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PHYTOEXTRACTOR SPECIES AND DOLOMITIC LIME AS STRATEGIES TO MANAGE CD IN CACAO (*THEOBROMA CACAO* L.) AND SPINACH (*SPINACIA OLERACEA*)

A Thesis in

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ABSTRACT

European Commission Regulation (2006) and European Food Safety Authority (EFSA) (2012) legislation setting cadmium (Cd) human ingestion thresholds in food products is now affecting cacao bean (*Theobroma cacao* L.) production in Latin American countries via the rejection of food products exceeding maximum ingestion threshold levels. Cacao producers must be proactive and minimize Cd uptake by commodities and such strategies must be affordable for low income communities (or countries) where agricultural development is critical to stabilizing socio-political-economic systems.

Two potential options for producers include: (a) the use of phytoextractor species, which can remediate some soil pollutants; and (b) metal immobilization via soil pH adjustment. We hypothesize that the use of a Cd phytoextractor species in an intercropping scheme, with the addition of dolomitic lime (to raise soil pH and decrease soil Cd availability), will decrease total Cd uptake in cacao and in spinach plants. The effectiveness of four phytoextractor species was evaluated: *Helianthus annuus* (sunflower), *Brassica napus* (oilseed rape), *Chyrsopogon zizanioides* (vetiver), and *Heliconia psittacorum* (heliconia). The efficacy of a combined phytoextraction and immobilization (per the use of dolomitic lime to raise soil pH) strategy was assessed.

This research is divided in five chapters. The first chapter is the introduction and problem statement. The second chapter is the literature review. The third chapter describes three Cd dose-response experiments. Experiment 1 and 2 were conducted at The Pennsylvania State University (US) and evaluated the Cd extraction ability of sunflower, oilseed rape, and vetiver cultivated in a soil-less media (play sand and perlite) artificially spiked with Cd. Experiment 2 followed the same scheme as the first, but plants were cultivated in a manufactured soil matrix (soil and perlite) at Penn State. Experiment 3 was conducted at Universidad del Valle (Cali, Colombia) and

used sunflower, vetiver, and heliconia cultivated in a natural soil artificially contaminated with Cd. Experiments 1, 2 and 3 were used to determine the most suitable Cd phytoextractor. Results from the experiments reveal that the sunflower presented a high plant aerial tissue total Cd and a high resistance to pest and diseases, in comparison to the other plant species, therefore it is recommended as a suitable Cd phytoextractor.

In chapter four, a new set of three experiments was developed. The aim of these experiments was to assess the effect of an intercropping scheme with sunflower (as Cd phytoextractor) and dolomitic lime application (as soil pH modifier) on cacao and spinach Cd accumulation. Results indicate that dolomitic lime application increased soil matrices pH reducing Cd accumulation in cacao (variety IMC-67), but neither the addition of dolomitic lime nor the intercropping scheme with sunflower resulted in a significant reduction on total Cd in plant aerial tissue of spinach (alpha = 0.1).

Chapter 5 presents a summary of results and future research.

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Chapter 1

Introduction

Statement of problem

Cacao (*Theobroma cacao* L.) is a critical commodity for many countries throughout South America and Africa, being typically used worldwide as a main ingredient for chocolate production. However, the marketing of cacao beans from South America could soon become difficult due to contamination of cacao beans by cadmium (Cd) taken up from soils (Barraza et al., 2017; Mrmošanin et al., 2018).

Cadmium is a naturally occurring heavy metal derived from volcanic rocks, sedimentary rocks, and alluvial sediments, but Cd can also accumulate from mining activities (as a byproduct of Zn, Pb or Cu extraction) (Faroon et al., 2012; Wei et al., 2017), sewage sludge additions to soil (Kabata-Pendias, 2011), from industrial processing (Houben et al., 2012), and from phosphate (P) fertilizers (Jiao et al., 2012; Faroon et al., 2012). Although plant species do not require Cd to survive, plants can inadvertently absorb Cd (Rascio & Navari-Izzo, 2011). Accumulation of Cd in cacao occurs in leaves, pod husks and beans (Arévalo-Gardini et al., 2017; Barraza et al., 2017; Valiente et al., 1996).

Due to its pronounced toxicity and prevalence in nature, the U.S. Environmental Protection Agency (USEPA) has classified Cd as one of the 126 priority pollutants (Jancic & Stosic, 2014), while the American Agency for Toxic Substances and Disease Registry (ATSDR). (2017) ranked Cd as seventh on the list of 275 primary dangerous substances. On January 1, 2019 the European Commission (2014) began enforcing a limit of 0.6 mg Cd kg^{-1} of cocoa powder ready for consumption. Given the potential effect of commodity Cd level restrictions to U.S. and

European markets, Cd management in soils and commodities is a primary concern of export countries. Cd management in soil and commodities is especially important in Colombia given cacao has been used as a replacement crop for coca (*Erythroxylum coca*). Maintaining the decline in coca production via other commodities is a primary goal for many nations and international aid organizations. For example, the USAID-USDA Cacao for Peace project has been a driver of cacao development in Colombia. Projects aimed to evaluate the dynamics of Cd in soil and its relationship with Cd present in cacao beans have been developed in Peru, Ecuador, Trinidad and Tobago, Honduras, and Bolivia (Arévalo-Gardini et al., 2017; Argüello et al., 2019; Gramlich et al., 2017; Gramlich et al., 2018; Ramtahal et al., 2016).

Need for study

The global demand for high quality cacao is increasing (Boeckx et al., 2018). Latin America is known for having naturally high levels of natural Cd in soils, which can accumulate in cocoa beans (Boeckx et al., 2018). Cd exposure in humans occurs primarily through smoking and food intake (Faroon et al., 2012). Once in the body, Cd can be retained in the kidney and liver for 15 to 30 years (Castelli et al., 2005; Boeckx et al., 2018) causing renal tissue nephrotoxicity (Jaishankar et al., 2014), severe pulmonal and gastrointestinal irritation (Tchounwou et al., 2012). The EFSA (2012) established a tolerable weekly human intake of 2.5 μg Cd kg^{-1} body weight to avoid toxicity effects. Health surveys have shown that in the U.S., the average daily food intake is 18.9 μg Cd kg^{-1} body weight (Jancic and Stosic, 2014). In January 2019, the European Commission (2014) introduced maximum limits in the concentration of Cd in chocolate products (Table 1-1). Guerra-Sierra et al. (2018) indicate that the maximum allowable level of Cd in dry cacao beans is 0.1 mg kg^{-1}.

Table **1-1**: Maximum permitted levels of Cd in cacao derived products established by the European Commission (2014).

Specific cacao and chocolate products	Max. permitted Cd (mg kg^{-1})
Milk chocolate with < 30 % tdcs	0.1
Chocolate with <50 % tdcs; milk chocolate with ≥ 30 % tdcs	0.3
Chocolate with ≥ 50 % tdcs	0.8
Cocoa powder or as ingredient in sweetened cocoa powder sfc	0.6

tdcs: total dry cacao solids; sfc: sold to the final consumer

The chocolate industry in Colombia provides jobs for more than 38,000 small-scale farmers (Colombian Ministry of Agriculture, 2018). Because of the presence of Cd in soils and the maximum limits enforced by the European Union (EU), Colombian high-quality cacao beans, with high Cd, could become unexportable. Measures must be taken to lower Cd uptake by cacao (Boeckx et al., 2018). Cacao is also the flag-crop promoted by the Colombian Government as a suitable substitute to coca (*Erythroxylum coca*), whose leaves are used to produce cocaine. Cocaine from Colombia accounts for 92% of what is seized on the streets of the US (US Drug Enforcement Administration DEA, 2017).

Objectives

Research experiments were designed to accomplish the following main objectives:

Objective 1. Evaluation of the phytoextraction capacity of four plant species: sunflower, oilseed rape, vetiver, and heliconia under greenhouse conditions.

Objective 2. Assessment of the effect of an intercropping scheme (Cd phytoextractor determined in objective 1 and cacao; Cd phytoextractor determined in objective 1 and spinach) with dolomitic lime application on the accumulation of Cd in cacao and spinach under greenhouse conditions.

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Chapter 2

Literature review

Cadmium in soils

Heavy metals naturally occur in many soils; however, they are considered a soil pollutant when their levels are associated with acute or chronic toxicity (Bhadkariya et al., 2014). Metals in the soil environment may exist in several forms as follows: (1) free metal ions and soluble metal complexes in the soil solution, (2) metal ions occupying ion-exchangeable sites and specifically adsorbed on inorganic soil constituents, (3) organically bound metals, (4) precipitated or insoluble compounds, particularly in the form of oxides, carbonates, and hydroxides, and (5) metals in structure of some silicate minerals (Manisha & Mohan 2017).

Cadmium is a toxic heavy metal, and it is frequently encountered in industrial wastewaters from plating and Cd–nickel battery production processes, phosphate fertilizers, mining, pigments, stabilizers used to retard the degradation processes which occur in polyvinylchloride (PVC) and related polymers on exposure to heat and ultra violet light (sunlight) (Mohammadi et al., 2015), and from the petroleum refining industry (Peláez-Peláez et al., 2016). Soil Cd pollution, from both anthropogenic and geogenic sources, has posed an increasing challenge to soil quality and food security as well as to human health. Cd-rich soils exist in many nations and require management to limit crop Cd intake (Paul & Chaney, 2017). In soils considered non-polluted, Cd concentrations range from 0.01 to 1.1 mg kg^{-1} with an average of 0.41 mg kg^{-1} (Kabata-Pendias, 2011; Singh & McLaughlin, 1999). Cadmium is typically more soluble and mobile than other heavy metals due to its weak affinity for soil colloids and hence is more easily transferred to the crops (Sarwar et al., 2010; Smolders, 2013). Approximately 70% of the Cd retained in soils is through electrostatic forces and Cd will often move to deeper soil

layers once bound (He et al., 2015a). In general, the total Cd distribution in soil decreases with depth (Caridad-Cancela et al., 2007).

Kabata-Pendias (2011) indicates that during weathering processes Cd forms simple compounds, such as CdO, $Cd(OH)_2$, $CdCl_2$, and CdF_2, which are easily mobilized, especially via sedimentation processes. Cd compounds are known to be isotypic with corresponding compounds of cations such as Zn^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}, Mg^{2+}, and, in some cases Ca^{2+}. Chemically, Cd is similar to Zn with both metals being in the +2-oxidation state, having an identical crystal structure (both hexagonal) and similar electronegativity (Zn 1.65, Cd 1.69) (Ming et al., 2016). As Chaney (2010) pointed out, Cd and Zn should always be considered together, because they have similar biogeochemical behavior in soils and accumulation in organisms.

In soils, the bioavailability of Cd is controlled by several factors such as total metal content, pH, redox potential, soil organic matter, cation exchange capacity, clay content, presence of other nutrients, Fe and Mn oxides (Corguinha et al., 2012; He et al., 2005; Kirkham, 2006; Smolders, 2013), parent material, soil texture (Kabata-Pendias, 2011), root exudates, types, and cultivars of crop plants (Brus et al., 2005; Cieśliński et al., 1996), and management practices (Corguinha et al., 2012).

Soil pH is an important factor affecting Cd availability (Barančíková et al., 2004; Golia et al., 2008; Kabata-Pendias, 2011; McBride, 2002). There is an indirect linear relationship between soil pH and bioavailability, or plant uptake of Cd (Tudoreanu & Phillips, 2004). As pH decreases, Cd uptake by plants increases (Chavez et al., 2016a; Kim et al., 2009; Kirkham, 2006). While some Cd is naturally released into soils through weathering of rocks, which typically contain Cd at concentrations < 0.2 mg kg^{-1}, high inputs originating from anthropogenic sources such as mining, smelting, microelectronics manufacturing, and phosphate fertilizers have significantly increased Cd contents in many soils worldwide (He & Singh, 1994; He et al., 2015a; Mahar et al., 2016). Sánchez et al. (2011) determined the adsorption capacity of Cd in four

Venezuelan agricultural soils with different textures, concluding that soils with the highest clay content, soil organic matter, and acid pH conditions were those that showed higher adsorbing Cd capacity.

Cd soil bioavailability has been documented to be related to phosphate (P) fertilizers use (Nartey et al., 2012). P fertilizers are of great importance because P is the most limiting macronutrient for crop growth in tropical soils due its retention in oxidic clays (Corguinha et al., 2012). Application of P fertilizers is considered one of the major inputs of Cd in agricultural soils, with Cd levels ranging from 130 mg Cd kg^{-1} (Chen et al., 2007; Jiao et al., 2012; Khwaja et al., 1997) to 300 mg Cd kg^{-1} (Grant, 2011). Application of P fertilizers like monoammonium phosphate may also increase the solubility of Cd by lowering soil pH (He et al., 2015a). Cd can also be added to soils via inputs with mineral fertilizers, farmyard manure, organic residues and other materials (Grant et al., 1999; Rankin et al., 2005; Zhang et al., 2012).

The fraction of the soil Cd concentration available to plants is called bioavailable Cd (Sarwar et al., 2010). Since not all forms of metals present in soil are available to plants, it is important to estimate bioavailable Cd rather than the total Cd concentration in order to assess potential toxic effects and metal amount available for plant uptake (He et al., 2015b).

Cadmium in plants

Cd is a trace metal that does not have an essential biological function (Gramlich et al., 2017). Cd can accumulate in roots, shoots and edible plant parts such as grains and cacao beans (Rascio & Navari-Izzo, 2011; Zarcinas et al., 2004). Cd also bio-accumulates in organisms and is carcinogenic at low concentrations (Gratão et al., 2012).

Plants absorb Cd as a divalent cation, which is the most predominant and mobile form of Cd in soil and the environment (Chavez et al., 2015). Cd affects plants by inhibiting

photosynthesis and respiration, reducing water and nutrient uptake, altering gene and protein expression, inducing and inhibiting enzymes, enhancing accumulation of reactive oxygen species, enhancing lipid peroxidation, and disturbing metabolism (Sandalio et al., 2001; Semane et al., 2010; Tanhan et al., 2007). Reduction of Cd uptake by plants, and the resultant translocation to edible parts, is thus an important strategy for the agronomic use of moderately Cd-contaminated soils (Singh & McLaughlin, 1999).

Cadmium phytoextraction

Conventional techniques to remediate heavy metals from polluted soils (vitrification, soil incineration, excavation and landfill, soil flushing, solidification, and stabilization of electro-kinetic systems) (Dermont et al., 2008) have limitations i.e. intensive labor, high cost, disturbance of indigenous soil micro-flora, and irreversible changes in soil physicochemical properties (Ali et al., 2013; Ghosh et al., 2005; Mahar et al., 2016). Phytoremediation is considered a green, alternative solution to the problem of heavy metal pollution (Ali et al., 2013). Phytoextraction of heavy metals is a potential phytoremediation uptake mechanism for extracting heavy metals in soils (Bhadkariya et al., 2014; Ghosh et al., 2005; Salaskar et al., 2011; Witters et al., 2012a). The terms phytoextractor or hyperaccumulation may be used to describe the plants with a strong uptake of a heavy metal. For Cd phytoextraction, the hyperaccumulator plant species should be capable of accumulating more than 100 mg Cd kg^{-1} in shoots in a dry weight basis (Baker & Brooks, 1989; Baker et al., 2000; Manisha & Mohan 2017).

Selection of suitable Cd phytoextractor species should consider local availability, cost, agronomic requirements (temperature, humidity, soil properties, irrigation, fertilization), as well as physiological features of the plant (growth rate, rooting depth, photoperiod, heavy metal tolerance, pests and diseases, vigor, evapotranspiration rates and possible symbiotic

relationships with other organisms) (Mahar et al., 2016; Kumar et al., 2018; Zhao et al., 2003), biomass production (Robinson et al., 2015), exposure time (Rizwan et al., 2016), and ease of cultivation and harvesting (Ali et al., 2013).

Multiple options for Cd phytoremediation exist, particularly by using edible plant species (Puschenreiter et al., 2005), although examples of commercial scale phytoextraction operations were not found. Most of what has been reported corresponded to greenhouse or field experiments. A brief summary of plant species used in soil phytoextraction investigations is presented in Table 2-2.

Table **2-1**: Species used in phytoextraction processes.

Plant species	Observation	Reference
Swiss chard (*Beta vulgaris* L. var. cicla)	Planted in a paddy soil with a Cd content in the top 20 cm that ranged 2.82-3.17 mg kg^{-1}. After harvest, swiss chard extracted a maximum of 20.1 mg Cd kg^{-1} in the aerial biomass for the best of three planting densities analyzed.	Song et al. (2012)
Indian mustard (*Brassica juncea*)	High ability to tolerate and accumulate Cd. The highest amount of Cd was found 45.23, 31.95 and 12.72 mg Cd kg^{-1} in roots, stems and leaves respectively when exposed to 0.09M Cd $(NO_3)_2$. $4H_2O$.	Bhadkariya et al. (2014)
Spinach (*Spinacia oleracea*)	No visual Cd toxicity effects on spinach and no significant reduction in the dry matter yield at 20 mg Cd kg^{-1} in soils when applied as $CdCl_2 \cdot 2½ H_2O$ salt.	Salaskar et al. (2011)
Lettuce (*Lactuca sativa* L.)	Considered a good indicator species for derivation of critical soil Cd concentrations. Used in a first-tier site risk assessment.	Swartjes (2011)
Radish (*Raphanus sativus* Linn. cv. Qianxi No. 2)	Radish accumulated more Cd than Chinese cabbage and tomato when a 1.5 kg mg^{-1} salts of $CdSO_4$ concentration in a Chinese calcareous soil was applied.	Li et al. (2014)
Noccaea caerulescens (syn. *Thlaspi caerulescens* J&C Pres)	Accumulated up to 235 mg Cd kg^{-1} in 3 months. The soils where it was planted include non-mined farm soils and alkaline coal mine soils from Illinois, U.S.	Broadhurst et al. (2015)
Arabidopsis (*Arabidopsis thaliana*)	The plants growing on the subsoil with levels of 0, 2, 10, and 30 mg kg^{-1} $3CdSO_4 \cdot 8H_2O$ with *Trichoderma* addition contained less Cd than those grown without it. The maximum total Cd in Arabidopsis was 54.06 mg Cd kg^{-1}.	Marchel et al. (2016)
Heliconia (*Heliconia psittacorum*)	Study results presented an average removal efficiency ranged from 92 to 98% Cd^{2+} when planted in constructed wetlands treating synthetic landfill leachate under tropical conditions. It accumulated 2.3 mg Cd^{2+} kg^{-1} in roots.	Madera-Parra et al. (2015)

Oilseed rape (*Brassica napus* L. var. oleifera, cv. Drakkar)	Oilseed rape was grown from seeds on a reconstituted soil contaminated with 100 mg Cd kg^{-1}. Compared with roots and stems, leaves accumulated high amounts of Cd. The whole leaves accumulated 263.1 mg Cd kg^{-1}.	Carrier et al. (2003)
Wildcane (*Gynerium sagittatum*)	Study results presented an average removal efficiency ranged from 92 to 98 % Cd when planted in constructed wetlands treating synthetic landfill leachate under tropical conditions. It extracted 5.98 mg Cd kg^{-1} in shoots.	Madera-Parra et al. (2015)
Signal grass (*Brachiaria decumbens* cv. Basilisk)	Study results presented high susceptibility to Cd under greenhouse conditions with 2, 4 and 12 mg kg^{-1} in pure $CdCl_2$ in comparison to *Panicum maximum* Jacq. cv. Aruana and cv. Tanzania; *Brachiaria brizantha* cv. Xaraés and cv. Marandu in a Brazilian dystrophic Oxisol. The highest and lowest concentrations were 497.5 and 58.5 mg Cd kg^{-1}, both recorded for Tanzania. None of the grasses was capable of limiting Cd soil absorption.	Silva et al. (2016)
English ryegrass (*Lolium perenne*)	Study results presented the smallest decrease in biomass and the largest Cd accumulation in roots at 30 mg kg^{-1} $CdCl_2$ in comparison to *Poa pratensis* and *Festuca rubra* grasses under greenhouse conditions in a soil with a pH of 5.4 and 23.47 % fine texture soil particles. None of the grasses is suitable for Cd phytoextraction.	Gołda & Korzeniowska (2016)
Maize (*Zea mays*), sunflower (*Helianthus annuus*) and tobacco (*Nicotiana tabaccum*)	All tested crops are realistic options to be used in a sustainable phytomanagement on a heavy-metal contaminated soil containing 540 mg Cu kg^{-1}, 680 mg Zn kg^{-1} and 1.4 mg Cd kg^{-1}. The heavy metals accumulated in the leaves and/or roots, rather than the seeds. Thus, sunflower seeds or maize grain could be safely used as food or animal feed.	Fässler et al. 2010

Limitations of phytoextraction

The use of edible plants to remove soil Cd represents a great health risk. Farmers can consume, exchange, produce compost, sell and/or feed animals with plants presenting toxic levels of Cd. The use of non-edible species to extract Cd from soils, that could provide positive ecosystem services (enhancement of soil nitrogen fixation, soil biota and soil porosity), as well as a possible extra income to the farmer is not well documented in the literature, especially by using tropical plant species in phytoextraction operations. When the limitations of phytoextraction are not considered, overestimation of effectiveness can result in misallocation of resources.

Phytoextraction is an environmentally friendly strategy to manage soils polluted by heavy metals (Boeckx et al., 2018; Manisha & Mohan 2017; Rizwan et al., 2016), but the time it takes to remove significant amounts of a heavy metal is a major limitation of phytoextraction (Thewys et al., 2010; Vangronsveld et al. 2009). On the other hand, only a fraction of the heavy metal would be available for plant uptake. While phytoextraction is occurring, the bioavailable pool, and accordingly plant concentrations and removal rates, could decrease. The bioavailable pool is likely to be replenished with time, but such resupply processes may be very slow and not significant within the phytoextraction period (Tack & Meers, 2010). Microbial synergies, amendments and reagents can be used to improve the performance of phytoremediation species; in this regard, several studies have examined *B. napus* (Dąbrowska et al., 2017; Ehsan et al., 2014; Pan et al., 2017; Wang et al., 2009).

The successful deployment of phytoextraction in competition with chemical or physical methods for cleansing trace elements from contaminated soil, requires this strategy to be less costly than the best alternative technology and importantly, less expensive or more viable than the cost of inaction (Robinson et al., 2003). For low-value land, such as that which occurs in rural settings, the cost of phytoextraction may be greater than the land value. In such cases, only regulators can force landowners to remediate the land (Robinson et al., 2007) and regulators need to be convinced that phytoextraction will provide a solution (Conesa et al., 2012).

