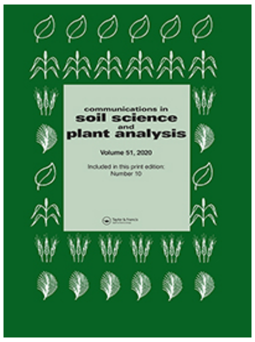

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Effect of Cattle Slurry on the Growth of Spinach Plants in Cd-contaminated Soil

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ABSTRACT

In this work the effect of the addition of different amounts of cattle slurry (CS) to a Cd contaminated soil, was studied regarding its effect in spinach plants. Two levels of Cd contamination (2 and 10 mg/kg) and three levels of CS addition were evaluated (2.5, 5 and 10 g CS/100 g soil). Spinach was shown to be a tolerant species, able to accumulate relatively high amounts of Cd (up to 367.7 mg/kg in the leaves), exceeding the limits established by European regulations for leaf vegetables. The addition of 2.5 and 5 g CS/100 g to soil containing 2 mg/kg Cd did not reduce the uptake of this metal but allowed the plants to grow as much as the control. The addition of 10 g CS/100 g lead to a reduced Cd uptake but also to a lower plant growth compared to the lower CS levels. The combined effects of Cd and CS changed element content in the plant, but without causing severe toxicity or deficiency effects.

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Cadmium; mineral content; spinach; manure; abiotic stress

Introduction

Contamination of agricultural soils is a worldwide problem that can be caused by the deposition of metals and metalloids due to the misuse of pesticides and fertilizers, application of industrial effluents as water source for irrigation of crop plants, mining and smelting activities, and emissions from industries and transport vehicles (Nagajyoti, Lee, and Sreekanth 2010). Cadmium has a relatively high solubility and is readily taken up by crop plants, contaminating the food chain (Choppala et al. 2014; Clemens et al. 2013). These compounds can accumulate in different body organs leading to potential adverse effects on human health. A high percentage of trace elements in human bodies living in urban areas resulted from consuming contaminated foods rather than air pollution. In this context FAO/WHO recommended, for Cd, a Provisional Tolerable Weekly Intake of 7 µg/kg body weigh per week (EFSA 2009).

Leaf vegetables are an important part of the human diet and growing plants in contaminated soils is an important pathway for entry of toxic pollutants into human body. Mean values of Cd contents in soils worldwide vary between 0.2 and 1 mg/kg but can be much higher in contaminated soils (Clemens et al. 2013). Naturally, the ability of plants to grow in contaminated soils is highly dependent on environmental conditions and plant species (Mourato, Reis, and Martins 2012).

Spinach is a highly consumed leafy vegetable and also an important source of nutrients. The Food and Agriculture Organization (FAO) estimates that in 2017 more than 27000 Mt of spinach was produced all over the world, the vast majority in China and this value underestimate the real production as it does not include small and familiar gardens (FAO 2015). Spinach is especially sensitive to metal contamination as is known to be able to grow healthily and at the same time accumulating high amounts of different metals (Pinto et al. 2017). In a study comparing the growth of nine different vegetables in waste-amended soils,

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Atkinson et al. (2012) reported that spinach accumulated more heavy metals than other vegetables (except lettuce that accumulated more Cd than spinach). Sinha et al. (2007) also found that spinach plants had good yields in contaminated soils with high accumulation of metals in the edible parts.

Several techniques are used to remediate soils contaminated with PTE (Mulligan, Yong, and Gibbs 2001). Organic matter application is one of the lower cost techniques that can be used to control metal mobility in soils that can also have the added benefit of a positive effect on the growth and yield of crops, promoting the restoration of soils (Mohamed et al. 2010; Morsch and Martins 1999). However the influence of organic substances on the availability of the PTE depends on the nature of these metals, soil types and the organic matter properties (Gautam and Agrawal 2019; Mohamed et al. 2010).

The humic substances, such as humic acid (HA), fulvic acids (FA) and humin are important fractions of soil organic matter, and its metal-cation binding capacity plays an important role in their mobility (Hernandez-Soriano and Jimenez-Lopez 2012). However, some conflicting results have emerged, since organic matter can reduce the availability of metals to plants, but can otherwise increase it in certain circumstances (Bai et al. 2012).

Li et al. (2008) describes how the incorporation of pig manure in a contaminated soil decreased the concentration of available Cu and Cd by 76.1% and 25.7 %, respectively. In another study (Liu et al. 2009), the application of chicken manure decreased the concentration of soluble Cd by 71.8–95.7 %, but increased the values of inorganic precipitated Cd and organic-bound Cd. The application of cattle slurry and manure to improve crop growth and reduce heavy metal toxicity has also been reported in several studies. Gul et al. (2016) studied the effect of the application of composted manure on maize growing in contaminated soils and concluded that plant growth was improved, and heavy metal uptake decreased (with a more pronounced effect the longer the composting time). In a study of the long-term effect of the application of cattle slurry (and other amendments) to agricultural soils the authors concluded that an increase in the soil content on certain metals was detected but this did not present a health hazard (Couto et al. 2018). Nunez-Delgado, Lopez-Periago, and Diaz-Fierros-Viqueira (2002) studied the leaching of contaminants in soil columns after cattle slurry application and concluded that there was a risk of heavy metals leaching and subsequent water pollution. In a plot-scale experiment Peyton et al. (2016) also detected elevated runoff losses of different metals through the application of cattle slurry and different biosolids to a grassland soil.

