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EFFECTIVENESS OF *Azospirillum brasilense* AND *Pseudomonas fluorescens* IN SUGARCANE DEVELOPMENT

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Abstract

The use of bio-inputs has been highly effective in cultivating large crops. *Azospirillum brasilense* and *Pseudomonas fluorescens* are referred to as plant growth-promoting rhizobacteria (PGPR), which act through both direct mechanisms, providing nutrients, and indirect mechanisms, such as biocontrol. This study aimed to evaluate the effectiveness of bio- inputs based on *A. brasilense* and *P. fluorescens* in sugarcane development. These bio-inputs were produced in bioreactors in isolated and combined fermentation. The test was conducted in a plant growth chamber with controlled temperature, photoperiod, and humidity. Pots received a sugarcane bud, and the bio-inputs were applied. The treatments consisted of T1 = control, T2 = *A. brasilense*, T3 = *P. fluorescens*, T4 = *A. brasilense* + *P. fluorescens* (combined fermentation) 0 hours (shelf life), and T5 = *A. brasilense* + *P. fluorescens* (combined fermentation) 180 hours (shelf life). The biocontrol action of bio-inputs against phytopathogenic fungi, the influence on soil microbiota, and the development of sugar cane were evaluated. The strains did not show biocontrol activity. In terms of microbiological parameters, the study revealed a significant increase in microbial biomass and soil biological activity, confirming the importance of fertility and nutrient availability. Positively impacting plant development. Thus, bio-inputs based on *Azospirillum brasilense* and *Pseudomonas fluorescens*, isolated or by combined fermentation, improved soil quality, soil biological activity, and the availability of plant nutrients.

Keywords: Bio-inputs. Bioreactor. PGPR. Rhizobacteria. Sustainable agriculture.

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1. Introduction

Sugarcane is a representative Brazilian crop cultivated in over 100 countries on different continents (America, Africa, Asia, and Oceania). Despite the number of producing countries, only 10 account for 80% of global production (FAO 2019). Brazil stands out as the world's largest sugarcane producer, with an estimated 678.67 million tons in the 2024/25 harvest. That is 4.8% lower than the previous harvest (CONAB 2024), which corresponds to approximately 39% of global production of this crop, followed by India (19%), China (6%), and Thailand (6%) (Silalertruksa and Gheewala 2018).

Sugarcane is a perennial crop. It is cut several times before being replanted. Its production cycle is, on average, six years with five cuts. In Paraná, non-mechanized logging areas are allowed to be burned, which further aggravates the negative impact of the crop on human health and the environment. The key technologies with potential to contribute to sugarcane productivity and sustainability are linked to genetic improvement, agricultural management, planting techniques, crop treatments, and harvesting (Borges et al. 2020).

Sustainable agriculture has been decisive in light of the growing challenges of food security, preservation of natural resources, and combating climate change. As the world population continues to grow, the need for agricultural practices that increase productivity and minimize environmental impacts becomes increasingly urgent (FAO 2023). In this context, the integration of bio-inputs, also known as biological inputs, represents an innovative and promising approach to face these challenges. These biological products often derive from living microorganisms, such as bacteria and fungi. They are designed to enhance soil fertility, stimulate plant growth, and decrease conventional chemical inputs (Vidal and Dias 2023).

Bio-input application aims to strengthen natural processes in agroecosystems, improve soil health, and promote crop resilience to biotic (pathogens, parasites) and abiotic (drought, extreme temperatures) stresses. These products play a key role in favorably modifying the soil's biological and biochemical dynamics, contributing to more fertile and productive soils. Among bio-inputs, microbial inoculants such as *Pseudomonas fluorescens* and *Azospirillum brasilense* are particularly effective in stimulating plant growth due to their nitrogen fixation, phosphate solubilization, and phytohormone production capacity, which favors root development (Hungria et al. 2010).

Plant growth-promoting rhizobacteria (PGPR) offer numerous environmental benefits and stimulate plant development through symbiotic interactions. They are also promising solutions for fungal and pest biocontrol (Azevedo et al. 2023). Direct mechanisms include improving plant nutrition and the absorption of elements such as P, K, Zn, Fe, and other vital minerals. They also favor the regulation of phytohormones such as IAA, produced by *Azospirillum* spp. (Gouda 2018). They indirectly produce biological agents, such as fungi and bacteria, which protect plants against pathogens and create a healthy environment (Santos et al. 2020; Dutta et al. 2023).

