ACTA UNIVERSITATIS AGRICULTURAE ET SILVICULTURAE MENDELIANAE BRUNENSIS

Volume LXI 163 Number 5, 2013
http://dx.doi.org/10.11118/actaun201361051471

CHARACTERISATION OF ACTINOMYCETES COMMUNITY FROM THE HEAVY METALS-POLLUTED SOIL

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Received: June 4, 2013

Abstract

VÍTĚZOVÁ MONIKA: *Characterisation of actinomycetes community from the heavy metals polluted soil.* Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis, 2013, LXI, No. 5, pp. 1471–1478

The isolation of actinomycetes was performed from soil samples influenced by car-traffic. The acute toxicity of soil leaches was tested by the help of Microtox® bioassay testing system which uses freeze dried luminescent bacteria *Photobacterium phosphoreum* as the test organisms. The content of heavy metals in biomass of soil microorganisms and in whole soil samples was determinate. 115 strains of actinomycetes were isolated and their total numbers in soil samples were estimated. The acute toxicity of soil influenced the total numbers of actinomycetes. By the help of DNA-DNA reassociation procedure the generic diversity of bacteria was estimated. The identification and differentiation of streptomycetes from the total isolated actinomycetes was made using specific morphological criteria and the gas chromatography-fatty acid methyl ester (GC-FAME) analysis. FAME method is adequate only for differentiation of members of genus *Streptomyces* from other actinomycetes because of their characteristical profile of fatty acids.

soil microorganisms, actinomycetes, streptomycetes, heavy metals, FAME method, API ZYM

It is well known fact that the traffic is taking part in pollution of roads surrounding environment. The car-traffic is an important source of fluid and solid emissions, they are getting loose into the air especially in combustion processes (Hlavňa *et al*., 2000). Automobile exhaust gases are source of carbon dioxide, carbon monooxide, nitrogen oxides, ammonia, partially oxidized hydrocarbons, aldehydes, lead compounds and other heavy metals. The air-polluting substances enter into the soil and affect the soil microflora. The contamination by heavy metals causes a serious problem because they cannot be naturally degraded like organic pollutants and they accumulate in different parts of the food chain. Several parameters of microbial activity could be used as good indicators of increasing concentration of heavy metals in soil (Šmejkalová *et al*., 2003). Soil is a natural environment for actinomycetes (Zhang *et al*., 2013). Streptomycetes, one group of order *Actinomycetales*, represent one of the biggest societies in soil microflora. Streptomycetes are well adapted to this environment because of their high enzymatic activity (Hoskisson *et al*., 2012). Streptomycetes embodies the high enzymatic activity in soil decontamination proceses, they produce many hydrolytic enzymes which allow them to obtain important nutrients from hardly utilizable substrates. Streptomycetes are common inhabitants of the soil environment and are regarded as the most numerous actinomycetes isolated from soil (Williams *et al*., 1984).

Streptomycetes in close cooperation with other soil organisms degrade biopolymers from vegetable and animal residuals. Predominantly found as spores, streptomycetes can germinate and grow in contaminated soils into a mycelial state form brief periods of time when nutrients become available (Mayfield *et al*., 1972).

The aim of our study was to determine the influence of car-traffic on the structure of soil microbial communities.

MATERIAL AND METHODS

Soil sampling

Soil samples were collected at three grassy sites near the rush cross-roads in the centre of the city with approximately 400 000 inhabitants. Two of them were close to the source of pollution (No. 1 and 2), one was distant (control site No.3). All soil samples were comparable from the vegetation cover point of view. Samples were taken from the top layer of soil (100 mm depth) and passed through a 2 mm sieve. Samples were homogenized and dried in the thin layer (10 mm) for 24 hours at the room temperature (Zaitlin *et al*., 2004). Analyses were performed in three replications and average values are presented. Dry soil samples were stored in closed glass vessels at the room temperature.

