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Article

Characterization of the Diversity of Microbial Communities in the Rhizosphere of *Solanum betaceum* in Response to Inoculation with Microbial Consortia Under Cultivation Condition

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ABSTRACT

Bioinoculation with native or introduced microorganisms is a promising alternative to using synthetic chemicals in agriculture, although its impact on the microbial communities of the rhizosphere is still poorly understood. This study analyzed the microbial community of the rhizosphere of *Solanum betaceum* (tree tomato) over 120 days, using a metagenomic approach to evaluate microbial diversity and the persistence of *Trichoderma spp.* through PCR. The predominant phyla identified were *Pseudomonadota* and *Actinomycetota*, and the most abundant genera were *Streptomyces*, *Nocardioides*, *Bradyrhizobium*, *Sphingomonas*, *Rhodanobacter*, and *Micromonospora*. While the microbial composition changed over time, diversity indices did not show significant differences before and after inoculation, and the establishment of *Trichoderma spp.* in the rhizosphere was not observed. These findings provide insight into the complex interactions between residents and introduced microorganisms, offering a basis for future studies. The results suggest that frequent inoculation alone was insufficient to change the diversity of rhizosphere microbial communities noticeably. It is recommended that research be expanded to other geographical conditions to deepen the understanding of the microbial profile of the *S. betaceum* rhizosphere and its application in different crops.

Keywords: Bioinoculation, rhizosphere, microbial communities, *Solanum betaceum*, metagenomics.

INTRODUCTION

The rhizosphere is the soil region that surrounds plant roots, and it is home to a wide variety of microorganisms and invertebrates. It is considered one of the most significant microbial hotspots determining nutrient dynamics and cycling; therefore, plant-microbe interactions are highly intense in this area [1]. In recent years, the relationship between rhizosphere microbial communities and plants has been extensively studied for biocontrol purposes, plant development, and the analysis of their influence on biogeochemical cycles. Therefore, it is essential to understand their diversity better so that beneficial microorganisms can be optimally used in plant growth, biocontrol, and bioinoculants [2,3].

In the face of the challenges posed by the conventional model of agricultural intensification, which severely impacts the environment and biodiversity due to its association with the use of synthetic chemicals, the use of native or introduced microorganisms represents an alternative that can be applied as a biological inoculant (bioinoculants). Furthermore, it can enhance microbial communities, constituting a strategy to harness the beneficial effects of certain groups of microorganisms on crops, which can substantially contribute to increasing agricultural productivity, resilience to global change, profitability, and sustainability while significantly reducing chemical inputs. However, its consistent replicability is often complex, as its effectiveness is linked to the specific edaphoclimatic conditions of each region [4,5].

Although bioinoculants are used to improve crop productivity, there is still limited information regarding their effects on the microbial communities in the rhizosphere of crops, and there is no report on the monitoring of the inoculated microorganisms during their establishment and/or post-inoculation development. This justifies the need to develop characterization studies of complex bioinoculants under local field conditions in specific cropping systems to initiate the development of processes that optimize the biological potential of agricultural soils for the sustainable intensification of agriculture in Ecuador.

Regarding crops in Ecuador, the Andean region is the native home to many little-studied fruits with high potential for commercial development, such as the tree tomato (*Solanum betaceum*), internationally known as tamarillo. In addition to its significant nutritional value, it is considered an alternative for the production, diversification, and marketing of non-traditional products [6,7]. Some research has been carried out on this species and its cultivation [8–12]; however, information regarding the dynamics and characterization of soil microbial communities is scarce, neglecting, in agronomic terms, its usefulness for the development of specific bioinoculants for *S. betaceum*, which is considered of great interest for production and commercialization in the national and international markets.

Based on this background, a field characterization was proposed to study the changes produced in the microbial communities of the *S. betaceum* rhizosphere in response to the enrichment of the native soil microbiota with microbial consortia, using metagenomics. The objective was to determine whether inoculation with beneficial microorganisms induced changes in the diversity of the resident microbial communities in the *S. betaceum* rhizosphere. Additionally, the persistence of *Trichoderma spp*. was estimated by specific PCR, as it was introduced as part of the microbial consortium in the crop's rhizosphere under field conditions. The information provided could be considered as a basis for designing integrated nutrient management strategies that would enhance soil nutrient acquisition, which, along with other factors (such as the irrigation system or fertilizer application), could be used to establish specific strategies for a given crop.

