



EVALUATION OF THE RHIZOSPHERIC MICROBIOTA FROM SOILS DEGRADED BY MINING ACTIVITIES

Simona DUNCA¹, Stefan MARIUS¹, Tanase CATALIN¹, Ana COJOCARIU¹

¹ "Alexandru Ioan Cuza" University of Iasi, Faculty of Biology, Bd. Carol I, no. 20 A, 700505, Romania, e-mail: sduanca@uaic.ro

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SYNOPSIS

Our researches aim to the quantitative analysis of the rhizospheric microbiota and estimation of rhizosphere effect in some soils degraded in mining activities by taking into account the theoretical importance of the interactions between microorganisms and plant roots and also the incontestable practical implications in bioremediation. The rhizospheric soil samples was collected from Dumitreleu, Pinului and Ilva Dumps (Calimani National Park, Romania), from the rhizosphere of the species *Salix caprea*, *Picea abies*, *Vaccinium myrtillus*, *Pinus mugo* and associations *Salix caprea* - *Deschampsia cespitosa*, *Picea abies* - *Pinus mugo*, *Salix caprea* - *Picea abies*, *Pinus mugo* - *Pinus cembra* and *Picea abies* - *Pinus cembra*. For the quantitative analysis of free/rhizospheric soil microbiota, we have used soil samples for dilutions and the results were expressed in the number of colony-forming units (CFU) in a gram of soil sample. The quantitative analysis of rhizospheric microbiota shows some differences between the studied species. We have revealed a low microbial load in the association *Salix caprea* – *Deschampsia cespitosa* (Dumitreleu Dump) – CFU/g soil of 29×10^3 , and comparatively the microbiota from *Picea abies* (Pinului Dump) – 61×10^4 CFU/g soil. The R/S rapport present high values on the most soil samples and is explained by the benefic effect of the plants roots on the rhizospheric microorganisms caused by the radicular exudates.

INTRODUCTION

Plant roots are favourable media for the growth of microorganisms, numerous and different populations of which being found on, as well as around, them. The interactions between microorganisms from the soil and the roots of the plant fulfil important nutritive needs, both for the plant and for the associated microorganisms.

This is demonstrated by the large number of microorganisms found in the rhizoplane and in the rhizosphere (Lim, D.L., 1998). Although some aspects of the interaction between microorganisms and plants are still unknown or controversial, there are observations that plead for a series of beneficial effects, without excluding however, in some cases, the possibility of negative consequences. The plants developed in common, non-sterile, soil, are more vigorous and the production of vegetal biomass is larger than that of witness plants grown in sterile soil. The best studied positive effects of rhizospheric microorganisms on the roots are: nitrogen fixation, mycorrhiza-type associations, plant growth stimulation, degradation of the nutrients which are inaccessible to plants, the cycle of the nutrients. At the level of the rhizosphere there is a large number of microorganisms, included in different taxonomical groups, their density decreasing as the distance from the roots of the plants increases (Giri, B., Giang, P.H., Kumari, R., Prasad, R., Varma, A., 2005). Bacteria are the most numerous microorganisms in the rhizosphere, being present in an approximate number of $10^6 - 10^9/g-1$ soil (Sylvia, D.M., Fuhrmann, J.J., Hartel, P.G., Zuberer, D.A., 1999). However, they represent just a small part of the total biomass because of their small scale. Among the species identified as predominant are mentioned those in the genus *Achromobacter*, *Agrobacterium*, *Alcaligenes*, *Corynebacterium*, *Pseudomonas* (the most numerous of species), *Rhizobium*, *Xanthomonas*; *Azotobacter chroococcum* is absent, or, according to other authors, present in a very number (Lugtenberg, B.J.J., Dekkers, L., Bloemberg, G.V., 2001). Actinomycetes represent approximately 10-30 % of the total rhizospheric microbiota, their number being influenced by season or by nutrients supply.

Due to the theoretical importance, but especially due to the incontestable practical implications in fields such as agriculture, bioremediation, pharmaceutical industry etc., the study of the interactions between microorganisms and the plant roots has now a particular impetus, bringing together top fields of the modern biology (Brimecombe, M.J., De Leij, F.A., Lynch, J.M., 2001). In the last decades, we have witnessed a permanent diversification of the studied research directions and a growing interest towards the use of rhizospheric microorganisms for the bioremediation of media contaminated with different pollutant substances. Another interesting aspect of the plant-rhizosphere interaction is the possibility of influencing the number and the type of rhizospheric microorganisms through the genetic modification of the plants (Akkermans, A.D.L., Van Elsas, J.D., 1995). Ideally, these modifications can be made in order to increase the presence and the activity of some beneficial microorganisms in the rhizosphere, such as nitrogen-fixing organisms or antibiotics producers or to decrease the presence and activity of microorganisms less beneficial from the rhizosphere (Madigan, M.T., Martinko, J.M., Parker, J., 2000).

