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Improving Sustainable Field-Grown Wheat Production With *Azospirillum brasilense* Under Tropical Conditions: A Potential Tool for Improving Nitrogen Management

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Sustainable intensification of cropping systems requires to increase productivity and nutrients use efficiency while reducing negative impacts of agricultural management practices on ecosystem and environment. Plant growth-promoting rhizobacteria (PGPR) inoculations are considered one of the most promising and safe strategy to alleviate environmental alterations in context of climatic extremes to improve plant nutrition while reducing dependency of nitrogen (N) fertilizer application. This study investigated the interactive effects of N levels and inoculation with *A. brasilense* on plant biomass, grain yield, agronomic efficiency (AE) of applied N, apparent N-fertilizer recovery (AFR) and N content in plant targeting economic feasibility of wheat production system. The field trial tested 4 N application levels applied in side-dressing (control, low, average and high; named 0, 50, 100 and 200 kg N ha^{-1}) and two inoculations (without and with *A. brasilense* seed inoculation). The results exhibited that inoculation with *A. brasilense* enhanced AE, AFR and N uptake in wheat plants with increased root and shoot N accumulation and grain N accumulation under average and high N application levels. In addition, inoculation increased root and shoot biomass, leading to a yield increase of 10.3% compared with non-inoculated plants. Wheat plant inoculation associated with application of the average N level provided the greatest profitability. Furthermore, results showed that reducing N fertilization from 100 to 50 kg N ha^{-1} along *A. brasilense* inoculation led to an increase in operating profit of 10.5%. In view of low economic cost, ease of application, and high probability of a positive response by wheat crops, even associated with different N application levels, the inoculation with *A. brasilense* prone to be a key sustainable management practice to improve wheat production under tropical conditions. This practice has the potential to increase wheat grain yield, N use and uptake, and overall farm profitability.

Keywords: *Triticum aestivum*, sustainable crop production, tropical agriculture, plant growth promoting rhizobacteria, improved nitrogen management

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the essential global staple foods that contributes around 20% dietary protein for human nutrition on daily basis (Zheng et al., 2021). Although Brazil is one the world's largest cereal producers but still importing more than half of wheat from other countries for its consumption (Conab, 2021). The growing demand of wheat consumption has become a threatened to food security in many South American, African, Caribbean and Pacific countries. Increasing population growth, rise and distribution of income, low prices and alteration in food preference are the contributory factors that increase cereal import in tropical countries faster than the ability to pay. Major cereal producers are North America and Europe, and Asia in the developing world, particularly India and China (Grote et al., 2021). However, consumption largely outpaces production in Africa and Asia, which are making these two regions major net importers of wheat (Grote et al., 2021). Therefore, crop failure in tropical and sub-tropical regions may cause a great deficit to wheat production. Thus, increasing wheat cultivation to tropical and sub-tropical regions could be handled as a matter of food security around the world.

The global demand for agricultural crops is expected to roughly double by 2050. The higher food security and production demand lead to an over-application of agricultural inputs (Kopittke et al., 2019). However, these overuses of agricultural inputs could increase methane (CH_4), nitrous oxide (N_2O) and carbon dioxide (CO_2) emissions which are a major source of total anthropogenic greenhouse gas (GHG) that lead to global warming and climate changes (Mateo-Marín et al., 2020; Wang et al., 2021). In addition, these GHG emissions are also expected to threaten sustainability of crop production with increasing environmental concerns worldwide (Mateo-Marín et al., 2020). Therefore, sustainable intensification of cropping systems requires increasing yields within optimized use of agricultural inputs.

Nitrogen (N) is considered one of the most limiting resources for potential crop growth and yield (Xu et al., 2020). Although, excessive N supply could directly contribute to soil acidification, ammonia (NH_3) and nitrogen oxide (N_2O, NO_2 and NO) gases emission and N leaching with extended consequences on environmental pollution and global warming (Sainju et al., 2019). The N application to crops has around 60% contribution to global N_2O anthropogenic emissions (Mateo-Marín et al., 2020). In addition, the application of N to wheat and corn through side-dressing technique in Brazilian Savannah has reported in almost 15% of their total operational profit (Galindo et al., 2017, 2019). Over the last decades, several management practices have been developed to enable farmers to reduce the application of N-based fertilizers in cereal crops, in order to limit potential environmental negative impacts and cost factors resulting from heavy chemical N fertilization (Brusamarello-Santos et al., 2017). The integrated N management techniques should imperatively contribute to greater N agronomic efficiency (AE) and recovery in tropical regions, especially for wheat cycle which is highly N demanding crop (Tsujimoto et al., 2019; Galindo et al., 2021).

The inoculation of plant growth-promoting rhizobacteria (PGPR) has quite significant role in alleviation of environmental changes under the context of climate extremes and excessive use of fertilizer in agricultural soils (Backer et al., 2018). The inoculation these microbes is recognized one of the best and alternative strategy for ecofriendly crop-management techniques as it could improve plant nutrition while reducing the dependance of N-fertilizer application (Koskey et al., 2021). The PGPR with several growth promoting attributes can improve AE, plant growth with greater grain yield of cereals in tropical environments (Hungria et al., 2010, 2018; Fukami et al., 2018; Galindo et al., 2019, 2020; Cassán et al., 2020). The free-living rhizobacteria (genus *Azospirillum*) are able to colonize more than one hundred plant species in almost every living soil on earth for being enhancing crop growth, development and production (Pedrosa et al., 2020). The *Azospirillum brasilense* Ab-V5 and Ab-V6 strains are officially authorized inoculants for increasing wheat and maize productivity under field conditions in Brazil (Hungria et al., 2010; Santos et al., 2021). To date, the additive hypothesis best addresses *A. brasilense* operating principle, in which multiple mechanisms of plant growth-promotion works in convergence or in sequence (Bashan and de-Bashan 2010; Cassán and Diaz-Zorita 2016; Cassán et al., 2020). For example, *A. brasilense* has been reported to promote plant growth by increasing the production of phytohormones like gibberellins, auxins and cytokinins (Fukami et al., 2018), increasing root development leading to greater nutrients and water acquisition (Caires et al., 2020), increasing N use efficiency from applied N-fertilizer (Galindo et al., 2020), enhanced nitrate reductase activity (Ferreira et al., 1987; Pereira-Defilippi et al., 2017), solubilization of phosphates (Rafi et al., 2019), among others. Although the adoption of *A. brasilense* inoculation by Brazilian farmers has increased in recent years, it is not yet a consolidated management practice in agricultural production systems such as *Bradyrhizobium* sp. inoculation in soybean [*Glycine max* (L.) Merr.].

