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Methylobacterium symbioticum Applied as a Foliar Inoculant Was Little Effective in Enhancing Nitrogen Fixation and Lettuce Dry Matter Yield

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Abstract: Nitrogen (N) is a limiting ecological factor for plant growth in most agroecosystems. Biological N fixation, especially from nodulated legumes, has been promoted in recent decades as an alternative or complement to industrially synthesized N fertilizers. The possibility of utilizing N-fixing organisms from the phyllosphere that demonstrate effectiveness across a wide range of crops is particularly exciting. In this study, we examined the N-fixing capacity and the impact on lettuce growth of an inoculant recently introduced to the market, which contains the microorganism *Methylobacterium symbioticum* and is recommended for various cultivated species. A pot experiment was conducted using a factorial design, which included the inoculant (No and Yes) and four N rates (0 (N0), 25 (N25), 50 (N50), and 100 (N100) kg ha^{-1} of N), with four replicates, over four lettuce growing cycles. The inoculant had a significant effect on dry matter yield (DMY) only during the second of the four growing cycles. The mean values of the four growing cycles ranged from 9.9 to 13.7 g pot^{-1} and 9.9 to 12.6 g kg^{-1} in pots that received and did not receive the inoculant, respectively. On the other hand, plants exhibited a robust response to N applied to the soil, showing significant increases in both DMY and tissue N concentration across all growing cycles. Mean values of DMY in the treatments N0 and N100 ranged from 5.6 to 8.9 g pot^{-1} and 12.5 to 16.1 g pot^{-1}, respectively. N concentration in tissues varied inversely with DMY, indicating a concentration/dilution effect. The difference in N concentration between treated and untreated plants, used as an estimate of fixed N, was very low for each of the soils' applied N rates, assuming average values for the four growing cycles of −1.5, −0.9, 2.4, and 6.3 kg ha^{-1} for N0, N25, N50, and N100, respectively. This study emphasized the low amount of N supplied to lettuce by the inoculant and its limited effect on DMY. Generally, in biological systems with N-fixing microorganisms, achieving high fixation rates requires a high level of specificity between the microorganism and host plant, a condition that seems not to have been met with lettuce. Considering the importance of the subject, is imperative that further studies be conducted to determine more precisely in which crops and under what growing conditions the inoculant proves to be a valuable input for farmers and an effective method for reducing N mineral fertilization.

Keywords: plant biostimulant; beneficial microorganism; foliar spray; biological nitrogen fixation; apparent nitrogen recovery

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1. Introduction

Nitrogen (N) is an ecological factor that determines plant productivity in natural and agricultural ecosystems. In the second half of the 20th century, the widespread use of N as a fertilizer was an important component of the green revolution, along with the use of

improved varieties and advances in irrigation techniques and crop protection [1]. Furthermore, the costs of using industrially synthesized N in agriculture have been increasing, mainly because the energy consumption in its manufacture remains high [2]. In addition, the use of N fertilizers tends to have low N-use efficiency. On a global scale, it is estimated that no more than 50% of N applied to crops is recovered in harvests, with the remainder lost to the environment [3]. N loss from agricultural fields in its inorganic form has a major impact on water contamination, contributing to its eutrophication with harmful algal blooms [4,5]. Significant amounts of N can also be lost from the soil through denitrification, contaminating the atmosphere with important greenhouse gases such as N oxides [5,6].

Organic amendments, in turn, were, for centuries, the main way to maintain soil fertility. In addition to supplying N and other nutrients after their mineralization by soil microorganisms, organic amendments have the potential to enhance overall plant growth conditions by promoting aeration in clay soils, water holding capacity in sandy soils, and, in general, soil biological activity, which is the driving force behind nutrient cycling [7,8]. In recent decades, the specialization of agricultural activity and the mechanization of farming practices have greatly reduced the availability of animal manure in vast regions of the world [5,9]. In addition, waste from agro-industrial activities, municipal solid waste, and other organic materials, although they have been valued as organic amendments [10,11], are available for agriculture also in limited quantities, which leads farmers to find other ways to fertilize their crops.

Biological N fixation has the potential to enhance soil fertility and decrease the requirement for external inputs. Organisms capable of fixing N from the atmosphere, collectively called diazotrophs, are those possessing a nitrogenase complex, which allow them to separate the two N atoms of the dinitrogen molecule (N_2) with the formation of ammonia [12,13]. N-fixing organisms can live in terrestrial or aquatic ecosystems, be aerobic or anaerobic, heterotrophic, or photosynthetic, and can be found free-living or in association with higher plants, sometimes forming a highly specific symbiotic relationship with the host plant [12,13].

Regarding biological N fixation, symbiotic associations play a major role in N fixation in agricultural fields, accounting for about 50% of total biological fixation in terrestrial ecosystems [3]. Nodulated legumes, in association with bacteria belonging to the Rhizobiaceae family, may have access to atmospheric N in sufficient quantities so that they do not need to receive N fertilizers to reach high productivity levels. In addition, N fixed by legumes can be available for other non-legume crops when integrated in intercropping [9,14,15] or in rotation with legumes [16,17]. The symbiotic relationship between the aquatic fern of the genus *Azolla* and the cyanobacterium *Anabaena azollae* is also a system with a high capacity for N fixation, with the fern commonly used as green manure in rice (*Oryza sativa* L.) cultivation [18–20]. High N fixation in agricultural fields can also be achieved from the relationship between some tropical grasses and N-fixing microorganisms, which may or may not be endophytic associations; perhaps the most striking example is the relationship between sugarcane (*Saccharum officinarum* L.) and some N fixers, such as *Gluconoacetobacter diazotrophicus* and *Azospirillum brasilense*, which are able to satisfy almost all sugarcane N requirements [18,21,22].

