Azospirillum brasilense inoculation and nitrogen fertilization in a 3-year maize and black oat yield in succession

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ABSTRACT

The objective of this work was to assess the effects of *A. brasilense* inoculation on seeds on the yield performance of maize and black oat as a function of nitrogen rate over a 3-year succession. The experiment was conducted from 2015 to 2018 under field conditions. Maize was grown for three growing seasons in rotation with two seasons of black oat at the same experimental site. A randomized block design with a split-plot arrangement was used. Main plots consisted of *A. brasilense* inoculation (inoculated and uninoculated plants) and subplots of four nitrogen rates (0, 40, 80, and 120 kg ha^{-1}). Maize plants were evaluated for biometric parameters, yield, and yield components at flowering. Black oat plants were analyzed for dry matter yield at flowering. The inoculation of *A. brasilense* in maize and black oat seeds in succession does not promote productivity nor does it favor the nitrogen fertilization of these cultures.

Keywords: *Zea mays* L; *Avena strigosa;* nitrogen; biological nitrogen fixation.

INTRODUCTION

Beneficial interactions between plants and microorganisms may improve crop performance. Historically, one of the most successful examples of plant-microbial interactions is the positive effect of nitrogen-fixing bacteria of the genus *Bradyrhizobium* on legumes, particularly soybean (Siampitti & Salvagiotti, 2018). Studies examining the association between diazotrophic microorganisms and plants revealed that bacteria of the genus *Azospirillum* have plant growth-promoting activity on grasses (Cassán & Diaz-Zorita, 2016) and co-inoculation with *Bradyrhizobium* and *Azospirillum brasilense* increases the yield of legumes (Rego *et al.,* 2018).

Free-living facultative endophytes have both direct and indirect effects on plants, including improved water and nutrient absorption and enhanced resistance to stressful environments (Backer *et al.,* 2018). Several reports of the beneficial effects of plant growth-promoting bacteria on plant metabolism under abiotic stress and nutrient-poor conditions are known (Cassán *et al.,* 2013; Cassán & Diaz-Zorita, 2016; Furlan *et al.,* 2017; Zeffa *et al.,* 2018; Bulegon *et al.,* 2019; Fioreze *et al.,* 2020). These bacteria have the ability to identify signals emitted or received by stressed plants, triggering joint responses that enhance plant tolerance to numerous stresses (Cohen *et al.,* 2009).

Although *Azospirillum* bacteria can fixate nitrogen, this does not seem to be their main mechanism of action (Cassán *et al.,* 2013). Whereas some investigations indicated that inoculation offers the potential to reduce nitrogen fertilizer

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rates applied to maize crops (Fukami *et al.,* 2016; Leite *et al.,* 2019), studies on maize (Besen *et al.,* 2019) and wheat (Ribeiro *et al.,* 2018) in southern Brazil found little or no evidence that inoculation increases these crop yield. Field results are contradictory (Zeffa *et al.,* 2018), and, to date, it has not been possible to draw definite conclusions regarding the benefits of biological nitrogen fixation in cropping systems. The relationship between nitrogen-fixing bacteria and nitrogen supply to crops is controversial, especially in high-productivity systems.

Longitudinal studies of the interaction of *A. brasilense* with nitrogen fertilization can provide a better understanding of residual effects on subsequent crops. Given that the biological efficiency of microbial agents decreases at low temperatures (Kaushik *et al.,* 2001), it is especially important to assess plant–microbial interaction effects in colder, montane environments of southern Brazil. The objective of this work was to assess the effects of *A. brasilense* inoculation on seeds on the yield performance of maize and black oat as a function of nitrogen rate over a 3-year succession.

MATERIAL AND METHODS

The experiment was conducted under field conditions from 2015 to 2018 in southern Brazil. Maize was sown in the 2015/16, 2016/17, and 2017/18 growing seasons in rotation with black oat in the winters of 2016 and 2017. The experimental field is located at 27°16′26.55″S 50°30′14.11″W and 987 m of altitude. The experimental soil is classified as clayey Inceptisol (Soil Survey Staff, 2014). The climate is classified as Cfb in the Köppen–Geiger system, with average temperatures ranging from 15 to 25 °C and an average annual precipitation of 1500 mm (Climate-Data, 2021). Average temperatures and accumulated rainfall during the study period are in Figure 1.

