Effects of conservation agriculture and bioinoculation on the microbiota of a soil cultivated with maize

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ABSTRACT

Objective: To evaluate the influence of conservation agriculture practices and bioinoculation on the soil microbiota in maize cultivation systems from the Frailesca region, Chiapas, Mexico, using 16S rRNA gene amplicon sequencing.

Design/methodology/approach: Microbial diversity was characterized in soils cultivated with maize white grain (*Zea mays* L.) under the influence of conservation agriculture (CA) and seed bioinoculation. Two experimental fields (A and B) were selected, each divided into two plots (P). Field A contained plot P1 (control) and P2 (CA management), while field B contained plots P3 (seed bioinoculation) and P4 (CA + bioinoculation). Soil samples were collected four months into the crop cycle to characterize microbial communities using 16S rRNA gene amplicon metabarcoding.

Results: The results showed changes in the microbial composition of the soil. The beta diversity analysis (PCoA-Bray-Curtis) showed differences in the microbial communities between the plots, where a separation of P4 (CA + bioinoculation) from P1, P2 and P3 was observed. The soil microbiota was dominated by the genera *Candidatus Koribacter* (P3>P4>P2>P1), *Gemmatimonas* (P4>P1>P2>P3) and *Candidatus Solibacter* (P4>P1>P3>P2), which are characteristic of the soil, with the ability to metabolize a wide range of simple and complex carbohydrates.

Limitations on study/implications: This study was conducted in a specific region and agricultural cycle; future research could consider seasonal variations and other crops to broaden our understanding of these microbial patterns.

Findings/conclusions: These results suggest that the combination of conservation agriculture and bioinoculation can promote specialized microbial communities, contributing to the maintenance of relevant ecological functions in agricultural soils.

Keywords: Biofertilizer; microbial diversity; regenerative agriculture.

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INTRODUCTION

A vast area of the Mexican state of Chiapas is used for the cultivation of corn (56% of the planted area). In the Frailesca region, 16% of the total production of Chiapas is reported,

while the municipality of Villaflores contributes with 40% of the region's production (SIAP, 2023). However, the intensive use of agrochemicals (particularly nitrochemicals) for the cultivation of corn has caused a significant loss of soil fertility. This has led to a growing interest in agroecological methods, such as conservation agriculture (CA) (minimal movement of the soil, retention of residues from the previous crop on the surface of the land, and rotation / diversification of crops) and the bioinoculation of corn seeds with Azospirillum and mycorrhizae (Rodríguez Larramendi *et al.*, 2017; Goddard *et al.*, 2022; Mutuku, Kassam and Mkomwa, 2020).

There is evidence that CA and bioinoculation are agroecological techniques that can improve crop yields, although little is known on their effect over soil microbial communities. While there are no studies regarding this specific subject in Chiapas, López Báez *et al.* (2019) evaluated the physical-chemical characteristics of soils cultivated with corn in Villaflores and concluded that it is necessary to increase the quality of these soils. To achieve this quality increase, it is first required to study the soil microbiota and to determine how it is affected by different agroecological techniques.

Soil microbes play important roles in biogeochemical cycles (Challacombre *et al.*, 2011; Emmerling *et al.*, 2002) such as the recycling of nutrients, the degradation of organic matter (Ye *et al.*, 2016) and xenobiotic compounds (Tejeda-Agredano *et al.*, 2013), carbon sequestration (Lian *et al.*, 2017), and prevention of diseases in crops (Tao *et al.*, 2015; Lim *et al.*, 2013). Moreover, agricultural practices influence soil microbial diversity, thus proper agricultural management could promote the settling of microbial communities that improve soil quality and increase plant productivity (Hartmann *et al.*, 2015; Liao *et al.*, 2019; Shrestha *et al.*, 2020).

The objective of this study was to evaluate the influence of conservation agriculture practices and bioinoculation on the soil microbiota in maize cultivation systems from the Frailesca region, Chiapas, Mexico, using 16S rRNA gene amplicon sequencing.

