

SHORT COMMUNICATION

Co-inoculation of *Beijerinckia indica* subsp. *indica* and *Cunninghamella elegans* spp. enriches cattle manure and promotes the growth of pepper (*Capsicum baccatum*)

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Abstract

Purpose This study aimed to evaluate the effects of different organic residues and beneficial microorganisms on the growth and yield of pepper (*Capsicum baccatum*).

Method Cattle manure (CM), earthworm humus (EH), and sugarcane filter cake (SFC) were enriched with *Beijerinckia indica* subsp. *indica* and *Cunninghamella elegans* spp. Chemical analysis revealed that CM had the highest contents of nitrogen (N), magnesium (Mg), carbon (C), and organic matter. The potential of CM contribution to the growth and yield of pepper was assessed using a pot experiment.

Results Enriched CM, which had the highest values of N, and Mg, linearly increased both morphological and nutritional parameters, enhancing plant growth and accumulation of N, phosphorus (P), and potassium (K) in both the shoots and roots of pepper.

Conclusion The enrichment of organic wastes with *B. indica* and *C. elegans* could be an alternative biological strategy to improve the quality of these wastes. Particularly, the enriched CM contributed to the increase in pepper growth.

Keywords Chemical properties, Diazotrophic bacteria, Microbial fertilizer, Nutrient input

Introduction

Pepper (*Capsicum* spp.) is an important horticultural crop that is widely cultivated and consumed worldwide (Safari et al. 2017; Pathirana 2013). In Brazil, pepper is cultivated in an estimated area of

15,000 ha, and approximately 334 t of pepper is produced every year (Santos et al. 2020). Small-holder farmers, who do not use fertilizers in their normal farm cultivation, are the main producers of pepper (Moreno-Cornejo et al. 2017). Chemical fertilizers enhance nutrient availability to plants, but the intensive use can promote soil pollution, acidification, and degradation, and consequently reduce soil quality and inhibit plant growth (Lu and Tian 2013; Tian and Niu 2015). Moreover, there is an economic cost associated with chemical fertilizers, which calls for the development of sustainable and economical alternatives.

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Organic wastes, such as cattle manure, are easily available and can be used in agriculture to replace chemical fertilizers and promote sustainability (Tejada et al. 2006). Moreover, organic wastes reduce the cost of production and improve soil properties and plant growth (Cherubin et al. 2018; Xie et al. 2014; Thangarajan et al. 2013; Chaparro et al. 2012; Seufert et al. 2012). However, nutrients from organic wastes are slowly supplied to plants depending on the soil's microbial activities (Gmach et al. 2020). Interestingly, the enrichment of organic waste with plant growth-promoting microorganisms (PGPM) has been reported to accelerate the nutrient release and improve plant growth (Gopi et al. 2020). PGPM are known to accelerate nutrient release when inoculated on organic wastes using different strategies, such as solubilization and mineralization, contributing to plant growth (Jana and Yashi 2020; Mitra et al. 2020). PGPM, such as *B. indica* and *C. elegans*, have the potential of co-inoculation with organic wastes (Stamford et al. 2019, 2020) to accelerate the release of nutrients and ensure high plant growth and yield. Earlier studies have reported that PGPM enrich the organic waste and improve the growth of horticultural crops (Berger et al. 2013; Silva et al. 2016; Oliveira et al. 2017a; Sousa et al. 2018; Souza et al. 2018; Stamford et al. 2019, 2020). Although these studies have reported the enrichment potential of organic wastes with PGPM, little is known about the enrichment of different organic wastes, such as cattle manure, earthworm residues, and sugarcane filter cake, with *B. indica* subsp. *Indica* and *C. elegans* spp., and their effect on pepper growth. Therefore, this study examined the nutrient content of cattle manure, earthworm residues, and sugarcane filter cake enriched with *B. indica* and *C. elegans* spp. In addition, the residue with the highest nutritional value was assessed for its effect on the growth and yield of pepper.