The objective of this work is to study the effect of the addition of different amounts of cattle slurry (CS), 2.5, 5 and 10 g CS/100 g soil, obtained from a Portuguese dairy farm near Lisbon, to soils contaminated with cadmium, in relation to its effects in the growth of spinach plants and element content.

Materials and methods

Plant material, growth conditions and Cd treatment

Spinach plants were first germinated in substrate cylinders (Jiffy-7) for 35 days, with regular watering, a 12 h photoperiod at a temperature between 22 and 25°C. The plants were then transferred to pots containing 2 kg of soil (three plants per pot), with two concentrations of Cd (designated LC – Low Concentration with 2 mg Cd/kg and HC – High Concentration with 10 mg Cd/kg) and four levels of Cattle Slurry with 0, 2.5, 5 and 10 g CS/100 g (designated CS0, CS2.5, CS5 and CS10, respectively). Including the four controls (No Cd and 0, 2.5, 5 and 10 g CS/100 g) and considering 4 pots per treatment, there were a total of 48 pots, in a completely randomized design.

The Cd containing soils had been previously contaminated with the adequate Cd concentrations (2 and 10 mg/kg), using a $CdCl_2$ solution, six months prior to the experiment. The CS was added two months prior to the experiment.

The soil was fertilized in three batches (just before cultivation and 26 and 33 days after planting) with 36.10 mg K/kg of soil (added as KH_2PO_4 and KNO_3 and corresponding to 79.9 mg KNO_3/kg soil), 4.09 mg P/kg of soil (added as KH_2PO_4 and corresponding to 17.96 mg KH_2PO_4/kg soil) and

33.28 mg N/kg of soil (added as KNO_3 and $Ca(NO_3)_2$, corresponding to 130.0 mg $Ca(NO_3)_2$/kg soil). The pots were irrigated periodically (to 70% water capacity) and were kept in a greenhouse under normal daylight for the duration of the experiment. Seventy days after germination the plants were collected for further analysis, as described below. Soil samples were also collected at the same time.

Determination of spinach weight, essential elements and Cd content

Spinach leaves, stems and roots were collected and immediately weighed to obtain the fresh weight (FW). Samples were washed with deionized water and dried at 70°C until constant weight to obtain the dry weight (DW).

Subsamples with 0.5 g were digested with 10 mL concentrated nitric acid in a digestion block (DigiPREP MS) for 100 min. The solutions were then analyzed for Cd, sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), zinc (Zn), manganese (Mn), iron (Fe) and copper (Cu) by atomic absorption spectrophotometry (Unicam Solaar M) as described previously (Pinto et al. 2017).

Soil analysis

Soil samples from each pot (approximately 100 g of a representative sample was collected from the whole pot) were dried at 50°C for 5 days in order to perform soil analysis. The soil used in this experiment was a sandy soil with, before the addition of Cd, CS or any fertilizer, a pH(H_2O) of 5.67 ± 0.09, pH(KCl) of 5.05 ± 0.10, <0.01% organic matter, and <5 mg/kg K (equivalent to <6 mg K_2O/kg) and <5 mg/kg P (equivalent to <11.5 mg P_2O_5/kg). Nitrogen content was analyzed using a segmented flow auto analyzer (Skalar) according to Houba, Van der Lee, and Novozamsky (1995). The sample solution was obtained by stirring for 60 min, 6 g of dry soil with 30 mL of 2 M KCl, followed by a 10 min centrifugation at 3500 rpm. The supernatant was then transferred to the reading tubes. Phosphorus and K were extracted using a solution of ammonium lactate and acetic acid buffered to pH 3.75. To 2 g of dried soil was added 40 ml of extraction solution and stirred for 2 h (Sherrell 1970). The solution was filtered and K was quantified by flame emission photometry. Phosphorous was determined by adding to 10 mL of sample solution, 2 ml of molybdic photo-rex reagent and 1 mL solution of stannous chloride in HCl 6 M. After allowing the color to develop for 30 min, the absorbance was read in a molecular absorption spectrophotometer at 775 nm.

For the determination of exchangeable bases (Ca, Mg, K and Na), 2 g of dry soil were stirred with 30 mL of ammonium acetate 1 M (pH 7) over 15 minutes (Schollenberger and Simon 1945). After centrifugation for 10 min at 3500 rpm, each element was quantified by flame atomic absorption spectrophotometry (Unicam Solaar M). The micronutrients Cu, Fe, Zn and Mn were determined by stirring 4 g of dry soil with 40 mL of extracting solution (acetic acid 0.5 M, ammonium acetate 0.5 M and EDTA 0.02 M) over 15 minutes (Lakanen and Ervio 1971). After centrifugation for 10 min at 3500 rpm, each element was quantified by flame atomic absorption spectrophotometry (Unicam Solaar M).

Soil pH was determined in a soil/water suspension (pH H_2O) and soil/solution of 1 M KCl (pH KCl) in the proportion 1: 2.5 (w/v), after 1 hour of contact, using a Metrohm pH meter model 632. Total organic carbon (TOC) was determined in a Skalar TOC Analyzer, using 1.5 g of dry soil. The soil characteristics are presented in Table 1.

Cattle slurry analysis

The CS used was collected from a dairy farm near Lisbon, Portugal, where the animal food was based on feed and silage. The CS was preserved at 4 °C in plastic barrels before application. The CS Characteristics (dry matter, organic matter, pH, conductivity, total N, total P and content of K, Na, Mg, Ca, Fe, Cu, Zn and Mn) were determined following the procedures described by Fangueiro et al. (2009) and are presented in Table 2.