Acute toxicity test

The acute toxicity of soil leaches was tested by the help of bacterial bioluminiscence toxicity test Microtox™ (CSN EN ISO 11348/2000, DIN 38 412 part 34/1991). The Microtox™ assay uses freeze dried luminescent bacteria (*Photobacterium phosphoreum* LX-1) as the test organism (Kahru, 1993; Kafka and Punčochářová, 1999). Luminometer Lumino M90a (Spinex, CZ) was used for all tests. The loss of light emission was measured after 15 and 30 minutes of incubation at 15 °C after the contact of bacterial cells with soil leach. The pH value of soil leaches was adjusted to 7 by HCl. The samples were diluted to 1:1; 1:2.5; 1:5; 1:10 and 1:20 by 2 % NaCl. The acute toxicity was expressed by $\Gamma = (I_{t0} - I_{t)} / I_t$. The sample embodies the acute toxicity if $\Gamma > 1$. Two paralel samples were evaluated and maintained.

Heavy metal analysis

Heavy metal concentrations (Pb, Cd and Zn) in soil were measured with atomic adsorption spectrophotometer PHILIPS PU-9200 (CSN EN ISO 15586; Čurdová and Tvrdíková, 1994; Komárek, 2000). Heavy metals concentrations in 2M HNO_3 extract were defined by using dry way mineralization in apparatus Apion (CSN EN ISO 465735 – EPA3052). Every measurement was performed twice.

The isolation of actinomycetes

1g of dried sample of soil was placed in 9 ml sterile demineralized water and vortexed vigorously at 2000 rpm for 3 minutes. Each sample was diluted by sterile demineralized water from 10^{-1} to 10^{-4} and then plated on Actinomycete isolation agar (AIA, HiMedia, Inc., Bombay, India). The plates were incubated at 26 °C for 14 days. The colonies of typical appearance were isolated by restreaking on Streptomyces agar (yeast extract 4g, malt extract 10 g, D-glucose 4 g, agar 1.5 g, deionized water 1000 ml) and stored on slants at 4 °C–8 °C. For identification and differentiation of streptomycetes from other isolated actinomycetes special morphological criteria were respected. The analysis LUCIA G version 4.61 (Laboratory Imaging s.r.o., Prag, CZ) together with Olympus BX50 microscope (Olympus Corp., Tokio, Japan) was used.

DNA-DNA reassociation method

There is a general consensus among taxonomists that all taxonomic information about a bacterium is incorporated in the complete nucleotide sequence of its genome (Stackebrandt *et al*., 2002). DNA-DNA reassociation technique is described by Rosselló-Mora (2006). DNA-DNA reassociation techniques, also known as DNA-DNA hybridization techniques, are based on an attempt to make raw comparisons of whole genomes between different organisms in order to calculate their overall genetic similarities.

FAME-analysis

Cellular fatty acids analysis was performed on automatic system Microbial Identification System Sherlock (MIDI, Inc., USA) consisting of gas chromatograph Hewlett-Packard HP6890 equipped with flame ionization detector (GC + FID) and an autosampler. Preparation of sample involved saponification, methylation, extraction and base wash (Sasser, 1990; Sasser and Wichman, 1991). The FAME profiles of samples were matched with profiles recorded in identification libraries ACTIN1 ver. 3.81 or TSBA 50. The data output includes fatty acids composition report in the table and sample chromatographic run. The software also computes "Sim index" which gathers values 0–1 in accordance of matching sample FAME profile with profiles in the identification library.

