

University of São Paulo
“Luiz de Queiroz” College of Agriculture

Carbon and nitrogen pools in the soil profile after seven years of nitrogen management and cover crop rotation in an Oxisol from Southern-Brazil

Acácio Bezerra de Mira

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

Piracicaba
2020

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**Carbon and nitrogen pools in the soil profile after seven years of nitrogen management
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versão revisada de acordo com a resolução CoPGr 6018 de 2011

Advisor:
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2020

Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Mira, Acácio Bezerra de

Carbon and nitrogen pools in the soil profile after seven years of nitrogen management and cover crop rotation in an Oxisol from Southern-Brazil / Acácio Bezerra de Mira. - - versão revisada de acordo com a resolução CoPGr 6018 de 2011 - - Piracicaba, 2020.

62 p. : il.

Tese (Doutorado) - - USP / Escola Superior de Agricultura "Luiz de Queiroz".

1. Adubação nitrogenada 2. Carbono lábil 3. Nitrogênio dissolvido 4. Acúmulo de nitrogênio no subsolo I.

CDD 631.4
M671c

ACKNOWLEDGEMENTS

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001 and for Agrisus Foundation – Sustainable Agriculture – Project number 2251/17.

I am thankful to “Luiz de Queiroz” College of Agriculture (ESALQ-USP), to the Department of Soil Science, and to the Soil Science and Plant Nutrition Graduate Program for providing me the opportunity of study both, master’s and doctoral course, and offering me indispensable resources during the Doctorate program.

I am grateful to the ABC Foundation (in the person of Dr. Gabriel Barth) and to the International Plant Nutrition Institute - IPNI (in the person of Dr. Luis Ignacio Prochnow and Dr. Eros Francisco) to have provided access to the experimental field and support with soil sampling.

I would like to thank Professor Marcos Kamogawa for have provided me overtime access to his laboratory and have helped me with the Flow Injection Analysis.

I also express my gratitude to Dr. João Luis Nunes Carvalho and his crew at the Brazilian Biorenewables National Laboratory (LNBR) for performing the total carbon and nitrogen analysis in soil samples.

I am grateful to Caio Diniz, Thales Sattolo, Catia Simon, Douglas Viana, Brena Ferneda, Bianca Machado, and Mirella Campos for the help provided in field and laboratory activities.

I am also deeply grateful to Ana Preczenhak for the indispensable support provided in the finalization of this manuscript.

I would like to express my truly gratitude to my advisor Professor Rafael Otto, for the receptivity, support, kindness, and trust to me dedicated since I came to the University of São Paulo, and for the invaluable advices and knowledge transmitted to me, which significantly contributed to my scientific and personal development.

Last, and above all, I am very grateful to the Lord, to my family and to my all friends (specially the closest ones Aijanio, Jhonny, Dener, Doug, Baiano, Fred, Ruan, Josimar, Galo-missioneiro, Bráci-k, Bãozin, Alpino, Promovido and Safena) for keeping me motivated, giving me support, relieving my load, and keeping my heart joyful, even in my darkest moments. I would have not made it through without them.

EPIGRAPH

May God bless and keep you always
 May your wishes all come true
 May you always do for others
 And let others do for you
 May you build a ladder to the stars
 And climb on every rung
 May you stay forever young
 (...)

May you grow up to be righteous
 May you grow up to be true
 May you always know the truth
 And see the light surrounding you
 May you always be courageous
 Stand upright and be strong
 May you stay forever young
 (...)

May your hands always be busy
 May your feet always be swift
 May you have a strong foundation
 When the winds of changes shift
 May your heart always be joyful
 May your song always be sung
 And may you stay forever young
 Bob Dylan, Forever Young

What song the Sirens sang, or what name
 Achilles assumed when he hid himself among
 women, although puzzling questions are not
 beyond all conjecture.

Sir Thomas Browne, Urn-Burial

My name is Nobody.

Homer, The Odyssey

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RESUMO

Frações de carbono e nitrogênio no perfil do solo após sete anos de manejo de adubação nitrogenada e rotação de culturas de cobertura em um Latossolo Vermelho no Sul do Brasil

O cultivo do milho na região sul do Brasil baseia-se uso intensivo de adubação nitrogenada para maximizar a produtividade. A aplicação excessiva de nitrogênio (N) pode afetar negativamente as dinâmicas do carbono (C) do N no solo. No entanto ainda existe controvérsia na literatura a respeito do efeito da adubação nitrogenada sobre a matéria orgânica do solo (MOS), e o resultado a longo prazo da aplicação contínua de doses fixas de N na distribuição de C e N no perfil do solo é ainda pouco estudado em Latossolos subtropicais. Neste estudo investigamos o efeito de sete anos de adubação nitrogenada na distribuição de frações de C e N no perfil de um solo sob diferentes rotações de cultura em sistema de plantio direto. Nossa hipótese é que a aplicação de elevadas doses de N promove o acúmulo de N no subsolo e reduz o acúmulo de C no perfil do solo sob sistema de plantio direto, e que esse efeito pode ser intensificado quando leguminosas são usadas como cultura de cobertura. O experimento de campo tem sido conduzido há sete anos sob o delineamento de blocos casualizados em um arranjo de parcelas subdivididas. Duas rotações bianuais cultivadas sob as seguintes sucessões inverno/verão foram alocadas nas parcelas: (i) aveia preta/milho, trigo/soja; e (ii) ervilha forrageira/milho, trigo/soja. Sempre que o milho foi cultivado, doses de N (0, 70, 140, 210 kg ha⁻¹ N) foram aplicadas em cobertura (estádio V4). Amostras de solo até 1.0 m de profundidade foram analisadas para N total (TN), C orgânico total (TOC), N inorgânico solúvel (SIN), N orgânico solúvel (SON), C mineralizável (MINC) e C oxidável por permanganato (POXC). A densidade do solo foi medida para calcular os estoques C e N. Apesar do potencial de fixação biológica de N da ervilha forrageira, a adubação nitrogenada aumentou o acúmulo de SIN no subsolo apenas na rotação com aveia preta. A rotação com ervilha forrageira também apresentou menor acúmulo médio de N no subsolo. Isto provavelmente está associado à maior extração de N devido às maiores produtividades do milho cultivado após a ervilha forrageira. A adubação nitrogenada resultou em maiores estoques de C na camada superficial do solo em ambas as rotações, mas reduziu o acúmulo de C nas camadas mais profundas na rotação com ervilha forrageira. A distribuição homogênea de SON no perfil do solo em ambas as rotações e a redução nos teores de POXC abaixo de 0.2 m de profundidade indicam que os menores estoques de C no subsolo estão mais associados a um enraizamento mais superficial quando doses elevadas de N são aplicadas, do que a um possível *efeito priming*. A inclusão de uma leguminosa como cultura de cobertura antes do milho em rotações de milho/soja tem potencial de aumentar a produtividade do milho e reduzir a demanda por adubação nitrogenada logo no primeiro ano de cultivo. Esta redução é recomendada, uma vez que a aplicação contínua de altas doses de N pode reduzir o acúmulo de C e aumentar o acúmulo de formas inorgânicas de N no subsolo de Latossolos sob plantio direto.

Palavras-chave: Adubação nitrogenada, Carbono lábil, Nitrogênio dissolvido, Acúmulo de nitrogênio no subsolo

ABSTRACT

Carbon and nitrogen pools in the soil profile after seven years of nitrogen management and cover crop rotation in an Oxisol from Southern-Brazil

Maize production in Southern Brazil relies in intensive nitrogen (N) fertilization to maximize maize yield. Excessive N fertilization may negatively influence soil carbon (C) and N dynamics. However, there is still controversy in literature about the effect of N fertilization on soil organic matter (SOM), and the long-term effect of continuous N rates on C and N distribution in soil profile is still understudied in subtropical Oxisols. In this study we investigate the effect of seven-year N fertilization on C and N distribution in a no-till soil profile under different cover crop rotations. We hypothesize that applying high N rates in maize promotes subsoil N accumulation and depletes soil C storage, and this effect may be more intense when legumes are used as cover crop. A field experiment has been conducted for seven years, under a randomized block design, in a split-plot arrangement. Two biannual crop rotations were allocated to the main plots with the following winter/summer successions: (i) black oat/maize, wheat/soybean; and (ii) field pea/maize, wheat/soybean. Nitrogen rates (0, 70, 140, 210 kg ha⁻¹ N) were top-dressed to the subplots whenever maize was grown (V4 stage). Soil samples until 1.0-m deep were analyzed for total N (TN), total organic C (TOC), soluble inorganic N (SIN), soluble organic N (SON), mineralizable C (MINC), and permanganate oxidable C (POXC). Soil bulk density was measured to calculate C and N stocks. Despite field pea's potential of biological N fixation, top-dress N fertilization increased subsoil SIN accumulation only in black oats' rotation. Field pea's rotation also showed a lower average subsoil N accumulation. This is probably associated to a higher N extraction due to the improved grain yields of maize grown after field pea. Nitrogen fertilization resulted in higher topsoil C stocks in both crop rotations but decrease subsoil C storage in field pea's rotation. The homogeneous SON distribution in soil profile in both crop rotations and the reduced POXC concentration below 0.2 m deep indicate that lower subsoil C stocks are more related to a shallower plant rooting when high N rates are applied than to a possible *priming effect* induced by extra N availability. Including a legume as winter cover crop before maize in maize/soybean rotations has potential to improve maize grain yield and reduce N fertilizer demand right in the first year. This reduction is recommended since applying high N rates may decrease soil C storage and increase subsoil N enrichment in Oxisols under no-tillage.

Keywords: Nitrogen fertilizer, Labile carbon, Dissolved nitrogen, Subsoil nitrogen enrichment

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LIST OF ABBREVIATIONS

BaCl ₂ – Barium chloride	mL – Milliliter
BaCO ₃ – Barium carbonate	mM – Millimolar
C – Carbon	mm – Millimeter
°C – Celsius degree	MINC – Mineralizable carbon
CaCl ₂ – Calcium chloride	N – Nitrogen
CO ₂ – Carbonic gas	N ₂ – Nitrogen gas
DOC – Dissolved organic carbon	NAGase – <i>N</i> -acetyl-β-D-glucosaminidase
DOM – Dissolved organic matter	NaOH – Sodium hydroxide
DON – Dissolved organic nitrogen	NH ₄ ⁺ – Ammonium
FIA – Flow injection analysis	NH ₃ – Ammonia
g – gram	nm – Nanometer
<i>g</i> – gravitational force equivalent	NO ₃ ⁻ – Nitrate
HCl – Hydrochloric acid	NO ₂ ⁻ – Nitrite
H ₃ BO ₃ – Boric acid	NH ₄ NO ₃ – Ammonium nitrate
HSD – Honest significant difference	O-M-W-S – Oat, Maize, Wheat, Soybean
KMnO ₄ – Potassium permanganate	P-M-W-S – Pea, Maize, Wheat, Soybean
K ₂ SO ₄ – Potassium sulfate	P – Phosphorus
K ₂ S ₂ O ₈ – Potassium persulfate	POXC – Permanganate Oxidable Carbon
h – Hour	SIN – Soluble inorganic nitrogen
ha – Hectare	SOM – Soil organic matter
kg – Kilogram	SON – Soluble organic nitrogen
L – Liter	TOC – Total organic carbon
m – Meter	TN – Total soil nitrogen
M – Molar (mol L ⁻¹)	TSN – Total soluble nitrogen
Mg – Megagram	WHC – Water-holding capacity
mg – Milligram	

1 RATIONALE

Excessive nitrogen (N) fertilization is widely known to promote N leaching and subsoil N enrichment (Ladha et al., 2005). However, in recent years major concerns and contentious discussions have arisen whether N fertilization may negatively affect carbon (C) sequestration and soil organic matter (SOM) storage in long term. While some authors claim that the increase in biomass production promoted by N fertilization result in soil C accumulation (Deng et al., 2018; Ladha et al., 2011) another authors state that N surplus boost microbial activity, stimulate SOM mineralization and deplete soil C stocks (Heitkötter et al., 2017; Khan et al., 2007; Mulvaney et al., 2009). There is also reports of no changing effect of N fertilization on soil C stocks (Kintché et al., 2014; Sattolo et al., 2017) and even authors that associated application of high N rates to a decrease in soil C storage threshold (Gao et al., 2013). Most disagreements about the effect of N fertilization on soil C stocks occur when deep soil layers are investigated. Since N is involved in concurrent parallel process of SOM accrual and depletion, a broad range of physical, chemical, biological and physiologic phenomena can be used to support both hypotheses.

Studies reporting no-change and negative effect of N fertilization on SOM claims that although adequate N nutrition improve plant growth, extra litter production little contributes to soil C accrual. In highly fertilized, fields plants usually allocate most of extra biomass to aboveground growth (Fageria and Moreira, 2011; Lu et al., 2011) and reduce subsoil root density, because of the natural behavior of plants increasing rooting in well fertilized zones (Drew, 1975; Otto et al., 2009). This change may be disadvantageous to soil C improvement since root biomass is known to be more effective than aboveground biomass in promoting soil C accumulation (Balesdent and Balabane, 1996; Bolinder et al., 1997; Santos et al., 2011). In subsoil lower oxygen levels, physical protection, and sorption to mineral surfaces contribute to a greater stabilization and long-term soil C storage (Jastrow et al., 2007; Lehmann and Kleber, 2015).

High N fertilization is also reported to increase dissolved organic matter (DOM) translocation to deeper soil horizons (Salazar et al., 2019). This process may contribute to long-term SOM depletion since some organic acids and labile C molecules present in DOM can release more stabilized C from the mineral bounds and make it available to microbial mineralization (Keiluweit et al., 2015; Kleber et al., 2015). Concomitantly, easily decomposable C molecules existing in DOM or in root exudates provide the activation energy

to C-limited microbial communities metabolize more stable SOM pools in deep soil layers (Bernal et al., 2016; Chen et al., 2014; Fontaine et al., 2007; Heitkötter et al., 2017; Shahzad et al., 2018). This effect, known as stoichiometric decomposition theory (Chen et al., 2014), is more expressive in subsoil horizons with high SOM levels (Shahzad et al., 2018), may negatively impact soil C storage (Khan et al., 2007) and is potentialized by soluble N availability in both organic or inorganic forms (Heitkötter et al., 2017; Jones et al., 2018). However, in a review on mineral-organic association dynamics Kleber et al. (2015) suggests that when soil aggregation favors preferential downwards waterflow, DOM can migrate largely unaltered through soil profile to be adsorbed by highly reactive amorphous mineral phases at depth and contribute to soil C accrual.

In contrapose to stoichiometric decomposition theory, several studies reported that microbial biomass decomposes part of the stable SOM pool to acquire N for metabolize labile C only if there was a shortage in N availability from more labile soil N pools. This trend is known as microbial N mining theory (Chen et al., 2014) and suggest that subsoil N enrichment may reduce soil C depletion by alleviating the microbial demand for N bound in stabilized SOM pools (Blagodatskaya and Kuzyakov, 2008; Kuzyakov, 2010; Kuzyakov et al., 2000). In addition, excess of inorganic N may also alter microbial community structure and induce a decline in soil microbial biomass, enzyme activities, and potential respiration with a consequent reduction in mineralization of stabilized soil C pools (Jones et al., 2018; Ramirez et al., 2012; Treseder, 2008). Based on the combination of those effects, Li et al. (2017) concluded that N-fertilization may increase the efficiency of C sequestration in soil.

Although N is usually limiting to microbial activity and may promote SOM mineralization if added alone to soil (Blagodatskaya and Kuzyakov, 2008; Kuzyakov et al., 2000) a number of studies report that balanced apports of crop residues and inorganic N did not induce priming effect and even reduced SOM mineralization (Bernal et al., 2016; Li et al., 2017; Qiu et al., 2016). In a global meta-analysis with 570 observations in a wide range of soil, climate and land use type in China, Deng et al. (2018) reported that the extra biomass production due to subsoil N enrichment, despite have increased C output by increasing soil respiration, resulted in a net increase of labile and total soil C pools. There are evidences that alteration of turnover time of different fractions of SOM does not necessarily implies in reduction of mean residence time of soil C (Paul, 2016) and N-induced increases in microbial activity and SOM mineralization may not result in soil C depletion (Deng et al., 2018).

In recent years, techniques yielding results with high molecular and spatial resolution have brought new understandings about the nature and properties of SOM and demonstrated that microbial transformation is an important pathway for stabilize fresh C inputs in SOM pools with longer residence time (Cotrufo et al., 2019; Haddix et al., 2020; Kögel-Knabner and Rumpel, 2018; Lehmann and Kleber, 2015; Stockmann et al., 2013). Liang et al. (2017) demonstrate that in long term, the microbial “entombing effect” can overcome the short-term priming effect and once the microbial products are stabilized by the interaction with soil minerals it significantly contributes to SOM accrual. Additionally, Cotrufo et al. (2019) has demonstrated that the process of persistent C sequestration in more stable SOM pools is strongly dependent of N availability in a wide range of forest and grassland soils.

