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Calcium oxalate crystals in cacao trees and their interactions with cadmium

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ABSTRACT

Cadmium (Cd) concentrations in cacao beans from Latin America often exceed limits for trading. A better understanding of the mechanisms of Cd accumulation in *Theobroma cacao* L. trees is necessary to advance mitigation strategies. Recent studies on a high Cd accumulating cultivar of cacao revealed that calcium oxalate (CaOx) crystals were involved in Cd accumulation in the branches. The purpose of this study was to quantify soluble and crystalline oxalate in cacao compartments, to characterize their morphology and distribution in the tissues, and to evaluate the relationship between CaOx and Cd total concentrations in each plant compartment. Two representative cultivars from Latin America (CCN-51 and Nacional) were studied. CCN-51 trees grew on soils with low and high total Cd contents (0.120 ± 0.002 and 2.59 ± 0.48 mg kg⁻¹, respectively), and Nacional trees grew on soils with low Cd content (0.188 ± 0.005 mg kg⁻¹). Oxalate was present in all organs of the two cultivars. In mature leaves, oxalate content exceeded the limit of 5% per dry weight used to define extreme oxalate accumulators. The crystalline form predominated in branches and mature leaves (82–92%), whereas the soluble form predominated in nibs (67–82%). Calcium oxalate crystal size varied from <1 µm (generally agglomerated as crystal sand) to a few tens of µm (faceted crystals). Log CaOx and Cd concentrations were positively correlated in branches ($R^2 = 0.77$, $p = 0.002$) and roots ($R^2 = 0.71$, $p = 0.005$), whereas in nibs, the oxalate content was almost constant among conditions. The possible roles of CaOx crystals in the cacao plant, including Ca regulation, protection against herbivory, tissue stiffening and Cd detoxification are discussed.

1. Introduction

Cadmium is toxic for most forms of life (Maret and Moulis, 2013). Its presence in the environment leads to its transfer along trophic chains and to human exposure. Food is the main route of exposure to Cd for the general population (EFSA, 2009; EFSA, 2011; EFSA, 2012). The main contributors are potatoes and wheat-derived products. Chocolate products are estimated to account for 4% of total dietary Cd exposure for adults and 9% for children, so they represent a higher risk for vulnerable populations such as children (EFSA, 2012). For this reason, the EU set a limit for Cd concentration in chocolates and cacao powders between 0.1 and 0.8 mg Cd kg⁻¹, depending on the cacao solids content of the product (ref No 488/2014). The cacao processing industry has translated the EU limits into 0.5–1.1 mg Cd kg⁻¹ in beans (Vanderschueren et al., 2021), and the value of 0.6 mg Cd kg⁻¹ is generally used in publications (Arguello et al., 2019) (and refs therein). This new

regulation threatens the sustainability of cacao farmers in South America, especially Ecuador, the third largest exporter worldwide of cacao beans (Vázquez-deCastro et al., 2023).

Various mitigation strategies are currently being evaluated to limit the transfer of Cd at various stages of its journey from the soil to the chocolate bar (Vanderschueren et al., 2021). Cultivar selection and plant breeding are one of them, but more knowledge on the mechanisms involved in Cd accumulation and detoxification is required. A recent study on a high Cd accumulating cacao cultivar showed that the branches were a major compartment for Cd accumulation (Blommaert et al., 2022). Cadmium in branches was primarily bound to carboxyl-ligands (60–100%) and mainly localized in the phloem rays and phelloderm of the bark. Calcium oxalate crystals were observed in branches, roots and leaves (Blommaert et al., 2024). A fraction of Cd has been suggested to associate with these crystals, so a role in Cd detoxification was suggested.

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Calcium oxalate (CaOx) crystals exert various functions in plants, including calcium regulation, improvement of the tissue stiffness, light reflectance and photosynthesis, and protection against herbivory (Franceschi and Nakata, 2005; Khan et al., 2023). In plant tissues, oxalate generally occurs in soluble form and in crystalline form, i.e., CaOx mono and dihydrate (He et al., 2024). In food products, oxalate is considered as an anti-nutritional factor because it limits the absorption of essential nutrients such as Ca and Fe (He et al., 2024; Noonan and Savage, 1999). In addition, oxalate-rich food increases the risk of formation of kidney stones (He et al., 2024; Noonan and Savage, 1999). Calcium oxalate crystals have been reported in cacao tissues (Blommaert et al., 2024; de Oliveira et al., 2007), and cacao bean and cacao powder are known to be rich in oxalate (Aremu et al., 1995; Nguyen et al., 2018; Schroder et al., 2011; Siener et al., 2021). However, there is no data on the oxalate and CaOx content in the various parts of the cacao plant, and on their relationship with Cd.

The purpose of this study was to quantify the role of oxalate and CaOx crystals in cacao trees, and in particular to evaluate their role in Cd storage and detoxification. Two cultivars of major economic importance in Ecuador, *Nacional* and *CCN-51* (Jaimez et al., 2022) were studied. Elemental concentration, as well as oxalate concentrations in soluble and crystalline form were determined in the cacao tree organs (roots, branches, and leaves) and cacao nib. The relationship between oxalate and Cd contents was evaluated. The morphology and distribution of CaOx crystals was studied at the scale of the tissues and cells. The importance of CaOx crystals in cacao trees and their putative roles were then discussed.

2. Materials and methods

2.1. Sites description and sampling on sites

The sampling was carried out in September 2022, in two neighbouring farms (Hacienda 1 and 2) in Guayas province, in Ecuador. Hacienda 1 produces the cultivar *Castro Naranjal Collection* (CCN-51), a hybrid variety very productive and used on a large scale in Ecuador (Rottiers et al., 2019). Hacienda 2 produces the variety *Nacional* (Nac), famous for its fine flavour and appreciated on international markets (Rottiers et al., 2019). Three conditions were studied: CCN-51 and Nac trees growing in soils with low total Cd content and CCN-51 trees growing in soil with high total Cd content (CCNG, Nac and CCNY, respectively, total soil Cd content: 0.102 ± 0.002 , 0.188 ± 0.005 and 2.59 ± 0.48 mg kg⁻¹, mean $\pm$ standard deviation of replicates). For comparison, a national survey of cacao farms in Ecuador showed a mean Cd concentration (digested in boiling aqua regia) of 0.44 mg kg⁻¹, a median of 0.33 mg kg⁻¹, and a range from 0.02 to 6.90 mg kg⁻¹ (Arguello et al., 2019). For comparison, 0.3 mg kg⁻¹ is also the mean Cd concentration in non-polluted European agricultural soils (McLaughlin et al., 2021). For each condition, we sampled three biological replicates (trees), about 5–10 m apart. For each replicate, the following samples were collected: topsoil (0–30 cm) at 50 cm from the trunk using an auger, roots (<5 mm in diameter) collected with a shovel, branches (4–8 mm in diameter), young reddish leaves called Flush-2 (F2) leaves and mature leaves called Interflush-3 (IF3) leaves based on the growth rhythm of cacao trees (Greathouse et al., 1971), and cacao nibs (seeds after removal of the envelope also called testa). Roots were rinsed in 20 mM EDTA solution for 10 min and rinsed with water, to remove elements externally adsorbed and/or precipitated. Branches and leaves were rinsed with ultrapure water. Fresh weights were determined. Plant and soil samples were then dried at 65 °C, weighted, and shipped to France.

