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Potential for the Combined Application of Bacterial Inoculants and Micronutrients: An Evaluation of Microbiological Compatibility

Potencial de aplicação conjunta de inoculantes bacterianos e micronutrientes: uma avaliação da compatibilidade microbiológica

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ABSTRACT

The simultaneous application of inoculants and micronutrients has become an increasingly common practice in soybean production, particularly in in-furrow application systems, to optimize sowing operations. However, information regarding the compatibility between these inputs remains limited. Therefore, the present study aimed to evaluate the viability of *Bradyrhizobium japonicum* and *Azospirillum brasilense* following exposure to commercial formulations containing boron (B), cobalt (Co), molybdenum (Mo), and nickel (Ni). The assays were conducted in vitro using serial decimal dilutions, spread plating on potato dextrose agar (PDA), and colony-forming unit (CFU) enumeration. Data were subjected to analysis of variance (ANOVA), and treatment means were compared using the Scott–Knott test ($p \leq 0.05$). No significant differences were observed in the CFU counts of *A. brasilense* or *B. japonicum* among the evaluated treatments, indicating that the commercial micronutrient formulations did not compromise bacterial viability. Visual assessment of colony development corroborated the quantitative results, showing uniform bacterial growth

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across all treatments. The results indicate that the evaluated commercial formulations did not reduce the viability of Bradyrhizobium japonicum and *Azospirillum brasilense* during short-term in vitro exposure, suggesting microbiological compatibility under the laboratory conditions evaluated. Further greenhouse and field studies are required to confirm whether this compatibility is maintained under practical agricultural conditions.

Keywords: Biological nitrogen fixation; inoculants; microbiological compatibility; micronutrients; *Bradyrhizobium japonicum*; *Azospirillum brasilense*.

RESUMO

A aplicação simultânea de inoculantes e micronutrientes tem se tornado uma prática cada vez mais frequente na cultura da soja, principalmente em sistemas de aplicação via sulco, visando otimizar as operações de semeadura. Entretanto, a compatibilidade entre esses insumos ainda é pouco conhecida. O presente estudo teve como objetivo avaliar a viabilidade de *Bradyrhizobium japonicum* e *Azospirillum brasilense* quando expostos a formulações comerciais contendo boro (B), cobalto (Co), molibdênio (Mo) e níquel (Ni). Os ensaios foram conduzidos **in vitro**, utilizando diluição seriada decimal, plaqueamento em meio batata-dextrose-ágar (BDA) e contagem de unidades formadoras de colônias (UFC). Os dados foram submetidos à análise de variância e as médias comparadas pelo teste de Scott-Knott ($p \leq 0,05$). Não foram observadas diferenças estatísticas significativas na contagem de UFC de *A. brasilense* e *B. japonicum* entre os tratamentos avaliados, indicando que as formulações comerciais testadas não comprometeram a viabilidade celular dos inoculantes. A avaliação visual das colônias corroborou os resultados quantitativos, evidenciando crescimento uniforme em todos os tratamentos. Os resultados demonstram que as formulações comerciais contendo B, Co, Mo e Ni são compatíveis com *Bradyrhizobium japonicum* e *Azospirillum brasilense* nas condições avaliadas, indicando potencial para aplicação conjunta em sistemas de inoculação via sulco e contribuindo para a otimização operacional do manejo da cultura da soja.

Palavras-chave: fixação biológica de nitrogênio; inoculantes; compatibilidade microbiológica; micronutrientes; *Bradyrhizobium japonicum*; *Azospirillum brasilense*.

1. INTRODUCTION

Brazil has evolved from being a food importer in the 1940s to becoming one of the world's largest producers and exporters of agricultural commodities (Artuzo et al., 2018). Within this context, soybean (*Glycine max* (L.) Merrill) stands out as Brazil's leading export commodity, playing a strategic role in both the national economy and agribusiness (Morais et al., 2022). According to CONAB (2024), Brazilian soybean production in the 2023/24 growing season was estimated at 147.38 million tons, cultivated over an area of 46.03 million hectares.

The remarkable increase in soybean productivity achieved in recent decades is closely associated with the adoption of technologies that improve production efficiency, particularly inoculation with biological nitrogen-fixing bacteria (BNF)

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(Prando et al., 2022). Soybean requires approximately 80 kg of nitrogen for every ton of grain produced (Sfredo; De Oliveira, 2010), and this demand is almost entirely supplied through biological nitrogen fixation. Since soybean is not native to Brazil, Brazilian soils do not naturally harbor sufficient populations of symbiotic bacteria capable of establishing this association, making inoculation at sowing an essential management practice (Meyer, 2022).

Among the microorganisms used for this purpose, species of the genus *Bradyrhizobium* are the most important due to their ability to establish symbiosis with soybean roots and efficiently fix atmospheric nitrogen. According to Mendes, Dos Reis Junior and Da Cunha (2010), these bacteria can fix up to 300 kg ha^{-1} of nitrogen, virtually replacing nitrogen fertilization, reducing production costs, and contributing to environmental sustainability. Among the most agronomically important species are *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* (Hungria; Campo; Mendes, 2007).

