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Mitigation of heavy metal soil contamination: A novel strategy with mycorrhizal fungi and biotransformed olive residue

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ABSTRACT

This study investigates a novel phytoremediation approach in, employing a synergistic treatment involving arbuscular mycorrhizal fungi (AMF) and biotransformed dry olive residue (DOR) to address heavy metal (HM) contamination in soils. The research focused on soil from the Guadiamar Green Corridor area (Aznalcóllar), characterized by bar soils with residual metal pollution (Co, As, Cr, Cd, Pb, Ni, Cu, and Zn). The findings highlight a significant improvement in soil physicochemical properties following the application of DOR, including a 3-unit increase in pH (from 4 to 7), an increase in $CaCO_3$ content from nearly 0 to 4, and a rise in organic matter content (from 0.92 % to 1.88 %). Additionally, the activity of all four enzymatic activities studied -dehydrogenase, β-glucosidase, phosphatase, and urease- was markedly enhanced, leading to improved biological properties. These changes led to a subsequent increase in vegetative response, as reflected in a 273 % rise in biomass, a 41 % increase in stomatal conductance, and a 47 % improvement in photosystem efficiency. Although mycorrhizal inoculation provided moderate benefit, the native species, *Rhizoglomus* sp. (Azn), emerged as the most effective, achieving a mycorrhization percentage of 28 % and an 80 % increase in root biomass compared to other treatments when combined with DOR. This study proposes an innovative, circular economy-driven approach to address diffuse pollution sources in the studied area by recommending the use of DOR and inoculation with sp. This approach proves superior in both soil and plant systems. Additionally, employing native inoculants and agricultural by-products, the research not only contributes to the valorization of local resources but also promotes economic growth while supporting environmental conservation efforts.

1. Introduction

Human activities have disrupted the geochemical cycles of metals, an illustrative example of was the Aznalcóllar (Southern Spain) metal mining spill occurred in 1998. The rupture of a reservoir containing residues from a complex sulfide mine led to the discharge of approximately 2 to 4 million m^3 of sludge and acidic water containing high amounts of heavy metals (HMs) (López-Pamo et al., 1999). Subsequent remediation measures were implemented in the affected area (Ayala-Carcedo, 2004), transforming it at the present time into an ecological corridor known as the "Green Corridor of the Guadiamar River". Nevertheless, there are still contaminated zones requiring unavoidable remediation (Sierra et al., 2019). In a study by Martín-Peinado et al. (2015), conducted fifteen years after the spill, elevated levels of heavy metals (HMs) were detected. However, these metals exhibited reduced mobility and availability, indicating the effectiveness of the remediation measures applied. Nevertheless, approximately 7 % of soils within a 12-kilometer radius from the spill site remained unrecovered. These soils presented characteristics such as an acidic pH and elevated metal concentrations, posing risks to both the environment and human health due to the potential dispersal of contaminants through runoff pathways.

One of the most ecological techniques for immobilizing pollutants is vegetation cover or restoration. The ability of plants to grow in highly contaminated soils with HMs can be enhanced by arbuscular mycorrhizal fungi (AMF), which increase the host plant's nutrients and water absorption, provide protection against pathogens, and mitigate the toxicity of certain mineral elements, including HMs (Turk et al., 2006;

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Riaz et al., 2021). This symbiotic interaction improves soil resources and enables plants to effectively response to environmental limitations. Arbuscular mycorrhizal fungi induce plant tolerance to HMs both in the rhizosphere and within plants through various mechanisms. One crucial mechanism involves HMs immobilization through chelation by AMF, which secrete glomalin—a stable, insoluble, and heat-resistant protein substance (Malekzadeh et al., 2016). Additionally, AMF have the capacity to bioaccumulate heavy metals in their structures due to the presence of acidic polysaccharides, such as chitin and chitosan, and functional groups like amine, phosphate, and hydroxyl (Tamjidi et al., 2022). In a study by Göhre and Paszkowski (2006), it was demonstrated that AMF could immobilize heavy metals in the soil (phytostabilization) and enhance their uptake and root-to-shoot transport (phytoextraction). The effectiveness of these processes depends on the plant host species, AMF activity and composition, metal concentration, and soil microbial structure and activity. Furthermore, AMF indirectly contribute to plant HM tolerance by influencing root morphology, enhancing mineral absorption, increasing aerial biomass, and activating antioxidant defence mechanisms in the plant (Janeeshma and Puthur, 2019).

Another effective method to enhance plant establishment involves incorporating organic amendments into contaminated soils. Low concentrations of organic matter (OM) have detrimental effects on soil fertility impacting water retention capacity, mechanical strength, aggregation, and overall structure (Eden et al., 2017). It is crucial to increase OM content in metal-contaminated soils, as this facilitates the formation of stable complexes between contaminants and organic ligands, moderating their mobility and associated processes (Sharma and Nagpal, 2018). Moreover, organic amendments influence the soil's redox potential and pH, with the latter playing crucial role in the mobility of HMs. Soils exposed to HMs for extended periods often exhibit low microbial activity, restricted to metal-resistant groups, with limited functional capacity (Siles et al., 2022). The addition of OM aids in restoring the state of indigenous microbial communities, which are also essential for OM decomposition, nutrient cycling, soil structure, and symbiotic relationships with plants, significantly influencing ecosystem functioning (Jiwan and Kalamdhad, 2011).

In the realm of soil remediation, the application of organic amendments emerges as a promising strategy to alleviate the impact of heavy metal contamination. Studies by Venegas et al. (2016) and Sharma and Nagpal (2018) have advocated for the use of such amendments to reduce metal mobility and enhance soil microbial activity, presenting a potential pathway for ecosystem restoration. A particularly compelling avenue for organic amendments involves the utilization of stabilized waste materials, specifically in Southern Spain, where residues from the prevalent two-phase olive oil extraction process are abundant. This process, recognized as the most widespread and efficient olive oil extraction methods (Vicario-Modroño et al., 2022), aligns with the principles of a circular economy by effectively repurposing and recycling waste within ecosystem. One of the wastes generated in the two-phase extraction process is "alperujo" (Vera et al., 2019), a semi-solid organic waste challenging to manage due to its high moisture content and viscous texture. Typically, it undergoes further treatment to become a more manageable residue known as dry olive residue (DOR) (Aranda et al., 2007; J.A. Siles et al., 2014). Spain alone generates around six million tons of DOR during a single olive harvest season (Lozano-García et al., 2011; J.A. Siles et al., 2014). The produced DOR, rich in minerals and organic matter, holds potential as an organic amendment (Aranda et al., 2007). However, its high phenol content, imparts phytotoxic and antimicrobial properties, discouraging its direct use (Sampedro et al., 2011; J.A. Siles et al., 2014). Therefore, DOR requires transformation before potential application as a soil amendment. Biotransformation of DOR with saprophytic fungi has been identified as one of the most effective techniques to reduce its concentration of phytotoxic compounds. This process stabilizes the organic matter content, improves the C/N ratio, and reduces the phenolic fraction of the residue (Sampedro et al., 2005, 2009). The basidiomycete *Coriolopsis rigida*, a saprophytic fungus is used as a potential agent for DOR bioremediation. This fungus secretes the ligninolytic enzyme laccase, responsible for oxidizing phenolic compounds and aromatic amines using molecular oxygen as an electron acceptor. Consequently, this detoxifies and transforms DOR, making it suitable for use as an organic amendment (Aranda et al., 2006).

The combined use of AMF and organic amendments has been previously studied (Medina and Azcón, 2010; Wang et al., 2013), demonstrating its effectiveness in soils contaminated with heavy metals and supporting the establishment of plant populations. However, this approach had never been tested in the context of Aznalcóllar. Notably, many of these studies often use mycorrhizal fungi that are not native to the application area, which can pose ecological risks. Introducing exotic fungal species may disrupt local microbial and plant communities, outcompete native mycorrhizal fungi, or create imbalances in soil biodiversity (Schwartz et al., 2006). To mitigate these risks and ensure sustainable ecosystem integration, it is crucial to prioritize the use of local mycorrhizal fungi. Additionally, while previous studies have primarily focused on the physicochemical properties of soils, their biological properties often remain underexplored. In this study, we emphasizes soil enzymatic activities as a key indicator of the recovery of ecological functionality.

Hence, the exploration of a combined treatment involving arbuscular mycorrhizal fungi (AMF) and organic amendments to enhance plant growth under adverse conditions, facilitating the accumulation of substantial amounts of heavy metals (HMs), holds significant promise in phytoremediation efforts (García-Sánchez et al., 2017; Siles et al., 2022). This study aims to investigate the potential utilization of agro-industrial residue, transformed by saprophytic fungi, in conjunction with arbuscular mycorrhizal symbiosis for remediating HMs contaminated soils, as an environmentally friendly and promising approach. The main objective is to assess the impact of DOR and various mycorrhizal treatments on both soil and plant systems by, i) evaluating vegetative response through the examination of antioxidant enzymatic activity (catalase, ascorbate peroxidase, glutathione reductase) and biomass; ii) analyzing soil microbial activity via enzymatic assays; and iii) assessing metal concentrations in both soil and plants.

2. Material and methods

2.1. Contaminated soil

The study utilized soil from the Guadiamar Green Corridor area (Aznalcóllar), specifically selected from bar soils within the first km from the mining spill site (37°29′35.5″N 6°13′13.5″W), where current vegetation was absent (García-Carmona et al., 2019). Soil samples were collected at a depth of 10 cm from a various bar sites and homogenized as a unified sample. Subsequently, the soils were sieved through a 5 mm mesh, dried in open air, and stored at room temperature. The characterization of contaminated soil confirmed elevated metal contents, especially for As, Pb, Cu, Zn, and Ni, along with acidic pH and high salinity.

