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GOLD

Growing energy crops on contaminated land for biofuels and soil remediation

D1.4

Application of best performing phytoremediation practices on pilot small-scale field trials (1st deliverable month 36)

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1. Document history

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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
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HUNAN - Hunan Agricultural University, China
UDES - Université de Sherbrooke, Canada
IBFC - Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences, China
CTD – Society for Economic and Social Studies, Center for Technology and Development, India

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Executive Summary

Soil pollution with organic and inorganic compounds, due to global anthropogenic and geogenic activities, is a worldwide concern and the number of contaminated sites is increasing year by year. One of the objectives of GOLD project is to exploit contaminated lands by cultivating selected high-yielding lignocellulosic energy crops for feedstock and biofuel production 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 soils from these sites were used for the pot experiments whose results were shown in D1.2. The 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 two best performing treatments for each site were selected to be subsequently tested in pilot small-scale field trials, as part of task 1.3, aiming at optimising the growth of selected high-yielding lignocellulosic energy crops in order to increase their potential for phytoextraction and/or bioaugmentation of different pollutants. Here, the two selected phytoremediation practices were tested among the seven sites for two growing seasons to assess their phytoextraction potential, in terms of plant growth and metal(loid) accumulation. Overall, the application of treatments did not result in any significant effect in the crops; however, biomass dry weight yields for the studied crops were generally higher in year 2, as compared to year 1, which ultimately resulted in higher metal(loid) bioaccumulation/uptake rates by plants. Moreover, sorghum plants showed higher accumulation/uptake values of Cd and Zn, in the French and in the polish sites, as well as in the AUA Greek site in year 2, demonstrating its phytoextraction capacity and its adaptability to a wide range of edaphoclimatic conditions (from Meridional locations, in the Greek sites, to the northern latitudes of the French site). These results demonstrate the phytoremediation potential of sorghum, hemp and miscanthus for phytoremediation of contaminated sites, ultimately allowing 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. Current estimates indicate the presence of over 10 million major contaminated sites worldwide, with approximately a quarter of these situated in Europe (Mench et al., 2018). Notably, contamination by metal(loids) constitutes the majority, comprising over 37.0% of cases, followed by mineral oil at 33.7%, polycyclic aromatic hydrocarbons at 13.3%, and various other pollutants (European Environment Agency, 2014). Concerningly, analysis of EU agricultural land reveals that approximately 6% (equivalent to around 137,000 km^2) exceeds permissible limits for metal(loid) concentrations, necessitating remediation measures.

To date, conventional approaches to remediate severely contaminated soils primarily involve excavation and landfilling practices, commonly referred to as "dig and dump". Supplementary techniques such as pump and treat, soil washing, soil flushing, physical and chemical stabilization, and electro-kinetic methods have seen limited application. While certain methodologies offer rapid and efficient remediation, they are often hindered by drawbacks including exorbitant expenses, labour-intensive requirements, and potential for irreversible alterations to geomorphological features and soil properties. Additionally, these methods can lead to significant disruption of indigenous soil microflora and compromise the agricultural productivity of affected land.

Within the framework of the GOLD project, and in WP1 in particular, the efficiency of two remediation methodologies—phytoextraction and bioaugmentation—is being investigated and refined for wider implementation in contaminated regions. Phytoextraction is an *in situ* technique in which metal(loid)s 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). The growing attention towards lignocellulosic energy crops stems from the transition away from fossil fuels towards biofuels, reflecting initiatives aimed at mitigating climate change and tackling the challenges of global warming (European Commission, 2015). 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 organic xenobiotic compounds are degraded by microbial communities, including bacteria or mycorrhizal fungi, or transformed into less dangerous forms (Ma et al., 2022).

This project aims to optimize crop phytoremediation through innovative agronomic practices, specifically mycorrhiza fungi and biostimulants. Mycorrhiza fungi establish symbiotic relationships with plant roots, enhancing nutrient uptake, water acquisition, and resistance to environmental stresses. They promote plant growth, root development, and metal transfer from soil to roots, facilitating metal(loid) removal from soil. Additionally, mycorrhizal inoculation is anticipated to enhance organic pollutant degradation. Biostimulants such as protein hydrolysates and fulvic/humic acids further support plant productivity by modulating signaling pathways, promoting stress resistance, and enhancing nutrient assimilation and water use efficiency. These practices collectively augment phytoremediation potential, offering a comprehensive approach for remediation efforts.

The main objective of WP1 is to optimize selected high-yielding lignocellulosic energy crops for phytoremediation, targeting different classes of soil pollutants. The specific objectives are (i) to compare different phytoremediation practices on contaminated soils polluted with organic and inorganic pollutants when growing selected high-yielding lignocellulosic energy crops, with the aim of selecting the best performing practices for each partner (results shown in Deliverable D1.2); and (ii) to apply these best performing phytoremediation practices on pilot small scale field trials. The polluted sites used for the field trials are situated in two continents and 5 countries (7 partners), representing the main agroclimatic zones and farming systems, as described in detail in Deliverable D1.1 (Figure 1).

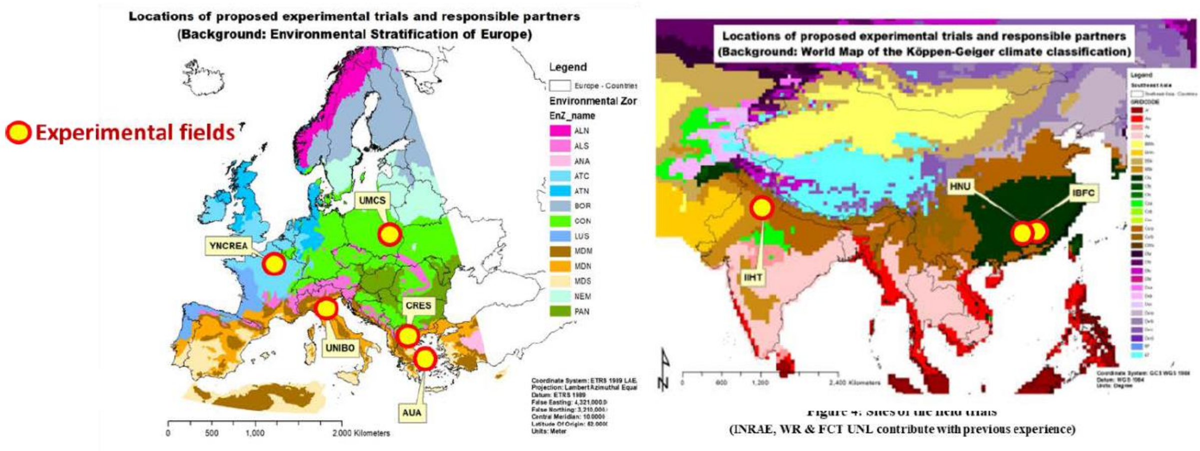


Figure 1. Location of the field trials involved in WP1 in Europe and Asia

The pilot trials of the perennial crops were established in the 1st year of the project implementation (establishment year of the perennial grasses), while the pilot small-scale fields for both annuals were established in the 2nd year and will be repeated in the 3rd year. At the end of each growing period, representative parts of each plot were harvested for biomass yield estimations. Metal(loid) concentrations in the shoots were also assessed, so estimations on metal(loid) bioaccumulation/uptake were also calculated based on the shoot DW yield and on the shoot metal(loid) concentrations obtained. The results from the pilot small scale field trials from both growing seasons are reported in this deliverable D1.4. Selected biomass feedstock per energy crop will feed the conversion processes in WP2. Moreover, all the results of this task will be given to WP3 for further process.

II. Materials & methods

Information regarding the site characterization, the climatic data, and the soil properties and contamination of each one of the partners' sites can be found in detail in Deliverable 1.1. Generally, the contamination of the sites is composed of several metal(loid)s, with some low concentrations of organic contaminants (pesticides and their metabolites) detected in the experimental fields of UMCS, CREES and UNIBO (Table 1).

Table 1. Contaminants exceeding legal thresholds in each partner's site.

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.

In the frame of GOLD, four high-yielding lignocellulosic energy crops were considered: two perennial crops and two annual crops:

- miscanthus: micro-propagated plants of *Miscanthus x giganteus* purchased from Rhizosfer© (France)
- switchgrass (*Panicum virgatum* L.): seeds of the variety KANLOW that CRES already acquired.
- sorghum (*Sorghum sudanense x bicolor* Moench) variety BULLDOZER, obtained from UNIBO, Italy.
- hemp (*Cannabis sativa* L.) variety FUTURA 75, obtained from CRES, Greece.

The biostimulants investigated in the frame of the project for treatment application were:

- Protein hydrolases (SIAPTON, Company: Agrology, Greece)
- Fulvic/humic acids (LONITE 80 SP, Company: Alba Milagro, Italy)
- Mycorrhiza (SYMBIVIT, Company: Symbiom, Czech Republic)

Therefore, the resulting treatments to be applied for each crop were:

1. B1: protein hydrolysate (Siapton®)

2. B2: fulvic/humic acids (Lonite®)
3. M: mycorrhizal inoculum (Symbivit®)
4. B1xM
5. B2xM
6. Control: no treatment applied

Each partner carried out a pot experiment for three of the above-mentioned crops, depending on the climatic zone corresponding to the field trial. All the six treatments were tested. Based on the results, the best two performing treatments were selected to be applied in the field trial, as reported in the Deliverable 1.2 (Table 2).

Table 2. Selection of treatments per partner for the field experiment, based on the pot experiment results (Deliverable 1.2).

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

The three different crops were tested over the period of two years in combination with three treatments. For this, all partners used the same plant material and products for treatment application, and the same protocols that had been agreed through technical meetings.

As indicated in the project proposal, the field trial experiments lasted two growing seasons, 2022 and 2023. For this, some preliminary operations were carried out in order to prepare the field trials for the experimental design. These preliminary operations included ploughing, chemical or mechanical weeding, and inorganic and/or organic fertilization, prior to each growing season. In the case of some sites, some extraordinary operations were carried out before the very first growing season, such as excavation, stripping and harrowing, and soil refinement (Figure 2).

Figure 2. Establishment of the field trials.

The experimental layout was a completely randomized experimental design with three replications (Figure 3), the size of each trial being approximately 0.2 ha per crop (100 m^2 each plot). Each partner carried out pilot trials for three energy crops, apart from the Chinese partners HUNAN and IBFC that worked with two crops each. Therefore, for the establishment of the field trials, plots of 81 m^2 were created (9 x 9 m), except for the UNIBO site, where plots had a surface of 10 m^2 (2.5 x 4m) due to the Chiarini site's constraints. For each crop, plots were created to accommodate the two selected treatments (plus the untreated control) with three replicates, accounting for a total of 9 plots. The type and quantity of fertilization was adjusted to the needs of each site separately, as well as the frequency and dose of irrigation (if needed).

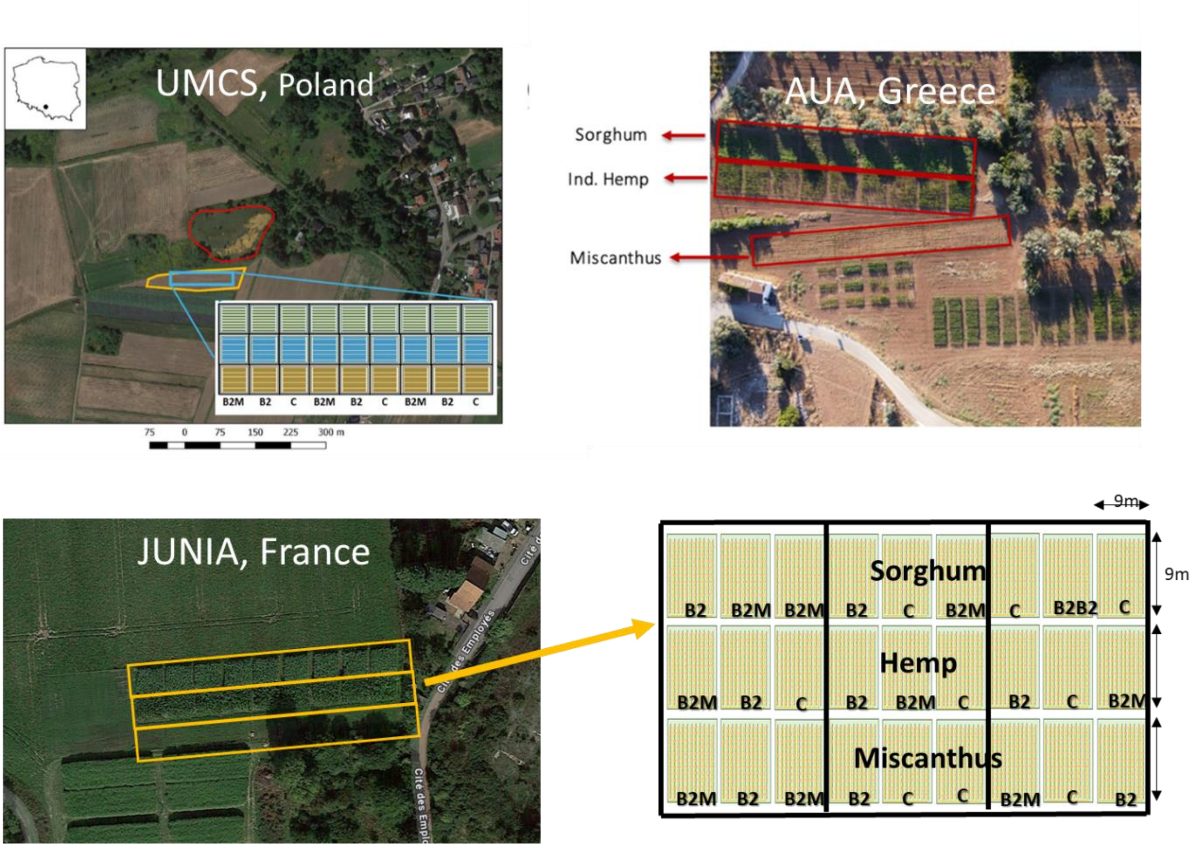


Figure 3. View of the experimental design in sites UMCS Poland, AUA Greece and JUNIA, France.

<u>Sowing and planting protocols</u>

For the establishment of the field trials in the first growing season, sowing of hemp and sorghum was done at 5 cm deep manually, either by hand or using a manual seeder, in May 2022 (Table 3). Seeds were placed 5 cm deep along each one of the 17 rows separated by 50 cm. For hemp, a distance of 8-10 cm between plants was left, making up a plant density of 1360-1700 plants per plot. For sorghum, a distance of 12-15 cm was maintained between plants, making up a density of 910-1130 plants per plot. In each plot, the outer perimeter of 50 cm remained unplanted.

Table 3. Dates for the sowing or planting and harvesting of the selected energy crops for both growing seasons.

ENERGY CROPS	DATES			
	2022		2023	
	Sow/transplant	Harvest	Sow/emerge	Harvest
AUA				
Hemp	23 May	10 October	20 April	21 October
Sorghum	23 May	26 October	20 April	21 November
Miscanthus	18 May		16 March (emerge)	13 December
CRES				
Sorghum	3 May	6 October	6 May	24 October
Miscanthus	10 May	15 December	Early April (emerge)	7 December
Switchgrass	3 June	2 December	2nd half of March (emerge)	6 December
UMCS				
Hemp	28 May	24 September	27 May	29 September
Sorghum	29 May	24 September	27 May	29 September
Miscanthus	28 May		Mid April (emerge)	29 September
UNIBO				
Hemp	10 May	10 August	12 April	26 July
Sorghum	19 May	7 October	5 May	11 October
Miscanthus	18 May	31 January	24 March (emerge)	29 November
JUNIA				
Hemp	4 May	29 September	15 May	28 September
Sorghum	8 May	4 October	25 April	3 October
Miscanthus	2 May	-	2 May (replanting)	-
IBFC				
Sorghum	22 April (pot experiment)	22 October	12 July	23 November
Kenaf	22 April (pot experiment)	22 October	12 July	23 November

For the second growing season (year 2), some preliminary operations were no longer needed which, along with the acquired experience of the previous year, allowed to certain adaptation in the sowing period in each site, ranging between April and May 2023. At the IBFC site, the establishment of the field trials for the first growing season was in 2023, taking place in July to adapt to the prevailing climate.

Miscanthus was planted also in May 2022. As a perennial crop, in 2023 no replanting was needed and it emerged naturally between the months of March and April. Rhizomes were planted along each one of the 11 rows separated one another by 70 cm. A distance of 70 cm was kept between plants, reaching a density of 121 plants per plot.

Treatment application protocols

The arbuscular **mycorrhizal fungi** were applied using a commercially available inoculum known as SYMBIVIT from Symbiom Ltd., located in Sazava 170, Czech Republic, Europe. This inoculum consists of reproductive particles from five distinct species of beneficial Arbuscular Mycorrhizal Fungi: *Rhizophagus irregularis*, *Funneliformis geosporum* BEG199, *Funneliformis mosseae*, *Claroideoglomus lamellosum* and *Septoglomus deserticola*. The inoculum mix is in granular form. The minimum number of spores was 200 per g (Figure 4).

