SEMINA
Ciências Agrárias

DOI: 10.5433/1679-0359.2021v42n1p43

Co-inoculation and inoculation methods of plant growth-promoting bacteria in wheat yield performance

Coinoculação e métodos de inoculação de bactérias promotoras de crescimento de plantas no desempenho produtivo da cultura do trigo

Luiz Júnior Perini[1]*; Douglas Mariani Zeffa[2]; William Rafael Roesler[3]; Claudemir Zucareli[4]; Leandro Simões Azeredo Gonçalves[4]

Highlights

Inoculation with *A. brasilense* does not always change wheat grain yield.
Post-emergence inoculation provided higher grain yield.
Post-emergence inoculation can replace nitrogen topdressing fertilization.

Abstract

Several studies have reported the beneficial effects of inoculation of *Azospirillum brasilense* in wheat, but only a few of them have related the co-inoculation of *A. brasilense* and *Rhizobium* sp. and the evaluation of different inoculation methods. This study aimed *i*) to verify the efficiency of plant growth-promoting bacteria (PGPB) in subtropical environments, *ii*) to verify the efficiency of co-inoculation of *A. brasilense* and *Rhizobium* sp., and *iii*) to verify the efficiency of the management of different inoculation methods in the wheat crop. The experiments were carried out in Londrina and Apucarana, State of Paraná, Brazil, under a complete randomized block design, with four replications and nine treatments: T1) absence of nitrogen (N) topdressing, T2) 30 kg ha^{-1} of N topdressing, T3) 60 kg ha^{-1} of N topdressing, T4) *A. brasilense* Ab-V5 in the seeds, T5) *A. brasilense* Ab-V5 in post-emergence, T6) *A. brasilense* Ab-V5 + *Rhizobium* sp. 53GRM1 in the seeds, T7) *A. brasilense* Ab-V5 + *Rhizobium* sp. 53GRM1 in post-emergence, T8) commercial inoculant (*A. brasilense* Ab-V5 + Ab-V6) in the seeds, and T9) commercial inoculant (*A. brasilense* Ab-V5 + Ab-V6) in post-emergence. The number of ears per linear meter, number of spikelets, number of grains per spikelet, number of grains per ear, thousand-grain weight, number of spikelets to ears ratio, leaf nitrogen content, and grain

---

[1] Discente do Curso de Doutorado do Programa de Pós-Graduação em Agronomia, Departamento de Agronomia, Universidade Estadual de Londrina, UEL, Londrina, PR, Brasil. E-mail: luiz.j.perini@outlook.com

[2] Discente do Curso de Doutorado do Programa de Pós-Graduação em Genética e Melhoramento de Plantas, Departamento de Agronomia, Universidade Estadual de Maringá, UEM, Maringá, PR, Brasil. E-mail: douglas.mz@hotmail.com

[3] Discente do Curso de Engenharia Agronômica, Departamento de Agronomia, UEL, Londrina, PR, Brasil. E-mail: willian_roesler@hotmail.com

[4] Profs. Drs., Programa de Pós-Graduação em Agronomia, Departamento de Agronomia, UEL, Londrina, PR, Brasil. E-mail: claudemircca@uel.br; leandrosag@uel.br

* Author for correspondence

Received: Nov. 21, 2019 - Approved: Nov. 17, 2020

yield were evaluated. Leaf N content and yield components showed no alterations due to the inoculation and co-inoculation performed both in the seed and in the post-emergence of seedlings. Treatments T3, T7, and T9 showed the highest means of grain yield (2077.50, 1743.12, and 1660.62 kg ha^{-1}, respectively), demonstrating that co-inoculation with *A. brasilense* Ab-V5 + *Rhizobium* sp. 53GRM1 and inoculation with *A. brasilense* Ab-V5 + Ab-V6, both in post-emergence of seedlings, have the potential to replace the topdressing nitrogen fertilization in wheat.

Key words: *Azospirillum brasilense.* Nitrogen. *Rhizobium* sp. *Triticum aestivum*.

Resumo

Em trigo, embora diversos estudos já tenham sido relatados sobre os efeitos benéficos da inoculação de *Azospirillum brasilense*, são escassos na literatura trabalhos relacionados ao uso de coinoculação de *A. brasilense* e *Rhizobium* sp., bem como a avaliação de diferentes métodos de inoculação. Os objetivos do estudo foram: *i*) verificar a eficiência de bactérias promotoras do crescimento de plantas (BPCP) em ambientes subtropicais; *ii*) verificar a eficiência da coinoculação de *A. brasilense* e *Rhizobium* sp.; e *iii*) verificar a eficiência do manejo de diferentes métodos de inoculação na cultura do trigo. Os experimentos foram conduzidos nos municípios de Londrina e Apucarana, Paraná, sob o delineamento experimental de blocos completos ao acaso, com quatro repetições e nove tratamentos: T1) ausência de nitrogênio (N) em cobertura; T2) 30 kg ha^{-1} de N em cobertura; T3) 60 kg ha^{-1} de N em cobertura; T4) *A. brasilense* Ab-V5 nas sementes; T5) *A. brasilense* Ab-V5 em pós-emergência; T6) *A. brasilense* Ab-V5 + *Rhizobium* sp. 53GRM1 nas sementes; T7) *A. brasilense* Ab-V5 + *Rhizobium* sp. 53GRM1 em pós-emergência; T8) Inoculante comercial (*A. brasilense* Ab-V5 + Ab-V6) nas sementes; e T9) Inoculante comercial (*A. brasilense* Ab-V5 + Ab-V6) em pós-emergência. Foram avaliadas as seguintes características: número de espigas por metro linear, número de espiguetas, número de grãos por espigueta, número de grãos por espiga, massa de mil grãos, relação número de espiguetas e espigas, teor de nitrogênio nas folhas e produtividade de grãos. O teor de N foliar e os componentes do rendimento não foram alterados pela inoculação e coinoculação realizada tanto na semente quanto em pós-emergência das plântulas. Os tratamentos T3, T7 e T9 apresentaram as maiores médias de produtividade de grãos (2077,50; 1743,12 e 1660,62 kg ha^{-1}, respectivamente), demonstrando que a coinoculação com *A. brasilense* Ab-V5 + *Rhizobium* sp. 53GRM1, bem como a inoculação com *A. brasilense* Ab-V5 + Ab-V6, ambos em pós-emergência das plântulas, apresentam potencial para substituir a adubação nitrogenada de cobertura na cultura do trigo.

