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Azospirillum brasilense Inoculation in a Maize–*Urochloa*–Rice Cropping System Promotes Soil Chemical and Biological Changes and Increases Productivity

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Abstract: Large quantities of cover crop residues in the soil, combined, or not, with the inoculation of seeds with diazotrophic bacteria, can increase organic matter (OM) and protect soil microorganisms, such as arbuscular mycorrhizal fungi (AMF) and dark septate endophytic (DSE) fungi. Thus, the use of these sustainable biotechnologies can benefit microbial interactions, soil fertility and rice production in the Brazilian Cerrado region. In this study, we evaluated the effects of maize and *Urochloa ruziziensis*, intercropped or individually, as cover crops and an inoculation of *Azospirillum brasilense* on the chemical (fertility) and biological (C–microbial biomass and C–CO_2 released) attributes of soil and the effects of root colonization by AMF and DSE on the yield of rice grown in succession in highlands. The experiment was conducted under field conditions, in a typical dystrophic Red Oxisol. The experimental design consisted of randomized blocks arranged in strips, incorporating a combination of eight residual cover crops: ((1) maize, (2) maize–I (I = inoculation of seeds with *A. brasilense*), (3) *Urochloa* (*U. ruziziensis*), (4) *Urochloa*–I, (5) maize + *Urochloa*–I, (6) maize + *Urochloa*–I, (7) maize–I + *Urochloa* and (8) maize–I + *Urochloa*–I). This was accompanied by two treatments of rice as a successor crop (inoculated or not with *A. brasilense*), with four replicates, totaling 64 experimental units. A cover crop and rice seed inoculation prompted increases in OM and AMF relative to DSE, while the inoculation of rice, regardless of the cover crop treatment, increased the soil's P content. The combination of maize + *Urochloa*–I and inoculated rice as the next crop generated increases in its sum of bases (SBs) and cation exchange capacity (CEC). There was a 19% increase in rice grain yields when the seed was inoculated.

Keywords: arbuscular mycorrhiza; dark septate endophytes; microbial activity; *Oryza sativa* L.; sustainable agriculture

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1. Introduction

Brazil is a global leader in upland rice (*Oryza sativa* L.) production [1]. In the 2022/23 crop year, Brazilian upland rice production reached approximately 742.5 tons, with an average productivity of 2553 kg ha^{-1}. However, it is estimated that the planted area of upland rice will be reduced by approximately 15.5% compared to that of previous crop years, mainly due to weather and the significant increase in the price of its inputs [2]. Therefore, sustainable alternatives that enable its production under adverse weather conditions or even allow for a reduction in input costs are desirable.

Conservation management helps in soil preservation, enabling more sustainable food production. In crop rotations, residues of the various plant species cultivated remain on the soil surface, ensuring the maintenance of the system [3]. This practice helps to maintain the productive capacity of soils. Straw raises the organic matter (OM) content, reduces soil erosion and compaction, provides nutrients to subsequent crops in the medium and long term and promotes the sequestration of carbon (C), thus reducing the emission of greenhouse gases [4–6]. Additionally, cover crops can provide a wide range of physical, chemical and biological soil benefits, including increases in nutrient content, porosity and water retention, microbial activity and soil organic carbon [6].

The use of *Urochloa ruziziensis* as a cover crop, in association with other species or individually, is a desirable practice due to its vigorous growth, biomass production, good soil cover and deep root system, which allows for the efficient cycling of soil nutrients [7]. The retention of organic compounds associated with the increase in the aggregation of soil particles physically protects the soil OM, decreasing variations in temperature and humidity and positively influencing the maintenance of soil microbial populations [8,9].

In the search for more sustainable production, techniques such as the inoculation of beneficial microorganisms into plants are becoming increasingly popular and may supplement or even replace the use of some fertilizers throughout the crop cycle [10]. Bacteria, such as those of the genus *Azospirillum*, have been recommended for their ability to synthesize plant growth phytohormones such as auxins, gibberellins and cytokinins, in addition to acting on phosphate solubilization and inducing the intrinsic systemic resistance of the plant to abiotic and biotic stresses [11,12]. The increase in the root system due to the synthesis of phytohormones allows plants to exploit a larger area of soil for water and nutrients, increasing crop productivity [13,14].

Azospirillum brasilense may stimulate other interactions between the plant and autochthonous soil microorganisms, such as arbuscular mycorrhizal fungi (AMF) and dark septate endophytic (DSE) fungi, which form symbioses with a wide range of plants [15,16]. As their hyphae colonize the soil beyond the root growth area, they provide water and minerals to plants, fulfilling their needs and enabling their survival and growth in environments with unfavorable conditions [17,18]. Several crops have been shown to benefit from these two symbioses, including rice [18,19].

In the present study, we hypothesized that an inoculation of *A. brasilense,* due to its action as a growth promoter [13], increases the root system of both maize and *U. ruziziensis* cover crops and of the rice grown subsequently, with increases in water and nutrient acquisition by the plants. The increased growth of the root system favors root colonization by the autochthonous soil AMF and DSE, enhancing the absorption of water and nutrients through the hyphal network, soil microbial activity and productivity. Thus, our results may provide a new perspective for the sustainable cultivation of upland rice in the Cerrado region. We evaluated the effects of maize and *U. ruziziensis* as cover crops and of an inoculation of *A. brasilense* on the soil's chemical and biological properties, in addition to the yield of a subsequent upland rice crop.

2. Materials and Methods

2.1. History and Initial Characterization of the Experimental Area

The study was conducted in the field in the municipality of Selvíria/MS, Brazil (51°24′11.34″ west and 20°20′35.32″ south), at an average elevation of 358 m. The area is located in the Cerrado biome (Figure 1) and the soil is classified as typical clayey Oxisol (Oxisol) [20] or Rhodic Hapludox [21].

