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Metagenomics and vegetative growth of *Salvia hispanica* inoculated with *Trichoderma harzianum*

Metagenômica e crescimento vegetativo de *Salvia hispanica* inoculada com *Trichoderma harzianum*

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Abstract

The soil is a dynamic environment, influenced by abiotic and biotic factors, which can result in changes in plant development. This study aimed to assess the impact on vegetative growth of chia (*Salvia hispanica* L) inoculated with *Trichoderma harzianum* and on the rhizosphere microbiome. The experimentation was conducted in a greenhouse under controlled conditions growing chia plants in pots containing soil with a clayey texture. Different concentrations of *T. harzianum* (0; 2.5; 5.0; 10.0; 20.0 µL. g^{-1} of seed) were applied to the chia seeds before planting. Morphological parameters, including plant height (cm), number of branches, stem diameter (mm), number of days to flowering and shoot and root dry masses (g) were quantitatively assessed. After the cultivation period, soil samples from the rhizosphere region were collected for subsequent chemical and metagenomic analyses. These samples were also compared with the control soil, collected before installing the experiment. The results showed that increasing doses of *T. harzianum* promoted a significant increase in the diameter of the stem, number of branches, dry biomass of the root system and the number of days to flowering, without modifying the overall height of the plants. Soil metagenomics indicated that *T. harzianum* inoculation modified the microbial diversity of the rhizosphere environment, with more pronounced effects observed in samples treated with higher concentrations of the inoculant. Furthermore, there were changes in the chemical composition and enzymes related to soil quality in correlation with the concentrations of the applied inoculant. This study demonstrated that inoculating chia seeds with *T. harzianum* not only promotes specific morphogenetic characteristics of the plant, but it also has a significant impact on the microbial diversity and biochemical functionality of the soil, including an observed increase in the populations of *T. harzianum* and *T. asperellum*.

Keywords: chia, growth promotion, microbiome, antagonists, phytopathogens.

Resumo

O solo é um ambiente dinâmico, influenciado por fatores abióticos e bióticos, e que pode resultar em modificações do desenvolvimento vegetal. Este estudo teve como objetivos avaliar o impacto da inoculação de sementes de chia (*Salvia hispanica* L) com *Trichoderma harzianum* sobre o desenvolvimento vegetativo, e verificar a influência desta inoculação no microbioma rizosférico. O estudo consistiu no cultivo de plantas de chia em solo com textura argilosa contido em vasos sob condições controladas de casa-de-vegetação. Para tal, diferentes concentrações de *T. harzianum* (0; 2.5; 5.0; 10.0; 20.0 µL.g^{-1} de semente) foram aplicadas às sementes antes do plantio. Parâmetros morfológicos, incluindo altura das plantas (cm), número de ramos, diâmetro do caule (mm), número de dias para florescimento e para massas secas da parte aérea e radicular (g) foram quantitativamente avaliados. Após o período de cultivo, amostras de solo da região rizosferica foram coletadas para posterior análises química e de metagenômica. Essas amostras foram comparadas também com o solo controle, coletado antes da instalação do experimento. Os resultados revelaram que doses crescentes de *T. harzianum* promoveram um aumento significativo do diâmetro do caule, do número de ramificações, da biomassa seca do sistema radicular e do número de dias para o florescimento, sem modificar a altura global das plantas. A metagenômica do solo indicou que a inoculação de *T. harzianum* alterou a diversidade microbiana do ambiente rizosférico, com efeitos mais pronunciados observados nas amostras tratadas com maiores concentrações do inoculante. Além disso, verificou-se alterações na composição química e em enzimas relacionadas a qualidade do solo em correlação com as concentrações do inoculante aplicado. Em síntese, este estudo sugere que a

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inoculação de sementes de chia com *T. harzianum* não apenas promove características morfogenéticas específicas da planta, mas também exerce um impacto significativo sobre a diversidade microbiana e a funcionalidade bioquímica do solo, incluindo um incremento observado nas populações de *T. harzianum* e *T. asperellum*.

Palavras-chave: chia, promoção do crescimento, microbioma, antagonistas, fitopatógenos Introduction.

1. Introduction

Plants and the soil microbiome are in dynamic interaction and are directly or indirectly influenced by abiotic factors and by other organisms. This interaction ends up determining physical-chemical and biological characteristics of the soil, which can result in changes in plant growth. In this context, in recent years, studies have been intensified to prove the relevance of plant-microorganism interaction in crop yield (Fu et al., 2020; Hang et al., 2022) which can contribute to more sustainable agriculture (Antoszewski et al., 2022).

The culture of chia (*Salvia hispanica*) is quite widespread among farmers in countries such as Argentina and Paraguay (Orona-Tamayo et al., 2017). In Brazil, chia cultivation is recent (Radke et al., 2018; Costa et al., 2022), occurring in the West of Paraná and Northwest of Rio Grande do Sul (Migliavacca et al., 2014). It has seeds with nutraceutical importance that are rich in fatty acids, proteins, fiber, vitamins (Jamboonsri et al., 2012; Knez Hrnčič et al., 2019) and substances with antioxidant effect (Pellegrini et al., 2018; Dziadek et al., 2022).

Chia plants are highly influenced by temperature and photoperiod conditions and planting time and/or latitude can substantially limit grain production (Jamboonsri et al., 2012; Rodríguez-Abello et al., 2018). According to Jamboonsri et al. (2012), chia is a short-day plant with a critical photoperiod for floral induction of approximately 12 h. In Western Paraná, chia produces inflorescences at the beginning of April (autumn) (Pereira et al. 2020). Therefore, plantings at the beginning of autumn can result in a reduction in the vegetative growth period, leading to a reduction in productivity, while those carried out in summer result in taller and more productive plants (Pereira et al., 2020). Nevertheless, chia lodging is recurrent, especially in taller plants, making harvesting difficult and facilitating exposure to the occurrence of microorganisms in the seeds (Goergen et al., 2018).