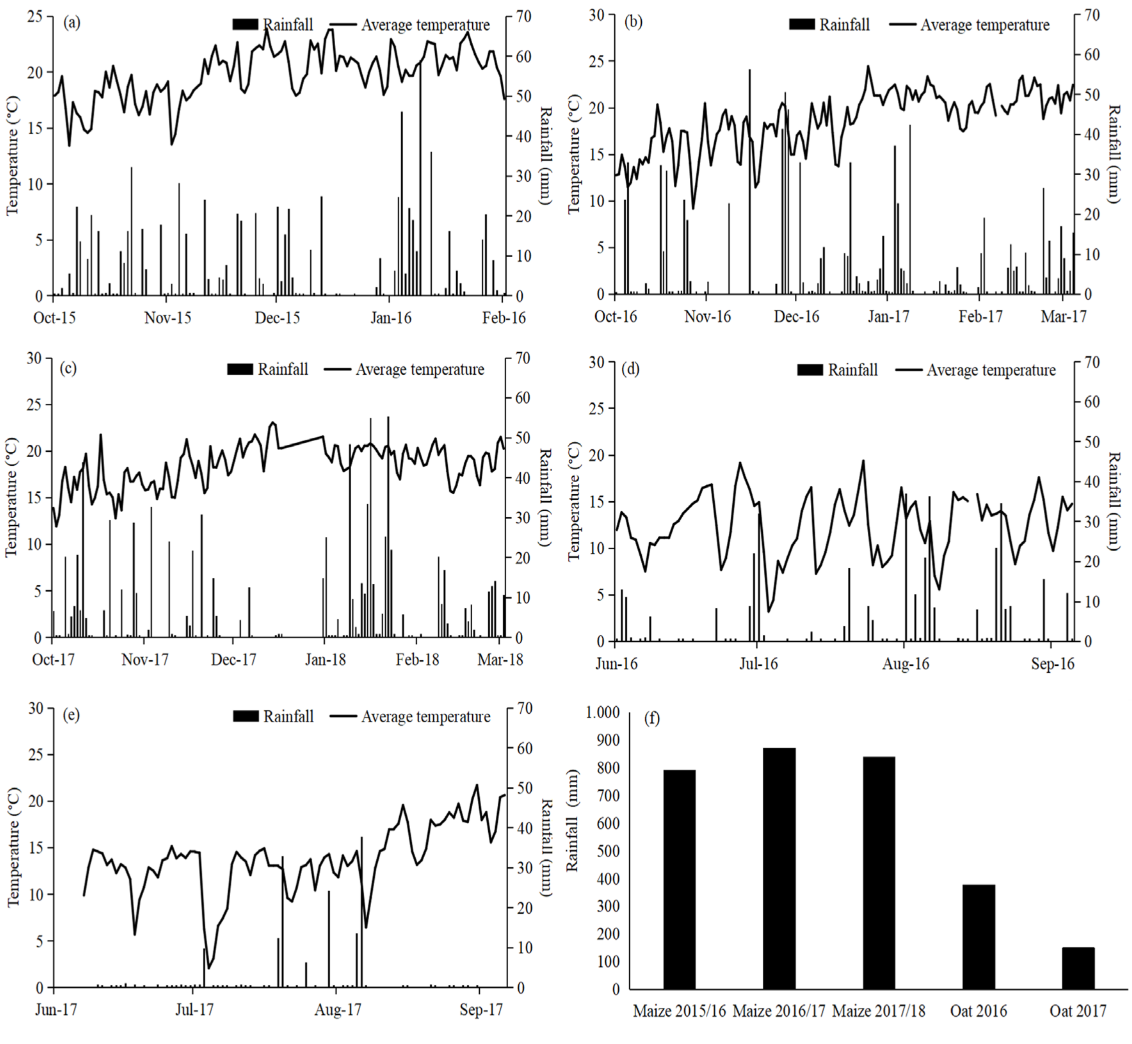

Figure 1: Average temperatures and rainfall for the (a) 2015/16, (b) 2016/17, and (c) 2017/18 maize seasons and the (d) 2016 and (e) 2017 black oat seasons, (f) Accumulated rainfall during each cropping season.

