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Extracellular Enzyme Activity Patterns Associated with Soil Properties and Culturable Microbial Taxa in Shade-Grown Coffee Orchards of Tropical Montane Cloud Forest

Enrique Alarcón-Gutiérrez [1], Mario del Roble Pensado-Leglise [2,*] and Merlyn A. Salazar-Huerta [3]

[1] Instituto de Biotecnología y Ecología Aplicada (INBIOTECA), Universidad Veracruzana, Av. de las Culturas Veracruzanas No. 101 Col. Emiliano Zapata, Xalapa 91090, Veracruz, Mexico
[2] Instituto Politécnico Nacional, Centro Interdisciplinario de Investigaciones y Estudios sobre Medio Ambiente y Desarrollo (CIIEMAD), Calle, 30 de Junio de 1520 s/n, La Laguna Ticoman, Gustavo A. Madero, Ciudad de México 07340, Estado de México, Mexico
[3] Instituto Politécnico Nacional, Escuela Nacional de Ciencias Biológicas (ENCB)-Unidad Profesional Adolfo López Mateos, Zacatenco, Av. Wilfrido Massieu 399, Col. Nueva Industrial Vallejo, Alcaldía Gustavo A. Madero, Ciudad de México 07738, Estado de México, Mexico; masalazarh@ipn.mx
* Correspondence: mpensado@ipn.mx

Abstract

The objective of this study was to analyze the patterns of extracellular enzyme activity in the soil of shade-grown coffee orchards (SCOs) and their association with soil physicochemical properties and selected microbial taxa in Tropical Montane Cloud Forest (TMCF) landscapes at 38 sites in the Sierra Norte de Puebla, Mexico. Correlation analyses and a redundancy analysis (RDA) were used to examine the relationships between edaphic variables, enzymatic activities, and the presence of culturable microorganisms. Protease activity was positively associated with nitrate concentration, whereas acid phosphatase activity showed no clear relationship with available phosphorus. A negative association between protease and acid phosphatase activities represented the strongest relationship observed, indicating contrasting patterns of enzyme activities across sites. Multivariate analysis indicated that soil variables, including pH, available phosphorus, electrical conductivity, nitrate concentration, and soil moisture, were associated with variations in enzyme activity patterns and selected microbial taxa. In addition, the abundance of genera such as *Bacillus*, *Cladosporium*, and *Pseudomonas* was associated with soil variability, suggesting ecological associations between microbial indicators and soil conditions. Overall, the results indicate that soil physicochemical variability is associated with differences in enzyme activity patterns and selected microbial taxa in SCO systems. These findings are broadly consistent with patterns described in ecoenzymatic framework, although interpreted here as associative relationships.

Keywords: extracellular enzymes; ecoenzymatic stoichiometry; shade-grown coffee agroforestry; tropical montane cloud forest; redundancy analysis; culturable microorganisms

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1. Introduction

Soil microorganisms play a central role in biodiversity conservation, ecosystem functioning, and agricultural productivity. In the context of climate change, their relevance has increased due to their contribution to plant acclimatization and the regulation of key biogeochemical processes [1,2]. Soil microbial communities mediate fundamental ecosystem

functions through metabolic processes such as rhizodeposition, biofertilization, biological control, soil aggregation, and plant biostimulation [3]. Diverse microbial functional groups have been documented in coffee, including plant growth-promoting rhizobacteria (*Azospirillum*, *Bacillus*, and *Acetobacter*), nitrogen-fixing bacteria (*Rhizobium* and *Bacillus*), arbuscular mycorrhizal fungi (*Glomus*, *Acaulospora*, and *Rhizophagu*), plant growth-promoting fungi (*Trichoderma*), and microbiota associated with fruit and fermentation processes (*Pseudomonas*, *Bacillus*, *Acetobacter*, *Aspergillus*, *Penicillium*, and *Fusarium*) [4,5]. The ecological functioning of these soil microorganisms must include their nutrient-acquiring functional strategies. In this context, the ecoenzymatic stoichiometry approach provides a framework to analyze these processes by examining the relative activity patterns of enzymes involved in carbon (C), nitrogen (N), and phosphorus acquisition [6,7]. Microorganisms respond to nutrient limitations by adjusting the production of extracellular enzymes that catalyze the decomposition of organic substrates and release limiting nutrients [8]. In this study, extracellular enzyme activity was interpreted within the ecoenzymatic stoichiometry framework as an indicator of microbial processes associated with carbon, nitrogen, and phosphorus cycling. Enzyme activities were considered in relative terms to explore patterns across sites; however, they were not used as direct measures of microbial resource allocation or metabolic optimization. Despite the increasing knowledge on microbial diversity and functions in coffee agroecosystems, the relationships between soil physicochemical gradients, extracellular enzyme activity patterns, and microbial taxa in shade-grown coffee systems remain insufficiently understood. In particular, how enzyme activities associated with carbon, nitrogen, and phosphorus acquisition vary across soil conditions in tropical montane cloud forest landscapes has not been clearly established [9,10]. Within this context, the study of soil culturable microbial groups in coffee agroecosystems has gained increasing relevance. Research has focused on understanding the composition, diversity, and ecological function of microbial communities associated with *Coffea* spp., particularly in SCOs, where interactions between plants, soil microorganisms, and environmental conditions shape ecosystem processes [11,12]. SCOs are commonly managed as agroforestry systems that maintain a multilayered vegetation structure similar to that of a natural forest. By conserving shade trees and organic litter inputs, these systems partially mimic forest ecological processes, including microclimatic regulation and nutrient cycling, which support diverse soil microbial communities and biological activity [13–16]. The production of extracellular enzymes is central to these processes because microbial communities regulate nutrient acquisition [17]. The production of extracellular enzymes represents a key interface between microbial metabolism and ecosystems' nutrient dynamics, as these enzymes control the depolymerization of organic matter and the mobilization of nutrients essential for plant productivity [18–21]. The ecoenzymatic stoichiometry approach provides a framework to analyze these processes by examining the relative activity patterns of microbial communities in enzymes involved in carbon, nitrogen, and phosphorus acquisition. According to this theory, the relative activity of enzymes targeting different nutrient pools has been interpreted in literature as being associated with microbial nutrient demand. Changes in enzyme patterns can, therefore, reveal shifts in microbial nutrient demand and ecosystem functioning under different environmental conditions, including land-use dynamics. This perspective is relevant in the context of Tropical Montane Cloud Forests (TMCFs), which are high-biodiversity and high-moisture ecosystems characterized by persistent cloud cover, high levels of endemism, and a complex biogeochemical cycle. Despite their ecological importance, TMCF ecosystems are among the most threatened tropical habitats due to land-use change, agricultural expansion, and climate variability [22,23]. Microbial communities contribute to multiple ecosystem services in SCO systems established within these landscapes, including soil fertility, nutrient cycling, and biodiversity maintenance [24].