MATERIAL AND METHODS

Study Site. The *S. betaceum* crop was located at the "El Romeral" farm of the University of Cuenca, situated at km 10 of the Paute–Guachapala road (2°45'50" N, 78°42'51" E). The farm is located within the Paute River sub-basin, has a subtropical climate, and an ecological formation of dry forest – Lower Montane (bs. MB). It is 2200 meters above sea level, with an annual precipitation ranging from 500 to 1000 mm, and the yearly

average temperature fluctuates between 15°C and 18°C. The plot's soil where the experimental plants were grown had a loamy-sandy texture with a pH of 6.3, and organic and chemical fertilization was received during its development and irrigated by an automated drip irrigation system.

Inoculum Preparation. The fungi *Beauveria bassiana, Paecilomyces spp*., and *Trichoderma spp*. were selected, and suspension mixtures (microbial consortia) were prepared following the manufacturer's recommendations: 105 mL per hectare of each product.

Experimental Design and Soil Sample Collection

Soil samples were collected in October 2019 during the productive phase of a homogeneous *Solanum betaceum* plantation. A completely randomized experimental design was used, selecting experimental units consisting of three consecutive plants with similar characteristics within the same crop row. The study evaluated a single treatment involving the monthly application of a consortium of beneficial microorganisms while control plants remained untreated. Both groups received identical agronomic management, including irrigation, fertilization, and foliar disease control. For the metagenomic analysis, soil samples were collected at two-time points: before the first inoculation (D1) and 120 days post-inoculation (D120).

Additionally, to assess the persistence of one of the introduced microorganisms (*Trichoderma spp.*), samples were collected before the first inoculation and at 90 and 210 days after it. In each sampling, approximately 10 g of rhizosphere soil was collected from each experimental plant. These were placed in polypropylene tubes and transported in a cooler to the laboratory, where they were stored at -20°C until DNA extractions were performed.

Sequencing and Metagenomic Analysis. To characterize and identify the microbial community associated with the *S. betaceum* rhizosphere, the workflow began with the extraction of genomic DNA using the PowerSoil kit (QIAGEN) following the manufacturer's instructions. Quality and concentration were quantified by fluorometry with the Qubit™ dsDNA HS and BR Assay kit (ThermoFisher) and by spectrophotometry. For library preparation, the Nanopore Native Barcoding genomic DNA protocol (version: NBE_9065_v109_revJ_23May 2018) was applied with the Native Barcoding Expansion 1-12 (EXPNBD104, replaced by EXPNBD103) and 13-24 (EXP-NBD114) kits provided by Oxford Nanopore Technologies (ONT) with the Ligation Sequencing kit (SQK-LSK109). Following the manufacturer's instructions, DNA repair, end preparation, adapter ligation, and library preparation were performed. The resulting library was sequenced with MinION Mk1B using an R9.4.1 flow cell, FLO-MIN106, for 87 hours. Once sequencing was completed, the raw data in fast5 format were used for real-time base-calling in the MinKNOW v21.06.10 software (Guppy v. 3.4.5 integrated) in super high accuracy mode (minimum qscore=10). The data resulting from this process were used for taxonomic assignment through Minimap2 (v. 2.25 (r1173)) using the Kraken2 database.

Persistence of the microorganisms introduced through the microbial consortium. *Trichoderma* was selected as the indicator organism to monitor the persistence of the introduced microorganisms. To calibrate the PCR monitoring assay, 8 µL of the *Trichoderma* liquid formulation was extracted and inoculated onto Petri dishes with fungal growth medium. These were stored at 23°C ± 2, with growth monitored every 24 hours until colony appearance. The colonies were then extracted and plated on PDA for 10 days at 25 ± 2°C under dark conditions. Morphological identification was performed by recognizing its macroscopic and microscopic structure using a series of taxonomic keys [13–15]. For DNA extraction, the protocol of [16] with

