Zamorano University

Agricultural Science and Production

B.S. in Agricultural Sciences

Special Graduation Project

Testing the Feedback Inhibition Hypothesis of Soil Enzymes: N Fertilization Impacts on N-hydrolytic Enzyme Activities in Tropical Agroecosystems

Student

Osman Asael Gonzalez Hernández

Advisors

Ricardo Peña Ph.D.

Andrew J. Margenot Ph.D.

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Authorities

KEITH L. ANDREWS

President i.a.

ANA M. MAIER ACOSTA

Vice President and Academic Dean

CELIA O. TREJO RAMOS

Director of Agricultural Science and Production

JULIO NAVARRO

Secretary General

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Abstract

Soil enzymes drive nutrient cycles and serve as an indicator of soil health. This study investigated the activity of seven soil enzymes involved in nitrogen cycling (N-acetyl-D-glucosaminidase (NAG), leucine aminopeptidase (LAP), alanine aminopeptidase (ALA), methionine aminopeptidase (MET), glycine aminopeptidase (GAP), glutamic acid aminopeptidase (GLU), and deaminase) after synthetic N fertilizations under field conditions. Twenty-one coffee fields, divided into 4 plots each subjected to one of four treatments: nitrogen (N) in the form of UREA at a rate of 285 kg N ha^{-1}, potassium (K) in the form of muriate of potash (MOP) 350 kg K ha^{-1}, nitrogen and potassium (NK) as a combination of the two individual rates, and no fertilization (UCL). This study aimed to evaluate the feedback inhibition of soil enzymes after fertilization treatments. It was hypothesized as a suppression of enzymatic activity due to the increased availability of N. Fertilization interaction findings did not provide field-based evidence that fertilization can suppress or stimulate soil N-hydrolytic enzymatic pathways refuting the feedback inhibition hypothesis. Additionally, an interaction between sampling timepoint and soil N-hydrolytic enzymes was found evidencing additional factors affecting enzyme activity in soils. Understanding these biochemical responses enhances our ability to optimize fertilization strategies, balancing productivity with long-term soil health.

Keywords: Deaminase; Feedback inhibition; Fertilization; Microbial activity; Nitrogen cycle Soil Enzymes

Resumen

Las enzimas del suelo impulsan los ciclos de nutrientes y sirven como indicadores de la salud del suelo. Este estudio investigó la actividad de siete enzimas del suelo involucradas en el ciclo del nitrógeno: N-acetil-β-D-glucosaminidasa (NAG), leucina aminopeptidasa (LAP), alanina aminopeptidasa (ALA), metionina aminopeptidasa (MET), glicina aminopeptidasa (GAP), acido glutámico aminopeptidasa (GLU) y desaminasa, después de fertilizaciones sintéticas con nitrógeno en condiciones de campo. El experimento se llevó a cabo en 21 cafetales, cada uno dividido en cuatro parcelas sometidas a uno de los siguientes tratamientos: nitrógeno (N) en forma de urea a una dosis de 285 kg N ha^{-1}, potasio (K) en forma de cloruro de potasio (MOP) a 350 kg K ha^{-1}, una combinación de nitrógeno y potasio (NK), y un control sin fertilización (UCL). El objetivo del estudio fue evaluar la posible inhibición por retroalimentación de la actividad enzimática del suelo tras los tratamientos de fertilización, bajo la hipótesis de que una mayor disponibilidad de nitrógeno suprimiría dicha actividad. Sin embargo, los resultados obtenidos en campo no respaldaron esta hipótesis, ya que la fertilización no suprimió ni estimuló consistentemente las rutas enzimáticas N-hidrolíticas del suelo. Además, se observó una interacción significativa entre el momento de muestreo y la actividad enzimática, lo que evidencia que otros factores también influyen en la actividad enzimática del suelo. Comprender estas respuestas bioquímicas mejora nuestra capacidad para optimizar las estrategias de fertilización, equilibrando la productividad con la salud del suelo a largo plazo.

Palabras clave: Actividad microbiana; Ciclo del Nitrógeno; Deaminasa; Enzimas del Suelo; Fertilización; Inhibición por retroalimentación

Introduction

Nitrogen (N) is an essential macronutrient and the primary limiting nutrient for crop production (Wang et al., 2024). N is an essential component of amino acids, proteins, nucleic acids, and chlorophyll; as such, it is fundamental for plant development and has a direct influence on crop yields therefore, is stated as the largest input of mineral (Sinha & Tandon, 2020). Despite its importance, increasing efficiency of N use is a persistent challenge in crop production, often addressed with the application of inorganic fertilizers. Soil enzymes catalyze the catabolic degradation of organic matter by cleaving covalent bonds, including C-N bonds, and thus play a key role in nutrient cycling and organic matter decomposition (Daunoras et al., 2024; Margenot & Daughtridge, 2022) These soil enzymes, play a key role in nutrient cycling and organic matter decomposition by facilitating the depolymerization and mineralization of organic nitrogen into ammonium-N. However, limited information exists on how endogenous factors, such as organic matter, temperature and pH, may influence the enzyme activity of soils. This project evaluates the feedback inhibition of seven soil enzymes over inorganic N fertilizers contributing to the reduction of the knowledge gap, regarding enzyme activity regulation under short- term nutrient inputs in tropical soils.

Soil is fundamental to sustainability in agriculture as the basis for food production. Soil health has been defined by (Doran & Zeiss, 2000)as “the capacity of a soil to function as a vital living system within ecosystems and land use boundaries to sustain plant and animal production, maintain or enhance water and air quality, and promote plant and animal health.” The soil hosts the Earth’s most diverse and intricate microbiome (Daunoras et al., 2024). More than 40 soil microbiome functions have been identified that directly or indirectly impact soil nutrient supply to crops. Among these functions, nutrient cycling has relevance for this study as it plays a central role in the regulation of N hydrolyzation.

Nitrogen may be present in different chemical forms such as chitin, a natural polysaccharide composed of repeating unites of N-acetyl glucosamine, and proteins, but plants are selective and can

accept only certain forms of nutrients (Dotaniya et al., 2019). Plant roots and microbiome are both a key drivers of nutrient hydrolysis due to their major contribution of soil enzymes (Greenfield et al., 2020; Staszel et al., 2022). Mineralization processes of nitrogen are largely catalyzed by soil extracellular enzymes, the activity of which can be collectively assayed as soil enzyme activity. Soil enzymes are secreted into the soil matrix by soil microbes as well as by plant roots, where they hydrolyze organic N into plant-available ammonium-N (Dotaniya et al., 2017).

Excessive accumulation of N from exogenous sources, such as un high fertilization rates, may trigger feedback inhibition of the enzymatic activity in the soil (Nieland et al., 2024). The feedback inhibition was described by (Gerhart & Pardee, 1962), as a biological mechanism by which an organism regulates the rates of a biosynthetic pathway through the end-product's inhibition of enzymatic activity within that pathway. This is a fundamental biological regulatory mechanism that evades the waste of essential nutrients. As described by (Olander & Vitousek, 2000), found that N addition suppressed N-acetyl-D-glucosaminidase (NAG) activity in nitrogen limited soils, indicating feedback inhibition caused by the addition of the synthetic N.

In the soil N cycle, two main pathways are involved in N supply to crops from organic matter (Daughtridge & Margenot, 2024; Kasmerchak et al., 2024). Both pathways include the processes of depolymerization and mineralization. N Depolymerization has two known independent but parallel paths. Proteolytic pathways starts with the breakdown of proteins by proteases into oligopeptides that subsequently are hydrolyzed into free amino acids by aminopeptidases(Fujii et al., 2020; Nannipieri & Eldor, 2009; Vinolas et al., 2001)**.** Chitinolytic constitutes the depolymerization of chitin polymers by chitinases into N-acetyl glucosamine (Cretoiu et al., 2013; Kielak et al., 2013). After both depolymerization processes a final step of mineralization takes place where deaminase (Veliz et al., 2017) hydrolyses both products into ammonium-N. The rates of hydrolysis of each process step can be estimated by assaying enzyme activities (Margenot et al., 2018).

