BABEŞ-BOLYAI UNIVERSITY of CLUJ-NAPOCA

Faculty of Biology and Geology

STUDIES ON MICORRHIZAE IN ORCHIDS

-Abstract of the PhD thesis-

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INTRODUCTION

Symbiosis is a general process that extends to the whole biosphere and consists of an interdependence relationship between individuals of the same species, between species of the same kingdom, as well as between species of different kingdoms.

During the course of evolution, living beings have come into contact with one another, establishing and developing relationships conditioned by environmental factors, as well as by the morphophysiological and biochemical particularities of each of them.

A key role in the functioning of natural and human altered ecosystems is played by the establishment of symbioses between photosynthesizing plants and soil microorganisms, particularly those situated in the rhizosphere.

Given the small number of Romanian studies on the genera and species of native or cultivated orchids, as well as on the symbiosis of these plants with specific mycorrhizal fungi, we chose to study the presence of this relationship between the two types of organisms, one considered as lower plants (fungi), the other as higher plants, among the most evolved plants (angiosperms, monocotyledons).

We also aimed to clarify, at least partially, the microbiota of soils in which orchids develop. For this, we chose 5 areas for the collection of samples, three of which being areas where orchids are present and two other being soils without orchids.

This topic was chosen because there are no data on the correlation between mycorrhizal fungi-orchids and the nature of soils specific for the different areas populated with these plants. The enzymatic processes in these soils that allow to assess the evolution of these types of soil have not been studied so far.

In this context, the main objectives were the following:

Chemical characterization of soils from different areas populated with orchids;

Evaluation of enzymatic activities in the soils concerned (by the quantitative and qualitative assessment of enzymatic activities) in order to assess the evolution of these soils;

Establishment of the abundance, density, diversity and ecological significance of bacteria involved in the biogeochemical nitrogen cycle (aerobic heterotrophic, ammonifying, nitrifying, denitrifying, nitrogen-fixing bacteria) in the soil samples in relation to environmental factors;

Determination of the enzymatic indicator of soil quality (EISQ) and of the bacterial indicator of soil quality (BISQ), which allow the comparison and hierarchization of the analyzed samples;

Determination of the main groups of fungi in orchid soils compared to soils without orchids;

The relationship between EISQ and BISQ in orchid soils;

Statistical analysis of the studied enzymological parameters, in order to observe the way in which these can influence the presence of orchids in the analyzed soils.

Through the studies carried out by us for the first time in Romania, we aimed to evidence the presence of mycorrhizae in orchids and also, to perform microbial ecology studies on the soils in which these mycorrhizae develop. These microorganisms applied to soils poor in organic substances increase the supply of plants with the necessary nutritive substances, so that they are considered real biofertilizers of the soil.

I. MYCORRHYZAL SYMBIOSIS.

Mycorrhizae are the result of symbiotic associations that develop between the roots of most of the plants and specific fungi, during the course of their growth periods.

This type of associations are complex mutual relationships that serve to the optimization of the reserve of mineral substances for the host plant and provides organic compounds and ecological shelter to fungi.

Mycorrhizal symbiosis as a mutual relationship brings advantages to both organisms involved in this relationship.

Mycorrhizae differ depending on the morphology, physiology and taxonomy of the partners, which manifest a high degree of specificity. The following types are found: ectomycorrhizae, endomycorrhizae, ectendomycorrhizae and peritrophic mycorrhizae [Zamfirache and Toma, 2000].

Endomycorrhizae are characterized by the fact that fungi are capable to penetrate the root cells of the host plant without any reaction from the part of the host plant to the invasion of the fungus [Zamfirache and Toma, 2000].

The group of endomycorrhizae includes three subgroups, ericoid mycorrhizae, orchid mycorrhizae, and vesicular-arbuscular mycorrhizae.

II. ELEMENTS OF MICROBIAL ECOLOGY.

II.3. Physical interactions between bacteria and mycorrhizal fungi.

Many rhizobacteria have proved to be excellent root colonizers [Lugtenberg and Dekkers, 1999; Barea et al., 2002], a process that is influenced by a number of external factors that play an important role in physical interactions between these bacteria and the roots of plants [Bianciotto and Bonfante, 2002]. Some bacteria have proved to be good root colonizers, for example, some *Pseudomonas* species, which are capable to adhere to the surface of fungal hyphae.

A benefit of this relationship between bacteria and mycorrhizal fungal hyphae is the fact that certain metabolic interactions are facilitated, such as the exchange of nutrients and carbon, due to the close contact between bacteria and their fungal partners.

II.8. Future perspectives on the relationship between fungi and bacteria.

Rhizobacteria can improve the growth of plants, nitrogen fixation, the production of hormones, plant nutrition and also, the control of diseases in plants. Different rhizobacteria such as *Azospirillum*, *Bacillus*, *Pseudomonas* and *Enterobacter* have been used for their beneficial effects on the growth of plants [Kloepper et al., 1992, Hoβlich et al., 1994]. Many biofertilizers are mainly based on rhizobacteria, which exert beneficial effects on the development of plants, frequently related to the increase of the availability of nutrients for plants [Vessay, 2003].

The synergistic interactions between mycorrhizal fungi and nitrogen-fixing bacteria such as *Azotobacter chroococcum*, *Azospirillum* spp. and *Acetobacter diazotrophicus* have been studied by Suresh and Bagyaraj [2002]. Mycorrhizal fungi and rhizobacteria complete one another in their role of nitrogen fixation, phytohormone production, solubilization of phosphorus and increase of the absorption surface.

III. MATERIALS AND METHODS.

III.1. Materials and methods used in the study for the identification of the presence of mycorrhizae in orchids.

The plant material used for the research consisted of the roots of 6 orchid species, of which 3 belong to the Romanian flora and the other 3 are plants that have been cultivated for many years in the greenhouses of the ”Alexandru Borza” Botanical Garden of Cluj-Napoca.

All orchid species in the world currently benefit from a special status, a protective status conferred by their presence on the CITES list, or which prevents trade with plants taken from nature, from the place of origin, and limits even seed exchanges for some very rare or endangered species [Convention on International Trade in Endangered Species of wild fauna and flora – online, conventions.coe.int. – online, O.G., 2000].

Of the orchids of the Romanian flora, we studied the following three species: *Cypripedium calceolus* L., *Dactylorhiza fuchsii* (Druce) Soó, and *Epipactis palustris* (L.) Crantz. The species from the greenhouses of the Botanical Garden were represented by *Paphiopedilum insignae*(Wall. ex Lindl.) Pfitz., *Anoectochilus regalis* Blume, and *Epidendrum radicans* Pav.ex Lindl.. The samples included in the study were subjected to both optical microscopy observations and electron microscopy analysis.