modifications. In a 2 mL Eppendorf tube, approximately 100 mg of fresh mycelium was added and 500 µL of TE buffer (homogenized with vortex for 10 seconds), then centrifuged at 16,000 RCF for 5 minutes. The supernatant was discarded, and 300 µL of lysis buffer (1% SDS, 0.5% Tween 20, 10 mM Tris-HCl pH 8, and 1 mM EDTA) and 4 metal beads were added. The solution was homogenized for 3 minutes (twice) using a TissueLyser LT (QIAGEN). The sample was incubated in a water bath at 65°C for 15 minutes, then 150 µL of sodium acetate (5M) was added and stored at -80°C for 10 minutes. The samples were manually thawed and centrifuged. The supernatant was transferred to a new tube, and isopropanol (1:1) was added, incubating for 5 minutes at room temperature. The DNA was precipitated by centrifugation, and the supernatant was discarded. The pellet was washed with 500 µL of 70% ethanol and a final centrifugation was performed to remove all ethanol (dried at 65°C). Finally, the pellet was dissolved in 100 µL of TE buffer, and the DNA quality and concentration were determined.

Trichoderma spp. was monitored at different times during the development of the project, for which the ITS1-ITS2 region was amplified using the specific primers uTf (5'-AACGTTACCAAACTGTTG-3') and uTr (5'-AAGTTCAGCGGGTATTCCT-3') that have been reported in the literature[17], A fragment of 540 bp was expected. Each reaction was prepared in a final volume of 10 µL (5 µL of Platinum High Fidelity Master Mix - Invitrogen, 1 µL of each primer at 10 mM, 1 µL of genomic DNA ≥100ng, and 2 µL of Milli-Q water). The reaction was run in a thermocycler with the following program: 95°C for 3 minutes, 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, a final extension at 72°C for 10 minutes, and a final cooling at 4°C. Finally, agarose gel electrophoresis (1%) was performed, mixing the DNA amplification products (7 µL) with 2 µL of loading buffer and 0.5 µL of ethidium bromide. 3 µL of a 1Kb molecular weight marker (Plus DNA ladder, Invitrogen) were used; the samples were run under a constant voltage of 100 volts, with a DNA fragment migration speed of approximately 0.00556 cm/s. The DNA fragments were visualized using a photo-documentation system with ultraviolet light.

Statistical Analysis

Metagenomic data analysis was conducted using R v.4.2.3, utilizing various specialized packages for microbial ecology and data visualization. The study incorporated phyloseq v.1.42.0, microeco v.1.0.0, ampvis2 v.2.8.9, ape v.5.7-1, GUniFrac v.1.7, permute v.0.9-7, and picante v.1.8.2. Graphical representations were generated using ggplot2 v.3.5.1. To assess the structural diversity of the microbial community, Shannon-Weiner and Simpson diversity indices were calculated. Additionally, Chao1 was used to estimate species richness, while the inverse Simpson index was applied to evaluate dominance, employing the vegan v.2.6-4 package.

RESULTS

Microbial community composition of the *S. betaceum* rhizosphere. The dataset analysis generated 3,749 reads from 6 samples, with a total output of 1.433 GB. This dataset was evaluated at the phylum and genus levels, excluding taxa corresponding to the genera *E.coli* and *Homo sapiens*, as they were identified as potential contaminants or experimental artifacts. The identified taxa were classified into 6 distinct kingdoms (Eubacteria, Archaea, Plantae, Protozoa, Chromista, Fungi) and viruses and were distributed across 40 phyla, 64 classes, 147 orders, 311 families, and 868 genera. Of these, 93% corresponded to bacteria, totaling 3,505 reads. The dominant phyla were *Pseudomonadota* (formerly known as *Proteobacteria*) with 47% of the total

reads, and *Actinomycetota* (formerly *Actinobacteria*) representing 32%, followed by *Bacteroidota* (formerly *Bacteroidetes*) and *Bacillota* (formerly *Firmicutes*).

Microbial community distribution at the genus level, 10 taxa were the most abundant across all samples, with the genera *Streptomyces, Nocardioides, Bradyrhizobium, Sphingomonas, Rhodanobacter, Lysobacter,* and *Micromonospora* standing out. Additionally, it was found that the samples from both conditions (D1 and D120) maintained a similar pattern in terms of taxonomic abundance, meaning the microbial composition was homogeneous at both the phylum and genus levels. Figure 1 shows the relative abundance of the microbial composition at the phylum level, while Figure 2 displays the genera with the highest relative abundance, classified by their respective phyla.