In addition to *Bradyrhizobium*, the genus *Azospirillum* also possesses the ability to fix atmospheric nitrogen (Santos; Nogueira; Hungria, 2021). However, compared with *Bradyrhizobium*, *Azospirillum brasilense* contributes only a small fraction of the nitrogen required by soybean plants (Meyer, 2022). Its primary benefit lies in the production of phytohormones that stimulate root development, thereby enhancing water and nutrient uptake while increasing plant tolerance to abiotic stresses (Santos; Nogueira; Hungria, 2021).

In this context, co-inoculation consists of the simultaneous application of two or more microorganisms with complementary mechanisms of action to maximize their beneficial effects on crop performance (Bárbaro et al., 2009). The association between *Bradyrhizobium* spp. and *Azospirillum brasilense* represents one of the most successful examples of this technology, as these microorganisms act synergistically by combining highly efficient biological nitrogen fixation with enhanced root growth and plant development (Meyer, 2022).

Alongside the use of inoculants, the application of micronutrients has gained increasing attention in soybean production systems. Elements such as boron (B), cobalt (Co), molybdenum (Mo), and nickel (Ni) play essential roles in several

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physiological and metabolic processes, although they are required in relatively small quantities (Silva, 2022). These micronutrients can be supplied through soil, seed treatment, or foliar applications to correct or prevent nutritional deficiencies and promote optimal crop development (Comiran et al., 2020).

In recent years, the widespread adoption of in-furrow directed-jet application systems has encouraged the simultaneous application of biological inoculants and micronutrients in the same spray tank, reducing field operations and improving sowing efficiency (Meyer, 2022). However, this practice may expose beneficial microorganisms to chemical compounds capable of reducing their survival and viability, thereby compromising the effectiveness of biological nitrogen fixation.

Despite the increasing commercial adoption of this practice, studies evaluating the compatibility between inoculants and commercial micronutrient formulations remain limited. Therefore, the present study aimed to evaluate the viability of *Bradyrhizobium japonicum* and *Azospirillum brasilense* when combined with commercial formulations containing boron (B), cobalt (Co), molybdenum (Mo), and nickel (Ni), simulating mixture conditions commonly employed in soybean crop management.

2. MATERIALS AND METHODS

2.1 Experimental site

The study was conducted at the Biological Control Laboratory of the Federal University of Technology – Paraná (UTFPR), Dois Vizinhos Campus, Paraná State, Brazil (25°42′13″ S, 53°05′47″ W).

2.2 Biological products and micronutrients

Commercial inoculants containing *Bradyrhizobium japonicum* (SEMIA 5079 and SEMIA 5080; 5.0 × 10^9 CFU mL^{-1}) and *Azospirillum brasilense* (AbV5 and AbV6; 1.8 × 10^8 CFU mL^{-1}) were evaluated. Both inoculants were tested at the manufacturers' recommended application rate of 300 mL ha^{-1}.

Four commercial micronutrient formulations commonly used in soybean production were selected for the compatibility assays. Two formulations contained

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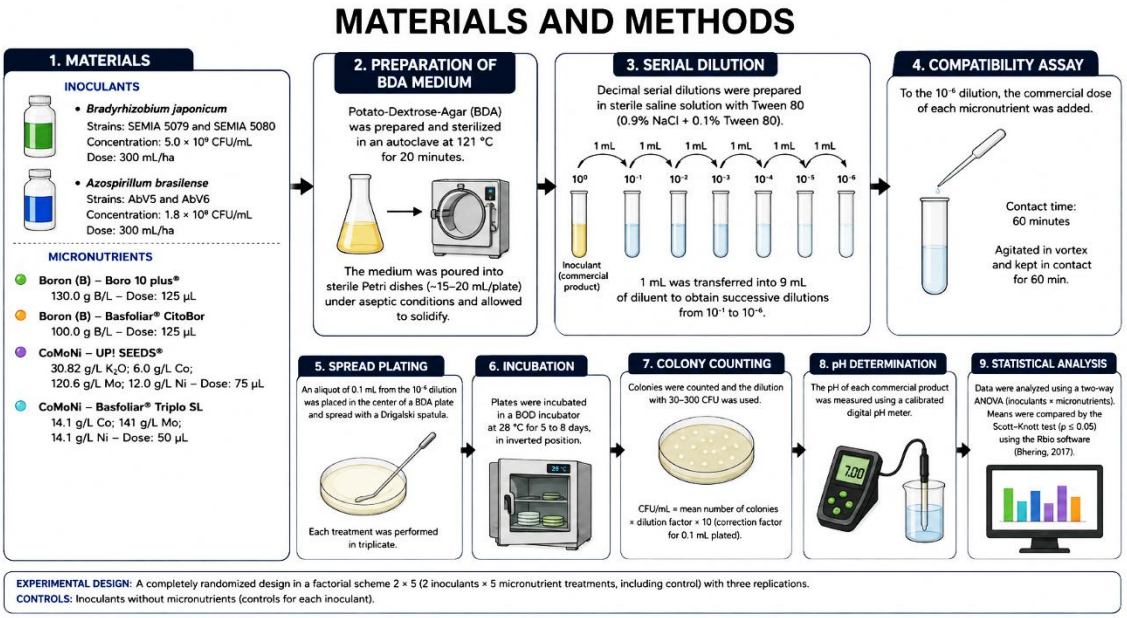
boron (130.0 g B L^{-1} and 100.0 g B L^{-1}), while the remaining products contained cobalt (Co), molybdenum (Mo), and nickel (Ni) at the concentrations specified by the manufacturers.

