

Biosynthetic Potential of Actinomycetes in Brown Forest Soil on the Eastern Coast of the Aegean Sea

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Abstract—The taxonomic and functional structures of the actinomycetal complex in the litter and upper horizon of the brown forest soil was studied in a *Pinus brutia* var. *pendulifolia* forest on the eastern coast of the Aegean Sea. The complex of actinomycetes included representatives of the *Streptomyces* and *Micromonospora* genera and oligosporous forms. Streptomycetes predominated (73.8%) in the soil, and micromonosporous (66.7%) were dominants in the litter. Thirty isolates of ten *Streptomyces* species from five series and three sections prevailed. In the upper soil horizon, species of the Helvolo-Flavus Helvolus section predominated (48%); the *S. felleus* species occurred most frequently. Among the isolated cultures, the *S. globisporus* and *S. sindenensis* species capable to produce antitumor antibiotics were found. The testing of the antimicrobial activity of the natural isolates showed that five strains inhibit the growth of pathogenic *Fusarium* sp., *Alternaria* sp., *Acremonium* sp., and *Bipolaris sorokiniana* fungi. When testing the effect of streptomycetes on the production of cellulases, a high-efficient strain belonging to the *S. noboritoensis* species was revealed. All the streptomycetes isolated from the brown forest soil produced auxins at the rate of 7.8 to 19.7 µg of indole acetic acid/mL of the liquid medium in the presence of 200 mg/L of tryptophan. Twelve isolates of streptomycetes were transferred to the collection of biotechnologically promising cultures for studying their properties.

Keywords: Cambisols, *Pinus brutia* var. *pendulifolia*, soil actinomycetes, structure of the complex, antimicrobial activity, hydrolysis of cellulose, synthesis of auxins

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INTRODUCTION

Actinomycetes are spore-forming, gram-positive bacteria capable to form branching mycelium. They are typical representatives of the soil microbiota and belong to the class of actinobacteria [15]. Actinomycetes are characterized by the capacity to synthesize a great amount of diverse secondary metabolites (antibiotics, antitumor preparations, vitamins, ectohydrolases, phytohormones, etc.) that are widely used in different fields of modern economy [13, 14, 27]. The use of actinomycetes in biotechnological production stimulates the interest to their distribution in nature, since the formation of actinomycetes complexes is closely related to their origin and properties of the soils and entire ecosystems [3].

Studies of the actinobiota in the soils of different natural zones and geographical regions allow us to assess the regional potential of actinomycetes for its use in modern biotechnologies. In this regard, the so-called “hot spots of biodiversity” are of special interest. Those are territories with a significant concentration of endemic species and the absence of strong transformation of their natural habitats. The eastern

coast of the Aegean Sea (western Anatoly) is included in one of the twenty-five global “hot spots” with the high natural diversity and concentration of endemic species [21]. Under coniferous–broadleaved and coniferous forests, brown soils of different composition are widespread [6]. Specific features of soil-forming processes related to a well-pronounced summer moisture deficit and domination of an endemic variety of *Pinus brutia* var. *pendulifolia* in the vegetation cover are also responsible for the specificity of the local actinobiota.

Previously, actinomycetes in this region were studied in specific ecotopes, such as karst caves [31, 32], hydrothermal springs [12], and marine bottom sediments [17, 23]. These studies made it possible to identify a considerable percentage (from 25% [23] to 62% [32]) of strains with the antimicrobial activity among the local isolates of actinomycetes. About 34% of species from the *Streptomyces* [22, 24] and *Streptosporangium* [25] genera isolated from the soils were characterized by the antimicrobial activity relative to some pathogenic and phytopathogenic species. A significant portion of actinomycetes—antagonists with the

wide spectrum and unusual patterns of activity made it possible to consider the local actinobiota as a potential source of new antibiotics [22].

In some works performed in western Anatoly, streptomycetes with the keratinolytic activity promising for their use in tanning industry [19], streptomycetes producing lignocellulases suitable for their use in paper and pulp industry [30], and actinomycetes, including those of the *Thermoactinomyces* genus, producing alkaline protease for the industrial use [12, 17] were identified. The authors frequently noted the thermal stability of physiologically active substances produced by local actinomycetes [17, 19, 24, 32].

