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Potential Use of Copper-Contaminated Soils for Hemp (*Cannabis sativa* L.) Cultivation

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Abstract: To mitigate climate change, reducing greenhouse gas emissions can be achieved by decreasing the use of fossil fuels and increasing that of alternative sources, such as energy crops. However, one of the most important problems in the use of biomass as a fuel is that of changing soil use and consumption, leading to competition with food crops. We addressed the topic by evaluating the possibility to exploit contaminated areas for energy crops cultivation. Indeed, soil contamination makes land inappropriate for cultivation, with damaging consequences for ecosystems, as well as posing serious health hazards to living beings. Specifically, this work aimed to evaluate the ability of hemp (*Cannabis sativa* L.) plants to grow on a copper (Cu)-contaminated medium. In addition, the effectiveness of an environment-friendly treatment with sulfate in improving plant ability to cope with Cu-induced oxidative stress was also explored. Results showed that plants were able to grow at high Cu concentrations. Therefore, hemp could represent an interesting energy crop in Cu-contaminated soils. Although the response of Cu-treated plants was evidenced by the increase in thiol content, following modulation of sulfur metabolism, it remains to be clarified whether the use of exogenous sulfate could be an agronomic practice to improve crop performance under these edaphic conditions.

Keywords: biofuel; *Cannabis sativa* L.; climate change; copper; sulfur; sustainability

1. Introduction

The limitation of greenhouse gases emission is considered a priority for the mitigation of global warming and climate change. In order to meet this challenge, a remarkable replacement of fossil fuels for energy purposes in favor of renewable sources, like energy crops, as also recommended by the European community with the Directive 2009/28/EC [1], appears to be crucial. However, the cultivation of energy crops poses several issues, such as land-use change. In fact, when virgin land areas are considered, land conversion from natural ecosystems is clearly counter-productive, considering the environmental impact and specifically the loss of biodiversity in the soil-plant system [2]. In addition, soil consumption can be unequivocally considered as one of today's major challenges of agriculture. Indeed, the greater the demand for biomass from energy crops to achieve the EU goal, the larger the land surface to be dedicated to the cultivation of these plant species, thus giving rise to a sort of competition in the use of arable land for food or non-food purposes [3].

Facing the challenge of food security and safety for a growing population while preserving the environment highlights how crucial are these choices. However, the possibility of using abandoned or impaired fertility soils (i.e., so-called marginal soils) for energy crops could undoubtedly contribute to reducing the food/energy conflict in agricultural production [4]. In fact, it is well-known how some industrial activities and/or agricultural

practices (e.g., the excessive use of agrochemicals for crop fertilization or protection against pathogens as well as the massive application of low-quality soil amendments), have sometimes compromised soil fertility, making soils partially or completely inappropriate for staple food production [5].

In particular, soil contamination with heavy metals, including both essential and non-essential metals, is a widespread issue posing environmental, animal, and human health risks [6]. A typical example is represented by copper (Cu), an essential micronutrient with fundamental roles played in plants [7] as well as a component of different agrochemicals applied for the control of fungal pathogens of different crops, particularly perennial ones [8].

The continuous and extensive application of Cu-based agrochemicals has led to a massive, and even alarming, long-term accumulation of this heavy metal in soils [9]. Specifically, in vineyard soils, Cu concentrations often exceed the limits set by European legislation for agricultural soils (ranging from 150–200 mg kg^{-1}) [10,11], even reaching levels up to approximately ten times higher [12].

In this respect, although levels of metal availability to plants in soil are strictly dependent on soil properties (e.g., cation exchange capacity, pH, organic matter, and clay content) [13], it should be highlighted that the above-mentioned Cu levels could undoubtedly represent a significant threat to soil use [14]. In fact, a range of toxic phenomena, leading to reduced crop yield and quality, have been described in soils with Cu bioavailability significantly higher than 20 mg kg^{-1} [15].

Excess Cu induces the production of high levels of reactive oxygen species (ROS), which cause oxidative degradation of cell membrane lipids, resulting in the leakage of the cell contents, and affects photosynthetic functions [16]. Furthermore, excess Cu interferes with the acquisition processes of other nutrients [17–21], leading to nutritional imbalances [22]. For instance, being sulfur (S) directly involved in the oxidative stress response, plants exposed to high Cu concentrations usually show an enhanced demand for S to sustain the increased production of thiols (mainly glutathione), which are the main plant antioxidants and heavy metal chelators implicated in detoxification [8,23]. Therefore, it is very likely that a higher S availability in soil could be a valuable strategy allowing plant to counteract oxidative stress induced by high Cu levels.

Based on these premises, the present work aimed at evaluating the possibility of exploiting Cu-contaminated soils for the cultivation of energy crops. Hemp (*Cannabis sativa* L.) was selected as a model plant for this study due to its high tolerance to biotic and abiotic stresses, leading it to good adaption to different soils and climatic conditions, and high biomass production.

In the last twenty years, several studies have investigated the possible use of hemp to clean up highly polluted soils [24]. These studies reported that hemp is extremely efficient to take up and accumulate heavy metals such as cadmium, lead, nickel, and zinc; however, very few studies described the use of hemp for the phytoremediation of Cu-contaminated soils [25,26].