MATERIALS AND METHODS

Description of the study area and soil sampling

The experimental fields were established on Frailesca region at the southeast of the state of Chiapas, Mexico at the common land Las Graditas (MasAgro module), located in Ejido Calzada Larga, in the Villaflores municipality (16° 20' 30.46453" N and 93° 19' 4.02390" W) (Figure 1). The predominant soil is luvisol, with acidic conditions (pH 4.9-6.6).

Two experimental fields (A and B) were established and divided into plots (P), where white grain maize (*Zea mays* L., PIONEER4082) was grown under different conditions: Field A: P1, control; P2, conservation agriculture (CA). Field B: P3, seed bioinoculation; P4, CA + seed bioinoculation.

Inoculation consisted of applying biofertilizers based on Azospirillum and commercial arbuscular mycorrhizal fungi (Azofer® + Mycorrhizafer®) to the seeds prior to sowing following the manufacturer's instructions.

After four months of cultivation, soil samples were collected at a depth of 10-15 cm (the region closest to the plant roots) using a soil core sampler (5 cm in diameter). For each plot, soil samples were collected at five subsites, one at each corner and one in the center,

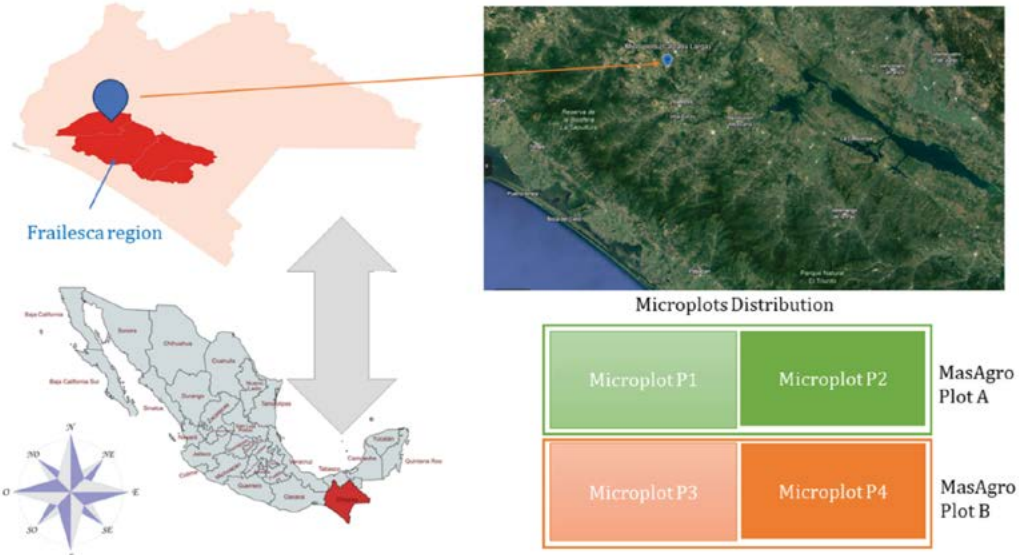

Figure 1. Location of the sampling area. Map prepared with picture Google Earth app.

to generate a composite sample for each plot (n=4). The samples were placed in sterile 50 ml tubes (Falcon®) and stored at 4 °C until processing at the Ecogenomics Laboratory (UNAM-Yucatán).

Characterization of microbial communities: 16S rRNA gene amplicon sequencing

To characterize microbial communities, total DNA was extracted using the methodology described by Rojas-Herrera *et al.* (2008) with some modifications, such as the addition of lysozyme without prior washing. The obtained DNA was used to amplify the V3-V4 regions of 16S rRNA gene using the primers S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and R: S-D-Bact-0785-a-A-21 (5´-GACTACHVGGGTATCTA ATCC-3´). The PCR conditions were carried out according to reported by Klindworth *et al.* (2013). The DNA quality was verified by agarose gel (1%) electrophoresis and quantified with a fluorometer (Quantus™ Promega). The PCR products were purified and paired-end sequenced in an Illumina MiSeq platform (2x250) (Illumina, San Diego, CA, USA) at CINVESTAV-Mérida.

