

# *Azospirillum Brasilense* And Zinc Rates Affecting Fungal Root Colonization and Yield of Cereal Crops in Succession Under Brazilian Cerrado Conditions

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## Research Article

**Keywords:** Arbuscular mycorrhiza, Dark Septate Endophytes, Zea mays L, Triticum aestivum L, Zinc fertilization, Microbiomes

**Posted Date:** September 24th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-897135/v1>

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# Abstract

Soil and plant beneficial microbes capitalize plant nutrition through successful colonization in roots rhizosphere which could sustainably increase cereal production. Zinc (Zn) is intensively reported a deficient nutrient for maize and wheat production in Brazilian Cerrado, which could be sustainably managed by beneficial microorganisms and their symbiotic association with other microorganisms like arbuscular mycorrhizal fungi (AMF) and dark septate endophytes (DSE). The objective of this study was to evaluate the effect of *Azospirillum brasilense* inoculation and residual Zn rates on root colonization and grain yield of maize and wheat in succession under Brazilian Cerrado conditions. These experiments were conducted in a randomized block design with four replications and arranged in a 5x2 factorial scheme. The treatments were consisted of five Zn rates (0, 2, 4, 6 and 8 kg ha<sup>-1</sup>) applied from zinc sulfate in maize and residual on wheat, and without and with seed inoculation of *A. brasilense*. Both crops were evaluated for root colonization of AMF and DSE, number of spores of AMF, quantification of CO<sub>2</sub>-C and grain yield. Colonization by AMF and DSE were significantly increased with interaction of Zn rates and inoculation treatments. The inoculation of *A. brasilense* favored root AMF and DSE colonization while increasing Zn rates by 4 kg ha<sup>-1</sup> for maize and while 2 and 4 kg ha<sup>-1</sup> Zn in residual for wheat reduced these colonizations. The inoculation did not influence spore production and CO<sub>2</sub>-C in both crops while maize-wheat yield were increased with Zn rates up to 4 kg ha<sup>-1</sup> in edaphoclimatic condition of Brazilian Cerrado.

# Introduction

Brazilian crop production system is dominated by soybean and maize cropping where maize (*Zea mays* L.) is the second large produced crop. Wheat (*Triticum aestivum* L.) yield is still low beyond consumption, therefore imported in a huge amount (Conab 2020). The cereal production system of Cerrado savannah is interrupted by several factors like soil-borne diseases, soil erosion and low nutrient and water use efficiency, rhizosphere microbiome and functional of soil existing microorganisms (Hatfield et al. 2017; Yang et al. 2020). However, cereal cultivation could be treated as a matter of food security due to their short life cycle. Cereals could also contribute to carbon (C) sequestration and soil organic matter which can improve nutrients pools, soil efficiency and productivity (Cherubin et al. 2018).

The intense weathering of tropical soils have devastating effects on organic matter and nutrients use efficiency especially Zn and therefore, can harm cereal productivity and soil fertility (Galindo et al. 2021). Zinc deficiency is a worldwide recognized micronutrient deficiency that limit crop growth and productivity (Jalal et al. 2020b). The low availability of Zn has drastic effects enzymatic activities and protein synthesis. Zinc is one of the fundamental constituents in the synthesis of tryptophan, indole acetic acid (IAA), proteinases, peptidases, dehydrogenases and phosphohydrolases which could improve soil and plant health (Marschner 2012; Castillo-González et al. 2018). The deficit or excess of Zn may have harsh consequences on plant health and yield therefore, proper Zn management is a wise agronomic strategy to improve soil health and crop productivity (Cakmak and Kutman 2017; Jalal et al. 2020a).

The interaction of soil-plant-microorganisms is alternative strategy that could contribute to soil-plant health and productivity (Jalal et al. 2021). The PGPBs especially the genus *Azospirillum* has the ability to fight for colonization site in above and below soil parts of several cereal crops and synthesis of phytohormones (Fukami et al. 2018a; Galindo et al. 2021; Karimi et al. 2021). The combination of *Azospirillum brasilense* and tryptophan as a precursor of Zn can increase IAA production and other hormones involved in plant growth (Housh et al. 2021). These beneficial activities of PGPBs can alter soil microbiota by root colonization as result of symbiosis between plants and certain fungi (Frey-Klett et al. 2007; Berta et al. 2014). These symbiotic fungi may be obligated biotrophs, arbuscular mycorrhizal (AMF) and facultative biotrophs, melanized septate endophytic (dark septate endophytic - DSE) that colonize in plant root system by spreading hyphae in soil to absorb water and nutrients, contributing to plant performance (Brundrett and Tedersoo 2018). These fungi (AMF and DSE) are coexisting in cortical region of plant to produce soluble metabolites that can increase germination, growth and implications of AMF hyphae (Vergara et al. 2018). These symbionts can indirectly influence host establishment by providing tolerance to biotic and abiotic stresses (Santos et al. 2017; He et al. 2019).

Since these symbionts are considered sensitive indicators and can exhibit behavioral alterations under different environmental or nutritional changes therefore, it is essential to adopt Zn management and biotechnology to minimize environmental damage and increase agricultural production in Brazilian Cerrado soils. The hypothesis of this research was whether Zn residual fertilization in association with inoculation of diazotrophic bacteria could benefit soil microbiota and population of AMF and DSE, thus increasing maize and wheat productivity. Therefore, this study aimed to evaluate the effect of *Azospirillum brasilense* inoculation and residual Zn rates on root colonization and grain yield of maize and wheat in succession under Brazilian Cerrado conditions.

## Material And Methods

### Site description

The study was conducted under field conditions in Selvíria (Brazilian Cerrado region), State of Mato Grosso do Sul, Brazil (20°22'S and 51°22'W, 335 m above sea level (Fig. 1) during 2013/14 (maize) and 2014 (wheat).

The soil was classified as Rhodic Haplustox (clayey Oxisol) according to Soil Survey Staff (2014). Twenty random soil samples were collected from the entire experimental site with a soil core type cup auger (0.10 m × 0.40 m - cup diameter and length respectively) from 0.00-0.20 m depth before initiation of field trial. The soil samples collected from each depth were homogenised, air-dried, sieved (2 mm), and stored at room temperature. The soil chemical attributes (Raij et al. 2001) and granulometry characterization (Teixeira et al. 2017) are summarized in Table 1.

Table 1

Soil chemical attributes in 0-0.20 m layer before field trial beginning.

Soil chemical attributes	0-0.20m layer
P (resin)	13.0 mg dm <sup>-3</sup>
S (SO <sub>4</sub> )	6.0 mg dm <sup>-3</sup>
Organic matter	23.0 g dm <sup>-3</sup>
pH (CaCl <sub>2</sub> )	4.8
K	2.6 mmol <sub>c</sub> dm <sup>-3</sup>
Ca	13.0 mmol <sub>c</sub> dm <sup>-3</sup>
Mg	8.0 mmol <sub>c</sub> dm <sup>-3</sup>
H + Al	42.0 mmol <sub>c</sub> dm <sup>-3</sup>
Al	2.0 mmol <sub>c</sub> dm <sup>-3</sup>
B (hot water)	0.24 mg dm <sup>-3</sup>
Cu (DTPA)	5.9 mg dm <sup>-3</sup>
Fe (DTPA)	30.0 mg dm <sup>-3</sup>
Mn (DTPA)	93.5 mg dm <sup>-3</sup>
Zn (DTPA)	0.5 mg dm <sup>-3</sup>
Cation exchange capacity (pH 7.0)	65.6 mmol <sub>c</sub> dm <sup>-3</sup>
Bases saturation	36%
Soil granulometry	0-0.20 m layer
Sand	438 g kg <sup>-1</sup>
Silt	90 g kg <sup>-1</sup>
Clay	472 g kg <sup>-1</sup>
<i>Azospirillum</i> sp. most probably number	1.65×10 <sup>4</sup> CFU g <sup>-1</sup> soil
<i>n</i> = 20, DTPA = diethylenetriaminepentaacetic acid	

The experimental area had been cultivated with annual leguminous and cereal crops for over 28 years. In addition, the area has been under no-tillage cultivation system for the last 13 years. The crop sequence prior to field trial was fallow until 2013 and black oats (*Avena strigosa* Schreb.) in 2013. Maximum, average and minimum temperatures and rainfall observed during the field trial are presented in Fig. 2.