Palavras-chave: *Azospirillum brasilense.* Nitrogênio. *Rhizobium* sp. *Triticum aestivum*.

Introduction

Wheat (*Triticum aestivum*) is one of the most important cereals for human consumption worldwide and locally. Brazil has a deficit of approximately 60% between production and consumption (Companhia Nacional de Abastecimento [CONAB], 2020), making it dependent on imports from countries such as Argentina, Canada, and the United States. Among the main obstacles to the development of wheat cultivation in Brazil are the lack of public policies, adverse climate conditions, high production costs, and low profitability (Galindo et al., 2018). Chemical fertilizers, especially nitrogen fertilizers, represent a high cost in production, reaching up to 30% of the total costs (CONAB, 2020). In addition, the indiscriminate use of nitrogen fertilizers has caused serious environmental problems, including water eutrophication, soil acidification, and air pollution (Chen et al., 2018).

Nitrogen (N) is considered the most absorbed and exported nutrient by the wheat plant, which is why its deficiency constitutes a limiting factor and directly influences the number of tillers, number of ears, spikelet development, grain weight, and, consequently, grain yield (Vaghar & Ehsanzadeh, 2018; Ul-Allah et al., 2018). According to Ladha et al. (2016), the two main strategies to reduce the dependence on nitrogen fertilizers in wheat cultivation is by increasing its use efficiency by plants and the use of diazotrophic bacteria. These bacteria can supply nitrogen to plants through non-symbiotic nitrogen fixation. Also, these microorganisms can favor nutrient uptake by plants, acting as biofertilizers, phyto-stimulators, and mitigators of abiotic and biotic stresses (Lugtenberg & Kamilova, 2009; Pii et al., 2015; Zeffa et al., 2018).

The genus *Azospirillum* spp. has been widely studied in the wheat crop (Piccinin et al., 2013; Fukami, Nogueira, Araujo, & Hungria, 2016; Di Salvo, Ferrando, Fernández-Scavino, & Salamone, 2018; Galindo et al., 2020), and it is the constituent of commercial biofertilizers. Veresoglou and Menexes (2010) found that inoculation with *Azospirillum* spp. in wheat crop increased grain yield by 8.9% in a meta-analysis study based on 59 scientific articles. However, an increase of 14% in yield was observed when considering only the experiments with no topdressing nitrogen fertilization. In Brazil, inoculants containing the elite strains of *Azospirillum brasilense* (Ab-V5 and Ab-V6) have been exponentially employed by farmers in recent years, mainly in corn, wheat, sugarcane, and brachiaria crops, in addition to being used as co-inoculants in soybean and bean (Hungria, Araujo, Silva, Barbosa, & Zilli, 2017; Zeffa et al., 2019, 2020).

Several studies have shown the benefits of co-inoculation of bacteria of the genera *Azospirillum* and *Rhizobium* in the wheat crop (Kavimandan, 1985; Galal, El-Ghandour, & El-Akel, 2001; Yanni et al., 2016). In general, bacteria of the genus *Rhizobium* are used as inoculants in legume crops, as there is a symbiotic association between the plant and the bacterium, leading to the formation of nodules in the roots (Ohyama, 2017). The benefits of *Rhizobium* for legume plants are mainly due to the N supply through the biological N_2 fixation (BNF) process (Hoang, Tóth, & Stacey, 2020). Bacteria of the genus *Rhizobium* can be associated with the rhizosphere of wheat plants, characterizing external colonization and promoting benefits through the production and release of auxins, gibberellins, siderophores, phosphorus solubilization, and biocontrol of several root diseases (Patil, Kale, Ajane, Sheikh, & Patil, 2017).

Standard inoculation with *A. brasilense* in wheat, recommended by most manufacturers, is performed by mixing the inoculant with the seeds in pre-sowing. However, seed treatment with fungicides can provide toxicity to non-target microorganisms and influence the efficiency of *Azospirillum* spp. and other plant growth-promoting bacteria (PGPB) (Yang, Hamel, Vujanovic, & Gan, 2011). Thus, post-emergence inoculation can be considered a viable alternative to circumvent this problem (Fukami et al., 2016).

Although several studies have already reported the beneficial effects of inoculation of *A. brasilense* in wheat, few of them have related the co-inoculation of *A. brasilense* and *Rhizobium* sp. and the evaluation of different inoculation methods. Thus, this study aimed *i*) to verify the efficiency of plant growth-promoting bacteria (PGPB) in subtropical environments, *ii*) to verify the efficiency of co-inoculation of *A. brasilense* and *Rhizobium* sp., and *iii*) to verify the efficiency of the management of different inoculation methods in the wheat crop.