III. 2. Materials and methods used in soil microbiology studies.

The sample collection areas were the following: soil from Valea Morii, Botanical Garden soil cultivated with *Cypripedium*, greenhouse soil cultivated with orchids, grassland soil from Chinteni, and greenhouse soil uncultivated with orchids. The last two served as control samples, orchids being absent from them.

III.2.1.1. Collection of samples.

The soil samples for various analyses were collected according to methodological norms established in ”Ghidul pentru prelevare a probelor de sol” (”Guide for the collection of soil samples”) (SR ISO 10381-2/2002) and were processed according to standard norms SR ISO 10381-6/1993.

The samples were collected using a sterile spatula and were introduced in sealed sterile bags. Each bag containing a soil sample had a label indicating the place from where the sample was taken, the day and hour of the collection, the depth at which the sample was collected.

III.2.2. Methods for the chemical analysis of soil samples.

The agrochemical analysis of the soil is performed in order to characterize (assess) the fertility of soils, to establish the required nutritive elements and even to determine the degree of soil pollution. It is considered that humus and the mobile (accessible) forms of macro- and microelements in the soil can provide a satisfactory picture of the general state of soils.

III.2.3. Enzymological methods

III.2.3.1. Quantitative enzymatic methods consisted of the carrying out of the following stages:

III.2.3.1.1. Determination of actual and potential dehydrogenase activity.

It is used as a global indicator of the biological activity of organisms, but also as an ecotoxicological test for evaluating the effects of pollutants on the soil microbiota.

III.2.3.1.2. Determination of phosphatase activity.

Phosphorus is one of the essential nutrients for the growth of plants. In the cycle of this element, inorganic and organic forms are associated through mineralization and immobilization processes, mediated by abiotic and biotic activities. In mineralization processes, organic phosphorus fractions that represent a large amount of soil and sediment phosphorus are converted to inorganic forms that are used by plants as a result of the action of phosphatases [Gianfreda şi Bollag, 1996].

III.2.3.1.3. Determination of catalase activity.

Catalase activity is determined by expressing the decomposition intensity of oxygenated water that is formed during the process of respiration of aerobic microorganisms. Catalases are found

in almost all animal cells and in smaller amounts in higher plants. In the case of microorganisms, catalases are only found in aerobic ones [Regelsberger et al., 2002].

III.2.3.2.2. Determination of the enzymatic indicator of soil quality.

The determination of enzymatic activities in the soil represents a research tool for assessing the biochemical processes in these natural environments and for finding soil quality indicators [Drăgan-Bularda et al., 2004].

The higher the enzymatic indicator, the higher the enzymatic potential of the soil. The enzymatic indicator of soil quality (EISQ) gives a general view of the enzymatic potential of the soil, being calculated based on a calculation formula elaborated by Muntean et al. [1996]:

$$EISQ = \frac{1}{n} \bullet \sum_{i=1}^{n} \frac{V_r(i)}{V_{\max}(i)}$$

where: EISQ = enzymatic indicator of soil quality;

n = number of activities;

V_r (i) = individual real value;

V_{max} (i) = maximum theoretical individual value.

III.2.4.7. Determination of the bacterial indicator of soil quality.

The bacterial indicator of soil quality allows the global determination of the abundance of microorganisms in the soil, the evaluation of the microbial potential of soils, the establishment of the seasonal variations of microorganisms, the estimation of the biological quality of terrestrial habitats.

The bacterial indicator of soil quality (BISQ) is determined based on the calculation formula proposed by Muntean (1995-1996):

$$BISQ = \frac{1}{n} \bullet \sum \log_{10} N$$

where: BISQ – bacterial indicator of soil quality;

n – number of ecophysiological groups of bacteria;

N – number of bacteria belonging to each ecophysiological group.

III.3. Methods used for the statistical interpretation of results: two tests were used: the Mann-Whitney test and logistic regression [Wilcox, 2009].

IV. RESULTS AND DISCUSSION.

IV.1. Evidencing of mycorrhizae in the studied orchids.

Figure 23 shows how fungal hyphae traverse the absorbing hairs of the root of the *Anoectochilus regalis* species. The rhizodermal layer is not very well defined in this image because there are many soil residues that adhere to rhizodermal cells very well. The image also demonstrates the fact that the contact between fungal hyphae and the root cortex is mainly established through the absorbing hairs.

Fig. 23. Cross section of the *Anoectochilus regalis* root (40x), unstained (photo C. Mocan)

In figure 25, the section being through the root of a terrestrial orchid, the velamen radicum (typical of aerial root orchids) is absent; the unstratified rhizodermis can be seen, which is continued with the exodermis, elongated cells with multiple suberin thickenings are noted. In the first cortical layers, there are inclusions that seem to be of lipid nature considering the brownish color, but these are residues of mycelial hyphae digested through the talyphophagy phenomenon (Figs. 26, 38) [Zamfirache and Toma, 2000].

What is exceptionally well seen here is the endodermis; the external tangential walls are seen, which remain cellulosic, while the others are impregnated with lignin (horseshoe thickenings). From place to place, usually at the level of the ligneous bundles, passage cells are visible (cells that maintain their cellulose walls, and the elaborated sap circulates through diffusion towards the cortical cells). Ligneous bundles with the metaxylem towards the center of the central cylinder are seen exceptionally well and the medullary rays are highly lignified towards the exterior, but the medulla in the center of the central cylinder remains cellulosic.