Material and methods

Experiment design and setup

The study consisted of two experiments: the first in the laboratory (to study the nutrient content in different wastes enriched with microorganisms), and the second in a greenhouse (to evaluate the enriched waste with the highest nutritional value on the growth and yield of pepper). Both experiments were conducted at the Center for Agricultural Sciences of the Federal University of Piauí, Brazil (5°2'58.48"S; 42°46'57.13"W; 86 m).

Microbial fertilizer

B. indica and *C. elegans* were obtained from the Biological Nitrogen Fixation Center of the Federal Rural University of Pernambuco (UFRPE; Recife, PE). *B. indica* was grown in Erlenmeyer flasks containing 50% tryptic soy broth liquid medium under orbital shaking (220 rpm; 120 h) at 28 °C. The bacterial inoculum was used at 10⁹ CFU mL⁻¹ (exponential growth phase). After the attainment of turbidity in the culture medium, the growth was recorded on a spectrophotometer at 560 nm, and the concentration of the bacterial inoculum was standardized at 10⁸ CFU mL⁻¹. *C. elegans* was cultivated in potato dextrose medium under orbital shaking (120 rpm, 120 h) at 30 °C until the features of mycelial mass appeared (Stamford et al. 2007).

Production of microbial enriched residues

In this experiment, three different organic wastes were enriched with *B. indica* and *C. elegans*. The experiment was conducted in a completely randomized design with three replicates in each of the seven treatments (single and mixed organic waste) under two microbiological conditions (with and without enrichment). The groups of single and mixed organic waste were as follows: 1, cattle ma-

nure (CM); 2, earthworm humus (EH); 3, sugarcane filter cake (SFC); 4, EH_{50%} + SFC_{50%}; 5, EH_{50%} + CM_{50%}; 6, CM_{50%} + SFC_{50%}; 7, CM_{33.3%} + EH_{33.3%} + SFC_{33.3%}.

Single and mixed organic wastes were added to the experimental pots (5 L). Afterward, 140 mL of *B. indica* bacterial suspension was added, mixed with each organic waste, and incubated for 35 days. Thereafter, 150 mg of *C. elegans* fungal biomass was added to each pot, mixed, and incubated for 30 days. After incubation (65 days for *B. indica* and 30 days for *C. elegans*), samples from each treatment were subjected to chemical analysis to determine contents of carbon (C), nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), total organic matter, and C:N ratio (EMBRAPA 2009) (Supplementary Table 1).

Pepper cultivation, growth and yield

The experiment was conducted in a greenhouse under a randomized block design with five enriched CM doses (0, 20, 40, 60, and 80 t ha⁻¹; dry basis) and four replicates. All doses were incorporated into the soil in the experimental pots (5 L) five days before seedling transplantation. The soil at 0–20 cm depth was used for analysis and had the following chemical parameters: pH (water), 5.7; organic matter, 1.21%; available phosphorus (P), 1.05 mg dm⁻³; exchangeable potassium (K), 15.64 mg dm⁻³; exchangeable calcium (Ca), 270.54 mg dm⁻³; exchangeable magnesium (Mg), 103.33 mg dm⁻³; base saturation (V), 57%. This soil, which has low pH, Ca, and K, and moderate Mg and V% values, was characterized as a soil with low capacity to provide nutrients to plants (Sobral et al. 2015). The experiment was carried out for 120 days (period of pepper growth), and the soil moisture was maintained at 60% via manual water application. At harvest, the stem diameter (cm) and plant height (cm) were measured using a digital caliper (Insize® Mod. SL-1112) graduated in millimeters

(accuracy of 0.01 mm). In the laboratory, the shoots were separated from the roots at ground level using pruning shears to facilitate handling of plant components and then were individually washed in 1% hydrochloric acid and distilled water. Subsequently, they were packed in kraft paper bags and placed in a drying oven (model SL - 102/630) with forced air circulation (65 °C, 72 h), and the dry mass of shoots and roots was weighed (Prado 2021). All the fruits were counted and weighed to determine the total number of fruits and mass of fresh fruit/plant. The dry mass of shoots and roots was initially verified using a precision balance (Bel Engineering, M5202) and then the parts were crushed in a Tecnal® Willye type TE-650/1 knife mill to determine N, P, and K using the method proposed by Miyazawa et al. (2009). The accumulation of nutrients (N, P, and K) in each of the plant parts (shoots and roots) was determined by multiplying the nutrient content with the corresponding dry mass (Mattar et al. 2018).

