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Transcriptomic and physiological effects of polyethylene microplastics on *Zea mays* seedlings and their role as a vector for organic pollutants

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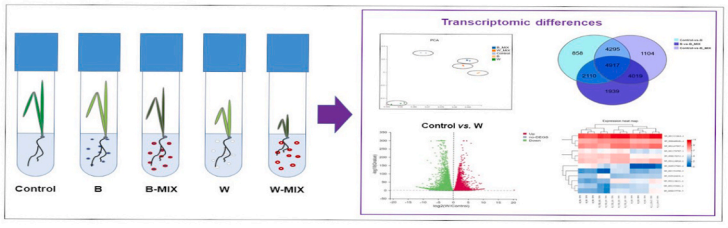
HIGHLIGHTS

- Microplastics (MPs) have a physiological effect on maize seedlings.
- There is a differential plant response according to the type of MPs.
- MPs have a role as organic contaminant vectors.
- W-MPs had the most negative effect on maize in our study.
- RNA-seq is a valuable tool to assess transcriptomic changes due to MPs exposure.

GRAPHICAL ABSTRACT



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ABSTRACT

The widespread employment of plastics in recent decades has resulted in the accumulation of plastic residues in all ecosystems. Their presence and degradation into small particles such as microplastics (MPs) may have a negative effect on plant development and therefore on crop production. In this study, the effects of two types of polyethylene MPs on *Zea mays* seedlings cultured *in vitro* were analysed. In addition, four organic pollutants (ibuprofen, simazine, sertraline, and amoxicillin) were adsorbed by the MPs to evaluate their capacity as other contaminant vectors. The development of the plants was negatively affected by MPs alone or with the organic compounds. The strongest effect was observed in the W-MPs treatments, with a reduction in leaf and root length near 70%. Chlorophyll content was also differentially affected depending on the treatment. Transcriptome analysis showed that MPs affected gene expression in the roots of maize seedlings. As observed in the physiological parameters analysed, some gene expression changes were associated with specific treatments, such as changes in sugar transport genes in the B-MIX treatment. These results contribute to a better understanding of the molecular mechanisms of plants in regard to plastic stress responses.

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1. Introduction

Since the mid-twentieth century, plastics have provided great benefits to humans and have symbolized modern life. In 2018, the global amount of plastic production reached 359 million tons (PlasticsEurope, 2019). Due to its abundance and persistence, plastic waste has been accumulating in ecosystems, and, in recent years, the study of the impact of plastics on the environment has become an emerging area of research.

According to the European Food and Safety Authority (EFSA), microplastics (MPs), are defined as synthetic polymer particles or fibres with a diameter of 0.1–5000 μm (Triebskorn et al., 2019) and may be the result of plastic degradation or manufactured for industrial purposes (e.g., 3D printing, adhesives, paint, electronics, personal care products, etc.) (Lehner et al., 2019). Together with the small size of MPs, which facilitate their ingestion by biota and therefore their incorporation into the food chain, another emergent problem of these particles is their capability to adsorb other pollutants (e.g. heavy metals, organic compounds, etc.) which may be transported to other habitats and even incorporated into organisms, imposing more severe toxic effects (Atugoda et al., 2021).

Although studies on the effects of MPs and nanoplastics (NPs) on terrestrial ecosystems have increased in recent years, they are still scarcer than those addressing aquatic ecosystems (de Souza Machado et al., 2018; Rillig and Lehmann, 2020). The results of existing studies suggest that the effects of MPs on terrestrial plants depend on the plant species, MPs properties and environmental factors (Li et al., 2022). Most of these studies have pointed to a negative effect of MPs on plant development, although the specific processes involved remain unclear. In addition to direct physical mechanisms such as blocking pores and light or releasing additives, in regard to indirect mechanisms (i.e., changes in soil properties that can also affect microbiota composition and their relationship with plants), studies have suggested the importance of changes in gene expression in the negative effects of MPs/NPs on plants (Li et al., 2022).

Among the studies that found a negative effect of MPs on plant development, a study by Urbina et al. (2020) on the effect of polyethylene (PE) microspheres on hydroponic maize found a significant decrease in transpiration, nitrogen content and growth of seedlings. Similarly, a reduction in growth, chlorophyll content and photosynthetic capacity was observed in *Nicotiana tabacum* seedlings exposed to different concentrations of PE-MPs (Teng et al., 2022). Other studies have similarly focused on chlorophyll degradation (Dong et al., 2020), a decrease in root development (Chae and An, 2018; Jiang et al., 2019) and oxidative damage or seed germination (see Li et al., 2022). On the other hand, Guo et al. (2022) reported no PE-MPs effect on *Triticum aestivum*. Even, other studies have found a positive effect of MPs, reporting an increase in biomass, as described in wheat under polystyrene MPs exposure (Lian et al., 2020).

Massive amounts of MPs can reach the soil environment from different sources, with PE being among the most common plastics found (Kasirajan and Ngouajio, 2012; Kang et al., 2015).

Diverse organism responses have been observed in previous studies, which is, in part, related to the specific characteristics of MPs (Qi et al., 2018). MPs exhibit great diversity due to their different sizes, compositions, surface morphologies and various added chemical substances. These particles can impose an impact *per se* on the ecosystem, which may be increased considering the capacity of MPs to adsorb other contaminants, making them pollution vectors.

We have also considered the role of MPs as vectors of organic contaminants: ibuprofen (IB), one of the nonprescription drugs most used worldwide, sertraline (SRT), one of the most prescribed antidepressants, which are two abundant polar pharmaceuticals detected in wastewater treatment plants (WWTPs) (Barra-Caracciolo et al., 2015; Gornik et al., 2020); together with simazine (SZ) and amoxicillin (AMX). SZ is a persistent organic pollutant widely used in agriculture and forestry worldwide for more than fifty years. Although its use has been banned in Europe, its high persistence in soil means that it is still currently found in WWTPs in rural and suburban areas (Münze et al., 2017). AMX is a beta-lactam antibiotic with broad specificity and is one of the most commonly used antibiotics. The association between MPs and antibiotics is especially interesting due to their potential impacts on the microbial community as well as the potential transference of resistance genes after the release of MPs into the soil environment (Yan et al., 2020; Yang et al., 2022). These organic pollutants were previously studied as adsorbed compounds in polyethylene MPs, resulting in a greater toxic effect on the development of roots of maize seedlings than the MPs alone (Fajardo et al., 2022).

The molecular mechanisms associated with the ecotoxicological impacts of MPs have not been sufficiently highlighted (Pehlivan and Gedik, 2022). Transcriptomic analyses can reflect the different functional groups involved in the metabolic pathways affected by these pollutants (Lian et al., 2022; Wang et al., 2022; Wu et al., 2022).

The objective of this study was to investigate the impacts of two types of PE-MPs and their interactions with a mixture of organic pollutants, ibuprofen (IB), sertraline (STR), amoxicillin (AMX) and simazine (SZ), on several endpoints of *Zea mays* plants growing under *in vitro* conditions. To better understand the molecular mechanisms underlying the effects of MPs and their combination with organic pollutants, RNA-seq analysis was used to detect transcriptomic changes in seedlings. These analyses may supply information about the metabolic pathways affected by MPs and provide potential biomarkers for the risk assessment of crops exposed to MPs.