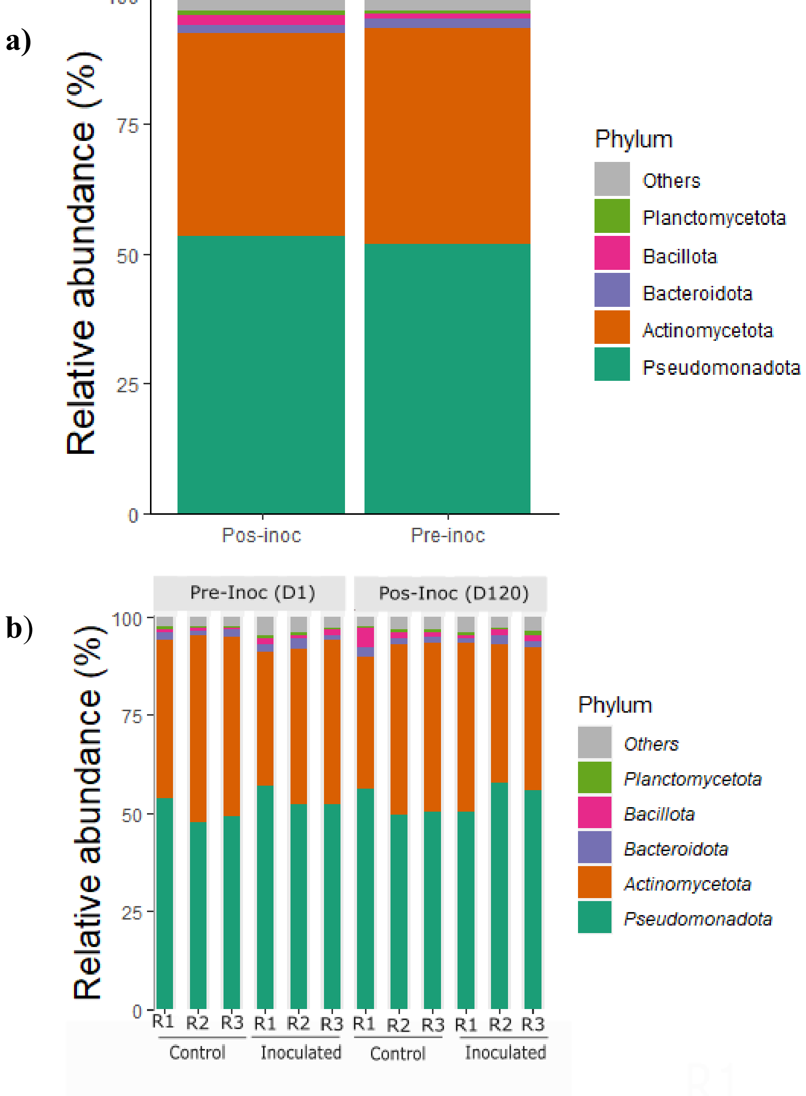

Figure 1. Microbial community composition of the *S. betaceum* rhizosphere in soils inoculated with microbial consortia. The bar charts display the top 5 most abundant phyla in the 12 samples analyzed, classified by the evaluated conditions (Pre-Inoculation and Post-Inoculation). The profiles of three replicates (R1-3) are shown for both control and inoculated soil plots. (a) Shows the phyla according to the evaluated conditions, (b) Shows the phyla of each sample.

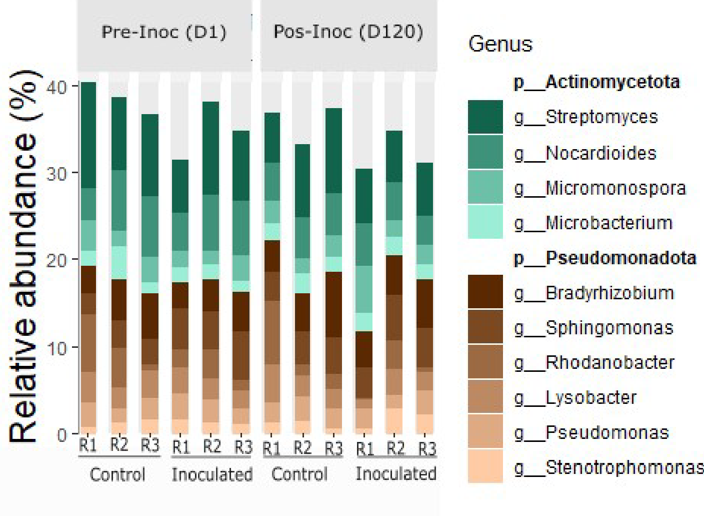

Figure 2. Microbial community composition of the *S. betaceum* rhizosphere in soils inoculated with microbial consortia. The bar charts display the top 10 most abundant genera (B) in the 12 samples analyzed, classified by the evaluated conditions (Pre-Inoculation and Post-Inoculation). The profiles of three replicates (R1-3) are shown for both control and inoculated soil plots.