2.2. Biological transformation of dry olive residue (DOR)

Dry olive residue (DOR), provided by Aceites del Sur (Granada, Spain), was sieved, subjected to multiple autoclave cycles, and stored at 4 °C until use. The initial levels of phytotoxic compound in DOR, such as phenolic compounds, were determined following the methodology described by Sampedro *et al.* (2009), which includes chemical characteristics and phytotoxicity assays. The fungus *Coriolopsis rigida* (EEZ-92) was selected from the collection of the Biofertilization and Rhizospheric Fungi Bioremediation research group at the Zaidín Experimental Station, belonging to the Spanish National Research Council (CSIC). The fungus was cultured on malt extract (2 %) agar plates for 2 weeks at 28 °C. After this period, the fungus was crushed in 40 ml of distilled water to prepare the fungal inoculum. Aliquots of 15 ml were added to various flasks containing 18 g of barley as a substrate for fungal growth and 30 ml of distilled water. The flasks were incubated at 28 °C for one week. Following this incubation, 20 g of DOR were added to each flask for biotransformation. The mixture was kept at 28 °C for 4 weeks, during which solid-state static fermentation transformed the DOR by *C. rigida*. After the process, barley was manually separated from the transformed DOR after fermentation, which was subsequently dried at room temperature, autoclaved multiple times for complete sterilization, sieved (2 mm), homogenized, and stored at 4 °C until use. The levels of phytotoxicity compounds in the biotransformed DOR were re-evaluated using the same chemical and phytotoxicity assessment techniques as those applied prior to the fermentation process, confirming the elimination of phytotoxicity and its safety for use as an organic amendment (J.A. Siles et al., 2014).

2.3. Arbuscular mycorrhizal fungi (AMF) inoculation

During seedling transplantation, inoculation with AMF was carried out by adding a 1 cm^3 in vitro AM inoculum (Cano et al., 2008), which contained AMF propagules (spores, active external hyphae and sterile root segments colonized by intraradical mycelium) from the different strains used. The inoculum was prepared in sterile conditions to avoid contamination and ensure the purity of the fungal strains. A small cavity was made in the planting substrate near the seedling roots, and the AMF inoculum was carefully placed to ensure close contact between the propagules and the roots system, for rapid colonization. The mycorrhizal inocula utilized were as follows: *Rhizoglomus* sp. (Azn, MUCL47213), isolated from the surviving vegetation in the Guadiamar Green Corridor after the Aznalcóllar and provided by Cusal Ingenio S.L.); *Rhizoglomus custos* (Custo, MUCL47214), isolated from the banks of the Rio Tinto River near Nerva in Huelva (Spain) (Cano et al., 2009), and *Rhizophagus irregularis* (Intra, *Glomus irregulare* DAOM 197,198, s.f.). All three mycorrhizal fungi were selected for their demonstrated ability to support plant survival and growth in polluted soils (Silva-Castro et al., 2022).

2.4. Experimental design

The experiment was conducted in polypropylene pots, each filled with approximately 1 kg of Aznalcóllar contaminated soil, complemented with 5 % marble sludge, a waste material from the mining area of Macael (Almería, Spain), which has been shown to effectively raise pH (Paniagua-López et al., 2023). The experimental design involved two main factors: the addition of biotransformed DOR by *C. rigida* (5 %) and the inoculation with three mycorrhizal fungi, *Rhizoglomus sp* (Azn), *Rhizoglomus custos* (Custo), and *Rhizophagus irregularis* (Intra). Tested as follow: 1) polluted soil (C); 2) polluted soil + marble (Mar 5 %); 3) polluted soil + marble + DOR (DOR 5 %); 4) polluted soil + marble + DOR + Azn (DOR+Azn); 5) polluted soil + marble + DOR + Custo (DOR+Custo); 6) polluted soil + marble + DOR + Intra (DOR+Intra); 7) polluted soil + marble + Azn (Azn); 8) polluted soil + marble + Custo (Custo); 9) polluted soil + marble + Intra (Intra). Seven repetitions ($n = 7$) were established for the nine treatments. After 10 days of growth in a vermiculite seedbed, three wheat plants (*Triticum aestivum* L.) were planted in each pot, since it reaches maturity within the study period and it is easily comparable and replicable. The experiment was carried out under greenhouse-controlled conditions (supplementary light, 25–19 °C, 50 % relative humidity) and regular irrigation.

After a 3 month growth period, in vivo measurement of the stomatal conductance and Photosystem II efficiency was determined. Subsequently, the plants were harvested, and study variables were analyzed in both the plant and the soil. Soil samples were taken from each pot under the same treatment, homogenized, and sieved (2 mm mesh). The soil samples were then divided into two subsamples: one for the analysis of enzymatic activities (β-glucosidase, dehydrogenase, phosphatase, urease), and another for the determination of the physicochemical characteristics of the soil including heavy metal content. Simultaneously, wheat plants were harvested, with roots, aerial parts, and spikes separated into subsamples for the determination of plant biomass, mycorrhizal percentage (only root), antioxidant enzymatic activity (catalase, ascorbate peroxidase, glutathione reductase) (only aerial), and heavy metal accumulation.

2.5. Soil analysis

After 3 months, the following characterization of soil samples was performed: pH in water extract (1:2.5 m:V) with a 914 pH/conductivity-meter Metrohm (Metrohm AG, Herisau, Switzerland); electrical conductivity (EC) in water extract (1:5 m:V) with an Eutech CON700 conductivity-meter (Oakton Instruments, Vernon-Hills, IL, USA); organic carbon (OC) content by wet oxidation (Tyurin, 1951); calcium carbonate ($CaCO_3$) content by volumetric gases using a modified Bernard calcimeter (Barahona, 1984); total nitrogen (N) content by dry combustion with the elemental analyser TrueSpec LECO© (St. Joseph, MI, USA).

To determine the total metal content (Co, As, Cr, Cd, Pb, Ni, Cu, and Zn), soil samples were digested using a Mars© XP1500 Plus microwave digestion system. A mixture of 9 ml of HCl and 3 ml of HNO_3 (3:1) was added to 0.1 g of soil, and the digestion was carried out for 1 hour at 200 °C and 800 W. The bioavailable fraction of metals was extracted by adding 50 ml of 0.05 M EDTA extractant solution to 5 g of soil and shaking for 1 hour. The water-soluble fraction was obtained by adding 25 ml of distilled water as the extractant solution to 10 g of soil, followed by shaking for 24 h. Metals concentration in the resulting extracts were measured using an Avio® 500 Inductively Coupled Plasma-Optical 190 Emission Spectrometer (ICP-OES) (Shelton, CT, USA). To verify the accuracy of the method, Standard Reference Material SRM2711 Montana Soil (US NIST, 2003), was analyzed ($n = 6$). The recovery rates for the 8 study elements in the reference material were 89±26 %.

Soil enzymatic activity, β-glucosidase and phosphatase activities were determined following the methods described by Eivazi and Tabatabai (1988). Dehydrogenase activity was quantified according to the Casida et al. (1964) method. To determine urease activity, the method described by Kandeler and Gerber (1988) was followed.

2.6. Plant analysis

At the end of the 3 month experiment, prior to harvesting, the stomatal conductance and photosynthetic II efficiency of the three uppermost leaves in each treatment were determined. This was achieved using a Porometer AP4 (Delta-T Devices Ltd., Cambridge, UK) for stomatal conductance and a FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic) for photosynthetic II efficiency using in the in vivo plants.

Following harvest, wheat plants were divided into roots, aerial parts, and spikes. Each component was weighed on a precision scale to determine its dried biomass (70 °C for 24 h). Aerial samples were used to quantify antioxidant enzymatic activity. Ascorbate peroxidase (APX) and glutathione reductase (GR) activities were determined following the methods proposed by De Gara et al. (2003). For catalase (CAT) activity determination, the method proposed by Beaumont et al. (1990) was followed. The effectiveness of the inoculation was confirmed by measuring the percentage of mycorrhization in wheat plant roots using the staining method of Phillips and Hayman (1970) and the counting method by Giovannetti and Mosse (1980).

The remaining plant samples (root and aerial parts) were used for the determination of total heavy metals (Co, As, Cr, Cd, Pb, Ni, Cu, and Zn). Acid digestion was performed in a digestion block (Perkin Elmer SPB 50–48) with 9 ml of HCl, 3 ml of HNO_3 and 3 ml of H_2O_2 (4:1:1) for 1 hour at 85 °C. Metals concentration was measured in Avio® 500

Inductively Coupled Plasma-Optical 190 Emission Spectrometer (ICP-OES) (Shelton, CT, USA). Method accuracy was confirmed by analyzing Standard Reference Plant Material (ERM-CD281 Rye Grass) (n = 6). Recoveries for the 8 study elements were 87 ± 11 %.

Using data on total metals concentrations in soil and plants, the root and aerial Bioaccumulation Factor (BAF) and Translocation Factor (TF) were calculated to assess the uptake and movement of heavy metals in the soil-plant system. The BAF was determined as the ratio of the heavy metal concentration in the plant to its concentration in the soil, calculated using the formula: BAF = (Concentration in organism) / (Concentration in environment). The TF was calculated as the ratio of the heavy metal concentration in the plant's aerial parts to that in the roots, using the formula: TF = (Concentration in aerial part) / (Concentration in roots). These indices were used to evaluate the plant's potential for heavy metal accumulation, exclusion, and/or translocation, which are critical for understanding the plant's role in phytoremediation.