2.2. Soil characterization, elemental content in soil and in cacao organs

Soil samples were manually ground with a mortar and pestle, sieved through a 2 mm mesh, and finally ground to a fine powder with a

mechanical grinder (Pulverizette 7 Premium Line, Fritsch, Germany). Soil pH was measured in 0.01 M CaCl₂, 1:5 soil to liquid (w:v). Total and organic C (%C_{tot} and %C_{org}) were determined with a Vario MAX cube (Elementar, France) and a Vario TOC cube (Elementar, France), respectively. Soil samples were digested in several steps (nitric acid (HNO₃) + hydrofluoric acid (HF)) before Inductively Coupled Plasma Mass Spectrometry analysis (Agilent 7900 ICP-MS, Agilent, Germany). For more information on the soil digestion, the reader is referred to section 1.1 in the SI.

Dry branches, roots, and young leaves (F2 leaves) and mature leaves (IF3 leaves) were ground to a fine powder like for the soils. The cacao nibs were ground in a coffee mill and sieved with 2 mm-mesh. Plant samples were digested in several steps before ICP-MS analysis. For more information on the plant digestion, the reader is referred to section 1.2 in the SI.

Solutions from soil and plant digestion were then analysed (Cd, Zn, Mn, Ni, Ca, P and Fe) with an ICP-MS. For quality assurance, elemental concentrations were determined in the certified reference materials (CRM: lichen: IAEA-336, cabbage: BCR-679, and San Joaquin soil: SMR-2709a) (Tables S1A and B).

2.3. Oxalate content in cacao organs

Soluble oxalate and total oxalate concentrations were determined in the various cacao organs based on a protocol adapted from (Ross et al., 1999; Holloway et al., 1989). 40 mg of ground and dry samples were mixed with 2 mL distilled water (for soluble oxalate) and with 2 M HCl (Hydrochloric acid, for total oxalate), following a 1:50 plant to liquid (w: v) ratio. The water and HCl suspensions were placed for 20 min in a water bath at 80 °C with regular mixing, then centrifuged for 15 min. The supernatant was then filtered (0.45 µm, Sterile Syringe Filter, Membrane Solution®), and analysed by High Performance Ionic Chromatography (Dionex Integron HPIC, Thermo Scientific, USA). Standard solutions containing 0, 1, 10 and 50 mg L⁻¹ of oxalic acid (Aldrich) were used for calibration. Standards were also prepared in 2 M HCl. Calibration curves with standards prepared in aqueous solution and in 2M HCl were identical, so there was no matrix effect due to the 2 M HCl extractant. For quality assurance, oxalic acid (Aldrich) powders from two different packages were used, one for the preparation of the standards and the other for the preparation of the controls (Table S1C). The concentration in crystalline oxalate in the plant samples was calculated with the following equation (1), as detailed in (Holloway et al., 1989):

$$[\text{Oxalate}]_{\text{crystal}} = [\text{Oxalate}]_{\text{total}} - [\text{Oxalate}]_{\text{soluble}} \quad (1)$$

In this article, oxalate concentrations are expressed in mass or molar concentrations of oxalate per dry weight (DW), mass concentrations of oxalate per fresh weight (FW) (calculated based on FW/DW ratios), and % dry weight for the crystalline oxalate, supposing it is CaOx monohydrate (146.1 g mol⁻¹) or dihydrate (164.1 g mol⁻¹).

2.4. Observations of plant organs and crystals by SEM-EDX

Cross-sections of branches and roots, 1–2 mm thick, were made with a microtome blade. For Scanning Electron Microscope Energy Dispersive X-ray (SEM-EDX Vega3, Tescan, Czech Republic), sections were placed on a SEM stub, and covered by a thickness of 20–30 nm of carbon from a carbon metallizer (Cressington Carbon coater 108 carbon/A), before being observed by SEM coupled to an EDS analyser (SMAX), with an acceleration voltage of 16 keV. We analysed a cross section of root and of branch of each biological replicate. The abundance of crystals observed was quantified by observation on all SEM images (Table 2).

Atomic ratios Mg/Ca, P/Ca and K/Ca in the CaOx crystals and sands were calculated with the following equation (in the case of Mg) (2), based on the atomic percentages calculated from an internal standard.

$$[\text{A}\%]_{\text{Mg/Ca}} = [\text{A}\%]_{\text{Mg}} / [\text{A}\%]_{\text{Ca}} \quad (2)$$

2.5. Statistical analyses

The Shapiro-Wilk test was used to test the normal distribution of residuals and the O'Brien and Brown-Forsythe tests for equality of variances. One-way analysis of (ANOVA) was used to compare means between conditions. Statistical significance was set at an alpha of 5%. For validated cases, the posthoc Tukey test was applied to differentiate between groups. Simple linear regression ($p < 0.05$) was used to test the relationship between Cd and CaOx concentrations. All statistical analyses were carried out using JMP® (v 18.0.1).

3. Results

3.1. Soil characteristics

The soils from the two farms differed in pH, from acidic for CCNG (pH 4.01 ± 0.14) to slightly alkaline for Nac (pH 6.50 ± 0.28) and alkaline for CCNY (pH 7.34 ± 0.04) (Table 1). CCNY soil was characterised by a high Cd concentration (2.59 mg kg^{-1} compared to 0.10 and 0.19 mg kg^{-1} for CCNG and Nac, respectively), and a high Ca concentration (32.6 g kg^{-1} compared to 3.4 and 9.2 mg kg^{-1} for CCNG and Nac, respectively). In brief, CCNY soil has a higher phytoavailability for Cd and Ca, a lower phytoavailability for the essential micronutrients Fe, Mn and Ni than CCNG and Nac soils, and a similar phytoavailability of Zn than CCNG but lower than Nac.