Different studies showed the presence of fungal contaminants in chia seeds, some of which are phytopathogenic fungi that can be transmitted to seedlings, such as *Fusarium* sp. (Witkovski et al., 2021), *Penicillium* sp. and *Aspergillus* sp (Jermnak et al., 2020; Witkovski et al., 2021), and a larger part made up of non-phytopathogenic species, but which can cause problems to human health due to the production of mycotoxins (Freire et al., 2007). In the absence of the main host these fungi have different survival strategies associated with resistance structures and permanence in alternative hosts or in soil organic matter for long periods (Kerdraon et al., 2019). The reduction in microbial diversification of soils, caused by conventional cultivation systems, tends to increase vulnerability to the invasion of these phytopathogens (Samaddar et al., 2021).

Different strategies to control these soil microorganisms have been suggested, including crop rotation, soil solarization, use of resistant cultivars and biological control agents (Panth et al., 2020). In this context, several antagonistic fungi have great potential for use in biocontrol (Sood et al., 2020; Nascimento et al., 2022), such as species of the genus *Trichoderma*, especially *T. harzianum*, which has been widely used in the biocontrol of several phytopathogens (Fraceto et al., 2018). Furthermore, different strains can adopt different antagonism mechanisms, which include antibiosis, competition and mycoparasitism (Asad, 2022), and could stimulate growth (Stewart and Hill, 2014; Fu et al., 2020; Hang et al., 2022) or induce plant systemic resistance to diseases (Meyer et al., 1998; Sabbagh et al., 2017; Ilham et al., 2019).

Studies using *Trichoderma* spp in chia crops are scarce (El-Kaed et al., 2021; Witkovski et al., 2021; Abdel-Aty et al., 2022), not having been published to date research that verifies the effect of its inoculation in seeds on the growth and development of this crop, and its rhizosphere.

This research aims studying the effect of inoculating chia seeds with *T. harzianum* on the growth of plants and the diversity of their rhizosphere microbiome.

2. Material and Methods

2.1. Genetic material

The Chia cultivar CH03 (MaisGenes Sementes LTDA, Toledo, Paraná, Brazil) was used in the experiments conducted under laboratory and greenhouse conditions at Faculdade Educacional de Medianeira (UDC Medianeira), Medianeira, Paraná, Brazil.

2.2. Seed sanitary analysis

The sanitary analysis was performed according to the Brasil (2009) protocol, consisting of four replications with 100 seeds each. The seeds were placed on two sheets of sterile germtest paper, previously moistened with an autoclaved solution of dichlorophenoxyacetic acid (2,4-D) at a concentration of 5 ppm. These preparations were placed in Gerbox-type polypropylene boxes and subjected to asepsis with 70% alcohol. Subsequently, the boxes were hermetically sealed with parafilm and incubated in a BOD chamber under controlled conditions of 25°C and a photoperiod of 12:00 h, at intervals of 7 and 14 days. The taxonomic identification of microorganisms associated with the seeds was carried out through morphological assessment with the aid of an optical microscope. Fungi belonging to the genera *Aspergillus, Penicillium* and *Rhizopus* were identified in 1%, 0.25% and 0.75% of the seeds, respectively.

2.3. Inoculation with Trichoderma harzianum and installation of the experiment

Chia seeds were inoculated with doses of *Trichoderma harzianum*, using the commercial strain CCT 7589, which

contained $1x10^9$ UFC.L^{-1} of the fungi. The treatments consisted of T1 (control), T2 (2.5 µL.g^{-1} seed), T3 (5.0 µL.g^{-1} seed), T4 (10.0 µL.g^{-1} seed) and T5 (20.0 µL.g^{-1} seed), having as reference the spray volume of 20 mL. kg^{-1} of seeds.

For treatments with the fungi, inoculation was carried out using 1 g of chia seeds placed in a becker filled with 20 µL of the solution, followed by homogenization for 15 s. After that the seeds were removed from the solution, dried at room temperature (25ºC) for 15 min and sowed at a depth of 1 cm using four seeds per pot containing substrate. Thinning was performed seven days after sowing, leaving one plant per pot.

The substrate used to cultivate the plants in pots (20 L capacity) was made up of a mixture of soil, sand, and poultry litter in a volumetric ratio of 3:2:1, respectively. After mixing, substrate samples were collected to perform chemical and metagenomic analyses. The fertilization of the substrate was prescribed based on its chemical composition (LABAGRO, Serranópolis do Iguaçu, PR, Brazil), 20 kg.ha^{-1} of N, 80 kg.ha^{-1} of P_2O_5 and 40 kg.ha^{-1} of K_2O.

2.4. Experimental design

The experiment was conducted in a greenhouse with controlled temperature conditions, 28 ± 4ºC, and water regime of 10 mm.day^{-1}. The experimental design used was completely randomized (DIC) with five treatments, which are described above, and ten replications. The experimental unit consisted of one plant per pot.

Plant height was measured periodically from 14 days after sowing until the beginning of flowering. The following characteristics were evaluated: number of days to flowering (NDF), number of branches (NB), stem diameter (SD), dry mass of the shoot (MSA), and root system (RDM).

At the same time of the final experimental evaluation, a soil sample was taken from the rhizosphere region, from each of the five treatments. These samples were placed in plastic bags and subjected to freezing at a temperature of -20ºC, until they were sent for chemical and metagenomic analyses.

