Comunicata Scientiae
Horticultural Journal

ORIGINAL ARTICLE
published: 29 Fevereiro 2024
https://doi.org/10.14295/CS.v15.4235

Lettuce yield response to application of rhizobacteria and nitrogen to the growth substrate

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Abstract

Rhizobacteria of agronomic interest can promote plant growth, increase productivity, and reduce the demand for nitrogen (N) fertilizers by improving the N use efficiency in crops. The objective of this study was to assess the effects of rhizobacteria and nitrogen on the yield of lettuce plants (cultivar Vera). The experiment was carried out in a greenhouse, using a completely randomized design in a 2×2×2×2 factorial arrangement, with five repetitions. The factors consisted of single and combined applications of liquid inoculants (1 mL) based on *Azospirillum brasilense*, *Bacillus subtilis*, and *Bradyrhizobium japonicum*, as well as nitrogen (75 kg ha^{-1}) in the growth substrate. The variables assessed were total and commercial shoot fresh and dry weights, root dry weight, total dry weight, and numbers of total and commercial leaves, and shoot nitrogen content. Lettuce yield components improved with nitrogen fertilizer application. The single application of rhizobacteria did not increase lettuce yield, as the plants had decreases in root weight in response to *Bradyrhizobium japonicum* and decreases in total and commercial shoot dry weights, root dry weight, and total dry weight when *Bacillus subtilis* was used. However, the combined application of *Azospirillum brasilense*, *Bacillus subtilis*, and nitrogen increased the lettuce number of commercial leaves and shoot nitrogen content when one or another microbial specie was associated with nitrogen fertilizer.

Keywords: *Azospirillum brasilense, Bacillus subtilis, Bradyrhizobium japonicum*, PGPR

Introduction

An adequate supply of nutrients, mainly nitrogen, is essential for ensuring the growth and yield of lettuce (*Lactuca sativa*) crops, which have significant economic and social importance in Brazil. However, nitrogen in soils or other growth substrates is often not readily available in an assimilable form or in sufficient quantities to meet the plants' demand; therefore, applications of nitrogen fertilizers are necessary (Amirouche et al., 2019; Cardoso et al., 2015; Milhomens et al., 2015; Sylvestre et al., 2019).

Considering the economic, environmental, and operational challenges associated with nitrogen fertilizers, the search for alternatives that can minimize their use and enhance the effects of applying lower quantities of N fertilizer is essential to improve the N use efficiency in crops. Plant growth-promoting rhizobacteria (PGPR) are a group of microorganisms that can contribute to both aspects (Andrade et al., 2023; Lima et al., 2017; Santos et al., 2019; Venancio et al., 2019).

PGPR have nitrogen-related effects as they are biological N-fixing bacteria. Additionally, these microorganisms provide other benefits to plants, such as production of siderophores, synthesis of phytohormones, solubilization of phosphates, and suppression of pathogens (Castillo et al., 2015; Hashem et al., 2019). Interest in using PGPR for vegetable crops is growing due to positive results that have been found not only for lettuce (Ferreira et al., 2013; Schlindwein et al., 2008; Segato et al., 2016) but also for carrot (Flores-Félix et al., 2013), sweet pepper (Blanco et al., 2018), and tomato crops (MangMang et al., 2015).

Leaf vegetables are a group of plant species that can benefit from PGPR due to their multiple mechanisms of action, which can directly and indirectly promote plant growth, minimize biotic and environmental stresses, and reduce the use of synthetic fertilizers, mainly nitrogen, and agrochemicals. Thus, the objective of this

Received: 20 October 2023
Accepted: 21 November 2023

work was to assess the isolated and combined effects of rhizobacteria (*Azospirillum brasilense*, *Bacillus subtilis*, and *Bradyrhizobium japonicum*) and nitrogen in the growth substrate on the yield of lettuce plants of the cultivar Vera.

Material and Methods

The experiment was carried out in a greenhouse at the experimental area of the Federal University of Acre, in Rio Branco, AC, Brazil (9°57'34.1"S, 67°52'08.9"W, and 150 m of altitude) from June to August 2018. The cultivar Vera lettuce (Crespa group) was used as plant test.