3.2. Total elemental and oxalate concentrations in the plant parts

The total Cd concentrations in the cacao plant parts have been measured for CCNG, CCNY and Nac (Fig. 1). The cacao trees in the CCNY conditions show markedly higher Cd accumulation compared to CCNG and Nac (Table S2). Branches are the mains compartment for CCNG, branches, nibs and mature leaves are the mains compartment for CCNY and branches and mature leaves are the mains compartment for Nac. CCNY branches and mature leaves (5.70 ± 0.43 , $5.92 \pm 0.53 \text{ mg kg}^{-1}$) have 10- to 13-fold higher Cd than CCNG (0.52 ± 0.24 , $0.46 \pm 0.12 \text{ mg kg}^{-1}$) and Nac (0.46 ± 0.03 , $0.44 \pm 0.08 \text{ mg kg}^{-1}$). The biggest difference in Cd concentrations was observed between the CCNY nibs (4.83 ± 0.92) with 32-fold higher Cd than CCNG ($0.15 \pm 0.02 \text{ mg kg}^{-1}$) and Nac ($0.15 \pm 0.02 \text{ mg kg}^{-1}$). For CCNG, Cd concentrations decrease in the order: branches > roots = mature leaves = young leaves > soils = nibs, whereas for CCNY, it follows the order of branches = nibs = mature leaves > young leaves > soils = roots, and for Nac, it follows the order of branches = mature leaves > young leaves > roots > soils = nibs.

Crystalline and soluble oxalate were present in all the cacao organs analysed (Fig. 2). For the three conditions, total oxalate content decreased in the order: branches and mature leaves > young leaves > roots > nibs. The total oxalate content in mature leaves (89.8 ± 17.6 , 63.3 ± 9.5 , and $87.9 \pm 5.0 \text{ g kg}^{-1}$ DW for CCNY, CCNG, and Nac, Fig. 2, Tables S4 and S5) was above the limit of 5% DW defined for extreme oxalate accumulators (Libert and Franceschi, 1987). In branches and mature leaves, crystalline oxalate was predominant (84–92% and 82–92% of total oxalate, respectively, Table S4). The roots of CCNY and Nac also contained more crystalline oxalate (78 and 73% or total

Table 2

Qualitative summary of crystalline CaOx crystals in the branches and roots of the three conditions.

	Nac	CCNG	CCNY
Branches			
Periderm	++	++	+++
Phloem	++	++	+++
Xylem	+	+	++
Medulla	+	+	+
Roots			
Periderm	+	+	+
phloem	+	+	+
xylem	0+	0	0+

The abundance of the crystals, evaluated by observation of two cross sections of branch or root for two biological replicates, ranged from 0 (absent) to 0+ (almost absent), + (few), ++ (abundant) and +++ (very abundant).

oxalate, Table S4). The roots of CCNG and the young leaves for all conditions contained similar amounts of both forms. In the nibs, soluble oxalate was predominant (67–82% of total oxalate, Table S4), and relatively constant among conditions (4.9 ± 0.2 , 4.5 ± 0.3 and $3.8 \pm 0.3 \text{ g kg}^{-1}$ DW for CCNG, CCNY, and Nac, respectively, Table S4). The comparison between the three conditions showed that the roots and branches of CCNY contained significantly (2–3 times) more CaOx than those of CCNG and Nac (values in Table S4).

The relationship between oxalate (either in crystalline or in soluble form) and Ca was evaluated (Fig. 3). Soluble oxalate did not correlate with Ca ($R^2 = 0.03$, $p = 0.27$). In contrast, a strong correlation between crystalline oxalate and Ca was found ($R^2 = 0.92$, $p < 0.0001$). The fitted function was close to a $y = x$ function ($y = 1.14x - 40.08$) (Fig. 3), which corresponds to the stoichiometry of CaOx (monohydrate and dihydrate), with one oxalate molecule per Ca atom. This observation strongly suggests that oxalate crystals correspond to CaOx. For the points situated below this $y = x$ function, Ca is in excess compared to the composition of CaOx. For the points situated above the $y = x$ function, Ca is in deficit compared to the composition of CaOx. It is hypothesized that crystalline oxalate measured in this study contains Ca but also other cations (see SEM-EDX analyses).

Contrary to Ca and oxalate, no linear correlation was found between Cd and oxalate when all organs were considered together (Fig. S1). It should be noted that oxalate and Cd molar concentrations differ by three to five orders of magnitude (Tables S2 and S3). However, correlations were found with the log transformed concentrations, in each organ separately (Fig. 4). A positive correlation was observed between crystalline oxalate and Cd in roots ($R^2 = 0.71$, $p = 0.005$) and branches ($R^2 = 0.77$, $p = 0.002$). There was no correlation in young leaves ($R^2 = 0.15$, $p = 0.300$), mature leaves ($R^2 = 0.24$, $p = 0.183$), and nibs ($R^2 = 0.21$, $p = 0.215$). Likewise, a positive correlation was observed between Ca and Cd in roots ($R^2 = 0.79$, $p = 0.001$), branches ($R^2 = 0.81$, $p = 0.001$), and mature leaves ($R^2 = 0.51$, $p = 0.032$), not in young leaves and nibs (Fig. S2). Overall, we conclude that oxalate, Ca and Cd concentrations were positively correlated in roots and branches. It is important to note that the correlation was found on a limited number of data, forming roughly two groups. Further investigations with more data would be necessary to confirm these correlations.

Table 1

Total elemental concentrations, pH and total organic carbon (%Corg) in the soils. n = 3 replicates. SD = standard deviation.

Condition	Cd, mg kg^{-1}		Zn, mg kg^{-1}		Mn, mg kg^{-1}		Ni, mg kg^{-1}		Ca, g kg^{-1}		P, g kg^{-1}		Fe, g kg^{-1}		pH ^a		%Corg	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
CCNG	0.102	0.002	120	40	185	42	34.9	3.5	3.4	0.1	0.67	0.03	56.2	5.5	4.01	0.14	0.90	0.07
CCNY	2.59	0.48	156	5	319	36	33.7	0.6	32.6	7.9	0.61	0.27	41.4	1.5	7.34	0.04	1.53	0.21
Nac	0.188	0.005	71	3	389	51	26.0	0.2	9.2	0.1	1.27	0.10	33.3	1.8	6.50	0.28	1.41	0.12

^a pH was measured in 0.01 M CaCl₂, 1:5 soil to liquid (w:v) ratio.