We also tested the hypothesis that an environment-friendly treatment with exogenous sulfate could enhance plant tolerance to Cu toxicity, mainly by improving plant ability to cope with oxidative stress under Cu contamination.

2. Materials and Methods

2.1. Plant Growing Conditions

Hemp (*Cannabis sativa* L. cv Tisza) seeds were soaked in distilled water for 1 h and allowed to germinate in moistened filter paper in the dark at 28 °C for 7 days. Homogeneous seedlings were selected and transferred in hydroponic culture in 2-L black pots (6 seedlings/pot), containing a nutrient solution (NS) with the following composition (mM): Ca(NO$_3$)$_2$ × 4H$_2$O 2.0, KCl 0.1, KH$_2$PO$_4$ 0.1, K$_2$SO$_4$ 0.7, MgSO$_4$ × 7H$_2$O 0.5, CuSO$_4$ × 5H$_2$O 0.2 × 10^{-3}, Fe^{3+}–EDTA 40 × 10^{-3}, H$_3$BO$_3$ 1 × 10^{-3}, MnSO$_4$ × H$_2$O 0.5 × 10^{-3}, (NH$_4$)$_6$Mo$_7$O$_{24}$ × 4H$_2$O 0.01 × 10^{-3}, and ZnSO$_4$ × 7H$_2$O 0.5 × 10^{-3}. After 14 days of

growth, hemp seedlings were exposed to excess Cu and/or sulfate treatment, as follows: control (C = 1.2 mM sulfate and 0.2 μM $CuSO_4$); sulfate treatment (ES = 2.4 mM sulfate and 0.2 μM $CuSO_4$); excess Cu (+Cu = 1.2 mM sulfate and 20 μM $CuSO_4$); excess Cu and sulfate treatment (ES+Cu = 2.4 mM sulfate and 20 μM $CuSO_4$). Applied sulfate concentrations were chosen based on previous study [27]. Plants were grown in a climate chamber with the following conditions: day 16 h, 28 °C; night 8 h, 20 °C; light intensity of 200 μE m^{-2} s^{-1}. The NS was renewed every 3 days. After 21 days of growth in hydroponics, plants were harvested, the shoot and root fresh weights (FWs) were recorded, and the following experiments were performed.

2.2. Chlorophyll Content

At harvest (21 days of growth), chlorophyll content was measured in attached leaves of hemp plants using a non-destructive portable apparatus, the Soil Plant Analysis Development (SPAD—502 Plus, Konica Minolta, Osaka, Japan). Three independent measurements within each treatment were detected on the youngest fully expanded leaf, averaged, and expressed as SPAD units.

2.3. Determination of Nutrient Concentration

The nutrient concentration was determined according to the method described by [28]. Briefly, shoot and root samples were oven-dried at 65 °C for 24 h and subsequently finely ground and homogenized with a ball mill (Mixer Mill, MM400, RETSCH, Pedrengo, BG, Italy). Approximately 0.3 g dry weight (DW) of each ground sample was mineralized in 4 mL concentrated ultrapure HNO_3 (650 mL L^{-1}; Carlo Erba, Milan, Italy), using a single reaction chamber microwave digestion system (UltraWAVE, Milestone, Shelton, CT, USA). After cooling, the digested samples were diluted with Milli-Q water to 20 mL. The concentrations of nutrients (i.e., calcium—Ca, copper—Cu, iron—Fe, magnesium—Mg, manganese—Mn, molybdenum—Mo, phosphorus—P, sulfur—S, and zinc—Zn) were measured three times, 12 s each, with 60 s of pre-running, using an inductively coupled plasma—optical emission spectroscopy (ICP—OES, Spectro Arcos, Spectro Ametek, Kleve, Germany) instrument, with an axial torch and high salinity kit. The ICP—OES operating conditions are summarized in Table 1. The Ca signal was measured at 184 nm, the Cu at 325 nm, the Fe signal at 260 nm, the Mg at 279 nm, the Mn at 258 nm, the Mo at 380 nm, the P at 178 nm, the S at 182 nm, and the Zn at 214 nm. Spinach leaves (SRM 1570a) and tomato leaves (SRM 1573a) were used as external certified reference materials. The detection limits for each nutrient are reported as follows (μg L^{-1}): Ca 7.000, Cu 1.170, Fe 0.135, Mg 2.000, Mn 0.034, Mo 20.2, P 11.000, S 11.000, and Zn 0.362.

Table 1. ICP—OES operating conditions.