The hypothesis is that AE and apparent N-fertilizer recovery (AFR) in wheat plant could be significantly improved by inoculation with *A. brasilense*. The increased AE and AFR may provide increased N plant accumulation and shoot and root development, which may lead to greater grain yield when compared with non-inoculated plants. This research provides a novel view for fomenting wheat cultivation under tropical conditions by allowing reduce N-fertilizer application rates without compromising grain yield. In addition, it would favor sustainable cereal production under tropical conditions with an increase in farming profitability. Therefore, the objective of this study was to investigate the combined effects of N application levels (control, low, average and high levels) and seed inoculation with *A. brasilense* on: 1) plant biomass, grain yield, AE and AFR; 2) inorganic ($N\text{-}NH_4^+$ and $N\text{-}NO_3^-$) and total N content in wheat plant; and 3) economic analysis and operating profit for each individual treatment.

MATERIALS AND METHODS

Field Experimental Characterization

The field experiment was conducted at the São Paulo State University Experimental Station (20°22′S and 51°22′W, 335 m above sea level) in Selvíria, Mato Grosso do Sul- Brazil, in the wheat growth seasons of 2016 and 2017. The site of experiment was being kept no-till for 15 years with annual cultivation of cereal and legume crops. The crop sequence prior to wheat was corn in both growth seasons. Climate classification was Aw according to Köppen-Geiger classification - tropical Savannah climate with dry winter. Daily temperature and rainfall measurements during the experiment were obtained from a meteorological station, located at experimental station (**Supplementary Figure S1**). Soil was classified as clayey, Rhodic Haplustox (Soil Survey Staff, 2014) with 47% sand, 9% silt and 44% clay, respectively. Chemical attributes of the 0–0.20 m soil depth were determined according to van Raij et al. (2001) and are presented in **Supplementary Table S1**. The N total was evaluated with semi-micro Kjeldahl method (Bremner and Breitenbeck, 1983) (**Supplementary Table S1**).

Experimental Design and Treatments

Four N application levels × 2 *A. brasilense* inoculations were factorially arranged in a randomized complete block experimental design with four replications. Each experimental plot was consisted of twelve 0.17 m wide rows with length of 6 m long. The useful area was the eight central rows, excluding 0.5 m at the end of each wheat row.

Nitrogen levels refers to side-dress N application as a control (0 kg N ha^{-1}), low (50 kg N ha^{-1}), average (100 kg N ha^{-1}) and high (200 kg N ha^{-1}) levels. These levels were selected based on previous studies with N management in wheat crop under Brazilian tropical Savannah conditions (Teixeira Filho et al., 2010). The applied N source was urea (45% of N) at wheat tillering decimal growth stage GS21 (Zadoks et al., 1974).

The two inoculations treatments consisted of with *A. brasilense* and absence of inoculation. The seeds of wheat were treated with *A. brasilense* strains (Ab-V5 and Ab-V6) selected from the Collection of Diazotrophic and Plant Growth Promoting Bacteria of Embrapa Soja (WFCC #1213 and WDCM #1054 with guarantee of 2×10^8 colony forming units mL^{-1}). A 300 ml liquid inoculant was manually coated and mixed per 50 kg of wheat seeds in plastic bags an hour before sowing. To check if inoculation was well succeeded (presence of at least 1×10^6 colony forming units g^{-1} soil), rhizosphere soil was randomly collected from wheat cultivated area at decimal growth stage GS619 (Zadoks et al., 1974) to analyze *Azospirillum* sp. colonization. The most probable number (MPN) mechanism with dilutions and inoculations in flasks having a medium of semisolid NFb without N addition and followed by growth at 35°C for 48 h (Döbereiner et al., 1995) was used and the obtained results were presented in **Supplementary Table S2**.

Wheat Management

The wheat cultivar CD 1104 was sown in both cultivated years (2016 and 2017) at a density of 412 viable seeds m^{-2} with a no-till drill. Also, basal fertilization application was performed at sowing for all treatments with 275 kg ha^{-1} of the granular fertilizer 08-28-16 (N-P_2O_5-K_2O) in the sowing furrows based on soil analysis and crop requirements (Cantarella et al., 1997). During this nutrient application, 22 kg N ha^{-1} was applied to the entire experimental area. Therefore, the total amount of N applied in each treatment was the amount of N applied at the sidedress (0–200 kg N ha-1, as indicated above) in addition to the basal application of 22 kg N ha^{-1}. As mentioned before, seed inoculation for the treatments with *A. brasilense* was performed at sowing. In addition, N treatment application in side-dressing (N application levels) was performed manually to uniformly distribute fertilizer on soil surface without incorporation during wheat tillering. The crop was irrigated with a center pivot sprinkling system with water depth of 14 mm depending on crop moisture requirement. The crop was cultivated during third May 2016 to 8 September 2016 (harvested 120 days after emergence, DAE) and 10 May 2017 to 12 September 2017 (harvested 117 DAE). The pre- and post-emergence herbicides were used for weeds control during crop cycle.

Samplings and Analysis

Wheat plants from an area of 0.17 m^2 (0.17 m—width of the row × 1.0 m) were manually harvested by cutting plants at the ground level during flowering for shoot collection. At the same time, a side trench of approximately 0.40 m depth was dug opened for roots collection which were then washed with deionized water. In addition, 30 wheat flag leaves per plot were collected for nutritional analysis according to Cantarella et al. (1997). Shoot, root, and leaves were dried for 90 h in a forced-air oven at 60°C. Then, shoot and root were weighed for dry mass (kg ha^{-1}). The N concentration was determined following the methodology of Malavolta et al. (1997), with sulfuric digestion and semi-micro Kjeldahl analysis method. Inorganic N concentration (N-NO_3^- and N-NH_4^+) in plant tissue was determined following Tedesco et al. (1995). Briefly, 1 g of plant tissue was extracted with 1 mol KCl L^{-1} (ratio of 1: 15, plant tissue: solution, w/v), distilled with MgO (N-NH_4^+) and Devarda's alloy and titrated with 2.5 mmol H_2SO_4 L^{-1} (N-NO_3^-). The accumulated total N, NH_4^+ and NO_3^- in shoot and root was obtained by the product of total N and inorganic N concentrations in tissue and produced dry biomass. The grain yield was obtained by collecting spike from the useful area of each plot which were mechanically harvested and grains were weighted (kg ha^{-1} and adjusted to a 13% moisture content in wet basis). Grain N accumulation was determined following the same procedures as shoot and root N accumulation. Agronomic efficiency of applied N-fertilizer (AE) was calculated following the methodology of Fageria et al. (2005) according to equation :

$$\text{AE}\left(\text{kg grain kg N applied}^{-1}\right) = \frac{(\text{grain yield at Nx} - \text{grain yield at N0})}{\text{N level applied}} \quad [1]$$

Where Nx is the N level applied and N0 is the control treatment (without N applied in side-dressing).

