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Long-Term Winter Cover Crops Alter the Soil Microbial Biomass and Enzyme Activities in Brazilian Oxisols

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Abstract

The diversification of cover crops grown in soils with granulometric variability may potentially enhance microbial community and enzyme activities. Thus, the main goal was to evaluate the effect of autumn/winter cover crop sequences and cash crops in spring/summer on soil microbial biomass and enzyme activities. The experiment was conducted in open-field microplots (10 m × 1 m × 0.7 m), containing soils from B horizon of five Oxisols with granulometric variability and clay content ranging from 17 to 80 dag kg^{-1}. The treatments were three cover crops and a winter fallow with a completely randomized experimental design with three replicates. Soil samples from the 0–10 cm layer were collected to analyze soil microbial biomass of carbon and nitrogen, enzyme activities of the acid phosphatase, arylsulfatase, urease, and fluorescein diacetate hydrolysis. The number of nodules in soybean roots was average 63 ± 14.42 nodules per plant and dry mass of nodules was 169 ± 13.74 mg $plant^{-1}$. Soybean nodulation and N uptake ensured the supply of nitrogen to the soybean plants with 331 ± 82 mg $plant^{-1}$. Overall, diversified autumn/winter cover crop sequences provide plant residue, containing nutrients, and different carbon/nitrogen, which alters microbial biomass, the ratio of C_{mic}/N_{mic}, basal respiration, and soil enzyme activities within each Oxisol with different particle size distribution.

Keywords: biophysical interactions; granulometric variability; *Glycine max*; biochemical index activity; acid phosphatase; arylsulfatase; urease; fluorescein diacetate hydrolysis

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1. Introduction

The concept of soil fertility involves the chemical, physical, and biological properties of the soil. In (sub)tropical environments, crops cultivated during the autumn and winter seasons to manage soil health, diseases, pests, fitonematodes, and weeds are known as winter cover crops. In contrast, crops cultivated in spring and summer—such as maize (*Zea mays* L.) and soybean (*Glycine max* L.) —are considered cash crops. Winter cover crops in succession with these cash crops play an important role in the chemical, physical, and microbiological properties in tropical and subtropical soils. Soil health data measured on a laboratory scale can be used to improve crop yields through land management decisions, enhancing the sustainability of agroecosystems [1].

Environmental factors such as soil water content, aeration, temperature, and mechanical impedance directly affecting plant growth [2] and increase nodulation and biological N_2 fixation (BNF) of soybean [3]. Temperatures between 20 °C and 30 °C are optimal for

symbiotic nitrogen fixation by *Bradyrhizobium japonicum*, whereas temperatures outside this range—either lower or higher—exert inhibitory effects [3].

Cover crops incorporated into agricultural practices provide various environmental benefits, such as maintaining soil and ecosystem health, as well as increasing the yield of main crops, possibly due to improvements in various soil processes [4,5]. However, specific local factors, such as climate, soil type, cover crop species, and cultivation practices, must be considered to optimize the benefits of cover crops.

A 30-year double cropping system of soybean and maize in a Oxisol (Latossolo Vermelho Distrófico) with granulometric variability proved effective in improving aggregation indices and distribution of macro and microaggregates, but not in enhancing organic carbon levels [6]. In five very clayey texture Oxisol-derived igneous rocks, from (sub) tropical soils of the State of Paraná, Southern Brazil, total organic carbon content was the most important for the stabilization of 4–8 mm aggregates and iron oxide (Fe_2O_3) for aggregate resistance against breakdown [7].

Conservation agriculture practices, such as no-till systems in Brazil, are widely adopted; however, there is no guarantee of sustainability if there are no efforts to transfer and adopt technology by farmers related to the other two principles of ecological agriculture, which are the permanent soil cover with biomass and the diversification of crops through rotations/sequences in the production system. Previous studies using data from long-term field experiments have shown that the use of agronomic practices such as no-till farming and crop rotation with legumes will contribute not only to agricultural sustainability but will also help maintain the population and diversity of symbiotic *Bradyrhizobium* of soybean [8] and also increase the profitability of soybean grain production when inoculated with *Bradyrhizobium* [9].

Microbial biomass is a sensitive bioindicator of changes in the soil [10,11], and the activities of enzymes related to nutrient cycles can provide information on how soil management influences the potential to carry out processes such as decomposition and nutrient cycling [1,12,13]. Similar to the number of microorganisms in the soil, microbial biomass and enzymatic activity show seasonal variation [14] due to factors such as moisture, temperature, aeration, soil structure, and organic matter content.

Negative effects of excessive soil compaction on microbial attributes, in an Oxisol in Londrina, Paraná, were observed when the mean weight diameter was increased from 3 mm to 8 mm, which led to a decrease in labile carbon, microbial biomass carbon, cellulase activity, and basal respiration [15].

Oxisols make up approximately 8% of the world's frost-free land surface, predominantly consisting of acidic soils containing low-activity minerals such as quartz, kaolinite, and iron oxides, with low natural fertility and low capacity to retain additions of lime and fertilizers [16]. In our study, the choice of the five Oxisols (Latosols) was due to its representativity; these soils occur in approximately 30% of the surface area of the state of Paraná [17].

In our study, we tested the hypothesis that winter cover crop sequences cultivated in Oxisols with granulometric variability influences the microbial community and its activity. Thus, the main goal was to evaluate soil microbial biomass and enzyme activity in microplot long-term experiments of autumn/winter cover crop sequences and cash crops in spring/summer.

2. Material and Methods

2.1. Geographical Location, Climate, and Soil Description

The experiment was conducted in open-field microplots (10 m × 1 m × 0.7 m), which were built at the Research and Innovation Station of the Rural Development Institute

of the State of Paraná, IAPAR-EMATER (IDR-Paraná), in Londrina, Paraná State, Brazil (23°21′30″ S, 51°10′17″ W). These microplots contained soils collected in 2004, from the Bw horizon at a depth of 1.0 m to 1.2 m from five Oxisols (latosols), classified according to the Brazilian Soil Classification System [18], from different municipalities in the state of Parana, Brazil. The experiments were set up and conducted without machine traffic, with sowing, weeding, pest control, and disease management operations carried out manually, with details reported by Lima et al. [19].

Air temperature and rainfall information during the conduction of the study were obtained from the weather (meteorological) station located 200 m from the experimental area. In the period before the soybean sowing, from 1 November 2023 to 12 November 2023, there was no rainfall, and the maximum temperatures were above 30 °C while the minimum temperatures ranged from 12.5 to 26.7 °C. On 5 December 2023, there was a high rainfall of 61.2 mm and the maximum air temperature was 24.0 °C (Figure 1).