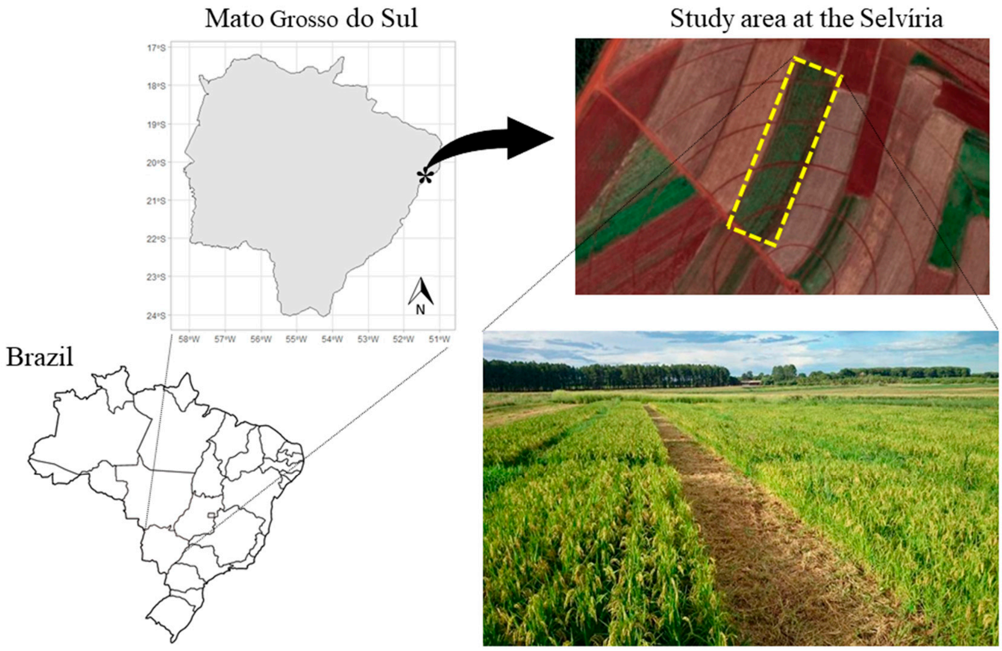

Figure 1. Experimental area located in the municipality of Selvíria, Mato Grosso do Sul State—MS, Brazil.

The climate type of the region is Aw, according to the Köppen classification system, and it is characterized by rainy summers and dry winters [22]. The average temperature and rainfall during the experimental period are shown in Figure 2.

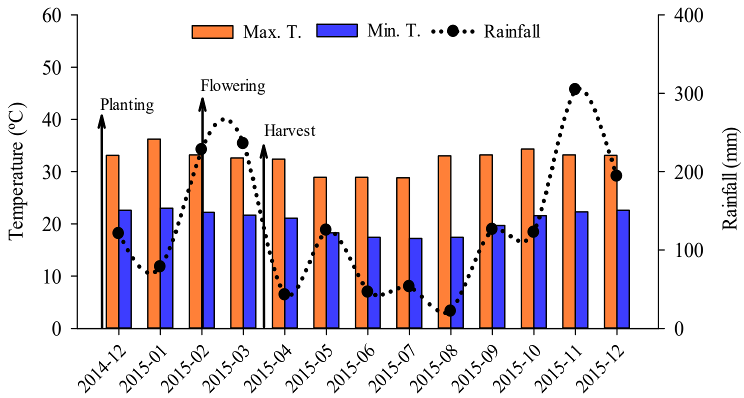

Figure 2. Mean values of maximum and minimum temperature (°C) and precipitation (mm), throughout the experimental year (December 2014 to December 2015). Source: Climate Monitoring System—UNESP Ilha Solteira.

The experimental area is part of a consolidated no-tillage system (for more than 10 years), and in the five years preceding the experiment, the following crops were cultivated: summer 2010/11, soybean; summer 2011/12, maize and *U. ruziziensis*; winter 2012, common bean/wheat; summer 2012/13, maize and *U. ruziziensis*; winter 2013, common bean; summer 2013/14, maize and *U. ruziziensis*; and winter 2013/14, common bean, with pearl millet used as vegetative cover from August to November.

Before the beginning of the experiment, a soil sample was collected at a depth of 0.0–0.2 m and used for the analysis of the soil's chemical properties [23]. The soil characteristics were as follows: P = 45.2 mg kg^{-1}, OM = 34.5 g dm^{-3}, $pHCaCl_2$ = 5.6, K = 0.29 cmol kg^{-1}, Ca = 4.7 cmol kg^{-1}, Mg = 3.7 cmol kg^{-1}, H + Al = 3.4 cmol kg^{-1}, sum of bases (SB) = 8.8 cmol kg^{-1}, CEC = 12.2 cmol kg^{-1} and base saturation (BS) = 72%.

2.2. Experimental Design

The experimental design consisted of randomized blocks arranged in strips, incorporating a combination of eight residual cover crops: (1) maize, (2) maize–I (I = inoculation of seeds with *A. brasilense*), (3) *Urochloa* (*U. ruziziensis*), (4) *Urochloa*–I, (5) maize + *Urochloa*, (6) maize–I + *Urochloa*, (7) maize + *Urochloa*–I and (8) maize–I + *Urochloa*–I, along with two treatments of rice as a successor crop (inoculated or not with *A. brasilense*), with four replicates, totaling 64 experimental units.

2.3. Implementation and Execution of the Experiment

The experiment was planted in the spring/summer harvest period (November 2014). Each plot comprised 10 rows, each 10 m long and spaced 0.35 m apart, with the central area containing 5 rows, with 0.50 m left unutilized at each end of the rows.

Maize and *Urochloa ruziziensis* were cultivated either intercropped or individually as cover crops in the area, as part of the study conducted by [24]. We evaluated the residual effects of cultivating these cover crops on the subsequent cultivation of rice, whether inoculated with *A. brasilense* or not.