## Experimental design and treatments

The experimental was designed in a randomized complete block with four replicates, arranged in a 5 × 2 factorial scheme. The treatments were consisted of five Zn rates (0, 2, 4, 6 and 8 kg Zn ha<sup>-1</sup>) applied from zinc sulphate (20% Zn and 10% S) and two seed inoculation with *A. brasilense* (without or with). The total area of each experimental plot was 13.5 m<sup>2</sup>, comprised of six maize rows of five meters long at row space of 0.45 m. Wheat were cultivated in twelve rows of five meters and 0.17 m apart with a plot total size of 10.2 m<sup>2</sup>. The useful area of maize-wheat plot were central rows (10 m<sup>2</sup>).

The seeds of maize and wheat were treated with insecticide and fungicide before inoculation. The seeds of both crops were treated with fungicides [carbendazim + thiram at an active ingredient (a.i.) of 45 g + 105 g per 100 kg seed] and insecticides [imidacloprid + thiodicarb at (a.i.) of 45 g + 135 g per 100 kg seed] before inoculation. This is a general practice used by cereals growing farmers however, the influence of chemical seed treatment on inoculation efficiency of PGPB is still controversial (Munareto et al. 2018; Silva et al. 2018; Cardillo et al. 2019). The inoculation with *A. brasilense* of maize or wheat seeds was carried out by mixing and coating inoculant manually in plastic bags before an hour of plantation.

The *A. brasilense* strains Ab-V5 and Ab-V6 [CNPSO 2083 and CNPSO 2084 respectively, guarantee of 2 × 10<sup>8</sup> colony forming unity (CFU) mL<sup>-1</sup>] were applied to maize seeds (24 kg) at a rate of 200 mL liquid inoculant ha<sup>-1</sup> and 300 mL ha<sup>-1</sup> to 150 kg of wheat seeds. These strains under similar conditions (specifically Brazilian Cerrado) showed positive results on maize and wheat development (Galindo et al. 2016, 2020a, b; Alves et al. 2017; Martins et al. 2018). The draft genome sequences of *A. brasilense* strains Ab-V5 and Ab-V6 carry similar *fix* and *nif* genes which are linked to biological N fixation (Hungria et al. 2018). These strains have different hormones production capacity however, sharing the same gene for auxin production (Fukami et al. 2017, 2018a). In addition, Ab-V5 and Ab-V6 have the capacity to induce expression of genes associated to abiotic and biotic stress tolerance in plants (Fukami et al. 2017; 2018c).

Zinc rates (0, 2, 4, 6 and 8 kg ha<sup>-1</sup>) were manually applied to soil surface at even distribution in maize crop. The calculated amount of fertilizer (zinc sulphate) per plot was applied in between rows at V<sub>2</sub> stage of maize (with two leaves completely unfolded). The experimental area was irrigated with central pivot irrigation system (14 mm) soon after side-dress Zn application to incorporate fertilizer in soil. The Zn fertilizer was not applied in wheat crop in order to analyse residual effect of treatments.

## Field management

### Maize

The area was broadcast applied with limestone (composed of 28% CaO and 20% MgO with an effective neutralizing power of 88%) at the rate of 2.5 Mg ha<sup>-1</sup>, 65 days before maize sowing. The amount of lime applied was based on initial soil analysis and base saturation to 70%, following Eq. 1.

$$LN = \frac{CEC (V2 - V1)}{10 \times ENP} \quad \text{Equation 1}$$

Where LN = Limestone required in Mg ha<sup>-1</sup>, CEC = cation exchange capacity, V2 = bases saturation to be achieved, V1 = current based saturation and ENP = effective neutralization power.

Pre-experiment weeds were controlled by spraying 2, 4-D (670 g ha<sup>-1</sup> a.i.) and glyphosate (1800 g ha<sup>-1</sup> a.i.). A maize triple hybrid DKB 350 VT PRO was sown on 4<sup>th</sup> December, 2013 by placing 3.3 seeds per meter. The NPK 08-28-16 (32, 112 and 64 kg ha<sup>-1</sup> of N, P<sub>2</sub>O<sub>5</sub> and K<sub>2</sub>O respectively) was applied at a dose 400 kg ha<sup>-1</sup> in plantation, based on soil analysis. The crop was irrigated with a center pivot irrigation system at 14 mm water supply. Seeds were emerged five days after sowing in both growing seasons. The recommended N (150 kg N ha<sup>-1</sup> as ammonium sulfate, which contains 21% N and 23% S) was applied manually in side-dress on V6 growth stage. The post emergence weeds during crop development were controlled by the application of atrazine (1000 g ha<sup>-1</sup> a.i.) and tembotrione (84 g ha<sup>-1</sup> a.i.) along with vegetable oil adjuvant (720 g ha<sup>-1</sup> a.i.). In addition, triflururon (24 g ha<sup>-1</sup> a.i.) and methomyl (215 g ha<sup>-1</sup> a.i.) were used for controlling insects. The plants were harvested manually at 108 DAE (27<sup>th</sup> March, 2014).

## Wheat

Wheat planting was carried out on exact area of preceding crop (maize) to analyze residual effect of Zn applied treatments in wheat (successor crop). The wheat genotype CD 116 was sown (80 seeds per meter) on 16<sup>th</sup> May, 2014. A basal fertilization of 350 kg ha<sup>-1</sup> of N-P-K (08-28-16) applied at plantation was equivalent to 28, 98 and 56 kg ha<sup>-1</sup> of N, P<sub>2</sub>O<sub>5</sub> and K<sub>2</sub>O. Seedling were emerged five days after sowing. The area was irrigated with a center pivot irrigation system adjusted to 14 mm water depth at the intervals of 72 h approximately. Nitrogen (120 kg N ha<sup>-1</sup> as ammonium sulfate, which contains 21% N and 23% S) was manually applied at a growth stage GS21 (Zadoks et al. 1974) in an even distribution of fertilizer to all treatments on soil surface. The post-emergence weeds were controlled by metsulfuron-methyl (3 g ha<sup>-1</sup> a.i.). The crop was manually harvested at 110 DAE (9<sup>th</sup> September, 2014).

## Measurements

The microbiological evaluations were performed by collecting four soil samples with maize or wheat roots at the depth of 0.00-0.10 m in each experimental plot. The collected roots were washed and stored in a 50% alcohol solution. One gram of root per plot was clarified in KOH 10% and HCl 1% solution, stained with trypan blue 0.05% and stored in lactoglycerol to assess root AMF and DSE colonization

(Phillips and Hayman 1970). Root colonization was determined by evaluating 100 segments of fine roots per plot.

The soil samples were homogenised and respiratory activity were determined by quantifying carbon released as CO<sub>2</sub>-C in 100 g fresh soil per plot, following the methodology of Anderson and Domsch (1993).