Figure 4. Application of treatments (from left to right, from top to bottom) in UNIBO Italy (mycorrhiza, Siapton and Lonite application), AUA Greece (Lonite application), and JUNIA France (mycorrhiza and Lonite application).

The application of mycorrhiza occurred after initial field preparation steps, including tillage, fertilization, and plot separation. In the case of industrial hemp and sorghum, furrows were created along the each one of the planting rows. Along the entire length of each furrow, mycorrhiza inoculum was incorporated in a thin line, at an estimated dose of 20 g per meter. Subsequently, seeds were placed on the mycorrhiza, and the furrows were filled with soil. For miscanthus, a slightly different approach was taken: small dimples were created, and 10 g of mycorrhizal inoculum was applied within these dimples. Rhizomes of miscanthus were then positioned on top of the mycorrhiza, and the dimples were filled with soil.

Humic/ fulvic acids were applied using a commercially available product known as LONITE 80 SP from Milagro, located in Via Filippo Corridoni 19, Milan, Italy, Europe. LONITE 80 SP represents a novel and innovative activator composed of Leonardite humic extract, distinguished by its elevated levels of humic and fulvic acids. The humic extracts present in LONITE 80 SP contribute to enhancing various aspects, including the efficiency of nutrient absorption, soil structure, plant metabolism, and biochemical processes in soil flora.

Lonite application was implemented twice throughout the cultivation period (Figure 4). The initial application took place when the plants exhibited 4-6 true leaves, in the case of hemp, sorghum and switchgrass; while for miscanthus it was first applied when the plants were well established. The second application was done one month later. The process involved manual application directly into the rows of cultivation. Lonite comes as a powder so it was generally diluted in water and applied via irrigation, at a dose of 5 g of product per m^2 of soil. Subsequently, the plants were irrigated to facilitate the dilution of the product.

Protein hydrolysates were applied using the commercial product SIAPTON, a plant bio-activator/nutrient of natural origin, consisting of balanced free amino acids and short-chain peptides. This product activates specific plant genes, boosting anti-stress reactions for quicker recovery of plant growth. It improves yield levels (both quantity and quality) under all conditions, mitigates yield losses under stress conditions, and is quickly absorbed and translocated.

Siapton was applied twice per cultivation period. The first application for sorghum and hemp was done when the plants had 4-7 leaves, and for miscanthus and switchgrass, it was done 15-20 days after transplantation or when the plants had adequate aerial biomass. The second application was made 15 days after the first application for all crops. It was sprayed on the plant foliage at a dose of 250 cm^3 per 100 liters of H_2O.

<u>Havesting</u>

Harvesting was carried out for both growing seasons during the peak flowering period, when cellulose and hemicellulose components are maximized (Figure 5). For Miscanthus, the harvesting corresponding to year 1 was done in winter, by the end of January, and in year 2 when the crop started the senescence phase in autumn, between October and December. This difference was due to the need to allow during the first biological cycle the complete translocation of nutrients from the aboveground biomass to the root system. This agronomic practice is considered fundamental in order to complete a correct establishment of the perennial crop and allow a vigorous re-sprouting in the following year so to enhance productivity in the first year after establishment.

At the time of harvesting, the following parameters were measured:

- Plant height and stem diameter (in some sites)
- Yield (in a dry matter bases)
- Metal(loid)s concentrations in shoots
- Metal(loid) uptakes or bioaccumulation, i.e. removal of contaminants from the soil
  The term bioaccumulation or uptake of metal(loid)s in the above-ground parts of crops refers to the ability of each crop to remove in the harvested shoots the soil contaminants, providing an indication of the contaminant amounts that can be removed per year. This characteristic depends on various factors, including the type of crop, soil composition, metal(loid) bioavailability, environmental

conditions, and cultivation practices. For its determination, apart from considering the total amount of each metal(loid) accumulated in the above-ground parts of crops per hectare, the DW yield produced per crop is also taken into consideration. Within the frame of GOLD project and for the case study of Lavrion, the uptake potential of the crops per year is presented below.

Figure 5. Harvesting campaign.

III. Results

1. UMCS, Poland

The field experiment, both in 2022 and 2023, began in the last week of May due to frosts occurring in Poland until mid-May. The plants were harvested at the end of September. The plants did not show any signs of phytotoxicity during the whole growth season. Sorghum and miscanthus bloomed, while hemp showed the first symptoms of aging at harvest – plants shed their leaves, which probably resulted in a reduction in biomass (Figure 1.1).

Sorghum sudanense x bicolor variety *Bulldozer* | *Cannabis sativa* L. | *Miscanthus x giganteus*

Figure 1.1. Sorghum, hemp, and miscanthus in the field trials at the time of harvest in 2023 (29.09.2023).

Of all three plant species tested, sorghum was characterised by the highest shoot production ranging from 15 to 21 ton of dry weight (DW) per ha (Fig. 1.2A). Application of humic substances (B2) and humic substances combined with mycorrhiza (B2xM) resulted in higher biomass of the above-ground parts of sorghum (Figure 1.2 A). The mean dry biomass of hemp ranged from 6.5 to 7.5 ton ha^{-1} and increased after application of B2 in comparison to non-treated control plants (Fig. 1.2B). The growth (shoot biomass and height) of miscanthus was only determined in the second growth season (2023) since the first one was to establish its plantation. Its biomass ranged from 5.4 to 6.1 ton DW ha^{-1} and was not affected by any treatment (Fig. 1.2C).

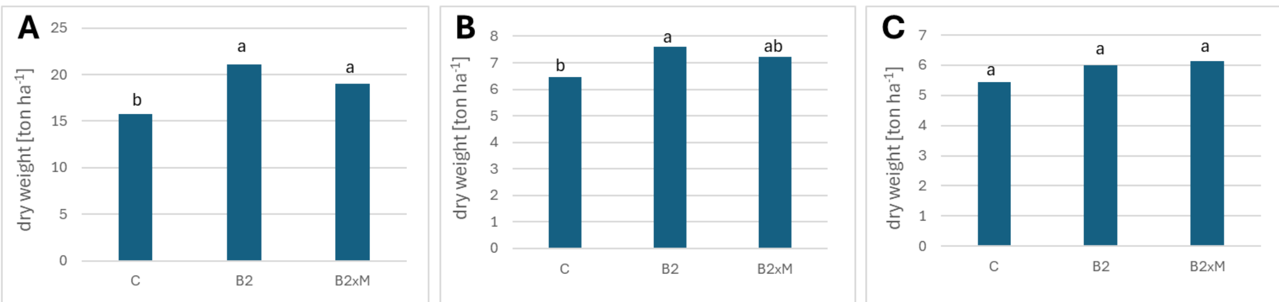


Figure 1.2. Shoot DW yield (t DW ha^{-1}) of sorghum (A), hemp (B), and miscanthus (C) per treatment. Values are mean from 2022 and 2023, in case of miscanthus the data of the second growth season are presented only.

There were no differences in the height (maximum shoot length) of plants grown in different conditions in 2022 and 2023 (Figure 1.3). Sorghum was the highest, up to 3.9 m in average, corresponding to the highest biomass production.

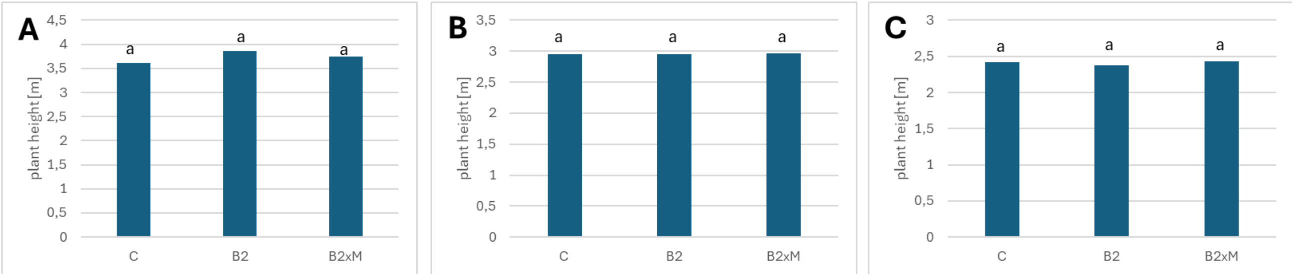


Figure 1.3. Plant height (m) of sorghum (A), hemp (B), and miscanthus (C) per treatment. Values are mean from 2022 and 2023, in case of miscanthus the data of the second growth season are presented only.

The concentration of Zn, Cd and Pb in the shoots did not differ among the treatments in any of the cultivated species (Fig. 1.4-1.6). In sorghum, the mean shoot Zn concentrations ranged from 210 to 219 mg kg^{-1} DW and were almost two times higher than these found in hemp and miscanthus. Also, the shoot Cd was the most efficient in sorghum (appr. 12 mg kg^{-1} DW) as compared to miscanthus (appr. 1.5 mg kg^{-1} DW) and hemp (appr. 1 mg kg^{-1} DW). However, hemp was characterised by the highest shoot Pb concentrations (appr. three-fold higher than in the other crops).

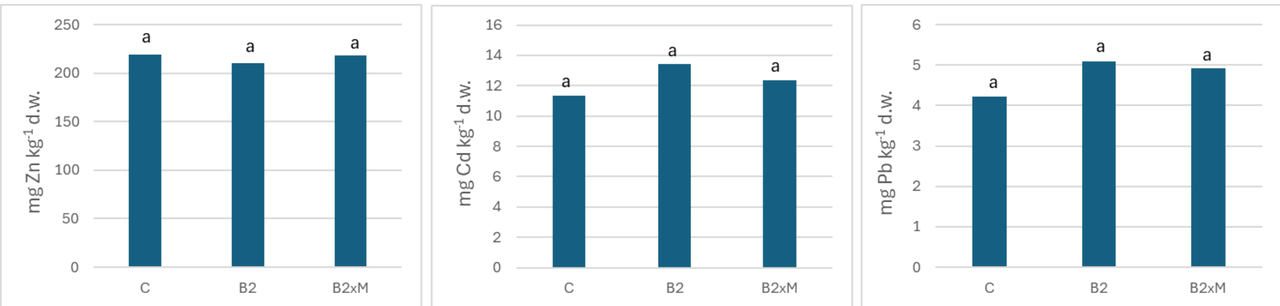


Figure 1.4. Metal concentration (mg kg^{-1} DW) in the shoots of sorghum. Values are mean from 2022 and 2023.

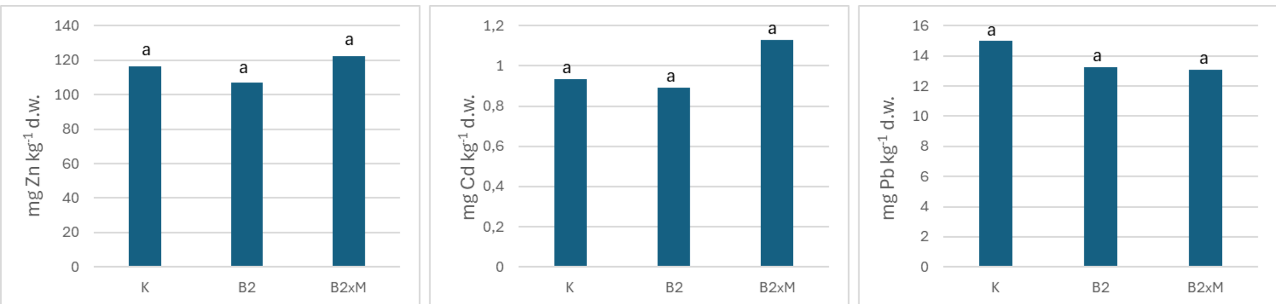


Figure 1.5. Metal concentration (mg kg^{-1} DW) in the shoots of hemp. Values are mean from 2022 and 2023.

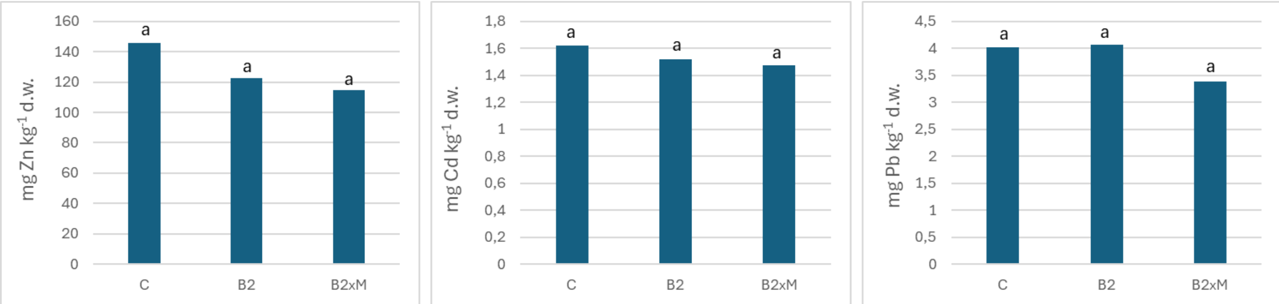


Figure 1.6. Metal concentration (mg kg^{-1} DW) in the shoots of miscanthus. Values are mean from 2023.

Based on the results of biomass production and metal concentrations in the shoots, metal uptake/removal from the soil was calculated for each crop showing their potential for phytoextraction of the contaminated soils under the experimental field conditions (Figures 1.7 – 1.9). Due to high variability in plant biomass and metal concentrations in the shoots of all tested crops between treatments, no significant effect of treatment application on metal removal from the soil was found in any case. The best crop for metal phytoextraction was sorghum. In this case an increasing trend, although not statistically significant, of metal removal was observed following B2 and B2xM application. Based on our results, harvesting the biomass of sorghum one could remove from the soil up to 4440 g of Zn, 280 g of Cd, and 107 g of Pb per ha (Fig. 1.7). Comparable level of Pb removal was also recorded for hemp; however, Zn and Cd removal from the soil was much less efficient – 5-fold less in case of Zn and 35-fold less in case of Cd (Fig. 1.8). Miscanthus was the least efficient crop in metal removal for all metals (Fig. 1.9).

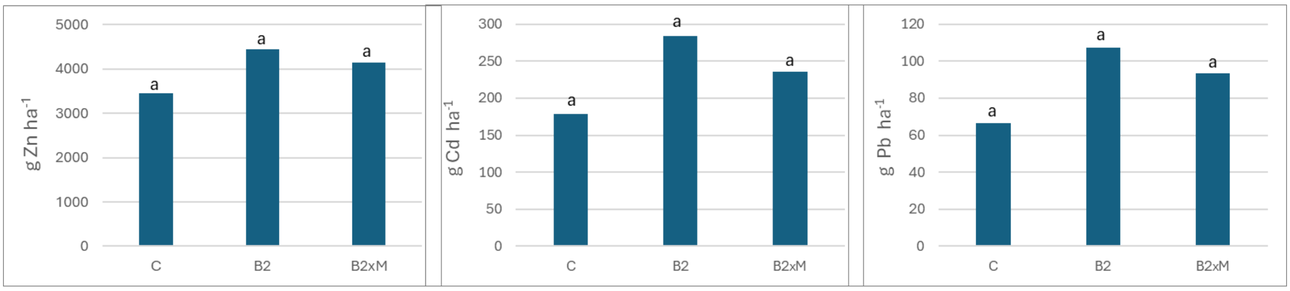


Figure 1.7. Shoot metal uptake (g ha^{-1}) per treatment in sorghum plots. Values are mean from 2022 and 2023.

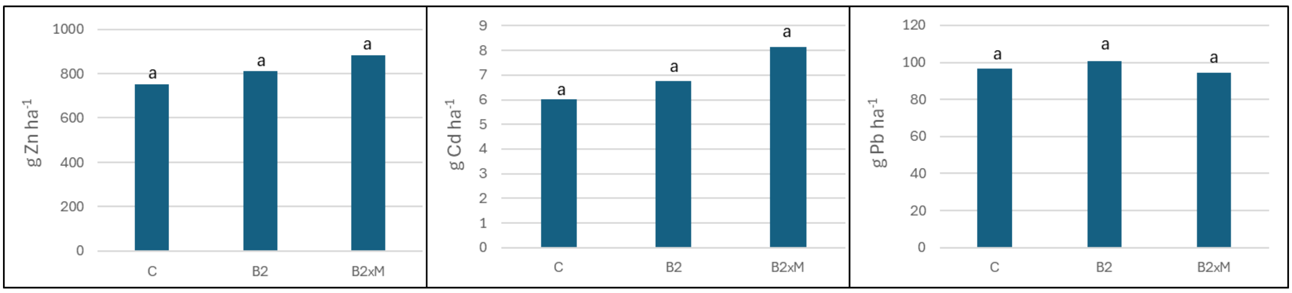


Figure 1.8. Shoot metal uptake (g ha^{-1}) per treatment in hemp plots. Values are mean from 2022 and 2023.