Few authors mention that the phytoextraction costs would be absorbed by the farmer (Thewys et al., 2010). The economic benefits and by-products generated from multi-purpose phytoextraction plant species should be considered. For example, non-edible plants can serve as energy crops (Licht & Isebrands, 2005; Pandey et al., 2016; Simmons et al., 2015; Witters et al., 2012a), as CO_2 abatement crops (Witters et al., 2012b), as timber (Liu et al., 2013; Srivastava, 2016), as fiber for paper production (Dickinson et al. 2009; Lebeau et al. 2008), to produce fragrances, or as an ornamental plant (Jelusic & Lestan, 2015; Liu et al. 2008; Nakbanpote et al.,

2016). Thewys et al. (2010) investigated the effect on dairy cattle farmers' income in the Belgian and Dutch Campine region (0.5 to 12 mg Cd kg^{-1} soil), when they switched from growing fodder maize to energy maize for biogas production. In this way, soil Cd remediation is demoted to a secondary objective with sustainable risk-based land use as primary objective. Therefore, the alternative use and valuation of the produced biomass, rather than considering it as a waste product of soil remediation, may become a prerequisite for field-scale application of phytoextraction as a remediation technique (Domínguez et al. 2008; Vangronsveld et al., 2009; Vassilev et al., 2004).

It is challenging to select one unique plant or combination of plants to extract a specific contaminant from soils because of the broad variety of phytoextractor species reported in the literature (a detailed list of phytoextractors can be found in: Bothe 2011; Mahar et al., 2016; Nakbanpote et al., 2016), and because of the reaction that each plant would have under different types of soils, climate conditions, concentration and combination of bioavailable heavy metals within the soil. In practice, very few soils are contaminated with only one type of pollutant and multiple contamination of soils is common (Wu et al., 2012).

If achievable, phytoextraction for soil cleansing would compete directly with other soil rehabilitation technologies, i.e. chemical or physical processes that either clean up the soil, bury, or remove the contaminated layers (Robinson et al., 2015). A detailed list of advantages and disadvantages of phytoremediation can be found in Mani & Kumar (2014)

Soil amendments

Although the cost of phytoremediation is lower than the conventional remediation technologies, it is time-consuming (Wei et al., 2017) and typically cannot satisfy the urgency of land development (Dermont et al., 2008). Innovative *in situ* technologies that require low inputs, and are low cost, are urgently required to meet the needs for soil remediation and

community acceptance (Lombi et al., 2002). Therefore, phytoremediation operations could be supported with the use of *in situ* immobilization/stabilization remediation technologies to achieve significant reductions of Cd concentrations in the plant-root zone. Chemical immobilization is based on alteration of contaminant and soil characteristics by the addition of soil amendments (Lee et al., 2004; Woldetsadik et al., 2016).

Numerous amendments including biochar (Mehmood et al., 2018; Puga et al., 2015; Woldetsadik et al., 2016), industrial by-products (Chavez et al., 2016b; Hamidpour et al., 2010; Kirkham 2006; Li et al., 2014; Lombi et al., 2002), liming materials (USEPA. -OSRTI, 2007; Kim et al., 2016; Lee et al., 2004; Mondlane & Maret 2016; Radziemska et al., 2018; Tlustoš et al., 2006; Trakal et al., 2011; Vrînceanu et al., 2017), organic materials (Ok et al., 2011; Rolka 2015; Shan et al., 2016), Zn-based fertilizers (Li et al., 2014; Paul & Chaney, 2017), among others, have been examined for precipitating Cd, increasing adsorption of Cd, or providing competition with Cd uptake by roots (Paul & Chaney, 2017).

Lee et al. (2004) used calcium carbonate in a natural sandy soil in northern Taiwan cultivated with a wheat crop under greenhouse conditions. The addition of calcium carbonate, calcium carbonate mixed with zinc oxide, and calcium carbonate mixed with compost significantly decreased diethylenetriaminepentaacetic acid (DTPA) extractable Cd and Pb concentration. The authors also report that the concentration of total Cd in soil extracts significantly decreased from 35.0 ± 2.70 mg Cd kg^{-1} to 8.90 ± 0.62 mg Cd kg^{-1} when treated with calcium carbonate.

Li et al. (2014), used a combination of red mud (SiO_2 20 %, Fe_2O_3 28 %, Al_2O_3 21 %, CaO 6.2 %, MgO 1.3 %, TiO_2 3.3 %, K_2O 0.26 % and Na_2O 11 %) and corn raw straw in naturally Cd contaminated soils, located in the experiment station of the Chinese Academy of Agricultural Sciences, Dezhou city, Shandong Province, China. Spinach, tomato and Chinese cabbage were grown under field conditions. As a result of this treatment, the recorded

reduction of Cd ranged 37 % to 76 % for spinach, 34 % to 63 % for tomato, and 59 % to 76 % for Chinese cabbage, and 61 % to 77 % for radish.

Contreras et al. (2012) evaluated the effect of soil application of $CaCO_3$ in greenhouse conditions on Cd accumulation in cacao leaves at Miranda State (Venezuela). The $CaCO_3$ was mixed with 1 kg of soil and incubated for a 16 day-period. Soils were reported to have a maximum of 2.05 mg Cd kg^{-1} and a minimum of 1.32 mg Cd kg^{-1} while cacao leaves ranged from 12 to 75 mg Cd kg^{-1}. Treatments of $CaCO_3$ (800 kg $CaCO_3$ ha^{-1}) diminished the quantity of exchangeable Cd of the soils compared to the control and resulted in 48 % reduction of Cd in cacao leaves. A digestion was made with sulfuric acid and hydrogen peroxide. The Cd determination was carried out by atomic absorption spectrophotometry.

Liming contaminated soils is the most widely used remediation treatment for reduction of heavy metals bioavailability (Lee et al., 2004) because it can lead to precipitation of metals as metal-carbonate (Mench et al., 2000).

It is important to select cost-effective and feasible amendments to immobilize Cd by specific sorption (Li et al., 2014), which depends on the soil type, the chemical species, the contact time, and the environmental conditions (Colzato et al., 2018). Soil amendments should be inexpensive or free, regionally abundant, show some ability to reduce bioavailable heavy metals (Trenouth & Gharabaghi, 2015), and culturally accepted by the farmers. Finally, physical-chemical properties of the amendments such as pH, cation exchange capacity (CEC), exchangeable cations, and porosity should be evaluated, since its significant effect on the immobilization of soluble Cd in soil (Hamidpour et al. 2010).

Thresholds for Cadmium in food crops

Three regulations are closely related to the research project:

1) The General Standard for Contaminants and Toxins in Food and Feed established in the Codex Alimentarius (food standards set by the Food and Agriculture Organization and World Health Organization) for leafy green vegetables which is set at 0.2 mg kg^{-1} fresh weight (~4 mg kg^{-1} dry weight) (FAO & WHO, 2015a).
2) The proposed draft for maximum levels for Cd in chocolate and cocoa-derived products established in the Codex Alimentarius, for cocoa powder critical level, which is set at 1.50 mg kg^{-1} (FAO & WHO, 2015b).
3) The EU Cd content in cacao powder (ready for consumption) critical level which is set at 0.6 mg kg^{-1}, to be enforced in January 2019 (European Commission, 2014).

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Chapter 3

Cadmium dose-response relationships of four plant species grown on artificially contaminated growing media

Abstract

Sunflower, oilseed rape, vetiver, and heliconia were grown in a series of greenhouse pot experiments to assess the accumulation of total cadmium (Cd) in plant aerial tissue (PAT) (stem, leaves, flower) under different Cd addition treatments. Three dose-response experiments were evaluated. Experiments 1 and 2 evaluated the total Cd accumulation in *Helianthus annuus* (sunflower), *Brassica napus* (oilseed rape), and *Chyrsopogon zizanioides* (vetiver) cultivated in two different growing media: a soil-less media of play sand plus perlite for experiment 1, and a soil matrix mixture of natural soil plus perlite for experiment 2. Experiment 3 assessed the total Cd accumulation in sunflower, *Heliconia psittacorum* (heliconia), and vetiver in a natural soil matrix. All matrices had different concentrations of Cd added (experiments 1 and 2: 0.0, 1.0, 2.5, and 5.0 mg Cd kg^{-1}; experiment 3: 0.0, 3.0, and 5.0 mg Cd kg^{-1}). Different Cd concentrations, types of soil, Cd-extraction methods, and plant varieties were used with the aim of establishing dose-response relationships for study plant species. Due to growing condition requirements of the study species, experiments 1 and 2 were conducted at The Pennsylvania State University (State College PA, United States of America) and experiment 3 was completed at the Universidad del Valle (Cali-Colombia). Plant Cd concentrations increased significantly with increasing Cd concentrations in the growing media. A significant effect was detected in the three experiments on total Cd accumulated in PAT. Total Cd in soil matrix, total Cd in soil, and BCF were found significantly different in experiments 2 and 3. PAT total Cu and Zn were found significantly different experiment 1, and soil pH was found significantly different in

experiment 3. In experiment 1, the total accumulation of Cd in PAT of sunflower (26.7 mg Cd kg^{-1}) and oilseed rape (25.6 mg Cd kg^{-1}) was found to be nonsignificant, however, in experiment 2, the total accumulation of Cd in PAT of oilseed rape (10.8 mg Cd kg^{-1}) was significantly higher than the other plant species, and in experiment 3, the total accumulation of Cd in PAT of sunflower (2.2 mg Cd kg^{-1}) was significantly higher than other species. Comparing the growing media used in experiments 1 and 2 (soil-less vs soil matrix), the soil-less media resulted in a higher Cd absorption in all plant species. More research is required to determine a) differences in the accumulation of Cd in heliconia and vetiver plant parts, and b) the practicality of using oilseed rape and sunflower as Cd phytoextractors (cultural acceptance, financial investment, multipurpose potential, growing period, and resistance to plagues and diseases).

Introduction

Cadmium is a toxic heavy metal whose presence in soils occurs naturally, but also due to anthropogenic pollution (Mohammadi et al., 2015; Kumar et al., 2018). Cadmium is typically more soluble and mobile than other heavy metals due to its weak affinity for soil colloids and hence is more easily transferred to the crops (Sarwar et al., 2010; Smolders, 2013). The threat of cadmium to human and animal health is aggravated by its bioaccumulation in the human body (Castelli et al., 2005; Boeckx et al., 2018). Hence, management of cadmium in soils, and its uptake by commodities used for food production, is of utmost importance in order to reduce human bioaccumulation.

Phytoextraction is the use of the plant roots to translocate metal pollutants from the soil to the harvestable plant parts (McGrath & Zhao, 2003), and many potential species can act in this capacity; detailed lists of phytoextractor species can be found in Bothe (2011),

Mahar et al. (2016), Mani & Kumar (2014), Nakbanpote et al. (2016), and Puschenreiter et al. (2005).

Selection of suitable Cd phytoextractor species should consider local availability, cost, agronomic requirements (temperature, humidity, soil properties, irrigation, fertilization), as well as physiological features of the plant (growth rate, rooting depth, photoperiod, heavy metal tolerance, pests and diseases, vigor, evapotranspiration rates and possible symbiotic relationships with other organisms) (Mahar et al., 2016; Kumar et al., 2018; Zhao et al., 2003), biomass production (Robinson et al., 2015), exposure time (Rizwan et al., 2016), and ease of cultivation and harvesting (Ali et al., 2013). The economic benefits or by-products generated by multi-purpose phytoextraction plant species are important to consider and may include: energy crop production (Licht & Isebrands, 2005; Pandey et al., 2016; Simmons et al., 2015; Witters et al., 2012a); CO_2 abatement crops (Witters et al., 2012b), as timber (Liu et al., 2013; Srivastava, 2016); fiber for paper production (Dickinson et al. 2009; Lebeau et al. 2008); fragrance production; or ornamental plant production (Jelusic & Lestan, 2015; Liu et al. 2008; Nakbanpote et al., 2016).

We evaluated four plant species that were previously reported as cadmium phytoextractors: sunflower (Fässler et al. 2010; Rizwan et al., 2016), oilseed rape (Carrier et al., 2003; Rizwan et al., 2018) vetiver (Aibibu et al., 2010; Kumar et al., 2018; Zhang et al., 2014), and heliconia (Madera-Parra et al., 2015). The goal of this study was to determine the Cd phytoextraction capability of each species under greenhouse conditions. For this purpose, the plants were grown in different growing media and total Cd was measured in aerial parts (or above ground biomass) of sunflower, oilseed rape, vetiver, and heliconia.

Materials and Methods

Potting media content and preparation

A sand (Quikrete brand, USA) was used in experiment 1. A Paleudalf soil (Hagerstown, soil series) from the Pennsylvania State University Russell E. Larson Agricultural Research Center (40°42'43.0" N 77°56'17.0" W) was used in experiment 2. The Paleudalf soil was selected because of its high iron oxides and clay content, which resembles some Colombian tropical soils. A Dystrudept soil from the Universidad del Valle research farm (3°22'22.57"N 76°31'47.57"W) was used in experiment 3. Soils used in experiments 2 and 3 were excavated from the surface to a depth of 20 cm. The collected soil was air dried for ~5 days and then passed through a 5 mm sieve. To improve root growth, and following Alaboudi et al. (2018), perlite grade 2 (GROW!T, USA) was mixed with play sand for experiment 1, and with soil for experiment 2. In both cases, the proportion used was one-part perlite per five parts of sand or soil (weight/weight), respectively. The soil collected for experiment 3 was not mixed with perlite because it presented suitable conditions for plant growth. The pots for all three experiments were filled with 3 kg of soil matrix material. Each pot was considered a replicate. Experiments 1 and 2 had five replicates, and experiment 3 had four replicates.

Cadmium treatments consisted of different concentrations of Cd as $CdCl_2$ salt (Thermo Fisher Scientific, Geel-Belgium). Container capacity was used to determine the volume of distilled water required to saturate the 3 kg of growing material (Figure 3-1).

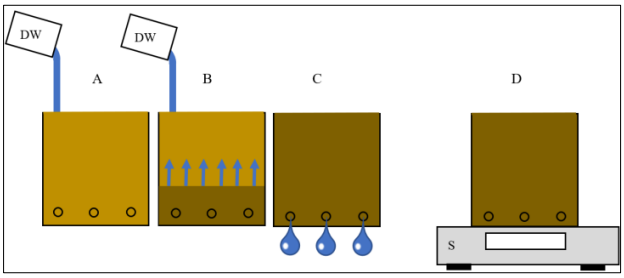

Figure **3-1**: Determination of container capacity. A. Distilled water (DW) was added to pots containing growing media; B. DW was added to saturate the growing media; C. Once the pots were fully saturated, they were left to drain excess water; D. Total weight was measured two times using scale (S): at one hour and at 24 hours after left to drain. With this information it was possible to determine the volume of water required to saturate the pots without having leaching.

The Cd solution was freshly prepared by dissolving $CdCl_2$ in deionized water as described in similar studies (Domínguez et al., 2011; Li et al, 2005; Lopes-Júnior et al., 2014; Nereida, 2011; Turgut et al., 2004). Cd solution was added to the growing media only one time. Distilled water was added to saturate Cd treated media. Polyethylene pots (2 gallons) were filled with the different study growing media, placed in a greenhouse in a larger polyethylene pot (2.5 gallons) with a 3 mm plastic layer in between, thus preventing leachate drainage (Figure 3-2).

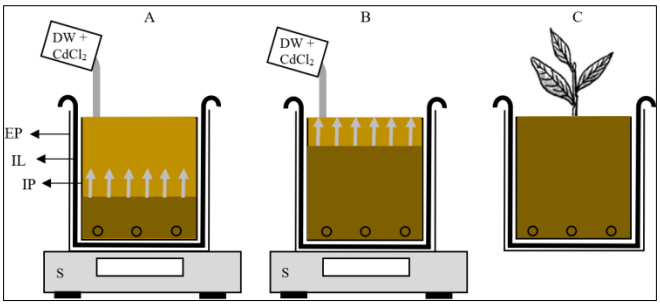

Figure **3-2**: One-time application of $CdCl_2$ solution. A, B: The solution of distilled water (DW) and $CdCl_2$ was added to the correspondent pots containing growing material until saturation using a scale (S). The growing material was incubated during 15 days under greenhouse conditions; C. plant material was transplanted to the growing material artificially contaminated with Cd. An internal pot (IP), an external pot (EP), and a plastic layer (3 mm thick) in between (IL) was used to avoid any leaching.

Experiments 1 and 2 consisted of 0.0, 1.0, 2.5, and 5 mg Cd per kg of growing media and for experiment 3, 0.0, 3.0, and 5.0 mg Cd per kg of growing media. The chosen Cd concentrations represent a range of concentrations around the current soil Cd in Latin American and Central American countries (Chavez et al., 2016; Gramlich et al., 2017; Huamaní et al., 2012; Ramtahal et al., 2016; Rodríguez, 2017; Tantalean & Huauya, 2017). All growing media was incubated for 15 days.

Following Hamidpour et al. (2010), Puga et al. (2015), Shan et al. (2016) and Yang et al. (2015), the water content of pots was maintained at 65-70 % of maximum water holding capacity, which was determined by weighing the pots weekly and adding water only as required and avoiding anoxic conditions. Hoagland's solution was added weekly to provide plant nutrients (Epstein, 1972).

Three subsamples of growing media were collected from each pot at different depths (1, 10, 30 cm) and combined. For experiment 2, soil matrix samples were oven dried for 5 days at 45 °C and ground using a laboratory mill (Thomas Wiley Mill No. 3, USA) fitted with a 1 mm sieve at the exit of the mill. After grinding each sample, the mill was cleaned with pressurized air repeatedly to avoid cross contamination. Soil samples in experiment 3 were oven dried for 3 days at 45 °C.

Soil analytical methods

Growing media pH was determined in distilled water extracts (1:1 weight vs. volume) through potentiometry (SB70P VWR, USA in experiment 1 and 2; Sartorius PT-10, Germany in experiment 3). In experiments 1 and 2, the cation exchange capacity (CEC) was determined using a 1 M CH_3COOH extraction method (Sumner & Miller, 1996). Soil organic matter (OM) was detected by the Walkley-Black method (Nelson & Sommers, 1982). Total P

and K content were determined by inductively coupled plasma-atomic emission spectroscopy, S was determined by US EPA 3050B method, Mn, Fe, Cu, B, Al, Zn, and Na were determined with saturation of CEC (Busenberg & Clemency, 1973). Particle size distribution was determined with the Pipette method (Miller & Miller, 1987). Cd concentration in growing media was determined by inductively coupled plasma-optical emission spectrometry following aqua regia digestion (Kim et al., 2016).

In experiment 3, CEC was determined using 1 N, pH 7 ammonium acetate (Colombian Technical Standard 5268). OM was measured per the Walkley-Black method (Nelson & Sommers, 1982). P was determined by Bray II modified (Colombian Technical Standard 5350). K, Mg, Ca were measured using ammonium acetate 1 N, pH 7 (Colombian Technical Standard 5349). Zn and Cu were determined by Mehlich I modified (Colombian Technical Standard 5526). S was determined by calcium phosphate (Agrilab S.A.S. internal method) and particle size distribution was determined by the hydrometer method with pre-treatments (Gee & Bauder, 1986).

Plant material

For the three experiments, sunflower was sown from seed for two weeks. Uniform sunflower plants (10 cm height) were selected and transplanted to the pots. For the three experiments, vetiver was ~1-month old and 20 cm height when transplanted. For experiments 1 and 2, oilseed rape was sown from seeds for two weeks. Uniform oilseed rape plants (5 cm height) were selected and transplanted to the pots. For experiment 3, heliconia was ~2-months old and 20 cm height when transplanted to the pots. A description of the plants used in each experiment, the variety and the sowing time is presented in Table 3-1 and Table 3-2.

Table **3-1**: Species of the selected plants and the sowing time for experiments 1 and 2.

Plants	Sowing time (Growth period in weeks)
Sunflower (*Helianthus annuus* cv Mammoth Gray Stripe)	August 1st (9)
Oilseed rape (*Brassica napus* cv Wichita)	August 1st (10)
Vetiver (*Chyrsopogon zizanioides* (L.) Roberty)	August 22nd (16)

Plant species were sown in a soil-less media (experiment 1) and in a soil matrix (experiment 2).

Table **4-2**: Species of the selected plants and the sowing time for experiment 3.

Plants	Sowing time (Growth period in weeks)
Sunflower (*Helianthus annuus* cv Domino)	October 1st (8)
Heliconia (*Heliconia psittacorum* cv Pajarito)	October 1st (16)
Vetiver (*Chyrsopogon zizanioides* (L.) Roberty)	October 1st (16)

All plant species were cultivated in soil.

After plants were transplanted and following the respective growing period per plant species (Table 3-1, Table 3-2), plant aerial tissue (or above ground biomass) were cut to the growing media's surface. Harvested plant material (leaves, stem, flower) in experiment 1 and 2 was cleaned with distilled water, cut into 5 cm size pieces, and weighed immediately (Ohaus Ranger 300, USA) (fresh weight) as described by Khan et al. (2013) and Zhang et al. (2014).

The fresh plant aerial tissue weight, but not the dry weight, was recorded in experiments 1, 2, and 3. Published conversion factors between plant aerial tissue fresh weight and dry weight were used for dry weight calculations in experiments 1, 2, and 3 (Table 3-3).

Table **5-3**: Fresh weight (FW) (g) and dry weight (DW) (g) reported in the scientific literature.

Plant species	Fresh weight	Dry weight	TCF (FW/DW)	n	Reference
Sunflower	26.1	3.9	6.7	3	Alaboudi et al., 2018
Oilseed rape	134.0	11.0	12.2	3	Ehsan et al., 2014
Vetiver	8.1	2.0	4.1	5	Kumar et al., 2018
Heliconia	197.5	43.98	4.5	80	Pinedo, 2010

TCF: Theoretical conversion factor. Data reported corresponds to control treatments provided by the references.

Knowing the theoretical dry weight and the total Cd in plant aerial tissue, it was possible to calculate a rate of Cd uptake in mg Cd per pot in each experiment.

For experiments 1 and 2, plant material was oven dried for 5 days at 45 °C and ground using a laboratory mill (Thomas Wiley Mill No. 4, USA) fitted with a 1 mm sieve at the exit of the mill. Plant and growing media samples (Experiments 1 and 2) were sent to the Agricultural Analytical Laboratory at Penn State (USA). Fresh aerial plant parts and soil samples from experiment 3 were sent to the Agrilab Laboratory S.A.S. (Bogotá, Colombia).

A summary of the experiments is presented in Table 3-4.

Table **6-4**: Summary of experiments.

Experiment	Plants evaluated	Cd source	Cd concentrations	Growing media	Location
1	Sunflower Oilseed rape Vetiver	$CdCl_2$	0.0 mg Cd kg^{-1} 1.0 mg Cd kg^{-1} 2.5 mg Cd kg^{-1} 5.0 mg Cd kg^{-1}	Sand and perlite Proportion 5:1 w/w (soil-less media) 3 kg/pot	Penn State (USA)
2	Sunflower Oilseed rape Vetiver	$CdCl_2$	0.0 mg Cd kg^{-1} 1.0 mg Cd kg^{-1} 2.5 mg Cd kg^{-1} 5.0 mg Cd kg^{-1}	Soil and perlite Proportion 5:1 w/w (soil matrix) 3 kg/pot	Penn State (USA)
3	Sunflower Heliconia Vetiver	$CdCl_2$	0.0 mg Cd kg^{-1} 3.0 mg Cd kg^{-1} 5.0 mg Cd kg^{-1}	Soil 3 kg/pot	Universidad del Valle (Colombia)

In experiment 1, three plant species x five replicates x four Cd concentrations resulted in sixty experimental units. For experiment 2, three plant species x five replicates x four Cd concentrations resulted in sixty experimental units. Finally, for experiment 3, three plant species x four replicates x three Cd concentrations resulted in thirty-six experimental units.

