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RESEARCH ARTICLE

Study of the behavior of Cellulolytic microorganisms and Phosphate solubilizers associated with Rhizospheric soil of *Espeletia grandiflora* in two zones with different degree of intervention in Páramo Ocetá, Colombia

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Abstract

Objective: To evaluate the behavior of cellulolytic microorganisms and phosphate solubilizers associated with rhizospheric soil of *Espeletia grandiflora* in two areas with different degrees of intervention in the Ocetá paramo. **Method:** In the field phase 25 samples were taken from each zone. Pre-enrichment of the samples was performed and subsequently isolation for cellulolytic microorganisms and phosphate solubilizers on CMC and SRS agar; colonies were identified macroscopically and microscopically and characterized by means of biochemical tests. For data analysis, normality tests, t-student, and principal component analysis were performed. **Results:** Twelve microorganisms from cellulolytic groups and phosphate solubilizers were identified. The genus Oerskovia sp. and Cryptococcus sp. were the most frequent with a percentage of 31% and 33% respectively for the conserved area and for the intervened area with a value of 45% and 26%, this is due to their cellulolytic action in relation to *Espeletia grandiflora*. Regarding the two least frequent microorganisms, *Fusarium* sp. and Aspergillus sp. which are pathogenic for the plant, indicate that the frailejones are in a good state of growth. For the comparison of the frequencies of species in each zone, there was no significant difference. The principal component analysis yielded a relationship between the vectors and some species, possibly due to the variability that exists for each one, taking into account their needs for growth.

Keywords: Microbial ecology; cellulolytics; solubilizers; frailejones

1 Introduction

In Colombia, the páramos are located in the high tropical mountains, where they play an important role as generators, regulators and storage of water resources. They are vital ecosystems to maintain the water cycle, because they allow the transformation

of the mist into a water resource. Its most representative species, the frailejón, is of great ecological importance, because it contributes to regulating the hydrological cycle, produces most of the biomass in these ecosystems, prevents soil erosion and has key associations with more than 125 animal species. In addition to being important spaces for the wealth of microbiota associated with its soils, this being the basis of sustenance for the establishment and development of the communities found there[1].

The microbial community is the most important functional component of soil biota, as it plays an important role in energy flow, nutrient transformation and cycling of elements in the environment[2]. This microbiota intervenes in processes that stimulate nutrients such as nitrogen fixation, phosphate solubilization and the degradation of carbon compounds; it influences the content of organic matter, the stability and fertility of the soil, favoring the establishment and development of plant communities[3].

Considering the relevant role of the microbiota in soil formation and in the different factors that act on plant growth, the behavior of cellulite microorganisms and phosphate solubilizers associated with rhizospheric soil of *Espeletia grandiflora* in two areas with different degrees of intervention was evaluated. in the Ocetá páramo, Boyacá.

2 Materials and method

The work was carried out in the Ocetá paramo, geographically located in the Cordillera Oriental to the northeast of the municipality of Monguí, in the department of Boyacá, Colombia. The paramo is located at an altitude of 3,800 meters above sea level and at an average annual temperature of 10 ° C. It comprises an approximate area of 57.71 km2.

2.1 Field phase

Four field trips were made, between the months of May to October of the year 2018. Two sampling areas were identified, identifying a conserved area that did not have visitor access and had not presented anthropic disturbance; and an intervened area where previously it was confirmed the anthropic intervention with respect to burns, agriculture and livestock. A quadrant of 50 x 50 m was made in each previously identified area (Conserved and intervened area). For sampling in each quadrant, X-transects were made and 5 samples were taken in each corner and in the center of the quadrant, for a total of 25 samples for each area. 200 g of rhizospheric soil were taken at a depth of 20 cm with a hole, each sample was subsequently labeled and stored at 4 ° C in hermetic bags for microbiological analysis[4].

2.2 Laboratory phase

The rhizospheric soil samples were taken to the El Bosque University laboratory, where they were processed and purified on a 2mm sieve and refrigerated at 4°C. To take the pH, 12 g of rhizospheric soil were weighed and mixed with 30 ml of distilled water, (1: 2.5), stirred for 5 minutes and allowed to settle, with a pH meter the value was obtained[5]. The percentage of humidity was determined by weighing 10 g of each rhizospheric soil sample and placing the samples in a drying oven at 110°C for 24 hours, and then re-weighing all the samples. The percentage of humidity was determined[6].

For the pre-enrichment of cellulolytic bacteria, 10 g of the rhizospheric soil sample were taken and added to 90 ml of cellulolytic bacteria nutrient broth, which was left for 1 to 3 weeks in the dark at room temperature. From the pre-enrichment of each sample, sowing by striation or exhaustion was performed on carboxymethylcellulose agar (CMC Agar) at 1% (w / v). The boxes were incubated at 35°C for 48 hours. They were identified, adding Congo Red 1% (w / v) as a developer to the colonies present in the media, after 15 minutes the excess was removed and NaCl at 0.1M was added, allowing to stand for 15 minutes to then count and identification of colonies with hydrolysis halos. Genres with Gram staining were confirmed[7].From the obtained microorganisms, 1% (w / v) CMC agar peaks were made from the colonies that presented cellulolytic activity, also incubating at 35°C for 48 hours and carrying out the same procedure for their revelation.