Material and Methods

Experimental conditions

The experiments were conducted in the experimental area of the State University of Londrina (UEL) (23°20′23″ S, 51°12′32″ W, 532 m altitude) and the farm Estância Natureza, located in Apucarana (23°31′17″ S and 51°27′10″ W, 790 m altitude), during the 2016 agricultural year. The soil in the UEL experimental area is classified as eutrophic Red Latosol, while the soil on the farm Estância Natureza is classified as eutrophic Red Nitosol. Both municipalities are in the State of Paraná, Brazil, and the climate is classified as Cfa, i.e., a subtropical humid mesothermal climate, according to Köppen classification. The data of precipitation and maximum and minimum temperatures were obtained from the Agronomic Institute of Paraná (IAPAR) for Londrina and the Meteorological System of Paraná (SIMEPAR) for Apucarana (Figure 1). Soil chemical analyses (0–20 cm) were carried out before the experiments were set up, presenting the following results in Londrina and Apucarana, respectively: pH ($CaCl_2$) = 4.17 and 5.24, P = 7.53 and 12.97 mg dm^{-3}, K$^+$ = 0.22 and 1.15 cmol$_c$ dm^{-3}, Ca^{2+} = 3.75 and 8.95 cmol$_c$ dm^{-3}, Mg^{2+} = 0.65 and 2.04 cmol$_c$ dm^{-3}, effective CEC = 4.62 and 12.14 cmol$_c$ dm^{-3}, and V% = 38.53 and 70.99.

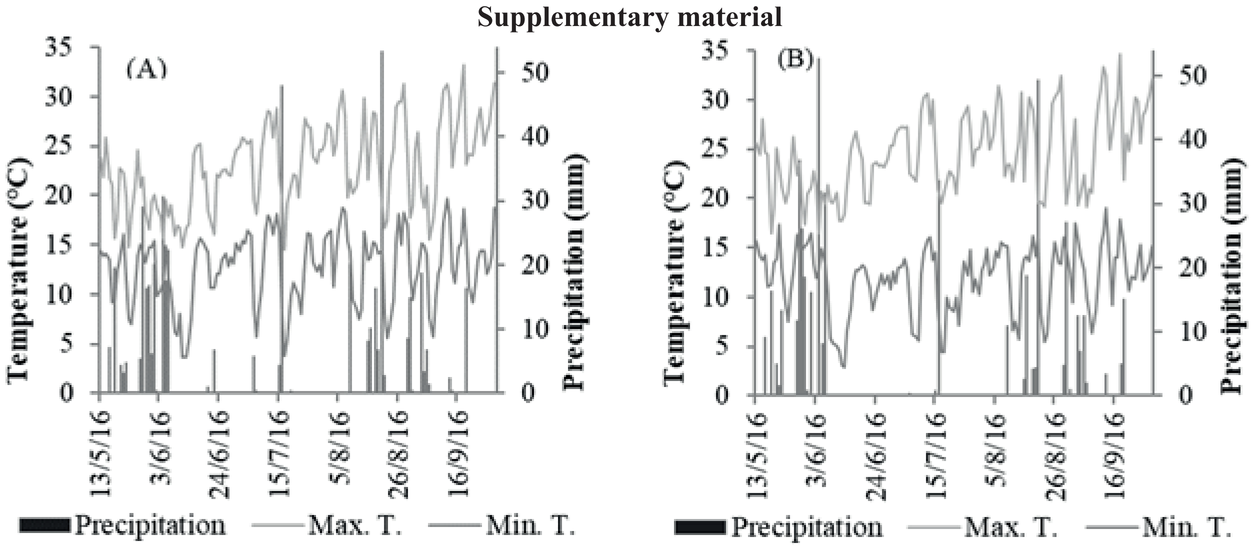

Figure 1. Daily data of precipitation and maximum and minimum temperature during the experimental period in Londrina (A) and Apucarana (B) in the 2016 agricultural year.

The experimental design was complete randomized blocks, with four replications and nine treatments: T1) absence of nitrogen (N) topdressing, T2) 30 kg ha^{-1} of N topdressing, T3) 60 kg ha^{-1} of N topdressing, T4) *A. brasilense* Ab-V5 in the seeds, T5) *A. brasilense* Ab-V5 in post-emergence, T6) *A. brasilense* Ab-V5 + *Rhizobium* sp. 53GRM1 in the seeds, T7) *A. brasilense* Ab-V5 + *Rhizobium* sp. 53GRM1 in post-emergence, T8) commercial inoculant (*A. brasilense* Ab-V5 + Ab-V6) in the seeds, and T9) commercial inoculant (*A. brasilense* Ab-V5 + Ab-V6) in post-emergence.

The plots consisted of 28 rows 5 m long, with an inter-row spacing of 0.17 m and a density of 441 seeds m^{-2}. The useful area of the plots was defined as the 26 central rows without the 0.5 m from each end, which corresponded to 17.68 m^2. The wheat cultivar used in the experiments was CD 150 (Coodetec), which has a small size, early cycle, high production potential in soils with good fertility, good sanity, and blast tolerance, being used as flour-improving wheat in the food industry. The fungicide pyraclostrobin and the insecticides thiophanate-methyl and fipronil were used for seed treatment at a dose of 2 mL kg^{-1} of seeds. Seed treatment was carried out five days before sowing, while seed inoculation with PGPB was carried out during sowing. Sowing was carried out on May 13, 2016, in Londrina and May 23, 2016, in Apucarana. Sowing fertilization consisted of 200 kg ha^{-1} of the NPK formulation 10–15–15, considering the soil chemical analyses and crop recommendations (Silva, Bassoi, & Foloni, 2017).

