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Evaluating Different Methodologies for Bioprospecting Actinomycetes in Canary Islands Soils

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Abstract

Actinomycetes are a wide group of Gram positive prokaryotes, the soil being their most characteristic habitat, where they play important ecological functions. Their immense biotechnological potential as producers of bioactive molecules of great commercial and industrial interest is exemplified by most antibiotics for clinical use being derived from this group of bacteria. In this work several methodologies and culture media were tested for the isolation of actinomycetes in soils from three different edafoclimatic areas of Tenerife (Canary Islands, Spain): an arid zone of the southeast coast of the island, a humid area in the laurel forest and the Canary high mountain. The results of this work evidenced that just an air drying of the sample during 7 days (pretreatment) produces high counts of actinomycetes versus non-actinomycetes in the three soils and in any of the culture media studied, making unnecessary post physical and chemical treatments. However, the only method that produced an exclusive isolation of actinomycetes was the use of 0.22-µm filters as a physical barrier, and hence being a tool of unquestionable utility to estimate actinomycetes populations in soil.

The analysis by BOX rep-PCR determined that the richness and genetic diversity of the isolates of these localities are very high. The greatest similarities were, with one exception, found between strains from the same locations. Izaña was one that obtained the highest diversity. Regarding the biotecnology potential as antibiotic produces isolates from Güimar soils are the most promising as 7 out of 10 isolates showed some antibiotic activity.

Introduction

Actinomycetes are a wide group of Gram positive prokaryotes characterized by the presence of filamentous morphology that grow developing a mycelium like fungi [1]. They produce spores from specialized hyphae that confers them resistance to multiple types of abiotic stress, such as dehydration. The actinomycetes (Phylum Actinobacteria) are bacteria widely distributed in natural environments, although their most characteristic habitat is soil, where they represent between 20 and 60% of the microbial population [2]. These bacteria have an immense biotechnological potential, since they are producers of numerous secondary metabolites with commercial and industrial interest, such as antimicrobials, antivirals, and anticancer agents, among others [3, 4]. Many of these are produced as part of their survival strategy because of their slow growth rate makes them a less competitive group. Genus *Streptomyces* stand out for the large part of bioactive molecules they synthesize. It is estimated that this genus is the producer of about two-thirds of the known naturally obtained antibiotics and more than 90% of those used in clinic have their origin in this group [5].

The repeated use of conventional isolation techniques inevitably entails the isolation of the same organisms [3, 6]. From a biotechnological point of view, this implies that the probabilities of isolating new microbial compounds are now too low [7]. However, Watve et al. [8] estimate that antimicrobial compounds production capacity of only the genus *Streptomyces* is of the order of 100,000, which shows the decreases in the discovery of new antibiotics seems to be due rather to a decrease in the detection efforts than to a

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compounds depletion. The importance of this search for new antibiotic substances that can be used in therapy is further increased due to the appearance of strains resistant (multiresistant) to one or several current antibiotics.

Several studies of biodiversity and biogeography, based on classical cultivation processes, as well as derivatives from metagenomic analysis (phylogenetic and functional), have shown clear evidence that environment particular conditions are strong selection factors for microbial populations and decisively influence the abundance and diversity of the microbiota on an ecosystem [9]. This suggests that novel unknown bacterial species or genetically distinct strains, including actinomycetes, can still be found in many ecosystems that have not yet been screened.

The Canary Islands are part of one of the most notable "hotspots" of biodiversity on the planet. A large percentage of the indigenous animals and plants of the Canary Islands are also endemic species, being the region of Spain that has the highest number of endemisms per square meter, 545 plant species and 3165 animal species [10]. Terrestrial environment in different islands have great diversity of habitats which set up favorable conditions for isolation of new microbial diversity. However, until now, a bioprospecting study has never been carried out to explore the biodiversity of the actinomycetes in the islands. Therefore, we hypothesized that, as it happens with higher taxon the Canary Islands can also be a good source of new, yet unknown, microbial biodiversity, which could hosted a great potential from a biotechnological point of view.

The objective of this work was to evaluate the efficiency of different treatments and culture media on the percentage and diversity of actinomycetes isolated. For this purpose we selected soils from three ecosystems in the island of Tenerife which had not previously been explored from this point of view. As expected, results from this work show that it exists a large number and richness of actinomycetes and great possibilities to find novel diversity with new biotechnological potential. In addition, as a first step in the characterization, a phenotypic description of isolated strains was carried out and as an approximation to the evaluation of their biotechnological potential their antimicrobial activity was assessed.

Materials and Methods

Locations and Collection of Soil Samples

To carry out this work, soils were collected from three different ecosystems on the island of Tenerife. Samples were taken from the first superficial centimeters (2–10 cm):

I. *Laurel forest soil (Las Mercedes, 28° 32′ 07.0″ N 16° 17′ 46.8″ W)*. These are high developed and humid soils. The laurel forest is a subtropical forest characterized by mild temperatures throughout the year [11], and high relative humidity, mild summer due to the influence of the trade winds [12]. Most representative species of the Canary laurel forest are *Laurus novocanariensis, Picconia excelsa, Ocotea foetens, Prunus lusitanica, Erica arborea, Sonchus acaulis*, etc.

II. *Coastal soil (Güimar, 28° 18′ 32.3″ N 16° 22′ 40.5″ W)*. Taken from a recent basaltic flow, this corresponds to superficially altered or unaltered rocks, usually covered only by lichens or shrubby vegetation [13]. Subjected to high temperatures throughout the year (> 22 ºC) [11] and have scarce rainfall (203.5 mm^3 average annual precipitation), under the influence of sea salts [12], which makes this vegetation consisted of halophytic and halotolerant represented by species like *Plocama pendula, Lavandula canariensis, Launaea arborescens, Hyparrhenia hirta, Atriplex halimus*, etc.

III. *High mountain soil (Izaña 28° 19′ 02.7″ N 16° 29′ 38.4″ W)*. Soils in this area have high content of recent volcanic material, mainly pyroclastic products such as ash and lapillis [13]. These soils are subjected to great thermal differences between day and night, as well as between winter and summer [11]. In addition, due to the altitude (2.200 m), rainfall is scarce [12] and there is high insolation. The most representative vegetation of the ecosystem are species adapted to low temperatures and high radiation like *Spartocytisus supranubius*, *Pterocephalus lasiospermus* and *Erysimum scoparius*.