2.3 Experimental design

The experiment was conducted under laboratory conditions using a completely randomized design in a 2 × 5 factorial arrangement, consisting of two bacterial inoculants (*B. japonicum* and *A. brasilense*) and five micronutrient treatments (control, two boron formulations, and two Co–Mo–Ni formulations), with three replicates per treatment.

A simplified overview of the experimental workflow, including bacterial suspension preparation, serial dilution, compatibility assay, colony quantification, pH determination, and statistical analysis, is presented in **Figure 1.**

Figure 1. Simplified schematic representation of the experimental design adopted to evaluate the compatibility between commercial inoculants containing *Bradyrhizobium japonicum* and *Azospirillum brasilense* and commercial micronutrient formulations. The workflow illustrates bacterial suspension preparation, serial dilution, compatibility assay, spread plating on potato dextrose agar (PDA) medium, incubation, colony-forming unit (CFU) quantification, pH determination, and statistical analysis.



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2.4 Preparation of bacterial suspensions and compatibility assay

Bacterial suspensions were prepared using sterile saline solution containing Tween 80 (9.0 g NaCl and 1 mL Tween 80 per liter of distilled water). Serial decimal dilutions were prepared according to Campo and Hungria (2007), obtaining suspensions from 10^{-1} to 10^{-6}.

The 10^{-6} dilution was selected for the compatibility assay. Subsequently, commercial doses of each micronutrient formulation, previously adjusted to the experimental volume, were added to the bacterial suspensions. The mixtures were homogenized using a vortex mixer and maintained in contact for 60 min under laboratory conditions before microbiological evaluation.

2.5 Microbiological evaluation

The spread plate technique described by Campo and Hungria (2007) was employed to determine bacterial viability. Aliquots of 0.1 mL from each treatment were uniformly distributed onto Petri dishes containing Potato Dextrose Agar (PDA) medium using a sterile Drigalski spatula.

The plates were incubated in a biochemical oxygen demand (BOD) incubator at 28 °C for 5–8 days in the inverted position. Following incubation, colony-forming units (CFU) were counted, selecting plates containing between 30 and 300 colonies for quantification.

Bacterial concentration was calculated using the equation proposed by Campo and Hungria (2007):

$$\mathbf{CFU\ mL^{-1} = N \times D \times 10}$$

Where *N* is the mean number of colonies and *D* is the dilution factor. The correction factor of 10 corresponds to the plated volume (0.1 mL).

2.6 pH determination

The pH of each commercial micronutrient formulation was determined using a calibrated digital pH meter (TECNAL®, model TEC-3MP). Measurements were

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performed immediately before the compatibility assays to support the interpretation of bacterial viability after exposure to each formulation.

2.7 Statistical analysis

The obtained data were subjected to analysis of variance (ANOVA) considering the factorial arrangement (2 × 5) at a significance level of 5%. When significant differences were detected, treatment means were grouped using the Scott–Knott test ($p \leq 0.05$). Statistical analyses were performed using the RBio software (Bhering, 2017).