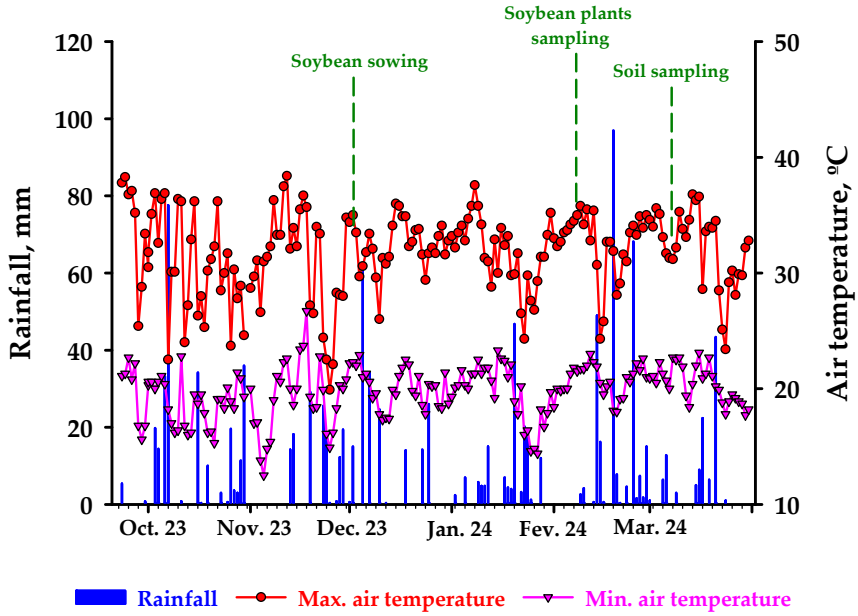


Figure 1. Rainfall and maximum and minimum air temperature during the spring/summer of 2023/2024. Data recorded at the meteorological station of the IDR-Paraná in Londrina County, State of Paraná, located 200 m from the experimental area.

2.2. Soil Physical Analysis

Physical characterization of the soil samples, collected from topsoil layer (0–10 cm), in 2015, was performed by determining soil particle density using a volumetric flask [20]. Particle-size analysis to obtain granulometric distribution of clay (<2 μm), silt (2–50 μm), and sand (>50 μm) fractions was performed using the pipette method [21,22], with chemical dispersion using a 10 mL 1 N sodium hydroxide solution in contact with the samples for 24 h. Mechanical dispersion was accomplished over 16 h [23] in a reciprocating shaker that shakes 180 times per minute with a 38 mm amplitude [24]. Water-dispersible clay was determined by mechanical shaking in water for 2 h [25]. Total porosity was calculated according to Flint and Flint [26]. The site, soil classification, and physical characterization of these soil samples are given in Table 1.

Table 1. Site, soil classification, and physical characterization of the soil's surface layer (0–10 cm depth).

Soil Properties \ Country	Londrina (LDA)	Mauá da Serra (MSA)	Arapongas (ARP)	Mauá da Serra (MSA)	Ponta Grossa (PTG)
Soil class [1]	LVdf	LAd	LVAd	LVd	LAd
Soil class [2]	Rhodic Haplustox	Typic Haplustox	Typic Haplustox	Typic Haplustox	Rhodic Haplustox
Geographical coordinates [3]	23°11′19″ S 51°09′19″ W	23°53′70″ S 51°11′30″ W	23°22′32″ S 51°26′41″ W	23°21′17″ S 51°11′31″ W	25°06′70″ S 50°10′30″ W
Pd, kg dm^{-3}	2.99	2.69	2.68	2.77	2.61
Clay, dag kg^{-1}	80	17	41	73	48
Silt, dag kg^{-1}	10	2	3	8	2
Sand, dag kg^{-1}	10	81	56	19	50
Texture	Very clayey	Sandy loam	Sand clay	Very clayey	Sand clay
WDC, dag kg^{-1}	24	5	6	6	5
DF, %	70	69	85	92	88
TP, dm^3 dm^{-3}	0.67	0.44	0.53	0.64	0.58
Bd, kg dm^{-3}	1.00	1.51	1.25	1.00	1.09
DC, %	68	84	77	69	70

[1] Soil classification according to the Brazilian Soil Classification System [18] (LVdf: LATOSSOLO VERMELHO Distroférrico; LAd: LATOSSOLO AMARELO Distrófico; LVAd: LATOSSOLO VERMELHO AMARELO Distrófico; LVd: LATOSSOLO VERMELHO Distrófico; [2] Soil classification according to in the USA soil taxonomy [27]. [3] Local geographical coordinates: where soil samples were extracted in B horizon the year 2004. Pd: particle density; WDC: water-dispersible clay; DF: degree of flocculation, TP: total porosity; Bd: soil bulk density; DC: degree of compactness calculated by ratio (Bd/max bulk density) × 100.

2.3. Soil Chemical Analyses

In May 2022, for soil chemical characterization, 100 days after gypsum application and before maize cultivation in cropping season 2022/2023, samples were collected from the topsoil layer (0–10 cm) with the aid of the auger, which were dried at 60 °C for 48 h in a forced-draft oven, milled to pass a 2 mm sieve. Soil pH was determined in a solution of 0.01 mol L^{-1} $CaCl_2$ at a soil/solution ratio of 1:2.5. Exchangeable calcium, magnesium, and aluminum were extracted with neutral 1 mol L^{-1} KCl in a 1:10 soil solution ratio and analyzed by atomic absorption spectrophotometry. The available phosphorus and potassium exchangeable contents were extracted with a mixture of sulfuric and hydrochloric acid (Mehlich-1), the phosphorus being determined by colorimetry with molybdenum blue and potassium by flame photometry. Extraction H + Al was carried out with $Ca(OAc)_2$ 0.5 mol L^{-1}, pH 7, at a ratio of 5 to 75 cm^3, TFSA mL extractor 10 min stirring, and decanting for 16 h. The cation-exchange capacity (CEC at pH 7.0) was obtained by the sum of Ca + Mg + K + (H + Al). Total organic carbon (TOC) content was determined with wet combustion by the Walkley–Black method with organic carbon oxidation of 5 mL of $K_2Cr_2O_7$ (potassium dichromate) 0.167 mol L^{-1} and 10 mL of concentrated H_2SO_4 (sulfuric acid) concentrate. All the soil chemical attributes were determined according to Pavan et al. [28].

2.4. Treatments, Experimental Design and Conduction

In each experiment, the treatments were the sequences of cover crops in autumn/winter and cash crops in the summer that were conducted during the years 2015 to 2023 as described in Table 2. The crops used were canola (*Brassica napus* L. var. oleífera), crambe (*Crambe abyssinica*), white-oat (*Avena sativa* L.) cv IPR Afrodite, black-oat (*Avena strigosa* Schreb.), cv Iapar 61 Ibiporã, forage pea (*Pisum sativum* L.) cv IAPAR 83, white lupine (*Lupinus albus*), and vetch (*Pisum sativum* L.), while soybean crops (*Glycine max*), cv BMX Lótus IPRO, or maize (*Zea maize* L.) híbrido AGN 2M 40 VT PRO4 (Agromen), or dry beans (*Phaseolus vulgaris* L.) cv IPR sabiá were culti-

vated for spring/summer. The experimental design was completely randomized, with three repetitions.

Table 2. Treatments, autumn/winter cover crop sequences (CCS) and spring/summer cash crops in each cropping season.

Cropping Seasons	Treatments					
	CCS 1	CCS 2	CCS 3	CCS 1	CCS 2	CCS 3
	Autumn/Winter			Spring/Summer		
	Cover Crop Sequences					
2015/2016	Canola	White-oat	Crambe	Soybean	Soybean	Soybean
2016/2017	Canola	White-oat	Crambe	Soybean	Soybean	Soybean
2017/2018	Black-oat	Black-oat	Black-oat	Soybean	Soybean	Soybean
2018/2019	Black-oat	Black-oat	Black-oat	Dry bean	Dry bean	Dry bean
2019/2020	White-oat	White-oat	White-oat	Soybean	Soybean	Soybean
2020/2021	Black-oat	Black-oat	Black-oat	Corn	Corn	Corn
2021/2022	Black-oat	Black-oat	Black-oat	Soybean	Soybean	Soybean
2022/2023 *	Forage Pea	White lupine	Vetch	Corn	Corn	Corn
2023/2024 **	Fallow ***	Fallow ***	Fallow ***	Soybean	Soybean	Soybean

CCS = cover crop sequences; * winter plant biomass sampling and ** soil sampling; *** predominance of Brachiaria.