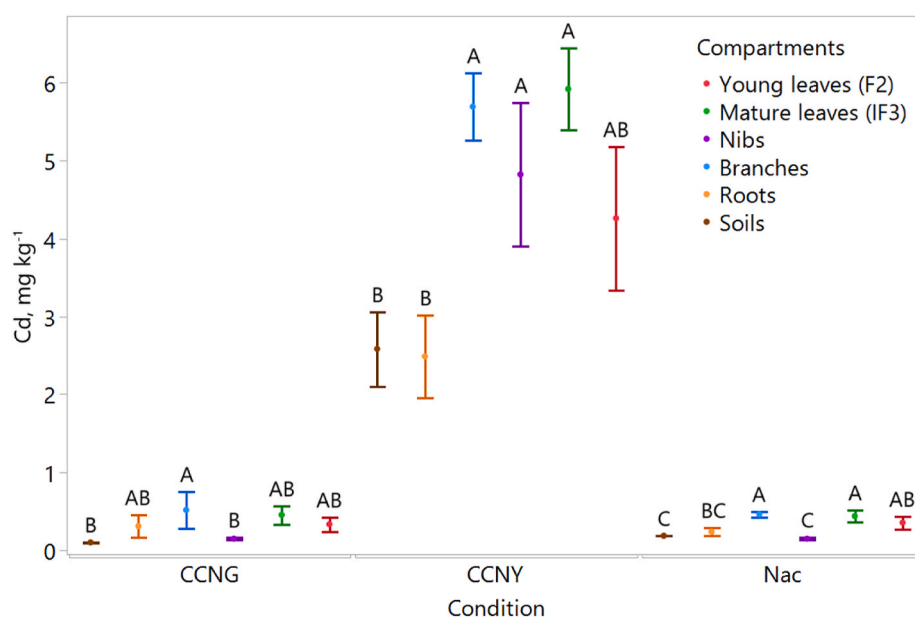


Fig. 1. Mean and standard deviation of total Cd concentrations in the soil and in the cacao organs, in mg kg^{-1} , for the three conditions. $n = 3$ biological replicates. Letters A, B and C denote, for each condition, different statistical groups among compartments (Table S3A and B).

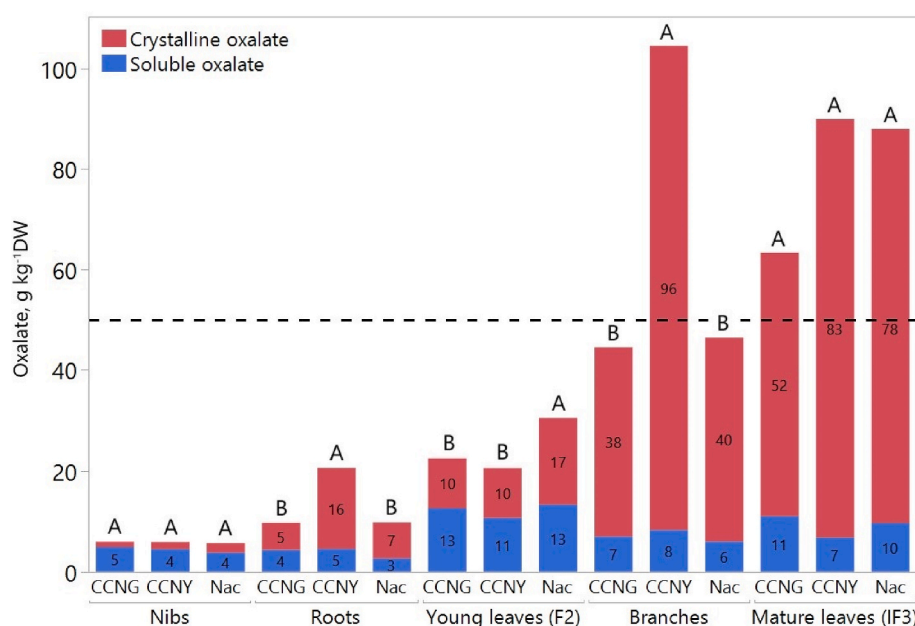


Fig. 2. Distribution of the concentration of oxalate, in g kg^{-1} DW, in the different organs of the three conditions (mean values are indicated in the bars, and mean and SD are given in Table S4). The dashed line represents the limit of 5% DW defined for extreme oxalate accumulators (Libert and Franceschi, 1987). Letters A and B denote different statistical groups among cultivars for total oxalate concentration in a given organ (Table S5A and B).

3.3. SEM-EDX observations of branches and roots

The distribution and morphology of CaOx crystals in branches and roots was studied by SEM-EDX. The CaOx crystals appear as bright particles in the images in backscattered electron mode due to the contrast in atomic number compared to the organic matrix. In the branches, they were observed in all tissues: in the phloem region (both along phloem rays and in phloem cells), in the periderm, in the xylem (generally along xylem rays) and in the medulla (Fig. 5).

Table 2 summarises the abundance of the CaOx crystals and sands (clusters of small crystals, typically $<3 \mu\text{m}$) in the various tissues of the branches. For the three conditions, the visual abundance decreases in

the order periderm > phloem > xylem > medulla. The abundance is similar in Nac and CCNG, and higher for CCNY. This is consistent with oxalate concentrations measured by HPIC. At the cellular level, crystals were observed inside cells (e.g., Fig. S5), but also in the cell wall of vascular bundles (e.g., Fig. S3). Calcium oxalate crystals had various sizes and morphologies (Fig. 6 and Figs. S4 and S5). Their size ranged from $<1 \mu\text{m}$ to about $20 \mu\text{m}$. Larger ones were faceted, sometimes rhombohedral (e.g., Fig. 6 and S5C). Druse crystals (spherical aggregates of individual crystals) were also observed (Fig. S5). Smaller crystals appeared as crystalline sands. Larger crystals were either isolated or embedded in crystalline sand. Needle shaped crystals (raphides) were never observed. The EDX analysis showed that most crystals were