16S rRNA gene sequence analyses

The bioinformatic analysis was performed in the MG-RAST platform. Low-quality sequences were removed to achieve a 99% accurate identification.

The diversity of each plot was analyzed in terms of alpha and beta diversity. Alpha diversity was assessed in terms of species richness (S) and the Margalef index, and diversity structure was evaluated using equity indices (Shannon-Wiener index (H) and Pielou index (J) and dominance indices (Simpson index (Λ), and Simpson-based diversity index (1-λ). Beta diversity was evaluated using the Bray-Curtis index.

Statistical analyses

The statistical and diversity analyses were carried out using the ecological statistics program PRIMER-e v7 (Plymouth Routines in Multivariate Ecological Research, version 7). The beta diversity analysis was performed using a principal coordinate analysis (PCO) and a CLUSTER hierarchical analysis (Group average and LINKTREE) (Marti, Gorley y Clarke 2008; Clarke, Somerfield y Gorley 2008). The SIMPROF (similarity profile, which consisted of 999 permutations at 95% confidence) and SIMPER (percentage of similarity and dissimilarity of one way, with only higher-contribution variables cut-off 70% at 95% confidence) analyses were conducted to assess the richness and abundance of each Operational Taxonomic Unit (OTU). Both statistical tests are based on Bray-Curtis dissimilarity metrics.

RESULTS AND DISCUSSION

Microbial diversity

A total of 207,329 16S rRNA gene reads were obtained after quality checks and were grouped in 2,004 OTUs: 481 in P1, 511 in P2, 471 in P3 and 541 in P4. The alpha diversity indicators revealed slight variations among the plots (P1-P4). Species richness (S) ranged from 194 to 227, with the highest value observed in P4, indicating a greater number of taxa in this plot (Table 1). The OTU distribution (N) was highest in P4 (11,021), suggesting a larger community size. The dominance indices (D and Λ) were relatively low (0.097-0.12), while the Simpson's diversity index (1-λ) remained high (0.87-0.90), reflecting a balanced community structure with no strong dominance of a few taxa. Shannon's index (H′) showed similar values across treatments (3.06-3.20). The Pielou's evenness (J′) ranged from 0.58 to 0.60, this index ranges from 0 to 1, with 0 representing an absence of evenness in abundances and 1 indicating that all species (in this case, genera) are equally abundant (Pumasupa Banda *et al.*, 2021), suggesting moderate evenness in species distribution. Considering all indices together, the combination of conservation agriculture and bioinoculation (P4) exhibited the highest richness and total abundance, along with the highest diversity, indicating a slightly more diverse and evenly distributed community (Table 1).

The remaining indices demonstrated a high degree of uniformity in the distribution of species within a community (Table 1). Some authors suggest that AC such as crop rotation and the utilization of biofertilizers influenced the microbial biomass, rather than the composition or richness of the communities (Song *et al.*, 2022). An equity index must

Table 1. Microplot diversity indices.

Microplots	**S**	**N**	**D**	**J′**	**H′**	**Λ**	**1-λ**
P1	194	8389	21.36	0.60	3.19	0.10	0.89
P2	217	7179	24.32	0.58	3.16	0.11	0.88
P3	194	7855	21.51	0.58	3.06	0.12	0.87
P4	227	11021	24.28	0.59	3.20	0.097	0.90

S: Total OTUs; N: Total reads; D: Margalef index; J′: Pielou uniformity index; J':Shannon-Wiener (log) index; Λ: Simpson dominance index, 1-λ: Simpson-based diversity index.

be independent of richness. The sensitivity of each index is of great importance in the evaluation of changes in the environment. Among the equity indices evaluated here, the most sensitive is Pielou (Barona-Narváez , 2021).

More than 50% of the dominance was attributed to the first four bacterial genera, including the unidentified genera, as illustrated in Figure 2. Additionally, the plot reveals that the representative genera of each microplot are comprised of the first 20 OTUs, which can be seen on the horizontal axis of Figure 2.

