

Rootstock, Scion, and Microbiome Contributions to Cadmium Mitigation in Five Indonesian Cocoa Cultivars

Jennifer Else Schmidt^{1*}, Mikael Dorman¹⁾, Andrew Ward¹⁾, and Alana Firlalana¹⁾

¹⁾PT. Effem (Mars Symbioscience) Indonesia, Jl. Andi Tadda, Luwu, Sulawesi Selatan, Indonesia

*Corresponding author: jennifer.schmidt@effem.com

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Abstract

Reducing levels of heavy metals such as cadmium (Cd) in cocoa beans has become an important priority for cocoa production in Indonesia. Current mitigation strategies revolve around breeding and the use of soil ameliorants, and in the future, the soil microbiome may also have the potential to reduce Cd bioavailability and uptake by cocoa trees. However, there remains a need for locally specific recommendations for low-Cd-accumulating cocoa cultivars and knowledge of native beneficial bacteria and fungi. In a greenhouse study using field soil supplemented with Cd, five cocoa clones (MCC 01, MCC 02, Sulawesi 1, Sulawesi 2, and ICCRI 09) were grafted in a fully factorial design to the same open-pollinated half sibling rootstocks, plant uptake was measured before and after the addition of cadmium nitrate, and rhizosphere microbial communities were characterized. Rootstock, scion, and graft combinations all significantly affected plant Cd levels, but the ranking of clones differed between low and high Cd soils. Twenty-six bacterial taxa and one fungal taxon were associated with Cd uptake. These results highlight the continued importance of breeding as a cadmium mitigation strategy and support the potential for the soil microbiome to contribute to reducing cadmium uptake in cocoa.

Keywords: *Theobroma cacao*, cadmium, breeding, microbiome

INTRODUCTION

Cocoa (*Theobroma cacao*) is an important agricultural commodity for Indonesia, accounting for 1.48 million hectares of land in 2022 (PDSIP, 2022). The majority of Indonesian cocoa is sold as cocoa butter and unsweetened cocoa powder, which account for 40% and 26% of exports, respectively. Despite its importance to the economy, Indonesian cocoa production has fallen in recent years to an estimated 707,000 tonnes in 2021 and 732,000 tonnes in the 2022 production year (PDSIP, 2022). Aging trees, declines in soil fertility, increase pests and pathogens attacks such as the cocoa pod borer, and the conversion of cocoa plantations to

palm oil and rice have all contributed to this decline (Fahmid *et al.*, 2018; Fahmid *et al.*, 2022).

Soil cadmium (Cd) poses an additional threat to the future of Indonesian cocoa. Cadmium occurs naturally in soils around the world at concentrations of 0.01-1 mg kg⁻¹ (Kubier *et al.*, 2019). It is unevenly distributed geographically and occurs at higher concentrations in soils formed from black shale and phosphorites (Liu *et al.*, 2017). Naturally occurring soil Cd levels can also be increased through atmospheric deposition, industrial or mining runoff, or application of contaminated phosphate fertilizers or manure (Meter *et al.*, 2019). Although Cd is not an essential

plant nutrient, the divalent cation Cd^{2+} is taken up by cocoa roots through zinc and iron transporters and a fraction is translocated into the beans.

Cadmium bioaccumulates in the human body, causing detrimental health impacts on the kidneys, bones, and other organs as well as increasing cancer risk when ingested above certain levels (Bernard, 2008). To reduce exposure to Cd, some regulatory bodies have imposed limits on Cd in cocoa and chocolate products and others are considering doing so. In 2009, the Indonesian government set a limit of 0.5 ppm in cocoa beans (SNI 7386-2009) (BSN, 2009). In 2018, a European Union regulation went into effect lowering allowable concentrations in finished chocolate products to 0.10-0.80 ppm depending on the type of product (EC No 488/2014, 2014). These regulations have heightened interest in farm-level mitigation practices that could potentially reduce Cd uptake by cocoa plants and could be feasibly implemented by the small-holder farms that make up 95% of Indonesian cocoa farms (Fahmid *et al.*, 2018).

Current Cd mitigation strategies on cocoa farms revolve around soil management, with the potential in the future for further reductions through breeding and microbiome optimization. Liming can raise soil pH above the level at which Cd is most available for uptake by cocoa roots but is not always effective deep in the root zone and requires repeated applications.

In Indonesia, most commercial cocoa trees consist of a clonal scion grafted onto hybrid rootstock derived from specific clones selected for a range of agronomic properties. Selection of low-Cd-accumulating rootstocks onto

which desirable scions could be grafted would allow continued production of beans that meet regulatory thresholds even on soils where Cd is present. Alternatively, low-Cd-accumulating scions may be able to be selected. However, little is currently known about Cd uptake by commercially available Indonesian cocoa cultivars and their suitability as rootstocks or scions on high-Cd soils. Manipulation of the soil microbiome to reduce Cd bioavailability is an active area of research in Latin America (Meter *et al.*, 2019), but has not been adequately addressed in the Indonesian context.

This study sought to fill knowledge gaps related to future breeding and microbiome-related Cd mitigation strategies. Cadmium uptake was measured in five commercially grown Indonesian cocoa clones, both ungrafted and as grafted rootstocks and scions, in field soil before and after augmentation with cadmium nitrate. Root-associated microbial communities were characterized and tested for associations with plant Cd uptake to identify potentially beneficial prokaryotes and fungi.

