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Plant testing with hemp and miscanthus to assess phytomanagement options including biostimulants and mycorrhizae on a metal-contaminated soil to provide biomass for sustainable biofuel production

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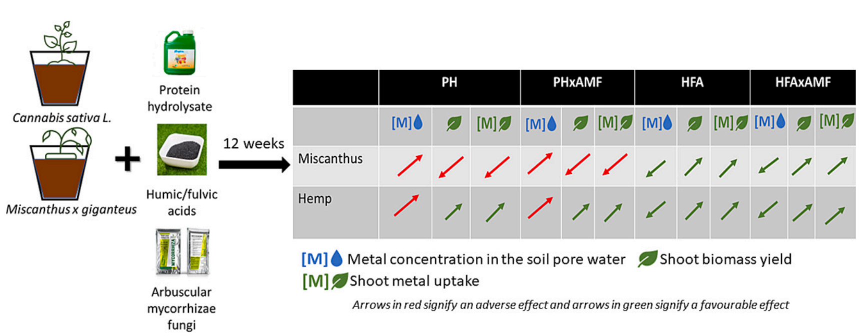
HIGHLIGHTS

- Miscanthus and hemp grow well on a metal-contaminated soil.
- Protein hydrolysate treatments increased Cd and Zn concentrations in the soil pore water.
- Soil acidification and metal exposure induced by protein hydrolysate reduced miscanthus biomass.
- Protein hydrolysate combined with mycorrhizae improved metal uptake by hemp.
- Mycorrhizae-associated humic/fulvic acids improved hemp biomass.

GRAPHICAL ABSTRACT



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ABSTRACT

The need of biofuels from biomass, including sustainable aviation fuel, without using agricultural land dedicated to food crops, is in constant demand. Strategies to intensify biomass production using mycorrhizal fungi, biostimulants and their combinations could be solutions for improving the cultivation of lignocellulosic plants but still lack well-established validation on metal-contaminated soils. This study aimed to assess the yield of Miscanthus x giganteus J.M. Greef & Deuter and *Cannabis sativa* L. grown on a metal-contaminated agricultural soil (11 mg Cd, 536 mg Pb and 955 mg Zn kg^{-1}) amended with biostimulants and/or arbuscular mycorrhizal fungi,

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Cadmium
Lead
Zinc
Phytoremediation

and the shoot Cd, Pb and Zn uptake. A pot trial was carried out with soil collected from a field near a former Pb/Zn smelter in France and six treatments: control (C), protein hydrolysate (a mixture of peptides and amino acids, PH), humic/fulvic acids (HFA), arbuscular mycorrhizae fungi (AMF), PH combined with AMF (PHxAMF), and HFA combined with AMF (HFAxAMF). Metal concentrations in the soil pore water (SPW), pH and electrical conductivity were measured over time. Miscanthus and hemp shoots were harvested on day 90. Both PH and PHxAMF treatments increased SPW Cd, Pb, and Zn concentrations (e.g. by 26, 1.9, and 22.9 times for miscanthus and 9.7, 4.7, and 19.3 times for hemp in the PH and PHxAMF treatments as compared to the control one, respectively). This led to phytotoxicity and reduced shoot yield for miscanthus. Conversely, HFA and HFAxAMF treatments decreased SPW Cd and Zn concentrations, increasing shoot yields for hemp and miscanthus. Shoot Cd, Pb, and Zn uptakes peaked for PH and PHxAMF hemp plants (in μg plant-1, Cd: 310–334, Pb: 34–38, and Zn: 232–309 for PHxAMF and PH, respectively), while lowest values occurred for PH miscanthus plants mainly due to low shoot yield. Overall, this study suggested that humic/fulvic acids can be an effective biostimulant for increasing shoot biomass production in a metal-contaminated soil. These results warrant further investigations of the HFAxAMF in field trials.

1. Introduction

Soil contamination by metal(loid)s poses risks to human and ecosystem health and impacts worldwide arable lands (Haller and Jonsson, 2020; Moreira et al., 2021). In the European Union (EU), 1.86 million ha are figured as metal(loid)-contaminated (Panagos et al., 2013; Payá Pérez and Rodríguez Eugenio, 2018; Van Liedekerke et al., 2013, 2014). As the human population grows and consumes more resources (food, fuel, etc.), pressure on arable land increases, necessitating the remediation and use of contaminated land (Schröder et al., 2022). The cultivation of high-yielding lignocellulosic crops on contaminated soils is gradually becoming relevant due to its associated low indirect land use change (ILUC) risk. Interest in these crops has increased as they have proven to be competitive to produce bioenergy (Finnan and Styles, 2013; Pogrzeba et al., 2019) and biofuel such as sustainable aviation fuel, freeing up arable land for food production (Mellor et al., 2021). Phytomanagement, i.e. the use of plant species, their associated microorganisms, with either soil amendments, macro- and mesofauna, bioaugmentation or other agricultural practices, for both producing usable biomass and ecologically, progressively, remediating the multifunctionality of polluted soils, is a favored management technique for contaminated land. This is due to its environmental friendliness, cost-effectiveness, and social acceptance. It is considered as a relevant option to valorize contaminated sites (e.g. biomass production). It can also serve as temporary non-destructive use (Burges et al., 2018; Evangelou et al., 2015; Krzyżak et al., 2022; Moreira et al., 2021; Rheay et al., 2021). When the long-term use on such marginal land with contaminated soil is primarily non-food crops using metal(loid) excluders, there is generally no time constraint to implementation (if edaphic conditions are suitable).

High-yielding lignocellulosic plant species, commonly used for phytomanagement, include *Miscanthus* x *giganteus* J.M. Greef & Deuter, its new hybrids, switchgrass, and *Cannabis sativa* L. (Al Souki et al., 2017; Amabogha et al., 2023; Krzyżak et al., 2022; Nsanganwimana et al., 2015; Rheay et al., 2021). These plant species are tolerant to high metal(loid) exposures, able to improve soil properties, and have biomasses that can be locally processed in the north-west of Europe (Interreg New-C-land AgriWasteValue, 2022). In their reviews, Amabogha et al. (2023), Krzyżak et al. (2022), Moreira et al. (2021), Nsanganwimana et al. (2014) and Pidlisnyuk et al. (2020) highlighted the dual use of miscanthus as an attractive option for sustainable development, as it not only helps to remediate polluted soils, but also provides a source of clean energy or biochar production. Hemp also is a suitable crop for phytoremediating metal(loid)-contaminated soils, and a competitive option for bioenergy and fiber production (Cleophas et al., 2022; Rheay et al., 2021). Additionally, both plant species require low agricultural inputs, making them a cost-effective and sustainable option for phytomanaging metal(loid)-contaminated land (Pandey et al., 2016; Rheay et al., 2021).

To improve plant growth, increase biomass yield, and concomitantly reduce contamination linkages by favoring either metal(loid) uptake from the topsoil or their immobilization, arbuscular mycorrhizal fungi (AMF) and biostimulants such as protein hydrolysates (PH) and humic/fulvic acid (HFA) mixtures can be used as alternative treatments (Calvo et al., 2014; Malécange et al., 2023). Biostimulants would promote plant growth on low-fertility soils and/or improve plant resistance to biotic and abiotic stresses by the regulation of physiological processes, nutrient-use efficiency and plant antioxidant mechanisms (Canellas et al., 2015; Colla et al., 2015; Francesca et al., 2021).