Most of controversy found in literature about the effect of N fertilization on SOM must be derived from the ambiguous effect of N on agricultural soils, where N inputs alters the turnover dynamics of different SOM pools at the same time that it increases C inputs through biomass production. There are several good reviews on the mechanisms of SOM building or consumption (Jastrow et al., 2007; Kögel-Knabner and Rumpel, 2018; Kuzyakov, 2010; Kuzyakov et al., 2000; Tiemann and Grandy, 2015) showing that the effect of N fertilization on those mechanisms are far more complex than established by individual studies and very influenced by several factors like climate, management, pedogenetic process, soil mineralogy, fertilization timing, N source, crop rotation, and residues quality. Most studies use short incubations and quantify enzymatic activities and CO₂ emission to investigate the effect of readily available compounds on the SOM pools. However, conclusions about the effect of N fertilization soil C stocks based on short time analysis under controlled conditions may often be biased by the study approach since this poorly represents the complex site dynamics in field conditions (Li et al., 2017). Disrupting the structure of subsoil samples before incubation affects subsoil microbial communities and significantly increases SOM mineralization which may lead to overestimated results (Salomé et al., 2010). Moreover, under field conditions plant growth affect SOM transformations not only by root exudation but also by competing directly with microorganisms for available N (Jones et al., 2018; Kuzyakov and Xu, 2013), absorbing soil water, changing water flow, inducing simultaneous formation and breakdown of soil aggregates, and influencing drying and rewetting dynamics. Additionally, the N content in crop residues may affect microbial communities composition (Frasier et al., 2016), regulate the dynamics of N immobilization/mineralization (Deng and Tabatabai, 2000; Vargas et al., 2005) and N mining from SOM pools (Sanchez et al., 2002).

Depending on the crop species, plant rooting may influence the movement of N through soil profile (Kušlienė et al., 2015; Salazar et al., 2019) and affect soil C and N dynamics through distinct mechanisms. Legumes root system inputs high quality residues into the soil profile below the fertilized layer and hold high microorganism biodiversity in rhizosphere which contribute to stabilize C through soil aggregation, biochemical transformation, and interaction with soil matrix (Frasier et al., 2016; Tiemann et al., 2015). Moreover, legumes provide large inputs of biological fixed N (Boddey et al., 1997; Ladha et al., 2005) and allow subsequent crops to explore the soil below the usual fertilized layer which stimulates N uptake from deeper soil layers and contributes to C deposition into the soil profile. Poaceous, in its turn, are widely reported to be more efficient than legumes in scavenge N and can effectively recover N stored below the surface layer (Albuquerque et al., 2015; Gabriel et al., 2016; Ladha et al., 2005; Salazar et al., 2019; Thapa et al., 2018; van Kessel et al., 2009). Poaceous also allocate proportionally more belowground biomass in its bulky fasciculate root system than tap-rooted species does (Redin et al., 2018) which may contribute to build larger soil C stocks. However, those are not strict rules since environmental factors like weather and soil texture may affect crop rooting and, consequently, C inputs and N depletion efficiency (Thapa et al., 2018; Thorup-Kristensen et al., 2003).

The belowground C and N dynamics are strongly influenced by soil chemical and physical features, land use, and regional climate (Deng et al., 2018; Kleber et al., 2015; Lehmann and Kleber, 2015) and the same management practices may lead to very different responses between distinct agroecosystems. In carbonaceous soils, like Mollisols and certain Inceptisols, acidification may disrupt soil aggregates and release occluded SOM and calcium stabilized organic molecules. In such conditions subsoil N enrichment by nitrate (NO_3^-) induce an intense mineralization of native SOM and depletion of soil C stocks in deep soil layers (Kögel-Knabner and Rumpel, 2018; Mulvaney et al., 2009; Rowley et al., 2018; Wuddivira and Camps-Roach, 2007). In opposite, in highly weathered Oxisols and Ultisols, the positively charged oxidic clays can adsorb NO_3^- ions, thus reducing N leaching, and the acidification promoted by surface N fertilization must be beneficial to C stabilization by stimulating the formation of strong innersphere bonds between OM and mineral surfaces with variable charge (Kleber et al., 2015; Rowley et al., 2018; Treseder, 2008). However, in highly weathered soils significant N losses may take place as dissolved organic N (DON) (Lehmann et al., 2004; van Kessel et al., 2009) which once transported to deep soil layers is hardly recovered by crop roots, especially in soils under no tillage systems, where the subsoil soil acidity hinders deep root

growth, and surface liming is not efficient to neutralize soil acidity below the superficial layer (Caires et al., 2016; Ernani et al., 2004; Melgar et al., 1992; Sierra et al., 2003). Therefore, under those conditions, inadequate N fertilization boosts the risk of subsoil N enrichment without proportional root biomass deposition which can induce SOM depletion if subsoil microbial communities were N limited.

Most of studies exploring the effect of N fertilization on SOM are performed in maize (*Zea mays* L.) fields or in crop rotations where maize is included. In modern maize farms with yields above 10 Mg ha⁻¹, N uptake by maize plants ranges from 250 to 340 kg ha⁻¹ (Bender et al., 2013; Resende et al., 2016). Even though SOM mineralization is known to be the major N source to maize in one growing season, (Abdelrahman et al., 2001; Gava et al., 2006; Kamukondiwa and Bergström, 1994), native N pools and atmospheric inputs are often insufficient to attain maize demands and large inputs of N fertilizers have been necessary to meet the shortage in soil-N supply and ensure high maize yields worldwide. In the past 30 years, the use of new maize hybrids with greater yield potential and shorter growth period have exponentially improved maize yield in tropical countries and increased the N fertilizer demand to supply the increased nutrient extraction. Brazil became the third largest maize producer in the world (USDA, 2020) and Brazilian Southern region holds the country's highest productivities in rainfed maize fields. In Southern Brazil, maize grain yield averaged 8.1 Mg ha⁻¹ in 2018/19 crop year (CONAB, 2020) with selected fields under adequate management practices supporting yields as high as 13 Mg ha⁻¹ (IPNI, 2017). Without effective means to estimate SOM mineralization most of maize growers in Brazil usually ignore the soil N pool and follow yield-based N fertilization programs in order to minimize the risk of N deficiency. In addition, to compensate the low recovering rates and eventual N losses, a surplus of N fertilizer is applied to meet maize absorption rate which may easily lead to excessive N fertilization and negatively impact soil C and N dynamics.

The long-term effect of different levels of N fertilization on SOM pools have been well documented in field trials under temperate climate. However, as reviewed by Ladha et al. (2011) and Lu et al. (2011), there are few studies investigating the long-term effect of N rates on the distribution soil C and N pools in Oxisols under subtropical climate where the mild temperatures and interactions between SOM and oxidic clay minerals must lead to different C and N dynamics when compared to temperate climates. In this study we explore the effect of seven-year N fertilization on C and N pools in an Oxisol profile under no-tillage which has been cultivated with crop rotations where legumes or non-legumes used as winter cover crops.

We hypothesized that long-term application of high N rates in maize increase subsoil N accumulation and depletes soil C storage, and this effect may be more intense in crop rotations where a legume is grown as winter cover crop before maize.

2 MATERIALS AND METHODS

2.1 The experimental site

The experimental site is part of IPNI Global Maize Project (IPNI, 2008) and has been conducted since 2011 in Southern Brazil at the experimental station of ABC Foundation for Agricultural Assistance and Technical Divulcation, in Ponta Grossa-PR, Brazil (25°00'46"S 50°09'06"W, 885 m). The climate at the site is Cfb according the Köppen–Geiger classification, a subtropical climate with cold winter (June–September), warm summer (December–March) and without dry season with annual precipitation from 1400 to 1550 mm. The annual mean air temperature is 17 °C, with monthly mean temperatures ranging from 13 °C in the coldest month, July, to 22 °C in the warmest, January (Aparecido et al., 2016). The soil is a Rhodic Kandiodox (Soil Survey Staff, 2014) with a clay content increasing from 324 to 447 g kg⁻¹ across the 1-m soil profile (Table 1).

Table 1. Average chemical and physical attributes of a no-till soil profile under seven-year crop rotations and N management in Southern Brazil.

Soil attributes	Soil depth (m)					
	0.0-0.1	0.1-0.2	0.2-0.4	0.4-0.6	0.6-0.8	0.8-1.0
pH†	4.7	4.5	4.7	5.0	5.2	5.3
SOC [g dm ⁻³]‡	24	17	14	10	9	7
P [mg dm ⁻³]§	54	60	8	<3	<3	<3
S [mg dm ⁻³]	5	6	9	12	26	27
Ca [mmol _c dm ⁻³]	23	12	7	8	9	8
Mg [mmol _c dm ⁻³]	11	3	3	4	5	4
K [mmol _c dm ⁻³]	3.6	1.9	1.3	1.6	1.4	1.0
Al [mmol _c dm ⁻³]	6	2	4	2	2	2
H+Al [mmol _c dm ⁻³]	52	58	47	34	28	25
CEC [mmol _c dm ⁻³]¶	89.6	74.9	58.3	47.6	43.4	38.0
BS [%]#	42	23	19	29	35	34
Sand [g kg ⁻¹]	660	598	576	541	487	533
Silt [g kg ⁻¹]	16	27	23	34	58	19
Clay [g kg ⁻¹]	324	376	402	426	455	447
BD [Mg m ⁻³]††	1.26	1.40	1.35	1.28	1.32	1.27

Chemical analysis following Raij et al. (2001)

† Soil pH by CaCl₂ 0.01 mol L⁻¹

§ P extracted by anion-exchange resin

BS, base saturation

‡ SOC, soil organic carbon

¶ CEC, cation exchange capacity

†† BD, bulk density

The field has been cultivated under no-till since 2007 with black-oat (*Avena strigosa* Schreb.), maize (*Zea mays* L.), wheat (*Triticum aestivum* L.) and soybean (*Glycine max* (L.) Merr.) in the following winter/summer succession: 2007-08 black-oat/soybean, 2008-09 black-oat/maize, 2009-10 wheat/soybean and 2010-11 black-oat/soybean. In April 2011, after soybean harvest, experimental plots were established to assess the effect of crop rotations on maize productivity and maize yield response to N fertilization. Two crop rotations were chosen to perform our studies, with the following winter / summer succession (Figure 1):

- (i) **O-M-W-S:** biannual rotation: *year 1* - black-oat (cover crop) / maize (grain yield)
year 2 - wheat (grain yield) / soybean (grain yield)
- (ii) **P-M-W-S:** biannual rotation: *year 1* - field-pea (cover crop) / maize (grain yield)
year 2 - wheat (grain yield) / soybean (grain yield)

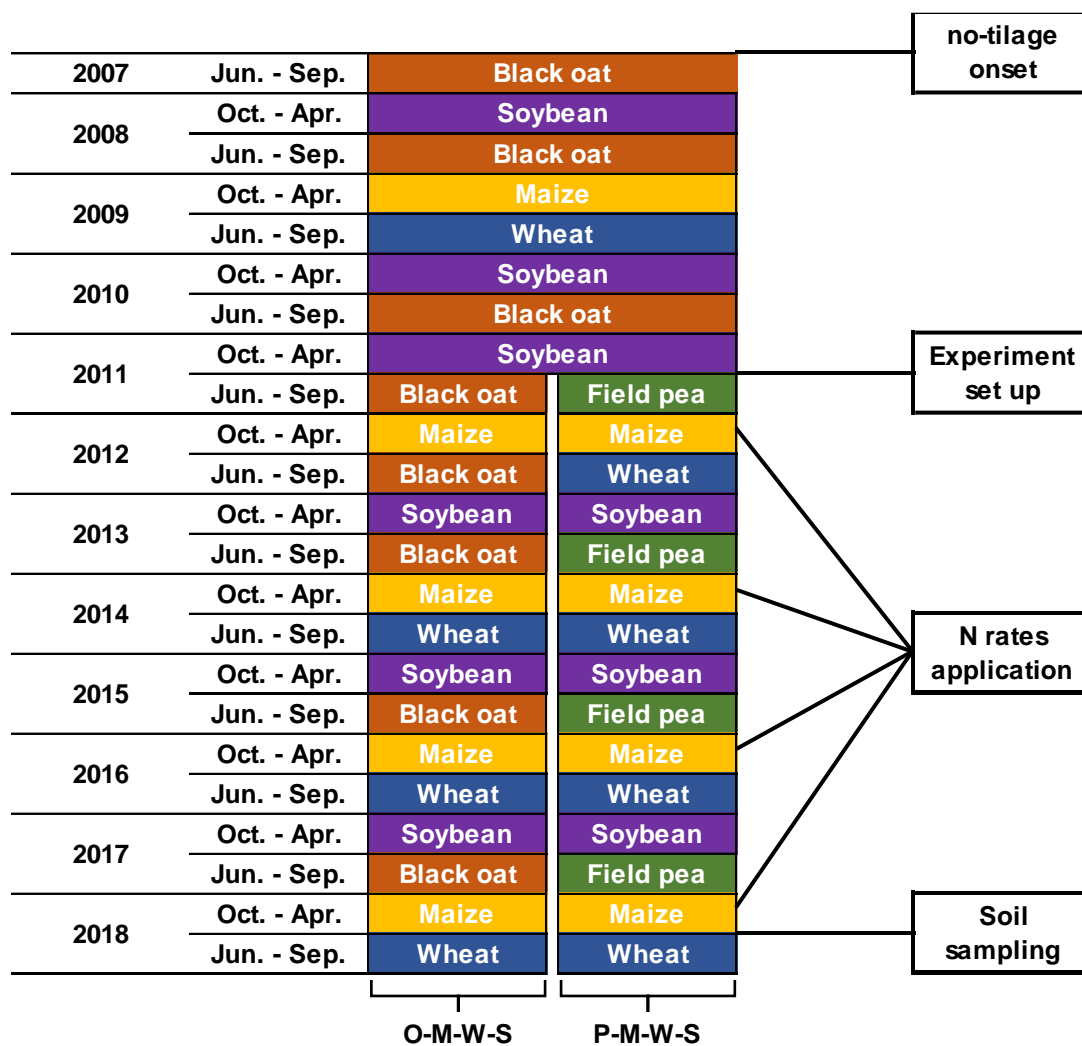


Figure 1. Timeline of crop rotation systems since no-tillage implementation in an agricultural field in Southern Brazil, where maize is grown biannually after different winter cover crops (black oat and field pea) and under four N top-dressing rates (0, 70, 140, and 210 kg ha⁻¹ N).

The plots were laid out under a split-plot arrangement, with four replications. The crop rotation models were allocated to the main plots (9.5 m wide by 36 m long) while the subplots (9.5 m wide by 9.0 m long) consisted in three top-dressed N rates (70, 140 and 210 kg ha⁻¹ N) plus a control without N top-dressing, totaling 192 subplots. The N rates were broadcast to maize at V4 stage in a single application every season when maize was grown using urea as N source and without incorporation. Additionally, all maize plots received 40 kg ha⁻¹ N (urea) in-furrow at sowing.

Maize and soybean (the summer crops) were sown with a spacing between rows of 0.8 m and 0.4 m, respectively. The winter crops were sown in the inter-row space of summer crops with a 0.17-m spacing between rows. The cultural traits (weed, pests and disease control) and fertilization for each crop along the years followed the regional pattern and usual recommendations to promote adequate plant development. In the winter season of 2012-13 crop year, O-M-W-S plots were exceptionally cropped with black oats instead wheat. In the following years, wheat was grown in the winter before soybean and black oats grown with catch crop purpose previous to maize (cash crop) every two years adding crop residues with C:N ratio higher as 35:1 (Aita et al., 2001), in a low natural N input but a high C input. P-M-W-S rotation in its turn stands as a large natural C and N input system with the field pea (*Pisum sativum* var. arvense (L.) Poir), a legume cover crop with green manure purpose, grown every two years previously to maize. Field pea has a potential to add from 30 to 180 kg ha⁻¹ N in the soil through biological N fixation (Carranca et al., 1999; Rennie and Dubetz, 1986).

2.2 Soil sampling

Soil sampling was performed right after the maize harvest in April 2018 to minimize the effect of the previous cover crop and quantify the N left in soil after the maize cultivation. Soil samples were collected in each and subplot in three positions (under maize row, at 0.2 m and 0.4 m away from the maize row) at the depths of 0.0-0.1, 0.1-0.2, 0.2-0.4, 0.4-0.6, 0.6-0.8 and 0.8-1.0 m. To make each composite sample until 0.6 m deep, 12 points were sampled using probes. From 0.6-1.0 m deep, three points were sampled using Dutch augers. The fresh samples were packed frozen at -18 °C at the end of the day, transported to the laboratory in boxes containing ice, and kept frozen (-18 °C) until analysis.