While synthetic N fertilization enhances crop productivity, it may also trigger the biological regulatory mechanism, feedback inhibition, where the accumulation of nutrient end products may suppress the enzymatic activity. The objective of this study was to evaluate the activity of key soil enzymes involved in the nitrogen cycle in response to N synthetic fertilization.

Materials and Methods

Experimental Site

On- farm omission trials were established in the township of Hermogenes Montellano (14°29'57.88"N; 90°59'31.47"O) in the south-central Guatemalan department of Chimaltenango in April 2022. The region has a sub-tropical highland climate (22 to 28°C mean annual temperature) and a rainy season from May to October (2,500 – 3,000 mm mean annual precipitation) with a dry season from November to April. The study landscape has coarse-textured soils formed in the ejecta of the nearby (<15km) active Fuego stratovolcano(Allen Asensio et al., 2024)**.** Fields were selected from a preliminary soil fertility and plant nutrition baseline survey of 36 fields conducted in 2020-2021. All 21 plots had varied planting densities, the average spacing was 2 × 2 m (2,500 plants ha^{-1}). Approximately half of the fields (n=10) were planted with the H1 Centro Americano Variety and the other half (n=11) had American varieties as Typica Bourbon or varieties derived from crosses between Caturra and Timor hybrid.

Experimental Design

The experiment was established as a randomized complete block design with 21 blocks (i.e., experimental replicates). Each block was subdivided into four subplots, each randomly assigned to a fertilization treatment: nitrogen (N) as urea at a rate of 285 kg ha^{-1}, potassium (K) as muriate of potash (MOP) at a rate of 350 kg ha^{-1}, nitrogen and potassium (NK) at the same rates applied in combination and control without fertilization. This model results in a total of 84 experimental subplots (21 × 4). To eliminate potential confounding results due to a phosphorus deficiency, all the 84 subplots received phosphorus fertilization as triple superphosphate (TSP) at a rate of 350 kg ha^{-1}. Composite soil samples were collected from each subplot within two time points (before and after), to test the hypothesized feedback inhibition by N fertilization on the activities of seven N-hydrolytic enzymes: N-acetyl-β-D-glucosaminidase (NAG), leucine aminopeptidase (LAP), alanine aminopeptidase (ALA), methionine

aminopeptidase (MET), deaminase, glycine aminopeptidase (GAP), and glutamic acid aminopeptidase (GLU).

Soil Sampling

The soil sampling was carried out by Dr. Andrew Margenot and Heidi Allen from the Soils Lab at the University of Illinois at Urbana – Champign. The first sampling (August 2022) was conducted during the cherry development stage. The second sampling (November 2022) was done during the initiation of harvest of the first wave of ripening coffee cherries. Soils were sampled at 0-15 cm depth approximately 20 to 35 cm linear distance from the tree trunk under the coffee canopy drip line using a hand auger. Samples were air dried and ground to pass a <2mm sieve.

Enzyme measurements

The enzyme activities for each soil sample were determined through the Standard Operating Procedures (SOPs) determined by the Soils lab from the University of Illinois at Urbana Champaign. For each soil sample, analysis duplicates of each enzyme activity were conducted to ensure reliability and quality.

NAG activity was measured using *p*NP-N-acetyl-β-D-glucosaminide a *para*-nitrophenyl (*p*NP) substrate that when hydrolyzed by soil enzymes releases para-nitrophenol (*p*NP). Each soil sample was weighed in 50mL centrifuge tubes twice per timepoint giving a total of 168 samples per timepoint. The substrate used in this enzyme had a concentration of 10mM using water to dilute the substrate not buffer.

To incubate each sample, a water bath was preheated to 37°C with a water level, each incubation included one abiotic hydrolysis (contains only substrate and no soil samples), and one true blank (only water); using two racks that could hold up to 25 centrifuge tubes per rack. Additionally in each rack, under the centrifuge tube position had a whole perforated to ensure a uniform temperature in the centrifuge tube.

Five mL of substrate was added to each centrifuge tube, then a plastic wrap cover was placed over the two racks. The two racks were placed in the water bath and maintained soaked using two extra racks on top and two heavy items (jugs filled with water). After a one-hour incubation, the samples were alkalized using 4 mL of 0.1M TRIS at pH 12 and 1mL of Calcium Chloride ($CaCl_2$), then let sit for ~5min to flocculate. Subsequently, 1mL of the supernatant is extracted and moved into a 1.5mL microcentrifuge to be centrifuged at 14,000 rpm for 1min 45s. In the next step, making sure the sedimented pellet in the microcentrifuge tube is not disturbed, 200µL were transferred to a 96-well microplate to be read in a microplate spectrophotometer at a 410nm absorbance.

Furthermore, a standard curve was prepared using a substrate of *para*-nitrophenol (*p*NP) at an initial concentration of 5 mM. To prepare the standard curve, 5mL of the substrate was diluted with 7 mL with water resulting in a concentration of 0.3125 mM. Then, 5mL of the diluted substrate were transferred into a clean centrifuge tube and subsequently, 4 mL of 0.1M TRIS and 1mL of $CaCl_2$. In the next step, the final solution was serially diluted into 7 microcentrifuge tubes at different dilution factors (1×, 2×, 3×, 4×, 5×, 10×, and 20×). Finally, 200 µL from each microcentrifuge tube was extracted and placed into the 96-well microplate to be read in a microplate spectrophotometer at a 410 nm absorbance.

GAP, LAP, ALA, MET, LYS, and GLU activities were measured using the SOP determined by the University of Illinois at Urbana-Champaign used for the quantification of rates of several aminopeptidases. This protocol uses *para*-nitroanilide (*p*NA)-linked substrates such as glycine 4-nitroanilide (for GAP), L-Leucine 4-nitroanilide (for LAP), L-Alanine 4-nitroanilide hydrochloride (for ALA), L-Methionine 4-nitroanilide (for MET), L-Lysine *p*-nitroanilide dihydrobromide (for LYS), and L-Glutamic acid y-*p*NA hydrochloride (for GLU). As the SOP indicates, each soil sample was weighed in 50mL centrifuge tubes twice per timepoint giving a total of 168 samples per timepoint. The substrate used in this enzyme had a concentration of 1 mM using water to dilute the substrate not buffer.

To incubate each sample, a water bath was preheated to 37°C. Each incubation included one abiotic hydrolysis (contains only substrate and no soil samples), and one true blank (only water); using two racks that could hold up to 25 centrifuge tubes per rack. Additionally in each rack, under the centrifuge tube position had a whole perforated to ensure a uniform temperature in the centrifuge tube.

Five mL of substrate was added to each centrifuge tube, then a plastic wrap cover was placed over the two racks. The two racks were placed in the water bath and maintained soaked using two extra racks on top and two heavy items (jugs filled with water). After a 24 hours incubation, the samples were alkalized using 4 mL of 0.1M TRIS at pH 12 and 1mL of Calcium Chloride ($CaCl_2$), then let sit for ~5min to flocculate. Subsequently, 1mL of the supernatant is extracted and moved into a 1.5mL microcentrifuge to be centrifuged at 14,000 rpm for 1min 45s. In the next step, making sure the sedimented pellet in the microcentrifuge tube is not disturbed, 200µL were transferred to a 96-well microplate to be read in a microplate spectrophotometer at a 380nm absorbance.

Furthermore, a standard curve was prepared using a substrate of para-nitroanilide (pNA) at an initial concentration of 1 mM. Using 5 mL of this stock solution, 4 mL of 0.1M TRIS and 1mL of $CaCl_2$ was added. In the next step, the final solution was serially diluted into 7 microcentrifuge tubes at different dilution factors (1×, 2×, 3×, 4× ,5×, 10×, and 20 ×). Finally, 200 µL from each microcentrifuge tube was extracted and placed into the 96-well microplate to be read in a microplate spectrophotometer at a 380 nm absorbance.