2.5. Metagenomics analysis

For this analysis, soil samples derived from T1 to T5 treatments were collected at the end of the experiment, plus the sample obtained before the start of cultivation, providing a temporal comparison regarding the microbial composition of the soil. The metagenomics analyzes were carried out by the company LAGBio – Análises Genômicas e Biotecnologia (Toledo, PR, Brazil). For this purpose, the samples were initially subjected to total DNA extraction, using the extraction kit DNEASY PowerSoil Pro – Qiagen, and the total DNA concentration and quality was verified using the dsDNA HS Assay Kit Qubit® - Life Technologies. With the use of 1 µg of DNA per soil sample, metagenomic libraries were constructed using the Nextera XT kit (Illumina, San Diego, CA, USA). Sequencing was carried out using MiSeq equipment (Illumina, San Diego, CA, USA).

After complete sequencing, the sequence reads were pre-processed using the FastQC program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). To check the quality of sequence readings and remove low-quality ones, the Sickle software was used (Joshi and Fass, 2011). In this process, all sequences smaller than 50 bp and with a Phred score lower than 20 were removed from the data set. In this way, all sequence readings with undetermined bases were removed.

To analyze the structure of microbial communities, including the study of taxonomic diversity, the Kraken 2 program was used (Wood et al., 2019). For functional analysis of the sequences, the MG-RAST online platform (version 4.0.3) was used (Keegan et al., 2016). The sequences were submitted to the platform and processed using the standard pipeline, which consisted of E-value 10^5 and cut-off with a minimum of 60% of similarity between sequences. To verify the possible metabolic routes present in the set of microorganisms in this study, the SEED Subsystems level 1 function was used, with direct plugging to the KEGG Pathway (Kanehisa and Goto, 2000). The results obtained from MG-RAST were subsequently analyzed using the Statistical Analysis of Metagenomic Profiles program, STAMP (version 2.1.3) (Parks et al., 2014).

2.6. Statistical analysis

The data collected throughout the development of chia plants, except for growth analysis, were subjected to normality and homogeneity of variance tests, followed by analysis of variance for regression, considering the 5% of probability by using the F test.

For the plant height variable, whose measurements were collected until flowering, the data were analyzed using Generalized Linear Mixed Models (GLMM). The gamma distribution was the best representation of the data. The quality of the models was assessed using the values of AIC (Akaike's information criterion) or QIC (Quasi likelihood under Independence model Criterion) as selection criteria. It was also verified that the individual (ID) is a random factor according to the AIC criterion. Therefore, the model with the best adherence considers data with gamma distribution, ID as a random factor and use of the AR1 covariance matrix. All parametric statistical analyzes were performed using the SPSS programs (IBM Corp. Released, 2020), JAMOVI (Navarro et al., 2020) and SISVAR (Ferreira, 2011).

3. Results and Discussion

3.1. Growth and development of chia plants

Under greenhouse temperature conditions, 28 ± 4ºC, the emergence of chia seedlings occurred between 3 and 4 DAP (days after planting), regardless of the dose of *T. harzianum*. The first lateral shoots occurred from 36 DAP, while the first inflorescences, also called clusters or spikes, were observed between 62 and 73 days after planting. The plants reached an average height of 116 ± 3.8 cm at the end of the vegetative stage. The emergence of seedlings between 3 and 4 DAP demonstrates the seed vigor and the capacity for rapid initial establishment, essential for short-cycle crops.

The GLMM analysis for the plant height variable showed a significant increase over time as indicated by the AIC

criterion (-473.974) and F value of 4921.787; ($p < 0.001$), however, it was not found any effects of the *Trichoderma* dose or Time x *Trichoderma* interaction (Figure 1). The lack of effect of the *Trichoderma* dose suggests that the influence of this fungus is not necessarily manifested in terms of height in chia plants. This strain is recommended for the biocontrol of *Rhizoctonia solani* and *Sclerotinia sclerotiorum*, with root growth stimulation being one of its modes of action (Bettiol et al., 2019). Generally, there is strong evidence for the influence of indole acetic acid (IAA) synthesized by microorganisms on plants. For Trichoderma, Stewart and Hill (2014) suggest that growth stimulation may be associated with the establishment of a balance of hormones such as IAA, gibberellic acid, and ethylene. Thus, the absence of a dose effect of CCT 7589 on the growth of chia plants' aerial parts is likely associated with genetic-environmental factors not investigated in the present study. This observation is relevant and highlights the need for future research as to explore the underlying mechanisms of this relation.

Although the treatment of chia seeds with *T. harzianum* did not result in changes in plant height, it was found a linear increase in the magnitude of the variables: number of days to flowering (NDF), number of branches per plant (NB) and stem diameter (SD) up to the maximum dose (20 µL.g^{-1} seed) of the inoculant (Figures 2A, 2B and 2D). For the variable root dry mass (RDM), a quadratic regression equation was adjusted, in which the maximum estimated point for root biomass accumulation was reached with the application of 17.08 µL.g-1 seed (Figure 2C), suggesting that inoculant doses above this may not offer additional benefits. Furthermore, by avoiding excessive doses, the risk of possible adverse effects on the soil ecosystem is minimized, contributing to a more sustainable agriculture.

