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Microbial richness in sandy-silty soil Tunisia and its role in arid zone fertilization

Rim Werheni Ammeri[1,2]*, Salma Kloula[2] & Abdennaceur Hassen[1]

[1]*Eremology and combating desertification (LR16IRA01), arid regions institute of Medenine, Tunisia*

[2] *Laboratoire de Traitement et Recyclage des Eaux, Centre des Recherches et Technologies des Eaux (CERTE), Technopole Borj-Cédria, Tunisie.*

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*** Corresponding author**
rimwerheni@gmail.com

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Abstract

Arid zone are stressful environments, typified by alkaline soils low in organic matter, with biologically limiting extremes in water availability, temperature, and UV radiation. This study aims to analyze the microbial diversity of forest soil and its evolution considering climate change in a typical Mediterranean forest of cork oak (*Quercus suber* L.) and its importance in arid soil fertilization. The investigation was conducted in forest at north-western Tunisia. The study of the quality of the forest soil was based on physicochemical (pH, C, N, EC...) and microbiological (fungi and bacteria) analyses. A collection of strains has been isolated and identified morphologically (form, gram ...) and biochemically (enzymatic activity: catalase, oxidase ...). According to the results obtained, the forest soil has a pH of about 5.03 ± 0.2, C/N ratio 39.82±1.02. The count and fungi, non-filamentous and non-filamentous bacteria on solid soil show a great diversity that confers the fertility of the soil. An important number of strains of actinomycetes (45 isolates) and non-filamentous bacteria (82 isolates) showing distinctive morphological characteristics. The isolates enzymatic activity showed an important value. These obtained results give an idea on the microbe diversity that can be used in the field of bio-fertilization of poor microbial structure soils as the arid zones and oasis system.

1. INTRODUCTION

The forest system is a fundamental element of life (Bouzidi et al 2020). The proliferation of forests through time has composed the air we breathe, perpetuated soil fertility, and generated much of the energy we consume each day (Remacle, 2005). Cork oak (*Quercus suber* L.) is the main native tree species. Over the years, these forests Suffer a degradation that is still poorly understood (Campos et al., 2007). Forest trees was permanently confronted with a range of microorganisms (fungi, bacteria and viruses) (Nelson, 2004, Vasanthakrishna et al., 1995, Rajan et al., 2000). In recent decades, the microbial community in tailings has aroused great interest among many microbiologists. Little was known about the microbial diversity of PG in Tunisia (Trifi et al., 2019). In forest ecosystems, these microorganisms play a determining role on the vigor and health status of trees and thus on the composition and sustainability of these forest ecosystems (Ghazouani et al., 2020). Given the importance of the forestry sector in Tunisia and the lack of knowledge of the microbial population living in the rhizosphere of these forests, it is essential to conduct a study on these populations in order to have a detailed knowledge that takes advantage of the beneficial effects that can be expected and that anticipates or minimizes the adverse effects that can be feared. Thus, it is essential to know their diversities, to study the interactions of these microorganisms with their hosts and to see the effect of abiotic conditions on the development and distribution of these fungi (Werheni et al., 2016). It seems essential to be better able to characterize the diversity of these microorganisms in forest ecosystems (Schimann, 2008). Preceding works of microbial community biomass and structure in dryland systems recommends these features are strongly

correlated with differences in microhabitat (Bates and Garcia-Pichel, 2009; Steven et al., 2013, 2014), which can, in turn, shift function and subsequently alter microbial responses to changes in climate such as altered precipitation (Johnson et al., 2012; Delgado-Baquerizo et al., 2014). For this reason, it is necessary to use a forest soil microbial community to fertilize the agricultural arid soil in different depth. Biofertilizers are substances containing living organisms and organic materials that can be utilized to increase soil nutrients availability and promote plant growth and productivity. They are also considered an eco-friendly way toward sustainable agriculture because they do not cause pollution (Cocking, 2000). Biofertilizers have become a preferable alternative or supplement to organic and inorganic fertilizers (Shariati et al., 2013).

The objective of this study was to determine the microbial richness of forest soil (0-20, 20-40 and 40-60 cm). Different soil samples were subjected to physicochemical (pH, C, N, EC...) and microbiological (filamentous and non-filamentous bacteria) analyses. Thus, some fungal and bacterial isolates were identified.

2. MATERIAL AND METHODS

1. Soil physical-chemical characterization

We collected one 1000 g soil sample from each plot during each sampling period, for a total of 7 samples. Soil samples were taken in Z in a humid forest area covered by cork oaks at the Rajel site in Tabarka. The samples (SI1, SI2, SI3, SII1, SII2, SII3, SIII) were taken at three different depths: 0-10, 10-20 and 20-40 cm (fig 1). The soil samples were well mixed to be homogenized and stored aseptically at -20°C in sterile plastic bags. Before characterization, the soils were dried, gently crushed and sieved to <2 mm (Manuel et al., 2020). The pH, EC, CEC, C total organic nitrogen, soil organic matter, and CaCO3 were assessed according to the Soil Sampling and Methods of Analysis 2 (Carter and Gregorich, 2007). For the soil pH measurements, a 1:2.5 soil water suspension was prepared and left standing overnight. SOM was determined by the Walkley and Black dichromate oxidation method (Jackson, 1958).