The potential activity of deaminase was determined using the SOP provided by the Soils laboratory at the University of Illinois at Urbana-Champaign. The protocol states the usage of a 4-nitro-o-phenylenediamine substrate at a concentration of 0.392mM. The substrate was dissolved using water and not a buffer chemical.

To incubate each sample, a water bath was preheated to 25°C. Each incubation included one true blank (only water); using one rack that can hold up to 25 centrifuge tubes per rack. Additionally

in each rack, under the centrifuge tube position had a whole perforated to ensure a uniform temperature in the centrifuge tube.

Ten mL of substrate was added to each centrifuge tube, then a plastic wrap cover was placed over the racks. The racks were placed in the water bath and maintained soaked using one extra rack on top and two heavy items (jugs filled with water). After a 24-hour incubation, the samples were taken from the water bath and, under a fume hood, 15 mL of methanol was added to each sample. After sealing all the tubes with their caps, they were placed in a horizontal shaker at low speed (~150 rev min^{-1}) for 15 minutes. After, the tubes were placed in a centrifuge at 4000 rev min^{-1} for 5 minutes. The next step is filtering the slurry sample through a Whatman No. 1 filter paper until the remnant is moist soil only. The true blank sample was not filtered.

For the second extraction, under the fume hood 15 mL of methanol is added to the remnant of the first extraction including the abiotic hydrolysis. The tubes were resealed using the caps and shook for 15 more minutes at a low speed (~150 rev min^{-1}). After the shaker, the tubes were moved to the centrifuge at 4000 rev min^{-1} for 5 minutes. Subsequently the samples were filtered through a Whatman No. 1 filter paper until the remnant is moist soil only.

After the two extractions were done, they were mixed into a new tube with a final volume of ~40mL. Finally, 200µL were transferred to a 96-well microplate to be read in a microplate spectrophotometer at a 405 nm absorbance.

The standard curve was generated upon five diluted solutions (1×, 2×, 4×, 8×, and 16×) using a stock solution of 4-nitro-o-phenylenediamine substrate at a concentration of 0.392 mM and 18.2MΩ-cm water. All five dilutions, including a centrifuge tube containing only water had equally added 30 mL of 100% methanol.

Table 1

Description of enzymes and substrates evaluated for potential activity under different fertilization treatments.

Enzyme		Substrate	Concentration (mM)
N-acetyl-β-D-glucosaminidase	NAG	pNP-N-acetyl- β -D-glucosaminide	10
Glycine aminopeptidase	GAP	L-Glycine 4-nitroanilide	1
Leucine aminopeptidase	LAP	L-Leucine 4-nitroanilide	1
Alanine aminopeptidase	ALA	L-Alanine-4-nitroanilide	1
Glutamic Acid-aminopeptidase	GLU	Glutamic acid y-pNA hydrochloride	1
Methionine- aminopeptidase	MET	L-Methionine 4-nitroanilide	1
Deaminase		4-nitro-o-phenylenediamine	0.392

Although each enzyme activity was estimated following its respective SOP, the calculation of each one follows the same principle. First, using Excel and the concentrations established by each SOP we generated a scatter graph using the concentrations in the x-axis and the concentrations in the y-axis. A linear trend line was generated setting the intercept to the absorption of the true blank. Next, we selected to display de r-squared value on the chart, this value is used just to check for quality of our data it should be <0.99 and the equation generated from the graph.

The equation generated was a slope intercept model equation [1],

$$y = mx + C \quad [1]$$

Due to the order of our axis (absorption is on the y axis) we resolved for x using as y the value of absorbance of our sample [2],

$$x = \frac{y-C}{m} \quad [2]$$

Using the same equation [2], we calculated the value for the abiotic hydrolysis. When we resolve for x, we convert our absorptions to mM.

In the next step we found the difference between the sample concentration (mM) and the abiotic hydrolysis concentration (mM). We multiplied the difference between the last result times 10

to convert them to µM. Finally, the product is divided into the product of the weight of the samples and the incubation time.

The result of this equation will give us the potential enzymatic activity [3] in the case of NAG µmol pNP g^{-1} soil h^{-1}, aminopeptidases µmol pNA g^{-1} soil h^{-1}, and deaminase µmol 1,2-DANB g^{-1} soil h^{-1}.

$$\boldsymbol{Potential\ Activity} = \frac{[Sample\ Concentration\ (mM) - Abiotic\ Hydrolisis\ Concentration\ (mM)] * 10}{Soil\ weight * incubation\ time} \quad [3]$$

Statistical Analysis

All statistical analyses were conducted using the software JMP® Pro 18.0.1. Soil enzyme activity assay duplicates were averaged for statistical analysis. Enzymatic activities were first tested for normality using the Shapiro Wilk test, however, five (NAG, ALA, GAP, GLU and MET) did not meet the requirements for normality therefore were treated as non-parametric. Two (LAP and Deaminase) were tested for homoscedasticity, with the Levene test and were treated as parametric. For parametric data, were tested through one-way ANOVA and a two-way ANOVA, followed by a Tukey's HONESTLY Significant Difference (HSD) test for multiple comparisons. Non-parametric data were analyzed using the Kruskal Wallis test, followed by Dunn's post hoc test for pairwise comparisons.