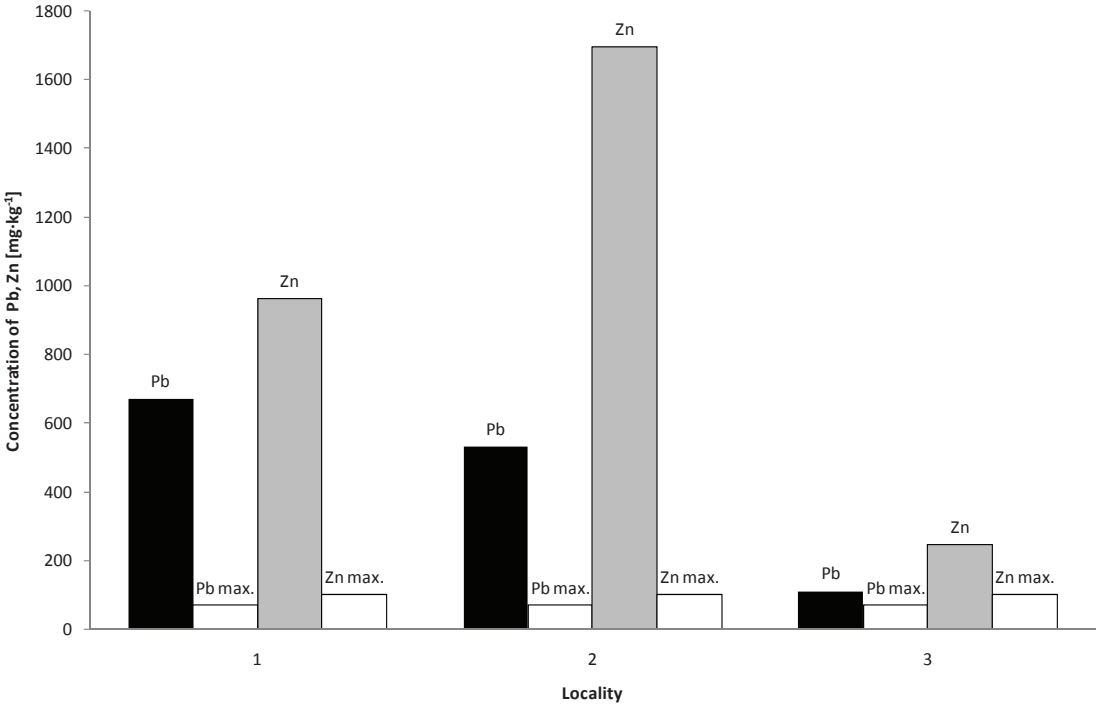
The estimation of enzymatic activity

Enzymatic activity was tested using microassay kit API ZYM (BioMérieux, France) capable to detect 19 enzymes (alkaline and acid phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase).

