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INVESTIGATIONS ON RHIZOPLANE ACTINOBACTERIA COMMUNITIES OF PAPYRUS (*CYPERUS PAPYRUS*) FROM AN EGYPTIAN WETLAND

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Wetlands have important global ecological functions, which include carbon storage and water interception. Wetland contributes to the maintenance of regional and global biodiversity. Though many important wetland ecological functions are based on microbial metabolism, we have scanty knowledge on microbial diversity in wetlands.

Plant rhizoplane habitats are considered to harbor highly diverse bacterial communities. Most of the floating mats on river Nile are dominated by papyrus (*Cyperus papyrus*). Papyrus root samples were collected from a floating mat at the "Gold Island" inside the Nile River at Cairo, Egypt in February 1996 and May 1997 in order to investigate the rhizoplane actinobacteria communities. The root-tip regions were cut off, repeatedly washed, macerated and plated.

Using the plate-count technique with three actinobacteria media, an average of 2.1×10^4 $CFUg^{-1}$ root actinobacteria were obtained. All actinobacteria colonies were isolated, purified and investigated by classical and molecular methods. In the papyrus rhizoplane *Streptomyces anulatus, Micromonospora* sp., *Rhodococcus luteus, Verrucosispora gifhornensis* and *Aureobacterium liquefaciens* dominated, moreover *Actinoplanes utahensis*, and *Str. diastaticus* were also present. The physiological traits of the members of dominant groups revealed that these bacteria might be active in the rhizoplane and can be present there in their vegetative forms.

Keywords: rhizoplane actinobacteria, *Cyperus papyrus*, wetland

Introduction

Wetlands are important components of the biosphere and play a significant role in the global cycling and geobiochemical balance of water, carbon, nitrogen, and sulfur [1]. Wetland plant root–microbe interaction sites are of paramount interest since most of microbial activities cumulate in the root environment. Aerobic microhabitats are formed by the oxygen-"releasing" aerenhymatous plants affecting the composition of the microbial community in the water-logged "pseudo"-soil. Not only easily degradable organic compounds (e.g. root exudates) as electron donors are available on the root surface, but the energetically most favored terminal electron acceptor O_2 is also present. Literally a microbial biofilm is formed on surface of aerenhymatous young roots. From the aerobic cuticle layer towards the totally anaerobic peaty "pseudo"-soil of a wetland (with redox-potential values lower than –200–250 mV), an extremely complex, intricate and intercalated mosaic of habitats can be found. The different layers of patches are characterized by an array of combinations of electron donors and acceptors. This may explain the fact that high numbers of microbes, which encompass many genera, are found in wetlands indicating that conditions are favorable for microbial growth [2]. Moreover, wetland habitats may be an important source of organisms with biochemical activities expressed over a wide range of environmental conditions. However, scanty information exists on actinobacteria present in wetlands. The streptomycetes, and other sporulating actinomycetes such as *Micromonospora* are good examples of organisms that are able to cope with the changing conditions of wetland pseudo-soils [3]. Other actinomycetes can be present also [4], though it is generally accepted that not all actinobacteria in aquatic systems are metabolically active [4]. Excepting these generalized statements, no detailed studies on wetland actinobacteria are available. This work attempts to characterize the quantitative and qualitative aspects of the rhizoplane actinobacteria of papyrus (*Cyperus papyrus*).

Materials and methods

Samples were taken on February 1996 and May 1997 from a floating mat dominated by papyrus (*Cyperus papyrus*) at the "Gold Island" inside the Nile River at Cairo, Egypt. A root woven pseudo-soil block (20×20×30 cm) was cut and transferred to laboratory. The lower parts of branching roots of papyrus were freed mechanically, and then root tips with a diameter of 1–3 mm were cut off in a maximum of 5 cm length. Root tips were serially washed (6 times) in sterile distilled water. Washed root mass was aseptically macerated in a mortar and an aliquot of the homogenate was serially diluted using physiological saline and plated on three different "actinobacteria