3. RESULTS AND DISCUSSION

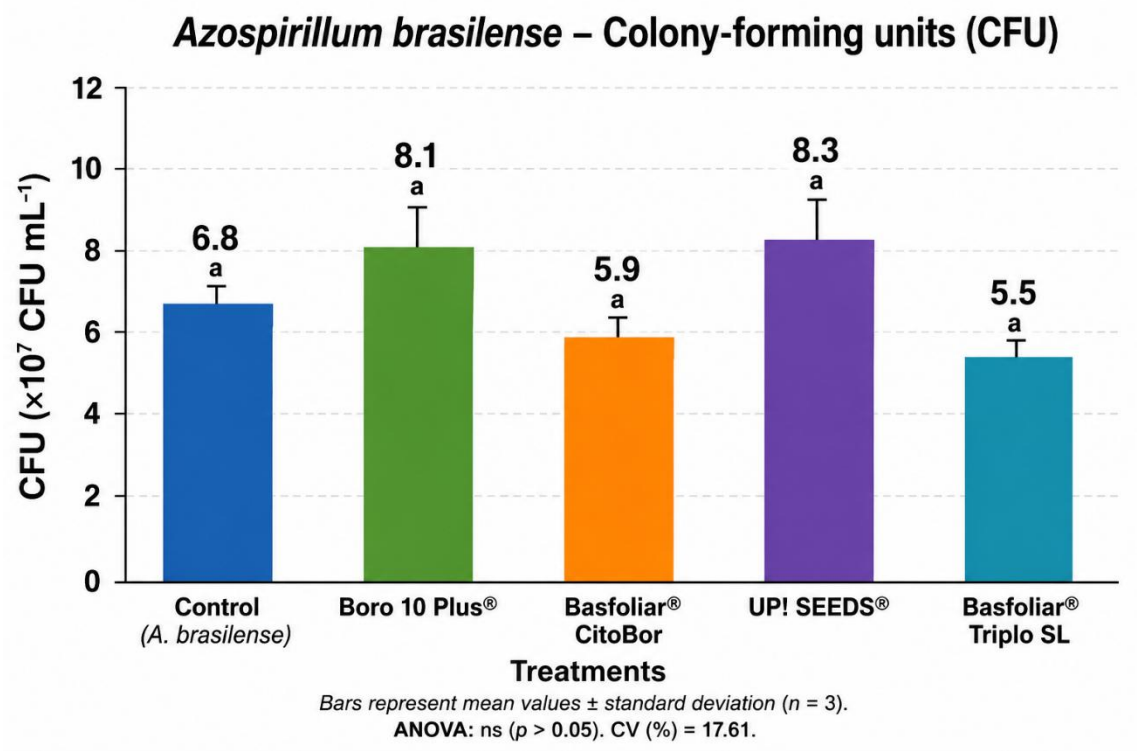
3.1 Colony-forming units of *Azospirillum brasilense*

The compatibility assay demonstrated that exposure of *Azospirillum brasilense* to the evaluated commercial micronutrient formulations did not significantly affect bacterial viability, as evidenced by the absence of statistical differences in colony-forming unit (CFU) counts among treatments. Likewise, the graphical representation of the results (Figure 2) confirms that all treatments maintained bacterial populations comparable to the untreated control, indicating that the tested formulations did not exert an immediate toxic effect capable of reducing cell survival.

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Figure 2. Colony-forming units (CFU) of *Azospirillum brasilense* following exposure to commercial micronutrient formulations. Bars represent the mean values of three replicates. Means followed by the same lowercase letter do not differ according to the Scott–Knott test ($p \leq 0.05$). Error bars represent the standard deviation.



Source: The authors.

The maintenance of bacterial viability following exposure to boron- and Co–Mo–Ni-based formulations suggests that these products are compatible with *A. brasilense* under the experimental conditions adopted. Although slight numerical differences were observed among treatments, all means belonged to the same statistical group, indicating that the micronutrients neither inhibited bacterial growth nor impaired colony establishment on the culture medium.

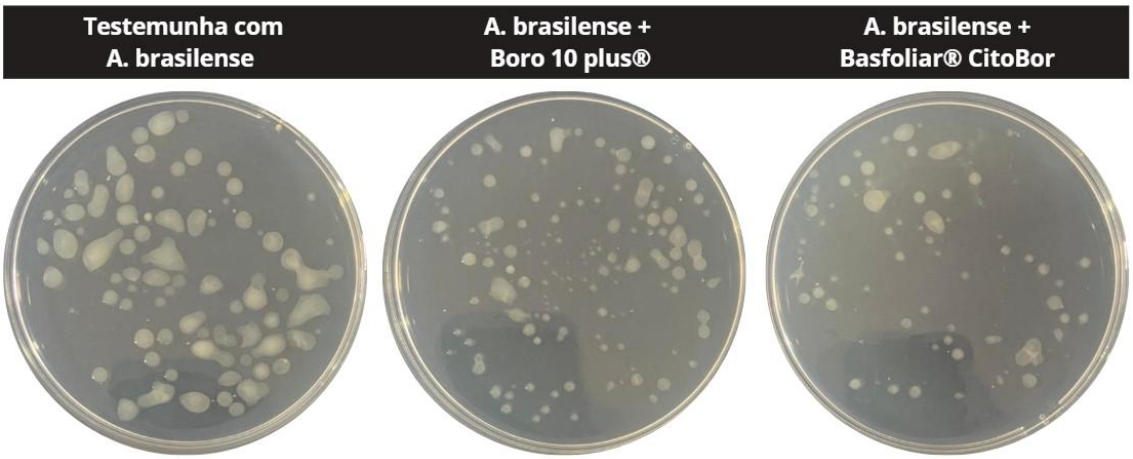
These findings agree with those reported by Galindo et al. (2017), who also demonstrated the successful association between micronutrients and bacterial inoculants under field conditions. In their study, seed treatment with cobalt and molybdenum combined with *Azospirillum brasilense* inoculation increased leaf nitrogen concentration and soybean grain yield, demonstrating that the simultaneous use of these technologies can generate agronomic and economic benefits.

