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Research Article

Inoculation with growth-promoting bacteria and arbuscular mycorrhizal fungi reduce the demand for fertilizers in lettuce[1]

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ABSTRACT

The use of plant growth-promoting microorganisms is an alternative to reduce costs with mineral fertilizers and increase sustainability in agriculture. This study aimed to investigate the effect of co-inoculation with plant growth-promoting bacteria and arbuscular mycorrhizal fungi on the growth and yield of lettuce under reduced NPK fertilization and greenhouse conditions, using five treatments (100 % of fertilization; 50 % of fertilization; 50 % of fertilization + *Azospirillum brasilense* inoculation; 50 % of fertilization + *Rhizophagus clarus* inoculation; 50 % of fertilization + co-inoculation), with eight replications. The inoculation with *R. clarus* increased plant height by 79.5 %, while, for *A. brasilense*, this increase reached 68 %, as compared to NPK fertilization. The single inoculation of both microorganisms increased the stem mass, but only *A. brasilense* was associated to the higher number of leaves, although it was not accompanied by an increase in the fresh and dry leaf matter. The co-inoculation increased the leaf fresh matter by 24.6 % and the number of leaves by 25.3 %.

KEYWORDS: *Lactuca sativa*, *Azospirillum brasilense*, *Rhizophagus clarus*.

RESUMO

Inoculação com bactérias promotoras de crescimento e fungos micorrízicos arbusculares reduzem a demanda de fertilizantes em alface

O uso de micro-organismos promotores de crescimento de plantas é uma alternativa para reduzir custos com fertilizantes minerais e aumentar a sustentabilidade na agricultura. Objetivou-se avaliar o efeito da coinoculação de bactérias promotoras de crescimento e fungos micorrízicos arbusculares no crescimento e produção de alface sob fertilização reduzida de NPK, em casa-de-vegetação, utilizando-se cinco tratamentos (100 % de adubação; 50 % de adubação; 50 % de adubação + inoculação com *Azospirillum brasilense*; 50 % de adubação + inoculação com *Rhizophagus clarus*; 50 % de adubação + coinoculação), com oito repetições. A inoculação com *R. clarus* resultou em aumento de 79,5 % na altura das plantas, enquanto *A. brasilense* promoveu aumento de 68 %, em relação à fertilização NPK. A inoculação com ambos os micro-organismos de maneira isolada aumentou a biomassa do caule; porém, apenas *A. brasilense* esteve associado ao maior número de folhas, apesar de não ter sido acompanhado de aumento na biomassa fresca e seca das folhas. A coinoculação aumentou a biomassa fresca foliar em 24,6 % e o número de folhas em 25,3 %.

PALAVRAS-CHAVES: *Lactuca sativa*, *Azospirillum brasilense*, *Rhizophagus clarus*.

INTRODUCTION

The inoculation of crops with plant growth-promoting bacteria (PGPB) has been one of the most successful techniques applied worldwide to improve yield and reduce production costs (Hungria et al. 2013, Hungria et al. 2022).

In Brazil, inoculation benefits result in an average economy of US$ 15 billion annually, due to reduced - or even eliminated - input of fertilizers promoted by PGPB (Telles et al. 2023).

Brazil is the world's largest consumer of triple superphosphate (619,3 t) and potassium chloride (6,814.7 t), while the world consumption of urea is led by India (16,433.6 t), followed by China (8,034.4 t) and Brazil (3,224.9 t) (IFA 2024). In Brazil, from 2000 to 2021, the consumption of nitrogen fertilizers increased by 208 %, followed by 190 % of potassium fertilizers and 175 % of phosphorus fertilizers (IFA 2024).

Mineral fertilization is one of the main sources of soil, water and air pollution (Yahaya et al. 2023), and NPK fertilization can reduce the abundance and diversity of the soil microbial community (Castle et al. 2021). Furthermore, a substantial fraction of N is lost to the environment, causing adverse

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effects on water and air quality (Ju & Zhang 2017), through nitrate leaching into groundwater. It is also known that nitrogen-based fertilizers are a major source of reactive N species in the atmosphere, like nitrous oxide (Wang et al. 2021). Like N, the excessive release of P fertilizers into water bodies leads to eutrophication, causing a serious threat to biodiversity (Huang et al. 2017).

The main benefits of PGPB inoculation to soybean or corn include increased plant biomass (Santos et al. 2021), resistance to biotic and abiotic stresses (Chakraborty et al. 2024) and improved yield (Zilli et al. 2021, Ramírez-Cariño et al. 2024).

