GOLD

Growing energy crops on contaminated
land for biofuels and soil remediation

D1.2

Comparison of phytoremediation practices for growing selected high-yielding energy crops on contaminated soils

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Editors/Authors: WOJCIK Małgorzata, VANGRONSVELD Jaco, JAROS Karolina, PAPAZOGLOU Eleni G., KOTOULA Danai, OUSTRIERE Nadège, OFORI-AGYEMANG Félix, BURGES Aritz, WATERLOT Christophe, MENCH Michel, ZEGADA-LIZARAZU Walter, PERONI Pietro, MONTI Andrea, WANG Yufu, ZHAO Xinlin, IQBAL Yasir, IORDANOGLOU Konstantinos, TSIPAS George, ALEXOPOULOU Efthymia

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Partners

CRES – Centre for Renewable Energy Sources and Saving Foundation, Greece
AUA – Geoponiko Panepistimion Athinon, Greece
TUM – Technische Universität München, Germany
RE-CORD – Consorzio per la Ricerca e la Dimostrazione sulle Energie Rinnovabili, Italy
ETA – ETA Energia, Trasporti, Agricoltura, Italy
Uni-Lublin – Uniwersytet Marii Curie-Sklodowskiej, Poland
TNO – Nederlandse Organisatie Voor Toegepast Natuurwetenschappelijkonderzoek TNO, Netherlands
CERTH – ETHNIKO KENTRO EREVNAS KAI TECHNOLOGIKIS ANAPTYXIS, Greece
UNIBO – Alma Mater Studiorum – Università di Bologna, Italy
INRAE – Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France
YNCREA HDF – Junia, France
UNL – Universidade Nova de Lisboa, Portugal
ICL – Imperial College of Science Technology and Medicine, United Kingdom
WR – Stichting Wageningen Research, Netherlands
METE S.A. – METE AE METALLEFTIKI EMPORIKI TEHNIKI AE*MINING TRADING TECHNICAL SA, Greece
HUNAN - Hunan Agricultural University, China
UDES - Université de Sherbrooke, Canada
IBFC - Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences, China

Statement of Originality
This deliverable contains original unpublished work except where clearly indicated otherwise. Acknowledgement of previously published material and of the work of others has been made through appropriate citation, quotation or both.

Executive Summary

Soil pollution with organic and inorganic compounds is one of the greatest concerns among the threats to soil resources in Europe and globally. One of the GOLD objectives is to exploit contaminated lands by cultivating selected high-yielding lignocellulosic energy crops and getting feedstock for advanced biofuels, and, in long-term, to return these lands back to the agricultural production. To this point, seven -contaminated sites have been selected in Greece (two), Italy, France, Poland, and China (two), characterised mainly by polymetallic pollution, and to a lesser degree, by organic pollution. Comprehensive characteristics of the contaminated sites were given in **D1.1**. The polluted soil obtained from the contaminated sites or fields was used for the pot experiments of task 1.2 aiming at optimisation of the growth of selected high-yielding lignocellulosic energy crops in order to increase their potential for phytoextraction and/or bioaugmentation of different pollutants. Effect of two different biostimulants (fulvic/humic acids and protein hydrolysates) and mycorrhiza fungi applied separately or in combinations (five treatments + untreated control) on growth and heavy metal and metalloid [metal(loid)] accumulation of four energy crops (two perennial grasses: miscanthus and switchgrass and two herbaceous annuals: sorghum and industrial hemp) was tested. The applied compounds did not significantly change the soil metal phytoavailability. Based on the results obtained (mainly the highest shoot biomass and height combined with the highest metal(loid) concentration in shoots), the best two treatments for each tested crop have been selected by each partner for the pilot scale field trials (Task 1.3). The most efficient treatment was the combination of the humic/fulvic acids and mycorrhiza – this treatment is being further tested in the field experiments by all partners. The second efficient treatment for UMCS-Poland and JUNIA-France was fulvic/humic acids application, for AUA-Greece mycorrhiza, for CRES-Greece protein hydrolysates and mycorrhiza and for UNIBO-Italy, depending on the crop, protein hydrolysates, fulvic/humic acids, or protein hydrolysates combined with mycorrhiza. Optimisation of plant growth combined with enhanced metal accumulation in shoots and/or organic pollutants degradation will allow to produce high feedstock quantities for biofuel production on polluted lands (ensuring low ILUC effects), while contributing to their cleaning-up.

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I. INTRODUCTION

Soil pollution is one of the greatest concerns among the threats to soil resources in Europe and globally. It is a growing problem causing vast areas of land to become unexploited and hazardous for both wildlife and human populations. It is estimated that there are more than 10 million major contaminated sites worldwide, of which about 25% are located in Europe (Mench et al., 2018). Among these, soils contaminated with heavy metals and metalloids [metal(loid)s] account for more than 37.0% of the cases, followed by 33.7% for contamination with mineral oil, 13.3% with polycyclic aromatic hydrocarbons and others (European Environment Agency, 2014). As for the EU agricultural land, it has been found that in 6% (approximately 137 000 km^2) the concentration of metal(loid)s were increased above the allowed limits and remediation actions should be taken (https://esdac.jrc.ec.europa.eu/ESDB_Archive/eusoils_docs/other/EUR26102EN.pdf).

In the frame of GOLD project, and especially in WP1, two remediation techniques, namely phytoextraction and bioaugmentation are tested and optimised for a broader application on the polluted areas. **Phytoextraction** is an *in situ* technique in which heavy metals and/or metalloids are removed from the substrate through their uptake by plants and are accumulated in the aboveground biomass that is subsequently harvested on maturity (Suman et al., 2018). One of the phytoextraction options involves the use of energy crops that are fast-growing, high-biomass yielding and offer an added value as a raw material for the production of biofuels and bioenergy (Werle et al., 2019). Lignocellulosic energy crops (like the perennial grasses miscanthus and switchgrass, and the annual herbaceous crops biomass sorghum and industrial hemp) are considered as ideal feedstock for advanced biofuel production with low indirect land-use change (ILUC) risks. **Bioaugmentation** is an environmentally friendly and potentially economic technology in which indigenous or allochthonous microorganisms are applied to the polluted soils in order to accelerate the removal of inorganic contaminants or to effectively reduce the organic contaminant load (Gao et al., 2022; Simmer and Schnoor, 2022). The toxic organic compounds are degraded by microbial communities, including bacteria or mycorrhizal fungi, or transformed into less dangerous forms (Ma et al., 2022).

The main objective of WP1 is to **optimise the growth of selected high-yielding lignocellulosic energy crops** in order to increase their potential for phytoextraction and/or bioaugmentation of different classes of pollutants (metal(loid)s, organic). The polluted sites used for the field trials are situated in two continents and 5 countries (7 partners), representing the main agro-climatic zones and farming systems, as described in details in **Deliverable D1.1** (Figure 1).

AUA	Agricultural University of Athens, Greece
CRES	C entre for Renewable Energy Sources and Saving Foundation, Greece
HUNAN	Hunan Agricultural University, China
IBFC	Institute of Bast Fiber Crops, China
UMCS	Maria Curie-Skłodowska University, Poland
UNIBO	University of Bologna, Italy
YNCREA	Junia, France + INRAE, France

Figure 1. Locations of WP1 field trials in Europe and Asia.

The optimization of crop phytoremediation capacity can be achieved, among others, by the implementation of **innovative agronomic practices**, including **application of biostimulants and mycorrhizae fungi**. These phytoremediation practices have been selected and applied to GOLD pot and field trials because they can enhance the plant growth and yields, the nutrition efficiency, abiotic stress tolerance and/or crop quality traits (du Jardin, 2015; Bartucca et al., 2022; Shahrajabian et al., 2022; Tiwari et al., 2022). They may either directly interact with the plant signalling cascades or act through stimulation of endophytic and non-endophytic bacteria and fungi to produce molecules of benefit to the plant. Biostimulants are generally classified into three major groups: humic substances (HS), amino acid containing products (AACP), and hormone containing products (HCP) (du Jardin, 2015). They have been successfully applied in agriculture and horticulture bringing positive effect on plant growth and fitness. Humic substances, including **humic/fulvic acids**, are formed by chemical and biological transformations of plant and animal matter and from microbial metabolism, and represent the major pool of organic carbon at the earth's surface (Canellas et al., 2015). **Protein hydrolysates** are based on a mixture of peptides and amino acids, and are mainly produced by enzymatic and/or chemical hydrolysis of proteins from animal- or plant-derived raw materials (Colla et al., 2015). Both groups may promote plant growth through the enhancement of nutrient uptake and nutrient-use efficiency, stimulation of carbon and nitrogen metabolism, modifying the level of plant hormones, stimulating beneficial plant microbiomes, and alleviating the negative effects of abiotic stress caused by salinity, drought or heavy metals (Colla et al., 2017; Jindo et al., 2020; Martín et al., 2022). **Mycorrhiza fungi** are plant-associated microorganisms that form a network of filaments associating with plant roots. Such symbiosis benefits for the plants in improved water and mineral nutrients acquisition, protection against soil-borne pathogens, and increased tolerance to other environmental stress factors, including drought, salinity or pollution, resulting in a better growth and general plant condition (Ma et al., 2022; Sakthieaswari et al., 2022).

In the GOLD project, in frame of WP1, **the optimizing practices are tested at two levels**: (i) pot trials, where a number of phytoremediation techniques (different biostimulants, mycorrhiza) are being tested under controlled conditions and (ii) pilot small-scale field trials, in which the best optimizing practices selected from the pot experiments will be tested in the contaminated sites of each partner in order to obtain higher plant biomass and contaminant remediation for WP2 to gain reliable results from the conversion methods.

This deliverable **D1.2** "**Comparison of phytoremediation practices for growing selected high-yielding energy crops on contaminated soils**" is concentrated on the **outcome of the pot trials** (**Task 1.2**) and aims in **proposing best optimisation practices to be tested in the field conditions.**

II. MATERIAL & METHODS – Experimental setup and protocols

Due to the fact that the project started on May 1st, 2021, it was too late to successfully establish the pot experiments outdoors and to get reliable results. In order to not lose completely the first year of the project implementation and to obtain results for the treatments to be applied in the field trials of the next spring (2022), all partners had to establish their pot experiments **in greenhouses**. The experiments started in September 2021 (UMCS, AUA, YNCREA, CRES) / October (UNIBO) 2021 or March (HUNAU) / April (IBFC) 2022 and lasted for 3 months. The temperature and photoperiod in the greenhouses were adjusted to the spring-summer season of each partner. **All partners used the same plant material, the same products for the treatments and the same protocols** that had been established during on-line technical meetings of WP1 before the beginning of the pot experiments.

The soil used per partner for the pot experiment was collected from the corresponding contaminated site that the field trials of task 1.3 will be established (Table 1, described in details in Deliverable 1.1). The soil samples were characterised mainly by multi-metal(loid) contamination, posing the biggest problem in the experimental fields. However, low concentrations of organic contaminants (pesticides: insecticides and their metabolites – tefluthrin, cypermethrin, DDT and DDE; herbicides and their metabolites – glyphosate and AMPA) were detected in the experimental fields of UMCS, CRES, and UNIBO (Table 1).

Table 1. Location of experimental fields and their main contaminations.

Partner	**Experimental field location**	**Soil contamination**	
		Metal(loid)s*	**Organics****
UMCS, Poland	Piekary Śląskie, Upper Silesia 50°21’19” N, 19°00’17” E	Pb, Zn, Cd, As	anthraquinone (0.10) p,p'-DDE (0.011) p,p'-DDT (0.01)
AUA, Greece	Lavreotiki peninsula, SE Attica 37°43’59'' N, 24°02’40'' E	Pb, Zn, Ni, Cd, As, Sb	no organic pollution
CRES, Greece	Kozani 40° 08' 45" N , 21° 55' 57" E	Ni, Cr, As	cypermethrin (0.035)
UNIBO, Italy	Chiarini, near Bologna 44° 50' N, 11° 28' E	Pb, Zn, Ni, Cu, Sn	tefluthrin (0.072) anthraquinone (0.10) p,p'-DDE (0.024) p,p'-DDT (0.018) glyphosate (0.136) AMPA (0.207)
YNCREA, France	Evin-Malmaison 50°26'17.3" N, 3°01'05.8" E	Pb, Zn, Cd, Cu	no organic pollution
IBFC, China	Yonghe Town, Hunan 28°16’42" N, 113°55’21" E	Cd	no organic pollution
HUNAU, China	Paishangcun, Hunan 27.72708 N, 113.180581 E	Cd	no organic pollution

* Metal(loid)s of which total concentrations are above the permissible thresholds for agriculture soils.

** Data not presented in Deliverable 1.1 (being under analysis when D1.1 was submitted), values in parentheses expressed in mg kg^{-1} soil DW.

The experimental setup is presented in Figure 2.

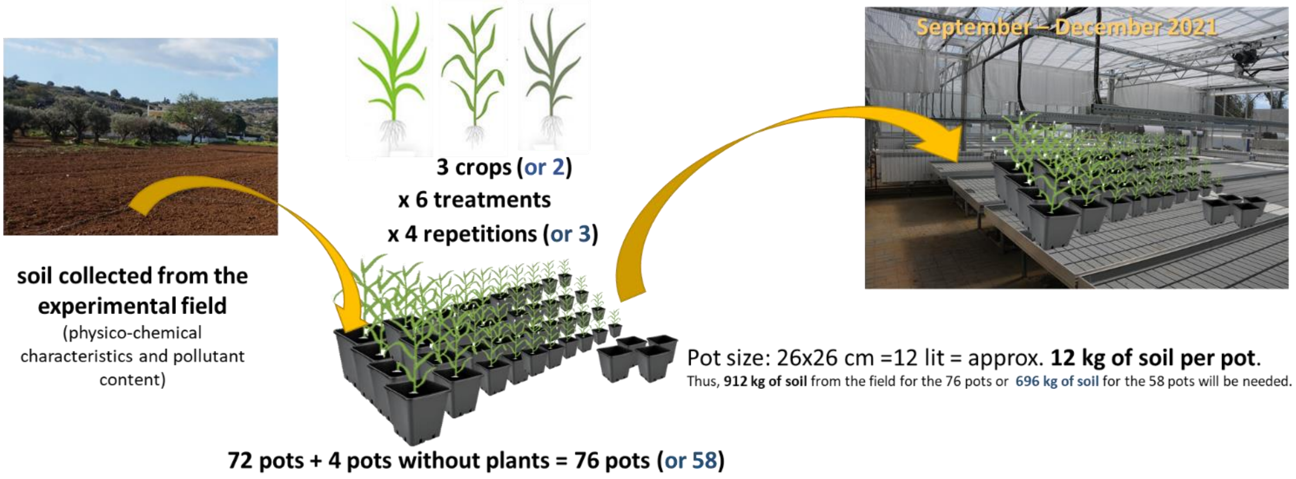

Figure 2. Experimental setup.

