



Soil microbial responses to perennial forage plant cultivation in degraded Ethiopian soils – a greenhouse experiment

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Abstract

Aims Ethiopian soils are severely degraded and nutrient depleted, calling for effective remediation strategies. Enhancing soil biological activity through the cultivation of perennial forages may improve soil nutrient cycling and ameliorate soils. The aim of this study was to evaluate specific forage species as to their ability to improve soil biological functions.

Methods We set up a fully factorial greenhouse experiment with Ethiopian soils from two regions differing in mineralogy, soil type and climate and tested the effect of two grass species *Urochloa* cv. ‘Cayman’, *Megathyrsus maximus* (Mombassa), and two legumes, *Desmodium intortum* (Greenleaf), *Stylosanthes guianensis* (Ubon) grown in single stands and mixtures on soil chemical and microbial variables. After 12 weeks of unfertilized growth, we measured

soil mineral nitrogen (N), respiration, exoenzyme activities, microbial biomass N and phosphorus and the symbiotic performance of legumes.

Results Soils from lower altitude Sidama region had 24% higher soil microbial activity than those from higher altitude Amhara. Aboveground N yield and shoot:root ratios were good indicators for stimulating effects on soil microbial functions, with *S. guianensis* having the strongest effect. Mixtures did not perform better than single stands. Legumes induced a 15% increase in acid phosphatase (AP) and 34% increase in N-enzyme activity which improved P-supply in three of the four soils.

Conclusions AP-activity was stimulated by legumes in all soils but the overall ameliorative effect of perennial forage species appeared to be highly soil dependent. Plant effects on soil biological functions are more pronounced in less acidic soils with higher extractable P.

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Keywords Forage species · Sub-Saharan Africa · Exoenzyme activity · Plant-soil feedback · Nutrient cycling · Legumes

Abbreviations	BG	β-1, 4-glucosidase
	NAG	N-acetyl-glucosaminidase
	LAP	Leucine aminopeptidase
	AP	Acid phosphatase
	EEA	Exoenzyme activity
	N _{mic}	Microbial biomass N
	P _{mic}	Microbial biomass P

Ndfa	Nitrogen derived from atmosphere
BNF	Biological nitrogen fixation

Introduction

Ethiopia faces severe soil degradation due to loss of organic carbon (C), nutrient depletion and erosion (Teku and Derbib 2025). The country has the highest livestock population in Africa, resulting in intense grazing pressure on crop residues in arable small holder agriculture. Paired with limited application of fertilizers, this has resulted in a country-wide decline of soil fertility and productivity (Gebreselassie et al. 2016; FAO 2019) and calls for cultivation strategies that ameliorate soil health while improving animal nutrition (Abegaz et al. 2020). Intermittent cultivation of perennial crop species in cereal cropping (crop rotation or intercropping) has been the norm before the advent of mineral fertilizers and now sees a resurgence for soil amelioration in countries with little access to external input factors (Huss et al. 2022). Particularly perennial grass mixtures, planted as leys, are known to provide multiple benefits connected to deep root systems that tolerate environmental stress, such as drought and low soil nutrient availability while producing high yields, which can be used as forage (Fornara and Tilman 2008; Mueller et al. 2013). In the specific context of mixed smallholder farming in the Ethiopian Rift Valley, intercropping grass mixtures may help in two ways. First, by reducing the grazing pressure on arable fields by providing high-quality feed from increased forage production and quality (Schaub et al. 2020). Second, by ameliorating degraded soils through input of organic matter (plant litter, rhizodeposition) thereby preventing erosion.

Plants impact soil chemical and physical properties by litter input and through constant exchange of matter across the root interface (Dijkstra et al. 2013; Bergmann et al. 2016; Joshi et al. 2024), fueling soil microbiological activity and functions (Potter et al. 2023; Zhou et al. 2024; Wang et al. 2024). Soil microbes are crucial for soil functioning, decomposing organic matter, mineralizing nutrients, and effectively driving soil nutrient cycles. However, soil microbial activity in degraded soils may be nutrient limited, impeding microbial nutrient turnover (Zeng

et al. 2021; Zhou et al. 2024). This is a major issue in weathered and acidic subtropical soils in which phosphorus (P) availability is limited by excessive binding to clay, aluminum oxides and iron oxides (Beyene 2023). Phosphorous binding also depends on the mineralogical composition of the soil, affecting the effective surface area and thereby nature and speciation of free metals (Guzman et al. 1994; Bellani et al. 2020; Amadou et al. 2022). Introducing diverse plant mixtures can relieve microbial nutrient limitation through root deposition (Furey and Tilman 2021) and stimulate microbial activity and nutrient turnover. This creates positive feedback to plants by improving soil nutrient availability (Chen et al. 2014; Zheng et al. 2022).

Soil microbes are most abundant and active in the rhizosphere, where exudates and rhizodeposits rich in carbon and nitrogen (N) stimulate microbial growth. These inputs increase the microbial activity in the vicinity of roots and trigger cascading effects that increase nutrient mineralization, decomposition of organic matter and nutrient cycling (Chen et al. 2014; Potter et al. 2023; Von Haden et al. 2024). However, the content of root exudates is not constant, as both composition and quantity are influenced by soil moisture, soil type and plant species (Maurer et al. 2021). Initial nutrient availability also plays a critical role; for example, increased N input (Jiang et al. 2025) and high soil organic C content (Maurer et al. 2021) have been shown to increase C and N exudation from roots.

Plants exert distinct effects on soil processes, which may vary between functional plant groups. Leguminous plants, for example, fix atmospheric N, acidify the rhizosphere (Yan et al. 1996) and mediate N-transfer from legumes to grasses in mixtures, thereby increasing overall N-yield (Tzanakakis et al. 2017). On the other hand, certain grass species are known to exude compounds inhibiting biological nitrification (BNI), thus retaining more N as NH_4^+ in the soil (Vázquez et al. 2020). Mixtures of species with complementary root traits can result in better root colonization with consequences for N uptake, as diversified growth strategies promote enhanced resource acquisition through interspecific competition (Byers et al. 2025). Thus, mixtures of species from different functional plant groups, such as grasses and legumes, can improve soil structure, enhance nutrient cycling and increase biodiversity.

Soil microbial activity involves synthesis and exudation of hydrolytic and oxidative exoenzymes, which facilitate the decomposition and mineralization of organic substrates for microbial uptake. This exoenzyme activity (EEA) is regulated by both resource availability and microbial metabolic demands (Sinsabaugh et al. 2009). EEA is thus closely linked to metabolic theory and ecological stoichiometry through threshold element ratios, which are defined as the critical ratio of C to N or P at which metabolic limitation shifts from energy conservation to nutrient acquisition (Sinsabaugh et al. 2009). Because the synthesis of exoenzymes is influenced by the balance between nutrient and energy limitation, EEA can be used to assess microbial nutrient limitation in the soil. Moorhead et al. (2016) suggested a vector-based model that utilizes proportional enzyme activities to estimate C-limitation and to identify whether N or P are the primary limiting nutrients. Several studies have demonstrated that EEA respond dynamically to soil amendments, with microbes adjusting enzyme production towards nutrients that are most limiting as resource constraints are alleviated (Chen et al. 2014; Zeng et al. 2021; Piñeiro et al. 2023; Rumeau et al. 2024). Furthermore, root derived inputs of C and N stimulate microbial activity in the rhizosphere, creating a feedback loop in which increased EEA promotes nutrient mineralization, consequently increasing nutrient availability for both plants and microbes (Chen et al. 2014; Zhou et al. 2024; Yang et al. 2024).

Direct observation of plant-soil-plant feedbacks in situ is challenging and requires long-term experimentation. To investigate plant effects putatively associated with soil amelioration in controlled short-term growth experiments, we conducted a climate chamber experiment using changes in microbial EEA, N:P stoichiometries and soil respiration, as short-term indicators for potential plant-soil feedbacks. We tested both single plants and mixed species in a greenhouse pot trial, by growing them in nutrient poor soils from four field locations in the Ethiopian Rift Valley without fertilizer addition. To increase the sensitivity of our assay, we embedded a limited amount of the native soils in washed and sterilized quartz sand and grew plants for 12 weeks in a climate chamber. By using four soils from two pedoclimatic zones in sub-Saharan Africa, we addressed the following questions: (i) Do belowground responses to different plant species vary with initial soil chemical

and mineralogical properties? (ii) Do legumes have larger belowground impacts than grasses? (iii) Are relative changes in soil enzymatic activity and microbial nutrient limitation associated with root biomass and inclusion of legumes? (iv) Do species mixtures result in increased root biomass and enhanced EEA? We hypothesized that (i) species mixtures would produce greater total aboveground and belowground biomass than single stands, regardless of soil, (ii) single stand legumes would have the most pronounced effect on belowground soil microbial functions, and (iii) these effects would significantly depend on soil chemical properties.

Materials and methods

Sites and soils

The soils were collected from four different Farmer Training Centers (FTCs), i.e. government backed centers that serve as platforms for agricultural extension in Ethiopia. These fields were selected due to their accessibility and local management support. The fields are managed by local farmers and soils are generally nutrient poor. The sites hosted field experiments on forage mixtures in the years 2021–2023 (Marit Jørgensen, *pers. comm.*). Soils were sampled prior to establishing field treatments. Two of the fields were situated in the Amhara region in northern Ethiopia, at Kudmi (11°23'21"N, 37°06'41"E) and Abiyot Fana (11°19'02"N, 37°08'44"E), and two in the Sidama region in southern Ethiopia, at Boricha (6°58'17"N, 38°20'14"E) and Bilate Zuria (7°00'09"N, 38°14'08"E). Recommended fertilization rates in Ethiopia are 64 kg ha⁻¹ urea-N (CO(NH₂)₂) grade 46–0–0 (N–P–K), and 20 kg P ha⁻¹ added as Diammonium phosphate (DAP). However, farmers at the FTCs commonly apply only 40–50% of the recommended rate (Shimelis Raji, *pers. comm.*), which also was the case for our four field sites. The mean annual rainfall in Amhara is between 1500 to 2200 mm and the mean annual temperature between 24 °C and 27 °C. The average rainfall in Sidama is between 650 and 960 mm, and the average annual temperature between 22 °C and 30 °C. All sites were used for maize production before the experiment, with yields ranging from 2.5 to 3.5 tons ha⁻¹ in the Amhara region and 3.2 to 3.5 tons ha⁻¹ in the Sidama region.

