Effect of nitrogen fertilization and shade type in soil quality and nitrogen assimilation in Ecuadorian Amazon *Coffea canephora* agroecosystems

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Abstract. In the Amazon basin, Robusta (*Coffea canephora*) is produced in contrasting agroecosystems of low input, shade-based smallholder production, or in intensively managed monoculture plantations that differ in nitrogen (N) availability and productivity. This study aimed to determine whether the shade trees and proper addition of N-fertilizer in coffee plantations may provide soil fertility benefits and contribute to soil quality and crop productivity. Full sun had a positive effect on enzyme activities, potentially mineralizable N (PMN), available ammonium (NH_4-N), leaf %N and leaf N:P. Enzyme activities as well as NH_4-N were greater under low input, whereas high input yielded greater PMN, leaf N and leaf N:P. Protease was positively associated with PMN ($R = 0.60$, $P = 0.003$) but unrelated to the activities of the aminopeptidases, and carbon (C) mineralizing enzymes. Permanganate oxidizable C (POXC) and nitrate (NO_3-N) were similar across input and shade managements but more variable under shade relative to full sun. Low input offers limited improvements in soil N cycling increasing by 53% the enzymatic activity and N availability by 31% relative to high input under Robusta coffee production in this region. Whereas, high input improves PMN and Robusta N uptake (Leaf N). Relatively lower response in enzymatic activities, C and N stocks, and leaf N under shade management suggests that although it is a common smallholder practice in this region, leguminous tree-based agroforestry may not close N needs of Robusta as effectively as higher N inputs.

Key words: Carbon and nitrogen dynamic, enzymatic activities, N-mineralizing, shade-based.

Resumen. En la Amazonía, el café Robusta (*Coffea canephora*) se produce a pequeña escala, en sombra o en monocultivos que difieren en disponibilidad de nitrógeno (N) y en productividad. El objetivo de este estudio fue determinar si los árboles de sombra y la adición de fertilizantes nitrogenados pueden proporcionar beneficios sobre la calidad del suelo y la productividad del café. Pleno sol influyó positivamente en las actividades enzimáticas, potencial de mineralización de N (PMN), amonio disponible (NH_4-N), %N en hojas y N:P en hojas. La actividades enzimática, y el NH_4-N, fueron mayores en baja fertilización, mientras que alta fertilización produjo mayores valores de PMN, %N en hojas y N:P en hojas. La proteasa se correlacionó positivamente con PMN ($R = 0.60$, $P = 0.003$) pero no se relacionó con las actividades de enzimas mineralizadoras de carbono (C) y N. El carbono oxidizable por permanganato (POXC) y el nitrato (NO_3-N) fueron similares entre fertilización y tipo de sombra, pero más variables bajo sombra en relación con pleno sol. La baja fertilización ofrece mejoras limitadas en el ciclo del N, aumentando en un 53% la actividad enzimática y en un 31% la disponibilidad de N, con relación a la alta fertilización para producción en esta región, sin embargo, la alta fertilización mejora la absorción de PMN y la asimilación de N. Una respuesta relativamente menor en actividades enzimáticas, reservas de C y N, y asimilación de N bajo sombra sugiere que, aunque es una práctica muy común en esta región, la asociación de café con árboles leguminosos puede no suplir las necesidades de N de Robusta de manera tan efectiva como la alta fertilización de N.

Palabras clave: Actividades enzimáticas, dinámica de carbono y nitrógeno, mineralización de nitrógeno, sombrío.

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1. INTRODUCTION

Coffee is an important cash crop in the developing tropics thought to be the livelihood of 20 million smallholder households (Lewin, Giovanucci, & Varangis, 2004). Globally, *Coffea arabica* (Arabica) is the most produced species (60%) with *Coffea canephora* (Robusta) accounting for 40% of remaining global production (Canet et al., 2016). Greater heat and drought tolerance of Robusta has led to its suggestion as a more climate-change resilient species that may replace Arabica in the coming decades (Bunn, Läderach, & Ovalle, 2015; Magrach & Ghazoul, 2015), and is why Robusta is more suitable for cultivation in the lowland humid tropics (Camargo & Marcelo, 2009). In Latin America, Robusta production is concentrated in the Amazon basin (Pérez et al., 2014) both in industrialized large-scale monoculture operations such as in Brazil (Hoffman, 2018; Volsi et al., 2019) or in smallholder and often agroforestry systems such as in northeastern Ecuador (Viteri-Salazar, 2013; Viteri-Salazar, Ramos-Martín & Lomas, 2018).

Nitrogen (N) availability in coffee crops is a determining factor for productivity. N is the most demanded nutrient by the plant, since it is essential for structural development, cell growth and fruit formation. Peak nutrient demand occurs in the fruit formation phase (Neto et al., 2011). However, in this and other crops the contributions of nitrogen are not proper and, therefore, it is not used efficiently. Since the green revolution, there has been a trend of overuse of nitrogen fertilizers due to the belief that the greater the amount of nitrogen applied, the higher the yield per unit area (Albornoz, 2016). However, the nitrogen dynamics in a soil-plant relation not only depends on the amount of N added to the soil but is mainly affected by the microbial activity responsible for the organic nitrogen mineralization processes. Moreover, a higher addition to the needs of the crop can have an impact on the decrease in the quality of the products and yields, causing economic losses for the producers (Altieri & Nicholls, 2003) and more importantly, contributing to the environmental impact. Excess N in the soil increases losses by leaching, volatilization, denitrification and erosion (Matson, Lohse, & Hall, 2002). These losses could be minimized by synchronizing the entry and availability of nitrogen with the nutritional demand of the crop (Panek, Matson, Ortiz-Monasteiro, & Brooks, 2000) and its relationship with the environmental and structural characteristics of soils in agroecosystems.

As mentioned, N is a key determinant of coffee yield and a driver of global coffee yield gaps, especially in input-limited smallholder systems (Bhattarai et al., 2017; Rahn et al., 2018; Wang et al., 2015). In Ecuador, the province of Orellana is one of the most important producers of Robusta (6,858 Mg of green coffee beans y^{-1}) (Viteri-Salazar et al., 2018). Largely produced by smallholders with low or zero N fertilizer inputs, and instead relying on leguminous N fixing trees, with an average yield of 0.45 Mg ha-1 y^{-1}. In Vietnam, intensively grown Robusta coffee has been shown to be very responsive to high NPK (Nitrogen, Phosphorus, Potassium) inputs, yielding up to 2.5 tons of coffee ha^{-1} $year^{-1}$ when applied 1.5 tons ha^{-1} $year^{-1}$ of NPK fertilizer (Marsh, 2007). Given that coffee green bean contains 35 kg N per Mg (Ahn, 1993), at least 15.8 kg N Mg ha^{-1} could be needed to meet N export under these yield conditions interacting with other soil dynamics like microbial degradation, legume N_2-fixation if association with legumes is present, soil structural characteristics soil organic matter and soil fertility. However, given relatively low nitrogen use efficiency (NUE) of coffee (Capa, Pérez-Esteban, & Masaguer, 2015;

Salamanca-Jiménez et al., 2017) (e.g., < 20%), at least 80 kg Mg ha^{-1} y^{-1} are likely needed, which is below the common application of 90 kg N ha^{-1} for the more intensive Ecuadorian region of coffee production of Guayas (Borbor-Cordova et al., 2006). Commonly used leguminous agroforestry species such as *Erythrina* spp can fix up to 35 to 60 kg N ha^{-1} y^{-1} (Nair, Buresh, Mugendi, & Latt, 1999), suggesting that many Robusta systems have deficiency in available N. On the other hand, with repeated pruning of *Erythrina*, net N inputs via soil-applied residues of up to 227 kg N ha^{-1} y^{-1} (Russo & Budowski, 1986), although seasonal cessation of the N-fixing nodules (Nair et al., 1999) means that N_2-fixation inputs thru *Erythrina* spp can vary substantially.