MATERIALS AND METHODS

Location and Climate

The present study was conducted at a cocoa research field station at Tarengge, Wotu subdistrict, Luwu Timur regency, South Sulawesi, Indonesia. This site is characterized by a humid tropical climate, with an average mean temperature of 28 °C and average annual precipitation of 2900 mm in 2021-2022. The soil at this site is an Entisol and soil physical and chemical properties are presented in Table 1.

Table 1. Compositied soil physical and chemical properties

Sand	Silt	Clay	pH		C	N	C/N	P ₂ O ₅	K ₂ O	P ₂ O ₅	K ₂ O	Ca	Mg	K	Na	CEC	
%	%	%	H ₂ O	KCl	%	%		Extract	HCl 25%	Olsen/Bray-I				meq/100g			
								(mg/100g)		(mg/100g)				(ppm)			
28.3	37.3	34.5	5.61	4.34	1.93	0.14	13.0	72.0	84.5	69.3	236	28.6	1.11	0.50	0.85	24.6	

Experimental Design and Plant Growth

Field soil naturally high in Cd was homogenized thoroughly and mixed with compost before potting into polybags. Each polybag contained 10 kg of the soil/compost mixture, with a mean of 0.331 ppm Cd according to soil tests (Table 2). Open-pollinated seeds derived from five Indonesian cocoa clones commonly grown commercially (MCC 01, MCC 02, Sulawesi 1, Sulawesi 2, ICCRI 09) were germinated in covered trays. Admixture percentages for these clones based on DNA analysis of leaf tissue are given in Table 3. At radical emergence, they were transplanted into polybags and maintained in a glasshouse. Three months after transplant, rootstock seedlings were cleft grafted in a fully factorial design with budwood of the same clones (see Table 2 for all rootstock-scion combinations). Ungrafted seedlings were kept as a control. Due to low availability of ICCRI 09 budwood, only three replicates (each consisting of five plants) per graft combination were possible ($n = 105$ plants), but all other graft combinations had five replicates of five plants each ($n = 155$ plants per rootstock, total $n = 725$ plants).

Two months after grafting, soil samples were taken from each polybag. After sub-sampling for microbial analysis, these samples were combined to result in one composite sample per graft combination and ungrafted control ($n = 30$). Samples for microbial analysis were stored at $-20\text{ }^{\circ}\text{C}$ until they could be shipped on ice, and composite samples were sent to an external lab for Cd analysis. At three months after grafting, 30 leaf samples were taken from each clone/rootstock combination ($n = 140$, lower-Cd soil sampling date). Leaf samples were taken because Cd levels in leaf tissue have been shown to be correlated to bean Cd (Ramtahal *et al.*, 2016), and because leaves can be sampled non-destructively in contrast to root or trunk tissue. A cadmium nitrate solution was then applied to each polybag to achieve a final soil concentration of 2 ppm Cd. Cadmium nitrate dissociates to Cd^{2+} and NO_3^{2-} , and Cd^{2+} is the form taken up by the plant. Three months after the application, 30 soils and 140 leaf samples were taken again (higher-Cd sampling date), and seedlings were transplanted into the field.

Table 2. Soil Cd (ppm) test results prior to seedling transplant. Samples from all replicates of each graft combination were composited prior to analysis. SD = standard deviation of the mean.

Scion	Rootstock					Scion mean	Scion SD
	MCC 01	MCC 02	Sulawesi 1	Sulawesi 2	ICCRI 09		
MCC 01	0.394	0.400	0.143	0.402	0.331	0.334	0.111
MCC 02	0.338	0.370	0.219	0.467	0.390	0.347	0.091
Sulawesi 1	0.328	0.162	0.408	0.417	0.194	0.302	0.119
Sulawesi 2	0.358	0.144	0.373	0.464	0.466	0.361	0.131
ICCRI 09	0.772	0.381	0.311	0.430	0.394	0.458	0.181
Ungrafted	0.156	0.125	0.125	0.125	0.341	0.174	0.094

Table 3. Admixture percentages showing the contribution of each cocoa genetic group to each clone used in this study

Clone	Genetic group										
	Amelonado	Contamana	Criollo	Curaray	Guiana	Iquitos	Maranon	Nacional	Nanay	Purus	Bolivian
Sulawesi 2	0.01	0.00	0.41	0.00	0.00	0.00	0.56	0.00	0.00	0.00	0.00
Sulawesi 1	0.19	0.03	0.20	0.00	0.01	0.00	0.02	0.00	0.51	0.00	0.05
MCC 01	0.51	0.14	0.05	0.02	0.00	0.11	0.01	0.01	0.01	0.00	0.14
MCC 02	0.44	0.23	0.22	0.01	0.01	0.01	0.01	0.02	0.03	0.00	0.03
ICCRI 09	0.30	0.01	0.21	0.00	0.00	0.04	0.03	0.00	0.40	0.00	0.01

Soil and Leaf Analysis

Leaves were sampled from clones from which the budwood was derived and 96 SNP markers were sequenced, and the data were analyzed with Structure software v.2.3.4 to calculate admixture percentages as described in Duval *et al.* (2017). Because different markers and clustering algorithms were used as compared to the 15 SSR markers and fewer reference groups in Dinarty *et al.* (2015), reported admixture percentages may differ from those published elsewhere.

