Special Supplement: Pulses

Rhizobacteria and bokashi-type biofertilizer interaction in common bean crop[1]

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ABSTRACT

Common bean has the potential to interact with nitrogen-fixing bacteria, especially of the *Rhizobium* genus. This study aimed to evaluate the effect of the interaction between two rhizobia endogenous isolates with bokashi-type biofertilizer, in order to promote biological nitrogen fixation in common bean crop. The experiment was conducted in a greenhouse, using a completely randomized design with the factors fertilization (mineral and organic) and inoculation (RBZ14 and RBZ15 isolates and CIAT899 strain). The microbial composition of the biofertilizer was also determined by sequencing. The RBZ14 isolate increased the fresh and dry root mass accumulation, when compared to the CIAT899 strain, as well as yield, if compared to the control and CIAT899 inoculation, when combined with the bokashi-type biofertilizer, indicating its capacity to stimulate the common bean growth and suggesting its potential use as an inoculant, especially when associated with the biofertilizer.

KEYWORDS: Endogenous bacteria, organic fertilizer, biological nitrogen fixation, microbiome.

RESUMO

Interação entre rizobactérias e biofertilizante tipo bokashi na cultura do feijão

O feijoeiro pode interagir com bactérias fixadoras de nitrogênio, especialmente do gênero *Rhizobium*. Objetivou-se avaliar o efeito da interação entre dois isolados endógenos de rizóbio com biofertilizante tipo bokashi, a fim de promover a fixação biológica de nitrogênio na cultura do feijoeiro. O experimento foi conduzido em casa-de-vegetação, utilizando-se delineamento inteiramente casualizado, com os fatores fertilização (mineral e orgânica) e inoculação (isolados RBZ14 e RBZ15 e estirpe CIAT899). A composição microbiana do biofertilizante também foi determinada por sequenciamento. O isolado RBZ14 aumentou o acúmulo de massa de raiz fresca e seca, em comparação com a inoculação da estirpe CIAT899, bem como a produtividade, em comparação com o controle e a inoculação com CIAT899, quando combinado com o biofertilizante tipo bokashi, indicando capacidade de estimular o crescimento do feijão comum e sugerindo seu uso potencial para inoculante, especialmente associado ao biofertilizante.

PALAVRAS-CHAVE: Bactérias endógenas, fertilizante orgânico, fixação biológica de nitrogênio, microbioma.

INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) crop holds significant economic and social importance in Brazil, which ranks third in global production, only behind India and Myanmar (Conab 2023). In 2023, 3.06 million tons of bean were harvested in Brazil, on 2.69 million hectares, achieving an average yield of 1,138 kg ha^{-1} (Conab 2023). Among the Brazilian states, Santa Catarina ranked eighth in total bean production and second in black bean production, reaching 80.7 thousand tons (Conab 2023).

Nitrogen (N) availability is a determining factor for the potential production of common bean, primarily due to the crop's high nutritional requirements and short cycle. The main sources of N for bean include mineral N fertilizers, organic N fertilizer sources such as livestock manure, organic amendments and biological N fixation (Shibata et al. 2017). Common bean establishes symbiotic interactions with N_2-fixing bacteria generically called rhizobia (Baraúna et al. 2016, Silva et al. 2020a), especially with species of the *Rhizobium* genus (Pelegrin et al. 2009). Common bean is relatively less efficient in symbiotic N_2 fixation (Moxley et al. 1986),

[1] Received: Dec. 07, 2024. Accepted: Mar. 06, 2025. Published: Apr. 23, 2025. DOI: 10.1590/1983-40632025v5581195.

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Editor: Luis Carlos Cunha Junior

when compared to soybean. These legumes typically require large amounts of nutrients, especially N, what increases production costs (Soratto et al. 2017). In addition, excessive N inputs can cause negative environmental impacts (Franciscon et al. 2014).