The remaining of collected soil was air-dried, sieved (2 mm) and stored at room temperature. The number of AMF spores were determined from 100 g dry soil sample per plot. The spores were separated from the soil according to methods of decantation and wet sieving (Gerdemann and Nicolson 1963), centrifugation and sucrose flotation (Jenkins 1964). Acrylic plate with concentric rings were used to count the spores under a stereoscopic microscope (40x).

Grain yield was determined by spikes collection in useful lines of each maize and wheat plots. The grains were quantified after mechanical threshing and the data processed in kg ha<sup>-1</sup> to 13% (humidity).

## Statistical analysis

All data were initially tested for normality using Shapiro and Wilk (1965) test and Levene's homoscedasticity test ( $p < 0.05$ ) which showed the data to be normally distributed ( $W \geq 0.90$ ). The data was then analyzed by ANOVA (F test) in a 2-way factorial design with Zn application rates and *A. brasilense* inoculation, their interaction was considered fixed effects in the model while block was considered a random variable. Mean separation was done for significant of main or interaction effects using Tukey test. Regression analysis was also performed to assess whether there is a linear or non-linear response to Zn rates using R software (R Development Core Team, 2015).

## Results

### Treatments effect on maize

Maize root AMF colonization was higher in plants treated with inoculation of *A. brasilense* and 4 kg Zn ha<sup>-1</sup> as compared to non-inoculated plants (Fig. 3A). The Root AMF colonization responded non-linearly to increasing Zn rates in inoculated (up to 3.5 kg Zn ha<sup>-1</sup>) and non-inoculated plants (up to 4.4 kg Zn ha<sup>-1</sup>) (Fig. 3A). In contrast, root DSE colonization was higher in non-inoculated treatment with 2 and 6 kg Zn ha<sup>-1</sup> and in inoculated plants with 8 kg Zn ha<sup>-1</sup> (Fig. 3B). Root DSE colonization was linearly adjusted to increasing Zn rates in inoculated treatments and non-linearly (up to 4.3 kg Zn ha<sup>-1</sup>) in non-inoculated treatments (Fig. 3B). Number of AMF spores and released CO<sub>2</sub>-C were not affected by Zn rates or inoculation (Fig. 3C and F).

The maize grain yield was significantly influenced by Zn rates and *A. brasilense* inoculation (Sup. Table 2), showing a non-linearly response (up to 3.8 kg Zn ha<sup>-1</sup>) with increasing Zn rates (Fig. 3G). Also, it was

observed that inoculated treatments provided greater grain yield ( $8286 \text{ kg ha}^{-1}$ ) compared to non-inoculated treatments ( $7943 \text{ kg ha}^{-1}$ ). Inoculated plots were noted with an increase of 4.3% (Fig. 3H).

## Treatments effect on wheat

Wheat root AMF colonization was higher in treatments with *A. brasilense* inoculation and absence of residual Zn application or with of 2 and 4  $\text{kg Zn ha}^{-1}$  as compared to non-inoculated treatments (Fig. 4A). Root AMF colonization was linearly decreased in inoculated treatments as Zn rates increased (Fig. 4A). While, root DSE colonization was higher in non-inoculated and without Zn residual treatments or with 2 and 6  $\text{kg Zn ha}^{-1}$  (Fig. 4B). Root DSE colonization responded linearly to increasing Zn rates regardless of inoculation (Fig. 4B). Number of AMF spores decreased linearly to increasing residual Zn rates (Fig. 4C). It was also observed that AMF sporulation increased ( $38 \times 100 \text{ g dry soil}$ ) with *A. brasilense* inoculation as compared to non-inoculated treatments ( $34 \times 100 \text{ g dry soil}$ ), an increase of 11.8% was observed (Fig. 4D). Released  $\text{CO}_2\text{-C}$  responded non-linearly to increasing Zn residual rates (up to  $3.6 \text{ kg Zn ha}^{-1}$ ) (Fig. 4E). In addition, released  $\text{CO}_2\text{-C}$  was higher with *A. brasilense* inoculation ( $13.5 \mu\text{g g}^{-1} \text{ soil}$ ) compared to non-inoculated treatments ( $12.7 \mu\text{g g}^{-1} \text{ soil}$ ) with an increase of 6.3% (Fig. 4F). Wheat grain yield responded non-linearly to increasing residual Zn rates (up to  $4.7 \text{ kg Zn ha}^{-1}$ ) (Fig. 4G), but was not affected by inoculation of *A. brasilense* (Fig. 4H).

## Discussion

In the present study, the root AMF colonization was increased with  $4 \text{ kg Zn ha}^{-1}$  in maize while 2 and 4  $\text{kg Zn ha}^{-1}$  in residual form for wheat in succession along with inoculation of *A. brasilense* (Fig. 3A and 4A). Plants mainly absorb Zn in divalent form ( $\text{Zn}^{2+}$ ) (Fernandes et al. 2018) which can also be required for several enzymatic activities, among them synthesis of indole acetic acid (IAA) via tryptophan route (Taiz and Zeiger 2013). Martínez-de la Cruz et al. (2015) reported that auxin has positive impact on branching and volume of plant root system. Our results indicated that Zn rates in association with bacteria has ability to produce IAA and enhance plant growth. In addition, this hormone promoted root system which can indirectly stimulate symbiotic relationship between AMF population and their hosts (Liu et al. 2018).

Bidondo et al. (2011) indicated that bacteria can interact with root microbial community by mycorrhizosphere, inside spores or mycelia as observed *in-vitro* study. It was also reported that diazotrophic bacteria promoted AMF colonization and spores number that lead to higher acquisition of nutrients especially P and Zn with improved rhizospheric environment (Jangra et al. 2019). Several strains of *Azospirillum* spp. and *Pseudomonas* spp. had been reported in degradation of biopolymers (Turrini et al. 2018) and along with Zn application increased root development, formation of new points of infection and auxin synthesis that could lead to higher spores production in successive wheat cultivation (Fig. 4D) and signal AMF colonization (Ludwig-Müller and Güther 2007).

The root architecture system of wheat has low root mycorrhization as observed in the present study (Sup. Table 2). The colonization of AM in wheat roots can be change during growth stages which may have influence on nutritional demand (Ma et al. 2019). In addition, an increase in root colonization was observed from seedling to maturity stage with an increase uptake of  $20 \text{ g kg}^{-1}$  of P and  $2 \text{ mg kg}^{-1}$  of Zn in wheat plants. Such increases were not observed in the present study that probably resulting from already exist optimal levels of soil fertility.

The colonization of DSE in root system of wheat or maize and its interaction with other endophytic microorganisms is still need to be addressed. The DSE colonization occur simultaneously with AMF in plant roots (Ranelli et al. 2015) to deal with biotic and abiotic factors in optimum levels of soil fertility or even increasing doses of zinc that can affect colonization of this fungal group (Lugo et al. 2018). A previous study reported that Zn application may alter DSE (*Exophiala pisciphila*) colonization in maize root system (Li et al. 2011). The low concentration of Zn provided a non-mutualistic relationship between plant and DSE which may result in low biomass production regardless of inoculation. Although, higher doses of Zn fertilizer increased root colonization and biomass production which indicated that alteration in DSE behavior may be due to increasing Zn rates which is verified in the present study (Fig. 3A, B and Fig. 4A, B).