Soil biological activity, i.e., the set of processes performed by living organisms in the soil, is an important indicator of soil health and fertility. Soil microorganisms, including bacteria, fungi, and microfauna, are crucial to decompose organic matter. This activity releases nutrients and forms humus, essential for plant growth (Bhaduri et al. 2022). Integrating bio-inputs into agricultural practices reinforces these natural processes, increasing the diversity and activity of soil microbial communities, which directly affect crop productivity.

Cassán et al. (2020) affirm that bacterial colonization and biofilm formation contribute to plant growth, particularly grasses. Several species of bacteria are used in agriculture. *Bradyrhizobium* spp., *A. brasilense*, and others are employed as biofertilizers, with commercial formulations applied to seed coatings or mixed with the soil. This reduces dependence on chemical fertilizers, decreases production costs, and mitigates environmental impacts (Cassán et al. 2020). Another important bacterium is *Pseudomonas fluorescens*, which is applied in suspension to coat seeds or spray crops. It helps reduce the use of chemical pesticides and represents a more sustainable alternative (Berg 2009).

This study aimed to evaluate the influence of bio-inputs based on *Pseudomonas fluorescens* and *Azospirillum brasilense* on soil microbial activity and the development of sugarcane.

2. Material and Methods

The study was conducted at the Soil Microbiology Laboratory of the State University of Northern Paraná, Campus Luiz Meneghel, in a plant growth chamber (Type – Growth) with controlled temperature, humidity, and photoperiod.

The tested bio-inputs were cultivated in two ways: 1) Isolated multiplication of *Pseudomonas fluorescens* ATCC 13525 and *Azospirillum brasilense* Ab-V5 and Ab-V6 in bioreactors; 2) Associated multiplication of *A. brasilense* + *P. fluorescens* cultivated in the same bioreactor. This associated multiplication yielded cultures with 0 (zero) and 180 days of storage. Storage was at room temperature and in 1.5 L plastic bags.

Biocontrol test

The antifungal activity of the bio-inputs was evaluated in Petri dishes by the paired culture technique against *Fusarium solani*, *Macrophomina phaseolina,* and *Sclerotinia sclerotiorum* in potato dextrose agar (PDA) culture medium. An 8mm diameter disc containing the mycelium of the phytopathogenic fungus was centered on the plate, and the bacteria were inoculated at four equidistant points. The test consisted of measuring the mycelial growth of the fungus daily to obtain the Mycelial Growth Speed Index (MGSI). The plates were maintained in a biological oxygen demand (BOD) oven at 25 °C until the fungal mycelium from the control treatment reached radial growth throughout the plate. The test was performed in quadruplicate.

Evaluation of sugarcane development in pots

To prepare the pots, the Eutroferric Red Latosol (EMBRAPA 2018) was collected and mixed with sand in a 3:1 ratio. A physical-chemical analysis of the soil was performed to assess the need for correction. Moisture was adjusted to 60% of field capacity. The soil presented the following initial characteristics: organic matter (OM) = 30.5 g kg^{-1}, pH = 5.0, Phosphorus (P) = 6.5 mg dm^{-3}, Potassium (K) = 0.40 mol_c dm^{-3}, Calcium (Ca) 7.0 mol_c dm^{-3}, Magnesium (Mg) = 0.7 mol_c dm^{-3}, Aluminum (Al) = 1.7 mol_c dm^{-3}, H+Al = 5.5 mol_c dm^{-3}, Cation Exchange Capacity (CEC) = 8.0 mol_c dm^{-3}, and base saturation (V%) = 31.2%. The tests were conducted in pots containing 9 kg of soil.

Each pot received only 1 pre-germinated bud, totaling 25 pots. They were subjected to five treatments (T1 = Control, T2 = *A. brasilense*, T3 = *P. fluorescens*, T4 = *A. brasilense* + *P. fluorescens* 0 days, and T5 = *A. brasilense* + *P. fluorescens* 180 days), with five replicates. All treatments received 5 mL of the bio-input, and distilled water was added to the control treatment.

