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## Tracing cadmium in cacao : from soil to bean

Hester Blommaert

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**Traçage du cadmium dans le cacao : du sol à la fève**

**Tracing cadmium in cacao: from soil to bean**

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# **TRACING CADMIUM IN CACAO: FROM SOIL TO BEAN**

Hester BLOMMAERT

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G. Sarret

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June 2023



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## Summary

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The consumption of chocolate products contributes significantly to the human cadmium (Cd) intake. Worldwide regulations have sparked initiatives to reduce Cd in cacao beans. The main mitigation strategies proposed are i) amendments to reduce soil Cd bioavailability, ii) cultivar selection and grafting, and iii) treatments during bean fermentation. The development of these strategies is hampered by the lack of knowledge about the biochemical processes that control the Cd migration from soil to bean, and the fate of Cd during fermentation. This work was set-up to increase the understanding of the pathways and chemical forms (speciation) of Cd in its journey from soil to beans in trees and during the fermentation.

First, we studied the pathways of Cd in cacao trees. Cacao is a large cauliflorous tree with fruits developing on the trunk, the loading of Cd in the beans may thus be atypical. We compared a high and a low Cd accumulating cultivar and a grafted cultivar, all from a field with similar soil bioavailable Cd. The grafted had the same scion as the low, but was propagated on another rootstock. We combined Cd stable isotope analyses at natural abundance with speciation techniques and elucidated distribution and storage forms of Cd by imaging techniques in micro to nano-scale. The total tree uptake of Cd was factor 2.9 (high) and 1.7 (grafted) higher than in the low cultivar. The Cd distribution within the tree was similar among cultivars. Thus, the main difference was at the entry of Cd in the plant. The total elemental uptake suggested similar uptake mechanisms for manganese (Mn) and Cd. Within the trees, Cd was separated from Mn along the transpiration pathway from roots to leaves, and mostly retained in the branches (57% of total tree Cd). The incorporation of Cd in Ca-oxalate crystals (roots and branches) together with binding to S-ligands (roots) were the major mechanisms of Cd retention observed in the high Cd cultivar. The weight of evidence indicated that the Cd pathway from branches to nibs was phloem mediated and differed from that from branches to the mature leaves.

The phloem mobility of Cd was subsequently analyzed in an experiment with cacao seedlings. A split-root experiment was set up with a stable isotope tracer ( $^{108}\text{Cd}$ ). Roots of cacao seedlings were split into two compartments, either enriched in tracer  $^{108}\text{Cd}$  (+) or not (-). The isotopic abundance of  $^{108}\text{Cd}$  readily increased in the shoot compartments but also increased in the root of the compartment without tracer from 11 days after labelling, which evidenced that Cd was phloem mobile and redirected to developing tissues. However, the dominant Cd transport pathway was the transpiration pathway (xylem) from root to leaves.

Cacao beans are fermented prior to further processing into chocolate products. We identified changes in Cd speciation in beans during fermentation. We used samples collected from farms where Cd concentrations were high or low. In unfermented beans, Cd speciation was dominated by sulfur (S) and oxygen (O)-ligands in the high and low Cd beans respectively. After incubation at  $>45^{\circ}\text{C}$  and in acidic conditions ( $\text{pH} < 5$ ), nib Cd concentration decreased up to a factor 1.5, and Cd was bound to O ligands in both beans. A combination of pH changes and/or phytate breakdown may explain the enhanced Cd mobility during fermentation and the migration of Cd from the nib to the outer solution.

This work confirmed that grafting and cultivar selection may mitigate Cd uptake in the nibs on the medium to long term. Soil amendments reducing soil Cd bio-availability may be ineffective in the first years after application, because of the large reservoir of Cd in the trees. On the short term, the optimization of fermentation processes is a more viable mitigation strategy.

## Sommaire

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La consommation de produits chocolatés est une source significative d'absorption de cadmium (Cd) chez les humains. Des réglementations mondiales ont entraîné des initiatives visant à réduire la teneur en cadmium des fèves de cacao. Les principales stratégies proposées sont i) l'amendement des sols visant à réduire la biodisponibilité du Cd, ii) la sélection des cultivars et la greffe, et iii) les traitements pendant la fermentation des fèves. Le manque de connaissances sur les processus impliqués entrave le développement de ces stratégies. Ce travail vise à améliorer la compréhension des voies et des formes chimiques (spéciation) du Cd lors de son transfert du sol aux fèves, et pendant la fermentation.

Tout d'abord, cette étude s'est intéressée aux voies du Cd dans les cacaoyers. Des cultivars à forte et faible accumulation de Cd et un cultivar greffé (faible accumulateur greffé sur un porte greffe différent) ont été comparés. L'analyse des concentrations et des rapports isotopiques du Cd ont été combinées avec des techniques de spéciation et d'imagerie micro et nanométrique pour élucider la distribution et les formes de stockage du Cd. L'absorption totale de Cd était 2,9 et 1,7 fois plus élevée chez le fort accumulateur par rapport au faible et au greffé, respectivement. La principale différence se situe à l'entrée du Cd dans la plante car les distributions entre organes sont similaires entre cultivars. Les données suggèrent des mécanismes d'absorption similaires pour le manganèse (Mn) et le Cd. Par contre, le Cd absorbé se distribue différemment du Mn dans la plante, et est principalement retenu dans les branches (57% du Cd total de l'arbre). L'incorporation du Cd dans les cristaux d'oxalate de Ca (racines et branches) ainsi que la liaison aux ligands S (racines) sont les principaux mécanismes de rétention observés dans le cultivar à forte accumulation. Dans tous les cultivars, les données suggèrent que le Cd est transféré dans les fèves via le phloème.

Ensuite, la mobilité du Cd dans le phloème a été analysée en hydroponie avec un traceur isotopique stable ( $^{108}\text{Cd}$ ). Les racines de plantules de cacao ont été divisées en deux compartiments, enrichis en traceur  $^{108}\text{Cd}$  ou non enrichis. L'abondance isotopique du  $^{108}\text{Cd}$  augmente rapidement dans les parties aériennes, mais aussi dans la racine du compartiment sans traceur après 11 jours. Le Cd est remobilisé dans les tissus en développement via le phloème. Cependant, la fraction remobilisée est faible par rapport au flux lié à la transpiration (xylème) des racines aux feuilles.

Enfin, nous avons identifié des changements de spéciation du Cd dans les fèves au cours de la fermentation. Nous avons étudié des fèves faiblement et fortement concentrées en Cd. Dans les fèves non fermentées, la spéciation du Cd était dominée par les ligands soufrés (S) et oxygénés (O)

dans les fèves à forte et faible concentration, respectivement. Après incubation à plus de 45°C et dans des conditions acides (pH <5), la concentration de Cd dans les fèves était divisée par 1,5, et le Cd était lié à des ligands O dans les deux fèves. Une combinaison de changements de pH et/ou de décomposition du phytate peut expliquer la mobilité accrue du Cd pendant la fermentation et la migration du Cd de la fève vers la solution.

Ce travail a confirmé que la greffe et la sélection des cultivars sont des stratégies d'atténuation prometteuses à moyen et à long terme. Les amendements du sol risquent d'être inefficaces les premières années suivant l'application, en raison de l'important réservoir de Cd dans les arbres qui est la source de Cd dans les fèves. À court terme, l'optimisation des processus de fermentation est une stratégie d'atténuation plus viable.

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## Context and objectives

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Food safety concerns us all. Among the challenges towards 'safe food', is the control or reduction of contaminants in food systems. One of the common contaminants is cadmium (Cd). For over 70 years, this element has raised concerns to public health after a first discovery of the *itai-itai* disease, a bone disease in subsistence farmers who had eaten Cd contaminated rice. Food safety organizations have stressed for over decades that efforts should be undertaken to reduce the dietary Cd intake to lower risks. As a consequence, since 1972, regulations have been established worldwide on maximal dietary Cd intake and later on maximally permitted levels for Cd in foodstuffs. Regulations were first made for staple crops that largely contributed to Cd exposure, i.e. rice, potatoes and wheat. More recently, cacao products were added to that list.

Chocolate and chocolate products are estimated to be a minor but significant contributor of Cd in the human diet and were consequently targeted by regulations in Europe, Australia, New Zealand, Russia, USA, and countries within the Southern Common Market. These regulations have sparked initiatives to reduce Cd in cacao beans, which are the basis for chocolate production. The main mitigation strategies proposed are the following:

- Short-term agronomic approaches, such as the application of soil amendments to reduce plant-availability of soil Cd, are generally advocated as the most promising mitigation method. However, to date there are no consistent results that prove the reduction of bean Cd concentrations by application of soil amendments.
- The selection of low Cd accumulating cultivars has proven its value in other crops. Scientific advances have led to the release of rice cultivars that accumulate less Cd. In the field, low and high Cd accumulating cultivars have been observed. A low Cd cacao innovation is nonetheless a distant dream for the moment as the knowledge on the mechanisms of Cd accumulation in cacao trees is in its infancy. Cacao is a large, perennial tree that displays cauliflorous flowering. Thus, the plant likely exerts different accumulation mechanisms than the widely studied staple crops like rice. Understanding the processes that control the allocation of Cd into cacao beans is a prerequisite before the creation of low Cd-cultivars.
- Post-harvest mitigation strategies, such as the fermentation or washing of cacao beans may be more applicable. Research has already reported promising results. Yet, the mechanisms behind the transport of Cd out of the beans into the fermentation/washing solutions remain unclear. This knowledge gap hinders the development of a general approach that can be readily implemented in each farm.

Briefly, for all mitigation strategies, more knowledge regarding the biochemical processes that control the Cd migration from soil to plant, bean and fermented beans are necessary. For the agronomic measures, we need to identify where bean Cd is originating from; either from direct root uptake, or from the internal stock in the plant. For the postharvest strategies, we need to identify the storage form of Cd in the bean initially, to reveal how fermentation and washing processes can be optimized to ensure an efficient migration of Cd outward of the beans. For the creation of low Cd cultivars, we need to understand how high and low Cd accumulating cultivars differ in terms of Cd accumulation.

This work was set-up to increase the understanding of the pathways and chemical forms (speciation) of Cd in intact cacao trees on its journey between soil and beans. A multi-analyses approach, including field and laboratory studies, was used to address the following specific questions:

- What are the main storage compartments in the cacao tree, and in which chemical form does Cd accumulate in each compartment?
- What are the Cd pathways in cacao plants and do they differ between a low and a high Cd accumulating cultivar?
- How does Cd speciation (chemical form) in beans change during fermentation?

To answer these research questions the following objectives were set:

- To dissect potential mechanisms that control the accumulation and partitioning of Cd in a high and a low Cd accumulator genotype of cacao grown in field conditions at environmentally relevant soil Cd concentrations (Chapter 3 and 4)
- To assess the effect of a different rootstock on the Cd uptake and transfer to aerial tissues (Chapter 4).
- To identify circulation of Cd in cacao seedlings, and to what extent Cd is mobile in the phloem of cacao (Chapter 5).
- To reveal the storage forms of Cd in cacao beans before and after incubation to enhance the understanding of mechanisms behind the mobilization of Cd in cacao beans during fermentation (Chapter 6).

The first chapter will take the reader briefly through the current knowledge regarding cadmium and cacao separately. The properties of Cd, as well as its toxic nature and the regulations to reduce human Cd consumption are reviewed. Thereafter, generalities about the crop cacao in an agricultural context are summarized. Finally, we end the chapter with a discussion about the current research available concerning Cd transfer pathways in plants. Chapter 2 is a detailed

review of the journey of Cd from soil to chocolate bar with due attention to possible mitigation strategies and their potential. Chapter 3 is the first experimental work in this thesis dissertation and elucidates the pathway of Cd in a high Cd accumulating cultivar together with its storage forms. In chapter 4, we extend this knowledge by comparison of these Cd pathways with the Cd dynamics in a low Cd accumulating cultivar. Furthermore, we also aimed to study the effect of a different rootstock on these Cd pathways within mature trees. In a next step, we wanted to confirm the observations in mature trees by short-term Cd stable isotope labelling experiments which assess the Cd circulation within cacao seedlings (Chapter 5). Chapter 6 assessed storage forms of Cd in cacao beans before and after fermentation. Finally, in Chapter 7, we wrap up our findings and reflect upon their implications for breeders, agronomists, and the cacao-industry, and propose some perspectives for future research.

## Chapter 1: Introduction

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## 1.1 Cadmium

Cadmium (Cd) is a potentially toxic metal. The element occurs naturally in rocks, soils and waters, due to volcanic input or weathering of Cd-containing parent material (Smolders and Mertens, 2013). The World Health Organization (WHO) was the first institution to set a threshold on the human intake for Cd in 1972 (Joint FAO/WHO Expert Committee on Food Additives, 1972). Ever after, the toxic nature of this element has raised concerns to the public and has resulted in numerous research and further policies to reduce Cd consumption by humans.

### 1.1.1 Chemical properties of Cd

Cadmium (atomic number 48) is located in group 12 and period 5 in the Periodic System of the Chemical Elements between zinc (Zn) and mercury (Hg). The most abundant speciation of Cd in the environment is  $\text{Cd}^{2+}$ . The divalent cation has a completely filled d-shell ( $d^{10}$ ) as electronic configuration, which results in a flexible coordination and an absence of redox chemistry. According to the HSAB (Hard-soft-Lewis acid-base) principle, Cd is a soft Lewis acid (Welch and Norvell, 1999). This indicates that  $\text{Cd}^{2+}$  has an electron cloud that is polarizable and an intermediate ionic radius of 0.95 Å (in six fold coordination in crystals (Shannon, 1976)). Cadmium can hence participate in covalent binding with surfaces and form inner-sphere complexes with high selectivity. The metal has a similar ionic radius as the calcium ion ( $\text{Ca}^{2+}$ , 1.00 Å (six fold coordination)), but  $\text{Cd}^{2+}$  has a higher affinity for sulfur ligands than  $\text{Ca}^{2+}$ , which is a hard cation with noble gas electron configuration. As a consequence,  $\text{Cd}^{2+}$  forms less stable complexes with oxygen and nitrogen ligands than  $\text{Ca}^{2+}$ . Therefore, the biochemistry of Cd involves strong complexation with proteins and biomolecules like glutathione, which have sulfur (S) ligands (Sigel *et al.*, 2013). Divalent zinc ( $\text{Zn}^{2+}$ ) (group 4) is classically seen as the nutrient analogue of Cd and belongs to the same group ( $d^{10}$  electronic configuration), yet  $\text{Zn}^{2+}$  has a smaller ionic radius (0.74 Å (six fold coordination) than Cd (Shannon, 1976). The manganese ion  $\text{Mn}^{2+}$  is a  $d^5$  element of period 4 and has an ionic radius of 0.83 Å. Manganese is thus more similar to  $\text{Cd}^{2+}$  compared to Zn in terms of ionic radius. This element will appear throughout this work as an element that follows the fate of Cd generally better than Zn.

### 1.1.2 Toxicology of Cd

Cadmium interferes with numerous transporters, signalling molecules and metalloproteins. One of the hypotheses for the toxicity is that Cd interferes with the cellular Zn homeostasis. Indeed, the log K (equilibrium complexation constant) values of metal cysteine (9.2 for  $\text{Zn}^{2+}$  and 11.0 for  $\text{Cd}^{2+}$ ) indicate that Cd can replace Zn in enzymes with S ligands (Sigel *et al.*, 2013). Especially when

Zn deficiency occurs, this mechanism will come to expression and potentially block critical biological pathways (EFSA, 2009).

### 1.1.2.1 Human toxicology

Cadmium is classified as carcinogenic to humans (Group 1) (International Agency for Research on Cancer (IARC)) (IARC, 2012) and is associated with increased risk of cancer in the bladder, lung, pancreas, endometrium, and breast. Other health effects that may be caused by Cd are: renal dysfunction, osteoporosis, diabetic nephropathy, hypertension, periodontal disease, age-related macular degeneration, and cardiovascular diseases (Agency for Toxic Substances and Disease Registry, 2012). The toxicity of Cd is more of a chronic than an acute nature as the doses for acute Cd intoxication rarely occur (Clemens *et al.*, 2013; Chaney, 2015). Consequently, the lifetime exposure is the best indicator to estimate the effects of Cd (Smolders and Mertens, 2013).

The biological half-life of Cd in the human body is 20 to 30 years, where it accumulates in the organs (particularly in the kidneys). The kidney in humans is the main target organ where Cd causes cell dysfunction (Sigel *et al.*, 2013). However, also the liver, respiratory system, and bones can be affected. One of the rare examples of a fatal Cd intoxication is the *itai-itai* disease. The symptoms are severe spinal and leg pains (Aoshima, 2016). The *itai-itai* disease manifested in residents of the Toyama Prefecture in Japan around 1950. Here, residents consumed rice that was irrigated with Cd contaminated water from a Zn mining area (Nordberg, 2009). It is now recognized that the occurrence of the *itai-itai* disease was due to a rice-based diet high in Cd and deficient in other nutrients (e.g. Fe and Zn), which are essential to reduce the bioavailability of Cd to humans (Chaney, 2015). For non-smoking humans, the main source of Cd is originating from foodstuffs. Food items with relatively higher concentrations of Cd are shellfish, offal, grains, and seeds (EFSA, 2009). The intake of Cd is generally higher in less-developed countries where rice is a staple food (Song *et al.*, 2017).

### 1.1.2.2 Environmental toxicology

The concentrations of Cd which cause toxicity on soil dwelling biota are commonly higher than the concentrations affecting humans. Soil biota and plants can thus have Cd concentrations that are already a risk for mammals whilst showing no physical disorders (Smolders and Mertens, 2013). Hence, the greatest ecotoxicological risk of Cd exists in the accumulation of Cd in the food chain. Cadmium accumulation has been reported in grasses, food crops, earthworms, poultry, cattle, horses, and wildlife (EFSA, 2009). There are only scarce studies of Cd toxicity for mammals and birds. A study on kidney Cd concentrations suggested a health risk for moles, shrews and

beavers in contaminated sites (IARC, 2012). In a study of Cd in the food web and tissues of white-tailed ptarmigan in Colorado, they measured biomagnification of Cd in the food chain, mostly through Cd accumulation in willow and consumption of this plant by herbivores like ptarmigan. This led the authors to the conclusion that Cd toxicity may be more common among natural populations and that Cd toxicity in the environment is often undetected or unrecognized (Larison *et al.*, 2000).

### 1.1.3 Regulating the human intake of Cd

The WHO was first to set a threshold on the intake of Cd in 1972 (tolerable daily intake (TDI) of 1  $\mu\text{g Cd kg}^{-1}$  body weight) (Joint FAO/WHO Expert Committee on Food Additives, 1972). In Europe, the European Food Safety Agency (EFSA) suggested in 2009 a TDI of 0.36  $\mu\text{g Cd kg}^{-1}$  b.w (EFSA, 2009). For a person weighing 60 kg, this is a tolerable intake of 21.6  $\mu\text{g day}^{-1}$  which is close to the average dietary intake for an adult (8-20  $\mu\text{g day}^{-1}$ ) (Smolders and Mertens, 2013). As a consequence, there is a small gap between the average Cd consumption and the tolerable intake of the human body, as defined by EFSA. Four subgroups (vegetarians, children, people living in Cd-contaminated areas and smokers) were even found to be two times above the TDI of 0.36  $\mu\text{g/kg b.w.}$  (Pisanello, 2014). WHO reviewed in 2010 the TDI and suggested a permissible monthly intake limit (PMTI) of 25  $\mu\text{g Cd kg}^{-1}$  body weight (equivalent to a TDI of 0.83  $\mu\text{g Cd kg}^{-1}$  b.w.), which is, in contrast to the EFSA limit, well above the average dietary intake values of the population (UN FAO/WHO, 2011).

The 2012 report of EFSA indicated that most of the Cd in the average European diet comes from staple foods, such as grain and grain products (27%), vegetables and vegetable products (16%), and roots and tubers (13%) (EFSA, 2012). A recent survey in the French population indicated that the urinary Cd concentrations in France in 2014-2016 were higher in adults compared to the levels in 2004-2006. A major result of this study was that 47.6% of the adult population (18-60 years) has Cd urinary levels exceeding the critical limit defined by the national food safety agency of France (ANSES). They suggested moreover that the body burden of Cd in the French population had risen with 40% over 10 years, and claimed that the increase in Cd intake was related to the consumption of shellfish, offal, sweet, savory biscuits or cereal bars and chocolate (Oleko *et al.*, 2021). EFSA reported indeed in their risk assessment of 2012 that chocolate and chocolate products had a relevant contribution to the total dietary Cd exposure, i.e. 4% for adults and over 9% for children. These estimations resulted in strict regulation of Cd in cacao products (see next section). A recent study confirmed that about 9% of the total dietary Cd intake in an average Belgian diet originates from chocolate and chocolate products (Vanderschueren, Doevenspeck, *et al.*, 2023).

Controversy exists about the risk assessments on which many regulations are based. A main critique is that the different metal bioavailability in different food matrixes is not accounted for in risk assessments. In the case of cacao, the bioaccessibility of Cd in cacao-derived products has been shown to be lower than that of wheat flour (Vanderschueren, Doevenspeck, *et al.*, 2023). If regulations would account for such differences in bioaccessibility between cacao and staple foods like wheat, this could have a large impact on the international cacao market.

### 1.1.3.1 Legislation of Cd in cacao

The dietary intake limits of WHO and EFSA described in previous paragraph are used to derive food limits. For the EU this is the 'Commission Regulation No 1881/2006', the framework EU legislation which sets maximum levels for chemical contaminants in foodstuffs (EFSA, 2011). The World Trade Organization (WTO) recognizes the standards of the Codex Alimentarius (or 'Food Code'), based on the WHO/FAO limits, to protect the consumers. The standards of the Codex Alimentarius can thus be interpreted as the international Cd standards for trade in foodstuff (Codex Alimentarius Commission, 2018).

The European Union reviewed in 2014 existing maximum levels of Cd in foodstuff (EC No. 488/2014) (European Commission, 2014). The review focused especially at the protection of infants and young children. Chocolate was targeted by these new maximum levels, since the consumption of chocolate was shown to be a substantial contribution to the intake of Cd by children. Three maximum levels in chocolate were set (Table 1.1) and entered into force on 1 January 2019. The strictest levels are imposed on chocolates that are most consumed by children (like milk chocolate) (EFSA, 2011). Similar regulations have been, and are expected to be, implemented worldwide, e.g. in Australia and New Zealand (Australia New Zealand Food Standards Code, 2017), Russia (Ministry of Health of the Russian Federation, 2011), and the countries within the Southern Common Market (Mercosur, 2011). In July 2018 the commission of the Codex Alimentarius has set as well limits for Cd in different types of chocolate (Codex Alimentarius Commission, 2018).

There are no official Cd limits in ready-for-sale beans. That is because the risk assessments have used the final product on the market (chocolate) as the basis. This situation is clearly different compared to staple crops such as rice and wheat for which the limits are on the raw materials and, hence, the effects of the limits on trade are straightforward. For cacao, the limits of Table 1.1 can be recalculated to bean limits but that requires assumptions that can vary. Consequently, in practice, cacao buyers insist on a relatively low cadmium content of the cacao bean with informal bean limits ranging between 0.5 and 1.1 mg Cd/kg bean (Meter *et al.*, 2019).

**Table 1.1:** Maximum Cd concentrations (mg kg<sup>-1</sup> wet weight) on specific cocoa and chocolate products (EFSA, 2011; Codex Alimentarius Commission, 2018).

Description	Regulation	mg Cd kg <sup>-1</sup>
Milk chocolate with <30% total dry cocoa solids	EC No. 488/2014	0.10
Chocolate with <50% total dry cocoa solids; milk chocolate with >30% total dry cocoa solids	EC No. 488/2014	0.30
Chocolate with >50% total dry cocoa solids	EC No. 488/2014	0.80
Cocoa powder sold to the final consumer or as an ingredient in sweetened cocoa powder sold to the final consumer (drinking chocolate)	EC No. 488/2014	0.60
Chocolate with >50% to <70% total dry cocoa solids	Codex Alimentarius 2018	0.80
Chocolate with >70% total dry cocoa solids	Codex Alimentarius 2018	0.90

## 1.2 The crop cacao: the food of the Gods

*Theobroma cacao* L. delivers its scientific name from the Greek words for Gods (theos) and food (broma). Cacao is a perennial tropical tree of the family *Sterculiaceae* (*Malvaceae sensu lato*), order *Malvales* (Boza *et al.*, 2014). The 'cacao belt' is located between latitudes 10°S and 20°N. The climate in this belt satisfies the conditions to grow the cacao tree: i) rainfall of 1250-3000 mm per annum with a dry season of no more than three months with less than 100 mm rain per month ii) maximum and minimum temperatures varying between 30-32°C and 18-21°C iii) and no persistent strong winds (Wood, 1980). Full-sunlight monocultures and agroforestry systems are the most relevant farming methods for cacao trees. In agroforestry systems palm, timber and fruit trees are grown together with the cacao trees (Schneider *et al.*, 2017).

Traditionally, *Theobroma cacao* was divided in three genetic groups: Criollo, Forastero, and Trinitario. Forastero is the most produced and is used for basic cacao products. Criollo is known for its fine flavour, but is more susceptible to diseases than Forastero. Trinitario is a hybrid of Forastero and Criollo, and is described to be more disease resistant and a fine flavour cacao type (Afoakwa *et al.*, 2008). However, these main genetic groups were defined based on morphological traits and geographical origins and did not provide a sound classification. Motamayor *et al.* (2008)

proposed a new classification of cacao germplasm into 10 genetic clusters: Marañón, Curaray, Criollo, Iquitos, Nanay, Contamana, Amelonado, Purús, Nacional and Guiana. This new classification reflects more accurately the genetic diversity now available for breeders, rather than the traditional classification as Criollo, Forastero or Trinitario.

### 1.2.1 Ecophysiology of the cacao tree

The type of soils in cacao plantations is diverse (clayey highly eroded soils, volcanic sands and silty soils with pH varying between 4 and 7). For optimal growth, cacao needs well drained clay loamy soils with high organic matter content (Quiroz and Agama, 2006). Soils in cacao plantations commonly have a top layer which accumulates soil organic matter (SOM) due to litterfall (Wood, 1980). Cacao plantations are often on Alfisols, typically having a subsoil with an argillic horizon where there is a significant accumulation of translocated layer silicate clay (Sumner, 2000).

A cacao tree is a perennial tree which can reach up 3 to 5 m under cultivation. The roots of cacao are characterised by a large tap root and a mat of surface lateral roots in the topsoil (Toxopeus *et al.*, 2008). The latter are most responsible for the uptake of water and nutrients (Hartemink, 2005). The tree exposes dimorphic growth with a vertical, orthotropic stem and fan branches that grow plagiotropic (De Almeida and Valle, 2007; Toxopeus *et al.*, 2008). Cacao trees show an asynchronous growth, i.e., not all trees of the same area grow simultaneously. The growth rhythm is divided in two flushing (growth) followed by three interflush (dormant) periods. The whole cycle lasts about 60 days in field conditions (Greathouse *et al.*, 1971; Abo-Hamed *et al.*, 1983). The flush cycle is as follows: terminal bud swelling (F1), leaf expansion and stem elongation (F2), maximum leaf area and greening (IF1), mature green leaves with green stem (IF2), and mature green leaves with brown stem (IF3) (Figure 1.1). During a flush, there is translocation of nutrients from the older to new leaves, which leads to shedding of the older leaves (Toxopeus *et al.*, 2008). During the dormancy period, the length of the shoot is constant and there is no leaf expansion. Synchronous flowering occurs soon after the dry season end. A tree enters the adult stage when it starts reproductive growth (De Almeida and Valle, 2007). Cacao is a cauliflorous tree, where flowers are formed on all branches, whilst pods are generally formed on the trunk and main branches (Figure 1.1) (De Almeida and Valle, 2007; van Vliet and Giller, 2017). Flowering occurs after the end of the dry season. After pollination, pods start to develop from the flowers. The young pods are known as 'cherelles'. A cherelle is still susceptible to wilt, which results in shivering and blackening of young cacao fruits. Developing pods are classified as immature pods until they reach full size. During the active metabolism phase, lipids, proteins, and anthocyanins accumulate in the seed. When the pods become mature, the ripening starts which takes about 20 to 30 days to complete. The total development of the fruit takes about six months from pollination (Toxopeus

*et al.*, 2008). The cacao fruit or pod is made up of a large woody outer pod husk filled with 20–50 cacao beans (seeds) embedded in a white sugary mucilaginous pulp. The color, thickness and level of lignification of pod husk varies considerably between cultivars. The cacao beans are connected to the plant through a central tissue called the placenta, and each cacao bean comprises of two cotyledons (the nib), a germ or embryo, the radicle and an outer shell (the testa). The nib is the only part of the cacao bean that is retained during chocolate processing.



**Figure 1.1:** (Left) Flushing cacao tree. Red leaves are in their expansion phase (F2) and the green leaves are mature (either IF2 or IF3 leaves). (Right) Cacao is a cauliflorous tree. Pods are generally formed on the trunk and main branches. Photos by the author.

### 1.3 Cadmium transfer pathways from soil to plants

All plants take up Cd to some extent, but cacao is rather effective in doing so (Chapter 2). Accumulation of Cd into the edible parts of plants involves several processes; i) Cd uptake from soil into root cells, ii) radial transport across root cells and iii) loading into the xylem, iv) subsequent long-distance transport via the xylem and/or phloem and v) final transfer into seeds or other types of storage organs (Clemens and Ma, 2016). The current knowledge on Cd accumulation by plants originates mostly from model plants like hyperaccumulators and cereals. Especially in the model monocotyledonous species rice (*Oryza sativa*) and the model dicotyledonous plant *Arabidopsis thaliana*, the knowledge of the pathway of Cd dynamics inside the plant is in a very advanced stage compared to cacao. Therefore, in next section, the overwhelming literature describing the different factors that control the Cd transfer in plants are

briefly discussed, while in chapter 2 the current literature concerning these processes in cacao is reviewed.

### 1.3.1 Occurrence of Cd in agricultural soils

Cadmium is naturally present in soils. The background Cd concentration in soils (away from local contaminations) is typically 0.10-1 mg/kg dry weight (dw) and is a function of the parent material, the localization and the land use of the soil (ECHA, 2007; Hooda, 2010; Smolders and Mertens, 2013). Cadmium originates mainly from Zn minerals (e.g. sphalerite (ZnS)) (Hooda, 2010). Concentrations of Cd in agricultural soils have risen due to anthropogenic activities like the use of phosphate fertilizers, application of sewage sludge on soils and atmospheric pollution (Welch and Norvell, 1999). The retention times for Cd in the upper soil layers are roughly around hundreds of years. Hence, in classical agricultural systems, the fluxes of Cd out of the topsoil are small compared to the Cd content in the soil (McLaughlin *et al.*, 2021). Cd is distributed over different soil compartment; the soil solution (aqueous species, complexes, suspended colloids) and the soil solid phase (weakly adsorbed to clays, strongly adsorbed to Fe, Mn, and Al oxides, sorption with organic matter, sparingly soluble precipitates). Cadmium concentrations in crops depend on the availability of soil. The availability of soil Cd increases with increasing total soil Cd, increasing soil acidity, decreasing soil organic matter, and in some cases, zinc deficiency and chloride salinity. The relationship between soil Cd availability and cacao bean Cd concentrations are further discussed in Chapter 2.

### 1.3.2 Uptake and transport by roots

The first step in the accumulation of Cd in edible parts of a plant involves the uptake into the root cell and the subsequent radial transfer towards the root stele (Welch and Norvell, 1999; Clemens and Ma, 2016; Sterckeman and Thomine, 2020). Plant root cells have a negatively charged plasma membrane which drives cation (and thus Cd<sup>2+</sup>) absorption into the root apoplast. The absorption drives Cd diffusion to the root surface from the soil solution. Both passive and active transport have been identified to play a role in the uptake of Cd across the root cells. Passive Cd absorption in the roots is driven by the electrochemical potential difference between the activity of Cd in the cytoplasm and the activity in the root apoplasm (McLaughlin *et al.*, 1997; Sterckeman and Thomine, 2020). This negative membrane potential is relatively large and produces sufficient energy to drive Cd<sup>2+</sup> uptake even when the activity of Cd in the apoplasm is low. However, Cd is more likely to enter the cells via an active, energy-dependent influx mediated by protein transporters (Sterckeman and Thomine, 2020; McLaughlin *et al.*, 2021). Most known transporters belong to the Zinc Iron-regulated Proteins (ZIPs) or the Natural Resistance-Associated Macrophage

Proteins (NRAMPs) (McLaughlin *et al.*, 2021). These proteins transport divalent transition metals such as Fe(II), Zn, Mn, and Cd (Clemens and Ma, 2016). In *Arabidopsis thaliana*, the AtIRT1 (of the ZIP family) has been identified as a key transporter for Cd uptake into the roots, particularly under Fe deficient conditions (Vert *et al.*, 2002). Under Fe deficient conditions, AtNRAMP1 was shown responsible for the uptake of Cd in *Arabidopsis thaliana*. In rice, several transporters were found to be related to Cd uptake (OsIRT1, OsIRT2, OsNRAMP1, OsNRAMP5) (McLaughlin *et al.*, 2021). More specifically, the OsNRAMP5 Mn-transporter has been proven to be the main pathway for Cd into rice roots (Sasaki *et al.*, 2012). However, we should also consider that the Cd uptake rate is much faster than its supply in soil by the water influx. As a result, diffusive Cd concentrations gradients occur in the rhizosphere (Smolders *et al.*, 2020). Several models have predicted a transport-controlled uptake by plants and a negligible effect of root ion uptake kinetics on Cd uptake from soil (Sterckeman *et al.*, 2004; Lin *et al.*, 2016) Short distance transport to the stele

After uptake in the epidermis or exodermis, Cd is transported through the cortex, endodermis, and the pericycle cells (Clemens and Ma, 2016). Cadmium moves its way through either a symplastic (intracellular) or apoplastic (extracellular) pathway. This flow can be hampered by sequestration in the vacuoles of the root cells. This transport from the cytosol into the vacuole can increase Cd tolerance of the plant (Sterckeman and Thomine, 2020). In *A. thaliana*, AtHMA3 is found to be a tonoplast transporter for Zn, Co, Cd and Pb (Morel *et al.*, 2009). In rice, this role is taken by OsHMA3 (Sasaki *et al.*, 2014). OsHMA3 was found to limit Cd translocation from roots to shoots by sequestering Cd into root vacuoles (Ueno *et al.*, 2010). In the cytosol and vacuole, Cd forms complexes with organic ligands (Sterckeman and Thomine, 2020). This complexation is considered to be a detoxification process (see section 1.3.4 Cadmium speciation inside plants).

### 1.3.3 Transport of Cd from root to shoot

The Cd that is still mobile in the roots, is loaded into the xylem and exported into the shoot. The xylem loading needs an active transport system, as the Cd transport is against its electrochemical gradient. In *A. Thaliana*, AtHMA4 and AtHMA2 were shown to load Cd into the xylem (Mills *et al.*, 2003; Verret *et al.*, 2004; Wong *et al.*, 2009). In rice, knock out of the homologue OsHMA1 resulted in lower root-to-shoot translocation and grain content (Ueno *et al.*, 2011). Hence, Cd translocation to shoots is thus determined by following three processes: i) sequestration in vacuoles of root cells, ii) transport in the symplast to the root stele, and iii) xylem loading. In most plants, Cd accumulates more in the roots than in the shoots (see review Sterckeman and Thomine (2020)). Nonetheless, some plants showed shoot Cd concentrations that were higher than root Cd concentrations when grown in low-Cd contaminated soils. Yet, when the plants were grown in

higher Cd conditions, the root concentration overpassed the shoot Cd concentration (John, 1973). The internal translocation depends thus on plant species and Cd exposure.

The subsequent transport through the xylem is driven by transpiration (Van Der Vliet *et al.*, 2007). Cadmium can migrate as a free ion or form complexes with organic and inorganic bases, and/or adsorb on cell walls of xylem vessels (Welch and Norvell, 1999; Sterckeman and Thomine, 2020). The Cd ions can then migrate through xylem cell walls into those of stems and leaves, still driven by the transpiration flow. Once in the leaf/stem apoplast, Cd is transported into the cytosol via membrane transporters (Leitenmeier and Küpper, 2011). In the cytosol, Cd can be stored into vacuoles. In *A. Thaliana*, NcHMA3 was shown to be responsible for Cd sequestration in leaf cell vacuoles (Mishra *et al.*, 2017). Several mechanisms of Cd detoxification with different speciation and/or localization have been observed in plants. Sorption in the apoplast, together with vacuolar sequestration and further translocation, are examples of such processes.

#### 1.3.3.1 Transport of Cd through xylem or phloem to storage organs

Part of the Cd in the leaves can be redistributed to other plant organs, like the reproductive tissues, via the phloem. The phloem can also be involved in Cd transport before reaching leaves, due to exchanges from xylem to phloem in the stem (Clemens and Ma, 2016; Sterckeman and Thomine, 2020). In rice, most Cd is exported to the transpiring organs like the leaves. During the development of the grain, Cd can originate from two sources: direct transfer from the roots (xylem route) or remobilization from plant organs that store mineral nutrients during vegetative plant growth (phloem route). In rice, it was estimated that 91-100% of Cd accumulated in the grain originated from the phloem (Tanaka *et al.*, 2007). In wheat, it was shown that half of the grain Cd originated from Cd taken up during grain filling, while the other half from Cd remobilized (Yan *et al.*, 2019). For potato, a crop with low rate of transpiration (i.e. reduced xylem flow), it was shown that Cd was rapidly distributed via the phloem to all tissues (Reid *et al.*, 2003). This suggested that Cd has high mobility in both xylem and phloem.

#### 1.3.4 Cadmium speciation inside plants

During the different steps of Cd accumulation in plants, Cd binds to functional groups of biomolecules (Sterckeman and Thomine, 2020). The speciation or chemical form of a metal is a key parameter that controls its bioavailability and its toxicity (Aucour *et al.*, 2023). Throughout this manuscript we make the distinction between S-ligands (like glutathione, phytochelatins, cysteine rich polypeptides) and O-ligands (e.g., organic bases like carboxylates, and phosphoryl-containing ligands). The soft acid character of Cd<sup>2+</sup> (see paragraph 1.1.1 Chemical properties of

Cd) leads to a strong affinity for soft sulfur donor groups, like thiolates (R-S<sup>-</sup>). These thiolate groups originate from the amino-acid cysteine which is the building block for peptides such as glutathione, phytochelatins and metallothioneins (Rennenberg and Herschbach, 2014). Glutathione (GSH) and phytochelatins (PCs) are peptides that have been shown to be involved in Cd detoxification in numerous plant species (Clemens and Ma, 2016; Sterckeman and Thomine, 2020). Glutathione is essential in all plant cells. When exposed to metal excess, PCs are synthesized from GSH. The synthesis of PCs by exposure to Cd has been shown in several plant species. In some species, phytochelatin has been shown to increase plant tolerance to Cd (for more details, see (Sterckeman and Thomine, 2020)). Another form of S-ligands is metallothioneins (MTs) and other cysteine-rich polypeptides (Rennenberg and Herschbach, 2014). MTs are cysteine-rich, low molecular weight proteins. They play an important role in metal homeostasis and tolerance in plants (Prasad *et al.*, 1999). In the group of O-ligands, carboxylates have been shown to contribute to Cd complexation in root and shoot tissues (Sterckeman and Thomine, 2020). Proven ligands are citrate, malate, and oxalate. The complexation of Cd by carboxylates has more importance in epidermal cells, where the vacuoles make over 99% of the symplast volume (Tian *et al.*, 2017). Another binding of Cd with O-ligands is the binding by polysaccharides from cell walls (Isaure *et al.*, 2006). Currently, it is quite unclear how the status of the plant in terms of nutrition and toxicity affects the binding of Cd with biomolecules. The proportion of S-ligands relative to O-ligands has been shown to increase with Cd exposure and decrease with Cd accumulation trait (Isaure *et al.*, 2015). Nonetheless, both S- and O-ligands have been observed in systems with high Cd concentration like in the studies of Pongrac *et al.* (2018) and where Cd was mainly associated with S ligands in the roots, and with O-ligands in the shoots.

## Chapter 2: Mitigating the level of cadmium in cacao products: a review

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### Summary

The new EU regulation on cadmium (Cd) in cacao-derived products affects the cacao market worldwide. This chapter reviews the journey of Cd from soil to chocolate bar and collates current data on the topic, giving due attention to data quality. Cacao bean Cd concentrations are typically about a factor two larger compared to the soil on which the cacao trees grow, this is high but not unusual and, therefore, the cacao plant is not classified as a Cd hyperaccumulator. Average Cd concentrations in cacao beans range between 0.02 and 12 mg kg<sup>-1</sup>, and are markedly higher in Central and South America, where more than half of cacao bean samples exceed the commonly applied industry threshold for export to the EU (0.60 mg kg<sup>-1</sup>). This regional enrichment is related to relatively high soil Cd concentrations in the young soils of Central and South America. The source of Cd is, in general, likely geogenic rather than derived from phosphate fertilisers or contamination. A meta-analysis of 780 soil-plant paired data shows that soil Cd, soil pH and soil organic carbon largely explain bean Cd concentrations. Detection of effects of cultivars, soil amendments or agronomic practices are strongly hampered by the spatial variability in phytoavailable soil Cd concentrations. Application of lime or biochar has the potential to lower bean Cd in acid soils. In the long term, breeding low Cd cultivars likely provides high potential for mitigation but genetics and breeding research is currently limited by the lack of understanding of how Cd is loaded into the developing fruit of the cauliflorous cacao tree. In the short term, mixing of cacao from different origins may be the most feasible strategy to meet the EU limits.

## 2.1 Introduction

Cadmium (Cd) is a potentially toxic trace metal that has no known biological function in humans. Cadmium accumulates in the human body and has a biological half-life of 10 – 35 years. Chronic Cd exposure has been related to several adverse health effects, including renal tubular dysfunction and osteomalacia (World Health Organization, 2010). The human diet is the main source of body burden Cd in the non-smoking population and staple foods such as rice, wheat grain products or potatoes contribute largely to dietary Cd exposure because of their high consumption. Certain luxury foods such as chocolate, although ingested in smaller quantities, can also contribute to dietary Cd exposure due to their elevated Cd concentrations. To protect consumers, the European Commission approved a new regulation in 2014 (in force from 2019), which sets the maximum allowed Cd concentration in cacao powder at 0.60 mg Cd kg<sup>-1</sup>, while the thresholds for chocolates range between 0.10 and 0.80 mg Cd kg<sup>-1</sup> depending on the cacao solids content of the product (European Commission, 2014). Similar regulations have been, and are expected to be, implemented worldwide, e.g. in Australia and New Zealand (Australia New Zealand Food Standards Code, 2017), Russia (Ministry of Health of the Russian Federation, 2011), and the countries within the Southern Common Market (Mercosur, 2011). In California (USA), products with elevated Cd concentrations must have a warning on the packaging (Meter *et al.*, 2019). The Cd-cacao regulations have also been adopted by the Codex Alimentarius (Codex Alimentarius Commission, 2018). These regulations have fuelled research worldwide to monitor and mitigate Cd accumulation in cacao. The number of research papers on Cd in cacao was only 29 up to 2014 but rose almost exponentially to > 100 by 2020.

Cadmium in cacao products originates from the cacao beans, rather than from contamination during processing. This is demonstrated by the strong correlations between the Cd concentration in the final chocolate product and its cacao content, and between the Cd concentration in chocolate and the geographical origin of the cacao (Villa *et al.*, 2014; Yanus *et al.*, 2014; Abt *et al.*, 2018; Lo Dico *et al.*, 2018). While all plants take up Cd to some extent, the cacao tree (*Theobroma cacao* L.) is rather effective in doing so. The oldest study reporting Cd concentrations in cacao beans and chocolates dates back to 1979 and that study already highlighted that elevated Cd concentrations are typically found in cacao and chocolates from Central and South American origin, as well as in specialty origin chocolates (Knezevic, 1979). The recently enforced EU limits and the Codex Alimentarius recommendations apply to the final product sold to consumers, not to the cacao beans. Therefore, the processing industry has translated the EU limits to requirements regarding the maximum Cd concentrations in the beans purchased from their suppliers. Those unofficial industry limits vary among companies and have been reported to range between 0.50 and 1.10

mg Cd kg<sup>-1</sup> (CBI Ministry of Foreign Affairs, no date; Meter *et al.*, 2019), but some cacao farmers are confronted with purchaser thresholds as low as 0.10 – 0.30 mg Cd kg<sup>-1</sup> (personal communication). To facilitate the discussion, 0.60 mg Cd kg<sup>-1</sup> was used as a threshold throughout this dissertation.

Mitigation of Cd accumulation has been intensively studied for several food crops including durum wheat, spinach, potatoes and rice (McLaughlin *et al.*, 2021), but for cacao this research is in its infancy. This chapter summarises the existing knowledge on the various factors influencing Cd concentrations in cacao-derived products at all stages of the production process. That includes soil Cd phytoavailability, Cd uptake and translocation within the plant, postharvest processing and the effect of dietary intake through chocolate consumption on the Cd body burden. A meta-analysis of the currently available Cd-cacao studies is presented with due attention given to the quality of the analytical data, thereby only selecting data for which the quality of chemical analyses met predefined quality criteria (more details below and in Appendix I). Research gaps are identified and potential mitigation strategies at the different stages of the cacao value chain are discussed.

## 2.2 Cadmium in soils and its relation to cacao plants

### 2.2.1 Origin of Cd in cacao beans: soil, air, fertiliser and irrigation water

Generally, most of the Cd in plant tissues originates from the soil through root uptake, while only a small fraction is derived from air through foliar uptake. This is likely also true for cacao, first because of the positive associations reported between soil Cd and bean and leaf Cd concentrations (see below) and, second because of the large Cd concentrations found in cacao leaves and beans, which are well above what can be reasonably expected from foliar uptake. The contribution of airborne Cd to crops can be measured using isotopes that indicate the Cd provenance, and can be quantified using Air Accumulation Factors (AAFs, m<sup>3</sup> g<sup>-1</sup>), calculated as the ratio of the Cd concentration in the plant to that in the air. For trace metals in general, the AAF is estimated at 20 m<sup>3</sup> g<sup>-1</sup> for plant leaves, with a range of 2 – 100 m<sup>3</sup> g<sup>-1</sup> depending on the type of plant and trace metal (McLaughlin *et al.*, 2011). Reported air Cd concentrations in the rural areas where cacao is grown range from 0.07 to 0.57 ng Cd m<sup>-3</sup> [cacao production areas near oil activities (Barraza *et al.*, 2017)]. Leaf Cd concentrations derived from air in these cacao growing areas will thus range between 0.001 and 0.011 mg Cd kg<sup>-1</sup> (considering an AAF of 20 m<sup>3</sup> g<sup>-1</sup>), with a maximum of 0.06 mg Cd kg<sup>-1</sup> (AAF of 100 m<sup>3</sup> g<sup>-1</sup>), which is only 6 % of the typical leaf Cd concentrations measured in cacao plants in Central and South America (> 1 mg Cd kg<sup>-1</sup>, see below).

Cadmium occurs naturally in the environment, with background soil concentrations ranging from 0.1 to 1.0 mg Cd kg<sup>-1</sup> and estimated global averages of 0.1 to 0.3 mg Cd kg<sup>-1</sup> (Smolders and Mertens, 2013). In contrast, polluted soils (e.g. near smelting sites) can contain Cd concentrations up to three orders of magnitude higher than the background (He *et al.*, 2015). The soil Cd concentrations in cacao producing areas differ worldwide and tend to be higher in the geologically young soils of Central and South America, with a range of reported averages between 0.22 and 10.8 mg Cd kg<sup>-1</sup> (Chavez *et al.*, 2015; Ramtahal *et al.*, 2016; Arévalo-Gardini *et al.*, 2017; Barraza *et al.*, 2017, 2018; Gramlich *et al.*, 2017, 2018; Lewis *et al.*, 2018; Argüello *et al.*, 2019; Rodríguez Albarracín *et al.*, 2019; Engbersen *et al.*, 2019; Scaccabarozzi *et al.*, 2020) and 0.12 to 0.85 mg kg<sup>-1</sup> in Asia (Fauziah *et al.*, 2001; Zarcinas *et al.*, 2004). Unfortunately, reliable information about Cd concentrations in soils of cacao producing areas in Africa remains scarce. The majority of global cacao is produced in West Africa, where soils are highly weathered and are expected to have low Cd concentrations. Indeed, a study in different crops of Ghana reported an average total soil Cd concentration of 0.026 mg Cd kg<sup>-1</sup> (Bortey-Sam *et al.*, 2015). It is important to note that, while soil Cd concentrations are thus generally reported to be higher in Central and South America compared to other continents, soil Cd concentrations in Central and South American cacao producing areas are mostly < 1 mg Cd kg<sup>-1</sup> (Figure 2.2), and thus not categorised as contaminated. Multiple researchers have indicated large geographical differences in cacao bean Cd, with elevated Cd concentrations found in cacao from Central and South American origin compared to origins, e.g. Africa (Bertoldi *et al.*, 2016; Abt *et al.*, 2018). The strong effect of geographical origin on bean Cd concentrations is outlined below and suggests that Cd in cacao generally does not originate from anthropogenic activity, as anthropogenic activities occur worldwide. Indeed, three independent studies by Gramlich *et al.* (2018), Argüello *et al.* (2019) and Scaccabarozzi *et al.* (2020) found that high soil Cd concentrations in cacao farms were associated with alluvial soils from sedimentary materials, which can be explained by the higher Cd concentrations usually found in sedimentary rocks compared to igneous rocks (Thornton, 1981; Birke *et al.*, 2017). However, the substrate or alluvium source is likely also of importance, as alluvial soils in Africa show different Cd concentrations compared to their Central and South American counterparts. Nevertheless, there are specific cacao producing areas in Central and South America where soil Cd concentrations are exceptionally high (Rodríguez Albarracín *et al.*, 2019), potentially due to historical enrichment related to mining activities.

The use of mineral P-fertilisers has been suggested to be at the origin of high Cd in cacao beans (Zug *et al.*, 2019). However, it is highly unlikely that the use of P-fertilisers can explain elevated cacao bean Cd concentrations on a large scale. Gramlich *et al.* (2017) measured the Cd concentration in P-fertilisers in a long term system comparison trial on an experimental cacao

farm in Bolivia and found an average Cd concentration of 102 mg Cd kg<sup>-1</sup> P<sub>2</sub>O<sub>5</sub>, which is high compared to fertilisers available in Europe [average 28 mg Cd kg<sup>-1</sup> P<sub>2</sub>O<sub>5</sub>, < 10 % of samples contained 60 mg Cd kg<sup>-1</sup> P<sub>2</sub>O<sub>5</sub> (Verbeeck *et al.*, 2020)]. At a sustained annual dose of 20 kg P<sub>2</sub>O<sub>5</sub> ha<sup>-1</sup> year<sup>-1</sup> with that local fertiliser, fertilisation would add about 2 g Cd ha<sup>-1</sup> year<sup>-1</sup>, equivalent to a net addition to soil of 0.001 mg Cd kg<sup>-1</sup> soil year<sup>-1</sup> at 15 cm incorporation depth or an accumulation of only 0.1 mg Cd kg<sup>-1</sup> soil after 100 years. This mineral fertiliser dose is unrealistically high, as most of the cacao production worldwide is in the hands of low-income smallholders who do not commonly use fertilisers (Snoeck *et al.*, 2016; Vaast *et al.*, 2016). This worst-case scenario calculation hence indicates that mineral P-fertilisers are not the main source of Cd in cacao plantations, at least not at a large scale.

Irrigation water contains Cd and moderate Cd contamination in the water may be a significant source of Cd in soils and plants. An annual irrigation application of 500 mm with irrigation water containing 1 µg Cd L<sup>-1</sup> is equivalent to 5 g Cd ha<sup>-1</sup> year<sup>-1</sup> and leads to an accumulation of 0.25 mg Cd kg<sup>-1</sup> soil, at a sustained irrigation over 100 years and 15 cm incorporation depth. Such accumulation is not unlikely in areas affected by mining activities. For example, in the Puyango-Tumbes river basin at the border between Ecuador and Peru, dissolved Cd concentrations range between 1 and 10 µg L<sup>-1</sup> due to upstream gold mining (Tarras-Wahlberg *et al.*, 2001; Carling *et al.*, 2013; Marshall *et al.*, 2018). The elevated Cd concentrations in these rivers have been mentioned as a potential source of Cd to nearby cacao fields (Chavez *et al.*, 2015).

Several soil profile studies have reported that soil Cd concentrations in cacao fields decline with increasing soil depth. The Cd concentration in the top 0 – 15 cm of the soil is about a factor 1.5 larger than the Cd concentration at depths 15 – 60 cm (Chavez *et al.*, 2015; Arévalo-Gardini *et al.*, 2017; Barraza *et al.*, 2017; Gramlich *et al.*, 2018). Although this might be interpreted as evidence for anthropogenic sources, it is more plausible that this concentration profile reflects the cycling effect caused by decomposition of litter from the aerial biomass (Reimann *et al.*, 2019). The dry leaf biomass production of cacao is about 3.9 Mg ha<sup>-1</sup> y<sup>-1</sup> (Heuveloop *et al.*, 1988) with an average leaf Cd concentration of 2.6 mg kg<sup>-1</sup> dry weight (dw) (n = 762, derived from the meta-analysis described below). Hence, the annual Cd flux returned to the soil by leaf litter decomposition is about 10 g Cd ha<sup>-1</sup> y<sup>-1</sup>, which is much larger than the worst-case inputs calculated from application of contaminated P-fertilisers or irrigation water as indicated above. Enriching the topsoil from a background soil concentration of 0.89 mg Cd kg<sup>-1</sup> [estimated by the subsoil Cd concentrations reported by Gramlich *et al.* (2017)] to 1.09 mg Cd kg<sup>-1</sup> [topsoil Cd concentrations reported by Gramlich *et al.* (2017)] would thus require approximately 50 years of leaf litter decomposition, which is not an unrealistic age for the many cacao orchards in the hands of small-scale farmers. Indeed, an economic survey conducted in Ecuador (Vazquez *et al.*, in preparation) reported that

the age of cacao orchards ranged from 1 to 100 years, with an average of 13.8 years ( $n = 1534$ ), and 11 % of the orchards surpassed the age of 30. Barraza et al. (2019) determined the stable isotope ratio ( $\delta^{114/110}\text{Cd}$ ) in cacao leaves, litter and topsoil from cacao plantations. While the isotopic ratios in topsoil differed among the different sites, there was a consistent fractionation between leaves and soil ( $\Delta_{\text{leaf-soil}}$ ), indicating that the topsoils were likely enriched with heavier Cd isotopes originating from the leaf litter. Considering all the above, the main Cd source in cacao producing soils is likely geogenic rather than anthropogenic, and local enrichments may be related to point sources such as mining activities, input of sedimentary materials or the use of contaminated irrigation water. The annual Cd cycling due to plant uptake and leaf litter decomposition can explain the larger Cd concentrations in the surface soil compared to the subsoil.

### 2.2.2 Bean Cd concentrations and their relationship with soil properties: a meta-analysis

Cacao bean Cd concentration data reported in different studies have been collated here to identify general trends (Table 2.1). Studies included in this table were selected based on the quality criteria elaborated in Appendix I, i.e. quality assurance of the chemical analysis combined with a minimal number ( $n \geq 4$ ) of different samples to characterise the region, as well as information on sample treatment (drying, peeling and digestion procedures). Studies that used Flame Atomic Absorption (AAS) or older Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) instruments were excluded from this compilation because of their poor detection limits for Cd in plant tissues ( $0.1 - 0.5 \text{ mg Cd kg}^{-1} \text{ dw}$ ). Average or median bean Cd concentrations clearly vary by geographical origin, with cacao beans originating from Central and South America showing Cd concentrations four to six times higher compared to beans from Asia or Africa (Table 2.1, data from Central and South America marked in grey). While most included studies have reported the data as bean Cd concentrations, sample treatments before analyses differ strongly among studies. Processing steps such as fermentation, peeling or roasting may influence the Cd concentration but require further research as explained in section 2.4.3.

Cadmium concentrations in crops depend on the availability of soil Cd, which increases with increasing total soil Cd, increasing soil acidity, decreasing soil organic matter, and, in some cases, Zn deficiency and Cl salinity (Smolders and Mertens, 2013). These relationships have also been corroborated for cacao (Gramlich *et al.*, 2018; Argüello *et al.*, 2019). To better illustrate these general trends, the raw data of different soil-plant studies were collated. Over 400 studies on soil-plant Cd relationships in cacao (including all plant tissues) were found. However, only seven passed all quality criteria elaborated in Appendix I (Table I.1). A total of 785 paired soil-plant data points were gathered from these seven datasets, which were all based on studies in Central and

South America (six published studies and one unpublished dataset Appendix I, Table I.2). The selected studies reported total soil Cd, soil pH, soil organic carbon (SOC) and bean Cd, among other variables. In studies I and VII, bean Cd referred to the Cd concentration in the peeled bean or nib; while in studies II, III, IV, V and VI, samples were not peeled and bean Cd thus referred to the total bean Cd concentration. The frequency distribution of these data shows that bean Cd concentrations are 1.08 mg kg<sup>-1</sup> (arithmetic mean) and 0.63 mg kg<sup>-1</sup> (median; n = 779) with distinct differences among studies (Figure 2.1). A total of 403 samples (52 % of the compiled data) showed bean Cd concentrations exceeding 0.60 mg kg<sup>-1</sup>, the threshold commonly used for export to the EU (Figure 2.1). Per study, the number of observations exceeding this threshold ranged between 7 and 100 %. This clearly emphasises the magnitude of the impact of the new EU Cd regulations on cacao producers.

**Table 2.1:** Average cacao bean Cd concentrations ( $\text{mg kg}^{-1}$ ) from several studies across the world clearly illustrate geographical differences, with the highest bean Cd concentrations reported for cacao grown in Central and South American countries (rows marked in grey). Incomplete information on analytical data quality is indicated by superscripts. The variation is indicated either by the standard deviation or the range min-max; P = peeled; UP = unpeeled; F = fermented; UF = unfermented; R = roasted.

Sample origin	Bean Cd [ $\text{mg kg}^{-1}$ ]			Processing	Study
	Average	Variation	n		
Ghana <sup>a,c</sup>	0.02	0.003	3	P, F	(Vītola and Ciproviča, 2016)
Nigeria <sup>a,c</sup>	0.02	0.003	3	P, F	(Vītola and Ciproviča, 2016)
Ghana <sup>a,c</sup>	0.05	0.045–0.058	30	UP, F	(Nnuro <i>et al.</i> , 2020)
Cameroon <sup>a,c</sup>	0.05	0.01	3	P, F	(Vītola and Ciproviča, 2016)
Ghana <sup>a,c</sup>	0.05	0.005–0.095	20	UP, F	(Amankwaah <i>et al.</i> , 2015)
Ivory Coast <sup>a,c</sup>	0.05	0.04	9	P, F	(Yapo <i>et al.</i> , 2014)
West Africa	0.09	0.04	21	UP, F	(Bertoldi <i>et al.</i> , 2016)
Brazil <sup>a</sup>	0.10	/	1	P, F	(Knezevic, 1979)
Ivory Coast <sup>a</sup>	0.12	0.09–0.14	3	P, F	(Knezevic, 1979)
Sao Thomas and Principe <sup>a</sup>	0.12	0.09–0.15	4	P, F	(Knezevic, 1979)
Dominican Republic	0.13	0.031	-	UP, F	(Kruszewski <i>et al.</i> , 2018)
Tanzania <sup>a</sup>	0.13	/	1	P, F	(Knezevic, 1979)
Ghana <sup>a</sup>	0.14	0.09–0.18	8	P, F	(Knezevic, 1979)
Brazil (Bahía) <sup>a</sup>	0.19	0.09–0.29	8	P, F	(Knezevic, 1979)
Ecuador <sup>a,c</sup>	0.20	0.04	3	UP, F	(Vītola and Ciproviča, 2016)
Bolivia <sup>a</sup>	0.21	0.02	64	P, F	(Gramlich <i>et al.</i> , 2017)
New Guinea <sup>a</sup>	0.22	0.14–0.29	8	P, F	(Knezevic, 1979)
Samoa <sup>a</sup>	0.22	/	1	P, F	(Knezevic, 1979)
Malaysia <sup>a</sup>	0.25	0.01–1.27	86	/	(Mohamed <i>et al.</i> , 2020)
Sri Lanka <sup>a</sup>	0.26	0.24–0.27	2	P, F	(Knezevic, 1979)
Ghana <sup>a,c</sup>	0.3	0.248–0.336	67	P, F	(Takrama <i>et al.</i> , 2015)
Mexico <sup>a</sup>	0.28	/	1	P, F	(Knezevic, 1979)
Asia	0.33	0.18	8	UP, F	(Bertoldi <i>et al.</i> , 2016)
Ecuador	0.35	0.24	50	UP, UF	(Acosta and Pozo, 2013)
East Africa	0.51	0.59	8	UP, F	(Bertoldi <i>et al.</i> , 2016)
Indonesia (Sumatra) <sup>a</sup>	0.52	/	1	P, F	(Knezevic, 1979)
Central America	0.54	0.30	10	UP, F	(Bertoldi <i>et al.</i> , 2016)
Malaysia <sup>a</sup>	0.55	0.26	10	/	(Fauziah <i>et al.</i> , 2001)
Brazil	0.55	0.10–1.50	36	P, F	(De Araujo <i>et al.</i> , 2017)
Caribbean <sup>a</sup>	0.57	/	1	P, F	(Knezevic, 1979)
Central and South America	0.62	0.38	7	P, F, R	(Abt <i>et al.</i> , 2018)
Ecuador	0.63	0.067	-	UP, F	(Kruszewski <i>et al.</i> , 2018)
Malaysia <sup>a</sup>	0.67	0.20–1.68	5	/	(Zarcinas <i>et al.</i> , 2004)

**Table 2.1:** continued

Sample origin	Bean Cd [mg kg <sup>-1</sup> ]			Processing	Study
	Average	Variation	n		
Trinidad & Tobago <sup>a</sup>	0.68	/	1	P, F	(Knezevic, 1979)
Ecuador	0.75	0.27–1.72	81	UP, F	(Romero-Estévez <i>et al.</i> , 2019)
Indonesia (Java) <sup>a</sup>	0.76	/	1	P, F	(Knezevic, 1979)
Grenada <sup>a</sup>	0.77	/	1	P, F	(Knezevic, 1979)
Ecuador	0.78	0.12–1.52	4	UP, UF	(Barraza <i>et al.</i> , 2018)
Ecuador	0.90	0.09–3.51	31	UP, UF	(Barraza <i>et al.</i> , 2017)
Ecuador	0.90	0.03–10.4	560	P, UF	(Argüello <i>et al.</i> , 2019)
Malaysia (Sabah) <sup>a</sup>	0.94	0.59–1.29	2	P, F	(Knezevic, 1979)
Ecuador <sup>c</sup>	0.94	0.02–3.00	19	P, UF	(Chavez <i>et al.</i> , 2015)
Jamaica <sup>a</sup>	0.95	/	1	P, F	(Knezevic, 1979)
Peru <sup>a</sup>	0.96	0.34	72	UP, UF	(Rosales-Huamani <i>et al.</i> , 2020)
Trinidad & Tobago	0.98	0.50–2.34	45	P, F	(Ramtahal <i>et al.</i> , 2015)
Trinidad & Tobago <sup>b</sup>	1.00	0.17–2.31	100	UP, UF	(Lewis <i>et al.</i> , 2018)
Costa Rica <sup>a</sup>	1.02	/	1	P, F	(Knezevic, 1979)
Honduras	1.10	0.10	110	P, UF	(Gramlich <i>et al.</i> , 2018)
Malaysia <sup>a</sup>	1.14	0.45–1.83	9	P, F	(Knezevic, 1979)
Peru <sup>a,d</sup>	1.13	0.11–6.30	70	UP, UF	(Arévalo-Gardini <i>et al.</i> , 2017)
Peru <sup>a</sup>	1.31	1.26–1.36	3	P, F	(Knezevic, 1979)
South America <sup>a</sup>	1.39	1.09	14	UP, F	(Bertoldi <i>et al.</i> , 2016)
Venezuela <sup>a</sup>	1.96	1.75–2.17	3	P, F	(Knezevic, 1979)
Costa Rica <sup>a,c</sup>	2.20	0.56–8.7	24	UP, UF	(Furcal-Beriguete and Torres-Morales, 2020)
Trinidad & Tobago	2.27	1.78	402	P, UF	(Ramtahal <i>et al.</i> , 2016)
Ecuador (Arriba) <sup>a</sup>	2.45	0.55–4.34	6	P, F	(Knezevic, 1979)
Honduras	2.56	0.81–10.6	60	UP, UF	(Engbersen <i>et al.</i> , 2019)
Ecuador <sup>d</sup>	2.68	1.26–3.92	5	UP, UF	(Barraza <i>et al.</i> , 2019)
Colombia <sup>a</sup>	12.0	6.94	57	UP, F	(Rodríguez Albarracín <i>et al.</i> , 2019)

<sup>a</sup> No information was found on whether certified reference materials (CRM) were included in the analysis or not.

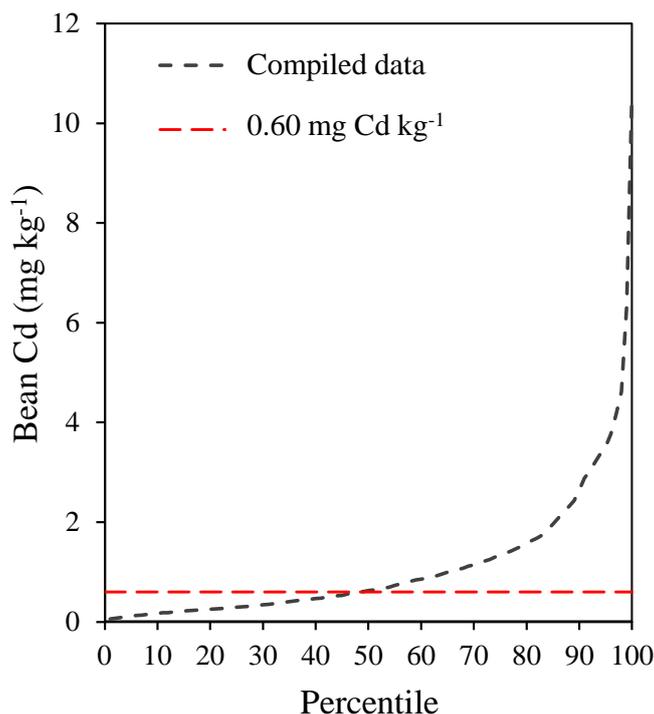
<sup>b</sup> CRM included with certified Cd concentrations above the reasonable range of cacao Cd concentrations.

<sup>c</sup> Reported sample Cd concentrations lower than the limit of detection of the equipment used for Cd analysis.

<sup>d</sup> Mean and variation were calculated from the raw data which was kindly provided by the authors of these studies.

The average total topsoil Cd concentrations of each study (Appendix I, Table I.2), as well as the overall average ( $\pm$  standard deviation, stdev) of the compiled dataset ( $0.43 \pm 0.41$  mg Cd kg<sup>-1</sup>, n = 776) are within the range for non-polluted soils. Only 50 soil samples across all studies exceeded 1.0 mg Cd kg<sup>-1</sup>, such “hotspots” thus represent only 6 % of the compiled data. Multivariate regression analysis was first applied to each individual dataset (details on methods can be found

in Appendix I). The results of this multivariate analysis (Table 2.2 and Appendix I, Table I.3) show a generally emerging trend: bean Cd increases with increasing total soil Cd, with decreasing pH (except in study II) and with decreasing SOC (except in study VI). The effect of organic carbon can be explained by the high affinity of Cd for sorption sites in organic matter (Christensen, 1989). The effect of pH on Cd availability is due to the competition between  $\text{Cd}^{2+}$  and protons for soil sorption sites, as  $\text{Cd}^{2+}$  binds to carboxylic or phenolic groups present in organic matter and oxyhydroxides (Smolders and Mertens, 2013).



**Figure 2.1:** Percentile distribution of cacao bean Cd concentrations of the compiled meta-analysis data ( $n = 780$ , overall mean  $1.08 \text{ mg Cd kg}^{-1}$ ), with indication of the unofficial industry threshold of  $0.60 \text{ mg Cd kg}^{-1}$ . Cacao beans were either peeled or not depending on the study. A detailed description of the studies included in the meta-analysis can be found in Appendix I.

Total soil Cd alone might not be the best predictor for crop Cd concentrations as the majority of Cd is sorbed to different soil particles and is not directly available for plant uptake. Therefore, plant Cd concentrations are better predicted if soil pH is included in the prediction equation, as it is the key soil property controlling the availability of soil Cd. Available soil Cd has been proposed as a proxy to predict crop Cd concentrations. Available soil Cd often refers to the soluble fraction in the soil and is operationally defined by the extraction method used to measure that soluble fraction (McLaughlin *et al.*, 2000). Study VII reported available Cd in the topsoil (measured either by simple extraction with ammonium-acetate-EDTA or using Diffusive Gradients in Thin film,

DGT), and available soil Cd was identified as a significant predictor for bean Cd for that study (Table 2.2 and Appendix I, Table I.3). Soil pH, which affects the soluble fraction of Cd in the soil, is logically not a significant variable when available soil Cd rather than total soil Cd is used. Multivariate regression was also applied to the compiled dataset including data from all seven studies, with correction for the disproportionate weight of their sample sizes ( $n = 334$ , methods are described in Appendix I). Total soil Cd, pH and SOC explained > 41 % of the variance in bean Cd concentrations within the compiled dataset.

The equation of the model reads as follows:

$$\log_{10}[\text{Bean Cd}] = 1.34 + 0.86 * \log_{10}[\text{Total Soil Cd}] - 0.18 * \text{pH} - 0.25 * \log_{10}[\text{SOC}]$$

where bean Cd and total soil Cd are expressed in  $\text{mg kg}^{-1}$ , pH is standardised as  $\text{pH}_{\text{CaCl}_2}$  using the equation proposed by Kissel et al. 2009 , and SOC is expressed as a percentage. This significant multivariate model suggests (i) that bean Cd increases almost proportionally to total soil Cd; (ii) that bean Cd increases by a factor of 1.5 per unit decrease in soil pH (Figure 2.2); and (iii) that doubling SOC reduces bean Cd by a factor of 1.8. The Cd-cacao issue is thus clearly not related to a single factor such as total soil Cd, but rather to an interaction of several factors, i.e. total soil Cd, soil pH and SOC, which control the solubility of Cd and, therefore, also its availability to the cacao plant.

**Table 2.2:** Predictors for bean Cd concentrations for each of the included studies, determined by multivariate regression analysis. All variables were log transformed before analysis except for pH. The sign in brackets corresponds to the sign of the coefficient of that predictor. Top = Topsoil (0 – 20 cm), Sub = Subsoil (20 – 40 cm), Av = Available, SOC = Soil Organic Carbon, DGT = Diffusive Gradient Thin film.

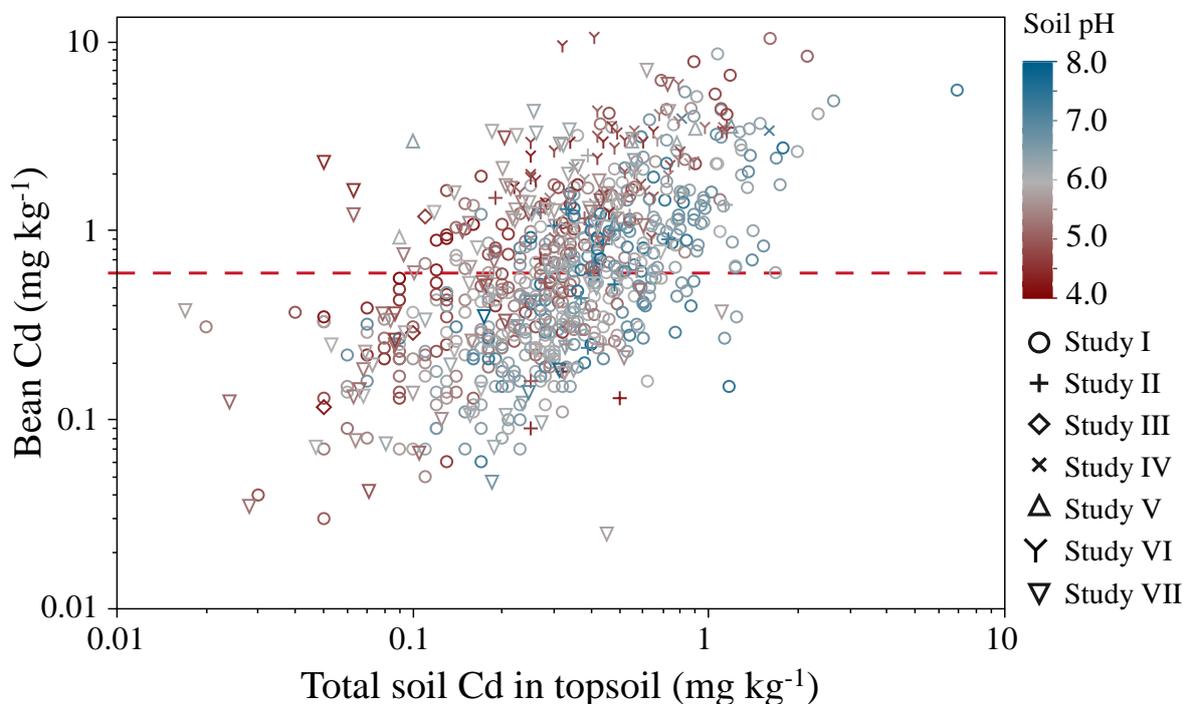
Study	n <sup>‡</sup>	Significant predictors	R <sup>2</sup>
I	559	Total Cd Top (+); pH Top(-); SOC Top(-)	0.57
II a <sup>§</sup>	28	SOC Sub (-); Total Zn Sub (+); Total Mn Top (+)	0.64
II b <sup>§</sup>	28	Total Cd Top (+); SOC Sub(-)	0.49
VI	60	Total Cd Top (+); pH Top (-)	0.23
VII a <sup>§</sup>	107	Total Cd Top (+); pH Top(-); SOC Top(-)	0.36
VII b <sup>§</sup>	106	Av Cd Top (+); SOC Top (-); Av Zn Sub (+); Av Mn Top (-)	0.41
VII c <sup>§</sup>	105	DGT Cd Top (+); SOC Top (-)	0.43

<sup>§</sup> There are three regression models for study VII as the variables Total Cd Top, Av Cd Top and DGT Cd Top were highly correlated thus running only one regression with all variables included would have violated the assumption of no multicollinearity. For study II, two models are included because Total Cd Top was significantly correlated to Total Zn Sub and Total Mn Top.

<sup>‡</sup> The number of observations included in each regression can differ from the number of observations mentioned in Appendix I (Table I.2) due to missing data for some of the predictors.

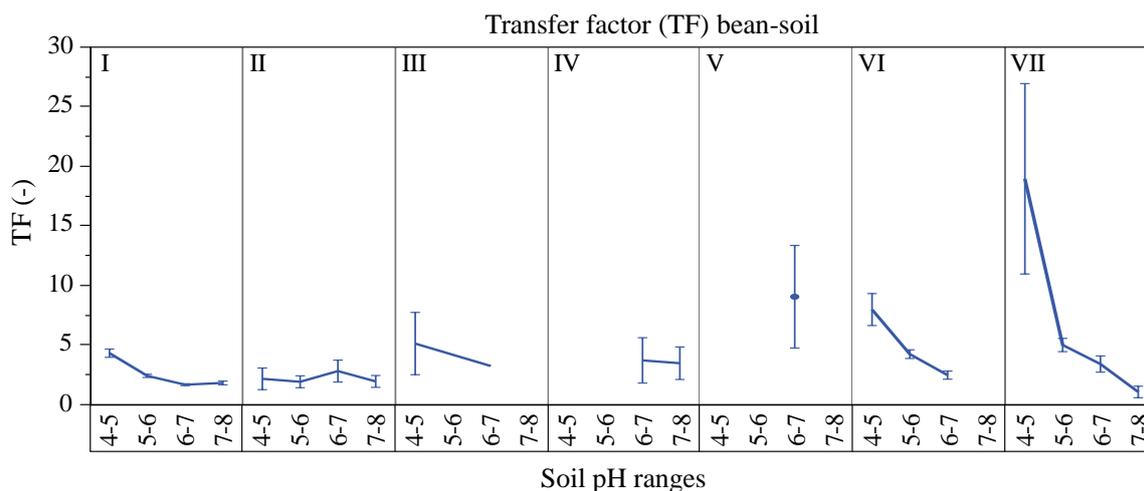
Because Zn and Cd are considered analogues, low available soil Zn has been suggested to trigger plant Cd uptake (Oliver *et al.*, 1994; Chaney *et al.*, 2006; Chaney, 2010). In a recent greenhouse study, dos Santos *et al.* (2020) reported that increasing soil Zn inhibited the uptake and translocation of Cd by CCN 51 cacao plants. However, this effect was only found in soils spiked to Cd concentrations well above environmentally relevant soil Cd concentrations. Argüello *et al.* 2020 found that Cd uptake in cacao from deeper soil layers was enhanced by surface liming and the authors related this to increased activity of deeper roots to cope with micronutrient deficiency in the topsoil (i.e. Zn) caused by surface liming. The multivariate regressions presented here did not identify total soil Zn as a significant predictor for bean Cd concentrations (with exception of study II, Table 2.2), even when using leaf Zn concentrations as regressors (details not shown). The overall average leaf Zn concentration in the meta-analysis was  $97 \pm 68 \text{ mg kg}^{-1}$  ( $n = 700$ , data from studies I, II, IV and VII) which falls within the range reported as optimal ( $> 20 \text{ mg Zn kg}^{-1} \text{ dw}$ ) for plant development (Fageria *et al.*, 2002). Only a small fraction of samples ( $< 4 \%$ ) presented suboptimal leaf Zn concentrations, which may explain why no significant effect of soil Zn was identified in the regression analysis. The lack of Zn deficiency within the included datasets may also indicate that Zn deficiency is not a widespread issue in Central and South American cacao production areas, where Cd accumulation is considerable. However, study II showed a significant effect of total subsoil Zn and total topsoil Mn on bean Cd (regression II a, Table 2.2). Similarly, study VII showed a significant effect of available subsoil Zn and available topsoil Mn on bean Cd,

when available soil Cd was used as a predictor instead of total soil Cd (regression VII b, Table 2.2). Additional research regarding the effect of available Zn and Mn is required to unravel their potential interaction with Cd and their effect on Cd accumulation in cacao.



