


# Synergistic effects of biochar and biostimulants on nutrient and toxic element uptake by pepper in contaminated soils

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## Abstract

**BACKGROUND:** Nowadays a significant amount of land contaminated with toxic elements is being used for agriculture, posing a serious risk of crop contamination and toxicity. Several methodologies are being used to remediate soil contamination, including the use of amendments such as biochar. This work evaluated the effects of biochar combined with different fertirrigations (water, a conventional fertilizer solution, or a fertilizer solution with a commercial biostimulant derived from leonardite) on the availability of toxic elements and nutrients for pepper cultivated in a soil contaminated with As, Cd, Pb, and Zn.

**RESULTS:** Irrigation with fertilizer solutions improved plant growth regardless of the biochar amendment. Biochar decreased the bioavailability of Cu and Pb in soil and the Cu content in pepper leaves. Combined with fertilization, biochar also decreased plant As and Pb content. Biochar combined with biostimulant decreased the bioavailable content of Cd in soil and its uptake by pepper plants.

**CONCLUSION:** The use of biochar and biostimulant presented advantages for plant production in a non-suitable scenario of nutrient scarcity and contamination.

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**Keywords:** biostimulant; biochar; *capsicum annuum*; toxic elements; plant growth; leonardite

## INTRODUCTION

More and more food is required to sustain the world population.<sup>1</sup> This implies not only an increase in the consumption of fertilizers and water but also the farming of new lands, increasing the pressure on the environment.<sup>2,3</sup> Some of these new croplands are contaminated with toxic elements (TEs) due to anthropogenic environmental changes.<sup>4</sup> Plants growing in TE-polluted sites exhibit altered metabolism, growth reduction, lower biomass production, and TE accumulation.<sup>5</sup> Soil properties such as pH, organic matter (OM), and clays can affect TE mobility and bioavailability.<sup>4,6,7</sup> Some soil organic amendments originating from organic wastes from human activity have been proven to restore and reclaim degraded soils effectively by maintaining organic matter and sustaining soil fertility for agricultural production.<sup>8</sup> Among them, biochar has shown clear potential for the reduction of a wide variety of organic and inorganic soil contaminants.<sup>9</sup>

Biochar is an alkaline recalcitrant C-rich material produced from the pyrolysis of biomass under oxygen-limited conditions.<sup>10</sup> Biochar may represent a low-cost and effective adsorbent because

the production of biochar is cheaper with lower energy requirements than other adsorbents.<sup>11</sup> Biochar is not a homogeneously structured material; it possesses a range of chemical structures and a heterogeneous elemental composition, which makes it capable of adsorbing contaminants like metals.<sup>12,13</sup> Biochar

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amendment has been shown to increase soil organic C and promote C stabilization when co-applied with other organic fertilizers through the formation of organo-mineral complexes,<sup>14</sup> to increase soil water retention and cation exchange capacity,<sup>15,16</sup> and effectively enhance plant growth and alleviate salt stress injuries.<sup>17</sup> However, biochar alone does not directly enrich the soil with nutrients and may cause N immobilization by elevating the C/N ratio. Thus, biochar should be used in combination with other nutrient supplements like fertilizers.<sup>18</sup>

The use of organic fertilizers to increase crop productivity has been proven to be an alternative way to reduce the agricultural pressure on the environment.<sup>19</sup> Good agricultural practices, especially proper use of mineral and organic fertilizers, may contribute to decrease heavy metal availability to plants. Due to their sorption capacity, organic fertilizers may be particularly effective to bind trace elements in soil.<sup>20</sup> This kind of product, including the so-called biostimulants, is intended to replace conventional fertilizers by providing greater efficiency and being more respectful to the environment.<sup>21</sup> Humic substances are considered to be biostimulants and are the product of the biochemical transformation of organic materials<sup>22</sup> with physical and chemical characteristics that depend on the type of the original organic material from which they derive.<sup>23</sup> They may exert positive effects on crops like an improvement in photosynthetic efficiency by increasing organic carbon levels and available nutrients.<sup>24,25</sup> Other proposed mechanisms consisted of 'indirect action' on the metabolism of the microbial population and the physical conditions of the soil, which result in a better nutrient uptake.<sup>26,27</sup>

The objective of this work was to assess the development of pepper (*Capsicum annuum* L.) in an agricultural soil contaminated with toxic elements, and to investigate the effects of biochar and a commercial biostimulant derived from leonardite on pepper growth, nutrient status and toxic element content. This use of materials could provide an innovative approach to restore contaminated soils for agriculture.

## MATERIALS AND METHODS

### Soil, biochar, fertilizers and biostimulant

The soil sample used in this work was collected from the topsoil (0–20 cm depth) of a potato cropland located in Murcia (Spain), which was contaminated with several toxic elements due to the mining activity of precious metals (e.g., silver) carried out near the area. Fifty subsamples of 1 kg of soil were taken following a zig-zag pated and mixed to form the soil sample. The composite soil sample was air dried and sieved to 2 mm. The biochar was supplied by PiroEco Bioenergy SL (Málaga, Spain) and was produced from milled holm oak (*Quercus ilex*) pruning (< 8 mm) pyrolyzed at 500 °C. The biochar was used as supplied.

