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Effects of Heavy Metal from Polluted Soils on the *Rhizobium* Diversity

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Abstract

Heavy metals adversely influence microorganisms, affecting their growth, abundance, genetic diversity, nodulation ability and efficacy. The aim of this study was to isolate and characterize free-leaving *Rhizobium* from soil which were artificially polluted with Cu (100, 250, and 500 mg kg^{-1} soil), Zn (300, 700, and 1500 mg kg^{-1} soil) and Pb (50, 250, and 1000 mg kg^{-1} soil), but also with a mixture of all these metals, and cultivated with red clover (*Trifolium pratense* L.), and to compare them with bacteria isolated from similar type of soil, but unpolluted. Rhizobia from soil were isolated on YMA medium with or without bromothymol blue (0.00125%) as a pH-change indicator and the morpho-physiological characteristics of the colonies were examined. The number of *Rhizobium* was estimated using the most probable number method. Compared to the control, a decrease of rhizobia number and an increase of the metal concentration were observed. Several decameric primers (Operon Technology type) were used and a reduced polymorphism among isolated bacteria was observed. Moreover, significant differences were observed among these strains and the collection strains used as reference. Also, when primers nodCF/nodCI for detection of *nod* genes were used, several amplicons were obtained, different from the results obtained with similar strains isolated from unpolluted soil. These results suggest that the survival „price" of the *Rhizobium* in such polluted area was the alteration of some genes, including those involved in symbiosis and, probably, in nitrogen fixation.

Keywords: heavy metals, pollution, *Rhizobium leguminosarum*, genetic diversity, soil

Introduction

Heavy metal contamination is now widespread (Nriagu, 1990). Different anthropogenic activities, such as mining, smelting, power station industry, sewage water and sewage sludge land spreading, application of metal-containing pesticides and fertilizers may contribute to soil pollution with heavy metals (Chaudhary *et al.*, 2004; Gimeno-García *et al.*, 1996; Lăcătuşu *et al.*, 1996; Purchase *et al.*, 1997; Robinson *et al.,* 2001; Turgut, 2003). In Romania there are several regions where the level of soil pollution with heavy metals increased due to industrial activity (Lăcătuşu *et al.*, 1996). It was shown that heavy metals adversely influence microorganisms, affecting their growth, abundance, morphology and activities (Castro *et al.*, 1997; Chaudhary *et al.*, 2004; Lakzian *et al.*, 2002; Smith, 1997).

There is an increasing interest in gaining knowledge on environmental factors influencing diversity and structure of soil microbial communities since biodiversity has been assumed to guarantee ecosystems stability, productivity and resilience towards disturbance (Mader *et al.*, 2002). *Rhizobium* spp. are gram-negative soil bacteria that have a profound scientific and agronomic significance due to their ability to establish nitrogen-fixing symbiosis with leguminous plants, which is of major importance for the maintenance of soil fertility (Somasegaran and Hoben, 1994; Yates *et al.*, 2008). Symbiotic nitrogen fixation has been demonstrated as being sensitive to heavy metals in soil (Chaudri *et al.*, 1993; McGrath *et al.*, 1988). This has received particular attention because N_2-fixation by clover can contribute more than 200 kg N ha^{-1} yr^{-1} to agricultural soils (Broadbent *et al.*, 1982). Many animal husbandry systems rely heavily on input of plant available N for their N-fertility (Horswell *et al.*, 2003). Given the importance of legumes in animal and human consumption and their use in maintaining soil fertility, some attention has been given to the effects that heavy metals exert on *Rhizobium* isolates (Pereira *et al.*, 2006).

The present study investigated the effects of the increasing levels of three heavy metals (Cu, Zn and Pb) on indigenous *Rhizobium* populations isolated from soils subjected to an artificial pollution. In order to reach this goal, an experiment was carried out in greenhouse conditions, using natural soil taken from a farm which was not subject of heavy metals pollution. The aim was to establish a relationship between the tolerance levels and the natural conditions experienced by the populations in their place of origin. These approaches can provide tools that could help to predict the impact of such activities on the microflora from the neighboring soils. Furthermore, *Rhizobium* can be used as an indicator organism to several toxic chemicals, including heavy metals as referred by Botsford (1999).