2. Material and methods

2.1. Microplastic and contaminant characteristics

Two types of PE-MPs were used in this study: blue microbeads (B) (1.08 g cm^{-3}), and white microbeads (W) (1.35 g cm^{-3}), which, how reported by the manufacturer, have a dominant particle size of 250–300 μm. The MPs were purchased from Cospheric LLC (Santa Barbara, CA, USA). Previous SEM analyses revealed that B and W microbeads appeared as spherical particles with smooth surfaces and similar average sizes (236 ± 7.4 μm and 281 ± 14 μm for B and W, respectively) (Martín et al., 2021; Fajardo et al., 2022).

The toxicity of each type of MPs selected for this study (B and W) was tested separately at a concentration of 0.4 mg mL^{-1}. MPs were directly included in the experimental medium.

To evaluate the carrier potential of the microbeads, each MPs (0.4 mg mL^{-1}) was incubated in a solution containing 15 μM of each organic contaminant (OC) tested in this study: IB (3.09 mg L^{-1}), AMX (5.48 mg L^{-1}), STR (5.14 mg L^{-1}) and SZ (2.75 mg L^{-1}). The incubation of MPs with the OC combinations (hereafter called MIX) was maintained in the dark for 72 h (120 rpm) at 25 °C. Thereafter, the MPs were removed (filtered) and included in the experimental media.

2.2. In vitro culture of Zea mays

Seeds of *Z. mays* L. (popcorn variety) were sterilized and aseptically transferred to tubes containing MS medium (Murashige and Skoog, 1962) without growth regulators as previously described in Martín et al. (2021). The corresponding MPs, and MIX for each treatment were added under aseptic conditions after sterilization of the medium when the temperature was low enough to avoid possible alterations of the MPs or OCs.

One seed was sown per tube and incubated at 25 °C with a 16 h photoperiod and photosynthetic photon flux density (PPFD) of 50 μmol m^{-2} s^{-1} from cool white fluorescent tubes.

Ten seeds were individually sown per treatment and control (culture medium without MPs or OCs).

After 2 weeks, germination and development were evaluated. Maximum leaf length and maximum root length were measured to

analyse growth. The effect of the treatments was evaluated by the percentage of germination and the percentages of leaf and root growth inhibition, [(Average Max. Leaf/Root length Control – Average Max. Leaf/Root length Treatment)/Average Max. Leaf/Root length Control] *100.

2.3. Chlorophyll determination

Chlorophyll (Chl) was extracted from 50 mg of frozen leaf tissue from the control and each of the treatments tested (B, W, B-MIX and W-MIX) following the protocol of Richardson et al. (2002) based on the dimethyl sulfoxide (DMSO) Chl extraction technique of Hiscox and Israelstam (1979). Optical density (OD) values at 645 and 663 nm of the chlorophyll extracts were read in a spectrophotometer (Beckman, Indianapolis, U.S.A) against a DMSO blank. The Chl content was calculated following the equations used by Arnon (1949):

Chlorophyll *a* (Chl_a) ($mg \cdot g^{-1}$ fresh weight) = [(12.7 *A_{663}) – (2.69 *A_{645})]* (V/1000*W); Chlorophyll *b* (Chl_b) ($mg \cdot g^{-1}$ fresh weight) = [(22.9 *A_{645}) – (4.68 *A_{663})]* (V/1000*W); Total Chlorophylls (Tot Chl) ($mg \cdot g^{-1}$ fresh weight) = [(20.08 *A_{645}) + (8.02 *A_{663})]* (V/1000*W); and Carotenoids + xanthophylls ($mg \cdot g^{-1}$ fresh weight) = (1000* A_{470} – 1.9 Chl_a – 63.14 Chl_b/214) * (V/1000*W); where 'V' is the extract volume (ml), and 'W' is the fresh weight of the sample (g).

2.4. Total free radical determination

The intracellular oxidative stress level (reactive oxygen species (ROS) and reactive nitrogen species (RNS)) was determined by using the OxiSelect™ *in vitro* ROS/RNS assay kit (Cell Biolabs, San Diego, USA). The kit was used according to the recommendations of the manufacturer. To measure the total free radicals, 50 mg of leaf and 50 mg of root tissues from control plants (C), plants cultivated in media containing one of the two MPs tested (B or W) and plants cultivated in the presence of the MP-OCs complexes (B-MIX and W-MIX) were homogenized in liquid nitrogen (LN), resuspended in 1x phosphate buffered saline (PBS; per litre, NaCl 8 g, KCL 0.2 g, Na_2HPO_4 1.42 g, and KH_2PO_4 0.24 g), and centrifuged at 10,000×*g* for 5 min. The supernatant was transferred to a new tube and stored at −80 °C until determination. All further steps were carried out according to the manufacturer's protocol. A Tecan Genios plate reader (Tecan Group Ltd., Switzerland) was used for the read out (excitation wavelength 485 nm; emission wavelength 535 nm).

2.5. RNA extraction

Total RNA was isolated from the leaf and root tissues (100 mg) from each sample replicate using an RNeasy Mini Kit (Qiagen, Iberia S.L) following the manufacturer's protocols. Each RNA sample was incubated with RNase-free DNaseI (Qiagen, Iberia S.L.) to eliminate the genomic DNA. Extracted RNAs were quantified in a NanoDrop One spectrophotometer and checked for RNA integrity in a Bioanalyzer 2100B (Agilent Technologies).

2.6. RT–qPCR of antioxidant-related genes in leaves

Antioxidant gene expression in leaf samples was assessed through RT–qPCR analyses. Specific reverse transcription (RT) for each target mRNA and cDNAs were prepared according to Fajardo et al. (2022).

Relative quantification was performed measuring the expression levels of the target genes (superoxide dismutase, (*SOD* 1), catalase (*CAT* 1), and glutathione-S-transferase 1 (*GST* 1)) compared to the expression levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The primer sets for the target and reference genes are listed in Table 1.

Table 1
Primers used for real time RT- PCR assays.

Target gene	Primer	Sequence	Reference
GAPDH	*GAPDH_A_F*	*5′-TTGGTACGACAACGAGTGGG-3′*	Fajardo et al. (2022)
	GAPDH_A_R	*5′-AGATGGTCTGCGACCAAAGG-3′*	Fajardo et al. (2022)
SOD-1	*SOD-1A_A_F*	*5′-CGGGTGACCTGGGAAACATT-3′*	Fajardo et al. (2022)
	SOD-1A_A_R	*5′-GCCCCCTTTCCCCAAATCAT-3′*	Fajardo et al. (2022)
CAT-1	*CAT1_F*	*5′-TGTTCTAACAGGCTGTCGTAGAG-3′*	Zhao et al., 2017;
	CAT1_R	*5′-TGTCAGTGCGTCAACCCATC-3′*	Fajardo et al., 2022
GST-1	*GST1_A_F*	*5′-CGAGCAATCTGCAAGTACGC-3′*	Fajardo et al. (2022)
	GST1_A_R	*5′-TAGCCTCCACCTCGATCCAA-3′*	Fajardo et al. (2022)

2.7. RNA sequencing of expressed genes in roots

Three biological replicates from root samples were sent to the Beijing Genomics Institute (BGI, Shenzhen, China, https://www.bgi.com) where the cDNA library was carried out using the DNBseq sequencer and pair-end sequencing (PE100, 20 M) was generated. Clean data were generated from the raw data and quality control (QC) was then performed on the raw reads to determine whether the sequencing data were suitable for subsequent analysis. After quality control, clean reads were filtered by removing adapter sequences and low quality reads (defined as reads with bases with a quality score less than 15 as the proportion of total bases in the reads that were greater than 20%) using filtering SOAPnuke software developed by BGI, and were aligned to the maize reference genome (GCF_000005005.2_B73_RefGen_v4). After alignment, the statistics of the mapping rate and the distribution of reads on the reference sequence were used to determine whether the alignment results passed the second QC of alignment. From them, gene quantification analysis and other analyses based on gene expression (principal component, correlation, differential gene screening, etc.) were carried out. Significant enrichment analysis of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functions on differentially expressed genes (DEGs) among the screened samples, significance enrichment analysis of pathways, and clustering, were carried out using a data mining system, Dr. Tom, which is a network platform of BGI (https://biosys.bgi.com/#/report/login).

