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Genetic bases of corn inoculated with *Azospirillum brasilense* via seed and foliar application

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ABSTRACT. Nitrogen is available in insufficient quantities in Brazilian soils, and is the nutrient that raises the most cost of production of maize. Nowadays there is a more interest in the use of alternative nitrogen sources, such as biological nitrogen fixation, to supply plant demand. Among the most used bacteria in association with grasses is *Azospirillum brasilense*. In this way this study aimed to establish a relationship between the genetic bases of corn and the forms of *A. brasilense* application and the effects on the plant morphological components, grain yield, and relative nitrogen contribution. Experiments were conducted during the 2013/14 and 2014/15 crop seasons. The experiments included two single hybrids, two double hybrids, and two triple hybrids inoculated with *A. brasilense* via seed treatment; foliar application during phenological stages V2, V2 + V4, and V4; and a control treatment without bacteria. The response varied with the hybrid and the year. In the 2013/14 crop, a significant difference was observed in the grain yield for the single hybrid AG9045 inoculated with *A. brasilense* via seed and foliar application in V2. In the 2014/15 crop, inoculation with *A. brasilense* via seed application resulted in significant increases in the grain yield for the triple hybrid BG7051. Foliar application of *A. brasilense* is an efficient alternative for the improvement of plant morphological traits, and inoculation via seed and with foliar application in V2 increases the relative contribution of N.

Keywords: *Zea mays* L.; biological nitrogen fixation; diazotrophic bacteria; foliar application.

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Introduction

Nitrogen (N) is the most important mineral element and a limiting element of the corn crop yield (*Zea mays* L.). N directly participates in the biosynthesis of proteins and chlorophylls. The management of N is complex due to its dynamics in the soil, which makes it the most expensive element of the production system (Bastos, Cardoso, Melo, Ribeiro, & de Andrade Júnior, 2008); for instance, 28 kg of N are estimated to be required for the production of a ton of corn grain. Thus, the economic and environmental costs related to nitrogen fertilization have stimulated the search for alternatives that can decrease the use of this fertilizer without reducing grain production (Dotto, Lana, Steiner, & Frandoloso, 2010).

One possible method to enable production with a lower cost and less environmental impact is to use the genetic potential of plants in combination with soil biological resources, such as diazotrophic bacteria (Fukami, Cerezini, & Hungria, 2018). Yield increases in the field have been obtained with these bacteria, which are found in association with grasses and contribute to biological nitrogen fixation (BNF) (Verma, Yadav, Tiwari, & Lavakush, 2012).

Plant-bacterium interactions can occur in the rhizosphere, in which plant growth-promoting bacteria (PGPB) are attracted to root exudates (Compant, Clément, & Sessitsch, 2010). The exudate composition depends on the soil type, nutrient availability, genotype, and biotic and abiotic factors. Additionally, changes in the patterns along the radicular system result in differences in the exudate composition and the associated bacterial communities (Zhang, Subramanian, Stacey, & Yu, 2009).

Among the bacterial groups associated with the roots, the endophytic diazotrophic bacteria have an advantage due to their ability to colonize the interior of the plant roots and establish niches that offer more suitable conditions for effective nitrogen fixation and later transfer to the host plant (Reinhold-Hurek & Hurek, 2011). The diazotrophic microorganisms found in grass roots include species of the genus *Azospirillum* (Ilyas & Bano, 2010), which exist naturally in most soils (Ardakani, Mazaheri, Mafakheri, & Moghaddam, 2011).

Azospirillum sp. can be inoculated in plants of agricultural interest to stimulate growth via multiple mechanisms, including the synthesis of plant hormones, improved nitrogen nutrition, stress mitigation, and the biological control of pathogenic microbiota (Bashan & De-Bashan, 2010). The BNF in the plant during the plant-bacterium association is the process by which the bacteria provide fixed N (Chubatsu et al., 2012), which partially meets the corn crop demand; thus, the inoculant does not fully replace nitrogen fertilization but promotes better absorption and utilization of soil N (Saubidet, Fatta, & Barneix, 2002). To obtain economic returns with the crop, nitrogen supplementation is essential using a dose of N that provides good plant development and does not hinder the BNF (Oliveira, Silva, de Arruda, do Nascimento, & Alves, 2003).