For the isolation of phosphate-solubilizing bacteria, the SRS medium was used. A pre-enrichment was performed in SRS medium without pH indicator taking 5 g of each rhizospheric soil sample in 45 ml of SRS broth. The pre-enrichments were left for 72 hours at room temperature. Then the pre-enriched sample was sown on SRS agar, which did have the pH indicator (Purple Bromocresol), in order to see the reaction and the color change in the phosphate solubilizing microorganisms. The boxes were incubated at 30°C for 72 hours[8]. The identification was made with the count of the colonies that grew, acidifying the culture medium and forming a transparent halo around the colony, indicating solubilizing activity or production of organic acids acidifying the medium. Microscopic features were confirmed with Gram staining.

For macroscopic identification, the colony morphology, size, color, border, and photographic records were described. On the other hand, for microscopic identification, Gram staining or lactophenol blue staining was performed. Peptides were

performed on nutrient agar of the bacteria identified in the selective agar, to carry out the characterization using the BBL Crystal biochemical test kit, and the oxidase, catalase and coagulase tests.

For the fungi identified in the selective agar, PDA agar was required and lactophenol blue staining was performed to confirm their genera, taking into account the macro and microscopic characteristics reported in taxonomic codes.

2.3 Statistical analysis

A descriptive statistic in the SPSS 2.0 program was used to establish the characteristics of the two sampling areas. Then a normality test and analysis of variance were applied to corroborate the fulfillment of assumptions. The ANOVA test and an average comparison test corresponding to the T-student test were implemented to establish if there is a significant difference between the two sampling areas and the frequency of cellulolytic microorganisms and phosphate solubilizers. A multivariate statistical analysis was performed, for this case a Principal Component Analysis (PCA) to find the relationship of the sampling areas with the microorganisms found and the different environmental variables

3 Results and Discussion

According to the microscopic and macroscopic characteristics (Figure 1) for cellulolytic microorganisms and phosphate solubilizers, 12 microorganisms were identified, distributed in 11 genera, 10 families and 8 orders (Table 1).

The following microorganisms are reported as cellulolytic: *Oerskovia* spp., *Bacillus brevis, Aspergillus* spp., *Streptococcus porcinus, Pseudomonas* spp., *Cryptococcus aureus* and for the phosphate-solubilizing microorganisms the following are reported: *Corynebacterium aquiatium, Micrococcus lylae, Micrococcus luteus, Fusarium spp., Streptomyces spp., Cryptococcus terrícola.*

Table 1. Identification of cellulolytic microorganisms and phosphate solubilizers associated with rhizospheric soil of *Espeletia grandiflora*.

Order	Family	Specie
Actynomicetales	Corynebacteriaceae	*Corynebacterium aquiaticus*
	Micrococcaceae	*Micrococcus lylae*
		Micrococcus luteus
	Cellulomonadacea	*Oerskovia* spp.
Bacillales	Bacilliaceae	*Bacillus brevis*
Eurotiales	Trichocomaceae	Aspergillus spp.
Lactobacillales	Streptococcaceae	*Streptococcus porcinus*
Nectriaceae	Hypocreales	*Fusarium* spp.
Pseudomonadales	Pseudomonadaceae	*Pseudomonas* spp.
Streptomycetales	Streptomycetaceae	*Streptomyces* spp.
Tremellales	Tremellaceae	*Cryptococcus aureus*
		Cryptococcus terrícola