The aim of our investigations were quantitative analysis of the rhizospheric microbiota and estimation of rhizosphere effect in some soils degraded in mining activities by taking into account the theoretical importance of the interactions between microorganisms and plant roots and also the incontestable practical implications in bioremediation.

SUBJECT AND METHOD OF RESEARCH

Collecting root samples: The classical method of collecting roots consists of cutting a soil monolith with the dimensions 20 x 20 x 20 cm, which should comprise the plant together with its roots. In order to be certain that you collect representative samples for roots, Stefanic (1966) suggested the technique of collecting the monolith only from one side of the symmetry plane that crosses the axis of the row of plants. In this way, there shall be collected also younger roots from the periphery of the radicular system. Disregard to these conditions led to very different and sometimes spurious assessments regarding the intensity of the rhizosphere effect upon the dynamics of the vegetative development of the plant.

Collecting soil samples: The way in which the collection of soil samples is achieved is particularly important for the precision of the results. When collecting samples, one should consider the fact that microorganisms are not spread uniformly in the soil. Thus, there are collected as many samples as possible in order to cover as much surface as possible from the ground to study. For the collection, there was established an initial delimitation of operation area, and in this area, randomly, there were chose different points at a depth between 5-10 cm, after clearing off the superficial layer of the soil, comprising mainly of vegetal residues.

Processing the root and soil samples: The root samples were introduced in a sterile mortar which contained a few grams of quartz sand. They were carefully triturated, without crushing the roots too much and then the whole content of the mortar was put in an Erlenmeyer flask that contained distilled water. After stirring the content for 15 minutes, we proceeded to consecutive dilution. The soil samples were sifted through a sifter with 1mm holes, which was previously sterilized. After an elaborate homogenization performed under aseptic conditions, a 10 g sample was weighed.

Isolating the microorganisms in root-free soil and rhizospheric soil: From the samples collected from the root-free soil and from the rhizospheric soil, there were performed consecutive suspensions-dilutions in sterile water, using a dilution factor of 10 (10⁻¹, 10⁻² etc. dilutions). For developing the microorganisms, it was used an agarised soil extract (Bunt-Rovira medium). On the surface of the medium on the plate, 2-3 drops from the previously prepared suspension were placed with a dropper, and then the inocul was exposed on the entire surface of the plate with a buckled glass rod. The inoculated plates were incubated at 28°C for seven days. At the end of the incubation period, the colonies became visible on the surface of the culture medium.

Obtaining the pure cultures and elaborating the microorganisms collection: After seven days of incubation, on the surface of the plates with the developed colonies, there were isolated several stems, replicated with a sterile anse, in test tubes with the Bunt-Rovira medium tilted, having the same composition as the initial medium used for isolation. The test tubes sown through the technique of describing striations were incubated at 28°C for seven days. In order to obtain the pure cultures, the isolated cultures had to be checked first. To this end, all the cultures have been

examined microscopically to check their purity.

Rhizosphere analysis method. Assessment of the rhizosphere effect: The indirect method increased the quantity of information by establishing the number of germs in the rhizosphere in comparison with the one in the non-rhizospheric soil (edafosphere). Moreover, the method provided data about the taxonomic structure of the microbiocenoses, as well as about the quantitative ratios between them. Knowing the global quantitative ratio and the ratio between different species of microorganisms in the rhizosphere, on the one hand, and from the edafosphere, on the other hand, led to the idea to express the influence that the roots exercise upon the microbiological balance of the soil through the notion of "rhizosphere effect" (Katznelson, 1946, quoted by Eliade, Ghinea, Stefanic, 1975). At the level of the rhizosphere, the plants roots have a direct influence on the composition and the density of the microbial community in the soil, influence known as rhizosphere effect. This effect can be quantified by calculating the ratio between the number of microorganisms in the rhizospheric soil (R) and the corresponding number of microorganisms in the root-free soil (S) –R/S ratio. In order to assess the rhizosphere effect and to calculate the R/S ratio, respectively, it was used the method of plate cultures. This method uses the calculation of the colonies developed after sowing on the Bunt-Rovira medium from the Petri dishes, of the dilutions obtained from the two samples subject to analysis (root-free soil, rhizospheric soil). This technique is based on the fact that each viable cell is the origin of a colony and thus, the number of colonies developed in the Petri dishes indicates the number of microorganisms contained by the sample, able to grow in the conditions provided by the culture medium employed. Because it is possible that a colony could have as origin not a cell, but a group of viable cells, to minimize the error that might occur during the calculation, it is preferred to express the final results in colony forming units (CFU) and not in the number of microorganisms.