*A. brasilense*is typically applied via seed treatment. Bacterial inoculation via foliar application is an effective alternative for different corn hybrids, but the conditions in which the bacterium can promote more benefits for the plants must be identified (Martins et al., 2012). *A. brasilense* inoculation is an inexpensive technology with a low environmental impact. However, its technical recommendation needs to be improved by taking into account the level of investment in the farm, among other factors (Sangoi et al., 2015).

The beneficial activity of bacteria is well established (Fukami et al., 2018). However, the interaction with different genotypes still needs to be researched because this interaction plays an important role in bacterial colonization. Thus, the study of corn hybrids with a positive agronomic response to inoculation is required (Quadros et al., 2015). The alternatives are regional studies using commercial hybrids with an aim of selecting hybrids that present good adaptability and stability prior to their recommendation (Cardoso et al., 2009).

Regarding hybrids, breeding studies have revealed an average superiority of single hybrids compared to triple and double hybrids (Arnhold, Pacheco, de Carvalho, Silva, & de Oliveira Júnior, 2010). Differences were found in experiments with early hybrids, with single hybrids showing superior performances compared to double and triple hybrids, thus highlighting the importance of heterosis in these populations (Silva & Miranda Filho, 2003).

The main barrier for the use of *A. brasilense* in the corn crop is the inconsistency of research results, which can vary according to the soil and climatic conditions, the study methodology, and the genetic basis (Bartchechen, Fiori, Watanabe, & Guarido, 2010). Thus, this study aimed to establish the relationship between the genetic bases of corn and the forms of *A. brasilense* application and the effects on the plant morphological components, grain yield, and relative nitrogen contribution.

Material and methods

The experiments were set up in the field under a direct sowing system during the 2013/14 and 2014/15 crop seasons in the Experimental Farm of the Plant Science Department at the Federal University of Santa Maria (UFSM) in Santa Maria, state of Rio Grande do Sul, Brazil. The region is subtropical with a temperate rainy climate and an annual average temperature of 19.4°C (Heldwein, Buriol, & Streck, 2009) and is classified as Cfa (humid subtropical) according to the Köppen classification (Peell, Finlayson, & Mcmahon, 2007).

The experimental soil is classified as sandy loam Typic Paleudalf (Embrapa, 2013) with the following characteristics at depths of 0 to 0.20 m: for the 2013/14 crop season: clay, 23% weight per volume (w/v); Shoemaker, Maclean, and Pratt (SMP) index, 5.5; P, 17.25 mg dm^{-3}; K, 84 mg dm^{-3}; and organic matter (OM), 2.2% w/v; for the 2014/15 crop season: clay, 24% w/v; SMP index, 6.0; P, 12.6 mg dm^{-3}; K, 108.0 mg dm^{-3}; and OM, 2.4% w/v. To correct the soil fertility, 250 kg ha^{-1} of formula 0-23-30 (N-P-K) was applied to both crops (Comissão de Química e Fertilidade do Solo, 2004).

In both crops, the experiments were composed of six commercial hybrids, including two single (SH), two double (DH), and two triple (TH) hybrids. The hybrids were inoculated with *A. brasilense* (strains AbV5 + AbV6 with a guarantee of 2 x 10^8 CFU mL^{-1}) via seed treatment (ST) for a dose of 2.50 mL kg^{-1} of seed and via foliar application in phenological stages V2 and V4 or as a combination application in V2 and V4 (V2 + V4) (Ritchie, Hanway, & Benson, 1993), for 300 mL ha^{-1} dose; the experiment also included a control treatment (without bacteria).The hybrids used in the 2013/14 crop were SH AG9045, SH AG8025, DH AG2040, DH

AG1051, TH AG5011, and TH AG8011, whereas the hybrids used in the 2014/15 crop were SH AG9045, SH AG8025, DH AG2040, DH Feroz, TH BG7051, and TH BG7060. The experimental design was randomized blocks with four replicates. Each experimental unit consisted of two seven-meter rows with half-meter margins on each end. The spacing between the rows was 0.45 m, and three plants were placed per linear meter for a total estimated population of 66,666 plants ha^{-1}.

The crop management and practices followed the technical recommendations for corn crops. The applications were made using a backpack sprayer with flat fan spray tips spaced 0.50 m apart with an operating pressure of 30 psi, which provided a spray solution flow rate of 200 L ha^{-1}.

