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Combined application of humic substances and PGPR inoculated and co-inoculated in plants of *Phaseolus lunatus* (L.) and *Leucaena leucocephala* (Lam.) de Wit

Aplicación conjunta de sustancias húmicas y PGPR inoculadas, y co-inoculadas en plantas de *Phaseolus lunatus* (L.) y *Leucaena leucocephala* (Lam.) de Wit

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Lima bean greenhouse experiment.
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ABSTRACT

The objective of this research was to evaluate the effect of inoculation and co-inoculation of rhizobia and *Azospirillum brasilense* combined with humic substances (HS) in growth promotion of *Phaseolus lunatus* (lima bean) and *Leucaena leucocephala* (leucaena). For this, experiments in a greenhouse with the cultivation of each plant species were carried out. A randomized complete block experimental design with five repetitions was followed. Plant seeds were sown and then inoculated with rhizobia and co-inoculated with *A. brasilense*. Subsequently, HS were added at the dose recommended by the manufacturer. In the experiments with both plants, control treatments with the addition of nitrogen (N) with or without HS were used. After 45 days, the shoot dry mass (SDM), root dry mass (RDM), shoot-accumulated N (Nac) and relative efficiency index (REI) were determined. In addition, the mass of dry nodules (MDN) in the lima bean plants and the number of nodules (NN) in leucaena plants were determined. The results showed that in the lima bean and leucaena plants there was a greater increase in SDM, RDM and Nac in treatments that received HS and co-inoculation with rhizobia and *A. brasilense* than in treatments that were inoculated only with rhizobia and HS and in treatments that received N and HS compared to addition of N and isolated inoculation of rhizobia. The combined application of HS and rhizobia in co-inoculation with *A. brasilense* had a greater effect on the increase of MDN in lima bean and NN in leucaena, than in treatments where only rhizobia with HS were added. These results indicate the existence of potential interaction of the use of HS with the co-inoculation of rhizobia and *A. brasilense*, showing promise for the production of sustainable agricultural crops.

Additional key words: humic substances; co-inoculation; rhizobia; *Azospirillum*; biostimulants; sustainability.

RESUMEN

El objetivo de esta investigación fue evaluar el efecto de la inoculación y co-inoculación de rizobios y *Azospirillum brasilense* combinados con sustancias húmicas (SH) en la promoción del crecimiento de *Phaseolus lunatus* (frijol lima) y *Leucaena leucocephala* (leucaena). Para ello se realizaron experimentos en invernadero con el cultivo de cada especie vegetal. Se siguió un diseño experimental de bloques completos al azar con cinco repeticiones. Se sembraron las semillas de plantas y luego se inocularon con rizobios y se coinocularon con *A. brasilense*. Posteriormente se agregaron SH con la dosis recomendada por el fabricante. En los experimentos con ambas plantas se utilizaron tratamientos control con adición de nitrógeno (N) con o sin SH. Después de 45 días, se determinó la masa seca de la parte aérea (MSPA), la masa seca de la raíz (MSR), el N acumulado en la parte aérea (Nac) y el índice de eficiencia relativa (IER). Además, se determinó la masa de nódulos secos (MNS) y en las plantas de leucaena el número de nódulos (NN). Los resultados mostraron que en las plantas de frijol lima y leucaena hubo un mayor aumento de MSPA, MSR y Nac en los tratamientos que recibieron SH y co-inoculación con rizobios y *A. brasilense* que en los tratamientos que fueron inoculados solo con rizobios y SH, y en tratamientos que recibieron N y SH en comparación con la adición de N y la inoculación aislada de rizobios. La aplicación combinada de SH y rizobios en co-inoculación con *A. brasilense* tuvo un mayor efecto en el aumento de MNS en frijol lima y NN en leucaena, seguido de los tratamientos donde solo se agregaron rizobios con SH. Estos resultados indican la existencia de una interacción potencial del uso de SH con la co-inoculación de rizobios y *A. brasilense*, lo que resulta prometedor para la producción de cultivos agrícolas sostenibles.

Palabras clave adicionales: sustancias húmicas; co-inoculación; rizobios; *Azospirillum*; bioestimulantes; sostenibilidad.

INTRODUCTION

Lima bean (*Phaseolus lunatus* L.) is the second most important species of the genus *Phaseolus*, after the common bean (*P. vulgaris*). This crop is important in Brazil due to its protein content, serving as a food source for the Brazilian population (Santos *et al*., 2009; Araujo *et al.*, 2015). In addition, this crop is a source of income for small producers in the northeastern region of the country and the state of Rio Grande do Sul (Franco *et al*., 2002). Additionally, although there is disagreement about its use in agriculture (Costa and Durigan, 2010), *Leucaena* *leucocephala* (Lam.) de Wit (leucaena) is a protein-rich legume used for animal production, for the development of silvopastoral and agroforestry systems in several Latin American countries such as Colombia, Panama, Costa Rica, Mexico, among others (Murgueitio *et al*., 2016), as well as for the recovery of degraded areas and reforestation in tropical soils (Barreto *et al*., 2010; Bueno and Camargo, 2015; Nicodemo *et al*., 2018; Kant *et al*., 2019).

In recent years, there has been growing interest in the production of food for human and animal food in a sustainable manner, with lower environmental impact and greater food security, especially with regard to the development of ecological intensification strategies, such as the efficient use of nutrients, biostimulant products for plant growth, the reduction of the need for disease and pest control, among others (Tittonell, 2014; Canellas *et al*., 2015). Plant growth biostimulants are substances or microorganisms that, when applied or inoculated in the rhizosphere or in plants, improve nutrient absorption, nutritional efficiency, tolerance to abiotic stress and crop quality. Examples include humic substances (HS) and plant growth-promoting rhizobacteria (PGPR) (Yakhin *et al*., 2017).

HS are the colloidal fraction of soil organic matter, being classified as humic acids (HA), fulvic acids (FA) and humines based on their solubility in acid or alkaline pH. Sources of HS include vermicompounds, sewage, peat, lignocellulose residues from refineries for ethanol production and coal production residues such as lignites and leonardite (Silva *et al*., 2000; Canellas *et al*., 2000; Canellas and Façanha 2004; Aguirre *et al*., 2009; Giannouli *et al*., 2009; Canellas and Olivares, 2014; Cubillos-Hinojosa *et al*., 2015; Spaccini *et al*., 2019). These latter sources are considered rich in HA and fulvic acids FA, obtained by the classical method of extraction with alkaline solutions (Senesi *et al*., 2007; Chassapis and Roulia, 2008; Giannouli *et al*., 2009), with leonardite the most used source for HS extraction in the HS-based product manufacturers industry.