The technique implies covering several stages: *carry out the dilutions* – the sample for analysis is diluted in order to obtain on a Petri dish 50-300 colonies; *inoculation* – for each obtained dilution in the 10 ratio, it is recommended the inoculating of 2-3 Petri dishes, then the average of the developed colonies is calculated; *incubation* – the inoculated plates are put at a thermostat at 28°C, for seven days; *counting the colonies* – the bacterial colonies are counted after seven days of incubation at thermostat; the counting is achieved using the unaided eye or with the use of a magnifying glass. In case a large number of colonies are developed, the counting is achieved by using some plates with a network of squares engraved in glass. The best dilution is the one that allows the development of a number of 50-300 colonies (Dunca, Simona, Ailiesei, Octăvița, Nimițan, Erica, Ștefan, M., 2004).

The formula for calculating the number of colony forming units (CFU) in a gram of sample (Angle, S., Weaver, R.W., Botztomley, P., Bezdicek, D., Smith, S., Tabatabai, A., Wollum, A., 1994) is:

$$\text{CFU/gram of soil} = a \times 10^n / V$$

where: a – number of colonies;

10ⁿ – dilution in which the calculation was carried out;

V – inocul volume.

RESULTS WITH DISCUSSIONS

Quantitative determination of the soil and rhizospheric microbiota

In order to determine the quantitative soil and rhizospheric microbiota from Calimani National Park, the samples were collected in October 2006, and June and September 2007. The samples of rhizospheric soil have been collected in October 2006 from two Dumps: Dumitreleu and Pinului, using as species *Salix caprea*, the *Salix caprea* and *Deschampsia cespitosa* association, *Picea abies* and *Pinus mugo* (Dumitreleu Dump), *Salix caprea* and *Picea abies*, respectively (Pinului Dump). In order to assess the rhizospheric microbiota in June 2007, there have been collected samples of *Picea abies* (Ilva Dump), *Pinus mugo* and *Pinus cembra* (Dumitreleu Dump) and *Vaccinium myrtillus* (Pinului Dump). In September 2007 the collection of the rhizospheric soil samples came from *Pinus cembra* and *Picea abies* (Ilva Dump) and *Pinus mugo* (Dumitreleu Dump), also compared with control samples pertaining to the same species.

For the quantitative assessment of the soil microbiota, it was necessary to count the colonies in the Petri dishes sown with the dilutions obtained (10⁻¹ - 10⁻⁶) from the root-free and the rhizospheric soil in October 2006, June and September 2007. The results have been expressed in colony forming units/g of soil (CFU).

The quantitative analysis of the rhizospheric microbiota in October 2006 (*Table I*) shows a series of differences between the species under study. In the case of the association *Salix caprea* – *Deschampsia cespitosa* (Dumitreleu Dump) - (*Photo 1*) a slight microbial charge turns out, where the CFU/g soil value was of 29 x 10³, in comparison with the microbiota from *Picea abies* (Pinul Dump) – (*Photo 2*), where it turned out 61 x 10⁴ CFU/g soil.

Table I. CFU/g soil values for rhizospheric soil samples (October, 2006)

Sample no.	Species (point of collection)	CFU/g soil	
		Root-free soil	Rhizospheric soil
1.	<i>Salix caprea</i> (Dumitreleu Dump)	32 x 10 ⁴	96 x 10 ³
2.	<i>Salix caprea</i> + <i>Deschampsia cespitosa</i> (Dumitreleu Dump)	113 x 10 ²	29 x 10 ³
3.	<i>Picea abies</i> (Dumitreleu Dump)	52 x 10 ⁴	321 x 10 ²
4.	<i>Pinus mugo</i> (Dumitreleu Dump)	60 x 10 ⁴	67 x 10 ³
5.	<i>Salix caprea</i> (Pinului Dump)	126 x 10 ⁴	565 x 10 ²
6.	<i>Picea abies</i> (Pinului Dump)	132 x 10 ³	61 x 10 ⁴

The rhizospheric microbiota is quantitatively well represented in June, 2007 (*Table II*) in the case of species *Picea abies* (Ilva Dump), with a CFU/g soil of 220 x 10² (*Photo 3*) and *Vaccinium myrtillus* (182 x 10² CFU/g soil), at the opposite side being *Pinus cembra* (Dumitreleu Dump), with 81 x 10² CFU/g soil – *Photo 4*.