In stage R1 (blooming), the leaf chlorophyll index was estimated using the portable ClorofiLOG® chlorophyll meter (model CFL 1030 - Falker). In this stage, the leaf area index (LAI) was determined by measuring the lengths and widths of all leaves of five randomly selected plants from the useful area of each plot with a ruler.

The harvest was performed at stage R6 of physiological plant maturity. The numbers of total plants and ears harvested were counted to obtain the final population and the number of ears per plant. Ten ears were used to perform the ear length measurement and to count the grains per row and the number of rows per ear to estimate the number of grains per ear. After the ears were threshed and classified, we determined the thousand-grain weight and plot weight after correcting the humidity to 13%; the value was converted to kg ha^{-1} to estimate the grain yield.

From the observation of the interaction between the hybrids and the treatment applications, the relative contribution of N (kg ha^{-1}) was estimated as described by Dourado Neto and Detomini (2005) as the amount of N required to increase the grain yield relative to the control treatment. The resulting accumulated N was converted into dollars ($) based on the current price of one kg of N (US$ 8.01) (CONAB, 2016) as follows:

$$QN = \frac{YD}{HI.EF}\,[GPC.PNC.HI + (1 - HI).NCOP].(1 - SN)$$

where: YD refers to the difference in the corn yield for treatments with and without inoculation, HI refers to the harvest index (0.5) (Doorembos & Kassam, 1994), GPC refers to the grain protein content (0.12) (Oliveira, Chaves, Duarte, Brasil, & Ribeiro, 2007), PNC refers to the protein nitrogen content (0.17) (Dourado Neto & Detomini, 2005), NCOP refers to the nitrogen content in other plant parts (0.01) (Dourado Neto & Detomini, 2005), SN refers to the soil nitrogen (0.6) (Urquiaga & Zapata, 2000), and EF refers to the efficiency of nitrogen utilization by the plants (0.42) (Urquiaga & Zapata, 2000).

The experimental data were subjected to analysis of variance (F-test), and the means of the treatments were compared with the Scott-Knott test using the statistical program SISVAR (Ferreira, 2011) with a significance level of 5%.

Results and discussion

In both crops, a significant effect of the interaction of the hybrids with the application forms of *A. brasilense* was detected for some of the analyzed variables. No significant association was detected between the bacterium and the genetic basis of the evaluated hybrids, which was similar to the results of other studies (Bertolini, Gamero, Salata, & Piffer, 2008).

In the experiments conducted during the first crop season, the leaf area index (LAI) was significant only for double hybrid (DH) AG1051, which presented an increase of 1 m² with the application of *A. brasilense* during V4 (Figure 1a). During the 2014/15 crop season, the double hybrids DH AG2040 and DH Feroz and the single hybrid (SH) AG9045 responded to inoculation of *A. brasilense* in the seed without significant differences compared to the control treatment (Figure 2a). The foliar applications during V2 and the combination V2 + V4 treatment increased the LAI of the triple hybrid (TH) BG7051 and SH AG8025.

The LAI of the plant depends on the spacing, number and size of the leaves, stage of plant development, soil fertility, climate conditions, and genetic material (Fancelli & Dourado Neto, 2000). In this study, the LAI increased with *A. brasilense* application. Thus, plants inoculated with the bacterium have a greater ability to explore the space available for their development and exhibit increased photosynthetic activity. The increases in the LAI are larger in plants that reach the maximum LAI more quickly and have leaf areas that remain active for a longer period of time.