Total Cd extraction methods for experiments 1 and 2

Total Cd, P, K, Ca, Mg, S, Mn, Fe, Cu, B, Al, Zn, and Na in plant aerial tissue was digested via the US EPA method 3050B (US EPA, 1996) and the US EPA method 6010 (US

EPA 2014). Digestion of Cd in the soil-less media (sand plus perlite) and in the soil matrix (soil plus perlite) followed the US EPA method 3050B and the US EPA method 6010.

US EPA method 3050B uses HNO_3, H_2O_2, and heat to digest samples (US EPA, 1996). US EPA method 6010 is a spectrometric technique used to determine trace elements in aqueous solutions, prior to analysis, aqueous and solid samples are solubilized or digested using an acid digestion procedure (US EPA 2014). Metal analysis is made through inductively coupled plasma atomic emission spectrometry (ICP-AES) or inductively coupled plasma-optical emission spectrometry (ICP-OES) and the detection limits of 3050B range between 0.0001 to 0.5 mg Cd kg^{-1} (Da Silva et al., 2014; Enamorado-Báez et al., 2013; Nham, 2006).

Total Cd extraction method for experiment 3

Total Cd and N, P, K, Ca, Mg, S, Fe, Mn, Cu, Zn, B, and Na in plant aerial tissue was digested via the US EPA method 200.9 (US EPA, 1994). Digestion of Cd in the soil followed the US EPA method 3051A (US EPA, 2007) and the US EPA method 200.9 (US EPA, 1994). The plant aerial tissue was digested via the method US EPA 3051.

US EPA method 3051A is a microwave extraction method designed to mimic extraction using conventional heating with HNO_3 or alternatively HNO_3 and HCl. Since this method is not intended to accomplish total decomposition of the sample, the extracted analyte concentrations may not reflect the total content in the sample. US EPA method 200.9 provides procedures for the determination of dissolved and total recoverable elements by graphite furnace atomic absorption in water (ground water, industrial, domestic), sediments, sludge, and soil. For total recoverable analysis of a solid or an aqueous sample containing undissolved material, analytes are first solubilized by refluxing with HNO_3 and HCl. The

analysis is made through inductively coupled plasma atomic emission spectrometry (ICP-AES) or inductively coupled plasma-optical emission spectrometry (ICP-OES). The detection limits of 3051A range between 0.0001 to 0.5 mg Cd kg^{-1} (Da Silva et al., 2014; Enamorado-Báez et al., 2013; Nham, 2006).

Bioconcentration factor

The bioconcentration factor (BCF), defined as the ratio of metal concentration in the aerial part of the plant to the total metal concentration in soil (Alloway et al., 1990, Li et al., 2017), was used to evaluate the transfer potential of Cd from the soil matrix in each experiment to the corresponding plant species. A BCF > 1 indicates a high accumulation of an element in the plant shoot (Baker et al., 1981; Kötschau et al., 2014). The BCF is not a constant value, as it may vary for even the same element in soils with different chemical properties (Boim et al., 2016; Swartjes et al., 2013).

Experimental design

This study was designed to determine if the total Cd in plant aerial tissue differed based on the plant species and the level of added Cd to the growing media. A completely randomized design was established, and experiments followed a two-way analysis of variance (ANOVA). The dependent variable was "total Cd in plant aerial tissue" measured in mg Cd kg^{-1}, and the two independent variables were "plant species (in experiments 1 and 2: sunflower, oilseed rape, and vetiver grass; in experiment 3: sunflower, heliconia, and vetiver grass)" and "level of added Cd into the growing media (experiments 1 and 2: 0.0, 1.0, 2.5, and 5.0 mg Cd kg^{-1}; experiment 3: 0.0, 3.0, and 5.0 mg Cd kg^{-1})".

The following abbreviations were used to describe treatments: Sf (sunflower), O (oilseed rape), V (vetiver), and H (heliconia). In experiments 1 and 2, five replicates were used and for experiment 3, four replicates were used.

Statistical analysis

Graphs and tables were constructed using Microsoft Excel 365 (Microsoft Corp., 2019). Descriptive statistic was calculated using Minitab, version 17.3.1 software package (Minitab Inc., 2016). P, K, Ca, Mg, S, Mn, Fe, Cu, B, Al, Zn, Cd and Na levels in plant tissue were evaluated using the Anderson-Darling test for normality and Breusch-Pagan test for equality of variances. Differences in total Cd concentrations for plant aerial tissue and growing media measured throughout the experiments were compared between treatment groups (Cd contaminated treatments and the control) with a two-way ANOVA (analysis of variance) test at an alpha of 0.1. Tukey's post hoc test was used for the multiple comparisons of means between treatments at an alpha of 0.1. Multifactorial linear regression (MLR) analysis was applied to experiments 1 and 2 to determine if total Cd was related to other plant tissue elements via a backward elimination method. Linear regressions were used to evaluate the relationship between total Cd detected in plant aerial tissue and the growing media. Data are presented as the mean ± standard deviation (SD) unless stated otherwise.

Results

Experiment 1. Cadmium dose-response relationships of three plant species cultivated in an artificially Cd-contaminated soil-less media

To create the treatments, a soil-less media was prepared (play sand and perlite grade two in a 5:1 proportion w/w). A soil-less media helped minimize potential matrix adsorption interference and facilitated a clearer assessment of Cd dose concentration differences and plant aerial tissue (PAT) responses across three plant species: sunflower (Sf), oilseed rape (O), vetiver (V). The soil-less media used in experiment 1 had a high concentration of boron (B), Zn and Cu relative to levels found in agricultural soils (Horneck et al., 2011) and the Mn concentration is higher than 5 mg kg^{-1}, which is considered sufficient for plant growth (Horneck et al., 2011) (Table 3-5).

A two-way ANOVA was run on a sample of 60 plants sown in a soil-less media to examine the effect of Plant species and Added Cd on PAT fresh weight, PAT total Cd, PAT total Cu, and PAT total Zn. A significant interaction was found between the effects of Plant species and Added Cd on fresh weight of PAT ($F (6, 48) = 2.07$, $p = 0.074$), in PAT total Cd ($F (6, 48) = 12.88$, $p < 0.001$), in PAT total Cu ($F (6, 48) = 3.49$, $p = 0.006$), and in PAT total Zn ($F (6, 48) = 3.22$, $p = 0.01$) (Table 3-6).

Differences in Plant species and Added Cd levels are presented in Table 3-7. A significant difference was found across Plant species with V having a significantly lower total Cd, and total Zn than the other species. In addition, PAT total Cu was found to be significantly different between the three species with Sf having the highest levels and V the lowest. A significant difference was found across Plant species with Sf reporting the highest Cd uptake, followed by O and V (Table 3-7).

Differences in Added Cd levels and PAT total Cd, Cu, and Zn are presented in Table

3-7. In the Added Cd treatment, significant differences were found in the PAT total Cd of all species, across different concentrations of added Cd. The highest concentration of added Cd corresponded to the highest PAT total Cd, regardless of species. In the Added Cd treatment, the 5.0 mg Cd kg^{-1} level resulted in a significant difference in total Cu and total Zn across all species in contrast to the 0.0, 1.0, and 2.5 mg Cd kg^{-1} levels. A significant difference at all levels was found in Cd uptake, which increased as the level of added Cd was increased (Table 3-7).

Modeling results suggest PAT total Zn was the best predictor of PAT total Cd of Sf and O (Table 3-8). The best predictor of total Cd in V was Mn; however, the relationship was weak (Table 3-8). A significant linear relationship in PAT total Cd by each plant species, with respect to the Added Cd treatment levels, is presented in Figure 3-3. As the Added Cd was increased PAT total Cd significantly increased (Table 3-9). The main effect of each plant species was not significant (Table 3-9). Oilseed rape leaf and stem damage attributed to Cd toxicity was observed in all treatments, particularly in the 5.0 mg Cd kg^{-1} treatment (fungus and insect attack was also observed) (Figure 3-4). Damage was not seen in sunflower or vetiver.

Table **7-5**: Chemical properties of soil-less media in experiment 1.

Element	Units	Value
P	%	0.012
K	%	0.031
Ca	%	0.014
Mg	%	0.006
S	%	0.004
Mn	mg kg^{-1}	24
Fe	mg kg^{-1}	3745
Cu	mg kg^{-1}	6.1
B	mg kg^{-1}	7.8
Al	mg kg^{-1}	270
Zn	mg kg^{-1}	4
Na	mg kg^{-1}	228
Cd	mg kg^{-1}	0.2

Table **8-6**: Analysis of variance of fresh weight (FW) (g) of PAT, total Cd (mg kg^{-1}) in PAT, total Cu (mg kg^{-1}) in PAT, and total Zn (mg kg^{-1}) in PAT in experiment 1.

Effect	df	FW of PAT	Total Cd in PAT	Total Cu in PAT	Total Zn in PAT
		p-value			
Plant species	2	0.005	<0.001	<0.001	<0.001
Added Cd	3	0.929	<0.001	0.05	0.002
Plant species*Added Cd	6	0.074	<0.001	0.006	0.01

PAT: Plant aerial tissue; df: degrees of freedom.

Table **9-7**: Tukey post hoc analysis for the means of total Cd (mg kg^{-1}) in PAT, Cd uptake* (mg Cd per pot), total Cu (mg kg^{-1}) in PAT, and total Zn (mg kg^{-1}) in PAT in experiment 1.

Factor	Total Cd in PAT	Cd uptake	Total Cu in PAT	Total Zn in PAT
Plant species				
Sf	26.7 ± 21.6 a	0.2 ± 0.1 a	54.9 ± 16.4 a	71.1 ± 18.3 a
O	25.6 ± 23.2 a	0.1 ± 0.1 b	44.7 ± 13.3 b	71.8 ± 9.6 a
V	4.9 ± 4.5 b	0.04 ± 0.04 c	11.0 ± 2.9 c	26.0 ± 4.3 b
Added Cd				
0.0	0.1 ± 0.05 a	0.001 ± 0.1 a	41.9 ± 30.3 a	62.7 ± 31.4 a
1.0	11.6 ± 7.4 b	0.1 ± 0.04 b	39.1 ± 22.0 ab	60.0 ± 24.5 a
2.5	24.3 ± 15.1 c	0.1 ± 0.1c	34.9 ± 18.9 ab	53.2 ± 23.0 ab
5.0	40.1 ± 24.2 d	0.2 ± 0.0003 d	31.5 ± 17.3 b	49.3 ± 18.4 b

Values are presented as mean ± standard deviation (SD); means followed by the same letter within columns are not significantly different and data not reported are not significantly different (alpha = 0.1). * See Table 3-3. Added Cd in mg kg^{-1} soil-less media.

Table **10-8**: MLR for total Cd (mg kg^{-1}) in PAT by treatment in experiment 1.

Plants	Model	R^2	R^2 (Adj)	E (C) *
Sf	Cd = 8.0 + 139.5 P - 1.1 Zn	0.48	0.41	Zn (0.47), P (0.01)
O	Cd = 134.1 + 1.4 Cu – 2.8 Zn + 0.003 Na	0.65	0.52	Cu (0.06), Zn (0.36), Na (0.23)
V	Cd = -3.8 + 0.1 Mn	0.10	0.04	Mn (0.10)

* Element and in parenthesis the contribution to the MLR (n = 5; alpha = 0.1). PAT: Plant aerial tissue.

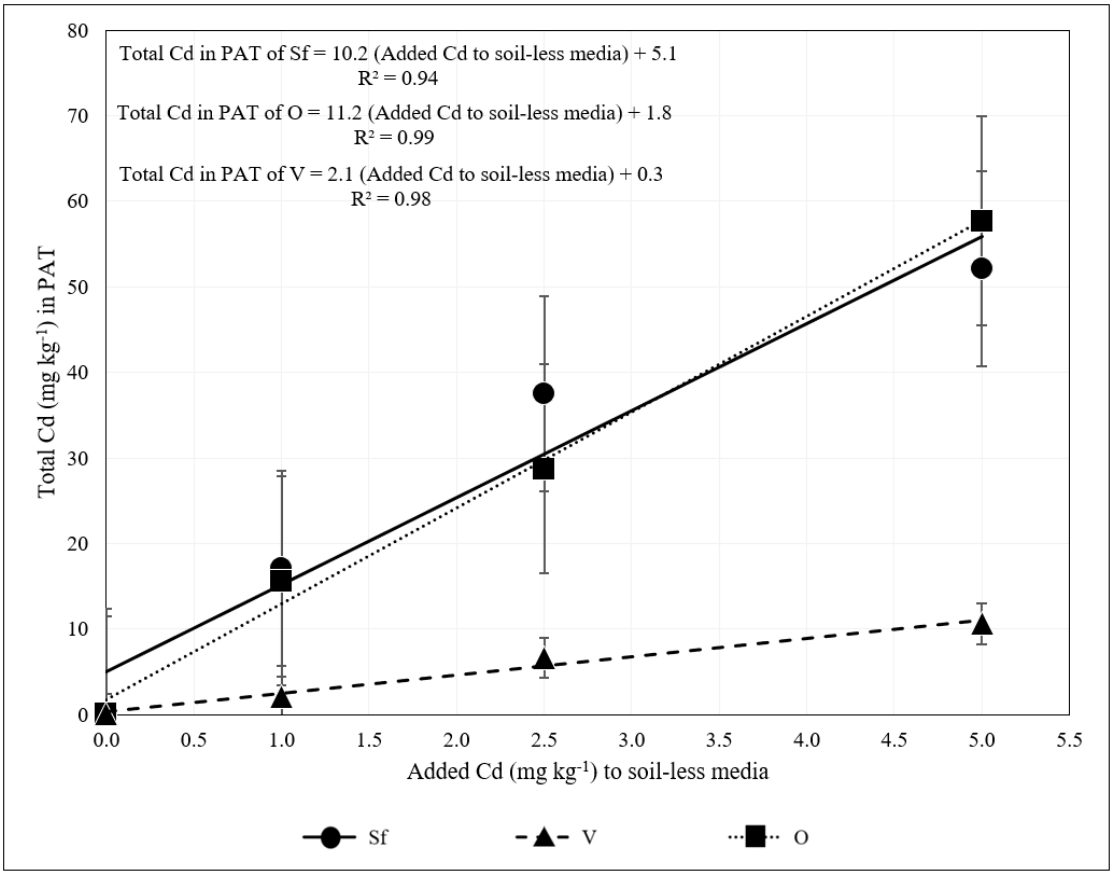

Figure **3-3**: Total Cd in plant aerial tissue (PAT) by added Cd to soil-less media in experiment 1. Vertical lines represent means ± standard error (SE) (n = 5) and lines are the fitted linear regression lines per plant (alpha = 0.1).

Table **11-9**: Constant, slope coefficients, p-value, and confidence intervals of coefficients for total Cd (mg kg^{-1}) in PAT in experiment 1.

Term	Coefficient	p-value	Confidence interval*
Constant	2.4	0.1	(-0.5, 5.2)
Added Cd	7.8	< 0.001	(6.8, 8.8)
Plant species			
O	-0.6	0.8	(-4.6, 3.4)
Sf	2.7	0.2	(-1.3, 6.7)
V	-2.1	0.3	(-6.1, 2.0)

*Alpha = 0.05.

Figure **3-4**: Plant species growing in soil-less media in the 5.0 mg Cd kg^{-1} treatment. A1: vetiver one day after transplanted; A2: vetiver one day before harvest; B1: sunflower one week after transplanted; B2: sunflower one day before harvest; C1: oilseed rape five days after transplanted; C2: oilseed rape two weeks before harvest; C3: oilseed rape one day before harvest.

Experiment 2. Cadmium dose-response relationships of three plant species cultivated in an artificially Cd-contaminated soil matrix

A mixture of soil and perlite (grade 2), in a 5:1 proportion w/w, was used to improve root development in sunflower (Sf), oilseed rape (O), and vetiver (V) due to clay content in the soil (Table 3-12). The soil used to prepare the soil matrix in experiment 2 (Table 3-10) was found to be slightly acidic with a high concentration of phosphorous (P) and potassium (K), and a medium concentration of magnesium (Mg) and sulfur (S) relative to an average agricultural soil. Zn and Cu were found adequate for plant growing (Horneck et al., 2011).

A two-way ANOVA was run on a sample of 60 plants sown in a soil matrix to examine the effect of Plant species and Added Cd on PAT fresh weight, PAT total Cd, PAT total Cu, PAT total Zn, soil matrix total Cd, and BCF (Table 3-11). A significant interaction was found between Plant species and Added Cd ($F(6, 48) = 83.21$, $p < 0.001$), total Cd in soil matrix ($F(6, 48) = 7.47$, $p < 0.001$), and BCF ($F(6, 48) = 17.43$, $p < 0.001$). Simple main effects analysis showed that Plant species had a significant effect on fresh weight of PAT (p

< 0.001), total Cu in PAT ($p < 0.001$), and total Zn in PAT ($p < 0.001$) in comparison to Added Cd (Table 3-11).

Differences between plant species are presented in Table 3-12. A significant difference across Plant species was found with V having a significantly lower total Cd, total Zn, and BCF than the other species (Table 3-12). In addition, PAT total Cu was found to be significantly different in Sf in comparison to O and V, with Sf having the highest levels and V the lowest. A significant difference was found across Plant species with Sf reporting the highest Cd uptake, followed by O and V (Table 3-12). BCF values were found to be significantly different with Sf and O having values greater than one.

Differences in Plant species and Added Cd levels are presented in Table 3-12. Significant differences were found in PAT total Cd (all species) between all added Cd levels. Generally, higher amounts of added Cd to the soil matrix resulted in significantly higher PAT total Cd. In addition, A significant difference was found in PAT total Cu between the 0.0 and 2.5 mg kg^{-1} added Cd treatments. A significant difference in the soil matrix total Cd was found between the control and 2.5 mg kg^{-1} Added Cd treatment. A significant difference at all levels was found in Cd uptake, which increased as the level of added Cd was increased (Table 3-12). A significant difference in BCF values was found between the control and the 1.0 mg kg^{-1} Added Cd treatment, and between the control and the 2.5 mg $kg^{-1}$and 5.0 mg kg^{-1} treatments.

Modeling results suggest that plant tissue Zn was the best predictor of total Cd in Sf. The best predictor of total Cd in O was Cu, while the best predictor of total Cd in V was Ca (Table 3-13). A significant linear relationship was found between the three plant species in PAT total Cd and Added Cd (Figure 3-5). The relationship between Added Cd and total Cd in PAT was statistically significant (Table 3-14). The main effect of each plant species was not significant, which suggest that the difference between the three constants was not

statistically significant (Table 3-14). A significant linear relationship between the soil matrix total Cd with respect to the Cd treatment levels and plant species growing in that media is shown in Figure 3-6. The relationship between Added Cd and total Cd in soil matrix was found statistically significant because the p-value for Added Cd was < 0.001 (Table 3-15). The main effect of V was significant; however, the main effect of Sf and O was not significant, which indicated that difference among these two constants was not statistically significant (Table 3-15). Leaf and stem damages attributed to Cd toxicity, fungus, and insect attack were observed in oilseed rape, particularly in the 5.0 mg Cd kg^{-1} treatment (Figure 3-7).

Table **12-10**: Properties of soil in experiment 2.

Parameter	Units	Value
pH		6.4
P	mg kg^{-1}	71
K	mg kg^{-1}	294
Mg	mg kg^{-1}	115
Ca	mg kg^{-1}	1470
CEC	meq/100 g	11.1
Zn	mg kg^{-1}	3.4
Cu	mg kg^{-1}	4.4
S	mg kg^{-1}	14.3
OM	%	2.16
Sand	%	16.91
Silt	%	54.15
Clay	%	28.94
Soil textural class		Silty Clay Loam

Table **13-11**: Analysis of variance of fresh weight (FW) (g) of PAT, total Cd (mg kg^{-1}) in PAT, total Cu (mg kg^{-1}) in PAT, total Zn (mg kg^{-1}) in PAT, total Cd (mg kg^{-1}) in soil matrix, and BCF in experiment 2.

Effect	df	FW of PAT	Total Cd in PAT	Total Cu in PAT	Total Zn in PAT	Total Cd in soil matrix	BCF
				p-value			
Plant species	2	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Added Cd	3	0.667	<0.001	0.075	0.254	<0.001	<0.001
Plant species*Added Cd	6	0.506	<0.001	0.146	0.527	<0.001	<0.001

PAT: Plant aerial tissue; df: degrees of freedom; BCF: Bioconcentration factor.

Table **14-12**: Tukey post hoc analysis for the means of total Cd (mg kg^{-1}) in PAT, total Cd (mg kg^{-1}) in soil matrix, Cd uptake* (mg Cd per pot), total Cu (mg kg^{-1}) in PAT, total Zn (mg kg^{-1}) in PAT, and BCF in experiment 2.

Factor	Total Cd in PAT	Total Cd in soil matrix	Cd uptake	Total Cu in PAT	Total Zn in PAT	BCF
Plant species						
Sf	9.3 ± 8.3 a	1.9 ± 1.2 a	0.1 ± 0.1 a	29.9 ± 5.3 a	65.5 ± 9.1 a	4.1 ± 2.9 a
O	10.8 ± 9.7 b	1.5 ± 0.7 b	0.1 ± 0.1 b	7.8 ± 0.9 b	40.9 ± 4.6 b	6.6 ± 4.5 b
V	1.4 ± 1.1 c	1.3 ± 0.7 b	0.01 ± 0.01 c	5.9 ± 1.0 b	19.8 ± 3.2 c	0.8 ± 0.5 c
Added Cd						
0.0	0.2 ± 0.1 a	0.74 ± 0.2 a	0.002 ± 0.001 a	15.9 ± 13.0 a		0.3 ± 0.1 a
1.0	2.9 ± 1.7 b	1.1 ± 0.3 a	0.04 ± 0.02 b	15.1 ± 12.7 ab		3.2 ± 2.2 b
2.5	9.7 ± 6.1 c	1.8 ± 0.3 b	0.1 ± 0.1 c	13.4 ± 10.2 b		5.8 ± 4.1 c
5.0	16.0 ± 9.7 d	2.7 ± 0.9 c	0.2 ± 0.1 d	13.7 ± 10.6 ab		6.1 ± 4.3 c

Values are presented as mean ± SD; means followed by the same letter within columns are not significantly different and data not reported are not significantly different (alpha = 0.1). * See Table 3-3. Added Cd in mg kg^{-1} soil matrix. PAT: Plant aerial tissue; BCF: Bioconcentration factor.

Table **15-13**: MLR for total Cd (mg kg^{-1}) in PAT by treatment in experiment 2.

Plants	Model	R^2	R^2 (Adj)	E (C) *
Sf	Cd = 51.6 - 128.4 P - 1.2 Cu + 0.9 Zn	0.60	0.51	P (0.1), Cu (0.1), Zn (0.4)
O	Cd = 5.7 - 9.1 K - 2.2 Zn + 15.8 Cu	0.54	0.45	K (0.07), Zn (0.12), Cu (0.35)
V	Cd = 9 - 19.4 Ca - 0.001 Al	0.40	0.33	Ca (0.24), Al (0.16)

* Element and in parenthesis the contribution to the MLR (n = 5; alpha = 0.1). PAT: Plant aerial tissue.