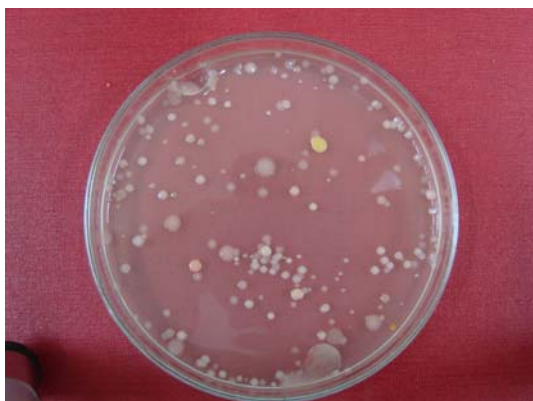


Photo 1. Macromorphological appearance of colonies isolated from *Salix caprea* – *Deschampsia cespitosa* (Dumitrellu Dump) - October, 2006

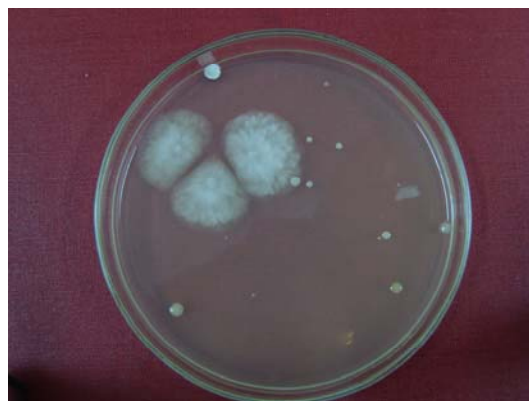


Photo 2. Macromorphological appearance of colonies isolated from *Picea abies* (Pinului Dump) - October, 2006

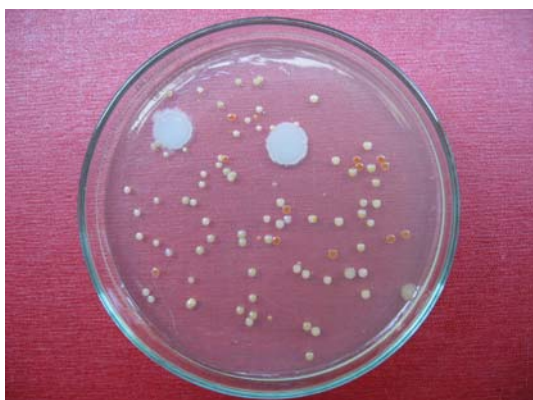


Photo 3. Macromorphological appearance of colonies isolated from *Picea abies* (Ilva Dump) - June, 2007



Photo 4. Macromorphological appearance of colonies isolated from *Pinus cembra* (Dumitrellu Dump) - June, 2007

Table II. CFU/g soil values for rhizospheric soil samples (June, 2007)

Sample no.	Species (Point of collection)	CFU/g soil	
		Root-free soil	Rhizospheric soil
1.	<i>Picea abies</i> (Ilva Dump)	88 x 10 ²	220 x 10 ²
2.	<i>Pinus mugo</i> (Dumitrellu Dump)	66 x 10 ²	217 x 10 ²
3.	<i>Pinus cembra</i> (Dumitrellu Dump)	170 x 10 ²	81 x 10 ²
4.	<i>Vaccinium myrtillus</i> (Pinului Dump)	362 x 10 ²	189 x 10 ²

The CFU/g soil values in the case of the rhizospheric microbiota identified in September, 2007 (Table III) were similar in the case of the species *Pinus cembra* (Pinului Dump) – Photo 5, *Picea abies* (Ilva Dump) and *Pinus mugo* (Dumitreleu Dump), but much smaller in comparison with those of the control samples (Photo 6).