2.7. Statistical analysis

To assess the distribution of metal content in soils and plant material, descriptive statistics were employed. This included the computation of individual histograms, mean, median, minimum and maximum values, as well as quartiles to examine the normality of the data. Additionally, Levene's test was used to evaluate the homogeneity of variances among the datasets and where necessary logarithmic transformation was applied to ensure normality. Statistical analysis was performed using the Statgraphics Centurion XVI software. Mean values and standard deviations were computed for each sample set after confirming normal distribution. Subsequently, a one-way analysis of variance (ANOVA) was conducted to compare means across multiple groups. Post-hoc analysis using the LSD (Least Significant Difference) test was applied to identify specific differences between individual groups. Statistical significance was set at a probability level of $p < 0.05$. To elucidate and visually represent relationships between treatments and variables, Principal Component Analysis (PCA) was employed. The observation of the results in the PCA facilitated the reduction of dimensionality and exploration of underlying patterns or correlations among the different treatments and variables.

3. Results and discussion

3.1. SOIL

3.1.1. Physicochemical properties

The physicochemical properties of the soil were analysed at the end of the experiment to determine the influence of the tested treatments, and the results are shown in Table 1.

The initial pH of the study soil, influenced by existing contamination, was highly acidic (4.03). Notably, the application of various treatments, including amendments and arbuscular mycorrhiza, resulted in a substantial increase in pH of approximately 3 units, reaching values close to neutrality in all cases. The addition of marble (Mar) slightly lowered the pH to 6.87, while other treatments showed values between 7.21 and 7.39. Generally, at acidic pH levels, certain metals tend become more soluble and mobile in the soil, posing greater risks of toxicity to plants and other organisms (Simón et al., 2002). Increasing the pH to values close to neutrality significantly mitigates toxicity issues in the area. This pH increase was linked to changes in $CaCO_3$ content. The% of $CaCO_3$ increased by more than 2.8 points in all treatments compared to the Control (contaminated soil without treatment). The presence of $CaCO_3$ in the soil can affects the total content and activity of heavy metals, either enhancing or restricting their absorption by plants. Alkaline soils rich in $CaCO_3$, according to He et al. (2020), facilitate the formation of hydrated hydroxides and insoluble carbonates or adsorption by soil colloids, reducing the mobility of these metals. In terms of salinity, the selected treatments did not significantly alter the salinity of contaminated soil, maintaining consistently high levels of electrical conductivity (EC close to values of 3 dS/m) in all cases, indicating moderate salinity. This fact is noteworthy, as high levels of soluble salts (Cl^-, NO_3, etc.) are associated with increased mobility of certain heavy metals (Cd, Pb, Ni, Cu), as they form complexes that can be more easily absorbed by plants (Usman et al., 2005). Regarding the addition of DOR led to higher expected values for percentage organic carbon (OC) in the soils. These treatments showed more balanced C/N ratios, indicating a more favourable nitrogen release to carbon content compared to treatment without DOR. However, the C/N ratio was generally low in all cases, indicating a high nitrogen content relative to carbon. Given short stabilization period (3 months) after adding the treatments to the soil, the decomposition time of organic matter was limited, while total nitrogen inputs remained high.

Table 1
Physicochemical characteristics of soil subjected to different treatments. C (Control soil); Mar (marble); DOR (biotransformed dry olive residue); Azn (*Rhizoglomus* sp.); Custo (*Rhizoglomus custos*); Intra (*Rhizophagus irregularis*); EC (electrical conductivity); OC% (organic carbon).

Treatment	pH	$CaCO_3$ (%)	EC (dS/m)	OC (%)	C/N
C	4.03	0.47	3.07	0.92	7.70
Mar	6.87	4.08	3.00	0.95	7.45
DOR	7.21	4.72	2.81	1.77	9.71
DOR+Azn	7.38	2.76	2.98	1.88	9.25
DOR+Custo	7.28	4.97	3.12	1.65	8.98
DOR+Intra	7.27	1.30	2.88	1.62	8.93
Azn	7.35	3.42	3.15	0.88	5.87
Custo	7.38	3.25	2.98	0.96	7.33
Intra	7.39	3.32	2.99	0.91	6.39

3.1.2. Enzymatic activity

The biological quality of soils was assessed using the enzymatic activities, including β-glucosidase, dehydrogenase, phosphatase, and urease as illustrated in Table 2.

Dehydrogenase activity was significantly higher in soils after addition of DOR and inoculation with Azn mycorrhiza, compared to mycorrhizal treatments with Custo and Intra. However, these values showed only a slight significantly different from the Control (C) and

Table 2
Enzymatic activity of dehydrogenase, β-glucosidase, phosphatase, and urease in soils subjected to different treatments. C (Control soil); Mar (marble); DOR (biotransformed dry olive residue); Azn (*Rhizoglomus* sp.); Custo (*Rhizoglomus custos*); Intra (*Rhizophagus irregularis*). Data are presented as mean ± standard deviation, n = 7. For each enzyme, lowercase letters indicate significant differences according to LSD test ($p < 0.05$).

Treatment	Dehydrogenase (μg TPF/g·day)	β-glucosidase (μg p-nitrofenol/g·h)	Phosphatase (μg p-nitrofenol/g·h)	Urease (μg NH_4–N/g·h)
C	10.4 ± 2.05 ab	0.49 ± 0.2 ab	1.43 ± 0.67 b	20.60 ± 12.46 a
Mar	9.5 ± 0.75 ab	0.38 ± 0.13 a	0.52 ± 0.3 a	140.35 ± 10.46 e
DOR	14.18 ± 2.75 b	10 ± 1.71 d	2.58 ± 0.41 d	159.27 ± 18.77 f
DOR+Azn	15.03 ± 3.73 b	14.15 ± 0.98 e	3.45 ± 0.76 e	157.79 ± 4.63 f
DOR+Custo	13.98 ± 3.56 b	8.33 ± 1.23 c	3.65 ± 0.39 e	162.31 ± 20.84 f
DOR+Intra	13.74 ± 3.59 b	14.47 ± 0.97 e	2.09 ± 0.7 c	175.16 ± 6.22 g
Azn	13.16 ± 4.79 b	0.97 ± 0.11 ab	0.74 ± 0.37 a	95.51 ± 9.26 d
Custo	7.55 ± 2.59 a	1.23 ± 0.1 b	0.67 ± 0.19 a	77.95 ± 9.45 c
Intra	6.09 ± 2.43 a	0.58 ± 0.09 ab	0.6 ± 0.29 a	52.70 ± 9.72 b

marble-treated (Mar) soils (increases in activity ≥32 %). The dehydrogenase enzyme is found in all live microbial cells, playing a crucial role in their respiratory processes (Bolton et al., 1985). This enzyme serves as indicator of soil metabolic activity since it is inactive independently of microbial cells as extracellular enzymes (de Oliveira and Pampulha, 2006). In the study by de Oliveira and Pampulha (2006), heavy metal contamination decreased dehydrogenase activity (80 %), demonstrating a negative correlation between the activity of this enzyme and heavy metal contamination. In our case, there is just a slight difference between the untreated contaminated soil (C) and the other treatments, potentially attributed to the sustained high concentrations of heavy metals, as we will discuss in Section 3.3.1.

In treatments where DOR was added, β-glucosidase activity exhibited a significantly increase, particularly in those inoculated with Azn and Intra (28-folds higher). Conversely in the treatments without DOR, β-glucosidase activity is maintained in values similar or around two-times higher than in control contaminated soil. β-glucosidase, along with cellulose, is part of the process of decomposing organic matter (a source of C) into simple sugars like glucose, which soil microorganisms can use as a source of energy (Deng and Tabatabai, 1994; Ahmed et al., 2017). Therefore, it is clear that soil microorganisms responded to the addition of DOR by increasing β-glucosidase activity, using DOR as a source of carbon source for energy.

Phosphatase activity also experienced a notable increase in soils where DOR was added, particularly in those inoculated with Azn and Custo. The inoculation of Azn and Custo helped the bioavailability of phosphorus supplemented with the addition of DOR, obtaining the highest values of this enzyme with these treatments. Phosphatase are a group of enzymes that play a fundamental role in the phosphorus cycle by catalysing the hydrolysis of esters and anhydrides of phosphoric acid, serving as reliable indicators of soil fertility (Schmidt and Weary, 1962; Dick et al., 2000). Again, the no addition of DOR did not help to increase phosphatase activity.

The total N content is a major environmental driver of phosphatase activity in soils. Given the close coupling between the N and P cycles, the large amounts of N require for phosphatase biosynthesis contribute to this relationship (Margalef et al., 2021). Consequently, when comparing phosphatase and urease activity, we observe very similar profiles.

Urease activity showed a significant increase in soils only with the marble addition. Furthermore, adding DOR leads to even greater activity, and when combined with inoculation with Intra, it results in significantly the highest urease activity. Among the solely mycorrhizal treatments, Azn achieved the highest values. Urease, responsible for hydrolysing urea into CO_2 and ammonia, play a vital role in the nitrogen cycle and regulates N supply to plants (Makoi and Ndakidemi 2008). The experiment's results align with the finding of Stocks-Fischer et al. (1999), indicating that urease activity is influenced by pH, with higher activity observed in treatments with raised pH levels, achieved by addition of marble in our experiments (Table 1). This pH modulation not only affects urease performance but also influences microbial metabolisms, enhancing their ability to decompose urea (Tang et al., 2020).