Even so, in agricultural fields, access to relevant amounts of atmospheric N is restricted to nodulated legumes and few other species, as mentioned above. Many important crops, such as lettuce (*Lactuca sativa* L.), do not have access to biologically fixed N. Recently, a commercial plant biostimulant (BlueN®, SYMBORG BUSINESS DEVELOPMENT S.L.U., Murcia, Spain), containing a N-fixing microorganism (*Methylobacterium symbioticum*) that promises to be able to thrive in the phyllosphere of most plants, was launched on the market. The bacterium *M. symbioticum* sp. Nov. (strain SB0023/T) was isolated from *Glomus iranicum* var. *tenuihypharum* spores [23]. Species of the genus *Methylobacterium* are ubiquitous in nature and can be present in a wide range of environments, including soil, air, water, and plants [24–26]. Other bacteria of the genus *Methylobacterium* have been recognized as capable of fixing N in interactions with plants. *M. nodulans* and *M.*

radiotolerans, for instance, are reported to be N-fixing bacteria, but by forming nodules on legume roots [27]. Regarding *M. symbioticum*, a recent study showed that the application of the inoculant resulted in decreases of 50% and 25% in the amount of N that would be required for maize and strawberry, respectively, accompanied by an increase in production compared to treatments of equivalent rates of nitrate-N applied, but without the application of the inoculant [28].

In fact, the possibility of a microorganism having the ability to fix N living in the phyllosphere of most plants, without an apparent specific relationship with the host plant, and being able to be applied as a foliar spray, gives it an unlimited potential for use in agriculture. Thus, it is of extraordinary importance that more studies emerge to evaluate the fixation capacity of this inoculant across a wide range of crops and cultivation conditions. The objective of this study was to evaluate the capacity of N fixation by *M. symbioticum*, commercial inoculant BlueN®, when applied to lettuce, measuring DMY, and estimating the apparent N recovery and apparent N fixation from four lettuce growing cycles. The hypotheses raised were about whether the bacterium can supply N to the crop in relevant amounts during its cropping cycle and whether the amount of fixed N depends on the nutritional status of lettuce, for which the commercial product was applied to plants subjected to different rates of mineral N-fertilizer.

2. Materials and Methods

2.1. Establishment of Pot Experiments

Four independent pot experiments were conducted during the 2021 and 2022 spring/summer seasons in Bragança, northeastern Portugal. The region benefits from a Mediterranean climate, concentrating its precipitation in winter (October to March) and presenting hot and dry summer months (June to September). The average air temperature is 12.3 °C, and the annual precipitation is 758.3 mm [29]. The four growing seasons began and ended in June–July 2021, August–October 2021, May–June 2022, and August–October 2022, respectively.

The experiment was arranged as a factorial, with the application of plant biostimulant (Yes and No) and four N rates (0, 25, 50, and 100 kg ha^{-1} of N) and four replicates (4 pots) of each treatment. N was split into two applications, half the rate at planting and the other half at phenological stage 43, with "30% of expected head size reached" [30]. Each pot received the fertilization corresponding to a single plant, assuming a typical commercial-lettuce planting density of 140,000 plants ha^{-1}. Thus, each pot received 0, 0.179, 0.357, or 0.714 g N, which corresponds to the field applications of 0, 25, 50, or 100 kg ha^{-1} of N. Ammonium nitrate 27% N (50% NH_4^+, 50% NO_3^-) was the fertilizer used. The pots also received phosphorus (P) and potassium (K) applied at planting, at rates corresponding to 50 kg ha^{-1} of P_2O_5 (as superphosphate, 18% P_2O_5) and K_2O (as potassium chloride, 60% K_2O). The plant biostimulant was applied at the time of N side-dressing application. The commercial product (Blue N®) contains 3×10^7 colony-forming units (CFUs g^{-1}) of *M. symbioticum*. The leaf spray was prepared at the concentration recommended by the manufacturer, 333 g ha^{-1}, diluted in 80 to L of water. Once again, it was considered that 1 ha represents 140,000 lettuces, with each lettuce receiving a fraction corresponding to the individual dose (2.37 mg, 1.5 mL water). The foliar spray was applied with a small household sprayer used to care for indoor plants, wetting the adaxial and abaxial sides of the leaves.

The pots (0.160 m mean diameter, 0.135 m height) were filled with 3 kg of dried (40 °C) soil, sieved through a 2 mm mesh, and obtained from the 0–0.20 m layer of a plot that had been fallow for a year. The soil was a Regosol [31] of colluvial origin, with a sandy clay loam texture (soil separates: 242, 217, and 541 g kg^{-1} clay, silt, and sand, respectively). It contained 11.7 g kg^{-1} of organic carbon (C) (Walkley–Black), had a $pH_{(H2O)}$ of 6.8, and P and K levels (Egnér–Riehm) of 85.7 mg kg^{-1} (P_2O_5) and 94.0 mg kg^{-1} (K_2O), respectively. The cation exchange capacity (ammonium acetate) was 17.9 $cmol_+$ kg^{-1}. The pots, both treated and untreated with the inoculant, were kept at 50 m from each other to prevent any potential cross-contamination.

2.2. Crop Management

The seedlings of lettuce (cv. Summer Wonder) were prepared in propagation trays with a square top measuring 0.025 × 0.025 m^2 and 0.04 m deep. A suitable commercial substrate for seed germination (Siro Agro 1®) (Leal e Soares, S.A., Mira, Portugal) was used. It was prepared with pine bark, blond peat (*Sphagnum* sp.), coconut peat, and NPK fertilizer (13:40:13) (1 kg m^3). The substrate had a granulometry of 0–8 mm, pH ($CaCl_2$) 5.0–6.0, and a conductivity of 150 to 200 µs cm^{-1}. The seedlings were transplanted approximately three weeks after sowing, at phenological stage 13, with the 3rd true leaf unfolded [30].

After planting the lettuces, the pots were surrounded with a wooden plank structure to prevent solar radiation from falling directly on the sides of the pots and excessively increasing the temperature at the level of the rhizosphere. The spatial arrangement of the pots was regularly changed to ensure they received uniform radiation exposure.

During the growing season, the lettuces were watered as many times as necessary. Considering that the treatments resulted in different lettuce growth, leading to different amounts of water transpired, and that some environmental variables, namely temperature and radiation, also lead to different water consumption, the amounts of water applied to each pot were managed daily by observing the apparent hydration level of the soil and the lettuces. After planting, the emergence of weeds was also monitored, which were promptly eliminated in the cotyledonary phase.

2.3. Crop Harvest, Sampling, and Laboratory Analysis

The plants were harvested by cutting them close to the ground. Some dirt on basal leaves was removed by gently washing them with water. Then, lettuces were dried in an oven at 70 °C until a constant weight was reached and ground (1 mm mesh) for laboratory analysis.