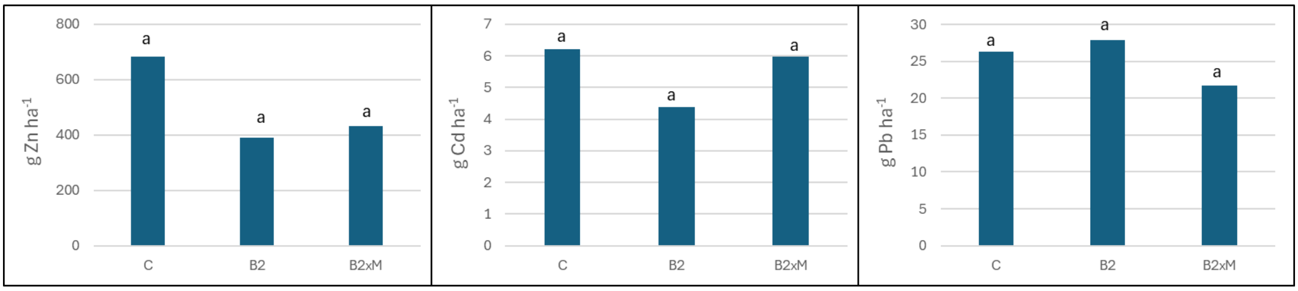


Figure 1.9. Shoot metal uptake (g ha^{-1}) per treatment in miscanthus plots. Values are mean from 2023.

2. AUA, Greece

Hemp, sorghum and miscanthus were well established for both years in the contaminated site of Lavrion (Figure 2.1), despite the high total soil metal(loid) concentrations. However, their growth and yields were reduced compared to the growth of the same crops and varieties in a non-contaminated site of an AUA field located 200 km NW of Lavrion. It is worth to note that in year 2, 2023, both hemp and sorghum showed significantly improved establishment and growth, producing taller and more vigorous plants, consequently leading to higher yields as well.

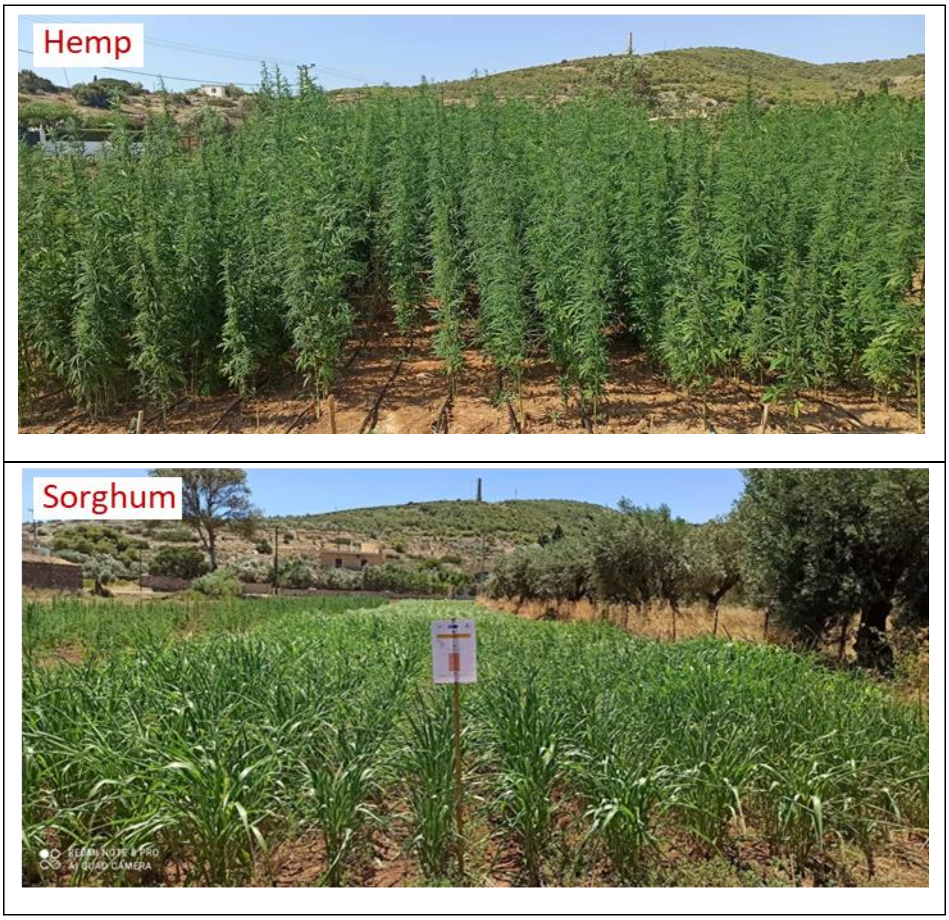


Figure 2.1. Hemp, sorghum and miscanthus plantations in the heavily contaminated site of Lavrion, Attika, Greece.

Plant height (m) and stem diameter (mm)

The results for plant height and stem diameter for the two years 2022 and 2023 are presented in Figures 2.2 and 2.3 respectively. Generally, year 2 was characterized by elevated mean values for the three measured parameters for both hemp and sorghum. For miscanthus, the year 2022 was the establishment year, and therefore, the measurements were not going to be representative of the plant growth. For this reason, data collection and analysis for this crop were conducted solely in 2023.

Figure 2.2. Average plant height (m) at harvest of Industrial Hemp (A), Sorghum (B) and Miscanthus (C) per treatment.

Figure 2.3. Average stem diameter (mm) at harvest of Industrial Hemp (**A**), Sorghum (**B**) and Miscanthus (**C**) per treatment.

Industrial hemp: the average height and stem diameter are presented in Figures 2.2 (A) and 2.3 (A) respectively. No significant differences were observed among treatments. The average plant height for 2022 was 0.99 m (mycorrhiza treatment), 0.93 m (B2xM treatment) and 0.92 m (control plants), while the corresponding values for 2023 were 1.39 m (M), 1.42 m (B2xM) and 1.43 m (control).

The mean diameters showed an increasing trend between the two studied years, although no significant differences were observed among treatments. In 2022, the M treatment presented the largest diameter compared to the control and B2xM treatments, with diameters ranging between 5.57-5.99 mm. In 2023, the B2xM treatment gave thicker shoots reaching up to 7.53 mm, surpassing both the control and M treatments, with diameter values up to 7.30 and 7.25 mm, respectively.

Sorghum: the plant height and stem diameter per year are presented in Figures 2.2 (B) and 2.3 (B). It is evident that in 2023, the plants displayed enhanced vigour, characterized by significantly increased height and thickness compared to 2022. In the first year, the plant height and stem diameter did not differ among treatments. However, in 2023, the control plants had a significantly greater height than the treated ones, reaching the 2.21m. The stem diameter of the mycorrhiza treated plants was significantly lower than the corresponding of control and B2xM plants.

Miscanthus: in 2022, which marked the first cultivation period, it was considered an establishment year, and consequently, no measurements were conducted. The results for height and stem diameter are presented in Figures 2.2 (C) and 2.3 (C) respectively. Treatments M and B2xM, had a positive impact on plant growth. All plants treated with B2xM exhibited a significant increase in height and diameter compared to control and mycorrhiza-treated plots. Specifically, plants treated with mycorrhiza showed an increase of 21.7% in height and 4.5% in diameter, while B2xM treated plants demonstrated a 23.9% increase in height and an 11.3% increase in shoot diameter compared to the control treatment.

Shoot dry weight (DW) yields (ton DW Ha^{-1})

Generally, the second year is characterized by greater mean values in the shoot DW yields of both hemp and sorghum (Figure 2.4).

Figure 2.4. Shoot DW yields (ton DW ha^{-1}) of Industrial Hemp (A), Sorghum (B) and Miscanthus (C) per treatment.

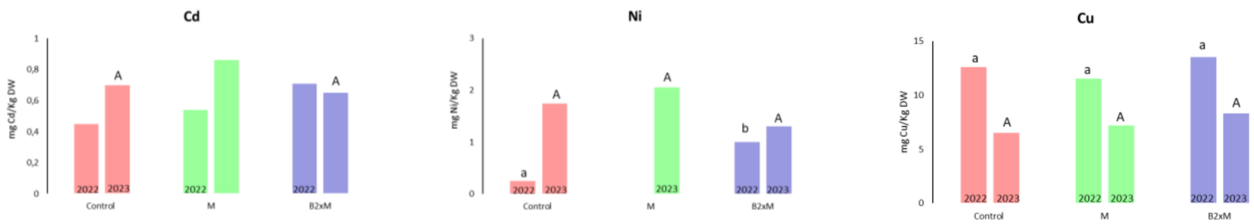
<u>Industrial hemp:</u> For both years the control plants gave highest biomass than the treated ones (Figure 2.4). However, the statistical analyses detected significant differences only for 2023, between control and M treatments. The mycorrhiza treatment had a moderate biomass, and it slightly increased from 2022 to 2023. The B2xM treatment, while having the lowest biomass in 2022, experienced a significant increase in biomass in 2023. Average biomass for control plots for 2022 and 2023 were 1.63 ton ha^{-1} and 2.78 ton ha^{-1}, respectively.

<u>Sorghum:</u> the plants displayed increased shoot DW yields under the B2xM treatment for both years when compared with control and mycorrhiza treated plots; however, no significant differences were observed (Figure 2.4 (B)). The shoot DW yield values for the B2xM treatment were up to 12.50 ton ha^{-1} for 2022 and 30.81 ton ha^{-1} for 2023 (an increase of 246%).

<u>Miscanthus:</u> no significant differences were observed among treatments (Figure 2.4 (C)). Notably, the highest shoot DW yield was found in the B2xM treated plots, reaching 5.1 ton ha^{-1}.

Metal(loid) concentrations in the above-ground plant parts

<u>Hemp:</u> the concentrations of metals and antimony (Cd, Ni, Cu, Pb, Zn, and Sb) in the above-ground biomass of this crop are presented in Figure 2.5. No significant differences in shoot metal(loid) concentrations were observed among treatments for both cultivation years, except for Ni in 2022. These results indicate that the applied treatments did not affect significantly the metal and Sb concentrations in the hemp shoots. Notably, the used variety Futura 75 demonstrated the ability to concentrate soil contaminants in the following order: Zn > Pb > Cu > Sb > Ni > Cd.



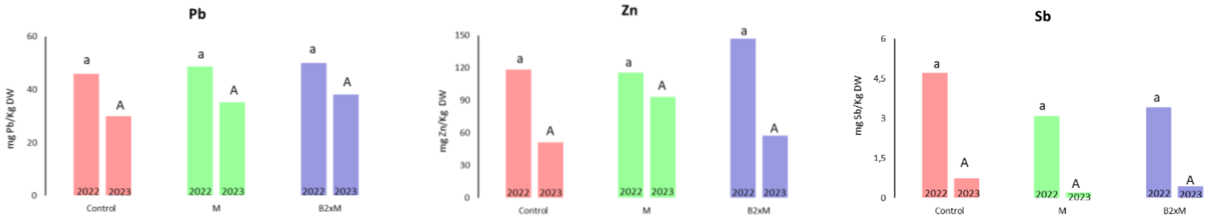


Figure 2.54. Metal(loid) concentrations in shoots (mg kg^{-1} plant DW) per treatment and per year in hemp.

<u>Sorghum:</u> In Figure 2.6 the concentrations of metals and Sb in sorghum shoots are presented. Significant differences in shoot metal(loid) concentrations were only evident for the year 2023, specifically for Cu and Zn. In year 1 (2022), sorghum treated with mycorrhiza fungi exhibited higher metal concentrations for Pb, Cu, and Zn in the order Zn > Pb > Cu, but differences were not significant. Ni was exclusively accumulated in the control plants of sorghum. Notably, the highest shoot Cd and Sb concentrations (numerically) were found in the B2xM plots. In year 2 (2023), higher shoot Cu and Zn concentrations were detected in the B2xM plots. Higher shoot Pb concentrations (albeit not statistically significant) were measured in the M plots, while Ni was below the detection limit for all tested treatments.

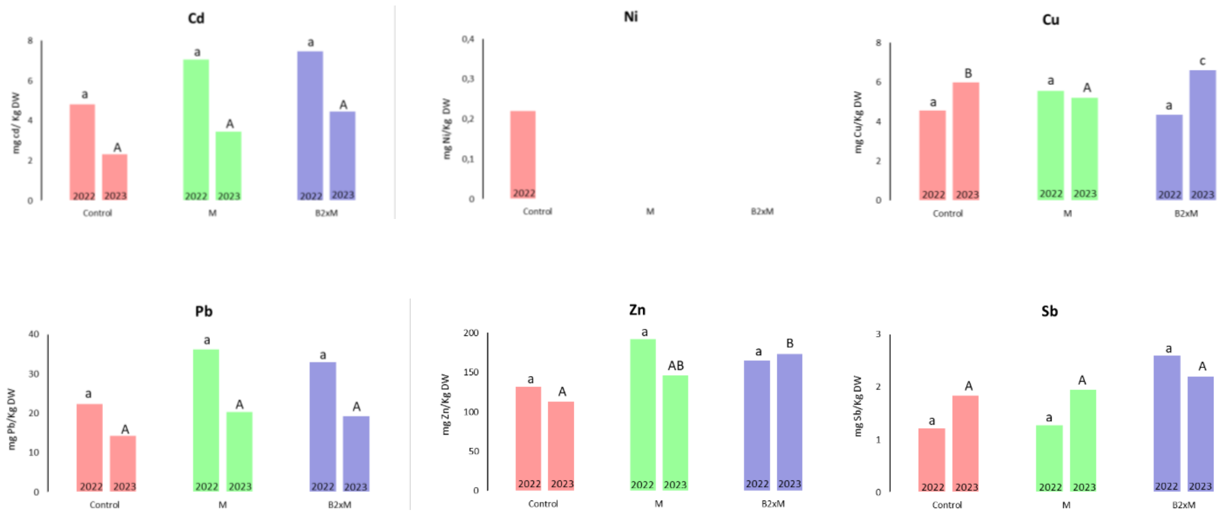


Figure 2.6. Metal(loid) concentrations in shoots (mg kg^{-1} plant DW) per treatment and per year in sorghum.

<u>Miscanthus:</u> The shoot metal(loid) concentrations of miscanthus plants are presented in Figure 2.15. Control plots exhibited higher values for Cd, Cu, and Sb, although no significant differences were observed among treatments except for Cu. The application of mycorrhiza fungi led to a significantly higher shoot Pb concentration, while the mycorrhiza fungi paired with humic/fulvic acid (B2xM) resulted numerically in higher Zn values, although no significant differences were found. Ni was below the detection limit of the ICP-OES. These results shows that the miscanthus variety Bulldozer demonstrated the ability to concentrate in its shoots soil contaminants in the following order: Zn > Pb > Cu > Sb > Cd > Ni.

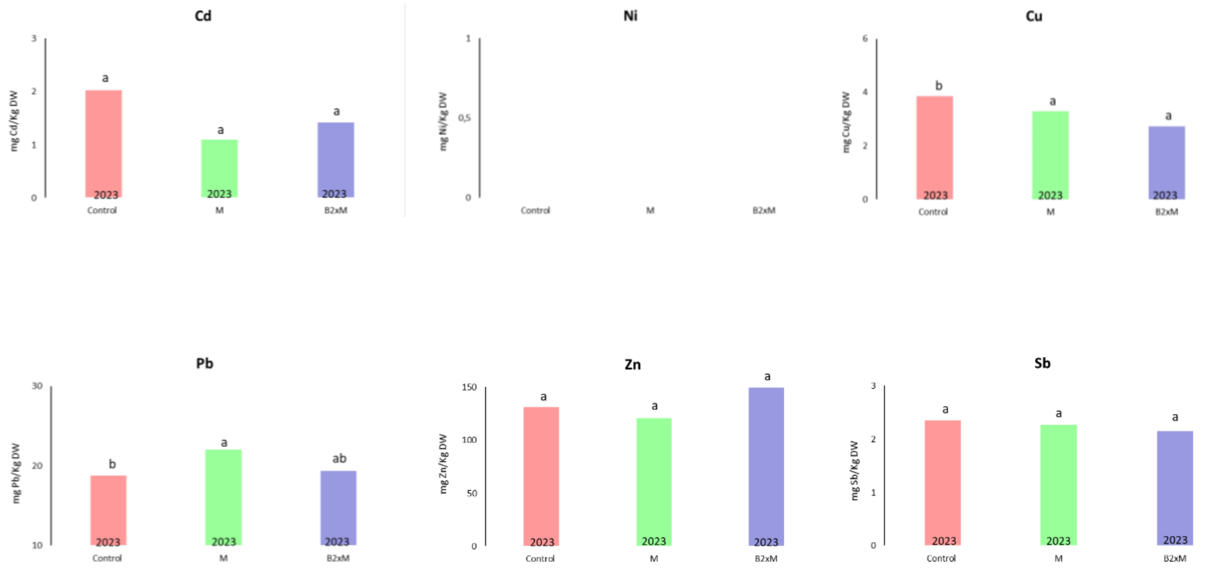


Figure 2.7. Metal(loid) concentrations in shoots (mg kg^{-1} plant DW) per treatment and per year in miscanthus.

