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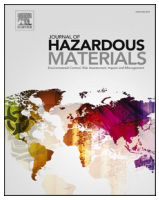

Polyethylene microplastics alter root functionality and affect strawberry plant physiology and fruit quality traits

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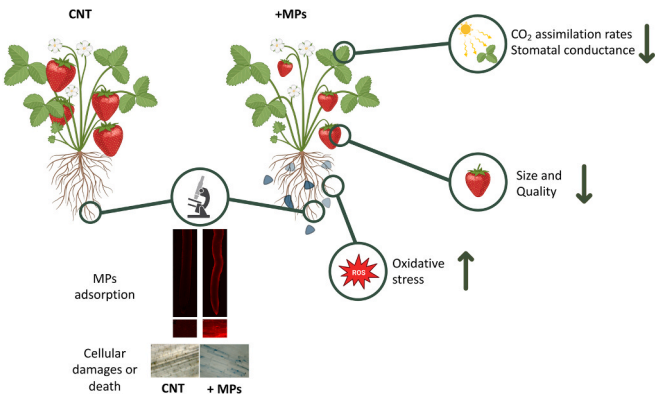
HIGHLIGHTS

- Polyethylene microplastics (PE-MPs) threaten strawberry physiology and fruit quality.
- ø35 μm/0.2% PE-MPs had the most severe impact on the roots, increasing oxidative stress.
- ø35 μm/0.2% PE-MPs impaired net CO2 assimilation due to stomatal limitations.
- ø35 μm/0.2% PE-MPs led to reduced fruit size, soluble solids, and anthocyanin content.

GRAPHICAL ABSTRACT



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ABSTRACT

Strawberry, a globally popular crop whose fruit are known for their taste and health benefits, were used to evaluate the effects of polyethylene microplastics (PE-MPs) on plant physiology and fruit quality. Plants were grown in 2-L pots with natural soil mixed with PE-MPs at two concentrations (0.2% and 0.02%; w/w) and sizes (ø 35 and 125 μm). Plant physiological responses, root histochemical and anatomical analyses as well as fruit biometric and quality features were conducted. Plants subjected to ø 35 μm/0.2% PE-MPs exhibited the most severe effects in terms of CO_2 assimilation due to stomatal limitations, along with the highest level of oxidative stress in roots. Though no differences were observed in plant biomass, the impact on fruit quality traits was severe in ø 35 μm/0.2% MPs treatment resulting in a drop in fruit weight (–42%), soluble solid (–10%) and anthocyanin contents (–25%). The smallest sized PE-MPs, adsorbed on the root surface, impaired plant water status by damaging the radical apparatus, which finally resulted in alteration of plant physiology and fruit

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quality. Further research is required to determine if these alterations also occur with other MPs and to understand more deeply the MPs influence on fruit physio-chemistry.

1. Introduction

The Anthropocene era is distinguished by the uncontrolled use of plastic, the reason for which it is often referred to as the 'plastic age' [1]. The utilization of plastic became prevalent around the mid-20th century, marked by a rapid and excessive increase in production [2]. Durability is arguably the most advantageous attribute of plastic in terms of performance, yet it can also be regarded as the most detrimental, as it contributes to environmental issues. Indeed, exposure of plastic to environmental agents results in the generation of plastic fragments with a wide range of particle size [3,4]. These plastic fragments are referred to as microplastics (MPs) when their size falls within the range of 1–5000 μm, while fragments smaller are called nanoplastics (NPs) [4,5]. Both MPs and NPs accumulate in aquatic and terrestrial environments, and they can even be released into the atmosphere, representing therefore a global concern for the biosphere [3,6,7]. The scientific community has primarily focused its attention on the accumulation and effects of MPs pollution in aquatic environments [8,9], their presence in drinking water [10], their accumulation in the sea [11], and their impacts on marine organisms and ecosystems [12]. However, it is imperative to also consider the effects of MPs accumulation in agricultural soils and to depict their effects on plant metabolism. Indeed, it is highly probable that larger MPs, rather than NPs, accumulate in heavily anthropized terrestrial environments, such as agricultural and urban areas [8,13], although the amount of NPs may be underestimated due to the technical difficulties involved in their quantification, especially in a complex matrix such as soil [14].

MPs can enter agricultural soil through various pathways. One significant route is through the application of plastic film and mulches used in usual farming practices. These materials degrade over time due to UV radiation, mechanical abrasion, and microbial activity, breaking down into smaller fragments that persist in soil for extended periods [15]. Another possible source is irrigation with contaminated water or the application of sewage sludges, which can contain MPs from domestic and industrial sources [15].

MPs possess the capability to engage in specific interactions with plant physiology [16–18]. They can be adsorbed over the root surfaces or alternatively absorbed and translocated to plant tissues [19]. In fact, very small (⌀<5 μm) plastic materials can enter plant tissue through the roots and be translocated to the shoots, leading to direct and indirect damage characterized by increased oxidative stress, cytotoxicity, and genotoxicity [5,20,21]. For instance, Gentili et al. [13] documented a negative impact of MPs on plant biometric traits and photosynthetic efficiency in *Senecio inaequidens* and *Centaurea cyanus* plants cultivated in soil contaminated with polyvinyl chloride (PVC) MPs. Moreover, also Colzi et al. [22] observed a significant dose-dependent reduction of the photosynthetic efficiency and the chlorophyll content in *Cucurbita pepo* plants grown in soil contaminated with PVC and polyethylene (PE) MPs. These observed effects may arise because, once MPs accumulate in the rhizosphere, they can limit water infiltration and alter nutrient absorption, resulting in diminished plant transpiration, reduced nitrogen uptake, and ultimately hampered overall plant growth [18–23].

Strawberry (*Fragaria* × *ananassa* Duch) is a species belonging to the Rosaceae family and developed three centuries ago through spontaneous hybridization of two ecotypes: *Fragaria chiloensis* subsp. *chiloensis* from South America and *Fragaria virginiana* subsp. *virginiana* from North America [24]. Strawberry fruit is appreciated for its organoleptic properties and its richness in bioactive phytochemicals [25]. Strawberry fruit are often shielded from pathogens and weeds using mulching films, preventing their direct contact with the soil and thereby reducing horticultural product loss [16,24]. This agricultural technique, commonly employed in both open fields and greenhouses, predominantly involves the use of plastic films, especially those based on PE. The high durability of these PE-based products can be attributed to PE's high molecular weight and hydrophobicity [24,26]. Once used in open field, PE mulching films can break down into smaller fragments due to physical and chemical agents, ultimately reducing the MPs size [15,16]. Since previous studies found some negative impacts of PE-MPs on plant physiology [22,23,27], the intricate relationship between physiology and PE-MPs, particularly concerning root functionality and leaf gas exchange, has yet to be comprehensively understood, especially when plants grow in a real soil system.