Beta diversity

By performing the principal coordinates analysis, the Beta diversity of the samples taken from the four microplots was characterized. The first important result is that two new variables or components (PCO1 and PCO2) were formed, which were able to explain 88.5% of the total variance. In this case, PCO1 made the largest contribution with 56.8% of the total variance (Figure 3). Beta diversity analysis showed a similarity of 74% between microplots, with P4 being the most distant (Figure 3).

Microbial communities composition

The dominant phyla were Acidobacteria, Actinobacteria, Proteobacteria, Verrucomicrobia, and Firmicutes, along with a significant proportion of unclassified bacteria that varied between microplots. Jeanbille *et al.* (2016) reported that Acidobacteria

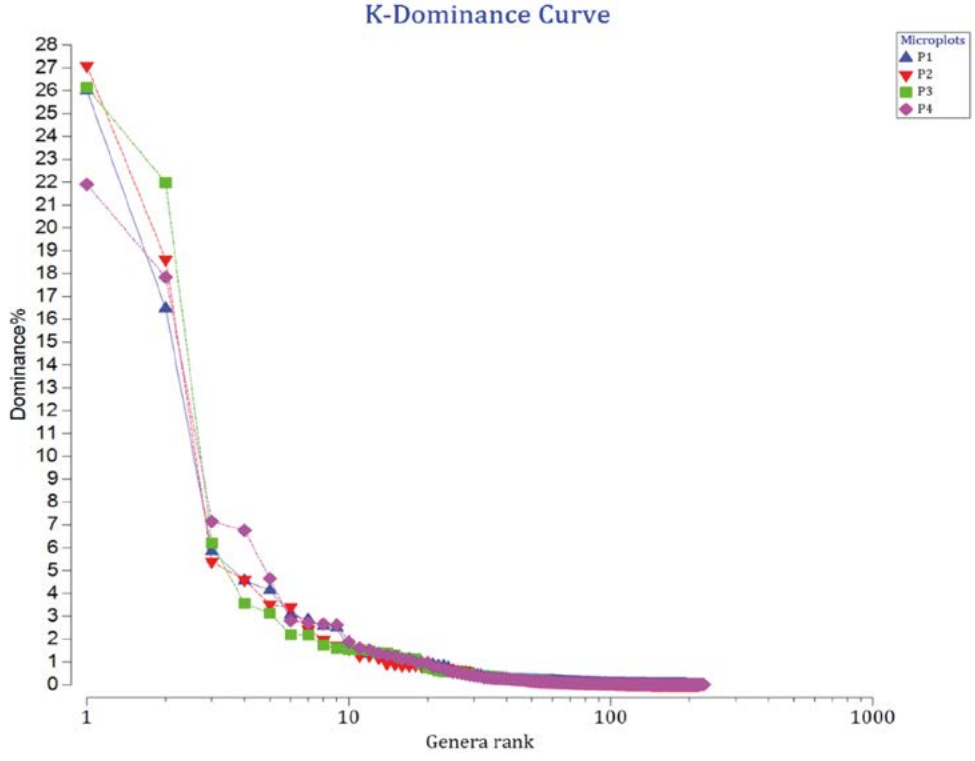

Figure 2. Dominance curve of the genera identified in each plot. P1=Witness, P2=First year of implementing Conservation Agriculture (CA) practices, P3=Bioinoculation in seed (by six year) and stop CA practices (it was done for five years) and P4=CA with biofertilizer (both for six years).

