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The effects of plant biostimulant and mycorrhiza applications on industrial hemp (*Cannabis sativa* L.) growth and phytoremediation performance

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Abstract Industrial hemp (*Cannabis sativa* L.) is a fast-growing lignocellulosic crop suitable for the phytoremediation of heavy metals, radionuclides, and organic contaminants on polluted marginal lands. The objective of this study was to evaluate the effects of plant biostimulant and mycorrhiza applications on the phytoremediation performance and growth of industrial hemp under greenhouse conditions. A 4-month pot experiment was performed in a Completely Randomized Design with six treatments (replicated three times): control (untreated), biostimulants B1 and B2 i.e., protein hydrolysates and fulvic/humic acids, respectively, mycorrhiza (M), and two biostimulant-mycorrhiza combinations, namely B1 × M, and B2 × M. Treatments affected plant height and number of leaves. Mycorrhiza application resulted in 30 and 37% higher fresh and dry aboveground biomass, respectively, compared to control plants. This treatment increased Cd accumulation; B1 and B2 × M increased Ni uptake (≥50%) when compared to the untreated plants. Significant were the effects of treatments on the accumulation of Pb, Zn, and Sb on plant shoots. Further research is required to evaluate more combinations of biostimulants, mycorrhiza and other natural environmentally friendly tools to optimize the use of industrial hemp and other multipurpose lignocellulosic crops for phytoremediation purposes.

Keywords: phytoextraction; protein hydrolysates; humic acids; lignocellulosic crop; heavy metals

1. Introduction

Soil pollution is a global problem occurring due to intensive industrial activities as inadequate waste disposal, mining, extensive use of agrochemicals, combustion of fossil fuels, etc. (Cachada et al., 2018). To mitigate this environmental threat, phytoremediation poses a sustainable and cost-effective strategy using plants to cleanse the soil from inorganic and organic pollutants (Cristaldi et al., 2017). Phytoextraction refers to the in-situ uptake of contaminants from the soil by plant roots and their translocation into harvestable plant parts, providing long-term cleanup of the soil environment from heavy metals and radionuclides (Suman et al., 2018).

Fast-growing lignocellulosic crops with high biomass production and adaptability to marginal/degraded lands are considered ideal for phytoextraction since they are tolerant to the majority of metals and xenobiotics (von Cossel et al., 2019). On the same time, they can be utilized as bioenergy feedstocks in areas where food crops cannot be grown relieving society of the fuel versus food debate (Pancaldi and Trindade, 2020). Industrial hemp is a short-day species with a 4- to 8-month life cycle (Adesina et al., 2020). It adapts to different soil types and has low nutrient demands; almost no pesticides are required for its cultivation (Tang et al., 2017). It has been reported that hemp plants tolerate stresses induced by heavy metal accumulation, and that the crop' biomass yields in polluted lands range between 10−18 t ha^{-1} (Blandinières and Amaducci 2022; Testa et al., 2023).

Biostimulants are defined as natural compounds, substances, microorganisms, enzymes etc. that can improve plant tolerance to abiotic stresses while enhancing crop productivity and quality (Palacio-Márquez et al., 2022). There is evidence that their applications can boost the phytoremediation potential of plants (Calvo et al., 2014). Biostimulants assisting phytoremediation strategies include humic substances, protein hydrolysates, inorganic salts, beneficial bacteria and cyanobacteria, algae, plant extracts, and fungal species (Bartucca et al., 2022).

To date, there is a research gap on the potential role of plant biostimulants and arbuscular mycorrhizal fungi (AMF) for improving the phytoextraction performance of industrial hemp. The objective of this study was to evaluate the effects of different biostimulants and AMF applied alone or in combinations on industrial hemp growth in soil polluted with different classes of heavy metals/metalloids and its capacity for phytoextraction. The results of this preliminary work, conducted under controlled greenhouse conditions, are expected to provide feedback for optimizing the use of this selected lignocellulosic crop for phytoremediation purposes.