The soil collected from the experimental fields was homogenized, sieved through a 10 mm mesh, mixed thoroughly with the fertilizer (in the proportion of 20 g of 20-5-10 N-P-K per pot), and placed into pots (12 kg of soil per pot) (Figure 3).

UMCS, Poland

AUA, Greece

CRES, Greece

YNCREA, France

HUNAU, China

Figure 3. Excavation and preparation of the soil for pot experiments by selected partners.

Four high-yielding lignocellulosic energy crops have been selected for the scope of GOLD; two perennial grasses: **miscanthus** and **switchgrass** and two herbaceous annuals: **sorghum** and **industrial hemp**. Each partner was supposed to carry out pot and field trials for three (or two in China) of the selected energy crops (depending on the climatic zone).

Uniform plant material was used by each partner:

- **miscanthus**: micro-propagated plants of *Miscanthus x giganteus* purchased from Rhizosfer© (France)
- **switchgrass** (*Panicum virgatum* L.): Immediately after the beginning of the project, CRES and the other partners did a thorough investigation worldwide to find switchgrass seeds but without any success. For this reason, and in order to avoid losing the cultivation period of 2021, it was decided to use seeds of the variety KANLOW that CRES already acquired, knowing that their germination rate was quite low. Meanwhile, the efforts to find seeds continued.
- **sorghum** (*Sorghum sudanense x bicol*or) variety BULLDOZER, obtained from UNIBO, Italy
- **hemp** (*Cannabis sativa* L.) variety FUTURA 75, obtained from CRES, Greece.

These plant species/varieties were chosen for the trial due to their ability to grow on metal-contaminated soils and to withstand harsh environmental conditions; in addition, they have low agricultural requirements and they are high yielding, giving biomass suitable for biofuel production.

Micro-propagated plants of **miscanthus** were transplanted in the potted soils, one plantlet per pot (Figure 4 A, B). Five seeds of **hemp** were sowed per pot and 15 days later the best grown seedling was selected and kept (Figure 4 C, D), while the other hemp seedlings were manually thinned. Similar protocol was applied to **sorghum**.

Figure 4. Miscanthus micro-propagated plants and transplantation (A, B); hemp cultivation in the greenhouse (C, D). Pictures: A-C from AUA, Greece; D from YNCREA, France.

Knowing the quite low germination capacity of **switchgrass**, 15-50 seeds were sowed per pot. Indeed, the germination was unsuccessful or limited (less than 10 %) and the seedlings were very week, unable to grow (Figure 5). Different partners tried to solve this problem using different strategies of germination and growing of young seedlings, including: (i) different germination substrates (moisture paper, mixture of peat and perlite, commercial gardening soil, Figure 5 A, B, C, D, E, F), (ii) special nurseries (e.g. petri dishes, mini greenhouses, Figure 5 C), (iii) selecting the heaviest and thus potentially viable seeds by their sedimentation in the pots filled with water, (iv) stratification (keeping the seeds under low temperature in order to induce germination). All these efforts resulted in a very limited number of seedlings that hardly survived (Figure 5 G) or died after transplanting to the polluted soil, independently if they were a few days or a few weeks old. Therefore, any reliable result on switchgrass could not be obtained and, thus, only the results for the other three plant species – miscanthus, hemp and sorghum – are further presented in this report.

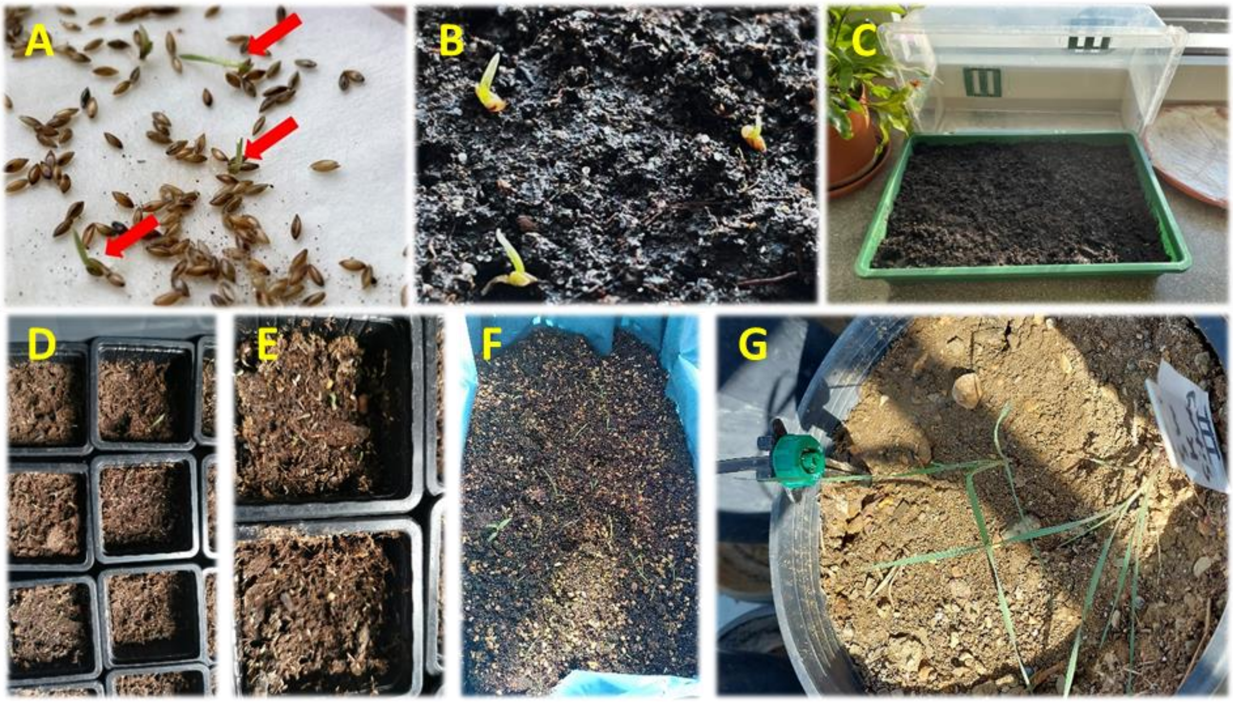

Figure 5. Germination of switchgrass seeds on different substrates (A-F) and weak seedlings hardly surviving after transplanting onto contaminated soil (G). Pictures: A, B, C from UMCS, Poland; D, E from YNCREA, France; F, G from AUA, Greece.

The **biostimulants** used in pot experiments were:

- protein hydrolysates (SIAPTON, Company: Agrology, Greece)
- fulvic/humic acids (LONITE 80 SP, Company: Alba Milagro, Italy)
- mycorrhiza (SYMBIVIT, Company: Symbiom, Czech Republic)

Six treatments were tested for each plant species:

1. **B1** (protein hydrolysate – Siapton)
2. **B2** (fulvic/humic acids – Lonite)
3. **M** (mycorrhiza – Symbivit)
4. **B1 x M**
5. **B2 x M**
6. **Control** (no treatment)

<u>**Application protocols**</u>

Protein hydrolysate (B1): the treatment was applied by diluting 13.5 mL in the irrigation water per pot. This dose was reduced to 3 mL per litre of spray liquid when the plants had adequate leaf area for foliar spraying. The first application was applied 3 days after the transplantation of miscanthus and when the hemp and sorghum plants had a height of 10 cm. The application was repeated every 10 days (Figure 6 A, B, C).

Humic/fulvic acids (B2): they were applied by diluting 0.5 g per pot in the irrigation water. Four weeks after the first application, this dose was increased to 0.7 g per pot. The first application was done one week after transplantation of miscanthus plantlets and when the hemp and sorghum plants had the first 3 – 6 leaves. The application of humic/fulvic acids was done every week.

Mycorrhizae fungi (M): before the transplantation of miscanthus rhizomes and the sowing of the hemp and sorghum seeds, 15 g per pot of the mycorrhizae fungi were added (Figure 6 D).

Figure 6. Foliar protein hydrolysate application on the three crops: sorghum (A), miscanthus (B) and hemp (C); application of humic/fulvic acids (irrigation) and mycorrhizae fungi application in a pot for the treatments M, B1XM, and B2XM. Pictures: A, B, C from UNIBO, Italy; D from HUNAU, China; E from AUA, Greece.

During the trial, the plants were monitored for phytotoxicity symptoms (chlorosis, necrosis, changed pigment contents, etc.) and for their growth (by measuring their height, number of leaves and tillers) (Figure 7).

Figure 7. Greenhouse experiment at the beginning (21.09.2021) and after 11 weeks (1.12.2021); UMCS, Poland.

The following parameters were determined for each crop at plant harvest:

- fresh and dry weight of aerial plant parts (during the 1st technical meeting of WP1 partners it was decided that the root biomass will not be analysed as not relevant in a pot experiment),
- plant total height, number of leaves, number of tillers, number of inflorescences,
- overt phytotoxicity symptoms,
- metal(loid) concentration of aboveground plant parts,
- the biomass quality characteristics (ash content, calorific value, etc.),
- extractable metal(loid) concentration in the soil (following 0.01 M $Ca(NO_3)_2$ extraction), soil pH (H_2O), organic pollutant concentrations.

Based on the results obtained (mainly the highest shoot biomass and height combined with the highest metal(loid) concentration), **the best two treatments should be selected by each partner for the pilot scale-small field trials (Task 1.3)**.

III. RESULTS – Comparison of phytoremediation practices for growing selected high-yielding energy crops on contaminated soils

In the following sections the description of the obtained results is presented separately per each partner. The detailed description of the contaminated sites per partner and of the corresponding soil characteristics are presented in Deliverable 1.1. "Site description and characterisation". However, in the following paragraphs the results from the analyses accomplished in the collected soil for the pot experiments is presented.

1. UMCS, POLAND

1.1. Soil parameters

The total concentrations of the metals Zn (average 8057.1 mg kg-1), Pb (average 2939.7 mg kg-1), Cd (average 51.56 mg kg-1), and the metalloid As (average 94.13 mg kg-1) in the soil samples exceeded their respective permissible limits for soils used for agriculture. At the beginning of the experiment, the extractable forms of Zn ranged from 1.54 to 4.17 mg kg^{-1} (average 2.85 mg kg^{-1}) and these of Cd varied from 0.15 to 0.30 mg kg^{-1} (average 0.19 mg kg^{-1}). The rate of extractable forms of Pb was the lowest in comparison to the total soil Pb, with their concentrations ranging from 0.22 to 0.44 mg kg^{-1} (average 0.29 mg kg^{-1}).

In general, the values of $Ca(NO_3)_2$-extractable, and thus potentially bioavailable forms of Zn, Cd and Pb in the soil samples did not differ significantly from the original values in the soil before starting the experiment. They also did not vary between the treatments (Table 1.1). This suggests that application of treatments tested does not result in a significant increase in metal phytoavailability.

The analyses of organic contaminants in the soil at the end of the pot experiment are in progress.

Table 1.1. Mean $Ca(NO_3)_2$-extractable concentrations of metals (mg kg^{-1}) in soil samples at the end of the pot experiment using miscanthus and industrial hemp after application of different treatments.

	Miscanthus x giganteus (mg kg^{-1})			*Cannabis sativa* L. (mg kg^{-1})		
	Zn	**Cd**	**Pb**	**Zn**	**Cd**	**Pb**
C	5.18	0.34	0.49	3.78	0.31	0.27
B1	4.36	0.26	0.58	5.38	0.35	0.66
B2	4.71	0.31	0.64	4.53	0.31	0.52
M	6.09	0.42	1.00	5.15	0.36	0.58
B1 x M	5.32	0.35	0.71	4.91	0.34	0.54
B2 x M	3.94	0.24	0.48	4.36	0.31	0.45

1.2. Plant growth and metal accumulation

No special toxicity symptoms were visible in miscanthus and industrial hemp during the experiment. The growth of individual plants, and especially hemp, within a treatment was highly variable (Figure 1.1). No significant differences in plant shoot fresh weight were found after application of different biostimulants and mycorrhiza (Figure 1.1). Significantly higher biomass production was only observed in miscanthus treated with fulvic/humic acids combined with mycorrhiza (B2xM) (Figure 1.1 and 1.2). Miscanthus plants from this treatment were also significantly higher and had bigger number of leaves and tillers in comparison to control or application of other treatments (Table 1.2).

