels: I% ($p < 0.001$), H ($p = 0.021$), and B ($p = 0{,}034$). I% showed higher value when plants are exposed to PE or PE+PVC; while controls showed I% below 10%. Similarly, shoot heights (H) are more influenced by PE and PE+PVC treatments, while controls showed the highest mean value (0.378±0.05 cm). Although not statistically significant, leaf number showed lower values for PE and PE+PVC treatments. Noteworthy, the biomass production (B) showed a slightly different trend from the other biometrical parameters, in fact only the treatment with PE confirmed other parameters showing a lower value compared to control; while all other treatments showed biomass production higher than control.

Chronic toxicity experiments (21 days of exposure). Results are reported in **Table 2**; statistically significant differences were highlighted only for I% ($p < 0.001$) and biomass ($p < 0.001$) production. Different types of microplastics affect differently these two biometrical parameters. I% of plants treated with PE and PP resulted most affected showing values higher than controls and the other treatments. The same trend was observed for biomass production, in fact the same treatments showed the lowest biomass production recorded. Conversely, plants H and #L are not statistically significant for all tested microplastic treatments. Although leaf number is not statistically significant, compared to control (C), PE+PVC treated plants showed similar values; while PVC, PE, and PP showed lower values. This trend is in agreement with results on biomass and I%. On the contrary, observed trend is different compared to plants height, because higher value is shown by PVC treated plants, while lower value is reported for PE+PVC treated plants.

3.2. Reactive oxygen species and antioxidants

The hydrogen peroxide and antioxidants concentration (**fig.** 1 a,b,c), in acute toxicity, showed a statistically significant differences between treatments. The ANOVA analyses show a significant interaction effect between each treatment and H_2O_2 ($p < 0.001$), AsA ($p = 0.002$) and GSH ($p <$

0.001) contents. The H_2O_2 production during acute toxicity (6 days of treatment) highlighted a significantly higher hydrogen peroxide accumulation in the PE and PE+PVC treatments (**fig.** 1a); while PVC and PP showed levels comparable to control. The ascorbic acid content (AsA, **fig.** 1b) is significantly lower than controls in all treatments with the exception of PP. The glutathione (GSH) concentration (**fig.** 1c) showed the highest concentration for untreated plants followed by PVC treated plants, all the other treatments showed significantly lower levels with low and similar values in PE, PP, and PE+PVC.

In the ANOVA analyses, carried out in chronic exposure (21 days of treatment), a significant interaction has been found for each treatment and H_2O_2 ($p < 0.001$), AsA ($p = 0.003$) and GSH ($p = 0.011$). A significant H_2O_2 production is recorded (**fig.** 2a), pointing out that PE and PVC showed higher values than others microplastic types tested. Interestingly PE+PVC and PP treatments are showing the same lower C treatment values. At the end of chronic exposure, antioxidant compounds showed a statistically significant differences between treatments; AsA (**fig.** 2b) showed higher values for PE+PVC and PP treated plants in comparison with control plants, while PE and PVC individually treated plants showed lower values compared to control plants. GSH (**fig.** 2c) showed a different trend in comparison with the other antioxidant compounds, in this case, C and PE+PVC treated plants showed lower levels, such as H_2O_2, conversely PP treated plants have higher values such for ascorbic acid production. Comparing effects produced after acute and chronic exposure, acute toxicity showed hydrogen peroxide values always higher than levels recorded after chronic exposure tests in all treatments. AsA resulted always lower in acute than after chronic exposures; GSH, instead, showed higher values in C plants after acute toxicity, while for the other treatments, except for PVC, higher values are showed in chronic exposure. Noteworthy, PVC treatment showed similar H_2O_2 and GSH values both in acute and in chronic toxicity.

3.3. Pigments and their precursor

Exposure to different types of microplastics over a prolonged period of time, is reflected in a statistically significant different concentrations of photosynthetic pigments in *L. sativum* leaves (**fig.** 3). The ANOVA analyses show that a significant interaction between treatment is found for pigments, particularly, Chl-a ($p < 0.001$), Chl-b ($p < 0.001$) and carotenoids ($p < 0.001$). Chlorophyll-a levels (Chl-a, **Fig.** 3a) are always higher than chlorophyll-b (Chl-b) levels as attended, while carotenoid (Car) showed similar or slightly lower concentrations than Chl-b. Chl-a level is significantly higher in PP treated plants, followed by PVC and PE treated plants; conversely, lower values of this photosynthetic pigment are recorded in PE+PVC treated plants and in controls. A similar trend is showed for the other two pigments. After 21 days of exposure to microplastic-induced stress, aminolaevulinic acid produced showed statistically significant differences between treatments ($p = 0.005$) (**fig.** 3b). Most affected treatments are PVC and PP treated plants (PVC>>PP) showing higher aminolaevulinic acid content, while lower values are showed by the other treatments.

3.4. Proline

During chronic toxicity exposure tests, the proline production (**fig.** 4) showed statistically significant differences between different microplastics supplied ($p = 0.004$). Plants treated with PVC showed the highest proline concentration; while PE+PVC treated plants showed the lowest proline value compared with control plants.