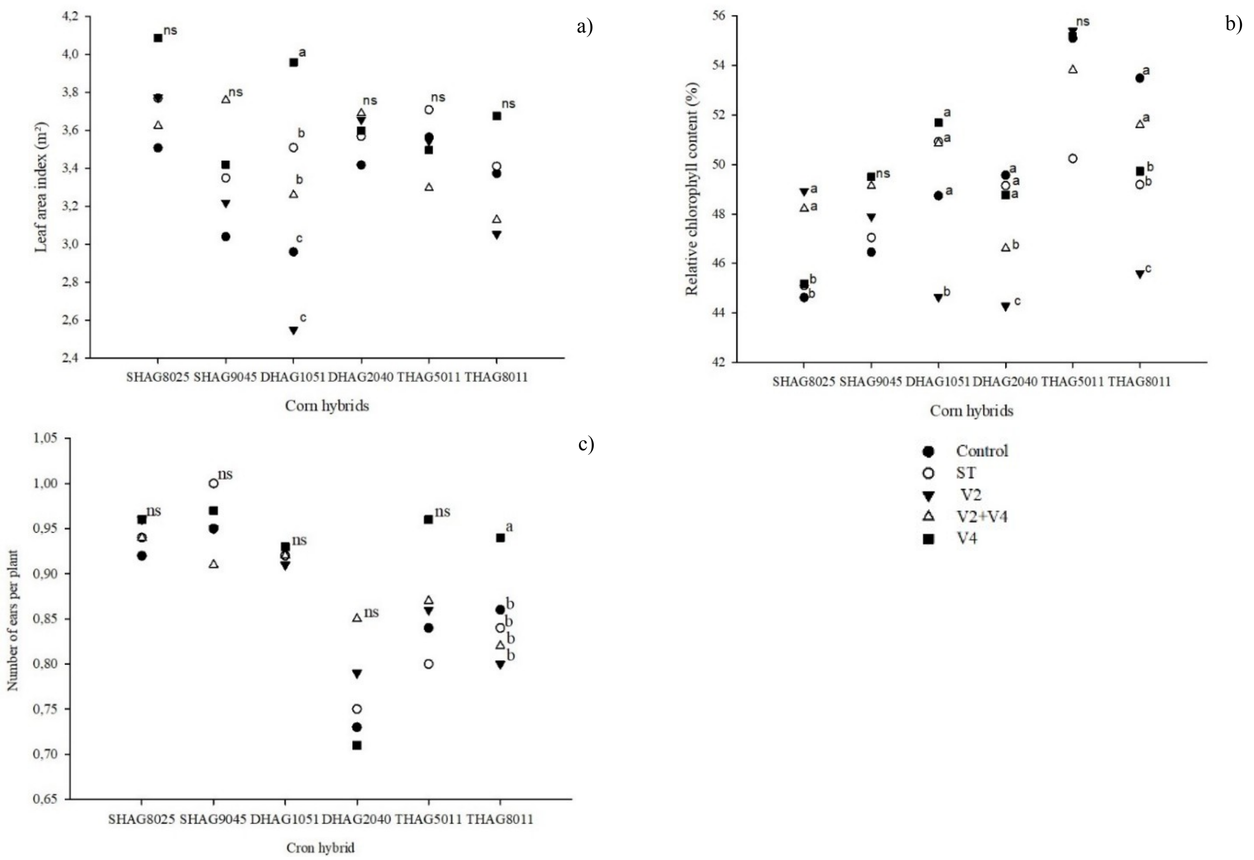

Figure 1. Leaf area index (a), relative chlorophyll content (b), and number of ears per plant (c) of corn inoculated with *A. brasilense* in the 2013/14 crop.