2.8. Statistical analyses

Studied variables were analysed using a linear model with treatment as a factor. The assumptions of normality and homogeneity of the variance were verified using the residual plots. For variables, leaf maximum length and root maximum length, a square-root transformation was performed to meet these assumptions.

After a significant effect for treatment in the ANOVA table, a pairwise comparison of treatments with the control was performed using Dunnett's test, and pairwise comparisons among treatments, except the control, were performed using Tukey's test.

3. Results

3.1. In vitro development of Z. mays

Seed germination was high in all tested treatments, especially in those with B-MPs (alone or MIX). In these treatments, the percentage of germination was 100% similar to the control. However, germination rates were lower for those treatments including W-MPs, reaching 70%

when MPs were alone and only 50% in the W-MIX treatment, although these differences were not statistically significant.

The growth parameters of the *Z. mays* seedlings (maximum leaf and root length) were compared to those obtained in the control treatment, and the corresponding inhibition indexes were calculated (Fig. 1). Leaf growth inhibition was detected in all treatments, although the level of inhibition was different depending on the type of MPs and on the presence of OCs (MIX treatments). B-MPs showed lower inhibition values than W-MPs, with the inhibition being practically negligible in the B-MPs alone treatment and reaching 26% in the B-MIX treatment. However, the inhibition values in the W-MPs treatments were considerably higher, even in the treatment with W-MPs alone (33% leaf inhibition), and reached almost 73% when MPs had adsorbed the OCs (W-MIX). A significant effect of the type of MPs was detected ($p < 0.05$), although only in the MIX treatments, for both types of MPs, showing a significant reduction in leaf length compared to the control. Leaf development in the B-MPs treatment was similar to that observed in the control treatment, while in W-MPs and W-MIX it was clearly reduced (Fig. 1).

In contrast, MPs alone (B or W) did not show a negative effect on root growth (Fig. 1). Only MIX treatments showed a detrimental effect on root length, being negligible in the B-MIX treatment (near 7%) but significantly different in the W-MIX treatment, with a much higher value of inhibition (near 63%) according to the inhibition also observed in leaf length. As it was also detected in the leaf length parameter, there was a significant effect of the type of MPs on root length.

3.2. Chlorophyll content

The contents of chlorophylls *a and b* in the leaves of maize seedlings grown under *in vitro* conditions in the presence of MPs alone (B or W) were reduced compared to the control samples (Fig. 1). The content of Chl *a* in the B-MPs treatment represented a 30% decrease with respect to the control, a percentage that increased to a 38% reduction for the W-MPs treatment. The percentage of reduction for both treatments was near 30% in Chl *b* content. However, when OCs were adsorbed on the MPs (B-MIX and W-MIX treatments), the content of Chl *a* and *b* increased 50% and 40%, respectively, in the B-MIX samples. More significantly, the Chl content increased in the W-MIX treatment, which was 2-fold higher that in the control (Fig. 1). Instead of the differences found, none of these parameters were statistically significant due to the high diversity of the data.

Total carotenoid and xantophyll data in the tested treatments showed lower values than the control, except for the W-MIX, although, as was observed in Chl content, values corresponding to MIX treatments were higher than the MPs alone, even for B-MPs (Fig. 1). Similar to the chlorophyll analysis, these results were not statistically different.

3.3. Total free radical determination

The total intracellular oxidative stress level in leaf samples was higher in the W-MPs and W-MIX treatments than in the control and the other treatments, although the differences were not significant (Fig. S1). Likewise, slight differences were observed among the control and treatments in the root sample, although values corresponding to B-MPs (alone or MIX) were higher than the control, especially B-MIX, without significant differences (Fig. S1).

3.4. Relative expression of antioxidant-related enzymes in Z. mays leaves

The expression levels of genes encoding antioxidant enzymes in leaves from maize seedlings were determined through qPCR analyses (Fig. 2). The relative expression of the *CAT* 1 gene did not show differences between the two treatments containing W-MPs (W and W-MIX), although their values were slightly higher than those of the control samples. However, B-MPs showed different results: a significant reduction in *CAT* 1 expression was observed in the B-MPs alone treatment, while the B-MIX treatment did not show differences with respect to the control. The relative expression of the *GST* 1 gene did not show differences in leaf samples in the B treatment and only a slight reduction in the other type of MPs alone, W-MPs, although both types of MPs combined with OCs showed an increase in gene expression. As was observed for *CAT* 1 relative gene expression, samples corresponding to the B treatment showed a reduction in the *SOD* 1 relative expression, but not so drastic, while B-MIX had similar values to the control. Additionally, in W-MPs the adsorption of OCs led to an increase in gene expression, although in this case, it was more significant than that in B-MPs, with a relative expression in the W-MIX treatment greater than 4-fold than that in the control (Fig. 2).

3.5. Transcriptional responses to MPs treatments

On average, the RNA-Seq experiments yielded 47.48 million paired-end reads per sample and between 48.8 and 45.3 million paired-end reads per sample clean reads. Over 96% of the clean reads had quality scores at the Q20 level (a base quality greater than 20 and an error probability of 0.01). A high proportion of clean reads, 83.6–87.82% were readily mapped to the maize reference genome sequence.

Principal component analysis (PCA) showed a strong relationship among the samples within the treatment (Fig. 3). The first two principal components explained more than 90% of the variability found. Seedlings cultivated with W-MPs were more different from the control samples than the other treatments and showed great differences from these other treatments.

Transcriptomic analysis based on the RNA-Seq results showed between 16,402 and 12,180 differentially expressed genes (DEGs) among the different treatments (Fig. 4). When compared to the control, the highest number of DEGs corresponded to the W-MPs. Among all comparisons, the lowest number of DEGs was found between the control and B-MPs treatments. The results showed that MPs, alone or combined with organic contaminants (MIX), can affect gene transcription in maize seedlings developed under *in vitro* culture. The distribution of up- and down-regulated DEGs and the degree of variation for the studied treatments were observed in the volcano plots (Fig. S2). Comparisons of each type of MPs *vs.* control, and MPs alone *vs*. MPs-MIX were considered. More significant differences and a higher level of variation were observed in W-MPs when compared to the control than that observed in B-MPs. Likewise, differences between the two types of MPs (B and W) were not as wide as those observed between the control and W-MPs.

Analysing separately the two types of MPs studied, the results of Venn diagrams showed 858 DEGs that only affected the control and B-MPs (Fig. S3a), from which 624 were up- (72.7%) and 234 were down-regulated (27.3%). In this group of treatments, there was a larger number of DEGs that only affected the comparison between B-MPs and B-MIX (1939 DEGs), of which 1154 were up-regulated (59.5%) and 785 were down-regulated (40.5%).