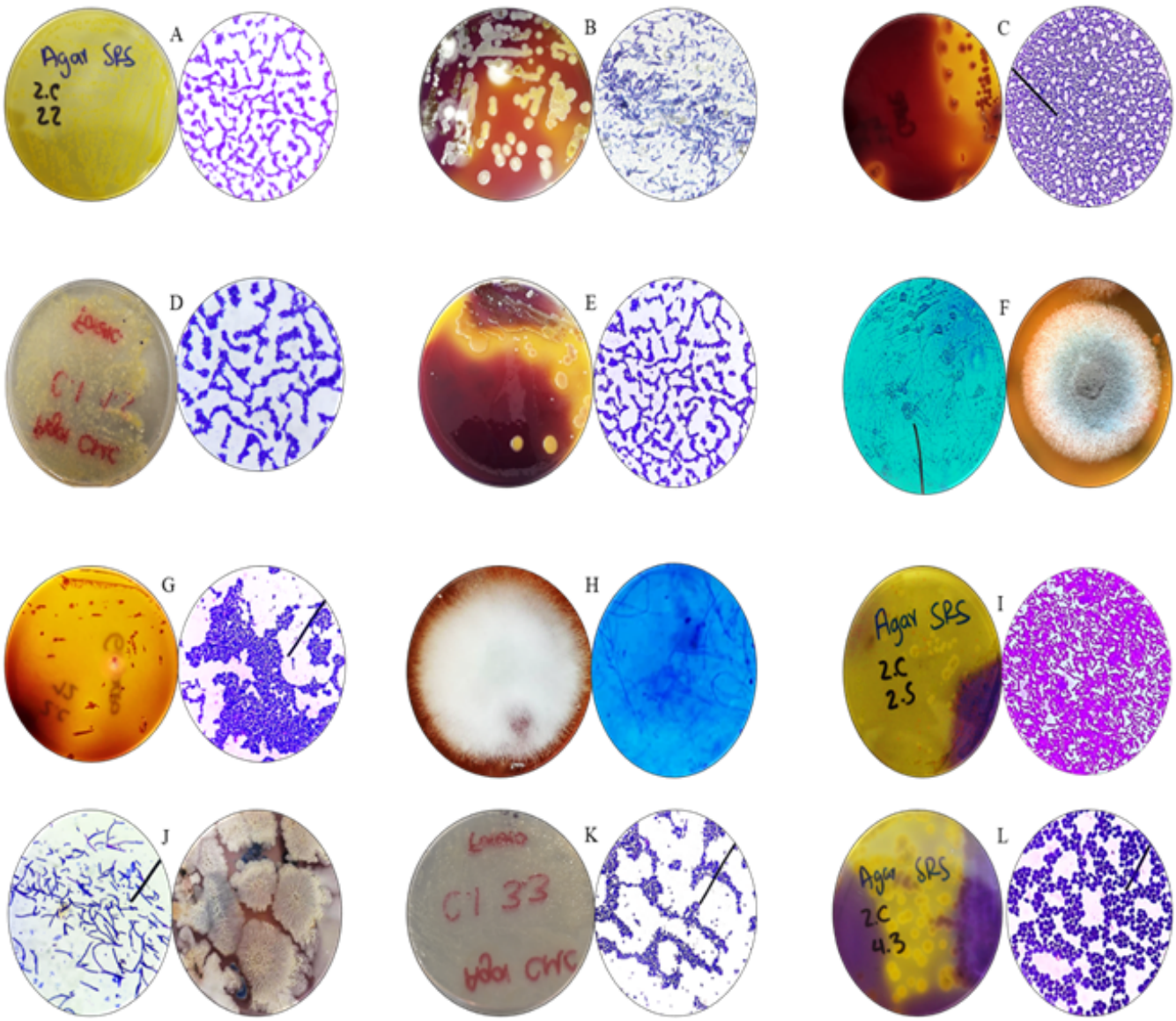

Fig 1. Macroscopic and microscopic characteristics of the isolated microorganisms. A. *Corynebacterium aquaticum*. B. *Micrococcus lylae*.C. *Micrococcus luteus*. D. *Oerskovia* spp. E. B*acillus brevis*. F. *Aspergillus* spp. G. *Streptococcus porcinus*. H. *Fusarium* spp. I. *Pseudomonas* spp. J. *Streptomyces* spp. K. *Cryptococcus aureus*. L.*Cryptococcus terricola*.

In all the isolates on CMC agar there was growth, but only in 111 boxes the hydrolysis halo was visualized with a variable amplitude for each one (Figure 2), likewise, 123 colonies were reported as positive for the Congo red test, thus demonstrating that cellulolytic bacteria in tropical environments (including paramos), like those isolated in temperate regions, differ in their ability to degrade cellulose [9]

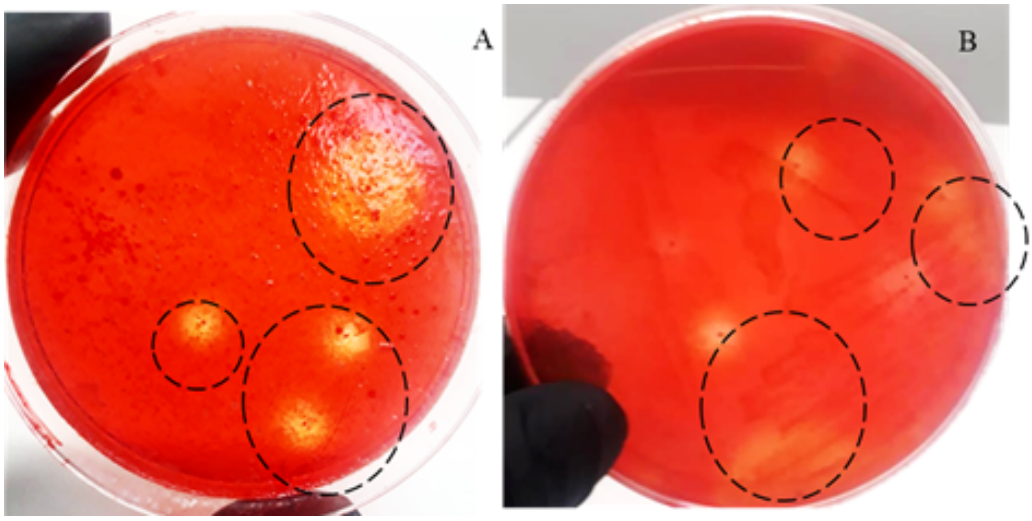

Fig 2. A - B Color change in CMR Agar after Congo Red test, indicating hydrolysis in cellulolytic microorganisms