A split-plot randomized block design was used in all growing seasons to study the effect of treatments over time. Main plots were sown with inoculated or uninoculated seeds. Subplots consisted of an area of 12 m^2 (4 × 3 m), with four nitrogen rates (0, 40, 80, and 120 kg ha^{-1}) in four replications.

Seeds of hybrid maize cultivars NS 50 PRO, AG 9040, and AG 9025 PRO3 were sown on October 29, 2015, October 2, 2016, and October 4, 2017, respectively, at a density of 65,000 plants per hectare and at 0.5 m of rows spacing. Seeds of black oat cv. BRS 139 Neblina were sown at 80 kg seeds ha^{-1} on June 15, 2016, and June 21, 2017, in 0.17 m rows spacing. Crops were established in no-tillage with prior glyphosate desiccation.

Soil properties at the beginning of the experiment were as follows: organic matter, 49.6 g dm^{-3}; pH ($CaCl_2$ 1:1), 5.9; Ca, 10.2 $cmol_c$ dm^{-3}; Mg, 3.1 $cmol_c$ dm^{-3}; Al, not detected (0.0 $cmol_c$ dm^{-3}); K, 0.18 $cmol_c$ dm^{-3}; P (extracted with Mehlich^{-1} solution), 20.8 mg dm^{-3}; and base saturation, 82%. A basal fertilization with 00-18-18 NPK was applied at 280 kg ha^{-1} before maize and black oat sowing, according to the local fertilizer recommendation manual (CQFS-RS/SC, 2004). Nitrogen fertilization maize was applied one-third at seedling emergence and two-thirds when plants were at the V3–V4 stage and at tillering of black oats (CQFS-RS/SC, 2004), using urea (45% nitrogen) as the nitrogen source.

Before maize and black oat sowing, seeds were treated with a commercial preparation of *A. brasilense* (AzoTotal®, Total Biotecnologia Indústria e Comércio Ltd., Curitiba, Paraná, Brazil) at 100 mL ha^{-1}, as per the manufacturer's instructions. The product is a suspension of *A. brasilense* strains AbV5 and AbV6 at 200 × 10^6 cells/mL. Inoculation was performed manually, and seeds were sown immediately after.

Maize plants in the flowering stage (Ritchie *et al.,* 1993) were evaluated for stem diameter (measured at the second internode), plant height, and leaf nitrogen content (except for the first crop). Total nitrogen was determined in the leaf opposite to the main ear by the semi-micro Kjeldahl method (Tedesco *et al.,* 1995). At the end of the crop cycle, maize plants were evaluated for ear height and yield components (ear length, number of rows per ear, number of grains per row, and number of grains per year) in 10 ears randomly collected from each subplot. The central area of subplots (6 m^2) was then harvested for determination of thousand grain weight and grain yield at 14% moisture. Black oat plants at full flowering were sampled from an area of 0.25 m^2, dried in a forced-air oven, and analyzed for shoot dry matter yield.

Data were subjected to analysis of variance (*F*-tests) at $p < 0.05$. When significant effects were detected, Student's *t*-test was used to assess differences between inoculation treatments ($p < 0.05$) and polynomial regression analysis was used to describe the effects of nitrogen fertilizer rates. All statistical analyses were performed using Sisvar (Ferreira, 2011).

RESULTS AND DISCUSSION

Maize height and leaf nitrogen content increased linearly with increasing nitrogen rates (Tables 1, 2, and 3). The same relationship was observed for maize yield components and grain yield during the three cropping cycles and for black oat dry matter yield during the two cropping cycles (Table 2, 3 and 4).