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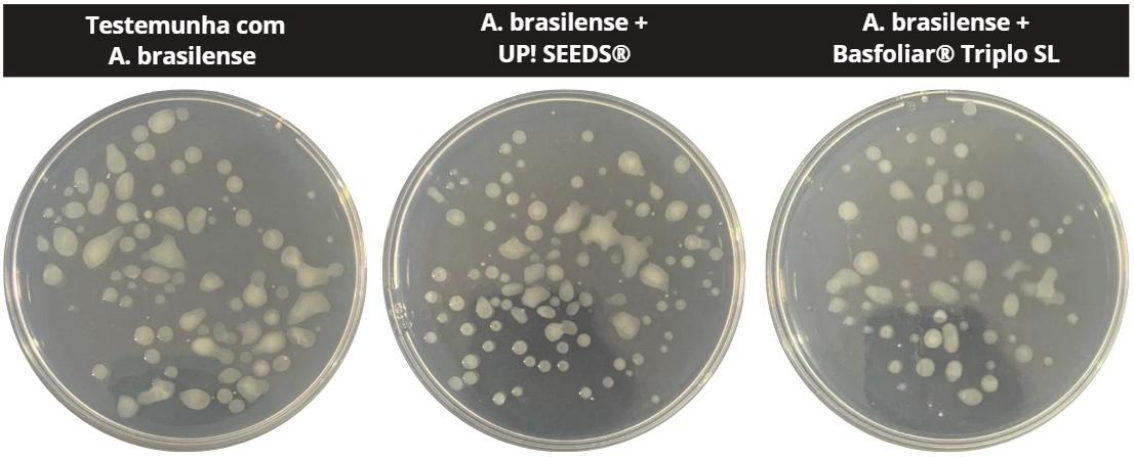
Visual assessment of the representative Petri dishes further corroborates the quantitative results. As shown in Figures 3 and 4, bacterial colonies developed uniformly in all treatments, with no evident reduction in colony density or marked alterations in colony morphology relative to the control. The consistency between the microbiological counts and the visual appearance of the colonies reinforces the absence of detrimental effects caused by the evaluated micronutrient formulations.

Figure 3. Representative Petri dishes showing the growth of *Azospirillum brasilense* after exposure to the control treatment, Boro 10 Plus®, and Basfoliar® CitoBor formulations. Bacterial suspensions were plated on potato dextrose agar (PDA) medium after 60 min of contact with each micronutrient formulation and incubated at 28 °C for 5–8 days.



Source: The authors.

Figure 4. Representative Petri dishes showing the growth of *Azospirillum brasilense* after exposure to the control treatment, UP! SEEDS®, and Basfoliar® Triplo SL formulations. Plates were prepared using the spread plate technique on potato dextrose agar (PDA) medium and incubated at 28 °C for 5–8 days.



Source: The authors.

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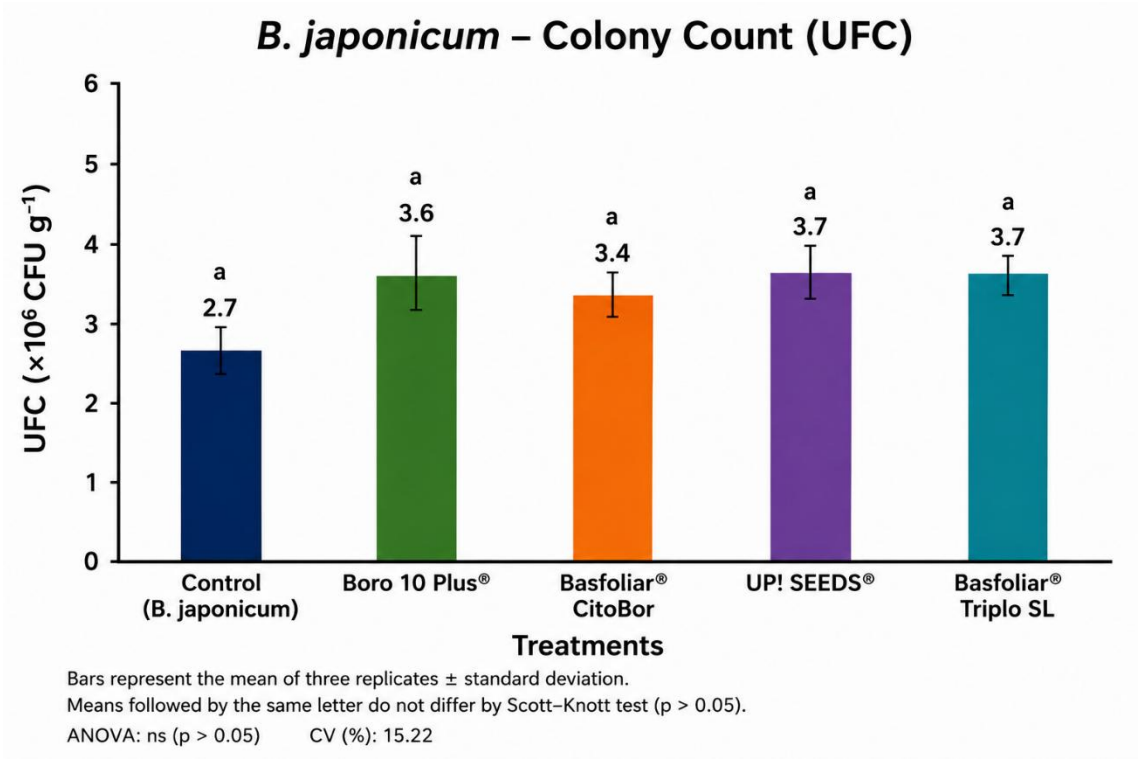
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From a practical perspective, these results indicate that the simultaneous application of *A. brasilense* inoculants and micronutrients, such as in directed in-furrow application systems, may represent a viable strategy to simplify sowing operations without compromising inoculant quality. Consequently, this practice has the potential to improve operational efficiency while maintaining the viability of beneficial microorganisms.