The bacterium *A. brasilense* has the ability to produce siderophores and other molecules like salicylic acid that may decrease mycelial growth (Kumar et al. 2018). The present study also showed a decrease in DSE colonization in roots of wheat (Fig. 3B and 4B). However, there has not been reported any antagonistic effect between *A. brasilense* and DSE in root/ soil system (Newsham 2011). Despite this, Santos et al. (2017) reported a mutualistic association between these microorganisms in most situations but can also develop a pathogenic characteristic in others. The mycelia growth can be inhibited by bacteria action, generating colonization inhibitory effects as observed in the present study for wheat. However, the interactions are still unclear and there is need for further studies on the influence of molecules produced by *A. brasilense* in root DSE colonization.

The  $\text{CO}_2\text{-C}$  released in maize root did not show significant effect with inoculation or Zn fertilization (Fig. 4E, F). While, inoculation of *A. brasilense* under residual Zn rates increased respiratory activity of wheat and thus increasing  $\text{CO}_2\text{-C}$  (Fig. 4E, F). The reason might be due to influence of crop and soil management on microorganisms activities. The respiratory activities of soil microorganisms (bacteria, fungi and other) are responsible for the release of  $\text{CO}_2\text{-C}$  and being act as sensitive indicators for soil quality (Saurich et al. 2019). In addition, different crop practices or even external inoculation of microorganism can generate changes in soil microbial activities. Bera et al. (2018) observed an increasing trend in microbial respiratory activities in wheat succession to rice under no-tillage. The respiratory activity was higher from the time of sowing to flowering while decreasing in later maturity. The present results reflected similar behavior for wheat succession to maize at 110 days after emergence. In addition, there was no significant differences in  $\text{CO}_2\text{-C}$  release at wheat maturity in succession to maize during 24 hours observation (Li et al. 2019). These results corroborate with the low values of  $\text{CO}_2\text{-C}$  as

verified in present study (Fig. 3F and 4F), which could lead to stabilized environment where a higher carbon as microorganism biomass is incorporated into soil and a low value of CO<sub>2</sub>-C is released lost to atmosphere.

The grain yield of maize was significantly different with Zn fertilization and inoculation and adjusted to a non-linear function with increasing Zn rates up to 3.8 kg ha<sup>-1</sup>. The initial soil Zn content medium (Table 1) which could meet plant needs and also explain the decreasing trend in productivity with further increase in Zn rates (Fig. 3G). In addition, inoculation with *A. brasilense* showed an increase in grain production (Fig. 3H). It is possible that *A. brasilense* favored the development of root system with higher absorption of nutrients and water that has a positive influence on nutritional status of plant (Gómez-Godínez et al. 2019; Galindo et al. 2021).

The assimilation of water and nutrients to spike and shoot are directly related to plant nutritional status (Galindo et al. 2019) and therefore leading to higher grain productivity. Our results showed that grain yield of wheat was not statistically influenced by inoculation of *A. brasilense* (Sup. Table 1; Fig. 4G, H) but still higher (1637 kg ha<sup>-1</sup> in Table 1) than average wheat production (900 kg ha<sup>-1</sup>) of State of Mato Grosso do Sul (Conab 2014). Kazi et al. (2016) stated that inoculation of *A. brasilense* did not significantly influence grain yield of different wheat genotypes. The reinoculation of these microorganisms was reported an essential management to increase their population and colonize in environment and can compete with less efficient native species (Cassán and Diaz-Zorita 2016).

## Conclusion

Root colonization by AMF and DSE was positively increased with interaction of Zn rates and *A. brasilense* inoculation via maize seeds. The inoculation of *A. brasilense* favored colonization by AMF and reduced by DSE at 4 kg Zn ha<sup>-1</sup> in maize, while 2 and 4 kg ha<sup>-1</sup> of residual Zn in wheat in succession. It was also concluded that *A. brasilense* inoculation increased maize grain yield and number of AMF spores and CO<sub>2</sub>-C released in wheat in succession.

## Declarations

### Funding

Authors would like to thanks CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), award number 312359/2017-9.

### Conflicts of Interest

The authors declare no conflict of interest.

### Authors' contributions

Conceptualization: F.S.G., M.C.M.T.F.; Methodology: P.S.T.S., F.S.G., A.M.R.C.; Investigation: P.S.T.S.; Writing – original draft: P.S.T.S., F.S.G.; Writing – review and editing: A.M.R.C.; F.S.G., A.J., M.C.M.T.F.; Supervision: A.M.R.C., M.C.M.T.F.

### **Ethics declarations / Ethics approval**

Not applicable.

### **Consent to participate**

Not applicable.

### **Consent for publication**

Not applicable.

### **Conflict of interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### **Data availability**

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

### **Code availability**

Not applicable.

## **References**

1. Alves CJ, Arf O, Ramos AF, Galindo FS, Nogueira LM, Rodrigues RAF (2017) Irrigated wheat subjected to inoculation with *Azospirillum brasilense* and nitrogen doses as top-dressing. R Bras Eng Agr Amb 21:537–542
2. Anderson TH, Domsch KH (1993) The metabolic quotient of CO<sub>2</sub> ( $qCO_2$ ) as a specific activity parameter to assess the effects of environmental conditions, such pH, on the microbial biomass of forest soils. Soil Biol Biochem 25(3):393–395
3. Bera T, Sharma S, Thind HS, Singh Y, Sidhu HS, Jat ML (2018) Changes in soil biochemical indicators at different wheat growth stages under conservation-based sustainable intensification of rice-wheat system. J Integr Agric 17(8):1871–1880
4. Berta G, Copetta A, Gamalero E et al (2014) Maize development and grain quality are differentially affected by mycorrhizal fungi and a growth-promoting pseudomonad in the field. Mycorrhiza 24:161–170. <https://doi.org/10.1007/s00572-013-0523-x>

5. Bidondo LF, Silvani V, Colombo R, Peérgola M, Bompadre J, Godeas A (2011) Pre-symbiotic and symbiotic interactions between *Glomus intraradices* and two *Paenibacillus* species isolated from AM propagules. *in vitro* and *in vivo* assays with soybean (AG043RG) as plant host. *Soil Biol Biochem* 43:1866–1872
6. Brundrett MC, Tedersoo L (2018) Evolutionary history of mycorrhizal symbioses and global host plant diversity. *New Phytol* 220(4):1108–1115
7. Cakmak I, Kutman UB (2017) Agronomic biofortification of cereals with zinc: a review. *Eur J Soil Sci* 69:172–180. <https://doi.org/10.1111/ejss.12437>
8. Cardillo BES, Oliveira DP, Soares BL, Martins FAD, Rufini M, Silva JS, Ferreira Neto GG, de Andrade MJB, Moreira FMDS (2019) Nodulation and yields of common bean are not affected either by fungicides or by the method of inoculation. *Agron J* 111:694–701. <https://doi.org/10.2134/agronj2018.06.0389>
9. Cassán F, Diaz-Zorita M (2016) *Azospirillum* sp. in current agriculture: From the laboratory to the field. *Soil Biol Biochem* 103: 117–130, 2016
10. Castillo-González J, Ojeda-Barrios D, Hernández-Rodríguez A, González-Franco AC, Robles-Hernández L, López-Ochoa GR (2018) Zn metalloenzymes in plants. *Interciencia* 43(4):242–248
11. Cherubin MR, Oliveira DMS, Feigl BJ, Pimentel LG, Lisboa IP, Gmach MR, Varanda LL, Moraes MC, Satiro LS, Popin GV, Paiva SR, Santos AKB, Vasconcelos ALS, Melo PLA, Cerri CEP, Cerri CC (2018) Crop residue harvest for bioenergy production and its implications on soil functioning and plant growth: a review. *Scient Agric* 75:255–272. <https://doi.org/10.1590/1678-992x-2016-0459>
12. Conab. Companhia Nacional de Abastecimento (2020) Grains report—October 2020. Brasília: Conab (in Portuguese). <https://www.conab.gov.br/info-agro/safras>
13. Conab. Companhia Nacional de Abastecimento (2014) Acompanhamento da safra brasileira grãos. Safra 2013/14 – Sexto Levantamento, Brasília, 2014. p. 1–88. (in Portuguese)
14. Fernandes MS, Souza SR, Santos LA (2007) Nutrição mineral de plantas, 2 edn. SBCS, Viçosa, p 670
15. Frey-Klett P, Garbaye J, Tarkka M (2007) The mycorrhiza helper bacteria revisited. *New Phytol* 176:22–36
16. Fukami J, Cerezini P, Hungria M (2018a) *Azospirillum*: benefits that go far beyond biological nitrogen fixation. *AMB Express* 8:73. <https://doi.org/10.1186/s13568-018-0608-1>
17. Fukami J, De La Osa C, Ollero FJ, Megías M, Hungria M (2018c) Coinoculation of maize with *Azospirillum brasilense* and *Rhizobium tropici* as a strategy to mitigate salinity stress. *Functional Plant Biol* 45:328–339
18. Fukami J, Ollero FJ, Megías M, Hungria M (2017) Phytohormones and induction of plant-stress tolerance and defense genes by seed and foliar inoculation with *Azospirillum brasilense* cells and metabolites promote maize growth. *AMB Express* 7:153–163
19. Galindo FS, Teixeira Filho MCM, Buzetti S, Pagliari PH, Santini JMK, Alves CJ, Megda MM, Nogueira TAR, Andreotti M, Arf O (2019) Maize yield response to nitrogen rates and sources associated with *Azospirillum brasilense*. *Agron J* 111:1985–1997

