University of São Paulo
"Luiz de Queiroz" College of Agriculture

# Cover crops affects soil nitrogen fractions and maize responsiveness to nitrogen in the Cerrado region

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Thesis presented to obtain the degree of Doctor in Science. Area: Soil and Plant Nutrition.

Piracicaba
2023

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# Cover crops affects soil nitrogen fractions and maize responsiveness to nitrogen in the Cerrado region

versão revisada de acordo com a resolução CoPGr 6018 de 2011

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# DEDICATORY

To all post-graduate students
for being brave and resilient.

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# EPIGRAPH

*Blackbird singing in the dead of night*
*Take these broken wings and learn to fly*
*All your life*
*You were only waiting for this moment to arise*

*Blackbird singing in the dead of night*
*Take these sunken eyes and learn to see*
*All your life*
*You were only waiting for this moment to be free*

*Blackbird, fly*
*Blackbird, fly*
*Into the light of the dark black night*

*(…)*

*(Paul McCartney/John Lennon)*

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# RESUMO

## Plantas de cobertura afetam as frações de nitrogênio do solo e a responsividade do milho ao nitrogênio na região do Cerrado

O nitrogênio (N) é o nutriente mais requerido pela maioria das culturas e o mais consumido mundialmente. A dinâmica do N no solo envolve processos de perdas e transformações, como por exemplo, lixiviação e desnitrificação, respectivamente, que tornam o manejo da adubação desse nutriente mais complexo e oneroso. O uso racional de fertilizantes nitrogenados é uma das formas de minimizar impactos econômicos e ambientais decorrentes desses processos. A estimativa do potencial de mineralização do N do solo, ou o N prontamente mineralizável, pode ser empregada nos boletins de recomendação, para que seja contabilizada a contribuição dessa fração de N orgânico na nutrição das culturas, utilizando um método de determinação prático e simples. Dependendo do sistema de manejo, especialmente no sistema plantio direto, que tem como um dos pilares a rotação de culturas, a cultura principal apresentará resposta distinta ao N-fertilizante dependendo da cultura antecedente. Diante desse contexto, o estudo foi conduzido em experimento de longa duração no bioma Cerrado com o cultivo de milho na primeira safra (período chuvoso) e plantas de cobertura (leguminosas e não leguminosas) na entresafra, para avaliação de: i) índices químicos e bioquímicos associados à mineralização de N que se correlacionem com componentes de produção de milho, como a produtividade de grãos; (ii) o método (Illinois Soil Nitrogen Test - ISNT), como forma de quantificar o N prontamente mineralizável, com a proposta de modificar a concentração da extração alcalina do método original para condições de solos de clima tropical, visando melhorar a correlação com componentes de produção do milho; (iii) contribuição de sistemas de rotação com plantas de cobertura no aumento da produtividade do milho e o efeito desses sistemas no acúmulo de N nos compartimentos da planta de milho (folhas + colmos, palha + sabugo e grãos), além da recuperação do N-fertilizante e eficiência de uso do N por meio da técnica do isótopo estável $^{15}$N. O experimento de longa duração vem sendo conduzido em um Latossolo Vermelho distrófico em Planaltina/DF, desde 2010, em delineamento em blocos casualizados com parcelas subdivididas, em que as parcelas são representadas por nove culturas de cobertura e o pousio (testemunha) e as subparcelas são representadas pelos dois manejos de nitrogênio no milho (com e sem N em cobertura). Dentre os índices químicos e bioquímicos, destacaram-se ISNT na molaridade 0,5 M NaOH, além da atividade enzimática do solo, especificamente das enzimas arilsulfatase e β-glicosidase. Isso é um indicativo que estes métodos são sensíveis em quantificar a variação das frações de N do solo e a responsividade do milho ao N, demonstrando potencial para adoção em boletins de recomendação de N para a cultura do milho. Houve aumento da produtividade do milho rotacionado com plantas de cobertura em dois anos agricolas (2018/2019 e 2019/2020), porém o acúmulo de N nos compartimentos da planta de milho, a recuperação do $^{15}$N-fertilizante e a eficiência de uso de N não diferiram do controle (pousio). Os resultados mostram que os benefícios da introdução de plantas de cobertura em sistemas de rotação com milho não estão relacionados somente à ciclagem de N e outros nutrientes, mas também a serviços ecossistêmicos que permitem aumento da produtividade da cultura principal. Os resultados deste estudo indicam potencial de adotar métodos químicos e bioquímicos para estimar o N prontamente mineralizável e a resposta do milho à adubação nitrogenada.

**Palavras-chave:** Nitrogênio mineralizável; *Zea mays*; Recuperação do nitrogênio fertilizante; Eficiência de uso do nitrogênio fertilizante

# ABSTRACT

## Cover crops affects soil nitrogen fractions and maize responsiveness to nitrogen in the Cerrado region

Nitrogen (N) is the most required nutrient for the major crops and is globally consumed. The soil N dynamic involves losses and transformations, for example, leaching and denitrification, respectively, which makes the N fertilization management more complex and costly. The rational use of N fertilizers is one of the strategies to minimize economic and environmental impacts. The soil potentially mineralizable N estimation, or the readily mineralizable N estimation, can be used in N fertilizer recommendation to account the contribution of the organic N fraction in crop nutrition, adopting a pratical and simple method. Depending on the management, especially in no-tillage systems, which has crop rotation as one of its pillars, the main crop will present a different response to N-fertilizer depending on the previous crop. The study was carried out in a long-term experiment in Cerrado biome with maize as first-crop (rainy season) and leguminous and non-leguminous cover crops as second-crop (off-season). The evaluations performed were: i) chemical and biochemical indices associated with N mineralization that correlates with maize productions component such as grain yield; (ii) the Illinois Soil Nitrogen Test (ISNT) as a tool to quantify the readily mineralizable N, testing different concentrations of the alcaline extraction, aiming to improve the correlation with maize components production in tropical agroecosystems; (iii) contribution of rotation systems with cover crops on maize yield and the effect in N accumulation in maize compartments (stalk + straw, cob + husk, and grains), N fertilizer recovery and N use efficiency using the $^{15}N$ stable isotope technique. The long-term experiment is located in Planaltina-DF in a Typic Haplustox (clayey texture) since 2010, in a randomized block design with subdivided plots, in which the plots are represented by nine cover crops and fallow (control) and subplots are represented by N managements (with or without N topdressing). Soil enzymatic activity and ISNT 0.5 M NaOH were the indices that showed correlation with maize yield. This is an indication that the indices are sensitive to quantify the variation on soil organic N and the maize responsiveness to N, with potential to adopt in N fertilizer recommendation for the crop. There was an increase in maize grain yield in maize-cover crops rotation systems in two seasons (2018/2019 and 2019/2020), but the N accumulation in maize compartments, the $^{15}N$ fertilizer recovery and N use efficiency did not differ from the control (fallow). The results shows that the benefits of the introduction of cover crops in rotation with maize are not only related to N cycling and other nutrients, but different ecosystem services that allows an increase in crop yield. The results indicate the potential of adopting indices to estimate readily mineralizable N and the maize response to N fertilization.

**Keywords:** Mineralizable N; *Zea mays*; N fertilizer recovery; N fertilizer use efficiency

# 1 INTRODUCTION

Nitrogen (N) is required by plants in high amounts, especially by *Poaceae* species like the maize crop, but N requirement is not fully attended by mineralization of soil organic N. Despite the contribution of organic N in plant nutrition can exceeding 80% (Stevens et al., 2005; Wu et al., 2008; St. Luce et al., 2011), the application of N fertilizer is essential to supply N demand by maize, especially in early stages of development, therefore maximize yields. An alternative to improve plant nutrition is to combine the application of mineral fertilizers and crop rotation or sucession by using cover crops (Amado & Mielniczuk, 2000; Amado et al., 2002).

Agricultural practices such as cover crop rotation with main crop promotes the increase of soil carbon (C) and N and is an important strategy of soil management. Cover crop rotation has the potential to increase the cycling of N in the soil, providing long-term release of organic N compounds into the soil-system, theoretically reducing the demands for N fertilization over the long term. In the same direction, previous studies demonstrated that crop residues supply N in slower rates than N fertilizers and the mineralization of organic N fractions can meet crop N requirements (Cabezas & Couto, 2007; Osterholz et al., 2017). Consequently, the application of N fertilizer can be reduced under crop rotation systems, depending on the cover crop specie (Otto et al., 2016; Coombs et al., 2017; Marcillo & Miguez, 2017), reducing the unwanted effects of N fertilizers in the contamination of air and water bodies (Rosolem et al., 2017; Ghiberto et al., 2015).

The positive effect of crop rotation in maize crop yield in tropical environments is described by several studies (Burle et al., 1999; Rosolem et al., 2004; Maltas et al., 2009; Carvalho et al., 2015; Silva et al., 2020; Bettiol et al., 2022), showing increments ranging from 0.8 to 2.4 Mg ha$^{-1}$ comparing with fallow. These studies have observed enhances in N use efficiency, N uptake and N accumulation by maize plants in rotation systems with grasses and legumes and reported changes in N availability in the soil. The next step is to identify chemical or biochemical indexes that quantifies this increment in soil N availability, and that are potentially related to the crop response to N fertilization, allowing the fine tuning of the current N recommendation methods.

Despite the N recommendation in Brazilian Cerrado being based in grain yield expectations and soil organic matter content, including maize crop (Sousa & Lobato, 2004; Cantarella, 2007) the potentially mineralizable soil N, provided by labile organic fractions, must be considered to improve the efficiency of N fertilization (Mulvaney et al., 2001; Wang et al., 2001; Otto et al., 2013). The potentially mineralizable N refers to the amount of soil organic N that is available to plant absorption during the growing season (Ros et al., 2011).

Chemical tests have been developed to access potentially mineralizable N, for example the Illinois Soil Nitrogen Test (ISNT) (Khan et al., 2001), that determine hydrolysable amino sugar-N, a readily decomposable fraction of the organic-N. The results of many studies shows that this fraction is correlated to N-supply for crops by soil organic N mineralization (Sharifi et al., 2007; McDonald et al., 2014; Otto et al., 2013). However, some authors have shown high correlations between ISNT and soil organic matter content (Osterhaus et al., 2008; Mariano et al., 2017) and total soil N (Barker et al., 2006; Spargo et al., 2009; Mariano et al., 2017), suggesting that ISNT extracts a fraction of the soil organic matter or total soil N, not being able to predict a mineralizable fraction of the soil N. The original method for analyzing ISNT is to heat the sample for 5 h with a 2 M NaOH solution, quantifying the N released by titration. One hypothesis is that 2 M NaOH solution is too strong to identify a labile soil organic N fraction, especially for the highly weathered soils of the tropics.

There are other chemical or biochemical methods used to quantify labile fractions of soil organic N. Identifying methods able to estimate potentially mineralizable soil N and, most importantly, that shows correlation with maize responsiveness to N, could be a tool to improve N recommendation and consequently N use efficiency in agricultural systems. Methods that measure labile soil organic matter and soil enzymatic activity could also play a role to better understanding

N mineralization process. (Mengel et al., 2006; Cameron et al., 2013; McDonald et al., 2014; Bettiol et al., 2022).

Access the soil labile organic C can also improve the prediction of soil N supply and crop response (Culman et al., 2012). Allied to that, understanding the activity of soil enzymes is a priority to comprehend organic matter decomposition and nutrient transformation. In this sense, biochemical methods of soil analysis were currently launched in Brazil and showed correlation to crop grain yields (Mendes et al., 2019), being already available for routine soil analysis. Enzymatic activity, particularly β-glucosidase and arylsulfatase, is affected by soil management which, consequently, affects the C and N mineralization (Acosta-Martínez et al., 2008), being potentially correlated to the crop response to N fertilization.

In this thesis, we evaluated the effects of crop rotation with cover crops in maize response to N fertilization and, consequently, N use efficiency in a long-term experiment established in the Brazilian Cerrado. The hypothesis is that crop rotation improves soil N cycling and soil N availability affecting N fertilizer efficiency, reduce maize responsiveness to N fertilization in the long term, and can be potentially quantified by chemical or biochemical methods. A second hypothesis is that the original ISNT method might be modified to a less strong solution to better correlate to maize responsiveness to N under crop rotation systems. The objective was to improve our comprehension of soil N contribution under crop rotation systems, measuring the N use efficiency in this environment and to evaluate chemical and biochemical soil N tests that correlates to N response in the field to improve the current recommendation system of N management. The thesis was separated into two chapters with different approaches as follow:

- In Chapter 2 we correlated the soil chemical and biochemical analyses with maize response to N in different cover crop rotations, in nine years of maize cultivation, to access tests that can predict N mineralization for improve N recommendations, with emphasis in varying the concentration of NaOH of the original ISNT method.

- In Chapter 3 we used $^{15}N$ labeled urea to evaluate N recovery from fertilizer by maize after ten years of cultivation under fallow or cover crops, to understand the effect of grass or legumes cover crop cultivation on the fate of N fertilizer by maize crop.