Our results are consistent with the meta-analysis conducted by Pokharel et al. (2020), which demonstrated a significant increase in urease, phosphatase, and dehydrogenase activities with the application of organic matter. Drawing from these findings, we can reasonably conclude that the addition of DOR serves not only as a source of organic matter but also as a general soil conditioner, leading to an improvement in biological activity. This enhancement is reflected in increased enzymatic activity associated with carbon and nutrient cycles. The collective impact contributes to an overall improvement in soil quality and health, aligning with the broader understanding of the positive effects of organic matter on soil ecosystems (Verheijen et al., 2010).

3.2. Plant

3.2.1. Mycorrhization percentage

Arbuscular mycorrhizal fungi (AMF) enhance the ability of plants to absorb water and nutrients (Riaz et al., 2021) and confer resistance to the toxicity of elements, including as heavy metals (Janeeshma and Puthur, 2019; Dhalaria et al., 2020). The treatment that achieved the highest mycorrhization percentage was the one with DOR and inoculated with Azn (28.2 %), followed by DOR inoculated with Custo (15.4 %). In treatments without DOR, Azn mycorrhiza did also exhibit the highest percentage (12.2 %), followed by Custo (9 %), Intra (7.2 %) and DOR+Intra (2.4 %). Notably, treatments without mycorrhizal fungi inoculation (C, Mar, DOR), showed no mycorrhization percentage due to absence of native AMF in the heavily metal contaminated soil (Table SI.1).

The AMF coevolves with plants in their habitats, resulting in the adaptation of both AMF and plants to specific regions (Gosling et al., 2006). Therefore, in our study, AMF *Rhizoglomus sp* (Azn), isolated from surviving vegetation in the study area post-disaster and adapted to adverse conditions, showed a significantly higher mycorrhization percentage than the other two fungi. *Rhizoglomus custos* (Custo), isolated from the heavy metal rich banks of the Rio Tinto River, (Nelson and Lamothe, 1993; De La Fuente et al., 2009), obtained the second-highest mycorrhization percentage. This aligns with findings in the study by Quatrini et al. (2003), where plants inoculated with native AMF obtained more mycorrhization than those inoculated with non-native species, *Glomus mosseae*.

Furthermore, we observe that mycorrhization percentages of Azn and Custo were higher in treatments with added DOR. The increase can be attributed to improved soil conditions resulting from the addition of organic matter and achieving a neutral pH (Table 1). The elevated soil enzyme activity in treatment with added DOR, as shown in Table 2, stimulate bacterial communities, as reported by Johansson et al. (2004), promoting spore germination and increasing the extent and rates of AMF colonization. The significant increase in soil enzymatic activity with the addition of DOR, aligns with the higher mycorrhization percentage observed in these treatments.

3.2.2. Vegetative response

To study the growth and development of wheat plants in the different treatments, we analysed biomass (Fig. 1), stomatal conductance, and the efficiency of photosystem II (Fig. 2).

In Fig. 1, a notable increase in biomass is observed in both roots and aerial parts, including the spike, with the addition of DOR, compared to treatments without this transformed residue as an amendment. Among treatments with DOR, there are a greater biomass, but no significant differences, in the aerial part and root among the types of inoculated mycorrhiza. Whereas, a significant increase in spike was observed in the DOR+Custo treatment.

Native AMF species hold an advantage over allochthones ones, having adapted to the conditions of their environment over time (as mentioned in Section 3.2.1). This aligns with finding from Quatrini et al. (2003), where the final harvest of *Citrus limon (*L.*)* showed significantly higher root and aerial weights in treatments with native mycorrhizas compared to *Glomus mosseae* (non-native). With *Glomus mosseae*, the growth variables of the treated plants were equal to or lower than the non-inoculated controls. These results are also consistent with the mycorrhization percentage (Table SI.1), which was higher in treatments inoculated with Azn, followed by Custo, mirroring the higher root weight in the Azn-inoculated treatment followed by the Custo-inoculated treatment.

On the other hand, Fig. 2 illustrates stomatal conductance, revealing that the treatments with the highest stomatal conductance were those with the addition of DOR, particularly noteworthy in the case of the inoculated treatment with Intra. This mycorrhiza also exhibited the highest conductance among treatments solely inoculated. Stomatal

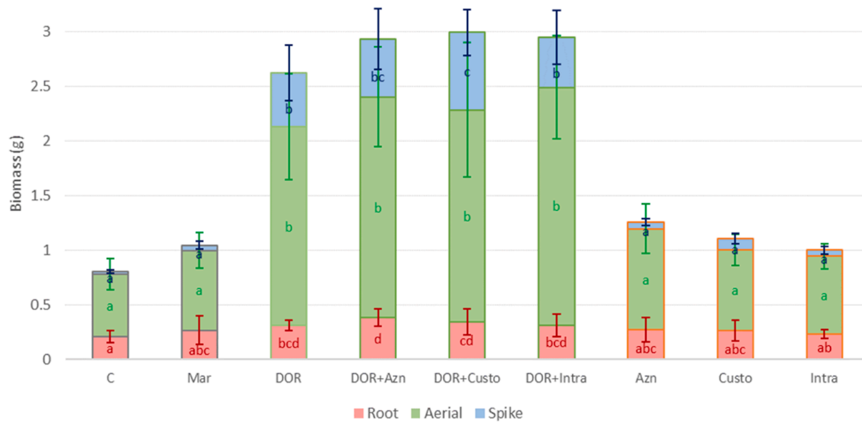

Fig. 1. Biomass of root, aerial and spike plant growth in soil subjected to different treatments. C (Control soil); Mar (marble); DOR (biotransformed dry olive residue); Azn (*Rhizoglomus* sp.); Custo (*Rhizoglomus custos*); Intra (*Rhizophagus irregularis*). Data represent the mean of biomass data. Error bar represent standard deviation ($n = 7$). For each micronutrient, data followed by different letter are significantly different according to LSD test ($p < 0.05$).

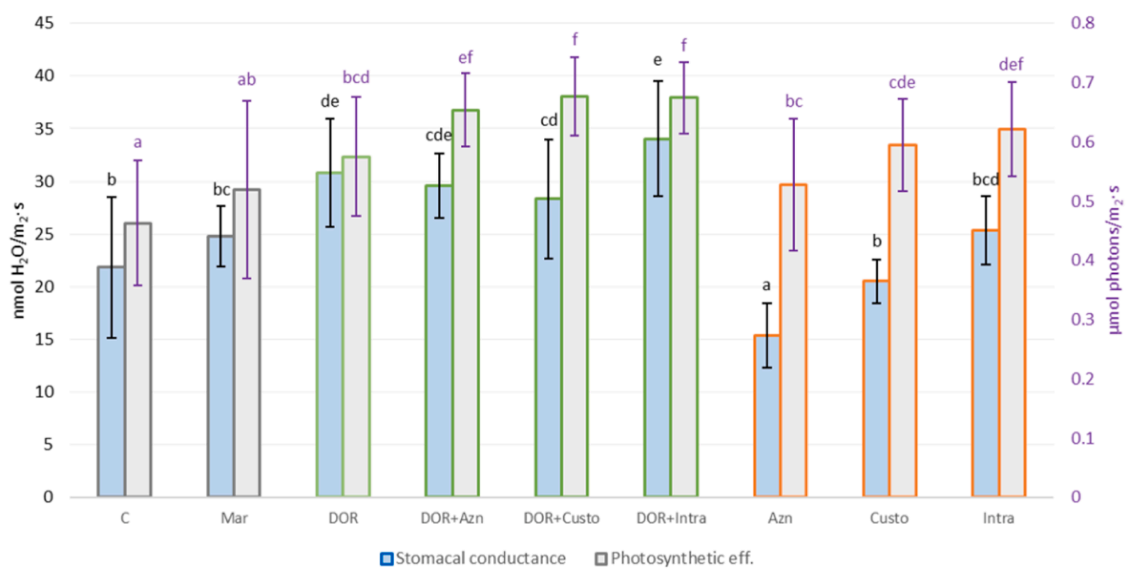

Fig. 2. Stomatal conductance and efficiency of photosystem II of plants growth in soil subjected to different treatments. C (Control soil); Mar (marble); DOR (biotransformed dry olive residue); Azn (*Rhizoglomus* sp.); Custo (*Rhizoglomus custos*); Intra (*Rhizophagus irregularis*). Data represent the mean of stomatal conductance and efficiency of photosystem II data. Error bar represent standard deviation ($n = 7$). For each micronutrient, data followed by different letter are significantly different according to LSD test ($p < 0.05$).

conductance represents the gas exchange capacity of stomata, regulated by stomatal pore opening and density (Zhu et al., 2018). Symbiosis with AMF alters stomatal behaviour through biochemical and/or biophysical mechanisms, such as stomatal concentration in tissues, root-to-shoot hydraulic signalling, tissue hydration, or plant size (Augé, 2000; Augé et al., 2016; Chitarra et al., 2016). As observed in the study by Zhu et al. (2018), under certain stress conditions, stomatal conductance significantly decreased, but to a lesser extent in mycorrhizal plants.

Regarding the efficiency of photosystem II (Fig. 2), treatments with higher values were significantly those treated with DOR and inoculated with mycorrhizas. Photosystem activity depends on its ability to capture light energy and the efficiency of the capturing excitation energy for photochemistry (Baker, 1991). Under stress conditions, photosystem efficiency reduces to prevent photooxidation, increasing the dissipation of excitation energy in pigment (Pippucci et al., 2015). Photosynthesis is highly sensitive to stress induced by heavy metal toxicity; the presence of divalent cations, such as Pb^{+2} or Cd^{+2}, competes for the active sites of enzymes responsible for chlorophyll synthesis (Sharma and Dubey, 2005). The higher availability of these metals reduce photosystem efficiently, as discussed in Section 3.3.2. Control, being the treatment with the highest solubility of these metals, significantly has the lowest efficiency. AMF also increase photosynthesis by increasing chlorophyll content (Begum et al., 2019). It is known that the rate of photosynthesis decreases when stomatal conductance decreases (Miyashita et al., 2005). By comparing the profiles in Fig. 2, we observe that they are similar.