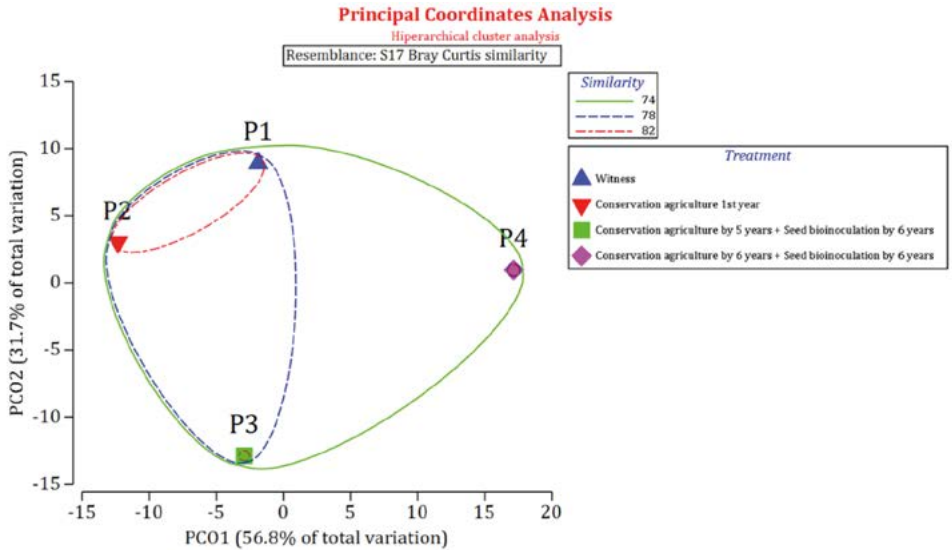

Figure 3. Principal coordinate analysis of the results of the microbiota in soils. The colored lines represent similarity percentages of diversity (based on Bray-Curtis). The figures and colors represent the treatments of the soils.

and Proteobacteria are more abundant in acidic and nutrient-poor soils, characteristics of our study area. Wang *et al.* (2022) concluded that Actinobacteria and Proteobacteria are the dominant phyla in subtropical soils, usually acidic. This is further evidence that the pH of a soil can entail an important effect on the structure of soil microbial communities. Rivera-Rivera and Cuevas (2020) reported that the dominant bacteria in a tropical soil were Proteobacteria and Actinobacteria, followed by Bacteroidetes, only to change during the dry season, when the presence of Actinobacteria increased significantly, while that of Proteobacteria and Bacteroidetes decreased.

The SIMPER analysis at the taxonomic family level revealed a 76.3% similarity between plots P1 and P3 (without CA practices) and a similarity of 67.5% between P2 and P4 (with CA practices). There was a dissimilarity of 24.0% between CA and non-CA plots.

The bacterial families with the highest abundance in CA plots included Solibacter, Verrucomicrobia sub 3, Bacillaceae, Acidobacteriaceae, Planctomycetaceae, Thermomonosporaceae, Clostridiaceae, Nitrospiraceae, and Ktedonobacteriaceae. Non-CA plots showed higher abundance of Pseudomonadaceae, Conexibacteraceae, Nitrosomonadales (unclassified), and Nocardioidaceae

The relative abundance of the dominant bacterial genera varied among the four microplots (Figure 4). *Candidatus Koribacter* was the most abundant taxon across all samples, ranging from 16.47% in P1 to 26.15% in P3. On the other hand, *Candidatus Solibacter* was the second most abundant genus, reaching its highest value in P4 (6.76%), while lower proportions were observed in the other plots (3.40-4.14%). Both genera belong to the phylum Acidobacteria, indicating that this group dominated the microbial communities across all sites. This phylum is abundant in soil samples (Paz and Menjivar, 2019). Candidatus Koribacter is a carbon-monoxide oxidizing bacterium, and C. Solibacter can degrade complex compounds such as hemicellulose, cellulose, and others. Both species