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# 2 HOW DO COVER CROPS AFFECTS SOIL NITROGEN FRACTIONS AND MAIZE RESPONSIVENESS TO NITROGEN IN A LONG-TERM STUDY IN CERRADO

## Abstract

Brazil is one of the major producers of maize and cultivates this crop under three crop seasons along the year. An adequate management of nitrogen (N) fertilizers is required to maximize the production with limited impact to environment. There are strategies to improve N recommendation, such as the use of chemical or biochemical soil N tests to quantify potentially mineralizable N allowing a fine-tuning of N recommendation. In this study, we evaluated, at the nineth year of a long-term maize experiment in the Brazilian Cerrado, the responsiveness of maize to N fertilization in different rotation systems and tested biochemical and chemical methods to identify mineralizable soil N fractions. The field experiment was established in 2010 in a Typic Haplustox in Planaltina/DF and harvested annually for maize yield components measurements and cover crop biomass quantification. The experimental design consisted of maize cultivation following nine cover crops species (*Cajanus cajan*, *Canavalia brasiliensis*, *Crotalaria juncea*, *Mucuna aterrima*, *Pennisetum glaucum*, *Raphanus sativus*, *Sorghum bicolor*, *Triticum aestivum*, *Urochloa ruziziensis*), plus a control treatment (fallow), and subplots with or without N side dress application in the maize season (130 kg ha$^{-1}$ N). Soil samples were collected in the 0-20 cm soil layer in October/2018, after nine years of field establishment, and maize grain yield was quantified at harvest time. The soil chemical indices analyzed were total C, total N, mineral N, dissolved organic N (DON), permanganate oxidizable carbon (POXC) and Illinois Soil Nitrogen Test (ISNT) with four NaOH concentrations (0.25, 0.5, 1.0, and 2.0 M NaOH). The soil biochemical indices analyzed were the β-glucosidase and arylsulfatase activities. The maize yield over 9 years of experimentation was compared and showed differences of maize response to cover crop rotation and N fertilization. The chemical and biochemical indices were compared in a multivariate analysis (principal component analysis – PCA). Results shown the ISNT in the 0.5 M of NaOH concentration and soil enzymes were the variables that explain most the differences between treatments. The relative importance analysis show that β-glucosidase and ISNT at 0.5 M NaOH are the variables that mainly explained the response of maize to N fertilization. Our results shows that cover crop rotation can provide N in readily decomposable organic forms to the cash crop, while soil enzymes, especially β-glucosidase, and the adapted version of the ISNT method (0.5 M NaOH) showed potential to quantify maize responsiveness to N and be used in the future for fine-tuning of N recommendations.

Keywords: ISNT, Arylsulfatase, Beta-glucosidase, Soil enzymes

## 2.1. Introduction

Brazil consolidated the position of the world's third largest producer of maize, with 116 million Mg in 2021/2022 crop-year, divided in two main seasons of cultivation, the first crop planted between October and December and the second crop (knows as "safrinha") planted between January and March. Second season maize is usually cultivated after soybean growth in the summer period and is already the preferential system of maize cultivation in Brazil with the largest area than the first crop. The production of maize expanded faster in the last decades (Allen & Valdes, 2016; USDA, 2021), and is associated to interactions between environment (climate and soil) and management, with highlight for plant nutrition (Lobell et al., 2009).

To achieve high maize yields is mandatory to apply N because of the crop high demand. The estimative is that 100 Tg of N from Haber-Bosch process is produced per year and 16% of this amount is applied in maize crop (Galloway et al., 2004; Fowler et al., 2013; Ladha et al., 2016). However, the losses of anthropogenic N to atmosphere, transport to water bodies or percolation to groundwater can represent more than 70% of the total N applied depending on factors such as soil type (Schlesinger, 2009).

Besides the environmental issues, the cost of N fertilizers is a global concern and needs to be considered in crop management focusing on rational use. Global events like first oil crisis, the 2008 market boom and crash, the Coronavirus disease pandemic and Russia-Ukraine war causes uncertainties and volatility of fertilizer market (Eisa et al., 2022). In addition, it is important to consider that N recovery is still limited under cereal crops cultivation. Data accessed from more than 800 studies shown that on average, only 51% of the N fertilizer is recovery by the plant (Cassman et al., 2002; Dobermann & Cassman, 2002; Ladha et al., 2005).

One of the strategies to increase N use efficiency by cash crops is to improve the N recommendation by considering the estimative of soil N mineralization potential in different management systems and climate conditions (Silva & Souza, 2020). The main objective of an adequate N recommendation system is one that accurately estimate the crop demand considering the N provided by soil and the plant N requirement (Morris et al., 2018). The supply of N to plants from soil organic matter decomposition is often underestimated in agroecosystems (Griffin, 2015). The intensification of agriculture to attend the increasing food demand by a growing world population involve the maximization of crop productivity and need to account for the contribution of cover crops in rotation systems (Carciochi et al., 2021).

Several chemical and biological methods have potential to estimate soil mineralizable N, a fraction of soil organic N that can be taken up by plants during the growing season. However, many of these methods are time-consuming and complex to apply in laboratory routine soil analysis (Martínez et al., 2018). Among the chemical methods that determine soil mineralizable N fractions, the Illinois Soil Nitrogen Test (ISNT) gained attention in the previous years, because of its potential to quantify a readily mineralizable soil organic N fraction, the amino sugar N, that was correlated to crops responsiveness to N (Mulvaney et al., 2006). The ISNT was intensively tested under temperate conditions presenting a mix of positive (Sharifi et al., 2007; McDonald et al., 2014; Otto et al., 2013) or negative results (Barker et al., 2006; Osterhaus et al., 2008; Spargo et al., 2009; Mariano et al., 2017). The main criticism is the fact that ISNT was positively correlated to total soil N content, not being able to quantify a mineralizable fraction of soil organic N by some authors (Barker et al., 2006; Spargo et al., 2009; Mariano et al., 2017). This method was tested for tropical soils under sugarcane cultivation. In the first study, Otto et al. (2013) found a positive correlation between ISNT content and sugarcane response to N fertilization. In the study of Mariano et al. (2017), none of the chemical methods evaluated (including ISNT) were able to identify a readily plant available N fraction and correlation to sugarcane N responsiveness. More recently, Otto et al. (2019) demonstrated that ISNT was able to quantify the soil N supplying power, and adequately identified non-responsive sites to N fertilization. The unsuccess of the ISNT method, as well as its correlation to total soil N content, can be associated to the strong alkaline hydrolysis promoted by the original 2 M NaOH solution. This is an indicator that a weaker extraction might provide more positive results, improving the current ISNT method, especially for the low-soil organic content of the highly weathered soils from the tropics.

Recently, in Brazil, Mendes et al. (2019) launched a method for biochemical soil analysis that was related to crop yields and soil management history. This method was based on the estimation of the activity of two soil enzymes arylsulfatase, and β-glucosidase, associated respectively to the sulfur (S) cycle and soil C decomposition. The relationship of enzymatic activity and N responsiveness to N fertilization was not evaluated so far under tropical conditions. Exploring the potential of biological methods in estimating a soil mineralizable N fraction is consistent with previous evidence that soil enzymes activity and soil labile organic C and N fraction can be correlated to labile N fractions and crop responses to N (Tian et al., 2017; Martínez et al., 2018).

The hypotheses tested in this study were: (i) the maize response to N vary in rotation systems with cover crops; (ii) chemical and biochemical methods present correlation with readily mineralizable N and maize yield response to N; (iii) reducing the concentration of NaOH in the original ISNT method is required to increase the accuracy to identify maize responsiveness to N in tropical agroecosystems. To evaluate those hypotheses, we evaluated maize grain yield and soil samples collected in a long-term field experiment in Cerrado biome, to assess the effect of cover crops cultivation (nine crop species plus a control – fallow) in maize grain yield in the presence and absence of side dress N fertilization in first-season maize.

## 2.2. Material and methods

### 2.2.1. Site description and experimental design

Soil samples were collected in 2018 in a long-term experiment established in 2010 at the experimental area of Brazilian Agricultural Research Corporation (EMBRAPA Cerrados), located in Planaltina, Federal District (15° 35’ 30” S, 47° 42’ 00” W and 990 m asl), in the central western region of Brazil (Figure 1). According to Köppen-Geiger’s classification, the climate is classified as Aw (tropical savannah), with dry period (winter) and rainy period (summer) (Beck et al., 2018).

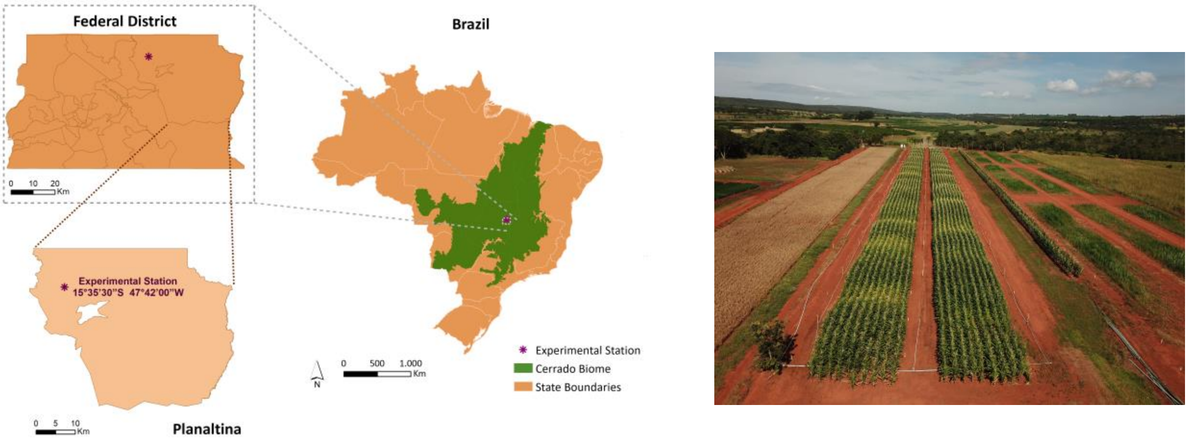

**Figure 1.** Location of experimental station (left) and aerial image of the area (right) taken in 2019.

The soil was classified as a Typic Haplustox (clayey texture) according to Soil Taxonomy (Soil Survey Manual, 2017). Analysis performed in 2018 identified the soil physicochemical characteristics of the area as follows (0-20 cm soil layer): 513 g kg$^{-1}$ clay, 186 g kg$^{-1}$ silt, 301 g kg$^{-1}$ sand, pH $_{(H2O)}$ = 5.9, SOM = 26.3 g kg$^{-1}$, exchangeable $Al^{3+}$ = 0.7 mmol$_c$ dm$^{-3}$, $Ca^{2+}$+$Mg^{2+}$ = 36 mmol$_c$ dm$^{-3}$, $K^+$ = 2 mmol$_c$ dm$^{-3}$, and $P_{Mehlich}$ = 8.3 mg dm$^{-3}$.

The experimental design was randomized complete block arranged in split-plots with three replications. The plots (principal treatments) were established in 2010, with maize-cover crop succession with 9 cover species (*Cajanus cajan*, *Canavalia brasiliensis*, *Crotalaria juncea*, *Mucuna aterrima*, *Pennisetum glaucum*, *Raphanus sativus*, *Sorghum bicolor*, *Triticum aestivum*, and *Urochloa ruziziensis*) and fallow (spontaneous vegetation emergence). The subplots (secondary treatments) were with two N management strategies: low N (20 kg ha$^{-1}$ N at planting), and high N (20 kg ha$^{-1}$ N at planting plus 130 kg ha$^{-1}$ N at side dress application, totaling 150 kg ha$^{-1}$ N). N fertilization at planting was applied as monoammonium phosphate, while N at side dress was supplied as urea applied over the soil surface, in two application of 65 kg ha$^{-1}$ N each (one in V4 stage and the other in V8 stage). The plots measured 12 x 8 m and the subplots, 6 x 8 m.

### 2.2.2. Field management

The cover crops were sown in a no-till system in April 2018 (beginning of the dry season). To avoid issues in cover crops establishment and development, an irrigation blade was applied to simulate rainy season (period comprehend between February and March). The plant density was 20 plants $m^{-1}$ for *Cajanus cajan*, *Crotalaria juncea*, *Sorghum bicolor*, *Triticum aestivum* and *Urochloa ruziziensis*; 40 plants $m^{-1}$ for *Pennisetum glaucum* and *Raphanus sativus*; and 10 plants $m^{-1}$ for *Cajanus cajan* and *Mucuna aterrima*. The spacing between plant rows was 0.5 m for all plant species (Amabile & Carvalho, 2006).

No-till maize was sown at the beginning of the rainy season (October 2018). At planting, 20 kg $ha^{-1}$ of N, 150 kg $ha^{-1}$ of $P_2O_5$, 80 kg $ha^{-1}$ of $K_2O$, 2 kg $ha^{-1}$ of Zn ($ZnSO_4.7H_2O$) and 10 kg $ha^{-1}$ FTE BR 12 as the micronutrient source (3.2 % S, 1.8% B, 0.8% Cu, 2.0% Mn, 0.1% Mo and, 9.0% Zn) were applied to all treatments.