4. DISCUSSION

Different types of microplastics tested in this study affect differently biometrical traits of garden cress. In plants treated with polyethylene (PE), after acute exposure (6 days), each of the biometric traits studied resulted negatively affected. Also plants exposed to PE+PVC are negatively affected for all trait considered with the exception of the biomass production. Noteworthy, is that these last two treatments showed a lowest germination compared to others. During chronic exposure experiments (21 days), PP and PE affected negatively germination rate, leaf number, and biomass

produced, while height is mostly affected by PE+PVC. Notable, is that PE treated plants in both acute and chronic exposure experiments, showed all considered parameter affected indicating that this toxicity is the first to coming out when the observation is performed after a short period of exposure; while for the other microplastics toxicity, always at biometrical traits level, coming out in the long period.

As far as we know, the only studies that have addressed the plant-microplastic interaction, are those that involved the effects of mulching films, such as polyethylene and biodegradable microplastics; furthermore, existing studies are focused only on assess the impact induced on plants in terms of yield and biomass produced without investigate more deeply: in other words, existing studies that have a more agronomic than physiological imprint. Previous studies on cotton carried out that the boll weight, yield, and biomass decreased when film residue density increased (Dong et al., 2015). Researches performed on *Triticum aestivum* found that starch-based biodegradable microplastics negatively affect biometric traits such as plant height, leaf number, and biomass producing more strongly effects than low-density polyethylene microplastics (Qi et al., 2018). On the contrary, on turnip, radish, cress, and monocotyledonous oat the poly-(butylene adipate-co-terephthalate), a plastic mulch film known as PBAT, highlighted no effect on the growth of tested plants (Muroi et al., 2016; Rychter et al., 2010).

Recent studies (Machado et al., 2018; Wan et al., 2019; Rillig et al., 2019; Rillig et al., 2017) have focused only on movement, consequences and fate of microplastics in soil, without investigate their effect at physiological level on exposed plants. In other words, for the plants, no yet answer was found if there is an effect in terms of ROS and antioxidants production when microplastics are added to the soil. Furthermore, is it right to say that microplastics are "considered" as abiotic stress factor? Increase in the reactive oxygen species production (ROS) is the key response of plants to stressful environments; ROS can be formed in plants through many reactions in which oxygen (O_2) undergoes reduction to superoxide or hydrogen peroxide (H_2O_2). Superoxide can be chemically

reduced or dismutated to H_2O_2, a reaction that is accelerated by superoxide dismutase (SODs) (Noctor et al., 2018). Results obtained in this study concerning the production of hydrogen peroxide showed a generally higher H_2O_2 concentration in acute than chronic toxicity. Specifically, plants exposed to acute experiments and treated with PE, showed the highest concentration than all the other plastics treatments; and this is in agreement with biometrical traits results. However, interestingly, PVC treated plants kept the same levels in either acute or chronic tests. This latter treatment, after chronic exposure, highlighted the highest H_2O_2 production, followed by PE-treated plants. Therefore, not only from a biometric but also from a biochemical point of view, different microplastics, supplied to plants for different time exposure, act in different ways in terms of quantity production. In fact, PE-exposed plants during short-term experiments, showed higher H_2O_2 production, while after long-term exposure, higher H_2O_2 production was shown by PVC-treated plants. This latter treatment, it is the only one that did not show different concentration between the acute and chronic exposure; this can be explained by the fact that the PVC-induced stress is "detected" before than the other microplastic stressors tested in this study, and that during the time lapse of the chronic exposure, plants are not able to counteract PVC toxicity. On the other hand, a reaction by plant was in some cases recorded, such as, for PE+PVC and PP treatments that showed the same concentrations recorded in control at the end of the chronic exposure.

The high production of H_2O_2 in plants is due both to its function as a signalling molecule and to the multiple enzymes that uses it as a substrate (Noctor et al., 2018; Foyer et al., 2016; Noctor et al., 2002), and consequently, they trigger the production of low molecular weight compounds with antioxidant action (Noctor et al., 2015; Couèe et al., 2006). The ascorbate and glutathione play an important role, as they react rapidly with H_2O_2 through specific enzymes, the peroxidases, meanwhile their oxidized forms are regenerated by reductases. This trait allows the redox cycle to repeat itself which in turn regulates the cellular redox state (Foyer and Noctor, 2011). The glutathione plays multiple roles: as an antioxidant in counteracting ROS, as a reducing agent for superoxide and as a substrate for other enzymes (Polle, 2001; Tarrago et al., 2009). Foyer and

Noctor (2011) highlighted that the main role of glutathione in H_2O_2 metabolism may be in regenerating ascorbate from dehydroascorbate (DHA), or via dehydroascorbate reductases (DHAR). The most important role exerted by ascorbate, on the other hand, is the ability to directly remove ROS (Noctor et al., 2011). The results obtained in this study on antioxidants showed that, at the end of the acute exposure tests, the concentration of ascorbic acid (AsA) is lower than the levels measured at the end of the chronic exposure for all tested microparticles types. Conversely, glutathione (GSH), showed an opposite production only for PVC treated plants and controls. From a more in-depth analysis, it emerged that the plants treated with PE and PVC showed lower AsA values compared to other treatments, under both after acute and chronic exposures. This result is in agreement with hydrogen peroxide trends, in fact the depletion of the antioxidant AsA could be explained by its counteracting action performed against this ROS. Concerning the other treatments, in chronic toxicity PE+PVC and PP showed almost the same levels of AsA compared to controls, also in agreement with the H_2O_2 results. On the contrary, in acute tests, there was a trend slightly different from chronic exposure. In fact, only the plants treated with PP showed the same AsA values shown by the control and PE+PVC had lower values, indicating that, in this case, AsA could have been involved to counteract the beginning of the oxidative burst. Differently, in chronic experiments, PP-treated plants showed the highest GSH concentrations, such as for AsA. On the contrary, in PE- and PVC-treated plants, higher GSH associated to lower AsA values, can have a dualistic effect: both to directly counteract the oxidative stress following to the depletion of AsA, and to regenerate the ascorbate from the cycle of Haliwell-Asada (Foyer and Noctor, 2011). In acute exposure tests, a further different scenario is recorded: controls and PVC-treated plants showed the highest concentrations of both GSH and, in minor way, of AsA. In the case of plants treated with PVC, measured levels resulted similar to levels recorded during the chronic exposure for the hydrogen peroxide. This may support the hypothesis that stress induced by PVC is "detected" earlier by plants than stress induced by other types of microplastic considered in this