Although the nitrogen rates used in the current study could be optimized for increased fertilization efficiency, it was clear that nitrogen fertilization exerted positive effects on maize yield, in agreement with extensive previous research (Morris *et al.,* 2018). Nitrogen rates of 80 and 40 kg ha^{-1} resulted in adequate leaf nitrogen contents in the 2016/17 and 2017/18 cropping seasons, respectively. However, to maximize yields, it is recommended to maintain leaf nitrogen contents between 35 and 40 g kg^{-1} (Gott *et al.,* 2014), which was obtained using rates of 120 and 80 kg N ha^{-1} in the 2016/17 and 2017/18 seasons, respectively.

It was observed that maize grain yield and leaf nitrogen content increased not only as a function of nitrogen rates but also as a function of time. These results can be explained by differences in the yield potential of maize cultivars or by the cumulative effects of nitrogen fertilization over five growing seasons or by residues from previous crops provided nutrients and organic carbon to the soil over time (Stewart *et al.,* 2016). Black oat showed an increase in dry matter yield in both seasons (Table 4), even though accumulated rainfall amounts were lower in the 2017 season (Fig. 1f). It should be noted that maize cultivars differed between the three seasons, whereas the same black oat cultivar was used in the two seasons.

Table 1: Biometric parameters and yield components of maize (cv. NS 50 PRO) as a function of seed inoculation with *Azospirillum brasilense* and nitrogen fertilization in the 2015/16 cropping season

Treatment	**SD (mm)**	**PH (cm)**	**EH (cm)**
Inoculation			
Inoculated plants	19.74	1.95 a	0.85 a
Uninoculated plants	18.37	1.83 b	0.78 b
p-value	0.08	0.01	0.00
LSD	1.57	0.08	0.03
Nitrogen rate			
0 kg ha^{-1}	17.03	1.70	0.70
40 kg ha^{-1}	21.37	1.74	0.93
80 kg ha^{-1}	17.84	2.04	0.69
120 kg ha^{-1}	19.98	2.07	0.95
p-value	0.04	0.00	0.00
Best-fit equation	ns	$y = 0.0035x + 1.6771$	ns
R^2	ns	0.86**	ns
Inoculation × N fertilization (*p*-value)	0.97	0.48	0.31
CV_a (%)	14.49	5.13	13.56
CV_b (%)	10.71	5.46	5.77
Treatment	NRE	NGR	TGW (g)
Inoculation			
Inoculated plants	14.05 a	33.06 a	325.39
Uninoculated plants	13.49 b	30.94 b	319.21
p-value	0.01	0.03	0.55
LSD	0.42	1.94	21.73
Nitrogen rate			
0 kg ha^{-1}	13.20	27.8	299.5
40 kg ha^{-1}	13.55	30.6	313.3
80 kg ha^{-1}	14.03	33.0	330.6
120 kg ha^{-1}	14.31	36.6	345.7
p-value	0.00	0.00	0.01
Best-fit equation	$y = 0.0095x + 13.2$	$y = 0.0725x + 27.645$	$y = 0.3898x + 298.91$
R^2	0.99**	0.99**	0.99**
Inoculation × N fertilization (*p*-value)	0.52	0.78	0.45
CV_a (%)	3.03	9.28	6.72
CV_b (%)	3.91	7.85	8.75

SD, stem diameter; PH, plant height; EH, ear height; NRE, number of rows per ear; NGR, number of grains per row; TGW, thousand grain weight; *p*-value, *F*-test probability value; LSD, least significant difference at $p < 0.05$; R^2, coefficient of determination; ns, not significant; **, significant at $p < 0.01$; CV_a, coefficient of variation for plots (seed inoculation); CV_b, coefficient of variation for subplots (nitrogen rates). Means followed by the same letter do not differ by the Student test ($p < 0.05$).