In Brazil, commercial inoculants for bean are predominantly produced with *Rhizobium tropici*, SEMIA 4077 (= CIAT899) and SEMIA 4088 (= H 12) strains, and *R. freirei* SEMIA 4080 (= PRF 81) strain (Coelho et al. 2021). *R. tropici* can fix approximately 20-30 % of the N required by the plant, being equivalent to up to 40 kg ha^{-1} of N (Soares et al. 2016), potentially replacing N fertilizer applications under certain conditions (Sousa et al. 2021, Sousa et al. 2022, Teixeira et al. 2022). However, the biological N fixation efficiency has limitations, such as the adaptation of inoculant bacteria to edaphoclimatic conditions of each region and competition with native (endemic) rhizobia. It constitutes a significant barrier to introduced strains, increasing the complexity of symbiotic relationships (Cavalcante et al. 2017).

A promising strategy to overcome these barriers involves searching for rhizobia isolates adapted to each condition, enhanced by biofertilizers (Sousa et al. 2022). In this context, bokashi-type formulations stand out for their potential to improve soil physical-chemical and biological conditions, stimulating biological N fixation (Batubara & Hapsoh 2015). This biofertilizer originated from an ancient technique of recycling organic materials, introduced in Brazil in the late 1980s (Silva et al. 2020b). It consists of an aerobic and thermophilic semi-decomposition process of a balanced mixture of organic materials from plant and animal origins and minerals, mediated by a diverse pool of microorganisms described as efficient microorganisms (Siqueira & Siqueira 2013). The bokashi-type biofertilizer functions as a complex biological matrix that operates through multiple complementary mechanisms, enhancing crop yield and N_2-fixation in leguminous crops (Olle 2020, Evcim & Gümüş 2022, Kruker et al. 2023).

This study aimed to evaluate endogenous rhizobia isolates for promoting common bean growth and yield, as well as the synergistic effects combined with a bokashi-type biofertilizer.

MATERIAL AND METHODS

The experiment was conducted in a greenhouse at the Universidade Federal de Santa Catarina, in Curitibanos, Santa Catarina state, Brazil, during the 2023/2024 season.

The experiment was developed in a completely randomized design, with a 3 × 2 factorial arrangement. The first factor included three inoculated treatments [two endogenous rhizobia isolates (RBZ14 and RBZ15) and a commercial strain (CIAT899)] and a non-inoculated control, whereas the second factor had two fertilization methods (mineral and bokashi-type biofertilizer) and a non-fertilized control, each one with five replicates.

For the experiment, pots received 9 kg of soil with no agrochemical or mineral fertilizers applications, collected from the 0.00-0.20 m depth layer. The soil was classified as Humic Cambisol (Santos et al. 2018), equivalent to Inceptisols (USDA 2014), which was air-dried and then sieved (4-mm mesh). A sample was taken for chemical analysis (Table 1).

The mineral fertilization followed the standardized recommendations for common bean (CQFS 2016), whereas the nitrogen (N) application rates were based on the soil organic matter content, with 50 kg ha^{-1} of N (urea - 0.52 g pot^{-1}). The phosphorus (P) and potassium (K) application rates were based on recommendations for low-fertility soils in no-tillage systems: 80 kg ha^{-1} of P_2O_5 (triple superphosphate - 0.82 g pot^{-1}) and 80 kg ha^{-1} of K_2O (potassium chloride - 0.62 g pot^{-1}).

Table 1. Soil chemical analysis from the experimental site.

Sample	Organic matter	P	K	Ca	Mg	Al	H + Al	
	%	mg dm^{-3}				$cmol_c$ dm^{-3}		
Humic Cambisol	3.67	7.70	62.40	9.41	4.59	0.00	4.28	
	pH ($CaCl_2$)	Cu	Mn	Zn	Fe	V	CEC (pH7)	SB
		mg dm^{-3}				(%)	$cmol_c$ dm^{-3}	
	5.60	4.70	1.10	1.90	20.88	76.79	18.44	14.16

CEC: cation exchange capacity; SB: sum of bases.