Table 1. Main characteristics of the soils at each experimental condition (value ± standard deviation). The asterisk indicates significant differences ($p < .05$) between the 0% CS and other CS levels, for each Cd concentration.

Parameter	Cd Level	CS (g/100 g)			
		0	2.5	5	10
pH(H_2O)	No Cd	5.03 ± 0.12	5.80 ± 0.12*	6.75 ± 0.13*	7.73 ± 0.35*
	LC	6.48 ± 0.10	6.49 ± 0.11	6.98 ± 0.10*	7.83 ± 0.21*
	HC	6.58 ± 0.10	6.40 ± 0.08*	7.13 ± 0.17*	7.90 ± 0.20*
Conductivity (mS/cm)	No Cd	0.48 ± 0.01	0.59 ± 0.04	0.50 ± 0.06	0.69 ± 0.04*
	LC	0.43 ± 0.07	0.51 ± 0.09	0.47 ± 0.08	0.75 ± 0.04*
	HC	0.54 ± 0.05	0.48 ± 0.04	0.54 ± 0.04	0.75 ± 0.02*
Total carbon (%)	No Cd	0.16 ± 0.01	0.20 ± 0.02*	0.25 ± 0.01*	0.38 ± 0.02*
	LC	0.17 ± 0.02	0.21 ± 0.01*	0.26 ± 0.01*	0.41 ± 0.02*
	HC	0.17 ± 0.01	0.22 ± 0.02*	0.28 ± 0.02*	0.41 ± 0.02*
NH_4^+ (mg/kg)	No Cd	69.35 ± 3.90	18.05 ± 3.45*	2.30 ± 0.55*	0.85 ± 0.05*
	LC	87.90 ± 10.20	27.60 ± 3.05*	3.40 ± 0.25*	1.60 ± 0.30*
	HC	59.50 ± 2.80	7.80 ± 0.55*	2.10 ± 0.30*	5.45 ± 0.45*
NO_3^- (mg/kg)	No Cd	486.6 ± 63.4	466.9 ± 75.9	243.1 ± 12.6*	265.9 ± 17.4*
	LC	562.5 ± 41.4	398.7 ± 32.6*	176.5 ± 14.5*	223.9 ± 19.5*
	HC	657.0 ± 93.3	438.3 ± 15.1*	257.4 ± 2.9*	178.0 ± 1.1*
P (mg/kg)	No Cd	52.5 ± 2.3	129.4 ± 13.7*	243.5 ± 24.7*	446.6 ± 53.6*
	LC	65.4 ± 15.8	150.5 ± 23.9*	241.2 ± 26.7*	480.7 ± 43.8*
	HC	51.6 ± 12.4	139.5 ± 16.8*	232.6 ± 17.8*	410.2 ± 32.8*
K (mg/kg)	No Cd	160.2 ± 44.0	258.1 ± 51.1*	383.1 ± 46.8*	831.2 ± 127.8*
	LC	160.2 ± 16.6	323.6 ± 38.7*	389.7 ± 24.8*	408.8 ± 39.0*
	HC	205.1 ± 35.8	425.2 ± 55.6*	513.8 ± 83.6*	727.3 ± 73.0*
Na (mg/kg)	No Cd	70.1 ± 17.9	125.4 ± 10.3*	129.3 ± 26.2*	289.1 ± 31.3*
	LC	82.3 ± 22.9	153.7 ± 24.1*	139.9 ± 14.7*	264.0 ± 37.8*
	HC	58.2 ± 12.9	116.6 ± 18.9*	168.4 ± 16.9*	230.8 ± 26.5*
Ca (mg/kg)	No Cd	99.8 ± 16.8	143.4 ± 33.0	158.8 ± 24.7*	280.3 ± 18.4*
	LC	109.5 ± 20.3	143.1 ± 21.7	175.8 ± 9.8*	271.8 ± 26.1*
	HC	121.0 ± 10.5	153.5 ± 25.7	189.9 ± 13.6*	251.2 ± 11.0*
Mg (mg/kg)	No Cd	39.7 ± 6.8	50.1 ± 9.2	59.3 ± 8.2*	130.9 ± 11.9*
	LC	26.9 ± 6.7	49.6 ± 6.4*	59.8 ± 5.2*	123.8 ± 12.6*
	HC	30.7 ± 3.5	50.8 ± 9.2*	70.7 ± 9.5*	114.7 ± 13.6*
Fe (mg/kg)	No Cd	6.6 ± 1.0	9.3 ± 0.8*	12.9 ± 0.9*	21.3 ± 1.3*
	LC	5.7 ± 0.6	9.0 ± 0.3*	12.4 ± 0.8*	23.2 ± 2.6*
	HC	4.7 ± 0.3	10.3 ± 0.9*	12.0 ± 1.1*	23.7 ± 2.8*
Cu (mg/kg)	No Cd	1.1 ± 0.1	1.2 ± 0.0	1.3 ± 0.0*	1.5 ± 0.1*
	LC	1.2 ± 0.1	1.3 ± 0.0	1.3 ± 0.1	1.4 ± 0.1*
	HC	1.2 ± 0.0	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.1*
Zn (mg/kg)	No Cd	0.7 ± 0.2	1.5 ± 0.2*	2.2 ± 0.4*	3.6 ± 0.1*
	LC	0.8 ± 0.0	2.0 ± 0.4*	2.7 ± 0.3*	4.3 ± 0.5*
	HC	1.0 ± 0.2	2.4 ± 0.5*	3.0 ± 0.6*	3.7 ± 0.5*
Mn (mg/kg)	No Cd	1.5 ± 0.2	3.6 ± 0.8*	3.6 ± 0.3*	6.3 ± 0.7*
	LC	1.6 ± 0.5	3.5 ± 0.8*	3.8 ± 0.8*	6.9 ± 0.7*
	HC	1.8 ± 0.5	3.0 ± 0.5*	4.1 ± 0.6*	5.9 ± 0.6*

Statistical analysis

Statistical analysis was performed using the software SPSS Statistics 23.0 (IBM, 1989–2015). A two-way ANOVA was conducted to examine the effects of Cd and CS on biomass ($p < .05$). To analyze the results of element content, the results were subjected to a one-way ANOVA using the Tukey test to check for significant differences between means ($P < .05$). All the experimental determinations described above were performed in triplicate.