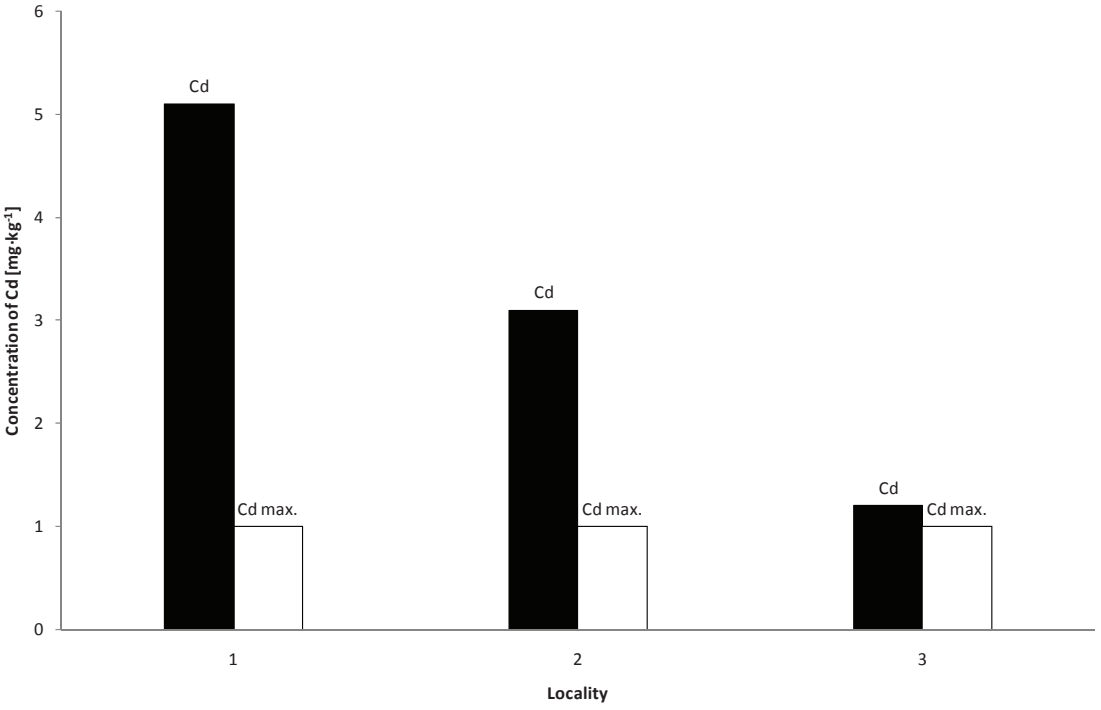
RESULTS AND DISCUSSION

Soil samples were taken from localities with the same soil type and vegetation cover. This fact is important in view of further comparison of soil samples taking into account that these factors are affecting total counts and distribution of actinomycetes in the soil (Korn-Wendisch and Kutzner, 1992).

Heavy metals are cumulating in the soils of grassy localities near rush road junctions (soil samples 1 and 2). Maximal permissible content of cadmium in other soil (2M HNO_3 leaching) is 1.0 $mg \cdot kg^{-1}$, content of lead is 70 $mg \cdot kg^{-1}$ and content of zinc is 100 $mg \cdot kg^{-1}$ (Decree No. 13/1994 Coll.). Overall, the estimated heavy metals content in these



1: *Concentrations of zinc and lead in soil samples in comparison with the maximum permissible values in Decree No. 13/1994 Coll.*



2: *Concentrations of cadmium in soil samples in comparison with the maximum permissible values in Decree No. 13/1994 Coll.*

localities was 6.6times higher than in control non-contaminated locality (soil sample 3) (Fig. 1, Fig. 2, Tab. I).

Obtained values of acute toxicity correspond with the heavy metals content in soils. Locality 1 showed acute toxicity ($\Gamma_1 = 1.12 \pm 0.011$) only in dilution 1:1 and 30 min. of incubation. Value Γ_2 of locality 2 was in the same conditions lower (0.85 ± 0.007) and the lowest value of Γ was recorded at the control locality 3 ($\Gamma_3 = 0.5 \pm 0.003$, dilution 1:1, 30 min. of incubation).

Based on our results we can state that increasing value of acute soil toxicity is negative affecting total counts of streptomycetes and diversity of

I: *Selected characteristics of soil microflora*

Estimation	Soil sample		
	1	2	3
Heavy metals	Concentration of heavy metals ($mg \cdot kg^{-1}$ dry biomass)		
Cd	5.2	3.4	1.2
Pb	671.8	532.9	112.2
Zn	962.8	1694.9	245.4
Actinomycetes (CFU/g dry soil)	$1.13 \cdot 10^5$	$5.22 \cdot 10^5$	$7.83 \cdot 10^5$
Acute toxicity of soil (Γ)	1.12 ± 0.011	0.85 ± 0.007	0.5 ± 0.003
Biomass of soil microorganisms ($\mu gC_{bio} \cdot g^{-1}$ dry soil)	411.2	377.4	520
Number of genetically different bacteria	505	430	710

streptomycetes. Similar negative effects on soil microflora are caused by heavy metals (Kafka and Punčochářová, 2002). Giller *et al.* (1998) observed overall decrease in total counts of bacteria, actinomycetes and free-living nitrogen fixators in the heavy metals contaminated localities. Actinomycetes were isolated from soil samples 1, 2 and 3. The highest number of total counts of actinomycetes, $7.83 \cdot 10^5$ CFU/g of dried matter, was in the control locality 3. This was approximately 7times higher than in the locality 1, which had been showing the highest value of Γ and heavy metals content (Tab. I). The generic diversity of actinomycetes is higher in control locality than in heavy metals influenced localities (Tab. I). Although the proportion of metal tolerant bacteria in the heavy metals contaminated localities is rising, total amount of bacterial biomass is descending. Kizilkaya *et al.* (2004) are in the conviction that some microbiological characteristics can be used as indicators for evaluation of farmlands heavy metals contamination. In soils with increased heavy metals content is often found out decreased basal respiration of microorganisms (Nordgren *et al.*, 1988; Zhou *et al.*, 2009).