Materials and methods

Experiment design and treatments

Four experiments were carried out in 2010, in greenhouse conditions, in 8 kg Mitscherlich type vessels using a soil from a permanent arable land. The soil was treated with different heavy metals solutions for an artificial pollution, as follows: for the first experiment the soil was treated with copper sulphate solution ($CuSO_4 \cdot 5H_2O$); for the second experiment the soil was treated with zinc sulphate solution ($ZnSO_4 \cdot 7H_2O$); for the third experiment the soil was treated with lead acetate [$Pb(CH_3COO)_2 \cdot 3H_2O$)]; for the fourth experiment the soil was treated with a mixture of the three metal salts in order to obtain a cumulative effect of them. 12 treated variants were obtained. A control with unpolluted soil was kept. All variants were made in triplicate. Details of the treatments are given in Tab. 1.

For these experiments the amounts of heavy metal (Cu, Zn and Pb) salts were calculated based on the normal values, on the fixed values of the alert threshold, on the values of the intervention threshold for sensitive and less sensitive soil uses, and on the maximum admissible limits for soils according with the Order 756/1997 regarding the environmental pollution assessment (published in the Official Journal of Romania, Part I, No. 303/06.09.1997).

Tab. 1. Treatments with heavy metals (2010)

Treatment	Metal source (mg kg^{-1} soil)		
	Cu	Zn	Pb
+V_0	Untreated / Unpolluted soil		
V_1	100		
V_2	250		
V_3	500		
V_4		300	
V_5		700	
V_6		1500	
V_7			50
V_8			250
V_9			1000
V_{10}	40	150	40
V_{11}	100	300	50
V_{12}	250	700	250

Tab. 2. The references values (mg kg^{-1}) for heavy metals used in experiments

Element	Normal values	Alert threshold / Soil use type		Intervention threshold / Soil use type	
		Sensitive	Less sensitive	Sensitive	Less sensitive
Cu	20	100	250	200	500
Zn	100	300	700	600	1500
Pb	20	50	250	100	1000

The references values (mg kg^{-1}) for heavy metals used in these experiments are given in Tab. 2.

The maximum admissible limits for Cu, Zn and Pb correspond to the alert threshold for the sensitive soil use, which are, respectively, 100 mg Cu kg^{-1} soil, 300 mg Zn kg^{-1} soil, and 50 mg Pb kg^{-1} soil. Thus, the variants V_1, V_4 and V_7 correspond to 100 mg Cu kg^{-1} soil, 300 mg Zn kg^{-1} soil, and 50 mg Pb kg^{-1} soil.

This order defines the significance of the alert and the intervention thresholds, as described below:

- alert threshold: the pollutant quantity in air, water, soil or emissions/discharges, which may warn the authorities about a potential risk for the environment and may require complementary monitoring actions and/or actions to reduce the pollutant quantity from emissions or discharges;
- intervention threshold: the pollutant quantity in air, water, soil or in emissions/discharges, which may determine the authorities to adopt appropriate solutions to reduce the emissions or discharges of pollutants.

The regulations regarding soils refer to the sensitive and less sensitive land uses that are identified as follows:

- sensitive land use: it refers to the use of land for residential, leisure or agriculture purposes and to the use of areas preserved or restricted for sanitary reasons;
- less sensitive land use: it refers to all kinds of industrial and commercial use of lands, and the lands that should have a similar destination in the future.

The Experiment 4 was carried out in order to study the cumulative effect of the three metals and their quantities were calculated so that they may range from normal values to maximum admissible limits. Thus, the rates of Cu, Zn and Pb for V_{10} were: 40 mg Cu kg^{-1} soil, which is a value little higher than the normal one; 150 mg Zn kg^{-1} soil, which is also a value higher than the normal one (100 mg Zn kg^{-1} soil is considered as a normal value); 40 mg Pb kg^{-1} soil, which is a value higher than the normal one, which is 20 mg Pb kg^{-1} soil, but below the maximum admissible limit, which is 50 mg Pb kg^{-1} soil. For the variant V_{11}, the rates of Cu, Zn and Pb that were used are similar to the values representing the Maximum Admissible Limits, which are 100 (Cu), 300 (Zn) and respectively 50 (Pb) mg heavy metal kg^{-1} soil. Otherwise, these values coincide with the values of the alert threshold for the sensitive type soils uses. For the variant V_{12}, there was used a treatment mixture of different rates of Cu, Zn and Pb, which are identical to the alert threshold for the less sensitive soil uses, 250 mg Cu kg^{-1} soil, 700 mg Zn kg^{-1} soil and 250 mg Pb kg^{-1} soil.