In addition to varying magnitude of net N inputs to coffee agroecosystems, leguminous trees can impact coffee N status by influencing soil N cycling, specifically mineralization. Nitrogen mineralization is influenced by the amount and form of N added to the soil, as well as the enzymatic activity responsible for N mineralization from this organic stock, available C, and therefore C:N ratios. Mineralization of organic N forms such as proteins into crop-available inorganic N is catalyzed by a suite of extracellular enzymes. In the proteolytic pathway of N mineralization, protein is degraded to oligopeptides via protease activity, and oligopeptides are then hydrolyzed into amino acids by aminopeptidases (Vranova, Rejsek, & Formanek, 2013). Rapid deamination or ammonification yields ammonium, which can nitrify into nitrate (Crohn, 2004; Mendieta & Rocha, 2007). A similar process occurs for C mineralization, following the cellulolytic pathway in which goes cellulose is degraded into oligosaccharides and ultimately monosaccharides (e.g., glucose) that contribute to soil labile C. Another N mineralization pathway is degradation of N-rich fungal biomass, namely the polymer chitin, which degrades via action of chitinases such as N-acetyl-glucosaminidase (Mori, 2020). Integrating these biochemical measures of soil N mineralization, potentially mineralizable N (PMN) determined by anaerobic incubation offers a metric of N that can become available through the decomposition of organic N (Bremner & Keeney, 1966; Waring & Bremner, 1964).

Quantifying key determinants of N inputs and its mineralization, such as the shade trees and N-fertilizer addition , just as interactive influences on N mineralization can help establish guidelines for N use efficiency (Clark et al., 2019; Ribaudo et al., 2011), particularly in low N input systems. Full-sun coffee production (i.e., no shade) or usage of shade trees can induce contrasting scenarios of N mineralization. For example, leguminous tree (*Inga densiflora*) used for shading in Costa Rican smallholder systems accelerated soil N mineralization rates by two-thirds compared to that from the monoculture or full sun system, thought to reflect a combination of altered soil microclimate (e.g., temperature) and non-coffee litter contributions (Borbor-Cordova et al., 2006). Decomposition of shade tree litter can improve nutrient uptake from coffee by synchronizing the rates of nutrient release from decomposed biomass with coffee nutrient demand. Litter contributed by shade trees can further stimulate N cycling by providing organic matter (OM) to soil microbial biomass, which is mineralized by extracellular enzymes (Bornemisza, 1982; Chander, Goyal, Nandal, & Kapoor, 1998; Rodrigues et al., 2015; Vallejo, Roldan, & Dick, 2010).

This study assessed biochemical drivers of soil N and C mineralization in different agroforestry and fertilization managements of Robusta, in the Amazon region of northeastern Ecuador. The purpose of this study was to determine whether the shade trees and N-fertilizer appropriate addition in coffee plantations may provide soil fertility benefits and contribute to soil quality and crop productivity. The objectives of the study were:

- To evaluate the effect of shade trees and the addition of N fertilizer in coffee agroecosystems on enzymatic activity related to the Nitrogen and Carbon mineralization in the soil.

- To determine the effect of shade trees and the addition of N fertilizer on potentially mineralizable soil organic N (PMN), inorganic N available, and labile organic C in soil.

- To evaluate the effect of shade trees and the addition of N fertilizer on coffee plant N uptake.

- To determine the relationships between enzyme activities, PMN and inorganic N stocks, and labile organic C in soil.

2. MATERIALS AND METHODS

Experimental site and design

An experimental coffee trial was established in 2015 by the "Instituto Nacional de Investigaciones Agropecuarias" (INIAP) of Ecuador, in collaboration with the University of Illinois in Urbana-Champaign (UIUC). The study site is located in the Amazon region of northeastern Ecuador (0°21'31.9'S, 76°51'51.1'W), in the province of Orellana that is a regional hotspot of Robusta production by smallholders according to Salazar & Ramos-Martín (2017) and Viteri-Salazar et al. (2018). At 265 meters above sea level, mean annual temperature (MAT) is 24 °C and mean annual precipitation (MAP) is 3,217 mm $year^{-1}$. The soil at the study site is Alfisol (USDA Soil Taxonomy), developed on alluvium from the nearby (< 10 km) Napo River, a tributary of the Amazon River. The texture of this soil is loam, with 50% sand, 30% clay and 20% silt. Soil organic matter (SOM) at the study site is 9.26% (mean value) and the pH of this soil is 5.2 (± 0.06). The cationic exchange capability is 17.5 $cmol_c$ kg^{-1} (mean value).

Four treatments representing a full factorial of two shade types and two fertilization combinations were evaluated thru a split block design. For each treatment, three replicates (blocks) were made, obtaining a total of 12 experimental plots in which two pairs of soil samples and two pairs of coffee trees were sampled to evaluate soil N and C dynamics and coffee leaf N. The shade types were a shade coffee system resulting from the combination of "porotillo" (*Erythrina velutina*) as an autochthonous leguminous shade tree and Robusta coffee plants, and sun-grown coffee plants as the full sun system. The fertilization combinations were high conventional fertilizer input (HI) in synthetic nitrate forms as 114 kg ha^{-1} $year^{-1}$ for 2017 and 123 kg ha^{-1} $year^{-1}$ for 2018 and 2019. Low input entailed N inputs in organic matter forms (compost) as 33 kg ha^{-1} $year^{-1}$ for 2017 and 40 kg ha^{-1} $year^{-1}$ for 2018 and 2019 (Table 1). Each plot includes a three-row buffer of coffee plants surrounding 36 coffee plants in the main plot. Though these treatments differ in the form and the amount of N, they represent distinct management strategies and reflect realistic rates and practices in this region. Phosphorus (P) and potassium (K) fertilization details will be mentioned below (Chart 1) but their dynamics will not be analyzed by this study.

Table 1. Nitrogen fertilization management per year in a 3-year Robusta coffee trial in the Amazon region of Northeastern Ecuador.