In the case of W-MPs, the number of specific DEGs affecting the W-MPs and control treatments was higher, nearly twofold than that for B-MPs, although the percentages of up- and down-regulated DEGs were similar. Of these 1769 DEGs, 1298 were up-regulated (73.4%) and 471 were down-regulated (26.6%) (Fig. S3b). However, the proportion of up- and down-regulated DEGs that were common to W-MPs and W-MIX was different from those found for B-MP and its MIX treatment. There were 1600 DEGs specific to W-MPs *vs*. W-MIX, with 1136 up-regulated (71%) and 464 down-regulated (29%) DEGs.

To identify significant pathways involved in the response of maize seedlings to the MPs and MIX treatments, enrichment analyses were conducted. GO enrichment analysis (Fig. 5) showed that DEGs could be categorized mainly into the terms ‘Cellular process’ (78.4%) and ‘Metabolic process’ (72.5%) within the class “Biological process”. The main terms in the class “Cellular Component” were ‘Cell’ (80%), ‘Cell part’ (79.5%) and ‘Organelle’ (60.7%). In the class “Molecular function”

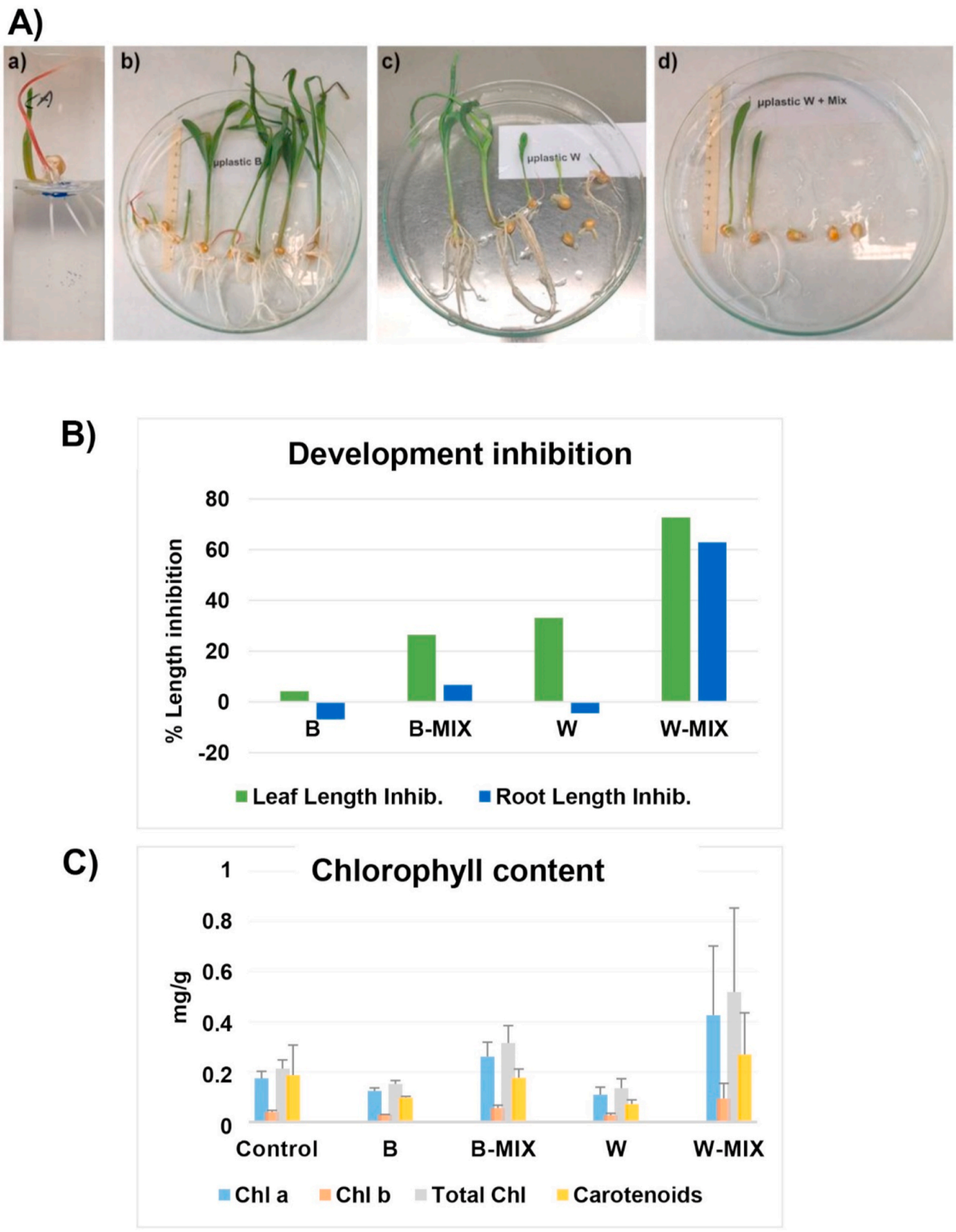

Fig. 1. A) Seedlings of *Z. mays* grown under *in vitro* conditions in media containing MPs and MPs with adsorbed OCs (MIX): a) detail of the culture media containing B-MPs and the emerging seedlings; b) seedlings obtained from the B-MP treatment; c) seedlings obtained from the W-MP treatment; and d) seedlings and germinated seeds obtained from the W-MIX treatment. **B)** Effects of B and W-MPs and their corresponding MIX on the inhibition rate of leaf and root growth in *Z. mays* seedlings. **C)** Effects of MPs (alone or MIX) on photosynthetic pigments content in leaf samples of *Z. mays* seedlings grown under *in vitro* culture. Chlorophyll content: chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and total chlorophyll (Total Chl). Carotenoid and xantophyll contents: Carotenoids. Data are expressed as the mean ± SD.

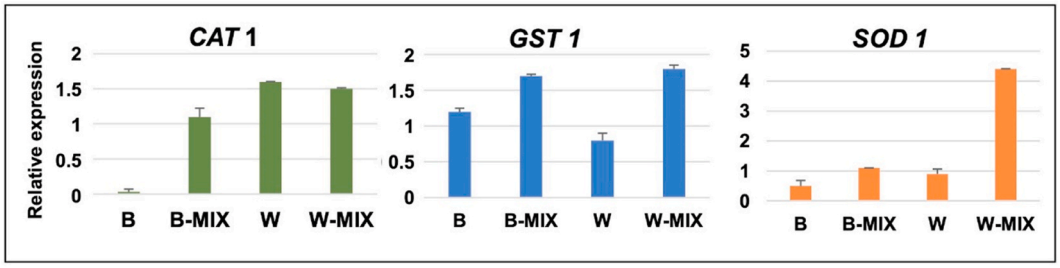

Fig. 2. - Effects of MPs and MIX treatments on the expression levels of the genes CAT 1, GST 1 and SOD 1, encoding antioxidant enzymes, on leaves of maize seedlings (mean ± SD, n = 3).