In vessels, the soil was seeded with a cultivar of red clover (*Trifolium pratense* L.) from the Research Institute for Pastures from Braşov.

Soil origin, physicochemical analyzes, and heavy metal content

These experiments used a *Phaeozem* type soil (FAO classification) taken from Farm fields of Moara Domnească, which is a farm of the University of Agronomic Sciences and Veterinary Medicine (USAMV) of Bucharest. This soil is not subject to any anthropological activities generating pollution. The soil was collected from 5-15 cm depth after carefully removing the top 5 cm of soil covered by spontaneous plants. The soil was sieved and stabilized for 7 days. Before preparing the soil, in order to set up the experiments, samples were collected for chemical and physical analyzes. Soil samples were mixed and homogenized and four laboratory samples of 1 g soil each, in four replicates, were used for analyzes. There were analyzed the pH, the organic carbon, the total nitrogen, the extractable forms of phosphorus and potassium in acetate-lactate, the total salt content in aqueous extract of 1:5, expressed in mg to 100 g dry soil, and the electric conductivity (EC).

Soil analyzes were made by the Laboratory for Soil Pollutant Control from the Research Institute for Pedology and Agrochemistry of Bucharest, Romania (RIPA). All soil samples were initially air-dried and sieved (2 mm size). Only the fraction < 2 mm, which amounted to 98% of the total soil weight, was used for further analysis. Soil pH measurements were made in H_2O suspension (1:2.5) using the potentiometric method. The organic carbon content was determined using the Walkley-Black (1934) method, modified by Gogoaşă (1959). The total nitrogen was determined by the Kjeldahl method. The available phosphorus and potassium were determined by the RIPA methodology and the Egnèr-Riehm-Domingo (1960) method. The heavy metal content of the soil was analyzed by atomic absorption spectrometry after mineralization in an acid mixture of nitric acid (HNO_3), perchloric acid ($HClO_4$), and sulphuric acid (H_2SO_4).

Microbiological analysis

Bacterial strains

The experiments used both new strains isolated from red clover or white clover nodules and a reference strain, *Rhizobium leguminosarum* biovar *trifolii* LMG 8820. Rhizobia were isolated from plants nodules. Nodules were surface sterilized for 10 min in a solution of 90% (v/v) ethanol followed by 10 min in a solution of Na hydrochloride at 0.5% (v/v). Nodules were then washed three times with sterile distilled water. Nodules were crushed in approximately 0.1 ml of sterile distilled water to release bacteroides. The bacteroid suspensions were plated on Mannitol-Yeast Extract-Congo red agar to obtain single colonies (Delorme *et al.*, 2003). In order to identify new isolates, the Api 20 NE kit and the BIOLOG system were used, using *R.leguminosarum* biovar *trifolii* LMG 8820 as reference.

Culture conditions

For *in vitro* studies, bacteria were cultivated in different media: yeast manitol agar (YMA) (10.0 g l^{-1} mannitol; 1.0 g l^{-1} yeast extract; 0.5 g l^{-1} K_2HPO_4; 0.2 g l^{-1} $MgSO_4.7H_2O$; 0.1 g l^{-1} NaCl; 0.3 g l^{-1} $CaCO_3$; 15.0 g l^{-1} agar, pH 7.0). YMA supplemented with 0.0025% Congo red or 0.00125% bromthymol blue. The enumeration of soil bacteria was carried out by using the Most Probable Number Method (Vincent, 1970) and determining the viable plate account (CFU/ml). The morphological traits evaluated comprised mucous production and colony morphology, pH change of the medium during growth of the isolates and growth rate (Somasegaran and Hoben, 1985).

Analysis of bacterial DNA from soil

The DNA from soil samples was isolated by ZR Soil Microbe DNA kit (Zymo Research). Preliminary examination of bacterial diversity was conducted by DGGE technique, using INGENYphorU system, according to Dilly *et al.* (2004). The primers used for amplification, and the amplification conditions were recommended by Stark *et al.* (2007) for α-proteobacteria: F203a (CCG CAT ACG CCC TAC GGG GGA AAG ATT TAT) / R1494 (CTA CGG YTA CCT TGT TAC GAC) for the first PCR reaction and F984GC (AAC GCG AAG AAC CTT ACC GCC CGG GGC GCG CCC CGG GCG GGG CGG GGG CAC GGG GGG)/R1378 (CGG TGT GTA CAA GGC CCG GGA ACG) for the second one. DNA bands were stained with a solution of 0,5 μg/ml ethidium bromide and examined under UV light with BioDocIt UVP.