Humic and fulvic acids are complex biomolecules that can offer several benefits when used in phytomanagement. They may improve nutrient uptake by increasing the solubility and availability of essential nutrients, while also promoting soil fertility through the growth of beneficial microorganisms, thus favoring nutrient cycling in the soil (Song et al., 2023). In addition, humic and fulvic acids can enhance plant stress tolerance and crop yield, providing a cost-effective alternative to mineral fertilizers (Bartucca et al., 2022; Wiszniewska et al., 2016).

Protein hydrolysates are a mixture of peptides and amino acids of plant or animal origin (Calvo et al., 2014). They can promote plant growth by increasing nutrient availability in the soil solution through enhanced microbial activity and nutrient complexation by peptides and amino acids (Colla et al., 2017; Rouphael et al., 2021). Protein hydrolysates also improve plant tolerance to abiotic stress by enhancing the plant's antioxidant activity thanks to the scavenging of free radicals by certain nitrogen compounds (proline and glycine betaine) present in protein hydrolysates (Del Buono, 2021; du Jardin, 2015; Malécange et al., 2023).

Arbuscular mycorrhizal fungi (AMF) are beneficial microorganisms associated with plant roots. They have been used extensively in association with plants for phytomanagement (Ciadamidaro et al., 2017; Nsanganwimana et al., 2015; Phanthavongsa et al., 2017; Szada-Borzyszkowska et al., 2022). Arbuscular mycorrhizal fungi improve the soil rhizosphere, macro and micronutrient and water uptake due to the extension of fungi mycelium from the roots (Lounés-Hadj Sahraoui et al., 2022). They can also increase metal(loid) availability in the soil involving chelation and/or solubilization by organic molecules excreted by the fungi. This may increase metal(loid) transfer from soil to roots and may enhance their translocation to shoots, especially for essential ones while the non-essential ones are retained in the hyphae and roots (Coninx et al., 2017).

Strategies to intensify biomass production using mycorrhizae, biostimulants, and their combinations have been studied for several plant species (Ciadamidaro et al., 2017; Colla et al., 2015; Vargas et al., 2016). However, their effects on selected lignocellulosic crops used to phytomanage metal-contaminated soils still lack well-established validation with various edaphic conditions. This study aimed at assessing in a pot experiment the effects of biostimulants (PH and HFA) and arbuscular mycorrhizal fungi, alone and in combination, on two high-yielding lignocellulosic crops (*Miscanthus* x *giganteus* J.M. Greef, Deuter ex

Hodk., Renvoize 2001 and *Cannabis sativa* L.) prior to subsequently establish a field trial for the phytomanagement of an agricultural metal-contaminated area. Accordingly, the biostimulant and arbuscular mycorrhizal fungi influence on (i) the concentration of bioavailable metals in the soil, (ii) the production of shoot biomass, and (iii) shoot metal (Cd, Pb and Zn) uptake were studied.

2. Materials and methods

2.1. Soil

The soil was collected (0–25 cm) from a 12-year-old miscanthus plantation (Nsanganwimana et al., 2015) located 700 m from the former Metaleurop Nord Pb/Zn smelter, in Northern France (50°26′15.5″N 3°01′04.7″E) using a stainless spade, sieved through a 1 cm mesh, homogenized, and kept fresh. The smelter activities had heavily contaminated the surrounding agricultural soils with Cd, Pb and Zn, respectively up to 26, 14 and 13 times higher than the regional geochemical background levels (Sterckeman et al., 2002). Our contaminated soil is a clay sandy loam soil dominated by silt and sand (Nsanganwimana et al., 2015) whereas Aligon and Douay (2011) reported a clay sandy loam texture dominated by silt for local uncontaminated soils (Table 1). The other physico-chemical parameters of the contaminated and local uncontaminated soils are quite similar (Table 1).

2.2. Plants and amendments

Micro-propagated plantlets of *M. giganteus* were purchased from Rhizosfer© (Brienne-sur-Aisne, France) and transplanted (approximately 20 cm long) in individual pots. For hemp, seeds (industrial hemp, Futura 75) were obtained from CRES (Dr. Efi Alexopoulou), Greece. In each pot, five seeds were sown. One plantlet was selected and the other ones thinned 15 days after the sowing. Before seeding, soil humidity was determined, and equivalent of 12 kg of dry soil were potted in plastic pots (14 L, 28.5 × 28.5 × 28.4 cm). The trial, arranged in a fully randomized block, lasted 90 days and was carried out in a greenhouse under controlled conditions (16/8 h light/darkness; 65 ± 5 % relative humidity; 25 ± 2 °C). Pots were weighed every two days and maintained at 70 % of water holding capacity (WHC) using tap water and cups without loss from drainage.

Table 1
Physico-chemical properties of the contaminated soil as compared to an unpolluted regional agricultural soil.

Parameters	Units	Contaminated soil	Uncontaminated agricultural soil
Clay	g kg^{-1}	180.6 ± 8.8	180[1]
Fine silt		191.6 ± 10.2	245[1]
Coarse silt		364.8 ± 7.1	447[1]
Fine sand		224.3 ± 11.3	93[1]
Coarse sand		39.0 ± 2.9	35[1]
pH		7.91 ± 0.21	6.8[1]
EC	μS cm^{-1}	122 ± 17	–
CEC Metson	$cmol^{+}$ kg^{-1}	13.6 ± 0.8	–
Organic Carbon	g kg^{-1}	21.02 ± 3.97	18.6[1]
Organic matter	g kg^{-1}	36.38 ± 6.86	32.17[1]
Total Carbonates	g kg^{-1}	4.18 ± 4.25	4[1]
Total Nitrogen	g kg^{-1}	1.32 ± 0.18	2.02[1]
C/N		15.88 ± 0.88	9.2[1]
Exchangeable K_2O	g kg^{-1}	0.36 ± 0.08	0.19[1]
Exchangeable MgO		0.14 ± 0.02	0.21[1]
Exchangeable CaO		5.63 ± 0.95	4.04[1]
Exchangeable Na_2O		0.02 ± 0.01	0.03[1]
Extractable P_2O_5 (Olsen)		0.11 ± 0.03	0.22[1]
Total Cu	mg kg^{-1}	25 ± 3	16.7[2]
$Ca(NO_3)_2$-extractable Cu		1.65 ± 0.77	–
Total Cd		11 ± 2	0.42[2]
$Ca(NO_3)_2$-extractable Cd		0.71 ± 0.16	–
Total Pb		536 ± 70	38.4[2]
$Ca(NO_3)_2$-extractable Pb		5.13 ± 1.84	–
Total Zn		955 ± 151	73.7[2]
$Ca(NO_3)_2$-extractable Zn		4.02 ± 3.22	–

EC: electrical conductivity; CEC: cation exchange capacity. Results from this study are expressed as mean ± standard deviation ($n = 4$). $[Ca(NO_3)_2] = 0.01$ M.
[1] Aligon and Douay, 2011.
[2] Sterckeman et al., 2002.