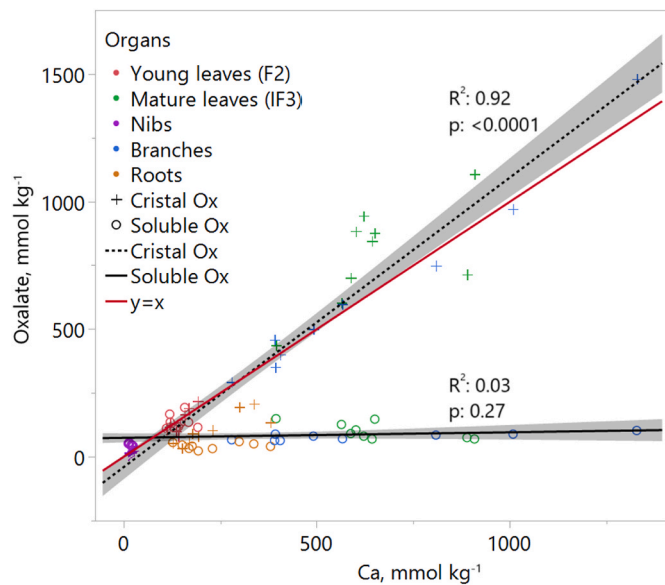


Fig. 3. Relationship between the concentration of oxalate in crystalline and soluble form, as a function of the concentration of total Ca, in mmol kg^{-1} , in the various organs and for the three conditions. R^2 and p -value of the F-test for the linear correlation between the concentration of oxalate crystals and Ca (dashed line, $y = 1.14x - 40.08$) and soluble oxalate and Ca (plain lines, $y = 0.02x + 74.19$) are indicated. Grey zones correspond to the confidence region for each linear fit ($\alpha = 0.05$).

mainly composed of C, O and Ca with traces of K (K/Ca: [0–6.2 %] in the branches, data not shown) (Fig. 6). Some crystals also contained Mg (Mg/Ca atomic ratio: [0–1 %] in the branches, data not shown) and P (P/Ca: [0–0.1%] in the branches, data not shown). (Figs. S3 and S5). The presence of Mg as a Ca substituent in CaOx (He et al., 2012, 2024) can explain the fact that oxalate was in excess relative to Ca in Fig. 4. Other metals such as Cd, Zn, Fe, Mn, and Ni were not detected by EDX, which means that their concentration was lower than 1% weight. In the roots, CaOx crystals and sands were less abundant than in branches, as expected from the HPIC results. They were mostly present in the periderm and phloem (Figs. S6 and S7, Table 2). Only a few crystals were observed in the xylem. The crystals had a more globular shape, and the crystal sands were much smaller in size (up to 30 μm in their larger dimension,

compared to a few hundreds of μm for the branches). The leaves were not investigated by SEM-EDX, but our study using nano X-ray fluorescence (nanoXRF) on the leaves of another cultivar of cacao (NA 312 of the genetic group Nanay) showed the presence of rhombohedral crystals in the mesophyll (Blommaert et al., 2022, 2024) (Fig. S8).

4. Discussion

Previous studies have found high amounts of oxalate in cacao nibs (Aremu et al., 1995; Nguyễn et al., 2018; Noonan and Savage, 1999; Schroder et al., 2011; Siener et al., 2021), and a study detected CaOx crystals in stem and meristem of cacao (de Oliveira et al., 2007). The present study is the first one quantifying soluble and crystalline oxalate in various cacao organs (roots, branches, young and mature leaves, and nibs). All of them contained soluble and crystalline oxalate, in the three conditions studied.

The total concentration in oxalate in mature leaves (6.3 ± 1.0 , 8.8 ± 0.5 and $9.0 \pm 1.8\%$ DW for CCNG, Nac and CCNY, respectively, Tables S4 and S5) was above 5%, defined by (Libert and Franceschi, 1987) as the threshold for extreme CaOx accumulators (Fig. 2). Thus, cacao tree can be considered as an extreme CaOx accumulator. The total oxalate concentration in cacao nibs (recalculated on a fresh weight basis for comparison with literature data) was consistent with previous studies ($341\text{--}358 \text{ mg.}100 \text{ g}^{-1} \text{ FW}$, compared to $300\text{--}900 \text{ mg.}100 \text{ g}^{-1} \text{ FW}$ in previous studies, Fig. 7 and Table S6). The cacao branches ($1381\text{--}3236 \text{ mg.}100 \text{ g}^{-1} \text{ FW}$) and mature leaves ($2596\text{--}3683 \text{ mg.}100 \text{ g}^{-1} \text{ FW}$) had comparable oxalate content as the richest crop reported to our knowledge, tea leaves ($300\text{--}2000 \text{ mg.}100 \text{ g}^{-1} \text{ FW}$) and licorice roots ($1137\text{--}1290 \text{ mg.}100 \text{ g}^{-1} \text{ FW}$, Fig. 7 and Table S6). Calcium oxalate monohydrate (whewellite) and CaOx dihydrate (weddelite) are the dominant states of crystalline oxalate in plants (Franceschi and Nakata, 2005; He et al., 2024). Based on the concentrations in crystalline oxalate determined in this study, we calculated the contribution of CaOx mono and dihydrate on the biomass of each organ. If one supposes that oxalate crystals are CaOx monohydrate, this mineral would account for 6–16% of the dry biomass of the branches, depending on the condition, and 9–14% of the dry biomass of the mature leaves (Table S3). With the assumption that oxalate crystals are CaOx dihydrate, percentages go to 7–18% for the branches, and 10–15 % for the leaves. Thus, CaOx crystals represent a major component of these cacao plant tissues. These percentages are higher than the average of 105 plant species (not only crops), containing CaOx (6.3 % of the dry biomass, although extreme