In May 2022, a surface application of agricultural gypsum was carried out (calcium sulfate—$CaSO_4 \cdot 2H_2O$), in the dose calculated according to Souza et al. [29], and Quaggio and van Raij [30]. Cultural practices were recommended for each crop. Before the soybean sowing, in October and December 2023, the herbicides glyphosate (3 L ha^{-1}) and carfentrazona ethyl (0.1 L ha^{-1}) were applied. Soybean cultivar BMX Lotus IPRO was sown in December 2023 at a density of 12 seeds per linear meter, spaced 0.45 m apart. The liquid inoculant containing strains of *Bradyrhizobium japonicum* (SEMIA 5079 e SEMIA 5080) was applied to the soil surface at a rate six times the recommended dose. During the initial development of the soybean, shade nets were installed on the soil surface to protect the seeds and seedlings. Before soybean planting, moisture and temperature sensors were installed.

Temporal fluctuations in soil physical properties at 0–5 cm depth, volumetric water content (θv), temperature (St), and bulk electrical conductivity (EC) were measured with Teros 12® (METER Group, Inc., Pullman, WA, USA) capacitance-based sensors (70 MHz oscillating wave), according to Cominelli et al. [31], connected to a ZL6 4G® datalogger (METER Group, Inc.) and data transmitted through to Zentra Cloud platform. The dataset spanned a period 15 days after soybean sowing from 2 December 2023 to 16 December 2023.

2.5. Plant and Soil Microbial Analysis

In February 2024, root nodulation and N uptake in the shoot of soybean were performed. In March 2024, microbial analysis was carried out on fresh soil samples collected from the 0–10 cm layer, which were sieved through 2 mm sieves and kept at temperatures of 6 to 10 °C until the analyses.

2.5.1. Soybean Nodulation and Nitrogen Uptake

At full flowering of soybean (R2), the plants were collected to determine the dry matter of the shoot at 65 °C and to evaluate nodulation, the number, and dry weight of nodules, according to Cardoso et al. [32]. The N concentration was determined in the dry matter of the shoot [33]. Nitrogen uptake was calculated using the dry matter of the shoot production per plant and N concentration [34].

2.5.2. Soil Microbial Analysis

In March 2024, soil samples from the layer 0–10 cm were collected, sieved through sieves 2 mm and kept at temperatures of 6 the 10 °C for microbiological analysis. All microbial data were expressed on a dry soil basis.

The microbial biomass C (MBC) and microbial biomass of N (MBN) were determined by the fumigation–extraction method according to Vance et al. [35] and calculated using a correction factor (kc) of 0.33 [36] for MBC and using 0.54 for MBN as conversion factor [37]. The ratio between the MBC and the MBN was calculated.

The basal soil respiration (BSR) was determined using the methodology described elsewhere [38–40] with adaptation, in which soil samples were incubated at 26 ± 2 °C for 10 days and measured by the amount of C-CO_2 released. Soil metabolic quotient (qCO_2) was calculated by dividing soil basal respiration by the C of the soil microbial biomass.

Activity of soil enzyme arylsulfatase (i.e., arylsulfate sulfohydrolase, EC 3.1.6.1) and acid phosphatase (EC 3.1.3) were measured after soil samples were incubated for 60 min at 37 °C in buffer solution, with *p*-nitrophenol sulfate or *p*-nitrophenol phosphate, respectively, with reading in a spectrophotometer at 410 nm [41]. Activity of soil urease (urea amidohydrolase EC 3.5.1.5) was determined according to procedures described by Tabatabai and Bremner [42] with reading at 690 nm, after a 30 min incubation at room temperature. The fluorescein diacetate (FDA) activity was determined by method described elsewhere [43] with absorbance reading at 490 nm. The biochemical index activity (BIA) was calculated based on a modified procedure that included enzyme activities related on total organic C (TOC) content and clay content (Wyszkowska et al., 2013) [44].

2.6. Statistical Analyses

The experimental data were subjected to the normality test (Shapiro–Wilk) and homogeneity of variance (Bartlett) and descriptive and variance analysis were performed in each experiment. To perform the joint analysis, the homogeneity of residual variances test was applied [45]. Only the number of nodules and dry mass of nodules per plant presented homogeneous residual variances and were subjected to joint analysis. The means were compared by Tukey's test at 5% probability.

3. Results

The data from this study refer to the agricultural years of 2022/2023 and 2023/2024 with soybean as the cash crop in the summer (Table 2).

3.1. Physical and Chemical Attributes

Soil texture for the five soils was sandy loam, sand clay, and very clayey. The clay content ranging from 17 dag kg^{-1} (sandy loam texture) for the LATOSSOLO AMARELO Distrófico from Mauá da Serra and 80 dag kg^{-1} (very clayey texture) for the LATOSSOLO VERMELHO Distroférrico típico de Londrina (Table 1).

As can be seen in Figure 1, on 5 December 2023, there was a high intensity precipitation of 61.2 mm and the maximum air temperature of 24.0 °C. The daily soil temperature decreased sharply between December 9 and 10, 2023. However, the daily soil temperature increased linearly on December 10 and 16, 2023 (Figure 2). Daily soil temperatures between 13 and 17 December 2023 exceeded 30 °C, a threshold known to exerted inhibitory effects for symbiotic nitrogen fixation by *Bradyrhizobium japonicum* [3].

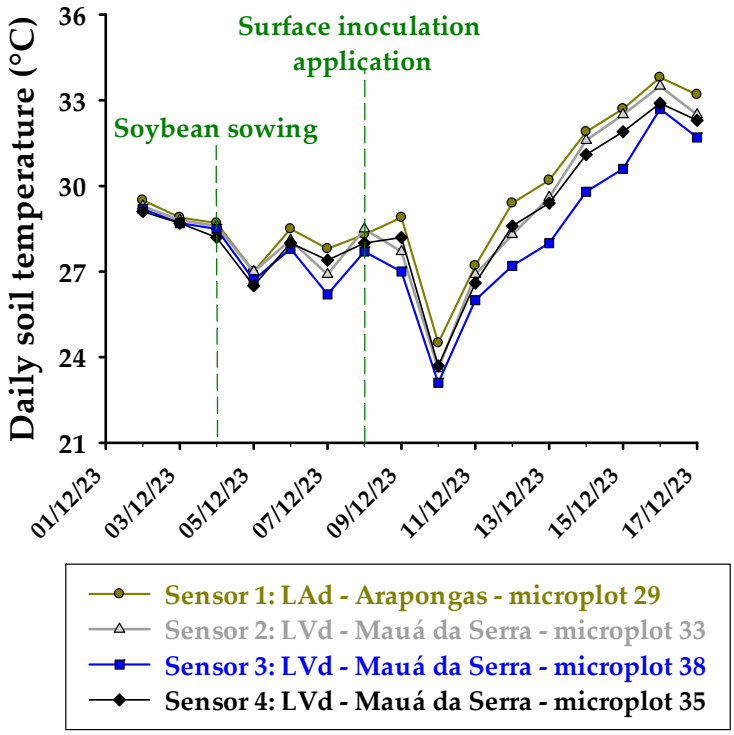


Figure 2. Average daily soil temperature over 15 days in the cropping season 2023/2024.

