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# *Bacillus thuringiensis*, *B. fungorum* and *B. bombysepticus*: species that enhance nutrient absorption and maize productivity

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**Abstract:** The objective was to evaluate the agronomic efficiency of the inoculant PRO-MIC-023-23B in different application modes and in association with fixed nitrogen doses corresponding to 50% and 100% of the soil requirement, in promoting the growth and productivity of maize crops. Five field experiments were conducted during the 2023/2024 growing season across different edaphoclimatic regions under rainfed conditions. A randomized block design was adopted, with ten treatments and ten replicates, with two controls using only chemical nitrogen fertilizer (50 and 100% of the dose) and two controls using the standard commercial inoculant. The inoculant PRO-MIC-023-23B is a pre-commercial liquid formulation composed of *Bacillus thuringiensis* isolate CMFAM035, *Bacillus fungorum* isolate CMFAC001, and *Bacillus bombysepticus* isolate CMFPN027, applied in seed treatment, sowing furrow and at $V_3$ and $V_6$ phenological stages. Joint analysis of variance was performed considering nested blocks. For variables showing treatment-by-environment interaction, the genotype plus genotype-by-environment (GGE) model was used. The GGE Biplot for mean performance versus stability and genotype ranking explained a high proportion of the variation in all measured variables. Results indicated that *Bacillus* application was significant for improving soil nitrogen use, offering a sustainable alternative. *Bacillus* species demonstrated synergistic effects in promoting growth and enhancing nutrient accumulation in leaves and grains, leading to increases up to 5.75% of maize grain yield. Complementing nitrogen fertilization in maize with the inoculation of PRO-MIC-023-23B in the sowing furrow associated with a dose of 100% nitrogen showed the greatest stability and the greatest grain yield potential among all the treatments tested.

**Keywords:** Plant growth-promoting rhizobacteria, *Zea mays* L., Nutrient use efficiency, Sustainable agriculture, GGE Biplot.

## Introduction

Nitrogen (N) is one of the nutrients widely used in most production systems (Lassaletta et al., 2014), a practice that seeks to meet the nutritional demand of crops and increase yields (Yang et al., 2015). However, several literatures indicate that N use efficiency is relatively low in most regions of the world, particularly in regions where the nutrient is applied in its synthetic form (Guo et al., 2010; Lassaletta et al., 2014; Swaney et al., 2018). This reduction in efficiency is caused by high temperatures and low soil moisture, which causes N losses through volatilization (Scremin et al., 2020). Furthermore, excess nitrogen in some cases leads to potential environmental risks, such as groundwater contamination due to nitrate leaching (Kim et al., 2015), acidification-induced soil degradation (Guo et al., 2010), and greenhouse gas ($N^2O$) emissions (Wang et al., 2017).

The current demand for high productivity in grain crops, especially maize (*Zea mays* L.), triggers the need to improve technologies to be adopted in management. During its development, this crop exhibits changes in the dynamics of nutrient requirements, with a progressive increase in N absorption and accumulation throughout the cycle (Yang et al., 2024). N deficiency in maize reduces root and shoot development, biomass production, number of grains per ear and, consequently, grain yield (Qi et al., 2020). Given this, it is necessary to seek ways and technologies to better use nutrients, where, once this use increases, farmers can reduce production costs and, consequently, increase profitability with less potential for environmental degradation.

In response to the needs of agricultural and environmental development, plant growth-promoting rhizobacteria (PGPR), a cost-effective and sustainable plant inoculant, have attracted great attention in recent years (Mitra et al., 2021). As the main focus of this study, the use of these beneficial microorganisms can lead to a reduction in the need for chemical fertilizers, especially nitrogen and phosphate fertilizers (Igiehon and Babalola, 2017). In this context, *Bacillus* is one of the most abundant genera in the rhizosphere, and this region, defined as a thin layer of soil around the roots, represents the primary site for nutrient absorption, as well as a region where important physiological, chemical, and biological activities occur (Hashem et al., 2019). The different species of this genus have the ability to produce metabolites, which in addition to stimulating plant growth, prevent infection by pathogens (Radhakrishan et al., 2017). These microorganisms act through association with roots and other parts of the plant, being identified as PGPR for maize because they perform biological nitrogen fixation, phosphate solubilization and phytohormone production, with emphasis on auxin, cytokinin and gibberellin (Kuan et al., 2016).

Given the known PGPR potential of species of the genus *Bacillus*, the objective was to evaluate the agronomic efficiency of the inoculant PRO-MIC-023-23B (*Bacillus thuringiensis* isolate CMFAM035; *Bacillus fungorum* isolate CMFAC001 and *Bacillus bombysepticus* isolate CMFPN288), in different application modes and in association with fixed nitrogen doses corresponding to 50% and 100% of the soil requirement, in promoting the growth and productivity of maize crops.

**Results and Discussion**

***Population of diazotrophic bacteria and meteorological variables***

The population of diazotrophic bacteria at the time of sowing presented values of $2.2x10^5$ NMP $g^{-1}$ in Bandeirantes-PR, $5.7x10^4$ NMP $g^{-1}$ in Ponta Grossa-PR, $1.7x10^4$ NMP $g^{-1}$ in Ouro-SC and $6.6x10^4$ NMP $g^{-1}$ in Artur Nogueira-SP. It is noted that these bacteria had a low influence on the results of the experiment, since the effects observed with the inoculation of the tested product were compared with the controls with nitrogen fertilization and with the standard inoculation treatment, which were also exposed to the same population of bacteria present in the soils.

Understanding the dynamics of meteorological variables (Figure 1) during the experimental development period is crucial to explain the results obtained. In general, it is observed that air temperature was not a limiting factor for the growth and development of maize, with the highest frequency of observations between 22 and 30 °C, and was also favorable for the propagation of PGPR. Askari-Khorasgani et al. (2019) reported that the optimal temperature for the development of rhizobacteria varies between 25 and 30 °C, while Zhang et al. (2023) found that some genera of PGPR, including *Bacillus*, can tolerate temperatures above 40 °C. For maize, the optimal temperature varies between 25 and 33 °C (Waqas et al. 2021). Regarding precipitation, there was a certain water restriction during the reproductive period in Bandeirantes/PR and Ponta Grossa/PR, which may have impacted grain yield. However, it can be considered that, in general, precipitation accumulations are adequate for the maize cycle.