Table III. CFU/g soil values for rhizospheric soil samples (September, 2007)

Sample no.	Species (point of collection)	CFU/g soil	
		Root-free soil	Rhizospheric soil
1.	<i>Pinus cembra</i> (Control)	578 x 10 ²	82 x 10 ²
2.	<i>Pinus mugo</i> (Control)	56 x 10 ²	54 x 10 ²
3.	<i>Picea abies</i> (Control)	88 x 10 ²	47 x 10 ²
4.	<i>Pinus cembra</i> (Pinului Dump)	200 x 10 ²	33 x 10 ²
5.	<i>Picea abies</i> (Ilva Dump)	8 x 10 ²	22 x 10 ²
6.	<i>Pinus mugo</i> (Dumitreleu Dump)	44 x 10 ²	21 x 10 ²



Photo 5. Macromorphological appearance of colonies isolated from - *Pinus cembra* (Pinului Dump) - September, 2007



Photo 6. Macromorphological appearance of colonies isolated from control sample - *Pinus cembra* (Pinului Dump) - September, 2007

The observed differences demonstrate that there is a real microbial diversity. The abundance of the microorganisms in a place depends, first of all, upon the availability of carbon and energy sources and upon the presence of other essential nutrients.

The results confirm the data in the technical literature, according to which, in soils, microorganisms are spread especially in the superficial layers. But the number and the composition of the microbiota can vary according to the type and the particularities of the soil.

QUANTIFICATION OF THE RHIZOSPHERE EFFECT. CALCULATING THE R/S RATIO

The number of microorganisms in the rhizospheric area is very important for determining the rhizosphere effect which is measured by the R/S ratio. This ratio was created due to the quantification differences of the number of microorganisms, instrumental for the assessment of the rhizospheric competence, considered to represent the capacity of an organism to colonize the plant roots. The rhizosphere stimulates the multiplication of bacteria and fungi. Using a simulation programme for the diffusion of the organic substances, Newman, 1978 (quoted by Sylvia, 1999) demonstrated that to a tenfold difference in exudation correspond a hundredfold difference in the abundance of the microorganisms. Experimentally, Bowen, G.D. and Rovira, A.D., (1976) demonstrated that the intact roots can exudates sufficient organic material to allow the development of a large number of microorganisms.

In order to assess the rhizosphere effect, it was necessary to count the colonies in the Petri dishes sown with dilutions made of root-free soil and rhizospheric soil in October 2006, and June and September 2007. It can be observed that in the case of the *Salix caprea* + *Deschampsia cespitosa* (Dumitrellu Dump) and *Picea abies* (Pinului Dump) samples, the number of rhizospheric microorganisms is larger in comparison to those present in root-free soil (*Table IV*). This difference between the two populations of microorganisms can be explained through the beneficial effect that the plant roots exercise upon the microorganisms in the rhizosphere, effect due to the radicular exudations.

Table IV. The assessment of the R/S ratio for the samples collected in October, 2006

Sample no.	Species (point of collection)	R/S ratio
1.	<i>Salix caprea</i> (Dumitrellu Dump)	0.3
2.	<i>Salix caprea</i> + <i>Deschampsia cespitosa</i> (Dumitrellu Dump)	2.566
3.	<i>Picea abies</i> (Dumitrellu Dump)	0.061
4.	<i>Pinus mugo</i> (Dumitrellu Dump)	0.111
5.	<i>Salix caprea</i> (Pinul Dump)	0.044
6.	<i>Picea abies</i> (Pinul Dump)	4.621

The assessed value of the rhizosphere effect is close to the values mentioned in the technical literature, where the rhizosphere effect can be quantified through a R/S ratio of approximately 3 (Sylvia, D.M., 1999, calculates the R/S ratio for a number of rhizospheric microorganisms of $61,4 \times 10^7$ / g).

The number of rhizospheric microorganisms is larger in comparison with those present in the root-free soil in June 2007 for the species *Picea abies* (Ilva Dump) and *Pinus mugo* (Dumitrellu Dump), the values are close to the normal one, mentioned the technical literature (*Table V*).

Table V. The assessment of the R/S ratio for the samples collected in June, 2007

Sample no.	Species (point of collection)	R/S ratio
1.	<i>Picea abies</i> (Ilva Dump)	2.5
2.	<i>Pinus mugo</i> (Dumitreleu Dump)	3.28
3.	<i>Pinus cembra</i> (Dumitreleu Dump)	0.47
4.	<i>Vaccinium myrtillus</i> (Pinului Dump)	0.52

The R/S ratio in September 2007 presents over a unit values (*Table VI*) in the majority of samples, except for the species *Picea abies* (Ilva Dump) where the ratio has a value of 0,36.