Lettuce tissues were subjected to elemental chemical analysis and a determination of nitrate concentration. Elemental tissue analyses were performed by Kjeldahl (N), colorimetry (B and P), flame emission spectrometry (K), and atomic absorption spectrophotometry (Ca, Mg, Cu, Fe, Zn, and Mn) methods after the nitric digestion of the samples [32]. Nitrate concentrations in lettuce tissues were determined according to Baird et al. [33] by UV-vis spectrophotometry in a water extract (dry matter/water, 1:50 *m*/*v*).

Initial soil samples were analysed for pH (soil: solution, 1:2.5), cation-exchange capacity (ammonium acetate, pH 7.0), organic C (wet digestion, Walkley–Black method) and extractable P and K (Egner–Riehm method, ammonium lactate extract). Soil separates (clay, silt, and sand) were determined by the Robinson pipette method. These analytical procedures are described in detail in Van Reeuwijk [34].

2.4. Data Analysis

Statistical analysis was performed using SPSS Statistics software (version 25, IBM SPSS, Armonk, NY, USA). The data were firstly tested for normality and homogeneity of variances using the Shapiro–Wilk test and Bartlett's test, respectively. After ANOVA examination, the means of the N treatments with significant differences ($\alpha < 0.05$) were separated by a Tukey HSD test ($\alpha = 0.05$).

Apparent N recovery (ANR) was used as an index of the N-use efficiency of the soil-applied N. The ANR was estimated according to the following equation: ANR (%) = 100 × [(N recovered in the fertilized treatments—N recovered in the control treatment)/N applied as a fertilizer].

Apparent N fixation (ANF) was used as an index of the effectiveness of N fixation by the microorganism. The ANF was determined by the difference between N recovered by plants that were and were not treated with the N-fixing microorganism separately for each rate of N applied to the soil: ANF = N recovered in treated plants—N recovered in untreated plants.

3. Results

3.1. Lettuce Dry Matter Yield

The DMY was minimally influenced by the application of the inoculant (Figure 1). Nonetheless, in four lettuce growing cycles, significant differences among treatments occurred in the second cycle, with the highest average values recorded in pots receiving the microorganism. The lettuce's response to N applied to the soil was clear and unequivocal, with significant differences in any of the growing cycles and an increase in average values from N0 to N100, although the results tended to show a saturation curve with an increasing N rate. Comparing treatments N0 and N100, the average values varied between 8.4 and 12.9 g plant^{-1}, 8.9 and 15.5 g plant^{-1}, 6.3 and 16.1 g plant^{-1}, and 5.9 and 12.5 g plant^{-1}, respectively, in the first, second, third, and fourth growing cycles. In general, there was no significant interaction between the factors. Only in the fourth growing cycle was the probability of interaction slightly lower than 0.05 (p = 0.0499). Indeed, the overall results suggest a substantial impact of N application as a fertilizer and a constrained effect of the inoculant on lettuce DMY.

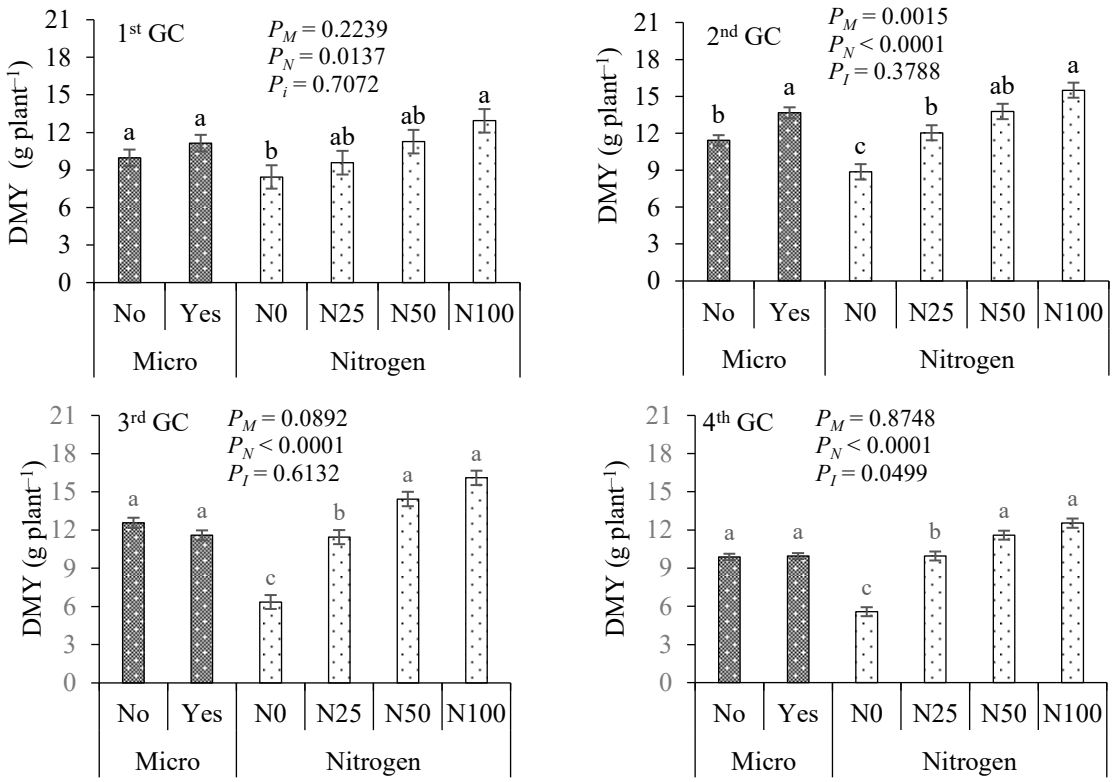

Figure 1. Lettuce dry matter yield (DMY) in the first, second, third, and fourth growing cycles (GCs) within a factorial experiment involving microorganism application (Micro, No or Yes) and nitrogen rates [0 (N0), 25 (N25), 50 (N50), and N100 (N100) kg ha^{-1} of N]. *PM*, *PN*, and *PI* represent the probability of the two-way ANOVA associated with microorganism application, N rate, and interaction, respectively. Error bars indicate standard errors. Within Micro or Nitrogen means followed by the same letter are not signficantly different at $p < 0.05$.