20. Galindo FS, Teixeira Filho MCM, Buzetti S, Santini JMK, Alves CJ, Nogueira LM, Ludkiewicz MGZ, Andreotti M, Bellotte JLM (2016) Corn yield and foliar diagnosis affected by nitrogen fertilization and inoculation with *Azospirillum brasilense*. R Bras Ci Solo 40:e015036
21. Galindo FS, Bellotte JLM, Santini JMK et al (2021) Zinc use efficiency of maize-wheat cropping after inoculation with *Azospirillum brasilense*. Nutr Cycl Agroecosyst 120:205–221.  
<https://doi.org/10.1007/s10705-021-10149-2>
22. Galindo FS, Buzetti S, Rodrigues WL, Boleta EHM, Silva VM, Tavanti RFR, Fernandes GC, Biagini ALC, Rosa PAL, Teixeira Filho MCM (2020a) Inoculation of *Azospirillum brasilense* associated with silicon as a liming source to improve nitrogen fertilization in wheat crops. Sci Rep 10:6160.  
doi:10.1038/s41598-020-63095-4
23. Galindo FS, Pagliari PH, Buzetti S, Rodrigues WL, Santini JMK, Boleta EHM, Rosa PAL, Nogueira TAR, Lazarini E, Teixeira Filho MCM (2020b) Can silicon applied to correct soil acidity in combination with *Azospirillum brasilense* inoculation improve nitrogen use efficiency in maize? Plos One 15:e0230954.  
doi:10.1371/journal.pone.0230954
24. Gerdemann JW, Nicolson TH (1963) Spores of micorrhizal endogone species extracted from soil by wet sieving and decanting. Trans Brit Mycol Soc 46:234–244
25. Gómez-Godínez LJ, Fernandez-Valverde SL, Romero JCM, Martínez-Romero E (2019) Metatranscriptomics and nitrogen fixation from the rhizoplane of maize plantlets inoculated with a group of PGPRs. Syst Appl Microbiol 42:517–525
26. Hatfield JL, Sauer TJ, Cruse RM (2017) Chapter one—soil: the forgotten piece of the water, food, energy Nexus. In: Sparks DL (ed) Advances in agronomy. Academic Press, Cambridge, pp 1–46
27. He YM, Fan XM, Zhang GQ, Li B, Li TG, Zu YQ, Zhan FD (2019) Effects of arbuscular mycorrhizal fungi and dark septate endophytes on maize performance and root under a high cadmium stress. S Afr J Bot 000:1–9
28. Housh AB, Benoit M, Wilder SL, Scott S, Powell G, Schueller MJ, Ferrieri RA (2021) Plant-growth-promoting bacteria can impact zinc uptake in *Zea mays*: An Examination of the Mechanisms of Action Using Functional Mutants of *Azospirillum brasilense*. Microorganisms 9(5):1002.  
<https://doi.org/10.3390/microorganisms9051002>
29. Hungria M, Ribeiro RA, Nogueira MA (2018) Draft genome sequences of *Azospirillum brasilense* strains Ab-V5 and Ab-V6, commercially used in inoculants for grasses and legumes in Brazil. Genome Announc 6:e00393–e00318. doi:10.1128/genomeA.00393-18
30. Jalal A, Galindo FS, Boleta EHM, Oliveira CEDS, Reis ARD, Nogueira TAR, Moretti Neto MJ, Mortinho ES, Fernandes GC, Teixeira Filho MCM (2021) Common bean yield and zinc use efficiency in association with diazotrophic bacteria co-inoculations. Agron 11(5):959.  
<https://doi.org/10.3390/agronomy11050959>
31. Jalal A, Shah S, Teixeira Filho MCM, Khan A, Shah T, Hussain Z, Younis M, Ilyas M (2020) Yield and phenological indices of wheat as affected by exogenous fertilization of Zinc and Iron. Braz J Agric Sci 15(1). DOI:10.5039/agraria.v15i1a7730

32. Jalal A, Shah S, Teixeira Filho MCM, Khan A, Shah T, Ilyas M, Rosa PAL (2020) Agro-biofortification of zinc and iron in wheat grains. *Gesunde Pflanzen* 72(3):227–236. <https://doi.org/10.1007/s10343-020-00505-7>
33. Jangra E, Yadav K, Aggarwal A (2019) Arbuscular mycorrhizal fungal-associated bacteria affect mycorrhizal colonization, essential oil and plant growth of *Murraya koenigii* L. *Acta Sci Pol Hortorum Cultus* 18(5):39–48. Doi:10.24326/asphc.2019.5.4
34. Jenkins WR (1964) A rapid centrifugal-flotation technique for separating nematodes from soil. *Plant Soil* 73:288–300
35. Karimi N, Goltapeh EM, Amini J et al (2021) Effect of *Azospirillum zea* and seed priming with zinc, manganese and auxin on growth and yield parameters of wheat, under dryland farming. *Agric Res* 10:44–55. <https://doi.org/10.1007/s40003-020-00480-5>
36. Kazi N, Deaker R, Wilson N, Muhammad K, Trethowan R (2016) The response of wheat genotypes to inoculation with *Azospirillum brasilense* in the field. *Field Crops Res* 196:368–378
37. Kumar P, Thakur S, Dhingra GK, Singh A, Pal MK, Harshvardhan K, Dubey RC, Maheshwari DK (2018) Inoculation of siderophore producing rhizobacteria and their consortium for growth enhancement of wheat plant. *Biocatal Agric Biotechnol* 15:264–269
38. Li P, Shi K, Wang Y, Kong D, Liu T, Jiao J, Liu M, Li H, Hu F (2019) Soil quality assessment of wheat-maize cropping system with different productivities in China: Establishing a minimum data set. *Soil Till Res* 190:31–40
39. Li T, Liu MJ, Zhang XT, Zhan HB, Sha T, Zhao ZW (2011) Improved tolerance of maize (*Zea mays* L.) to heavy metals by colonization of a dark septate endophytes (DSE) *Exophiala pisciphila*. *Sci Total Environ* 409:1069–1074
40. Liu CY, Wang P, Zhang DJ, Zou YN, Kuca K (2018) Mycorrhiza-induced change in root hair growth is associated with IAA accumulation and expression of *EXPs* in trifoliolate orange under two P levels. *Sci Horti* 234:227–235
41. Ludwig-Müller J, Güther M (2007) Auxins as signals in arbuscular mycorrhiza formation. *Plant Signal Behav* 2(3):194–196
42. Lugo MA, Menoyo E, Allione LR, Negritto MA, Henning JA, Anton AM (2018) Arbuscular mycorrhizas and dark septate endophytes associated with grasses from the Argentine Puna. *Mycologia* 110(4):654–665
43. Ma X, Luo W, Li J, Wu F (2019) Arbuscular mycorrhizal fungi increase both concentrations and bioavailability of Zn in wheat (*Triticum aestivum* L) grain on Zn-spiked soils. *Appl Soil Ecol* 135:91–97
44. Martínez-de la CE, García-Ramírez E, Vázquez-Ramos JM, Cruz HR, López-Bucio J (2015) Auxins differentially regulate root system architecture and cell cycle protein levels in maize seedlings. *J Plant Physiol* 176:147–156
45. Martins MR, Jantalia CP, Reis VM, Döwich I, Polidoro JC, Alves BJR, Boddey RM, Urquiaga S (2018) Impact of plant growth-promoting bacteria on grain yield, protein content, and urea-<sup>15</sup>N recovery by maize in a Cerrado Oxisol. *Plant Soil* 422:239–250. doi:10.1007/s11104-017-3193-1