Isolation of Actinomycetes: Treatments and Inoculation

Pretreatment

Samples were air-dried for 7 days [14]. Without any other type of treatment, it was considered as control samples to compare growths with the other treatments.

To inoculate the agar plates, serial dilutions were prepared as follows: 10 g of dry soil were diluted in 90 ml of 0.85% saline and then shaken for 30 min to prepared 10^{-1} dilution. The remaining of dilutions were made with fresh sterile 0.85% saline.

Physical Treatment

Pretreatment dilutions were subjected to a thermal shock at 60 °C for 20 min in water bath (adapted from Hayakawa [15]).

Chemical Treatment

1 g of dry soil was diluted in 9 ml of 5 mM phosphate buffer (pH 7.0). This dilution was vortexed for one minute and allowed to stand for another minute. 0.5 ml was taken and added to 4.5 ml of 5 mM phosphate buffer (pH 7.0) with 0.1% SDS [16]. This dilution was introduced in a water bath at 40 °C for 20 min to enhance chemical agent action.

Enrichment Treatment

0.5 g of calcium carbonate ($CaCO_3$) was added to 5 g of soil and placed in a Petri dish with a wet sterile filter paper and incubated 2 weeks at 26 ºC, keeping moist atmosphere adding sterile water when required [15].

Samples Culture

After each treatment, 100 µl of the serial dilutions were plated in duplicate in the following culture media for the isolation of actinomycetes:

- *Plate Count Agar (PCA)* (Scharlab, Spain).
- *Yeast Malt Agar (YMA)* (Scharlab, Spain). This medium was also prepared with soil extract from each sample [17], substituting half of the corresponding water volume, abbreviating YMA+EX.
- *Tap Water Yeast Extract (TWYE)*: [18] is a medium with high shortage in nutrients and consequently greatly reduces the growth of microorganisms. To fulfill this purpose, it was not supplemented with soil extract.
- *Starch-Casein agar (AC)* [19] is widely used for actinomycetes isolation. It was also prepared supplementing with soil extract from each type sample soil, substituting half of the corresponding water volume, abbreviating as well as AC+EX.

Cicloheximide (+CH) at a final concentration of 100 µg/m was added to all medial. Plated media were incubated at 26 °C for 15 days.

Specific Actinomycetes Isolation Based on the Use of 0.22-µm Filters

0.22-µm nitrocellulose membrane filters were placed on Starch-Casein medium and 100 µl of the sample was inoculated, carefully extending to not to reach the filter edge. Plates were incubated at 26 °C. After 4 days the filter was removed and the plate was re-incubated to allow the growth of bacteria that had managed to cross it [20].

Statistical Analysis

Statistical analysis of the data was performed using the SPSS 22.0 program. The comparison between counts obtained with the different methods and culture media was carried out using the one-way Anova test. To evaluate the effect of the different media and treatments used on actinomycetes counts, a factorial ANOVA was used. All differences were considered significant when $P < 0.05$.

Fingerprint and Biodiversity Analysis

DNA from 41 strains was analyzed by rep-PCR fingerprinting. BOX fingerprinting has been widely used in the characterization of strains of the group of actinobacteria due to its high reproducibility and its high correlation with DNA–DNA hybridization tests [21] and 16S rRNA gene sequences [22].

Strains were growth in starch-casein broth for 1 week and DNA extraction was carried out by adding 5 µl of proteinase K 10 ng/µl (AppliChem) and incubated for 3 h at 56 °C. After this, washed sand of 0.1–0.6 mm diameter was added and subjected to three cycles of 1 min vortex followed by 1 min of ice bath. DNA was precipitated with isopropanol, resuspended in fresh Mili-Q water and normalized to 20 ng/µl. Each 25 µl PCR reaction contained: 1 × reaction Buffer, 1 µl DMSO 100%, 1.25 mM of each dNTP, 5.2 mM $MgCl_2$, 2 µM BOXA1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′), 0.12 U/µl Taq polymerase and 2 ng of DNA sample. Temperature profile used was: 95 °C 10 min, 30 cycles of 95 °C 1 min, 52 °C 1 min, 72 °C 8 min, and a final extension step at 72 °C 13 min.

PCR products were run in 1.5% agarose gels prepared with 0.5 × TAE and visualized using Midori Green DNA stain. Gel images were captured with Gel Doc EZ System (Bio-Rad, USA) and analyzed with GelCompar II version 4.00 (Applied Maths NV). Pairwise Pearson product–moment correlation coefficient was used to calculate similarity matrices of densitometric curves, and dendrograms were constructed using the Unweighted Pairgroup Method with Arithmetic Averages Algorithm (UPGMA), optimization value was automatically calculated for each case.

The strain richness index at each location was calculated by dividing the number of strains (unique BOX-fingerprint type) identified by the number of total isolates in each location. The frequency of occurrence of each genetic fingerprint was used to clause genetic diversity at a site (h) using a formula based on multilocus enzyme electrophoresis analysis, as $h = \left[1 - \sum pi^2\right]\left[NT/(NT-1)\right]$[23], where "pi" is the frequency of each unique BOX-fingerprint type (ni/NT), "ni" is the number of isolates with the same fingerprint, NT is the total of isolates per location, and [NT/(NT − 1)] is a correction for small samples.

Phenotypic Characterization of Isolated Actinomycetes

Selected strains were plated in four culture media, two of routine use in this work, YMA and PCA, and two commonly used for the description of actinomycetes, more specifically for the genus *Streptomyces*, ISP4 and Oat Meal Agar [24]. Strains were described after 6 and 15 days of incubation according to the following criteria: color of vegetative mycelium and spores; production of diffusible pigments; morphology of the colony, taking into account the presence of lobes, folds or diverse structures, such as plant-trichome like. For microscopy characterization protocol was adapted from that used for fungi by Pérez-Hernández et al. [25]: 5 mm agar plugs were cut from 1 week growth culture in YMA medium, and placed upside down in a slide. These slides were incubated for another week in a Petri dish with a sterile moist filter paper. Agar plugs were removed and samples were fixed and stained with crystal violet. The isolates were grouped into different approximate genera according to the observed characteristics [24, 26, 27].

Evaluation of the Antimicrobial Potential of Isolated Actinomycetes

The ability of each strain to inhibit the growth of test microorganisms was evaluated against Gram positive and negative prokaryotes, *Escherichia coli* ATCC13706, *Staphylococcus aureus* ATCC 25923 and *Salmonella* enteritidis ATCC 13076 and the yeast *Candida albicans*.