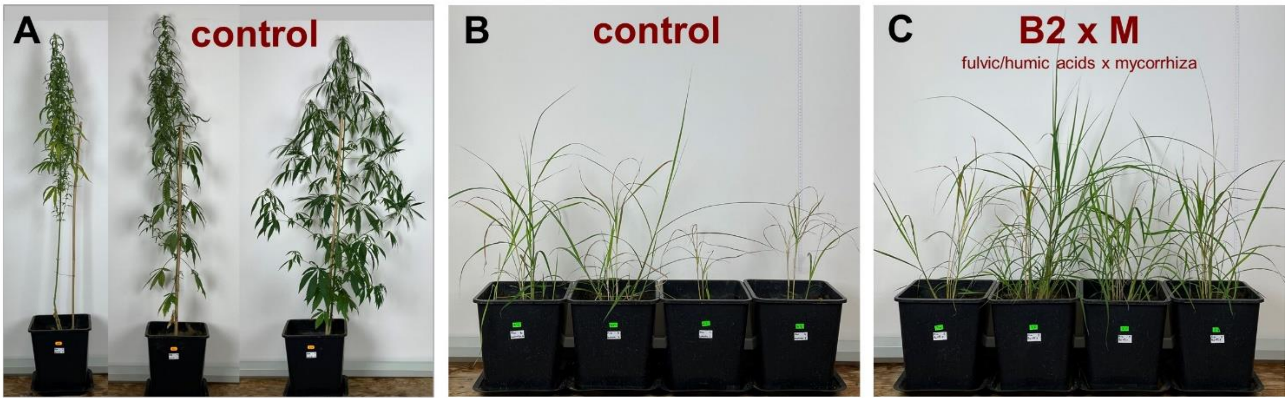

Figure 1.1. Various growth and appearance of hemp (A) and miscanthus (B, C) individuals within a treatment.

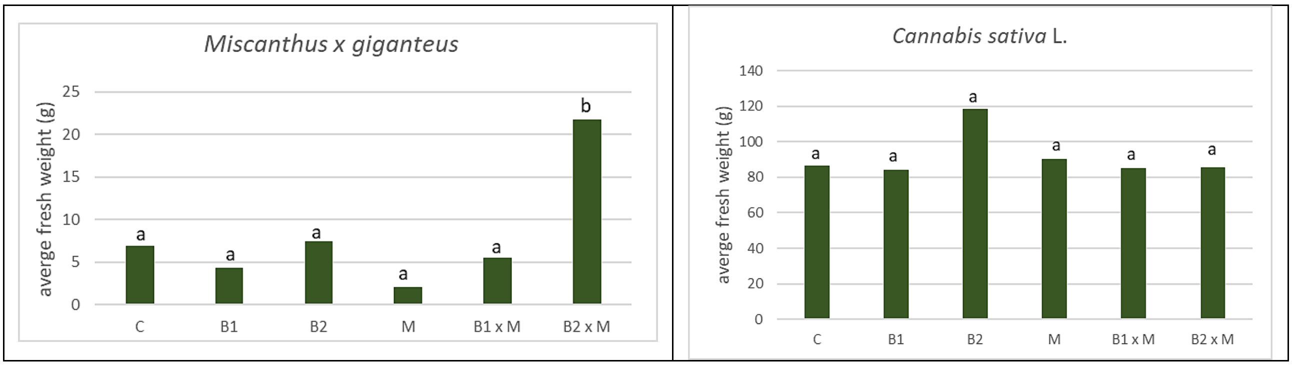

Figure 1.2. Fresh weight of miscanthus and industrial hemp per treatment.

Table 1.2. Plant morphometric parameters at harvest.

	Miscanthus x giganteus			***Cannabis sativa* L.**		
	Height (cm)	Leaves (N°)	Tillers (N°)	Height (cm)	Leaves (N°)	Tillers (N°)
Control	59.0	27	5	117.5	61	0
M	46.8	15	3	136.1	63	0
B1	48.5	25	5	138.3	56	0
B2	54.4	38	8	145.0	53	0
B1xM	50.6	22	5	138.0	47	0
B2xM	70.0	58	14	145.6	46	0

The effect of biostimulant application on metal(loid) concentration in plant shoots is presented in Figures 1.3 and 1.4. In case of miscanthus, the highest concentrations of Pb and As were found in plants treated with fulvic/humic acids (B2). Relatively highest Zn and Cd concentrations were recorded in B2 and B2xM treatments. However, there were no significant differences in metal(loid) accumulation in shoots of industrial hemp after treatment application.

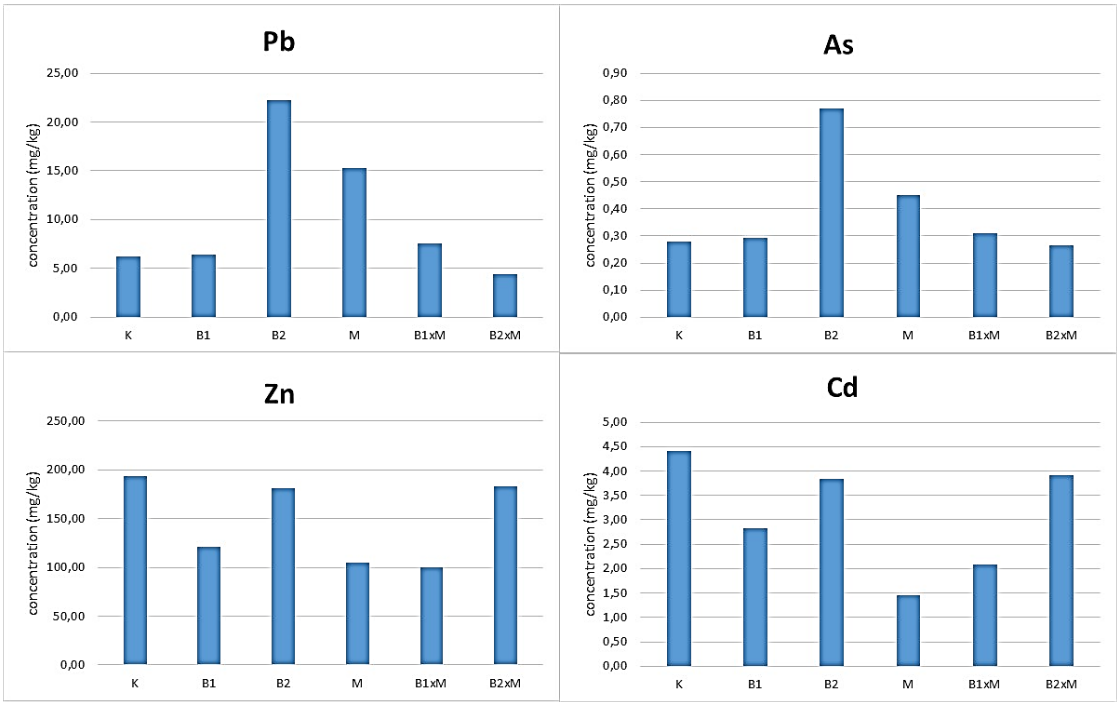

Figure 1.3. Metal(loid) concentrations (mg kg^{-1} dry weight) in the shoots of miscanthus at the end of the experiment.

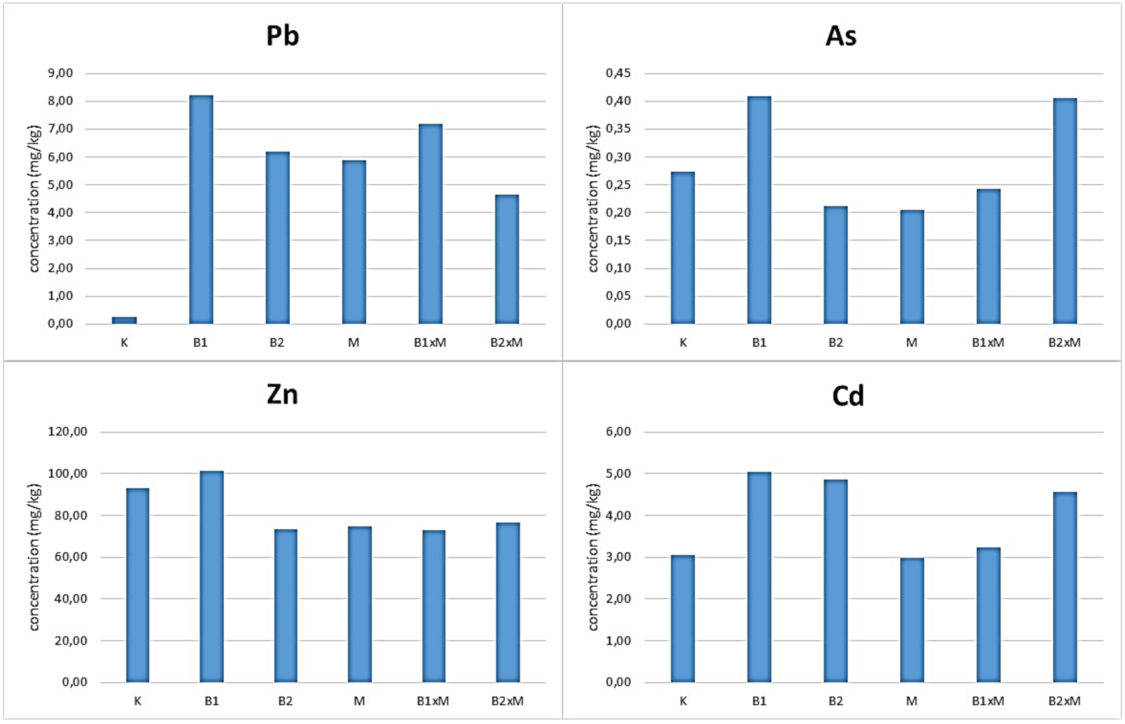

Figure 1.4. Metal(loid) concentrations (mg kg^{-1} dry weight) in the shoots of industrial hemp at the end of the experiment.

2. AUA, GREECE

2.1. Soil parameters

The results for both total and $Ca(NO_3)_2$-extractable concentrations before planting the pots and after harvest did not differ significantly. Total soil concentrations at the beginning of the trial were up to 4782 mg Pb/kg, 3602 mg Zn/kg, 196 mg Ni/kg, 12 mg Cd/kg, 453 mg As/kg and 75 mg Sb/kg. The initial $Ca(NO_3)_2$-extractable concentrations were up to: 58 mg Pb/kg soil, 153 mg Zn /kg soil, 18 mg Ni/kg soil, 3 mg Cd/kg soil, 31 mg As/kg soil and 10 mg Sb /kg soil. At the end of the experiment the $Ca(NO_3)_2$-extractable concentrations were similar and are presented in Figure 2.1. No organic pollution was found in the analysed soil samples.

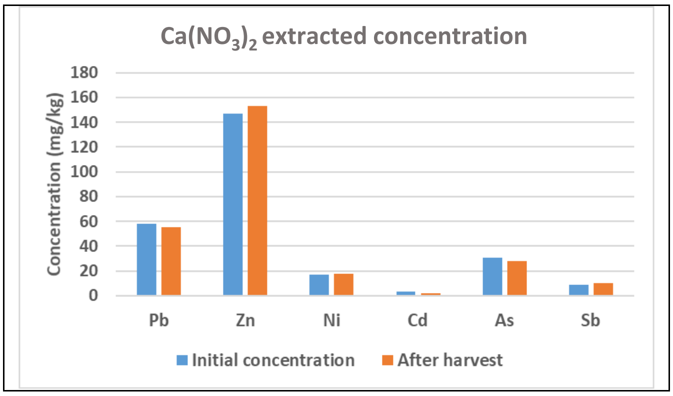

Figure 2.1. $Ca(NO_3)_2$-extractable metal(loid) concentrations in the soil of the pots.

2.2. Plant growth and metal accumulation

During the experimental period the growth and development of miscanthus and hemp plants were affected by the soil contamination. All plants of both species remained small, and for this reason their aerial biomass was not partitioned in shoots, leaves and inflorescences for further analyses as their dry matter was not adequate for the planned analyses.

Miscanthus: 47 days after transplantation (03/11/2022) the highest plants were observed in treatments M (mycorrhizae fungi) and B2xM (humic/fulvic acids + mycorrhizae fungi), reaching the 26.8 cm and 23.3 cm respectively (Figure 2.2). In addition, several leaves were desiccated. The same observations were made one month later (09/12/2022), when the maximum shoot length was measured in the same treatments and was up to 29.8 cm and 26.1 cm respectively. The final plant height, number of leaves and number of tillers at harvest are shown in Table 2.1.

miscanthus industrial hemp

Figure 2.2. Miscanthus and industrial hemp plants on 03/11/2022 (left panels) and 09/12/2022 (right panels).

Table 2.1. Plant morphometric parameters at harvest.

	Miscanthus* x *giganteus			***Cannabis sativa* L.**		
	Height (cm)	Leaves (N°)	Tillers (N°)	Height (cm)	Leaves (N°)	Tillers (N°)
Control	18.3	25	6	42.5	78	0
M	33.0	34	6	54.3	133	0
B1	25.3	37	5	57.7	73	0
B2	27.5	21	4	21.7	108	0
B1xM	12.5	12	2	47.7	138	0
B2xM	30.7	39	6	32.7	142	0

Hemp: 45 days after transplantation (03/11/2022) the plants started already to form inflorescences and to complete their growth cycle. That is a common reaction of plants when they face strong abiotic stresses as they try to preserve and protect their existence on earth by producing seeds. On 9/12/2022 in most of the plants the inflorescence was totally developed, even though they were short and with an inadequate growth (Figure 2.2). The final plant height, number of leaves and number of tillers at harvest are presented in Table 2.1. It has to be noted that the increased number of leaves shown in table 2 is because the leaflets of the inflorescences were included in the measurement. The highest plants were observed in the M (mycorrhizae fungi) and B1 (protein hydrolysate) treatments.

The fresh weight of both crops per treatment is presented in Figure 2.3.