**Figure 2.2:** Cacao bean Cd concentrations as influenced by total soil Cd and soil pH for the selected studies in the meta-analysis. Soil pH values for all studies are standardised to  $\text{pH}_{\text{CaCl}_2}$  (Kissel et al. 2009). The dashed line indicates the industry threshold of  $0.60 \text{ mg Cd kg}^{-1}$  for export of cacao beans to the EU. Details of the studies can be found in Appendix I.

Transfer factors (TF) indicate to what extent a crop accumulates Cd in its edible parts and are calculated as the ratio of the Cd concentration in the plant leaf or the edible part over the total Cd concentration of the soil. Average TFs for cacao (i.e. bean Cd over soil Cd) in the different studies range from 0.26 to 19 and are significantly affected by soil pH (Figure 2.3). Transfer factors for plants grown in acid soils are higher compared to TF for plants grown in more neutral or alkaline conditions, confirming the importance of soil pH for plant Cd availability. The TF expression shows that average bean Cd will remain below the  $0.60 \text{ mg Cd kg}^{-1}$  industry threshold if soil Cd is below  $0.32 \text{ mg kg}^{-1}$  (soil pH 7.0 – 8.0), below  $0.29 \text{ mg kg}^{-1}$  (pH 6.0 – 7.0), below  $0.19 \text{ mg kg}^{-1}$  (pH 5.0 – 6.0) or below  $0.10 \text{ mg kg}^{-1}$  for the most acid soils (pH < 5.0). Transfer factors are also influenced by plant genetics and allow comparison of Cd accumulation among crops (see below).



**Figure 2.3:** Average transfer factors (dry weight bean Cd concentrations divided by dry weight-based soil Cd concentrations, dimensionless) vs soil pH range per study (I – VII). Bars represent the standard error of the average. For study III, no error bars are given in pH range 6 – 7 as only one observation was available. Details of the studies can be found in Appendix I.

### 2.2.3 Agronomic factors affecting Cd uptake in cacao

Some researchers have argued that Cd uptake is affected by cacao management, e.g. monoculture vs agroforestry or organic vs conventional mineral fertilisation (Maddela *et al.*, 2020). However, there is not enough information available to date to assess the effect of those agronomic practices on Cd in cacao, and the scarce existing information is not standardised among studies. Two studies investigated the effect of cropping systems (monoculture and agroforestry) on Cd uptake in cacao. Argüello *et al.* (2019) found no significant effect of cropping systems on bean Cd concentrations in their large national study in Ecuador. Gramlich *et al.* (2017) also found no such effect on bean Cd concentrations in a field trial but did find higher leaf Cd concentrations when cacao was grown as a monoculture compared to agroforestry. Gramlich *et al.* (2017) suggested that the higher plant density in agroforestry systems may be the reason for the lower leaf Cd concentration in cacao plants in this cropping system. Higher plant density could be translated to higher competition for nutrients, water and light, which may slow down the growth rate of cacao. The authors proposed that plants with a lower growth rate take up less Cd, because lower growth rate infers lower nutrient uptake and Cd is usually taken up as a hitchhiker element along with essential nutrients such as Zn.

The effect of fertiliser application, i.e. organic vs conventional fertilisers, on Cd uptake in cacao has not been widely studied. Three studies presented the effect of fertiliser application on Cd uptake with inconsistent results. On one hand, Gramlich *et al.* (2017) found no significant

differences in soil and bean Cd concentrations between soils treated with either organic or conventional fertilisation, but this could be related to the low number of observations. On the other hand, Argüello et al. (2019) reported higher Cd concentrations in cacao beans from trees that received organic compared conventional fertilisers, whereas Zug et al. (2019) found higher Cd concentrations in cacao beans from trees that received conventional N-fertilisation but no effect of P-fertilisation. Even though the results are contradictory, both situations could be plausible. First, compost is the most common source of nutrients in organic farming. As compost is primarily made with vegetal residues, the quality of the compost product, i.e. amount of trace metals, is directly related to the composition of the raw materials. The quality of compost and the high rates of application could result in higher Cd concentration in organically managed cacao as reported by Argüello et al. (2019). Second, increased N application has been mentioned to enhance Cd uptake and accumulation in plants (Yang *et al.*, 2020). As N-fertilisation increases aboveground vegetative growth of plants, it stimulates active nutrient uptake and thus uptake of hitchhiker elements such as Cd will also increase. Additionally, N-fertilisation can lead to soil acidification and reduced soil pH can mobilise Cd, as explained above.

## 2.3 Uptake, translocation and partitioning of cadmium in the cacao tree

### 2.3.1 Soil-plant transfer of Cd in cacao compared to other plant species

Soil-plant transfer factors (TFs) allow identification of Cd accumulating characteristics, although they are influenced by genotype and external conditions such as soil Cd availability, as mentioned in section 2.2.2. Soil-plant TFs reported in cacao based on both leaves and beans are higher compared to TFs reported for most other crops (Table 2.3). The soil-leaf TFs for Cd in cacao are close to those reported for other woody species such as willow (Van Slycken, Witters, Meiresonne, Meers, *et al.*, 2013) and poplar (Laureysens *et al.*, 2004). The soil-bean TFs for cacao range between 2 and 6 depending on the study and are distinctly larger than TF reported for wheat and rice, but of similar magnitude as TFs reported for sunflower (Table 2.3). The Cd accumulation potential of the cacao plant is thus high, but not unusual. Cacao has been described as a Cd accumulator because of its high soil-plant TF, but this term lacks a clear definition. Several criteria indicate whether a plant can be classified as a Cd hyperaccumulator: (i) in natural conditions the plant should be able to accumulate  $\geq 100 \text{ mg Cd kg}^{-1}$ ; (ii) both the TF and the internal shoot-to-root translocation factor ( $ITF_{shoot-root} = Cd_{shoot}/Cd_{root}$ ) should be larger than one; and (iii) extreme metal tolerance should be achieved through efficient biochemical detoxification (van der Ent *et al.*, 2013). While cacao does not meet the first criterion,  $TF > 1$  have been reported (Table 2.3). The average Cd concentrations in the vegetative tissues of the cacao plant reported in different field studies are collated in Table 2.4, and these data suggest a relatively homogeneous

Cd distribution between roots, scions and leaves. Engbersen et al. (2019) reported a similar  $ITF_{\text{stem-root}}$  for cacao (0.99) compared to  $ITF_{\text{stem-root}}$  reported for known moderately Cd accumulating woody species, i.e.  $ITF_{\text{stem-root}} \approx 1$  in poplar and cotton (Vollenweider *et al.*, 2011; Chen *et al.*, 2015). To decide on the third criterion and to allow classification of cacao, additional research on the speciation of Cd in cacao tissues is crucial to elucidate the molecular detoxification mechanisms. Nonetheless, the Cd uptake potential of the cacao tree can be conceived as rather high compared to other agricultural crops, and more similar to woody species used for phytoextraction such as willow and poplar. Therefore, it is proposed to term cacao a moderate Cd accumulator.

It is unlikely that the accumulation of Cd in cacao results in Cd toxicity in the field. Different critical Cd toxicity levels have been proposed for various crops in mature leaves, e.g. 6 – 10 mg Cd kg<sup>-1</sup> (Krämer, 2010) or 5 – 30 mg Cd kg<sup>-1</sup> (Kabata-Pendias, 2010). Leaf Cd concentrations in cacao exceed 10 mg Cd kg<sup>-1</sup> in only 3 % of the data included in the meta-analysis, suggesting that Cd toxicity to cacao plants is unlikely to occur at a large scale. There is only one study available to date discussing Cd toxicity in cacao seedlings (Pereira de Araújo *et al.*, 2017). However, the soils in that study were spiked to high Cd concentrations (50 and 100 mg Cd kg<sup>-1</sup> soil) and the reported leaf Cd concentrations (230 – 390 mg kg<sup>-1</sup>) were not in the range of leaf Cd concentrations found in most cacao fields.

**Table 2.3:** Average cadmium transfer factors (TF) (min-max) for cacao and other selected agricultural crops, i.e. Cd concentration ratios between leaf or seed (e.g. cacao bean) and soil. The TFs predicted from soil-plant regression models used median values of the soil properties from the studies that yielded the corresponding models. Additional information regarding specific study conditions (e.g. study location and soil Cd concentrations) can be found in Appendix I (Table I.4).

<b>TF calculated as concentration ratio</b>				
<b>Plant</b>	<b>TF (leaf – soil)</b>	<b>TF (seed – soil)</b>	<b>n</b>	<b>Reference</b>
Cacao	4.5 (0.34–42.8)	1.6 (0.13–12.5)	560	(Argüello <i>et al.</i> , 2019)
	3.4 (0.56–20.1)	2.0 (0.26–7.8)	28	(Barraza <i>et al.</i> , 2017)
	4.9 (1.9–29.2)	5.6 (1.4–29.8)	60	(Engbersen <i>et al.</i> , 2019)
	7.1 (1.9–21.1)	2.7 (0.5–16.3)	108	(Gramlich <i>et al.</i> , 2018)
Willow	(1.6–10.3)		8	(Van Slycken <i>et al.</i> , 2013)
Oak	0.26 <sup>§</sup>		2	(Sevel <i>et al.</i> , 2009)
Poplar	(5.0–40.0)		13	(Laureysens <i>et al.</i> , 2004)
Leafy vegetables	0.19 (0.001–4.5)		170	(Zhang <i>et al.</i> , 2014)
Sunflower		2.2 (1.8–3.4)	200	(Li <i>et al.</i> 1995)
Cotton		0.46 <sup>§</sup>	6	(Chen <i>et al.</i> , 2015)
Wheat	0.27 (0.06–0.64)	0.15 (0.03–0.34)	40	(Puschenreiter and Horak, 2000)
Rye	0.11 (0.03–0.29)	0.04 (0.01–0.16)	40	(Puschenreiter and Horak, 2000)
Pistachio		0.03 (0.01–0.04)	220	(Shirani <i>et al.</i> , 2017)
<b>TF calculated from predicted crop Cd concentration model based on soil properties</b>				
<b>Plant</b>		<b>TF (seed – soil)</b>	<b>n</b>	<b>Reference</b>
Cacao		1.76	334	This chapter
Indica rice		0.76	1043	(Römken <i>et al.</i> , 2009)
Japonica rice		0.30	2155	(Römken <i>et al.</i> , 2009)
Wheat		0.27	246	(Adams <i>et al.</i> , 2004)

<sup>§</sup>No range available, only average is given.

### 2.3.2 Uptake and translocation of Cd within the cacao plant: trends and mechanisms

Cadmium is known as a hitchhiker element, using transporters for essential elements such as Zn, Fe, Mn and Ca at all steps of these nutrient pathways, from root uptake to grain or seed loading (Clemens and Ma, 2016). Important transporter gene families for the uptake and translocation of Cd in plants are Zinc-Iron Permease (ZIP), Natural Resistance Associated Macrophage Proteins (NRAMPs) and Heavy Metal transporting ATPases (HMAs). These proteins transport divalent transition metals such as Fe(II), Zn, Mn and Cd. The limited information available to date regarding the role of these transporter genes for Cd uptake in cacao is discussed below. For a more profound synthesis about the genetic and molecular mechanisms of Cd uptake in plants in general, the reader is referred to Chapter 1 and specialised reviews by (Clemens and Ma, 2016; Shahid *et al.*, 2016; Sterckeman and Thomine, 2020). The identification of key genes involved in Cd uptake and translocation in cacao is of great importance as it can be used to develop mitigation strategies by

using these genes as targets for genetic engineering, or through marker-assisted breeding programs.

### 2.3.2.1 Uptake of Cd in the cacao root system

Ullah *et al.* (2018) identified five genes from the NRAMP family in cacao and demonstrated that TcNRAMP5 can encode for a protein which transports Cd, Mn(II) and Fe(II). It has been reported that NRAMP5 plays a role in the entry of Cd in the root cells for rice (Ishikawa *et al.*, 2012; Sasaki *et al.*, 2012), barley (Wu *et al.*, 2016), Polish wheat (Peng *et al.*, 2018) and tobacco (Tang *et al.*, 2017). More specifically, Ullah *et al.* (2018) showed that the TcNRAMP5 transcript was expressed in the roots of cacao seedlings. Usually, the expression of NRAMP transporter genes is up-regulated under divalent metal deficiency. In cacao seedlings, expression of the TcNRAMP5 transporter genes in the roots was down-regulated in the presence of Cd and up-regulated under Fe deficiency, while Zn or Mn deficiency did not affect gene expression. To link the expression pattern to its function, Ullah *et al.* (2018) cloned five TcNRAMP genes and expressed them in yeast strains. The yeast cells expressing TcNRAMP5 accumulated up to three times more Cd compared to the empty vector control cells. Considering the above, TcNRAMP5 likely plays a major role in the regulation of Cd uptake in cacao plants (Figure 2.4) and may be targeted for the development of mitigation strategies through induction of loss-of-function mutations. Yet, extrapolation of these findings to relevant cacao soil-plant systems is limited. First, activities used in the hydroponic experiment are about 100 times higher than natural soil Cd concentrations. The observed expression pattern may thus differ from natural field conditions. Second, uptake in yeast may not be suitable to predict uptake in cacao plants.

The isotopic signature of Cd in plants is increasingly used to infer biogeochemical processes but only few studies have reported Cd isotope fractionation data for cacao. Several processes can generate stable isotope fractionation during the transfer of Cd from soil to plant tissues, such as membrane transport or chemical binding (see Chapter 1). (Moore *et al.*, 2020) compared the isotopic shift of TcNRAMP5 transporter expressed yeast relative to the empty vector control cells ( $\Delta^{114/110} \text{Cd}_{\text{trans-ev}} \approx -0.8 \text{ ‰}$ ), with the isotopic shift in the cacao seedlings relative to the hydroponic solution ( $\Delta^{114/110} \text{Cd}_{\text{tot-sol}} = -0.22 \pm 0.08 \text{ ‰}$ ). Because both systems showed a shift toward lighter isotopes, and because the expressed TcNRAMP5 proteins were localised in the external plasma membrane of the yeast cells, the authors confirmed that TcNRAMP5 transporters can be a major pathway for Cd uptake in cacao. However, these observations cannot exclude the possibility that other transporters can also be involved in Cd uptake. Compared to other species, the total isotopic fractionation due to absorption of Cd from hydroponic solutions is less pronounced in cacao seedlings, but it is similar to the isotopic shift reported for Cd tolerant and

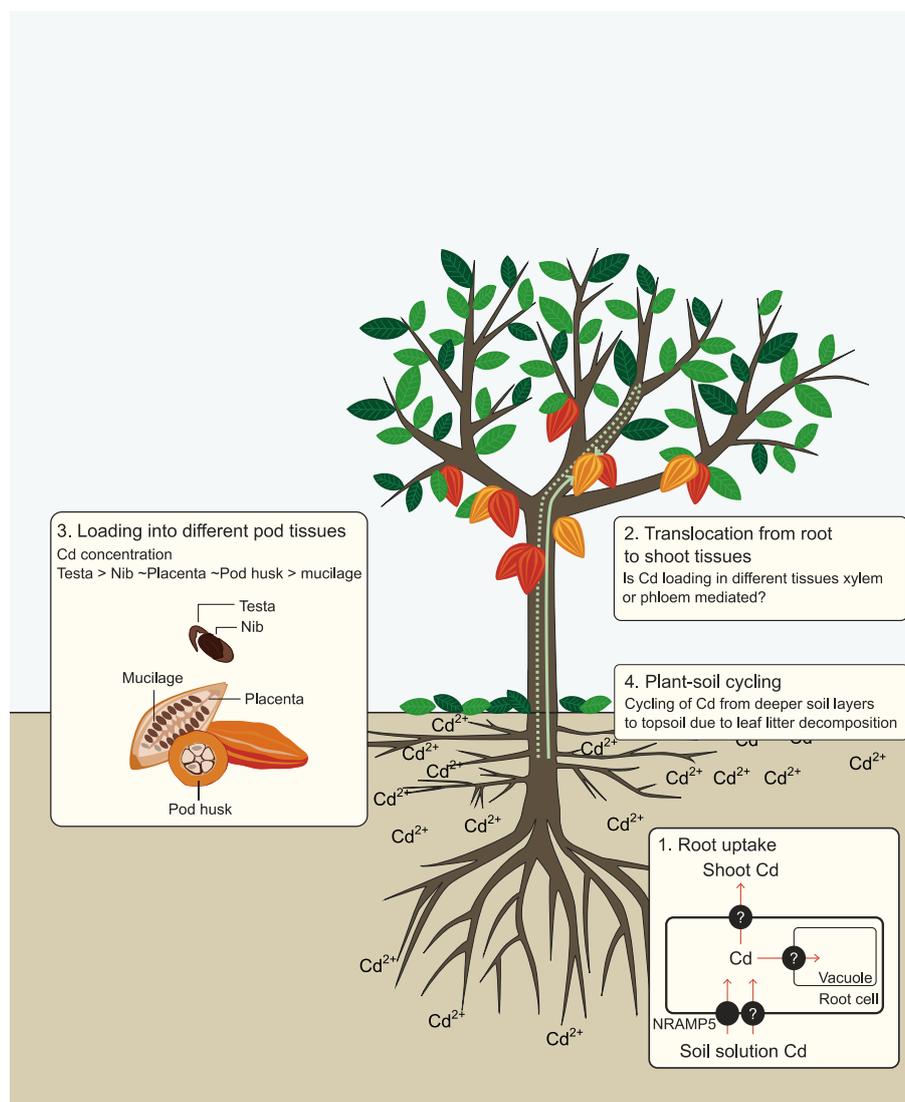
accumulator species (Wei *et al.*, 2016). The reported fractionation towards lighter isotopes is similar to the fractionation from soil solution to plants in cereals and rice, although there is some variation in the rate of fractionation (Table 2.5). It is noteworthy that the cacao plants used in the hydroponic study of Moore *et al.* (2020) had leaf Cd concentrations of about 200 mg Cd kg<sup>-1</sup> which far exceeds the Cd toxicity threshold of 10 mg Cd kg<sup>-1</sup> and the relevant concentrations in the field (1 – 10 mg Cd kg<sup>-1</sup>). This may have influenced the Cd uptake and translocation system due to activation of internal systems to cope with Cd toxicity.

Experiments with identification or induction of loss-of-function mutations in the TcNRAMP5 gene are indispensable to confirm the role of TcNRAMP5 for Cd uptake in cacao. Moreover, other proteins that were previously identified as major Cd transporters in other crops [e.g. AtIRT1 in *A. Thaliana*, and OsIRT1 and OsIRT2 in rice (McLaughlin *et al.* 2021)] should be studied, since information regarding the role of these transporters in cacao is not available to date. Knowledge on such transporters is important to predict potential effects of soil Zn concentrations on Cd uptake. Recent work with cacao seedlings showed that leaf Cd concentrations can be reduced by soil Zn addition (Souza dos Santos *et al.* 2020), but these effects were only observed in soils spiked to Cd concentrations well above the environmentally relevant range. Follow-up experiments with more realistic soil Cd concentrations are required.

### 2.3.2.2 Partitioning and translocation of Cd inside the cacao plant

From plant roots, Cd is generally transported radially across the root cells and loaded into the xylem, followed by long-distance transport via xylem and phloem and transfer into the plant organs (Clemens and Ma, 2016). The rate of translocation of Cd from roots to above-ground tissues depends on vacuolar sequestration, xylem loading, and intervascular and xylem-to-phloem transfer (Figure 2.4). Cadmium can be sequestered in vacuoles during the radial transport through the root cells, as a protective measure to reduce Cd translocation to other tissues. A key transporter in this vacuolar sequestration is the Heavy Metal transporting ATPase 3 (HMA3) influx transporter, which is located at the membrane of vacuoles and which has been proven to control the sequestration of Cd in the roots of rice (Wang *et al.*, 2019), soybean (Wang *et al.*, 2012), and Chinese cabbage and pak choi (L. Zhang *et al.*, 2019). For a discussion of the further translocation of Cd, loading in the xylem, root-to-shoot translocation and intervascular transfer in plants in general, the reader is referred to Chapter 1, and the reviews by Clemens and Ma (2016), McLaughlin *et al.* (2021) and Sterckeman and Thomine *et al.* (2020). The role of the HMA family in Cd sequestration in cacao has not yet been determined to date. Moore *et al.* (2020) transformed yeast with HMA family transporters to compare the isotope fractionation pattern due to Cd sequestration in cacao seedlings. The data were, however, not conclusive on the specific role of

HMA transporters in controlling Cd transport in cacao seedlings. In both rice and durum wheat, root-to-shoot Cd translocation via the xylem has been identified as a major determinant for shoot Cd accumulation (Harris and Taylor, 2004; Uraguchi *et al.*, 2009). These observations highlight the need for studies focused on unravelling the mechanisms for Cd translocation from roots to aboveground tissues.



**Figure 2.4:** Uptake and translocation of Cd within the cacao plant-soil system. (1) Cadmium uptake in a simplified cacao root cell. TcNRAMP5 has been identified as an important transporter for Cd uptake from soil solution, but it is likely not the only transporter. Potential further pathways include sequestration in vacuoles and root-to-shoot translocation. (2) Translocation of Cd from root to cacao pod may occur through direct uptake from the xylem or through remobilisation of Cd from the leaves and transportation through the phloem. (3) Loading of Cd in the different pod tissues. (4) Cycling of Cd from deeper soil layers to the topsoil through leaf litter decomposition.

**Table 2.4:** Average (min-max) dry weight-based Cd concentrations (mg Cd kg<sup>-1</sup>) for different cacao plant tissues, only including unfermented cacao beans. Dashes indicate that the parameter was not reported for this specific study.

Reference	Tissue Cd (mg kg <sup>-1</sup> )					
	Root	Scion	Leaf	Nib	Testa	Pod husk
Argüello et al. (2019)	/	/	2.62 (0.13–55.5) n = 560	0.90 (0.03–10.4) n = 560	/	/
Barraza et al. (2017)	/	/	1.99 (0.19–7.9) n = 28	0.90 (0.09–3.5) n = 28	/	0.98 (0.08–4.4) n = 31
Engbersen et al. (2019)	2.44 (0.68–9.70) n = 60	2.25 (0.41–13.0) <sup>§</sup> n = 60	2.31 (0.64–9.3) n = 60	2.56 (0.81–10.6) n = 60	/	/
Gramlich et al. (2018)	/	/	2.64 (0.06–28.0) n = 110	1.06 (0.03–7.1) n = 109	/	1.12 (0.04–10.2) n = 108
Lewis et al. (2018)	/	/	2.18 (0.48–5.2) n = 198	0.99 (0.17–2.3) n = 137	/	/
Ramtahal et al. (2016)	/	/	3.56 (0.45–17.4) n = 482	2.27 (0.48–9.3) n = 402	2.55 (0.33–15.4) n = 441	2.55 (0.46–8.0) n = 241

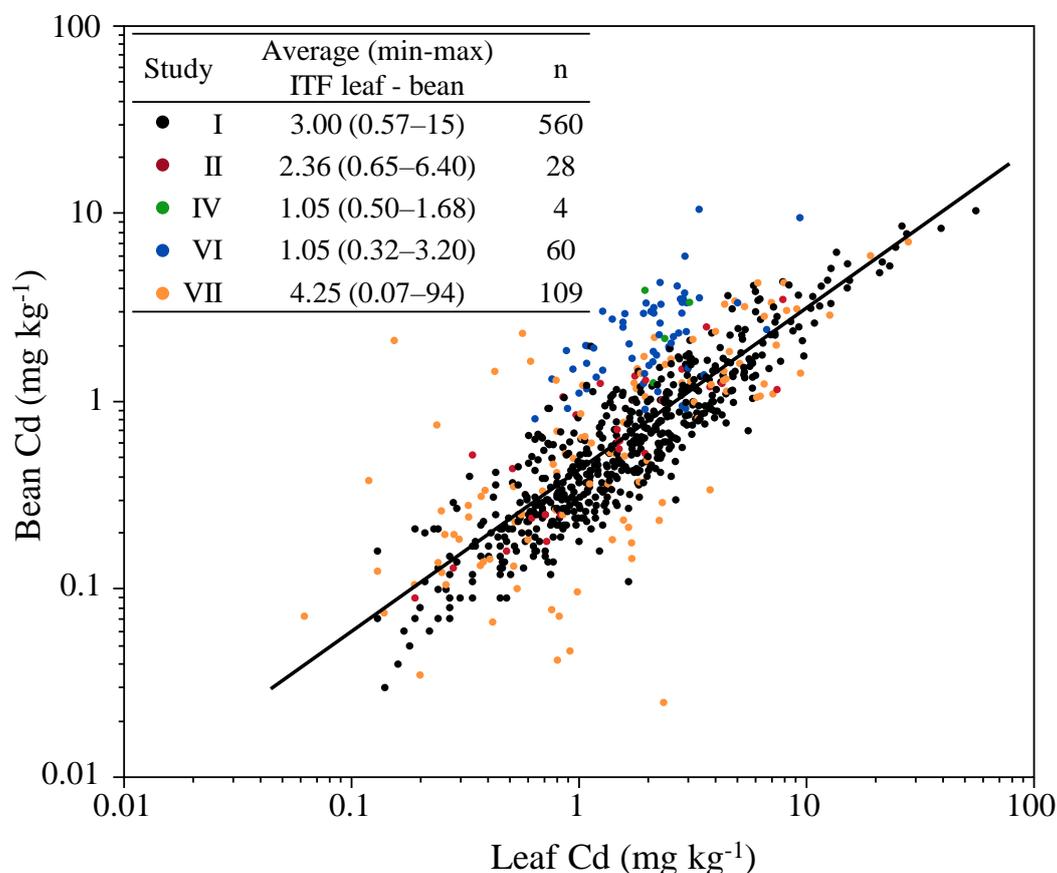
<sup>§</sup> Analysis of scion wood cores

An approach to study the translocation of Cd within plants, is to quantify isotope discrimination among different tissues (Table 2.5). In the hydroponic system of Moore et al. (2020), cacao leaves were strongly enriched in heavy Cd isotopes compared to the roots ( $\Delta^{114/110} \text{Cd}_{\text{leaf-root}} = 0.26 \text{ ‰}$ ). Similar hydroponic studies with *Ricinus communis* and *Solanum nigrum* (Wei et al., 2016) showed a less systematic fractionation than reported in the cacao study of Moore et al. (2020). This may indicate that cacao seedlings exert mechanisms to cope with Cd that differ from the coping mechanisms found in Cd accumulator plants. A larger fractionation between roots and aboveground tissues may indicate that additional Cd retention mechanisms are invoked to inhibit the translocation of Cd to aerial plant parts, as discussed before. In most plants, Cd becomes enriched in heavy isotopes in the order roots < stem < leaf < seed (Table 2.5).

Cacao is a cauliflorous tree and mechanisms and pathways for Cd loading into the developing cacao beans thus may be different from other plant species discussed in chapter 1. First, it was suggested that Cd can be transported directly from roots to fruit (Barraza et al., 2019, Engbersen et al., 2019), rather than indirectly via redistribution from the leaves as in most other plants. Engbersen et al. (2019) reported that bean Cd concentrations correlated more strongly with Cd concentrations in the scion and rootstock, than with Cd concentrations in the leaves. The authors suggested substantial direct loading of Cd from the stem to the beans with a transfer from xylem to phloem at the level of the fruit, without first passing through the leaves. Additional evidence for direct xylem loading came from a study on the natural stable isotope signature of Cd in cacao (Barraza et al., 2019). This pilot field study in Ecuador reported an average trend towards lighter isotopes in the cacao beans compared to the leaves ( $\Delta^{114/110} \text{Cd}_{\text{bean-leaf}} = -0.27 \text{ ‰}$ ). That different isotopic signature may be related to the cauliflorous character of cacao, and hints again to the hypothesis that Cd is directly transferred from xylem to phloem in developing cacao beans, without first passing through the leaves. However, a recent stable isotope ( $^{108}\text{Cd}$ ) pulse-chase experiment showed that there was a rather slow rise in  $^{108}\text{Cd}$  in the beans compared to the leaves. This may suggest that Cd is not directly loaded in to the nibs, but that Cd may be re-allocated from other tissues to some extent (Vanderschueren, Wantiez, et al., 2023). This study was nonetheless limited to one environment (soil/climate) and one cultivar, grafted on an unknown rootstock.

Most studies report leaf Cd concentrations to be higher than corresponding bean Cd concentrations (Table 2.4; Figure 2.5). The data from the meta-analysis (section 2.2.2) shows a markedly positive correlation between bean and leaf Cd concentrations, with average ITF<sub>leaf-bean</sub> ranging from 1.1 to 4.2 among studies (Figure 2.5). This clear correlation may justify the use of leaf Cd concentration measurements as a proxy for bean Cd because leaf sampling is convenient as it does not depend on fruit availability. However, the use of leaf Cd measurements as a proxy for bean Cd may only be justified when considering a genetically homogeneous sample set, as both

Engbersen et al. (2019) and Lewis et al. (2018) reported only moderate correlations between leaf and bean Cd concentrations in their genetically diverse studies.



**Figure 2.5:** Cadmium concentrations in cacao leaves and beans are significantly correlated (Pearson correlation coefficient  $r = 0.82$ ) and Cd concentrations are generally higher in leaves compared to beans. Studies III and V are not included as they did not include leaf Cd measurements. Details of the studies can be found in Appendix I. ITF = internal transfer factor calculated as the ratio of leaf Cd over bean Cd concentrations.

### 2.3.2.3 Distribution of Cd within the cacao fruit

Inside the cacao fruit, reported average Cd concentrations are 0.90 – 2.56 mg kg<sup>-1</sup> in the nibs, 0.99 – 2.55 mg kg<sup>-1</sup> in the testae and 0.98 – 2.55 mg kg<sup>-1</sup> in the pod husks (Table 2.4). There is no data available thus far regarding the Cd content of the mucilage. Lewis et al. (2018) studied the partitioning of Cd between testa and nib, and found that testa Cd concentrations were, on average, twice as large as nib Cd concentrations. However, the authors reported that the concentration ratio (testa over nib Cd) varied depending on the genotype, ranging from 1.2 to >7. The testa

accounts for only a small part of the total bean weight and is thus likely also responsible for only a small fraction of total bean Cd, despite its elevated Cd concentration.

The outer pod husk has been reported to be a storage organ providing nutrients to the developing cacao beans through the mucilage (Toxopeus 1985), but its role in Cd transport to the cacao beans is not known thus far. Several studies have compared Cd concentrations between nibs and pod husks, and all reported similar Cd concentrations in both tissues, i.e.  $ITF_{\text{husk-nib}}$  close to one (Ramtahal et al. 2016; Barraza et al. 2017; Gramlich et al. 2018). It has been reported that different nutrient distribution pathways exist between cacao pod husks and beans (De Araujo *et al.*, 2020). Engbersen et al. (2019) stated that Cd partitioning within the cacao fruit may change during maturation of the fruit on the tree. Their results showed decreasing Cd concentrations in the pod husk and increasing Cd concentrations in the nibs with maturation, which indicates that there may be remobilisation of Cd occurring from the husk to the bean. However, additional research is required to corroborate this hypothesis, as the observed differences may have been related to the variation in Cd concentration among fruits on one tree, rather than to an effect of maturation.

**Table 2.5:** Average (min/max) or average  $\pm$  stdev apparent Cd isotope fractionation ( $\Delta^{114/110}Cd_{A-B} = \delta^{114/110}Cd_A - \delta^{114/110}Cd_B$ ) (‰) for different plants. Positive values indicate that tissue A is enriched in heavy isotopes compared to tissue B.

Plant	$\Delta^{114/110}Cd_{A-B}$ (‰)							Reference
	Plant-substrate	Plant-soil	Shoot-root	Leaf-stem	Leaf-root	Grain-stem	Grain-leaf	
Hydroponics								
<i>Ricinus communis</i>	-0.38 <sup>a</sup> (-0.46/-0.32)		0.03 (-0.08/0.19) <sup>s</sup>	-0.11 (-0.18/-0.04)	-0.05 (-0.26/0.15)			(Wei <i>et al.</i> , 2016)
<i>Solanum nigrum</i>	-0.41 <sup>a</sup>		0.02 <sup>s</sup>	0.09	0.11			(Wei <i>et al.</i> , 2016)
Cacao	-0.22 <sup>a</sup> (-0.34/0.01)				0.33 (0.13/0.9)			(Moore <i>et al.</i> , 2020)
Pot experiments								
Wheat	-0.07 <sup>b</sup> (-0.21/0.03)	0.23 (0.13/0.39)	0.29 (0.21-0.41)			0.30 (0.1/0.5) <sup>s</sup>		(Wiggenhauser <i>et al.</i> , 2016)
Wheat	(-0.36/-0.20) <sup>c</sup>	(0.31/0.46)	0.26 (0.19/0.35)			0.32 (0.16/0.46) <sup>s</sup>		(Imseng <i>et al.</i> , 2019)
Barley	(-0.1/-0.06) <sup>c</sup>	(0.51/0.55)	0.34 (0.27/0.45)			0.59 (0.44/0.82) <sup>s</sup>		(Imseng <i>et al.</i> , 2019)
Rice	-0.30 $\pm$ 0.01 <sup>c</sup>		0.16 $\pm$ 0.03					(Wiggenhauser, A. M. Aucour, <i>et al.</i> , 2021)
Rice	-0.43 $\pm$ 0.01 <sup>c</sup>		-0.02 $\pm$ 0.05					(Wiggenhauser, A. M. Aucour, <i>et al.</i> , 2021)
Rice			0.28	0.19 <sup>\$\$</sup>	0.44 <sup>\$\$</sup>		0.51	(Wiggenhauser, A. Aucour, <i>et al.</i> , 2021)
Field experiments								
Cacao	0.34 (0.22/0.41)						-0.27 (-0.40/-0.08)	(Barraza <i>et al.</i> , 2019)
Rice	0.45							(Zhang <i>et al.</i> , 2020)

<sup>s</sup>Shoot = stem; <sup>\$\$</sup>Leaf = flag-leaves; <sup>a</sup> Source: Hydroponic solution; <sup>b</sup> Source: Ca(NO<sub>3</sub>)<sub>2</sub> soil extract; <sup>c</sup> Source: Soil solution.

### 2.3.2.4 Cultivar-related differences in Cd uptake and partitioning

The cultivar effect on Cd uptake in cacao was first reported in 2018, with a factor 13 variation in bean Cd and a factor 7 variation in leaf Cd concentrations observed among cultivars grown on the field (Lewis *et al.*, 2018). The data suggest a differential partitioning of Cd between vegetative (leaf) and reproductive (bean) tissues, favouring the hypothesis that there are cultivar-specific differences in xylem-to-phloem transfer of Cd to those different tissues. In their genetically diverse database, Engbersen *et al.* (2019) reported that available soil Cd was closely related to Cd in vegetative parts ( $R^2 = 0.50$ ) but to a minor extent to bean Cd ( $R^2 = 0.26$ ), which was attributed to a cultivar effect in the loading of Cd into the beans. The authors used this as indirect evidence suggesting that cultivar-related differences in bean Cd concentrations are mainly related to cultivar-specific differences in xylem-to-phloem transfer. While Cd transfer to the leaves is probably mostly governed by the xylem, the contributions of either the xylem or the phloem pathway for bean Cd loading are yet to be revealed and may differ among cultivars. However, the cacao trees in the study of Engbersen *et al.* (2019) were grafted onto rootstocks of unknown genetic identity, which may have affected the uptake of metals and the further translocation from the root, obscuring the cultivar effect. The design of that study also did not allow to account for potential effects of local spatial variability, which may have influenced Cd uptake and partitioning. Barraza *et al.* (2019) reported a genotype effect in the isotopic fractionation between cacao leaves and beans (Nacional:  $\Delta^{114/110} \text{Cd}_{\text{bean-leaf}} = -0.34$  to  $-0.40$  ‰, CCN 51 hybrid:  $\Delta^{114/110} \text{Cd}_{\text{bean-leaf}} = -0.08$  ‰). Similarly, distinctly different isotope fractionation patterns have also been reported between shoots and roots of an excluder ( $\Delta^{114/110} \text{Cd}_{\text{shoot-root}} = 0.19$  ‰) and a non-excluder type of rice ( $\Delta^{114/110} \text{Cd}_{\text{shoot-root}} = -0.02$  ‰) (Wiggenhauser *et al.*, 2020). The consistent isotope fractionation between cacao leaves and soil reported by Barraza *et al.* (2019) is intriguing and indicates clear differences in the soil-bean fractionation among cultivars. This confirms the previous hypothesis that the translocation and sequestration of Cd in different cacao tissues varies among cultivars. This pilot study has some limitations due to the limited number of observations but confirms that the isotope approach can provide insights on Cd pathways in the cacao plant and pave the road for further studies in the field.

## 2.4 Mitigation strategies to lower the cadmium concentration in the final product

The current Cd-cacao regulations apply to the final product, not the cacao beans. Therefore, mitigation strategies can be applied at all stages of the production process, from tree to chocolate bar. Mitigation practices at different production steps will likely have to be combined to achieve a final product that complies with the new EU regulations. Mitigation practices can be based on soil amendments to reduce Cd uptake into the plant; plant-based strategies, i.e. selection of cultivars

with reduced translocation of Cd to the cacao beans; or postharvest processing. Agronomic practices have also been suggested to influence Cd concentrations in cacao but additional research on their potential effects is required (see section 2.2.3).

### 2.4.1 Mitigating Cd uptake in cacao using soil amendments

While several soil amendment techniques exist, lime, biochar, gypsum and Zn supplementation are the only amendments discussed here, as these are the only amendments that have been or are being investigated for the mitigation of Cd in cacao. A reduction factor (RF), defined as the ratio between the crop Cd concentration in the control (no treatment) and the Cd concentration in the treatment was calculated to quantify the effect of each amendment. A RF > 1 indicates that crop Cd was reduced by the treatment.

#### 2.4.1.1 Lime

The application of liming materials [i.e.  $\text{CaCO}_3$ ,  $\text{CaO}$ ,  $\text{Ca(OH)}_2$  or  $\text{CaMg(CO}_3)_2$ ] has often been recommended as an agronomic practice to reduce the uptake and accumulation of Cd in edible parts of crops. Liming decreases the solubility of soil Cd and thus decreases available soil Cd, by increasing the soil pH and through competition between  $\text{Ca}^{2+}$  and  $\text{Cd}^{2+}$  at root uptake sites (Christensen, 1984; Bolan, Adriano and Curtin, 2003). However, increasing the solution concentration of  $\text{Ca}^{2+}$  can also result in competitive desorption of surface bound  $\text{Cd}^{2+}$  (Christensen, 1984). The latter is one of the plausible reasons why liming does not consistently decrease Cd concentrations in plant tissues (Bolan, Adriano, Duraisamy, *et al.*, 2003). Significant reductions in crop Cd by the application of lime have been reported in field experiments with paddy rice in China: the application of  $7.5 \text{ Mg ha}^{-1} \text{ CaCO}_3$  led to RF 3 – 4, and this was sufficient to also have an effect on rice grain Cd grown in the 2<sup>nd</sup> year crop (H. Chen *et al.*, 2018); while application of  $1.5 \text{ Mg ha}^{-1}$  burned lime (75 %  $\text{CaO}$ ) resulted in a RF of 1.5 (Zhu *et al.*, 2016). However, it is unclear if such high RFs can also be reached with lime application for cacao.

A recent study by Ramtahal *et al.* 2019 evaluated the effect of  $\text{Ca(OH)}_2$  application at different rates ( $0 - 6 \text{ Mg ha}^{-1}$ ) on Cd uptake in cacao plants grown in pots (6 month old cuttings) and in the field (30 year old trees). Results from the pot trial showed effectively reduced leaf Cd concentrations 6 months after lime application, with a RF of 3. In the field trial, however, leaf Cd concentrations initially decreased by a factor 2 (measured 4 months after lime application), after which they started to increase in both control and limed treatments so that for the last sampling at 9 months after lime application, leaf Cd in the limed treatment was only reduced by a factor 1.1. Interestingly, soil pH remained at the target pH of 7.0 up until the 9<sup>th</sup> month after application.

Similar contrasts in liming effects between field trials and pot experiments have been reported previously. Earlier work conducted in potatoes (Maier *et al.*, 1997; Sparrow and Salardini, 1997), sunflower kernels (Li *et al.*, 1996) and peanuts (McLaughlin *et al.*, 1997) suggested the difficulty in fully incorporating lime to deeper soil layers as an explanation for the lack of effect and/or limited effectiveness of liming in reducing crop Cd concentrations in the field. The lack of effect of liming in the cacao field trial may thus be related to Cd being taken up by deeper rooted soil horizons that were not affected by the lime treatment. In established plantations, lime incorporation to deeper soil horizons is not possible without damaging the root system, which may compromise the efficacy of the amendment. Argüello *et al.* (2020) showed in a pot experiment with cacao seedlings that liming the topsoil compartment only, as would be the case in field liming, enhanced Cd uptake from the non-limed bottom compartment.

#### 2.4.1.2 Gypsum

The effectiveness of lime in reducing soil Cd availability to cacao is limited by its shallow penetration into the soil. Amendments such as gypsum may be able to overcome such limitations because gypsum can reach deeper soil layers. The mechanism by which gypsum reduces soil Cd availability is yet to be revealed; it is not a liming material and thus does not increase the soil pH. Nevertheless, studies have shown that adding gypsum as a soil amendment reduces Cd uptake in different crops: RF 1.9 with application of 3 % (w/w) in a pot trial with *Angelica gigas* (Kim *et al.*, 2018); RF 2.5 with application of 0.15 g S kg<sup>-1</sup> soil in a pot experiment with rice (D. Zhang *et al.*, 2019); and RF 2 – 3 with application of 0.8 % (w/w) in a field trial with wheat and rice (Rehman *et al.*, 2015). Based on the data available in other crops, gypsum might be a plausible amendment to ameliorate Cd accumulation in cacao beans. However, research is needed to reveal the potential of gypsum as a soil amendment for Cd mitigation in cacao.

#### 2.4.1.3 Biochar

Biochar has received a lot of attention as an effective material for soil immobilisation of metals such as Cd. The sorption properties of this carbon rich material are related to its high specific surface area, high cation exchange capacity (CEC), its alkaline pH and the presence of carboxylic, hydroxyl or phenolic surface functional groups (Li *et al.*, 2017). Because of the complex composition of biochar, the exact mechanism of metal sorption is not yet completely understood. However, results from laboratory experiments indicate that, at least in the short term, metal immobilisation is controlled by an increase in soil pH due to alkalisation processes and by intra-particle diffusion of the elements within the biochar pores (Rees *et al.*, 2014). The effectiveness of biochar application also depends on soil properties, the type of crop and the type of biochar used.

According to the meta-analysis of D. Chen *et al.* (2018), positive effects in reducing plant Cd are expected when biochar is applied in soils with acid pH, coarse texture and intermediate organic carbon content.

In cacao, Ramtahal *et al.* (2019) evaluated the effect of a commercially available biochar (charcoal green®) on the phytoavailability of soil Cd in pot and field experiments. Biochar was applied at rates of 0, 326, 489, 652 kg ha<sup>-1</sup> and the materials were incorporated at 20 cm depth. In the greenhouse study, biochar significantly increased the soil pH by about one pH unit and reduced the Cd concentration in the leaves of cacao seedlings, and this effect was dose dependent. Results from the field experiment with 30-year-old cacao trees showed that biochar did not increase the soil pH in contrast to the pot trial. Biochar applied at the highest dose in the field trials reduced leaf Cd by a factor 1.9 compared to the control, and the effect was more consistent over time than the effect of lime (last sampling done 6 months after biochar application). Results of studies with other crops are promising but the effectiveness of the treatment varies: RF 2 with application of 20 – 40 Mg ha<sup>-1</sup> in a field study with rice and the effect of biochar application on grain Cd could still be observed three years after application to the soil (Bian *et al.*, 2013, 2014); and RF 1.2 determined one year after biochar application with doses up to 40 Mg ha<sup>-1</sup> in a field study with wheat on alkaline soil, but this effect was no longer present or highly variable in the second and third year after application (Sui *et al.*, 2018). Such high application rates of soil amendments would likely not be cost effective in cacao farming.

#### 2.4.1.4 Fertiliser management: Zn supplementation

Although no studies in cacao to date have reported the effect of Zn fertilisation in reducing Cd uptake in the field, this topic is briefly described here because, based on information collected in other crops, the application of soil or foliar Zn can effectively reduce Cd concentrations in crops. The effect of Zn fertilisation on crop Cd depends on the status of Zn in the soil, with largest effects reported in Zn deficient soils: RF 2 with application of up to 5 kg Zn ha<sup>-1</sup> in wheat grain (Oliver *et al.*, 1994); RF 2 with application of 13 kg Zn ha<sup>-1</sup> in rice (Fahad *et al.*, 2015); and a linear decrease in Cd concentration in grain and shoots of yellow lupin with increasing Zn doses up to 6.4 kg Zn ha<sup>-1</sup> (Brennan and Bolland, 2014). It has been suggested that the effect of Zn application on plant Cd can be explained by competition between Zn and Cd at the root uptake sites, or that the application of Zn reduces the Zn deficiency of the plant, thereby preventing loss of integrity of the root cells which can facilitate non-selective Cd uptake through mass flow (Grant *et al.*, 1999). In studies conducted in soils where soil Zn levels were not deficient, Zn fertilisation did not significantly affect the Cd concentration in wheat grain (Grant and Bailey, 1998; Rojas-Cifuentes *et al.*, 2012; Forster *et al.*, 2018). An exception is the study on potatoes by McLaughlin *et al.* (1995),

which showed that the addition of very large doses of Zn at planting (up to 100 kg Zn ha<sup>-1</sup>) in non-deficient soils could significantly decrease Cd in the tuber (RF 2) in one of the studied sites.

#### 2.4.1.5 Factors complicating the use of soil amendments to remediate Cd in cacao

*Theobroma cacao* L. is a large perennial tree and it is thus essential to test the efficacy of soil amendments in field conditions. The root system of the cacao tree is most dense in the near-surface soil (Nygren *et al.*, 2013; Niether *et al.*, 2019), which complicates effective incorporation of soil amendments. The poorly understood complexity of rhizosphere processes in the roots of cacao may also explain the lack of observed effect for some soil amendments and additional research is needed to better understand how cacao roots shape the local biochemical environment in the rhizosphere. In addition, high spatial variability is a major obstacle to detect effects of soil treatments on bean Cd concentrations in the field. A power analysis was performed based on the variation in bean Cd within fields reported by Argüello *et al.* (2019) (Appendix I). Beans from only 5 trees per treatment are required to identify a statistically significant effect of treatment if a reduction factor (RF) of 2 is expected and if the field has an average spatial variability. For fields with a realistic worst-case variability, that number increases to 15 trees per treatment. In contrast, an unfeasibly large number of 198 trees are required to identify significant effects if the treatments yield only a RF of 1.2 and if the variability is a realistic worst case. A RF of 1.5 can have large consequences on the economy of the cacao sector but it is clear that detection of such moderate but realistic RFs is challenged by soil and bean Cd variability within fields.

#### 2.4.2 Genetics as a potential mitigation strategy for Cd in cacao

The research on the potential of genetic mitigation strategies, i.e. traditional genetic selection, plant breeding techniques, marker-assisted molecular breeding and/or genetic engineering, is still in an early phase as the genetic and molecular mechanisms for Cd uptake and partitioning in cacao are yet to be revealed. However, there are indications that genetics-based techniques could offer effective and sustainable strategies for Cd mitigation in cacao. As discussed above, the work by Ullah *et al.* (2018) and Moore *et al.* (2020) identified TcNRAMP5 as a potentially important gene for Cd uptake in cacao, and thus as a potential target for genetic selection or modification. A recent greenhouse experiment with 53 wild and domesticated cacao genotypes proposed 11 cacao clones as low Cd accumulators which can be potentially exploited for future work (Arévalo-Hernández *et al.*, 2020). However, soils in that study were spiked to extreme Cd concentrations (25 mg Cd kg<sup>-1</sup>). A follow-up experiment with more environmentally relevant soil Cd concentrations is needed to truly confirm those cultivars as low Cd accumulators. Lewis *et al.* (2018) found significant differences in cacao bean and leaf Cd concentrations among cultivars

grown in the field (up to a factor 13) and authors claimed that soil properties were homogeneous throughout the field, despite its large size (34 ha). Conversely, Engbersen et al. (2019) observed only a factor 2 – 3 difference in bean Cd among cultivars. The differences in bean Cd concentrations among cultivars observed in these studies might be related to other factors such as soil variability and other site conditions, e.g. specific growth dilution or differences in flushing and pod development cycles, rather than cultivar differences in Cd uptake and translocation. Future studies of the cultivar effect on Cd in cacao should evaluate multiple sites and consider large genotype-environment interactions.

#### 2.4.2.1 Grafting as a short-term genetic approach for Cd mitigation

Grafting is a typical shortcut to breed tree orchards and has been proposed as a short-term Cd mitigation strategy where economically interesting scions are grafted on low cadmium accumulating rootstocks. This technique has been used in other crops to mitigate plant stress due to adverse soil chemical conditions in the root environment, e.g. heavy metal uptake and accumulation (Savvas *et al.*, 2010). The effect of rootstock on above-ground Cd concentrations appeared to be species dependent. For example, Arao et al. (2008) demonstrated that grafting eggplants onto *Solanum torvum* rootstock reduced Cd concentrations in the eggplants by 63 – 74 %, compared to grafting onto *Solanum melongena* or *Solanum integrifolium*. In addition, the Cd concentration measured in the xylem sap was significantly lower in *S. torvum* compared to *S. melongena*. This suggests that *S. torvum* can limit translocation of Cd from the root to the shoot, by preventing Cd loading into the xylem. In contrast, studies conducted on potato showed that the rootstock regulates the total Cd uptake by the plant but that the scion regulates the distribution of Cd between the shoots and the tubers (Mengist *et al.*, 2018).

The use of cacao rootstocks with low Cd uptake/translocation potential could thus be the basis to renovate cultivars and eventually reduce Cd concentrations in cacao while maintaining the flavour of specific cultivars. A recent greenhouse experiment did not reveal any consistent rootstock effect on the shoot Cd concentrations in grafted cacao seedlings (Fernández-Paz *et al.*, 2021). The role of the rootstock versus the scion in Cd accumulation in cacao beans is not yet known and this is a key knowledge gap for breeding.

## 2.5 Postharvest processing

There are four main approaches within the postharvest process that may affect the Cd concentration in the final cacao-derived product: testa removal, choice of product recipe, bean mixing and fermentation (Figure 2.6). First, optimal removal of the testa may reduce the Cd

concentration in the final product, as the testa Cd concentration is approximately twice that of the nib [Table 2.4 and (Lee and Low, 1985; Yanus *et al.*, 2014; Ramtahal *et al.*, 2016; Lewis *et al.*, 2018)]. The testa is removed after roasting in a process called *breaking and winnowing*, after which the nibs are ground to obtain cacao liquor. However, testa removal is not complete and the intermediate product cacao liquor is allowed to contain up to 5 % (m/m) residual testa and/or germ (also referred to as radicle) on a fat-free dry weight basis or approximately 2.5 % on a total dry weight basis (Codex Alimentarius, 2014). Considering that the testa comprises approximately 10 % of the dry weight of a cacao bean, complete testa removal can reduce the Cd concentration by a factor 1.16 from intact cacao beans to cacao liquor. This also indicates that cacao beans may be unnecessarily rejected by the industry because of elevated Cd concentrations if this decision is based on analysis of total bean Cd (nib and testa) instead of nib Cd.

Second, Cd is mostly partitioned in the non-fat cacao solids (Mounicou *et al.*, 2003; Yanus *et al.*, 2014; Kruszewski *et al.*, 2018). Therefore, products made from the same cacao beans can have different Cd concentrations depending on the product type, i.e. Cd concentrations in white chocolate are negligibly small while fat-reduced cacao powder contains much higher Cd concentrations. Indeed, Cd concentrations are reported to be higher in cacao powders compared to cacao liquor and/or beans (Mounicou *et al.*, 2002; Kruszewski *et al.*, 2018). Considering this, it can be relevant for processing companies to take the intended final product into consideration when setting Cd requirements for their suppliers (Figure 2.6). In addition, the new EU Cd thresholds differ depending on the cacao solids content of the final product. To produce a typical milk chocolate with 35 % cacao solids (EU limit 0.30 mg Cd kg<sup>-1</sup>), cacao beans can be used with nib Cd concentrations up to 0.86 mg Cd kg<sup>-1</sup>, not considering effects of fermentation and/or testa removal. For dark chocolate with 70 % cacao solids (EU limit 0.80 mg Cd kg<sup>-1</sup>), beans with nib Cd concentrations up to 1.14 mg Cd kg<sup>-1</sup> could be used, which is nearly double the unofficial industry requirement of 0.60 mg Cd kg<sup>-1</sup>. A webtool was recently created which allows farmers to easily calculate the acceptable bean Cd concentrations based on the cacao solids content of the intended product (<https://www.chocosafe.org>).

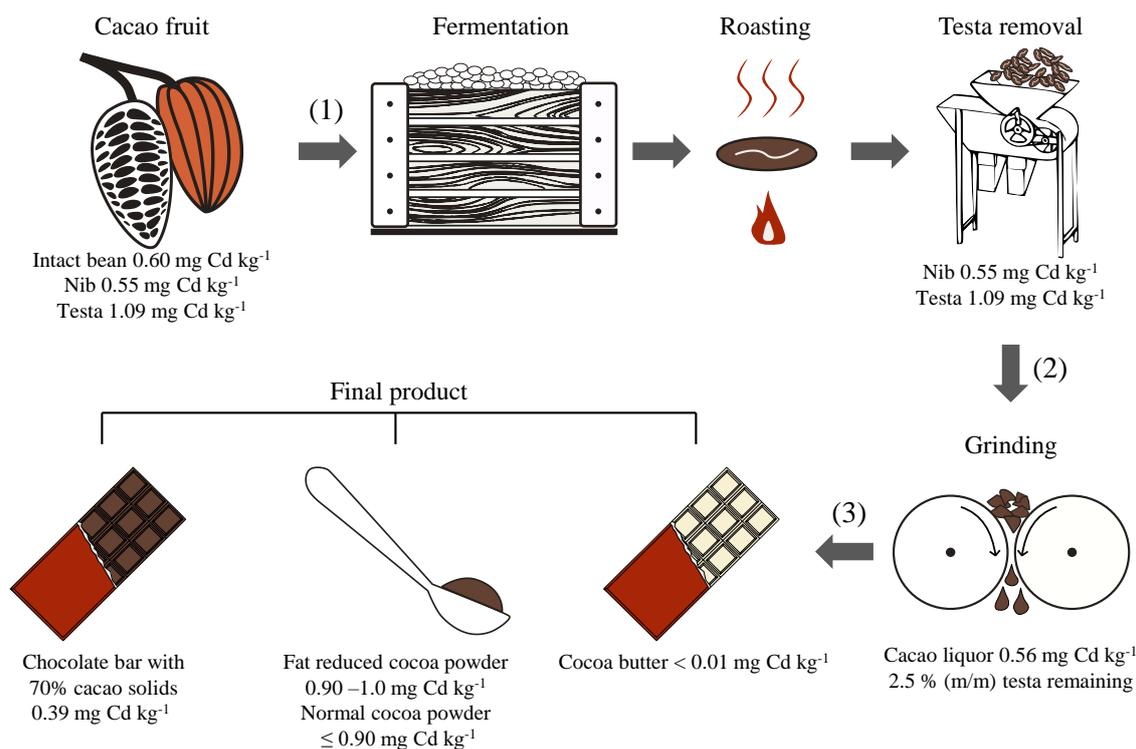
Third, it is common practice to mix cacao from different geographical sources during the production process. Considering the large geographical differences in cacao Cd concentrations (Table 2.1), beans from different origins can be mixed to ensure acceptable Cd concentrations in the final product, e.g. mixing cacao from West Africa which is generally reported to contain low Cd concentrations, with cacao from Central or South America where Cd concentrations are higher. This strategy can also offer viable solutions on a national scale, which is especially relevant for countries specialising in fine flavour origin chocolates. For example, based on the bean Cd concentrations measured by Argüello *et al.* (2019) in their nationwide study, and on the cacao

production per province reported by the Ecuadorian government between 2014 and 2019 (Instituto Nacional de Estadística y Censos - Gobierno de La República del Ecuador, 2019), the average bean Cd concentration per province exceeds the recommended value of 0.60 mg Cd kg<sup>-1</sup> in 10 of the 23 Ecuadorian provinces. This corresponds to 32 000 metric tonnes or 15.5 % of the national production. The extensive database of Argüello et al. (2019) indicates an overall average bean Cd concentration of 0.90 mg Cd kg<sup>-1</sup>, with 48 % of fields exceeding the guideline of 0.60 mg Cd kg<sup>-1</sup>. Mixing all production at a national scale would result in an average bean Cd concentration of 0.59 mg kg<sup>-1</sup> and the product would thus comply with the Cd requirements (Table 2.6). Although mixing on a national scale is likely not practically feasible, there is potential for mixing strategies on smaller scales. The cacao market in many Central and South American countries is already set up in a way that would allow structural mixing of cacao from different sources, as larger cooperatives purchase the cacao from small scale farmers and then sell the (mixed) product to their clients.

**Table 2.6:** The effect of cacao mixing as a strategy to comply with the 0.60 mg Cd kg<sup>-1</sup> threshold, using Ecuador as an example. The distribution of Cd concentrations without mixing is based on the national study of Argüello et al. (2019), which included 159 fields. The national and provincial estimates were calculated with spatial explicit data from that study and weighed by provincial production, details on the calculations can be found in Appendix I.

Produce is not mixed	Average cacao bean Cd concentration (mg kg <sup>-1</sup> )	Fields with average bean Cd > 0.60 mg kg <sup>-1</sup> (%)
All included data (Argüello et al. 2019)	0.90	48
1 % of fields with largest average bean Cd concentration excluded	0.81	47
5 % of fields with largest average bean Cd concentration excluded	0.71	43
10 % of fields with largest average bean Cd concentration excluded	0.64	38
13 % of fields with largest average bean Cd concentration excluded	0.59	34
Produce is mixed at national level <sup>£</sup>	Average cacao bean Cd concentration (mg kg <sup>-1</sup> )	Production lost (%)
All provinces included	0.59	0
Excluding provinces with average > 1 mg Cd kg <sup>-1</sup>	0.53	8
Excluding 10 provinces with highest bean Cd concentrations	0.51	15

<sup>£</sup> Average bean Cd concentrations per province estimated by combining data from the national agricultural land use map (Sistema de Información Pública Agropecuaria, 2016) with the map of spatial distribution of Cd in cacao beans from Argüello et al. (2019). Average total annual production calculated based on the survey of continuous agricultural production and surface area (Instituto Nacional de Estadística y Censos - Gobierno de La República del Ecuador 2019). More detailed information on this calculation can be found in Appendix I.



**Figure 2.6:** Effect of postharvest processing steps on Cd concentrations in (intermediate) cacao-derived products, considering an initial total bean Cd concentration of  $0.60 \text{ mg Cd kg}^{-1}$  (the unofficial industry threshold). Only the processing steps which can potentially reduce the Cd concentration in the final product are given: (1) the effect of fermentation is not yet revealed; (2) current regulations allow  $2.5 \%$  (m/m) testa material to remain in cacao liquor, more efficient testa removal may lower the Cd concentrations due to high testa Cd (i.e. in this case complete testa removal would result in cacao liquor with  $0.55 \text{ mg Cd kg}^{-1}$ ); (3) Cd is contained in the non-fat cacao solids and thus Cd concentrations in the final product depend on product type.

Fourth, the potential impact of fermentation on Cd in cacao and the need for research on this topic has been indicated (Meter *et al.*, 2019). Some authors have reported Cd concentrations measured in unfermented beans and intermediate products after fermentation. For example, Barraza *et al.* (2017) measured Cd in unfermented unpeeled cacao beans ( $1.02 - 1.37 \text{ mg kg}^{-1}$ ) and in cacao liquor ( $1.47 - 3.88 \text{ mg kg}^{-1}$ ), i.e. suggesting some Cd enrichment after fermentation and processing. However, those samples did not originate from the same location and the observed difference in Cd concentrations was thus likely related to sample variation rather than a processing effect. Yanus *et al.* (2014) reported Cd concentrations in unfermented nibs ( $0.072 \pm 0.001 \text{ mg kg}^{-1}$ ) and testae ( $0.085 \pm 0.001 \text{ mg kg}^{-1}$ ), and in cacao powder ( $0.125 \pm 0.011 \text{ mg kg}^{-1}$ ), again suggesting enrichment which could be related to fat removal during cacao powder production. However, it is again unclear to what extent this trend is related to variability of samples or to processing.

During fermentation, the microbial community in the mucilage produces metabolites such as ethanol and acetic acid, which are known to penetrate the testa and thus migrate inward from the mucilage to the nib. In contrast, polyphenols and methylxanthines have been reported to migrate outward from the nib to the testa, in the form of exudates. Vanderschueren et al. (2020) showed that conventional fermentation practices can reduce Cd concentrations in the cacao nib by up to a factor 1.3. This reduction of nib Cd concentration has been attributed to a combination of high temperature (>45°C) and acidic pH (<5). Such conditions may mobilise Cd within the different cacao tissues, i.e. nib, testa or mucilage. Although the biochemical mechanisms behind this mobilization remain unclear, it was suggested that Cd in cacao nibs was mobilized by a release of Cd from the phytate complex due to acidification and/or phytate breakdown (Vanderschueren, Helsen, *et al.*, 2023).

## 2.6 From chocolate bar to body burden

While different regulatory bodies are implementing limitations regarding the maximum allowed Cd concentration in chocolate and other cacao-derived products, controversy remains regarding tolerable intake levels, and regarding the relation between dietary intake and body burden. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) set a provisional tolerable monthly intake of 25 µg Cd kg<sup>-1</sup> body weight (bw), which corresponds to a tolerable daily intake of 0.83 µg Cd kg<sup>-1</sup> bw (FAO/WHO 2010). Conversely, the European Food Safety Authority (EFSA) set a stricter tolerable weekly intake of 2.5 µg Cd kg<sup>-1</sup> bw or a daily tolerable intake of 0.36 µg Cd kg<sup>-1</sup> bw (EFSA 2011). Children and infants have been identified as a high risk group within the population, due to differences in their diet compared to adults, and because they have a higher food consumption per unit body weight. However, as adverse effects due to dietary Cd intake are mostly related to accumulation in the human body over a lifetime of exposure, focussing on these young age groups may not be relevant. Although research has indicated that only a small part of dietary Cd is absorbed in the human body (European Chemicals Bureau 2007), regulations and health based guidelines are based on dietary Cd intake rather than on the Cd body burden. In contrast to the assumption in the risk assessment models, most dietary Cd studies show that the Cd body burden increases less than proportional with increasing dietary intake, and this is related to lower Cd bioavailability and/or differences in micronutrient status with diet when relying on high Cd diets (Vahter *et al.*, 1996; Reeves *et al.*, 2001). Only limited information is currently available regarding the gastro-intestinal absorption rate of Cd from cacao-derived products and studies thus far have focussed only on *in vitro* experiments. Mounicou et al. (2002 and 2003) studied the *in vitro* bioaccessibility of Cd in cacao liquor and cacao powder and found accessible fractions ranging between 10 and 50 %. Conversely, Barraza et al. (2017) determined the gastric

bioaccessibility of Cd using the unified method from the Bioaccessibility Research Group of Europe and reported a gastric bioaccessibility of > 90 % in cacao liquor from Ecuador. A recent study showed that the bioaccessibility of Cd in cacao-derived products is factor 2-5 lower than that of wheat flour (Vanderschueren, Doevenspeck, *et al.*, 2023). They moreover suggested that the Cd intake from cacao consumption is underestimated in risk assessment because of hidden cacao in non-chocolate food categories, but that the true exposure is overestimated due to the lower bioavailability compared to staple foods. However, considering the strong influence of dietary status (e.g. dietary Zn, Fe and Ca) on the gastro-intestinal absorption of Cd (Reeves and Chaney, 2008), it remains to be demonstrated if long term high chocolate consumption truly increases the Cd body burden to levels of concern. *In vivo* duplicate diet studies with increased dietary Cd through chocolate consumption would offer vital insights to assess the assumptions behind the existing and future food regulations.

## 2.7 Conclusions

Recent (and upcoming) regulations on the maximum allowed Cd concentrations in cacao-derived products are threatening cacao producers worldwide and are especially causing concern in Central and South America. Elevated soil Cd concentrations found in cacao production areas are likely related to geogenic rather than anthropogenic origin. Bean Cd concentrations are generally larger in cacao plants grown on acid soils with low soil organic carbon, when considering equal total soil Cd. Several mitigation strategies are being explored to deal with elevated Cd concentrations in cacao. However, mitigation strategies are not a cure-all and their use comes at a cost, either due to the financial cost of implementing the strategy or due to adverse effects on the final product quality. First, soil amendments can limit Cd plant uptake by reducing the phytoavailable soil concentration (e.g. biochar, lime or gypsum) or by saturating uptake mechanisms through which Cd can otherwise enter the plant (e.g. Zn fertilisation). Application of lime and biochar in cacao field trials has been reported to yield reduction factors (RF) up to 2, but as both treatments are based on a liming effect (i.e. increasing soil pH), these amendments are mostly effective in acid soils. While soil amendments could offer a readily applicable medium term solution, their incorporation is limited due to the dense rooting system of the perennial cacao tree. Second, root uptake and root-to-shoot translocation of Cd within the plant may be targeted for selection of low Cd accumulating cultivars. While the research on Cd uptake and translocation mechanisms in cacao is only in its early stages, genetics-based mitigation strategies can be highly valuable in the future. The available work on cacao indicates potential RF ranging from 2 to 13. However, to avoid current uncertainties related to the different soils under the cultivars, experiments on the same soil (i.e. same location, also termed common garden experiments)

should be performed to identify low Cd accumulating cultivars and their potential RF. The use of specific rootstocks in grafting should also be explored as this may offer the benefit of genetics-based mitigation (selection of a low Cd uptake rootstock) while maintaining the flavour profile of the original cultivar in the scion. There is a need to better characterise the pathways of Cd loading into cacao beans and to reveal the role of the xylem and phloem pathways. Third, postharvest mitigation strategies may offer the benefit of control for industrial scale cacao processors but have not yet been explored, and the effect of such strategies on the flavour quality of the final product should always be taken into consideration. On the short term, a realistic and easily implementable strategy is mixing of cacao from different origins (and thus with different bean Cd concentrations), both on larger and smaller scales. The intended final product should be kept in mind when setting Cd requirements, as current industry thresholds may be overly strict. For example, to produce dark chocolate with 70 % cacao solids, cacao beans with nib Cd concentrations up to 1.14 mg kg<sup>-1</sup> could be used, which is almost double the currently common requirement of 0.60 mg Cd kg<sup>-1</sup>. Finally, considering the indications that cacao is a moderate Cd accumulator, farmers may be recommended to change to a lower Cd accumulating crop if mitigation for cacao is not feasible.

# Chapter 3: From soil to cacao bean: Unravelling the pathways of cadmium translocation in a high Cd accumulating cultivar of *Theobroma cacao* L.

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## Summary

The research on strategies to reduce cadmium (Cd) accumulation in cacao beans is currently limited by a lack of understanding of the Cd transfer pathways within the cacao tree. In this chapter, we elucidated the transfer of Cd from soil to the nib (seed) in a high Cd accumulating cacao cultivar, through Cd stable isotope fractionation, speciation, and localization. The plant Cd concentrations were 10-28 higher than the topsoil Cd concentrations and increased as placenta < nib < testa < pod husk < root < leaf < branch. The retention of Cd in the roots was low, and light Cd isotopes were retained in the roots and heavier Cd isotopes were transported to the shoots ( $\Delta^{114/110}\text{Cd}_{\text{shoot-root}} = 0.27 \pm 0.02 \text{‰}$  (weighted average  $\pm$  standard deviation)). Leaf Cd isotopes were heavier than Cd in the branches ( $\Delta^{114/110}\text{Cd}_{\text{IF3 leaves-branch}} = 0.18 \pm 0.01 \text{‰}$ ), confirming typical trends observed in annual crops. Nibs and branches were statistically not distinguishable ( $\Delta^{114/110}\text{Cd}_{\text{nib-branch}} = -0.08\text{‰} \pm 0.06 \text{‰}$ ), contrary to the leaves and nibs ( $\Delta^{114/110}\text{Cd}_{\text{nib-IF3 leaves}} = -0.25\text{‰} \pm 0.05 \text{‰}$ ). These isotope fractionation patterns alluded to a more direct transfer from branches to nibs rather than from leaves to nibs. The largest fraction (57%) of total plant Cd was present in the branches where it was primarily bound to carboxyl-ligands (60-100%) and mainly localized in the phloem rays and phelloderm of the bark. Cadmium in the nibs was mainly bound to oxygen ligands (60-90%), with phytate as the most plausible ligand. The weight of evidence suggested that Cd was transferred like other nutrients from root to shoot and accumulated in the phloem rays and phelloderm of the branches to reduce the transfer to foliage. Finally, the data indicated that the main contribution of nib Cd was from the phloem tissues of the branch rather than from leaf remobilization. This study extended the limited knowledge on Cd accumulation in perennial, woody crops and revealed that the Cd pathway is markedly different than in annual crops, which has implications for mitigation strategies.

### 3.1 Introduction

Cadmium (Cd) is a potentially toxic trace metal that has no known biological function in humans. It is known as a hitchhiker element, using transporters for essential elements such as Zn, Fe, Mn and Ca at all steps of these nutrient pathways, from root uptake to grain or seed loading (Clemens and Ma, 2016). Consequently, Cd eventually ends up in the human food chain. To protect consumers, the European Commission and the Codex Alimentarius approved new regulations, which set the maximum allowed Cd concentration in chocolates and cacao powders between 0.10 and 0.80 mg Cd kg<sup>-1</sup> (EU, 2014; Codex Alimentarius Commission, 2018). These regulations have fueled research worldwide to monitor and mitigate Cd accumulation in cacao. There are indications that in the short-term agronomic changes like soil amendments could offer effective and sustainable strategies for Cd mitigation in cacao (Vanderschueren *et al.*, 2021). In the long-term, breeding low Cd cultivars likely provides the highest potential. However, the genetics and breeding research is currently limited by the lack of understanding of how Cd is loaded into the developing cacao fruit of the tree.

*Theobroma cacao* L. is a perennial, woody tree that displays cauliflorous flowering, i.e. flowers and pods develop on the trunk and thicker branches of the plant (Toxopeus *et al.*, 2008). Cacao is considered a moderate Cd accumulator, as the Cd uptake potential of the cacao tree can be conceived as rather high compared to other agricultural crops, and more similar to woody species used for phytoextraction such as willow and poplar (Vanderschueren *et al.*, 2021). Nonetheless, large differences (factor 13) in cacao bean Cd concentrations were found between cultivars grown in field conditions with comparable available soil Cd (Lewis *et al.*, 2018). Some cultivars express thus high or low Cd accumulating properties. Due to its different anatomy, the mechanism of Cd accumulation may differ from other, more studied crops, like hyperaccumulators and cereals. In rice, the model plant for cereals, the knowledge of the pathway of Cd from soil to grain is in a very advanced stage compared to cacao. Numerous studies have shown the importance of membrane transport and pathways (e. g. xylem versus phloem transport), in the control of Cd in cereals. In summary, Cd is first taken up by root metal transporters for nutrients (McLaughlin *et al.*, 2021). The roots are a major compartment for Cd accumulation since in the roots Cd is being sequestered into vacuoles. The Cd that is still mobile in the root is loaded into the xylem and exported into the shoot, and more particularly to the transpiring organs like the leaves (Clemens and Ma, 2016). When the grains develop, they can accumulate Cd from potentially two sources: direct transfer from roots or remobilization from plant organs that store mineral nutrients during vegetative plant growth (Yan *et al.*, 2018). It is estimated that in rice, 91-100% of Cd accumulated in the grain is delivered via the phloem (Tanaka *et al.*, 2007). Hence, Cd is remobilized into the grains from

senescing leaves or stems via the phloem and/or in its pathway from root to shoot, Cd is transferred from xylem to phloem, which occurs likely in the rice nodes (Tan *et al.*, 2019). Storage forms of Cd (i.e. its chemical speciation) have also been proven to play an important role in the control of the mobility of Cd in rice (Wiggenhauser *et al.*, 2021). Such knowledge of translocation pathways and storage forms of Cd within cacao would be an important step forward in understanding Cd accumulation in cacao beans.

In cacao, the overall pathway from soil to nib remains unclear. Moore *et al.* showed that Natural Resistance Associated Macrophage Protein 5 (NRAMP5) transporters may constitute an important pathway for uptake of Cd by cacao seedlings (Moore *et al.*, 2020). They further suggested that the HMA-family proteins may contribute to Cd sequestration in root vacuoles, as shown in rice. A study on partitioning of Cd in cacao trees indicated that the transfer of Cd from root to the shoot tissues is large (Engbersen *et al.*, 2019) compared to other crops where Cd accumulates mostly in the roots (Clemens *et al.*, 2013; Vanderschueren *et al.*, 2021). Based on concentration data they further proposed that Cd can be directly loaded from stems and branches into cacao beans without passing through leaves. This was corroborated by a study in mature cacao trees where the isotope fractionation suggested that Cd remobilization from the leaves is not an important pathway for Cd in the nibs, as has been shown for Cd loading into the rice grains (Barraza *et al.*, 2019). However, the mechanism of Cd translocation in cacao trees remains unclear since organs such as roots and branches that may be important organs for storage and translocation were not analyzed. Little data on chemical Cd species has yet been obtained in cacao tissues. Synchrotron X-ray absorption spectroscopy (XAS) revealed that Cd in the nibs is coordinated to O, but S binding also occurred (Vanderschueren *et al.*, 2020). However, information on Cd speciation, especially in important organs for storage and translocation is lacking.

The objectives of this study were to dissect potential mechanisms that control the accumulation and partitioning of Cd in a high Cd accumulator genotype of cacao grown in field conditions at environmentally relevant soil Cd concentrations. We determined the main compartments of Cd accumulation at the scale of the whole plant, using stable isotope and mass balance approaches. For the first time, the Cd isotope composition was measured in the roots, branches, and testa of a cacao tree. The corresponding fractionations helped in identifying plant uptake and translocation mechanisms. Speciation analyses in the branches, testa, and nibs and microscopic imaging of branches and roots provided complementary snapshots of major forms of Cd. In addition, interelement correlations were studied to elucidate nutrient pathways in the cacao tree. Finally, these data were combined to propose Cd transport pathways in cacao trees.

## 3.2 Materials and methods

Sampling was conducted in January 2020 during the main harvest season, from November to January. Samples were collected at the International Cocoa Genebank, Trinidad (ICGT), a field cacao collection with approximately 2400 cacao genotypes, also further described as cultivars. A cultivar with high Cd accumulating properties, NA 312 of the genetic group Nanay (Motamayor *et al.*, 2008), was selected for this study. The selection was based on the Cd levels in beans and leaves as reported in a previous study at the ICGT (Lewis *et al.*, 2018). Three biological replicates, i.e. genetically identical trees, generated by rooted cuttings (T2, T4 and T6), were sampled.

### 3.2.1 Location

The International Cocoa Genebank, Trinidad (ICGT) is situated in Centeno (Location using UTM – 685617E, 1,169,849N). The mean annual rainfall at this site is 2000 mm, and the mean annual maximum and minimum temperatures are 33 °C and 22 °C. The dry season takes place from January to May and the wet season from June to December. The soil in the ICGT is bordered by the Caroni river and is subject to repeated wet season floodings, which periodically bring sediments over the whole plot. The clay-loam soil (subgroup: Aquic Hapludalfs) is formed from non-calcareous silty claystone and its alluvial derivatives (Ahmad, 2011).

### 3.2.2 Soil and tree sampling

Around each tree, a composite soil sample of the surface soil (0-20 cm) and the subsoil (20-40 cm) was collected with an auger around the dripzone of the cacao tree (1-2 m from trunk) by mixing three soil cores. The soil samples were afterward homogenized and weighed prior to oven drying (65°C). Around the surface roots (top 0-10 cm), the soil was dug out with a shovel (1-2 m from trunk) on three locations. Here, roots of different sizes were collected (with a diameter of 0.5-10 mm) (Figure II.1). Branches, containing leaves of different maturation stages, were collected with pruning shears. The leaves were stripped off the branch and aliquots of branch tissue with a diameter of 3-7 mm were selected for further analyses (Figure II.1). The leaves were categorized according to their maturation into Interflush-2 (IF2) and Interflush-3 (IF3) leaves. Interflush-2 leaves are leaves that have attained the deep green of a mature leaf, and have a green stem bearing the leaves. The maturation stage of Interflush-3 leaves begins when the stem bearing the leaves turns brown (Greathouse *et al.*, 1971). About ten leaves of each age were collected (Figure II.1). The cacao fruit or pod is made up of a large woody outer pod husk, filled with 20–50 cacao beans (seeds) (Figure II.1). The cacao bean consists of a nib and an outer shell, the testa. The nib is the only part of the cacao bean that is retained during chocolate processing. Healthy fruits were

collected and because of the limited number of fruits in the trees, mature but also intermediate and immature pods were collected. All plant samples (roots, branches, leaves, pods) were rinsed using baths with deionized water, and finally dried with a paper towel. First bulk root measurements indicated a contamination from soil particles (as seen by the high Mn and Fe content). To avoid contamination of soil particles in further analyses of the roots, the roots were peeled with a scalpel and remeasured. We assumed that the removed root part represented the epidermis and a minor part of the root cortex. Considering the minor biomass fraction that has been removed, the peeling did not affect the bulk concentration and isotope composition measurements in the roots. The intact pod husk was opened and all the different tissues (pod husk, placenta, testa, nib (Figure II.1)) were separated and weighed. Immediately after separation, all plant samples were divided into subsets, apropos the further processing of the samples for the different techniques. For the X-ray absorption spectroscopy (XAS) analysis, an aliquot of the fresh tissues was milled with a pestle and a mortar in liquid N<sub>2</sub> and stored in cryotubes in a freezer at -80°C until speciation analysis took place to preserve Cd speciation. Frozen-hydrated samples were shipped in a dry shipper filled with liquid N<sub>2</sub>. Aliquots for the concentration and isotope analyses were weighed for fresh weight/dry weight determination, homogenized, oven dried (65°C), shipped, and ground to powder with a grinder (Pulverisette7, Fritsch).