3.2. Nitrogen and Nitrate Concentration in Plant Tissue

The N concentration in lettuce tissues varied significantly with inoculant application in two out of the four lettuce growing cycles (Figure 2). However, the average values were significantly lower in the lettuces that received the microorganism in the second growing cycle, and significantly higher in the third growing cycle. Another noteworthy aspect is that the average tissue N concentration varied in the opposite direction compared to DMY in response to inoculant application.

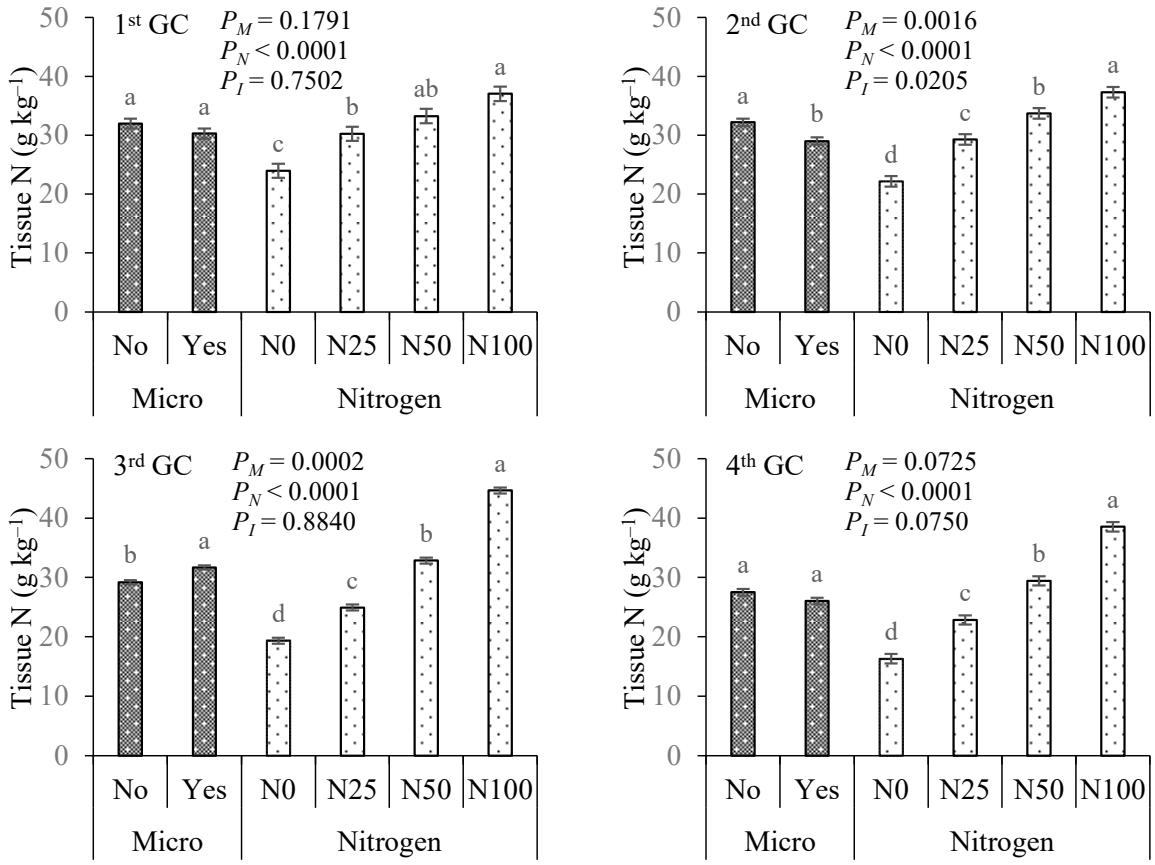

Figure 2. Tissue nitrogen (N) concentration in the first, second, third, and fourth growing cycles (GCs) within a factorial experiment involving microorganism application (Micro, No or Yes) and nitrogen rates [0 (N0), 25 (N25), 50 (N50), and N100 (N100) kg ha^{-1} of N]. *PM*, *PN*, and *PI* represent the probability of the two-way ANOVA associated with microorganism application, N rate, and interaction, respectively. The means of the N rate factor were separated using the Tukey HSD test (α = 0.05). Error bars indicate standard errors. Within Micro or Nitrogen means followed by the same letter are not signficantly different at $p < 0.05$.

The N concentration in tissues varied significantly with the N application in all four lettuce growing cycles (Figure 2). Except for the first growing cycle, the N25 treatment resulted in significantly higher values than the control, the N50 treatment significantly higher values than N25, and N100 significantly higher values than N50. This result highlights the strong response of tissue N concentration to N applied to the soil, contrary to what was observed with the application of the inoculant. On the other hand, significant interaction occurred only in the second growing cycle of the lettuce.

The nitrate concentration in tissues varied significantly with the application of the inoculant in only one out of the four sampling dates, the second one (Figure 3). The values from the treatment without the microorganism were significantly higher than those from the treatment with the microorganism.

The nitrate levels in tissues varied significantly with the application of mineral N to the soil in all lettuce growing cycles (Figure 3). However, the increase in average nitrate values in tissues with the application of mineral N to the soil was not as striking as the total N concentration in tissues.