For biological fertilization, 47 g of bokashi-type bio compost were incorporated into each pot, being equivalent to a rate of 10 Mg ha^{-1} (Kruker et al. 2023). The bokashi formulation consisted of a mixture of broiler and poultry manure, screened soil, wheat and rice bran, Varvito® soil remineralizer, charcoal, wood biomass ash, molasses, plant extract-based biofertilizer, purified efficient microorganisms and water.

The RBZ14 and RBZ15 isolates were selected through previous laboratory and preliminary field analyses (Botelho et al. 2023). They were inoculated in flasks containing 10 mL of liquid Luria Bertani medium (Miller 1987) at 27.5 ºC, for 48 hours. Then, a 10-mL aliquot of each suspension was transferred to flasks containing 1 g of sterilized peat, whose amount was calculated based on recommendations for commercial inoculants. The flasks were kept for 72 hours at 27.5 ºC, until the day of sowing.

For seed inoculation (20 seeds per treatment), the inoculated peat was transferred to plastic bags with 5 mL of sugar solution (10 %), based on recommendation for commercial inoculants. The population of each isolate inoculum was estimated at 10^7 colony-forming units g^{-1}. The control was submitted to the same conditions, without inoculation. For the *R. tropici* (strain CIAT899) treatment, the manufacturer's recommendations were performed.

The used common bean cultivar was IPR Uirapuru, developed by Iapar (2000). This cultivar exhibits a shrubby, indeterminate type II growth habit, with a mean flowering period of 43 days and a complete cycle from emergence to harvest of approximately 86 days.

Seeds were manually sown and each pot received three seeds. Thinning was performed at 15 days after sowing (DAS), leaving two seedlings per pot. Irrigation was standardized to maintain approximately 80 % of field capacity, with regular water replenishment based on the crop needs.

At 60 DAS, when the plants reached the R6 stage, one plant from each pot was harvested for analysis of root and shoot fresh and dry masses and shoot nitrogen content. The total nitrogen content was performed using the Kjeldahl method (Tedesco et al. 1995), after dried shoots were crushed and sieved (250-mm mesh).

After 90 DAS, the remaining plants were harvested, and the grains were extracted to estimate yield. The grains were dried and, after adjusting the moisture content to 15 %, the yield was calculated in g pot^{-1}.

The bokashi was produced following standardized protocols, ensuring that its physical and chemical properties complied with the regulatory standards for classification as a Class "A" organic compound fertilizer, according to the Brazilian legislation (Brasil 2020) (Table 2).

Regarding the microbiological taxonomic profile of the bokashi-type biofertilizer, genomic sequencing of extracted DNA was performed for a complete analysis of fungal and bacterial genes through the Next Generation Sequencing (NGS) at Biome4All - Biotechnology Company (São Paulo - SP). After the DNA extraction and sequencing by the Illumina HiSeq platform, libraries were assembled, and the metagenome was reconstructed. Regarding the sequencing information, the target gene for bacteria was RNAr16S, region v3-v4, volume (reads) 66677, average length (reads) 2 x 250 bp. For fungi, the target gene was ITS2, with volume (reads) 65031 and average length (reads) 2 x 250 bp. The QIIME2 package was used to define amplicon sequence variants with the DADA2 algorithm, with the standard protocol of the PICRUSt2 software. Biome4all Agri-Analysis (v.2.1) was used as a tool for interpreting the genetic data of the microbiota. Regarding the bacteria taxonomic profile, the number of sequences classified at the species level was 32,221 and the total number of identified operational

Table 2. Chemical profile of the bokashi-type biofertilizer.