### 2.2.3. Soil sampling

Before the maize sowing, in October 2018, soil samples were collected at 0-0.2 m depth, except the samples collected for biochemical analyses, sampled at 0-0.1 m depth as suggested by Lopes et al. (2015). Five samples were taken in row and five samples in inter-row positions in each subplot with low N application and mixed to obtain the composite sample. Part of the samples was maintained at freezing temperatures until ammonium and nitrate determination and another part were air dried and sieved in 10-mesh (2 mm) sieve for the other chemical analysis. The soil samples taken to determine the enzymes activity (β-glucosidase and arylsulfatase), previously maintained at 4° C were dried and sieved according proposed by Lopes et al. (2015).

### 2.2.4. Soil biochemical analyses

The activity of soil enzymes, β-glucosidase and arylsulfatase were determined according to Tabatabai (1994), a colorimetry method of p-nitrofenol determination released after soil incubation at 37°C for 1h in a specific substrate (buffered solution of p-nitrophenyl-β-D-glucopyranoside for β-glucosidase and buffered solution of p-nitrofenil sulphate for arylsulfatase) (Supplementary Figure 1).

### 2.2.5. Soil chemical analyses

The soil N and C total concentration were determined in a LECO CHN elemental analyzer (LECO Corporation, Michigan, USA). The soil samples (sieved in a 10-mesh (2 mm) sieve) were milled to pass a 100-mesh (0.149 mm) sieve to perform this analysis.

The soil ammonium ($N\text{-}NH_4^+$) and nitrite and nitrate ($N\text{-}NO_2^-$ and $N\text{-}NO_3^-$) contents were determined by colorimetric method according Mulvaney (1996) and Miranda et al. (2001), respectively, and each sample was analyzed in triplicate. The extract obtained was placed in a microplate with 96 cells and absorbance reading were performed in an absorbance microplate reader Sunrise™ (Tecan Group Ltd., Männedorf, Switzerland) (Supplementary Figure 2). To obtained N mineral, all data were counted as ($[N\text{-}NH_4^+] + [N\text{-}NO_2^-] + [N\text{-}NO_3^-]$).

The ammonium determination occurs in three steps as follows: (1) $NH_3$ reaction with hypoclorite to form monochloramine ($NH_2Cl$); (2) $NH_2Cl$ reacts with salicylate to form benzoquinone monoimine; (3) Benzoquinone monoimine and salycilate couples and forms emerald green color (absorbance measured at 667 nm).

The nitrite and nitrate determination consists in addition of Griess reagents and occurs as follows: (1) vanadium (III) chloride ($VCl_3$) reduces $N\text{-}NO_3^-$ to $N\text{-}NO_2^-$; (2) $N\text{-}NO_2^-$ reacts with sulfanilamide in acid solution; (3) the product reacts with N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD – $C_{10}H_{14}N_2$ 2 HCl) forming intense red color (absorbance measured at 540 nm).

The soil dissolved organic N (DON) was determined according to Cabrera & Beare (1993). Briefly, the soil DON in the samples were oxidized to $N\text{-}NO_3^-$ and then reading were performed in an absorbance microplate reader Sunrise™ (Tecan Group Ltd., Männedorf, Switzerland). To calculate DON, the N-$NH_4^+$ and N-$NO_3^-$ concentration before the oxidation was discounted of the N-$NO_3^-$ concentration after the oxidation process.

The determination of permanganate oxidizable carbon (POXC) was performed as described by Weil et al. (2003) and Culman et al. (2012). The active soil C was oxidized by a potassium permanganate solution ($KMnO_4$) and then the samples were placed in an UV-spectrophometer (BEL Engineering®, Monza, Italy) and the absorbance measured at 550 nm (Supplementary Figure 3).

The Illinois Soil Nitrogen Test (ISNT) was determined according to (Khan et al., 2001), in triplicate. Briefly, the dry soil samples (< 2 mm) were placed in a Mason-jar with an adaptation in the lead to support 5 ml of a 4% boric acid solution. The samples were heated at 53-54ºC for a 5h period in an adapted grill, and the mineralizable N was oxidized by the 2 M NaOH solution. In this study, the original method was modified testing other three NaOH concentrations (0.25; 0.5; and 1 M NaOH) (Supplementary Figure 4).

### 2.2.6. Plant analyses

At harvesting time, four rows with four meters each were sampled in subplots (low and high N). The grains sampled were weighted and then oven-dried at 65ºC to determine grain moisture. Maize grain yield was determined after correct grain moisture content to 13% (w.b.) and the results were expressed in Mg $ha^{-1}$. The Δ maize yield was expressed as the difference between the maize grain yield in subplots with high application of N and low application of N. The N responsiveness (NR) was obtained as:

NR = [(Yield in sublot with high N – Yield in subplot with low N)/ Yield in subplot with low N] x 100

In addition to the data obtained in the 2019 season (current study), we also analyzed the date obtained in the previous 8 years, totaling 9 seasons (2010-2019), to improve data quality and to consider the long-term effect of crop cultivation in maize responsiveness to N.

### 2.2.7. Statistical analyses

The analysis of variance (ANOVA) was performed on maize data average in the long term (2010-2019) to reveal the differences between treatments response to N side dress application. Means were compared with Tukey test at 5% of probability. The N responsiveness data was transformed to log (x+1).

Before the principal components analysis (PCA), measures of sampling adequacy were performed using the Kaiser-Meyer-Olkin (KMO) and Bartlett's sphericity test ($p<0.05$). KMO values greater than 0.5 to 1.0 are considered acceptable for PCA application (Hair, 2011). Bartlett's sphericity test checks the independency of variables tested. PCA was performed to group the dataset into new variables that resume the information in principal components (PC). The analysis also allows avoid the multicollinearity between the original variables. The PC has the objective to explain most of the variation in original variables.

The relative importance of chemical and biochemical methods tested over maize yield was carried out using *lmg* method from *relaimpo* package in R software ($R^2$ decomposition by averaging orders). All statistical analyses were performed in R (R Development Core Team, 2022).

## 2.3. Results

### 2.3.1. Maize response to crop rotation and N side dress application

Maize yield response to cover crop rotation and side dress application of N (high N and low N, respectively), as well as the difference between yield in subplots with low or high N (Δ maize yield) and N responsiveness (%) are described on Table 1.

**Table 1.** Maize yield (Mg ha$^{-1}$), Δ maize yield (high N – low N) (t ha$^{-1}$) and N responsiveness (%) in a maize-cover crop rotation experiment conducted for 9 years. Planaltina, DF. Means represent the average of 9 crop-seasons.

| Cover crops | Maize yield (Mg ha$^{-1}$) | | Δ maize yield (High N - Low N) (t ha$^{-1}$) | N responsiveness (%) |
|---|---|---|---|---|
| | Low N | High N | | |
| *Crotalaria juncea* | 8.0abB | 9.8aA | 1.8bc | 21.3cd |
| *Canavalia brasiliensis* | 8.6aB | 10.0aA | 1.5c | 18.2d |
| *Cajanus cajan* | 8.2abB | 9.8aA | 1.9bc | 26.0bcd |
| *Pennisetum glaucum* | 8.1abB | 9.8aA | 2.6ab | 40.9ab |
| *Mucuna aterrima* | 7.3bcdB | 9.9aA | 1,8bc | 23.9bcd |
| *Raphanus sativus* | 7.8abcB | 9.9aA | 2.2abc | 32.8bcd |
| *Sorghum bicolor* | 7.0cdB | 9.3aA | 2.4abc | 37.4abc |
| *Triticum aestivum* | 7.5bcdB | 9.4aA | 2.0bc | 27.9bcd |
| *Urochloa ruziziensis* | 6.8dB | 9.8aA | 3.0a | 53.7a |
| Fallow | 7.5bcdB | 9.6aA | 2.2abc | 30.4bcd |
| Mean | 7.7 | 9.7 | 2.1 | 31.3 |
| p-value | 0.0157[1]<br>$p<0.0001$[2] | | $p<0.001$ | $p<0.001$ |
| CV (%) | 4.4[1]<br>2.9[2] | | 15.2 | 5.9 |

Means not followed by the same lowercase letter in the columns and uppercase letter in the lines differ by Tukey's test ($p>0.05$). [1] cover crops; [2] N management.

The maize-cover crop rotation with high N differs of maize-cover crop rotation with low N for all cover species. In treatments with high N, the maize yield does not differ between cover crop rotation systems, whereas in treatments with low N, it was observed differences in maize yield response according to cover crop species. Lowest maize yield in the treatment with low N was obtained for *Urochloa ruziziensis* (6.8 Mg ha$^{-1}$). The yield increase, expressed by Δ maize yield, differed between cover crop rotation systems. The treatments with legume cover crop in rotation with maize had smaller responses to N application (lower responsiveness), with highlight to *Canavalia brasiliensis*. As expected, in treatments with grass cover crops in rotation, N responsiveness was increased. Highest N responsiveness was obtained for *Urochloa ruziziensis* (53.7%), followed by *Pennisetum glaucum* (40.9%) and *Sorghum bicolor* (37.4%) (Table 1).

### 2.3.2. Soil chemical and biochemical analyses correlation with mineralizable N

All chemical and biochemical analyses data are described on Table 2. The treatments do not differ in analyses performed, except mineral N and enzymatic activity (arylsulfatase and β-glucosidase) in a statistical univariate approach (variance analysis). The cover crops that provide higher amounts of mineral N were *Canavalia brasiliensis* and *Urochloa ruziziensis*. The same behavior was observed for enzymatic activity. Despite the reduction in ISNT content levels with the reduction in the NaOH concentration, as well as the lowest ISNT contents for fallow treatment, mean values of ISNT content did not differ between cover crop species (Table 2).

**Table 2.** Chemical and biochemical analyses in a maize-cover crop rotation experiment conducted for 9 years. Samples collected in the 0-20 cm soil layer in October 2018, before maize sowing. Planaltina, DF.

| Cover crops | Total N | Total C | Mineral N | DON | Arylsulfatase | β-glucosidase |
|---|---|---|---|---|---|---|
| | g kg$^{-1}$ | | mg kg$^{-1}$ | | µg p-nitrofenol g$^{-1}$ soil h$^{-1}$ | |
| *Cajanus cajan* | 1.51 | 21.17 | 22.15d | 24.25 | 66.15ab | 138.22abc |
| *Canavalia brasiliensis* | 1.50 | 21.38 | 36.29a | 22.87 | 80.94ab | 184.63a |
| *Crotalaria juncea* | 1.52 | 21.78 | 28.88bc | 23.92 | 62.66ab | 144.48abc |
| *Pennisetum glaucum* | 1.50 | 21.78 | 23.74cd | 23.37 | 80.12ab | 169.22ab |
| *Mucuna aterrima* | 1.48 | 20.89 | 24.73cd | 22.33 | 59.27ab | 136.39abc |
| *Raphanus sativus* | 1.51 | 20.98 | 23.16cd | 22.50 | 74.99ab | 154.15abc |
| *Sorghum bicolor* | 1.55 | 21.95 | 27.92bcd | 22.01 | 90.96a | 165.50abc |
| *Triticum aestivum* | 1.49 | 21.14 | 21.99d | 23.20 | 50.60b | 126.81bc |
| *Urochloa ruziziensis* | 1.51 | 21.56 | 31.76ab | 24.48 | 87.70a | 165.92abc |
| Fallow | 1.56 | 21.68 | 23.37cd | 21.70 | 80.44ab | 113.92c |
| Mean | 1.51 | 21.43 | 26.40 | 23.06 | 73.38 | 149.91 |
| p-value | 0.98 $^{ns}$ | 0.98 $^{ns}$ | $p < 0.0001$ | 0.20 $^{ns}$ | 0.01 | $p < 0.01$ |
| CV (%) | 5.61 | 5.86 | 7.90 | 5.70 | 16.63 | 11.91 |

Means not followed by the same letter in the columns differ by Tukey's test ($p>0.05$).Total C: Soil total carbon; Total N: Soil total nitrogen; Mineral N: $[N\text{-}NH_4^+]+[N\text{-}NO_2^-]+[N\text{-}NO_3^-]$; ISNT 2M: Illinois Soil Nitrogen Test NaOH 2M; ISNT 1M: Illinois Soil Nitrogen Test NaOH 1M; ISNT 0.5M: Illinois Soil Nitrogen Test NaOH 0.5 M; ISNT 0.25M: Illinois Soil Nitrogen Test NaOH 0.25M.