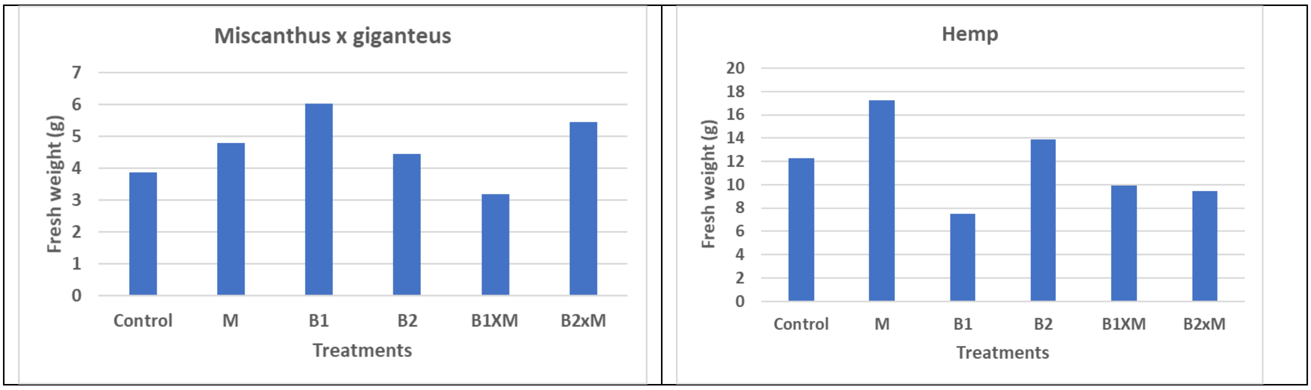

Figure 2.3. Fresh weight of miscanthus and hemp per treatment.

The effect of treatments on the heavy metal and metalloid concentrations in the shoots of miscanthus (Figure 2.4) and industrial hemp (Figure 2.5) was determined. The measurements of As concentrations in plant parts of both crops are in progress.

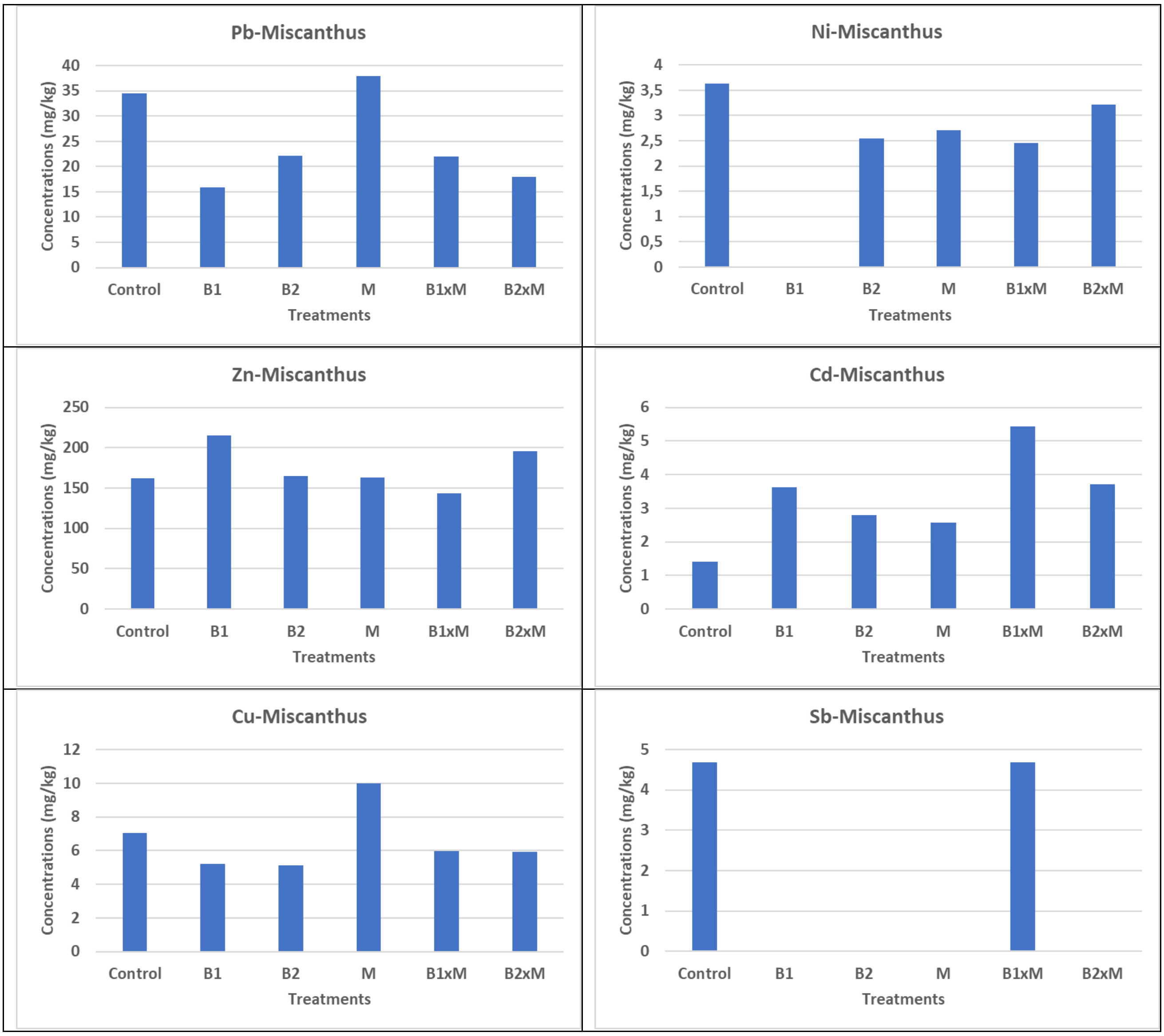

Figure 2.4. Metal(loid) concentrations (mg kg^{-1} dry weight) in the shoots of miscanthus at the end of the experiment.

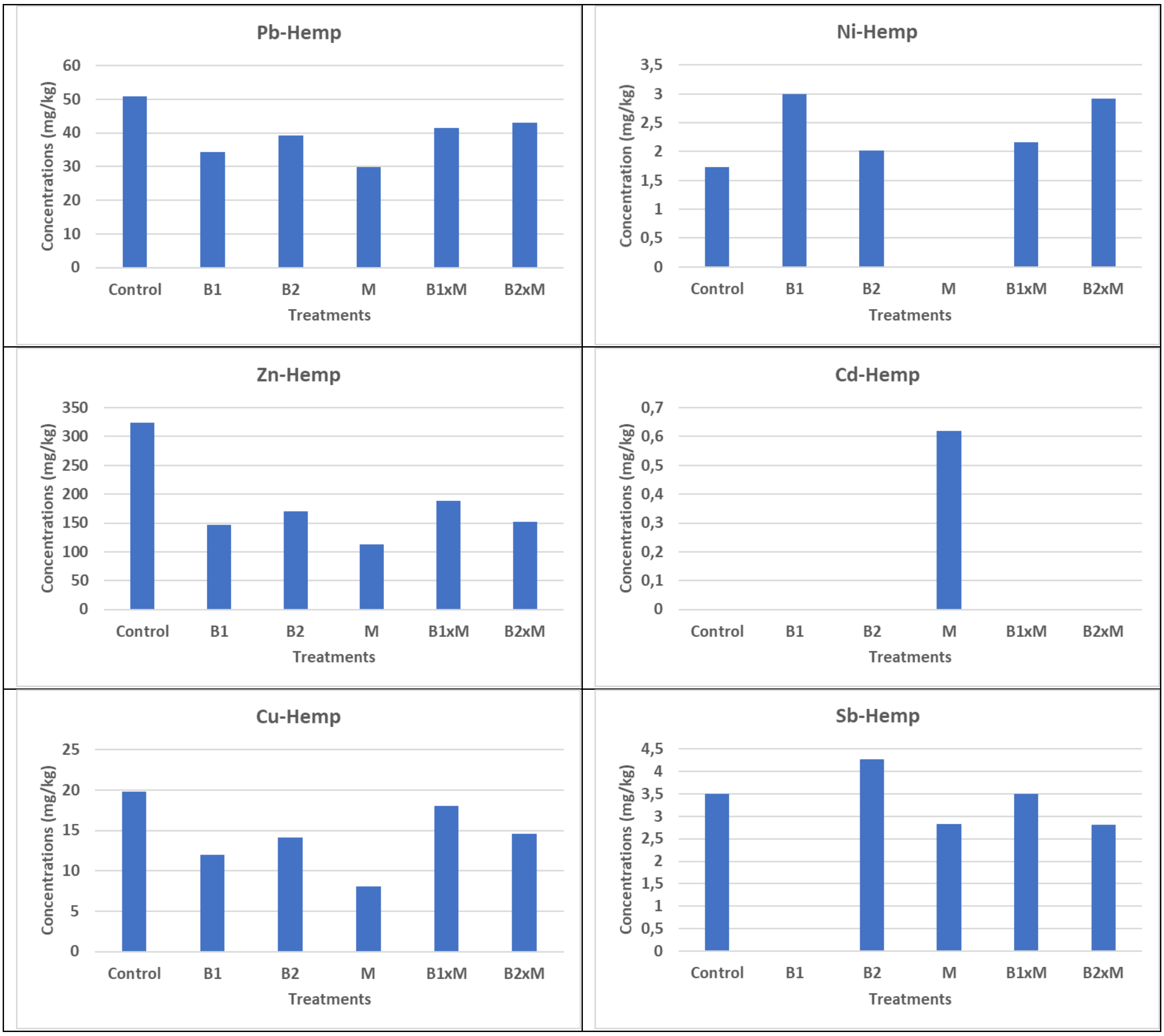

Figure 2.5. Metal(loid) concentrations (mg kg^{-1} dry weight) in the shoots of industrial hemp at the end of the experiment.

3. CRES, GREECE

3.1. Soil parameters

The soil collected from the field of CRES & METE company in Kozani was analysed for the determination of both total and $Ca(NO_3)_2$-extractable concentrations. Soil samples were taken before planting the pots and after harvest. The initial total concentrations of Cd and Sb were below the detection limit of the ICP, while only Ni concentration was much higher than the normal values usually found in soils, and was up to 984.33 mg kg^{-1} at the beginning and 967.30 mg kg^{-1} after harvest. Zinc, copper, lead and arsenic reached the 46.63 mg kg^{-1}, 14.40 mg kg^{-1}, 7.20 mg kg^{-1} and 1.17 mg kg^{-1} respectively. The $Ca(NO_3)_2$-extractable concentrations were up to: 3.2 mg Zn /kg soil, 43.66 mg Ni/kg soil, and 0.2 mg As/kg soil. The corresponding measurements for Cu, Pb, Cd and Sb were below the detection limit of the ICP. Generally, the initial and the after-harvest concentrations did not differ significantly and the results of the bioavailable fraction are presented in Figure 3.1.

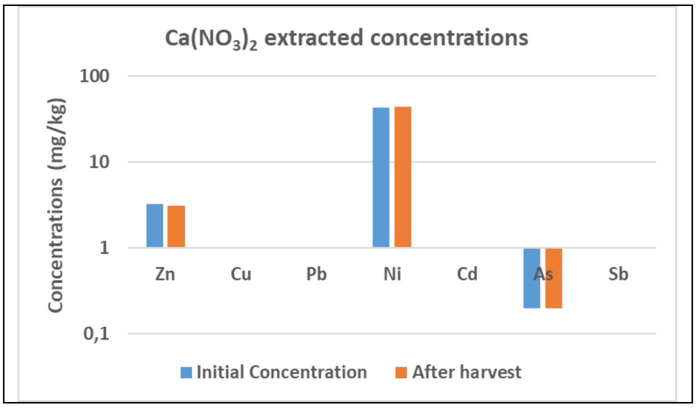

Figure 3.1. Ca(NO$_3$)$_2$-extractable metal(loid) concentrations in the soil of the pots.

3.2. Plant growth and metal accumulation

No overt phytotoxicity symptoms were observed in both plant species. However, miscanthus plants remained generally small for all treatments (Figure 3.2).

For miscanthus the highest plants were developed for the treatments B2 and B2xM with plant height 25.8 cm and 33.9 cm, respectively (Table 3.1). The highest fresh weight was measured in the pots treated with B2xM (26.87 g), while the lowest fresh weight was recorded in the pots of the B1 treatment (6.97 g) (Figure 3.3).

The highest sorghum plants were recorded in B2 and B1xM treatments, with heights of 152.1 cm and 158.0 cm, respectively (Table 3.1). The sorghum fresh weights for all treatments are presented in Figure 3.3. The highest fresh weight was recorded the control pots, while among the other treatments the highest values were recorded in B1xM treatment. The lowest values were recorded in B2 treatment.

Figure 3.2. Sorghum (left) and miscanthus (right) before harvest.

Table 0.1. Plant morphometric parameters at harvest.

	Miscanthus x giganteus			**Sorghum**		
	Height (cm)	Leaves (N°)	Tillers (N°)	Height (cm)	Leaves (N°)	Tillers (N°) (apart the main)
Control	18.8	114	12	150.7	10	1
M	19.3	89	11	136.3	9	0
B1	18.9	73	8	137.3	9	1
B2	25.8	119	12	152.1	9	3
B1xM	19.1	113	14	158.0	7	1
B2xM	33.9	166	12	145.0	10	0

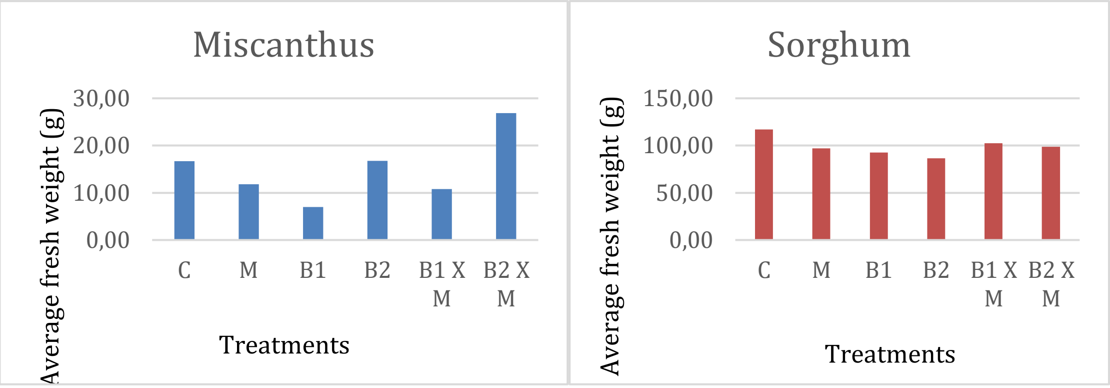

Figure 3.3. Average fresh weight of miscanthus (left) and sorghum (right) at the time of harvest.