Fertilization management																
Year	**Fertilization**	**Applied fertilizer (kg ha-1 year-1)**												**Total N**	**Total P**	**Total K**
		Gallinaza (Ecoabonaza ®)			**KNO3**		**$Mg(NO_3)_2$**	**20-7-10 (Yaramila actyva ®)**			**19-4-19 (Yaramila hydran ®)**					
		N	**P**	**K**	**N**	**K**	**N**	**N**	**P**	**K**	**N**	**P**	**K**			
2017	Low	33.8	19.4	22.0										33.8	19.4	22.0
	High				9.0	25.2	16.3	89.0	31.2	44.5				114.3	31.2	69.7
2018	Low	40.0	23.0	26.0										40.0	23.0	26.0
	High				18.4	51.5	11.4	83.8	29.3	41.9	9.7	2.0	9.7	123.2	31.3	103.1
2019	Low	40.0	23.0	26.0										40.0	23.0	26.0
	High				18.4	51.5	11.4	83.8	29.3	41.9	9.7	2.0	9.7	123.2	31.3	103.1

Soil and plant sampling

Soil and plant samples were obtained in May 2019, during a period of lower rainfall. For each plot, two pairs of coffee plants were selected within the main plot. To address variability in the shade treatment, one pair of the sampled plants were adjacent to the shade tree and the other pair in the row between shade trees. Soil samples were collected at 0 - 7.5 cm depth on each side of the coffee plant (180°) at a 60 cm distance from the base of the tree. For each selected plant, two soil samples were obtained on opposite sides of the canopy and then were mixed for a composite sample. Samples were stored, labeled and transported on ice to the laboratory at UIUC, were they were stored at 4 °C until further analyses, which would likely change enzymatic activities in terms of absolute values, but may help to calculate estimates with the accurate corrections. Leaf samples were collected as a composite sample per each of two pair of trees, by selecting the first pair of mature leaves from the four lateral stems for each tree. Leaves were air-dried and ground before analysis.

Enzyme assays

Soil protease activity was determined using the method of Ladd and Butler (1972) using the protein casein as a natural substrate. In one set 50 ml centrifuge tubes, 0.5 g of oven-dried equivalent of soil, 1.25 ml of 0.2 M Tris (pH 8.0) and 1.25 ml of 2% (m/v) sodium caseinate solution were added. In a second set of tubes, 0.5 g of oven-dried equivalent of soil and 1.25 ml of 0.2 M Tris (pH = 8), this set serve as a soil control. Both sets of tubes were incubated at 50 °C for 2 hours. After incubation, 5 ml of 10% trichloroacetic acid (TCA) was added to the first set, and 1.25 ml of Na-caseinate and 5 ml of 10% TCA was added to the control set (soil only). A clear supernatant was obtained by centrifugation at 13,000 rpm (60 sec). Then, 0.50 ml of sample supernatant or tyrosine standards were transferred to a second set of micro-centrifuge tubes and treated with 0.75 ml of 1.4 M Na_2CO_3 and 0.5 ml of Folin-Ciocalteu reagent. An aliquot of each reaction was measured for absorbance at 650 nm and protease activity was calculated as µmol tyrosine g^{-1} h^{-1}.

To evaluate soil N as well as C mineralization, the activities of six additional hydrolytic enzymes were determined using *para*-nitrophenyl-linked substrates (Table 2). The concentration of *para*-nitrophenol (*p*NP) released is determined with colorimetry to determine the potential maximum enzyme activity. In 50 ml centrifuge tubes, 1 g of oven-dried equivalent of soil, 4 ml of 18.2 MΩ water and 1 ml of *p*NP-linked substrate solution at the appropriate concentration (Chart 2) were added. For each enzyme, the tubes were incubated using a water bath at a temperature of 37 °C for 1 hour, except for leucine aminopeptidase which was incubated for 2 hours to ensure generation of sufficient *p*NP product. Reactions were terminated using 4 ml of 0.1 M 2-amino-2-(hydroxymethyl) propane-1,3-diol (Tris; pH 12) to alkalize the solution for colorimetry and to recover hydrolyzed *p*NP, and 1 ml of 2 M $CaCl_2$ to flocculate sediments. A clear supernatant was obtained by centrifugation at 14,000 rpm (105 sec). An aliquot was measured for absorbance at 410 nm by spectrophotometry. To maximize reliability of enzyme activity values, samples were corrected for dissolved OM interference according to the methodology developed by Margenot et al. (2018), incomplete *p*NP recovery by Cervelli et al. (1973), and abiotic hydrolysis of substrate blank according to Neal et al. (1986). Potential activity was calculated in µmol *p*NP g^{-1} soil hr^{-1} by Margenot et al. (2018).

Table 2. Description of enzymes and substrates evaluated for potential activities.

Enzyme		Element cycle(s)	Substrate	[Substrate] (mM g-1)
β-Glucosidase	BG	C	pNP-β-glucopyranoside	10
Cellobiohydrolase	CBH	C	pNP-β-D-cellobioside	10
N-Acetyl- β -D-glucosaminidase	NAG	C and N	pNP-N-acetyl- β -D-glucosaminide	10
Glycine-aminopeptidase	GAP	N	L-Glycine 4-nitroanilide	2
Leucine-aminopeptidase	LAP	N	L-Leucine 4-nitroanilide	2
Alanine-aminopeptidase	AAP	N	L-Alanine-4-nitroanilide	2

Labile N and C stocks

Available NH_4-N and NO_3-N concentrations were determined with colorimetry using potassium chloride (KCl) extracts. In 50 ml centrifuge tubes, 5 g of oven-dried equivalent of soil were extracted in 25 ml of 2 M KCl (1:5 m/v) by shaking horizontally at 150 rpm for 1 hour and then centrifuged at 4,000 rpm for 10 minutes. Colorimetric quantification of NH_4-N and NO_3-N in KCl extract were performed as described by Griffin et al. (2017) using the salicylate-hypochlorite according to the methodology developed by Verdouw, Van Echteld and Dekkers (1978) and vanadium (III) chloride reduction method by Doane and Horwath (2003), respectively.

Potentially mineralizable N (PMN) was determined by anaerobic incubation according to Drinkwater (1994). In 50 ml centrifuge tubes, 5 g of oven-dried equivalent of soil were added. Then, the tubes were purged with N_2 to obtain anaerobic conditions, and incubated at 23 °C, the mean annual temperature of the study site, for 7 days. After incubation, the samples were extracted with 2 M KCl and NH_4 extracted was quantified with colorimetry following the same procedure described above. PMN was calculated as the difference in ammonium between non-incubated and incubated soil.

Permanganate oxidizable carbon (POXC) was determined using the method described by Culman (2012). In 50 ml centrifuge tubes, 1.25 g of oven dried equivalent of soil, 1 ml of 0.2 M $KMnO_4$ and 9 ml of ultra-pure water were added. The mixture was shaken at 150 rpm for exactly 2 minutes and then allowed to settle for 10 minutes. The supernatant was diluted (1:50) and absorbance at 550 nm quantified by spectrophotometry. POXC was calculated assuming 9,000 mg C oxidized per mol permanganate according to Weil et al. (2003).