Despite the significant biotechnological potential of the actinomycetes isolated in this region, there were no special studies of the complexes of soil mycelial prokaryotes associated with endemic plant species in the given area.

Our study was aimed at the determination of the structure of the actinomycetal complex in the brown forest soil under the *Pinus brutia* var. *pendulifolia* forest and the assessment of the biosynthetic potential of *Streptomyces* species.

OBJECTS AND METHODS

Samples of forest litter and upper soil horizons collected in April 2015 in a forest near the settlement of Ichlemer (36°48'4" N, 28°13'54" E) in the Mugla province (southwestern Turkey) were examined. The soil cover of the forest consists of brown forest soils (Cambisols according to the WRB) eroded to a different extent due to the intensive surface runoff. In some places, a shallow stony horizon is exposed. The litter thickness varies from 0.5 to 3 cm; it is friable, weakly decomposed; needles predominate in its composition. The litter reaction is slightly acid (pH_{KCl} 5.0–5.5); the C_{org} content is 7.2%.

Samples were taken under the crown of *Pinus brutia* var. *pendulifolia* from the litter (0–2 cm) and upper soil horizon (2–10 cm). Two mixed samples from five individual ones collected on the area of 100 m² were analyzed.

The composition and number of colony-forming units (CFU) of actinomycetes was studied by the inoculation of diluted substrate suspensions on agar media with sodium propionate and casein–glycerin agar [4]. Before the inoculation, the substrates were heated at 70°C for 4 h to limit the growth of nonmycelial bacteria. The samples were incubated at 28°C for two weeks. Colonies of different morphotypes were counted separately and inoculated on slant agar. After purification, the cultures were identified according to standard guides [1, 8].

The generic structure of the complexes on the medium with sodium propionate and the species structure of the *Streptomyces* genus on the casein–glycerin agar (CGA) were characterized on the basis of

data on the percentage and occurrence frequency of some taxa.

The identification of the isolated actinomycetal cultures as representatives of the *Streptomyces* genus was based on their characteristic morphological properties: contiguous mycelium, long chains of spores on the aerial mycelium, and the absence of spores on the substrate mycelium. Actinomycetes having single spores on the substrate mycelium and those deprived of or with poorly developed sterile aerial mycelium and contiguous mycelium were preliminarily identified as representatives of the *Micromonospora* genus. Actinomycetes forming single spores on the aerial mycelium or short chains of larger spores than those of streptomycetes on the branches of aerial or/and substrate mycelium were identified as oligosporous actinomycetes [8]. The species of streptomycetes were identified according to the guide by Gause et al. [1] on the basis of their morphological (shape of spore chains) and cultural (color of aerial and substrate mycelia, the presence of soluble and melanoid pigments on diagnostic media) characteristics.

When assessing the biosynthetic potential of the isolated cultures, the cellulolytic activity was determined on the Hutchinson medium with carboxymethyl cellulose (CMC) [29]. The surface of the medium with the developed colonies of streptomycetes was poured with a 0.1% water solution of Congo red for 15 min. Then, the colorant was taken away, and 1 M NaCl was added to the surface; the plates were exposed for 10 min. Taking into account the fact that the products of cellulose destruction are not stained by the colorant, the cellulolytic activity was estimated by the size of a bleached zone around the tested microorganism.

The antagonistic activity of streptomycetes was investigated by the method of agar blocks [2]. Four cultures of phytopathogenic micromycetes and four cultures of bacteria were used as test cultures.

The phyto regulatory activity of the isolates was estimated by their capability for the production of auxins. For this purpose, streptomycetes cultures were grown for four days on a shaker (120 rpm) in flasks (250 mL) with 50 mL of Czapek's medium and 200 µg/mL of tryptophan as a predecessor of auxin. Cells of microorganisms were removed from the cultural liquid using a centrifuge at 6000 g for 10 min. In the supernatant liquid, the concentration of auxins was determined using the Sal'kovsky reagent [20] and a Shimadzu spectrophotometer (model Uvmini-1240) at the wavelength of 540 nm. Dilutions of the standard solution of indole acetic acid (IAA) (Fluca, Switzerland) were used for the construction of a calibration curve. The control was the non-inoculated medium with the added reagent.