3.2 Colony-forming units of *Bradyrhizobium japonicum*

Similarly, no statistically significant differences were observed in the population of *Bradyrhizobium japonicum* among the evaluated treatments. The graphical presentation of the data (Figure 5) shows that bacterial viability remained stable regardless of the commercial micronutrient formulation used, indicating that exposure to boron and Co–Mo–Ni formulations did not adversely affect bacterial survival.

Figure 5. Colony-forming units (CFU) of *Bradyrhizobium japonicum* following exposure to commercial micronutrient formulations. Bars represent the mean values of three replicates. Means followed by the same lowercase letter do not differ according to the Scott–Knott test ($p \leq 0.05$). Error bars represent the standard deviation.



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Source: The authors.

Although the treatments containing micronutrients presented numerically higher CFU values than the untreated control, these differences were not statistically significant. Therefore, the results indicate that the tested formulations were compatible with the inoculant and did not exert a bactericidal or bacteriostatic effect capable of reducing the bacterial population.

These findings differ from those reported by Ferreira et al. (2022), who observed superior agronomic performance when cobalt and molybdenum were applied separately via foliar spraying rather than mixed with inoculants during sowing. It is important to emphasize, however, that the present study evaluated bacterial viability under laboratory conditions, whereas the study conducted by Ferreira et al. (2022) assessed crop responses under field conditions. Thus, although bacterial survival was not affected by the mixtures evaluated herein, additional field studies remain necessary to determine whether this compatibility is maintained throughout the crop cycle and reflected in agronomic performance.

In contrast, Lai et al. (2025) reported high toxicity and rapid reductions in rhizobial populations following exposure to formulations containing copper (Cu) and zinc (Zn). Unlike those micronutrients, the formulations evaluated in the present study contained boron, cobalt, molybdenum, and nickel, elements that are not commonly associated with severe toxicity to rhizobia at the tested concentrations. This difference in chemical composition probably explains the absence of significant reductions in bacterial viability. Moreover, bacterial tolerance to micronutrients is strain dependent, and *Bradyrhizobium japonicum* may exhibit greater resistance to these formulations than the bacterial species evaluated by Lai et al. (2025).

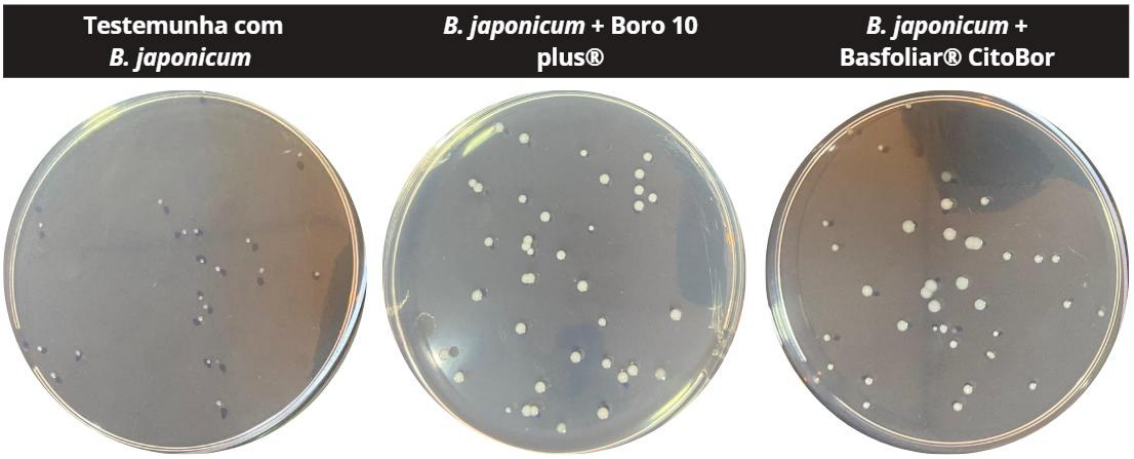
The representative plates shown in Figures 6 and 7 support these observations. Colonies remained abundant and well developed in all treatments, with no visual evidence of growth inhibition. Interestingly, colonies exposed to some micronutrient formulations appeared slightly larger than those of the control, particularly in treatments containing boron. According to Bolanos, Brewin and Bonilla (1996), boron plays an important role in maintaining the structural stability of bacterial surface interactions through the organization of cell-surface