Statistical analyses

The statistical program R v 4.1.3 (R Core Team 2022) was used to analyze the experimental data. The parametric assumptions of normality and homoscedasticity in the analysis of variance (ANOVA) were evaluated using Shapiro-Wilk and Levene's tests, respectively, for the data from the first experiment. The results were analyzed using ANOVA followed by the Scott-Knott test at 5% probability ($p < 0.05$). The data were subjected to multivariate analysis using non-metric multidimensional scaling (NMDS) and linear discriminant analysis. NMDS, a rank-based multivariate analysis, was then applied to correlate the dissimilarities of treatments (based on chemical characteristics) and elucidate the relationships among non-inoculated and co-inoculated microbes in organic waste. All the data from the second experiment fulfilled assumptions of normality and homoscedasticity.

The results of macronutrient accumulation (N, P, and K in the aerial parts and roots) were subjected to ANOVA followed by the Scott-Knott test at the 5% probability ($p < 0.05$). The regression models for the morphological and production data of pepper plants were adjusted when the effect was significant based on the F test at 5% probability.

Results and discussion

Cattle manure enriched with *B. indica* subsp. *indica* and *C. elegans* spp. had the highest N, Ca, and Mg (Fig. 1), whereas non-enriched CM had the

highest C and organic matter. Sugarcane filter cake and earthworm humus enriched with *B. indica* subsp. *indica* and *C. elegans* spp. showed higher P and C: N ratios, respectively, compared to the non-inoculated residues. Compared to non-enriched organic wastes, enriched EH, EH + SFC, and EH + CM had higher organic matter.

When all the enriched organic wastes were compared, CM presented higher contents of N (18.1 g kg^{-1}), Mg (3.5 g kg^{-1}), C (20.4 g kg^{-1}), and organic matter (52.8 g kg^{-1}), and SFC and EH showed higher P (21.7 g kg^{-1}) and C: N (15.6 g kg^{-1}) ratios, respectively.