Regarding the identification of phosphate solubilizing microorganisms, 119 colonies were reported with the presence of solubilizing activity, observing the metabolic behavior in the SRS medium due to the presence of the pH indicator, bromocresol purple (Figure 3), which allows the reaction to be evidenced. by color change in the medium, (change to yellow color), for those phosphate solubilizing microorganisms, indicating the consumption of tricalcium phosphates (Calcium phosphate)[10]. The solubilizing phosphate activity is determined by the microbial biochemical capacity to produce organic acids that through the carboxylic groups bind to phosphates converting them into soluble forms, although the production of organic acids is one of the main blame in this process, it is not the unique mechanism to solubilize phosphates.

Taking into account the acidification of the medium, insoluble phosphates that cannot be assimilated by plants can be brought to soluble forms by the action of many microorganisms. The main route of solubilization is through the production of organic acids such as: acetic, lactic, oxalic, citric, butyric, succinic, malic, gluconic, fumaric and 2-ketogluconic[11].

On the other hand, bacteria also transform insoluble phosphates to soluble forms by the action of different direct or indirect mechanisms. These include: the action of organic acids produced by microorganisms, chelation of the elements responsible for the insolubility of the phosphates present and direct assimilation of insoluble phosphates by microorganisms that accumulate it in their cells and subsequently release them[12].

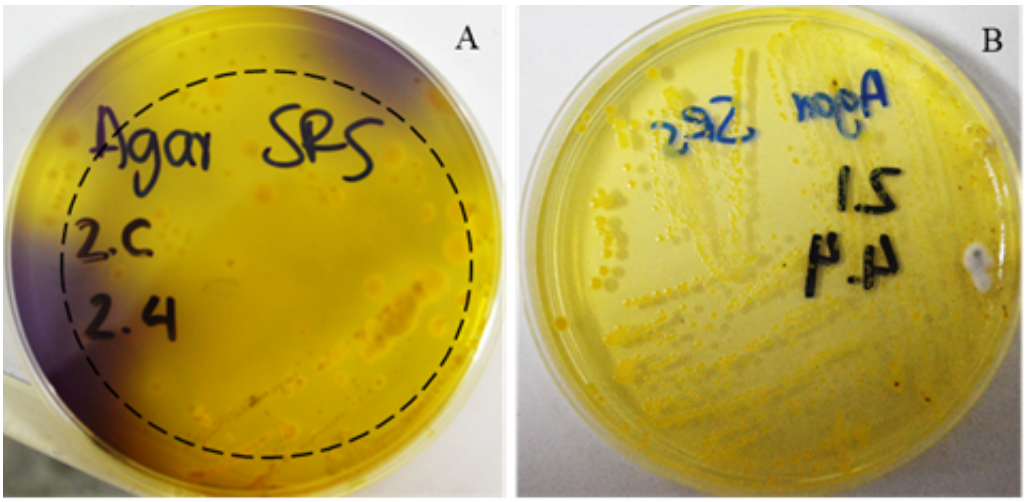

Fig 3. Battery isolation on SRS agar. A. Color change in the isolation of a sample for the conserved quadrant. B. Solubilizing activity throughout the box insulation of the operated quadrant.

Phosphorus is a vital element, along with nitrogen, and its deficiency crucially limits the growth of plants[13]. Phosphate solubilizing bacteria solubilize insoluble phosphates in soils and make them available to plants[14], in addition to this, they provide them with carbonaceous compounds that are metabolized for microbial growth and also root exudates and plant debris supply the substrate energy that favors its solubilizing activity.

It should be noted that some microorganisms that solubilize phosphates in the soil may also have other plant growth promotion activities, such as the production of gibberellic acid, hydrogen cyanide (HCN), cytokinins, ethylene, asbiotic fixation of nitrogen, and resistance to pathogenic organisms of the soil[13].

Most organic acids produced by some phosphate-solubilizing genera are aliphatic, these genera modify the pH causing the dissolution of insoluble phosphates in the soil[14].