selective" isolation medium: Starch-Casein (SC) [5] and Malt-Yeast extract (MY) agars supplemented with cycloheximide [6] and Difco-Actinomycete Isolation Agar (DA). Following incubation at 28 °C for 2–3 weeks total and actinobacteria counts were read and actinobacteria were isolated, then subjected to purification. Based on their macro- and micromorphological characteristics, the isolates were divided into 3 groups: (i) "streptomycetes", (ii) "micromonosporae" and (iii) "other actinobacteria". Phenotypical tests (morphological–biochemical–physiological and chemotaxonomic investigations) were performed as described previously [7, 8]. Data were coded for cluster analysis. Similarity calculations were made using the simple matching or Jaccared coefficients [9], trees were generated by the UPGMA algorithm. The SPSS for Windows, release 6.0 statistical software group was used to generate phenograms. In order to help our determinative work in case of a set of representative strains selected by ARDRA analysis [10] partial or full 16S rDNA sequences were determined. DNA isolation, 16S rDNA amplification, cycle sequencing reactions were performed as described earlier [11]. The sequences were automatically determined by an ABI PRISM 310 Genetic Analyser (PE Applied Biosystems). The data were aligned (automatically and controlled manually) to the ARB data base (updated with most recent sequences from Ribosomal Database Project) and processed for similarity calculations by the ARB programme [12].

Results and discussion

The results of germ counting are summarized in Fig. 1. Total germ counts ranged from a maximum of 1.4×10^6 $CFUg^{-1}$ to a minimum of 5.8×10^4 $CFUg^{-1}$. The only comparative data from the literature is that of Das and coworkers [13], who determined the total germ count of rice rhizosphere soil to be 141.7×105 $CFUg^{-1}$. This figure is only 10× higher compared to our results. We have to consider that rice plants were grown in a "true"-soil in their experiment and not in a peaty "pseudo"-soil.

In case of February 1996 sample total germ counts were practically equal on all media. By May 1997 the total germ count values on SC agar increased, but on MY agar decreased. These changes may be explained by the difference in the vegetational period of the plant in Egypt. Papyrus will grow out at the end of December and continuously grow till June/July, then brings flower and will start to senesce in August. In case of a February sample ("early spring") the rising metabolic activity, the growing root tips confer new organic material to the bacteria. In May ("late spring") root growth is slower, cells have thicker walls, thus higher cellulose content and lower exudate release are present. This in turn may cause a structural change in the bacterial communities.

Speculation on effect of seasonality must be corroborated later by analyzing more samples.

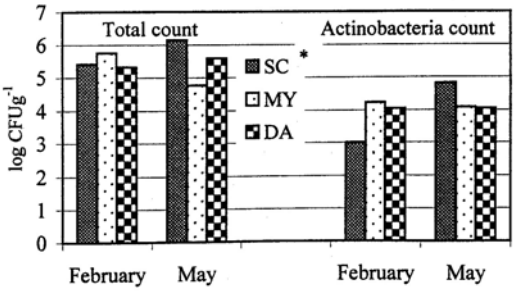

Fig. 1. Results of papyrus rhizoplane total and actinobacteria counts of February 1996 and May 1997 samples (Abbreviations: SC, Starch-Casein agar with cycloheximide; MY, Malt-Yeast extract agar with cycloheximide and DA, Difco-Actinomycete Isolation agar)

The average actinobacterial count of papyrus rhizoplane was 2.4×10^4 $CFUg^{-1}$. Significant changes in figures were observed only on SC agar: the minimum (1.0×10^3 $CFUg^{-1}$) was obtained in the sample collected at February, and the maximum (6.5×10^4 $CFUg^{-1}$) in May. Actinobacteria constitute a significant component of the microbial population in most soils and a count over 1 million per gram is commonly obtained. In case of cattail rhizoplane [14] a similar actinobacteria count was recorded.

One hundred and seven isolates were obtained and characterized. The distribution of different actinobacteria "morphotypes" in samples is shown in Fig. 2. It is interesting to note that in both samples streptomycetes were numerous whereas *Micromonospora*-like and other actinobacteria strains were less abundant.

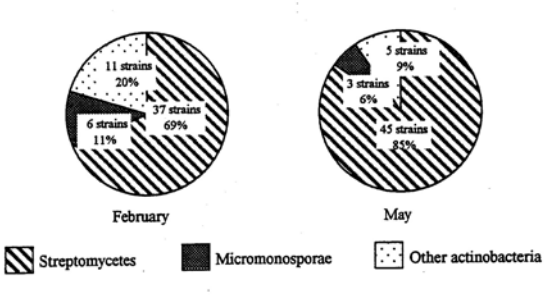

Fig. 2. Distribution of actinobacteria strains isolated from papyrus rhizoplane samples into actinomycete morphogroups

This may be explained by the fact that streptomycetes probably prefer the polysaccharide (cellulose, lignified cellulose, starch, etc.) rich environment or at least

have a selective advantage compared to other actinobacteria. Similar data were obtained in case of cattail rhizoplane (*Typha angustifolia*) [14] but streptomycetes were much less abundant there (in average 35.5%). Besides the relatively good O_2 supply around the roots in papyrus [15] the high streptomycete numbers might be due to the higher amount of native plant debris in the "pseudo"-soil under papyrus strands.