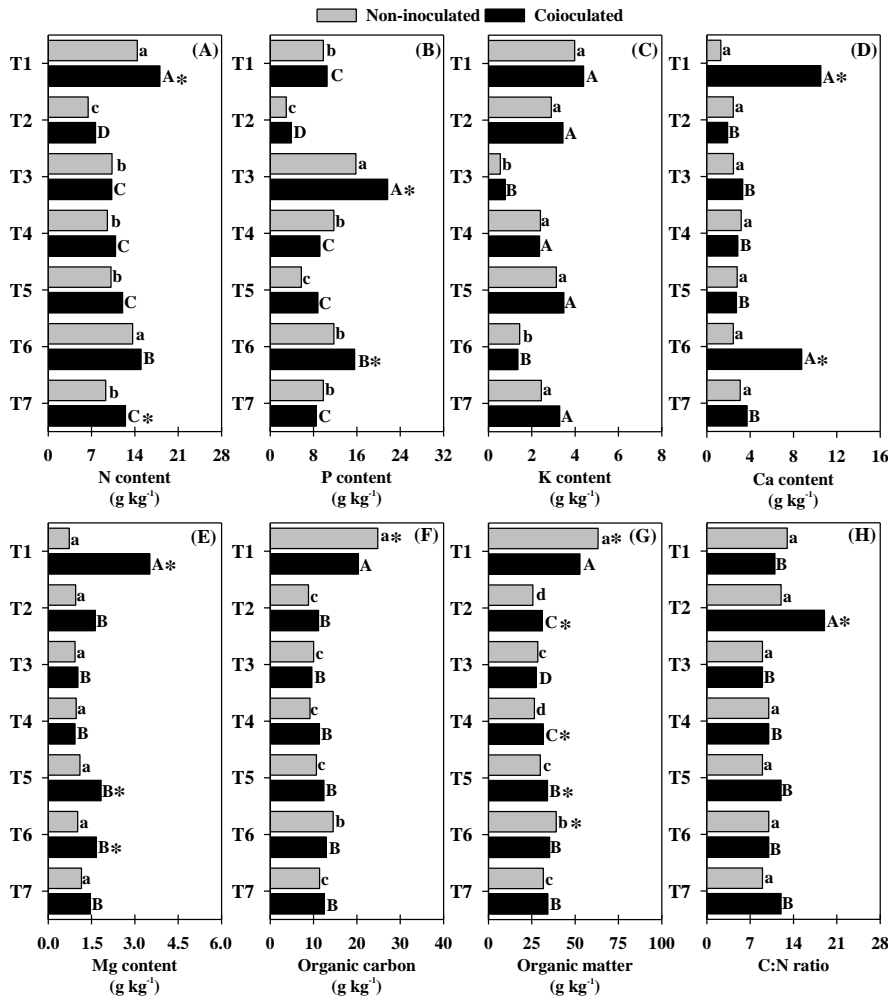


Fig. 1 Chemical evaluation of organic waste non-inoculated and co-inoculated with *B. indica* and *C. elegans* T1: cattle manure (CM); T2: earthworm humus (EH); T3: sugarcane filter cake (SFC); T4: EH50% + SFC50%; T5: EH50% + CM50%; T6: CM50% + SFC50%; T7: CM33.3% + EH33.3% + SFC33.3%. Different letters represent significant differences among different types of organic waste non-inoculated (lowercase letters) or co-inoculated (capital letters). The asterisk (*) indicates significant differences between non-inoculated and co-inoculated in each treatment (Scott-Knott' test; $p \leq 0.05$)

Non-metric multidimensional scaling showed a broader effect of both *B. indica* and *C. elegans* on the chemical parameters of organic waste (Fig. 2A). Linear discriminant analysis explained 98.3%

of the total variation distributed in axis 1 (82.9%) and axis 2 (15.4%). Interestingly, there was a clear and significant difference between CM and SFC and EH (Fig. 2B).

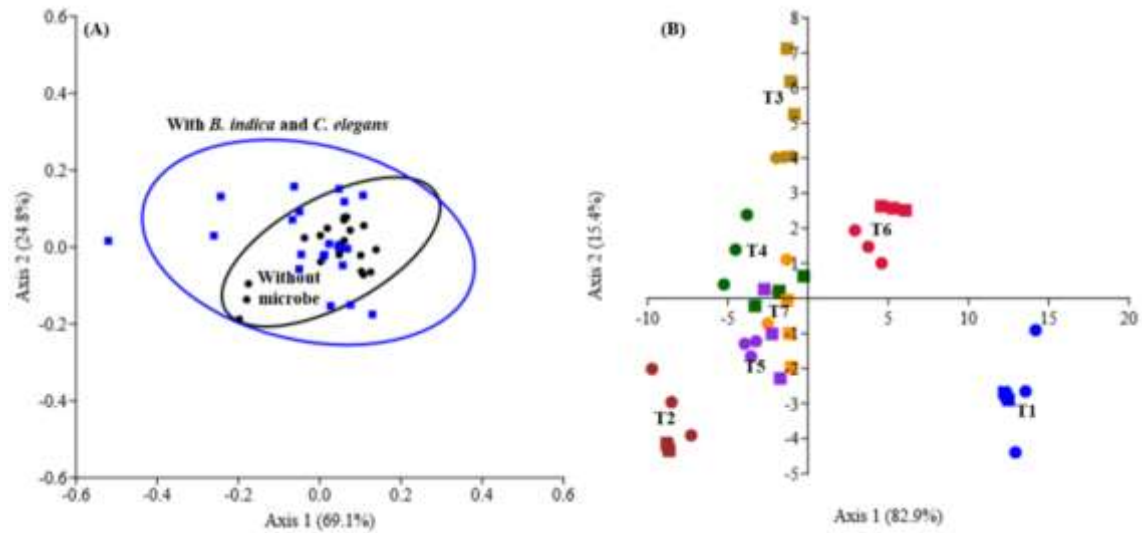


Fig. 2 Non-metric multidimensional scaling (A) and linear discriminant analysis (B) of the chemical attributes by organic residues non-inoculated and co-inoculated with *B. indica* and *C. elegans*