Results and discussion

Soil physicochemical analyzes and heavy metal content

The pH has an average value of 5.80 (Tab. 3a), which belongs to the moderate acid class; it has a medium content in total nitrogen (0.156%), a very low content in soluble phosphorus (7.72 mg/kg), and a low content in soluble potassium (96 mg/kg). The soil content in soluble salts was very low (8 mg/100 g soil, in average), and the electric conductivity was 25.07 μS cm^{-1}. The soil was unsaturated in basic cations (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}), that is characterized by the degree of bases saturation (V<100) V% = 76,4%, a lower sum of exchangeable bases (SB) (14,79 me/100 g sol), and a medium (4,44 me/100 g sol) hydrolytic acidity (Ah) as it may be observed in Tab. 3b. The soil has a texture of medium clay (LL), without carbonates (Tab. 3c). The heavy metal content of soil (Tab. 3d) was within the normal limits in the unpolluted soils (Castro *et al.*, 1997; Lindsay, 1979; Răuță and Cîrstea, 1983).

Effect of metals on Rhizobium

Two months after seeding, the red clover plants (T*rifolium pratense*) were well developed in the control variant (V_0) but showed a relative reduced number of root nodules. The CFU (colonies forming units) ml^{-1} of free-leaving bacteria in the soil was 8 x 10^6 CFU ml^{-1}, with a

Tab. 3. Soil chemical and physical properties and heavy metal content

a. Chemical properties

	pHH_2O	C %	Nt %	PAL mg k^{g-1}	KAL mg kg^{-1}	EC µS cm^{-1}
x̄	5.80	1.18	0.156	7.72	96	25.07

b. Content in exchangeable cations of soil

	SB me 100 g^{-1} sol	Ah me 100 g^{-1} sol	T (SB+Ah) me 100 g^{-1} sol	V %
x̄	14.79	4.44	19.23	76.4

c. Granulometric fractions (mm) (% from the mineral mass of soil)

	Coarse sand 2.0-0.2	Sand 0.2-0.02	Dust 0.02-0.002	Clay <0.002	Texture
x̄	1.8	35.1	31.6	31.5	LL

d. Soil heavy metal content

	Pb	Cu	Zn	Cd	Mn	Ni	Co
	(mg kg^{-1} dry soil)						
x̄	20.15	12.62	59.5	0.278	528	22.4	5.1

small diversity and a small number colonies with mucoid aspect (Fig. 1).

The number of bacteria in the variants treated with Cu were smaller than in the control (V_0), 2.8 x 10^6 CFU ml^{-1} in V_1 (100 mg Cu kg^{-1} soil), 2.5 x 10^6 CFU ml^{-1} in V_2 (250 mg Cu kg^{-1} soil), and 2.1 x 10^6 CFU ml^{-1} in V_3 (500 mg Cu kg^{-1} soil), respectively. But, in V_1 the plants were well developed, with big nodules developed on the roots. In V_2, the plants were also well developed, with numerous nodules on the roots, but in a small number compared to V_1. In V_3 the plants were small, without visible nodules on the roots and the number of soil bacteria was 2.1 x 10^6 CFU ml^{-1}.

In V_4 (300 mg Zn kg^{-1} soil), the plants were smaller compared to the plants from the experiment with Cu but, the nodules were big and well visible on the roots but, some of them were lobated and frequently grouped in bundles. The CFU ml^{-1} value was smaller than in the control variant, 3.03 x 10^6, respectively, and the colonies on the plates were diverse, including mucoid colonies (Picture 1).

In the V_5 variant (700 mg Zn kg^{-1} soil) the plants were small, without nodule which may suggest the toxic effect of the amount of Zn used for the treatment. That is in accordance with Smith (1997) who related that the absence of nodulation was indicative of either a very small rhizobial population, below the detection limit of the plant infection assay, or the complete absence of rhizobia from soil. Renella *et al.* (2002) reported also that the general order of inhibition for single metals was: Zn>Cu. The CFU number was always small (3.2 x 10^6) compared to the control (V_0), and the colonies was diverse. These data suggest that the number of rhizobia could be reduced in treated soils. In the V_6 variant (1500 mg Zn kg^{-1} soil) the plants were absent due to the high level of Zn and its toxic effect but the number of bacteria in the soil was higher than in the previous variant (4.1 x 10^6 CFU ml^{-1}). There was a reduced diversity of colonies with a dominance of mucous mobile colonies.