Table 2: Biometric parameters, yield, and yield components of maize (cv. AG 9040) as a function of seed inoculation with *Azospirillum brasilense* and nitrogen fertilization in the 2016/17 cropping season

Treatment	**SD (mm)**	**PH (cm)**	**EH (cm)**	**N_L (g kg^{-1})**
Inoculation				
Inoculated plants	22.73	183.20 b	93.28 b	27.81
Uninoculated plants	22.82	185.20 a	93.81 a	27.12
p-value	0.65	0.04	0.05	0.89
LSD	0.52	1.78	0.51	14.70
Nitrogen rate				
0 kg ha^{-1}	17.96	170.16	86.89	17.85
40 kg ha^{-1}	21.36	180.51	90.50	24.26
80 kg ha^{-1}	23.29	187.71	95.50	30.68
120 kg ha^{-1}	28.48	198.44	101.30	37.09
p-value	0.00	0.00	0.00	0.00
Best-fit equation	$y = 0.0837x + 17.75$	$y = 0.2301x + 170.4$	$y = 0.1206x + 86.31$	$y = 0.1603x + 17.85$
R^2	0.97**	0.99**	0.99**	0.99**
Inoculation × N fertilization (*p*-value)	0.48	0.22	0.08	0.89
CV_a (%)	2.01	0.86	0.48	47.58
CV_b (%)	2.02	1.00	1.07	19.88
Treatment	NRE	NGR	TGW (g)	Yield (kg ha^{-1})
Inoculation				
Inoculated plants	15.13	29.1 a	284.9 a	7390.9
Uninoculated plants	14.98	27.3 b	275.8 b	7015.1
p-value	0.29	0.04	0.04	0.65
LSD	0.38	1.60	8.20	2359.2
Nitrogen rate				
0 kg ha^{-1}	14.3	19.9	281.0	3579.4
40 kg ha^{-1}	15.3	27.3	274.6	5880.8
80 kg ha^{-1}	15.2	32.2	284.5	9523.4
120 kg ha^{-1}	15.5	33.5	281.4	9828.5
p-value	0.00	0.00	0.87	0.00
Best-fit equation	$y = 0.0086x + 14.54$	$y = 0.1146x + 21.3$	ns	$y = 55.975x + 3844.5$
R^2	0.72**	0.92**	ns	0.92**
Inoculation × N fertilization (*p*-value)	0.65	0.72	0.38	0.36
CV_a (%)	2.21	5.18	2.63	29.11
CV_b (%)	3.42	10.74	8.69	22.05

SD, stem diameter; PH, plant height; EH, ear height; N_L, leaf nitrogen content; NRE, number of rows per ear; NGR, number of grains per row; TGW, thousand grain weight; *p*-value, *F*-test probability value; LSD, least significant difference at $p < 0.05$; R^2, coefficient of determination; ns, not significant; **, significant at $p < 0.01$; CV_a, coefficient of variation for plots (seed inoculation); CV_b, coefficient of variation for subplots (nitrogen rates). Means followed by the same letter do not differ by the Student test ($p < 0.05$).

Table 3: Biometric parameters, yield, and yield components of maize (cv. AG 9025 PRO3) as a function of seed inoculation with *Azospirillum brasilense* and nitrogen fertilization in the 2017/18 cropping season