Cadmium concentrations in soil and leaf samples were tested using inductively coupled plasma-mass spectrometry (ICP-MS) protocols. Soil samples were prepared using an acid digest (U.S. EPA, 1996) and analyzed according to APHA Method 3120 (American Public Health Association, 1992). Leaf samples were analyzed according to AOAC Official Method 2015.01 Heavy Metals in Food.

To test cocoa genetic effects on Cd uptake, two generalized linear models were constructed with leaf Cd content measured in either lower- or higher-Cd soil as the response variable and rootstock, scion, and their interaction as fixed effects. Type III ANOVA was used to generate analysis of deviance tables for these models, and effects were considered significant at $p < 0.05$. χ^2 values were calculated for each model as the difference of null and residual deviance and converted to p values using three degrees of freedom from the three predictor variables.

Microbial Community Analysis

DNA was extracted from two technical replicates (0.25 g soil each) of each polybag-level soil sample using DNeasy PowerSoil Pro kits (QIAGEN, Germantown, MD, USA). After checking DNA quality, technical replicates were combined and paired-end amplicon sequencing of the 16S rRNA gene and ITS region of fungal ribosomal DNA was conducted on an Illumina NovaSeq

platform. Prokaryotic communities were characterized using the primers 515F (5'-GTGCCAGCMGCCGC GGTA-3') and 806R (5'-GGACTACHV GGGTWTCTAAT-3'), which are specific to the V4 region (Caporaso *et al.*, 2011). The ITS region was targeted with the primers ITS1F (5'-CTTGGTCATTTAGAGGAAG TAA-3') and ITS2R (5'-GCTGCGTTCTTCATCGAT GC-3') (Gardes & Bruns, 1993; White *et al.*, 1990). Downstream analysis was conducted in R software 4.2.2 to determine microbial alpha and beta diversity as well as identify specific taxa associated with cadmium accumulation. The dada2 package v.1.26.0 was used for additional read processing (Callahan *et al.*, 2016; R Core Team, 2022). 16S rRNA gene forward and reverse reads were truncated to 240 bp and 160 bp, respectively, as determined by read quality profiles, and all reads were filtered and trimmed using the parameters $\text{maxEE} = 2$ and $\text{truncQ} = 2$. ITS reads were not truncated to a specific length, as the length of this region is highly variable, and filtering and trimming was conducted with the parameters $\text{maxEE} = 2$ and $\text{truncQ} = 11$. Taxonomy of bacterial and archaeal amplicon sequence variants (ASVs) was assigned to the genus level where possible using the SILVA reference database v.138.1 (Quast *et al.*, 2013; Yilmaz *et al.*, 2014) and fungal taxonomy was assigned to the species level where possible using the UNITE data base (April 2, 2020 release) (Abarenkov *et al.*, 2020; Nilsson *et al.*, 2019).

Alpha (within-sample) diversity of microbial communities was calculated using the vegan package v.2.6-4 (Oksanen *et al.*, 2022). Taxonomy-based alpha diversity metrics included richness, the Shannon index, and the Pielou index. A generalized least squares linear model was constructed to test for differences among rootstocks using the nlme package v.3.1-161 (Pinheiro & Bates, 2022). Potential relationships between cadmium uptake and microbial diversity

metrics were tested using a linear model and type II ANOVA.

Beta (between-sample) diversity of microbial communities was assessed by ordinating Bray-Curtis distance matrices of microbial abundance data using canonical analysis of principal coordinates (CAP). In light of previous research showing that rootstock, but not scion, affected cocoa rhizosphere microbial community composition (Schmidt *et al.*, 2021), only rootstock effects on beta diversity were tested using permutational multivariate analysis of variance (PERMANOVA) with 5000 permutations.

Generalized linear models were used to screen for associations between microbial communities and plant Cd uptake from lower- and higher-Cd soil using the R package MaAsLin2 v.1.12.0 (Mallick *et al.*, 2021). Separate univariate models were constructed for prokaryotic and fungal communities with leaf Cd in lower-Cd and higher-Cd soil as fixed effects. For each model, the minimum prevalence threshold was set at 25% and maximum q value at 0.01 (i.e. controlling the Bonferroni-Holm-adjusted false discovery rate at 0.01).

RESULTS AND DISCUSSION

Cocoa Genetic Effects on Cd Uptake

Generalized linear models with rootstock, scion, and the rootstock:scion interaction as fixed factors were useful for predicting leaf Cd both in lower-Cd soil ($\chi^2 = 29.1$, $df = 3$, $p < 0.001$) and higher-Cd soil ($\chi^2 = 473.1$, $df = 3$, $p < 0.001$). Rootstock, scion, and the interaction all had significant effects on uptake from lower-Cd soil at the $\alpha = 0.05$ level, while only rootstock and the rootstock:scion interaction had significant effects on uptake from higher-Cd soil. Deviance values were an order of magnitude greater in both models

for rootstock than scion, indicating that rootstock identity is likely a stronger driver of Cd uptake than scion identity (Table 4).