This test was carried out by the cross streak method (adapted from Undabarrena et al. [28]) in Mueller–Hinton medium (Scharlab, Spain) cultivating the central streak for 1 week at 26 ºC. After this, the pathogenic microorganisms were inoculated in perpendicular streaks, from a TSB cultures at 0.5 McFarland, and incubated at 37 °C for 24 h, after which the inhibition of the growth of the test-pathogen microorganisms was observed, in the case of existing.

Results

To select the protocol that produced the greatest success in terms of actinomycetes counts, a combination of several physical and chemical treatments combined with plated on different culture media (as described in the Isolation of Actinomycetes section) were evaluated. The results of the average values for each treatment and medium are shown in Fig. 1.

Isolation of Actinomycetes

Pretreatment

The average counts of actinomycetes obtained from pretreatment of the samples on the different culture media were similar, except between the culture media TWYE+CH (4.40

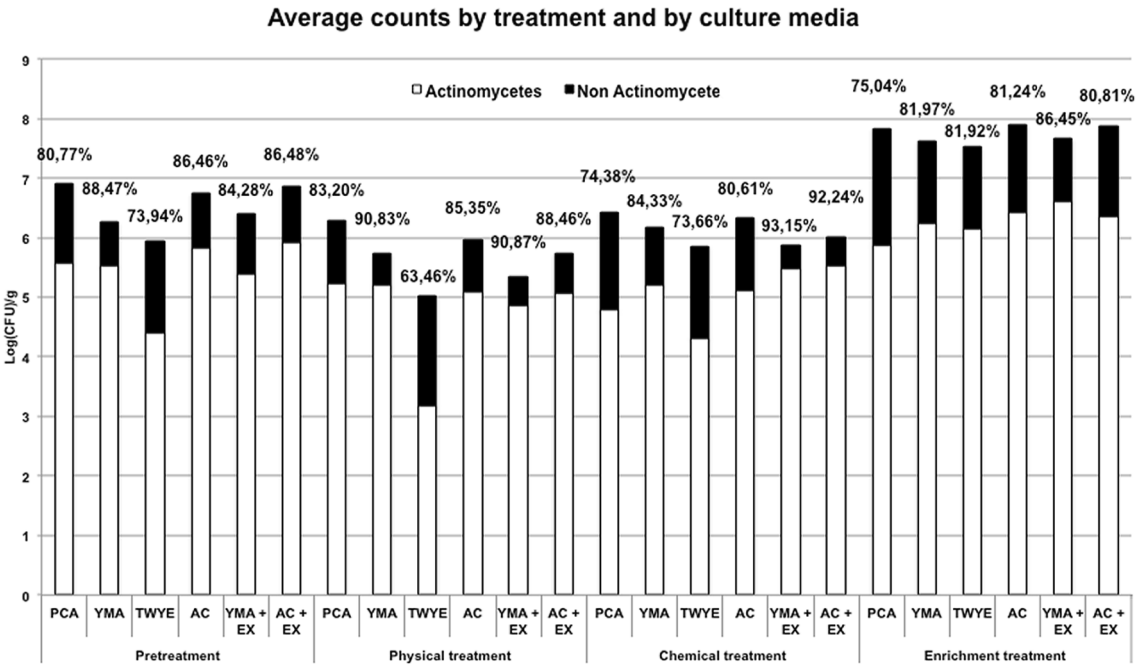


Fig. 1 Average counts by treatment and by culture media. Numbers refer to the percentage of isolated Actinomycetes

logCFU/g of soil) and AC+CH+EX (5.92 logCFU/g soil) ($F=3.13$, $P=0.049$), which were the two media with lowest and highest counts, respectively (Fig. 1).

The counts per kind of soil obtained in this treatment are shown in Fig. 2. Comparing the counts obtained in PCA, actinomycetes in Las Mercedes soils represent 6.38 logCFU/g of soil, and 5.46 logCFU/g and 4.90 logCFU/g for Güimar and Izaña, respectively. The best isolation of actinomycetes for the soil of Las Mercerdes was achieved with YMA+CH (92.05%), YMA+CH+EX for Izaña (88.63%) and AC+CH+EX for Güimar (87.17%).

Physical Treatment

With this treatment, the soil samples analyzed showed similar average counts of actinobacteria in all the tested culture media, although in the case of the TWYE+CH medium, lower counts (3.19 logCFU/g of soil) were obtained and slightly higher in PCA and YMA+CH(5.23 logCFU/g of soil) and (5.20 logCFU/g of soil) (Fig. 1).

The results of this treatment are summarized in Fig. 3, where an apparent reduction in the number of total bacteria and actinomycetes can be observed in all samples compared to pretreatment. The highest actinomycetes percentages were obtained for Las Mercedes on YMA+CH (99.28%), while for Güimar soil was TWYE+CH and YMA+CH+EX (88.88% and 87.51%), and in the case of the Izaña soil, all media used had recovery percentages higher than 90%, except in PCA (87.17%) and TWYE+CH media, in which 0% actinomycetes was obtained. Thus, this treatment with an 83.7% average recovery of actinomycetes against other bacteria, was the one producing the best results in this study.

Chemical Treatment

The average counts of actinomycetes obtained from chemically treated soil samples on the different culture media were different ($F=3.84$, $P=0.026$), giving an average count on TWYE+CH lower (4.31 logCFU/g of soil) than on AC+CH+EX (5.54 logCFU/g of soil), ($P=0.039$), and on YMA+CH+EX (5.48 logCFU/g of soil) ($P=0.029$). This is the only treatment that substantially improves with the addition of soil extract, being the combination of treatment and cultural-medium that represent the highest percentage of actinomycetes isolated (Fig. 1).

The best results for Las Mercedes soil are obtained with the medium AC+CH+EX and YMA+CH+EX for Güimar and Izaña soils (Fig. 4).