Sample	FA	HA	DM	C_{org}	OM	Total N	P_2O_5	K_2O	Ca	Mg	Na	S
	%											
Bokashi-type biofertilizer	3.28	0.48	79.81	6.22	23.44	0.45	0.93	0.16	1.61	0.40	1.2	0.1
	B	Cu	Mn	Zn	Fe	Ds	pH	pH		C/P	C/N	CEC
	mg kg^{-1}				%	g cm^{-3}	(H_2O)	($CaCl_2$)				$mmol_c$ kg^{-1}
	28.07	50.52	414.8	92.48	17.58	0.80	7.3	6.97		6.69	13.8	126.01

FA: fulvic acid; HA: humic acid; DM: dry matter; OM: organic matter; Ds: density; CEC: cation exchange capacity.

taxonomic units (OTUs) was 522. For fungi, there were 48,774 sequences classified at the species level and 51 OTUs.

The data were subjected to the homogeneity and normality tests (Levene and Shapiro-Wilk tests, respectively), to the F test at 5 % of probability, and, when there was statistical difference, to mean analysis using the Tukey test at 5 % of probability by the RStudio 2024.12.1 Build 563 for Windows. A principal component analysis (PCA) was also performed to verify the relationship between the bean yield and the inoculation treatments by the Canoco 5.0 software. The figures were created using the SigmaPlot 12.5.

Table 4. Effect of rhizobacteria inoculation and fertilization on shoot and root masses of the IPR Uirapuru bean cultivar.

Treatment	SFM (g)	SDM (g)	RFM (g)	RDM (g)
		Inoculation		
CIAT899	32.46 c	5.59 c	3.01 b	0.46 c
RBZ14	77.40 a	11.98 a	6.00 a	1.19 a
RBZ15	41.40 bc	6.82 bc	3.07 b	0.64 bc
Control*	58.23 b	9.94 ab	4.28 ab	0.98 ab
		Fertilization		
Bokashi	70.49 a	11.30 a	4.99 a	1.07 a
NPK	50.52 b	8.43 ab	4.25 ab	0.80 ab
Control**	36.10 b	6.01 b	3.01 b	0.58 b

Averages followed by the same letter do not differ statistically using the Tukey test at 5 % of significance. * Without inoculation; ** without fertilization. SFM: shoot fresh mass; SDM: shoot dry mass; RFM: root fresh mass; RDM: root dry mass.

RESULTS AND DISCUSSION

The analysis of variance revealed significant differences for both factors (bacterial inoculation and fertilization), individually and in interaction, except for plant height (Table 3). Additionally, a significant interaction between these two factors was observed for shoot nitrogen content and yield, indicating that the combination of bacterial inoculants and fertilizers had a synergistic effect on these parameters.

Among the inoculation treatments, the RBZ14 isolate reached the highest average for shoot fresh mass, significantly surpassing the other treatments (Table 4). RBZ14 showed average increases of 46.51 and 58.06 %, when compared to RBZ15 and CIAT899, respectively, which did not differ from each other. The control differed from the RBZ15 isolate, but it was superior to CIAT899 (Table 4), suggesting that RBZ14 was more effective in enhancing shoot growth, while the control treatment performed better than the CIAT899 inoculation.

Regarding shoot dry mass, RBZ14 did not differ statistically from the control, followed by the other two treatments (RBZ15 and CIAT899). Dry matter accumulation is related to nutrient absorption by the plant (Fageria et al. 2008). The stimulation to shoot dry mass accumulation is significant, since part of this will be translocated to the grain's formation, thus increasing the plant's production (Taiz et al. 2017). The bean shoot dry mass indicates its growth and is directly related to grain yield (Taiz et al. 2017). In previous experiments carried out by Botelho et al. (2023), it was also observed a higher performance of the RBZ14 isolate, when compared to the commercial inoculant treatment (CIAT899 strain), for the shoot and root dry masses, corroborating these results. In laboratory tests, those authors found that RBZ14 and RBZ15 isolates performed calcium phosphate solubilization and production of indole acetic acid (IAA), a phytohormone of the auxin class, which stimulates lateral and adventitious root growth, increasing the area for water and nutrient absorption. Both mechanisms may aid in the absorption of nutrients by plants, allowing biomass to increase. Currently, the selection of new rhizobia already considers other mechanisms that can increase the rhizobia-plant interaction (Wekesa et al. 2021). Raklami et al. (2019) observed that two *Ensifer meliloti* isolates produced between 38.08 and

Table 3. Analysis of variance of inoculation and fertilization effect on the performance of the IPR Uirapuru bean cultivar.