The statistic processing of the data was made by traditional methods using the STATGRAPHICS and EXCEL 5.

Table 1. The number and structure of actinomycetal complexes in the brown forest soil under the *Pinus brutia* var. *pendulifolia* crown in the vicinity of the settlement of Ichmeler

Parameter	Substrates under <i>Pinus brutia</i> var. <i>pendulifolia</i> crown	
	litter	soil
Total number of prokaryotes grown on casein–glycerin agar, CFU/g	10 ± 0.21	66.6 ± 22.5
Portion of actinomycetes in prokaryote complex, %	1.9–4.8	12.8–51.6
Number of sections and series of the <i>Streptomyces</i> genus	1	5
Number of actinomycetes grown on medium with sodium propionate, thousands of CFU/g	25 ± 14.2	167 ± 74.7
Number of genera isolated on medium with sodium propionate	2	3
Abundance of representatives of genera in the complex, %		
<i>Streptomyces</i>	33.3	73.0–73.8
<i>Micromonospora</i>	66.7	16.1–16.6
Oligosporus actinomycetes	0	10.1–10.4

RESULTS AND DISCUSSION

The study performed in the *Pinus brutia* var. *pendulifolia* forest showed that the number of soil actinomycetes in the litter and upper soil horizons (tens of thousand CFUs in 1 g of the substrate) was an order of magnitude lower than that under the crowns of *P. sylvestris* in central Russia [5] and *P. koraiensis* in eastern Asia [7]. In addition, the number of mycelial prokaryotes in the brown forest soil on the Aegean Sea coast, unlike in geographically remote pine biomes, significantly exceeded the number of actinomycetes in the forest litter (Table 1). These differences in the distribution of actinomycetes appear to be related to the Mediterranean climate with its strongly pronounced summer deficit of moisture. Therefore, the main place of the actinomycetes concentration was not the litter, which was air-dry in the studied period, but the soil due to some moisture reserves accumulated in it. One can expect that in warm and moist winter periods, under more favorable conditions for the decomposition of plant falloff, the distribution pattern of actinomycetes should be opposite. The maximum population of actinomycetes participating in processing of the most difficultly decomposable polymers should be found (as in other forest biomes) in the litter against the background of a general increase in their population in both substrates.

The higher taxonomic diversity in the soil than that in the litter was related with the predominant localization of actinomycetes in the droughty summer period. At the genus level, along with streptomyces and micromonosporas found in both substrates, oligosporus forms were identified in the soil with a relatively high abundance (>10% of the total number of isolated species). In the soil, streptomyces predominated (≥73%), whereas in the litter, micromonosporas were dominants (66.7%). In the litter composed of the *P. brutia* var. *pendulifolia* falloff, the predominance of micro-

monospores could be a local feature, which distinguished the actinomycetal complex of the soil under *Pinus brutia* var. *pendulifolia* from the actinomycetal complex in the soils under other pine species and in other geographical regions [5, 7].

The diversity of streptomyces species was higher in the brown forest soil than in the litter. Whereas cultures of only one species (*S. candidus* from the Albus series) were identified in the litter, the streptomyces complex of the soil was represented by species of three sections and five series: Albus, Albus Albocoloratus, Helvolo–Flavus Helvolus, Cinereus Chromogenes, and Cinereus Achromogenes. The Helvolo–Flavus Helvolus (48% of the isolates) section predominated. The *S. felleus* species, from which the pikromycin and proactinomycin A were produced, occurred most frequently [1], as well as a new macrolide antibiotic, namely, alirininimycin C, [10] used for plant protection against phytopathogenic fungi. Among the isolates from the brown forest soil under the *P. brutia* var. *pendulifolia* forest, a considerable portion (16.6% each) belonged to *S. globisporus* and *S. sindenensis* strains that are known for their capacity to synthesize a number of antitumor antibiotics (Table 2). A significant potential in the production of antitumor preparations and biopesticides characterizes the *S. xanthocidicus* species [28] and some other species isolated from the studied soil. For instance, *S. hydrogenans*, due to its larvicidal effect is considered promising for the Asian moth control, an agricultural pest of more than 150 crops in Indo-Australian region [18]. Cinerubin [1], an antiparasitic antibiotic, which is produced by *S. niveoruber*, was patented in 1975 in the USA as an effective agent in the treatment of poultry coccidiosis [26].