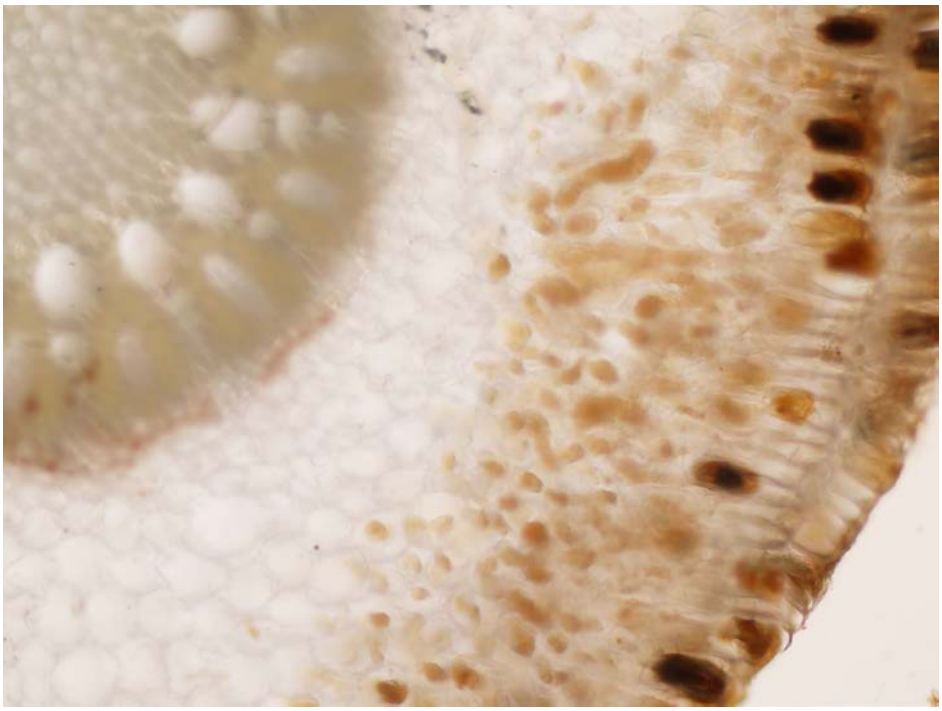

Fig. 25. Cross section of the *Cypripedium calceolus* root (20x), unstained (photo C. Mocan);
yellow arrow – pelotons in the cortical cells

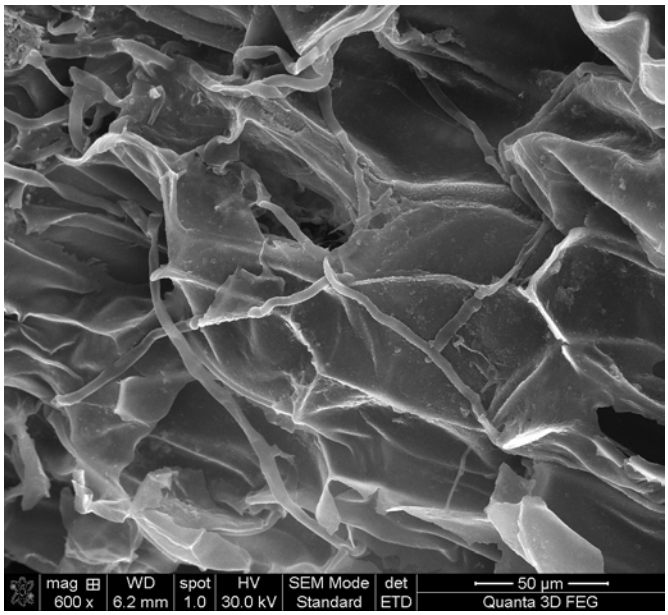

Fig. 53. Septate hyphae on the external surface of root cells (photo C. Mocan)

The scanning electron microscopy observations made on mycorrhizae are presented in Figures 50, 51, 52 and 53.

The last two figures show how the hyphae penetrate the root cells (Fig. 52), as well as the fact that they have 90^0 ramifications (Fig. 53).

The analysis of the preparations using the transmission electron microscope evidenced the presence of mycorrhizal mycelial hyphae inside the cortical cells, found at different development stages. TEM images also revealed the presence of endophytic bacteria inside the root cells, which entered the cells along with mycorrhizal fungi.

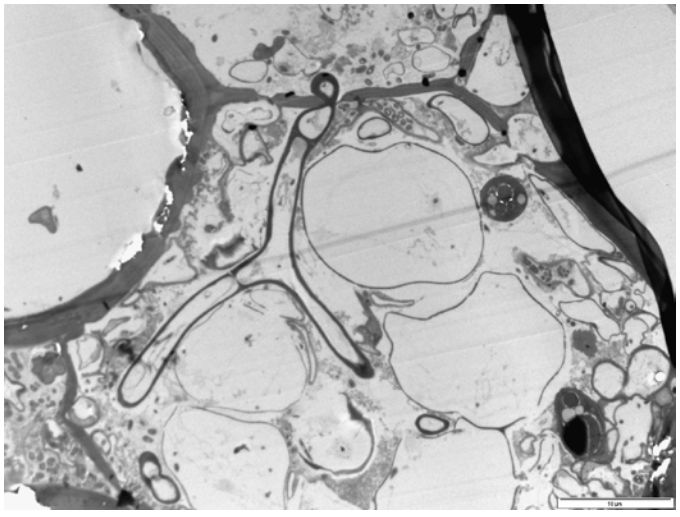

Fig. 54. Septate hypha inside cortical cells (photo C. Mocan)

The root cortical cell in the image shown in Figure 54 is invaded by fungal hyphae of different sizes, some of which are septated. The cell wall with a punctuate pattern is also seen.

V. Microbiological data on orchid soils.

In the 5 soil samples, the following enzymatic activities were quantitatively determined: actual dehydrogenase activity (ADA) (reduction of 2,3,5-triphenyltetrazolium chloride – TTC in samples without glucose addition) and potential dehydrogenase activity (PDA) (with glucose addition), phosphatase activity (PA) and catalase activity (CA). Because the different soil samples may have a variable water content, which would influence the expression of enzymatic activities in relation to the weight of the soil, humidity was determined and dry substance was established in parallel to the preparation of the samples for analysis [Atlas, 2004,Cuşa, 1996].

V.2.2.1.1. Determination of actual and potential dehydrogenase activity.

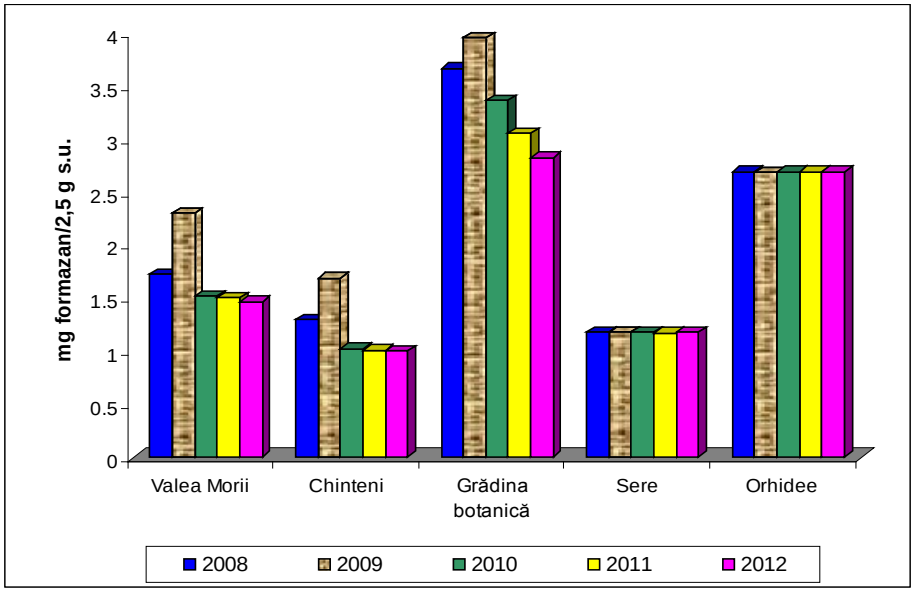

Fig. 66. Evolution of actual dehydrogenase activity (ADA) (mg formazan/g d.s.) recorded in the analyzed soils, in the period 2008-2012.