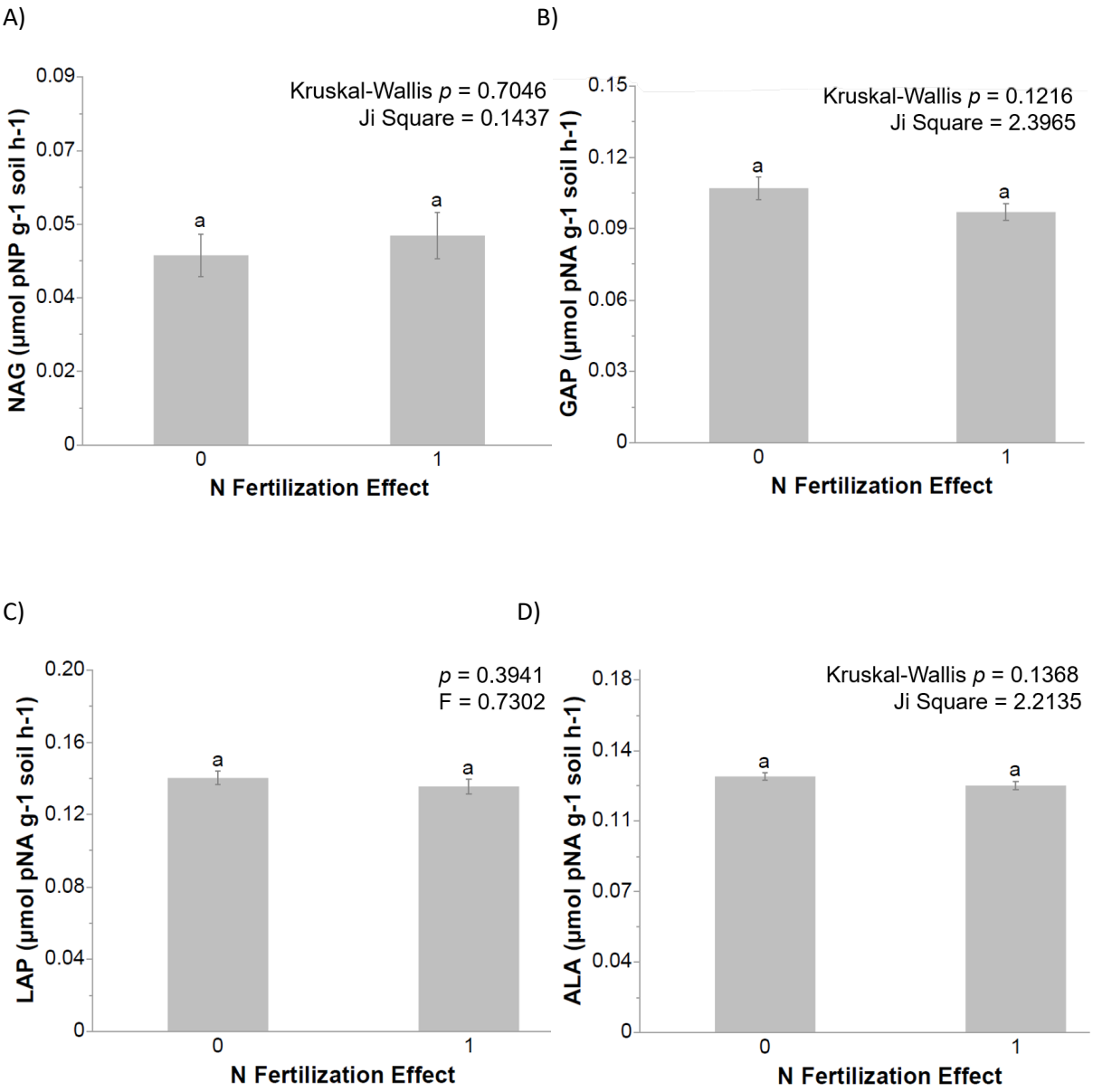
Results and Discussion

In these coffee production systems, protein inputs are likely derived from leaf litter decomposition and compost tea, both contribute with organic N to the soil in proteinaceous forms(Schulten & Schnitzer, 1997). Protease activity, as the upstream enzyme step responsible for the initial depolymerization of proteins into oligopeptides, was not assessed. Consequently, it remains uncertain how proteases responded to the N fertilization to the system. However, proteolysis is not generally rate limiting for downstream N mineralization under field relevant protein inputs (Greenfield et al., 2020). Thus, while omission of protease activity limits mechanistic inference across the full proteolytic pathway, it is unlikely that it may represent a major bottleneck influencing N enzyme activity.

Figure 1 shows the influence of N inorganic fertilization (N and NK) effect of the seven N-hydrolytic soil enzymes involved in the N cycle. In the graphs, “1” refers the applications including UREA and “2” refers to the applications that didn’t included UREA (K and UCL). No statistical differences ($p>0.05$) were found for any of the seven soil enzymes measured among the four fertilization treatments, as indicated by the post hoc test results showed by the shared letters above the error bars.

Figure 1

Effect of N fertilization treatments on (A) N-acetyl-β-D-glucosaminidase (NAG) activity in soil samples; (B) Glycine Aminopeptidase (GAP) activity in soil samples; (C) Leucine aminopeptidase (LAP) activity in soil samples; (D) Alanine aminopeptidase (ALA) activity in soil samples; (E) Glutamic acid aminopeptidase (GLU) activity in soil samples; (F) Methionine aminopeptidase (MET) activity in soil samples and, (G) Deaminase. Error bars represent ±SE. Means with different letters are significantly different at p < 0.05 according to Dunn's and Tukey's post hoc test.



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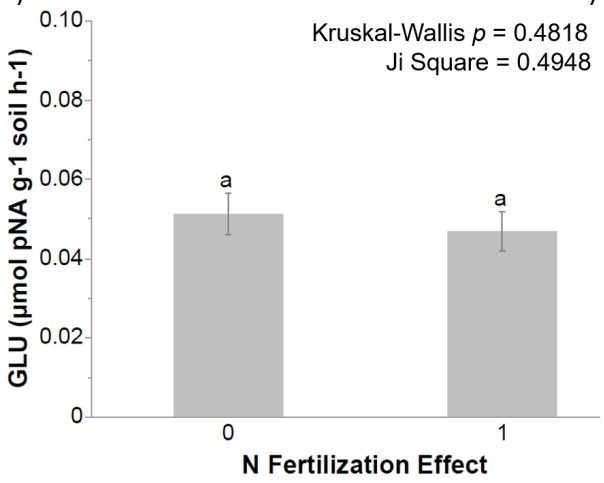

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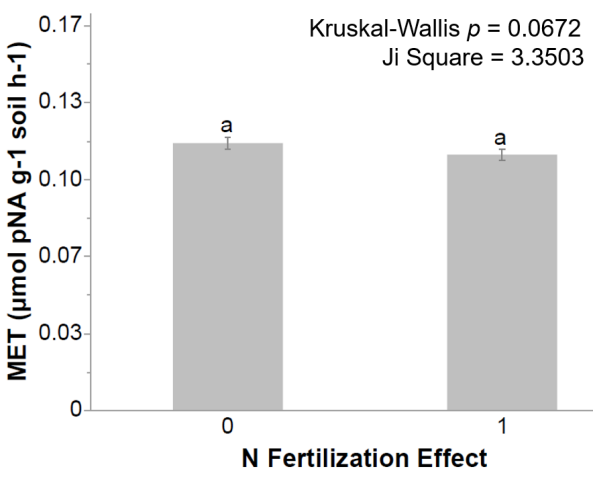

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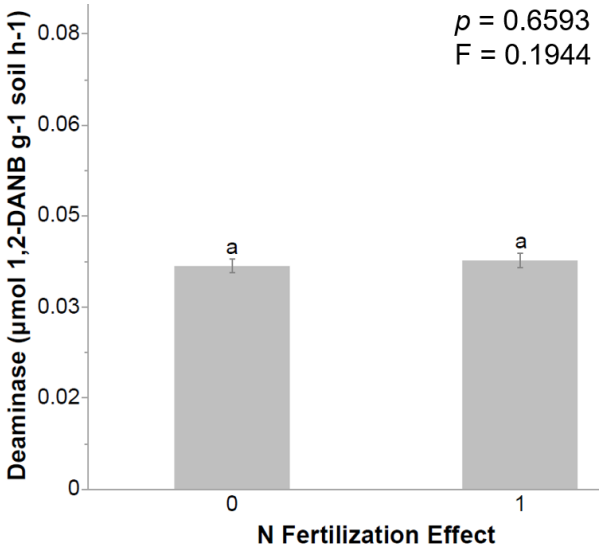


These results indicate that under the conditions tested the application of inorganic N fertilizers did not induce any measurable changes in N-hydrolytic enzyme activities. Furthermore, it suggests that N fertilization alone, as an independent factor, does not suppress hydrolytic enzyme activity. This refutes the traditional hypothesis of feedback inhibition where accumulation of inorganic ammonium-N downregulates extracellular N-acquiring enzymes (Liu et al., 2022; Y. Zhang et al., 2019b).

The lack of differences in N-hydrolytic enzyme activity between N fertilization treatments suggests that neither a single application of N, nor its combination with K, (NK) impacted enzyme

activities. These results are consistent with previous studies indicating that short-term or single-dose fertilization may be insufficient to elicit measurable shifts in enzymatic activity (Yang et al., 2023), especially in biologically buffered systems.

The seven N-hydrolytic enzymes measured in this study are associated with nitrogen acquisition, and their regulation is often sensitive to inorganic nitrogen availability through feedback inhibition. For example, studies have shown that sustained nitrogen enrichment can reduce the activity of N-acquiring enzymes as microbes down-regulate their production when inorganic N is abundant (Fujita et al., 2018; X. Zhang et al., 2016). However, in this study, enzyme activities remained consistent across N and NK treatments, suggesting that the nutrient inputs were either not high enough, or not sustained long enough, to trigger feedback regulation.