2. Soil microbiological characterization

2.1. Bacterial enumeration on solid media

The soil suspension-dilution technique was used for the global estimation of the concentration of soil microorganisms (Hatimi et al., 2007). A series of decimal dilutions was performed from the mother suspensions of different soil samples (10 g of soil in 90 ml of sterile distilled water). Mechanical agitation at 420 rpm for 2 hours will allow the desorption and release of the main groups of microorganisms. These dilutions are inoculated in duplicate and at a rate of 0.5 ml per 90 mm Petri dish containing a culture medium Glycerol-Arginine-Agar, Nutrient Agar, PCA, R2A, ISP2, SCA, Malt Extract and PDA respectively (Mokni, 2009) to achieve a total enumeration of forest soil microorganisms. The total number of colonies (CFU) is determined and all colonies with specific morphological characteristics of actinomycetes are isolated. They are then purified and stored in 25% glycerol as colonies or in a suspension of tweened water with 20%

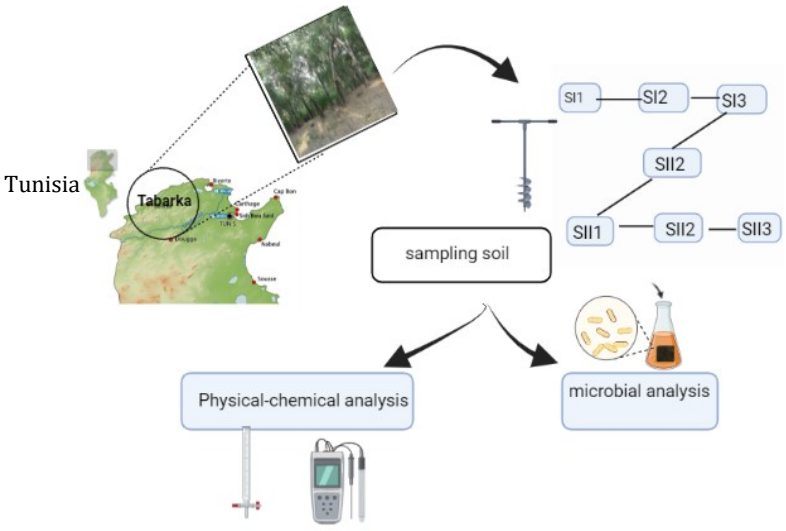

Fig 1. Soil sampling sites and analysis of the Ras Rajel forest in Tabarka (S: sampling site)

glycerol as spores. The isolates are classified into taxonomic groups based on morphological characteristics studied by Cross (1994) and Chevalier (1994) in the 9th edition of the Bergeys manual. Bacterial diversity was often studied by direct counting of viable bacteria on specific solid media (Cherif, 2009).

2.2 Isolation of strains

Following the realization of the counts during 7, 15 and 30 days on the various culture media, the isolation of a dominant bacterial grouping was carried out, on the basis of the morphological aspect. Thus, different colonies were collected and consecutively subcultured on the same medium, until their purification. The strains thus obtained, considered pure, were plated in TSB medium and incubated at 30°C. The cultures obtained were used as biological study material and a part is stored in a freezer at -80°C.

2.3. Phenotypic identification

The isolated bacterial strains were identified according to classical methods, by studying their morphological, physiological and biochemical characteristics. The different strains studied are subjected to gram staining. The cells are fixed and then stained according to the classical method of Zermane (2008). Gram positive cells (very thick peptidoglycans) retain a purple color after this treatment. Gram-negative cells (thin peptidoglycans) are discolored by alcohol and appear pink.

The oxidase test may involve the enzyme cytochrome oxidase, which catalyzes the oxidation of cytochrome C while reducing oxygen to form water (Gerhardt et al., 1981). The catalase test was used to detect the presence of the enzyme catalase in a given bacterial strain. The enzyme catalase serves to neutralize the bactericidal effects of hydrogen peroxide. Catalase accelerates the decomposition of toxic hydrogen peroxide (H_2O_2) into water and oxygen with rapid bubble formation.

2.4 Testing the enzymatic activity of isolates on solid media

The culture media used to test CMCase, xylanase, amylase and cellulase activities are realized for the different isolates. The different tests was made in mineral salt medium supplemented with organic substrate. The mineral salt medium composition was (mg/L): 800, Na_2HPO_4, 200 $MgSO_47H_2O$, 10 $CaCl_22H_2O$, 500 mg NH_4Cl and 1ml trace de metal constitute (mg/ml) : 5 FeSO47H2O , 4 Zn SO_4H2O, 0.2 MnSO4 4H2O, 0.1 NiCl6H2O, 0.1 H_3BO_3, 0.5 $CoCl_26H_2O$, 0.25 $ZnCl_2$, 2.5 EDTA. The substrate and reagents for the revelation of each enzymatic activity have been described by Mokni (2009).