Plasma Power (kW)	1.4
Pump Speed (Rpm)	30
Coolant Flow (L min^{-1})	14
Auxiliary Flow (L min^{-1})	0.8
Nebulizer Flow (L min^{-1})	0.95

2.4. Determination of Malondialdehyde Content

The level of lipid peroxidation was expressed as malondialdehyde (MDA) content and was determined as 2-thiobarbituric acid (TBA) reactive metabolites according to [29]. Briefly, frozen shoot and root samples (0.5 g FW) were finely ground with liquid nitrogen and homogenized in 5 mL of an extraction solution containing 0.25% (*w/v*) TBA dissolved in 10% (*w/v*) trichloroacetic acid (TCA). The resulting extracts were heated at 95 °C for 30 min and then quickly cooled on ice. After centrifugation at 10,000× *g* for 10 min, the absorbance of the supernatant was measured at 532 nm. Correction of non-specific turbidity was obtained by subtracting the absorbance value detected at 600 nm. Finally, the level of lipid peroxidation was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.5. Non-Protein Thiols Extraction and Determination

Water soluble non-protein thiol compounds were determined colorimetrically with 5,5′dithio-bis-(2-nitrobenzoic acid) (DTNB), following the procedure reported by [30]. Briefly, both shoot and root tissues (1 g FW) were ground in liquid nitrogen and extracted in 3 mL of a solution composed of 80 mM TCA, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.15% (*w/v*) ascorbic acid, and 10% (*w/v*) polyvinylpolypyrrolidone (PVP). After a centrifugation step (30 min at 4000× *g* and 4 °C), the supernatant was recovered, and the concentration of DTNB-reactive compounds was detected spectrophotometrically at 415 nm.

2.6. Extraction and Assay of OASTL Activity

Frozen shoot and root tissue (1 g FW) of hemp plants were powdered in liquid nitrogen. Cold extraction buffer, containing 50 mM HEPES-KOH (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 10% (*v/v*) glycerol, 0.1% (*v/v*) Triton X-100, 5 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, and 1% (*w/v*) PVP, was added in a ratio of 1:7 (*w/v*) for shoots and of 1:3 (*w/v*) for roots. Following extraction steps were performed according to the method described by [30].

The extractable *O*-acetylserine(thiol)lyase (OASTL; EC 4.2.99.8) enzyme activity was assayed in shoot and root samples by detecting cysteine production as described by [30]. The protein content was estimated in shoot and root extracts by the protein-binding Coomassie Brilliant Blue G-250 dye method using bovine serum albumin as standard [31].

2.7. Statistical Analysis

The results are presented as mean ± standard deviation (SD) of measurements carried out in triplicate (*n* = 3) and obtained from four independent treatments. All data were statistically analyzed by one-way analysis of variance (ANOVA) with least significant difference (LSD) test at $p < 0.05$ by using the statistical software CoStat Version 6.45 (CoHort, Berkeley, CA, USA). Principle component analysis (PCA) was performed with PAST (PAleontological STatistics) software (Version 4.05 for Windows).

3. Results

3.1. Plant Growth Rate, Chlorophyll Content and Nutritional Status

Figure 1 shows the effect of Cu excess in the growing medium and/or sulfate treatment on the growth rate of hemp plants in terms of shoot and root fresh weights. The plant exposure to Cu stress (+Cu condition) significantly inhibited the shoot biomass accumulation (−58%), as also clearly visible in the picture below Figure 1, compared to the control plants (C condition) (Figure 1). When Cu stress was coupled with sulfate treatment (ES+Cu condition), shoot growth inhibition was also clearly detected (although to a lesser extent: −40%) compared to the C condition even if no significant difference were noted between the ES+Cu and +Cu conditions (Figure 1). Plants exposed to the sole sulfate treatment (ES condition) did not significantly exhibit changes in growth rate at both shoot and root level when compared to the control (Figure. 1).

Leaf chlorosis is one of the earliest and distinct symptoms of plant Cu toxicity (Figure 1). In the present study, we found that exposure of hemp plants to Cu excess (sole or in combination with sulfate treatment) reduced the chlorophyll content and led to visible leaf chlorosis (Figure 1 and picture below Figure 1). As observed for plant growth, the ES condition did not significantly affect the chlorophyll content with respect to the control (Figure 1).

It is well-known that excess Cu interferes with nutrient uptake, leading to nutritional imbalances.