Due to the production of antibiotics, streptomyces can be regulators of microbial communities restricting the number of phytopathogens on plant roots and determining natural suppressive soil proper-

Table 2. The species composition of streptomycetes isolated from the brown forest soil under *Pinus brutia* var. *pendulifolia*

Section	Series	Species, strains	Antibiotics (from [1, 10, 26, 28])
Helvolo–Flavus	Helvolus	<i>S. globisporus</i> 1T – 8, 3T – 3.1, 3T – 2, 1T – 3, 1T – 4	Actinoxanthine, an antitumor antibiotic C 1027,7, landomycin E
		<i>S. felleus</i> 3T – 1, 3T – 13, 3T – 7, 3T – 2.1, 3T – 8, 1T – 5, 3T – 11, 3T – 12, 1T – 6	Pikromycin, proactinomycin A, alirinomycin C
Cinereus	Chromogenes	<i>S. xanthocidicus</i> 1T – 11	Xanthocydine
		<i>S. noboritoensis</i> 1T – 14	Gygomycin, noboritomycin
	Achromogenes	<i>S. wedmorensis</i> 1T – 12, 1T – 2.1, 1T – 2	Phosphomycin, antibiotics 280
		<i>S. hydrogenans</i> 1T – 10	Not described
Albus	Albocoloratus	<i>S. niveoruber</i> 3T – 5	Cinerubin
		<i>S. sindenensis</i> 3T – 10, 1T – 1.1, 3T – 9, 1T – 7, 1T – 1	Actinomycin D, amicetin
	Albus	<i>S. candidus</i> 2T – 1, 1T – 4.1, 3T – 3	Lemonomycin, nigericin

Table 3. The size (mm) of inhibition zones for the test cultures by streptomycetes from the brown forest soil

Strains	Inhibition zones (mm) for test cultures							
	1	2	3	4	5	6	7	8
<i>S. candidus</i> 1T – 4.1	0	0	0	0	0	0	24	0
<i>S. candidus</i> 3T – 3	0	0	0	25	0	0	0	0
<i>S. felleus</i> 1T – 5	0	0	0	30	0	0	0	0
<i>S. felleus</i> 3T – 1	0	28	0	0	0	0	0	0
<i>S. felleus</i> 3T – 2.1	0	0	0	20	0	0	0	0
<i>S. felleus</i> 3T – 7	0	0	0	32	0	0	24	0
<i>S. felleus</i> 3T – 8	0	23	0	0	0	0	0	0
<i>S. felleus</i> 3T – 12	30	29	25	0	0	0	0	0
<i>S. felleus</i> 3T – 13	0	33	0	26	0	0	0	0
<i>S. globisporus</i> 1T – 3	27	22	0	20	0	0	24	0
<i>S. globisporus</i> 1T – 4	0	30	24	30	0	0	0	0
<i>S. globisporus</i> 1T – 8	0	23	0	26	0	0	0	0
<i>S. globisporus</i> 3T – 2	0	0	0	30	0	0	0	0
<i>S. globisporus</i> 3T – 3.1	0	0	0	0	0	0	33	0
<i>S. sindenensis</i> 1T – 1	0	0	0	18	0	0	26	0
<i>S. wedmorensis</i> 1T – 2	28	30	35	30	0	0	0	0
<i>S. wedmorensis</i> 1T – 12	28	29	29	36	0	0	0	0
<i>S. xanthocidicus</i> 1T – 11	0	0	0	20	0	0	0	0

1—*Fusarium* sp. K-15; 2—*Alternaria* sp.; 3—*Acremonium* sp.; K-15; 4—*Bipolaris sorokiniana*; 5—*Pseudomonas putida*; 6—*E. coli* K17; 7—*Erwinia herbicola*; 8—*E. rhapontici*.

ties. Checking the activity of isolates from the brown forest soil showed that 60% of them inhibited the growth of at least one of eight test cultures. About 17% of the streptomycetes cultures, namely strains of the *S. wedmorensis*, *S. globisporus*, *S. felleus* species, had

an extended spectrum (against three–four test cultures). They suppressed the growth of the *Fusarium* sp., *Alternaria* sp., *Acremonium* sp., and *Bipolaris sorokiniana* fungi. Some isolates of the same species and also of *S. candidus* and *S. sindenensis* were active against

the isolates of the phytopathogenic *Erwinia herbicola* bacteria. At the same time, none of isolated cultures of streptomycetes did inhibit the growth of gram-positive phytopathogenic bacteria (*Pseudomonas putida*, *E. rhapontici*, and *Escherichia coli*).