The tests were conducted under controlled conditions (Plant Growth Chamber – Type – Growth) with temperature control simulating daytime variations from 20 to 38 °C, constant humidity of 70%, and a photoperiod of 16 hours of light and 8 hours of dark. Each pot received 300 mL of water daily to ensure ideal conditions for plant growth.

Microbiological soil assessment

Microbial biomass carbon (MBC)

Microbial biomass carbon was assessed using the fumigation-extraction method, as proposed by Vance et al. (1987).

Basal soil respiration (BSR)

Basal soil respiration was determined as proposed by Silva et al. (2007).

Metabolic quotient (qCO_2) and microbial quotient (qMIC)

The metabolic quotient was determined using the ratio between BSR and MBC. The microbial quotient was determined with the ratio between MBC and total organic carbon (TOC).

Analysis of agronomic parameters

The plants were evaluated at 60 days for the parameters of height (cm), shoot fresh and dry mass (g), as well as root length (cm), volume (m/v), and fresh and dry mass (g), according to EMBRAPA (2009). Plant height was measured using a tape measure from the crown (collar) to the tip of the tallest stem; shoots were then cut at crown level.

The same procedure was used for the roots, measuring them from the base to the tip of the plants. The shoot fresh mass (SFM) was determined by weighing the plants on a semi-analytical balance, placing them in paper bags, and drying them in a forced-air circulation oven at 60 °C. The plants were weighed daily until achieving stability to obtain shoot dry mass (SDM). The same procedure was performed to determine the root fresh (RFM) and dry (RDM) mass (EMBRAPA 2009). The root volume was determined with a water-filled graduated cylinder by submerging the root and observing the displacement of the water column.

Statistical analysis

The statistical analysis was performed using RStudio software (R Core Team 2023). The assumptions of analysis of variance (ANOVA) were evaluated, including normality and homoscedasticity of variables. After confirming them, ANOVA and Tukey's post hoc test were performed (5% probability).

3. Results

Biocontrol test

None of the tested treatments presented control over the phytopathogenic fungi *Sclerotinia sclerotiorum* and *Macrophomina phaseolina*. However, the fungus *Fusarium solani* showed slight inhibition, reaching 30.82% in T1, 17.12% in T5, and 13.70% in T3 (Table 1).

Table 1. Percentage of growth inhibition of *Sclerotinia sclerotiorum, Fusarium solani,* and *Macrophomina phaseolina.*

Treatment	*S. sclerotiorum*		*F. solani*		*M. phaseolina*	
	MGSI	% INIB	MGSI	% INIB	MGSI	% INIB
T1	1.28		1.04		1.92	
T2	1.26	1.56	0.72	30.82	1.92	0
T3	1.26	1.56	0.90	13.70	1.92	0
T4	1.28	0	1.04	0	1.92	0
T5	1.26	1.56	0.86	17.12	1.92	0

Note: MGSI = Mycelial Growth Speed Index; % INIB = Percentage of growth inhibition [T1] = Control; [T2] = *A. brasilense*; [T3] = *P. fluorescens*; [T4] = *A. brasilense* + *P. fluorescens* 0 day; [T5] = *A. brasilense* + *P. fluorescens 18*0 days.

Soil microbiological analysis

All treatments that used microorganism applications presented better results in all evaluated biological attributes. The MBC showed a significant increase compared to the control.

The *q*MIC considerably improved using bio-inputs. Although *q*MIC in T2 did not significantly differ from the control, in T3 it showed an optimal value for organic matter mineralization and nutrient provision to the plant. In the treatments with *A. brasilense* + *P. fluorescens* at 0 and 180 days of shelf life, the increase in *q*MIC indicates the immobilization of carbon in organic form (Table 2).

The BRS demonstrates the release of CO_2 by the total metabolic respiration of the microbial community. In comparison to the other treatments, T1 presented better respiration (0.16), which does not

differ between treatments T2, T3, and T4 in absolute values. However, these treatments comprise a much larger population. Respiration was higher in T5 than in T1, and the microbial population was much larger. The respiration results reflect the qCO_2, which measures the relationship between BRS and MBC, i.e., the release of CO_2 per MBC unit. T1 presented a higher value (1.10), demonstrating high metabolic stress compared to the other treatments (Table 2).

Table 2. Soil microbiological attributes.