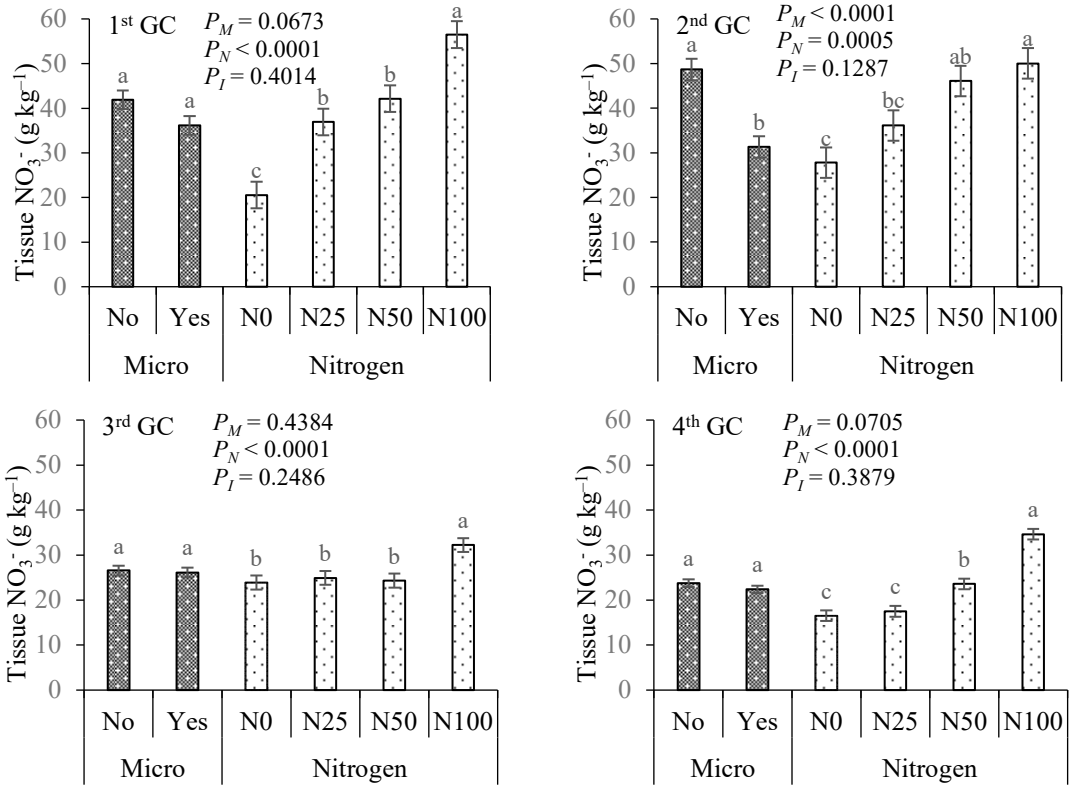

Figure 3. Tissue nitrate (NO_3^-) concentration in the first, second, third, and fourth growing cycles (GCs) within a factorial experiment involving microorganism application (Micro, No or Yes) and nitrogen rates [0 (N0), 25 (N25), 50 (N50), and N100 (N100) kg ha^{-1} of N]. *PM*, *PN*, and *PI* represent the probability of the two-way ANOVA associated with microorganism application, N rate, and interaction, respectively. The means of the N rate factor were separated using the Tukey HSD test (α = 0.05). Error bars indicate standard errors. Within Micro or Nitrogen means followed by the same letter are not signficantly different at $p < 0.05$.

3.3. Apparent Nitrogen Recovery and Apparent Nitrogen Fixation

The apparent N recovery is an indicator of N-use efficiency, and, therefore, in this study, it was calculated from the results of the pots that did not receive the inoculant. Although the result is not clear in all lettuce growing cycles, there was a tendency for lower N-use efficiency as the N rate increased (Table 1). The result became clear when observing the average values of the four growing cycles, where the apparent N recovery values were 73.5%, 66.4%, and 54.0% in the N25, N50, and N100 treatments, respectively.

Table 1. Apparent nitrogen recovery of soil-applied N (±standard deviation, n = 4) by treatment [25 (N25), 50 (N50), and 100 (N100) kg ha^{-1} of N] and growing cycle (GC).

	Apparent Nitrogen Recovery (%)				
N Treatment	**1st GC**	**2nd GC**	**3rd GC**	**4th GC**	**Average**
N25	31.3 ± 26.7	81.7 ± 10.5	88.7 ± 22.9	92.1 ± 5.4	73.5
N50	33.4 ± 12.9	60.6 ± 17.5	91.1 ± 7.0	80.5 ± 14.3	66.4
N100	32.8 ± 11.8	43.9 ± 8.4	86.7 ± 9.2	52.4 ± 3.3	54.0

Apparent N recovery (%) = 100 × [(N recovered in the fertilized treatments − N recovered in the control treatment)/N applied as a fertilizer].

The apparent N fixation was calculated by the difference between the values of the treatments receiving and not receiving the inoculant for each of the N rates applied to the soil. The values expressed in kg ha^{-1} were consistently very close to zero, although there was a slight tendency for positive average values in treatments that received more

N applied to the soil (Table 2). This is a result that should be emphasized, as it clearly demonstrated the reduced capacity of the inoculant to fix atmospheric N.

Table 2. Apparent nitrogen fixation (±standard deviation, n = 4) by treatment [0 (N0), 25 (N25), 50 (N50), and 100 (N100) kg ha^{-1} of N] and growing cycle (GC).

N Treatment	Apparent Nitrogen Fixation (kg ha^{-1})				
	1st GC	2nd GC	3rd GC	4th GC	Average
N0	−5.4 ± 2.7	−3.2 ± 1.1	1.0 ± 0.7	1.4 ± 0.9	−1.5
N25	2.5 ± 6.1	−2.0 ± 1.4	2.4 ± 5.4	−6.4 ± 3.6	−0.9
N50	9.9 ± 6.5	10.2 ± 7.8	−1.6 ± 6.5	−8.9 ± 4.7	2.4
N100	7.0 ± 10.3	16.9 ± 5.7	−5.2 ± 6.8	6.5 ± 6.7	6.3

Apparent N fixation = N recovered in treated plants − N recovered in untreated plants.

3.4. Concentration of Other Nutrients in Lettuce Tissues

In the first lettuce growing cycle, no significant differences were observed with the application of the inoculant or between rates of mineral N application for any of the macronutrients, P, K, Ca, or Mg (Table 3). On the other hand, the B concentration in tissues varied with the application of mineral N to the soil, with average values decreasing as the applied N rate increased. Among the metallic micronutrients, only the Zn concentration in tissues varied significantly with the application of the inoculant, with the highest average value observed in treated pots. The Mn concentration varied significantly with the treatments of mineral N application to the soil, but without a clear trend with the N rate, with the lowest average value observed in the N25 treatment.

Table 3. Tissue nutrient concentration in the first growing cycle within a factorial experiment involving microorganism application (Micro, No or Yes) and nitrogen rate [0 (N0), 25 (N25), 50 (N50), and N100 (N100) kg ha^{-1} of N]. *PM*, *PN*, and *PI* represent the probability of the two-way ANOVA associated with microorganism application, N rate, and interaction, respectively.