Microbial diversity and richness. Microbial counts at the genus level were initially explored to determine if the overall data showed separation between them and with the treatment. Alpha diversity metrics Chao1, Shannon, Simpson, and the inverse Simpson were used to evaluate richness, diversity, and evenness within and between the groups of microbial communities corresponding to the controls, treatment control, and treatment (Figure 3). Overall, microbial diversity showed that the rhizosphere microbiota was not significantly different ($p > 0.05$).

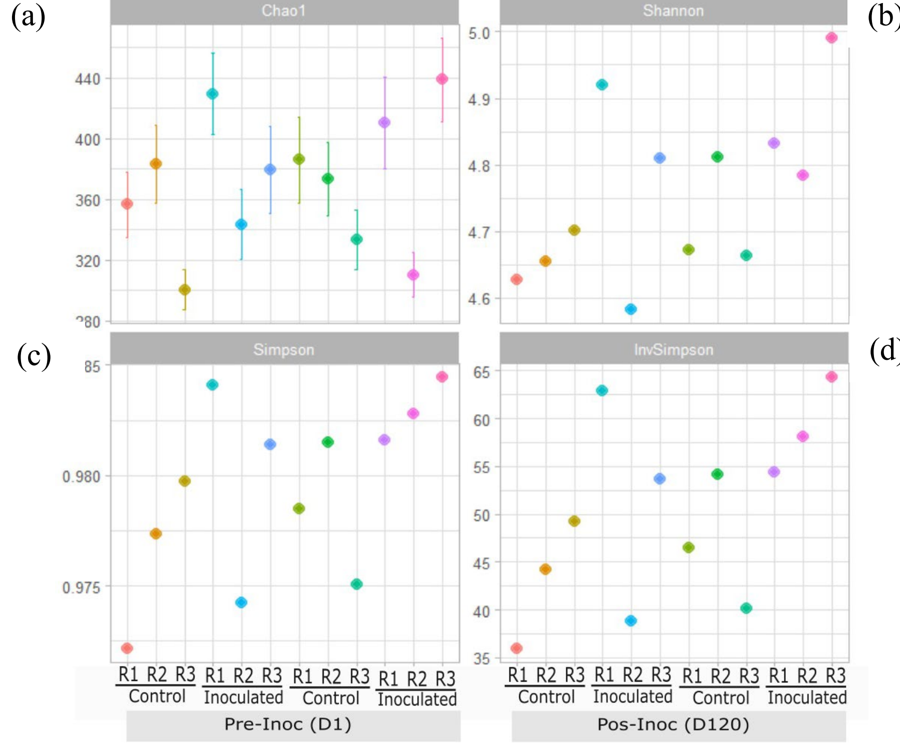

Figure 3. Alpha diversity indices of the microbial community in the *S. betaceum* rhizosphere in soils inoculated with microbial consortia at the genus level. (a) Chao index, (b) Shannon index, (c) Simpson index, (d) Inverse Simpson index.

Furthermore, no significant differences were found between the microbial communities in the *S. betaceum* rhizosphere based on the PERMANOVA analysis.

Persistence of *Trichoderma spp*. at the field level.

Macroscopic and microscopic morphological characterization. Based on macroscopic observation (Figure 4a), the isolates exhibited a regular shape (spreading from the center to the edges of the Petri dish, the mycelium remained in a flat elevation, with pigmentation ranging from white to greenish; colony formation was rapid, taking five to seven days to colonize the plate, including the reverse fully. Around the fifth day, pale white, light green, and creamy shades appeared, resulting in 3 isolates with different macroscopic characteristics. Regarding the microscopic observation and characterization results (Figure 4b), in general, the isolates displayed hyaline hyphae with globose conidia and some slightly ovoid, smooth-walled hyaline conidia, both individual and grouped, with long conidiophores, broad at the center and elongated at the base, clustered in bunches.