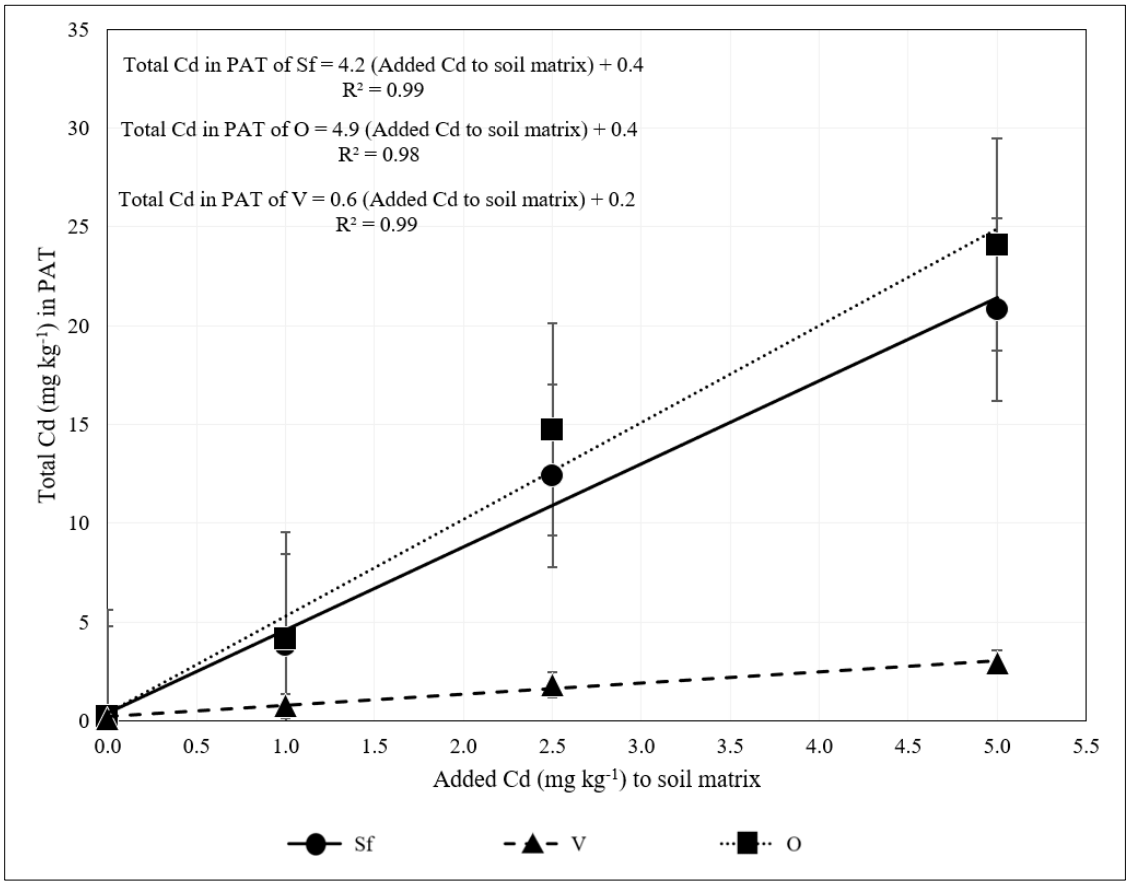

Figure **3-5**: Total Cd in plant aerial tissue (PAT) by added Cd to soil matrix in experiment 2. Vertical lines represent means ± SE (n = 5) and lines are fitted linear regression lines per plant (alpha = 0.1).

Table **16-14**: Constant, slope coefficients, p-value, and confidence intervals of coefficients for total Cd (mg kg^{-1}) in PAT in experiment 2.

Term	Coefficient	p-value	Confidence intervals*
Constant	0.3	0.3	(-0.3, 0.9)
Added Cd	3.2	< 0.001	(3.0, 3.4)
Plant species			
O	0.1	0.9	(-0.8, 0.9)
Sf	0.1	0.9	(-0.8, 0.9)
V	-0.1	0.8	(-0.9, 0.7)

*Alpha = 0.05.

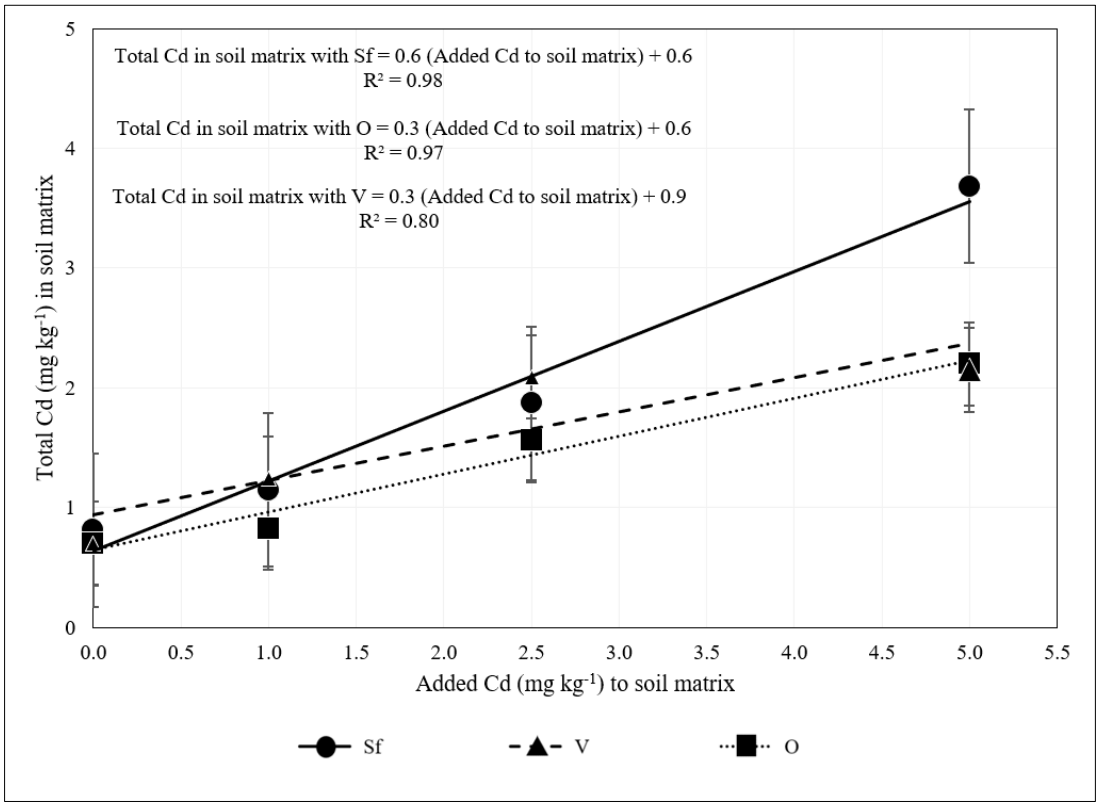

Figure **3-6**: Total Cd in soil matrix by added Cd to soil matrix in experiment 2. Vertical lines represent means ± SE (n = 5) and lines are the fitted linear regression lines per plant (alpha = 0.1).

Table **17-15**: Constant, slope coefficients, p-value, and confidence intervals of coefficients for total Cd (mg kg^{-1}) in soil matrix in experiment 2.

Term	Coefficient	p-value	Confidence intervals*
Constant	0.7	< 0.001	(0.6, 0.9)
Added Cd	0.4	< 0.001	(0.3, 0.4)
Plant species			
O	-0.1	0.4	(-0.3, 0.1)
Sf	-0.1	0.3	(-0.3, 0.1)
V	0.2	0.1	(-0.02, 0.4)

*Alpha = 0.05.

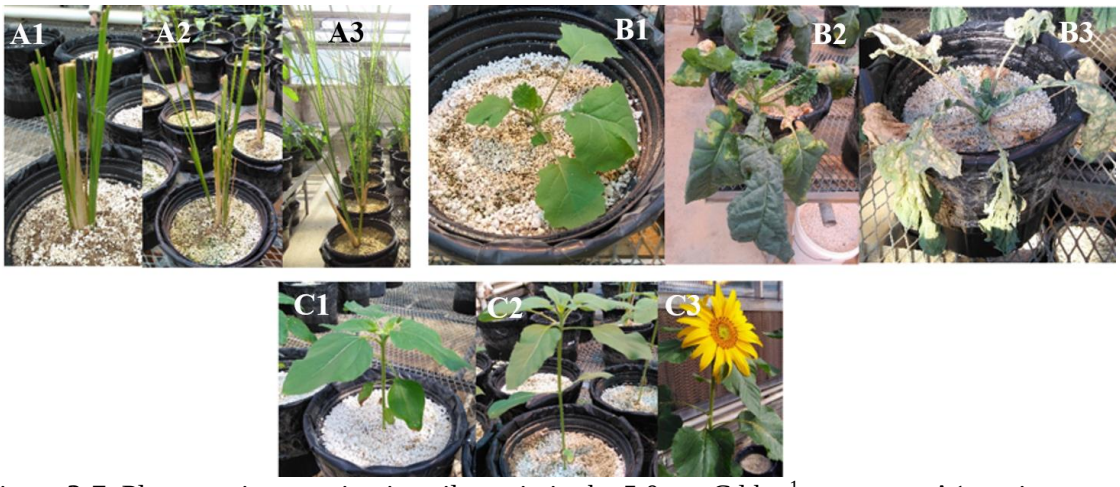

Figure **3-7**: Plant species growing in soil matrix in the 5.0 mg Cd kg^{-1} treatment. A1: vetiver one day after transplanted; A2: vetiver two weeks after transplanted; A3: vetiver one day before harvest; B1: oilseed rape one week after transplanted; B2: oilseed rape one week before harvest; B3: oilseed rape one day before harvest; C1: sunflower five days after transplanted; C2: sunflower four weeks after transplanted; C3: sunflower one day before harvest.

Experiment 3. Cadmium dose-response relationships of three plant species cultivated in an artificially Cd-contaminated soil

The plant species used were sunflower (Sf), heliconia (H), and vetiver (V). Soil used in this experiment was not mixed with any other material. Table 3-16 shows the properties of the soil. Soil used in experiment 3 (Table 3-16) was moderately acidic. The soil's cation-exchange capacity corresponded with its finer texture (clay). A two-way ANOVA was run on a sample of 36 plants sown in soil to examine the effect of Plant species and Added Cd on fresh weight of PAT, Total Cd in PAT, Total Cd in soil, BCF, and soil pH. A significant interaction was found between Plant species and Added Cd for: FW of PAT ($F(4, 27) = 102.11$, $p < 0.001$); Total Cd in PAT ($F(4, 27) = 30.43$, $p < 0.001$); Total Cd in soil ($F(4, 27) = 50.68$, $p < 0.001$); BCF ($F(4, 27) = 6.89$, $p = 0.001$); and soil pH ($F(4, 27) = 4.77$, $p < 0.005$) (Table 3-17).

PAT total Cd was significantly higher in Sf; no difference was found between H and V (Table 3-18). Total Cd in the soil was significantly different between the three species and highest in V and lowest in Sf. BCF was significantly higher in Sf; no difference was found between H

and V. Soil pH was significantly lower in V; no difference was found between H and Sf. A significant difference was found across Plant species with Sf reporting the highest Cd uptake, followed by V and H; however, V and H did not present a significant difference (Table 3-18).

The Added Cd treatment compared the effect of the different concentrations of Cd added to soil on PAT total Cd, total Cd in soil, BCF, and soil pH, without considering the difference due to plant species (Table 3-18). In the Added Cd treatment, a significant effect was found on PAT total Cd, and total Cd in soil at all treatment levels, with the highest Cd addition corresponding with the highest PAT Total Cd, and Total Cd in the soil. A significant difference at all levels was found in Cd uptake, which increased as the level of added Cd was increased (Table 3-18). Soil pH in the control was significantly higher in comparison to either Cd additions (Table 3-18).

Significant differences were found within and across species in PAT Total Cd, Total Cd in the soil, BCF, and Soil pH (Table 3-18). PAT total Cd in Sf was significantly greater than other species and highest in PAT with the highest Added Cd. Total Cd in soil was typically higher with greater added Cd, but only significantly higher between all levels with Sf; H and V exhibited significant differences between the control and the two Cd additions. Sf BCF was significantly higher with higher Added Cd.

Modeling results suggest show significant, positive relationship exists between plant total Cd for the 3 species with respect to Cd treatments (Figure 3-8). A regression for H is not presented because the total Cd concentration of both the control and the 3.0 mg kg^{-1} Cd treatment was non-detectable. The relationship between Added Cd and total Cd in PAT was found statistically significant (Table 3-19). The main effect of each plant species was not significant, which indicated that difference between the two constants was not statistically significant (Table 3-19). A significant, positive linear relationship in soil total Cd with respect to the Cd treatment levels and plant species growing in the respective soil was found (Figure 3-9). The relationship between Added Cd and total Cd in soil was found statistically significant (Table 3-20). The main

effect of each plant species was not significant, which indicated that difference among the three constants was not statistically significant (Table 3-20). Leaf and stem damages attributed to Cd toxicity, fungus, and insect attack were observed in H, particularly in the 5.0 mg Cd kg^{-1} treatment (Figure 3-10). No damage was seen in Sf and V treatments.

Table **18-16**: Chemical properties of soil in experiment 3.

Properties	Units	Value
pH		5.5
P	mg kg^{-1}	110
K	mg kg^{-1}	194
Mg	mg kg^{-1}	805
Ca	mg kg^{-1}	510
CEC	meq/100 g	9.9
Zn	mg kg^{-1}	10.8
Cu	mg kg^{-1}	9.5
S	mg kg^{-1}	77
OM	%	6.29
Sand	%	32
Silt	%	28
Clay	%	40
Soil textural class		Clay

Table **19-17**: Analysis of variance of fresh weight (FW) (g) of PAT, total Cd (mg kg^{-1}) in PAT, total Cd (mg kg^{-1}) in soil, BCF, soil pH in experiment 3.

Effect	df	FW of PAT	Total Cd in PAT	Total Cd in soil	BCF	Soil pH
		----------p-value----------				
Plant species	2	<0.001	<0.001	<0.001	<0.001	<0.001
Added Cd	2	<0.001	<0.001	<0.001	0.1	<0.001
Plant species*Added Cd	4	<0.001	<0.001	<0.001	0.001	0.005

PAT: Plant aerial tissue; df: degrees of freedom; BCF: Bioconcentration factor.

Table **20-18**: Tukey post hoc analysis for the means of total Cd (mg kg^{-1}) in PAT, total Cd (mg kg^{-1}) in soil, Cd uptake* (mg Cd per pot), BCF, and soil pH in experiment 3.

Factor	Total Cd in PAT	Total Cd in soil	Cd uptake	BCF	Soil pH
Plant species					
Sf	2.2 ± 1.8 a	1.2 ± 0.8 a	0.02 ± 0.01 a	1.6 ± 0.7 a	4.8 ± 0.2 a
H	0.02 ± 0.03 b	1.4 ± 0.8 b	0.0004 ± 0.001 b	0.01 ± 0.01 b	4.8 ± 0.1 a
V	0.3 ± 0.3 b	2.3 ± 1.5 c	0.002 ± 0.002 b	0.2 ± 0.2 b	4.6 ± 0.1 b
Added Cd					
0.0	0.1 ± 0.1 a	0.3 ± 0.8 a	0.001 ± 0.001 a		4.9 ± 0.1 a
3.0	0.8 ± 1.0 b	2.1 ± 0.8 b	0.01 ± 0.01 b		4.7 ± 0.03 b
5.0	1.5 ± 2.0 c	2.5 ± 1.5 c	0.01 ± 0.01 c		4.7 ± 0.2 b

Values are presented as mean ± SD; means followed by the same letter within columns are not significantly different and data not reported are not significantly different (alpha = 0.1). * See Table 3-3. Added Cd in mg kg^{-1} soil; PAT: Plant aerial tissue; BCF: Bioconcentration factor; n.d.: not detected.

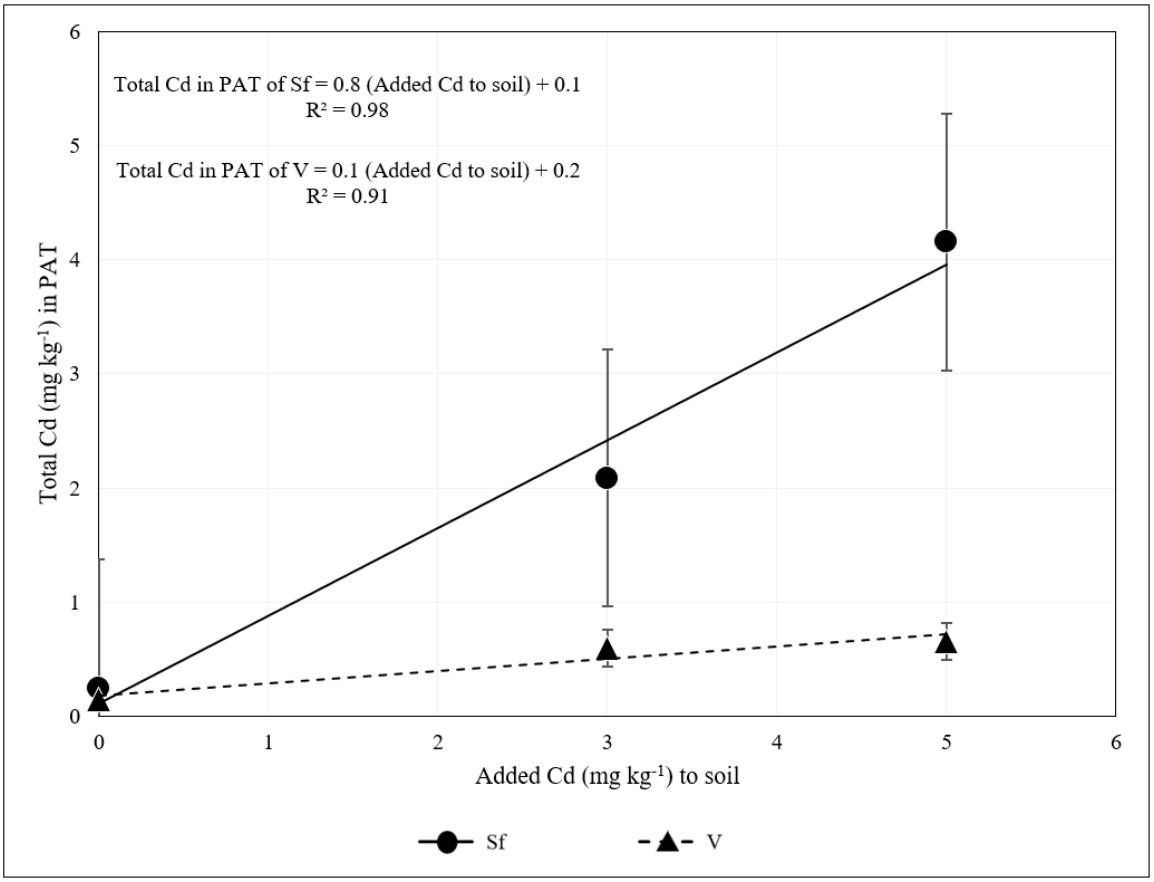

Figure **3-8**: Total Cd in plant aerial tissue (PAT) by added Cd to soil in experiment 3. Vertical lines represent means ± SE (n = 4) and lines are the fitted linear regression lines per plant (alpha = 0.1).

Table **21-19**: Constant, slope coefficients, p-value, and confidence intervals of coefficients for total Cd (mg kg^{-1}) in PAT in experiment 3.

Term	Coefficient	p-value	Confidence intervals*
Constant	0.1	0.4	(-0.1, 0.3)
Added Cd	0.3	< 0.001	(0.2, 0.3)
Plant species			
Sf	0.02	0.9	(-0.3, 0.3)
V	0.07	0.6	(-0.2, 0.4)

*Alpha = 0.05; Total Cd in PAT of H was not detected.

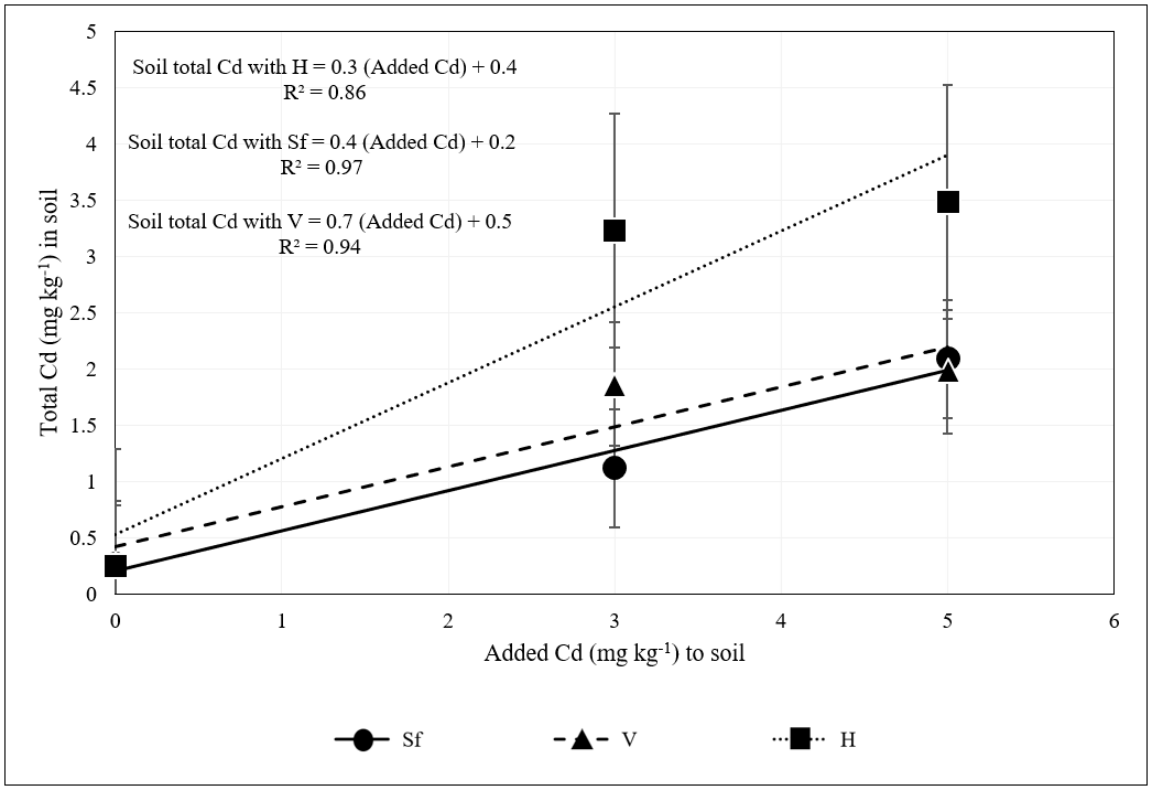

Figure **3-9**: Total Cd in soil by added Cd to soil in experiment 3. Vertical lines represent means ± SE (n = 4) and lines are the fitted linear regression lines per plant (alpha = 0.1).