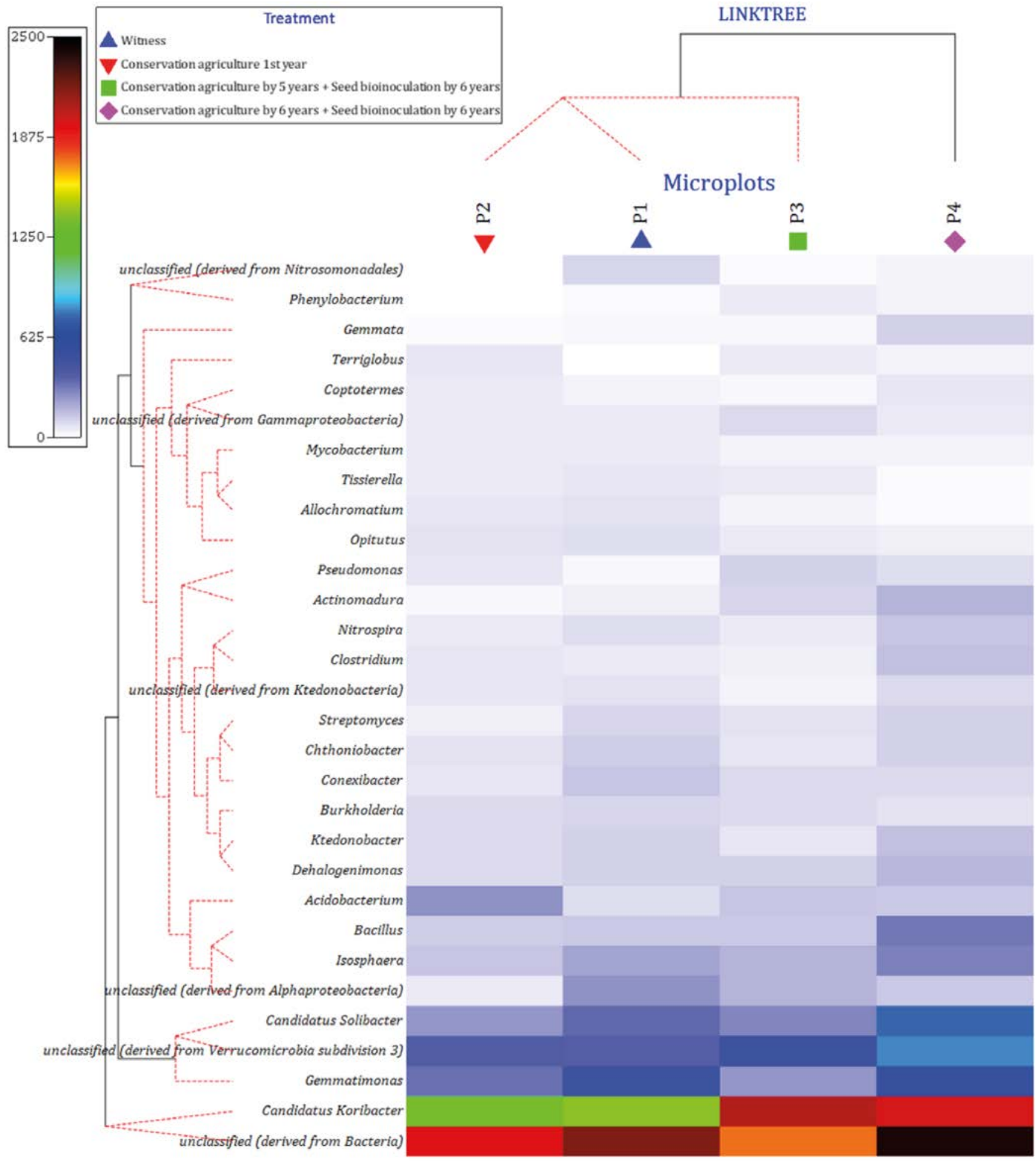

Figure 4. Shade plot of the samples (columns) according to the genera identified (30 most abundant). The depth of the spectrum shading is linearly proportional to an abundance of reads. The dendrograms correspond to the groupings by similarity of abundances of the biotic variables according to the CLUSTER LINKTREE analysis. Red lines correspond to the statistical grouping according to the SIMPROF analysis.

account with resistance potential against desiccation (Challacombre *et al.*, 2011), which could significantly influence the structure of microbial soil communities. Microplots P3 and P4 are where there is the greatest abundance of them, and they are where AC practices have been applied for the longest time.

Among the remaining taxa Verrucomicrobia-bacterium showed a gradual increase from P1 (4.55%) to a maximum in P4 (7.15%), whereas the *Bacillus* genus reached its highest abundance in P4 (2.80%), highlighting it as another prominent taxon in this plot. Regarding *Gemmatimonas* genus, displayed high relative abundance in P1 (5.85%) and P4 (4.65%), while *Isosphaera* reached its maximum in P4 (2.66%) (Figure 5). The *Gemmatimonas* genus is related to the reduction of N_2O, a compound that is stored in soils (Oshiki *et al.*, 2022). Moreover, *Isosphaera* is usually found in aquatic habitats and wastewater. However, some studies have reported both genera in soils and compost leachates (Wang *et al.*, 2002).