The bioactivity of HS in the promotion of plant growth has been widely reported in several studies, which show that HS stimulates plant growth and development, induction of root proliferation by modifying root system architecture, leaf development, increased absorption of nutrients and regulation of enzymes important for plant metabolism, such as H^+-ATPase, VATP-ase, nitrate reductase, and auxinic (Chen and Avaid, 1990; Pinton *et al*., 1992; Façanha *et al*., 2002; Nardi *et al*., 2005; Chen *et al*., 2004; Zandonadi *et al*., 2007; Dobbs *et al*., 2010; Trevisan *et al*., 2010; Zandonadi and Busato, 2012; Zandonadi *et al*., 2013, Zandonati *et al*., 2014, Canellas and Olivares, 2014; Canellas *et al*., 2015; Shah *et al*., 2018).

These effects of HS on plant development depend on source, dose and genotype of plant (Vaughan and Malcolm, 1985; Rodda *et al*., 2006; Zandonadi *et al*., 2014). In lima bean plants, there are only reports of the effects of HS on the modification of potassium absorption kinetics, assimilation of mineral elements such as phosphorous and nitrogen, plant growth and nutrient concentration in common beans (*P. vulgaris*) (Rosa *et al*., 2009; Aydin *et al*., 2012). However, there were no reports on these effects in *P. lunatus* and *L. leucocephala*.

Plant growth-promoting rhizobacteria (PGPR) are a very diverse group of bacteria that have the capacity to promote plant growth (Kloepper and Schroth, 1978) by means of several mechanisms such as: biological nitrogen fixation (BNF) (Iniguez *et al*., 2004; Montañez *et al*., 2009), the production of auxins, cytokinins, gibereline strain and ethylene inhibition (Arshad and Frankenberger, 1992), antagonism against phytopathogens by the production of siderophores (Scher and Baker, 1982), the induction of systemic acquired resistance (Pieterse *et al*., 2014), or increased availability of nutrients such as phosphorus (Sturz *et al*., 2000; Sessitsch *et al*., 2002). These effects of PGPR on the growth of common bean, lima bean and leucaena plants have been documented in several studies, with some strains of PGPR used in the formulation of commercial inoculants (Hungria *et al*., 2000; Hungria *et al*., 2003; Yadegari *et al*., 2008; Bueno and Camargo 2015; Aguirre-Medina *et al*., 2015; Cubillos-Hinojosa *et al*., 2019, Cubillos-Hinojosa *et al*., 2020, Cubillos-Hinojosa *et al*., 2021). In common bean, the co-inoculation of rhizobia and *A. brasilense* is recommended, generating an increase in nodulation and crop yield (Hungria *et al*., 2013). However, despite some studies showing that several cultivars of lima bean can establish symbiotic association with rhizobia efficient in the biological nitrogen fixation (Ormeño *et al*., 2007; Santos *et al*., 2009; Antunes *et al*., 2011; Santos *et al*., 2011; Servín-Garcidueñas *et al*., 2014; Duran *et al*., 2014; Ormeño-Orrillo *et al*., 2017; Cubillos-Hinojosa *et al.*, 2021; Cubillos-Hinojosa *et al.*, 2023), there are no reports of the use of co-inoculation rhizobia and *A. brasilense* in either lima bean and the same in leucaena plants.

The combined use of PGPR and humic substances (HS) is reported in some studies on corn, tomato, pineapple, and potato crops, showing increases in crop yield and stress mitigation in plants when compared with the isolated application (Melo *et al*., 2017; Ekin, 2019). However, there are no reports yet for fava beans and leucaena. In common bean plants (*Phaseolus vulgaris*

L.), a reduction in water stress was observed with the combined application of HS extracted from vermicompost and co-inoculation of *Rhizobium tropici* strains BR322, BR520 and BR534 and *Herbaspirillum seropedicae* (Melo *et al*., 2017). Despite these findings, there is still a lack of studies on the effects of the combined application of HS and efficient rhizobia in the biological fixation of nitrogen co-inoculated with *A. brasilense* in lima bean and leucaena plants.

The objective of this research was to evaluate the effect of inoculation or co-inoculation of rhizobia and *A. brasilense* in combined application with humic substances (humic and fulvic acids, extracted from leonardite) on the growth promotion of lima bean and leucaena plants.

MATERIALS AND METHODS

During the summer in January and February (2019), two experiments were carried out with plants of *P. lunatus* and *L. leucocephala* in the greenhouse of the Department of Soils of the Federal University of Rio Grande do Sul (UFRGS), Brazil. Seeds and bacterial crops were processed at the Soil Microbiology Laboratory of the Faculty of Agronomy of UFRGS.

Seed disinfection

Seeds of "olho de cabrapreto" variety of lima bean (*P. lunatus*) plants were used, supplied by producers from northeastern Brazil. These seeds were disinfected by successive immersion in alcohol (70%) for 30 s, sodium hypochlorite (2.5%) for 30 s, followed by six consecutive washes with sterile distilled water (Vincent, 1970). Then, the seeds were sowed in plastic pots with a capacity of 1.5 L that had been washed and flamed with 99% ethyl alcohol previously. The pots were filled with a sterile mixture, vermiculite and sand in proportion (2:1) in autoclave.

For the experiment with leucaena plants (*L. leucocephala* [Lam.] De Wit), seeds from the same plant at the Agronomic Experimental Station (EEA) of the Federal University of Rio Grande do Sul (UFRGS) were collected. These seeds were scarified with sandpaper Nº 100 for 1 min, then disinfected by the Vincent (1970) method as previously described for lima bean. In this experiment, plastic pots with a capacity of 700 mL that had been previously disinfected with 99% ethyl alcohol were used, rinsed successively with sterile distilled water and filled with a mixture of vermiculite and sand in proportion (2:1) sterilized in autoclave. Three seeds were sown in each pot.

Production of the inoculums of the isolates and strains of growth-promoting rhizobacteria

For the experiment with lima bean plants, the rhizobia Plu03 and Plu14 isolates and the SEMIA 4077 strain (*Rhizobium tropici* CIAT 899) were evaluated. This strain, released by the "Ministério da Agricultura, Pecuária e Abastecimento – MAPA" of Brazil to produce commercial inoculants for common bean (*Phaseolus vulgaris* L.), was obtained from the "SEMIA" collection of rhizobia of the Departamento de Diagnóstico e Pesquisa Agropecuária (DDPA), Rio Grande do Sul State, Brazil. These isolates and strains were selected for their ability to establish symbiosis and fix nitrogen in a previous experiment in lima bean plants of the "olho de cabrapreto" variety (Cubillos-Hinojosa *et al*., 2021).