Despite substantial variation among graft combinations and individual plants, clear patterns were observed for specific rootstocks in lower- and higher-Cd soil (Figure 1). In lower-Cd soil, the ungrafted rootstock treatment had a significant negative coefficient, indicating lower Cd uptake relative to other treatments ($\beta = -0.936$, $p = 1.20e^{-7}$) while rootstock Sulawesi 1 had a significant positive coefficient, indicating higher Cd uptake ($\beta = 0.638$, $p = 1.88e^{-4}$). Scions MCC 01 ($\beta = -0.376$, $p = 0.245$), Sulawesi 1 ($\beta = -0.410$, $p = 0.0144$), and Sulawesi 2 ($\beta = -0.520$, $p = 0.00209$) all had significant negative coefficients at this sampling date. The highest accumulation was observed in graft combinations Sulawesi 1-MCC 01, ICCRI 09-Sulawesi 2, Sulawesi 1- , and Sulawesi 1-ICCRI 09 (rootstock listed first), while the lowest accumulation was observed in ungrafted MCC 02, MCC01, Sulawesi 1, and Sulawesi 2 seedlings followed by MCC 02-Sulawesi 2, MCC 02-Sulawesi 1, and MCC 02-MCC 01 (Figure 1A).

In higher-Cd soil, rootstocks ICCRI 09 ($\beta = 3.765$, $p = 2.00e^{-7}$) and Sulawesi 2 ($\beta = 3.254$, $p = 2.10e^{-7}$) had significant positive coefficients, while the ungrafted rootstock treatment had a significant negative coefficient ($\beta = -2.402$, $p = 8.15e^{-05}$). The highest accumulation was observed in ICCRI 09-MCC 02, Sulawesi 1-ICCRI 09, and Sulawesi 2-MCC 02, while the lowest accumulation was observed in the ungrafted seedlings, followed by MCC 01 grafted on MCC 01, MCC 02, and Sulawesi 1 (Figure 1B). The difference in rankings of rootstocks and graft combinations before and after soil Cd levels were artificially increased may indicate a genotype by environment interaction in which uptake depends on the soil Cd level (Figure 1). Recommendations for desirable graft combinations

Table 4. Analysis of deviance tables for plant Cd uptake. Type III ANOVA was used to test generalized linear models of leaf Cd in lower- or higher-Cd soils against rootstock, scion, and their interaction. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

	Lower-Cd soil		
	G ²	df	p
Rootstock	101.61	5	<2.2e-16 ***
Scion	17.27	4	0.0017 **
Rootstock:scion	93.90	20	1.5e-11 ***
Higher-Cd soil			
	G ²	df	p
Rootstock	134.57	5	<2e-16 ***
Scion	9.45	4	0.051
Rootstock:scion	143.72	20	<2e-16 ***

could thus be optimized for specific locations based on the results of soil Cd testing, e.g. MCC 02-Sulawesi 2 for low-Cd soils and MCC 01-MCC 01 for high-Cd soils. However, it is important to note that Cd levels varied among polybags at the lower-Cd sampling date and were standardized at the higher-Cd sampling date, potentially affecting the ranking of genotypes. Furthermore, not all graft combinations tested may be commercially viable even if Cd uptake was low, due to lack of seed availability or *Phytophthora* susceptibility, for example. In light of evidence showing that cadmium levels in leaf and bean tissues are highly correlated (Ramtahal *et al.*, 2016), we assume that rankings of graft combinations would not change if bean Cd, the metric of interest for cocoa producers and sourcers, were measured instead.

Due to within-field variability in soil Cd or other factors, farmers may instead desire a recommendation for a single rootstock to plant throughout the farm. MCC 02 may be a good candidate for Cd mitigation, based on its low baseline uptake and low increase in leaf Cd in the higher-Cd soil relative (Figures 1, 2). Seedlings grafted onto Sulawesi 2 tended to have a large relative increase in Cd between conditions, meaning that this clone should be planted with caution in high-Cd soils, and seedlings grafted onto Sulawesi 1 were highly variable, with both the highest and lowest relative increases in Cd (Figure 2).

Few other studies have compared Cd uptake across these specific cultivars. A comparison of Sulawesi 1 and Sulawesi 2 as ungrafted seedlings found that Sulawesi 1 had much higher root uptake of Cd across treatments of 0-8 ppm Cd but that shoot content did not differ between clones (Zakariyya *et al.*, 2022). While root uptake was not measured in the present study, that ranking is consistent only with uptake from the lower-Cd soil (Figure 1A); uptake was highly variable across seedlings grafted onto Sulawesi 1 in the higher-Cd soil (Figure 1B). Variability in uptake could be attributed in part to the hybrid nature of the rootstocks. Field trials across multiple locations in Indonesia that incorporated genotyping of rootstocks and scions would be a valuable extension of this work and could help validate the existence of a potential GxE interaction for uptake based on soil Cd levels as well as the influence of genetic variation on uptake.

Lower uptake by ungrafted seedlings is unexpected given that grafting (even self-grafting) has been observed to reduce Cd uptake in other crops such as tomato (Kumar *et al.*, 2015; Xie *et al.*, 2020). Orthotropic seedlings would also be expected to take up more Cd from the soil due to faster growth rates and an unobstructed transpirational stream given the lack of a graft junction. Contaminated budwood is

