Surface soil liming reduces cadmium uptake in cacao (Theobroma cacao L.) seedlings but is counteracted by enhanced subsurface Cd uptake

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CORE IDEAS

- Superficial liming effectiveness depends on Cd availability in the subsurface soil.
- Neither liming nor soil source affected cacao seedling root distribution.
- Superficial liming may enhance Cd uptake due to increased activity of deeper roots.
- Cacao seedlings may trigger a compensating effect to cope with micronutrient deficiency.

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ABSTRACT

Cadmium concentrations in cacao beans from South America often exceed trade limits. Liming soil is advocated as a remediation option, but amendments cannot be incorporated in the entire root zone without harming the trees. An experiment was set up to identify how Cd uptake varies within the root zone when surface and subsurface soil layers are either limed or not. The experiment used 22 cm height pots with top and bottom layers using surface and subsurface soil samples from a cacao field. The potted soils were either or not surface limed or fully limed and layers spiked with stable ^{108}Cd isotope in various combinations to trace the plant Cd provenance. The root distribution was neither affected by liming nor by soil source; 70% of root biomass was present in the top layer. Plants grown on the fully limed surface soil had 1.7 times lower Cd concentrations in leaves than in unlimed treatments, whereas this was factor 1.2 when only the top layer was limed (surface soil used in both layers). The isotope dilution data showed that surface soil liming enhanced Cd uptake from the unlimed bottom layer compared to that in unlimed soil, suggesting compensating mechanisms. The pots containing surface soil over subsurface soil also showed that compensating effect but, due to lower phytoavailable Cd in the subsurface soil, surface liming still effectively reduced foliar Cd. We conclude that liming might be a feasible mitigation strategy, but its effectiveness is limited when Cd phytoavailability remains untreated in the subsurface layer.

Keywords: Ecuador, heavy metal, agricultural limestone, compensating effect, pot experiment.

ABBREVIATIONS

%OC	Soil organic carbon
AG-$CaCO_3$	Agricultural Limestone
ANOVA	Analysis of Variance
Cd_r	Cadmium concentration in the rhizosphere
Cd_{UPT}	Cadmium uptake per plant
CPS	Counts per second
CRM	Certified Reference Materials
CV	Coefficient of variation
DIW	Deionized Water
DM	Dry matter
ECEC	Effective cation exchange capacity
Eq.	Equation
ESPOL	Escuela Superior Politécnica del Litoral
EU	European Union
f	Fraction
ha	Hectare
IA	Isotopic abundance
ICP-OES	Inductively Coupled Plasma Optical Emission Spectroscopy
ICP-MS	Inductively Coupled Plasma Mass Spectroscopy
ISO	International Standard Organization
L	Limed
LOQ	Limit of Quantification
NL	Non-limed
SD	Standard deviation
WHC	Water Holding Capacity
Zn_r	Zinc concentration in the rhizosphere
Zn_{UPT}	Zinc uptake per plant

INTRODUCTION

Cadmium is one of the most toxic trace metals in the environment. Chronic exposure to Cd has been related to kidney proximal tubular dysfunction and osteoporosis in humans

(Åkesson & Chaney, 2019). The consumption of food with high levels of Cd is the primary source of intake for non-smokers (Järup & Åkesson, 2009). In 2014, the European Union (EU) adopted Cd limits for chocolate products (EC–European Commission, 2014) because its consumption was identified as an important contributor to the Cd dietary exposure for the European population, especially children (European Food Safety Authority, 2012). These new EU Cd limits range from 0.1-0.8 mg Cd kg^{-1} chocolate product; the restrictions vary with the percentage of cocoa solids present in the final product. The Codex Alimentarius Commission also adopted limits for chocolate 0.8-0.9 mg Cd kg^{-1} depending on the percentage of cocoa (Joint FAO/WHO Codex Alimentarius Commission, 2018). The average concentrations of Cd measured in several surveys range from 0.9 to 1.4 mg kg^{-1} dry matter in cacao (*Theobroma cacao* L.) beans from Central (Gramlich et al., 2018) and South American countries, particularly Colombia, Ecuador (Argüello et al., 2019; Barraza et al., 2017; Chavez et al., 2015) and Peru (Arévalo-Gardini, Arévalo-Hernández, Baligar, & He, 2017). The implementation of the new regulation is seen as a threat to the sustainability of the cacao industry. Since the EU announcement, research to better understand, predict and reduce the soil-plant transfer of Cd in cacao has intensified.

Soil pH is the key property governing Cd solubility in soils (Christensen, 1984). Christensen (1984) showed that Cd sorption strength in sandy soils increases three-fold for each unit increase on soil pH. The higher solubility of Cd in acidic pH is due to the competition with hydrogen ions for sorption sites on the reactive groups of organic matter or oxyhydroxides, which are the main adsorbents for Cd in soils (Smolders & Mertens, 2013). In general, higher Cd concentrations in the soil solution are related to higher Cd availability. The role of soil pH on Cd concentrations in cacao was corroborated in a survey of bean Cd across Ecuador (Argüello et al., 2019). Multivariate analysis showed that increasing soil pH 1.0 unit is

associated with 1.6-fold lower bean Cd concentrations, all other factors such as total soil Cd, organic carbon and type of cultivar being constant (Argüello et al., 2019).

Managing soil pH by liming has been investigated as a mitigation strategy to reduce Cd uptake by many crops grown in acidic soils. Several researchers have reported significant reductions in Cd uptake in different plants, mostly in pot trials. For example, Lee, Lai, and Chen (2004) noted that the addition of lime on potted soils at rates equivalent to a field dose of 10 and 20 Mg of lime ha^{-1} reduced Cd concentration in wheat grains by between 60% for sandy and 90% for clay soils compared to corresponding control values. Similarly, Maier et al. (1997) observed reductions of up to 72% in Cd concentrations in potato tubers by adding 20 Mg ha^{-1} of calcitic limestone in glasshouse conditions. Han and Lee (1996) reported that liming soil increased the pH from 5.7 to 7.1 and reduced Cd concentration in the shoots (66%) and roots (40%) of radishes without compromising yield.

In contrast, field experiments are generally inconclusive about the success of liming on mitigating crop Cd concentrations. For example, Li, Chaney, Schneiter, and Johnson (1996) found no significant differences between control and limestone treatments in Cd concentrations of kernels and sunflower leaves in the three different locations tested. The authors suggested that the depth of incorporation of limestone was likely not deep enough (only 15 cm) to reach soil layers where sunflower roots were active. Furthermore, in near-neutral pH soils, chloride was the key factor affecting Cd accumulation. Similarly, Maier et al. (1997) reported no effects of liming on Cd concentrations in potato tubers after the addition of lime at various rates up to 15 Mg ha^{-1} in field trials. The authors attributed that such lack of effect is related to the long-time needed for the reaction of lime with soil, the variable moisture status of the soil profile, competitive desorption of Cd^{2+} by Ca^{2+}, reduced penetration of liming material to deeper soil horizons where active roots take up the metal, and Cl^- controlled Cd phytoavailability in their test soils (Maier et al., 1997). The first report

on liming to reduce Cd accumulation in cacao showed inconclusive results as both limed and control treatments presented a reduction in leaf-Cd concentration over the experimental period (Ramtahal et al., 2018). Ramtahal, Umaharan, Hanuman, Davis, and Ali (2019) reported only minor effects of liming soils with up to 6 Mg $Ca(OH)_2$ ha^{-1}. The liming in a pot test showed a 3-fold reduction in leaf Cd concentrations in cacao seedlings but were less than 1.2, nine months after application in the field (Ramtahal et al., 2019).