Overall, these findings imply that N fertilization alone, without the interaction of time or repeated application, may not function as a strong regulator of soil N-hydrolytic enzyme activity. Thus, we refute the hypothesis of feedback inhibition by N fertilizer in coffee agroecosystems, suggesting that N-hydrolytic enzyme activities are not as sensitive as thought to inorganic N fertilizers (Davies et al., 2022; Grandy et al., 2022)**.**

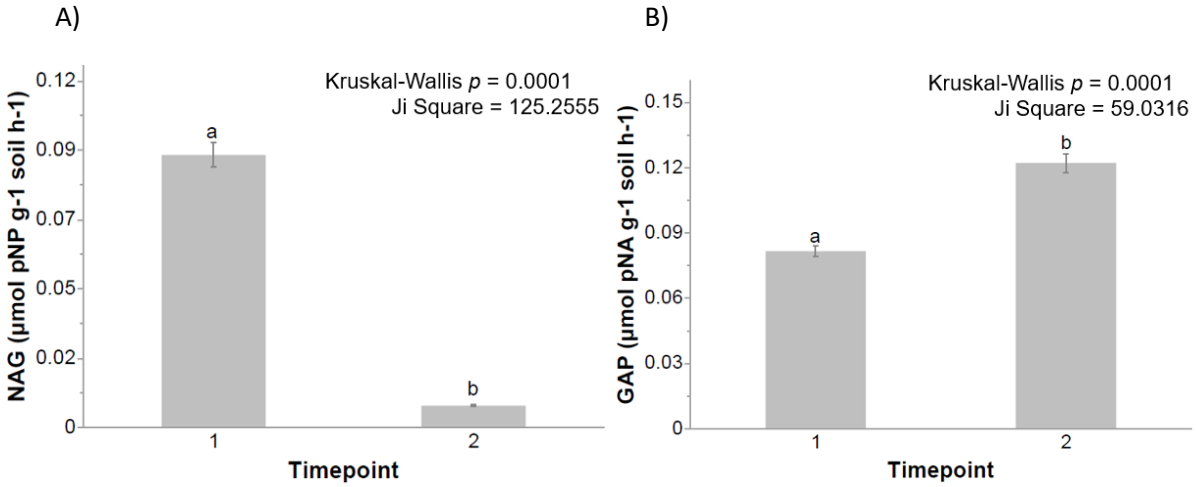
Figure 2 illustrates the activity of seven soil enzymes measured based on timepoint, August 2022 (Timepoint 1) and November 2022 (Timepoint 2) both after fertilization. Significant temporal differences were observed in six out of the seven enzymes, indicating a strong overall effect of time on enzymatic responses.

N-acetyl-β-D-glucosaminidase (NAG; Figure 2A) activity decreased sharply ($p<0.05$) following fertilization, with values dropping from approximately 0.09 to near 0.01 µmol pNP g^{-1} soil h^{-1}. An increase was observed for glycine aminopeptidase (GAP; Figure 2B), which showed a significant raise from 0.08 to around 0.12 µmol pNA g^{-1} soil h^{-1}. Similarly, leucine aminopeptidase (LAP; Figure 2C) exhibited a significant increase after fertilization, suggesting a stimulation of proteolytic activity over time.

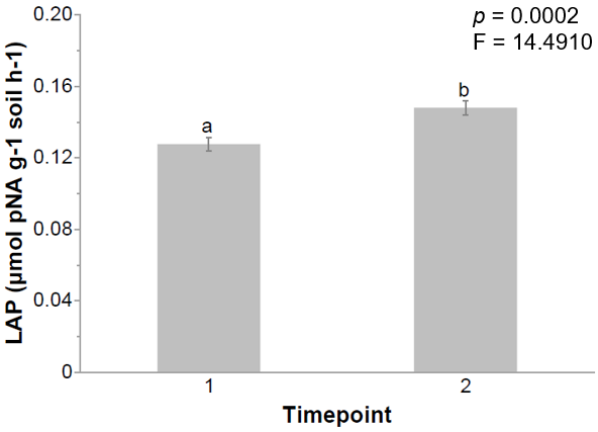
Alanine aminopeptidase (ALA; Figure 2D) showed no significant differences between timepoints (p = 0.1896), indicating temporal stability in this enzymatic function. Meanwhile, glutamic acid aminopeptidase (GLU; Figure 2E) showed a significant difference as a reduction in enzymatic activity dropping from approximately 0.09 to 0.01 µmol pNA g^{-1} soil h^{-1}. Methionine aminopeptidase (MET; Figure 2F) increased significantly, like GAP and LAP. Deaminase activity (Figure 2G) increased significantly post-fertilization, rising from approximately 0.04 to 0.05 µmol 1,2-DANB g^{-1} soil h^{-1}.

Figure 2

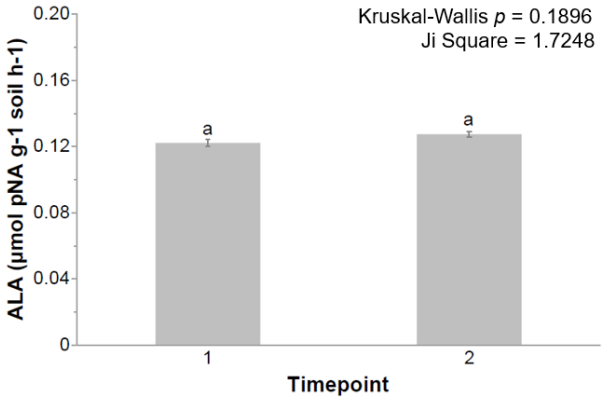
Effect of sampling timepoint, August 2022 (1) and November 2022 (2), on (A) N-acetyl-β-D-glucosaminidase (NAG) activity in soil samples; (B) Glycine Aminopeptidase (GAP) activity in soil samples; (C) Leucine aminopeptidase (LAP) activity in soil samples; (D) Alanine aminopeptidase (ALA) activity in soil samples; (E) Glutamic acid aminopeptidase (GLU) activity in soil samples; (F) Methionine aminopeptidase (MET) activity in soil samples and, (G) Deaminase. Error bars represent ±SE. Means with different letters are significantly different at p < 0.05 according to Dunn's and Tukey's post hoc test.



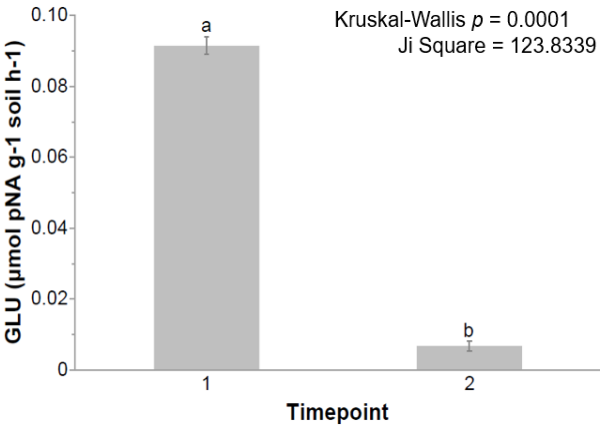
C)


p = 0.0002
F = 14.4910
LAP (µmol pNA g-1 soil h-1)
0.20
0.16
0.12
0.08
0.04
0
a
b
1
2
Timepoint

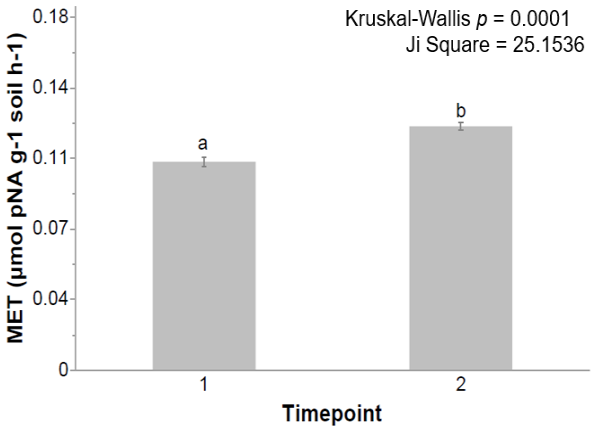
D)