Enrichment Treatment

The average counts of actinobacteria were similar in all the investigated media, although we can observe a greater affinity with the YMA+CH+EX medium that improves counts

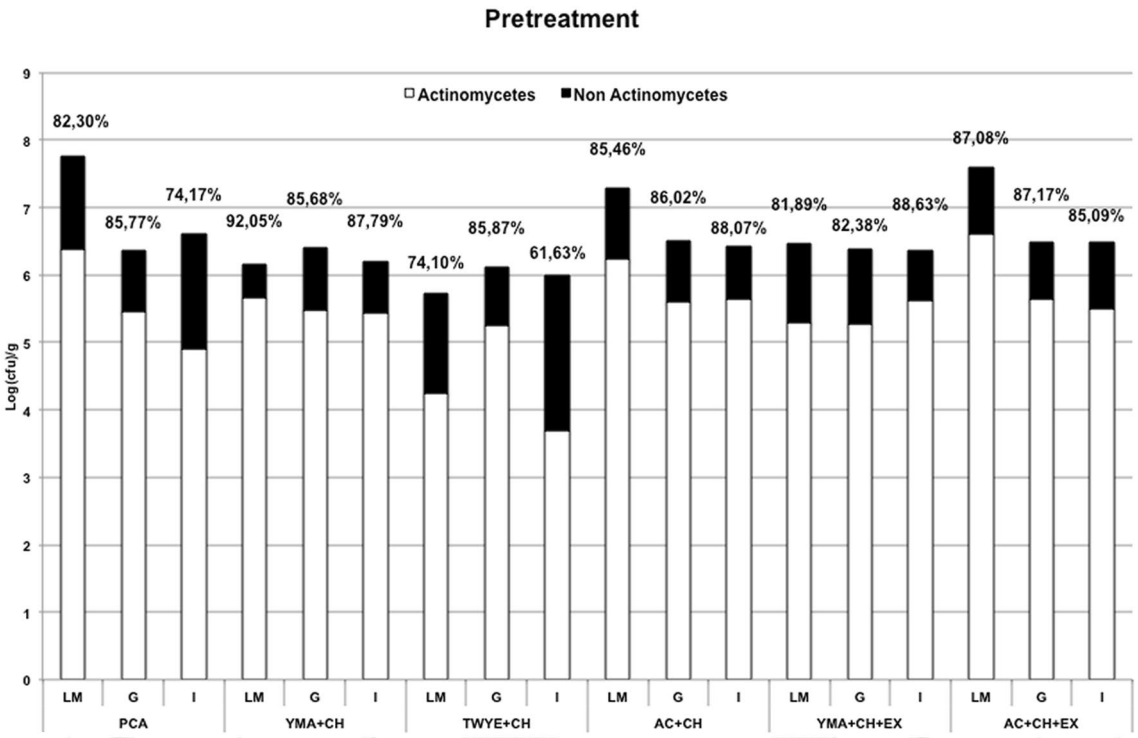


Fig. 2 Pretreatment counts by culture media and by soil. Numbers refer to the percentage of isolated Actinomycetes

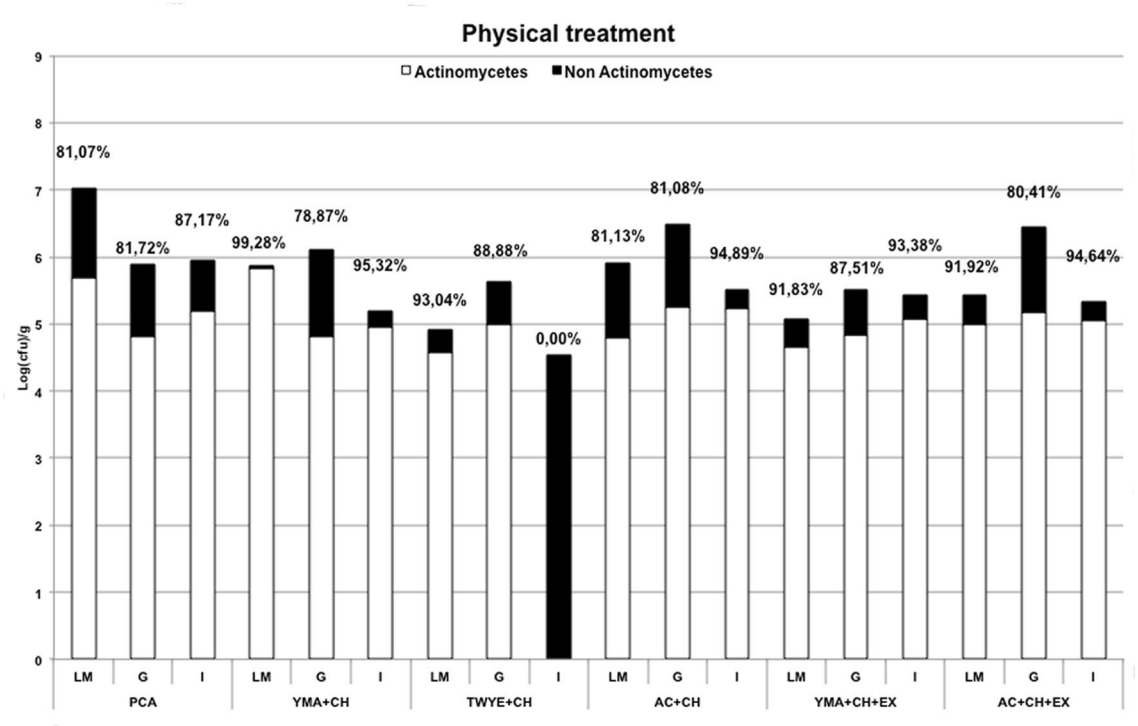


Fig. 3 Physical treatment by culture media and by soil. Numbers refer to the percentage of isolated Actinomycetes