Soil sampling and seeds

Five soil cores (0–25 cm) were taken with a soil corer (10 cm dia, 27 cm length) on each field on June 16, 2021, one in each corner and one in the middle of the field which had an approximate size of 750 m² in the Amhara region and 530 m² in the Sidama region. The samples were pooled. Larger roots and stones were removed before air-drying and shipping the soil to the Norwegian University of Life Science. Upon arrival, the soils were characterized for soil texture, extractable nutrients, organic C and total N content and acidity (Table 1). Grass seeds were obtained from Tropical seeds, LLC (Papalotla group) with *U. cv.* Cayman sent from Mexico and *M. maximus* sent from Spain, and legume seeds from Alliance Biodiversity & CIAT in Kenya (*D. intortum*) and from Isaan seeds LTD in Thailand (*S. guianensis*). Seeds were kept refrigerated at 4 °C until use.

Soil mineralogy

Soil mineralogy was measured in bulk soil as well as in the clay fraction. Air-dried soil was ground in an agate mill and the soil for the bulk sample was packed directly into disks and analyzed by X-ray diffraction (XRD). The clay fraction was prepared using a modified protocol from Hauff et al. (1984) and Moore and Reynolds (1989). Clay was separated by gravitational sedimentation, and the suspension was arranged in oriented aggregates by pressing the suspension with 5 bar pressure onto ceramic disks. The clay fraction underwent four treatments, two high temperature treatments of 500 °C and 300 °C, one ethylene-glycol treatment and one air dried treatment to identify the minerals based on their behaviors to the respective treatments. The XRD patterns was measured on an D8 ADVANCE XRD (Bruker AXS, Germany) equipped with a LYNXEYE-EX-T

Table 1 Soil chemical characteristics, texture, and mineral composition in the bulk soil. Texture, CEC, mineralogy and extractable nutrients were determined in composite samples. Soil pH and C:N ratio are the averages of five technical replicates ($n = 5$) with standard deviation (SD). Extractable nutrients were measured using the Mehlich III method

			Amhara		Sidama	
			Kudmi	Abiyot Fana	Boricha	Bilate
Altitude	m.a.s.l.		1983	2043	1837	1819
Soil texture			Silt Clay	Silt Loam	Silt Loam	Loam
Sand	%		7.00	10.7	36.6	41.7
Silt			46.7	66.3	54.4	49.0
Clay			46.3	23.0	9.00	9.30
Qtz	wt%		18.2	13.6	33.7	31.0
Fsp			-	-	49.3	53.2
Ill			25.6	26.7	17.0	15.8
Hem			4.30	8.20	-	-
Gth			13.5	8.80	-	-
Ant			7.10	6.50	-	-
Kln			31.3	36.2	-	-
pH			5.53 ± 0.17	5.26 ± 0.092	6.26 ± 0.10	6.20 ± 0.041
C:N ratio			10.9 ± 0.32	11.2 ± 0.38	11.9 ± 0.36	11.8 ± 0.29
CEC	cmol kg ⁻¹		33.4	36.4	48.5	37.3
P	mg kg ⁻¹		1.30	1.00	15.0	16.0
K			5400	6300	5700	5500
Mg			540	230	240	220
Al			930	1200	640	580
S			9.60	24.0	6.40	6.90
Mn			120	190	110	56.0
Fe			150	100	140	120
Ni			4.50	1.30	1.30	0.06
Cu			6.10	4.40	0.26	0.13
Zn			1.60	1.10	7.60	8.50

Abbreviations are given according to the International Mineralogical Association (IMA); Qtz (Quartz), Fsp (Feldspar), Ill (Illite), Hem (Hematite), Gth (Goethite), Ant (Anatase) and Kln (Kaolinite). CEC: cation exchange capacity

detector (1D mode, 0.3° divergence slit and 2.5° Soler slits) and CuK α radiation (1.54 Å, 40 mA, 40 kV). The patterns were obtained from 5° to 70° 2 θ , with a step size of 0.01° and 2.5 s/step. We used the Bruker DIFFRAC.EVA software (v. 5.1) in conjunction with the Crystallographic Open Database (COD) for phase identification.

Experimental design and growth conditions

A general problem when studying below ground effects of plants is the low signal-to-noise ratio of soil nutrient pools and microbial transformation rates; this is partly because of high background values and small-scale heterogeneity. To create a more sensitive system with respect to detecting plant effects on soil nutrient pools and microbial functions in a short-term growth experiment, we opted for a soil inoculum approach (Petipas et al. 2020), deliberately diluting the background concentrations and activities of the native soils. We placed 40 mL air-dried native soil into sterilized sand with a pH of 6.5. This was done to provide the same volume of native soil for root growth in each pot. The added soil dry weight (DW) varied across the four soils, with 40.27 g for Kudmi, 41.74 g for Abiyot Fana, 39.29 g for Boricha and 40.13 g for Bilate. Nutrient concentrations in two rounds of sand leachates were assessed by ICP-MS (8800 ICP-MS Triple Quad, Agilent, USA). For each leaching, 20 mL of de-ionized water was percolated through 10 g of sand. Nutrient contents in the percolate were low (Table S1). The sand was sterilized in a drying cabinet (B8420, Termaks, Norway) at 200 °C for 4 h before use. Each pot (20 cm tall, 13 cm diameter at the rim, slightly conical, 2 L volume) was filled with sand (Fig. 1). A shallow and flat depression (ca. 3 cm deep, 7 cm in diameter) was prepared at the surface before adding 40 mL of dry field soil and covering it with 1 cm sand. The soil was rewetted carefully and incubated overnight to allow microbial activity to stabilize before planting. We set up a fully factorial design with four soils and six plant treatments, each replicated seven times, resulting in a total of 168 pots.

Four different forage species, representing distinct functional groups and growth characteristics were selected: two grasses, *Urochloa* hybrid cv. ‘Cayman’ and *Megathyrsus maximus* (cv. Mombassa), and two legumes, *Desmodium intortum* (cv. Greenleaf) and

Stylosanthes guianensis (cv. Ubon). The species were selected based on contrasting above- and below-ground growth habits and their ability for symbiotic N fixation, thus representing different ecological niches (Fig. 1B). Both grasses are C4 species native to sub-Saharan Africa, while the legumes are C3 species originating from Central and South America. All four species are highly palatable and are widely used as forages in South America and increasingly adopted in sub-Saharan Africa. The selected species were established in six plant treatments consisting of four single stands and two mixtures, one grass-grass (*U.* cv. Cayman and *M. maximus*) and one grass-legume (*U.* cv. Cayman with *D. intortum*) across all four soils. The species used for the grass-legume mixture were selected based on their complementary growth habits: *U.* cv. Cayman is semi-erect and decumbent and has a rhizomatous growth while *D. intortum* is creeping with a strong taproot. To minimize soil disturbance, four seeds were initially sown in each pot at 1 cm depth directly into the soil. After germination, seedlings were thinned to keep two viable plants per pot. In the case of species mixtures, care was taken to ensure that one individual of each species remained in the pot. To account for dead plants or unsprouted seeds, seedlings pre-grown in sterile sand where transplanted to maintain two plants per pot. The pots were placed in a climate chamber at Holt, Tromsø (69°39'7"N, 18°57'12"E), Norway, under controlled conditions: 25 °C during the day and 15 °C at night, with a day/night cycle of 12/12 h and a light intensity of 180 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were watered daily by spraying (ca. 30 mL pot $^{-1}$).

Plant and soil sampling

The experiment was terminated after 12 weeks. Plants were cut 1 cm above the soil surface and placed in paper bags. In pots with two species (mixtures), individual species were sampled separately. The plants were dried at 60 °C for 3 days before determining their dry weight. The upper 4 cm of sand and soil was sampled from each the pot. This was done to ensure that a maximum amount of native soil was recovered, since it was likely that some of the added soil was displaced to the surrounding sand. To account for the dilution of soil by sand, the soil samples were weighed immediately after collection, and a sand-to-soil dilution ratio was calculated

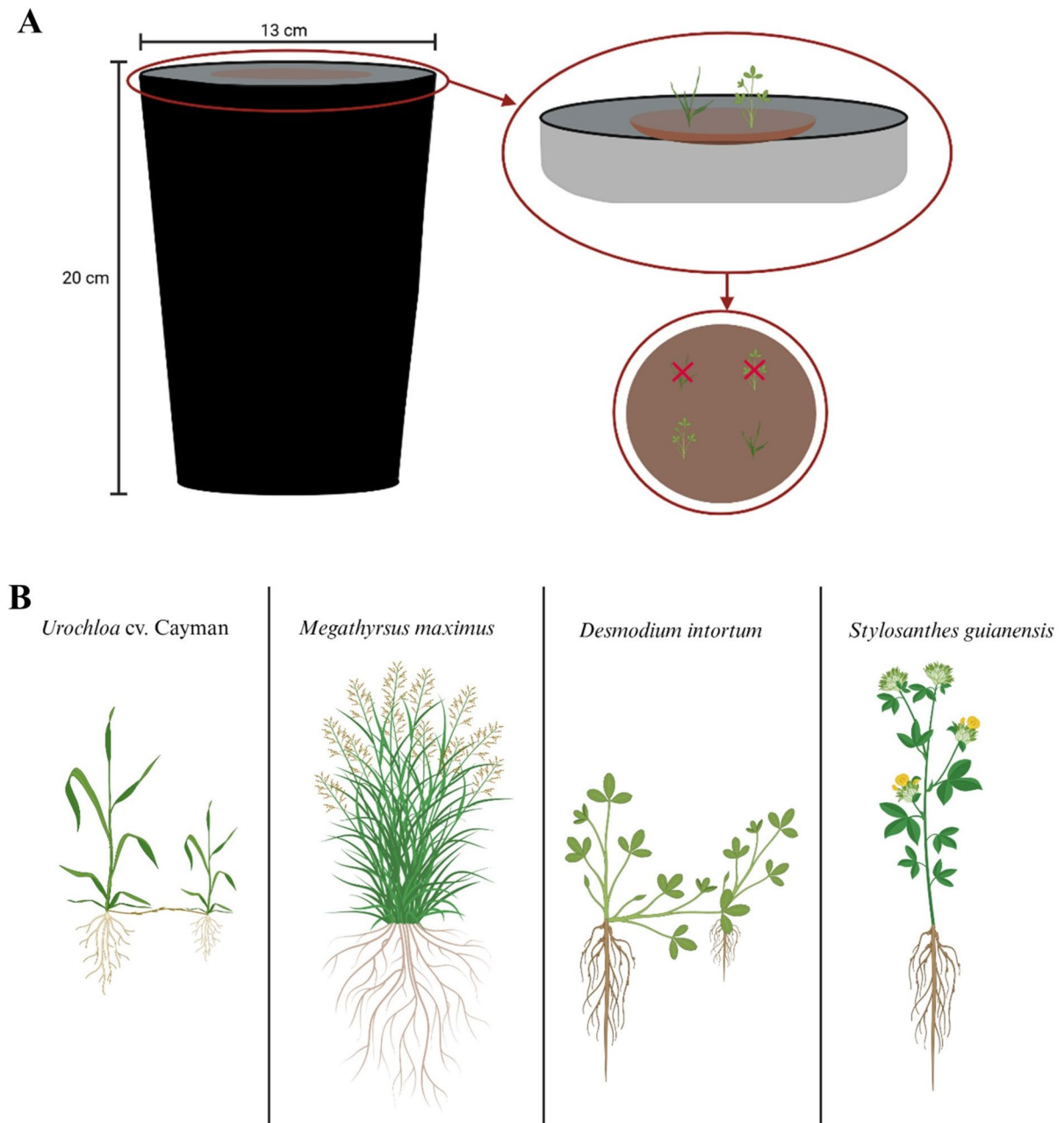


Fig. 1 (A) Illustration of the pot design. (B) Rooting and growth patterns of the plant species used in the experiment (figure created with Biorender)