The phenogram of the streptomycete strain group of both February, and May samples is depicted in Fig. 3. The dominant clusters were identified according to the identification scheme of Szabó and coworkers [16] (and taking into consideration the system of Williams and coworkers [7]) as *Str. anulatus*. This "griseus" species is widespread in nature where decaying organic matter is present. Their dominant presence in the rhizoplane actinobacteria community is however surprising, primarily because streptomycetes prefer aerobic environments. However, most of the strains are capable of anaerobic (NO_3^-) respiration. The presence of this species in cattail rhizoplane [14] seems to corroborate this result. From the dominant groups a number of strains were subjected to ARDRA analysis, resulting in 5 groups (1: Nes37, Nes30, Nls15, Nes24, Nls41; 2: Nes20, Nes15; 3: Nls12; 4: Nls22; 5: Nes11). As it is evident from Fig. 4, where the phylogenetic relationship of our strains is depicted, they cluster into three main groups. Strain Nls22 showed a complete identity with species *Str. lavendulae*. Strain Nes11 has been enrolled into a cluster containing two *Streptomyces* sp. with over 99% similarity. We can suppose that Nes11 together with the earlier deposited ones from the database represents new species within the genus *Streptomyces*, close to *Str. griseus*, and *Str. setonii*. Unfortunately, we could not obtain any information on the two streptomycetes present in the ribosomal DNA database, excepting that they were isolated at Palace Leas site, Cockle Park Experimental Farm, Northumberland, UK [17].

Both of Nes20 and Nes30 seem to be closest to *Str. setonii* with an average similarity of ≈99%. This result confirms our previous findings, because *Str. setonii* is a nomenspecies belonging to *Str. anulatus* based on the phenotypical scheme of Williams and coworkers [7]. Phenotypically, *Str. griseus* is a member of *Str. anulatus* (sensu [7]), too. Unfortunately, there is no *Str. anulatus* reference strain sequenced yet, but its close phenotypic relation to *Str. griseus* and *Str. setonii* probably refers to a close genetic relatedness, which is highly possible according to our results. We can assume and repeat our opinion that some strains present in the root environment of papyrus and represented in the phylogenetic tree with strain Nes11, moreover with Nes20 and Nes30 possibly comprise new species. These strains present on the root surface of papyrus are physiologically adapted to this environment, they are phenotypically fairly variable, and have a role in recycling of nutrients. Strains represented by Nes30 were present both in the February and May root samples. They

are assumed as constant partners of papyrus, present in high numbers. Whereas the others were characteristic either in May or in February sample, similarly to other minor phena, being representatives of *Str. lavendulae, Str. violaceus, Str. diastaticus* and some unidentified *Streptomyces* spp.

In the phenogram of "non-streptomycete actinobacteria" from the February 1996 sample (Fig. 5), the strains are divided into two parts. The first part of the phenogram is composed of phena with "coryneform/nocardioform" morphology. The first cluster is designated only as *Corynebacteriaceae*. The taxonomic resolution of the phenetic data determined was inadequate for more precise delineation. The second cluster comprises *Microbacterium* strains. Originally, the genus *Corynebacterium* was created to accommodate the "diphtheria bacillus" and some other animal-pathogenic species [18]. Later the genus became a wastebasket of Gram positive irregular rod forming bacteria with no very special distinctive characters. With the development of chemotaxonomic methods and lately by using genotypic techniques the genus could be grouped into over 15 new genera. By sequencing representative strains from the groups, one seemed to be member of species *Microbacterium (Aureobacterium) liquefaciens* but in the other case no genus level resolution could be obtained. Collins and coworkers [19] reclassified a part of plant originating coryneform bacteria (*Corynebacterium* spp.) with others in a new genus *Aureobacterium* based mainly on chemotaxonomic markers. In 1998, the genus *Aureobacterium* was unified with *Microbacterium*, under the redefined genus *Microbacterium* [20]. Microbacteria seem to be typical soil organisms, though they appear to be present in dairy environment in insects, and sewage, too. One species (*M. testaceum*) has been isolated from a rice paddy. Unfortunately, we have no information on their function in different habitats and have no idea about their importance in papyrus rhizoplane. Strains Nes46, Nes55, and Nes4l, respectively were not identified at species level.