The effects of biostimulants on heavy metal accumulation for both crops are presented in Figures 3.4, 3.5 and 3.6. Since the plants of miscanthus remained small, there was not an adequate quantity of biomass to separate the leaves from the shoots and for this reason the aerial biomass was analyzed as a whole. The highest accumulation of Cu was measured in B2 treatment, followed by B2xM treated plants. For Pb, treatments B2xM and M were the most effective in terms of lead accumulation in the aerial biomass; Zn concentrations were higher in treatments B1xM and B1, while the highest Ni concentrations were observed in B2xM and B1xM treatments (Figure 3.4)

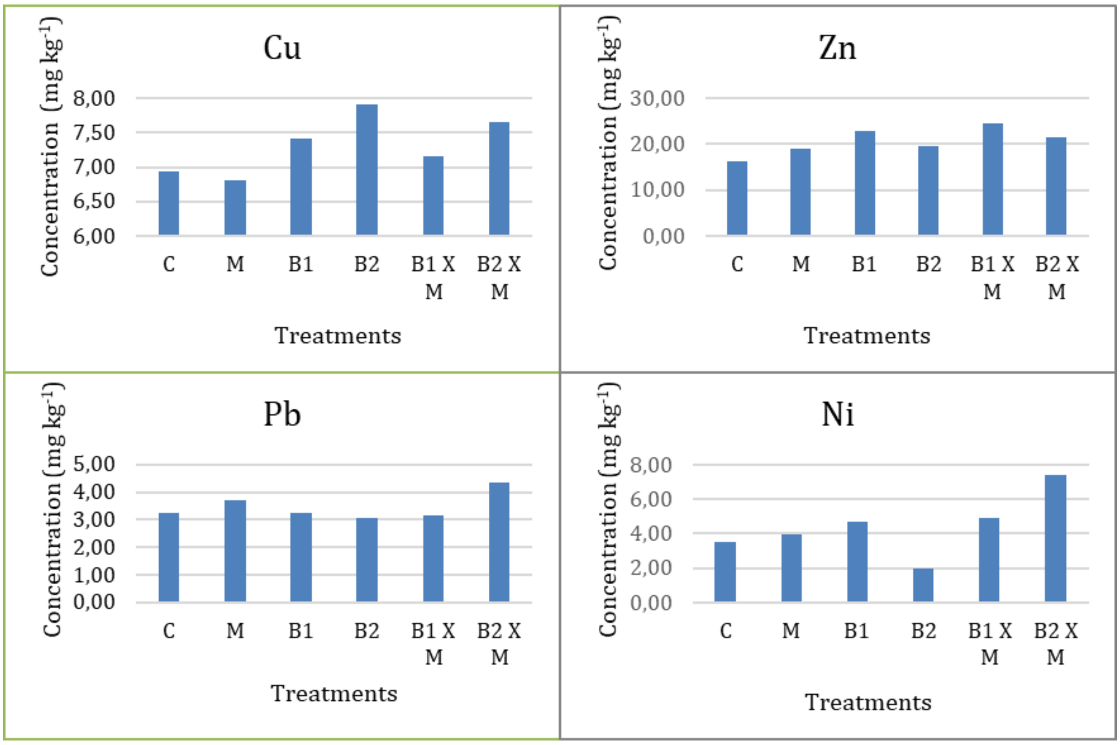

Figure 3.4. Heavy metal concentrations in the aerial biomass of miscanthus plants.

Sorghum plants were divided into two aliquots, leaves and shoots. In leaves, under the treatments B2xM and B2 the higher concentrations of Cu and Zn were observed, while Pb was measured in higher concentrations in treatment B2 and B1, and Ni in B1xM and B2xM (Figure 3.5). In shoots, treatments B1xM and B2 resulted in higher Cu and Zn concentrations. Lead was concentrated in higher amounts in M and B1xM treatments and Ni with a significant difference in B2 treatment followed by M and B1xM (Figure 3.6).

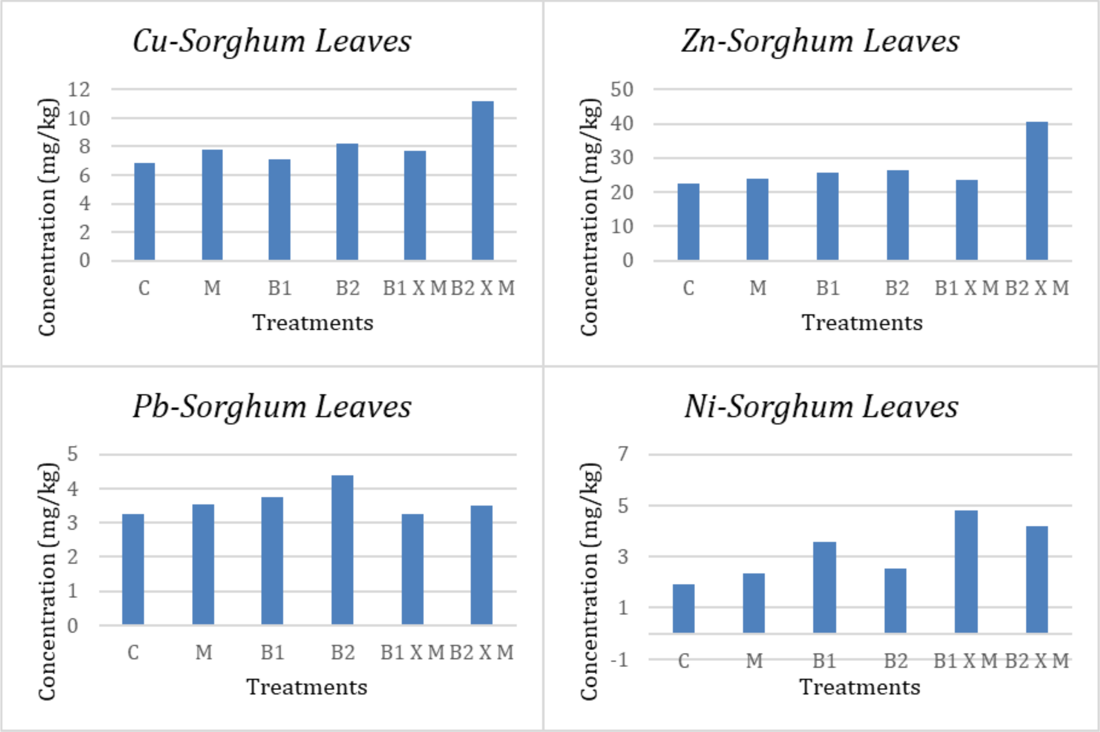

Figure 3.5. Heavy metal concentration (mg kg^{-1}) of sorghum leaf biomass.

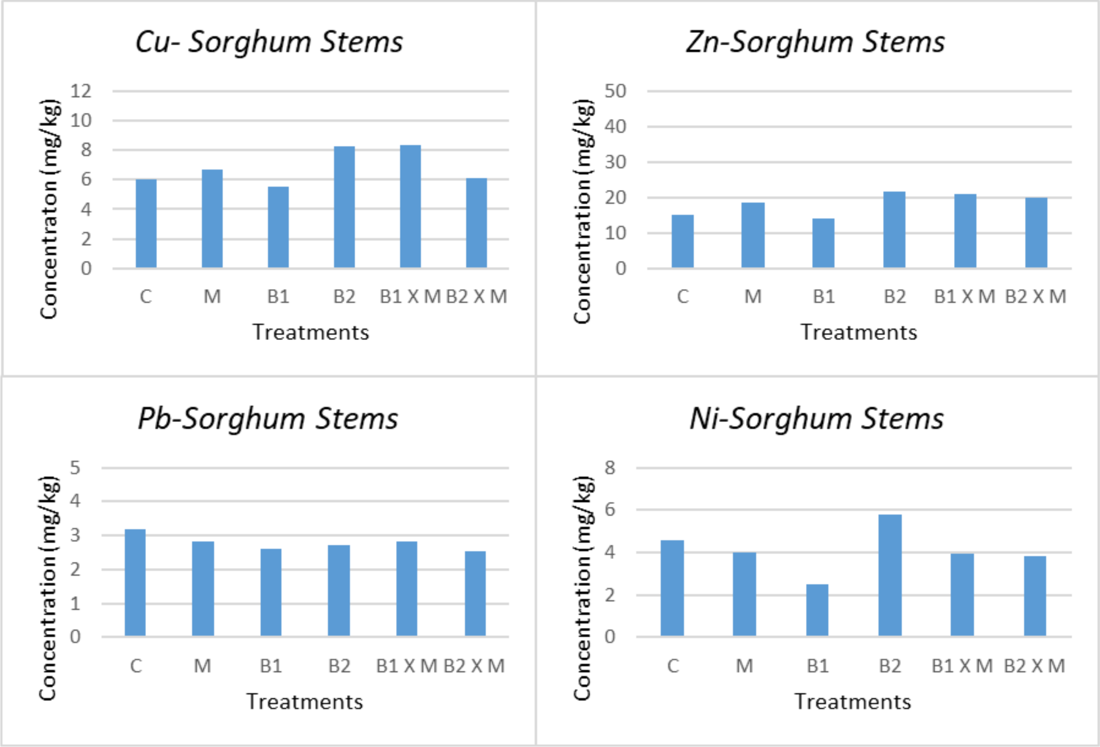

Figure 3.6. Heavy metal concentration (mg kg^{-1}) of sorghum stem biomass.

4. UNIBO, ITALY

4.1. Soil parameters

The total heavy metals concentration at the beginning and end of the pot trial was determined. No significant differences were found between both periods (Figure 4.1). Before planting the concentrations were 209 mg kg^{-1} (Ni), 160 mg kg^{-1} (Pb), 137 (Cu), 455 (Zn) and 9 mg kg^{-1} (Sn). The determination of the bioavailable fractions by extraction with $Ca(NO_3)_2$ at the beginning of the trial did not give any results above the detection limit (Table 4.1), therefore the analysis at the end of the trial was unnecessary.

Since no significant concentrations of heavy hydrocarbons (C>12) were detected while the PCB values were slightly above the legal threshold, it was decided to deepen such analysis directly in the field trial as the soil collection process to fill the pots may have affected the real concentration of organic compounds.

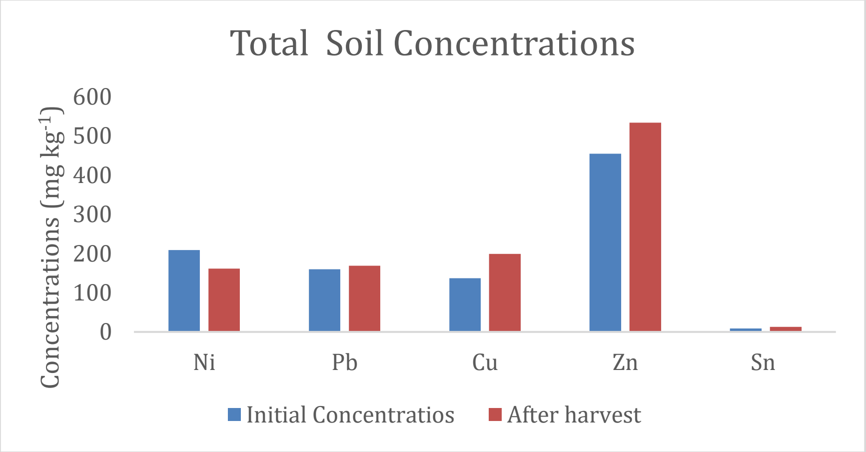

Figure 4.1. Total metal(loid) concentrations in the soil of the pots.

Table 4.1. Potentially bioavailable metal(loid) concentrations determined by calcium nitrate extraction in the soil at the beginning of the trial.

BIOAVAILABLE CONCENTRATIONS			
Parameter	**Result**	**U. M.**	**L.Q.**
Ni	< L.D.	mg kg^{-1} DM	0.5
Pb	< L.D.	mg kg^{-1} DM	0.5
Cu	< L.D.	mg kg^{-1} DM	0.5
Zn	< L.D.	mg kg^{-1} DM	0.5
Sn	< L.D.	mg kg^{-1} DM	0.5

4.2. Plant growth and metal accumulation

No significant differences in fresh biomass production between treatments were found in hemp and miscanthus (Figure 4.2). The treatments with the highest average value were MxB2 and B1 for hemp and B2 and MxB2 for miscanthus. On the other hand, sorghum treatments resulted in significant differences with lower productivity in C and M and higher values in MxB2, B2, MxB1, while B1 was found to be comparable with both the first and second groups.