Frozen soil samples were defrosted under room temperature and divided into two portions. The first was used fresh under field moisture to perform soluble N analysis. To express

the element concentration over a dry basis, soil moisture was estimated by measuring the weight loss of a 10-g sub-sample of each fresh sample after a 24-h oven dry at 105 °C. The second portion was oven-dried at 65 °C for 72 h, passed through a 2-mm sieve and stored in PVC screw-capped flasks for POXC and MINC analysis. To characterize soil attributes in the overall experimental, 5-g aliquots from each one of the 192 stored soil samples were combined per depth and sent to the laboratory. A 10-g aliquot from the stored dry soil was finely ground and sieved (<0.149 mm), before submitted to total C and total N analysis.

Bulk density for each soil layer was measured using the core method (Grossman and Reinsch, 2002). For this purpose, undisturbed soil cores were collected 0.2 and 0.4 m away from the maize row at one subplot of each main plot, totaling 16 points in the whole field trial. In each point the soil cores were taken by inserting 5-cm high stainless-steel cylinders (98 cm³ of inner volume) centered in the depths of 0.05, 0.15, 0.3, 0.5, 0.7 and 0.9 m to represent the layers 0.0-0.1, 0.1-0.2, 0.2-0.4, 0.4-0.6, 0.6-0.8 and 0.8-1.0 m respectively. The soil inside each cylinder was carefully removed, stored in small paper boxes, oven-dried at 105 °C for 24 h and weighed in a precision scale (error = 0.01 g). The dry soil mass was divided by the inner core volume and the bulk density calculated and expressed in Mg m⁻³.

2.3 Laboratorial analysis

2.3.1 Soluble N

For the extraction of soluble N forms ($\text{N-NH}_4^+ + \text{N-NO}_3^- + \text{N-NO}_2^- + \text{SON}$), 8.0-g fresh samples were shaken with 40 mL of 0.5 M K_2SO_4 solution in 50-mL screw-capped polypropylene tubes (Falcon tubes) placed horizontally on a horizontal shaker at 180 rotation min⁻¹ for 30 min. After shaking, the mixture was centrifuged at $2.5 \times g$ for 5 min. The supernatant was filtered through a Nalgon® #3552 blue-ribbon quantitative paper filter previously leached with 5 mL of 0.5 M K_2SO_4 solution, in order to remove eventual NH_4^+ contamination. Since both soluble inorganic N (SIN) and soluble organic N (SON) were quantified in the same extract, we choose using 0.5 M K_2SO_4 instead of 1 M KCl as extractant because chloride interferes in SON determination by persulfate-oxidation (Cabrera and Beare, 1993) and 0.5 M K_2SO_4 is a well established SIN extractant (Mulvaney, 1996) adequate for both purposes. In addition, to prevent further chemical or biological transformations of SON forms in solution (Rousk and Jones, 2010) the extracts were stored at -80 °C until analysis.

After defrosting, soluble NH_4^+ concentration in extract was determined colorimetrically by Flow Injection Analysis (FIA), with an automated sample injection analyzer (ASIA, Ismatec, Zurich, Switzerland), according to the methods described by Kamogawa and Teixeira (Kamogawa and Teixeira, 2009). Peristaltic pumps pull 2 mL of the liquid sample and stores 50 μL of it in a sample loop. A multichannel three-way valve drives the 50- μL aliquot to a confluence where it is mixed with a 1 M NaOH solution inside an extended reaction loop where the elevated pH converts NH_4^+ into NH_3 . At the end of reaction loop, the mixture reaches a diffusion chamber where NH_3 diffuses through a polypropylene membrane into an indicator solution of bromocresol purple ($\text{pH} \approx 6.5$) flowing parallelly through of the diffusion chamber upper part. The NH_3 income raises the pH of the indicator solution and bromocresol purple turns from ruby-colored to purple. The indicator solution flows into a quartz cuvette where color intensity is measured by a photometric detector in absorbance mode ($\lambda = 605 \text{ nm}$). The N-NH_4^+ concentration was estimated by correlation between the area under the transient absorption peaks of the sample and area under the peaks of a calibration a curve of NH_4NO_3 with concentrations ranging from 0.5 to 10.0 $\text{mg L}^{-1} \text{ N}$.

As the method previously described quantifies exclusively NH_4^+ , for measuring SIN ($\text{NH}_4^+ + \text{NO}_3^- + \text{NO}_2^-$) concentration in extracts, a Zn+Cu reduction column was attached before the reaction loop in order to reduce all $\text{NO}_3^- + \text{NO}_2^-$ in solution to NH_4^+ . The same NH_4NO_3 standard curve was used for NH_4^+ and SIN quantification. In the first case, only N-NH_4^+ concentration was considerate, and the target concentrations ranged from 0.025 to 5.0 $\text{mg L}^{-1} \text{ N}$. In the last case, all N in solution was considered and the target concentrations ranged from 0.5 to 10.0 $\text{mg L}^{-1} \text{ N}$. The $\text{NO}_3^- + \text{NO}_2^-$ concentration was calculated as the difference between the SIN reading and NH_4^+ reading in the same extract. The soil N concentration under the respective SIN fractions was calculated as follows:

$$xSN_{(\text{mg kg}^{-1})} = \left[a \text{ mg of N L}^{-1} \right] \times \left(\frac{0.04 \text{ L solution} + \left(0.008 \text{ kg of moist soil} \times \frac{U}{100} \right)}{0.008 \text{ kg of moist soil} \times \left(1 - \frac{U}{100} \right)} \right) \quad (1)$$

were xSN is the N concentration in soil over a dry basis in the form of the respective analyzed fraction (NH_4^+ or $\text{NO}_3^- + \text{NO}_2^-$), $a \text{ mg of N L}^{-1}$ is N concentration in solution under the respective analyzed fraction, 0.04 L is the volume of 0.5 M K_2SO_4 used to extract N from soil sample,

0.008 kg is the amount of field moist soil reacted with K_2SO_4 solution, and U is the moisture in percentage of each individual sample.

SON was determined by the persulfate-oxidation procedure as described and optimized by Cabrera and Beare (1993) in which all N in extract is converted to NO_3^- . Briefly, 5 mL of 0.5 M $K_2S_2O_8$ + 0.375 M NaOH + 3% (m/v) H_3BO_3 solution were added to 5.0 mL of extract in 20-mL glass tubes. Tubes were immediately sealed tightly with screw caps containing intern soft seals, weighed, and autoclaved at 120 °C for 30 min. After cooling, tubes were weighed again to verify the necessity of concentration correction due to water loss. Weight alterations after autoclaving step were negligible and N recovery as NO_3^- of a 10-mg L^{-1} urea and ammonium nitrate standards yielded from 1.00 to 1.02 and from 1.06 to 1.10, respectively. Recoveries above 1.00 are common for this method and are attributed to oxidation of N_2 atmospheric in tube headspace.

The $N-NO_3^-$ concentration in persulfate-oxidized extracts was measured colorimetrically by FIA as $N-NH_4^+$ after a chemical reduction in a Zn+Cu reduction column, similar to the procedure adopted for SIN determination. The total soluble N (TSN) concentration was calculated similarly as SIN fractions as follows:

$$TSN_{(mg\ kg^{-1})} = \left[a\ mg\ of\ N\ L^{-1} \right] \times 2.0 \times \left(\frac{0.04\ L\ solution + \left(0.008\ kg\ of\ moist\ soil \times \frac{U}{100} \right)}{0.008\ kg\ of\ moist\ soil \times \left(1 - \frac{U}{100} \right)} \right) \quad (2)$$

where TSN is the soil N extractable by a 1-M K_2SO_4 solution ($NH_4^+ + NO_3^- + NO_2^- + SON$), expressed as a concentration in soil over a dry basis, 2.0 is the dilution factor to correct the 1:1 mixture $K_2S_2O_8$ oxidant solution. $a\ mg\ of\ N\ L^{-1}$ is N concentration in solution, 0.04 L is the volume of 0.5 M K_2SO_4 used to extract n from soil sample, 0.008 kg is the amount of field moist soil reacted with K_2SO_4 solution, and U is the moisture in percentage of each individual sample measured before the N extraction. The SON concentration in soil was calculated as the difference between TSN and SIN in the same sample, as follows:

$$SON_{(mg\ kg^{-1})} = TSN - SIN \quad (3)$$

where SON is the N concentration in the form of soluble organic N in soil over a dry basis, TSN is the total N concentration of extractable N ($NH_4^+ + NO_3^- + NO_2^- + SON$) and SIN is the N concentration in the form of soluble inorganic N ($NH_4^+ + NO_3^- + NO_2^-$) in soil over a dry basis.

2.3.2 Permanganate oxidizable C

Analysis of POXC were performed accordingly the assumptions and method developed by Blair, Lefroy and Lisle (1995) with the modifications of Weil et al. (2003) and Lucas and Weil (2012). Since KMnO_4 is highly photo-oxidizable, all steps were performed in dark. First, 20 mL of $0.02 \text{ mol L}^{-1} \text{ KMnO}_4 + 0.1 \text{ mol L}^{-1} \text{ CaCl}_2$ were added into 50-mL polypropylene screw-top centrifuge tubes containing 2.5 g air-dried soil. The tubes were shaken for exactly 2 min on a horizontal shaker at $180 \text{ rotation min}^{-1}$ and allowed to settle for exactly 10 min. Then, 0.5 mL of the supernatant was transferred into a second 50-mL centrifuge tube and mixed with 49.5 mL of deionized water. The solution absorbance at $\lambda = 550 \text{ nm}$ was measured in a single quartz-cuvette spectrophotometer and compared to a curve of standard concentrations ($0.005, 0.010, 0.015$ and 0.02 M KMnO_4) constructed using unreacted KMnO_4 solution plus ultra-pure deionized water, with the absorbance in the x axis and the KMnO_4 concentration in the y axis. POXC concentration was calculated as described by Weil et al. (2003) with the assumptions of Blair, Lefroy and Lisle (1995) as follows:

$$\text{POXC}_{(\text{mg kg}^{-1})} = \left[0.02 \text{ mol L}^{-1} - (a + bz) \right] \times (9000 \text{ mg C mol}^{-1}) \times \left(\frac{0.02 \text{ L solution}}{0.0025 \text{ kg soil}} \right) \quad (4)$$

where 0.02 mol L^{-1} is the initial concentration of the KMnO_4 reactant, a is the intercept of the standard curve, b is the slope of the standard curve, z is the sample absorbance, 9000 mg is the mass of 0.75 mol of C oxidized by 1 mol of MnO_4 , changing Mn^{7+} to Mn^{4+} (Blair et al., 1995) and 0.0025 kg soil is the amount of soil reacted with KMnO_4 .

2.3.3 Mineralizable C

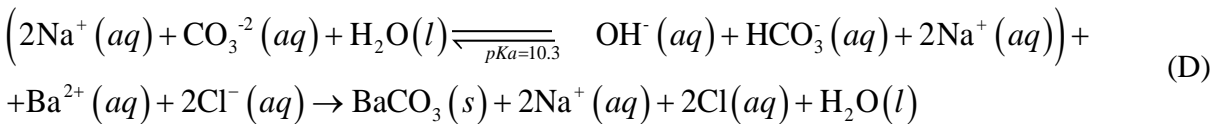
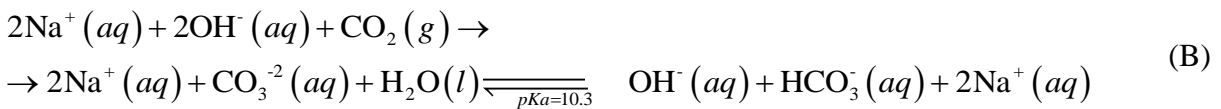
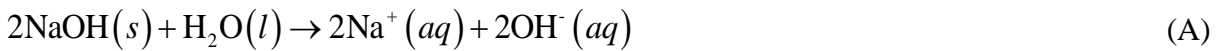
Short-term mineralizable C was determined by measuring CO_2 flush during a 72-h incubation using the method proposed by Franzluebbers et al. (2000), with adaptations based on the observations of Franzluebbers (2016) and Hurisso and Culman (2016). As well as other observations, these studies have shown that water-filled pore space strongly influences the CO_2 flush of rewetted soil. For this reason, in our study soil water-holding capacity (WHC) was measured for each soil depth. WHC was determined as the amount of water retained by a 50 g of 2.0-mm sieved air-dried soil samples, disposed in 80-mL bottom-pierced flasks (with small holes at the bottom) that were laid out onto soaked sand to rewet from bottom to top by

capillarity. After reaching the equilibrium (24 h), the amount of water retained in the soil sample was calculated by discounting initial weight of air-dried soil sample (50 g) from the weight of the moist soil sample.

To measure short-term C mineralization, 50 g of 2-mm sieved air-dried soil samples were placed into 1-L glass Mason-jars and rewetted to 50% WHC. Open 50-mL vials containing 10 mL of 0.5 M NaOH solution to trap CO₂ was hanged to the jar lids upon the rewetted soil samples into the Mason-jars using modified structures as described in Mulvaney et al. (1997). Mason-jars were tightly closed and incubated in dark at 25 °C for 72 h. At the end of incubation, the vials of NaOH were forthwith removed from the jar lids and screw-capped until titration. After sealed, each vial of NaOH was opened one at time and received 1 mL of 1.5 M BaCl₂ solution to precipitate bicarbonate in a less soluble form as BaCO₃. Thereafter, the NaOH traps were titrated with a 1 M HCl solution (previously standardized through titration against tris(hydroxymethyl)aminomethane) with an auto titrator Metrohm 848 Titrino plus (Metrohm, Herisau, Switzerland) until the endpoint pH (pH = 9.3). For every batch of 12 soil samples, a vial of 1 M NaOH was incubated without soil and used as a blank. The amount of C-CO₂ evolved from a sample was calculated according Franzluebbers (2016) as follows:

$$\text{C-CO}_{2(\text{mg kg}^{-1})} = \left(L_{[\text{blank}]} - L_{[\text{sample}]} \right) \times 0.5 \text{ mol}_c \text{ L}^{-1} \times \frac{6000 \text{ mg C mol}_c^{-1}}{0.05 \text{ kg of soil}} \quad (5)$$

where $L_{[\text{blank}]}$ and $L_{[\text{sample}]}$ are the volume of acid to reach the endpoint pH for blanks and samples respectively, $0.5 \text{ mol}_c \text{ L}^{-1}$ is the normality of the acid used in titration, 0.05 kg is the mass of dried soil material incubated, and 6000 mg is the mass of C that reacted with NaOH and consumed 1 mol_c of OH⁻ after BaCl₂ addition, resulting in a decrease of pH proportional to the amount of CO₂ captured for NaOH alkali traps, as show in the following A to D reactions:



2.3.4 β -glucosaminidase activity

The activity of the enzyme *N*-acetyl- β -D-glucosaminidase (EC 3.2.1.30) (from now on referred as NAGase) was measured to provide a better understanding about the effect of N fertilization on SOM mineralization dynamics. For being expensive and developed preferentially for topsoil samples, in this study NAGase activity was only measured in the first 0.2 m deep in control subplots and subplots where maize received 210 kg ha⁻¹ N in top-dress.

NAGase activity was measured accordingly Parham and Deng (2000). Briefly, one gram of 2-mm sieved field-moist soil was thoroughly mixed with 4 ml of 0.1 M acetate buffer (pH 5.5) and 1 ml of 10 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide solution, as substrate, into 50-mL glass Erlenmeyer flasks. The flasks were placed into a water bath incubator at 37 °C for 1 h. After incubation, 1 ml of 0.5 M CaCl₂ and 4 ml of 0.5 M NaOH were added to stop the reaction and the samples were swirled and filtered through a Nalgon® #3552 blue ribbon quantitative paper filter. Controls were performed with the substrate being added after the reaction were stopped. The yellow color intensity of the filtered extracts resultant from *p*-nitrophenol production was measured in a single quartz-cuvette spectrophotometer in absorbance mode ($\lambda = 405$ nm). The *p*-nitrophenol content of the filtrates were then calculated by comparing readings to a standard curve with *p*-nitrophenol concentrations ranging from 0.0 to 0.6 mg L⁻¹. Moisture was determined as for soluble N analysis and all results were expressed over dry weight basis, as follows:

$$\text{NAGase}_{(\text{activity})} = \left[a \text{ mg } \rho\text{-nitrophenol L}^{-1} \right] \times 0.001 \text{ L} \times \left(\frac{\left(0.001 \text{ kg of moist soil} \times \frac{U}{100} \right)}{0.001 \text{ kg of moist soil} \times \left(1 - \frac{U}{100} \right)} \right) \quad (6)$$

where NAGase_(activity) is the mass of *p*-nitrophenol formed per kg of soil over a dry basis after the incubation time (mg *p*-nitrophenol kg⁻¹ h⁻¹), *a* is the *p*-nitrophenol concentration in filtered extracts, 0.001 L is the total volume of reagents added to each soil sample, 0.001 kg is the amount of field moist soil incubated, and *U* is the moisture in percentage of each individual sample measured before incubation.