From these results, we can conclude that the vegetative response of plants in heavy metals contaminated soil is significantly more favourable in treatments where DOR was added. When combined with symbiosis with AMF, the response is partially improved.

3.2.3. *Enzymatic activity*

Antioxidant enzymatic activity was investigated in the aerial part of the plants, focusing on the enzymes catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR), as shown in Table 3. These enzymes are part of the plant's mechanisms against oxidative stress caused by the production of reactive oxygen species (ROS) induced by the presence of heavy metals. Superoxide dismutase (SOD) initiates the

Table 3
Enzymatic activity of catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) in the aerial part of the plant growth in soil subjected to different treatments. C (Control soil); Mar (marble); DOR (biotransformed dry olive residue); Azn (*Rhizoglomus* sp.); Custo (*Rhizoglomus custos*); Intra (*Rhizophagus irregularis*). Data are presented as mean ± standard deviation, $n = 9$. For each enzyme, lowercase letters indicate significant differences according to LSD test ($p < 0.05$).

Treatment	CAT (μmol/gr protein·min)	APX (μmol/gr protein·min)	GR (μmol/gr protein·min)
C	0 a	0.0065 ± 0.0018 ab	0.0015 ± 0.0005 a
Mar	0.0023 ± 0.0008 b	0.0067 ± 0.002 ab	0.0034 ± 0.0006 cd
DOR	0.0040 ± 0.0009 c	0.0138 ± 0.0034 cd	0.0036 ± 0.0009 d
DOR+Azn	0.0027 ± 0.0005 b	0.0081 ± 0.002 b	0.0025 ± 0.0007 b
DOR+Custo	0.0043 ± 0.0009 c	0.0169 ± 0.0054 d	0.0030 ± 0.0005 bcd
DOR+Intra	0.0032 ± 0.0007 bc	0.0124 ± 0.0024 c	0.0011 ± 0.0003 a
Azn	0.0022 ± 0.0008 b	0.0056 ± 0.0006 ab	0.0034 ± 0.0003 cd
Custo	0.0043 ± 0.0003 c	0.0039 ± 0.0012 a	0.0027 ± 0.0002 bc
Intra	0.0067 ± 0.0014 d	0.0046 ± 0.001 ab	0.0024 ± 0.0006 b

process by dismutating $\cdot O_2^-$ into H_2O_2, and the excess H_2O_2 is then eliminated by CAT and APX enzymes through the ascorbate-glutathione cycle, with GR being a crucial component. This cycle maintains redox reactions, preserving the NADP/NADPH ratio and cellular redox status, preventing ROS formation, and protecting the photosynthetic electron transport (Hashem et al., 2018).

In CAT activity, the mycorrhizal treatment with Intra stands out significantly, showing the highest activity, in contrast to the non-treated soil (C) where no activity was detected. In the other treatments, CAT activity was observed. Ascorbate peroxidase (APX) activity was significantly higher in plants treated with DOR, with the exception of the DOR+Azn treatment, which exhibited activities similar to the other treatments. Glutathione reductase (GR) activity was significantly lower in the non-treated soil (C) and the combined treatment with DOR and Intra, while in the other treatments, this activity was higher. In comparison with the untreated soil (C), there is a notable increase in these enzymatic activities in most treatments. Although APX activity increases significantly with the addition of DOR, no differences were observed in CAT and GR activities between the presence or absence of DOR. The increase activities of antioxidant enzymatic suggests an activation to counteract the increased production of ROS. Non-detection or a decrease in this activity may be attributed to the concentration of ROS exceeding the enzymes' capacity or even inactivating them (Wang et al., 2019; Silva-Castro et al., 2022), as in the case of CAT, where no activity was detected in the untreated soil (C), indicating that the plant does not need to produce them due to absence of ROS generation.

There is ample literature on how symbiosis with AMF enhances the activity of antioxidant enzymes through the growth and improvement of nutrition in mycorrhizal plants (Ruiz-Lozano, 2003; Wu et al., 2006; Evelin and Kapoor, 2014; Hashem et al., 2018; Mitra et al., 2021), providing resistance to exposure to heavy metals. Arbuscular mycorrhizal fungi play a vital role in the absorption and transport of Zn and Cu (Cavagnaro, 2008; Subramanian et al., 2011), which are structural components of these enzymes (Natasha et al., 2022), highlighting SOD, a metalloenzyme with different isoforms depending on its metallic cofactor: Fe-SOD, Mn-SOD, and Cu-Zn SOD (Alscher et al., 2002). Although the results obtained indicate an increase in these enzymatic activities in all treatments compared to the non-treated soil (C), there is no clear increase when the plants are mycorrhized, as the Mar treatment (non-mycorrhized) obtains similar values in CAT and GR activity to the mycorrhized treatments. Only in APX activity is there a significant increase when adding DOR compared to the other treatments.

3.3. Heavy metals

3.3.1. Heavy metals in the soil

To determine whether the concentrations of HMs are anomalous, the obtained results are graphically compared with the average amount of heavy metals in similar uncontaminated soils from a nearby area located 42 km in a straight line (Zuluaga et al., 2017). The concentration of cobalt (Co) (Fig. 3A) and nickel (Ni) (Fig. 3C) show values similar to the average value of the reference soil for all treatments. In contrast, the concentrations of the other studied metals, Cu (Fig. 3B), Zn (Fig. 3D), As (Fig. 3E), Pb (Fig. 3F), Cd (Fig. 3G), Cr (Fig. 3H), are notably higher than the average of the reference in all the treatments.

In addition to determining the total concentration of heavy metals in the soil, studying their solubility and bioavailability is crucial, as these factors are related to their risk of dispersion in the ecosystem and absorption by organisms (Kim et al., 2015). Soils contaminated with heavy metals pose a significant concern because these metals can accumulate in crops, entering the food chain and posing risks to the health of animals and humans (Shahid et al., 2017). Metal availability for plants depends on soil properties, especially pH and organic matter, as well as the chemical speciation of each metal (Basta et al., 2005; Micó et al., 2008).

The solubility of metals in the treatments, determined through water extraction, is presented in Table 4A. The values obtained through this method indicate the fraction of metals with a higher risk of short-term dispersion (Quevauviller et al., 1998). The general trend observed for all metals, except for As, is a reduction in solubility compared to the Control (C). For As, there is no clear trend. The addition of marble to the soil leads to a clear reduction in the solubility of the metals studied, as it is linked to increasing pH (Table 1). Increasing pH favours certain oxidation states in metals, making them less mobile and, therefore, less available. The only exception is arsenic, which remains highly toxic and mobile over a wide pH range, with As(III) being 60 times more toxic and mobile than As(V) (Giri and Patel, 2012). Similar results were obtained by Chuan et al. (1996).

We also conducted an extraction with EDTA (Table 4B). EDTA has the ability to extract elements from the soil that are soluble, exchangeable, and weakly adsorbed, forming complexes with humic or carbonated substances. These elements constitute the fraction that could be available for living organisms in the medium to long term (bioavailable elements) (Quevauviller et al., 1998; Álvarez et al., 2006). In Table 4B, the general trend in this case is an increase in bioavailability compared to the Control (C), except for Ni and Zn, which tend to decrease. With the addition of marble and the rise of pH (Table 1), bioavailability tends to increase for most metals. Mitsios *et al.* (2005) also found negative correlations between the amount of extractable Zn and soil pH, while positive correlations were found in the case of Cd. Zeng et al. (2011) observed that when pH ranged from 5 to 7, the values of extractable metals were approximately the same, but when pH increased above 7, the values decreased significantly. The pH of our treatments after the addition of marble ranges from 6.87 to 7.39. In this study, the content of organic matter was also positively correlated with the amount of extractable metals with EDTA. In our results, this trend seems to be observed only in Co and As. Organic matter reduces the availability of metals by adsorbing or forming complexes with humic substances (Liu et al., 2009), but it also supplies organic chemicals to the soil that act as chelators and can increase the availability of metals (Vega et al., 2004; McCauley et al., 2009).

For both soluble and bioavailable metals, the concentrations of Pb were below the detection limits of the spectrophotometer used. Lead forms precipitates with PO_4^{3-} and CO_3^{2-} ions within the pH range of the soils in our treatments (5.5–7), making it highly unavailable (Blaylock et al., 1997). Additionally, some concentrations were so low that they were also below the detection limits, preventing the calculation of standard deviations for all measurements. Further details can be found in Table SI.2.