Results

Spinach shoot fresh mass obtained at the end of the experiment, 70 days after germination (DAG), is presented in Figure 1. There was a statistically significant interaction between Cd concentration and CS level for the Biomass, $F(6, 88) = 14.118$, $p < .001$. The highest amount of biomass was obtained for spinach plants growing in non-contaminated soil with CS5 (7.1 g per plant), while the lowest value

Table 2. Main characteristics of the Cattle Slurry (CS) used in the experiment (value ± standard deviation).

Parameter	Value
Dry matter (g/kg)	11.74 ± 0.55
Organic matter (g/kg)	7.20 ± 0.48
pH	8.23 ± 0.11
Conductivity (mS/cm)	17.25 ± 0.70
N (g/kg)	3.45 ± 0.86
P (g/kg)	0.85 ± 0.03
Na (mg/kg)	1022.4 ± 40.3
K (mg/kg)	3567.8 ± 252.9
Ca (mg/kg)	2820.1 ± 225.0
Mg (mg/kg)	665.3 ± 15.6
Fe (mg/kg)	373.3 ± 38.6
Cu (mg/kg)	2.87 ± 0.14
Zn (mg/kg)	18.62 ± 0.26
Mn (mg/kg)	27.41 ± 1.96

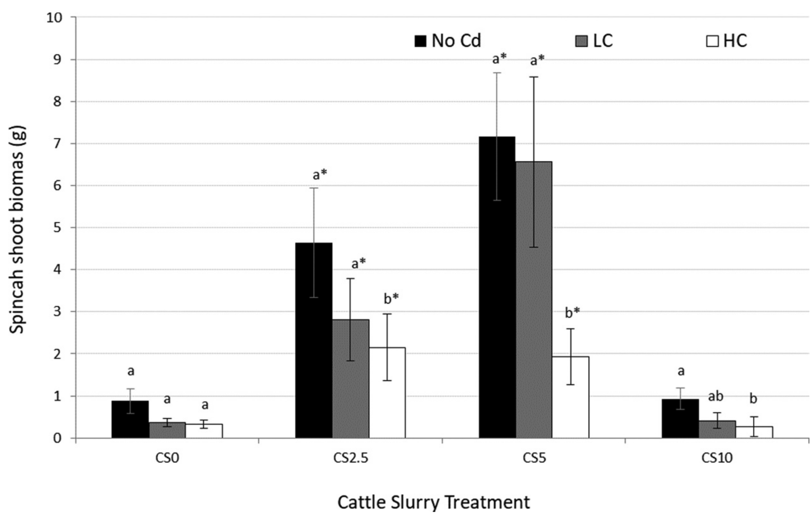

Figure 1. Spinach shoot biomass obtained seventy days after germination. Error bars represent ± standard deviation and the asterisk indicates significant differences ($p < .05$) between the different CS treatments in relation to control. Different lowercase letters represent significant differences ($p < .05$) between the Cd treatments at each CS concentration.

(0.3 g per plant) was measured in the Cd-contaminated soils (10 mg/kg) with CS10. No significant differences were detected between the control and LC soils, but plant growth was significantly affected in HC soils for all the tested CS concentrations.

In Table 3, Cd content in the different plant parts is presented. In the leaves the maximum Cd content was 367.7 mg/kg DW in HC soil with CS2.5, while at higher CS levels the amount of Cd was reduced. In the roots, the maximum measured Cd content was 520.5 mg/kg DW in HC soil with CS5, although this was not significantly different from the CS2.5 treatment.

In Figure 2 the percentage of total Cd that is accumulated in the leaves, stems and roots for both Cd concentrations is presented. It can be seen that with higher CS content there is a larger percentage of Cd accumulating in the roots and a lower percentage in the leaves.

The effect of Cd contamination and CS application in the uptake of other essential elements is shown in Table 3 for roots and leaves, for Na, K, Ca, Mg, Fe, Cu, Mn and Zn. There is a large variation in the levels of the different elements but globally the changes are not enough to cause deficiency or toxicity symptoms.

Table 3. Element concentration of spinach leaves (L), roots (R) and stems (S), only for Cd, at each experimental condition (value ± standard deviation), on a dry matter basis. Different lowercase letters represents significant differences ($p < .05$) between the Cd treatments at each CS concentration, for each plant part.