**Cont. Table 2.** Chemical and biochemical analyses in a maize-cover crop rotation experiment conducted for 9 years. Samples collected in the 0-20 cm soil layer in October 2018, before maize sowing. Planaltina, DF.

| Cover crops | POXC | ISNT 2M | ISNT 1M | ISNT 0.5M | ISNT 0.25M |
|---|---|---|---|---|---|
| | mg kg$^{-1}$ | | | | |
| *Cajanus cajan* | 401.17 | 252.45 | 202.89 | 159.20 | 143.48 |
| *Canavalia brasiliensis* | 467.44 | 236.36 | 204.38 | 167.69 | 134.16 |
| *Crotalaria juncea* | 451.93 | 244.13 | 190.00 | 160.88 | 132.50 |
| *Pennisetum glaucum* | 448.55 | 246.24 | 196.05 | 171.09 | 143.05 |
| *Mucuna aterrima* | 439.01 | 236.53 | 200.09 | 158.95 | 135.90 |
| *Raphanus sativus* | 422.61 | 272.28 | 228.45 | 187.23 | 133.06 |
| *Sorghum bicolor* | 419.97 | 261.90 | 212.87 | 162.50 | 138.61 |
| *Triticum aestivum* | 442.54 | 242.99 | 198.85 | 174.36 | 144.29 |
| *Urochloa ruziziensis* | 416.35 | 240.38 | 205.64 | 162.95 | 135.38 |
| Fallow | 466.99 | 205.31 | 161.05 | 131.24 | 120.20 |
| Mean | 437.66 | 243.86 | 200.03 | 163.61 | 136.06 |
| p-value | 0.53 $^{ns}$ | 0.64 $^{ns}$ | 0.33 $^{ns}$ | 0.34 $^{ns}$ | 0.94 $^{ns}$ |
| CV (%) | 9.16 | 14.32 | 13.34 | 13.72 | 15.06 |

Means not followed by the same letter in the columns differ by Tukey's test ($p>0.05$). Total C: Soil total carbon; Total N: Soil total nitrogen; Mineral N: [N-$NH_4^+$]+[N-$NO_2^-$]+[N-$NO_3^-$];ISNT 2M: Illinois Soil Nitrogen Test NaOH 2M; ISNT 1M: Illinois Soil Nitrogen Test NaOH 1M; ISNT 0.5M: Illinois Soil Nitrogen Test NaOH 0.5 M; ISNT 0.25M: Illinois Soil Nitrogen Test NaOH 0.25M.

The Pearson's correlation between chemical and biochemical analyses are described in Table 3. Dissolved organic N (DON) and permanganate oxidizable carbon (POXC) were not suitable for factor analysis. The KMO indices were 0.20 and 0.11 respectively.

**Table 3.** Pearson's correlation matrix of soil chemical and biochemical analyses with mineralizable N.

| Chemical and biochemical analyses | Total N | Total C | Mineral N | Arylsulfatase | β-glucosidase | ISNT 2M | ISNT 1M | ISNT 0.5M | ISNT 0.25M |
|---|---|---|---|---|---|---|---|---|---|
| Total N | 1 | | | | | | | | |
| Total C | 0.81*** | 1 | | | | | | | |
| Mineral N | 0.052 | 0.026 | 1 | | | | | | |
| Arylsulfatase | 0.11 | 0.14 | 0.46* | 1 | | | | | |
| β-glucosidase | -0.077 | 0.013 | 0.55** | 0.47* | 1 | | | | |
| ISNT 2M | 0.08 | 0.026 | -0.22 | -0.29 | 0.17 | 1 | | | |
| ISNT 1M | -0.13 | -0.21 | -0.07 | -0.15 | 0.27 | 0.83*** | 1 | | |
| ISNT 0.5M | -0.26 | -0.30 | -0.096 | -0.28 | 0.26 | 0.78*** | 0.85*** | 1 | |
| ISNT 0.25M | 0.10 | -0.17 | -0.13 | -0.33 | 0.17 | 0.69*** | 0.62*** | 0.55** | 1 |

*** P values less than 0.001; ** P values less than 0.01; *P values less than 0.05. Total C: Soil total carbon; Total N: Soil total nitrogen; Mineral N: [N-$NH_4^+$]+[N-$NO_2^-$]+[N-$NO_3^-$]; Aril: Arylsulfatase; Beta: β-glucosidase; ISNT 2M: Illinois Soil Nitrogen Test NaOH 2M; ISNT 1M: Illinois Soil Nitrogen Test NaOH 1M; ISNT 0.5M: Illinois Soil Nitrogen Test NaOH 0.5 M; ISNT 0.25M: Illinois Soil Nitrogen Test NaOH 0.25M.

The total N and total C had a statistically significant positive correlation ($r=0.81$, $p<0.001$). Mineral N had positive correlation between soil enzymes, arylsulfatase ($r=0.46$, $p<0.05$) and β-glucosidase ($r=0.55$, $p<0.01$). Soil enzymes also present positive correlation between each other ($r=0.47$, $p<0.05$). However, soil enzymes had no correlation between total N, total C and ISNT in none of the four-molarities tested.

The ISNT determined using four molarities of NaOH presented correlation between each other, with higher correlation between the test performed with 2 M and 1 M ($r=0.83$, $p<0.001$) and lower correlation between the test performed with 2 M and 0.25 M ($r=0.55$, $p<0.01$). The ISNT, test performed to access readily mineralizable N does not correlate with total N, total C, mineral N and soil enzymes in none of the four-molarities tested.

The PCA indicates that 75.65% of the original data could be explained by the three principal components (Table 4; Figure 2). As noted, the four molarities of ISNT analyzed are strongly correlated (Figure 2). The soil enzymes are correlated with mineral N, showing the ongoing process of soil organic matter decomposition.

The N potentially mineralizable accessed by ISNT, in the four-molarities tested, showed the higher values of loadings at PC1 axis, with highlight to ISNT NaOH 0.5M. In PC 2 axis, the higher values of loadings were related to soil enzymes and mineral N. Soil total C and N had higher values of loadings in PC3 axis.

**Table 4.** Summarization of Principal Component Analysis (PCA).

| | | PC1 | PC2 | PC3 |
|---|---|---|---|---|
| Eigen value | | 3.44 | 1.99 | 1.82 |
| Variability (%) | | 38.28 | 22.12 | 20.25 |
| Cumulative (%) | | 38.28 | 60.4 | 75.65 |
| Total N | Factor loadings | -0.225 | 0.088 | 0.927 |
| Total C | | -0.334 | 0.102 | 0.874 |
| Mineral N | | -0.219 | 0.793 | -0.114 |
| Arylsulfatase | | -0.401 | 0.722 | -0.043 |
| β-glucosidase | | 0.195 | 0.876 | -0.085 |
| ISNT 2M | | 0.884 | 0.047 | 0.338 |
| ISNT 1M | | 0.905 | 0.198 | 0.065 |
| ISNT 0.5M | | 0.911 | 0.119 | -0.069 |
| ISNT 0.25M | | 0.778 | 0.023 | 0.231 |

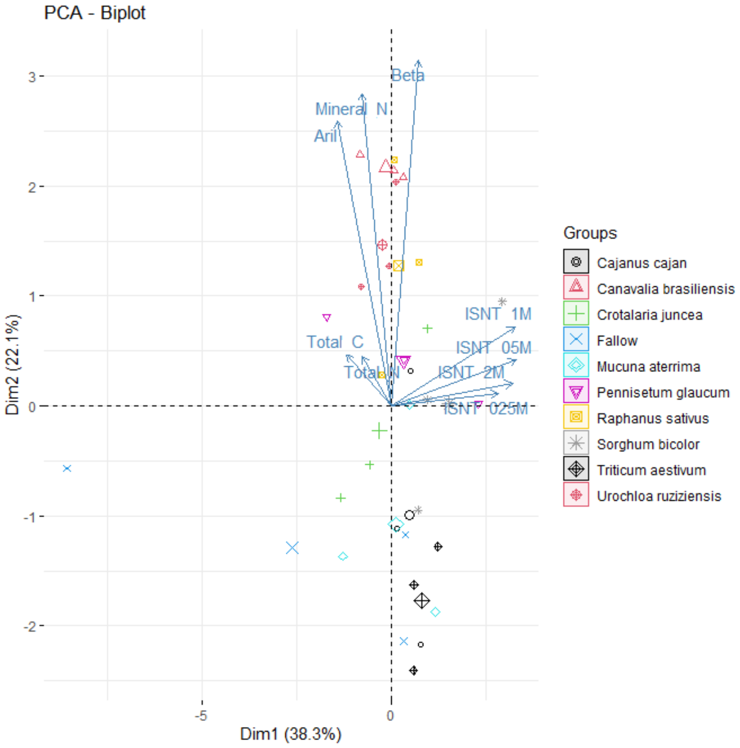

**Figure 2.** PCA biplot showing PC1 and PC2 scores, arrows indicating the loadings of the chemical and biochemical variables, and points indicating cover crop rotation systems. Total C: Soil total carbon; Total N: Soil total nitrogen; Mineral N: $[N\text{-}NH_4^+]+[N\text{-}NO_2^-]+[N\text{-}NO_3^-]$; Aril: Arylsulfatase; Beta: β-glucosidase; ISNT 2M: Illinois Soil Nitrogen Test NaOH 2M; ISNT 1M: Illinois Soil Nitrogen Test NaOH 1M; ISNT 0.5M: Illinois Soil Nitrogen Test NaOH 0.5 M; ISNT 0.25M: Illinois Soil Nitrogen Test NaOH 0.25M.

### 2.3.3. Relative importance of variables on maize yield

The relative importance of the chemical and biochemical variables over maize yield in subplots with low N in surface soil samples (Figure 3) was observed in a linear model that shows soil total N, β-glucosidase and ISNT NaOH 0.5M the mainly variables explaining maize yield, with 30%, 18% and 16% of contribution, respectively. These results, especially for the β-glucosidase and ISNT NaOH 0.5M, shows the contribution of soil organic fractions on plant nutrition.

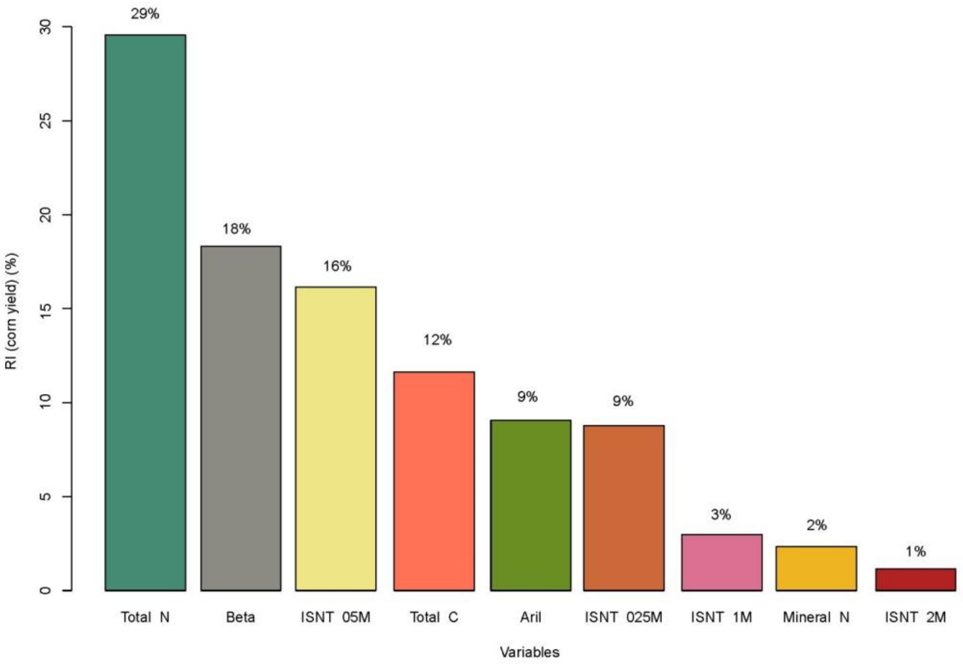

**Figure 3.** Relative importance (%) of chemical and biochemical variables over maize yield based on a linear model of $R^2$ decomposition. Total C: Soil total carbon; Total N: Soil total nitrogen; Mineral N: [N-$NH_4^+$]+[N-$NO_2^-$]+[N-$NO_3^-$]; Aril: Arylsulfatase; Beta: β-glucosidase; ISNT 2M: Illinois Soil Nitrogen Test NaOH 2M; ISNT 1M: Illinois Soil Nitrogen Test NaOH 1M; ISNT 0.5M: Illinois Soil Nitrogen Test NaOH 0.5 M; ISNT 0.25M: Illinois Soil Nitrogen Test NaOH 0.25M.

## 2.4. Discussion

### 2.4.1. Effects of cover crop rotation systems in maize yield response

The Δ maize yield (high N- low N) shows that N mineralization of organic matter provides part of the N that crops needed (St. Luce et al., 2011). The average maize grain yield in treatment with high N was 9.7 t $ha^{-1}$, whereas in the low N was 7.7 t $ha^{-1}$, representing a gain of 2 t $ha^{-1}$ considering the mean of all cover crop species. Indeed, the responsiveness of maize to N is lower or higher depending on the cover crop in the rotation system. Higher responsiveness was observed for maize followed by the grass species *Urochloa ruziziensis, Pennisetum glaucum and Sorgum bicolor*. Cover crop residues that had higher concentrations of soluble fractions of structural C (e.g. hemicellulose) and lower C/N ratio, like *Canavalia brasiliensis*, *Crotalaria juncea* and *Cajanus cajan*, promotes great accumulation of labile fractions on soil organic matter (Carvalho et al., 2022) and might reduce responsiveness to N

The effect of legume cover crops in maize yield was related in a meta-analysis by Marcillo & Miguez (2017), in which the yield was 21% higher under cover crop compared to fallow. Santos Júnior et al. (2019) showed in an experiment with maize-legumes cover crop rotation that the grain yield improvement provided by legumes cover crop depends on the specie adopted in the succession and can be higher or neutral compared to the treatment without cover crop.