In the experiment with leucaena plants, the rhizobia studied were the isolate leu01 and strains SEMIA 4081 and SEMIA 6361, obtained from leucaena plants from the rhizobia collection of the DDPA. Both the isolate Leu01 and strains SEMIA 4081 and SEMIA 6361 were selected for their efficiency in BNF in a previous experiment (Cubillos-Hinojosa *et al.*, 2020).

In the production of bacterial inoculums, the bacteria were inoculated separately in falcon tubes with 30 mL of liquid mannitol yeast culture medium (Vincent, 1970) and kept in an orbital incubator at 28°C±2 with agitation of 120 rpm until reaching a concentration of 10^8 cells/mL. In the case of *Azospirillum brasilense*, a commercial product containing the AbV5 and AbV6 strains of *A. brasilense* with concentration of 10^8 cells/mL was used.

Humic substances

Two commercial biostimulators rich in humic substances (HS) extracted from leonardite coal were used: one of the products was based on fulvic acids (FA) at a concentration of 1.35%, referred to as FA. The other product was based on humic acids (HA) at a concentration of 1.44%, referred to as HA. The HS were applied at the dose recommended by the manufacturer of 4 L ha^{-1}, and 10 mL of a solution was added (7.5 mL L^{-1}).

Design of the lima bean experiment in a greenhouse

A randomized complete block design with 23 treatments and five replications was established, with two isolates (Plu03 and Plu14) and the strain (SEMIA 4077) inoculated or co-inoculated with *A. brasilense*, an addition of mineral N (control) and the combined application of humic acids (HA) and fulvic acids (FA), as presented in table 1.

One of the non-inoculated control treatments received nitrogen (N) in the form of NH_4NO_3 at a dose equivalent to the addition of 100 kg ha^{-1} (control+N) and two treatments included the addition of N in conjunction with the HS (control+N+HA and control+N+FA) (Tab. 1). Nitrogen was added fractionally in 5 applications, with a solution (0.015 g L^{-1}) of NH_4NO_3 added weekly in 10 mL pots.

The pots of the treatments inoculated with the rhizobia received 2 mL of bacterial broth at sowing. The treatments co-inoculated with rhizobia and *A. brasilense* and those inoculated with only *A. brasilense* received 1 mL of the commercial product containing the strains AbV5 and AbV6 of *A. brasilense* in the sowing.

Table 1. Identification and description of experiment treatments in lima bean plants.

Treatment	Description
Control-N	Not inoculated, no N
Control+N	Not inoculated, with N
N+HA	Not inoculated, with N + humic acids
N+FA	Not inoculated, 100% N + fulvic acids
Az	Inoculation *A. brasilense*
Plu03	Inoculation of rhizobia isolate Plu03
Plu03+Az	Co- inoculation rhizobia isolate Plu03 and *A. brasilense*
Plu03+FA	Inoculation rhizobia isolate Plu03 + fulvic acids
Plu03+HA	Inoculation rhizobia isolate Plu03 + humic acids
Plu03+Az+FA	Co- inoculation rhizobia isolate Plu03 + *A. brasilense* + fulvic acids
Plu03+Az+HA	Co- inoculation rhizobia isolate Plu03 + *A. brasilense* + humic acids
Plu14	Inoculation of rhizobia isolate Plu14
Plu14+Az	Co- inoculation rhizobia isolate Plu14 and *A. brasilense*
Plu14+FA	Inoculation rhizobia isolate Plu14 + fulvic acids
Plu14+HA	Inoculation rhizobia isolate Plu14 + humic acids
Plu14+Az+FA	Co- inoculation rhizobia isolate Plu14 + *A. brasilense* + fulvic acids
Plu14+Az+HA	Co- inoculation rhizobia isolate Plu14 + *A. brasilense* + humic acids
SEMIA 4077	Inoculation rhizobia SEMIA 4077
SEMIA 4077+Az	Co- Inoculation rhizobia SEMIA 4077 and *A. brasilense*
SEMIA 4077+FA	Inoculation rhizobia SEMIA 4077 + fulvic acids

SEMIA 4077+HA	Inoculation rhizobia SEMIA 4077 + humic acids
SEMIA 4077+Az+FA	Co-Inoculation rhizobia SEMIA 4077 + *A.brasilense* + fulvic acids
SEMIA 4077+Az+HA	Co-Inoculation rhizobia SEMIA 4077 + *A.brasilense* + humic acids

HA: humic acids; FA: fulvic acids; Az: *Azospirillum brasilense*; N: nitrogen; Plu03 and Plu14: rhizobia isolates; SEMIA 4077: rhizobia strain.

The humic substances (HA and FA) were added one day before seeding and nitrogen in the form of NH_4NO_3 was added 3 days after seeding. All plants were irrigated with nutrient solution of Hoagland and Arnon (1950), modified by Silveira *et al*. (1998). One week after seeding, thinning was performed, leaving one plant per pot.

Design of the leucaena experiment in a greenhouse

The experimental design in randomized blocks with five replications was conducted. Twenty-three treatments were established, with one isolate Leu01 and two strains SEMIA 4081 and SEMIA 6361, separately or co-inoculated, with addition of N (control) and combined application with humic acids (HA) and fulvic acids (FA) (Tab. 2).

Table 2. Identification and description of experiment treatments with leucaena plants.

Treatment	Description
Control-N	Not inoculated, no N
Control+N	Not inoculated, with N
N+HA	Not inoculated, with N + humic acids
N+FA	Not inoculated, 100% N + fulvic acids
Az	Inoculation *A. brasilense*
Leu01	Inoculation of rhizobia isolate Leu01
Leu01+Az	Co- inoculation rhizobia isolate Leu01 and *A. brasilense*
Leu01+FA	Inoculation rhizobia isolate Leu01 + fulvic acids
Leu01+HA	Inoculation rhizobia isolate Leu01 + humic acids
Leu01+Az+FA	Co- inoculation rhizobia isolate Leu01 + *A. brasilense* + fulvic acids
Leu01+Az+HA	Co- inoculation rhizobia isolate Leu01 + *A. brasilense* + humic acids
SEMIA 4081	Inoculation rhizobia SEMIA 4081

SEMIA 4081+Az	Co- inoculation rhizobia SEMIA 4081 and *A. brasilense*
SEMIA 4081+FA	Inoculation rhizobia SEMIA 4081 + fulvic acids
SEMIA 4081+HA	Inoculation rhizobia SEMIA 4081 + humic acids
SEMIA 4081+Az+FA	Co- inoculation rhizobia SEMIA 4081 + *A. brasilense* + fulvic acids
SEMIA 4081+Az+HA	Co- inoculation rhizobia SEMIA 4081 + *A. brasilense* + humic acids
SEMIA 6361	Inoculation rhizobia SEMIA 6361
SEMIA 6361+Az	Co- Inoculation rhizobia SEMIA 6361 and*A. brasilense*
SEMIA 6361+FA	Inoculation rhizobia SEMIA 6361 + fulvic acids
SEMIA 6361+HA	Inoculation rhizobia SEMIA 6361 + humic acids
SEMIA 6361+Az+FA	Co- Inoculation rhizobia SEMIA 6361 + *A.brasilense* + fulvic acids
SEMIA 6361+Az+HA	Co- Inoculation rhizobia SEMIA 6361 + *A.brasilense* + humic acids

HA: humicacids; FA: fulvic acids; Az: *Azospirillum brasilense*; N: nitrogen; Leu01: rhizobia isolate; SEMIA 4081: rhizobia strain; SEMIA 6361: rhizobia strain.