3. RESULTS AND DISCUSSION

3.1. Study area and physical-chemical analysis

The area of study is in Tabarka forest zone Ras el rajel. Its geographical coordinates are 33° 53′ 0″ North, 10° 7′ 0″ East. The climate is humid, soils are sampled at four different sites. It is well known that the physico-chemical characteristics of the soil (pH, organic matter concentration) greatly influence the absorption and desorption of pollutants such as PCP (Xunchi, 2005); for this reason, we made sure to study the soil parameters such as pH, conductivity, moisture and C/N ratio. The precise measurement of soil pH at Ras Rajel site in Tabarka recorded an acidic pH of about 5.03 ± 0.2. Thus, it can be said that forest soils have a slightly acidic to neutral pH (Mejri, 2009). This acidic pH favors the uptake of PCP into the contaminated soil (Diez, 2010). The measured electrical conductivity is about 39.7 ± 0.3 µs/cm in the studied soil. The relative humidity values obtained for each soil horizon in Ras Rajel was 13.26% (Table 2). Generally forest soils have an average moisture content that did not exceed 16% (Mejri, 2009). The forest soil was rich in organic matter (13.38%), this richness is represented by the high percentage of C/N ratio (Table 1). The

Table 1. Physicochemical characterization of the forest soil Tabarka (Ras Rajel)

Parameters	Humidity %	N %	C/N	pH	EC (µs/cm)	Ni	Cu
Forest soil (Tabarka, Tunisia)	13.26	0.22±0.01	39,82±1.02	5.03 ± 0.2	39.7 ± 0.3	71,54±10.96	65,57±1.25

C: carbon, N: nitrogen, Ni: nickel, Cu : copper, EC : electrical conductivity

studied soil was more fertilize than arid zone soil (Ben Hassine et al., 2017).

3.2. Microbiological characterization of the soil

A microbiological study of the forest soil contaminated by PCP, with the aim of highlighting the micro-organisms that grow or survive in this type of soil, was conducted.

The results of bacterial enumeration performed on the six culture media tested were recorded in Fig 2. From this histogram, we showed that PCA, AGA and GN media were found to be the best for bacterial growth. The PCA medium allowed enumeration during a short incubation period at 30˚C, so this medium provides good growth in a very short time of 48 hours. In the case of enumeration of actinomycetes, AGA and ISP_2 media were the most nutritious for the growth of these bacteria. The growth of these bacteria is slow, the colonies start to grow after 3 days. According to the histogram, their number is significant but still lower than that recorded for the other bacteria (Fig. 2). To enumerate the fungi, 8 culture media were used. The GEM and PDA media are the most favorable for the development of fungi. Their appearance requires a period of 10 days of incubation at 30°C (Fig. 2). If we consider the results recorded after the enumeration of microorganisms on the AGA medium, we notice that the number of bacteria and actinomycetes appears is always higher in the contaminated soil compared to that recorded in the control soil. This result can be attributed to the high carbon composition of the forest soil of Tabarka. This richness has the effect of slowing down the inhibitory effect of PCP on the microbial biomass. This is not the case for fungi. Since they represent close values for the control soil and the contaminated soil.

The results obtained are in agreement with work done by Mejri (2009) who showed that the forests of northwest Tunisia, given the richness of these forests in natural species, are richer in microorganisms than cultivated land (Global et al., 2003). After enumeration, a collection of bacterial strains from the contaminated soil and the control soil was isolated and identified.

3.3. Effect of depth on the number of bacteria

Depth is an ecological variable that significantly affects the survival of microorganisms. In the