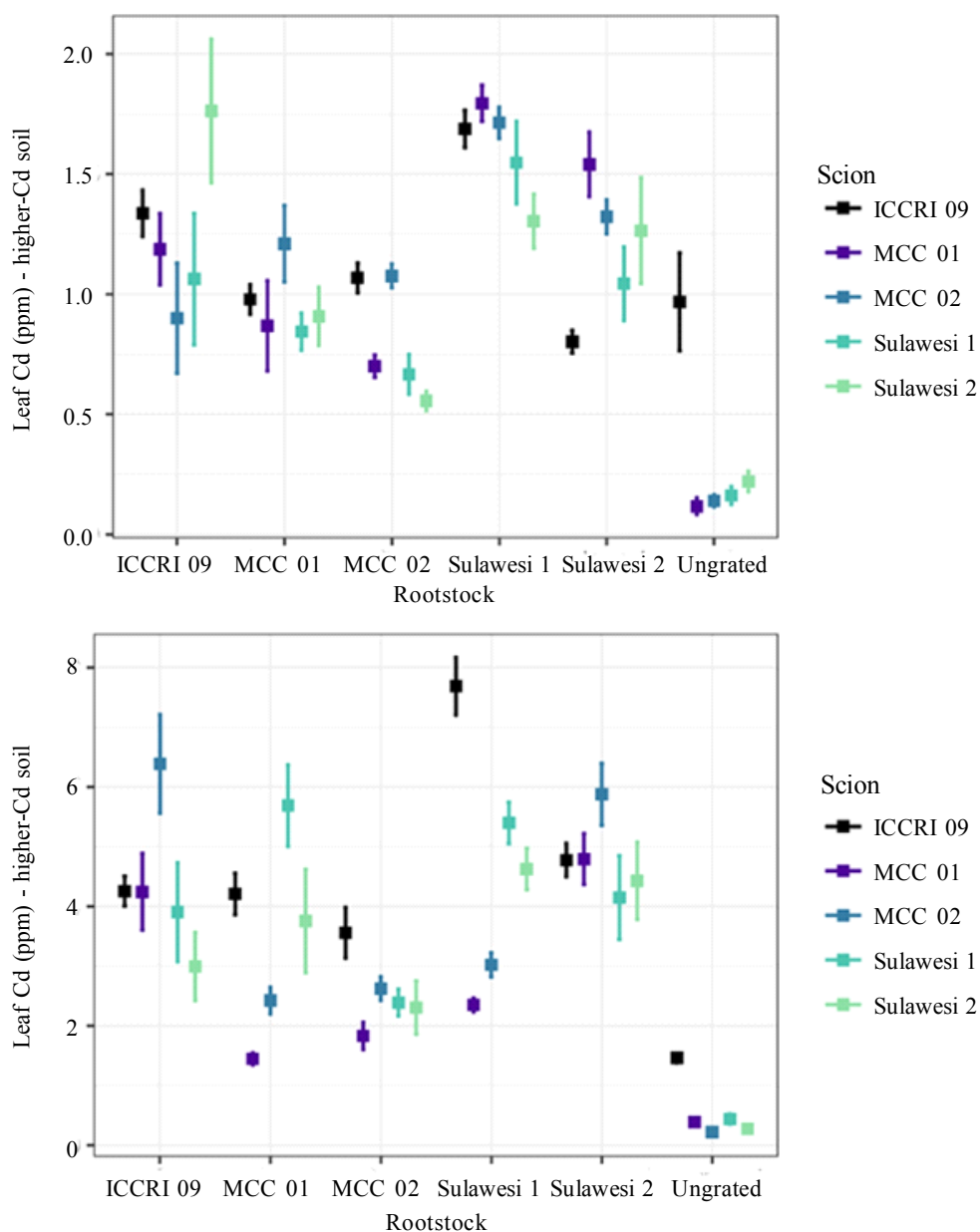


Figure 1. Leaf cadmium of cocoa graft combinations (A) in field soil mixed with compost and (B) three months after soil was augmented with cadmium nitrate to reach 2 ppm Cd; Bars represent standard error

one possible explanation, although the trees from which budwood was taken were grown on a soil low in Cd. A follow-up study analyzing Cd levels in the budwood would be warranted.

Microbial Diversity and Community Composition

Prokaryotic alpha diversity tended to be lower in ungrafted seedlings than that of grafted rootstock treatments across all diversity metrics (Figure 3A). Seedlings grafted onto ICCRI 09 had