One of the non-inoculated control treatments received N in the form of NH_4NO_3 at a dose equivalent to the addition of 100 kg ha^{-1} (control+N) and two treatments included the addition of N in conjunction with the HS (control+N+HA and control+N+FA) (Tab. 2). Nitrogen was added weekly in 6 applications, with 10 mL of a solution (1.43 g L^{-1}) of NH_4NO_3 placed in the pots.

The treatments that were inoculated with rhizobia received 2 mL of bacterial broth in the seeds sowing. The treatments co-inoculated rhizobia and *Azospirillum* as well as the treatment inoculated only with *A. brasilense* received 1 mL of the commercial product containing the strains AbV5 and AbV6 of *A. brasilense* atsowing. The humic substances (HA and FA) were added one day before seeding and nitrogen in the form of NH_4NO_3 was added 3 d after seeding. All plants were irrigated with nutrient solution of Sarruge (1975). One week after, thinning was performed, leaving one plant per pot.

Measured variables

After 45 days of cultivation, the lima bean and leucaena plants were collected, and the aerial part of the root system was separated. Shoot dry mass (SDM), root dry mass (RDM), mass of dry nodules (MDN), and subsequently, total accumulated N (Nac) in shoots and relative efficiency index (REI) of biological nitrogen fixation of rhizobia were determined according to Brockwell *et al*. (1966). The aerial part was packed in paper bags and dried in the forced circulation oven at 65°C for three days and then ground for the quantification of N by the method described by Tedesco *et al*. (1995). All roots were washed to remove particles from adhered substrate and dried with an absorbent paper towel to remove excess water. Then, the nodules of the treatments inoculated with rhizobia in lima bean were removed from the roots and dried in paper bags under the same conditions and weighed to determine the MDN. In leucaena plants nodules of the treatments inoculated with rhizobia were removed and quantified.

The relative efficiency index (REI) in biological nitrogen fixation was calculated using the equation (1) (Brockwell *et al*., 1966)

$$REI\ (\%) = ((NT - NT\text{-}N) / (NT{+}N - NT\text{-}N)) \times 100 \qquad (1)$$

where, *NT* was total nitrogen from the plant of the inoculated treatment, *NT-N* the total nitrogen non-inoculated control without nitrogen and, *NT+N* the total nitrogen of the non-inoculated control with nitrogen supplementation.

Data analysis

The data obtained were submitted to variance analysis, and the comparison of means was performed by the Tukey test (P<0.05), using the statistical program SPSS 15.

RESULTS AND DISCUSSION

Effect of the combined application of HS and rhizobia inoculated and co-inoculated with *A. brasilense* on lima bean plants

The results of the variables evaluated in the growth promotion of lima plants in response to the treatment with rhizobia inoculated and co-inoculated with *A. brasilense* in conjunction with HS are presented in table 3.

For the dry mass of the plants, the treatments Plu14+Az+FA and Plu14+Az+HA showed the highest increases (8.49 and 8.61 g, respectively), compared with the other treatments and the control treatment (control+N), which obtained 4.68 g (Tab. 3). The greatest increases were when plants were co-inoculated with rhizobia and *A. brasilense* in combined application with HS (treatments Plu03+Az+FA; Plu03+Az+HA; SEMIA 4077+Az+FA and SEMIA 4077+Az+HA), followed by plants inoculated with rhizobium with HS (Plu03+HA; Plu03+FA; Plu14+HA; Plu14+FA; SEMIA 4077+HA; SEMIA 4077+FA). There were no differences with plants co-inoculated with rhizobia and *A. brasilense* and plants treated with N with HS (N+HA and N+FA). All these treatments were superior to the control+N treatment and the isolated inoculation of rhizobia except for N+HA and N+FA treatments, which were similar to the treatment inoculated with rhizobia Plu03 but superior to the control+N treatment.

Table 3. Effect of inoculation and co-inoculation of rhizobia (Plu03, Plu14 and SEMIA 4077) and *A. brasilense* (Az) combined with humic acids (HA) and fulvic acids (FA), in the promotion of growth of lima bean plants.

Treatments	SDM	RDM	MDN	Nac
	(g)			(mg)
Control+N	4.68 f	1.20 f	0 h	174.30 h
Plu14 + Az+ HA	8.61 a	2.18 a	1,298 a	461.97 a
Plu14 + Az+ FA	8.49 a	2.06 a	1,276 a	429.92 b
SEMIA 4077 + Az+ HA	7.90 b	1.87 b	984 c	385.66 c
Plu03 + Az+ HA	7.85 b	2.07 a	1,140 b	422.66 b
SEMIA 4077 + Az+ FA	7.85 b	1.89 b	980 c	383.09 c
Plu03 + Az+ FA	7.71 b	2.06 a	1,100 b	383.43 c
Plu14 + HA	7.22 c	1.86 b	1,106 b	341.53 d
Plu14 + Az	7.13 c	1.69 c	884 d	339.19 d
Plu14 + FA	7.04 c	1.65 cd	1,088 b	330.14 d
Plu14	6.53 d	1.41 e	762 e	292.76 d
Plu03 + HA	6.49 d	1.88 b	996 c	299.27 d
Plu03 + Az	6.48 d	1.66 cd	850 d	300.83 d
Plu03 + FA	6.46 d	1.83 b	970 c	288.14 d
SEMIA 4077 + Az	6.29 d	1.63 cd	652 f	276.87 d
SEMIA 4077 + HA	6.18 d	1.61 cd	876 d	252.19e
SEMIA 4077 + FA	6.17 d	1.57 cd	854 d	247.55 ef
N+HA	5.64 e	1.66 cd	0 h	226.04 fg
Plu03	5.60 e	1.40 e	724 ef	225.98 efg
N + FA	5.39 e	1.56 d	0 h	211.92 g
SEMIA 4077	4.52 f	1.26 f	570 g	174.15 h

Az	1.69 g	0.81 g	0 h	19.70 i
Control-N	1.84 g	0.78 g	0 h	15.47 i

N: nitrogen; SDM: shoot dry mass; RDM: root dry mass; MDN: mass of dry nodules; NAC: nitrogen accumulated in the shoot. *Equal letters in the same column do not differ statistically (P<0.05) according to the Tukey test (n=5).