The lower part of the phenogram comprises strains belonging to *Micromonosporaceae*. By using the determinative keys of Bergey's Manual no identification at species level could be obtained [21]. Sequence analysis revealed the presence of *Micromonospora* sp. group, and *Verrucosispora gifhorenesis*. *Micromonospora* species are normal inhabitants of the soil and the sediments of aquatic environments, where they play an important role in the mineralization of organic matter. Members of the *Micromonospora* sp. cluster (close to *M. carbonacea*) utilize a wide variety of sugars and other carbon sources. Their microaerophilic manner should be emphasized, i.e. they are capable of tolerating low O_2 levels and to perform NO_3^- respiration. *Verrucosispora gifhorenensis* was originally isolated from a German peat bog sample [22] near Gifhorn. Accordingly its presence in the root environment of a wetland plant is not surprising.

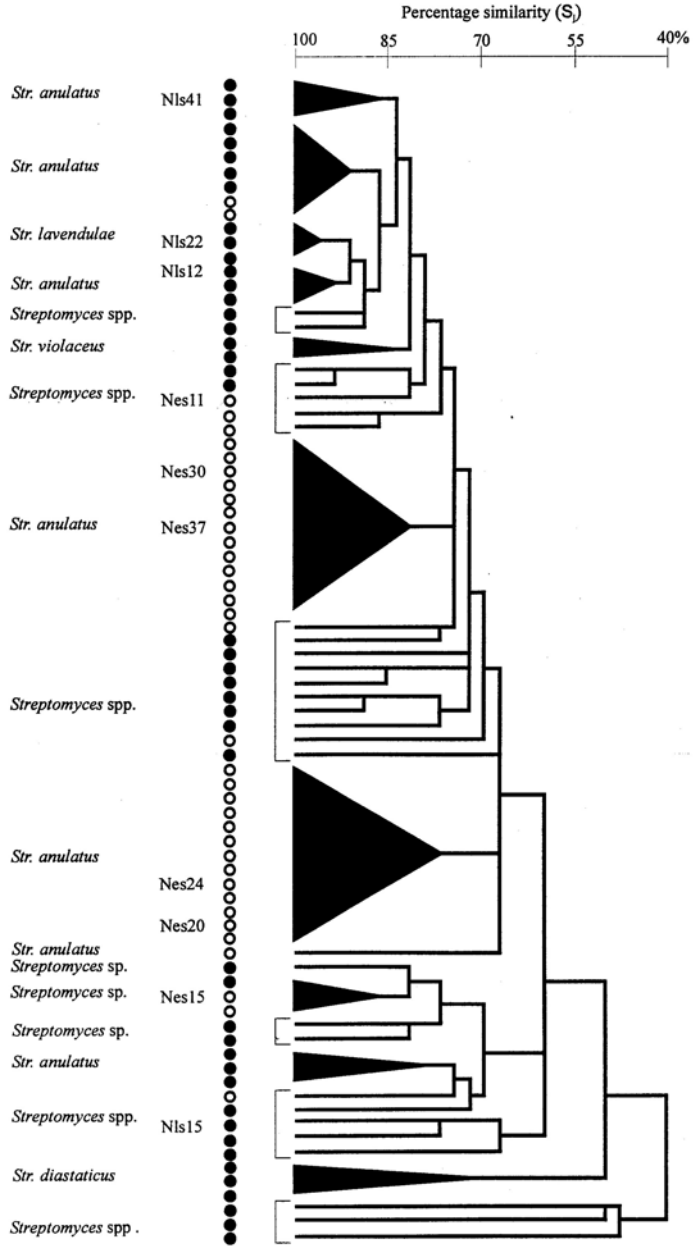

Fig. 3. Phenogram of papyrus rhizoplane streptomycete strains of February 1996 (o) and May 1997 (•) samples. Similarity calculations were made with Jaccared coefficient, the UPGMA algorithm was used to generate the tree (Strains marked with numbers were sequenced)