93 isolates of actinomycetes were analyzed by cellular fatty acids analysis (FAME). Actinomycetes isolated from soil were identified through morphological criteria. 88 percent of strains were matched as *Streptomyces* sp. Therefore it could be assumed that streptomycetes are numerously predominant population of cultivable actinomycetes in soils. The major and common cellular fatty acids for tested streptomycetal isolates were saturated *iso/anteiso-* branched fatty acids with C_{15}–C_{17} long chain. Obtained FAME profiles were also used for cluster analysis (Fig. 3 and 4). Graphically displayed relatedness between organisms was expressed by Euclidian distance (ED), which means the distance in multi-dimensional space between two strains when their fatty acid compositions are compared.

Cluster containig isolates identified other than *Streptomyces* sp. was delineated at 60 ED from the group of clusters containig *Streptomyces* sp. (Fig. 3). Clusters with isolates matched as *Streptomyces* sp. were delineated at 15–20 ED (Fig. 4).

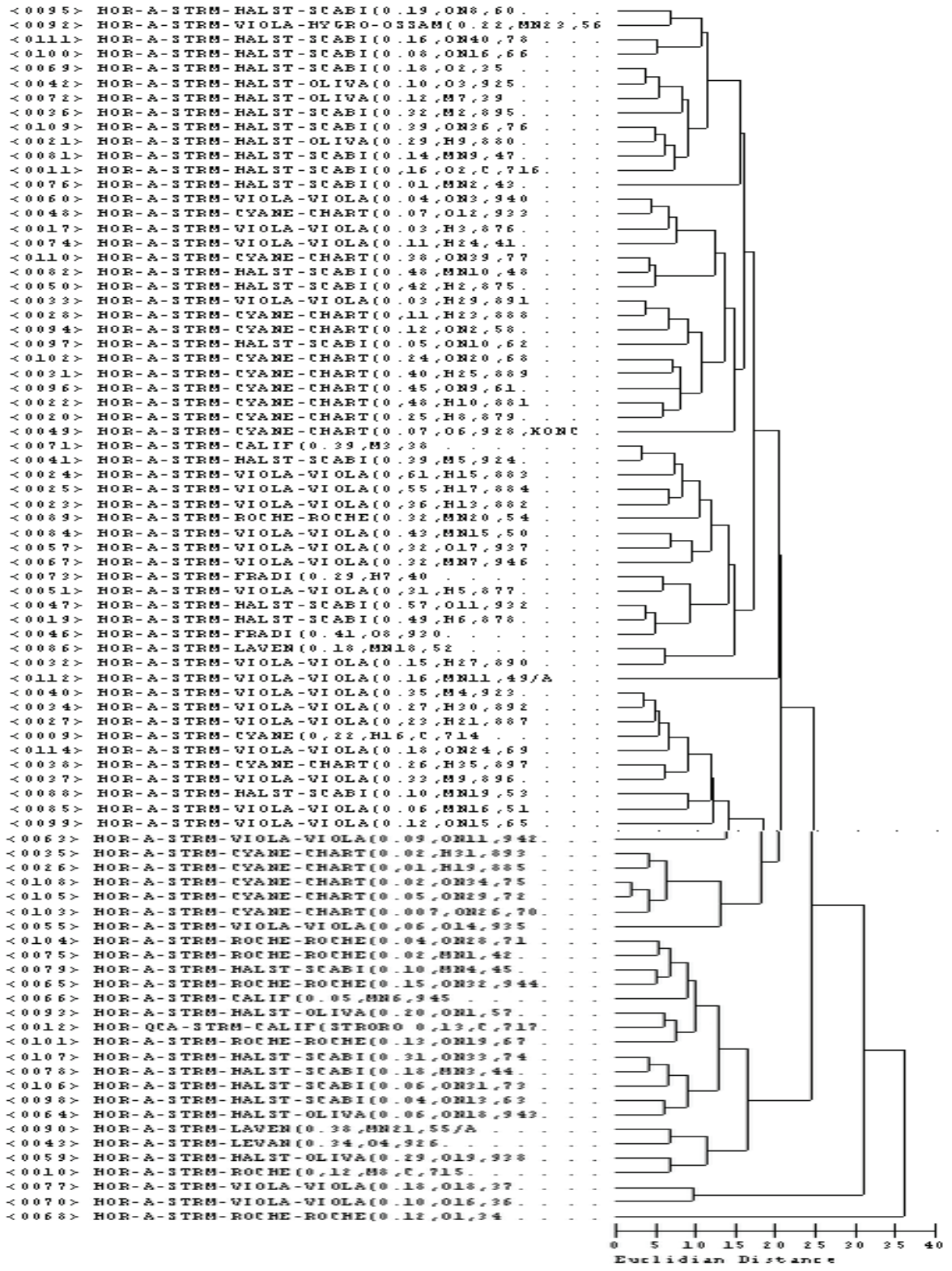
Overall, the identification to genus level using FAME method failed. Only 5% of strains were identified, 16.5% were atypical and for 67% of isolates was Sim index lower than 0.300. This fact means that there is no record in the database matching the tested strain. In our work FAME analysis was useful to distinguish between streptomycetes and other related genera of actinomycetes. According Korn-Wendisch and Kutzner (1992) the main patterns (more than 80%) in chromatograms of streptomycetes are *iso-/anteiso-* branched saturated fatty acids with C_{14}–C_{17} long chain. We found as dominant components *iso-/anteiso-* branched saturated fatty acids with C_{15}–C_{17} long chain. These findings are in congruence with statement above, same as with the results of Sahín *et al.* (2001).