In this study, the effect of grass cover crops in maize yield response was neutral when compared to fallow. The same results were described in a meta-analysis, showing neutral effect of grass cover crops in maize yields (Marcillo & Miguez, 2017). Maltas et al. (2009) testing maize preceded by *Pennisetum glaucum* + *Urochloa ruziziensis* did not observed effect on grain yield in comparison to bare fallow.

As observed in this study, the effect of cover crops in systems with high N was lower than in treatments with low N. This result was also observed by Maltas et al. (2009) in maize-cover crops systems in Brazilian Cerrado. Pott et al. (2021) in an experiment with a maize-hairy vetch rotation observed that the effects of cover crop were largest for low N rates with reflects in high contributions of vetch to maize N nutrition. In this study, cover crop species differentiated maize grain yield only in the low N supply, without differences in the high N treatments. It seems that the high N treatment leveled the yield of maize independently of the cover specie cultivated. In addition, the cultivation of grass species increased the responsiveness to N, reaching values higher than 40%. In opposite, most of the legume species presented lower responsiveness to N, with mean values approaching 20%. This indicate that cultivation of grass or legume species modify the responsiveness of the main crop (maize) to N fertilization, suggesting modification in the N management according to the specie cultivated. The excessive application of N fertilizer in less responsiveness areas can lead to losses to the atmosphere and water (Ti et al., 2015) and decreases the N-use efficiency (Quaggio et al., 2014).

However, the application of low N rates can lead to soil organic N depletion and C loss, with consequences to cash crop yield in the long term. The negative N soil balance could affect the system sustainability and a deficit of N for the crops in succession. Rocha et al. (2020) in an experiment with maize-grass rotation system showed that the N balance was negative in all treatments analyzed except in the treatment with application of higher dose of N (210 kg $ha^{-1}$) and maize-palisade grass rotation system. In this study, the lower and higher N rates differed largely (20 vs 150 kg $ha^{-1}$ N), but both are rates lower than the recommendation.

### 2.4.2. Effects of cover crop on chemical and biochemical variables

In this study, the only parameters affected by cover crop cultivation was mineral N content, as well as arylsulfatase and beta glucosidase. The introduction of cover crops in agricultural systems alters the N dynamic in soil, especially after decomposition of cover residues increasing N supply depending on the crop specie (Carciochi et al., 2021). Carvalho et al. (2022) showed that legume cover crop *Canavalia brasiliensis* has high contents of N in the shoots, lower C/N ratio and consequently higher decomposition rates, which affects the soil N mineralization and soil mineral N concentration. Douxchamps et al. (2014) in an experiment testing *Canavalia brasiliensis* in crop-livestock systems in tropical region showed that the introduction of the legume cover specie as green manure increases soil fertility and consequently allows an application of lower rates of N fertilizer.

Carvalho et al. (2022) observed that *Urochloa ruziziensis* has high N contents in the shoots and, despite being a grass cover crop, the specie produces more readily decomposable organic matter. Veras et al. (2016) showed that *Canavalia brasiliensis* and *Urochloa ruziziensis* had low C/N ratios and consequently higher amounts of N are released, similar to the results obtained in this study. In contrast, Maltas et al. (2009), testing legume and grass as cover crops, did not observed differences in mineral N concentration in soil.

Several authors have showed the effect of cover crops in the enzyme activity, that were also observed in this study, specifically arylsulfatase and β-glucosidase activity. Sanchez et al. (2019) in an experiment with maize-cover crops rotation showed that conservation tillage and cover crops enhances C, N and S cycling enzymes. Ma et al. (2021) in a meta-analysis study observed that microbial biomass C increases 28% and consequently the extracellular enzymes activity increases 14-39% in systems with green manure.

Klose et al. (1999) in an evaluation of arylsulfatase activity in a long-term field experiment have observed significant effect of crop rotation and plant cover on enzyme activity. The maize-oats and soybean-oats rotation systems presented higher arylsulfatase activity than the systems with continuous crop (maize or soybean). Sanchez et al. (2019) showed an increasing in arylsulfatase activity in soils sampled after the management of cover crops, similar to the results obtained in this study. Bonini Pires et al. (2020) related an increase in β-glucosidase activity with the incorporation of cover species in a no-till system, also observed by Tyler (2019). The activity of this enzyme is related to crop residue quality and the management practices adopted, which increases in no-till systems (Pandey et al., 2014; Adetunji et al., 2017).

### 2.4.3. Correlation of chemical and biochemical variables with potentially mineralizable N and N mineralization

Unexpectedly, both dissolved organic N (DON) and labile soil C, determined by the permanganate oxidizable C analysis (POXC), when correlated with other variables in this experiment condition were not suitable for factor analysis.

Our results show no correlation between the ISNT in the four-molarities tested and soil total N. Many studies reported high correlation between soil total N and ISNT. McDonald et al. (2014) obtained, analyzing 35 soil samples, a correlation between the total soil N and ISNT levels of 0.927. Spargo et al. (2009) in experiments on-farm with maize, also obtained correlation superior of 0.9 between both indices. This high correlation observed by the authors with soil total N suggests that ISNT extracts some fraction that is not the labile. This was not the trend observed in our study, since ISNT did not presented significant correlation to total soil C or total soil N, for anyone of the NaOH concentrations evaluated. In addition, the PCA analysis show the highest loadings in the first principal component related to ISNT in four-molarities tested, suggesting the importance of the N fraction extracted by the method to understand the variance between treatments.

The positive correlation between mineral N (expressed by the sum of all forms of N mineral) and soil enzymes activity is a result that may allow a biological approach in N recommendation. The effect of soil health, which can be expressed by many factors including soil enzymes activity (Das & Varma, 2010), is related with gains in maize yield as observed by Wade et al. (2020). These authors found that soil biological health corresponds to 18% of the magnitude of the fertilization effect in maize yield response, and this result supports the importance to account the processes mediate by microorganisms to improve N supply to crops.

Sainju et al. (2022) did not observed association of arylsulfatase and β-glucosidase with soil inorganic N, in contrast from those obtained in this study. Other authors have obtained positive correlation with enzymes activity and N mineralization, similar to the results of this study. Balota & Chaves (2010) in an experiment with coffee and green manure have showed the effect of legume cover crops in arylsulfatase and consequently in N mineralization. Nevins et al. (2020) in a maize - cover crop rotation system experiment observed effects of cover crops on β-glucosidase activity and, consequently, N mineralization. However, it was also noted that part of the N remains immobilized in soil microorganisms, and the impact of immobilization need to be accounted in N fertilization management. Geisseler & Horwath (2009) have observed in an incubation experiment that extracellular enzyme activity, with highlight to β-glucosidase, had correlation with N mineralization.

### 2.4.4. Contribution of the chemical and biochemical variables to maize yield response

The relative importance analysis was a tool to access information that helps to understand the contribution of the predictors tested in maize yield response. The main objective of this analysis is comprehending the role played by each variable in a regression equation (Tonidandel & LeBreton, 2011). The analysis was able to show that the variance of maize yield response can be explained especially by total N, activity of β-glucosidase and potentially mineralizable N accessed by 0.5 M NaOH concentration by the ISNT method.

Despite of the weak correlation between total soil N and ISNT 0.5 M, and the strong correlation between NaOH extractions tested in four-molarities, the relative importance analysis showed contributions of these variables to maize yield. This behavior was also observed by Williams et al. (2007), that achieved correlation between ISNT and maize yield response ($r = 0.70$), indicating that ISNT has potential to access mineralizable N and can improve maize N recommendation. In the other hand, Osterhaus et al. (2008) did not found that ISNT was a good predictor of mineralizable N. These lack of consensus between authors about the potential of ISNT to predict mineralizable N can be partially explained by other factors such as soil types and environmental conditions. In this study, using a highly weatherized tropical soil, the reduction in NaOH concentration compared to the original ISNT method showed potential in better discriminating differences between treatments.

The activity of β-glucosidase showed high contribution on the 10-year average maize yield. It is outstanding that nutrient cycling is affected by soil microbial activity. These findings of contribution of enzymatic activities, especially in C, N and S cycling was also observed by Sanchez et al. (2019).

## 2.5. Conclusion

Maize cultivation in succession with cover crop species was favored under limited N supply, but similar maize yields was obtained under high N rates independently of cover crop cultivation. Under limited N supply, *Urochloa ruziziensis* resulted in the lowest maize grain yield.

N responsiveness of maize is affected by cover crop species cultivated in succession. Maize cultivated under fallow presented N responsiveness of 30%, whereas maize cultivated following grass species or legume species presented N responsiveness varying from 40 to 20%, respectively.

Soil enzymes, especially β-glucosidase, and ISNT (NaOH 0.5 M) most impacted maize grain yield under our experiment conditions, demonstrating potential to identify labile fractions of soil organic N for maize cultivation. Reducing the concentration of the ISNT method to 0.5 M showed potential in improving the predictive capacity compared to the original ISNT method (2 M NaOH), based on our results.

The study evaluated nine years od crop rotation with cover crops (legumes, grasses, and cruciferous) in rotation with maize cultivated in the summer, with low (20 kg ha$^{-1}$ N) or high (150 kg ha$^{-1}$ N) rates application. In the last two years, a decline in maize grain yield was experienced and this might be related to intensification of diseases in the maize crop, suggesting that a more diversified crop system should be adopted.

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# 3 $^{15}$N-FERTILIZER RECOVERY BY MAIZE AFTER NINE YEAR OF CROP ROTATION IN A CERRADO OXISOL

## Abstract

Nitrogen (N) is required in large amounts by crops and the use of N fertilizer is mandatory to achieve high yields, especially in cereal production. However, the efficiency of N fertilization is still low in agricultural systems, due to N-fertilizer immobilization in organics forms or losses as volatilization, leaching or denitrification. We hypothesized that long-term crop rotation will increase $^{15}$N-fertilizer recovery by maize plants by modifying N transformation and reducing N losses. Therefore, we evaluated the effect of nine years of rotation systems with leguminous and non-leguminous in a Cerrado Oxisol on maize crop yield, N uptake, N in the plant derived from fertilizer, N derived from other sources, 15-N fertilizer recovery, and N use efficiency. The treatments consisted by plots of maize cultivated after the cover species: *Cajanus cajan*, *Crotalaria juncea*, *Pennisetum glaucum*, *Urochloa ruziziensis*, and the control (fallow), and subplots with low (20 kg ha$^{-1}$ N at sowing) or high N application (20 kg ha$^{-1}$ N at sowing and 130 kg ha$^{-1}$ N on side dress application). Labeled urea (2.07% atom $^{15}$N) was applied on side-dress application and maize plants sampled for total N and $^{15}$N-abundance quantification at harvest time. Highest maize yield was observed in systems with *Cajanus cajan*, and lowest maize yield was observed under fallow or *Urochloa ruziziensis* cultivation. High N treatment provided higher yields compared to low N treatment, but no interaction between crop specie *vs* N management was identified. N uptake and N derived from other sources did not differ between cover crop species, but on average, the treatments with leguminous presented higher values compared to the treatments with non-leguminous. The N in the plant derived from fertilizer did not differ between cover species, but on average, it was lower in treatments with leguminous cultivation. There was no effect of cover crops species on N fertilizer recovery and N fertilizer use efficiency. The lack of response can be explained by N immobilization in organic forms and by the decrease in grain yield in the experiment in the last two growing seasons (2018/2019 and 2019/2020) due to weather restrictions. The introduction of cover crops in rotation with maize can improve N inputs in the systems, especially with leguminous species. However, the benefits to maize yield are also related to the ecosystems services provided by cover crops and the system diversification rather than an improvement in $^{15}$N-fertilizer recovery promoted by cover crops cultivation.

Keywords: *Cajanus cajan*, *Urochloa ruziziensis*, N accumulation, N fertilizer use efficiency

## 3.1. Introduction

Nitrogen (N) is the most required nutrient by majority crops and the limited supply of N to plants can lead to pronounced growth reductions (Marschner, 2011). Therefore, N-fertilizer is a key agricultural input that allows farmers to achieve high crop yields to satisfy the increasing demand of food products driven by population growth. More than half of the world population consume food resources that grows with synthetic N fertilizers application (Zhang et al., 2015; Asibi et al., 2019).

Maize is the crop that receives the highest amount of N fertilizer, representing 20% of the world consumption (IFA, 2022), although the N use efficiency is still low under maize crop cultivation (36%) (Yu et al. 2022). Proper application of N fertilizer is required to achieve profitable maize yields. However, excessive N-fertilizer application can lead to environmental issues such as nitrous oxide emission, a potent greenhouse gas, air pollution by N oxides and ammonia, eutrophication of rivers, lakes, oceans, and contamination of groundwater, affecting aquatic life and water quality (Good & Beatty, 2011; Zhang et al., 2015).

Since the use of synthetic N is indispensable for high yield agricultural production, some important actions to reduce the impact of N pollution need to be taken, with highlight to improve N-fertilizer use efficiency (NUE) in crop and livestock systems (Bodirsky et al., 2014). The adoption of soil management practices, such as inclusion of cover crops in rotation systems, are strategies to improve NUE, reducing costs of production, losses of N species to the environment, and increasing yield gains (Fageria & Baligar, 2005; Rosolem et al., 2017).