2.3.5 Total organic C and total N

Total organic C (TOC) and total N (TN) in soil samples were determined by dry combustion with a CHN-2000 auto analyzer (LECO Corp., St. Joseph, MI, USA). This method is based in the conversion of any soil C or N into CO₂ or N₂ gases, which are passed through an infrared detector and through a thermal conductivity detector to determinate the content of C and N, respectively (Nelson and Sommers, 1996). TOC and TN concentration was expressed as mg kg⁻¹ soil, over a dry weight basis. TOC and TN were determined in 60-mesh dry soil samples.

2.4 Bulk density and stocks calculation

Soil stocks of chosen element pools for each soil layer were calculated accordingly to Ellert and Bettany (1995) and the results were expressed in Mg ha⁻¹. Since similar soil densities were measured for all treatments, there was no necessity of depth correction calculations. However, in order to avoid undesirable variability, the soil stocks in each soil layer for each crop rotation were calculated through multiplying the element concentration by the mean of soil bulk densities measured in the respective rotation instead using a global soil density for the whole experiment. Therefore, elements stocks were calculated as follows:

$$SS_{(\text{Mg ha}^{-1})} = \frac{a \text{ kg of element}}{\text{Mg of soil}} \times b \text{ m soil depth} \times \frac{c \text{ Mg of soil}}{\text{m}^3} \times \frac{10000 \text{ m}^2}{\text{ha}} \times \frac{0.001 \text{ Mg}}{\text{kg}} \quad (7)$$

where SS is the soil stock of the chosen element (Mg ha⁻¹) in the specific soil layer, a is the element concentration on a dry mass basis (kg Mg⁻¹), b is the thickness (m) and c is the bulk density (Mg m⁻³) of the sampled soil layer.

In order to perform a clear discussion, the soil profile was separated in three major soil layers for performing the soil C and N stocks comparisons, namely topsoil (0.0-0.2 m), subsoil (0.2-0.6 m) and deep soil (0.6-1.0 m). This arrange aggregates soil layers in a way that better reflects the soil C and N dynamics and attenuates the lack of robustness of statistical analysis by natural variability of element concentration into the soil profile.

2.5 Statistical procedures

Means of C and N stocks for grouped soil layers were analyzed under a split plot design through contrasts comparisons ($P < 0.1$) using the software Sisvar version 5.7 (Ferreira, 2011). The R software (R Team Core, 2017) was used to perform all other statistical procedures.

In order to check ANOVA assumptions, model residues were obtained through *aov()* and *residuals()* commands and tested using the functions *bartlett.test()*, *shapiro.test()*, *tukeyNonaddTest()*, and *dwt()* in R. The possibility of performing a split split-plot analysis using soil depths as split split-plot was initially cogitated. However, since several assumptions were not validated due to disparity between soil layers features, the analysis were performed individually under split plot arrangement for each soil layer and for grouped soil layers using the *split2.rbd()* function of ExpDes package in R. Tukey's HSD test ($P < 0.1$) was used to perform paired comparisons.

Linear relationships between POXC and TOC, POXC and MINC were fitted via a regression model obtained with *lm()* function, graphed with the *stat_smoth()* function, and tested with *stat_fit_tb()* function in R. The effects of N rates on maize yield was graphed with the *stat_smoth()* function and tested with *stat_fit_tb()* function in R. The effect was fitted to the linear plateau -linear regression model using a function developed in R environment as follows:

$$> \text{function}(X, A, B1, X0) \text{ ifelse}(\text{test} = X < X0, \text{yes} = A + B1 * (X - X0), \text{no} = A) \quad (8)$$

where X is the top-dress N rate (kg ha^{-1}), A is the yield plateau (Mg ha^{-1}) above which maize grain yield is not increased by N fertilization, $B1$ is function slope in the response zone within which maize grain yield is linearly increased by N fertilization, and $X0$ is the top-dress N rate (kg ha^{-1}) in which yield plateau was reached. Yield responses that could not be modeled due significant lack of fit of interpretable models were compared through Tukey's HSD test ($P < 0.1$) using the *split2.rbd()* function of ExpDes package in R.

The variables relationships and least squared regression lines were graphed with the package ggplot2 in R. Geometric bars charts were graphed with the software package Office 365 (Microsoft corporation, Redmond, WA, USA).

3 RESULTS AND DISCUSSION

3.1 Effect of cover crops on soil C and N stocks

After seven years, crop rotations modified the C stock distribution into the soil profile (Figure 2). Comparable C stocks were observed in topsoil and deep soil for both crop rotation, but O-M-W-S showed higher C stock than P-M-O-S in the 0.2-0.6 m layer.

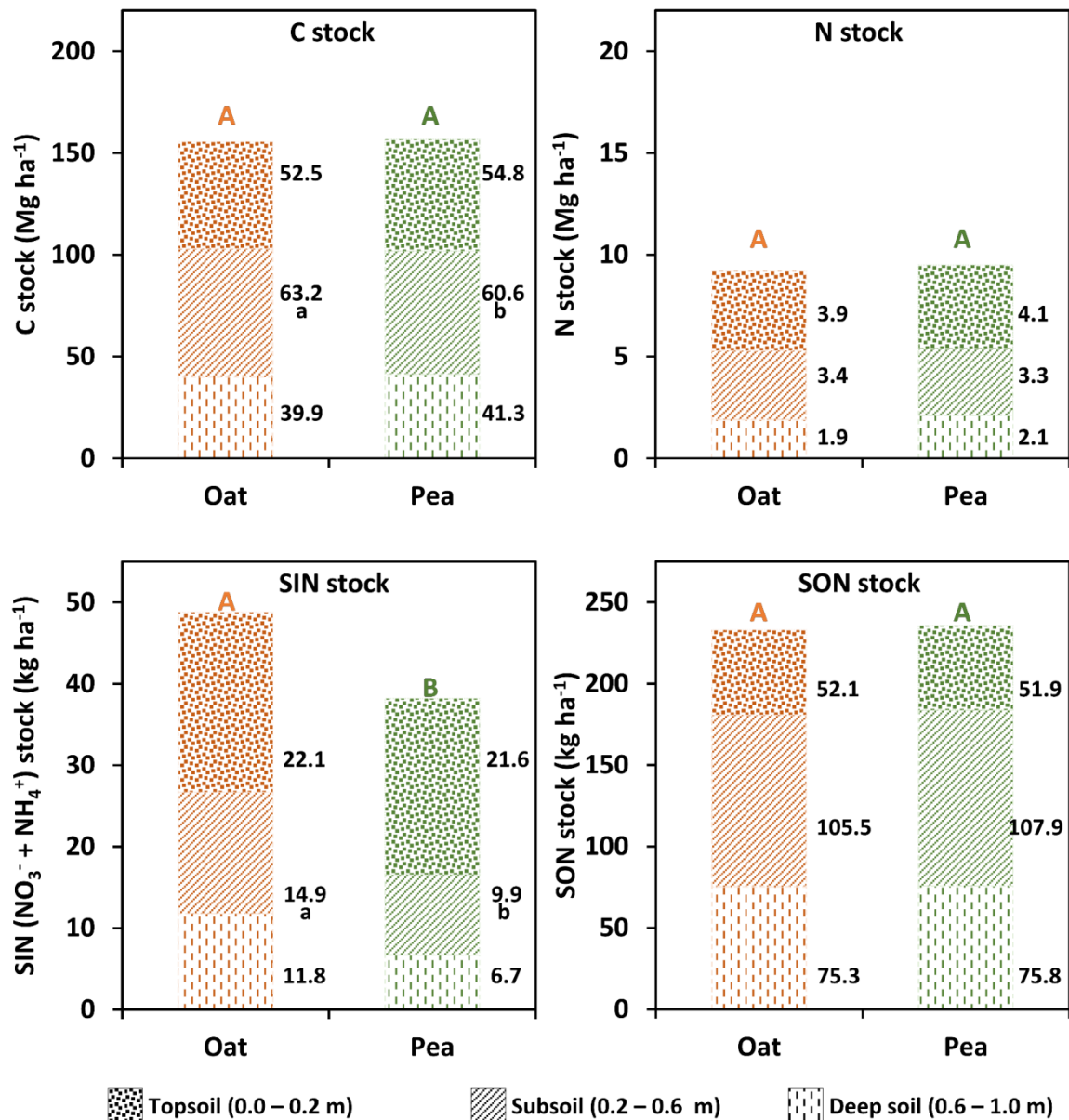


Figure 2. Soil stocks of organic C (TOC), total N (TN), soluble inorganic N (SIN), and soluble organic N (SON) in grouped layers of a no-till soil profile under seven-year crop rotations in Southern Brazil, where maize is grown biannually after different winter cover crops (black oat and field pea) and under four N top-dressing rates (0, 70, 140, and 210 kg ha⁻¹ N). Stocks' means for cover crops were averaged between N rates. Layers' stocks not sharing a lowercase letter inside the same layer or cumulative stocks not sharing an uppercase letter are significantly different by Tukey's HSD test ($P < 0.1$).

The higher subsoil C stocks when black oat was used as winter cover crop was expected since Poaceae usually have a greater rooting capacity when compared to legume crops (Redin et al., 2018). The efficiency of black oat in improve soil C storage was reported by Santos et al. (2011) and Albuquerque et al. (2015) after evaluating 17-y and 21-y effect of crop rotation under similar conditions of this study. Between the crop rotations evaluated by those authors, one was identical to O-M-W-S rotation of our study and the other was similar to P-M-W-S rotation, but with vetch (*Vicia villosa* Roth) instead of field pea as legume cover crop. Both authors reported higher biomass C addition in subsurface layer, when black oat was used as winter cover, and supported the hypothesis that root biomass was the major C source to SOM building, which suggests a similar trend has occurred C in our study.

Despite O-M-W-S had built higher C stock in the depth of 0.2-0.6 m, both crop rotations showed similar 1-m soil C stocks. Same trend was observed by Santos et al. (2011) and Albuquerque et al. (2015). The most probable explanation is the higher C input through maize root and shoot biomass when its grown after field pea in the topsoil layer. In the crop season 2011-12, 2013-14 and 2017-18, average maize grain yield has been, respectively, 1.3, 1.0, and 0.5 Mg ha⁻¹, greater in plots where maize was cropped after field pea, than in plots where black oat was grown in winter (data not shown). Maize aboveground biomass is directly correlated to maize grain yield (Donald and Hamblin, 1976), and this can be extrapolated to root biomass (Bolinder et al., 1997). Therefore, the higher C input through maize biomass in P-M-W-S may have compensated the higher C input by black oat's biomass in the 0.2-0.6 m layer of O-M-W-S rotation and balanced 1-soil C stocks between crop rotations.

There are evidences that higher maize yields in P-M-W-S relies on a better N nutrition and improved root development of maize grown after field pea. The importance of the C addition by maize roots, particularly in crop rotations where maize is grown after legume cover crop has been emphasized by several studies (Balesdent and Balabane, 1992; Lovato et al., 2004; Zanatta et al., 2007) and is attributed either to fixed-N and non-N effects, also known as cropping effects or rotation effects (Stevenson and Kessel, 1996; Thorup-Kristensen et al., 2003). Legumes leave root channels replete with high quality residues that can release nutrients (specially biological fixed N) in a rate that match to maize requirement (Boddey et al., 1997; Ladha et al., 2005) and support a high microorganism biodiversity (Frasier et al., 2016; Tiemann et al., 2015). This stimulate maize root to grow faster and wider, explore the soil volume, and access more water and nutrients, which reflects in higher grain yields and biomass production.

Increases in soil C stocks beneath surface layer has been often associated with downwards transportation of dissolved organic C (DOC) in coarse textural soils under agricultural and grasslands in temperate climate (Salazar et al., 2019; van Kessel et al., 2009). The possibility of DOC accumulation in the subsoil layer was taken in consideration in this study. However, as the DOC and SON are directly related and derived from the same organic matter pool (van Kessel et al., 2009), the lack of significant difference between SON stocks between crop rotations and across soil profile (Figure 2) is a solid evidence that the increase in soil C stocks in the 0.2-0.6 m layer of O-M-W-S rotation was more related to root C input rather than DOC accumulation.

SON fraction accounted for 83% of the total soluble N (SIN+SON) in O-M-W-S rotation and 86% in P-M-W-S rotation, averaging 2% of the soil N stock in both systems, a contribution five times greater than the SIN pool. However, in spite of SON has been reported as a significant pathway of N transport throughout maize-cropped Oxisol profiles (Lehmann et al., 2004) in our study SON pool was not sensitive neither to cover crops, nor to top-dress N fertilization (Figure 2). SON concentration slightly decreased from 22 to 13 mg kg⁻¹ with soil depth (Table 2). The constant concentration of SON throughout soil profile and the lack of response of SON to the crop rotations and N management, may be related to the different metabolization dynamics of the labile and more stable fractions of SON (Mariano et al., 2016; van Kessel et al., 2009). Under adequate conditions, there is a rapid microbial consumption and subsequently conversion of the most labile hydrophilic SON forms to NO₃⁻, while the hydrophobic dissolved organic matter with higher C/N ratios is more likely adsorbed in uppermost soil (Mariano et al., 2016). In this scenario, inorganic N forms the most significant pathway of N transport within subtropical soil profiles (van Kessel et al., 2009).

SIN pool was much more sensitive to crop rotation than other N pools in our study. After maize harvest, O-M-W-S rotation showed a 1-m SIN stock about 10 kg ha⁻¹ higher than P-M-W-S rotation (Figure 2) and subsoil N enrichment with SIN was observed in deeper soil layers when higher N rates were applied in O-M-W-S plots (Table 2). This finding was opposite to expected, since legume biological N fixation should improve soluble N content in soil profile and non-legumes are reported to be more efficient than legumes in reducing NO₃⁻ leaching (Thapa et al., 2018).

Table 2. Soluble inorganic N ($\text{NH}_4^+ + \text{NO}_3^- + \text{NO}_2^-$ – SIN) and soluble organic N (SON) concentrations in a no-till soil profile under seven-year crop rotations in Southern Brazil, where maize is grown biannually after different winter cover crops (black oat and field-pea) and under four N top-dressing rates (0, 70, 140 and 210 kg ha⁻¹ N).