Identification of steptomycetes to the genus level was not sufficient due to low values of obtained Sim indexes of analyzed streptomycetal strains and their bad division. The fail in taxonomic assignment based upon FAME can be explained by low ACTIN1 database range consisting of only 24 streptomycetal species. This low number is highlighted by the fact that Hain *et al.* (1997) is mentioning about 464 validly described species and 45 subspecies of the genus *Streptomyces*.

Taxonomy within this genus is still unresolved problem. Many strains are insufficient or incorrect described (Giller *et al.*, 1998; Korn-Wendisch and H. J. Kutzner, 1992). This FAME identification method matched 88% of tested isolates as *Streptomyces* sp. This showed us that major part of culturable actinomycetes belongs to genus *Streptomyces*. Similar findings were reached by Sahín *et al.* (2001) or Huddleston *et al.* (1997) which also found that most of isolated actinomycetes are members of genus *Streptomyces*.

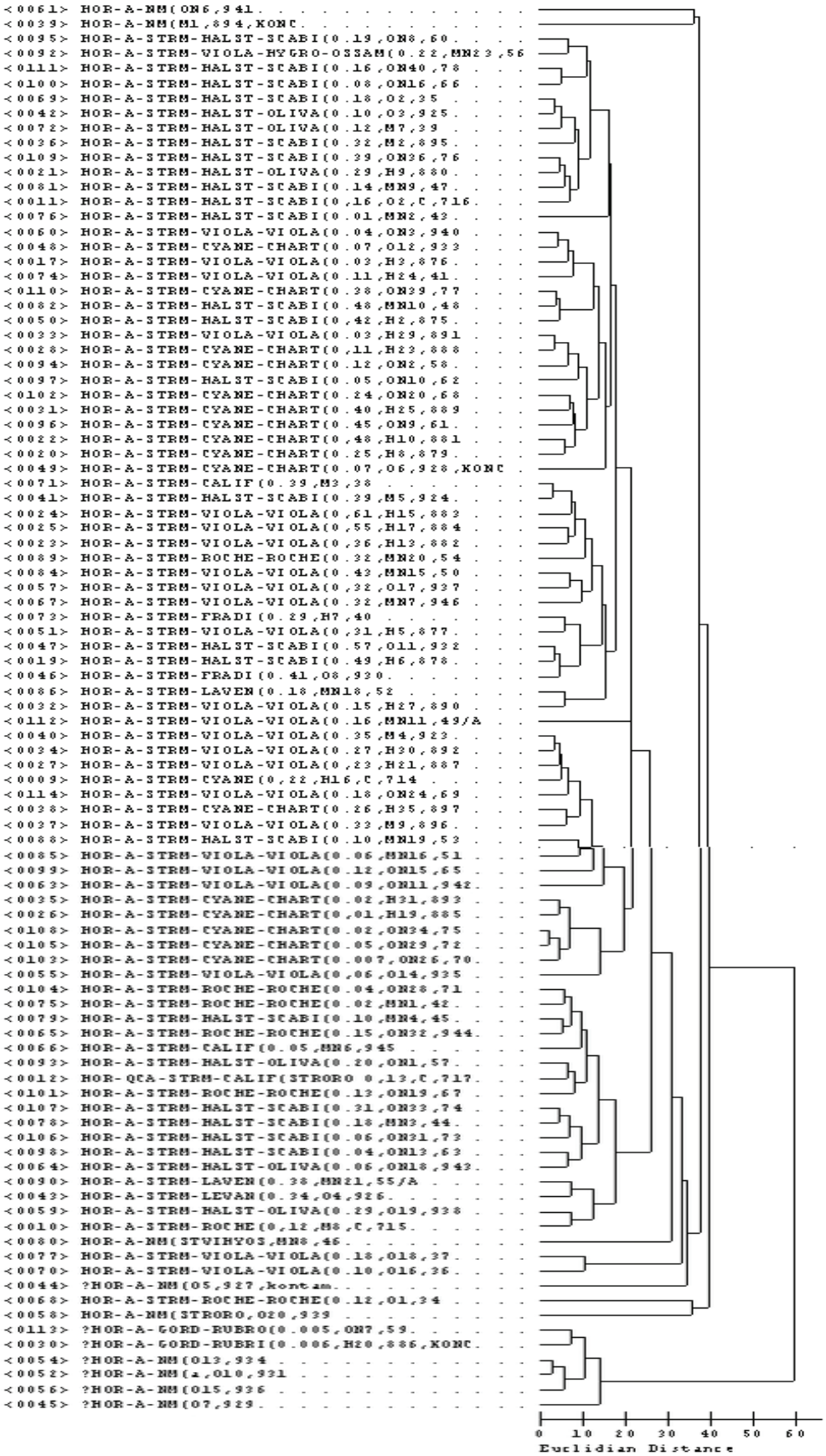
Enzymatic activity of strains considered to be streptomycetes was analyzed. The enzymatic activity of 19 enzymes was estimated. Common enzymes found at all tested strains were leucin arylamidase and acid phosphatase, 89 percent of strains showed activity of valine arylamidase. Contrary, the least occuring enzyme was β-glucuronidase, which was found only at 3.6 percent of analyzed strains.

Some of isolates showed excesive enzymatic activity, others had only narrow spectrum of



3: *The dendrogram of all isolated actinomycetes; Euclidian distance of similarity*

produced enzymes. A huge enzymatic variability was discovered within the range of isolated streptomycetes. No characteristic set of enzymes common for streptomycetes was revealed, only leucin arylamidase and acid phosphatase were distributed within all isolated streptomycetes. Using API ZYM is providing the advantage of easy and fast determination between two or more isolates