Soil temperature is affected, among other factors, by solar radiation, soil surface cover, crop type, soil physical properties, soil water content, and the soil's thermal conductivity. The peak volumetric water content in the soil can be seen 0.37–0.38 cm^3 cm^{-3}.

After the precipitation peak on 5 December 2023, it is observed through the electromagnetic sensor installed in the 0–5 cm deep layer at LVd, Mauá da Serra (very clayey texture), that the crop sequence comprising the summer maize (2022/2023 harvest), black-oat cultivar IAPAR 61 Ibiporã (winter 2023), and soybean cultivar BMX Lótus IPRO (2023/2024 harvest) provided higher volumetric water contents in the surface layer with a volumetric water content of 0.25 cm^3 cm^{-3} on 16 December 2023.

On the other hand, it is observed through the sensors installed in LVAd, Arapongas, where the crop sequence comprised summer maize (2022/2023 harvest), black-oat cultivar IAPAR 61 Ibiporã (winter 2023), and soybean cultivar BMX Lótus IPRO (2023/2024 harvest), that the surface layer had the lowest volumetric water contents of 0.16 cm^3 cm^{-3} (close to water retention at −10 kPa matric potential) on 16 December 2023.

The higher clay content in LVd (73 dag kg^{-1} of clay, Mauá da Serra) when compared to LVAd (Arapongas: 41 dag kg^{-1} of clay) may be one of the causes of the higher water content observed. Furthermore, the highest soil density value is 1.52 kg dm^{-3} in LAd [19], which can reduce total porosity and, consequently, water infiltration and retention by the soil. Soil temperature and volumetric water contents in the surface layers, can be due to biophysical interactions between granulometric variability, soil structure, root systems, and microbial attributes.

The chemical characterization was performed in soil samples collected 100 days after gypsum application and before maize cultivation in the cropping season 2022/2023 (Table 3).

Table 3. Chemical characterization of the soil's surface layer (0–10 cm depth). Soil sampling in cropping season 2022/2023.

Chemical Characterization \ Country	Londrina (LDA)	Mauá da Serra (MSA)	Arapongas (ARP)	Mauá da Serra (MSA)	Ponta Grossa (PTG)
pH, $CaCl_2$	6.3 (±0.12)	6.3 (±0.36)	6.2 (±0.41)	6.6 (±0.13)	6.6 (±0.15)
$H^+ + Al^{3+}$, $cmol_c\ dm^{-3}$	2.75 (±0.22)	2.01 (±0.35)	2.63 (±0.56)	2.62 (±0.27)	2.51 (±0.25)
Al^{3+}, $cmol_c\ dm^{-3}$	0.0 (±0.0)	0.0 (±0.0)	0.00 (±0.00)	0.0 (±0.0)	0.0 (±0.0)
TOC, $g\ dm^{-3}$	15.64 (±2.9)	12.7 (±2.15)	20.4 (±2.09)	21.67 (±2.17)	16.99 (±2.39)
P, $mg\ dm^{-3}$	83.2 (±54.5)	101.4 (±52.5)	103.1 (±45.1)	58.90 (±31.6)	49.74 (±22.0)
Ca^{2+}, $cmol_c\ dm^{-3}$	7.3 (±0.86)	3.9 (±0.85)	6.95 (±0.70)	7.30 (±0.80)	5.88 (±1.02)
Mg^{2+}, $cmol_c\ dm^{-3}$	2.4 (±0.25)	1.5 (±0.27)	2.54 (±0.46)	2.59 (±0.32)	2.72 (±0.47)
K^+, $cmol_c\ dm^{-3}$	0.6 (±0.11)	0.16 (±0.05)	0.28 (±0.09)	0.18 (±0.08)	0.16 (±0.05)
SB, $cmol_c\ dm^{-3}$	10.3 (±0.97)	5.6 (±1.11)	9.76 (±1.13)	10.08 (±0.66)	8.76 (±1.45)
CEC, $cmol_c\ dm^{-3}$	13.3 (±1.06)	7.6 (±0.94)	12.39 (±0.70)	12.69 (±0.63)	11.27 (±1.35)
BS, %	78.9 (±1.7)	73.1 (±6.7)	78.59 (±5.41)	79.36 (±2.28)	77.43 (±3.61)

TOC: Total organic carbon; SB: sum of bases = ∑ (Ca, Mg, K, and H + Al); CEC: cation-exchange capacity = ($\sum cmol_c\ dm^{-3}$ of Ca, Mg, K, Al, H); BS: base saturation = (Ca, Mg, K) × 100/CEC; values represent means of nine samples. Numbers between brackets are the standard deviation (Std Dev).

3.2. Soybean Nodulation and N Uptake

For nodule number and dry weight of nodule, a significant difference was observed for the interaction between the Oxisols (latosols) and the crop sequence, with the cover crop sequence being split into each latosol. The number of nodules in soybean root ranged from 43 to 90 $plant^{-1}$, with a mean of 63 ± 14.42 nodules $plant^{-1}$ without (Figure 3a). The autumn/winter cover crop sequences did not influence the number of nodules in soybean cultivated in the following soils: LVdf (Londrina), LVAd (Arapongas), and LAd (Ponta Grossa). In contrast, in both Oxisols (LAd and LVd) from Mauá da Serra municipality, the cultivation of crambe, black-oat, white-oat, black-oat, and vetch (CCS 3) had different effects on soybean nodulation; for instance, the number of nodules per soybean plant in the LVAd soil decreased while it increased in the LVd soil (Figure 3a).

Soybean nodule mass increased in the LVAd (Arapongas) and LVd (Mauá da Serra) soils when cultivated in the autumn/winter with the cover crop sequences, CCS1 and CCS3, respectively. Regardless of the cover crop sequences and soil types, the dry mass of nodules ranged from 99 to 295 mg $plant^{-1}$ with an average of 169 ± 13.74 mg $plant^{-1}$ (Figure 3b).

Overall, there was no effect of autumn/winter cover crop sequences on the concentration of N uptake in the soybean shoot, which varied from 159 to 442 mg $plant^{-1}$ with an average of 331 ± 82 mg $plant^{-1}$ (Figure 3c). However, in highly clayey soils (clay content > 60 dag kg^{-1}), specifically in the LVdf (LDA) and LVd (MSA) classes, the presence of CCS 1 was associated with reduced nitrogen uptake.

3.3. Soil Microbial Biomass, Basal Respiration and Enzyme Activities

The microbial C-biomass ranged from 179 to 565 μg g^{-1} of dry soil with an average of 362 μg g^{-1} of dry soil and standard deviation of 123.21 μg g^{-1} of dry soil (Figure 4a).

In the LVdf (Londrina), LAd (Mauá da Serra) and LVAd (Arapongas) soils cultivated with the cover crop sequences comprising canola, black-oat, white-oat, black-oat, pea (CCS1), and white-oat, the parts with black-oat, white-oat, black-oat, white lupine (CCS2) had the higher microbial C-biomass. Regardless of the crop sequences, the microbial C-biomass was lower in LVd (Mauá da Serra) and LAd (Ponta Grossa) soils than in the other soils.