Bacterial growth and inoculation

The bacteria used in the non-commercial inoculant are deposited in the collection of bacterial cultures of the Laboratory of Molecular Biochemistry of UEL (LBM–UEL). Colonies of Ab-V5 and *Rhizobium* sp. 53GRM1 were grown as pre-inoculum in liquid DYGS medium (2 g of glucose, 1.5 g of peptone, 2 g of yeast extract, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4$, and distilled water q.s. to 1 L, pH 6.0) for 24 hours. Then, they were multiplied in 250 mL of liquid M15 medium (Oliveira et al., 2017) and cultured for 48 hours under stirring in an orbital incubator (180 rpm at 28 °C). After this period, the cultures were interrupted and the cell concentration was determined by counting in a Neubauer chamber, with subsequent standardization of the cell suspension and dilution to a concentration of 1 × 10^8 bacterial cells mL^{-1} of the inoculant.

The inoculants were applied at a concentration of 1 × 10^8 bacterial cells mL^{-1} for treatments T4 and T6 using 30 mL of inoculant per kg of seed. Seed inoculation for treatment T8 was performed using the commercial product Qualyfix® (*A. brasilense* strains Ab-V5 + Ab-V6) using 6 mL of inoculant per kg of seed at a commercial concentration of 5 × 10^8 bacterial cells mL^{-1}. Treatments with post-emergence inoculation (T5 and T7) received 2.5 L ha^{-1} of inoculants at a concentration of 1 × 10^8 bacterial cells mL^{-1} using a knapsack sprayer at seven days after seedling emergence. The treatment commercial inoculant in post-emergence (T9) received 500 mL ha^{-1} of the Qualyfix® inoculant at a concentration of 5 × 10^8 bacterial cells mL^{-1}. Treatments T2 and T3 received 30 and 60 kg ha^{-1} of N topdressing, respectively, at the beginning of tillering, with ammonium sulfate as the N source.

Agronomic characteristics and nitrogen content

The following characteristics were evaluated: grain yield (GY, in kg ha^{-1}), corrected to 13% moisture; thousand-grain weight (TGW, in g), according to the rules for seed testing; number of ears per linear meter (NEM); number of grains per ear (NGE); number of grains per spikelet (NGS); number of spikelets to ears ratio (SER); and leaf nitrogen content (LNC, in g kg^{-1}). LNC was determined using 30 flag leaves collected from each plot, packed in paper bags, and taken to a forced-air ventilation oven at 65 °C until constant mass. Subsequently, the samples were ground, homogenized and aliquots of 0.1 g were taken for later N determination using the Kjeldahl method through digestion, distillation, and titration processes, as described by Bremner and Mulvaney (1982).

Data analysis

The data were subjected to the individual analysis of variance and, subsequently, to joint analysis of variance after detecting the homogeneity of residual variances by the Bartlett (1937) test. The means were grouped by the Scott and Knott (1974) clustering test at a 5% probability level when there was statistical significance ($P<0.05$). Statistical analyses were performed using the software R (http://www.r-project.org), using the *easyanova* package (Arnhold, 2013).

Results and Discussion

Most of the agronomic characteristics showed a significant effect of environment, except for SER and LNC. On the other hand, only GY presented a significant effect of treatment. Only the characteristic LNC had a significant effect ($P<0.05$) for the interaction treatments × environments (Table 1), indicating a differential behavior of treatments relative to environmental variations. Although Londrina and Apucarana showed similar variations in temperature and precipitation (Figure 1), a high difference was observed regarding the soil fertility of these environments. According to Castro-Sowinski, Herschkovitz, Okon and Jurkevitch (2007), the response to inoculation may vary according to the environmental conditions, soil chemical, physical, and/or biological characteristics, plant genotype, bacterial strain, and quality of PGPB cells used as inoculants.

Table 1

Summary of the joint analysis of variance and coefficient of variation (CV) of seven agronomic characteristics evaluated in the wheat cultivar CD 150 as a function of different forms of fertilization in experiments conducted in Londrina and Apucarana in the 2016 agricultural year

SV	DF	Mean square[1]						
		NEM	SER	NGS	NGE	TGW	GY	LNC
Block/Site	6	148.73	1.71	0.059	43.10	2.22	1814.88	13.90
Treatment (T)	8	26.91	0.75	0.073	48.29	3.30	537.26**	29.15
Environment (E)	1	1462.50*	2.41	2.456**	279.66*	356.00**	14113.01*	12.87
T × E	8	33.56	0.74	0.022	20.10	4.66	232.07	42.35*
Residual	48	20.08	0.92	0.099	33.43	2.38	122.91	16.69
Apucarana		39.90	16.16	3.31	51.64	36.57	1967.50	19.74
Londrina		30.88	16.52	2.94	47.70	32.12	1082.03	18.9
Overall mean		35.39	16.43	3.13	49.67	34.35	1524.76	19.32
CV (%)		12.65	5.86	10.05	11.64	4.49	22.96	21.14

[1] GY = grain yield; TGW = thousand-grain weight; NEM: number of ears per linear meter; NGE = number of grains per ear; NGS = number of grains per spikelet; SER = number of spikelets to ears ratio; LNC = leaf nitrogen content.

*, ** significant at the 5 and 1% probability level by the F-test, respectively.