The dot and square symbols represent organic residues non-inoculated and co-inoculated with microbe, respectively. T1 = 100% of cattle manure (CM). T2 = 100% of earthworm humus (EH). T3 = 100% of sugarcane filter cake (SFC). T4 = EH + SFC (50% of each). T5 = EH + CM (50% of each). T6 = CM + SFC (50% of each). T7 = CM + EH + SFC (33.3% of each). The PERMANOVA analysis confirm the difference between treatments ($F = 7.63$; $p = 0.0001$)

Enriched CM showed positive chemical parameters and promoted a linear increase in pepper growth (Fig. 3) and accumulation of N, P, and K in both shoots and roots (Fig. 4A, B, and C).

The results showed that the enrichment of organic wastes, particularly CM, promoted higher N, P, Ca, Mg, C, and organic matter in the waste.

Supplementary Table 1 reveals that enrichment resulted in lower C: N ratio (~11) and accelerated the process of mineralization with a consequent increase in the release of nutrients (Pan et al. 2012; Ribeiro et al. 2017). Previous studies have also shown that inoculation with *B. indica* and *C. elegans* enriches organic waste with nutrients (Oliveira et al. 2017b; Stamford et al. 2017). These results indicated that CM was the most effective when co-inoculated with both *B. indica* and *C. elegans* spp. (Fig. 1; Supplementary Table 1), although enriched SFC presented a higher P content

than enriched CM, which could be related to the higher value of P found in SFC waste. These results confirmed the positive effect of PGPM on organic waste. It has been reported that *C. elegans* has a high metabolic activity to mineralize several organic residues due to the secretion of a variety of enzymes (Zhang et al. 1996).

Furthermore, the addition of organic material with a high N content increases microbial activity by promoting the mineralization of the material, thus allowing an increase in the availability of nutrients in these organic substrates (Sousa et al. 2018).

The increase in degradation of organic matter and N release after inoculation with *B. indica* and *C. elegans* spp. promoted plant growth and production, suggesting the efficiency of using microbial enriched organic residues for soil fertilization.