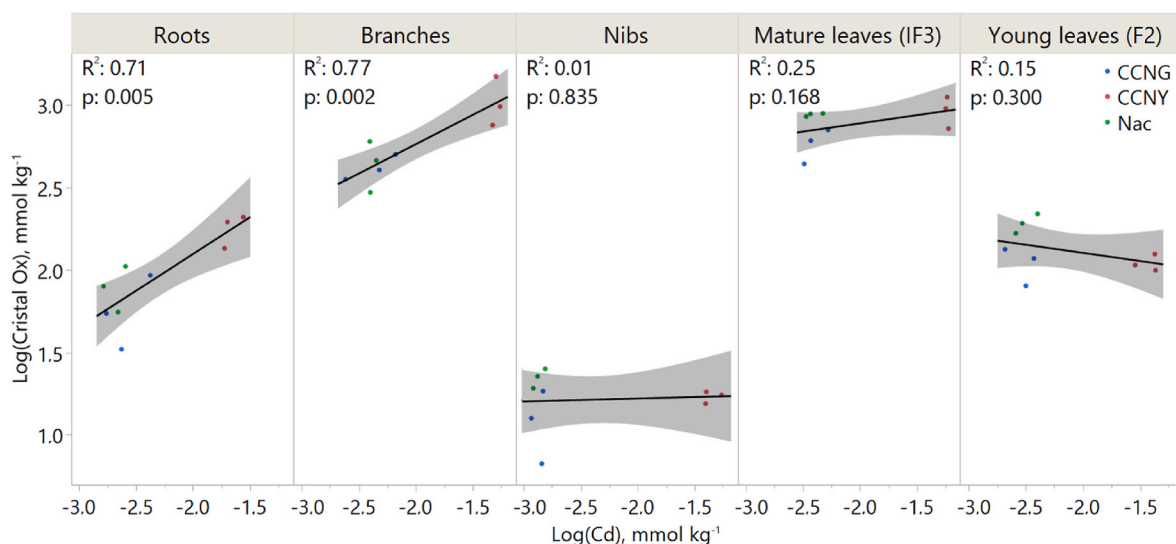


Fig. 4. Relationship between Log transformed concentration of crystalline oxalate and Cd, in mmol kg^{-1} , in each organ of the cacao tree, and for individual replicates. The R^2 and p -value of the F-test for the linear correlation is indicated for each organ, the grey zone is the confidence region for the linear fit ($\alpha = 0.05$).

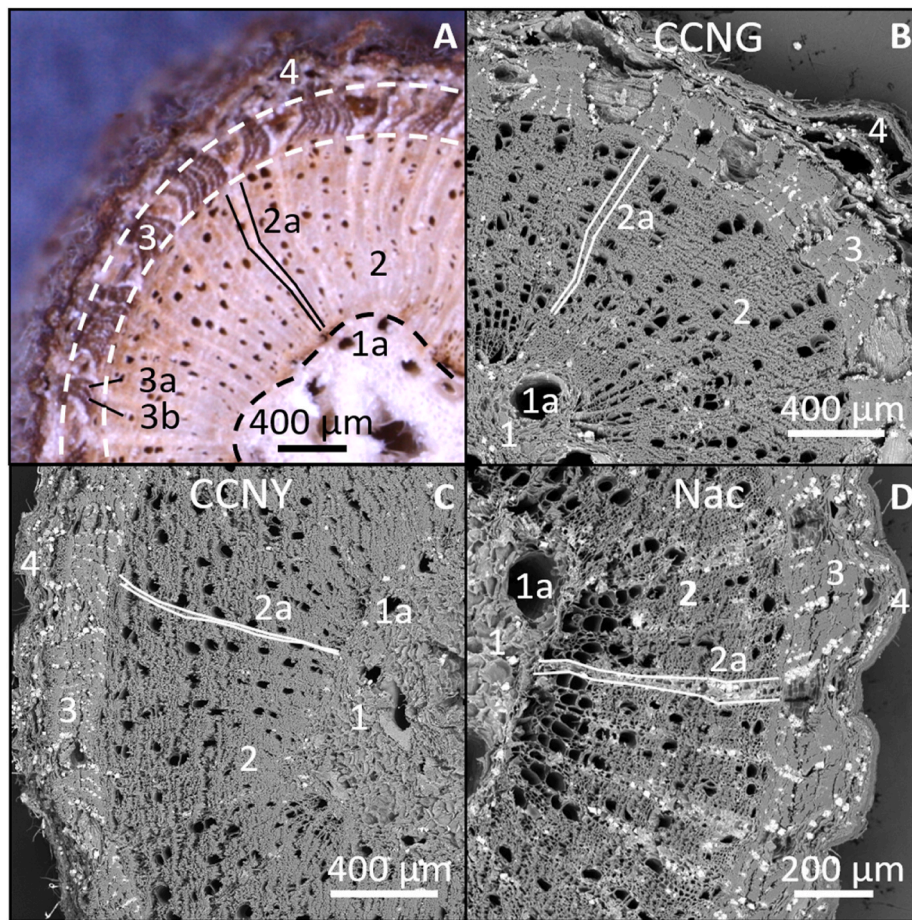


Fig. 5. A. Binocular observation of representative cross-sections of a cacao branch (Nac), with the various tissues indicated: 1. Medulla/pith, containing mucilage cavities (1a) in the central part, 2. wood (xylem) with the wood rays (2a), 3. phloem with phloem vessels (3a) and phloem rays (3b) and 4. Periderm. B–D: SEM images in back-scattered electron mode of the external parts of the branch (wood, phloem, and periderm) for the branches in the three conditions (B. CCNG, C. CCNY, and D. Nac). The bright spots correspond to CaOx crystals.

cases in cacti were reported with CaOx crystals representing 80–90% of the dry weight (Zindler-Frank, 1976)).

In the branches, CaOx crystals were particularly concentrated in the phloem and in the periderm, but they were also present in the medulla and xylem. In the roots, the phloem and periderm were also the richest compartments, although roots contained less crystals and less total oxalate. At the cellular level, crystals were observed inside cells but also in the cell wall of vascular bundles. Calcium oxalate crystal formation is initiated within vacuoles of specialized cells (Webb, 1999), but crystals are often deposited in cell walls (Franceschi and Nakata, 2005). Calcium oxalate crystals exert various functions in plants, including Ca regulation and protection against herbivory and metal detoxification (Franceschi and Nakata, 2005). They also can participate in photosynthesis, and improve plant tissue rigidity (Li et al., 2022). The protection against herbivory can be realized by raphide crystals, but also by layers or sheets of crystals that form a physical barrier around an organ or tissue such as phloem (Franceschi and Nakata, 2005). In the present study, acicular crystals or raphides were absent, but the alignment of crystals along the xylem and phloem rays may suggest a role in the protection of these tissues and/or in plant stiffening. The young leaves contained less CaOx crystals than the mature leaves. At the opposite, Finley et al. (Finley, 1999) showed that the young leaves of tropical plants contained more CaOx crystals than mature leaves, and suggested an enhanced protection against herbivory for these young leaves.

In the branches and mature leaves, the large majority of Ca was present as CaOx. Calcium has many functions in plants, including cell wall and membrane stability and signalling. It occurs in other forms than

CaOx, including Ca pectate in cell walls, Ca bound to proteins and free Ca^{2+} at very low concentration in the cytosol (0.1–0.2 μM) (Hawkesford et al., 2012; Thor, 2019). In some samples, the molar concentration in crystalline oxalate was in excess compared to Ca. This observation can be explained by the occurrence of Ca substituents in CaOx. Magnesium, detected by EDX in some crystals (Figs. S3 and S5), is a possible Ca substituent, as previously observed in *Acacia* species (He et al., 2012).