The means of GY of the treatments *A. brasilense* Ab-V5 + *Rhizobium* sp. 53GRM1 in post-emergence (1743.12 kg ha^{-1}) and commercial inoculant (*A. brasilense* Ab-V5 + Ab-V6) in post-emergence (1660.62 kg ha^{-1}) did not differ statistically from the treatment 60 kg ha^{-1} of N topdressing (2035.43 kg ha^{-1}) by the Scott and Knott test, which were higher than the other treatments (Figure 2). Thus, these treatments could completely replace N fertilization in the wheat crop. Some studies have observed that inoculation of wheat with *A. brasiliense* replaced topdressing N fertilization only partially (Piccinin et al., 2013; Veresoglou and Menexes, 2010). For instance, Pereira et al. (2017) studied the effect of different forms of inoculation of *A. brasilense* (seed, post-emergence, and sowing furrow) associated with topdressing N doses (0, 30, and 60 kg ha^{-1}) and reported that the use of half the N dose (30 kg ha^{-1}) associated with *A. brasilense*, regardless of the inoculation method, provided an increase in wheat yield compared to the application of half the N dose alone. On the other hand, the inoculation of *A. brasilense* was not able to replace the total topdressing N fertilization.

Vogel, Martinkoski, Jadoski and Fey (2015) found that the chemical treatment of seeds had a detrimental effect on inoculation with PGPB when it is carried out directly on the seeds. In this sense, the results observed in the present study have importance regarding the inoculation methods of PGPB, as the inoculation in post-emergence is a viable and effective alternative compared to the inoculation directly in the seeds. Fukami et al. (2016) evaluated different inoculation methods and found that the inoculant application via leaf spraying increased yield relative to the control treatments in wheat and corn crops. Pereira et al. (2017) reported that inoculation with *A. brasilense* in its liquid form via seed treatment, leaf application, or sowing furrow provided increases in wheat yield compared to the control treatment. On the other hand, Ferreira et al. (2017) observed increases in wheat yield when inoculated with *A. brasilense* via seed and post-emergence. However, the authors did not find significant increases when the inoculation was performed via leaf spraying.

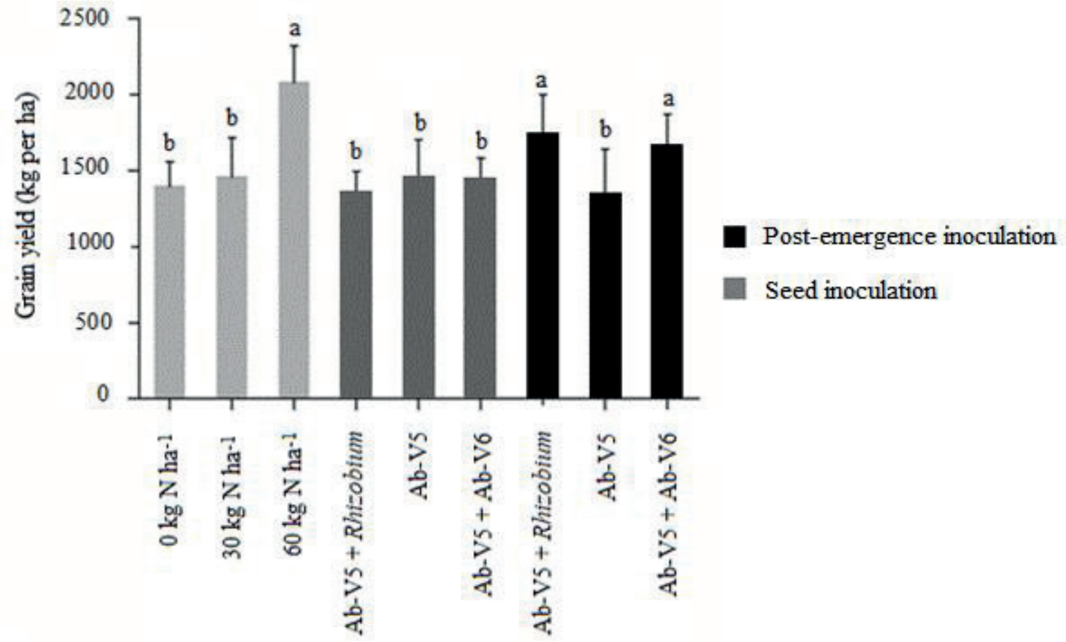

Figure 2. Means and their respective groups by the Scott and Knott (1974) test for grain yield of the wheat cultivar CD150 as a function of different inoculation methods. Means followed by the same letters constitute a statistically homogeneous group by the Scott and Knott (1974) test.

The treatment *A. brasilense* Ab-V5 + *Rhizobium* sp. 53GRM1 in post-emergence showed an increase of 17.42 and 15.21% in GY relative to treatments with no N topdressing and 30 kg ha^{-1} of N topdressing, respectively (Figure 2), indicating the inoculation potential of *A. brasilense* and *Rhizobium* sp. in wheat cultivation. Spolaor et al. (2016) studied the effect of co-inoculation of *A. brasilense* and *Rhizobium* sp. in popcorn and reported an increase of 26.1% in the co-inoculated treatment relative to the non-inoculated treatment in the absence of topdressing nitrogen fertilization. Regarding the treatment commercial inoculant (*A. brasilense* Ab-V5 + Ab-V6) in post-emergence (Figure 2), the inoculation with the strains Ab-V5 + Ab-V6 showed an increase of 16.12% relative to the inoculation with only the strain Ab-V5 in post-emergence. These results are corroborated by those observed by Hungria et al. (2010), who reported that the effect of strains Ab-V5 and Ab-V6 is enhanced when they are inoculated together. On the other hand, no statistical difference was observed between the treatments commercial inoculant (*A.*

brasilense Ab-V5 + Ab-V6) and the strain Ab-V5 inoculated in the seeds.