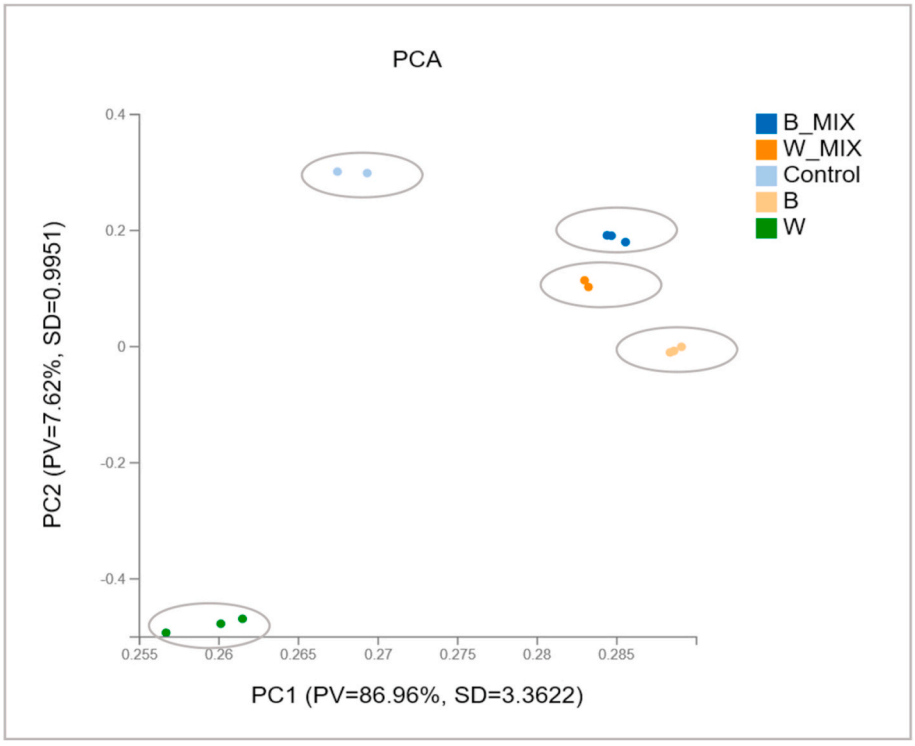

Fig. 3. - Principal Component Analysis (PCA) of transcriptome analyses obtained from maize samples under different treatments. The X and Y axes represent a new data set of the corresponding principal. Each point represents a sample, and the same colour represents the same group. PV is the proportion of variance; SD is the standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

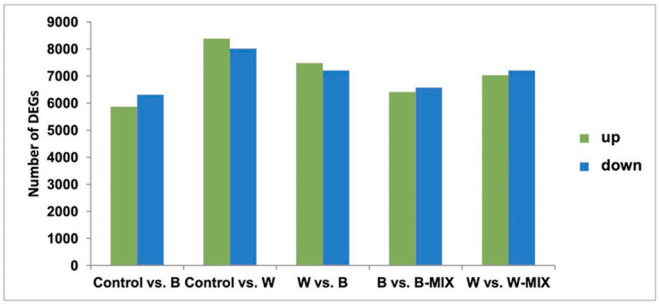

Fig. 4. - Number of up- and down-regulated Differentially Expressed Genes (DEGs) (Q value ≤ 0.05) among treatments (control, B-MPs, W-MPs, B-MIX and W-MIX).

there was a great difference between the two main functions and the others. The 'Binding' term comprised 64.7% of DEGs and 'Catalytic activity' 56.6%, followed by 'Transporter activity', with only 7.7%, and 'Transcription regulator' with 6.6% (Fig. 5).

According to the KEGG enrichment analyses, most genes (1801 genes) were assigned to the 'Global and overview maps' function within the Metabolism pathway, which presents global and overall pictures of metabolism. The other main categories for this analysis were 'Signal transduction' (772 genes) within Genetic Information Processing pathway, and 'Translation' function (756 genes) in Environmental Information Processing pathway (Fig. S4).

To obtain a better understanding of the effects of the MPs and their combination with OCs on the metabolism of maize seedlings, GO enrichment analyses for "Molecular function" among treatments were further studied (Fig. 5). Considering only those functions significantly affected (Q > 0.05), the numbers of most enriched functions for control *vs.* B, control *vs.* W, B-MPs *vs.* B-MIX, and W-MPs *vs.* W-MIX comparisons were 29, 18, 28 and 15 respectively. All of them corresponded to the GO term functions 'Catalytic activity', 'Binding', 'Transporter activity' and 'Structural molecular activity' (see Table S1). For the last term, only one function, 'Structural constituent of ribosome' (GO:0003735), was significantly affected, but it appeared in all comparisons between the treatments. Other functions were differentially enriched according to the treatment comparisons. From the term 'Binding', the function 'heme