study. This hypothesis will be verified by future further investigations sized to focus on specific molecular mechanisms involved.

Generally, abiotic stresses on plants (drought-, salt-, heavy metal-, chill-, heat- stress), cause damage to the biosynthesis of chlorophylls, this is reflected in the whole metabolism of tetrapyrrole (Dalal and Tripathy, 2012). In this study, microplastics did not affect the production of chlorophylls in chronic exposure tests and these findings are in agreement with literature (Qi et al., 2018). In this study, the treatments that showed lower pigment production were controls and plants treated with PE+PVC. This could be explained by the fact that a reduced production of pigments, caused in turn by a reduced production of aminolaevulinic acid (AlA), would down-regulate the transport of electrons to reduce the production of ROS (Dalal and Tripathy, 2012). In fact, both controls and plants treated with PE+PVC, did not show a significant amount of hydrogen peroxide compared to other treatments. On the contrary, plants treated with PE and PVC, showed a greater production of hydrogen peroxide. Furthermore, the latter, showed a higher production of AlA which, as mentioned above, could work not only to counteract ROS production, but also for the chlorophyll production pathway. The accumulation of proline and its protective function during abiotic stresses (i.e. osmo-protectant and against oxidative burst activities), have already been reported in many researches (Szabados and Savoure, 2010). Interestingly, both proline and aminolaevulinic acid have almost the same trend, as reported by the results collected in this study. In fact, plants exposed to PVC-induced stress, showed the highest concentration of both proline and aminolaevulinic acids. This is an interesting result, because a previous study, on salt-induced stress demonstrated that proline and aminolaevulinic acid compete for their production because of sharing the same biosynthetic precursor (glutamic acid), in their pathway (Xiong et al., 2018). In fact, when AlA decreases, proline increases (Averina et al., 2010). Our results suggest that during microplastics-induced stress there is no competition in favour of proline production, but on the contrary, an increase in both when stress is induced, as in the case of plants treated with PVC. At this point our

results showed that, for *L. sativum*, PVC showed the highest toxicity compared to other plastics materials, in fact both proline and AlA worked to counteract the production of hydrogen peroxide.

5. CONCLUSIONS

This research represents the first paper that highlights that different types of microplastics affect the growth of garden cress in a different way during acute and chronic exposure. Furthermore, the microplastics supplied in this study are able to produce oxidative burst in tested plant. On a biochemical level, it is found that the concentration of hydrogen peroxide is always higher in acute than chronic exposure in each treatment, with the exception of plants treated with PVC. Furthermore, concerning chronic exposure to PVC, both aminolaevulinic acid and proline concentrations showed higher levels than other microplastic types. On the basis of the results obtained we can support the hypothesis that garden cress is not able to counteract PVC, and in minor way, PE toxicities in long exposure time; as conversely, is happened for the other microplastics.

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Authors' contribution: S.P = performed experiment development, statistical analyses, and the first paper draft; A.B = performed laboratory analyses; M.R. = perform fund recruitment, experimental project plan, revision of the first draft for the production of the submitted version.

Authors declare no conflict of interest.

Authors declare the ethical compliance of the research to ethic guidelines for scientific researches.

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FIGURES

Figure 1. Biochemical responses to microplastic-induced stress obtained in *Lepidium sativum* plants exposed during acute toxicity experiments (6 days). Measured levels of Hydrogen peroxide (H_2O_2), ascorbic acid (AsA), and glutathione (GSH) in *L. sativum* leaves treated with different microplastics are reported respectively in figure A, B, and C. Data are expressed as mean ± standard error (SE, *n*=3). Different letters represent statistical differences between treatment for each tested chemical (Fisher-LSD multiple comparison, $p < 0.01$ level).