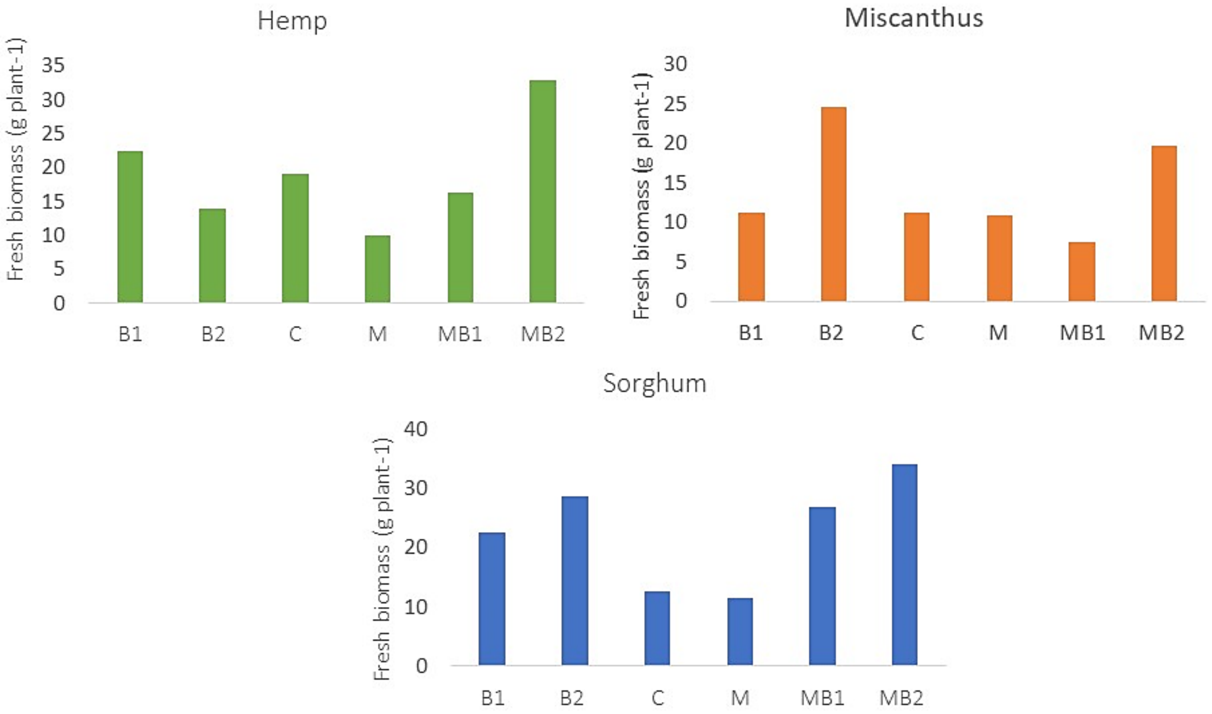

Figure 4.2. Fresh weight of hemp, miscanthus and sorghum.

As for the production of dry biomass, the most productive treatments were: Hemp: MB2, B1; Sorghum: MB2, MB1; Miscanthus: B2, MB2.

Regarding the height of the plants, only sorghum showed significant differences (Table 4.2), especially the MB1 treatment was found to be greater than C and M. The other treatments (MxB2, B2, B1) were comparable with both MxB1 and C, and M.

For hemp it can be noted that MxB2 was the treatment with a higher number of leaves comparable to other treatments, which showed no significant differences from each other (Table 4.2). Similar result was noted for miscanthus, with MxB2 treatment resulting in the largest number of leaves. More marked differences were found in sorghum: the treatments in which the radical biostimulant (B2, MxB2) was applied determined a greater number of leaves than the others.

No statistically significant differences were observed in the treatments of the three species for the concentration of Cu and Zn (Figure 4.3, 4.4, 4.5).

Table 4.2. Plant morphometric parameters at harvest.

		Cannabis sativa		
Treatment	Height	Leaves	Tillers	flowers
B1	104,7	170,0	0,0	68,7
B2	76,0	149,5	0,0	60,0
C	93,7	130,0	0,0	66,3
M	56,0	102,0	0,0	40,5
MB1	88,7	122,7	0,0	42,7
MB2	129,3	213,7	0,0	93,7
		Miscanthus x giganteus		
Treatment	Height	Leaves	Tillers	Flowers
B1	18,2	57,0	10,7	0
B2	17,2	63,3	15,0	0
C	22,5	40,7	7,7	0
M	22,3	44,7	7,7	0
MB1	16,8	49,0	10,7	0
MB2	23,3	70,0	14,0	0
		Sorghum bicolor		
Treatment	Height	Leaves	Tillers	Flowers
B1	28,7	10,0	0,0	0,0
B2	32,7	11,7	0,0	0,0
C	24,5	8,0	0,0	0,0
M	23,7	9,0	0,0	0,0
MB1	44,8	9,7	0,0	0,0
MB2	35,5	12,0	0,0	0,0

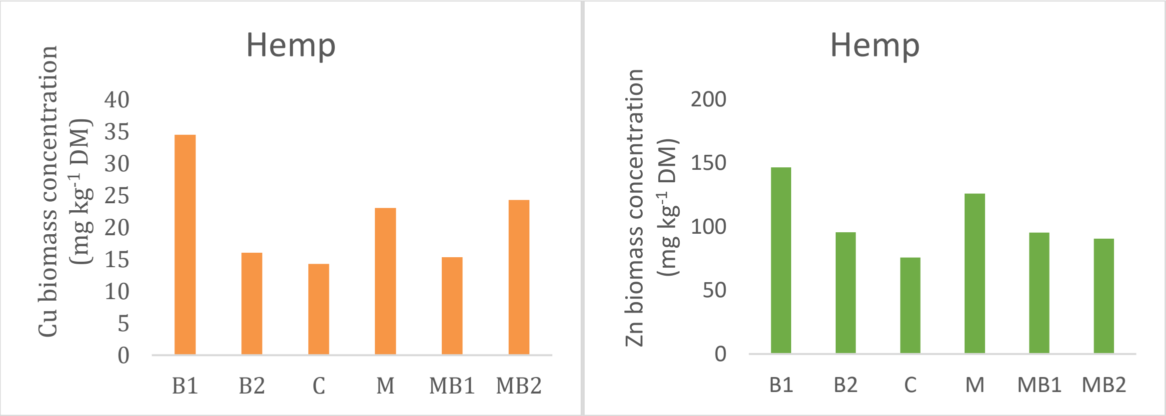

Figure 4.3. Cu and Zn concentrations (mg kg^{-1} dry weight) in hemp shoots at harvest.

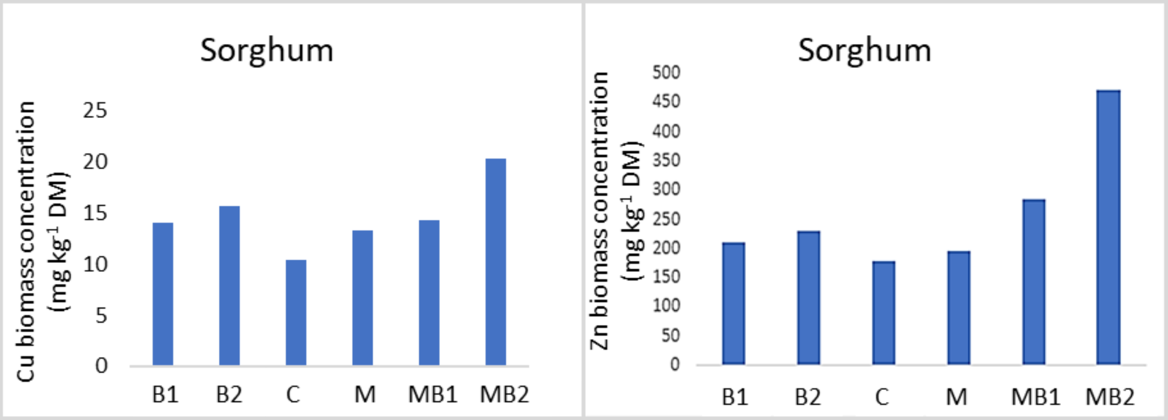

Figure 4.4. Cu and Zn concentrations (mg kg^{-1} dry weight) in sorghum shoots at harvest.

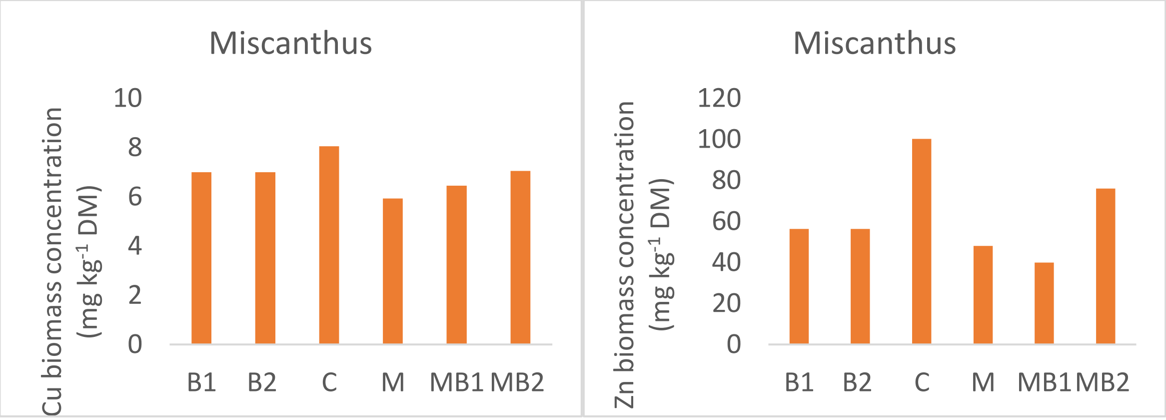

Figure 4.5. Cu and Zn concentrations (mg kg^{-1} dry weight) in miscanthus shoots at harvest

5. YNCREA, FRANCE

5.1. Soil parameters

The total concentrations of metals in the soil: Zn (average 955 mg kg^{-1}), Pb (average 536 mg kg^{-1}), Cu (average 25 mg kg^{-1}), and Cd (average 11 mg kg^{-1}) were higher that their pedogeological background in the North of France showing significant polymetallic pollution of the field site. The $Ca(NO_3)_2$-extractable forms of metals were relatively low compared to the total soil concentrations and their values were on average (in mg kg^{-1}): 4.02 for Zn, 5.13 for Pb, 1.65 for Cu and 0.71 for Cd.

At the end of the experiment, $Ca(NO_3)_2$-extractable Pb concentrations were below the detection limit (i.e. DL: 0.05 mg kg^{-1}) and no significant differences were observed between the treatments (Table 5.1). $Ca(NO_3)_2$-extractable Cd and Zn concentrations varied between < DL: 0.005 mg kg^{-1} and 0.17 mg kg^{-1} for Cd and 0.11 mg kg^{-1} and 1.55 mg kg^{-1} for Zn with significant highest concentrations in the B1 and B1xM treatments for both plant species. This suggests a remobilization of Cd and Zn in the soil following the application of the B1 treatments.

Table 5.1. $Ca(NO_3)_2$-extractable Cd, Pb and Zn concentrations in the soil of pots cultivated with *M. giganteus* or *C. sativa* at week 12.

Treatments	***Miscanthus x giganteus***			
	Cd (mg kg^{-1})	**Cu (mg kg^{-1})**	**Pb (mg kg^{-1})**	**Zn (mg kg^{-1})**
C	< DL: 0.005	0.10	< DL: 0.05	0.24
M	0.009	0.12	< DL: 0.05	0.15
B1	0.045	0.64	< DL: 0.05	0.8
B1xM	0.116	0.55	< DL: 0.05	1.55
B2	0.022	0.11	< DL: 0.05	0.2
B2xM	< DL: 0.005	0.11	< DL: 0.05	0.11
	Cannabis sativa			
	Cd (mg kg^{-1})	**Cu (mg kg^{-1})**	**Pb (mg kg^{-1})**	**Zn (mg kg^{-1})**
C	0.039	0.16	< DL: 0.05	0.34
M	0.015	0.11	< DL: 0.05	0.2
B1	0.178	0.42	< DL: 0.05	1.3
B1xM	0.101	0.37	< DL: 0.05	0.59
B2	0.022	0.09	< DL: 0.05	0.23
B2xM	< DL: 0.005	0.08	< DL: 0.05	0.13

Mean value for each treatment.
<DL :Treatments with values below the detection limit

5.2. Plant growth and metal accumulation

Before plant harvest, maximum shoot length, number of leaves, number of tillers (only for *Miscanthus)*, number of inflorescences (only for hemp), and toxicity symptoms (chlorosis, necrosis) were determined (Figure 5.1).

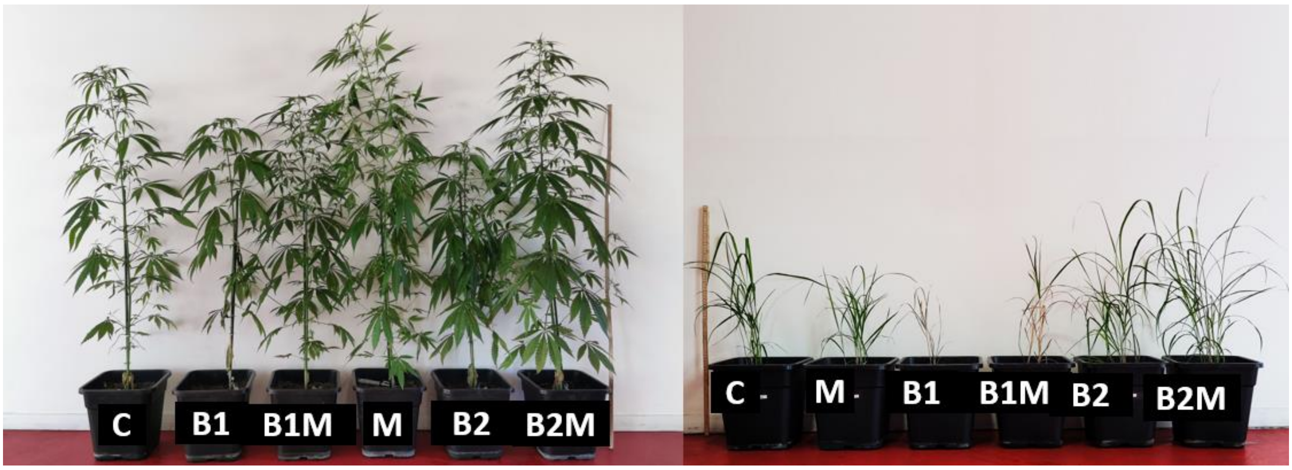

Figure 5.1. Hemp (left) and miscanthus (right) plants at the end of the experiment.

The shoot DW yield (in g DW plant^{-1}) for *M. giganteus* was reduced for the B1 treatment (1.1) as compared to the control one (3.0). No significant difference was found for the shoot DW yield of *M. giganteus* between the M, B1xM, B2, and B2xM treatment and the control (Figure 5.2). Shoot DW yield for *C. sativa* was increased (98 g DW plant^{-1}) for the B2xM treatment as compared to control (86 g DW plant^{-1}). The shoot DW yields of *C. sativa* for the M, B1, B1xM and B2 treatment did not significantly differ from that of the control.