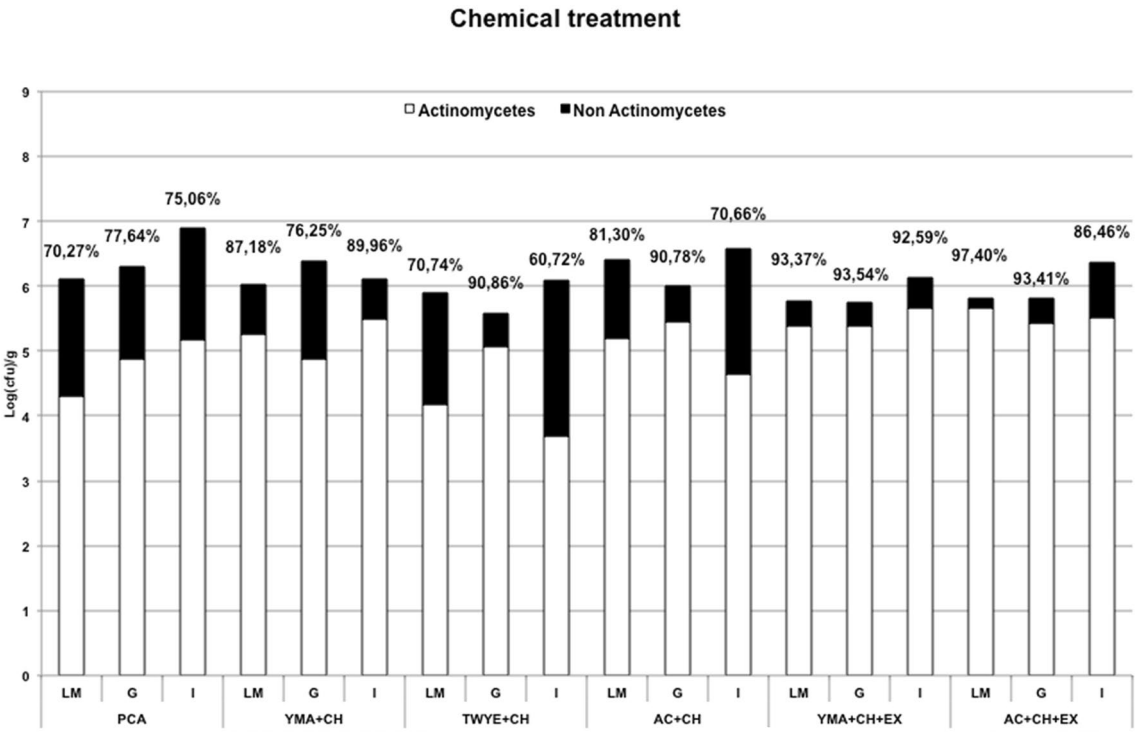


Fig. 4 Chemical treatment by culture media and by soil. Numbers refer to the percentage of isolated Actinomycetes

from 5.88 logCFU/g of soil in PCA to 6.62 logCFU/g (Fig. 1).

Although enrichment produced an increase of the CFU of actinomycetes, the same increase is observed for other bacteria (Fig. 5). Thus, when comparing these results on PCA with those of the pretreated samples (Fig. 2) we can see an increase of the total bacteria in 1.04, 1.08, and 0.64 logarithmic units for Las Mercedes, Güimar, and Izaña soils, respectively, but only in one of the 18 soil-culture medium combinations, a percentage of actinomycetes higher than 90% was obtained (Fig. 5).

Specific Actinomycetes Isolation Based on the Use of 0.22-µm Filters

The analysis of variance showed significant difference between the actinomycete counts of samples plated directly on the surface of plates with media and those with the filter ($F = 21.589$, $P = 0.002$). The average of actinomycete counts using the filters (5.02 logCFU/g of soil) was lower than those obtained when surface planting was used (5.83 logCFU/g of soil). This difference was more evident for the soil of Las Mercedes (4.81 versus 6.24 logCFU/g of soil), and less pronounced for soils of Güimar (5.24 versus 5.6 log CFU/g of soil) and Izaña (5.01 versus 5.65 logCFU/g of soil). However, this treatment is the only one where actinomycetes were exclusively isolated.

Fingerprint and Biodiversity Analysis

From a UPGMA dendrogram of BOX profiles including all actinomycetes isolated (Fig. 6) can be observed that isolates from different locations are disperse throughout the dendrogram. However, the majority of the clusters with similarities above 85% are made up of isolates from the same soil. Few exceptions were isolates g-17M and LM-74 (from Güimar and Las Mercedes, respectively), g-15 M and i-1Q (from Güimar and Izaña, respectively) and i-14, LM-11Q (from Izaña and Las Mercedes, respectively).

To compare diversity among isolates from the same sampled site, individual dendrograms were performed for each location (Supplementary Material, Figs. 1–3). Up to35 unique BOX-fingerprints were obtained from the 41 isolates. Redundant (clone) strains could be those with above 90% similar profiles, such as g-11E and g-15E (99% similar profiles), g68F and g-77M (95.1% similar), LM-111and LM-1M (92.9% similar), LM-87Q and LM-90 (92.6% similar),and g-12M and g-15F (92.3% similar).

The indices of richness and diversity for each location are shown in Table 1. The soil of Izaña was the one showing the highest indices, despite it is the location with less number of isolates. Thus, our results show that the diversity of actinomycetes in the soils of Tenerife is very high with mean diversity of 0.97.