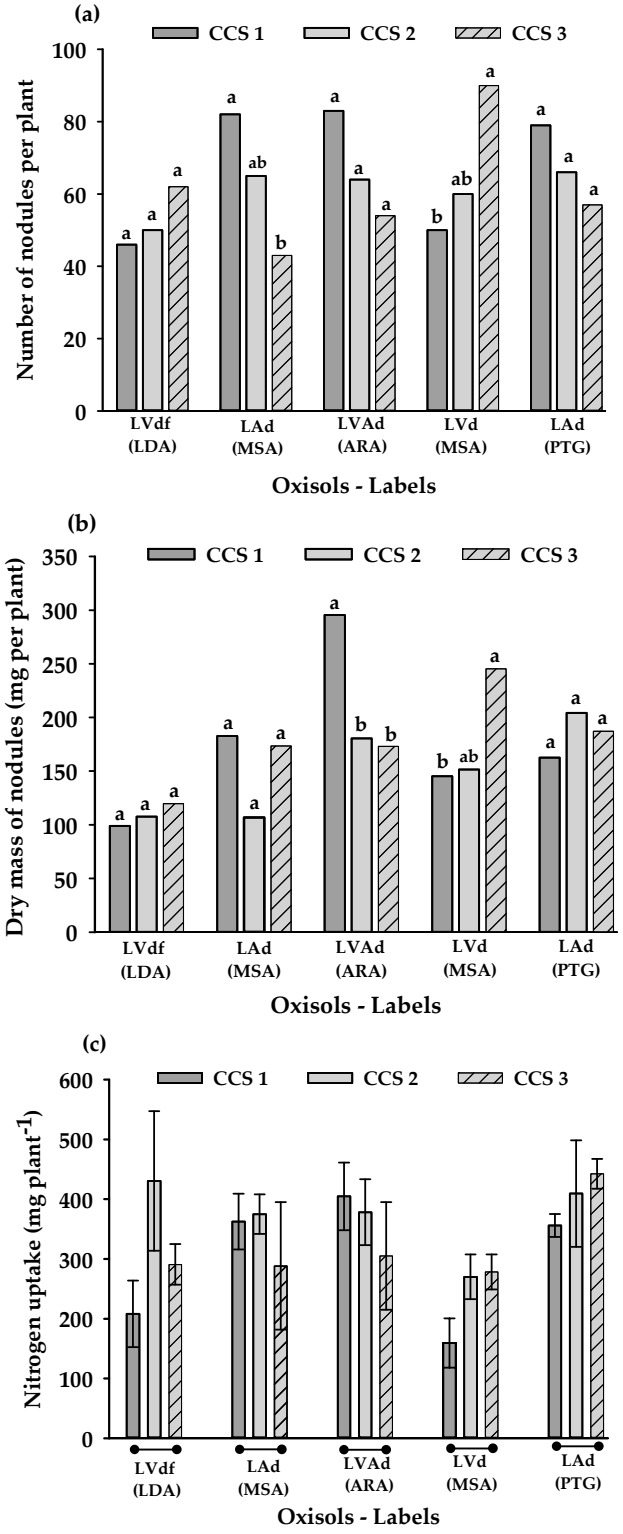


Figure 3. Nodulation: (**a**) number of nodules, (**b**) dry mass of nodules plant^{-1}, and (**c**) N uptake in the shoot of soybean cultivated in cropping season 2023/2024. Means that are followed by the same lowercase letters in each oxisol did not show significant differences according to the Tukey test ($p > 0.05$). Error bars represent standard error (SE); n = 9. **Legend:** cover crop sequences = CCS 1: canola, black-oat, white-oat, black-oat, forage pea; CCS 2: white-oat, black-oat, white-oat, black-oat, white lupine and CCS 3: crambe, black-oat, white-oat, black-oat, vetch. Oxisols labels: LVdf—(LDA); LAd—(MSA); LVAd—(ARA); LVd—(MSA): LAd—(PTG) as described in Table 1.

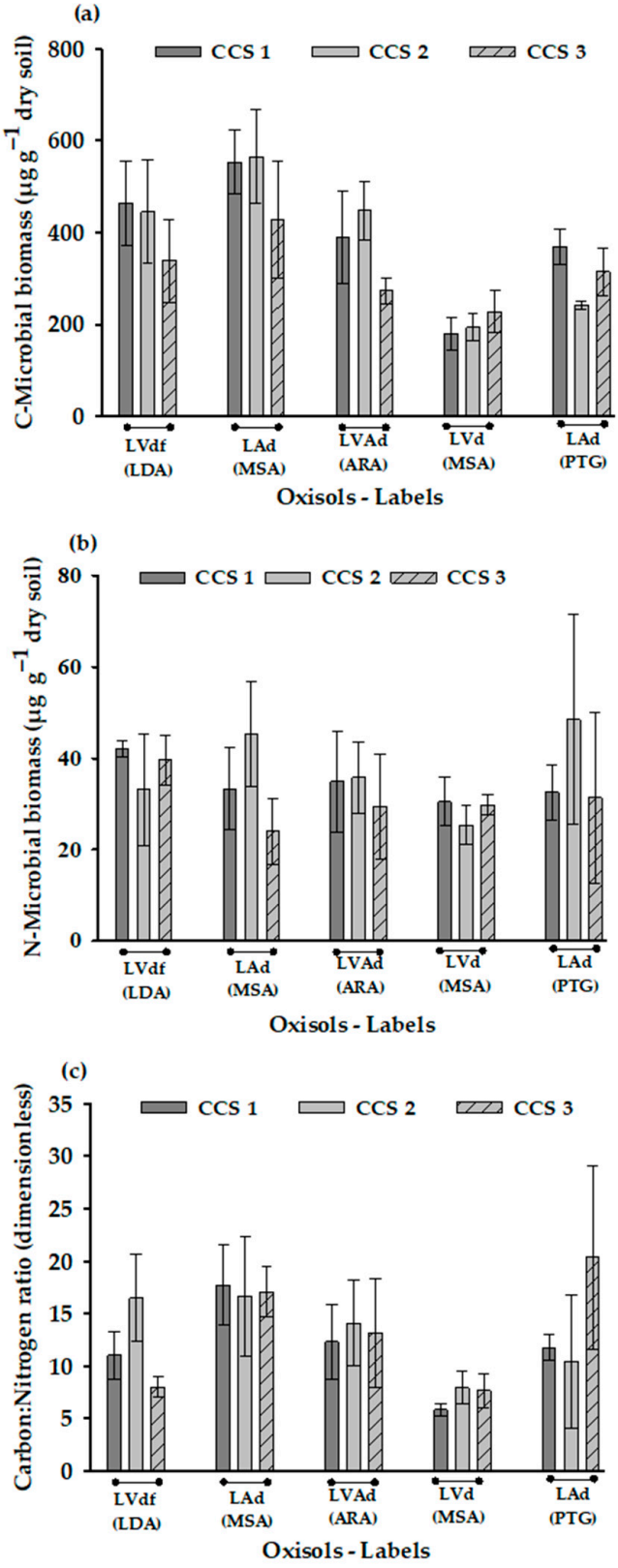


Figure 4. Cover crop sequences effect on (**a**) microbial biomass C, (**b**) microbial biomass N, and (**c**) Cmic/Nmic ratio. Soil sampling in cropping season 2023/2024. Error bars represent standard error (SE); n = 9. **Legend:** cover crop sequences = CCS 1: canola, black-oat, white-oat, black-oat, pea; CCS 2: white-oat, black-oat, white-oat, black-oat, white lupine; CCS 3: crambe, black-oat, white-oat, black-oat, vetch. Oxisols labels: LVdf—(LDA); LAd—(MSA); LVAd—(ARA); LVd—(MSA): LAd—(PTG) as described in Table 1.