Maximum shoot length and inflorescence numbers of hemp were not affected by the treatment while for *M. giganteus*, B1 and B1xM significantly decreased the maximum shoot length of plants (i.e. 20 and 23 cm) as compared to the control ones (i.e. 36 cm) (Table 5.2). Leaf numbers for *C. sativa* and *M. giganteus* shoot biomass of the B2 and B2xM plants did not significantly differ from the control ones but were higher than that for the B1.

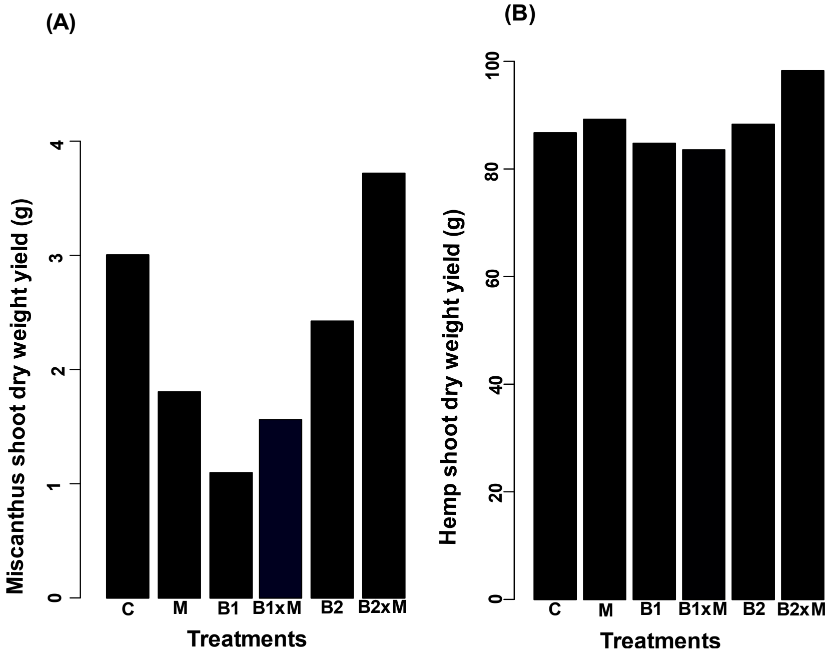

Figure 5.2. Shoot DW yields of *Miscanthus x giganteus* (A) and *Cannabis sativa* (B) after the 90-day growth period.

Table 5.2. Shoot functional traits of *M. giganteus* and *C. sativa* at week 12.

	Miscanthus x giganteus			***Cannabis sativa* L.**		
	Height (cm)	Leaves (N°)	Tillers (N°)	Height (cm)	Leaves (N°)	**Inflorescences** (N°)
Control	36	33	6.0	120	86	5.5
M	26	37	6.3	123	123	3.3
B1	20	15	3.3	107	73	9.0
B2	34	42	6.5	111	139	9.0
B1xM	23	20	4.5	94	64	3.0
B2xM	37	56	9.5	131	173	13.3

For the C treatment, the Cd, Pb and Zn shoot concentrations were 7.0, 7.0 and 115.0 mg kg^{-1} for miscanthus, and 2.1, 8.5 and 59.0 mg kg^{-1} for hemp, respectively (Table 5.3). Shoot Cd and Zn concentrations of *M. giganteus* were significantly lower for the B1 and B1xM treatments, while the B2, B2xM and C treatments had significantly higher shoot Cd and Zn concentrations (Table 5.3). Conversely, shoot Cd concentration for *C. sativa* L. was significantly higher for the B1 and B1xM treatments relative to the B2, B2xM and M ones. No significant differences were observed for shoot Pb concentrations of *M. giganteus* and *C. sativa.*

Table 5.3. Shoot ionome of *M. giganteus* and *C. sativa* at week 12.

Treatments	***Miscanthus x giganteus***			***Cannabis sativa***		
	Cd (mg kg^{-1})	**Pb (mg kg^{-1})**	**Zn (mg kg^{-1})**	**Cd (mg kg^{-1})**	**Pb (mg kg^{-1})**	**Zn (mg kg^{-1})**
C	7.0	7.0	115	2.1	8.5	59
M	5.7	7.3	100	0.9	10.9	54
B1	1.8	6.4	56	4.0	9.6	77
B1xM	2.1	6.1	48	3.7	8.8	60
B2	6.8	7.7	110	1.6	9.4	60
B2xM	8.4	7.3	120	0.9	8.5	51

6. IBFC, CHINA

Since growing industrial hemp is still illegal in China, hemp was replaced by kenaf because this crop has the same advantages as those of hemp.

6.1. Soil parameters

The soil used was contaminated with Cd and samples were analysed before and at the end of the experiment. The initial total Cd concentration of the soil was 1.96 mg kg^{-1}, while the 0.01 M $Ca(NO_3)_2$ extractable Cd concentration in the original soil and after finishing the pot experiment is under analysis.

6.2. Plant growth and metal accumulation

No significant difference in sorghum dry biomass between the six treatments was found (Figure 6.1). For kenaf, the addition of B1 treatment significantly increased the shoot biomass compared with the control plants (Figure 6.1). The addition of M and B2 singly could also elevate the biomass but no considerable changes were shown compared with other treatments.

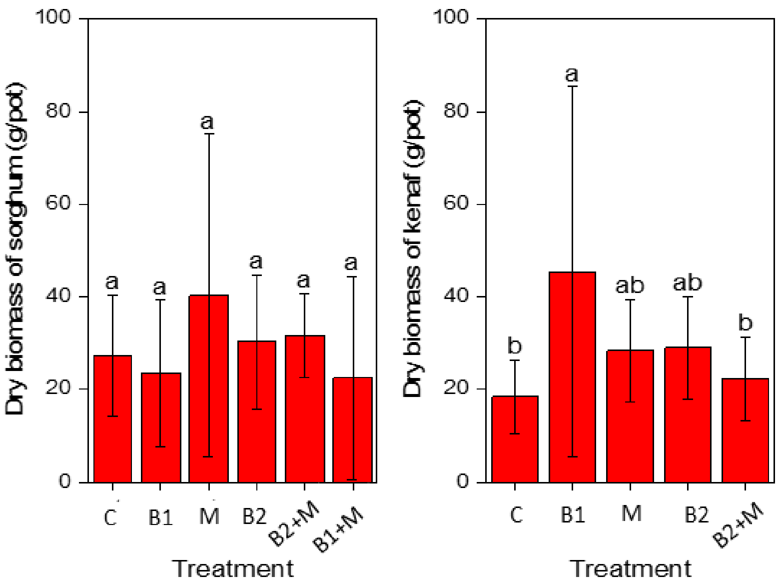

Figure 6.1. Dry biomass of sorghum and kenaf at different treatments.

The different treatments showed different effects on the plant height of sorghum and kenaf (Figure 6.2). Compared with the control soil, B1 treatment significantly decreased sorghum height, however, the other treatments showed no significant impacts on the plant height. Mycorrhiza and B2xM have the potential for increasing sorghum height. Compared with the control, all the test treatments could increase the plant height of kenaf to a different extent, however, no significant differences were found between the treatments.

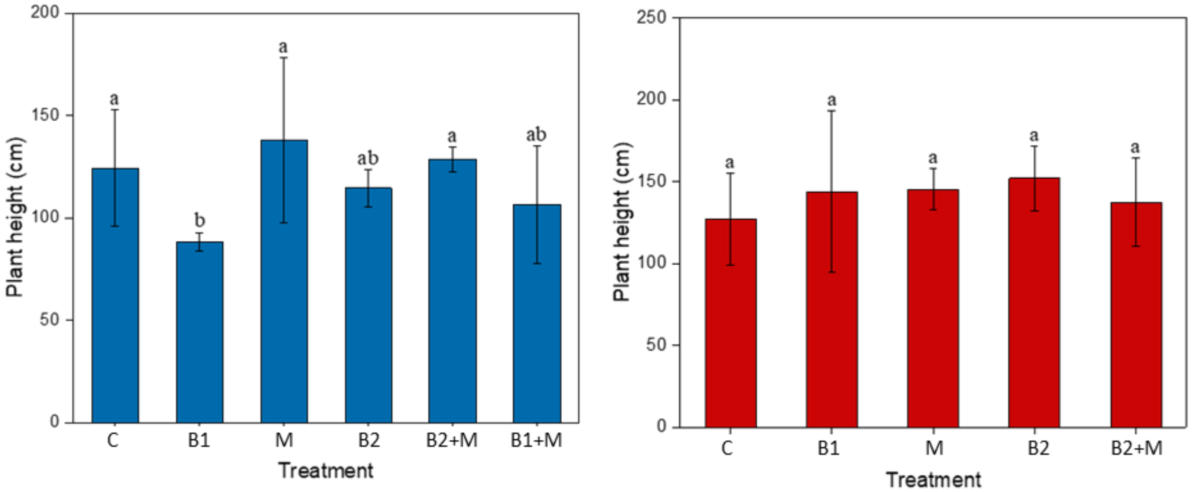

Figure 6.2. Plant height of sorghum (left) and kenaf (right) under different treatments.

Cd concentrations in plant samples are under analysis.

7. HUNAU, CHINA

Two plant species i.e., *Miscanthus* (wild species) and switchgrass (*Panicum virgatum* L. lowland Alamo ecotype) were tested in pot trials. For miscanthus rhizomes were dug out from the field and then directly planted to pots, whereas for switchgrass roots were used, collected from an already established switchgrass plantation (Figure 7.1). Roots were used because seed did not grow well.

Figure 7.1. Collection of plantation material for miscanthus (photo credit: Liu Qiao).

In case of miscanthus and switchgrass, manual thinning of the seedlings per pot was carried out in order to have one plant per pot. It was conducted when the plants had 2-4 leaves.

7.1. Soil parameters

The soil used for pot experiment was predominantly contaminated by Cd. The Cd content in case of $Ca(NO_3)_2$-extractable forms did not differ significantly across different treatments in the potted soil at the end of experiment. Contrary to this, the bioavailable Cd content differ significantly from the original soil values which indicates that all the treatments increased the bioavailability of Cd. The extractable forms of Cr ranged from 0 to 0.03, Cu varied from 0.09 to 0.27, whereas Pb ranged from 0.07 to 0.28, and Zn varied from 5.37 to 21.11 mg kg^{-1}. The mean values of extractable heavy metal content is presented in Table 7.1.

Table 7.1. Mean $Ca(NO_3)_2$-extractable heavy metal concentrations (mg kg^{-1}) in the potted soil for miscanthus and switchgrass under different treatments.

	Miscanthus					***Panicum virgatum* L.**				
	Cd	**Cr**	**Cu**	**Pb**	**Zn**	**Cd**	**Cr**	**Cu**	**Pb**	**Zn**
Control	0.75	0.01	0.09	0.14	7.35	0.73	0.02	0.09	0.14	6.98
M	0.58	0.01	0.12	0.12	5.37	0.58	0	0.10	0.07	7.57
B1	0.57	0.01	0.20	0.08	6.03	0.67	0.01	0.18	0.15	6.28
B2	0.70	0.02	0.12	0.15	8.36	0.84	0	0.13	0.17	10.35
B1xM	0.58	0.01	0.10	0.12	5.48	0.58	0.01	0.27	0.12	5.66
B2xM	0.62	0	0.13	0.10	21.12	0.70	0.01	0.10	0.28	8.83

7.2. Plant growth and metal accumulation

Pot trials were set up and during the whole period different morphological parameters such as plant height, number of leaves, tiller number, number of yellow and dead leaves were recorded. The results show that for miscanthus and switchgrass B2xM treatment has the highest number of leaves and tillers (Table 7.2). The number of yellow and dead leaves varied for each treatment.

Table 7.2. Plant morphological parameters at harvest.

	Miscanthus					***Panicum virgatum* L.**				
	Height (cm)	Leaves (Nº)	Tillers (Nº)	Yellow leaves (Nº)	Dead leaves (Nº)	Height (cm)	Leaves (Nº)	Tillers (Nº)	Yellow leaves (Nº)	Dead leaves (Nº)
Control	115	17	2	3	7	109.2	24	4	8	5
M	137	17	1	5	8	87	35	7	7	6
B1	137.2	25	2	6	9	75	16	3	4	1
B2	142	21	2	7	14	103	26	4	4	5
B1xM	135	18	1	2	11	116	14	2	7	2
B2xM	144.1	32	6	5	19	106.4	38	8	7	6

Fresh weight was recorded for both miscanthus and switchgrass at the harvest of pot trials (Figure 7.2). The results show that the application of mycorrhiza in combination with fulvic acid/humic acid has outperformed other treatments in terms of fresh biomass accumulation for both miscanthus and switchgrass.

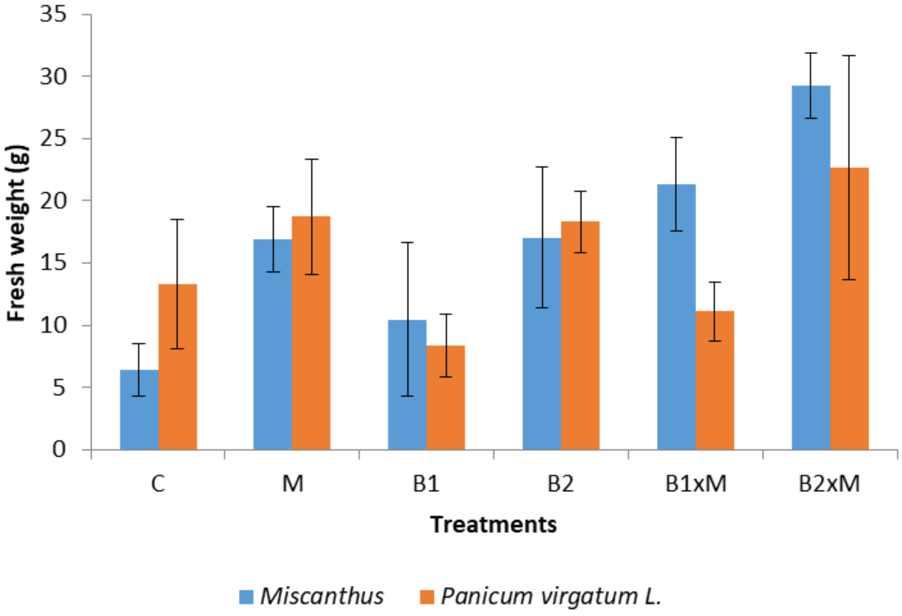

Figure 7.2. Fresh weight (g) of miscanthus and switchgrass, mean +/- SE.