Root distribution of a crop with depth in the soil likely affects the effectiveness of liming materials in reducing Cd uptake. The changes in soil pH occur where lime has been mixed, i.e., in the surface layer, however, deeper roots may still take up Cd from the deeper unlimed acid soil. Bell, McLaughlin, Wright, and Cruickshank (1997) studied Cd uptake in different cultivars of peanuts under greenhouse and field conditions. The Cd concentration in peanut kernels was higher under field compared to greenhouse conditions, despite the use of the same soil. The authors suggested that, under field conditions, plants have deeper roots, thus accessing Cd at deeper soil layers. McLaughlin, Bell, Wright, and Cozens (2000) reported that the main root system was the core path for Cd uptake in peanuts and deeper roots may indeed affect the peanut Cd concentrations more than shallow roots.

Cacao is a perennial crop with a deep rooting system (~2 m), although roots are most dense in the near-surface soil depth. Lime cannot be incorporated in such a system without harming roots. Hence, surface application is the only suitable option. This also means that the success of lime application in reducing Cd uptake may be affected by the relative distribution of the active roots and the soil physicochemical properties through the whole soil profile. It is unclear how the root distribution in a soil profile affects crop Cd accumulation. If Cd uptake is non-regulated, then crop Cd may be predicted from the root distribution and corresponding distribution of Cd phytoavailability; this is further termed the unregulated uptake model. In

contrast, if Cd uptake is regulated, e.g., indirectly by homeostatic control on uptake of its nutrient analog Zn, then reduced phytoavailability in one part of the root zone may be compensated by enhanced Zn and Cd uptake from the zone with higher phytoavailability. This concept is further termed the regulated uptake model. The latter model may explain why surface liming may yield smaller effects than that predicted from the unregulated model. Nutrient uptake is a regulated process, and such compensating effects logically occur in the root zone under heterogeneous supply, e.g., for N (Gojon, Nacry, & Davidian, 2009). From studies in hydroponics, arguments have been given that the uptake of the non-essential Cd is, perhaps surprisingly, a plant regulated process: when pruning roots of a hyperaccumulator plant, shoot-Cd concentration was unaffected suggesting that compensating effects on root uptake processes occur (Sterckeman, Goderniaux, Sirguey, Cornu, & Nguyen, 2015).

To date, no study has reported the contribution of different rooting soil layers on crop Cd in plants grown with vertical heterogeneities in Cd supply. It is known that Cd concentrations are higher in the surface soil than subsoils of cacao farms (Barraza et al., 2017; Chavez et al., 2015; Gramlich et al., 2017; Gramlich et al., 2018) which could be related to an effect of cacao plants themselves rather than the use of contaminated P-fertilizers (Barraza et al., 2019). For these reasons, this study was set up to determine the response of partial (top compartment) soil liming on Cd uptake by cacao plants. The responses are analysed with measurements of Cd provenance identified with stable isotope spiking of a selected soil layer in potted soils. The experiments were set up in such a way to test which of the models, regulated or unregulated, applies best to fit the observed Cd uptake.

MATERIALS AND METHODS

Experiments with 22 cm height pots were set-up to test the effect of partial (top compartment) or complete soil depth liming on Cd uptake by cacao seedlings, compared to

an unlimed soil. There were two scenarios: using the same soil layer source (surface soil) in the entire pot or using a combination of a soil sample collected from surface soil (0-15 cm) placed on top of a soil sampled from subsurface soil (15-30 cm) from the cacao farm. The collection of soil at different depths aimed to test the effect of soil with different chemical properties. This experiment is not designed to transfer its result to field conditions but to evaluate soils with different phytoavailable Cd either by liming or by using soils with lower total Cd. The soil sampling and chemical characterization (Table S1) is described in the Supplemental Information. In this study, the term "top or bottom layers" refers to the physical location of the soil samples within the same pot, whereas the term "surface or subsurface soil" refers to the depth of collection of the soils in the field. Soils were either enriched or not with a trace of ^{108}Cd isotope, and several reference treatments were set-up to measure the isotope abundance of the phytoaccessible Cd in the soil (see below for details).

Experimental Setup

The pot experiment was set-up to assess the source of Cd in cacao seedlings as related to uptake from the top or bottom layer. Treatments 1─12 (Table 1) tested the effects of liming in pots with the same surface soil samples. The soil in the top or both layers was amended with agricultural limestone (AG-$CaCO_3$) to assess the effect of liming and liming position on the accumulation of Cd; unlimed soil was designated as the control. For treatments with the contrasting soil samples in the same pot (treatment 18-22; excluding T19), only the top layer was limed. A subsurface over subsurface non-limed treatment was also added (T13 and T19). Finally, the soils in the different layers were either labeled or not labeled with ^{108}Cd in all possible combinations. Each of these treatments was deemed necessary to identify the source of Cd from the ^{108}Cd isotopic abundance (IA) in the plants. A lower number of treatments would only be possible by using assumptions, e.g., that the IA of phytoavailable Cd is unaffected by the liming treatments or by the location of the spiked soil. All treatments were

grown in triplicate, giving a total of 66 pots. Detailed information of ^{108}Cd spiking, amendment addition and incubation times are given in the supplemental information. A complete description of the different treatments is summarized in Table 1.

Pot experiment

The incubated soils were weighed (5 kg total dry wt. divided into equal amounts for top and bottom layers) into conical drained plastic pots (22.5 cm height, 17.5 cm lower and 23.5 cm upper diam.) following the treatment position described in Table 1. The bottom layer reached a height of 11 cm (17.5 cm lower diam.; 21 cm upper diam). The top layer was 9.5 cm tall (21 cm lower diam.; 23 cm upper diam). Six-week-old cacao seedlings (cultivar CCN-51) obtained by rooted cutting propagation were purchased from a commercial nursery. Cacao seedlings were removed from the nursery substrate, and the roots were washed by immersing in deionized water (DIW) to remove any adhering substrate particles. Noteworthy, seedlings had minimal root biomass; therefore, almost all new roots were exposed to enriched ^{108}Cd isotope conditions. One cacao seedling was planted per pot. Replicate washed seedlings were dried to measure the plant Cd concentration and quantity at the start. The experiment was carried out in a naturally lit greenhouse under a black shade mesh (to prevent direct sunlight) at ESPOL, Guayaquil, Ecuador from Aug. to Dec. 2018. The average temperature regime recorded in the greenhouse was 31°C during the day, 24°C at night. Pots were weighed and watered daily with DIW to maintain soil moisture at 70% WHC, and they were arranged in a completely randomized design. Seven d after planting, 16 plants were replaced as they showed signs of wilting. Fertilization details for this experiment are provided in the Supplemental Information.