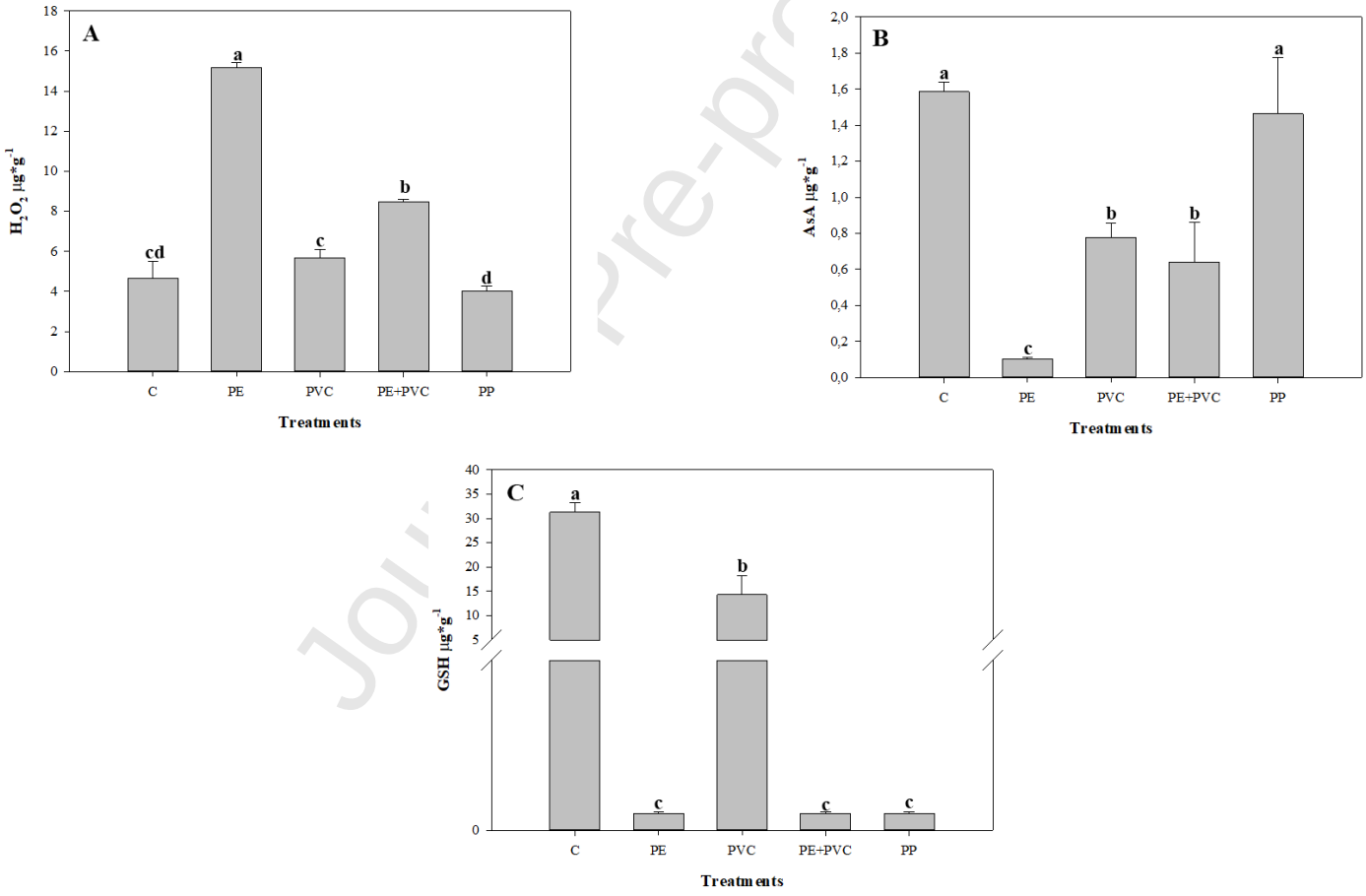


Figure 2. Biochemical responses to microplastic-induced stress obtained in *Lepidium sativum* plants exposed during chronic toxicity experiments (21 days). Measured levels of Hydrogen peroxide (H_2O_2), ascorbic acid (AsA), and glutathione (GSH) in *L. sativum* leaves treated with different microplastics are reported respectively in figure A, B, and C. Data are expressed as mean ± standard error (SE, *n*=3). Different letters represent statistical differences between treatment for each tested chemical (Fisher-LSD multiple comparison, $p < 0.01$ level).