3.1 Frequency of cellulolytic microorganisms and phosphate solubilizers

Of the 12 isolated and identified microorganisms, the genera with the highest presence were *Oerskovia* spp. y *Cryptococcus* spp.; the genera with the lowest presence were *Aspergillus* spp. and *Fusarium* spp. In the following graph we can see the relative frequency of the total number of isolated microorganisms, being *Oerskovia* spp. the most frequent for the two sampling areas. (Figure 4)

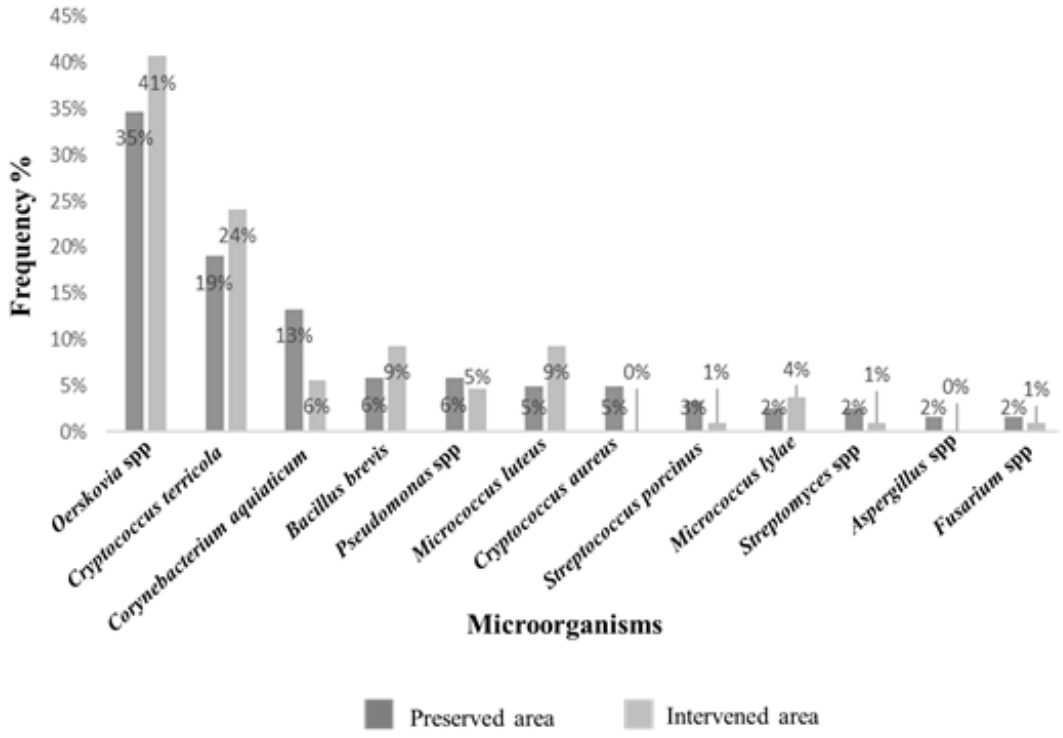

Fig 4. Relative frequency of the two sampling areas. Preserved and intervened

Oerskovia spp., is a Gram positive bacillus present in soil, decomposing plant materials, and sewage[15].

Taking into account the high frequency of this genus with respect to other microorganisms and with its cellulolytic action in relation to *Espeletia grandiflora*, it could be explained genetically, this genus has a gene that encodes 1,4-β-cellobiosidase cellulose and is present in the genome that converts cellulose to 1,4-β-D-glucan. 1,4-β-D-glucan would also be converted to β-D-glucose through the action of a β-glucosidase. Furthermore, cellulose can be converted to cellobiose, using endoglucanases, and can also be converted to β-D-glucose through the action of a β-glucosidase, in order to meet your carbon and energy needs, as well for carbon production for the cycle[16].

The *Cryptococcus terricola* species inhabits densely vegetated soils in regions with cold climates, where it occurs in litter and on mineral soil horizons, for example, in birch, fir or frailejones[17].

Corynebacteriumaquiaticum is a bacterium found in water and soil. The ability of this and other genera to degrade lignocelluloses implies that this group of bacteria has great potential to offer useful indicators of maturity in the rhizospheric soil of the plant [3] in this case for *Espeletia grandiflora*.

Bacillus brevis is found in the Bacilliaceae family, this genus is commonly found in soils and plants where they have an important role in the carbon and nitrogen cycle. It is a recommended microorganism for the production and development of biofertilizers. It was one of the most frequent microorganisms in the rhizospheric soil of, *Espeletia grandiflora*, for this reason it is of great help as a promoter of plant growth and carbon and energy production [18].

The *Micrococcusluteus* species was the fifth most frequent species in the rhizospheric soil of *Espeletiagrandiflora*. This species is found in diverse environments such as soil and water; In this case, the abundance of water that species such as *Espeletia grandiflora* have, found these types of bacteria adapted to survive these environments. This genus has properties that make it of great use for bioremediation and it is also closely related in terms of wastewater treatment and, mainly, in the rhizospheric soil of some plants to promote growth in these [19].

Pseudomonas spp., has the ability to degrade cellulose, improve phosphorus availability; in addition to being considered a genus that has the ability to promote growth in plants, being associated with studies of rhizospheric soils of *Espeletia grandiflora* [20].