Apparent N-fertilizer recovery (AFR) was calculated following Sieling and Kage, (2021) and presented in equation :

$$\text{AFR}\,(\%) = \frac{(\text{grain N accumulated at Nx} - \text{grain N accumulated at N0})}{\text{N level applied}} \quad [2]$$

Where Nx is the N level applied and N0 is the control treatment (without N applied in side-dressing).

Statistical Analysis

All data were initially tested for Levene's homoscedasticity test ($p \leq 0.05$) and normality using the Shapiro and Wilk test, which showed the data to be normally distributed ($W \geq 0.90$). Data were submitted to analysis of variance (F test) using repeated measures (with year as the repeated variable) using a compound symmetry model for the covariance parameter. When a significant main effect or interaction was observed by the F test ($p \leq 0.05$), then Tukey test ($p \leq 0.05$) was used for comparison of means of N application levels, inoculation, years of study and their interactions using the ExpDes package in R software (R Core Team, 2015).

Principal Component Analysis

Principal component analysis (PCA) was used to evaluate biomass, grain yield, AE, inorganic and total N content in wheat plant. The PCA was carried out with the help of FactoMineR and factoextra packages in R software (R Development Core Team, 2015). The selection of PCs were based on eigenvalue such as PCs with eigenvalues ≥1 were reserved while the rest were excluded. The selected PCs indicated a total variability of 70% or greater where the correlations between chosen PCs and observed variables were elaborated with factor loading which was estimated via equation:

$$\text{Factor loadings} = \text{Eigenvectors} \times \sqrt{\text{Eigenvalue}} \quad [3]$$

A factor loading of >0.30 was considered significant according to Lawley and Maxwell (1962). The biplot graphics indicated that PC1 (axis x) and PC2 (axis y) were plotted to separate inoculated and non-inoculated treatments.

Economic Analysis and Operating Profit

The structure of economic analysis was based on total operating expenses (TOE) that was adopted from Brazilian Institute of Agricultural Economics (BIEA), following Matsunaga et al. (1976). The cost-effectiveness of applied treatments were determined by profitability analyses, following Martin et al. (1998) and Galindo et al. (2019). Gross revenue, operating profit and average operating profit (average profit in both cropping years) were determined (in US\$ ha^{-1}). The mean expenses were estimated in Savannah region (Midwest region of Brazil) (average of the last 3 years—2019–2021). The selling price of wheat 60-kg sack was US\$ 13.09 per unit. The cost of urea fertilizer was US\$ 381.94 per Mg. The *A. brasilense* inoculant was priced for US\$ 1.88 per rate (100 ml). The average dollar exchange rate was R\$5.33 = US\$1.00 according to Central Bank of Brazil.

RESULTS

Wheat Biomass, Grain Yield, Agronomic Efficiency and Apparent N-Fertilizer Recovery

Root and shoot biomass were greater in 2016 than 2017 (**Supplementary Table S3**, **Figures 1A,B**). Interaction between inoculation and N levels significantly affected root biomass (**Supplementary Table S3**, **Figure 1A**). In the absence of N application and under low and average N applications, inoculated plots showed 15.9, 37.6 and 12.7% greater root dry mass (ctl: 494 kg ha^{-1}; low: 685 kg ha^{-1}; avg: 619 kg ha^{-1}) compared to non-inoculated plots (ctl: 426 kg ha^{-1}; low: 498 kg ha^{-1}; avg: 549 kg ha^{-1}) (**Supplementary Table S3**, **Figure 1A**). In addition, root biomass response to N application was different between inoculated and non-inoculated treatments (**Supplementary Table S3**, **Figure 1A**). When inoculation was performed, root biomass was greater when coupled with low and average N levels. Whereas, without *A. brasilense* inoculation, root biomass was greater when the average and high N levels were applied (**Supplementary Table S3**, **Figure 1A**).

Shoot biomass was 5.9% greater in inoculated plots (5,549 kg ha^{-1}) in comparison to without inoculation plots (5,239 kg ha^{-1}) (**Figure 1B**). The application of high N level provided greater shoot biomass compared with control treatment (absence of side-dressing N application) (**Supplementary Table S3**, **Figure 1B**).

Similarly, as verified in shoot biomass data, inoculated plots (4,062 kg ha^{-1}) showed 10.3% greater grain yield compared to without inoculation plots (3,682 kg ha^{-1}) (**Supplementary Table S3**, **Figure 1C**). In 2016, grain yield was greater under low and average N levels while in 2017, grain yield was greater under average N application level (**Supplementary Table S3**, **Figure 1C**). Under low N application, grain yield was greater in 2016 than 2017 however, grain yield under average N application was greater in 2017 than 2016 (**Supplementary Table S3**, **Figure 1C**).

Agronomic efficiency (AE) was 51.2% greater in inoculated plots (13.0 kg grain kg^{-1} N applied) in comparison to non-inoculated plots (8.6 kg grain kg N applied^{-1}) (**Supplementary Table S3**, **Figure 1D**). In 2016, AE was greater under low N application while in 2017, AE was decreased under high N application (**Supplementary Table S3**, **Figure 1D**). Agronomic efficiency under average N application was greater in 2017 than 2016 (**Supplementary Table S3**, **Figure 1D**). Similar trend was verified for apparent N-fertilizer recovery (AFR) (**Supplementary**