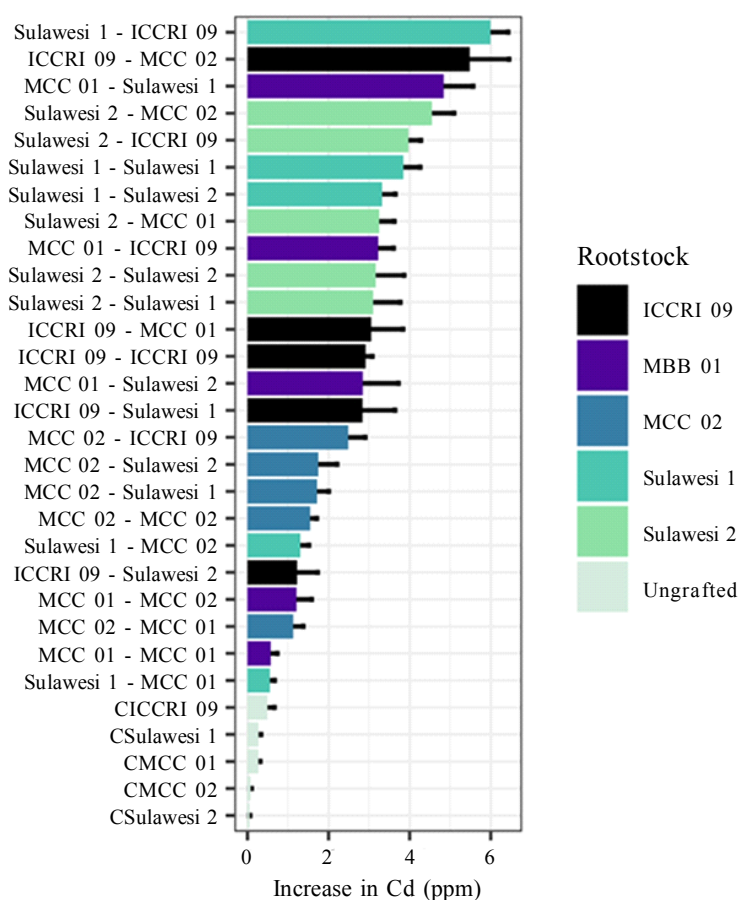


Figure 2. Absolute increase in leaf Cd three months after addition of cadmium nitrate. Rows indicate graft combinations, with rootstock listed before scion and ungrafted seedlings designated by “C” (for “control”); Bars represent standard error

higher richness than Sulawesi 1 or Sulawesi 2 rootstocks, and a higher Shannon index than Sulawesi 1, but there were no significant differences among other grafted treatments. Fungal alpha diversity differed among rootstocks only for richness, which was higher in rootstock MCC 02 than rootstock Sulawesi 2 (Figure 3B). These patterns suggest that microbial richness was more responsive to host genetic factors in this study than diversity metrics that also accounted for community evenness, but that rootstock effects on microbiome alpha diversity are minimal. Analysis of variance showed that Cd uptake from both lower- and higher-Cd soils was not significantly affected by any prokaryotic or fungal diversity metric (all $p > 0.05$).

Rootstock had a significant effect on beta diversity of prokaryotic communities (PERMANOVA $p < 0.001$). Constrained analysis of principal coordinates highlighted this clustering by rootstock, which accounted for ~13% of variance (CAP 1: 9.8%, CAP 2: 3.6%, Figure 4A). Samples from ungrafted seedlings were clearly separated from other samples along CAP 1, and the other rootstocks separated slightly along CAP 2. Beta diversity of fungal communities was also affected by rootstock (PERMANOVA $p < 0.001$). However, rootstock explained only ~7% of variation in fungal communities (CAP 1: 5.4%, CAP 2: 2.1%), and clustering by rootstock was not as clear (Figure 4B).

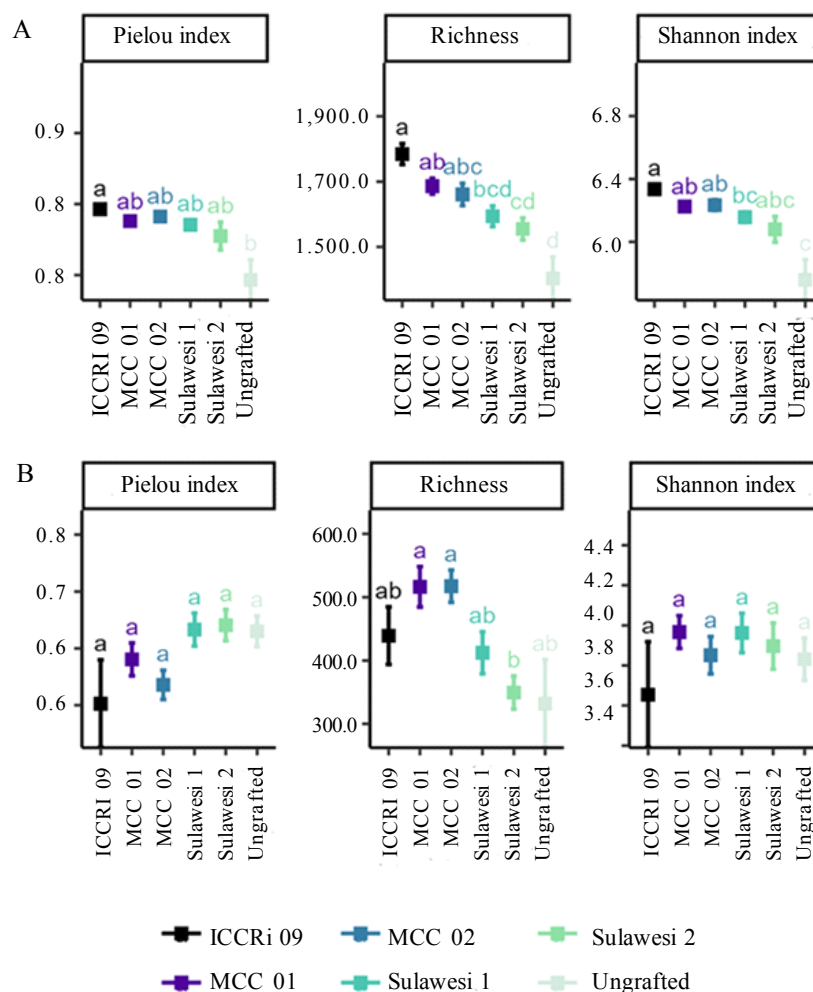


Figure 3. Alpha diversity of prokaryotic (A) and fungal communities by rootstock (B); Richness indicates the number of unique taxa, while the Pielou index and Shannon index integrate community evenness as well; Bars represent standard error

Ungrafted rootstocks had lower pro-karyotic alpha diversity and prokaryotic communities tended to cluster separately from the other treatments. Scion-to-rootstock transfer is not likely to be a significant source of rhizosphere microbial diversity, so this finding is unexpected. Differences in handling during grafting and seedling growth may be responsible. It is possible that the absence of certain taxa that make Cd more bioavailable in the rhizosphere could contribute to both the lower diversity and lower Cd uptake observed in this treatment, but further studies would be necessary to confirm this hypothesis.