Bradyrhizobium and *Azospirillum* species are recommended as the best growth-promoting bacteria for soybean and corn, and are used to produce commercial inoculants in several countries (Nishi et al. 1996). More recently, products containing arbuscular mycorrhizal fungi (AMF) that belong to the *Rhizophagus* genus have also been explored in agricultural practices (Stoffel et al. 2020, Shimoia et al. 2023). Overall, the economic benefits of microbial inoculants are well established, and the cultivation of soybean and other crops is routinely associated to inoculation with commercial inoculants in top-producing countries, such as the United States and Brazil (Santos et al. 2019).

Due to the outstanding effects of PGPB on soybean and corn, research has expanded to other crops such as bean, rice and wheat (Hungria et al. 2010, Hungria et al. 2013), which are representative grain crops. However, very few efforts have been registered on horticultural species, and a very limited number of records are found regarding leaf-producing species such as lettuce, arugula and cabbage (Yildirim et al. 2016, Gato et al. 2023, Oliveira et al. 2023).

The inoculation of lettuce has been investigated mainly at the seedling-producing stage (Shao et al. 2023). Studies about the effects of inoculation at later growth stages of lettuce are very important to reveal the benefits of PGPB on leaf growth and mass, and those aspects are related to economic value. Machado et al. (2022) tested the inoculation of lettuce with *Azospirillum brasilense*, *Bacillus subtilis*, *Bacillus megaterium* and *Pseudomonas fluorescens*, and found no plant response to any bacteria, when compared to the absence of PGPB. However, all treatments had the same amount of fertilization, what could compromise plant response, because the benefits of microorganisms to plants are often maximized at reduced nutrients levels (Mrkovacki et al. 2008, Johnson et al. 2010).

In natural soil conditions, plants have their roots colonized by arbuscular mycorrhizal fungi, which are known as beneficial due to the improved mineral nutrition already widely proved in field and greenhouse studies, including some carried out with lettuce (Baslam et al. 2011, Turrini et al. 2018). These organisms are also influenced by mineral fertilizers and can lose their main biologic function. Ma et al. (2021) described that the addition of nitrogen and phosphorus decreased the AMF Shannon index, species richness, total colonization, spore abundance and extraradical biomass, and marginally decreased hyphal length. Liu et al. (2020) also demonstrated that the high amount of NPK addition decreased the AMF Shannon index and changed the community composition.

Hence, this study aimed to investigate the effects of single and co-inoculation with *Azospirillum brasilense* and *Rhizophagus clarus* on lettuce growth and production under reduced NPK fertilization. The hypothesis was that the use of plant growth-promoting microorganisms can reduce the amount of fertilizers applied in lettuce, representing significant savings for farmers and promoting a more sustainable and environmental-friendly production.

MATERIAL AND METHODS

The experiment was carried out under greenhouse conditions at the Universidade Federal de Santa Catarina, in Curitibanos, Santa Catarina state, Brazil (27º16’58”S, 50º35’04”W and altitude of 987 m), between April and July 2022.

According to the Köppen-Geiger classification, the predominant climate of the region is temperate mesothermal humid with mild summer (Cfb-type), with average annual rainfall of 1,676 mm and air temperature of 15 ºC. The soil is classified as a Cambisol (6.4 % of organic matter, clay texture) (FAO 2015), and its pH was adjusted to 6.0 with $CaCO_3$ at rates of 21 t ha^{-1}. Pots were set up using 5 L of soil and kept incubated for 40 days, to allow for the $CaCO_3$ reaction. After that, the experiment was set up.

The experiment was performed under a completely randomized design, with five treatments (T1: 100 % of NPK fertilization; T2: 50 % of

NPK fertilization; T3: 50 % of NPK fertilization + inoculation with *Azospirillum brasilense*; T4: 50 % of NPK fertilization + inoculation with *Rhizophagus clarus*; T5: 50 % of NPK fertilization + co-inoculation with *A. brasilense* and *R. clarus*) and eight replications.

The *A. brasilense* strains used in the experiment were obtained from the Embrapa Soja (Ab-V5 and Ab-V6) and the *R. clarus* isolate from the Universidade do Estado de Santa Catarina, in Lages. 'Vera' was the lettuce cultivar used, and sowing was performed in styrofoam trays containing commercial substrate, which were irrigated every day in the early morning and late afternoon. The best seedlings were transplanted at 40 days after sowing, when the plants had four definitive leaves.