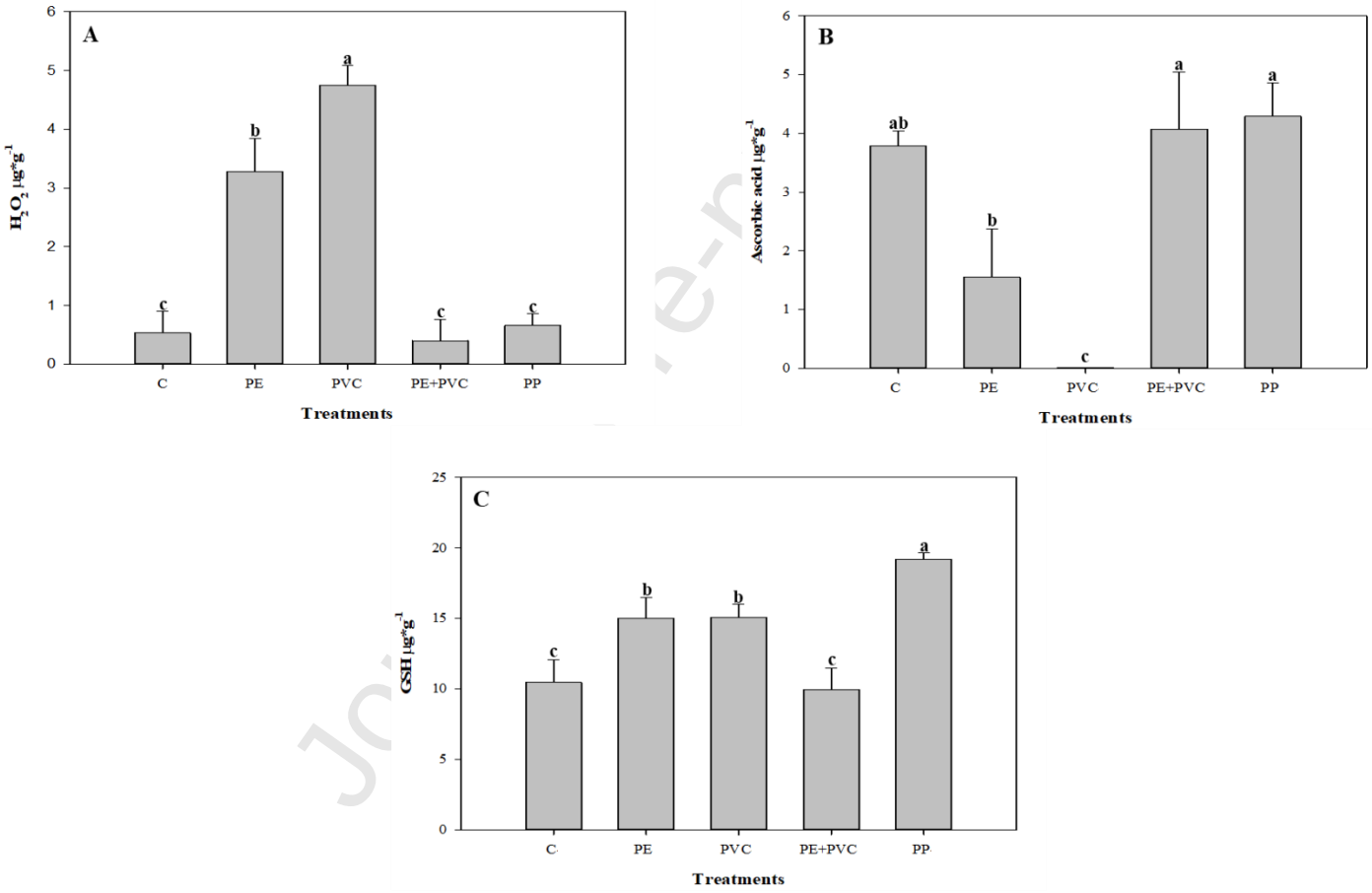


Figure 3. Pigments concentration in *L. sativum* leaves exposed to microplastic-induced stress during chronic toxicity experiments (21 days). Chlorophylls (Chl-a, Chl-b) and carotenoids (Car) are represented in Figure A, while aminolaevulinic acid concentrations are represented in Figure B. The values are expressed as mean ± standard error (SE, *n*=3). Different letters represent statistical differences between treatment for each compound (Fisher-LSD multiple comparison; $p < 0.01$ level).

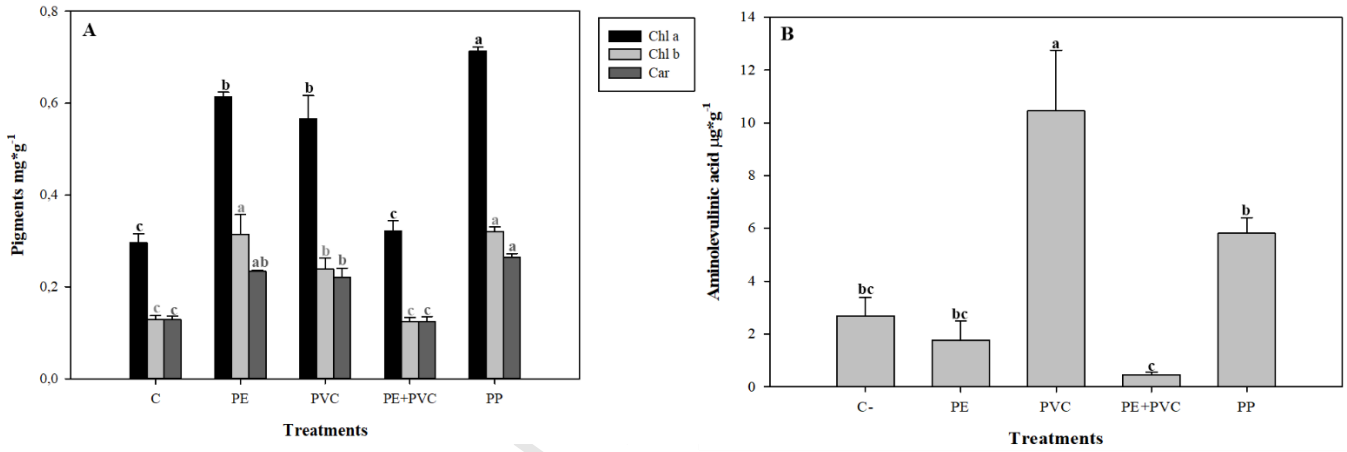
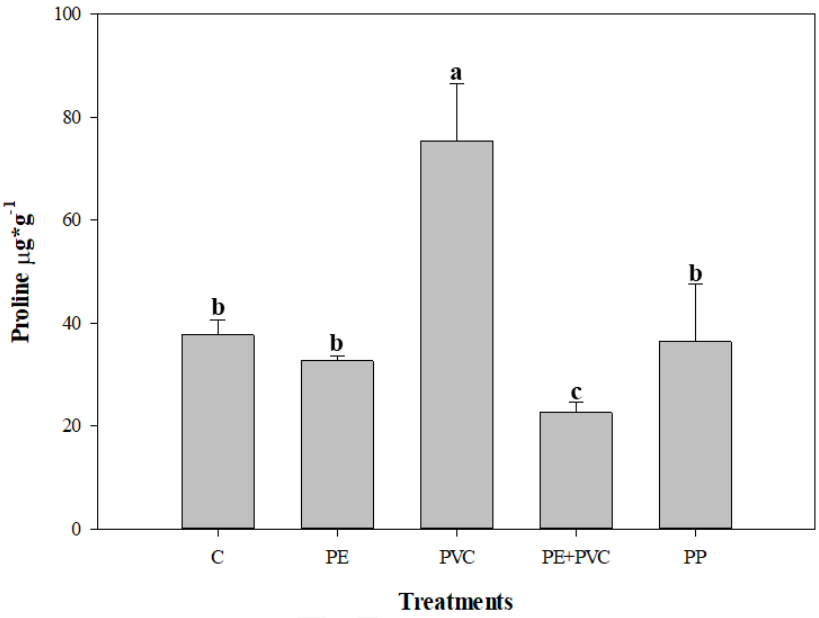