The *Micrococcus lylae* species is present in the soil and is capable of anaerobically producing glycerol, glucose, hydrolysis of esculin, and the conversion of nitrate to nitrite. On the other hand, for this species to have the solubilizing capacity it must depend on the source of nitrogen and potassium in some cases [21].

Figure 4 shows that the species with less frequency in the two sampling areas are *Cryptococcusaureus*, *Streptococcusporcinus*, *Streptomyces* sp., *Aspergillus* sp. and *Fusarium* sp., this may be due, in the case of *Cryptococcusaureus*, to the fact that these microorganisms are not of great help in the rhizospheric soil of *Espeletia grandiflora* and it is only found there to survive, but it is not providing any component so much for the carbon cycle as for the phosphorus cycle. Some species of the genus are capable of surviving only in soil and trees, as well as in woody plant remains [22].

The low frequency of the genus *Fusarium* indicates that the frailejones are in a good state of growth, since the mechanism of infection by this genus is effective when it penetrates weakened tissue and is pathogenic for the plant [23].

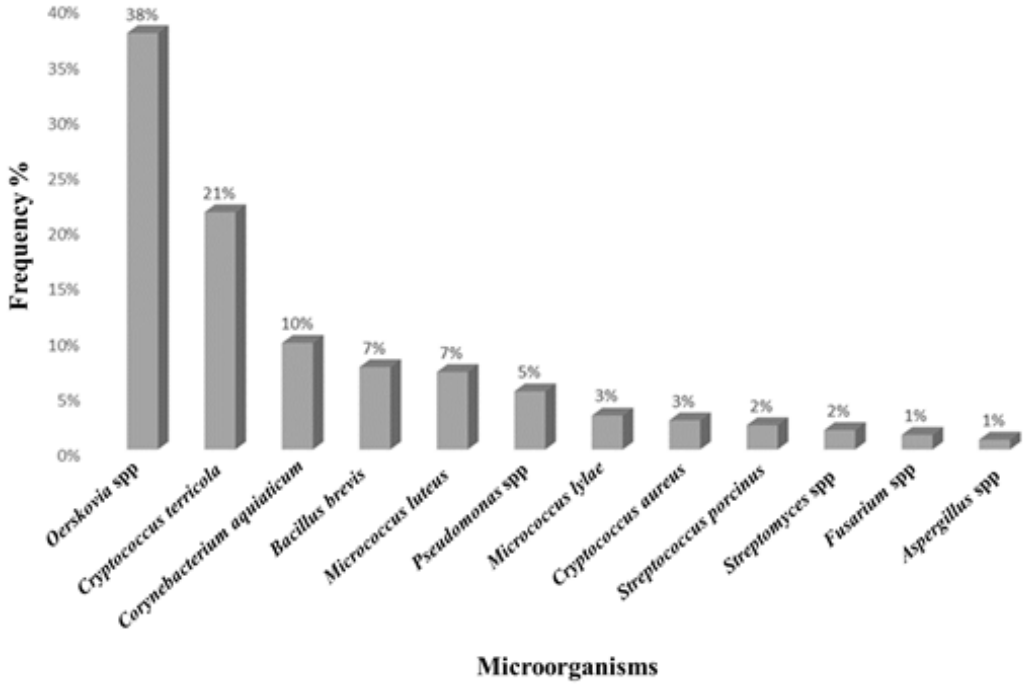

Fig 5. Relative frequency of the conserved zone vs. the intervened zone

The preserved area of the Ocetá paramo is not anthropically affected by any activity, this makes it an area where the soils and their components are very effective for the growth of frailejón, also the cellulolytic microorganisms and phosphate solubilizers found in this rhizospheric soil will be those that best provide suitable conditions to promote the growth of *Espeletia grandiflora.*

In Figure 5 we can see that the genera *Bacillus* and *Pseudomonas* had an average frequency compared to the other microorganisms, this agrees with Sylvia in 2005 [24], these genera are classified as microorganisms with high cellulolytic capacity and have been widely reported as effective agents. biocontrol of disease-causing fungi in various crops [24].

The intervened area of the Ocetá páramo was anthropically affected at the time, carrying out agriculture, burning and livestock activities; therefore, the soils of this area could perhaps have a lower yield for the growing conditions of *Espeletia grandiflora*, also for this area microorganisms were found that grow both in rhizospheric soil and also in contaminated soils, wastewater or even organic waste, possibly produced by this anthropic intervention that was done in this area of the moor.

For the *Cryptococcus aureus* species, no isolation was reported in the operated area. This species is associated with soils contaminated by the feces of different animals and taking into account that the intervened area is mainly affected by burning and agricultural activities, it is very rare that animals are found in it [25].