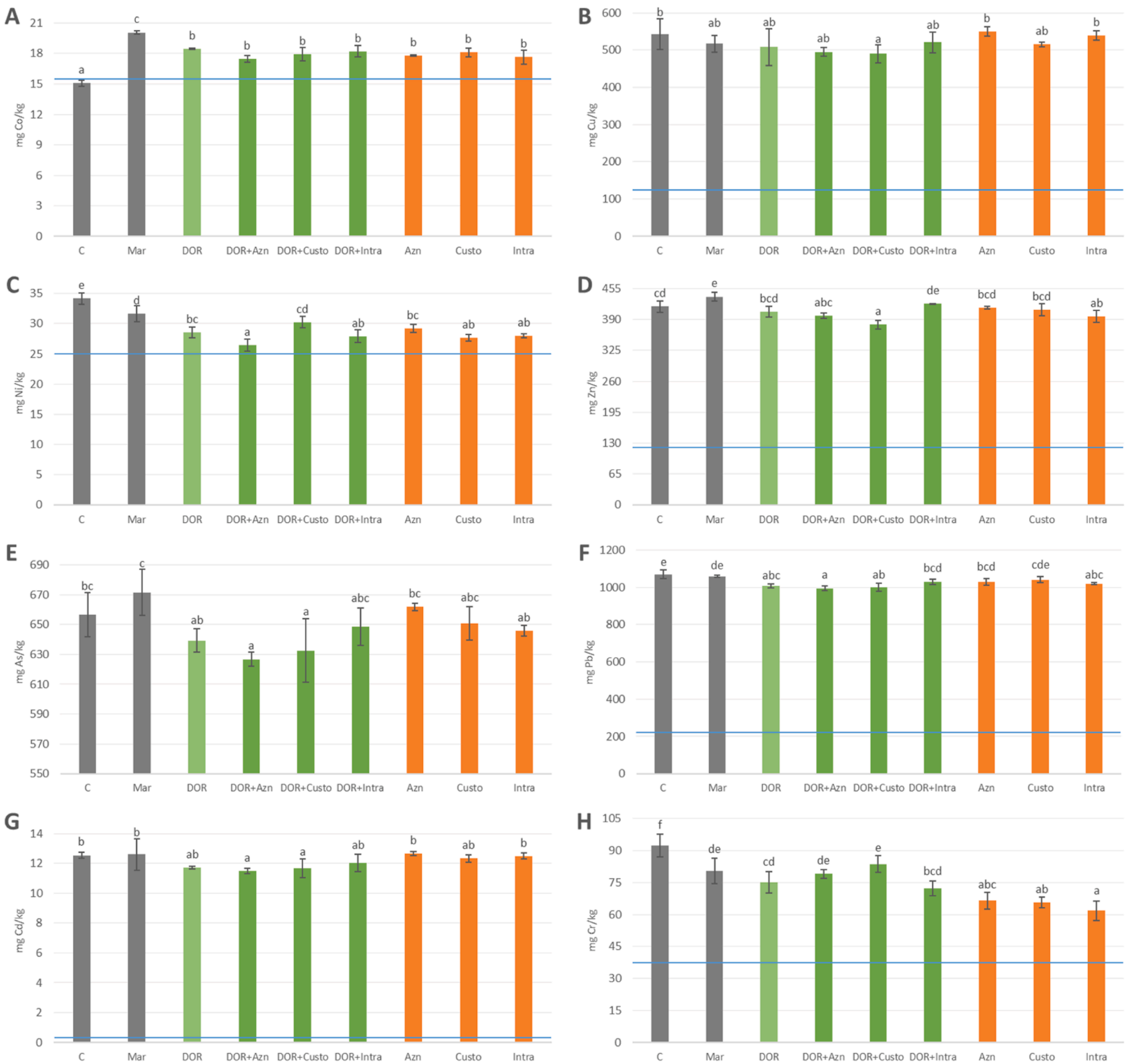

Fig. 3. Total cobalt (A), copper (B), nickel (C), zinc (D), arsenic (E), lead (F), cadmium (G), chromium (H) in soil subjected to different treatments. C (Control soil); Mar (marble); DOR (biotransformed dry olive residue); Azn (*Rhizoglomus sp.*); Custo (*Rhizoglomus custos*); Intra (*Rhizophagus irregularis*). Data represent the mean of micronutrient data. Error bar represent standard deviation ($n = 3$). For each micronutrient, data followed by different letter are significantly different according to LSD test ($p < 0.05$). The blue lines represent the average value in uncontaminated soils from a nearby area, being 15.5 for Co, 125 for Cu, 25 for Ni, and 120 mg/kg for Zn, 110.5 for As (below the values on the axis), 221 for Pb, 0.3 for Cd, and 37.5 mg/kg for Cr (Zuluaga et al., 2017).

Elevated levels of heavy metals in soils pose significant long-term ecological risks. These metals can disrupt soil microbial communities by inhibiting enzymatic activities (Table 2), which are essential for nutrient cycling, potentially leading to a decline in soil fertility and functionality over time (Karaca et al., 2010). Furthermore, heavy metals may bioaccumulate in plant tissues (as discusses in Section 3.3.2), potentially affecting plant growth (Fig. 1) and entering the food chain, where they could pose risks to animal and human health (Angon et al., 2024). The persistence of these contaminants in the environment could lead to lasting negative effects on biodiversity and ecosystem services.

3.3.2. Heavy metals in the plant

In the obtained values for cobalt (Co) (Fig. 4A) and nickel (Ni) (Fig. 4C), none of the treatments exceed concentrations beyond which it becomes toxic. In both cases, a clear significant difference is observed between the Control (C), which has elevated values, and the rest of the treatments, which have significantly lower amounts. In the case of copper (Cu) (Fig. 4B), the Control (C) again displays significantly higher values than the treatments, with those treated with DOR having the lowest concentrations, particularly the one inoculated jointly with Azn, which is significantly the lowest. The normal amount of Cu in cereals is typically around 0.3–13 mg/kg (Pendias and Kabata-Pendias, 2000). With the exception of treatments involving DOR, which are closer to a normal concentration, the rest of the treatments significantly exceeded these values. For zinc (Zn) values (Fig. 4D), the Control (C) show significantly higher amounts than the other treatments as well, being it the only one that exceeds non-harmful levels. Although essential, an excess of Co, Cu, Ni, and/or Zn can have toxic effects on plants, causing various symptoms, including growth inhibition, mineral nutrition disorders, disruption in sugar transport, photosynthesis, transpiration, and inducing chlorosis in leaves (Shahzad et al., 2018; Natasha et al., 2022). These toxicity mechanisms are attributed to the generation of free

Table 4
Concentration of: A) soluble metals extracted with water (1:2.5, soil:water) and B) Bioavailable metals extracted with EDTA (1:10, soil:EDTA) in soil subjected to different treatments. C (Control soil); Mar (marble); DOR (biotransformed dry olive residue); Azn (*Rhizoglomus* sp.); Custo (*Rhizoglomus custos*); Intra (*Rhizophagus irregularis*). Pb values are not shown because were below the detection limits.

Treatment	A) Water-Soluble metals (mg/kg)						
	Co	Cu	Ni	Zn	As	Cd	Cr
C	0.62	2.48	0.58	27.61	0.08	0.12	0.02
Mar	< 0.01	0.63	0.02	0.11	0.06	< 0.01	< 0.01
DOR	< 0.01	0.86	0.04	0.16	0.11	< 0.01	< 0.01
DOR+Azn	< 0.01	0.91	0.03	0.14	0.09	< 0.01	< 0.01
DOR+Custo	0.01	0.59	0.04	0.14	0.09	0.01	0.01
DOR+Intra	< 0.01	0.92	0.03	0.11	0.11	< 0.01	< 0.01
Azn	< 0.01	0.57	0.01	0.11	0.07	< 0.01	< 0.01
Custo	0.01	0.56	0.02	0.07	0.06	< 0.01	< 0.01
Intra	< 0.01	0.59	0.01	0.12	0.06	< 0.01	< 0.01
Treatment	**B) Bioavailable metals by EDTA extraction (mg/kg)**						
	Co	Cu	Ni	Zn	As	Cd	Cr
C	6.89	602.26	8.43	389.23	6.01	2.21	1.77
Mar	11.69	880.67	5.20	183.74	15.70	3.05	3.67
DOR	19.68	919.23	7.39	198.99	32.01	3.04	3.84
DOR+Azn	15.71	943.87	7.32	224.87	22.67	2.53	3.35
DOR+Custo	17.38	843.75	6.26	176.03	28.96	2.69	2.86
DOR+Intra	16.21	926.75	7.18	211.89	34.28	3.37	2.95
Azn	10.44	915.76	6.25	202.44	28.36	3.09	3.29
Custo	10.38	914.32	5.61	191.25	19.29	3.37	3.34
Intra	10.70	909.99	4.57	190.70	25.39	2.71	3.11

radical species, enzyme inactivation, and the increase in reactive oxygen species (ROS) (Hao et al., 2015; Natasha et al., 2022). We will further compare the obtained results graphically with normal values that are non-toxic for plant development and function.

In the case of arsenic (As) (Fig. 4E) and cadmium (Cd) (Fig. 4G), all treatments exhibit concentrations above the normal levels typically observed in plants. For lead (Pb) (Fig. 4F), treatments with significantly lower values were those where only DOR was added and where mycorrhization with Custo was done, with or without the addition of DOR. These treatments are also the only ones with Pb concentration below 2 mg/kg, a concentration from which harmful symptoms for plants have been observed (Pendias and Kabata-Pendias, 2000). The rest of the treatments exceed this limit, with the highest Pb concentrations found in the mycorrhized treatment with Azn. Chromium (Cr) (Fig. 4H) normal values in plants vary between 0.02–0.2 mg/kg (Pendias and Kabata-Pendias, 2000), and all treatments are around those levels, except for the one inoculated with Azn and the Control, which has significantly higher values.