2. Material and methods

2.1. Experimental setup and design

A pot experiment was conducted from September to December 2021 in the greenhouse of the Laboratory of Systematic Botany of Agricultural University of Athens. Surface soil (0-25 cm) was collected from the AUA experimental field, located in Thorikon of Lavreotiki peninsula (South Eastern Attica), in a distance of 3 km from the city of Lavrion, and transferred to AUA premises.

Soil type was sandy clay loam (SCL) with the following texture: 29.9% silt, 47.5% sand, 22.6% clay. The collected soil was also characterized by the following properties: 2.08% organic matter, 1.4 g kg^{-1} total N, 12.1 mg kg^{-1} available P, 330 mg kg^{-1} exchangeable K, 7.8 pH, 51.30 μS cm^{-1} electrical conductivity, 19.6% equal carbonate. The soil was sieved through a 10 mm mesh, homogenized and air-dried. Soil samples were taken for the determination of the inorganic pollutants. Total concentrations of heavy metals and metalloids were up to: 25 kg^{-1} for Cd, 182 kg^{-1} for Ni, 138 mg kg^{-1} for Cu, 10797 mg kg^{-1} for Pb, 4959 mg kg^{-1} for Zn, 92 mg kg^{-1} for Sb and 590 mg kg^{-1} for As, respectively.

The collected soil was mixed thoroughly with a complete 20-5-10 (N-P-K) fertilizer applied at a rate of 20 g pot^{-1}. A quantity of 12 kg of the prepared soil was potted in 12 L plastic pots and placed in the greenhouse. A plastic plate was placed under each pot to avoid drainage losses. Irrigation was adjusted to maintain 70% of water holding capacity (WHC) using tap water. Industrial hemp variety FUTURA 75 was the studied genotype obtained from Center for Renewable Energy Sources, Athens, Greece. Five seeds of hemp were sown per pot on 20 September, 2021, and 15 days later the best grown plant was selected and kept, while the other hemp seedlings were manually removed. The experiment was established in a completely randomized design with six treatments replicated three times to compare the following phytoremediation practices/treatments: two biostimulants namely protein hydrolysates (B1) and fulvic/humic acids (B2) and a mycorrhiza (M) applied singularly or combined (B1 × M and B2 × M). An untreated control was also included. At harvest the following growth parameters were determined: plant height, number of leaves per plant, fresh aboveground biomass per plant, and dry aboveground biomass per plant after air-drying at 70°C for 48 h. Heavy metal/metalloid concentration of aboveground biomass was also measured with HNO3 digestion, as described by Papazoglou and Fernando, 2017.

2.2. Treatment application protocols

Protein hydrolysate (B1) (Siapton® 10L, Company: Agrology S.A., Thessaloniki, Greece): The treatment was applied by diluting 13.5 ml of product pot^{-1} in the irrigation water. This application rate was reduced to 3 ml per litre of spray solution when the plants had adequate leaf area for foliar spraying. The first application was applied when industrial hemp plants had a height of 10 cm. The application was repeated every 10 days.

Humic/fulvic acids (B2) (Lonite 80SP, Company: Agripro G.P., Thessaloniki, Greece): The treatment was applied by diluting 0.5 g of product pot^{-1} in the irrigation water. Four weeks after the first application, this dose was increased to 0.7 g pot^{-1}. The first application was done when industrial hemp plants had developed their first 3–6 true leaves. The applications were repeated every week.

Mycorrhizae fungi (M) (Symbivit®, Symbiom, s.r.o., Lanškroun, Czech Republic): AMF applications were conducted before industrial hemp sowing at a rate of 15 g pot^{-1}.

2.4. Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) at a significance level of 0.05. Means were compared using the Fisher's Least Significance Difference (LSD) test. Statgraphics Centurion XVI (Statgraphics Technologies, Inc., P.O. Box 134, The Plains, VA, USA) was the statistical package used.