3.2 Comparison between the two sampling areas

There were no statistical differences (P <0.05) between the frequencies of species of cellulolytic microorganisms between the two sampling areas (Figure 6)

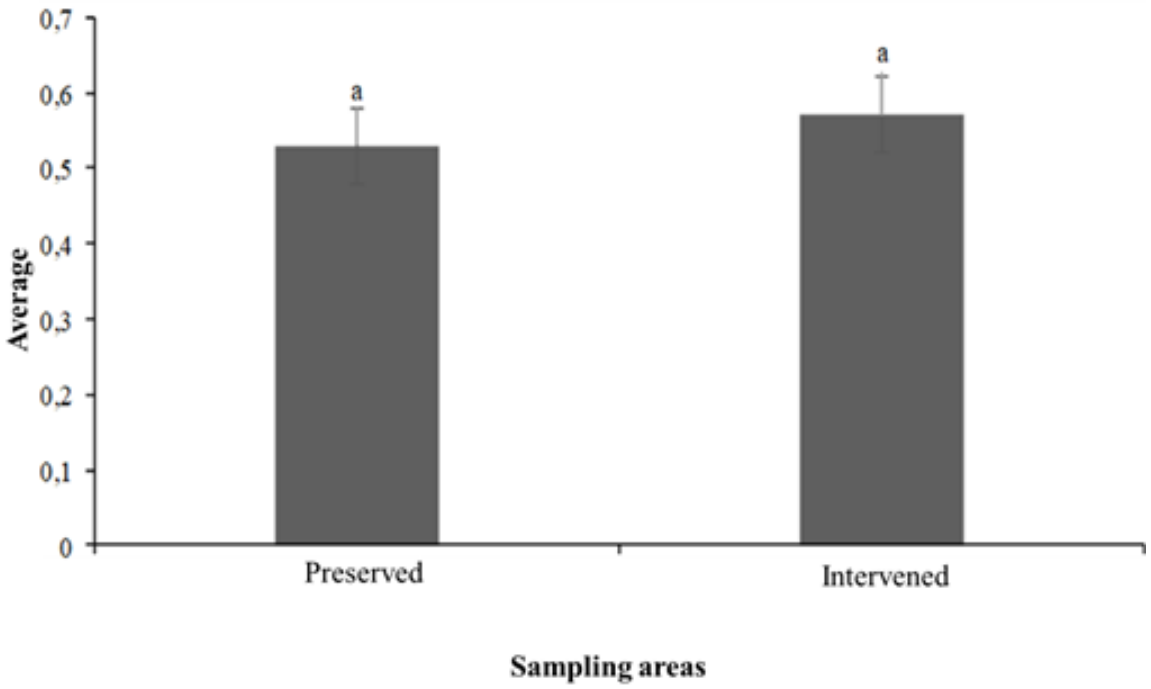

Fig 6. Average frequency of cellulolytic species for the conserved area and the intervened area.

The bars in the average indicate the standard error. Averages followed by equal letters, do not present statistical differences according to the t-student test.

Microbial growth depends on the influence of organic materials such as cellulose, which largely depends on the availability of carbon in the soil [26], taking into account how close the two sampling areas were, regardless of the fact that one is conserved and the other intervened, this could be a reason why the availability of carbon in the two soils was similar. On the other hand, the results are contrasted with the work carried out by Beltrán, in this case they were differentiating the microorganisms between two areas with different degrees of intervention and there were no significant differences in the count of microorganisms in two areas (p> 0.05) [26].

For the phosphate solubilizing microorganisms there were no statistical differences (P <0.05) between the species frequencies of these microorganisms between the two sampling areas (Figure 7).

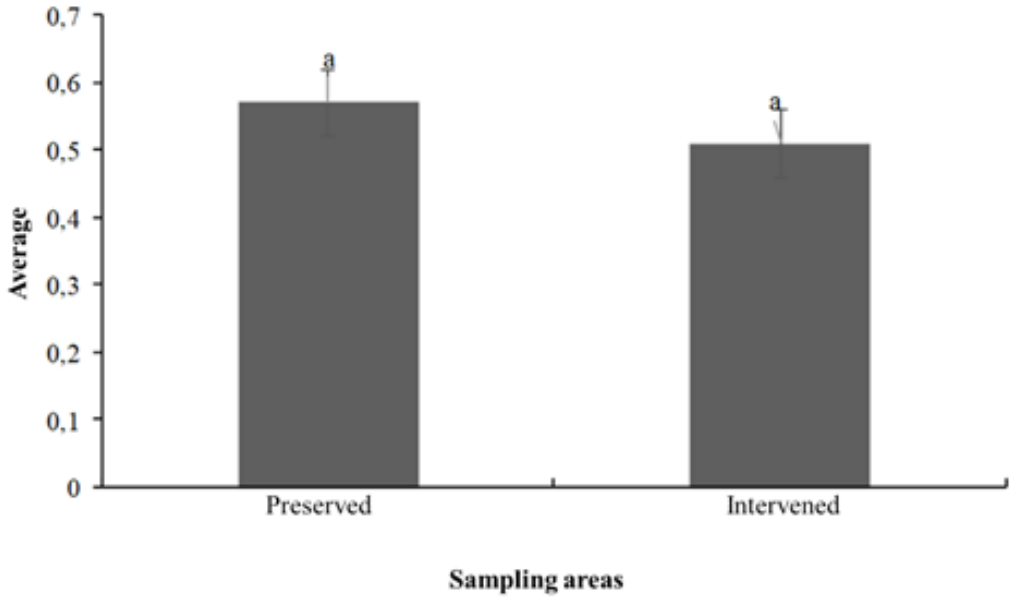

Fig 7. Average frequency of phosphate solubilizing species for the conserved zone and the intervened zone.