Six treatments were used based on previous studies (Canellas et al., 2015; Malécange et al., 2023; Phanthavongsa et al., 2017). Biostimulants and AMF inoculum were:

- A commercial arbuscular mycorrhizal fungi mix, Symbivit® provided by Symbiom (Czech Republic). The mix was in granular form containing five AMF fungi (*Rhizophagus irregularis, Funneliformis geosporum BEG199, Funneliformis mosseae, Claroideoglomus lamellosum* and *Septoglomus deserticola)*. The minimum number of spores was 200 per g.
- A commercial animal-derived protein hydrolysate (PH), Siapton® produced by Isagro S.p.A (Italy). The PH treatment, which was in liquid form, contained 79 g free amino acids and 543.5 g total amino acids per kg.
- Lonite 80SP® (a water-soluble powder-based humic/fulvic acid product, HFA) produced by Alba Milagro (Italy). The HFA product comprised both humic acid (75 % *w*/w) and fulvic acid (5 %).

Each biostimulant, i.e. HFA and PH, was used alone and in combination with AMF. The unamended soil was included to serve as a control. All the treatments used in the study are summarized below;

(1) Unamended soil, referred to as control (C)
(2) Mycorrhizae fungi (AMF)
(3) Protein hydrolysate (PH)
(4) Protein hydrolysate + mycorrhizae fungi (PHxAMF)
(5) Humic/fulvic acids (HFA)
(6) Humic/fulvic acids + mycorrhizae fungi (HFAxAMF)

Before miscanthus plantation, 15 g Symbivit® pot^{-1} were deposited in a U-shape of the soil, placed in contact with the roots. Extra soil was added to cover both the roots and the mycorrhizae. For hemp, the same Symbivit® amount was deposited in a 5–6 cm circle where the hemp seeds were further sown. The mycorrhizae were mixed carefully with a spatula into the topsoil. One week after miscanthus transplantation and when the hemp reached the 3–6 leaf stage (in virtually the same period), humic/fulvic acids were applied on top of potted soils by dissolving 0.5 g pot^{-1} of the product in the irrigation water. This dose was increased up to 0.7 g pot^{-1} four weeks after the first application and repeated weekly to reach a total of 11 applications (7.7 g of humic/fulvic acids per pot). Protein hydrolysate was first applied on top of the potted soils 3 days after the transplantation of miscanthus and when the hemp reached a maximum shoot length of 10 cm. The PH treatment application was done by diluting 13.5 mL of the product in 1 L of irrigation water per pot. This PH application was then changed to foliar application when the plants had adequate leaf surface area (6 weeks after miscanthus transplantation and sowing of hemp seeds). The dose was decreased to 3 mL L^{-1} of spray liquid per pot and repeated every 10 days to attain a total of eight applications (66 mL of protein hydrolysate per pot).

2.3. Soil pore water and soil physico-chemical analysis

The soil pore water (SPW) was collected after 0, 3, 7, 9 and 12 weeks (i.e. T0, T3, T7, T9 and T12), using Rhizon samplers (Eijkelkamp Agrisearch Equipment, The Netherlands) placed in each pot, and stored at

4 °C until analyses. The pH and electrical conductivity (EC) were determined using electrodes. The soil pH (H_2O) and EC were measured after stirring a mixture of soil and deionized water (1:5, *v*/v) for 1 h (ISO 10390). Some soil physicochemical parameters were performed in an external laboratory (SADEF, Aspach le Bas, France). They include total carbonates (NF ISO 10 693), organic matter (NF ISO 14235), total organic carbon (NF ISO 14235), total nitrogen (NF ISO 13878), CEC Metson (NFX 31-130), available P (P_2O_5) (NF ISO 11263-Olsen) and exchangeable Ca (CaO), Mg (MgO), K (K_2O), Na (Na_2O) were determined according to NFX 31-108. The pseudo-total soil Cd, Pb and Zn concentrations were determined after acid digestion in aqua regia (HCl: HNO_3, 3:1 v/v,6 mL) of 300 mg of soil (ground and sieved at 250 μm) using a digestion block at 95 °C for 75 min. After cooling, the volume was adjusted to 25 mL with double-distilled water and the solution was filtered (ash-free 0.45 mm cellulose acetate filters). The quality control of the extraction and analysis was provided by including two internal reference samples and a certified soil reference (CRM 141, IRMM, Belgium) in the analyzed series. The total Cd, Pb and Zn concentrations in the soil digests and SPW samples were measured by atomic absorption spectrometry (AA-6800, Shimadzu, Japan).

2.4. Plant sampling and analysis

For all plants, shoot biomass (stem and leaves) was harvested at T12, being cut 1 cm above the soil surface. The biomasses were then weighed, washed, and rinsed using deionized water. The biomass samples were then oven dried at 40 °C for three days. All dried shoot samples were grounded (<1 mm particle size, Retsch MM200). For each plant sample, a 300 mg DW aliquot was weighed into a digestion tube. Concentrated nitric acid (HNO_3, 70 %, 5 mL) was added to the sample and manually shaken. Afterwards, the aliquot was heated at 95 °C for 75 min using a digestion plate (HotBlock −36- Position, 50 mL, Environmental Express, USA). After cooling, hydrogen peroxide (H_2O_2, 30 %, 5 mL) was added and the mixture was heated again at 95 °C for 3 h. Each digested aliquot was adjusted to 25 mL using osmotic water and filtered using a 0.45 μm, cellulose acetate membrane. Certified reference material (Polish Virginia Tobacco Leaves, INCT-PVTL-6, Poland) was used to check the accuracy and precision of analytical metal (Cd, Pb, and Zn) determinations. Shoot metal uptake was calculated as: metal uptake (μg $plant^{-1}$) = shoot DW yield (g $plant^{-1}$) x shoot metal concentration (μg g^{-1} DW).

2.5. Statistical analysis

The influence of soil treatments on the SPW parameters, shoot DW yields, and shoot ionome of miscanthus and hemp were tested using a one-way analysis of variance (ANOVAs). Normality and homoscedasticity of residuals were met for all tests. Multiple comparisons of mean values were performed using post-hoc Tukey HSD tests when significant differences occurred between treatments. Analysis of co-variance (ANCOVA) was performed to investigate changes in metal concentrations in the SPW and the effects of treatments and time on the SPW metal concentrations. Principal component analysis (PCA) was conducted for SPW metal concentrations, shoot ionome, and soil pH. All statistical analyses were performed using R software (version 4.1.2, Foundation for Statistical computing, Vienna, Austria).

3. Results

3.1. Soil pore water

3.1.1. pH and EC

The SPW pH of the unamended soil for miscanthus was neutral and remained stable over the plant growth period (Table 2). A significant decrease in SPW pH was observed for the PH and PHxAMF soils (5.8 and 6.6, respectively) relative to the unamended soil (7.1). In contrast, the SPW pH of the AMF, HFA, and HFAxAMF soils (7.2 and 7.3, respectively) remained similar to the unamended soil. For hemp, SPW pH decreased for the unamended soils as well as the PH and PHxAMF soils. The SPW EC decreased for all treatments and plant species. At T12, the SPW EC for the PH and PHxAMF soils (7.8 and 7.2, respectively for miscanthus, and 6.9 and 6.1 for hemp) were higher as compared to the C soils (3.0 for miscanthus and 3.6 for hemp).

Table 2
Comparison of physico-chemical parameters of the soil pore water, 0 and 12 weeks after soil treatment.