Regarding LNC (Table 2), the treatments *A. brasilense* Ab-V5 in the seeds, *A. brasilense* Ab-V5 + *Rhizobium* sp. 53GRM1 in the seeds, *A. brasilense* Ab-V5 + *Rhizobium* sp. 53GRM1 in post-emergence, and commercial inoculant (*A. brasilense* Ab-V5 + Ab-V6) in post-emergence presented the lowest values in Apucarana environment. However, the Londrina environment showed no significant difference between treatments. The treatment commercial inoculant (*A. brasilense* Ab-V5 + Ab-V6) in the seeds had a higher LNC mean in Apucarana than Londrina, while the treatment *A. brasilense* Ab-V5 in the seeds was superior in Londrina than in the Apucarana environment. Galindo et al. (2017) reported that inoculation with *A. brasilense* alone showed no effect on yield and production components of the wheat crop. However, the authors observed an increase in LNC relative to non-inoculated treatments. Hungria et al. (2010) observed a mean increase of 31% of treatments inoculated with strains Ab-V5 and Ab-V6 compared to non-inoculated treatments, which corroborates with the results obtained in the present study, in which the mean increase was 20%. According to the authors, the positive effects of inoculation were attributed to general increases in the macro-and micronutrient uptake by inoculated wheat plants. Also, Camilios et al. (2014) observed changes in the expression of genes involved in the transport of nutrients of wheat plants inoculated with *A. brasilense*, directly reflecting in the uptake of nutrients such as N.

Table 2
Leaf nitrogen content (g kg^{-1}) in the wheat cultivar CD 150 as a function of different forms of fertilization in experiments conducted in Londrina and Apucarana in the 2016 agricultural year

Treatments[1/]	Site	
	Apucarana	Londrina
T1) Absence of N topdressing	20.03 ± 0.96[1] Aa[2]	16.97 ± 1.22 Aa
T2) 30 kg ha^{-1} of N topdressing	20.08 ± 2.1 Aa	19.95 ± 1.75 Aa
T3) 60 kg ha^{-1} of N top-dressing	22.13 ± 1.18 Aa	19.07 ± 1.92 Aa
T4) *A. brasilense* Ab-V5 in the seeds	18.37 ± 2.7 Bb	24.41 ± 3.65 Aa
T5) *A. brasilense* Ab-V5 in post-emergence	20.91 ± 2.0 Aa	17.41 ± 1.57 Aa
T6) *A. brasilense* Ab-V5 + *Rhizobium* sp. 53GRM1 in the seeds	16.27 ± 0.4 Ab	17.32 ± 0.96 Aa
T7) *A. brasilense* Ab-V5 + *Rhizobium* sp. 53GRM1 in post-emergence	17.06 ± 4.5 Ab	18.37 ± 0.98 Aa
T8) Commercial inoculant (*A. brasilense* Ab-V5 + Ab-V6) in the seeds	26.86 ± 2.3 Aa	17.41 ± 1.22 Ba
T9) commercial inoculant (A. brasilense Ab-V5 + Ab-V6) in post-emergence	15.22 ± 1.25 Ab	19.16 ± 1.13 Aa

[1] Means ± standard deviation. [2] Means followed by the same uppercase letters in the row (site) and lowercase letters in the columns (treatments) constitute a statistically homogeneous group using the Scott and Knott (1974) test.

According to Cassán and Diaz-Zorita (2016), more than 104 biological products containing *Azospirillum* spp. are commercially available in South America alone and 64% of them are registered for wheat cultivation. Although most of these biofertilizers are recommended for inoculation via seed, the results presented in the present study demonstrate that post-emergence inoculation can be considered an efficient strategy in co-inoculation of *A. brasilense* and *Rhizobium* sp. in the wheat crop, bypassing the problems caused by the chemical treatment of seeds. Several other studies have reported higher efficiency of post-emergence inoculation methods, which was corroborated by the results obtained in the present study.

Conclusion

Co-inoculation with *A. brasilense* Ab-V5 + *Rhizobium* sp. 53GRM1 and inoculation with *A. brasilense* Ab-V5 + Ab-V6, both in post-emergence of seedlings, showed grain yield similar to the fertilization of 60 kg ha^{-1} of N topdressing, indicating that these technologies have the potential to replace topdressing nitrogen fertilization in the wheat crop.

References

Arnhold, E. (2013). Package in the R environment for analysis of variance and complementary analyses. *Brazilian Journal of Veterinary Research and Animal Science*, *50*(6), 488-492. doi: 10.11606/issn.1678-4456.v50i6p488-492

Bartlett, M. S. (1937). Properties of sufficiency and statistical tests. *Proceedings of the Royal Society of London. Series A-Mathematical and Physical Sciences*, *160*(901), 268-282. doi: 10.1098/rspa.1937.0109

Bremner, J. M., & Mulvaney, C. S. (1982*). Nitrogen total 1. Methods of soil analysis. Part 2. Chemical and microbiological properties (methods of soil an 2)*.

Camilios, D., Neto, Bonato, P., Wassem, R., Tadra-Sfeir, M. Z., Brusamarello-Santos, L. C., Valdameri, G.,... Pedrosa, F. O. (2014). Dual RNA-seq transcriptional analysis of wheat roots colonized by *Azospirillumbrasilense* reveals up-regulation of nutrient acquisition and cell cycle genes. *BMC Genomics*, *15*(1), 378. doi: 10.1186/1471-2164-15-378

Cassán, F., & Diaz-Zorita, M. (2016). *Azospirillum* sp. in current agriculture: from the laboratory to the field. *Soil Biology and Biochemistry*, *103*, 117-130. doi: 10.1016/j.soilbio.2016.08.020

Castro-Sowinski, S., Herschkovitz, Y., Okon, Y., & Jurkevitch, E. (2007). Effects of inoculation with plant growth-promoting rhizobacteria on resident rhizosphere microorganisms. *FEMS Microbiology Letters, 276*(1), 1-11. doi: 10.1111/j.1574-6968.2007.00878.x