Figure 4. Proline concentration in *L. sativum* leaves exposed to microplastic-induced stress during chronic toxicity experiments (21 days). Reported values are expressed as mean ± standard error (SE, *n*=3). Different letters represent statistical differences between treatment for each compound (Fisher-LSD multiple comparison; $p < 0.01$ level).



TABLES

Table 1. **Biometrical parameters obtained in *Lepidium sativum* plants exposed during acute toxicity experiments (6 days).** Percentage of inhibition of germination (I%), shoots height (H cm), leaf number (#L), and shoots biomass (B g) exposed to different microplastics are reported as mean values ± Standard Error (SE; *n* = 10). One-way ANOVA was applied to determine significant differences between treatment and untreated controls C (*p*-level is given; * = p<0.05; ** = p<0.01; ***; *p* < 0.001; ns = not significant). Significant figures for each determination was defined according to the measurement resolution of each method (LOQ).

Acute Stress	I (%)		H (cm)		#L		B (g)	
	mean	SE	mean	SE	mean	SE	mean	SE
C	0.1	<0.01	0.38	0.05	1.8	0.2	0.02	<0.01
PE	55.0	<0.01	0.08	0.03	0.8	0.3	0.02	<0.01
PVC	22.4	<0.01	0.23	0.06	1.4	0.3	0.04	<0.01
PE+PVC	55.3	<0.01	0.18	0.07	0.8	0.3	0.03	<0.01
PP	33.0	<0.01	0.22	0.06	1.2	0.3	0.04	<0.01

***p*-level**	***	*	ns	*

Table 2. Biometrical parameters obtained in *Lepidium sativum* plants exposed during chronic toxicity experiments (21 days). Percentage of inhibition of germination (I%), shoots height (H cm), leaf number (#L), and shoots biomass (B g) exposed to different microplastics are reported as mean values ± Standard Error (SE; $n = 10$). One-way ANOVA was applied to determine significant differences between treatment and untreated controls C (p-level is given; * = p<0.05; ** = p<0.01; ***; $p < 0.001$; ns = not significant). Significant figures for each determination was defined according to the measurement resolution of each method (LOQ).

Chronic Stress	**I (%)**		**H (cm)**		**#L**		**B (g)**	
	mean	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**
C	0.0	<0.01	1.42	0.15	7.0	0.5	0.21	0.02
PE	7.1	<0.01	1.16	0.14	5.6	0.6	0.13	0.01
PVC	0.0	<0.01	1.58	0.13	6.0	0.5	0.14	0.01
PE+PVC	0.0	<0.01	1.00	0.12	7.0	0.5	0.22	0.01
PP	14.3	<0.01	1.48	0.19	5.6	0.6	0.12	0.01
***p*-level**	***		ns		ns		***	

Declaration of interests

x 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Authors' contribution:

S.P = performed experiment development, statistical analyses, and the first paper draft;

A.B = performed laboratory analyses;

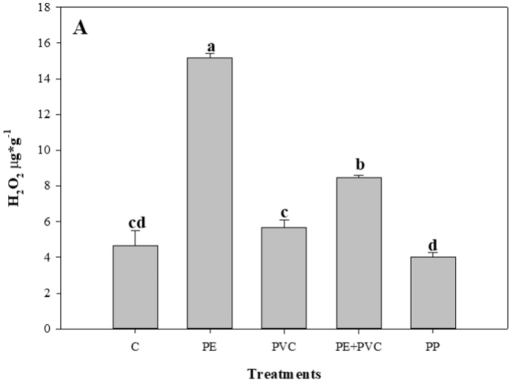
M.R. = perform fund recruitment, experimental project plan, revision of the first draft for the production of the submitted version; final revision of the manuscript.