Table **22-20**: Constant, slope coefficients, p-value, and confidence intervals of coefficients for total Cd (mg kg^{-1}) in soil in experiment 3.

Term	Coefficient	p-value	Confidence intervals*
Constant	0.4	0.001	(0.2, 0.6)
Added Cd	0.5	< 0.001	(0.4, 0.5)
Plant species			
H	0.04	0.8	(-0.3, 0.3)
Sf	-0.2	0.2	(-0.5, 0.1)
V	0.1	0.3	(-0.2, 0.4)

*Alpha = 0.05.

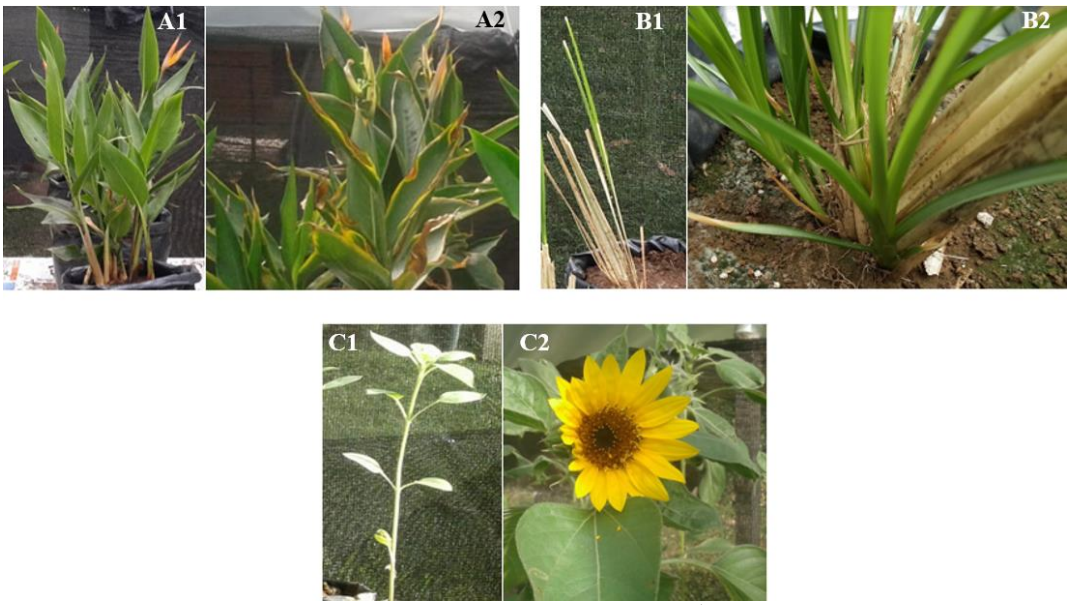

Figure **3-10**: Plant species growing in soil in the 5.0 mg Cd kg^{-1} treatment. A1: heliconia two weeks after transplanted; A2: heliconia one day before harvest; B1: vetiver one week after transplanted; B2: vetiver one day before harvest; C1: sunflower three weeks after transplanted; C2: sunflower one day before harvest.

Discussion

Across all experiments, total Cd in PAT increased with increasing Cd concentration in the soil matrix. Plants growing in the soil-less media (experiment 1), and highest Cd treatment to soils (5.0 mg Cd kg^{-1}), had the highest PAT total Cd (Table 3-7), particularly sunflower and vetiver presented the highest PAT total Cd without significant differences between each other in experiment 1; however, vetiver accumulated significantly less Cd in comparison to sunflower and oilseed rape in experiment 1 (Table 3-7). The inert nature of the growing media in experiment 1 likely facilitated high plant uptake of Cd given the lower tendency for Cd binding to exchange sites. A significant difference at all levels was found in Cd uptake, which increased as the level of added Cd was increased in all experiments (Tables 3-7, 3-12, 3-18). In all experiments, the highest Cd uptake was reported for sunflower (Tables 3-7, 3-12, 3-18). This could indicate that sunflower accumulates more Cd than the other plant species. In line with this analysis, the PAT in

vetiver accumulated a fifth of total Cd in more biomass as compared to sunflower and oilseed rape total Cd in the corresponding PAT (Table 3-7). These results could indicate that the vetiver did not accumulate a significant concentration of Cd in its PAT.

In experiment 2, the highest Cd treatment to soils (5.0 mg kg^{-1} total Cd) produced the highest PAT total Cd, but PAT total Cd values were approximately half of experiment 1: oilseed rape had the highest value and vetiver the lowest (Table 3-12). Similar to Carrier et al. (2003), the PAT total Cd in oilseed rape increased as the added Cd in soil was increased. Carrier et al. (2003) found Cd levels in oilseed rape leaves plateaued at 260 mg kg^{-1} after 50 mg kg^{-1} added Cd in soil.

In experiment 3, the lowest total Cd in PAT corresponded to heliconia. No significant difference was found in total Cd in soil between sunflower and heliconia. This could mean that heliconia extracted Cd, but this Cd was not allocated in PAT but in its root system (Table 3-18). The evaluation of total Cd in the root system of vetiver and heliconia vs the total Cd detected in the corresponding PAT is recommended to determine where the plant species allocate the Cd extracted from soil.

Plant varieties, soil matrices, and Cd extraction methods used in experiments 2 and 3 were different, and as a result, the total Cd reported in each plant species varied between experiments. Soils in experiment 3 were more acidic than those in experiment 2 (pH 5.5 and pH 6.4 respectively). The solubility of Cd is greatly affected by soil pH; in alkaline soil, precipitation is likely to account for Cd equilibria; and in acid soil, the Cd activity may largely be governed by soil organic matter and sesquioxides (Kabata-Pendias, 2011). Soils used in experiment 2 had a lower organic matter (OM) content (2.16 %) in comparison to soils in experiment 3 (6.29 %). Soil organic matter is a dominant factor affecting the availability of metals in soil (Huang et al., 2017), however studies relating Cd bioavailability with OM are contradictory. Some researchers have observed that Cd solubility and availability in soils increases with an increase in soil OM content (Pinto et al, 2004), while others have concluded the opposite (Cui et al., 2019). The increase in

Cd availability has also been related to the exudation of organic acids (Javed et al., 2017; Li et al., 2019; Montiel-Rozas et al., 2016).

The different plant total Cd levels found across this study's plant species are likely due to Cd interactions in soil across the different treatments and plant-specific metal absorption characteristics. For example, physiological or genetic differences are known to effect Cd uptake in grasses (Silva et al., 2016). Soils in experiment 3 had a clay texture, while soils in experiment 2 have a silty clay loam texture. Peris (2005) indicates that soil texture is of great importance in heavy metal retention due to the clay adsorption capacity.

PAT total Cd was found in this study to be a useful indicator for assessing the phytoremediation efficiency (Zhao et al., 2003). In experiment 1, while all three species exhibited significant increases in PAT total Cd with Cd additions to the soil-less media, only sunflower and oilseed rape acted as Cd hyperaccumulators (Ghosh & Singh, 2005; Rascio & Navari-Izzo, 2011). Zhang et al. (2002) suggest a suitable phytoremediator species should have a BCF value >1. Results from experiment 2 show that sunflower and oilseed rape have BCF values > 1 for Cd when was added to soil at 1.0, 2.5 and 5.0 mg kg^{-1}. Oilseed rape had a higher BCF in all Cd treatments but notably a 2x higher value in the 5.0 mg kg^{-1} Cd treatment. BCF trends for experiment 2 were: oilseed rape > sunflower > vetiver (Table 3-12). In experiment 3 only sunflower had a BCF value > 1 and this was consistent across all treatments; BCF trends were: sunflower > vetiver > heliconia (Table 3-18). No significant correlation was detected between BCF of PAT and pH of soil after plants were harvested in experiment 3 (Pearson correlation: 0.17; p-value: 0.3). BCF values found in this study for oilseed rape and sunflower are higher than reported values for other species. The maximum BCF value reported for *Pinus pinaster* was 1.85 (Andras et al., 2016), for carrot 0.62 (Yang et al., 2009), for sunflower 2.63 (Kötschau et al., 2014), for cucumber 4.8 (Li et al., 2017), for potato 0.28 (Swartjes et al., 2013), for spinach 2.29 (Swartjes et al., 2013), for rice 1.11 (Song et al., 2015), and for corn 0.16 (Hamidpour et al.,

2010). Interestingly, in experiment 3 vetiver's BCF value plateaued as the treatment Cd increased (Table 3-18). This result is somewhat consistent with findings reported by Zhang et al. (2014) who showed a decrease in the BCF in vetiver grown under greenhouse conditions following increasing treatment soil Cd. A plant's BCF could increase or decrease depending on biotic and abiotic conditions, especially in conjunction with other plants, which might ultimately change over time the bioavailability of Cd in soil (Kötschau et al., 2014). Further research should explore the interaction of target commodities with phytoextractors or amendments to reduce the propensity for toxic metal uptake.

Solís-Domínguez et al. (2007) stated that a Cd concentration in leaf tissue, in the range of 5 to 30 mg kg^{-1}, can be considered excessive or toxic. In addition, plant tissue damage can be an indication of Cd toxicity. Studies carried out in different plant species have revealed that Cd is strongly phytotoxic and causes growth inhibition and even plant death, although the mechanisms involved in its toxicity are still not completely understood (Sandalio et al., 2001). Cd affects plants by inhibiting photosynthesis and respiration, reducing water and nutrient uptake, altering gene and protein expression, inducing and inhibiting enzymes, enhancing accumulation of reactive oxygen species, enhancing lipid peroxidation, and disturbing metabolism (Semane et al., 2010; Shanmugaraj & Ramalingam et al., 2019; Tanhan et al., 2007). Plant tissue damage was observed in oilseed rape in experiments 1 and 2 (Figures 3-4 and 3-7). However, plant tissue damage could also have been due to insects and fungus observed at times on leaves over the course of the experiment. In experiment 3, heliconia exhibited some plant tissue damage due to the Panama and the eye spot fungal disease as described by Alarcón-Restrepo (2007). Many leaves and inflorescences in heliconia showed green areas neighboring very pale/yellow areas (Figure 3-10). Across all experiments, sunflower exhibited the least plant tissue damage, but was accumulating Cd at or above levels noted by Solís-Domínguez et al. (2007). Interestingly, across all experiments, vetiver did not exhibit observable plant tissue damage, but vetiver also did not

take up much Cd relative to the other study species. Previous research suggests vetiver has a high tolerance for Cd-rich soil (Kumar et al., 2018). To date, the status of vetiver as a Cd phytoextractor is mixed with some research suggesting the species is a Cd phytoextractor (Kumar et al., 2018) versus others suggesting it is a Cd excluder, where Cd concentrations in the aerial parts of a plant are maintained constant and low over a wide range of soil concentrations, up to a critical soil value above which the physiological plant mechanisms breaks down and unrestricted Cd transport results (Baker et al., 1981; Zhang et al., 2014); our results suggest vetiver is not a Cd phytoextractor.

Cadmium toxicity occurs in many plants due to cadmium's chemical similarity with essential elements, particularly Zn and Cu, deregulating the uptake and distribution of these elements or causing a displacement from proteins (Clemens, 2006; Shanmugaraj & Ramalingam et al., 2019; Verbruggen et al., 2009). Embedding itself into the enzymes associated with proteosynthesis and energy processes, Zn is necessary for maintaining the integrity of biomembranes and also plays an important role in the development of seeds and generative organs (Sturikova et al., 2018). Results from experiment 1 found that PAT Zn in sunflower was not significantly different from PAT Zn in oilseed rape, but PAT Zn in sunflower and PAT Zn in oilseed rape were significantly different in comparison to PAT Zn in vetiver (Table 3-7). In experiment 2, PAT Zn in sunflower was significantly different from PAT Zn in oilseed rape and vetiver PAT Zn (Table 3-14).

Cu acts as an essential cofactor of numerous proteins that play key functions in plant cell metabolism, such as the transport of electrons in mitochondria and chloroplasts, the regulation of the cellular redox state, the perception of ethylene, or the modification of the cell wall (Migocka & Malas, 2018). In experiment 1 PAT Cu in sunflower was not significantly different from total Cu in oilseed rape, but PAT Cu in sunflower and total Cu in oilseed rape were significantly different in comparison to PAT Cu in vetiver (Table 3-7). Results from experiment 2 indicate that

PAT Cu in sunflower was found significantly different from PAT Cu in oilseed rape and PAT Cu in vetiver (Table 3-12).

Macro and micro elements in plant tissue were used to analyze possible relationships between these elements and the total Cd in plant tissue using multiple linear regression (MLR) via a backward elimination method. The identification of plant elements as significant predictors of total Cd in PAT might serve to elucidate the dynamics of Cd within the plant. MLR models indicate that Zn in PAT was the most significant predictor of total Cd in sunflower in experiment 1 ($R^2 = 0.48$) and in experiment 2 ($R^2 = 0.40$) and oilseed rape in experiment 1 ($R^2 = 0.36$). Mn in PAT was the best predictor of total Cd in vetiver in experiment 1 ($R^2 = 0.10$). In experiment 2, MLR models indicates that Cu in PAT was the best predictor of total Cd in oilseed rape ($R^2 = 0.35$), and Ca in PAT was the best predictor of total Cd in vetiver in experiment 2 ($R^2 = 0.24$).

Fe is essential for plants and plays critical roles in important processes such as photosynthesis and respiration (Jeong & Connolly, 2009). Disparate values were found in vetiver tissue total Fe from experiment 3, which ranged from 2140 mg kg^{-1} in the control treatment (no added Cd) to 7890 mg kg^{-1} in the 5.0 mg kg^{-1} Cd treatment (Appendix). Total Fe in vetiver PAT from experiment 1 ranged from 165 mg kg^{-1} in the control treatment (no added Cd) to 238 mg kg^{-1} in the 5.0 mg kg^{-1} Cd treatment. Total Fe in vetiver PAT from experiment 2 ranged from 857 mg kg^{-1} in the control treatment (no added Cd) to 243 mg kg^{-1} in the 5.0 mg kg^{-1} Cd treatment. The difference in the results obtained in experiments 1 and 2, in contrast to results reported in experiment 3, is perhaps due to a possible cross contamination between soil particles and PAT. The vetiver used in experiments 1 and 2 was not sown from seed. The vetiver was approximately 1 month old when purchased for all experiments. In experiment 1 and 2 the vetiver was bought from *Molokai Seed Company* (Hawaii) and it took three weeks to arrive at Penn State. The vetiver used in experiment 3 was purchased in *Pastos, Henos & Ganado Company* (Caldas, Colombia) and it took 3 days to arrive at Universidad del Valle (Cali, Colombia). Both the crop management

(fertilization, phytosanitary control) and the stress that the grass suffered during its transportation might also have play important roles in its final elemental composition.

Soil pH is recognized as the most important factor affecting Cd bio-availability (Kabata-Pendias, 2011; McBride, 2002). Soil pH was found to be significantly more acid in soil under vetiver cultivation in the 5.0 mg Cd kg^{-1} interaction treatment in comparison to sunflower and heliconia that did not present significant difference between each other in soil pH in experiment 3 (Table 3-18). Plants may have been stressed due to the addition of Cd, producing acid exudates as a defense mechanism. Stress conditions can induce plant roots to secrete large amounts of organic acids; this process is viewed as an active adaptive response (Adeleke et al., 2017). The transformation of heavy metal solubility due to root exudates is one of the mechanisms responsible for heavy metal hyperaccumulation of plants (Li et al., 2019). Alternatively, soil acidification could be due to a soil reaction to the N contained in the Hoagland's solution (Shi et al., 2019) and salt effect pH. Note that total Cd in vetiver PAT in experiment 3 did not significantly increase in the 5.0 mg kg^{-1} Cd treatment, which corresponded to the most acidic soil (pH = 4.5), but it presented a significant difference in total Cd in soil at that pH level (Table 3-18). This could mean that the vetiver could have decrease the pH of the soil at a Cd addition of 5.0 mg Cd kg^{-1}, and this could lead to a higher mobility of Cd within the soil, thus, reporting a high total Cd in soil.

Across all treatments with added Cd, including the control without added Cd, soil pH decreased over the course of the experiment. Heliconia and vetiver, which grew in a tropical soil matrix in Colombia (experiment 3) had low soil pH values and the lowest plant tissue total Cd (Table 3-18). This is contrary to what might be expected given Cd bioavailability is typically greater in acid soils (Barančíková et al., 2004; Chavez et al., 2016; Golia et al., 2008; Kim et al., 2009; Sánchez et al., 2011). A theory that could explain this, is that heliconia and vetiver did extract Cd, but that Cd was stored in their root system instead of in their PAT.

Cadmium levels in this study's control matrices are similar to natural background levels. Cadmium levels in control matrices (treatments without added Cd) had a minimum soil matrix total Cd of 0.7 mg Cd kg^{-1} and a maximum of 0.8 mg Cd kg^{-1} in experiment 2, and a minimum soil total Cd of 0.2 mg Cd kg^{-1} and a maximum of 0.3 mg Cd kg^{-1} in experiment 3. Smolders & Mertens (2013) indicate that the mean Cd concentration in non-polluted European agricultural soils is 0.3 mg Cd kg^{-1} and median soil Cd in USA is 0.2 mg Cd kg^{-1}, although the authors do not indicate the extraction method used to establish these values.

Conclusions

Results from this study indicate that sunflower accumulated a significant total Cd concentration in plant aerial tissue and was not attacked by pests and diseases in comparison to the other plant species in any experiment. In contrast, Cd concentrations in heliconia and vetiver were low, and while the total Cd absorbed by oilseed rape was high, oilseed rape was observed to be very sensitive to insect infestation and disease. Results indicate that vetiver (experiment 2) and heliconia (experiment 3) could have extracted Cd from the corresponding soil matrix similarly as oilseed rape (experiment 2) or as sunflower (experiment 3), but that Cd was not accumulated in the plant aerial tissue but rather in the root system. Results suggest that sunflower accumulated a significant concentration of Cd in plant aerial tissue, and it was more resistant to plagues and diseases than oilseed rape and heliconia, therefore, is a suitable phytoextractor of low and moderate Cd pollution.

Technologies used to remediate moderately polluted soils must have the capability of simultaneously ensuring the safety of agricultural products and gradually reducing the heavy metal contents of soils. Ideally, these technologies should be able to remediate heavy metals while allowing the conduction of agricultural production activities at the same time (Lin et al.

2014), for instance, in an intercropping scheme.

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Chapter 4

Accumulation of cadmium in cacao and spinach sown in artificially contaminated soil matrices treated with sunflower and dolomitic lime

Abstract

Cacao and spinach are high-value commodity crops, which can accumulate cadmium (Cd) to high enough levels that human ingestion becomes a health threat. New European Union (EU) regulations governing Cd contents in food products to reduce human exposure could restrict EU importation of foods found in excess of Cd thresholds; similar regulations may soon come to the U.S. Reducing Cd uptake by commodities is one potential mechanism to ensure food products meet new EU regulations. Phytoextractor plant species, which can accumulate excess metals in plant parts, or soil pH adjustments via liming to reduce metal bioavailability, are two methods currently used for managing soils with high Cd levels. Sunflower (Sf) has been widely reported as a suitable Cd phytoextractor species, and dolomitic lime (L) has been used to immobilize Cd in soils. We combined these approaches in an intercropping scheme (sunflower plus cacao plus dolomitic lime; sunflower plus spinach plus dolomitic lime) and assessed their potential to reduce Cd accumulation in cacao and spinach versus a control without such techniques. Plant aerial tissue (PAT) and the bioconcentration factor (BCF) of sunflower, cacao, and spinach were used to evaluate the accumulation of Cd in each plant species. In experiment 1, a significant effect was found on total Cd in PAT and BCF of cacao (variety ICS-95) when planted in an intercropped scheme with a Sf. A significant effect was detected on soil matrix pH when both Sf and L were used. In experiment 2, a significant effect was found on total Cd in PAT of cacao (variety IMC-67) when both Sf and L were used. In experiment 3, a non-significant effect was found on total Cd in PAT of spinach when Sf and L were used. Results indicate that dolomitic lime

increased soil matrix pH, which in turn, led to a significant reduction of extractable Cd in experiment 2 (US EPA method 3050B). An increase in the soil matrix total Cd was correlated to a significant increase of total Cd in PAT of cacao (variety ICS-95), and an increase in the soil matrix pH was correlated to a significant decrease of total Cd in cacao PAT (variety ICS-95). The bioconcentration factor of cacao varieties ICS-95 and of IMC-67 were >1 and < 1 respectively. The use of sunflower with cacao or with spinach did not result in a significant reduction in cacao nor spinach total Cd, although a high and low plant total Cd concentration was noticed in cacao and spinach when planted with sunflower respectively. Additionally, results for spinach showed that an increase in the soil matrix pH with dolomitic lime did not correspond to a significant reduction in spinach total Cd. We conclude that the use of dolomitic lime is a suitable approach to reduce Cd uptake by cacao (variety IMC-67) versus a sunflower intercropping scheme. More research is needed to elucidate the effect of intercropping schemes in spinach total Cd.

Introduction

Heavy metal contamination of agricultural soils has posed a risk to the environment and human health. Cadmium (Cd) is a non-essential toxic metal, mostly found as a divalent cation, complexing with other anions (Hamid et al., 2018; Kabata-Pendias, 2011). In soils, the bioavailability of Cd is controlled by several factors such as total metal content, pH, redox potential, soil organic matter, cation exchange capacity, clay content, presence of other nutrients, Fe and Mn oxides (Corguinha et al., 2012; He et al., 2005; Kirkham, 2006; Smolders, 2013), parent material, soil texture (Kabata-Pendias, 2011), root exudates (Li et al., 2019; Muehe et al., 2015), types, and cultivars of crop plants (Brus et al., 2005; Cieśliński et al., 1996), and management practices (Corguinha et al., 2012). Soil pH is an important factor

affecting Cd availability (Barančíková et al., 2004; Golia et al., 2008; Kabata-Pendias, 2011; McBride, 2002). There is an indirect linear relationship between soil pH and bioavailability, or plant uptake of Cd (Tudoreanu & Phillips, 2004). As pH decreases, Cd uptake by plants increases (Chavez et al., 2016a; Kim et al., 2009; Kirkham, 2006).

While some Cd is naturally released into soils through weathering of rocks, which typically contain Cd at concentrations < 0.2 mg kg^{-1}, high inputs originating from anthropogenic sources such as mining, smelting, microelectronics manufacturing, phosphate fertilizers, and from the petroleum refining industry have significantly increased Cd contents in many soils worldwide (He & Singh, 1994; He et al., 2015; Mahar et al., 2016; Mohammadi et al., 2015; Peláez-Peláez et al., 2016).