Variation factor	DF	PH	SFM	SDM	RFM	RDM	SNC	Yield
Inoculation (I)	3	1.54^{ns}	24.38*	16.28*	15.08*	13.57*	3.72*	4.72*
Fertilization (F)	2	1.83^{ns}	18.49*	13.07*	7.25*	5.94*	2.59^{ns}	0.25^{ns}
I x F	6	1.09^{ns}	2.19^{ns}	1.97^{ns}	2.04^{ns}	1.29^{ns}	3.07*	7.69*
Residue	48	36	36	36	36	36	48	36
Average		6.4	7.47	3.29	2.07	0.92	45.46	5,653.79

* F test ($p < 0.05$); ^{ns} not significant. DF: degrees of freedom; PH: plant height; SFM: shoot fresh mass; SDM: shoot dry mass; RFM: root fresh mass; RDM: root dry mass; SNC: shoot N content.

290.64 µg mL^{-1} of IAA, and they were the largest producers. This phytohormone, stimulating root production, can also increase the root surface to rhizobia, stimulating nodulation and, consequently, the biological nitrogen fixation.

Regarding root fresh and dry mass, the RBZ14 isolate had the highest performance (Table 4), with increases of 48.83 and 46.22 %, respectively, when compared to the RBZ15 isolate. In comparison to CIAT899, the increase ranged respectively to 49.80 and 61.34 %. This stimulation to mass accumulation could be also related to the IAA production by the isolate (Botelho et al. 2023)

Regarding the fertilization effects (Table 4), a significant increase was observed, with the bokashi-type biofertilizer for the shoot fresh mass being 48.78 % higher than for the control, and 28.33 % higher than for the mineral fertilization. For shoot dry mass, the increases were 46.81 and 25.39 %, when compared to the control and NPK fertilization, respectively. For root fresh and dry mass, the bokashi fertilization did not differ from the NPK one.

Regarding the shoot N content, the treatments showed significant differences in interaction between inoculation and fertilization (Table 5). It was possible to observe that the type of fertilizer influenced the performance of the inoculated bacteria. The CIAT899 strain and the control did not show significant differences. However, the RBZ14 isolate obtained a higher shoot N content at the NPK treatment and differed from the control. The RBZ15 isolate reached a higher N content with the bokashi-type biofertilizer than with NPK.

Regarding the interaction with fertilization, the non-fertilized treatment showed a significant difference from the inoculation treatments (Table 5). It exhibited a higher average with the RBZ14 isolate, suggesting that this isolate stimulated the N translocation by biological N fixation and/or by increasing the root area. The isolate produced a significant amount of IAA (153.26 µg mL^{-1}) and increased the N accumulation (Botelho et al. 2023), corroborating the results in Table 5.

Nitrogen is one of the most essential nutrients required by plants. Its dry matter levels vary from 2 to 5 % (Malavolta 1980), whereas the adequate range for shoot N levels is 30-50 g kg^{-1} (Ambrosano et al. 1996). In this sense, all treatments were within this range. For common bean, the R6 stage is considered the N translocation peak from bacteria to plant (Lalonde et al. 2003). The three bacteria generally obtained higher averages to shoot N content, regarding the control, indicating efficiency in the N translocation, perhaps contributing to yield (Table 5). In a previous study under field conditions, a higher shoot N content was observed in the CIAT899 strain inoculation and, subsequently, for the RBZ14 and RBZ15 isolates inoculations (Botelho et al. 2023), indicating that these isolates are effective. However, it is necessary to carry out further analyses.