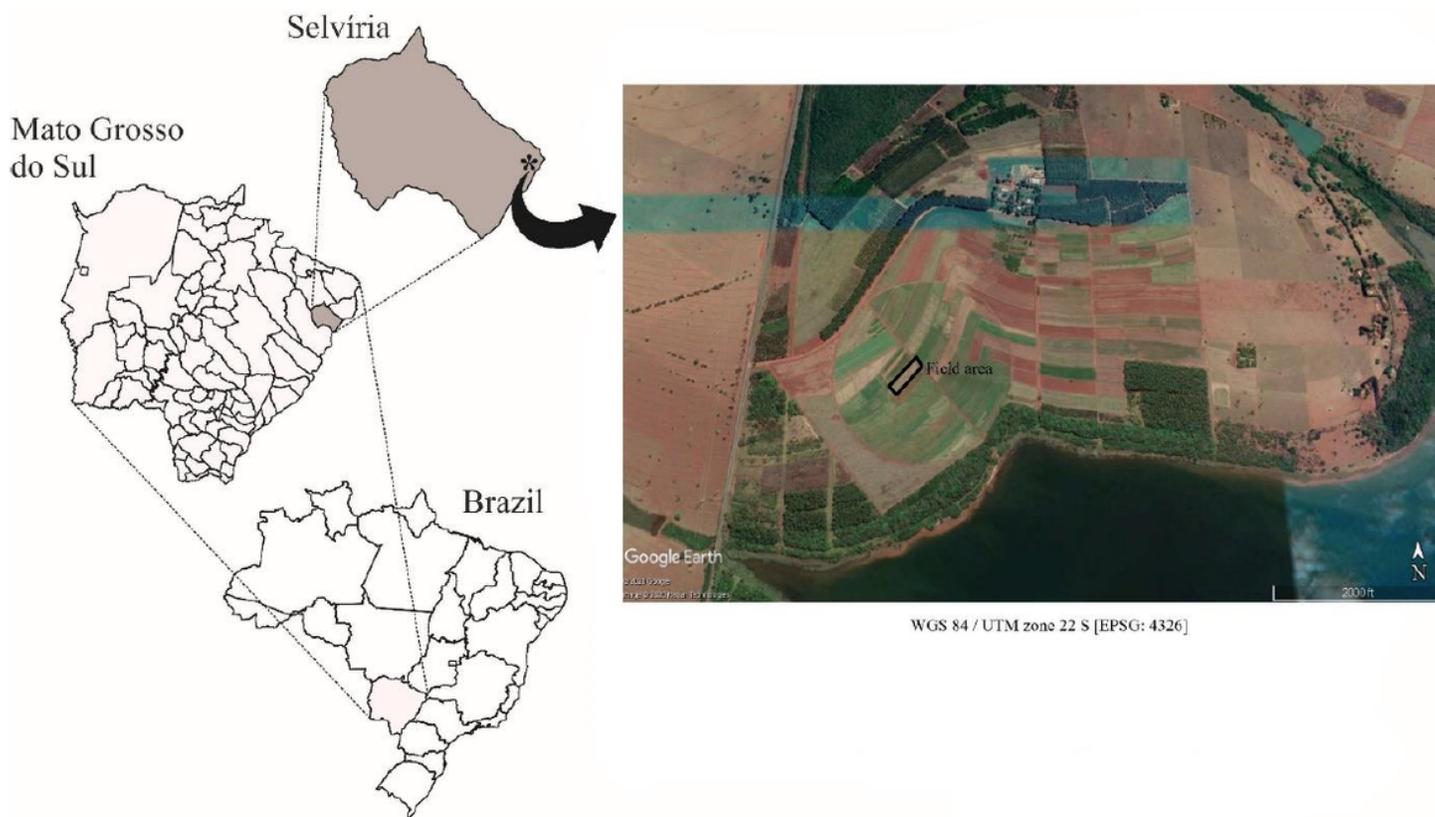
46. Munareto JD, Martin TN, Muller TM, Nunes UR, Rosa GB, Grando LFT (2018) Compatibility of *Azospirillum brasilense* with fungicide and insecticide and its effects on the physiological quality of wheat seeds. *Semina: Ci Agr* 39:855–864
47. Newsham KK (2011) A meta-analysis of plant responses to dark septate root endophytes. *New Phytol* 190:783–793
48. Phillips JM, Hayman DS (1970) Improved procedures for clearing roots for rapid assessment of infection. *Trans Brit Mycol Soc* 55:158–161
49. R Development Core Team (2015) R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>
50. Raj B van, Andrade JC, Cantarella H, Quaggio JA (2001) Chemical analysis for fertility evaluation of tropical soils. IAC, Campinas, p 285 p (in Portuguese)
51. Ranelli LB, Hendricks WQ, Lynn JS, Kivlin SN, Rudgers JA (2015) Biotic and abiotic predictors of fungal colonization in grasses of the Colorado Rockies. *Divers Distrib* 21(8):1–15
52. Santos SGD, Silva PRAda, Garcia AC, Zilli JE, Berbara RLL (2017) Dark septate endophyte decreases stress on rice plants. *Braz J Microbiol* 48:333–341, 2017
53. Saurich A, Tiemeyer B, Don A, Fiedler S, Bechtold M, Amelung W, Freibauer A (2019) Drained organic soils under agriculture – The more degraded the soil the higher the specific basal respiration. *Geoderma* 335:12. <https://doi.org/10.1016/j.geoderma.2019.113911>
54. Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples). *Biometrika* 52:591–611. doi:10.2307/2333709
55. Silva K, Silva EE, Farias ENC, Chaves JS, Albuquerque CNB, Cardoso C (2018) Agronomic efficiency of *Bradyrhizobium* preinoculation in association with chemical treatment of soybean seeds. *Afr J Agr Res* 13:726–732
56. Soil Survey Staff (2014) Keys to Soil Taxonomy. 12 Ed. USDA. Natural Resources Conservation Service, Washington, DC.
57. Taiz L, Zeiger E (2017) Plant physiology, 5 edn. Artmed, Porto Alegre, 918p. (in Portuguese)
58. Teixeira PC, Donagemma GK, Fontana A, Teixeira WG (2017) Manual of soil analysis methods. Centro nacional de pesquisa de solos, Embrapa, Rio de Janeiro (in Portuguese)
59. Turrini A, Avio L, Giovannetti M, Agnolucci M (2018) Funcional Complementarity of Arbuscular Mycorrhizal Fungi and Associated Microbiota: The Challenge of Translational Research. *Front Plant Sci* 9:1407. doi:10.3389/fpls.2018.01407
60. Velho JP, Zardin PB, Jobim CC, Haygert-Velho IMP, Alessio DRM, Giotto E, Conceição GM, Gehrke CR (2020) Meta-analysis of corn plants, green fodder (ensilage), and silages of different types of maize hybrids used in experimental conditions in Brazil. *Semina: Ciênc Agrária* 41(1):237–254
61. Vergara C, Araujo EC, Alves LS, Souza SR, Santos LA, Santa-Catarina C, Souza SR, Santos LA, Santa-Catarina C, Silva K, Pereira GMD, Xavier GR (2018) Contribution of dark septate fungi to the nutrient uptake and growth of rice plants. *Braz J Microbiol* 49:67–78