Plant tissues were harvested after 120 d. Leaves were washed with DIW to remove any dust, and oven-dried at 60°C before transporting to the KU Leuven laboratory in Belgium for

chemical analysis. Old leaves that came with the plant from the nursery were excluded. Only leaves that developed during the experiment were analyzed. Roots from the top and bottom layers of selected treatments (pots with isotope labeling in only one of the layers) were collected separately. In these treatments, pots were cut horizontally at the boundary of the upper and bottom layers to collect intact roots from each layer separately. First, a soil sample (100 g approx.) was collected from each compartment of all treatments. Then, the central and lateral roots were collected manually. Soil from different layers was sieved through a stainless steel mesh (<2 mm) to gather fine roots. Harvested roots were washed with DIW to remove adhered soil particles and subsequently oven-dried at 60°C overnight. Soils samples were air-dried for 72 h. Dried soil samples were sieved through a stainless steel mesh (<2mm) and stored in ziplock bags for further analyses. Dried roots collected from the two layers were weighed separately, and leaf, as well as root dry biomass, were recorded.

Chemical analysis

Cacao leaves were ground to a fine powder and digested in boiling concentrated Suprapure® nitric acid (HNO_3) for about 6 h in an open digestion block. Total elemental composition of the leaves and all Cd isotopes (^{106}Cd, ^{108}Cd, ^{110}Cd, ^{111}Cd, ^{112}Cd, ^{113}Cd, ^{114}Cd, and ^{116}Cd) were measured by ICP-MS (Agilent 7700x, Agilent Technologies, collision cell ORS^3), with helium as the collision gas.

The IA of a specific isotope is its amount relative to the total amount of all the isotopes of an element present in a sample. The IA ^{108}Cd in the cacao leaves was calculated from the mass-bias-corrected ^{108}Cd intensity (counts per second) relative to all Cd isotopes (Total Cd CPS), i.e.,

$$IA\ ^{108}Cd = \frac{^{108}Cd(cps)}{Total\ (cps)} \quad \text{Eq. (1)}$$

The mass-bias correction for each isotope was derived by analyzing a 100 µg Cd l^{-1} single element certified standard solution (Certipur® Merck 119777), which showed a mass discrimination of about 5.7% per mass unit. The measurement of the IA of ^{108}Cd may be affected by ^{108}Pd or by the interference of $^{92}Mo^{16}O$ (May & Wiedmeyer, 1998) or of $^{92}Zr^{16}O$. Along the same lines, the main isotopes of Cd (mass 110-114) might also be affected by Sn and In isotopes. The collision cell is very effective in removing oxides interferences (Boulyga, Dietze, & Becker, 2001; Koppenaal, Eiden, & Barinaga, 2004) and bioavailable Pd, Sn and In is very low in soil, hence in plants. This was verified by measuring the IA of plants grown on the non-spiked soils and comparing the measured isotopic ratio with expected, i.e., natural abundance. The average ±SD of the ^{108}Cd IA in the plants of the unspiked soils was 0.93±0.02% for unlimed samples and 0.91±0.03% for limed samples, i.e., very close to the natural abundance of 0.89% suggesting little enrichment with the interferents. The error in the IAs of these samples was small enough for this experiment because of the soil-spiking enriched soils to IAs of Cd to 5 or 8% (Table S3). The ^{108}Cd IAs in the plants grown on fully spiked soils were 5.3±0.1% (limed surface soil sample), 5.1±0.1% (unlimed surface soil sample), and 8.2±0.3% (unlimed subsurface soil sample), all closely matching the targeted IAs. We also compared an alternative method to detect the ^{108}Cd IA by only relying on the $^{108}Cd/^{111}Cd$ line and assuming that the ratio of other isotopes of Cd to ^{111}Cd is the natural one; the ^{111}Cd line has almost not interferent. That second method yielded almost identical results, the ^{108}Cd IA (ranging 0.008-0.0089 depending on treatments) calculated with the second method differed less than 0.0001 from the ones with the first method, that confirmed little effects of Sn and In isotopes on the measured ^{108}Cd IA with the first method.

The ICP-MS instrument calibration was validated using three certified aqueous solutions of trace elements (SRM-1643f, SPS-SW2, TM-35), and the limit of quantification (LOQ) for Cd

was 0.02 µg Cd l^{-1}. The corresponding LOQ for solid samples (soil or leaves) was 0.02 mg Cd kg^{-1} dry matter. For chemical analysis, duplicates were included every ten samples to evaluate reproducibility. The coefficients of variation for each set of duplicate samples ranged from 0.39 to 3.11% (avg. 1.58%, n=7). Certified reference materials (CRM) were included in each digestion batch for quality assurance. The CRM and certified Cd (±uncertainty) were NIST-1573a tomato leaves (1.52±0.04 mg Cd kg^{-1}) and BCR-679 white cabbage (1.66±0.07 mg Cd kg^{-1}). The recoveries (±SD) of Cd calculated relative to the certified concentration (in %) were 103±6.62 for NIST-1573a, and 114±0.25 for BCR-679.

Calculation of Cd in the leaf derived from top or bottom pot layers

The two-pool mixing model was used to calculate the proportion of Cd in cacao leaves that derived from the top or bottom layer (f_{top}, f_{bottom}) using the ^{108}Cd IA of the plants grown in labeled (fully spiked) and non-labeled pots. The fraction of leaf ^{108}Cd IA (IA_{leaf}) for a plant grown in a pot is calculated from the general Eq. 2:

$$f_{top} + f_{bottom} = 1 \quad \text{Eq. (2)}$$

The mass balance Eq. 2 with IA is:

$$IA_{leaf} = IA_{top} \times f_{top} + IA_{bottom} \times f_{bottom} \quad \text{Eq. (3)}$$

The fraction from the labeled layer, e.g., f_{bottom} ,was calculated by combining Eq. 2 and 3:

$$f_{bottom} = \frac{IA_{leaf} - IA_{top}}{IA_{bottom} - IA_{top}} \quad \text{Eq. (4)}$$

The IA of a given layer (top or bottom) was derived from the IA in the plant grown on a soil in which the top and bottom were identical and treated or not with agricultural limestone as in the corresponding layer. This means that treatments 1, 2, 6, and 12 served to calculate f_{top} or

f_{bottom} in all other treatments between no. 3-11. For example, the f_{top} in treatment of L* (Limed and spiked, *) over a non-limed NL soil layer used the IA of the L*/L* treatment to infer IA_{top} and the IA of the NL/NL treatment for the IA_{bottom}. Note that f_{top} for a given treatment can be calculated from two different treatments, e.g., the limed over unlimed soil can be inferred from L* over L or L over L* treatments; both should yield equal results if no confounding factors are involved, and both were combined here.

Statistical analysis

All statistical analyses were performed using the software JMP-Pro version 14.0. One-way analyses of variance (ANOVA) were conducted to determine if liming, amendment placement and soil source (layer) had a significant effect on leaf Cd. All limed treatments with the same soil sample (surface or subsurface soil samples) were taken together, irrespective of the addition of the ^{108}Cd spike or not because the spiking is expected to not affect the Cd phytoavailability, an assumption that was tested. Differences between treatments were analyzed by Student's t-test or Tukey HSD Post-hoc test at $P \leq 0.05$ significance level. All data were checked to comply with ANOVA's assumptions.

RESULTS

Leaf and root biomass

The number of leaves increased from an average of 4 leaves $plant^{-1}$ at the start of the experiment (6 weeks old seedling) to an average of 12 leaves $plant^{-1}$ after 4 months. There were no signs of nutrient deficiency or diseases. No significant differences in leaf biomass dry weight (DW) were observed between any of the treatments (data not shown). Similarly, no significant differences in total root biomass were observed within the different treatments.