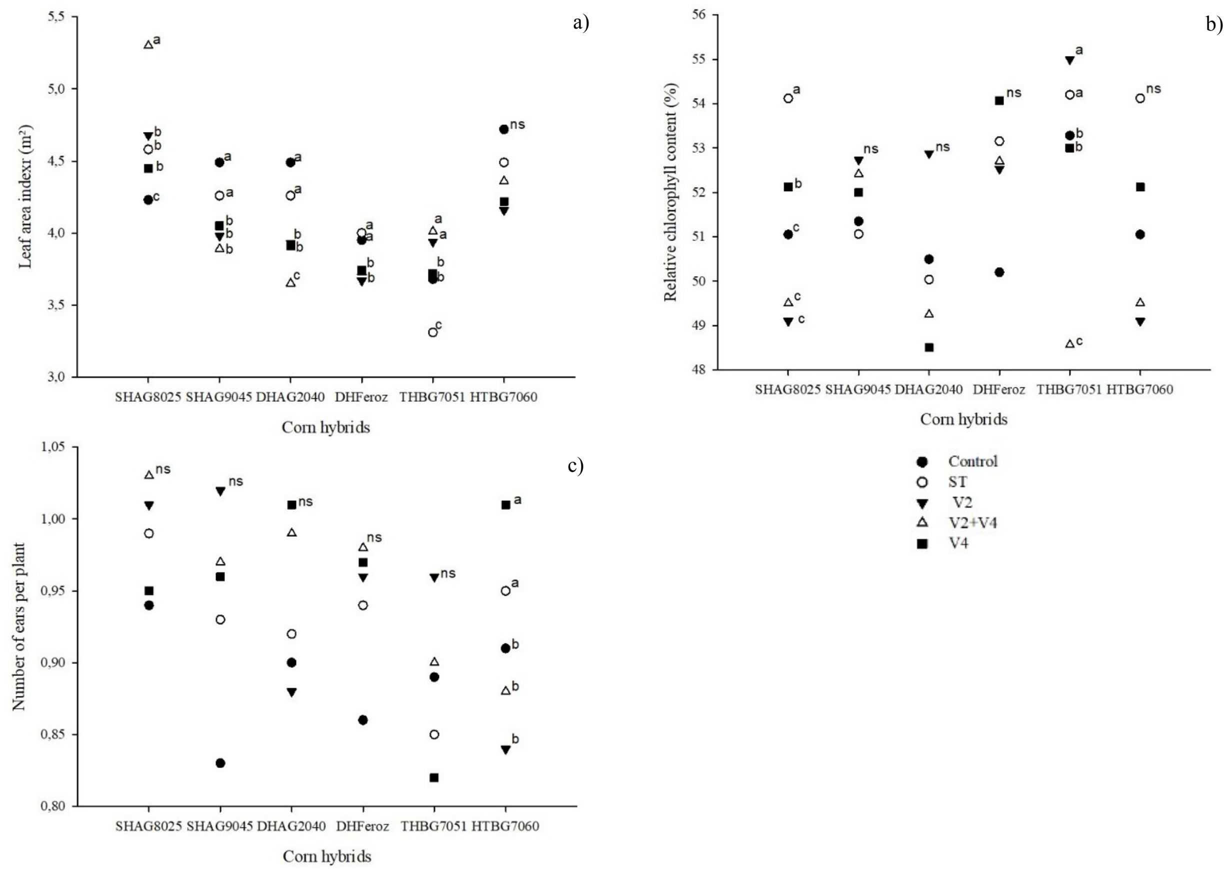

Figure 2. Analysis of the leaf area index (a), relative chlorophyll content (b), and number of ears per plant (c) variables during the 2014/15 crop season.

The variable most related to N absorption by the plant is the chlorophyll content (Argenta et al., 2003). The content values varied with the treatment and hybrid. In 2013/14, the inoculation via seed significantly reduced the chlorophyll content of DH AG1051 but increased the chlorophyll content of DH AG2040, showing the variation of the interaction between the genotype and the bacteria (Figures 1b and 2b). SH AG8025 had the largest chlorophyll content for the V4 application, whereas TH BG7051 had the largest mean chlorophyll content in treatments TS and V2 for the 2014/15 crop. The effect on the chlorophyll content is related to the BNF and the efficiency of absorbed N utilization, which represents the internal capacity of the plant to produce grains and dry matter (Reis Júnior, Machado, Machado, & Sodek, 2008).

The productive capacity of the plant is related to its ability to accumulate and transfer carbohydrates from the stem to ear formation and grain filling (Taiz & Zeiger, 2013). TH AG8011 (2013/14 crop season) and TH BG7060 (2014/15 crop season) presented the highest number of ears for foliar application during V4 (Figures 1c and 2c), indicating that the bacteria aided in the accumulation and transfer of carbohydrates.

The number of rows per ear is defined in stage V8, which is the phase with the greatest demand for nutrients, especially N (Repke, Cruz, Silva, Figueiredo, & Bicudo, 2013). In addition to affecting the dry mass of the corn ears, the ear components substantially affected the grain yield. The variable numbers of grains per ear presented positive responses to *A. brasilense* application for hybrid SH AG8025 with seed inoculation and foliar applications during stage V4 and the combination V2 + V4, whereas an increase in the number of grains for all forms of application of the bacteria in the 2013/14 crop was observed for SH AG9045 (Figure 3a). For the 2014/15 crop, the grains per ear variable was greater in hybrid SH AG8025 for the foliar application of the bacteria in V2 + V4, DH Feroz for all foliar applications, and TH BG7060 for the application in V4 (Figure 4a).

Nutrient deficiency up to R3 (the grain filling stage) can severely reduce the size of the ears (Repke et al., 2013). In both crops, an increase was observed in the ear length with *A. brasilense* application, with variation in the response of each hybrid to the treatments (Figures 3b and 4b).