62. Yang T, Siddique KHM, Liu K (2020) Cropping systems in agriculture and their impact on soil health - a review. *Glob Ecol Conserv* 23:e0111118. <https://doi.org/10.1016/j.gecco.2020.e011118>
63. Zadoks JC, Chang TT, Konzak CF (1974) A decimal code for the growth stages of cereals. *Weed Res* 14:415–421

## Supplemental Data

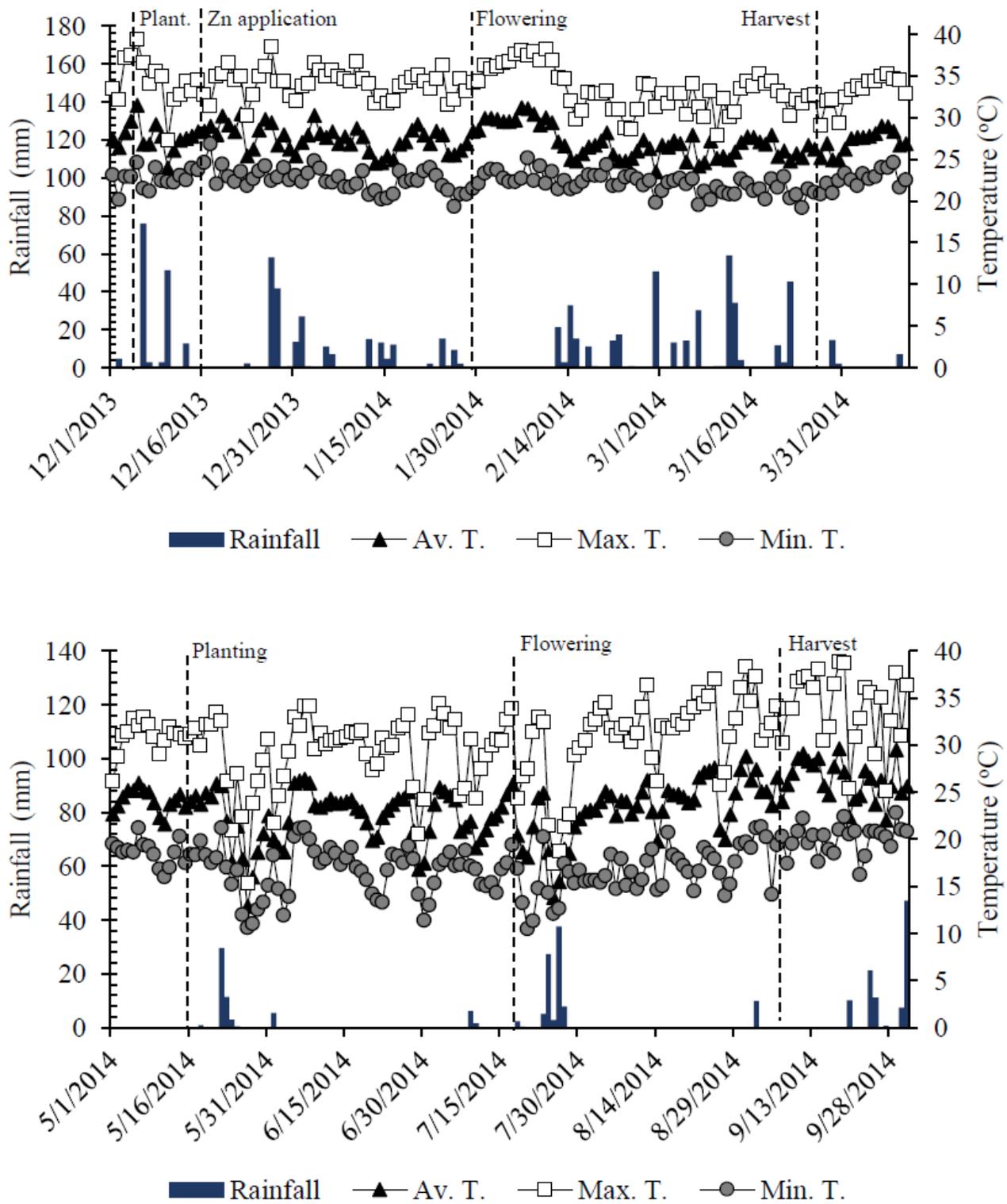
Supplemental Table 2 is not available with this version.