Microbial N-biomass showed an average of 34.39 ± 6.96 μg g^{-1} of dry soil, with values ranging from 24.00 to 48.56 μg g^{-1} of dry soil without significant effect from the winter/autumn cover crop sequences (Figure 4b).

The carbon/nitrogen (C:N) ratio showed an average of 12.70, standard deviation of 4.32, with values ranging from 5.83 to 20.35. The C:N ratio was altered in the LVdf soil (Londrina), with CCS 2 (white-oat, black-oat, white-oat, black-oat) having a ratio value greater than 18, and in the LAd soil (Ponta Grossa) with CCS 3 being lower on average and above 10 in most (Figure 4c). The LVd soil (Mauá da Serra) presented an average value of 7.14, regardless of the cover crop sequence.

The average soil basal respiration values were 0.51 μg C-CO_2 kg^{-1} h^{-1}, a standard deviation of 0.13 μg C de CO_2 kg^{-1} h^{-1}, with a minimum value of 0.28 μg C of C de CO_2 kg^{-1} h^{-1} and a maximum of 0.77 μg C of C de CO_2 kg^{-1} h^{-1} (Figure 5a). The effect on soil basal respiration was affected by the sequence of cover crops within each soil type.

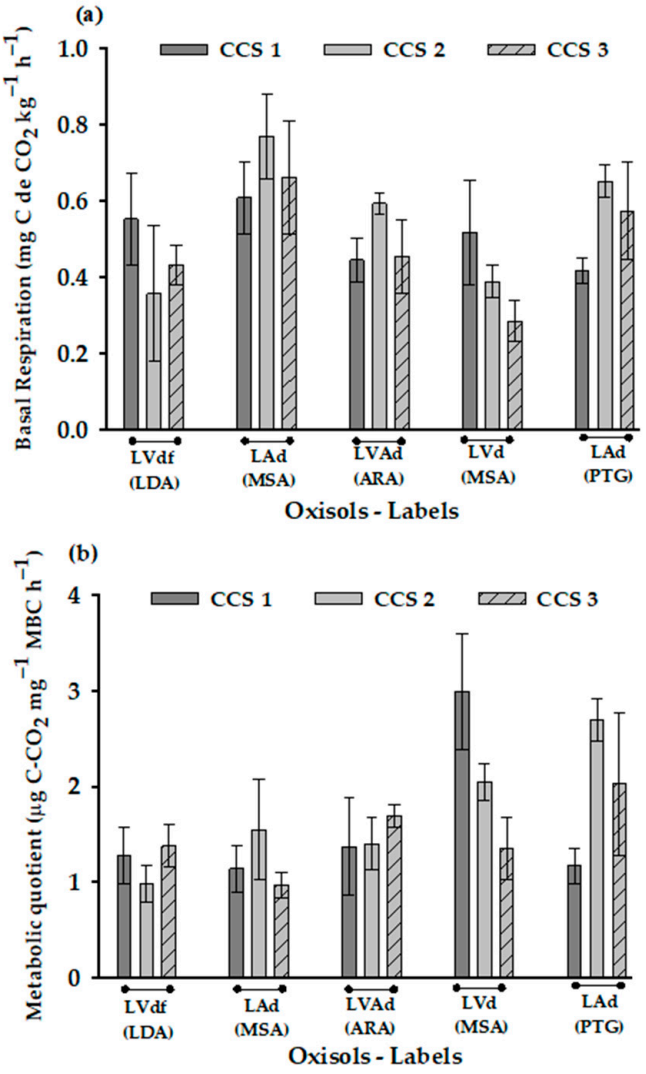


Figure 5. Effect of cover crop sequences on soil (**a**) basal respiration and (**b**) metabolic quotient in different oxisols. Soil sampling in cropping season 2023/2024. Error bars represent standard error (SE); n = 9. **Legend:** cover crop sequences = **CCS 1:** canola, black-oat, white-oat, black-oat, pea; **CCS 2**: white-oat, black-oat, white-oat, black-oat, white lupine; **CCS 3:** crambe, black-oat, white-oat, black-oat, vetch. Oxisols labels: LVdf—(LDA); LAd—(MSA); LVAd—(ARA); LVd—(MSA): LAd—(PTG) as described in Table 1.

The metabolic quotient in the soils ranged from 0.97 mg C-CO_2 mg^{-1} MBC h^{-1} under CCS3 in the LAd (MSA) soil to 2.99 mg C-CO_2 mg^{-1} MBC h^{-1} under CCS1 in the LVa

(MSA) soil. For the LAd (Ponta Grossa) soil, it was observed that the crop sequences canola, black-oat, white-oat, black-oat, pea (CCS 1) had the highest metabolic quotient value than that under the treatment (CCS 2) sequence of crops white-oat, black-oat, white (white-oat, black-oat, white lupine (Figure 5b).

The lowest value of urease enzyme activity 8.22 μg NH_4-N g^{-1} h^{-1} of dry soil was observed in the LVd (Mauá da Serra, very clayey soil) cultivated with the cover crop sequence CCS2, while the highest value, 168.50 μg NH_4-N g^{-1} h^{-1} of dry soil, was observed in the CCS3 (LVdf, Londrina), a very clayey soil (Figure 6a).