by dividing the DW of the added soil with the DW-corrected weight of the sand-soil mixture taken from each pot. This ratio was used to upscale the measured variables to the native soil. The estimated amount of native soil recovered accounted for $13.8\% \pm 1.8\%$ on a dry weight basis. During soil sampling, roots were

extracted and collected by brushing off soil and sand from the sampled top layer. After soil and root sampling, the remaining roots in the sand were extracted and all roots were washed with de-ionized water before drying them at $60\text{ }^{\circ}\text{C}$ for three days followed by weighing.

Laboratory analyses

Samples were sieved through a 2 mm mesh to homogenize the soil and to remove small stones, after which the soil samples were stored at 4 °C for one week to overcome the initial flush of microbial activity and allow microbial activity to stabilize before sample processing. Soil pH was measured in a 1:2.5 soil:water suspension using an Orion Star A211 pH meter with an Orion ROSS Sure-Flow combination electrode (Thermo Fisher Scientific, USA). All measurements described below were carried out on seven replicates (i.e. pots per plant-soil combination). For selected variables, additional technical replication was included (e.g. EEA; see below).

Soil respiration

Soil respiration was measured by placing 1 g of fresh soil into a 10 mL septum vial (Chromacol) crimp-sealed with a butyl rubber septum. The vials were flushed with synthetic air (21% O₂ and 79% N₂) by five rounds of evacuation and filling and incubated for 24 h at room temperature. After incubation, the vials were placed in an autosampler connected to a gas-chromatograph (GC model 7890A, Agilent, USA) to measure the accumulated CO₂. CO₂ was recalculated as mg C g⁻¹ DW soil and expressed as a production rate per hour.

Exoenzyme activities (EEA)

We evaluated the activities of four exoenzymes: β -1, 4-glucosidase (BG) as representative for C acquisition, N-acetyl-glucosidase (NAG) and leucine aminopeptidase (LAP) for N-acquisition, and acid phosphatase (AP) for P-acquisition using the method described by Saiya-Cork et al. (2002) and German et al. (2011) with modifications. Briefly, 0.5 g soil (DW equivalent) was suspended in 21 mL TRIS-HCl buffer (50 mmol L⁻¹, pH 6.35). Four replicates of 200 μ L of the suspension were pipetted into a 96-well microplate, and 50 μ L of the fluorometric substrate were added. 10 μ M of 4-methylumbelliferone was added as a quench control. Due to the weathered and oxidized state of the soil, it was difficult to achieve

quantifiable enzyme activity, and we increased the incubation time to 24 h at room temperature (22 °C). To terminate reactions, we added 10 μ L of 1 mol L⁻¹ NaOH. Fluorescence was measured using a Variskan Lux microplate reader (3020–471, Thermo Fisher Scientific, USA) with an excitation wavelength at 365 nm and an emission wavelength at 450 nm.

Microbial biomass

To focus on microbial nutrients known to be limiting in sub-Saharan soils, microbial biomass N and P were determined using the chloroform fumigation-extraction method (Brookes et al. 1982). For microbial biomass nitrogen (N_{mic}), 4 g of fresh soil was fumigated with chloroform for 24 h in a desiccator. After fumigation, 20 mL of 0.5 mol L⁻¹ K₂SO₄ was added, and the tubes were shaken for 1 h. The resulting suspensions were filtered through Schleicher & Schuell filter paper. A parallel, non-fumigated soil sample was extracted and filtered in the same way. 5 mL of potassium peroxidase was added to 10 mL of the extracts and autoclaved 120 °C for 30 min. Total N was measured using a Flow Injector Analyzer (FIA, FIAstar 5000 System, Foss A/S, Denmark). The N_{mic} was calculated from the fumigation excess of total N using a conversion factor of 0.54 (Brookes et al. 1985; Joergensen and Mueller 1996). For microbial biomass phosphorus (P_{mic}), we used 0.5 mol L⁻¹ NaHCO₃ as extractant. Total P was analysed by the molybdenum blue colorimetric method. P_{mic} was calculated from fumigation excess in total P with a conversion factor of 0.40 (Brookes et al. 1982).

Mineral N

Mineral N was measured in all soil samples using colorimetric assays. Soil suspensions were prepared with 5 g DW soil suspended with 6.25 mL of 2 mol L⁻¹ KCl in 15 mL falcon tubes (Byers et al. 2024). The suspensions were shaken at 125 rpm for 30 min. Aliquots of the supernatant were transferred to 1.5 mL Eppendorf tubes and centrifuged for 10 min before analyzing NO₃⁻ and NO₂⁻ by Griess reaction with V(III)Cl (Doane and Horváth 2003) and NH₄⁺ using the Berthelot reaction (Krom 1980).

Plant N and ^{15}N natural abundance

To estimate plant N yield and N derived from the atmosphere (Ndfa) in treatments with legumes, we determined the N content and ^{15}N natural abundance in the above ground plant material (Peoples et al. 1995). The dried plant material was clipped and placed in a ball mill (MM301, Retsch, Germany) and shaken at a frequency of 13 Hz for 4 min to grind the material into a fine powder. 5–10 mg of the powder was weighed into 6×3 mm tin capsules, which were combusted by a flash-combustion element analyzer (Thermo Flash EA 1112, Thermo Scientific, USA) coupled to a continuous flow isotope ratio mass spectrometer (IRMS, Delta XP, Thermo Finnigan MAT GmbH, Germany). N content was measured against a calibration curve with EDTA and multiplied with aboveground dry matter yield to determine N yield. ^{15}N natural abundance was measured against a certified standard (IAEA-N1) and used to estimate how much of the N was derived from the atmosphere according to Eq. 1:

$$\text{Ndfa (\%)} = \frac{\delta^{15}\text{N}_{\text{ref}} - \delta^{15}\text{N}_{\text{legume}}}{\delta^{15}\text{N}_{\text{ref}} - \text{B}} \times 100 \quad (1)$$

where N_{ref} is the measured ^{15}N abundance of the grasses (single stands) and N_{legume} the $\delta^{15}\text{N}$ value of the legume biomass. B is defined as the lowest measured $\delta^{15}\text{N}$ value in each legume (−8.6 for *S. guianensis* and −7.5 for *D. intortum*) (Hansen and Vinther 2001).

The amount of biological N fixed (BNF) by the legumes was as follows:

$$\text{BNF} = \text{Dry matter} \times \text{N content (\%)} \times \text{Ndfa (\%)} \quad (2)$$

where Dry matter is the sum of the shoot and root biomass calculated from dry matter yield and the N content is the % N in the plants. We assumed roots to have the same N content as measured in the above-ground biomass.

Calculations

Potential overyielding in the mixtures was calculated according to Eq. 3:

$$\text{Overyielding} = \text{Mixture} - \frac{(\text{Plant A} + \text{Plant B})}{2} \quad (3)$$

where Plant A and Plant B are the average dry matter yields of the single species grown in single stands, which is subtracted from the average DMY of the mixture. A positive number indicates overyielding.

We used the original vector model proposed by Moorhead et al. (2016) to calculate microbial nutrient balances based on measured EEA. Proportional ratios of the measured N- and P- EEA were calculated according to Eqs. 4 and 5, respectively:

$$x = C/(C + N) \quad (4)$$

$$y = C/(C + P) \quad (5)$$

where C represents BG enzyme activity, N the activity of the NAG and LAP enzymes and P the AP enzyme activity. These proportional activities were then used to calculate vector length and angle according to Eq. 6 and 7, respectively:

$$\text{Vector length} = \sqrt{(x^2 + y^2)} \quad (6)$$

$$\text{Vector angle} = \text{DEGREES}(\text{ATAN2}(x, y)) \quad (7)$$

C-limitation was estimated as the length of the vector, with increasing length indicating increasing C-limitation. The vector angle indicates whether the microbes are N or P limited, with 45° indicating nutrient balance and values above 45° P-limited and below 45° N-limitation.

Statistics

Differences in response variables between plants and soils and potential interactive effects were tested by two-way Anova. In case of significant soil effects, we tested each soil separately for plant effects using one-way Anova for effects on above- and below ground biomass, N yield, mineral N, soil pH, %Ndfa, soil microbial functions and the output of the vector analysis. Tukey HSD post hoc tests were used to identify significant differences between treatments. To check for potential collinearity between the variables, we used the cor_mat function in the rstatix package (Kassambara 2019) overlaid with the cor_pmat function from the ggcorrplot package (Kassambara 2016), thus testing for significant correlations between the measured soil variables and above- and belowground

biomass. We further subjected the data to principal component analysis (PCA) separately for each soil, using the `prcomp` function. All tests were performed using R studio v. 4.3.1 (Posit team 2025).

Results

Soil properties and mineralogy

Soil properties varied greatly between the two regions. Soils from the Amhara region were more clay-rich and acidic (pH 5.26–5.53) while soils from the Sidama region were loamier and less acidic (pH 6.20–6.26) (Table 1). Also, chemical properties varied between the regions, most prominently for Melich III-extractable P, which was 11 to 16 times higher in Sidama than Amhara soils. In contrast, Amhara soils had almost twice as much extractable aluminium (Al) compared to Sidama soils.