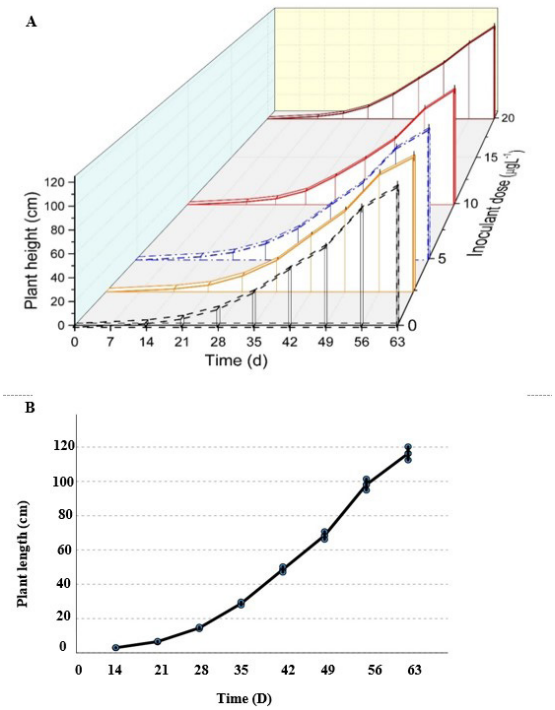

Figure 1. Marginal averages estimated for: A: Height of chia plants as a function of inoculant dose and assessment period in days after planting (D); A: Height of chia plants as a function of time. This variable was not affected by the inoculant dose.

The stimulation of root growth of chia plants promoted by *T. harzianum* is of great relevance for the crop, since this species has an incipient root system with frequent occurrence of lodging (Pereira et al., 2020). Studies carried out with different plant species have revealed that the *Trichoderma*-plant interaction causes changes in the root system (Contreras-Cornejo et al., 2009; Harman et al., 2012; Chagas et al., 2017), which can lead to improvement in the carrying capacity of the aerial part (Contreras-Cornejo et al., 2009). In addition, compounds produced by the fungus induce the formation of a greater volume of root cells, thus maximizing the absorption of water and nutrients by the plant, as well as participating in the decomposition of organic matter, consequently contributing to the availability of nutrients (Vergara et al., 2019).

Recent studies by Fu et al. (2020) and Hang et al. (2022) revealed that inoculation with fungi of the genus *Trichoderma* promoted changes in the soil microbiome, and that depending on the strain, they favored plant growth in maize and cucumber plants, respectively. However, to date, there are no research results available on chia.

The data obtained in this study show that the *T. harzianum* had no dose influence on the characteristics of germination and plant height. However, it had a significant impact, especially in optimizing root biomass. These results expand the understanding of plant-fungus interactions in chia cultures, highlighting the potential of *T. harzianum* as a growth-promoting agent. Identification of the effectiveness threshold of *T. harzianum* provides a basis for more sustainable and cost-effective agricultural practices. Nevertheless, it is imperative that further research is needed to understand the mechanisms of these interactions, and to verify the applicability of these findings in different agricultural contexts.

3.2. Metagenomics analysis

Through the exploratory analysis of metagenome data from soil samples from the rhizosphere region, we found a wide variability both in the number of obtained sequences, as well as in the diversity of genera and identified species. Nonetheless, it was verified that, independently of the soil sample, approximately 98% of sequences belonged to the Bacteria domain, with a predominance of the phyla Proteobacteria (52.94 to 65.77%), Actinobacteria (10.35 to 24.08%), Bacteroidetes (2.35 to 13.74%), Firmicutes (3.16 to 7.21%) and Plantomycetes (1.68 to 3.45%). For the Eukaria domain, which represents approximately 1% of the biological diversity of the samples, were predominantly detected the organisms from the phyla Ascomycota (0.23 to 0.63%), Streptophyta (0.12 to 0.19%) and Chordata. (0.1 to 0.13%).

In relation to the diversity of bacteria and fungi (Tables 1 and 2), it was found that, regardless of the soil sample analyzed, highly diverse microbial taxa were detected, which according to Igiehon and Babalola

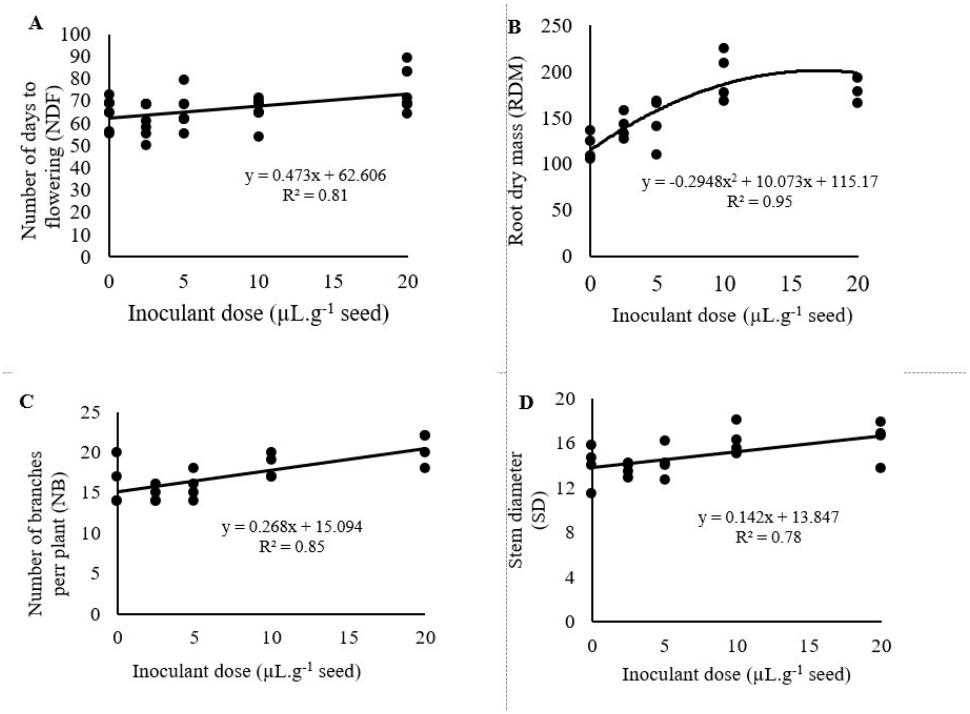

Figure 2. Regression analysis of the variables number of days to flowering (NDF), root dry mass (RDM), number of branches per plant (NB) and stem diameter (SD) of chia as a function of inoculant dose (*T. harzianum*).