On the whole, potential dehydrogenase activity proved to be much higher than actual dehydrogenase activity. This reflects the stimulating action of the carbon source (added glucose) on the synthesis of enzymes by microorganisms.

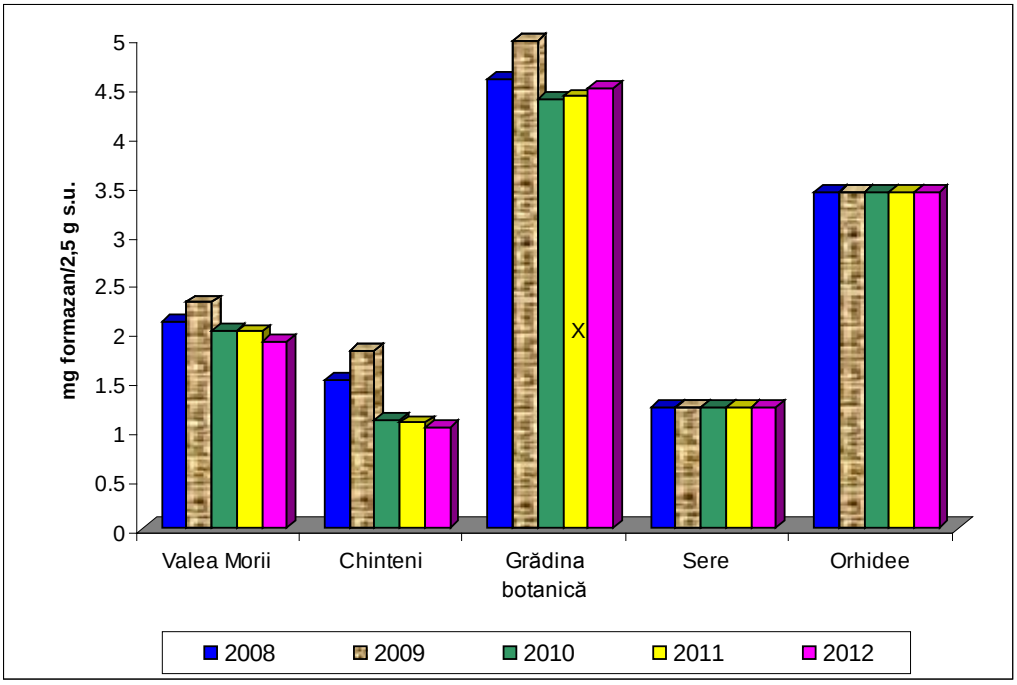

Fig. 67. Evolution of potential dehydrogenase activity (PDA) (mg formazan/g d.s.) recorded in the analyzed soils

Like in the case of actual dehydrogenase activity, potential dehydrogenase activity had elevated values in the soil samples cultivated with orchids compared to the other analyzed samples.

The highest values were found in the soil samples cultivated with orchids, the Botanical Garden and the Valea Morii soils.

However, it should be noted that the values of total dehydrogenase activity were relatively lower compared to those of agricultural soils, which demonstrates the presence of a lower microbial potential in the analyzed soils that were not subjected to organic and/or mineral fertilization.

V.2.2.1.2. Determination of phosphatase activity.

Phosphatase activity was detected in all 5 analyzed soil samples, the mean value distributions being illustrated in Tables 8, 9, 10, 11, 12 (in thesis), as well as in Fig. 68. Altogether, phosphatase activity was higher in the soil samples from Chinteni compared to the soil samples from Valea Morii and the soil cultivated with orchids in greenhouses, increased numerical values being recorded. The highest values recorded in the soil from Chinteni were due to the accumulation of plant residues in the soil at the end of the vegetation period [Reis et al., 2001], this soil being an undisturbed grassland soil.

This can be explained by the much more intense biological activity of the soil in the hot season. Another explanation can be given by the increase in the amount of organic matter stored in the soil.

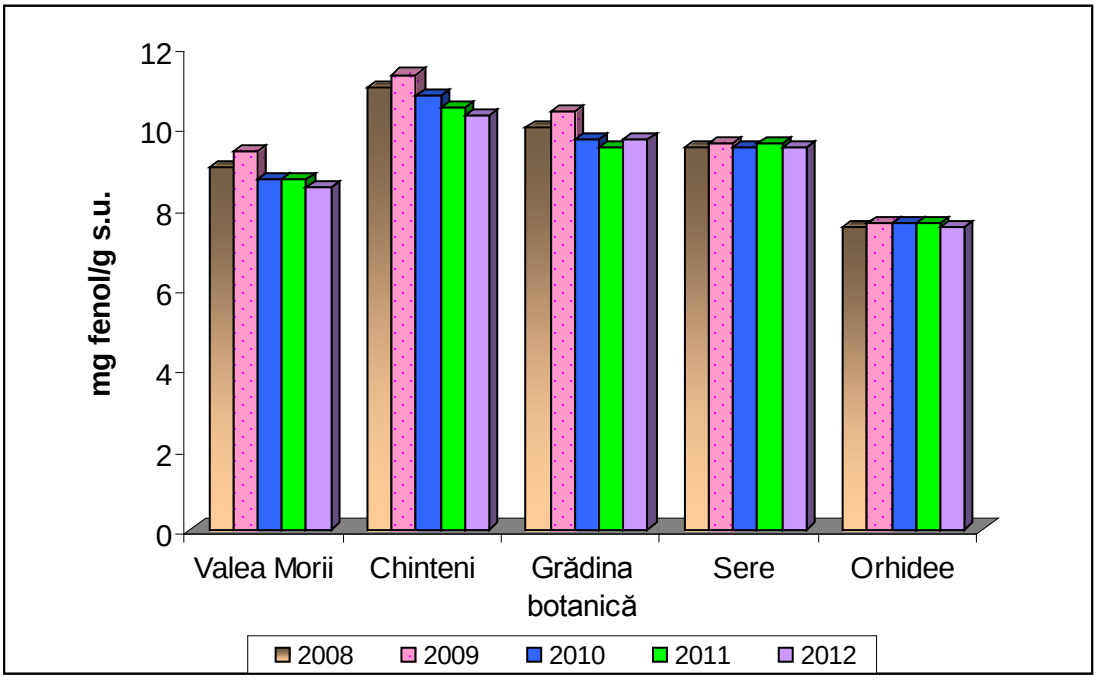

Fig. 68. Evolution of phosphatase activity (mg phenol/g d.s.) recorded in the analyzed soils

Figure 68 shows the evolution of phosphatase activity during the five study years in the five studied soils. It can be seen that the grassland soil from Chinteni had during the course of all five years monitored by us the highest phosphatase activity values, followed by the Botanical Garden soil. It is known that uncultivated soils may have important enzymatic activities, as they are not affected by human intervention [Kiss et al., 1975].