A completely randomized design was used, in a 2×2×2×2 factorial arrangement, with 5 repetitions. The factors consisted of single and combined applications of liquid inoculants (1 mL) based on *Azospirillum brasilense*, *Bacillus subtilis* and *Bradyrhizobium japonicum*, and nitrogen (75 kg ha^{-1}) in the growth substrate. The experimental units consisted of white polyvinyl chloride pipes, with a diameter and height of 14.5 cm, with capacity for 2.4 L, containing a commercial growth substrate composed of biostabilized *Pinus* bark, vermiculite, ground charcoal, and phenolic foam. The growth substrate was chemically characterized before the experiment, presenting the following results: pH (water) = 6.4; organic matter = 89 g dm^{-3}; P = 560 mg dm^{-3}; K = 33 mmol$_c$ dm^{-3}; Ca = 96 mmol$_c$ dm^{-3}; Mg = 27 mmol$_c$ dm^{-3}; H + Al = 18 mmol$_c$ dm^{-3}; S = 36 mg dm^{-3}; Fe = 66 mg dm^{-3}; Mn = 112 mg dm^{-3}; Cu= 0.4mg dm^{-3}; Zn = 40.9 mg dm^{-3}; B = 1.55 mg dm^{-3}; sum of bases = 190.7 mmol$_c$ dm^{-3}; and base saturation = 91.3%.

The seedlings used in the experiment were grown in 200-cell expanded polyethylene trays containing a mixture of sand (50%) and vermiculite (50%), using one seed per cell. Two seedlings were transplanted into each experimental unit 10 days after sowing, when they exhibited one pair of true leaves.

Commercial liquid inoculants based on *Azospirillum brasilense* (strains AbV5 and AbV6), *Bradyrhizobium japonicum* (SEMIA 5079 and 5080), and *Bacillus subtilis* (isolate UFPEDA 764) were used as sources of rhizobacteria. The viable cell number of each product was estimated using serial dilution and plate count technique (Tortora et al., 2017) in the following media: a) modified NFb medium for *Azospirillum brasilense* composed of malic acid (5 g), dipotassium phosphate (0.5 g), iron sulfate (0.5 g), manganese sulfate (0.01 g), and magnesium sulfate (0.2 g), sodium chloride (0.1 g), calcium chloride (0.02 g), sodium molybdate (0.002 g), bromothymol blue (0.002 g), agar (20 g), potassium hydroxide (4 g), and distilled water (1 L); b) modified 79 medium for *Bradyrhizobium japonicum* containing mannitol (10 g), yeast extract (1 g), sodium chloride (0.1 g), magnesium sulfate (0.2 g), dipotassium phosphate (0.5g), bacteriological agar (20 g), and distilled water (1 L); and c) nutrient agar for *Bacillus subtilis* containing peptone (5 g), meat extract (1.5 g), yeast extract (1.5 g), sodium chloride (5 g), agar (15 g), and distilled water (1 L). The results obtained after plate counting showed the presence of 2.01 × 10^{8}, 6.45 × 10^{9}, and 1.11 × 10^{9} viable cells per mL of *A. brasilense*, *B. japonicum*, and *B. subtilis*, respectively.

The treatments were applied directly to the growth substrate one day after seedling transplanting, under mild temperatures to minimize their effect on the survival and establishment of microorganisms. One milliliter of each rhizobacteria-based inoculant was manually applied, either isolated or in combination, to furrows between the two seedlings at an approximate depth of 1 cm. Urea (45%N) was used as source of nitrogen and was applied at a dose corresponding to 75 kg ha^{-1} (Milhomens et al., 2015; Yuri et al., 2016) and manually incorporated to the growth substrate to a depth of approximately 2 cm. Thinning was performed 10 days after transplanting, leaving the most vigorous plant in each experimental unit.

Irrigation was carried out regularly, focusing on maintaining the moisture of the growth substrate at approximately 70% in all experimental units. Additionally, temperature and air relative humidity were monitored during the experiment using a datalogger, and averaged 27.1 °C and 71.3%, respectively.

Assessments were carried out 49 days after transplanting, during the period of maximum lettuce vegetative development, before the bolting stage. Plants were cut at the substrate level, below the basal leaves, using a blade, and the following yield indicators were evaluated: total and commercial shoot fresh and dry weights, root dry weight, total dry weight, total number of leaves, number of commercial leaves, and shoot nitrogen content.

Total shoot fresh weight and total number of leaves were evaluated considering all leaves, regardless of their conditions. However, the commercial shoot fresh weight and number of commercial leaves were assessed disregarding senescent, spotted, and physically damaged leaves. Fresh weights were measured on a digital scale with precision of 0.01 g. Roots were removed from the growth substrate and washed in sieves for assessing their dry weights. The total dry weight was obtained by summing the total shoot and root dry weights.

The dry weights were obtained by placing the shoot and roots in paper bags and drying them in a forced air circulation oven at 65 °C until constant weight; they were then weighed on a digital scale with precision of 0.01 g. Shoot nitrogen content was quantified by wet digestion, following the Semimicro-Kjeldahl method described by Miyazawa et al. (2009).