	Soil treatments	Miscanthus		Hemp	
		pH	EC (mS cm^{-1})	pH	EC (mS cm^{-1})
SPW: T0	C	7.3 ± 0.1b	38.0 ± 2.2a	7.3 ± 0.3b	36.7 ± 10.0a
	AMF	7.5 ± 0.1b	42.5 ± 1.7a	7.5 ± 0.04ab	43.9 ± 8.9a
	PH	7.8 ± 0.2a	41.2 ± 7.6a	7.6 ± 0.1ab	40.6 ± 1.6a
	PHxAMF	7.7 ± 0.3a	37.9 ± 9.7a	7.7 ± 0.2a	41.7 ± 1.0a
	HFA	7.7 ± 0.1a	38.8 ± 7.8a	7.4 ± 0.1ab	37.2 ± 10.0a
	HFAxAMF	7.6 ± 0.1ab	40.3 ± 7.3a	7.4 ± 0.1ab	38.1 ± 4.2a
SPW: T12	C	7.1 ± 0.1a	3.0 ± 0.3b	6.6 ± 0.1abc	3.6 ± 0.2cd
	AMF	7.1 ± 0.1a	3.3 ± 0.8b	7.6 ± 0.2a	2.9 ± 0.1d
	PH	5.8 ± 0.9b	7.8 ± 0.7a	5.7 ± 1.0c	6.9 ± 0.1a
	PHxAMF	6.6 ± 0.5ab	7.2 ± 0.8a	6.1 ± 0.8bc	6.1 ± 0.6b
	HFA	7.2 ± 0.1a	2.9 ± 0.6b	7.3 ± 0.4ab	3.8 ± 0.2c
	HFAxAMF	7.3 ± 0.1a	2.9 ± 0.1b	7.5 ± 0.4ab	3.1 ± 0.1c

SPW: soil pore water, T0: week 0, T12: week 12; mean value ± SD for each treatment. Values with different letters differ significantly (one way ANOVA, *p*-value <0.05).

3.1.2. Total Cd, Pb and Zn concentrations in the soil pore water

The SPW Cd and Zn concentrations for the PH and PHxAMF soils increased throughout the trial. The SPW Cd concentrations for the PH and PHxAMF soils were 26 and 15 times higher, respectively, for miscanthus (Fig. 1) and 9.7 and 9.3 times higher, respectively, for hemp (Supplemental material 1 and 2) as compared to the unamended soils. A similar trend was observed for SPW Zn concentration for the PH and PHxAMF soils (22.9 times higher and 8.9 times higher for miscanthus and 19.3 and 8.0 times higher for hemp than the C soils, respectively). Conversely, the SPW Cd and Zn concentrations for the HFA and HFAxAMF soils decreased as compared to the C soils (HFA:1.3, 1.1, and HFAxAMF 2.4 and 2.1 times lower for miscanthus, respectively; HFA: 3.2, 3.7, and HFAxAMF: 8.1 and 8.3 times lower for hemp, respectively). No significant difference was observed between the soil treatments for the SPW Pb concentrations (Supplemental material 2). The SPW Cd and Zn concentrations depended on the treatment used, the trial duration and their interaction (Table 3). Indeed, both factors had a synergistic effect on the SPW Cd and Zn concentrations.

3.2. Shoot DW yields

The shoot DW yield (in g DW $plant^{-1}$) of miscanthus was reduced for the PH soils (1.10 ± 0.53) as compared to the unamended soil (3.00 ± 0.45) (Fig. 2A). No significant difference occurred between the shoot DW yield of miscanthus for the AMF, PHxAMF, HFA, and HFAxAMF soils and the C soils (Fig. 2A). Shoot DW yield of hemp was higher (98.23 ±

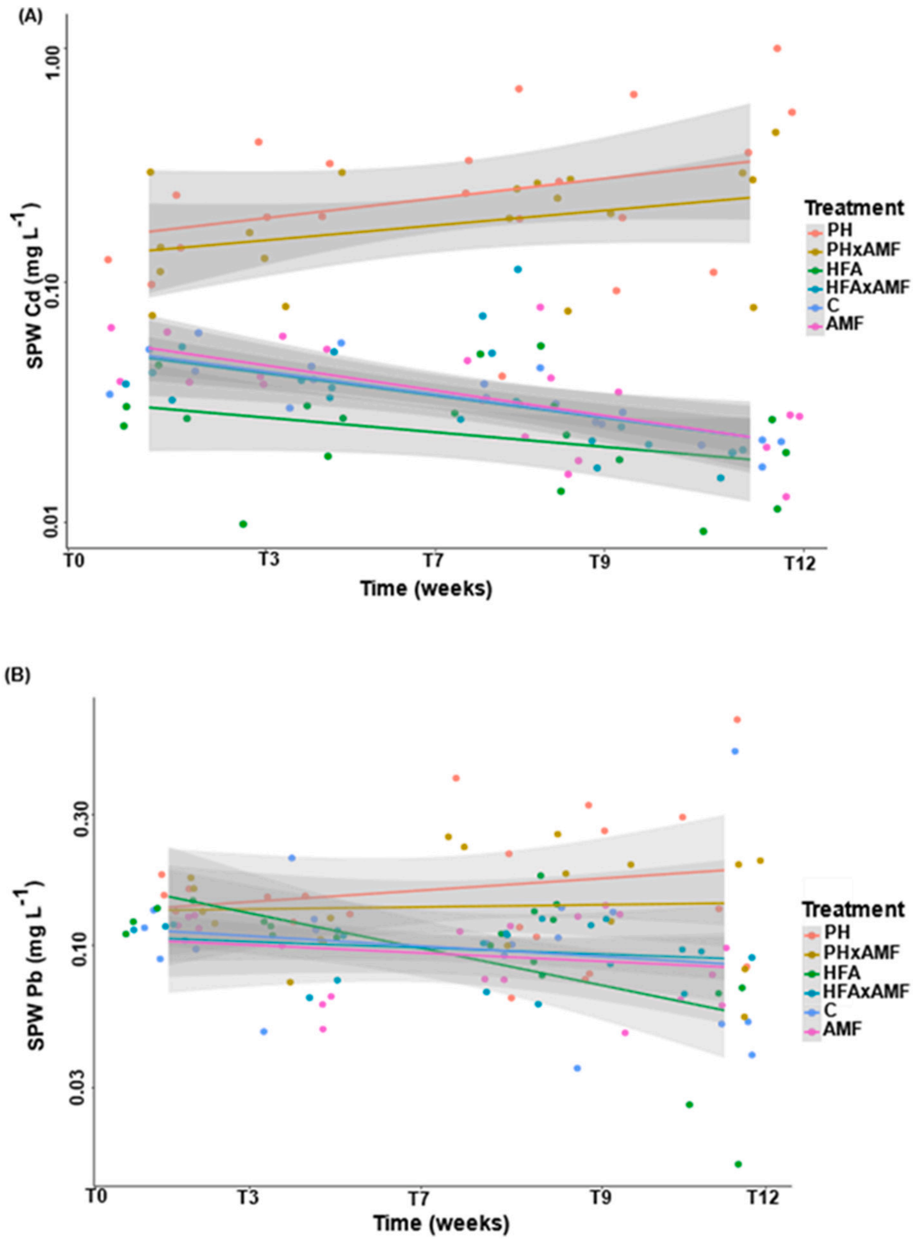

Fig. 1. ANCOVA plot for (A) Cd, (B) Pb, and (C) Zn concentrations in the SPW for the PH (pink), PHxAMF (gold), HFA (green), HFAxAMF (light blue), C (blue), and AMF (purple) treaments for miscanthus (for hemp see the supplemental material). Mean values per treatment (n = 4). The trendline power and shaded areas were obtained by the ggplot library of the R software. They represent model predictions and corresponding standards = error (95 %).