Against this backdrop, the primary objective of our study was to delve deeper into the potential adverse effects of PE-MPs on the physiology of strawberry plants, focusing particularly on their growth, photosynthetic traits, and root functionality. Moreover, given that the quality of fruit is an important attribute for consumers, we also sought to determine if MPs might lead to any discernible alterations in the quality of the produced strawberry fruit. For this investigation, we utilized PE-MPs with ø of 35 and 125 μm. To understand the range of impact, we introduced these MPs at both low (0.02% w/w) and high (0.2% w/w) concentrations. The findings from this research is important not only for the strawberry farming community but also for broader agricultural practices, as they try to shed light on the long-term implications of plastic pollutants in the soil.

2. Material and methods

2.1. Plant material, growth conditions and experimental design

The experiment was carried out from October to December 2022 in a greenhouse located at the Department of Agriculture, Food and Environment of the University of Pisa (43.711249° N, 10.412219° E). Strawberry plantlets (*Fragaria* × *ananassa* var. Alba) were purchased in a local nursery and transplanted into 2-L pots filled with natural soil collected from an uncontaminated natural park area close to Pisa (Regional Park Migliarino San Rossore Massaciuccoli, Pisa, Italy). Treated soils were prepared by mixing certified PE powder purchased from Goonvean fibers Ltd (Cullompton, Devon, UK) with the natural soil. Firstly, the soil was sieved using a stainless-steel test sieve (particle size $\leq$ 2 mm); thereafter, the soil was mixed with the different concentrations/sizes of PE-MPs and additionally passed through the sieve to ensure a homogeneous distribution of MPs. The experimental design consisted of five treatments with five plants each (n = 5). The control group (CNT) consisted of plants growing in uncontaminated soil, while treated plants received one of the following treatments: 0.02% (w/w) of 35 μm ø MPs (P1), 0.2% (w/w) of 35 μm ø MPs (P2), 0.02% (w/w) of 125 μm ø MPs (P3) or 0.2% (w/w) of 125 μm ø MPs (P4). Treated and CNT soils were fertilized with a slow-release fertilizer (8 g plant^{-1}). Physiological analyses were conducted when the plants reached full maturity (fruiting stage), approximately two months after transplantation, while destructive biometric measurements were performed at the fruit harvest. Strawberry fruit were harvested between the 59th and 73rd day after transplantation (commercial maturity stage), immediately ground and frozen in liquid nitrogen, and stored at –80 °C until biochemical analysis.

2.2. Plant and fruit biometric measurements and anatomical and histochemical analysis

The number of leaves and length of petioles were recorded in four different plants. Above- and below-ground fresh biomasses were

determined by weighing the entire aerial and hypogeal parts of four different plants. Above- and below-ground dry matters were obtained by drying the aerial and hypogeal parts of three different plants at 105 °C in a thermostatic oven (Memmert GmbH + Co. KG Universal Oven UN30, Schwabach, Germany) until a constant weight was reached.

Strawberry fruit were harvested at the commercial stage, ensuring that fruit from different treatments had reached a similar ripening level (75–90% redness). All the fruit were weighed to assess overall productivity. Each individual fruit was analyzed in terms of height and width, measured at the midpoint of the fruit.

Nile red dye was prepared from 9-(Diethylamino)− 5 H-benzo[*a*] phenoxazin-5-one powder (TCI America Division of Tokyo Chemical Industry). It was solubilized in acetone (500 mg mL^{-1}) and stored at a low temperature as a stock solution. Five roots at a similar growth stage were randomly selected from both control and treated plants for staining. These roots were thoroughly washed with deionized water to remove soil residues, and then immersed in a Nile Red working solution. The working solution was freshly prepared by adding 5 μL of the stock solution to 1 mL of 75% glycerol. After 15 min of staining, root analysis was conducted using a Leica DMLB fluorescence microscope equipped with an appropriate set of excitation/emission filters for red fluorescence (515–560 nm). The most representative images were captured using a Leica DFC7000 T camera.

Similarly, for Evans Blue staining, five roots at an equivalent growth stage were randomly chosen from both control and treated plants. These roots were thoroughly washed to remove any soil residues and then immersed in a 0.05% (w/v) Evans Blue solution (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for 30 min, following the method described by Bellini et al. [28]. Analyses were performed on the apical segment of the roots using a Leica Leitz DM RB microscope equipped with a Leica DFC420 digital camera.

2.3. Leaf gas exchange and chlorophyll (chl) a fluorescence analysis

Gas exchange analyses were measured (n = 6) using a portable infrared gas analyser, the *LI-6400* System (Li-Cor, Lincoln, NE, USA). Gas exchange measurements were conducted on randomly selected fully expanded leaves from 11:00 a.m. to 1:00 p.m. under an ambient light intensity of 500 μmol m^{-2} s^{-1}. Within the leaf chamber, the CO_2 concentration was maintained at 400 μmol mol^{-1} using a CO_2 mixer, the leaf temperature was set to approximately 20 °C, relative humidity was maintained at ~50%, and the flow rate was set at 500 μmol s^{-1}. Once a steady state was reached, net photosynthetic rate (P_n), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were determined. Chl *a* fluorescence parameters were measured using a Plant Efficiency Analyzer fluorimeter (Handy PEA, Hansatech Ltd, Norfolk, UK). Measurements (n = 6) were conducted at the same time with gas exchange analysis. Leaves were dark-adapted with leaf clips (4 mm diameter) for 20 min, then samples were lightened for one second with a saturating (up to 2700 μmol photons m^{-2} s^{-1}) red light pulse and fluorescence emission was recorded for one second. Then, the effects of the treatments were assessed by the maximum quantum yield of PSII photochemistry (F_v/F_m), where $F_v = (F_m - F_0)$ is the variable fluorescence, F_0 is the basal fluorescence prior saturation pulse extrapolated from the line of best fit determined through the initial data point recorded at the onset of illumination, and F_m is the maximum Chl *a* fluorescence after saturating light pulse.

2.4. Extraction and determination of H_2O_2 content and thiobarbituric acid reactive substances (TBARS)

The concentration of H_2O_2 in both roots and leaves was determined according to Jana and Choudhuri [29] method. Samples were homogenized in a phosphate buffer (50 mM, pH 6.5), and the resulting homogenate was subjected to centrifugation at 6000 × *g* for 25 min·H_2O_2 levels were assessed spectrophotometrically (Shimadzu - UV mini-1240 spectrophotometer) at 410 nm using a solution containing 0.1% titanium chloride in 20% (v/v) H_2SO_4. The H_2O_2 concentration was expressed as μmol g^{-1} fresh weight (FW), determined by reference to a standard curve. Lipid peroxidation in both roots and leaves was estimated by quantifying the levels of thiobarbituric acid reactive substances (TBARS), following the procedure outlined in Spanò et al. [30]. Briefly, roots and leaves were homogenized using 5% trichloroacetic acid (TCA), and the resulting extracts were mixed with a reagent composed of 5% (w/v) TCA and 0.5% (w/v) thiobarbituric acid (TBA). The mixture was heated at 95 °C for 30 min, allowed to cool for 15 min, and then subjected to centrifugation at 2000 *g* for 15 min. The concentration of TBARS, expressed as nmol g^{-1} FW, was determined by measuring the absorbance at 532 nm after subtracting the nonspecific absorbance at 600 nm. Calculations were performed using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.5. Fruit firmness, soluble solid content, titratable acidity and dry matter

Fruit firmness (n = 4) was measured by penetrating strawberry fruit with a Turoni penetrometer, model 53205 (T.R. Turoni, Forlì, FC, Italy). The compressive force (kg cm^{-2}) required to induce a 5 mm deformation in the fruit was recorded. Subsequently, each fruit (n = 4) under investigation was homogenized in a mortar to extract fruit juice for the analysis of soluble solid content (SSC) and titratable acidity (TA). The SSC was measured using a digital refractometer (Atago, Tokyo, Japan) and expressed as a percentage (%). For TA determination (n = 4), 1 g of fruit was diluted with 30 mL distilled H_2O, then titrated with 0.1 N NaOH until reaching pH 8. TA was expressed as citric acid %. Fruit dry matter (n = 4) was obtained by oven-drying the fruit at 105 °C until constant weight was reached.