The results were subjected to statistical analysis after assessing the presence of discrepant data (Grubbs, 1969), error normality (Shapiro & Wilk, 1965), and homogeneity of variance (Cochran, 1941). The results of the variables that did not meet the assumptions of analysis of variance were transformed. The F-test was applied to verify the significance of isolated (independent) and combined (interactions) effects of the factors, and for significant interactions ($p \leq 0.05$), the degrees of freedom of the treatments were unfolded, considering the effects of levels of one within the other. Higher-order interaction was considered for two or more significant interactions involving the same factors. Analysis of variance was performed using the statistical program Sisvar, developed by Ferreira (2011).

Results and Discussion

The application of nitrogen (N) fertilizer had isolated effect on total shoot fresh and dry weights, commercial shoot fresh and dry weights, root dry weight, total number of leaves, and shoot nitrogen content (**Table 1**). Positive responses of lettuce plants to additional application of nitrogen fertilizers have been reported in the literature (Amirouche et al., 2019; Cardoso et al., 2015; Milhomens et al., 2015; Sylvestre et al., 2019), denoting the need for this element for growing this vegetable species. In the present study, the application of N fertilizer at a dose equivalent to 75 kg ha^{-1} to the growth substrate contributed to increases in the evaluated yield components of lettuce plants. The primary commercial component of lettuce plants is the leaves in their aerial part; thus, the overall increase in yield obtained with N application, considering the variables shoot fresh weight (SFW) and commercial shoot dry weight (CSDW), was higher than 10% (Table 1). The increases resulting from N fertilizer application (Table 1) may be attributed to the insufficient amount of this nutrient available in the commercial substrate to meet the plants' demand for N until the harvest stage.

Although the growth substrate used had other fertility attributes considered adequate for lettuce crops, the lower yield performance of lettuce plants grown in this substrate without N fertilizer application may have been due to some characteristic of the substrate compounds, such as the C-to-N ratio, which could have compromised the N release to the plants. Additionally, N immobilization by the microbiota may have reduced the nutrient availability to the plants; thus, the application of N fertilizer contributed to making the growth substrate nutritionally more suitable, promoting better growth and yield for lettuce plants.

Bacillus subtilis had isolated effect on total and commercial shoot dry weights, total dry weight, and root dry weight (**Table 2**). The decreases in these variables due to the application of this rhizobacterium may be attributed to the competition between the plants and the microorganisms for nutrients in the substrate, mainly nitrogen, which is direct related to plant growth and production. In contrast with the findings of the present study, Segato et al. (2016) found that the use of *Bacillus subtilis* increased shoot and root fresh weights in lettuce plants. Ferreira et al. (2013) reported that this rhizobacterium improved the biometric parameters of seedlings when rates from 5 g to 20 g of inoculant per kg of commercial substrate were applied. However, positive effects will not always be obtained from the use of this microbial species, even under similar conditions to the present study, as several factors related to biological interactions—among microorganisms and between microorganisms and plants—are determining factors for increases, decreases, or no effects on lettuce growth and production or even no effects.

The single application of *Bradyrhizobium japonicum* decreased root dry weight (**Table 3**). However, this decrease in root growth does not necessarily indicate a negative effect of the application of this microbial species, provided that the total plant growth is not reduced; this decrease was not observed in the present study. Kozusny-Andreani & Andreani Junior(2014) reported

Table 1. Effect of nitrogen on total shoot fresh and dry weights (TSFW and TSDW, respectively), commercial shoot fresh and dry weights (CSFW and CSDW, respectively), total dry weight (TDW), root dry weight (RDW), total number of leaves (TNL), and shoot nitrogen content (SNC) of the cultivar Vera lettuce

Nitrogen	TSFW	CSFW	TSDW	CSDW	TDW	RDW[1]	TNL[2]	SNC
	.... g						unit	mg
Absence	195.73b	181.68b	15.71b	13.88b	17.71b	1.99b	34.38b	295.58b
Presence	216.11a	199.96a	17.75a	15.53a	19.94 a	2.19a	36.13a	327.66a
CV (%)	11.04	11.78	9.67	10.51	9.14	8.61	7.83	17.61
F-value	16.09**	13.23**	31.68**	22.85**	33.56**	22.53**	9.50**	6.83*

Means followed by the same letter in the columns do not differ (p>0.05) according to the F-test.

[1]Original results with data transformed into 1/x for analysis of variance as they do not meet the homogeneity of variances.