Soil mineralogy differed grossly between the two regions. ~50% of the bulk soil in Sidama consisted of feldspar, which was absent in Amhara soils. Conversely, hematite, goethite, anatase and kaolinite were found in Amhara, but not in Sidama soils (Table 1). The clay fraction in Amhara soils contained kaolinite, was high in Fe-oxides, and contained an interstratified phase of illite-vermiculite-smectite (IVS) which, together, indicated ongoing weathering (Fig. S1). In contrast, the clay fraction in Sidama soils lacked IVS and was dominated by vermiculite (Fig. S2).

Plant growth

Plant growth differed significantly between the soils ($p < 0.001$). Overall, plants grown in Sidama soils accumulated more biomass than in Amhara soils, both above- and belowground (Table 2). Legumes generally out-performed grasses and mixtures in DM production irrespective of soil. *S. guianensis* produced the largest above ground biomass, while *D. intortum* accumulated most belowground biomass. Belowground biomass and shoot:root ratios varied significantly between plant species, illustrating differences in growth strategy between the legumes; for instance, *S. guianensis* had a shoot:root ratio above 1 in all soils, while this ratio was below 1 for *D. intortum*. Grasses and mixtures had generally lower shoot:root ratios (0.510–0.910) except for

the *Urochloa* + *Desmodium* mixture in Boricha soil (1.92) and *M. maximus* in Bilate soil (1.99). A graphical presentation of dry matter yields and shoot:root ratios can be found figure S3. No overyielding of any significance was observed.

Soil mineral N, plant N yield and symbiotic performance

Soil mineral N content after the growth experiment was significantly smaller in Amhara than Sidama soils (Table 3). Sidama soils showed a huge variability in extractable NH_4^+ which was not observed in Amhara soils (Fig. 2A). Total mineral N was highest with legumes, except for Abiyot Fana soil, for which *Urochloa* + *Megathyrsus* had the highest value (Table 3).

Plant N yield varied widely between both species and soils, ranging from 0.0816 to 12.0 mg N pot^{-1} (Table 3, Fig. S3). Bilate soil produced most plant N, followed by Boricha soil and the Amhara soils. N yield varied significantly between plants with *Urochloa* + *Megathyrsus* having the lowest aboveground N yield irrespective of soil. Legumes had higher N yields than non-legumes and *S. guianensis* had significantly higher N yield ($p < 0.001$) than *D. intortum* irrespective of soil. N yields correlated positively with soil mineral N in all soils except Abiyot Fana (Fig. S4). In Sidama soil, mineral N was negatively related to soil pH, which was lower under legumes.

Symbiotic performance of legumes assessed as N derived from atmosphere (%Nd_{fa}) was generally high ($73.1 \pm 15.2\%$) and showed no significant differences between soils or legume-species mixture (Fig. 2B). A notable exception was a significantly higher %Nd_{fa} in *S. guianensis* than *D. intortum* in Bilate soil ($p = 0.001$), both in single stand and in mixture with *U. cv. Cayman*. By contrast, BNF varied significantly with the Sidama soils fixing more N than Amhara soils in absolute terms. BNF was largest with *S. guianensis* in Bilate soil (Fig. 2C).

Soil respiration

Soil respiration varied significantly ($p < 0.001$) between the soils, with the highest rates observed in Kudmi and Bilate and the lowest in Abiyot Fana (Table 3, Fig. S5). Soil respiration in Sidama soils

Table 2 Mean values of plant growth parameters and plant N yield per pot, with standard errors (SE), across the different plant treatments and soils. Levels of significance are indicated as follows: NS = not significant, * = $p < 0.05$ –0.01.

** = $p < 0.01$ –0.001 and *** = $p < 0.001$. Different superscript letters indicate statistically significant differences between plant treatments. Sample size for each treatment combination was $n = 7$

	Site	Treatment	g dry matter pot ⁻¹		Shoot:root ratio	mg N pot ⁻¹
			Shoot (DW)	Root (DW)		N yield
All soils	P-value	Plants	< 0.001***	< 0.001***	< 0.001***	< 0.001***
		Soils	< 0.001***	< 0.001***	< 0.001***	< 0.001***
		Plant × Soil	< 0.001***	< 0.001***	< 0.001***	< 0.001***
Amhara	Kudmi	P-value	< 0.001***	< 0.001***	< 0.001***	< 0.001***
		<i>Urochloa</i> + <i>Desmodium</i>	0.112 ± 0.012 ^{bc}	0.170 ± 0.019 ^b	0.697 ± 0.074 ^b	0.547 ± 0.064 ^c
		<i>Urochloa</i> + <i>Megathyrsus</i>	0.0882 ± 0.0040 ^d	0.183 ± 0.021 ^{ab}	0.510 ± 0.044 ^b	0.318 ± 0.010 ^d
		<i>Urochloa</i>	0.107 ± 0.011 ^c	0.168 ± 0.031 ^b	0.753 ± 0.10 ^b	0.635 ± 0.056 ^c
		<i>Megathyrsus</i>	0.0772 ± 0.0042 ^d	0.122 ± 0.023 ^b	0.755 ± 0.10 ^b	0.603 ± 0.038 ^c
		<i>Desmodium</i>	0.125 ± 0.011 ^b	0.247 ± 0.075 ^a	0.653 ± 0.092 ^b	1.77 ± 0.18 ^b
		<i>Stylosanthes</i>	0.170 ± 0.0066 ^a	0.130 ± 0.020 ^b	1.59 ± 0.28 ^a	2.97 ± 0.10 ^a
		Kudmi mean	0.113 ± 0.0059	0.170 ± 0.016	0.827 ± 0.078	1.14 ± 0.15
	Abiyot Fana	P-value	< 0.001***	< 0.001***	< 0.001***	< 0.001***
		<i>Urochloa</i> + <i>Desmodium</i>	0.122 ± 0.0082 ^b	0.162 ± 0.014 ^a	0.763 ± 0.039 ^b	0.561 ± 0.051 ^c
		<i>Urochloa</i> + <i>Megathyrsus</i>	0.0627 ± 0.0046 ^d	0.109 ± 0.017 ^b	0.634 ± 0.060 ^b	0.207 ± 0.033 ^d
		<i>Urochloa</i>	0.0878 ± 0.0085 ^c	0.125 ± 0.023 ^{ab}	0.774 ± 0.070 ^b	0.521 ± 0.051 ^c
		<i>Megathyrsus</i>	0.0479 ± 0.0017 ^d	0.0970 ± 0.039 ^b	0.788 ± 0.13 ^b	0.339 ± 0.013 ^d
		<i>Desmodium</i>	0.133 ± 0.0061 ^b	0.165 ± 0.012 ^a	0.828 ± 0.052 ^b	1.92 ± 0.051 ^b
		<i>Stylosanthes</i>	0.151 ± 0.012 ^a	0.143 ± 0.028 ^{ab}	1.71 ± 0.65 ^a	2.44 ± 0.19 ^a
		Abiyot Fana mean	0.101 ± 0.0066	0.134 ± 0.010	0.917 ± 0.12	1.00 ± 0.14
	Boricha	P-value	< 0.001***	< 0.001***	< 0.001***	< 0.001***
		<i>Urochloa</i> + <i>Desmodium</i>	0.199 ± 0.022 ^c	0.163 ± 0.034 ^{bc}	1.92 ± 0.60 ^b	0.963 ± 0.15 ^c
		<i>Urochloa</i> + <i>Megathyrsus</i>	0.0703 ± 0.0061 ^d	0.0936 ± 0.023 ^d	0.910 ± 0.13 ^c	0.0816 ± 0.0086 ^d
		<i>Urochloa</i>	0.0993 ± 0.0087 ^d	0.131 ± 0.019 ^{bcd}	0.836 ± 0.093 ^c	0.254 ± 0.026 ^d
		<i>Megathyrsus</i>	0.0844 ± 0.0082 ^d	0.102 ± 0.015 ^{cd}	0.885 ± 0.067 ^c	0.255 ± 0.022 ^d
		<i>Desmodium</i>	0.334 ± 0.021 ^b	0.519 ± 0.058 ^a	0.697 ± 0.074 ^c	4.81 ± 0.50 ^b
		<i>Stylosanthes</i>	0.411 ± 0.020 ^a	0.171 ± 0.019 ^b	2.65 ± 0.35 ^a	8.81 ± 0.39 ^a
		Boricha mean	0.200 ± 0.021	0.200 ± 0.026	1.32 ± 0.16	2.53 ± 0.51
	Bilate	P-value	< 0.001***	< 0.001***	< 0.001***	< 0.001***
		<i>Urochloa</i> + <i>Desmodium</i>	0.274 ± 0.031 ^c	0.349 ± 0.029 ^{bc}	0.799 ± 0.075 ^b	1.36 ± 0.35 ^c
		<i>Urochloa</i> + <i>Megathyrsus</i>	0.0924 ± 0.011 ^c	0.150 ± 0.020 ^{cd}	0.693 ± 0.11 ^b	0.298 ± 0.028 ^d
		<i>Urochloa</i>	0.115 ± 0.013 ^{de}	0.157 ± 0.032 ^{cd}	0.798 ± 0.053 ^b	0.923 ± 0.11 ^{cd}
		<i>Megathyrsus</i>	0.185 ± 0.072 ^d	0.111 ± 0.020 ^d	1.99 ± 0.88 ^a	1.73 ± 0.51 ^c
		<i>Desmodium</i>	0.444 ± 0.029 ^b	0.579 ± 0.094 ^{ab}	0.919 ± 0.15 ^b	7.00 ± 0.47 ^b
		<i>Stylosanthes</i>	0.600 ± 0.030 ^a	0.587 ± 0.25 ^a	1.71 ± 0.30 ^a	12.0 ± 0.59 ^a
		Bilate mean	0.285 ± 0.032	0.322 ± 0.055	1.15 ± 0.18	3.89 ± 0.68

was on average 19.4% larger than in Amhara soils. *S. guianensis* and in some cases *D. intortum* showed significantly ($p < 0.001$) higher soil respiration rates than other plant treatments in all soils except Abiyot Fana, in which the *Urochloa* + *Desmodium* mixture had

highest respiration. Soil respiration correlated positively with root biomass in the Sidama soils (Fig. S4). This was not observed in Amhara soils. By contrast, shoot biomass correlated positively with respiration in all four soils ($p < 0.001$). In Kudmi, Boricha and

Table 3 Mean values of soil biological variables, with standard errors (SE), across the plant treatments and soils. All values were corrected for sand dilution to ensure comparability across samples. Significance levels are indicated as follows: NS=not significant. *= $p<0.05$ –0.01. **= $p<0.01$ –0.001 and ***= $p<0.001$. Different superscript letters indicate statistically significant differences between plant treatments. Sample size was $n=28$ for the exoenzyme activities and $n=7$ for other measurements for each treatment combination