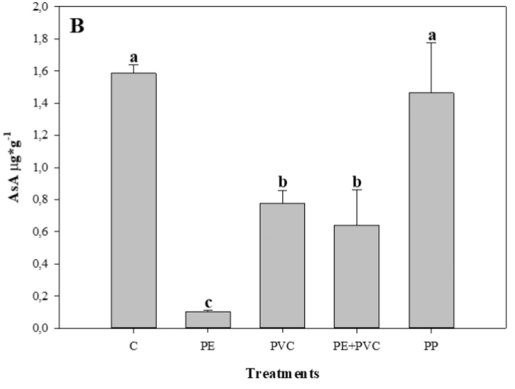
Graphical abstract

HIGHLIGHTS

- *L. sativum* was exposed to microplastics (PP, PE, PVC, PE+PVC)

- Acute and chronic effects due to different microplastics exposure were recorded
- Biometric and biomarkers of oxidative stress parameters were measured
- The occurrence of oxidative burst was highlighted in exposed plants
- PVC resulted the most toxic than other microplastics tested


A
H_2O_2 µg*g^{-1}
18
16
14
12
10
8
6
4
2
0
a
cd
c
b
d
C
PE
PVC
PE+PVC
PP
Treatments


B
AsA µg*g^{-1}
2,0
1,8
1,6
1,4
1,2
1,0
0,8
0,6
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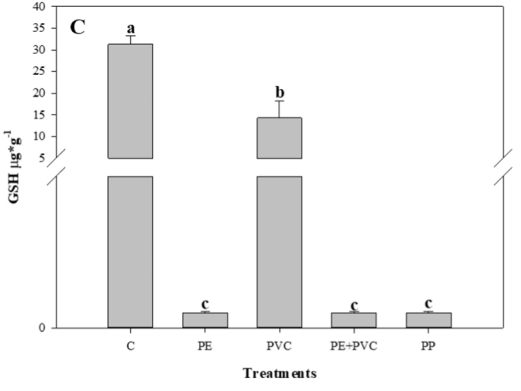
PE+PVC
PP
Treatments


C
GSH µg*g^{-1}
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35
30
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5
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C
PE
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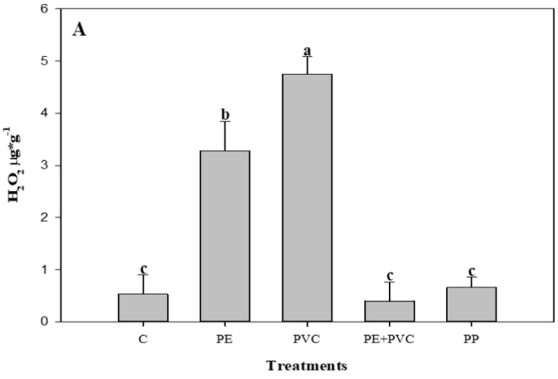
PE+PVC
PP
Treatments

Figure 1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H_2O_2 μg*g^{-1}
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1
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3
4
5
6
c
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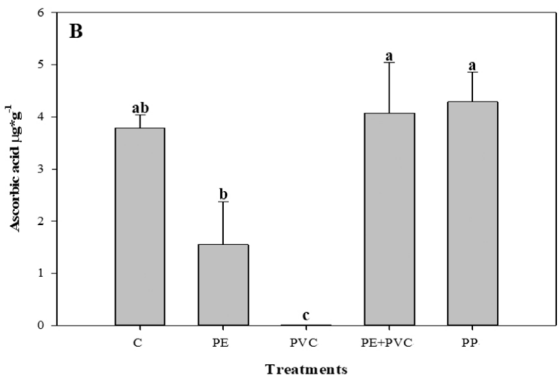
a
c
c
C
PE
PVC
PE+PVC
PP
Treatments


B
Ascorbic acid μg*g^{-1}
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ab
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c
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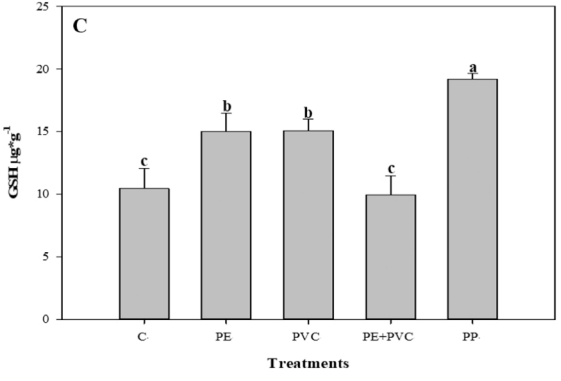
a
C
PE
PVC
PE+PVC
PP
Treatments


C
GSH μg*g^{-1}
0
5
10
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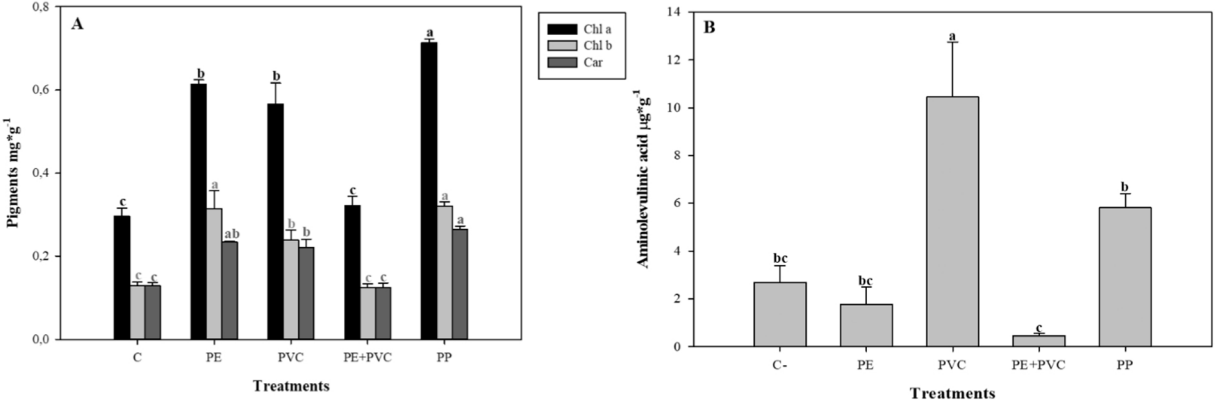
20
25
c
b
b
c
a
C
PE
PVC
PE+PVC
PP
Treatments