## Figures



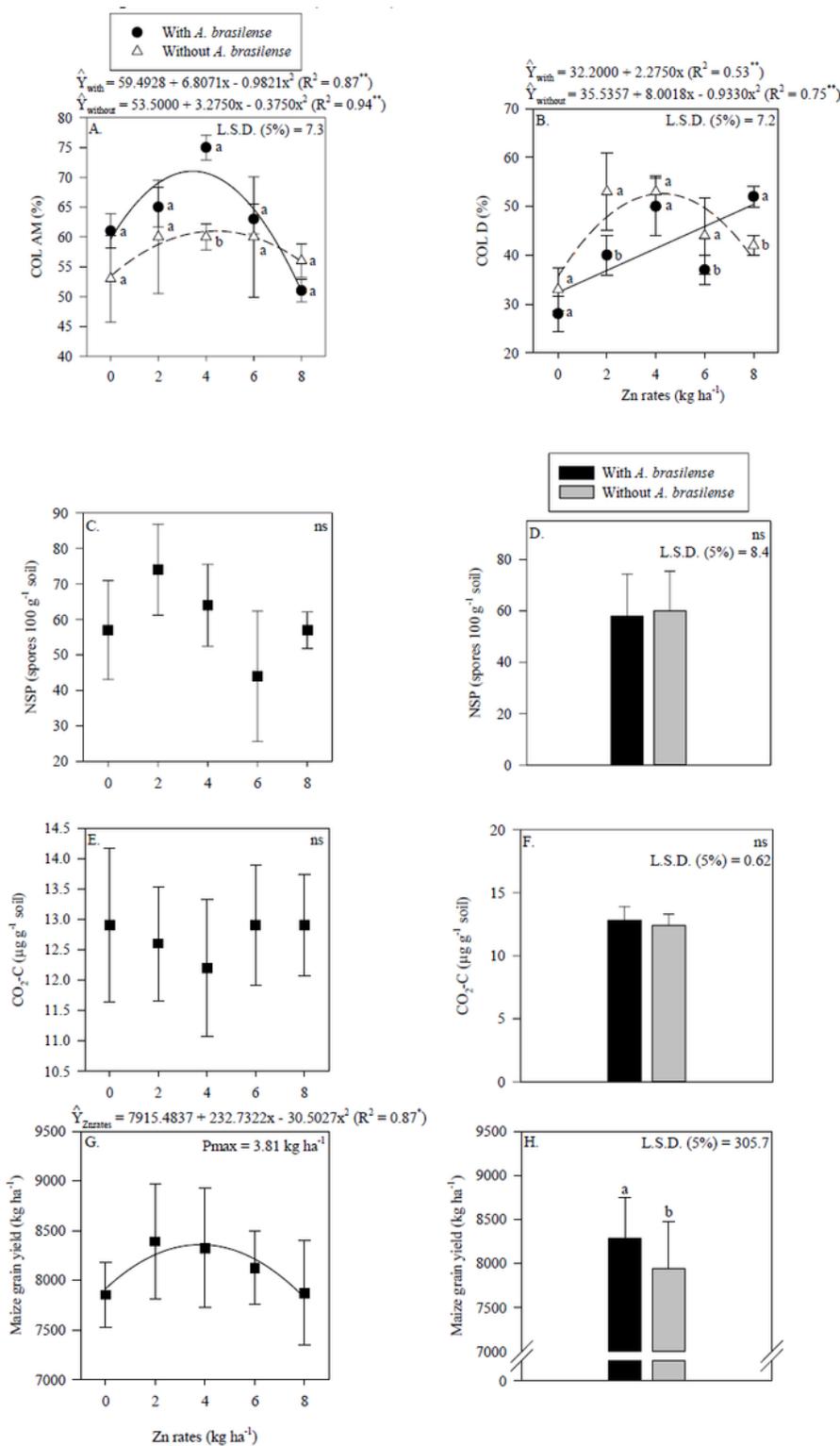
**Figure 1**

Study area at the Selvíria, State of Mato Grosso do Sul, Brazil (20°22'S, 51°22'W, the altitude of 335 m above sea level). Map created by using QGIS software and Google Earth program. QGIS Development Team (2019). QGIS Geographic Information System. Open Source Geospatial Foundation Project. <http://qgis.osgeo.org>. Image obtained in Google Earth program. Google company (2020).



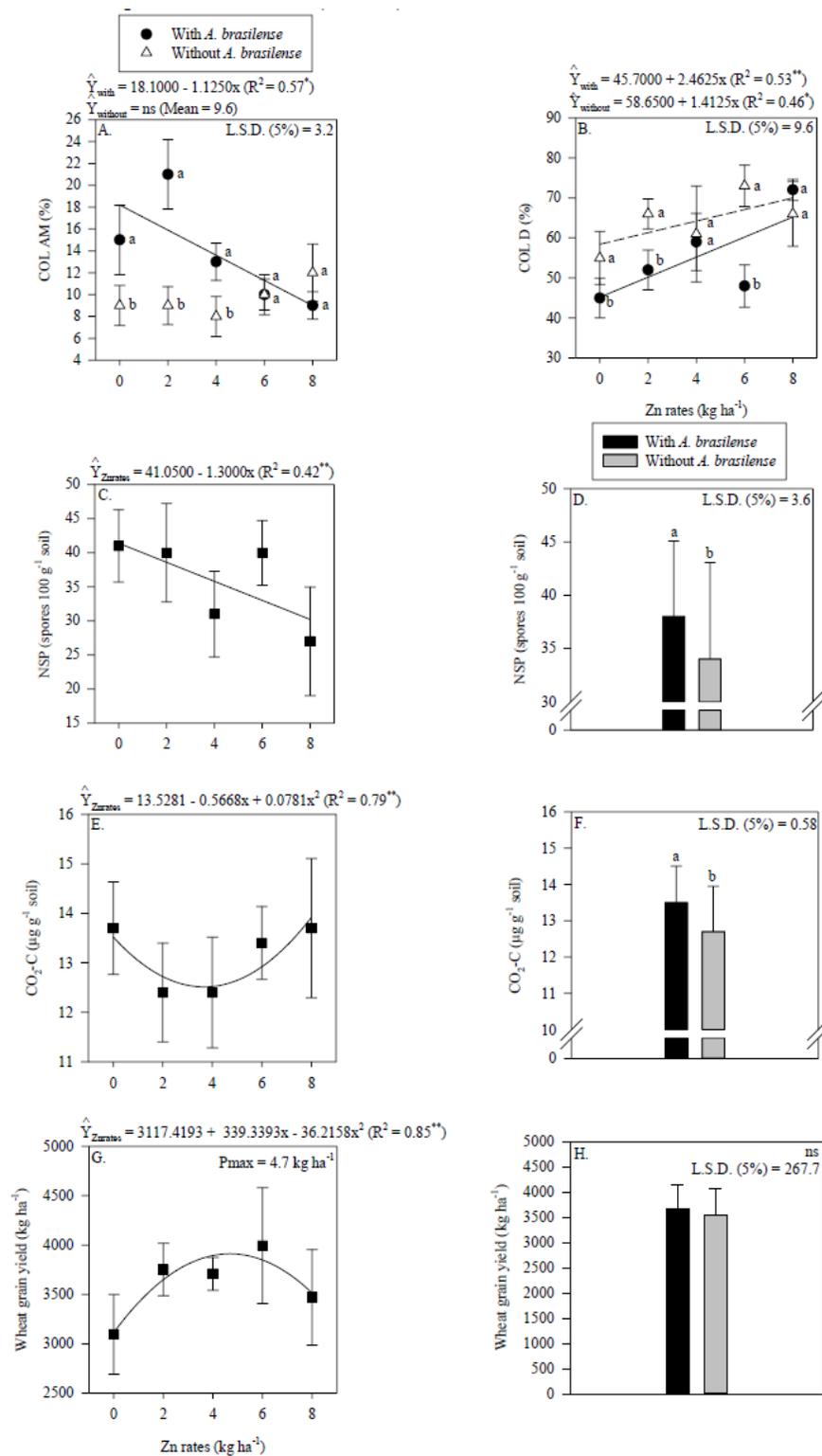
**Figure 2**

Rainfall, maximum, average and minimum temperatures obtained from the weather station located in the Education and Research Farm of UNESP during the maize (A) and wheat (B) cultivation.



**Figure 3**

Interaction between Zn rates and inoculation or not with *Azospirillum brasilense* in maize root colonization (COL AM) (A) and root colonization by endophytic fungi with septated, melanized hyphae ("dark septate" - COL D) (B), number of spores of arbuscular mycorrhizal fungi (NSP), released carbon from CO<sub>2</sub> (CO<sub>2</sub>-C) and maize grain yield as a function of single effect of Zn rates (C, E and G) and inoculation or not with *Azospirillum brasilense* (D, F, H).



**Figure 4**

Interaction between Zn rates and inoculation or not with *Azospirillum brasilense* in wheat root colonization (COL AM) (A) and root colonization by endophytic fungi with septated, melanized hyphae (“dark septate” - COL D) (B), number of spores of arbuscular mycorrhizal fungi (NSP), released carbon from CO<sub>2</sub> (CO<sub>2</sub>-C) and maize grain yield as a function of single effect of Zn rates (C, E and G) and inoculation or not with *Azospirillum brasilense* (D, F, H).

## Supplementary Files

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