A role of CaOx crystals in Ca regulation in cacao is suggested by the fact that the plant with the highest content in CaOx crystals was the one growing on the soil with the highest total Ca content (32.6 g kg^{-1} compared to 3.4 g kg^{-1} for CCNG and 9.2 g kg^{-1} for Nac). The increase in CaOx crystals in response to an increased Ca supply is typical of so-called ‘oxalate plants’ (Hawkesford et al., 2012). In these plants, the transfer of Ca in vacuoles and precipitation as CaOx crystals is the main strategy to buffer free Ca^{2+} in the cytosol.

The CCNY soil contained more Cd ($2.59 \pm 0.48 \text{ mg kg}^{-1}$) compared to CCNG and Nac (0.100 ± 0.005 and $0.188 \pm 0.005 \text{ mg kg}^{-1}$). Even though the CaOx molar concentration was five orders of magnitude higher than Cd molar concentration, the two parameters correlated in branches ($R^2 = 0.77$; $p = 0.002 < 0.05$) and in roots ($R^2 = 0.71$; $p = 0.005 < 0.05$). Thus, it can be hypothesized that CaOx crystals are involved in Cd detoxification in these organs. SEM-EDX was not sensitive enough to confirm this hypothesis by direct detection of Cd in the CaOx crystals. In a study on a cacao cultivar with high Cd accumulation (NA 312, genetic group Nanay), the analysis of the branches by nanoXRF showed that a certain fraction of Cd present in the branches was associated with the CaOx crystals (Blommaert et al., 2024). Calcium oxalate

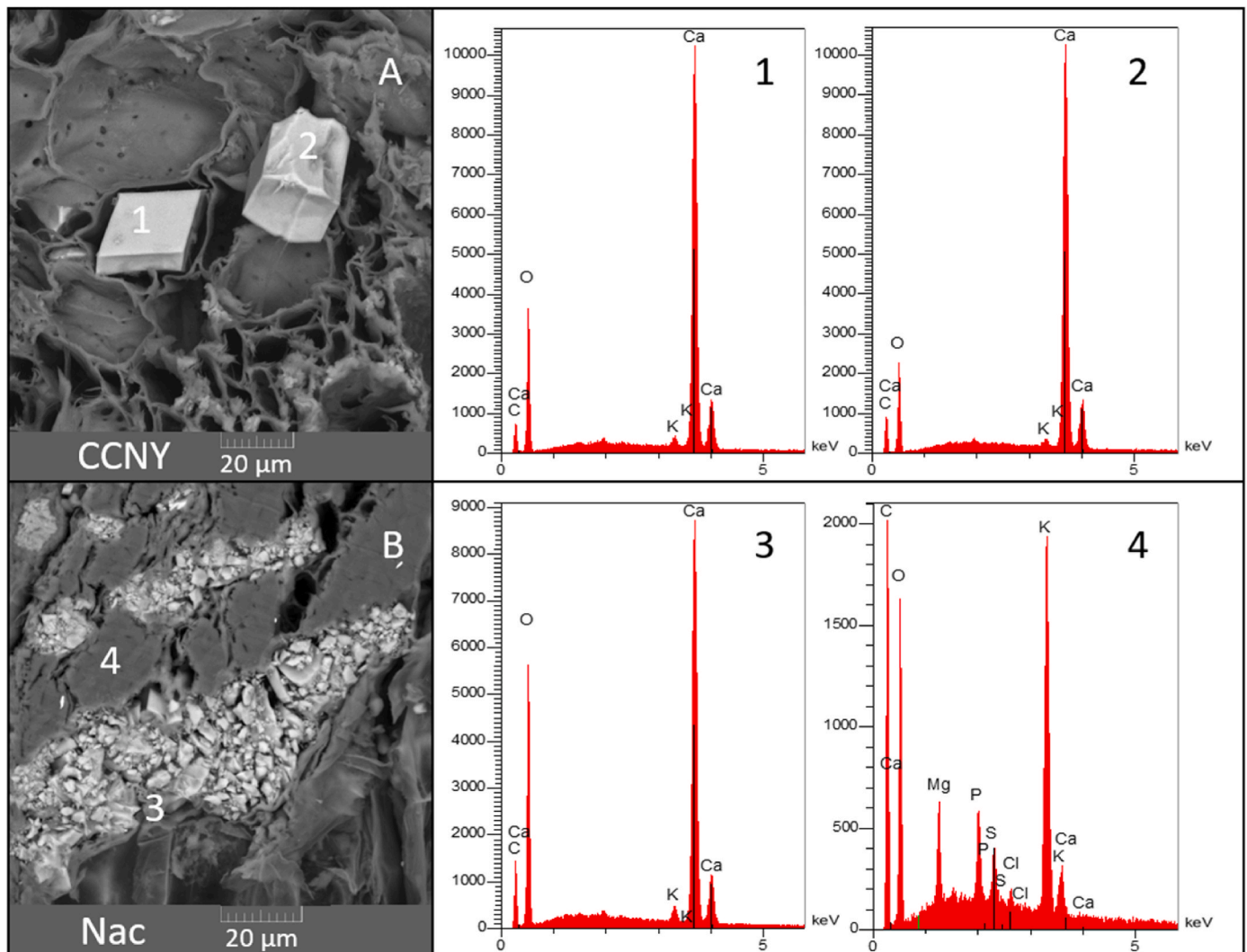


Fig. 6. Representative SEM images of A. a rhombohedral crystal, and a crystal with less defined shape and B. crystal sand in cacao branches. 1–4: EDS spectra of the crystal (1 and 2), crystal sand (3), and surrounding organic matrix (4).

is known to play a role in the detoxification of several metals including Al, Pb, Sr, Cu and Cd (Franceschi and Nakata, 2005; Khan et al., 2023). For Cd, this detoxification mechanism was observed in the bioindicator *Gomphrena claussenii* (Pongrac et al., 2018; Villafort Carvalho et al., 2015), in tomatoes (van Balen et al., 1980) and in water hyacinths (Mazen and El Maghraby, 1997). Despite a higher content in CaOx in roots, branches and mature leaves of cacao in the condition CCNY compared to CCNG, the Cd transfer to the nibs was not reduced. Instead, higher values for the ratios of nibs/branches and nibs/mature leaves were observed for CCNY (0.85 ± 0.10 and 0.81 ± 0.21) than for CCNG (0.29 ± 0.13 and 0.33 ± 0.06). Thus, the increased content in CaOx crystals does not seem to limit the transfer of Cd to the beans.