### 3.2.3 Chemical and physical analyses

#### 3.2.3.1 Soil and plant properties

Soil pH was measured in 0.01 M CaCl<sub>2</sub>, 1:5 soil to liquid (w:v). For organic carbon (expressed in %OC), the soil was acidified with 20-50 µL of 10% hydrochloric acid (HCl) and carbon was determined with elemental analyzer CarloErbaEA1108. The soil effective cation exchange capacity (eCEC) was determined using cobalt hexamine trichloride extractant solution as described in the standardized protocol ISO 23470:2018. Ammonium oxalate extractable aluminum, iron and manganese were measured in a 1:50 soil to oxalate (pH 3) extract ratio, where Al, Fe and Mn were analysed in the extract by ICP-OES (Schwertmann, 1964). For the elemental content analyses, 50 mg of soil or plant were digested in respectively boiling aqua regia (2 mL 1:3 Suprapur HCl:HNO<sub>3</sub>) and concentrated nitric acid (2 mL Suprapur HNO<sub>3</sub>) in an open digestion block for 8 h at a maximum temperature of 130 °C. The total elemental composition of the digest was measured by ICP-MS (Agilent7700x, Agilent Technologies) and ICP-OES (iCAP 7400 series, Thermo Scientific). For quality assurance, elemental concentrations were also determined in the certified reference materials BCR-142R (light sandy soil), NIST 1573a (tomato leaves), and NIST 2384 (baking chocolate). The Cd recovery of these samples ranged from 89 to 91% indicating that

the digestion processes were effective. A full overview including other elements is given in Table II.1.

Soil pools considered as phytoavailable Cd were extracted from the topsoil and subsoil using 0.05 M  $\text{Ca}(\text{NO}_3)_2$  (5 g soil: 50 mL  $\text{Ca}(\text{NO}_3)_2$ ), the extracts were shaken for 16 h, centrifuged, and then filtered (Rotilabo KH55.1 0.45  $\mu\text{m}$ , Carl Roth) (Gray, 1998). Extracts were digested in concentrated  $\text{HNO}_3$  and total element content was measured with ICP-MS prior to further purification for isotope analyses.

### 3.2.3.2 Cd isotope analyses in soil and cacao tissues

Plant ( $200 \pm 20$  mg) and soil ( $400 \pm 20$  mg) samples selected for isotope analyses were digested with a microwave digestion system (MARS 6, CEM, North Carolina, USA) with respectively 8 mL concentrated nitric acid and 8 mL aqua regia. The total digestion cycle, consisting of a heating, a holding and a cooling phase, took 70 minutes to complete with a 20 min holding phase at  $180^\circ\text{C}$ . The soils were subjected to an additional digestion step with 3 mL HF and 3 mL  $\text{HNO}_3$  on the hotplate to ensure complete dissolution of the metals. Prior to the isotope analysis, Cd was purified using an anion exchange chromatography procedure (Cloquet *et al.*, 2005; Pallavicini *et al.*, 2014) which was previously used for samples in contaminated environments (Wei *et al.*, 2016; Wiggerhauser *et al.*, 2021). Briefly, matrix elements (e.g. Ca, Zn, Sn) were removed from the sample using different concentrations of HCl while Cd was eluted from the column using 0.0012M HCl. Because the samples in this study were of environmentally-relevant conditions, the matrix contained a high element-to-Cd ratio. Thus, to achieve a better separation, it was necessary to perform the column chemistry twice. Some samples were purified three times if significant traces of Zn and Fe were still detected in the double purified Cd eluate. The yield of the purification procedure for certified reference materials can be found back in Table II.2. The procedural blank ( $n=10$ ) was  $<1.07\%$ . Cadmium isotope compositions were measured using a multiple collector ICP mass spectrometer (MC-ICP-MS, Neptune Plus, Thermo Scientific) using an Aridus desolvator for sample introduction. Each sample was measured at least three times if there was enough sample available. To correct for instrumental mass bias, 'standard-sample bracketing' was applied and isotope standards and samples were doped with silver (Cd:Ag ratio of 2:1). Frequent measurements of a certified isotope standard and soil and plant standards for interlaboratory comparison revealed robust precision and accuracy for Cd concentration and Cd isotope measurements (Table II.1 and II.2).

### 3.2.3.3 Phytate content of cacao nibs

The phytate content in the nibs was determined in triplicate with a phytic acid assay (McKie and McCleary, 2016). In the SI the procedure and principle are explained. For quality assurance, an internal reference material (KULeuven wheat flour) was analyzed in triplicate. The recovery ranged between 105 and 110%. The internal reference material contained 0.88 g phytate/(100 g DW) (based on the median measured concentration after 38 individual analyses of the material).

### 3.2.3.4 Speciation analyses

Frozen hydrated samples were milled in liquid N<sub>2</sub> using a cryo-grinder (Pulverisette23, Fritsch) and then pressed into pellets in cryo conditions (diameter 6 mm, thickness 3-5 mm). Cadmium K-edge XAS measurements were conducted at SAMBA beamline (SOLEIL synchrotron) and BM30 beamline (ESRF synchrotron). The X-ray absorption Absorption Near Edge Structure part (XANES), was analyzed since the diluted nature of the samples did not allow to retrieve valuable spectra from the Extended-X-ray Absorption Fine Structure part (EXAFS). The monochromator was a Si220 crystal, and acquisition was done in fly scan (SAMBA) and in step-by-step (BM30) mode. All samples were recorded at 20 K using a He cryostat, in fluorescence mode with a 35-element germanium detector (SAMBA) or a 13-element germanium detector (BM30). A Cd foil was recorded simultaneously in transmission mode for the energy calibration of XANES spectra.

A previously recorded database of Cd reference spectra recorded at 15 to 20K was used, containing Cd-cell wall, Cd-glutathione, Cd-phytochelatin, Cd-hydrated, Cd-malate, Cd-histidine, Cd phosphate. The preparation and measurements of this dataset are described in Huguet *et al.*, 2012, 2015. As additional standards, Cd-metallothionein extracted from mussels (Bataille *et al.*, 2005), Cd phytate (phytate/Cd molar ratio = 5), Cd-Ca-oxalate (Ca-oxalate/Cd molar ratio = 10 and 50) (McBride *et al.*, 2017) were measured at BM30 and included in the database. The absorption spectra of the references can be found in the SI (Figure II.2).

The raw XANES spectra were averaged, calibrated in energy and normalized, and treated by linear combination fits (LCFs) using a XAS software (Fastosh (Landrot, 2018)). LCF was performed by fitting the normalized XANES spectra in regions between -20 and 80 eV using the database of Cd reference compounds. The R-factor ( $= \sum[\mu_{\text{exp}} - \mu_{\text{fit}}]^2 / \sum[\mu_{\text{exp}}]^2$ ) was used to assess the goodness of fit. A combination of two reference compounds was used since the R-factor of the fit with three-reference compounds was not significantly smaller compared to the R-factor with two-reference compounds LCFs. For some spectra, there was not a unique solution. Particularly for the noisier spectra, the LCF procedure provided fits of equivalent quality. The fits of one XANES spectrum

were considered equivalent if the R-factor increased <10% compared to the best fit. For these spectra, several fits are shown.

### 3.2.3.5 Imaging

Branches of two biological replicates (T2 and T4) were oven dried (60 ° C) prior to cutting with a microtome blade and placed on a glass slide with Kapton tape. Elemental analyses of the branch sections were achieved by Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS). An Analyte Excite+ excimer laser ablation system (Teledyne) equipped with a 2-Volume HelEx II Cell was used. The ARIS (Aerosol Rapid Introduction System, Teledyne) was operated to connect the 2-volume cell with the 8900 Triple Quadrupole ICP-MS (Agilent). The sections were ablated using an excimer laser at 193 nm with a 50 Hz repetition rate, spot size 10 µm square laser beam, a fluence of 3.5 J cm<sup>-2</sup>, and a scan speed of 50 µm s<sup>-1</sup>. Cadmium and other elements (C, P, Ca, Mn, Ni, Cu, and Zn) were measured with He cell gas to reduce polyatomic interferences. The program HDIP 1.6 was used to build the maps of the different isotopes. The final editing of the images was done with Fiji ImageJ (version 1.53s). For further calculations, elements were normalized to <sup>13</sup>C in each pixel to correct for differences in tissue hardness and water content within the sample (Becker *et al.*, 2008).

### 3.2.4 Calculations and statistics

Isotope ratios of <sup>114</sup>Cd/<sup>110</sup>Cd in the soil and cacao samples were expressed relative to the <sup>114</sup>Cd/<sup>110</sup>Cd isotope ratio of isotope reference material NIST3108 as δ;

$$\delta^{114/110}\text{Cd} (\text{‰}) = \left[ \frac{\left(\frac{^{114}\text{Cd}}{^{110}\text{Cd}}\right)_{\text{sample}}}{\left(\frac{^{114}\text{Cd}}{^{110}\text{Cd}}\right)_{\text{NIST3108}}} - 1 \right] \times 1000$$

The weighted average Cd isotope ratio of Cd in the entire trees was calculated as a weighted arithmetic mean;

$$\delta^{114/110}\text{Cd}_{\text{tree}} = \frac{\sum_i m_i c_i \delta^{114/110}\text{Cd}_i}{\sum m_i c_i}$$

Where  $m_i$  denotes the dry weight (g),  $c_i$  the Cd concentration (mg kg<sup>-1</sup>) and  $\delta_i$  the corresponding isotope ratio of the different cacao tissues  $i$  (roots, stem, branch, leaves and fruits). For the dry biomass of roots, stem, branch and leaves, values were used estimated by (Nurafiza *et al.*, 2017)

from an average of 20 cacao trees (10 years old). In Table II.3 more information concerning the mass balance estimation can be found.

Apparent Cd isotope fractionation between two compartments A and B is expressed as 'Δ' by subtracting Cd isotope ratios from each other;

$$\Delta^{114/110}Cd_{A-B} = \delta^{114/110}Cd_A - \delta^{114/110}Cd_B$$

Transfer factors (TF) indicate to what extent a crop accumulates Cd in its edible parts and are calculated as the ratio of the Cd concentration in either the plant leaf or the edible part (mg (kg DW)<sup>-1</sup>) to the aqua regia-extractable Cd concentration of the soil (mg (kg DW)<sup>-1</sup>) as shown below for the nib.

$$TF_{nib} = \frac{Cd_{nib}}{Cd_{soil}}$$

Similarly, internal translocation factors (ITF) were calculated to indicate how Cd is partitioned inside the crop. They are calculated as the ratio of the Cd concentration in a tissue, over the Cd concentration in another tissue (mg (kg DW)<sup>-1</sup>), as shown in next example;

$$ITF_{nib-branch} = \frac{Cd_{nib}}{Cd_{branch}}$$

In the images, we compared the accumulation of Cd in the different zones of the branches. Certain regions of interest (ROIs) were defined. The average of the <sup>13</sup>C corrected-counts of Cd in these rois were normalized to the average <sup>13</sup>C corrected-counts Cd counts in the roi of the wood (Table 3.4). For example, the relative intensity of Cd in the pith was expressed as: (Cd<sub>pith</sub>/<sup>13</sup>C<sub>pith</sub>)/(Cd<sub>wood</sub>/<sup>13</sup>C<sub>wood</sub>). Additionally, the relation between Ca, Mn, Zn, Cu, Ni, and P was verified in these rois by making a regression prediction of the <sup>13</sup>C-corrected counts. The extent of correlation was determined by the R<sup>2</sup> of the bivariate fit of Y= log (E/<sup>13</sup>C) and X = log (Cd/<sup>13</sup>C).

The SD (standard deviation) reported throughout the study, was calculated with the biological replicates, i.e. the genetically identical trees. One-way analysis of variance (ANOVA) was conducted to compare means. Posthoc Tukey HSD tests were used when ANOVA revealed significant differences in the comparison of means (level of significance: *p*<0.05). Simple linear regression was used to test the relationship between Cd concentrations and nutrients (level of significance: *p*<0.05). The data sets were first tested for equality of variances with the O'Brien and Brown-Forsythe test (Table II.4). Additionally, the normal distribution of residuals was tested

with the Shapiro-Wilk test (Table II.4). If these prerequisites were not fulfilled, the data were log transformed prior to conducting an ANOVA, as was the case for the molar ratios of the nutrients versus Cd in the bulk and image analyses. Statistical analysis was conducted with JMP® Pro version 14.0.0 (SAS Institute 2018). For the Cd isotope measurements, the 2SD (two times standard deviation) of replicate measurements on the same sample was additionally shown to visualize analytical uncertainty. For the LA-ICP-MS analyses, only two replicates (T2 and T4) were measured due to high cost of analyses.

### 3.3 Results

#### 3.3.1 Characterization of the cacao-soil system

The soil was previously characterized as a clayey loam soil (Lewis *et al.*, 2018). The pH measured at the plot was  $4.1 \pm 0.1$  (mean  $\pm$  standard deviation of replicates), soil organic carbon (SOC) content was  $1.9 \pm 0.4\%$ , and the eCEC was  $11.6 \pm 0.12$  cmol<sub>c</sub>/kg. The overall variation in soil properties between the three plots was low (Table II.5). The aqua regia soluble ( $0.22 \pm 0.05$  mg kg<sup>-1</sup>) and total Cd ( $0.28 \pm 0.05$  mg kg<sup>-1</sup>) concentrations in the topsoil were at the lower end compared to soil Cd concentrations reported in cacao producing areas in Latin America and Caribbean. A meta-analysis of  $n=12$  studies indicated concentrations ranging from 0.22 to 10.8 mg Cd kg<sup>-1</sup> soil (either determined by aqua regia, or aqua regia + HF, or HNO<sub>3</sub> + H<sub>2</sub>O<sub>2</sub>) (Vanderschueren *et al.*, 2021). However, due to the strongly acidic nature of the soil and the rather low SOC, a large fraction (29%) of the Cd was extracted by Ca(NO<sub>3</sub>)<sub>2</sub> ( $0.08 \pm 0.02$  mg kg<sup>-1</sup>). The subsoil was lower in aqua regia soluble Cd ( $0.06 \pm 0.01$  mg kg<sup>-1</sup>) than the topsoil, a trend observed in many cacao plantations (Chavez *et al.*, 2015; Arévalo-Gardini *et al.*, 2017; Barraza *et al.*, 2017; Gramlich *et al.*, 2018).

Based on a comparison of proposed normal, low and deficient concentrations of nutrients in cacao leaves (van Vliet and Giller, 2017), the sampled cacao leaves had adequate levels of Ca ( $17.33 \pm 1.78$  g kg<sup>-1</sup> DW), but were moderately deficient in P ( $1.17 \pm 0.14$  g kg<sup>-1</sup> DW) and severely deficient in K ( $8.35 \pm 2.09$  g kg<sup>-1</sup> DW) (Table II.6). Phytate measurements revealed that 84% of total P in the nib was in the form of phytate-P. ( $4.40 \pm 1.00$  g phytate-P kg<sup>-1</sup>,  $n=5$ ) (Table 3.1). Similar concentrations of phytate have been reported in cacao nibs (1.89 – 4.53 g phytate-P kg<sup>-1</sup>) (Vanderschueren, Helsen, *et al.*, 2023) and are in the range of phytate concentrations reported for different foodstuffs (Schlemmer *et al.*, 2009).

Table 3.1: Phytate and relative elemental content (DW) in the nibs ( $n=5$ ).

		<b>mean</b>	<b>SD</b>
<b>Phytate*</b>	g kg <sup>-1</sup>	15.60	3.50
<b>P-phytate**</b>	g kg <sup>-1</sup>	4.40	1.00
<b>Cd</b>	mg kg <sup>-1</sup>	2.07	0.38
<b>P***</b>	g kg <sup>-1</sup>	5.28	1.15
<b>P-phytate/ P</b>	(g kg <sup>-1</sup> )/(g kg <sup>-1</sup> )	0.84	0.07
<b>phytate/ Cd</b>	(mol kg <sup>-1</sup> )/(mol kg <sup>-1</sup> )	1300	140
<b>phytate/(Cd+Zn+Fe)</b>	(mol kg <sup>-1</sup> )/(mol kg <sup>-1</sup> )	6.2	1.9

\*Myo- inositol 1,2,3,4,5,6 hexakisphosphate

\*\*Phosphorus originating from phytate

\*\*\*Bulk P concentration

### 3.3.2 Cadmium accumulation, partitioning and Cd isotope ratios in the cacao-soil system

All cacao organs were highly enriched in Cd relative to the soil, with tissue-soil transfer factors ranging 10-28 depending on tissue. The Cd concentrations in the organs were ranging from 2.05 (nib) to 6.12 (branch) mg Cd kg<sup>-1</sup> (Table 3.2). Branches were highly enriched in Cd, followed by the roots and leaves, and the lowest concentrations were found in the fruit tissues (testa, pod husk and nib).

The Cd mass fractions in the trees revealed that branches contained most Cd ( $0.57 \pm 0.01$  (=  $\text{mass}_{\text{Cd, branch}}(\text{mg})/\text{mass}_{\text{Cd, tree}}(\text{mg})$ ), (Table II.3, Figure II.3)). The stem followed ( $0.21 \pm 0.01$ ), assuming a similar Cd concentration as in the branches. Subsequently were the roots ( $0.14 \pm 0.01$ ), leaves ( $0.09 \pm 0.01$ ), and lastly the cacao fruit ( $<0.01$ ). The Cd mass balance within fruits was calculated based on the measured dry weights (Table II.3). The partitioning of Cd in the fruit was highly variable between the biological replicates but most Cd was found in the pod husk ( $0.62 \pm 0.15$ ), followed by the nib ( $0.29 \pm 0.13$ ) and only a minor fraction of fruit Cd was in the placenta ( $0.03 \pm 0.01$ ) and testa ( $0.06 \pm 0.02$ ) (Figure II.4).

**Table 3.2:** Cd concentrations and isotope compositions (as  $\delta^{114/110}\text{Cd}$ ) for the soil and different cacao plant organs. The standard deviation (SD) is calculated based on the mean of the biological replicates (genetically identical trees). Letters denote different statistical groups with the Tukey HSD test ( $p \leq 0.05$ ). Immature, intermediate, mature are samples with different maturation stages in the development of the fruit.

		Cd (mg/kg)			$\delta^{114/110}\text{Cd}$ (‰)				
		n	mean	SD	group	n	mean	SD	group
<b>Topsoil</b>	Total*	3	<b>0.28</b>	0.05	d	3	<b>-0.32</b>	0.05	bc
	Ca(NO <sub>3</sub> ) <sub>2</sub>	3	<b>0.08</b>	0.02	d	3	<b>-0.17</b>	0.03	ab
<b>Subsoil</b>	Total**	1	<b>0.10</b>		cd				
	Ca(NO <sub>3</sub> ) <sub>2</sub>	3	<b>0.03</b>	0.01	d	2	<b>-0.18</b>	0.03	ab
<b>Roots</b>		3	<b>3.21</b>	0.87	b	2	<b>-0.58</b>	0.02	d
<b>Branch</b>		3	<b>6.12</b>	0.44	a	3	<b>-0.33</b>	0.03	bc
<b>Pod husk</b>	Average	5	<b>2.76</b>	0.94	b	5	<b>-0.26</b>	0.06	ab
	Immature	1	3.23			1	-0.31		
	Intermediate	1	3.19			1	-0.31		
	Mature	3	2.46	1.20		3	-0.22	0.05	
<b>Testa</b>	Average	4	<b>2.36</b>	0.43	bc	4	<b>-0.28</b>	0.06	abc
	Immature								
	Intermediate	1	2.23			1	-0.21		
<b>Nib</b>	Mature	3	2.46	1.20		3	-0.30	0.05	
	Average	5	<b>2.05</b>	0.42	bc	3	<b>-0.41</b>	0.10	c
	Immature	1	2.61			1	-0.44		
	Intermediate	1	2.41			1	-0.41		
<b>IF2 leaves</b>	Mature	3	1.75	0.08		3	-0.40	0.13	
		3	<b>3.49</b>	1.12	b	3	<b>-0.15</b>	0.03	a
<b>IF3 leaves</b>		3	<b>3.09</b>	0.35	b	3	<b>-0.18</b>	0.02	ab

\*Total digestion (aqua regia with subsequent HF digestion)

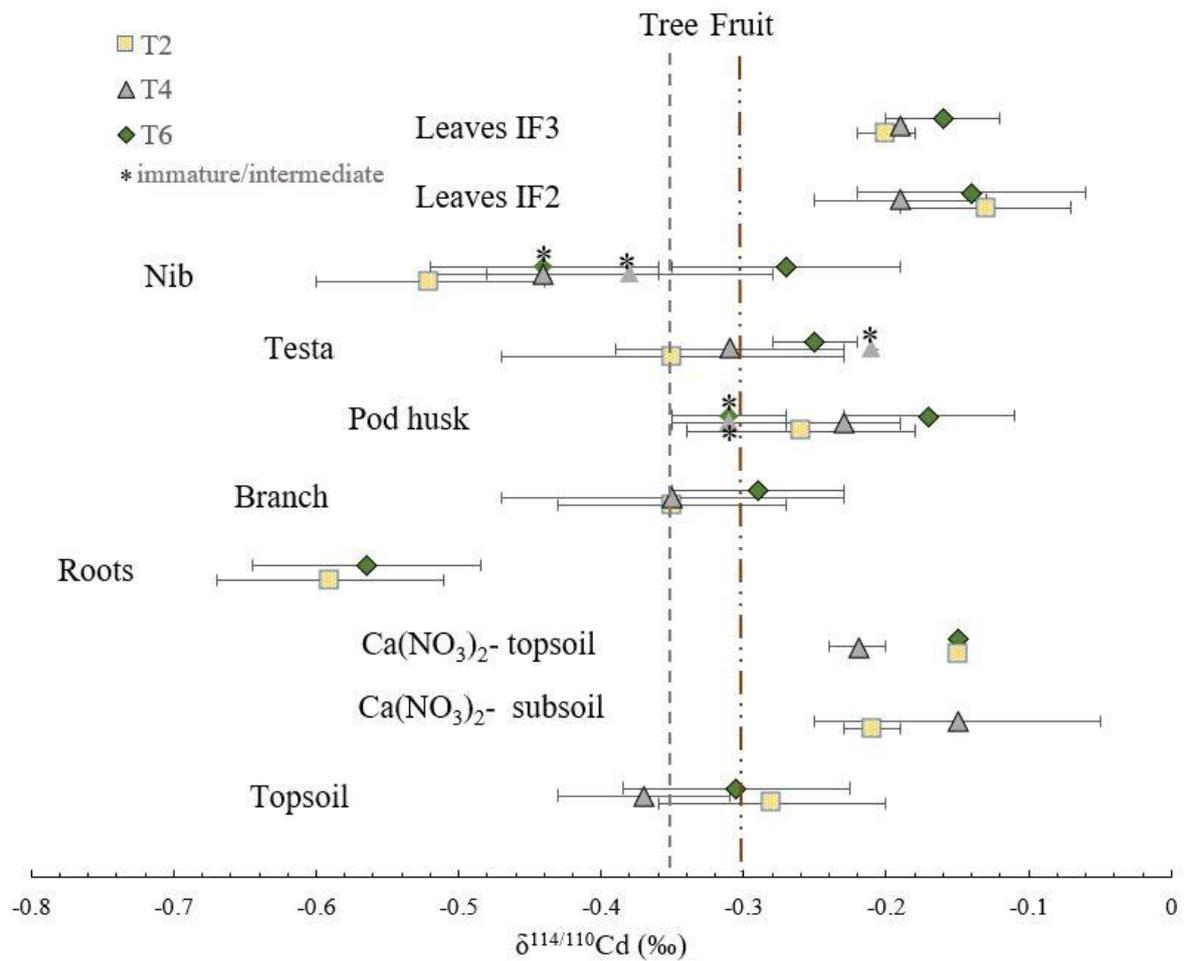
\*\*Aqua regia digestion

§ Ca(NO<sub>3</sub>)<sub>2</sub>-extract: 0.05 M Ca(NO<sub>3</sub>)<sub>2</sub> (5 g soil: 50 mL Ca(NO<sub>3</sub>)<sub>2</sub>)

The isotope compositions of total Cd in the topsoils of the three replicate trees were similar ( $\delta^{114/110}\text{Cd}_{\text{topsoil}} = -0.32 \pm 0.05$  ‰) (Figure 3.1, Table 3.2). A shift towards heavier isotopes was observed from the total topsoil Cd soil pool to the Ca(NO<sub>3</sub>)<sub>2</sub>-extractable pool ( $\delta^{114/110}\text{Cd}_{\text{extractable topsoil}} = -0.17 \pm 0.03$  ‰). The isotopic composition of the Ca(NO<sub>3</sub>)<sub>2</sub>-extractable pools of the subsoil and topsoil were similar ( $\delta^{114/110}\text{Cd}_{\text{extractable subsoil}} = -0.18 \pm 0.03$  ‰). The cadmium isotope composition varied widely within the plant. The average isotope composition of the whole cacao tree calculated based on biomass assumptions was  $\delta^{114/110}\text{Cd} = -0.35 \pm 0.02$  ‰ ( $n=2$ , weighted average). This composition was mostly governed by the isotope composition of the branches since

they represent the biggest reservoir of Cd. No isotope fractionation was observed from total soil Cd to tree Cd ( $\Delta^{114/110}\text{Cd}_{\text{tree-topsoil}} = -0.05 \pm 0.03 \text{ ‰}$ ). The trees were isotopically lighter than the  $\text{Ca}(\text{NO}_3)_2$ -available Cd source ( $\Delta^{114/110}\text{Cd}_{\text{tree-extractable soil}} = -0.20 \pm 0.01 \text{ ‰}$ ).

Aerial tissues were isotopically heavier than roots, which is reflected in positive  $\Delta^{114/110}\text{Cd}_{\text{shoot-root}} = 0.27 \pm 0.01 \text{ ‰}$  (Table 3.3). Within the shoot, Cd isotopes in the leaves were heavier than the branches ( $\Delta^{114/110}\text{Cd}_{\text{IF3 leaves-nib}} = 0.18 \pm 0.01 \text{ ‰}$ ). No fractionation occurred between the branch and the fruit ( $\Delta^{114/110}\text{Cd}_{\text{fruit-branch}} = 0.05 \pm 0.02 \text{ ‰}$ ). The Cd in the cacao nibs was isotopically lighter than in the leaves ( $\Delta^{114/110}\text{Cd}_{\text{leaf IF3-nib}} = 0.25 \pm 0.05 \text{ ‰}$ ). Within the fruit, Cd fractionated from the pod husk to the nibs ( $\Delta^{114/110}\text{Cd}_{\text{nib-pod husk}} = -0.19 \pm 0.05 \text{ ‰}$ ). The testa showed an isotope composition in between the nibs and pod husks ( $\Delta^{114/110}\text{Cd}_{\text{testa-pod husk}} = -0.08 \pm 0.01 \text{ ‰}$ ).



**Figure 3.1:** Cd isotope composition for the various organs in three trees (T2, T4 and T6). The first dashed line on the left represents the weighted average isotope composition of the whole cacao tree ( $\delta^{114/110}\text{Cd} = -0.35 \pm 0.02$  ‰ ( $n=2$ , biological SD)). The second dashed line represents the weighted average isotope composition of the cacao fruits ( $\delta^{114/110}\text{Cd} = -0.35 \pm 0.05$  ‰ ( $n=5$ , biological SD)). For the fruit tissues, the mature, intermediate and immature stages are indicated by depicting the immature and intermediate stages with a \*. Error bars represent 2 x standard deviations (2 SD) of technical replicates ( $n=3$ , analytical precision). If no error bar is shown, no measurements of technical replicates were done.

**Table 3.3:** Apparent Cd isotope fractionation  $\Delta^{114/110} \text{Cd}_{A-B}$  in the cacao tree with SD of three biological replicates. Fractionations that are significant according to Tukey HSD test ( $p < 0.05$ ) on isotope composition tissues in Table 3.2 are given in bold.

	<b>n</b>	<b>mean</b>	<b>SD</b>
tree-topsoil*	2	-0.05	0.03
tree- $\text{Ca}(\text{NO}_3)_2$ pool <sup>§</sup>	2	-0.20	0.01
shoot-root	2	0.27	0.01
fruit-branch	3	0.05	0.02
<b>branch-root</b>	<b>2</b>	<b>0.26</b>	<b>0.04</b>
pod husk-branch	3	0.07	0.05
testa-pod husk	3	-0.08	0.01
<b>nib-pod husk</b>	<b>3</b>	<b>-0.19</b>	<b>0.07</b>
nib-branch	3	-0.08	0.06
<b>IF3 leaves-branch</b>	<b>3</b>	<b>0.18</b>	<b>0.01</b>
<b>IF3 leaves-nib</b>	<b>3</b>	<b>0.25</b>	<b>0.05</b>
IF3 leaves-IF2 leaves	3	-0.03	0.03

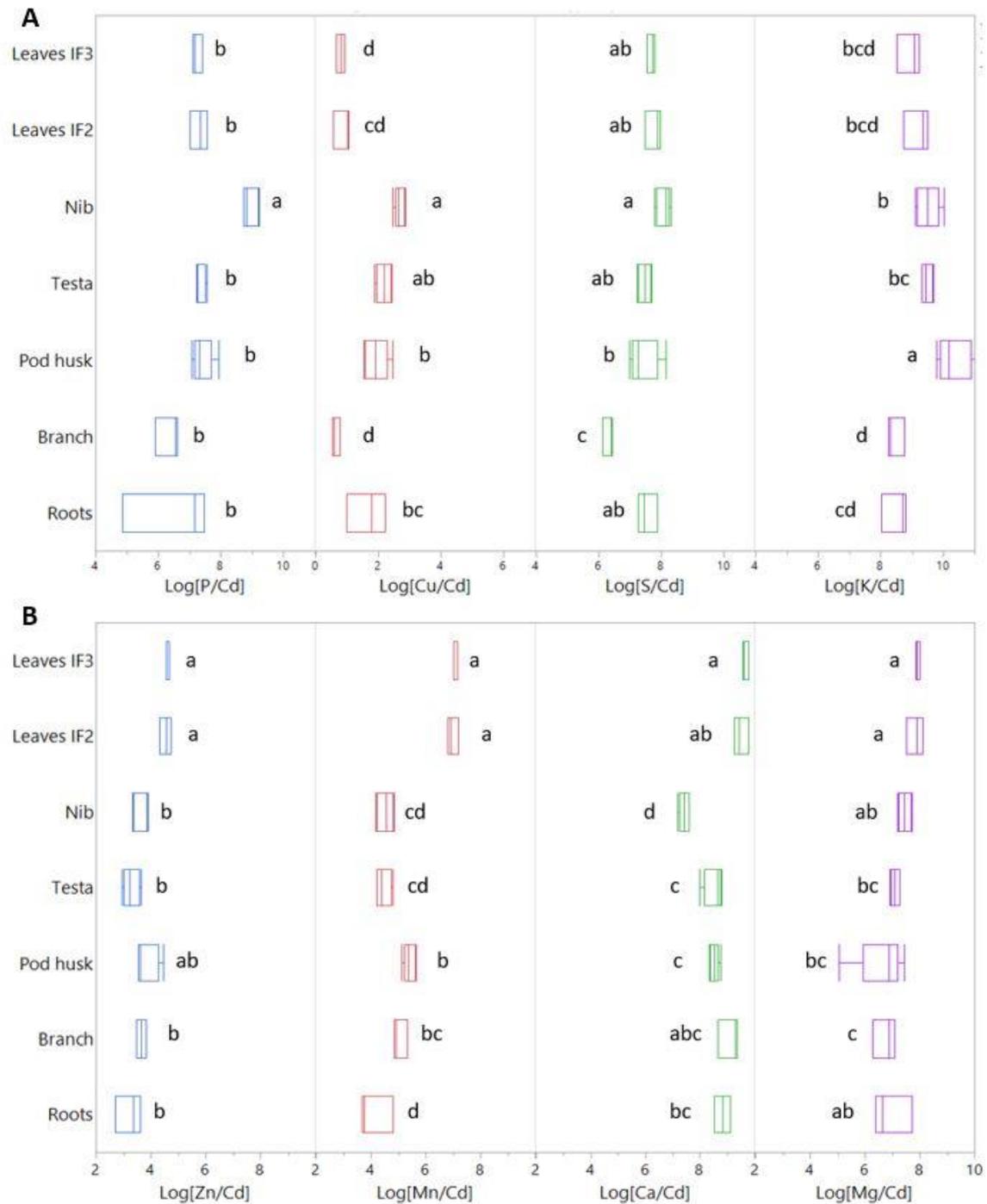
\*Total digestion (aqua regia with subsequent HF digestion)

<sup>§</sup>  $\text{Ca}(\text{NO}_3)_2$ -extract: 0.05 M  $\text{Ca}(\text{NO}_3)_2$  (5 g soil: 50 mL  $\text{Ca}(\text{NO}_3)_2$ )

### 3.3.3 Transfer of nutrients relative to Cd

The linear relationship between Cd and nutrients was evaluated using the log transform of bulk Cd and nutrient concentrations in the various organs (Figure II.5). Zinc, Mn, Ca and Mg (hereafter referred to as Group I) correlated positively with Cd (Table II.7). Contrasting trends were found for P, Cu, S and K (Group II) which correlated negatively to Cd.

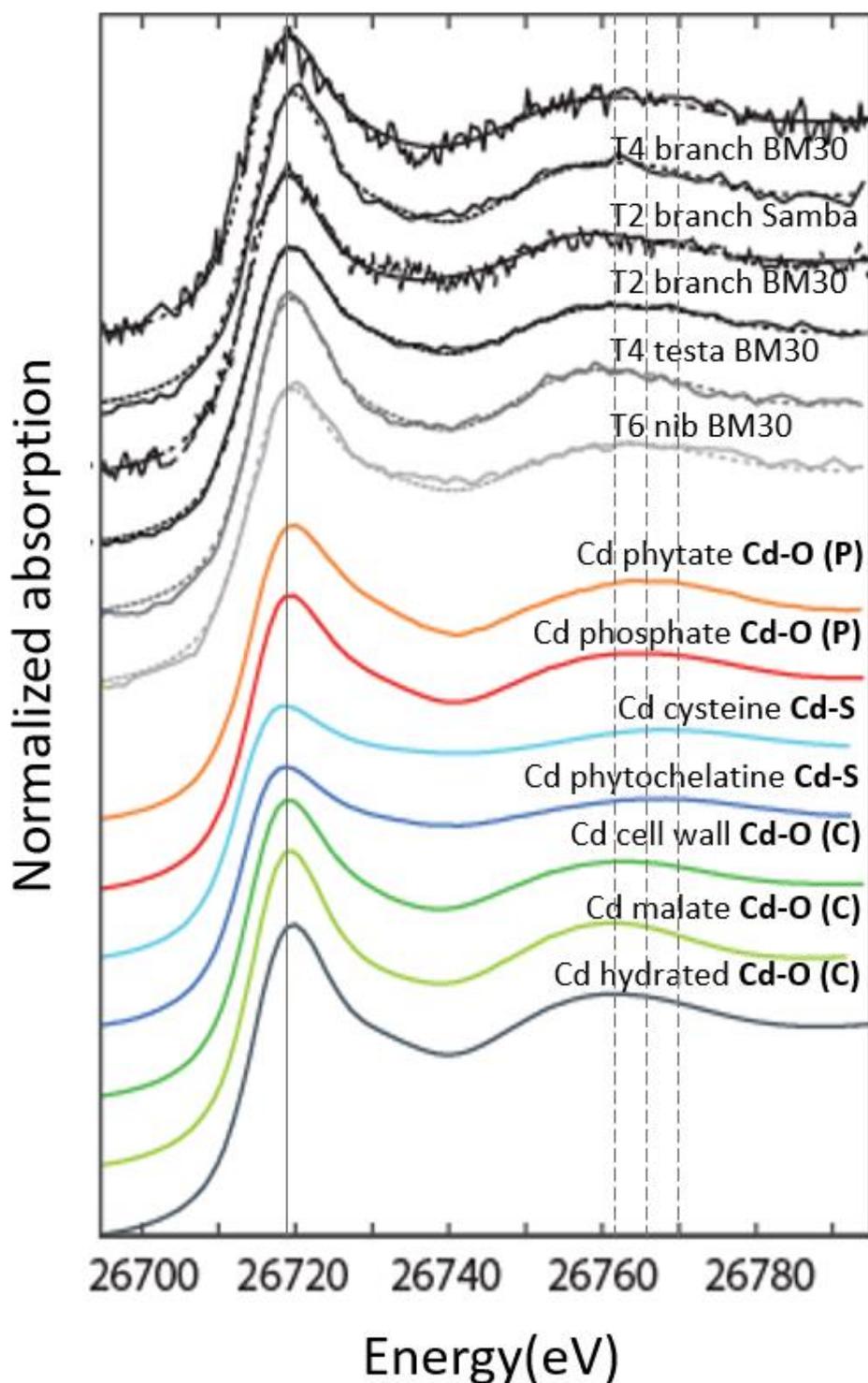
For Group I, the log transformed ratios of nutrient versus Cd concentrations (Figure 3.2) in the different organs revealed that there was no clear retention of Cd relative to other nutrients in the roots. Likewise, in the branches, pod husks, testa and nibs, the element ratios had similar values, indicating a similar accumulation of Cd compared to the other elements. However, the nutrients of Group I accumulated markedly more in leaves than in the other tissues. For Group II, Cu and S were retained more in the roots than Cd, and accumulated less strongly in the branches compared to the nutrients in Group I. P/Cd ratios were markedly higher in the nibs than in the other tissues, whilst the K/Cd ratio was remarkably high in the pod husk.



**Figure 3.2:** Bulk concentration ratios ( $\log[\text{nutrient}/\text{Cd}]$  ( $\text{mg kg}^{-1} \text{ DW}/\text{mg kg}^{-1} \text{ DW}$ )) for the different tree organs. The value of each treatment is shown ( $n=3$ , except for fruit tissues where  $n=5$ ). Letters denote statistical difference of the mean determined by Tukey HSD test ( $p < 0.05$ ). **(A)** Group I elements that correlate positively with Cd. **(B)** Group II elements that correlate negatively with Cd (Figure II.3).

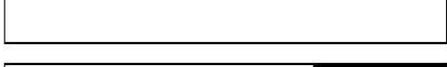
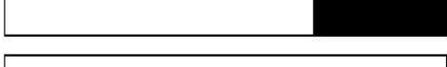
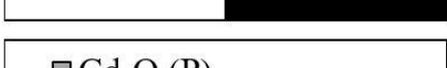
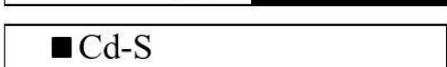
### 3.3.4 Cadmium speciation in cacao organs

In the cacao tree system studied, we identified three major groups of ligands that bind Cd in cacao; (i) O (C)-ligands represented by Cd-hydrated, Cd-cellulose, Cd-cell wall, Cd-Ca-oxalate and Cd-organic acids mix), (ii) O (P)-ligands constituting of R-PO<sub>4</sub>H<sub>2</sub> ligands represented by Cd-phosphate and Cd-phytate and (iii) S-ligands represented by Cd-phytochelatin, Cd-cysteine, Cd-glutathione and Cd-metallothionein. Groups (i) and (ii) represent O donors (Cd-O) and group (iii) S donors (Cd-S) in the first shell. For Cd-S, the amplitude of the white line (the intense absorption peak after the edge; Figure 3.3) is reduced compared to Cd-O. All spectra recorded resemble a first Cd coordination sphere constituted of O atoms (Cd-O). For these ligands, the second shell also contains valuable information as the oscillations for the O (P) and S-containing ligands shift to higher energy values than those for O (C)-ligands (Fig. 3.3) as shown in Isaure *et al.*, 2015. The branches and testa had similar energy of the first oscillation as the O (C)-ligands, while the first oscillation of the nib is shifted like the O (P)-ligands.



**Figure 3.3:** Cd K-edge XANES spectra of cacao tissues and of some Cd references. Cd-hydrated, Cd-malate, Cd-cell wall represent O (C)-ligands; Cd-phosphate and Cd-phytate represent O (P)-ligands, Cd-cysteine and Cd-phytochelatine represent S-ligands. The solid line is positioned at the white line energy (i.e. the intense absorption peak after the edge). The first vertical dashed line is at the first oscillation maximum for O (C)-ligands, the second dashed line for O (P)-ligands, and the third dashed line for S-ligands. Samples were either measured at ESRF, beamline BM30 or synchrotron Soleil, beamline SAMBA.

Linear combination fits revealed that Cd in nib T6 was mostly (60 - 90%) bound to O-ligands (Fig. 3.4), with S-ligands as minor species. The best fits were obtained with O (P)- ligands as major species, but also an equivalent fit was obtained with O (C)-ligands as major species (increase in R-factor of 4%). In testa T4, Cd was exclusively associated with O (C)- ligands (100 %). The branches T4 and T6 were recorded both at BM30 and SAMBA beamlines. The measurements at BM30 showed that the Cd speciation in the biological replicates varied (T4: 60% versus T6: 100% O (C)-ligands). The spectra obtained at SAMBA were noisier (higher R-factor) but resulted in equivalent fits for the same measurement, between 60 and 100% O (C)-ligands and S-ligands as minor ligands. Hence, XANES revealed that overall, Cd was mostly bound to O -ligands in the branches, testa and nibs.

Cd (mg kg <sup>-1</sup> )	Tissue	Replicate	Beamline	R-factor LCF	Fraction of total Cd					
					0	0.2	0.4	0.6	0.8	1
2.2	Nib	T6	BM30	0.00063						
				0.00066						
2.5	Testa	T4	BM30	0.00053						
1.9	Branch	T4	BM30	0.00023						
1.9	Branch	T4	Samba	0.00156						
				0.00156						
2.1	Branch	T6	BM30	0.00169						
2.1	Branch	T6	Samba	0.00242						
				0.00246						

**Figure 3.4:** Proportion of Cd species in cacao tissues determined by XANES. Linear combination fits were done in the  $[E_0-20; E_0+80]$  eV range. Fit quality was examined by the R-factor. If the R-factor increases  $<10\%$ , the fits are of equivalent quality and are shown in the figure.

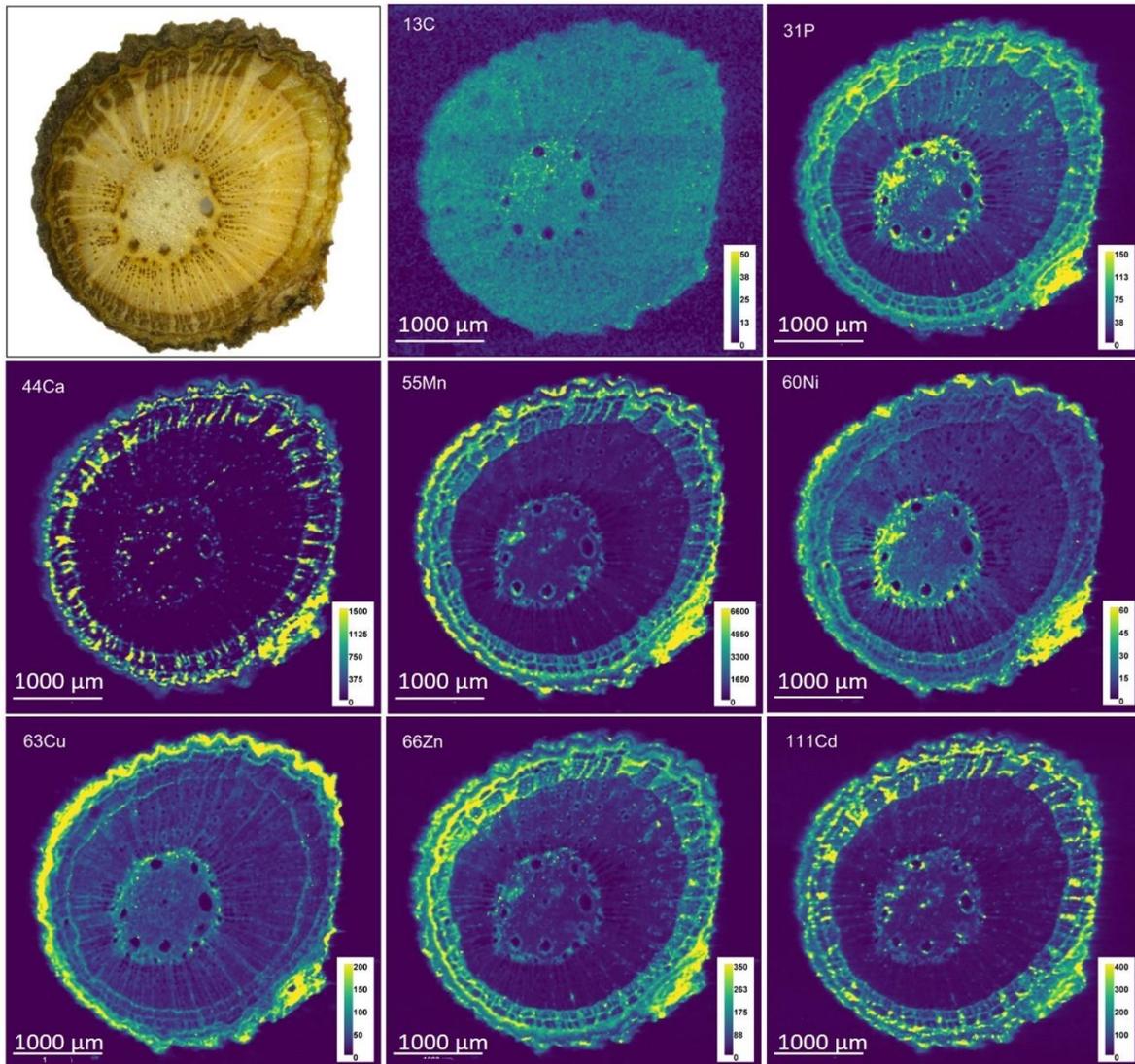
### 3.3.5 Cadmium localization in branches

Branch cross sections were analyzed for Cd, Zn, Cu, Mn, Ca, Ni and P with LA-ICP-MS. The elemental maps revealed the relative abundances of an element between each part of the branch (Figure 3.5, Figure II.7). In the branches of T2 and T4, the pith (medulla), wood and bark phloem, separated by the cambium could be identified (Figure II.6). The wood contains xylem vessels

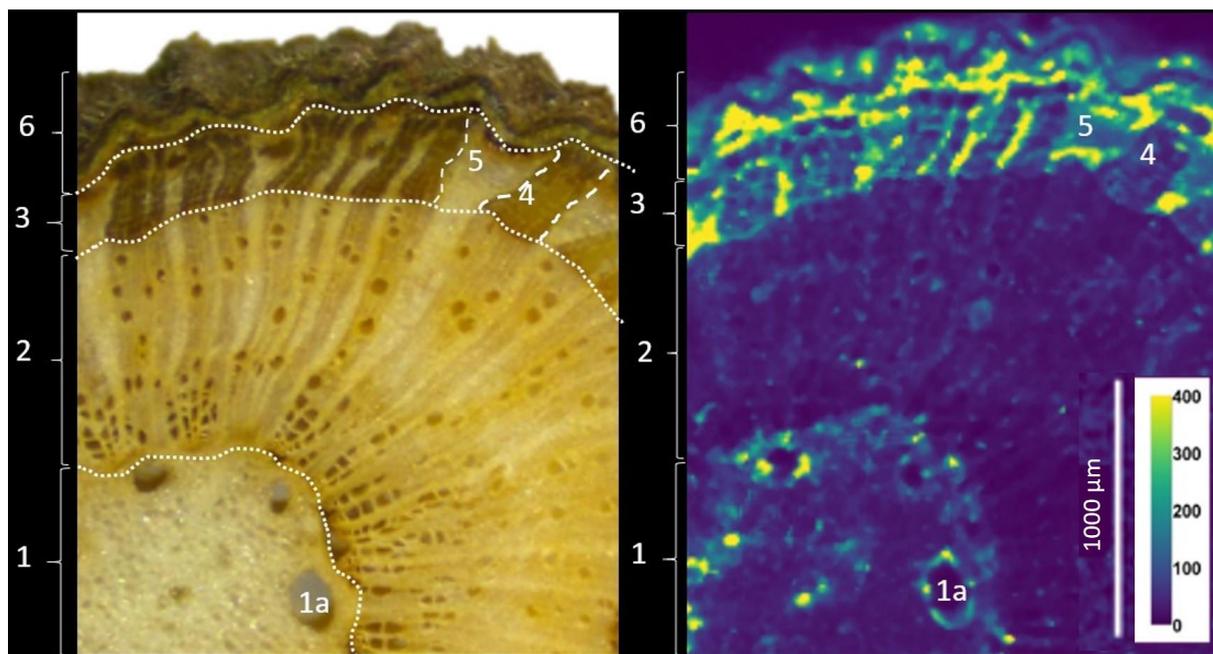
which are in close contact with ray cells. These xylem rays are abundant and continue into the phloem and are essential for the translocation of nutrients between phloem and xylem. Phloem rays separate the phloem vessels, which consist out of sieve tubes, companion cells, phloem fibers and phloem parenchyma. The bark is surrounded by the periderm including phelloderm, cork cambium and heavily suberized cork (Brooks and Guard, 1952).

Cadmium was fourfold more abundant in the bark tissues than in the wood (Figure 3.6, Table 3.4). More specifically, the Cd abundance in the periderm (especially the phelloderm), and the phloem rays was up to seven times higher than in the wood. The phloem rays contained three times more Cd than the phloem vessels. Cadmium was also present in the pith and seemed to be more localized around the lysigenous cavities (i.e. the large channels in the pith).

Overall, LA-ICP-MS revealed that the nutrients were not evenly distributed in the branch. The living cells of the phloem were richer in nutrients than the dead cells of the xylem (Plomion *et al.*, 2001), and consequently, the wood had generally the lowest intensities whereas the bark and pith were more concentrated in all elements investigated (Figure 3.5, Figure II.7). The cambial zone was distinguishable as it consists of a few layers of dense, thin-walled cells. The rays in the wood and bark could be visually differentiated too as they have a more concentrated mineral content than the vessels, which is consistent with their role in nutrient translocation. In every compartment Cd, Mn and Zn co-localized strongly, while the distribution of Cd in the tissues correlated less with Ca and P and especially weak correlations for Cu and Ni were found (Table 3.4). In addition, the correlations were weaker in the wood (and thus xylem) tissues, while in the bark, and especially in the phloem vessels the elements correlated highly. Cadmium accumulated more in the phloem rays than in the vessels compared to the nutrients. In the periderm, Cd strongly correlated with Zn and Mn ( $R^2=0.50$ ), while the correlations with P and Ca were weak ( $R^2<0.15$ ).



**Figure 3.5:** Microscope image and LA-ICP-MS elemental maps of cross section of branch NA T2. Cd, Ca, Mn and Zn co-localized, and less spatial correlation was found between Cd and P or Cu. The intensity is expressed in total counts (cts). The color scale is adjusted to the range of each element.



**Figure 3.6:** (Left) Microscope image of the branch section, and LA-ICP-MS image for Cd. The intensity is expressed in total counts (cts). From inside to outside: 1: Medulla/pith, including the secretory vessels (1a). 2: wood. 3 to 6: bark, including the phloem part (3) with phloem vessels (4) and phloem rays (5), and the periderm (6), composed of phelloderm and cork. (Right) Map of Cd counts (cts). Cadmium is particularly abundant in the phloem rays (5) and periderm (6).

**Table 3.4:** Intensity of Cd in the different structures of the branch (T2) and R<sup>2</sup> of regressions to intensities of other elements detected by LA-ICP-MS.

Tissue	Intensity <sup>\$</sup>	R <sup>2</sup> <sup>\$\$</sup>					
		Cd	Ca	Mn	Zn	Cu	Ni
<b>1 Medulla/pith</b>	2.87	0.32	0.44	0.36	0.12	0.26	0.34
<b>2 Wood</b>	1	0.27	0.36	0.40	0.31	0.21	0.32
<b>3 Bark phloem</b>	4.40	0.43	0.54	0.48	0.30	0.24	0.45
4 Phloem vessels	2.39	0.37	0.51	0.52	0.28	0.18	0.47
5 Phloem Rays	7.29	0.26	0.41	0.35	0.15	0.15	0.29
<b>6 Periderm</b>	7.17	0.13	0.49	0.5	0.24	0.34	0.11

Elements were normalized to <sup>13</sup>C to for differences in tissue hardness and water content within the sample.

<sup>\$</sup>The counts of Cd (cts) were normalized to the mean intensity of counts in wood to compare the accumulation of Cd in the different structures of the branch.

<sup>\$\$</sup> R<sup>2</sup> of bivariate fit of Y= log (E/<sup>13</sup>C) and X = log (Cd/<sup>13</sup>C) (with E=Ca, Mn, Zn, Cu, Ni or P). All fits were significant at  $p \leq 0.05$ .

### 3.4 Discussion

#### 3.4.1 High soil to plant transfer of Cd in cacao

The chosen cacao cultivar efficiently took up Cd from the soil as indicated by the leaf-soil transfer factor ( $TF_{\text{leaf-soil}} = 12.5$ ). A survey in Ecuador in cacao plantations ( $n=560$ ) revealed an average  $TF_{\text{leaf-soil}}$  of 4.5 (Argüello *et al.*, 2019). Likewise, compared to other woody species,  $TF_{\text{leaf-soil}}$  values were higher in our study. Willow and oak showed a lower transfer of Cd to foliar tissues compared to cacao (Sevel *et al.*, 2009; Van Slycken *et al.*, 2013), although in poplar a similar transfer efficiency for Cd has been described (Laureysens *et al.*, 2004)). The high transfer of Cd from soil to plant in our study can be partly explained by the low pH and the low % SOC of the soil that increases the soil Cd bioavailability (Smolders and Mertens, 2013). In addition to these soil properties, the high Cd accumulating properties of the chosen cultivar may have exacerbated the Cd concentrations in the nibs (2 mg Cd kg<sup>-1</sup>). Together, the properties of the soil and the cultivar led to a high Cd concentration in the cacao beans that exceeded the typical trade limits of 0.6 mg Cd kg<sup>-1</sup>.

Cadmium isotope fractionation was used to gain more information on the Cd uptake mechanisms of the cacao tree. The Cd in the cacao plant was lighter than in the Ca(NO<sub>3</sub>)<sub>2</sub>-extractable soil pool

(Figure 3.1, Table 3.3), indicating that preferentially light Cd isotopes were taken up from the soil. The isotope fractionation found in this study was similar to previous studies ( $\Delta^{114/110}\text{Cd}_{\text{plant-source}}$  0.00 to -0.45‰ (Wiggenhauser *et al.*, 2022)), even though these values origin not only from cacao and were obtained in soils with distinct soil properties, including soil pH. The similar isotope fractionation for plant Cd uptake in previous studies and our acidic soil-cacao system suggests that Cd uptake mechanisms may be very similar in distinct soil-plant systems. Currently, it is suggested that the major processes that induce Cd isotope fractionation during plant uptake are Cd complexation in solution by e.g., dissolved organic matter (Ratié *et al.*, 2021), sorption to the apoplast (Wiggenhauser *et al.*, 2021), and cross membrane transport (Moore *et al.*, 2020). Given the low pH in our study, other potential processes that fractionate Cd in the soil-plant interface such as complexation in the solution and sorption in the apoplast should be negligible (Degryse *et al.*, 2009). Consequently, TcNRAMP5, a Mn-transporter identified for Cd uptake in cacao (Ullah *et al.*, 2018) that preferentially takes up light isotopes (Moore *et al.*, 2020) may be major driver for the preferential uptake of light Cd isotopes in our study.

### 3.4.2 Limited retention of Cd in the transfer from root to the aerial tissues

The transfer of Cd from root to aerial tissues was high ( $\text{ITF}_{\text{leaf-root}} \approx 1$ , Table 3.2), as observed in other studies on cacao plantations (Vanderschueren *et al.*, 2021). The isotope fractionation revealed that the cacao surface roots in this study were enriched in light isotopes compared to above-ground parts (Figure 3.1), as observed in wheat, barley, rice, cacao, and a Cd accumulator plant (*Solanum nigrum*) ( $\Delta^{114/110}\text{Cd}_{\text{shoot-root}}$  0.00 to 0.50 ‰ (Wiggenhauser *et al.*, 2022)). Other crops such as cereals translocate much less Cd from root to shoot ( $\text{ITF}_{\text{root-shoot}} < 0.05$ ) (McLaughlin *et al.*, 2011). In the roots of cereals, Cd is separated from nutrients as a protective measure to reduce the translocation of Cd to other tissues (Wiggenhauser *et al.*, 2021). Briefly, during the radial transport towards the xylem, Cd is sequestered in vacuoles by the Heavy Metal transporting ATPase 3 (HMA3) influx transporter (Wang *et al.*, 2019) and subsequently bound to S-containing peptides forming stable complexes (Wiggenhauser *et al.*, 2021). Both processes have been proposed to drive isotope fractionation in the transfer from root to shoot in cereals. Vacuolar sequestration was also suggested to drive Cd fractionation from root to shoot in a contaminated hydroponic system with cacao seedlings (Moore *et al.*, 2020). However, the low retention in the roots of the cacao trees of our study indicates that vacuolar sequestration is rather employed to a small extent to detoxify Cd. Additionally, the observed smaller or similar bulk concentration ratio of Zn, Mn, Ca and Mg relative to Cd between roots and branches, suggests that there is no preferential sequestration of Cd in the roots compared to these nutrients (Figure 3.2). Taken together, we conclude that a small fraction of light Cd was retained in the surface roots and that

the majority of Cd was loaded into the xylem for further transport to the shoot together with nutrients (Figure 3.7).

### 3.4.3 Branches are the main accumulation compartment for Cd

The branches had the highest Cd concentration (Table 3.2) and stored 57% of the total Cd of the cacao tree (Figure II.3). Similarly, (Engbersen *et al.*, 2019) reported higher Cd concentration in the branches of cacao trees compared to the other tissues. This contrasts with other Cd-accumulating trees like willow, birch, and oak, where Cd concentrations were 2-3 times higher in foliage than in stems (Evangelou *et al.*, 2012). Cacao exploits thus a different strategy in the partitioning of Cd than these trees, and stores the majority of Cd in its woody parts. The imaging in our study revealed that Cd was mostly accumulating in the bark and more specifically in the phloem rays and the periderm of the branches (Figure 3.5). Indeed, bark concentrations of trace metals are reported to be consistently greater than concentrations in the wood (Pulford and Watson, 2003). Additionally, we observed that in the phloem rays there was a relative enrichment of Cd compared to the Zn and Mn, indicating that Cd was translocated more to the phloem rays, while Zn and Mn were translocated more by the xylem to the foliage (Figure 3.2). Hence, Cd seems to be partially separated from nutrients in the branches.

The fact that Cd isotopes in the leaves were heavier than in the branches may indicate a progressive retention of light isotopes in the branches. In cacao seedlings, light isotopes were retained in the roots and stem compared to the leaves (Moore *et al.*, 2020). The retention of light isotopes in our study probably occurred in the phloem rays and phelloderm of the branches, since these were the main compartments for Cd accumulation (Figure 3.6, Table 3.4), governing the isotopic composition of the bulk measurement. Based on the bulk XANES results, Cd was likely bound to O (C)-ligands in these tissues. This could be due to the complexation of Cd with the O-functional group of an organic ligand and/or cell wall components, as observed in Cd hyperaccumulating species like *Arabidopsis halleri* (Huguet *et al.*, 2012) and *Sedum alfredii* (Tian *et al.*, 2017). Taken together, light Cd was probably separated from heavy Cd and other nutrients like Mn and Zn in its transport from xylem to phloem rays and phelloderm where it finally complexed with an O (C)-ligand (Figure 3.7).

### 3.4.4 Differential partitioning of Cd between nibs and leaves

The leaf Cd concentrations were more than two times higher than the bean Cd concentrations (nib < IF2 leaf – IF3 leaf < branch, Table 3.2). This trend followed the general consensus that plants accumulate more Cd in leaves than in seeds (Clemens and Ma, 2016). Indeed, other studies in

cacao show leaf Cd concentrations to be higher than the corresponding bean Cd concentrations (Vanderschueren *et al.*, 2021). The mechanism in cacao that affects the partitioning of Cd into reproductive parts seems to be genetically controlled since the variation in bean Cd content has been shown to be much larger than the leaf Cd content (Lewis *et al.*, 2018). In our study, the higher variation in isotope composition observed in fruit tissues compared to vegetative tissues (Table 3.2) may be due to the sampling of the fruits at different maturation. However, the small sample size did not allow to conclude on any effect of maturation on isotope signature.

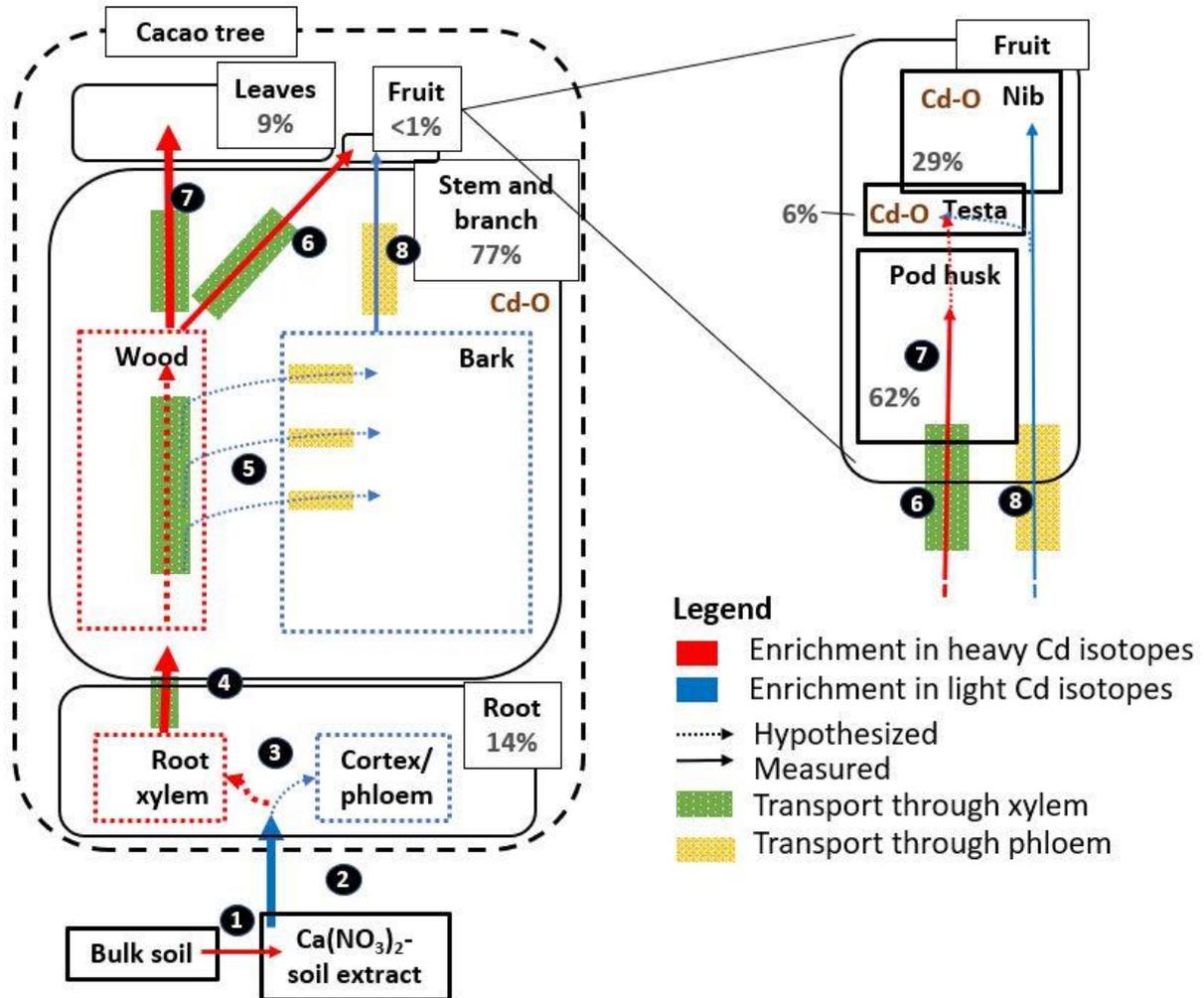
In the present study, nibs (seeds) were isotopically lighter than leaves (Table 3.3). This result is consistent with a previous study on cacao plants in the field (Barraza *et al.*, 2019). An opposite fractionation was observed in cereals, where Cd in the seeds were isotopically heavier than in the leaves (Imseng *et al.*, 2019; Wiggerhauser *et al.*, 2021; Zhong *et al.*, 2022). It was suggested that heavy Cd isotopes were favorably transported from xylem to phloem and finally to the seeds (Zhong *et al.*, 2022). In cacao, another mechanism seemed at stake which may be related to the cauliflorous properties of the tree. The similar isotopic composition of the branches and fruits (Figure 3.1) alluded to a more direct transfer of Cd from the branches to the fruits. As suggested before, in the branch, heavier isotopes were probably transferred to the phloem rays. Cadmium that accumulated in the stem and the branches, may thus be transported directly to the fruits by both xylem and phloem, while the leaves receive the heavier Cd in the xylem from the progressive retention of light isotopes in the stem (Figure 3.7).

#### 3.4.5 Allocation of Cd inside the cacao fruit

Fruits were estimated to be only a small sink of Cd in the tree (<1%, Figure II.3), and within the fruit the Cd was mostly divided between the nibs and the pod husk (Figure II.4). It has been suggested that different nutrient distribution pathways exist between cacao pod husks and nibs (De Araujo *et al.*, 2020). The main transport pathway for minerals to the nib has been reported to be the phloem. In contrast, it was suggested that the xylem was more important for the import of nutrients in the testa and the mucilaginous pulp (Humphries, 1944).

The Cd isotope fractionation between the nib and pod husk (Table 3.3) and different nutrient distribution (Figure 3.2) was consistent with this finding. The isotopic composition of the pod husk was in between that of the leaves and the branches (Figure 3.1). This result indicates that the pod husk received heavier Cd from the xylem, although the fractionation is less pronounced than in the leaves. The high K content in the pod husk corroborates the indications that Cd was transferred via xylem to the pod husk, since the K comprises 70% of all nutrients in the cacao xylem (De Almeida and Valle, 2007). For the testa, Cd had a similar isotope composition and

speciation as the branches and could thus be fed by the xylem and phloem from the branch. Hence, we suggest that Cd was mainly transported from the branches to the cacao fruit, and not remobilized from the leaves to the nibs. The nibs were lighter in isotopes due to the transfer of light isotopes from the phloem, while the pod husk received heavier isotopes from the xylem and the testa received Cd by both xylem and phloem (Figure 3.7).



**Figure 3.7:** Proposition of Cd pathways, in high Cd accumulator NA 312 of cacao. The figure includes information on Cd mass balance (% in grey, size of the boxes and arrows are not proportional to the but are indicative to the main Cd fractions and transport rates), Cd isotope fractionation, and Cd speciation. **1)** Desorbed Cd was isotopically heavier than adsorbed Cd. **2)** The uptake of Cd<sup>2+</sup> inside the plant favors light isotopes, most likely through cross membrane transport with the protein TcNRAMP5 (Moore *et al.*, 2020). **3)** Cd was weakly retained in roots, and the majority was loaded in the xylem for further transport to the shoot, together with nutrients. **4)** Preferentially heavy Cd isotopes were transported from root to shoot via the xylem. **5)** In the branch, Cd was horizontally transferred from the xylem to the phloem in the branches. Lighter Cd isotopes accumulated mostly in the bark (phloem vessels and periderm), while heavier Cd isotopes remained in the xylem. In the branches, Cd was bound to carboxyl ligands, probably to organic acids and/or cell wall components (Cd-O). **6)** Cd that accumulated in the branches (i.e. wood and bark), was transported directly to the fruits by both xylem and phloem. **7)** Leaves and pod husks received heavier Cd isotopes from the progressive retention of light Cd isotopes in the branches. **8)** The nibs were isotopically lighter than the pod husks due to transfer of lighter Cd from the phloem into the nibs. The testa received Cd from both xylem and phloem. The nib was a phloem sink where the majority of Cd was complexed to Cd-O, with phytate as the most plausible ligand. In the testa, Cd was complexed to Cd-O, and more likely to cell wall components or organic acids. Figure layout based on (Kiczka *et al.*, 2010) and (Aucour *et al.*, 2023).

### 3.4.6 Cadmium speciation in the nibs and its implications on bioaccessibility

The nibs had a five times higher P/Cd ratio than other tissues (Table 3.1, Figure 3.2). About 85% of the phosphorus measured in the nibs was in the form of phytate. The phytate/Cd and phytate/(Cd+Zn+Fe) molar ratio showed that phytate could be an abundant ligand for Cd. Together with the XANES result of binding of Cd with mostly O-ligands, and probably more specifically O (P)-ligands (Figure 3.4), we suggest that phytate is the most plausible ligand for Cd in the nibs. Information on the role of P and phytate in cacao nibs is scarce. Mounicou *et al.*, 2003 found that in cacao powders up to 20% of Cd was removed after treatment with phytase, indicating that phytate may be an important chelator of Cd. In wheat kernels (seeds), it has been shown that also about 85% of the phosphorus (P) is stored as phytate (Lemmens *et al.*, 2018) and that micronutrients like Fe and Zn can strongly bind to phytate, inhibiting the human absorption of these minerals. The same principle may apply to Cd (Schlemmer *et al.*, 2009), and may consequently have important implications on Cd absorption in human nutrition. However, speciation data in cereal grains currently indicates mostly a binding of Cd to S-ligands, despite the high phytate content (Gu *et al.*, 2020; Wiggerhauser *et al.*, 2021). It should be noted that these speciation measurements were in systems with higher seed Cd concentrations, which could have affected Cd speciation (Andresen *et al.*, 2013).

### 3.5 Implications and outlook

We combined for the first time total elemental concentrations, Cd stable isotope analyses, speciation, and imaging in cacao trees grown in field conditions at environmentally relevant soil Cd concentrations. We highlighted that compared to annual crops, the retention of Cd in the roots was limited. Cadmium accumulated mainly in the branches and more specifically in the phloem rays of the bark phloem where it was bound to carboxylic ligands. The weight of evidence suggests that in the high Cd accumulating cacao cultivar NA 312, Cd was transferred like other nutrients from root to shoot and accumulated in the phloem rays and phelloderm of the branches to reduce the transfer to foliage. The main contribution of Cd in the nibs came from the phloem tissues of the branch rather than from the remobilization of the leaves. Finally, in the nibs, Cd was stored by chelation with phytate.

Some limitations affected this study. *Theobroma cacao* L. is a perennial tree with a deep rooting system and evidently sampling in field conditions was constrained. For instance, the surface root samples collected may not represent the whole rooting system. Similarly, the thin branch samples may not represent the bigger branches and stem structures, and the mass balance had to be estimated based on literature. Further, this study was only limited to a high Cd accumulating

cultivar, which was deficient in K and P. More comparative studies on other genotypes and hybrids used agronomically are indispensable to extrapolate findings on different control points. We also suggest more speciation measurements as the low Cd concentrations touched the detection limits of XAS, which reduced the sample throughput severely. Despite its limitations, our study has important implications. We extended the limited knowledge on Cd accumulation in perennial, woody crops and revealed that the Cd pathway is markedly different than in annual crops, like the thoroughly studied cereals. The branch was identified as a major tissue for the regulation of the loading of Cd to the nibs. Additional studies which identify the corresponding regulating genes will allow breeding programs to produce low Cd accumulating cultivars in the long term. Grafting has been proposed as a short-term strategy where economically interesting scions are grafted on low cadmium accumulating rootstocks. However, as the scion may be the main regulator of Cd loading into the nibs, further studies must be conducted to validate the efficacy of this strategy. Postharvest mitigation research may benefit from the knowledge of the chemical speciation of Cd in nibs and testa as this is a first step in grasping the chemical processes behind the mobilization of Cd within cacao nibs observed during fermentation. A final piece of important information is the chelation of Cd with phytate which may reduce the absorption of Cd in humans and calls for follow-up studies on human absorption of Cd derived from cacao products.

# Chapter 4: Cadmium uptake, and translocation in mature cacao trees: the effects of cultivar and grafting

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## Summary

Cadmium (Cd) concentrations in cacao often exceed food limits. Novel insights regarding Cd pathways in cacao trees are necessary to advance Cd mitigation strategies. This study aimed i) to determine Cd distribution and speciation within organs of a high Cd accumulating cultivar and ii) identify whether the mechanisms of Cd uptake and translocation within trees vary based on the cultivar or rootstock. We collected samples from triplicate trees of a high and a low Cd accumulating cultivar and a grafted cultivar, all from the same field with similar soil bioavailable Cd. The grafted had the same scion as the low, but was propagated on another rootstock. We used Cd stable isotope analyses in all cultivars and elucidated the distribution of Cd and its storage forms by imaging and speciation techniques in micro to nano-scale in the high Cd accumulator. The total uptake of Cd was factor 2.9 (high) and 1.7 (grafted) higher than in the low cultivar and was highly related to manganese (Mn) uptake. Yet, Cd distribution among organs was similar for all cultivars. The isotope ratio  $\delta^{114/110}\text{Cd}$  of tree organs increased in the order roots < nibs (seeds) < branches < pod husks < mature leaves. The enrichment in heavy isotopes was related to a retention of Cd compared to nutrients along the xylem pathway from roots to leaves. Incorporation of Cd in Ca oxalate crystals was identified as a major mechanism of Cd sequestration in roots and branches of the high cultivar. The weight of evidence indicated that the Cd pathway from branches to nibs was phloem mediated and differed from that from branches to the mature leaves. Our results suggested that processes of plant uptake rather than translocation caused the main differences in nib Cd accumulation among cultivars, and highlight the potential of grafting to reduce bean Cd.

## 4.1 Introduction

Cadmium (Cd) is a potentially toxic trace metal that has no known biological function in humans and has been related to adverse health effects upon chronic exposure (FAO/WHO, 2010). To protect consumers, regulators have set limits on Cd concentrations in chocolate and cacao powders between 0.10 and 0.80 mg Cd kg<sup>-1</sup> (Mercosur, 2011; Ministry of Health of the Russian Federation, 2011; European Commission, 2014; Australia New Zealand Food Standards Code, 2017; Codex Alimentarius Commission, 2018). These regulations have fueled research worldwide to monitor and mitigate Cd accumulation in cacao (Meter *et al.*, 2019; Vanderschueren *et al.*, 2021). Agronomic approaches such as the application of soil amendments have been proposed as a mitigation strategy. However, recent research has indicated that after 30 months of application there is only a limited effect on Cd bean concentrations, while leaf Cd concentrations were reduced (Argüello *et al.*, 2023). Alternatively, differences in Cd accumulation have been observed between different cacao genotypes in the field (Arévalo-Gardini *et al.*, 2017; Lewis *et al.*, 2018; Engbersen *et al.*, 2019). These studies opened the potential of using genetic approaches to mitigate Cd, such as the selection of low Cd accumulating cultivars and/or the use of low Cd accumulating rootstocks in grafting. In the latter, cacao shoots can be grafted on a rootstock with low Cd uptake and/or translocation to the fruit. However, to date, it remains unknown why bean Cd concentrations differ among cultivars, which hampers the development of low Cd accumulating cultivars. In plants in general, Cd is in a first step taken up from the soil by root metal transporters for micronutrients (Clemens and Ma, 2016; Sterckeman and Thomine, 2020; McLaughlin *et al.*, 2021). In cacao, the Mn-transporter TcNRAMP5 has been shown to encode for a protein that transports Cd, Mn, and Fe in cacao seedlings (Ullah *et al.*, 2018). Differences in Cd uptake among cultivars were related to a different expression of specific transporter proteins (Moore *et al.*, 2020). Other transporters are probably also involved in the uptake of Cd, however, no other Cd uptake transporters have been reported in cacao. Once inside plant root cells, Cd can be either sequestered or loaded into the xylem (Clemens and Ma, 2016; Sterckeman and Thomine, 2020; McLaughlin *et al.*, 2021). Concentration measurements in mature cacao trees indicated that the roots are a minor compartment for Cd accumulation (Engbersen *et al.*, 2019; Blommaert *et al.*, 2022). Yet, it remains unclear where and how Cd is sequestered in the roots, and how and in which chemical form the mobile Cd is loaded into the xylem. After loading into the xylem, Cd is being transported to the transpiring organs like leaves and pod husks (Clemens and Ma, 2016). Barraza *et al.* (2019) measured the isotope composition of different organs and indicated that there are differences in the molecular mechanisms among cultivars that are employed for the translocation and sequestration of Cd between cacao leaves, pods and beans. In plants in general, when beans (seeds) develop, they are fed by the seed coat (testa) and can accumulate micronutrients and Cd

from potentially two sources: direct transfer from roots and branches or reallocation from plant organs that store mineral nutrients during vegetative plant growth (Waters and Sankaran, 2011). First, based on correlations of Cd concentrations between organs, Engbersen et al. (2019) suggested that Cd is transported directly from the roots to the nibs after a xylem-to-phloem transfer in the woody part of cacao trees, rather than indirectly via reallocation from the leaves. Isotope compositions combined with imaging data in a high Cd accumulating cultivar corroborated that the xylem-to-phloem transfer occurred in the branches and that Cd in the nibs was loaded from the phloem (Blommaert *et al.*, 2022). A follow-up enriched stable isotope ( $^{108}\text{Cd}$ ) pulse-chase experiment proposed that Cd in cacao nibs likely originates from a more complex mix of direct root uptake and reallocation from both the stem, branches and/or mature leaves (Vanderschueren *et al.*, 2023). From all studies together, it remained unclear how Cd is stored in the leaves and branches, and to which extent Cd is reallocated to other developing organs like the nib. More data on localization and speciation, coupled with isotope composition (Aucour *et al.*, 2023), will help to disentangle the pathways of Cd in cacao tree and the differences in these pathways among cultivars.

The objectives of this study were i) to determine the Cd distribution and speciation in a high Cd accumulator cultivar in the roots, branches, and leaves at micro- and nanoscale ii) to compare the Cd uptake and translocation mechanisms at organ scale between a high and a low Cd accumulating cultivar and iii) to assess the effect of a different rootstock on the Cd uptake and translocation to aerial tissues. For the latter objective, we compared the low Cd accumulating cultivar with a grafted cultivar that had the same scion as the low Cd accumulating cultivar, but was propagated on another rootstock.

## 4.2 Materials and methods

Sampling was conducted in January 2020 and October 2022. Samples were collected at the International Cocoa Genebank, Trinidad (ICGT), a field cacao collection with approximately 2400 cacao genotypes, also further described as cultivars. Two cultivars (further mentioned as a high and a low Cd accumulator) were selected for this study based on their Cd levels in beans and leaves as reported in a previous study at the ICGT (Lewis *et al.*, 2018). The high Cd accumulator was NA 312 of the genetic group *Nanay*, and was studied in a previous experiment (Blommaert *et al.*, 2022). The low accumulator was CL 10/3 of the genetic group *Refractario* (Zhang *et al.*, 2008). Of the CL 10/3 cultivar, there was also a plot of trees that were later introduced in the genebank and were propagated on a TSH 919 rootstock (*Trinidad Selected Hybrids*). These grafted trees were neither classified as a high Cd accumulator, nor as a low Cd accumulator. They were included in this study to investigate how a different rootstock affects uptake and translocation of Cd in the CL

10/3 cacao trees and are subsequently referred to as the grafted cultivar. In brief, the experiment consisted of three cultivars with each three biological replicates: high Cd accumulator (T2, T4, T6), low Cd accumulator (T1, T3, T15), and grafted (T1, T2, T3). The numbers correspond with the respective trees on the field (there were more than three trees on each plot). The biological replicates were genetically identical trees. In this study, we include data on total elemental concentrations and Cd isotope ratios already published for the high Cd accumulator to facilitate the comparison between the three cultivars. Results that were already published are indicated in each table.