Kruskal-Wallis p = 0.1896
Ji Square = 1.7248
ALA (µmol pNA g-1 soil h-1)
0.20
0.16
0.12
0.08
0.04
0
a
a
1
2
Timepoint

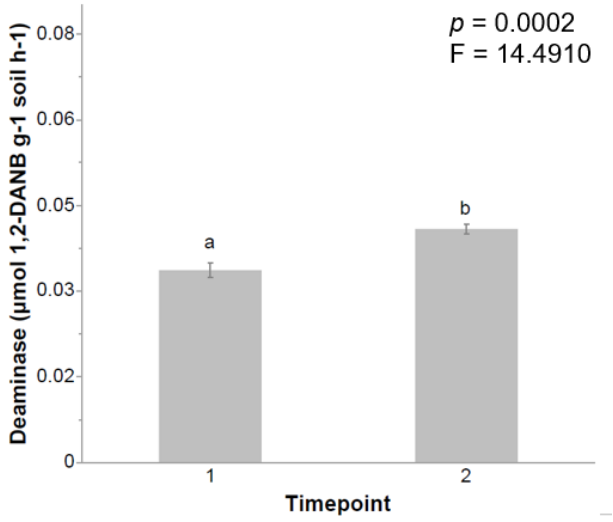
E)


Kruskal-Wallis p = 0.0001
Ji Square = 123.8339
GLU (µmol pNA g-1 soil h-1)
0.10
0.08
0.06
0.04
0.02
0
a
b
1
2
Timepoint

F)


Kruskal-Wallis p = 0.0001
Ji Square = 25.1536
MET (µmol pNA g-1 soil h-1)
0.18
0.14
0.11
0.07
0.04
0
a
b
1
2
Timepoint

G)


p = 0.0002
F = 14.4910
Deaminase (µmol 1,2-DANB g-1 soil h-1)
0.08
0.06
0.05
0.03
0.02
0
a
b
1
2
Timepoint

Temporal analysis of soil enzyme post-fertilization revealed contrasting enzymatic responses, coinciding with the increasing N demand from the coffee plant due to the development and filling of cherries. Notably, the significant post-fertilization declines in N-acetyl-β-D-glycosaminidase (NAG; Figure 2A) and glutamic acid aminopeptidase (GLU; Figure 2E) suggests a suppression of N-acquiring enzymatic activity, due to increased nitrogen availability in the soil. This suppression is consistent with the concept of feedback inhibition, where microbes downregulate, enzymes involved in nitrogen acquisition when inorganic nitrogen becomes abundant (Jian et al., 2016; Olander & Vitousek, 2000). The sharp decline in NAG activity (approximately 90%) parallels prior findings in fertilized soils, where N-acetyl-β-D-glucosaminidase activity was negatively correlated with ammonium levels (Allison & Vitousek, 2005).

In contrast, the significant increases in glycine aminopeptidase (GAP; Figure 2B), leucine aminopeptidase (LAP; Figure 2D), methionine aminopeptidase (MET; Figure 2F), and deaminase (Figure 2G) following fertilization suggest stimulation of proteolytic and nitrogen mineralization pathways. These enzymes play key roles in the depolymerization and mineralization of proteins and amino acids, releasing ammonium-N as a nitrogen source for plants and microbes. Their activation could reflect either increased substrate availability from root exudates or decaying biomass or shifts in microbial community structure favoring enzyme producers (Hill et al., 2012; Y. Zhang et al., 2019a). The enhancement of deaminase activity supports the idea that nitrogen mineralization processes were upregulated post-fertilization, a mechanism critical for nitrogen turnover in agroecosystems.

Interestingly, alanine aminopeptidase (ALA; Figure 1D) activity remained statistically unchanged between timepoints, suggesting that not all aminopeptidase activities respond similarly to fertilization events. The stability of ALA may be due to lower substrate turnover or less dynamic regulation in microbial expression, as suggested by studies of enzyme redundancy and specialization in diverse soil environments (Tripathi et al., 2012).

Collectively, these findings indicate that the temporal effect of fertilization on soil enzymes is enzyme-specific and driven by a combination of factors not necessarily related to fertilization. From a sustainable agriculture perspective, understanding such dynamics is essential for designing nutrient management strategies that maintain soil biochemical balance and functional capacity (Bowles et al., 2014). By identifying how enzymes are affected and how they independently respond to stimuli, it becomes possible to optimize fertilization inputs to sustain soil health, reduce nutrient losses, and enhance ecosystem resilience.

Figure 3 shows the interactive effect of fertilization and time point, August 2022 and November 2022, on the activity of seven soil enzymes. For most enzymes, activity patterns were predominantly influenced by sampling time, while fertilization treatments showed limited differential effects across timepoints. However, some enzyme-specific trends emerged when both factors were considered jointly.

N-acetyl-β-D-glucosaminidase (NAG; Figure 3A) showed a strong temporal suppression in all treatments, with activity significantly decreasing after fertilization regardless of the nutrient applied ($p = 0.0001$). No significant differences were observed among fertilization treatments at either timepoint, suggesting a universal decline in NAG activity over time.

Glycine aminopeptidase (GAP; Figure 3B) presented more nuanced behavior. At Timepoint 2, GAP activity was significantly higher in K and UCL treatments compared to NK and N, indicating a possible suppressive effect of nitrogen inputs. Notably, GAP was the only enzyme where fertilization treatments diverged significantly at both timepoints ($p = 0.0001$), showing interaction-driven variation.

Leucine aminopeptidase (LAP; Figure 3C) and alanine aminopeptidase (ALA; Figure 3D) did not exhibit significant differences between treatments or time points, reflecting consistent activity over time and across nutrient inputs.

Glutamic acid aminopeptidase (GLU; Figure 3E) displayed a significant overall decline in activity after fertilization, similar to NAG, with no clear treatment differentiation. In contrast, methionine aminopeptidase (MET; Figure 3F) showed a modest increase in the UCL treatment post-fertilization, although differences among fertilized plots were not statistically significant.

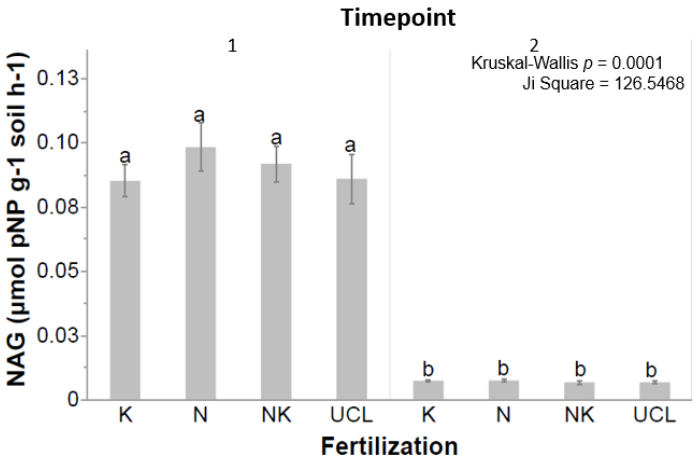
Finally, deaminase (Figure 3G) activity increased after fertilization across all treatments, with the most pronounced change observed in the N treatment. However, significant differences were only detected between timepoints, not among treatments, indicating a general stimulation effect rather than a nutrient-specific response.

Overall, the interaction between fertilization and sampling timepoint revealed enzyme-specific regulatory patterns. Most enzymes responded dominantly to time, while GAP and, to a lesser extent, MET and deaminase exhibited modest fertilization-related shifts. These results suggest that soil enzyme activity dynamics are primarily time-dependent, but certain enzymes may be more sensitive to the type of fertilization applied.