In the V_7 variant (50 mg Pb kg^{-1} soil) the plants of red clover were well developed, with numerous nodules, some of them with a curved form, and the bacteria was abundant (3.1 x 10^6 CFU ml^{-1}) The diversity of bacteria from soil was comparable to the control (V_0), and there were mucoid colonies with rounded convex shape (Picture 2).

In V_8 variant (250 mg Pb kg^{-1} soil) the plants were very vigorous but the number of nodules was low, some of nodules being hypertrophied (Picture 3). The bacterial colonies developed were generally confluent, with reduced diversity and rarely convex mucoid colonies. No visible modifications of the plant phenotype cultivated in the V_9

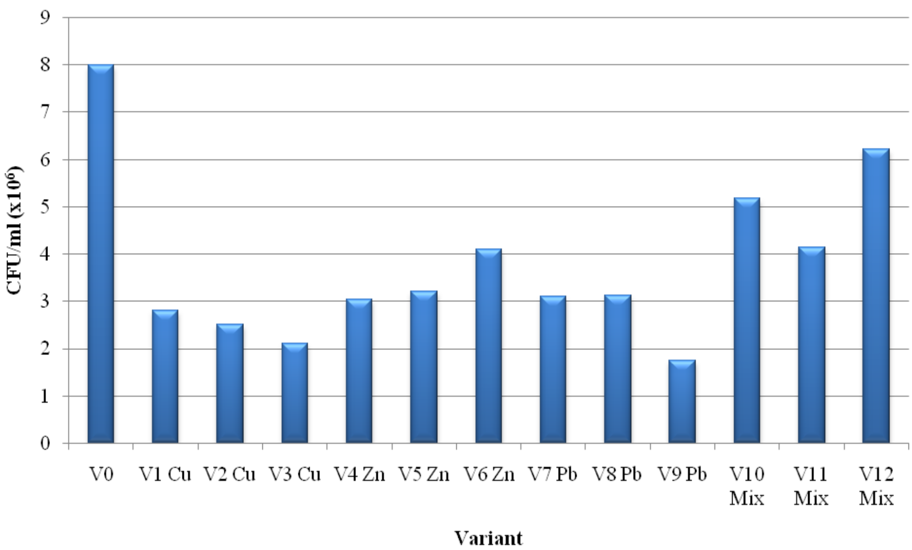
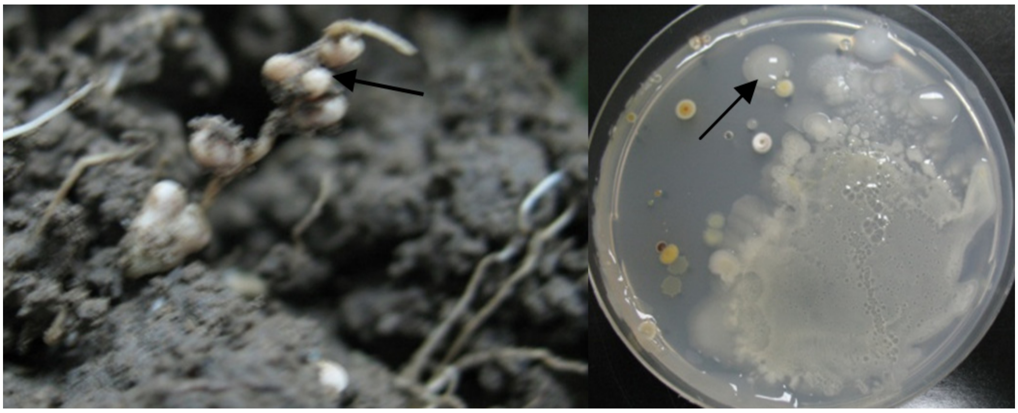


Fig. 1. The variation of CFU number in the soil treated with different amount of heavy metals



Picture 1. Root nodule and mucoid colonies in V_4 (300 mg Zn kg^{-1} soil)

Picture 2. Root nodules and colonies on the plates in V_7 (50 mg Pb kg^{-1} soil)

Picture 3. Hypertophied root nodule and convex mucoid colonies in V_8

were observed but the number of the bacterial colonies isolated from soil samples was reduced, compared with other experimental variants (1.75 x 10^6 CFU ml^{-1}). These results suggest an increased toxic effect of the lead concentration.