Actinomycetes can favor plant productivity not only due to the biological control of phytopathogens but also owing to their capacity to produce phytohormones that play an important role in establishing associative relations with plants, stimulating and improving their growth. The ability to synthesize indole acetic acid (IAA) and other auxins is characteristic of symbionts, inhabitants of the rhizosphere, and endophytes isolating up to 80–100 µg of IAA/mL of the cultural medium in the presence of tryptophan—an auxin predecessor [9]. Free-living microorganisms that are not associated with plants can also synthesize auxins and other phytohormones in considerably smaller amounts. When growing on the medium containing 200 µg of tryptophan, all the streptomycetes isolated from the brown forest soil produced auxins in the amounts from 7.8 (*S. felleus* 3T-11) to 19.7 µg of IAA/mL (*S. sindenensis* 3T-9). According to the auxin activity, the cultures were mainly differentiated at the strain level. In some species, the mean values of IAA production differed to a lesser extent varying from 8.1 to 13.6 µg/mL (Table 4). Within one species of bacteria, there were both highly active and slightly active strains. On the average, the auxin production by the cultures identified as *S. wedmorensis* (13.6 µg/mL) and *S. sindenensis* (13.1 µg/mL) was greater than by other species. The species of *S. niveoruber*, *S. candidus*, and *S. noboritoensis*, whose IAA synthesis did not exceed 8.9 µg/mL, could be conventionally attributed to slightly active species. The comparison of the obtained results with analogous data on streptomycetes isolated from podzols of the European northeast shows that these values are of the same order [11]. Using streak plating of streptomycetes isolates on agar medium with carboxymethylcellulose, a screening of the cultures that form bleached zones in the laboratory test with Congo red was performed. This fact points to their ability to hydrolyze cellulose (Fig. 1). Depending on the size of the zone of polymer destruction, the groups of strains with the weak (zones of less than 15 mm), moderate (15–25 mm), and high (CMC lysis zones exceed 25 mm) cellulolytic activity were distinguished. The microscopic analysis confirmed the destruction of the microcrystalline CMC structure in places of developing streptomycete mycelium (Fig. 2). More than a half of the strains studied (58.1%) were characterized by a moderate cellulolytic activity. The portions of weakly active and highly active strains amounted to 24.7 and 17.2%, respectively, of the total number of isolates from the brown forest soil. The maximum cellulolytic activity characterized the *S. noboritoensis* 1T-14 strain (CMC lysis zone is 40 mm). The ability of this species (Table 2) to synthesize cellulose along with antibiotics was noted earlier [16]. No cellulolytic activity was found in the

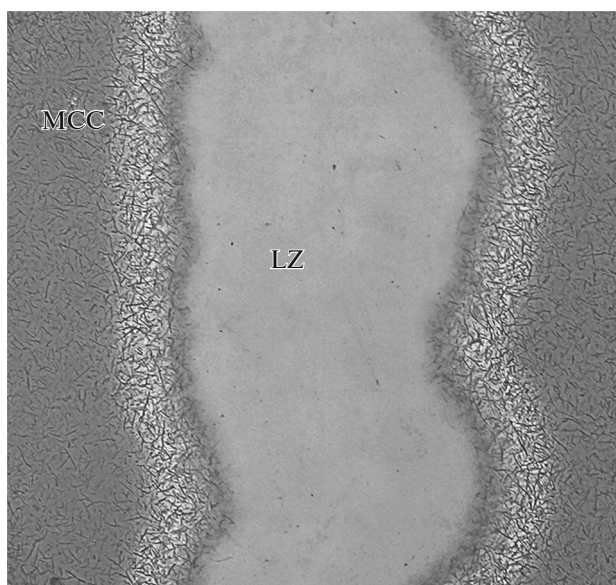


Fig. 1. The formation of bleached zones around *Streptomyces noboritoensis* 1T-14 on the medium with carboxymethylcellulose (CMC). Designations: MCC—microcrystals of cellulose, LZ—lysis zone.