In Figure 7.3 visual impressions are presented to describe that application of mycorrhiza in combination with fulvic acid/humic acid (B2xM) performed better than control treatment for both miscanthus and switchgrass.

Figure 7.3. Comparison of control with the best performing treatment for both miscanthus and switchgrass.

The comparison of the heavy metal content in aerial biomass of miscanthus and switchgrass under different treatments is presented in Figures 7.4 and 7.5. In miscanthus, Cd content was highest for B1 treatment followed by B2xM and B1xM, whereas in switchgrass highest Cd content was recorded for control followed by M, B1 and B2xM. In miscanthus, the Cu content was highest in B1 and B2xM treatments, whereas in switchgrass it was highest for B2xM.

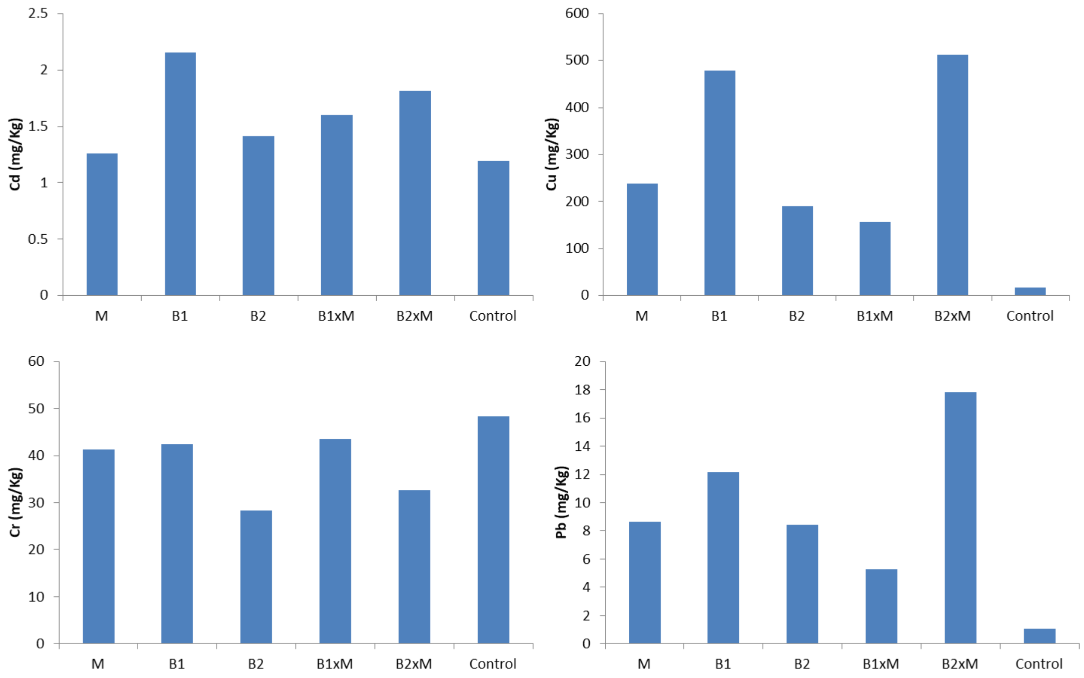

Figure 7.4. Heavy metal content in aerial biomass of miscanthus under different treatments at the end of pot experiment.

The Cr content in switchgrass was highest in aerial biomass for the pots treated with M, followed by B1xM and B2xM, whereas in miscanthus it was highest for control followed by B1xM, B1 and M. The Pb content was highest in aerial biomass of miscanthus and switchgrass in the B2xM treatment.

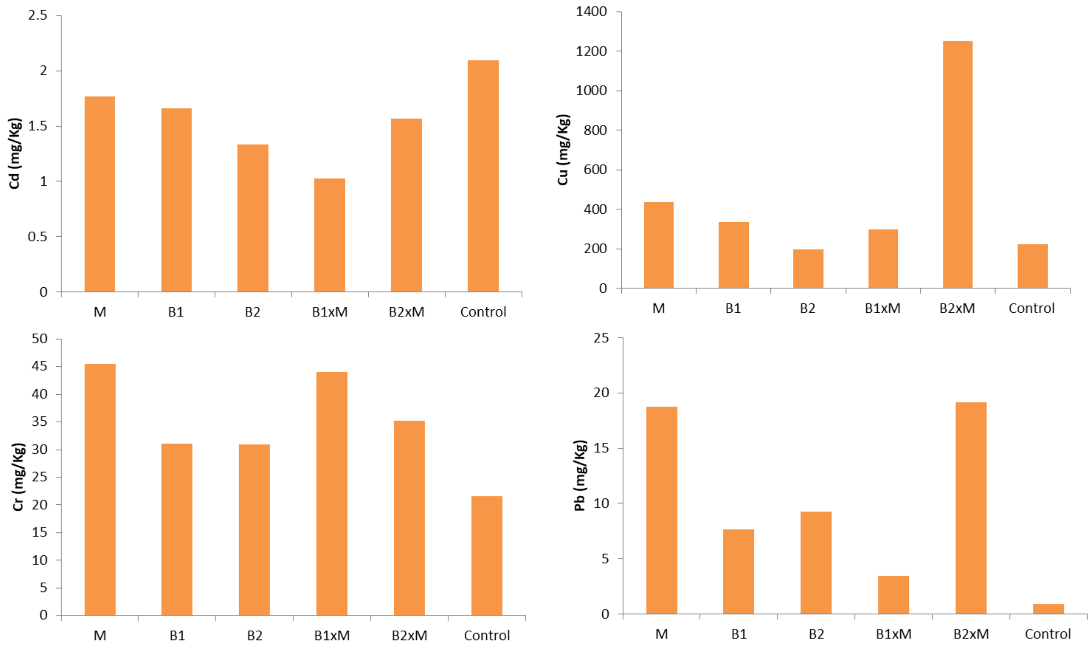

Figure 7.5. Heavy metal content in aerial biomass of switchgrass under different treatments at the end of pot experiment.

At the end of pot experiment, the harvested biomass of miscanthus and switchgrass was analysed to determine ash content for different treatments. The highest ash content for miscanthus and switchgrass was recorded for B2xM followed by B1xM. The results of ash content in miscanthus and switchgrass for all tested treatments are presented in Figure 7.6.

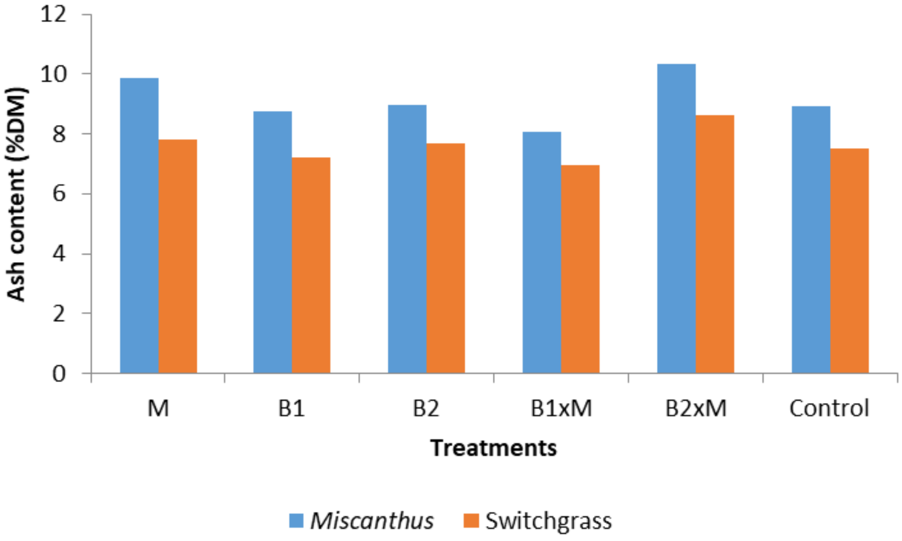

Figure 7.6. Ash content (%) in miscanthus and switchgrass for the treatments tested in pot trials.

KEY FINDINGS, SUMMARY and FURTHER STEPS

The 3-month pot experiments were performed by 7 partners (see Figure 1) in greenhouses from September 2021 to January 2022 (European partners) and from March to July 2022 (Chinese partners). The aim of these experiments was to check the effect of five treatments (two biostimulants and mycorrhiza applied singularly or in combinations) on the growth and metal accumulation capacity of four selected energy crops to find the best solutions to be tested subsequently in field conditions as foreseen within the GOLD project.

Four high-yielding lignocellulosic energy crops were tested in the pot experiments – two perennial grasses: miscanthus and switchgrass, and two herbaceous annuals: sorghum and industrial hemp (Table 2). However, the germination of seeds and surviving of seedlings of switchgrass was very weak as determined by all partners in the pot experiments. Therefore, during the technical meeting of WP1 it was decided, that the **switchgrass will be replaced by sorghum** to continue the research in the small scale field experiments. The seeds of sorghum (*Sorghum sudanense x bicolor*, var. BULLDOZER) were provided to all partners by UNIBO.

Six uniform treatments were applied by all partners in order to check the potential of biostimulants to improve plant growth and metal accumulation (optimization of crops for phytoremediation purposes):

1. **B1 (protein hydrolysate – Siapton)**
2. **B2 (fulvic/humic acids – Lonite)**
3. **M (mycorrhiza – Symbivit)**
4. **B1 x M**
5. **B2 x M**
6. **Control (no treatment)**

Plant growth parameters and metal(loid) concentrations in the aerial biomass were determined as the most relevant features determining the plant usefulness for phytoextraction purposes under various biostimulant applications. The pot experiment showed no influence of any treatment on the metal(loid) phytoavailability in the soil (in general, no significant changes in the extractable metal(loid) concentrations in the pot soil at the end of the experiment were noted). The biomass quality characteristics (ash content, calorific value, etc.) are in progress.

Based on the collective results of plant growth parameters (biomass, plant height) and metal accumulation in the shoots obtained in the pot experiments by European partners, principal component analysis (PCA) was performed. PCA is a useful statistical technique for analysing large datasets containing a high number of features per observation, increasing the interpretability of data while preserving the maximum amount of information. The eigenvalues of the first (the main principal component) and second axes indicate the presence of two gradients, within which the samples are differentiated in terms of the analysed characteristics. Most partners used miscanthus followed by hemp, therefore the PCA analysis was limited to these two crops. Since the polluted soils used by each partner were highly variable, it was not possible to perform one PCA analysis combining the results of all partners for a given crop. Therefore, the result of PCA ordination for each partner separately is shown in Figure 8.

Figure 8. PCA diagrams for hemp and miscanthus grouping the samples in terms of plant biomass (FW – fresh weight), height, and metal accumulation (Cd, Pb, Zn, Ni and Cu) in different treatments (B1 - protein hydrolysate, B2 - fulvic/humic acids, M – mycorrhiza, B1M – B1 x M, B2M – B2 x M, C – Control) from different partners (P – Poland, F – France, G – Greece, I – Italy, CH - China).

Summarizing, the data presented by the partners and the PCA analysis showed that the best effect on biomass production and metal accumulation was achieved with the application of **B2xM**. Therefore, it was decided by all partners to use this common treatment for the field experiments. Application of other treatments in different plant species did not provide such evident results (Figure 8). Based on own results and experience, and supported by the PCA analysis, each partner had to choose another treatment for his field experiments. **The treatments that will be used by each partner for each plant crop tested in pilot scale-small field trials (Task 1.3) are summarised/presented in Table 2.**

Table 2. Treatments selected by each partner for field experiments for task 1.3.

Partner	**Plant species**	**Treatments selected for field trials**					
		B1	**B2**	**M**	**B1xM**	**B2xM**	**Control**
UMCS, Poland	miscanthus		**X**			**X**	**X**
	industrial hemp		**X**			**X**	**X**
	sorghum		**X**			**X**	**X**
AUA, Greece	miscanthus			**X**		**X**	**X**
	industrial hemp			**X**		**X**	**X**
	sorghum			**X**		**X**	**X**
CRES, Greece	miscanthus				**X**	**X**	**X**
	sorghum				**X**	**X**	**X**
	switchgrass				**X**	**X**	**X**
UNIBO, Italy	miscanthus		**X**			**X**	**X**
	industrial hemp	**X**				**X**	**X**
	sorghum				**X**	**X**	**X**
YNCREA, France	miscanthus		**X**			**X**	**X**
	industrial hemp		**X**			**X**	**X**
	sorghum		**X**			**X**	**X**
IBFC, China	industrial hemp → **kenaf**	**X**				**X**	**X**
	sorghum			**X**		**X**	**X**
HUNAU, China	miscanthus				**X**	**X**	**X**
	switchgrass				**X**	**X**	**X**

Optimisation of plant growth in the field conditions combined with enhanced metal(loid) accumulation in shoots and/or organic pollutants degradation will allow to produce high plant biomass for biofuel production on polluted lands (ensuring low ILUC) while contributing to their cleaning-up. Such an approach will bring tangible benefits in several aspects: economic (production of biofuel), environmental (management and remediation of contaminated sites) and social (protection of human health by reducing the spread of pollution and alternative use of polluted land that might otherwise be irresponsibly used for food or feed production).

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