Microbial Taxa Associated with Cd Uptake

Linear modeling identified twenty-six prokaryotic ASVs whose abundance was associated with Cd uptake at either sampling date (Figure 5). Three of these ASVs had negative coefficients, indicating that an increase in their relative abundance in the rhizosphere was associated with lower leaf Cd (*Lysobacter* sp.: $\beta = -0.97$, $p < 0.001$; *Nocardioides* sp.: $\beta = -0.80$, $p < 0.001$; and *Sphingomonas* sp.: $\beta = -0.69$, $p < 0.001$). The relative abundance of these and twenty

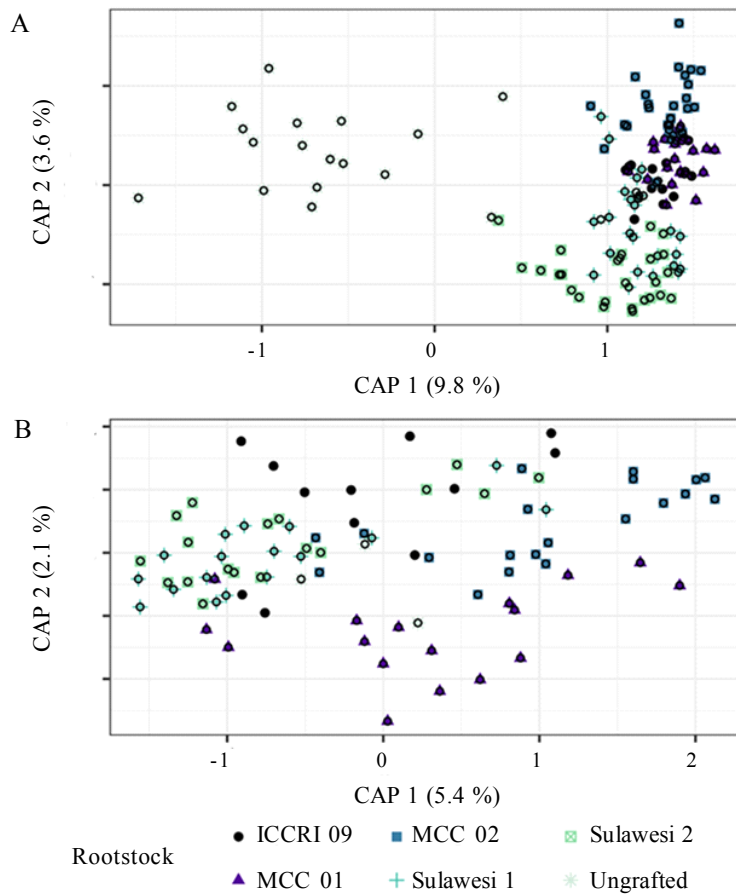


Figure 4. Constrained analysis of principal coordinates (CAP) of prokaryotic (A) and fungal communities by rootstock (B); Ordinations were based on Bray-Curtis distance matrices; Permutational analysis of variance with 5000 permutations found that rootstock effects were significant in both cases ($p < 0.001$)

other ASVs had significant relationships with leaf Cd in the lower-Cd soil, while three ASVs (*Microvirga* sp., *Pseudolabrys* sp., and *Luteimonas* sp.) were significantly positively related to Cd uptake in the higher-Cd soil (Figure 5). Only one fungal ASV, *Mycothermus thermophilus*, was associated with leaf Cd, with a negative coefficient for leaf Cd in the lower-Cd soil ($\beta = -0.99$, $p < 0.001$).

One goal of the present study was to identify microbial taxa with strong and significant negative relationships with leaf Cd for further investigation as potential bioamendments to reduce plant Cd uptake.

Of the four ASVs identified here, two come from genera for which some Cd-relevant research have been conducted. Numerous studies exist for the genus *Sphingomonas*, but the direction of the effect depends on the species: *S. paucimobilis* is capable of Cd biosorption (Tangaromsuk *et al.*, 2002) and *Sphingomonas* sp. C40 decreases Cd accumulation in rice (Cheng *et al.*, 2021), but *Sphingomonas* sp. SaMR12 increases Cd accumulation by *Sedum alfredii* (Chen *et al.*, 2014; Pan *et al.*, 2016). The abundance of *Lysobacter* sp. has been found to increase in response to soil Cd enrichment in the *Chrysopogon zizanioides* L. rhizosphere