Cd persists in soil for long durations due to its minimal microbial or chemical loss (Hamid et al., 2019a). Cd is ranked 7th among 20 toxins due to its high solubility and toxic nature (Hamid et al., 2019b; Yang et al., 2004). Cadmium exposure in humans occurs primarily through smoking and food intake (Faroon et al., 2012). Once in the body, Cd can be accumulated in the kidney and liver for 15 to 30 years (Castelli et al., 2005; Boeckx et al., 2018) causing renal tissue nephrotoxicity (Jaishankar et al., 2014), severe pulmonal and gastrointestinal irritation (Tchounwou et al., 2012). Excess Cd typically causes a number of toxic symptoms in plants such as growth retardation, leaf chlorosis, induction/inhibition of enzymes and altered stomatal function (Carrier et al., 2003).

Agricultural commodities such as cacao (*Theobroma cacao* L.) and spinach (*Spinacia oleracea*) are being affected from cadmium (Cd) accumulation in their edible parts (beans and leaves, respectively), which in turn, has elicited public concern leading to regulations that restrict the commerce of contaminated products worldwide, particularly in the European Union (EU) and in the United States (US) (Argüello et al., 2019; Boeckx et al., 2018; Chavez et al., 2016a, 2016b; Engbersen et al., 2019; Huang et al., 2017).

There are various physical, chemical or biological techniques which are helpful to minimize Cd risk in the food chain (Hamid et al., 2019a). One of those techniques is called phytoextraction. Phytoextraction or hyperaccumulation may be used to describe plants with a strong uptake of a heavy metal (Ali et al., 2013; McGrath & Zhao, 2003; Robinson et al.2015; Sarwar et al., 2017), and for Cd phytoextraction, a plant species should be capable of accumulating more than 100 mg Cd kg^{-1} in shoots on a dry weight basis (Baker & Brooks, 1989; Baker et al., 2000; Manisha & Mohan 2017). In most plant species, the Cd concentration is less than 3 mg Cd kg^{-1} but may reach 20 mg kg^{-1} or more in Cd enriched soils (Ehsan et al., 2009). A plant concentration equal or higher than 100 mg kg^{-1} may be regarded as exceptional, even on a Cd-contaminated soil (Reeves & Baker, 2000). Another criterion used to classify a plant species as a heavy metal accumulator is the bioconcentration factor (Kötschau et al., 2014). The bioconcentration factor (BCF) is defined as the ratio of metal concentration in the aerial part of the plant to the total metal concentration in soil (Alloway et al., 1990; Li et al., 2017), and it is a function of the properties of the metal itself, the soil properties and the genotype of the plant (Yang et al., 2009). A bioconcentration factor ≥ 1 indicates a high accumulation of an element in the plant shoot (Kötschau et al., 2014). However, the BCF is not a constant value, as it may vary for even the same element in soils with different chemical properties (Boim et al., 2016; Swartjes et al., 2013).

The successful deployment of phytoextraction in competition with chemical or physical methods for cleansing trace elements from contaminated soil, requires this strategy to be less costly than the best alternative technology and importantly, less expensive or more viable than the cost of inaction (Robinson et al., 2003). Selection of a suitable Cd phytoextractor species should consider local availability, cost, agronomic requirements (temperature, humidity, soil properties, irrigation, fertilization), as well as physiological features of the plant (growth rate, rooting depth, photoperiod, heavy metal tolerance, pests and diseases, vigor,

evapotranspiration rates and possible symbiotic relationships with other organisms) (Mahar et al., 2016; Kumar et al., 2018; Zhao et al., 2003), biomass production (Robinson et al., 2015), exposure time (Rizwan et al., 2016), and ease of cultivation and harvesting (Ali et al., 2013).

During the last years, many authors have concluded that phytoextraction is a "cost effective" (Ali et al., 2013; Kumar et al., 2018; McGrath & Zhao, 2003; Rizwan et al., 2018; Robinson et al.2015; Song et al., 2012; Sarwar et al., 2017), or a "low-cost" (Brown et al., 1995; Jabeen et al., 2009; Mench et al., 2009) method to manage soils contaminated with heavy metals. However, few authors recognize two critical factors, first, that in developing countries, such as Colombia, most farmers are poor small-scale land holders (DANE, 2014), and second, the phytoextraction costs would be absorbed by the farmer (Thewys et al., 2010). Economic benefit or by-product generation for multi-purpose phytoextraction plant species would help offset phytoextraction costs. For example, non-edible plants can serve as energy crops (Licht & Isebrands, 2005; Pandey et al., 2016; Simmons et al., 2015; Witters et al., 2012a), as CO_2 abatement crops (Witters et al., 2012b), as timber (Liu et al., 2013; Srivastava, 2016), as fiber for paper production (Dickinson et al. 2009; Lebeau et al. 2008), to produce fragrances, or as an ornamental plant (Jelusic & Lestan, 2015; Liu et al. 2008; Nakbanpote et al., 2016). Sunflower has been reported as a suitable option for Cd phytoextraction (Rizwan et al., 2016; Kötschau et al., 2014), and could be considered a Cd phytoextractor with potential as an energy crop (or biofuel crop).

Although the cost of phytoremediation is lower than the conventional remediation technologies, it is time-consuming (Wei et al., 2017) and typically cannot satisfy the urgency of land development (Dermont et al., 2008). Innovative *in situ* technologies that require low inputs, and are low cost, are urgently required to meet the needs for soil remediation and community acceptance (Lombi et al., 2002). Therefore, phytoremediation operations could be supported with the use of *in situ* immobilization/stabilization remediation technologies to

achieve significant reductions of Cd concentrations in the plant-root zone, this is known by some authors as phytomanagement (Rizwan et al., 2016; Vamerali et al., 2014).

Several soil amendments can reduce Cd uptake and enhance immobilization by adsorption, complexation, and precipitation processes (Hamid et al., 2019a). Amendments including biochar (Hamid et al., 2019a; Mehmood et al., 2018; Puga et al., 2015; Woldetsadik et al., 2016), industrial by-products (Chavez et al., 2016b; Hamidpour et al., 2010; Kirkham 2006; Li et al., 2014; Lombi et al., 2002), liming materials (USEPA-OSRTI, 2007; Kim et al., 2016; Lee et al., 2004; Mondlane & Maret 2016; Radziemska et al., 2018; Tlustoš et al., 2006; Trakal et al., 2011; Vrînceanu et al., 2017), organic materials (Ok et al., 2011; Rolka 2015; Shan et al., 2016), Zn-based fertilizers (Li et al., 2014; Paul & Chaney, 2017), among others, have been examined for precipitating Cd, increasing adsorption of Cd, or providing competition with Cd uptake by roots (Paul & Chaney, 2017). It has been reported that inorganic amendments are more effective than organic amendments in reducing metal bioavailability and toxicity in plants (Hamid et al., 2019b). This might be due to the fact that inorganic amendments provide more binding sites for metal (Arunakumara et al., 2013).

Dolomitic lime has been used as a suitable Cd amendment in soils (Vondráčková et al., 2017), being recognized as a pH change-induced Cd immobilizer (Kim et al., 2016). The presence of hydroxyl (OH^-) ions induced by the addition of dolomite in soil causes deprotonation on the soil surface and an increase in the net negative charged surface area, thus increasing the adsorption of heavy metals (Grybos et al., 2009). A detailed review of soil amendments used to reduce Cd phytoavailability and transfer to food chain can be found in Hamid et al. (2019b).

This study aimed to elucidate changes in total Cd accumulation in aerial parts of cacao and spinach in an intercropping scheme with a sunflower acting as Cd phytoextractor and dolomitic lime as a Cd immobilization material.

Materials and Methods

Potting media content and preparation

A Paleudalf soil (Hagerstown, soil series) from the Pennsylvania State University Russell E. Larson Agricultural Research Center (40°42'43.0" N 77°56'17.0" W) was used in experiments 1 and 3 The Paleudalf soil was selected because has high iron oxides and clay content, which resembles some Colombian tropical soils.

A Dystrudept soil from the Universidad del Valle research farm (3°22'22.57"N 76°31'47.57"W) was used in experiment 2. Soil samples were excavated from the surface to a depth of 20 cm. The collected soil was air dried for ~5 days and then passed through a 5 mm sieve. Perlite grade 2 (GROW!T, USA) was mixed with soil for experiment 1 and 3 to improve the root growing environment in this high-clay content soil (Alaboudi et al., 2018).

The prepared soil matrix was one-part perlite per five parts of soil (weight/weight), respectively. The soil collected for experiment 2 was not mixed with perlite because it presented suitable conditions for plant growth. The pots of all three experiments were filled with 3 kg of the correspondent soil matrix.

Cadmium treatments consisted of different concentrations of Cd as $CdCl_2$ salt (Thermo Fisher Scientific, Geel-Belgium). Container capacity was used to determine the volume of distilled water required to saturate the 3 kg of growing material (Figure 4-1).

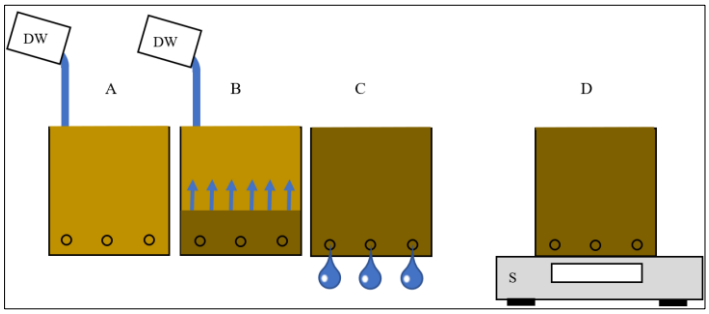

Figure **4-1**: Determination of container capacity. A. Distilled water (DW) was added to pots containing a soil matrix (soil, or soil and perlite); B. DW was added to saturate the soil matrix; C. Once the pots were fully saturated, they were left to drain excess water; D. Total weight was measured two times using scale (S): at one hour and at 24 hours after left to drain. With this information it was possible to determine the volume of water required to saturate the pots without having leaching.

The Cd solution was freshly prepared by dissolving $CdCl_2$ in deionized water as described in similar studies (Domínguez et al., 2011; Li et al, 2005; Nereida, 2011; Turgut et al., 2004). Cd solution was added to the soil matrices only one time. Distilled water was added to saturate Cd treated media. Polyethylene pots (2 gallons) were filled with the different study soil matrices, placed in a greenhouse in a larger polyethylene pot (2.5 gallons) with a 3 mm plastic layer in between, thus preventing leachate drainage (Figure 4-2).

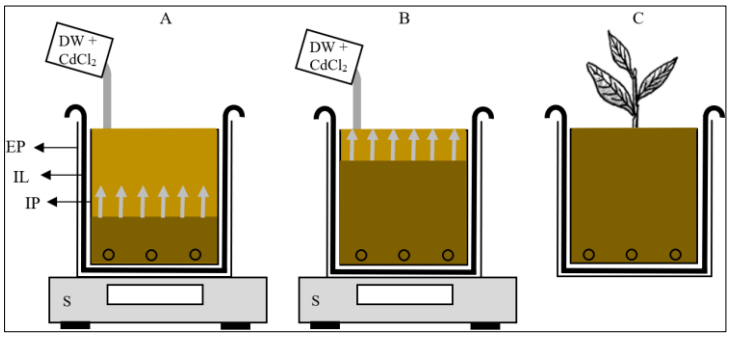

Figure **4-2**: One-time addition of $CdCl_2$ solution. A, B: The solution of distilled water (DW) and $CdCl_2$ was added to the correspondent pots containing a soil matrix (soil, or soil and perlite) until saturation using a scale (S). The soil matrices were incubated during 15 days under greenhouse conditions; C. plant material was transplanted to the soil matrices artificially contaminated with Cd. An internal pot (IP), an external pot (EP), and a plastic layer (3 mm thick) in between (IL) was used to avoid any leaching.

All experiments consisted of 0.0 and 5.0 mg Cd per kg of the corresponding soil matrix. The chosen Cd concentrations represent a range of soil Cd concentrations in cacao plantations across Latin American and Central American countries (Chavez et al., 2016; Gramlich et al., 2017; Huamaní et al., 2012; Ramtahal et al., 2016; Rodríguez, 2017; Tantalean & Huauya, 2017). All soil matrices were incubated for 15 days.

Dolomitic lime ($CaMg(CO_3)_2$) was added to the soil matrix and to the soil (5 g kg^{-1}, approximately 10 Ton ha^{-1}). The application rate was selected after reviewing the scientific literature regarding the use of dolomitic lime in different crops and its effect on soil pH. Ruiz (2011) found a significant difference in a Peruvian soil pH cultivated with cacao by an application of 1.5 Ton ha^{-1} dolomitic lime. Nazar (2010) found a significant difference in a Peruvian soil pH by a total application of 2.3 g of dolomitic lime per cacao plant (three months old) in a pot experiment. Vivanco (2016) grow cacao seeds in a five-months pot experiment to evaluate the effect of dolomitic lime, hydrated lime, and phosphate rock in plant height, foliar area, and dry matter. Vivanco (2016) used a 3.9 g dolomitic lime kg^{-1} dose in a Peruvian soil (equivalent to 7.9 Ton ha^{-1}) and found no significant difference in cacao plant height, foliar area, and dry matter in comparison to the results obtained with hydrated lime and phosphate rock in a five-months experiment; there was no report of soil pH results. Rosas-Patiño et al. (2017) used increasing doses of dolomitic lime (0, 1, 3, 5, 7, 9, and 11 Ton ha^{-1}) in a Colombian Typic Udorthent soil under field conditions with the aim of evaluating nutrient availability for cacao plants (seven years old). A 7 ton ha^{-1} dolomitic lime application resulted in a significant increase in soil pH within two months (4.4 to 6.0) and an increased nutrient availability (Ca, Mg, P and Zn) and a reduction on Al, Fe and Mn concentrations in the soil (Rosas-Patiño et al., 2017). Other authors have used different crops with dolomitic lime in different rates to evaluate its effect on plant nutrition and soil pH. Ullah et al. (2017) used 10 g per pot (each pot = 10 kg soil) in a polluted soil in Bangladesh

cultivated with rice. Vondráčková et al. (2017) used 21.6 and 68.1 g dolomitic lime kg^{-1} of two contrasting soils in Czech Republic to evaluate the growth of willows (*Salix* spp.) plants. Costa et al. (2016) used different dolomitic lime rates (0, 1, 2, and 4 Ton ha^{-1}) to evaluate soil acidity and soil fertility effects on soybean, and of black oat and sorghum in crop succession in a Brazilian clayey Oxisol under field conditions in a two-years experiment. Kovačević & Rastija (2010) used different dolomitic lime rates (0, 5, 10, and 15 Ton ha^{-1}) on a very acidic Croatian soil (pH_{KCl} = 3.78) in a five-year field experiment to evaluate the effect of liming on maize and spring barley grain yields and maize nutrient status.

We used 5 g of dolomitic lime, which was weighted (Ohaus Ranger 300, USA) and thoroughly mixed with the dry soil matrices in respective pots in order to ensure a homogeneous distribution. Dolomitic lime was selected due to its reported high Cd-adsorption capacity (Mohammadi et al., 2015), its local availability, its low economic cost, and its cultural acceptance by farmers. Each treatment, including control soil matrices (without dolomitic lime; without Cd), was carried out in six replicates in experiment 1 and 2, and in three replicates in experiment 3.

Following Hamidpour et al. (2010), Puga et al. (2015), Shan et al. (2016) and Yang et al. (2015), the water content of the pots was maintained at 65-70 % of maximum water holding capacity, which was determined by weighing the pots weekly and adding water only as required and avoiding anoxic conditions. Hoagland's solution was added weekly to provide plant nutrients (Epstein, 1972).

Each pot had 3 subsamples of soil matrix collected at different depths (1, 10, 30 cm) and combined. For experiments 1 and 3, soil matrix samples were oven dried for 5 days at 45 °C and softly chopped using a laboratory mortar and a pestle (agate) and then sieved to < 1 mm. After each sample chopping, the mortar and the pestle were washed twice with distilled water and dried with paper towels to avoid cross contamination. Soil samples in experiment 2

were oven dried for 3 days at 45 °C.

Soil analytical methods

Growing media pH was determined in distilled water extracts (1:1 weight vs. volume) through potentiometry (SB70P VWR, USA in experiment 1 and 3; Sartorius PT-10, Germany in experiment 2). In experiments 1 and 3, the cation exchange capacity (CEC) was determined using a 1 M CH_3COOH extraction method (Sumner & Miller, 1996). Soil organic matter (OM) was detected by the Walkley-Black method (Nelson & Sommers, 1982). Total P and K content were determined by inductively coupled plasma-atomic emission spectroscopy on samples prepared by microwave-assisted acid digestion, S was determined by US EPA 3050B method, Mn, Fe, Cu, B, Al, Zn, and Na were determined with saturation of CEC (Busenberg & Clemency, 1973). Particle size distribution was determined with the Pipette method (Miller & Miller, 1987). Cd concentration in growing media was determined by inductively coupled plasma-optical emission spectrometry following aqua regia digestion (Kim et al., 2016).

In experiment 2, CEC was determined using ammonium acetate1N pH 7 (Colombian Technical Standard 5268). OM detected by the Walkley-Black method. P was determined by Bray II modified (Colombian Technical Standard 5350). K, Mg, Ca were detected using ammonium acetate 1N pH 7 (Colombian Technical Standard 5349). Zn and Cu were determined by Mehlich I modified (Colombian Technical Standard 5526). S was detected by calcium phosphate (Agrilab S.A.S. internal method). Particle size distribution was determined by hydrometer (Gee & Bauder, 1986).

Plant material

Cacao and sunflower were cultivated in the soil matrix in experiment 1, and in soil in experiment 2. Spinach and sunflower were cultivated in the soil matrix in experiment 3. The varieties used are presented in Table 4-1.

Table **4-1**: Species of the selected plants.

Experiment 1	Company, Location
Cacao (*Theobroma cacao* (L.) cv ICS-95) [a]	USDA, Mayagüez, Puerto Rico
Sunflower (*Helianthus annuus* cv Mammoth Gray Stripe)	David's Garden Seeds, USA
Experiment 2	
Cacao (*Theobroma cacao* (L.) cv IMC-67) [b]	Las Cosechas Nursery, Colombia
Sunflower (*Helianthus annuus* cv Domino)	Las Cosechas Nursery, Colombia
Experiment 3	
Spinach (*Spinacia oleracea* cv Bloomsdale Heirloom)	Sweet Yards Seeds Co., USA
Sunflower (*Helianthus annuus* cv Mammoth Gray Stripe)	David's Garden Seeds, USA

[a] Cacao plantlings were 6-weeks old when transplanted into the soil matrix.
[b] Cacao plantlings were 4-monts old when transplanted to soil.; Growth period for all experiments: 9 weeks.

Experiments 1 and 2 had six replicates per treatment. Experiment 3 had three replicates per treatment. Replicates of the 6 cacao plants within experiments 1 and 2 were combined in pairs to ensure enough plant aerial tissue (PAT) for Cd extraction (Figure 4-3), leaving a replicate of three. The same was done for spinach plants (Figure 4-3). Each sunflower provided enough PAT for Cd extraction; thus, the six sunflower replicates in the corresponding treatment were processed separately for experiments 1 and 3. In experiment 2, the three sunflower replicates were processed separately for Cd extraction.

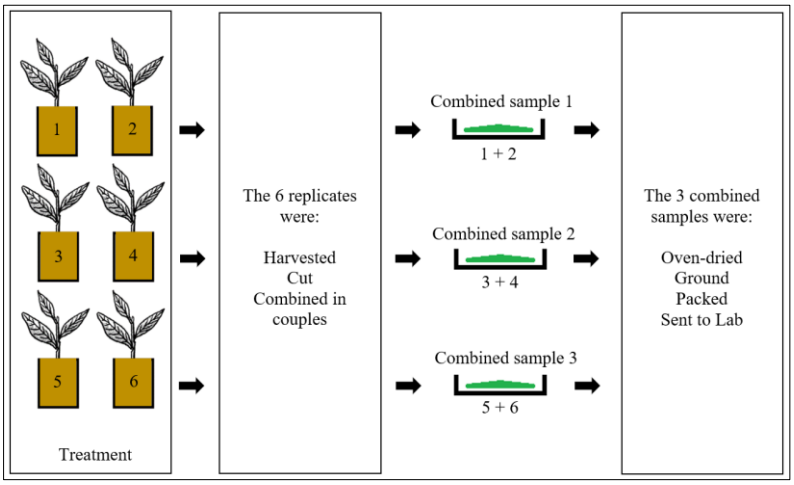

Figure **4-3**: Combined samples description.

For the three experiments, sunflower was sown from seed for two weeks. In experiment 3, spinach was sown from seed for two weeks. After sowing, uniform-sized plants were selected to grow in each pot (10 cm height for sunflower, 5 cm height for spinach). In experiment 1, cacao was ~6-weeks old and 20 cm height when transplanted. In experiment 2, cacao was ~4-months old and 35 cm height when transplanted. After plants were transplanted, and following 9 weeks of growth, plant aerial tissue (or above ground biomass) were cut to the soil matrices' surface, harvested (leaves, stem, flower), cleaned with distilled water and cut into 5 cm size pieces. For experiments 1 and 3, harvested plant material was oven dried for 5 days at 45 °C and then ground using a laboratory mill and 1 mm sieve (Thomas Wiley Mill No. 4, USA). Cacao, sunflower and soil matrix samples from experiments 1 and 3 were sent to the Agricultural Analytical Laboratory at Penn State (USA). Samples in experiment 2 of fresh, aerial cacao and sunflower parts, and dry soil samples, were sent to the Agrilab Laboratory S.A.S. (Bogotá, Colombia). A summary of the experiments is presented in table 3-3.

Table **4-2**: Summary of experiments.

Experiment	Plants evaluated	Cd source	Cd concentrations	Growing media	Location
1	Cacao ICS-95 Sunflower	$CdCl_2$	0.0 mg Cd kg^{-1} 5.0 mg Cd kg^{-1}	Soil and perlite Proportion 5:1 w/w (soil matrix) 3 kg/pot	Penn State (USA)
2	Cacao IMC-67 Sunflower	$CdCl_2$	0.0 mg Cd kg^{-1} 5.0 mg Cd kg^{-1}	Soil 3 kg/pot	Universidad del Valle (Colombia)
3	Spinach Sunflower	$CdCl_2$	0.0 mg Cd kg^{-1} 5.0 mg Cd kg^{-1}	Soil and perlite Proportion 5:1 w/w (soil matrix) 3 kg/pot	Penn State (USA)

Total Cd extraction methods for experiments 1 and 3

Total Cd, P, K, Ca, Mg, S, Mn, Fe, Cu, B, Al, Zn, and Na in plant aerial tissue was digested via the US EPA method 3050B (US EPA, 1996) with the US EPA method 6010 (US EPA 2014). Digestion of Cd in the soil matrices followed the US EPA method 3050B with the US EPA method 6010. US EPA method 3050B uses HNO_3, H_2O_2, and heat to digest samples. US EPA method 6010 is a spectrometric technique used to determine trace elements in aqueous solutions, prior to analysis, aqueous and solid samples are solubilized or digested using an acid digestion procedure. The analysis is made through inductively coupled plasma atomic emission spectrometry (ICP-AES) or inductively coupled plasma-optical emission spectrometry (ICP-OES). The detection limits of 3050B range between 0.0001 to 0.5 mg Cd kg^{-1} (Da Silva et al., 2014; Enamorado-Báez et al., 2013; Nham, 2006).