Maize (cv. 'AG 8088 PRO') was mechanically sown with a spacing of 0.90 m and 5.4 plants per meter of furrow, aiming to achieve a population of 60,000 plants ha^{-1}. The planter used for the no-tillage system was equipped with a shank-type furrower mechanism and a pneumatic seed distribution system (vacuum with perforated discs). The base fertilization in the seeding furrows of the cover crops was calculated following the recommendations of [25]. At sowing, mineral fertilization with 12, 39 and 25 kg ha^{-1} of N, P and K, respectively, was applied using 300 kg ha^{-1} of formulated N–P–K (04–30–10) following the recommendation of [26]. On the same day, single *U. ruziziensis* plants were manually sown using a rattle seeder with a spacing of 0.45 m and 20 kg of seeds ha^{-1}. Similarly, the intercropped grass was manually sown at 0.45 m between the maize rows, with a seed rate of 10 kg ha^{-1}.

The inoculation of the cover crops and rice seeds occurred after seed treatment with an insecticide. The Ab–V_5 and Ab–V_6 strains, with 2×10^8 colony-forming units (CFU) g^{-1} soil, were applied at a dose of 200 mL of inoculant per 25 kg of seeds. After a brief period of drying in the shade, sowing was conducted. The rice cultivar 'BRS Esmeralda' was chosen based on its type of grain, which is classified as long–fine ('agulhinha'). Mechanical sowing was performed in November to achieve 180 plants per m^2 [27]. Nitrogen topdressing fertilization, performed 30 days after sowing (DAS) the rice, was accomplished using ammonium sulfate as the N source at a rate of 60 kg ha^{-1} N. The fertilizer was distributed in small continuous strips, approximately 0.10 m away from the plant rows. Subsequently, 20 mm of water was applied to incorporate the fertilizer.

Pendimethalin and metsulfuron methyl were used at rates of 1400 and 2 g ha^{-1}, a.i., respectively, as postemergence herbicides to control broadleaf weeds. Any surviving plants were mechanically controlled with the aid of a hoe. Seedling emergence occurred at seven DAS, and, during the reproductive stage of the crop, between 65 and 84 DAS, the fungicide tebuconazole + trifloxystrobin was applied at a rate of 75 + 150 g ha^{-1} to manage diseases such as blast. These applications were performed using a tractor-operated boom sprayer equipped with flat-jet ("fan") nozzles and adjusted to apply 200 L ha^{-1} of spray solution. Water was supplied by a central pivot when necessary, with an average water depth of 13 mm, and rainfall was measured using a Ville de Paris rain gauge.

2.4. Evaluation of Soil Chemical and Biological Attributes

When the plants reached the R4 stage (grain filling stage), four soil samples were collected per plot and close to the plants, at a depth of 0.0–0.1 m, to create a composite sample. The soil samples were air-dried, sieved (9 mesh), homogenized and taken to the laboratory for the determination of their soil concentrations and chemical properties, including phosphorus (P_{resin}), OM, soil reaction ($pHCaCl_2$), exchangeable potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), sum of bases (SB), cation exchange capacity (CEC), base saturation (V%), aluminum (Al^{3+}) and potential acidity (H + Al), following the methodology described by [24]. The C present in the microbial biomass (MBC) was quantified using 10 g soil samples and the fumigation–extraction method [28]. The difference between the C released by the death of microorganisms via fumigation (fumigated and non-fumigated) was determined according to the methodology of [29], with reading performed using a spectrophotometer (495 nm).

To quantify the C from CO_2 (C–CO_2) released by the microbial activity in 100 g of soil, after incubation, the remaining sodium hydroxide was titrated with hydrochloric acid, following the method proposed by [29]. The soil incubation period was determined using the calibration curve resulting from monitoring the soil every other day using extra jars. From the values obtained, the microbial quotient (*q*MIC) and metabolic quotient (qCO_2) were calculated; the *q*MIC was calculated by the expression MBC/Organic C (OC) soil [30], while qCO_2 compared the loss of C by microbial activity relative to that retained in the soil in the form of MBC, expressed by C–CO_2 released/MBC [31].

To evaluate the root colonization of AMF and DSE, the roots of rice plants, collected with the soil and separated during sieving, were washed in running water, bleached in potassium hydroxide (10%), acidified with hydrochloric acid (1%), stained with trypan blue (0.05%) and maintained in lactoglycerol [32]. The percentage of colonization was determined by evaluating 100 fine root segments per sample and separately recording the root colonization and structures formed by AMF or DSE symbionts. The number of AMF spores was counted after their separation from the soil (100 g of dry soil) by means of decantation, wet sieving [33], centrifugation and flotation with 60% sucrose [34]. The collected spores were rinsed and placed on acrylic plates with concentric rings for counting under a stereoscopic microscope.

2.5. Rice Development and Productivity

At flowering, samples were collected from the plots at two points in the two central rows. The dry matter (MSPA) was obtained after drying the plants (10 plants from the central rows) at 65 °C until constant weights were reached. The samples were weighed and, based on a previous estimate of the plants m^2, the values were converted to kg ha^{-1}. Plant height (AP) was assessed as the average distance from the base of the panicle to the upper end of the highest panicle, determined during the maturation stage in plants, at random, in the ground area of each plot. The number of panicles (NPAN) was determined by counting the number of panicles in a 1.0 m plant row in the useful area of the plots.

The mass of 100 grains (M100) and their hectolitre weigh (MHEC) was evaluated by the random collection and weighing of two samples of 100 grains from each plot (13% wet basis), using two samples per plot. The grain yield (YIELD) was reported at 13% moisture.

2.6. Statistical Analyses

Statistical analysis was performed using R studio [35] 2022.02.0+443 software, and graphs were plotted with the aid of SigmaPlot 12.5 software. The hypothesis of the normality of errors was tested using the Shapiro–Wilk test [36]. To verify the differences between the levels of the factors and the interactions between them, analyses of variance with fixed effects (ANOVA) were used and the F test ($p \leq 0.05$) was applied. When significant differences were found, their means were compared using the Scott–Knott test ($p \leq 0.05$).