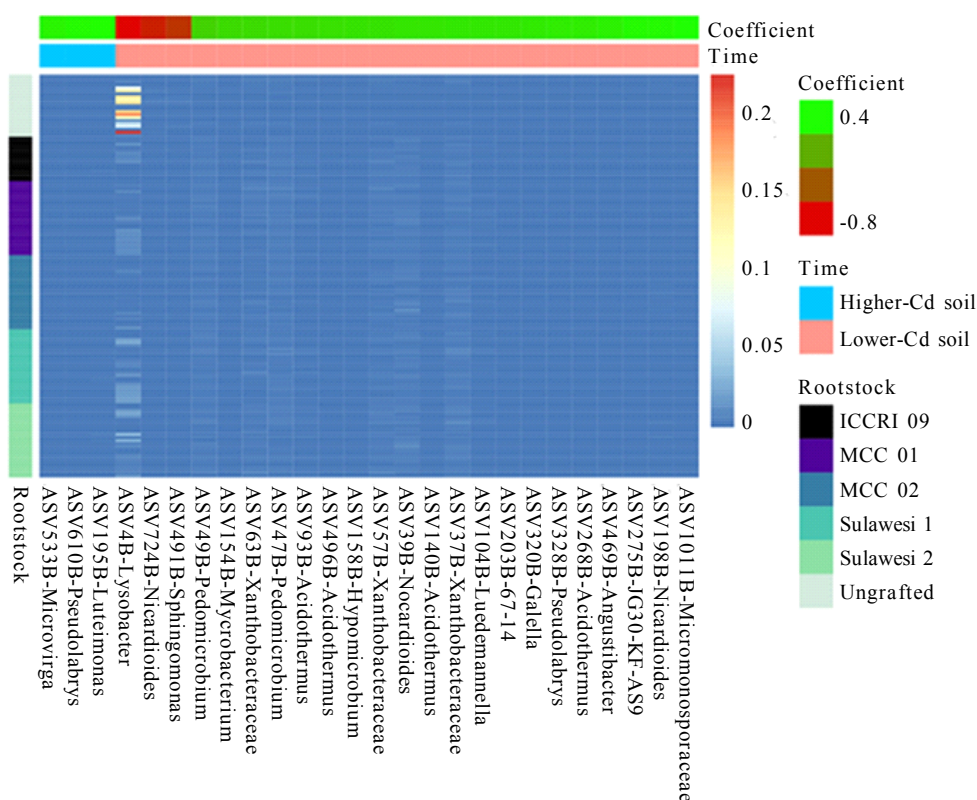


Figure 5. Heatmap of prokaryotic ASVs associated with leaf Cd. Rows represent microbiome samples, organized by rootstock according to the color legend to the left of the plot, and columns represent taxa; The color of the cell indicates the relative abundance of the ASV in that sample, according to the color scale to the right of the plot; Positive coefficients (green in upper legend) indicate a positive relationship between relative abundance and leaf Cd, while negative coefficients (red in upper legend) indicate a negative abundance-uptake relationship; Sampling date is indicated by the second color scale above the plot

(Wu *et al.*, 2022), but decrease in response to soil Cd enrichment elsewhere (Wyszkowska *et al.*, 2022). This genus was also associated with increased Cd accumulation in the rhizosphere of the hyperaccumulator *Arabidopsis helleri* (Muehe *et al.*, 2015). Relationships of *Nocardioidea* sp. to Cd are not well-documented. *Mycothermus thermophilus* is a commercially relevant fungus capable of breaking down diverse types of organic matter. Published genomes and secretomes of this species show that it produces at least three zinc/cadmium resistance proteins (Basotra *et al.*, 2016). It is possible to culture at least some

species of *Lysobacter*, *Sphingomonas*, and *Nocardioidea* as well as *Mycothermus thermophilus*, so future studies should seek to validate their potential mitigating effect on plant Cd uptake and identify the underlying physiological or metabolic mechanisms involved.

This study design, in which field soil was augmented with CdNO₃, may not have been ideal for identifying Cd-tolerant bacteria and fungi, although the soil was taken from the highest-Cd field available. Microorganisms in high-Cd environments face selective pressure to evolve tolerance

traits, increasing the likelihood of isolating potentially beneficial organisms from soils naturally high in Cd. Screening approaches that have been successful in other cocoa-growing regions (e.g. Bravo *et al.*, 2018; Cordoba-Novoa *et al.*, 2022; Guerra Sierra *et al.*, 2022; Quiroga-Mateus *et al.*, 2022;) could be reproduced in Indonesia to find locally-adapted Cd-tolerant microorganisms.

CONCLUSIONS

Breeding and root-associated microbiome optimization remain promising avenues for future Cd mitigation. This study may help inform recommendations for low-Cd-accumulating clones that could be planted as rootstock. The rootstock with the lowest absorption in both soils was MCC 02, and the lowest-absorbing scion was MCC 01. MCC 02 as rootstock with Sulawesi 2 as a scion absorbed the least Cd in the lower-Cd soil, and self-grafted MCC 01 absorbed the least Cd in the high-Cd soil. However, the discrepancy in rankings between graft combinations in soil with naturally occurring levels of Cd vs. augmented Cd also highlights that the potential for G x E interactions deserves further study. Better understanding of the genetic basis of Cd uptake and translocation in cocoa is required to facilitate breeding commercially available clones with even lower uptake. Although further from implementation, microbiome optimization strategies deserve further study. The four taxa identified here as negatively associated with plant Cd uptake (*Lysobacter* sp., *Sphingomonas* sp., *Nocardioides* sp., and *Mycothermus thermophilus*) were not rootstock-specific (i.e. they were found across samples). Potential future bioamendments may thus be applied to low-Cd cultivars, enabling synergy between breeding and management strategies to reduce Cd uptake in Indonesian cocoa.

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