The bars in the average indicate the standard error. Averages followed by equal letters, do not present statistical differences according to the t-student test.

In this case, there were no significant differences either, and this can be corroborated according to Nieto in 2017 27 who argues that agriculture is not an activity anthropically causing changes in the state of the soil or in the composition of nutrients, that is, these anthropic activities do not affect the composition of the soil and the change in its microbiota [27].

3.3 Canonical correspondence analysis. (CCA)

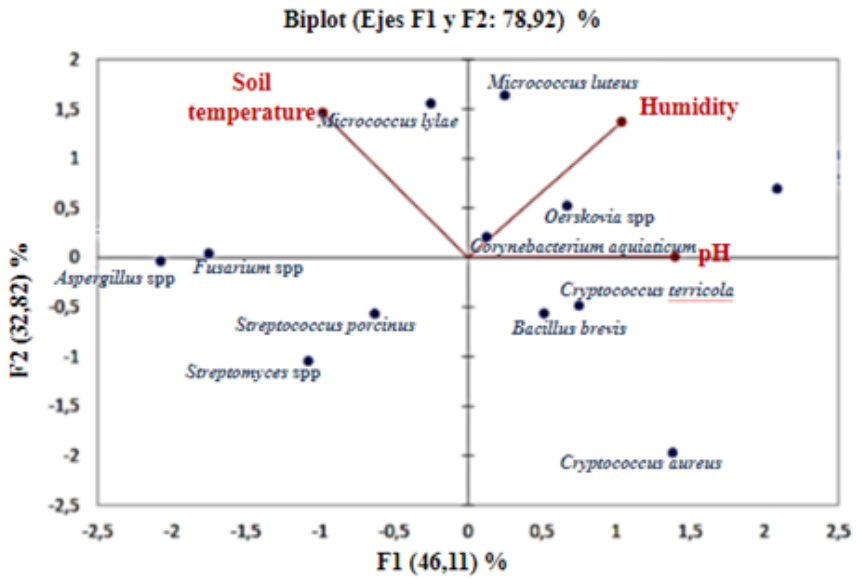

Fig 8. Principal component analysis, Triplot of the relationship between individuals and continuous variables

Figure 8 shows the analysis of main components of the relationship of the identified microorganisms with respect to variables; for the distance of the vectors, it approximates the variability between each variable. The distance between the points, in this case between the species, estimate the dissimilarity between them. The cosine of the angle approximates the correlation between the variables. The lengths of the 3 vectors are observed (humidity, pH, soil temperature): while the vector is closer to the variable, it means that there is variability between them, the length of the vector of the temperature of soil that is closely related to *Micrococcuslylae*. In the case of the humidity vector, a close relationship with *Micrococcusluteus* is observed. This is due to the rhizospheric soil conditions of *Espeletia grandiflora*, high humidity is required for this microorganism to be more frequent in the soil. A higher percentage of humidity favors the activity of some phosphate-solubilizing microorganisms in the soil [28].

Regarding the pH vector, it is related in this case to the microorganisms *Pseudomonasfluorecens*, *Oerskovia* spp., *Corynebacterium aquiaticum*. This is because these microorganisms need a suitable pH for their growth. Factors involved in microbial activity, such as temperature and pH influence the decomposition of organic matter for cellulolytic microorganisms [29].

4 Conclusion

Oerskovia was the most frequent genus in terms of cellulolytic microorganisms, giving *Espeletia grandiflora* the greatest cellulolytic action, thus the genus becomes the most important for plant growth by meeting the carbon and energy needs.

The two sampling areas showed a difference in the ecosystem in terms of the abundance of fraile zones that were observed, taking into account that the intervened area was greatly affected by anthropogenic intervention. However, no significant difference was found regarding the comparison of frequency and species of cellulolytic microorganisms and phosphate solubilizers in these two areas. On the other hand, different relationships were observed between the environmental variables and some microorganisms, this is due to the capacity of each of them for their growth. As for humidity, it is closely related to the *Micrococcus lylae* species, which needs a high percentage of humidity for its growth.

Studies of this type of microorganisms that promote nutrients and growth in *Espeletia grandiflora*, are of great importance in order to arrive at an estimate of which microorganisms could be introduced into intervened areas, so that, in this way, they help with a possible restoration thanks to their relationship in the rhizospheric soil.

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