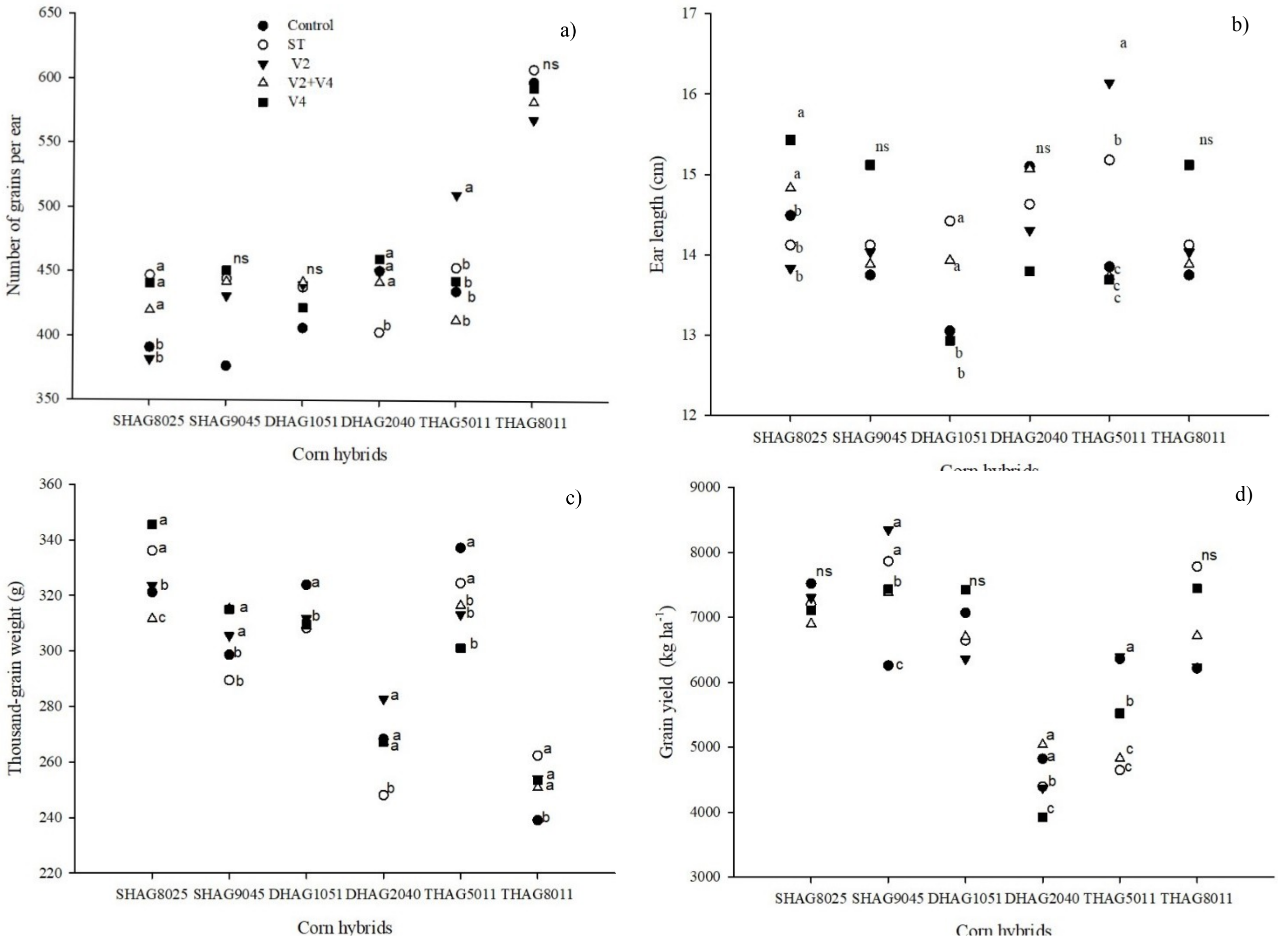

Figure 3. Scatterplot of the mean values for the number of grains per ear (a), ear length (b), thousand-grain weight (c), and grain yield (d) evaluated in corn hybrids as a function of *A. brasilense* application via seed and foliar application during the 2013/14 crop season.