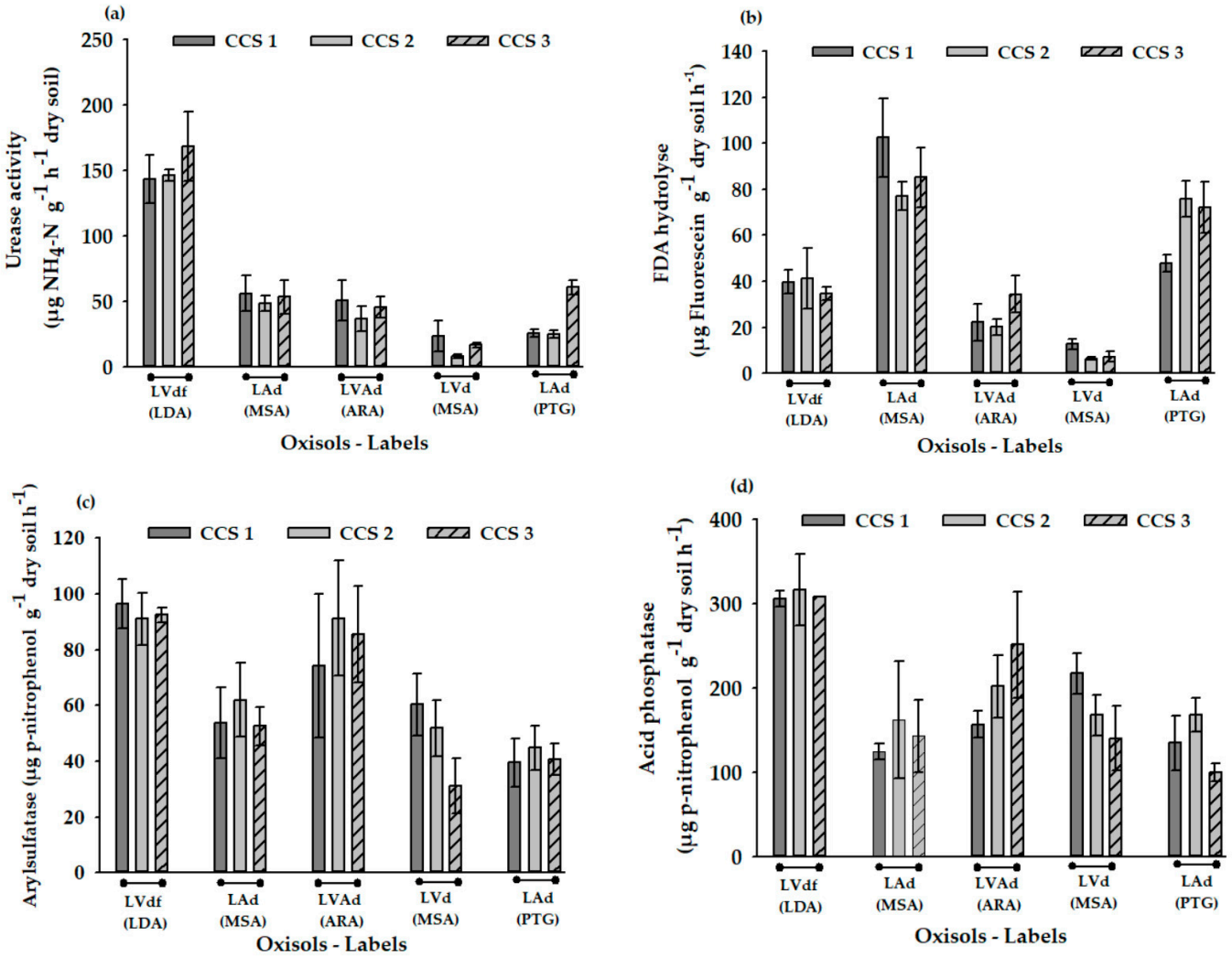


Figure 6. Effect of cover crop sequences on soil enzyme activities (**a**) urease, (**b**) fluorescein diacetate (FDA), (**c**) arysulfatase and, (**d**) acid phosphatase in different Oxisols. Soil sampling in cropping season 2023/2024. Error bars represent standard error (SE); n = 9. **Legend:** cover crop sequences = CCS 1: canola, black-oat, white-oat, black-oat, pea/CCS 2: white-oat, black-oat, white-oat, black-oat, white lupine/CCS 3: crambe, black-oat, white-oat, black-oat, vetch. Oxisols labels: LVdf—(LDA); LAd—(MSA); LVAd—(ARA); LVd—(MSA): LAd—(PTG) as described in Table 1.

The values of FDA hydrolyze enzyme activity in the soil ranged from 6.43 to 102.54 μg FDA g^{-1} dry soil h^{-1} of dry soil with a mean of 45.27 ± 30.47 μg FDA g^{-1} dry soil h^{-1} of dry soil (Figure 6b). The lowest value of FDA hydrolyze activity was observed for LVd (Mauá da Serra, very clayey for CCS2), while the highest value was observed for LAd (Mauá da Serra, sandy loam for CCS3).

The arylsulfatase enzyme activity, measured in μg of *p*-nitrophenol g^{-1} dry soil h^{-1}, ranged from 31.08 to 96.42 μg g^{-1} dry soil h^{-1} of dry soil with a mean of 64.50 ± 22.19 μg

(Figure 6c). The lowest value of arylsulfatase enzyme activity was observed for LVd (Mauá da Serra, very clayey for CCS3) while the highest value was observed for LVdf which had a very clayey texture (Londrina, for CCS3).

For all Oxisols, the acid phosphatase activity ranging from 100.58 to 316.66 μg *p*-nitrophenol g^{-1} dry soil h^{-1} with a mean 193.63 ± 71.05 μg PNF g^{-1} dry soil h^{-1} (Figure 6d). The highest activity of acid phosphatase was obtained for the LVdf soil with very clayey texture (Londrina) and the lowest activity was obtained for LAd (Ponta Grossa) CCS3.

Regardless of the autumn/winter cover crop sequence, it was found that values of biochemical index activity (BIA) based on C_{org} (%) and clay content (%) were higher in the LVdf Londrina soil (Figure 7a,b). There was no effect of autumn/winter cover crop sequence on the soil BIA calculated based on both total organic C (%) and soil clay content (%).

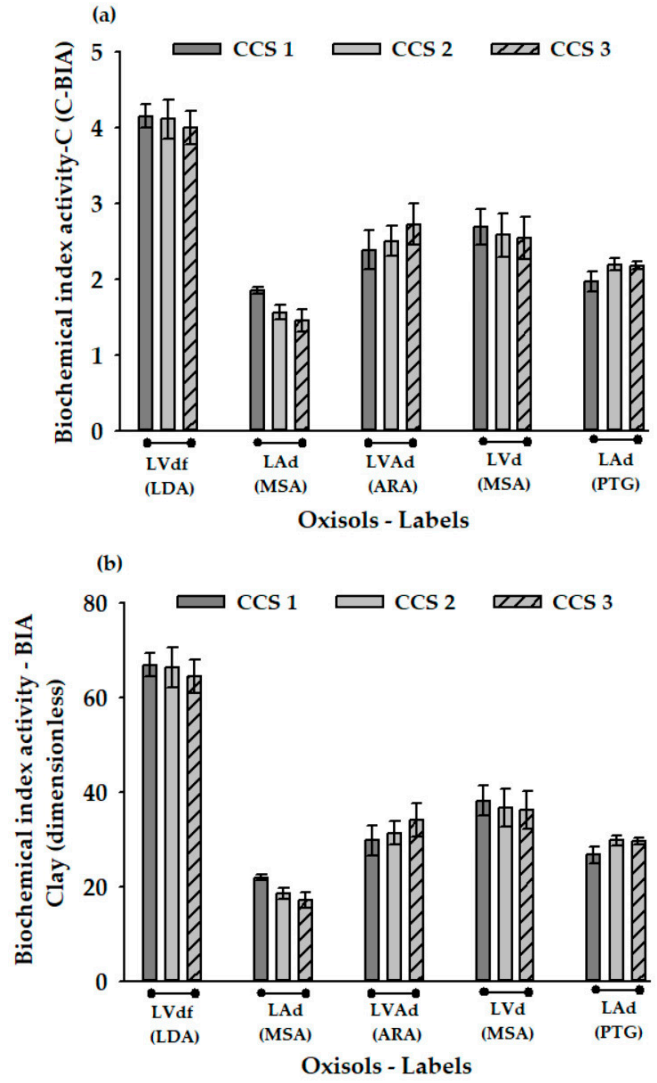


Figure 7. Biochemical index activity (BIA) based on (**a**) C content and (**b**) clay in different Oxisols. Soil sampling in cropping season 2023/2024. Error bars represent standard error (SE); n = 9. **Legend:** cover crop sequences = CCS 1: canola, black-oat, white-oat, black-oat, pea/CCS 2: white-oat, black-oat, white-oat, black-oat, white lupine/CCS 3: crambe, black-oat, white-oat, black-oat, vetch. Oxisols labels: LVdf—(LDA); LAd—(MSA); LVAd—(ARA); LVd—(MSA): LAd—(PTG) as described in Table 1.

4. Discussion

4.1. Soil Attributes

Previous studies have shown that soil and crop management affect the physical, chemical, and microbial properties of soil. For example, in an Aeric Ochraqualf soil in Columbus, Ohio, USA, the application of wheat straw mulching at rates up to 16 Mg ha^{-1} $year^{-1}$ increased the volumetric water content at low suctions, the field capacity moisture content, and the available water content, but the effects of crop residues on soil bulk density were highly variable [46]. The positive effects of using autumn–winter cover crops in a long-term study on an Oxisol in South of Brazil were associated with an increase in labile carbon content (carbon readily available for microbial activity and most rapid turnover times), and it was also suggested that the degree of soil compaction negatively impacts microbial biomass and its activity according to aggregate size [15].