2.6. Total phenolic content (TPC) and total anthocyanin content (TAC) assays

The TPC (n = 3) was determined following the method of Dewanto et al. [31]. The oxidation of phenolic compounds coupled with the reduction of metals within the phosphomolybdate-phosphotungstate solution of the Folin-Ciocalteu reagent were measured spectrophotometrically (Ultrospec 2100 Pro, GE Healthcare Ltd., Chalfont, Buckinghamshire, UK) at 760 nm. Results of the TPC were obtained using the following calibration curve (0–1000 μM gallic acid) and solving for x:

$$y = 0{\cdot}0008x + 0{\cdot}0025$$

Results were expressed as mg gallic acid equivalents (GAE) g^{-1} FW.

The TAC (n = 3) was determined using the pH differential method [32] with minor modifications. An amount (0.1 g) of fruit was homogenized in a mortar with 1 mL of acidified methanol (1% HCl; v/v). The homogenate was centrifuged at 10,000 × *g* for 10 min and the supernatant was recovered and used as extract. An aliquot (100 μL) of extract was incubated for 15 min with a 0.025 M potassium chloride buffer (pH 1.0) and, at the same time, another aliquot (100 μL) was incubated with a 0.4 M sodium acetate buffer (pH 4.5). The absorbance was recorded at 530 (A_{530}) and 700 nm (A_{700}) of both mixtures. Then, the final absorbance (A_f) of diluted samples was calculated as follows:

$$A_f = (A_{530} - A_{700})_{pH1{\cdot}0} - (A_{530} - A_{700})_{pH4{\cdot}5}$$

The calculation of TAC in the original sample was performed using the following equation:

$$TAC = (((A_f \times{\cdot} MW \times DF)/ \varepsilon \times l)^* mL_{ex})/g_s)$$

where MW is the molecular weight of pelargonidin-3-glucoside (468.8 g mol^{-1}), DF is the dilution factor, ε is the molar absorptivity (15600 L mol^{-1} cm^{-1}), l is the cuvette pathway length (cm), L_{ex} is the volume of the original extract expressed in (mL), and g_s is the sample mass. Results were expressed as mg pelargonidine-3-glucoside g^{-1} FW.

2.7. Total ascorbic acid content (ASA) assay

Fresh fruit material (about 0.1 g; n = 3) were homogenized with 6% (w/v) TCA. After the sample centrifugation (14,000 × *g* for 10 min at 4 °C), the supernatant was collected and used for the assay. ASA content was determined spectrophotometrically as described by Kampfenkel et al. [33] with minor modifications. Briefly, 50 μL of extract were incubated at room temperature for 10 min with 50 μL of 10 mM dithiothreitol (DTT) and with 100 μL Na-P buffer 0.2 M (pH 7.4). After 10 min, 50 μL of 0.5% (w/v) N-ethylmaleimide (NEM) were added and samples were vortexed vigorously. TCA (250 μL; 6% (w/v)), 200 μL of 42% (w/v) H_3PO_4, 200 μL of 4% (w/v) 2,2'-dipyridil and 100 μL of 3% (w/v) $FeCl_3$ were added to the samples and incubated at 42 °C for 40 min. The increase in absorbance at 525 nm was measured against a blank solution (without sample). Results were obtained using the following calibration curve (0–1000 μM ascorbic acid; ASA) and solving for x:

$$y = 0.1025x + 0.0169$$

Results were expressed as mg ascorbic acid (ASA) g^{-1} FW.

2.8. Antioxidant activity assays

The antioxidant activity was determined on the same extract utilized for TPC assay by using two different assays: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay as described by Brand-Williams et al. [34] with minor modifications, and the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical cation-based assay as described by Re et al. [35] with minor modifications.

For the DPPH assay (n = 3), 10 μL of sample extract were added to 990 μL of DPPH solution 3.12×10^{-5} M and incubated in the dark for 30 min at room temperature. The decrease in absorbance at 515 nm was measured against a blank solution (without extract). Results were obtained using the following calibration curve (0–1000 μM Trolox) and solving for x:

$$y = 0{\cdot}0003x - 0{\cdot}0208$$

Results were expressed as mg Trolox equivalents (TE) g^{-1} FW.

For the $ABTS^{\bullet+}$ assay (n = 3), 50 μL of suitably diluted extract sample were added to 950 μL of $ABTS^{\bullet+}$ working solution obtained by incubating 7 mM (w/v) ABTS buffer (5 mM (w/v) NaH_2PO_4-H_2O, 5 mM (w/v) Na_2HPO_4-2 H_2O, pH 7.4) with 2.5 mM (w/v) $K_2S_2O_8$ and the absorbance at 734 nm was monitored for 90 s. Results were obtained using the following calibration curve (0–1000 μM Trolox) and solving for x:

$$y = 0{\cdot}0013x - 0{\cdot}019$$

Results were expressed as mg TE g^{-1} FW.

2.9. Statistical analysis

All data (± standard deviation; SD), fruit yield, plant and fruit biometric measurements, leaf gas exchange and chl *a* fluorescence analysis, leaf and root TBARS assay, leaf and root H_2O_2 concentration and fruit organoleptic and antioxidant analyses, were analyzed by one-way analysis of variance (ANOVA) with different MPs treatments (P1, P2, P3 and P4) considered as source of variation. All the means were separated by Fisher's least significant difference (LSD) *post-hoc* test ($P \leq 0.05$). The normality of data was tested using Shapiro-Wilk test, whilst the homoscedasticity was tested using Bartlett's test. These statistical analyses were conducted using GraphPad (GraphPad, La Jolla, CA, USA).

3. Results

3.1. Effect of PE-MP concentrations on plant biomass and biometric features and anatomical and histochemical analysis on roots

The investigation of the effects of different treatments on plant biomass and biometric features highlighted no significant statistical differences among CNT, P1, P2, P3 and P4 treatments (Table 1). Indeed, across the parameters assessed, no discernible variations emerged among treatments for the number of leaves and petiole length. Moreover, the analysis of shoot and root biomass, as well as shoot and root dry matter, supported the trend of no differences across the treatments tested. Despite the diverse treatments analysed, no treatment-induced shifts in biomass allocation or dry matter accumulation were detected (Table 1).