		P	K	Ca	Mg	B	Fe	Mn	Zn	Cu
		g kg^{-1}				mg kg^{-1}				
Micro	No	5.8 a	94.2 a	8.0 a	4.4 a	51.1 a	730.8 a	62.2 a	102.7 b	14.5 a
	Yes	5.8 a	89.9 a	7.6 a	4.1 a	50.4 a	696.1 a	58.9 a	129.9 a	13.6 a
N rate	N0	5.6 a	89.7 a	7.6 a	3.9 a	54.6 a	673.2 a	61.5 ab	115.8 a	13.3 a
	N25	5.8 a	92.4 a	8.0 a	4.6 a	50.5 b	759.5 a	50.0 b	124.5 a	13.8 a
	N50	5.9 a	93.5 a	7.3 a	4.1 a	51.3 b	775.8 a	65.2 a	122.3 a	14.2 a
	N100	5.9 a	92.6 a	8.2 a	4.4 a	46.5 c	645.4 a	65.5 a	102.5 a	14.9 a
PM		0.7644	0.4179	0.2804	0.1244	0.3644	0.6523	0.3188	0.0041	0.0920
PN		0.3760	0.9595	0.3415	0.1085	<0.0001	0.5558	0.0079	0.2864	0.1693
PI		0.9950	0.8899	0.9205	0.8595	0.0039	0.0435	0.3236	0.0236	0.0222

In columns, separated by microorganism application and N rate; means followed by the same letter are not significantly different by Tukey HSD test (α = 0.05).

In the second lettuce growing cycle, among the macronutrients, only the K levels in tissues varied with the application of the inoculant and with the N rate (Table 4). Pots treated with the inoculant showed significantly lower K levels in tissues than untreated pots. Regarding the mineral N rate, the K levels in tissues decreased with the increase in the applied N. The B levels in tissues, like those of K, also exhibited a significant decreasing trend with the application of mineral N rates to the soil. Among metallic micronutrients, significant differences were only observed in Mn levels in tissues when comparing the results of pots that received the microorganism and those that did not, with higher levels in the former.

Table 4. Tissue nutrient concentration in the second growing cycle within a factorial experiment involving microorganism application (Micro, No or Yes) and nitrogen rate [0 (N0), 25 (N25), 50 (N50), and N100 (N100) kg ha^{-1} of N]. *PM*, *PN*, and *PI* represent the probability of the two-way ANOVA associated with microorganism application, N rate, and interaction, respectively.

		P	K	Ca	Mg	B	Fe	Mn	Zn	Cu
		g kg^{-1}				mg kg^{-1}				
Micro	No	5.3 a	72.8 a	6.3 a	3.9 a	42.6 a	624.2 a	56.8 a	184.5 a	13.3 a
	Yes	5.1 a	66.7 b	6.1 a	3.7 a	41.7 a	592.0 a	50.4 b	132.7 a	12.3 a
N rate	N0	5.2 a	75.4 a	6.1 a	3.6 a	47.5 a	622.4 a	59.0 a	160.7 a	12.5 a
	N25	5.3 a	76.6 a	6.1 a	3.9 a	43.8 b	645.0 a	52.4 a	213.4 a	12.3 a
	N50	5.4 a	68.8 b	6.0 a	3.7 a	39.0 c	554.2 a	50.0 a	127.9 a	13.4 a
	N100	5.1 a	58.1 c	6.7 a	4.0 a	38.4 c	610.9 a	53.1 a	132.4 a	13.1 a
PM		0.1509	0.0004	0.3792	0.2765	0.2814	0.6004	0.0379	0.2077	0.0601
PN		0.5827	<0.0001	0.0668	0.1139	<0.0001	0.7494	0.1868	0.4234	0.3990
PI		0.6720	0.0022	0.3373	0.4840	0.0010	0.4956	0.5370	0.2222	0.9338

In columns, separated by microorganism application and N rate; means followed by the same letter are not significantly different by Tukey HSD test ($\alpha = 0.05$).

In the third lettuce growing cycle, among macronutrients, significant differences were only observed for K levels in tissues when comparing the response to the applied mineral, N (Table 5). The K levels in tissues were significantly lower in the treatment that received the highest N rate. Among micronutrients, significant differences were observed in the concentration of Zn and Cu in tissues. Zn values were significantly higher in the treatment that did not receive the microorganism and tended to be higher in treatments corresponding to higher rates of N applied to the soil (N50 and N100). Cu values differed significantly depending on the N rate, showing a tendency to be significantly higher as the applied N rate increased.

Table 5. Tissue nutrient concentration in the third growing cycle within a factorial experiment involving microorganism application (Micro, No or Yes) and nitrogen rate [0 (N0), 25 (N25), 50 (N50), and N100 (N100) kg ha^{-1} of N]. *PM*, *PN*, and *PI* represent the probability of the two-way ANOVA associated with microorganism application, N rate, and interaction, respectively.

		P	K	Ca	Mg	B	Fe	Mn	Zn	Cu
		g kg^{-1}				mg kg^{-1}				
Micro	No	3.6 a	48.9 a	7.8 a	4.4 a	33.6 a	746.2 a	62.4 a	103.0 a	7.4 a
	Yes	3.5 a	48.3 a	7.6 a	4.5 a	34.1 a	711.0 a	62.6 a	85.1 b	7.9 a
N rate	N0	3.3 a	49.6 a	7.4 a	4.3 a	35.3 a	725.7 a	60.7 a	70.4 b	6.6 c
	N25	3.6 a	50.9 a	7.5 a	4.3 a	33.8 a	739.7 a	63.7 a	72.1 b	6.6 bc
	N50	3.7 a	49.1 a	7.8 a	4.6 a	34.0 a	699.4 a	65.3 a	113.5 a	8.2 ab
	N100	3.6 a	44.8 b	8.1 a	4.7 a	32.3 a	749.6 a	60.3 a	120.2 a	9.1 a
PM		0.2150	0.4803	0.6653	0.5821	0.6390	0.2976	0.9510	0.0019	0.2153
PN		0.0911	0.0007	0.3795	0.0873	0.1612	0.7285	0.5424	<0.0001	<0.0001
PI		0.0588	0.0822	0.5948	0.0205	0.0007	0.0143	0.8321	0.6639	0.2491

In columns, separated by microorganism application and N rate; means followed by the same letter are not significantly different by Tukey HSD test ($\alpha = 0.05$).