Other genera, including *Acidobacterium*, *Burkholderia*, *Conexibacter*, *Dehalogenimonas*, and *Ktedonobacter*, were present at lower abundances (<2-3%), showing no clear pattern across plots. In turn the genus *Streptomyces* remained relatively stable (~1%) across all plots (Figure 5).

Overall, microplot P4 was characterized by higher relative abundances of *Candidatus Solibacter*, *Verrucomicrobia*-bacterium, *Bacillus*, and *Isosphaera*, suggesting microenvironmental or management conditions that may specifically favor these taxa and distinguish P4 from the other plots in terms of microbial community composition (Figure 5).

Unique and Shared Soil Microbial Phylotypes Among Agricultural Treatments

A Venn diagram showed the distribution of unique phylotypes among the four plots (P1, P2, P3, and P4) (Figure 6). Plot P4 exhibited the highest number of exclusive phylotypes (56; 15%), followed by P2 with 47 (12%), P3 with 36 (9%), and P1 with 35 (9%). In addition, 92 phylotypes were shared among all four plots (24%), indicating the presence of a common core microbiota, along with a differentiated fraction associated with each management

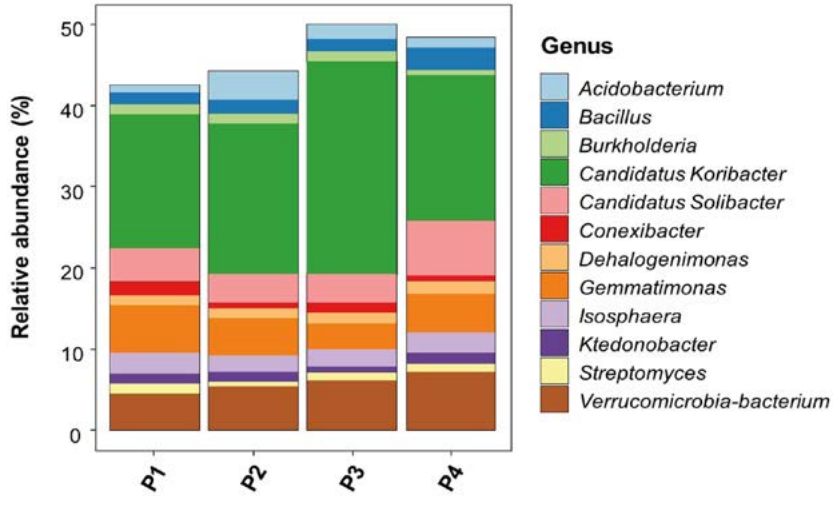

Figure 5. Relative abundance of dominant bacterial genera across microplots.