Analyses of the root biomass for the different pot layers indicated that, on average, 70% of the root biomass occurred in the top layer and 30% in the bottom layer (Figure S1).

Effect of contrasting soils on leaf-Cd concentrations

Liming increased soil pH by one unit from 5.2 to 6.5 in the surface soil sample at the start of the plant growth experiment. The soil pH after the final harvest was 5.2 in limed surface soil, and 5.3 in the unlimed soil. The drop in pH after liming might be related to the form of nitrogen present in the fertilizers. The mixed fertilizers contained NH_4-N, which upon oxidation causes acidification (Table S2). Soil pH in spiked soils was only 0.1 units below corresponding unspiked soils, and differences due to spiking were not significant in any of the surface or subsurface soil samples (Table S3).

Leaf-Cd concentrations

The shoot-Cd concentration in the seedlings was 1.09 mg kg^{-1} DW at the start and increased during the 120 d of growth to 10.1 mg kg^{-1} DW. Therefore, the stock of Cd in the seedlings was small and the shoot-Cd at the end of the experiment reflected Cd uptake from the soil during the greenhouse trial. The shoot-Cd concentrations in the fully ^{108}Cd spiked soils were not larger than corresponding values in fully unspiked soils ($p>0.05$). Hence, data of spiked and corresponding unspiked soils were merged, yielding up to 12 replicates per treatment. All raw data of treatments are given in Table S3.

Liming significantly reduced leaf-Cd concentrations (Table 2). The application of AG-$CaCO_3$ to both layers of the surface soil reduced leaf-Cd concentration by factor 1.7 compared to the non-limed treatments. AG-$CaCO_3$ applied only in the top layer decreased leaf Cd concentration only by a factor of 1.2, suggesting that some compensation from the bottom layer occurs (regulated Cd uptake model, see below). Cadmium concentration in cacao leaves

grown on the subsurface soil in both upper and lower positions (treatments 13 and 19) was half as high as in plants grown on the surface soil in both positions. This lower leaf-Cd concentration can be related to the much lower total soil Cd measured in the subsurface soil (Table S1). Liming the top layer, when different soils were in the upper and lower positions, reduced leaf-Cd by factor 1.7. Interestingly, Cd concentration in plants grown on the surface over subsurface soil was not statistically different from corresponding values in plants grown on the surface soil only.

Tracing the source of Cd in the plant

The ^{108}Cd isotope allowed tracing the source of Cd expressed as relative contributions from the top or bottom layer (Figure 1). About 56% of leaf-Cd was derived from the top layer in the non-limed control soil, whereas the top contained about 70% of root biomass; the difference is probably related to thicker roots at the base of the stem in the top layer. Liming the top layer reduced the fraction of Cd that derived from this layer to 36%. This agrees with the fact that for this treatment, Cd was more available in the bottom layer, which was not limed. Logically, liming the top layer significantly increased the fraction of Cd derived from the bottom layer. The increase was a factor of 1.5 ($p<0.05$) for treatments using surface soil samples. The increment was even more prominent (factor 2) in the treatments with two different soil layers ($p<0.01$). The shoot Cd derived from a given depth and expressed as leaf Cd concentration (mg Cd kg^{-1} leaf dry matter) derived from either the top or bottom layer was calculated from the fractions (Eq. 3) multiplied with the Cd content of the leaf. The mean shoot-Cd derived from the bottom in non-limed soil was 4.5 mg Cd kg^{-1} compared to 5.5 mg Cd kg^{-1} when the surface layer was limed, pointing to compensating effects. However, due to the large standard error of the means (0.6 mg Cd kg-1), no significant differences ($p=0.13$) were found between these treatments. On a closer analysis of the data, we could observe that

there was a significant spike position effect on the calculated fractions of Cd, i.e., the fraction derived from a given layer was consistently lower when the isotope was added in that layer. We speculate that the presence of trace levels of Cd coming from dust, in the amendment or even in the fertilizer may have further diluted the isotope in the top layer but not in the bottom layer. To overcome this bias and make a more robust analysis, we calculated the average Cd fraction derived from each layer for each treatment among all replicates irrespective of the spike position (Figure 1); we used that average fraction multiplied by the replicate data of the leaf Cd (n=6 per treatment) and used it to estimate the amount of Cd derived from the bottom layer of these treatments. The results from the previous calculation showed approximately equal mean values for the shoot Cd accumulated from the bottom layer (4.4 mg Cd kg^{-1} vs. 5.5 mg Cd kg^{-1}), but the standard errors of the mean were below 0.3 mg kg^{-1} and the difference was significant ($p<0.01$). This indicates a regulated Cd uptake and explains the minor effects of surface liming on leaf-Cd concentrations when the Cd in the deeper layer has high phytoavailability (acidic topsoil). The regulated Cd uptake was similar in the treatments with two different soil samples: liming the surface soil significantly enhanced Cd uptake from the lower Cd acidic subsurface soils, i.e., 2.3 mg kg^{-1} DM compared to 2.8 mg kg^{-1} DM ($p<0.05$).

Effect of liming and contrasting soil properties on the concentration of selected leaf

Liming reduced the availability of essential micronutrient cations (e.g., Mn and Zn) in the different soil layers (Table 3). Application of AG-$CaCO_3$ to both layers (surface soil, L/L) reduced shoot-Mn concentration by 44% and shoot-Zn concentration 20%. Liming the top layer (surface soil, L/NL) reduced leaf-Mn by 19% and leaf-Zn by 12%. Post-hoc analysis showed that leaf-Zn concentrations were statistically different between non-limed and fully limed treatments (Table 3). However, no significant differences were found between top

limed (surface soil, L/NL), and the other treatments. This result suggests that cacao plants were able to compensate for the lower phytoavailability of soil Zn in the top layer due to liming by taking Zn from the bottom layer. Shoot-Zn concentrations were significantly lower (1.3-factor) after surface layer liming in the surface over subsurface soil treatments.

DISCUSSION

Limestone application reduced the uptake of Cd in cacao seedlings, as shown in the concentrations of Cd in leaves (Table 2). Previous studies reported similar significant reductions in Cd uptake by liming in several crops (Han & Lee, 1996; Lee et al., 2004; Maier et al., 1997). Argüello et al. (2019) reported a strong positive correlation between leaf and bean Cd in cacao and that the soil pH reduced bean Cd by a factor of 1.6 for every pH unit increase, which is similar to the reduction (1.7) found in the fully limed treatments. These results suggest that liming has the potential to reduce Cd concentration in cacao plants. However, liming the top layer only had minor effects compared to liming both layers (Table 2). That reduction is logically smaller because the full liming used more AG-$CaCO_3$ than the top layer limed only, but it is also lower than that expected for a non-regulated Cd uptake model: by liming the top layer, shoot-Cd uptake derived from the bottom layer increased a factor 1.2. This counteracts the partial (top compartment) liming effects yielding a minimal (<1.2) treatment effect on leaf-Cd.