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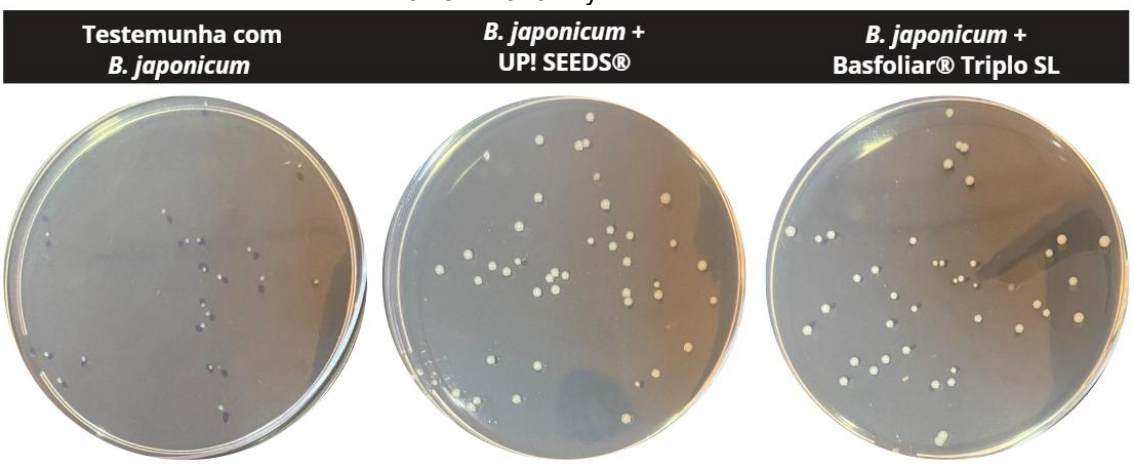
glycoconjugates. This physiological role may partially explain the vigorous colony development observed, although these visual differences were not reflected in statistically significant increases in bacterial population.

Figure 6. Representative Petri dishes showing the growth of *Bradyrhizobium japonicum* after exposure to the control treatment, Boro 10 Plus®, and Basfoliar® CitoBor formulations. Bacterial suspensions were plated on potato dextrose agar (PDA) medium after 60 min of contact with each micronutrient formulation and incubated at 28 °C for 5–8 days.



Source: The authors.

Figure 7. Representative Petri dishes showing the growth of *Bradyrhizobium japonicum* after exposure to the control treatment, UP! SEEDS®, and Basfoliar® Triplo SL formulations. Plates were prepared using the spread plate technique on potato dextrose agar (PDA) medium and incubated at 28 °C for 5–8 days.



Source: The authors.

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3.3 pH characterization of the evaluated products

The pH values determined for the evaluated commercial products ranged from 6.54 to 8.44, corresponding to 7.07 for Up Seed®, 6.54 for Boro 10 Plus®, 8.44 for Basfoliar® CitoBor®, and 7.05 for Basfoliar® Triplo SL®. Overall, the formulations exhibited pH values close to neutrality, with only Basfoliar® CitoBor® presenting a more alkaline profile.

The observed pH range may have contributed to maintaining bacterial viability throughout the compatibility assays. According to Baldani et al. (2015), species of the genus *Azospirillum* exhibit optimal growth under slightly acidic to neutral conditions (pH 5.5–7.5), whereas alkaline conditions may induce physiological and morphological changes, particularly when associated with additional stress factors. Although no statistical reduction in CFU counts was observed, Basfoliar® CitoBor®, which presented the highest pH (8.44), produced one of the lowest numerical CFU values and visually smaller colonies in Figures 3 and 4. These observations suggest that the alkaline environment may have reduced bacterial vigor without significantly affecting cell survival.

Similarly, Miguel and Moreira (2001) reported that *Bradyrhizobium japonicum* exhibited maximum growth and exopolysaccharide production at pH 6.0. Although the formulations evaluated in the present study presented slightly higher pH values, they remained within a range sufficiently close to neutrality to preserve bacterial viability, explaining the absence of significant reductions in CFU counts (Figure 5) and the uniform colony development observed in Figures 6 and 7.

Overall, the results indicate that the compatibility observed between inoculants and micronutrient formulations is likely associated, at least in part, with the favorable pH of the evaluated products. Because none of the formulations presented extreme acidic or alkaline conditions, bacterial survival was preserved throughout the compatibility assays, supporting the potential use of these products in combined application systems.