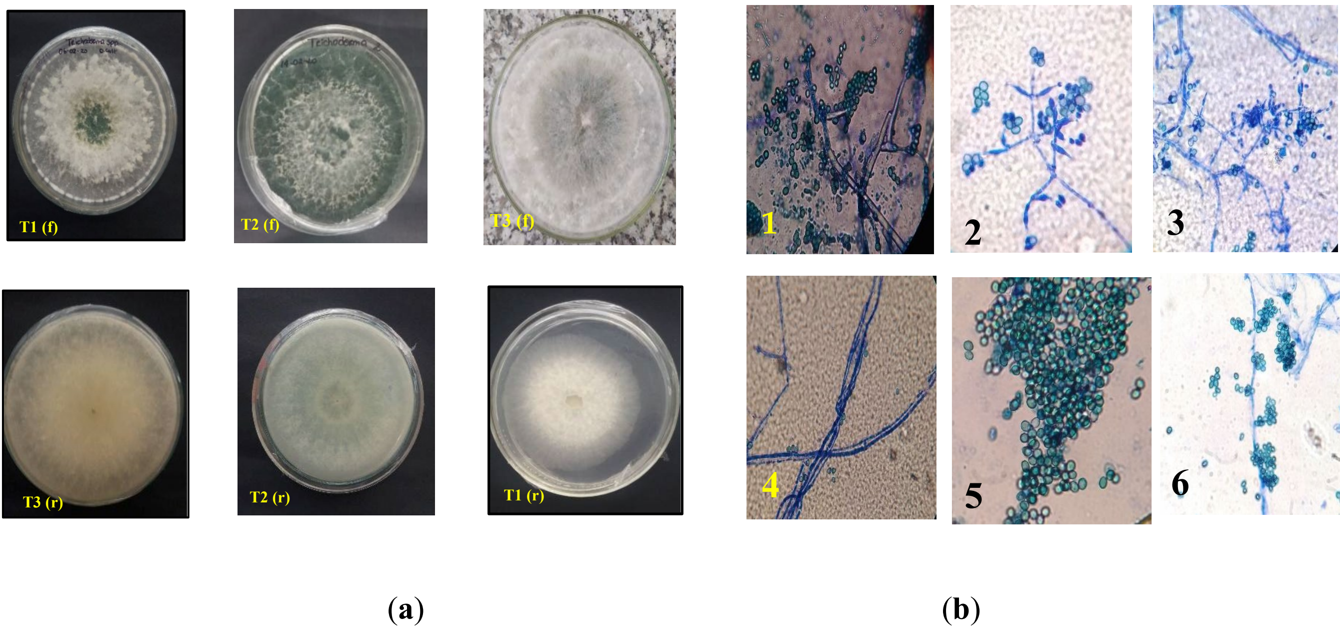

Figure 4. (a) Growth of three *Trichoderma spp.* Isolates on PDA medium 5 days after inoculation, showing the isolates' front (f) and reverse (r) sides. (b) Representation of the microscopic morphological characteristics of Trichoderma spp. Stained with lactophenol blue (1-3) conidiophores and conidia. (4) Conidiophores, (5) Conidia, (6) Conidia and hyphae. The observation was performed with a 100X objective.

Molecular Characterization (PCR Test). *Trichoderma spp*. was monitored in the S. betaceum rhizosphere at three points: before inoculation, 90 days, and 120 days post-inoculation. However, the pres
ence of Trichoderma was not detected at any of the evaluated time points (Figure 5).