Treatment	**SD (mm)**	**PH (cm)**	**EH (cm)**	**N_L (g kg^{-1})**
Inoculation				
Inoculated plants	25.64	2.44	1.00	36.86
Uninoculated plants	25.19	2.44	0.98	32.84
p-value	0.79	0.98	0.66	0.28
LSD	4.97	0.23	0.12	9.70
Nitrogen rate				
0 kg ha^{-1}	21.83	1.98	0.77	22.59
40 kg ha^{-1}	25.06	2.41	1.03	30.76
80 kg ha^{-1}	25.65	2.59	1.08	38.93
120 kg ha^{-1}	29.11	2.77	1.07	47.10
p-value	0.00	0.00	0.00	0.00
Best-fit equation	$y = 0.0561x + 22.04$	$y = 0.0064x + 2.06$	$y = 0.0024x + 0.85$	$y = 0.2043x + 22.59$
R^2	0.94**	0.95**	0.70**	0.96
Inoculation × N fertilization (*p*-value)	0.81	0.95	0.81	0.60
CV_a (%)	17.39	8.23	10.98	24.75
CV_b (%)	6.93	5.83	7.88	20.07
Treatment	NRE	NGR	TGW (g)	Yield (kg ha^{-1})
Inoculation				
Inoculated plants	13.81	29.68	396.1	7996.8
Uninoculated plants	13.98	28.96	368.1	7379.3
p-value	0.24	0.69	0.32	0.38
LSD	0.37	5.31	74.70	1892.6
Nitrogen rate				
0 kg ha^{-1}	11.94	19.56	313.8	2421.4
40 kg ha^{-1}	14.08	29.00	340.4	6148.7
80 kg ha^{-1}	14.93	33.35	426.7	9608.1
120 kg ha^{-1}	14.63	35.37	447.6	12573.9
p-value	0.00	0.00	0.00	0.00
Best-fit equation	$y = 0.0223x + 12.55$	$y = 0.1294x + 21.55$	$y = 1.2189x + 308.9$	$y = 84.792x + 2600.5$
R^2	0.73**	0.90**	0.94**	0.99**
Inoculation × N fertilization (*p*-value)	0.86	0.48	0.94	0.43
CV_a (%)	2.36	16.10	17.38	21.88
CV_b (%)	6.45	9.18	14.19	18.94

SD, stem diameter; PH, plant height; EH, ear height; N_L, leaf nitrogen content; NRE, number of rows per ear; NGR, number of grains per row; TGW, thousand grain weight; *p*-value, *F*-test probability value; LSD, least significant difference at $p < 0.05$; R^2, coefficient of determination; **, significant at $p < 0.01$; CV_a, coefficient of variation for plots (seed inoculation); CV_b, coefficient of variation for subplots (nitrogen rates). Means followed by the same letter do not differ by the Student test ($p < 0.05$).

Table 4: Dry matter yield of black oat (cv. BRS 139) as a function of seed inoculation with *Azospirillum brasilense* and nitrogen fertilization in the 2016 and 2017 seasons

Treatment	**Dry matter yield (Mg ha^{-1})**	
	2016	**2017**
Inoculation		
Inoculated plants	3.68	5.40
Uninoculated plants	3.81	5.89
p-value	0.67	0.33
LSD	0.83	1.32
Nitrogen rate		
0 kg ha^{-1}	2.23	4.08
40 kg ha^{-1}	3.18	4.88
80 kg ha^{-1}	5.08	6.27
120 kg ha^{-1}	4.49	7.34
p-value	0.00	0.02
Best-fit equation	$y = 0.0217x + 2.44$	$y = 0.0279x + 3.97$
R^2	0.76**	0.99**
Inoculation × N fertilization (*p*-value)	0.68	0.89
CV_a (%)	19.84	20.78
CV_b (%)	32.82	33.85

p-value, *F*-test probability value; LSD, least significant difference at $p < 0.05$; R^2, coefficient of determination; **, significant at $p < 0.01$; CV_a, coefficient of variation for plots (seed inoculation); CV_b, coefficient of variation for subplots (nitrogen rates).

Inoculation of *A. brasilense* in maize seeds had different effects on the morphological parameters of maize throughout the three growing seasons (Tables 1, 2, and 3). In the 2015/16 season, inoculation increased plant and ear height, in agreement with the results of a previous study on maize grown under the same soil–climatic conditions (Besen *et al.,* 2019). However, in the 2016/17 season, these parameters were lower plants that received *A. brasilense*. In the following season (2017/18), none of the morphological parameters were affected by *A. brasilense* inoculation. Leaf nitrogen content was not affected by seed inoculation in any season. Although *A. brasilense* increased the number of rows per ear and the number of grains per row in the 2015/16 harvest, grain yield and other yield components were not influenced by the inoculation. The lack of inoculation effects on grain yield and yield components was also observed in 2016/17 and 2017/18. The dry matter yield of black oats did not show a positive response to seed inoculation with *A. brasilense* in either growing season (Table 4).