Figure 2


A
Pigments mg*g-1
0,8
0,6
0,4
0,2
0,0
Chl a
Chl b
Car
c
b
b
a
c
c
a
ab
b
b
c
a
c
c
a
C
PE
PVC
PE+PVC
PP
Treatments
B
Aminolevulinic acid µg*g-1
14
12
10
8
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bc
bc
a
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C-
PE
PVC
PE+PVC
PP
Treatments

Figure 3

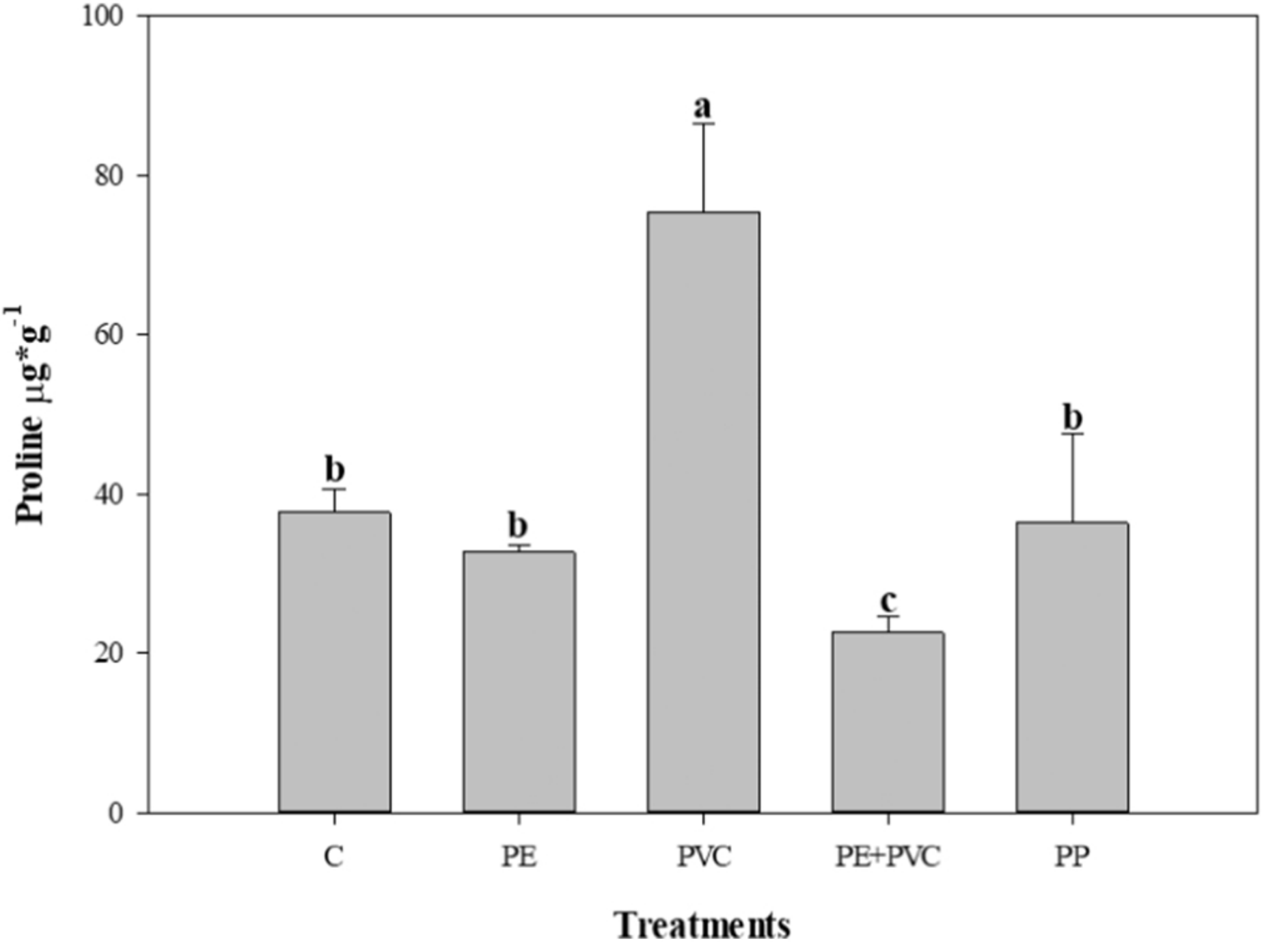
100
80
60
40
20
0
Proline µg*g-1
a
b
b
c
b
C
PE
PVC
PE+PVC
PP
Treatments

Figure 4