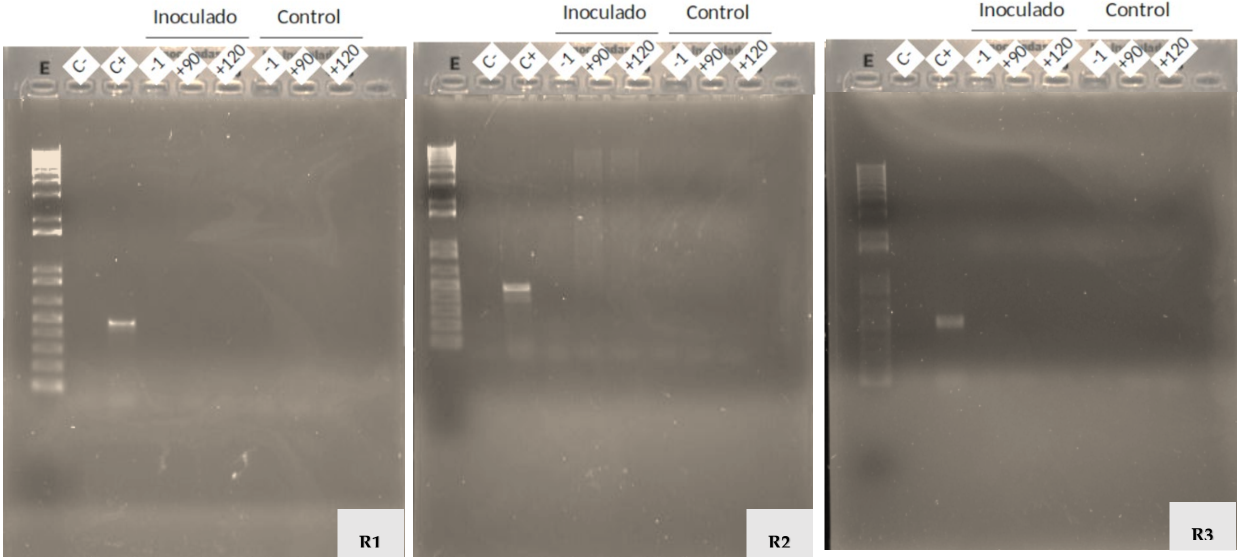

Figure 5. Agarose gel electrophoresis analysis (1%) of PCR products derived from amplifying the ITS1 and ITS2 regions of Trichoderma spp. The gels show the marker/ladder (E), negative control (C-), positive control (C+), pre-inoculation rhizosphere sample (-1), and rhizosphere samples 90 and 120 days post-inoculation (+90, +120).

DISCUSSION

This study aimed to analyze the changes occurring in the microbial communities of the rhizosphere of *Solanum betaceum* in response to inoculation with microbial consortia at the field level. Interestingly, both the treatments and controls, before and after inoculation, showed similar behaviors, behaving similarly, and the persistence of *Trichoderma spp.* in the samples could not be established. Some hypotheses may be responsible for the limited success of microbial inoculants at the field level.

Some studies have pointed out that introducing microorganisms into the rhizosphere does not always result in successful colonization due to competition with native communities [18–20]. This competition can manifest through various mechanisms, such as competition for nutrients, the production of antimicrobial substances by the resident microbiota, or competitive exclusion through interactions with the host plant. According to Kurkjian et al. [21], pre-existing microbial communities can resist the introduction of foreign species and even eliminate them. It is presumed that the introduction of the microbial consortium into the soil system may have served as a brief reservoir for the spores, only to be displaced by inhibitory substances that prevented the establishment of *Trichoderma spp.* and were not detected in the PCR analysis

Another relevant factor could be the influence of agricultural practices, such as fertilization and irrigation management. It is possible that the application of microbial consortia was carried out shortly after chemical fertilization, which may have negatively affected the survival of *Trichoderma spp*. Recent studies have shown that certain fertilizers can alter the physicochemical properties of the soil, modifying nutrient availability and affecting microbial composition [22].

An additional aspect to consider is irrigation management, which represents a key factor in microbial communities, as inadequate moisture levels can limit the biological activity of inoculants [23]. The literature indicates that the main changes in bacterial community parameters are closely related to soil pH, which significantly correlates with irrigation levels. Both pH and moisture have been associated with the relative abundance of dominant bacteria at the phylum and genus levels [24]. It has also been observed that these variations in microbial structure influence the *Solanum* genus [27,28], and it is concluded that changes induced by different irrigation treatments can improve the nitrogen cycle in the rhizosphere and favor nutrient catabolism. A reduced irrigation approach could, in this regard, increase microbial community richness and, in turn, improve fruit yield. This adjustment in irrigation management optimizes water efficiency and contributes to agricultural cost reduction.

It is also considered that the experimental design used in this study, based on a single treatment and location, may have conditioned the obtained results. Soil and climatic variability between different sites could influence the persistence capacity of *Trichoderma spp.*, so studies with a greater diversity of experimental conditions would allow for better evaluation of these effects. Additionally, the monitoring time was limited, which may have prevented the observation of long-term changes in the microbial community, as this behavior is considered normal. According to the literature, soil microbial communities can undergo significant changes over various periods, ranging from days to years [29–34]. Future research could include extended periodic evaluations over time to determine if colonization occurs more slowly or if there are temporal fluctuations in the abundance of the inoculated microorganism.