Total S concentration increased by 153% only in roots of plants exposed to sole sulfate treatment (ES condition) (Figure 2A). In addition, Cu treatment (sole or in combination with sulfate treatment) did not significantly impact S accumulation in both shoots and roots of hemp plants (Figure 2A).
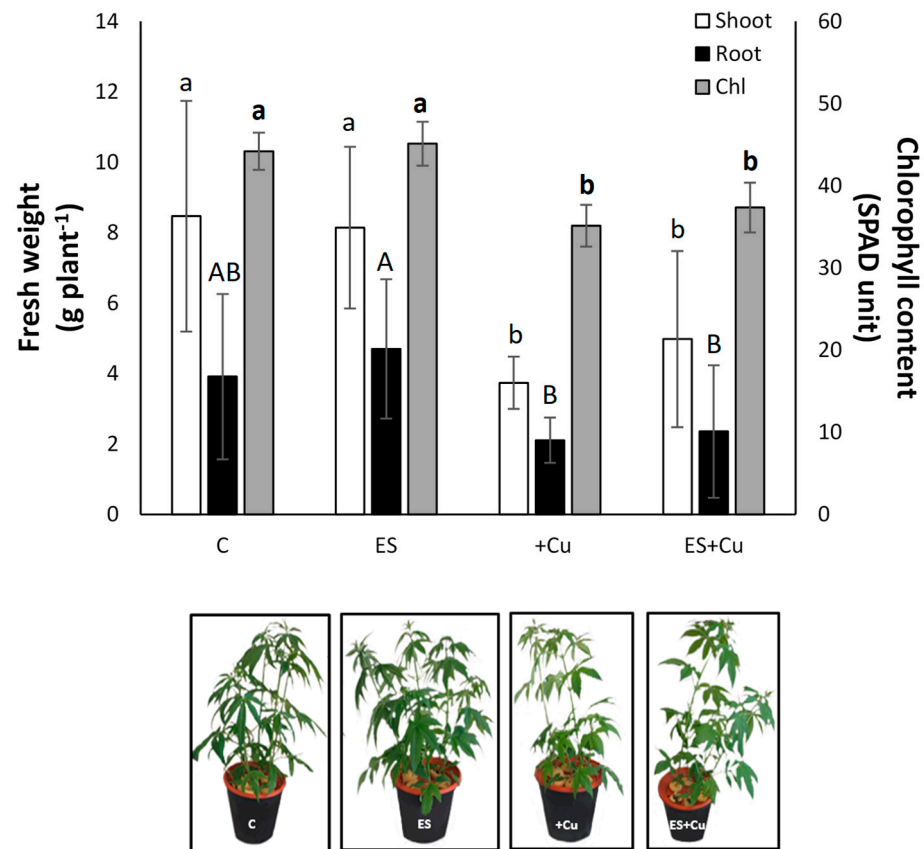
Figure 1. Fresh weight of shoots (white bars) and roots (black bars), and chlorophyll (Chl) content (grey bars) of hemp plants grown hydroponically in four different growth conditions: control (C = 1.2 mM sulfate and 0.2 μM CuSO$_4$); sulfate treatment (ES = 2.4 mM sulfate and 0.2 μM CuSO$_4$); excess Cu (+Cu = 1.2 mM sulfate and 20 μM CuSO$_4$); excess Cu and sulfate treatment (ES+Cu = 2.4 mM sulfate and 20 μM CuSO$_4$). Below graph, pictures of 21-day-old hemp plants exposed to the four different growth conditions. Data are reported as mean ± SD of three independent replicates (n = 3). The statistical significance was determined by one-way ANOVA analysis with LSD post hoc test (p < 0.05). Different lower-case, upper-case, and bold lower-case letters indicate significantly different values among the fresh weights in shoots and roots, and Chl content, respectively.

As expected, Cu concentration increased in plant exposed to Cu stress (sole or in combination with sulfate treatment) (Figure 2B). In particular, Cu accumulation was higher in the roots than in the shoots in both +Cu and ES + Cu condition (Figure 2B). Interestingly, the Cu excess treatment combined with the sulfate one (ES + Cu condition) led to lower shoot accumulation of Cu (−36% with respect to +Cu condition) (Figure 2B). On the other hand, no significant difference in Cu accumulation was observed between ES condition and C condition both in shoots and in roots (Figure 2B).

The Cu-treated plants showed a lower Fe accumulation in the shoots (−34% and −37% in +Cu and ES+Cu conditions, respectively) as compared to the control (Figure 2C). Conversely, the Fe concentration increased significantly in the shoots of ES plants (+18% with respect to control C) (Figure 2C).

Figure 2D shows that treatment with Cu also significantly affected Mn accumulation. In particular, a significant drop of Mn concentration was observed in the roots of +Cu plants (−67%) as well as in the shoots of ES+Cu plants (−44%) compared to the values measured in control plants.
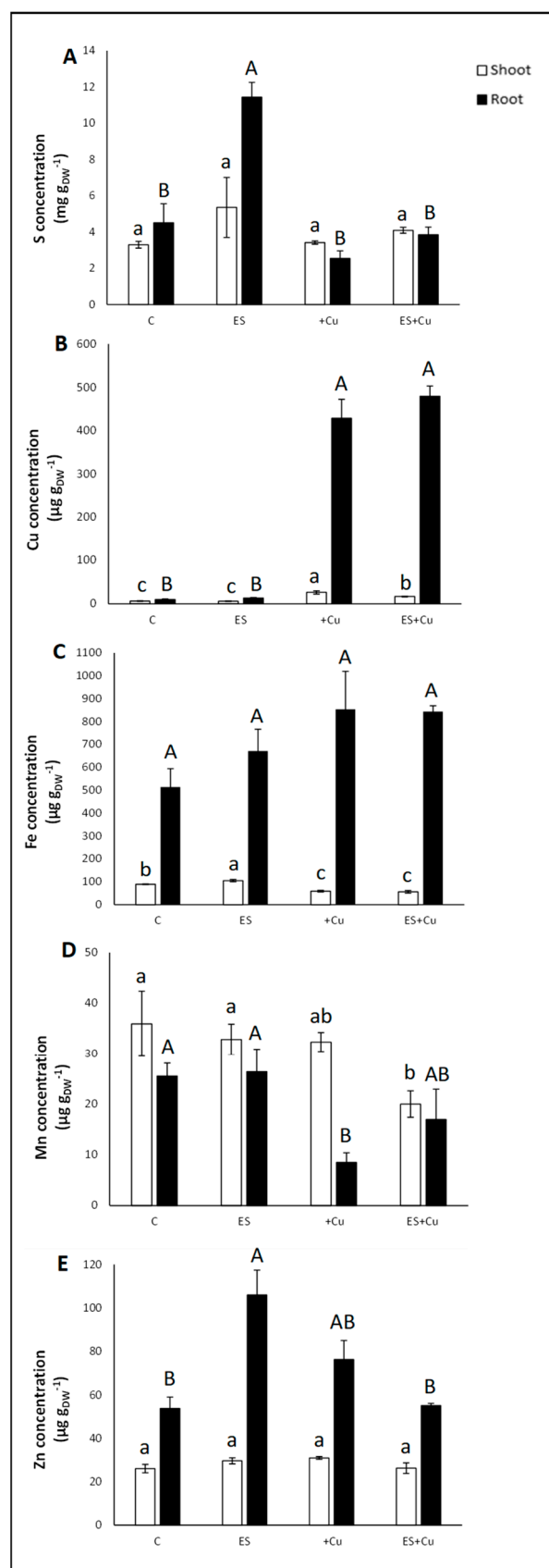
Figure 2. Sulfur (**A**), Cu (**B**), Fe (**C**) Mn (**D**), and Zn (**E**) concentration detected in shoots (white bars) and roots (black bars) of hemp plants grown hydroponically as described in Figure 1. Statistics as in Figure 1.