Chen, J., Lü, S., Zhang, Z., Zhao, X., Li, X., Ning, P., & Liu, M. (2018). Environmentally friendly fertilizers: a review of materials used and their effects on the environment. *Science of the Total Environment, 613*, 829-839. doi: 10.1016/j.scitotenv.2017.09.186

Companhia Nacional de Abastecimento (2020). *Safra brasileira de grãos*. Recuperado de https://www.conab. gov.br/index.php/info-agro/safras/graos

Di Salvo, L. P., Ferrando, L., Fernández-Scavino, A., & Salamone, I. E. G. de. (2018). Microorganisms reveal what plants do not: wheat growth and rhizosphere microbial communities after Azospirillum brasilense inoculation and nitrogen fertilization under field conditions. *Plant and Soil, 424*(1-2), 405-417. doi: 10. 1007/s11104-017-3548-7

Ferreira, J. P., Nunes, R. F., Silva, R. B., Dal Bem, E. A., Garcia, D. P., Sabundjian, M. T., & Souza, F. M. L. de. (2017). *Azospirillum brasilense* via foliar e doses de nitrogênio em cobertura na cultura do trigo na região de Itapeva-SP/ spraying with *Azospirillum* on wheat leaf and nitrogen coverage rates in Itapeva-SP. *Revista Brasileira de Engenharia de Biossistemas*, *11*(2), 154-163. doi: 10.18011/bioeng2017v11n2p154-163

Fukami, J., Nogueira, M. A., Araujo, R. S., & Hungria, M. (2016). Accessing inoculation methods of maize and wheat with *Azospirillum brasilense*. *AMB Express*, *6*(1), 3. doi: 10.1186/s13568-015-0171-y

Galal Y.G.M., El-Ghandour I.A., El-Akel E.A. (2001) Stimulation of wheat growth and N fixation through *Azospirillum* and *Rhizobium* inoculation: A field trial with 15N techniques. In: W. J .Horst (Ed.), *Plant Nutrition*. Developments in Plant and Soil Sciences, vol 92. Springer, Dordrecht.

Galindo, F. S., Teixeira, M. C. M., Fº., Buzetti, S., Santini, J. M. K., Alves, C. J., & Ludkiewicz, M. G. Z. (2017). Wheat yield in the Cerrado as affected by nitrogen fertilization and inoculation with *Azospirillumbrasilense*. *Pesquisa Agropecuária Brasileira*, *52*(9), 794-805. doi: 10.1590/s0100-204x20170 00900012

Galindo, F. S., Teixeira, M. C. M., Fº., Buzetti, S., Santini, J. M. K., Boleta, E. H. M., & Rodrigues, W. L. (2020). Macronutrient accumulation in wheat crop (Triticum aestivum L.) with *Azospirillum brasilense* associated with nitrogen doses and sources. *Journal of Plant Nutrition, 43*(8), 1057-1069. doi: 10.1080/ 01904167.2020.1727511

Galindo, F. S., Teixeira, M. C. M., Fº., Tarsitano, M. A. A., Buzetti, S., Santini, J. M. K., Ludkiewicz, M. G. Z., & Alves, C. J. (2018). Technical and economic feasibility of irrigated wheat as a function of nitrogen doses, sources, and inoculation with *Azospirillum brasilense*. *Semina: Ciências Agrárias*, *39*(1), 51-66. doi: 10.5433/1679-0359.2018v39n1p51

Hoang, N. T., Tóth, K., & Stacey, G. (2020). The role of microRNAs in the legume–*Rhizobium* nitrogen-fixing symbiosis. *Journal of Experimental Botany, 71*(5), 1668-1680. doi: 10.1093/jxb/eraa018

Hungria, M., Araujo, R. S., Silva, E. B. Jr., & Zilli, J. É. (2017). Inoculum rate effects on the soybean symbiosis in new or old fields under tropical conditions. *Agronomy Journal*, *109*(3), 1106-1112. doi: 10.2134/agronj 2016.11.0641

Kavimandan, S. K. (1985). Root nodule bacteria to improve yield of wheat. *Plant and Soil*, *86*(1), 141-144. doi: 10.1007/BF02185034

Ladha, J. K., Tirol-Padre, A., Reddy, C. K., Cassman, K. G., Verma, S., Powlson, D. S.,... Pathak, H. (2016). Global nitrogen budgets in cereals: a 50-year assessment for maize, rice, and wheat production systems. *Scientific Reports*, *6*(1), 19355. doi: 10.1038/srep19355

Lugtenberg, B., & Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annual Review of Microbiology*, *63*(1), 541-556. doi: 10.1146/annurev.micro.62.081307.162918

Oliveira, A. L., Santos, O. J., Marcelino, P. R., Milani, K. M., Zuluaga, M. Y., Zucareli, C., & Gonçalves, L. S. (2017). Maize inoculation with *Azospirillum brasilense* Ab-V5 cells enriched with exopolysaccharides and polyhydroxybutyrate results in high productivity under low N fertilizer input. *Frontiers in Microbiology*, *8*, 1873. doi: 10.3389/fmicb.2017.01873

Ohyama T. (2017) The Role of Legume-Rhizobium Symbiosis in Sustainable Agriculture. In: S. Sulieman, & L S Tran. (Eds.), *Legume Nitrogen Fixation in Soils with Low Phosphorus Availability* (pp.1-20). Springer, Cham. doi: 10.1007/978-3-319-55729-8_1

Patil, A., Kale, A., Ajane, G., Sheikh, R., & Patil, S. (2017). Plant growth-promoting Rhizobium: mechanisms and biotechnological prospective. In A. Patil, A. Kale, G. Ajane, R. Sheikh, & S. Patil (Eds.), *Rhizobium biology and biotechnology* (pp. 105-134). Cham: Springer.