The potential benefits of the combined effects of rhizobia and *A. brasilense* co-inoculation and HS on the growth of lima bean plants were evidenced. The bioactivity of the application of HS to promote plant growth has been reported, such as the stimulation of photosynthesis by mechanisms similar to phytohormones, proliferation and elongation of roots due to H^+-ATPase activity that induces cell division (Nardi *et al*., 2005; Dobbs *et al*., 2010; Trevisan *et al.*, 2010; Canellas *et al*., 2011; Trevisan *et al*., 2011) and the assimilation of nutrients thanks to the presence of hydroxyl (OH) in carboxylic and phenolic groups (Muscolo *et al*., 2013). In a study conducted with faba bean plants (*Vicia faba*) treated with commercial HA applied on the leaves, increases in plant growth were observed (El-ghamry *et al*., 2009), In the common bean, stimulation of HA and FA on peroxidase activity has been reported to stimulate the growth and development of leaves. In common bean plants, the benefits of co-inoculation of rhizobia and *Azospirillum* in increasing crop growth and productivity have also been reported (Burdman *et al*., 1997; Hungria *et al*., 2013), while in lima beans, increases in SDM were observed in this study (Tab. 3).

Similar results of combined application of HS in conjunction with PGPR were observed in the common bean (*Phaseolus vulgaris*), where HA extracted from vermicompost in co-inoculation with *Rhizobium tropici* BR322, BR520 and BR534 strains and *H. seropedicae* strain HRC 54 in *P. vulgaris* plants cv. Grafite and cv. Bonus (originated from Brazil and Mozambique) submitted to water deficit, showed increases in SDM of plants both submitted or not to water stress compared to the non-inoculated control treatment that received nitrogen fertilization (Melo *et al*., 2017).

In the root dry mass (RDM) of lima bean plants (Tab. 3), the highest values were observed in plants co-inoculated with rhizobia and *A. brasilense* using the rhizobia isolate Plu14 and Plu03 in combined application with HA and FA (treatments Plu14+Az+HA and Plu14+Az+FA), followed by treatments SEMIA 4077+Az+HA and SEMIA 4077+Az+FA. The highest increases were in plants co-inoculated with rhizobia and *A. brasilense* (Plu03+Az; Plu14+Az; SEMIA4077+Az) and plants inoculated with rhizobia and HS (Plu03+HA; Plu03+FA; Plu14+HA; Plu14+FA;

SEMIA4077+HA; SEMIA 4077+FA). These treatments showed no differences between them, but differed compared with the isolated inoculation of rhizobia and the control+N treatment. In addition, increases were observed in non-inoculated treatments that received N with HS (N+HA and N+FA) compared with control+N and isolated inoculation of rhizobia.

These results showed that co-inoculation of the rhizobia isolates (Plu14 and Plu03) and *A. brasilense* in conjunction with HS obtained higher increases in RDM in lima bean plants. This bioactivity of HS that induces elongation and proliferation of roots that modify the architecture of the root system by increasing H^+-ATPase activity has been reported (Chen and Avaid, 1990; Façanha *et al*., 2002; Nardi *et al*., 2005; Zandonadi *et al*., 2007; Dobbs *et al*., 2010; Trevisan *et al*., 2010; Canellas *et al*., 2011; Canellas and Olivares, 2014; Canellas *et al*., 2015). Similar effects were evidenced in plants of the same family of fava beans (*P. lunatus*) as common bean (*P. vulgaris*) (Aydin *et al*., 2012; Melo *et al*., 2017). Similar results of co-inoculation of rhizobia and *A. brasilense* have been reported in common beans (Burdman *et al*., 1997; Hungria *et al*., 2013) and the combined application of HA and PGPR employing HA extracted from vermicompost in co-inoculation with the strains of *Rhizobium tropici* BR322, BR520 and BR534 and *H. seropedicae* strain HRC 54 in plants of *Phaseolus vulgaris* cv. Grafite and cv. Bonus subjected to water deficit. Significant increases were also evidenced in RDM both submitted or not to water stress compared to the non-inoculated control treatment that received nitrogen fertilization (Melo *et al*., 2017).

Regarding the mass of dry nodules (MDN) in lima bean plants, the greatest increases in MDN were seen in the treatments plu14+Az+HA and Plu14+Az+FA, followed by treatments Plu14+HA; Plu14+FA; Plu03+Az+HA and Plu03+Az+FA compared with the treatments inoculated only with the rhizobia isolates (Plu14 and Plu03). The greatest increases were seen in both the treatments of co-inoculation rhizobia and *A. brasilense* (treatments Plu03+Az; Plu14+Az and SEMIA 4077+Az) and the inoculation of rhizobia with HS (treatments Plu03+HA; Plu03+FA; Plu14+HA; Plu14+FA; SEMIA 4077+HA and SEMIA 4077+FA) compared to isolated inoculation of rhizobia.

These results show the potential of both the combined application of HS in co-inoculation with rhizobia and *A. brasilense* and the application of rhizobia with HS in increasing MDN in lima bean plants. There are reports on the bioactivity of HS in legumes such as soybean obtaining an increase in the number of nodules (Tilba and Sinegovskaya, 2012) and efficacy in triggering the expression of nod genes in mung bean plants (*Vigna radiata*) (Ahmad *et al*., 2012). The influence of co-inoculation of rhizobia and *A. brasilense* on nodules in common bean has been reported (Hungia *et al*., 2013).

Regarding the combined use of HS and PGPR, similar reports in soybean showed that inoculation with rhizobia in conjunction with sodium humate and ammonium molybdate in field increases significantly the number of nodules (Tilba and Sinegovskaya, 2012). However, in a study in common bean plants where *Rhizobium tropici* BR322, BR520 and BR534 and *H. seropedicae* HRC 54 strains in conjunction with HA (extracted from vermicompost) were co-inoculated, there were no increases in MDN (Melo *et al*., 2017), contrasting with what was evidenced in soybean plants and in the experiment with lima bean (Tab. 3).

In non-inoculated treatments that received nitrogen fertilization, with or without joint addition of HS (control+N; N+HA; N+FA) and N-Control, no nodules were observed, which ensured that the experiment did not cross-contaminate with rhizobia used in the other treatments.