Total Cd extraction method for experiment 2

Total Cd and N, P, K, Ca, Mg, S, Fe, Mn, Cu, Zn, B, and Na in plant aerial tissue was digested via the US EPA method 200.9 (US EPA, 1994). Digestion of Cd in the soil followed the US EPA method 3051A (US EPA, 2007) with the US EPA method 200.9 (US

EPA, 1994). The plant aerial tissue was digested via the method US EPA 3051. US EPA method 3051A is a microwave extraction method designed to mimic extraction using conventional heating with HNO_3 or alternatively HNO_3 and HCl (US EPA, 2007). Since this method is not intended to accomplish total decomposition of the sample, the extracted analyte concentrations may not reflect the total content in the sample (US EPA, 2007). US EPA method 200.9 provides procedures for the determination of dissolved and total recoverable elements by graphite furnace atomic absorption in water (ground water, industrial, domestic), sediments, sludge, and soil (US EPA, 1994). For total recoverable analysis of a solid or an aqueous sample containing undissolved material, analytes are first solubilized by refluxing with HNO_3 and HCl (US EPA, 1994). The analysis is made through inductively coupled plasma atomic emission spectrometry (ICP-AES) or inductively coupled plasma-optical emission spectrometry (ICP-OES) (US EPA, 1994). The detection limits of 3051A range between 0.0001 to 0.5 mg Cd kg^{-1} (Da Silva et al., 2014; Enamorado-Báez et al., 2013; Nham, 2006).

Bioconcentration factor

The *bioconcentration factor*, defined as the ratio of metal concentration in the aerial part of the plant to the total metal concentration in soil (Alloway et al., 1990), was used to evaluate the transfer potential of Cd from the soil matrix (experiments 1 and 3) and soil (experiment 2) to the corresponding plant species. A bioconcentration factor ≥ 1 indicates a high accumulation of an element in the plant shoot (Baker et al., 1981; Kötschau et al., 2014).

Experimental design

A completely randomized design was established. The experiments followed a three-way analysis of variance (ANOVA). Therefore, the dependent variable was "total Cd in plant aerial tissue (experiments 1 and 2: cacao; experiment 3: spinach)" measured in mg Cd kg^{-1}, and the three independent variables were "added Cd (0.0 or 5.0 mg Cd kg^{-1})", "sunflower (with or without)", and "dolomitic lime (with or without)". In particular, the study was designed to determine if the total Cd in plant aerial tissue (cacao or spinach) differed based on the presence of a sunflower in an intercropping scheme (sunflower with cacao; sunflower with spinach), the addition of dolomitic lime to the soil matrix, and the addition of Cd into the soil matrix.

The following abbreviations were used to describe treatments: Sf (sunflower), C (cacao), L (dolomitic lime), and S (spinach).

Statistical analysis

Graphs and tables were constructed using Microsoft Excel 365 (Microsoft Corp., 2019). Descriptive statistic was calculated using Minitab, version 17.3.1 software package (Minitab Inc., 2016). P, K, Ca, Mg, S, Mn, Fe, Cu, B, Al, Zn, Cd and Na levels in plant tissue were evaluated using the Anderson-Darling test for normality and Breusch-Pagan test for equality of variances. Differences in total Cd concentrations for plant aerial tissue of cacao, sunflower, spinach, and soil matrices measured throughout the experiments were compared between plant treatment groups (Cd contaminated treatments and the control; added lime and the control) with a three-way ANOVA (analysis of variance) test at an alpha of 0.1. Tukey's post hoc test was used for the multiple comparisons of means between treatments at an alpha

of 0.1. Multifactorial linear regression (MLR) analysis was applied to experiments 1 and 3 to determine if total Cd was related to other plant aerial tissue elements via a backward elimination method. Linear regressions were used to evaluate the relationship between total Cd detected in plant aerial tissue and the soil matrix (experiments 1 and 3) and soil (experiment 2). Data are presented as the mean ± standard deviation (SD) unless stated otherwise.

Results

Experiment 1. Accumulation of cadmium (Cd) in cacao and sunflower cultivated in an artificially Cd-contaminated soil matrix

Plant species used in experiment 1 were cacao (C) and sunflower (Sf). A soil matrix (Table 4-3) was created from a mixture of a Paleudalf soil from State College, USA and grade 2 perlite (5:1 proportion) (perlite was added to improve root development).

Cacao results

A three-way ANOVA was run on a sample of 24 cacao plants (C) sown in a soil matrix to examine the effect of Added Cd (0.0 or 5.0 mg Cd kg^{-1}), Sf (with or without), and L (with or without) on total Cd in PAT of C, total Cd in soil matrix, BCF of C, and soil matrix pH (Table 4-4). Simple main effects analysis indicates that Added Cd had a significant effect on total Cd in PAT of C ($p < 0.001$) and total Cd in soil matrix ($p < 0.001$). Simple main effects analysis indicates that Sf had a significant effect on total Cd in PAT of C ($p = 0.034$) and soil matrix pH ($p = 0.003$). Simple main effects analysis indicates that L had a significant effect on soil matrix pH ($p < 0.001$). A significant interaction was found between Added Cd and Sf on total Cd in PAT of

C ($F(1, 16) = 5.55$, $p = 0.032$) and BCF ($F(1, 16) = 5.13$, $p = 0.038$). A significant interaction was found between Sf and L on soil matrix pH ($F(1, 16) = 6.34$, $p = 0.023$).

A Tukey post hoc analysis for C is summarized in Table 4-5. In the Added Cd treatment, a significant difference was found in Total Cd in PAT of C and on Total Cd in soil matrix, having in both cases a higher concentration of Cd in the 5.0 mg Cd kg^{-1} level in comparison to the control level (0.0 mg Cd kg^{-1}). In the Sf treatment, a significant difference was found in Total Cd in PAT of C and in soil matrix pH. Interestingly, cacao grown with Sf in an intercropping scheme reported the highest total Cd in PAT of C in comparison to cacao grown without Sf. Cacao grown with Sf had a less acidic pH in comparison to cacao grown without Sf. In the L treatment, a significant difference was found in soil matrix pH, with a less acidic soil pH with L. Growing cacao without Sf and L resulted in the most acidic soil matrix pH (Table 4-5).

Total Cd in PAT of C was positively, and significantly related to the soil matrix total Cd with 83 % of the variability in cacao total Cd accounted for by the soil matrix total Cd (Table 4-6). A significant positive Pearson correlation was found between total Cd in PAT of C and the total Cd in soil matrix. No significant correlation was found between total Cd in PAT of C and soil matrix pH (Table 4-7). Total sulfur in PAT of C (mg kg^{-1}) was the best predictor of total Cd in PAT of C (backward elimination method), however, the relationship was weak ($R^2 = 0.19$) (Table 4-8).

Sunflower results

A three-way ANOVA was run on a sample of 36 sunflower plants (Sf) sown in a soil matrix to examine the effect of Added Cd (0.0 or 5.0 mg Cd kg^{-1}), C (with or without), and L (with or without) on total Cd in PAT of Sf (Table 4-9). Simple main effects analysis indicates that Added Cd had a significant effect on total Cd in PAT of Sf ($p<0.001$); C had a

significant effect on total Cd in PAT of Sf ($p=0.015$); and L had a significant effect on total Cd in PAT of Sf ($p<0.001$) (Table 4-9). The Tukey post hoc analysis for Sf is summarized in Table 4-10. In the added Cd treatment, a significant difference was found in total Cd in PAT of Sf, having a higher accumulated Cd in PAT of Sf in the 5.0 mg Cd kg^{-1} level. In the C treatment, a significant difference was found in total Cd in PAT of Sf between the With C and the Without C levels, having a higher accumulated Cd in PAT of Sf in the Without C level. In the L treatment, a significant difference was found on total Cd in PAT of Sf between the With L and the Without L levels, having a higher accumulated Cd in PAT of Sf in the Without L level.

Total Cd in PAT of Sf was positively and significantly related to soil matrix total Cd, with 69 % of the variability in sunflower total Cd explained by the soil matrix total Cd (Table 4-11). A significant positive Pearson correlation was found between total Cd in PAT of Sf and the total Cd in soil matrix. A significant negative Pearson correlation was found between total Cd in PAT of Sf and soil matrix pH (Table 4-12). Backward elimination found that total Fe in PAT of Sf (mg kg^{-1}) and total Mn in PAT of Sf (mg kg^{-1}) were the best predictors of total Cd in PAT of Sf ($R^2 = 0.63$) (Table 4-13).

Table **4-3**: Properties of the Paleudalf soil in experiment 1.

Parameter	Units	Value
pH		6.4
P	mg kg^{-1}	71
K	mg kg^{-1}	294
Mg	mg kg^{-1}	115
Ca	mg kg^{-1}	1470
CEC	meq/100 g	11.1
Zn	mg kg^{-1}	3.4
Cu	mg kg^{-1}	4.4
S	mg kg^{-1}	14.3
OM	%	2.16
Sand	%	16.91
Silt	%	54.15
Clay	%	28.94
Soil textural class		Silty Clay Loam

Table **4-4**: Analysis of variance of total Cd (mg kg^{-1}) in PAT of cacao (C), total Cd (mg kg^{-1}) in soil matrix, BCF of C, and soil matrix pH in experiment 1.

Effect	df	Total Cd in PAT of C	Total Cd in soil matrix	BCF	Soil matrix pH
		----------p-value----------			
Added Cd	1	<0.001	<0.001	0.273	0.870
Sf	1	0.034	0.675	0.322	0.003
L	1	0.633	0.893	0.918	<0.001
b	1	0.032	0.710	0.038	0.736
Sf * L	1	0.180	0.181	0.683	0.023

PAT: Plant aerial tissue; df: degrees of freedom; BCF: Bioconcentration factor.

Table **4-5**: Tukey post hoc analysis for the means of total Cd (mg kg^{-1}) in PAT of cacao (C), total Cd (mg kg^{-1}) in soil matrix, and soil matrix pH in experiment 1.

Factor	Total Cd in PAT of C	Total Cd in soil matrix	Soil matrix pH
Added Cd			
0.0	1.2 ± 0.4 a	0.30 ± 0.04 a	
5.0	21.0 ± 8.0 b	4.8 ± 1.5 b	
Sf			
With Sf	13.3 ± 14.0 a		6.2 ± 0.5 a
Without Sf	8.9 ± 8.5 b		5.7 ± 0.8 b
L			
With L			6.5 ± 0.4 a
Without L			5.5 ± 0.6 b
Sf * L			
With Sf * Without L			5.9 ± 0.4 b
With Sf * With L			6.6 ± 0.4 a
Without Sf * With L			6.4 ± 0.3 ab
Without Sf * Without L			5.0 ± 0.3 c

Values are presented as mean ± standard deviation (SD); means followed by the same letter within columns are not significantly different and data not reported are not significantly different (alpha = 0.1). Added Cd in mg kg^{-1} soil matrix; PAT: Plant aerial tissue.

Table **4-6**: Predictive relationship between total Cd (mg kg^{-1}) in PAT of cacao (C) and total Cd (mg kg^{-1}) in soil matrix in experiment 1.

Model	R^2	Max. total Cd in soil matrix	Max. total Cd in PAT of C
Total Cd C = 0.02 + 4.8 Total Cd in soil matrix	0.83	6.80	38.75

n = 3; alpha = 0.1. PAT: Plant aerial tissue.

Table **4-7**: Correlations among soil matrix pH, total Cd (mg kg^{-1}) in soil matrix, and total Cd (mg kg^{-1}) in PAT of cacao (C) in experiment 1.

	Soil matrix pH	Total Cd in soil matrix
Total Cd C	0.08 (0.70)	0.91 (<0.001)

Data presented as Pearson correlation (p-value); n = 3. PAT: Plant aerial tissue.

Table **4-8**: MLR for total Cd (mg kg^{-1}) in PAT of cacao (C) in experiment 1.

Model	R^2	R^2 (Adj)	E (C) *
Total Cd C = -39.6 + 212.7 S	0.19	0.15	S (0.19)

* Element and in parenthesis the contribution to the MLR (n = 3; alpha = 0.1). S (sulfur); PAT: Plant aerial tissue.

Table **4-9**: Analysis of variance of total Cd (mg kg^{-1}) in PAT of sunflower (Sf) in experiment 1.

Effect	df	Total Cd in PAT of Sf
		---------p-value---------
Added Cd	1	<0.001
C	1	0.015
L	1	<0.001
Added Cd * C	1	0.015
Added Cd * L	1	<0.001

PAT: Plant aerial tissue; df: degrees of freedom.

Table **4-10**: Tukey post hoc analysis for the means of total Cd (mg kg^{-1}) in PAT of sunflower (Sf) in experiment 1.

Factor	Total Cd in PAT of Sf
Added Cd	
0.0	0.5 ± 0.002 a
5.0	7.7 ± 2.5 b
C	
With C	3.7 ± 3.8 a
Without C	4.6 ± 3.0 b
L	
With L	2.8 ± 2.6 a
Without L	5.4 ± 4.7 b

Values are presented as mean ± SD; means followed by the same letter within columns are not significantly different and data not reported are not significantly different (alpha = 0.1). Added Cd in mg kg^{-1} soil matrix; PAT: Plant aerial tissue.

Table **4-11**: Predictive relationship between total Cd (mg kg^{-1}) in PAT of sunflower (Sf) and total Cd (mg kg^{-1}) in soil matrix in experiment 1.

Model	R^2	Max. total Cd in soil matrix	Max. total Cd in PAT of Sf
Total Cd Sf = 0.4 + 1.1 Total Cd soil matrix	0.69	8.04	10.68

n = 6; alpha = 0.1. PAT: Plant aerial tissue.

Table **4-12**: Correlations among soil matrix pH, total Cd (mg kg^{-1}) in soil matrix, and total Cd (mg kg^{-1}) in PAT of sunflower (Sf) in experiment 1.

	Soil matrix pH	Total Cd in soil matrix
Total Cd Sf	-0.68 (0.02)	0.90 (<0.001)

Data presented as Pearson correlation (p-value); n = 6. PAT: Plant aerial tissue.

Table **4-13**: MLR for total Cd (mg kg^{-1}) in PAT of sunflower (Sf) in experiment 1.

Model	R^2	R^2 (Adj)	E (C) *
Total Cd Sf = 11.6 - 0.2 Fe + 0.01 Mn	0.65	0.63	Fe (0.35); Mn (0.30)

* Element and in parenthesis the contribution to the MLR; n = 6; alpha = 0.1. PAT: Plant aerial tissue.

Experiment 2. Accumulation of Cd in cacao and sunflower cultivated in an artificially Cd-contaminated soil

Plant species used in experiment 2 were cacao (C) and sunflower (Sf). The Dystrudept soil from Cali, Colombia (Table 4-14) used in this experiment was not mixed with perlite due to the lower clay content than the Paleudalf used in Experiment 1.

Cacao results

A three-way ANOVA was run on a sample of 24 cacao plants (C) sown in soil to examine the effect of Added Cd (0.0 or 5.0 mg Cd kg^{-1}), Sf (with or without), and L (with or without) on total Cd in PAT of C, total Cd in soil, BCF of C, and soil pH (Table 4-16). Simple main effects analysis indicates that Added Cd had a significant effect on total Cd in soil ($p < 0.001$) and BCF of C ($p < 0.001$) (Table 4-15). Simple main effects analysis indicates that Sf had a significant effect on total Cd in PAT of C ($p = 0.077$); L had a significant effect on total Cd in PAT of C ($p = 0.005$), BCF of C ($p = 0.007$), and soil pH ($p < 0.001$). A significant interaction was found between Added Cd, Sf, and L on total Cd in PAT of C ($F (1, 16) = 3.64$, $p = 0.075$). A significant interaction was found between Sf and L on total Cd in PAT of C ($F (1, 16) = 4.5$, $p = 0.05$). A significant interaction was found between Added Cd and L on BCF of C ($F (1, 16) = 6.96$, $p = 0.018$).

The Tukey post hoc analysis for C is summarized in Table 4-16. In the added Cd treatment, Total Cd in soil was significantly greater at the 5.0 mg kg^{-1} level than the control and BCF significantly lower. The Total Cd in PAT of C was significantly greater when grown with Sf than without. The Total Cd in PAT of C was significantly lower when grown with a lime addition, the BCF was significantly lower and the soil pH significantly higher. Growing cacao with Sf but no L produced significantly higher Total Cd in the PAT of C compared to other

levels. The 5.0 mg kg^{-1} Cd addition to cacao grown with Sf, but not with L, resulted in significantly greater total Cd in PAT of C.

A poor predictive relationship was determined between total Cd in PAT of C and total Cd in soil (R^2=0.03) (Table 4-17). A negative Pearson correlation was found between total Cd in PAT of C and soil pH. No significant correlation was found between cacao total Cd and soil total Cd (Table 4-18).

Sunflower results

A three-way ANOVA was run on a sample of 18 sunflower plants (Sf) sown in soil to examine the effect of Added Cd (0.0 or 5.0 mg Cd kg^{-1}), C (with or without), and L (with or without) on total Cd in PAT of Sf (Table 4-19). Simple main effects analysis indicates that Added Cd had a significant effect on Total Cd in PAT of Sf ($p < 0.001$); C had a significant effect on Total Cd in PAT of Sf ($p = 0.003$); and L had a significant effect on Total Cd in PAT of Sf ($p = 0.007$). A significant interaction was found between Added Cd and C on Total Cd in PAT of Sf ($F (1, 12) = 10.6$, $p = 0.007$). A significant interaction was found between Added Cd and L on Total Cd in PAT of Sf ($F (1, 12) = 16.14$, $p = 0.002$) (Table 4-19).

The Tukey post hoc analysis for Sf is summarized in Table 4-20. In the Added Cd treatment, Sf accumulated significantly more Cd in the 5.0 versus 0.0 mg kg^{-1} level. When grown with C, Sf accumulated significantly more Total Cd in PAT. When grown with a lime addition, Sf accumulated significantly less Total Cd in PAT. Growing Sf with C and, at the 5.0 mg kg^{-1} level, resulted in significantly more Total Cd in PAT than growing Sf without C.

Total Cd in PAT of Sf was positively and significantly related to soil total Cd, with 61% of the variability in sunflower total Cd explained by the soil total Cd (Table 4-21). A significant positive Pearson correlation was found between total Cd in PAT of Sf and the total

Cd in soil. A significant negative Pearson correlation was found between total Cd in PAT of Sf and soil matrix pH (Table 4-22).

Table **4-14**: Properties of the Dystrudept soil in experiment 2.

Properties	Units	Value
pH		5.4
P	mg kg^{-1}	110
K	mg kg^{-1}	194
Mg	mg kg^{-1}	805
Ca	mg kg^{-1}	510
CEC	meq/100 g	9.9
Zn	mg kg^{-1}	10.8
Cu	mg kg^{-1}	9.5
S	mg kg^{-1}	77
OM	%	6.29
Sand	%	32
Silt	%	28
Clay	%	40
Soil textural class		Clay

Table **4-15**: Analysis of variance of total Cd (mg kg^{-1}) in PAT of cacao (C), total Cd (mg kg^{-1}) in soil, BCF of C, and soil pH in experiment 2.

Effect	df	Total Cd in PAT of C	Total Cd in soil	BCF	Soil pH
		----------p-value----------			
Added Cd	1	0.139	<0.001	<0.001	0.116
Sf	1	0.077	0.108	0.515	0.152
L	1	0.005	0.138	0.007	<0.001
Added Cd * Sf	1	0.410	0.118	0.518	0.310
Added Cd * L	1	0.123	0.162	0.018	0.323
Sf * L	1	0.050	0.809	0.231	0.246
Added Cd * Sf * L	1	0.075	0.750	0.316	0.304

PAT: Plant aerial tissue; df: degrees of freedom; BCF: Bioconcentration factor.

Table **4-16**: Tukey post hoc analysis for the means of total Cd (mg kg^{-1}) in PAT of cacao (C), total Cd (mg kg^{-1}) in soil, BCF of C, and soil pH in experiment 2.

Factor	Total Cd in PAT of C	Total Cd in soil	BCF	Soil pH
Added Cd				
0.0		0.3 ± 0.04 a	5.6 ± 1.7 a	
5.0		7.3 ± 1.9 b	0.3 ± 0.1 b	
Sf				
With Sf	1.8 ± 0.8 a			
Without Sf	1.4 ± 0.4 b			
L				
With L	1.3 ± 0.3 a		2.3 ± 2.3 b	5.3 ± 0.4 a
Without L	1.9 ± 0.8 b		3.5 ± 3.5 a	4.5 ± 0.1 b
Added Cd * L				
5.0 * With L			0.2 ± 0.1 a	
5.0 * Without L			0.3 ± 0.1 a	
0.0 * With L			4.5 ± 0.6 b	
0.0 * Without L			6.7 ± 1.8 c	
Sf * L				
With Sf * Without L	2.3 ± 0.9 a			
With Sf * With L	1.3 ± 0.3 b			
Without Sf * With L	1.3 ± 0.4 b			
Without Sf * Without L	1.6 ± 0.3 ab			

Values are presented as mean ± standard deviation (SD); means followed by the same letter within columns are not significantly different and data not reported are not significantly different (alpha = 0.1). Added Cd in mg kg^{-1} soil; PAT: Plant aerial tissue; BCF: Bioconcentration factor.

Table **4-17**: Predictive relationship between total Cd (mg kg^{-1}) in PAT of cacao (C) and total Cd (mg kg^{-1}) in soil in experiment 2.

Model	R^2	Max. total Cd in soil	Max. total Cd in PAT of C
Total Cd C = 1.52 + 0.03 Total Cd in soil	0.03	10.4	3.65

n = 3; alpha = 0.1. PAT: Plant aerial tissue.

Table **4-18**: Correlations among soil pH, total Cd in soil, and total Cd in PAT of cacao (C) in experiment 2.

	Soil pH	Total Cd in soil
Total Cd C	-0.48 (0.02)	0.17 (0.42)

Data presented as Pearson correlation (p-value); n = 3. PAT: Plant aerial tissue.

Table **4-19**: Analysis of variance of total Cd (mg kg^{-1}) in PAT of sunflower (Sf) in experiment 2.

Effect	df	Total Cd in PAT of Sf
		---------p-value---------
Added Cd	1	<0.001
C	1	0.003
L	1	0.007
Added Cd * C	1	0.007
Added Cd * L	1	0.002

PAT: Plant aerial tissue; df: degrees of freedom.

Table **4-20**: Tukey post hoc analysis for the means of total Cd (mg kg^{-1}) in PAT of sunflower (Sf) in experiment 2.

Factor	Total Cd in PAT of Sf
Added Cd	
0.0	0.9 ± 0.5 a
5.0	9.1 ± 4.2 b
C	
With C	6.2 ± 5.8 a
Without C	3.8 ± 2.0 b
L	
With L	4.0 ± 3.5 a
Without L	6.1 ±7.1 b

Values are presented as mean ± SD; means followed by the same letter within columns are not significantly different (alpha = 0.1). Added Cd in mg kg^{-1} soil; PAT: Plant aerial tissue.