***Joint analysis of variance***

The joint analysis of variance (Table 1) shows a significant effect for the treatment x environment interaction (TxE) for all measured variables, except for shoot length, root length and thousand grain weight. In addition, a significant effect of environment and treatment was observed on the variables fresh root weight, shoot dry weight, shoot length, root length and thousand grain weight. However, the main effects of these factors will not be reported in this study, taking into account only the variables with significant TxE interaction. The coefficient of variation ranged between 2.34 and 7.92% among the measured variables, which provides reliability to the information obtained. Given the existence of TxE interaction, it is possible to implement the GGE model to verify the performance of the treatments and environments tested.

***GGE Biplot for mean versus stability analysis***

The GGE Biplot of mean performance versus stability explained a high proportion of the variation for all measured variables (Figure 2). An explicability of the information of 87.84% (PC1: 74.92%; PC2: 12.92%) and 96.80% (PC1: 94.72%; PC2: 2.08%) was obtained for the variables fresh weight of aerial part and dry weight of root. The total explanatory power was 92.83% (PC1: 80.84%; PC2: 11.99%), 84.40% (PC1: 60.45%; PC2: 23.95%) and 80.74% (PC1: 63.16%; PC2: 17.58%) for the accumulation of nitrogen, phosphorus and potassium in the leaves, respectively. In the same sense, the percentages were 94.37%, 93.30 and 93.57% for nitrogen in the grains, protein in the grains and grain yield. In this analysis, the higher the value of the X axis (PC1) the more productive the treatment, while the Y axis (PC2) describes the stability of the genotype (Yan et al., 2000).

As described in the study by Klein et al. (2024), the straight line passing through the origin of the Biplot is called Average Environmental Coordination (AEC), used to classify treatments based on performance (Yan and Kang, 2003). The arrow on the abscissa of the AEC indicates a higher average performance, classifying the genotypes accordingly (Yan et al., 2007). A line perpendicular to the AEC represents performance stability, in which the greater distance from the origin indicates greater instability (Yan, 2011). Thus, treatment T7 (PRO-MIC-023-23B at $V_3$ and $V_6$ + 100% N) obtained the best performance for shoot fresh weight (Figure 2a), with an average of 512.67 g and good stability. There was little difference for treatments T9 (Utrisha™ N + 100% N), T3 (PRO-MIC-023-23B in seed treatment, $V_3$ and $V_6$ + 100% N) and T5 (PRO-MIC-023-23B in seed furrow, $V_3$ and $V_6$ + 100% N), with averages of 511.47, 508.47 and 507.60 g, respectively. The results show that the performance of the tested inoculant was similar to the commercial inoculant, regardless of the application method. A similar ranking of treatments was attributed to root dry weight (Figure 2b), with averages of 128.10, 127, 125.90 and 124 for T9, T7, T5 and T3, respectively. However, there was little difference between the treatments with inoculation and T1 (100% N) for both variables, which does not justify the complementary use of inoculation in this case. Nitrogen accumulation in leaves (Figure 2c) showed superior performance for T6 (PRO-MIC-023-23B in furrow, $V_3$ and $V_6$ + 50% N), which obtained good stability when confronted with the different environments, with an average of 15.30 g $kg^{-1}$. The subsequent treatments were T4 (PRO-MIC-023-23B in seed treatment, $V_3$ and $V_6$ + 50% N) and T5, with average values of

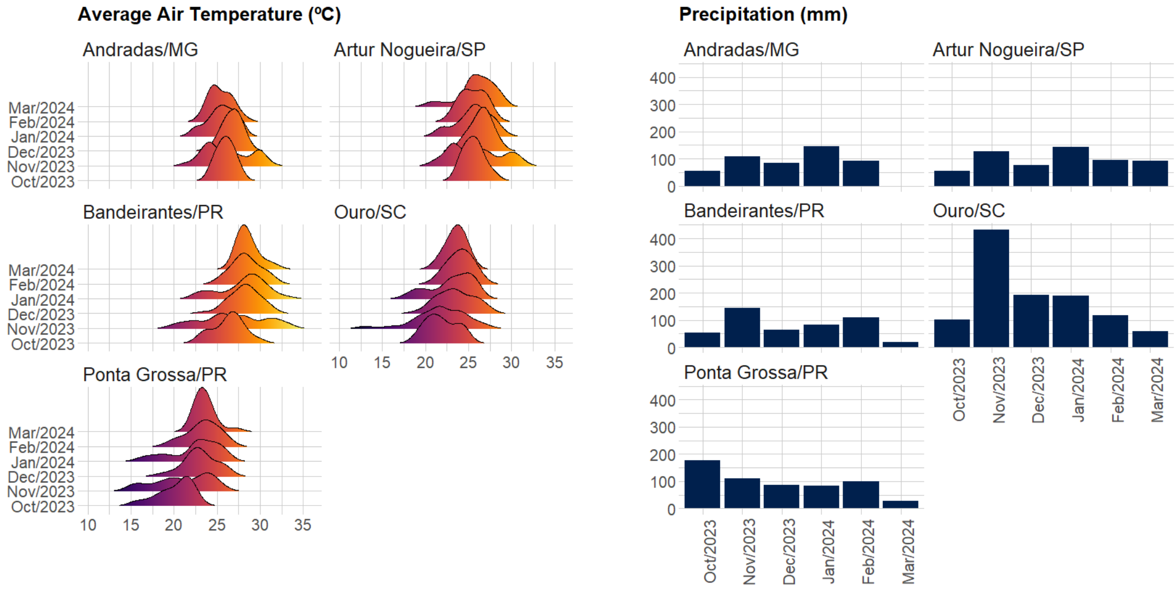


**Fig 1.** Meteorological data on mean air temperature (ºC) and accumulated precipitation volume (mm) for the maize development cycle in the environments of Andradas/MG, Artur Nogueira/SP, Bandeirantes/PR, Ouro/SC and Ponta Grossa/PR.