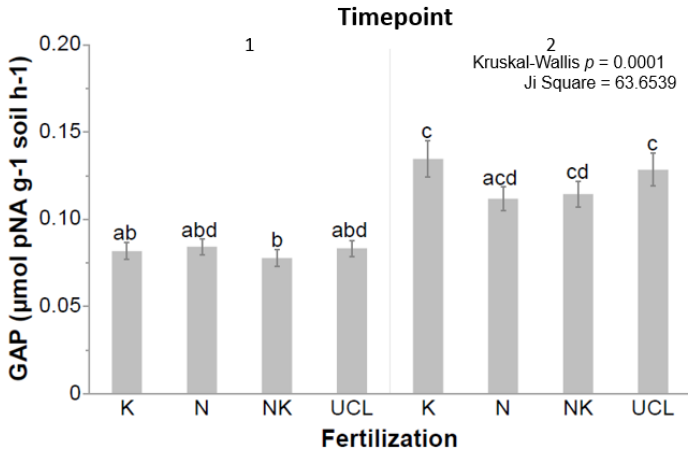
Figure 3

Effect of sampling timepoint, August 2022 (1) and November 2022 (2), and fertilization treatment on (A) N-acetyl-β-D-glucosaminidase (NAG) activity in soil samples; (B) Glycine Aminopeptidase (GAP) activity in soil samples; (C) Leucine aminopeptidase (LAP) activity in soil samples; (D) Alanine aminopeptidase (ALA) activity in soil samples; (E) Glutamic acid aminopeptidase (GLU) activity in soil samples; (F) Methionine aminopeptidase (MET) activity in soil samples and, (G) Deaminase. Error bars represent ±SE. Means with different letters are significantly different at $p < 0.05$ according to Dunn's and Tukey's post hoc test.

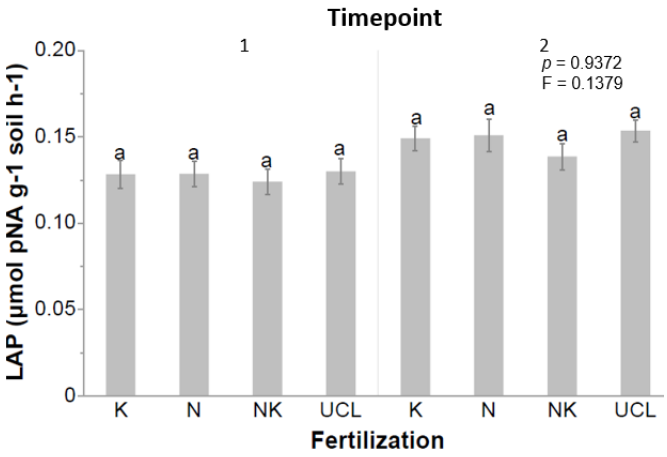
A)


Timepoint
1
2
Kruskal-Wallis p = 0.0001
Ji Square = 126.5468
NAG (µmol pNP g-1 soil h-1)
0.13
0.10
0.08
0.05
0.03
0
a
a
a
a
b
b
b
b
K
N
NK
UCL
K
N
NK
UCL
Fertilization

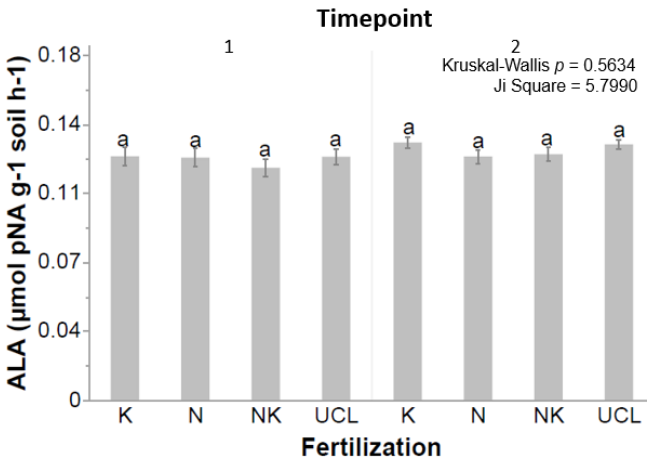
B)


Timepoint
1
2
Kruskal-Wallis p = 0.0001
Ji Square = 63.6539
GAP (µmol pNA g-1 soil h-1)
0.20
0.15
0.10
0.05
0
ab
abd
b
abd
c
acd
cd
c
K
N
NK
UCL
K
N
NK
UCL
Fertilization

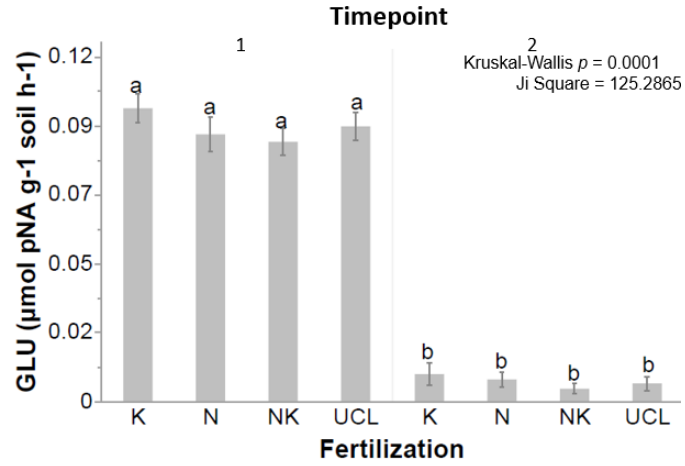
C)


Timepoint
1
2
p = 0.9372
F = 0.1379
LAP (µmol pNA g-1 soil h-1)
0.20
0.15
0.10
0.05
0
a
a
a
a
a
a
a
a
K
N
NK
UCL
K
N
NK
UCL
Fertilization

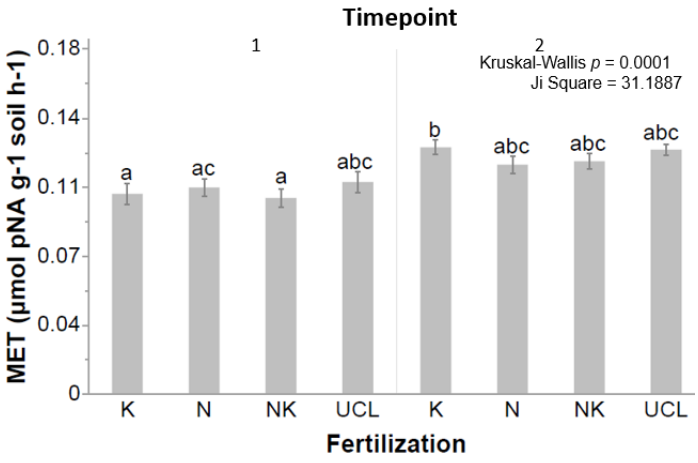
D)


Timepoint
1
2
Kruskal-Wallis p = 0.5634
Ji Square = 5.7990
ALA (µmol pNA g-1 soil h-1)
0.18
0.14
0.11
0.07
0.04
0
a a a a a a a a
K N NK UCL K N NK UCL
Fertilization

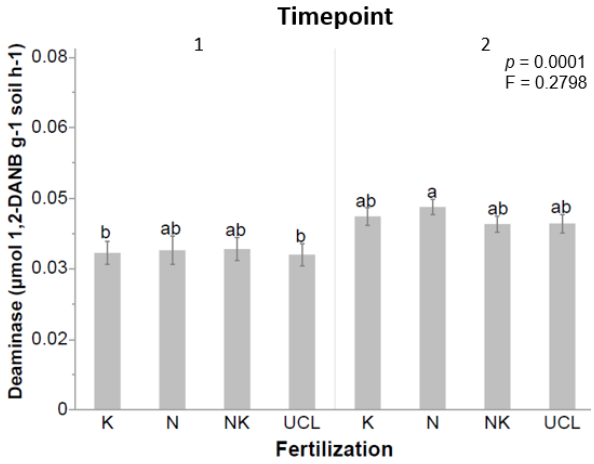
E)


Timepoint
1
2
Kruskal-Wallis p = 0.0001
Ji Square = 125.2865
GLU (µmol pNA g-1 soil h-1)
0.12
0.09
0.07
0.05
0.02
0
a a a a b b b b
K N NK UCL K N NK UCL
Fertilization

F)


Timepoint
1
2
Kruskal-Wallis p = 0.0001
Ji Square = 31.1887
MET (µmol pNA g-1 soil h-1)
0.18
0.14
0.11
0.07
0.04
0
a ac a abc b abc abc abc
K N NK UCL K N NK UCL
Fertilization

G)



The combined analysis of fertilization treatment and sampling timing highlights temporal dynamics as the dominant factor influencing enzyme activity, with treatment-specific effects appearing only in select enzymes. Notably, N-acetyl-β-D-glucosaminidase (NAG; Figure 3A) and glutamic acid aminopeptidase (GLU; Figure 3E) declined sharply post-fertilization across all treatments, underscoring a time-dependent suppressive trend rather than treatment-specific regulation. This finding is consistent with earlier reports indicating that increased inorganic nitrogen availability leads to widespread feedback inhibition of N-acquiring enzymes (Jian et al., 2016; Olander & Vitousek, 2000).