Table **4-21**: Predictive relationship between total Cd (mg kg^{-1}) in PAT of sunflower (Sf) and total Cd (mg kg^{-1}) in soil in experiment 2.

Model	R^2	Max. total Cd in soil	Max. total Cd in PAT of Sf
Total Cd Sf = 1.03 + 0.99 Total Cd soil	0.61	10.4	15.20

n = 3; alpha = 0.1. PAT: Plant aerial tissue.

Table **4-22**: Correlations among soil pH, total Cd (mg kg^{-1}) in soil, and total Cd (mg kg^{-1}) in PAT of sunflower (Sf) in experiment 2.

	Soil pH	Total Cd in soil
Total Cd Sf	-0.54 (0.02)	0.78 (<0.001)

Data presented as Pearson correlation (p-value); n = 3. PAT: Plant aerial tissue.

Experiment 3. Accumulation of Cd in spinach and sunflower cultivated in an artificially Cd-contaminated soil matrix

The plant species used in experiment 3 were spinach (S) and sunflower (Sf) and the same Paleudalf soil matrix from State College, USA used in experiment 1 was used (Table 4-3).

Spinach results

A three-way ANOVA was run on a sample of 24 spinach plants (S) sown in a soil matrix to examine the effect of Added Cd (0.0 or 5.0 mg Cd kg^{-1}), Sf (with or without), and L (with or

without) on total Cd in PAT of S, total Cd in soil matrix, BCF of S, and soil matrix pH (Table 4-23). Simple main effects analysis indicates that Added Cd had a significant effect on total Cd in PAT of S ($p < 0.001$), in the soil matrix ($p<0.001$), and BCF ($p < 0.001$) (Table 4-23). Significant interactions were found between the effects of Added Cd and Sf on soil matrix pH ($F (1, 16) = 8.17$, $p = 0.011$); between Added Cd and L on total Cd in soil matrix ($F (1, 16) = 6.03$, $p = 0.026$); between Sf and L on soil matrix pH ($F (1, 16) = 6.92$, $p = 0.018$); and Added Cd, Sf, and L on soil matrix pH ($F (1, 16) = 10.65$, $p = 0.005$).

A Tukey post hoc analysis for S is summarized in Table 4-24. Adding 5.0 mg kg^{-1} Cd to soil that S was grown in versus 0, resulted in a significantly more Total Cd in PAT of S, significantly more total Cd in soil matrix, and a significantly lower BCF (<1). Growing S with Sf resulted in a significantly greater soil matrix pH. Growing S with L resulted in significantly more Total Cd in the soil matrix and a significantly higher soil matrix pH. Spinach grown with Sf at the 5.0 mg kg^{-1} level resulted in significantly higher soil matrix pH than at the 0.0. mg kg^{-1} level. Growing S with Sf and L resulted in significantly higher soil matrix pH.

Total Cd in PAT of S was positively, and significantly related to total Cd in soil matrix, and 65 % of the variability of total Cd in PAT of S can be explained by the total Cd in soil matrix (Table 4-25). A significant positive Pearson correlation was found between total Cd in PAT of S and total Cd in soil matrix. No significant correlations were found between total Cd in PAT of S and soil matrix pH (Table 4-26). Total Al in PAT of S (mg kg^{-1}) and total K in PAT of S (%) were the best predictor of total Cd in PAT of S ($R^2 = 0.64$) (Table 4-27).

Sunflower results

Simple main effects analysis indicates that Added Cd had a significant effect on total Cd in PAT of Sf ($p < 0.001$) (Table 4-28). The Tukey post hoc analysis for Sf is summarized in Table 4-30. In the Added Cd treatment, a significant difference was found on total Cd in PAT of Sf between the 0.0 and the 5.0 mg Cd kg^{-1} levels, with more accumulated Cd in the 5.0 mg Cd kg^{-1} level (Table 4-29). Total Cd in PAT of Sf was positively, and significantly related to total Cd in soil matrix and model results indicate that 83 % of the Sf total Cd variability can be explained by the total Cd in soil matrix (Table 4-30). A significant positive Pearson correlation was found between total Cd in PAT of Sf and the total Cd of soil matrix. No significant Pearson correlation was found between total Cd in PAT of Sf and soil matrix pH (Table 4-31). Total sulfur in PAT of Sf (mg kg^{-1}) and total K in PAT of S (%) were the best predictors of total Cd in PAT of Sf, however, results were weak ($R^2 = 0.32$) (Table 4-32).

Table **4-23**: Analysis of variance of total Cd (mg kg^{-1}) in PAT of spinach (S), total Cd (mg kg^{-1}) in soil matrix, BCF of S, and soil matrix pH in experiment 3.

Effect	df	Total Cd in PAT of S	Total Cd in soil matrix	BCF	Soil matrix pH
		----------p-value----------			
Added Cd	1	<0.001	<0.001	<0.001	0.155
Sf	1	0.556	0.154	0.931	0.001
L	1	0.543	0.025	0.763	<0.001
Added Cd * Sf	1	0.570	0.147	0.199	0.011
Added Cd * L	1	0.547	0.026	0.784	0.536
Sf * L	1	0.561	0.188	0.932	0.018
Added Cd * Sf * L	1	0.614	0.168	0.133	0.005

PAT: Plant aerial tissue; df: degrees of freedom; BCF: Bioconcentration factor.

Table **4-24**: Tukey post hoc analysis for the means of total Cd (mg kg^{-1}) in PAT of spinach (S), total Cd (mg kg^{-1}) in soil matrix, BCF of S, and soil pH in experiment 3.

Factor	Total Cd in PAT of S	Total Cd in soil matrix	BCF	Soil matrix pH
Added Cd				
0.0	1.5 ± 0.1 a	0.3 ± 0.04 a	5.0 ± 0.6 a	
5.0	5.9 ± 2.0 b	8.1 ± 2.4 b	0.8 ± 0.4 b	
Sf				
With Sf				5.4 ± 0.4 a
Without Sf				5.1 ± 0.3 b
L				
With L		4.9 ± 5.0 a		5.5 ± 0.4 a
Without L		3.5 ± 3.6 b		5.1 ± 0.1 b
Sf * L				
With Sf * Without L				5.1 ± 0.1 a
With Sf * With L				5.7 ± 0.2 b
Without Sf * With L				5.3 ± 0.3 a
Without Sf * Without L				5.0 ± 0.1 a

Values are presented as mean ± SD; means followed by the same letter within columns are not significantly different and data not reported are not significantly different (alpha = 0.1). Added Cd in mg kg^{-1} soil matrix; PAT: Plant aerial tissue; BCF: Bioconcentration factor.

Table **4-25**: Predictive relationship between total Cd (mg kg^{-1}) in PAT of spinach (S) and total Cd (mg kg^{-1}) in soil matrix in experiment 3.

Model	R^2	Max. total Cd in soil matrix	Max. total Cd in PAT of S
Total Cd S = 1.6 + 0.5 Total Cd soil matrix	0.65	10.96	9.69

n = 3; alpha = 0.1. PAT: Plant aerial tissue.

Table **4-26**: Correlations among soil matrix pH, total Cd (mg kg^{-1}) in soil matrix, and total Cd (mg kg^{-1}) in PAT of spinach (S) in experiment 3.

	Soil matrix pH	Total Cd soil matrix
Total Cd S	0.10 (0.65)	0.80 (<0.001)

Data presented as Pearson correlation (p-value); n = 3. PAT: Plant aerial tissue.

Table **4-27**: MLR for total Cd (mg kg^{-1}) in PAT of spinach (S) in experiment 3.

Model	R^2	R^2 (Adj)	E (C) *
Total Cd S = -2.6 - 0.1 K + 0.1 Al	0.64	0.61	Al (0.40); K (0.24)

* Element and in parenthesis the contribution to the MLR (n = 3; alpha = 0.1). PAT: Plant aerial tissue.

Table **4-28**: Analysis of variance of total Cd (mg kg^{-1}) in PAT of sunflower (Sf) in experiment 3.

Effect	df	Total Cd in PAT of Sf
		----------p-value----------
Added Cd	1	<0.001
S	1	0.649
L	1	0.858

PAT: Plant aerial tissue; df: degrees of freedom.

Table **4-29**: Predictive relationship between total Cd (mg kg^{-1}) in PAT of sunflower (Sf) and total Cd (mg kg^{-1}) in soil matrix in experiment 3.

Model	R^2	Max. total Cd in soil matrix	Max. total Cd in PAT of Sf
Total Cd Sf = 0.7 + 0.7 Cd soil matrix	0.83	13.08	9.96

n = 6; alpha = 0.1. PAT: Plant aerial tissue.

Table **4-30**: Correlations among soil matrix pH, total Cd (mg kg^{-1}) in soil matrix, and total Cd (mg kg^{-1}) in PAT of sunflower (Sf) in experiment 3.

	Soil matrix pH	Total Cd in soil matrix
Total Cd Sf	0.03 (0.90)	0.91 (<0.001)

Data presented as Pearson correlation (p-value); n = 6. PAT: Plant aerial tissue.

Table **4-31**: MLR for total Cd (mg kg^{-1}) in PAT of sunflower (Sf) in experiment 3.

Model	R^2	R^2 (Adj)	E (C) *
Total Cd Sf = -17.9 + 3.3 K + 0.01 Fe	0.32	0.23	K (0.12); S (0.20)

* Element and in parenthesis the contribution to the MLR; n = 6; alpha = 0.1. PAT: Plant aerial tissue.

Discussion

Uptake of Cd by cacao, sunflower, and spinach occurred in all experiments and was greatest in experiment 1 for cacao, in experiment 2 for sunflower and in experiment 3 for spinach. Cadmium uptake was not only reflected in plant total Cd but also in the amount of Cd left in soils at the end of the experiment; experiment 1 had the lowest total Cd in the soil matrix and experiment 3 the highest in the 5.0 mg Cd kg^{-1} treatment. The availability of Cd for uptake should theoretically be greater in experiment 2 soils given the Dystrudept soil pH is lower. The concentration of Cd in cacao (variety ICS-95) and sunflower in experiment 1, sunflower in experiment 2, spinach and sunflower in experiment 3 was significantly and positively related to the total soil Cd concentration.

As evident by the cacao, spinach versus sunflower total Cd and bioconcentration results, and the soil matrix total Cd results at the end of the experiment, sunflower is an effective phytoextractor. However, when sunflower was grown with cacao, sunflower appears to promote Cd uptake in cacao (experiment 1 results were significant but experiment

2 results were not significant) but when grown with spinach decreases Cd levels in spinach. The effect of this interaction on cacao in the Colombian Dystrudept soil was to double uptake and in the USA Paleudalf soil was to increase uptake 1.6 times. Further research should investigate this result.

The effect of lime additions to soils to increase soil pH was successful, but the effect on lessening Cd uptake presented mixed results depending on the species. Lime had no significant effect on reducing spinach, sunflower or cacao Cd uptake alone except in experiments 1 and 2 with sunflower. Adding lime to a soil also appears to be less effective at reducing plant uptake of Cd when two crops are grown together (see experiment 2 results). The effect of lime, and a phytoextractor like sunflower, on reducing Cd uptake by another species, appears to be soil dependent too. For example, in experiments 1 and 2, growing cacao and sunflower together significantly lowered cacao Cd uptake but adding lime to cacao and sunflower when grown together did not significantly lower Cd uptake in cacao; a similar result was found for spinach. Bioconcentration factor results also suggest lime reduces the effectiveness of sunflower as a phytoextractor. In experiments 1 and 2, the addition of lime reduces the bioconcentration factor of sunflower, when grown with another species, to less than 1 when grown with cacao, and near to 1 in experiment 2.

The increased sorption of Cd to soils at elevated pH values should theoretically reduce the solution Cd concentration and thus decrease Cd phytoavailability (Liang et al., 2013). An increase of soil matrix pH corresponded to a decrease in cacao total Cd, thus affecting the distribution of Cd between the soil solid and solution phase (Eriksson, 1990, Naidu et al., 1994); metal uptake was increased in the soil matrix with the increase in pH (Mohammadi et al., 2015; Hamid et al., 2018). Vondráčková et al. (2013) investigated the effect of quick lime (lime) and dolomitic lime application on the immobilization of Cd, Zn, Pb, As, Fe, and Mn in weakly acidic and alkaline soils in Czech Republic and found that

dolomitic application in an alkaline soil did not produce a significant effect on plant-available Cd concentrations, but it did decrease plant-available Cd in an acidic soil.

Note that in Chapter 3 a total Cd level was found in sunflower plant aerial tissue of 20.8 mg Cd kg^{-1} in a treatment with Sf alone (with added Cd). Comparing this chapter's experiment 1 results with the previous chapter 3 results suggests that cacao removed more than a half of the total Cd that Sf would have absorbed if planted alone.

Engbersen et al. (2019) concluded that total Cd accumulation in cacao is a function of the cultivar. Thus, not all cacao varieties may act as a phytoextractor. Cacao varieties present genetic variation in bioaccumulation and partitioning of Cd (Lewis et al., 2018). The IMC-67 cacao variety (experiment 2) has been classified as a low Cd accumulator (Lewis et al., 2018). Zug et al. (2019) reported differences in Cd accumulation rates in Peruvian farms with CCN-51 (lower Cd concentrations in leaves and beans) in comparison to Peruvian farms with a combination of CCN-51 and ICS-95 (experiment 1) (higher Cd concentrations in leaves and beans). The cacao varieties used in this research exhibited different bioconcentration factors for similar Cd additions. For example, the bioconcentration factor of cacao in experiment 1 was greater than 1 but in experiment 2 was less. Interestingly, sunflower total Cd in experiments 1 and 2, while belonged to different varieties (Mammoth Gray Stripe in experiment 1 and 3, and Domino in experiment 2), had plant total Cd levels in a similar range. It appears that the sunflower varieties used in this study presented consistent plant total Cd results, even when different Cd extraction methods were used.

A possible source of variation in this study could be due to the way cacao plants were grown. In experiment 1, ICS-95 seeds from three pods were donated by USDA and they were sown from seed under a controlled environment, but it is not possible to affirm that the pods came from the same tree, this could signify that the genetic material is not identical, in comparison to IMC-67 plants bought in a specialized cacao nursery in Colombia, in this case,

all plants were clones with identical genetic characteristics. It is possible that ICS-95 cacao in experiment 1 was a more efficient Cd phytoextractor than IMC-67 cacao plants.

The relationship between total Cd in plant aerial tissue of cacao and cacao elemental composition revealed that Cd concentration in cacao tissue was weakly predicted with its sulfur content ($R^2 = 0.19$). The relationship between total Cd plant aerial tissue in spinach and spinach elemental composition revealed that Cd concentration in spinach tissue was predicted with its aluminum and potassium content ($R^2 = 0.64$). The relationship between total Cd in plant aerial tissue of sunflower and sunflower elemental composition revealed that Cd concentration in sunflower tissue was predicted weakly with its sulfur and potassium content ($R^2 = 0.32$).

Total Cd in plant aerial tissue of spinach was positively and significantly related to soil matrix total Cd which agrees with results of other studies (Liang et al., 2013). No significant correlation was found between total Cd in plant aerial tissue of spinach and soil matrix pH. The bioconcentration factor of spinach was not significantly different between treatments with and without added Cd, only S treatment had a bioconcentration factor > 1. Total Cd in plant aerial tissue of sunflower and total Cd in soil matrix had a positive and significant correlation and also a significant predictive relationship. Total Cd in plant aerial tissue of sunflower and soil matrix pH had no significant correlation.

In experiments 1 and 2, the presence of Cd did not cause visual toxicity symptoms on cacao or in sunflower leaves, such as necrosis or chlorosis, nor in experiment 3 for spinach and sunflower. During the nine-week growing period, cacao plants were largely free of insect, aphid, fungus or other observable pest/disease incidents.

Conclusions

We investigated the effectiveness of the combined use of Cd phytoextraction (via sunflower) and Cd immobilization via liming on cacao and spinach total Cd accumulation in aerial parts. An increase in soil matrices pH due to the application of dolomitic lime corresponded to an increase in soil matrices total Cd. As pH increased, cacao total Cd was reduced (variety IMC-67). The addition of dolomitic lime has been recommended to reduce cacao total Cd. However, the growing of cacao and sunflower resulted in the highest cacao total Cd, thus the use of sunflower to manage soil Cd is not recommended. Results indicate that the use of sunflower and/or dolomitic lime with spinach was not effective in reducing total Cd in spinach. This study was the first to evaluate total Cd accumulation in an intercropping scheme with dolomitic lime application. Further research, however, is necessary for a comprehensive understanding of the dynamic relationship between Cd in agricultural soils and Cd accumulation in cacao and spinach tissue, and in particular with new Cd adsorbents/immobilization materials in sub-surface soil layers and the time needed for the amendments to modify plant total Cd.

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Chapter 5

Summary and future research

In this study, the phytoextraction ability of four plant species under greenhouse conditions was evaluated. Sunflower significantly accumulated cadmium (Cd) in its aerial parts in comparison to vetiver, and heliconia, and showed more tolerance to Cd toxicity. Although oilseed rape total Cd was high, this plant was severely attacked by insect pests and diseases during the duration of the experiment, reducing evaluation of its suitability. Sunflower was observed to be more resistant to insect and fungus attack. Considering the plant total Cd accumulated in aerial parts and the resistance to insects and diseases, sunflower is concluded to be a suitable Cd phytoextractor.

Intercropping results (sunflower and cacao; sunflower and spinach) with dolomitic lime application suggest that lime additions to soil are not as effective at reducing Cd uptake in cacao and spinach as is growing either species with a phytoextractor. The use of plants for Cd-phytoextraction purposes has been widely described. While a variety of plant species have been reported as suitable Cd phytoextractor species, plant total Cd uptake was the most important criterion in this study. Current knowledge of Cd phytoextraction, while understood, remains perhaps less practical; we suggest research focus on phytoextractor selection criteria covering cost-efficiency, cultural acceptance, logistic practicality, multi-purpose plant ability, and the availability of local phytoextraction species.

Recommendations for future research include:

Determination of Cd phytoextractor species optimal days of growth to reach a maximum total soil and total plant Cd extraction. Although time spans are very long, the use of successive Cd phytoextraction phases in low to moderately Cd contaminated areas for

growing multipurpose Cd phytoextractor plant species (for instance, bioenergy crops), still seems a promising approach (Kötschau et al., 2014).

Evaluation of Cd uptake by plants under conditions of contamination by multiple metals, such as in real fields. Various metal stresses alone and in combination are required to identify the mechanisms of tolerance in plants under single as well as combined metal stresses (Rizwan et al., 2016).

Evaluation of new cacao and spinach varieties to Cd accumulation. The uptake and translocation of Cd may depend on soil conditions and crop management factors. Thus, studies focused on breeding and selecting appropriate genotypes to mitigate Cd accumulation in cacao and spinach should integrate a climatic and geochemical approach. Results from Argüello et al. (2019) and Engbersen et al. (2019) show that selection of cultivars with low Cd transfer from vegetative parts into the beans has high potential to keep Cd accumulation in cacao beans at levels that are safe for consumption.

Assessment of non-Cd-hyperaccumulator plants as energy crops in intercropping schemes. Plant species used in phytoextraction have extended from hyperaccumulators to non-hyperaccumulator plants due to showing significantly higher biomass productivity and a more rapid growth rate than hyperaccumulating plants (Zhang et al., 2014). Although ambitious, studies comparing different crop management schemes and inputs are required to determine the optimal combination of practices to reduce soil total Cd. The distribution and content of trace elements, such as Cd, in agricultural soils may be altered by cultivation practices including applications of fertilizers and micronutrients, irrigation, pesticide uses, organic waste amendments, and re-incorporation of crop residues (Chang & Page, 2000).

Combination of organic and inorganic soil amendments in the reduction of Cd in cacao and spinach. The combination of organic and inorganic soil amendments such as lime material, phosphate, zeolite, bentonite, clay, Fe and Mn oxides, and organic matter could

promote sorption, ion exchange, and precipitation to convert soluble and preexisting potentially soluble solid phase forms of heavy metals to more geochemically stable solid phases, reducing the heavy metal pool for root uptake in soil (Cruz-Paredes et al., 2017; Hamid et al., 2019).

Determination of optimal time of Cd sorption and its duration by different soil amendments. Immobilized Cd ions may become again mobile and plant-available with time, therefore, the desorption and plant-availability of adsorbed Cd ions on soil amendments need to be investigated (Hamidpour et al., 2010).

Evaluation of different liming application rates in different soil profiles and different exposition times. A future study must be designed with higher dolomitic lime application rates so as to detect a maximal possible decrease of plant-available Cd concentrations after dolomitic lime application as well as to evaluate its dynamic in different soil profiles in different time periods. The major challenges of using lime are to achieve liming effect in sub-surface soil layers and the time needed for the amendments to modify soil pH (Argüello et al., 2019).

Development of models that could predict Cd in soils and its later uptake by cacao and spinach. Methods that are able to simulate plant accumulation could provide a quantitative understanding of the underlying mechanisms both in soil and plant tissue (Zhu et al., 2018). Chen et al. (2009) developed a predictive model assessing the effect of long-term crop cultivation on distribution of Cd in the root zone. Non-linear methods, such as artificial neural networks, might be used to model the dynamic of heavy metals in soils (Anagu et al., 2009; Hattab et al., 2013), in plant tissue (Hattab & Hambli, 2014), and in the heavy metal-soil-plant interaction (Hou et al., 2018).

Development of a soil properties correction factor for the bioconcentration factor (BCF). The BCF is a function of soil minerology and plant species, therefore, it should reflect

this edaphoclimatic variability in order to reduce uncertainties in the derivation of risk concentrations in soils through a “soil properties correction factor” (Boim et al., 2016; Swartjes et al., 2013).

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Appendix

Plant elemental composition in experiment 3, chapter 3

Treatment	N [a]	P [b]	K [c]	Ca [c]	Mg [c]	S [d]	Fe [c]	Mn [c]	Cu [c]	Zn [c]	B [e]	Na [c]
	%						mg kg^{-1}					
H (Control)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
H (3.0 mg kg^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
H (5.0 mg kg^{-1})	2.5	0.18	4.55	0.96	0.385	0.18	486	383	49	25	23	203
V (Control)	2.1	0.14	1.92	0.42	0.225	0.14	2140	295	28	124	25	186
V (3.0 mg kg^{-1})	1.6	0.16	2.25	0.34	0.155	0.14	1510	213	12	36	23	144
V (5.0 mg kg^{-1})	1.9	0.15	1.96	0.52	0.23	0.12	7890	285	24	88	102	287
Sf (Control)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sf (3.0 mg kg^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sf (5.0 mg kg^{-1})	3.3	0.48	3.649	2.03	0.567	0.27	344	668	28	67	56	455

n.d.: not detected; [a] Micro-Kjeldahl (NTC 5167); [b] nitric and perchloric acid (NTC 234); [c] nitric and perchloric acid; [d] nitric and perchloric acid (NTC 1154); [e] nitric and perchloric acid (NTC 1860); NTC: Colombian Technical Standard.

Due to the loss of plant aerial tissue from experiment 3at Agrilab S.A.S, the control and the 3.0 mg kg^{-1} Cd treatments have missing values for H and Sf.