5.65) for the HFAxAMF soils as compared to C soils (86.69 ± 3.79) and far exceeded the shoot biomass of miscanthus (Fig. 2B). The shoot DW yield of hemp for the AMF, PH, PHxAMF, and HFA soils did not significantly differ from that for the C soils (Fig. 2B).

3.3. Shoot ionome

Shoot Cd and Zn concentrations of miscanthus were significantly lower for the PH and PHxAMF plants as compared to the C plants (Table 4). Inversely, shoot Cd concentration of hemp significantly increased for the PH and PHxAMF plants (4.0 ± 0.3 and 3.7 ± 1.2 μg g^{-1}, respectively) relative to the HFA, HFAxAMF and AMF ones. No significant differences between treatments were evidenced for shoot Pb concentrations of miscanthus and hemp and shoot Zn concentration of hemp.

3.4. Metal (Cd, Pb and Zn) uptake in shoots

The uptake of Cd and Zn by miscanthus shoots was 9.2 and 5.8 lower respectively for the PH plants, and 4.7 and 4 times lower for the PHxAMF plants as compared to the C plants (Fig. 3A). Shoot Pb uptake of miscanthus was higher for the HFAxAMF plants relative to the PH and PHxAMF ones due to higher shoot DW yield (Fig. 2A). The Cd uptake by hemp shoots was 1.8 and 1.7 times higher for the PH and PHxAMF plants as compared to the C plants (Fig. 3B). The shoot Zn uptake was 2.4 times increased for the PH plants relative to the C plants. For shoot Pb uptake of hemp, no significant difference was found between the treatments and the control but shoot Pb uptake was higher for the PH and PHxAMF plants relative to the AMF and HFAxAMF (Fig. 3B).

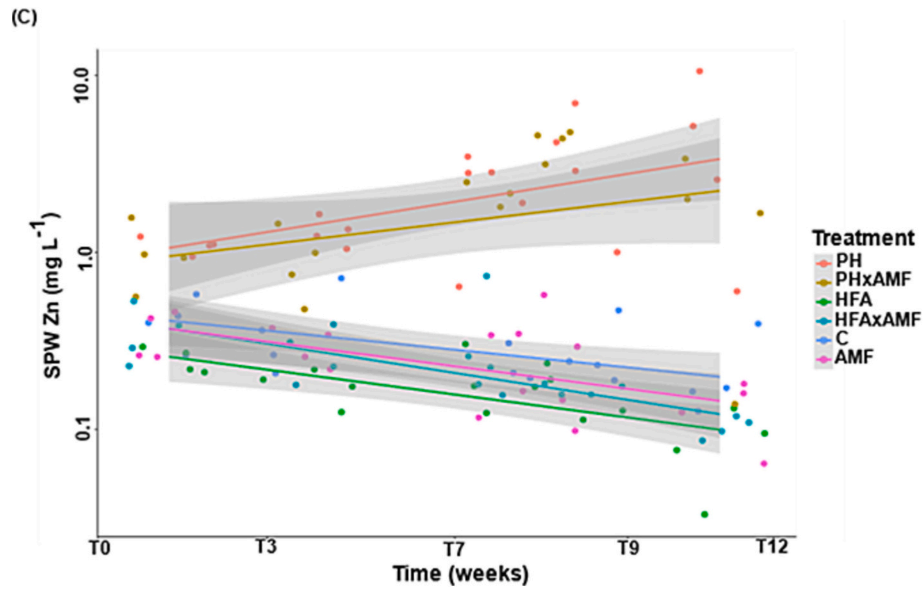

Fig. 1. (*continued*).

Table 3
Effects of treatments, time and their interactions on the Cd, Pb and Zn concentrations in the soil pore water for Miscanthus, analyzed by ANCOVA.

Parameters	Df	Sum of squares	F-value	*p*-value
SPW Cd				
Time	1	0.2307	5.0275	0.027*
Treatment	5	18.5462	80.8437	10^{-16}***
Time x treatment	5	1.1295	4.9234	0.0004***
Residuals	108	4.9552		
SPW Pb				
Time	1	0.1226	2.8243	0.0957
Treatment	5	1.1334	5.2203	0.0002***
Time x treatment	5	0.4326	1.9928	0.0854
Residuals	108	4.6896		
SPW Zn				
Time	1	0.2364	4.0643	0.0463*
Treatment	5	22.4615	77.2436	10^{-16}***
Time x treatment	5	2.4399	8.3907	10^{-7}***
Residuals	108	4.6896		

Significance levels: '***' 0.001 '**' 0.01 '*' 0.05; Df: degree of freedom; F-value: Fisher value; *p*-value: probability value; SPW Cd, SPW Pb and SPW Zn: Cd, Pb and Zn concentrations in the soil pore water, respectively.

3.5. PCA on pH in the soil pore water and on metal concentrations in the soil pore water and shoots

The X canonical weights of the Principal Component Analysis (PCA) accounting for the pH and metal (Cd, Pb and Zn) concentrations in the SPW and shoot metal concentrations of miscanthus explained 81.2 % of the total variance (Fig. 4A). The first axis (66.8 %) was characterized by the SPW metal concentrations, SPW pH as well as shoot metal concentrations. The first axis opposed the HFA, HFAxAMF and AMF soils as well as the C soil, characterized by low SPW metal concentrations, high shoot metal concentrations and high SPW pH, to the PH and PHxAMF soils characterized by high SPW metal concentrations, low SPW pH, and low shoot metal concentrations. A different trend was exhibited by hemp plants (Fig. 4B). The first two axes explained 80.1 % of the total variance. The first axis (64.1 %) separated the PH and PHxAMF treatments characterized by low SPW pH and high SPW metal concentrations as well as high shoot metal concentrations from the HFA, HFAxAMF, AMF and C treatments with high SPW pH, low SPW metal concentrations, and low shoot metal concentrations.

4. Discussion

Due to the soil texture, it was difficult to collect the roots and to remove metals sorbed on the iron plaque covering the root epidermis, and with a view to using the biomass for biofuel production, the aboveground plant parts were mainly of interest. Thus, this study focused on the influence of biostimulants (protein hydrolysate and humic/fulvic acids) and arbuscular mycorrhizal fungi on the shoot DW yield of miscanthus and industrial hemp, metal (Cd, Pb, and Zn) concentrations and uptake in shoots, and a proxy of root exposure to metals, i.e. metal concentrations in the soil pore water.

4.1. Total Cd, Pb and Zn concentrations in the soil pore water

In the studied soil, the historic contamination through atmospheric deposition dated back more than one century. Long-term reactions of metal(loid)s with soil bearing phases (i.e. ageing) and past liming have resulted in low soil metal(loid) availability (Nsanganwimana et al., 2015), which corroborates the low $(CaNO_3)_2$-extractable metal concentrations (Table 1). This soil is slightly alkaline and rich in organic matter (i.e. 36.4 ± 6.8 g kg^{-1}). The SPW Cd and Zn concentrations however increased for the PH and PHxAMF soils in line with a decrease in SPW pH ($R^2 = 0.98$). This can be explained by a significant increase in solubility of Cd and Zn induced by the decreased pH (Table 2) (Kabata-Pendias and Szteke, 2015). This result corroborated the findings of Chwil et al. (2016), who reported acidic pH values (5.9–6.2) after the application of Hemozym (protein hydrolysates) on white mustard. Conversely, a progressive decrease in SPW Cd and Zn concentrations was determined for HFA, HFAxAMF and AMF soils for both plant species. In line, the SPW pH for these three treatments remained neutral throughout our study. This decrease in SPW Cd and Zn concentrations could also be attributed to the promoted uptake of Cd and Zn from the labile pool when humic/fulvic acids and mycorrhiza fungi are used (Gao et al., 2022; Phieler et al., 2015). For all treatments and both plant species, SPW Pb concentration remained steady throughout the trial. Kabata-Pendias and Szteke (2015) reported that Pb was more bound in soils with high soil organic matter, pH, clay minerals, carbonates and Mn-oxides. A low Pb solubility was expected in our studied soil, rich in organic matter and slightly alkaline. Therefore, Pb would be bound with the soil OM due to its strong affinity for soil organic matter.