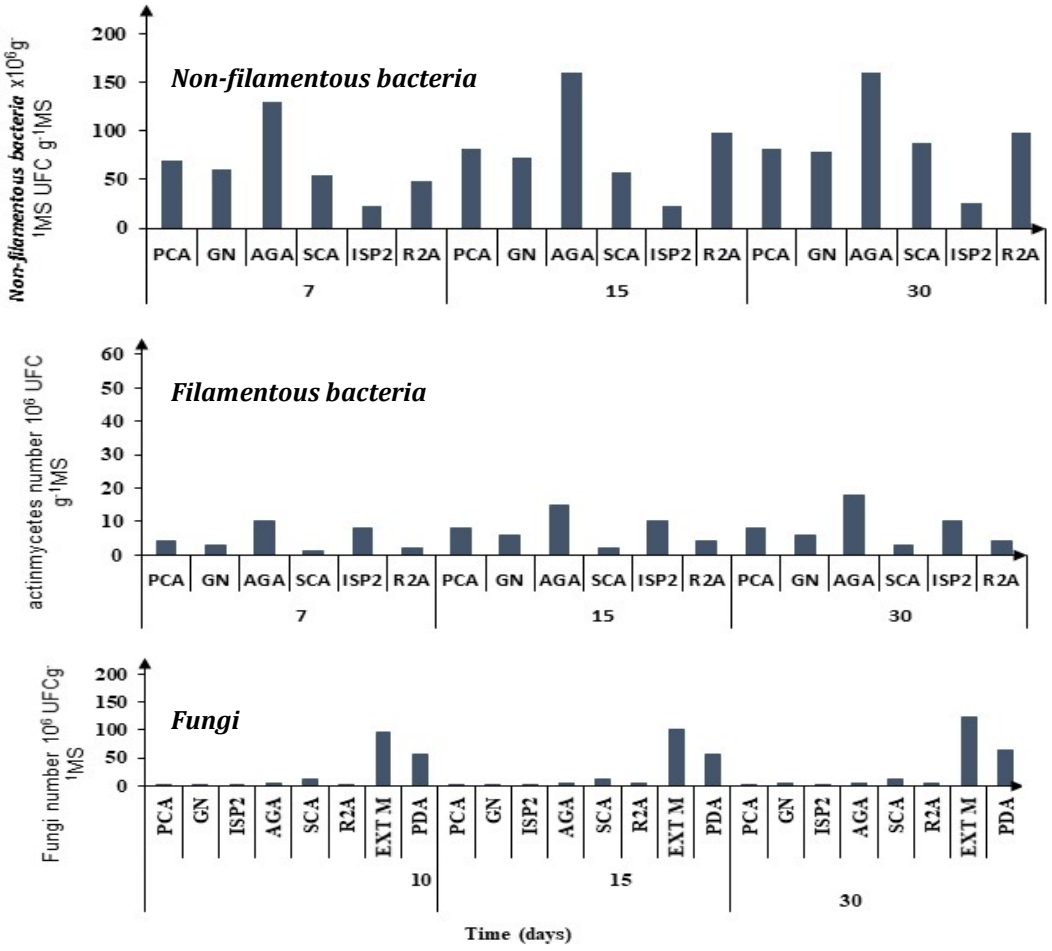

Fig 2. Enumeration of the different types of microorganisms in the control soil and the soil of Tabarka the legend realized by using the different culture media at a growth temperature of 30°C.

evaluation of the number of bacteria in the Ras Rajel forest soil, the depth was not a very interesting factor. Indeed, the enumeration on the PCA medium shows that we have a number of bacteria in the horizon 0-10 cm, it is comparable to that observed by the horizon 10-20 of the order 3.42 .10^8 CFU g^{-1} MS, this number increases in the horizon 10-20 cm, it reaches 4.59.108 CFU g-1 MS, and it has decreased slightly to 20-40 cm to reach 1.43.108 CFU g^{-1} MS for the sampling site SII2 (Fig 3) Bacteria numbers were always slightly higher in the 10-20 cm horizon than in the 0-10 and 20-40 cm horizons. Given, that the effect of O2 availability and abundant nutrients in the superficial soil horizon (0-10 cm) may be at the origin of this distribution of microbial communities.

In temperate zones, if a large part of the microorganisms were concentrated in the first meter of the surface layer, it is in fact the first centimeters that contain the greatest number (Crosinier, 1999) which is not the case in our sample for the 0-10 cm depth. This difference can be explained by the texture, the composition in organic matter and the contamination of the soil.

Indeed, the enumeration remains selective and is not representative of the total microbial flora in the soil. It can only provide a limited idea of the cultivable fraction of bacteria, knowing that many microorganisms require specific conditions (incubation temperature, pH, composition of the culture medium, incubation period, etc.) for their development (Torsvik et al., 1998).

3.4. Morphological and biochemical characterization of the isolates

During the enumeration, we isolated several colonies with distinctive morphologies typical of bacteria from the different samples. This isolation allowed to collect a collection with an important number of strains of actinomycetes (45 isolates) and non-filamentous bacteria (82 isolates) showing distinctive morphological characteristics (Table 2). Once the collection of strains was purified and preserved in glycerol and tweened water at -80°C, we performed a classification based on the morphological and biochemical characteristics of these different isolates.

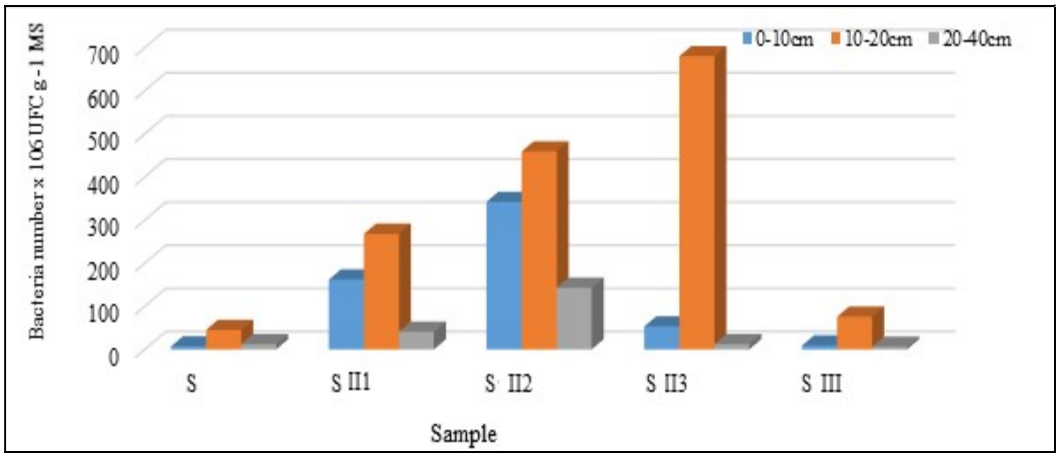

Fig 3. The effect of depth on the enumeration of microorganisms in the three horizons studied 0-10, 10-20 and 20-40 cm on the PCA medium.

Macroscopic and microscopic morphology

Morphological and cultural characteristics are determined using ISP2 and GN culture media. These sterilized media are plated in series with one drop of each inoculum placed on the edge of the agar plate. After 48 hours of incubation at 30°C, we evaluated the phenotypic characteristics of each isolate. Isolates representing the same characteristics were grouped together. The results recorded in Table 2 showed the presence of two main phenotypic groups.