Many coffee-producing regions located within TMCF landscapes in Mexico are managed by smallholder farmers whose livelihoods are highly vulnerable to climate variability and coffee market instability [25]. Declining profitability often promotes land-use change, deforestation, and degradation, generating feedback loops that threaten both ecosystem services and local economies [26,27]. Within this context, strategies based on an inclusive bioeconomy, supported by local capacities and responsible use of biotechnology, have been proposed as potential pathways for sustainable rural development. Within TMCF coffee agroecosystems, the characterization of enzyme patterns may reveal how microbial communities respond to environmental gradients, nutrient availability, and management practices. Such information can contribute to understanding how microbial processes support plant nutrition, soil fertility, and ecosystem resilience in these fragile landscapes. However, despite advances in understanding microbial processes in coffee agroecosystems, it remains unclear whether extracellular enzyme activity patterns are consistently associated with soil physicochemical gradients in shape-grown coffee systems under tropical montane cloud forest conditions. Therefore, this study aimed to analyze the patterns of extracellular enzymatic activities and their association with soil physicochemical properties and microbial taxa across tropical montane cloud forest landscapes of the Sierra Norte of Puebla, México. Based on the ecoenzymatic stoichiometry framework, we hypothesized that extracellular enzyme activity patterns would be associated with soil nutrient gradients in SCO systems. Specifically, we expected that enzyme activities associated with nitrogen and phosphorus acquisition would vary across sites in relation to soil physicochemical conditions, and that protease and acid phosphatase activities would show contrasting patterns across soil gradients.

2. Materials and Methods

2.1. Participatory Action Research Framework

This study was conducted using a participatory action research (PAR) approach based on the research experience developed by the Instituto Politécnico Nacional (IPN) in the Sierra Norte de Puebla region since 2012. PAR is conceived as a collaborative research process in which stakeholders actively participate in decision making and the execution of the different phases of the research aimed at addressing socio-ecological challenges [28,29].

The IPN, through the Centro Interdisciplinario de Investigaciones y Estudios sobre el Medio Ambiente y Desarrollo (CIIEMAD), has implemented PAR initiatives in the municipalities of the Sierra Norte de Puebla, where TMCFs predominate. These initiatives aimed to promote the sustainable management of SCOs [30]. In 2023, researchers from the IPN and the Scottish Rural College (SRUC, the University of Edinburgh) obtained funding from the UK Medical Sciences Association under the project "The use of orphan crops in a sustainable, diversified portfolio for food security in Mexico", Project GCRFNGR8\1498, IPN-SRUC [31]. These collaborations facilitated the implementation of the SECIHTI-CF2023-G-1519 project in participating coffee-growing communities and supported local collaboration during site selection and sampling.

2.2. Study Area

This study was conducted in shade-grown coffee orchards (SCOs) located in municipalities in the Sierra Norte de Puebla, Mexico (Figure 1), in territories with a predominance of TMCF (Figure 2). These ecosystems are characterized by high biodiversity, persistent cloud cover, and complex nutrient cycling processes.