(2018) may be involved in neutral, beneficial as well as phytopathogenic associations. In the metagenomic analysis of a soil sample pre-cultivation of chia, a prevalence of DNA sequences associated with the genera *Pseudomonas*, *Streptomyces* and *Mesorhyzobium* was found, involved, respectively, in growth promotion (Sah et al., 2021), antagonism (Pengproh et al., 2023) and atmospheric nitrogen fixation (Knežević et al., 2022). As for fungi, there was a predominance of the biological control agent *Trichoderma* spp. and the genera related to etiological agents of plant diseases such as *Fusarium*, *Rhizoctonia*, *Sclerotium*, *Macrophomina* and *Aspergillus*.

In relation to the fungus *Trichoderma* spp, a greater number of sequence readings were detected in the T2 sample (2.5 µL. g^{-1} seed) when compared to the other treatments (Table 2). Through the analyzes of the *Trichoderma* species present in the soil (Table 3), there was a predominance of *T. reesei* and *T. virens*, followed by *T. asperellum* and *T. harzianum* in all samples. In the T1 treatment, soil without seed inoculation with *T. harzianum*, it was detected a lower percentage of *Trichoderma* DNA sequences (1.06%), in relation to other treatments, as well as for the species that was inoculated to the seeds (11.3%). The increase in readings of *Trichoderma* spp. in the T2 sample suggests a positive response to the applied treatment, which may provide strategies for crop management. However, the lower reading of T1 sequences in relation to other treatments demonstrates the importance of inoculation in ensuring the presence and activity of this beneficial fungus.

The diversity of the genus species of *Trichoderma* revealed in this research (Table 3), associated with the role of this biological control agent as an antagonist and/or growth promoter presented in the literature, allows us to suggest a probable influence on the growth and development of chia plants by modifying the soil microbiome.

Through the comparison of the bacteria genera in rhizospheric soil after plant cultivation (Table 1), there was a wide variation between treatments for *Mesorhyzobium*, *Pseudomonas* and *Streptomyces*. For the genus *Pseudomonas*, it was originally detected 6.6%, being that after cultivation with chia there was a wide variation between treatments T5 (1.75%) and T2 (7.4%). In relation to the genus *Mesorhyzobium*, T1 (4.1%) and T5 (1.7%) had, respectively, the highest and lowest abundance among all treatments. For the genus *Streptomyces*, the opposite occurred, that is, T5 (6.2%) was higher than T1 (2.1%). The variation observed between the treatments for bacteria after chia cultivation highlights the dynamic intervention of agricultural practices on the soil microbiome. Chia, as a crop, may be influencing the abundance of specific bacterial genera, which could have repercussions on soil health and plant productivity. For example, fluctuations in *Pseudomonas* and *Mesorhyzobium* may reflect changes in nutrient availability or in the plant-microbe interactions (Nadarajah and Abdul Rahman, 2021).

Using the estimated Pearson correlation coefficients, we detected no association between the abundance of *Mesorhyzobium*, *Pseudomonas*, *Streptomyces* and *Trichoderma* and the increase in doses of the inoculant based on *T. harzianum.* Yet, a strong negative correlation was estimated between the abundance of *Trichoderma*

Table 1. Diversity of bacteria for the main genera of agronomic importance in different soil samples: Before (BC) and after (T1 to T5) cultivation with chia, in the % of reading sequence of DNA per gram of soil.

Agronomic Importance	Genus	Abundance (%)					
		BC	T1	T2	T3	T4	T5
Antagonist	*Bacillus*	0.298	1.031	0.416	0.941	1.964	0.674
	Chromobacterium	0.036	0.039	0.046	0.05	0.041	0.04
	Saccharopolyora	0.053	0.015	0.052	0.069	0.09	0.085
	Streptomyces	4.281	2.142	3.974	5.968	5.906	6.186
Denitrifier	*Nitrosomonas*	0.102	0.207	0.053	0.101	0.119	0.09
Diazotrophic	*Achromobacter*	0.505	0.548	0.311	0.261	0.295	0.285
	Azopirillum	0.185	0.538	0.265	0.365	0.377	0.367
	Azorhizobium	0.01	0.01	0.013	0.028	0.034	0.031
	Bradyrhizobium	0.732	1.722	2.349	2.764	2.546	3.513
	Burkholderia	0.378	0.252	0.572	0.603	0.616	0.691
	Cupriavidus	0.217	0.341	0.293	0.381	0.336	0.406
	Mesorhizobium	1.728	4.15	3.25	3.617	3.524	3.22
	Paraburkholderia	0.276	0.123	0.289	0.358	0.377	0.375
	Rhizobium	0.843	0.686	0.833	0.892	0.788	0.821
	Sinorhizobium	0.11	0.138	0.13	0.147	0.105	0.135
Phytopathogenic	*Acidovorax*	0.43	1.239	0.177	0.267	0.34	0.375
	Agrobacterium	0.113	0.079	0.102	0.122	0.142	0.085
	Clavibacter	0.07	0.099	0.074	0.072	0.09	0.071
	Curtobacterium	0.24	0.173	0.196	0.205	0.194	0.291
	Erwinia	0.017	0.025	0.008	0.017	0	0.014
	Pantoea	0.049	0.005	0.028	0.026	0.015	0.054
	Pectobacterium	0.01	0	0.022	0.006	0.004	0.011
	Ralstonia	0.083	0.109	0.066	0.097	0.105	0.096
	Spiroplasma	0	0	0	0	0.004	0
	Xanthomonas	0.685	0.183	0.138	0.207	0.179	0.172
	Xylella	0.006	0	0.001	0.002	0	0
Growth promoter	*Kosakonia*	0.009	0.02	0.001	0.006	0.004	0.014
	Lactobacillus	0.067	0.025	0.101	0.038	0.078	0.051
	Neorhizobium	0.153	0.03	0.068	0.084	0.071	0.062
	Paenibacillus	0.264	0.168	0.333	0.5	0.474	0.356
	Pseudomonas	6.607	5.596	7.455	2.17	2.606	1.752
	Serratia	0.061	0.025	0.026	0.041	0.03	0.042