The highest accumulation of N (Nac) in lima bean plants (Tab. 3) and higher relative efficiency (REI) of BNF (Fig. 1) was evidenced in plants that were co-inoculated with rhizobia and *A. brasilense* in combined application with HS (Plu14+Az+HA; Plu14+Az+FA; Plu03+Az+HA; Plu03+Az+FA; SEMIA 4077+Az+HA; SEMIA 4077+Az+FA), with higher values when HA was employed. In addition, increases in Nac and REI of plants were observed both in co-inoculation of rhizobia and *A. brasilense* and in the inoculation of rhizobia in combined application with HS.

These results suggest that the co-inoculation of rhizobia and *A. brasilense* in combined application with HS exert greater potential in the accumulation of N in the shoot of lima bean plants. The low amount of N contained in the HS applied would not be the cause of the increase of N of the plants. Similar studies of rhizobia inoculation have reported contributions in the accumulation of N in lima bean plants by BNF (Ormeño *et al*., 2007; Santos *et al*., 2009; Antunes *et al*., 2011; Santos *et al*., 2011; Duran *et al*., 2014; Servín-Garcidueñas *et al*., 2014; Ormeño-Orrillo *et al*., 2017).

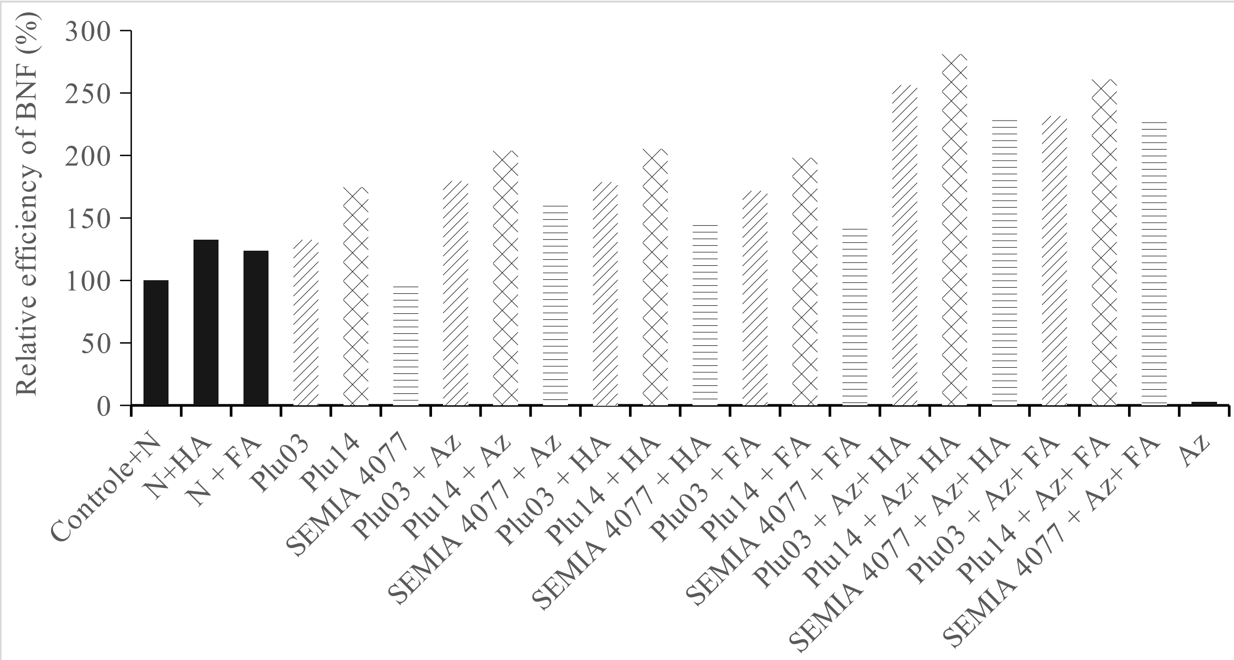

Figure 1. Relative efficiency index of biological nitrogen fixation by inoculation or co-inoculation of rhizobia (Plu03, Plu14 and SEMIA 4077) and *A. brasilense* (Az) combined with humic substances (humic acids [HA] and fulvic acids [FA]) in lima bean plants.

Regarding the co-inoculation of rhizobia and *Azospirillum,* some studies with legumes such as common bean, soybean (Hungria *et al*., 2013) and lentils (Kumar and Chandra, 2008) reported increases in Nac in plants. Similarly, the effects of HS have been reported as modulators of metabolic processes in plants, such as increased H^+-ATPase activity, alteration of nitrogen metabolism, and photosynthesis (Canellas *et al*., 2013).

Similar results of combined application of HS in co-inoculation of rhizobia with *A. brasilense* obtained in this study in Nac in lima bean plants (Tab. 3) were observed in soybean, where inoculation with rhizobia in the presence of sodium humate (humic acids with sodium) and ammonium molybdate in the field significantly increased the Nac in the plants (Tilba and Sinegovskaya, 2012).

Non-inoculated treatments that received N with HS (N+HA; N+FA) increased Nac in plants when compared to the control treatment with N (control+N) (Fig. 1). The contribution of HS in the accumulation of N in plants may have occurred by inducing HS in the modulation of metabolic processes such as N absorption through membrane permeability facilitated by H+-ATPase activity (Canellas *et al*., 2013).

Cubillos-Hinojosa, J.G., E.L.S. Sa, A.W Muniz, and D.P. Dick. 2024. Combined application of humic substances and PGPR inoculated, and co-inoculated in plants of *Phaseolus lunatus* (L.) and *Leucaena leucocephala* ([Lam] of Wit). Revista Colombiana de Ciencias Hortícolas 18(2), e17621. Doi: https://doi.org/10.17584/rcch.2024v18i2.17621

Effect of the combined application of HS and co-inoculation of rhizobia and *A. brasilense* in leucaena plants

The results of the variables evaluated in the growth promotion of leucaena plants in response to the treatment with rhizobia inoculated or co-inoculated with *A. brasilense* in conjunction with HS are presented in table 4.

Table 4. Effect of inoculation and co-inoculation of rhizobia (Leu01, SEMIA 4081, SEMIA 6361) and *A. brasilense* (Az) combined with humic acids (HA) and fulvic acids (FA) in the promotion of growth of leucaena plants.