In the V_{10} variant (40 mg Cu kg^{-1} soil + 150 mg Zn kg^{-1} soil + 40 mg Pb kg^{-1} soil), polluted with a mixture of heavy metals, the plants were vigorous, with well developed root system but with a small number of nodules. Surprisingly, the number of bacteria (5.18 x 10^6 CFU ml^{-1}) and their diversity was similar to the control number, but the number of mucoid and convex colonies was reduced. In the V_{11} variant (100 mg Cu kg^{-1} soil + 300 mg Zn kg^{-1} soil + 50 mg Pb kg^{-1} soil) the plants were very small, and not well developed. The number of root nodules and the bacteria diversity on the plates were very low. In the V_{12} variant (250 mg Cu kg^{-1} soil + 700 mg Zn kg^{-1} soil + 250 mg Pb kg^{-1} soil) the plants were absent, but the number of bacteria in the soil was closed to control (6.21 x 10^6 CFU ml^{-1}). The growth of CFU number may be due to the genes conferring specific adaptation to pollutants, including heavy metals, which are often plasmid-borne. The correlation between the selection pressure due to pollutants and the existence of some plasmids suggest that plasmids play a major role in the adaptation of bacteria to xenobiotics and the acquisition of new genetic traits due to pollution. That is why many soil microorganisms can develop resistance in response to toxic concentrations of heavy metals and in gram-negative bacteria this is frequently mediated by plasmids (Silver, 1992; Silver and Misra, 1988).

The results of these experiments highlight a variation in the number of CFU/ml, which suggests the toxicity of heavy metals concentrations not only on plants, but also on soil microorganisms. Also, in general, inhibitory effects on nodule number correlates with rhizobia decrease in soil samples. Compared to other reported results, data obtained here are somehow surprising, especially for zinc. Chaudri *et al.* (2008) observed the decrease of rhizobia number that may nodulate the clover when Zn is present in big concentration, while copper has less obvious effects on *R.leguminosarum* biovar *trifolii* population size.

In these experiments, the most important inhibitory effects on the overall number of bacteria were found in the case of lead, although these effects were low, and the evidence is the presence of nodules on roots clover in all the concentrations of lead used. In contrast, zinc caused stronger effects on the ability nodulation of rhizobia. Similar issues were highlighted by Ibeke *et al.* (1997) who reported that soil contamination with various types of heavy metals does not directly affect the nodules, while soil pH has more important effects. Also, although the effects of the heavy metal mixture were expected to have a clearly negative impact on soil bacterial populations, the results showed little effect on CFU number, although diversity was affected. Regarding the presence and especially the nodulation ability of rhizobia, mixed heavy metals determined the reduction of the nodule number ($V_{10}<V_{11}$) compared to the control but also with other variants.

The results are generally consistent with others reported in literature. Thus, the presence of certain concentrations of heavy metals in the short term does not significantly affect populations of *Rhizobium*, which are rather influenced by the crops technologies (Laguere at *al.*, 2006). Thus, the rhizobia may be tolerant to certain levels of heavy metals (e.g. copper), although numerically, at the population level, there are decreases. Moreover, not only that the rhizobia were detected in the presence of the big concentrations of heavy metals used in the experiments but also their ability to form nodule, apparently active, was not significantly affected.

The possible influence of heavy metals on *R. leguminosarum* biovar *trifolii* genome was examined. Eight strains of *R. leguminosarum* biovar *trifolii* were isolated from clover nodules (varints V_0, V_1, V_2, V_4, V_7, V_8, V_{10} and V_{11}) and identified by specific culture characteristics and by API and BIOLOG systems using as reference the strain *R. leguminosarum* biovar *trifolii* LMG 8820. DNA isolated from all the nine strains was subjected to RAPD analysis with 8 decameric primers: OPA3, OPA7, OPA9, OPC12, OPC19, OPE 02, OPO 13 and OPP 07. Generally, no significant differences were observed among the bacterial strains isolated from nodules and the reference. However, with the primer OPA9 some variations in electrophoretic profile of DNA fragments were detected, especially for the strains isolated from variants V_8, V_{10} and V_{11} (Fig. 2).

These results are supported by the aspect of the nodules developed on the clover roots grown in increased and combined concentrations of heavy metals. They could be also associated with the symbiotic and nitrogen fixation abilities of these strains but these affirmations need to be confirmed by further experiments.