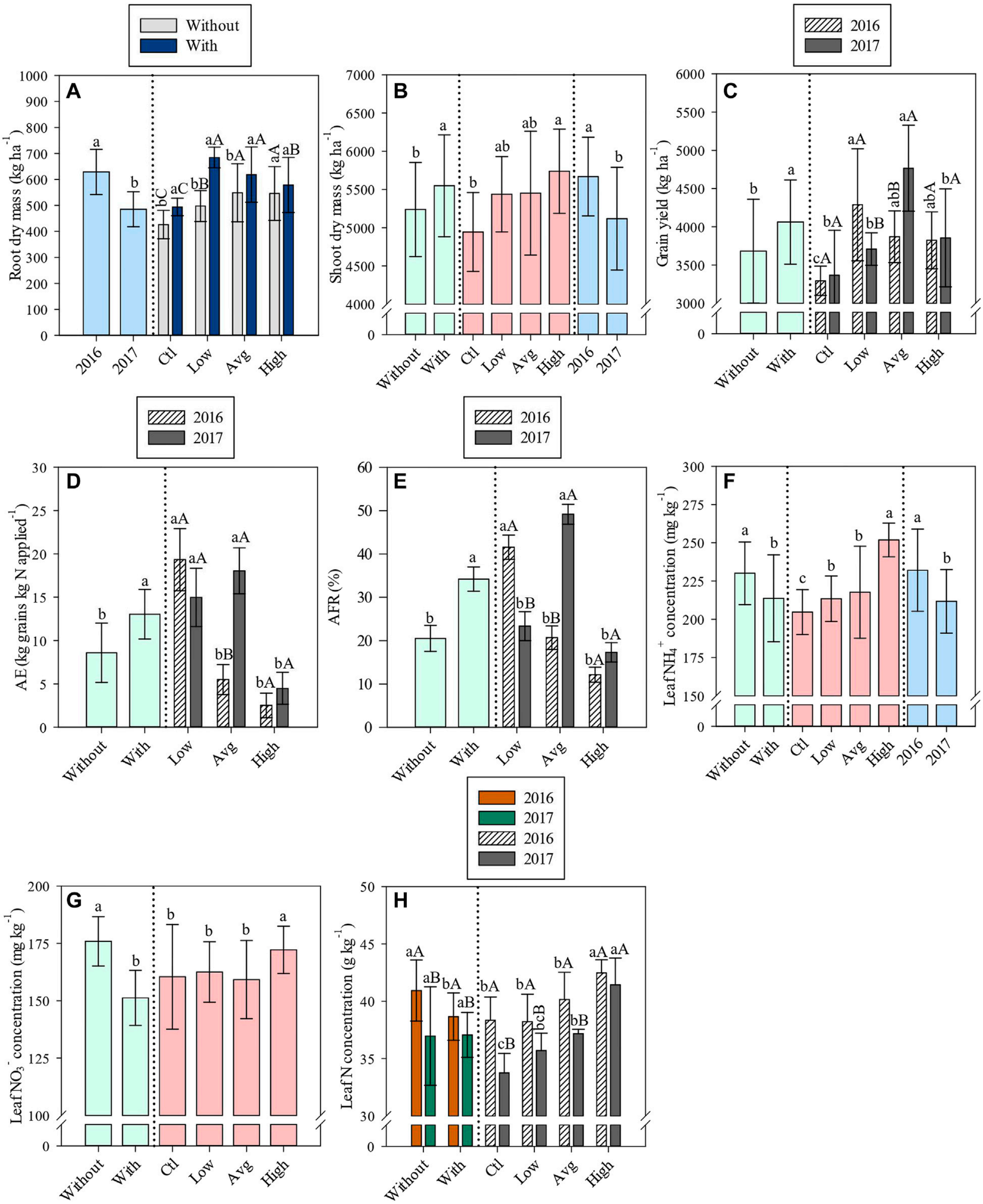

FIGURE 1 | Root dry mass **(A)**, shoot dry mass **(B)**, grain yield **(C)**, agronomic efficiency **(D)**, apparent N-fertilizer recovery **(E)**, leaf NH_4^+ concentration **(F)**, leaf NO_3^- concentration **(G)** and leaf N concentration **(H)** affected by inoculation with *A. brasilense*, N levels, years of study and/or their interactions.