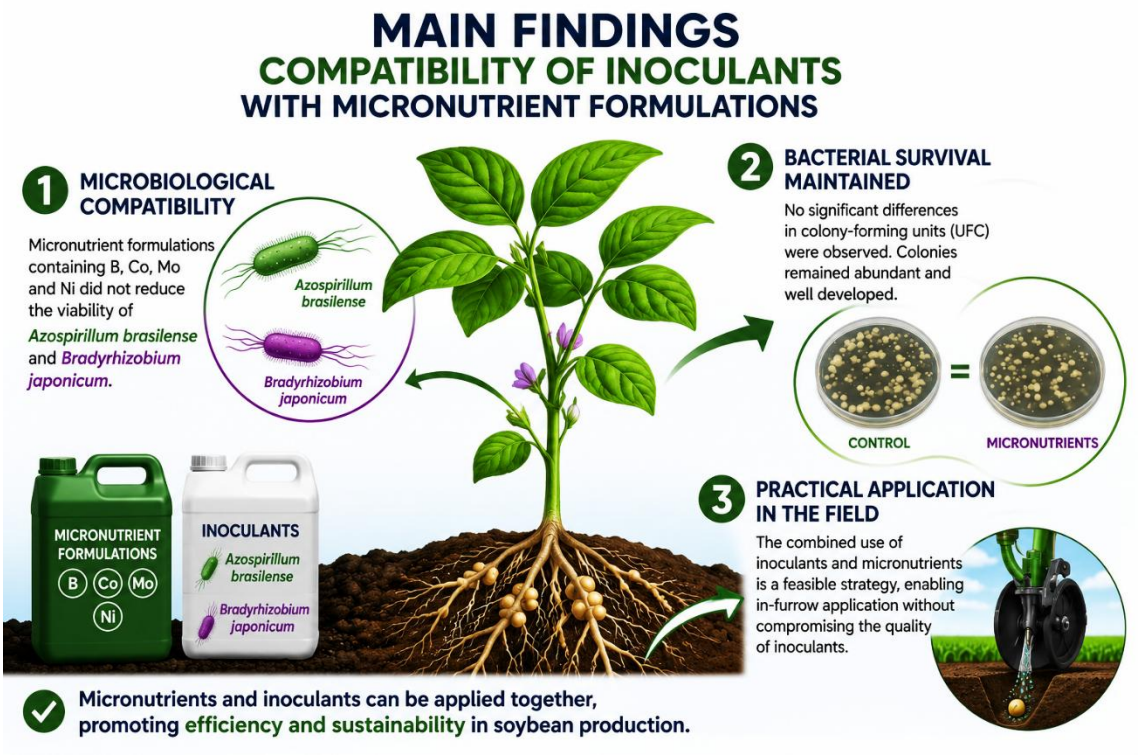
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3.4 Summary of the main findings

The schematic representation presented in figure 8 summarizes the principal findings obtained in this study, integrating the microbiological, physiological, and practical implications of the compatibility assays. Overall, the evaluated commercial micronutrient formulations did not compromise the viability of either *Azospirillum brasilense* or *Bradyrhizobium japonicum*, demonstrating that bacterial populations remained stable after exposure to boron (B), cobalt (Co), molybdenum (Mo), and nickel (Ni). These observations are supported by both the colony-forming unit (CFU) quantification and the visual assessment of colony development, reinforcing the compatibility between the evaluated products.

Figure 8. Schematic summary of the main findings obtained in the compatibility assays between commercial inoculants containing *Azospirillum brasilense* and *Bradyrhizobium japonicum* and commercial micronutrient formulations. The figure highlights the maintenance of bacterial viability following exposure to boron (B), cobalt (Co), molybdenum (Mo), and nickel (Ni) formulations, the preservation of colony development and colony-forming units (CFU), and the potential applicability of the combined use of inoculants and micronutrients in in-furrow soybean management.



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An additional contribution of this work is the demonstration that the preservation of bacterial viability was consistently observed for both inoculant species, suggesting that the tested formulations did not exert immediate bactericidal effects under the evaluated conditions. The maintenance of abundant and well-developed colonies indicates that the micronutrients did not interfere with bacterial establishment on the culture medium, corroborating the microbiological results obtained throughout the compatibility assays.

From an agronomic perspective, the results have important practical implications for soybean production systems. The compatibility observed between inoculants and micronutrient formulations supports the possibility of their simultaneous application, particularly through in-furrow directed spraying, a practice that has become increasingly common due to its operational advantages. The combined use of these inputs may simplify sowing operations, reduce application costs, and improve field efficiency without compromising inoculant quality or bacterial survival.

Although the present study was conducted under controlled laboratory conditions, the results provide robust evidence that the evaluated commercial formulations are microbiologically compatible with both bacterial inoculants. Nevertheless, complementary field experiments remain essential to confirm whether the observed compatibility is maintained under environmental conditions and whether it translates into improvements in nodulation, biological nitrogen fixation, nutrient uptake, and soybean productivity.

4. CONCLUSION

The evaluated commercial micronutrient formulations containing boron (B), cobalt (Co), molybdenum (Mo), and nickel (Ni) did not result in statistically detectable reductions in the viability of *Bradyrhizobium japonicum* or *Azospirillum brasilense* after short-term exposure under the laboratory conditions adopted in this study. These findings indicate microbiological compatibility under the evaluated *in vitro* conditions, suggesting that the tested formulations did not exert immediate deleterious effects on bacterial survival.

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However, because the present study was limited to laboratory compatibility assays, the results should not be interpreted as direct evidence of agronomic compatibility or field performance. Additional studies evaluating longer exposure periods, bacterial persistence, root colonization, nodulation, biological nitrogen fixation, greenhouse performance, and field conditions are necessary to determine whether the microbiological compatibility observed *in vitro* is maintained under practical agricultural conditions.

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