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Follow up characterisation of rhizoplane streptomycetes isolates of *Cyprus papyrus* from an Egyptian wetland and their antimicrobial activities

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During an investigation of actinomycetes from papyrus rhizoplane samples from Egyptian wetland, 120 streptomycetes were recovered, morphologically characterised and assessed for their antimicrobial activity. The dominant rhizoplane streptomycetes were grey colour group, which represent *Streptomyces anulatus*. Most of the isolates (80%) were active against one or more of the organisms tested (two Gram negative bacteria, three Gram positive bacteria, two yeasts and two filamentous fungi). Most of the antibiotic- producing isolates possessed white or grey colour. Strong antibiosis was exhibited against *Staphylococus aureus*, *Bacillus subtilis* and *Aspergillus niger* (71, 60 and 48%, respectively), while only 8% of isolates displayed an activity against *Escherichia coli*.

Keywords: Antimicrobial activity, Egypt, papyrus, streptomycetes, rhizoplane, wetland.

Introduction

Actinomycetes are among the most widely distributed group of microorganisms in nature. They are found in various regions throughout the world, abundantly in all soils both cultivated and uncultivated, fertile and infertile (Goodfellow & Simpson 1987). Their major function is the decomposition of plant and animal residues (Williams & Vickers 1988). This group encompasses genera covering a wide range of morphology, extending from the coccus and rod-coccus cycle through fragmentation hyphal forms to genera with permanent and highly differentiated-branched mycelium (Piepersberg 1993).

Since the discovery of actinomycin, actinomycetes have provided many important bioactive compounds of high commercial value and new bioactive substances are continued to be routinely screened. Approximately two thirds of naturally occurring antibiotics, including many of medical importance, have been isolated from actinomycetes (Okami & Hotta 1988, Takisawa et al. 1993). At least 2700 antibiotics are known to be produced by the genus *Streptomyces sp.* (Watve et al. 2001). In many academic as well as industrial laboratories, the search for novel products is now focused on rare genera or on well-characterised species that are found in unusual environments. The list of novel actinomycetes and products found in microbiologically unexplored environments around the world suggest that a careful exploration of new habitats might continue to be useful (Nolan & Cross 1988, Courtois et al.

2003). In fact, the data concerning the distribution of actinomycetes in papyrus rhizoplane from Egyptian habitats are rare (Rifaat et al. 2002).

The aim of the present work was to undertake a follow up characterisation of streptomycetes isolated from papyrus rhizoplane and to assess their antimicrobial activities.

Material and Methods

Sampling, isolation and characterisation of streptomycetes

Cyprus papyrus root samples were taken in June (2001) from a floating mat at Dahab Island in River Nile, Egypt. The rooting rhizomes of papyrus were freed mechanically in original wetland water and the root tips with a diameter of 1–3 mm were cut off to a maximum length of 5 cm. The roots were washed in sterilised distilled water for six times. The washed roots were aseptically homogenised in water and the macerate was serially diluted (10^0–10^{-6}) and plated (Rifaat et al. 2000). The selective media used were: Starch – casein agar with cycloheximide (SC) (Kuster & Williams 1964), Malt-yeast extract with cycloheximide (MY) (Pridham et al. 1956–57) and Difco-actinomycetes isolating agar (DA). The plates were incubated at 28 ^{o} C for 1–2 weeks. The streptomcetes were isolated and subjected to purification. Adequate phenotypical test set and chemotaxonomical investigations were used for the identification of strains following the method given by International Streptomycetes Project (ISP) (Shrilling & Gottlieb 1966) and (Williams et al. 1983). In order to confirm identified streptomycetes isolates, a selected set of isolates were subjected to partial or full 16S rDNA technique (Pospiech & Neumann 1995). The obtained data were analysed according to de Soete (1983) and Ludwig & Strunk (1997).