Metal(loid) bioaccumulation/uptake in the shoots

Hemp: The bioaccumulation potential for industrial hemp was calculated and presented in Figure 2.8. Industrial hemp succeeded higher mean values, in control plots for all the studied soil contaminants. Shoot metal(loid) uptakes by hemp did not differ across the treatments for both cultivation years, except for Ni in 2022 in the control treatment compared to the B2xM one. These results indicate that hemp phytoextraction potential is not significantly affected by the applied treatments. The elemental removal from the soil by the harvested shoots follows the order: Zn > Pb > Cu > Sb > Ni > Cd.

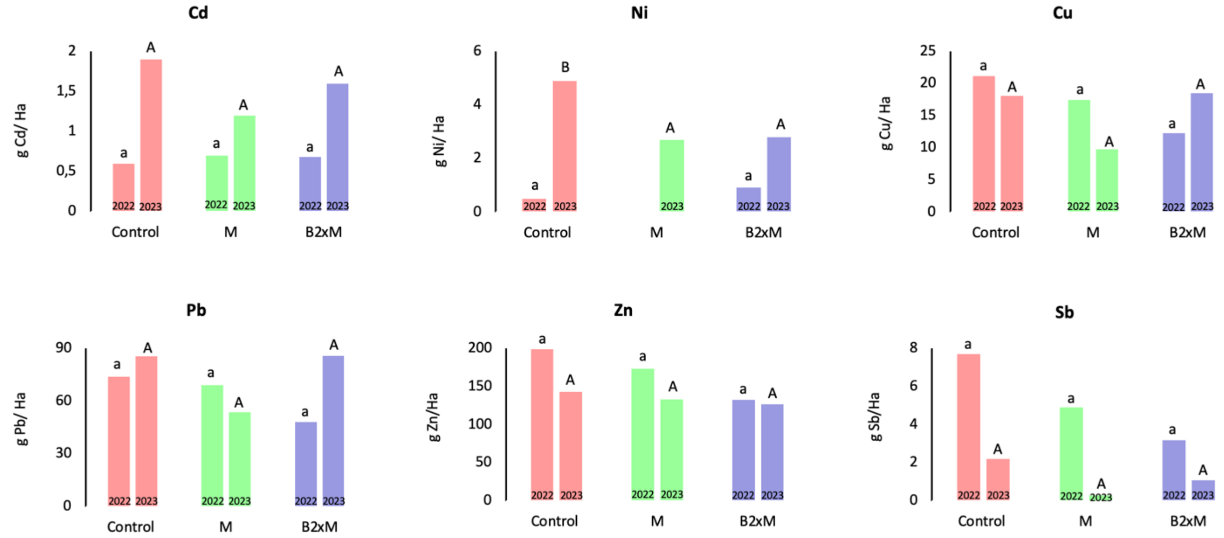


Figure 2.8. Shoot metal(loid) uptake (g Ha^{-1}) per treatment and per year in hemp plots.

Sorghum: Figure 2.9 illustrates the bioaccumulation capacity of sorghum plants. Sorghum can uptake significant Cd, Cu, Pb, Zn, and Sb amounts, except Ni in the M and B2xM treatments. In 2022, sorghum's accumulation potential increased with mycorrhiza treatment for Cu and Zn, while B2xM treatment resulted in higher removal from soil of Cd, Pb and Sb. However, no statistically significant differences were observed among treatments. In 2023, significantly higher results were noted for all elements with the B2xM treatment, except for Ni. The elemental removal from the soil follows the order: Zn > Pb > Cu > Cd > Sb > Ni.

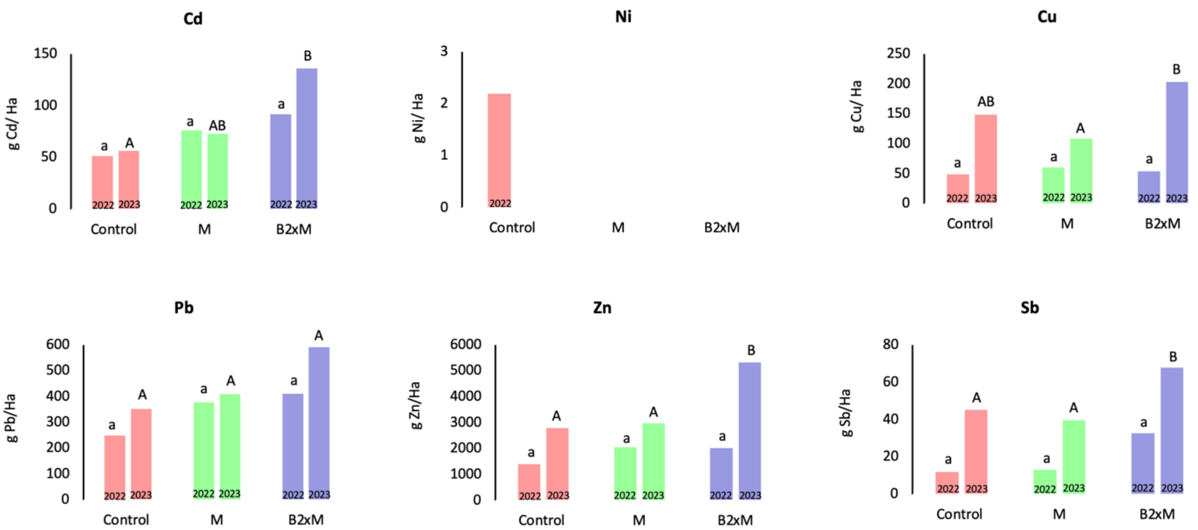


Figure 2.9. Shoot metal(loid) uptake (g Ha^{-1}) per treatment and per year in sorghum plots.

<u>Miscanthus:</u> The bioaccumulation capacity of miscanthus is illustrated in Figure 2.10. Generally, the application of the M and B2xM treatments tends to enhance the uptake potential of miscanthus for most of the metals and Sb, although no significant differences were observed among treatments. Cadmium accumulation was higher in control plants, while miscanthus acted as an excluder for Ni. Specifically, miscanthus demonstrated the ability to accumulate metal(loid)s in shoots in the order: Zn > Pb > Cu > Sb > Cd.

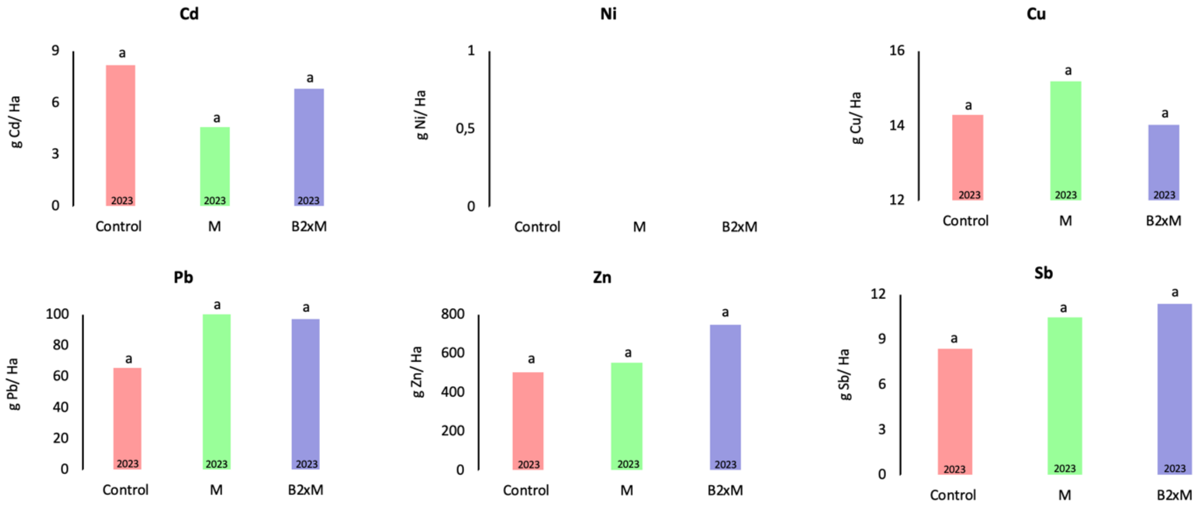


Figure 2.50. Shoot metal(loid) uptake (g Ha^{-1}) per treatment and per year in miscanthus plots.

3. CRES, Greece

The three studied crops had a good establishment and development in Kozani (Mete premises). In 2022 the establishment was done by seeds for sorghum and switchgrass and by rhizomes for miscanthus. At the establishment year the sowing of sorghum was done in the 1st half of May (variety: Bulldozer) and for switchgrass in the 1st half of June (due to late seeds arrival, variety: Blackwell). The establishment of miscanthus was done at the end of May 2022. In Figure 3.1 presented the three crops at the end of the 1st growing period. Some lodging problems had been recorded in some plots of switchgrass.

Figure 3.1: View of sorghum at several stages of growth (3 weeks from sowing, 2 months from sowing and at the harvesting time

Sorghum: Both years the crop had very good establishment and development (Figure 3.1) and reached high biomass yields. As presented in Figure 3.3 the shoot DW yields were to 24 t ha^{-1} in 2022, while in following year come up to 34 t ha^{-1} (40% higher in the 2nd year). Among both tested treatments (MXB1 and MXB2) higher yields had been recorded by MXB2 (Figure 3.2) but this superiority was statistically significant only in the 1st growing period. In Figure 3.2 the effect of both treatments over the control is presented for both years including growth characteristics (plant height, stem diameter, and number of leaves/plant) and dry matter yields. In the 2nd, year higher value had been recorded for all parameters but differences among the tested parameters weren't statistically significant. In both years, the highest values had been recorded in the plots amended by MXB2.

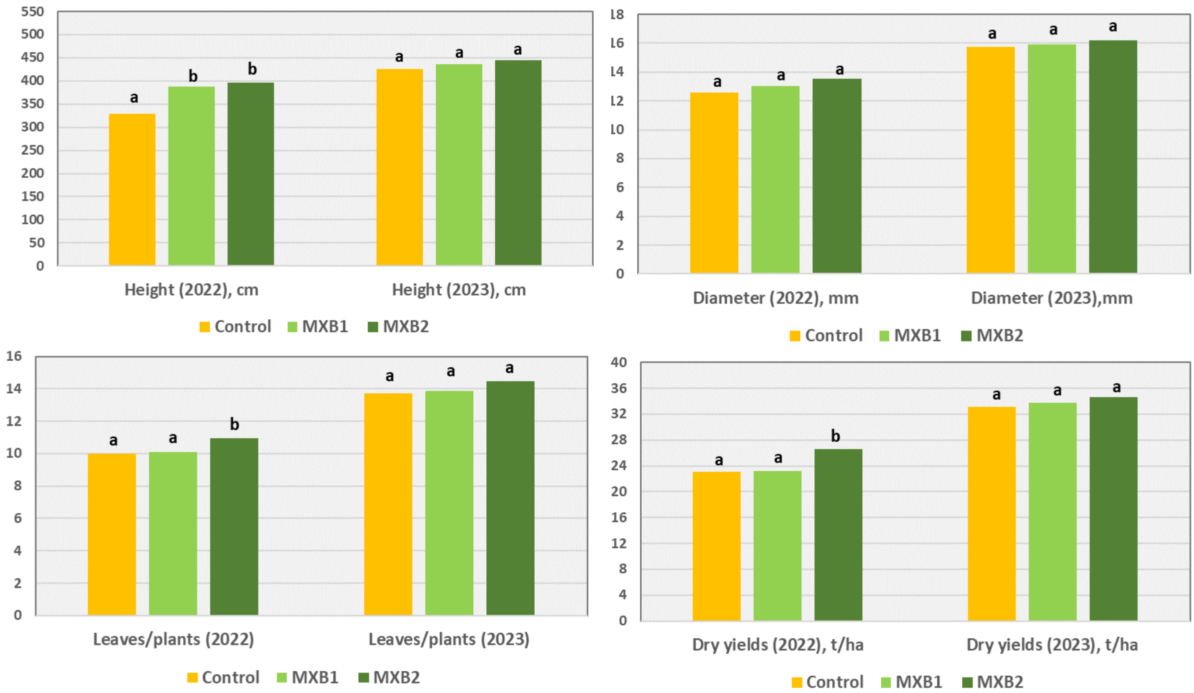


Figure 3.2. Effect of both treatments (MXB1 and MXB2) on sorghum for both years including growth characteristics and yields.

Miscanthus: The crop was established by rhizomes and weed control was needed at the early stages of growth. In the beginning of July, the crops were able to compete the weeds and the soil coverage was quite good. In Figure 3.3 the miscanthus trial is presented in July in September and at the final harvest (December). No lodging problems had been recorded in both years.

Figure 3.3. View of miscanthus at vegetative phase and at the final harvest.

Miscanthus yields in the 2nd year were more than 4 times higher compared to establishment year (Figure 3.4). The highest shoot DW yields had been measured in the plots where MXB2 was applied (>33 t/ha) and statistically significant difference were recorded between both treatments and the control. In terms of growth characteristics higher values had been recorded in the 2nd year, and between the two treatments higher values were obtained for MXB2 plots. Some statistically significant differences were recorded for height and yields in 2023 and for number of leaves in 2022 as presented in Figure 3.4.

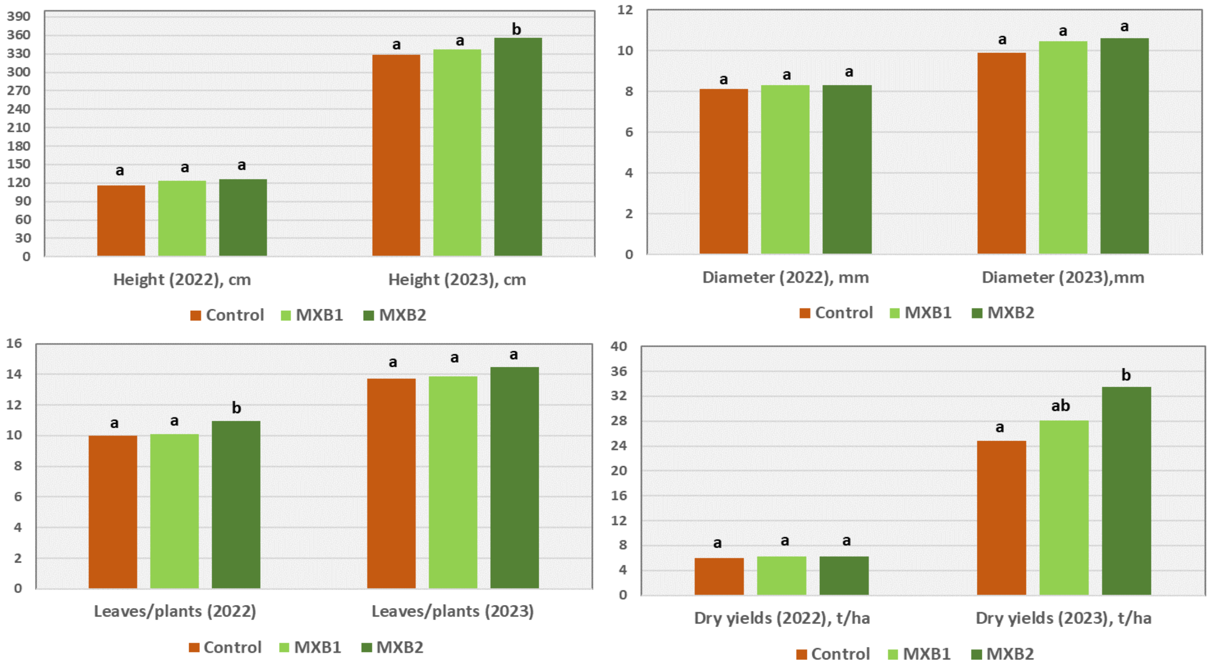


Figure 3.4: Effect of two tested treatments (MXB1 & MXB2) on Miscanthus for both years including growth characteristics and yields.

Switchgrass: The establishment of switchgrass needed special attention at the year of establishment till the middle of summer. Switchgrass is a crop is rather slow development at the establishment year and needs a cleared field from weeds. As it is presented in Figure 3.5, the development was quite good and the crop was ready for harvest in the beginning of December.

Figure 3.5: View of switchgrass at vegetative phase and at the final harvest.

The yields of switchgrass doubled in the 2nd year (14.5 t/ha vs 7 t/ha in the establishment year). The MXB2 treatment gave the highest biomass yields (Figure 3.6) and statistically significant differences were recorded between MXB1 and MXB2. Significant differences were observed in the 2nd year in terms of plant height and DW yields. As it was recorded for the studied crops (sorghum and miscanthus) the MXB2 treatment was the one that gave the best results in terms of growth and yields. For both perennial crops (miscanthus and switchgrass) the growth characteristics and yields were significantly increased from the establishment year to the 2nd year. For sorghum, higher yields were also obtained in the 2nd year (the sowing took place earlier than the 1st year).