A P-group: the bacteria generally have a soft appearance and lack a mycelium. These are bacteria with a microscopic coccoid (Fig 4) or bacillus (Fig 4) shape.

A group G: the bacteria present a hard aspect and most of the colonies are covered by a cottony layer of variable color corresponding to the aerial mycelium (Fig 4). This mycelium may be attached to the agar, detachable or slimy. Most colonies secrete pigments on the surface of

the culture medium (Fig 5). Microscopic observation of these bacteria shows a filamentous form. The filaments are in most cases long and tangled forming clusters (Fig 4).

The isolates give a panoply of shape, arrangement, and Gram stain that is necessary for their morphological classification. The comparison between the characteristics of the isolates obtained and the criteria for the determination of bacteria described in "Bergey's Manual of Determinative Bacteriology" (8th edition, Lechevalier et al., 1989) allowed us to identify approximately the genera studied and to conclude that the P phenotype represents bacteria that can belong to Streptococcus, Bacillus, Citrobacter and the G phenotype are bacteria that can be affiliated to the genus Streptomyces.

3.5. Results enzymatic activity of isolates on solid media

Table 2. Aspect of the colonies of the different isolates obtained

Under Groups		Form	Color	Aspect	Pigment diffuse	Aerial mycelium	Vegetative mycelium	Diameter (mm)
P	**P1**	R	Bl. Y	Mo	-	-	-	0.9
	P2	R	Bl	Mo	-	-	-	0.9
	P3	R	Cr	Mo	-	-	-	0.1
	P4	R	Bl	Mo	-	-	-	0.1
	P5	Ir	Bl	Mo	-	-	-	2
	P6	R	Bl	Mo	-	-	-	3
	P7	R	Cr	Mo	+	-	-	0.9
	P9	R	Or	Mo	-	-	-	0.9
	P10	R	Y	Mo	-	-	-	0.9
	P11	R	Cr	Mo	-	-	-	0.9
	P12	R	Bl	Mo	-	-	-	0.9
	P13	R	Bl	Mo	-	-	-	0.1
	P14	R	Ro	Mo	-	-	-	0.9
	P15	R	Dark	Mo	-	-	-	0.9
	P16	R	Bl	Mo	-	-	-	0.1
	P17	R	Bl	Mo	-	-	-	0.9
	P18	R	Ro	Mo	-	-	-	0.9
	P19	R	Y	Mo	-	-	-	0.9
	P20	R	Ml	Mo	-	-	-	0.9
G	**G1**	Ir	Bl	D	+	+	+	6
	G2	R	Bl	D	+	+	+	4
	G3	Ir	M	D	+	+	+	10
	G4	R	Cr	D	-	+	-	4
	G5	R	Ma	D	-	+	-	4
	G6	R	B	D	+	+	-	1
	G7	Ir	Bl	D	+	+	+	4
	G8	Ir	B	F	-	+	-	2
	G9	Ir	G	D	+	+	+	2
	G10	Ir	B	D	-	-	-	2
	G11	R	Bl	D	-	+	-	10
	G12	R	Bl	F	-	+	-	4
	G13	R	Bl	D	-	+	+	2
	G14	R	B	F	-	+	-	4
	G15	Ir	B	D	+	+	-	4
	G16	Ir	Ma	D	+	+	+	4
	G17	R	P	V	-	+	-	4
	G18	Ir	G	D	+	+	+	4
	G19	R	Ma	F	+	+	+	6
	G20	Ir	Bl	D	+	+	+	4
	G21	Ir	Ma	D	+	+	+	2
	G22	Ir	Ma	D	+	+	+	10
	G23	Ir	Ma	D	+	+	+	4
	G24	R	Ma	D	+	+	-	4
	G25	Ir	Bl	D	-	+	-	2
	G26	Ir	B	F	+	+	-	2
	G27	Ir	B	D	-	+	-	4
	G28	Ir	Ma	D	+	+	+	10

(+) : Positive result; (-) : Negative result; R: Regular; Ir: Irregular; Bl: White ; Y: Yellow; Cr: Creamy; P: Pink; B: Beige; G: Grey; Ma : Brown; M: Purple; Viscous : V ; Mo: soft; D : Hard; F: Friable ; Ml: Milky