#### 4.2.1 Soil and tree sampling

The procedure of soil and tree sampling is described extensively in Chapter 3. Briefly, surface soil (0-20 cm), subsoil (20-40 cm), roots, branches, IF2 leaves (mature green leaves with green stem), IF3 leaves (mature green leaves with brown stem) were collected for all replicates of the three cultivars. In addition, leaves in the flushing 2 phase (F2) were also sampled for the low and grafted cultivar. Flushing 2 leaves are young, developing leaves in their expanding phase and are characterized by a red color (Greathouse *et al.*, 1971). In the high Cd accumulator, no F2 leaves were available at the time of sampling. In all trees, healthy fruits were collected and because of the limited number of fruits on the trees, mature as well as intermediate mature and immature pods were collected. The grafted cultivar bore only one fruit in only one tree at the time of sampling. An overview and illustration of the samples collected can be found in appendix III (Table III.1 and Figure III.1).

All plant samples (roots, branches, leaves, pods) were washed and rinsed with deionized water, and finally dried with paper towels. The intact pod husk was opened and all different tissues (pod husk, placenta, testa, nib (Figure III.1)) were separated and weighed. Intact branches (diameter 2-5 mm) were oven dried and stored for further sample preparation for imaging analyses. Aliquots of the samples for concentration and isotope analyses were weighed for fresh weight/dry weight determination, homogenized, oven dried (65°C), shipped, and ground to powder with a grinder (Pulverisette7, Fritsch).

#### 4.2.2 Chemical analyses

##### 4.2.2.1 Soil and plant properties

For the plant elemental content analyses, 50 mg of plant were digested in concentrated nitric acid (2 mL Suprapur HNO<sub>3</sub>) in an open digestion block for 8 h at a maximum temperature of 130 °C. For the soils, an aliquot was taken from the aqua regia + HF digest prepared for isotope analysis

(see next section). The total elemental composition of the digest was measured by ICP-MS (Agilent7700x, Agilent Technologies) and ICP-OES (iCAP 7400 series, Thermo Scientific). For quality assurance, elemental concentrations were also determined in the certified reference materials BCR-142R (light sandy soil), NIST 1573a (tomato leaves), and NIST 2384 (baking chocolate). The Cd recovery of these samples ranged from 89 to 91% indicating that the digestion processes were effective. A full overview including other elements is given in appendix II. Soil pH, organic carbon (expressed in %OC), soil effective cation exchange capacity (eCEC), ammonium oxalate extractable aluminum, iron and manganese, and 0.05 M  $\text{Ca}(\text{NO}_3)_2$  (5 g soil: 50 mL  $\text{Ca}(\text{NO}_3)_2$ )-extractable minerals were determined with the protocols described in Chapter 3.

#### 4.2.2.2 Cadmium isotope analyses

Prior to the isotope analysis, plant ( $200 \pm 20$  mg) and soil ( $400 \pm 20$  mg) samples were digested and subsequently purified using an anion exchange chromatography procedure as described in Blommaert *et al.* (2022). Cadmium isotope compositions were measured with a multiple collector ICP mass spectrometer (MC-ICP-MS, Neptune Plus, Thermo Scientific). To correct for instrumental mass bias, 'standard-sample bracketing' was applied and isotope standards and samples were doped with silver (Cd:Ag ratio of 2:1). Frequent measurements of a certified isotope standard and soil and plant standards for interlaboratory comparison revealed robust precision and accuracy for Cd concentration and Cd isotope measurements (Table II.1 and Table II.2).

#### 4.2.3 Imaging and speciation analyses

##### 4.2.3.1 LA-ICP-MS

Branches of two biological replicates of the low Cd cultivar and two IF2 leaf cross sections (Table III.1) were analyzed for elemental content (C, P, Ca, Mn, Ni, Cu, Zn, and Cd) by Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS). The branches with a diameter of ca. 5 mm were oven dried ( $60^\circ\text{C}$ ), cut with a microtome blade and placed on a glass slide with Kapton tape prior to measurement. The thin sections of leaves were prepared by cryosectioning frozen-hydrated leaf sections embedded in OCT, with a cryomicrotome (Leica CM 1950). The sections were freeze-dried in a vacuum for 20-30 minutes, then moved to a cabinet for 10 minutes to warm. Afterwards, the sections were airdried and finally attached to a glass slide with Kapton tape prior to measurement. For more information on the operating conditions and parameters of LA-ICP-MS measurement the reader is referred to Chapter 3 (section 3.2.3).

#### 4.2.3.2 MicroXRF imaging and Cd L<sub>III</sub>-edge microXANES analyses

Additional samples of the branches and roots of the high Cd accumulating cultivar were collected in October 2023 to obtain frozen-hydrated tissues. Plant tissue samples in the frozen-hydrated state are the closest possible to relevant biological conditions and minimize the risk of elemental redistribution (van der Ent *et al.*, 2018). The newly sampled branches (Fig. III.1, C), sized about 2-5 mm (diameter), were in an earlier development stage than the previously sampled branches (e.g. the ones used for LA-ICP-MS and hard X-rays imaging (Fig. III.1, E)). These newly collected branches will henceforth be mentioned as the young branches. The freshly sampled root and branch tissues were washed with distilled water to rinse of soil and dust particles. The roots were afterwards submerged in Na<sub>2</sub>-EDTA solution for 20 minutes, and finally rinsed with distilled water. Intact pieces of freshly sampled and washed root and branch tissue (diameter of 1-5 mm) were put in an OCT (Optimal Cutting Temperature) resin in a PCR tube to facilitate subsequent cryosectioning. Afterward the PCR tubes containing the sample embedded in OCT were fast frozen in isopentane to preserve the state of the fresh tissue (Castillo-Michel *et al.*, 2017). Frozen-hydrated samples were shipped in a dry shipper filled with liquid N<sub>2</sub> to the laboratory facilities in France.

Micro-XRF and Cd L<sub>III</sub>-edge  $\mu$ XANES were performed on the ID21 scanning X-ray microscope at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) on transversal thin sections (1-5 mm) of roots and branches. For more information on the parameters of the measurement the reader is referred to section III.1 in appendix III. Because plants contain a large amount of potassium, the K K $\alpha$  fluorescence emission line (3313.8 eV) overlaps the most intense Cd line, Cd L $\alpha$ 1 (3133.7 eV). Consequently, phosphorus (P), sulfur (S), chloride (Cl), magnesium (Mg), strontium (Sr) and cadmium (Cd) maps were recorded using an incident energy below the absorption edge of potassium (3550 eV). Large maps were obtained by scanning the samples with a 2  $\mu$ m step and a counting time of 100 ms per point. The hyperspectral images were analyzed with the PyMCA software package. XRF data were energy calibrated, normalized by the incoming photon flux, and batch-fitted to extract elemental distributions. However, with the model already implemented in the PyMCA software, it was not possible to fit two background contributions that interfered with the Cd L $\alpha$ 1 emission peak. These contributions were attributed to the sample matrix and to the K inelastic scattering. Therefore, we adopted an *ad hoc* fitting strategy that consists in the addition of three Gaussian contributions (see section III.2 and Figure III.2 and III.3). Finally, to allow a visual comparison between different maps, we fixed the maximum value of photon counts for each element (Cl: 10 000, Mg: 600, P: 5000, S: 10 000, Sr: 300, Cd: 300).

In the Cd hotspots observed in the branches and roots, Cd L<sub>III</sub>-edge XANES spectra were collected in fluorescence mode with a silicon drift detector (SDD) in the energy range 3530-3600 eV. Ten to fifty scans of 3 min each in a different spot were averaged using the Orange data mining software (Demšar et al., 2013). A database of Cd reference spectra recorded previously (Isaure et al., 2006) containing Cd-O species (Cd-cell wall, Cd-malate, Cd-oxalate, and Cd-pectin), and Cd-S species (Cd-cysteine and Cd-glutathione) was used for linear combination fitting. Cd-cell wall was remeasured during the measurement to calibrate the samples on the previously recorded database. As additional standard Cd-Ca-Oxalate (Cd-O) (McBride *et al.*, 2017) was measured and included in the database. After averaging, calibrating in energy, and normalization, the XANES spectra were treated by linear combination fits (LCFs) using a XAS software (Fastosh (Landrot, 2018)). LCF was performed by fitting the normalized XANES spectra in regions between -20 and 55 eV (around the absorption edge), using the database of Cd reference compounds. The R-factor (in %) ( $= \frac{\sum[\mu_{\text{exp}} - \mu_{\text{fit}}]^2}{\sum[\mu_{\text{exp}}]^2} * 100$ ) was used to assess the goodness of fit. For some spectra, in particular the noisiest ones, different combinations of reference compounds provided equal goodness-of-fit. The fits of one XANES spectrum were considered of equivalent quality if the R-factor increased < 10% compared to the best fit. For these spectra, several fits are shown.

#### 4.2.3.3 NanoXRF imaging

A selection of branches and leaves of the high Cd accumulator was subjected to high resolution imaging with hard X-rays (2639 keV above Cd K-edge) (see Table III.1). The mature branches collected for LA-ICP-MS imaging were embedded in an OCT-resin and cut with a microtome to obtain transversal cross sections. Freeze-dried leaf thin sections were cryo-sectioned similarly as for LA-ICP-MS. The thin sections were fixed between ultralene foils prior to measurement. At the ID16B beamline (ESRF), maps with higher resolution were recorded (50 nm beam size) at 26.9 keV incident energy, i.e. above the Cd K-edge. For more details on the measurement, see section III.3 in appendix III. XRF data were energy calibrated, normalized by the incoming photon flux, and batch-fitted to extract spatially resolved elemental distributions. No XANES data were recorded, as several attempts showed a high degree of radiation damage due to the non-cryogenic conditions.

#### 4.2.4 Calculations and statistics

Cadmium mass balances were calculated based on the biomass distribution of cacao trees that were 10 years old (average of 20 cacao trees (roots: 31.50 kg, stem: 22.50 kg, branch: 61.90 kg, leaves IF2: 7.75 kg and leaves IF3: 7.75 kg) (Nurafiza *et al.*, 2017)). The trees in that study were sampled out of cacao peak fruiting season, hence the authors did not consider fruits in the biomass

distribution. Therefore, to include the reproductive tissues, the dry weights of the sampled fruits ( $51 \pm 18$  mg DW (average  $\pm$  SD,  $n=11$ ), 1 or 2 fruits on each tree (Table S1)) in this study were added to calculate the Cd mass distribution in the total trees. The stems were not sampled, thus the same Cd concentration as in the branches was assumed. An example of the mass balance calculation for the high Cd accumulator can be found in can be found in Table II.3.

Isotope ratios of  $^{114}\text{Cd}/^{110}\text{Cd}$  in the soil and cacao samples were expressed relative to the  $^{114}\text{Cd}/^{110}\text{Cd}$  isotope ratio of isotope reference material NIST 3108 as  $\delta$ ;

$$\delta^{114/110}\text{Cd} (\text{‰}) = \left[ \frac{\left(\frac{^{114}\text{Cd}}{^{110}\text{Cd}}\right)_{\text{sample}}}{\left(\frac{^{114}\text{Cd}}{^{110}\text{Cd}}\right)_{\text{NIST3108}}} - 1 \right] \times 1000$$

The weighted average Cd isotope composition and Cd concentration of in the entire tree, shoot, and fruit was calculated as a weighted arithmetic mean (shown for a tree);

$$\delta^{114/110}\text{Cd}_{\text{tree}} = \frac{\sum_i m_i c_i \delta^{114/110}\text{Cd}_i}{\sum m_i c_i}$$

Where  $m_i$  denotes the dry weight (g),  $c_i$  the Cd concentration ( $\text{mg kg}^{-1}$ ) and  $\delta_i$  the corresponding isotope composition of the different cacao organs  $i$  (roots, stem, branch, leaves and fruits).

The difference in isotope composition (often mentioned as apparent isotope fractionation) between two compartments A and B is expressed as ' $\Delta$ ' by subtracting Cd isotope ratios from each other;

$$\Delta^{114/110}\text{Cd}_{A-B} = \delta^{114/110}\text{Cd}_A - \delta^{114/110}\text{Cd}_B$$

Transfer factors (TF) indicate to what extent a crop accumulates Cd in its organs and were calculated as the ratio of the Cd concentration in either the plant root/leaf or the edible part ( $\text{mg (kg DW)}^{-1}$ ) to the total Cd concentration of the soil ( $\text{mg (kg DW)}^{-1}$ , determined by aqua regia and HF digestion) as shown below for the nib.

$$TF_{\text{nib}} = \frac{Cd_{\text{nib}}}{Cd_{\text{soil}}}$$

Similarly, internal translocation factors (ITF) were calculated to indicate how Cd is partitioned inside the crop. They are calculated as the ratio of the Cd concentration in a tissue, over the Cd

concentration in another tissue ( $\text{mg (kg DW)}^{-1}$ ), as shown in next example;

$$ITF_{nib-branch} = \frac{Cd_{nib}}{Cd_{branch}}$$

The SD (standard deviation) reported in this study was calculated with biological replicates. One biological replicate is defined as an individual tree. All technical replicates or sampling replicates (e.g. different fruits) were first averaged per tree before calculating the SD. The difference in isotope composition, TF and ITF were always calculated on data of the corresponding tree (paired data). One-way analysis of variance (ANOVA) was conducted to test whether the means of different groups/cultivars (e.g., high, low, grafted) differed significantly from each other. Posthoc Tukey HSD tests were used when ANOVA revealed significant differences in the comparison of means (level of significance:  $p < 0.05$ ). Simple linear regression was used to test the spatial relationship between Cd concentrations and nutrients in the LA-ICP-MS images (level of significance:  $p < 0.05$ ). The data sets were first tested for equality of variances with the O'Brien and Brown-Forsythe test. Additionally, the normal distribution of residuals was tested with the Shapiro-Wilk test. If these prerequisites were not fulfilled, the data were log transformed prior to conducting an ANOVA or linear regression analyses. Such a data transformation was required for the molar ratios of the nutrients versus Cd in the fully digested samples. Statistical analysis was conducted with JMP® Pro version 17.0.0 (SAS Institute 2022).

## 4.3 Results

### 4.3.1 Soil characteristics

The soil on the plot of the high Cd accumulator was characterized in Chapter 3 as strongly acidic, rather low in %OC, and total Cd compared to other cacao plantations (Vanderschueren *et al.*, 2021). The total soil Cd ( $0.26 \pm 0.07 \text{ mg Cd kg}^{-1}$  (average  $\pm$  SD,  $n=9$ )), pH ( $4.20 \pm 0.09$ ), SOC ( $2.10 \pm 0.38 \%$ ), and  $\text{Ca}(\text{NO}_3)_2$ - extractable Cd ( $0.08 \pm 0.02 \text{ mg Cd kg}^{-1}$ ) did not differ significantly among the soils of the different plots ( $p < 0.05$ ) (Table 4.1 and III.2). However, the topsoil of the grafted cultivar had a slightly higher CEC, and higher content of exchangeable bases, ammonium oxalate extractable Fe, Al, and Mn, and a lower  $\text{Ca}(\text{NO}_3)_2$ - extractable Mn content than the high Cd accumulating cultivar. In all plots, the subsoil was more acidic than the topsoil ( $3.79 \pm 0.09$ ), and lower in total Cd ( $0.10 \pm 0.01 \text{ mg Cd kg}^{-1}$ ) and  $\text{Ca}(\text{NO}_3)_2$ - extractable Cd ( $0.03 \pm 0.01 \text{ mg Cd kg}^{-1}$ ). Most subsoil characteristics were similar for the three cultivars. However, the subsoils of the grafted cultivars had again a higher CEC, and a higher  $\text{Ca}(\text{NO}_3)_2$ - extractable Zn compared to the subsoils of the non-grafted plants (Table III.2).

**Table 4.1:** Total, 0.05 M Ca(NO<sub>3</sub>)<sub>2</sub>-extractable soil Cd concentrations and plant organ Cd concentrations of the three cacao cultivars. The grafted cultivar had the same scion as the low cultivar but was propagated on another rootstock. Letters a-c denote different statistical groups with the post hoc Tukey HSD test ( $p < 0.05$ ).

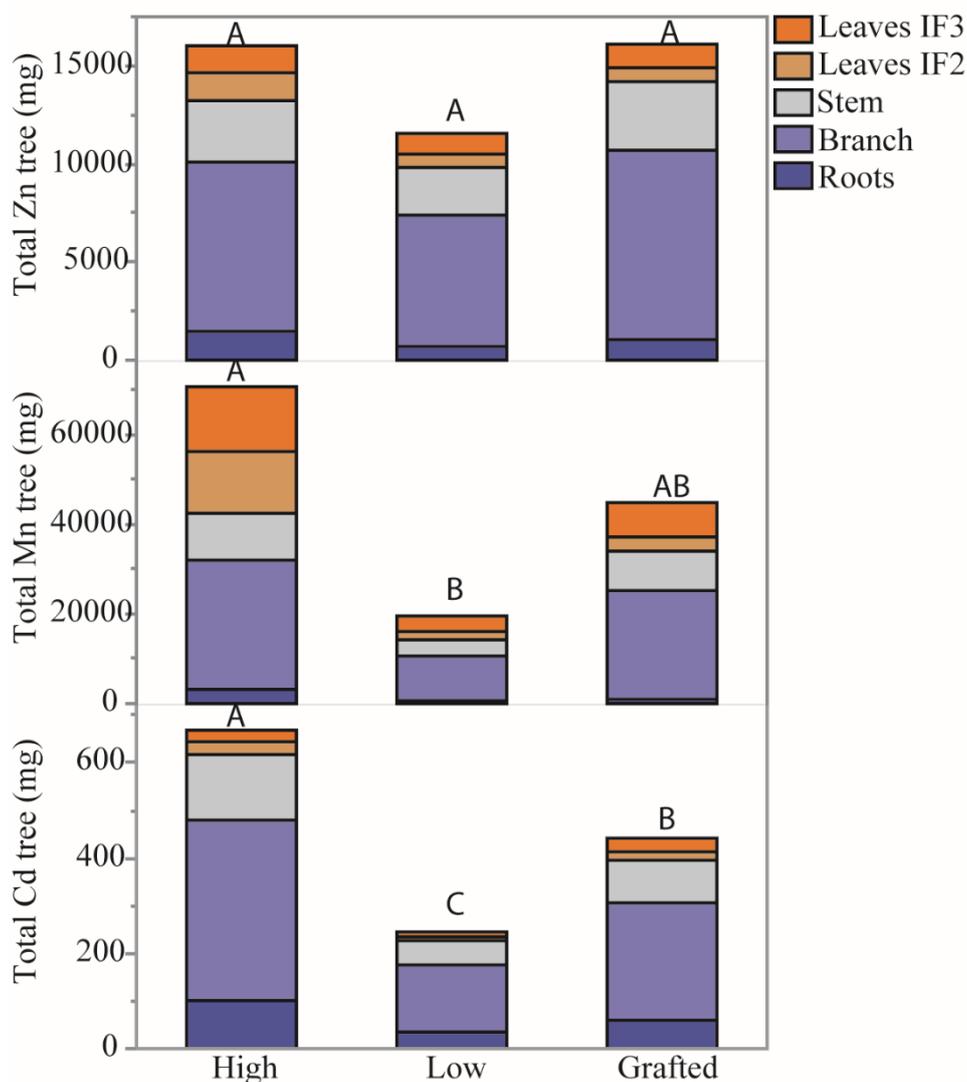
Cultivar:		Cd concentration (mg kg DW <sup>-1</sup> )								
		High			Low			Grafted		
		Mean	SD	n	Mean	SD	n	Mean	SD	n
Topsoil (0-20 cm)	Total*	<b>0.28<sup>a</sup></b>	<i>0.06</i>	3	<b>0.19<sup>a</sup></b>	0.02	3	<b>0.32<sup>a</sup></b>	0.07	3
	Ca(NO <sub>3</sub> ) <sub>2</sub> <sup>§</sup>	<b>0.06<sup>a</sup></b>	<i>0.02</i>	3	<b>0.07<sup>a</sup></b>	0.01	3	<b>0.10<sup>a</sup></b>	0.01	3
Subsoil (20-40 cm)	Total*	<b>0.10<sup>a</sup></b>		1	<b>0.10<sup>a</sup></b>		1	<b>0.10<sup>a</sup></b>		1
	Ca(NO <sub>3</sub> ) <sub>2</sub> <sup>§</sup>	<b>0.03<sup>a</sup></b>	<i>0.01</i>	2	<b>0.03<sup>a</sup></b>	0.01	2	<b>0.04<sup>a</sup></b>	0.01	3
Root		<b>3.21<sup>a</sup></b>	<i>0.87</i>	3	<b>1.11<sup>b</sup></b>	0.37	3	<b>1.75<sup>ab</sup></b>	0.37	3
F2 leaves					<b>1.50</b>	0.32	2	<b>0.50</b>		1
IF2 leaves		<b>3.49<sup>a</sup></b>	<i>1.12</i>	3	<b>0.99<sup>b</sup></b>	0.21	3	<b>2.27<sup>ab</sup></b>	0.18	3
IF3 leaves		<b>3.10<sup>a</sup></b>	<i>0.35</i>	3	<b>1.36<sup>b</sup></b>	0.45	3	<b>3.67<sup>a</sup></b>	0.70	3
Branches		<b>6.11<sup>a</sup></b>	<i>0.44</i>	3	<b>2.27<sup>c</sup></b>	0.77	3	<b>3.89<sup>b</sup></b>	0.51	3
Nib		<b>2.00<sup>a</sup></b>	<i>0.27</i>	3	<b>0.54<sup>b</sup></b>	0.18	3	<b>1.42</b>		1
Testa		<b>2.35<sup>a</sup></b>	<i>0.40</i>	3	<b>0.67<sup>b</sup></b>	0.23	3	<b>0.97</b>		1
Pod husk		<b>2.92<sup>a</sup></b>	<i>0.72</i>	3	<b>0.87<sup>b</sup></b>	0.54	3	<b>1.59</b>		1
Placenta		<b>1.24<sup>a</sup></b>	<i>0.34</i>	3	<b>0.25<sup>b</sup></b>	0.09	3	<b>0.40</b>		1

The data in italic stems from Chapter 3. The immature, intermediate, mature samples of the fruit organs were pooled together per biological replicate, i.e. individual tree. The standard deviation (SD) is calculated based on the means of the biological replicates. <sup>§</sup>Total digestion (aqua regia with subsequent HF digestion). <sup>§§</sup> 0.05M Ca(NO<sub>3</sub>)<sub>2</sub>- extract: (5g soil: 50 mL Ca(NO<sub>3</sub>)<sub>2</sub>). \*Flushing 2 leaves are young, developing leaves. \*\*Interflush-2 and interflush-3 leaves are mature green leaves.

#### 4.3.2 Cadmium uptake and partitioning in the three cultivars

In all three cultivars, the tree organs were enriched in Cd compared to total soil Cd concentrations (transfer factor (TF) > 1, Table 4.2). The branches were most enriched in Cd, followed by the roots and leaves, and the lowest concentrations were found in the organs of the fruit (testa, pod husk and nib) (Table 4.1). The transfer factors from soil to organ revealed significant differences between the high and the low cultivar (Table 4.2). Consequently, in all organs, Cd concentrations were significantly higher in the high cultivar than in the low cultivar while those in the grafted plants were in between both in most tissues. Cadmium mass estimations (Fig. 4.1) showed that total Cd uptake was a factor 2.7 higher in the high cultivar compared to the low Cd cultivar. The differences in Cd concentration between the grafted and low cultivar were statistically not significant except for the branches and IF3 leaves (Table 4.1). The total Cd uptake was a factor 1.8 higher in the grafted cultivar than in the corresponding low cultivar that was ungrafted ( $p < 0.05$ ).

Despite differences in Cd accumulation, we observed that the internal translocation factors (ITFs) did not differ between the high and the low cultivar (Table 4.2). Only the  $ITF_{IF3leaf-IF2leaf}$  differed significantly between the high ( $0.93 \pm 0.17$ ) and the grafted ( $1.63 \pm 0.36$ ) cultivar. The Cd mass fractions, calculated under the assumption that the three cultivars have similar biomass distribution, showed that the Cd partitioning between the organs was similar within each cultivar (Figure III.4).



**Figure 4.1:** Total Cd, Mn, and Zn (mg) in the organs of the trees calculated with assumptions on tree biomass (see materials and methods). The Cd, Mn, Zn concentration in the stem was estimated to be equal to that in the branches. Letters A, B, and C denote different statistical groups among cultivars for total Cd uptake. IF2 leaves are mature green leaves with green stem, IF3 leaves are mature green leaves with brown stem. The biomass of the fruits and developing (F2)-leaves was negligible compared to the total tree biomass.

**Table 4.2:** Cadmium transfer and internal translocation factors for the different cultivars. The grafted cultivar had the same scion as the low cultivar but was propagated on another rootstock. Letters a-b denote different statistical groups with the posthoc Tukey HSD test ( $p < 0.05$ ). The standard deviation (SD) is calculated based on the mean of the biological replicates.

	<i>High</i>		Low		Grafted	
	<i>Mean</i>	<i>SD</i>	Mean	SD	Mean	SD
<b>Transfer factor</b>						
root/total soil*	<b><i>11.52<sup>a</sup></i></b>	<i>1.11</i>	<b>5.85<sup>b</sup></b>	1.9	<b>5.59<sup>b</sup></b>	1.66
IF2 leaf/total soil*	<b><i>13.11<sup>a</sup></i></b>	<i>5.23</i>	<b>5.16<sup>b</sup></b>	0.83	<b>7.20<sup>ab</sup></b>	1.04
nib/ total soil*	<b><i>7.38<sup>a</sup></i></b>	<i>0.80</i>	<b>2.89<sup>b</sup></b>	0.83	<b>5.35</b>	.
<b>Internal translocation factor</b>						
branch/root	<b><i>1.97<sup>a</sup></i></b>	<i>0.22</i>	<b>2.04<sup>a</sup></b>	0.05	<b>2.30<sup>a</sup></b>	0.22
nib/root	<b><i>0.64<sup>a</sup></i></b>	<i>0.12</i>	<b>0.49<sup>a</sup></b>	0.10	<b>0.69</b>	.
IF2 leaf/root	<b><i>1.17<sup>a</sup></i></b>	<i>0.58</i>	<b>0.91<sup>a</sup></b>	0.16	<b>1.33<sup>a</sup></b>	0.25
nib/branch	<b><i>0.33<sup>a</sup></i></b>	<i>0.03</i>	<b>0.24<sup>a</sup></b>	0.04	<b>0.32</b>	.
IF2 leaf/branch	<b><i>0.58<sup>a</sup></i></b>	<i>0.21</i>	<b>0.45<sup>a</sup></b>	0.07	<b>0.58<sup>a</sup></b>	0.07
nib/IF2 leaf	<b><i>0.61<sup>a</sup></i></b>	<i>0.20</i>	<b>0.54<sup>a</sup></b>	0.08	<b>0.64</b>	.
IF3 leaf/IF2 leaf	<b><i>0.93<sup>b</sup></i></b>	<i>0.17</i>	<b>1.36<sup>ab</sup></b>	0.20	<b>1.63<sup>a</sup></b>	0.36

The data in italic comes from Chapter 3. \*Total digestion of the soil was conducted with aqua regia with subsequent HF digestion. Transfer factor (TF) = ratio of the Cd concentration in a plant organ ( $\text{mg (kg DW)}^{-1}$ ) to the total Cd concentration of the soil ( $\text{mg (kg DW)}^{-1}$ ). Internal translocation factor (ITF) = ratio of the Cd concentration in an organ ( $\text{mg (kg DW)}^{-1}$ ), over the Cd concentration in another organ ( $\text{mg (kg DW)}^{-1}$ ). IF2 leaves are mature green leaves with green stem, IF3 leaves are mature green leaves with brown stem.

#### 4.3.3 Differences in isotope composition from soil to cacao nib and leaves

The isotope compositions of total Cd in the topsoils of the three plots did not differ among cultivars and were, on average,  $-0.32 \pm 0.05$  ‰ (mean  $\pm$  SD,  $n=8$ ). A shift towards heavier isotopes was observed from the total topsoil Cd soil pool to the  $\text{Ca(NO}_3)_2$ - extractable pool ( $\delta^{114/110}\text{Cd}_{\text{extractable topsoil}} = -0.17 \pm 0.06$  ‰). The isotope composition of the  $\text{Ca(NO}_3)_2$ -extractable pools of the subsoil and topsoil were similar ( $\delta^{114/110}\text{Cd}_{\text{extractable subsoil}} = -0.18 \pm 0.06$  ‰). The average weighted isotope composition of the entire cacao tree was calculated based on biomass assumptions for the three cultivars (see Materials and Methods). All sampled trees absorbed preferentially the lighter Cd isotopes from the  $\text{Ca(NO}_3)_2$ -extractable Cd soil pool ( $\Delta^{114/110}\text{Cd}_{\text{tree-Ca(NO}_3)_2\text{-extractable}} = -0.18 \pm 0.04$  ‰, Table 4.3).

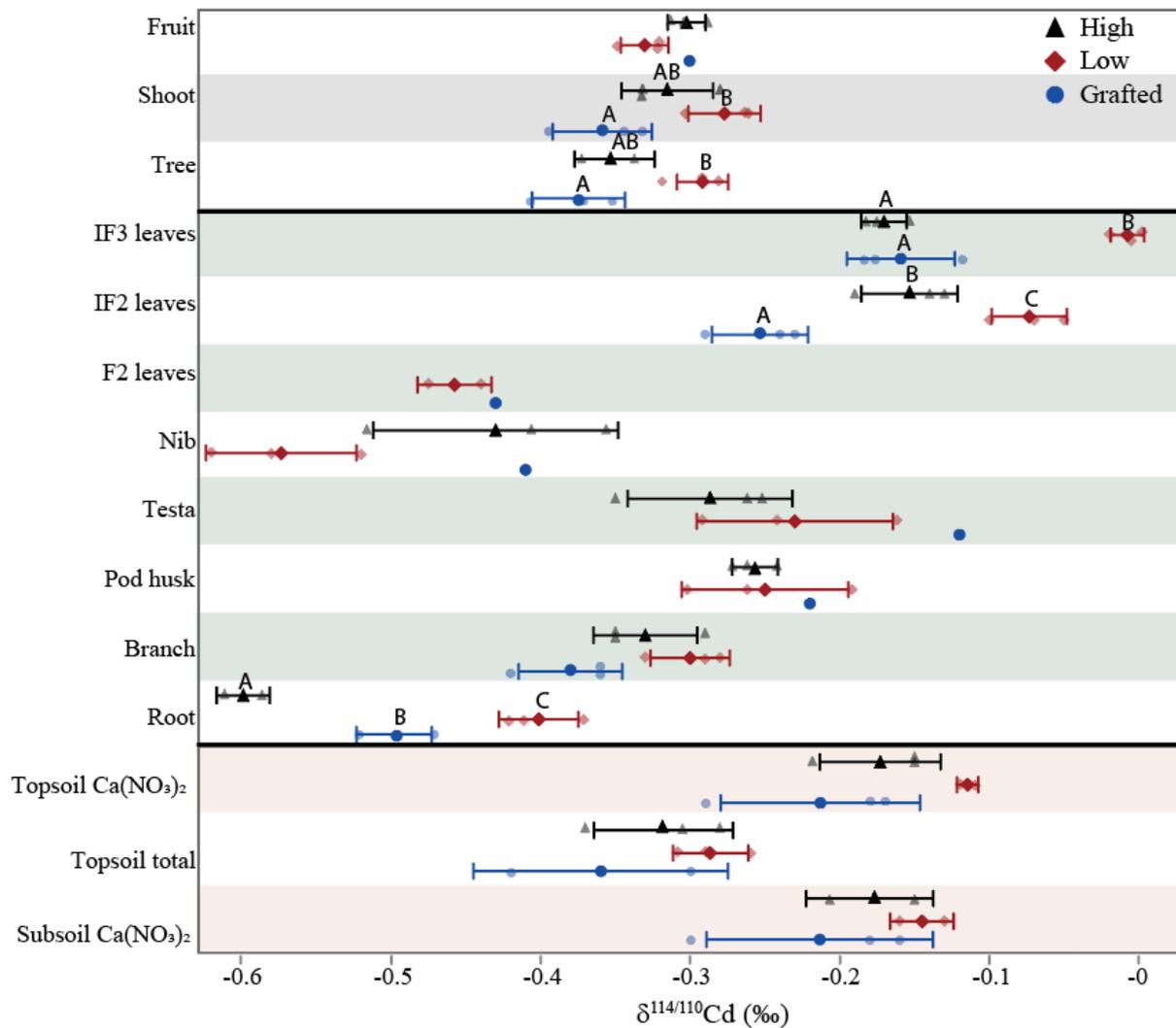
The difference in isotope composition was significant from roots to branches for the high Cd cultivar ( $\Delta^{114/110}\text{Cd}_{\text{branches-root}} = 0.26 \pm 0.02$  ‰), but not for the low and the grafted cultivar (Figure 4.2, Table 4.3). The mature leaves (IF2 and IF3) were enriched in heavy isotopes compared to the

roots and branches in all cultivars. The Cd in the nibs was significantly lighter in isotopes compared to IF2 and IF3 leaves for the high and low Cd accumulator, with larger differences in the low ( $\Delta^{114/110}\text{Cd}_{\text{nib-IF2 leaves}} = -0.28 \pm 0.10 \text{ ‰}$  (high),  $\Delta^{114/110}\text{Cd}_{\text{nib-IF2 leaves}} = -0.50 \pm 0.06 \text{ ‰}$  (low)). The low Cd accumulator showed a similar isotope composition between the F2 (young, developing) leaves and the nibs. In general, the isotope composition within trees followed a similar pattern among cultivars and increased in the order roots<nibs<branches<pod husks<IF2 and IF3 leaves.

**Table 4.3:** Differences in isotope composition between soil or plant parts ( $\Delta^{114/110}\text{Cd}_{\text{A-B}}$ ) in the cacao trees. Significant differences are indicated in bold and with \*. The isotope composition between two compartments is significantly different when the 95% confidence interval (CI) does not encompass 0.

Cultivar	$\Delta^{114/110}\text{Cd}_{\text{A-B}}$ (‰)								
	High			Low			Grafted		
	Mean	SD	95% CI	Mean	SD	95% CI	Mean	SD	95%CI
tree-topsoil	-0.06	0.04	-0.46/0.03	-0.01	0.02	-0.04/0.03	-0.03	0.11	-1.05/0.99
tree-topsoil extract	-0.2	0.03	-0.44/0.04	<b>-0.22</b>	0.06	-0.36/-0.07	-0.16	0.08	-0.35/0.03
branch-root	<b>0.26*</b>	0.02	0.04/0.48	0.08	0.05	-0.05/0.21	0.1	0.04	0-0.20
shoot-root	<b>0.27*</b>	0.02	0.09/0.46	0.1	0.05	-0.02/0.23	<b>0.12</b>	0.04*	0.03/0.21
IF2 leaf-root	<b>0.44*</b>	0.02	0.22/0.66	<b>0.31*</b>	0.01	0.28/0.34	<b>0.22</b>	0.02*	0.19/0.26
IF3 leaf-root	<b>0.40*</b>	0.01	0.30/0.49	<b>0.37*</b>	0.04	0.27/0.47	<b>0.32</b>	0.03*	0.25/0.39
nib-root	0.14	0.1	-0.72/1.00	-0.19	0.08	-0.38/-0.01	0.04		
pod husk-root	<b>0.33*</b>	0	0.30/0.36	<b>0.13*</b>	0.03	0.06/0.20	0.4	0.15	
IF2-nib	<b>0.28*</b>	0.1	0.03/0.52	<b>0.50*</b>	0.08	0.31/0.69	0.18		
nib-branch	-0.1	0.06	-0.25/0.05	<b>-0.27*</b>	0.03	-0.35/-0.20	-0.05		
pod husk-branch	<b>0.07*</b>	0.02	0.02/0.13	0.05	0.08	-0.15/0.25	0.14		
IF2 leaf-branch	<b>0.18*</b>	0.04	0.08/0.27	<b>0.23*</b>	0.05	0.10/0.35	0.13	0.06	-0.01/0.26
IF3 leaf-branch	<b>0.15*</b>	0.02	0.11/0.18	<b>0.29*</b>	0.02	0.25/0.32	<b>0.22*</b>	0.05	0.11/0.34
testa-pod husk	-0.03	0.05	-0.16/0.10	0.02	0.01	0.00/0.04	0.1		
testa-nib	<b>0.14*</b>	0.03	0.07/0.22	<b>0.34</b>	0.12*	0.06/0.63	0.29		
IF3 leaf-IF2 leaf	-0.03	0.04	-0.12/0.06	0.06	0.04	-0.03/0.15	<b>0.10*</b>	0.04	0.01/0.18
fruit-branch	0.03	0.02	-0.03/-0.06	-0.03	0.01	-0.06/0.00	0.06		
F2 leaf-nib				0.11	0.05	-0.30/0.52			

&Total digestion (aqua regia with subsequent HF digestion).<sup>§</sup>0.05M  $\text{Ca}(\text{NO}_3)_2$ - extract: (5g soil: 50 mL  $\text{Ca}(\text{NO}_3)_2$ ).<sup>#</sup>The isotope composition of the tree, shoot and fruit was calculated based on biomass assumptions/measurements (see section 4.2). The grafted cultivar had the same scion as the low cultivar but was propagated on another rootstock.



**Figure 4.2:** Cd isotope compositions ( $\delta^{114/110}\text{Cd}$ ) for the soil and different cacao plant organs (in ‰). The measurement of each biological replicate is depicted with transparent markers and the mean of the biological replicate with a non-transparent marker. The error bars represent the standard deviation (SD), calculated based on the means of the biological replicates if available. Letters A-C denote different statistical groups in Cd isotope composition of tree parts/organs among cultivars with the post hoc Tukey HSD test ( $p < 0.05$ ). Topsoil total: total digestion (aqua regia with subsequent HF digestion) and 0.05M  $\text{Ca}(\text{NO}_3)_2$ -extract: (5g soil: 50 mL  $\text{Ca}(\text{NO}_3)_2$ ). The isotope composition of the tree, shoot and fruit was calculated based on biomass assumptions (see materials and methods).

#### 4.3.4 Uptake and translocation of nutrients relative to Cd

The Cd and nutrient (P, Cu, S, K, Zn, Mn, Ca, Mg) concentrations measured in each organ (Table 4.1 and Table III.3) were used to calculate the total uptake of Cd and nutrients (Figure 4.1 and Table III.4) and the log transform of the ratio of bulk nutrient versus Cd concentration (Table III.5). The total uptake of Cd and Mn was significantly higher in the high Cd accumulator compared to the low Cd accumulator. Total tree K was lower in the high accumulator than in grafted cultivar, and

total S was lower in the high accumulator than in the low accumulator (Table III.4). For all other nutrients measured, the total uptake did not differ among cultivars.

For all nutrients, except S, Mn, and Ca, the log nutrient/Cd concentration ratio was similar for roots and branches, and this was true for all cultivars (Table III.5). The Mn/Cd and Ca/Cd ratios were higher in the branches than in the roots. Thus, there was a separation of Ca and Mn from Cd from root to the branches. For S on the contrary, less S was exported relative to Cd from roots to branches. With the log nutrient/Cd concentration ratios we could also observe that there was, relatively to Cd, a larger accumulation of P, Cu, S, and K in the tissues of the fruit than in the branches. The P and Cu concentrations were especially high in the nibs, while the K concentration was high in the pod husks (Table III.3). In the IF2 and IF3 leaves of the high Cd accumulator there was a pronounced enrichment in Zn, Mn, Ca and Mg relative to Cd. In all cultivars there was a large Mn internal translocation factor between IF2 leaves and roots ( $ITF_{IF2 \text{ leaves-roots}} = 18$  (high), 12 (low), and grafted (13), ratios calculated from Table III.3). The log nutrient/Cd concentration ratios of the F2 leaves were similar to those of the nibs which was illustrated by the increase in P, Cu, S, and K relative to Cd.

#### 4.3.5 Imaging and speciation at micro to nano-scale

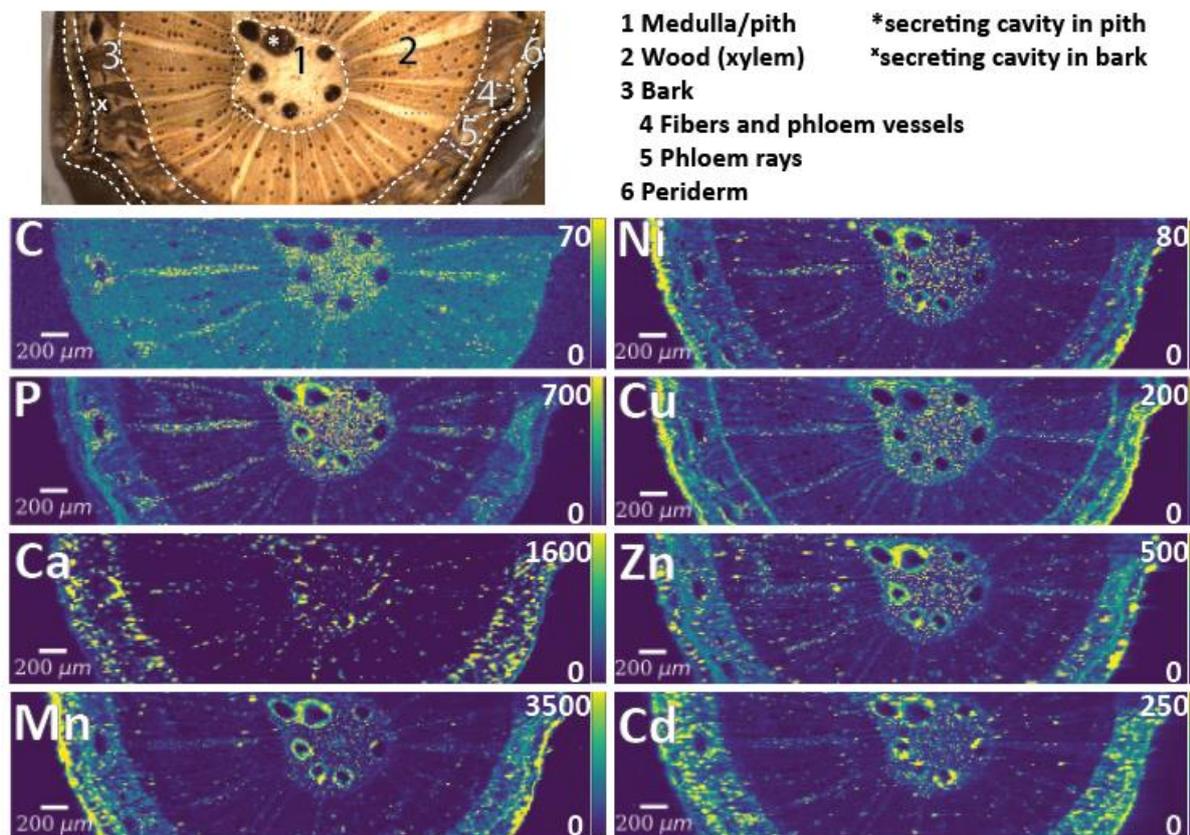
##### 4.3.5.1 Cadmium localization in branches

Two dried branch cross sections of the low Cd accumulator (T3 and T15, Table III.1) were mapped with LA-ICP-MS. The branches (T2 and T4) of the high Cd accumulator were previously characterized for the same elements (Blommaert *et al.*, 2022). The LA-ICP-MS images allowed to visualize the relative abundance of an element between each tissue of the branch (pixel size: 10  $\mu\text{m}$ ). The pith, wood, bark (with phloem vessels and phloem rays), and periderm could be clearly identified (Figure 4.3 and Figure III.5). The xylem rays in the wood continued into the phloem rays. The pith had large secreting cavities (Figure 4.3, indicated with \*) (Brooks and Guard, 1952; Angyalossy *et al.*, 2016). These cavities are known to transport mucilage within the cacao tree. Mucilage is a water-soluble edible pulp that contains carbohydrates and uranic acids (Tosif *et al.*, 2021). In the bark, other large cavities were observed, which have also been reported to transport mucilage in cacao trees (Figure 4.3, indicated with x) (Brooks and Guard, 1952; Angyalossy *et al.*, 2016).

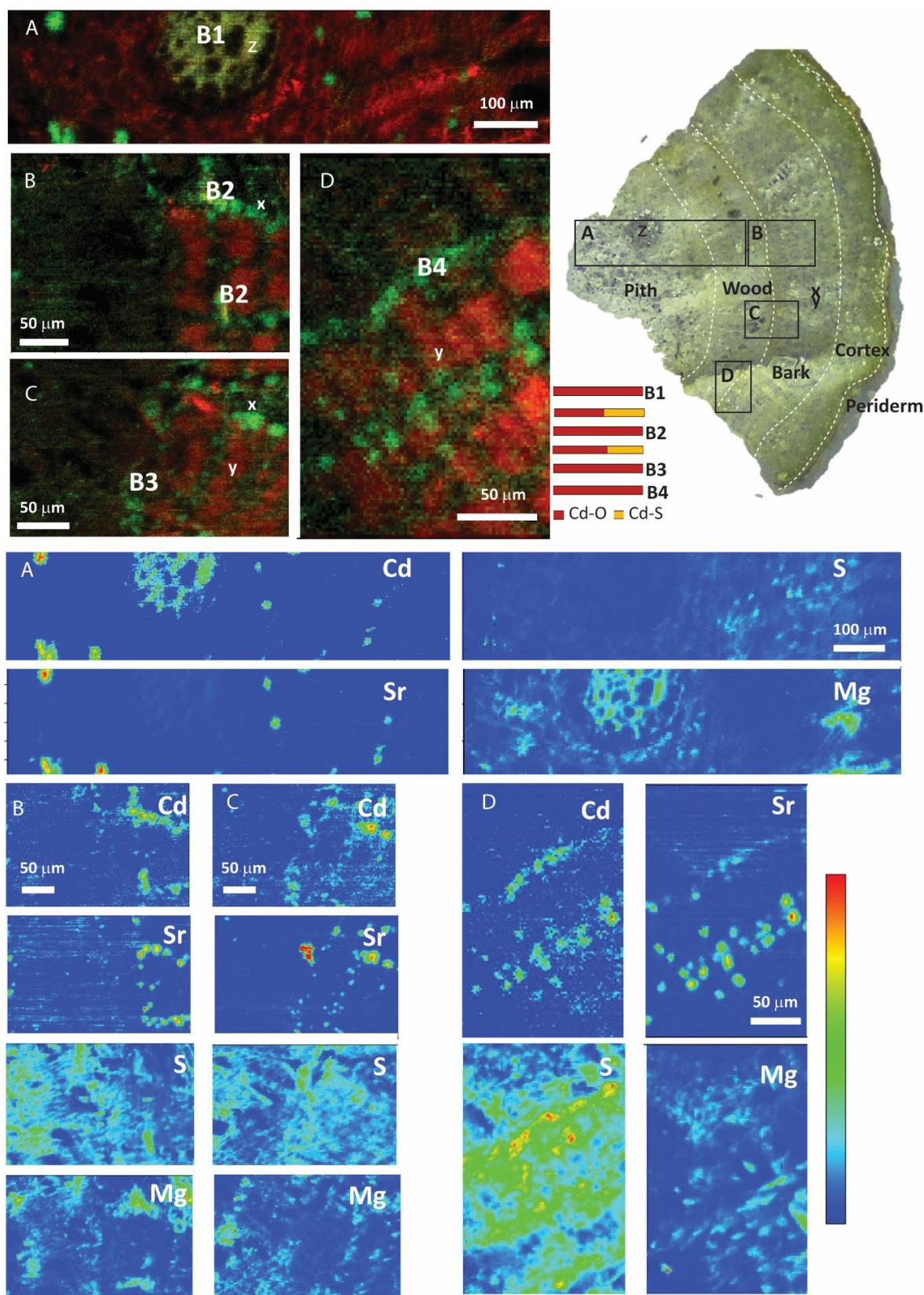
The comparison of pixel intensities in the different ROIs indicated a similar Cd distribution as in the high Cd accumulator (Table III.6), i.e. an enrichment of Cd in the bark versus the wood. Cadmium was particularly enriched in the phloem rays and the periderm. Cadmium in the pith was heterogenous and accumulated around the large secreting cavities in the pith. There was no

enrichment of Cd in the secreting cavities of the bark. The bark was richer in most macro and micronutrients than the wood/pith. In the different ROIs, Cd correlated most strongly with Mn followed by Zn and Ca, and less with P, Cu and Ni (Table III.6), except for the periderm. In the latter, there was a high co-localization with P as well. The maps showed a clear accumulation of Mn and Cu in the periderm which was less pronounced for Cd. Just like for Cd, there was an enrichment of Mn, Zn and Ca around the big cavities in the pith (Figure 4.3).

In the frozen hydrated young branch cross sections, we imaged the distribution of Cd, Sr, S, Mg, Cl and P by  $\mu$ XRF (Figure 4.4, Figure III.6). The  $\mu$ XRF maps had a higher resolution (pixel size: 1 to 2  $\mu$ m) than the LA-ICP-MS and allowed to localize elements more precisely within the tissues. Similar to the older branches (Figure 4.3), we could distinguish between the pith, wood, bark, and periderm (Figure 4.4 and Figure III.6). The cortex, a primary tissue between the vascular system and the periderm (Angyalossy *et al.*, 2016) was also present in these younger branches, whereas in the older branches the cortex was strongly modified and reduced in size. In the pith we observed large vessel structures (indicated with z, Figure 4.4 and III.6), which were possibly the origin of the big, lysigenous resin cavities observed in the older branches (indicated with \* in Figure 4.3). The phloem rays were less visible than in the older branches, however it was sometimes possible to distinguish between the rays (x) and the phloem vessels (y), as the rays consisted frequently out of dilated parenchymatous tissues (Angyalossy *et al.*, 2016) (Figure 4.4 and III.6). In the pith, wood, bark and periderm, the  $\mu$ XRF imaging showed the presence of Cd (Figure III.6). In the pith of the branch, an enrichment in Cd was observed in a big vessel structure (z in Figure 4.4 and III.6). Here, Cd was colocalizing strongly with Mg. Other hotspots of Cd were also observed in the pith, and these spots were strongly correlating with Sr (Figure 4.4). These Cd-Sr correlating hotspots were observed in the pith, wood, bark and periderm. Especially in the bark close to the cambium region on the bark phloem side, there was an enrichment of these spots (B, C and D in Figure 4.4). Most of the Cd was located in parenchymatous tissues of the rays, however some spots were also observed in the region containing the phloem vessels (image D).



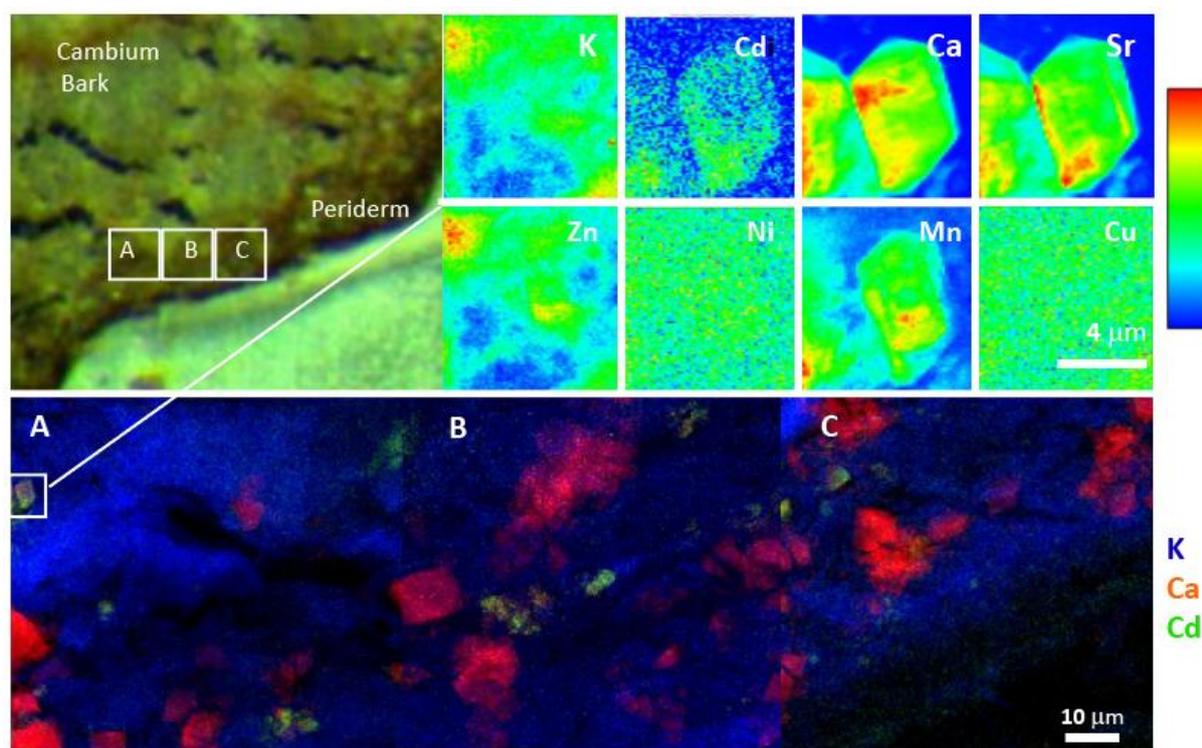
**Figure 4.3:** Microscope image and LA-ICP-MS elemental maps of cross section of branch of the low cultivar (replicate T15). The color scale bar indicates the intensity expressed in total counts and is adjusted to the range of each element. Cadmium was particularly enriched in the phloem rays and the periderm. The Cd in the pith accumulated around the large secreting cavities in the pith (\*).



**Figure 4.4:** Top figure: Cd localization and speciation in branch tissues of the high Cd accumulator (T6) (green = Cd, red = Cl (shown to display cellular structure)). B1, B2, B3 and B4 indicate the Cd spots where XANES spectra were recorded (Figure 4.9). The Cd-O (red bar), and Cd-S (yellow bar) fractions were fitted

with linear combination fits (in % fitted fraction). In map A (pith), Cd accumulated in a vessel structure (z). In map B and C, Cd accumulated mostly in phloem rays (x) close to the cambium. Cadmium also accumulated around the phloem vessels (y) (map D). Bottom heatmaps: distribution of Cd and Sr, S, and Mg in map A, B, C, and D from top figure. The color scale (blue = minimum and red = maximum photon counts) is adjusted to the range of each element (see materials and methods).

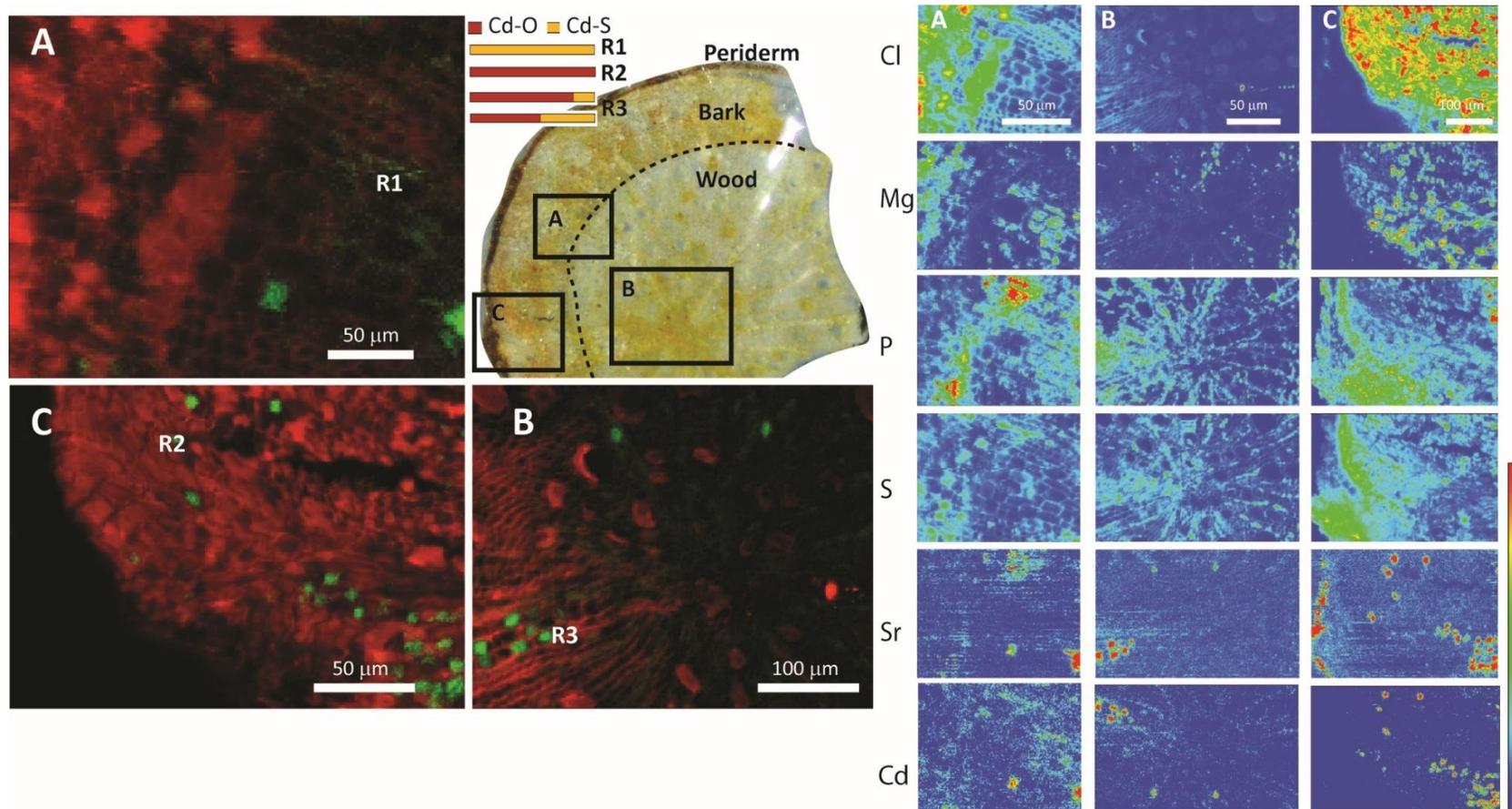
In a next step, nanoXRF maps at high energy were obtained to elucidate the distribution of K, Ca, S, Cd, Zn, Mn and Ni in dried cross sections of a mature branch of the high Cd accumulator (T2 and T4) on a smaller scale (pixel size 0.1  $\mu\text{m}$ ). We mapped regions in the periderm (Figure 4.5), in the bark and cambium (Figure III.7), and in the wood (Figure III.8). In all mentioned regions we could observe Cd. Calcium and Sr were colocalized in prismatic crystals, and also in less crystalline structures (Figure 4.5, III.7 and III.8). Cadmium did sometimes colocalize with the crystals (Figure III.5). Yet, no systematic pattern of colocalization was found. In a similar manner, Mn and Ni were sometimes incorporated within the crystals.



**Figure 4.5:** NanoXRF image of the bark and periderm of the branch of the high Cd accumulator. Different forms of Ca-oxalate crystals are observed (red), and in some of them, Cd (green) was included. K was displayed to show the cellular structure of the periderm. On the top right a twin prismatic oxalate crystal is shown, where there was also an enrichment in Sr, Mn and Cd. Nickel, Zn and Cu were not observed within the crystal. The color scale (blue = minimum and red = maximum photon counts) is adjusted to the range of each element.

#### 4.3.5.2 Cadmium localization in roots

Frozen-hydrated cross sections of the roots of the high Cd accumulator were imaged with  $\mu$ XRF. We distinguished the following tissues: wood, bark, and periderm (Figure 4.6 and III.9). In the center of the wood (Figure 4.6 B), a group of Cd-Sr spots was observed parallel to the rays (indicated with R3). Close to the cambium, but at the side of the wood (Figure 4.6 A, R1), Cd was dispersed, and located within dilated (i.e. elongated) cells. There was no co-localization with other measured elements in this area. In the bark a strong co-localization between Cd and S was noted (Figure III.9 B). In the cambium and in the bark of the roots Cd colocalized with Sr, however the number of Cd-Sr spots was less abundant than in the cambium of the branches. In the periderm, the XRF measurements did not show any presence of Cd.

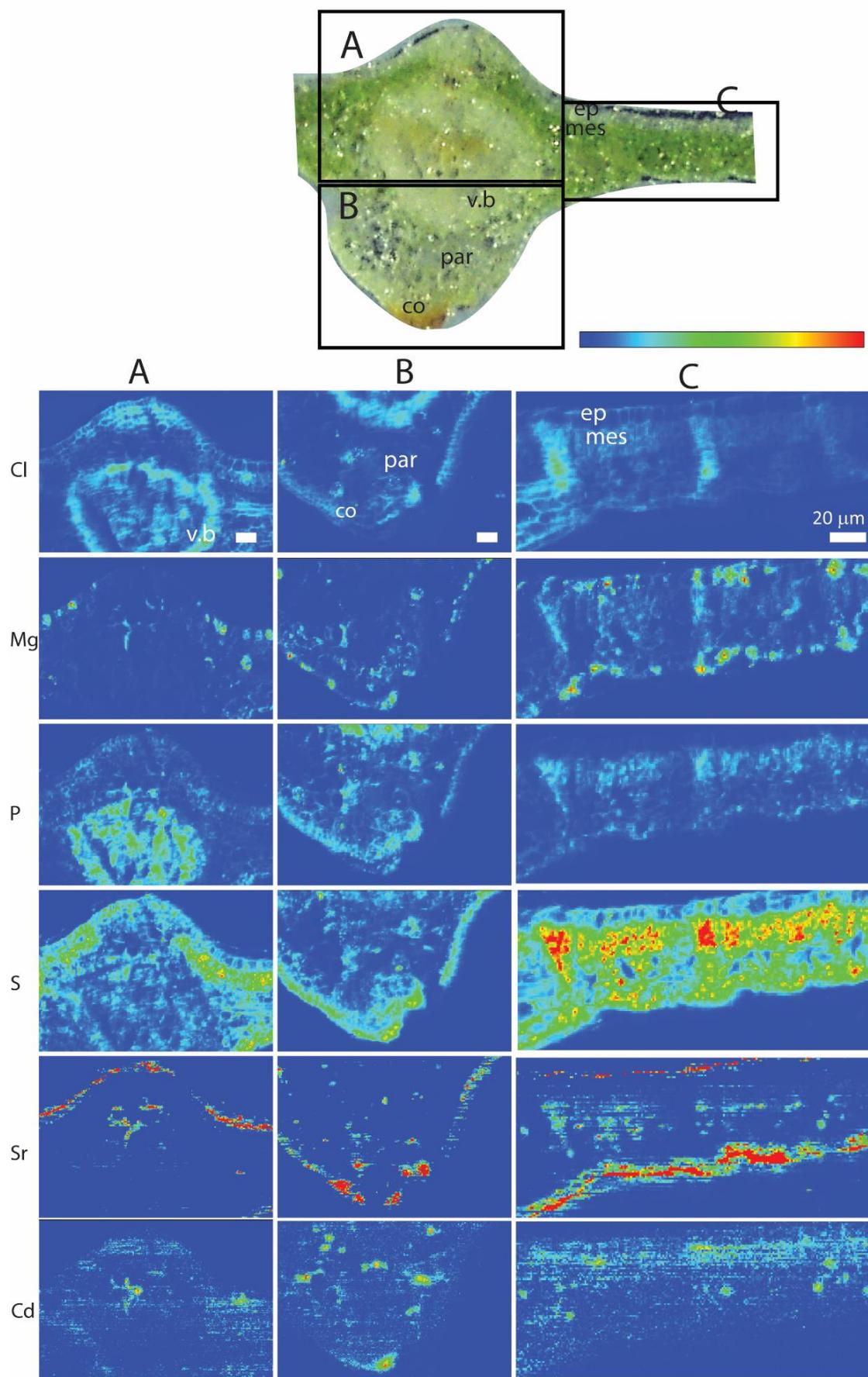


**Figure 4.6:** Left figure: Cd localization and speciation in root tissues of the high Cd accumulator (T6) (green = Cd, red = Cl (shown to display cellular structure)). R1, R2, and R3 indicate the spots where the speciation of Cd was recorded with XANES. The Cd-O (red bar), and Cd-S (yellow bar) fractions were fitted with linear combination fits (in % fitted fraction (top left). For more detail see Figure 4.8). Right figure: heatmaps of Cl, Mg, P, S, Sr, and Cd in map A, B, C, and D from left figure. The color scale (blue = minimum and red = maximum photon counts) is adjusted to the range of each element (see materials and methods). In map A (cambium), Cd was dispersed around dilated cells in the wood and was coordinated with Cd-S. In map B (wood), Cd colocalized with Sr crystals. In the bark (map C), Cd-colocalizes with Sr, and was complexed with oxygen ligands.

#### 4.3.5.3 Cadmium localization in leaves

In the images of frozen-hydrated IF2 leaf of the high Cd accumulator recorded with  $\mu$ XRF we could observe a vascular bundle, that contained the xylem and phloem, which was surrounded by parenchymatous cells. In the leaf blade we could distinguish between the epidermis and the mesophyll (Viet Ha *et al.*, 2016). Cadmium was not detected in the vascular bundle (Figure 4.7). In the parenchymatous cells, there were local enrichments of Cd, and some of them correlated with Sr, other ones were more located on cell walls (Figure 4.7 A). There were also some enrichments around glands (g). In the collenchyma cells (co, Figure 4.7 B), we observed an enrichment of Cd. However, this enrichment in the collenchyma did not show a systematic presence. In the leaf blade, we noticed a Cd enrichment in the upper side of the mesophyll compared to the epidermis and lower side of the mesophyll. Compared to the branches and roots, there was less localization of Cd with Sr in the leaves, as Sr was strongly enriched in the upper epidermis.

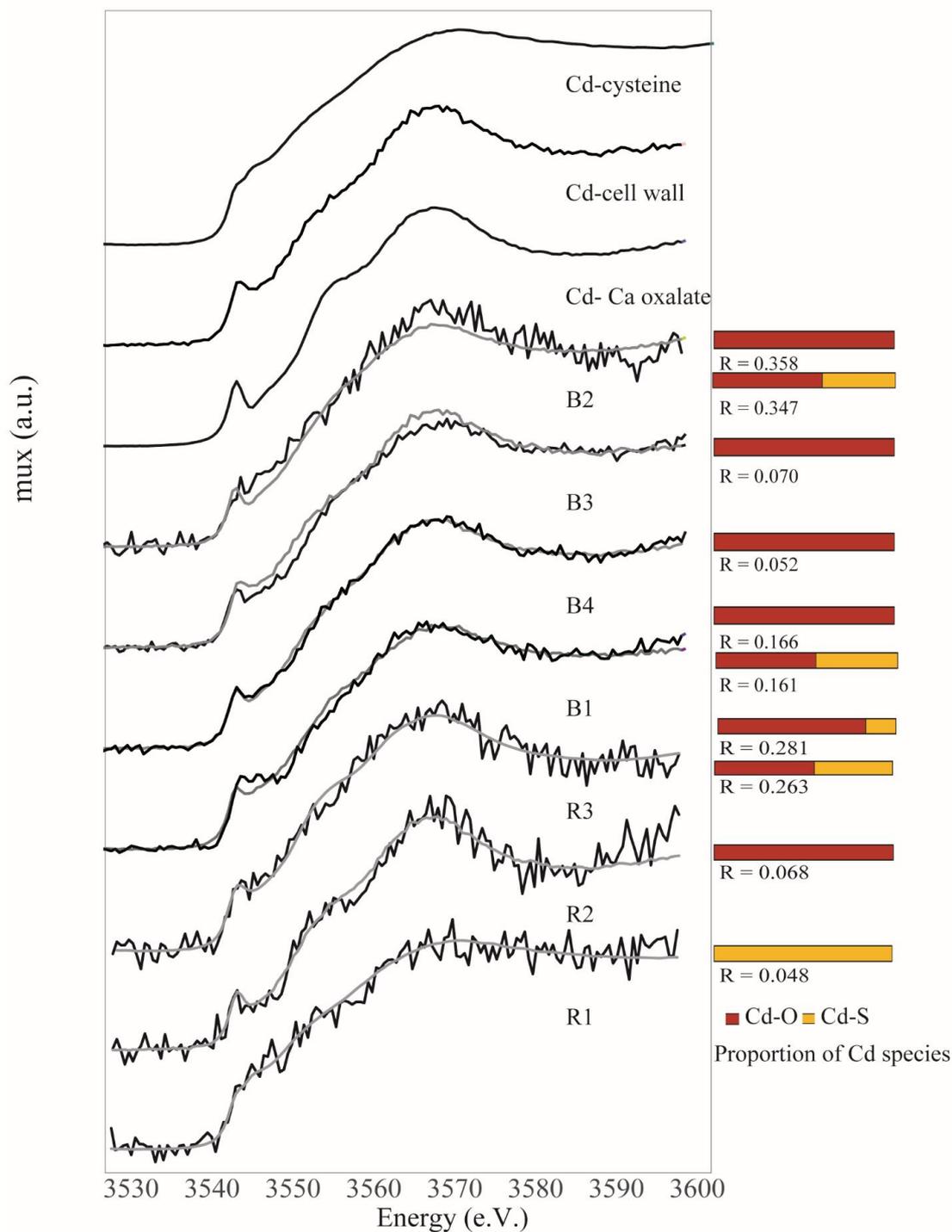
With the nanoXRF resolution it was possible to zoom in on the epidermis and mesophyll on freeze-dried tissues of IF2 leaves (Figure III.10 and III.11). These images confirmed that Cd was more enriched in the mesophyll than in the upper epidermis (Figure III.10). Moreover, many Ca crystals accumulated in the mesophyll, that did not show a colocalization with Cd. Furthermore, Cd was enriched around a gland in the collenchyma (Figure III.11). Two freeze-dried cross sections of IF3 leaves of the high and low Cd accumulator were mapped with LA-ICP-MS (Figure III.12 and III.13), and confirmed the distribution previously described in the frozen-hydrated leaf, and showed moreover similar Cd leaf distributions between the cultivars.



**Figure 4.7:** Cl, Mg, P, S, Sr, and Cd distribution in IF2 leaves (mature green leaves with green stem) of the high Cd accumulator (T4). Vb: vascular bundle, ep: epidermis, mes: mesophyll, co: collenchyma, par: parenchyma. Cadmium was more enriched in the parenchyma compared to the vascular bundle. In the leaf blade, Cd was more abundant in the upper mesophyll than in the epidermis. All scalebars indicate 20  $\mu\text{m}$ . The color scale (blue = minimum and red = maximum photon counts) is adjusted to the range of each element (see materials and methods).

#### 4.3.5.4 Cadmium chemical forms in branches and roots

Regions with high Cd signal were selected to study Cd speciation by recording Cd  $L_{III}$ -edge  $\mu\text{XANES}$  spectra (Figure 4.8). In the branches, the XANES in the big vessel (B1) revealed that Cd was either bound with oxygen-ligands, or partly (up to 40%) with sulfur-ligands (spectrum B1, Figure 4.8). In the Cd-Sr spots (B2, B3, B4), Cd was 100% bound to oxygen-ligands, except for B2, where some fits also indicated Cd-S as species (up to 50%). Speciation measurements in around the cambium in the roots indicated that Cd was fully bound to sulfur ligands (Figure 4.8). In the bark of the roots, many more Cd- Sr spots were observed and here, Cd was also bound to oxygen-ligands (R2, Figure 4.6 C). In the center of the roots (Figure 4.8), the Cd in the crystals mostly bound to oxygen ligands (R3, 85-100%), with a minority bound to sulfur-ligands. All spectra recorded in the roots of T4 indicated that Cd was mostly bound to oxygen ligands (85-100%) while Cd-S was a minor species (Figure III.9).



**Figure 4.8:** Cd  $L_{III}$ -edge XANES spectra of the Cd spots defined in Figure 4.4 (B1, B2, B3, B4 in the branches) and Figure 4.6 (R1, R2, and R3 in the roots). Cd-cysteine represents the Cd-S references, and Cd-cell wall and Cd-Ca oxalate represent the Cd-O references (see materials and methods). The proportion of Cd species is indicated on the right with the bar plots. The fit quality was examined by the R-factor (indicated below the corresponding bar plot). For spectra B1, B2, and R3, the R-factor of two linear combination fits differed with less than 10%. The fits were thus considered to have equivalent quality and were both shown in the figure.

## 4.4 Discussion

### 4.4.1 Soil characteristics that determine Cd bioavailability are similar in the three plots

Total soil Cd, pH and SOC were similar in all plots (Table III.2). A meta-analysis of  $n=12$  studies in Latin America and Caribbean indicated that these parameters explained  $>41\%$  of the variance in bean Cd concentrations (Vanderschueren *et al.*, 2021). As these parameters and the  $\text{Ca}(\text{NO}_3)_2$ -extractable Cd did not differ significantly in the topsoil and subsoil of the three plots (Table III.2), we assume a similar Cd soil bioavailability in the plots of all cultivars. However, it should be noted that the CEC and Fe, Al, and Mn- ammonium oxalate extractable content were higher in the topsoil and subsoil of the grafted cultivar than in the soils of the other cultivars. An increase in sorbing phases would decrease Cd bioavailability and thus reduce Cd uptake (Smolders and Mertens, 2013).

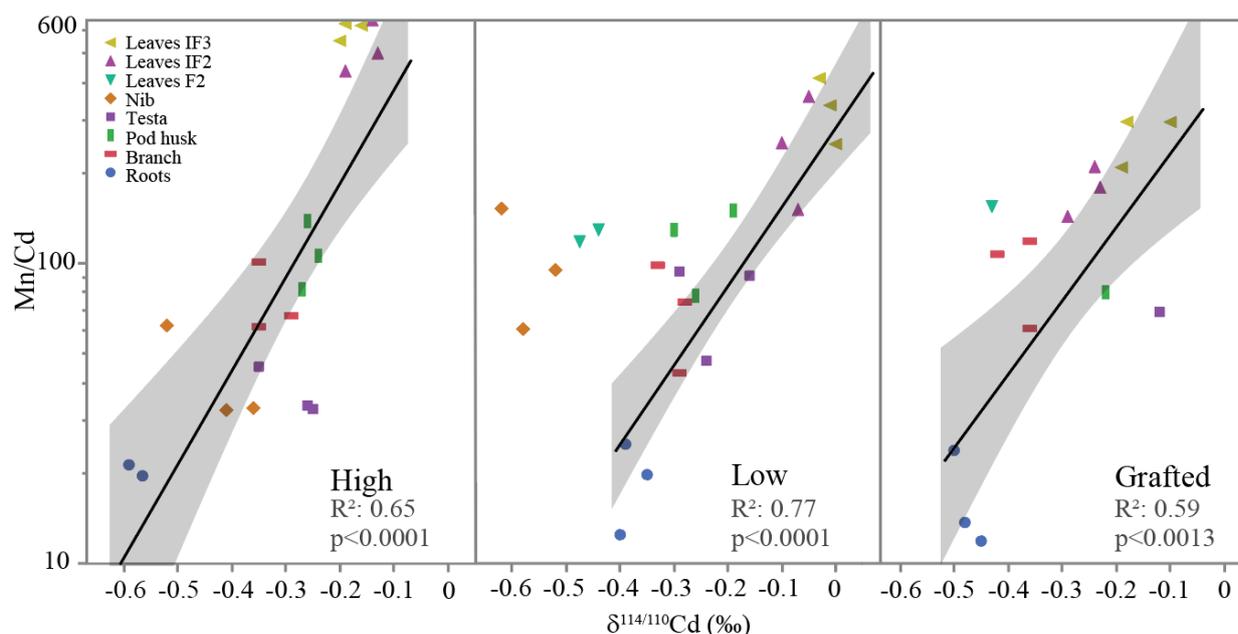
### 4.4.2 Cadmium and manganese uptake differs among cultivars

Despite the similar soil properties, there was a clear difference in Cd concentrations among the three different cultivars (Table 4.1). The high Cd accumulator had a particularly high Cd transfer from soil to the leaves ( $\text{TF}_{\text{leaf IF2-soil}}=13.1$ ) (Blommaert *et al.*, 2022). The transfer factors for the grafted ( $\text{TF}_{\text{leaf IF2-soil}}=5.2$ ) and low ( $\text{TF}_{\text{leaf IF2-soil}}=7.2$ ) cultivar were closer to the average  $\text{TF}_{\text{leaf-soil}}$  (4.5) observed in a survey in Ecuador ( $n=560$ ) (Argüello *et al.*, 2019). The chosen cultivars represented thus well the variability among cacao trees in Cd uptake.

The high and grafted cultivar absorbed factor 2.7 and 1.8 more Cd than the low cultivar (Figure 4.1, Table III.4). The Mn uptake followed a similar trend and was factor 3.6 and 2.3 times higher in the high and grafted cultivar compared to the low cultivar. Cadmium isotope compositions were used to trace the processes causing the differences in the uptake of Cd from the soil among the three cultivars. The trees in our study absorbed preferentially light Cd isotopes from the soil  $\text{Ca}(\text{NO}_3)_2$ -extractable fraction (Figure 4.2). The TcNRAMP5-transporter (a Mn, Fe, and Zn-transporter) has been shown to preferentially transport light Cd isotopes in cacao (Moore *et al.*, 2020). Thus, the concentration and isotope data point in the direction that i) there are similar Cd uptake processes among the cultivars and that ii) Cd may have been taken up through the TcNRAMP5-transporter which has high affinity for Mn. In summary, we propose that the higher uptake rate for Mn in the high Cd accumulator and grafted cultivar may have also increased the amount of Cd hitchhiking through these transporters.

#### 4.4.3 The separation of Cd from nutrients is associated with an enrichment in Cd heavy isotopes in the transpiring organs

For all cultivars, the transfer of Cd from root to shoot was high and in a similar range (ITF branch-root ranged from 1.9 to 2.2 and ITF leaf-root from 0.91 to 1.33 (Table 4.2)). In addition, all internal translocation factors and Cd mass distributions indicated a similar partitioning in root and shoot organs for all cultivars (Table 4.2, Figure 4.2). Hence, in our study there were no cultivar related differences in the Cd distribution in trees, as was observed in the field (Lewis et al., 2018; Engbersen et al., 2019). We observed in all cultivars that the Mn/Cd ratio increased in the order roots < branches < pod husks < IF2 and IF3 leaves (Table III.5). This increase was related with an enrichment in heavy isotopes in these organs (Figure 4.9). These organs are along the transpiration stream (via the xylem). Hence, there is a mechanism along the transpiration pathway that is retaining Cd compared to nutrients which leads to an enrichment in heavy isotopes in the organs that are at the end of the transpiration pathway (like the mature leaves). The F2-leaves and nibs did not follow this trend in the low Cd accumulator. The two latter organs are phloem sinks for nutrients because they are developing organs (Page *et al.*, 2006; Waters and Sankaran, 2011). The combination of different nutrient/Cd ratios and isotope composition in these organs suggests that the unloading is different than in the organs along the transpiration stream, and that Cd is probably originating from the phloem in these tissues.