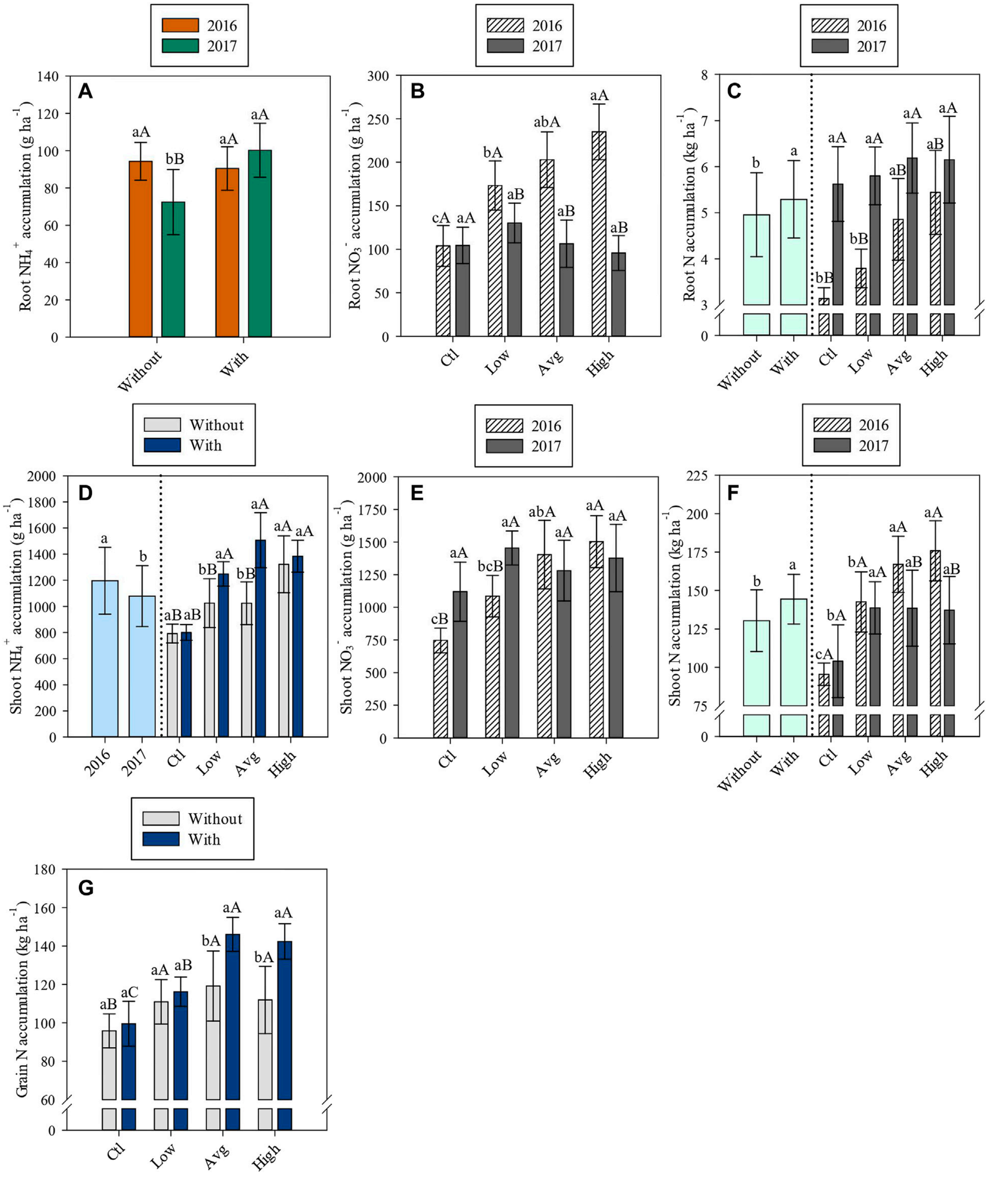

FIGURE 2 | Root NH_4^+ accumulation **(A)**, root NO_3^- accumulation **(B)**, root N accumulation **(C)**, shoot NH_4^+ accumulation **(D)**, shoot NO_3^- accumulation **(E)**, shoot N accumulation **(F)** and grain N accumulation **(G)** affected by inoculation with *A. brasilense*, N levels, years of study and/or their interactions.

Table S3, **Figure 1E**). Apparent N-fertilizer recovery was 66.7% higher in inoculated plots (34.2%) in comparison to without inoculation plots (20.5%) (**Supplementary Table S3**, **Figure 1E**). In 2016, AFR was greater under low N application while in 2017, AFR was greater under average N application (**Supplementary Table S3**, **Figure 1E**). Apparent N-fertilizer recovery under low N application was greater in 2016 than 2017, whereas average N application was greater in 2017 than 2016 (**Supplementary Table S3**, **Figure 1E**).

Inorganic (N-NH_4^+ and N-NO_3^-) and Total N Content in Wheat Plants

The treatments without inoculation were observed with higher leaf NH_4^+ and NO_3^- concentration as compared to inoculated plots (**Supplementary Table S3**, **Figures 1E,F**). Both NH_4^+, NO_3^- and total N in leaf tissue tended to be greater when high N level was applied (**Supplementary Table S3**, **Figures 1E–G**). In addition, leaf NH_4^+ was greater in 2016 than 2017 (**Supplementary Table S3**, **Figure 1E**). Leaf total N concentration in 2016 was greater in non-inoculated plots compared to *A. brasilense* inoculated plots (**Supplementary Table S3**, **Figure 1G**). Leaf total N concentration was greater in 2016 than 2017, regardless of inoculations (**Supplementary Table S3**, **Figure 1G**).

In 2017, root NH_4^+ accumulation was 38.4% greater in inoculated plots (100.2 g ha^{-1}) compared to non-inoculated plots (72.4 g ha^{-1}) (**Supplementary Table S3**, **Figure 2A**). In the absence of inoculation, root NH_4^+ was greater in 2016 than 2017 while no significant differences were observed for inoculated plots in both 2016 and 2017 (**Supplementary Table S3**, **Figure 2A**).

Root NO_3^- accumulation tended to be greater with application of high N level in 2016 however, no significant differences in root NO_3^- due to N levels were observed in 2017 (**Supplementary Table S3**, **Figure 2B**). Under low, average, and high N application, root NO_3^- was greater in 2016 than 2017 (**Supplementary Table S3**, **Figure 2B**).

Root total N accumulation was 6.9% greater in inoculated plots (5.3 kg ha^{-1}) compared to non-inoculated plots (4.96 kg ha^{-1}) (**Supplementary Table S3**, **Figure 2C**). Root total N accumulation tended to be greater with application of high N level in 2016 however, it was not significantly influenced by N levels in 2017 (**Supplementary Table S3**, **Figure 2C**). In addition, root total N accumulation was greater in 2017 than 2016 (**Supplementary Table S3**, **Figure 2C**).

Shoot NH_4^+ accumulation was greater in 2016 than 2017 (**Supplementary Table S3**, **Figure 2D**). Under low and average N application, shoot NH_4^+ was 21.9 and 47.1% greater in inoculated plots (1,249 g ha^{-1} and 1,506 g ha^{-1}) compared to non-inoculated plots (1,025 g ha^{-1} and 1,024 g ha^{-1}) respectively (**Supplementary Table S3**, **Figure 2D**). In the absence of inoculation, the high N application level provided greater shoot NH_4^+ (**Supplementary Table S3**, **Figure 2D**). Whereas, when inoculation was performed, the low, average, and high N levels provided similar shoot NH_4^+ accumulation, being greater as compared with control (absence of side-dressing N application) (**Supplementary Table S3**, **Figure 2D**).

Shoot NO_3^- accumulation tended to be greater with high N application level in 2016 (**Supplementary Table S3**, **Figure 2E**), however, it was not significantly influenced by N levels in 2017 (**Supplementary Table S3**, **Figure 2E**). Without (control) and under low N application, shoot NO_3^- was greater in 2017 than 2016 (**Supplementary Table S3**, **Figure 2E**).

Shoot total N accumulation was 10.8% greater in inoculated plots (144 kg ha^{-1}) compared to non-inoculated plots (130 kg ha^{-1}) (**Supplementary Table S3**, **Figure 2F**). In 2016, application of the average and high N levels provided greater shoot N accumulation (**Supplementary Table S3**, **Figure 2F**). Whereas in 2017, all N levels provided similar shoot N accumulation, but still greater compared with control (**Supplementary Table S3**, **Figure 2F**). Under average and high N application levels, shoot total N was greater in 2016 than 2017 (**Supplementary Table S3**, **Figure 2F**).

Grain N accumulation when coupled with average and high N application levels was 22.7 and 26.8% greater in inoculated plots (146 kg ha^{-1} and 142 kg ha^{-1}) compared with non-inoculated plots (119 kg ha^{-1} and 112 kg ha^{-1}) (**Supplementary Table S3**, **Figure 2G**). In the absence of inoculation, all 3 N application levels provided similar grain N accumulation, being greater only when compared with control (absence of side-dressing N application) (**Supplementary Table S3**, **Figure 2G**).

Principal Component Analysis

The eigenvalues of extracted PCs were <1 which were grouped into a four-component model that accounts for 87 and 82% of data variation in plots without and with *A. brasilense* inoculation respectively (**Supplementary Table S4**).