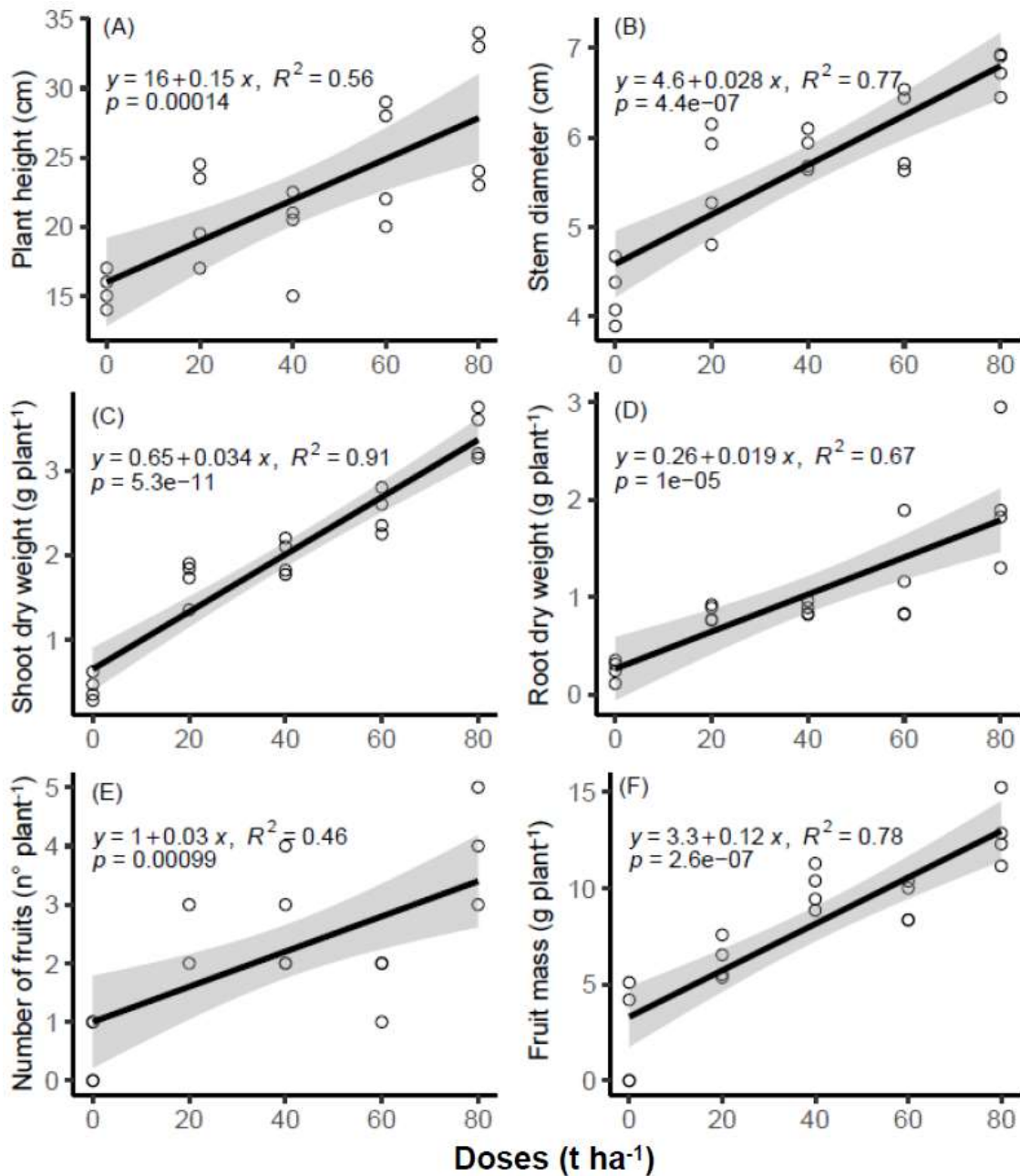


Fig. 3 Plant height (A), stem diameter (B), shoot dry weight (C), root dry weight (D), number of fruits (E) and fruit mass (F) of the pepper plants in response to the amendment of microbially enriched CM

The gray band represents the 95% confidence interval for the slope of the regression line

Aerobic bacteria from the genus *Beijerinckia* are non-symbiotic and have the ability to fix N from the atmosphere (Becking 2006). Particularly, *B. indica*, which can excrete amino acids as a product of N fixation (Pati et al. 1994), has been found to enrich biofertilizers, suggesting it to be an effective alternative in reducing the use of chemical fertilizers (Oliveira et al. 2017b). *C. elegans* produces

chitosan, a natural polysaccharide with biological activity and chemical characteristics (Stamford et al. 2007). Besides, chitosan is an interesting biological stimulant for crops (Malerba and Cerana 2016) and increases the growth of tomatoes, lettuce, radish, and pepper (Chibu and Shibayama 2003; Chookhongkha et al. 2012; Xu and Mou 2018).