A possible explanation for the more substantial fraction of Cd derived from the bottom layer could be that cacao plants increased the activity of roots in this layer to search for essential nutrients to compensate for the deficit of such nutrients in the top layer. Indeed, liming reduces the availability of essential nutrients. For instance, Zn uptake is highly pH-dependent, and its solubility could be reduced 100-fold by every unit of pH increase (Fageria, Baligar, & Clark, 2002). Chaney, Filcheva, Green, and Brown (2006) reported that over-

liming might induce Zn deficiency, consequently promoting Cd accumulation in lettuce. Here, liming reduced Zn concentration in cacao leaves (Table 3). However, shoot-Zn did not show a significant reduction in the single-layer limed soils as it did when both layers were limed. The compensation effect indicates that regulated Cd uptake could be a physiological mechanism triggered by cacao plants to cope with Zn deficiency. As reported by Clemens (2006), Cd can enter plant cells through Ca, Fe, and Zn transporters of low specificity, particularly in conditions of micronutrients deficiency where there is an induced expression of the transporters involved in the uptake of such nutrients (Cohen, Fox, Garvin, & Kochian, 1998; Cohen, Garvin, & Kochian, 2004). Upon top layer liming, more Cd and more Zn may be absorbed from the high Cd and Zn acid soil located at the bottom of the pots. If that bottom layer is not an abundant source of available Zn as in the case of the subsurface soil sample, less compensating effects may occur. Hence, the following equation is proposed to describe the linear relationship between Zn and Cd:

$$Cd_{UPT} = Zn_{UPT} * \left(\frac{Cd_r}{Zn_r}\right) \quad \text{Eq. (5)}$$

Where Cd_{UPT} is the Cd uptake per plant [µg plant^{-1}], Zn_{UPT} is the Zn uptake per plant [µg plant^{-1}], Cd_r is the Cd concentration in the rhizosphere [mg kg^{-1}], and Zn_r is the Zn concentration in the rhizosphere [mg kg^{-1}]. By liming, soluble Zn and Cd concentrations in the rhizosphere may be reduced to the same extent, i.e., the Cd/Zn ratio in the rhizosphere might be unaffected. However, higher Zn uptake from that layer due to homeostatic control mechanisms acting systemically might enhance Cd uptake. Based on the proposed equation, Cd_{UPT}/Zn_{UPT} did not show significant differences between unlimed and top limed surface soil samples (the ratio is 0.11 in both treatments), suggesting that the plants increased Cd and Zn uptake from the bottom layer to the same extent. Rhizosphere Zn and Cd concentration measurements are needed to confirm the proposed equation. Not surprisingly, leaf-Cd and

leaf-Zn concentrations decrease similarly upon liming and are, hence, tightly positively correlated across almost all treatments. Noteworthy, the decrease in Cd and Zn concentrations stops at a lower Zn level in the leaves (about 50 mg Zn kg^{-1}) below which, Cd uptake is even enhanced by further lowering leaf-Zn (Fig S2). Alternatively, the regulated Cd uptake may also be related to changes in the rhizosphere condition, i.e., by excreting H^+ or organic anions to compensate for the unbalance created by the excessive amount of cations that enter the roots (Hinsinger, Plassard, Tang, & Jaillard, 2003). Plassard, Meslem, and Souche (1999) reported that the exudation of H^+ did not occur regularly along with the roots of maize, and it was more identifiable in the subapical zone. This change in root activity might also be triggered by Zn deficiencies (Marschner, 1993). Therefore, cacao roots might be able to modify their activity depending on the availability of essential nutrients such as Zn. More research is needed to unravel a possible correlation between the H^+ fluxes and Zn uptake in cacao.

Even though these results cannot be extrapolated to field conditions, due to intrinsic differences in pot trials vs. field trials (De Vries & Tiller, 1978), they suggest that three factors might be the key to understand why superficial liming might not effectively reduce Cd uptake in cacao crops. First, root depth. Sexually propagated cacao trees can develop roots up to 200 cm depth (Wood & Lass, 2008). Second, limited lime penetration. Caires, Joris, and Churka (2011) reported that in a long-term study in Brazil, surface application of dolomitic lime (4.5 Mg ha^{-1}) successfully increased the soil pH to a depth of 60 cm after 8 yr, which is less than 30% of the total cacao root length. Third, Cd-surface/Cd-subsurface ratio. Chavez et al. (2015) found that soil Cd concentration decreased with depth in a Cd hotspot on the southern coast of Ecuador. Barraza et al. (2017) found a similar trend at contaminated sites on the northern Ecuadorian Amazon, but this trend was not always present at unpolluted sites

on the central coast of Ecuador as some sites showed a relatively constant Cd concentration down the soil profile. The interaction of deep roots, poor alkalinity penetration after surface application, and relatively constant Cd concentration down the soil profile could intensify the compensation effect, which might be related to a reduction in Zn availability due to liming. A plausible option to increase the effectiveness of superficial liming could be a simultaneous application of Zn, especially in cases where shoot-Zn concentrations are low or below the inversion point of the leaf Cd vs. Zn plot (Figure S2).

CONCLUSIONS

Liming soil may reduce Cd uptake in cacao, factors found here are 1.2 and 1.7 with modest doses of 2 and 4 Mg ha^{-1} where the AG-$CaCO_3$ was thoroughly mixed with the soil layer. However, the effectiveness of such a strategy will be more than proportionally related to the depth of incorporation of the liming material. Therefore, it is necessary to search for liming materials that are able to change the chemical environment of deep soil layers. The possible changes in root activity due to a deficiency of essential nutrients such as Zn suggests that limestone+Zn supplementation could be a plausible mitigation strategy. However, more research is needed to corroborate the results of this experiment. Finally, this study shows that surface liming may be ineffective when the subsurface soils are a substantial source of Cd and micronutrients which presence affects Cd uptake.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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Legend of images

Figure 1. Fractions of shoot Cd derived from either top or bottom layer in the pot filled with either the surface soil sample or the combination of a surface over a subsurface soil sample based on the IA of leaf Cd. Error bars show the standard deviation for the fractions. L: Limed with agricultural limestone (AG-$CaCO_3$), NL: Non-Limed, B: Subsurface soil.