Data reported here highlight that the Cu treatment did not significantly affect Zn accumulation in hemp plants regardless of sulfate supply (Figure 2E). However, plants grown in the ES condition exhibited a higher capability to accumulate Zn at root level (+97% as compared to the control) (Figure 2E).

The PCA analysis was applied for each set of nutrient concentration data (Ca, Cu, Fe, Mg, Mn, Mo, P, S, and Zn) measured in the shoots and roots of hemp plants grown under the four growth conditions, with the aim of distinguishing different metal accumulation performances as a function of the growth condition (Figure 3A,B).
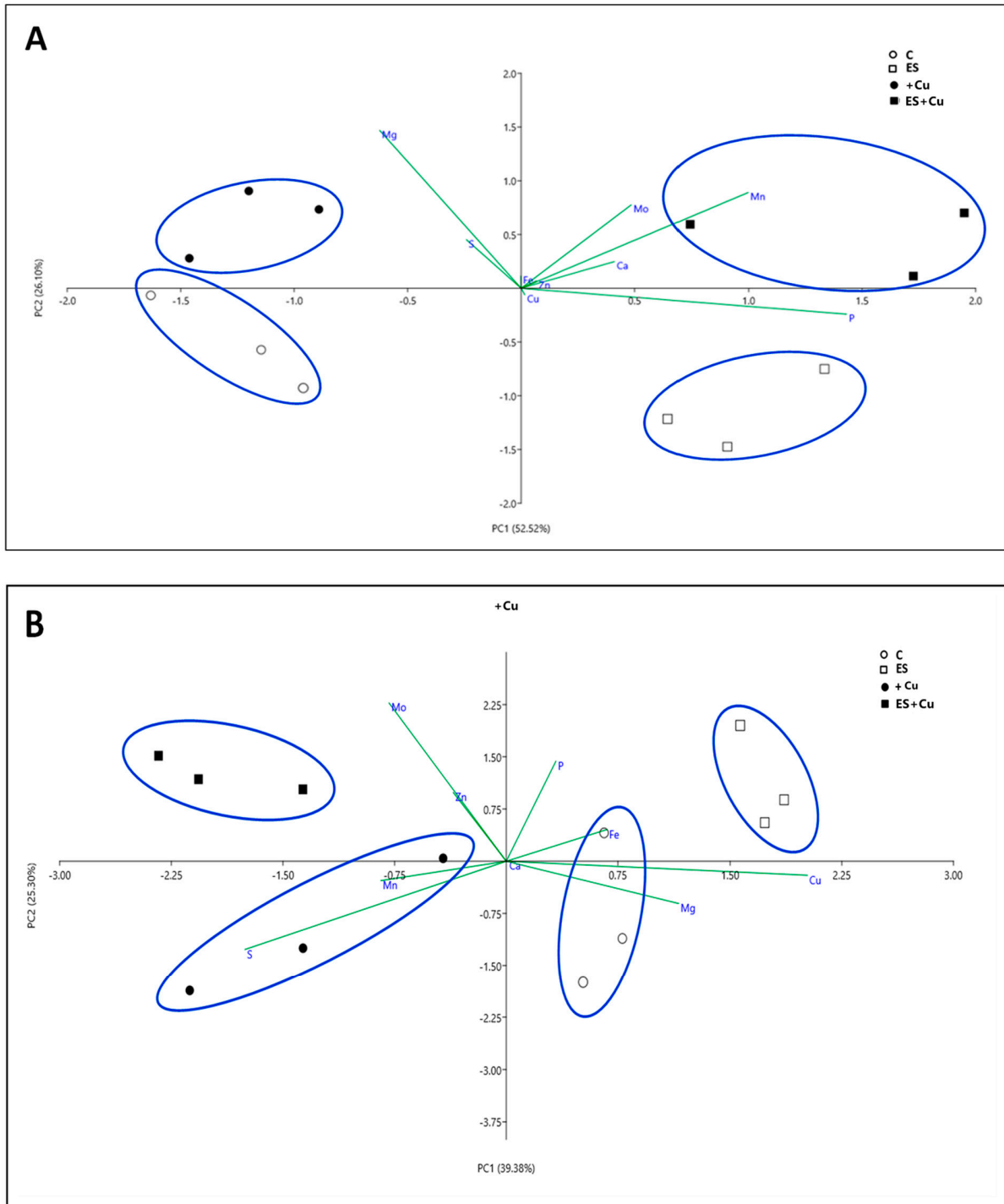