**Table 1.** Joint analysis of variance for the effect of ten treatments and five environments on the agronomic traits of maize crops.

| SV | DF | Mean Square | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | SFW | RFW | SDW | RDW | SL | RL | LN |
| ENV | 4 | 188749* | 5879.4* | 10380.9* | 1547.2* | 6196.7* | 516.94* | 363.27* |
| REP(ENV) | 25 | 279 | 54.3 | 104.00 | 56.6 | 29.4 | 1.7 | 0.423 |
| TREAT | 9 | 6608* | 1737.8* | 317.6* | 1112.5* | 189.1* | 11.0* | 16.34* |
| TREAT x ENV | 36 | 620* | 66.3 | 51.2 | 187.9* | 40.5 | 3.58 | 5.43* |
| Residuals | 225 | 400 | 88.7 | 57.8 | 43.7 | 29.3 | 3.02 | 0.323 |
| CV (%) | - | 4.04 | 7.94 | 6.58 | 9.92 | 2.95 | 6.09 | 4.04 |
| Overall Mean | - | 495.25 | 118.56 | 115.62 | 66.63 | 183.31 | 28.55 | 14.06 |

| SV | DF | Mean Square | | | | | |
|---|---|---|---|---|---|---|---|
| | | LP | KL | NG | GP | TGW | GY |
| ENV | 4 | 31.61* | 167.89* | 11.43* | 13.42* | 38377.1* | 41491284* |
| REP(ENV) | 25 | 0.06 | 0.44 | 0.41 | 0.23 | 250.3 | 86489 |
| TREAT | 9 | 0.79* | 25.68* | 2.44* | 1.32* | 177.8 | 541889* |
| TREAT x ENV | 36 | 0.20* | 13.01* | 0.92* | 0.46* | 86.8 | 135651* |
| Residuals | 225 | 0.06 | 0.42 | 0.15 | 0.09 | 151.4 | 37101 |
| CV (%) | - | 7.92 | 4.19 | 4.06 | 4.05 | 4.31 | 2.34 |
| Overall Mean | - | 3.21 | 15.48 | 9.52 | 7.20 | 285.20 | 8236.28 |

SV: source of variation; DF: degrees of freedom; SFW: fresh shoot weight (g); RFW: fresh root weight (g); SDW: dry shoot weight (g); RDW: root dry weight (g); SL: shoot length (cm); RL: root length (cm); LN: leaf nitrogen (g kg$^{-1}$); LP: leaf phosphorus (g kg$^{-1}$); LK: leaf potassium (g kg$^{-1}$); NG: grain nitrogen (g kg$^{-1}$); GP: grain protein (%); TGW: thousand grain weight (g); GY: grain yield (kg ha$^{-1}$); ENV: environment effect; REP(ENV): nested block effect; TREAT: treatment effect; TREAT x ENV: treatment × environment interaction effect; CV: coefficient of variation.

15.02 and 14.59 g kg$^{-1}$, where T4 was less stable compared to the aforementioned treatments. These are promising results, as they differed from both the nitrogen control, with 13.49 and 12.84 g kg$^{-1}$ for T1 and T2 (50% N), and the control with commercial inoculant, which presented averages of 13.81 and 13.75 g kg$^{-1}$ for T9 and T10 (Utrisha™ N + 50% N), respectively. These findings corroborate the study by Kuan et al. (2016), who concluded that plants inoculated with PGPR obtained an increase in N absorption, which resulted in green leaves for longer, a higher concentration of indole-3-acetic acid (IAA) and, consequently, a higher grain yield.

The evaluation of phosphorus and potassium accumulation in the leaves (Figures 2d; 2e) is not directly related to the nitrogen chemical fertilization applied in the treatments, but rather to the potential of the microorganisms tested to enhance the accumulation of these nutrients. Thus, greater effectiveness of T3 was observed in both phosphorus and potassium accumulation, with averages of 3.35 and 16.82 g kg$^{-1}$, respectively. However, there was some instability of this treatment in the case of phosphorus when compared to T9 and T5, with average performance of 3.31 and 3.32 g kg$^{-1}$. The multiple capacity to increase the absorption of these nutrients, not only nitrogen, is linked to other PGP abilities, such as phosphate solubilization and production of phytohormones that promote root development, where in addition to IAA, cytokinin and