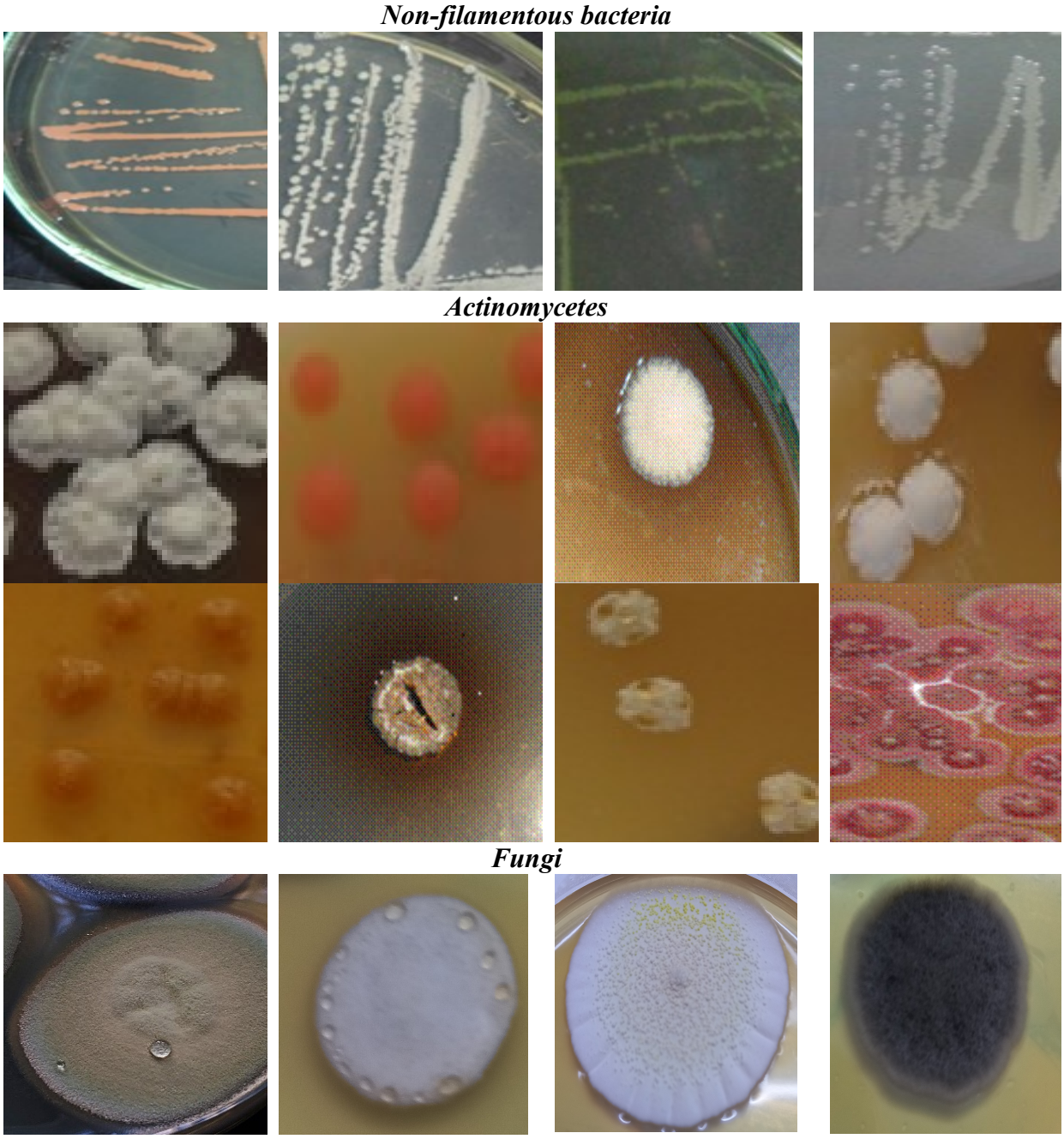

Fig 4. Morphological diversity of different isolates obtained from the forest floor of Ras Rajel of Tabarka.

The identification of soil microbial isolates was based on enzymatic tests such as amylase, xylanase, cellulase, catalase and oxidase. This enzyme has been used in systematic bacteriology for the identification of bacteria. It consists in putting in contact the bacteria to be studied in the presence of hydrogen peroxide. An effervescence (due to the release of oxygen) indicates the presence of a catalase. The results of this experiment showed that 68% of isolates were catalase positive (Fig 6).

The oxidase test is a fundamental test to orient the identification of bacteria. After the addition of a drop of the oxidase reagent N-methylated paraphenylenediamine, a purple coloration appears if the bacterium is able to synthesize oxidase. The results obtained indicate that 51% of the isolates are oxidase positive. In the amylase activity after incubation for 5 days at 30˚C of the strains, amylase activity was revealed using lugol, which reveals transparent areas around the colonies (Fig 6). These colonies correspond to amylase-producing strains. We recorded the presence of 23% of the amylase-producing strains. The xylanase activity was determined by staining with Congo Red, clear halos appear around the colonies, these colonies are able to degrade xylan (xylose polymer,