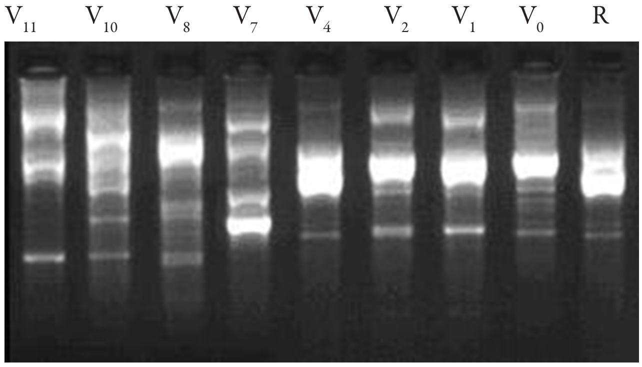


Fig. 2. Electrophoretic pattern of the amplicons obtained with OPA 9 primer

Preliminary analysis of the bacterial diversity in soils under different concentration of heavy metals

Another studied aspect was the possible effects of heavy metals on the microbial communities, especially on bacterial diversity. In the experiments differences in electrophoretic pattern of amplification products obtained with primers specific for α-proteobacteria (the genus *Rhizobium* belongs to this group of bacteria) were examined. DNA from 12 soil samples containing various concentrations of heavy metals (variants V_1-V_{12}) and control variant (V_0) was isolated and subjected to PCR amplification, according to

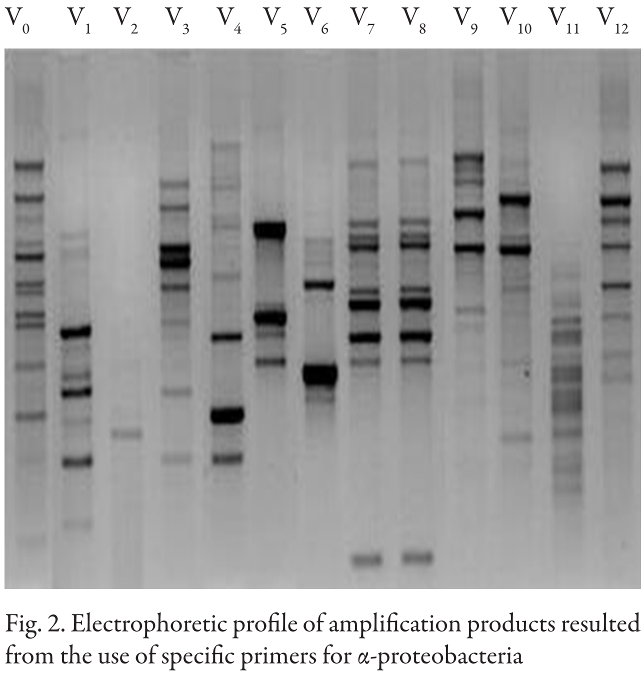


Fig. 2. Electrophoretic profile of amplification products resulted from the use of specific primers for α-proteobacteria

Stark *et al.* (2007). Differences in the amplicons number and size were observed after DGGE analysis (Fig. 3).

Compared to the control (Fig. 3, lane1), the profiles of the V_1, V_2 and V_3 variants (with different concentrations of copper) are different, but some common amplicons could be detected. These aspects are correlated with the data obtained from the macroscopic examination of the bacterial diversity occurring on the culture medium. The amplification products obtained from DNA isolated from soils with different concentrations of Zn (V_4-V_6) presented different profiles comparing with the control, as well as other samples. The amplicons obtained from DNA isolated from variants V_7 and V_8 were very similar, but different comparing with V_9, where larger fragments were observed. The most interesting results were observed for the variants containing mixtures of heavy metals: there are many DNA bands, suggesting a greater diversity for the variant V_{12}. The same issue was highlighted in case of the macroscopic observations. Also, the fact that there is no perfect correlation between the data obtained by different test methods could be explained by the fact that, for the DGGE analysis, specific primers were used only for a certain class of bacteria, α-proteobacteria, while other types of bacteria could also grow on the Lazareva plates. For these reason, extensive studies are necessary in order to identify the presence of certain *Rhizobium* species among other bacteria by DGGE technique. Moreover, the reduced number of amplicons detected in the experiments, compared with data from literature suggests that some optimizations of the method are also necessary for a better evaluation of the diversity.

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