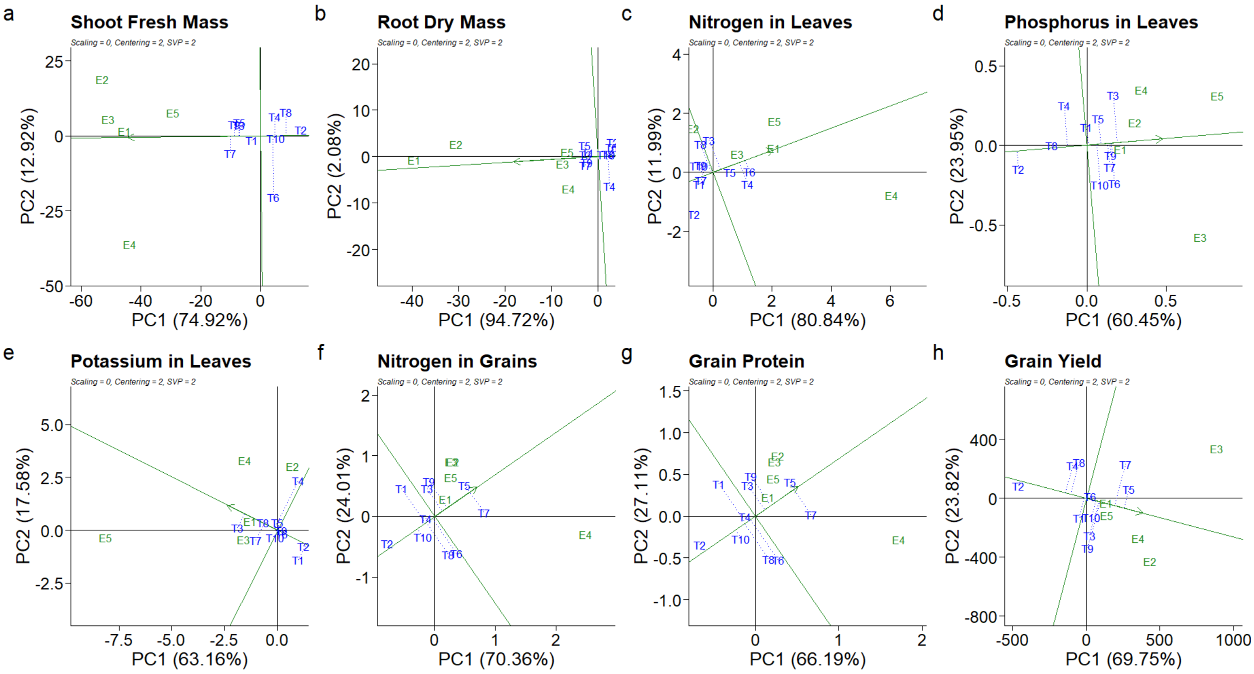


**Fig 2.** Biplot of treatment-environment interaction on mean performance vs. stability of ten inoculation treatments tested in five environments for the expression of agronomic components of maize crop. T1: 100% N; T2: 50% N; T3: PRO-MIC-023-23B in seed treatment, $V_3$ and $V_6$ + 100% N T4: PRO-MIC-023-23B in seed treatment, V3 and V6 + 50% N; T5: PRO-MIC-023-23B in furrow, $V_3$ and $V_6$ + 100% N; T6: PRO-MIC-023-23B in furrow, V3 and V6 + 50% N; T7: PRO-MIC-023-23B in $V_3$ and $V_6$ + 100% N; T8: PRO-MIC-023-23B in V3 and V6 + 100% N; T9: Utrisha™ N + 50% N; T10: Utrisha™ N + 100% N; E1: Bandeirantes/PR; E2: Ponta Grossa/PR; E3: Andradas/MG; E4: Artur Nogueira/SP; E5: Ouro/SC.

gibberellin also stand out (Kuan et al., 2016; Wu et al., 2009). This instability was not observed to the same extent for potassium accumulation, where T3 was 15.82 and 17.84% higher compared to T1 and T2, respectively, with emphasis on the T7 treatment, with accumulation of 16.39 g $kg^{-1}$.

Due to the way it was calculated, it is defined that grain protein is dependent on the nitrogen content in the grains, that is, the performance of the treatments is identical, with a change only in the magnitude of the observed values (Figure 2f; 2g). Treatments T5 and T7 presented practically identical average values, which resulted in averages of 9.92 and 7.49% for nitrogen and protein in the grains, respectively, where T5 was more stable under environmental variation. From these results, greater stability is verified with complementary inoculation in the sowing furrow, when compared only with $V_3$ and $V_6$. It can be inferred that the greater viability of bacteria through application via the sowing furrow may have caused greater absorption of N and subsequent translocation, also in greater quantities, to the grains. Wang et al. (2021) found a very strong linear correlation between nitrogen accumulation in the leaves and yield and protein content of maize grains. This highlights how promising the complementary effect of inoculation is to improve the use of nitrogen in the soil, showing its superiority in relation to the isolated application of nitrogen fertilization.

These treatments also stood out in grain yield (Figure 2h), where T5 was again more stable than T7, with averages of 8405.37 and 8353.91 kg $ha^{-1}$, respectively. Compared to the control with nitrogen fertilization only, T5 and T7 achieved yield increases of 5.74% and 5.15%. The lowest stability was conferred to treatments T9 and T3, which shows that, even with averages of 8292.86 and 8308.45 kg $ha^{-1}$, they were more sensitive to environmental variation. The variability observed for the expression of grain yield may not be related only to the instability of certain treatments, but to the high environmental influence and genotype-environment interaction (GxE) of the hybrids used. The genetic effect on the expression of maize grain yield is less than 25% (Nardino et al., 2016), with environmental variations being more relevant for the variation and instability of the trait (Carvalho et al., 2018). However, the results are favorable to enhance the use of nitrogen fertilization, where most treatments were superior to the control with nitrogen fertilization. The grain yield values obtained were higher than those obtained in other studies for the evaluation of PGPR bacteria and nitrogen fertilization, which refers to 6261 kg $ha^{-1}$ obtained from the use of *Azospirillum brasilense* (Smaniotto et al., 2023) and 6476 kg $ha^{-1}$ from the use of 300 kg $ha^{-1}$ of N-fertilizer (Albert et al., 2023).

### *GGE Biplot for evaluating and ranking environments*

The environment comparison analysis (Figure 3) shows the ranking of the experimental sites in comparison to an environment considered "ideal" for each measured variable, which is represented by the circle on the abscissa of the arrow (Klein et al., 2024). The explainability of the information was high for all measured characters, being: 87.84% (PC1: 74.92%; PC2: 12.92%) for fresh weight of aerial part, 96.80% (PC1: 94.72%; PC2: 2.08%) for dry weight of root, 92.83% (PC1: 80.84; PC2: 11.99%) for nitrogen in leaves and 84.40% (PC1: 60.45%; PC2: 23.95%) for phosphorus in leaves. Furthermore, an explainability of 80.74% (PC1: 63.16%; PC2: 17.58%) was verified for potassium in leaves, 94.37% (PC1: 70.36%; PC2:

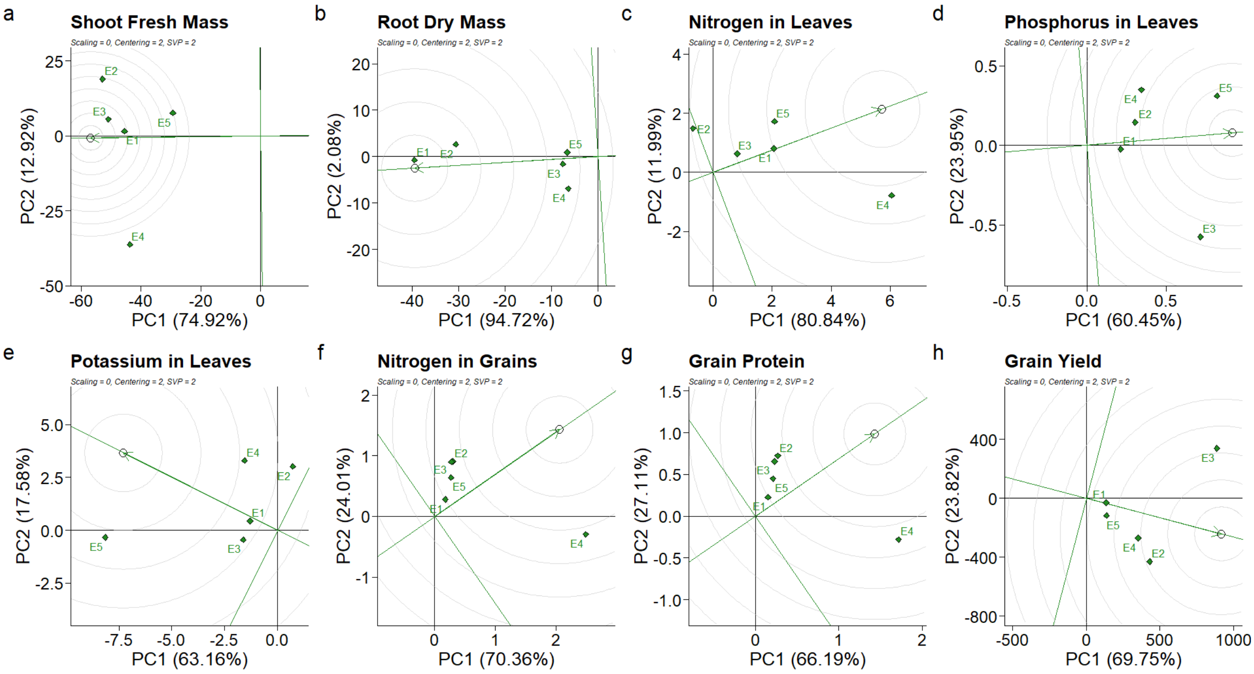


**Fig 3.** Biplot of treatment-environment interaction in the ranking and representation of the ideal environment for the expression of agronomic components of maize crop. E1: Bandeirantes/PR; E2: Ponta Grossa/PR; E3: Andradas/MG; E4: Artur Nogueira/SP; E5: Ouro/SC.

24.01%) for nitrogen in grains, 93.30% (PC1: 66.19; PC2: 27.11%) for grain protein and 93.57% (PC1: 69.75%; PC2: 23.82%) for grain yield.

The high proportion of explained variance across variables reinforces the robustness of the model in capturing both treatment performance and environmental effects. This analysis was essential for identifying how environmental variation influenced the expression of treatment effects across environments. By using the GGE biplot, it was possible to visualize the stability and adaptability of the inoculant-based treatments across contrasting environments, highlighting both favorable and limiting conditions for each agronomic trait evaluated.

The ideotype and the ideal environment are represented by the center of the circle and, therefore, the most desirable treatments and environments are those plotted within or in the circles close to the ideotype (Yokomizo et al., 2020). Since the study does not aim to identify desirable environments for future experiments, the discussion of this analysis should be carried out from the point of view of identifying which environments were most favorable for the expression of each variable studied. Thus, it was identified that E3 (Andradas/MG) and E1 (Bandeirantes/PR) were the most favorable for fresh weight of aerial parts (Figure 3a), while E4 (Artur Nogueira/SP) was the least favorable. Environment E1 was very close to the ideal environment for dry weight of roots (Figure 3b), as it presented little variation in the values observed in all treatments. There was no environment considered ideal for nitrogen accumulation in leaves (Figure 3c), with E1, E4 and E5 (Ouro/SC) being the closest, while E2 (Ponta Grossa/PR) was the least desirable environment.

Phosphorus accumulation in leaves showed environment E5 as the most favorable (Figure 3d), while the other environments were distant and grouped in the same division of the GGE, with E3 being the most distant. A similar behavior was observed for potassium accumulation in leaves (Figure 3e), however, with E5 being less close to the ideal environment. E2 was the environment with the lowest contribution, and it can be inferred that there was great variability in the treatments in this case. For nitrogen in grains and protein in grains (Figures 3f and 3g), again, none of the environments were favorable, with E1 being the least desirable. Likewise, there was no desirable environment for grain productivity (Figure 3h), where E2 was the only environment that approached the ideal environment.

Overall, the environmental variability observed highlights the importance of testing the inoculant under diverse conditions. The consistent performance of certain treatments across environments indicates good stability and adaptability, reinforcing its potential for use in different production scenarios.

### *GGE Biplot for evaluating and ranking treatments*

The treatment ranking (Figure 4) also defines a treatment considered "ideal" for comparison with the treatments used. This is only symbolic and serves as a reference to evaluate the other treatments (Oliveira et al., 2019). The explainability of the information with the principal components was high for all traits, being: 87.84% (PC1: 74.92%; PC2: 12.92%) for fresh weight of aerial part, 96.80% (PC1: 94.72%; PC2: 2.08%) for dry weight of root, 92.83% (PC1: 80.84%; PC2: 11.99%) for nitrogen in leaves, 84.40% (PC1: 60.45%; PC2: 23.95%) for phosphorus in leaves. Furthermore, the explainability was 80.74% (PC1: 63.16%; PC2: 17.58%) for potassium in leaves, 94.37% (PC1: 70.36%; PC2: 24.01%) for nitrogen in grains, 93.30% (PC1: 66.19%; PC2: 27.11%) for protein in grains and 93.57% (PC1: 69.75%; PC2: 23.82%) for grain yield.