Treatments	SDM	RDM	NN	Nac
	(g)			(mg)
Control+N	1.61 l	0.22 f	0 f	29.35 h
N + HA	2.00 ij	0.44 cd	0 f	42.55 f
N + FA	1.94 jk	0.37 de	0 f	40.52f
Leu01 + Az+ HA	3.00 a	0.63 ab	57 a	73.13 ab
SEMIA 6361 + Az+ HA	2.93 ab	0.67 ab	67 a	74.93 a
Leu01 + Az+ FA	2.92 ab	0.60 b	56 a	69.82 bc
SEMIA 4081 + Az+ HA	2.81 bc	0.69 a	56 a	71.02 ab
SEMIA 6361 + Az+ FA	2.73 cd	0.61 ab	61 a	66.48 c
SEMIA 4081 + Az+ FA	2.61 d	0.64 ab	59 a	61.84 d
Leu01 + FA	2.37 e	0.42 cde	42 bc	51.48 e
Leu01 + Az	2.36 ef	0.46 c	42 bc	51.59 e
Leu01 + HA	2.35 ef	0.46 c	46 b	51.93 e
SEMIA 4081 + Az	2.27 efg	0.45 c	40 c	52.59 e
SEMIA 4081 + HA	2.23 fgh	0.42 cde	43 bc	50.48 e
SEMIA 6361 + Az	2.18 gh	0.44 cd	40 bc	48.99 e
SEMIA 6361 + HA	2.14 hi	0.44 cd	41 bc	48.68 e
SEMIA 4081 + FA	1.91 jk	0.37 de	38 c	43.06 f
SEMIA 6361 + FA	1.86 k	0.35 e	41 bc	40.37 f
Leu01	1.67 l	0.26 f	28 d	35.44 g
SEMIA 4081	1.63 l	0.25 f	24 d	35.42 g
SEMIA 6361	1.58 l	0.25 f	27 d	31.10 h
Az	0.31 m	0.07 g	0 f	0.44 i

Controle-N	0.31 m	0.06 g	0 f	0.43 i

N: nitrogen; SDM: shoots dry mass; RDM: root dry mass; NN: number of nodules; NAC: nitrogen accumulated in the aerial part. *Equal letters in the same column do not differ statistically (P<0.05) according to the Tukey test (n=5).

This experiment showed the that the greatest increase in SDM in leucaena plants occurred when they were co-inoculated with rhizobia and *A. brasilense* in combined application with HS (treatments Leu01+Az+HA; Leu01+Az+FA; SEMIA 4081+Az+HA; SEMIA 4081+Az+FA; SEMIA 6361+Az+HA and SEMIA 6361+Az+FA), followed by inoculation of rhizobia with HS (treatments Plu03+HA; Plu03+FA; Plu14+HA; Plu14+FA; SEMIA 4077+HA and SEMIA 4077+FA) and co-inoculation rhizobia and *A. brasilense* (Leu01+Az; SEMIA 4081+Az; SEMIA 6361+Az), and finally, the addition of N with HS (N+HA; N+FA), when compared to control+N (Tab. 4).

These results showed the beneficial effects of the combination of rhizobia and *A. brasilense* co-inoculation and the bioactivity of HS in the growth of leucaena plants, which can be explained by the benefits of the application of both HS and PGPR that have been reported in several studies. Regarding the effects of HS, there are reports that show that they promote plant growth by stimulating photosynthesis by mechanisms similar to phytohormones, root proliferation and elongation due to H^+-ATPase activity that induces cell division (Nardi *et al*., 2005; Trevisan *et al*., 2010; Dobbs *et al*., 2010, Canellas *et al*., 2011; Trevisan *et al*., 2011), and the assimilation of nutrients due to the presence of OH in the carboxylic and phenolic groups (Muscolo *et al*., 2013).

In leucaena plants, it was reported that the application of HA extracted from lignite contributed to the increase of SDM of plants (Murgas and Falla, 2016). As well, some studies showed the efficiency of rhizobia in the BNF and benefits in promoting growth in leucaena plants (Bueno and Camargo 2015; Aguirre-Medina *et al*., 2015). The results of this experiment (Tab. 4) also showed increases in SDM of leucaena plants by co-inoculation of rhizobia and *A. brasilense*, when compared with the isolated inoculation of rhizobia and control+N, similar to the increases reported in studies in soybean and common bean plants co-inoculated with rhizobia and *A. brasilense* (Hungria *et al*., 2013).

In relation to the combined application of HS and PGPR, similar results have been reported in other legumes. In the case of common bean (*P. vulgaris*), HA extracted from vermicompost were tested in co-inoculation with PGPR in *P. vulgaris* plants submitted to water deficit. Increases were evidenced in SDM of plants both submitted or not to water stress compared to the non-inoculated control treatment that received nitrogen fertilization (Melo *et al*., 2017). In soybean, sodium humate was applied in conjunction with rhizobia in the seed, showing increases in plant growth compared to the control (Tilba and Sinegovskaya, 2012).

Regarding the dry mass of the root (RDM) of leucaena plants, the highest increases were observed in the treatments co-inoculated with rhizobia and *A. brasilense* in combined application with HS (HA and FA) (Leu01+Az+HA; Leu01+Az+FA; SEMIA 4081+Az+HA; SEMIA 4081+Az+FA; SEMIA 6361+Az+HA; SEMIA 6361+Az+FA), followed by treatments co-inoculated with rhizobia and *A. brasilense*, treatments inoculated with rhizobia with HS (HA and FA), and treatments that received N with SH (N+HA and N+FA); compared with the non-inoculated control treatment that received N (control+N) and treatments inoculated alone with rhizobia.

These results showed the beneficial effects exerted by the co-inoculation of rhizobia and *A. brasilense* in combined application with HS (HA and FA) on the growth of the roots of leucaena plants. The bioactivity of HS has been reported to induce the elongation and proliferation of roots that modify the root system architecture by increasing H+-ATPase activity which induces elongation and cell division (Chen and Avaid, 1990; Façanha *et al*., 2002; Nardi *et al*., 2005; Zandonadi *et al*., 2007; Dobbs *et al*., 2010; Trevisan *et al*., 2010; Canellas *et al*., 2011; Canellas and Olivares, 2014; Canellas *et al*., 2015). Similar studies of combined application of HS and PGPR have been reported in legumes such as common bean (*P. vulgaris*), where HA were tested in co-inoculation with *Rhizobium tropici* BR322, BR520 and BR534 strains and *H. seropedicae* strain HRC 54, showing increases in RDM both in plants submitted or not to water stress (Melo *et al*., 2017).

In leucaena plants, the highest number of nodules (NN) was observed in plants that were co-inoculated with rhizobia and *A. brasilense* in combined application with HS (Leu01+Az+HA; Leu01+Az+FA; SEMIA 4081+Az+HA; SEMIA 4081+Az+FA; SEMIA 6361+Az+HA; SEMIA 6361+Az+FA), followed by plants that were co-inoculated with rhizobia and *A. brasilense* (Leu01+Az; SEMIA 4081+Az; SEMIA 6361+Az) and plants inoculated with rhizobia in combined application with HS (Leu01+HA; Leu01+FA; SEMIA 4081+HA; SEMIA 4081+FA; SEMIA 6361+HA; SEMIA 6361+FA), compared with plants inoculated with rhizobia alone.