V.2.1.4. Determination of the enzymatic indicator of soil quality (EISQ)

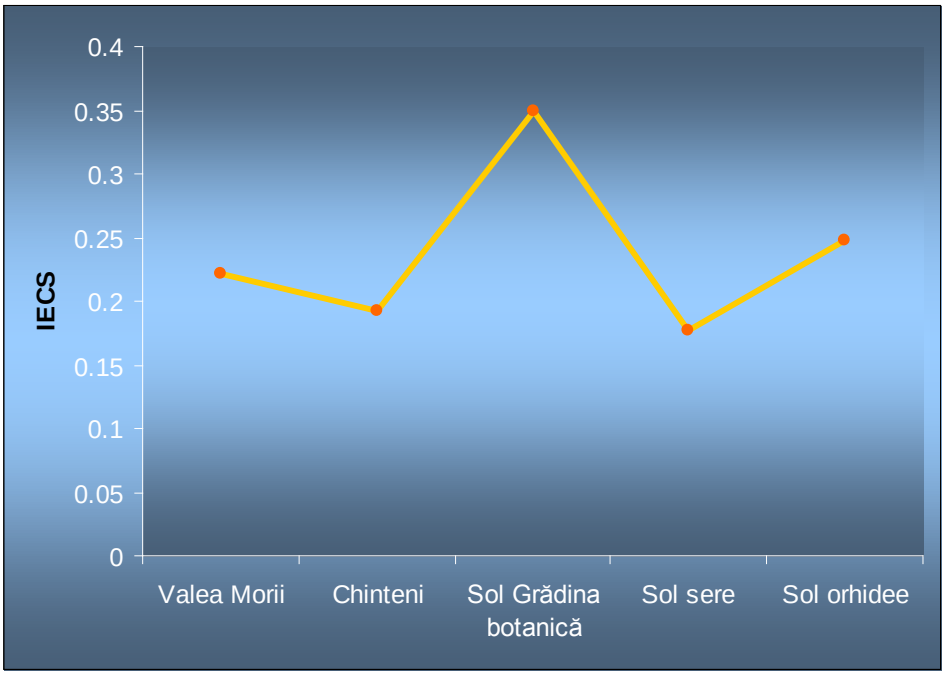

Fig. 70. Values of the enzymatic indicator of soil quality

The determination of enzymatic activities in soils is a research tool for evaluating the functional diversity of the microbiota and of biochemical processes in these habitats.

Based on the results obtained and compared to the literature data [Paşca et al., 1993; Drăgan-Bularda et al., 1995], it can be estimated that some analyzed soils have a considerable biological potential.

Compared to literature data [Zborovschi et al., 1989; Drăgan-Bularda et al., 1995], it can be concluded based on our data that phosphatase activity and particularly, catalase activity have higher values compared to dehydrogenase activity. This outcome can partly be explained by the fact that the accumulated enzymes are the result of a soil microbiota activity for tens of years, while enzymes due to the proliferating microbiota are more affected by the climate factors manifesting during the five study years.

The higher the EISQ, the better the quality of a soil [Muntean et al., 2004].

V.2.2.Qualitative enzymatic analysis.

V.2.2.1. Evidencing of the activity of some oligases and polyases (polysaccharidases).

In the five soil samples taken from the five areas, the following enzymatic activities were qualitatively determined: two oligase activities – saccharase (invertase) activity (SA), lactase (β-galactosidase) activity (LA) and three polyase activities: - amylase activity (AA), dextranase activity (DA) and cellulase activity (CelA). These enzymatic activities were determined by paper chromatography, the circular technique [Drăgan-Bularda, 2000]. The reducing hydrolytic products are evidenced using the paper chromatography method. The more intense the spot of the hydrolytic products, the higher the activity of oligases and polyases. During the course of examination, the spots of the tested samples are compared to those of control samples.

Oligase activities (SA, LA) were present in all the analyzed soil samples.

The polysaccharidase (polyase) activities of the soils are the main component of the enzymatic potential of a soil, because polysaccharides are the most important substances from the plant residues that reach the soil. Sugars that reach the soil following root secretion also contribute to enzyme synthesis [Kiss et al., 1975].

Of the analyzed polysaccharidase activities, two – amylase and cellulase activities proved to be important (Figs. 73 and 74). Amylase activity (AA) is directly proportional to the amount of humus and the cation exchange capacity [Eliade et al., 1975]. Interestingly, the analysis of the chromatogram in Fig. 64 shows that amylase activity was extremely high in all soil samples, without significant qualitative differences depending on the amount of humus that varied considerably according to the collection area.

The cellulase activity of the five soils included in the study reveals that this was present in soil samples 1-5, through the formation from the enzymatic substrate (cellulosi pulvis) of the end hydrolysis product, glucose. The glucose spot of the Valea Morii soil was similar to that of the Chinteni soil and to that of the Botanical Garden and the orchid greenhouse soils. It was less intense in the greenhouse soil. Based on the spot intensity, it can be estimated that the accumulated cellulase was considerable, given that the enzyme was detected in a very small reaction mixture amount.

Dextranase activity was present particularly in the first four soil samples, with the highest values in the Botanical Garden and the orchid greenhouse soils, and lower values in the greenhouse soil sample, which could also be attributed to its presence in small undetectable amounts in the analyzed fluid. The end product resulting following dextran hydrolysis was glucose.

V.3. Microbiological analysis.

This study on the dynamics of microbial populations involved in the nitrogen cycle in soils cultivated with orchids is a necessity under the conditions in which there are no data regarding the role, function and dynamics of these bacteria in these soils. So, this study analyzes for the first time the evolution and the diversity of these bacterial populations in orchid soils compared to soils without orchids. The microbiological analyses by which the spatial distribution of the density of aerobic heterotrophic bacteria and bacteria involved in the biogeochemical nitrogen cycle was determined were carried out in the 5 analyzed types of soils.