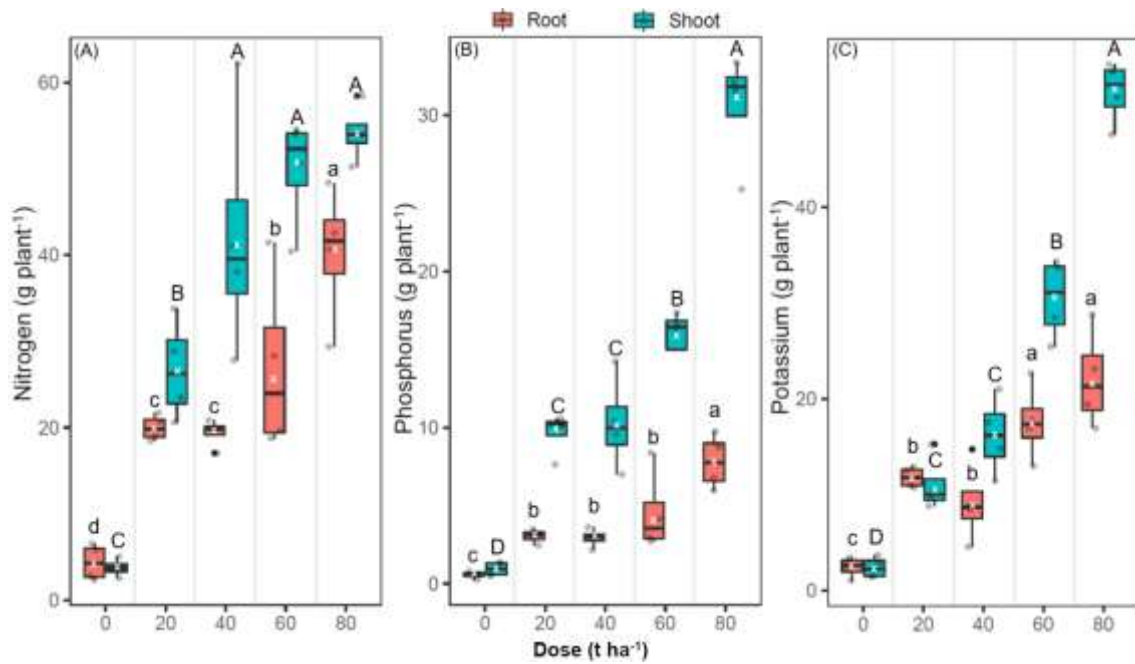


Fig. 4 Box plot with accumulation (g plant^{-1}) of Nitrogen (A), Phosphorus (B), and Potassium (C) in the shoots and roots of pepper under different doses of microbially enriched CM

Different letters represent significant differences among different doses of CM in root (lowercase letters) or shoot (capital letters) by the Scott-Knott test ($p \leq 0.05$). The x (white color) in each box represents the mean of repetitions ($n = 4$)

In the present study, CM promoted greater growth and production of pepper compared to other treatments, indicating CM is rich in nutrients and combining it with beneficial microorganisms increased its efficiency. This finding indicates the benefit of using microbial enriched CM to promote pepper production. Previous studies have also reported the positive effects of high rates of cattle manure on the growth and production of pepper (Mabuza et al. 2019; Ribeiro et al. 2020). Ribeiro et al. (2020) reported an increase in plant height, number of leaves, stem diameter, and productivity of pepper after the application of 20 t ha^{-1} of cattle manure.

Conclusion

Application of enriched organic waste with *B. indica* subsp. *Indica* and *C. elegans* spp. to low fertility soil can increase pepper growth and yield. In particular, this study demonstrated a significant positive impact of enriched cattle manure on the soil, contributing to the growth and yield of pepper.

Chemical fertilizers are among the major inputs that increase the cost of production, and their partial or full replacement by organic fertilizers will be beneficial to smallholder farmers. This study recommends the use of enriched cattle manure without the addition of chemical fertilizer to promote the growth and production of pepper.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest associated with this study.