The evaluation of the adsorption of MPs in root tissue has been performed using Nile Red, a fluorescent stain commonly used for lipid tissue staining, and recently recommended for effective MPs identification [20,36]. In comparison to CNT, the red fluorescent intensity was well detectable and brighter in the treated samples, especially in the P2 roots (Fig. 1), where the stain was distinctly visible in the columella region (Fig. 1c). Additionally, MP aggregates were observed as adsorbed materials on the external root tissue (Fig. 1b, c, d, e).

To investigate whether the presence of PE-MPs in the growing medium led to cell death or damage in the root cells, Evans Blue staining was applied. This dye did not penetrate living cells with intact membranes, as occurred in the CNT roots (Fig. 2). Conversely, root tips from the treated plants showed localized regions within the tegmental tissues where groups of cells, often elongated in shape, displayed positive staining (Fig. 2b, c, d, e). Furthermore, the root cup of P2 samples showed heightened responsiveness to the dye (Fig. 2c).

3.2. Influence of different PE-MP concentrations and size on gas exchanges and chl a fluorescence on strawberry leaves

In terms of gas exchange analysis, statistically significant differences were only observed in strawberry leaves exposed to the smallest MPs (ø 35 μm) at the highest concentration in the soil (2% w/w) when compared to the CNT. Specifically, the P_n parameter exhibited a significant decrease of 29% in P2 plants compared to CNT plants (Fig. 3A). A similar trend was observed for g_s, which decreased by 23% in P2 plants compared to CNT plants (Fig. 3B). No significant variations were observed in C_i and F_v/F_m values when comparing plants treated with MPs to those of CNT group (Fig. 3C, D).

Table 1
Number of leaves, petiole length, shoot biomass, root biomass, shoot dry matter, root dry matter measured in plants of *Fragaria* × *ananassa* var. Alba grown in uncontaminated soil (CNT), or treated soil with 0.02% (w/w) 35 μm ø polyethylene- (PE) microplastics (MPs; P1), 0.2% (w/w) 35 μm ø PE-MPs (P2), 0.02% (w/w) 125 μm ø PE-MPs (P3) and 0.2% (w/w) 125 μm ø PE-MPs (P4). Means with different letters are significantly different after Fisher's LSD *post-hoc* test ($P \leq 0.05$).

	CNT	P1	P2	P3	P4
Number of leaves (n° plant^{-1})	6.25 ± 2.99^a	5.25 ± 1.89^a	6.25 ± 2.63^a	6.00 ± 2.16^a	6.00 ± 1.63^a
Petiole lenght (cm)	15.70 ± 1.38^a	16.15 ± 1.74^a	15.33 ± 3.97^a	16.83 ± 1.05^a	14.89 ± 2.99^a
Shoot biomass (g plant^{-1})	10.05 ± 3.11^a	8.95 ± 4.89^a	9.49 ± 5.78^a	12.18 ± 4.70^a	9.71 ± 4.94^a
Root biomass (g plant^{-1})	14.85 ± 3.58^a	13.58 ± 6.86^a	15.96 ± 6.86^a	16.22 ± 4.58^a	14.13 ± 8.49^a
Shoot dry matter (%)	24.44 ± 1.56^a	24.58 ± 0.21^a	26.15 ± 0.38^a	24.71 ± 0.52^a	24.85 ± 1.53^a
Root dry matter (%)	19.44 ± 1.08^a	20.99 ± 1.83^a	21.07 ± 1.56^a	21.40 ± 0.46^a	19.26 ± 1.79^a

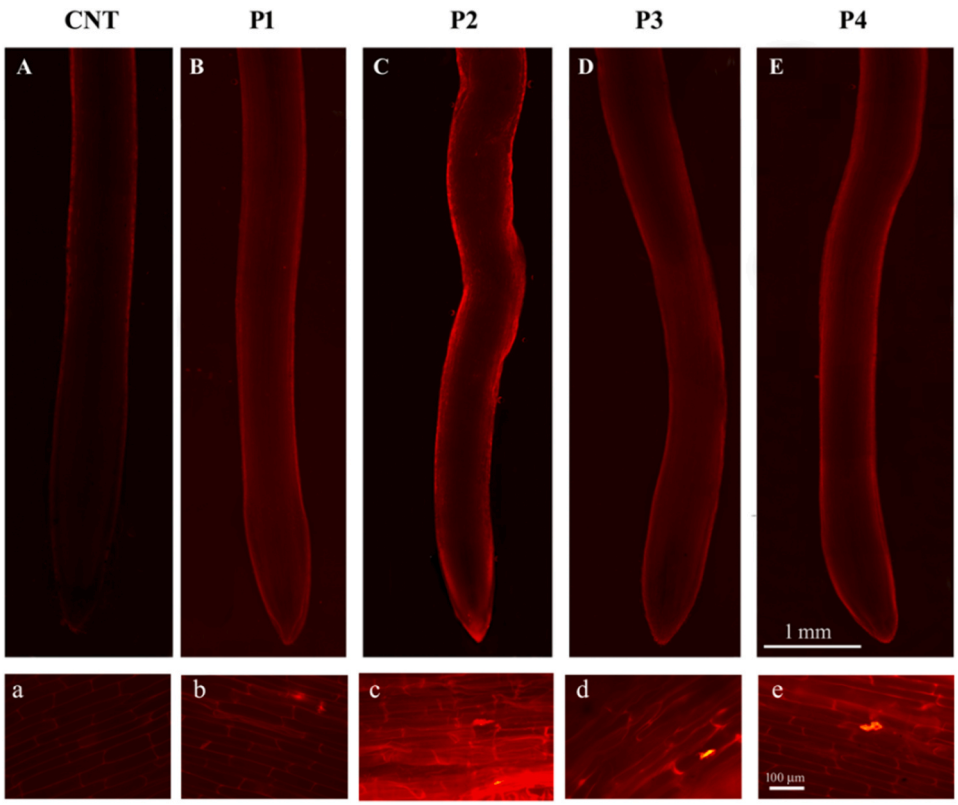

Fig. 1. Nile red staining in roots of *Fragaria* × *ananassa* var. Alba grown in uncontaminated soil (CNT; A,a), or treated soil with 0.02% (w/w) 35 µm ø polyethylene- (PE) microplastics (MPs; P1; B,b), 0.2% (w/w) 35 µm ø PE-MPs (P2; C,c), 0.02% (w/w) 125 µm ø PE-MPs (P3; D,d) and 0.2% (w/w) 125 µm ø PE-MPs (P4; E,e).