Element	Plant part	Treatment											
		CS0			CS2.5			CS5			CS10		
		No Cd	LC	HC	No Cd	LC	HC	No Cd	LC	HC	No Cd	LC	HC
Na (g/kg)	L	2.80 ± 0.36^{a}	5.34 ± 0.30^{b}	4.66 ± 0.14^{b}	3.68 ± 0.17^{a}	5.98 ± 0.25^{b}	6.10 ± 0.08^{b}	4.96 ± 0.29^{a}	6.08 ± 0.19^{b}	7.59 ± 0.23 ^{c}	9.65 ± 0.57^{a}	11.34 ± 1.46^{a}	10.09 ± 1.09^{a}
	R	0.80 ± 0.09^{a}	1.45 ± 0.39^{a}	1.45 ± 0.39^{a}	3.39 ± 0.31^{a}	3.42 ± 0.20^{a}	2.41 ± 0.22^{b}	4.98 ± 0.24^{a}	4.01 ± 0.20^{a}	4.65 ± 0.67^{a}	9.93 ± 1.55^{a}	8.63 ± 0.71^{a}	8.15 ± 0.37^{a}
K (g/kg)	L	52.0 ± 2.3^{a}	35.4 ± 2.0^{b}	35.8 ± 2.0^{b}	72.3 ± 8.0^{a}	65.6 ± 0.9^{a}	63.4 ± 3.5^{a}	80.6 ± 5.8^{ab}	85.1 ± 4.6^{a}	70.4 ± 4.4^{b}	80.2 ± 1.1^{a}	80.8 ± 4.6^{a}	71.5 ± 1.7^{b}
	R	7.88 ± 1.87^{a}	8.42 ± 2.51^{a}	6.48 ± 3.0^{a}	39.3 ± 2.7^{a}	29.6 ± 3.6^{b}	17.0 ± 4.3 ^{c}	47.9 ± 2.5^{a}	28.6 ± 3.0^{b}	25.3 ± 4.9^{b}	45.8 ± 3.7^{a}	41.8 ± 5.1^{a}	29.5 ± 1.0^{b}
Ca (g/kg)	L	2.77 ± 0.10^{a}	2.36 ± 0.06^{b}	2.73 ± 0.20^{a}	1.85 ± 0.20^{a}	1.88 ± 0.07^{a}	2.68 ± 0.04^{b}	1.22 ± 0.09^{a}	1.35 ± 0.15^{a}	1.92 ± 0.21^{b}	0.96 ± 0.09^{a}	1.07 ± 0.10^{a}	1.04 ± 0.10^{a}
	R	0.75 ± 0.04^{a}	2.22 ± 0.30^{b}	1.71 ± 0.28^{b}	2.31 ± 0.20^{a}	2.37 ± 0.28^{a}	2.35 ± 0.23^{a}	2.29 ± 0.07^{a}	2.35 ± 0.09^{a}	2.92 ± 0.24^{b}	2.82 ± 0.12^{a}	3.57 ± 0.31^{b}	3.56 ± 0.25^{b}
Mg (g/kg)	L	5.47 ± 0.38^{a}	4.70 ± 0.26^{b}	4.92 ± 0.38^{ab}	8.47 ± 0.51^{a}	7.85 ± 0.20^{a}	6.80 ± 0.22^{b}	7.24 ± 0.24^{ab}	7.86 ± 0.28^{a}	6.95 ± 0.36^{b}	6.42 ± 0.43^{a}	4.44 ± 0.05^{b}	5.01 ± 0.25^{b}
	R	1.02 ± 0.11^{a}	2.35 ± 0.39^{b}	1.31 ± 0.28^{a}	6.79 ± 0.14^{a}	4.56 ± 0.39^{b}	3.60 ± 0.07 ^{c}	7.45 ± 0.25^{a}	5.47 ± 0.03^{b}	6.04 ± 0.12 ^{c}	6.85 ± 0.29^{a}	5.68 ± 0.07^{b}	5.65 ± 0.17^{b}
Fe (mg/kg)	L	172 ± 17^{a}	219 ± 15^{b}	209 ± 9^{b}	106 ± 8^{a}	112 ± 13^{a}	107 ± 7^{a}	95 ± 18^{a}	99 ± 8^{a}	83 ± 8^{a}	104 ± 9^{a}	211 ± 28^{b}	198 ± 35^{b}
	R	1397 ± 18^{a}	2391 ± 283^{b}	2205 ± 130^{b}	1169 ± 115^{a}	1475 ± 82^{b}	1502 ± 62^{b}	1030 ± 109^{a}	1303 ± 53^{b}	1395 ± 46^{b}	1241 ± 121^{a}	1853 ± 217^{b}	2789 ± 435 ^{c}
Cu (mg/kg)	L	6.8 ± 0.7^{a}	6.5 ± 1.5^{a}	5.9 ± 0.7^{a}	8.8 ± 0.6^{a}	10.9 ± 0.5^{b}	7.6 ± 0.7^{a}	9.2 ± 0.9^{a}	10.7 ± 0.4^{b}	10.9 ± 1.0 ^{c}	10.9 ± 1.0^{a}	10.9 ± 0.1^{a}	10.0 ± 1.4^{a}
	R	7.7 ± 1.7^{a}	28.1 ± 3.4^{b}	19.6 ± 2.2 ^{c}	23.3 ± 1.6^{a}	29.3 ± 2.1^{b}	26.2 ± 0.7^{ab}	23.1 ± 0.8^{a}	25.5 ± 0.5^{b}	31.9 ± 0.9 ^{c}	42.8 ± 3.0^{a}	43.3 ± 5.4^{a}	56.8 ± 5.8^{b}
Mn (mg/kg)	L	127.6 ± 13.0^{a}	92.012.5^{b}	259.1 ± 5.9 ^{c}	56.9 ± 8.7^{a}	116.6 ± 5.6^{b}	201.0 ± 6.7 ^{c}	53.4 ± 10.1^{a}	88.2 ± 1.5^{b}	98.9 ± 10.4^{b}	113.3 ± 11.9^{a}	107.5 ± 11.4^{a}	104.2 ± 14.7^{a}
	R	44.0 ± 3.0^{a}	32.1 ± 10.7^{a}	103.1 ± 15.4^{b}	129.1 ± 12.3^{a}	255.8 ± 10.6^{b}	236.1 ± 9.6^{b}	222.2 ± 26.6^{ab}	257.4 ± 5.9^{a}	187.5 ± 7.1^{b}	400.4 ± 44.0^{a}	391.2 ± 67.5^{a}	467.3 ± 81.5^{a}
Zn (mg/kg)	L	133.4 ± 8.9^{a}	56.4 ± 4.7^{b}	53.0 ± 1.2^{b}	97.4 ± 8.7^{a}	74.7 ± 4.0^{b}	44.6 ± 0.3 ^{c}	118.4 ± 8.1^{a}	102.7 ± 8.3^{a}	40.9 ± 1.2^{b}	60.0 ± 3.6^{a}	87.0 ± 5.2^{b}	65.3 ± 5.6^{a}
	R	53.5 ± 5.1^{a}	6.8 ± 32.7	15.0 ± 3.1^{b}	114.7 ± 10.4^{a}	61.7 ± 9.3^{b}	44.8 ± 5.6^{b}	133.9 ± 8.8^{a}	97.8 ± 11.7^{b}	45.7 ± 4.3 ^{c}	68.2 ± 5.7^{a}	62.1 ± 4.5^{ab}	51.7 ± 3.4^{b}
Cd (mg/kg)	L	0.8 ± 0.1^{a}	87.3 ± 12.1^{b}	317.4 ± 2.6 ^{c}	0.6 ± 0.1^{a}	184.4 ± 4.1^{b}	367.7 ± 6.4 ^{c}	0.6 ± 0.2^{a}	140.8 ± 4.8^{b}	304.5 ± 35.4 ^{c}	0.4 ± 0.1^{a}	26.6 ± 1.0^{b}	63.1 ± 2.2 ^{c}
	S	0.7 ± 0.1^{a}	89.7 ± 9.4^{b}	240.2 ± 9.9 ^{c}	0.5 ± 0.1^{a}	150.7 ± 1.9^{b}	211.7 ± 14.1 ^{c}	0.7 ± 0.0^{a}	127.8 ± 3.1^{b}	173.0 ± 12.1 ^{c}	0.5 ± 0.0^{a}	41.7 ± 5.1^{b}	67.6 ± 2.6 ^{c}
	R	1.2 ± 0.2^{a}	152.6 ± 10.7^{b}	340.8 ± 49.7 ^{c}	2.8 ± 0.7^{z}	330.6 ± 10.1^{b}	502.4 ± 15.5 ^{c}	2.5 ± 0.4^{a}	285.5 ± 10.1^{b}	520.5 ± 20.1 ^{c}	0.9 ± 0.1^{a}	82.2 ± 1.8^{b}	222.9 ± 15.7 ^{c}