The main characteristics of soil and biochar were determined as follows: pH was measured in 1:5 aqueous suspensions; electrical conductivity (EC) in 1:2.5 aqueous extracts; OM by calcination in a muffle furnace at 450 °C for 16 h for biochar and titration of potassium dichromate for soil; soil texture by the Boyoucos method; carbonate content with the Bernard calcimeter; and total C, N, and S content using a LECO Element Analyzer CHNS-932 (St Joseph, MI, USA). The biochar was analyzed by the Fourier-transform infrared (FTIR) spectroscopy KBr pellet method using a Bruker IFS66v spectrometer (Billerica, MA, USA). Total elements in soil and biochar were extracted by wet digestion assisted by microwave oven using a pressurized closed-vessel microwave system (CEM Mars X Press, Matthews, NC, USA).

Samples were first milled and weighed to 250 mg ( $\pm$  0.1 mg), placing them in microwave polyfluoroacetylene vessels. Then, 12 mL of aqua regia (9 mL of 35% HCl + 3 mL of 69% HNO<sub>3</sub>) was added to the vessels and subjected to a digestion consisting of a 15 min gradual increase to 200 °C, a 15 min step at 200 °C, 1200 W, and then a cooling stage.<sup>28</sup> After cooling, the suspension was filtered through an ashless Whatman 42 filter, diluted to 50 mL with Milli-Q water, and stored in polyethylene bottles at 4 °C for analyses. Blanks were treated using the same procedure. The element concentration in the digestates was determined by inductively coupled plasma mass spectrometry (ICP-MS). The accuracy of the soil digestion was evaluated using the Certified Reference Material soil NCSDC 73031 (CS Testing Technology Co., Ltd, Shenzhen, China). Recovery percentages were 75–92%.

The fertilizer solutions were prepared with analytical grade salts (Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O, KH<sub>2</sub>PO<sub>4</sub>, K<sub>2</sub>SO<sub>4</sub>, MgSO<sub>4</sub>·7H<sub>2</sub>O, (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, CuSO<sub>4</sub>·5H<sub>2</sub>O, ZnSO<sub>4</sub>·H<sub>2</sub>O, H<sub>3</sub>BO<sub>3</sub>, MnSO<sub>4</sub>·H<sub>2</sub>O (Panreac, Barcelona, Spain), KNO<sub>3</sub> (Merck & Co., Inc., Kenilworth, NJ, USA) and Fe-EDDHA (Quimioprox, Barcelona, Spain) and had the following nutrient composition: 15, 2, 9, 10, 3 mmol L<sup>-1</sup> of N, P, K, Ca and Mg, respectively, and 2, 1, 0.1, 4, 0.5, 0.05 mg L<sup>-1</sup> of Fe, Mn, Cu, Zn, B, and Mo, respectively.<sup>29</sup> The biostimulant HUMA GRO® X-Tend (6–2-0, 6.02% organic matter) was provided by Bio Huma Netics, Inc. (Gilbert, AZ, USA).<sup>30</sup> This product consists of a concentrated liquid produced from humic acid derivatives obtained after biological digestion of leonardite from Arizona (USA).

### Pot experiment

Pepper plants (*Capsicum annuum* L.), Brocanto cultivar (Surinver Coop. V., Murcia, Spain), were cultivated in plastic pots (one plant per plot) containing 2 kg of soil under the following treatments: irrigation with distilled water (W); irrigation with distilled water and amendment with biochar at a rate of 5% (w/w, WB); irrigation with conventional fertilizer solution (C); irrigation with conventional fertilizer solution and amendment with biochar at a rate of 5% (w/w, CB); irrigation with conventional fertilizer solution and biostimulant at 0.2% v/v (X); or irrigation with conventional fertilizer solution and biostimulant (0.2% v/v) and amendment with biochar at a rate of 5% (w/w, XB). Four replicates were used for each treatment.

The experiment was performed in a temperature and humidity-controlled culture chamber and terminated after 35 days when severe nutritional stress in plants under the W treatment was observed. The daily growth cycle was 14 h, 23 °C and 40% relative humidity during the day, and 10 h, 19 °C and 60% relative humidity at night. Irrigation was adjusted by an automatic system using pumps and self-compensating drippers of 4 L h<sup>-1</sup> for 5 min twice per day.

### Sampling and analysis

#### Plant sampling and analysis

Soil and plant analysis development (SPAD) readings were taken on fresh leaves 35 days after transplanting. The SPAD index is proportional to the amount of chlorophyll in the leaf.<sup>31</sup> The leaves were analyzed by radiometry, registering the entire ultraviolet-visible (UV-visible) spectrum using a portable ultraviolet-visible near infrared (UV-visible-NIR) spectrometer (Stellarnet Inc., Tampa, FL, USA). Photochemical Reflectance Index (PRI), a physiological index sensitive to the epoxidation state of the xanthophyll cycle pigments and photosynthetic efficiency, was calculated as the standardized difference of the 530 nm band related to the

absorption of xanthophyll pigments and a reference band located at 570 nm [ $PRI = (R_{531} - R_{570}) / (R_{531} + R_{570})$ ].<sup>32</sup>

Plant growth was determined by weighing fruits, leaves, stems, and roots of the pepper plants. Plant roots were washed with distilled water before the analysis. The plant material was dried in an oven at 60 °C until constant weight. Nutrients and TEs were analyzed after high-pressure acid-oxidant digestion. In particular, 250 mg of milled plant samples (fruits, leaves, stems, and roots) was digested with 4 mL of H<sub>2</sub>O Milli-Q, 1.5 mL of HNO<sub>3</sub> (97%), and 1 mL of H<sub>2</sub>O<sub>2</sub> (30%) in autoclave at 125 °C and 1.5 kPa for 30 min,<sup>33</sup> brought to 25 mL, and the digestates analyzed by ICP-MS (NexION 300XX, Perkin-Elmer, Waltham, MA, USA). The accuracy of the plant material digestion was evaluated using the Certified Reference Material citrus leaves NCSZC 73018 (CS Testing Technology Co., Ltd, Shenzhen, China). Recovery percentages were in the 89–123% range.