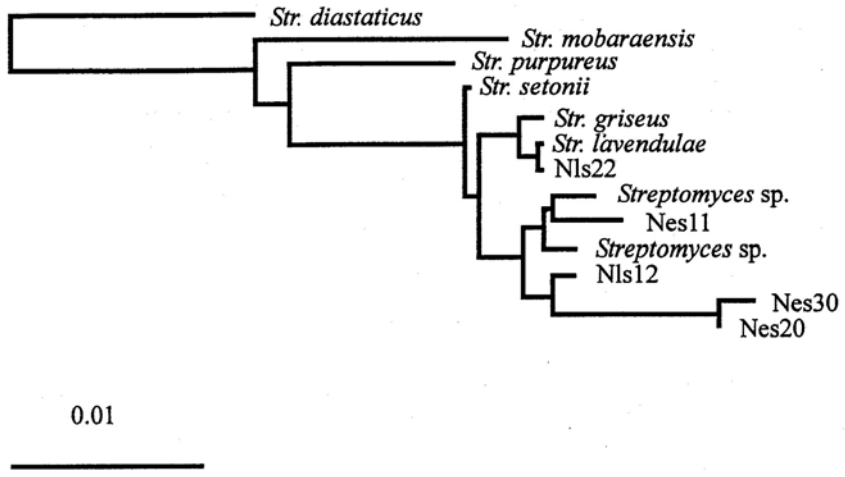

Fig. 4. Phylogenetically ordered dendogram of selected *Streptomyces* strains clustered together with their closest database relatives based on their 16S rDNA sequence comparison. Neighbor joining treeing, evolutionary distances were calculated by Jukes–Cantor correction method. The bar represents 1% dissimilarity (1 substitution per 100 bases) between 16S rDNA sequences

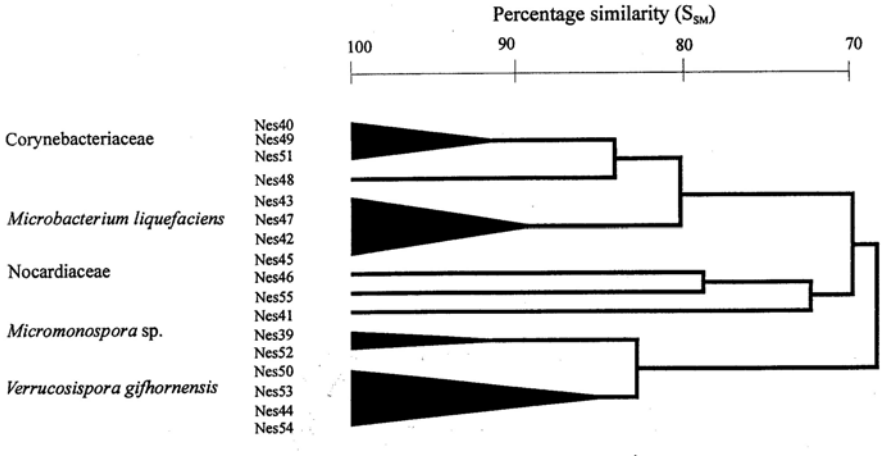

Fig. 5. Phenogram of papyrus rhizoplane "non streptomycete actinobacteria" strains isolated from the February 1996 sample. Tree was generated by UPGMA algorithm, based on simple matching similarity calculations

In case of May 1997 sample, we obtained only two clusters. The first cluster (3 strains) was identified phenotypically as member of *Micromonosporaceae*. It shows typical phoenetic characteristics and chemotaxonomic markers, and based also on its sequence similarity it was delineated as *Actinoplanes utahensis* [23, 24]. The second cluster (3 strains) was identified as *Rhodococcus* sp. After applying 16S rDNA sequencing, the selected strain showed identity with *Rhodococcus luteus*. Representatives of the genus *Rhodococcus* are widely distributed in streams and rivers contaminated by farm effluents [25] and they are common in soils beneath grazed pastures and accumulate in high numbers in lake or river mud. According to Cross [4] this genus represents an example of "wash-in" organisms that can be recovered from aquatic habitats. Our strains, however, are typical autochthonous organisms in aquatic biofilms. Their presence in the root environment is interesting.

The interesting set of our results reveals important similarities and differences compared to data obtained on the actinobacteria community of cattail (*Typha angustifolia*) rhizoplane dominating in a Hungarian wetland [14]. Streptomycetes are overdominant in our case. This phenomenon is less characteristic in case of cattail, though *Str. anulatus* forms a dominant portion of cattail streptomycetes. Concerning the monosporic actinobacteria it is evident that members of *Micromonosporaceae* are present in both papyrus (*Micromonospora* sp., *Verrucosispora gifhornensis, Actinoplanes utahensis*) and also in case of cattail (*Micromonospora carbonacea, M. chalcea, Verrucosispora gifhornensis*). We can suppose that these organisms are well adapted to survive under the special conditions found in wetland plant rhizoplane. Among the actinobacteria in papyrus *Aureobacterium* sp. and *Rhodococcus luteus* were characteristic whereas in cattail *Brevibacterium, Gordona, Dietzia*, and *Micrococcus* species were present.

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