3. Results and discussion

Treatments affected the plant height and the number of leaves per plant. The tallest plants were observed for B1 biostimulant and its combination with mycorrhiza (B1 × M). Industrial hemp had more leaves in five out of six treatments compared to the control (Table 1).

Table 1. Hemp growth parameters and aboveground biomass at harvest.

Treatment	Growth parameters		Biomass	
	Height (cm)	Leaves (no.)	Fresh weight (g)	Dry weight (g)
Control	42abc[1]	78cd	12.30bc	3.27bc
B1	58a	73d	7.50c	2.59c
B2	22c	108bc	13.87ab	3.42b
M	54a	133ab	17.60a	5.22a
B1 × M	48ab	139ab	9.90bc	3.79b
B2 × M	33bc	142a	9.43bc	2.64c
$LSD_{0.05}$	21	31	5.19	0.75
p-value	0.024	<0.001	0.0136	<0.001

[1] Different lowercase letters between the same column highlight significant differences.

Mycorrhiza application resulted in 30 and 37% higher fresh and dry aboveground biomass, respectively, compared to control plants. These results agree with the recent results by Sun et al., (2023) who reported the promoting impact of AMF on industrial hemp growth and biomass production under stress induced by heavy metals on polluted soil. Single biostimulant applications (B1 and B2) did not result in significantly higher aboveground biomass compared to the untreated plants. However, B1 × M tended to improve the dry weight of plants. This is in agreement with other studies highlighting the beneficial effects of mycorrhiza-biostimulant combinations on crop growth (Torun and Toprak, 2020).

Significant were the effects of treatments on the accumulation of Pb, Zn, and Sb on industrial hemp shoots. Relatively Ni highest concentrations were detected in plants treated with B1 × M and B2 × M. Highest concentrations of Sb were found in plants treated with B2 and in control. In addition, B1 and B2 × M increased Ni uptake (≥50%) compared to the untreated plants (Table 2).

Table 2. Heavy metal concentration (mg kg^{-1}) in the shoots of industrial hemp at the end of the experiment.

Treatment	Heavy metal concentration (mg kg^{-1})				
	Pb	**Cd**	**Zn**	**Ni**	**Sb**
Control	50.95a[1]	<D.Lb[2]	324.68 a	1.73b	3.5ab
B1	34.38bc	<D.Lb	146.53c	3a	<D.Lc
B2	39.19b	<D.Lb	170.45bc	2.01ab	4.28a
M	29.85c	0.62a	112.96d	<D.Lc	2.83b
B1 × M	41.56b	<D.Lb	187.97b	2.17ab	3.50ab
B2 × M	43.13ab	<D.Lb	151.49c	2.92a	2.82b
$LSD_{0.05}$	9.07	0.07	26.13	1.07	1.29
p-value	0.0047	<0.001	<0.001	<0.001	<0.001

[1] Different lowercase letters between the same column highlight significant differences. [2] D.L.; Detection Limit.

Our findings agree with Bartucca et al. (2022) who also documented the ability of biostimulants to counteract the deleterious effects of pollutants on plants, thus increasing the phytoremediation efficiency of some species. Similar were the results by Omara et al. (2019) on sweet sorghum. In addition, Fiorentino et al. (2017) reported that biostimulated giant reed achieved higher shoot and root biomass, and accumulated significantly higher Zn amounts compared to the untreated plants. As for mycorrhiza application (M), it increased Cd accumulation as also shown in other related studies indicating that mycorrhiza has the capacity to assist in phytoremediation techniques (Cabral et al., 2015).

To summarize, the preliminary results of this work, conducted under controlled greenhouse conditions, are expected to provide feedback for optimizing the use of industrial hemp for phytoremediation purposes. Further research is required to evaluate more combinations of biostimulants, mycorrhiza and other natural environmentally friendly tools to optimize the use of industrial hemp and other multipurpose lignocellulosic crops for phytoremediation purposes, not only in screening pot experiments but also in more robust field trials repeated under different soil and climatic conditions.

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