Arsenic, Pb, Cd, and Cr do not play any biological function in plant metabolism; they lack specific mechanisms for their entry into plants. Nevertheless, they are actively absorbed and transported by plants through phosphate or sulfate transporter proteins or other essential elements due to their structural similarity. Alternatively, they can passively enter, governed by the concentration gradient between soil and plant cells (Shanker et al., 2005; Panda et al., 2010; de Oliveira et al., 2014; Neidhardt et al., 2015, 2016; Green et al., 2017; Niazi et al., 2017; Abbas et al., 2018). Once inside plants, although they exhibit some tolerance mechanisms such as binding to the cell wall, compartmentalization into inert parts, chelation, or enhancement in certain enzymatic activities (Rizwan et al., 2016), they induce toxic effects in morphological, physiological, and biochemical processes when certain limits are exceeded. While the specific mechanisms and transporters responsible for the uptake and translocation of metals like arsenic, lead, cadmium, and chromium in wheat plants are crucial for understanding their potential role in phytoremediation, this complex topic will be explored in future studies (Page and Feller, 2015; Zhou and Zheng, 2022). The studied metals severely impact on plants, reducing growth and development due to the decrease in photosynthetic rate, chlorophyll content, stomatal conductance, transpiration rate, and relative leaf water content. They also affect germination, early crop growth, nutrient absorption and assimilation, water relations, enzymatic activity, and carbon assimilation (Sharma and Dubey, 2005; Tiwari et al., 2009; Sarwar et al., 2010; Chandrakar et al., 2016; Anjum et al., 2016; Kushwaha et al., 2018; Ullah et al., 2019). This toxicity is also associated with an increase in the quantity of reactive oxygen species (ROS) (Talukdar, 2013; Rajput et al., 2021). Therefore, it is essential to relate the effect of the concentration of these elements to the biochemical responses of the study plant.

3.4. Bioaccumulation index and translocation factor

The Bioaccumulation Factor (BAF) represents the relationship between metal concentrations in the soil and a plant fraction. It is calculated as the quotient of the metal concentration in the plant divided by its concentration in the soil expressing the plant's efficiency in absorbing and accumulating metals in its different structures. A coefficient >1 indicates the plant as an accumulator, while <1 is considered an excluder (Hobbs and Streit, 1986; Van der Ent et al., 2012). In Table SI.3 BAF values for the roots indicate that none of the treatments have values above 1, suggesting that the species is an excluder for the studied metals. Comparing the values for the study treatments, a consistent trend is observed: the treatments decrease the BAF coefficient compared to the Control. Similarly, Table SI.3 displays BAF values for the aerial part, where once again, none of the treatments have values above 1, supporting the idea that this species excludes the studied elements. An increase in BAF in Azn-inoculated treatments for some metals (Co, As, Cr, Cd, Cu) seems to be observed, but without reaching values of accumulator.

After the addition of marble and the increase in pH (Table 1), root BAF decreases in all treatments due to the decrease in their solubility (Table 2). The aerial BAF seems to reflect the same trend. *Rhizoglomus sp* (Azn) mycorrhized plants tend to have a higher aerial BAF than the other treatments for some metals (Co, Ni, As, Pb, Cr). This trend is also evident in the concentration of Ni, Pb, As, and Cr in plant (Fig. 4). Since this fungus was isolated from the study area, it likely acquired greater tolerance or a more accumulator strategy against these metals (Gosling et al., 2006).

The Translocation Factor (TF) provides insight into the plant's ability to mobilize heavy metals from roots to the aerial part, valuable for understanding its phytoremediation capacity. It is calculated as the quotient of the metal concentration in the aerial part divided by its concentration in the roots. Coefficients >1 indicate accumulation, while coefficients <1 indicate exclusion (Van der Ent et al., 2012). In Table SI.3, it is observed that the only metal with a coefficient >1 is zinc. For the rest of the metals, TF is lower than 1.

The translocation of heavy metals to the aerial biomass is a strategy adopted by certain plants to minimize potential damage to the physiology and/or biochemistry of their roots. Conversely, other plants may prevent or limit the translocation of these metals, and their strategy can vary depending on environmental conditions (Mehes-Smith et al., 2013; Zacchini et al., 2009). Chelation of metals to ligands facilitates this translocation from roots to the aerial part (Zacchini et al., 2009). Among the metals studied, zinc (Zn) is the only one that achieves an TF index >1, with an even higher index in mycorrhizal treatments. This is attributed to the fact that AMF catalyze the absorption of Zn among other nutrients (Cavagnaro, 2008). The enhanced translocation of zinc to the aboveground parts in mycorrhizal treatments underscores the potential role of AMF in facilitating nutrient uptake and translocation

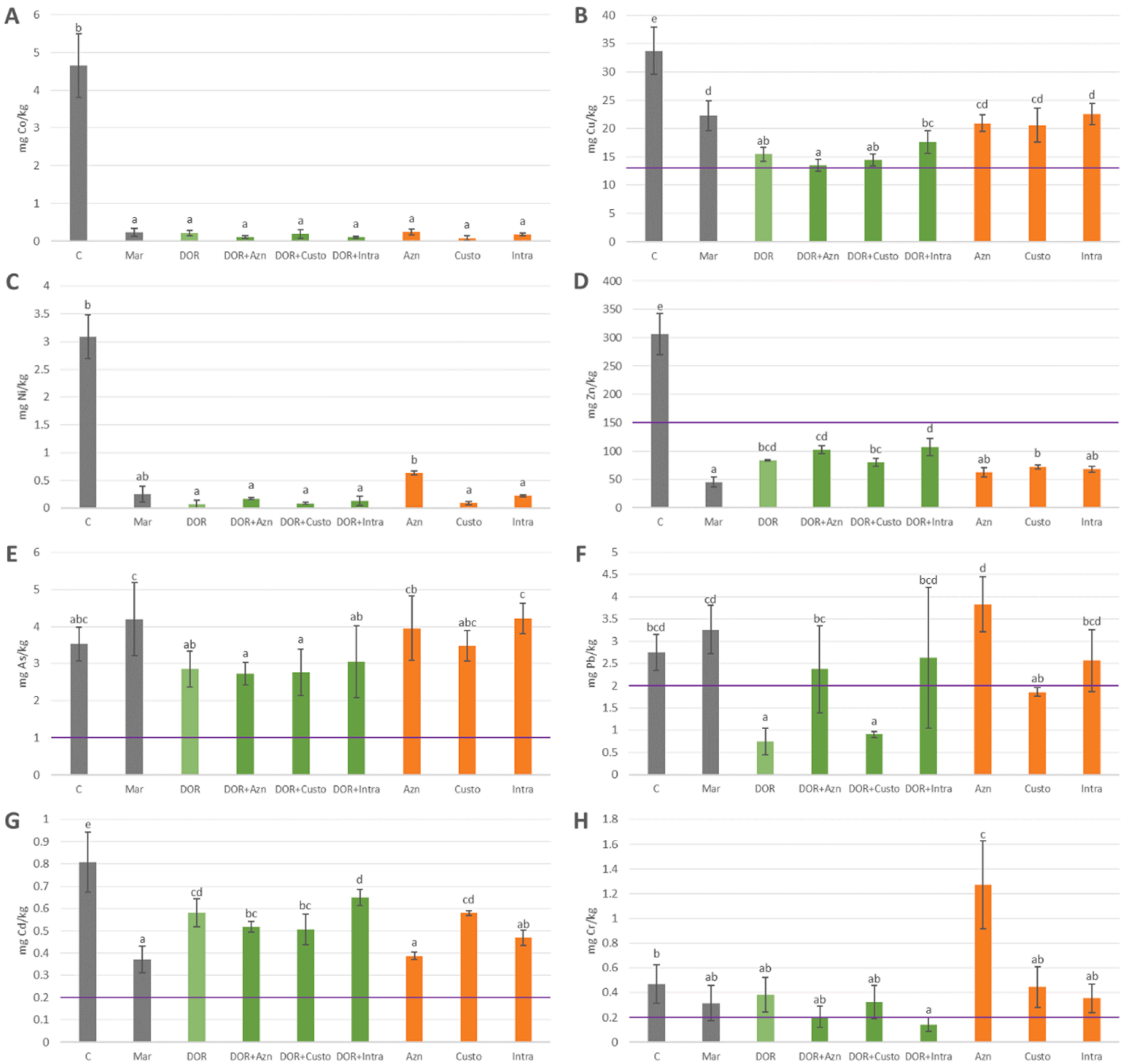

Fig. 4. Total cobalt (A), copper (B), nickel (C), zinc (D), arsenic (E), lead (F), cadmium (G), chromium (H) in the aerial part of the plant growth in soil subjected to different treatments. C (Control soil); Mar (marble); DOR (biotransformed dry olive residue); Azn (*Rhizoglomus* sp.); Custo (*Rhizoglomus custos*); Intra (*Rhizophagus irregularis*). Data represent the mean of micronutrient data. Error bar represent standard deviation (n = 3). For each micronutrient, data followed by different letter are significantly different according to LSD test ($p < 0.05$). The purple lines represent normal and/or non-harmful levels for plants, being 50 mg/kg for Co (above the axis values), 13 mg/kg for Cu, 10 mg/kg for Ni (above the axis values), 150 mg/kg for Zn 1 mg/kg for As, 2 mg/kg for Pb, 0.2 mg/kg for Cd, and 0.2 mg/kg for Cr (Wagner, 1993; Adriano, 2001; Pendias and Kabata-Pendias, 2000; Kabata-Pendias, 2011; Hassan et al., 2019).

within the plant, thereby influencing its metal accumulation patterns.

Research on wheat (*Triticum aestivum* L.) as a phytoaccumulator of heavy metals has produced mixed results. While some studies suggest that wheat can tolerate and accumulate metals such as zinc and cadmium from contaminated soils (Shumaker and Begonia, 2005; Liu et al., 2009), others indicate it may not be an effective accumulator (Brunetti et al., 2012). Our study's results support this finding; we observed the highest TFs for Zn and Cd, while the values for the other metals remained well below 1 (Table SI.3). Although certain wheat cultivars exhibit excluder properties for specific metals (Gramss and Voigt, 2016), they can still accumulate concentrations exceeding safety thresholds, as observed in our study (Fig. 4).