Treatment	MBC	*q*MIC	BSR	qCO_2
	mg C kg^{-1}	(%)	mg C-CO_2 Kg^{-1} h^{-1}	RBS C_BMS^{-1}
T1	149.61^{c}	0.76^{c}	0.16^{b}	1.10^{a}
T2	270.28^{b}	1.07^{c}	0.18^{b}	0.67^{bc}
T3	360.70^{a}	1.73^{b}	0.15^{b}	0.42^{c}
T4	356.79^{a}	2.18^{a}	0.16^{b}	0.45^{c}
T5	367.96^{a}	2.39^{a}	0.28^{a}	0.78^{ab}
C.V. (%)	10.83	14.76	14.48	24.95

Note: T1 = Control; T2 = *Azospirillum brasilense*; T3 = *Pseudomonas fluorescens*; T4 = *Azospirillum brasilense* + *Pseudomonas fluorescens* 0 day; T5 = *Azospirillum brasilense* + *Pseudomonas fluorescens* 180 days; Microbial Biomass Carbon (MBC); Microbial Quotient (*q*MIC); Basal Soil Respiration (BSR); Metabolic Quotient (qCO_2). Averages followed by the same lowercase letters in the column do not differ at a 5% probability.

Evaluation of sugarcane agronomic parameters

The parameters evaluated for plant shoot, including height and fresh and dry mass, were all superior compared to T1 (control), with 31% more fresh mass and 36% more dry mass in T2. Shoot dry mass did not increase in T5, probably due to the smaller root volume and consequently smaller root fresh and dry mass, which was still superior to T1 (control). Treatments T3 and T4 yielded the best results for root parameters, with an increase of more than 120% in volume, 140% in fresh mass, and 75% in dry mass (Table 3).

Table 3. Agronomic parameters of sugarcane.

Treatment	HGT	SFM	SDM	RLGT	RVOL	RFM	RDM
	m	g	g	cm	mL	g	g
T1	1.37^{b}	57.16^{b}	11.45^{b}	53.25^{c}	38.75^{b}	30.47^{c}	8.3^{c}
T2	1.56^{a}	72.13^{ab}	13.57^{ab}	83.50^{b}	67.50^{a}	59.31^{b}	10.2^{b}
T3	1.66^{a}	75.82^{a}	15.71^{a}	101.25^{a}	86.90^{a}	73.24^{a}	14.8^{a}
T4	1.58^{a}	66.61^{ab}	13.82^{ab}	75.75^{b}	80.00^{a}	67.95^{ab}	14.2^{a}
T5	1.67^{a}	73.16^{ab}	11.46^{b}	98.70^{a}	67.00^{a}	57.14^{b}	11.3^{b}
C.V.(%)	4.62	11.21	10.92	7.98	13.84	10.25	11.40

Note: T1 = Control; T2 = *Azospirillum brasilense*; T3 = *Pseudomonas fluorescens*; T4 = *Azospirillum Brasilense* + *Pseudomonas fluorescens* 0 day; T5 = *Azospirillum Brasilense* + *Pseudomonas fluorescens* 180 days); HGT (Height); SFM (Shoot Fresh Mass); SDM (Shoot Dry Mass); RLGT (Root Length); RVOL (Root Volume); RFM (Root Fresh Massa); RDM (Root Dry Mass). Averages followed by the same lowercase letters in the column do not differ at a 5% probability.

4. Discussion

Biocontrol test

The lack of biocontrol activity of the bio-inputs against phytopathogenic fungi in this study warrants further investigation. *Pseudomonas* spp. are known for efficiently colonizing the rhizosphere, promoting plant development, and occupying infection sites by competing with other microorganisms and blocking pathogen entry points, thereby contributing to biological control (Silva et al. 2022).

Pseudomonas spp. control plant pathogens and play an effective role in disease suppression through direct and indirect mechanisms. These mechanisms include competition for siderophores, nutrient provision (nitrogen, phosphorus, and potassium), and the production of phytohormones, lytic enzymes, volatile compounds, and secondary metabolites. These metabolites also induce systemic resistance in plants (Mehmood et al. 2023). They are commonly applied as biocontrol agents and biofertilizers via seed coating

or crop spraying, which helps reduce chemical pesticide use and offers a more sustainable and environmentally friendly alternative (Berg 2009).