In the analyzed soil samples, the bacteria involved in the biological nitrogen cycle were present in rather small amounts (Tables 13, 13a, 13b), in 3 cases the highest values reaching tens of thousands. Of the studied bacteria, AMB had higher values, while NiB had lower values in all soils.

Aerobic heterotrophic bacteria were present in high numbers compared to the other groups, and they were found in the highest numbers in the soils with the highest dehydrogenase and catalase activity (Botanical Garden soil, orchid soil, and Valea Morii soil).

In 2009, the number of all studied groups of bacteria increased slightly, similarly to the increase in the value of the studied enzymatic activities, with the exception found in greenhouse soils, where environmental conditions were maintained somewhat constant, and thus, there were no significant variations in the number of bacteria in the soil.

The groups of bacteria studied in 2010 had lower values compared to the first year (2008) and particularly, the second year (2009). This decrease was in accordance with the reduction of enzymatic activities, maily due to less favorable weather conditions.

In 2011, the situation of the previous years was maintained, but numerical values slightly decreased. The same picture is shown in Table 13d, presenting the results obtained in 2012, but the values found were close to those of the last three study years.

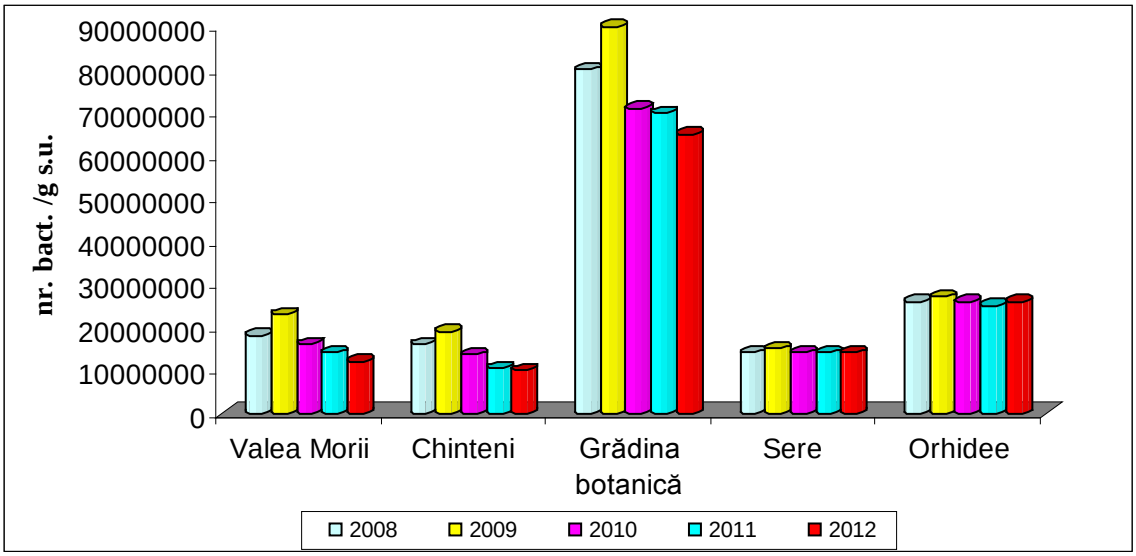

Fig. 76. Numerical distribution of aerobic heterotrophic bacteria (AHB) in the analyzed soils

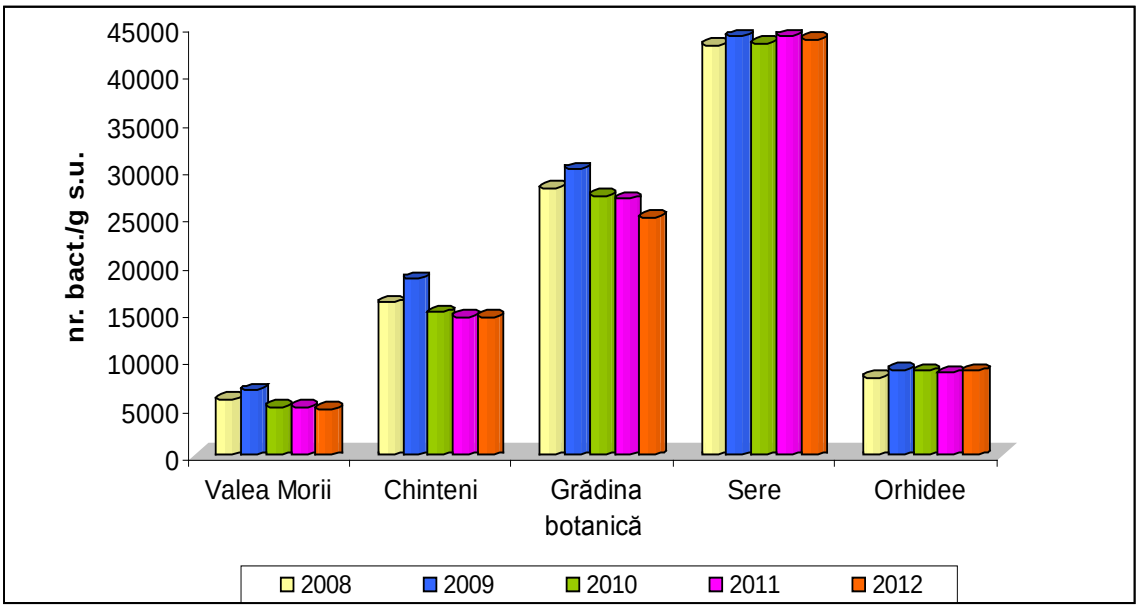

Fig. 77. Numerical distribution of ammonifying bacteria (AMB) in the analyzed soils