3. Results

The *Urochloa*–I treatment with cover crops provided the highest OM (51.3 g kg^{-1}) (Table 1). The inoculation of rice seeds with *A. brasilense* (I–rice), regardless of the cover crop treatment, provided increases in OM, P and soil pH (Table 1).

Table 1. Soil chemical attributes under a no–till system for more than 10 years as a function of cover crop treatments (maize—M and *Urochloa ruziziensis*—U) and the inoculation (I) of subsequent upland rice seeds with *Azospirillum brasilense*.

Treatments	OM	P_{resine}	pH $CaCl_2$	H + Al	SB	CEC
	g kg^{-1}	mg kg^{-1}		——	cmol kg^{-1}	——
Cover crops (CC)						
Maize	42.6 c	68.3 a	6.3 a	2.0 a	18.0 b	20.1 b
Inoculated maize (I)	43.7 c	66.5 a	6.6 a	1.7 a	21.3 a	23.0 a
Urochloa	47.4 b	81.6 a	6.3 a	1.9 a	18.6 b	20.5 b
Inoculated *Urochloa* (I)	51.3 a	75.4 a	6.4 a	1.8 a	20.9 a	22.7 a
Maize + *Urochloa*	47.8 b	82.4 a	6.3 a	2.0 a	17.3 b	19.4 b
Maize–I + *Urochloa*	44.4 c	74.9 a	6.3 a	2.4 a	19.8 a	22.3 a
Maize + *Urochloa*–I	48.5 b	75.1 a	6.3 a	1.9 a	19.8 a	21.8 a
Maize–I + *Urochloa*–I	47.2 b	83.1 a	6.5 a	1.8 a	21.2 a	23.0 a
Plant in succession (PS)						
Rice–I	48.0 a	78.8 a	6.5 a	1.9 a	20.6 a	22.6 a
Rice	45.2 b	73.0 b	6.3 b	2.0 a	18.6 a	20.6 a
General mean	46.6	75.9	6.4	1.9	19.6	21.6
F values						
CC	7.54 **	2.38 ^{NS}	1.07 ^{NS}	1.76 ^{NS}	2.70 *	2.28 ^{NS}
PS	23.6 *	31.3 *	24.2 *	0.03 ^{NS}	2.23 ^{NS}	2.60 *
CC × PS	1.90 ^{NS}	2.03 ^{NS}	1.66 ^{NS}	0.94 ^{NS}	4.52 **	6.42 **
Coefficient of variation (%)						
CC	6.5	5.4	1.7	22.2	15.0	30.5
PS	4.9	15.1	4.6	25.8	29.1	12.7

Means followed by the same letter, in the same column, for each variable, do not differ by the Scott-Knott test at a 5% probability, ** and *: significant at 1 and 5%, respectively, ^{NS}: not significant.

Among the treatments with significant interactions, rice–I grown in succession exhibited the highest levels of SB (26.3 cmol kg^{-1}) and CEC (29.0 cmol kg^{-1}) in the maize + *Urochloa*–I treatment (Figure 3B,D).

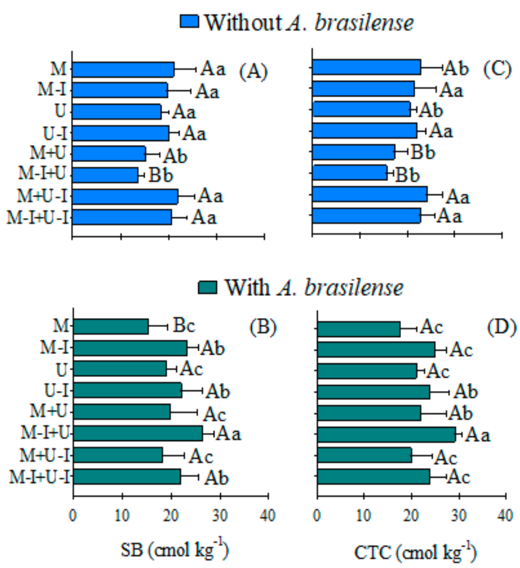

Figure 3. Significant interactions between cover crops and seed inoculation of subsequent upland rice plants with *Azospirllum brasilense* for the sum of bases (**A**,**B**) and cation exchange capacity of the soil (**C**,**D**). The means of each variable followed by the same uppercase letter, for inoculation (without and with), and lowercase letter, for cover crops, do not differ in a Scott–Knott test at 5% probability, (M: maize, MI: maize–I (Inoc), U: *Urochloa ruziziensis*, UI: *Urochloa*–I, M + U: maize + *Urochloa*, MI + U: maize–I + *Urochloa*, M + UI: maize + *Urochloa*–I, MI + UI: maize-I + *Urochloa*–I).

The highest values of MSPA (9161 kg ha^{-1}) and AP (0.99 m, Table 2) were observed in the maize–I+ *Urochloa* and *Urochloa* treatments with cover crops.

Table 2. Shoot dry biomass, plant height, number of panicles, hundred-grain mass, hectolitric mass and yield of upland rice as a function of the cover crop treatments (maize—M and *Urochloa ruziziensis*—U) and the inoculation (I) of subsequent upland rice seeds with *Azospirillum brasilense*.