**Figure 4.9:** An increased separation of Mn versus Cd along the transpiration pathway (roots-branches, pod husks, leaves IF2 and IF3) leads to a heavier isotope composition in the high, low and grafted cultivar. In the low Cd accumulating cultivar, the nibs and (developing) F2-leaves did not follow this trend and were excluded from the regression fit. The F2 leaves are young, developing leaves. IF2 and IF3 leaves are mature green leaves. The grey zone is the confidence region for the linear fit ( $\alpha=0.05$ ). The  $R^2$  and  $p$ -value of the F-test for the linear correlation between the log (Mn/Cd) ratio and the Cd isotope composition is indicated for each cultivar on the graph.

#### 4.4.4 Cadmium retention in roots of the high Cd accumulator by binding with by S-ligands and incorporation into Ca-oxalate crystals

In rice roots, it was suggested that the sequestration of lighter isotopes with S-ligands in the vacuole does progressively remove light isotopes during the radial transport towards the xylem (Wiggenhauser, A. M. Aucour, *et al.*, 2021). To investigate the currently unknown storage mechanisms of Cd in cacao roots, we combined  $\mu$ XRF mapping with  $\mu$ XANES to localize Cd in the roots and determine the corresponding speciation (i.e. chemical form). The maps of the high Cd accumulator demonstrated Cd accumulation in different zones and forms in the roots of the high Cd accumulator (Figure 4.6 and III.9). In the dilated cells of the wood (R1), in proximity to the cambium, there was a disperse accumulation of Cd around cell walls. Here, Cd was bound to S-ligands. However, the maps did not allow the localization of Cd within the cellular structures, it remains thus unclear if these Cd-S complexes reside within the vacuole or not, and if they contributed to Cd root sequestration. In the wood and bark regions of the root, Cd was observed in the form of crystal-shaped structures (Figure 4.6 A, B and C), which were determined to be Ca-oxalate crystals because of their prismatic forms and their strong colocalization with Sr (and Ca) (Figure 4.5). It is known that the incorporation of Sr in Ca oxalate crystals is a common feature in

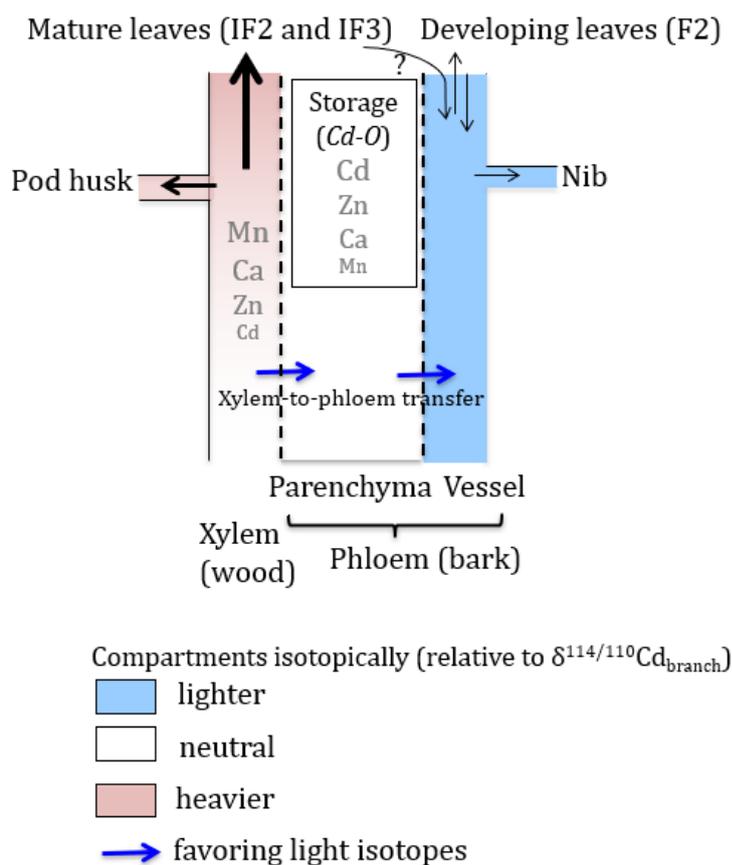
plant cells (Kim and Heinrich, 1995). Calcium oxalate crystals have been observed previously in stems of *Theobroma cacao* L. (de Oliveira Ceita *et al.*, 2007). These crystals play a central role in a variety of functions, including tissue calcium regulation, protection from herbivory, and metal detoxification (Nakata, 2003; Hawkesford *et al.*, 2011). To our knowledge, Cd colocalization with Ca in crystals has been reported for tobacco (Isaure *et al.*, 2010), tomato (Van Balen *et al.*, 1980), water hyacinth (Mazen and El Maghraby, 1997) and the bioindicator *Gomphreni clausenii* (Villafort Carvalho *et al.*, 2015; Pongrac *et al.*, 2018). However, all these studies were hydroponic studies with high (toxic) tissue Cd accumulation. This study is the first to show the incorporation of Cd in Ca-oxalate crystals at natural field Cd concentrations. Speciation measurements moreover revealed that the Cd in these crystals was bound with O-ligands (Figure 4.8). The best linear combination fits were obtained with reference spectra from Cd-cell wall, Cd-oxalate, and Cd-Ca-oxalate. A similar Cd speciation was observed in *Gomphreni clausenii*, where the authors suggested a role for the Ca-oxalate crystals in the detoxification of Cd (Pongrac *et al.*, 2018). Taking the isotope, concentration, imaging and speciation data in the roots of the high Cd accumulator together, indicated that (a major) part of Cd is still mobile and loaded into the xylem, while (a minor) part of the Cd sequestered, possibly with S-ligands or within the Ca-oxalate crystals. The processes of retention in the roots could lead to an enrichment in Cd light isotopes compared to the shoots.

#### 4.4.5 Cadmium accumulation in branches of high Cd accumulator: the role of Ca oxalate crystals and xylem-to-phloem transfer of Cd

The speciation measurements corroborated previous bulk Cd XANES that indicated that Cd was mostly bound to O(C)-ligands in the high Cd accumulator (Chapter 3). The  $\mu$ XRF mapping in the branches of the high Cd accumulator revealed that like in the roots, various Ca-oxalate crystals were present in the branches (in the periderm, cortex, bark, wood, and pith (Figure 4.4, 4.5, III.6, III.7 and III.8)). A study on cacao seedlings demonstrated that Ca-oxalate crystals were mostly located in the cortex, but also occurred in the pith (de Oliveira Ceita *et al.*, 2007). In stems of *G. clausenii*, crystals were observed exclusively inside pith cells and the periderm (Villafort Carvalho *et al.*, 2015; Pongrac *et al.*, 2018). Part of Cd was also present in non-crystalline structures (Figure 4.4, B1). Here, Cd was bound to oxygen, with sulfur-ligands as minor species and correlated strongly with Mg. The Cd in this vessel is probably more mobile than the Cd in the crystals, and can be transported to other organs.

#### 4.4.6 Cadmium storage and limited remobilization in the mature leaves of the high Cd accumulator

The largest level of separation of Cd from nutrients was in the mature IF2 and IF3 leaves (Table III.5). This separation was associated with an enrichment in heavy isotopes in all cultivars (Figure 4.9). A significant amount of Cd was loaded into the leaves (Figure 4.1). It has been shown in other plants that after transport with other nutrients in the xylem, the nutrients and Cd are loaded into the leaf tissues (Sterckeman and Thomine, 2020). Cadmium in leaves is compartmentalized to reduce damage to important proteins, especially the ones involved in photosynthesis (Leitenmaier and Küpper, 2013). Hence, the Cd concentration in the epidermis should be higher than in the mesophyll that contains photosynthesizing cells. This Cd coping strategy has been observed in many hyperaccumulator species (Sterckeman and Thomine, 2020). However, our study showed the inverse, with an enrichment of Cd in the mesophyll versus the epidermis (Figure 4.7 and III.13). This phenomenon was also observed for Cd in the Zn hyperaccumulator *Arabidopsis halleri* (Kupper *et al.*, 2000; Leitenmaier and Küpper, 2013; Isaure *et al.*, 2015). In willow, a woody species, Cd was accumulating in the collenchyma cell walls (Vollenweider *et al.*, 2011). Our study indicated that there was a local enrichment in the collenchyma (Figure III.11), but this was not as systematic as in the case of willow. Overall, Cd was more heterogeneously spread throughout the leaf tissues than in willow, where the Cd was more compartmentalized. The willow was nonetheless exposed to higher Cd concentrations. The Ca-oxalate crystals that were abundant in the roots and branches in the high Cd accumulator were again observed in the leaves (Figure III.10). Yet, there was a smaller degree of Sr (and Ca)-Cd co-localization than in the branches and roots. Regulation of Cd through incorporation into the Ca-oxalate crystals played thus a minor role in the cacao leaves studied. There was an accumulation of Cd and nutrients around the glands in the parenchyma (Figure III.11). These glands possibly transport mucilage (Garcia *et al.*, 2014). To our knowledge, there is no literature on the transport of nutrients by these glands in *T. cacao*. The absence of Cd in the phloem in our study suggested that at the time of sampling, the Cd reallocation from leaves to other organs through phloem was limited.



**Figure 4.10:** Proposed Cd transport pathways and compartments for accumulation in the branches. We observed a preferential retention of light Cd isotopes in the phloem of the branches which was associated with an enrichment in heavy Cd isotopes in the transpiration pathway to the leaves via the xylem. Along this pathway, Cd was separated from other nutrients, especially Mn and Ca. Part of Cd was stored into the branches in the form of Cd-sorbed or substituted Ca-oxalate crystals, and/or Cd complexes with other oxygen-ligands. Isotope and nutrient/Cd ratios indicated that Cd in the pod husk was loaded via xylem, and Cd was loaded in the developing leaves (F2) and nibs via the phloem originating (partly) from the branches. The question mark (?) indicates that part of Cd from the mature leaves is possibly loaded in the phloem for reallocation to the developing organs, however, at the time of sampling, we did not observe Cd in the phloem of the mature leaves.

#### 4.4.7 Similar Cd transfer pathways in the low and high Cd accumulating cultivar

As the grafted cultivar bore only one fruit, we mostly focus on the high and the low accumulator in following section. For both cultivars, the difference in isotope composition was significant between IF2/IF3 leaves and nibs (Table 4.3), with a larger difference in isotope composition for the low cultivar. The Cd partitioning and differences in isotope composition between organs were similar for the three cultivars, suggesting similar processes within the plant. The isotope composition between leaves and seeds is opposite to that in cereals (Imseng *et al.*, 2019;

Wiggenhauser, A. Aucour, *et al.*, 2021; Zhong *et al.*, 2022) but similar to cacao plants in the field (Barraza *et al.*, 2017). The isotope compositions of the low and high Cd accumulator, together with the images suggested that Cd was actively transferred from the xylem into the branch tissues, where it was partly stored into Ca-oxalate crystals, and partly transferred to the phloem (Figure 4.10). This mechanism drove an enrichment in heavy Cd isotopes in the transpiration pathway from roots to the leaves via the xylem. The nutrient/Cd ratios and isotope data suggested that the Cd was loaded into the pod husk via the xylem. The similar isotope composition of the nibs and young, developing leaves corroborated that Cd was loaded into the nibs via the phloem in both cultivars.

#### 4.4.8 Cultivar selection and grafting and their potential for mitigating Cd in cacao

We observed differences in Cd accumulation between a high (NA 312 of the genetic group *Nanay*) and a low Cd accumulator (CL 10/3 of the genetic group *Refractario*). We highlighted that the main difference in Cd concentrations in the nibs of these cultivars is related to the difference in the overall uptake of Cd rather than to differences in internal distribution. Interestingly, we observed that Cd and Mn uptake were highly correlated. This main outcome can be used to identify cross membrane transporters that are responsible for both Mn and Cd uptake (such as the TcNRAMP5-transporter). The branches served as the main organ for Cd accumulation in the cacao tree. In the high Cd accumulator, we could observe that part of Cd was transferred from xylem-to-phloem transfer in the branches and part of Cd was stored in Ca-oxalate crystals. These observations should be confirmed by verifying that the small branches analyzed in the present study are representative for all branches constituting the tree.

Our study highlighted the potential of grafting as we observed that the uptake of Cd was influenced by the rootstock. The low Cd accumulator accumulated less Cd than the grafted cultivar, while both cultivars had the same scion. Again, the uptake of Cd was highly related with Mn uptake. Further tests are necessary to research whether a combination of a rootstock of a low Cd accumulating cultivar and an agronomically interesting scion is a viable strategy to mitigate Cd in cacao.

# Chapter 5: Evidence of cadmium transport via the phloem in cacao seedlings

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## Summary

Cacao is a tropical woody tree and Cd (cadmium) pathways in the plant can differ from model plant species studied for Cd accumulation. Cadmium isotope compositions in mature cacao trees suggested that the transfer of Cd from stem to leaves and seeds in cacao indeed differs from other species, such as cereals. These differences could be related to a different mobility of Cd in the xylem and phloem of cacao compared to other species. In fact, it is currently unknown to what extent Cd is mobile in the phloem of cacao. A split-root experiment with a stable isotope  $^{108}\text{Cd}$  tracer was set up to address this question. Roots of cacao seedlings were split into two parts, each growing in separate containers. The compartments contained an equal volume of nutrient solution enriched in  $^{108}\text{Cd}$  (+) or not (-) with an environmentally relevant total Cd concentration (19 nM). The seedlings were harvested at different times during two weeks. At day 1, there was an instantaneous rise in the  $^{108}\text{Cd}$  isotopic abundance in the roots in the labelled compartment. In the shoot organs, the  $^{108}\text{Cd}$  isotopic abundance remained at natural abundance. After 7 days, about 75% of the tracer  $^{108}\text{Cd}$  in the plant was translocated to the shoot tissues. The stems accumulated the most of the total Cd (45%). The isotopic abundance of  $^{108}\text{Cd}$  in the roots in the unlabelled compartment increased significantly after day 11, demonstrating that Cd was phloem mobile and redirected to developing tissues. The isotopic abundance of  $^{108}\text{Cd}$  in the new, developing leaves was similar to the stems and older leaves, suggesting that Cd originates from these tissues rather than from the roots.

## 5.1 Introduction

Cadmium (Cd) is a non-essential trace metal that is highly toxic to most living organisms. Food is the main source of Cd human exposure for the non-smoking population. Chocolate and chocolate products contribute significantly to the total dietary Cd exposure in the European population (EFSA, 2012). To protect consumers, regulators have established limits on Cd concentrations in chocolate and cacao powders between 0.10 and 0.80 mg Cd kg<sup>-1</sup> (Mercosur, 2011; Ministry of Health of the Russian Federation, 2011; European Commission, 2014; Australia New Zealand Food Standards Code, 2017; Codex Alimentarius Commission, 2018). These regulations have fuelled research worldwide to reduce Cd concentrations in cacao beans (Meter, Atkinson and Laliberte, 2019; Vanderschueren *et al.*, 2021). The creation of cultivars with low Cd accumulating properties has raised interest as differences in Cd accumulation among different genotypes have been observed in the field (Arévalo-Gardini *et al.*, 2017; Lewis *et al.*, 2018; Engbersen *et al.*, 2019). However, the selection and creation of low Cd cultivars is still in its infancy. A major bottleneck that hampers their development, is that the knowledge concerning the genetic and molecular mechanisms for Cd uptake and translocation in cacao is small compared to other plant species.

The current knowledge regarding Cd mobility within plants originates from herbaceous model plant species such as *Arabidopsis thaliana* L. and rice. In these two species only, striking differences in uptake mechanisms, as well as mechanisms of distribution in the plant are observed. Hence, Cd pathways in plants can thus differ according to species (Sterckeman and Thomine, 2020). The Cd pathways from roots to above-ground tissues depend on vacuolar sequestration, xylem loading, and intervascular and xylem-to-phloem transfer (Clemens and Ma, 2016; Sterckeman and Thomine, 2020; McLaughlin *et al.*, 2021). Cacao is a tropical woody tree and consequently has a markedly different physiology from that of rice and *A. Thaliana*. Thus, Cd pathways in cacao may differ vastly from these species. Cadmium concentrations are often higher in the roots than in the shoots (Sterckeman and Thomine, 2020). In cacao the contrary is observed. Several studies showed a high transfer of Cd from root to shoots (Engbersen *et al.*, 2019; Scaccabarozzi *et al.*, 2020; Vanderschueren *et al.*, 2021; Blommaert *et al.*, 2022). Within the aerial tissues, two studies on Cd isotope compositions in mature cacao trees (Barraza *et al.*, 2019; Blommaert *et al.*, 2022) suggested that the transfer of Cd from stem to leaves and seeds in cacao is different compared to other species, such as cereals (Wiggenhauser *et al.*, 2016, 2021). These differences were ascribed to the cauliflorous character of cacao, i.e. the flowers and fruits develop directly on the trunk. However, it remains unclear what these differences exactly are and if they are related to differences in the mobility of xylem and phloem of Cd compared to other species. Several studies have shown that Cd is reallocated from leaves to fruits, and grains through the

phloem flow. In rice, it was estimated that 91-100% of Cd in the grains was loaded from the phloem (Tanaka *et al.*, 2007). Cadmium has been shown in white lupin and wheat to be less phloem-mobile than Zn, but more than Mn and Fe (Page and Feller, 2005, 2015; Page, Weisskopf and Feller, 2006). Especially Mn and Ca have been shown to have poor mobility in the phloem compared to other nutrients and Cd. In the Cd hyperaccumulator *S. Nigrum*, Cd was loaded efficiently into the xylem, and was not or only slowly redistributed via the phloem (Wei, Anders and Feller, 2014). This study was set up to identify the circulation of Cd in cacao, and to what extent Cd is mobile in the phloem of cacao. To this end, we used a split-root approach with cacao seedlings. In this setup, the nutrient solution of one root compartment was labelled with  $^{108}\text{Cd}$  stable isotope to trace the fate of  $^{108}\text{Cd}$  in the plant.

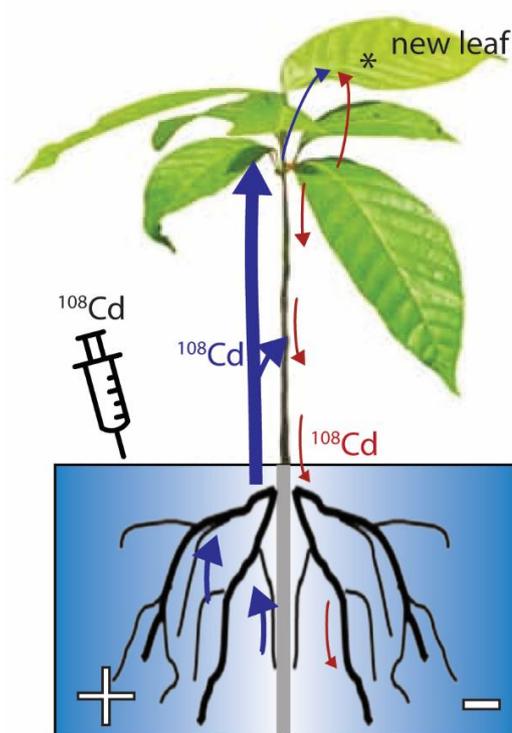
## 5.2 Materials and methods

### 5.2.1 Experimental design

A split-root hydroponic system was set up with five-week-old *Theobroma cacao* L. seedlings. Split-root experiments have been used in the past to reveal the circulation of trace elements in plants (McLaughlin *et al.*, 2000; Haslett, Reid and Rengel, 2001; Qin *et al.*, 2013; Feller, Anders and Wei, 2015; Valentinuzzi *et al.*, 2020; Wei *et al.*, 2022). The roots were divided into two separate containers (also mentioned as root 1 and root 2) (Figure 5.1, Figure IV.1). The containers had an equal volume (330 mL) of nutrient solution either enriched with the stable isotope  $^{108}\text{Cd}$  (+) or not (-). There were three different treatments; (-/-), (+/-), and (+/+). In treatment (+/-), the nutrient solution in the root 1 compartment was labelled with isotope  $^{108}\text{Cd}$  as a tracer. The other compartment (root 2) had an identical solution, with the same Cd concentration but no  $^{108}\text{Cd}$  tracer. In treatment (-/-), both root 1 and 2 compartments contained the nutrient solution without  $^{108}\text{Cd}$  tracer. In (+/+) treatment, both compartments were labelled with  $^{108}\text{Cd}$  tracer. We included these unlabelled and fully labelled solutions as reference treatments. The experiment took fourteen days. At days 0, 1, 7, 11, and 14, four plants per treatment were harvested (Table 5.1). In total, the experiment consisted of  $n=40$  split-root seedlings.

**Table 5.1:** Experimental design and sampling dates after transfer of seedlings to the experimental solutions. The split-root compartments were either both spiked with  $^{108}\text{Cd}$  (+/+), one spiked (+/-) or not spiked (-/-). All other solution components, including total dissolved Cd concentration, were equal between the compartments. Each treatment had four biological replicates, which resulted in  $n=40$  split-root seedlings.

Sampling time (days after transfer to solution)				
0	1	7	11	14
(-/-)				(-/-)
	(+/-)	(+/-)	(+/-)	(+/-)
	(+/+)	(+/+)	(+/+)	(+/+)



**Figure 5.1:** A split-root hydroponic system with cacao seedlings. In treatment (+/-), the nutrient solution in the + compartment was labelled with stable isotope  $^{108}\text{Cd}$  as a tracer. The other compartment (-) had an identical solution, with the same Cd concentration but no  $^{108}\text{Cd}$  tracer. The results of our study indicated that the  $^{108}\text{Cd}$  absorbed in the roots is readily transported via xylem (blue) to the aerial organs, where it accumulates primarily in the stems. A minor contribution of  $^{108}\text{Cd}$  was redirected to the roots *via* the phloem (red). Cadmium in the new developing leaves (\*) was rather derived from the leaves and stems than from root uptake.

## 5.2.2 Plant growth

Three-week-old seedlings (Trinitario variety) were purchased from a nursery (Cacaobomen.nl, Harmelen, The Netherlands), where they germinated from seed. At arrival in the laboratory facilities, the seedlings were removed from their nursery substrate. Their roots were washed by immersion in deionized water to remove any adhering substrate particles. The seedlings were transplanted into 9-liter preculture vessels with continuously aerated nutrient solution for a two weeks preculture in hydroponics before the split-root experiment started. The nutrient solution contained the following.  $\text{Ca}(\text{NO}_3)_2$ , 2.05 mM;  $\text{MgSO}_4$ , 0.50 mM;  $\text{KNO}_3$ , 1.24 mM;  $\text{KH}_2\text{PO}_4$ , 0.09 mM;  $\text{H}_3\text{BO}_3$ , 0.015 mM;  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$ , 0.10  $\mu\text{M}$ ;  $\text{MnSO}_4\cdot\text{H}_2\text{O}$ , 4.81  $\mu\text{M}$ ;  $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$ , 1.42  $\mu\text{M}$ ;  $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$ , 5.80  $\mu\text{M}$ ;  $\text{NaCl}$ , 25  $\mu\text{M}$ ; MES (2-(*N*-morpholino)ethanesulfonic acid) 2 mM;  $\text{NaOH}$ , 1 mM;  $\text{FeNaEDTA}$ , 0.01 mM. Cadmium was added as  $\text{Cd}(\text{NO}_3)_2\cdot 4\text{H}_2\text{O}$  together with nitrilotriacetic acid (NTA) (19.6 nM Cd). The pH was set at 6.14 and kept constant during plant growth with the help of a MES buffer (2 mM). Speciation calculations predicted that most Cd is complexed with EDTA (37%) and NTA (61%) ligands (Visual Minteq version 3.1). This complexation ensured that free  $\text{Cd}^{2+}$  was buffered at low activity (0.38 nM) and environmentally relevant concentrations.

The culture vessels with the seedlings were installed in a growth chamber with a constant air humidity of 70 % and a 12h/12h (25°C/20°C) day/night cycle with 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity. The vessels were covered from light to prevent algal growth. After two weeks of preculture, individual plants were transferred from the 9 L containers to the split root device (Figure IV.1) of twice 330 mL of continuously aerated solution. The lateral roots were gently split and each part transferred into separate compartments (as described in Saiz-Fernández *et al.*, 2021). Preliminary tests with three seedlings in a (+/-) treatment showed undetectable  $^{108}\text{Cd}$  enrichment in the unlabeled compartment 72 hours after labelling the other compartment. During the course of the experiment, the nutrient solution was not replaced. On day 7, deionized water was added to each compartment to restore the initial volume of 330 mL.

## 5.2.3 Cadmium stable isotope labelling

A stock solution (6.3 g Cd L<sup>-1</sup>) with an isotopic abundance of  $^{108}\text{Cd}$  of 71.1% was prepared from an enriched  $^{108}\text{Cd}$  stable isotope metal (Trace Sciences International, Delaware, USA) (for details on the preparation, see Argüello *et al.* (2020); Vanderschueren *et al.* (2023)). In Table IV.1 the isotopic abundance of natural Cd and the enriched Cd sources is depicted (Berglund and Wieser, 2011). For the preparation of the labelled (enriched in  $^{108}\text{Cd}$ ) nutrient solution (+), 260  $\mu\text{L}$  of the enriched stock solution was added to 9L of nutrient solution (19.6 nM Cd) and well mixed. The final (measured)  $^{108}\text{Cd}$  isotopic abundance in the (+) nutrient solution was 6.51% (Table IV.1).

Depending on the treatment, the compartments were filled with either  $^{108}\text{Cd}$  enriched (+) or natural Cd (-) nutrient solution and at day 0 the plants were immersed in the nutrient solution and the isotope labelling experiment started.

#### 5.2.4 Sample collection

After each harvest (day 0, 1, 11, and 14 (Table IV.1)), plant organs were gently separated into root 1, root 2, crown (which consisted out of the interface in between the split roots and the stem), stem, leaves, and newly developing leaves if available (Figure IV.2). Special care was taken to ensure that there was no cross-contamination from one root compartment to the other. The roots were immersed in a  $\text{CaCl}_2$  solution (2 mM) for 20 minutes to remove apoplastically bound Cd. All parts of the plant were rinsed in baths with deionized water and dried with paper towels to remove excess water. All plant tissues were oven-dried overnight (60 °C) and weighed for mass balance calculations. A subsample of each sample was ground and sieved through a 2 mm stainless steel mesh to obtain a homogenous powder. Crowns were not included in further analyses.

#### 5.2.5 Chemical analysis

Leaves and stems ( $400 \pm 20$  mg) were acid digested with 8 mL  $\text{HNO}_3$  (NORMATOM® 67–69 % w/w, VWR International) in a microwave digestion system (MARS 6, CEM, Matthews, NC, USA). Roots and new leaves (50 mg) were digested with 2 mL  $\text{HNO}_3$  in an open block digestion with 10 mL glass tubes. The dry mass of the roots and new leaves was not sufficient for microwave digestion that requires minimally 200 mg. Cadmium isotopes ( $^{108}\text{Cd}$ ,  $^{111}\text{Cd}$ ) were measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Agilent 7700x, Agilent Technologies, collision cell ORS3), with He as the collision gas.  $^{111}\text{Cd}$  is commonly the preferred isotope for Cd detection with ICP-MS as it has almost no isobaric interferences (Rehkamper *et al.*, 2012).

The mass bias of the isotopic ratio (IR)  $^{108}\text{Cd}/^{111}\text{Cd}$  was determined during two different ICP-MS measurement sessions. A Cd single element standard ( $10 \mu\text{g Cd L}^{-1}$ , Certipur Merck 119,777) was measured and the measured IR ( $\text{IR}_{\text{measured}}$ ) was compared with the theoretical IR ( $\text{IR}_{\text{theory}}$ ) to obtain the mass bias correction factor  $f_{MB}$  (Nolan *et al.*, 2004).

$$f_{MB} = \frac{\text{IR}_{\text{theory}}}{\text{IR}_{\text{measured}}}$$

The mass bias factor was 1.11 in the first measurement session and 1.18 in the second session. For each session, the corresponding value was selected as the linear mass bias correction factor. The limits of quantification (LOQs) were determined as ten times the standard deviation of the

measured signal in all procedural blanks ( $n=22$ ) and were 15 cps for  $^{108}\text{Cd}$  and 42 cps for  $^{111}\text{Cd}$  (expressed as signal). The signal in the measured plant samples in our study was on average 868 cps for  $^{108}\text{Cd}$  and 4019 cps for  $^{111}\text{Cd}$ . The IA (isotopic abundance) of  $^{108}\text{Cd}$  was determined on the mass-bias corrected isotopic ratio of  $^{108}\text{Cd}/^{111}\text{Cd}$  and the isotopic ratios of all other Cd isotopes relative to  $^{111}\text{Cd}$ ;

$$IA^{108}\text{Cd} = \frac{\left(\frac{^{108}\text{Cd}}{^{111}\text{Cd}}\right)_{\text{measured}}}{\frac{^{106}\text{Cd}}{^{111}\text{Cd}} + \left(\frac{^{108}\text{Cd}}{^{111}\text{Cd}}\right)_{\text{measured}} + \frac{^{110}\text{Cd}}{^{111}\text{Cd}} + 1 + \frac{^{112}\text{Cd}}{^{111}\text{Cd}} + \frac{^{113}\text{Cd}}{^{111}\text{Cd}} + \frac{^{114}\text{Cd}}{^{111}\text{Cd}} + \frac{^{116}\text{Cd}}{^{111}\text{Cd}}}$$

Only the  $^{108}\text{Cd}$  and  $^{111}\text{Cd}$  isotopes were measured, thus we used the isotope ratios of  $^{106}\text{Cd}$ ,  $^{110}\text{Cd}$ ,  $^{112}\text{Cd}$ ,  $^{113}\text{Cd}$ ,  $^{114}\text{Cd}$  and  $^{116}\text{Cd}$  relative to  $^{111}\text{Cd}$  at natural abundance (Berglund and Wieser, 2011). This decision was justified because the change in the isotopic ratios of these isotopes with respect to  $^{111}\text{Cd}$  is negligible at the maximum IA of  $^{108}\text{Cd}$  (6.51%) in this study (Table IV.1). The analytical quality of the ICP-MS  $^{108}\text{Cd}$  measurements was assessed by measuring a series of 1  $\mu\text{g Cd L}^{-1}$  solutions with increasing  $^{108}\text{Cd}$  IA. These solutions were prepared by mixing a certified reference Cd solution with the  $^{108}\text{Cd}$  stock solution used for labelling. The measured IA was compared with the theoretical IA and the data closely followed the 1:1 line, which indicated accurate IA measurements (see SI (Vanderschueren *et al.*, 2023)). The average  $\pm$  SD of the measured  $^{108}\text{Cd}$  IA in the plant material grown without  $^{108}\text{Cd}$  isotope in nutrient solution ( $n=30$ ) was  $0.93 \pm 0.19$  % (theoretical natural abundance  $^{108}\text{Cd}$ : 0.89%). The precision (SD) and accuracy (mean) of this measurement of natural abundance depended on the sample matrix (roots  $1.00 \pm 0.20$  % ( $n=12$ ), leaves  $0.91 \pm 0.17$  ( $n=8$ ), stem  $0.84 \pm 0.05$  % ( $n=8$ )).

Cadmium concentration measurements with ICP-MS typically use one isotope only (here:  $^{111}\text{Cd}$ ) and are calibrated with calibration standards at natural abundance. The  $^{111}\text{Cd}$  was at non-natural abundance in the labelled (+) treatments. The Cd concentrations were not corrected for the change in isotopic abundance of  $^{111}\text{Cd}$  in the samples at non-natural abundance. This decision was justified because the difference in isotope abundance of  $^{111}\text{Cd}$  between natural abundance samples (12.80%) and samples that were maximally enriched with  $^{108}\text{Cd}$  (12.15%) was considered as negligible (Table IV.1). Plant certified reference materials were included in each ICP-MS measurement session. The % total Cd recoveries ranged from 86-95 % (min-max) with on average 90 % for NIST-1573a Tomato Leaves (certified concentration:  $1.52 \pm 0.07$  mg Cd  $\text{kg}^{-1}$ ),  $n=8$  and from 91-100 % with on average 95 % for BCR-679 White Cabbage (certified concentration:  $1.66 \pm 0.07$  mg Cd  $\text{kg}^{-1}$ ),  $n=2$ .

## 5.2.6 Calculations and data analysis

Mass balances combined with isotope abundances allowed to calculate the fraction of tracer in each plant organ at the different harvest time points. Total Cd in each organ  $i$  (root 1 and root 2, crown, stem, leaf, new leaf) was calculated by multiplying the Cd concentration measured with the biomass of that organ. The sum of total Cd in each organ  $i$  represents the total Cd taken up by the plant. Crown Cd concentrations (not measured) were estimated to be similar as the stem Cd concentrations.

$$(Total\ Cd_i\ (ng) = Cd_i\ (mg\ kg^{-1}) * biomass_i\ (mg\ DW))$$

The total Cd in each organ  $i$  was multiplied with the  $^{108}\text{Cd}$  IA corrected for the natural  $^{108}\text{Cd}$  in that organ to obtain the mass of tracer derived Cd in that tissue.

$$^{108}\text{Cd, tracer}_i\ (ng) = total\ Cd_i\ (ng)[IA\ ^{108}\text{Cd}_i - IA\ ^{108}\text{Cd}_{i,nat}]$$

with  $IA^{108}\text{Cd}_{i,nat}$  the average natural abundance measured in organ  $i$  in the (-/-) treatment. The mass of tracer ( $^{108}\text{Cd}$ ) in each organ  $i$  was added up to determine the total plant  $^{108}\text{Cd}$  ( $^{108}\text{Cd, tracer}_{plant}\ (ng)$ ). Subsequently the  $^{108}\text{Cd}$  in each organ  $i$  was divided by the total tracer  $^{108}\text{Cd}$  in the respective plant to obtain the fraction of tracer in each plant organ.

$$f_{tracer} = ^{108}\text{Cd, tracer}_i\ (ng) / ^{108}\text{Cd, tracer}_{plant}\ (ng)$$

All statistical analyses were performed with the software JMP-Pro version 17.0. The mean and standard deviation (SD) reported throughout the study were calculated with biological replicates ( $n=4$ ), unless otherwise specified. One-way analysis of variance (ANOVA) was conducted to determine if there were changes in Cd concentrations and  $^{108}\text{Cd}$  isotopic abundances among the treatments on the same day or among different days for the same treatment. Differences were tested by Student's t-test or Tukey's HSD post-hoc test at  $p<0.05$  significance level. All data were checked to comply with ANOVA assumptions.

## 5.3 Results

### 5.3.1 Plant growth

Labelling of the nutrient solution with  $^{108}\text{Cd}$  had no effect on total plant biomass (DW) (Figure IV.3). The biomasses of all treatments (-/-), (-/+), and (+/+) were thus grouped together to infer plant growth. The plant biomass approximately doubled between day 0 ( $850 \pm 160\ \text{mg}$  ( $n=4$ )) and day 14 ( $1630 \pm 260\ \text{mg}$ ,  $n=12$ ). On day 0, the largest biomass fraction was in the leaves ( $50 \pm 5\%$

of DW, mean  $\pm$  SD of all treatments ( $n=4$ ), followed by the stem ( $37 \pm 4\%$ ), crown ( $10 \pm 1.8\%$ ), and roots (separated in root 1 ( $1 \pm 1\%$ ) and root 2 ( $2 \pm 1\%$ )). At the end of the experiment, the stem was the largest organ ( $38 \pm 1\%$  ( $n=12$ )), followed by the leaves ( $33 \pm 2\%$ ), crown ( $16 \pm 1\%$ ), root 1 ( $6 \pm 1\%$ ) and root 2 ( $7 \pm 1\%$ ), and newly developing leaves ( $2 \pm 1\%$ ). New leaves started to develop on day 7 of the experiment.

### 5.3.2 Cadmium concentrations in plant organs

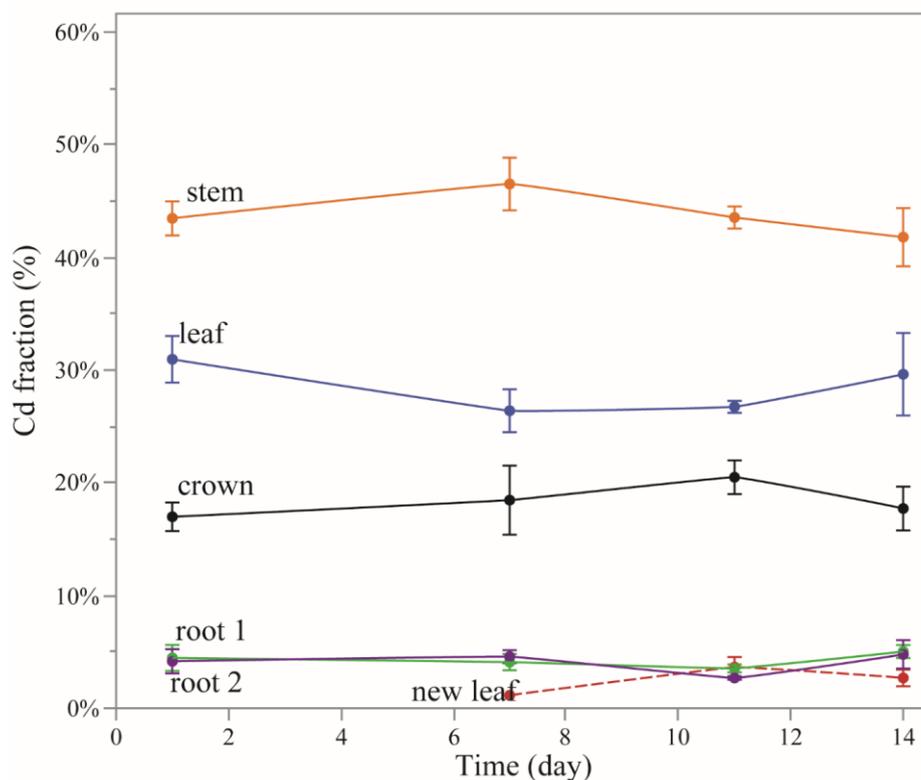
Cadmium concentrations were measured in both root 1 and 2, leaves, stems and, if available, newly developing leaves (Table 5.2). Regardless of treatment, there were no differences in Cd concentrations between the two root compartments (1 and 2), except for day 7 in treatment (+/+), where the Cd concentration was significantly higher in root 1 ( $2.87 \pm 1.25$ ) than in root 2 ( $1.26 \pm 0.11$ ) ( $p=0.04$ ) (data not shown). Further ANOVA tests revealed no significant differences in Cd concentrations among the different treatment at each time point in the organs ( $p>0.05$ ). We can thus conclude that the isotope enrichment in the (+) nutrient solution did not affect the uptake of Cd.

Before the start of the experiment, the seedlings had already grown two weeks in a preculture nutrient solution that contained around 20 nM Cd. Therefore, on day 0, there was already a relevant Cd accumulation in the roots ( $1.52 \pm 0.71$  mg kg<sup>-1</sup>), leaves ( $0.55 \pm 0.10$  mg kg<sup>-1</sup>) and stems ( $1.07 \pm 0.54$  mg kg<sup>-1</sup>) (Table 5.2), and we observed that the accumulation was larger in the roots than in the leaves and stems. Over the 14 days the concentrations in the leaves and stems increased relatively to the roots and on day 14, the Cd concentrations were similar in all organs (Table 5.2).

At the end of the experiment, the total plant Cd uptake did not differ significantly among the three treatments ( $1.15 \pm 0.54$   $\mu$ g (-/-),  $1.59 \pm 0.43$   $\mu$ g (+/-), and  $1.90 \pm 0.07$   $\mu$ g Cd (+/+)). The fraction of total Cd in each organ remained similar over the course of the experiment (Figure 5.2) and ranged from 5 to 11% in the roots, from 12 to 20% in the crown (under the assumption that the crown contains the same Cd concentration as the stem), from 26 to 35% in the leaves and from 39 to 46% in the stems. The new developing leaves contained about 4% of all plant Cd.

**Table 5.2:** Cd concentrations ( $\text{mg kg}^{-1}$ ) in different organs at each sampling day (t). The Cd concentrations of the different treatments (-/-), (-/+), and (+/+) were averaged as there were no differences in organ Cd concentrations among the different treatments. Letters within each row denote different statistical groups that were determined using the Tukey HSD test ( $p < 0.05$ ).

t	N	Root 1		Root 2		Leaf		New leaf		Stem					
		Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD			
0	3	<b>1.52</b>	0.71	2	<b>1.54</b>	0.29	4	<b>0.55</b>	0.1	0		4	<b>1.07</b>	0.54	
1	8	<b>1.49<sup>ab</sup></b>	0.64	8	<b>2.06<sup>a</sup></b>	1.35	8	<b>0.50<sup>b</sup></b>	0.1	0		8	<b>0.90<sup>b</sup></b>	0.23	
7	8	<b>2.16<sup>a</sup></b>	1.32	8	<b>1.16<sup>ab</sup></b>	0.45	8	<b>0.85<sup>a</sup></b>	0.18	1	<b>1.34</b>	8	<b>1.61<sup>ab</sup></b>	0.4	
11	8	<b>0.52<sup>b</sup></b>	0.11	6	<b>1.36<sup>ab</sup></b>	1.27	8	<b>1.0<sup>ab</sup></b>	0.16	8	<b>1.51<sup>a</sup></b>	0.48	8	<b>1.46<sup>ab</sup></b>	0.26
14	12	<b>1.28</b>	0.93	10	<b>0.65</b>	0.26	12	<b>0.90</b>	0.33	7	<b>0.89</b>	0.19	12	<b>1.02</b>	0.4

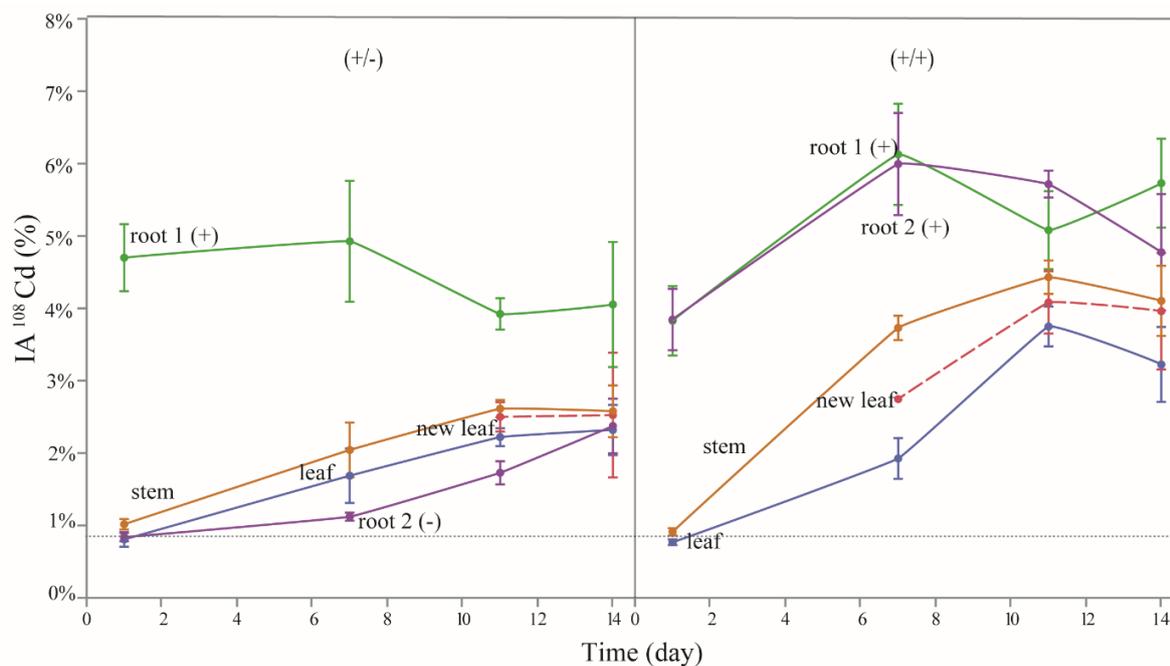


**Figure 5.2:** Mass fraction of total Cd in different organs ( $\text{Cd}_{\text{organ}} (\text{ng}) / \text{Cd}_{\text{plant}} (\text{ng}) * 100\%$ ). The partitioning of Cd remained similar over the course of the experiment. Most Cd accumulated in the stem. The error bars represent the standard error of the mean.

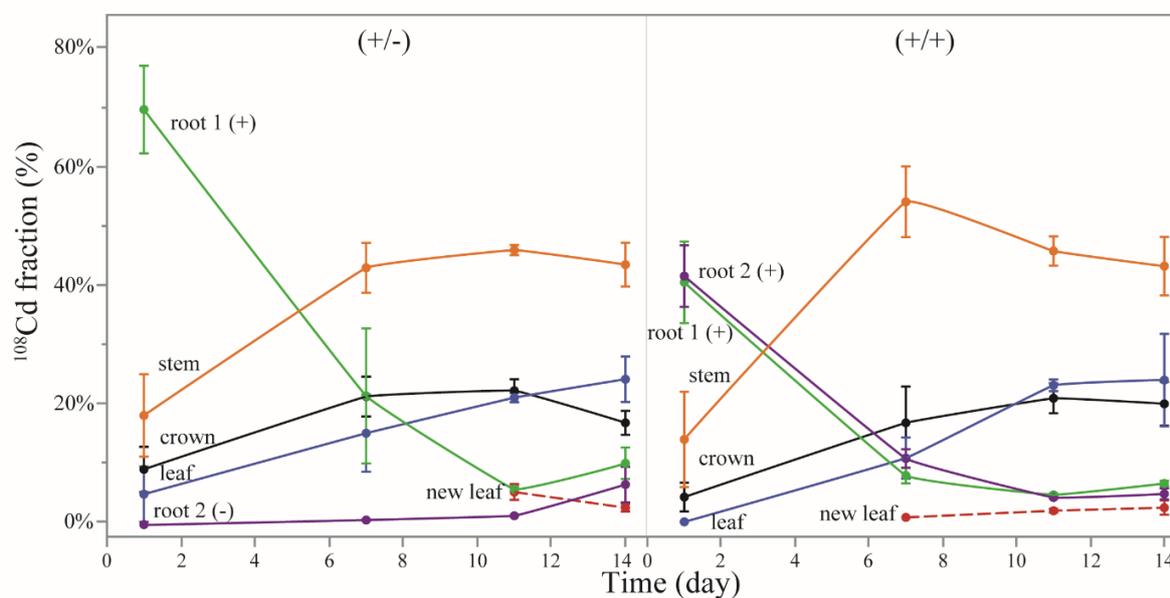
### 5.3.3 Circulation of $^{108}\text{Cd}$ tracer in the split-root cacao plants

In the (+/-) treatment, there was a clear increase in IA  $^{108}\text{Cd}$  in the root 1 (labelled) ( $4.70 \pm 0.93\%$ ) at day 1 compared to the natural abundance and the other organs (Figure 5.3, Table IV.4). Therefore, the tracer  $^{108}\text{Cd}$  was thus absorbed readily within 24 h and accumulated primarily in root 1 (+). This fast root absorption and accumulation was also reflected by the fraction of  $^{108}\text{Cd}$  tracer in the root 1 (+) (66%) (Figure 5.4). At day 7, the IA  $^{108}\text{Cd}$  increased in the leaves ( $1.69 \pm 0.75\%$ ) and stems ( $2.04 \pm 0.76\%$ ) compared to day 1 and we observed that most of the  $^{108}\text{Cd}$  tracer was translocated to the shoot tissues and accumulated in the stems (43 % in (+/-)). In contrast, the IA  $^{108}\text{Cd}$  showed that the tracer was not yet translocated to the roots of the unlabelled compartment (root 2 (-)), as the IA  $^{108}\text{Cd}$  remained at natural abundance. On day 11, the IA  $^{108}\text{Cd}$  in root 2 (-) increased ( $1.73 \pm 0.23\%$ ) and the fraction of tracer  $^{108}\text{Cd}$  was 1% of the total plant tracer  $^{108}\text{Cd}$ . The newly developing leaves ( $2.50 \pm 0.41\%$ ) had an IA  $^{108}\text{Cd}$  similar to that of the stems ( $2.61 \pm 0.24\%$ ), and leaves ( $2.22 \pm 0.25\%$ ). Finally, on day 14, the IA  $^{108}\text{Cd}$  of the stems, leaves, new leaves and root 2 were similar (ranging from 2.32 to 2.58 %). The fraction of  $^{108}\text{Cd}$  tracer indicated that most tracer Cd was accumulating in the stems (around 46%), and the leaves (21%).

In the (+/+) treatment, the general trends in the circulation of the  $^{108}\text{Cd}$  tracer were similar to the (+/-) (Figure 5.3, Table IV.4). On day 1, both roots had already absorbed  $^{108}\text{Cd}$  (IA  $^{108}\text{Cd}$  in root 1:  $3.83 \pm 0.96\%$  and root 2:  $3.84 \pm 0.85\%$ ) and accumulated most of the tracer  $^{108}\text{Cd}$  in the plant (Figure 5.4) as all other tissues had an IA  $^{108}\text{Cd}$  around natural abundance at day 1. At day 7, the IA  $^{108}\text{Cd}$  in the roots (root 1:  $6.13 \pm 1.40\%$  and root 2:  $5.99 \pm 1.40\%$ ) was similar as the IA  $^{108}\text{Cd}$  of the nutrient solution (6.51 %). The increase of IA  $^{108}\text{Cd}$  in the stems was faster than that in the leaves ( $3.73 \pm 0.34\%$  in the stem versus  $1.93 \pm 0.56\%$  in the leaves). Thus, most of the tracer  $^{108}\text{Cd}$  was accumulating in the stem (54%, Figure 5.4). The new leaves sampled on day 11 had an isotopic abundance in between the stems and leaves ( $4.08 \pm 0.86\%$ ). On day 14 there were no significant differences in IA  $^{108}\text{Cd}$  among the different organs. At the end of the experiment, the partitioning of the  $^{108}\text{Cd}$  was very similar as the partitioning of the total Cd (Figure 5.2). The new leaves contained on average less than 2.4%, the roots less than 10%, the crown 20%, the leaves 24%, and the stem 43%.



**Figure 5.3:** The isotopic abundance (IA) of <sup>108</sup>Cd (%) for the (+/-) and (+/+) treatment as function of time after spiking the solutions with <sup>108</sup>Cd. The background (natural) IA in unspiked plants is given as the horizontal dashed line. Error bars represent the standard error of the mean. Note that the IA in the spiked root of the +/- treatment decreases after 7 days unlike in the +/+ treatment, likely as a result of isotope dilution with Cd imported from the unspiked compartments.



**Figure 5.4:** Fraction of <sup>108</sup>Cd tracer (%) in each organ relative to the total spiked derived <sup>108</sup>Cd in the plant. The error bars represent the standard error of the mean.

## 5.4 Discussion

### 5.4.1 High Cd translocation from root to shoot tissues

The Cd leaf concentrations in the plant organs were in a similar range as the average leaf Cd concentration observed in a meta-analysis in cacao plantations (2.6 mg kg<sup>-1</sup> dry weight,  $n=762$ ) (Vanderschueren *et al.*, 2021). The concentrations observed were thus well below critical Cd toxicity levels that were proposed for various crops in mature leaves (6-19 mg Cd kg<sup>-1</sup> (Krämer, 2010) and 5-30 mg Cd kg<sup>-1</sup> (Kabata-Pendias, 2010). The Cd dose of ca. 20 nM Cd in hydroponic solution can thus be considered as environmentally relevant. The Cd distribution observed can thus be extrapolated to field conditions. During the experiment, the Cd mass fraction distribution (%) remained constant and most Cd accumulated in the shoot tissues, and less than 10% of Cd accumulated in the roots (Figure 5.2). The fraction of freshly added <sup>108</sup>Cd tracer, was larger in the labelled roots in the beginning of the experiment than those in the shoot organs (Figure 5.4). At the end of the experiment, most tracer <sup>108</sup>Cd was translocated to the shoots. Cadmium was thus only slightly retained in the roots and mostly translocated from root to transpiring organs (the leaves). On the contrary, the general trend described in other plants is that Cd concentrations are often higher in the roots than in the shoots, which is considered to be an excluder strategy (Sterckeman and Thomine, 2020). Thence, in the root-shoot translocation cacao follows more an accumulator strategy where metals are transported to above-ground parts (van der Ent *et al.*, 2013). Cadmium is known to use nutrient pathways and to be transported with the transpiration stream from roots to transpiring organs, which are the leaves in cacao seedlings. Yet, most Cd accumulated in the stems (39 to 46%, Figure 5.2), and not in the leaves. This suggests that there was a sequestration of Cd in the stems during their translocation to the transpiring leaves and/or that part of Cd was remobilized to the stems from the leaves. The sequestration could be driven by interactions with cell walls in the xylem or selective removal from the xylem sap, while remobilization would require transport by the phloem (Page and Feller, 2015). The root-shoot translocation observed in this study, with the limited retention of Cd in the roots and possible sequestration in stems corresponds well with observations in mature trees in the field (Engbersen *et al.*, 2019; Chapter 3). Here, the branches were a main organ for Cd accumulation.

#### 5.4.2 Cadmium transport via the phloem to the sink tissues (roots and new developing leaves)

The isotopic labelling split-root setup was exploited to identify Cd circulation fluxes in the (+/-) treatment. In the unlabelled roots there was an increase in IA  $^{108}\text{Cd}$  after day 11 and the tracer  $^{108}\text{Cd}$  fraction in the unlabelled roots finally increased to 6%. Along the same lines, the IA  $^{108}\text{Cd}$  in root 1 (+) decreased between day 7- 14 after an initial rise on day 1, suggesting transport of natural Cd in the opposite direction. Cadmium movement from the labelled to the non-labelled root compartment can only occur *via* the phloem, either by transport through phloem between root segments or by a downward flux of Cd from the shoot (Welch *et al.*, 1999). Consequently, we evidenced the mobility of Cd in the phloem of cacao seedlings. The earlier rise in IA  $^{108}\text{Cd}$  in the shoot tissues compared to the non-labelled roots suggested moreover that Cd is first transported to the upper shoot and then back transported to the roots from the aerial tissues (Figure 5.1, 5.3). The back transport to the roots is small compared to the Cd loading in the transpiration stream because only 6% of Cd is redirected to one root compartment whilst over 75% of Cd is transported to shoot organs. In wheat and white lupin, Cd redistribution via the phloem was observed, but likewise only to a small extent (Page and Feller, 2005; Riesen and Feller, 2005; Page, Weisskopf and Feller, 2006). On the contrary, in the hyperaccumulator *S. Nigrum*, there was a rapid transfer to the shoot and an important accumulation in the leaves and stems of Cd, and was only poorly redistributed to other organs via the phloem. The behaviour of the cacao seedlings in our study lies in between the two. Like in *S. Nigrum*, Cd was loaded efficiently into the xylem in the roots, and transported to the transpiring leaves (Wei, Anders and Feller, 2014). However, in the cacao seedlings there was a significant redistribution via the phloem, like in white lupin and wheat. A study on split-root *Ricinus communis* seedlings, demonstrated the Cd transfer from roots of one compartment to another. However, contrary to our study, there was a root-to-root transfer before there was an upward translocation to the leaves (Wei *et al.*, 2022). Nevertheless, the Cd concentrations in the hydroponic solution of that study were around 500 times higher than in our study. Hence, Cd detoxification mechanisms such as strong sequestration of Cd in root tissues, may have played a more important role in that study than in our study. New developing leaves are considered to be a phloem sink due to their need for nutrients for growth (Page and Feller, 2015). The new developing leaves did not contain any Cd from the preculture. Therefore, the similar IA $^{108}\text{Cd}$  of stem, leaves, and new developing leaves indicated that Cd in these growing sinks was primarily derived from the leaves and stems (Figure 5.3, (+/+) treatment). If the main source of Cd was originating directly from the roots, we would observe that the IA $^{108}\text{Cd}$  was more like that of the roots and nutrient solution, which was not the case.

In conclusion, we evidenced that Cd was mobile in the phloem of cacao seedlings that were grown under environmentally relevant Cd conditions. Through the phloem, Cd was translocated from shoot tissues to the roots and newly developing leaves. The dominant transport pathway of Cd was nonetheless the transpiration pathway (xylem) from roots to leaves, with a large accumulation in the stems.

## **5.5 Conclusions**

We evidenced that Cd was mobile in the phloem of cacao seedlings that were grown under environmentally relevant Cd conditions. Through the phloem, Cd was translocated from shoot tissues to the roots and newly developing leaves. The dominant transport pathway of Cd was nonetheless the transpiration pathway (xylem) from roots to leaves, with a large accumulation in the stems.

# Chapter 6: Cadmium speciation in cacao beans changes during a fermentation-like incubation

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This chapter is submitted to Food Chemistry and is currently under review.

## Summary

Cadmium (Cd) concentrations in cacao often exceed food limits. Recently, it was shown that cacao bean fermentation enhances Cd solubility, opening potential for Cd mitigation in cacao products. This study was set-up to identify changes in Cd speciation during fermentation. X-Ray absorption spectroscopy (XAS) complemented with speciation calculations, were used on samples collected from high and low Cd farms, that were subjected to a fermentation-like incubation that reached high temperatures ( $>45^{\circ}\text{C}$ ) and acidic pH ( $<5$ ). Incubation decreased nib Cd concentration up to a factor 1.5 and changed Cd complexation in high Cd beans from sulphur to oxygen ligands, likely due to pH changes. In beans with lower Cd concentrations, Cd was complexed before and after incubation with oxygen-ligands. A combination of pH changes and/or phytate breakdown may explain the migration of Cd outward from the nib. XAS and speciation calculations proved complimentary techniques and indicated similar speciation changes during fermentation.

## 6.1 Introduction

The European Commission and the Codex Alimentarius enforced a new regulation which limits the allowed concentration of cadmium (Cd) in cacao-derived consumer products, such as chocolate (European Commission, 2014; Codex Alimentarius Commission, 2018). These limits range between 0.10 and 0.80 mg Cd kg<sup>-1</sup> depending on the type of product and the percentage of cocoa solids in the final product. To comply with those limits, the cacao processing industry has introduced unofficial Cd requirements that apply to the cacao beans they purchase. Those unofficial industry limits vary between 0.10 and 1.10 mg Cd kg<sup>-1</sup> bean dry weight (CBI Ministry of Foreign Affairs n.d.; Meter et al. 2019; Vanderschueren et al. 2021). To facilitate the discussion below, 0.60 mg Cd kg<sup>-1</sup> bean dry weight is further used as a threshold. These Cd-cacao thresholds can strongly impact the cacao industry, especially in Central and South America, where bean Cd concentrations are higher compared to those in other cacao producing regions (Chapter 2). Studies on mitigation strategies for Cd in cacao have mostly focussed on agronomic practices, e.g., use of soil amendments or cultivar selection. While results of that research are promising, the practical implementation of such mitigation strategies is not yet relevant, probably because it takes a longer time before effects are detected (Vanderschueren *et al.*, 2021).

Postharvest mitigation strategies focussing on fermentation may offer a valid short-term alternative to address the Cd contamination in cocoa products. Previous work has indicated that conventional fermentation practices can reduce the Cd concentration in the cacao nib (the inner part of the cacao beans that is used in chocolate production) by up to a factor 1.3 (Vanderschueren *et al.*, 2020). This reduction of nib Cd concentration has been attributed to a combination of high temperature (>45°C) and acidic pH (<5). The study showed that Cd mobilized from the nib to the testa (seed envelope that is discarded for chocolate production). However, the biochemical mechanisms behind this mobilization remain unclear.

Cadmium complexation with ligands i.e., Cd speciation, has been studied in non-fermented cacao beans. The few existing speciation data indicates that Cd in nibs is bound to a combination of carboxylate, phosphate and thiol (sulphide) ligands (Vanderschueren, 2021) (Chapter 3). An abundant phosphate ligand in seeds is phytate. This molecule is the typical storage for P in seeds and serves as storage site for K and Mg, and sometimes also Ca, Fe and Zn (Hawkesford *et al.*, 2012; Silva *et al.*, 2021). The chemical similarity between Cd and Zn suggests that Cd may also be bound to phytate (Schlemmer *et al.*, 2009). Data on Cd speciation in other plant seeds is currently limited, and mostly restricted to plants that cope with high exposure levels of Cd due to the current detection limits of synchrotron X-Ray Absorption Spectroscopy (XAS) (within 1 to 10 mg Cd kg<sup>-1</sup> (Gu *et al.*, 2020; Yan *et al.*, 2020; Wiggerhauser, A. Aucour, *et al.*, 2021)). For cereals, speciation

data indicated primarily a binding of Cd to S-ligands in rice (Gu *et al.*, 2020; Wiggerhauser, A. Aucour, *et al.*, 2021), and a mixture of O and S ligands in durum wheat (Yan *et al.* 2020) despite the high phytate content in these cereal grains. Sulphur is a constituent of the amino acid cysteine and acts as a functional group (R-SH, thiol group) which has very high affinity for Cd. Plant cells respond to metal exposure by synthesis of the tripeptide glutathione, and/or of more complex thiol-containing polypeptides including phytochelatins and metallothioneins (Hawkesford *et al.*, 2012; Clemens, 2019).

Taken together that i) phytate is a plausible Cd ligand in the cacao nibs (Chapter 3) and ii) that fermentation conditions may activate phytase in the nibs, which may hydrolyse phytate and thereby mobilize chelated metals, it was suggested that Cd in the cacao nibs was reduced after fermentation by a release of Cd from the phytate complex due to acidification and/or phytate breakdown (Vanderschueren, Helsen, *et al.*, 2023). Cadmium speciation after fermentation may also have implications for the food chain risk of cacao-derived Cd because phytate can inhibit the gastro-intestinal absorption of Cd in humans similarly as it does for Zn (Schlemmer *et al.*, 2009). To our knowledge, there is no Cd speciation data in fermented nibs. This data may help to decipher the biochemical mechanisms behind the Cd mobilization outward from the nib.

The main objective of this study was to reveal the changes in chemical properties and Cd speciation in cacao nibs and testa before and after fermentation. We used a controlled incubation setup to mimic fermentation (see Vanderschueren *et al.* 2023 for original reference).

## 6.2 Materials and methods

### 6.2.1 Harvest of cacao pods and experimental setup

Eight ripe cacao pods of the Nacional cultivar were collected in Sucumbíos province (Ecuador). The samples were sourced from a Cd contaminated location previously identified as a hotspot for Cd accumulation (Argüello *et al.* (2019)) and are further mentioned as the high Cd beans (H1-H4). The pods were cut open using a kitchen knife and the beans from each pod were divided in two subsamples of equal size: one subsample that was not incubated (*untreated*) and the other that was incubated (*treated*). The subsamples consisted of 6 to 25 beans depending on the pod. The subsamples for incubation weighed  $40 \pm 22$  g (mean  $\pm$  standard deviation, wet weight without removing mucilage). The beans were incubated in 300 mL deionized water or acetic acid solution ( $20 \text{ g L}^{-1}$ ) for 72 hours at 25°C, 45°C or 65°C. The experimental setup was a reduced setup derived from the incubation experiment of 44 runs previously described by Vanderschueren *et al.* (2023), where the effect of temperature (T), ethanol, and acetic acid on the Cd mobilisation in the nibs was assessed. The reduced set-up in this study included four treatments that presented the lower

and upper range of effects on Cd mobilisation observed in Vanderschueren et al. (2023). The treatments consisted of: deionized water at 45°C (H1); 20 g L<sup>-1</sup> acetic acid at 25°C (H2); 20 g L<sup>-1</sup> acetic acid at 45°C (H3); and 20 g L<sup>-1</sup> acetic acid at 65°C (H4) (Table 6.1). Each treatment was performed on two different pods (i.e. *biological replicate*, *n*=2). From the same pod the incubation was performed in duplicate to determine the coefficient of variation (CV) for the Cd concentration measurements in the testa and nibs (i.e. *process replicate*, *n*=2). To minimise the effect of sample variation, beans from different pods were never mixed.

Two additional samples from the setup of Vanderschueren et al. 2023 were included in this study. These pods were sourced from soils with natural concentrations of Cd in Guayas (Argüello *et al.*, 2019). These beans are further mentioned as the low Cd beans (L1 and L2). The low Cd beans were incubated in duplicate (*process replicate*, *n*=2) in 20 g L<sup>-1</sup> acetic acid and 45 °C (L1) or 40 g L<sup>-1</sup> acetic acid and 65 °C (L2) (Table 6.1).

## 6.2.2 Sample processing and chemical analyses

### 6.2.2.1 Incubation solutions

The pH of the incubation solutions was measured before and after incubation for each biological replicate. A 10 mL subsample was taken from each biological replicate before and after incubation. Subsamples were diluted 10 (samples before incubation) or 100 times (samples after incubation), acidified to 1.0 % (v/v) HNO<sub>3</sub> (NORMATOM ® 67 – 69 % w/w, VWR International, Radnor, PA, USA) and analysed by inductively coupled plasma mass spectrometry to measure the elemental content (ICP-MS, Agilent 7700x, Agilent Technologies, Santa Clara, CA, USA).

### 6.2.2.2 Nib pH

Three cacao beans were randomly selected from each pod (untreated and treated). The mucilage on the beans was removed and the beans were oven-dried for 72 h at 70 °C. After drying, the shell or testa of the beans was removed manually, and peeled nibs were ground in an electric mill (IKA, Staufen, Germany). The ground nibs were sieved to obtain a more homogeneous particle size (800 µm test sieve, VWR International) and incubated in an end-over-end shaker at a 1:20 (g:mL) solid:deionized water ratio for 24 h at 20°C. Samples were then centrifuged for 10 minutes at 2000 g (Heraeus Multifuge X3R, Thermo Scientific, Waltham, MA, USA) and the pH of the supernatants was measured to determine the nib pH.

### 6.2.2.3 Nib and testa elemental composition

Two beans were selected randomly from each biological and process replicate (untreated and treated) and dried and peeled as described above. Individual nibs were ground in the electric mill and 300 ( $\pm 1$ ) mg subsamples were acid digested in 8 mL HNO<sub>3</sub> (NORMATOM ® 67 – 69 % w/w, VWR International) using a microwave digestion system (MARS 6, CEM, Matthews, NC, USA). Nib digests were then diluted to 200 mL with Milli-Q water (18.2 M $\Omega$  cm<sup>-1</sup>) prior to ICP-MS analysis. Intact testa samples (154  $\pm$  96 mg, not ground) were acid digested in 3.0 mL HNO<sub>3</sub> (NORMATOM ® 67 – 69 % w/w, VWR International) for 8 h in an open digestion block at a maximum temperature of 135 °C. Testa digests were then brought to a volume of 10.0 mL and diluted ten times with Milli-Q water prior to ICP-MS analysis. Blank samples (triplicate for the microwave digestion and quadruplicate for the open block) and certified reference material NIST 2384 baking chocolate (in triplicate) were included in each digestion batch. The limit of quantification was 0.01 mg Cd kg<sup>-1</sup> dry weight, and the Cd recovery of the certified reference materials was 107  $\pm$  18 % (n = 6). The average coefficient of variance between the process replicates (CV) was 7% for both nib and testa Cd analyses.

### 6.2.2.4 Phytate analysis

The phytate content was determined in oven-dried (60°C ) nibs (treated and untreated) after extraction with 0.66 M HCl (incubation overnight in an end-over-end shaker at 20°C), with the Megazyme K-Phyt assay (Megazyme, Bray, Ireland). Extracts were treated or not with phytase and alkaline phosphatase to convert polyphosphates to monophosphate and the phosphate content was then colorimetrically measured using the molybdenum blue method (Murphy and Riley, 1962). The colorimetric measurements were performed at a wavelength of 655 nm using a UV/VIS spectrophotometer (Lambda 25, PerkinElmer, Waltham, MA, USA). The P present as phytate (phytate-P) content in the nibs was then calculated from the difference in phosphate between extracts with and without enzyme treatment, thereby assuming that all P released from enzymatic hydrolysis originated from phytate. An internal reference wheat flour sample was included in duplicate in the phytate analysis and had a recovery of 95% (based on the median measured phytate concentration after 36 individual analyses of that material).

### 6.2.3 Speciation analysis: synchrotron X-ray absorption spectroscopy (XAS)

Speciation analysis was performed on the untreated and treated nib and testa samples from H3, H4, L1 and L2. For H3 and H4, two biological replicates were measured for each treatment (n=2). The mucilage was manually removed from the cacao beans (minimum four beans), and the beans

were peeled to separate nib and testa without drying. Those fresh nib and testa samples were flash-frozen in liquid N<sub>2</sub> and stored at -80°C until further processing. Frozen hydrated samples were milled in liquid N<sub>2</sub> using a cryo-grinder (Pulverisette23, Fritsch) and then pressed into pellets in cryo conditions (diameter 6 mm, thickness 3-5 mm). In addition to frozen hydrated samples, subsamples of H4 (biological replicate 1), H3 (biological replicate 2), L1, and L2 were oven-dried for 48 h, ground, and pressed into pellets of the same size.

Cadmium K-edge XAS measurements were conducted at SAMBA beamline (SOLEIL synchrotron, Saint Aubin, France) and BM30 beamline (ESRF synchrotron, Grenoble, France). The X-ray absorption near edge structure (XANES) part, was analysed. The low Cd concentrations in the samples did not allow to retrieve valuable spectra from the extended-X-ray absorption fine structure (EXAFS) part. The monochromator was a Si220 double crystal, and acquisition was done in fly scan (SAMBA) and in step by step (BM30) mode. The acquisition time for one scan at SAMBA was 4.5 minutes, and on average 50 scans were necessary for one sample. At BM30, one scan took 17 minutes, and around 5 scans were needed for the high Cd beans, and 20 for the low Cd beans. All samples were recorded at 20 K using a He cryostat, in fluorescence mode with a 35-element germanium detector (SAMBA) or a 13-element germanium detector (BM30). A Cd foil was recorded simultaneously in transmission mode for the energy calibration of XANES spectra.

The raw XANES spectra were averaged, calibrated in energy and normalized, and treated by linear combination fits (LCFs) using the XAS software Fastosh (Landrot, 2018). Linear combination fitting was performed by fitting the normalized XANES spectra in regions between -20 and 80 eV with the use of a database of Cd reference compounds. A previously recorded database of Cd reference spectra recorded at 15 to 20K was exploited that contained Cd-cell wall, Cd-glutathionine, Cd-phytochelatin, Cd-hydrated, Cd-malate, Cd-histidine, Cd-phosphate, Cd-phytate, Cd-metallothionein. The preparation and measurements of this dataset is described in (Huguet *et al.*, 2012, 2015). The R-factor ( $= \frac{\sum[\mu_{\text{exp}} - \mu_{\text{fit}}]^2}{\sum[\mu_{\text{exp}}]^2}$ ) assessed the goodness of fit. A combination of two reference compounds was used. For some spectra, particularly the noisier ones, there was not a unique solution since the LCF procedure provided fits of equivalent quality (R-factor increased <10% compared to the best fit). For these spectra, several fits are shown.

#### 6.2.4 Modelling Cd speciation in nibs before and after incubation

Cadmium speciation in the nib was modelled with Visual MINTEQ software (version 3.1, available online at <https://vminteq.lwr.kth.se/>). Three main ligand groups were defined; cysteine (Cd-S; i.e. sulphide ligands), phytate (Cd-O (P)), and organic acids (citric acid, acetic acid, oxalic acid, and lactic acid (Cd-O (C); i.e. carboxylate ligands). Metal-phytate and metal-cysteine complexation

data were added into Visual MINTEQ after consulting literature data that was corrected to infinite dilution (see section V.1 in appendices).

Cadmium speciation was modelled for untreated and treated nibs (H3-H4 and L1-L2). The input for the model can be found in Table V.1. The nib pH and phytate content measured before and after incubation were used as input parameters. Dry weight element concentrations measured were converted to liquid concentrations based on the solid:liquid ratio measured in the cacao nibs of 1:0.09 g mL<sup>-1</sup>. The model considers the nib thus as a single compartment, the cell plasma, containing all elements. The ionic strength was fixed at 0.1 M. The possible precipitation of Ca-oxalate, Mg-oxalate, magnetite and ferrihydrite was allowed in the model to avoid an oversaturated solution. An estimation of the amount of organic acids in cacao beans was derived from Watson et al. 2013. From Calvo et al. 2021, we derived that the acetic acid concentration in the nib increased with 60% after incubation, and that the concentration of lactic acid increased from 0 to 1 g/kg nib. The concentrations of cysteine and glutathione in nibs are reported to be respectively 4.3 mg kg<sup>-1</sup> dry weight and 11.67 mg kg<sup>-1</sup> dry weight (Minyaka *et al.*, 2007). These values were used as input concentrations for the nibs of the low Cd beans. As only cysteine was added into the software to represent all S-ligands, the sum of molar liquid concentrations of glutathione and cysteine (0.82 mM) was used as input for cysteine in the model. For the high Cd beans, it was estimated that the higher Cd concentrations induced the synthesis of thiol ligands with a factor 10, and the cysteine input was thus estimated at 8.2 mM. The Cd complexation was very sensitive to the occupation of Mg on the phytate complex (Fig. V.1). Therefore, two different scenarios were modelled at two different Mg input concentrations, details are given in the result section (Mg = 1.8 M (scenario A) and Mg = 1.0 M (scenario B), Table V.1).

### 6.2.5 Calculations and statistics

The mean and SD (standard deviation) reported throughout the study were calculated with the biological replicates of each treatment. The coefficient of variation (CV %) was calculated with the process replicates for Cd nib and testa concentrations. Concentration ratios for different elements were calculated in the nibs and testa (exemplified for Cd:  $\frac{Cd_{untreated\ sample}}{Cd_{treated\ sample}}$  (mg kg<sup>-1</sup> DW/mg kg<sup>-1</sup> DW)). The SD of these ratios was calculated by using the formula for error propagation for multiplication. Statistical analysis was conducted with JMP® Pro version 18.0.0 (SAS Institute, 2022).

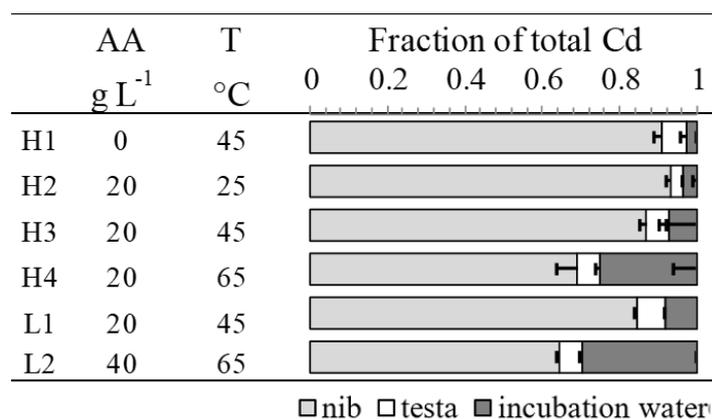
## 6.3 Results

### 6.3.1 Characterization of untreated cacao beans

For the high Cd beans (H1-H4), the average Cd concentration before the incubation treatment was  $9.1 \pm 2.6$  mg Cd kg<sup>-1</sup> in the nibs and  $8.2 \pm 2.0$  mg Cd kg<sup>-1</sup> in the testa ( $\pm$  standard deviation,  $n=8$ ). For the low Cd treatments (L1 and L2), the average Cd concentrations in the nib were  $2.5 \pm 0.4$  mg Cd kg<sup>-1</sup> and  $2.8 \pm 0.6$  mg Cd kg<sup>-1</sup> in the testa ( $n=2$ ). Testa concentrations were generally lower than the corresponding nib Cd concentration in H1-H4, and higher in treatments L1-L2 (Table 6.1). The mean nib pH was  $6.7 \pm 0.1$  (H1-H4,  $n=4$ ) and  $7.0 \pm 0.0$  (L1-L2,  $n=2$ ). The mean nib phytate content was  $5.2 \pm 0.5$  g phytate-P kg<sup>-1</sup> (H1-H4,  $n=4$ ) and was not measured in L1-L2 (Table 6.1).

### 6.3.2 Effect of incubation on cacao bean composition

Incubation decreased nib pH. The pH decrease was more pronounced with increasing temperature and acetic acid concentrations (Table 6.1). Nib Cd concentrations decreased mostly in treatments with highest temperature and with acetic acid addition, i.e. H4 and L2. In these treatments, nib Cd concentration was about factor 1.5 lower than the corresponding original, non-treated beans (Table 6.1). The testa concentrations increased after incubation, and the increase was most pronounced for treatment H1 (Table 6.1). The Cd mass distribution between nibs, testa, and incubation solution after incubation confirmed that there was a gradient in Cd migration from nib into testa and incubation solutions with increasing temperature and decreasing pH (Figure 6.1).