#### Soil sampling and analysis

The soil in each pot was separated from the plant, dried, and extracted with aqua regia as described above. The Community Bureau of Reference sequential extraction scheme, usually called the Community Bureau of Reference (BCR) method, was used to determine the modifications in the distribution pattern of TEs. This extraction procedure consisted of an initial acetic acid extraction (0.11 mol L<sup>-1</sup>, 16 h) to obtain an acid soluble fraction, a second extraction with hydroxylamine hydrochloride (0.5 mol L<sup>-1</sup>, pH 1.5, 16 h) to obtain a reducible fraction (Fe–Mn oxides), a third extraction with hydrogen peroxide (8.8 mol L<sup>-1</sup>, 2 × 1 h, 85 °C), followed by ammonium acetate (1 mol L<sup>-1</sup>) to obtain an oxidizable fraction (OM and sulfides), and a fourth extraction with aqua regia to obtain residual fractions strongly associated with crystalline structures of minerals. All the extracts were analyzed by ICP-MS (NexION 300XX, Perkin-Elmer). The accuracy of the soil digestion methodology used was evaluated using the Certified Reference Material soil NCSDC 73031 provided by CS Testing Technology Co., Ltd (China). Recovery percentages were in the range of 75–92%.

#### Data analysis

Data were evaluated statistically by one-way and two-way ANOVAs followed by *post hoc* Duncan tests with a level of significance of 95% ( $P \leq 0.05$ ) using the IBM SPSS v20 (Armonk, NY, USA).

## RESULTS AND DISCUSSION

### Physico-chemical characteristics of soil and biochar

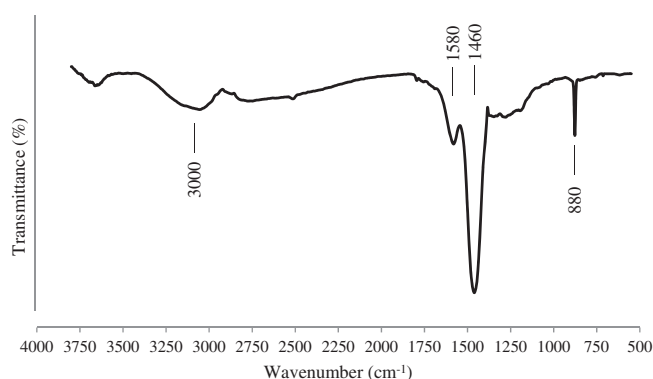
The soil had a sandy clay loam texture, slightly alkaline pH, and low organic matter content (Table 1). The P, K, and Mg content was relatively high,<sup>34</sup> which may be related to the historical fertilization applied to the soil. The As, Cd, Pb, and Zn content was over the threshold values of agricultural soils.<sup>35</sup>

The biochar had an alkaline pH due to acidic functional group removal and enrichment with alkali and alkaline earth elements during pyrolysis<sup>36</sup> (Table 1). The low O/C and H/C ratios indicated that the biochar was highly stable (minimum life estimated at 1000 years).<sup>12</sup> The relatively high polarity index ((O + N) / C ratio) indicated a high affinity for polar compounds, such as metal compounds.<sup>37</sup> Pyrolytic temperature had a considerable influence on the surface functional groups of biochar, as indicated by the low intensity or absence of a number of FTIR bands commonly found in untreated biomasses<sup>11</sup> (Fig. 1). In particular, the FTIR spectrum

**Table 1.** Physical and chemical characteristics of the contaminated soil and the biochar used in this work (mean ± standard deviation, n = 3)