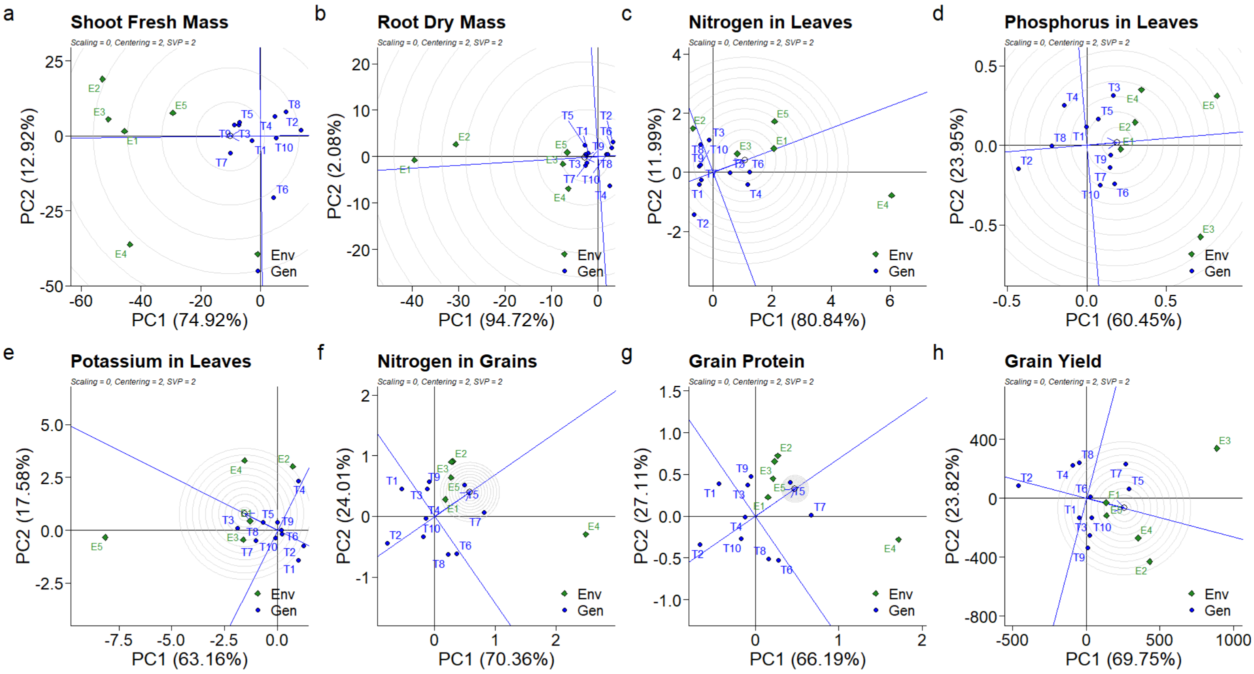


**Fig 4.** Biplot of treatment-environment interaction in the ranking and representation of the ideal treatment for the expression of agronomic components of maize crop. T1: 100% N; T2: 50% N; T3: PRO-MIC-023-23B in seed treatment, $V_3$ and $V_6$ + 100% N T4: PRO-MIC-023-23B in seed treatment, $V_3$ and $V_6$ + 50% N; T5: PRO-MIC-023-23B in furrow, V3 and V6 + 100% N; T6: PRO-MIC-023-23B in furrow, $V_3$ and $V_6$ + 50% N; T7: PRO-MIC-023-23B in V3 and V6 + 100% N; T8: PRO-MIC-023-23B in $V_3$ and $V_6$ + 100% N; T9: Utrisha™ N + 50% N; T10: Utrisha™ N + 100% N; E1: Bandeirantes/PR; E2: Ponta Grossa/PR; E3: Andradas/MG; E4: Artur Nogueira/SP; E5: Ouro/SC.

When analyzing the fresh weight of aerial parts (Figure 4a), the GGE shows treatments T1, T3, T5, T7 and T9 within the circle of the ideal treatment, which states that the proposed inoculant obtained similar performance to the nitrogen control and the standard inoculant for 100% N. There was little difference between the treatments for root dry weight (Figure 4b), outlined only by the difference in N dose, and, with the exception of treatment T10, which had 50% N, all treatments with 100% N were grouped together with the ideal genotype. Inoculation in the sowing furrow (T5 and T6) showed promise for nitrogen accumulation in the leaves (Figure 4c), while the nitrogen controls were the treatments furthest from the center. In addition, they were also superior in relation to the standard inoculant, which defines the potential for nitrogen accumulation promoted by the PGPR species tested in this study.

It is important to highlight those treatments varied not only in inoculant presence but also in application timing and method, including seed treatment, in-furrow, foliar, and combined approaches. These differences influenced nutrient uptake patterns and overall crop performance, as explored in the subsequent analyses. Notably, treatments combining early inoculation (seed or in-furrow) with foliar application, such as T3 and T5, tended to cluster closer to the ideal genotype, suggesting additive or synergistic effects of sequential applications. Treatments relying solely on foliar application (T7 and T9) also performed well, particularly in traits related to nutrient accumulation.

The ranking T9>T7>T5>T1>T6 was observed for phosphorus accumulation in the leaves (Figure 4d), with T9 being the treatment closest to the center, which shows that, through this analysis, the application of inoculants via foliar application was more favorable for phosphorus absorption. For potassium accumulation in the leaves (Figure 4e), T3>T5>T8 was found to be the order of promising treatments, which shows that inoculation at the time of maize sowing, both in the seed treatment and in the furrow, was more effective. The analysis for nitrogen and protein content in the grains (Figure 4f; Figure 4g) highlights the T5 treatment as the most favorable, being plotted in the circle with the ideal treatment, in addition to presenting affinity with most of the environments tested, with T7 being the only treatment to present similar performance for these traits. Inoculation in the sowing furrow (treatment T5) obtained the best performance for grain yield, being the genotype closest to the center, while T2, T4 and T8 were the least desirable treatments.