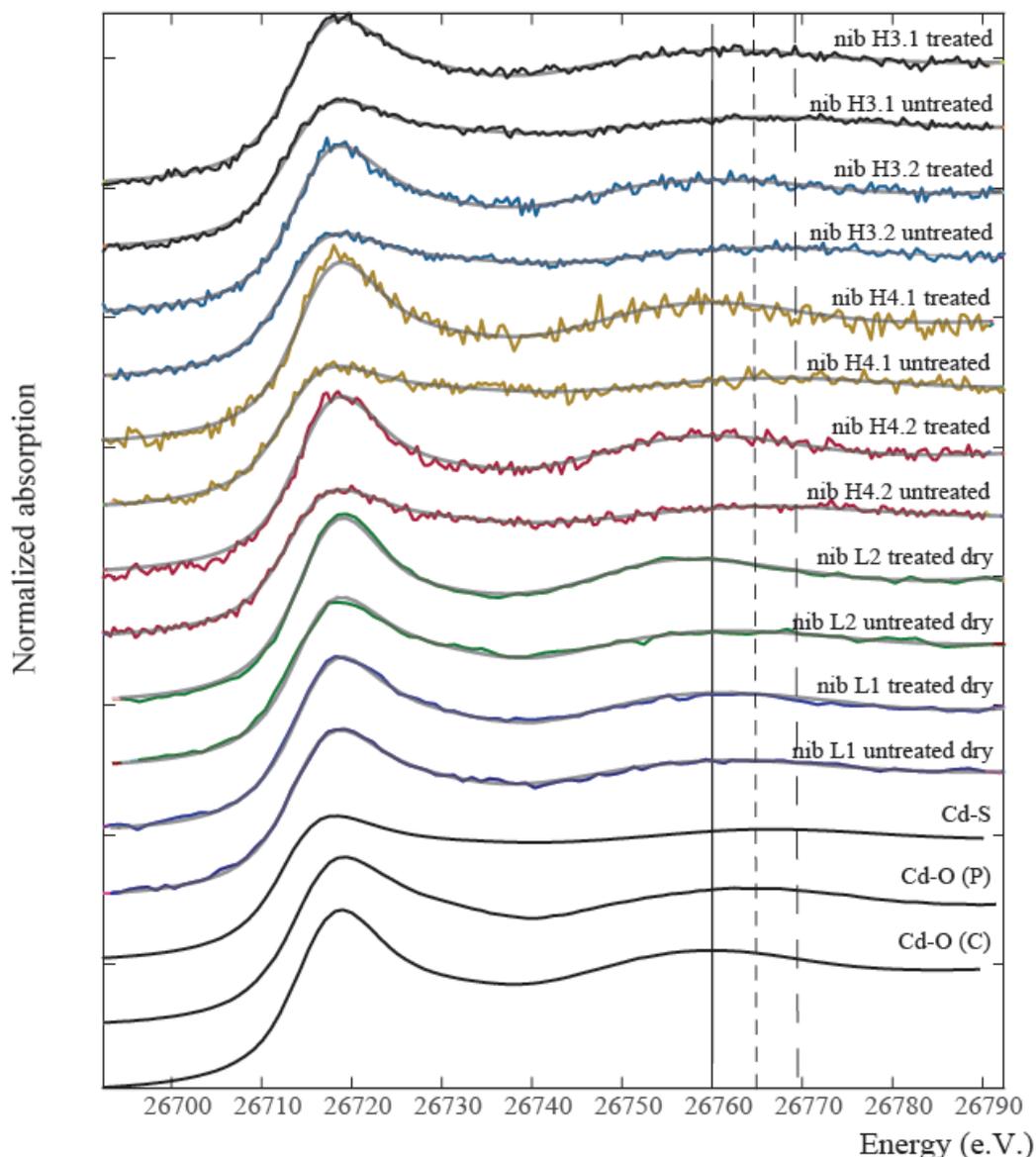


**Figure 6.1:** Cadmium distribution between the nib, testa, and incubation solution after incubation. Higher temperature/acetic acid conditions promote mobilization of Cd from the nib to the incubation solution. AA= dose acetic acid and T= incubation temperature. The error bars indicate the standard deviation of the replicate measurements ( $n=2$ ).

For other elements, the nib elemental concentrations decreased mostly for treatments H4 and L2 after incubation (Table V.2). The decrease of nib elemental concentrations after incubation was especially pronounced for K. For Ca and Sr, incubation did not affect the concentration ratio. The testa was overall enriched in the element after incubation (except for Ca, Sr, and Mo) (Table V.3). The increase in testa elemental content due to incubation was variable and could be less related to incubation conditions. Testa Ca and K were not affected during incubation, and in general for the other elements the largest increase in testa elemental composition was in treatment L1 (Table V.3). Incubation at higher temperatures and higher acetic acid concentrations (H3 and H4) lowered the phytate concentrations in the nibs with a factor 1.4-2.4 (Table 6.1), which was similar to the experiment with the low Cd beans (Vanderschueren, Helsen, *et al.*, 2023).

### 6.3.3 Cadmium speciation analyses in cacao beans

We identified two major groups of ligands that complex Cd in cacao beans; (i) O -ligands represented by the references of Cd-hydrated, Cd-cellulose, Cd-cell wall, Cd-Ca-oxalate, Cd-malate and Cd bound to phosphate ligands (i.e. Cd-phosphate and Cd-phytate), and (ii) S-ligands represented by Cd-phytochelatin, Cd-cysteine, Cd-glutathione and Cd-metallothionein. Group (i) represents O donors (Cd-O) and group (ii) S donors (Cd-S) in the first shell. For Cd-S, the amplitude of the white line (the intense absorption peak after the edge; Figure 6.2) is reduced compared to Cd-O. The references with phosphate (Cd-O (P)) and carboxylate (Cd-O (C)) ligands display spectral differences (the first oscillation maximum of O (P)-ligands is slightly shifted towards higher energy), yet the quality or signal to noise ratio of the spectra obtained in this study was not sufficient enough to differentiate between these two O-ligands.



**Figure 6.2:** Cd K-edge XANES spectra of cacao nibs before and after incubation. Their corresponding linear combination fits are shown in grey. The black spectra (bottom) represent the reference spectra. The first vertical line is at the first oscillation maximum for O (C)-ligands, the second line for O (P)-ligands, and the third for S-ligands. Dry specifies that the samples were measured in dried state. If not specified, samples are measured in frozen hydrated state.

### 6.3.3.1 Effect of sample preparation on Cd speciation

Cadmium speciation was compared between samples prepared in frozen hydrated and oven-dried state between treatments H4 (replicate 1) and H3 (replicate 2). The absorption spectra recorded for the frozen hydrated and dried samples were alike, although the spectra of dried samples showed less noise (Figure V.2). Linear combination fitting confirmed minimal differences in speciation. However, the noisier spectra in frozen-hydrated state of nibs (Figure V.2) and testa

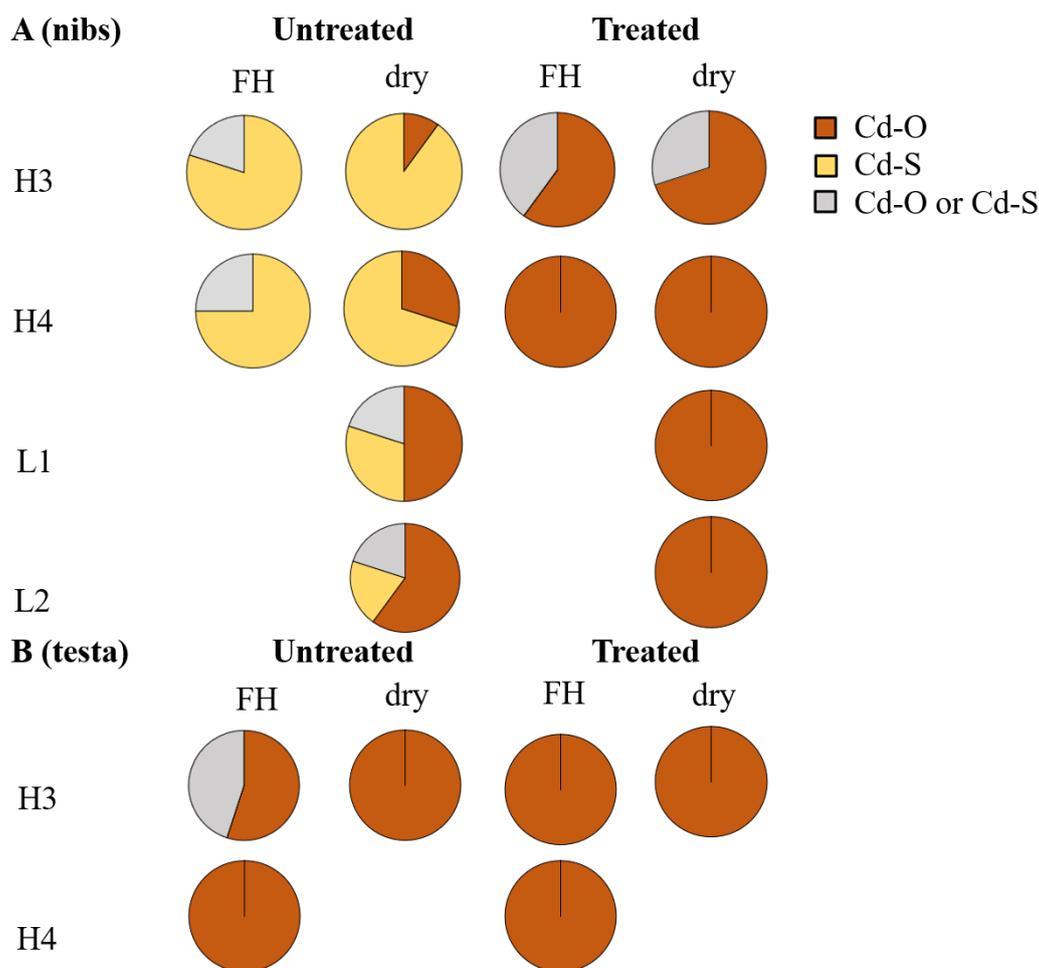
(Figure V.3) gave equivalent fits with slight changes in speciation (see below), whereas with the spectra of dried samples the low R-factor allowed to fit a unique solution.

### 6.3.3.2 Effect of Cd concentration on Cd speciation in untreated beans

The spectra of the untreated nibs of the high Cd treatments (H3-H4), resembled mostly the Cd-S reference spectra (Figure 6.2). The linear combination fits also confirmed that Cd was mostly coordinated with sulphur donors ((75-100%), Figure V.2 and Figure 6.3A). The spectra of the lower Cd beans were noisier than the high Cd beans (lower R-factor) due to the lower abundance of Cd in the sample. The linear combination fits in the nibs indicated that for L1-L2 Cd was mostly bound with O-ligands (60-100%), and that S-ligands could occur as minor species (0-40 %).

### 6.3.3.3 Effect of incubation on Cd speciation in cacao beans

The XANES spectra revealed clear differences in speciation between the untreated and treated cacao nibs (Figure 6.2) in treatments H1-H4. Cadmium was mainly bound to S-ligands before incubation (varying from 70-100%, Figure 6.3). After incubation, most Cd was bound to O-ligands (60-100%) with Cd-S as possible minor ligand. In the low Cd beans, Cd speciation seemed less impacted by incubation as the Cd coordination remained mainly with O-ligands (Figure 6.3A). Similarly, in the testa no systematic shift in speciation was observed (Figure 6.3B). Before incubation, Cd was mostly bound to O ligands, with possibly a fraction of Cd-S. After incubation, Cd-O was the only species found.



**Figure 6.3:** The fraction of Cd species in the cacao nibs (A) and testa (B) determined by XANES for treatments H3-H4, L1-L2. The grey zone represents the variation in the replicate measurements or the quality of the linear combination fit. For more detailed fits of each run, see Figure V.3 and V.4. FH=frozen hydrated.

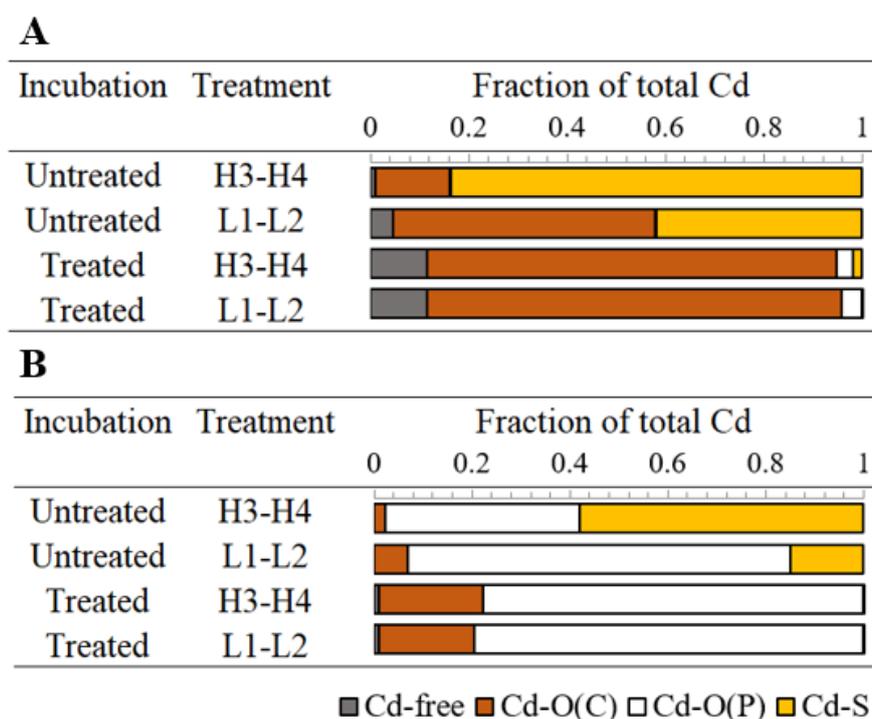
#### 6.3.4 Modelling Cd speciation in nibs before and after incubation

The modelled Cd speciation was sensitive to the assumed Mg concentrations (Fig. V.1). The Mg concentrations in the mean dry bean Mg concentration corrected to the water content was equivalent to 1.8 M. At that concentration, Mg binding to phytate was outcompeting Cd (Figure V.1). At a lower Mg concentration, here arbitrarily reduced to 1.0 M, complexation of Cd with free phytate became relevant. Therefore, two scenarios with different Mg concentrations were modelled (Mg = 1.8 M (*scenario A*) and Mg = 1.0 M (*scenario B*), Table V.1).

In scenario A, the low Cd beans prior to incubation (L1-L2) were mainly bound to carboxylate ligands (54% Cd-O (C)) and sulphide-ligands (42% Cd-S) (Figure 6.4A). After incubation, 85% of Cd was associated with the organic carboxylate anions, 10% was free, 3% was on the phytate

complex, and 2% was associated with cysteine. With lower Mg competition (Scenario B, Figure 6.4B), most Cd was coordinated with phytate (78%), and the remainder was complexed with sulphide (15%) and carboxylate ligands (7%). After incubation, most Cd was still complexed with phytate (78%), but the remainder was complexed with carboxylate ligands (21%) and free Cd<sup>2+</sup> (1%).

In the high Cd beans (H3-H4), the predictions showed that most Cd was coordinated with S-ligands before incubation, at both scenarios A (84% Cd-S) and B (58% Cd-S) (Figure 6.4). In scenario A, the remainder was complexed with carboxylate (15%), and in scenario B with phytate (40%). After incubation, the model predicted that the proportion of Cd bound to S-ligands decreased to less than 2% in both scenarios. The remaining ligands complexing Cd were O-ligands, and more specifically carboxylate ligands in scenario A (Figure 6.4A), and phytate ligands in scenario B (Figure 6.4B).



**Figure 6.4:** Simulated Cd speciation in the nibs before and after incubation. Simulations used Visual Minteq in which the complexation database was complemented with stability constants for phytate and cysteine ligand (see appendix, V.1). Cadmium is either free or complexed with carboxylate ligands (Cd-O(C)), phytate (Cd-O(P)) and sulphide ligands (Cd-S). The predicted Cd speciation was sensitive to the assumed Mg concentrations (Fig. S1), therefore two scenarios are shown here. **A:** available Mg in the nibs estimated from total nib Mg concentration and **B** available Mg in the nibs estimated factor 1.8 lower than in A to illustrate how partial complexation of Mg<sup>2+</sup> to unaccounted other ligands may lower Mg-phytate complexation and

enhance Cd-phytate complexation. In the SI, the input for the different treatments and conditions can be found.

## 6.4 Discussion

### 6.4.1 Cadmium speciation and chemical composition in the untreated nibs

The Cd concentrations in both treatments H1-H4 and L1-L2 (Table 6.1) exceed the threshold commonly used for processing of cacao beans of  $0.60 \text{ mg Cd kg}^{-1}$  and the median bean Cd concentration of  $0.63 \text{ Cd mg kg}^{-1}$  reported in a meta-analysis in Latin America ( $n=785$ , (Vanderschueren *et al.*, 2021)). These samples with relatively high Cd content were selected for this study as the detection limit for Cd XANES currently ranges between one and a few  $\text{mg kg}^{-1}$  (depending on matrix) at the beamlines used in this study.

The nib phytate content ( $5.2 \pm 0.5 \text{ g phytate-P kg}^{-1}$ ) prior to incubation was in a similar range as in studies with lower bean Cd concentrations ( $4.4 \text{ g phytate-P kg}^{-1} \pm 1.00$  (Chapter 3),  $4.5 \pm 0.3 \text{ g phytate-P kg}^{-1}$  (Vanderschueren, *et al.*, 2023)). The phytate content seems thus not to be affected by higher Cd concentrations in the nib. The Cd/phytate molar ratio is only 5.1% on average and maximally 9.1% in the H1-H4 beans, illustrating that even in contaminated beans, phytate is in large excess for Cd.

Cadmium speciation in the nibs was clearly impacted by the concentration of Cd in the beans (Figure 6.2 and 6.3). In the high Cd beans (H3-H4), Cd was mainly bound to S-ligands. In plant cells it is well documented that to cope with elevated Cd concentrations, a common mechanism to maintain low concentrations of  $\text{Cd}^{2+}$  in the cytoplasm is metal complexation or chelation by low-molecular-weight compounds like the thiol-containing ligands cysteine, glutathione (GSH), and phytochelatins (PCs) (Seth *et al.*, 2012; Clemens, 2019). The Minteq model indeed clearly confirmed that thiol-containing compounds have high affinity for Cd, as the majority of Cd was bound to cysteine in the high Cd beans. In rice grains, the current speciation data indicates mainly a binding of Cd to S-ligands, although these data represent a variety of Cd concentrations ( $0.98 \text{ mg kg}^{-1}$  (Wiggenhauser, *et al.*, 2021),  $3.51\text{-}11.4 \text{ mg kg}^{-1}$  (Gu *et al.*, 2020)). Rice thus stores Cd differently in the seeds and/or detoxification by thiol-containing compounds is already triggered at lower Cd concentrations in rice than in cacao nibs. Previous studies in cacao with similar nib Cd concentrations as the low-Cd conditions ( $2\text{-}3 \text{ mg kg}^{-1}$ ) confirmed binding of Cd with O-ligands (Chapter 3). In chapter 3, it was hypothesized that Cd was mostly bound with phytate. In our study, the quality of the spectra did not allow differentiation between phosphate and carboxylate ligands. It is thus also possible that carboxylate ligands like the anions of organic acids (Watson *et*

*al.*, 2013), and/or cell wall components like pectines (Huguet *et al.*, 2012) may be complexing Cd in the cacao nibs.

Our study revealed that in the testa of H3 and H4, Cd was mostly coordinated with O-ligands in unfermented state (Figure 6.3). Hence, speciation in the testa differed vastly from their corresponding nibs. This may indicate that detoxification mechanisms (by chelation with S-ligands) are more activated in the nibs than in the testa and/or that in the testa other mechanisms are at stake, like the binding of Cd with a cell wall component as has been observed in other plant tissues (Huguet *et al.*, 2012; Tian *et al.*, 2017).

To further disentangle the speciation of the low Cd nibs, the Minteq model was used, as the model could differentiate between Cd-O (P) and Cd-O (C) complexation. With relatively higher Mg concentrations in the system (1.8 M, Figure 6.4A), the modelled Cd speciation predicted a predominant binding to carboxylate ligands (mostly acetate, not shown) for L1-L2. This was mostly due to the occupation of the phytate complex by Mg. Phytate is known to have high affinity for Mg (Hawkesford *et al.*, 2012). The relative concentration of available Mg in the plasma was probably largely overestimated in this model. When Mg concentrations were lowered from 1.8 M to 1.0 M, most of Cd was complexed with phytate (about 78%, Figure 6.4B). Briefly, the model indicated that the modelled metal-phytate and metal-organic acid interaction depended strongly on the concentrations of other competing elements. With higher Mg competition, less Cd was associated with phytate, and more with organic acids. It has been reported that proportions of K, Mg and Ca associated with phytate vary considerably among plant species and between tissues of a seed (Hawkesford *et al.*, 2012).

#### 6.4.2 Cadmium mobilization and changes in Cd speciation during incubation

After incubation, the concentration of elements increased in the testa and decreased in the nibs, except for Ca (Table V.2 and V.3), as observed in Vanderschueren *et al.* 2020. In the conditions with low pH and high T, around 30% of Cd was mobilized into the incubation solution (Figure 6.1). The measured Cd speciation in the left-over Cd fraction in the nibs indicated for all treatments that Cd was bound to O-ligands (Figure 6.3) and this was confirmed by the calculated speciation when there was lower Mg competition (Figure 6.4B).

Our model indicated that for samples H3-H4 the mobilization of nib Cd can be mostly explained by pH dependent interactions between Cd and thiol groups (i.e. cysteine or glutathione) in the nibs. The pKa of thiol groups is estimated around 9.6 (Tummanapelli and Vasudevan, 2015), and of carboxyl groups around 4 (Visual Minteq database). Decreasing the pH increased  $\text{Cd}^{2+}:\text{H}^+$

competition on the functional binding groups of thiol groups, by which the binding sites on the cysteine complex become unavailable whereas the carboxylate and phosphate groups are well accessible (unless in the very acid pH range). Further, as the complexation with the organic acids is less strong, part of the Cd may be mobilized towards the testa and diffuse into the incubation solution.

For the low Cd beans (L1-L2) in scenario B, where the phytate complex is less occupied by Mg, Cd is complexed with phytate before complexation and remains bound to phytate after incubation (Figure 6.4B). This model thus suggests that Cd releases from the phytate complex due to phytate breakdown by phytase activation. This is corroborated by the fact that up to 58% of phytate broke down in the nib during incubation (Table 6.1). Furthermore, we observed that the breakdown of phytate has a large impact on the migration of Mg and K towards the nib and outer solution. Before and after incubation, Mg and K in the nibs are mostly complexed with phytate due to their high affinity for phytate (Hawkesford *et al.*, 2012), and these elements are largely mobilized into the incubation solution (Figure V.5). When the competition with Mg is higher (scenario A, Figure 6.4A), the model indicated that about 54% of Cd was bound to carboxylate before and the remainder with thiol groups. After incubation, almost all Cd was bound to carboxylate. For the Cd bound to thiols, a similar analogy can be drawn as in the case of high Cd beans of scenario A; i.e. the release of Cd is related to the decrease in pH. For the Cd bound to carboxylate, a possible ligand could be the polysaccharides of the cell wall (Cd-O (C)-ligand) (Isaure *et al.*, 2015; Tian *et al.*, 2017), and fermentation could cause a disruption of these cells by which Cd releases and binds with organic acids that are available. This mechanism could lead to a partial mobilization of Cd into the incubation solution.

## 6.5 Implications, limitations and outlook

This study provided new insights on the chemical form of Cd in cacao beans and on the mechanisms responsible for the decrease of nib Cd concentrations during fermentation. However, some limitations affected this study. The conditions H3-H4-L1-L2, had pH ranging between 3.8 and 4.6 after incubation. It has been reported that pH between 4.8 and 5.2 indicates a good fermentation process (Calvo *et al.*, 2021). The incubation conditions in this study were thus more acidic than an ideal fermentation. Consequently, the processes observed may be more pronounced than in real conditions. Moreover, the Cd concentration in the low Cd beans in this study was still relatively high compared to the average Cd concentration reported in cacao beans. In general, samples for studies using XANES have higher metal content due to the detection limit. It is very plausible that other speciation may be detected at lower concentrations, since we observed that speciation depends on the metal concentration.

This study has its importance as postharvest mitigation strategies focussing on fermentation may offer a valid short-term alternative to manage the Cd-cacao issue. Our study indicates that Cd speciation in unfermented beans may differ, according to different bean Cd concentrations. However, in all treatments, the final Cd speciation after incubation was with O-ligands. This information can be used to decipher biochemical mechanisms behind the reduction of Cd during fermentation to optimize these processes. Lastly, the complexation of Cd after fermentation may have important implications for human nutrition studies.

**Table 6.1:** Bean compositions for the different treatments before (untreated) and after (treated) incubation.

	n	<b>H1</b>		<b>H2</b>		<b>H3</b>		<b>H4</b>		<b>L1 (1)</b>		<b>L2 (1)</b>						
		0 g L <sup>-1</sup> AA		20 g L <sup>-1</sup> AA		20 g L <sup>-1</sup> AA		20 g L <sup>-1</sup> AA		20 g L <sup>-1</sup> AA		40 g L <sup>-1</sup> AA						
		45 °C		25 °C		45 °C		65 °C		45 °C		65 °C						
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD					
<b>Nib Cd (mg kg<sup>-1</sup>)</b>																		
Untreated	2	10.1	4.76	2	8.58	1.18	2	8.86	1.49	2	9.03	2.41	1	2.19	/	1	2.83	/
Treated	2	9.68	5.17	2	9.07	1.54	2	7.47	1.69	2	6.09	2.51	1	1.96	/	1	1.8	/
<i>Ratio</i>		<b>1.04</b>	<b>0.74</b>		<b>0.95</b>	<b>0.21</b>		<b>1.19</b>	<b>0.33</b>		<b>1.48</b>	<b>0.73</b>		<b>1.12</b>	/		<b>1.57</b>	/
<b>Testa Cd (mg kg<sup>-1</sup>)</b>																		
Untreated	2	8.00	3.11	2	9.02	2.51	2	8.09	1.26	2	7.78	0.45	1	2.32	/	1	3.32	/
Treated	2	23.73	8.01	2	12.54	2.28	2	19.49	2.62	2	19.57	9.7	1	6.35	/	1	6.13	/
<i>Ratio</i>		<b>0.34</b>	<b>0.17</b>		<b>0.72</b>	<b>0.24</b>		<b>0.42</b>	<b>0.09</b>		<b>0.40</b>	<b>0.20</b>		<b>0.37</b>	/		<b>0.54</b>	/
<b>Testa/nib Cd ratio</b>																		
Untreated	2	0.82	0.50	2	1.04	0.32	2	0.91	0.21	2	0.91	0.25	1	1.08	/	1	1.18	/
Treated	2	0.85	0.54	2	1.38	0.34	2	2.66	0.70	2	3.13	2.02	1	3.24	/	1	3.41	/
<b>Nib phytate-P (g P kg<sup>-1</sup>)</b>																		
Untreated	2	5.19	0.97	2	5.12	0.29	2	5.34	0.39	1	5.18		0	/	/	0	/	/
Treated	2	5.04	0.51	2	5.33	0.00	2	3.78	0.23	1	2.19		0	/	/	0	/	/
<i>Ratio</i>		<b>1.03</b>	<b>0.22</b>		<b>0.96</b>	<b>0.06</b>		<b>1.41</b>	<b>0.13</b>		<b>2.37</b>							
<b>Nib pH</b>																		
Untreated	2	6.72	0.08	2	6.76	0.07	2	6.64	0.06	1	6.74		1	7.00		1	7.00	
Treated	2	6.71	0.08	2	5.19	0.10	2	4.59	0.12	1	4.10		1	4.40		1	3.80	

The mean and SD (standard deviation) were calculated from the biological replicates of each treatment. The SD of the ratios was calculated by using the formula of error propagation for multiplication.

(1) Dried nib samples from a previous experiment (Vanderschueren, Helsen, *et al.*, 2023). Treatments L1 and L2 each refer to a single experimental run in that previous study, and thus to a single cacao pod. Phytate content was only analysed in a subset of samples in that study, not including the experimental runs that were analysed here.

(2) Pod 7 contained only 13 beans. Hence, insufficient beans were available for replicate measurements of pH and phytate measurements.

## Chapter 7: Conclusions

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This work was set-up to increase the understanding of the pathways and chemical forms (speciation) of Cd in intact cacao trees on its journey between soil and beans. A multi-analyses approach, including field and laboratory studies, was used to address the following specific questions:

- What are the main storage compartments in the cacao tree, and in which chemical form does Cd accumulate in each compartment?
- What are the Cd pathways in cacao trees, and do these Cd pathways differ between a low and a high Cd accumulating cultivar?
- How does Cd speciation in beans change during fermentation?

To answer these questions, we studied mature trees and beans from the field and seedlings in laboratory-controlled conditions. We used a multi-analyses approach, including metal stable isotope compositions, isotope tracing, speciation, and imaging analyses. This chapter will conclude this research by summarizing the key findings in relation to the research aims and discuss their implications. We will also review the limitations of our studies and propose recommendations for future research.

## 7.1 Key findings

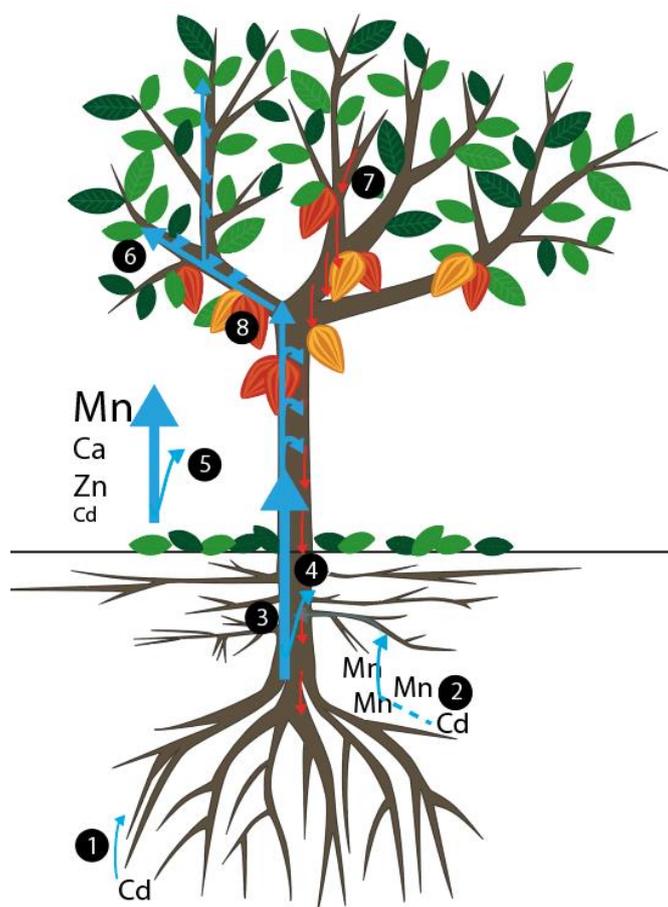
1. *At environmental (low Cd) conditions, Cd is readily absorbed by the plant.* The high Cd accumulator had a particular high Cd transfer from soil to the leaves ( $TF_{\text{leaf-soil}}=13.1$  (transfer factor TF: total Cd concentration in the plant leaf, over the total Cd concentration in the soil) (Chapter 3). The TFs in the grafted ( $TF_{\text{leaf-soil}}=5.2$ ) and low ( $TF_{\text{leaf-soil}}=7.2$ ) cultivar were closer to the range of average  $TF_{\text{leaf-soil}}$  (3.4-7.1) reported in several studies in Latin-America (Chapter 4). The Cd uptake potential of the cacao tree can be conceived as rather high compared to other agricultural crops, and more similar to woody species used for phytoextraction such as willow and poplar (Chapter 2).
2. *The three cultivars exhibited marked differences in Cd uptake.* The uptake was factor 2.9 higher in the high Cd accumulator and factor 1.7 higher in the grafted cultivar than in the low Cd accumulator. The grafted cultivar had the same scion as the low Cd accumulator, but a different rootstock. There was thus an effect of rootstock on the uptake of Cd. Similar to the Cd uptake, the Mn uptake was factor 3.6 and 2.3 times higher in the high and grafted cultivar compared to the low cultivar. Hence, an increased use of the Mn uptake pathway led to more Cd hitchhiking through this pathway (Chapter 4). This main outcome can be

used to identify cross membrane transporters that are responsible for both Mn and Cd uptake (such as the TcNRAMP5-transporter).

3. *The accumulation of Cd in the roots was limited in mature trees and in seedlings grown at low Cd conditions.* In the three cultivars the internal translocation factor from roots to aerial parts, for example the branches ( $ITF_{\text{branch-root}}$ ) ranged from 1.9 to 2.2 (Chapter 4). Similar high translocation factors between roots and aerial parts were observed in other studies on cacao plantations and in moderately Cd accumulating woody species such as poplar and cotton (Chapter 2). In other studied crops such as cereals, the retention of Cd in roots is generally larger than in cacao ( $ITF < 1$ ).
4. Despite the large Cd transfer from roots to shoots, *there was a retention of Cd compared to nutrients (mostly Mn and Ca) in the roots of the three cultivars studied.* Light Cd isotopes accumulated in the roots of the high Cd accumulator and heavy isotopes were transported to the shoot. The Cd storage forms observed in the roots of the high Cd accumulator were: i) the incorporation of Cd in Ca-oxalate crystals and ii) binding of Cd to sulfur-ligands that complex Cd (Chapter 4).
5. *The branches were the main storage compartment for Cd accumulation in all three cultivars (57% of all tree Cd) (Chapter 4).* In the high Cd accumulator, it was shown that Cd was primarily bound to carboxyl-ligands (60-100%) and mainly localized in the phloem rays and phelloderm of the bark. We suggested that Cd was mainly translocated to the bark through a xylem-to-phloem transfer to reduce the transfer to the leaves (Chapter 3). In the seedlings, the stems were the major compartment for Cd accumulation (39-46%) (Chapter 5).
6. *In all cultivars, there was a separation of Cd from nutrients (especially Mn) along the transpiration pathway from branches to leaves (xylem).* We could relate this separation of Cd from nutrients (especially Mn) along this pathway with a progressive enrichment of heavy isotopes in transpiring organs along the xylem (Chapter 4). The isotope ratio  $\delta^{114/110}\text{Cd}$  of tree organs increased in the order roots<nibs (seeds)<branches<pod husks<mature leaves.
7. A Cd tracing experiment with cacao seedlings showed that *cadmium is mobile in the phloem of cacao plants and can be transported from stems and older leaves to developing tissues, like flushing leaves, and developing roots* (Chapter 5). The dominant transport pathway of Cd was nonetheless the transpiration pathway from roots to leaves. The

circulation of Cd indicated a behavior intermediate between crop species and accumulator species.

8. The combination of isotope and nutrient/Cd ratios suggested that the *Cd pathway from branches to nibs was mainly phloem mediated and differed from that from branches to the mature leaves* in the low and the high Cd accumulator.
9. *Cadmium in nibs is bound to oxygen-ligands (most probably phytate) in low Cd concentration conditions and at higher environmental Cd exposure Cd is bound to sulfur-ligands* (Chapter 3 and 6). Cadmium speciation in the beans is thus dependent on the nib Cd concentration.
10. *When the beans are acidified and exposed to high temperatures, nib Cd reduces up to a factor 1.5. There is an outward migration of nib Cd into the testa (seed envelope) and the solution. After exposure to these conditions, Cd becomes bound to oxygen ligands* (Chapter 6).
11. *A combination of pH changes and/or phytate breakdown may explain the migration of Cd outward of the nib during fermentation* (Chapter 6).



**Figure 7.1:** Summary of the proposed Cd pathways in a cacao tree and the numbers refer to 9 different findings obtained in this work (see section above). The blue arrow indicates the Cd that is loaded in the xylem and transported to transpiring organs. The red arrows indicate that a minor contribution of Cd is remobilized to developing tissues *via* the phloem. In the branches, there is a separation of Cd relative to other nutrients (Mn, Ca, and Zn).

## 7.2 Implications for the field

### 7.2.1 Agronomic countermeasures face limitations

The application of soil amendments faces some limitations. On the one hand there are no consistent results that show a reduction in bean Cd after amending the soil, and on the other hand there are practical constraints regarding cost and labour. Our findings in this dissertation highlight that Cd in the beans can be loaded from an internal stock in the plant (Chapter 3 and 4). If we assume that Cd in the nibs is loaded from an entirely mixed pool in the trees, we can make an estimation of how long it would take before this source is replenished by estimating the residence time in above ground tissues. Data of standing biomass and annual litterfall in a classical plantation of 30 years old (Boyer, 1973) and a young agroforestry system of only 4.5 years old (Alpízar *et al.*, 1986; Heuvelop *et al.*, 1988), allowed to calculate the residence time of the above ground biomass, i.e. the time required to build the same biomass as the biomass returned to the soil as litter (=leaves + branches). This, combined with Cd concentrations in each plant part that was taken over from the high Cd cultivar, allowed to calculate the theoretical mean residence time of Cd in the above-ground tissues (Table 7.1). In the classical plantations the theoretical mean Cd residence time was 4.8 years, and 3.4 years in a young agroforestry system. These estimations are in line with recent work that showed that after 30 months of application of soil amendments, there was still no reduction of bean Cd (Argüello *et al.*, 2023), whilst there was already a reduction in leaf Cd. However, recent work with isotope pulse-chase tracing where the isotope was added with irrigation water in the soil (Vanderschueren, Wantiez, *et al.*, 2023) has shown that after  $\pm 6$  months half of the Cd in the beans was reflecting the isotope composition of the soil, which indicates a faster Cd turn-over in the beans (1 year) than when Cd in the nibs originates only from the fully mixed stock in the plant (4.8 (plantations) or 3.4 years (agroforestry), see above). Thus, probably Cd is not only loaded from the internal stock in the branches. However, there was a delay in the bean loading compared to the leaves, confirming that bean Cd is not originating only from directly taken up Cd by the roots. Most likely, soil amendments require more than one year to affect the entire root zone explaining the delayed response compared to that in a pulse-chase experiment. More isotope source tracing studies could greatly help our understanding concerning these fluxes of directly taken up versus re-

allocated Cd in the beans (see section 7.3.1). Amending the soil before establishing the orchard could be a way to bypass this internal stock problem, especially if the soil has high soil Cd bioavailability.

**Table 7.1:** Standing biomass and annual fluxes in a classical plantation versus an agroforestry system.

			<b>Cd concentration*</b>		<b>Mass Cd</b>			
	Plantation	Agroforestry			Plantation	Agroforestry		
<b>Standing biomass aboveground</b>								
wood	15.18	6.84	ton/ha	6.1	mg/kg	92.57	41.72	g Cd/ha
leaves	3.84	2.97	ton/ha	3.3	mg/kg	12.67	9.80	g Cd/ha
fruits+flowers	0.42	1.47	ton/ha	2.5	mg/kg	1.05	3.68	g Cd/ha
total reservoir	19.44	11.28	ton/ha			106.29	55.20	g Cd/ha
<b>Annual litterfall</b>								
leaves	3.84	3.8	ton/ha/year	3.3	mg/kg	12.67	12.54	g Cd/ha/year
wood litter	1.36	-	ton/ha/year	6.1	mg/kg	8.30	0.00	g Cd/ha/year
fruits	0.42	1.47	ton/ha/year	2.5	mg/kg	1.05	3.68	g Cd/ha/year
Total flux	5.62	5.27	ton/ha/year			22.02	16.22	g Cd/ha/year
<b>Residence time above ground Cd (reservoir/flux)</b>						<b>4.83</b>	<b>3.40</b>	<b>year</b>

The data of standing stock and fluxes in the classical plantation originates from (Boyer, 1973), in agroforestry system from (Alpizar *et al.*, 1986; Heuvelop *et al.*, 1988). \*The Cd concentrations of the high Cd accumulator (Chapter 3) were used for the Cd mass calculations.

## 7.2.2 Low Cd accumulating cultivars: a reality or a holy grail?

The creation of low Cd accumulating Cd varieties has been proposed as a long-term strategy to reduce Cd accumulation, especially in locations with high soil Cd bioavailability. For rice, low Cd innovations are about to be released in the market. The development of these genotypes is a product of the scientific advances of over decades. In sharp contrast is the research in cacao, that has just recently begun.

We highlighted that there were differences in Cd accumulation between a low (CL 10/3) and a high (NA 312) Cd accumulating cultivar. The uptake of Cd was 2.7 times higher in the high Cd accumulating cultivar than in the low. We related this difference mainly to an altered Mn uptake in the high Cd accumulating cultivar (Chapter 4). This main outcome can be used to identify cross membrane transporters that are responsible for both Mn and Cd uptake (such as the TcNRAMP5-transporter). Our study moreover revealed that in both cultivars, ~~most Cd in the nib was originating from an internal stock and~~ that the branches were a main regulatory tissue, that served both as a sink and a source (Chapter 3 and 4).

The development of low Cd accumulating cultivars now needs field studies comparing different commercial cultivars growing in the same conditions. To our knowledge, the most used agronomic cultivars are not yet screened for Cd accumulation characteristics. This screening will allow to identify and test promising cacao cultivars for low Cd accumulation. However, due to the slow-growing properties of cacao such experiment can take several years and is probably the main reason why these studies are not out there yet and that the creation of a low Cd cacao cultivar remains a 'holy grail' solution. To further advance our understanding of the transporters involved in Cd uptake and translocation there is necessity of gene expression and mutant based studies. To facilitate this challenging genetic research, we can benefit from the knowledge that already exists in crops like rice and in Cd hyperaccumulating species, and is in a far stage.

### 7.2.3 Grafting: a more feasible medium-term solution?

Grafting is the physical combination of two or more parts of living plant tissue through forced vascular connection. This procedure results in a single plant. It is a technique commonly used in cacao to propagate vegetatively. This technique can be a shortcut to breed cultivars by combining desirable agronomic traits of the rootstock with those of the scion. Hence, the use of cacao rootstocks with low Cd uptake/translocation potential can be the basis to renovate commercially used cultivars. Chapter 4 indicated that the rootstock indeed affects the overall Cd plant uptake. This leads to the suggestion that we could combine a rootstock of a low Cd accumulating cultivar with an agronomically interesting scion. However, to evaluate the effect of grafting on Cd uptake and distribution in the plant, we need specifically designed experiments. For example, cacao genotypes of low (L) and high (H) Cd accumulator genetic groups could be grafted in four combinations: H/L, L/H, and H/H and L/L (self-grafts included). This set-up allows to corroborate if the rootstock controls indeed Cd uptake.

### 7.2.4 Post-harvest mitigation to the rescue?

Conventional fermentation practices can reduce Cd concentrations in the cacao nib by up to a factor 1.3 due to processes of acidification and phytate breakdown (Chapter 5). If we can further optimize these processes, this is a relatively easy solution for the mitigation of Cd in cacao products due to its low-cost. However, the potential is limited to beans that exceed the Cd limits by only 30%. Mixing of beans with a high Cd concentration with beans with low Cd concentrations is a strategy readily applicable. However, more research is necessary regarding quality control of Cd measurements and the ideal mixing ratio. And a drawback of this strategy is that mixing is not an option for fine-flavor beans that originate from one location.

## 7.3 Future perspectives

### 7.3.1 A multi-analysis approach

We explored the potential of several state-of-the-art techniques (X-Ray Absorption Spectroscopy, Cd isotopes at natural abundance, stable isotope Cd as a tracer, imaging techniques) at low Cd concentrations. They rewarded us with complementary insights but also led to a reduced sample throughput, especially with regard to the different genotypes that we could analyze. There was moreover a disbalance in the potential between each technique in terms of measurements. The XAS analyses were most constraining because they had in general a higher limit of detection (a few mg kg<sup>-1</sup>, depending on the matrix and on the beamline). As a consequence, we were restricted to the organs of the high Cd accumulator and fermented/unfermented beans with a relatively high Cd content. The natural Cd isotope measurements proved more feasible at these low Cd concentrations, yet the number of samples was also limited due to a time-consuming purification procedure that had to be repeated because of the low Cd concentrations. Imaging techniques have generally a high cost and/or limited beamtime, and the process of data treatment is tedious and complex to automate which reduces the sample size severely. In our study, the  $\mu$ XRF measurements at the Cd L-edge were moreover challenging due to interferences of potassium, but here we had the advantage to couple  $\mu$ XRF with  $\mu$ XANES in frozen hydrated conditions. With nanoXRF at the Cd K-edge there was no interference issue, and we could also analyse transition metals (Zn, Mn, Ni, Co, Cu). However, nanoXANES in frozen hydrated conditions was not available. Synchrotron beamlines are in constant progress, and it is now possible, in some of them, to perform  $\mu$ XRF and  $\mu$ XAS at the Cd K-edge in cryo conditions. The stable Cd isotope labelling technique proved its value to study Cd circulation at low concentrations, but was limited to lab and hydroponic conditions, and we had to extrapolate source-sink findings from a vegetative state. Cacao trees only flower when they are four to six years old and it takes after flowering approximately 150 days before the fruits reach maturity.

A limitation in our study was that we only sampled small branches (5mm diameter). Our observations concerning the branches should be confirmed by verifying that the small branches analyzed in the present study are representative for all woody tissues constituting the tree. This research studied only two native cultivars (NA 312 and CL 10/3 5A). More comparative studies on other genotypes that are used commercially are indispensable to extrapolate our findings on the dynamics of Cd in cacao plants. Another limitation was that the speciation measurements did not allow to differentiate within the Cd-O ligands between phytate, organic bases, polyphenols, ... It is clear that we need advances in especially XAS-techniques to improve our understanding of metal cycling in environmental systems. It is thus timely that at this moment, new detection

modes such as HERFD-XAS are developed. These developments lower further the detection limits and will allow future researchers to focus on systems that are environmentally/agronomically relevant, as many research gaps considering the control of trace metals in these systems remain. The combined XAS-isotope-imaging approach can be a tool to study these systems but should be applied with careful consideration of the advantages and drawbacks of each technique.

### 7.3.2 The cacao tree: a challenging plant to study

Cacao is a large perennial tree. This large nature poses some challenges in terms of sample collection. First, we were not able to completely sample and weigh the tree. We had to assume a similar mass for each tree. Hence, our work did not consider an effect of 'vigor' (i.e. growth efficiency) on Cd uptake and translocation. Second, sampling of the roots was not evident, and the surface roots collected may not represent the whole rooting system. The thin branches sampled may also differ from big branches and stem structures which were not collected and for which we assumed a similar Cd concentration and isotope signature. We recommend further research on the branches and stems as we identified these organs as large Cd sinks and sources, playing a major role in the regulation of Cd. More long-term trials where the partitioning of Cd is studied in terms of flowering and leaf flushing is also recommended as their effects on Cd uptake and translocation remain unknown.

### 7.3.3 Final conclusion

The new worldwide regulations have joined the efforts of several actors of the cacao industry and research institutions to mitigate high Cd levels in the soil and beans. We contributed to these actions by opening a part of the black box regarding the biochemical mechanisms underlying the accumulation of cadmium in cacao trees. We hope that the methodology and findings of this work can be recycled in new research projects to understand the fate of metals in soils and soil-plant systems. Soil pollution is one of the main threats that affect soils and their ecosystem services. Some regions across the world face critically high trace metal soil concentrations, which increases contaminant exposure in crop plants and humans. At the same time, human deficiency in micronutrients is a major health issue. These two research areas that both focus on trace metals are highly interconnected with each other. Researchers, industry and policy makers need to balance between reducing contaminants in crops, while increasing micronutrient content. This delicate balance is a major challenge to sustain food security in the coming decades.

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## Appendices

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# Appendix I: Mitigating the level of cadmium in cacao products: a review

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## I.1 Meta-analysis: methods and description of included studies

### I.1.1 Selection of individual studies

First, a search in three databases (Scopus, Web of Science and PubMed) was performed using the following search words: cacao, cocoa, chocolate, cadmium and heavy metals. All the results from the databases using all different search word combinations were merged into a general database which was revised by removing duplicate results. After this revision, a total of 429 publications remained. The selection of studies was based on the following criteria:

- (i) *Bean Cd concentrations with indication of origin*: the publication must report Cd concentrations of cacao bean samples with information about the sample origin and the pre-processing before chemical analysis (i.e. fermentation, roasting, peeling, etc.).
- (ii) *Reported quality assurance and quality control protocols*: the article must report the use of suitable certified reference materials (CRM), as well as the percentage of recovery. The CRM should have certified Cd concentrations within reasonable range of the Cd concentrations expected in the samples.
- (iii) *Acceptable limit of quantification (LOQ) for relevant environmental Cd concentrations*: the acceptable LOQ were defined according to the instrument used for measurement, i.e. atomic absorption spectrometer (AAS)  $LOQ \geq 1.0 \text{ mg Cd kg}^{-1}$ ; inductively coupled plasma optical emission spectrometer (ICP-OES)  $LOQ \geq 0.1 \text{ mg Cd kg}^{-1}$ ; atomic absorption spectrometer with graphite furnace (AAS-GF)  $LOQ \geq 0.05 \text{ mg Cd kg}^{-1}$ ; and inductively coupled plasma mass spectrometer (ICP-MS)  $LOQ \geq 0.02 \text{ mg Cd kg}^{-1}$ .
- (iv) *Availability of paired soil and bean Cd information*: paired data were defined as bean and soil Cd measurements from the same tree.

#### *Bean cadmium data*

From a total of 439 publications gathered using the search words, 393 were rejected as their topic did not fit the objective of this review, i.e. experiments with cacao seedlings, chocolate quality, analytical techniques to measure Cd, etc. Only 46 articles passed the first selection criterium. From these 46 publications, 15 publications were rejected as they reported only soil Cd data (5), no bean Cd data (3), no data available online (1), reported Cd concentrations were below the limit of detection (4), or no Cd data was reported but other heavy metals were analysed (2). Thus, 31 publications were used to summarise Cd concentrations in cacao beans across the world (Table 2.1).

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*Paired soil and bean cadmium data*

To analyse the effect of soil properties and agronomic factors on the uptake of Cd in the cacao tree, it was necessary to have paired bean-soil Cd data, i.e. soil and bean samples from the same cacao tree. Starting with 31 publications, 25 of them were rejected as no paired data were reported (16), no CRM was reported (6), reported CRM was not suitable for the expected Cd concentrations (2), or pre-processing of bean samples was unconventional (1).

The selection process resulted in a total of six studies that fulfilled the listed criteria (Table I.1). To avoid data losses, corresponding authors from the selected studies were contacted to gather the original databases. Only for study VI, no contact was made with the authors as most of the information was already published. During the communication to gather the data, authors from study II provided an additional unpublished dataset that fulfilled the selection criteria and was added to this meta-analysis.

### I.1.2 Description of included studies

Study I (Argüello et al. 2019) is a nationwide study conducted in cacao producing areas of Ecuador where 560 paired bean-soil samples were collected. Study II (Barraza et al. 2017) is a localised study conducted in cacao producing areas in the Northern part of the Amazonian region of Ecuador where 31 paired bean-soil samples were collected. Study III (Barraza et al. 2018) is also a localised study conducted in the Northern part of the Amazonian region of Ecuador, focused on areas influenced by oil extraction activities. A total of 15 sites were sampled within study III but only four sites reported paired bean-soil samples, therefore, only these four observations were included in the meta-analysis. Study IV (Barraza et al. 2019) is a pilot field study focused on determining the isotope fractionation in the soil-cacao system. A total of five samples were collected from three farms in the Northern province of Sucumbíos in Ecuador. Study V is a localised study conducted in 15 small scale cacao farms, along the Pacific coast in the Northern part of Ecuador (Esmeraldas province). This study was realised in collaboration with the French cooperative Ethiquable. All cacao trees grew in an agroforestry system without any addition of fertilisers or other treatment. The monitoring of Cd concentrations in topsoils and cacao beans lasted 2 years. The results from study V have not been published. Study VI (Engbersen et al. 2019) is a localised study within the cacao plantation of the research station CEDEC-JAS in Northern Honduras where a total of 60 paired bean-soil samples were collected. Finally, study VII (Gramlich et al. 2018) is a nationwide study conducted in the cacao producing areas of Honduras, where a total of 110 paired bean-soil samples were gathered. Main differences in the methods to

determine pH, soil organic carbon, total soil Cd and bean Cd, as well as the equipment used to measure Cd concentrations, are presented in Table I.1.

## I.2 Standardisation of selected variables

All variables were standardised to the same units before analysis. Some studies collected soil samples at different depths which complicated the analysis and resulted in data losses during the different regressions. To simplify the analysis, two new variables were created, i.e. [Cd Top] and [Cd Sub]. The variable [Cd Top] was defined as the soil Cd concentration at a depth of 0 – 20 cm, whereas [Cd Sub] was defined as the soil Cd concentration at a depth of 20 – 40 cm. For the studies that gathered information in several soil depths (i.e. 0 – 5, 5 – 10, 10 – 20, etc.) a weighted average was used to calculate [Cd Top]. Likewise, to account for differences in applied methods between the studies,  $pH_{\text{water}}$  was converted to  $pH_{\text{CaCl}_2}$  using the equation described by Kissel et al. (2009). Based on a comparison of pH in water with pH in 0.01 M calcium chloride ( $\text{CaCl}_2$ ) for 1186 soil samples, the median difference in pH was 0.67. The pH measured in water was thus slightly higher than the pH measured in 0.01 M  $\text{CaCl}_2$ .

The equation used to convert  $pH_{\text{water}}$  to  $pH_{\text{CaCl}_2}$  proposed by Kissel et al. (2009) reads as follows:

$$pH_{H_2O} = 0.9228 * pH_{CaCl_2} + 1.1008$$

## I.3 Multivariate regression analysis on individual studies

For individual study regression analysis, studies III through V were excluded because of their small sample size. As the selected studies reported different variables, the analysis was based on the soil characteristics considered to be most important for Cd uptake, i.e. pH, SOC, % clay, total soil Cd, Zn, and Mn concentrations, and available soil Cd, Zn and Mn concentrations. Statistical data analysis was performed using JMP® Pro Version 15.1.0 (SAS Institute 2019). All variables were log transformed before analysis, except pH. Statistical significance was established throughout all analysis at P-value < 0.05. Not all the variables were present in all studies and, therefore, the predictors included in each regression varied among studies (Table I.3). For study VII, three different regressions were made because the variables Total Cd Top, Cd Av Top and DGT Cd Top were highly correlated and thus running a single regression including all those variables would violate the assumption of no multicollinearity. For the same reason, two different regressions were made for study II because the variables Total Cd Top, Total Zn Sub and Total Mn Top were significantly correlated. Stepwise regression analysis (forward and backward) was

conducted to select soil variables predicting Cd concentration in beans. The variables were selected based on the maximisation of the adjusted R-square and the minimisation of the Bayesian Information Criterion (BIC). Multivariate regression analysis using ordinary Least Square Model (LSM) was performed using the selected variables for different models. To fulfil the assumption of multivariate regression, the Variation Inflation Factor (VIF) was evaluated for all significant variables in each model.

### I.3.1 Multivariate regression applied to the compiled dataset

A new model was constructed using the compiled data ( $n = 334$ ), focusing on the most important soil variables identified in the multivariate regression analysis of the individual studies (pH, SOC and total soil Cd). The high number of observations in study I created a bias and any new model would thus be dominated by the results of this study. To overcome this issue, an unbiased subset of study I ( $n = 127$ ) was selected using the algorithm described by Ortell et al. (2019), which retains representative averages and standard deviations from the original dataset for the selected variables. As such, an equal number of observations (167) was randomly selected from the data of the two countries available in the dataset (Ecuador: study I,  $n = 127$ , study II,  $n = 28$ , study III,  $n = 4$ , and study V,  $n=8$ ; Honduras: study VI,  $n = 60$  and study VII,  $n = 107$ ).

**Table I.1:** Analytical methods used in the selected studies for soil and plant analysis.

Study	Reference	pH	SOC	Soil*	Leaves*	Beans*	Equipment
I	Argüello et al. (2019)	1 mM CaCl <sub>2</sub> 1:5 w/v water	combustion	Aqua regia	HNO <sub>3</sub>	HNO <sub>3</sub>	ICP-MS
II	Barraza et al. (2017)	1:5 w/v water	combustion	Aqua regia + HF	HNO <sub>3</sub> + H <sub>2</sub> O <sub>2</sub>	HNO <sub>3</sub> + H <sub>2</sub> O <sub>2</sub>	ICP-MS
III	Barraza et al. (2018)	1:5 w/v water	combustion	Aqua regia + HF	HNO <sub>3</sub> + H <sub>2</sub> O <sub>2</sub> + HF	HNO <sub>3</sub> + H <sub>2</sub> O <sub>2</sub> + HF	ICP-MS
IV	Barraza et al. (2019)	1:5 w/v water	combustion	Aqua regia + HF	HNO <sub>3</sub> + H <sub>2</sub> O <sub>2</sub> + HF	HNO <sub>3</sub> + H <sub>2</sub> O <sub>2</sub> + HF	ICP-MS & MC-ICP-MS
V	Maurice & Schreck (unpublished)	1:5 w/v water	combustion	Aqua regia + HF	HNO <sub>3</sub> + H <sub>2</sub> O <sub>2</sub>	HNO <sub>3</sub> + H <sub>2</sub> O <sub>2</sub>	ICP-MS
VI	Engbersen et al. (2019)	Water 1:2 w/v	Walkley & Black	Aqua regia	HNO <sub>3</sub>	HNO <sub>3</sub>	ICP-MS
VII	Gramlich et al. (2018)	water 1:2 w/v	Walkley & Black	HNO <sub>3</sub> + H <sub>2</sub> O <sub>2</sub>	HNO <sub>3</sub> +H <sub>2</sub> O <sub>2</sub>	HNO <sub>3</sub> +H <sub>2</sub> O <sub>2</sub>	AAS & AAS-GF

\* Method used for digestion to determine total elemental concentrations: HNO<sub>3</sub> = Nitric Acid, H<sub>2</sub>O<sub>2</sub> = oxygen peroxide; HF = hydrofluoric acid.

SOC = Soil Organic Carbon; AAS = Atomic Absorption Spectrometry; GF = Graphite Furnace; ICP-OES = Inductively Coupled Plasma Optical Emission Spectrometry; ICP-MS = Inductively Coupled Plasma Mass Spectrometry; MC-ICP-MS = Multi Collector Inductively Coupled Plasma Mass Spectrometry.

**Table I.2:** Average values (min-max) of selected soil characteristics from the topsoil (0 – 20 cm) and subsoil (20 – 40 cm) for each study. A detailed description of the analytical methods used in each study can be found in Table I.1. SOC = Soil Organic Carbon.

Study	Reference	Country	n	pH		SOC (%)		Total Cd (mg kg <sup>-1</sup> )	
				Topsoil	Subsoil §	Topsoil	Subsoil §	Topsoil	Subsoil §
I	Argüello et al. (2019)	Ecuador	560	6.0 (4.1–7.9)	/	2.59 (0.19–13.1)	/	0.44 (0.02–6.90)	/
II	Barraza et al. (2017)	Ecuador	31	5.9 (4.2–7.7)	6.0 (4.4–7.9)	0.86 (0.17–2.41)	0.69 (0.11–4.82)	0.46 (0.19–1.15)	0.40 (0.16–1.02)
III	Barraza et al. (2018)	Ecuador	4	4.9 (4.3–6.0)	5.5 (4.9–6.5)	0.96 (0.49–1.85)	0.32 (0.16–0.58)	0.18 (0.05–0.47)	0.15 (0.08–0.30)
IV	Barraza et al. (2019)	Ecuador	5	6.8 (6.3–7.1)	6.9 (6.9–7.0)	/	/	0.68 (0.26–1.60)	0.33 (0.25–0.40)
V	Maurice & Schreck (unpublished)	Ecuador	15	6.6 (6.1–7.1)	/	1.95 (0.61–3.09)	/	0.55 (0.09–1.19)	/
VI	Engbersen et al. (2019)	Honduras	60	5.2 (4.3–6.5)	/	2.04 (1.03–3.34)	/	0.53 (0.22–1.15)	/
VII	Gramlich et al. (2018)	Honduras	110	5.8 (4.6–7.8)	5.7 (4.6–7.8)	2.30 (0.97–4.56)	1.44 (0.16–2.74)	0.25 (0.02–1.11)	0.16 (0.01–0.82)

§ Dashes indicate that the parameter was not reported for this specific study.

**Table I.3:** Included variables and resulting prediction equation for the regression analysis for each separate study.

Study	Predictors included in the multivariate regression analysis <sup>§</sup>	Prediction equation for bean Cd
I	pH Top, SOC Top, Total Cd Top, Total Zn Top, Total Mn Top	$1.7 + 0.94 * \log_{10}[\text{Total Cd Top}] - 0.21 * [\text{pH Top}] - 0.94 * \log[\text{SOC Top}]$
II a	pH Top, pH Sub, SOC Top, SOC Sub, Total Cd Top, Total Cd Sub, Total Zn Top, Total Zn Sub, Total Mn Top, Total Mn Top	$-3.4 - 0.49 * \log[\text{SOC Sub}] + 1.0 * \log[\text{Total Zn Sub}] + 0.41 * \log[\text{Total Mn Top}]$
II b	pH Top, pH Sub, SOC Top, SOC Sub, Total Cd Top, Total Cd Sub	$-0.056 + 0.93 * \log[\text{Total Cd Top}] - 0.74 * \log[\text{SOC Sub}]$
VI	pH Top, SOC Top, Av Cd Top, Clay Top, Av Zn Top	$1.6 + 0.49 * \log [\text{Total Cd Top}] - 0.21 * [\text{pH Top}]$
VII a	pH Top, pH Sub, SOC Top, SOC Sub, Total Cd Top, Total Cd Sub, Clay Top, Clay Sub, Total Zn Top, Total Zn Sub	$2.2 + 0.84 * \log[\text{Total Cd Top}] - 0.25 * [\text{pH Top}] - 1.2 * \log[\text{SOC Top}]$
VII b	pH Top, pH Sub, SOC Top, SOC Sub, Av Cd Top, Av Cd Sub, Clay Top, Clay Sub, Av Zn Top, Av Zn Sub, Av Mn Top, Av Mn Sub	$1.2 + 0.55 * \log[\text{Av Cd Top}] + 0.49 * \log[\text{Av Zn Sub}] - 0.25 * \log[\text{Av Mn Top}]$
VII c	pH Top, pH Sub, SOC Top, SOC Sub, DGT Cd Top, DGT Cd Sub, Clay Top, Clay Sub, DGT Zn Top, DGT Zn Sub	$0.47 + 0.82 * \log[\text{DGT Cd Top}] - 0.73 * \log[\text{SOC Top}]$

<sup>§</sup> Top = topsoil (0 – 20 cm), Sub = subsoil (20 – 40 cm), Av = available, SOC = soil organic carbon, DGT = diffusive gradient thin film.

**Table I.4:** Soil-plant transfer factors for different crops, including cacao.  $TF_{\text{leaf-soil}} = \text{leaf Cd} / \text{soil Cd}$ ;  $TF_{\text{seed-soil}} = \text{seed Cd} / \text{soil Cd}$ . Additional information to support Table 2.3. Table continued on the following page ( \$ No range available, only average is given).

Transfer factor calculated as concentration ratio					
Plant	TF (leaf – soil)	TF (seed – soil)	n	Reference	Remarks
Cacao	0.34–42.8 (4.46)	0.13–12.5 (1.60)	560	Argüello et al. (2019)	Ecuador Soil Cd: 0.025 – 6.90 (0.44 average) mg kg <sup>-1</sup> , pH: 6.02
	0.56–20.1 (3.39)	0.26–7.84 (1.96)	28	Barraza et al. (2017)	Ecuador Soil Cd: 0.05 – 1.60 (0.46 average) mg kg <sup>-1</sup> , pH: 6.01
	1.88–29.2 (4.93)	1.44–29.8 (5.62)	60	Engbersen et al. (2019)	Honduras Soil Cd: 0.22 – 1.15 (0.53 average) mg kg <sup>-1</sup> , pH: 5.15
	1.9–21.1 (7.1)	0.5–16.3 (2.7)	108	Gramlich et al. (2018)	Honduras Soil Cd: 0.017 – 1.11 (0.25 average) mg kg <sup>-1</sup> , pH: 5.77
Willow	1.6–10.3		8	Van Slycken et al. (2013)	France Soil Cd 6.5 ± 0.8 mg kg <sup>-1</sup> ; soil Zn 377 ± 69 mg kg <sup>-1</sup> ; pH 6.6 ± 0.2
Oak	0.26 \$		2	Sevel et al. (2009)	Two oaks in tree forest ecosystem with soil Cd A horizon (0 – 35 cm) 0.15 mg kg <sup>-1</sup> , pH 5.2.
Poplar	5.0–40.0		13	Laureysens et al. (2004)	Short rotation coppice culture on a former waste disposal site. Soil Cd 0.05 – 1.60 mg kg <sup>-1</sup> . South China.
Leaf vegetables	0.001–4.5 (0.189)		170	Zhang et al. (2014)	Soil Cd: 0.012 – 1.18 mg kg <sup>-1</sup> Austria.
Wheat	0.06–0.64 (0.27)	0.03–0.034 (0.015)	40	Puschenreiter and Horak (2000)	Soil Cd: 0.13 – 0.44 (0.25 mean) mg kg <sup>-1</sup> Austria.
Rye	0.03–0.29 (0.11)	0.01–0.16 (0.04)	40	Puschenreiter and Horak (2000)	Soil Cd: 0.13 – 0.44 (0.25 mean) mg kg <sup>-1</sup> USA
Sunflower		1.76–3.4 (2.19)	200	Li et al. (1995)	Soil Cd 0.39 mg kg <sup>-1</sup> .

Table I.4 continued

Transfer factor calculated as concentration ratio					
Plant	TF (leaf - soil)	TF (seed - soil)	n	Reference	Remarks
Cotton		0.46 §	6	Chen et al. (2015)	Soil Cd $0.26 \pm 0.04$ mg kg <sup>-1</sup> ; TF calculated based on average seed Cd and average soil Cd.
Pistachio		0.01-0.04 (0.03)	220	Shirani et al. (2018)	Iran Soil Cd: 0.61 - 2.9 (1.6 average) mg kg <sup>-1</sup> , pH: 7.9
Transfer factor calculated from predicted crop Cd concentration model based on soil properties <sup>£</sup>					
Plant	TF (seed - soil)	Prediction model	n	Reference	Remarks
Cacao	1.76	$\log[\text{Bean Cd}] = 1.34 + 0.86 \log[\text{Total Soil Cd}] - 0.18 [\text{soil pH}] - 0.25 \log[\text{SOC}]$	334	Chapter 2 §	Ecuador and Honduras
Indica rice	0.76	$\log[\text{Grain Cd}] = 1.20 + 0.76 \log[\text{Cd Soil}] - 0.17 [\text{Soil pH}] - 0.32 \log[\text{CEC}]$	1043	Römkens et al. (2009)	Taiwan. Soil Cd ranges from 0.1 mg kg <sup>-1</sup> to 30 mg kg <sup>-1</sup> (i.e. from background to heavily polluted soils)
Japonica rice	0.30	$\log[\text{Grain Cd}] = 0.97 + 0.74 \log[\text{Cd Soil}] - 0.18 [\text{Soil pH}] - 0.43 \log[\text{CEC}]$	1043	Römkens et al. (2009)	Taiwan. Soil Cd ranges from 0.1 mg kg <sup>-1</sup> to 30 mg kg <sup>-1</sup> (i.e. from background to heavily polluted soils)
Wheat	0.27	$\log[\text{Grain Cd}] = 0.12 + 0.43 \log[\text{Cd Soil}] - 0.16 [\text{Soil pH}]$	246	Adams et al. (2004)	USA

§ No range available, only average is given

§ See paragraph 2.2.2.

£ Transfer factors were calculated with median values from studies I-VII (see above). pH 5.99, CEC 17.84 cmol<sub>c</sub> kg<sup>-1</sup>, soil Cd 0.33 mg kg<sup>-1</sup>.

#### I.4 Power analysis to test the effect of mitigation strategies

It is essential to test mitigation strategies in field conditions because cacao is a perennial tree. However, there is scarce experience testing the effect of soil amendments on the reduction of Cd uptake in perennial trees. A statistical power test can be used to estimate the minimum sample size needed to identify a significant treatment effect on bean Cd. In order to calculate such power analysis, it is necessary to know the standard deviation in the population of interest, as well as the plausible effect size of the applied treatments. Argüello et al. (2019) reported an average coefficient of variation of 39 % for bean Cd within 157 different cacao fields in Ecuador, with a 90<sup>th</sup> percentile of 70 %. Bean Cd data in that study were log-normally distributed and a power analysis for mitigation strategies can be readily calculated using the standard deviation of log-transformed bean Cd values. The standard deviation of log-transformed bean Cd values varied between 0.0027 and 0.53, with an average of 0.18 and a 90<sup>th</sup> percentile of 0.32. Assuming that the effect of any treatment would be a certain reduction factor (RF), defined as the ratio of the bean Cd concentration in the treatment over the bean Cd concentration in the control, a power analysis can be calculated. Table I.5 shows the results of a power analysis for a scenario with average bean Cd variability and a scenario with high bean Cd variability, as well as differences in the size of replicates, i.e. replicates corresponding to individual trees or replicates corresponding to plots of five or ten trees. All analyses were performed using the online tool developed by Rollin Brant [university of California (<https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html>)]. All data were rounded to two significant digits to maintain consistency in the calculations. To estimate the reduction in standard deviation due to increment of the number of trees per replicate, the following equation was used:

$$Stdev_{plot} = \frac{Stdev_{population}}{\sqrt{n}}$$

**Table I.5** Statistical power test to calculate the minimum number of replicates required to identify a significant effect of treatment in cacao field trials (with 80 % power and 5 % type I error, one side comparison of two averages and equal sample size in both) as affected by the variance and expected treatment effect on bean Cd concentration expressed as a reduction factor (RF).

RF	Number of replicates needed according to the expected factor reduction					
	>2.0*	>1.5*	>1.2*	>2.0**	>1.5**	>1.2**
Replicate = 1 tree	5	13	63	15	40	198
Replicate = 5 trees	1	3	13	3	8	38
Replicate = 10 trees	1	2	7	2	4	20

\* Assumed Stdev= 0.18 [average Stdev log(bean Cd) within fields in Ecuador]

\*\* Assumed Stdev = 0.32 [90<sup>th</sup> percentile of Stdev log(bean Cd) within fields in Ecuador]

### I.5 Calculations on cacao mixing in Ecuador

Data from the national agricultural land use map for Ecuador (Sistema de Información Pública Agropecuaria 2016) was combined with the map of spatial distribution of bean Cd in Ecuador reported by Argüello et al. (2019) to obtain an estimate for the average cacao bean Cd concentration in each province adjusted for province yield. Specifically, all the geographic sectors identified as cacao plantations from satellite data were singled out and the Cd distribution map reported by Argüello et al. (2019) was used to obtain expected bean Cd concentrations for each of their centroid coordinates. Assuming each sector had the same bean Cd concentration as its centroid, these values were then used to calculate an average for each province, weighted by plantation area. To calculate the national average bean Cd in a mixing scenario, these provincial averages were combined with the 5-year (2014 – 2019) average annual cacao production per province as reported in the national survey of continuous agricultural production and surface area (Instituto Nacional de Estadística y Censos - Gobierno de La República del Ecuador 2019). Provinces without reported cacao production during this five-year period, or for which no average bean Cd concentration was available in the study of Argüello et al. (2019), were excluded. This resulted in a total of 20 provinces with 5-year average annual production ranging 69 – 54566 metric tonnes and provincial average bean Cd concentrations ranging 0.26 – 1.41 mg Cd kg<sup>-1</sup>. While this short calculation can provide a first approximation for the average bean Cd in each province, it ignores any potential variability due to different cacao cultivars and harvesting seasons. Furthermore, this estimate assumes that any later expansion in cacao plantation areas is uniformly distributed within each province, so that the Cd average remains unchanged. More precise estimates can be obtained by accounting for these variables in future calculations.

## Appendix II: From soil to cacao bean: Unravelling the pathways of cadmium translocation in a high Cd accumulating cultivar of *Theobroma cacao* L.

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### II.1 Principle and procedure phytate analyses

The phytate content in the nibs was determined in triplicate with a phytic acid assay (McKie and McCleary 2016). Twenty mL 0.66 M HCl was added to ground cacao nibs (500 mg  $\pm$  20 mg) and incubated for 16h in an end-over-end shaker (150 rpm, 20°C). The extract was centrifuged, and the supernatant was neutralized by addition of NaOH (0.75 M). Two aliquots were taken from the supernatant, one for a 'total P' reaction and one for the 'free P' reaction. In the total P, an excess of phytase and alkaline phosphatase was added, for the breakdown of polyphosphates to monophosphate. Phosphate was measured in both aliquots colorimetrically by adding a color reagent solution consisting of ammonium molybdate, ascorbic acid, and sulfuric acid (Murphy and Riley 1962) [measurement at a wavelength of 655 nm using a UV/VIS spectrophotometer (Lambda 25, PerkinElmer, Waltham, MA, USA)]. The concentration of bound P in the supernatants was obtained by subtracting free P concentration from the total P concentration. The bound P was then converted to the amount of phytate under the assumption that the measured P released from enzymatic hydrolysis exclusively originates from phytic acid.

## II.2 Tables

**Table II.1:** Quality control of elemental analyses. The measured concentration, certified and indicative values are expressed in mg kg<sup>-1</sup> DW<sup>-1</sup>. Fe and Cr showed poor recovery results and were not considered in further analyses.

Sample type	Description		K	Ca	Cr	Cu	Mn	Ni	Pb	Zn	Cd	P	S	Mg	Fe
Soil TM3	Internal	Indicative value	2518.70	2125.60	26.17	11.10	441.64	12.31	19.36	44.81	0.31	559.82	-	2291.65	16465.45
	KULeuven	Measured conc.	2505.24	2096.63	26.06	10.67	432.80	12.58	18.38	42.16	0.28	501.94	119.29	2377.50	16007.59
	soil standard	SD <sup>a</sup>	38.25	50.77	1.01	0.53	30.74	0.71	0.78	4.29	0.03	20.83	0.39	40.34	439.92
	(n=4) <sup>c</sup>	<b>Recovery digestion (%)<sup>b</sup></b>	<b>99</b>	<b>99</b>	<b>100</b>	<b>96</b>	<b>98</b>	<b>102</b>	<b>95</b>	<b>94</b>	<b>91</b>	<b>90</b>	-	<b>104</b>	<b>97</b>
Baking chocolate	NIST 2384 (n=2) <sup>c</sup>	Certified value	8650.00	840.00	-	23.90	20.80	-	-	37.60	0.07	3330.00	-	2610.00	132.00
		Measured conc.	8666.07	899.61	0.71	24.08	20.30	6.07	-	36.13	0.07	3368.30	1428.44	2702.26	22.32
		SD <sup>a</sup>	46.99	22.29	0.04	0.20	0.16	0.59	-	0.22	0.00	26.48	10.69	20.39	0.63
		<b>Recovery digestion (%)<sup>b</sup></b>	<b>100</b>	<b>107</b>	-	<b>101</b>	<b>98</b>	-	-	<b>96</b>	<b>89</b>	<b>101</b>	-	<b>104</b>	<b>17</b>
Tomato leaves	NIST 1573a (n=2) <sup>c</sup>	Certified value	27000.00	50500.00	1.99	4.70	246.00	1.59	-	30.90	1.52	2160.00	9600.00	12000.00	368.00
		Measured conc.	26855.00	50664.44	1.65	4.76	238.60	1.61	0.52	28.72	1.42	2321.52	9903.57	11121.44	310.99
		SD <sup>a</sup>	469.30	700.35	0.00	0.04	2.35	0.18	0.00	0.07	0.02	27.55	163.37	199.93	3.71
		<b>Recovery digestion (%)<sup>b</sup></b>	<b>99</b>	<b>100</b>	<b>83</b>	<b>101</b>	<b>97</b>	<b>102</b>	-	<b>93</b>	<b>94</b>	<b>107</b>	<b>103</b>	<b>93</b>	<b>85</b>

<sup>a</sup>SD= standard deviation of the mean

<sup>b</sup>equal to [measured concentration]/[reference concentration]\*100

<sup>c</sup>number of processing replicates (individually digested, and measured)

**Table II.2:** Quality control of Cd isotope measurements

Sample Type	Description	Reference Cd conc		Measured Cd conc <sup>a</sup>			Recovery digestion <sup>b</sup>	Purification yield <sup>c</sup>		Ref Cd isotope ratio $\delta^{114/110}\text{Cd}$ (‰)		Measured Cd isotope ratio $\delta^{114/110}\text{Cd}$ (‰)		
		mean	SD	mean	SD	n <sup>d</sup>	mean	mean	SD	mean	SD	mean	SD	n
white cabbage	BCR-679	1.66	0.07	1.70	0.06	6	101	100	20	0.22 <sup>e</sup>	0.02	0.18	0.03	10
soil	NIST S Joaquim	0.36	0.01	0.35	0.07	4	93	89	5	-0.22	0.02	-0.22	0.04	4
Münster Cd	isotope standard	-	-	-	-	-	-	-	-	4.49 <sup>f</sup>	0.05	4.43	0.06	5

<sup>a</sup>measured in unpurified samples,

<sup>b</sup>equal to [measured concentration]/ [reference concentration]\*100,

<sup>c</sup>equal to [concentration purified sample]/[concentration non purified sample]\*100,

<sup>d</sup>number of processing replicates (individually digested, purified for isotope analyses, and measured for concentrations and isotope ratios),

<sup>e</sup>value obtained by Wiggerhauser et al. 2021,

<sup>f</sup>values obtained by Cloquet et al. 2005, Pallavicini et al. 2014 and Liu et al. 2019.

**Table II.3:** Calculation of Cd mass balances for cacao trees

<b>Tissue</b>	<b>Replicate</b>	<b>n**</b>	<b>Mass (kg)</b>	<b>Mass fraction</b>	<b>Total Cd (mg)</b>	<b>Cd fraction</b>
Roots	T2	1	31.50 <sup>\$</sup>	0.24	86.17	0.14
	T4	1	31.50 <sup>\$</sup>	0.24	84.15	0.13
	T6	1	31.50 <sup>\$</sup>	0.24	132.84	0.18
Stem <sup>#</sup>	T2	1	22.50 <sup>\$</sup>	0.17	130.98	0.21
	T4	1	22.50 <sup>\$</sup>	0.17	132.98	0.21
	T6	1	22.50 <sup>\$</sup>	0.17	148.92	0.20
Branch	T2	1	61.90 <sup>\$</sup>	0.47	360.34	0.58
	T4	1	61.90 <sup>\$</sup>	0.47	365.83	0.57
	T6	1	61.90 <sup>\$</sup>	0.47	409.70	0.56
Leaves IF2	T2	1	7.75 <sup>\$</sup>	0.06	23.40	0.04
	T4	1	7.75 <sup>\$</sup>	0.06	36.96	0.06
	T6	1	7.75 <sup>\$</sup>	0.06	20.77	0.03
Leaves IF3	T2	1	7.75 <sup>\$</sup>	0.06	22.47	0.04
	T4	1	7.75 <sup>\$</sup>	0.06	27.07	0.04
	T6	1	7.75 <sup>\$</sup>	0.06	22.33	0.03
Pod husk	T2	1	0.027 <sup>*</sup>	<0.01	0.10	<0.01
	T4	2	0.023 <sup>*</sup>	<0.01	0.07	<0.01
	T6	2	0.024 <sup>*</sup>	<0.01	0.06	<0.01
Placenta	T2	1	0.001 <sup>*</sup>	<0.01	<0.01	<0.01
	T4	2	0.001 <sup>*</sup>	<0.01	<0.01	<0.01
	T6	1	0.001 <sup>*</sup>	<0.01	<0.01	<0.01
Testa	T2	1	0.003 <sup>*</sup>	<0.01	0.01	<0.01
	T4	2	0.002 <sup>*</sup>	<0.01	<0.01	<0.01
	T6	1	0.002 <sup>*</sup>	<0.01	<0.01	<0.01
Nib	T2	1	0.011 <sup>*</sup>	<0.01	0.02	<0.01
	T4	2	0.014 <sup>*</sup>	<0.01	0.03	<0.01
	T6	2	0.014 <sup>*</sup>	<0.01	0.03	<0.01
<b>Fruit</b>	T2	1	0.042	<0.01	0.13	<0.01
	T4	2	0.04	<0.01	0.10	<0.01
	T6	2	0.045	<0.01	0.10	<0.01
<b>Tree</b>	T2	1	131.44	1	623.36	1
	T4	1	131.48	1	646.99	1
	T6	1	131.47	1	734.56	1

\*The masses of the roots, stem, branches and leaves were estimated based on the biomass distribution reported by (Nurafiza *et al.*, 2017).