Inoculation was performed near the roots using a precision pipette at the time of transplant, during a period with mild temperatures, to maximize the survival and establishment of microorganisms. First, both the *A. brasilense* strains were combined and the final concentration of the inoculant was 2×10^8 CFU mL^{-1}. Then, 0.5 mL of the inoculant was diluted in 19.5 mL of water. This suspension was carefully pipetted around seedlings and their substrate, when they were transplanted to the pots. The inoculation with *R. clarus* consisted of adding 33 g of inoculant (which delivered approximately 10 spores $plant^{-1}$) around the seedlings and their substrate during transplantation.

Regarding the 100 % of NPK fertilization treatment, base fertilization with 44.4 kg ha^{-1} of N (urea) and 355.5 kg ha^{-1} of P_2O_5 (triple superphosphate) was applied. For the topdressing fertilization, 35.5, 62.1 and 79.9 kg ha^{-1} of N were applied at 10, 20 and 30 days after the seedlings transplantation, respectively. Regarding KCl, 80, 140 and 180 kg ha^{-1} were applied at the same frequency as N. For the other treatments, the fertilizer doses applied were reduced by half. The fertilization procedures were carried out as recommended by the fertilization and liming manual for the Rio Grande do Sul and Santa Catarina state (SBCS 2016).

All pots were kept under greenhouse conditions at 28 ºC. They were weighed twice a week and watered as needed to keep the soil moisture at 50 % of the field capacity. The plant height (determined from the soil level to the shoot apical bud) and diameter (from the distance between the edges of different and opposite leaves; cm $plant^{-1}$), number of leaves (by manually counting), root length (from one to the other end of the root; cm $plant^{-1}$), root volume (by water displacement in a graduated cylinder of 100 mL, according to Scheffer-Basso 1999; cm^3 $plant^{-1}$), stem fresh and dry matter (g $plant^{-1}$), leaf fresh and dry matter (g $plant^{-1}$), root fresh and dry matter (g $plant^{-1}$) were evaluated at 47 days after transplantation. The aboveground structures of plants were divided manually into leaves and stems. The roots were carefully separated from the soil and thoroughly rinsed with tap water.

After weighing the fresh matter, the plant material was placed in paper bags and then dried in a forced air circulation oven at 70 ºC, for 48 hours. After that, measurements of stem, leaf and root dry matter were done.

All datasets were assessed for normality, and the plant variables were analyzed with one-way Anova. When significant variances were detected, the means were compared by the Scott-Knott test ($p < 0.10$). All statistical analyses were performed in the Sisvar software (Ferreira 2011).

RESULTS AND DISCUSSION

Significant differences ($p < 0.10$) among the treatments were observed for plant height, number of leaves, leaf fresh and dry matter (Table 1), and the highest value for plant height was registered when the lettuce plants were inoculated with *R. clarus* or with *A. brasilense*, if compared to 100 % of NPK fertilization (Table 2). When compared to 100 % of NPK fertilization, *R. clarus* promoted an increase of 79.5 % and *A. brasilense* of 68 % on plant height.

The highest number of leaves was observed with co-inoculation, as compared to 100 % of NPK fertilization (Table 2). Co-inoculation promoted increases of 25.3 % in the number of leaves (Figure 1). In a field experiment, Tahiri et al. (2022) showed that co-inoculation with PGPB (*Acinetobacter* sp. + *Rahnella aquatilis*) and AMF (*Glomus* sp. + *Sclerocystis* sp. + *Acaulospora* sp.) increased the number of leaves by 81 % and the total fresh weight by 63 %, when compared to the control treatment. Unlike *Acinetobacter* sp. and *Rahnella aquatilis*, *A. brasilense* is a bacterium proven to be non-pathogenic to humans and other plants (Alexandre 2017), emphasizing the importance of its study as an inoculant. Aini et al. (2019) showed that co-inoculation with a consortium of several strains of PGPB and *Glomus* sp. increased the total fresh weight of hydroponic lettuce by 14 %, when compared to the control.

The highest values for leaf fresh (60.8 g plant^{-1}) and dry matter (5.69 g plant^{-1}) were also observed when the lettuce plants were co-inoculated with *A. brasilense* and *R. clarus*, as compared to 100 % of NPK fertilization (48.8 g plant^{-1} and 4.22 g plant^{-1}, respectively) (Figure 2). When compared to 100 % of NPK, co-inoculation with *A. brasilense* and *R. clarus* promoted an increase of 24.6 % in the leaf fresh matter, which is one of the most important commercial traits for lettuce (Dalastra et al. 2020), and interactions between AMF and PGPB in the rhizosphere can result in a synergistic effect that enhances plant growth and quality, as shown by Bona et al. (2017). Emmanuel & Babalola (2020) also reported that co-inoculation is more beneficial for the yield and quality of horticultural crops than a single application of PGPB or AMF.