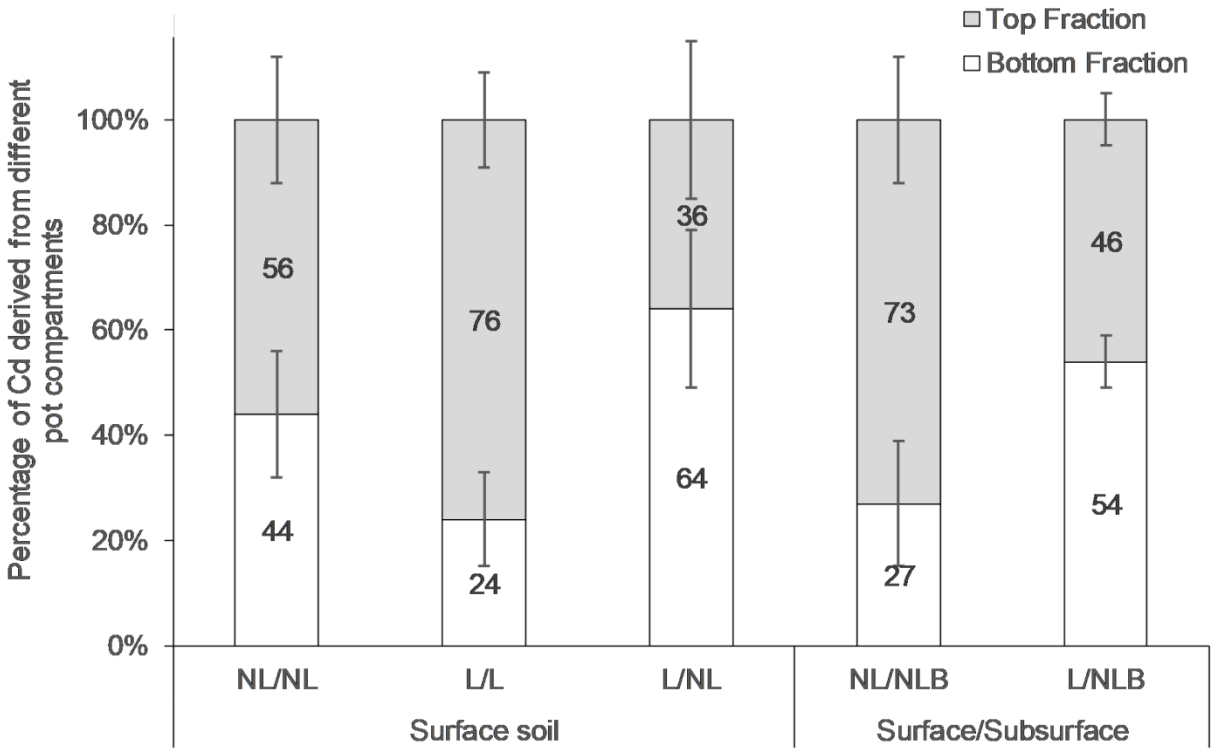

Table 1. Description of the 22 different treatments in the pot experiment to test effects of soil liming, either complete or surface only (T1-T3) and effects of surface liming with a different bottom layer (T14 and T18). Stable Cd isotope was added to the top or bottom layer to identify the provenance of Cd. The isotope signature of bioavailable Cd in these spiked soils was determined with the other treatments.

Pot Layer	Treatments [a]					
	T1	T2	T3	T4	T5	T6
Top	LS	NLS	LS	NL*S	NLS	NL*S

Bottom	LS	NLS	NLS	NLS	NL*S	NL*S
	T7	T8	T9	T10	T11	T12
Top	L*S	LS	L*S	L*S	LS	L*S
Bottom	NLS	NL*S	NL*S	LS	L*S	L*S
	T13	T14	T15	T16	T17	T18
Top	NLB	NLS	NL*S	NLS	NL*S	LS
Bottom	NLB	NLB	NLB	NLB*	NLB*	NLB
	T19	T20	T21	T22		
Top	NLB*	L*S	LS	L*S		
Bottom	NLB*	NLB	NLB*	NLB*		

[a] T: treatment, L: Limed with agricultural limestone (AG-$CaCO_3$), NL: Non-Limed, *: ^{108}Cd labeled; S: surface soil; B: subsurface soil.

Table 2. Mean Cd concentrations in the leaf for the different treatments. Different letters within a column denote significant differences ($p \leq 0.05$) with Tukey Post-hoc analysis. Small letters within a column indicate the comparison of the effect of limestone and limestone position among treatments with surface soils only. Capital letters show the effect of different soil layers. Underlined letters show the effect of liming the upper soil layer vs. liming the subsurface layer.

Treatment combinations		Treatment code*	N	Mean mg Cd kg^{-1}			
Surface soil only	No lime	NL/NL	12	10.1	a	A	
	Limed top	L/NL	12	8.54	b		
	Limed top/bottom	L/L	12	5.84	c		
Surface over subsurface soils	No lime	NL/NLB	12	9.16		A	a
	Limed top	L/NLB	12	5.42			b
Subsurface soil only	No lime	NLB/NLB	6	5.17		B	

* L: Limed with agricultural limestone (AG-$CaCO_3$), NL: Non-Limed, B: Subsurface soil.

Table 3. Mean Mn, Zn, and Fe concentrations in the leaf for the different treatments. Different letters within a column denote significant differences ($p \leq 0.05$) with Tukey Post-hoc analysis. Small letters within a column indicate the comparison of the effect of limestone and limestone position within treatments of equal (surface) soil samples. Capital letters show the effect of different soils. Underlined letters show the effect of superficial liming on the surface over subsurface treatments.

Treatment combinations		Label *	N	Mn [g kg^{-1}]				Zn [mg kg^{-1}]				Fe [mg kg^{-1}]			
Surface soil	No lime	NL/NL	12	1.28	a	B		87.1	a	A		120	ns	NS	
	Limed top	L/NL	12	1.04	b			76.8	ab			147	ns		
	Limed top/bottom	L/L	12	0.72	c			69.8	b			177	ns		
Surface over subsurface soil	No lime	NL/NLB	12	1.43		AB	a	72.9		B	a	138		NS	ns
	Limed top	L/NLB	12	0.94			b	53.8			b	115			ns
Subsurface soil only	No lime	NLB/NLB	6	1.58		A		36.1		C		209		NS	

* L: Limed with agricultural limestone (AG-$CaCO_3$), NL: Non-Limed, B: Subsurface soil.
** ns: no significant (P>0.05)

Surface soil liming reduces cadmium uptake in cacao (*Theobroma cacao* L.) seedlings but is counteracted by enhanced subsurface Cd uptake

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Soil sampling and chemical characterization

The soils were sampled at a cacao farm in Ecuador that was selected based on soil pH and soil Cd concentration observed in a previous soil survey (Argüello et al., 2019). Acidic surface (0─15 cm, pH 5.2) and subsurface (15─30 cm, pH 5.5) layers of an Oxiaquic Udifluvents were collected under 12 to 15 different trees. About 450 kg of surface soil and 150 kg of subsurface soil were collected. Soil samples were air-dried and sieved (<2 mm) before chemical analysis and use for the pot experiment. The soil physicochemical properties are shown in Table 1.; under these conditions, elevated bean-Cd concentrations (4.37 mg/kg) had been measured in our previously reported survey (Argüello et al., 2019). Soil pH [in deionized water (DIW)] was measured in a 1:5 (w/v, g:ml) soil to solution suspension. Soil organic carbon (%OC) was determined with an elemental analyzer (Carlo Erba EA1108) from a soil subsample previously ground to a powder and acidified with 20 to 50 µL of 10% v/v hydrochloric acid (HCl) to remove any carbonates. The cobalt hexamine trichloride extractant solution method was used to determine the effective cation exchange capacity (ECEC) as described in the protocol ISO-23470 (International Standard Organization, 2007). Hydrous iron (Fe_{OX}), aluminum (Al_{OX}), and manganese (Mn_{OX}) oxyhydroxides were determined in a 1:50 soil to ammonium oxalate (pH 3) solution in the dark (Schwertmann, 1964). The concentrations of Al, Fe, and Mn in the extract were analyzed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, iCAP 7400 series, Thermo Scientific). The total elemental concentrations of the soils were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent 7700x, Agilent Technologies), after digestion in boiling aqua regia (1:3 Suprapur® HCl:HNO_3) for approximately four h.

The Certified Reference Soil Material and certified Cd (±uncertainty) was BCR-142R light sandy soil with *aqua regia* soluble Cd 0.25±0.01 mg kg^{-1}. The recovery (±SD) of Cd calculated relative to the certified concentration was 90.0±4.75%.