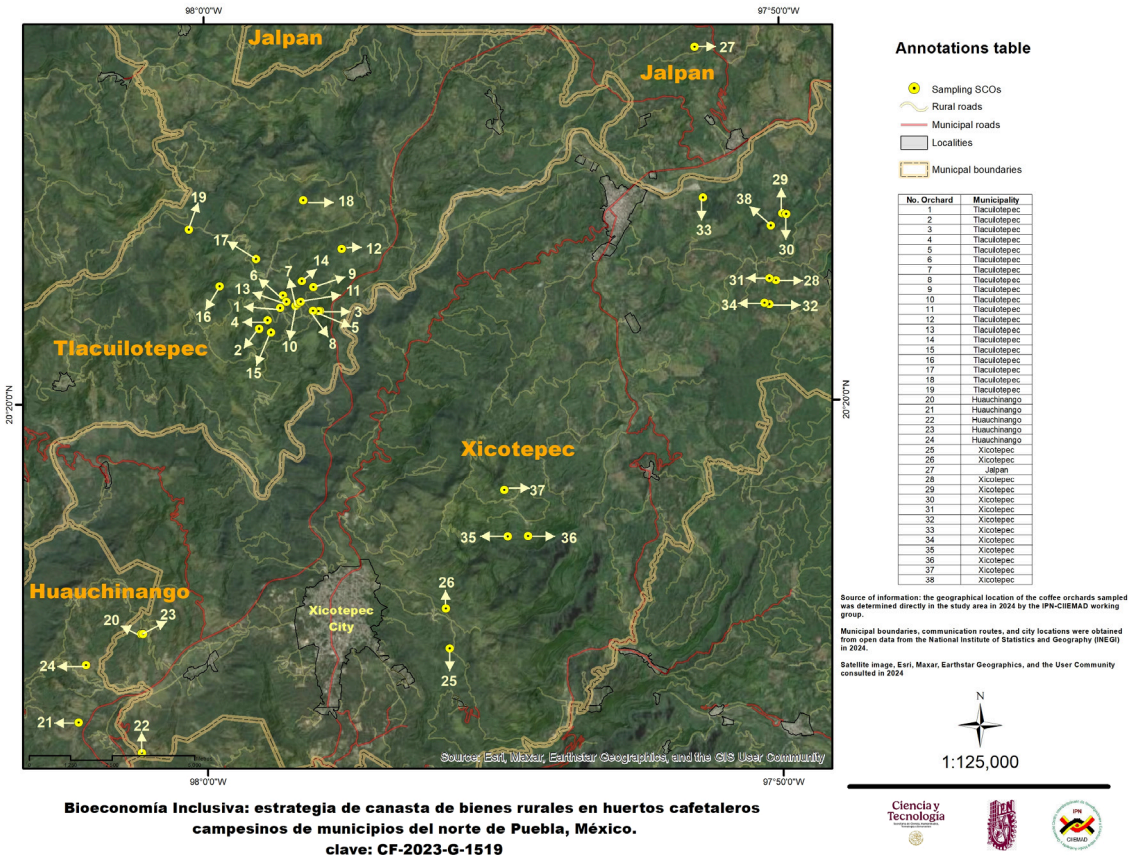


Figure 1. Locations of sampling sites.

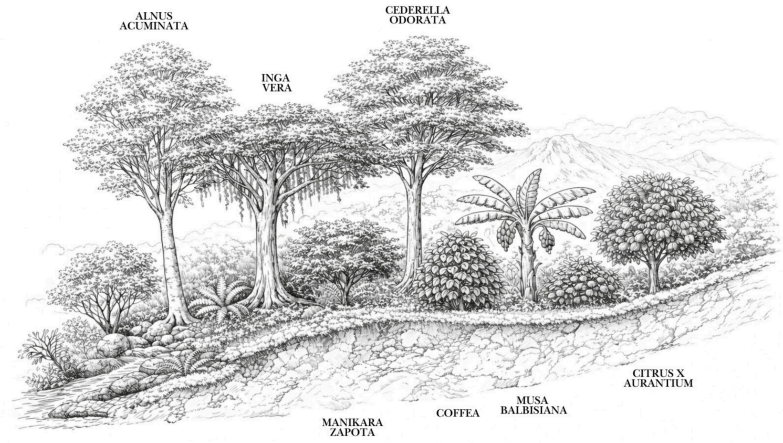


Figure 2. Vegetation stratification in a shaded-grown coffee agroecosystem. Source: Authors' own design.

The objective of soil sampling was to analyze the relationships between soil physico-chemical variables, enzymatic activity, and the presence of key culturable microorganisms (bacteria, fungi, and actinomycetes) to better understand enzyme patterns within the soil culturable microbial groups.

The study sites correspond to traditional SCO agroforestry systems embedded within TMCF landscapes. These systems are characterized by a multilayer vegetation structure composed of canopy shade trees, coffee shrubs, and a litter-rich soil surface (Figure 2).

2.3. Site Selection and Soil Sampling

The sampling sites were selected via participatory collaboration with coffee producers in each locality. Farms were prioritized if they had not received chemical fertilizers,

agrochemicals, or other external inputs for at least 18 months before sampling. Farms with longer periods without external inputs (≥2 years) were included in the sampling gradient to capture variability in management conditions. These sites were not treated as experimental controls but as part of the observational design.

Sampling was conducted between mid-April and mid-May 2024 during a period of relatively low precipitation and elevated temperatures. Two field teams were formed, each consisting of a lead researcher, a field coordinator, a graduate student from CIIEMAD-IPN, and agricultural engineering students from the University of Xicotepetl, Puebla, completing their social service requirements. Soil samples were collected using standardized instruments for measuring chemical fertility, culturable microbial taxa, and enzymatic activity. The sampling protocol incorporated the participation of local producers, whose knowledge of the territory contributed to identifying representative sampling sites. A chain of custody was established from sample extraction to laboratory delivery to preserve the integrity of the chemical and biological variables analyzed.

At each sampling site, soil was collected from the top 0–30 cm layer after removing surface litter. Five subsamples were collected within each plot following a random sampling scheme and subsequently homogenized to form a single composite sample per site. Each site was represented by a single composite sample (sampling unit), corresponding to an individual coffee plot, no within-site replication was considered in the statistical analysis. All subsequent analyses were performed at the site level (n = 38).

2.4. Soil Physicochemical Analysis

Soil characterization was performed by GISENA-labs. The analyses included pH, electrical conductivity (dS m^{-1}), soil moisture (%), bulk density (g cm^{-3}), texture (based on the proportion of sand, silt, and clay), the concentration of micronutrients (i.e., Cu, Fe, Mn, and Zn (mg kg^{-1})), the concentration of contaminant ions (i.e., Cd, Pb, and Ni (mg kg^{-1})), percentage of organic matter (%), NO_3^- (mg kg^{-1}), P (mg kg^{-1}), B (mg kg^{-1}), Ca (cmol kg^{-1}), Mg (cmol kg^{-1}), Na (cmol kg^{-1}), K (cmol kg^{-1}), and cation exchange capacity (cmol kg^{-1}). Soil moisture was determined gravimetrically as the percentage of water content relative to dry soil mass.

2.5. Microbial Analysis

Fungal identification was conducted by GISENA-labs using dilution extraction followed by comparison with reference schemes and species (method approved by SADER SENASICA, LAB-190123-02-MICO-002, based on NOM-021-RECNAT-2000). Likewise, the most abundant bacteria were isolated via dilution-seeding in King B medium and rapid hypersensitivity testing (method approved by SADER SENASICA, LAB-190123-02-BACT-002). The results obtained for fungi and bacteria were reported in CFU g^{-1} soil.