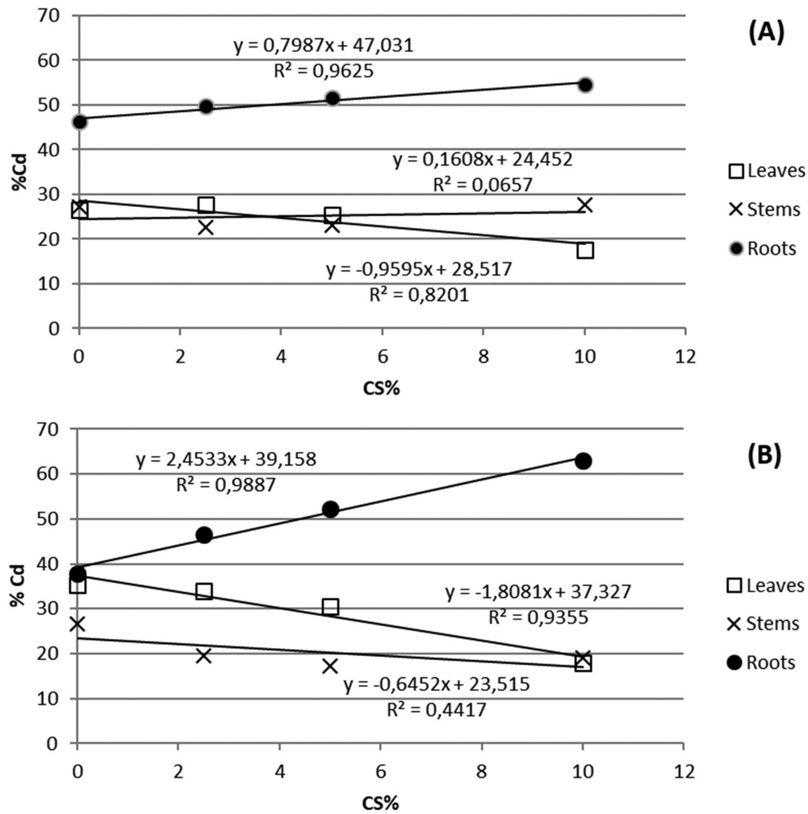

Figure 2. Percentage of Cd in each plant part (leaves, stems and roots), in relation to the total, as a function of CS treatment for both Cd treatments, A – LC and B – HC.