Site	Plant treatment	nmol g ⁻¹ h ⁻¹			µg C g ⁻¹ DW soil h ⁻¹			mmol kg ⁻¹			µg N g ⁻¹ DW soil		
		BG	NAG+LAP	AP	Respiration	N _{mic}	P _{mic}	N _{mic} :P _{mic} ratio	Mineral N	pH			
All soils	Plant	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	0.018*	<0.001***	<0.001***	<0.001***	<0.001***	
	Soil	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	
	Plant × Soil	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	
	P-value	<0.001***	<0.001***	0.103 ^{NS}	<0.001***	<0.001***	<0.001***	0.018*	<0.001***	<0.001***	<0.001***	<0.001***	
Amhara	Kudmi	<i>Urochloa</i> + <i>Desmodium</i>	0.586 ± 0.052 ^{bc}	9.50 ± 0.52 ^b	655 ± 13	0.827 ± 0.035 ^b	2.65 ± 0.51 ^b	0.394 ± 0.11 ^b	14.5 ± 5.4 ^{ab}	18.4 ± 1.3 ^{bc}	5.97 ± 0.016 ^a	5.97 ± 0.016 ^a	
		<i>Urochloa</i> + <i>Megathyrsus</i>	0.901 ± 0.062 ^a	7.22 ± 0.36 ^b	614 ± 19	0.854 ± 0.040 ^b	1.05 ± 0.16 ^c	0.285 ± 0.13 ^b	9.13 ± 3.1 ^b	22.6 ± 4.2 ^b	5.96 ± 0.029 ^a	5.96 ± 0.029 ^a	
	<i>Urochloa</i>	0.781 ± 0.068 ^{ab}	8.86 ± 0.69 ^b	665 ± 16	0.875 ± 0.017 ^b	2.53 ± 0.37 ^b	0.240 ± 0.050 ^b	17.7 ± 6.9 ^a	21.0 ± 3.8 ^{bc}	5.89 ± 0.024 ^b	5.89 ± 0.024 ^b		
		<i>Megathyrsus</i>	1.02 ± 0.10 ^a	9.39 ± 0.45 ^b	671 ± 13	0.867 ± 0.026 ^b	2.78 ± 0.16 ^b	0.308 ± 0.000 ^b	9.03 ± 0.53 ^b	14.2 ± 0.74 ^c	5.81 ± 0.014 ^c	5.81 ± 0.014 ^c	
	<i>Desmodium</i>	0.520 ± 0.040 ^c	7.46 ± 0.38 ^b	662 ± 19	0.822 ± 0.042 ^b	2.51 ± 0.30 ^b	0.191 ± 0.014 ^b	14.3 ± 2.5 ^{ab}	16.5 ± 1.4 ^{bc}	5.80 ± 0.016 ^c	5.80 ± 0.016 ^c		
		<i>Stylosanthes</i>	0.938 ± 0.25 ^{abc}	12.7 ± 0.84 ^a	681 ± 21	1.02 ± 0.040 ^a	5.82 ± 0.54 ^a	0.877 ± 0.21 ^a	10.7 ± 3.2 ^{ab}	29.5 ± 5.5 ^a	5.83 ± 0.026 ^c	5.83 ± 0.026 ^c	
	Kudmi mean		0.791 ± 0.051	9.19 ± 0.27	658 ± 7.1	0.877 ± 0.017	2.86 ± 0.27	0.377 ± 0.057	12.6 ± 1.7	20.4 ± 1.5	5.88 ± 0.014	5.88 ± 0.014	
		P-value	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	0.009*	0.004***	<0.001***	<0.001***	<0.001***
	Abiyot Fana	<i>Urochloa</i> + <i>Desmodium</i>	0.774 ± 0.084 ^{ab}	3.92 ± 0.27 ^c	320 ± 7.8 ^c	0.514 ± 0.073 ^a	6.01 ± 0.39 ^a	0.429 ± 0.043 ^{bc}	14.7 ± 1.4 ^{ab}	16.0 ± 4.8 ^{ab}	5.88 ± 0.019 ^{ab}	5.88 ± 0.019 ^{ab}	5.88 ± 0.019 ^{ab}
		<i>Urochloa</i> + <i>Megathyrsus</i>	0.403 ± 0.033 ^c	4.78 ± 0.22 ^c	329 ± 12 ^{bc}	0.390 ± 0.014 ^c	5.97 ± 0.48 ^a	0.531 ± 0.14 ^{abc}	16.1 ± 3.9 ^{ab}	19.2 ± 6.1 ^a	5.86 ± 0.024 ^{bc}	5.86 ± 0.024 ^{bc}	5.86 ± 0.024 ^{bc}
<i>Urochloa</i>		0.484 ± 0.048 ^{bc}	4.02 ± 0.37 ^c	377 ± 18 ^b	0.434 ± 0.020 ^{bc}	4.73 ± 0.39 ^b	0.713 ± 0.10 ^{ab}	7.58 ± 1.1 ^b	9.38 ± 1.7 ^b	5.91 ± 0.020 ^a	5.91 ± 0.020 ^a	5.91 ± 0.020 ^a	
<i>Megathyrsus</i>		0.823 ± 0.11 ^{ab}	4.57 ± 0.40 ^c	356 ± 15 ^{bc}	0.400 ± 0.023 ^c	3.77 ± 0.70 ^c	0.387 ± 0.14 ^{bc}	16.2 ± 3.4 ^{ab}	13.2 ± 2.6 ^{ab}	5.83 ± 0.026 ^c	5.83 ± 0.026 ^c	5.83 ± 0.026 ^c	
<i>Desmodium</i>		0.568 ± 0.079 ^{bc}	7.48 ± 0.44 ^b	369 ± 8.1 ^{bc}	0.383 ± 0.020 ^c	2.40 ± 0.23 ^d	0.841 ± 0.37 ^a	10.5 ± 6.7 ^{ab}	15.2 ± 2.1 ^{ab}	5.84 ± 0.021 ^{bc}	5.84 ± 0.021 ^{bc}	5.84 ± 0.021 ^{bc}	
<i>Stylosanthes</i>		1.02 ± 0.16 ^a	10.9 ± 0.45 ^a	478 ± 14 ^a	0.487 ± 0.033 ^{ab}	3.12 ± 0.32 ^{cd}	0.338 ± 0.15 ^c	17.0 ± 5.8 ^a	12.8 ± 0.99 ^{ab}	5.71 ± 0.015 ^d	5.71 ± 0.015 ^d	5.71 ± 0.015 ^d	
Abiyot Fana mean		0.685 ± 0.042	5.95 ± 0.25	371 ± 6.5	0.435 ± 0.016	4.33 ± 0.28	0.540 ± 0.079	13.7 ± 1.8	14.1 ± 1.5	5.84 ± 0.012	5.84 ± 0.012	5.84 ± 0.012	

Table 3 (continued)

Site	Plant treatment	nmol g ⁻¹ h ⁻¹		µg C g ⁻¹ DW soil h ⁻¹		mmol kg ⁻¹		µg N g ⁻¹ DW soil		pH
		BG	NAG + LAP	AP	Respiration	N _{mic}	P _{mic}	N _{mic} :P _{mic} ratio	Mineral N	
Sidama	P-value	<0.001***	<0.001***	0.001**	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***
	<i>Urochloa</i> + <i>Desmodium</i>	0.812 ± 0.066 ^{bed}	11.4 ± 0.91 ^a	400 ± 33 ^{ab}	0.629 ± 0.041 ^c	4.01 ± 0.22 ^b	1.06 ± 0.48 ^a	17.1 ± 7.0 ^b	29.7 ± 3.6 ^a	6.61 ± 0.026 ^a
	<i>Urochloa</i> + <i>Megathyrus</i>	0.754 ± 0.049 ^{cd}	9.96 ± 0.64 ^{ab}	454 ± 22 ^a	0.668 ± 0.055 ^c	3.87 ± 0.41 ^b	0.145 ± 0.035 ^b	39.4 ± 8.2 ^a	22.7 ± 0.80 ^b	6.62 ± 0.014 ^a
	<i>Urochloa</i>	0.599 ± 0.076 ^d	8.45 ± 0.89 ^{abc}	364 ± 19 ^b	0.658 ± 0.023 ^c	3.95 ± 0.23 ^b	0.186 ± 0.047 ^b	33.0 ± 12 ^{ab}	22.4 ± 2.2 ^b	6.63 ± 0.024 ^a
	<i>Megathyrus</i>	0.960 ± 0.050 ^{abc}	11.4 ± 0.55 ^a	428 ± 12 ^{ab}	0.653 ± 0.024 ^c	5.00 ± 0.16 ^a	0.228 ± 0.047 ^b	32.2 ± 9.4 ^{ab}	30.4 ± 2.7 ^a	6.55 ± 0.017 ^b
	<i>Desmodium</i>	1.26 ± 0.11 ^a	8.22 ± 0.93 ^{bc}	473 ± 21 ^a	0.793 ± 0.030 ^b	4.39 ± 0.38 ^{ab}	0.322 ± 0.065 ^b	19.4 ± 4.9 ^b	33.9 ± 3.3 ^a	6.34 ± 0.030 ^c
Bilate	<i>Stylosanthes</i>	1.04 ± 0.058 ^{ab}	6.56 ± 0.54 ^c	477 ± 14 ^a	1.03 ± 0.037 ^a	3.94 ± 0.33 ^b	0.186 ± 0.022 ^b	22.5 ± 2.2 ^b	33.0 ± 3.2 ^a	6.22 ± 0.018 ^d
	Boricha mean	0.905 ± 0.033	9.36 ± 0.33	432 ± 9.2	0.738 ± 0.026	4.19 ± 0.14	0.354 ± 0.095	27.0 ± 3.3	28.7 ± 1.3	6.50 ± 0.026
	P-value	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***
	<i>Urochloa</i> + <i>Desmodium</i>	1.17 ± 0.053 ^{abc}	4.63 ± 0.46 ^c	363 ± 8.6 ^c	0.837 ± 0.060 ^c	4.11 ± 0.47 ^a	0.365 ± 0.077 ^b	17.0 ± 4.6 ^{abc}	19.4 ± 2.5 ^{abc}	6.46 ± 0.029 ^b
	<i>Urochloa</i> + <i>Megathyrus</i>	1.16 ± 0.10 ^{abc}	5.73 ± 0.57 ^c	404 ± 23 ^{bc}	0.785 ± 0.020 ^c	4.22 ± 0.55 ^a	0.284 ± 0.022 ^{bc}	15.3 ± 1.8 ^{bc}	18.2 ± 2.0 ^{bc}	6.58 ± 0.017 ^c
	<i>Urochloa</i>	0.941 ± 0.054 ^c	9.70 ± 0.93 ^b	357 ± 19 ^c	0.805 ± 0.048 ^c	2.93 ± 0.51 ^b	0.231 ± 0.080 ^{bc}	30.4 ± 10 ^{ab}	18.2 ± 1.1 ^{bc}	6.62 ± 0.018 ^c
Bilate	<i>Megathyrus</i>	1.04 ± 0.10 ^{bc}	9.44 ± 0.40 ^b	339 ± 8.4 ^c	0.802 ± 0.019 ^c	1.78 ± 0.29 ^c	0.559 ± 0.14 ^a	5.74 ± 2.0 ^c	16.5 ± 1.3 ^c	6.60 ± 0.021 ^c
	<i>Desmodium</i>	1.45 ± 0.074 ^a	13.6 ± 0.78 ^a	446 ± 22 ^{ab}	0.952 ± 0.071 ^b	2.20 ± 0.27 ^{bc}	0.166 ± 0.063 ^c	29.3 ± 9.7 ^{ab}	22.6 ± 2.1 ^a	6.45 ± 0.025 ^b
	<i>Stylosanthes</i>	1.55 ± 0.11 ^{ab}	15.5 ± 0.72 ^a	489 ± 11 ^a	1.15 ± 0.057 ^a	2.72 ± 0.36 ^b	0.190 ± 0.051 ^c	32.4 ± 12 ^a	22.1 ± 2.3 ^{ab}	6.20 ± 0.028 ^c
	Bilate mean	1.22 ± 0.039	9.77 ± 0.40	400 ± 7.8	0.889 ± 0.028	3.53 ± 0.22	0.275 ± 0.038	24.0 ± 3.5	24.0 ± 0.85	6.49 ± 0.024