N rate (kg ha ⁻¹)	Cover crop			Cover crop		
	Oat	Pea	Mean	Oat	Pea	Mean
	SIN (mg kg ⁻¹)			SON (mg kg ⁻¹)		
0.0 - 0.1 m						
0	11.7±2.0	14.5±2.0	13.1±1.4	19.9±2.2	21.5±0.8	20.7±1.1
70	13.7±1.8	13.5±1.1	13.6±1.0	21.2±1.1	20.8±0.5	21.0±0.6
140	14.6±2.4	13.9±1.5	14.3±1.3	20.4±2.7	23.3±0.6	21.8±1.4
210	13.2±1.9	12.7±0.2	13.0±0.9	22.9±1.9	22.3±0.8	22.6±1.0
Mean	13.3±0.9	13.6±0.6		21.1±1.0	22.0±0.4	
P-value	Cover (C) = 0.752; N rate (R) = 0.418; C×R = 0.158			Cover (C) = 0.645; N rate (R) = 0.140; C×R = 0.167		
CV (%)	Cover = 20.1; N rate = 12.3			Cover = 23.0; N rate = 7.9		
0.1 - 0.2 m						
0	3.1±0.2 ^{bA}	3.5±0.6 ^{aA}	3.3±0.3	19.5±0.8	16.8±0.9	18.2±0.8
70	4.0±0.4 ^{abA}	3.2±0.7 ^{aB}	3.6±0.4	17.0±0.6	17.4±0.9	17.2±0.5
140	4.3±0.6 ^{aA}	2.8±0.4 ^{aB}	3.6±0.5	20.2±1.4	17.7±0.3	18.9±0.8
210	4.1±0.7 ^{abA}	3.5±0.5 ^{aA}	3.8±0.4	16.8±1.3	17.5±1.2	17.2±0.8
Mean	3.9±0.3	3.2±0.3		18.4±0.6	17.4±0.4	
P-value	Cover (C) = 0.078; N rate (R) = 0.032; C×R = 0.045			Cover (C) = 0.139; N rate (R) = 0.255; C×R = 0.185		
CV (%)	Cover = 19.8; N rate = 17.5			Cover = 8.0; N rate = 11.0		
0.2 - 0.4 m						
0	2.6±0.5	2.0±0.4	2.3±0.3	21.8±1.8	21.5±1.4	21.7±1.0
70	3.1±0.6	2.2±0.5	2.6±0.4	19.0±1.8	20.3±1.4	19.7±1.1
140	2.6±0.5	1.6±0.5	2.1±0.4	22.8±2.2	22.2±0.6	22.5±1.0
210	3.2±0.4	2.7±0.5	3.0±0.3	20.7±1.8	22.0±1.6	21.4±1.1
Mean	2.9±0.2 ^A	2.1±0.2 ^B		21.1±0.9	21.5±0.6	
P-value	Cover (C) = 0.020; N rate (R) = 0.213; C×R = 0.910			Cover (C) = 0.639; N rate (R) = 0.192; C×R = 0.807		
CV (%)	Cover = 19.2; N rate = 33.1			Cover = 11.4; N rate = 11.8		
0.4 - 0.6 m						
0	2.1±0.6	1.7±0.5	1.9±0.4	20.3±1.8	20.3±0.6	20.3±0.9
70	2.9±0.8	1.6±0.4	2.3±0.5	18.9±1.2	19.3±0.9	19.1±0.7
140	2.9±1.0	1.5±0.6	2.2±0.6	19.3±1.8	18.7±0.3	19.0±0.8
210	3.1±0.4	1.8±0.7	2.4±0.4	17.9±1.6	19.8±0.3	18.8±0.8
Mean	2.8±0.3 ^A	1.6±0.2 ^B		19.1±0.8	19.5±0.3	
P-value	Cover (C) = 0.033; N rate (R) = 0.686; C×R = 0.681			Cover (C) = 0.704; N rate (R) = 0.339; C×R = 0.526		
CV (%)	Cover = 38.1; N rate = 42.5			Cover = 14.2; N rate = 9.0		
0.6 - 0.8 m						
0	2.1±0.2 ^{bA}	1.6±0.3 ^{aA}	1.8±0.2	15.8±0.4	15.4±1.4	15.6±0.7
70	2.9±0.5 ^{abA}	1.0±0.2 ^{aB}	2.0±0.4	15.9±0.7	16.4±1.5	16.2±0.8
140	2.7±0.7 ^{abA}	1.2±0.4 ^{aB}	2.0±0.5	15.5±0.8	15.1±1.2	15.3±0.7
210	3.4±0.1 ^{aA}	1.2±0.2 ^{aB}	2.3±0.4	15.5±1.1	17.1±1.2	16.3±0.8
Mean	2.8±0.2	1.3±0.1		15.7±0.4	16.0±0.6	
P-value	Cover (C) = 0.006; N rate (R) = 0.580; C×R = 0.085			Cover (C) = 0.798; N rate (R) = 0.321; C×R = 0.277		
CV (%)	Cover = 30.3; N rate = 32.3			Cover = 20.7; N rate = 7.2		
0.8 - 1.0 m						
0	1.3±0.3 ^{bA}	1.4±0.1 ^{aA}	1.4±0.1	14.3±1.3	13.3±0.9	13.8±0.8
70	1.6±0.4 ^{abA}	1.4±0.2 ^{aA}	1.5±0.2	12.4±0.4	13.8±1.6	13.1±0.8
140	1.9±0.5 ^{abA}	1.3±0.3 ^{aB}	1.6±0.3	13.8±0.8	13.4±0.7	13.6±0.5
210	2.2±0.3 ^{aA}	1.2±0.3 ^{aB}	1.7±0.3	13.2±1.0	12.6±0.2	12.9±0.5
Mean	1.7±0.2	1.3±0.1		13.4±0.5	13.3±0.5	
P-value	Cover (C) = 0.131; N rate (R) = 0.490; C×R = 0.085			Cover (C) = 0.784; N rate (R) = 0.732; C×R = 0.559		
CV (%)	Cover = 38.0; N rate = 27.5			Cover = 11.8; N rate = 13.2		

† Standard errors of mean ($n = 16$ for cover crops means and $n = 8$ for N rates means and $n = 4$ for doses means inside covers).

‡ Means not sharing a lowercase letter in the column or an uppercase letter in the row are significantly different by Tukey's HSD test ($P < 0.1$).

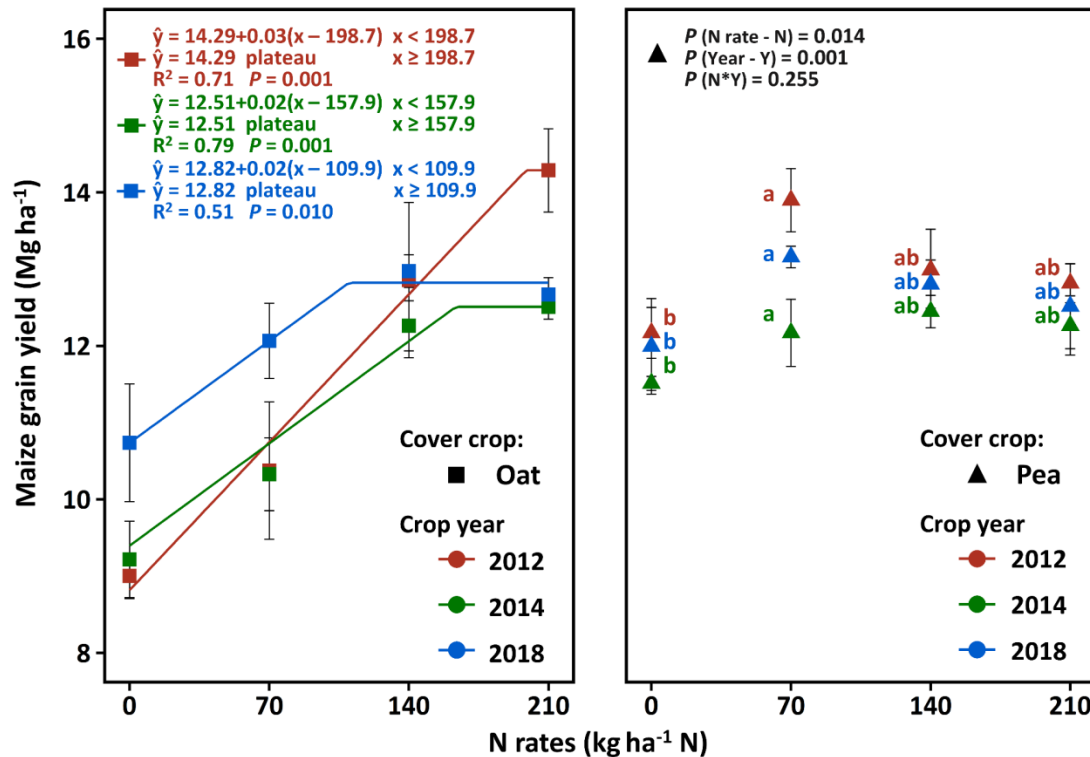


Figure 3. Grain yield response to N rates top-dressed in V4 stage of maize plots grown biannually after different winter cover crops (black oat and field-pea) under no-till in Southern Brazil. Error bars' range represents two standard errors of mean ($n = 4$). The linear-plateau model was fitted to yields responses with significant linear pattern. Yield responses that could not be modeled due significant lack of fit of interpretable models were compared through Tukey's HSD test. For yield response of maize after cover crop pea, means not sharing a lowercase letter between N rates inside the same crop year are significantly different by Tukey's HSD test ($P < 0.05$). Data available in IPNI GMP reports (IPNI, 2017). Data from 2016 crop season is lacking.

The more plausible explanation for the lower SIN subsoil accumulation in P-M-W-S rotation may reside in the higher maize yields in P-M-W-S plots as compared to O-M-W-S rotation under similar N management (Figure 3). For every 1000 kg of grain produced, modern maize hybrids accumulates from 23.8 to 28.4 kg N in aboveground biomass, from which 14.1 kg are exported in grains (Bender et al., 2013; Resende et al., 2016). Therefore, the reduction in SIN content in the soil profile accompanied by an increase in maize yields in P-M-W-S rotation (Figure 3) indicates that more N must have been extracted to support the higher maize yields and removed from the field (in grains) and contributed to reducing SIN levels in soil after harvest when maize was grown after field pea.

The potential of legume crops in providing N to subsequent cash crops is well established under subtropical conditions (Aita et al., 2001; Amado et al., 2002, 1999; Silva et al., 2006). However, low soil inorganic N levels in crops grown after legumes are also reported in tropical soils. Along several evaluations, Tenelli et al. (2019), reports higher sugarcane (*Saccharum* spp.) yields and lower SIN levels in plots established after a legume crop

(*Crotalaria spectabilis*) as compared to fallow. The lower SIN stocks observed when maize was grown after a legume may relies on a better capacity of maize roots to explore the soil in earlier stages of maize grown taking advantage of rotation effects and N-related benefits provided by field pea's residues mineralization to attain higher grain yields. These results evidence the importance of previous soil management on growing plant capacity of acting as a sink of inorganic N and reducing subsoil N enrichment. A possible role of field pea as catch crop cannot be ignored either. Even though field pea acquires N through biological N fixation, part of the N accumulated in filed pea's biomass is obtained by uptaking SIN from soil. Thorup-Kristensen et al. (2003) observed that when temperature is a limiting factor, grasses cover crops showed a slower and shallower rooting and, even though broadleaf cover crops were found to allocate a much smaller fraction of their biomass to the root system, the later presented higher nitrate uptake rates than monocots

Maize yield was maximized when 70 kg ha⁻¹ N has been top-dressed in P-M-W-S rotation, demonstrating that maize was responsive to N rates above that limit. In opposite, in the O-M-W-S rotation, maize yield fitted a linear-plateau model, showing a linear response to N rates until reaching the equilibrium (Figure 3). For O-M-S-W-S rotation, maximum maize yields were obtained with 198, 158, and 110 kg ha⁻¹ N respectively in 2012, 2014, and 2018 crop seasons. In addition, higher yields were obtained when maize was grown after field pea, especially in the lower N rates. These data indicate that P-M-W-S rotation is providing much more N to maize in comparison to O-M-W-S rotation. Also, it has to be noted that, even though P-M-W-S rotation was more efficient in deplete subsoil SIN and provide N to maize, growing black oats as winter catch crop also showed potential in supply N to maize in the long term. The reduction the amount of N required to optimize maize yield in O-M-W-S rotation over the years indicates that non-legumes also has potential to preserve N into system and release N to the cash crops, reducing the N fertilizer requirement for profitable maize production.

3.2 Effect of N rates on soil C and N stocks

Paired mean comparison inside each soil depth was not efficient in detect interaction effects between cover crops and N rates on TOC, TN and SIN stocks (Figure 4). This was expected as consequence of the nonlinear biomass allocation of maize in response to N fertilization (Fageria and Moreira, 2011; Santos et al., 2011), and to the complex dynamics of N movement in soil profile (Melgar et al., 1992).

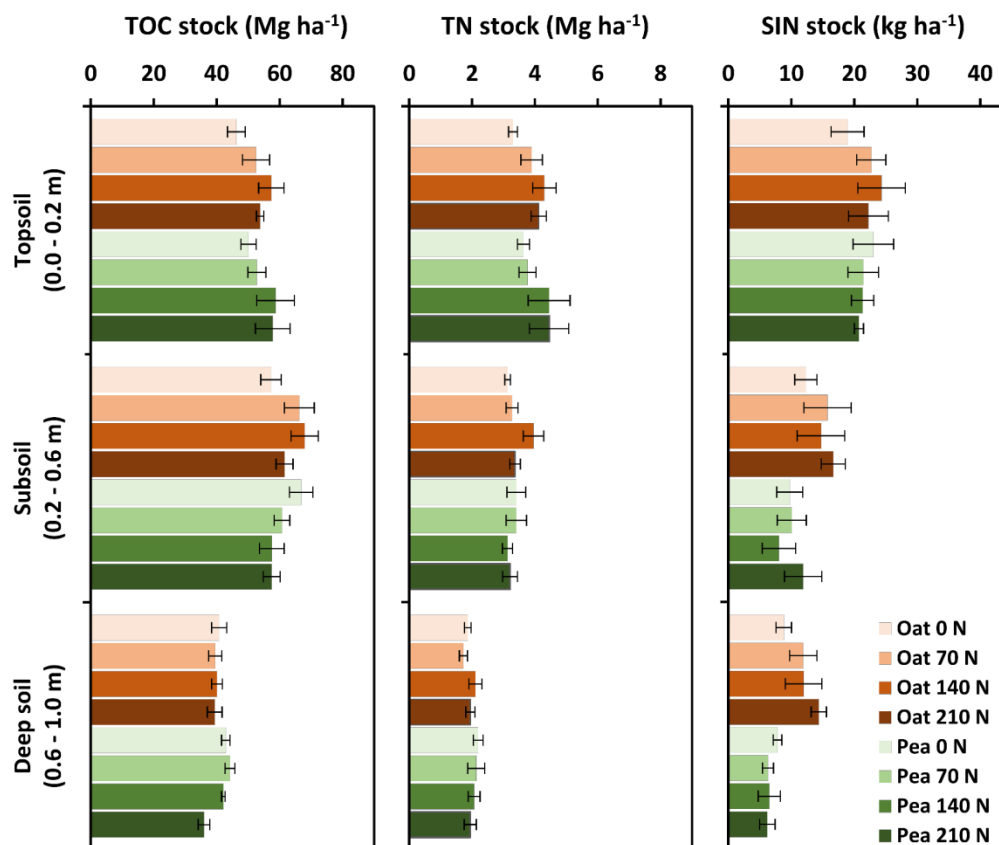


Figure 4. Soil stocks of organic C (TOC), total N (TN), and soluble inorganic N (SIN) in grouped layers of a no-till soil profile under seven-year crop rotations in Southern Brazil, where maize is grown biannually after different winter cover crops (black oat and field pea) and under four N top-dressing rates (0, 70, 140, and 210 kg ha⁻¹ N). Error bars' range represents two standard errors of mean ($n = 4$).

Orthogonal contrasts analysis was more adequate to investigate the effect of N fertilization on soil C and N stocks since most of variation was observed between non-fertilized and fertilized plots (Table 3). The N stock in 1-m soil profile increased with N rates in both O-M-W-S and P-M-W-S rotation systems (Table 3). Maize plots that received N in top-dress showed soil N stocks 2.2 Mg ha⁻¹ higher in the entire 1-m profile compared to non-fertilized plots. Furthermore, increasing N rates above 70 kg ha⁻¹ increased 1-m soil N stock by 1.3 Mg ha⁻¹, in average. Most of this variation occurred in the first 0.2 m.

In both crop rotations, independently of the amount of N applied, N fertilization resulted in average C stock 22 Mg ha⁻¹ higher in the first 0.2 m when compared to non-fertilized plot (Table 3). However, at the depth of 0.2-0.6 m, soil C stocks in O-M-W-S rotation was in average 24 Mg ha⁻¹ lower in the control plots when compared to the fertilized plots while in P-M-W-S, in opposite, N fertilization reduced C stocks by 24.7 Mg ha⁻¹. These conflicting results show that in each crop rotation N fertilization affect soil C and N stocks building in different ways.

Table 3. Differences in soil C and N stocks in the soil profile as effect of N rates (0, 70, 140, and 210 kg ha⁻¹ N) top-dressed to maize grown biannually after different cover crops (black oat and field-pea) in seven-year crop rotations under no-till in Southern Brazil. Values presented are differences between means obtained through orthogonal contrasts.