Discussion

It is known that organic matter (OM) incorporation in soils can cause a positive effect on the growth and yield of crops (Mohamed et al. 2010; Sinha et al. 2007). Our results indicate an increase in biomass production for plants grown in both contaminated and non-contaminated soils with CS2.5 and CS5 (Figure 1) compared to the control. In the contaminated soils, although there was a decrease in biomass with increasing Cd content, the presence of CS2.5 and CS5 was shown to have a positive effect in avoiding the Cd toxic effect. It is evident that both CS2.5 and CS5 can counteract part of the harmful effect of Cd, mainly at the lower concentration of 2 mg/kg. This is no longer valid for the highest Cd concentration in soil, since the optimum ratio of OM/Cd could be exceeded.

One possible cause for the positive effect of the added CS to the soil was the increase in pH (Table 2). Spinach plants grow better at a pH around or above 7, and as the pH of the initial soil was acidic the differences between the control and CS2.5 and CS5 experiments can be partially explained by the pH increase. The added CS, at those levels, did also improve nutrient provision for the plants, facilitating its growth (Beesley et al. 2014). With the CS10 treatment the biomass production reverts to the control levels and the beneficial effects in plant grow observed for the lower CS levels are not visible anymore. This reduction in biomass production with increased organic matter can be due to different complex factors associated with the source and nature of the organic matter (Bai et al. 2012; Inaba and Takenaka 2005; Narwal and Singh 1998). Also, the C/N ratio is important as higher values of this parameter could cause the immobilization of nitrogen (and also of phosphorous), affecting plant productivity (Fangueiro et al. 2014). However, the C/N ratio of the CS used in this study (2.1) was much lower than in the organic matter used by other authors, like a C/N ratio of 55 reported for farmyard manure or 6.2 for chicken manure (Citak and Sonmez 2009). Bai et al. (2012) observed a decrease in rice plant biomass with increasing levels of straw applied to the soil. They also attributed the decrease in biomass to the putative effect of toxic substances in the organic matter, and this might also be a factor to explain the results obtained in this work.

The results presented in Table 3 confirm that the toxic effect observed in the CS10 experiment was not due only to Cd uptake by spinach plants. In fact, only at this concentration of CS was observed a significant decrease in Cd uptake in all the analyzed plant parts. The addition of CS2.5 and CS5, for both Cd concentrations studied (LC and HC), does not cause a significant decrease in the uptake of Cd by spinach plants. However, these levels of CS are sufficient to improve the biomass production of the plant indicating that the positive factors that affect plant growth overcome the toxic effect induced by Cd. Actually, a significant increase in the uptake of Cd by the roots was measured at both CS2.5 and CS5 treatments. This might be due to increased solubility of Cd in the presence of increased amounts of dissolved carbon, as has been reported earlier (Antoniadis and Alloway 2002). The increase in soil humic acids content by the addition of the CS can also increase Cd uptake by plants as has been shown by other authors (Evangelou, Daghan, and Schaeffer 2004). Another explanation could be the formation of soluble $CdCl^+$ complexes that lower the affinity of Cd to the organic matter (Adriano et al. 2004). On the other hand, the opposite effect was observed when the CS concentration reached 10%. As the metal concentration in soil solution is dependent on pH and on the nature and amount of both organic and inorganic ions (Bolan et al. 2014), the higher pH of the soil with CS10 can increase Cd chelation by organic matter leading to a reduce Cd uptake by the plant (Adriano et al. 2004). The lower CS concentrations of 2.5 and 5% apparently are not high enough to trigger this effect.

The level of CS also affected the distribution of Cd in the plant parts. In Figure 2 the percentage of total Cd that is accumulated in the leaves, stems and roots in relation to the total, is presented. The relationship between the % of Cd uptake and CS level shows a linear correlation, with R^2 values higher than 0.9 for leaves and roots (except for leaves for the higher content of Cd, where R^2 = 0.82). As can be seen, plants growing in soils containing a higher percentage of CS show an increase in the levels of Cd in the roots (as shown by the positive values of the respective slopes) concomitant with a decrease in the leaves (with negative slopes), that is, the translocation of Cd between roots and leaves decreased linearly with higher CS%. This effect is more pronounced in the HC treatment than in the LC, as can be confirmed by the larger values of the positive slopes and lower for the negative slopes. The explanation of this decreased translocation, and thus of a higher Cd retention in the roots, is probably related to the increased tolerance of spinach to Cd under higher levels of CS. We postulate that the addition of CS to the soil leads to increased availability of sulfur compounds leading to higher glutathione and phytochelatins (PC) synthesis in the roots (Khan et al. 2015). These compounds are known to be very important in plant resistance to PTE toxicity, especially Cd, as they can form complexes with this metal and transport them to the vacuoles (Seth et al. 2012). This can increase the tolerance of the plant, and can be another part of the explanation for the obtained results of better plant growth under higher Cd concentrations when grown in soils containing CS. Moreover, this can also explain the decreased translocation of Cd between roots and leaves, as an increase in PC synthesis will likely lead to increased retention of Cd-PC complexes in the root vacuoles. Pinto et al. (2004) reported an increased Cd accumulation in shoots in relation to roots of sorghum plants, with increased organic matter but these results were obtained in nutrient solution and the authors explained it due to a decreased Cd bioavailability.

As spinach is reported to be a relevant food source of mineral elements (Citak and Sonmez 2009) it is important to evaluate how the different growth conditions affect their concentrations. It has been reported that Cd can be taken up by the plants via the transporters of essential cations, like Ca, Zn, Fe, Mg and Cu, and thus affecting the element homeostasis in the plant (Gallego et al. 2012). However these effects are highly dependent on plant species and metal (Martins et al. 2013). In Table 3 the concentrations of essential elements in leaves and roots are presented.