The results show that the application of *Bacillus* was significant for the use of nitrogen in the soil, being a sustainable alternative. The species *B. thuringiensis*, *B. fungorum* and *B. bombysepticus* showed synergism in promoting growth and increasing the accumulation of nutrients in the leaves and grains and, consequently, increased the grain yield of maize.

## Materials and Methods

### *Experimental design and plant material*

Five experiments were carried out during the 2023/2024 harvest (Figure 5), in different edaphoclimatic regions without the presence of an irrigation system (Bandeirantes/PR, Ponta Grossa/PR, Andradas/MG, Artur Nogueira/SP, Ouro/SC), to evaluate the effect of inoculation with the pre-commercial product called PRO-MIC-023-23B on the agronomic traits of maize. The full description of the climate, soil type, location, sowing and harvest dates of each environment can be found in

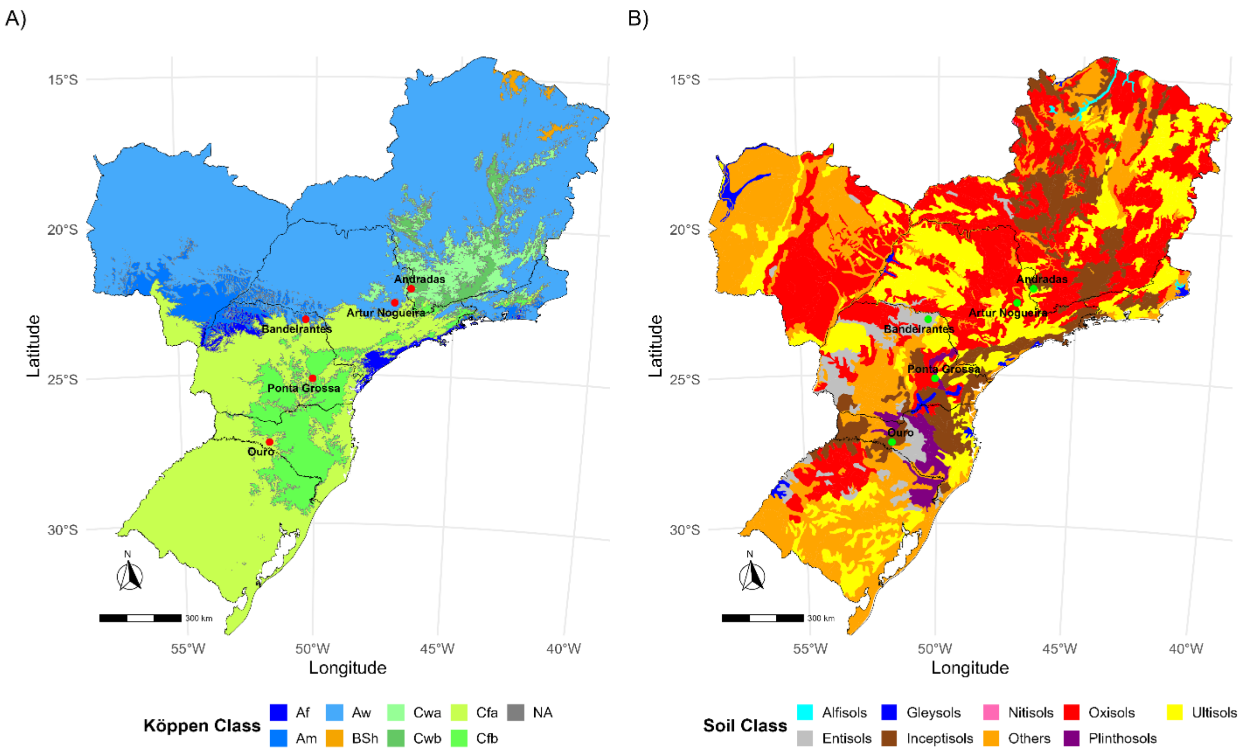


**Fig 5.** Edaphoclimatic characterization of the study area, showing the Köppen climate classification (A) and soil types (B), with the geographical location of the experimental environments indicated. Köppen Raster Source: Beck et al. (2023); Soil Class Raster Source: Santos et al. (2024).

Supplementary Table 1. Before installing the experiments, the population of diazotrophic bacteria present in the soil was determined using the Most Probable Number (MPN) methodology described by Döbereiner et al. (1995). For this, a soil sample from each experimental area was sent for analysis to an accredited laboratory.
The experimental design used was randomized blocks with ten treatments and ten replicates. Each experimental unit had a total area of 21.0 $m^2$ (3.0 m x 7.0 m), with a useful area of 5.0 $m^2$. The maize hybrid NK Feroz Viptera 3 was used in the environments of Bandeirantes/PR, Andradas/MG, and Artur Nogueira/SP, with characteristics of early cycle, hard orange grain, indicated for grain and silage production, good tolerance to the stunting complex, and low nematode reproduction factor. In the environments of Ponta Grossa/PR and Ouro/SC, the hybrid DKB 235 PRO3 was used, characterized by presenting a super early cycle, yellow-orange grain, indicated for silage production, 864 GDU for flowering, and tolerance to *Turcicum*. Both cultivars were sown with a spacing of 0.5 m between rows and 3.1 plants per linear meter, with a desired population of 62 thousand plants per hectare. The base fertilizer used was 300 kg $ha^{-1}$ of the 00-20-20 formula, with no nitrogen in its formulation. Phytosanitary treatments were performed preventively, in order to reduce the effect of biotic factors on the results of the experiment.