Abbreviations: β-1, 4-glucosidase (BG), N-acetyl-glucosaminidase (NAG), leucine aminopeptidase (LAP), acid phosphatase (AP), microbial biomass N (N_{mic}), microbial biomass P (P_{mic}), microbial biomass N and microbial biomass P ratio (N_{mic}:P_{mic} ratio)

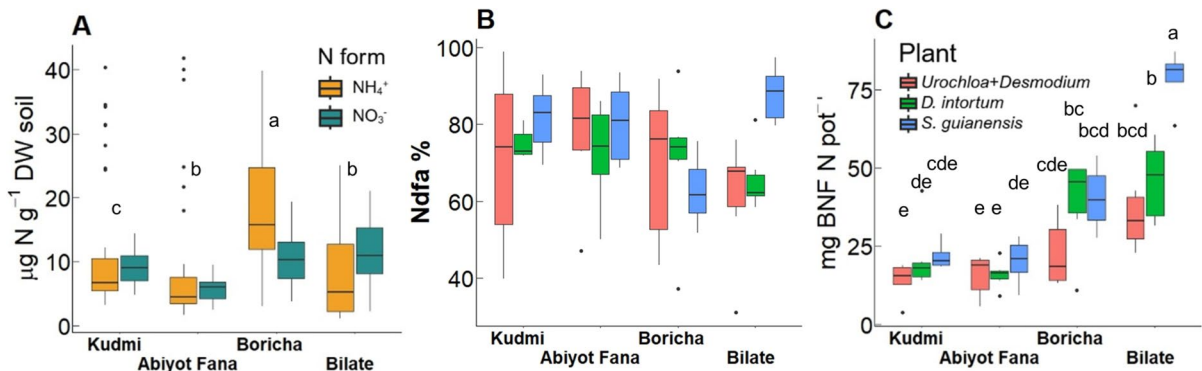


Fig. 2 Boxplots of N parameters in the four soils and under different legume input. (A) Content of KCl-extractable NO_3^- and NH_4^+ -N (mineral N). (B) Percentage of N derived from atmosphere (Ndfa) and (C) biological N fixation (BNF) by legumes in the three treatments including legumes. Each box in the plot shows the upper and lower quartiles, with horizontal lines indicating the median values. The vertical lines represent

the minimum and maximum values, and dots indicate outliers. Different letters indicate statistically significant differences between the treatments obtained from Tukey HSD post hoc test. In plot A, each box is calculated from each soil, with a sample size of 42 per box. For plots B and C the boxes represent each leguminous plant in the soils, with a sample size of 7 per box

Bilate soil, respiration was additionally correlated with plant N yield.

Enzyme activity and microbial N:P stoichiometry

Exoenzyme activity varied significantly between the soils ($p < 0.001$, Table 3, Fig. S5). In Kudmi soil, EEA was highest under single stand legumes, with *S. guianensis* having the highest observed NAG+LAP and AP activity. *M. maximus* in single stand or in mixture with *U. cv. Cayman* had the highest BG activity. While there was no significant plant effect on AP in Kudmi soil, AP activity was highest with *S. guianensis*. Also in Abiyot Fana soil, highest EEAs were measured with legumes, in particular with *S. guianensis*. In Borichia soil, plant treatment effects on EEA varied. *D. intortum* had the highest BG activity, *M. maximus* and *Urochloa-Desmodium* mixture the highest NAG+LAP activities and *Urochloa+Megathyrsus* and the legumes the highest AP activity. Interestingly, the legumes had the lowest NAG+LAP activity. In Bilate soil, EEA was highest under legumes. *D. intortum* had the highest BG activity and *S. guianensis* the highest AP activity, while both legumes had the highest NAG+LAP activity.

When comparing EEA across soils, Bilate soil had the highest measured BG and NAG+LAP activities, while the highest measured AP activity was

in Kudmi soil. EEA was lowest in Abiyot Fana soil for all enzymes. On average, BG and NAG+LAP activities were higher in Sidama than Amhara soils. By contrast, AP activity was on average higher in Amhara than Sidama soils, while differences in AP activity were larger in Amhara than Sidama soils, ranging from 320 (Abiyot Fana) to 681 (Kudmi) $\text{nmol g}^{-1} \text{h}^{-1}$. Across all soils, *D. intortum* showed the largest variability in BG and NAG+LAP activities, with BG activities ranging from 0.491 to 1.46 $\text{nmol g}^{-1} \text{h}^{-1}$, and NAG+LAP activities from 6.15 to 14.0 $\text{nmol g}^{-1} \text{h}^{-1}$. On the other hand, *U. cv. Cayman* had the largest variability in AP activity, ranging from 322 to 695 $\text{nmol g}^{-1} \text{h}^{-1}$.

Plants had contrasting effects on N_{mic} and P_{mic} (Table 3, Fig. S5). In Kudmi soil, *S. guianensis* supported highest N_{mic} and P_{mic} . In Abiyot Fana soil, both mixtures had the highest N_{mic} and *D. intortum* had the highest P_{mic} . In Borichia soil, *M. maximus* had the highest N_{mic} and *Urochloa+Desmodium* mixture the highest P_{mic} . In Bilate soil, both mixtures had the highest N_{mic} and *M. maximus* the highest P_{mic} .

Across the soils, Abiyot Fana soil had both the highest N_{mic} and P_{mic} . N_{mic} was lowest in Kudmi soil. P_{mic} was lowest in Bilate soil. On average, N_{mic} was higher in Sidama soils than Amhara soil. By contrast, P_{mic} was on average higher in Amhara than Sidama soils.

The microbial N:P ratio showed no consistent pattern between the soils or with plant species (Table 3,

Fig. S5). Single stand grasses and mixtures had some of the lowest observed ratios. In Kudmi soil the lowest ratio was under *M. maximus* and *Urochloa* + *Megathyrus* mixture. In Abiyot Fana soil the lowest ratio was under *U. cv. Cayman*. In Boricha soil the lowest ratio was under legumes and the *Urochloa* + *Desmodium* mixture and in Bilate soil under *M. maximus*.

The microbial N:P ratio was on average lower in Amhara soils, 12.6 in Kudmi and 13.7 in Abiyot Fana, than in Sidama soils which had ratios of 27.0 in Boricha and 24.0 in Bilate.

Vector model

There were significant variations in vector angle ($p < 0.001$) and vector length ($p < 0.001$) between the soils (Fig. 3). High vector angles ($> 45^\circ$) suggested that the microbes were P-limited, indicated by vector angles around 89° for all four soils. The model suggested that microbial C-limitation was low in the soils.

In Kudmi, Abiyot Fana and Bilate soils, the model indicated that the legumes reduced the microbial P-limitation, albeit only significant in Kudmi and Bilate soil. Contrasting, the microbial P-limitation was indicated to be higher under legumes in Boricha soil. In this soil, the lowest microbial P-limitation was observed under *Urochloa* + *Desmodium*.

With respect to vector length, values across soils and treatments were generally low (0.0279–0.196) and no consistent plant effects emerged: three of four soils (Kudmi, Abiyot Fana, Bilate) showed a tendency for greater microbial C-limitations under mixtures, most so in Bilate (Fig. 3). In Boricha soil the model indicated that the microbes under the legumes had significantly greater C-limitation while microbes under the grasses and mixtures had lower C-limitations. The mixtures in Amhara soils showed contrasting effects; *Urochloa* + *Megathyrus* had the greatest microbial C-limitation in Kudmi soil and the smallest in Abiyot Fana soil, while the *Urochloa* + *Desmodium* mixture had a greatest microbial C-limitation in Abiyot Fana soil and was smallest in Kudmi soil. In Kudmi soil, the greatest microbial C-limitation was found for *M. maximus*.