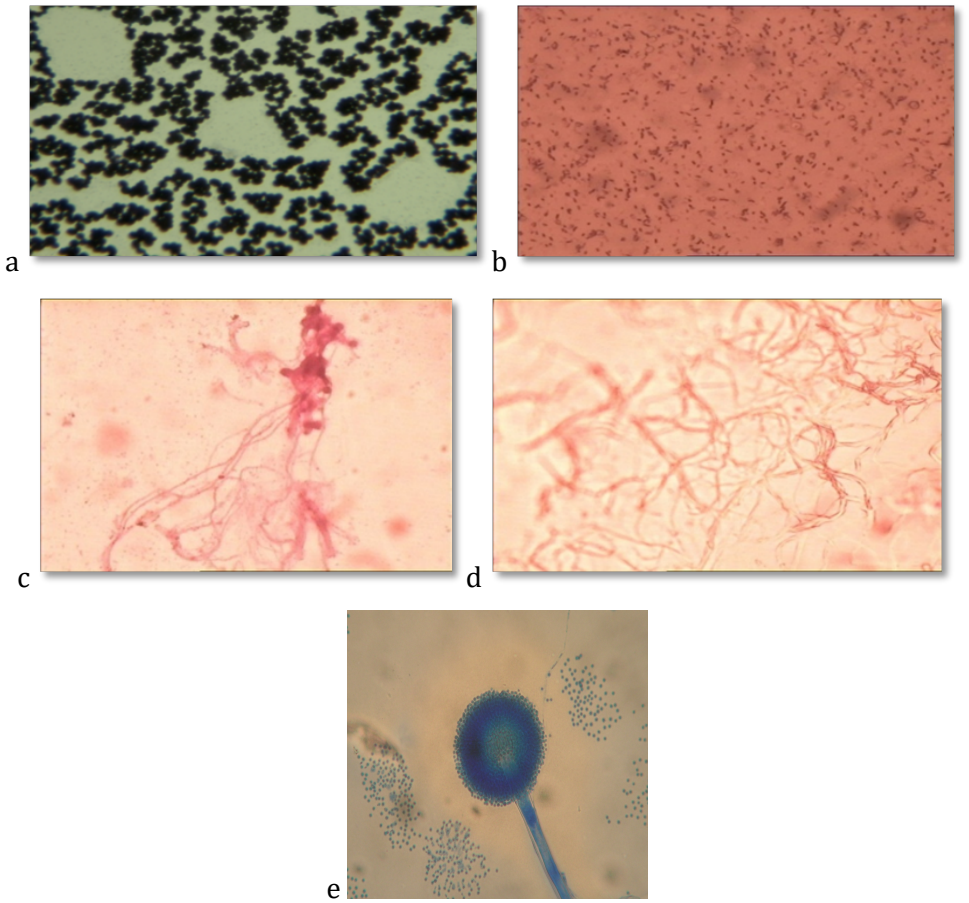

Fig 5. Gram staining results of bacteria as S105 cocci (a), bacilli (b), actinomycete filaments of strain S72 (c), image of actinomycete filament spawning by the scotch method (d), and aspergillus fungi (e) GX40.

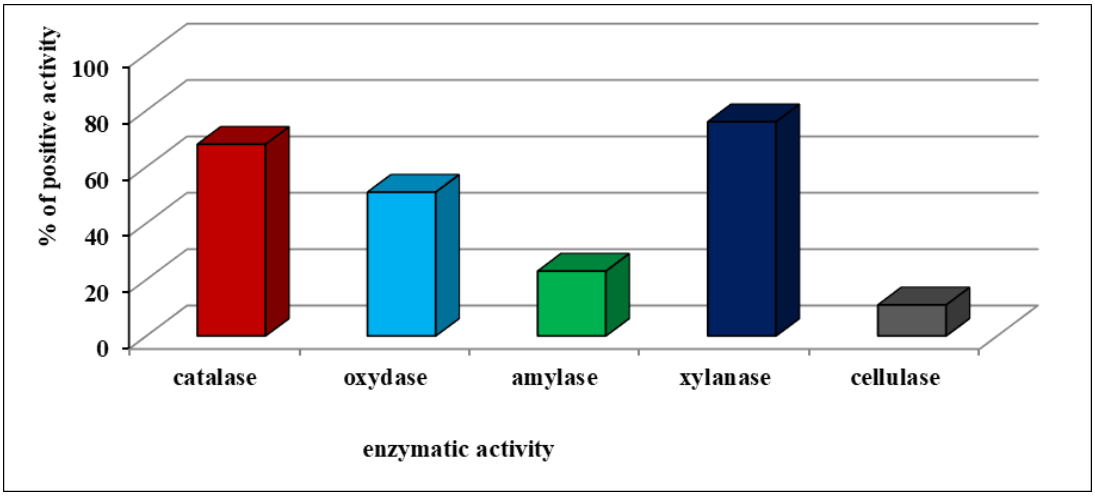

Fig 6. Percentages of active bacteria isolated from the soil of Ras Rajel of Tabarka3

present in the hemicelluloses of wood and food plants) by the xylanase enzyme. According to the results obtained, 76% of strains are xylanase producers. The revelation of cellulase activity was done by Congo red and clear halos appear. 11% of isolates showed positive cellulase activity.

These biochemical tests are a classical approach for bacterial identification. Among the enzymatic activities, the percentages of catalase (68%) and xylanase (76%) activities are the most important (Fig 6). Generally, forest soils was covered by debris of plant origin mainly leaves of cork oaks. This debris constitutes an organic support for the stimulation of the enzymatic activities

(essentially cellulase and xylanase) of the autochthonous soil microorganisms.

2.3 Classification of bacterial isolates obtained from Ras Rajel soil

The classification of soil bacteria is very interesting to get a general idea about the microbial diversity of the studied soil. This classification was based on the morphological and biochemical characters of the isolates. According to the results obtained from this classification we could distinguish two phenotypes P and G.

The analysis of polymorphism of morphological characters, obtained by macro and microscopic observations as well as by enzymatic tests, was carried out thanks to a numerical taxonomy, by the program "MVSP 3.13n" (Multi Variate Statistical Package) using the method of UPGMA (Unweighted Pair-Group Method Algorithm) and the Pearson coefficient.