Figure 3. Principal component analysis (PCA) scatter plot of shoot **(A)** and root **(B)** ionome of hemp plants grown hydroponically as described in Figure 1.

Results for shoots are reported in Figure 3A, where the first two components, used to build the scatter plot, represented 78.62% of the total variance. The four different growth conditions (C, ES, +Cu, and ES + Cu) clustered in four distinct groups (Figure 3A). Along the first component (PC1), representing 52.52% of the total variance, the groups were distinguished according to the sulfate supply in the NS (i.e., +Cu vs. ES + Cu and C vs. ES). Along the second component (PC2), representing 26.10% of the total variance, the groups were distinguished in function of the Cu concentration in the NS (i.e., +Cu vs. C and ES + Cu vs. ES) (Figure 3A). Considering PC1, the positive contribution to the separation was given mostly by P (Figure S1). Only Mg and S concentrations contributed negatively to the separation along PC1 (Figure S1). Considering PC2, mainly Mg drove the distribution along the positive direction of the axis, while only P and Cu concentrations were the drivers for the negative direction of the axis (Figure S2).

When the PCA analysis was carried out with the ionome profile of hemp roots, the first two components described 64.68% of the total variance and the corresponding scatter plot is shown in Figure 3B. The PCA still clearly displays the separation of the four different growth conditions (C, ES, +Cu, and ES + Cu) in four distinct clusters (Figure 3B). In this case, PC1, representing 39.38% of the total variance, separated the different growth conditions as a function of the Cu concentration in the NS (i.e., ES + Cu vs. ES and +Cu vs. C). On the other hand, PC2, representing 25.30% of the total variance, separated the different growth conditions according to the sulfate concentration in the NS (i.e., ES + Cu vs. +Cu and ES vs. C) (Figure 3B). Along PC1, the positive influence for the distribution was given to a large extent by Cu, while the negative influence primarily by S concentration (Figure S3). Along PC2, the highest positive and negative influence for the distribution by nutrients was given by Mo and S, respectively (Figure S4).

3.2. Malondialdehyde Content

Malondialdehyde is one of the most common biomarkers of lipid peroxidation, an oxidative damage affecting the functionality of cell membranes. Figure 4 shows that the MDA content in shoots and roots was not significantly influenced neither by the Cu toxicity nor by the sulfate availability in the NS, except for shoots from plants exposed to ES condition, where this parameter was increased by 13% compared to the control shoots (Figure 4).
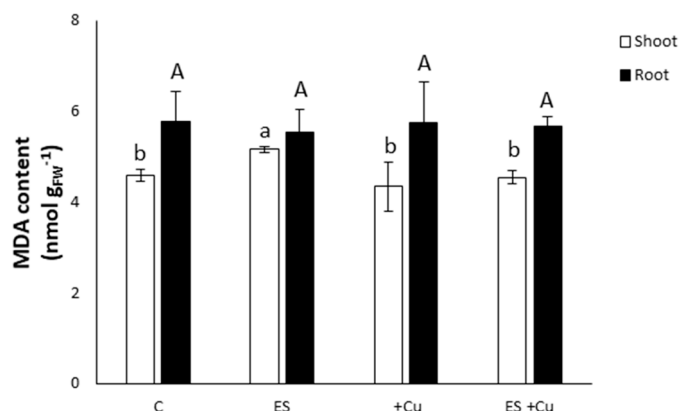

Figure 4. Malondialdehyde (MDA) content in shoots (white bars) and roots (black bars) of hemp plants. Growth conditions and statistics as in Figure 1.