Treatments	**MSPA**	**AP**	**NPAN**	**M100**	**MHEC**	**YIELD**
	kg ha^{-1}	**m**	**n° m^{-2}**	**g**	**kg/100 L**	**kg ha^{-1}**
Cover crops (CC)						
Maize	6485 c	0.98 a	290 a	2.58 a	51.88 a	5481 a
Inoculated maize (I)	5868 c	0.92 b	305 a	2.60 a	52.99 a	4506 a
Urochloa	7042 b	0.99 a	294 a	2.60 a	51.20 a	5757 a
Inoculated *Urochloa* (I)	6984 b	0.96 b	309 a	2.56 a	53.23 a	5449 a
Maize + *Urochloa*	7385 b	0.97 b	301 a	2.59 a	51.61 a	5006 a
Maize–I + *Urochloa*	8240 b	0.91 c	287 a	2.64 a	54.08 a	4214 a
Maize + *Urochloa*–I	9161 a	0.96 b	293 a	2.59 a	52.59 a	5382 a
Maize–I + *Urochloa*–I	7997 b	0.93 b	300 a	2.55 a	53.13 a	4233 a
Plant in succession (PS)						
Rice–I	7217 a	0.96 a	298 a	2.48 b	52.62 a	5439 a
Rice	7573 a	0.95 a	297 a	2.53 a	52.55 a	4568 b
General mean	7395	0.95	297	2.50	54.60	5004
F values						
CC	3.76 *	4.18 *	0.30 ^{NS}	0.92 ^{NS}	1.20 ^{NS}	12.69 ^{NS}
PS	2.21 ^{NS}	0.71 ^{NS}	0.01 ^{NS}	1.08 *	0.02 ^{NS}	1.74 *
CC × PS	0.21 ^{NS}	0.33 ^{NS}	0.67 ^{NS}	0.32 ^{NS}	0.97 ^{NS}	1.94 ^{NS}
Coefficient of variation (%)						
CC	21.2	4.5	13.4	3.7	4.7	26.1
PS	13.2	4.2	8.5	1.2	4.1	20.0

Means followed by the same letter, in the same column, for each variable, do not differ by the Scott–Knott test at a 5% probability, *: significant at 5%, ^{NS}: not significant.

The M100 did not exhibit differences among the cover crop treatments; however, when *A. brasilense* was applied to the seeds of rice grown in succession, the M100 was higher (2.53 g) than that of the inoculated treatment (2.48 g). The NPAN and MHEC did not respond to treatments with cover crops and/or rice inoculation in succession, while rice–I increased the YIELD, resulting in a 19% increase (5439 kg ha^{-1}) compared to the treatment without inoculation (4568 kg ha^{-1}). A higher percentage of AMF (74.37%) was detected in the *Urochloa*–I treatment than in the maize (59.12%), maize–I + *Urochloa*–I (58.50%) and maize–I + *Urochloa*–I (58.0%) treatments, which had the lowest values. Rice–I grown in succession increased the values of AMF (66.12%) compared to treatments without inoculation (61.84%) (Table 3).

In assessing the interactions present, DSE (82.7%) was higher in the treatment consisting only of maize followed by rice, both uninoculated, than in the maize–I (58.5%) and maize–I + *Urochloa* (54.7%) and maize+ *Urochloa*–I (54.5%) treatments, which exhibited the lowest DSE percentages (Figure 4C). The rice–I, *Urochloa* (69.5%), maize+ *Urochloa* (68.0%) and maize–I+ *Urochloa*–I (63.5%) treatments exhibited higher percentages of colonization than the other treatments of predecessor plants (Figure 4D).

Table 3. Soil biological attributes of soil under a no-till system for more than 10 years as a function of cover crop treatments (maize–M and *Urochloa ruziziensis*–U) and the inoculation (I) of subsequent upland rice seeds with *Azospirillum brasilense*.

Treatments	AMF	DSE	NESP	C–CO_2	MBC	OC	qCO$_2$	qMIC
	——— % ———		100 g Soil	µg C–CO_2 g^{-1} Soil	µg MBC g^{-1} Soil	g kg^{-1}	µg C–CO_2 g^{-1}/µg MBC g^{-1} Soil	µg MBC g^{-1}/OC Soil–%
Cover crops (CC)								
Maize	59.12 d	70.62 a	67.75 a	15.91 a	82.31 d	18.57 c	0.23 a	1.48 b
Inoculated maize (I)	66.87 b	57.00 c	50.75 b	15.52 a	103.98 c	19.07 c	0.20 b	1.86 b
Urochloa	62.62 c	67.12 a	65.25 a	15.91 a	121.32 b	20.66 c	0.26 a	1.98 b
Inoculated *Urochloa* (I)	74.37 a	66.50 a	70.50 a	16.43 a	121.03 b	22.48 a	0.22 a	1.85 b
Maize + *Urochloa*	69.00 b	60.62 b	62.87 a	16.18 a	114.24 c	20.87 b	0.24 a	1.82 b
Maize–I + *Urochloa*	58.50 d	53.62 c	58.37 b	16.11 a	142.81 a	19.36 c	0.22 a	2.48 a
Maize + *Urochloa*–I	63.37 c	63.25 a	70.50 a	16.76 a	111.11 c	21.17 b	0.20 b	1.73 b
Maize–I + *Urochloa*–I	58.00 d	64.25 a	57.37 b	16.60 a	104.99 c	20.61 b	0.20 b	1.71 b
Plant in sucession (PS)								
Rice–I	66.12 a	60.56 b	62.03 a	16.30 a	110.28 a	20.95 a	0.22 a	1.78 b
Rice	61.84 b	65.18 a	63.81 a	16.05 a	115.17 a	19.75 b	0.22 a	1.96 a
General mean								
F values								
CC	42.05 **	37.34 **	0.55 **	1.29 NS	2.86 **	24.23 *	3.03 *	23.83 *
PS	18.73 **	10.87 **	2.68 NS	1.12 NS	8.38 NS	7.43 **	0.09 NS	5.95 **
CC × PS	2.04 NS	13.19 **	3.67 **	1.93 NS	23.11 **	1.91 NS	1.81 NS	12.34 **
Coefficient of variation (%)								
CC	6.2	8.3	19.5	6.8	10.2	5.0	19.2	8.3
PS	4.4	5.2	15.0	6.4	15.2	6.7	16.2	17.4

Means followed by the same letter, in the same column, for each variable, do not differ by the Scott–Knott test at a 5% probability, ** and *: significant at 1 and 5%, respectively, NS: not significant.