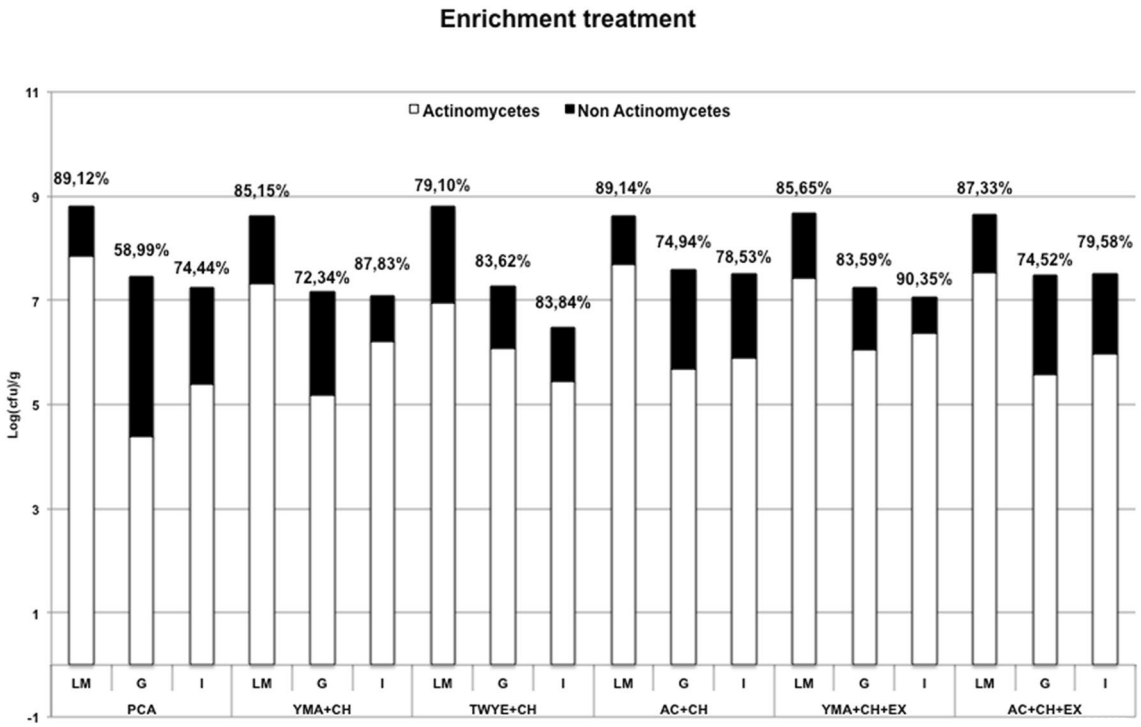
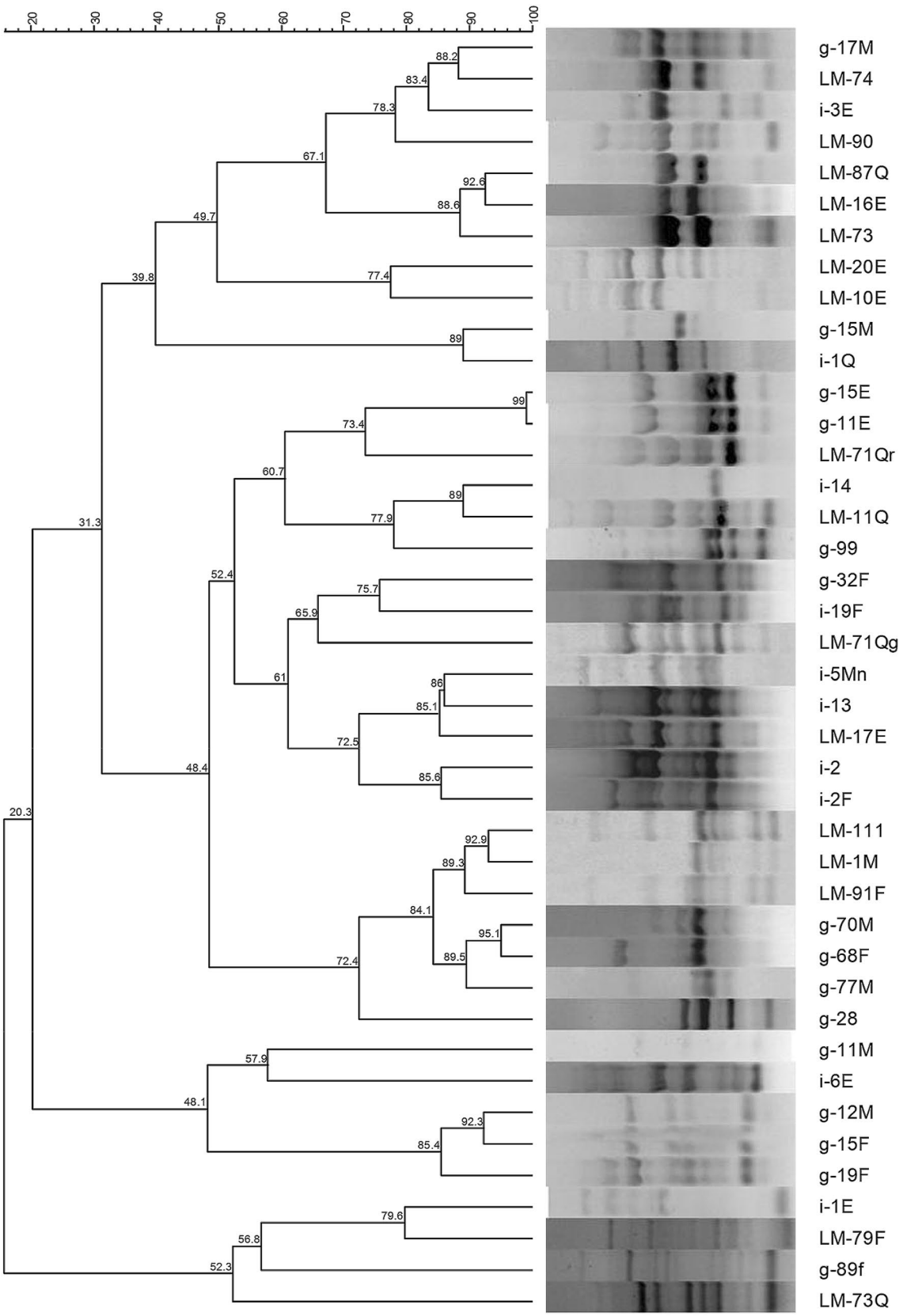


Fig. 5 Enrichment treatment by culture media and by soil. Numbers refer to the percentage of isolated Actinomycetes


20
30
40
50
60
70
80
90
100
88.2
83.4
78.3
67.1
92.6
88.6
49.7
39.8
77.4
89
99
73.4
60.7
89
77.9
31.3
52.4
75.7
65.9
61
86
85.1
72.5
48.4
85.6
20.3
92.9
89.3
84.1
95.1
89.5
72.4
57.9
48.1
92.3
85.4
79.6
56.8
52.3
g-17M
LM-74
i-3E
LM-90
LM-87Q
LM-16E
LM-73
LM-20E
LM-10E
g-15M
i-1Q
g-15E
g-11E
LM-71Qr
i-14
LM-11Q
g-99
g-32F
i-19F
LM-71Qg
i-5Mn
i-13
LM-17E
i-2
i-2F
LM-111
LM-1M
LM-91F
g-70M
g-68F
g-77M
g-28
g-11M
i-6E
g-12M
g-15F
g-19F
i-1E
LM-79F
g-89f
LM-73Q

◂**Fig. 6** Dendrogram of BOX rep-PCR fingerprints. First letter of isolate code correspond with the location: g = Güimar, i = Izaña and LM = Las Mercedes. Last capital letter corresponds with treatment: nothing = pretreatment, F = physical, Q = chemical, E = enrichment, M = 0.22 filter. Clusters were determined using UPGMA with the Pearson correlation coefficient. The similarity percentage is shown in each branch node

Although the number of isolates per treatment is very low to obtain definitive conclusions, it seems that there is no clear redundancy between the strains from the same treatment and that for finding actinomycetal diversity the variable locality is more important than the treatment.

Phenotypic Characterization of Isolated Actinomycetes

Twenty one actinomycetes were selected according their differential morphological characteristic from the total of isolates, trying to avoid clones redundant. Characteristics studied were taken for the presumptive strains identification is shown in Supplementary Material, Table 1. This was based mainly on the microscopic characteristics, according to Fig. 7.