S. hydrogenans species and some *S. felleus* strains (Table 4). For some strains, several kinds of biosynthetic activity were manifested simultaneously. For example, these were the synthesis of cellulases and antibiotics by the *S. felleus* 3T-12 strains, or the pro-

Table 4. Parameters of biosynthetic activity in streptomycete isolates from the brown forest soil

Species	IAA, µg/mL	Zones of CMC destruction, mm
<i>S. globisporus</i>	<u>11.6</u> 8.0–13.7	<u>23</u> 10–30
<i>S. felleus</i>	<u>11.3</u> 7.8–14.7	<u>15</u> 0–30
<i>S. xanthocidicus</i>	11.0	23
<i>S. noboritoensis</i>	8.8	40
<i>S. wedmorensis</i>	<u>13.6</u> 10.2–18.5	<u>26</u> 24–28
<i>S. hydrogenans</i>	9.1	0
<i>S. clavuligerus</i>	11.6	20
<i>S. niveoruber</i>	8.1	25
<i>S. sindenensis</i>	<u>13.1</u> 9.8–19.7	<u>19</u> 14–20
<i>S. candidus</i>	<u>8.4</u> 7.9–8.9	<u>23</u> 10–30

Above the line—mean values, under the line—minimum and maximum values for the species.

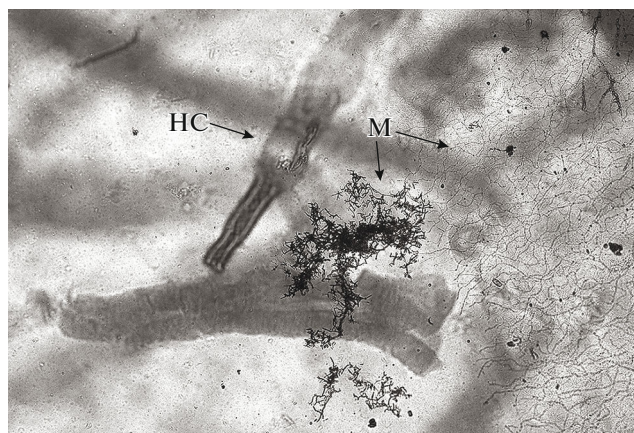


Fig. 2. The microscopic pattern: the growth of *Streptomyces noboritoensis* 1T-14 on CMC and its destruction. Designations: M is the substrate and aerial bacterial mycelium, HC is hydrolyzed cellulose.

duction of auxins and antibiotics by the *S. wedmorensis* 1T-12 strains. As a result of the primary screening, a total of 12 streptomycetes were included into a working collection of biotechnologically promising cultures for investigation of their properties.

CONCLUSIONS

In our study of actinobiota in the brown forest soil under the *P. brutia* var. *pendulifolia* (a species endemic for the eastern coast of the Aegean Sea) forest, some specific features of the taxonomic structure of the complex of mycelial prokaryotes were revealed. These features distinguish this complex from the complexes of actinomycetes characteristic of the soils under pine forests composed of other species of the *Pinus* genus and in other soil-geographical conditions. In the studied brown forest soil, some streptomycetes were identified, and the potential of their biosynthetic activity was shown.

The study of the antimicrobial and cellulolytic activity of the isolates allowed us to identify streptomycetes strains promising in terms of their use in biotechnologies. Primary screening permitted us to select five strains belonging to *S. wedmorensis*, *S. globisporus*, and *S. felleus* species with the capacity to suppress the growth of phytopathogenic fungi, as well as *S. noboritoensis* 1T-14 species with the high cellulolytic activity. The capacity of almost all the streptomycetes isolated from the brown forest soil to synthesize auxins participating in the regulation of plant growth and development has been shown.

The results obtained testify to the considerable potential of streptomycetes from the brown forest soil under the *P. brutia* var. *pendulifolia* forest for the production of agricultural preparations with antifungal, cellulose, and phyto regulatory effects.

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