3.3. Non-Protein Thiols Content and OASTL Activity Rate

Copper excess in the NS resulted in thiols levels 92% and 48% higher than control in both shoots and roots, respectively (Figure 5A). However, when Cu stress was imposed together with the sulfate treatment (ES + Cu condition), there was no accumulation of thiols in both shoot and root tissues, showing values similar to those of control (C condition) samples (Figure 5A).
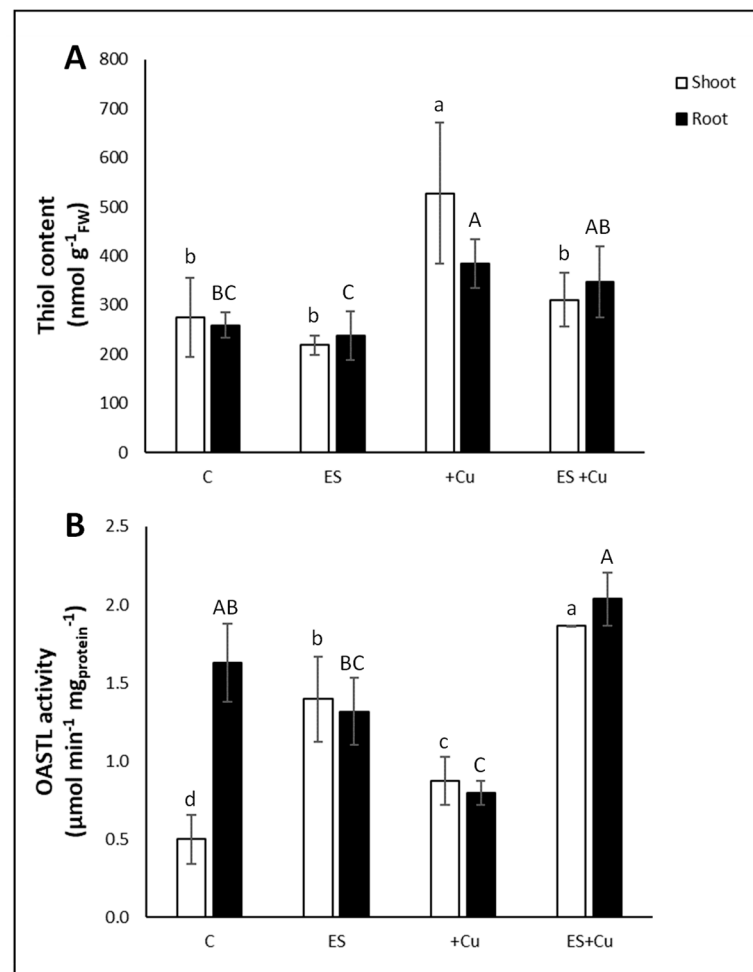
Figure 5. Thiol content (**A**) and *O*-acetylserine(thiol)lyase (OASTL) activity (**B**) in shoots (white bars) and roots (black bars) of hemp plants. Growth conditions and statistics as in Figure 1.

In shoots, OASTL activity was stimulated in all the three different growth conditions in the following order: ES + Cu condition (+274%) > ES condition (+180%) > +Cu condition (+75%), compared to C condition (Figure 5B). On the other hand, OASTL activity in roots of plants from both ES and ES + Cu conditions was consistently similar to that of control roots, whereas in +Cu roots it was significantly decreased (−51%) (Figure 5B).

4. Discussion

It is widely demonstrated that an excess of Cu availability in the growing medium negatively impacts plant growth and development, making Cu-contaminated soils unsuitable for the cultivation of food crops [8,9,15]. Considering the relevant and urgent challenges of the agricultural sector (i.e., transition towards sustainability as well as food safety and security for an increasing human population), here we evaluated the possibility of exploiting Cu-contaminated soils for the cultivation of energy crops, such as hemp [32,33]. Furthermore, being S directly involved in plant response to oxidative stress and nutritional disorders [34], the hypothesis that sulfate treatment could trigger plant Cu tolerance has been tested [8,23].

The results presented for the growth of hemp plants (measured in terms of change in fresh weight of shoots and roots after exposure to the four different treatments) clearly indicated that only shoot growth was strongly reduced by high Cu concentration (both +Cu and ES + Cu condition) (Figure 1). This result seems to be consistent with the suggestion that underground stresses usually lead to a decrease in biomass allocation to shoots in favor of roots, in order to compete more effectively for water and soil nutrients [35].

Leaf chlorosis is the most frequent and the first symptom appearing of Cu toxicity [8,17]. Accordingly, in the present work, chlorophyll content was significantly reduced in the leaves of both plants from +Cu condition and from ES + Cu one. However, a lower decrease could be observed between ES + Cu and control plants (−15%) than between +Cu and control plants (−20%) (Figure 1). This effect might be considered a protective action of sulfate at the level of chlorophyll biosynthesis against Cu toxicity [27,36].

As expected, Cu concentration increased in plants exposed to Cu treatment (Figure 2B). Interestingly, excess Cu combined with sulfate treatment (ES + Cu condition) resulted in a lower shoot Cu accumulation with respect to the +Cu condition (Figure 2B), suggesting a role of S in Cu allocation within the plant and its homeostasis.

Excess Cu also impairs the acquisition processes of other nutrients, leading to plant nutritional imbalances [17,20,22,37], due to either limited uptake, known as "phenomenon of inducible deficiency", or increased acquisition [37]. For instance, plant capability to accumulate S and Zn was not affected by Cu treatment (both single and combined with sulfate) (Figure 2A,E, respectively). On the other hand, exposure to excess Cu reduced Fe accumulation in shoot tissues (Figure 2C). The finding that root Fe concentration was not affected by Cu treatment could be explained considering that a huge Fe amount is present in this tissue in the apoplasm as an extraplasmatic pool [38,39]. However, the existence of competition phenomena among nutrients affecting Fe uptake and, then, its translocation to the shoot cannot be excluded [40,41].