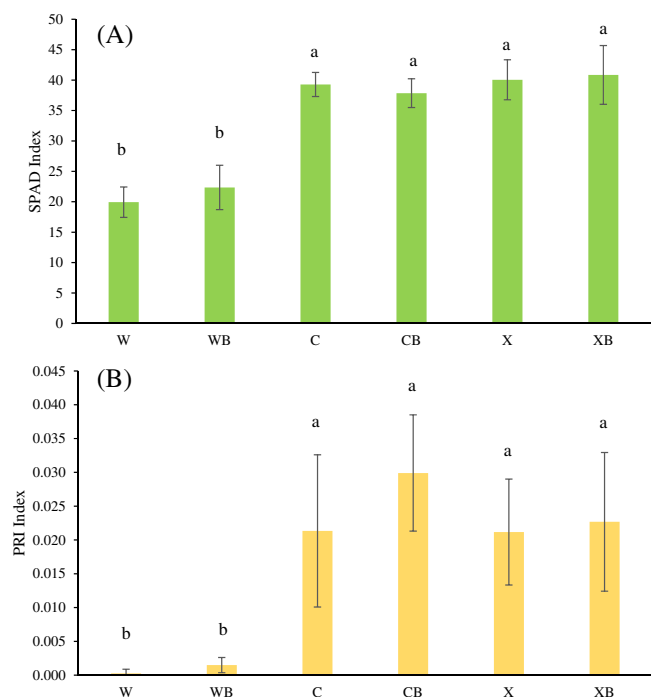
	Soil	Biochar
Sand (%)	52	-
Silt (%)	16	-
Clay (%)	32	-
pH	7.34	9.29
E.C. 25 °C (dS·m <sup>-1</sup> )	0.449	0.49
Total limestone (%)	5.01	-
Total O.M. (%)	1.69	79.6 ± 0.4
C (%)	0.979	78.19
Total N (%)	0.110	0.02
O (%)	-	5.73
H (%)	-	1.88
C/N	8.9	3910
P (mg·kg <sup>-1</sup> )	302 ± 60	740 ± 127
K (g·kg <sup>-1</sup> )	11.8 ± 0.3	7 ± 2
Mg (g·kg <sup>-1</sup> )	8.2 ± 0.3	2.8 ± 0.1
Ca (g·kg <sup>-1</sup> )	18 ± 2	40 ± 8
S (mg·kg <sup>-1</sup> )	1993 ± 330	146 ± 13
Si (mg·kg <sup>-1</sup> )	1019 ± 570	44 ± 8
B (mg·kg <sup>-1</sup> )	43 ± 30	13 ± 3
Cu (mg·kg <sup>-1</sup> )	72 ± 3	12 ± 3
Co (mg·kg <sup>-1</sup> )	11.1 ± 0.2	1.5 ± 0.3
Fe (g·kg <sup>-1</sup> )	57 ± 1	0.3 ± 0.2
Mn (mg·kg <sup>-1</sup> )	1892 ± 157	489 ± 59
Mo (mg·kg <sup>-1</sup> )	1.03 ± 0.09	0.11 ± 0.09
Zn (mg·kg <sup>-1</sup> )	5215 ± 125	39 ± 18
Al (g·kg <sup>-1</sup> )	102 ± 2	1.2 ± 0.8
As (mg·kg <sup>-1</sup> )	141 ± 6	0.19 ± 0.02
Cd (mg·kg <sup>-1</sup> )	16.6 ± 0.3	N.D.
Cr (mg·kg <sup>-1</sup> )	48 ± 2	N.D.
Ni (mg·kg <sup>-1</sup> )	40 ± 12	8 ± 1
Pb (mg·kg <sup>-1</sup> )	6608 ± 206	2 ± 1

E.C., electric conductivity; O.M., organic matter.



**Figure 1.** Fourier transfer infrared spectrum of the biochar.

of the biochar that was examined was dominated by an intense band at 1580 cm<sup>-1</sup>, characteristic of aromatic rings' skeletal vibration.<sup>38</sup> The FTIR spectrum also has a band at 1460 cm<sup>-1</sup>, derived from aromatic C–C stretching,<sup>14</sup> a broad absorption at 3000 cm<sup>-1</sup>, attributable to aromatic C–H and indicative of incomplete combustion of cellulose,<sup>39</sup> and a peak at 880 cm<sup>-1</sup>,



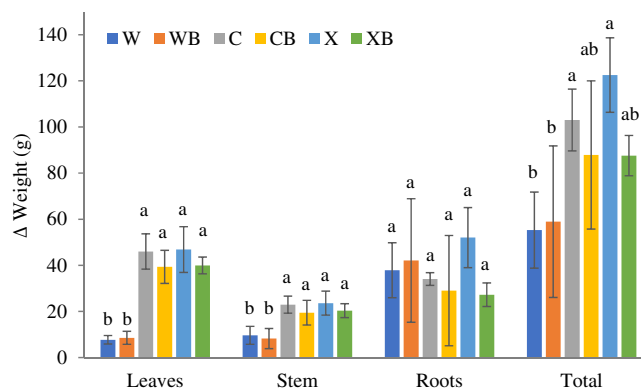
**Figure 2.** Soil plant analysis development chlorophyll index (A) and photochemical reflectance index (PRI) (B) of pepper leaves from plants irrigated with water (W), conventional fertilizer solution (C) and biostimulant solution (X) in unamended or biochar amended (B) soils. Mean  $\pm$  SD. Different letters indicate significant differences between treatments ( $P < 0.05$ ,  $n = 4$ ).

attributed to C—H aromatic vibrations<sup>40</sup> (Fig. 1). The high pH of the biochar indicated that carboxylic and phenolic hydroxyl groups were mostly deprotonated and available for metal complexation.

### Effects on plant

The SPAD index and PRI were significantly lower for pepper plants irrigated with water regardless of biochar treatment (Fig. 2(A) and (B)). These treatments resulted in deficient nutrition because the soil did not have enough nutrients for an optimal growing cycle and xanthophyll cycle conversion.<sup>41</sup> We found no significant differences among plants irrigated with conventional fertilizer solutions (C and CB) and biostimulant fertilizer solutions (X and XB) regardless of biochar amendment. Biostimulants had higher effects on roots than on above-ground biomass as reported,<sup>42</sup> which may explain limited effects on chlorophyll activities.

We found significant differences in weight increments of aerial biomass, leaves, and stems between plants irrigated with the two nutritional solutions (C and X) and those irrigated with water (W) (Fig. 3). The nutritional deficiency under W and WB treatments, compared to the fertilizer treatments (C and X), resulted in lower growth of the aerial part of the pepper plants but a similar root growth. Amendment with biochar (WB, CB, and XB) did not exert significant effects on pepper growth with respect to the analogous treatments without biochar (W, C, and X). Several studies have shown that the ability of biochar to increase plant growth depends strongly on soil factors such as pH and nutritional status.<sup>16,18</sup> In general, the biostimulant (X) induced the greatest increases in weight. No significant differences were found in fruit production (data not shown) among treatments.



**Figure 3.** Increments of fresh weights (mean  $\pm$  SD) of pepper plants irrigated with water (W), conventional fertilizer solution (C) and biostimulant solution (X) in unamended or biochar-amended (B) soils. The increment was calculated using fresh weights at the beginning and at the end of the experiment. Different letters indicate significant differences between treatments ( $P < 0.05$ ,  $n = 4$ ).