Statistical analyses

Two-way analysis of variance (ANOVA) was performed to evaluate the effect of N- fertilizer inputs (high and low) and shade type (full-shade and no shade) on enzyme activities, POXC, potentially mineralizable N and available inorganic N ($P \leq 0.05$). Tukey's HSD test was used to determine significance difference in treatment or treatments means the greatest enzyme activities, POXC, PMN and available inorganic N. Pearson's correlation coefficient test was used to measure the relationships among variables. Statistical analyses were performed using the Statistical Analysis Software (SAS) version 9.4 program (Cary Institute, NC).

3. RESULTS AND DISCUSSION

Hydrolytic enzyme activities

Potential activities of N and C cycling enzymes tended to be higher under low input and full sun, contrary to the hypothesis that increased organic matter additions under the leguminous shade tree *Erythrina* spp would lead to elevated enzymatic activities. Greater cellulolytic enzyme activities occurred under low input and were greater in full sun. However, no fertilization × shade type interaction was found to be significantly different. Low input increased the activity of BG (Figure 1) by 43% (P = 0.043), and CBH (Figure 2) by 50% (P = 0.0014) relative to high input management. Full-sun elevated CBH activity by 32% (P = 0.0247). Nevertheless, BG was similar between shade types. Shade trees can be an important input of OM (De Souza et al., 2012; Fontes, Barrios & Six, 2010; Gama-Rodrigues E., Gama Rodrigues A., & Nair, 2011; Manlay, Feller, & Swift, 2007), in turn fomenting greater microbial biomass, which is an important source of enzymes in soil (Vallejo et al., 2010; Udawatta, Kremer, Adamson & Anderson, 2008; Yadav et al., 2011). Therefore, greater potential enzyme activities in shade grown coffee systems would be expected (Asuming-Brempong et al., 2008; Chaer, Fernandes, Myrold, & Bottomley, 2009). Though hydrolytic enzyme activities tend to scale with soil OM (Min, Kang, & Lee, 2011; Sinsabaugh et al., 2008) a significant differences were observed in enzyme activities despite no differences in either labile POXC, that will be described later, or total soil OM, indicating that management may impact turnover rates of OM without necessarily altering concentrations of the measured stocks.

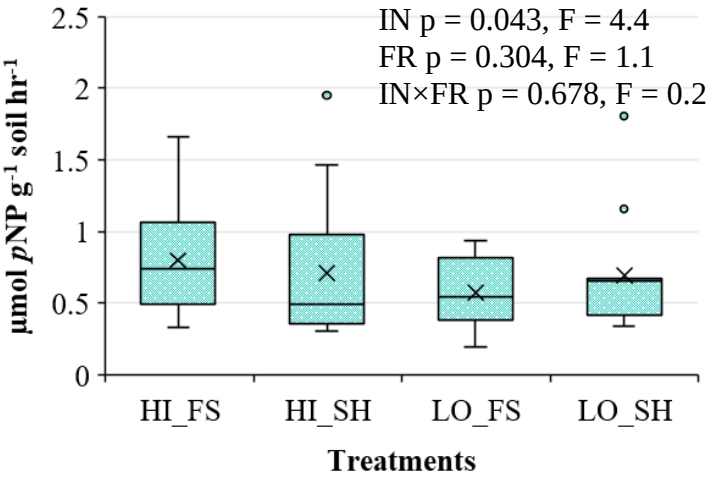

Figure 1. β-Glucosidase activity under different shade types, shade (SH) and full sun (FS), and fertilization practices, low input (LO) and high input (HI).

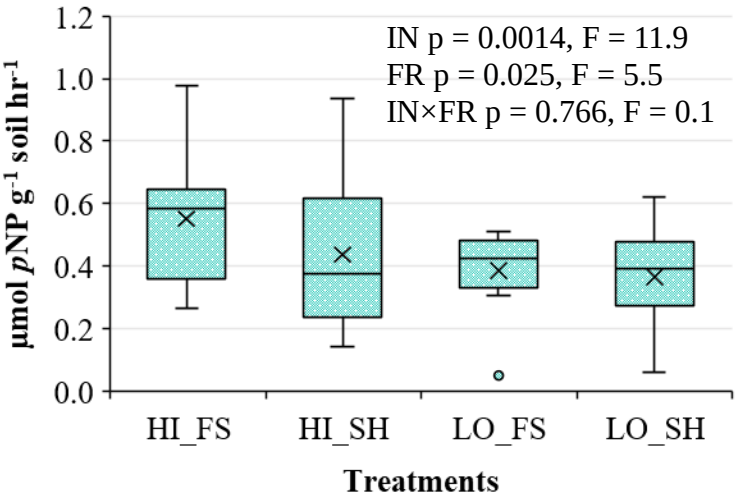

Figure 2. Cellobiohydrolase activity under different shade types, shade (SH) and full sun (FS), and fertilization practices, low input (LO) and high input (HI).

A metric of both C- and N-cycling, NAG activity (Figure 3) responded similarly as C- and N-cycling enzymes to fertilization and shade type. High input repressed the activity of NAG by 71% (P = 0.0372) relative to low input. However, shade and full sun responded similarly. In our study, the activity of NAG was repressed by high inputs, as in Yu et al. (2016), who reported that fungal biomass and NAG activity decreased due to high N fertilization.

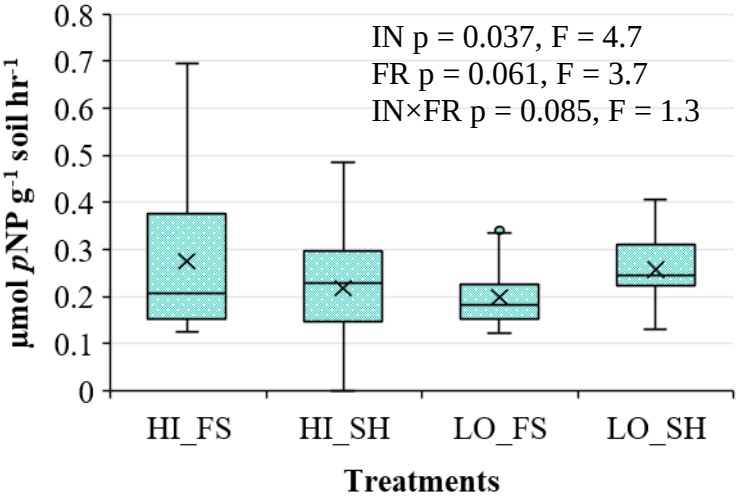

Figure 3. N-Acetyl-β-D-glucosaminidase activity under different shade types, shade (SH) and full sun (FS), and fertilization practices, low input (LO) and high input (HI).