2.6. Enzymatic Activity

The enzymatic activities of four enzymes were determined to further characterize the SCO in TMCF. The enzymatic activities analyzed were selected based on the major enzyme types commonly present in soils, specifically β-1,4-glucosidase, exoglucanase, protease, and acid phosphatase, which collectively reflected the overall biological capacity of the soil. These enzymes were measured as indicators of microbial participation in carbon (C), nitrogen (N), and phosphorus (P) acquisition processes in soils. Exoglucanase catalyzes the cleavage of cellulose chains by releasing cellobiose units from the end of cellulose polymers and, therefore, plays an important role in the initial depolymerization of plant-derived structural carbohydrates during soil organic matter decomposition [32]. β-1,4-glucosidase catalyzes the hydrolysis of cellobiose and other β-glucosides into glucose, representing a key step in the final stages of cellulose degradation and microbial carbon

acquisition. Together, these enzymes reflect microbial participation in the decomposition of cellulose-derived organic carbon and the mobilization of carbon sources available for microbial metabolism.

Protease activity is consistent with the depolymerization of proteins into peptides and amino acids and is, therefore, associated with the mineralization and mobilization of organic nitrogen in soils. Acid phosphatases catalyze the hydrolysis of organic phosphate esters, releasing inorganic phosphate available for microbial and plant uptake. It is commonly used as an indicator of microbial phosphorus acquisition under conditions of limited P availability [33]. Enzymatic activity was determined using the adjusted method previously employed. The substrates added to the enzyme activity assay were 4-MUF β-D-cellobioside for exoglucanase activity; 4-MUF β-D-glucopyranoside for β-glucosidase activity; 4-MUF phosphate for phosphatase activity; and leucine-aminomethylcoumarin for protease activity.

Soil samples were analyzed 3 to 4 weeks after collection because additional material from the sampling campaign was still being processed and delivered. To prevent enzyme degradation, the samples were kept at 4 °C, and the extraction process was carried out immediately before the enzymatic activity test, following recommendations showing that cold storage (2–7 °C) best preserves soil enzyme activities compared with freezing or drying. Soil suspensions were prepared by placing 1 g of soil in a 50 mL centrifuge tube and adding 40 mL of sodium acetate buffer (50 mM; pH 5.5). The soil suspension was homogenized with a vortex mixer for 2 min and then centrifuged at 4000 RCF for 5 min. The supernatant was transferred to a new tube and frozen at −20 °C until use. MUF was used to calibrate phosphatase, cellulase, and glucosidase activities, while AMC was used to calibrate protease activity.

Enzymatic reactions were carried out by adding 600 µL of a 2 mM substrate solution to 2400 µL of soil extract. After 1 h of incubation at 20 °C, the relative fluorescence was measured at 365 nm excitation and 450 nm emission using a fluorescence spectrophotometer (Shimadzu RF-6000-Japan, Kyoto, Japan, purchased in 2024 by the SECIHTI CF-2023-G-1519 project). The activity unit was considered as 1 µM of 4-methylumbelliferone (4-MUF) released per minute. The enzymatic activity test was measured from the aqueous extract made from each mixed soil sample.

2.7. Data Preparation and Database Construction

All soil, microbial, and enzymatic data were compiled into a unified database. The sampling sites were identified using numeric plot codes (e.g., 1–38), each corresponding to a unique composite soil sample. The dataset included soil variables (pH, available phosphorus concentration, electrical conductivity (dS m^{-1}), nitrate concentration, and soil moisture), microbiological variables (relative abundances of *Bacillus*, *Pseudomonas*, Actinomycetes, *Pythium*, *Cladosporium*, *Mucor*, and *Fusarium*), and biochemical variables representing enzyme activities. Before conducting multivariate analysis, the variables were standardized to comparable scales for redundancy analysis (RDA). Collinearity among soil variables was assessed using variance inflation factors (VIFs); values below 5 were considered acceptable. Although electrical conductivity (Method AS-18, based on NOM-021-RECNAT-2000) and nitrates shared partial information, the VIF values remained within the permitted ranges and were, therefore, included in the multivariate models. The final database was used for both bivariate and multivariate analyses.

2.8. Statistical Analysis

All statistical analyses were conducted using the sampling sites as the unit of analysis (n = 38). In this study, enzyme activities were analyzed individually rather than using

integrated indices such as the geometric mean of enzyme activities (GMean). This approach was adopted to preserve the interpretation of relative differences among enzymes associated with C, N, and P cycling, which are relevant for evaluating patterns of enzyme activity associated with C, N, and P cycling.

Correlation analyses were performed to explore bivariate relationships between soil variables, enzyme activities, and the presence of microorganisms. The relationships were graphically represented using scatter plots, which allowed us to identify trends among the variables, such as the trade-off between proteases and acid phosphatases, the relationship between pH and *Bacillus*, and the association between phosphorus and *Cladosporium*.

A redundancy analysis (RDA) was applied to comprehensively evaluate the effect of soil properties on the joint structure of enzymatic and microbial variables. In this analysis, soil variables were considered explanatory, while enzymatic activities and the presence of microorganisms were used as response variables. The explanatory variables included in the RDA were soil pH, nitrate concentration, available phosphorus, electrical conductivity, and soil moisture. These variables were selected based on their relevance to soil physicochemical conditions and their potential influence on enzyme activity and microbial indicators. The overall significance of the RDA model and canonical axes was evaluated using Monte Carlo permutation tests, with 999 permutations. The proportion of variance explained by the constrained fraction and the significance of each axis were used to identify general patterns of association between soil variables, enzyme activities, and microbial indicators. The RDA was interpreted as an exploratory multivariate approach rather than as a predictive or mechanistic model [34,35].

2.9. Software and Computational Environment

Data processing, analysis, and visualization were performed in Python (version 3.13) using the Google Colaboratory environment [36]. Database management and preprocessing were carried out with the pandas and numpy libraries, while statistical and correlation analyses were performed with scipy.stats. Visualizations were generated with Matplotlib (version 3.10) and Seaborn (version 0.13.2). Redundancy analysis (RDA) was implemented in Python using scikit-learn, and its statistical significance was evaluated using a Monte Carlo simulation. Collinearity between edaphic variables was verified using the variance inflation factor (VIF) with the statsmodels library.