Azospirillum spp. are classified as plant growth-promoting rhizobacteria (PGPR) because of their multiple mechanisms, including phytohormone production, phosphate solubilization, siderophore production, bioremediation of xenobiotics, increased tolerance to abiotic stress, and biocontrol of phytopathogens. However, their primary use as biofertilizers is associated with biological nitrogen fixation and plant growth promoters through phytohormone synthesis (Pedraza et al. 2020).

These functional microbial groups interact in complex and synergistic ways to maintain soil health, promote plant growth, and support agricultural productivity. Sustainable soil management practices that promote and preserve microbial diversity are vital for enhancing the resilience of agricultural ecosystems to climate change and intensive land-use practices (Matsumoto et al. 2005).

The absence of biocontrol activity in the strains evaluated in this study may be due to the lack of specific genes associated with biocontrol mechanisms. These characteristics are not universally present in all bacterial strains (Belen et al. 2024).

Soil microbiological analysis

The biological aspects of soil are often the most dynamic indicators of quality. The diversity and activity of microorganisms such as bacteria, fungi, and nematodes, among others, are crucial for nutrient cycling and decomposition of organic matter. These microorganisms regulate fundamental ecological processes, facilitating the release of nutrients to plants and contributing to the formation and stabilization of soil aggregates (Alves et al. 2021).

Biological indicators such as microbial respiration, microbial biomass carbon (MBC), and enzyme activities are used to evaluate biological activity and soil health. High microbial activity is often equivalent to efficient decomposition of organic matter, which is essential for soil fertility and productivity (Matsumoto and Marques 2015; Liu et al. 2020). Microbiological soil analysis revealed important information about soil health, as microorganisms play a central role in the decomposition, nutrient cycling, and the suppression of pathogens of organic matter.

The MBC is an indicator of microbial community in the soil that reflects the amount of active microorganisms and suggests intense microbial activity, which is usually a sign of good soil health. Increased microbial biomass is often associated with more efficient decomposition of organic matter and the rapid release of nutrients essential for plant growth (Anderson and Domsch 2010).

Soil management practices may affect soil microbial biomass. Conventional management systems have considerably reduced biomass due to soil disturbance, which directly damages microorganisms by exposing them to variations in temperature and humidity. Conservative systems that employ bio-inputs and no-tillage indicate that changes in communities of microorganisms and their activity directly affect the biological and biochemical processes of the soil, agricultural practice, and consequent agroecosystem sustainability (Ferreira et al. 2017).

Basal soil respiration (BSR) is a direct indicator of microorganism metabolic activity, expressed in CO_2 production (Vieira, 2011). The observed BSR values were relatively low, which may indicate moderate metabolic activity and balanced organic matter decomposition. Excessive basal respiration might indicate recent disturbance or rapid organic matter decomposition, often associated with soil disturbance or a high content of easily decomposable organic matter (Totola and Chaer 2002).

The metabolic quotient (qCO_2) measures the efficiency of microorganisms in using available carbon resources. The relatively low qCO_2 values suggest efficient use of available resources, soil stability, and fewer disturbances. Low qCO_2 is often associated with a stable and resource-efficient microbial community, which is a sign of healthy soil (Matsumoto and Marques 2015). The use of microbial biofertilizers or bio-inputs reduces soil stress.

The microbial quotient (qMIC) represents the ratio between microbial carbon and total organic carbon. It reflects the efficiency of the microbial community in assimilating soil carbon. Soils with qMIC between 1.5 and 1.8 have higher carbon assimilation efficiency, which may suggest a healthier soil with an active microbial community. A lower index indicates little microbial activity due to an excess or lack of

microorganisms in the soil for organic matter decomposition. This means that the carbon in the soil is lost. Conversely, indices above 1.8% mean that soil carbon is immobilized in organic form. However, in microbial death, carbon is readily available (Baretta et al. 2005).

Land management practices considerably influence soil quality. Intensive cultivation, excessive use of chemical fertilizers, and monoculture may degrade soil quality by reducing organic matter, promoting acidification, and decreasing microbial biodiversity. This increases the risk of erosion, compaction, and nutrient loss, compromising the long-term sustainability of agricultural systems (Montgomery, 2018). However, sustainable agricultural practices, such as regenerative agriculture, cover crops, and crop rotations, benefit soil quality. They favor organic matter accumulation, reduce erosion, and increase microbial diversity, which strengthens soil resilience to environmental disturbances and climate change (Pittelkow et al. 2015).