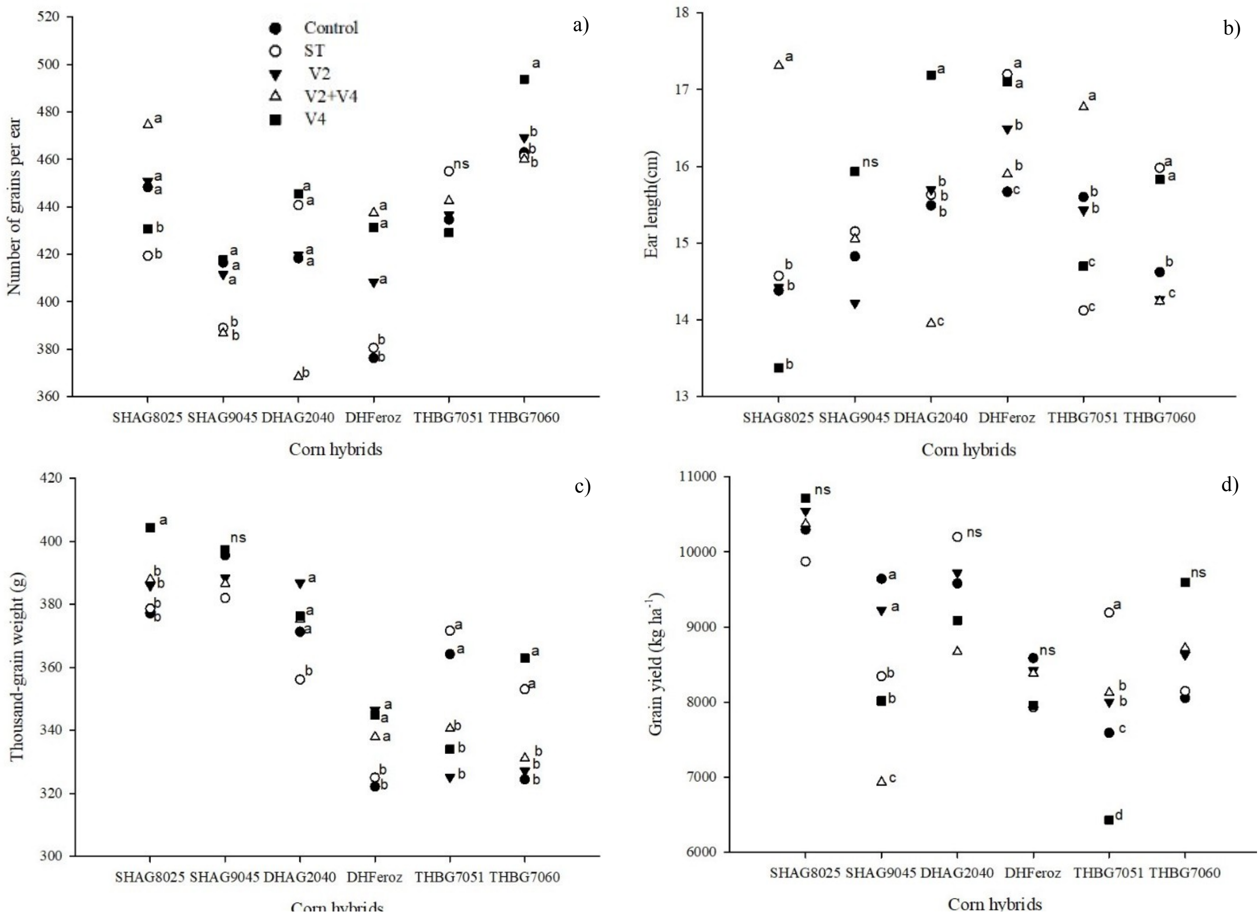

Figure 4. Number of grains per ear (a), ear length (b), thousand-grain weight (c), and grain yield (d) observed in corn hybrids with *A. brasilense* application via seed and leaf during the 2014/15 crop season.

The genotype-environment interaction exerts a great influence on the expression of quantitative traits (Schmildt, Nascimento, Cruz, & Oliveira, 2011). The thousand-grain weight varied with the year and the hybrid, with the bacteria proving to be effective in the treatments with foliar applications for some hybrids (Figures 3c and 4c). This result might be related to the BNF and the greater availability of N for the plant in the initial stages, which promote greater accumulation of dry mass in the plant and transport to the ear sand result in an increased thousand-grain weight.

However, some studies have shown no increase in the grain weight with *A. brasilense* inoculation (Araújo, Mercante, & Vitorino, 2015; Bracccini et al., 2012).