3. Results

3.1. Soil Physicochemical Characteristics

The physicochemical characteristics of the soils across the sampled shade-grown coffee sites are presented in Table 1. The soils were generally acidic, with pH values ranging from 3.95 to 6.10. Electrical conductivity values were relatively low (0.09 to 0.39 dS m^{-1}), suggesting low salinity across the sites. Nitrate concentrations ranged from 5.66 to 55.0 mg kg^{-1}, while available phosphorus showed high variability (0.00 to 66.4 mg kg^{-1}), indicating heterogeneous nutrient availability among sites. Similarly, CEC varied substantially (1.00 to 27.10 $cmol^{+}$ kg^{-1}), suggesting differences in soil fertility and nutrient retention capacity across the sampled coffee agroecosystems.

Meanwhile, given the exploratory nature of the enzymatic and soil variables, the relationship described below should be interpreted as an association rather than a causal effect.

Table 1. Soil physicochemical characteristics of the 38 sampling sites in SCOs.

Site	pH	Electrical Conductivity (dS·m^{-1})	Organic Matter (%)	Nitrates (mg kg^{-1})	Available P (mg·kg ds^{-1})	Ca (cmol^{+} kg^{-1})	Mg (cmol^{+} kg^{-1})	Na (cmol^{+} kg^{-1})	K (cmol^{+} kg^{-1})	CEC (cmol^{+} kg^{-1})
1	4.2	0.36	4.19	55	4.68	2.4	0.47	0.04	0.16	3
2	5.04	0.33	7.5	54.6	3.01	13.1	2.27	0.05	0.77	16.2
3	4.8	0.3	5.12	43.6	2.52	8.5	2.23	0.05	0.16	10.9
4	4.76	0.39	6.94	46.6	3.16	10.1	1.36	0.07	0.23	11.8
5	3.95	0.23	3.36	43	6.38	6.2	1.47	0.04	0.11	7.8
6	4.49	0.16	4.1	21.1	18.3	4.2	0.88	0.04	0.14	5.3
7	4.69	0.12	3.96	14.1	4.75	2.6	1.11	0.04	0.1	3.8
8	4.37	0.13	3.48	14.5	1.22	0.9	0.48	0.03	0.08	1.5
9	4.14	0.16	3.87	13.5	5.11	0.6	0.26	0.03	0.15	1
10	4.35	0.21	8.61	34.2	2.82	0.8	0.27	0.04	0.12	1.2
11	5.19	0.22	5.13	46.1	2.57	12.4	1.36	0.06	0.15	13.9
12	4.79	0.24	8.3	33.6	8.04	4.9	0.69	0.04	0.14	5.7
13	5.59	0.19	4.52	24.5	6.41	13.5	1.18	0.04	0.2	15
14	5.19	0.19	3.64	26.3	29	7.1	1.24	0.03	0.31	8.7
15	5.07	0.2	3.62	37.6	2.39	11.3	1.65	0.05	0.32	13.3
16	5.18	0.14	3.83	15	1	14.9	1.9	0.06	0.13	17
17	6.04	0.16	6.02	25.3	5.77	22.9	1.99	0.06	0.23	25.2
18	5.06	0.12	5.69	12.4	6.27	8.2	1.14	0.04	0.33	9.7
19	4.37	0.13	3.56	11.9	1	1.8	0.91	0.03	0.19	3
20	4.32	0.09	6.08	6.33	7.55	0.8	0.5	0.03	0.15	1.5
21	4.78	0.26	5.69	32.2	66.4	9.4	1.59	0.05	0.48	11.6
22	4.84	0.16	6.88	17.7	1.75	6.7	0.44	0.04	0.12	7.3
23	4.2	0.09	4.62	5.66	3.75	0.7	0.33	0.03	0.09	1.1
24	4.52	0.1	5.61	11.7	1	1.8	0.69	0.05	0.12	2.7
25	4.21	0.13	5.51	17.5	1.12	0.8	0.42	0.04	0.14	1.4
26	4.29	0.11	4.5	10.8	1	1.4	0.29	0.04	0.1	1.8
27	4.21	0.16	7.57	30.6	3.12	0.9	0.45	0.05	0.15	1.5
28	4.27	0.09	6.58	8.09	0	0.6	0.27	0.05	0.13	1
29	4.3	0.1	6.3	8.3	1.27	0.9	0.27	0.04	0.09	1.3
30	6.1	0.18	5.24	19	2.95	19.9	1.24	0.13	0.09	21.6
31	6.1	0.19	7.03	54.5	5.9	24.8	1.9	0.07	0.34	27.1
32	4.75	0.14	4.56	24.1	1.61	4.1	1.91	0.04	0.11	6.1
33	4.43	0.17	3.43	21.8	3.06	2.2	0.66	0.03	0.21	3.1
34	4.9	0.13	4.61	15.7	4.62	3.6	0.46	0.05	0.11	4.2
35	5.02	0.35	5.36	34.7	2.94	15.1	1.67	0.06	0.15	17
36	4.64	0.11	3.51	11.5	0	3.4	0.73	0.04	0.12	4.3
37	4.4	0.1	8.13	8.58	1.15	1.1	0.31	0.05	0.07	1.5
38	4.35	0.16	2.93	24	5.62	2.8	0.45	0.03	0.11	3.4

3.2. Patterns of Extracellular Enzyme Activity

As shown in Table 1, nitrate and available phosphorus concentrations varied across sampling sites, providing clear soil nutrient gradients. These gradients were associated with differences in extracellular enzyme activities.

Protease activity was positively associated with nitrate concentration (Figure 3A), indicating that sites with higher nitrogen availability tended to exhibit higher protease activity. In contrast, acid phosphatase activity showed no clear relationship with available phosphorus (Figure 3B), suggesting that the measured phosphorus pool does not fully capture the variability of phosphorus dynamics in these soils.