Therefore, to improve the persistence of *Trichoderma spp*. and optimize its field use, it is recommended to isolate and use native strains, as these may exhibit better adaptation to the soil and local microbiota [4,31], since the origin of an inoculant strain can provide important information about its ecological behavior relevant to field application [35–37]. Additionally, it would be beneficial to evaluate the effectiveness of different doses and combinations of microbial consortia and test alternative application methods, such as seed pre-treatment or co-inoculation with other beneficial microorganisms. It is also essential to consider environmental factors such as soil pH, crop history, water availability, and the use of agrochemicals, as these influence the interaction between native microbiota and introduced microorganisms [17,38–40].

Regarding the microbial composition found in the rhizosphere of *S. betaceum*, this study presents preliminary data, identifying the most abundant taxa at the phylum and genus levels. The most abundant phyla are *Pseudomonadota* and *Actinomycetota* (47% and 32%, respectively), which have been reported as dominant bacterial taxa in agricultural soils related to soil and root health, suggesting that this could be a general pattern in the interaction of these microorganisms in the farm environment [41–43]. As of the submission of this manuscript, no reports have been found with information regarding microbial communities in *S. betaceum* crops to suggest that these phyla could be associated with this plant or even considered endophytes. However, some studies, although not on the same species under investigation, have evaluated the rhizosphere microbial composition in plants of the Solanum genus and have cited the presence of these phyla [44–47].

When analyzing the microbial composition at the genus level, the dominant groups were Streptomyces, *Nocardioides, Bradyrhizobium, Sphingomonas, Rhodanobacter,* and *Micromonosporas*, which play fundamental roles in the rhizosphere and soil. Although the presence of these genera has not been reported in studies related to *S. betaceum*, their benefits, and roles have been documented in some crops, many of which belong to the Solanum genus. Streptomyces bacteria contribute to the decomposition of organic matter, nutrient cycling, and plant protection by producing phytohormones and bioactive compounds and participating in the bioremediation of pollutants [48–51]. On the other hand, *Bradyrhizobium* is key for nitrogen fixation and

plant growth, improving soil fertility and reducing the dependence on synthetic fertilizers [52–56]. *Nocardioides* also participate in nutrient cycling and promote plant growth by protecting them from pathogens [57,58]. *Sphingomonas* stands out for its ability to degrade pollutants, produce phytohormones, and facilitate bioremediation processes [59,60]. *Rhodanobacter* contributes to the degradation of toxic compounds in plant growth and enhances plant resistance under stress conditions [61,62]. Finalmente, *Micromonospora* produce compuestos bioactivos que controlan patógenos del suelo y participa tanto en la descomposición de materia orgánica como en la biorremediación. [63–65].

CONCLUSIONS

The microbial communities in the rhizosphere of *Solanum betaceum* remained relatively stable in the medium term following inoculation with microbial consortia, indicating that the soil's microbial diversity was not significantly altered. Additionally, the absence of *Trichoderma spp.* in post-inoculation samples suggests that this microorganism did not establish itself at detectable levels.

This study provides pioneering insights into the microbial communities associated with *S. betaceum*, identifying predominant phyla and genera, some of which had not been previously reported in the crop's rhizosphere. The results underscore the importance of investigating the factors that affect the persistence of microbial consortia in soil, particularly the interactions between introduced and native microorganisms.

A key novel finding is the detailed characterization of microbial diversity using metagenomic tools, which revealed the dominance of *Pseudomonadota* and *Actinomycetota*, as well as the presence of *Streptomyces, Nocardioides, Bradyrhizobium, Sphingomonas, Rhodanobacter*, and *Micromonospora*, suggesting potential beneficial interactions in *S. betaceum*. The study also highlights the potential resistance mechanisms of native microbiota, which may prevent the establishment of introduced microorganisms like *Trichoderma spp.*

Furthermore, this research contributes to understanding microbial resilience in agroecosystems, reinforcing the need for long-term monitoring and using native microbial strains that may better adapt to local soil conditions. Future research should explore broader geographical conditions, assess microbial consortia compositions, and optimize inoculation strategies to enhance microbial integration and effectiveness. These findings lay the groundwork for developing sustainable bioinoculant applications tailored to the Andean region, promoting soil health and agricultural productivity while reducing reliance on chemical inputs.

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