The foliar content of macronutrients was adequate for K, low for P, and high for Mg and Ca for all the treatments<sup>43</sup> (Table 2). The most variable nutrient was P, for which there was a significant interaction between fertilization and biochar application (Table 2). The highest concentration of P was found in the plants irrigated with water (W and WB), which was statistically not different from that of plants under the CB treatment. This suggests that biochar mobilizes non-bioavailable forms of P or act as P storage in conventional fertilization. Previous studies have shown that biochar is able to increase the recovery of phosphate by chemisorption and precipitation.<sup>44</sup> The K content in leaves was lower for plants irrigated with water where no nutrients were supplied. This explains the differences previously observed for the SPAD activities (Fig. 2) since K is strongly related to SPAD index.<sup>45</sup> The highest content of Ca in leaves was found in plants irrigated with water. This could be related to the ability of plants to solubilize soil Ca through organic acid root exudates in nutritional stress scenarios<sup>46</sup> or to the antagonism of K versus (Ca + Mg). There were no significant differences in leaf Mg content among treatments.

Concerning the micronutrient content of leaves, significant differences were found for B, Cu, Fe, Mo, and Zn but not for Co, Mn, and Si (Table 2). Leaves of plants grown in soils amended with biochar exhibited the lowest Cu content. For all the treatments, Cu leaf content was below deficit limits.<sup>47</sup> In contrast, B, Mo and Zn content was at adequate levels for the fertilized treatments (X and C) or even higher for the unfertilized ones (W and WB), which could be related to the ability of plants to mobilize nutrients from soil in stress scenarios by exuding organic acids.<sup>48</sup> Fe showed an opposite pattern, with lower foliar values for the W and WB treatments, which was consistent with the SPAD index because Fe is an essential element for chlorophyll synthesis.<sup>49</sup>

Concerning the toxic elements, significant differences were found in the foliar content of As, Cd, and Pb among treatments, whereas the foliar contents of Al, Cr, and Ni were not affected by the fertilization nor the biochar application (Table 2), probably because these elements typically have low availability and are strongly adsorbed in soil. The highest concentration of As was found for W and WB treatments, whereas C and X treatments resulted in less content of As in leaf. Pb followed the same pattern with higher values for unfertilized treatments and lower for treatments with fertilizers. Irrespectively of the fertilization treatment,

**Table 2.** Content of macronutrients, micronutrients, and toxic elements (mean  $\pm$  SD,  $n = 4$ ) in pepper leaves as affected by irrigation irrigated with water (W), conventional fertilizer solution (C), and biostimulant solution (X) in unamended or biochar amended (B) soils. A two-way ANOVA was performed to evaluate the effect of fertilization (F) and biochar application (B) and their interaction