Antimicrobial activity

The antimicrobial activity was determined by the plate diffusion method (Bauer et al. 1966) against bacteria (*Escherichia coli*, *Pseudomonas aureginosa*, *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*), yeasts (*Candida albicans*, *Saccharomyces cerevisiae*) and fungi (*Aspergillus niger, Fusarium oxysporium*). Inhibition zones were determined after 24 h for bacteria and yeasts and after 48 h for fungi.

Results and Discussion

Distribution of streptomycetes

Total bacteria and streptomycete counts ranged from 2.4×10^5 to 1.7×10^6 CFU g^{-1}, and 1.3×10^4 to 1.5×10^5 CFU g^{-1}, respectively (Table 1). In relation to the total bacteria counts, percentage of streptomycetes ranged from 0.9 to 8.8. Streptomycete counts varied in the different isolation media used which is in accordance with the results of Rifaat et al. (2002) and Kovacs et al. (1997). Such variation may be due to the presence of NO_3^- as N source in the (CS) medium which favour streptomycetes. Due to changes in nitrification activity, water of the river Nile has a

Table 1. Number of total bacteria and streptomycetes count at different selective media

Name of media	Total bacteria count (CFU*/g)	Streptomycetes count (CFU/g)	Percentage of streptomycetes
SC**	1.7×10^6	1.5×10^5	8.8
MY***	1.5×10^6	1.3×10^4	0.9
DA****	2.4×10^5	1.6×10^4	6.6

*CFU = Colony forming unit
**SC = Starch-casein agar with cycloheximide
***MY = Malt-yeast extract with cycloheximide
***DA = Difco-actinomycetes isolating agar

typical yearly cycle in NO_3^- level reaching a characteristic peak in June. A relative NO_3^- abundance may probably also result from the aerobic conditions which lead to an increase in the nitrification, these are favoured by the active root respiration processes.

Characterisation of streptomycete isolates

In the present study, 120 streptomycete isolates were recovered from the papyrus root samples. These isolates were divided into six clusters (Table 2). Some of the clusters, however, are single member phenons. According to the identification scheme of Williams et al. (1983), the dominant cluster was identified as *S. anulatus* which is in agreement with the results of Rifaat et al. (2002) and Kovacs et al. (1997). The other clusters were identified as *S. lavandulae, S. lydicus, S. rimosus* and *S. antibioticus.* Rifaat et al. (2002) detected *S. lavendulae* in papyrus rhizoplane, whereas the last three clusters are newly reported in the present study. The rest of the isolates could only be identified as *Streptomyces* species. The differences in colour of aerial mycelia of the isolates may indicate the diversity of streptomycete isolates. Distributuion of streptomycetes clusters indicate differences in the degree of adaptation to the root rhizoplane environment among the clusters. Physiologically, these groups differ only in minor characteristics; however, from a broad spectrum of dominant cells present in the root environment members of many different genera could grow into population of significant size.

A selected set of isolates, from different streptomycete clusters, were investigated based on 16S rDNA sequence. Because of the high phenotypical variability of *S. anulatus* and the lack of reliable genotypical data on streptomycetes we chose only two strains from the dominant group for partial 16S rDNA sequencing. The first and second strains showed a complete identity with *S. lipmanii* and *S. griseus*, respectively. This result confirmed the previous results because *S. lipmanii* and *S. griseus* are a nomen species belonging to *S. anulatus* based on the phenotypical scheme of Williams et al. (1983). From the second cluster, which is determined as *S. rimosus*, one strain was chosen for 16S rDNA sequencing. It showed identity with *S. anandii*. This result was also confirmed because *S. anandii* is a nomen species belonging to *S. rimosus* (Waksman & Lechevalier 1953). In case of *S. antibioticus* and *S. lydicus*, the phenotypical markers are highly characteristic for these given species and give a precise identification. One selected strain from *S. lavendulae* cluster was selected for 16S rDNA sequencing. This strain showed a complete identity with *S. toxytricini*, which is a nomenspecies of *S. lavendulae* (Waksman & Curtis 1916). The last cluster could only be identified as *Streptomyces* species and the molecular characterisation of these isolates based on 16S rDNA sequences are under investigation.