Soil spiking with ^{108}Cd and soil liming

Cadmium metal with enrichment of 71.1% in ^{108}Cd isotope was dissolved in concentrated Suprapure® nitric acid (HNO_3, 65% v/v). The ^{108}Cd concentrated solution was diluted with Milli-Q water (18.2 MΩ cm^{-1}) (hereafter DIW) to obtain a 0.1 M HNO_3 stock solution with a measured Cd concentration of 1630 mg L^{-1} and measured ^{108}Cd abundance of 71.2%. For spiking the soils, 5 ml of the prepared ^{108}Cd stock solution was diluted in DIW to a final volume of 7.7 L.

Approximately 7.5 kg of air-dried and sieved soil for each treatment was placed in plastic bags to facilitate homogenization of the ^{108}Cd spike solution and amendment application. the ^{108}Cd-treatments, a total volume of 350 ml of the spike solution was sprayed onto and thoroughly mixed with soil. The ^{108}Cd-enriched treatments were allowed to equilibrate for 3 d. as it is the most common time used in isotopic exchange studies (Hamon, Parker, & Lombi, 2008). The same procedure was done for the unspiked treatments using only DIW. Total Cd concentration added via the spike to the soil was 0.049 mg kg^{-1}; this was a 5% ^{108}Cd enrichment for the surface soil and 8% for the subsurface soil which contained lower Cd. The addition of ^{108}Cd did not significantly change the measured total soil Cd concentration for surface ($p=0.078$, t-test) or subsurface ($p=0.06$, t-test) soil samples.

Commercially available AG-$CaCO_3$ sieved through a mesh # 325 (or 45 μm) was added at a rate of 1.54 g per kg of soil equivalent to either 4 Mg ha^{-1} for the fully limed treatment or 2 Mg ha^{-1} for treatments with only one layer amended. At this rate, more than one unit of soil pH_{DIW} rise was achieved (target pH_{DIW} ~6.5). Limed and unlimed soils were moistened with DIW to reach 50%

of water holding capacity (WHC) and were incubated for 3 d. After equilibrium, soil subsamples were taken to measure soil pH. The final soil pH of the limed surface soil sample was confirmed at 6.5 (pH_{DIW} 1:5 ratio soil/DIW). oil moisture content in all bags was finally adjusted to 70% of WHC before placing 5 kg (dry wt.) of soil in individual pots.

Experimental Fertilization details

Seedlings received weekly applications of commercial foliar fertilizer sprayed on the shoots (5.5 mg kg^{-1} soil, containing 24% N, 7.8% P, 5.3% K, and 0.05% Mg) and cytokinins (2.7 mg kg^{-1} soil) for 30 d. Thereafter, every 30 d, a mix of two commercial granular fertilizers, 80% of NPK (16% N, 7% P, and 6.6% K) and 20% of NPK + micronutrients (12% N, 10.5% P, 5% K, 1.2% Mg, 1% S, 0.04% B and 0.02% Zn) were applied to the surface of each pot to supply nutrients equivalent to 67 mg N, 15 mg P, 23 mg K, 0.64 mg Mg, 0.88 mg S, 35 µg B and 18 µg Zn per kg of soil. The specific chemical forms of the fertilizers added are reported in Table S2.

Table S1. Selected chemical properties of the surface (0-15 cm) and subsurface (15-30 cm) soil samples before the amendment application (Mean±SD).

Characteristic	Surface	Subsurface
pH [a]	5.2	5.5
Organic carbon (%)	2.62	1.45
Oxalate-extractable Fe (g kg^{-1})	10.3	6.54
Oxalate-extractable Al (g kg^{-1})	2.03	2.09
Oxalate-extractable Mn (g kg^{-1})	3.73	2.37
ECEC ($cmol_c$ kg^{-1})	16.6	14.6
Exchangeable Ca ($cmol_c$ kg^{-1})	11.4	9.48
Exchangeable Mg ($cmol_c$ kg^{-1})	4.70	3.63
Exchangeable K ($cmol_c$ kg^{-1})	0.36	0.24
Total P (mg kg^{-1}) *	494 ± 60.6	293 ± 14.9
Total Cd (mg kg^{-1}) *	1.38 ± 0.26	0.77 ± 0.13
Total Zn (mg kg^{-1}) *	124 ± 7.32	115 ± 7.04
Total Mn (g kg^{-1}) *	3.34 ± 0.32	3.25 ± 0.40
Total Ca (g kg^{-1}) *	3.52 ± 0.27	2.81 ± 0.17
Total Mg (g kg^{-1}) *	3.41 ± 0.54	3.60 ± 0.32
Total Fe (g kg^{-1}) *	60.8 ± 4.27	72.6 ± 5.16
Maximum WHC (ml kg^{-1})	430	300
FAO Soil classification (MAG-SIGTIERRAS, 2017)	Fluvisol	

[a] 1:5 w:v soil: DIW, (*) aqua regia soluble; ECEC: effective cation exchange capacity; WHC: water holding capacity

Table S2. List of the chemical form of fertilizers added during the experiment.

Fertilizer Type	Ingredients	Percentage in commercial mixture*
NPK	Ammonium nitrate	30 – 35
	Potassium chloride	15 – 20
	Ammonium chloride	7 – 10
	Ammonium dihydrogen orthophosphate	7 – 10
	Potassium nitrate	7 – 10
	Calcium hydrogen orthophosphate	7 – 10
	Diammonium hydrogen orthophosphate	3 – 5
	Calcium fluoride	2.5 – 3
NPK + Micronutrients	Ammonium dihydrogen orthophosphate	35 – 45
	Potassium chloride	20 – 25
	Ammonium nitrate	20 – 25
	calcium dyhydrogen orthophosphate	7 – 10
	Ammonium sulphate	3 – 5
	calcium hydrogen orthophosphate	2.5 – 3
	Boric Acid	0.3 – 1
	Disodium tetraborate pentahydrate	0.1 – 0.2

* Ranges are reported by the seller in order to protect the confidentiality or due to variations between lots.

Table S3. Effect of soil treatments and limestone treated layer on Cd concentrations in leaves and isotopic abundances of ^{108}Cd (mean and standard error), and soil pH (at harvest) in sample per soil type per pot compartment at harvest (120 days after transplant).