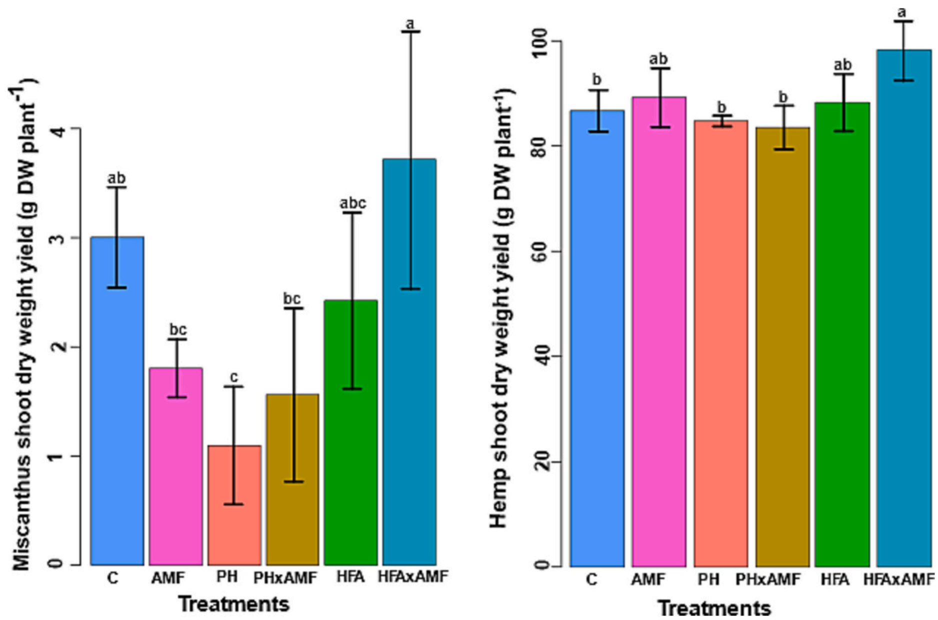

Fig. 2. Shoot DW yields (g DW plant-1) of miscanthus (A) and hemp (B) after the 90-day growth period. Untreated soils (C), arbuscular mycorrhizae (AMF), protein hydrolysate treament (PH), protein hydrolysate combined with mycorrhizae (PHxAMF), humic/fulvic acids (HFA), and humic/fulvic acids combined with mycorrhizae (HFAxAMF). Mean values per treatment (n = 4). Values with different letters differ significantly (one-way ANOVA, *p*-value <0.05).

Table 4
Shoot ionome of miscanthus and hemp at week 12 ($n = 4$).

Treatments	Miscanthus			Hemp		
	Cd (μg g^{-1})	Pb (μg g^{-1})	Zn (μg g^{-1})	Cd (μg g^{-1})	Pb (μg g^{-1})	Zn (μg g^{-1})
C	7.0 ± 2.5a	7.0 ± 1.8a	115 ± 21a	2.1 ± 0.1b	8.5 ± 0.1a	59 ± 3a
AMF	5.7 ± 1.6ab	7.3 ± 2.6a	100 ± 11a	0.9 ± 0.2c	10.9 ± 1.9a	54 ± 18a
PH	1.8 ± 0.9b	6.4 ± 1.6a	56 ± 14b	4.0 ± 0.3a	9.6 ± 1.1a	77 ± 15a
PHxAMF	2.1 ± 2.6b	6.1 ± 2.5a	48 ± 21b	3.7 ± 1.2a	8.8 ± 1.3a	60 ± 20a
HFA	6.8 ± 1.3a	7.7 ± 2.4a	110 ± 12a	1.6 ± 0.4c	9.4 ± 0.8a	60 ± 4a
HFAxAMF	8.4 ± 2.1a	7.3 ± 1.7a	120 ± 18a	0.9 ± 0.2c	8.5 ± 0.3a	51 ± 12a

Mean value ± SD for each treatment (μg g^{-1} DW). Values with different letters differ significantly (one way ANOVA, *p*-value <0.05).

4.2. Effects on shoot DW yield and shoot metal concentrations

4.2.1. Shoot DW yield

The studied soil showed relatively good soil properties, except metal (loid) excess (Table 1) and supported the growth of miscanthus and hemp (Fig. 2). This confirmed Nsanganwimana et al. (2015) and Bidar et al. (2019). However, the shoot yield of PH miscanthus plants was the most reduced (−63 %) compared to the unamended ones (Fig. 2). This could be attributed to a potential phytotoxicity induced by the high SPW Cd and Zn concentrations due to the high dose of Siapton (protein hydrolysate (PH) of animal origin) used at the early trial stages. Cerdán et al. (2009) evidenced a severe reduction of plant (tomato) growth after the foliar and root application of amino acids from animal origin. Lisiecka et al. (2011) also reported a significant decrease in the weight of strawberry plants after the application of animal-derived protein hydrolysate. Such PH inhibitory effect is likely due to excessive leaf uptake of free amino acids thereby causing an intracellular amino acid imbalance, inhibition of nitrate uptake, increased cell susceptibility to apoptosis, energy drain caused by active transport of amino acids, and potential negative influence on root morphology (Bonner and Jensen, 1997; Malécange et al., 2023). Shoot DW yield of hemp plants was however not affected by the high SPW Cd/Zn concentrations in the PH soils as compared to the C ones. Hemp has been shown to be tolerant to Cd, Pb and Zn excess. It accumulates more Cd, Pb and Zn in its roots than its shoots thereby limiting impairment of key physiological plant activities such as photosynthesis (Guidi Nissim et al., 2023; Pietrini et al., 2019). These two plant species differ in other traits: e.g. miscanthus is a C4 plant with a rhizome, while hemp is a C3 plant, strategies for uptake of Fe and other micronutrients, etc. Differences in nitrate transport system, nitrogen assimilation processes, and repression of lateral root initiation in response to nutritional cues might be involved (Malécange et al., 2023).

The shoot DW yield was highest for the HFAxAMF treatments and hemp. This could be attributed to a synergistic effect of humic/fulvic acids and mycorrhizae, e.g. the increased solubility and availability of essential nutrients by HFA coupled with the improved uptake of nutrients and water by the roots due to extended AMF mycelium. The tests carried out to verify root mycorrhizal colonization showed colonization for the HFAxAMF and PHxAMF treatments (Supplementary material 4). Humic/fulvic acids and mycorrhizal fungi increased nutrient uptake, plant growth, and shoot yield as shown in Ciadamidaro et al. (2017), Coninx et al. (2017), El-Nemr et al. (2012) and Tahir et al. (2011).