In the fourth lettuce growing cycle, K levels in tissues were higher in the treatment that received the inoculant compared to the treatment that did not, and were significantly lower in the treatment corresponding to the highest N rate compared to the other treatments (Table 6). Among macronutrients, Mg values were significantly lower in the N0 and N25 treatments compared to treatments receiving more N, N50 and N100. Mn concentrations in tissues varied significantly among soil N treatments, tending to be higher in treatments corresponding to higher N rates. The Cu values also exhibited significant variation among

N fertilization treatments, although no clear trend was observed with respect to the applied rates. In this growing cycle, a significant interaction was observed between the factors for P, Fe, and Mn. However, this result was not consistent with what was observed in previous growing cycles, in which significant interactions also occurred, but not always involving the same nutrients.

Table 6. Tissue nutrient concentration in the fourth growing cycle within a factorial experiment involving microorganism application (Micro, No or Yes) and nitrogen rate [0 (N0), 25 (N25), 50 (N50), and N100 (N100) kg ha^{-1} of N]. *PM*, *PN*, and *PI* represent the probability of the two-way ANOVA associated with microorganism application, N rate, and interaction, respectively.

		P	K	Ca	Mg	B	Fe	Mn	Zn	Cu
		g kg^{-1}				mg kg^{-1}				
Micro	No	2.7 a	35.8 b	7.8 a	5.3 a	31.1 a	841.4 a	61.4 a	61.7 a	7.8 a
	Yes	2.8 a	38.6 a	7.5 a	5.4 a	32.6 a	797.6 a	64.3 a	69.2 a	8.8 a
N rate	N0	2.6 a	39.7 a	7.9 a	5.0 b	30.8 a	992.7 a	56.9 b	65.2 a	8.6 ab
	N25	2.8 a	39.7 a	7.4 a	4.9 b	31.9 a	720.1 a	57.7 ab	65.8 a	6.9 b
	N50	3.0 a	37.1 a	7.3 a	5.7 a	32.8 a	865.6 a	68.5 a	66.2 a	8.3 ab
	N100	2.8 a	32.3 b	8.1 a	5.9 a	31.8 a	699.6 a	68.4 a	64.8 a	9.4 a
PM		0.3527	0.0188	0.1972	0.5517	0.1008	0.6219	0.4170	0.1055	0.0516
PN		0.0638	0.0002	0.1053	0.0002	0.4816	0.0892	0.0404	0.9960	0.0059
PI		0.0117	0.6067	0.5319	0.9850	0.8389	0.0205	0.0066	0.1076	0.0576

In columns, separated by microorganism application and N rate; means followed by the same letter are not significantly different by Tukey HSD test ($\alpha = 0.05$).

4. Discussion

4.1. Effect of the Inoculant on Dry Matter Yield and Apparent Nitrogen Fixation

The application of the biological inoculant significantly increased DMY in the second lettuce growing cycle (Figure 1). However, considering all four growing cycles, the effect was inconsistent, as in some cycles the average values were higher in lettuce that did not receive the microorganism. Additionally, the application of the inoculant did not consistently increase leaf N concentration, with values even being significantly lower in untreated lettuce in the second growing cycle (Figure 2). Nitrate levels in lettuce tissue did not increase with the inoculant application. In the second growing cycle, the values were even significantly lower than those in the untreated control. A concentration/dilution effect seems to have occurred, where, when DMY was lower, N levels in the tissues were higher, and vice versa. The phenomenon of concentration/dilution has long been established [35,36] and has continued to be observed in more recent studies [11,37]. In this study, it seems that the small fluctuations observed in DMY due to the application of the microorganism were poorly related to N availability for the plant. Also, the nitrate content in the tissues, sometimes seen as an indicator of high sensitivity to N availability because it is an unmetabolized form that accumulates in tissues [38], varied similarly to the total N concentration in the leaves (Figure 3). Thus, this variable also did not reveal any influence on the plant's N nutritional status due to the microorganism application, perhaps because in biological fixation, the plant accesses N in the form of ammonia [12,13,28].

The average value of apparent N fixation was consistently very low, slightly negative for lower mineral N rates, and slightly positive for higher mineral N rates (Table 2). This inoculant is presented to farmers as having the potential to be used for all crops [39], supported by the fact that microorganisms of the *Methylobacterium* genus are ubiquitous in nature and colonize most plants [24,26]. However, the N-fixing capacity typically depends on highly specific relationships between diazotrophic microorganisms and host plants, reaching its maximum exponent in endophytic relationships that occur in nodulated legumes [12,13]. In the case of legumes, it is well established that in N-poor soils, the plant secretes secondary metabolites called flavonoids, which are recognized by the bacteria, eliciting the release of lipochitooligosaccharides known as nodulation factors. These

nodulation factors, in turn, are recognized by the legume [40]. Subsequently, the bacteria penetrate the root cells, preferably through the root hairs. As the bacteria advance within the root hair, their multiplication occurs along with that of the cortical cells, initiating nodule growth. The bacteria transform into bacteroids as nitrogenase and leghemoglobin synthesis takes place, enabling N fixation at high rates due to the supply of photosynthates to the nodule via the phloem and the protection conferred by leghemoglobin to nitrogenase activity [12,13].

The type of specificity established with *M. symbioticum* and plants is not sufficiently clear. It is known that some *Methylobacterium* species are involved in N fixation during interactions with plants and have been reported as putative endophytes, maintaining a symbiotic relationship with plants [27]. The microorganism used in this study, although isolated from soil, from an arbuscular mycorrhizal fungus [23], appears in a commercial product for application to the shoots [39]. Supposedly, the microorganism invades the phyllosphere, where it will have privileged access to various products released by the plant such as methanol, which it uses as a C source, but also soluble carbohydrates, amino acids, organic acids, and many other compounds, which may increase its fixation capacity compared to free-living microorganisms [20,24,27,41].