3.5. Principal components analysis

Finally, after collecting all the study data, a Principal Components Analysis (PCA) was conducted (Fig. 5) to comprehensively described the data and determine the effectiveness of the different treatments. Each axis or principal component is ordered based on the amount of original variance it describes. In our case, the PC1 accounts for 44.4 % of this variance, and PC2 accounts for 27.6 %, resulting in a cumulative total of 72 % of the original variability. Given the extensive dataset, we have represented only the variables with the most significant influence. To further elucidate the relationships between parameters, we have included the PCA weights in the support information (Table SI.4).

In the upper right quadrant, treatments where DOR was added are clustered, clearly associated with vegetative response, enzymatic activity, and physicochemical properties of the soil. In the lower left

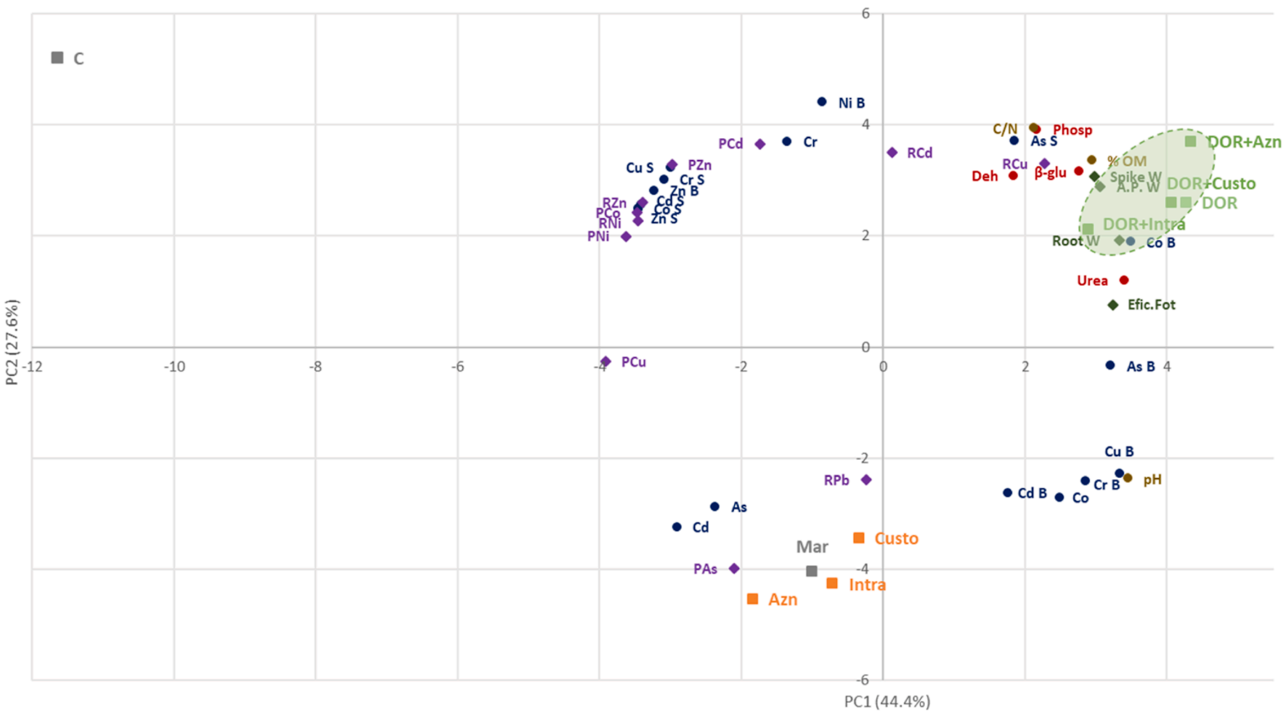

Fig. 5. Principal Components Analysis (PCA). The treatments (C, Mar, DOR, DOR+Azn, DOR+Custo, DOR+Intra, Azn, Custo, Intra) are shown as squares; soil enzymatic activity (β-glucosidase, dehydrogenase, phosphatase, urease) as red circles; soil physicochemical properties (pH,% organic matter, C/N) as brown circles; vegetative response variables (photosystem II efficiency, root weight, aerial weight, spike weight) as green diamonds; soil metals [bioavailable (Cd B, Co B, As B, Cr B, Ni B, Cu B, Zn B), soluble (As S, Cr S, Cu S, Co S, Cd S, Zn S), total (Cr, Co, As, Cd)] as blue circles; and plant metals [aerial (PCo, Pas, PNi, PCu, PCd, PZn), root (RPb, RNi, RZn, RCd, RCu)] as purple diamonds.

quadrant, mycorrhizal treatments and the addition of marble are grouped, associated with non-essential metals As, Cd, and Pb. In the upper left quadrant, very distant from the rest of the treatments, the Control (C) is found, associated with the quantity of metals in soil and plant.

The Control (C) is notably distant from the rest of the treatments, linked to heavy metal concentrations in soil and plants. Across all treatments, heavy metal concentrations in soils exceed the average reference value (Section 3.3.1), mostly at levels harmful to organisms. Despite heavy metal concentrations still exceeding reference values after adding marble to the soil and raising the pH (Table 1), their solubility (3.3.2) is reduced, meaning their short-term dispersion is minimized, thereby decreasing the plants' ability to absorb them, as evidence in Section 3.3.3, where the concentration of heavy metals (Co, Cu, Ni, Zn, Cd) in the aerial part significantly decreases in treatments where marble was added compared to untreated soil (C). The PCA results show a correlation between soil pH and the bioavailability of certain metals (Cu, Cr, Co, Cd, As), suggesting that the pH of the soil plays a key role in regulating the availability of these metals for plant uptake. This elucidates why the treatment where only marble was added (Mar) is distant from the Control.

Grouped with Mar, mycorrhizal treatments with Azn, Custo, and Intra are also situated. This grouping suggests that mycorrhization does not have a significantly different effect when added to marble. Even further away, we find treatments where DOR was also added, associated with vegetative response, physicochemical properties, and enzymatic activity of the soil. Although the pH increase itself significantly elevated urease activity, the addition of DOR further significantly increased this and also the activity of β-glucosidase and phosphatase (Table 2). Results reveal an association between soil organic matter, enzymatic activities (dehydrogenase, β-glucosidase, phosphatase) and plant biomass. This suggests that soils with higher organic matter content and more active enzymatic processes tend to support better plant growth. Specifically, the breakdown of organic matter and the availability of nutrients, as indicated by enzymatic activities, appear to be key factors driving plant development, as reflected in the increased plant weight. This enhancement in soil biological quality reflects the improvement in its physicochemical properties, such as the percentage of organic carbon (%OC) and the C/N ratio (Table 1) in DOR treatments. Although not evaluated in the study, the addition of organic matter to the soil is known to improve water retention, aggregate stability, generate greater nutrient availability (N, P, K), and increase cation exchange capacity (Walsh and McDonnell, 2012; Murphy, 2015), generally creating more optimal conditions for microorganisms and their activity. This improvement with the addition of DOR is also reflected in the vegetative response of our plants, which significantly exhibit higher biomass in root, stem, and spike in these treatments (Fig. 1), higher stomatal conductance, and photosystem II efficiency (Fig. 2).

Within the DOR treatments, it is evident that they are distanced from each other: DOR+Azn being the furthest, followed by DOR and DOR+Custo, and DOR+Intra. This difference is also observed in the mycorrhization percentage (Table SI.1), where Azn has the highest percentage, followed by Custo, and finally, Intra.

4. Conclusions

The application of biotransformed DOR has demonstrated a significant positive impact on the physicochemical properties and enzymatic activity (biological properties) of soils. These improvements resulted in notable enhancements in plant biomass, stomatal conductance, and photosynthesis, indicating an overall boost in vegetative response. When combined with mycorrhizal inoculation, marginal improvements were observed, with the most favourable results achieved using *Rhizoglomus* sp. (Azn), a native species from the study area, which exhibited the highest percentage of mycorrhization and root biomass. The next most effective species were *Rhizoglomus custos* (Custo) and *Rhizophagus irregularis* (Intra). Although metallic pollutants were not removed from the soil, their translocation to the aerial parts of the plants was significantly limited.

These findings highlight a sustainable and effective strategy for mitigating diffuse pollution sources in the region, promoting vegetative cover establishment through the combined use of marble sludge, DOR, and inoculation with *Rhizoglomus* sp. (Azn). Furthermore, the valorization of local resources offers significant socio-economic and environmental benefits, aligning with circular economy principles. Future studies should focus on optimizing the dosage and timing of DOR and mycorrhizal inoculum applications to maximize their benefits under a range of environmental conditions. Investigating the long-term effects on soil health and pollutant dynamics will also be crucial. Additionally, exploring the potential of integrating other native microbial communities or amendments could enhance the remediation potential of this approach. Lastly, field-scale trials are essential to validate the scalability and practical applicability of the proposed method for broader implementation.

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CRediT authorship contribution statement

María Higueras-Valdivia: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Gloria Andrea Silva-Castro:** Writing – review & editing, Visualization, Validation, Investigation, Data curation. **Mario Paniagua-López:** Visualization, Validation, Investigation, Data curation. **Ana Romero-Freire:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Inmaculada García-Romera:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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Data availability

Data will be made available on request.

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