Pereira, L. C., Piana, S. C., Braccini, A. L., Garcia, M. M., Ferri, G. C., Felber, P. H.,... Dametto, I. B. (2017). Rendimento do trigo (*Triticum aestivum*) em resposta a diferentes modos de inoculação com *Azospirillum brasilense*. *Revista de Ciências Agrárias*, *40*(1), 105-113. doi: 10.19084/RCA16089

Piccinin, G. G., Braccini, A. L., Dan, L. G., Scapim, C. A., Ricci, T. T., & Bazo, G. L. (2013). Efficiency of seed inoculation with *Azospirillum brasilense* on agronomic characteristics and yield of wheat. *Industrial Crops and Products*, *43*, 393-397. doi: 10.1016/j.indcrop.2012.07.052

Pii, Y., Mimmo, T., Tomasi, N., Terzano, R., Cesco, S., & Crecchio, C. (2015). Microbial interactions in the rhizosphere: beneficial influences of plant growth-promoting rhizobacteria on nutrient acquisition process. A review. *Biology and Fertility of Soils*, *51*(4), 403-415. doi: 10.1007/s00374-015-0996-1

Scott, A. J., & Knott, M. (1974). A cluster analysis method for grouping means in the analysis of variance. *Biometrics*, *30*(3), 507-512. doi: 10.2307/2529204

Spolaor, L. T., Gonçalves, L. S. A., Santos, O. J. A. P. D., Oliveira, A. L. M. D., Scapim, C. A., Bertagna, F. A. B., & Kuki, M. C. (2016). Plant growth-promoting bacteria associated with nitrogen fertilization at topdressing in popcorn agronomic performance. *Bragantia*, *75*(1), 33-40. doi: 10.1590/1678-4499.330

Silva, S. R., Bassoi, M. C., & Foloni, J. S. S. (2017). *Informações técnicas para trigo e triticale-safra 2017.* Brasília: EMBRAPA Soja-Livro Técnico (INFOTECA-E). Recuperado de https://www.infoteca.cnptia.embrapa.br/infoteca/bitstream/doc/1064344/1/TecnicasparaTrigoeTriticaleSafra2017OL.pdf

Ul-Allah, S., Iqbal, M., Maqsood, S., Naeem, M., Ijaz, M., Ashfaq, W., & Hussain, M. (2018). Improving the performance of bread wheat genotypes by managing irrigation and nitrogen under semi-arid conditions. *Archives of Agronomy and Soil Science*, *64*(12), 1678-1689. doi: 10.1080/03650340.2018.1450974

Vaghar, M., & Ehsanzadeh, P. (2018). Comparative photosynthetic attributes of emmer and modern wheats in response to water and nitrogen supply. *Photosynthetica*, *56*(4), 1224-1234. doi: 10.1007/s11099-018-0825-5

Veresoglou, S. D., & Menexes, G. (2010). Impact of inoculation with *Azospirillum* spp. on growth properties and seed yield of wheat: a meta-analysis of studies in the ISI Web of Science from 1981 to 2008. *Plant and Soil*, *337*(1-2), 469-480. doi: 10.1007/s11104-010-0543-7

Vogel, G. F., Martinkoski, L., Jadoski, S. O., & Fey, R. (2015). Efeitos na combinação de *Azospirillum brasilense* com fungicidas no desenvolvimento de trigo. *Brazilian Journal of Applied Technology for Agricultural Science/Revista Brasileira de Tecnologia Aplicada nas Ciências Agrárias*, *8*(2), 73-80. doi: 10.5935/PAeT.V8.N3.08

Yang, C., Hamel, C., Vujanovic, V., & Gan, Y. (2011). Fungicide: modes of action and possible impact on nontarget microorganisms. *ISRN Ecology*, *2011*, 1-8. doi: 10.5402/2011/130289

Yanni, Y. G., Dazzo, F. B., Squartini, A., Zanardo, M., Zidan, M. I., & Elsadany, A. E. Y. (2016). Assessment of the natural endophytic association between *Rhizobium* and wheat and its ability to increase wheat production in the Nile delta. *Plant and Soil*, *407*(1-2), 367-383. doi: 10.1007/s11104-016-2895-0

Zeffa, D. M., Fantin, L. H., Koltun, A., Oliveira, A. L. de, Nunes, M. P., Canteri, M. G., & Gonçalves, L. S. (2020). Effects of plant growth-promoting rhizobacteria on co-inoculation with *Bradyrhizobium* in soybean crop: a meta-analysis of studies from 1987 to 2018. *PeerJ*, *8*, e7905. doi: 10.7717/peerj.7905

Zeffa, D. M., Fantin, L. H., Santos, O. J. A. P. D., Oliveira, A. L. M. D., Canteri, M. G., Scapim, C. A., & Gonçalves, L. S. A. (2018). The influence of topdressing nitrogen on *Azospirillum* spp. inoculation in maize crops through meta-analysis. *Bragantia*, *77*(3), 493-500. doi: 10.1590/1678-4499.2017273

Zeffa, D. M., Perini, L. J., Silva, M. B., Sousa, N. V. de, Scapim, C. A., Oliveira, A. L. M. de,... Goncalves, L. S. A. (2019). *Azospirillum brasilense* promotes increases in growth and nitrogen use efficiency of maize genotypes. *PloS One*, *14*(4), e0215332. doi: 10.1371/journal.pone.0215332