In non-inoculated plots, PC1 represented 42% of variance which indicated shoot and root dry mass, leaf N and NH_4^+ concentration, shoot N and NH_4^+ accumulation, root NO_3^- accumulation and AFR as positively concordant (**Supplementary Table S4**). The PC2 exhibited a positive correlation between shoot N, NH_4^+ and NO_3^- accumulation, root N accumulation and AFR (**Supplementary Table S4**). Conversely, leaf NO_3^- concentration was negatively correlated with the PC2 components (**Supplementary Table S4**). There was a cumulative variance of 61.4% between PC1 and PC2 (**Supplementary Table S4**). The rest of two extracted factors were least important in terms of eigenvalues and variability (**Supplementary Table S4**). Principal component 3 showed a positive correlation between grain yield and grain N accumulation and AE however, a negative correlation between root NH_4^+ accumulation and the PC3 components were verified (**Supplementary Table S4**).

In *A. brasilense* inoculated plots, PC1 represented 39.3% of the variance and indicated positive correlation between shoot and root dry mass, shoot and grain N, NH_4^+ and NO_3^- accumulation and AFR (**Supplementary Table S4**). Principal component 2 exhibited that leaf N, NH_4^+ and NO_3^- concentration and root N accumulation positively correlated however, root dry mass was negatively correlated with both leaf total N and inorganic N forms concentration and root N accumulation (**Supplementary Table S4**). Both PC1 and PC2 indicated 57.9% of the cumulative variance (**Supplementary Table S4**. Similarly, as observed in non-inoculated plots, the other two extracted factors are of minor importance in terms of both eigenvalues and explained variability (**Supplementary Table S4**). Principal component 3 showed grain yield, root N accumulation and AE as positively concordant (**Supplementary Table S4**). In contrast, leaf NH_4^+ concentration was negatively correlated with all previously reported parameters (**Supplementary Table S4**).

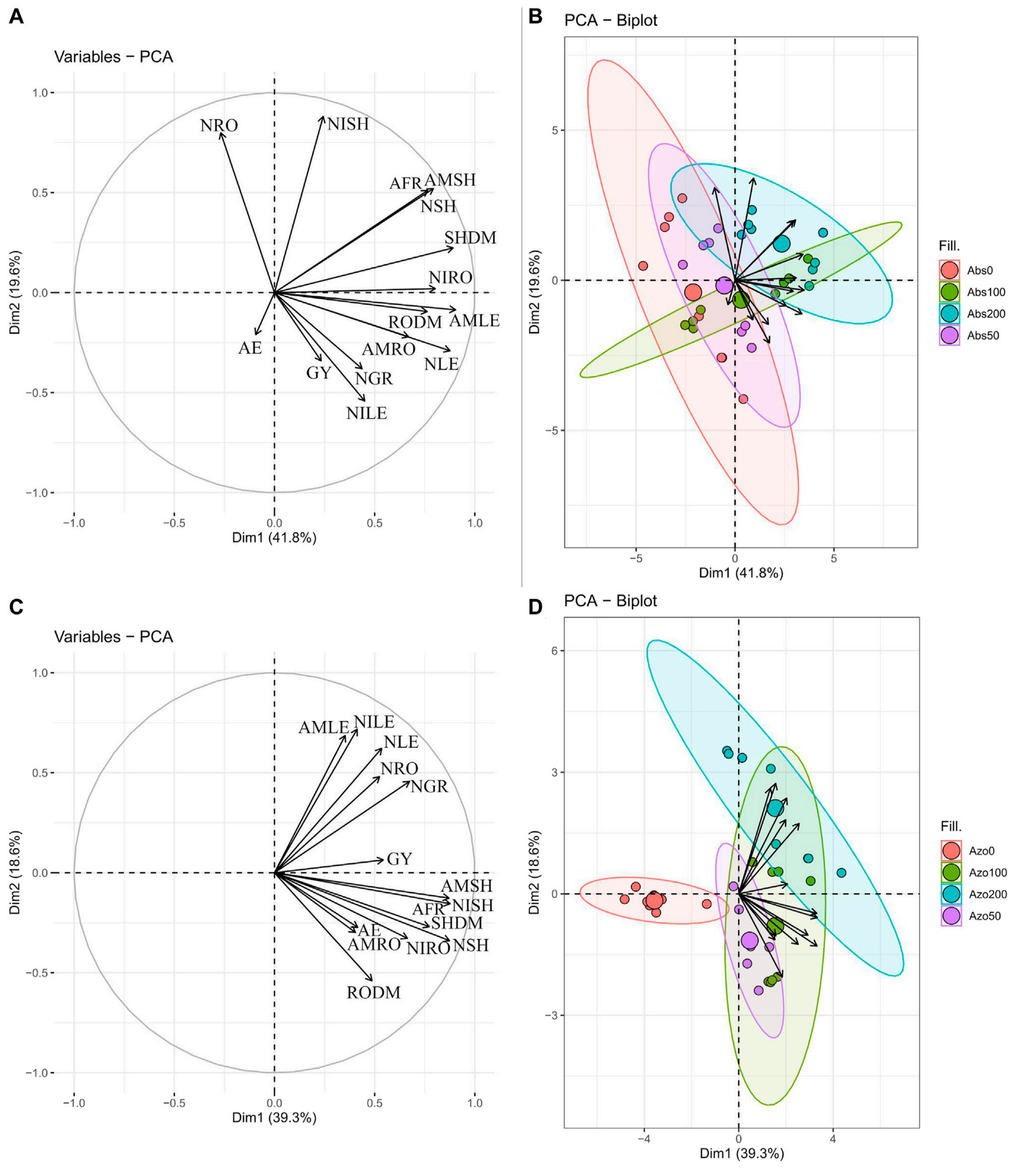

FIGURE 3 | Loadings and biplot graphics of principal component analysis among the relationship between wheat shoot dry mass (SHDM), root dry mass (RODM), grain yield (GY), leaf N concentration (NLE), leaf NH_4^+ concentration (AMLE), Leaf NO_3^- concentration (NILE), Shoot N accumulation (SHN), Shoot NH_4^+ accumulation (SHAM), Shoot NO_3^- accumulation (SHNI), Root N accumulation (RON), Root NH_4^+ accumulation (ROAM), Root NO_3^- accumulation (RONI), Grain N accumulation (GRN), agronomic efficiency (AE) and apparent N-fertilizer recovery (AFR) evaluated in non-inoculated (absence) **(A,B)** and *A. brasilense* inoculated treatments **(C,D)**.