Similar studies in legumes such as soybeans where only HA were applied reported increases in the number of nodules (Tilba and Sinegovskaya, 2012) and in *V. radiata*, the efficacy of HA in

Cubillos-Hinojosa, J.G., E.L.S. Sa, A.W Muniz, and D.P. Dick. 2024. Combined application of humic substances and PGPR inoculated, and co-inoculated in plants of *Phaseolus lunatus* (L.) and *Leucaena leucocephala* ([Lam] of Wit). Revista Colombiana de Ciencias Hortícolas 18(2), e17621. Doi: https://doi.org/10.17584/rcch.2024v18i2.17621

triggering nod gene expression (Ahmad *et al*., 2012). In relation to the co-inoculation of rhizobia and *A. brasilense* in leucaena plants (Tab. 4), the results showed increases in the number of nodules, similar to that reported in some studies in common bean, soybean (Hungria *et al*., 2013), and lentils (Kumar and Chandra, 2008). In addition, similar results obtained by inoculation or co-inoculation of PGPR in the presence of HS were reported in soybean (Tilba and Sinegovskaya, 2012). On the other hand, the results obtained in this study (Tab. 4) differ from those found in common beans, where there was no evidence of increases in NN by co-inoculation of rhizobia and *H. seropedicae* (Melo *et al*., 2017).

The results of the accumulated nitrogen (Nac) determined in the shoot of the leucaena plants showed that the highest accumulation of N (Tab. 4) was reflected in the highest relative efficiency index (REI) of the BNF (Fig. 2) in the plants that were co-inoculated with rhizobia and *A. brasilense* in combination with HS (treatments Plu14+Az+HA; Plu14+Az+FA; Plu03+Az+HA; Plu03+Az+FA; SEMIA 4077+Az+HA and SEMIA 4077+Az+FA). This was followed by plants that were co-inoculated with rhizobia and *A. brasilense*, then by plants inoculated with rhizobia with HS, which increased Nac and REI (there were no differences between these treatments), compared with plants inoculated with rhizobia alone and the uninoculated control that received N (control+N).

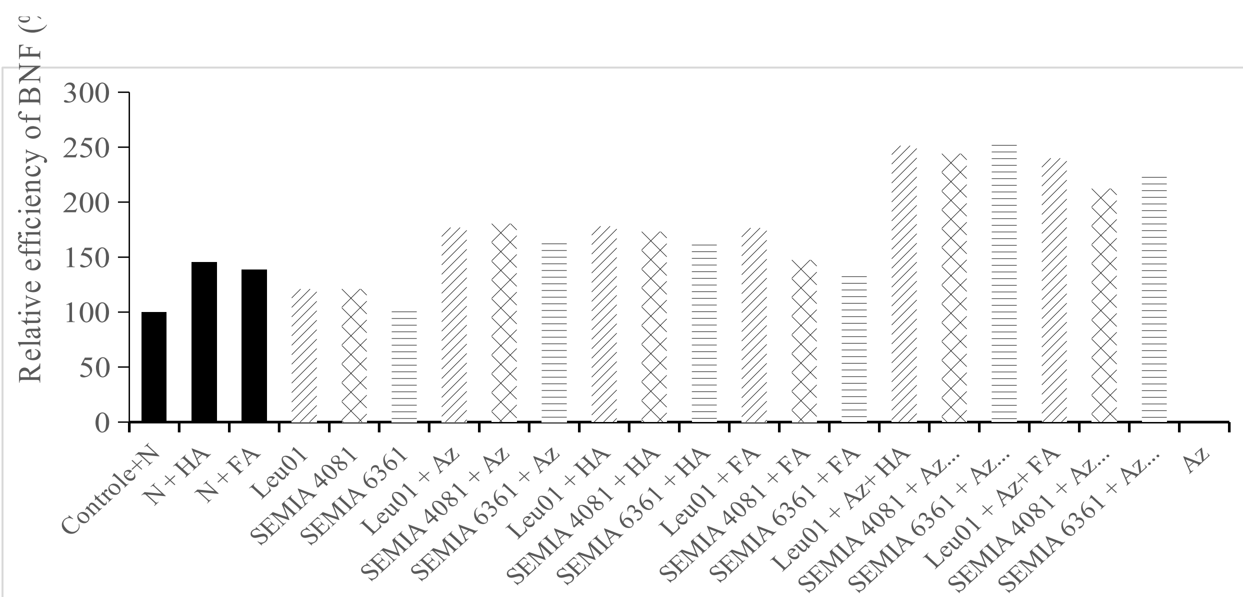

Figure 2. Relative efficiency index of biological nitrogen fixation by inoculation or co-inoculation of rhizobia and *A. brasilense* in conjunction with humic substances in leucaena plants.

These results showed the efficiency of combining the effects of co-inoculation of rhizobia and *A. brasilense* with HS, which contributed to the greatest stimulation of Nac in the shoots of leucaena plants and higher relative efficiency of BNF. Regarding these effects, there are reports of the contributions of isolated rhizobia inoculation in Nac and REI of BNF in leucaena plants (Aguirre-Medina *et al*., 2015; Bueno and Camargo 2015). There are also reports that show the effects of HS as modulators of metabolic processes in plants, such as increased H^+-ATPase activity, alteration of nitrogen metabolism, and photosynthesis (Canellas *et al*., 2013). Similar results in the increase of Nac and REI of BNF by the combined application of HS in co-inoculation with rhizobia have been reported in soybean crops. In these crops, significant Nac increases were observed when inoculated with rhizobia in the presence of sodium humate (humic acids with sodium) and ammonium molybdate in the field (Tilba and Sinegovskaya, 2012).

The results obtained in these two experiments in lima bean and leucaena plants showed that the co-inoculation of rhizobia and *A. brasilense* in combined application with HS could be considered a potential tool in the promotion of plant growth, not only as an economic alternative but as a sustainability strategy.

CONCLUSION

The co-inoculation of rhizobia and *A. brasilense* in combined application with humic substances (humic acids and fulvic acids) promotes greater growth of fava bean plants, stimulating an increase in shoot dry mass, root dry mass, nitrogen accumulation in the shoot and the relative efficiency index of biological nitrogen fixation of the plants. This highlights the significance of the inoculation of the rhizobia isolate Plu14 and the application of HA.

The growth promotion of leucaena plants is maximized when the plants are co-inoculated with rhizobia and *A. brasilense* in conjunction with humic substances (humic acids and fulvic acids). This increases shoot dry mass, root dry mass, and nitrogen accumulation in the shoot, as evidenced by the enhanced relative efficiency index of biological nitrogen fixation.

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