\*The trees in this study were sampled out of cacao peak fruiting season, hence the authors did not consider fruits in the biomass distribution. Therefore, to include the reproductive tissues, the dry weights of the sampled fruits in this study were added to calculate the Cd mass distribution in the total trees.

#As no measurement of the stem was done, the same Cd concentration as in the branches was assumed. \*\* Some trees contained two fruits, others only one.

$$\text{Mass fraction} = \text{mass}_{\text{tissue}} / \text{mass}_{\text{tree}}$$

$$\text{Total Cd (mg)} = \text{mass}_{\text{tissue}} (\text{kg}) * \text{Cd}_{\text{tissue}} (\text{mg kg}^{-1})$$

$$\text{Cd fraction} = \text{total Cd}_{\text{tissue}} / \text{total Cd}_{\text{tree}}$$

**Table II.4:** *p*-values of statistical tests performed to verify the assumptions of ANOVA and *p*-value for ANOVA tests.

X	Y	Shapiro-Wilk	O'Brien	Brown-Forsythe	ANOVA
Tissue	Cd (mg kg <sup>-1</sup> )	0.11	0.07	0.07	<0.0001
Tissue	δ <sup>114/110</sup> Cd (‰)	0.14	0.29	0.46	<0.0001
Tissue	log (Zn/Cd) (mM)	0.76	0.46	0.79	<0.0001
Tissue	log (Mn/Cd) (mM)	0.30	0.17	0.70	<0.0000
Tissue	log (Ca/Cd) (mM)	0.21	0.62	0.91	<0.0001
Tissue	log (Mg/Cd) (mM)	0.60	0.16	0.59	0.0014
Tissue	log (S/Cd) (mM)	0.18	0.57	0.80	<0.0001
Tissue	log (K/Cd) (mM)	0.51	0.66	0.89	<0.0001
Tissue	log (Cu/Cd) (mM)	0.61	0.20	0.36	<0.0001
Tissue	log (P/Cd) (mM)	0.00	0.08	0.39	<0.0001

**Table II.5:** Soil properties of the soil of the accession plot of NA 312 ( $n=3$ ).

		Topsoil		Subsoil	
		mean	SD	mean	SD
pH		<b>4.13</b>	0.11	<b>3.74</b>	0.06
Organic Carbon	%	<b>1.86</b>	0.36		
Aqua regia soil Cd	mg/kg	<b>0.22</b>	0.05	<b>0.06</b>	0.01
Available Cd*	mg/kg	<b>0.08</b>	0.02	<b>0.03</b>	0.01
Total Cd**	mg/kg	<b>0.28</b>	0.05		
eCEC	cmol <sub>c</sub> /kg	<b>11.65</b>	0.12	<b>11.16</b>	0.31
Oxalate extractable Al	g/kg	<b>1.00</b>	0.05	<b>1.01</b>	0.11
Oxalate extractable Fe	g/kg	<b>4.86</b>	0.20	<b>3.20</b>	0.89
Oxalate extractable Mn	g/kg	<b>0.83</b>	0.11	<b>0.45</b>	0.22

\*Ca(NO<sub>3</sub>)<sub>2</sub>-extract: 0.05 M Ca(NO<sub>3</sub>)<sub>2</sub> (5 g soil: 50 mL Ca(NO<sub>3</sub>)<sub>2</sub>)

\*\* Total digestion (aqua regia with subsequent HF digestion)

The soil at the ICGT was previously characterized as a clayey loam soil (Lewis et al., 2018). The measured pH was around the minimum reported in a survey of cacao plantations in Ecuador (4.1-7.9, mean: 6.0) (Argüello et al., 2019). Organic carbon content was on the lower end of the range, while ECEC, oxalate extractable Al, Fe and Mn followed the average trends found in the studied Ecuadorian farms.

**Table II.6:** Mean and SD of concentrations of nutrients in the various tissues (DW).

	<b>Zn (mg kg<sup>-1</sup>)</b>		<b>Mn (mg kg<sup>-1</sup>)</b>		<b>Ca (g kg<sup>-1</sup>)</b>		<b>Mg (g kg<sup>-1</sup>)</b>	
	mean	SD	mean	SD	mean	SD	mean	SD
roots	46.80	10.24	101.10	54.52	7.61	1.08	3.56	2.16
branch	139.20	25.15	465.10	114.25	20.86	8.16	5.54	2.14
pod husk	71.40	6.92	308.40	134.74	4.98	1.67	3.15	0.65
placenta	33.40	4.79	58.70	35.09	1.51	0.30	1.59	0.44
testa	36.10	6.87	99.70	28.09	4.53	1.69	2.86	0.53
nib	46.70	4.90	93.60	24.77	1.24	0.22	3.51	0.27
leaves IF2	183.80	21.52	1776.90	287.47	16.12	2.31	8.54	0.46
leaves IF3	177.60	15.65	1863.60	306.21	17.33	1.78	8.53	0.56

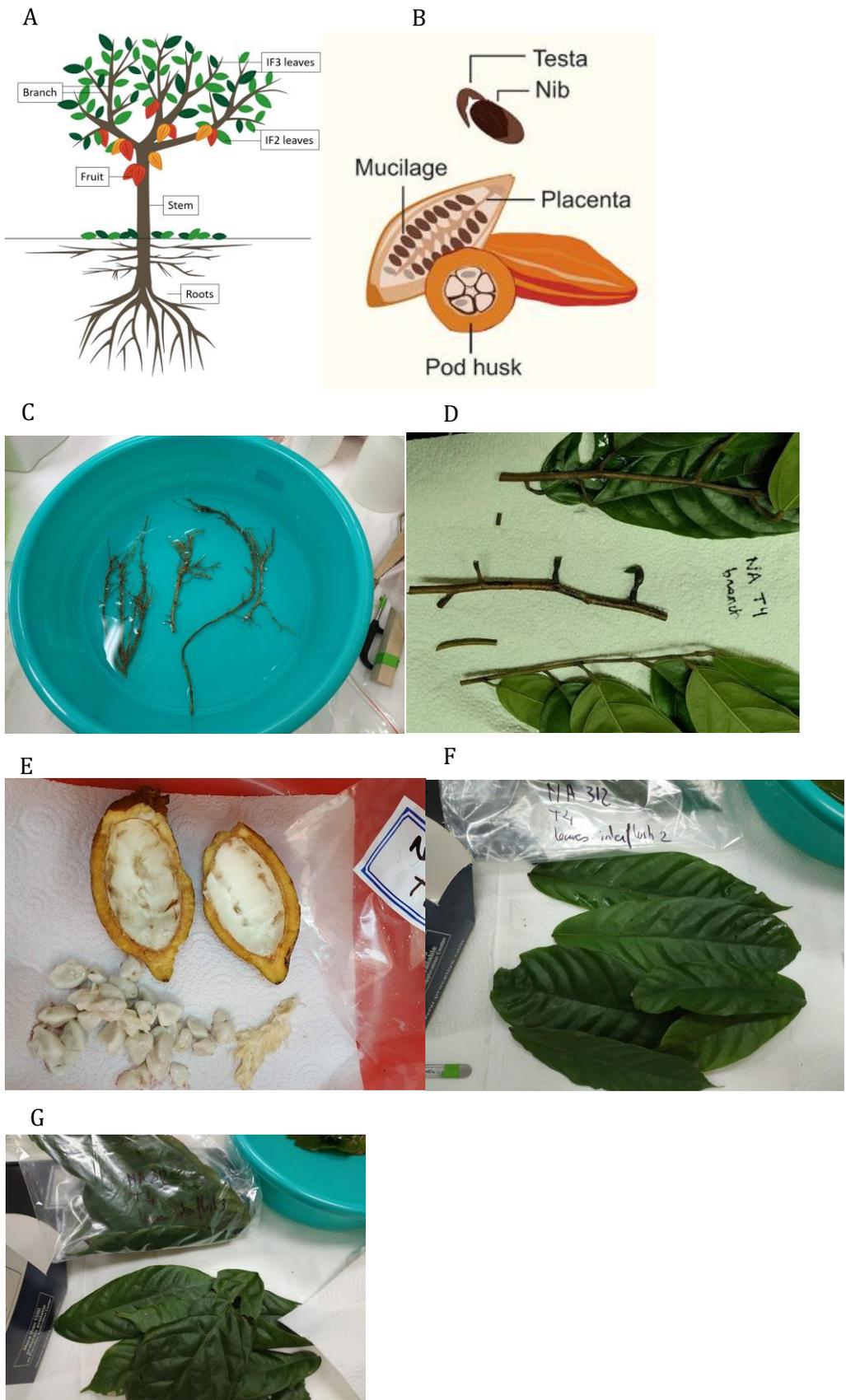
  

	<b>P (g kg<sup>-1</sup>)</b>		<b>Cu (mg kg<sup>-1</sup>)</b>		<b>S (g kg<sup>-1</sup>)</b>		<b>K (g kg<sup>-1</sup>)</b>	
	mean	SD	mean	SD	mean	SD	mean	SD
roots	0.99	0.78	9.80	3.70	1.69	0.33	5.44	0.87
branch	1.04	0.40	6.50	1.44	1.00	0.21	10.12	3.82
pod husk	1.18	0.13	10.10	1.59	1.27	0.08	28.57	5.20
placenta	1.47	0.10	9.60	3.96	1.38	0.27	15.71	3.12
testa	0.99	0.17	11.50	1.54	1.17	0.07	10.46	0.37
nib	4.24	0.53	16.80	1.82	1.88	0.09	9.50	2.19
leaves IF2	1.39	0.08	4.60	0.25	2.32	0.09	11.58	2.01
leaves IF3	1.17	0.14	3.80	0.26	1.93	0.08	8.35	2.09

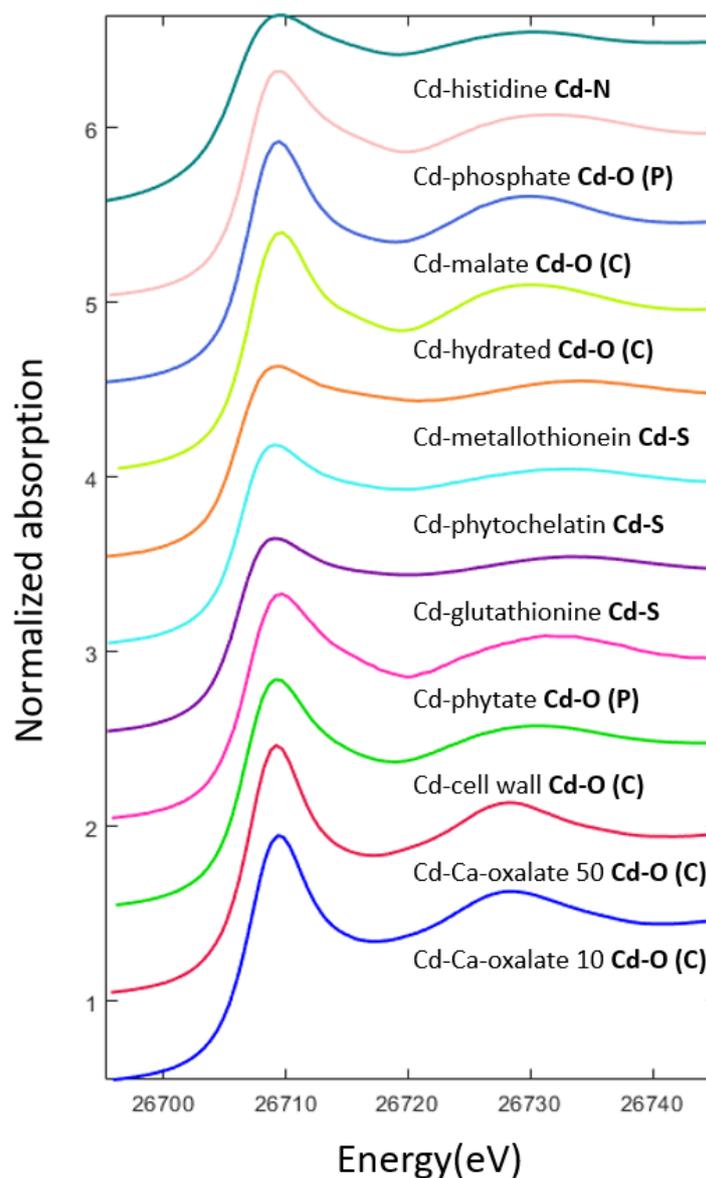
**Table II.7:** R<sup>2</sup> and F-test for the linear correlation between the log transform of bulk Cd concentrations and nutrients in the various organs (Figure II.3), all values measured are shown for the n=3 trees.

<b>Element</b>	<b>R<sup>2</sup></b>	<b>F(1,28)</b>	<b>p-value</b>	<b>equation</b>
<b>log Zn</b>	0.38	17.41	0.0003	3.49+0.78x
<b>log Mn</b>	0.38	17.31	0.0003	3.97+1.54x
<b>log Ca</b>	0.62	46.52	<0.0001	7.01+1.62x
<b>log Mg</b>	0.41	19.8	<0.0001	7.55+0.82x
<b>log P</b>	0.00	1.12	0.29	7.46-0.27x
<b>log Cu</b>	0.10	3.22	0.08	2.45-0.31x
<b>log S</b>	0.01	0.14	0.72	7.35-0.038x
<b>log K</b>	0.01	2.72	0.11	9.64-0.30x

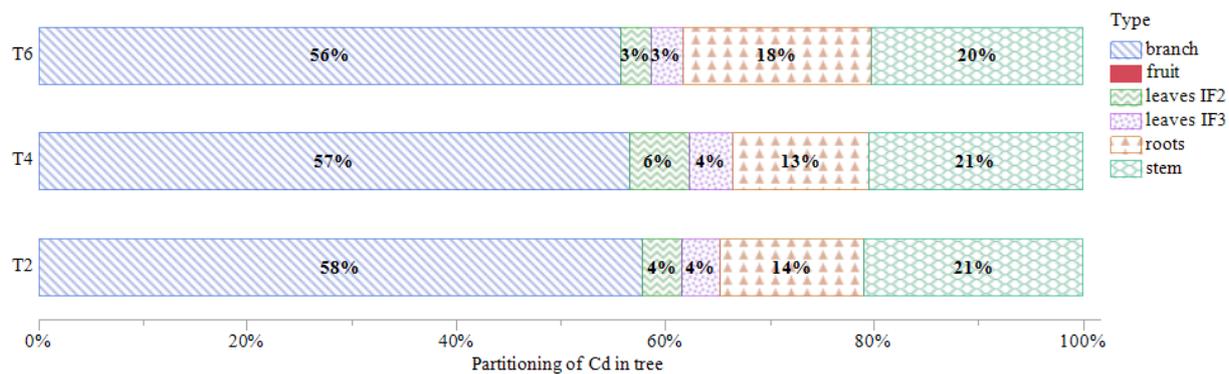
II.3 Figures



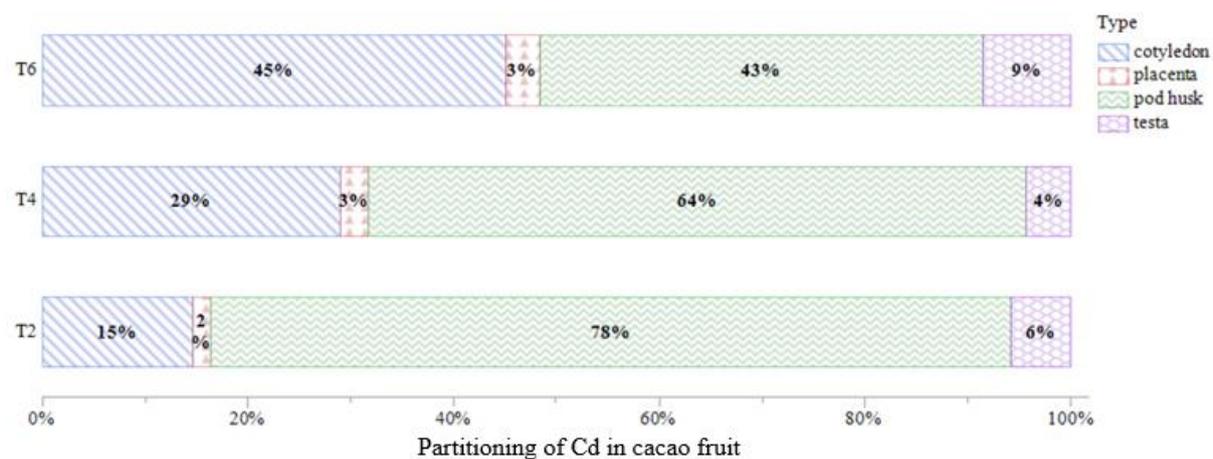
**Figure II.1:** (A) Terminology of the sampled organs of the cacao tree. (B) Tissues in a cacao fruit (Vanderschueren *et al.*, 2021). The cacao fruit or pod is made up of a large woody outer pod husk, filled with 20–50 cacao beans (seeds). The cacao bean consists of a nib and an outer shell, the testa. The nib is the only part of the cacao bean that is retained during chocolate processing. (C) Example of roots collected (diameter 1-10 mm). (D) Example of branch sampled (diameter 3-7 mm). (E) Example of fruit tissues sampled. (F) Example of IF2-leaves. (G) Example of IF3 leaves.



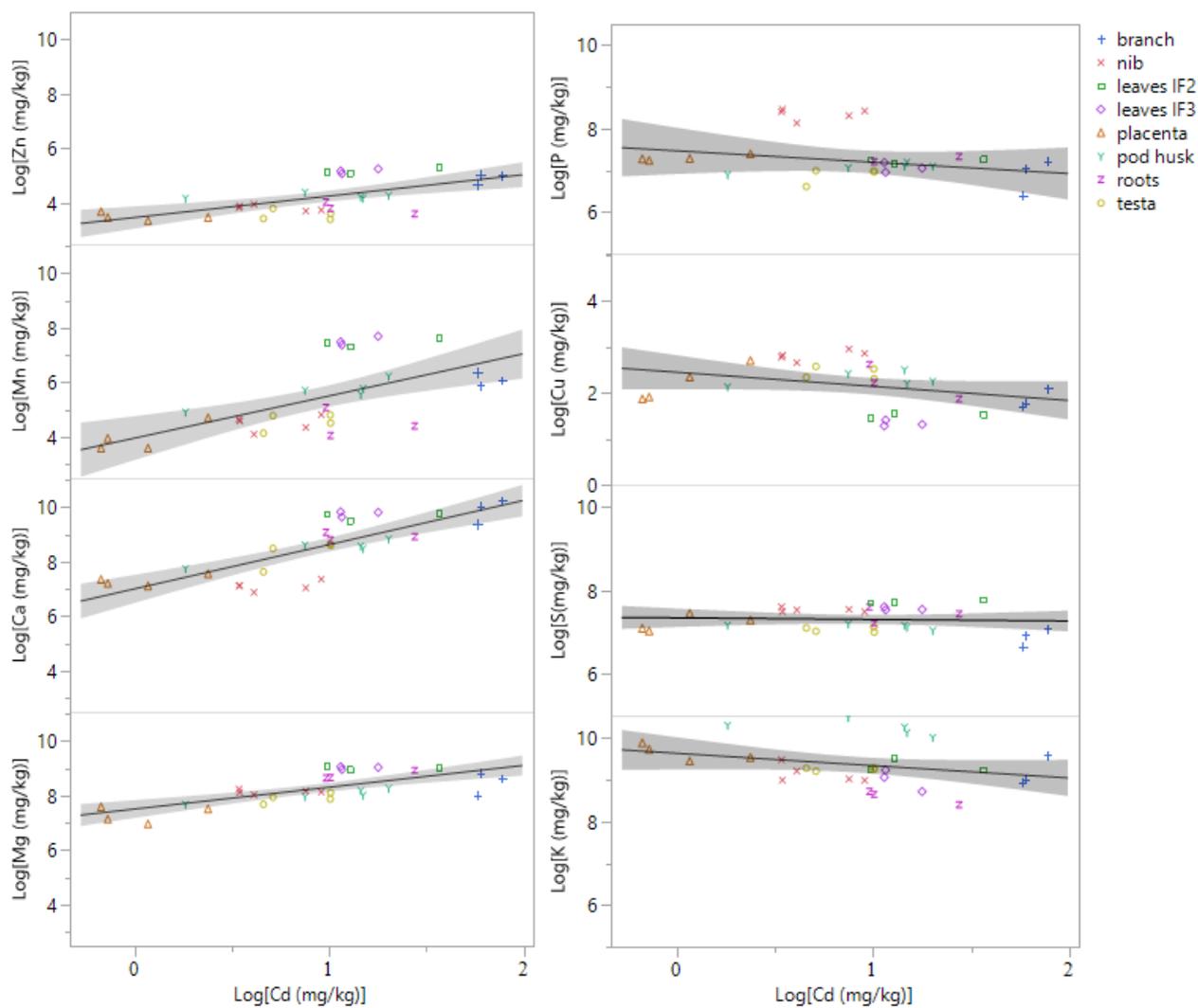
**Figure II.2:** Cd K-edge XANES spectra of selected reference materials. A previously recorded database of Cd reference spectra recorded at 15 to 20K was used, containing Cd-cell wall, Cd-glutathione, Cd-phytochelatin, Cd-hydrated, Cd-malate, Cd-histidine, Cd phosphate. The preparation and measurements of this dataset is described in (Huguet *et al.*, 2012, 2015). As additional standards, Cd-metallothionein extracted from mussels (Bataille *et al.*, 2005), Cd phytate (phytate/Cd molar ratio = 5), Cd-Ca-oxalate (Ca-oxalate/Cd molar ratio = 10 and 50) (McBride *et al.*, 2017) were measured at BM30 and included in the database.



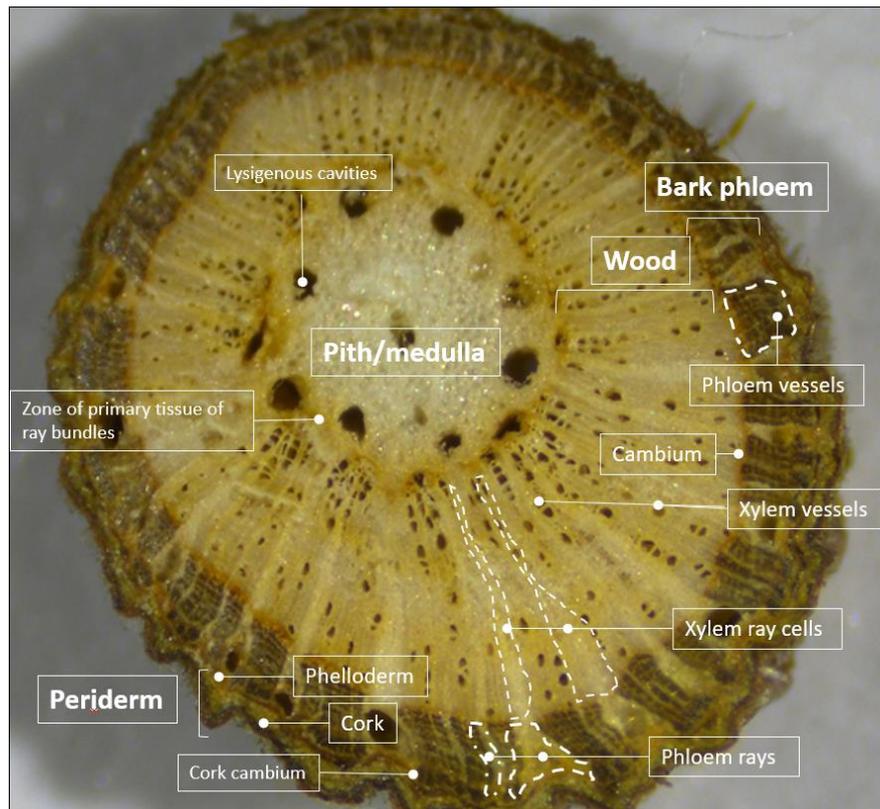
**Figure II.3:** Partitioning of Cd in the three trees assuming a tree biomass as described by (Nurafiza *et al.*, 2017). Assumed biomass of the different tissues: roots 31.5 kg, stem: 22.5 kg, branch: 61.9 kg, IF2 leaves: 7.75 kg, IF3 leaves: 7.75 kg, fruit: 0.05 kg



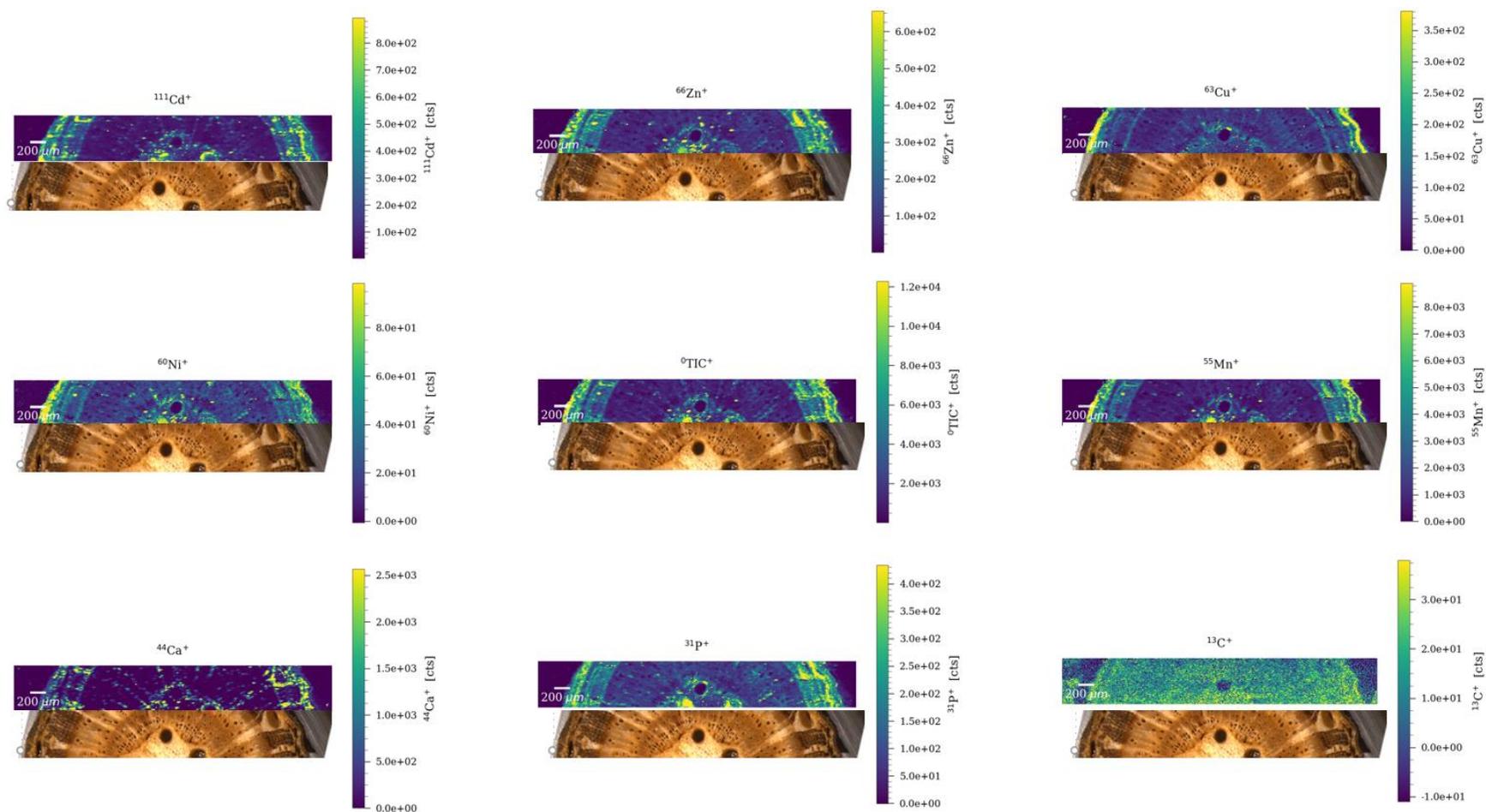
**Figure II.4:** Partitioning of Cd in the cacao fruit of NA312.



**Figure II.5:** Relationships between the log transform of Cd concentrations and nutrients in the various organs, all values measured are shown for the  $n=3$  trees. The grey zone is the confidence region for the linear fit ( $\alpha = 0.05$ , Table 3.4). Left: Nutrients in group I correlate positively with Cd, Right: Group II elements correlate negatively with Cd in the various tissues.



**Figure II.6.** Anatomy of transverse cross section of cacao branch (NA T2). The pith in the center is surrounded by the wood, which is enclosed by the bark phloem and the cork.



**Figure II.7:** Microscope image and LA-ICP-MS elemental maps of cross section of branch NA T4. The intensity is expressed in total counts (cps). The color scale is adjusted to the range of each element. The trends were the same as observed in NA T2. Cd, Ca, Mn and Zn co-localized, and less spatial correlation was found between Cd and P or C

## Appendix III: Cadmium uptake, and translocation in mature cacao trees: the effects of cultivar and grafting

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### III.1 Parameters for $\mu$ XRF measurement

Transversal thin sections (1-5 mm) of roots and branches were prepared by cryosectioning small frozen intact roots embedded in OCT, with a cryomicrotome (Leica RM2265/LN22). Samples were transferred to the sample holder immediately after cutting and kept frozen during measurement. The X-ray beam was monochromatized with a Si(111) double-crystal monochromator and focused using Kirkpatrick-Baez mirrors on the sample with a lateral resolution of 0.6 mm (V) x 0.8 mm (H). The incident energy of the photon beam was 3.55 keV. Measurements were done in vacuum and in cryogenic conditions (110°K) using a liquid N<sub>2</sub> passively cooled cryostat. The fluorescence signal was collected with a Silicon Drift Diode (SDD) XRF detector (SGX Sensortech with an active area of 80 mm<sup>2</sup>) and normalized by the incident photon intensity.

### III.2 Procedure to build Cd $\mu$ XRF maps

In a normal procedure, a batch fitting file is created which attributes each XRF peak to an element based on its respective emission lines and defines the corresponding ROIs (Castillo-michel *et al.*, 2017). However, the batch fitting routine with the model implemented in the PyMCA software, was not able to fit two background contributions, which were attributed to the sample matrix and to the K inelastic scattering. As a consequence, the batch fitting file was not able to fit the Cd peak well (at 3537.6 eV). On the one hand, in the Sr-hotspots there was a contribution on the left side of the peak which was originating from background scatter (related to the matrix of the Ca-crystals) (Figure III.2 A). On the other hand, in the vessel structures, there was a contribution on the right side of the peak originating from K inelastic scatter (Figure III.2 B). To extract only the Cd peak, we defined manually three gaussians (Figure III.3). The first gaussian represents the background scatter from the crystals. The second gaussian is the real Cd signal and the third gaussian is the inelastic scatter. By fitting the three gaussians manually, we could extract the second gaussian and build the Cd XRF maps with this peak.

### III.3 Parameters for nXRF measurement

The beam was focused using Kirkpatrick-Baez mirrors down to 50 x 50 nm<sup>2</sup>. The fluorescence emission from the sample was recorded using two 3-element SDD arrays positioned at 13° from the sample. High resolution maps were recorded at room temperature, raster scanning the sample

in the X-ray focal plane with  $100 \times 100 \text{ nm}^2$  step size and 500 ms dwell time/pixel. The hyperspectral images were analyzed with the PyMCA software package.

### III.4 Tables

**Table III.1:** Sampling scheme and samples selected for image analyses on the samples. Some tissues were not sampled, as they were unavailable at the time of the sampling.

Cultivar: Biological reps.:	High			Low			Grafted		
	T2	T4	T6	T1	T3	T15	T1	T2	T3
Roots	1	1 <sup>\$</sup>	1 <sup>\$</sup>	1	1	1	1	1	1
Branches	1*	1 <sup>&amp;*</sup>	1 <sup>&amp;</sup>	1	1*	1*	1	1	1
Pod husk									
immature	0	0	1	1	1	0	0	0	1
intermediate	0	1	0	1	0	1	0	0	0
mature	1	1	1	0	1	1	0	0	0
Testa									
immature	0	0	0	1	0	0	0	0	1
intermediate	0	1	0	1	0	1	0	0	0
mature	1	1	1	0	1	1	0	0	0
Nib									
immature	0	0	1	1	1	0	0	0	1
intermediate	0	1	0	1	0	1	0	0	0
mature	1	1	1	0	1	1	0	0	0
Leaves									
F2	0	0	0	0	1	1	0	0	1
IF2	1	1 <sup>\$</sup>	1	1	1	1	1	1	1
IF3	1 <sup>*&amp;</sup>	1	1 <sup>&amp;</sup>	1	1*	1	1	1	1

1 = tissue sampled, 0 = tissue not sampled; \*LA-ICP-MS imaging; <sup>\$</sup>μXRF imaging coupled with μXANES &nXRF imaging.

**Table III.2:** Soil properties in the top and subsoil for the different cultivars. Letters denote different statistical groups with the posthoc Tukey HSD test ( $p < 0.05$ ).

<b>Topsoil</b>		<b>High</b>		<b>Low</b>		<b>Grafted</b>	
		Mean	SD	Mean	SD	Mean	SD
pH		<b>4.13</b>	0.11	<b>4.20</b>	0.09	<b>4.27</b>	0.04
OC	%	<b>1.86</b>	0.36	<b>2.02</b>	0.23	<b>2.44</b>	0.36
CEC	cmol <sub>c</sub> /kg	<b>12.16<sup>b</sup></b>	0.12	<b>11.65<sup>b</sup></b>	0.16	<b>14.31<sup>a</sup></b>	0.47
Exchangeable Ca	cmol <sub>c</sub> /kg	<b>6.23<sup>c</sup></b>	0.58	<b>7.32<sup>b</sup></b>	0.23	<b>10.11<sup>a</sup></b>	0.34
Exchangeable Mg	cmol <sub>c</sub> /kg	<b>2.72<sup>a</sup></b>	0.36	<b>2.05<sup>b</sup></b>	0.06	<b>2.28<sup>ab</sup></b>	0.06
Exchangeable K	cmol <sub>c</sub> /kg	<b>0.22<sup>b</sup></b>	0.00	<b>0.21<sup>b</sup></b>	0.00	<b>0.31<sup>a</sup></b>	0.02
Exchangeable Na	cmol <sub>c</sub> /kg	<b>0.14</b>	0.01	<b>0.11</b>	0.01	<b>0.14</b>	0.02
Base saturation	%	<b>76.44</b>	6.83	<b>83.11</b>	2.35	<b>89.78</b>	2.90
Oxalate extractable P	mg/kg	<b>157<sup>b</sup></b>	25.72	<b>196<sup>b</sup></b>	34.47	<b>465<sup>a</sup></b>	100.19
Oxalate extractable Al	mg/kg	<b>1000<sup>b</sup></b>	54.76	<b>937<sup>b</sup></b>	39.66	<b>1104<sup>a</sup></b>	14.26
Oxalate extractable Fe	mg/kg	<b>4859<sup>b</sup></b>	203.08	<b>5542<sup>b</sup></b>	614.01	<b>9073<sup>a</sup></b>	984.84
Oxalate extractable Mn	mg/kg	<b>832</b>	106.90	<b>617</b>	94.22	<b>678</b>	138.99
Ca(NO <sub>3</sub> ) <sub>2</sub> - Mn	mg/kg	<b>248<sup>a</sup></b>	27.99	<b>197<sup>ab</sup></b>	5.92	<b>155<sup>b</sup></b>	25.08
Ca(NO <sub>3</sub> ) <sub>2</sub> - Zn	mg/kg	<b>3.64</b>	0.37	<b>4.15</b>	0.95	<b>6.10</b>	2.98
Ca(NO <sub>3</sub> ) <sub>2</sub> - Cu	mg/kg	<b>0.08</b>	0.01	<b>0.08</b>	0.02	<b>0.07</b>	0.01
Ca(NO <sub>3</sub> ) <sub>2</sub> - Cd	mg/kg	<b>0.08</b>	0.02	<b>0.07</b>	0.01	<b>0.10</b>	0.01
<b>Subsoil</b>							
pH		<b>3.74</b>	0.12	<b>3.84</b>	0.06	<b>3.78</b>	0.06
CEC	cmol <sub>c</sub> /kg	<b>11.47<sup>b</sup></b>	0.39	<b>11.16<sup>b</sup></b>	1.22	<b>14.04<sup>a</sup></b>	0.31
Exchangeable Ca	cmol <sub>c</sub> /kg	<b>4.07<sup>b</sup></b>	0.69	<b>4.82<sup>b</sup></b>	0.96	<b>7.27<sup>a</sup></b>	0.18
Exchangeable Mg	cmol <sub>c</sub> /kg	<b>2.54</b>	0.18	<b>2.26</b>	0.50	<b>2.81</b>	0.22
Exchangeable K	cmol <sub>c</sub> /kg	<b>0.23<sup>b</sup></b>	0.01	<b>0.25<sup>b</sup></b>	0.04	<b>0.39<sup>a</sup></b>	0.01
Exchangeable Na	cmol <sub>c</sub> /kg	<b>0.19<sup>b</sup></b>	0.00	<b>0.17<sup>b</sup></b>	0.02	<b>0.33<sup>a</sup></b>	0.03
Base saturation	%	<b>61.34<sup>b</sup></b>	4.02	<b>66.7<sup>ab</sup></b>	6.55	<b>76.74<sup>a</sup></b>	1.31
Oxalate extractable P	mg/kg	<b>85.67<sup>b</sup></b>	34.82	<b>111<sup>ab</sup></b>	28.54	<b>178<sup>a</sup></b>	33.47
Oxalate extractable Al	mg/kg	<b>1007</b>	17.58	<b>1157</b>	51.30	<b>1032</b>	108.47
Oxalate extractable Fe	mg/kg	<b>3202</b>	500.03	<b>4141</b>	891.62	<b>5045</b>	887.93
Oxalate extractable Mn	mg/kg	<b>445</b>	132.15	<b>502</b>	166.01	<b>173</b>	219.54
Ca(NO <sub>3</sub> ) <sub>2</sub> - Mn	mg/kg	<b>109</b>	43.63	<b>130.04</b>	40.97	<b>67.94</b>	3.52
Ca(NO <sub>3</sub> ) <sub>2</sub> - Zn	mg/kg	<b>4.00<sup>b</sup></b>	0.49	<b>4.17<sup>b</sup></b>	0.93	<b>6.59<sup>a</sup></b>	4.17
Ca(NO <sub>3</sub> ) <sub>2</sub> - Cu	mg/kg	<b>0.13</b>	0.06	<b>0.10</b>	0.03	<b>0.15</b>	0.01
Ca(NO <sub>3</sub> ) <sub>2</sub> - Cd	mg/kg	<b>0.03</b>	0.01	<b>0.03</b>	0.00	<b>0.04</b>	0.01

OC: organic carbon (%), CEC: cation exchange capacity, ex: exchangeable bases, ox: ammonium oxalate extractable Fe, Mn and Al.

Ca(NO<sub>3</sub>)<sub>2</sub> – extraction: 0.05 M Ca(NO<sub>3</sub>)<sub>2</sub> (5g soil: 50 mL Ca(NO<sub>3</sub>)<sub>2</sub>).

**Table III.3:** Nutrient concentrations measured in the organs of the cacao trees for the different cultivars.

	High			Low			Grafted		
	N	Mean	Std Dev	N	Mean	Std Dev	N	Mean	Std Dev
P (g kg <sup>-1</sup> )									
Roots	3	0.34	0.28	3	0.62	0.17	3	1.08	0.85
Branch	3	0.85	0.29	3	1.26	0.27	3	1.23	0.15
Pod husk	5	1.21	0.12	6	0.91	0.27	1	0.76	
Testa	4	1.00	0.16	5	1.44	0.35	1	1.45	
Nib	5	4.45	0.61	6	5.67	0.45	1	5.49	
Leaves F2	0			2	5.59	0.38	1	4.73	
Leaves IF2	3	1.23	0.08	3	1.45	0.12	3	1.73	0.26
Leaves IF3	3	1.03	0.13	3	1.05	0.05	3	1.24	0.16
Cu (mg kg <sup>-1</sup> )									
Roots	3	9.82	3.70	3	6.29	1.21	3	7.66	6.77
Branch	3	6.55	1.44	3	8.77	1.52	3	10.21	2.33
Pod husk	5	10.15	1.59	6	7.78	1.39	1	9.30	
Testa	4	11.52	1.54	5	11.46	4.60	1	15.54	
Nib	5	16.80	1.82	6	19.55	2.11	1	19.53	
Leaves F2	0			2	24.25	0.02	1	13.82	
Leaves IF2	3	4.58	0.25	3	5.28	0.47	3	6.29	1.32
Leaves IF3	3	3.81	0.26	3	3.98	0.49	3	5.41	1.28
S (g kg <sup>-1</sup> )									
Roots	3	1.69	0.33	3	2.05	0.26	3	1.37	0.09
Branch	3	1.00	0.21	3	1.60	0.05	3	1.54	0.31
Pod husk	5	1.27	0.08	6	1.53	0.32	1	1.94	
Testa	4	1.17	0.07	5	1.00	0.11	1	1.21	
Nib	5	1.88	0.09	6	1.53	0.08	1	1.64	
Leaves F2	0			2	3.57	0.00	1	3.05	
Leaves IF2	3	2.32	0.09	3	2.11	0.19	3	2.32	0.35
Leaves IF3	3	1.93	0.08	3	2.42	0.52	3	2.16	0.54
K (g kg <sup>-1</sup> )									
Roots	3	5.44	0.87	3	5.62	1.63	3	9.30	0.17
Branch	3	10.12	3.82	3	15.76	4.30	3	22.00	1.07
Pod husk	5	28.57	5.20	6	25.75	6.30	1	34.58	
Testa	4	10.46	0.37	5	15.55	1.43	1	30.00	
Nib	5	9.50	2.19	6	10.22	1.27	1	9.76	
Leaves F2	0			2	21.91	3.94	1	14.05	
Leaves IF2	3	11.58	2.01	3	15.95	2.82	3	20.28	1.78
Leaves IF3	3	8.35	2.09	3	9.88	0.97	3	15.37	1.90

Zn (mg kg <sup>-1</sup> )									
Roots	3	46.80	10.24	3	22.27	5.25	3	33.10	2.40
Branch	3	139.22	25.15	3	108.12	31.96	3	155.90	72.34
Pod husk	5	71.41	6.92	6	51.42	12.06	1	64.16	
Testa	4	36.14	6.87	5	33.92	7.09	1	66.77	
Nib	5	46.70	4.90	6	38.88	4.17	1	45.77	
Leaves F2	0			2	85.21	11.76	1	66.85	
Leaves IF2	3	183.82	21.52	3	87.14	23.29	3	90.74	7.83
Leaves IF3	3	177.57	15.65	3	136.57	61.01	3	154.06	46.22
Mn (mg kg <sup>-1</sup> )									
Roots	3	101.15	54.52	3	19.97	4.32	3	30.68	10.42
Branch	3	465.08	114.25	3	160.87	71.42	3	391.08	164.57
Pod husk	5	308.42	134.74	6	98.44	66.24	1	128.18	
Testa	4	99.71	28.09	5	52.82	24.02	1	66.82	
Nib	5	93.58	24.77	6	49.09	16.60	1	62.95	
Leaves F2	0			2	184.44	28.13	1	77.11	
Leaves IF2	3	1776.86	287.47	3	239.08	69.06	3	406.20	108.08
Leaves IF3	3	1863.56	306.21	3	445.00	152.67	3	990.83	314.77
Ca (g kg <sup>-1</sup> )									
Roots	3	8.55	1.13	3	3.66	1.31	3	4.59	1.07
Branch	3	18.71	6.62	3	16.80	2.90	3	22.28	5.87
Pod husk	5	5.00	1.71	6	5.90	2.40	1	3.92	
Testa	4	4.45	1.67	5	6.61	2.11	1	4.87	
Nib	5	1.24	0.24	6	1.62	0.32	1	1.29	
Leaves F2	0			2	3.57	0.81	1	4.01	
Leaves IF2	3	15.28	2.53	3	7.97	1.75	3	7.54	0.97
Leaves IF3	3	16.21	1.52	3	14.51	0.91	3	13.92	0.36
Mg (g kg <sup>-1</sup> )									
Roots	3	6.32	1.04	3	3.24	0.84	3	4.80	0.87
Branch	3	5.10	1.92	3	5.46	1.09	3	4.34	1.63
Pod husk	5	3.12	0.67	6	3.37	1.18	1	3.12	
Testa	4	2.73	0.48	5	3.67	0.68	1	3.78	
Nib	5	3.44	0.26	6	3.61	0.18	1	3.64	
Leaves F2	0			2	4.62	0.28	1	4.03	
Leaves IF2	3	8.37	0.52	3	4.93	0.87	3	4.30	0.33
Leaves IF3	3	8.25	0.41	3	7.01	0.63	3	6.83	0.84
Ni (mg kg <sup>-1</sup> )									
Roots	2	7.84	2.39	3	2.21	0.44	3	5.22	2.57
Branch	0			0			1	14.05	
Pod husk	5	10.23	1.45	6	7.77	2.7	1	7.29	
Testa	4	12.05	4.26	5	27.93	7.01	1	46.14	
Nib	5	16.35	2.64	6	12.49	2.7	1	12.28	
Leaves F2	0			2	13.59	0.41	0		
Leaves IF2	3	41.18	7.69	2	22.17	1.58	2	14.36	2.01
Leaves IF3	3	44.53	5.89	3	34.15	10.82	3	24.23	7.77

**Table III.4:** Estimation of total uptake of Cd and nutrients for each cultivar (in g).

	<b>High</b>		<b>Group</b>	<b>Low</b>			<b>Grafted</b>		
	Mean	SD		Mean	SD		Mean	SD	
Cd	0.67	0.06	a	0.25	0.08	c	0.44	0.05	b
Mn	70.65	4.42	a	19.51	7.56	b	44.80	16.39	ab
K	1180.28	289.41	b	1707.79	426.31	ab	2425.83	107.32	a
S	170.37	26.23	b	235.19	13.39	a	208.21	30.85	ab
Ni	0.83	0.22		0.45	0.17		0.82	0.76	
P	100.17	20.97		144.71	27.44		161.03	36.85	
Ca	2092.18	612.34		1707.64	278.15		2191.34	460.79	
Cu	0.93	0.09		1.01	0.15		1.19	0.21	
Zn	16.02	2.47		11.56	3.49		16.10	6.55	

Total uptake was calculated based on biomass assumptions (see materials and methods). Letters a, b, and c differences in Cd/nutrient uptake among cultivars (posthoc Tukey HSD test,  $p < 0.05$ ).

**Table III.5:** Bulk concentration ratios ( $\log[\text{nutrient}/\text{Cd}]$  ( $\text{mmol kg}^{-1} \text{ DW}/\text{mmol kg}^{-1} \text{ DW}$ )) for the different tree organs. Letters x, y, z denote different statistical groups within the same row, letters a, b, c, d denote different statistical groups within the same column (posthoc Tukey HSD test,  $p < 0.05$ ).

	High			Low			Graft		
	Mean	SD	Group	Mean	SD	Group	Mean	SD	Group
<b>log (P/Cd)</b>									
Roots	5.53	1.27	c	7.61	0.19	d	7.45	0.78	c
Branch	6.18 <sup>x</sup>	0.35	bc	7.62 <sup>y</sup>	0.33	d	7.02 <sup>y</sup>	0.20	c
Pod husk	7.40	0.37	b	8.35	0.80	bcd	7.44	.	bc
Testa	7.33 <sup>x</sup>	0.15	b	8.92 <sup>y</sup>	0.31	bc	8.6 <sup>y</sup>	.	abc
Nib	8.97 <sup>x</sup>	0.17	a	10.66 <sup>y</sup>	0.47	a	9.55 <sup>xy</sup>	.	ab
F2 leaves	.	.		9.52	0.29	ab	10.45	.	a
IF2 leaves	7.18 <sup>x</sup>	0.30	b	8.59 <sup>y</sup>	0.15	bcd	7.92 <sup>z</sup>	0.23	bc
IF3 leaves	7.09 <sup>x</sup>	0.18	b	7.97 <sup>y</sup>	0.33	cd	7.11 <sup>x</sup>	0.06	c
<b>log (Cu/Cd)</b>									
Roots	1.66	0.62	bc	2.32	0.30	bcd	1.73	0.89	bc
Branch	0.62 <sup>x</sup>	0.14	d	1.94 <sup>y</sup>	0.43	cd	1.5 <sup>y</sup>	0.13	bc
Pod husk	1.87	0.42	b	2.89	0.64	bc	2.33	.	abc
Testa	2.16 <sup>x</sup>	0.24	ab	3.34 <sup>y</sup>	0.32	b	3.34 <sup>y</sup>	.	ab
Nib	2.68 <sup>x</sup>	0.15	a	4.27 <sup>y</sup>	0.49	a	3.19 <sup>xy</sup>	.	ab
F2 leaves	.	.		3.36	0.22	ab	3.89	.	a
IF2 leaves	0.87 <sup>x</sup>	0.28	cd	2.26 <sup>z</sup>	0.14	bcd	1.57 <sup>y</sup>	0.28	bc
IF3 leaves	0.78 <sup>x</sup>	0.14	d	1.67 <sup>y</sup>	0.41	d	0.95 <sup>y</sup>	0.09	c
<b>log(S/Cd)</b>									
Roots	7.53 <sup>x</sup>	0.31	ab	8.8 <sup>y</sup>	0.45	ab	7.84 <sup>x</sup>	0.03	bc
Branch	6.33 <sup>x</sup>	0.17	c	7.85 <sup>y</sup>	0.33	b	7.21 <sup>z</sup>	0.10	d
Pod husk	7.41 <sup>x</sup>	0.52	b	8.86 <sup>y</sup>	0.72	ab	8.35 <sup>xy</sup>	.	b
Testa	7.47 <sup>x</sup>	0.20	ab	8.54 <sup>y</sup>	0.37	ab	8.38 <sup>xy</sup>	.	b
Nib	8.09 <sup>x</sup>	0.22	a	9.32 <sup>y</sup>	0.48	a	8.3 <sup>xy</sup>	.	b
F2 leaves	.	.		9.04	0.22	ab	9.98	.	a
IF2 leaves	7.78 <sup>x</sup>	0.27	ab	8.94 <sup>y</sup>	0.29	ab	8.18 <sup>x</sup>	0.22	b
IF3 leaves	7.69 <sup>x</sup>	0.13	ab	8.76 <sup>y</sup>	0.56	ab	7.62 <sup>x</sup>	0.06	c
<b>log (K/Cd)</b>									
Roots	8.5 <sup>x</sup>	0.42	cd	9.59 <sup>y</sup>	0.27	b	9.56 <sup>y</sup>	0.12	bc
Branch	8.42 <sup>x</sup>	0.28	d	9.91 <sup>y</sup>	0.23	b	9.68 <sup>y</sup>	0.16	d
Pod husk	10.26 <sup>x</sup>	0.58	a	11.47 <sup>y</sup>	0.62	a	11.04 <sup>xy</sup>	.	b
Testa	9.47 <sup>x</sup>	0.18	abc	11.09 <sup>y</sup>	0.36	a	11.4 <sup>y</sup>	.	b
Nib	9.49 <sup>x</sup>	0.37	ab	11.01 <sup>y</sup>	0.51	a	9.89 <sup>xy</sup>	.	b
F2 leaves	.	.		10.65	0.04	ab	11.30	.	a
IF2 leaves	9.18 <sup>x</sup>	0.40	bcd	10.75 <sup>y</sup>	0.11	ab	10.15 <sup>y</sup>	0.16	b
IF3 leaves	8.94 <sup>x</sup>	0.37	bcd	9.98 <sup>y</sup>	0.44	b	9.4 <sup>xy</sup>	0.09	c

Table III.5 continued.

	<b>High</b>			<b>Low</b>			<b>Graft</b>		
	Mean	SD	Group	Mean	SD	Group	Mean	SD	Group
<b>log (Zn/Cd)</b>									
Roots	3.23	0.46	c	3.55	0.19	b	3.40	0.17	c
Branch	3.65 <sup>x</sup>	0.17	c	4.41 <sup>y</sup>	0.14	ab	4.14 <sup>xy</sup>	0.35	b
Pod husk	3.79	0.45	bc	4.74	0.58	a	4.23	.	abc
Testa	3.27 <sup>x</sup>	0.29	c	4.44 <sup>y</sup>	0.33	ab	4.77 <sup>y</sup>	.	ab
Nib	3.67 <sup>x</sup>	0.29	c	4.93 <sup>y</sup>	0.47	a	4.01 <sup>xy</sup>	.	bc
F2 leaves	.	.		4.59	0.08	ab	5.44	.	a
IF2 leaves	4.53 <sup>xy</sup>	0.21	ab	5.01 <sup>x</sup>	0.30	a	4.23 <sup>y</sup>	0.07	b
IF3 leaves	4.59 <sup>x</sup>	0.06	a	5.12 <sup>y</sup>	0.14	a	4.25 <sup>x</sup>	0.22	b
<b>log (Mn/Cd)</b>									
Roots	4.09	0.63	d	3.62	0.35	d	3.47	0.37	d
Branch	5.02	0.27	bc	4.93	0.42	c	5.23	0.36	bc
Pod husk	5.37	0.22	b	5.40	0.42	bc	5.09	.	abc
Testa	4.43 <sup>x</sup>	0.28	cd	4.99 <sup>y</sup>	0.28	c	4.94 <sup>xy</sup>	.	bc
Nib	4.52 <sup>x</sup>	0.30	cd	5.29 <sup>y</sup>	0.40	c	4.5 <sup>xy</sup>	.	cd
F2 leaves	.	.		5.53	0.06	abc	5.76	.	abc
IF2 leaves	6.97 <sup>x</sup>	0.20	a	6.19 <sup>y</sup>	0.44	ab	5.88 <sup>y</sup>	0.19	ab
IF3 leaves	7.11 <sup>x</sup>	0.07	a	6.5 <sup>y</sup>	0.25	a	6.29 <sup>y</sup>	0.20	a
<b>log(Ca/Cd)</b>									
Roots	8.82 <sup>x</sup>	0.31	bc	9.94 <sup>y</sup>	0.29	b	8.81 <sup>y</sup>	0.22	c
Branch	9.11 <sup>x</sup>	0.39	abc	10.03 <sup>y</sup>	0.34	a	9.7 <sup>xy</sup>	0.17	ab
Pod husk	8.47 <sup>x</sup>	0.10	c	9.88 <sup>y</sup>	0.29	a	8.8 <sup>x</sup>	.	bcd
Testa	8.53 <sup>x</sup>	0.36	c	10.17 <sup>y</sup>	0.31	a	9.54 <sup>xy</sup>	.	abc
Nib	7.43 <sup>x</sup>	0.19	d	9.15 <sup>y</sup>	0.31	b	7.87	.	d
F2 leaves	.	.		8.88	0.45	b	10.11 <sup>x</sup>	.	a
IF2 leaves	9.49 <sup>xy</sup>	0.28	ab	10.12 <sup>x</sup>	0.41	a	9.19 <sup>y</sup>	0.12	abc
IF3 leaves	9.66 <sup>x</sup>	0.12	a	10.55 <sup>y</sup>	0.40	a	9.34 <sup>x</sup>	0.20	abc
<b>log (Mg/Cd)</b>									
Roots	8.46 <sup>x</sup>	0.71	ab	10.00 <sup>y</sup>	0.21	b	9.47 <sup>xy</sup>	0.09	b
Branch	8.29 <sup>x</sup>	0.42	c	9.38 <sup>y</sup>	0.23	b	8.51 <sup>x</sup>	0.32	c
Pod husk	8.56 <sup>x</sup>	0.27	bc	9.90 <sup>y</sup>	0.47	ab	9.09 <sup>xy</sup>	.	bc
Testa	8.64 <sup>x</sup>	0.15	bc	10.15 <sup>y</sup>	0.27	ab	9.82 <sup>y</sup>	.	ab
Nib	9.00 <sup>x</sup>	0.24	ab	10.50 <sup>y</sup>	0.48	a	9.41 <sup>xy</sup>	.	abc
F2 leaves	.	.		9.62	0.30	ab	10.59	.	bc
IF2 leaves	9.37 <sup>x</sup>	0.29	a	10.11 <sup>y</sup>	0.39	ab	9.11 <sup>x</sup>	0.15	bc
IF3 leaves	9.47 <sup>x</sup>	0.08	a	10.29 <sup>y</sup>	0.51	ab	9.09 <sup>xy</sup>	0.06	bc

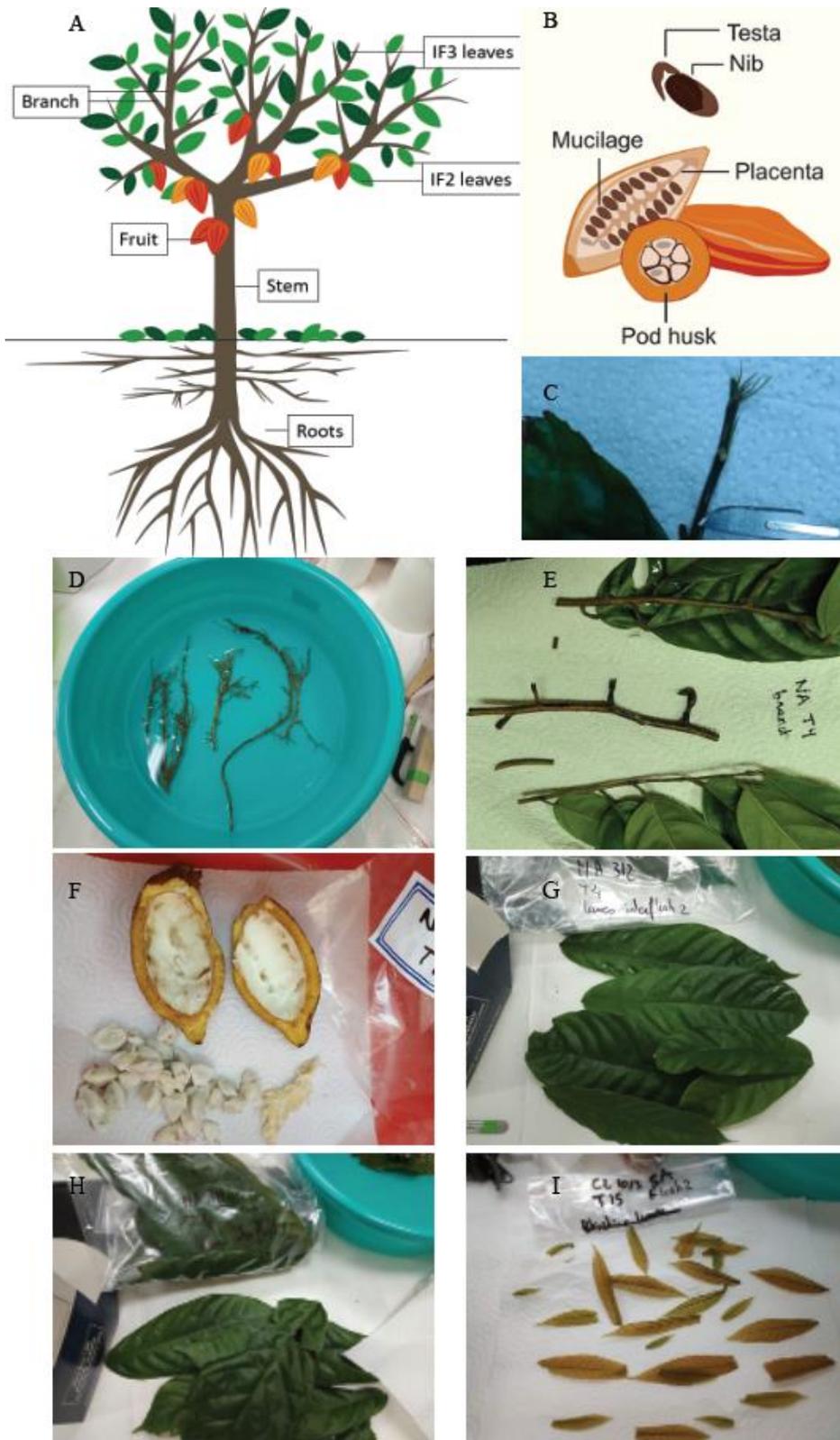
**Table III.6:** Intensity of Cd signal detected by LA-ICP-MS in the different structures of the branch of the low Cd accumulating cultivar (replicates T15, shown in Figure 4 of main paper and T3) and R<sup>2</sup> of regressions to intensities of other elements.

	Intensity <sup>§</sup>		R <sup>2</sup>											
	Cd		Ca		Mn		Zn		Cu		Ni		P	
	T15	T3	T15	T3	T15	T3	T15	T3	T15	T3	T15	T3	T15	T3
<b>1 Medulla/pith</b>	8.74	1.73	0.42	0.21	0.44	0.28	0.47	0.32	0.17	0.27	0.28	0.28	0.27	0.19
<b>2 Wood</b>	1.00	1.00	0.28	0.29	0.36	0.27	0.37	0.16	0.25	0.16	0.16	0.12	0.29	0.18
<b>3 Bark phloem</b>	9.43	3.74	0.48	0.45	0.55	0.54	0.32	0.38	0.2	0.25	0.22	0.22	0.36	0.42
4 Phloem vessels	8.93	3.58	0.54	0.45	0.59	0.48	0.34	0.31	0.25	0.19	0.26	0.13	0.42	0.3
5 Phloem rays	10.50	6.38	0.45	0.42	0.55	0.46	0.34	0.31	0.21	0.12	0.21	0.09	0.32	0.25
<b>6 Periderm</b>	11.12	5.68	0.31	0.32	0.42	0.48	0.33	0.4	0.18	0.09	0.24	0.1	0.35	0.35

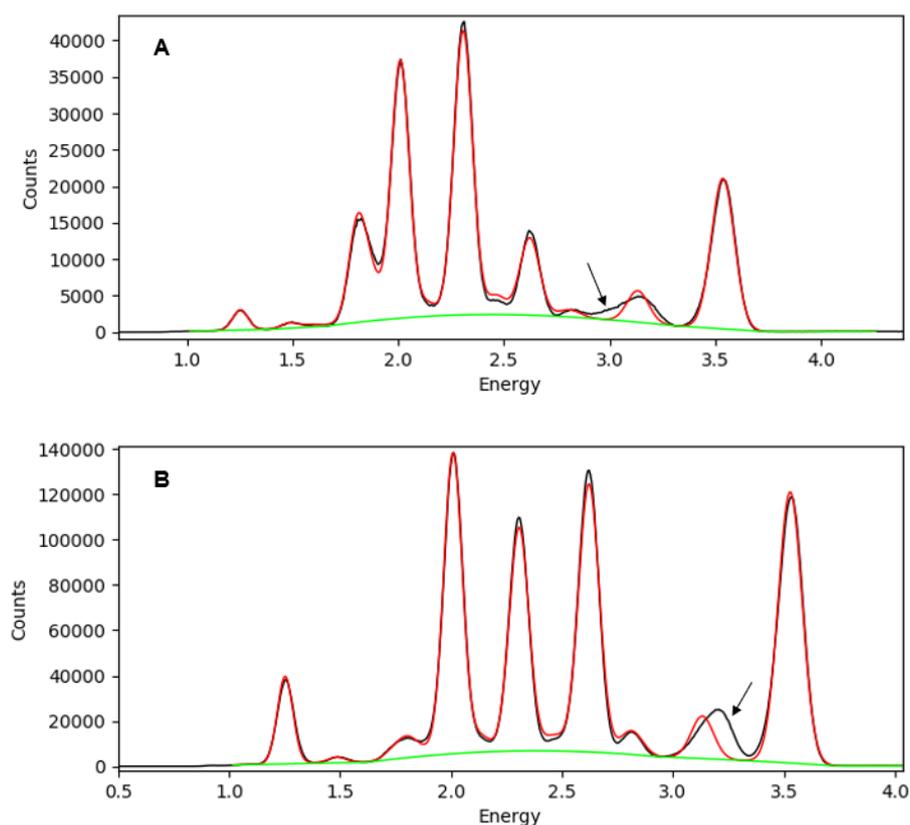
Elemental counts (cts) of each element were normalized to <sup>13</sup>C to account for differences in tissue hardness and water content within the sample. <sup>§</sup>The Cd counts were normalized to the mean Cd counts in wood to compare the accumulation of Cd in the different structures of the branch.

In the different ROIs, Cd correlated most strongly with Mn followed by Zn and Ca, and less with P, Cu and Ni (Table S6), except for the periderm. In the latter, there was a high co-localization with P as well.

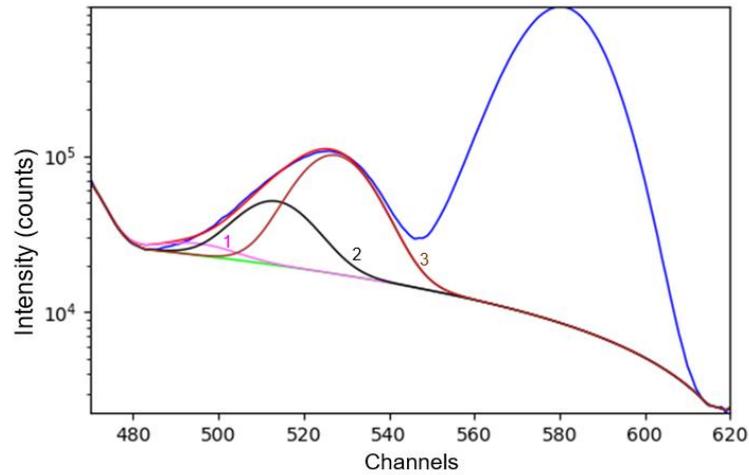
III.5 Figures



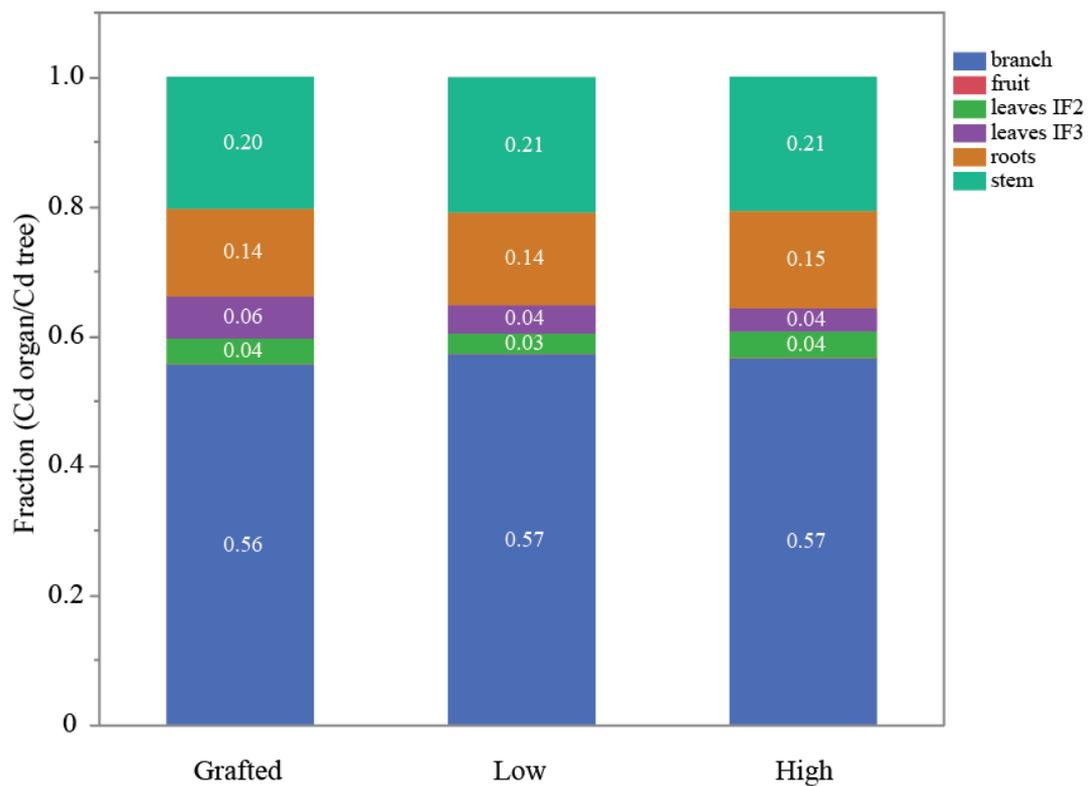
**Figure III.1:** (A) Terminology of the studied organs of the cacao trees in this study. (B) Tissues in a cacao fruit (Vanderschueren *et al.*, 2021). The cacao fruit or pod is made up of a large woody outer pod husk, filled with 20–50 cacao beans (seeds). The cacao bean consists of a nib and an outer shell, the testa. The nib is the only part of the cacao bean that is retained during chocolate processing. (C) Example of young branch sampled and analyzed for soft X-Ray imaging (diameter 2-5 mm). (D) Example of roots collected (diameter 1-10 mm). (E) Example of branches sampled (diameter 3-7 mm). (F) Example of fruit tissues sampled. (G) Example of IF2 leaves. (H) Example of IF3 leaves. (I) Example of F2 leaves.



**Figure III.2:** Example of XRF spectra. A) There is a background contribution which overlaps with the Cd emission line which is probably originating from the Ca crystals. B) There is a background contribution from the inelastic scattering of K.

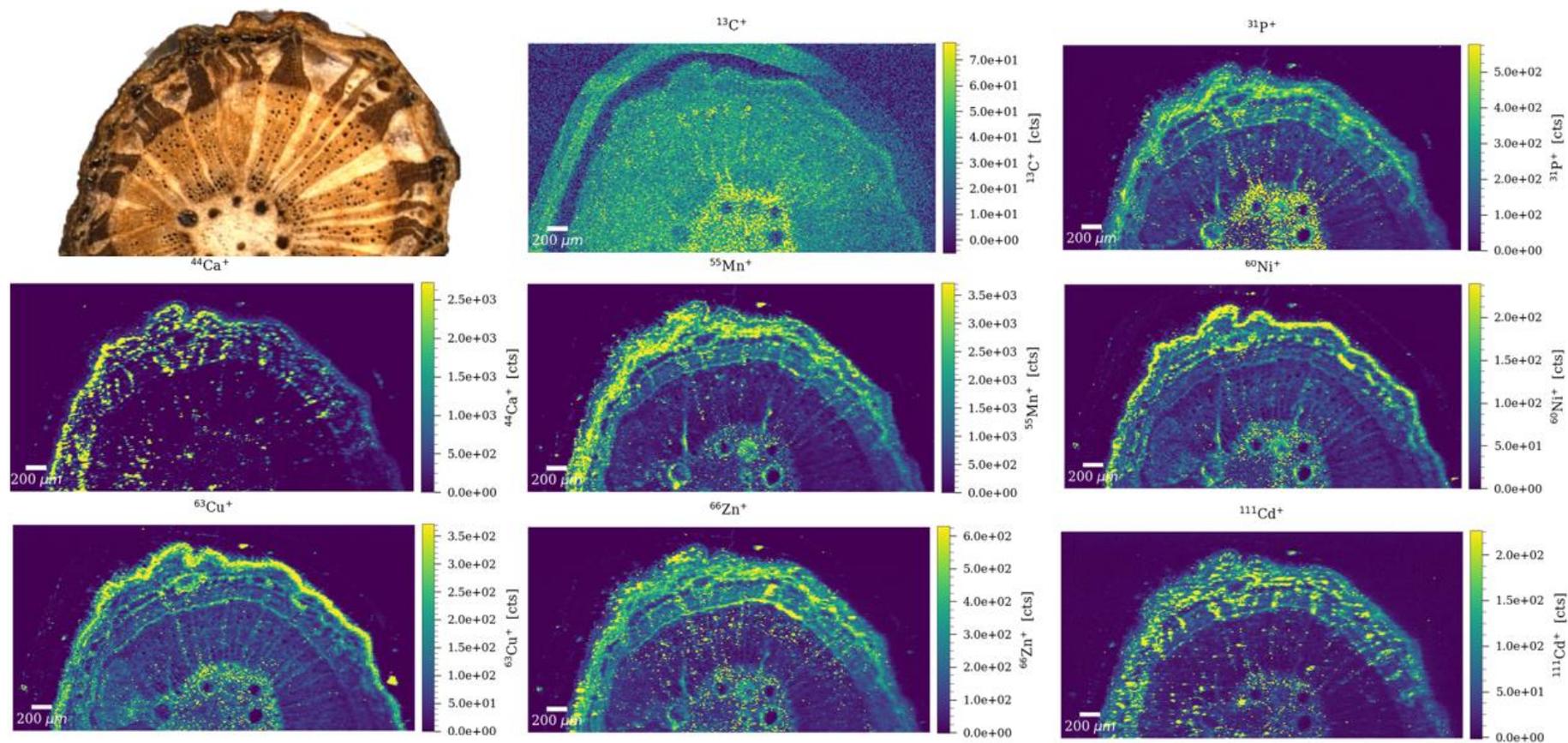


**Figure III.3:** The three different gaussians defined. Gaussian 1 is the background originating from Ca crystals, gaussian 2 is the Cd peak, gaussian 3 is the contribution from K inelastic scattering. The Cd peak was used to build the Cd XRF maps.

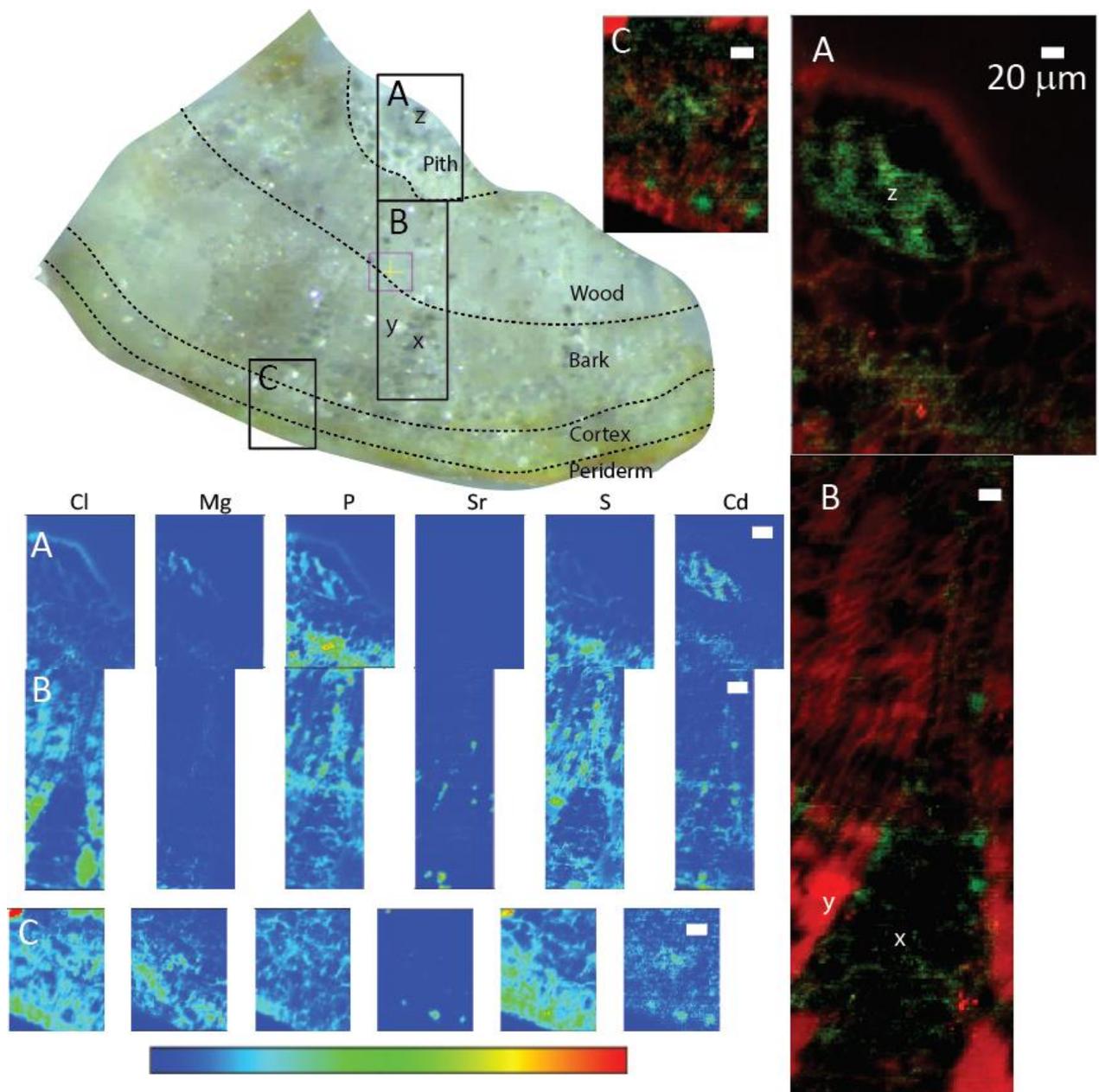


**Figure III.4:** Fraction of Cd accumulation in each organ relative to the Cd taken up by the tree (total Cd organ (mg)/total Cd tree (mg)). Total Cd was calculated with assumptions on tree biomass (see materials and methods).