Contrasts	Cover crop			Cover crop			Cover crop		
	Oat	Pea	Mean	Oat	Pea	Mean	Oat	Pea	Mean
	— Δ C stock (Mg ha ⁻¹) —			— Δ N stock (Mg ha ⁻¹) —			— Δ SIN stock (kg ha ⁻¹) —		
Topsoil (0.0 - 0.2 m)									
0 N <i>vs</i> 70 N, 140 N, 210 N	-24.9	-19.0	-22.0* †	-2.4	-1.8	-2.1*	-12.5**	5.6 ^{ns}	-3.5
70 N <i>vs</i> 140 N, 210 N	-6.2	-11.0	-8.6 ^{ns}	-0.6	-1.4	-1.0 [§]	-1.2 ^{ns}	0.8 ^{ns}	-0.2
140 N <i>vs</i> 210 N	3.6	0.9	2.2 ^{ns}	0.2	0.0	0.1 ^{ns}	2.1 ^{ns}	0.6 ^{ns}	1.3
<i>P</i> (Cover crops)	0.508			0.596			0.777		
<i>P</i> (N rates)	0.056			0.030			0.407		
<i>P</i> (Cover crops x N rates)	0.934			0.857			0.027		
CV (%)	Cover = 16.6; N rates = 12.9			Cover = 20.6; N rates = 15.4			Cover = 17.8; N rates = 10.2		
Subsoil (0.2 - 0.6 m)									
0 N <i>vs</i> 70 N, 140 N, 210 N	-24.0 [§]	24.7 [§]	0.4	-1.2 ^{ns}	0.7 ^{ns}	-0.3	-10.2	-0.7	-5.5
70 N <i>vs</i> 140 N, 210 N	3.0 ^{ns}	6.5 ^{ns}	4.7	-0.8 ^{ns}	0.5 ^{ns}	-0.1	-0.1	0.3	-0.2
140 N <i>vs</i> 210 N	6.0 ^{ns}	0.0 ^{ns}	3.2	0.6 [§]	-0.1 ^{ns}	0.3	-2.0	-3.8	-2.9
<i>P</i> (Cover crops)	0.089			0.595			0.018		
<i>P</i> (N rates)	0.774			0.643			0.348		
<i>P</i> (Cover crops x N rates)	0.112			0.081			0.753		
CV (%)	Cover = 4.8; N rates = 12.9			Cover = 17.9; N rates = 13.2			Cover = 23.8; N rates = 31.5		
Deep soil (0.6 - 1.0 m)									
0 N <i>vs</i> 70 N, 140 N, 210 N	3.3 ^{ns}	6.4 ^{ns}	4.8	-0.2	0.5	0.1	-11.8**	4.4 ^{ns}	-3.7
70 N <i>vs</i> 140 N, 210 N	-0.5 ^{ns}	10.3*	4.9	-0.6	0.3	-0.2	-2.6 ^{ns}	-0.1 ^{ns}	-1.3
140 N <i>vs</i> 210 N	-0.7 ^{ns}	6.2*	3.4	0.2	0.1	0.1	-2.4 ^{ns}	0.3 ^{ns}	-1.0
<i>P</i> (Cover crops)	0.499			0.469			0.013		
<i>P</i> (N rates)	0.077			0.542			0.433		
<i>P</i> (Cover crops x N rates)	0.150			0.185			0.047		
CV (%)	Cover = 12.3; N rates = 8.5			Cover = 30.0; N rates = 12.2			Cover = 29.1; N rates = 25.4		
Total (0.0 - 1.0 m)									
0 N <i>vs</i> 70 N, 140 N, 210 N	-45.6*	12.1 ^{ns}	-16.8	-3.8	-0.6	-2.2*	-34.4**	9.2 ^{ns}	-12.6
70 N <i>vs</i> 140 N, 210 N	-3.6 ^{ns}	5.8*	1.0	-2.0	-0.6	-1.3 [§]	-3.7 ^{ns}	1.0 ^{ns}	-1.3
140 N <i>vs</i> 210 N	10.6 ^{ns}	7.1*	8.9	0.9	0.0	0.5 ^{ns}	-2.3 ^{ns}	2.9 ^{ns}	-2.6
<i>P</i> (Cover crops)	0.853			0.769			0.005		
<i>P</i> (N rates)	0.274			0.051			0.189		
<i>P</i> (Cover crops x N rates)	0.211			0.255			0.020		
CV (%)	Cover = 9.8; N rates = 7.0			Cover = 20.6; N rates = 8.9			Cover = 9.1; N rates = 11.63		

† P-value of |t| for orthogonal contrasts (Pr > |t|): ns ($P \geq 0.1$); § ($P < 0.1$); * ($P < 0.05$); ** ($P < 0.01$).

The lower subsoil C stocks in the control plots of O-M-W-S rotation can be explained by a well-known temporary N shortage effect that occurs when maize is grown after poaceous. In order to process high lignin and high C:N ratio black oat residues, microbial biomass competes with maize for N in early maize stages and compromises maize grain yield, biomass production and, consequently soil C inputs (Aita et al., 2001; Ceretta et al., 2002). This hypothesis is corroborated by studies reporting maize as the major contributor to soil C inputs in most crop rotation systems, and the fundamental role of N fertilization at improving biomass

production (Lovato et al., 2004; Zanatta et al., 2007). Another hypothesis is the association between a root system with high C:N (black oat) with N fertilization, enabling microbial biomass to process the root C biomass and transform it in more stable SOM pools, increasing soil C stocks in O-M-W-S rotation. This emphasizes the importance of sizable top-dress N fertilization when maize is grown after black oat, not only to maximize grain yield, but also to avoid depletion in soil C stocks.

In opposite to what occurred in O-M-W-S rotation, the reduced subsoil C stocks in P-M-W-S fertilized plots suggests that the biological fixed N and legume crop rotation effects (non-N effect) support a bulky and deeper maize rooting in soil under low N fertilizer supply. However, when N fertilization inputs readily available SIN in topsoil layer, maize root growth may have been restricted to the first 0.2 m which may have reduced subsoil C inputs through root biomass and hindered SOM building in subsoil. The side effects of excessive N fertilization remained evident in P-M-W-S rotation between 0.6-1.0 m where N rates above 70 kg ha⁻¹ N have reduced soil C stocks by 10.3 Mg ha⁻¹ (Table 3). More importantly, a further reduction of 6.2 Mg ha⁻¹ occurred when N rate was increased from 140 to 210 kg ha⁻¹ N in the same layer. The increase of soil N stock only in topsoil in parallel to a decreased in soil C stock in the deep soil layers as consequence of N fertilization in P-M-W-S rotation, reinforces the possibility of a negative effect of excessive N application on root development and soil C storage subsoil, especially in crop rotations where maize accounts for a large share of the biomass inputs.

Similar to our findings, the effect of N fertilization on subsoil C stocks has been described in long-term trials under several crop rotations. Khan et al. (2007) and Mulvaney et al. (2009) reported a depletion of soil C stocks by continuous application of N fertilization. Khan et al. (2007) also found the depletion being larger in subsoil rather than surface soil layers and with inclusion of legume (soybean-corn) rather than poaceous (corn-corn) in the crop rotation system. According to those authors, the association of heavy N fertilization with biological N fixation by legume crops increase inorganic N availability in subsoil which may have stimulated subsoil microbial activity and promoted organic C mineralization with a consequent depletion of SOM stocks. However, in contraposition to stoichiometric decomposition theory defended by those authors our findings indicate that lower subsoil C stocks were not associated to an eventual priming effect due to inorganic N enrichment. In our study, subsoil C depletion was not associated to increased SIN stocks in P-M-W-S rotation and, contrarywise, higher C stock between 0.2-0.6 m were associated to an increase SIN stocks (Figure 2) as a result of N fertilization in O-M-W-S rotation (Table 3).

Top-dress N fertilization increased SIN stocks in topsoil and deep soil layers stocks after maize harvest in O-M-W-S rotation while in P-M-W-S plots N fertilization did not increased SIN even when high N rates were used (Table 3). This result reinforces the possibility that field pea, both by providing biological fixed N and though non-N rotation effects, may have supported an intensive rooting and a readily N uptake in the earlier stages of maize development overcoming N transportation to deep soil layers. A well-established root system may have improved maize capacity of uptake N provided by fertilizer N and, consequently, increased maize yield potential and lowered maize yield response to N fertilization O-M-W-S rotation (Figure 3). Corroborating this hypothesis, several studies report high fertilizer N uptake and greater N use efficiency by maize associated to a deep root system with appropriate architecture and higher stress tolerance when legumes are included in crop rotation (Bhattacharya, 2019; Gabriel et al., 2016; Thorup-Kristensen et al., 2003; Vogeler et al., 2019).

Even though P-M-W-S were more efficient in avoid SIN transportation to deep soil layers (Figure 1) the lack of response of SIN stock to N fertilization in 0.2-0.6 m layer of O-M-W-S rotation (Table 3) is an indicative that black oat rooting has been, in some extent, efficient in scavenging N until that 0.6 m deep and make it available to maize. This possibility is reinforced by the diminishing maize yield response to N fertilization in O-M-W-S over the years (Figure 3), and by the increase in 0.2-0.6 m soil C stock in this rotation (Figure 1). This finding reinforces the importance of adequate N management to ensure higher yields and build up SOM in crop rotations including maize and other poaceous crops. In opposite, maize N fertilization should be reduced in crop rotations including legumes, to avoid unnecessary supply of N that would reduce SOC storage in deep soil layers.

When soil layers were analyzed ungrouped is possible to observe that N rates significantly increased soil TN concentration only in the first 0.2 m, and soil C concentration only in the first 0.1 m (Figure 5). In both crop rotations higher N rates negatively affected C and N dynamics in deeper soil layers. N rates above 70 kg ha⁻¹ N promoted N accumulation at depth of 0.6-0.8 m in O-M-W-S rotation and reduced TOC concentration at the depth of 0.6-0.8 m soil in P-M-W-S rotation where increasing N rate from 70 to 210 kg ha⁻¹ N decreased TOC by 1.4 g kg⁻¹. In addition, N rates increased TN concentrations in the 0.0-0.2 m soil layer, with 140 and 210 kg ha⁻¹ N resulting in average TN concentrations of 2.1 and 2.2 g kg⁻¹, respectively, at the depth of 0.0-0.1 m; and 1.1 and 1.2 g kg⁻¹, respectively, at the depth of 0.1-0.2 m.

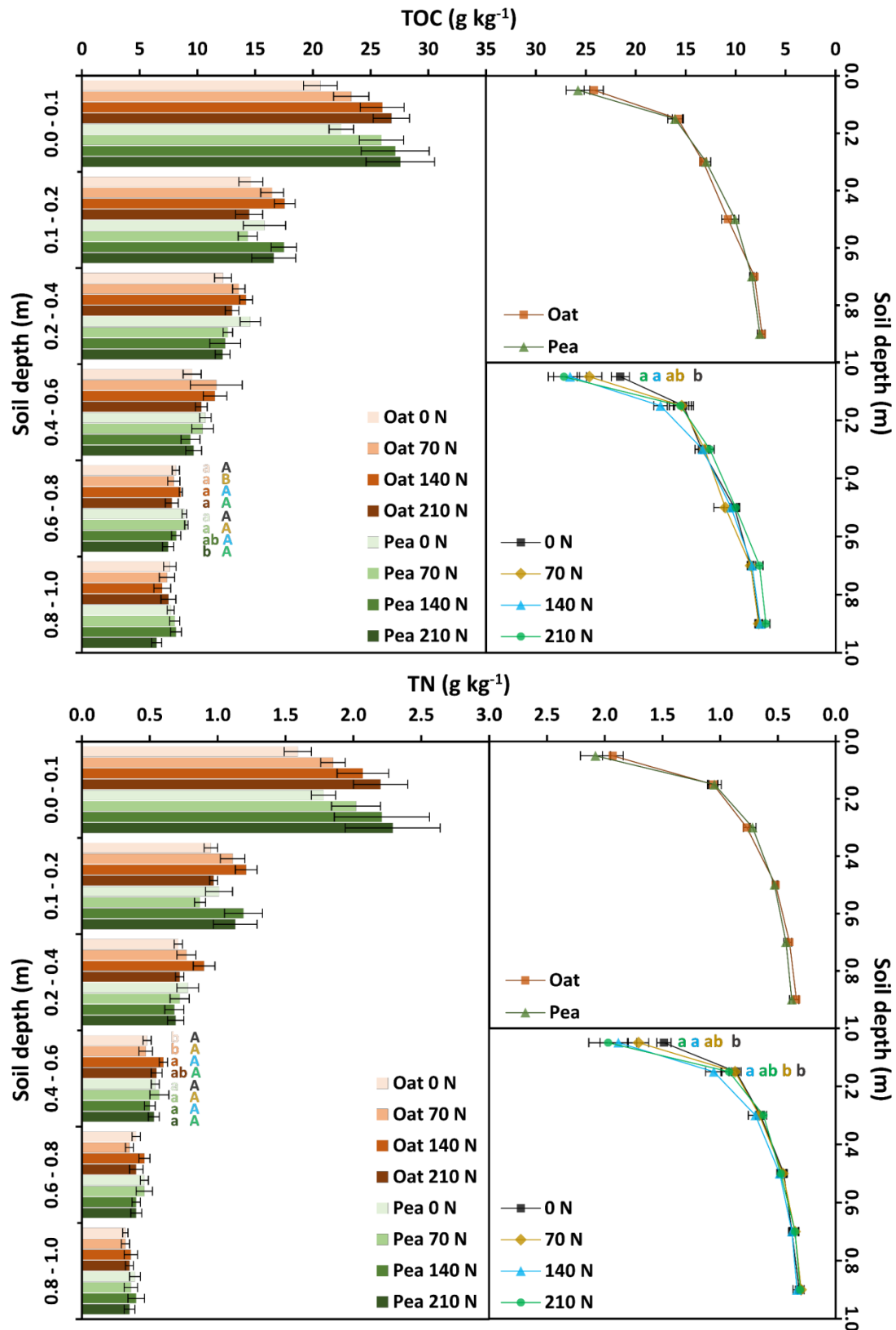


Figure 5. Total organic C (TOC) and total N (TN) concentrations in a no-till soil profile under seven-year crop rotations in Southern Brazil, where maize is grown biannually after different winter cover crops (black oat and field-pea) and under four top-dressing N rates (0, 70, 140, and 210 kg ha⁻¹ N). Error bars' range represents two standard errors of mean ($n = 16$ for cover crops means, $n = 8$ for N rates means and $n = 4$ for doses means inside covers). Means not sharing a lowercase letter between N rates inside the same cover crop, or an uppercase letter between cover crops inside the same N rate are significantly different by Tukey's HSD test ($P < 0.05$).

The reduction in TOC concentration in deep soil layers in the 210 kg ha⁻¹ N treatment and the linear increase of soil TN by N fertilization in P-M-W-S topsoil (Figure 5) corroborate the hypothesis that the rich N pool in topsoil created by higher N rates stimulated the concentration of root system in this layer and reduced root biomass input in subsoil. In addition, the increase in TN concentration at 0.4-0.6 m in O-M-W-S rotation when 140 and 210 kg ha⁻¹ N were top-dressed to maize, meets the hypothesis that N fertilization above the yield response range promotes subsoil N accumulation.

Top-dressing N fertilization was essential to increase soil C in the topsoil layer (Figure 5). In control plots (no top-dress N), TOC concentration in the first 0.1 m deep was 5 to 6 g kg⁻¹ lower than in fertilized plots. This phenomenon occurred for both O-M-W-S and P-M-W-S rotation. This findings corroborate the observations of Ladha et al. (2011) that N fertilization is essential for improving crop productivity, and reduce the rate of soil C declining in agricultural soils. Nevertheless, the reduced POXC concentration below 0.6 m by N fertilization in O-M-W-S rotation (Table 4) indicates that excessive N application may lessen plant root potential of exploring subsoil layers. Even though POXC represent a more stabilized pool of labile soil C, it is very sensitive to stabilized biomass amendments (Morrow et al., 2016) in a way that increases in POXC values below 0.1 m deep must be associated to root biomass inputs. O-M-W-S rotation showed higher POXC values than P-M-W-S until 0.6 m (Table 4) indicating a great rooting capacity of the first crop rotation. However, increasing N rates above 70 kg ha⁻¹ N reduced POXC concentration in O-M-W-S rotation below 0.6 m deep and resulted in similar concentrations between crop rotations. We believe that low POXC values in deep soil layers indicate that the side effects of excessive N fertilization is also taking place in O-M-W-S rotation, even though not so harsher as it was in P-M-W-S rotation, where the increase N fertilization was followed by a reduction soil C stock storage below 0.1 m (Table 3).

POXC was positively correlated with MINC, in topsoil layers in fertilized plots, but the relationship disappeared below 0.2 m (Figure 6) increased POXC concentrations were not accompanied increases MINC concentration. Positive relationship between POXC and MINC indicates a simultaneous mineralization/stabilization process was taken place (Hurisso et al., 2016). The absence of correlation between POXC and MINC is an evidence that soil C inputs below 0.2 m are better stabilized and have more potential to build a more resilient C stock. Therefore, higher subsoil POXC concentrations presented by O-M-W-S rotation highlights the potential of black oats in exploring soil volume, input root biomass and increase soil C stocks in subsoil layer.

Table 4. Permanganate oxidable C (POXC) and mineralizable C (MINC) concentrations in a no-till soil profile under seven-year crop rotations in Southern Brazil, where maize is grown biannually after different winter cover crops (black oat and field-pea) and under four N top-dressing rates (0, 70, 140 and 210 kg ha⁻¹ N).