Treatment	Soil Type	Amendment position	Spike Position	N	Cd-Leaf	IA ^{108}Cd	pH of compartment	
					[mg kg^{-1}]	[%]	Top	Bottom
T1	Surface	T/B	No	3	6.34 ± 0.41	0.91 ± 0.02	5.4	5.5
T2	Surface	No	No	3	11.1 ± 0.51	0.93 ± 0.01	5.1	5.1
T3	Surface	Top	No	3	9.20 ± 0.66	0.92 ± 0.02	5.1	5.0
T4	Surface	No	Top	3	10.6 ± 0.32	2.83 ± 0.08	4.9	5.0
T5	Surface	No	Bottom	3	9.60 ± 0.49	2.38 ± 0.26	4.9	5.0
T6	Surface	No	T/B	3	9.22 ± 0.53	5.04 ± 0.06	4.9	5.0
T7	Surface	Top	Top	3	8.54 ± 0.80	2.02 ± 0.12	5.2	5.0
T8	Surface	Top	Bottom	3	8.72 ± 0.59	3.08 ± 0.27	5.2	5.0
T9	Surface	Top	T/B	3	7.71 ± 0.18	5.50 ± 0.08	5.2	4.9
T10	Surface	T/B	Top	3	5.51 ± 0.60	4.15 ± 0.27	5.2	5.4
T11	Surface	T/B	Bottom	3	5.39 ± 0.53	1.88 ± 0.21	5.2	5.4
T12	Surface	T/B	T/B	3	6.13 ± 0.77	5.32 ± 0.08	5.2	5.4
T13	Subsoil	No	No	3	5.23 ± 0.28	0.99 ± 0.05	5.2	5.2
T14	Surf/Sub	No	No	3	11.2 ± 0.68	0.91 ± 0.01	5.0	5.0
T15	Surf/Sub	No	Top	3	7.80 ± 0.29	3.62 ± 0.17	4.9	4.9
T16	Surf/Sub	No	Bottom	3	10.2 ± 0.61	2.25 ± 0.36	5.0	5.0
T17	Surf/Sub	No	T/B	3	7.40 ± 0.47	5.66 ± 0.07	4.9	4.9
T18	Surf/Sub	Top	No	3	5.81 ± 0.21	0.97 ± 0.01	5.2	5.0
T19	Subsoil	No	T/B	3	5.11 ± 0.74	8.21 ± 0.20	5.0	5.0
T20	Surf/Sub	Top	Top	3	5.28 ± 0.35	2.94 ± 0.19	5.1	5.0
T21	Surf/Sub	Top	Bottom	3	5.20 ± 0.07	4.81 ± 0.05	5.2	5.0
T22	Surf/Sub	Top	T/B	3	5.41 ± 0.27	6.47 ± 0.02	5.2	5.1

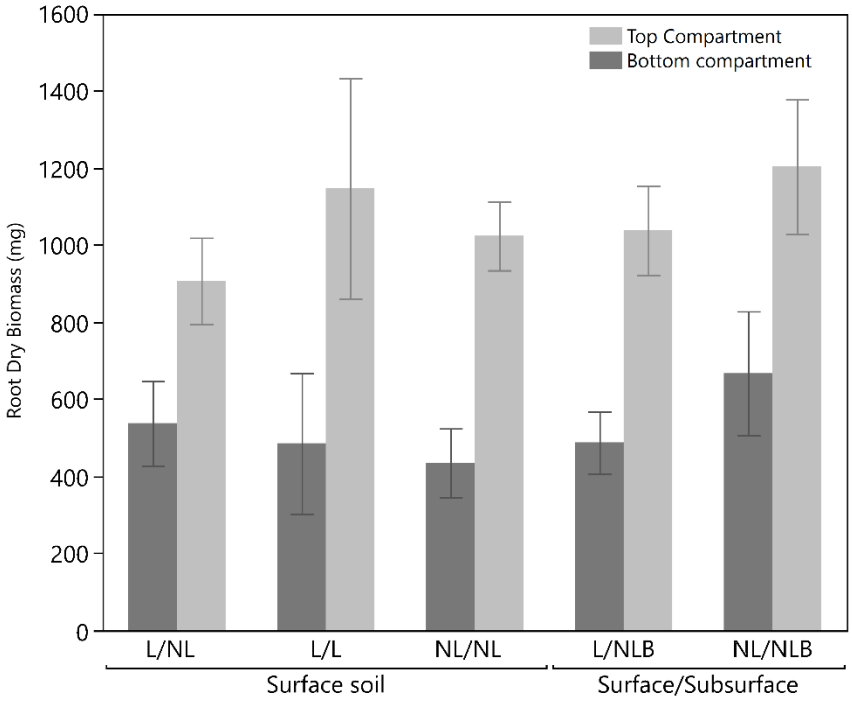

Figure S1. Average root biomass by layer for the different treatments. Error bars show the standard error using one standard error from the mean. L: Limed with agricultural limestone (AG-$CaCO_3$), NL: Non-Limed, B: Subsurface soil.

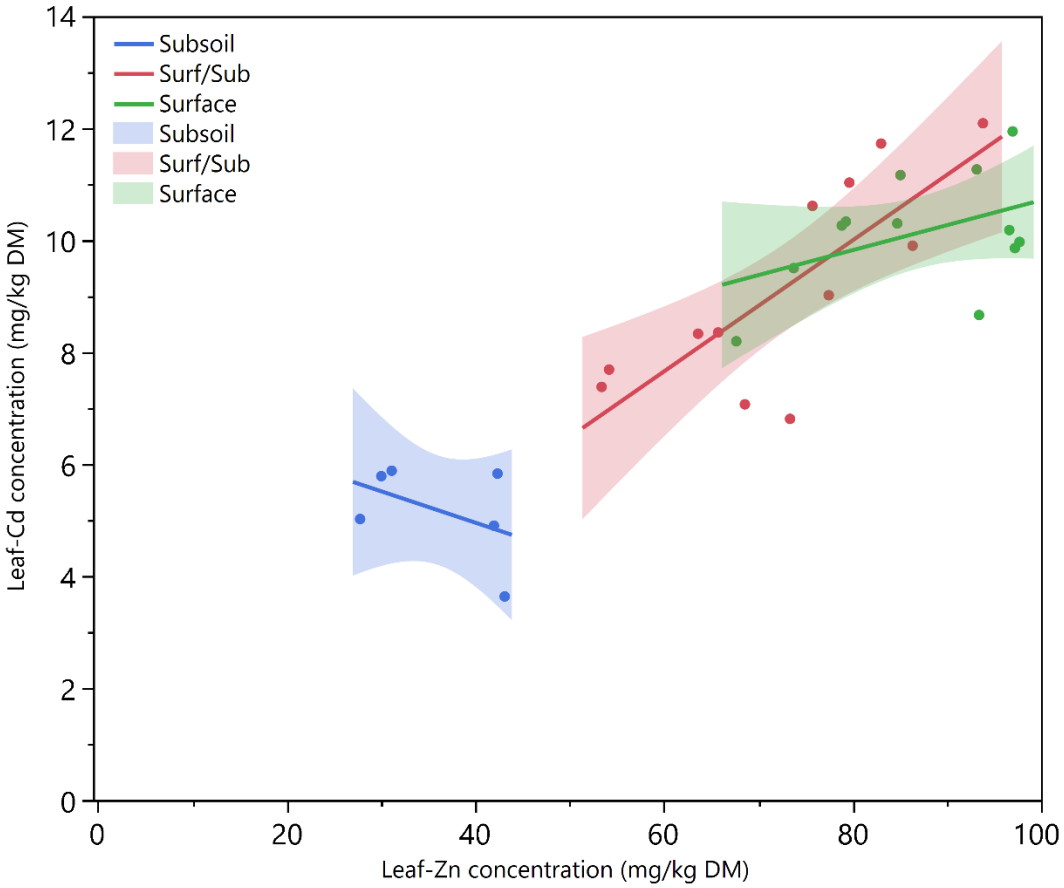

Figure S2. Relation between concentrations of Cd and Zn in the leaves for the different soil types. Lines represent a linear regression fitted to the different soil data. The shaded area represents the confidence interval for the predicted value at 95% confidence.

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