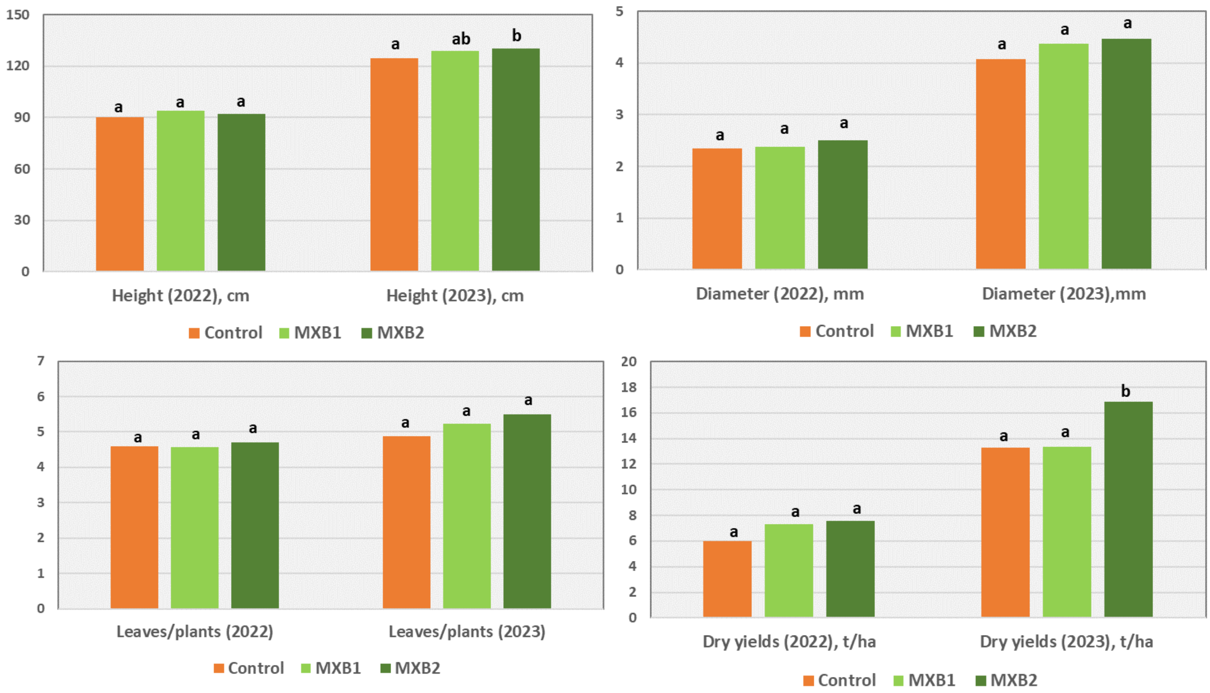


Figure 3.6: Effect of both tested treatments (MXB1 and MXB2) for both years including growth characteristics and yields.

Shoot Ni concentration: Higher Ni concentrations were recorded for leaves compared to stems for all crops in both years. The highest Ni concentration in shoots had been recorded in switchgrass followed by miscanthus and sorghum (Figure 3.7).

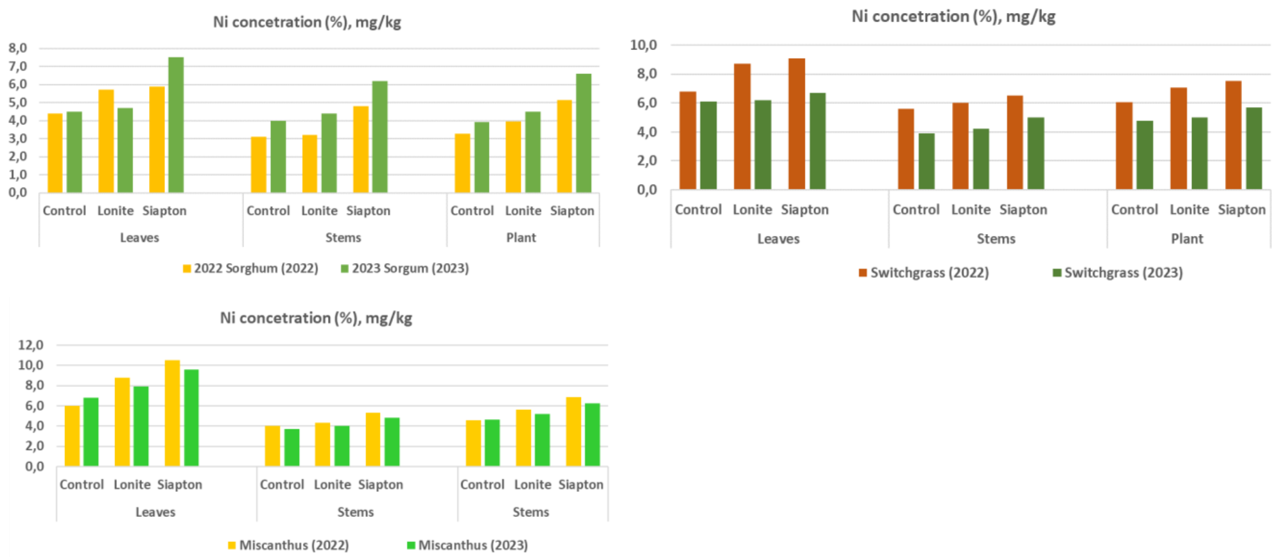


Figure 3.7. Ni concentration for the studied crops (sorghum, miscanthus, and switchgrass) and both plant fractions (stems, leaves) in both years (2022, 2023).

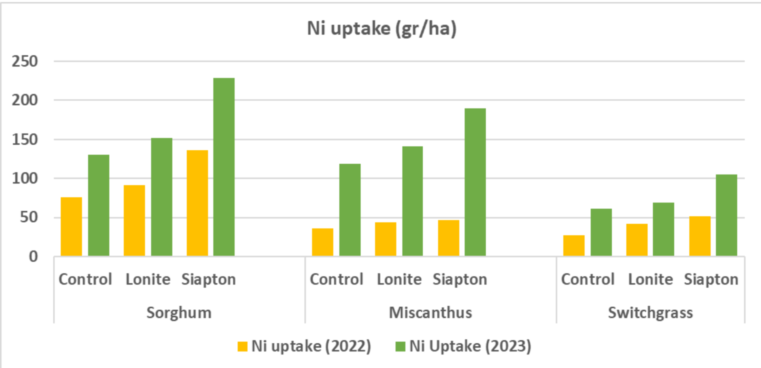


Figure 3.8: Ni uptake (mg ha^{-1}) for the studied crops (sorghum, miscanthus and switchgrass) in both years (2022, 2023) for both treatments (MXB1 and MXB2 vs. control).

Shoot Ni uptake: The highest Ni uptake was obtained by sorghum (MXB2; 228 gr/ha) followed by miscanthus (MXB2; 190 gr/ha). The lowest Ni uptake was recorded by switchgrass (MXB2; 105 gr/ha), although switchgrass had the highest Ni concertation among the three under study crops.

4. UNIBO, Italy

Hemp harvesting was carried out both seasons during the peak flowering period (i.e. late July/early August), when cellulose and hemicellulose components are maximized. For sorghum, the harvest time in both years corresponded with the end of the biological cycle (mid-October). While for miscanthus during the first growing season, harvesting took place in winter (end of January), and in the second year when the crop started the senescence phase (mid-November). This difference was due to the need to allow during the first biological cycle the complete translocation of nutrients from the aboveground biomass to the root system. This agronomic practice is considered fundamental in order to complete a correct establishment of the perennial crop and allow a vigorous re-sprouting in the following year so to enhance productivity in the first year after establishment. Generally, the plants at harvest and during both growing seasons were vigorous and without any particular problems (Fig 4.1). Only hemp showed potential phytotoxicity symptoms in both years, such as the formation of necrotic spots on the leaf apices and subsequent leaf desiccation (Fig. 4.2). However, these symptoms were found sporadically within the plots and no correlation was found with the shoot DW yield produced or the metal concentrations in the above-ground biomass.

Figure 5.1. Plants at harvest, top left hemp, top right miscanthus, bottom sorghum.

Figure 4.2. Necrosis formations on leaf apices and subsequent desiccation in hemp leaves.

Hemp dry biomass production (Fig. 4.3) was higher in the control than in the treatments in both years. However, in the first year this difference was not statistically significant, mainly due to the considerable variability between plots. In 2023 a significant difference was found between C (10.3 Mg/ha) and B1 and MB2 (6.9 and 7.1 Mg/ha, respectively). In year 2, differences were also found for the main biometric parameters monitored. Such biometric variability helps to explain the productive difference between treatments. In fact, plant height (Fig. 4.4) was higher in C than in MB2 (2.1 and 1.8 m, respectively in 2022, and 2.0 m and 1.7 m, respectively in the 2023). While the basal diameter (Fig. 4.5) in the C treatments was about 9% wider compared to B1 only in the second year with no difference detected in 2022.

For miscanthus in year 1, no significant differences were found for shoot DW yield (Fig 4.3) despite an increasing trend from C (1.4 Mg/ha) to MB2 (1.6 Mg/ha) and B2 (2.2 Mg/ha). In 2023 clear and significant differences were observed; B2 was the most productive treatment followed by MB2 and finally C (19, 14 and 11 Mg/ha respectively). The significant difference between the first and second year is due to the fact that miscanthus, as a perennial species, in the first year invests most of its resources in the growth of the root system at the expense of aboveground biomass production, which instead becomes maximum between the 2^{nd} and 3^{rd} year after planting. However, no differences were found for the height and basal diameter of the main stem (Fig. 4.4 and 4.5) in both growing seasons.

The biomass production of sorghum was significantly increased by the MB1 and MB2 treatments compared to C (Fig.4.3), especially in 2022 (+ 75 % and 120 % respectively), but also in 2023 (+ 56 % and + 44 % respectively). The most marked differences were therefore found in the first growing season, probably due to the adverse climatic conditions experienced during the emergence and stem elongation phase (above-average temperatures, prolonged drought). During 2023, more favourable conditions allowed higher yields to be achieved in all treatments, probably cushioning the production difference between control and biostimulants. For the plant height (Fig 4.4), a very similar trend to that found for biomass was evidenced. However, while in year 1 the plant height in MB2 was on average greater than in MB1 (2.2 m and 1.6 m respectively), in year 2 both treatments did not differ with an average height of 2 m both, confirming how the more favourable climatic conditions mitigated the difference between them. Finally, for basal diameter (Fig. 4.5) significant differences between MB2 and C were observed in year 1, but not in year 2.

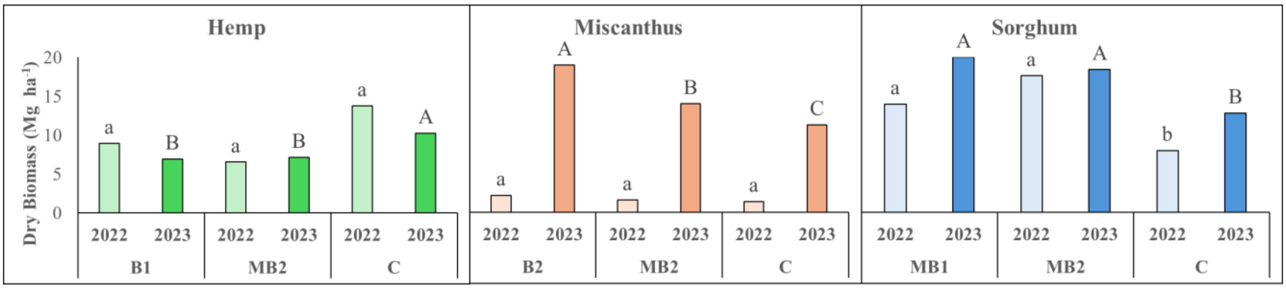


Figure 4.3. Shoot DW yield of hemp, miscanthus and sorghum per treatment

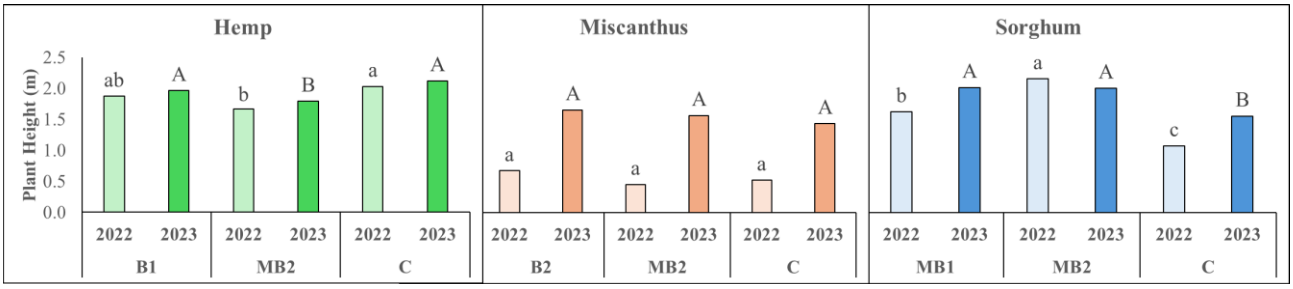


Figure 4.4. Plant height at harvest for hemp, miscanthus and sorghum per treatment.

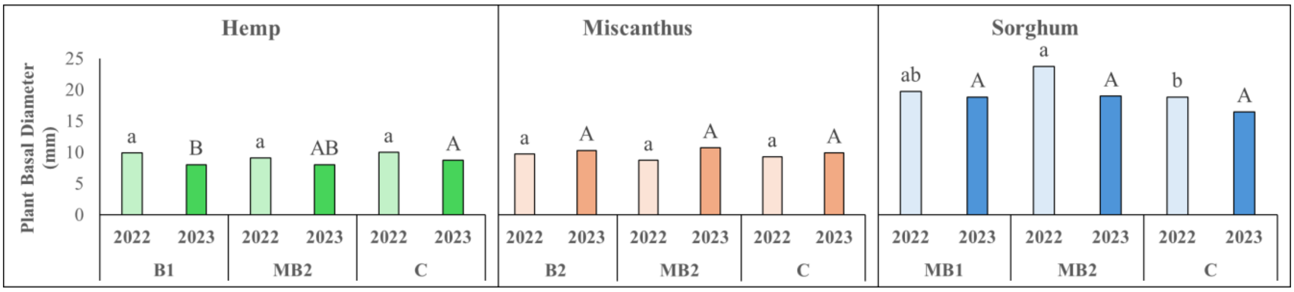


Figure 4.5. Plant basal diameter at harvest for hemp, miscanthus and sorghum per treatment.

For the Cu concentration in the harvested biomass (Fig. 4.6), only sorghum in 2023 showed treatment-related differences, as MB2 and C had higher concentrations than MB1 (+ 37% and + 44% respectively). In general, such concentrations were higher in the second year than in the first year for hemp (10.8 - 14.3 mg/kg vs 5.7 - 10.8 mg/kg), miscanthus (4.6 - 7.7mg/kg vs 3.9 - 4.0 mg/kg) and sorghum (5.7 - 8.0 mg/kg vs 2.7 - 4.2 mg/kg). With regard to shoot Zn concentrations (Fig. 4.7), only hemp in 2023 showed higher concentration in the C than in B1 (+ 58%). Again, there was a significant reduction in the metal concentrations in the three crops between the first and second year.

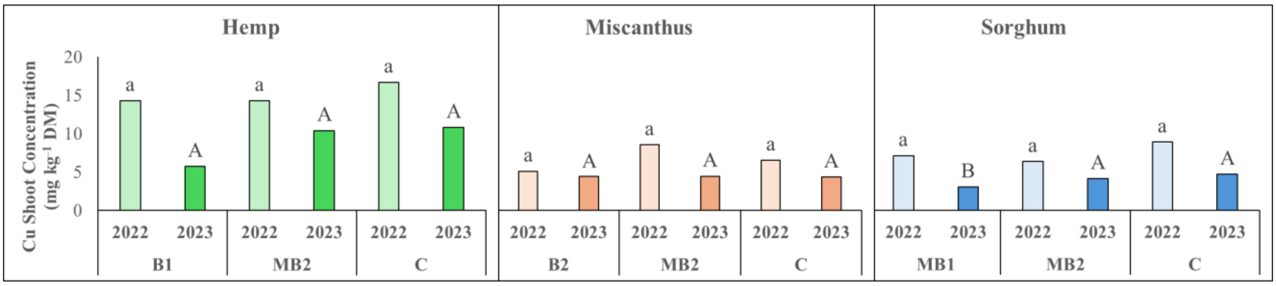


Figure 6.6. Shoot Cu concentration after harvest in hemp, miscanthus and sorghum per treatment.

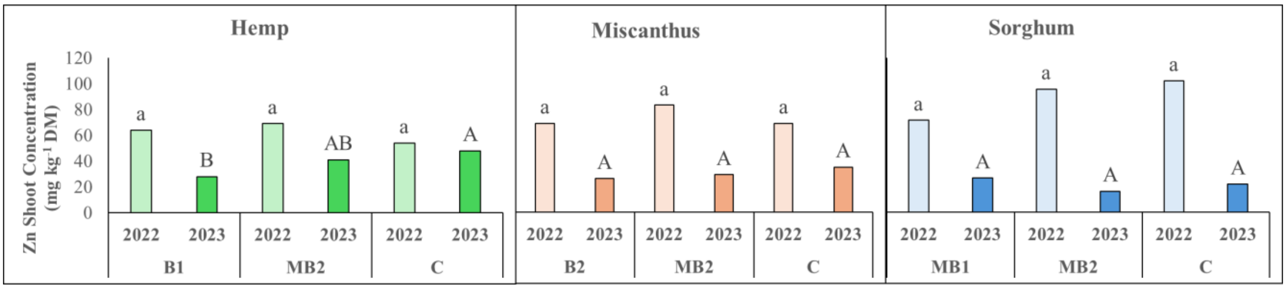


Figure 4.7. Shoot Zn concentration after harvest in hemp, miscanthus and sorghum per treatment.