The results showed that 10 out of 21 strains had characteristics compatible with the genus *Streptomyces*. The second genus in importance was *Actinomadura* with 4 strains. Finally, other minority genera were *Nocardioides* and *Nocardiopsis*. The relative predominance of these genera has also been previously described in other soils [7, 29]

Potential Antibiotic Evaluation of Isolated Actinomycetes

A preliminary assay was conducted to detect antibiotic activity against different types of pathogens (prokaryotes and eukaryotes) with a selection of 21 of the isolated actinomycetes. From the 21 isolated selected, 10 presented antibiosis against one or several of the tested pathogens and one isolate (g-68F, a presuntive *Actinomadura* sp. strain) inhibited the growth of the 4 indicator microorganisms (Supplementary Material, Table 2). Seven of these antibiosis-positive strains belonged to the soil of Güimar, two to Las Mercedes and one to Izaña.

Discussion

Isolation of Actinomycetes

The statistical treatment seems to reveal that, when observing the data of the three soils together, there is no a single treatment-medium combination that presents notable differences with the others, being impossible to determine a single effective isolation protocol. This suggests that the characteristics of each sample, either by its texture, amount of organic matter, the local microbiota itself, are important factors when selecting a particular treatment.

Treatments and Culture Media Evaluation

The average of the count values obtained only with the air-drying, or pretreatment, of the soil sample are very promising, obtaining, in all media, values higher than 70% actinomycetal colonies versus other bacteria (Fig. 1). Moreover, it is also probably the method that more diversity of actinomycetes would conserve from the original soil sample as it is the treatment that entails less manipulation. Only with this pretreatment, results are remarkably better than those obtained by Vieira and Nahas [30], where the CFU of actinomycetes account for around 26% of CFUs of total mesophiles, for three types of soil studied without using the pretreatment.

Regarding the physical treatment, the best results for actinomycetes were obtained for the soils of Las Mercedes and Izaña, with 99.28% and 95.32%, respectively, of colonies belonging to actinomycetes, and in both cases in YMA medium. The second best result was for Güimar soils with 88.88% (Fig. 3) of actinomycetes predominance. Therefore, this physical treatment being the one that shows in general higher percentages of actinomycetes. This isolation treatment is the more commonly referenced for the genus *Streptomyces* [15]. As this is the largest group of actinomycetes that can be isolated from the soil [1], this treatment is recommended for the counting and estimation of the total amounts of actinomycetes in a soil sample. Chemical treatment, that requires the preparation of the soil extract to obtain results comparable to the physical treatment (Fig. 4), would not be advisable to select it as a routine treatment for the intensive isolation of actinomycetes from the islands soils. The enrichment treatment it could be observed that it promotes the growth of actinomycetes, which agrees with previous observations of Tsao et al. [31] that show the positive effect of calcium carbonate, acting also as a carbon source for some actinomycetes and altering the pH in favor of the growth of actinomycetal propagules. In addition, calcium ion favors the growth of their aerial mycelium and consequently the formation of spores [32]. However, the growth of another type of microorganisms is also favored, making it necessary to carry out a greater number of dilutions, usually of one or two orders of magnitude greater than in those subjected only to pretreatment. This method, which adds up to a month from the collection of the sample to obtaining pure cultures of the actinomycetes, makes it not indicated for the isolation purpose.

The statistics revealed that there was no difference in the isolation between the culture media, so, as a whole, there is

Table 1 Diversity of BOX fingerprint in each location

Soil	NT	BFT	Richness	Diversity(h)
Güimar	15	12	0.80	0.97
Izaña	10	10	1.00	1.00
Las Mercedes	16	12	0.75	0.95

Values near 1 represent high richness and genetic diversity

BFT BOX fingerprint type

no real difference between the media supplemented with the soil extract and its versions without it. But in general it can be concluded, in order to achieve a good diversity of actinobacteria, we propose to choose the AC+CH medium, since together with its corresponding version with soil extract it presented the greatest morphological diversity, pigments production, etc. Likewise, the YMA medium presented one of the highest percentages of recovery of actinomycetes against non-actinomycetes microorganisms, in addition, in this medium, a greater development of actinomycetes was observed in several aspects such as size, production of pigments and characteristic aerial structures which greatly facilitates the recognition of the colonies of these bacteria as their subsequent isolation.

It is important to note that these observations emphasize the number of actinomycetes, but not the biodiversity of them. This characteristic can be inferred from the analysis of the BOX patterns, which did not show notable redundancy among the isolates from the same treatment, while the relationship were closer between isolates from the same location. Therefore, the selection of a single treatment is unwise if it is expected to obtain the greatest possible diversity of the sample.

Use of 0.22-µm Filters

For this treatment, the starch-casein culture medium was chosen because, due to its composition, it slows the growth of other bacteria (not actinomycetes), preventing them from masking the slow growth of actinomycetes [20]. Although our results show that with the filters smaller quantity of actinomycetes were recovered, this seems to be due to the fact that the system saturates with greater ease than surface plating, so that the use of very concentrated dilutions does not necessarily result in a greater number of actinomycetes colonies. This is more evident when we observe the differences in the counts between the filter and its equivalent surface plating (pretreatment in AC+CH), in Güimar and Izaña there is a difference of 0.36 and 0.64 LogCFU/g soil respectively between filter and surface plating, while for Las Mercedes, the soil with the highest bacterial counts, the difference was 1.42 LogCFU/g of soil.

Considering the biodiversity, this method could limit the isolation to certain species or genera that are able to cross the filter. However, according to the in situ culture system developed by Gavrish et al. [33] based on the use of membranes for the selective isolation of actinobacteria, it is capable of obtaining more diversity than the serial dilution plating method. Although we know that the system is not comparable to that used in the laboratory, this confirms that the membrane by itself is not selective for a few genera of actinomycetes.

So, in practice, this system can be interesting when estimating the amount of these bacteria as part of routine soil analysis protocols, using sufficiently diluted inoculum, since it allows the exclusive growth of actinomycetes without the need to apply a previous treatment or a specific medium, facilitating recount for the inexperienced.