TABLE 1 | Wheat total operating expenses (TOE), gross revenue, operating profit and average operating profit (based on the two wheat cropping seasons) affected by inoculation with *A. brasilense* and N levels.

Treatment	TOE	Gross revenue		Operating profit		Average profit
	(US$)					
	–	First year	Second year	First year	Second year	–
Without + Ctl	385	725	646	340	261	300
Without + Low	431	895	770	463	338	401
Without + Avg	477	831	963	354	485	420
Without + High	569	818	781	249	212	230
With + Ctl	403	713	823	310	420	365
With + Low	449	977	849	528	400	464
With + Avg	495	858	1,118	363	623	493
With + High	587	851	902	264	315	290

Without and With refers to absence and presence of A. brasilense *inoculation; Ctl, Low, Avg and High refers to absence, 50, 100 and 200 kg N ha^{-1}, applied in side-dressing.*

Analyzing the grouped PCA biplot graph (PC1 and PC2) in non-inoculated plots, it was verified that group formed by the high N application level best comprised most of the analyzed parameters (**Figure 3A**). However, the group formed by low N application level best comprised grain yield, AE, grain N accumulation and leaf NO_3^- concentration (**Figure 3A**). Whereas, when inoculation was performed, the group formed by average N application level was found to comprised better the total and inorganic N content in wheat plant, biomass, grain yield, AE and AFR (**Figure 3B**).

Economic Analysis and Operating Profit

As expected, TOE tended to increase with increasing N application levels and inoculation, from US$ 385 ha^{-1} (control) to US$ 569 ha^{-1} (high N level applied) without inoculation, and from US$ 403 ha^{-1} (control) to US$ 587 ha^{-1} (high N level applied) with *A. brasilense* inoculation (**Supplementary Table S5**, **Table 1**). However, the greater wheat grain yield provided by the tested N levels and inoculation lead to an increased gross revenue (**Supplementary Table S5**, **Table 1**). The relation between gross revenue and TOE (operating profit) indicated that inoculation of *A. brasilense* coupled with mean N application level provided greater profit (US$ 493 ha^{-1}), followed by inoculation with the low N application level (US$ 464 ha^{-1}) (**Table 1**). Similarly, in the absence of inoculation, the highest operating profit was observed with fertilization of an average N level (US$ 420 ha^{-1}) followed by application of the low N level (US$ 401 ha^{-1}) (**Table 1**).

DISCUSSION

The findings of current study exhibited that inoculation of *A. brasilense* enhanced agronomic efficiency (AE), apparent N-fertilizer recovery (AFR) and N uptake in wheat plants with increased root and shoot N accumulation and grain N accumulation under the average and high N application levels (**Figure 1D**; **Figure, 2C,F,G**). Agronomic efficiency is the product of N recovery efficiency from applied N and the efficiency of plants to use each additional acquired N unit, and usually range from 10 to 30 kg grain kg^{-1} N applied (Doberman, 2005) as verified in this study. Apparent N-fertilizer recovery indicates directly how well applied fertilizer is removed by the harvest products (Hawkesford and Griffiths, 2019), in this study wheat grains. Several concepts and technologies have been adopted and developed to increase N use efficiency (Sharma and Bali, 2018; Galindo et al., 2021) such as; 1) enhancing crop N requirement and uptake through improvements in genetic and management factors to eliminate restrictions in crop growth and N requirement and 2) adopting such management practices that improve soil health and N supply for plant uptake (Chien et al., 2009). The latter initially include more effective N application methods, site-specific N management and highly effective fertilizers (new N forms, modified fertilizers and inhibitors that lead to slow/controlled release). Based on our results, *A. brasilense* inoculation could fit in the first above-mentioned group. It is essential to understand but several technological choices had different impacts on crop yield in response to N which is often the combination of such practices that lead to several greater benefit (Doberman, 2005; Chien et al., 2009).

The benefits of *A. brasilense* inoculation on root development highlighted by the observed increase in root dry mass and N content in roots are likely to be the key mechanism to increase AE and AFR, enhancing wheat growth and grain yield. The positive correlations between root-shoot dry mass, shoot and grain N accumulations had strengthened current hypothesis (**Supplementary Table S4**). Several previous studies have reported benefits of *A. brasilense* in root hairs and lateral root growth in different crops (Rondina et al., 2020; Araujo et al., 2021; Barbosa et al., 2021). Studies reported that several rhizobacteria can alter plant physiology with effect on biomass distribution in plant like altering root architecture (Backer et al., 2018; Bavaresco et al., 2020; Araujo et al., 2021) which could influence plant ability to penetrate into soil for greater water and nutrients uptake (Li et al., 2016). The ability of *A. brasilense* to excrete molecules such as phytohormones and nitric oxide to root uptake or regulate hormone balance in the plants to boost growth has been considered the major factor affecting root architecture (Backer et al., 2018; Cassán et al., 2020; Barbosa et al., 2021). Strong root system is related to higher rhizo-deposition of organic forms of carbon (C) and N from soil that can favor the trophic interactions of rhizosphere biodiversity and benefit overall plants functions (Barbosa et al., 2021).

Interestingly, the increased shoot and root biomass provided a dilution effect verified by the reduced inorganic (N-NH_4^+ and N-NO_3^-) and total N concentration in leaf tissue (**Figures 1E–G**). Negative correlations among root dry mass and leaf N, NH_4^+ and NO_3^- concentrations in PC2 and between grain yield and leaf NH_4^+ concentration in PC3 when inoculation was performed, support this dilution effect hypothesis (**Supplementary Table S4**). Nonetheless, the total N accumulation occurred mainly due to the enhanced N-NH_4^+ accumulation provided by inoculation. It was verified as function of greater root NH_4^+ accumulation mainly in 2017 and increased shoot NH_4^+ when inoculation was performed coupled with the low and average N application levels (**Figures 2A,D**). Plausible explanations for the observed increase in NH_4^+ content in wheat plant could be related to 1) NH_4^+ derived from *Azospirillum*-BNF (Fukami et al., 2018; Pii et al., 2019) and/or 2) improved nitrate reductase activity, mainly due to increased root development provided by inoculation (Fereira et al., 1987; Pereira-Defilippi et al., 2017). Biological N fixation was the first mechanism to be identified that demonstrated the way in which *Azospirillum* sp. positively affects plant growth (Döbereiner and Day, 1976; Okon et al., 1983; Cassán et al., 2020; Pedrosa et al., 2020). It has been reported that *A. brasilense* strains (Ab-V5 and Ab-V6) are carrying similar *fix* and *nif* genes that are contributing in BNF (Hungria et al., 2018). Despite this, these strains had different capacity of phytohormones production (Fukami et al., 2018) but still sharing similar genes for auxin synthesis. Although evidence that N fixation can contribute to N balance of plants has been reported, the contribution of N fixation by *Azospirillum* sp. to plants is not the major role in plant growth promotion (Cassán et al., 2020). Additional plant growth promoting mechanisms were proposed, such as the production of phytohormones like gibberellins, auxins and cytokinins (Fukami et al., 2018), increases in root development leading to greater nutrient and water acquisition (Caires et al., 2020), increase in N use efficiency from applied N-fertilizer (Galindo et al., 2020), enhancement of nitrate reductase activity (Fereira et al., 1987; Pereira-Defilippi et al., 2017), solubilization of phosphates (Rafi et al., 2019), among others. Therefore, the additive hypothesis best addresses *A. brasilense* operating principle, in which multiple mechanisms of plant growth-promotion works in convergence or in sequence (Bashan and de-Bashan 2010; Cassán et al., 2020).