	W	WB	C	CB	X	XB	F	B	FxB
	mg·kg <sup>-1</sup>						Two-way ANOVA		
P	807 $\pm$ 111 <sup>ab</sup>	987 $\pm$ 195 <sup>a</sup>	618 $\pm$ 53 <sup>c</sup>	817 $\pm$ 98 <sup>ab</sup>	784 $\pm$ 121 <sup>bc</sup>	782 $\pm$ 18 <sup>bc</sup>	*	*	*
K	51 998 $\pm$ 1515 <sup>c</sup>	54 857 $\pm$ 2228 <sup>c</sup>	62 190 $\pm$ 3474 <sup>b</sup>	65 620 $\pm$ 1582 <sup>a</sup>	59 998 $\pm$ 1473 <sup>b</sup>	61 051 $\pm$ 2214 <sup>b</sup>	***	*	N.S.
Mg	9058 $\pm$ 1679 <sup>a</sup>	8284 $\pm$ 793 <sup>a</sup>	7956 $\pm$ 378 <sup>a</sup>	8128 $\pm$ 356 <sup>a</sup>	8040 $\pm$ 600 <sup>a</sup>	8198 $\pm$ 1108 <sup>a</sup>	N.S.	N.S.	N.S.
Ca	25 031 $\pm$ 3308 <sup>a</sup>	20 381 $\pm$ 3016 <sup>b</sup>	20 287 $\pm$ 273 <sup>b</sup>	19 552 $\pm$ 2242 <sup>b</sup>	20 171 $\pm$ 1832 <sup>b</sup>	18 979 $\pm$ 2509 <sup>b</sup>	*	*	N.S.
B	160 $\pm$ 40 <sup>a</sup>	114 $\pm$ 38 <sup>b</sup>	58 $\pm$ 11 <sup>c</sup>	55 $\pm$ 17 <sup>c</sup>	53 $\pm$ 37 <sup>c</sup>	76 $\pm$ 15 <sup>bc</sup>	***	N.S.	N.S.
Cu	8 $\pm$ 1 <sup>ab</sup>	6 $\pm$ 1 <sup>b</sup>	7.8 $\pm$ 0.7 <sup>ab</sup>	7.2 $\pm$ 0.3 <sup>b</sup>	9 $\pm$ 2 <sup>a</sup>	7 $\pm$ 1 <sup>b</sup>	N.S.	**	N.S.
Co	0.02 $\pm$ 0.02 <sup>a</sup>	0.03 $\pm$ 0.03 <sup>a</sup>	0.035 $\pm$ 0.007 <sup>a</sup>	0.04 $\pm$ 0.02 <sup>a</sup>	0.03 $\pm$ 0.02 <sup>a</sup>	0.03 $\pm$ 0.02 <sup>a</sup>	N.S.	N.S.	N.S.
Fe	39 $\pm$ 4 <sup>b</sup>	41 $\pm$ 6 <sup>b</sup>	72 $\pm$ 4 <sup>a</sup>	75 $\pm$ 3 <sup>a</sup>	81 $\pm$ 17 <sup>a</sup>	67 $\pm$ 6 <sup>a</sup>	***	N.S.	N.S.
Mn	77 $\pm$ 17 <sup>a</sup>	63 $\pm$ 30 <sup>a</sup>	47 $\pm$ 8 <sup>a</sup>	57 $\pm$ 24 <sup>a</sup>	75 $\pm$ 23 <sup>a</sup>	52 $\pm$ 11 <sup>a</sup>	N.S.	N.S.	N.S.
Mo	5 $\pm$ 2 <sup>a</sup>	5 $\pm$ 2 <sup>a</sup>	2.3 $\pm$ 0.6 <sup>b</sup>	1 $\pm$ 1 <sup>b</sup>	3 $\pm$ 1 <sup>ab</sup>	2.1 $\pm$ 0.5 <sup>b</sup>	**	N.S.	N.S.
Si	131 $\pm$ 115 <sup>a</sup>	213 $\pm$ 51 <sup>a</sup>	136 $\pm$ 23 <sup>a</sup>	129 $\pm$ 19 <sup>a</sup>	179 $\pm$ 56 <sup>a</sup>	176 $\pm$ 41 <sup>a</sup>	N.S.	N.S.	N.S.
Zn	120 $\pm$ 7 <sup>a</sup>	118 $\pm$ 17 <sup>a</sup>	86 $\pm$ 1 <sup>b</sup>	88 $\pm$ 12 <sup>b</sup>	85 $\pm$ 9 <sup>b</sup>	83 $\pm$ 8 <sup>b</sup>	***	N.S.	N.S.
Al	33 $\pm$ 32 <sup>a</sup>	14 $\pm$ 1 <sup>a</sup>	9 $\pm$ 2 <sup>a</sup>	11 $\pm$ 3 <sup>a</sup>	166 $\pm$ 248 <sup>a</sup>	35 $\pm$ 34 <sup>a</sup>	N.S.	N.S.	N.S.
As	0.93 $\pm$ 0.05 <sup>a</sup>	0.603 $\pm$ 0.06 <sup>b</sup>	0.31 $\pm$ 0.09 <sup>cd</sup>	0.27 $\pm$ 0.06 <sup>d</sup>	0.25 $\pm$ 0.07 <sup>d</sup>	0.39 $\pm$ 0.07 <sup>c</sup>	***	*	***
Cd	6 $\pm$ 1 <sup>bc</sup>	9 $\pm$ 2 <sup>a</sup>	5.1 $\pm$ 0.8 <sup>cd</sup>	6.8 $\pm$ 0.7 <sup>b</sup>	4.6 $\pm$ 0.6 <sup>de</sup>	3.2 $\pm$ 0.3 <sup>e</sup>	***	*	**
Cr	0.09 $\pm$ 0.05 <sup>a</sup>	0.08 $\pm$ 0.03 <sup>a</sup>	0.05 $\pm$ 0.006 <sup>a</sup>	0.05 $\pm$ 0.01 <sup>a</sup>	0.08 $\pm$ 0.08 <sup>a</sup>	0.06 $\pm$ 0.03 <sup>a</sup>	N.S.	N.S.	N.S.
Ni	0.03 $\pm$ 0.05 <sup>b</sup>	1 $\pm$ 1 <sup>a</sup>	0.02 $\pm$ 0.03 <sup>b</sup>	N.D.	0.5 $\pm$ 0.6 <sup>ab</sup>	0.05 $\pm$ 0.07 <sup>b</sup>	N.S.	N.S.	N.S.
Pb	3.5 $\pm$ 0.4 <sup>a</sup>	2.8 $\pm$ 0.6 <sup>b</sup>	1.8 $\pm$ 0.3 <sup>cd</sup>	1.6 $\pm$ 0.5 <sup>d</sup>	2.3 $\pm$ 0.1 <sup>bc</sup>	1.7 $\pm$ 0.4 <sup>cd</sup>	***	*	N.S.

Different letters indicate significant differences between treatments ( $n = 4$ ; Duncan's test,  $P < 0.05$ ). N.S., not significant; N.D., not detected.  
 \* $P < 0.05$ .  
 \*\* $P < 0.01$ .  
 \*\*\* $P < 0.001$ .

biochar reduced Pb foliar content, which may be related to the capacity of biochar to immobilize metals.<sup>11</sup> Similarly, the application of C or X fertilization produced a significant reduction in foliar Pb with respect to W and WB treatments. The foliar concentration of Cd was also significantly affected by fertilization and biochar. However, the use of conventional fertilization did not significantly decrease the foliar concentration of Cd with respect to W. In contrast, the application of biostimulant resulted in less foliar Cd content. The application of biochar produced a significant reduction of the foliar concentration of Cd with respect to the unamended soil, except in the plants fertilized with X treatment, denoting the significant interaction between biochar and fertilization treatments. In general, the application of biochar and fertilization resulted in higher content of foliar P and lower content of As, Pb and Cd. The use of biostimulant improved plant nutritional quality<sup>50</sup> and its application combined with biochar may be a promising way to safely use polluted soils for agriculture.

The contents of Cu, Fe and Zn in pepper fruit were slightly below optimal.<sup>43</sup> No significant differences were found in the concentration of Cu, Fe, and Zn in pepper fruit among the treatments (Table 3).