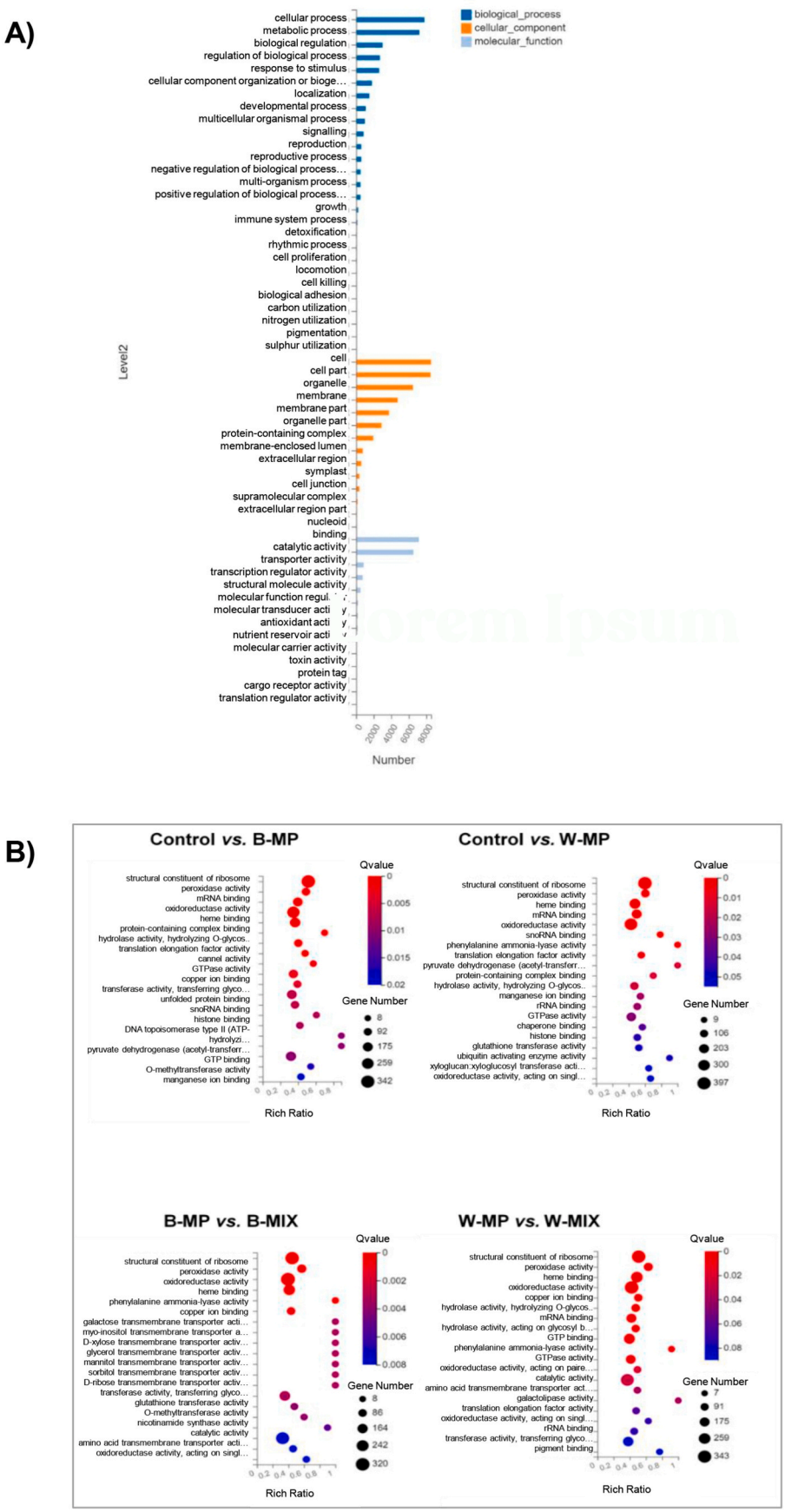

Fig. 5. A) GO enrichment analysis in maize seedling growth *in vitro* in the presence of MPs and MPs with adsorbed OCs (MIX). The X-axis represents the number of genes annotated to the GO entry, and the Y-axis represents the GO functional classification. **B)** GO enrichment analysis of the transcriptome in maize roots exposed to the MPs and MP-MIX treatments corresponding to the 'Molecular function' term. The X-axis is the enrichment ratio [the ratio of the number of genes annotated to an entry in the selected gene set to the total number of genes annotated to the entry in the species, calculated as Rich Ratio = Term Candidate Gene Num/Term Gene Num. The size of the bubble represents the number of DEGs annotated to a GO Term. The colour represents the enriched significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

binding' (GO:0020,037) was found in all comparisons; likewise, the functions 'phenylalanine ammonia-lyase activity' (GO:0045,548), 'peroxidase activity' (GO:0004601), and 'oxidoreductase activity' (GO:0016,491), from the GO function term 'Catalytic activity', were enriched in all compared treatments (Fig. 5).

The Rich ratio of the function 'phenylalanine ammonia-lyase activity' was above 0.75 in all the treatment comparisons. The analysis of the genes affected within this function revealed that the expression of the *PAL 3* gene (Gene ID 100273579) showed significant differences ($Q \ll 0.05$) among the studied treatments. Both types of MPs, W and B, showed a reduction in their expression compared to the control samples (Fig. 6a), which reached more than half for W-MPs. However, when the expression levels of MIX treatments were compared to their corresponding MPs, an increase was observed, although their expression levels were also lower than the control level (data not shown). A reduction in the expression level of the *PAL* 9 gene (Gene ID 100273579) was also observed for the MPs treatments compared to the control (Fig. 6a). In this case, both types of MPs showed similar expression levels, and a reduction in the expression level was observed for the W-MIX treatment compared to W-MPs alone, in contrast with *PAL* 3. Phenylalanine ammonia lyase (PAL) catalyses the deamination of phenylalanine, yielding *trans*-cinnamate (Fig. 6b). The effect of the treatments considered on the differential expression of genes related to phenylalanine ammonia-lyase activity was represented in a heat map (Fig. 6c).

The differential effect on gene expression related to OCs adsorption was analysed by comparing each type of MP with their corresponding MIX treatment. The GO terms 'Amino acid transmembrane transporter' (GO: 0015,171_Level 2: Transporter activity) and 'Catalytic activity' (GO: 0003824_Level 2: Catalytic activity) were the only two functions significantly represented (Q value < 0.05) in common for both MIX treatments. Only two functions were significantly enriched in the W-MIX treatment compared with W-MPs alone: 'Galactolipase activity' (GO: 0047,714_Level 2: Catalytic activity) and 'Oxidoreductase activity' (GO: 0047,714_Level 2: Catalytic activity). Interestingly, the functions exclusive to B-MIX *vs.* B-MPs corresponded to 'Transporter activity' (GO: 0005365), and were related to sugar transport: 'Carbohydrate: proton symporter activity' (GO: 0005351), 'Galactose transmembrane transporter activity' (GO:0005354), 'Glucose transmembrane transporter activity' (GO:0005355), 'Myo-inositol transmembrane transporter activity (GO:0005365), Monosaccharide transmembrane transporter activity (GO:0015,145), D-xylose transmembrane transporter activity (GO:0015,148), Glycerol transmembrane transporter activity (GO:0015,168), Mannitol transmembrane transporter activity (GO:0015,575), Sorbitol transmembrane transporter activity (GO:0015,576), and D-ribose transmembrane transporter activity (GO:0015,591). The GO annotation of these functions corresponding to Cellular Component was 'Plasma membrane' (GO: 0005886) and 'Integral component of membrane' (GO: 0016,021), and for Biological Process: 'Lateral root formation' (GO: 0010,311) and Glucose import (GO: 0046,323).

The Molecular functions affecting carbohydrate transport showed a rich Ratio equal to 1.0 (Q value = 0.03) (see Table S1). Seven genes (proton myo-inositol (Gene ID 100193474), sorbitol transporter (Gene ID: 100,281,055) and putative polyol transporters (Gene ID: 100273244, 100279532, 100381931, 103647781 and 100273244) were affected in these functions. All of them, except one (Gene ID 100279532), showed a higher expression level in B-MIX than in B-MPs samples. In particular, the proton myo-inositol gene and one of the putative polyol transporter (Gene ID: 103647781) were significantly up-regulated in the B-MIX treatment (Fig. S5). The expression levels of these two genes were not only significantly up-regulated compared to the B-MPs samples, but also to the control samples (data not shown).

4. Discussion

Knowledge about the impact on physiology and biological function in plant species is still scarce, together with the fact that terrestrial ecosystems have received less attention than aquatic ecosystems (Ng et al., 2018; Lian et al., 2020; Qi et al., 2020). In addition, the capacity of plastics (MPs and NPs) to bind organic pollutants has been stated (Martín et al., 2021), and the synergistic effect of MPs and organic pollutants was particularly relevant for corn seedlings (Fajardo et al., 2022). Therefore, in this study, we assessed the impact of two types of PE-MPs, alone or associated with organic pollutants, on corn seedlings at the physiological and molecular levels. According to the studies that reflected a wide range of responses to different types of MPs, in our study, it was observed that W-MPs led to different effects compared with B-MPs in physiological and transcriptomic analyses.

Our results showed that corn germination was negatively affected by W-MPs, alone or associated with organic pollutants (W-MIX); however, in a previous study with the same *Z. mays* genotype, no significant differences were observed when germination was carried out in soil (Fajardo et al., 2022), which could be related to a mitigating effect of the soil complex. Nevertheless, a similar negative impact of PE-MPs on *in vitro* germination was previously reported on *Lactuca sativa* exposed to PE fluorescent blue microbeads associated with SZ (Martín et al., 2021).

Although some studies have shown that plastic particles have no significant negative effect on plants (e.g., Qi et al., 2018; Colzi et al., 2022), many studies have reported a negative effect of MPs/NPs (see Li et al., 2022). In our study, when growth parameters were analysed (Fig. 1), it was found that leaf growth was reduced in all treatments, confirming the generalized negative impacts of MPs. Similarly, other authors found a decrease in leaf growth caused by exposure to MPs, as was observed in maize grown in soil in the presence of 10% polylactic acid (PLA) MPs (Wang et al., 2020) or in hydroponic lettuce culture grown under the PE-MPs effect (Gao et al., 2019). In addition, in our study, significant differences were observed due to the type of MPs: the strongest leaf inhibition was observed in the W-MPs respect to B-MPs treatments. This finding may be related to the TiO_2 content in W-MPs (Martín et al., 2021), which is related to the generation of eco- and genotoxicity in plants (Foltête et al., 2011). In fact, the strong effect of W-MPs was previously reported in lettuce exposed to different PE-MPs (Martín et al., 2021). In addition, MIXs showed a more negative effect than their corresponding MPs alone treatments (Fig. 1).