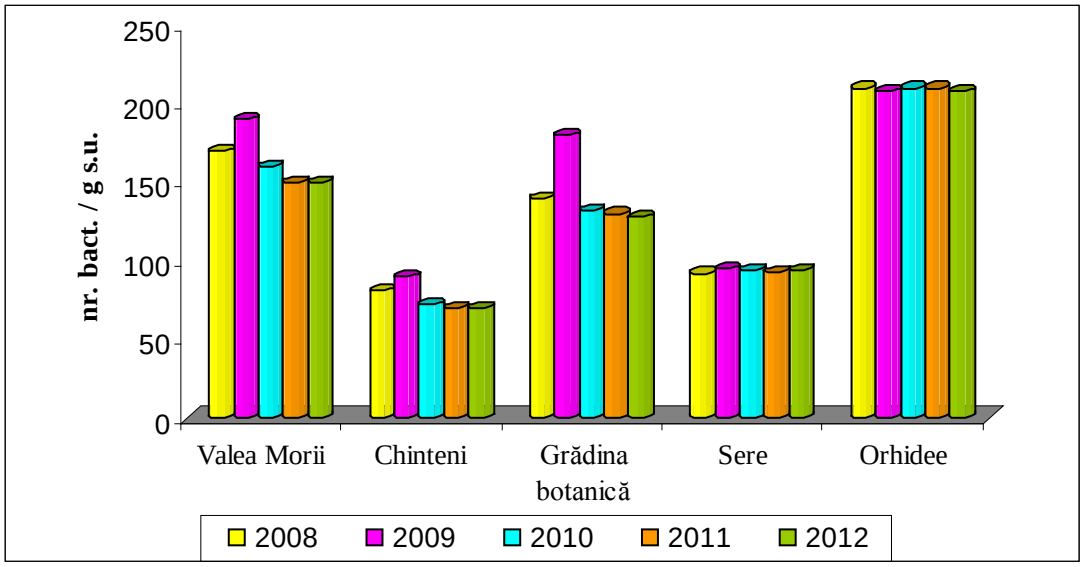

Fig. 79. Numerical distribution of nitrate bacteria (NaB) in the analyzed soils

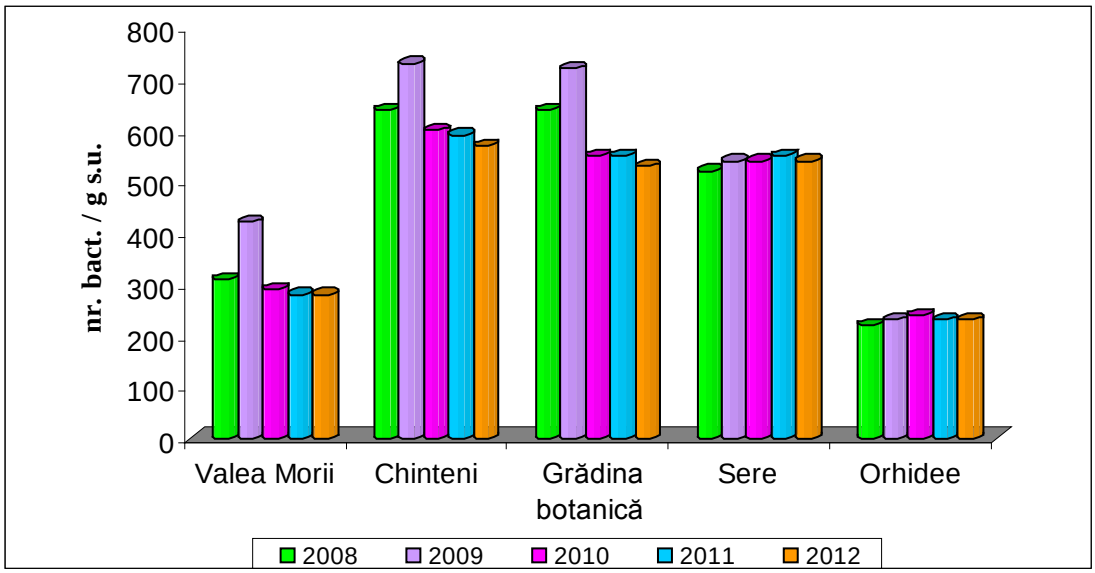

Fig. 80. Numerical distribution of denitrifying bacteria (DNB) in the analyzed soils

V.3.2. Percent distribution of bacteria groups involved in the nitrogen cycle according to collection areas.

In Valea Morii, ammonifying bacteria were present in the highest percentage (90%), followed by denitrifying bacteria, nitrate bacteria and nitrite bacteria.

It is known that in marsh and peat areas, microbial processes in the nitrogen cycle are more reduced.

For the Chinteni soil, the situation was similar to that of the Valea Morii soil, ammonifying bacteria being the most numerous, followed by denitrifying bacteria, while nitrifying bacteria were found in a proportion of less than 1% (Fig. 82).

The Botanical Garden soil (Fig. 83) had the highest percentage of ammonifying bacteria, 98%, the other bacteria being in a low proportion, 2%.

In the soil samples taken from greenhouses, soil uncultivated with orchids (Fig. 84), ammonifying bacteria were predominant, the other groups of bacteria being present in a very low percentage.

In the soil samples taken from greenhouses, soil cultivated with orchids (Fig. 85), there was a higher proportion of denitrifying and nitrifying bacteria compared to the soils from Chinteni, the Botanical Garden, and the greenhouse soil uncultivated with orchids.

This uneven distribution of the bacteria groups involved in the nitrogen cycle might be explained by the fact that the Valea Morii soil and the greenhouse soil cultivated with orchids have a structure that creates a favorable environment for the development of these bacteria. These soils are more humid and have a higher peat content. In these two soils, the percentage of nitrifying and denitrifying bacteria was higher.

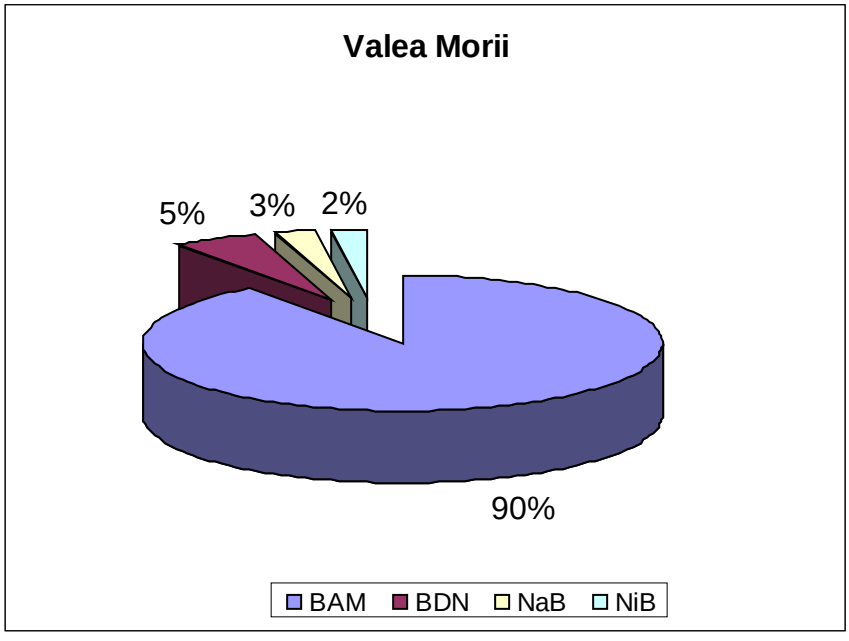

Fig. 81. Quantitative variation (%) of bacteria in the nitrogen cycle in the Valea Morii soil