Concerning the TEs in fruit, the treatments with no fertilization resulted in the highest As and Cd content, and biochar did not exert any significant effects. The concentrations of TEs in fruit were below toxic levels: 0.1 mg kg<sup>-1</sup> for As<sup>51</sup> and 0.1 and 0.05 mg kg<sup>-1</sup> for Pb and Cd respectively<sup>52</sup> (Table 3). Many factors may affect the bioaccumulation magnitude of TEs in fruits and vegetables, such as crop physiological properties<sup>53</sup> and dust deposition.<sup>54</sup> Vegetable contamination generally increases non-linearly in response to soil contamination level.

### Effects on soil

We examined the speciation of Cu, Fe, and Zn in soil by BCR sequential extraction (Fig. 4) as the foliar content of these micronutrients was found to be significantly affected by the treatments (Table 2). Copper was mainly associated with the residual fraction. However, appreciable percentages of Cu were associated with the reducible (5–6%) and oxidizable (5–6%) fractions, respectively. Although no differences were found in the pepper fruit, the foliar analysis showed significant lower concentrations with biochar application (Table 2). Biochar treatments decreased the occurrence of Cu in the most bioavailable fractions for plants (fraction 1 and 2). This was consistent with the relatively lower Cu foliar content found for biochar treatments.<sup>5</sup>

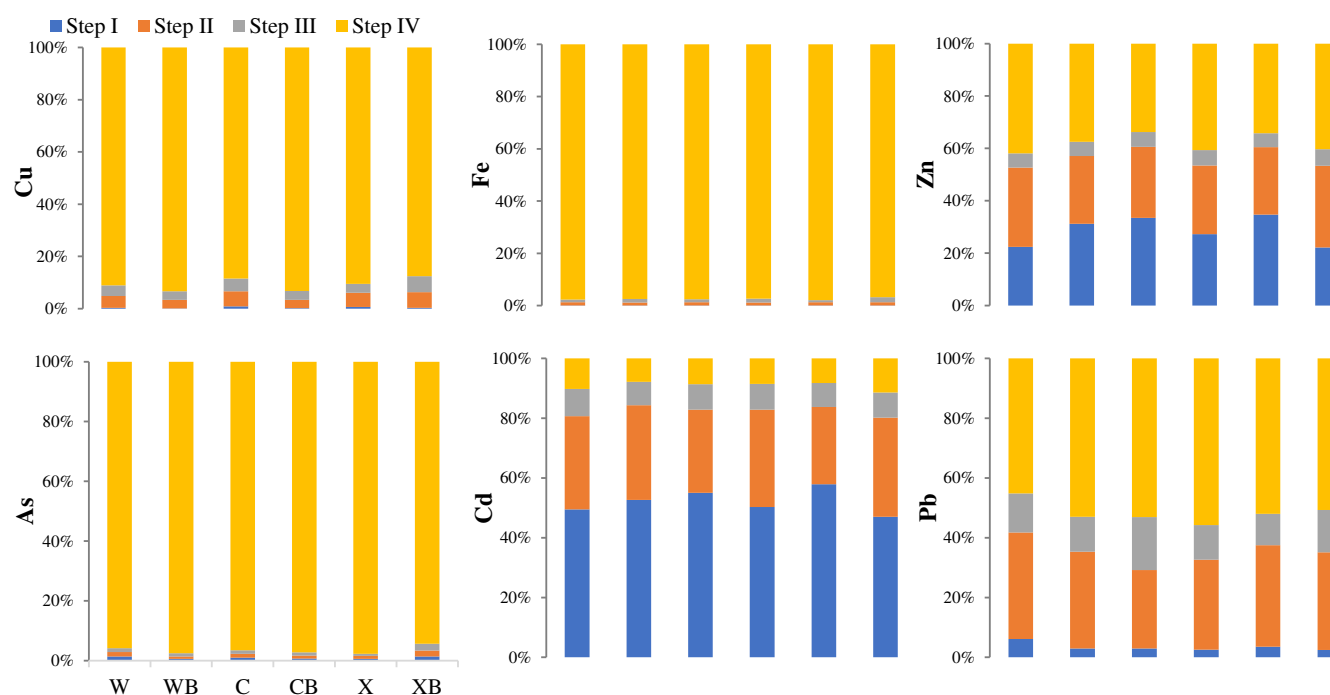
Iron, at the end of the experiment, was mainly found in the residual structural fraction (Fig. 4). Biochar and fertilization increased acid soluble and oxidizable fractions (steps 1 and 3) and decreased the reducible fraction, which may be related to the migration of native soil Fe mediated by biochar.

Zinc was mainly associated with the acid soluble (25–35%) and reducible (25–30%) fractions (Fig. 4). The high percentage of Zn associated with the first fraction indicated that Zn was the most available of the three micronutrients examined. The fertilized treatments (C and X) resulted in higher percentages of fraction 1 than W. In contrast, the application of biochar reduced the Zn associated with the fraction 1 of fertilized treatments (CB and XB) in comparison with WB. Hence, the combined use of biochar and fertilizers reduced the most available fraction of Zn. This immobilization of Zn was associated with the increment of fraction 4, denoting a strong immobilization of this element. The foliar content of Zn was significantly higher in W and WB than in the

**Table 3.** The content of micronutrients and toxic elements in pepper fruit as affected by irrigation with water (W), conventional fertilizer solution (C) and biostimulant solution (X) in unamended or biochar amended (B) soils. Two-way ANOVA was performed to evaluate the effect of fertilization (F) and biochar application (B) and their interaction