Cover crops cultivated in the off-season period provide a range of benefits for soil and for the main crop. The inclusion of these species in rotation systems decreases N leaching (Abdalla et al., 2019; Govindasamy et al., 2023), provides soil protection avoiding surface runoff and soil erosion (Du et al., 2022), increases soil organic carbon (SOC) (Jian et al., 2020; Muhammad et al., 2021), improves soil biological activity (Adetunji et al., 2020; Muhammad et al., 2021), further promoting a better nutrient cycling (Rosolem et al., 2017; Koudahe et al., 2022).

On regard to the N transformations, studies have reported the positive effect of introducing cover crop in rotation systems, especially leguminous cover crops, that provide N by the biological nitrogen fixation (BNF). Salazar et al. (2021) in an experiment with maize-leguminous rotation system, have shown that the introduction of cover crops contributed to increase the NUE, but without yield gains. Hu et al. (2023) have found in a long-term experiment that leguminous and non-leguminous cover crops increased the NUE by maize and also promoted better yields of the main crop.

An improvement of NUE or in the N-fertilizer recovery is thus expected due to potential effect of rotation systems in providing extra N to the systems, increasing N cycling, and reducing N losses. However, there is no consensus in the literature, since some studies have shown neutral or negative effects of cover crops on NUE. Gabriel et al. (2016) observed that even in a legume cover crop-maize rotation system, the amount of N derived from fertilizer was not improved compared to fallow and maize yield was not affected by the treatments. Rocha et al. (2019) in a maize-forage grasses rotation system, showed that non-legumes cover crops decreased maize grain yield and had no effect on N fertilizer recovery by main crop. This demonstrates that further studies are required to better elucidate the long-term effect of crop rotation on NUE by the main crop.

The hypotheses tested in this study was that long-term cultivation of cover crop in rotation improves N fertilizer use efficiency by maize cultivated as the main crop. To test the hypothesis, we evaluated, in a long-term field experiment in a Cerrado Oxisol, the effect of cover crop rotation in N accumulation on maize compartments (stalk + straw, cob + husk, and grains), N derived from fertilizer, N derived from other sources (from soil, native biological fixation, atmospheric deposition, and other), N recovery by fertilizer, and N fertilizer use efficiency.

## 3.2. Material and methods

### 3.2.1. Site description and experimental design

The long-term experiment (2010 – 2020) was carried out at the experimental area of Brazilian Agricultural Research Corporation (EMBRAPA Cerrados), based in Planaltina, Federal District (15° 35’ 30” S, 47° 42’ 00” W and 990 m), in the central western region of Brazil (Fig. 1). According to Köppen-Geiger’s classification, the climate is classified as Aw (tropical savannah), with dry period (winter) and rainy period (summer) (Beck et al., 2018).

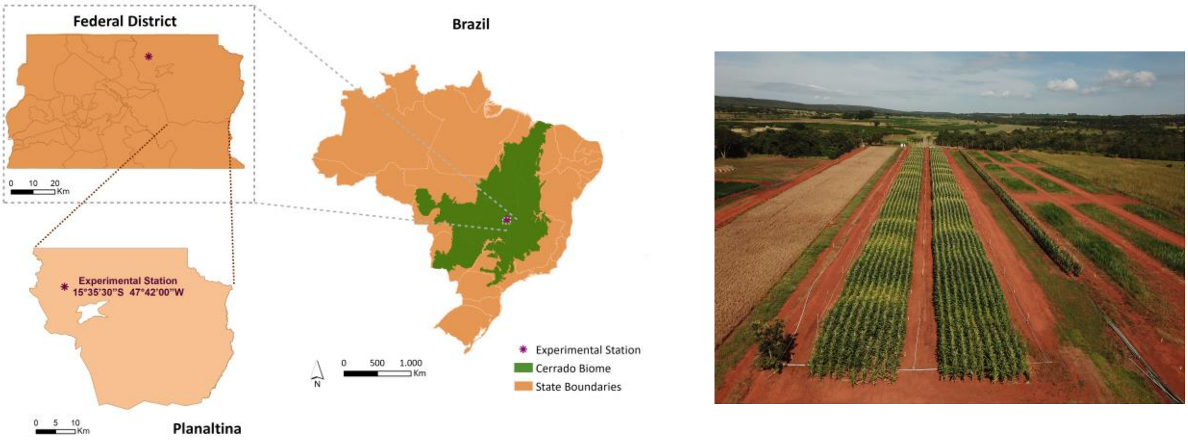

**Figure 1.** Location of experimental station (left) and aerial image of the area (right).

The soil was classified as Typic Haplustox (clayey texture) according to Soil Taxonomy (Soil Survey Manual, 2017). The soil physicochemical characteristics of the area were as follows: 513 g kg$^{-1}$ clay, 186 g kg$^{-1}$ silt, 301 g kg$^{-1}$ sand, pH $_{(H2O)}$ = 5.9, SOM = 26.3 g kg$^{-1}$, exchangeable $Al^{3+}$ = 0.07 cmol$_c$dm$^{-3}$, $Ca^{2+}$+$Mg^{2+}$ = 36 mmol$_c$dm$^{-3}$, $K^+$ = 2 mmol$_c$dm$^{-3}$, and $P_{Mehlich}$ = 8.3 mg dm$^{-3}$.

The experimental design was randomized complete block arranged in split-plots with three replications. The plots (principal treatments) were maize-cover crop rotation with 4 cover species (*Cajanus cajan*, *Crotalaria juncea*, *Pennisetum glaucum* and *Urochloa ruziziensis*), plus a fallow treatment (emergence of spontaneous vegetation). The subplots (secondary treatments) were low N (20 kg ha$^{-1}$ N at sowing) and high N application (20 kg ha$^{-1}$ N at sowing plus 130 kg ha$^{-1}$ N at side dress application). In the high N treatment, two application of urea was performed at V4 and V8 stage (65 kg ha$^{-1}$ N each). The subplots without side dressing application of N were used to access the natural abundance of $^{15}$N. The plots measured 12 x 8 m and the subplots, 6 x 8 m.

### 3.2.2. Field management

After maize harvest, in April 2019, the cover species were sown in a no-till system, directly on the maize residues and residual fertilization of the previously crop. The plant density was 20 plants m$^{-1}$ for *Cajanus cajan*, *Crotalaria juncea* and *Urochloa ruziziensis*; and 40 plants m$^{-1}$ for *Pennisetum* *glaucum*. The spacing between plant rows was 0.5 m for all plant species (Carvalho & Amabile, 2006). Another plot was maintained as fallow, only with the emergence of spontaneous vegetation. Cover crops grown during winter period, with herbicide application before maize sowing in the season period.

Maize was sown at the beginning of the rainy season (October 2019). The space between plant rows was 0.75 m. At planting, 500 kg ha$^{-1}$ of N-P-K 4-30-16, 2 kg ha$^{-1}$ of Zn ($ZnSO_4.7H_2O$) and 10 kg ha$^{-1}$ FTE BR 12 was applied to all treatments. In both N treatments (low N and high N), 20 kg ha$^{-1}$ N was applied as basal fertilization. In the high N treatment, 130 kg ha$^{-1}$ of N (no labelled urea) was applied twice (V4 and V8 stage) as urea in top dress application, except in the microplots with application of $^{15}$N-labelled urea. The fertilizer rates described was based on recommendations for maize sowed in the Cerrado region (Sousa & Lobato, 2004).

### 3.2.3. Topdressing fertilization

The side dressing application of $^{15}$N-labelled urea fertilizer (in the high N treatment) was manually performed in December 2019 (Supplementary Figure 5). The $^{15}$N-labeled urea (2.07% atom $^{15}$N) was diluted in deionized water to facilitate uniform application. The solutions were applied in one central row measuring 1 m (microplots). The application was split in V4 and V8 maize growth stages (Supplementary Figure 6). After the N fertilization, 15 mm of irrigation was manually applied to avoid losses by volatilization.

### 3.2.4. Plant sampling and analysis

The maize plants in the microplots (principal row) and the two adjacent rows, parallel to central row, were harvested separately. The plants of the adjacent rows (1 m each) were harvested separately but combined for biomass quantification and N analysis. The plants were split in three compartments: stalk + straw, grains, and cob + husk. The compartments were weighted to obtain the total green matter and placed in a forced-air oven at 65°C, until a constant weight. The material placed in paper bags were reweighted to obtain the total dry matter. After this process, samples of the dry matter were taken (Figure 2) and passed through a Wiley type knife laboratory mill. Since $^{15}$N-fertilizer was applied to the microplots, a carefully cleaning of the equipment (with deionized water and alcohol) was performed between each sample to avoid cross contamination. To determine maize yield, four lines with four meters each were sampled in subplots (low N and high N). The grains sampled were weighted and then oven-dried at 65ºC to determine grain moisture. Maize grain yield was determined after correct grain moisture content to 13% (w.b.) and the results were expressed in t ha$^{-1}$. The data of the 2019/2020 season was used in this study.

**Figure 2.** Samples of the compartments of maize plants. A. stalk + straw; B. grain; C. cob and husk.

The total N content and $^{15}$N abundance of the three compartments were determined at CENA/USP using a DELTA V Advantage isotope ratio mass spectrometer (Thermo Scientific, Massachusetts, USA). The N accumulation, N derived from the fertilizer, N derived from other sources (from soil, native biological fixation, atmospheric deposition, and other), N fertilizer recovery, and N fertilizer use efficiency were calculated using the following equations:

(a) Yield (obtained from the useful area of the plot)

Yield (kg ha$^{-1}$) = dry biomass obtained in 1 m x 13,333 (factor considering the row spacing of 0.75m)

(b) N accumulation in different plant compartments (NA, kg ha$^{-1}$):

$$NA\,(\frac{kg}{ha}) = (\frac{N\;x\;DM}{1000}),$$

where N is the total N concentration (g kg$^{-1}$) and DM is the total dry matter (kg ha$^{-1}$). In order to reduce variability, total dry matter was calculated using the mean value of N concentration and dry matter obtained in the central row (1m) and the adjacent row of the microplots (2m).

(c) Percentage of N derived from fertilizer ($N_{dff}$):

$$Ndff\;(\%) = \left(\frac{15N\;atoms\;(\%)\;in\;tissues - \;15N\;atoms\;(\%)\;natural\;abundance}{15N\;atoms\;(\%)\;in\;fertilizer - 15N\;atoms\;(\%)\;natural\;abundance}\right)\text{ x }100,$$

where $^{15}$N atoms (%) in tissues is the $^{15}$N atoms (%) in tissues samples in the central row; $^{15}$N atoms (%) natural abundance was equivalent to 0.368 atom (%) $^{15}$N (obtained as a mean value of eight subsamples), $^{15}$N atoms (%) in fertilizer was equivalent to 2.07 atoms (%) in fertilizer. Ndff (%) in central row and adjacent rows were calculated separately.

(d) Amount of N in the plant derived from fertilizer (Ndff, kg ha$^{-1}$):

$$Ndff\left(\frac{kg}{ha}\right) = \frac{\big((\%)\;Ndff\;x\;NA\big)}{100}$$

Ndff (%) is the amount of N in plant derived from fertilizer (%), NA is the N accumulation in different plant compartments (kg ha$^{-1}$). Ndff (kg ha-1) represents the sum of the Ndff (kg ha$^{-1}$) obtained in the central row and the Ndff (kg ha$^{-1}$) obtained in the adjacent row.

(e) Percentage of N derived from other sources ($N_{dfs}$, %)

$$Ndfs\;(\%) = \;100 - \;Ndff\;(\%)$$

Ndff (%) is the amount of N in the plant derived from fertilizer (%).

(f) Amount of N in the plant derived from other sources ($N_{dfs,}$ kg ha$^{-1}$)

$$Ndfs\left(\frac{kg}{ha}\right) = NA - Ndff\left(\frac{kg}{ha}\right)$$

NA, is the N accumulation in plant compartments (kg ha$^{-1}$); Ndff is the amount of N in the plant derived from other sources (kg ha$^{-1}$)

(g) Percentage of N fertilizer recovery ($N_R$):

$$Nr\ (\%) = \left(\frac{Ndff}{Nrate}\right) x\ 100,$$

Ndff is the amount of N in the plant derived from fertilizer (kg ha$^{-1}$); Nrate applied as side dress application (130 kg ha$^{-1}$ N).

The N fertilizer use efficiency was calculated using the following equation, according to (A. R. Dobermann, 2005):

(h) Nitrogen fertilizer use efficiency (NUE):

$$\text{NUE}\left(\frac{\text{kg grain}}{kg\ N\ applied}\right) = \frac{(Grain\ yield\ at\ high\ N\ treatment - Grain\ yield\ at\ low\ N\ treatment)}{Nrate},$$

Nrate is the N side dressing rate applied (130 kg ha$^{-1}$ N).

### 3.2.5. Statistical analysis

First, the data was subjected to Shapiro-Wilk test for normality and to O’Neill-Mathews test for variance homogeneity. The analysis of variance (ANOVA) and means comparison were performed using F test and Tukey test, respectively. All the tests were performed considering 0.05 probability level, using R software (R Development Core Team, 2022).