While influenced by both fertilization and shade type, activities of N-cycling enzymes were more positively influenced by fertilization than shade type, except for LAP (Figure 4), which was similar across treatments. Aminopeptidase enzyme activities were higher under low input by 86% for GAP (P = 0.0016) (Figure 5) and 57% for AAP (P = 0.0029) (Figure 6) relative to high input. In contrast, shade type was reported to be similar between combinations. Lee & Jose (2003), Treseder et al. (2008), and Wang, Liu and Bai (2018), reported that soil microbial biomass declined following N fertilization. The metabolic capabilities of soil microbial communities can be reduced due to the direct inhibition in microbes after high N fertilizer additions (Zhang, Chen, & Ruan, 2018), which agrees with the lower enzymatic activity in soils following N synthetic fertilizer additions. Protease activity (Figure 7) was similar for low and high input N-fertilizer treatments (P = 0.69) and was marginally influenced by shade type (P = 0.075) tending to be higher under full-sun. No difference between combinations was detected. However, under high input fertilization, protease activity under full sun was 16-fold (0.201 µmol tyrosine g^{-1} h^{-1}) than shade (0.012 µmol tyrosine g-1 h-1). Protease activity and microbial biomass have been reported to be greater in soils with higher C:N ratios and limited the most by N (Geisseler & Horwath, 2008), due to the N requirement of microbes and the need to transform this mineral form into available forms.

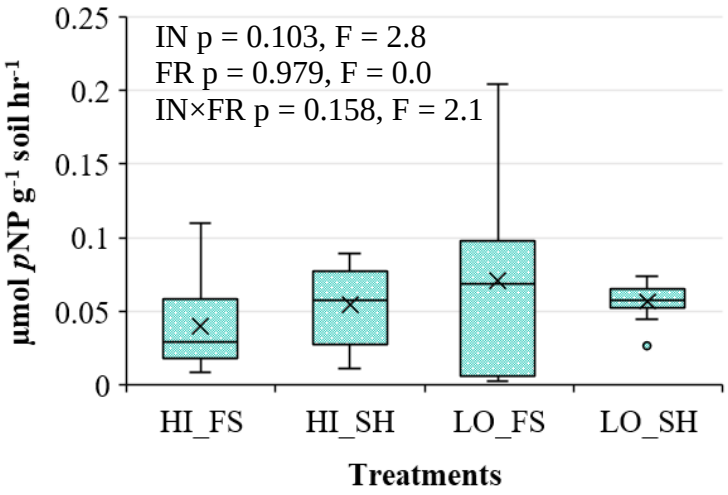

Figure 4. Leucine-aminopeptidase activity under different shade types, shade (SH) and full sun (FS), and fertilization practices, low input (LO) and high input (HI).

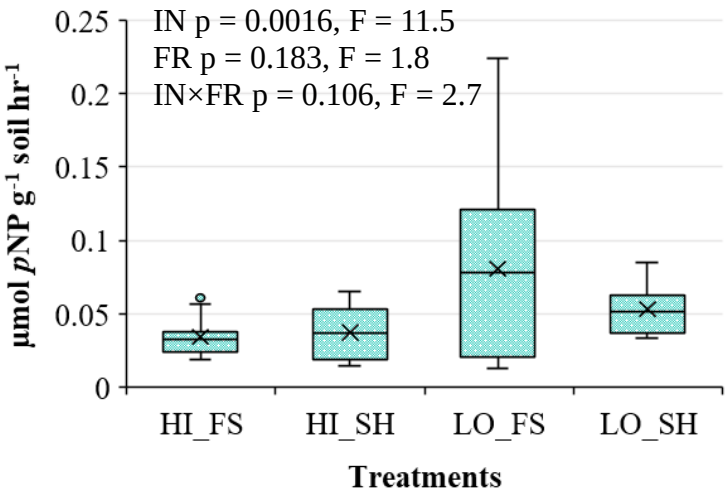

Figure 5. Glycine-aminopeptidase activity under different shade types, shade (SH) and full sun (FS), and fertilization practices, low input (LO) and high input (HI).

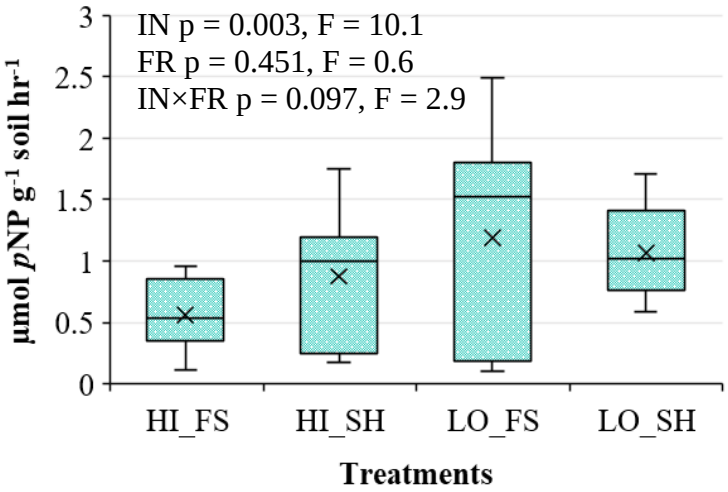

Figure 6. Alanine-aminopeptidase activity under different shade types, shade (SH) and full sun (FS), and fertilization practices, low input (LO) and high input (HI).

This study found no significant differences in LAP potential activity, consistent with the results of Sinsabaugh et al. (2008), who found that LAP was related with soil pH. However, in this study, pH was statistically similar between fertilization, shade type and treatments, suggesting that pH did not influence the activity of this enzyme. The lower activity of GAP relative to LAP identified here and elsewhere is thought to reflect lower dependence on organic sources of N from microbes (Allison & Jastrow, 2006) and sufficient available N stocks. In turn, low organic input entails OM additions that include organic N, when combined with no shade may have stimulated enzyme activities by reducing immediate N availability and stimulating the degradation of the nutrient forms required by microorganisms.

The stimulating effect of full sun on the activity of CBH (+ 32%) in this study is consistent with increased scavenging under lower conditions of lower C availability. Negative correlations between enzyme activity and nutrient availability have been previously observed because of the activity of enzymes being regulated by the supply of the specific nutrient that each enzyme mineralizes (Juma & Tabatabai 1978; Sinsabaugh, 1994). Chen, Li, Zhao, Xiao, & Wang (2018) reported that the activity of GAP decreases by 33 ± 18% under high N inputs, and that LAP remain similar across contrasting inputs, which agrees with the results of our study. High N additions and accumulation can repress N mineralizing enzyme activity in weathered soils in tropical forests (Olander & Vitousek, 2000). These authors evaluated the enzymatic activity of NAG in tropical systems, observing an increase in this activity in the more N limited soils and in contrast, NAG was more repressed in the long-term N enriched soils. Mori, Imai, Yokoyama and Kitayama (2018) described a similar tendency, in which microorganisms invested N into the production of P-acquisition enzymes to under P scarcity. It can be inferred then that either if C, P or N are limited, the less nutrient availability, the more enzymatic activity as compensation.

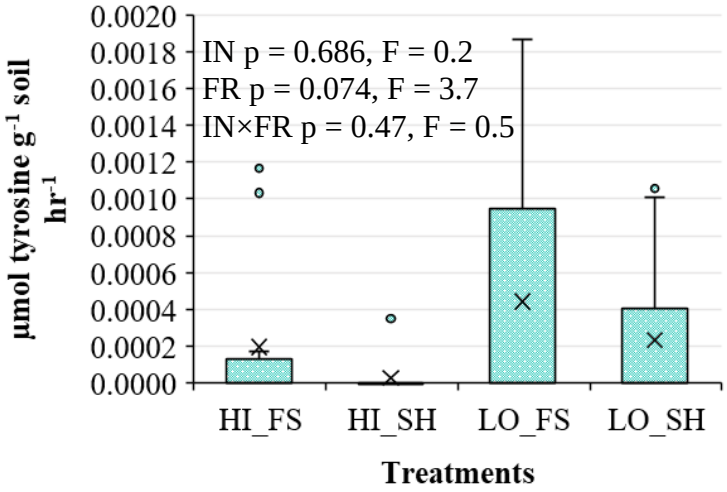

Figure 7. Protease activity under different shade types, shade (SH) and full sun (FS), and fertilization practices, low input (LO) and high input (HI).