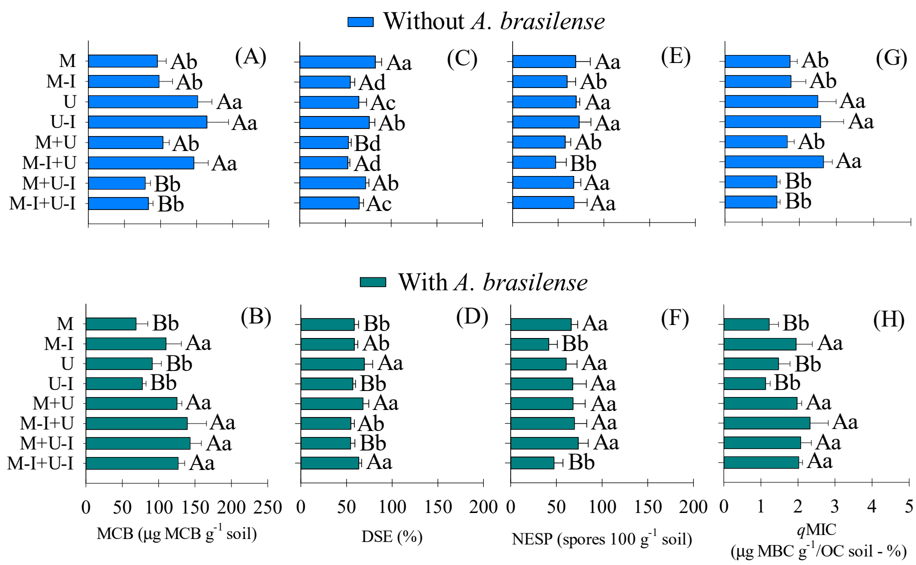

Figure 4. Significant interactions between cover crops and the seed inoculation of subsequent upland rice plants with *Azospirllum brasilense* in terms of their C–microbial biomass (**A**,**B**), root colonization by dark septate endophytes (**C**,**D**), number of mycorrhizal fungi spores (**E**,**F**) and microbial quotient (**G**,**H**). Means followed by the same uppercase letter, for inoculation (without and with), and lowercase letter, for cover crops, for each variable, do not differ by the Scott–Knott test at a 5% probability, (M: maize, MI: maize–I (Inoc), U: *Urochloa ruziziensis*, UI: *Urochloa*–I, M + U: maize + *Urochloa*, MI + U: maize–I + *Urochloa*, M + UI: maize + *Urochloa*–I, MI + UI: maize-I + *Urochloa*–I).

When there was no inoculation of the rice grown subsequently, the NESP (spores 100 g^{-1} soil) was higher in the maize (69.25), *Urochloa* (69.75), *Urochloa*–I (73.00), maize + *Urochloa*–I (67.00) and maize–I+ *Urochloa*–I (67.25) treatments (Figure 4E). With rice–I, the number of spores was higher in the cover crop treatments maize (66.25), *Urochloa* (60.75), *Urochloa*–I (68.00), maize+ *Urochloa* (68.25), maize–I+ *Urochloa* (67.00) and maize+ *Urochloa* –I (74.00) and smaller in maize–I (41.75) and maize–I+ *Urochloa*–I (47.50) (Figure 4F).

The MBC content in rice without an inoculation with *A. brasilense* was higher for the cover crop treatments *Urochloa*, *Urochloa*–I and maize–I + *Urochloa* (Figure 4A).

With rice–I, the maize–I, maize+ *Urochloa*, maize–I + *Urochloa*, maize + *Urochloa*–I and maize–I + *Urochloa*–I treatments showed the highest concentrations, while the lowest concentrations were observed for the maize, *Urochloa* and *Urochloa*–I treatments (Figure 4B). In the absence of the successive inoculation of rice, the treatments *Urochloa* (2.50 μg MBC g^{-1}/OC soil %), *Urochloa*–I (2.57 μg MBC g^{-1}/OC soil %) and maize–I + *Urochloa* (2.65 μg MBC g^{-1}/OC soil %) resulted in higher *q*MIC values (Figure 4G), which differed from those of the maize (1.75), maize–I (1.95 μg MBC g^{-1}/OC soil %), maize + *Urochloa* (1.97 μg MBC g^{-1}/OC soil %), maize + *Urochloa*–I (2.07 μg MBC g^{-1}/OC soil %) and maize–I + *Urochloa*–I (2.02 μg MBC g^{-1}/OC soil %) treatments, which were lower (Figure 4H).

The released C–CO_2 did not differ between the cover crops and/or successive rice inoculation treatments (Table 2). The qCO_2 was higher in the maize, *Urochloa*, *Urochloa*–I, maize+ *Urochloa* and maize–I + *Urochloa* treatments and lower in the maize–I, maize + *Urochloa*–I and maize–I + *Urochloa*–I treatments (Table 2).

4. Discussion

Maize and *U. ruziziensis* have deep root development, a morphological characteristic that allows them to efficiently absorb and cycle nutrients from the soil. The synthesis of growth phytohormones by *A. brasilense*, especially IAA, benefits the growth of root hairs and lateral roots in maize and *U. ruziziensis,* as reported by Li et al. [37] and Crusciol et al. [38]. Changes in root architecture positively influence the ability of these plants to discover resources and, consequently, absorb water and nutrients, which subsequently return to the soil through rhizodeposition or decomposition, participating in nutrient cycling [39]. Thus, the inoculation of maize and/or *U. ruziziensis* with *A. brasilense*, which is associated with high biomass production and a high C/N ratio, may contribute to increased soil fertility [40]. The C/N ratio of maize and *U. ruziziensis* slows down the decomposition rate of their straw, ensuring a slow and gradual release of nutrients into the soil, which can then be used by the crop grown subsequently.