The effects of inoculation with *Azospirillum* sp. on the nutrition and yield of corn depend on the hybrid used and the climatic conditions (Quadros et al., 2014). After inoculation of *A. brasilense,* increases of 4, 6, 9, and 17% were reported by some authors (Brum, Cunha, Stecca, Grando, & Martin., 2016; Hungria, Campo, Souza, & Pedrosa, 2010; Müller et al., 2016; Novakowiski et al., 2011). Other studies that evaluated *Azospirillum* spp.-based inoculants found no significant increases in the grain yield (Repke et al., 2013; Sangoi et al., 2015; Vogt, Balbinot Junior, Gallotti, Zoldan, & Pandolfo, 2014).

In the 2013/14 crop, the inoculation was significantly different from the control for the grain yield only for SH AG9045 with application via seed treatment and via leaf in V2, representing increases of 1,606 and 2,092 kg ha^{-1}, respectively (Figure 3d). Inoculation via seed resulted in an increase in the grain yield of 412 kg ha^{-1} for TH BG7051 during the 2014/15 crop season (Figure 4d); the lack of or the low increase in the hybrids is possibly a response to the lack of nitrogen fertilization. In studies on crop-livestock integration systems, Brum et al. (2016) observed that inoculation with *A. brasilense* had a positive effect on the plant morphological components and grain yield regardless of the hybrid used, which differed from the results obtained in this study.

Considering the increase in the hybrids calculated by the formula of Dourado Neto and Detomini (2005), we estimated an accumulation of 46.50, 60.60, and 46.40 kg ha^{-1} N with savings of 372.47, 485.41, and US$ 371.66 ha^{-1}, for SH AG9045 following applications via seed and leaf in V2 and for TH BG7051 via seed application, respectively. Despite the increases obtained with the application of the bacteria in the seed and leaf in V2, this technology is not yet consolidated as a management strategy to improve the agricultural performance of the corn crop (Sangoi et al., 2015).

The factors that interfere with the responses of the crops to *Azospirillum* inoculation have not yet been fully clarified. Three of the evaluated hybrids remained unchanged during the two years of the experiment. In contrast, the responses of the analyzed variables for hybrids SH AG9045, SH AG8025, and DH AG2040 varied between the two crops, confirming that the response of the bacteria was not necessarily linked to the genotype or the compatibility between the bacteria and the genotype but instead might be linked to the requirements and survival of the bacteria in the environmental and soil conditions. The positive results with increases in the corn-*Azospirillum* association in most situations are related to bacterial factors, such as the choice of strain, the optimal number of colony forming units (CFU) per seed and the bacterial viability (Mehnaz & Lazarovits, 2006).

Sangoi et al. (2015) concluded that the high level of organic matter (OM) in the soil used (50.0 g kg^{-1}) might have increased the availability of N to the plants, thereby mitigating the benefits of seed inoculation with *Azospirillum* sp. Based on the hypothesis of Sangoi et al. (2015), the experiments were conducted in soils with 24 and 26 g kg^{-1} of OM in the 2013/14 and 2014/15 crops, respectively. Thus, the reduced N availability should have influenced the activity of the bacteria, there by increasing the BNF and the availability of fixed N. In addition to OM, the pH of the soil, the soil type and the availability of nutrients influence the composition of the root exudates, thereby affecting the plant-bacterium interaction (Tadra-Sfeir et al., 2011).

The complete replacement of nitrogen fertilizers by *A. Brasilense* is not feasible for grasses due to the small contribution of the bacteria to the BNF (Fukami, Nogueira, Araujo, & Hungria, 2016). However, the combination of all of the small contributions of *Azospirillum* for the growth of plants can result in plants that more efficiently absorb water and nutrients from the soil, thereby improving plant nutrition and growth (Fipke et al., 2016). Although the responses were variable according to the treatments evaluated, the results show an influence of inoculation on the evaluated genotypes, resulting in a better response than the treatment without inoculation in some cases.

Conclusion

The foliar application of *A. brasilense* is an efficient alternative to improve plant morphological traits.

The seed inoculation of *A. brasilense* presents larger increases in the grain yield than the foliar applications.

No relationship was observed between the forms of *A. brasilense* application and the hybrid genetic basis.

Application of *A. brasilense* via seed and leaf in V2 increases the relative contribution of N.

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