Antimicrobial activity

The antimicrobial activity of the streptomycetes isolates from papyrus rhizoplane of Egyptian wetland was determined. Eighty percent of isolates were active against one or more of the test organisms (Table 3). Such percentage is higher than those described by many authors who studied the activity of rhizoplane actinomycetes (Rovasz et al. 1986, Saadoun et al. 1999). The highest percentage of active isolates was obtained against *St. aureus*, *B. subtilis* and *As. niger* (71, 60 and 48% respectively). On the other hand, the lowest percentage exhibited against *E. coli* (8%). The percentage of isolates exhibiting antibiosis against *C. albicans*, *Sac. cervisiae* and *F. oxysporium* were almost equal. Many authors have mentioned that streptomycetes isolates appear to be highly active against Gram-positive bacteria (Saadoun et al. 1999). However, the per-

Table 2. Distribution of streptomycete clusters in papyrus rhizoplane

Name of streptomycete clusters	Number of streptomycete isolates
Streptomyces anulatus	70
Streptomyces rimosus	12
Streptomyces lydicus	10
Streptomyces antibioticus	11
Streptomyces lavendulae	2
Streptomyces species	15

centage of isolates active against fungi seems to be equal to that previously investigated in actinomycetes screening studies (Crawford et al. 1993). About 20% of the streptomycete isolates showed no antibiotic activity towards the test organisms. The percentage of active isolates varied within each colour series (Table 3). The most antibiotic producing isolates belonged to the white and grey isoletes (90 and 81%, respectively) and only 64% of isolates producing no aerial mycelium were active against one or more of the test organisms. The comparison of the antimicrobial activity between all colour classes against the test organisms showed that isolates in the yellow series displayed the highest antibiosis against the Gram-negative organisms tested (*E. coli* and *P. aureginosa*). No activity was indicated in the green series against these test organisms. Arai et al. (1976) indicated that the most active species of streptomycetes were found in the grey and yellow series and no antibiotic producing species were described in the white and green series. Most of the streptomycetes from the yellow, green and white series inhibited the growth of Gram-positive bacteria, *St. aureus* and *B. subtilis*. Isolates of the yellow, green and white were found to be active against yeasts and fungi. This difference in the effect of antibiosis may imply that the investigated streptomycetes belong to different species or to the same one but they produce different bioactive compounds.

Conclusion

Papyrus rhizoplane from Egyptian wetland habitats is a rich source of diverse species of streptomycete clusters. The dominant cluster was *S. anulatus*, however *S. lydicus*, *S. rimosus*, *S. antibioticus* and *S. lavendulae* were also present in minor proportions. The papyrus rhizoplane from Egyptian wetland habitat provide streptomycete clusters, which probably possess antimicrobial properties. The large spectral and high level percentage of the activity showed by the isolated streptomycetes also provide evidence that these habitats harbour species that can produce useful secondary metabolites. In the same streptomycete clusters, most of the isolates showed different activity spectrum.

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Table 3. Antimicrobial activity of the streptomycetes isolates.

	Grey	White	Green	Yellow	No. of Aerial mycelium	Total	% of antimicrobial isolates
Number of isolates	90	10	5	4	11	120	
Number of active isolate	73	9	4	3	7	96	
% of active isolates	81	90	80	75	64	80	
Escherichia coli	2	4	0	2	2	10	8
Pseudomonas aureginosa	2	2	0	1	1	6	5
Bacillus subtilis	51	8	3	3	7	72	60
Bacillus cereus	33	5	2	2	7	49	40
Staphylococcus aureus	62	9	4	3	7	85	71
Candida albicans	28	3	1	2	2	36	20
Saccharomyces cerevesiae	17	3	1	2	1	24	20
Aspergillus niger	39	7	4	3	5	58	48
Fusarium oxysporium	16	5	1	1	3	26	22

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