and microorganisms of the genera Ralstonia ($r = -0.94$; p-value = 0.012) and Nigrospora ($r = -0.89$; p-value = 0.04). In this context, different studies have revealed antagonistic action of *Trichoderma* spp. against Ralstonia (Konappa et al., 2018; Okinda, 2022) and Nigrospora (Talapatra et al., 2016; Hamdia et al., 2020; Zhang et al., 2021), suggesting a possible influence of this biological control agent.

Although limited statistically significant associations were detected in this study, a pronounced increase in the abundance of *Trichoderma* was found in treatments with inoculation, especially T2 with a prevalence of 5.47% compared to the control treatment, 1.06%. According to Kaul et al. (2021), microorganisms introduced into the agroecosystem modify the soil microbiome in a targeted manner. This statement is corroborated by studies developed by Fu et al. (2020) and Hang et al. (2022), who investigated the inoculation of fungi of the genus *Trichoderma*.

Based on metagenomic analyzes focused on genes associated with cellular metabolism using the UPGMA

Table 2. Diversity of fungi and oomycetes for the main genera of agronomic importance in different soil samples: Before (BC) and after (T1 to T5) cultivation with chia, in the % of reading sequence of DNA per gram of soil.

Agronomic Importance	Genus	Abundance (%)					
		BC	T1	T2	T3	T4	T5
Antagonist	*Beauveria*	0.061	0	0.03	0.05	0.075	0.117
	Isaria	0.546	0.212	0.239	0.251	0.375	0.351
	Metarhizium	0.061	0	0.06	0.075	0.075	0.117
	Paraphaeosphaeria	0	0.085	0.06	0.025	0	0.117
	Pochonia	0.061	0	0	0.126	0	0
	Trichoderma	1.96	1.06	5.466	2.21	2.327	1.521
Phytopathogenic	*Alternaria*	0.02	0	0	0.025	0	0
	Aspergillus	6.607	0.678	2.061	4.496	5.555	4.389
	Bipolaris	0.02	0.085	0.03	0.126	0.075	0.117
	Cercospora	0	0.042	0.03	0.025	0	0
	Colletotrichum	0.323	0	0.06	0.176	0.075	0.351
	Corynespora	0.283	0.127	0.179	0.276	0.45	0.234
	Diaporthe	0.141	0.085	0.06	0.05	0.15	0.234
	Diplodia	0.081	0	0.09	0.251	0.15	0.234
	Exserohilum	0.02	0	0.03	0	0	0
	Fusarium	8.183	0.127	0.179	0.527	0.601	0.468
	Macrophomina	3.475	2.67	2.18	2.738	4.054	2.399
	Neurospora	0	0.042	0	0.05	0.15	0.059
	Nigrospora	0.02	0.297	0.03	0.151	0.225	0.176
	Penicillium	0.101	0.042	0.03	0.151	0	0.117
	Peronospora	0.242	1.865	1.344	1.658	2.552	1.053
	Phialophora	0.081	0	0.03	0	0	0
	Phomopsis	0.182	0.89	0.657	0.703	1.351	0.819
	Pseudocercospora	0.04	0.042	0	0.1	0.075	0.117
	Pyrenochaeta	0	0.042	0.09	0.05	0	0
	Pyrenophora	0.02	0	0	0	0	0.059
	Pythium	0.242	4.45	3.047	3.793	4.955	4.096
	Rhizoctonia	2.445	5.128	4.869	5.576	6.531	3.511
	Sclerotinia	0	0	0.06	0.025	0	0
	Sclerotium	1.273	2.67	2.27	2.914	4.354	2.224
	Septoria	0.222	0.085	0.03	0.05	0.15	0.176
	Ustilago	0.02	0	0.09	0.025	0.075	0
	Verticillium	0.141	0	0.09	0.1	0.3	0.059
	Zymoseptoria	0.04	0	0	0.075	0.075	0
	Phytophthora	0.222	0	0.179	0.377	0.3	0.41

method (Figure 3), greater genetic similarity was found between T3, T4 and T5 in relation to treatments T1 (control) and T2. These results suggest the existence of high genetic divergence of T1 (control) in relation to the other treatment samples after cultivation with *Salvia hispanica*, suggesting potential implications for the vegetative growth of the plant.