[2]Original results with data transformed into $\sqrt[5]{x}$ analysis of variance as they do not meet the normality of errors.

Table 2. Effect of *Bacillus subtilis* on total shoot dry weight (TSDW), commercial shoot dry weight (CSDW), total dry weight (TDW), and root dry weight (RDW) of the cultivar Vera lettuce

Bacillus subtilis	TSDW	CSDW	TDW	RDW[1]
	.... g			
Absence	17.11 a	15.10 a	19.26 a	2.15 a
Presence	16.35 b	14.32 b	18.39 b	2.04 b
CV (%)	9.67	10.51	9.14	8.61
F-value	4.47*	5.12*	5.13*	8.41**

Means followed by the same letter in the columns do not differ (p>0.05) according to the F-test.

[1]Original results with data transformed into 1/x for analysis of variance as they do not meet the homogeneity of variances.

Table 3. Effect of *Bradyrhizobium japonicum* on root dry weight[1] (g) of the cultivar Vera lettuce

Bradyrhizobium japonicum		CV (%)	F-value
Absence	Presence		
2.17 a	2.02 b	8.61	13.41**

Means followed by the same letter do not differ (p>0.05) according to the F-test. [1]Original results with data transformed into 1/x for analysis of variance as they do not meet the homogeneity of variances.

that *Bradyrhizobium japonicum* had no effect on root weight of lettuce plants and attributed the absence of significant effects on seedling growth to non-interaction between plants and the bacterial strain used. Blanco et al. (2018) also found that lettuce plants presented low sensitive to the action of rhizobial strains applied to a commercial substrate for seedling production, resulting in absence of growth-promoting effects from the tested microorganisms.

The interaction between *Bacillus subtilis* and *Bradyrhizobium japonicum* had a significant effect on commercial shoot fresh weight, number of commercial leaves, and shoot nitrogen content (**Table 4**). The application of *Bacillus subtilis* in the absence of *Bradyrhizobium japonicum* decreased commercial shoot fresh weight and shoot nitrogen content, whereas the application of *Bradyrhizobium japonicum* in the absence of *Bacillus subtilis* decreased the number of commercial leaves and shoot nitrogen content. However, when one of these rhizobacterial species was applied in the presence of the other, the negative effect caused by one of them was attenuated. Nevertheless, the use of rhizobia for leaf vegetable growth has been an alternative primarily used to reduce the application of nitrogen fertilizers, as these microorganisms can partially meet the nitrogen demand for plants according to Santos, Nogueira, and Hungria (2019). Furthermore, Flores-Félix et al. (2013) found increases in N and P contents and increased growth of lettuce and carrot plants, highlighting the potential of rhizobial application for growing non-legume crops.

The negative effects of the combined application of *Bacillus subtilis* and *Bradyrhizobium japonicum* on lettuce (Table 4) differ from positive results found in other studies evaluating the single use of these rhizobacteria. Pishchik et al. (2016), for example, found increases in fresh and dry weights, shoot nitrogen and chlorophyll content, as well as a decrease in nitrate levels when *Bacillus subtilis* was used. The authors attributed these effects to the production of auxins and a higher concentration of organic solutes (products of photosynthesis) in plant vacuoles. Schlindwein et al. (2008) also reported that the synthesis of indoleacetic acid by isolates of *Bradyrhizobium* sp. increased seedling vigor, dry weight, and root and shoot lengths in lettuce plants.

The triple interaction between *Azospirillum brasilense*, *Bacillus subtilis*, and nitrogen resulted in differentiated responses for the number of commercial leaves and shoot nitrogen content (**Table 5**). The application of *Azospirillum brasilense* in the absence of *Bacillus subtilis* and nitrogen decreased the number of commercial leaves, as found for the application of *Bacillus subtilis* in the absence of the other two factors. However, an increase in this variable was observed for the combined application of nitrogen and *Azospirillum brasilense* in the absence of *Bacillus subtilis*; this may be due to the presence of the microorganism, which enhanced the plant's capacity to use the N made available through fertilization. Lima et al. (2017) also verified that *A. brasilense* improved the availability of nitrogen applied to the soil 15 days before transplanting lettuce seedlings, resulting in no need for additional fertilizer applications. *Azospirillum brasilense* produces cytokinin, which is a plant hormone directly involved in the physiological maturation of chloroplasts, cell enlargement, and leaf expansion (Castillo et al., 2015); this microbial mechanism may have contributed to the increase in the number of commercial leaves in the lettuce plants evaluated in the present study. Additionally, this effect can also be attributed to the synthesis of indoleacetic acid by *Azospirillum brasilense*, as observed by MangMang et al. (2015) when evaluating germination and initial growth of lettuce and tomato plants.