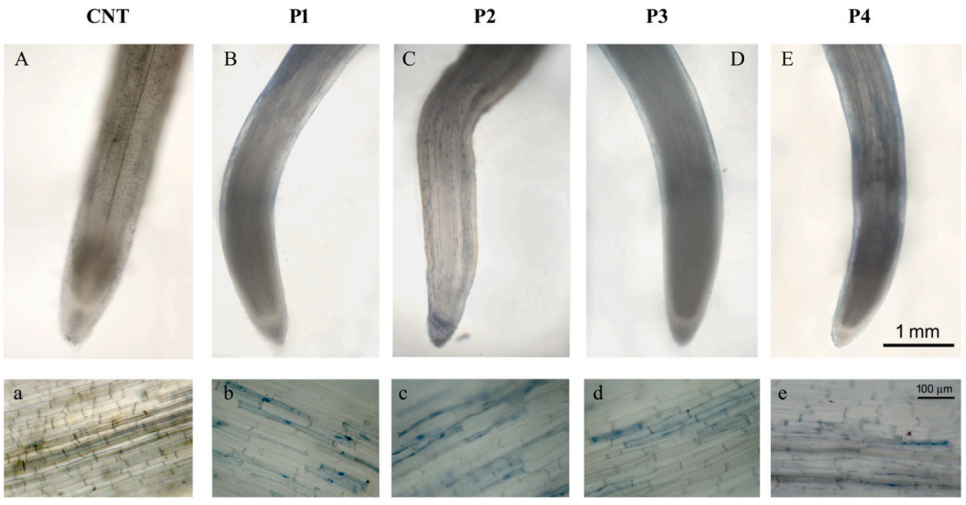

Fig. 2. Evans Blue staining in roots of *Fragaria* × *ananassa* var. Alba grown in uncontaminated soil (CNT; A,a), or treated soil with 0.02% (w/w) 35 µm ø polyethylene- (PE) microplastics (MPs; P1; B,b), 0.2% (w/w) 35 µm ø PE-MPs (P2; C,c), 0.02% (w/w) 125 µm ø PE-MPs (P3; D,d) and 0.2% (w/w) 125 µm ø PE-MPs (P4; E,e).

3.3. Effect of PE-MP concentrations and size on oxidative stress of plant tissues

The H_2O_2 content exhibited consistent patterns depending on the concentrations and size of MPs in both roots and leaves (Fig. 4A, B). Notably, the highest H_2O_2 levels were recorded in response to a 0.2% (w/w) concentration of ø 35 µm PE-MPs treatment. In the case of roots, the highest value of H_2O_2 content related to the highest oxidative damage, as indicated by the TBARS assay (Fig. 4C). Conversely, in leaves, the highest oxidative damage was observed in plants treated with MPs ø 125 µm (Fig. 4D).

3.4. Effect of PE-MP concentrations and size on fruit quality

In P3 plants, strawberry fruit exhibited the highest weight, whilst the lowest value was observed in fruit from P2 plants (Table 2). The fruit height was lower in fruit from P2 plants compared to those from both CNT plants and plants from the other treatments (Table 2). Similarly, the fruit width was lower in fruit from P2 and P4 plants in comparison to those from CNT, P1, and P3 plants. No significant differences were

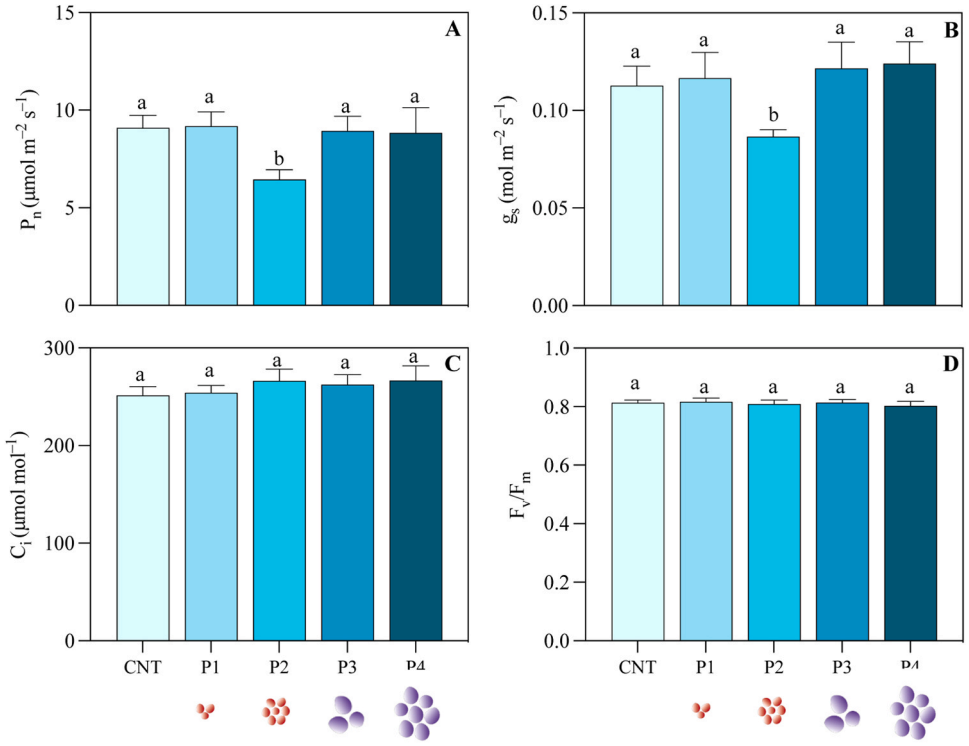

Fig. 3. Net photosynthetic rate (P_n; A); stomatal conductance (g_s; B); C: intercellular CO_2 concentration (C_i; C) and maximum quantum yield of primary PSII photochemistry (F_v/F_m; D) measured in plants of *Fragaria* × *ananassa* var. Alba grown in uncontaminated soil (CNT), or treated soil with 0.02% (w/w) 35 μm ø polyethylene- (PE) microplastics (MPs; P1), 0.2% (w/w) 35 μm ø PE-MPs (P2), 0.02% (w/w) 125 μm ø PE-MPs (P3) and 0.2% (w/w) 125 μm ø PE-MPs (P4). Means with different letters are significantly different after Fisher's LSD *post-hoc* test ($P \leq 0.05$).