	W	WB	C	CB	X	XB	F	B	FxB
			mg·kg <sup>-1</sup>				Two-way ANOVA		
Cu	5.25 ± 0.01 <sup>ab</sup>	2 ± 3 <sup>b</sup>	7 ± 0 <sup>a</sup>	-	7 ± 1 <sup>a</sup>	5 ± 0 <sup>ab</sup>	N.S.	N.S.	N.S.
Fe	33 ± 4 <sup>a</sup>	78 ± 53 <sup>a</sup>	32 ± 0 <sup>a</sup>	-	31 ± 2 <sup>a</sup>	29 ± 0 <sup>a</sup>	N.S.	N.S.	N.S.
Zn	16 ± 1 <sup>a</sup>	32 ± 15 <sup>a</sup>	20 ± 0 <sup>a</sup>	-	23 ± 4 <sup>a</sup>	20 ± 0 <sup>a</sup>	N.S.	N.S.	N.S.
As	0.081 ± 0.003 <sup>ab</sup>	0.14 ± 0.07 <sup>a</sup>	0.001 ± 0 <sup>b</sup>	-	0.03 ± 0.02 <sup>b</sup>	0.03 ± 0 <sup>b</sup>	*	N.S.	N.S.
Cd	1.5 ± 0.3 <sup>b</sup>	4 ± 2 <sup>a</sup>	0.9 ± 0 <sup>b</sup>	-	0.6 ± 0.2 <sup>b</sup>	0.4 ± 0 <sup>b</sup>	*	N.S.	N.S.
Pb	0.02 ± 0.03 <sup>b</sup>	0.19 ± 0.07 <sup>a</sup>	0.02 ± 0 <sup>b</sup>	-	0.11 ± 0.06 <sup>ab</sup>	0.05 ± 0 <sup>b</sup>	N.S.	N.S.	*

Different letters indicate significant differences between treatments (n = 4; Duncan's test, P < 0.05). N.S. not significant. \*P < 0.05.



**Figure 4.** Distribution of soil Cu, Fe, Zn, As, Cd, and Pb fractions (n = 3) as affected by irrigation with water (W), conventional fertilizer solution (C), biostimulant solution (X), and biochar (B). Step 1 refers to the acid soluble fraction, Step 2 refers to the reducible fraction. Step 3 refers to the oxidizable fraction and Step 4 to the residual fraction.

fertilized treatments (Table 2) even though W and WB did not induce the highest percentages of Zn in fraction 1. Pepper plants uptake high concentrations of Zn in leaves<sup>55</sup> so the addition of biochar could reduce the potential damage of the plants grown in Zn polluted soils.

The distribution of TEs in soil fractions was significantly affected by the treatments (Fig. 4). Arsenic was mainly found in the residual fraction (95–98%), Cd in the soluble (47–58%) and reducible (26–33%) fractions and Pb was mainly found in the residual (45–56%) and reducible (26–36%) fractions. The biochar together with water and conventional fertilization treatments (WB and CB) reduced the As percentage of the soluble, reducible, and oxidizable fractions. Biochar with biostimulant (XB) decreased the percentage of As in the mineral fraction and increased the rest of the fractions' percentages. However, this mobilization did not affect plants (Table 2). The highest As contents were measured

in plants irrigated only with water. This treatment resulted in a nutritional stress for the crop. Abiotic stresses triggered morphological, physiological, and biochemical changes in plants,<sup>56</sup> including the exudation of organic acidic compounds that mobilizes nutrients and may mobilize As well through, for example, the formation of As phytochelatin complexes.<sup>57</sup>

Cadmium was found mainly in the acid soluble fraction (50%), the most bioavailable fraction (Fig. 4). Fang *et al.*<sup>4</sup> attributed the high availability of Cd to the similar ionic radius of Cd<sup>2+</sup> (0.97 Å) and Ca<sup>2+</sup> (0.99 Å), resulting in a strong adsorption onto carbonates, making it a labile element.<sup>58</sup> The use of biochar and fertilization lowered the acid-soluble Cd percentage in soils. Specifically, the XB treatment resulted in the lowest percentage. This can be related to the lowest Cd tissue concentration found in plants fertilized with this treatment (Table 2). The combinatory effect of the adsorption ability of the biochar by chemisorption and

complexation mechanisms<sup>44</sup> and the biostimulant effect to promote nutrient uptake resulted in a better plant status with less Cd uptake.

The Pb was predominantly found in the reducible (26–36%) and residual fractions (45–56%). Biochar treatment increased the percentage of Pb in the residual fraction and lowered the percentage of Pb in the soluble fraction (Fig. 4). Biochar application may cause Pb immobilization by increasing soil pH<sup>10</sup> because Pb concentration in plants is strongly affected by the pH.<sup>51</sup> Plant Pb content was the lowest for the biochar and fertilization treatments.

The treatment XB, which contained both the biochar and the biostimulant, resulted in synergistic effects of both products. The nutritional status of the plants of this treatment was optimal and the content of TEs in the bioavailable fractions of the soil were lower than for the rest of the treatments. The combined use of those agronomic inputs represented improvements in the soil-plant system.

## CONCLUSIONS

As a whole, our results indicate that the use of biochar and biostimulant solution enhanced pepper cropping in contaminated soils by improving plant nutrition and protecting against TE accumulation. The synergic effect of biochar and biostimulant lowered TE content in plants, immobilizing them on non-available fractions of the soil. Our work suggests synergistic effects of different management practices for a safer and more sustainable management of agricultural production in contaminated soils. As the use of plant biostimulants is growing and several unsuitable soils are being used for agriculture, these results could be applicable in real scenarios.

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