All enzyme activities were positively correlated except for CBH, which was not associated with N-cycling enzymes of NAG (R = 0.27, P = 0.1971), LAP (R = 0.18, P = 0.3867) nor AAP (R = 0.37, P = 0.0723). The strongest associations occurred between the three aminopeptidases. Protease activity was strongly and positively associated with the C-cycling enzyme β-glucosidase (BG) (R = 0.51, P = 0.0098) and C- and N-cycling enzyme N-Acetyl-β-D-glucosaminidase (NAG) (R = 0.41, P = 0.0459) activities. However, protease activity was unrelated to aminopeptidase activities and inorganic N stocks (Table 3).

Table 3. Correlation coefficients (R-values) between surface soil (0–7.5 cm) of C and N cycling enzyme activities, potentially mineralizable N (PMN) and permanganate oxidizable carbon (POXC).

Pearson Correlation Coefficients												
	BG	**NAG**	**GAP**	**LAP**	**CBH**	**AAP**	**Protease**	**PMN**	**POXC**	**NH4-N**	**NO3-N**	**Leaf %N**	**Leaf N:P**
BG		0.80***	0.54**	0.59**	0.46*	0.48*	0.52**	-0.1	-0.21	0.59*	0.48*	0.09	0.18
NAG			0.55**	0.71***	0.27	0.45*	0.41*	-0.1	-0.19	0.62*	0.25	0.37	0.05
GAP				0.75***	0.54**	0.86***	0.09	-0.2	-0.17	0.29	0.01	0.17	0.16
LAP				0.19	0.83	0.06***	0.06	-0.3	-0.16	0.00	0.08	0.22	0.06
CBH						0.37	0.18	0.01	-0.08	0.53*	0.33	0.17	0.32
AAP							-0.11	-0.3	-0.14	0.03	0.09	0.09	0.11
Protease								0.60**	-0.15	0.27	0.19	0.09	0.22
PMN									-0.2	0.18	0.09	0.02	0.39
POXC										0.31	0.16	0.08	0.16
NH4-N											0.65**	0.13	0.3
NO3-N												0	0.03
Leaf %N													0.56
Leaf N:P													

Where *, **, *** indicate significance at $p<0.05$, 0.01, 0.001 level, respectively. Labels represent BG = β-Glucosidase, NAG = N-Acetyl- β -D-glucosaminidase, GAP = Glycine-aminopeptidase, LAP = Leucine-aminopeptidase, CBH = cellobiohydrolase, AAP = Alanine-aminopeptidase, PMN = Potentially mineralizable N, POXC = Permanganate oxidizable carbon.

Potentially Mineralizable Nitrogen and labile N stocks

Potentially mineralizable N (PMN) yielded the greatest values when full sun was combined with high input, also contrary to the hypothesis that shade and low input would favor mineralizable N and the soil enzymatic potential activity. In contrast, greater available N stocks were reported under low input.

Potentially mineralizable N (Figure 8) differed significantly by shade type (P = 0.003) and treatment (P = 0.011) but not by fertilization (P = 0.16). When combined with high inputs, full sun yielded the largest PMN (mean = 2.96 mg NH_4-N kg-soil^{-1} day^{-1}). Full sun increased PMN by 153% (P = 0.003) as well as variability (CV = 75.4%), relative to shade (CV = 52.7%). PMN was only positively associated to the activity of protease (R = 0.6, P = 0.003). The NH_4-N available to the plant (Figure 9) was 45% higher in low input compared to high input (P < 0.0001) and unaffected by shade type. The NO_3-N available in the soil (Figure 10) was similar across fertilization, shade type and treatment but nearly 3-fold more variable in shade (CV = 50%) relative to full sun (CV = 15%). Previous studies have shown that the amount of N mineralized is directly proportional to the quality and the size of available N stocks (Clark et al., 2019; Sierra, 1996; Wu, Ma, & Liang, 2008), making PMN sensitive to N inputs and a positive predictor of N mineralization in N-fertilized systems. Nitrogen mineralization is positively influenced by soil temperature and, to a point, with soil moisture (Clark et al., 2019; Kuzyakova & Stahr, 2003; Wu, Ma, & Liang, 2008). Nunan, Morgan, Scott and Herlihy (2000) reported that protease activity increased with temperature and was positively related to N mineralization in a similar climate as this study site (MAP 3,217 mm, MAT 24 °C). However, at our study site, soil moisture at the time of sampling was similar across treatments (mean = 62%), suggesting that shade type effects on PMN may not have been mediated by soil moisture.

Shade is often used in coffee systems to increase nutrient retention (Tully, Lawrence, & Wood 2013). Nitrogen inputs via pruning of leguminous trees can be an important source of N for coffee, especially in low-input systems (Daudin & Sierra, 2008). Snoeck, Zapata and Domenach (2000), reported that the percentage of N in coffee leaves increased when different species of leguminous trees were present (varying from 3% to 11% between species) and was directly proportional to the N_2-fixation capacity of each species. *Erythrina* spp is a legume N_2-fixing tree, but its N_2-fixing nodules can disappear for up to 10 weeks after pruning or during dry seasons, causing cessation of N fixation and competition for soil N with the coffee plant (Nair et al., 1999). Because of this, size of N stocks may have been reduced and hence the N_2-fixation by *Erythrina* spp. Higher coffee leaf N:P ratio reported by the present study without shade trees is consistent with shade tree competing for available N stocks.

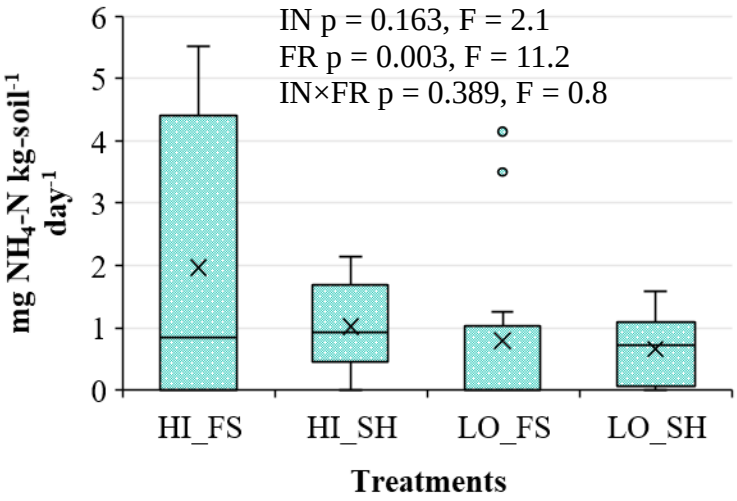

Figure 8. Potentially Mineralizable Nitrogen (PMN) under different shade types, shade (SH) and full sun (FS), and fertilization practices, low input (LO) and high input (HI).