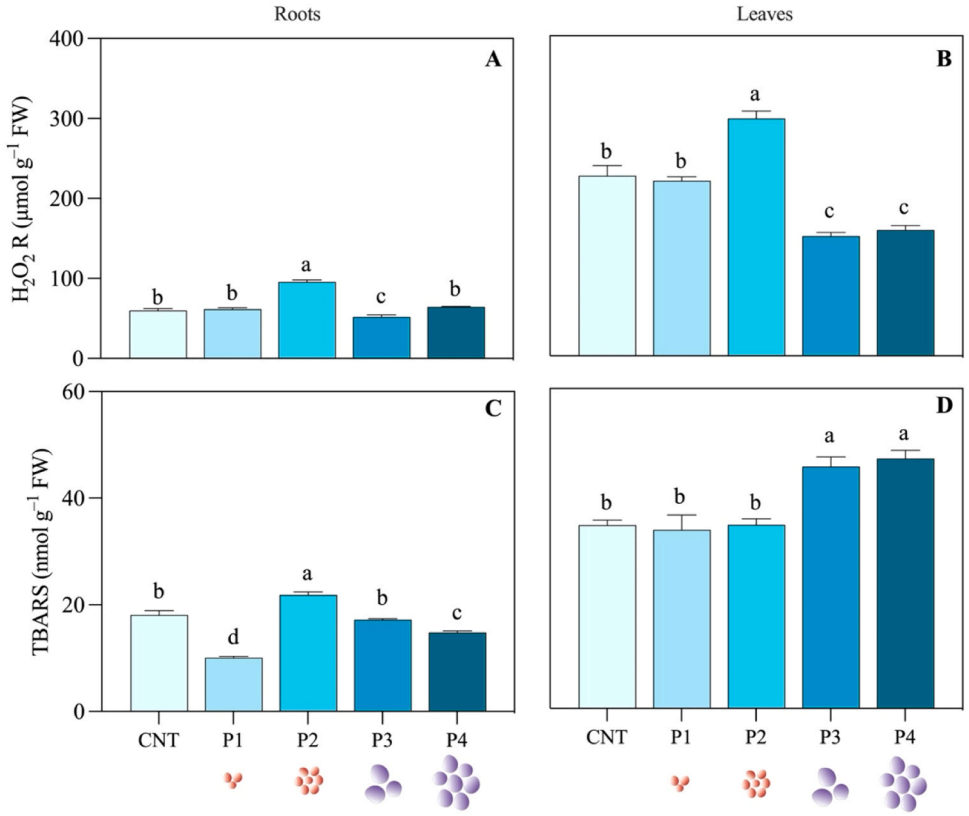

Fig. 4. Concentrations of H_2O_2 and thiobarbituric acid reactive substances (TBARS) in roots (A,C) and leaves (B,D) of *Fragaria* × *ananassa* var. Alba grown in uncontaminated soil (CNT), or treated soil with 0.02% (w/w) 35 μm ø polyethylene- (PE) microplastics (MPs; P1), 0.2% (w/w) 35 μm ø PE-MPs (P2), 0.02% (w/w) 125 μm ø PE-MPs (P3) and 0.2% (w/w) 125 μm ø PE-MPs (P4). Means with different letters are significantly different after Fisher's LSD *post-hoc* test ($P \leq 0.05$).

Table 2
Weight, biometric parameters, dry matter, solid soluble content (SSC), firmness, pH and titratable acidity (TA) of strawberry fruit of *Fragaria* × *ananassa* var. Alba collected by plants grown in uncontaminated soil (CNT), or treated soil with 0.02% (w/w) 35 μm ø polyethylene- (PE) microplastics (MPs; P1), 0.2% (w/w) 35 μm ø PE-MPs (P2), 0.02% (w/w) 125 μm ø PE-MPs (P3) and 0.2% (w/w) 125 μm ø PE-MPs (P4). Means with different letters are significantly different after Fisher's LSD *post-hoc* test ($P \leq 0.05$).

	CNT	P1	P2	P3	P4
Fruit weight (g)	6.56 ± 0.29^{b}	5.71 ± 0.09^{b}	3.81 ± 0.58^{d}	7.99 ± 1.25^{a}	5.22 ± 1.34^{c}
Fruit height (mm)	25.72 ± 3.83^{a}	24.75 ± 3.74^{a}	20.12 ± 1.55^{b}	27.19 ± 1.01^{a}	25.84 ± 3.18^{a}
Fruit width (mm)	21.35 ± 0.96^{a}	22.75 ± 1.69^{a}	18.86 ± 1.43^{b}	22.59 ± 1.11^{a}	19.26 ± 1.38^{b}
Fruit dry matter (%)	9.02 ± 1.75^{a}	8.86 ± 1.52^{a}	7.96 ± 2.23^{a}	7.26 ± 1.61^{a}	9.01 ± 1.91^{a}
SSC (%)	8.08 ± 0.44^{a}	7.7 ± 0.41^{a}	7.29 ± 1.01^{b}	7.71 ± 0.72^{a}	8.46 ± 0.39^{a}
Firmness (kg cm^{-2})	1.5 ± 0.31^{a}	1.15 ± 0.13^{a}	1.33 ± 0.29^{a}	1.15 ± 0.1^{a}	1.15 ± 0.26^{a}
pH	3.56 ± 0.15^{a}	3.37 ± 0.25^{a}	3.58 ± 0.31^{a}	3.26 ± 0.44^{a}	3.59 ± 0.40^{a}
TA (%)	0.95 ± 0.12^{b}	1.24 ± 0.29^{a}	0.93 ± 0.11^{b}	1.19 ± 0.06^{a}	0.95 ± 0.08^{b}

observed in fruit dry matter (Table 2). Fruit collected from P2 plants reported also lower SSC, while no significant differences were observed among treatments in terms of fruit firmness and pH (Table 2). Fruit collected from P1 and P3 plants showed higher TA percentages when compared to CNT, P2 and P4.

Although no significant differences were observed in terms of TPC (Fig. 5A), the TAC was higher in fruit from P1 plants, followed by those collected from CNT, P3 and P4 plants. The lowest TAC values were found in fruit from P2 plants (–20% than CNT; Fig. 5B). In contrast, ASA content of strawberries from P1 and P2 plants was similar to that of fruit from CNT plants, while fruit from P3 and P4 plants exhibited significantly lower ASA contents (Fig. 5C).

Although the results of the DPPH assay followed the TPC content, no significant differences among the MPs treatments were found (Fig. 6A); the ABTS assay results indicated the highest value in fruit from P3 plants, followed by fruit from P2 plants. Fruit from P1 and P4 plants did not exhibit significant differences; however, they were higher in value compared to fruit from CNT plants (Fig. 6B).

4. Discussion

The term MP encompasses a broad spectrum of compounds, and the presence of one type over another can trigger distinct responses in both plant and soil ecosystems. In this study, we specifically examined PE-MPs, as PE mulching films used in open fields are prone to degradation from both physical and chemical factors, potentially releasing MPs on the soil. MPs can interact with plant root apparatus, adversely affecting plant physiology and development [37–39]. The present experiment shows no significant variation in the biometric traits of plants subjected to PE-MPs. Colzi et al. [22] found that the application of PE-MPs to the soil did not impact the biomass of *Cucurbita pepo* plants, using MP particles with a ø of 40–50 μm at concentrations of 0.02 to 2% (concentrations similar to our study), in accordance with the results of the present study. Additionally, in a study conducted by Meng et al. [40] on *Phaseolus vulgaris* plants, it was found that the presence of PE-MPs, with particle ø ranging from 250 to 1000 μm, at concentrations ranging from 0.5% to 2.5% (w/w), did not yield significant effects on the shoot and root biomass. Interestingly, Colzi et al. [22] documented that PE-MPs (ø of 40–50 μm at concentrations of 0.02 to 2%), compared to other MPs of the same size and concentration (i.e., PVC-, and polyethylene terephthalate- [PET] based) had a milder adverse effect on plant biomass growth probably due to the lower impact on the soil structure compared to other MP types. However, Pinto-Poblete et al. [39], using the MPs derived by the shredding of a PE mulching film (a mix that ranged from ø 1 mm to 5 mm) at a concentration of 0.02%, found a significant reduction in total plant biomass in strawberry plants. This contradictory observation, concerning our results and those of Colzi et al. [22] and Meng et al. [40], was likely due to the larger PE-MPs used (ø 1 mm to 5 mm), which were able to reduce plant root length and volume thus altering the development of aerial biomass [39]. Therefore, though most studies did not show a significant impact on plant biomass, the effect of MP sizes and a MP species-specific interaction on plant biomass still needs to be fully elucidated in a broad range of crop species.