5. Conclusions and environmental implications

This study reports for the first time the abundance of oxalate and CaOx in the organs of the cacao tree. Total oxalate content in mature leaves was above the limit of 5% oxalate by dry weight for the three conditions tested (Libert and Franceschi, 1987). Thus, cacao tree can be considered as an extreme oxalate accumulator. Crystalline CaOx content accounted for 7–10% of the biomass of the mature leaves, and 6–14% of the biomass of the branches. Due to their abundance, CaOx probably enhances the rigidity of the tissues. Their distribution in branches and roots also suggests a protection role of the crystals for the phloem part.

Calcium oxalate and Cd concentrations were positively correlated in branches and mature leaves, which are the major Cd storage compartments in cacao trees, suggesting a role of CaOx in Cd detoxification. However, in the plant with the highest CaOx and Cd concentrations, Cd transfer from branches and mature leaves to the nibs was not reduced, instead it was enhanced. In this study, it was not possible to conclude about the specific role of Ca and Cd in the formation of CaOx crystals. Further studies, for example on cacao plants grown in a soil with high Ca and low Cd content and inversely, would be necessary to determine the effect of Ca and Cd on the enhanced production of CaOx crystals and their effect on Cd uptake and translocation in cacao. In addition, although the affinity of Cd for co-precipitation in CaOx crystals is known (McBride et al., 2017), further characterization of the CaOx crystals in plants using more sensitive techniques such as High-Resolution X-ray Fluorescence Mapping and High Energy Resolution Fluorescence Detected X-ray Absorption Spectroscopy, would be necessary to unravel the role of these crystals in Cd sequestration.

Oxalic acid is an anti-nutritional factor, and the consumption of chocolate contributes to the dietary intake of this compound (He et al., 2024; Noonan and Savage, 1999; Schroder et al., 2011). This study shows some specificities of cacao nibs compared to the other organs of cacao: (1) soluble oxalate was the predominant form in nibs, as opposed to CaOx in the other organs, and (2) the oxalate content in nibs was relatively constant among conditions.

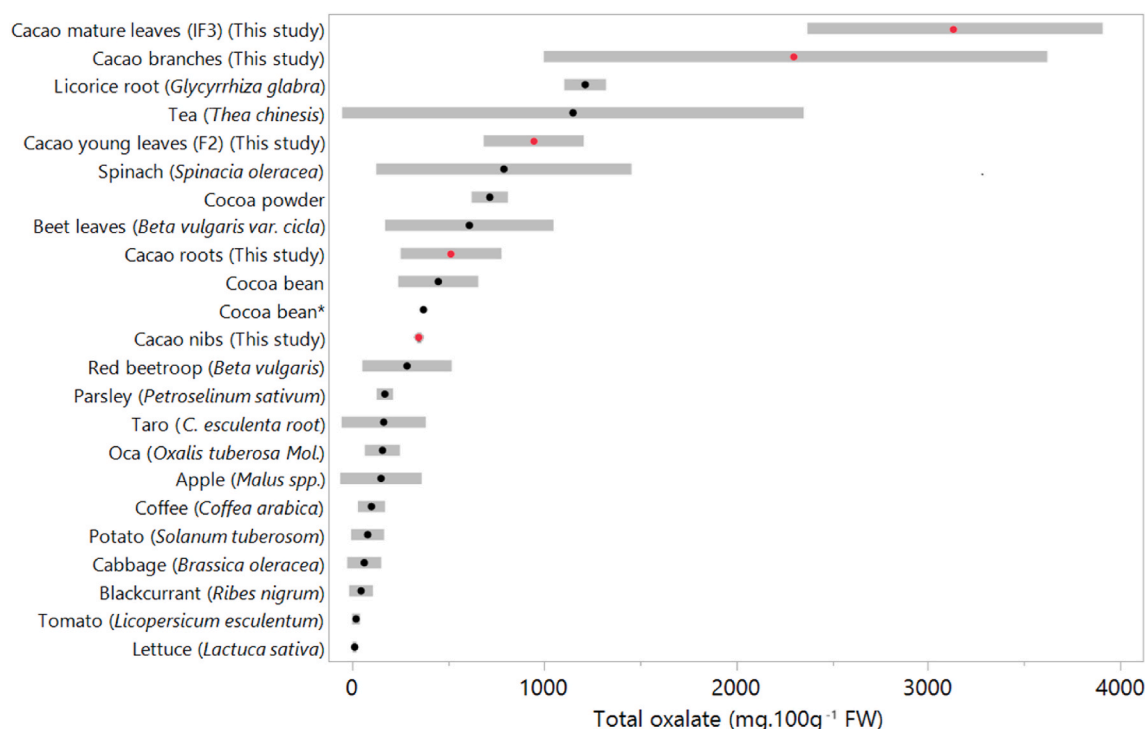


Fig. 7. Comparison of the mean and range in total oxalate in various crops and in cacao organs from this study (in red, the three conditions merged), and from literature data (Aremu et al., 1995; Holloway et al., 1989; Nguyễn et al., 2018; Noonan and Savage, 1999; Ross et al., 1999; Schroder et al., 2011; Siener et al., 2021, Table S6). *: Min and max calculated as min = mean – SD, max = mean + SD, and DW/FW set at 0.60. Values in this study recalculated based on DW concentrations, using the ratios DW/FW = 0.34 for roots, 0.31 for branches, 0.37 for F2 leaves, 0.41 for IF3 leaves, and 0.60 for nibs. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The CaOx crystals present in high concentrations in mature leaves are probably incorporated into the soil during leaf fall. This input of CaOx in the soil may favour the precipitation of carbonates and increase in soil pH through the action of oxalotrophic bacteria (Martin et al., 2012). The fate of CaOx in the soil of cacao plantations, and their impact on the bioavailability of Cd and micronutrients would require further investigations.

Author contributions

Conceptualization and methodology: GS and EC; validation: FL, DT, SC and GS; Resources: SD, DT, SC, RM and SS; Investigations: FL, EC, DT, SC, SS, RM, GS; Formal analysis: FL, SC, DT, HB and GS; visualization: FL, GS; writing original draft: FL; writing – review and editing: GS, EC, FL, HB, DT, SC, RM; Funding acquisition: GS. All authors approved the submitted version.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2025.109499>.

Data availability

Data will be made available on request.

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