Extracellular enzyme activities showed substantial variability across sites, with protease activity ranging from 0.00 to 87.96 U g^{-1} ds and acid phosphatase activity from 0.3 to 128.42 U g^{-1} ds. This variability occurred across a wide gradient of soil physicochemical conditions, including pH values ranging from 3.95 to 6.10, nitrate concentrations from 5.66 to 55.0 mg kg^{-1}, and available phosphorus from 0.00 to 66.4 mg kg^{-1} (Table 1).

Bivariate analysis revealed contrasting patterns of associations between enzyme activities and soil variables (Figure 3). Protease activity was positively associated with nitrate concentration (Figure 3A; r = 0.516 and $p < 0.001$), with higher protease values generally observed in sites with higher nitrate levels. In contrast, soil acid phosphatase, despite its wide range of variation, showed no clear relationship with available phosphorus (Figure 3B; r = −0.069 and $p = 0.681$). This result suggests that the measured phosphorus pool does not fully capture the variability of phosphorus dynamics in these soils. A negative correlation was observed between acid phosphatase and protease activities, with sites with higher protease activity simultaneously exhibiting lower acid phosphatase activity (Figure 3C;

r = −0.626 and $p < 0.001$). Sites 3, 4, 10, 12, and 8 had the highest protease activity and the lowest acid phosphatase activity.

This pattern suggests contrasting enzyme activity patterns associated with nitrogen and phosphorus cycling across the sampled soils.

Additionally, *Bacillus* abundance was positively associated with soil pH (Figure 3D; r = 0.336 and $p = 0.039$), with higher abundance observed in less acidic conditions.

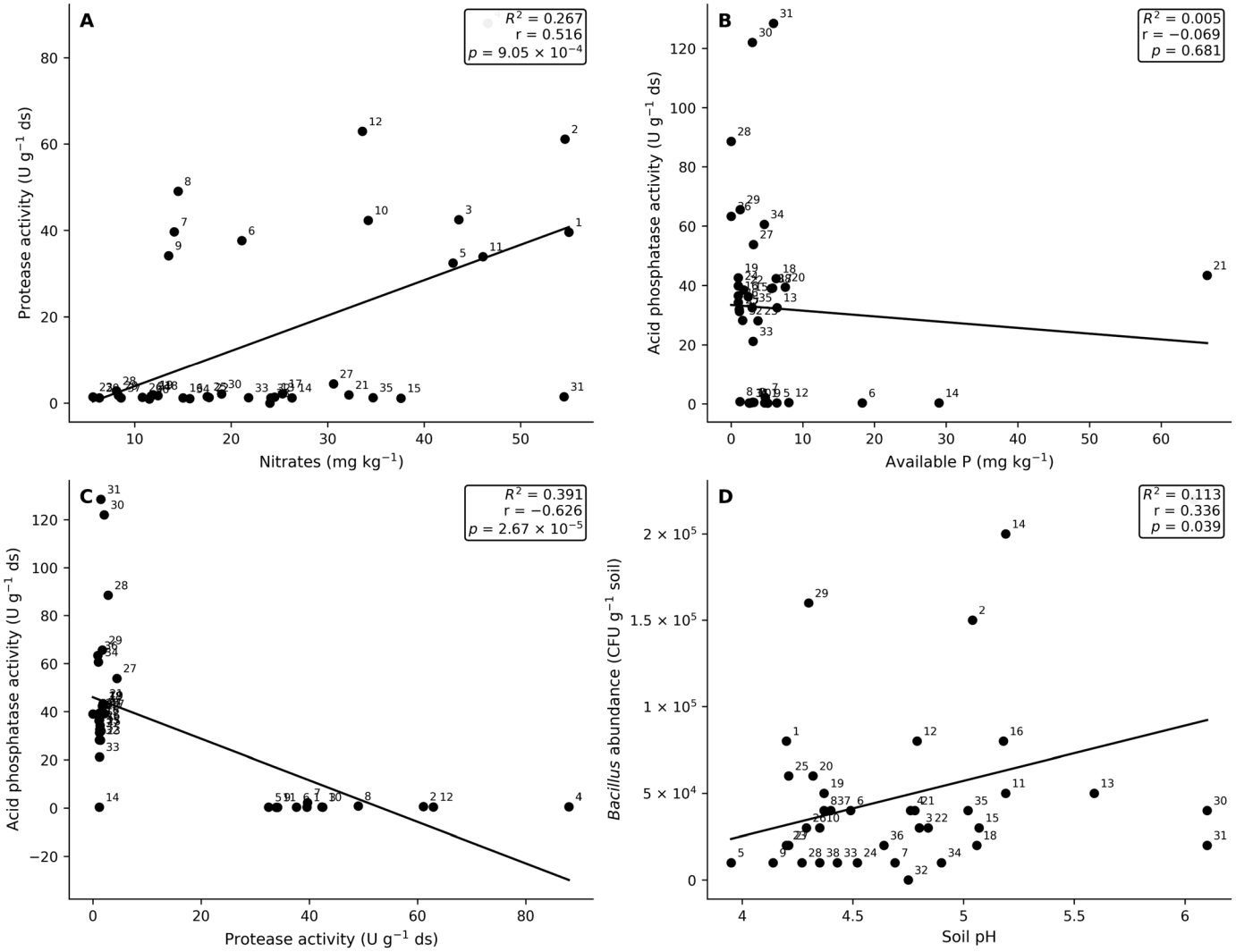


Figure 3. Relationships between extracellular enzyme activities and soil physicochemical variables across SCOs (n = 38). (**A**) Protease activity in relation to nitrate concentration. (**B**) Acid phosphatase activity in relation to available phosphorus. (**C**) Relationship between protease and acid phosphatase activities. (**D**) Relative abundance of *Bacillus* in relation to soil pH. Each point represents an individual sampling site. Numeric labels correspond to site identifiers (1–38). Lines represent linear trends for visualization purposes. These relationships illustrate patterns of association among variables within the observational dataset.