With regard to the overall Cu accumulation (Fig. 4.8) hemp and miscanthus showed no significant differences between treatments, but in general the average values obtained in 2022 and 2023 were within very different ranges. In the case of hemp, higher average values were obtained in the first year (93-193 g/ha) than in the second one (31-68 g/ha) similarly to what was observed for shoot Cu concentrations (Fig. 4.6). On the other hand, for miscanthus, the highest values were recorded in year 2 (50-73 g/ha) compared to year 1 (7-13 g/ha), despite the lower concentrations found on average in 2023, mainly due to the considerable increase in biomass obtained in year 2. With regard to Cu accumulation in sorghum, the range differences between both years were less evident, while in 2023 a clear significant difference emerged with MB2 (89 g/ha) showing higher values than MB1 and C (54 and 55 g/ha respectively).

Considering the Zn accumulation (Fig. 4.9) in hemp in the first year again showed values in a higher range (436-839 g/ha) than in the second (180-268 g/ha). In 2023, C showed lower values than B1(-27%) and MB2 (-33%), mainly due to the treatments effects on biomass production (Fig. 4.3). In miscanthus, the accumulation of Zn, similarly to what was observed for Cu (Fig. 4.8), did not lead to any significant differences between the treatments, but to very different average values along the years, mainly due to the higher biomass yield found in 2023. In sorghum the accumulation of Zn in 2022 was higher in MB2 than in C (1,696 g/ha and 861 g/ha respectively), while in 2023 no significant difference was found, but similarly to what was observed for hemp, a much lower range of values was noted in the first growing season (337- 525 g/ha).

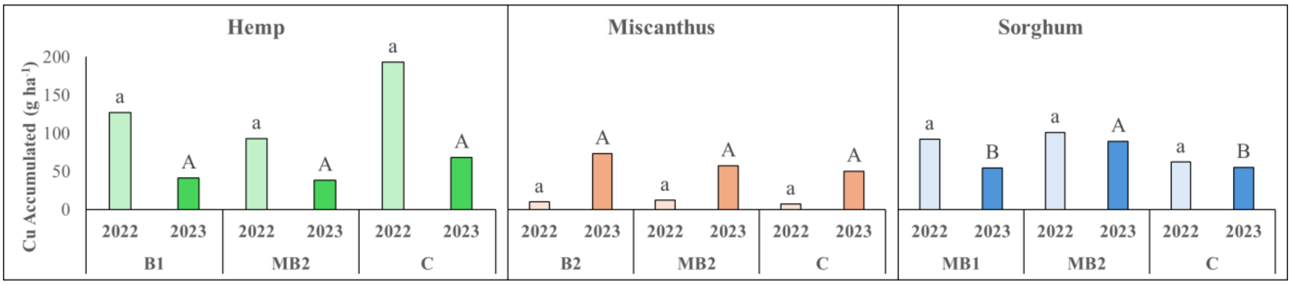


Figure 4.8. Shoot Cu accumulation in hemp, miscanthus and sorghum per treatment.

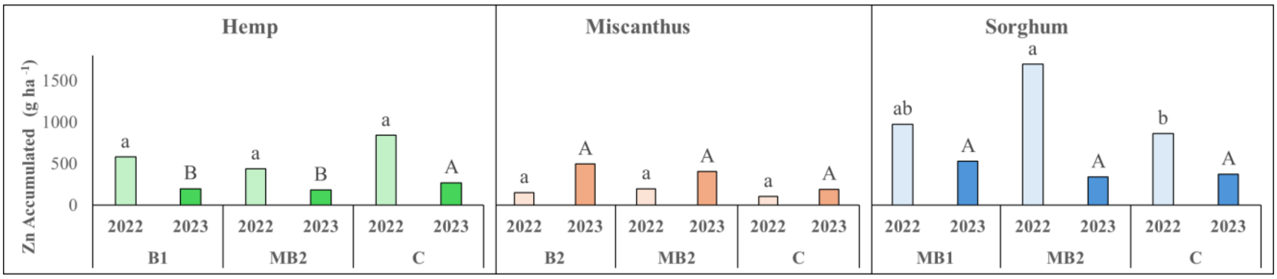


Figure 4.9. Shoot Zn accumulation in hemp, miscanthus and sorghum per treatment.

5. YNCREA, France

At the time of harvest (late September/early October) in year 2022, some visible phytotoxicity symptoms were found in the aerial parts of some plants: a red coloration in the leaves and stem of sorghum plants, and chlorosis in the leaves of hemp plants. In year 2023, however, hemp and sorghum plants did not show apparent phytotoxicity symptoms on the shoots (Figure 5.1). It is worth mentioning that in both years, the senescence had already began in hemp plants, indicating that the harvest time may have been late for hemp, a few weeks after the peak flowering. This situation may have resulted in a remobilization of nutrients from the leaves and a decrease in shoot biomass due to leaf biomass reduction and dropping.

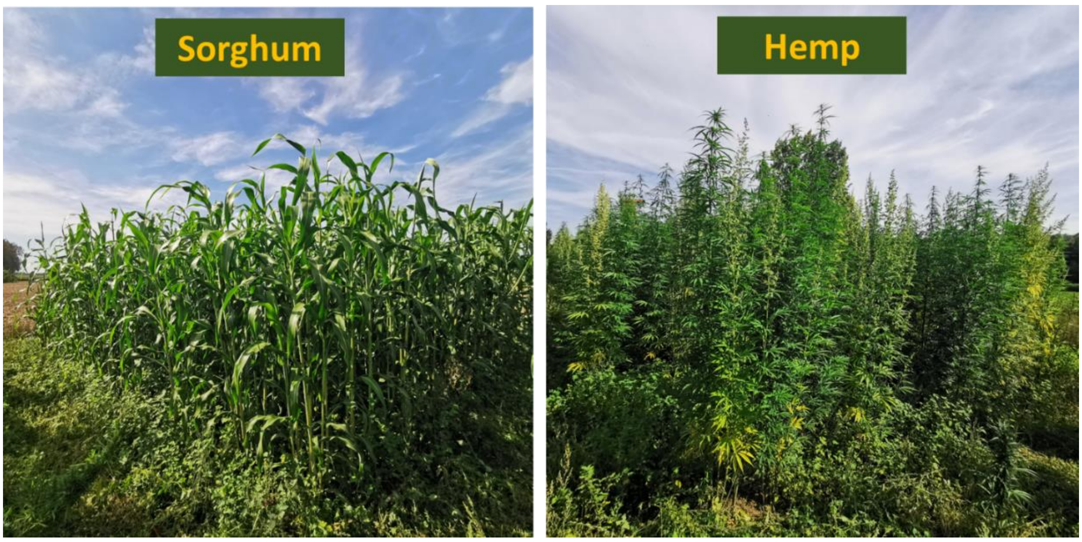


Figure 5.1. Sorghum and hemp plants in the field trials near the MetalEurop North smelter at the time of the harvest in year 2.

The treatment application did not significantly affect hemp biomass in year 1 in terms of shoot DW yield (Figure 5.2). In year 2, the combination of humic/fulvic acids and mycorrhiza (B2xM) resulted in higher values of shoot DW yield; however, this effect was not statistically significant. When compared to year 1, hemp plants in year 2 showed higher shoot DW yields, passing from average values of 10.7 ton DW ha^{-1} to 14.5 ton DW ha^{-1}. The average height (maximum shoot length) of the hemp plants ranged from 3.1 - 3.3 m in year 1 to 3.2 – 3.4 m in year 2 (Figure 5.3), accounting for an average increase of 0.1 m. This suggested that the substantial increase in hemp biomass may not result from an increase in plant height but from one in plant diameter. However, no data on plant diameter was available.

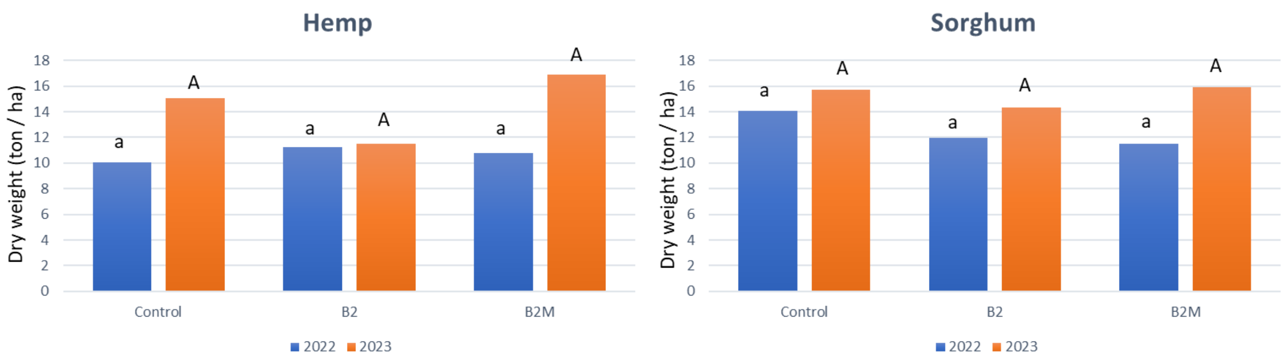


Figure 5.2. Shoot DW yields of hemp and sorghum per treatment.

In the case of sorghum, there were no significant differences detected among the treatments concerning shoot DW yield across both years of the field experiment (Figure 5.2). The shoot DW yield of sorghum in year 1 did not differ from that in year 2 for all treatments. Nonetheless, a discernible increasing trend was evident in the shoot DW yield recorded in year 2 as compared to year 1. The average shoot DW yield (ton DW ha^{-1}) ranged from 12.5 in year 1 to 15.3 in year 2. The average height (maximum shoot length) of the plants passed from 2.6 in year 1 to 3.3 m in year 2, accounting for an average increase in plant height of 0.7 m in year 2 (Figure 5.3). This showed a positive correlation between plant height and shoot DW yield for sorghum.

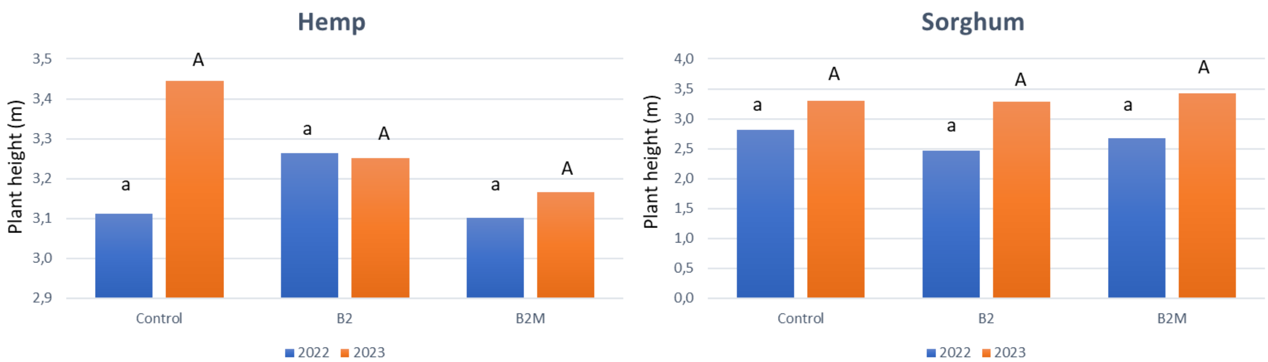


Figure 5.3. Plant height of hemp and sorghum per treatment.

The average shoot concentrations of Cd, Pb, and Zn in hemp varied between 0.3 – 0.5, 8.2 – 9.9, and 48.4 – 32.3 mg kg^{-1}, respectively, across both growing seasons (Figure 5.4). The treatment application did not have a significant effect on the shoot concentrations of these metals for both years. Nonetheless, values of shoot Cd concentrations were higher in year 2, while shoot Zn concentrations were lower. For sorghum, average values of 7.7 – 10.4, 7.4 – 11.6, and 88.9 – 119.8 mg kg^{-1} were found for shoot Cd, Pb and Zn concentrations, respectively, for both years (Figure 5.5). No significant differences were found among the treatments for both years. However, a decreasing trend was found in year 2 for the shoot Cd concentration, whereas shoot Pb and Zn concentrations increased for all treatments in year 2 as compared to year 1.

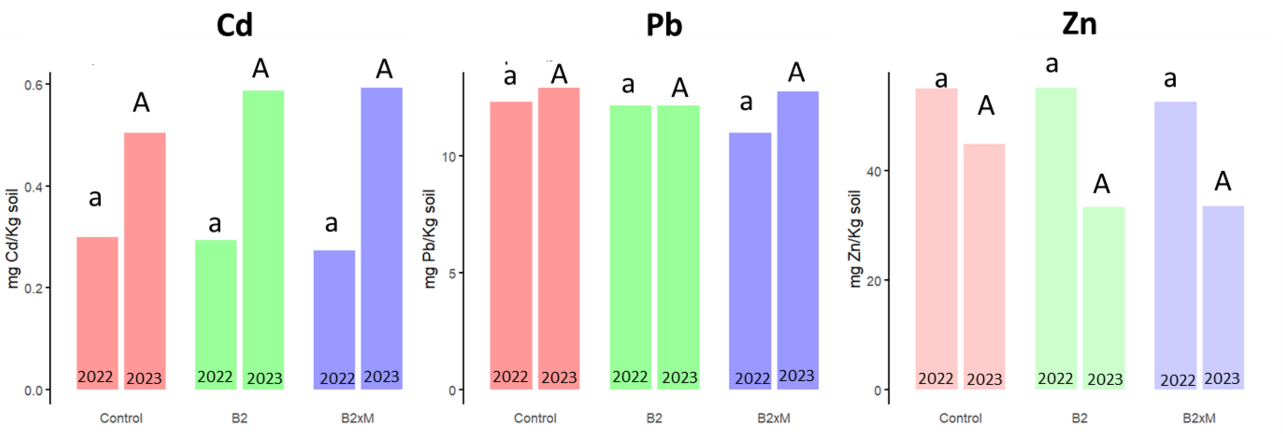


Figure 5.4. Shoot ionome (mg kg^{-1} plant dry weight) per treatment and per year in hemp.

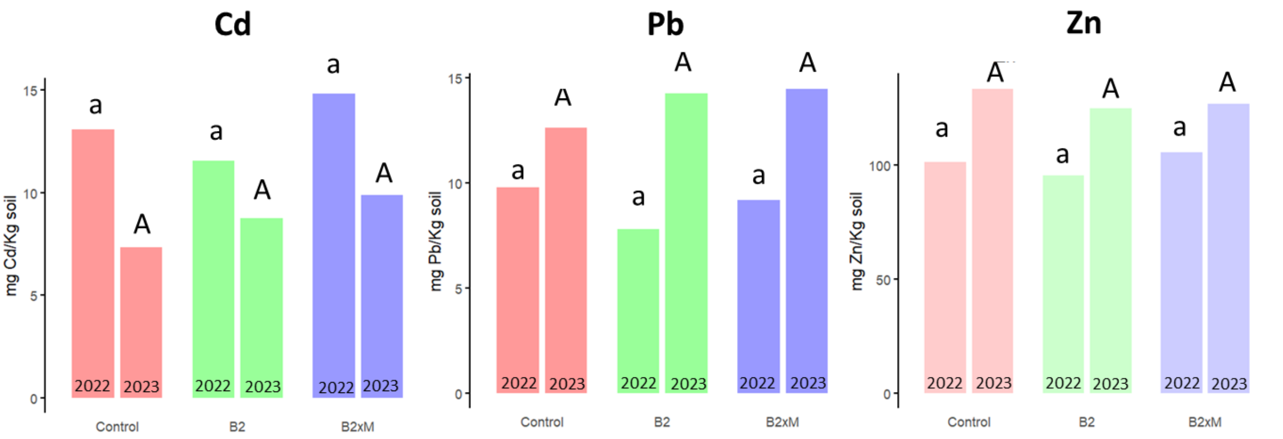


Figure 5.5. Shoot ionome (mg kg^{-1} plant dry weight) per treatment and per year in sorghum.