Principal component analysis (PCA)

Plant treatments clustered differently on PCA score plots combining plant and soil variables (Fig. 4). However, *S. guianensis* treatments clustered consistently along PC1, closely overlapping with *D. intortum*. Thus, legume treatments separated, while all other treatments strongly overlapped. Interestingly, the mixtures did not separate from single stands. The degree of variation explained by the first two

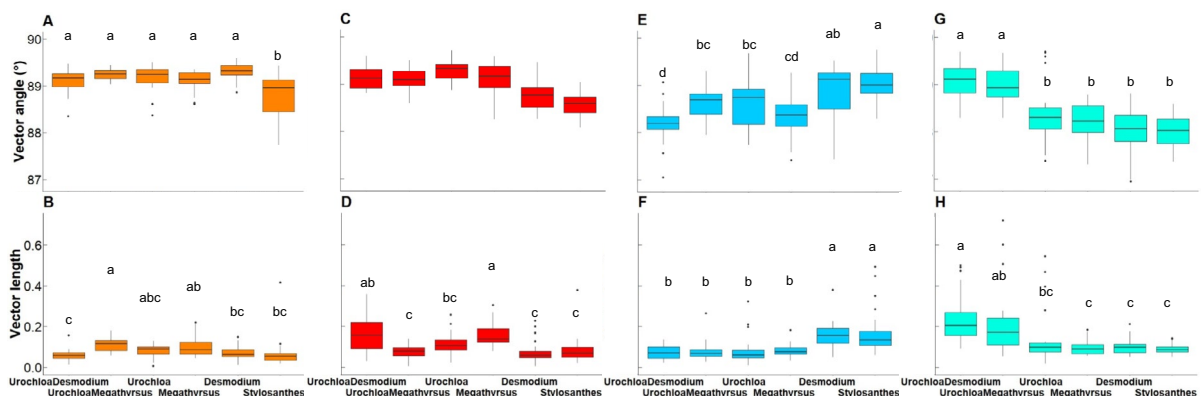


Fig. 3 Boxplots of the vector model outputs under the six plant treatments and the four soils. Vector angle ($^\circ$) (A, C, E, G) and vector length (unitless) (B, D, F, H) calculated from exoenzyme activity (EEA) in soils from Kudmi (A, B), Abiyot Fana (C, D), Boricha (E, F) and Bilate (G, H). The vector angle exceeds the threshold of 45° for all plots, indicating strong primary P-limitation across all soils. Each box in the plot shows the upper and lower quartiles, with horizontal lines

indicating the median values. The vertical lines represent the minimum and maximum values, and dots indicate outliers. Different letters indicate statistically significant differences between the treatments obtained from Tukey HSD post hoc test. Data for each box is derived from the model output based on seven biological replicates (pots) and four technical replicates per enzyme assay, yielding a total sample size of $n = 28$

components was higher for Sidama soils than for Amhara soils.

Loading plots revealed that the separation of legume treatments was driven by different, soil-specific factors. Soil pH appeared to have a strong antagonistic effect on legumes in all soils except Kudmi. Here, separation was associated with plant parameters (shoot:root ratio, N yield) rather than soil pH, which correlated positively with soil respiration and mineral N content, and negatively with vector angle and length (Fig. S4), indicating that *S. guianensis*, which had the highest N yields in Kudmi soil affected belowground microbial processes and EEA. By contrast, in the three other soils, soil pH was strongly negatively related to the plant parameters, and, in the Sidama soils, to mineral N and soil respiration. In Abiyot Fana soil, separation was associated with

plant parameters and pH, shoot:root ratio correlated positively with soil respiration and N yield negatively correlated with vector length, indicating that *S. guianensis*, which had the highest shoot:root ratio and N yield, influenced microbial processes. In Boricha soil, separation was associated with plant parameters, soil respiration, mineral N, vector angle and length and pH. N yield correlated positively with soil respiration, mineral N and vector length and angle, and correlated negatively with microbial N:P ratio and pH. Shoot:root ratio correlated positively with soil respiration and correlated negatively with microbial N:P ratio and pH. The highest shoot:root ratios and N yields were observed with *S. guianensis*, suggesting that there was a positive effect on belowground microbial processes and microbial nutrient status. In Bilate soil, separation was associated with N yield,

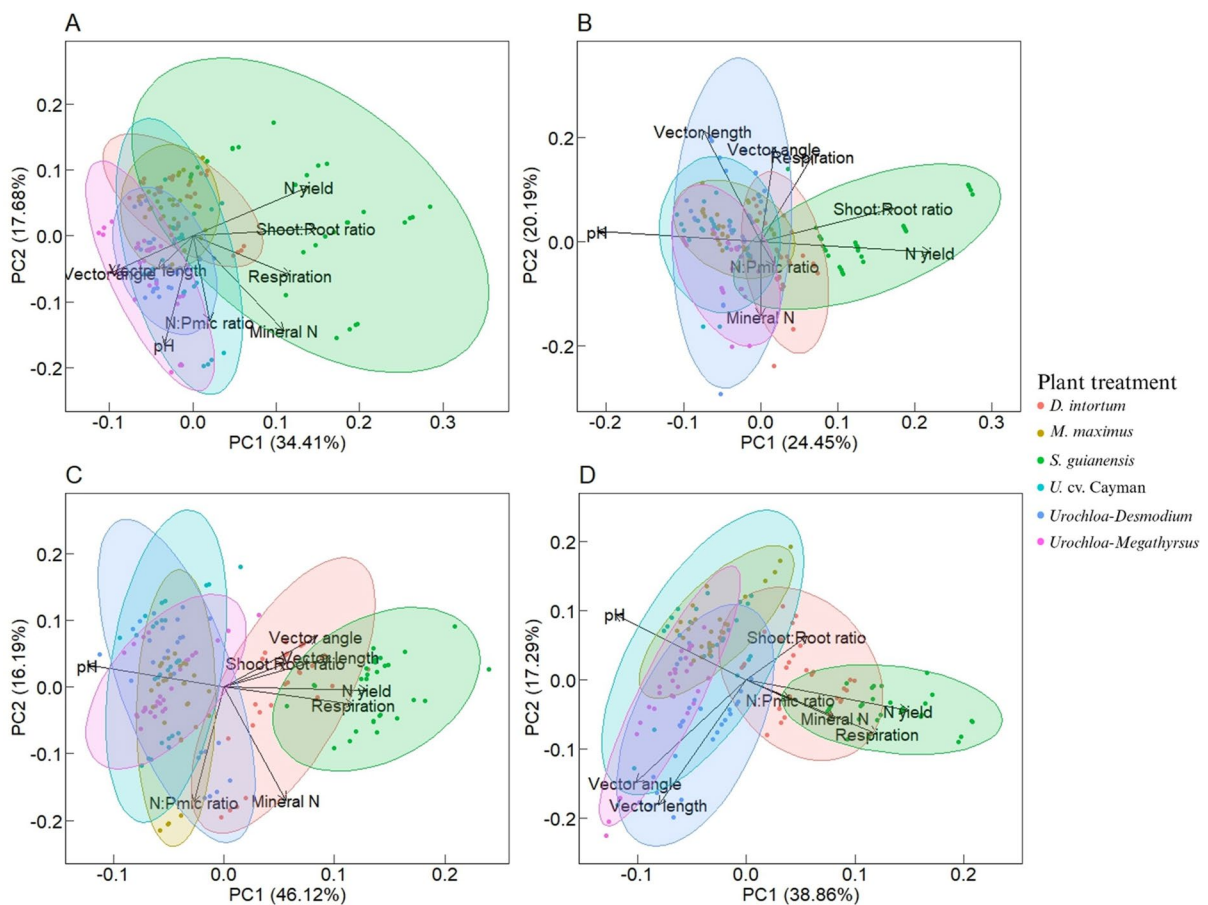


Fig. 4 Principal component analysis (PCA) of plant, soil biological and chemical variables in the four soils, Kudmi (A), Abiyot Fana (B), Boricha (C), and Bilate (D), across all plant treatments

soil respiration, mineral N and pH. Shoot:root and microbial N:P ratios had minor effects on the separation. N yield correlated positively with soil respiration, mineral N and microbial N:P ratio, and correlated negatively with pH and vector length and angle. Shoot:root ratio correlated positively with soil respiration and negatively with vector length and angle. *S. guianensis* had the highest shoot:root ratio and N yield, indicating a positive influence on belowground microbial processes and microbial nutrient balance.

Discussion

Experimental design

Our approach to evaluate potential soil ameliorating effects of plant species in short-term climate chamber experiments yielded partially successful outcomes. All four plant species established and grew in pots in the absence of fertilization and despite the substantial dilution of the native soils by quartz sand. The decision to inoculate the four indigenous Ethiopian soils into quartz sand was based on the idea to reduce the amount of soil interacting with the plants, thereby creating a system that would be more sensitive to detect plant-induced changes. However, this resulted in reduced analytical precision, as EEA measurements were, in some instances, near the detection limit. Our methodology was inspired by Petipas et al. (2020), who sampled soils and roots grown in smaller pots with a small soil corer, thus reducing the sand-soil dilution. Additionally, due to the absence of untreated controls, our results only allow for relative comparisons of plant effects, limiting the overall interpretation of plant input on the soils. Future applications of this approach should consider reducing both pot size and the proportion of sampled sand and include unplanted controls. Despite these limitations, we observed distinct soil chemical and biological patterns across the different soils and plant treatments, even after a short, 12 week growth period. These results suggest that with refinements, the method can be used to study plant-mediated effects on soil microbial processes associated with soil quality in short-term experiments.

Differences between the soils

The initial chemical and physical characteristics of the soils revealed basic differences, with the most

pronounced differences observed between the two regions. Amhara soils appeared to be inherently more P-limited due to their texture and mineral composition (Table 1). Specifically, these soils had a higher clay content and contained minerals such as goethite, hematite, anatase and kaolinite, which are known for their high P adsorption and fixation (Guzman et al. 1994; Bellani et al. 2020; Amadou et al. 2022). We therefore tentatively attribute the lower observed plant biomass production in these soils to P-limitation, which may have been exacerbated by high, potentially phytotoxic, aluminum concentrations (Wen et al. 2023). On the other hand, Amhara soils also contained illite, which has a low P adsorbing potential and is commonly regarded as a good potassium source for plants (Vetterlein et al. 2013), as well as an IVS phase in the clay fraction that contributes to N retention (Kome et al. 2019). The combination of P absorbing minerals and P fixation on clay may have resulted in a stoichiometrically imbalanced nutrient availability in Amhara soils. By contrast, the Sidama soils contained less clay, the majority of which was composed of illite. This combined with the presence of fewer P adsorbing minerals suggests a more lithologically balanced nutrient status in Sidama soils. Additionally, the presence of vermiculite in the clay fraction of Sidama soils is likely to enhance N retention (Pisa et al. 2020; Tzanakakis et al. 2021). However, the difference in extractable NH_4^+ between the two Sidama soils cannot be explained by mineralogy, as it is similar in both soils.