The absence of detrimental effects on plant biometric traits observed in our study does not necessarily exclude the potentially harmful effect of MPs on root functionality. This assertion is supported by recent studies highlighting the direct adverse effects on root functionality when hydroponically cultivated plants were exposed to PE-MPs [18,23,41]. In light of these findings, our study aimed to further examine the potential harm to plant root system functionality when PE-MPs were introduced into the soil, and consequently, to assess their impact on plant physiology. Firstly, the plant root system functionality and the presence of MPs aggregates on this organ were evaluated by the use of Nile Red staining. Indeed, Nile Red is widely used for selective fluorescent staining to identify various types of MPs, such as PE, polyurethane, polystyrene, and polypropylene in environmental samples [42] as well as in biological samples such as animal and plant tissues/organs [20,36,43]. The differences observed between the CNT and treated roots in our study can be attributed to the presence of MPs, which were adsorbed onto the root surface and were clearly detectable as distinct aggregates. In an experiment conducted by Shi et al. [44], it was reported that PE-MPs with a ø of 5 μm, a lower MPs size than those used in the present

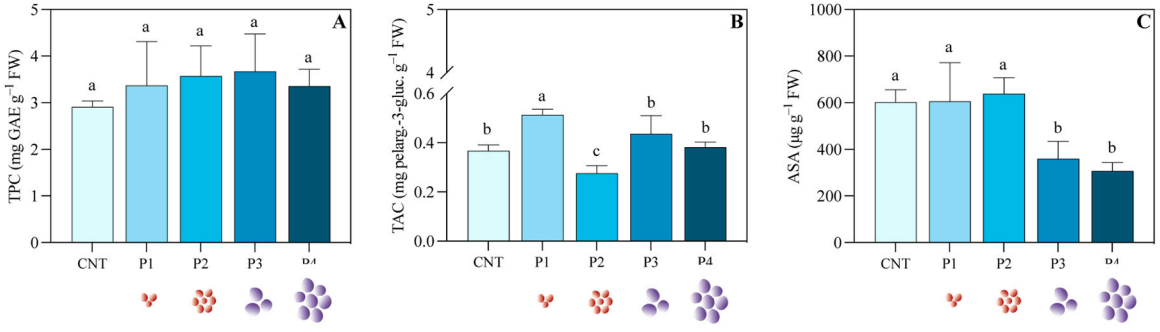

Fig. 5. Total phenol content (TPC; A); total anthocyanin content (TAC; B) and total ascorbic acid content (ASA, C) of fruit from plants of *Fragaria* × *ananassa* var. Alba grown in uncontaminated soil (CNT), or treated soil with 0.02% (w/w) 35 μm ø polyethylene- (PE) microplastics (MPs; P1), 0.2% (w/w) 35 μm ø PE-MPs (P2), 0.02% (w/w) 125 μm ø PE-MPs (P3) and 0.2% (w/w) 125 μm ø PE-MPs (P4). Means with different letters are significantly different after Fisher's LSD *post-hoc* test ($P \leq 0.05$).

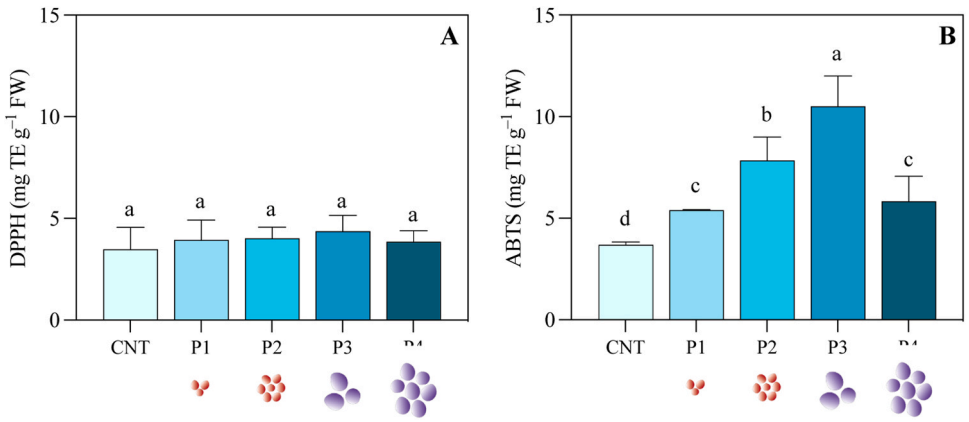

Fig. 6. Antioxidant activity determined by DPPH (A) and ABTS (B) assays of fruit from plants of *Fragaria* × *ananassa* var. Alba grown in uncontaminated soil (CNT), or treated soil with 0.02% (w/w) 35 μm ø polyethylene- (PE) microplastics (MPs; P1), 0.2% (w/w) 35 μm ø PE-MPs (P2), 0.02% (w/w) 125 μm ø PE-MPs (P3) and 0.2% (w/w) 125 μm ø PE-MPs (P4). Means with different letters are significantly different after Fisher's LSD *post-hoc* test ($P \leq 0.05$).

experiment, were even absorbed into the cortical tissues of roots and stems of sweet potato plants. In our experimental system, the treatment with PE-MPs with ø of 35 μm at the highest concentration (P2) was associated with the greatest intensity of whole Nile Red root staining, which also extended to the external root cup and to the columella cells. This suggests that smaller MPs exhibit greater mobility in the soil, facilitating their accumulation on both the root surface and within the root cup compared to their larger counterparts. Similar interaction patterns were observed in previous studies involving *Arabidopsis* and wheat exposed to polystyrene NPs and MPs with ø of 40 nm and 1 μm, respectively [45]. In our experimental system, the root cup, being rich in mucilage, may exert a protective role for root apical meristem (RAM) against environmental stresses, in this case entrapping MPs and preventing their interaction with RAM. However, we cannot exclude the possibility that Nile Red may have also reacted with the lipid fraction of the root tegmental layer, leading to a colour interference in terms of MPs discrimination. A good candidate in this respect could be the so-called "diffuse suberin" [46], which is occasionally observed in the primary wall of the root epidermis [47]. This polymer, composed of both phenolic-derived and fatty acid-derived monomers, reacts positively to the staining [48], and its impregnation within the cell wall could be considered a response mechanism against both biotic and abiotic stresses [46]. In any case, differences in staining intensity can be ascribable to a response associated with MP treatment. Indeed, a negative impact of MP particles on root tissues has been recorded, especially by the smaller and more concentrated plastic particles, as shown by Evans Blue staining results. Specific areas of the root surface showed damage, accompanied by alterations in cell morphology characterized by significant elongation, indicative of impending cell death. Direct damage to root cells confirms the results obtained in hydroponics with the same staining approach in peanut plants treated with polypropylene MPs, where membrane damage was extended to the whole root apex [49]. Alterations in root cell membrane integrity can be directly associated with an increase in reactive oxygen species (ROS) leading to oxidative stress. Indeed, plants treated with MPs have shown an overproduction of H_2O_2, suggesting MPs phytotoxicity [27,50]. Consistent with histochemical data, the highest oxidative stress and damage in roots were observed when exposed to P2 treatment. These results suggest that the observed root tissue damage was attributed to the excessive production of H_2O_2, induced by the accumulation of smaller-sized MPs on and within the root organ.