No significant differences in shoot metal uptake in hemp were observed among treatments in year 1 (Figure 5.6); however, in year 2 the combination of humic/fulvic acids and mycorrhiza resulted in significantly higher values of shoot Cd uptake by hemp, in parallel with the higher values of shoot DW yield obtained with this treatment. Moreover, an overall increasing trend was observed from year 2 to year 1. In year 1, hemp plots showed average values of shoot Cd, Pb and Zn uptakes of 2.9, 89 and 514 g ha^{-1}, respectively. In year 2, shoot uptake average values of Cd and Pb increased to 7.1 g ha^{-1} and 142 g ha^{-1}, respectively, whereas average values for Zn stayed relatively similar (479 g ha $^{-1}$). These results showed a very likely correlation between the increase in shoot metal uptake and the increment of the shoot DW yield in hemp plants. For sorghum, treatments did not significantly change the uptake of metals by shoots, but an increasing trend was observed over the growing seasons. Despite the decrease in shoot Cd concentration, shoot Cd uptakes remained similar across the growing seasons (126 and 117 mg h^{-1} in year 1 and 2, respectively). Shoot uptake values increased for Pb (94 and 178 mg ha^{-1} in year 1 and 2, respectively) and for Zn (1113 and 1819 mg ha^{-1}). This increase in shoot metal uptake occurred mainly due to a higher shoot DW yield (Figure 5.2).

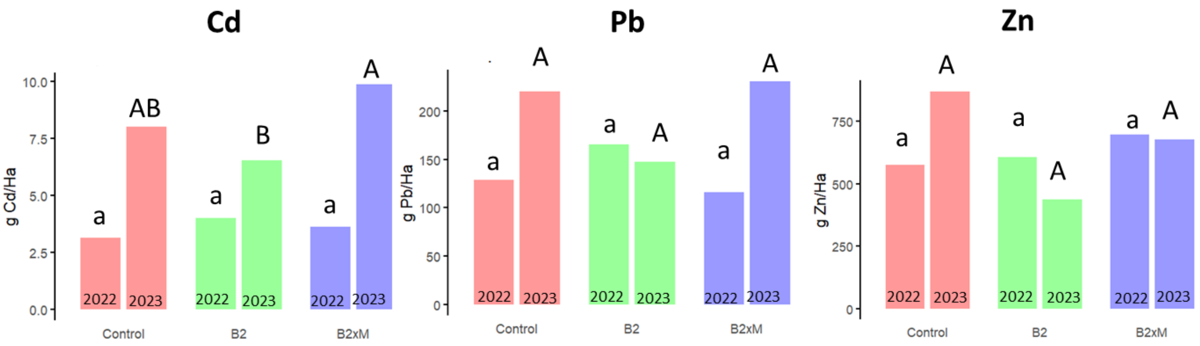


Figure 5.6. Shoot metal uptake (g ha^{-1}) per treatment and per year in hemp plots.

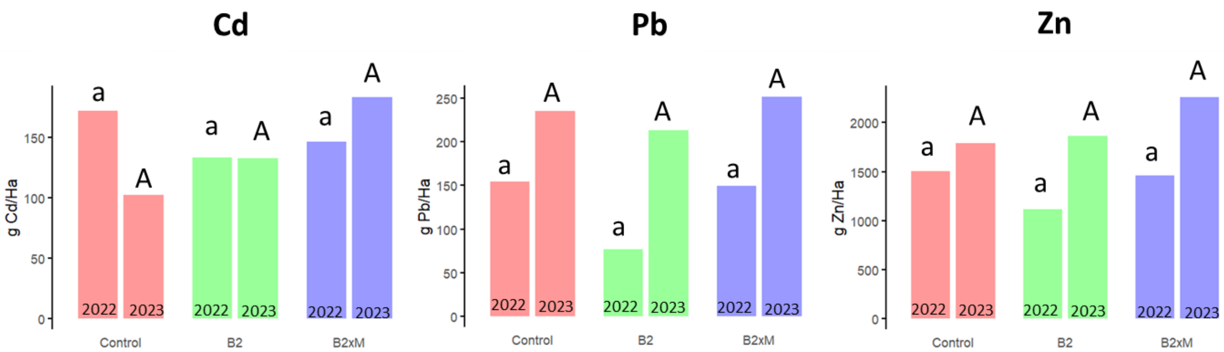


Figure 5.7. Shoot metal uptake (g ha^{-1}) per treatment and per year in sorghum plots.

6. IBFC, China

Figure 6.1: Kenaf and sorghum in field experiment at harvesting stage

Effect of treatments on sorghum

Different additives showed different effects on the plant height of sorghum (Figure 6.1). Compared with the control, the protein hydrolysate (SI) significantly decreased the plant height, however, the other treatments showed no significant impacts on the plant height. SY and SY+LO have the potential for increasing sorghum plant height (Figure 6.2).

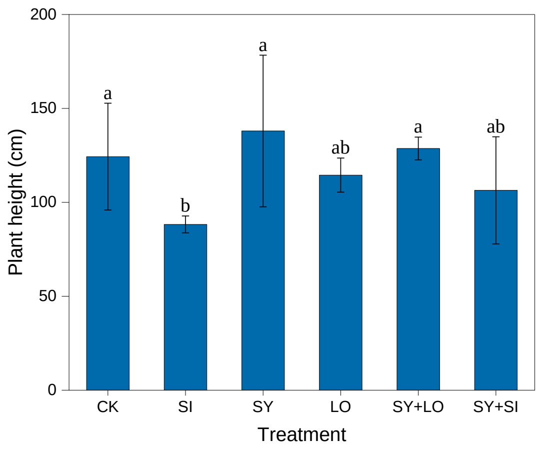


Figure 6.2: Plant height of sorghum under different treatments. Note: CK, no treatment applied; SY, symbivit (mycorrhiza inoculum); LO, Lonite (humic/fulvic acids); SI, siapton (protein hydrolysate); SY+LO, symbivit (mycorrhiza) + Lonite (humic/fulvic acid); SY+SI, symbivit (mycorrhiza) +siapton (protein hydrolysate).

Different additives did not significantly affect the stem diameter of sorghum (Figure 6.3). Compared with the control, the protein hydrolysate (SI) has the potential to decrease the stem diameter, however, the other treatments have the potential to increase sorghum stem diameter.

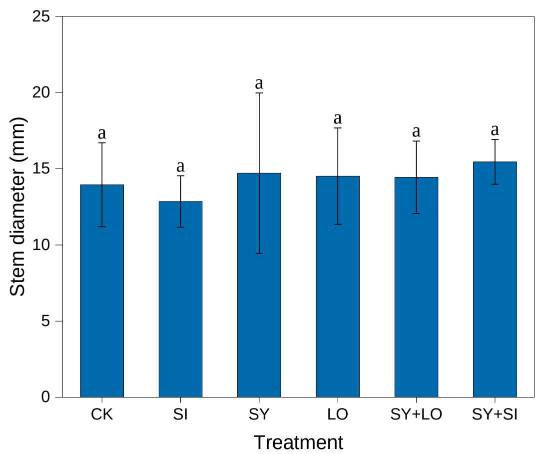


Figure 6.3: Stem diameter of sorghum under different treatments. CK, no treatment applied; SY, symbivit (mycorrhiza inoculum); LO, Lonite (humic/fulvic acids); SI, siapton (protein hydrolysate); SY+LO, symbivit (mycorrhiza) + Lonite (humic/fulvic acid); SY+SI, symbivit (mycorrhiza) + siapton (protein hydrolysate).

Effect of treatments on kenaf

Compared with CK, all of the test additives can increase the plant height of kenaf to different extent, however, no significant differences were found between the treatments (Figure 6.4).

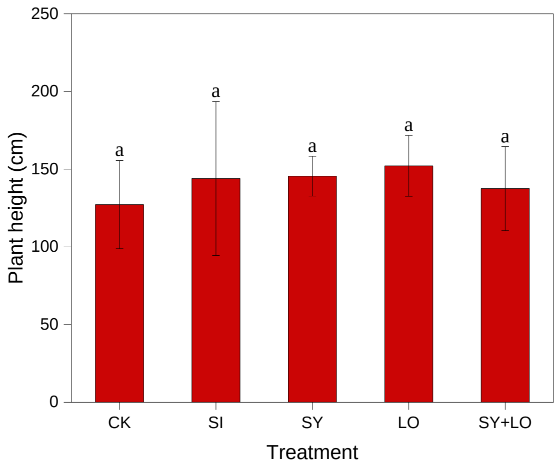


Figure 6.4: Stem diameter of kenaf under different treatments. CK, no treatment applied; SY, symbivit (mycorrhiza inoculum); LO, Lonite (humic/fulvic acids); SI, siapton (protein hydrolysate); SY+LO, symbivit (mycorrhiza) + Lonite (humic/fulvic acid); SY+SI, symbivit (mycorrhiza) + siapton (protein hydrolysate).

Compared with CK, SI significantly increase the stem diameter of kenaf (Figure 6.5). However, no significant differences were found between CK and the rest treatments. Furthermore, the stem diameter of SI was also significantly higher than those of SY, SY+LO and LO.

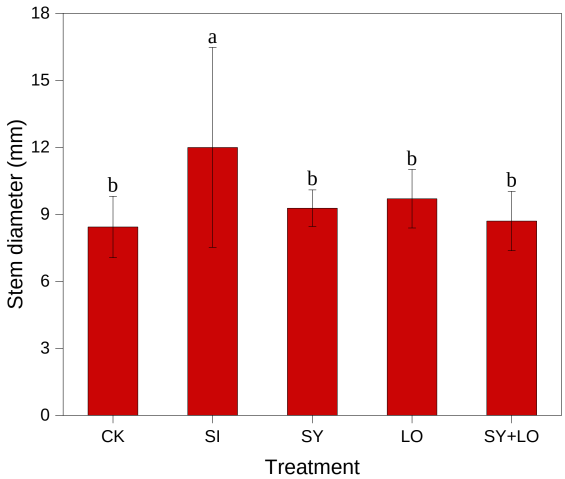


Figure 6.5: Plant height of kenaf under different treatments.

Metal accumulation in the shoots

Different treatments affected the Cd concentrations in the aboveground part of sorghum and kenaf (Figure 6.6). All of the additives can increase the shoot Cd concentration of sorghum compared with CK and SY+LO performed best.

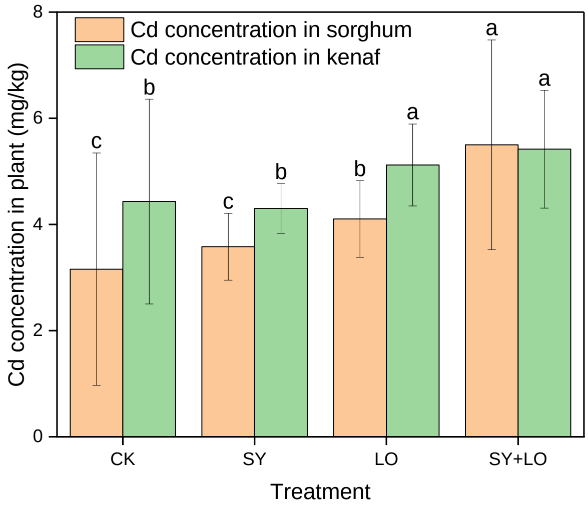


Figure 6.6. Cd concentrations in the aboveground part of sorghum and kenaf

7. HUNAU, China

Figure 7.1: Visual impressions of the field trials with miscanthus and switchgrass at HUNAU, China

Plant growth parameters such as plant height, stem diameter as well as dry biomass yield were estimated at the time of final harvest. The results show that the mean dry biomass yield of switchgrass was higher than miscanthus across all treatments. It is mainly because during first year of field trials, crops were destroyed due to persistent rains and flooding right after the setting-up of the field trials and extensive replantation was carried out in the following year. Thus, in reality it is first year of dry biomass yield for both crops. Miscanthus needs relatively longer time to establish than switchgrass, which is why this dry biomass yield difference was observed. Overall, for both crops no significance difference was recorded between treatments on dry biomass yield. For miscanthus, the best performing treatment was B2 with dry biomass yield well below 1 t ha^{-1}, whereas in switchgrass B2M treatment outperformed others with mean dry biomass yield of 4 t ha^{-1} (figure 7.2).

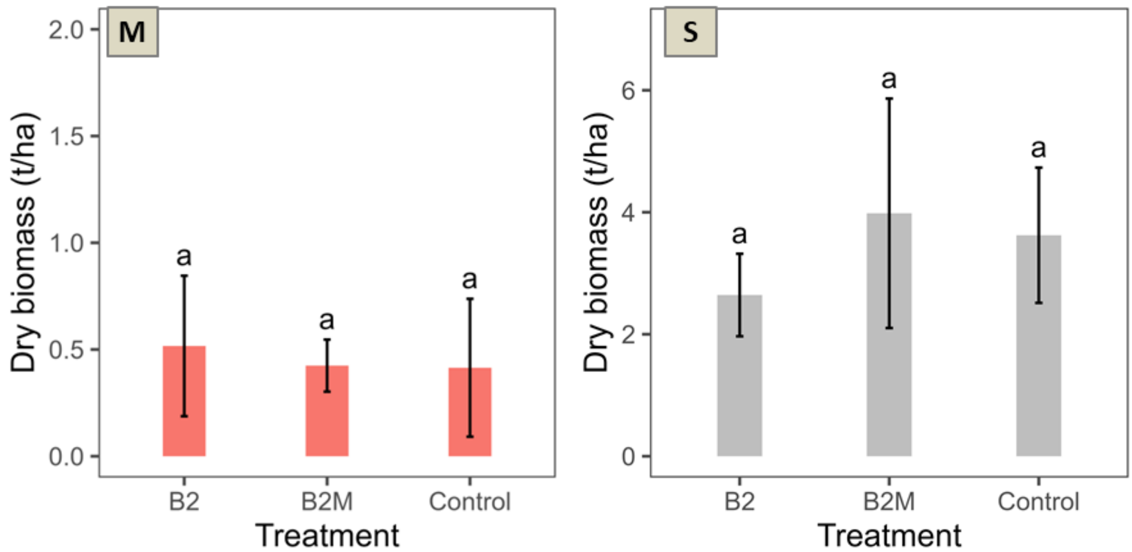


Figure 7.2: Dry biomass yield of miscanthus (M) and switchgrass (S) under three tested treatments. Same superscripts refer to no significant differences. Error bars are calculated for replications.

In addition to dry biomass yield, measurements about plant height and stem diameter were also carried out for both crops. The statistical analysis shows that there is no significant effect of treatments on plant height and stem diameter for miscanthus as was true for dry biomass yield. For switchgrass, the treatment affect was only significant for stem diameter. Across two crops, B2M treatment led to highest stem diameter and plant height, which is in line with the results of dry biomass yield. For miscanthus the plant height varied from 243 to 269 cm and stem diameter fluctuated between 1.37 and 1.47 cm, whereas plant height for switchgrass was ranged from 200 to 222 cm with stem diameter from 0.5 to 0.6 cm (figure 7.3).

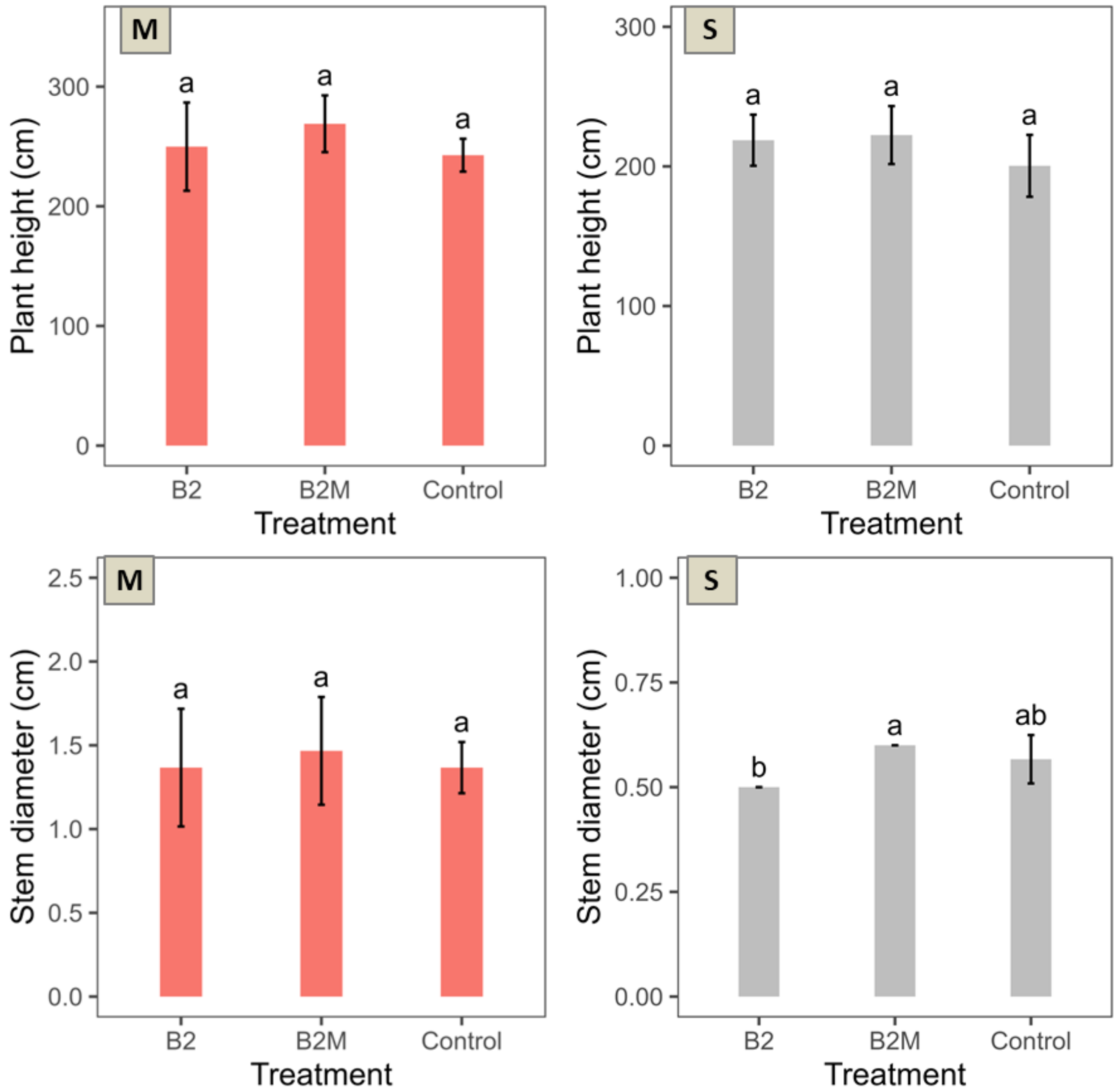


Figure 7.3: Growth parameters (plant height, stem diameter) of miscanthus (M) and switchgrass (S) under three tested treatments. Same superscripts refer to no significant differences. Error bars are calculated for replications.