N rate (kg ha ⁻¹)	Cover crop			Cover crop		
	Oat	Pea	Mean	Oat	Pea	Mean
	POXC (mg kg ⁻¹)			MINC (mg kg ⁻¹ 72h ⁻¹)		
0.0 - 0.1 m						
0	780.8±37.2	754.9±24.4	767.8±21.2	360.5±62.5 ^{aA}	158.1±10.8 ^{aB}	259.3±48.2
70	750.4±52.0	710.1±74.6	730.2±42.8	149.0±20.4 ^{bA}	144.3±17.9 ^{aA}	149.7±12.6
140	726.0±48.3	686.9±55.1	706.5±34.7	142.8±15.4 ^{bA}	133.2±11.3 ^{aA}	138.0±9.0
210	759.3±44.1	694.0±57.8	726.7±35.8	143.6±17.9 ^{bA}	125.0±9.7 ^{aA}	134.3±10.0
Mean	754.1±21.1 ^A	711.5±25.9 ^B		199.0±28.7	140.2±6.6	
P-value	Cover (C) = 0.098; N rate (R) = 0.155; C×R = 0.893			Cover (C) = 0.030; N rate (R) = 0.000; C×R = 0.000		
CV (%)	Cover = 6.9; N rate = 7.0			Cover = 25.1; N rate = 23.2		
0.1 - 0.2 m						
0	430.1±25.7	377.4±34.6	403.8±22.3	86.0±0.6	68.6±3.4	77.3±3.6 ^a
70	439.1±33.6	394.5±13.1	416.8±18.7	72.9±5.3	64.5±10.9	68.7±5.8 ^{ab}
140	384.6±26.5	364.9±41.1	374.8±22.9	69.7±5.2	64.8±11.0	67.3±5.7 ^b
210	429.3±15.9	388.2±26.9	408.8±16.4	72.8±3.7	61.9±6.4	67.3±4.0 ^b
Mean	420.8±12.9 ^A	381.3±14.1 ^B		75.4±2.5	65.0±3.9	
P-value	Cover (C) = 0.049; N rate (R) = 0.137; C×R = 0.817			Cover (C) = 0.149; N rate (R) = 0.053; C×R = 0.447		
CV (%)	Cover = 8.7; N rate = 8.9			Cover = 21.7; N rate = 11.0		
0.2 - 0.4 m						
0	280.3±15.0	246.4±19.1	263.4±12.9	44.2±4.5	45.9±5.4	45.0±3.3
70	304.5±24.0	260.7±12.8	282.6±15.1	51.5±17.9	49.4±7.5	50.5±9.0
140	257.2±8.1	260.9±16.8	259.1±8.7	49.4±5.2	32.2±10.0	40.8±6.1
210	272.3±6.2	270.6±15.5	271.4±7.7	47.2±3.7	38.8±8.8	43.0±4.7
Mean	278.6±8.0 ^A	259.7±7.6 ^B		48.1±4.4	41.6±4.0	
P-value	Cover (C) = 0.027; N rate (R) = 0.274; C×R = 0.183			Cover (C) = 0.486; N rate (R) = 0.524; C×R = 0.528		
CV (%)	Cover = 4.9; N rate = 9.2			Cover = 51.5; N rate = 29.7		
0.4 - 0.6 m						
0	187.5±16.1	151.8±14.7	169.7±12.1	32.1±6.1	23.9±5.1	28.0±4.0
70	182.2±12.9	166.1±8.2	174.2±7.7	35.4±14.5	25.1±6.1	30.2±7.5
140	155.4±7.5	164.3±19.3	159.9±9.7	28.0±8.3	25.0±6.1	26.5±4.8
210	164.3±13.8	166.2±10.9	165.2±8.2	26.6±8.2	27.1±4.3	26.9±4.3
Mean	172.4±6.7 ^A	162.1±6.4 ^B		30.5±4.5	25.3±2.5	
P-value	Cover (C) = 0.014; N rate (R) = 0.510; C×R = 0.132			Cover (C) = 0.345; N rate (R) = 0.847; C×R = 0.648		
CV (%)	Cover = 3.3; N rate = 11.6			Cover = 47.6; N rate = 33.1		
0.6 - 0.8 m						
0	121.5±8.2 ^{abA}	93.8±15.1 ^{aB}	107.7±9.5	20.1±5.1	29.2±8.2	24.6±4.8
70	133.2±7.3 ^{aA}	108.1±19.1 ^{aB}	120.6±10.6	31.6±5.6	22.3±7.3	26.9±4.6
140	93.9±8.8 ^{cA}	114.3±15.1 ^{aA}	104.1±9.0	19.2±4.2	15.0±0.0	17.1±2.1
210	104.6±11.6 ^{bcA}	117.0±12.8 ^{aA}	110.8±8.3	19.4±4.4	15.0±0.0	17.2±2.2
Mean	113.3±5.7	108.3±7.4		22.6±2.6	20.4±2.4	
P-value	Cover (C) = 0.637; N rate (R) = 0.208; C×R = 0.009			Cover (C) = 0.593; N rate (R) = 0.103; C×R = 0.261		
CV (%)	Cover = 24.2; N rate = 14.0			Cover = 48.9; N rate = 43.2		
0.8 - 1.0 m						
0	108.1±16.5 ^{abA}	84.0±13.6 ^{aB}	96.1±10.9	19.5±4.5	19.9±4.9	19.7±3.1
70	117.0±13.6 ^{aA}	79.6±12.3 ^{aB}	98.3±11.0	19.6±4.6	20.0±5.0	19.8±3.1
140	74.7±11.9 ^{cB}	97.6±8.0 ^{aA}	86.1±7.9	23.6±5.0	19.9±4.9	21.7±3.3
210	86.7±11.4 ^{bcA}	91.2±10.3 ^{aA}	88.9±7.1	23.8±6.2	27.8±4.6	25.8±3.7
Mean	96.6±7.4	88.1±5.3		21.6±2.4	21.9±2.3	
P-value	Cover (C) = 0.126; N rate (R) = 0.284; C×R = 0.002			Cover (C) = 0.953; N rate (R) = 0.581; C×R = 0.893		
CV (%)	Cover = 12.4; N rate = 15.1			Cover = 58.8; N rate = 45.6		

† Standard errors of mean ($n = 16$ for cover crops means, $n = 8$ for N rates means and $n = 4$ for doses means inside covers).

‡ Means not sharing a lowercase letter in the column or an uppercase letter in the row are significantly different by Tukey's HSD test ($P < 0.1$).

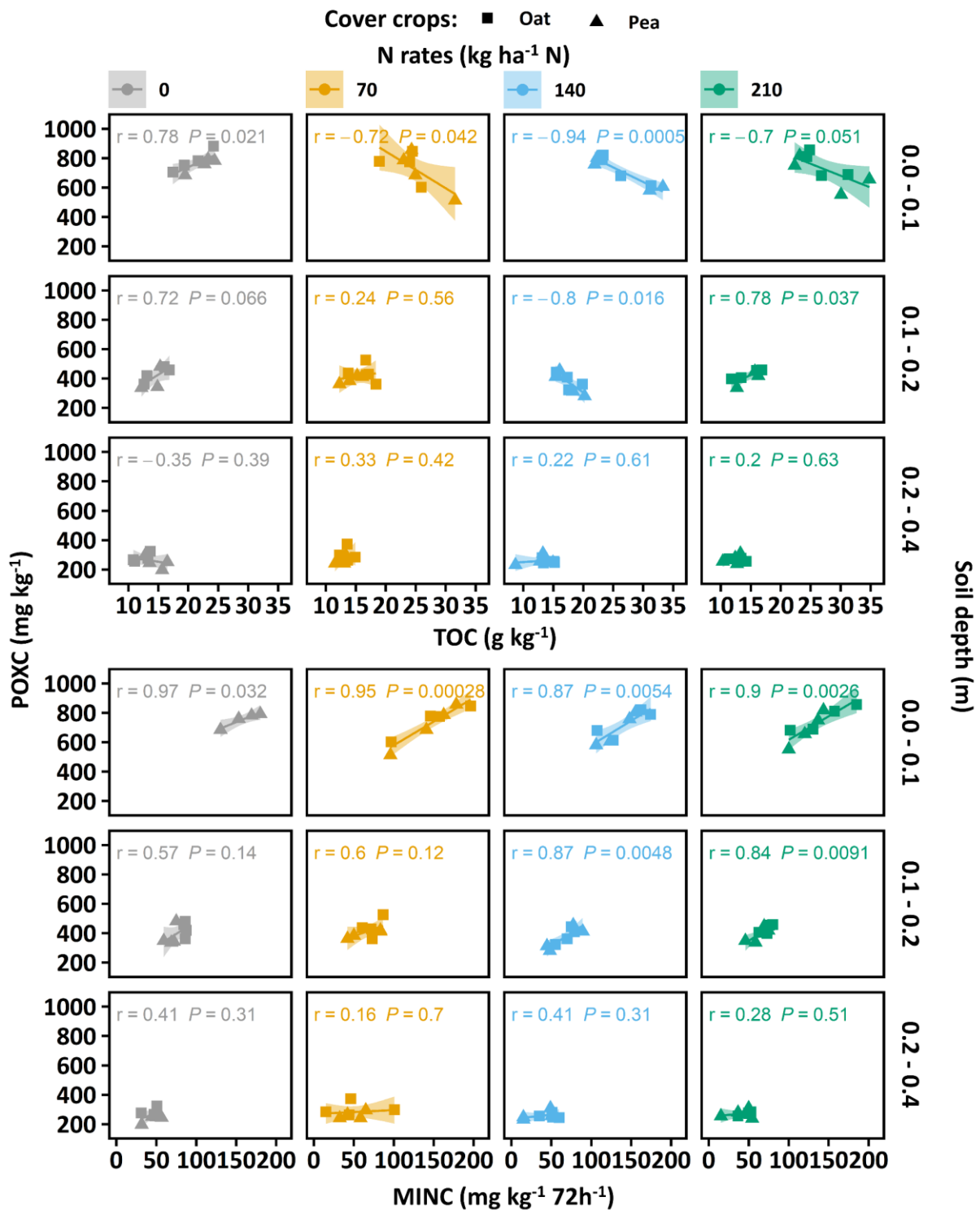


Figure 6. Relationship between permanganate-oxidizable C (POXC) and mineralizable C (MINC) and between POXC and total organic C (TOC) concentrations in three layers (0.0-0.1, 0.1-0.2 and 0.2-0.4 m) of a no-till soil under seven-year crop rotations in Southern Brazil, where maize is grown biannually after different winter cover crops (black oat and field-pea) and under four top-dressing N rates (0, 70, 140 and 210 kg ha⁻¹ N). Shaded areas near the regression lines stand for the 95% confidence interval of the model. MINC values above 300 mg kg⁻¹ were excluded from analysis due its overwhelming influence.

Relationships between POXC and TOC were not affected by cover crops in each soil layer but was significantly changed by N fertilization in the same soil layer. In the first 0.1 m POXC was negatively correlated to TOC in non-fertilized plots but was negatively correlated to TOC in fertilized plots (Figure 6). This relationship was less consistent as soil depth increased and disappeared below 0.2 m. Our findings contrapose several studies that report a strong positive correlation between TOC and POXC (Culman et al., 2012; Hurisso et al., 2016; Lucas and Weil, 2012; Zhong et al., 2015) and bring attention to an overlooked influence of depth and time of sampling on POXC in most of studies where this relationship is established (Culman et al., 2013, 2012). Studies that correlate POXC and TOC in soil samples from multiple sites and/or mixed soil depths in the same site are bound to find positive correlations and erroneously attribute TOC increase to C stabilization, whilst sampling depth and season are the dominant effect. Therefore, the only reasonable conclusion to be drawn for the negative correlation presented herein is that more stable pools of labile soil C (represented by POXC) have decreased as TOC concentration increased by N fertilization in topsoil layers. In other words, most of the TOC increase in topsoil layer due N fertilization occurs in more labile C pools with a lower turnover time that can be easily consumed if the biomass apporsts were discontinued management practices changed.

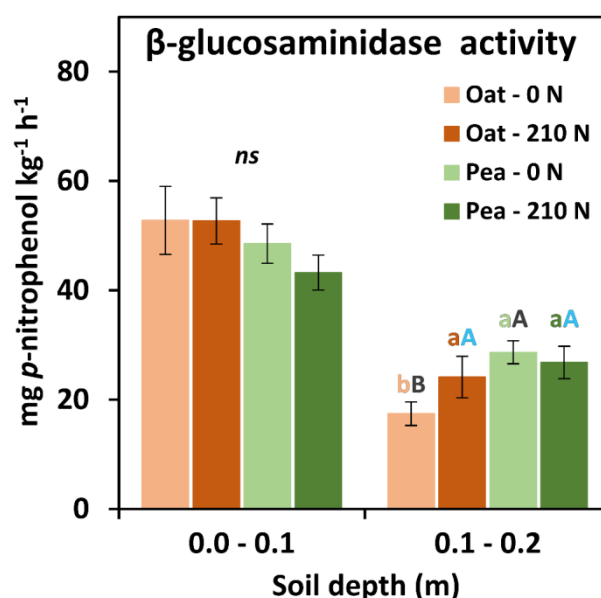


Figure 7. *N*- α -glucosaminidase (NAGase) activity in the topsoil layers (0.0-0.1 and 0.1-0.2 m) as effect of N rates (0 and 210 kg ha⁻¹ N) top-dressed to maize grown biannually after different cover crops (black oat and field-pea) in seven-year crop rotations under no-till in Southern Brazil. Error bars' range represents two standard errors of mean ($n = 4$). Means not sharing a lowercase letter between N rates inside the same cover crop, or an uppercase letter between cover crops inside the same N rate are significantly different by Tukey's HSD test ($P < 0.05$). ns = absence of significant difference between the means in the layer.

Soil samples from the depth of 0.0-0.1 m soil in non-fertilized O-M-W-S plots flushed $360 \text{ g kg}^{-1} \text{ C-CO}_2$ during the 72-h incubation, more than two-fold the average of all samples in the same layer (Table 4). In 0.1-0.2 m layer, higher MINC values were observed in both crop rotations in no-fertilized plots. NAGase activity was higher at the first 0.1 m deep, but differences between treatments were not observed in that layer (Figure 7). In the depth of 0.1-0.2 m, NAGase activity was improved by N fertilization in the black oat rotation, while there was no effect of N fertilization on NAGase activity in the field pea rotation.

NAGase acts in the earlier stages of N mineralization by breaking down chitin and peptidoglycan structures, a constituent of polymers in microbial cell walls, especially fungi (Ekenler and Tabatabai, 2002; Parham and Deng, 2000) and its activity has been suggested as an early indicator of TOC accrual, since this enzyme signalizes increments in chitin, which is more resistant to degradation in soils than cellulose and may contribute to greater soil C stocks (Margenot et al., 2017). The later CO_2 flux in soil sample from treatments with lower N inputs in association with the lower NAGase activity (Figure 7) in treatments under low SIN was available (Table 2) indicate a delayed residue mineralization and reduced C stabilization when N was a limiting component for microorganisms to process residues C and incorporate it in more stable SOM pools. Corroborating our proposition, Muruganandam et al. (2009) and Tiemann and Grandy (2015) verified that elevated NAGase activities were related to soil C accrual in $<0.5 \text{ mm}$ aggregates, and Cotrufo et al. (2019) reported that mycological communities has a higher nitrogen demand to sequester soil C in more persistent mineral-associated organic matter.

The results here presents reinforces the hypothesis raised by several authors that N is essential to build C stocks in topsoil layers in long term and cropping systems with negative N balance tend to emit more CO_2 and have their C stocks reduced over time and the opposite effect would be observed for the positive N balance (Boddey et al., 2010; Lal and Kimble, 1997; Zotarelli et al., 2012). Additionally, our findings indicate that, even though excessive N fertilization may deplete soil C storage in systems where a significant amount of N is already provided by biological N fixation, adequate N availability is essential to maximize SOM storage in agriculture systems. In this way, the capacity of field pea of making N available boosts a simultaneous process of plant biomass inputs and mineralization and soil C stabilization in short N budgets, reduces the dependence of N fertilizer to maximize maize yields and support mechanisms of soil C accrual in subtropical Oxisols.

4 CONCLUSIONS

Top-dress N fertilization did not alter organic N distribution in soil profile in both crop rotations but increased inorganic N accumulation in subsoil in black oats' rotation. Despite field pea's potential of biological N fixation, more N was accumulated in subsoil when maize was grown after black oats. We believe that a higher N extraction along the maize growing season as consequence of the higher maize yields is the major factor that contributed to reducing inorganic N left in the soil profile after maize harvest in field pea's rotation.

N fertilization increased soil C stocks in surface soil layers in both crop rotations but decrease soil C storage in subsoil in field pea's rotation. However, in opposite of observed in temperate conditions, our findings indicate that it is more related to a shallower plant rooting when high N rates are applied, than to a possible priming effect induced by extra N availability. Future studies investigating the impact of N fertilization on soil C and N storage should include root growth analysis in order to elucidate the mechanisms behind the SOM dynamics in agricultural systems.