The importance of bio-inputs in sustainable soil management is also related to their ability to influence microbial biomass and soil metabolic activity. These biological products significantly increase microbial biomass and improve parameters, such as soil respiration and microbial carbon. They also reduce the qCO_2, which indicates the metabolic efficiency of soil microorganisms (Verma et al. 2023). These improvements promote soil health, increase its capacity to retain water, limit erosion, and offer higher resilience to extreme climate conditions.

Bioprospecting involves discovering and exploring new bioactive molecules from microbial biodiversity. It has increased the potential of bio-inputs in the sustainable management of agroecosystems. The secondary metabolites produced by these microorganisms effectively regulate microbial interactions in the soil, favoring a balanced microbiome that is resilient against pathogens (Almeida 2017; Guo 2017). Bio-inputs not only stimulate microbial activity but also create favorable conditions for beneficial microbial populations, while reducing the prevalence of pathogens. This approach helps improve crop productivity and the sustainability of agricultural systems.

Assessment of sugarcane agronomic parameters

The findings of this study are supported by previous research demonstrating that *Pseudomonas* spp. enhance nutrient uptake and promote plant growth through mechanisms such as indole-3-acetic acid (IAA) production and phosphate solubilization (Bashan et al. 2014).

Treatments with *A. brasilense* + *P. fluorescens* combinations exhibit a greater dispersion of plant height data, indicating variability in plant responses. This may reflect the complexity of interactions between soil and plant microbial species, as well as the need to adjust proportions or doses of inoculants. Recent studies found that applying microbial consortia may lead to variable responses due to competition or synergy between microorganisms (El-Nahal et al. 2022).

A. brasilense alone showed the largest stem diameter, indicating more robust stems (data not shown). However, this improvement did not translate into overall optimal plant growth as in the *Pseudomonas* treatment. These findings confirm that bioinput effectiveness varies according to growth parameters. Some have a stronger influence on the structure, and others on nutrient uptake and hormonal regulation (Van Loon 2007).

The association between *Azospirillum* and *Pseudomonas* also promotes a beneficial synergy in root volume and length. These are key characteristics for water and nutrient absorption, as well as plant support. This combination maximizes the benefits of both bacteria, increasing biomass and the plant's ability to resist environmental stresses, as confirmed by several studies on the influence of growth-promoting rhizobacteria on the root system (Bhattacharyya and Jha 2012).

The number of leaves, as a reflection of photosynthetic capacity, is also a key indicator of plant performance. The pronounced effect of *Pseudomonas* on this parameter confirms its role in promoting growth through mechanisms that increase the plant's ability to produce energy and store resources for future growth (Vacheron et al. 2013). Conversely, the control treatment presents significantly lower results for all parameters, clearly demonstrating the impact of bio-inputs on improving plant performance.

Treatment with *P. fluorescens* alone showed the highest potential to improve root growth in sugarcane, while the combination with *A. brasilense* may be optimized depending on the application time.

These results confirm the essential role of PGPR in promoting plant growth, especially in sustainable production systems (Afrida, Syahril, and Tampubolon 2022).

The greater the root mass, the higher the potential of bio-inputs to control water stress. Additionally, plants with higher volume allow for searching for nutrients deeper in the soil (El-Nahal et al. 2022).

Bio-inputs are natural, non-chemically synthesized, and biodegradable products that contain living microorganisms, such as PGPR, capable of solubilizing phosphate and fixing atmospheric nitrogen (Paula et al. 2021). These microorganisms promote plant growth by increasing nutrient input, biomass, and root area. Some bio-inputs produce phytohormones such as IAA, which stimulate plant growth. They also produce substances that inhibit the proliferation of pathogens (Noumavo et al. 2015).

5. Conclusions

The bio-inputs *Azospirillum brasilense* and *Pseudomonas fluorescens* offer a promising alternative to improve sugarcane productivity. They may also be combined with chemical fertilizers to help promote sustainable agriculture. These microorganisms may increase soil microbial activity, consequently providing higher plant nutrient availability and promoting growth. This study provides a basis for future research evaluating additional strains and their combinations with other plant growth-promoting organisms, with the goal of maximizing their effectiveness in agricultural systems.

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