Stem and leaf samples for each crop were analysed separately to determine the metal content. Statistically, no significant difference was recorded among treatments for either crop. For miscanthus, the Cd content in leaf samples varied from 0.11 to 0.15 mg kg^{-1}, whereas the stem content ranged from 0.13 to 0.25 mg kg^{-1}. The Cd content in switchgrass leaf and stem varied from 0.14 to 0.17 mg kg^{-1} DW and from 0.08 to 0.09 mg kg^{-1}DW, respectively. The trend clearly indicates that switchgrass tend to accumulate more Cd in leaves than stems (figure 7.4).

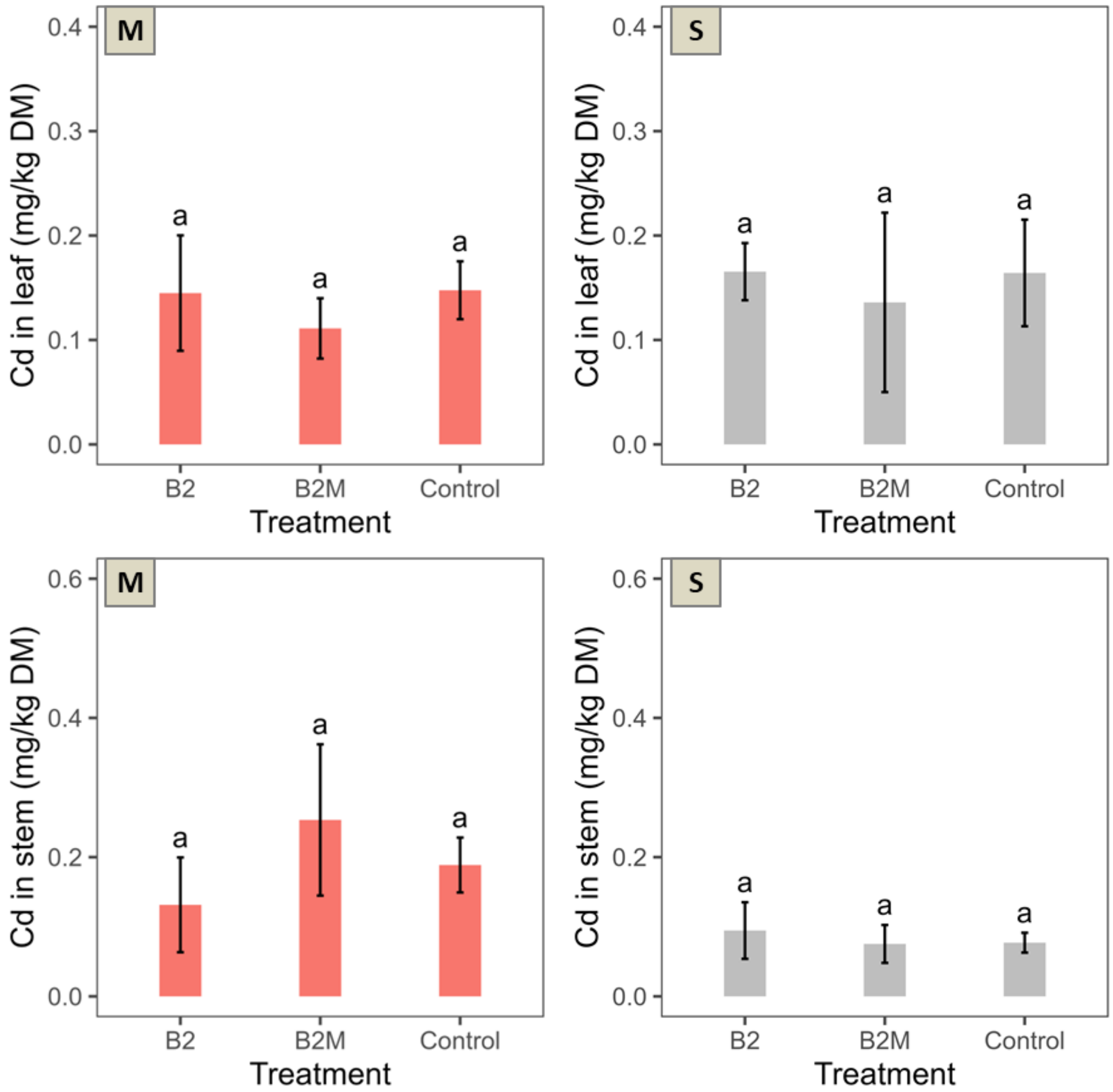


Figure 7.4: Cadmium (Cd) concentration in leaf and stem of miscanthus (M) and switchgrass (S) under three tested treatments. Same superscripts refer to no significant differences. Error bars are calculated for replications.

As for Cd, no significant difference was recorded among treatments for Cr as well for both crops. The Cr content in miscanthus leaf varied from 4.9 to 6.6 mg kg^{-1} DW and stem from 5.3 to 6.3 mg kg^{-1}DW. For switchgrass, the leaf Cr content ranged from 5.1 to 7.2 mg kg^{-1} DW, whereas for stem it varied from 3.4 to 5.0 mg kg^{-1} DW. The outcomes indicated that switchgrass prefers to accumulate higher Cr concentrations in leaves than stems, the trend, which is consistent with Cd (figure 7.5). For miscanthus, the differences are not very stark.

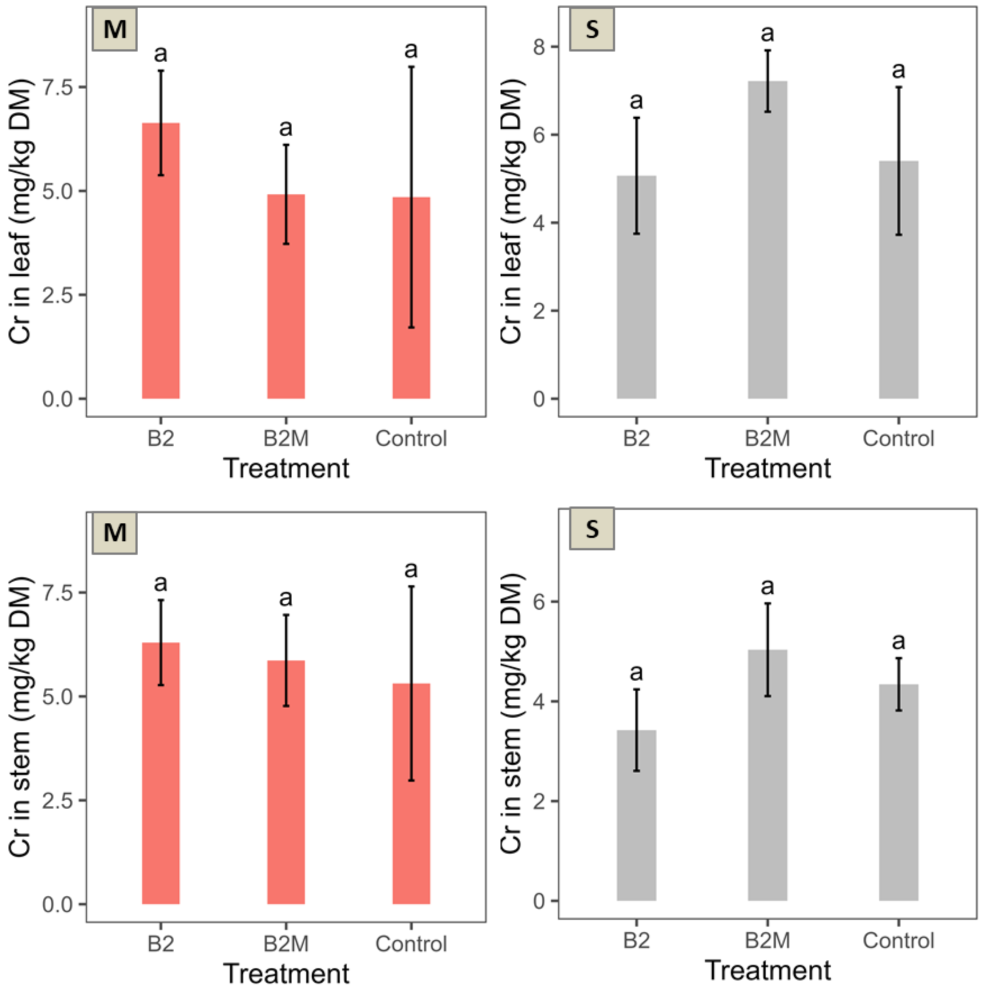


Figure 7.5: Chromium (Cr) concentration in leaf and stem of miscanthus (M) and switchgrass (S) under three tested treatments. Same superscripts refer to no significant differences. Error bars are calculated for replications.

Based on the metal content and total dry biomass, the metal uptake per hectare was calculated for each crop under the tested treatments (Table 7.1). For both crops, dry biomass yield was the main determinant of total metal uptake. As switchgrass outperformed miscanthus in dry biomass yield, the total metal uptake was also higher in switchgrass than miscanthus. The total Cd uptake per hectare for miscanthus varied from 43 to 108 mg ha^{-1} for leaf biomass, whereas for stem it was 34 to 49 mg ha^{-1}. For switchgrass, total Cd uptake in leaf and stem varied from 229 to 365 mg ha^{-1} and from 440 to 659 mg ha^{-1}, respectively. The total Cr uptake in leaf and stem of miscanthus ranged from 1212 to 2490 mg ha^{-1} and from 1172 to 2088 mg ha^{-1}, respectively. In switchgrass, the total Cr uptake for leaf varied from 9409 to 24392 mg ha^{-1}, whereas for stem it ranged between 13394 to 34975 mg ha^{-1}.

Table 7.1: Total metal uptake for miscanthus and switchgrass

Miscanthus				
	Cd (mg/ha)		Cr (mg/ha)	
	leaf	stem	leaf	stem
C	43.1	34.4	1211.8	1171.5
B2	54.5	49.1	2131.9	1986.2
MB2	107.6	47.2	2490.4	2088.0
Switchgrass				
	Cd (mg/ha)		Cr (mg/ha)	
	leaf	stem	leaf	stem
C	333.3	540.6	15731.8	16585.8
B2	229.2	440.4	9409.2	13394
MB2	364.8	658.9	24392.2	34975.1

Key findings and policy relevant messages

Pilot small-scale field trials were established at 7 sites in Europe and in Asia (Figure 1) where each partner applied the best performing phytoremediation practices obtained in their previous pot experiment (Table 2). These field trials are within the frame of the main objective of WP1, which is to optimize selected high-yielding lignocellulosic energy crops for phytoremediation, targeting various classes of soil pollutants. For this, four high-yielding lignocellulosic crops were selected for assessment, in combination with two phytoremediation practices, i.e. plant associated microorganisms and biostimulants, during two growing seasons (2022 and 2023). The trials were implemented for identifying the best combination of phytoremediation practices in terms of biomass yields and quality as well as uptake of metal(loid)s and biodegradation of organic pollutants, at some sites, under a broad range of soil pollutants. The ultimate objective was to develop optimised phytoremediation solutions for the selected crops in the form of lessons learnt.

Plant growth parameters and metal(loid) concentrations in the above-ground biomass were determined as the most relevant features determining the plant usefulness for phytoextraction purposes under various biostimulant applications.

Results indicate that hemp is a crop capable of growing well in soils contaminated with several metal(loid)s, as it is also the case for Miscanthus. This validates the previous results already obtained in the pot experiments (deliverable D1.2). Sorghum not only is a crop resistant to drought, as it was the case for most of the sites during the year 2022 with higher average temperatures and lower precipitation rates, but it is also adapted to a wide range of edaphoclimatic conditions. The biomass obtained in sites such as the North of France demonstrates that this crop can be grown at sites in locations far from the meridional latitudes where it is generally grown.

Overall, there was no significant effect of the applied treatments on the crops. Nevertheless, investigation of the sorghum roots for the site located in the North of France demonstrated a good mycorhization rate. In more details, it was evidenced, at some sites that the combination of humic/fulvic acids with mycorrhiza had a tendency to increase the biomass yield of sorghum and miscanthus plants. For hemp, the treatment showing the most beneficial effect was the application of humic/fulvic acids. Our collective results of plant growth indicate also that there was an overall increase in the shoot yields, expressed in biomass dry weight, in the second growing season compared to the first one. This positive effect may be due to the implementation of the phytoremediation practices in the first year, resulting in an improvement in the second year of the soil conditions in terms of nutrient availability or/and mitigation of the toxicity effect of the contaminants. A decrease in the $Ca(NO_3)_2$-extractable soil Cd, Pb and Zn concentrations (a proxy of the phytoavailable pool of metal in the surface soil) was evidenced after harvest in year 2 at the site in the North of France, suggesting a potential progressive decrease in root exposure to metal excess induced by the successive cultivations

Collective results on metal(loid) concentrations in the shoots generally showed no significant effect of the treatments on the studied crops. However, the combination of humic/fulvic acids with mycorrhiza slightly increased the shoot concentration of Cu and Zn for sorghum in year 2 at the site of AUA, Greece. This was remarkable as in contrast shoot Cd and Pb concentration of the AUA sorghum plants decreased in year 2 compared to year 1, likely due a dilution in the higher shoot biomass. Generally, lower critical threshold value for Cu is in the 2-5 mg Cu kg^{-1} DW range, and sorghum at the AUA site displayed the lowest shoot Cu concentration out of all sites. Application of humic/fulvic acids at the AUU site may have increase Cu binding to dissolved organic matter and root exposure, leading to a better root Cu uptake by sorghum. Overall, the lack of differences in shoot metal concentrations among the treatments may be explained by high variability, in terms of available contaminant concentration in the soil and soil physicochemical properties that is generally found at contaminated sites, particularly at agronomic scale. However, it was globally evidenced that sorghum plants showed higher values of shoot Zn and Cd concentrations, as compared to the hemp and miscanthus. There was difference for sorghum between sites. Clearly, highest shoot Cd concentrations were evidenced at the Polish and French sites, both being large areas contaminated by fallout from smelters. In contrast, hemp and miscanthus displayed a similar pattern (metal-excluder) for shoot Cd and Zn concentrations at all sites.

The phytoextraction potential of the studied crops was further assessed by calculating the metal(loid) bioaccumulation/uptake rate. The bioaccumulation or uptake of metal(loid)s in the shoots refers to the ability of crops to remove the contaminants from the soil and store them in the above-ground parts that are subsequently harvested. This characteristic depends on various factors, including the type of crop, soil composition, metal(loid) bioavailability, environmental conditions, and cultivation practices. This capacity is based on the shoot metal(loid) concentration and the shoot dry weight yield produced per hectare, for each crop. Overall, no significant effect of the applied treatments was found.

However, it was evidenced that metal(loid) bioaccumulation/uptake values obtained for sorghum were significantly higher than those for hemp and miscanthus. These results obtained in the different sites demonstrate the potential of this crop for Cd and Zn phytoextraction under a wide range of edaphoclimatic conditions and with different types of contamination. Again it is worth to notice the highest amounts of Cd and Zn were phytoextracted at the Polish and French sites, and also for Zn in year 2 at the AUA site.

Moreover, the metal(loid) bioaccumulation/uptake potential for the studied crops in year 2 was generally higher than the one observed in year 1. This is mainly due to the fact that the obtained shoot dry weight yields of crops in year 2 was in general higher than in year 1. We could hypothesize that this increment in the crops yields, resulting in higher metal(loid) bioaccumulation/uptake rates, may be ultimately due to an improvement in the physicochemical properties of soils. The amounts of Cd and Zn phytoextracted must be compared with their bioavailable amounts in the root zone.

Above all, the pool of these trials demonstrate that it is worth continuing to evaluate the effects of phytomanagement of contaminated soils with such lignocellulosic non-food crops over a number of years, extending it to include market and non-market ecosystem services.

For preparing this report the deliverables D1.1, D1.2 have been taken into consideration

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