Total organic carbon content (TOC) ranged from 6.81 to 24.05 g dm^{-3} with an average of 16.51 $\pm$ 4.95 g dm^{-3}. In general, the highest value of TOC was obtained in the LVd (MSA) soil and the lowest in the LAd (MSA) soil, which may be related to lower clay content and texture sandy loam in the Typic Haplustox soil.

4.2. Biological Nitrogen Fixation

Despite the maximum soil temperature reaching 50.4 °C on December 16 between 13:35 h and 13:45 h, a high number of nodules and nodule dry mass were observed in the LAd soil from Ponta Grossa, mainly in the cover crop sequence CCS 1.

Under the same conditions as our study in the very clayey soil of Londrina, a previous long-term experiment showed that the no-tillage system had a temperature lower by 6 °C compared to conventional cultivation (plowing) [8]. The effects of both soil compaction and mulching were outstandingly on nodule-size distribution in soybean plants [3]. Environmental factors such as soil temperature and moisture content during the initial days following soybean sowing (13–17 December 2023)—with daily soil temperatures exceeding 30 °C—were considered inhibitory to *Bradyrhizobium japonicum* and soybean root growth; however, nodulation and nitrogen uptake were sufficient to sustain early soybean nutrition and development. This outcome was likely due to a favorable balance of soil physical and chemical properties that exerted compensatory effects on biological activity.

4.3. Soil Microbial and Enzyme Activities

Variations in microbial dynamics induced by different winter crops in Oxisols with contrasting particle size distributions may also be influenced by shoot and root biomass inputs, as well as by the quantity and quality of plant residues and associated soil enzyme activities. Microbial biomass and activity, including soil enzymes may be helpful to establish effects of soil management and crop rotation/sequences under long-term [39,47,48] soil fertilizers [49]. In most of the soils, the metabolic quotient was lower than 2.0, except for LVd (MSA) with the crop sequences CCS1, CCS2 and for Lad (PTG) with CCS2 and CCS3.

The relationship between extracellular enzymes and soil quality is demonstrated, validating their use as a tool for soil quality monitoring [15,50,51]. In the surface layer (0–10 cm) of Italian clay loam soil, Pagliai and Nobili [52] observed a correlation between soil porosity in the range 30–200 μm and urease activity (μmol ammonium release h^{-1} g^{-1} soil).

In our study with different Oxisols and cover crop sequences, the activity of the enzyme urease measured in μg NH_4-N g^{-1} of dry soil h^{-1} had the lowest value (8.22), and the highest (168.50) was measured under the cover crop sequences (CCS2) with white-oat, black-oat, white-oat, black-oat, white lupine and (CCS3) with crambe, black-oat, white-oat, black-oat, vetch), respectively; both being very clayey soils suggests that the activity of the enzyme urease is not only affected by soil texture but also related to chemical attributes such

as organic matter, nutrient content, microbial community, and crop management. Urease activity is dependent on several soil factors that are likely to vary at different scales [53], not just on the distribution of soil particle size. These soil factors likely vary with the diversity of the microbial community and also with chemical properties, such as nitrogen levels and the quantity and quality of organic matter. For instance, land use type had a significant influence on several soil chemical and microbial attributes, including enzyme urease activities [54].

Arylsulphatase activity and acid phosphatase activity have been used to measure the effect of microclima on soil quality [55]. In our study, the soils presented arylsulfatase enzyme activity ranging from 31.08 to 96.42 μg g^{-1} dry soil h^{-1} of dry soil. Overall, these values were similar to those reported for long-term field experiments conducted on very clayey soils in the state of Parana. These include experiments on a typic Haplorthox (85% clay) soil evaluating crop rotation and soil tillage [12], on a Rhodic Hapludox (72% clay) soil that compared conventional with no tillage and several winter crops as treatments [56], and on a Haplustox (60% clay) soil with different doses of agricultural gypsum application [49]. In Cambodia, short-term no-tillage crop rotations in an Oxisol showed that permanent soil cover increased the soil enzyme activity due to a higher biomass C input and the absence of soil disturbance [13].

In our study, the acid phosphatase activity ranged from 100.58 to 316.66 μg *p*-nitrophenol g^{-1} dry soil h^{-1}) with a mean of 193.63 ± 71.05 (μg *p*-nitrophenol g^{-1} dry soil h^{-1}) with results lower than those reported by Balota et al. [56] in a long-term field study on a Rhodic Hapludox soil under no-tillage management. In a long-term land cultivated with maize crops at the Firenze, Italy on loam soil (17 dag kg^{-1} of clay), phosphatase activities were lowest under conventional deep tillage and the highest under minimum tillage: harrowing with a disk harrow to a depth 10 cm [57].

Arylsulphatase, acid phosphatase, and urease activity were used to calculated the biochemical indices to compare the effect of cover crop sequences in different Oxisols. There was no effect of autumn/winter cover crop sequence on the soil BIA calculated based on both total organic C (%) and soil clay content (%). The biochemical indices to compare soil quality should be based on the activity of enzymes involved in the processes of carbon and nitrogen transformations [44].

Soil microbial community plays a key role in the essential nutrient cycling that has positive impacts on soil quality [48,58]; pea and other legume crops are suitable for sustainable crop management. This study indicated that in Oxisols with granulometric variability, the balance between the physical and chemical attributes of soil was reached with liming, gypsum application, and no traffic machines; winter cover crop sequences with cash crops are recommended to help microbial activity during heat and water stress.

5. Conclusions

In our study, findings suggest that autumn/winter cover crop systems alter root soybean nodulation, nitrogen shoot uptake, microbial biomass, the ratio of Cmic/Nmic, and enzyme activity related to nutrient cycling, thus changing C, N, P, and S content in soil. Regardless of cover crop sequences, the highest value of urease and acid phosphatase enzymes and biochemical index activities were observed in very clayey soils.

The balance between the physical and chemical attributes of soil after 20 years of cultivation with winter crops and cash crops and the addition of lime, gypsum, and fertilizer exerts compensatory effects on biological activity during water and heat stress in the soil.

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analysis, A.D.R.M., C.F.A.-J., and D.S.A.; investigation, D.S.A., C.F.A.-J., and A.D.R.M.; resources, C.F.A.-J., D.S.A., and M.M.; data curation, A.D.R.M., C.F.A.-J., and D.S.A.; writing—original draft preparation, A.D.R.M., C.F.A.-J., and D.S.A.; writing—review and editing, D.S.A., A.D.R.M., and C.F.A.-J.; visualization, D.S.A., A.D.R.M., and C.F.A.-J.; supervision, D.S.A., C.F.A.-J., and A.D.R.M.; project administration, M.M. and C.F.A.-J.; funding acquisition, M.M., C.F.A.-J., and D.S.A. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

LVdf	LATOSSOLO VERMELHO Distroférrico
LAd	LATOSSOLO AMARELO Distrófico
LVAd	LATOSSOLO VERMELHO AMARELO Distrófico
LVd	LATOSSOLO VERMELHO Distrófico
CCS	cover crop sequences
LDA	Londrina
MSA	Mauá da Serra
ARP	Arapongas
PTG	Ponta Grossa
CCS 1	canola, black-oat, white-oat, black-oat, pea
CCS 2	white-oat, black-oat, white-oat, black-oat, white lupine
CCS 3	crambe, black-oat, white-oat, black-oat, vetch
FDA	fluorescein diacetate activity
BIA	biochemical index activity
TOC	total organic carbon

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