The adherence of MPs to plant root can have detrimental effects on the overall plant physiology [17,18,50,51]. Indeed, we suggest that the deposition of PE-MP particles over the root surface, along with alterations in root cellular integrity observed by Evans Blue staining, appears to alter water homeostasis in plants exposed to the P2 treatment by limiting the water uptake. The consequence of this disturbance was mainly detectable by a decline in leaf transpiration rates compared to control plants (data not shown), thus resulting in stomatal limitation to net CO_2 assimilation, as stomatal adjustments represent one of the first responses to reduced water availability [52,53]. In accordance with our results, in hydroponic conditions, Gao et al. [23] observed a decrease in P_n and g_s, along with a reduction in leaf evapotranspiration rate, in lettuce plants exposed to a PE-MP contaminated nutrient solution. Additionally, they noted a decrease in PSII photochemistry. Indeed, in response to limited water uptake and a decline in CO_2 assimilation rates, light conversion efficiency should be repressed. This is to avoid a large imbalance between light harvesting capacity and energy utilization, with the consequent formation of ROS [54,55]. However, our study highlights that PE-MPs exposure did not affect the PSII photochemistry of MPs-treated plants, reporting no reduction in light energy conversion efficiency as instead observed by Colzi et al. [22] and Gao et al. [23]. The constraints to CO_2 assimilation reduce the consumption of ATP and reducing power (NADPH), generating a high excitation pressure and restricting the utilization of light energy [56]. In this scenario, the absorbed light energy becomes excessive leading to possible alternative electron sink, i.e. the increase of Mehler reactions in the chloroplast with the generation of H_2O_2 [56,57]. This explains why, in the present experiment, leaves of P2-treated plants show high levels of H_2O_2, even though it did not result in damage to lipids of cell membranes, likely due to the elicitation of scavenging mechanisms [51]. Nonetheless, the presence of high values of TBARS under both treatments with larger size MPs suggests that oxidative damage could not be only dependent to H_2O_2 overproduction. Overall, the existing literature already demonstrates that, under hydroponic conditions, PE-MPs may be adsorbed by the root surface, inhibiting root functionality and limiting water and nutrient uptake and translocation to the plant aerial part via the xylem stream, altering plant metabolic processes [18,23,41]. However, to the best of our knowledge, this study is the first demonstration that in natural soil the accumulation of PE-MPs on root tissues results in root damage with a subsequent reduction in the plant transpiratory fluxes that reduced leaf CO_2 assimilation rate.

Analysing the path from the root through the leaf to fruit, if the reduction in net CO_2 assimilation did not significantly impact the vegetative plant development in P2 treatment, it affected the final fruit size. Indeed, in our observations, P2 treatment negatively influenced the fruit development, decreasing its final weight compared to the other treatments. Very little information is present in the literature about the influences of MPs on fruit development [38,58,59]. Dainelli et al. [38] observed a decrease in fruit weight in *Solanum lycopersicon* var. Micro-Tom plants grown with PET or PVC-MPs of ø between 40 and 50 μm. They suggested that PVC-MPs negatively influenced the plant water

status, reducing the tomato weight at harvest. Khalid et al. [37] further emphasized that MPs in the soil might impede the optimal absorption of water and nutrients by plants, potentially leading to macronutrient deficiencies in the fruit, as identified by Alharbi et al. [59]. Consequently, this could retard regular fruit ripening and growth, in line with the findings of Hernández-Arenas et al. [58] in tomato fruit from plants amended sewage sludge contaminated by MPs (MPs size and concentration not specified). Therefore, we suggest that our results hint at potential metabolic imbalances within the plant, stemming from alterations in the plant's water status with possible consequent nutritional deficiencies in the fruit, the organ where the transpiratory stream ends [38,58–60]. Moreover, treatment P2 not only led to a decrease in fruit development but also resulted in diminished fruit organoleptic quality (e.g., lower SSC and red colouration compared to the other treatments), a trend also observed in treatments P3 and P4 (e.g., low ASA contents). Few works analysed the influence of MPs on fruit qualitative properties, especially on antioxidant compounds [38,59]. Alharbi et al. [59] reported low concentrations of total phenols and flavonoids in pepper fruit grown in soil treated with PET-MPs ø of 11 μm at a concentration of 0.002% (w/w), due to the negative effects of MPs treatment on their precursors. Differently, in our experiment, treatment with PE-MPs did not significantly alter the total phenol content, in line with the results of Dainelli et al. [33] for PET-MPs. This result correlates with unchanged level of antioxidant activity (irrespectively to the treatments) detected by DPPH assay, as phenolic compounds are often the main determinant of the total antioxidant activity of plant tissue. However, in some cases other strong antioxidant compounds (e.g ascorbic acid, glutathione), with different affinity with different total antioxidant activity test (i.e. DPPH vs ABTS) might lead to different results. Indeed, ABTS assay reported the highest antioxidant activity in the P3 treatment. However, we observed a negative alteration of specific metabolite contents, i.e., anthocyanins and ASA. Given their implications for human health [25,61], any reduction in these compounds, especially in treatments P2 (low anthocyanin content) and P3 and P4 (low ASA content), indicates diminished fruit quality. As such, comprehensive research focusing on fruit quality, especially concerning potential secondary metabolite alterations in fruit grown in MP-contaminated soils, remains a pressing need, as the current body of literature remains scant on evaluating the quality of consumer-targeted products cultivated in MP-contaminated soils.

5. Conclusion

The presence of PE-MPs with a ø 35 μm at a concentration of 0.2% in natural soil had a deep effect on the physiological and biochemical health of *Fragaria* × *ananassa* plants. Specifically, this treatment induced a significant reduction in CO_2 assimilation due to stomatal limitations. This was linked to noticeable root tissue damage and a relative rise in oxidative stress in roots. This stress was attributed to the adherence of MPs on the root surface potentially altering the plant water status. Consequently, the same treatment led to loss of fruit quality, i.e., reduced fruit size and weight, and diminished levels of anthocyanin and soluble solid contents. Our results provide the first comprehensive insight into the physiological, anatomical, and biochemical changes induced by MPs on strawberry plant organs. Nevertheless, conducting more comprehensive research on the possible metabolic responses of fruit from plants grown in soils contaminated by MPs remains necessary.

Environmental implication

The Anthropocene is characterized by an unprecedented surge in plastic use, leading to global microplastic (MP) proliferation in terrestrial/aquatic environments. This study delves into the topic of MP interactions with terrestrial plants, by using a spread MP (polyethylene [PE]) in agricultural soil and strawberry, a globally strategic crop. Beyond physiological impacts, this study unveils potential alterations in strawberry fruit quality due to PE-MP contamination in a natural soil. Herein is demonstrated the potential damage of PE-MPs in relation to their size and concentration, providing scientific pieces of evidence about the risk exposure of strawberries cultivated in an MP-polluted soil-system.

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.

Data availability

Data will be made available on request.

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