Studies conducted by [41,42] also found that an inoculation of *A. brasilense* via the seeds of maize and *U. ruziziensis* led to an increase in the production of root biomass, with increases in the foliar N contents in maize and P and K in *U. ruziziensis*. Song et al. [43] reported increases in the concentrations of Ca, K and Mg in the shoots of rice in their pearl millet + Guandu + *U. ruziziensis* cropping system, with rice grown in succession. These results corroborate those found in the present study, where the treatment of preceding maize–I + *U. ruziziensis,* with I–rice inoculated with *A. brasilense* grown in succession, resulted in the highest content of SB (Figure 3B). Another important aspect related to the biomass produced by these plants is the increase in OM, which affects the maintenance and health of the soil, resulting in gains in nutrients, moisture and water retention. Baptistella et al. [44] and Rodrigues et al. [45] reported that cover crops increase the soil's OM content due to the constant addition of residues into the rotation system. In this study, the treatment using a predecessor plant, namely, *U. ruziziensis* inoculated with *A. brasilense*, exhibited the highest OM (Table 1). The results of the inoculation of the rice grown in succession was also satisfactory, with a higher OM content compared to that in non-inoculated rice (Table 1). In relation to P, there are reports that the cultivation of *U. ruziziensis* has increased the availability of P in the soil, evidencing its potential to cycle nutrients, especially P [46]. In this study, when rice was inoculated in succession, there was a greater availability of P in the soil (Table 1).

The growth-promoting ability of bacteria also includes P solubilization [47]. In the soil, P remains insoluble, and a large part of the inorganic phosphate applied in the form of fertilizers is quickly immobilized, becoming unavailable. In the present study, the inoculation of *A. brasilense* associated with the exudation of organic acids by the roots of the plant [48] may have contributed to stimulating microbial activity in the rhizosphere region and thus increasing the availability of this nutrient. Kumari et al. [49] also reported that the use of cover crops favored a P cycling mediated by microorganisms. In this context, the practice of reinoculation is essential for bacteria to increase their population and colonize the environment, successfully competing with autochthonous species, which are fewer [50].

The use of cover crops, with or without inoculation, also benefits other soil chemical properties, such as its CEC. It was found that the soil's CEC was affected by different cultivation practices, where the use of crop rotation increased this indicator in the soil [51]. In a study of different rice planting systems, it was reported that there was an increase in CEC in a no-tillage systems of cultivation compared to conventional cultivation [52], while other authors have found increases in CEC when intercropping with maize [53]. Similar results were found in the present study for the treatment with a cover crop of maize + *Urochloa*–I and maize–I + *Urochloa* without the inoculation of the rice cultivated in succession and maize–I + *Urochloa* with inoculated rice cultivated in succession (Figure 3D). It has also been shown that an increase in OM benefits the soil's chemical properties, especially its CEC [53,54]. In our study, there was an increase in CEC compared to the initial values measured at the start of the experiment. These results may be related to crop rotation and the permanence of straw in the soil, which increases the input of organic matter to the soil and consequently increases its CEC.

In the municipality of Selvíria–MS, Brazil, researchers observed a 19% increase in grain yield under an irrigation treatment for upland rice inoculated with *A. brasilense* compared to the treatment without inoculation [55]. In the same municipality, it was reported that the inoculation of seeds with *A. brasilense*, with or without fertilization at 50% of the recommended nitrogen dose for the crop (10 kg ha^{-1} at sowing + 40 kg ha^{-1} at topdressing), increased the grain yield of rice compared to the treatment without inoculation [56]. Similar results were found in the present study, which included topdressing with ammonium sulfate as the N source, at a dose of 60 kg ha^{-1}, and the inoculation of seeds with this plant-growth-promoting bacterium.

The average rice yield (Table 1) was higher than the national average expected for the cultivar 'BRS Esmeralda' [57]. Increases in plant height, 100-grain weight and yield for the 'BRS Esmeralda' were also observed when inoculated with *A. brasilense* [58]. These results may be related to the increase in and maintenance of these bacteria in the soil through the reinoculation of the subsequent crop, which in this case was rice. *A. brasilense* is capable of altering root structures, especially through the synthesis of growth hormones (auxins, cytokinins and gibberellins), ensuring the better development and nutrition of these plants [59]. Thus, a greater supply of dry biomass in the production system, which is reflected in the gains in the successor crop, can be obtained with the inoculation of growth-promoting microorganisms combined with intercropping between plants [60].

Brachiaria provides rapid soil cover, good chemical composition, excellent nutrient cycling, an ease of desiccation and uniform seed production [61]. The intercropping of maize and Brachiaria stands out in terms of its dry matter production, soil cover percentage and nutrient accumulation at the end of the off-season [62]. In this study, a similar result was found for maize–I + *Urochloa*, where an increase in the aerial part of the dry matter of rice not cultivated in succession was observed (Table 2). However, the increased vegetative growth of the plant due to inoculation or even competition with native strains in the soil may have contributed to the reduction in its 100-grain mass (Table 2).

An inoculation of *A. brasilense* may facilitate root colonization by several fungal groups. These symbiotic fungi can be obligate biotrophic, facultative biotrophic, or melanized septate endophytic. *A. brasilense* may contribute positively to mycorrhizal colonization due to its activity as a mycorrhizal auxiliary bacterium [63] and in promoting fungal propagation and germination, stimulating mycelial growth or altering root architecture [62], thus contributing to the mobilization of nutrients that could increase mycorrhizal performance, root colonization and crop productivity [64].

The inoculation of *A. brasilense* provided the highest AMF values and the lowest values for DSE (Table 2). Although AMF and DSE can simultaneously colonize plant roots, factors such as environmental conditions, different elevations and optimal levels of soil fertility can lead to the predominance of one fungal group or the ability of one group to outcompete another for the acquisition of nutrients [65]. Some studies report that the presence of DSE is greater in environments unfavorable to AMF [66].

During rice growth under field conditions, the variations in AMF and DSE reported by [67] showed an increasing trend up to 90 days after planting, followed by a decline, which may explain the low values of both fungal colonizations in the present study. The inoculation of grasses with *A. brasilense* that support the proliferation of AMF [38] may have favored the increase in the amount of inoculum in the area, contributing to the root colonization of the subsequent crop [68,69].