Including a legume as winter cover crop in maize/soybean rotations has potential in improve maize yield and reduce N fertilizer demand right in the first year. This reduction is highly recommended since applying high N rates may reduce soil C storage and increase subsoil N enrichment in agricultural Oxisols under no-tillage system.

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APPENDIX

APPENDIX A. Total organic carbon (TOC) and total nitrogen (TN) concentrations in a no-till soil profile under seven-year crop rotations in Southern Brazil, where maize is grown biannually after different winter cover crops (black oat and field-pea) and under four top-dress N rates (0, 70, 140 and 210 kg ha⁻¹ N).

N rate (kg ha ⁻¹)	Cover crop			Cover crop		
	Oat	Pea	Mean	Oat	Pea	Mean
	TOC (g kg ⁻¹)			TN (g kg ⁻¹)		
0.0 - 0.1 m						
0	20.65±1.46†	22.46±1.06	21.55±0.90 ^{b‡}	1.59±0.10	1.78±0.09	1.69±0.07 ^b
70	23.32±1.52	25.94±1.92	24.63±1.24 ^{ab}	1.85±0.09	2.02±0.18	1.94±0.10 ^{ab}
140	26.01±1.90	27.13±2.95	26.57±1.64 ^a	2.07±0.19	2.21±0.35	2.14±0.18 ^a
210	26.80±1.57	27.58±2.97	27.19±1.56 ^a	2.20±0.20	2.29±0.35	2.24±0.19 ^a
Mean	24.20±0.96	25.78±1.18		1.93±0.09	2.08±0.13	
P-value	Cover (C) = 0.321; N rate (R) = 0.038; C×R = 0.965			Cover (C) = 0.366; N rate (R) = 0.022; C×R = 0.991		
CV (%)	Cover = 15.1; N rate = 15.4			Cover = 20.1; N rate = 17.3		
0.1 - 0.2 m						
0	14.63±1.05	15.81±1.84	15.22±1.01	0.95±0.05	1.01±0.10	0.98±0.05 ^b
70	16.47±0.98	14.36±0.83	15.41±0.71	1.11±0.09	0.87±0.04	0.99±0.06 ^b
140	17.56±0.90	17.49±1.10	17.52±0.66	1.21±0.08	1.19±0.14	1.20±0.08 ^a
210	14.48±1.17	16.61±1.91	15.54±1.11	0.97±0.03	1.13±0.16	1.05±0.08 ^{ab}
Mean	15.78±0.57	16.06±0.73		1.06±0.04	1.05±0.06	
P-value	Cover (C) = 0.865; N rate (R) = 0.154; C×R = 0.264			Cover (C) = 0.927; N rate (R) = 0.048; C×R = 0.121		
CV (%)	Cover = 26.9; N rate = 13.6			Cover = 30.3; N rate = 15.3		
0.2 - 0.4 m						
0	12.21±0.73	14.58±0.89	13.39±0.70	0.71±0.03	0.78±0.08	0.74±0.04
70	13.59±0.54	12.63±0.41	13.11±0.36	0.77±0.07	0.72±0.07	0.75±0.04
140	14.21±0.56	12.41±1.33	13.31±0.75	0.90±0.08	0.68±0.07	0.79±0.07
210	13.00±0.59	12.18±0.64	12.59±0.43	0.72±0.03	0.69±0.06	0.71±0.03
Mean	13.25±0.33	12.95±0.47		0.77±0.03	0.72±0.03	
P-value	Cover (C) = 0.180; N rate (R) = 0.772; C×R = 0.101			Cover (C) = 0.278; N rate (R) = 0.652; C×R = 0.204		
CV (%)	Cover = 3.7; N rate = 12.8			Cover = 15.6; N rate = 17.0		
0.4 - 0.6 m						
0	9.55±0.78	10.69±0.49	10.12±0.48	0.48±0.03 ^{bA}	0.54±0.03 ^{aA}	0.51±0.02
70	11.64±2.24	10.45±0.93	11.04±1.15	0.47±0.05 ^{bA}	0.57±0.07 ^{aA}	0.52±0.04
140	11.52±1.02	9.40±0.82	10.46±0.73	0.60±0.03 ^{aA}	0.50±0.04 ^{aA}	0.55±0.03
210	10.34±0.51	9.68±0.69	10.01±0.42	0.55±0.04 ^{abA}	0.53±0.04 ^{aA}	0.54±0.03
Mean	10.77±0.63	10.05±0.36		0.52±0.02	0.53±0.02	
P-value	Cover (C) = 0.181; N rate (R) = 0.745; C×R = 0.461			Cover (C) = 0.841; N rate (R) = 0.595; C×R = 0.030		
CV (%)	Cover = 11.1; N rate = 19.6			Cover = 21.2; N rate = 12.0		
0.6 - 0.8 m						
0	8.13±0.32 ^{aA}	8.87±0.19 ^{aA}	8.50±0.22	0.40±0.03	0.46±0.03	0.43±0.02
70	7.96±0.53 ^{aB}	9.04±0.15 ^{aA}	8.50±0.33	0.35±0.03	0.46±0.06	0.40±0.04
140	8.56±0.13 ^{aA}	8.16±0.40 ^{abA}	8.36±0.21	0.46±0.04	0.40±0.03	0.43±0.03
210	7.78±0.56 ^{aA}	7.46±0.48 ^{bA}	7.62±0.35	0.40±0.05	0.40±0.04	0.40±0.03
Mean	8.11±0.20	8.38±0.22		0.40±0.02	0.43±0.02	
P-value	Cover (C) = 0.313; N rate (R) = 0.030; C×R = 0.060			Cover (C) = 0.580; N rate (R) = 0.800; C×R = 0.095		
CV (%)	Cover = 7.8; N rate = 7.6			Cover = 30.0; N rate = 15.7		
0.8 - 1.0 m						
0	7.61±0.55	7.69±0.29	7.65±0.29	0.32±0.02	0.39±0.04	0.36±0.02
70	7.36±0.66	8.03±0.45	7.70±0.39	0.32±0.03	0.36±0.05	0.34±0.03
140	6.95±0.73	8.14±0.47	7.54±0.46	0.36±0.05	0.40±0.06	0.38±0.04
210	7.49±0.64	6.46±0.44	6.98±0.41	0.35±0.03	0.35±0.04	0.35±0.02
Mean	7.36±0.30	7.58±0.25		0.34±0.02	0.38±0.02	
P-value	Cover (C) = 0.662; N rate (R) = 0.405; C×R = 0.134			Cover (C) = 0.379; N rate (R) = 0.466; C×R = 0.467		
CV (%)	Cover = 17.6; N rate = 12.4			Cover = 30.9; N rate = 14.2		

† Standard errors of mean ($n = 16$ for cover crops means, $n = 8$ for N rates means and $n = 4$ for doses means inside covers).

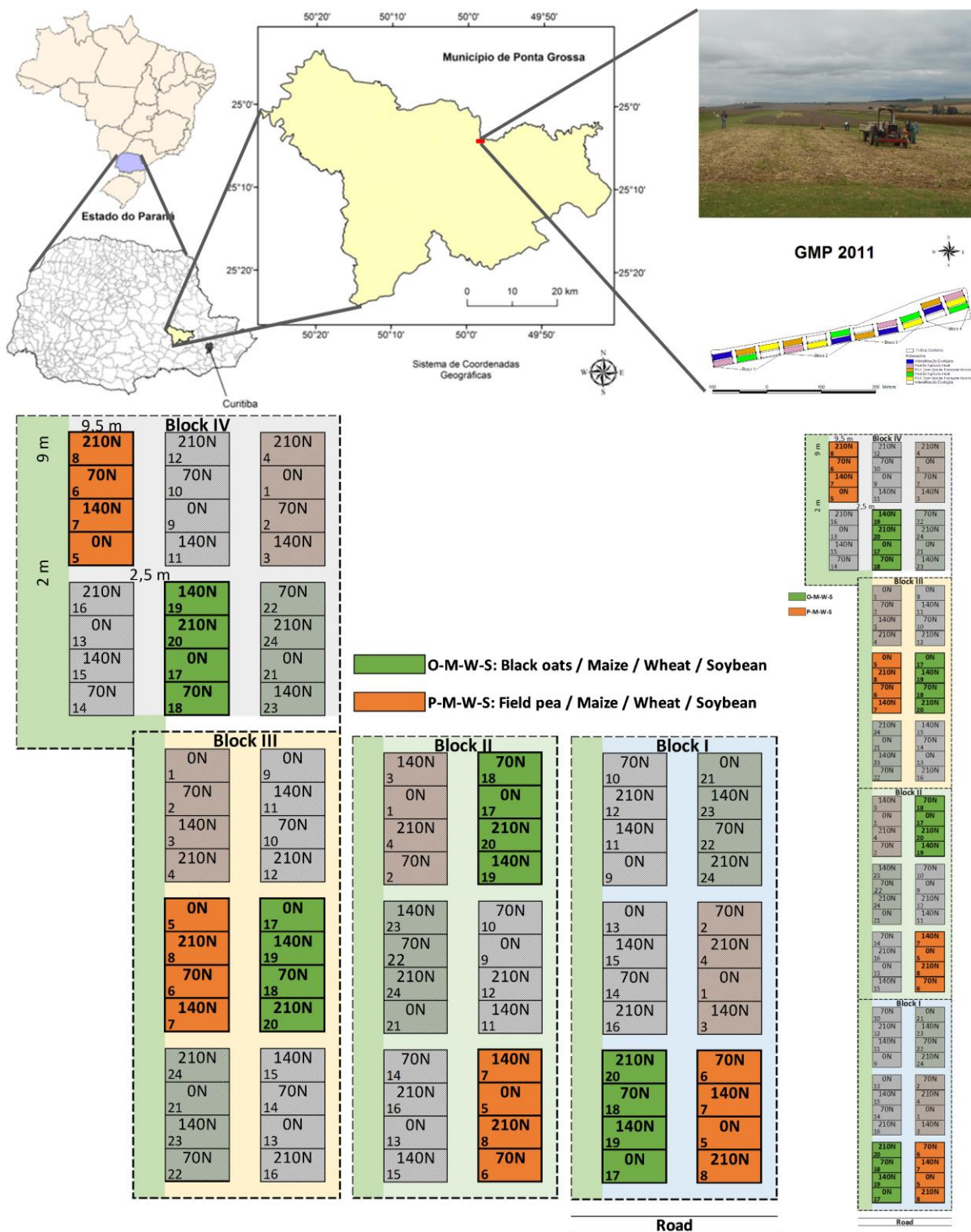
‡ Means not sharing a lowercase letter in the column are significantly different by Tukey's HSD test ($P < 0.1$).

APPENDIX B. Ammonium (NH_4^+) and nitrate plus nitrite ($\text{NO}_3^- + \text{NO}_2^-$) concentration in a no-till soil profile under seven-year crop rotations in Southern Brazil, where maize is grown biannually after different winter cover crops (black oat and field-pea) and under four top-dressing N rates (0, 70, 140 and 21 kg N).

N rate (kg ha ⁻¹)	Cover crop			Cover crop		
	Oat	Pea	Mean	Oat	Pea	Mean
	N-[NH ₄ ⁺] (mg kg ⁻¹)			N-[NO ₃ ⁻ +NO ₂ ⁻] (mg kg ⁻¹)		
0.0 - 0.1 m						
0	5.0±0.9†	4.9±0.5	4.9±0.5 ^{a‡}	6.8±1.2	9.5±1.6	8.1±1.1 ^b
70	4.0±0.9	3.4±0.7	3.7±0.5 ^{bc}	9.7±1.0	10.1±0.9	9.9±0.6 ^a
140	4.6±0.9	3.1±0.8	3.9±0.6 ^b	10.0±1.5	10.8±0.7	10.4±0.8 ^a
210	3.4±0.9	2.6±0.3	3.0±0.5 ^c	9.8±1.1	10.1±0.1	10.0±0.5 ^a
Mean	4.2±0.4	3.5±0.3		9.1±0.6	10.1±0.5	
P-value	Cover (C) = 0.197; N rate (R) = 0.000; C×R = 0.229			Cover (C) = 0.155; N rate (R) = 0.019; C×R = 0.247		
CV (%)	Cover = 32.0; N rate = 17.8			Cover = 16.7; N rate = 14.1		
0.1 - 0.2 m						
0	1.6±0.3	<1.0	-	1.4±0.2 ^{bB}	2.4±0.4 ^{aA}	1.9±0.3
70	1.6±0.6	<1.0	-	2.4±0.5 ^{aA}	1.9±0.5 ^{aA}	2.2±0.3
140	1.5±0.2	<1.0	-	2.9±0.4 ^{aA}	1.8±0.5 ^{aB}	2.3±0.4
210	1.2±0.3	<1.0	-	2.9±0.5 ^{aA}	2.5±0.5 ^{aA}	2.7±0.3
Mean	1.5±0.2	-		2.4±0.2	2.2±0.2	
P-value	Cover (C) = 0.064; N rate (R) = 0.384; C×R = 0.932			Cover (C) = 0.245; N rate (R) = 0.065; C×R = 0.011		
CV (%)	Cover = 32.1; N rate = 31.9			Cover = 20.8; N rate = 24.0		
0.2 - 0.4 m						
0	<1.0	<1.0	-	1.9±0.4	<1.0	-
70	<1.0	<1.0	-	2.2±0.5	<1.0	-
140	<1.0	<1.0	-	1.9±0.5	<1.0	-
210	<1.0	<1.0	-	2.3±0.3	<1.0	-
Mean	-	-		2.1±0.2 ^A	-	
P-value	Cover (C) = 0.151; N rate (R) = 0.141; C×R = 0.999			Cover (C) = 0.012; N rate (R) = 0.363; C×R = 0.873		
CV (%)	Cover = 21.8; N rate = 23.1			Cover = 28.6; N rate = 46.5		
0.4 - 0.6 m						
0	<1.0	<1.0	-	1.1±0.4	<1.0	-
70	<1.0	<1.0	-	2.0±0.5	<1.0	-
140	<1.0	<1.0	-	2.0±0.8	<1.0	-
210	<1.0	<1.0	-	2.2±0.2	<1.0	-
Mean	-	-		1.8±0.3 ^A	-	
P-value	Cover (C) = 0.193; N rate (R) = 0.891; C×R = 0.911			Cover (C) = 0.032; N rate (R) = 0.327; C×R = 0.480		
CV (%)	Cover = 25.3; N rate = 37.1			Cover = 54.5; N rate = 53.3		
0.6 - 0.8 m						
0	<1.0	<1.0	-	1.0±0.3	<1.0	-
70	<1.0	<1.0	-	1.7±0.5	<1.0	-
140	<1.0	<1.0	-	1.6±0.9	<1.0	-
210	<1.0	<1.0	-	2.4±0.2	<1.0	-
Mean	-	-		1.6±0.3 ^A	-	
P-value	Cover (C) = 0.247; N rate (R) = 0.753; C×R = 0.723			Cover (C) = 0.022; N rate (R) = 0.223; C×R = 0.136		
CV (%)	Cover = 59.7; N rate = 39.5			Cover = 74.9; N rate = 57.7		
0.8 - 1.0 m						
0	<1.0	<1.0	-	<1.0	<1.0	-
70	<1.0	<1.0	-	<1.0	<1.0	-
140	<1.0	<1.0	-	<1.0	<1.0	-
210	<1.0	<1.0	-	<1.0	<1.0	-
Mean	-	-		-	-	
P-value	Cover (C) = 0.899; N rate (R) = 0.776; C×R = 0.492			Cover (C) = 0.055; N rate (R) = 0.193; C×R = 0.354		
CV (%)	Cover = 38.1; N rate = 33.8			Cover = 57.3; N rate = 56.7		

† Standard errors of mean ($n = 16$ for cover crops means, $n = 8$ for N rates means and $n = 4$ for doses means inside covers).

‡ Means not sharing a lowercase letter in the column or an uppercase letter in the row are significantly different by Tukey's HSD test ($P < 0.1$).



APPENDIX D. Soil sampling and laboratorial analysis, where: *a.* Field team performing soil sampling for chemical analysis using soil probes and Dutch augers; *b.* Slicing the soil core inside soil probe and separating soil samples by depth; *c.* Undisturbed soil cores collected to calculate soil bulk density; *d.* Soil sample collected for chemical analysis being packed in labeled PVC bags; *e.* Filtration of soil extracts for Soluble N analysis; *f.* Soil weighting in precision scale; *g.* Solution absorbance being measured with a spectrophotometer in permanganate oxidable C analysis; *h.* Screw-capped glass tubes containing soil extracts being placed into an autoclave for persulfate-oxidation procedure; *i.* Quantification of N concentration in soil extracts by flow injection analysis; *j.* Titration of NaOH traps with an auto titrator in mineralizable C analysis.