When applying principal component analysis (PCA) with the metagenomic data of the arylsulfatase enzymes (AS), beta-glucosidase (BG) and alkaline phosphatase (FA), which act as bioindicators and are crucial in evaluating soil health with the chemical parameters of soil samples, it was verified a wide dispersion of treatments in the

Table 3. Diversity analysis of *Trichoderma* spp. (% of sequence of DNA/g of soil) in different soil samples before and after inoculation with *T. harzianuam*: BC: Before cultivation; T1: control; T2: 2.5 µL. g^{-1} seed; T3: 5.0 µL. g^{-1} seed; 10.0 µL. g^{-1} seed; 20.0 µL. g^{-1} seed.

Species	% of *Trichoderma* spp. sequences per gram of soil					
	BC	T1 (controle)	T2	T3	T4	T5
T. asperellum	11.4	7.0	18.4	19.3	21.6	18.8
T. harzianum	11.8	11.3	17.6	19.4	17.0	14.6
T. virens	17.6	23.9	21.2	23.3	22.7	20.8
T. reesei	26.3	26.8	28.2	25.6	27.3	27.1
Others	32.9	31.0	14.6	12.4	11.4	18.8
Trichoderma spp.	1.96	1.06	5.47	2.21	2.33	1.52

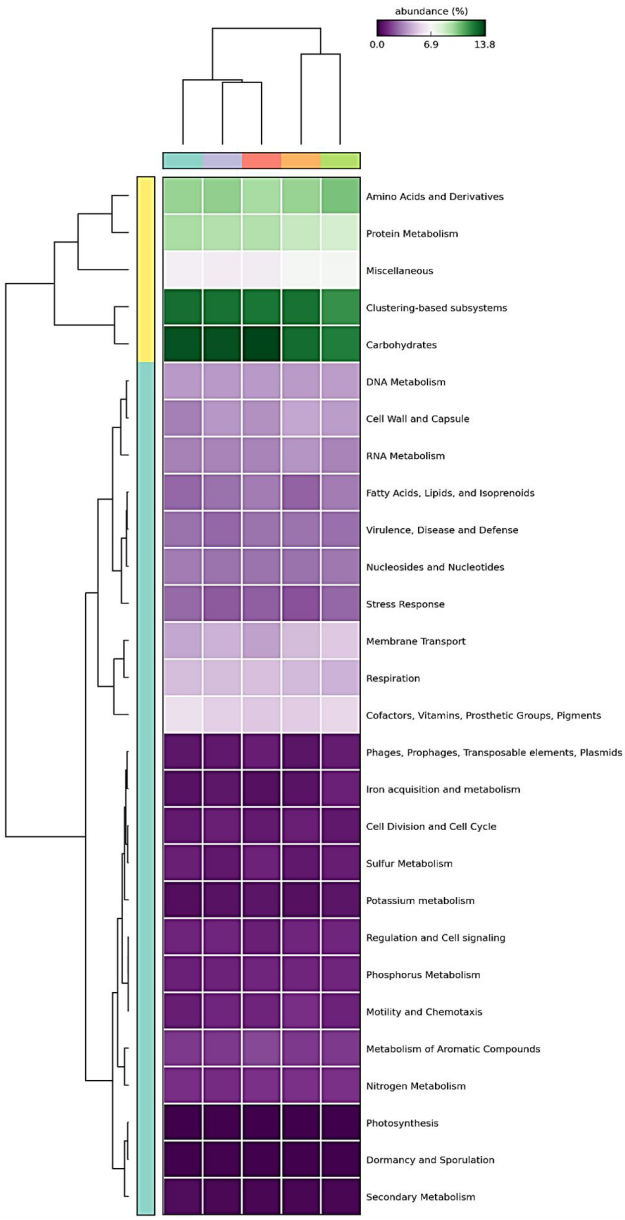

Figure 3. UPGMA analysis for genes related to metabolic pathways of metagenomes from soil samples using the Stamp program.

graph (Figure 4a). The variables Ca, S and MO content were identified as the main contributions to this variation (Figure 4b).

In relation to the biplot graph, the distribution of treatments demonstrated distinct relations with the variables studied. The samples from treatment T5 were distinctively characterized by high levels of Ca, Mg, K and P, in addition to presenting a high number of copies of metagenomic DNA associated with the arylsulfatase enzyme (Figure 4a), a key enzyme in sulfur metabolism (Tabatabai and Bremner, 1970; Ganeshamurthy and Nielsen, 1990). Treatments T1 (control) and T4, which are close together in the biplot graphical dispersion, were predominantly associated with high iron contents, and showed weaker correlations with the levels of copper, boron, organic matter, and sulfur. Treatments T2 and T3 exhibited weak associations with the alkaline phosphatase and beta-glucosidase enzymes, which are involved in hydrolysis reactions of organic phosphorus (Nannipieri et al., 2011) and glycosidic bonds (Bhatia et al., 2002), respectively. It is interesting to note that these enzymes did not show a direct association with the nutrients analyzed. This suggests the complexity and multifunctionality of the soil microbiome, where certain enzymatic functions can be influenced by factors beyond traditionally measured nutrients, reinforcing the need for more in-depth studies to understand their interactions and impacts on the edaphic ecosystem.