Regarding root inhibition, significant differences were found only in the MIX treatments; being the inhibition 10-fold more evident in the W-MIX than in the B-MIX treatments (Fig. 1). Consequently, we again observed significant differences related to the type of MPs, as has been previously reported (Qi et al., 2018; Li et al., 2022). A similar effect was described in different plant species: *Allium cepa* grown in hydroponic culture and in the presence of PS (Maity et al., 2020); *Cucurbita pepo* in soil culture and in the presence of PP, PE, PVC, and PET (Colzi et al., 2022); *L. sativa* and *Z. mays* grown in hydroponic cultures and in the presence of PS (Gong et al., 2021).

Another key physiological parameter to evaluate plant development is the chlorophyll content, which is generally positively correlated with the rate of plant photosynthesis. MPs exposure can affect the photosynthesis of vascular plants and reduce the chlorophyll content of plant leaves (Yin et al., 2021). Several studies have reported a significant decrease in chlorophyll content due to the effect of MPs, such as Dong et al. (2020) in *Oryza sativa*, and Pflugmacher et al. (2021) in *Lepidium sativum*. Similar results were found when the MPs used were PE (the same type employed in this study) in *L. sativa* (Gao et al., 2019), *C. pepo* (Colzi et al., 2022), and *Nicotiana tabacum* (Teng et al., 2022). In our study, the same response was observed in the MPs alone (B or W) treatments, although the differences were not significant. However, Meng et al. (2021) indicated that a high concentration of PE had no significant effect on the chlorophyll content in *Phaseolus vulgaris*. In the present study, an increase in the total amount of chlorophyll was also

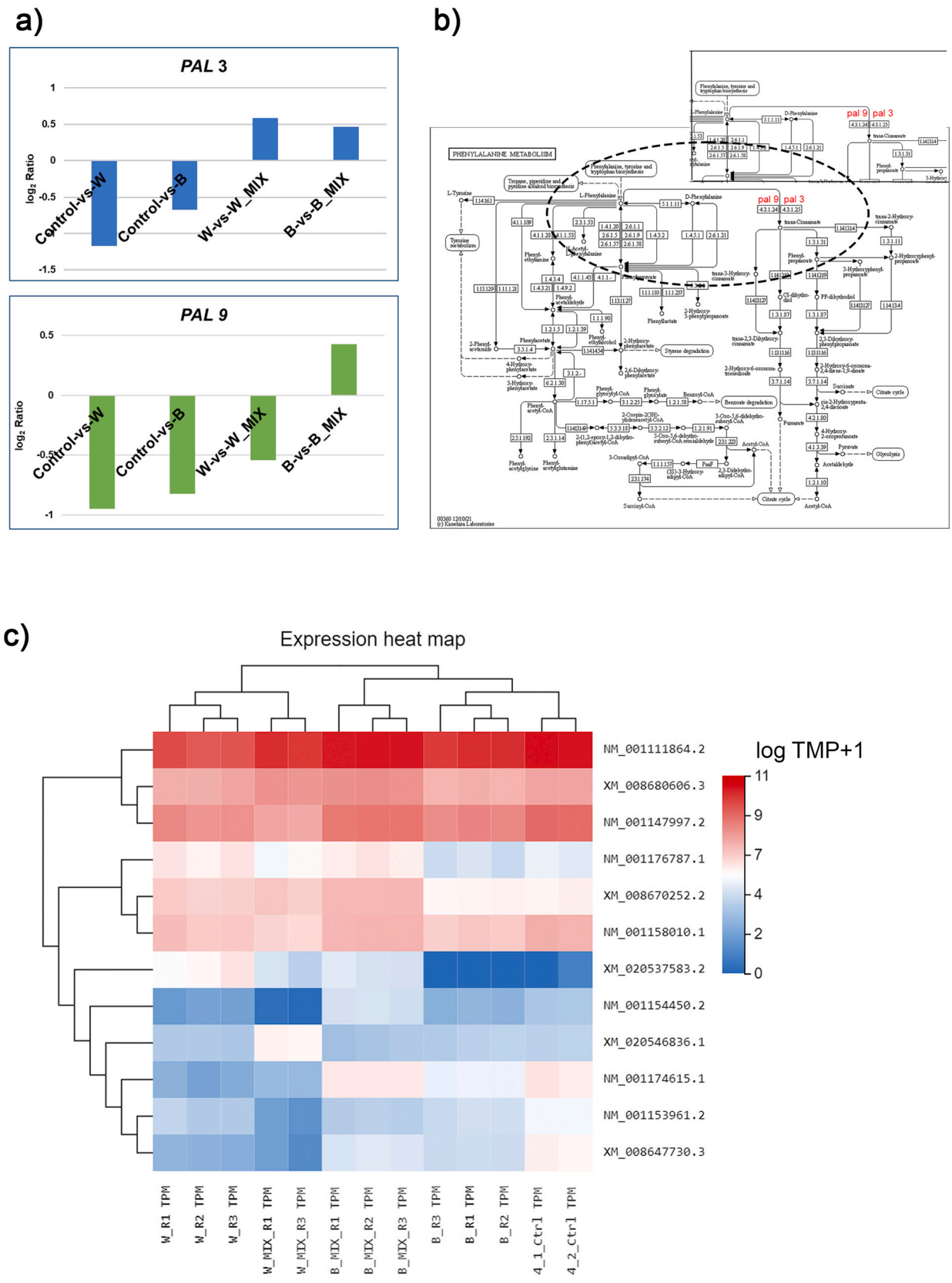

Fig. 6. - Effects of MPs and the MIX treatment on the Differentially Expressed Genes (DEGs) related to phenylalanine ammonia-lyase activity. a) Expression differences of PAL 3 and PAL 9 genes among control samples and the two types of MPs studied, and their corresponding MIX treatment. b) Scheme of phenylalanine metabolism and pal 3 and pal 9 function. c) Heat map of the DEGs related to phenylalanine ammonia-lyase activity.

observed in the treatments with adsorbed organic compounds, especially in W-MIX. This increase may be due to the specific presence of these compounds, since treatments with MPs alone showed a different response. In fact, the impact of stress on plants varies greatly according to the plant species and the type and/or intensity of the stressor (Li et al., 2022; Teng et al., 2022; Pignattelli et al., 2020). Additionally, it may also be considered that some studies have indicated an increase in chlorophyll content in the presence of MPs/NPs (Lian et al., 2021). Some authors interpreted the increase in chlorophyll content under stress as a tolerance strategy of the plant (Khayatnezhad et al., 2011). In conclusion, our results seem to indicate that chlorophyll content was affected by MPs treatments, and therefore, the photosynthesis mechanism may be disturbed.