Isolates interactions with the fertilizations for yield were also observed (Table 5). In general, the highest yields were observed with interaction between fertilization and inoculation. The CIAT899 strain reached higher averages with NPK fertilization and bokashi-type biofertilizer, and it was less effective without fertilization. The RBZ14 isolate reached a higher average without fertilizer, what could indicate its efficiency, even in the absence of fertilizers. The RBZ15 isolate did not differ statistically among the treatments.

The significant increase in some parameters of inoculated plants associated with the biofertilizer could be related to nutrient availability and bioactive compounds of the biofertilizer (Tana & Woldesenbet 2017). The principal component analysis showed a relationship between the root fresh and dry mass with the RBZ14 isolate inoculation and associated with the bokashi-type biofertilizer (Figure 1).

The plant height, shoot fresh and dry masses showed a higher relationship with the RBZ15

Table 5. Inoculation and fertilization effect on shoot N content and on yield of the IPR Uirapuru bean cultivar.

Shoot N content (g N kg^{-1} SDM)			
	Type of fertilization		
Inoculation	Bokashi	NPK	Control
CIAT899	42.91 aA	44.66 aA	38.47 bcA
RBZ14	36.45 aAB	44.92 aA	52.97 aB
RBZ15	44.60 aA	51.03 aB	37.71 cAB
Control*	48.65 aA	52.77 aA	50.45 abA
Yield (g pot^{-1})			
Inoculation	Bokashi	NPK	Control
CIAT899	19.72 bA	30.23 abA	20.02 bB
RBZ14	40.66 aB	21.64 bAB	29.87 abA
RBZ15	30.60 abA	28.97 abA	22.53 bA
Control**	21.03 bA	37.38 aA	38.13 aA

Averages followed by the same letter do not differ statistically, according to the Tukey test at 5 % of significance. Lowercase letters compare the inoculation effect in the column, whereas uppercase letters compare the fertilization effect in the row. SDM: shoot dry mass. * Without inoculation; ** without fertilization.

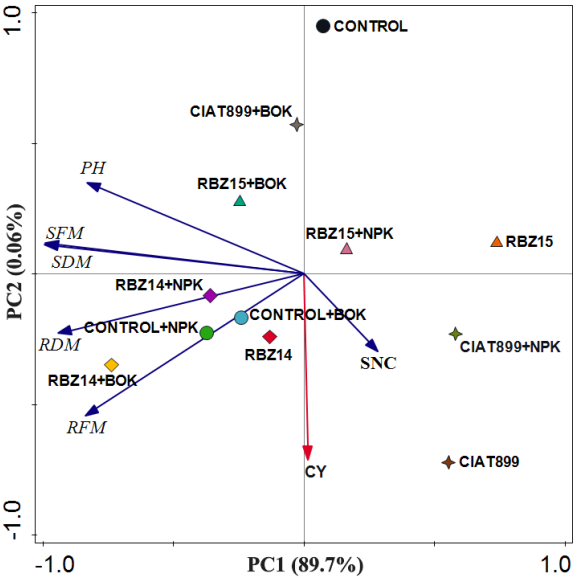

Figure 1. Principal component analysis (PCA) on yield, phytometric attributes and shoot N content of inoculated common bean and bokashi-type biofertilizer. PC1: principal component 1 (x-axis); PC2: principal component 2 (y-axis); PH: plant height; SFM: shoot fresh mass; SDM: shoot dry mass; RDM: root dry mass; RFM: root fresh mass; SNC: shoot N content; CY: crop yield. Control + Bok: uninoculated with bokashi; Control + NPK: uninoculated with NPK.

isolate, also associated with the bokashi-type biofertilizer.

The increase in nutrient availability and bioactive compounds can be correlated to the beneficial microbial communities introduced by the biocompost (Figure 2) (Kruker et al. 2023).