***Description of the inoculant, treatments and application methods***
The PRO-MIC-023-23B inoculant is a pre-commercial liquid product composed of the bacteria *Bacillus thuringiensis* isolate CMFAM035; *Bacillus fungorum* isolate CMFAC001 and *Bacillus bombysepticus* isolate CMFPN288, with a concentration of $8x10^7$ CFU $ml^{-1}$ and absence of contaminants. The commercial inoculant Utrisha™ N, used as standard treatment, is a solid product composed of the bacteria *Methylobacterium symbioticum* isolate SB23, with a concentration of $3.0x10^7$ CFU $g^{-1}$. (Corteva Agriscience, 2023). Treatments T1 and T2 consisted of the control with nitrogen fertilization, with doses of 100 and 50%, corresponding to 100 and 50 kg $ha^{-1}$ of urea (45% N), respectively. PRO-MIC-023-23B was applied in seed treatment (T3; T4), in the sowing furrow (T5; T6) as well as in phenological stages $V_3$ and $V_6$ (T3; T4; T5; T6; T7; T8), following the phenological scale of Ritchie *et al.* (1993). The T9 and T10 treatments are characterized by the application of the commercial inoculant Utrisha™ N at the $V_4$ phenological stage. The full description of the treatments used can be seen in Supplementary Table 2. The inoculation in the seed treatment was performed manually, using identified plastic bags, in which 1000 g of seeds and the equivalent volume of PRO-MIC-023-23B were deposited, with a spray volume of 500 mL per 100 kg of seeds. The application in the sowing furrow and via foliar of the treatments was carried out with a constant pressure backpack sprayer, propelled by pressurized $CO_2$, with a spray tip XR110:02 and spray volume of 60 and 150 L $ha^{-1}$, respectively.

***Measured variables***
Five complete plants were collected in the central area of each experimental unit, outside the area for productivity evaluation, when the crop was in the tasseling stage, to determine the shoot length (SL, cm), root length (RL, cm), shoot fresh weight (SFW, g), root fresh weight (RFW, g), shoot dry weight (SDW, g) and root dry weight (RDW, g). The dry weight

was measured after drying in an oven at 65 ºC until constant weight. Nutrient accumulation was determined in samples of indicator leaves collected in the tasseling stage, with nitrogen (NL, g kg$^{-1}$) determined by the semi-micro Kjeldahl method and phosphorus (PL, g kg$^{-1}$) and potassium (KL, g kg$^{-1}$) determined by nitric-perchloric solubilization followed by spectrometric reading with vanadate yellow, according to the methodology described by Carmo *et al*. (2000). The protein content (GP, %) in the grains was calculated by multiplying the nitrogen content in the grains (NG, %) by the correction factor 6.25. The grain yield (GY, kg ha$^{-1}$) and the thousand grain weight (TGW, g) were evaluated in 5.0 m$^2$ of each experimental unit, where the grains were cleaned and weighed, with moisture correction to 130 g kg$^{-1}$. Satellite data on mean air temperature (ºC) and precipitation were obtained with the aid of the NASA Power platform (NASA Power, 2023).

***Statistical analysis***

The data matrix obtained was subjected to the assumptions of normality of errors and homogeneity of variances by the Shapiro-Wilk and Bartlett tests, respectively. With the assumptions met, joint analysis of variance was performed considering nested blocks, following the following model: $Y_{ijk} = \mu + G_i + A_j + GA_{ij} + \frac{B}{A}_{jk} + \varepsilon_{ijk}$, where $Y_{ijk}$ is the random variable obtained in the experimental unit with the ith genotype in the jth environment in the kth block; μ is the general mean; $G_i$ is the effect of the ith genotype considered fixed; $A_j$ is the effect of the jth environment considered fixed; $GA_{ij}$ is the effect of the interaction of the ith genotype with the jth environment; $\frac{B}{A}_{jk}$ is the effect of the kth block within the jth environment considered random and; $\varepsilon_{ijk}$ is the random error.

The mean of ith genotype in jth environment is described by a general linear model, being: $\hat{y}_{ij} = \mu + \alpha_i + \beta_j + \phi_{ij}$, where $\hat{y}_{ij}$ is the estimate of the mean of ith genotype in jth environment; μ is the general mean; $\alpha_i$ is the main effect of ith genotype; $\beta_j$ is the main effect of jth environment and; $\phi_{ij}$ is the effect of the interaction between ith genotype and jth environment. In the model called genotype plus genotype environment interaction (GGE), the term $\alpha_i$ is excluded from the equation and the centralized environment matrix ($\phi_{ij}$) is subjected to singular value decomposition (SVD), based on the model described by Yan *et al*. (2007) and Wekai & Kang (2003), as follows: $\phi_{ij} = \hat{y}_{ij} - \mu - \beta_j = \sum_{k=1}^{p} \xi_{ik}^{\square}\eta_{jk}^{\square}$, being $\xi_{ik}^{\square} = \lambda_k^{\alpha}\xi_{ik\iota}\eta_{jk}^{\square} = \lambda_k^{1-\alpha}\eta_{jk}$, where $\lambda_k$ is the kth eigenvalue of the SVD; α is the singular value partition factor for the Principal Component (PC) k and; $\xi_{ik}^{\square}$ and $\eta_{jk}^{\square}$ are the PC scores for ith genotype in jth environment, respectively. The analyses were performed using the packages version 1.18.0 (Olivoto and Lúcio, 2020) and ggplot2 (Wickham, 2016), in the R software (R Core Team, 2024).

## Conclusion

Inoculation with PRO-MIC-023-23B in seed treatment and in the sowing furrow promoted nitrogen accumulation in the leaves higher than that observed for isolated chemical fertilization and for the standard commercial inoculant, with the potential to reduce the nitrogen fertilization dose by up to 50%. There was no difference between inoculation with PRO-MIC-023-23B and the standard commercial inoculant for the accumulation of phosphorus and potassium in the leaves, for applications in seed treatment and in the sowing furrow. The PRO-MIC-023-23B inoculant obtained the best performance for increasing protein in maize grains when applied via seed treatment and sowing furrow.

Complementing nitrogen fertilization in maize with the inoculation of PRO-MIC-023-23B in the sowing furrow associated with a dose of 100% nitrogen showed the greatest stability and the greatest grain yield potential among all the treatments tested.

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