As can be seen in this table, the levels of K decreased with Cd uptake. This effect is more pronounced in the roots, and in the leaves the plant manages to compensate K levels and only for the highest concentration of Cd is there a significant decrease in plants growing in CS-containing soil. However, in the control plants K levels in leaves are always lower in plants growing in Cd contaminated soils. As K has an osmotic function in plants, it may be substituted by Na in plants with considerable Na uptake potential, as has been reported for spinach (Mengel 2007). Our results are in agreement with this as we can observe an increase in Na levels in leaves of plants growing under Cd stress.

Although the levels of Ca decreased in leaves with increasing CS concentration, no reduction was detected due to Cd. On the contrary, an increase in Ca content was measured in spinach leaves with CS2.5 and CS5, for the HC Cd treatment. As spinach plants showed reduced growth under this Cd concentration, the observed increase in Ca content could be due to disturbances in the plasma membrane leading to increased Ca influx, as has been proposed by Michalska and Asp (2001) that detected similar effects in lettuce growing under Cd and Pb stress. A similar effect occurs with Fe and Mn, with a generally increased element uptake under Cd stress, more pronounced in CS10 soils for iron and in CS0 or CS2.5 for Mn. This could also be due to a disturbance in the uptake mechanisms of this element that happens with plants growing under CS10 and HC Cd concentration. Although several Cd toxicity studies report decreases in the uptake of several ions (DalCorso et al. 2008), the opposite effects, similar to the ones described in this work, have also been reported (de la Rosa et al. 2004; López-Millán et al. 2009).

As for Mg, the presence of Cd led to a decrease in the root content of this essential element, leading to the conclusion that Cd is affecting Mg uptake mechanisms by the roots. This effect is also reflected in the Mg levels in the leaves that are generally lower under Cd stress. However, this reduction is not high enough to cause deficiency problems in the plant. In relation to Cu, an increase in its content in roots was observed, but not in leaves, confirming that the translocation to this organ was affected, mainly at the higher Cd concentration.

Zinc was the element most clearly affected by Cd. For all the CS contents, the uptake of Zn by the spinach roots was reduced under Cd toxicity, although the effect was less pronounced for the highest CS level. This translated in lower contents of Zn in leaves. Citak and Sonmez (2009) reported a concentration of 135.5 mg/kg of Zn in spinach leaves but referred that these values can go as low as 28 mg/kg, depending on season. In the present work the lowest value of Zn in the leaves was still 40.9 mg/kg and so they are not low enough to induced serious deficiency effects. In another study with spinach plants in nutrient solution (Pinto et al. 2017), the same effect of apparent Cd competition with Zn was observed, confirming that for this plant, Cd uptakes affects the transport of Zn.

As can be seen in Table 3, although the roots showed the higher concentration of Cd, the above-ground parts, both stems and leaves, also accumulate considerable amounts of Cd, for both Cd treatments, LC and HC. The leaves of contaminated plants accumulated up to 367.7 ± 6.4 mg Cd/kg DW (corresponding 25.75 mg Cd/kg FW), exceeding the maximum value allowed to leaf vegetables (0.2 mg/kg FW) according to the European regulations (Commission Regulation (EC) 2006). Even at the lower Cd contamination evaluated in the present study spinach plants were able to absorb 12.91 mg Cd/kg FW, also exceeding the regulated value. Our results confirm that spinach has the potential to exceed these legal threshold levels, if the conditions (Cd concentration, growing medium and exposition period) are adequate. As such, the FAO recommended value of Provisional Tolerable Weekly Intake of 7 μg/kg body weight (EFSA 2009) can be quickly overcome with the consumption of highly contaminated spinach, in a diet that includes moderate consumption of this vegetable. Moreover, the present study confirms that the addition of CS will lead to increased plant growth but also to increased Cd uptake, in a contaminated soil. Thus, the plants might even look healthier than those growing without CS but at the same time containing high amounts of Cd in its edible parts. This constitutes an increased food safety hazard as the vegetables will look healthy, reducing the probability of rejection by the consumer.

Conclusions

The growth of Spinach plants in Cd-contaminated soil containing different amounts of cattle slurry has demonstrated different responses according to the CS concentration. At the CS2.5 and CS5 treatments plant growth has improved but Cd uptake was not decreased, as the type of organic matter probably increased Cd mobility in the soil. A higher concentration of CS (10%) caused a significant decrease in Cd uptake but also a reduction of plant growth, presumably due to the high pH or high C: N ratio, or the presence of other substances in the CS. This is evidence that there are different opposite factors at stake that are highly dependent on the level of CS applied to the soil.

Spinach plants with 70 days of development, at a similar development stage as used for human consumption, were able to tolerate Cd at a concentration of 25.75 mg/kg leaf FW while not showing visual evidence of stress, except for reduced biomass. These Cd levels are well above the maximum recommended values according to European regulations.

Spinach plants growing under Cd stress in different CS concentration showed affected essential metal ion uptake but not enough to cause either deficiency or toxicity problems. In reality an increase in the concentrations of some beneficial elements was detected in Cd-contaminated plants.

These results confirm that CS is not adequate to reduce the uptake of Cd by spinach plants as at the concentrations necessary for this decrease in uptake, other negative factors will prevail that will cause toxic effects and a decrease in the biomass production. Further studies on CS application to the soil should focus on possible corrective measures, like decreasing pH and changing the C:N ratio in order to improve CS ability to retain Cd in soil.

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