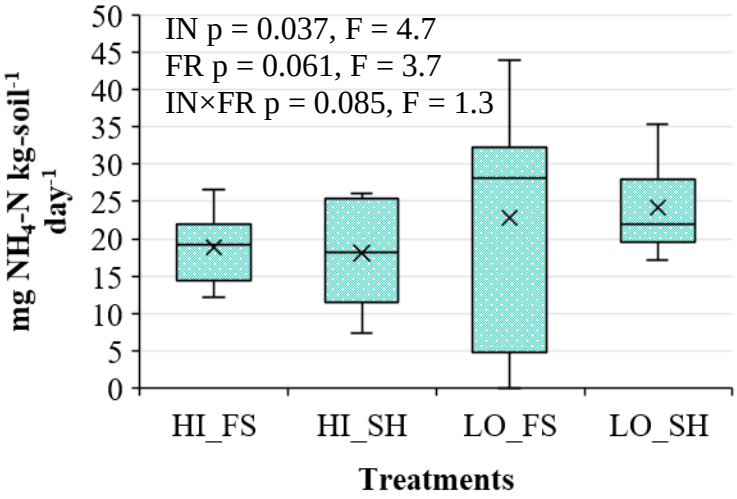

Figure 9. Available NH_4-N stocks under different shade types, shade (SH) and full sun (FS), and fertilization practices, low input (LO) and high input (HI).

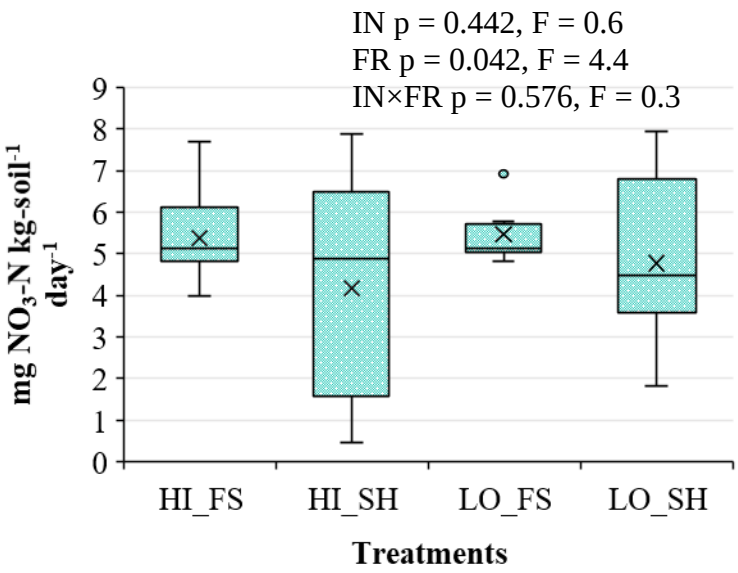

Figure 10. Available NO_3-N stocks under different shade types, shade (SH) and full sun (FS), and fertilization practices, low input (LO) and high input (HI).

Permanganate Oxidizable Carbon (POXC)

POXC was similar across fertilization and shade types (Figure 11). Under shade, high input yielded the greatest POXC (mean = 207.8 mg C kg^{-1} soil) while low input under full sun reported the lowest (mean = 156.8 mg C kg^{-1} soil). POXC was not reported to be significantly related with any enzyme activity. However, associations among soil labile C and corresponding C-hydrolytic enzymes have been identified previously in tropical agroecosystems. For example, in sub-humid tropical Kenya, Margenot et al. (2017), found strong and positive associations of POXC and C-cycling enzyme activities. In this study C-cycling enzyme activities and POXC greatest values occurred in the same fertilization treatment (low input) and were positively associated. Grandy et al. (2013) and Mahal et al. (2019), reported that total and labile C stocks, as well as POXC, were unaffected by differences in N fertilizer rates in annual cropping systems in the Midwestern USA, consistent with evidence that N fertilization impacts on soil C are indirectly mediated by biomass inputs and not priming.

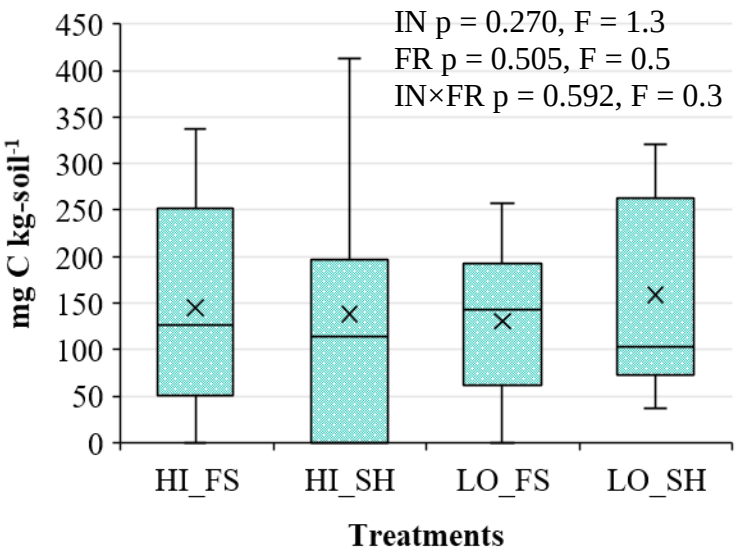

Figure 11. Permanganate oxidizable carbon (POXC) under different shade types, shade (SH) and full sun (FS), and fertilization practices, low input (LO) and high input (HI).

Coffee Leaf N and N:P ratio

Coffee leaf N content differed significantly by treatments, and more so by fertilization than shade type. Under shade, leaf N (Figure 12) was 6% higher relative to full sun. Leaf N was influenced by fertilization (P = 0.020) and not responsive to shade type (P = 0.078). High input increased leaf N by 9% relative to low input. Leaf N:P ratio (Figure 13) was highly sensitive to fertilization (P = 0.0008). Full sun increased N:P ratio by 14% relative to shade, while high input increased N:P by 45% relative to low input. Although inorganic N stocks and leaf N were not related in the present study, PMN was marginally and positively related to N:P ratio (R = 0.39, P = 0.0671) with high input full sun being the treatment with the greatest N:P ratio as well. Previous studies suggest that N:P ratio could be affected not just by N-fertilization, but by climatic conditions, latitude, and forest type (Delgado-Baquerizo et al., 2013). The N:P ratio within a given location becomes sensitive to non-location conditions and to fertility instead, increasing in response to climatic gradients such as a rise in temperature and precipitation (Chen, Li, & Yang, 2016) and N additions. Though PMN was influenced by shade type and was correlated with coffee N status (leaf N), shade type did not impact leaf N. This suggests that in this region and those with similar soil and climate conditions, the use of the leguminous shade tree *Erythrina* spp may not necessarily improve soil N availability.