This approach determines the similarity between the different isolates by comparing their characters based on the presence or absence of the character obtained. This analysis results in the construction of a dendrogram consisting of different groups (Fig 7).

If we consider the lowest similarity coefficient (0.3) we finally confirm the presence of two major phenotypic groups: a P group and a G group. The G group represents 35% of the bacteria and corresponds to the mycelial bacteria, they are the actinomycetes constituted of 25 phenons. The P group represents 65% of the bacteria and corresponds to the non-mycelian bacteria constituted of 29 phenons.

4. CONCLUSION

In this study, the microbiological analyses of the forest soil show a large diversity that is related

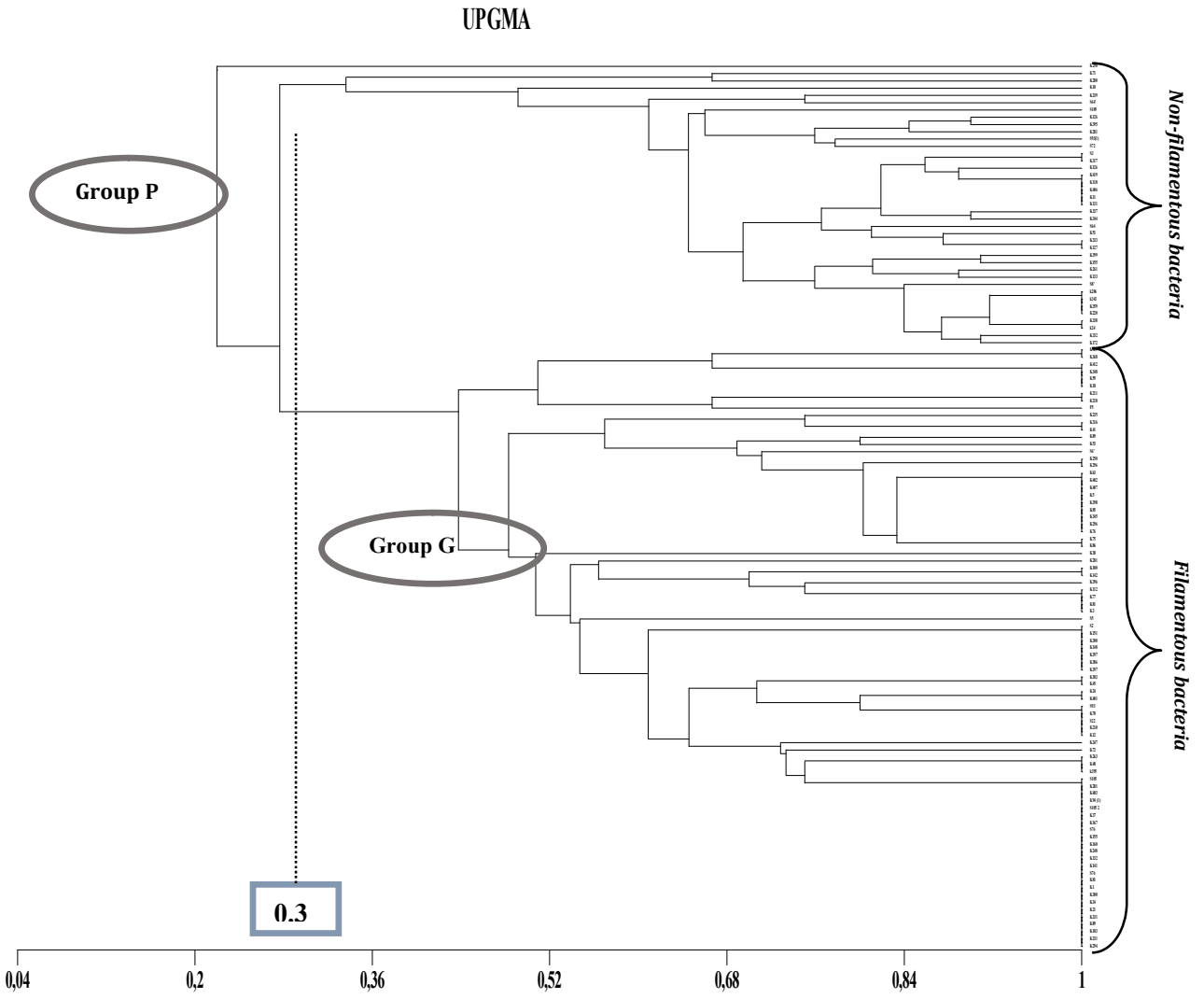

Fig 7. Dendrogram showing 54 phenotypes of isolated bacteria according to their morphological and biochemical characteristics using the MVSP 3 .13n program (Pearson coefficient/UPGMA algorithm).

to the organic richness of the soil. Indeed, the samples were taken at three depths: 0-10, 10-20 and 20-40 cm. After the enumeration, isolation and phenotypic identification of bacteria, we noticed that the soil of the 10-20 cm horizon represents a greater microbial richness compared to the 0-10 and 20-40 cm horizons. In addition, the fungal community is very intense and diversified compared to the bacteria due to the intense humidity in the areas of Tabarka. These results of our study give an idea on the microbe's richness that can be used in the field of bio-fertilization of soils of poor microbial structure as the arid zones.

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