Soil specific plant-soil-plant feedbacks

In all four soils, single-stand legumes produced the highest total plant biomass, although patterns of above- and belowground allocation differed between the two legume species (Table 2). *D. intortum* developed greater belowground biomass and yielded less N aboveground than *S. guianensis* which developed more aboveground biomass with a higher N yield. These growth differences are consistent with previously reported variations in growth habits between these legumes (Bryan 1969; Lambais and Cardoso 1990). In our pot experiment, root development was promoted by low nutrient availability but spatially constrained by the limited volume of soil inoculum, which facilitated the recovery of near intact root systems. Surprisingly, some of the most pronounced

belowground effects on soil biological parameters were observed in pots with *S. guianensis*, which had higher shoot biomass and lower root biomass compared to *D. intortum*. This suggests that belowground effects in our experiment were primarily driven by total photosynthetic assimilation, which was greatest in *S. guianensis* as it had the largest shoot biomass among all plant treatments, rather than by the extent of the root-soil interface, which was greater in *D. intortum* (Mellado-Vázquez et al. 2016). As a result, this may have overridden species-specific influences on belowground processes, as described by others. For example, species-specific root exudates have been shown to influence soil microbial community composition (Potter et al. 2023) and plant–microbe symbiosis (Wang et al. 2024). Along these lines, the pronounced belowground effects observed for *S. guianensis* may be attributed to enhanced recruitment of beneficial microbes in the rhizosphere.

Contrary to our expectations, we did not detect significant species effects on mineral N. Both *Urochloa* spp. and *M. maximus* are reported to inhibit nitrification (BNI; Subbarao et al. 2009; Villegas et al. 2020) thereby promoting N retention in the soil. However, in our experiment, pots containing grasses did not show elevated $\text{NH}_4^+:\text{NO}_3^-$ ratios (Fig. S6). This indicates that nitrification was not inhibited in our experiment. The absence of detectable BNI may be attributed to general low mineral N levels in our experiment, likely restricting nitrification and the fact that there was no leaching.

Mixing grass–grass and grass–legume (one plant each) produced few significant effects on belowground processes. However, there were notable exceptions: in Abiyot Fana and Bilate soil, the mixtures had highest N_{mic} values, and in Abiyot Fana soil the root DW and soil respiration was highest for the *Urochloa* + *Desmodium* mixture. Overall, species mixtures had no consistent effect on the soils. This may be due to the sparse plant growth in the unfertilized pots with only two individual plants per pots, which may be too few to induce active competition for nutrients and belowground niche separation. Overyielding with consequences for belowground C and N input and root distribution has been reported previously even in degraded agricultural soils (Fornara and Tilman 2008; Mueller et al. 2013; Wong et al. 2024). We therefore would have expected stronger effects of the species mixtures, given the small volume of native

soil inoculated in our experiment. Among the two mixtures, *Urochloa* + *Desmodium* developed more root biomass than *Urochloa* + *Megathyrsus*. This may be attributed to the inherently lower shoot:root ratio of *D. intortum*, as observed for the single species. The absence of significant mixture effects on DM production may also be explained by the short growth period, as most reported mixture effects have been observed in long-term field studies (Fornara and Tilman 2008; Mueller et al. 2013; Cong et al. 2014).

Higher plant N yields of legumes than grasses indicated active BNF, which was confirmed by %Ndfa values of $73.1 \pm 15.2\%$ (Fig. 2). The calculated BNF revealed significant difference between the soils, with the Sidama soils supporting a greater fraction of fixed N in plant biomass compared to the Amhara soils. This variation in BNF is consistent with previous studies demonstrating that soil conditions, such as N availability and P addition, constrains symbiotic performance in SSA soils (Raji et al. 2019; Vázquez et al. 2022). In our experiment, higher P-availability and mineral N content in Sidama soils may thus explain both the higher BNF and the increased N yields observed in these soils. By contrast, Amhara soils, which are rich in P-adsorbing minerals, exhibited lower N yields and BNF. However, this was mostly due to lower biomass accumulation and not due to systematically lower symbiotic performance. Across the studied legumes, *S. guianensis* consistently showed the highest N-yield irrespective of soil, indicating a greater N fixation capacity than *D. intortum*, although these differences were only significant in Bilate soil. We could not confirm N-transfer between legume and grass in the *Urochloa* + *Desmodium* mixture, likely owing to the short growth period of the experiment. Minor increases in pots N yields were primarily due to the legume component, which inherently has a greater N yield, as observed in the *D. intortum* single stand pots. However, N-transfer to non-legumes may take time as reported by Tzanakakis et al. (2017) who did not observe N-transfer until the fourth production year in a field study in a subsoil grass-clover ley.

Plant induced effects on soil microbial nutrient cycling and acquisition

We observed high AP-activity across all soils, suggesting substantial microbial P-deficiency and

intense microbial mining for P. Notably, AP-activity was highest in pots with legumes which may result from increased C and N inputs stimulating microbial metabolism and, consequently, increasing AP-activity as a response to intensified microbial P-stress (Zeng et al. 2021; Zhu et al. 2023; Yang et al. 2024). In Kudmi soil, AP-activity did not vary significantly across plant treatments, although this soil had the highest overall AP-activity. Among the Amhara soils, AP-activities varied significantly, with Kudmi soil showing the highest average activity and Abiyot Fana soil the lowest. In Amhara soils, P_{mic} emerged as a good indicator for AP-activity: Kudmi soil, with the lowest P_{mic} , exhibited the highest AP-activity, whereas Abiyot Fana soil, with the highest P_{mic} , showed the lowest AP-activity. This pattern suggests that microbial P availability influences the AP-activity. A similar trend was observed for N_{mic} and N-enzyme activity: Kudmi soil had the lowest N_{mic} but elevated N-enzyme activity, while Abiyot Fana soil had higher N_{mic} and lower N-enzyme activity. Yang et al. (2024) proposed the microbial N:P ratio as a predictor of microbial P-limitation. In our study, the microbial N:P ratios did not differ substantially across the two Amhara soils, nor did they correlate with vector angle in any of our soils. Our results suggest that the microbial N:P ratio alone does not suffice to explain microbial P-limitation in SSA soils.

We used the vector model introduced by Moorhead et al. (2016) to infer microbial nutrient status based on EEAs. The model output indicated that microbial communities were severely P-limited. While P-limitation was expected given the low levels of available P in our soils, particularly for Amhara, the extent of this limitation was more pronounced than expected. The strong P-limitation indicated by our study is likely attributable to the model structure itself. The vector model calculates nutrient limitation based on the proportional activities of C-, N- and P-acquiring enzymes, making it highly sensitive to both the total measured enzyme activity and the number of enzymes included in each nutrient category. In our study, only one C-acquiring enzyme was included, which exhibited low activity. This led to a disproportionate difference between the ratios of C- to N-enzyme activity and C- to P-enzyme activity, resulting in the model output being skewed toward extreme P-limitation. Still, the method might be suitable to infer relative differences between soils or plant treatments.

Vector angles indicated a reduction in P-limitation under legumes. This aligns with our expectations as previous studies have demonstrated that increased C input stimulates microbial metabolic activity and increases N demand, thereby promoting N-enzyme synthesis, skewing the vector angle towards N-limitation (Piñeiro et al. 2023; Rumeau et al. 2024). Interestingly, the opposite trend was observed in Boricha soil, where the vector angle increased in the legume pots. In contrast to the other three soils, N-enzyme activity in Boricha soil was substantially lower in the legume pots, whereas the AP activity was higher. This may be attributed to the influence of soil N availability on N-enzyme synthesis; for instance, low mineral N, particularly NH_4^+ , has been shown to enhance N-enzyme activity (Zhou et al. 2024), while NH_4^+ addition results in reduced N-enzyme activity (Chen et al. 2014). In our study, Boricha soil with legumes had the highest NH_4^+ concentrations, likely alleviating microbial N stress and consequently intensifying P-stress (Yang et al. 2024), thereby shifting the vector angle towards P-limitation.

BG activities were low in all soils. In general, legumes increased BG activity, which coincided with increases in soil respiration, indicating increased metabolic activity and energy demand. This increase is consistent with the anticipated higher C input from legumes and supports previous reports of enhanced C-enzyme activity following increase C availability in soils (Chen et al. 2014; Zhou et al. 2024; Rumeau et al. 2024). Despite this trend, overall C-enzyme activity remained low in our soils, which may be attributed to prevailing P- and N-limitations (Chen et al. 2014; Piñeiro et al. 2023).

Soil specific variability in microbial responses and plant input

The observed soil chemical and biological responses to growing plants in our study can largely be attributed to the inherent differences in soil characteristics. Although consistent patterns emerged across the different soils in response to specific plant species, the strength and direction of these effects depended on initial soil conditions. Variations in mineralogy and nutrient availability influenced both microbial activity and plant growth, with subsequent impacts on soil processes through root growth. Notably, the strongest responses were detected under *S. guianensis*, despite its lower root biomass compared to *D. intortum*,

suggesting that the observed effects were more closely associated with overall photosynthetic assimilation than with absolute root biomass. Belowground responses were highly variable across the soils, with Sidama soils being more responsive to *S. guianensis* than Amhara soils. This aligns with the observation that composition and amount of root exudates depend on soil nutrient availability. Maurer et al. (2021) reported increased C and N contents in rhizodeposits when plants were grown in nutrient rich solutions, whereas Jiang et al. (2025) found that low P availability enhanced root exudation. Based on these findings, we would expect root exudates in Sidama soils to be more C and N rich, while root exudates in Amhara soils may be quantitatively larger but lower in C and N.

Conclusions

We studied plant-soil interactions in short-term growth experiments with diluted native soils without nutrient addition. Our experimental setup was partially successful and could, with refinements, be used to study species-specific effects on soil microbial processes associated with soil functioning and health. Soil microbial responses to plants were, however, strongly influenced by parental soil conditions, particularly mineralogical composition and a priori nutrient availability. Among the tested plants, legumes exerted the most pronounced effects across all soils, as evidenced by the significant increases in soil respiration and exoenzyme activities (NAG+LAP and AP) alleviating microbial P-limitation. These effects appeared to be influenced by the availability of mineral N in the soil which, in turn, controlled microbial P demand. Under the minimal conditions imposed in our experiment, the impacts of plants on soil biological functions appeared to depend more on photosynthetic assimilation than on root-soil interactions. Our study highlights the critical role of parental soil conditions for plant-based soil amelioration and demonstrates that different plant species have different effects depending on location. This implies that forage species must be selected specifically after testing soils for mineralogy and nutrient status. However, for Ethiopian smallholder farmers, determining optimal plant mixtures may be constrained by practical considerations, such as forage yield.

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Author contributions NJW performed the greenhouse experiment and carried out laboratory work, processed and analyzed the data and wrote the initial draft. MJ planned and managed the greenhouse experiment together with NJW and assisted in data interpretation and text editing. PD conceived the study together with NJW and MJ, analyzed ¹⁵N abundances, assisted in data interpretation and contributed to writing and revising the manuscript.

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Data availability The datasets generated during and/or analyzed under the current study are available at zenodo.org, <https://doi.org/10.5281/zenodo.18254134>.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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