The application of *Bacillus subtilis* in the absence of *Azospirillum brasilense* and nitrogen decreased shoot nitrogen content (Table 5). A similar result was found for the application of *Azospirillum brasilense* in the presence of the

Table 4. Commercial shoot fresh weight (CSFW), number of commercial leaves (NCL), and shoot nitrogen content (SNC) of the cultivar Vera lettuce as a function of interaction between *Bacillus subtilis* and *Bradyrhizobium japonicum*

Variables	*Bacillus subtilis*	*Bradyrhizobium japonicum*		CV (%)	F-value
		Absence	Presence		
CSFW(g)	Absence	198.98Aa	190.02Aa	11.78	4.52^{*}
	Presence	180.92Ba	193.34Aa		
NCL(unit)	Absence	30.30Aa	28.45Ab	8.70	4.41^{*}
	Presence	29.05Aa	29.60Aa		
SNC(mg)	Absence	360.63Aa	285.79Ab	17.61	19.48^{**}
	Presence	283.28Ba	316.78Aa		

Means followed by the same lowercase letters in the columns and uppercase in the rows do not differ (p > 0.05) according to the F-test.

Table 5. Number of commercial leaves (NCL) and shoot nitrogen content (SNC) of the cultivar Vera lettuce as a function of the interaction between *Azospirillum brasilense*, *Bacillus* subtilis, and nitrogen

Variables	Rhizobacteria		Nitrogen		CV(%)	F-value
	A. brasilense	*B. subtilis*	Absence	Presence		
NCL (unit)	Absence	Absence	30.50 AAa	29.00AAa	8.70	5.59^{*}
	Presence	Absence	27.80 Bab	30.20Aaa		
	Absence	Presence	28.10 ABa	29.90AAa		
	Presence	Presence	9.50 Aaa	29.80Aaa		
SNC (mg)	Absence	Absence	320.51 AAa	319.57AAa	17.61	4.36^{*}
	Presence	Absence	313.51Aaa	339.25Aaa		
	Absence	Presence	266.85 ABb	356.49AAa		
	Presence	Presence	281.46 Aaa	295.32Baa		

Means followed by the same lowercase letters in the columns, uppercase in the rows, underlined uppercase in the columns for the first and third means and underlined lowercase in the columns for the second and fourth means, do not differ (p > 0.05) according to the F-test.

other two factors. However, the N fertilizer combined with *Bacillus subtilis* in the absence of *A. brasilense* resulted in higher shoot nitrogen content. The high potential for root colonization by *Bacillus subtilis* (Hashem et al., 2019) may have contributed to a better N use efficiency in the lettuce plants, increasing the shoot nitrogen content by 34%, indicating the benefit of the combined application of these biological and mineral sources to the growth substrate used. Venancio et al. (2019) evaluated the combined applications of *Bacillus subtilis* and nitrogen fertilizer and found increased lettuce yield using only 50% of the N rate recommended for the crop, reinforcing the results obtained in the present study, which indicate that the use of rhizobacteria can increase the availability of N to plants while reducing the amount of fertilizer applied for growing this leaf vegetable.

According to the results of the present study, the application of *Azospirillum brasilense*, *Bacillus subtilis*, *Bradyrhizobium japonicum*, and nitrogen fertilizer to commercial substrate can result in no effects, decreases, or increases in yield componentes of lettuce plants of the cultivar Vera (Tables 1 a 5). These results indicate the high variability of plant responses to the isolated or combined application of these biological and mineral sources for vegetable crops. However, it is important to further investigate the potential of these biological sources, typically used for growing Fabaceae and Poaceae species, since the action of plant growth-promoting rhizobacteria is not limited to plants from these botanical families. Therefore, conducting research studies of this nature is essential not only for the potential benefits to plant growth and production but also because the use of rhizobacteria can contribute to the mitigation of impacts and the reduction of production, operational, and environmental costs associated with the use of agrochemicals in traditional agricultural production systems.

Conclusions

The yield of Vera lettuce increases with the application of nitrogen fertilizer to commercial substrate with similar characteristics to that used in this study.

The single application of *Bacillus subtilis* or *Bradyrhizobium japonicum* decreases the performance of yield components of Vera lettuce plants, whereas the combination of these two rhizobacteria species has no effects in these variables.

The application of *Azospirillum brasilense*, *Bacillus subtilis*, and nitrogen to the growth substrate is beneficial for lettuce plants, but only when one or another microbial specie is combined with nitrogen fertilizer.

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