4.2.2. Shoot metal concentrations and plant metal uptake

Generally, the shoot Cd, Pb and Zn concentrations of miscanthus (Table 4) exceeded the previous values reported by Nsanganwimana et al. (2015) (1–1.7 μg Cd g^{-1}, 1.5–2 μg Pb g^{-1} and 80–137 μg Zn g^{-1} DW). These values surpassed the average concentrations of Cd, Pb and Zn (0.07–0.4, 0.4–4.6 and 12–47 μg g^{-1}, respectively) found in grasses (Kabata-Pendias and Szteke, 2015). For comparison purposes, maximum permitted concentrations in French green fodders are 1.13 μg Cd and 11.3 μg Pb g^{-1} DW (JORF, 2013). The shoot Cd and Zn concentrations for miscanthus and their uptake were significantly lower for the PH and PHxAMF treatments than the control one. This could be attributed to root exposure to high SPW Zn and Cd concentrations for both treatments (Fig. 1). The miscanthus roots would be affected in the PH and PHxAMF treatments by such high SPW Cd and Zn concentrations (corroborated by the low shoot DW yield of miscanthus for these treatments, Fig. 2) and

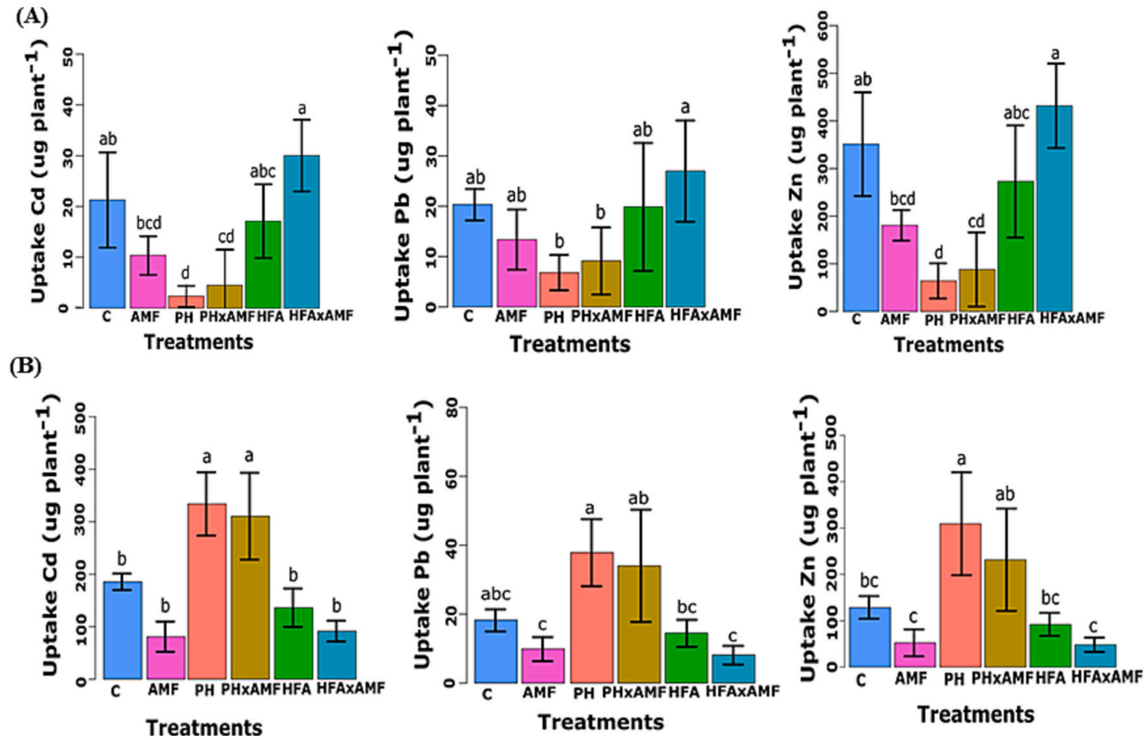

Fig. 3. Uptake of Cd, Pb and Zn in the shoots of miscanthus (A) and hemp (B). Untreated soils (C), arbuscular mycorrhizae (AMF), protein hydrolysate (PH), protein hydrolysate combined with mycorrhizae (PHxAMF), humic/fulvic acids (HFA), humic/fulvic acids combined with mycorrhizae (HFAxAMF). Mean values per treament ($n = 4$). Values with different letters differ significantly (one-way ANOVA, p-value <0.05).

hence Cd and Zn uptake would be reduced since roots are crucial in metal uptake by plants (Arif et al., 2016).

For hemp grown in a pot trial using a soil collected 500 m from the former Metaleurop site, the shoot Cd, Pb and Zn concentrations (Table 4) were within the range or slightly higher than those obtained by De Vos et al. (2022) (0.55–1.18 μg Cd g^{-1}, 5.48–14.46 μg Pb g^{-1}, and 15.02–32.70 μg Zn g^{-1} DW). Significantly higher shoot Cd concentrations for hemp were recorded with the PH and PHxAMF treatments as compared to the unamended ones (Table 4). In addition, PH and PHxAMF treatments promoted shoot Cd uptake and PH treatment enhanced shoot Zn uptake for hemp (Fig. 3B). Most hemp cultivars have a strong tolerance to Cd stress and can be cultivated in Cd-contaminated soils (Shi et al., 2012), which may partly explain why hemp performed better than miscanthus (Fig. 2). Our results with hemp agreed previous findings that the application of biostimulants and mycorrhizal fungi (in some cases depending on inoculum type, ecotype and plant species) can increase metal uptake by plants (Colla et al., 2015; Phieler et al., 2015; Wang et al., 2013). An increased uptake of Cd, Pb and Zn has been shown for hemp in presence of increased soluble/phytoavailable Cd, Pb and Zn (Rheay et al., 2021). Indeed, the PH and PHxAMF soils displayed the highest SPW Cd and Zn concentrations (Supplemental material 2) and a slightly acidic SPW pH (Table 2), which increased the solubility of Cd and Zn. This favored the Cd and Zn uptake by hemp. Also, hemp possesses metal ion transport mechanisms, such as natural resistance-associated macrophage proteins (NRAMP) and zinc/iron-regulated transporter like protein (ZIP), that facilitate the transport of Cd^{2+} and Zn^{2+} from the roots to the aboveground plant parts (Nevo and Nelson, 2006; Sterckeman and Thomine, 2020).

4.3. Practical application

Protein hydrolysate (PH) increased SPW Cd and Zn concentrations throughout the pot trial. This induced phytotoxicity for miscanthus, affecting both its growth and subsequently its shoot yield. This was not expected, but the composition of commercial protein hydrolysates is highly variable and application rates must be adapted to each plant species (Malécange et al., 2023). Conversely, a progressive decrease in SPW Cd and Zn concentrations was evidenced for humic/fulvic acids (HFA), arbuscular mycorrhizal fungi (AMF), and their combination (HFAxAMF). However, no significant effect was found on shoot DW yield when either HFA or AMF was used alone. The combination of HFA and AMF (HFAxAMF) increased the shoot DW yield for both miscanthus and hemp. An increasing trend of shoot Cd, Pb, and Zn uptake by miscanthus occurred when each biostimulant (PH and HFA) was combined with AMF, i.e. (PHxAMF and HFAxAMF). For hemp, the highest shoot Cd and Zn uptakes were evidenced for both PH and PHxAMF treatments. However, these last ones also increased the Cd and Zn concentrations in the soil pore water, which is not desirable due to potential leaching.