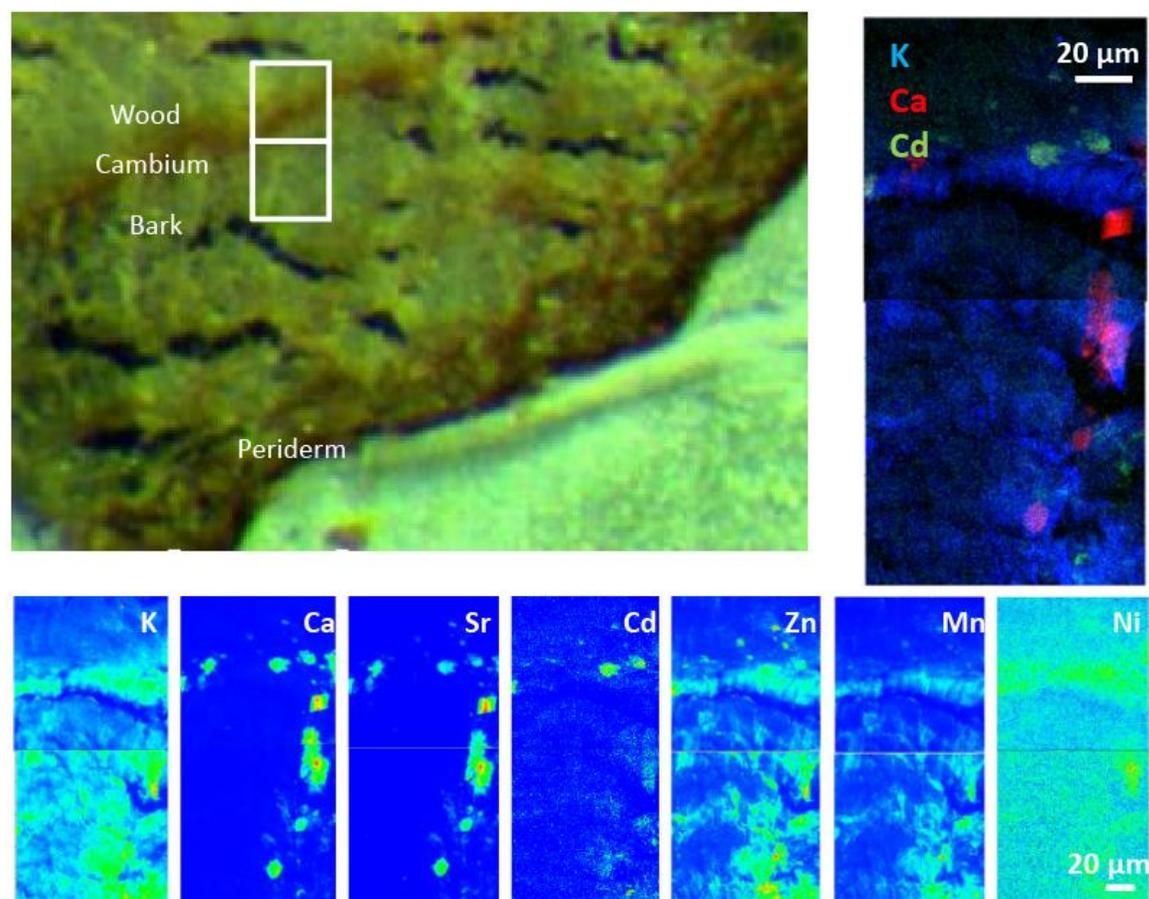




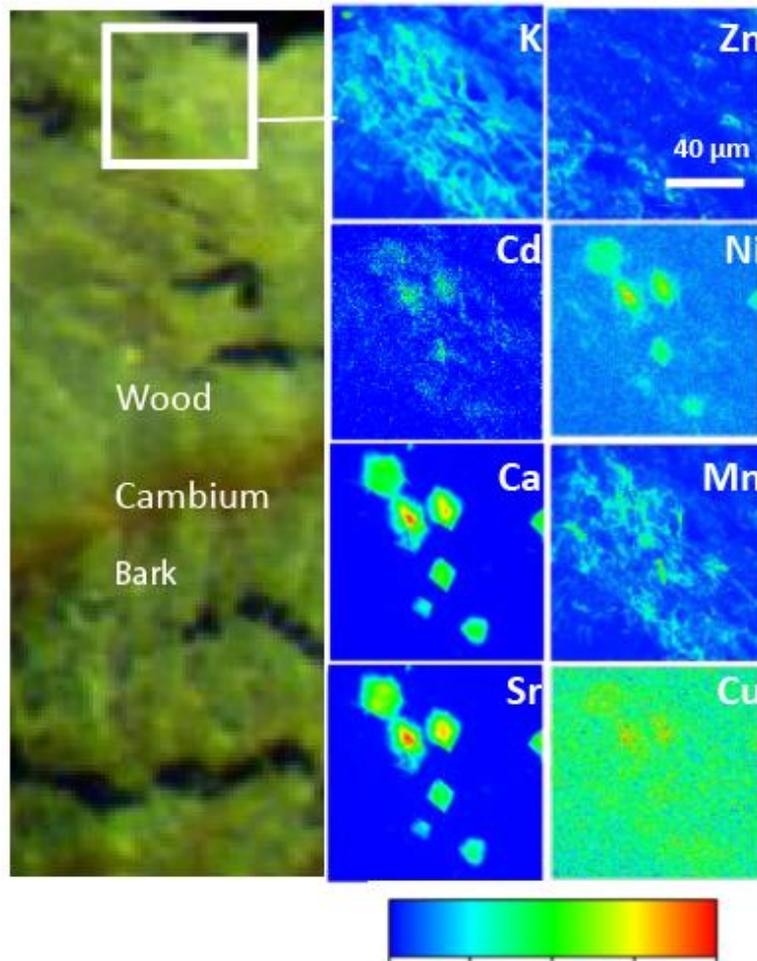
**Figure III.5:** Microscope image and LA-ICP-MS elemental maps of a cross section of branch CL 10/3 (replicate T3). The intensity is expressed in total counts (cts). The color scale is adjusted to the range of each element.



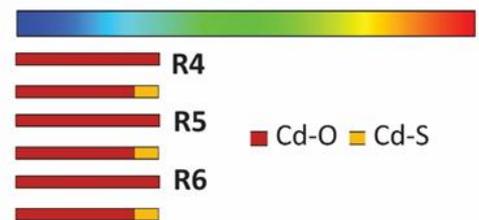
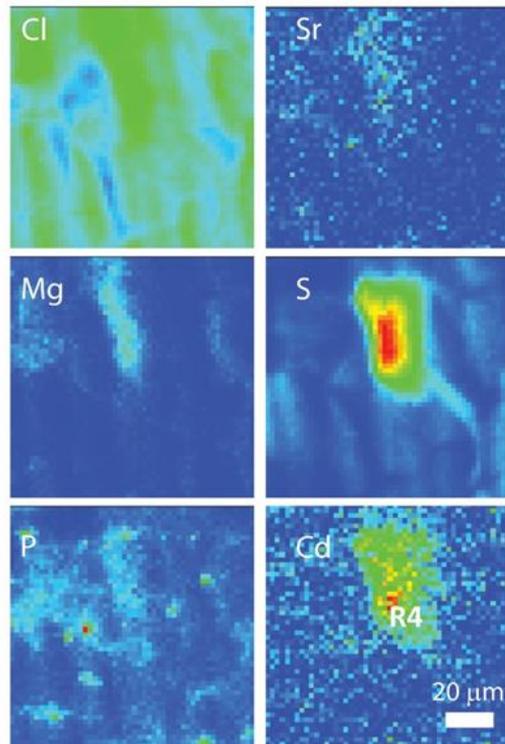
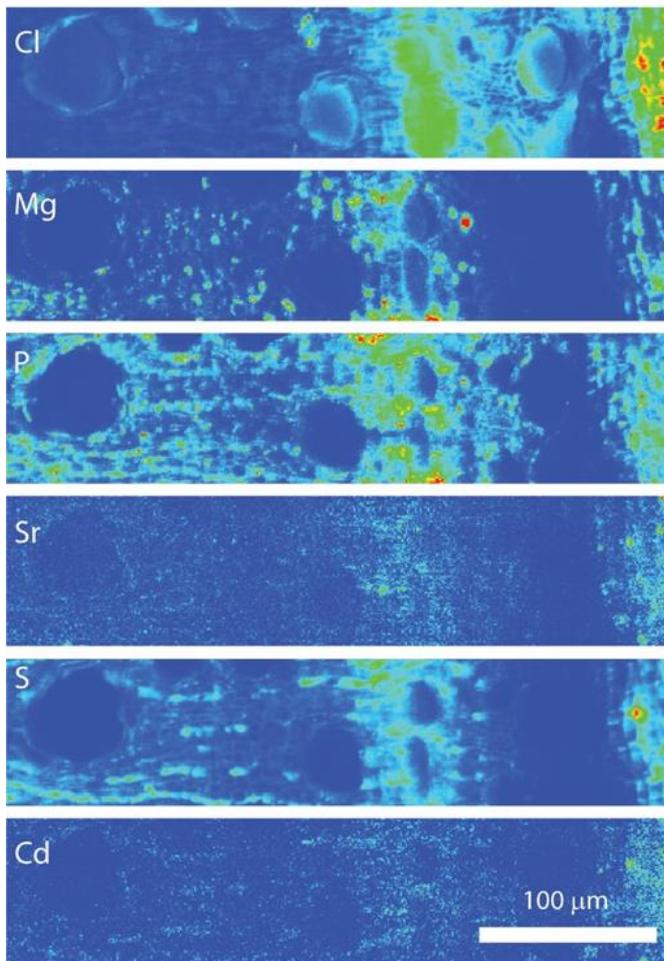
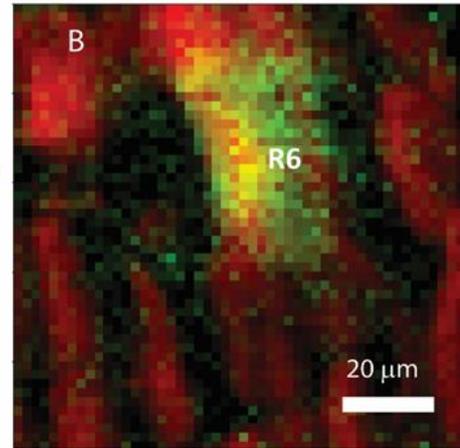
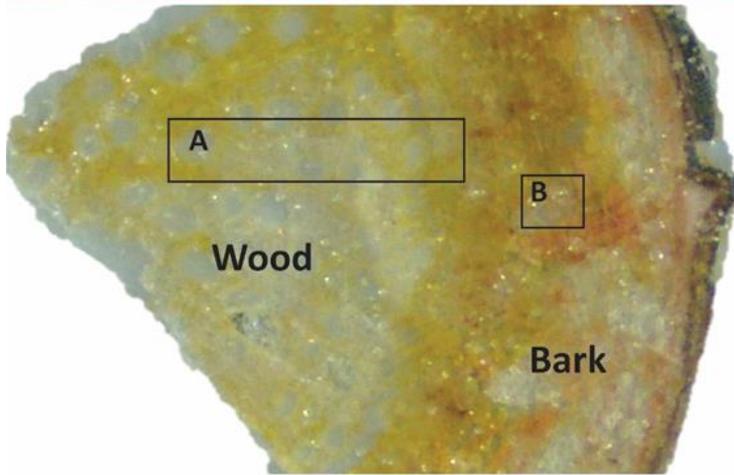
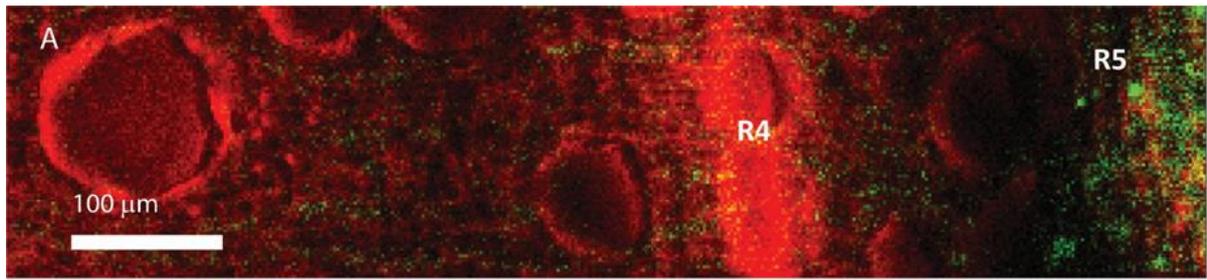
**Figure III.6:** Right figure: Cd localization in branch tissues of the high Cd accumulator (T4). Green = Cd, Red = Cl (shown to display cellular structure). In map A (pith), Cd accumulated in a vessel structure. In map B, Cd is localized in the phloem rays, and less in the phloem vessel. Map C indicates the periderm and cortex. Left figure: heatmaps for other elements. The color scale (blue = minimum and red = maximum photon counts) is adjusted to the range of each element (see materials and methods).



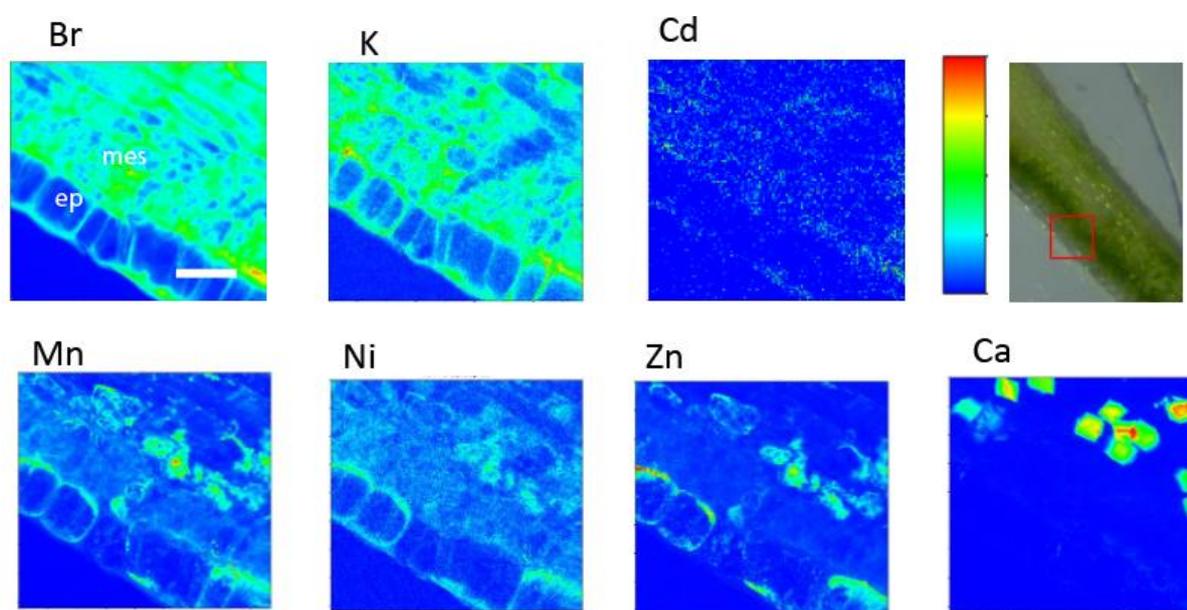
**Figure III.7:** NanoXRF image in the wood and cambium/bark of the branch. The image on the right top displays K (blue) to see the cellular structures, Ca (red), and Cd (green). Different forms of Ca-oxalate crystals are observed (red), and in some Cd is included (green). Bottom figures: heatmaps of K, Ca, Sr, Cd, Zn, Mn and Ni in selected wood and bark region from the figure above. Blue = minimum and red = maximum photon counts. Cadmium colocalized mostly with the Ca-oxalate crystals observed in the cambium.



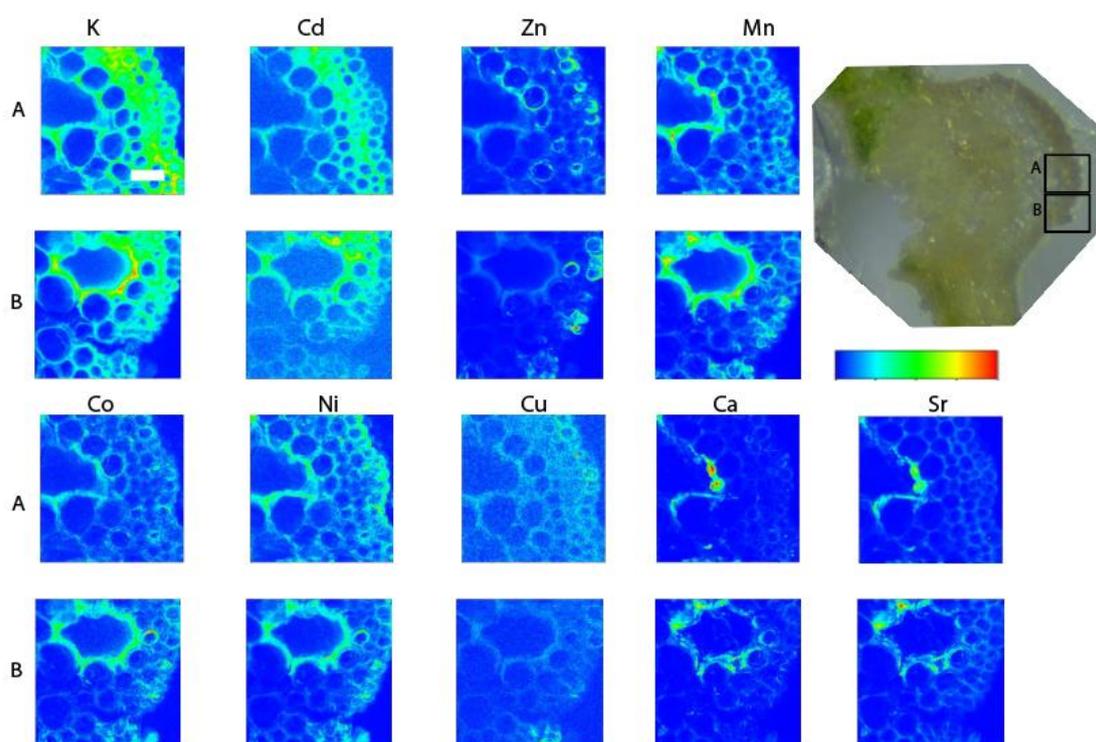
**Figure III.8:** Left: microscope image of branch thin section with the region of interest in the wood. Right: heatmaps with the distribution of K, Zn, Cd, Ni, Ca, Mn, Sr, and Cu in this region. The color scale (blue = minimum and red = maximum photon counts) is adjusted to the range of each element. A band of Ca-oxalate crystals are observed and, in some, Cd and Ni are included. Zinc and Mn were not observed in these crystals.



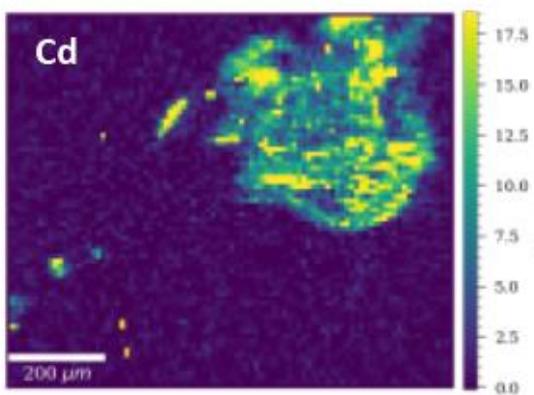
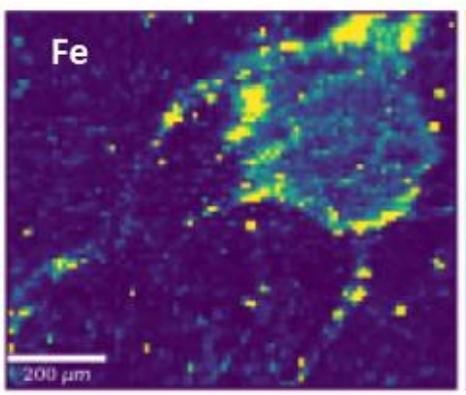
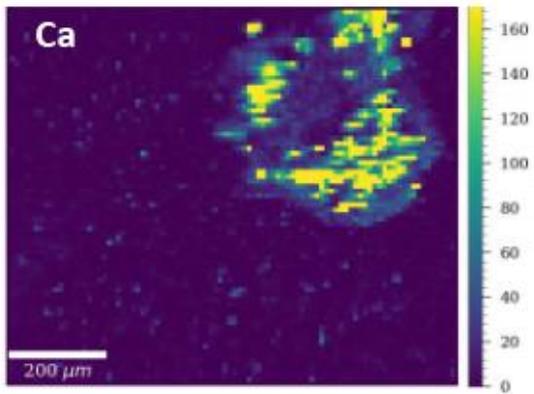
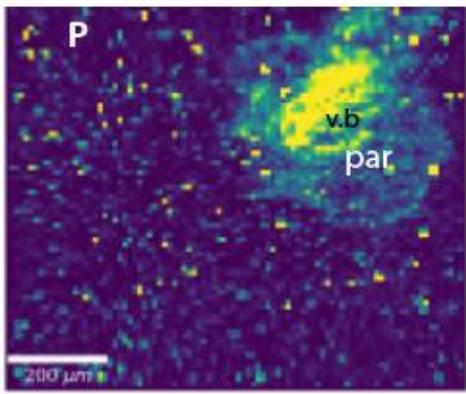
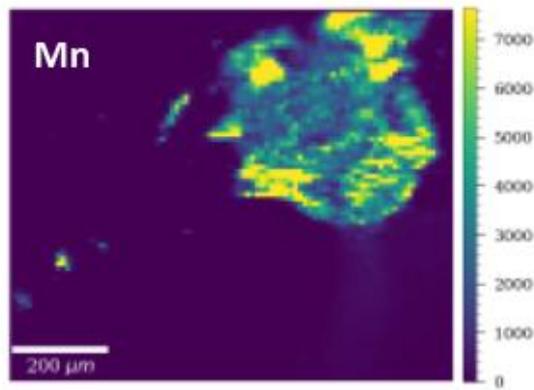
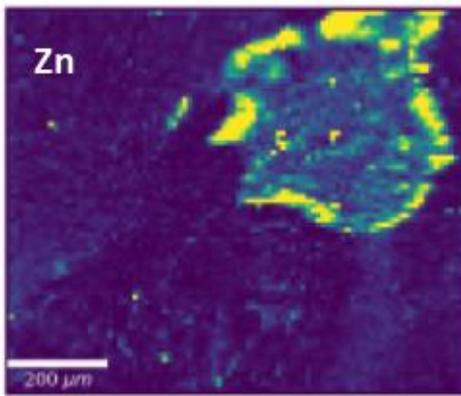
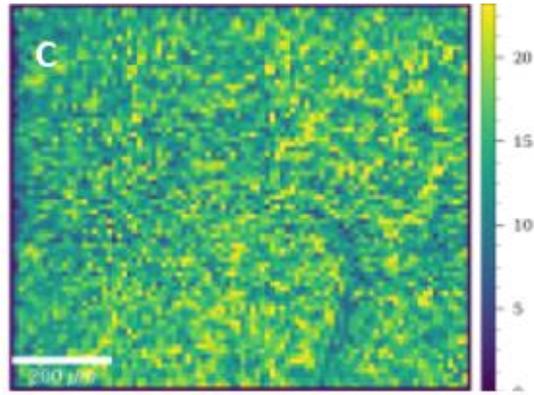
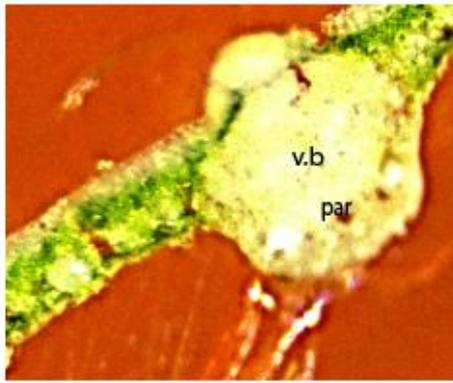
**Figure III.9:** Cadmium localization and speciation in frozen-hydrated root thin section of the high Cd accumulator (T4). Top figure: Green = Cd, Red = Cl (shown to display cellular structure). R4, R5, and R6 indicate Cd spots where XANES were recorded. The results of the linear combination fits are indicated in the right bottom (in % fitted fraction) (red bar = Cd-O, yellow bar = Cd-S). The fit quality was examined by the R-factor (indicated with the proportion). For spectra R4, R5, and R6, the R-factor of two linear combination fits differed with less than 10%. The fits were thus considered to have equivalent quality and were both shown in the figure. Bottom figure: heatmaps of Cl, Mg, P, Sr, S, and Cd of regions indicated in microscope image of root thin section. The color scale (blue = minimum and red = maximum photon counts) is adjusted to the range of each element (see materials and methods). In map A, the Cd distribution indicates that there is a slight enrichment in Cd around the cambium and bark compared to the wood. In map B a strong co-localization between Cd and S was noted.



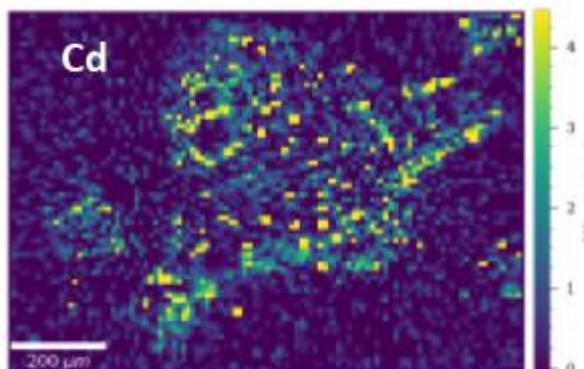
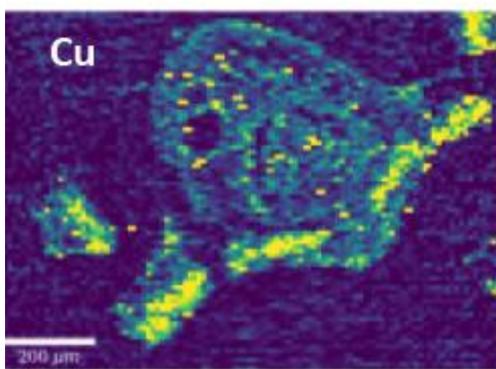
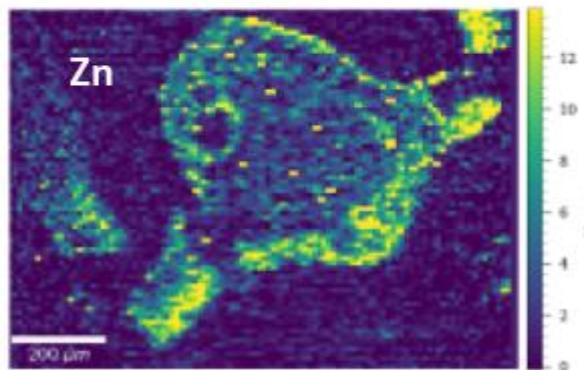
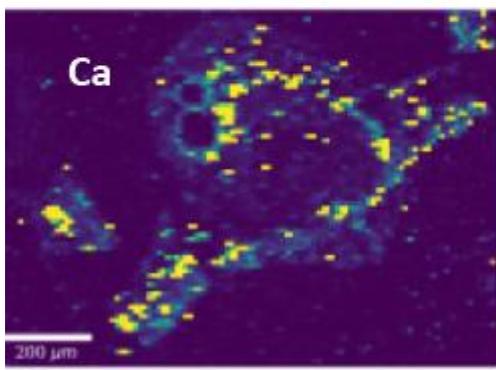
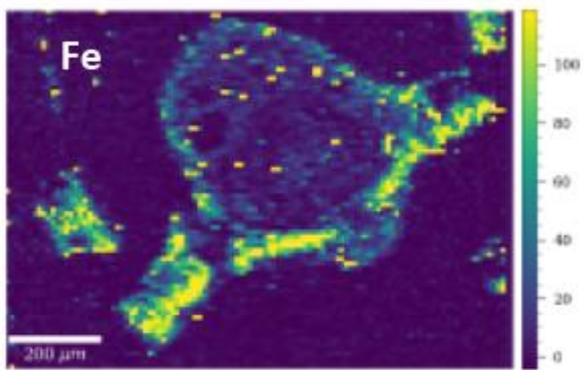
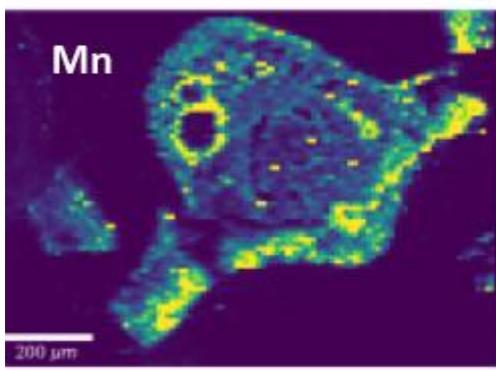
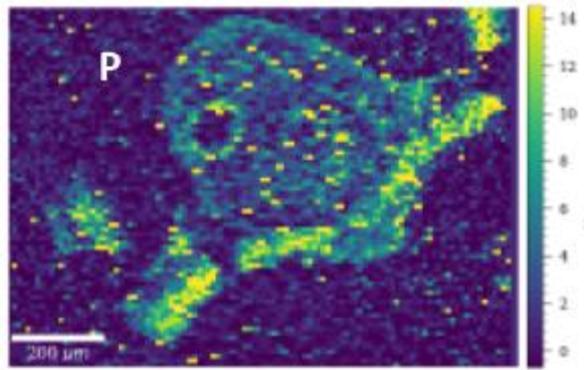
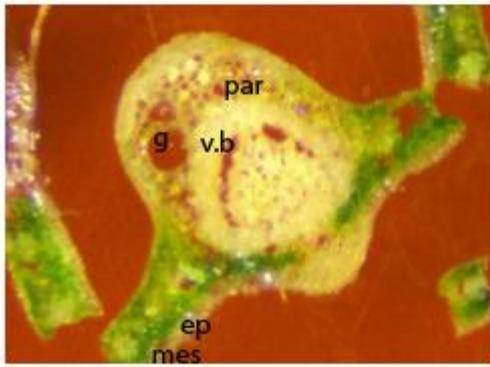
**Figure III.10:** NanoXRF image in freeze-dried leaf blade in the upper epidermis region (microscope image on the right) of the high Cd accumulator (T2). The color scale (blue = minimum and red = maximum photon counts) is adjusted to the range of each element. The Cd is absent in the epidermis and present in the mesophyll. A band of Ca-oxalate crystals are observed in the mesophyll. However, there is no particular Cd incorporation in these crystals. Scalebar: 20  $\mu$ m. Ep: epidermis, mes: mesophyll.



**Figure III.11:** NanoXRF image in freeze-dried IF2 leaf thin section (microscope image on the right) of the high Cd accumulator (T6). The color scale (blue = minimum and red = maximum photon counts) is adjusted to the range of each element. Map A shows the accumulation of Cd in collenchyma cells. The K map indicates the cells that did not collapse during sample preparation and have K inside. Cd is located inside these cells too, contrary to Mn and Zn. Ca and Sr localize on the walls of a big gland. Map B shows the enrichment of many nutrients and Cd around a gland in the collenchyma. Scalebar: 20  $\mu\text{m}$ .



**Figure III.12:** Microscope image and LA-ICP-MS elemental maps of a freeze-dried cross section of IF3 leaves of the high Cd accumulator. During measurement the leaf blade moved and consequently only the vein structure was imaged. The intensity is expressed in total counts (cts). The color scale is adjusted to the range of each element. V.b.: vascular bundle, par: parenchymous tissue. Cd was less enriched in the vascular bundles in the center compared to the surrounding parenchyma and dispersed throughout all parenchymatous tissue. Scalebar: 200  $\mu\text{m}$ .



**Figure III.13:** Microscope image and LA-ICP-MS elemental maps of a freeze-dried cross section of IF3 leaves of the low Cd accumulator. The intensity is expressed in total counts (cts). The color scale is adjusted to the range of each element. V.b.: vascular bundle, par: parenchymous tissue with g: gland, ep: epidermis, and mes: mesophyll. Cadmium intensities were similar in the vascular bundle and parenchymous tissues. There was a notable enrichment around a gland (g) in the parenchyma. In the leaf blade, Cd accumulated more in the mesophyll. Scalebar: 200  $\mu\text{m}$ .

## Appendix IV: Evidence of Cd remobilization via the phloem in cacao seedlings

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### IV.1 Calculation of the isotopic abundances of Cd in nutrient solution

When mixing two isotope sources with two distinct isotopic abundances, the isotopic abundances and ratios of all isotopes in the mixture change. To verify if the change in isotopic abundance was negligible for the  $^{108}\text{Cd}$  isotope enrichment in this study, we calculated the isotopic abundances and ratios theoretically in the nutrient solution with mixing equations (IAEA, 2009) (equation (S1) and (S2)). The two sources were natural Cd and the enriched  $^{108}\text{Cd}$  stock solution (Table S1). The IA values for all Cd isotopes of the enriched  $^{108}\text{Cd}$  stock solution were provided by the manufacturer (Trace Sciences International, Delaware, USA). The fraction of each source ( $f_{\text{natural}}$  and  $f_{\text{enriched}}$ ) was derived from the  $\text{IA}^{108}\text{Cd}$  measurement in the nutrient solution (6.51%). These fractions were then used to calculate the isotopic abundances of the other isotopes under this maximum enrichment of Cd (Table IV.1). The change in isotopic ratio of the isotopes was negligible as shown in Table IV.1 by comparison of the isotope ratios in the rows of 'natural abundance' and 'nutrient solution'. Hence, with a maximum IA  $^{108}\text{Cd}$  of 6.51%, we can safely calculate the isotopic abundance of IA  $^{108}\text{Cd}$  assuming the natural abundance for the non-measured isotopes, i.e. all but the  $^{108}\text{Cd}$  and  $^{111}\text{Cd}$ .

$$f_{\text{natural}} + f_{\text{enriched}} = 1 \quad (\text{IV.1})$$

$$\text{IA}_{\text{mixed}} = \text{IA}_{\text{natural}} \times f_{\text{natural}} + \text{IA}_{\text{enriched}} \times f_{\text{enriched}} \quad (\text{IV.2})$$

## IV.2 Tables

**Table IV.1:** Isotopic abundance (IA, %) and Isotope Ratio (IR, -) of Cd at natural abundance, the  $^{108}\text{Cd}$  enriched stock solution and theoretical values of the nutrient solution (Eqn. (IV.2)).

		$^{106}\text{Cd}$	$^{108}\text{Cd}$	$^{110}\text{Cd}$	$^{111}\text{Cd}$	$^{112}\text{Cd}$	$^{113}\text{Cd}$	$^{114}\text{Cd}$	$^{116}\text{Cd}$
IA (%)	Natural abundance	1.25	0.89	12.49	12.80	24.13	12.22	28.73	7.49
	Enriched stock	0.20	71.10	7.09	4.64	6.98	2.92	6.01	1.06
	Nutrient solution	1.17	<b>6.51*</b>	12.06	12.15	22.76	11.48	26.91	6.98
IR	Natural abundance	0.10	0.07	0.98	1.00	1.89	0.95	2.24	0.59
	Enriched stock	0.04	15.32	1.53	1.00	1.50	0.63	1.30	0.23
	Nutrient solution	0.10	0.54	0.99	1.00	1.87	0.94	2.22	0.57

IA: ( $^{108}\text{Cd}/\text{Cd}$ ), IR: ( $^{108}\text{Cd}/^{111}\text{Cd}$ ) \*The IA $^{108}\text{Cd}$  of the nutrient solution measured was 6.51%. All other values of the nutrient solution are calculated by mixing equations (equation IV.2). The enriched stock was prepared from an enriched  $^{108}\text{Cd}$  stable isotope metal (Trace Sciences International, Delaware, USA).

**Table IV.2:** Total Cd (ng) in each organ an in the plant (sum of total Cd in each organ).

Day		Roots 1			Roots 2			Crown*			Leaf		New leaf		Stem		Plant					
		N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD			
0	(-/-)	3	<b>19.9</b>	17.5	2	<b>19.7</b>	9.6	4	<b>88.1</b>	43.0	4	<b>235.6</b>	63.3	0		4	<b>322.5</b>	141.0	4	<b>680.1</b>	212.3	
1	(+/-)	4	<b>67.8</b>	37.7	4	<b>64.7</b>	54.8	4	<b>190.8</b>	81.5	4	<b>251.9</b>	47.0	0		4	<b>396.9</b>	137.9	4	<b>972.3</b>	312.5	
	(+/+)	4	<b>14.5</b>	8.1	4	<b>16.0</b>	12.1	4	<b>99.8</b>	31.0	4	<b>236.0</b>	50.3	0		4	<b>313.0</b>	76.6	4	<b>679.5</b>	155.1	
7	(+/-)	4	<b>89.7</b>	69.8	4	<b>65.7</b>	9.8	4	<b>462.3</b>	222.9	4	<b>545.8</b>	152.5	0		4	<b>910.9</b>	306.9	4	<b>2074.5</b>	698.6	
	(+/+)	4	<b>75.3</b>	42.8	4	<b>88.4</b>	22.1	4	<b>270.7</b>	203.4	4	<b>399.9</b>	115.8	1	<b>19.5</b>	4	<b>766.9</b>	202.8	4	<b>1601.3</b>	430.2	
11	(+/-)	4	<b>72.5</b>	21.0	2	<b>55.8</b>	20.6	4	<b>483.6</b>	151.8	4	<b>589.1</b>	72.0	4	<b>113.4</b>	59.2	4	<b>985.1</b>	120.8	4	<b>2271.8</b>	324.8
	(+/+)	4	<b>97.3</b>	36.8	4	<b>66.2</b>	8.7	4	<b>469.5</b>	125.6	4	<b>645.1</b>	60.6	4	<b>50.6</b>	24.4	4	<b>1034.1</b>	211.5	4	<b>2363.0</b>	355.8
14	(-/-)	4	<b>62.4</b>	37.5	3	<b>54.5</b>	4.5	4	<b>209.9</b>	199.9	4	<b>380.1</b>	120.5	2	<b>11.6</b>	6.0	4	<b>452.9</b>	349.1	4	<b>1149.5</b>	545.1
	(+/-)	4	<b>94.2</b>	38.9	3	<b>73.2</b>	54.2	4	<b>271.6</b>	119.1	4	<b>440.2</b>	82.5	2	<b>46.0</b>	8.7	4	<b>701.1</b>	301.3	4	<b>1585.1</b>	426.1
	(+/+)	4	<b>78.6</b>	1.7	4	<b>80.7</b>	46.8	4	<b>354.3</b>	126.1	4	<b>579.3</b>	267.6	3	<b>48.8</b>	39.1	4	<b>769.4</b>	168.6	4	<b>1899.0</b>	73.0

\*Total Cd in the crown was calculated under the assumption that the Cd concentration was the same as in the stem (Cd concentration was not measured in the crown).

**Table IV.3:** Fraction (in %) of biomass of each organ compared to total plant biomass (mg DW/mg DW\*100%).

Day		Roots 1			Roots 2			Crown*			Leaf		New leaf			Stem			
		N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
0	(-/-)	3	<b>1.2</b>	1.1	2	<b>1.7</b>	0.4	4	<b>9.9</b>	1.8	4	<b>50.3</b>	4.7				4	<b>37.4</b>	4.4
1	(+/-)	4	<b>3.1</b>	2.0	4	<b>2.3</b>	1.3	4	<b>16.7</b>	2.0	4	<b>42.6</b>	2.3				4	<b>35.3</b>	3.4
	(+/+)	4	<b>1.4</b>	0.7	4	<b>1.5</b>	1.0	4	<b>11.8</b>	1.5	4	<b>47.8</b>	3.5				4	<b>37.4</b>	1.6
7	(+/-)	4	<b>5.1</b>	4.6	4	<b>5.2</b>	3.0	4	<b>16.8</b>	3.1	4	<b>38.1</b>	4.0				4	<b>34.8</b>	2.3
	(+/+)	4	<b>2.6</b>	2.2	4	<b>5.9</b>	2.6	4	<b>12.6</b>	9.6	4	<b>39.9</b>	3.9	1	<b>1.0</b>		4	<b>39.0</b>	5.5
11	(+/-)	4	<b>8.1</b>	4.1	2	<b>5.1</b>	0.3	4	<b>17.7</b>	3.1	4	<b>30.0</b>	2.5	4	<b>5.0</b>	2.8	4	<b>36.7</b>	1.4
	(+/+)	4	<b>9.6</b>	4.1	4	<b>4.2</b>	3.4	4	<b>16.6</b>	4.6	4	<b>32.3</b>	1.6	4	<b>1.5</b>	0.6	4	<b>36.2</b>	3.2
14	(-/-)	4	<b>5.7</b>	3.4	3	<b>6.1</b>	2.6	4	<b>15.3</b>	5.1	4	<b>35.6</b>	7.4	2	<b>0.8</b>	0.1	4	<b>37.8</b>	1.0
	(+/-)	4	<b>6.4</b>	6.0	3	<b>8.4</b>	5.5	4	<b>15.1</b>	3.5	4	<b>31.4</b>	1.5	2	<b>3.1</b>	1.4	4	<b>39.3</b>	2.8
	(+/+)	4	<b>5.3</b>	1.9	4	<b>7.8</b>	5.2	4	<b>16.8</b>	3.5	4	<b>30.5</b>	2.0	3	<b>3.0</b>	2.2	4	<b>37.4</b>	2.1

**Table IV.4:** IA <sup>108</sup>Cd (%) in the different organs for each harvest day for each treatment.

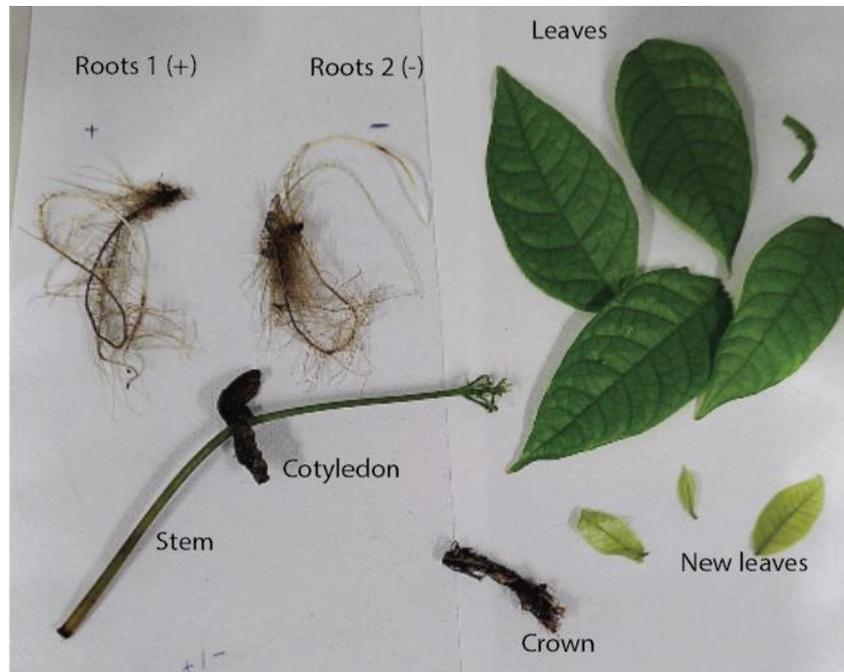
Day	Treatment	Roots 1			Roots 2			Leaf			New leaf		Stem			
		N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
0	(-/-)	3	<b>1.05</b>	0.33	2	<b>0.89</b>	0.26	4	<b>0.87</b>	0.20			4	<b>0.86</b>	0.08	
1	(+/-)	4	<b>4.70<sup>a</sup></b>	0.93	4	<b>0.84<sup>b</sup></b>	0.11	4	<b>0.81<sup>b</sup></b>	0.20			4	<b>1.02<sup>b</sup></b>	0.14	
	(+/+)	4	<b>3.83<sup>a</sup></b>	0.96	4	<b>3.84<sup>a</sup></b>	0.85	4	<b>0.77<sup>b</sup></b>	0.08			4	<b>0.91<sup>b</sup></b>	0.10	
7	(+/-)	4	<b>4.93<sup>a</sup></b>	1.67	4	<b>1.12<sup>b</sup></b>	0.11	4	<b>1.69<sup>b</sup></b>	0.75			4	<b>2.04<sup>b</sup></b>	0.76	
	(+/+)	4	<b>6.13<sup>a</sup></b>	1.40	4	<b>5.99<sup>ab</sup></b>	1.41	4	<b>1.93<sup>c</sup></b>	0.56	1	<b>2.75<sup>abc</sup></b>		4	<b>3.73<sup>bc</sup></b>	0.34
11	(+/-)	4	<b>3.92<sup>a</sup></b>	0.43	2	<b>1.73<sup>b</sup></b>	0.23	4	<b>2.22<sup>b</sup></b>	0.25	4	<b>2.50<sup>b</sup></b>	0.41	4	<b>2.61<sup>b</sup></b>	0.24
	(+/+)	4	<b>5.08<sup>ab</sup></b>	1.08	4	<b>5.72<sup>a</sup></b>	0.37	4	<b>3.75<sup>b</sup></b>	0.55	4	<b>4.08<sup>b</sup></b>	0.86	4	<b>4.43<sup>ab</sup></b>	0.46
14	(-/-)	4	<b>1.02</b>	0.14	3	<b>1.02</b>	0.16	4	<b>0.94</b>	0.14	2	<b>0.98</b>	0.53	4	<b>0.84</b>	0.04
	(+/-)	4	<b>4.05</b>	1.73	3	<b>2.37</b>	0.65	4	<b>2.32</b>	0.69	2	<b>2.52</b>	1.22	4	<b>2.58</b>	0.72
	(+/+)	4	<b>5.73</b>	1.23	4	<b>4.77</b>	1.61	4	<b>3.22</b>	1.03	3	<b>3.96</b>	1.39	4	<b>4.10</b>	0.97

Letters (a-c) denote different statistical groups with the Tukey HSD test ( $p < 0.05$ ).

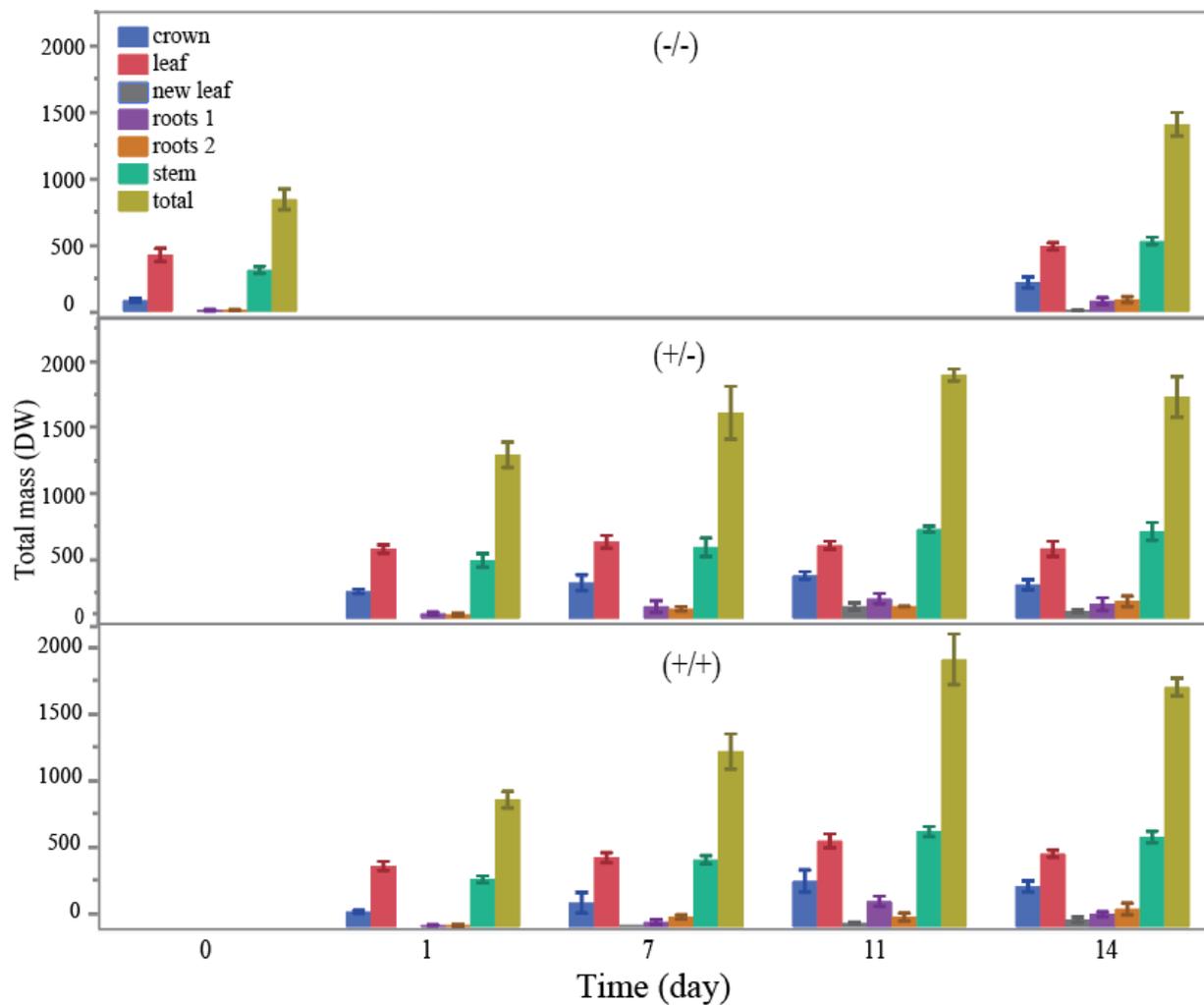
## IV.3 Figures



**Figure IV.1:** Split-root container. Each container contained 330 mL of nutrient solution at the start of the experiment.



**Figure IV.2:** Example of harvested seedling and the corresponding sampled organs for a (+/-) treatment at day fourteen. The cotyledon was discarded. The crown was the interface between stem and roots and where the roots could not be split. When available, newly developing leaves were collected separately from the leaves.



**Figure IV.3:** Biomass (mg/plant, mean,  $n=4$ ) for the different treatments. Error bars represent the standard deviation. The biomass of the total plant and for each organ was tested between the different treatments (for each day separately) with Student's t-test or Tukey HSD post-hoc test. There were no significant differences in growth among the three treatments (-/-), (+/-), and (+/+) ( $p < 0.05$ ).

## Appendix V: Cadmium speciation in cacao beans changes during a fermentation-like incubation

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### V.1 Addition of phytate and cysteine complexation data to the Visual MINTEQ database

Visual MINTEQ is a chemical equilibrium software developed for calculating the speciation, solubility and sorption of metals in natural waters. Phytate complexation data for H<sup>+</sup> was derived from (Bretti *et al.*, 2015). Vanderschueren 2021 describes in detail how the various complexes were entered in the speciation software VMinteq 3.1. The protonation and complexation constants for cysteine with Ca<sup>2+</sup>, Cd<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>, Ni<sup>2+</sup>, Zn<sup>2+</sup> were selected from the NIST SRD 46 database: “Critically Selected Stability Constants of Metal Complexes” (<https://n-hatada.github.io/stability-constant-explorer/english.html>). The complexation constants for Cu<sup>2+</sup>, Fe<sup>2+</sup>, and Fe<sup>3+</sup> were selected from (Berthon, 1995). The protonation and complexation constants, typically measured at I = 0.1 – 0.25 M, were corrected to infinite dilution with the Davies equation and its parameters at 298 °K with following equation:

$$\log K_i = \log K^0 + 0.5108 * z^* \left( \frac{\sqrt{I}}{1 + \sqrt{I}} - 0.3286 I \right)$$

$$z^* = \sum (charges)_{react}^2 - \sum (charges)_{prod}^2$$

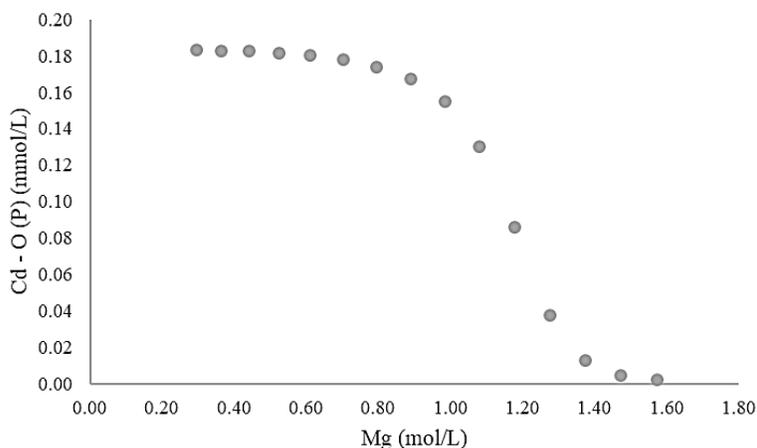
With K<sub>i</sub> the complexation constant at infinite dilution, and K<sup>0</sup> the raw complexation constant at the measured ionic strength. The corrected log K constants were entered into the speciation software VMinteq 3.1.

## V.2 Tables and Figures

**Table V.1:** Input concentrations (mM) for the chemical equilibrium calculation in Visual MINTEQ for the high Cd beans (H3-H4) and low Cd beans (L1-L2). The input was based on the average elemental composition of cacao nibs prior incubation ( $\text{mg kg}^{-1}$  DW). Dry weight element concentrations were converted to concentrations in solution based on the solid:liquid ratio measured in the fresh cacao nibs of  $1:0.09 \text{ g mL}^{-1}$ . The pH was based on measured nib pH (Table 6.1); i.e. for H3-H4 pH changed from 6.7 to 4.1 and for L1-L2 from 7.0 to 4.1. The phytate content before and after incubation was used for H3-H4. For L1-L2, the average P-phytate content was estimated as 85% of total P in the nibs is P-phytate as observed in (Chapter 3), and assuming a similar rate of phytate breakdown as in the H1-H2. The other assumptions for the input into the model can be found in the materials and methods section.

	H3-H4		L1-L2	
	$\text{mg kg}^{-1}$ DW	mM	$\text{mg kg}^{-1}$ DW	mM
Cd	8.90	0.88	1.89	0.19
Mn	31.00	6.26	14.00	2.83
Fe <sup>2+</sup>	15.50	3.15	11.00	0.39
Fe <sup>3+</sup>	15.50	3.15	11.00	4.37
Ni	4.40	0.86	5.40	1.05
Cu	24.00	4.17	18.00	3.13
Zn	45.00	7.69	41.00	7.01
Cysteine	89.00	8.20	15.97	0.82
	$\text{g kg}^{-1}$ DW	M	$\text{g kg}^{-1}$ DW	mM
Mg scenario <b>A</b> *	3.92	1.81	3.90	1.80
Mg scenario <b>B</b> *	2.16	1.00	2.16	1.00
K	10.75	3.06	11.69	3.33
Ca	1.03	0.29	13.55	0.38
Phytate before incubation	18.79	0.32	17.70	0.30
<i>Phytate after incubation</i>	98.99	0.17	11.77	0.20
Oxalate	4.50	0.56	4.50	0.56
Lactate before incubation	0.00	0.00	0.00	0.00
<i>Lactate after incubation</i>	1.00	0.12	1.00	0.12

\* The predicted Cd speciation was sensitive to the assumed Mg concentrations (Fig. V.1), therefore two scenarios with different Mg concentrations were modelled. **A**: available Mg in the nibs estimated from total nib Mg concentration and **B** available Mg in the nibs estimated factor 1.8 lower than in A.



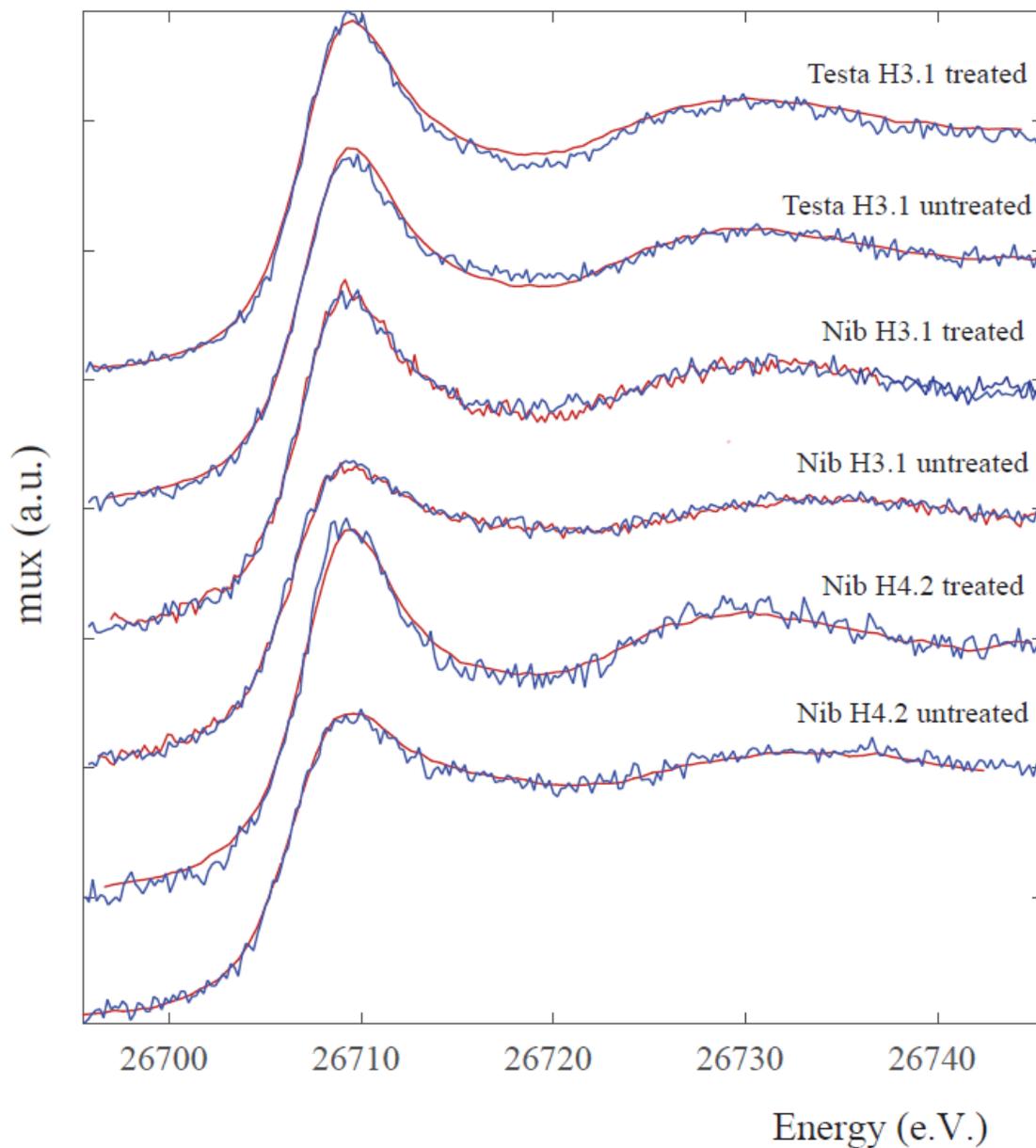
**Figure V.1:** Cd-phytate concentration in function of Mg concentration exemplified for the low Cd beans L3-L4 before incubation. There is a strong effect of Mg concentration on the complexation of Cd with phytate. Therefore, cadmium speciation was modelled at two different Mg concentrations (scenario A: 1.0 M and scenario B: 1.8 M).

**Table V.2:** Nib concentration ratios [treated (mg kg<sup>-1</sup> DW)/untreated (mg kg<sup>-1</sup> DW)] for all measured elements.

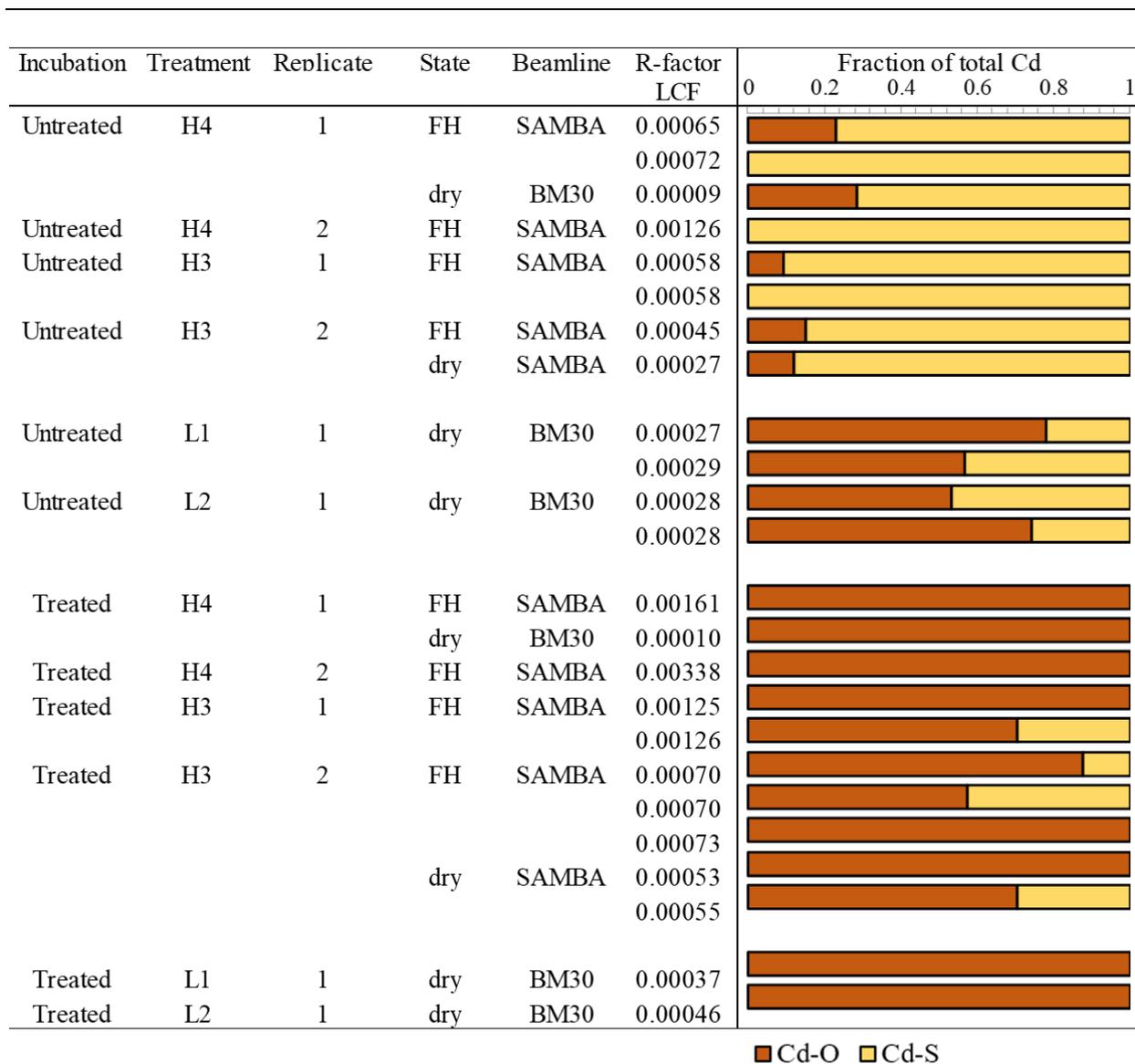
	H1		H2		H3		H4		L1		L2	
	Mean	SD										
Mg	<b>0.95</b>	0.09	<b>1.03</b>	0.06	<b>0.63</b>	0.11	<b>0.28</b>	0.07	<b>0.54</b>	0.09	<b>0.09</b>	0.01
P	<b>1.00</b>	0.09	<b>1.02</b>	0.06	<b>0.66</b>	0.10	<b>0.38</b>	0.04	<b>0.58</b>	0.08	<b>0.25</b>	0.04
S	<b>1.03</b>	0.07	<b>0.95</b>	0.02	<b>0.91</b>	0.07	<b>0.70</b>	0.12				
K	<b>0.77</b>	0.11	<b>0.99</b>	0.06	<b>0.46</b>	0.09	<b>0.06</b>	0.05	<b>0.33</b>	0.03	<b>0.09</b>	0.01
Ca	<b>1.05</b>	0.22	<b>1.05</b>	0.38	<b>1.10</b>	0.31	<b>1.51</b>	0.45	<b>1.02</b>	0.59	<b>1.25</b>	0.35
Mn	<b>1.19</b>	0.30	<b>0.93</b>	0.17	<b>0.80</b>	0.13	<b>0.55</b>	0.07	<b>0.77</b>	0.07	<b>0.33</b>	0.07
Fe	<b>1.10</b>	0.11	<b>0.99</b>	0.09	<b>0.80</b>	0.07	<b>0.76</b>	0.09	<b>1.19</b>	0.36	<b>0.98</b>	0.12
Co	<b>0.86</b>	0.27	<b>0.93</b>	0.17	<b>0.62</b>	0.10	<b>0.34</b>	0.18	<b>0.52</b>	0.10	<b>0.16</b>	0.05
Ni	<b>0.82</b>	0.12	<b>0.96</b>	0.09	<b>0.63</b>	0.04	<b>0.25</b>	0.08	<b>0.60</b>	0.20	<b>0.24</b>	0.02
Cu	<b>0.94</b>	0.15	<b>1.08</b>	0.04	<b>0.89</b>	0.12	<b>0.56</b>	0.11	<b>1.03</b>	0.04	<b>1.13</b>	0.08
Zn	<b>0.96</b>	0.17	<b>1.05</b>	0.09	<b>0.82</b>	0.07	<b>0.68</b>	0.12	<b>1.06</b>	0.31	<b>0.38</b>	0.08
Sr	<b>1.11</b>	0.37	<b>1.03</b>	0.37	<b>1.15</b>	0.28	<b>1.23</b>	0.45				
Mo	<b>1.06</b>	0.18	<b>0.93</b>	0.17	<b>1.00</b>		<b>0.93</b>	0.25	<b>1.42</b>	0.12	<b>1.31</b>	0.05
Cd	<b>0.94</b>	0.11	<b>1.05</b>	0.04	<b>0.84</b>	0.12	<b>0.66</b>	0.12	<b>0.9</b>	0.12	<b>0.64</b>	0.09

**Table V.3:** Testa concentration ratios [treated (mg kg<sup>-1</sup> DW)/untreated (mg kg<sup>-1</sup> DW)] for all measured elements.

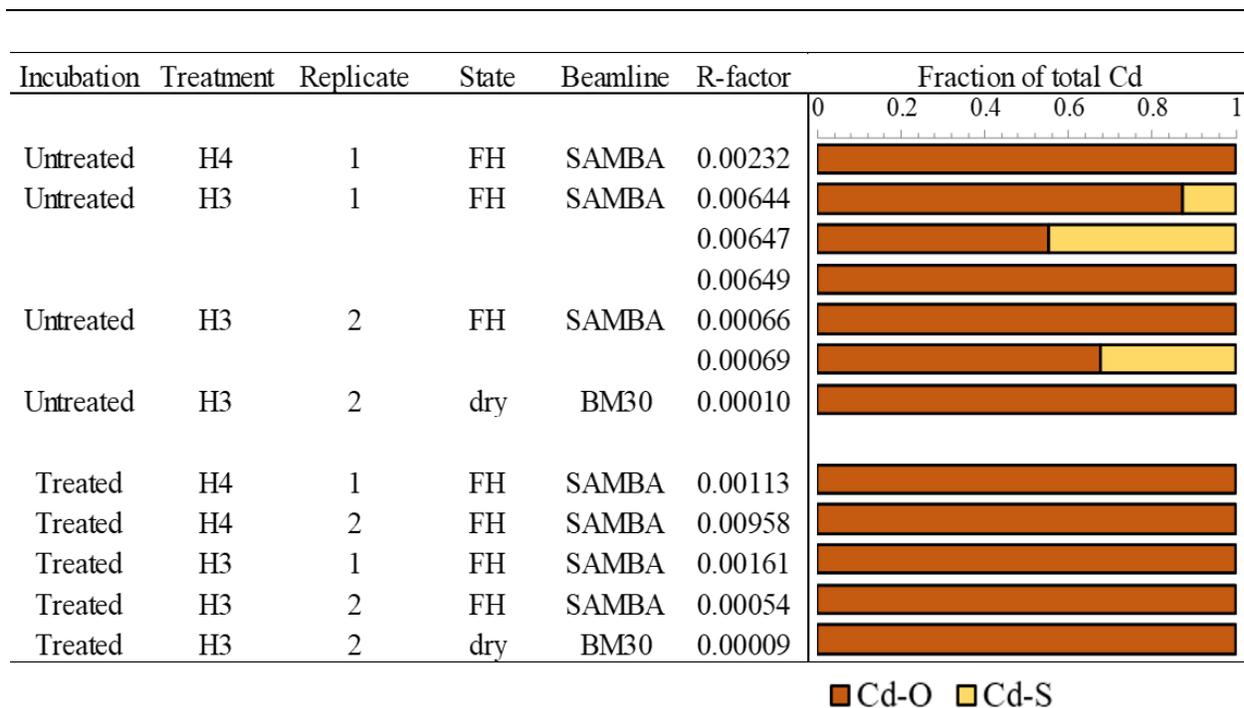
	H1		H2		H3		H4		L1		L2	
	Mean	SD										
Mg	<b>1.96</b>	0.3	<b>0.53</b>	0.15	<b>3.01</b>	0.71	<b>1.45</b>	0.96	<b>4.9</b>	0.61	<b>2.49</b>	0.27
P	<b>0.98</b>	0.23	<b>1</b>	0.48	<b>4.73</b>	0.27	<b>2.28</b>	0.18	<b>6.68</b>	1.96	<b>3.25</b>	0.02
K	<b>0.89</b>	0.17	<b>0.41</b>	0.06	<b>0.76</b>	0.17	<b>0.32</b>	0.21	<b>0.99</b>	0.12	<b>0.45</b>	0.06
Ca	<b>2.24</b>	0.62	<b>2.22</b>	1.02	<b>2.76</b>	0.89	<b>1.7</b>	0.35	<b>0.71</b>	0.08	<b>2.45</b>	1.82
Mn	<b>1.61</b>	0.27	<b>0.77</b>	0.27	<b>2.7</b>	0.73	<b>1.68</b>	0.86	<b>5.66</b>	0.61	<b>3.82</b>	0.57
Fe	<b>1.73</b>	0.35	<b>1.86</b>	0.41	<b>2.12</b>	0.84	<b>0.97</b>	0.16	<b>0.82</b>	0.05	<b>1.07</b>	0.22
Co	<b>4.05</b>	1.45	<b>1.2</b>	0.17	<b>2.64</b>	0.69	<b>3.21</b>	2.61	<b>5.68</b>	2.13	<b>3.34</b>	0.29
Ni	<b>3.46</b>	2.07	<b>1.22</b>	0.18	<b>3.01</b>	0.51	<b>3</b>	2.29	<b>3.4</b>	1.48	<b>2.11</b>	0.35
Cu	<b>3.01</b>	0.9	<b>2.47</b>	0.52	<b>3.21</b>	0.29	<b>2.58</b>	0.5	<b>2.02</b>	0.46	<b>1</b>	0.21
Zn	<b>1.72</b>	0.17	<b>1.72</b>	0.44	<b>3.55</b>	0.59	<b>4.21</b>	1.95	<b>3.54</b>	0.07	<b>4.61</b>	0.23
Sr	<b>2.13</b>	0.47	<b>1.98</b>	0.78	<b>2.69</b>	0.47	<b>1.31</b>	0.27				
Mo	<b>1.72</b>	0.16	<b>2.74</b>	0.73	<b>2.03</b>	0.57	<b>2.37</b>	1.5	<b>1.34</b>	0.37		
Cd	<b>3.04</b>	0.55	<b>1.43</b>	0.29	<b>2.42</b>	0.07	<b>2.49</b>	1.16	<b>2.77</b>	0.7	<b>1.84</b>	0.07



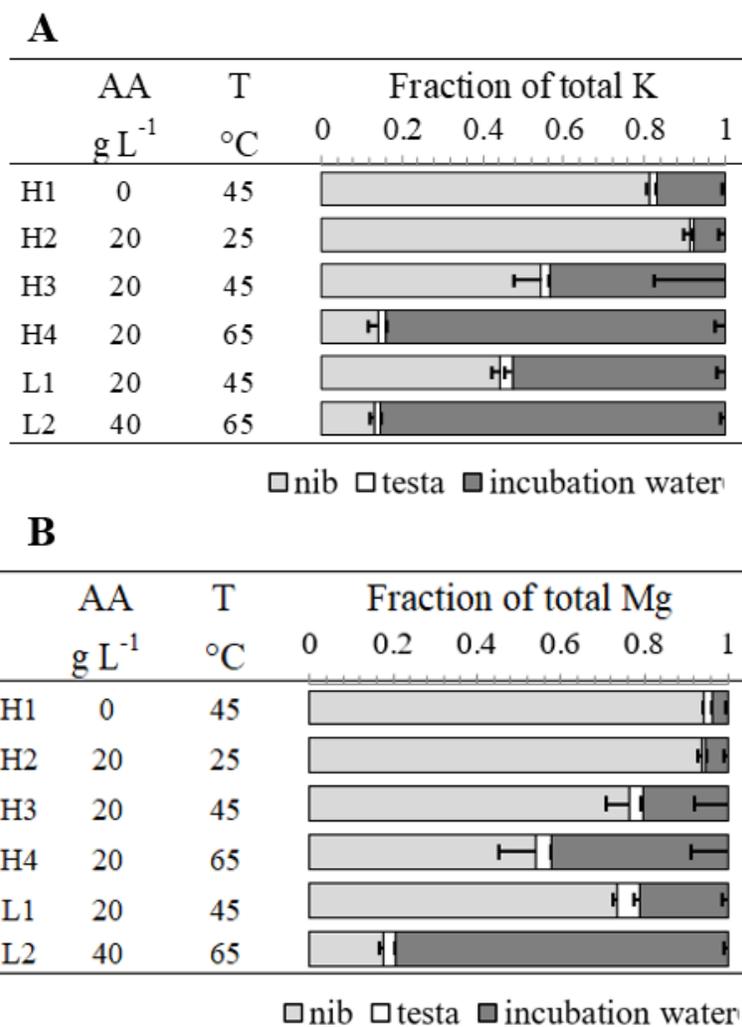
**Figure V.2:** Comparison of X-Ray Absorption Spectra of different sample preparation methods. Red spectra represent the normalized absorption spectra of oven-dried aliquots, blue spectra of the frozen-hydrated aliquots. The spectra for the dried samples show less noise than the spectra for the frozen hydrated samples.



**Figure V.3:** Proportion of Cd species in nibs determined by XANES. Dark brown represents Cd-O ligands, light yellow Cd-S ligands. Linear combination fits were done in the  $[E_0-20; E_0+80]$  eV range. Fit quality was examined by the R-factor. If the R-factor increases  $<10\%$ , the fits are of equivalent quality and are shown in the figure. Samples were either measured at ESRF, beamline BM30 or synchrotron Soleil, beamline SAMBA.



**Figure V.4:** Proportion of Cd species in testa determined by XANES. Dark brown represents Cd-O ligands, light yellow Cd-S ligands. Linear combination fits were done in the  $[E_0-20; E_0+80]$  eV range. Fit quality was examined by the R-factor. If the R-factor increases  $<10\%$ , the fits are of equivalent quality and are shown in the figure.



**Figure V.5:** Potassium (A) and Magnesium (B) distribution between the nib, testa, and incubation solution after incubation. Higher temperature/ acetic acid conditions promote mobilization of Mg and K from the nib to the incubation solution. AA= dose acetic acid and T= incubation temperature. The error bars indicate the standard deviation of the replicate measurements ( $n=2$ , H1-H4).

## List of Publications

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Aucour, A.M., Sarret, G., **Blommaert, H.** and Wiggemhauser, M. (2023) 'Coupling metal stable isotope compositions and X-ray absorption spectroscopy to study metal pathways in soil-plant systems: a mini review', *Metallomics*, p. mfad016. Available at: <https://doi.org/10.1093/mtomcs/mfad016>.

Argüello, D., Chavez, E., Gutierrez, E., Pittomvils, M., Dekeyrel, J., **Blommaert, H.** and Smolders, E. (2023) 'Soil amendments to reduce cadmium in cacao ( *Theobroma cacao* L.): A comprehensive field study in Ecuador.', *Chemosphere*, 324(March), p. 138318. Available at: <https://doi.org/10.1016/j.chemosphere.2023.138318>.

Vanderschueren, R., Wantiez, L., **Blommaert, H.**, Flores, J., Chavez, E. and Smolders, E. (2023) 'Revealing the pathways of cadmium uptake and translocation in cacao trees (*Theobroma cacao* L.): A <sup>108</sup>Cd pulse-chase experiment', *Science of The Total Environment*, 869(November 2022), p. 161816. Available at: <https://doi.org/10.1016/j.scitotenv.2023.161816>.

**Blommaert, H.**, Aucour, A., Wiggemhauser, M., Moens, C., Telouk, P., Landrot, G., Testemale, D., Pin, S., Lewis, C., Umaharan, P., Smolders, E. and Sarret, G. (2022) 'From soil to cacao bean : Unravelling the pathways of cadmium translocation in a high Cd accumulating cultivar of *Theobroma cacao* L.', *Frontiers in Plant Science*, (December), pp. 1–19. Available at: <https://doi.org/10.3389/fpls.2022.1055912>.

Vanderschueren, R.<sup>1</sup>, Argüello, D.<sup>1</sup>, **Blommaert, H.**<sup>1</sup>, Montalvo, D., Barraza, F., Maurice, L., Schreck, E., Schulin, R., Lewis, C., Vazquez, J.L., Umaharan, P., Chavez, E., Sarret, G. and Smolders, E. (2021) 'Mitigating the level of cadmium in cacao products: Reviewing the transfer of cadmium from soil to chocolate bar', *Science of the Total Environment*, 781, p. 146779. Available at: <https://doi.org/10.1016/j.scitotenv.2021.146779>.

Sarret, G., **Blommaert, H.** and Wiggemhauser, M. (2021) 'Comment on "Speciation and fate of toxic cadmium in contaminated paddy soils and rice using XANES/EXAFS spectroscopy"', *Journal of Hazardous Materials*, 401, pp. 2018–2020. Available at: <https://doi.org/10.1016/j.jhazmat.2020.123240>.

Wiggemhauser, M., Aucour, A., Telouk, P., **Blommaert, H.** and Sarret, G. (2021) 'Changes of Cadmium Storage Forms and Isotope Ratios in Rice During Grain Filling', *Frontiers in Plant Science*, 12(April), pp. 1–18. Available at: <https://doi.org/10.3389/fpls.2021.645150>

Argüello, D., Montalvo, D., **Blommaert, H.**, Chavez, E. and Smolders, E. (2020) 'Surface soil liming reduces cadmium uptake in cacao seedlings but subsurface uptake is enhanced', *Journal of Environmental Quality*, 49(5), pp. 1359–1369. Available at: <https://doi.org/10.1002/jeq2.20123>

Blommaert, M.A., Verdonk, J.A.H., **Blommaert, H.**, Smith, W.A. and Vermaas, D.A. (2020) 'Reduced Ion Crossover in Bipolar Membrane Electrolysis via Increased Current Density, Molecular Size, and Valence', *ACS Applied Energy Materials*, 3(6), pp. 5804–5812. Available at: <https://doi.org/10.1021/acsaem.0c00687>.

Quaghebeur, W., Mulhern, R.E., Ronsse, S., Heylen, S., **Blommaert, H.**, Potemans, S., Valdivia Mendizábal, C. and Terrazas García, J. (2019) 'Arsenic contamination in rainwater harvesting tanks around Lake Poopó in Oruro, Bolivia: An unrecognized health risk', *Science of the Total Environment*, 688(June), pp. 224–230. Available at: <https://doi.org/10.1016/j.scitotenv.2019.06.126>.