However, the phyllosphere microbiome exerts effects on the plant that can be neutral, negative, or positive, and in the latter case, may result from N fixation or other interactions, such as the production of plant hormones [24,25,42]. On the other hand, the phyllosphere is a potentially hostile environment influenced by biotic and abiotic factors. This causes the microbiome at the phyllosphere level to vary significantly with the cultivated species and within the same species, with the prevailing environmental conditions in each region, due to the strong competition for nutrients and space [20,43,44]. Although quantified effects of the microbiome on plants are poorly understood [20,24,45], it does not seem very plausible that a given microorganism remains strongly competitive in the phyllosphere of a wide diversity of plant species and environmental growth conditions.

Being ubiquitous in nature, the presence of microorganisms of the *Methylobacterium* genus in the phyllosphere can be expected regardless of their external application. It remains to be better clarified whether the selected strain of *M. symbioticum* used in this study is more effective in N fixation than others under such diverse conditions. The results of this study showed that in lettuce, under these cultivation conditions, the N fixed by the microorganism was very modest, not matching the very favourable results reported in maize and strawberries by the team that developed the commercial product [28,39]. The observed N fixation values in this study were so low (<6.3 kg ha^{-1} at the highest N rate) that they would hardly justify farmers' application of this commercial product. The observed values appear to align more closely with the N fixation capacity observed in free-living phyllosphere N-fixing microorganisms [18,46–48].

4.2. Effect of Nitrogen Applied to the Soil on Dry Matter Yield and Apparent Nitrogen Recovery

The application of N to the soil significantly influenced DMY, following a saturation curve (between 0 and 100 kg ha^{-1} of N), as typically occurs when increasing the applied N rate [5]. The N concentration in the tissues increased even more sharply than DMY, showing a direct relationship with the N applied to the soil. This occurs because N can be taken up by plants in luxury consumption, that is, in quantities above immediate metabolic needs, accumulating in tissues as reserves for future use [5].

Although with some variation between growing cycles, the apparent N recovery decreased with N application. The reduction in N-use efficiency by plants as the applied rate increases is one of the main aspects to consider in N fertilization management in crop fields [49,50]. It is estimated worldwide that no more than 50% of the applied N is used by growing plants [3]. The remaining N is lost from agricultural ecosystems, mainly through nitrate leaching into water bodies [4,5] and through denitrification, leading to atmospheric contamination with greenhouse gases such as N oxides [5,6]. In this study, the apparent N recovery values, considering the average of the four growing cycles, were

73.5%, 66.4%, and 54.0% for the applications corresponding to 25, 50, and 100 kg ha^{-1} of N, respectively (Table 1). These values can be tendentially higher than those frequently observed in field trials [49,50], and they are due to the better control of N losses through leaching and denitrification that can be achieved in pots compared to field trials.

4.3. Concentration of Other Nutrients in Lettuce Tissues

The application of the inoculant had a reduced effect on the concentration of other nutrients in plant tissues besides N. When there was an occasional significant difference, the result was not consistent with the other growing cycles. If the microorganism had any effect on the plant, it was not sufficient to affect the concentration of nutrients in its tissues.

The strong effect of N applied to the soil on DMY and N concentration in plant tissues exerted various synergistic and antagonistic interactions with other nutrients (Tables 4–6). A consistent reduction in K concentration in tissues was observed with increasing N rate, and, consequently, with the increase in N concentration in tissues and DMY. Since K is a nutrient taken up by plants in very high amounts, with sufficiency range values for most types of lettuce being higher than those of N [51], the likely cause for the reduction in K concentration in tissues with N applied was a dilution effect, due to the strong increase in biomass production [35,36].

In the case of B, a similar trend of decreasing concentration with increasing N rate seems to have been observed. Soluble B in the soil is primarily in the form of boric acid (H_3BO_3), which is the main form taken up by plants at pH levels between 5 and 9 [49,52], making it less likely for antagonistic or synergistic interactions to occur with N in absorption. On the other hand, soils low in organic matter and clay have a very limited capacity to supply B to plants [5,52], as has been demonstrated in many other studies in the region [53,54]. It was likely the limitation in B supply to plants that facilitated the occurrence of the dilution effect, as the N rate stimulated an increase in biomass production.

In the case of micronutrient metals, there were occasionally significant differences, but without clear consistency within the treatments. The bioavailability of these metals is highly dependent on the soil redox potential [55,56]. It is always challenging to maintain the same soil moisture conditions in pots, as treatments can cause some plants to grow more than others and transpire different amounts of water. Although efforts were made to maintain equivalent soil moisture levels in all pots during watering, it is likely that irrigation created small differences in soil redox potential throughout the growing cycle, which is the likely the cause for the sometimes-observed differences in metal concentrations in tissues.

Significant interactions were observed between factors in almost all growing cycles, particularly concerning the concentration of nutrients in tissues. However, consistency across results was lacking, as different nutrients exhibited varied responses in different growing cycles. The influence of one factor on another may be attributed to several factors: N fixation, which varied with the N applied to the soil; concentration/dilution phenomena, mentioned earlier; and environmental factors such as wetting/drying cycles in the pot. These environmental dynamics affect nutrient bioavailability, particularly that of micronutrients, for which significant interactions between factors were more frequently observed.

5. Conclusions

In a study where a strong response to applied N to the soil occurred, as typically observed in N fertilization trials, and where the response to applied N reduced N-use efficiency, the plant's response to the application of *M. symbioticum* to the shoots was weak for any of the N rates applied to the soil, but particularly for N0 and N25 treatments, even considering that four lettuce growing cycles were conducted. In biological systems, high N fixation typically occurs when there is a high degree of specificity between the microorganism and the host plant. Therefore, achieving a high capacity for fixation across a wide range of plant species is unlikely.

Certainly, the inoculant has the potential to be used in agriculture, providing benefits to farmers, but likely not across all crops. It seems necessary to establish a much better understanding of more specific conditions under which the benefits can be quantitatively more effective, reducing the risk of failure in agricultural fields. This is because it is an input factor with associated acquisition and application costs. Moreover, if the technology fails, it can lead to a loss of crop productivity due to N deficiency, reducing farmers' profits.

As far as we know, these are the first independent results of the use of this inoculant. It is important that more studies emerge in the coming years for a more accurate assessment of the potential use of this commercial product.

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