The valorization of plant biomass harvested at a site with contaminated soil depends on its compatibility with the intended end-use, i.e. sustainable production of clean biofuel in our case. The miscanthus and hemp biomass produced here would be potentially compatible with biofuel production and further studies are underway to assess this suitability (https://www.gold-h2020.eu/). For instance, Vintila et al. (2016) showed that trace concentrations of Cd, Pb and Zn were in the bio-oil fraction with the majority of metals remaining in the solid fraction during the conversion process of sorghum biomass cultivated on a metal-contaminated soil to bioethanol.

5. Conclusion

This study aimed at investigating the effect of two biostimulants, i.e. protein hydrolysate (PH) and humic/fulvic acid product (HFA), arbuscular mycorrhizal fungi (AMF), and their combination (PHxAMF and HFAxAMF) on miscanthus and hemp (i.e. shoot DW yield and shoot metal uptake) growing on an agricultural metal-contaminated soil collected in the vicinity of a former Pb/Zn smelter. After a 3-month growth period, Cd and Zn concentration in the soil pore water (SPW)

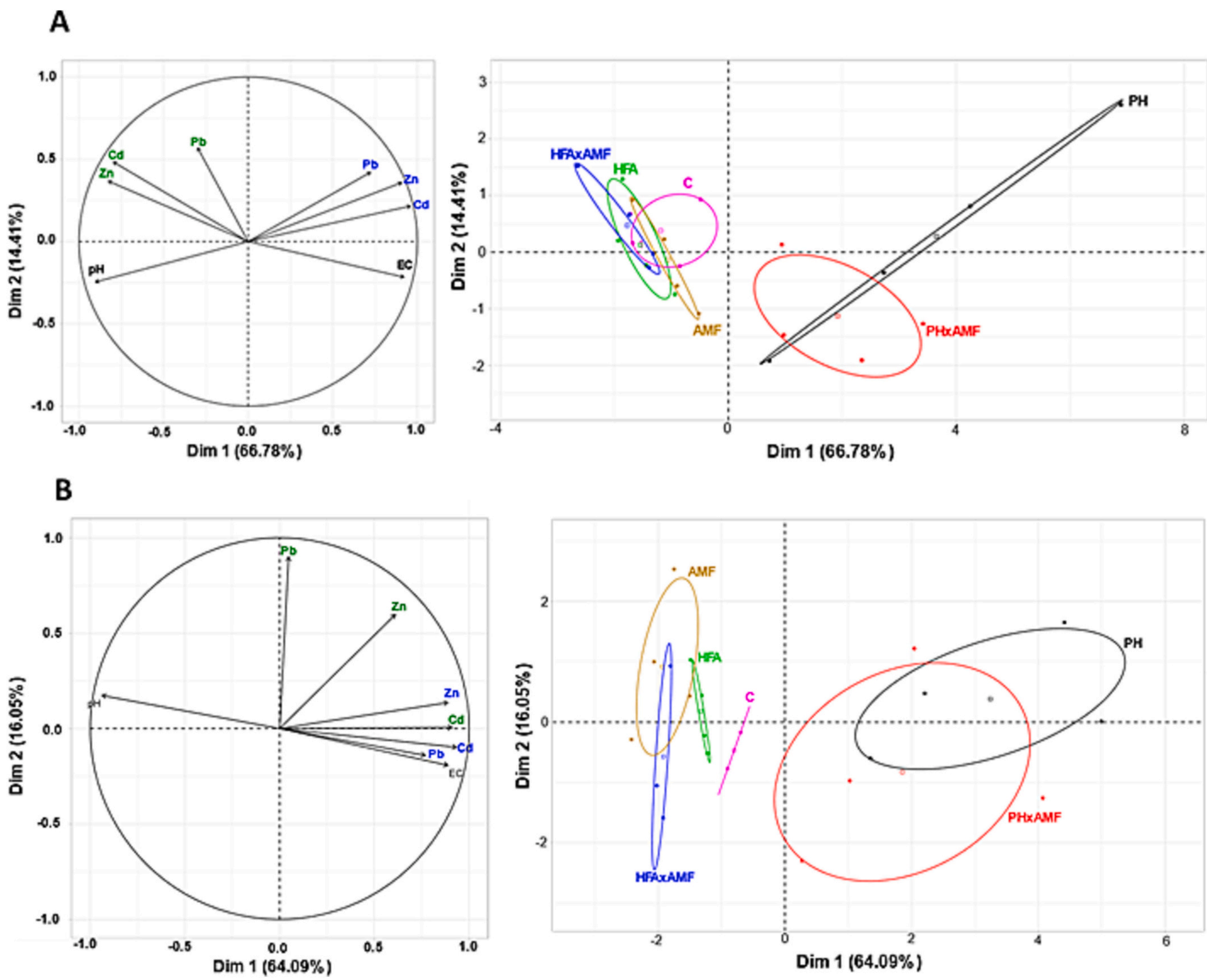

Fig. 4. Principal Component Analysis (PCA) on pH and metal concentrations in the soil pore water and shoot metal concentrations of miscanthus (A) and hemp (B). Green: metal concentration in shoots; blue: metal concentrations in the SPW.

increased for the PH and PHxAMF treated soils as compared to the untreated soil (C) and to the AMF, HFA and HFAxAMF treated soils for both plant species. Such an increase likely induced a phytotoxicity for miscanthus, reducing its shoot DW yield and shoot Cd/Zn uptake. Shoot Cd/Zn uptake of miscanthus decreased for both PH and PHxAMF treatments, due to reduced shoot DW yield and lower shoot Cd/Zn concentrations, while it increased for hemp. Regarding miscanthus, the PH product used did not satisfy our initial hypotheses. The HFAxAMF treatment significantly stimulated the shoot DW yield especially for hemp, while HFA and AMF alone did not result in such effect as compared to the unamended soil. This suggested hemp as a promising candidate as well as humic/fulvic acids and arbuscular mycorrhiza fungi as relevant treatments, notably in combination, which need to be assessed in a field trial to validate and optimize their effect on plant biomass production and metal (Cd, Pb and Zn) uptake. The determinants of such differential responses to biostimulants between miscanthus and hemp should also be investigated, including plant microbiome. Differential Cd transport and detoxification in Cd-tolerant hemp cultivars would be due to expression of key genes encoding some specific Cd transporter, defense system, and proteins (Huang et al., 2019).

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CRediT authorship contribution statement

Felix Ofori-Agyemang: Conceptualization, Data curation, Formal analysis, Writing – original draft. **Christophe Waterlot:** Supervision, Validation, Writing – review & editing. **James Manu:** Investigation, Resources. **Roman Laloge:** Investigation, Resources. **Romain Francin:** Investigation, Resources. **Eleni G. Papazoglou:** Funding acquisition, Project administration. **Efthymia Alexopoulou:** Funding acquisition, Project administration. **Anissa Lounès-Hadj Sahraoui:** Data curation, Formal analysis, Methodology, Supervision. **Benoît Tisserant:** Data curation, Formal analysis, Methodology. **Michel Mench:** Conceptualization, Funding acquisition, Project administration, Validation, Writing – review & editing. **Aritz Burges:** Investigation, Supervision, Validation, Writing – review & editing. **Nadège Oustrière:** Conceptualization, Methodology, Supervision, Validation, Writing – original draft, Funding acquisition.

Declaration of competing interest

The authors declare that there is no conflict of interest.

Data availability

Data will be made available on request.

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