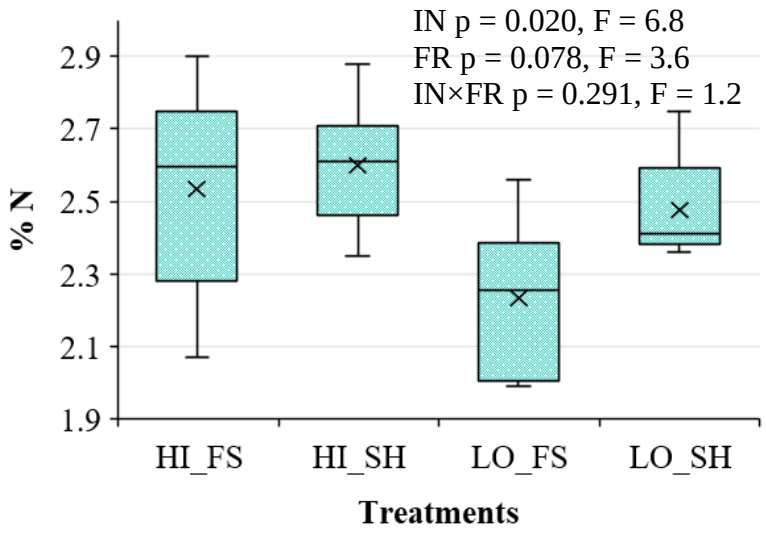

Figure 12. Coffee leaf nitrogen under different shade types, shade (SH) and full sun (FS), and fertilization practices, low input (LO) and high input (HI).

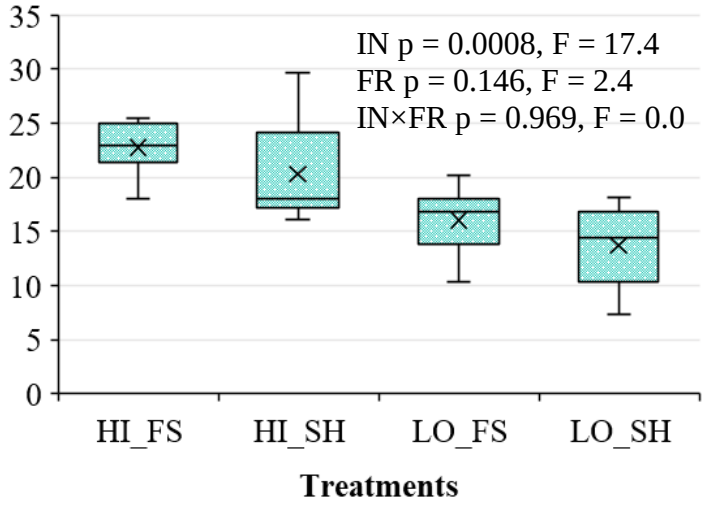

Figure 13. Coffee leaf N:P under different shade types, shade (SH) and full sun (FS), and fertilization practices, low input (LO) and high input (HI).

Fertilization and shade effect on N cycling

Contrary to the hypothesized, full sun enhanced N cycling effects by increasing the amount of mineralizable N and the activity of enzymes that catalyze its mineralization. In contrast, shade increased the size of available N stocks (NH_4-N and NO_3-N). As found by Beer, Muschler, Kass and Somarriba (1997) and Nair et al. (1999), excessive use of shade and seasonal prune-induced cessation of N- fixation by *Erythrina* spp may lead to competition for N with coffee. Potential cessation of N_2-fixation by *Erythrina* spp during some periods after pruning may explain why we observed higher available N stocks relative to shade at the study site. Results of the present study

were the opposite to those found for Arabica production systems assessed by Aranguren, Escalante and Herrera (1982), in which the use of shade management represented an important source of N and organic matter through litter deposition and N_2-fixation which were highly seasonally influenced, especially by rainfall. In the equatorial study site, however, precipitation is relatively consistent throughout the year and soil moisture was similar across treatments.

This study indicates that the assessed components of N cycling in this region of the Amazon and potentially the humid lowland tropics may not be responsive to fertilization and shade type in the same way as in seasonally cooler temperate regions. Though the relationship between PMN and protease activity is consistent with proteolysis as the initiating step of N mineralization and was more influenced by input levels, the ensuing step of aminopeptidase activity was more influenced by shade type. This may suggest that different managements impact different stages of the N mineralization pathway. This means mineralization could be more positively influenced by high inputs in the protein degradation step, though more positively influenced by low inputs and absence of shade in the aminopeptidase activity stage. Moreover, absence of relationships between enzyme activities and C and N stocks observed in temperate systems suggests decoupling of labile stocks with the activities of these catalysts of labile organic matter decomposition. This, in contrast to other legume tree-coffee associations with high correlation between C and N mineralization observed by Balota and Chaves (2010).

4. CONCLUSIONS

- Higher enzymatic activities and labile N and C stocks occurred without shade trees under full sun management, concurrent with greater leaf N uptake and leaf N:P. Regardless of shade types, the addition of high N inputs increased PMN, leaf N and leaf N:P.

- Whereas low rates of N fertilization and inorganic forms were associated with greater enzyme activities, inorganic N stocks, labile (POXC) and total OM were unaffected by low inputs. The positive correlation of soil protease and PMN was consistent with the hypothesized relationship of these N indicators in the proteolytic degradation pathway and the dominance of proteolysis in soil N mineralization. The fact that these were unrelated to activities of three aminopeptidases suggests decoupling of latter stages of this pathway.

- Higher N fertilization rates entailed greater soil PMN and translated to greater coffee N uptake (leaf N and N:P), but required lower enzymatic activity and inorganic N stocks, suggesting that this practice may not be the optimal for soil N cycling in this region.

- While benefits of *Erythrina* spp for coffee productivity and quality are well-documented in previous studies and offer additional ecosystem services such as erosion mitigation, in this study employing the leguminous shade tree *Erythrina* spp does not appear to offer appreciable benefits to soil N cycling or meeting Robusta N needs.

- In the studied Robusta systems of northeastern Ecuador, improved coffee N status as well as enhanced soil N cycling in these systems uptake appears best achieved using full sun (shade-free) management and higher N fertilization rates.

5. RECOMMENDATIONS

- The unexpected results in enzyme activity, PMN, POXC, and available N stocks suggest the need for additional research in tropical agroecosystems in order to understand better N and C cycling, in particular in high value tropical crop production systems such as coffee produced by resource-limited smallholders.

- To replicate this type of study in several tropical Robusta agroecosystems given different locations in order to contrast scenarios and determine if this factor is an important influence on N- and C- cycling.

- To perform additional research to contrast the use of *Erythrina* spp and other species of leguminous N_2-fixing trees that could be used for shade, to evaluate N_2-fixation rates and structural characteristics like nodule dynamics to determine if *Erythrina* spp may be actually representing competition for N.

- To perform more specific laboratory analyses that could explain with more detail the process of mineralization in its different degradation steps and explain the contrasting behaviors between the primary degradation steps, the cellulolytic and proteolytic steps, and the labile C- and N- stocks.

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