The bokashi microbiome profile showed the prevalence and diversity of bacteria. The bacterial profile was composed mainly of the Actinobacteria phylum, followed by Protobacteria, Chloroflexota, Gemmatimonadota, Firmicutes and Bacteriota. Among the most common bacterial genera were found: *Longimicrobium*, *Nitrolancea*, *Hyphomicrobium*, *Sphaerobacter*, *Micromonospora*, *Truepera*, *Streptomyces*, *Euzebya* and *Chryseolinea.* The fungal microbiome showed the Ascomycota phylum predominance. The three genera with the highest occurrence were *Chrysosporium*, *Mycochlamys* and *Petriella*.

Among the bacteria, some are described as significant to the organic matter degradation and to the plant growth promotion (Carter et al. 1990, Thanaboripat et al. 2015, Mahan et al. 2016). *Sphaerobacter* spp. is described as an enzyme producer that aids in the organic matter decomposition

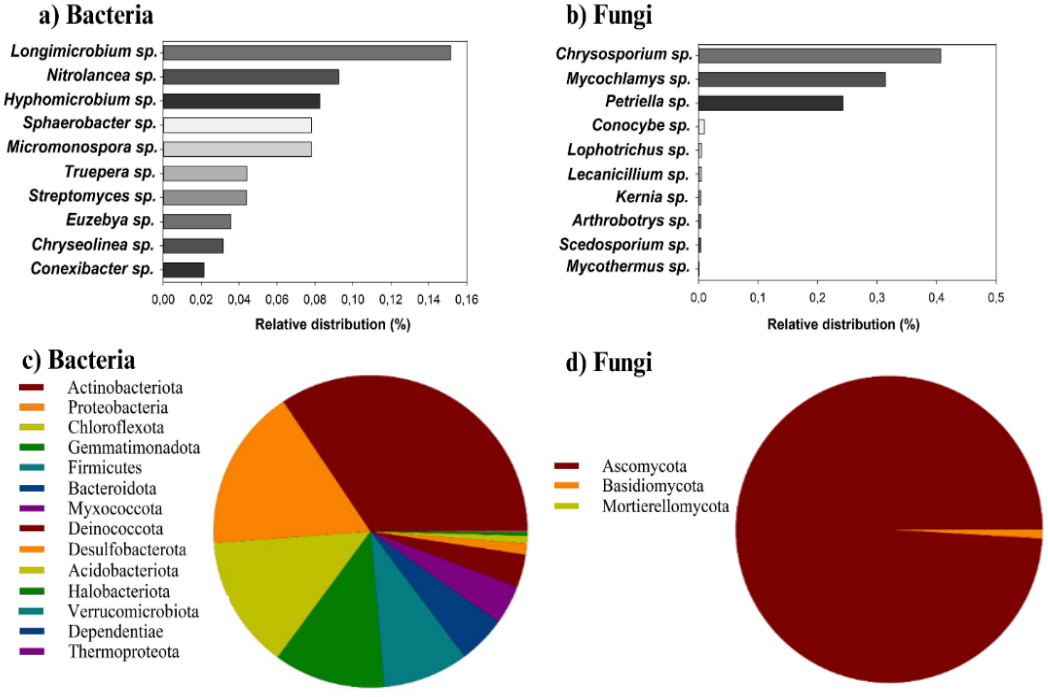

Figure 2. Metagenomic analysis of the bokashi-type biocompost, illustrating the distribution of predominant bacterial and fungal genera and phyla within its microbial taxonomic profile. a) and b) most common genera of bacteria and fungi; c) and d) most common phyla of bacteria and fungi.

(Kim et al. 2022). *Longimicrobium terrae* also plays a crucial role in decomposing organic matter and exhibits phosphatase activity, contributing to the P release for plant uptake (Pascual et al. 2016). The action of these microorganisms already present in the biocompost can maximize the effect of the tested isolates on beans.

CONCLUSIONS

Among the tested isolates, RBZ14 induced a higher accumulation of fresh and dry root mass by common bean. This isolate, with the bokashi-type biofertilizer, increased yield, when compared to the control and to the CIAT899 inoculation under controlled conditions.

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