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Supplementary Table 1 Descriptive analysis of chemical attributes of treatments with and without co-inoculation with *Beijerinckia indica* subsp. *indica* and *Cunninghamella elegans* spp

Treatment	Chemical attributes																
	N		P		K		Ca		Mg		C		C:N				
	NI	COIN	NI	COIN	NI	COIN	NI	COIN	NI	COIN	NI	COIN	NI	COIN			
T1 (CM)	Mean	14.4	18.1	9.8	12.8	4.0	6.4	1.3	11.5	0.7	3.5	24.8	20.4	63.3	52.8	13.0	11.0
	Max	15.4	20.1	10.8	15.5	5.0	8.4	2.3	15.5	0.7	4.5	25.8	21.4	65.3	54.8	16.0	14.0
	Min	13.4	16.1	8.8	10.5	3.0	4.4	0.3	8.5	0.7	2.5	23.8	19.4	61.3	50.8	10.0	8.0
T2 (EH)	Mean	6.5	7.7	3.0	3.9	2.9	3.4	2.4	1.9	1.0	1.6	8.9	11.2	25.7	31.1	12.0	19.0
	Max	7.5	9.7	4.0	4.9	3.9	4.4	3.4	2.9	1.0	2.6	10.9	13.2	26.7	32.1	14.0	20.0
	Min	5.5	5.7	2.0	2.9	1.9	2.4	1.4	0.9	0.9	0.6	6.9	9.2	24.7	30.1	10.0	18.0
T3 (SFC)	Mean	10.3	10.3	15.8	21.7	0.6	0.8	2.5	3.3	0.9	1.0	10.0	9.6	28.5	27.5	9.0	9.0
	Max	12.3	12.3	16.8	22.7	0.6	0.8	3.5	4.3	1.0	2.0	12.0	11.6	30.5	29.5	10.0	12.0
	Min	8.3	8.3	14.8	20.7	0.5	0.8	1.5	2.3	0.9	1.0	8.0	7.6	26.5	25.5	8.0	6.0
T4 (EH + SFC)	Mean	9.6	10.9	11.8	9.2	2.4	2.4	3.2	2.9	1.0	0.9	9.2	11.4	26.5	31.6	10.0	10.0
	Max	10.6	11.9	12.8	11.2	3.4	3.4	4.2	3.9	1.0	1.0	10.2	12.4	27.5	32.6	12.0	11.0
	Min	8.6	9.9	10.8	7.2	1.4	1.4	2.2	1.9	1.0	0.9	8.2	10.4	25.5	30.6	8.0	9.0
T5 (EH + CM)	Mean	10.2	12.0	5.7	8.8	3.1	3.5	2.8	2.7	1.1	1.8	10.7	12.4	30.0	34.0	9.0	12.0
	Max	11.2	14.0	6.7	9.8	4.1	4.5	3.8	3.7	2.1	2.8	12.7	13.4	31.0	36.0	10.0	13.0
	Min	9.2	10.0	4.7	7.8	2.1	2.5	1.8	1.7	0.1	0.8	8.7	11.4	29.0	32.0	8.0	11.0
T6 (CM + SFC)	Mean	13.7	15.0	11.8	15.6	1.4	1.4	2.4	8.8	1.0	1.7	14.5	13.0	39.1	35.3	10.0	10.0
	Max	14.7	17.0	12.8	17.6	2.4	2.4	3.4	10.8	2.0	2.7	16.5	14.0	40.1	37.3	12.0	12.0
	Min	12.7	13.0	10.8	13.6	0.4	0.4	1.4	6.8	1.0	0.7	12.5	12.0	38.1	33.3	8.0	8.0
T7 (CM + EH + SFC)	Mean	9.3	12.5	9.8	8.5	2.4	3.3	3.1	3.7	1.2	1.5	11.4	12.5	31.7	34.3	9.0	12.0
	Max	10.3	14.5	11.8	10.5	4.4	4.3	4.1	4.7	2.2	2.5	12.4	14.5	32.7	36.3	12.0	14.0
	Min	8.3	10.5	7.8	6.5	0.4	2.3	2.1	2.7	0.2	0.5	10.4	10.5	30.7	32.3	6.0	10.0

OM: organic matter; CM: cattle manure; EH: earthworm humus; SFC: sugarcane filter cake; NI: no inoculate; COIN: co-inoculate; N, P, K, Ca, Mg, C and OM (g kg⁻¹)

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