Factors such as climate, soil texture, management and soil use affect the composition and diversity of AMF species [70]. The concentrations of AMF propagules tend to be higher in soils with a sandy texture; however, the vegetation composition and the land use system can change the structure of AMF communities [71]. In Cerrado soils, the AMF population is naturally low, gradually increasing with the cultivation of plants, especially grasses [72]. These plants exhibit different degrees of mycorrhizal dependence and, consequently, may alter the AMF propagules in the soil, directly influencing the colonization of plants cultivated in succession [73].

We found that DSE was lower in the maize, *Urochloa*–I and maize+ *Urochloa*–I treatments when rice was inoculated than in its non-inoculated treatments. There are few studies on the interaction between *A. brasilense* and DSE; however, it was shown that the production of siderophores and other molecules by the bacterium was able to decrease the mycelial growth in vitro and in vivo in some fungal groups, including *Alternaria* and *Fusarium* spp. [74]. DSE fungi can be mutualistic in most situations, but develop pathogenic characteristics in others, thus requiring thorough selection [75]. However, the reasons for these patterns are still not clear, and there is a need for further studies on the behavior of these microorganisms in upland rice cultivation in the Cerrado.

Soil microorganisms contribute to the processes of OM decomposition, participating directly in important biogeochemical cycles. Higher rates of C incorporated as MBC and lower levels of C–CO_2 released into the atmosphere resulted in low levels of $q$$CO_2$ (Table 3). In agricultural areas, higher MBC and qMIC values and lower C–CO_2 and $q$$CO_2$ values are related to the maintenance and permanence of straw in the soil [76]. The MBC content refers to the amount of C that is immobilized in microbial cells and is considered a sensitive indicator of soil management because it is an important nutrient reservoir linked to the decomposition of soil OM [77]. In studying the use of cover crops in grain cultivation, there have been increases in the MBC in the soil after the cultivation of *U. ruziziensis* and *U. brizantha* [78]. Similar results were found in the present study for the *U. ruziziensis and U. ruziziensis*–I treatments without the inoculation of the rice cultivated in succession (Figure 4A) and for the maize+ *U. ruziziensis*, maize–I + *U. ruziziensis,* maize + *U. ruziziensis*–I and maize–I + *U. ruziziensis*–I treatments with rice–I grown in succession (Figure 4B). The inoculation of *A. brasilense* and its growth-promoting effect on roots throughout the plant growth period participate in the diversification of the soil microbial population with root deposition. In this context, the importance of rotating crops with different C/N ratios, helping to improve soil MBC stocks, was observed by [79], who also reported that the use of *U. ruziziensis* was beneficial for the upkeep of MBC in the cultivation of plants in Cerrado soil.

OC is an important indicator of the health of ecosystems and sustainability of land use in agriculture. The increase in the contribution of OC to the soil is related to the maintenance and decomposition of the straw from crop rotation and NTS. Some authors observed increases in soil OC in studies on the cultivation of perennial species intercropped with rice [80]. The authors report that the use of plants with different characteristics, such as deep and voluminous root systems, intercropped or not, contribute the OC to the soil due to the deposition of rhizospheric compounds and the decomposition and permanence of plant remains in the area. Similar results were observed in the present study, where there was an increase in OC in the *Urochloa*–I treatment when there was an inoculation of the rice cultivated in succession (Table 3). The effect of *A. brasilense* on the soil carbon supply may be indirectly related to its growth-promoting effect on plants and, consequently, to its interaction with other soil microorganisms, such as AMF [81]. According to a previous study [82], the presence of AMF hyphae (Figure 4A) is directly related to the contribution of OC to the soil through photoassimilates and the renewal of dead fungal tissue (fungal necromass), which occurs with certain periodicity. According to one paper, the maintenance of the hyphal network in the soil occurs mainly due to the diversity of the plants provided during crop rotation. Similar results were observed in this study [83].

Regarding the *q*MIC, other studies [84] reported that this index reflects how much C may be immobilized as MBC in the soil. Assessing the *q*MIC at the depth of 0.00–0.10 m, [84] reported higher values for the intercropped maize treatments that used dwarf pigeon pea or sunn hemp (1.6%) than for the cultivation of only maize (0.8%). These results are similar to those found in this study for the maize–I and maize–I + *Urochloa*–I treatments and demonstrate that the use of cover crops, either in intercropping or in rotation, can contribute to the incorporation of C into the soil as MBC, allowing greater efficiency of the use of organic compounds by microorganisms. The absence of soil inversion in the no-tillage system provides an increase in OM due to the deposition of plant material in the surface soil depths, which, together with liming and fertilization, provides favorable conditions for greater microbial activity. However, these results differed from those found in the present study. For these reasons, studies with plant growth-promoting bacteria such as *A. brasilense* should be performed under different production systems (for example, organic and conventional farming systems) and climatic conditions (tropical, subtropical and temperate environments) to investigate their interactions with soil properties and, consequently, food production.

5. Conclusions

Taking into account the low economic cost, ease of application and high probability of a positive response in upland rice cultivation, the inoculation of seeds with *A. brasilense* appears to be a key biotechnology for sustainable production in the savannah region of Rio de Janeiro. In our study, there was a 19% increase in the grain yield of upland rice when the seeds were inoculated. In addition to inoculation, the cultivation of cover crops, intercropped or not, increased the chemical and biological attributes of the soil. The inoculation of *A. brasilense* in cover crops (maize and *U. ruziziensis*) with upland rice cultivated in succession promoted increases in soil OM and AMF relative to DSE, while the inoculation of rice seeds, regardless of cover crop treatment, increased soil P levels. The treatment that used a combination of cover crops, namely, maize+ *Urochloa*–I, with an *A. brasilense* inoculation of the rice cultivated in succession, promoted increases in the SB and CEC in the soil. Thus, our study encourages future research that evaluates the effects of agronomic practices, the inoculation of plant growth-promoting rhizobacteria in different agroecosystems and their relationship with the soil, aiding sustainable food production.

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