3.3. Multivariate Integration Using Redundancy Analysis (RDA)

The bivariate relationships observed in Figure 3 and other variables (microbial presence, electrical conductivity, and soil moisture) were further integrated using redundancy analysis (RDA), allowing the joint evaluation of soil physicochemical variables, enzyme activities, and microbial taxa. The RDA showed a significant overall relationship between soil variables and microbial and enzymatic variables (Figure 4). The variance fraction explained 25% of the total variation (F = 2.77; $p = 0.002$), suggesting that variation in soil

chemistry is associated with microbial and enzymatic patterns across sites. In agreement with the patterns observed in Figure 3, nitrate and electrical conductivity were aligned with protease activity, whereas pH and phosphorus were associated with acid phosphatase activity (Figure 4). This multivariate structure is consistent with the contrasting enzyme activity patterns identified in the bivariate analysis. The first two canonical axes of the RDA were statistically significant (RDA 1: $p = 0.03$; RDA 2: $p = 0.002$), indicating two dominant soil gradients: one associated with pH and phosphorus, and another with electrical conductivity and nitrates. The collinearity between predictors remained within acceptable limits (VIF < 5). The gradients in phosphorus, pH, electrical conductivity, and nitrates accounted for part of the observed multivariate variability across sites (Figure 4).

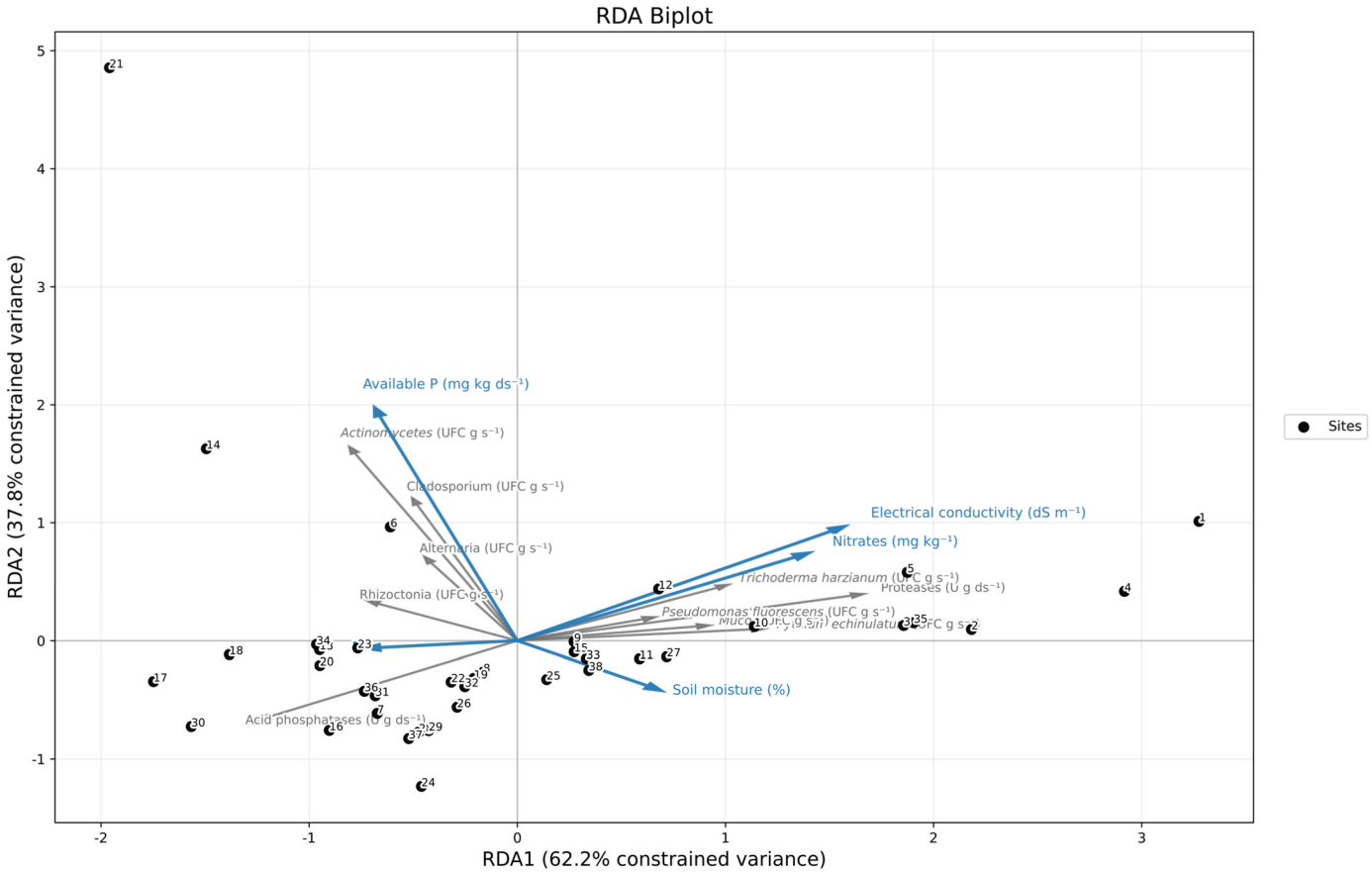


Figure 4. Redundancy analysis showing the relationships among soil physicochemical variables (blue arrows), extracellular enzyme activities, and selected culturable microbial taxa (grey arrows), at the sites analyzed (black dots).

4. Discussion

While this study did not include vector-based stoichiometry metrics or direct measurements of microbial nutrient limitation, the observed enzyme activity patterns could be discussed within the ecoenzymatic framework as showing contrasting enzyme activity patterns across sites. The most notable pattern observed in this study was the inverse relationship between protease and acid phosphatase activities across sampling sites. This pattern suggests contrasting activities of enzymes associated with nitrogen and phosphorus acquisition along soil gradients. According to the ecoenzymatic stoichiometry framework, microbial communities adjust extracellular enzyme production in response to an imbalance in carbon, nitrogen, and phosphorus availability. Proteases contribute to the depolymerization of proteins and the mobilization of organic nitrogen, whereas phosphatases release inorganic phosphate from organic phosphorus compounds. Therefore,

the observed trade-off between protease and phosphatase activity indicates contrasting enzyme activity patterns across sites, although these relationships should be interpreted as associative rather than mechanistic. The absence of a clear relationship between acid phosphatase activity and available phosphorus indicates that phosphorus dynamics cannot be robustly inferred from the current dataset. This limitation highlights the constraint of using a single phosphorus pool to interpret phosphorus-related enzyme activity.