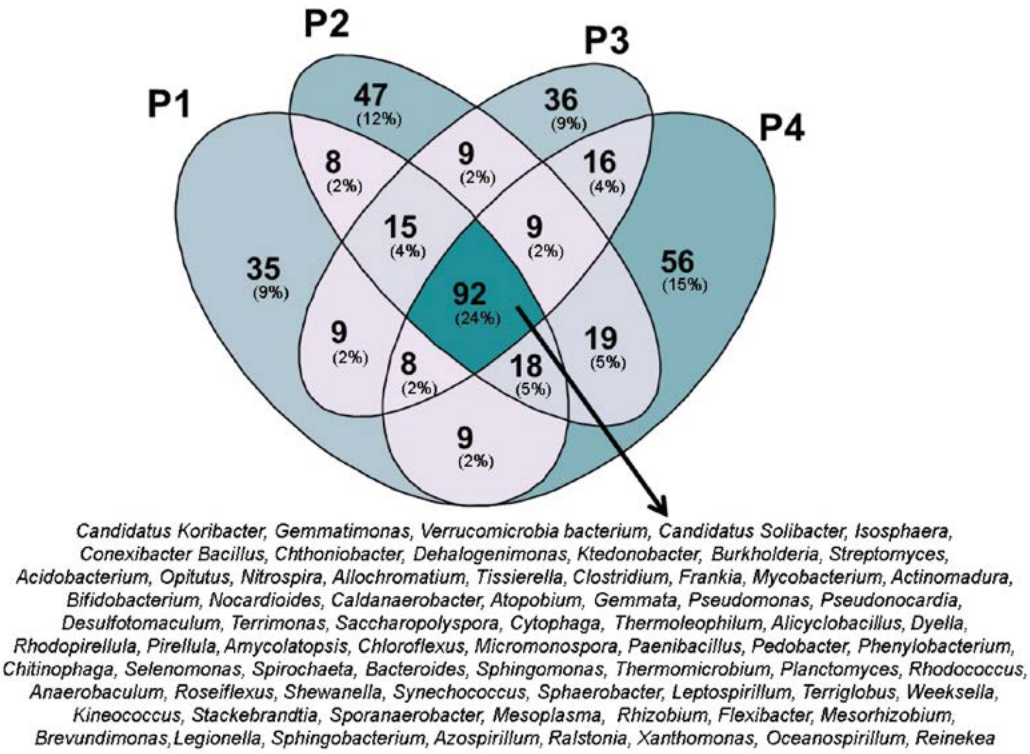

Figure 6. Distribution of unique and shared phylotypes among soil samples from plots. P1, P2, P3, and P4.

practice (Figure 6). These findings suggest that agricultural practices, particularly conservation agriculture and bioinoculation, influence the uniqueness and composition of soil microbial communities.

The shared bacterial genera represent the set of microbial taxa that remain stable and persistent across different environmental conditions or agricultural practices. These microorganisms are associated with the maintenance of the basal functioning of soil, acting as key components of microbial stability and resilience (Custer *et al.*, 2022). Their consistent presence suggests their involvement in essential microbial processes, such as nutrient cycling, organic matter decomposition, and the regulation of plant-microbe interactions. In this context, the core microbiota constitutes the functional backbone of the soil ecosystem, ensuring the stability of biogeochemical processes even under changes in agricultural management (Custer *et al.*, 2023).

Some bacterial genera shared among the four plots are related to plant growth promoting activities. Such is the case of the genera *Bacillus*, *Burkholderia*, and *Pseudomonas*, include some opportunistic pathogenic species of great importance to public health, species with bioremediation potential, and species that have been used as growth promoters (Hashem *et al.*, 2019; Madigan *et al.*, 2015). Regarding *Azospirillum* genus is a cosmopolitan genus found in soil samples and associated with roots in tropical, subtropical, and temperate regions. Some species of this genus have been used as bioinculants due to their plant growth-promoting capabilities, such as nitrogen fixation, hormone production, phosphorus solubilization, and siderophore production (Pedraza *et al.*, 2020).

Another bacterial genera with agricultural importance identified in this study included *Frankia*, *Paenibacillus*, *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Azorhizobium*, *Clostridium* and *Pseudomonas*, among others (De Souza *et al.*, 2015; Madigan *et al.*, 2015), corresponded to bacterial genera with potential as biofertilizers, which are widely used in agriculture. It has been shown that the use of conservation agriculture techniques can have a beneficial effect on soil microbial activity, thereby increasing the diversity of soil microbial populations. The combination of bioinoculation practices with conservation agriculture can have an even more beneficial effect, although proper selection of micro-organisms is required to achieve the expected effects. However, further research is needed to determine the stability of the soil microbiota in relation to the practices used, the microorganisms used as bioinoculants and the timing of their application to the soil.

CONCLUSIONS

The combination of conservation agriculture and bioinoculation appears to promote a more diverse and balanced soil microbial community, as evidenced by both alpha and beta diversity patterns. This suggests that management practices can play a key role in shaping microbial assemblages, potentially enhancing soil ecosystem functionality and resilience. These results highlight the potential of integrating bioinoculants with sustainable agricultural practices to support soil health.

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