4: *The dendrogram of strains determinated as streptomycetes, Euclidian distance of similarity*

showing significant appearance by means of the differences in their enzyme profiles.

On the other hand, there are doubts about usability of this method as a taxonomic tool.

Physiological variability and discontinuous distribution of enzymes were observed by Goodfellow *et al.* (1999). Reliable identification of isolated actinomycetes from soils should involve using a polyphasic taxonomic approach and employing a wide variety of phenotypic, biochemical and molecular techniques.

SUMMARY

The isolation of actinomycetes was performed from soil samples influenced by car-traffic. The acute toxicity of soil leaches was tested by the help of Microtox® bioassay testing system which uses freeze dried luminescent bacteria (*Photobacterium phosphoreum*) as the test organisms. The content of heavy metals in biomass of soil microorganisms was determinated. The generic diversity of bacteria was estimated by the help of DNA-DNA reassociation procedure. 115 strains of actinomycetes were isolated and their total numbers in soil samples were estimated. The acute toxicity of soil influenced the total numbers of actinomycetes. The identification and differentiation of streptomycetes from the total isolated actinomycetes was made using specific morfological criteria and the gas chromatography-fatty acid methyl ester (GC-FAME) analysis. FAME method is adequate only for differentiation of members of genus *Streptomyces* from other actinomycetes because of their characteristical profile of fatty acids.

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