## 3.3. Results

### 3.3.1. Maize response to rotation systems and N fertilizer

The maize response to rotation systems and N topdressing fertilization is described on Table 1. The systems with leguminous cover crop *Cajanus cajan*, showed the highest yield, 11% superior on average compared to fallow. On the other hand, the system with *Urochloa ruziziensis* showed the lowest yield, statistically similar to fallow. As expected, high N treatment presented highest yield in comparison to low N treatment, with an increase of 18.5% on average. The interaction between cover crops and N treatments was not significant, demonstrating that N response occurred independently of cover crop rotation specie.

**Table 1.** Maize yield (Mg ha $^{-1}$) in a long-term maize-cover crop rotation experiment in the 2019/2020 season. Planaltina, DF.

| Rotation system | Maize yield (Mg ha$^{-1}$) | | Means[1] |
|---|---|---|---|
| | Low N | High N | |
| *Cajanus cajan* | 8.26 | 10.23 | 9.24a |
| *Crotalaria juncea* | 8.53 | 9.23 | 8.88ab |
| *Pennisetum glaucum* | 7.63 | 9.66 | 8.64ab |
| *Urochloa ruziziensis* | 6.90 | 8.98 | 7.94b |
| Fallow | 7.25 | 9.21 | 8.23b |
| Means[1] | 7.71B | 9.46A | 8.59 |
| Cover crops (C)[2] | 0.0098 | | |
| N topdressing (N)[2] | < 0.0001 | | |
| CxN[2] | 0.2889$^{ns}$ | | |

[1]Means followed by the same lowercase letter in column and same uppercase letter in line do not differ by Tukey's test ($p < 0.05$). CV (cover crops) = 5.53%; CV (N topdressing) = 6.98%. [2]p-value of the factors and the interaction between factors.

### 3.3.2. N accumulated in maize compartments

The N uptake by maize in the stalk + straw did not differ between rotation systems and differed for the other two compartments (cob + husk, and grains). The total N uptake (the sum of three compartments) did not differ between rotation systems (Table 2; Figure 3).

**Table 2.** N accumulated (kg $ha^{-1}$) in maize compartments (stalk + straw, cob + husk, and grain) in a long-term maize-cover crop rotation experiment in the 2019/2020 season. Planaltina, DF.

| | Compartments | | | |
|---|---|---|---|---|
| Rotation system | Stalk + straw | Cob + husk | Grain | All compartments |
| | N uptake (kg $ha^{-1}$) | | | |
| *Cajanus cajan* | 56.93 | 13.25a | 131.18a | 201.36 |
| *Crotalaria juncea* | 62.94 | 9.37b | 100.72ab | 173.03 |
| *Pennisetum glaucum* | 54.84 | 9.99b | 104.94ab | 169.77 |
| *Urochloa ruziziensis* | 56.77 | 8.92b | 107.76ab | 173.45 |
| Fallow | 73.74 | 11.12ab | 96.66b | 181.52 |
| p-value | $0.45^{ns}$ | 0.052 | 0.033 | $0.30^{ns}$ |
| CV (%) | 21.67 | 9.67 | 10.12 | 10.18 |

Means followed by the same letters in the columns do not differ by Tukey test ($p < 0.05$).

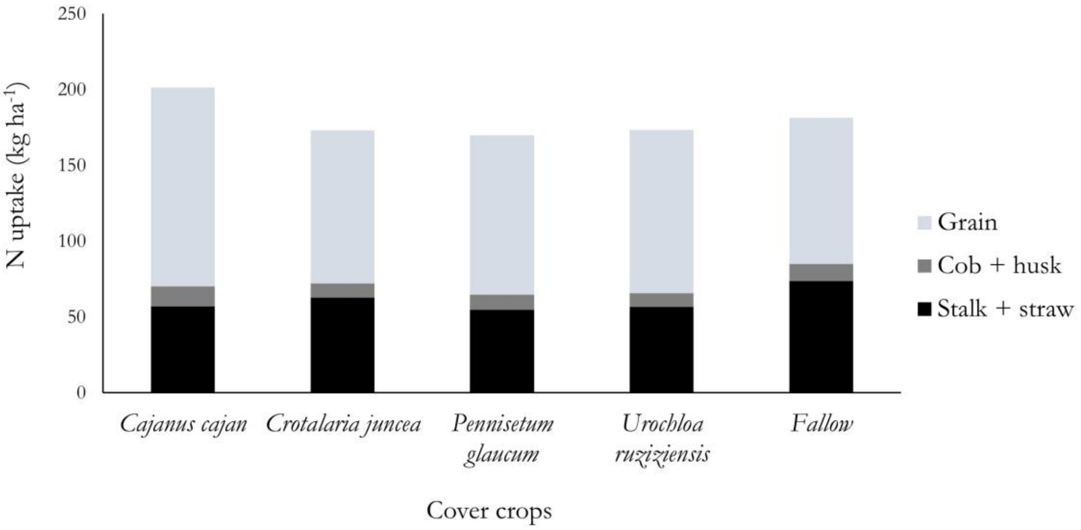

**Figure 3.** Total N uptake and N uptake in three compartments (stalk + straw, cob + husk, and grain) by maize plants, in a long-term cover crop rotation system in Cerrado region. Data was obtained in the 2019/2020 crop season.

### 3.3.3. Contribution of N fertilizer and N from other sources on maize nutrition

The N derived from fertilizer did not differ between leguminous and non-leguminous cover crops tested for the three compartments (Table 3; Figure 4).

**Table 3.** N derived from fertilizer (kg ha$^{-1}$) in maize compartments (stalk + straw, cob + husk, and grain) in a long-term maize-cover crop rotation experiment in the 2019/2020 season. Planaltina, DF.

| Rotation system | Compartments | | | |
|---|---|---|---|---|
| | Stalk + straw | Cob and husk | Grain | All compartments |
| | N derived from fertilizer (kg ha$^{-1}$) | | | |
| *Cajanus cajan* | 15.31 | 3.10 | 31.88 | 50.29 |
| *Crotalaria juncea* | 16.23 | 2.21 | 24.11 | 42.55 |
| *Pennisetum glaucum* | 18.98 | 2.82 | 30.00 | 51.80 |
| *Urochloa ruziziensis* | 20.96 | 2.79 | 34.44 | 58.19 |
| Fallow | 25.08 | 3.09 | 28.16 | 56.33 |
| p-value | 0.080$^{ns}$ | 0.442$^{ns}$ | 0.310$^{ns}$ | 0.180$^{ns}$ |
| CV (%) | 19.95 | 22.01 | 19.04 | 14.25 |

Means comparison was performed using Tukey test ($p<0.05$).

The N derived from other sources shown differences between treatments for stalk + straw and grain compartments. In cob + husk compartment, the Ndfs was higher in the system with *Cajanus cajan* (Table 4; Figure 4) compared to all remaining treatments. On average, the treatments with leguminous cover crops had higher Ndfs values when accounted all compartments, with an increase of 17% for *Cajanus cajan* and 7.4% for *Crotalaria juncea* as compared to fallow. The lower Ndfs values, on average, was observed in the system with *Urochloa ruziziensis,* with a decrease of 5.5% when compared to fallow.

**Table 4.** N derived from other sources (kg ha$^{-1}$) in maize compartments (stalk + straw, cob + husk, and grain) in a long-term maize-cover crop rotation experiment in the 2019/2020 season. Planaltina, DF.

| Rotation system | Compartments | | | |
|---|---|---|---|---|
| | Stalk + straw | Cob and husk | Grain | All compartments |
| | N derived from other sources (kg ha$^{-1}$) | | | |
| *Cajanus cajan* | 98.54a | 23.39a | 230.49a | 352.42 |
| *Crotalaria juncea* | 109.65a | 16.53b | 177.34ab | 303.52 |
| *Pennisetum glaucum* | 90.70a | 17.16b | 179.88ab | 287.74 |

| | | | | |
|---|---|---|---|---|
| *Urochloa ruziziensis* | 92.59a | 15.04b | 181.10ab | 288.73 |
| Fallow | 122.41a | 19.16ab | 165.16b | 306.73 |
| p-value | $0.508^{ns}$ | 0.001 | 0.033 | 0.267 |
| CV (%) | 23.52 | 8.55 | 10.98 | 11.75 |

Means followed by the same letters in the columns do not differ by Tukey test ($p < 0.05$).

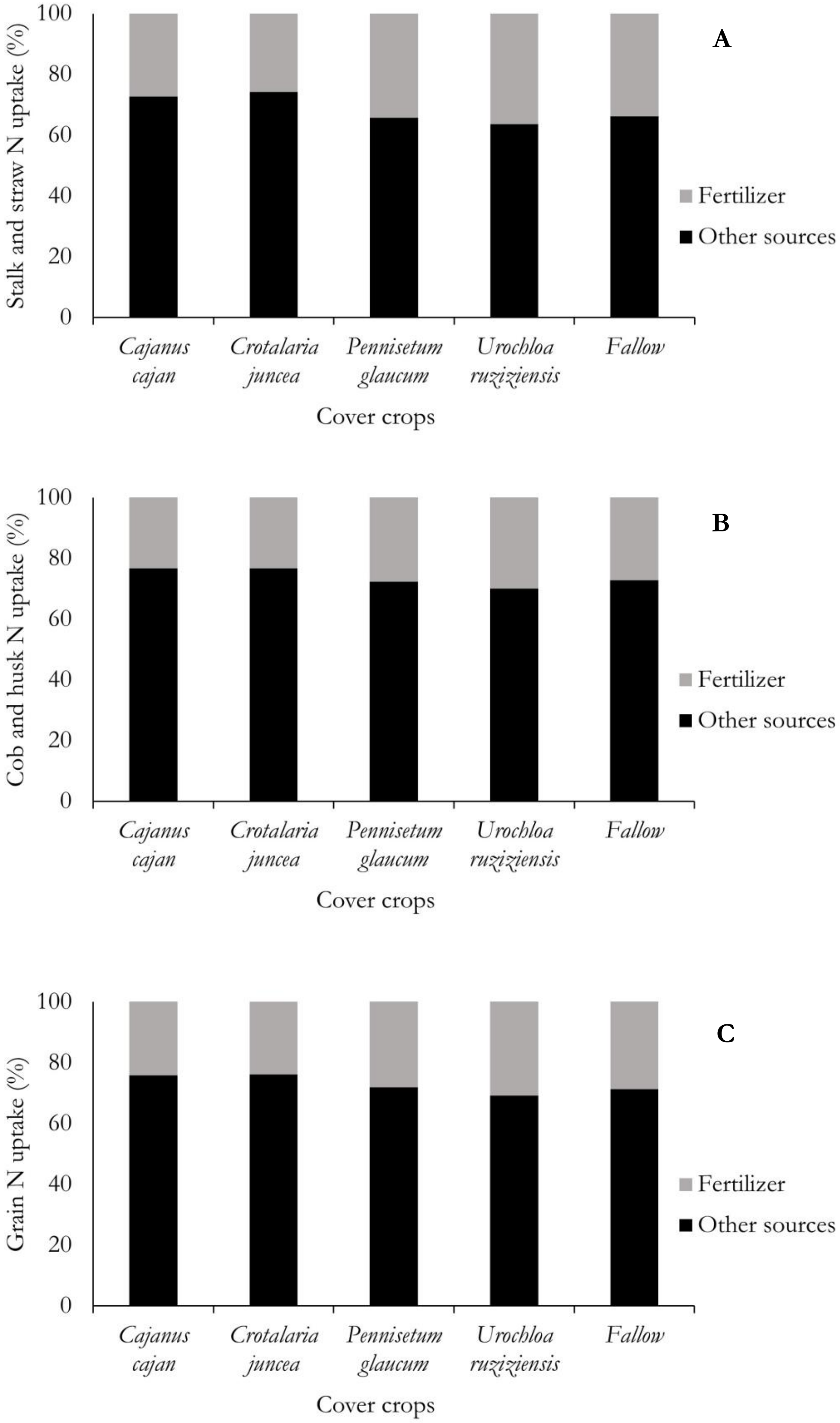

**Figure 4.** N derived from fertilizer and from other sources in three compartments: stalk + straw (A), cob + husk (B), and grains (C) in a long-term maize-cover crop rotation experiment in the 2019/2020 season.

### 3.3.4. N fertilizer recovery in maize compartments

Most of N fertilizer recovery was accumulated in grains, followed by stalk + straw, and cob + husk plant compartments. N fertilizer recovery did not differ between rotation systems with leguminous and non-leguminous cover crops (Table 5).

**Table 5.** Nitrogen fertilizer recovery (%) in maize compartments (stalk + straw, cob + husk and grain) in a long-term maize-cover crop rotation experiment in the 2019/2020 season. Planaltina, DF.

| Rotation system | Compartments | | | |
|---|---|---|---|---|
| | Stalk + straw | Cob and husk | Grain | All compartments |
| | N fertilizer recovery (%) | | | |
| *Cajanus cajan* | 11.78 | 2.39 | 24.52 | 38.69 |
| *Crotalaria juncea* | 12.48 | 1.70 | 18.55 | 32.73 |
| *Pennisetum glaucum* | 14.60 | 2.17 | 23.07 | 39.84 |
| *Urochloa ruziziensis* | 16.12 | 2.15 | 26.48 | 44.75 |
| Fallow | 19.29 | 2.37 | 21.66 | 43.32 |
| p-value | 0.080$^{ns}$ | 0.439$^{ns}$ | 0.310$^{ns}$ | 0.180$^{ns}$ |
| CV (%) | 19.93 | 21.99 | 19.04 | 14.25 |

Means comparison was performed using Tukey test ($p<0.05$).