It is important to note that enzyme activities were not normalized by microbial biomass or soil organic carbon. Consequently, part of the variability observed in enzyme activities may reflect differences in microbial biomass or total biological activity among soils. Nevertheless, the interpretations presented here are based on relative patterns of enzyme activity and their associations with soil variables, rather than on absolute rates or direct measures of microbial resource allocation. This distinction is essential for maintaining consistency between the dataset and the level of inference.

Previous studies have shown that soil enzyme activities are closely linked to soil physicochemical conditions and land-use practices, supporting their use as indicators of soil biochemical functioning. Extracellular enzyme activities are widely used as indicators of microbial function, but their interpretation as direct proxies of nutrient limitation remains constrained. Reviews of agroecological systems further emphasize that enzymatic activity profiles reflect soil health and biogeochemical processes under management regimes. In this sense, the patterns observed in this study are comparable to those reported in other agricultural and forest systems, where enzyme activities vary in response to environmental gradients rather than following fixed metabolic strategies [37].

The redundancy analysis (Figure 4) provides an integrated perspective of these relationships, showing that soil chemistry is associated with the combination of enzyme activities, and culturable microbial. Although the RDA explained 25% of the total variation, suggesting that additional environmental and biological factors may also influence microbial activity, two environmental gradients emerged: one associated with pH and available phosphorus, and another associated with electrical conductivity and nitrate concentration. These gradients are consistent with the bivariate relationships observed and suggest that multiple soil variables are associated with microbial and enzymatic patterns across sites.

Several of the culturable microbial genera detected in this study, including *Bacillus* and *Pseudomonas*, have been reported in the literature as taxa associated with plant growth promotion and other soil-related processes in agroecosystems. Their presence in the sample sites, therefore, provides ecological context for the observed associations, but does not by itself demonstrate functional roles in the studied sites. However, culturable counts represent only a fraction of total microbial diversity and do not allow inference at the community or functional level.

Soil pH was associated with variation in the composition of culturable microbial groups, including the relative abundance of *Bacillus*. Numerous studies have shown that pH strongly influences selected microbial taxa at regional and global scales [38]. Experimental evidence also indicates that changes in soil pH can alter microbial abundance and diversity in agricultural soils [39] and may influence enzyme activity measurements, particularly in acidic soils, where enzymatic reactions are highly sensitive to pH variations [40]. However, in this study, pH should be interpreted as one of several environmental factors associated with microbial patterns rather than the dominant structuring force.

Associations between culturable microorganisms and enzyme activities provide additional ecological context, although these relationships should be interpreted cautiously, given their correlative nature. The association between *Bacillus* abundance and soil pH observed in this study indicates that variations in pH were related to differences in the abundance of this genus across sites, which is consistent with its known ecological versatil-

ity and its role as a plant growth-promoting rhizobacterium [41]. Similarly, the association between [42] *Pseudomonas* and *Pythium* with higher protease activity is consistent with previous reports that these microorganisms can be involved in protein degradation and nitrogen-related processes in soils [43–45]. These observations provide an ecological context for the association [46–48], although causal relationships cannot be inferred from our study's dataset.

The gradient associated with electrical conductivity and nitrate concentration further indicates that soil chemical conditions may influence enzyme activity patterns. Previous studies have shown that salinity and nitrogen transformations can affect microbial metabolism and enzyme production in soils [49], supporting the interpretation that enzyme activities respond to environmental variability. However, the absence of a more comprehensive characterization of nutrient pools limits the ability to directly link these patterns to nutrient availability or limitation [50,51]. Accordingly, these results should be interpreted as associations with measured soil variables rather than as direct evidence of nutrient limitation. Beyond the biogeochemical interpretation, the patterns observed in this study are relevant for understanding soil functioning in shade-grown coffee agroecosystems embedded within the tropical montane cloud forest (TMCF). These systems maintain complex vegetation structures and organic inputs that are associated with conditions favorable to microbial activity and soil biochemical processes. In this context, extracellular enzymes can be interpreted as indicators of ongoing microbial processes that are consistent with soil biochemical processes relevant to fertility and ecosystem functioning. Shade-grown coffee agroecosystems have been recognized as agroforestry systems that support biodiversity and ecosystem services while maintaining agricultural productivity [52].

At the local scale, management practices such as shade-tree composition, litter inputs, and organic amendments influence soil microbial communities and biogeochemical processes [53,54]. Collectively, the results of this study are consistent with these findings, suggesting that soil microbial activity in SCO systems is closely linked to environmental conditions and management context.

5. Conclusions

Overall, the results show that variation in soil physicochemical properties is associated with differences in extracellular enzyme patterns across SCOs. In particular, the consistent negative association between protease and acid phosphatase activities suggests contrasting patterns of enzyme activity across sites. These patterns are consistent with expectations from ecoenzymatic stoichiometry, although they should be interpreted as associative evidence within the scope of the observational design. In addition, associations between enzyme activities and culturable microbial taxa such as *Bacillus*, *Pseudomonas*, and *Cladosporium* indicate potential associations between selected culturable taxa and soil biochemical processes. Environmental variables, including pH, nitrate concentration, and electrical conductivity, were associated with variations in enzyme activity patterns, although they explain only part of the observed variability. Taken together, these findings provide an exploratory framework for understanding enzyme activity patterns in SCO agroecosystems, embedded within TMCF landscapes, while highlighting the need for more comprehensive datasets to evaluate underlying mechanisms.

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