The verified results agreed with previous findings that *A. brasilense* inoculation associated with N fertilization management could increase wheat grain yield between 3 and 26% compared to N application without inoculation (Galindo et al., 2017; Munareto et al., 2019; Caires et al., 2020; Galindo et al., 2020). It has been broadly explored that multifaceted microbe-environment-plant interactions shifts with expansion of agronomic response to *A. brasilense* inoculation (Cassán and Díaz-Zorita 2016), including bacteria and plant genotypes (Bashan and de-Bashan, 2010; Cassán et al., 2020). Under field trial evaluations, crop responses to *Azospirillum* sp. inoculation have been related to harsh environmental conditions (e.g., dryland, limited fertilization and high temperatures), especially during the initial stages of plant growth (Cesari et al., 2019; Caires et al., 2020). In this study, although supplementary irrigation was performed, total rainfall was poor and not well distributed in all the wheat growing seasons (**Suppplementary Figures S1A,B**). In addition, high temperatures were observed during the field trial (maximum and minimum temperature above 30 and 15°C in most of the cases, respectively). Thus, plant growth response to inoculation with *A. brasilense* under tropical conditions would be more noticeable, as verified in this study. Nonetheless, considering that *Azospirillum* sp. are found in almost every living soil on the earth and able to colonize more than 100 plant species (Pedrosa et al., 2020), further investigation should be performed under subtropical and temperate climate conditions.

The higher gross revenue provided by *A. brasilense* due to the greater wheat grain yield overcame the increased expense in TOE with inoculation. It was verified that inoculation associated with the average N application level provided greater operating profit (US\$ 493 ha^{-1}), 17.3% higher than the most profitable treatment in the absence of inoculation (100 kg N ha^{-1} without inoculation - US\$ 420 ha^{-1}) (**Table 1**). The results showed that it would be possible to reduce N fertilization level to the low when inoculation is performed without compromising profitability. In fact, the operating profit obtained from the low N application level associated with inoculation (US\$ 464 ha^{-1}) was greater than the operating profit obtained by applying average N level without inoculation (US\$ 420 ha^{-1}). This suggests that it is possible to increase operating profit by 10.5% while reducing N application by 50% (**Table 1**). Moreover, the grouped PCA biplot graphs indicated that *A. brasilense* could reduce N application from the high level to average level benefiting N accumulation in shoot and root and biomass production; while enhance the potential for greater AE, grain yield and grain N uptake limited to low N application when inoculation is not performed (**Figures 3A–D**). It has been reported that inoculation with *A. brasilense* can generate an economy of 1.2 billion dollars per year with half substitution of N-based fertilizer application required by wheat and maize crops (Hungria et al., 2010). Similarly, Caires et al. (2020) concluded that since wheat seed inoculation *A. brasilense* has demonstrated economic viability, the strategy of side-dressing N application could be changed and a lower N level may be applied to wheat crops. For instance, it should be also highlighted that the obtained results should be carefully considered for the specific conditions tested (no-till, specific N fertilizer type, environmental conditions, wheat cultivar etc.) and do not allow general recommendations worldwide. However, the use of PGPR has quite significant role in alleviation of environmental changes under the context of climate extremes and excessive use of fertilizer in agricultural soils (Backer et al., 2018). In addition, the use of these beneficial microorganisms is considered one of the most promising alternatives for safe crop-management practices as it could improve plant nutrition while reducing the dependance of N-fertilizer application (Koskey et al., 2021). For these reasons, studies with PGPR such as *Azospirillum brasilense* should be performed under different production systems (e.g., organic and conventional farming systems) and climatic conditions (tropical, subtropical and temperate environments).

The prospective studies need to evaluate the effects of agronomic practices, climate changes and plant growth-promoting rhizobacteria inoculation under different agroecosystems. The combination of some refined scientific techniques (*e.g.,* metabolomics, isotopic and molecular techniques, among others) are needed to deepen the knowledge of multiple mechanisms and benefits of plant growth-promoting rhizobacteria in soil-plant-environment system.

CONCLUSION

In view of low economic cost, ease of application and with a high probability of response by wheat crop, even associated with different N application levels, the inoculation with *A. brasilense* prone to be a key management strategy to improve sustainable wheat production. This practice will also be needed to help fomenting wheat crop expansion under tropical conditions by reducing the dependency of N-fertilizer applications. In addition, *A. brasilense* inoculation could be a key pillar in combating hunger, promoting food security, and environmental sustainability. The results have revealed that inoculation with *A. brasilense* enhanced agronomic efficiency and apparent N-fertilizer recovery by wheat plants, benefiting shoot and root development and leading to an increased grain yield by 10.3%). The increased total N accumulation observed occurred mainly as a result of the enhanced N-NH_4^+ accumulation in wheat root and shoot. *Azospirillum brasilense* inoculation associated with application of average N application level provided the greatest operating profit compared with any other treatment. However, the results also showed that a reduction in the required amount of N needed for optimum profitability is possible when inoculation is combined with low N application level tested in this study. This represents a reduction of 50% in the amounts of N needed for optimum production.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Design the experiment: FSG and MCMTF. Obtain and process the data: FSG, GCF, WLR, and EHMB. Analyze the data: FSG. Wrote the paper: FSG, PHP, AJ, JL, and MCMTF with contribution of all co-authors. All authors confirm being contributor of this work and has approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.821628/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.