Likewise, oxidative damage has been related to the toxic effect of MPs and NPs on plants (Jiang et al., 2019). ROS are generated as products of different enzymatic reactions that trigger signalling molecules for oxidative stress cascades responsible for scavenging free radicals. Oxidative stress is a symptom of toxicity that occurs when ROS production and antioxidant detoxification/neutralization are imbalanced. In this study, we assessed the quantity of free radicals (ROS/RNS) in leaf and root tissues, and the results showed a slight increase in leaf tissues corresponding to the W-MPs and W-MIX treatments. These results could be related to the increase in *CAT* 1, *GST* 1 and *SOD* 1 gene expression levels in leaf tissue of seedlings from these treatments, especially for W-MIX, and was more significant in *SOD* 1 level (Fig. 2). SOD is one of the main antioxidant defences of the cell, catalysing the dismutation of the superoxide radical (O_2^-) into O_2 and H_2O_2. The toxic effect of W-MPs, and particularly when organic compounds are adsorbed (W-MIX), would lead to an increase of ROS which produced an increase in antioxidant enzyme gene expression. Nevertheless, it must be considered that the plant antioxidant system is very complex, and many other molecular and physiological processes may be involved in the defence of plant cells and tissues against oxidative damage. An increase in the *SOD* 1 gene expression level was also observed in leaf tissues of *Z. mays* seedlings cultivated in soil containing a combination of MPs (including W-MPs) and organic compounds (MIX), although the increase was not as high and decreased over time (Fajardo et al., 2022). The effects of MPs on plants are considered stronger in hydroponic than under soil culture conditions (Li et al., 2022). This effect may be related to the fact that MPs are more uniformly dispersed in aquatic media and their mobility is higher. In addition, soil structural complexity must be considered. *In vitro* tissue culture is not exactly a hydroponic culture, but some characteristics may be similar. In previous studies, we detected a stronger negative effect of PE-MPs on plants (lettuce and corn) in an *in vitro* experiment than in soil mesocosms (Martín et al., 2021; Fajardo et al., 2022).

The transcriptomic results of this study showed that MPs affected gene expression in different functions and processes, and that the type of MPs or MIX treatments may involve a difference in the plant response. Our results revealed that some metabolic functions were enriched in all treatments studied, pointing to a common response mechanism to the pollutants. Genes related to peroxidase and oxidoreductase activity were especially involved, which is in consistent with the oxidative damage described above. Likewise, Jiang et al. (2019) reported similar results related to the toxic effect of NPs/MPs on plants. A reduction in *PAL* genes (*PAL* 3 and *PAL* 9) was also observed in all studied treatments compared to the control. PAL (EC 4.3.1.5) is the enzyme at the entry point of the phenylpropanoid pathway. PAL catalyses the deamination of L-phenylalanine to form *trans*-cinnamic acid, a substrate common to the biosynthesis of different classes of phenylpropanoid products: anthocyanins, flavonoid pigments, ultraviolet (UV) protectants, antimicrobial furanocoumarins, isoflavonoid phytoalexins, lignin and wound phenolic esters (Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995). Due to the nature and function of these products, PAL activity and the activation of *PAL* genes under stress conditions have been considered part of a defence mechanism operating in stress-afflicted cells (Dixon and Paiva, 1995). In *Z. mays*, ten putative *PAL* genes have been described, although there is no detailed analysis or functional studies on them (Yuan et al., 2019). Most of them have been positively related to nematode infection (Starr et al., 2014), but *PAL* 3 was unresponsive to this infection. The effect of *PAL* knockdown on *Brachypodium* plants resulted in delayed development and reduced root growth, along with increased susceptibility to fungal pathogens (Cass et al., 2015). These studies agree with our results observed in maize seedlings, in which a reduction in *PAL* gene expression was observed in all treatments compared to the control, together with a reduction in leaf development, while an inhibition of root growth was observed only in the MIX treatments. The results seem to indicate that, in this case, the effect of MPs on *PAL* genes would not induce an activation of defence mechanisms to abiotic stress, but to a reduction of its activity that could affect the development of the plant. An analysis of the molecular responses of *Torreya grandis*, a conifeousr tree, to polystyrene NPs (Yu et al., 2022) found that *PAL* expression levels were significantly changed under NPs treatments, but similarly to our results, these genes were not up-regulated.

Our study showed differences in the molecular functions of maize seedlings related to the type of MPs they were exposed to, alone or associated with OCs. In addition to a differential response due to the type of MP tested, we also observed a different effect on corn seedling growth in the MIX treatments. Especially relevant was the transcriptomic difference found in the B-MIX treatment compared to the B-MPs treatment related to 'Transporter activity' (GO: 0005365) and, in particular, to sugar transport. More specifically, a significant increase in sugar transporter genes was observed in seedlings developed in the B-MIX treatment, although this increase was not observed in the other treatments (Fig. 5).

In plants, three types of sugar transporters have been identified: monosaccharide/polyol transporters (MSTs), sucrose transporters (SUTs or SUCs) and SWEETs (Sugars Will Eventually be Exported Transporter) (Julius et al., 2017; Zeng et al., 2022). The function of sugar transporters is involved in many developmental processes and physiological responses in plants. In fact, sugars provide the necessary energy for plant growth and development, but also act as signals to regulate the plant response to abiotic stress and to improve stress tolerance (Julius et al., 2017; Yadav et al., 2015; Zeng et al., 2022). Sugars responses to abiotic stresses have been demonstrated in many plant species, and it is widely recognized that stress conditions significantly change the expression levels of the involved genes (Williams et al., 2000; Yamada and Osakabea, 2018). Similarly, Zeng et al. (2022) observed in *Lilium* spp. that the expression levels of the sugar transporter genes increased significantly under cold, drought, salt stress and ABA stress conditions.

Therefore, our results confirm the capacity of MPs to act as contaminant vectors since the response of maize seedlings to MIX treatments is, in most of the studied physiological and transcriptomic aspects, different from those observed in the treatments with MPs alone. In particular, the strongest differential effect was detected under the W-MPs or W-MIX treatments, producing a negative effect on plant physiological and transcriptomic characteristics.

5. Conclusions

This study revealed the differential effect of two types of microplastic beads (W-MPs and B-MPs), alone or associated with other environmental pollutants, on the development of *Z. mays* seedlings sown under *in vitro* conditions. Our results indicate that MPs may act as pollutant carriers affecting plant physiology and transcriptomic pathways. The synergic effects between MPs and organic pollutants observed may vary depending on the type of MPs, which may make it difficult to predict plastic contamination consequences in natural ecosystems. Transcriptomic analyses can facilitate the understanding of plant responses to plastic exposure stress. In this context, the results obtained at the moment, as the differences observed in genes related to *PAL* and sugar

transport, could point to the development of biomarkers to detect changes associated with MPs or OCs carrier effects. Hence, further studies should be carried out to gather a wide range of plant metabolic responses under the emergent pollution of plastics and other associated contaminants.

In a climate-changing world, plastic degradation is predicted to be higher and to have a more significant impact. Therefore, understanding plastic pollution mechanisms and their effects on organisms, especially in crops, is crucial to guarantee sustainable agroecosystems and food security in the future.

Credit author statement

Carmen Martín: Conceptualization, Investigation, Writing -original draft. Michela Pirredda: Investigation, Writing -original draft. Carmen Fajardo: Conceptualization, Investigation, Writing -original draft. Gonzalo Costa: Investigation, Writing -original draft. Sebastián Sánchez-Fortún: Investigation, Writing -original draft. Mar Nande: Investigation. Gerardo Mengs: Investigation. Margarita Martín: Funding acquisition, Investigation, Supervision, Writing -original draft.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.138167.

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