Glycine aminopeptidase (GAP; Figure 3B) exhibited a more nuanced pattern: at Timepoint 2, GAP activities were significantly higher in the K-fertilized and control plots, while remaining lower in N and NK treatments. This suggests a specific suppressive effect of nitrogen inputs on proteolytic enzyme activity. Comparable suppressive trends were observed in prior studies where inorganic N reduced GAP activity by 30–50%, consistent with end-product inhibition(Norman et al., 2020). The significant interaction effect ($p = 0.0001$) underscores the enzyme's sensitivity to N-fertilization within a narrow temporal window.

Neither leucine aminopeptidase (LAP; Figure 3C) nor alanine aminopeptidase (ALA; Figure 3D) showed significant variation across treatments or timepoints, implying stable enzyme profiles that

may result from substrate redundancy or functional overlap within microbial communities (Tripathi et al., 2012).

Methionine aminopeptidase (MET; Figure 3F) displayed a slight upward trend in the UCL treatment post-fertilization but lacked statistical significance across treatments. Deaminase activity significantly increased after fertilization in all treatments, especially under N application, indicating enhanced amino acid turnover. This aligns with findings that nitrogen-driven community shifts can enhance deaminase expression, supporting nutrient cycling in agroecosystems (Hill et al., 2012).

Conclusions

This study assessed the hypothesized suppression of soil N-hydrolytic enzymatic activity under replicated, on-farm N fertilization, and changes in activities after synthetic N fertilizations.

We do not find evidence for and thus refute the hypothesis of feedback inhibition, as there were no differences in activities of N-hydrolytic enzymes regardless of synthetic N fertilization.

Additionally, we find that timing relative to fertilization influenced enzyme activity, with notable decreases and increases in certain enzymes in post-fertilization sampling. Suggesting the interaction of additional factors influencing enzymatic activity.

By testing a common ecological hypothesis of soil enzyme activity response to fertilization practices, this study contributes to a better understanding of N- hydrolytic soil enzymes in fertilized systems compared to an unfertilized and refutes feedback inhibition of N-cycling soil enzyme activities under field conditions in coffee agroecosystems. These results enrich our comprehension of N cycling in tropical agricultural soils and highlight the importance of monitoring enzymatic behavior as an indicator of soil health.

Recommendations

Future research should explore longer-term effects of repeated fertilization at control rated based on soil analysis, on N- and P hydrolytic enzyme activity to assess the degree to which the absent feedback inhibition is transient or persistent over time. It is recommended to include additional enzymes, especially those linked to phosphorus and carbon cycling, to develop a more comprehensive view of nutrient dynamics. By capturing a broader spectrum of soil enzyme functions, the knowledge gap would be less, and it could generate a better way to assess the interaction and tradeoffs among nutrient cycles. Moreover, repeating this study under different soil types, climates, or cropping systems could validate the generalizability of these findings across diverse agroecosystems. Additionally, implementing a similar study non-perennial crops in combination with a nitrogen fixing plant used in a rotational crop production system could provide valuable insight into their synergistic effect over N-hydrolytic enzymes. Finally, applying biological control organisms, such as *Trichoderma harzianum* providing insights in how the colonization of the plant's rhizosphere affects the production of extracellular enzymes and therefore enzyme activities.

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Appendices

Appendix A

Statistic

Enzyme	Factor	ANOVA/Kruskal-Wallis				
		F / χ^2	df	p	Level	Mean (± standard error)
NAG	Timepoint	125.256	1	<0.0001	1	0.090 ± 0.0041 a
					2	0.007 ± 0.0002 b
	Fertilization	0.5965	3	0.8972	K	0.046 ± 0.004 a
					N	0.053 ± 0.008 a
					NK	0.049 ± 0.007 a
					UCL	0.046 ± 0.007 a
	N Fertilization Effect	0.1437	1	0.7046	0	0.046 ± 0.005 a
					1	0.051 ± 0.005 a
GAP	Timepoint	59.032	1	<0.0001	1	0.082 ± 0.0024 a
					2	0.122 ± 0.0043 b
	Fertilization	2.5409	3	0.468	K	0.108 ± 0.007 a
					N	0.098 ± 0.004 a
					NK	0.096 ± 0.005 a
					UCL	0.106 ± 0.006 a
	N Fertilization Effect	2.3965	1	0.1216	0	0.106 ± 0.004 a
					1	0.097 ± 0.003 a
LAP	Timepoint	14.491	1	0.0002	1	0.128 ± 0.0037 a
					2	0.148 ± 0.0038 b
	Fertilization	0.6757	3	0.5681	K	0.139 ± 0.005 a
					N	0.140 ± 0.006a
					NK	0.131 ± 0.005 a
					UCL	0.142 ± 0.005 a
	N Fertilization Effect	0.7302	1	0.3941	0	0.140 ± 0.003 a
					1	0.136 ± 0.004 a
ALA	Timepoint	1.7248	1	0.1891	1	0.128 ± 0.0037 a
					2	0.148 ± 0.0038 a
	Fertilization	2.4637	3	0.4819	K	0.127 ± 0.003 a
					N	0.124 ± 0.003 a
					NK	0.122 ± 0.003 a
					UCL	0.127 ± 0.002 a
	N Fertilization Effect	2.2135	1	0.1368	0	0.127 ± 0.002 a
					1	0.123 ± 0.001 a
GLU	Timepoint	123.838	1	<0.0001	1	0.091 ± 0.0025 a
					2	0.007 ± 0.0013 b
	Fertilization	1.3023	3	0.7286	K	0.054 ± 0.008 a
					N	0.048 ± 0.007 a
					NK	0.046 ± 0.007 a
					UCL	0.049 ± 0.007 a
	N Fertilization Effect	0.4948	1	0.4818	0	0.051 ± 0.005 a
					1	0.045 ± 0.002 a
MET	Timepoint	25.1536	1	<0.0001	1	0.103 ± 0.0025 a
					2	0.121 ± 0.0019 b
	Fertilization	3.6185	3	3.6185	K	0.113 ± 0.004 a
					N	0.110 ± 0.003 a

					NK	0.109 ± 0.003 a
					UCL	0.116 ± 0.003 a
	N Fertilization Effect	3.3505	1	0.0672	0	0.115 ± 0.002 a
					1	0.110 ± 0.002 a
Deaminase	Timepoint	22.301	1	<0.0001	1	0.034 ± 0.0012 a
					2	0.041 ± 0.0009 b
	Fertilization	0.3399	3	0.7965	K	0.037 ± 0.002 a
					N	0.039 ± 0.002 a
					NK	0.037 ± 0.002 a
					UCL	0.036 ± 0.002 a
	N Fertilization Effect	0.1944	1	0.6593	0	0.037 ± 0.001 a
					1	0.038 ± 0.002 a