### 3.3.5. Nitrogen fertilizer use efficiency in a maize-cover crops rotation system

The N fertilizer use efficiency was not improved by none cover crop rotation tested (Table 6). On average, the treatments with highest yield per N applied were *Crotalaria juncea* and *Urochloa ruziziensis*, but without statistical difference to other treatments.

**Table 6.** Nitrogen fertilizer use efficiency in a long-term maize-cover crop rotation experiment in the 2019/2020 season. Planaltina, DF.

| Rotation system | N fertilizer use efficiency |
|---|---|
| | kg grain kg N applied$^{-1}$ |
| *Cajanus cajan* | 23.89 |
| *Crotalaria juncea* | 30.04 |
| *Pennisetum glaucum* | 25.70 |
| *Urochloa ruziziensis* | 30.04 |
| Fallow | 25.24 |
| Mean | 26.98 |
| p-value | 0.847 |
| CV (%) | 26.38 |

Means comparison was performed using Tukey test ($p<0.05$).

## 3.4. Discussion

### 3.4.1. Effect of cover crop rotation system and N management on maize

Cover crops play an important role in soil conservation, improving physical, chemical, and biological properties and consequently supporting to achieve high yields of the main crop. Many studies have reported the positive effects of cover crop rotation systems to main crop yield, as observed by *Cajanus cajan* in our experiment conditions. Hu et al. (2023) in a long-term maize-cover crop experiment had observed that maize yield in rotation with leguminous and non-leguminous cover crops increased by 12%, after 10 years, when compared with fallow. Fan et al. (2021) have showed, in a meta-analysis study, that cover crop increases by 9.7% on average the crop yields in comparison with fallow and these results are influenced by cover crop type (leguminous or non-leguminous), growing season and N input. The same authors observed that gains promoted by cover crop rotation are smaller with the increase in N fertilizer application.

The lack of effects of cover crop rotation on maize yield, observed in our conditions for the systems with *Crotalaria juncea, Pennisetum glaucum* and *Urochloa ruziziensis*, was also previously described. Kramberger et al. (2009), in an experiment with winter cover crops-maize rotation system, had observed that leguminous and non-leguminous species had no effect on maize yield in comparison to fallow, depending on the field experiment. Similar to our findings, Qin et al. (2021) in an experiment with maize-cover crop rotation system, had reported that legume cover crops did not affect maize yield.

Abdalla et al. (2019) describes, on a critical review study, the potential disadvantages of leguminous and non-leguminous cover species on main crop yield, observing a decrease of 4% on average. The authors suggest concern to management practices, especially to soil and climatic conditions. Cover crops can cause temporarily N deficiency for main crop, because of N immobilization, especially to those crops with residues with high C/N ratio, affecting main crop yield (Fageria et al., 2005). This might have occurred for the *Urochloa ruziziensis* tested in our conditions, showing a 34.5 kg ha$^{-1}$ N lower accumulation in grains compared to *Cajanus cajan* rotation. In the current study, *Urochloa ruziziensis* also showed the lowest yields in the 2019/2020 season, comparable to fallow and lower than the other cover crops systens.

Regarding this issue, management decisions about the introduction of cover crops must account for farm profitability and environmental sustainability (Kramberger et al., 2009), considering the ecosystem services provided by cover crop rotation systems (Bowles et al., 2017). The management of cover crop in rotation systems promote multifunctionality and the service interactions can be synergistic or antagonistic, with positive or negative effects on the main crop (Finney et al., 2017).

The N inputs are expected to affect crops productivity, since N fertilizer recovery efficiency is directly associated to basal and topdressing N application rate and timing (Shao et al., 2023). However, the N fertilizer use efficiency can decrease if the N rate was excessive, contributing to losses of N and environmental issues (Salazar et al., 2021), and must be avoided in agricultural systems.

The long-term experiment had shown, since the establishment, effect of the interaction between rotation systems with cover crops and N topdressing application. However, in the last two growing seasons with maize cultivation (2018/2019 and 2019/2020), it was observed a decrease in yield gains (comparison with treatments with N topdressing and without N topdressing), possibly due to long period testing the same cover species in the same plots (Supplementary Figure 7). The advantages of a rotation systems can be reduced since continuous cultivation of the same crop species is delivering a succession system instead an integrated rotation system.

### 3.4.2. Effect of cover crop rotation systems on N uptake by maize

The introduction of cover crops in rotation systems with maize, especially leguminous species, can improve the N uptake by the main crop. Gabriel & Quemada (2011) have reported increases in N uptake by maize and N content in grain in a rotation system with *Vicia villosa*. On the other hand, the positive effect of grass cover crop on N accumulation by maize is also described on literature. Rocha et al. (2020), in a maize-forage cropping system, have shown that the amount of N exported to grains was higher in a maize-*Panicum maximum* rotation system when compared to systems with *Urochloa ruziziesis* and *Urochloa brizantha*.

However, similar to our findings, other studies have shown neutral effects on N uptake by maize in cover crop rotation systems. Salazar et al. (2021) did not observed effect of cover crops on N content in maize compartments when compared to fallow. These results can be explained by the possible lack of synchrony of cover crops residue N release and maize N demand (Nevins et al., 2020). N is accumulating over the years as soil organic N due to current-year fertilization (Van Meter et al., 2016) and data from literature shows that N stabilization, release and uptake by crops is occurring in low rates (Yan et al., 2014).

Moreover, maize N uptake after cover crops cultivation, when the N fertilizer rate applied attends the crop's demand, usually does not present differences between rotation systems with cover crops and fallow (Miguez & Bollero, 2005). It's important to highlight that in the last two growing seasons, in our experimental conditions, the maize straw input was lower compared with the past years, with possibly decrease in N cycling.

### 3.4.3. Effect of cover crop rotation systems on N fertilizer recovery and N fertilizer use efficiency

The establishment of cover crops can enhance N recycling, with reduce of N losses (Rosolem et al., 2017), and can increase N availability to subsequent crop through N mineralization of the residues (Salazar et al., 2021). On the other hand, cover crops like ruzigrass (*Urochloa ruziziensis*), can decrease the yield and N uptake by the main crop, and consequently, reduce N fertilizer use efficiency due to N immobilization in organic N forms (Gabriel et al., 2016; Rocha et al., 2019).

In our experimental conditions, cover crops did not affect fertilizer N recovery by maize. This was associated to comparable values of N derived from fertilizer in all treatments. Similar to our results, Gabriel et al. (2016) did not observed effect of leguminous and non-leguminous cover crops rotation system on N fertilizer use efficiency. The authors showed that even if the cover crop is a leguminous, that incorporates N via biological N fixation, the amount of N derived from fertilizers was not improved when compared to treatment without cover crops (fallow) or with grass cover crops. A possible reason pointed by the authors is that higher N fertilizer use efficiency is obtained when the soil N availability is low, which does not apply to our experimental condition, with annual applications of N in maize crop according to recommendation.

## 3.5. Conclusion

In our experimental conditions, the maize-cover crops rotation did not improved N fertilizer use efficiency. The introduction of *Cajanus cajan* in rotation system improved maize yield and N accumulation in the grains compared to *Urochloa ruziziensis* or fallow. This effect can be associated to an improvement in N availability through N fixation and subsequent N mineralization, rather than an increase in the N fertilizer recovery by main crop. The reduction in maize yield obtained in the last two seasons in the current study, in addition to the adequate N rates supplied over the years (130 kg $ha^{-1}$ N), might be the reason for the lack of significant effect continuous cover crops cultivation on N fertilizer recovery and N fertilizer efficiency.

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# 4 FINAL CONSIDERATIONS

It’s well known the concerning about the economic and environmental issues caused by an unappropriated management of N fertilizers. The losses can affect the soil, air and water quality and can also affects crop yields, reducing gains, especially under cereal crops cultivation. The strategies to improve crop yields combined with rational use of inputs, specifically synthetic N fertilizers, need to be enforced to reduce the impacts caused by agriculture.

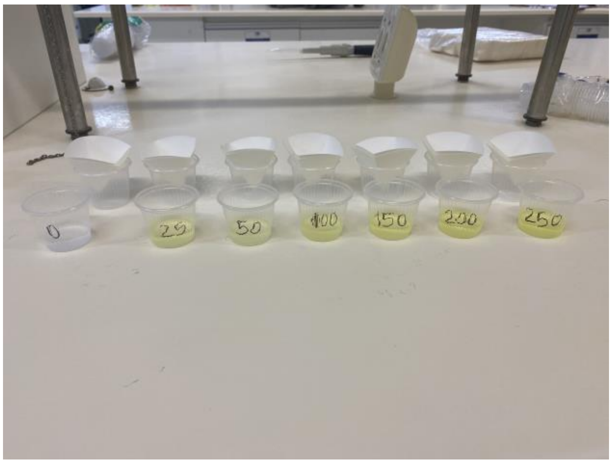
The introduction of leguminous or non-leguminous cover crops in rotation is one of the strategies that can help mitigate N losses and, consequently, provide a better fertilizer use efficiency. These species, when properly adopted, can impact on organic N input and N cycling, that affects in short and long-term crop yields allowing a reduction in N-fertilizer rates without yield losses. Moreover, there are many other advantages crop species diversification in agricultural systems, such as improvement of soil microbiota, reduction in losses, soil organic matter builds up, and hence the soil health.

The contribution of potentially mineralizable soil N in rotation systems with cover crops need to be accounted for to improve the N recommendation to main crops. This improvement has potential to reduce the spendings with N fertilizer inputs, considering the high contribution of soil N supply to crop nutrition, as demonstrated in this study and in many other in the literature.

We have shown here that biological soil analysis (specially the beta-glucose activity) and the modified ISNT-method, using a less strong alkaline solution (0.5 versus 2.0 M NaOH of the original method), were the best predictors of soil N mineralization and maize yield in a system with nine years of cover crop rotation. Both methods accounted for most of the variability of maize grain yield in the component principal analysis, demonstrating a potential to indicate a mineralizable fraction of soil organic N. However, our study did not find an improvement of N fertilizer recovery or N use efficiency by maize even under nine years of cover crop rotation cultivation. This is an indicator that cover crop cultivation might improve agricultural yields through other mechanisms rather than by improving the recovery of N fertilizers. One of the possibilities is by building up soil organic N reserves through biological N fixation, as well as other ecological services not evaluated in this study.

# APPENDIX

## APPENDIX A. Supplementary material



Supplementary Figure 1. Standard curve for p-nitrofenol.

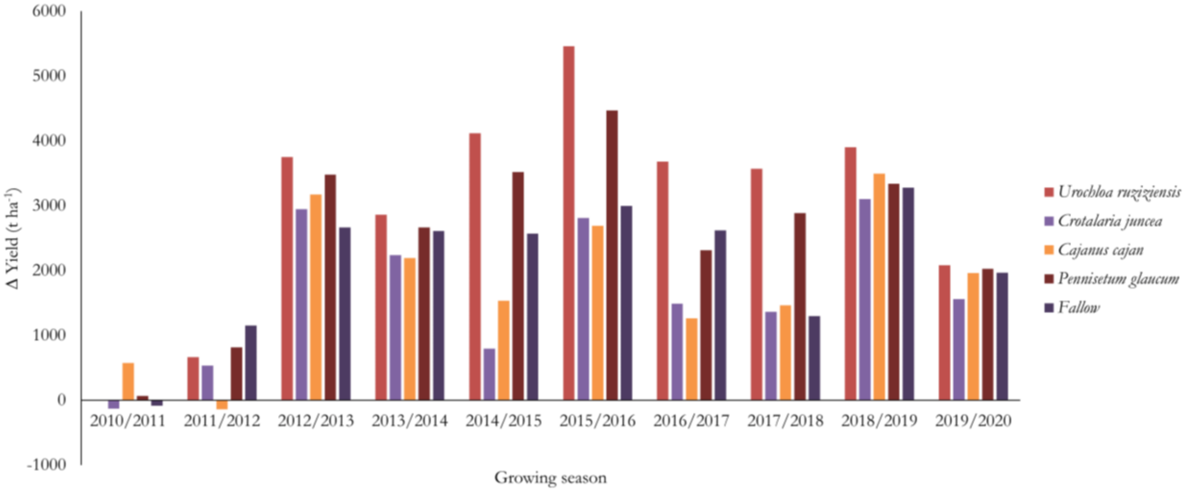
Supplementary Figure 2. Absorbance microplate reader performing nitrite and nitrate analysis.

Supplementary Figure 3. Samples of active soil carbon prepared to absorbance measurement.

Supplementary Figure 4. Alkaline digestion in Mason jars heated in an electric hot plate (ISNT analysis).

Supplementary Figure 5. Side dress application of urea$^{15}$-N.

Supplementary Figure 6. Central row defined to the application of $^{15}$N-labeled urea.



Supplementary Figure 7. Δ maize yield (with N topdressing application – without N topdressing application) (t ha$^{-1}$) in a maize-cover crop rotation experiment conducted for 10 years. Planaltina, DF.