The reported effects of seed inoculation with *A. brasilense* vary greatly. It is well accepted that seed inoculation promotes beneficial physiological effects, increasing plant dry matter and yield components, but exerts little or no effects on grain yield (Marini *et al.,* 2015; Alves *et al.,* 2017; Besen *et al.,* 2019; Souza *et al.,* 2019; Quatrin *et al.*, 2019; Fioreze *et al.,* 2020). The physiological responses are related to stimulation and secretion of growth hormones and not necessarily to biological nitrogen fixation (Cassán & Diaz-Zorita, 2016; Pii *et al.,* 2019). Such effects, therefore, may explain the increase in some yield components with microbial inoculation observed in the present study. The lack of positive effects on maize growth and yield in most seasons might be associated with the various biotic and abiotic factors affecting crop yield, as well as the interaction of bacteria with the maize cultivars used.

Most studies on the effects of *A. brasilense* inoculation were conducted using summer crops such as maize including studies that obtained positive results (Cassán & Diaz-Zorita, 2016). Few studies assessed the effects of this beneficial bacterium on winter cereals. Correa Filho *et al.*

(2017) found that inoculation of black oat seeds with *A. brasilense* favored crop yield with comparable efficiency to nitrogen fertilization. Inoculation of dual-purpose wheat promoted an increase in dry mass production but did not influence grain yield in two consecutive crops (Quatrin *et al.,* 2019). Inoculation with *A. brasilense* and *Pseudomonas fluorescens* increased root and shoot weights but not the yield of wheat (Naiman *et al.,* 2009). Ribeiro *et al.* (2018) found no effect of seed inoculation on the grain yield of wheat; the highest yields were obtained using nitrogen fertilization without inoculation.

In the present study, a lack of effect of microbial inoculation was observed even in the absence of nitrogen fertilization during the five growing seasons, contradicting many studies found in the literature. For wheat crops, for example, *A. brasilense* inoculation exerts positive effects on yield in about 10% of experiments, although there seems to be a pattern of positive responses in nutrient-restricted environments for several wheat species (Cassán & Diaz-Zorita, 2016).

It seems that inoculation efficiency may increase with decreasing nitrogen rates; however, literature results are not entirely conclusive. Fukami *et al.* (2016) observed a 25% reduction in the nitrogen requirements of maize inoculated with *A. brasilense* under field conditions. The experiment was conducted in three locations but without repetitions in different seasons, precluding analysis of the cumulative effects of treatments over time. Thus, the 25% reduction in nitrogen requirements might have been associated with a greater utilization of nitrogen and other soil minerals as a response to the increase in root surface. Coelho *et al.* (2017) observed that the interaction between maize and *A. brasilense* did not increase soil nitrogen content after harvest, evidence of the low nitrogen fixation efficiency of the bacterium. In the long term, a low biological nitrogen fixation potential can lead to reductions in soil nitrogen stocks.

The lack of significant effects on crop yield after three years of rotation shows that the interaction between bacteria and crops was not effective under the studied climatic conditions. Bacteria of the genus *Azospirillum* have optimum growth and activity at 37 °C (Tripathi & Klingmuller, 1992) and, therefore, probably have reduced efficiencies in cold, montane regions. The fact that Fukami *et al.* (2016) and Ribeiro *et al.* (2018) also observed a low efficiency of *A. brasilense* inoculation in wheat under Cfb climate conditions indicates that the bacterium may be affected by low temperatures. For wheat crops, the use of cultivars adapted to cold climates increased plant interactions with *A. brasilense* (Kaushik *et al.,* 2001). This relationship, however, should be further investigated, particularly in summer crops.

CONCLUSION

Seed inoculation with *A. brasilense* did not influence the yield performance of maize or black oat over a 3-year rotation, regardless of the nitrogen rate applied. On the other hand, nitrogen fertilization until 120 kg N ha^{-1} linearly improved maize and black oat yield.

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