Diversity of Actinomycetes from Fingerprint of Genomes

BOX fingerprinting has been widely used in the characterization of strains of the group of actinobacteria due to its high reproducibility [21, 22]. In his study with more than 400 strains, Lanoot et al. [21] determines that there is a large correlation between the relationships shown by the BOX patterns and the level of DNA-DNA homology, when this is greater than 80%. On the other hand, Maldonado et al. [22] observed a good consensus between the relationships obtained by BOX profiles and those obtained by the 16S rRNA. In both cases, the level of redundancy is established at around 87% when considering two strains as clones. Considering groups with more than 85% similarity (Fig. 6) we observed that they correspond mainly to isolates from the same soil. This result agrees with Davelos et al. [34], who show that in the spatial distribution of the *Streptomyces* genus the isolates tend to be grouped by isolation location. In our study the isolates with similarities above 90% show profiles visually almost identical, which suggest that they could be clones. Although the number of strains per treatment is too low, it is interesting that isolates within the five possible clonal groups, only 2 have strains were isolated with the same treatment. In the case of LM-73 and LM-74 it was expected a closer similarity due to their identical phenotypic growth on Petri dish. The soil with less number of redundant strains was the Izaña soil, none of its strains have a similarity greater than 86% (Supplementary Material, Fig. 2). We can conclude that both the richness and genetic diversity is high in all three soils (Table 1) when compared with the extractable data from other studies [34] and according to the values obtained for other groups of soil microorganisms [35], as expected from the high diversity within the genus *Streptomyces* in particular, and in the group of actinobacteria in general [21].

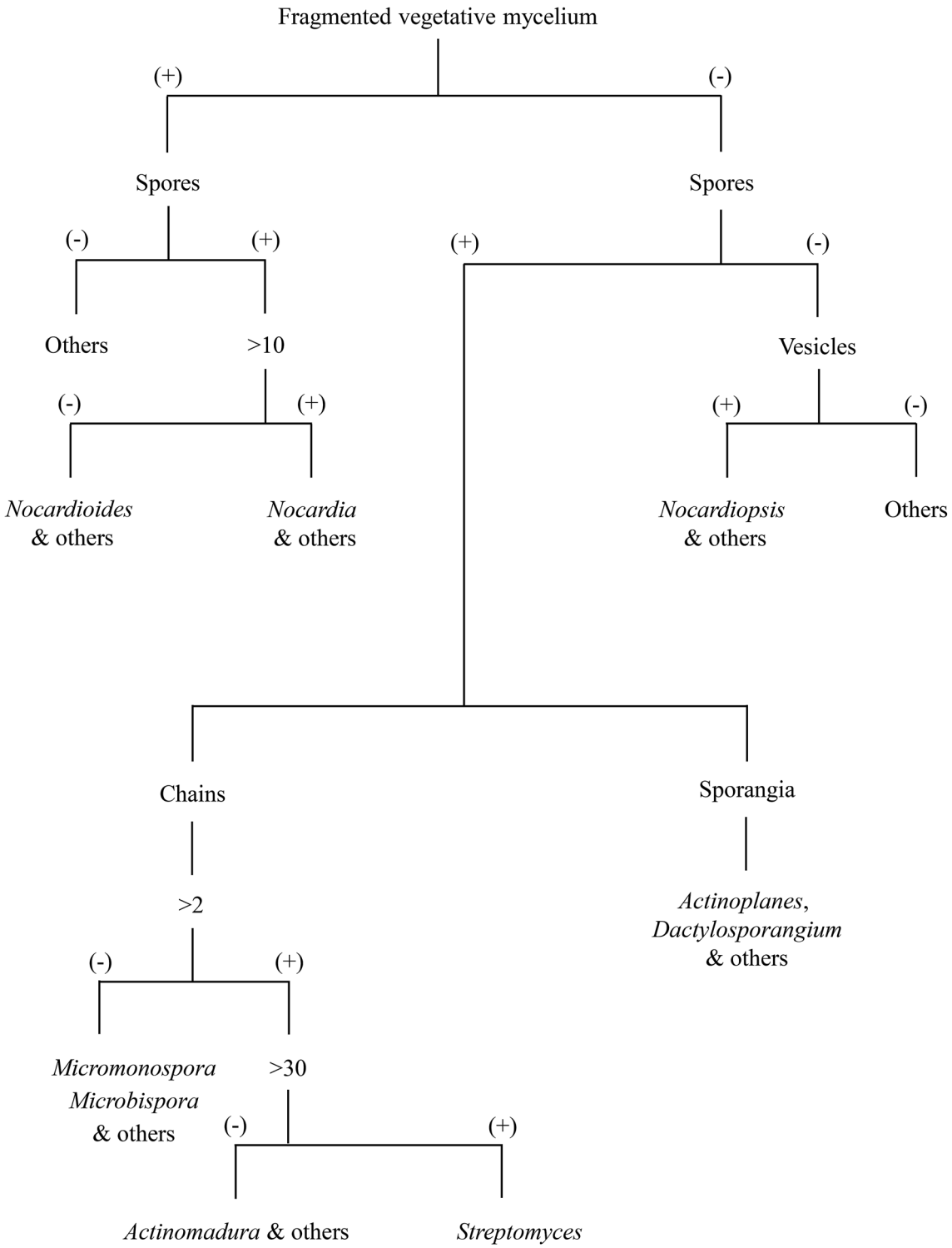


Fig. 7 Actinomycetes diferencial key

Potential Antibiotic Evaluation of Isolated Actinomycetes

Ten of the strains studied (47.62%) presented antibiosis against the indicator microorganisms, the majority isolated from Güimar. In this study, our observations confirm that this activity is more frequent in oligotrophic environments less rich in nutrients [36]. Most of these strains with activity

belonged to the genus *Streptomyces* (Supplementary Material, Table 1), which is consistent with the literature consulted [5, 37].

Conclusions

Results from this show that just a pretreatment of the sample, air-dried for 7 days, is a high effective method for the isolation of actinomycetes, avoiding further manipulation of the sample and more tedious methods. This pretreatment in combination with the rich media, such as AC and YMA, produced satisfactory results in terms of richness and diversity of actinomycetes, which, from their genomic profiles, was proved to be very high for these soils when compared with other similar studies. Among the different treatment here tested, the use of 0.22-µm filters is the only one exclusively recovering actinomycetes, and so it is proposed as the most suitable routine method for counting actinobacteria in soils. Furthermore, our preliminary characterization suggests that the isolated actinomycetal strains belong mostly to the genus *Streptomyces*, a fact that coincides with its great abundance and high number of species currently described. Because of the high capacity of secondary metabolites produced by the species of this genus [8], *Streptomyces* continues to be an interesting group when bioprospecting new ecosystems. Hence the large number of isolates that potentially new strain of genus *Streptomyces* are a great promise for finding new active molecules, which is supported by the relatively high number of isolates that showed antibiotic activity on the plate assays.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interest.

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