Table 1. Results of Anova for the lettuce growth variables. N = 40 (5 treatments x 8 replicates).

Variables	p-value	F value
Number of leaves (leaves plant^{-1})	0.0902*	2.191
Plant height (cm)	0.0010*	5.832
Diameter (cm)	0.3615^{ns}	1.123
Root length (cm)	0.1684^{ns}	1.717
Root volume (cm^3)	0.3935^{ns}	1.054
Leaf fresh matter (g)	0.0837*	2.248
Leaf dry matter (g)	0.1031*	2.090
Stem fresh matter (g)	0.3306^{ns}	1.194
Stem dry matter (g)	0.9365^{ns}	0.200
Root fresh matter (g)	0.6721^{ns}	0.590
Root dry matter (g)	0.2650^{ns}	1.368

* Significant at $p < 0.10$; ^{ns} not significant.

A. brasilense is the most thoroughly characterized PGPB, and its main mode of action is fixing nitrogen (El-Esawi et al. 2018), thus improving the photosynthetic efficiency and, as a result, fresh biomass (Oliveira et al. 2023). The benefits of *A. brasilense* for leaf vegetables have been related in some studies. Gato et al. (2023) showed an increase of 24.4 % in the shoot fresh matter of arugula plants (*Eruca sativa*), while Consentino et al. (2022) observed an increase of 47.8 % in the fresh weight of lettuce plants.

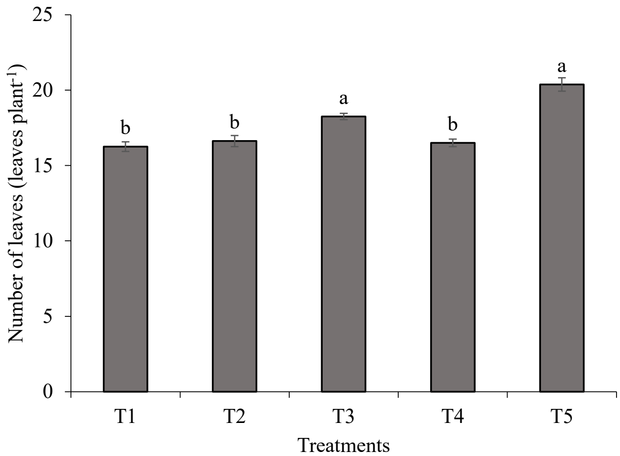

Figure 1. Number of leaves of lettuce plants under inoculation treatments with growth-promoting bacteria and arbuscular mycorrhizal fungi and fertilization, under greenhouse conditions. Different letters represent statistically significant differences according to the Scott-Knott test ($p < 0.10$). T1: 100 % of NPK fertilization; T2: 50 % of NPK fertilization; T3: 50 % of NPK fertilization + inoculation with *Azospirillum brasilense*; T4: 50 % of NPK fertilization + inoculation with *Rhizophagus clarus*; T5: 50 % of NPK fertilization + co-inoculation.

Table 2. Means and standard deviations (values between parentheses) of lettuce growth variables in response to inoculation treatments with growth-promoting bacteria and arbuscular mycorrhizal fungi and fertilization, under greenhouse conditions.

Variables	T1	T2	T3	T4	T5
Plant height (cm)	7.80(0.13) c[1]	10.70(0.18) b	13.10(0.38) a	14.00(0.40) a	10.50(0.19) b
Diameter (cm)	1,270.40(43.90) a	1,484.40(42.2) a	1,690.70(54.3) a	1,508.70(37.1) a	1,570.70(30.6) a
Root length (cm)	45.70(0.75) a	38.30(0.79) a	39.60(0.62) a	48.10(1.24) a	43.00(0.84) a
Root volume (cm^3)	30.00(1.55) a	23.10(1.19) a	23.10(0.65) a	20.60(0.49) a	21.20(0.87) a
Stem fresh matter (g)	2.09(0.10) a	1.64(0.09) a	2.28(0.09) a	1.69(0.06) a	2.44(0.09) a
Stem dry matter (g)	0.29(0.02) a	0.24(0.01) a	0.30(0.01) a	0.27(0.01) a	0.30(0.02) a
Root fresh matter (g)	21.00(0.88) a	22.80(1.05) a	25.00(0.69) a	20.80(0.86) a	26.40(0.95) a
Root dry matter (g)	0.80(0.02) a	1.10(0.05) a	1.30(0.06) a	1.50(0.07) a	1.30(0.07) a