Table VI. The assessment of the R/S ratio for the samples collected in September 2007

Sample no.	Species (point of collection)	R/S ratio
1.	<i>Pinus cembra</i> (Control)	3,39
2.	<i>Pinus mugo</i> (Control)	1,03
3.	<i>Picea abies</i> (Control)	1,87
4.	<i>Pinus cembra</i> (Pinului Dump)	6,06
5.	<i>Picea abies</i> (Ilva Dump)	0,36
6.	<i>Pinus mugo</i> (Dumitreleu Dump)	2,09

The R/S ratio varies, according to Clark, 1966 (quoted by Zarnea, 1994), in the rhizospheric soil existing 10-25 times more microorganisms than in the root-free soil, without roots. The ratio can equal 50 in the case of leguminous plants and can reach, in exceptional situation, values between 100 and 200. The R/S ratio of actinomycetes has much lower values in comparison to that of bacteria.

CONCLUSIONS

1. The quantitative analysis of the rhizospheric microbiota shows a series of differences between the species selected for study.

2. In the case of the association *Salix caprea* – *Deschampsia cespitosa* (Dumitreleu Dump) a slight microbial charge is emphasized – where the CFU/g soil value was of 29×10^3 , in comparison with the microbiota from the *Picea abies* (Pinului Dump) – where it turned out to be 61×10^4 CFU/g soil.

3. The rhizospheric microbiota is quantitatively well represented in June 2007, in the case of the species *Picea abies* and *Vaccinium myrtillus*.

4. The R/S ratio in September 2007 presents over-one-unit values in the majority of samples, explained through the beneficial effect that the roots of the plants exercise upon the microorganisms in the rhizosphere due to radicular exudation.

REFERENCES

- AKKERMANS, A.D.L., VAN ELSAS, J.D. (1995): Molecular microbial ecology manual. - *Kluwer Academic Publishers*, Dordrecht, The Netherlands, 177 pp.
- ANGLE, S., WEAVER, R.W., BOTZTOMLEY, P., BEZDICEK, D., SMITH, S., TABATABAI A., WOLLUM A. (1994): Methods of soil analysis, part 2 – Microbiological and biochemical properties. – *Soil Science Society of America, Inc.*, pp. 1121.
- BOWEN, G.D., ROVIRA, A.D. (1976): Microbial colonization of plant roots. - *Annual Review of Phytopathology* 14:121-144.
- BRIMECOMBE, M.J., DE LEIJ, F.A., LYNCH, J.M. (2001): The effect of root exudates on rhizosphere microbial populations. In: Pinto, R., Varanini, Z., Nannipierei, P. (Eds.), *The rhizosphere, Marcel Dekker, New York*, pp: 95-141.
- DUNCA, SIMONA, AILIESEI, OCTAVITA, NIMITAN, ERICA, STEFAN, M. (2004): Microbiologie aplicata. - *Ed. Tehnopress, Iasi*, 263 pp.
- ELIADE, G., GHINEA, L., STEFANIC, G. (1975): Microbiologia solului. – *Ed. Ceres, Bucuresti*, 218 pp.
- GIRI, B., GIANG, P.H., KUMARI, R., PRASAD, R., VARMA, A. (2005): Microbial diversity in soils. In: Buscot, F., Varma, S. (Eds), *Microorganisms in soils: roles in genesis and functions. - Springer-Verlag, Heidelberg, Germany*, pp: 195-212.
- LIM, D.L. (1998): Microbiology. - *McGraw-Hill Companies, Inc.*, 720 pp.
- LUGTENBERG, B.J.J., DEKKERS, L., BLOEMBERG, G.V. (2001): Molecular determinants of rhizosphere colonization by *Pseudomonas*. - *Annual Review of Phytopathology* 39:461.
- MADIGAN, M.T., MARTINKO, J.M., PARKER, J. (2000): Brock Biology of Microorganisms. - *Prentice Hall, Inc. Upper Saddle River, New Jersey*, 991 pp.
- NORRELL, S.A., MESSLEY, K.E. (1997): Microbiology laboratory manual, Principles and Applications. – *Prentice Hall, Inc. Upper Saddle River, New Jersey*, 301 pp.
- ROVIRA, A.D. (1969): Plant Roots Exudates. - *Botanical Review* 35: 35-57.
- SYLVIA, D.M., FUHRMANN, J.J., HARTEL, P.G., ZUBERER, D.A. (1999): Principles and applications of soil microbiology. - *Prentice Hall, Inc. Upper Saddle River, New Jersey*, 407 pp.
- ZARNEA, G. (1994): *Tratat de microbiologie generala. - Ed. Academiei Romane, vol.V, Bucuresti*, 1078 pp.