It is also interesting to note that a limited Mn accumulation has been measured in the presence of Cu in the NS in +Cu roots and in ES + Cu shoots, projecting a potential risk for the activation of endogenous crop defense systems [42] (Figure 2D).

Plant responses in terms of nutrient uptake may depend on the plant species, the metal concentration to which plants are exposed, and the mechanisms of competition among nutrients occurring at the root plasma membrane level [43]. The root uptake of Cu is essentially mediated by the *COPT1* transporter [44], although transporters belonging to the *ZIP* family [45] as well as the Fe transporter (*IRT1*) [46] are certainly involved in this process. Therefore, it cannot be excluded that competition phenomena with other essential elements (specifically Fe) can occur in case of increased Cu concentration in the rhizosphere [8].

Principal component analysis revealed a clear separation of the four different conditions with respect to Cu or S treatment, suggesting that there were significant ionomic differences among the groups (Figure 3A,B). Interestingly, Cu was positively correlated with the second axis (PC2) in the shoot ionome and negatively correlated with the first axis (PC1) in the root ionome (Figure 3A,B, respectively).

Although high Cu availability in the growing medium interferes with root Fe uptake, some studies have shown that the treatment with Fe could alleviate the deleterious effects of Cu [47]. On the other hand, antagonistic uptake phenomena between Mg vs. Cu and Ca vs. Cu have been reported, suggesting that treatments with Mg or Ca can mitigate Cu toxicity, thus promoting the growth and development of different plant species [48–50].

Most remarkably, a significant increase in both Fe (Figure 2C) and Zn (Figure 2E) accumulation was found in ES shoots and roots, respectively, confirming other recent studies where the S benefits for Fe and Zn accumulation in durum wheat plants have been demonstrated [34]. The enhancement of Fe accumulation in plants exposed to high S supply could originate from a response mechanism based on an increased availability of S-containing metabolites, which are precursors for the synthesis of nicotianamine, ethylene, and phytosiderophores playing a key role in the improvement of Fe uptake activity [51,52].

Copper stress leads to increased production and accumulation of ROS, such as the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) [53]. These free radicals are usually present in cells as intermediate products of various metabolic pathways, but they represent a serious problem under stress conditions since their increased concentration can damage biological structures and alter the functionality of the cells and tissues, specifically by oxidizing lipids of plasma membranes [54]. The oxidative action on lipids proceeds by a

radical chain mechanism known as lipid peroxidation. The main targets of this process are polyunsaturated fatty acids, which are present in high concentrations in phospholipids of plasma membranes [55]. One of the biomarkers commonly used for the evaluation of the level of lipid peroxidation is MDA, which is a highly toxic by-product generated by lipid peroxidation [56]. In the present study, Cu stress (both +Cu and ES + Cu condition) did not significantly affect MDA content in both shoots and roots of hemp plants (Figure 4), suggesting that hemp plants are able to cope efficiently with oxidative stress. Overall, plants have complex defense systems to avoid or, at least, limit the heavy metal-induced oxidative damage. These systems can be enzymatic and non-enzymatic [57]. Among the non-enzymatic systems, ascorbic acid and glutathione are the compounds primarily involved in defense mechanisms against oxidative stress. Glutathione is a non-protein thiol with antioxidant properties [58]. Consequently, exposure of hemp plants to Cu could require an enhanced metabolic demand for reduced S (i.e., thiols), through an increased sulfate assimilation rate. Accordingly, the thiol level reached the highest values in both shoots and roots of Cu-treated plants (+Cu condition) (Figure 5A). However, the combination of Cu treatment with sulfate one (ES + Cu condition) did not lead to improved thiol content compared to the control (Figure 5A). To evaluate whether the increased thiol production induced by the Cu stress alone was related to an increase in the plant ability to assimilate sulfate, changes in OASTL activity in shoots and roots of hemp plants were analyzed. This enzyme catalyzes the last S assimilation reaction, leading to the synthesis of cysteine from *O*-acetylserine and inorganic sulfide [59]. In our study, the activity of OASTL was increased by Cu treatment (both single and combined) in shoots, whereas it was unaffected in plants exposed to combined treatment and even decreased in +Cu roots (Figure 5B). This different behavior of OASTL activity between shoots and roots could be explained considering that the sulfate assimilation occurs mainly at shoot level [60]. Thus, our results are consistent with the hypothesis of thiols translocation from shoots to roots, where their accumulation is known to inhibit root sulfate uptake and assimilation [61,62].

5. Conclusions

In conclusion, the present results suggest that hemp plants possess a good ability to adapt to the presence of high Cu concentrations in the growth medium, suggesting that soils with impaired fertility, such as those characterized by high Cu contents, can represent a valid opportunity at least for the cultivation of energy crops.

Furthermore, although S metabolism is involved in the plant response to face Cu toxicity, it remains to be clarified whether the use of exogenous sulfate may represent an agronomic practice to improve crop performance under these edaphic conditions.

The agriculture transition toward sustainability and the Sustainable Development Goals proposed by United Nation in the 2030 Agenda (https://sdgs.un.org/goals accessed on 26 July 2021) is yet to be achieved, but these findings could represent a valuable opportunity contributing to realize this goal.

Conflicts of Interest: The authors declare no conflict of interest.

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