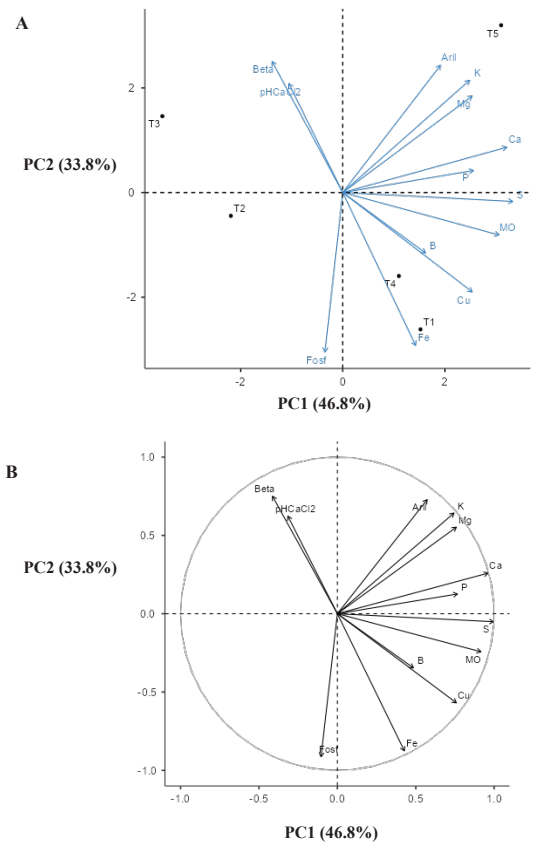

Figure 4. Main components of metagenomic analysis for the enzymes arylsulfatase (AS), beta-glucosidase (BG) and alkaline phosphatase (FA) and soil chemical parameters. A: Graphical dispersion of scores in relation to the first two main components (PC1 and PC2) resulting from the condensation of abundance data for 67 genera of agronomic importance; B: Relative contribution of the variables analyzed based on the first two main components (PC1 and PC2).

In the joint analyzes of the results of metagenomics and soil chemistry, it is possible to suggest that there was a differential modification of the environment with the cultivation of chia (*Salvia hispanica*). Several studies reveal that microbial diversity is associated with soil multifunctionality (Wagg et al., 2014; Li et al., 2019). However, this diversity does not in itself guarantee edaphic functionality (Shade, 2017). According to Zhao et al. (2020), the soil microbiome plays a crucial role in crop production and drives nutrient cycling. Soil interventions such as fertilization can lead to changes in the microbial community. Notably, microbial predators, when affecting the genetic variability of others, have substantial influence on the dynamics and functioning of soil. Through the metagenomic analysis for *Trichoderma* of soil samples collected before (AC) and after cultivation with chia, without seed inoculation with *T. harzianum* (T1), it was found that the predominant native species *T. asperellum*, *T. harzianum*, *T. virens* and *T. reesei*, represented respectively 67.1% and 69% of the total sequences per gram of soil for this microbial predator (Table 3). With the inoculation of seeds with *T. harzianum*, there was an increase in the contribution of the predominant species to 85.4% in T2, 87.6% in T3, 88.6% in T4 and 81.2% in T5. Thus, although there was no association detected between doses of the inoculant applied to the seeds and the abundance of species of this fungus present in the soil, significant correlations were found between *T. asperellum* (r = -0.96 p-value = 0.009) and *T. harzianum* (r = - 0.96 p-value = 0.02) with the contribution of other *Trichoderma* species. These results make it possible to suggest that the increase in *T. asperellum* and *T. harzianum* populations influenced reducing the contribution of other *Trichoderma* species.

The *Trichoderma* species, which include *T. asperellum* and *T. harzianum*, have the ability to cooperate with other beneficial soil microorganisms, thus promoting modification of the soil microbiome, which can result in growth promotion (Stewart and Hill, 2014; Fu et al., 2020; Hang et al., 2022) and to induce systemic plant resistance to diseases (De Meyer et al., 1998; Sabbagh et al., 2017; Ilham et al., 2019). In relation to the present study, a substantial increase in the root biomass of chia plants (Figure 2B) and in the populations of *T. asperellum* and *T. harzianum* (Table 3) was detected in the samples that received inoculant in relation to the control treatment (T1). Metagenomic analyzes revealed the significant presence of the phytopathogenic genera *Macrophomina*, *Rhizoctonia* and *Sclerotinia* in soil samples (Table 2). Despite this, no disease symptoms were observed in plants, although such pathogens have been associated with infection in chia (El-Kaed et al., 2021). In this way, it is plausible to state that the presence of *Trichoderma* species, particularly *T. asperellum* and *T. harzianum*, had a significant contribution to the

dynamics of the soil microbiome, and favored plant growth during the vegetative stage.

4. Conclusions

The inoculation of the *T. harzianum* strain into chia seeds promotes an increase in the number of branches per plant, stem diameter, root dry mass and the number of days to flowering. Besides, there is a change in soil microbial diversity after chia cultivation, and the soil in which plants were grown from seeds inoculated with *T. harzianum* result in a greater number of genera and species in relation to the control (T1). Metagenomic analysis reveals that, regardless of the sample analyzed, the soil is made up of beneficial and phytopathogenic microorganisms. Based on the results of the multivariate analysis of taxonomic data and genes related to metagenome metabolic pathways, it is possible to suggest that seeds inoculated with higher doses of *T. harzianum* (T3 to T5) result in the formation of a soil microbial community with greater genetic similarity than those of T1 (control) and T2 (lowest dose). Besides, the inoculation with a higher dose of *T. harzianum* resulted in a higher content of Ca, Mg, K, P, and in the number of soil metagenomic DNA copies for the arylsulfase enzyme. *T. asperellum*, *T. harzianum*, *T. virens* and *T. reesei* were the Trichoderma species in the soil sample, having occurred after the cultivation of plants that received inoculum, an increase in the abundance of *T. asperellum* and *T. harzianum* and a reduction in less relevant native species present in the soil. Finally, it is concluded that the community of Trichoderma species in the soil, especially *T. asperellum* and *T. harzianum*, has a significant contribution to the dynamics of the soil microbiome, and to the growth of chia plants during the vegetative stage.

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