[1] Different letters on the same line indicate statistically significant differences according to the Scott-Knott test ($p < 0.10$). T1: 100 % of NPK fertilization; T2: 50 % of NPK fertilization; T3: 50 % of NPK fertilization + inoculation with *Azospirillum brasilense*; T4: 50 % of NPK fertilization + inoculation with *Rhizophagus clarus*; T5: 50 % of NPK fertilization + co-inoculation.

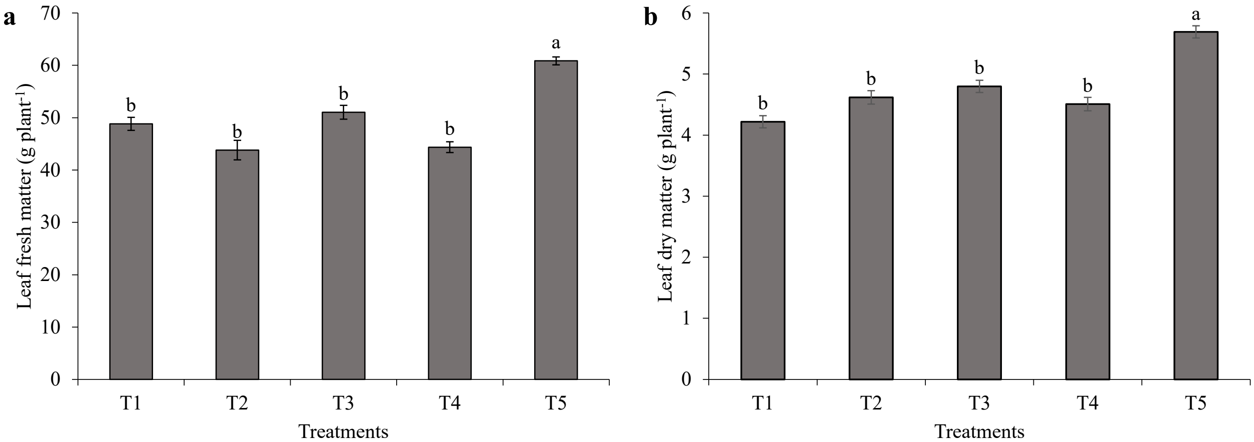

Figure 2. Leaf fresh and dry matter of lettuce plants under inoculation treatments with growth-promoting bacteria and arbuscular mycorrhizal fungi and fertilization, under greenhouse conditions. Different letters represent statistically significant differences according to the Scott-Knott test ($p < 0.10$). T1: 100 % of NPK fertilization; T2: 50 % of NPK fertilization; T3: 50 % of NPK fertilization + inoculation with *Azospirillum brasilense*; T4: 50 % of NPK fertilization + inoculation with *Rhizophagus clarus*; T5: 50 % of NPK fertilization + co-inoculation.

On the other hand, AMF increase water and nutrient absorption through hyphae, improving crops performance (Sagar et al. 2021), and has been proven a profitable practice for various horticultural plants (Basiru et al. 2020). These effects improve the agricultural production through increased yield and reduce the use of fertilizers, contributing to a sustainable agriculture (Zhu et al. 2022). Püschel et al. (2020) demonstrated that AMF (*Rhizophagus irregulars*) increase the water and nutrient uptake of alfalfa plants (*M. truncatula*), in addition to total dry weight, even in treatments with reduced supply of water. Moustakas et al. (2020) showed that the same AMF species increased the efficiency of photosynthesis in the *Salvia fruticose* herb, contributing to growth advantages by increasing leaf biomass. However, none of those studies were performed considering reduced fertilization. Furthermore, this is the first study to address the effect of *A. brasilense* (a commercially available bacterium strain in several countries) and AMF co-inoculation on lettuce growth. Those aspects represent feasible and economic-friendly tools that are both accessible to farmers and promising regarding production costs.

CONCLUSIONS

An increase was observed in height, number of leaves and leaf biomass of lettuce plants, even when reducing the NPK fertilization by 50 %, providing strong evidence that the co-inoculation with *Azospirillum brasilense* and *Rhizophagus clarus* represent a possibility of reducing mineral fertilizer inputs in large-scale lettuce production.

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