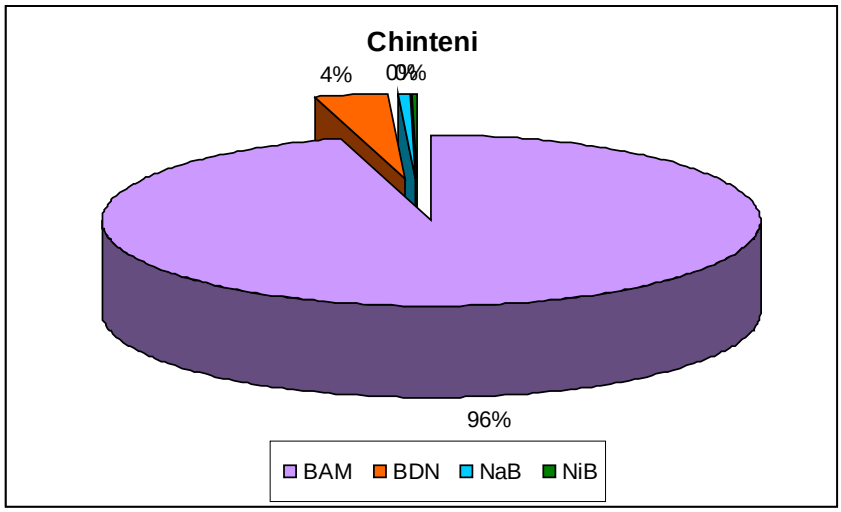

Fig. 82. Quantitative variation (%) of bacteria in the nitrogen cycle in the Chinteni soil

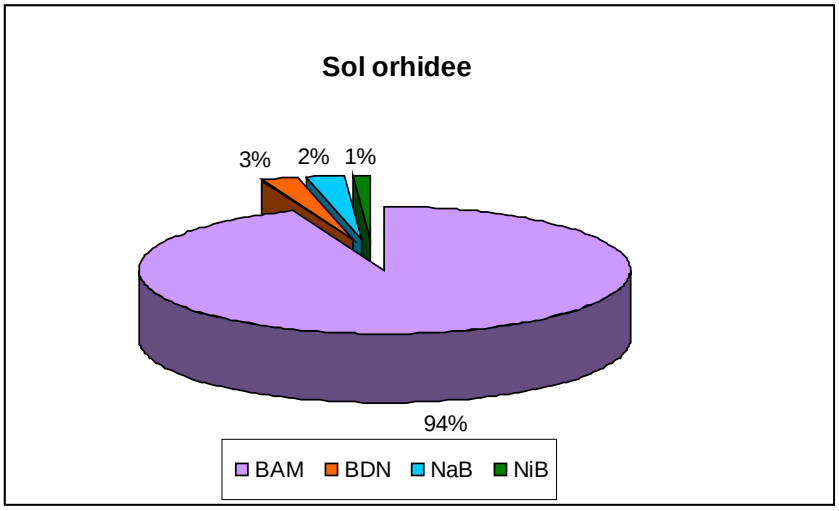

Fig. 85. Quantitative variation (%) of bacteria in the nitrogen cycle in the orchid greenhouse soil

V.3.3. Determination of the bacterial indicator of soil quality (BISQ).

In order to estimate the quality of the soil from various study areas, we used the five ecophysiological groups of bacteria: aerobic heterotrophic bacteria (AHB), ammonifying bacteria (AMB), nitrifying bacteria (nitrite bacteria – NiB and nitrate bacteria – NaB), and denitrifying bacteria (DNB). The microbial potential of the analyzed soils was moderate, with values ranging between 3.8-3.5. Thus, the Botanical Garden soil proved to have a relatively important bacteriological potential, being followed by the greenhouse soil and the greenhouse soil cultivated with orchids, the lowest potential being found in the Chinteni soil (partially degraded grassland) and the Valea Morii soil, a peat and marshy soil.

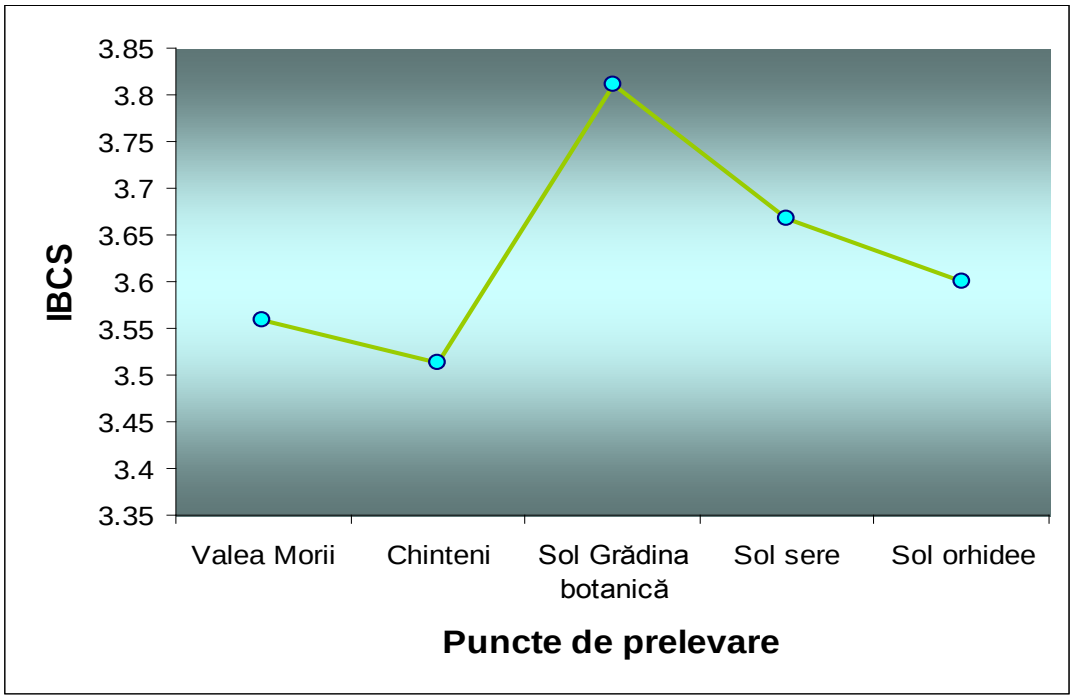

Fig. 86. Distribution of BISQ values for the different analyzed soils

Between BISQ and EISQ values, a positive correlation significant at $p < 0.05$ was established, which certifies the parallel between the bacterial and enzymatic potential of soils, regardless of the collection area.

V.4. Isolation of *Azotobacter* strains from the soil samples.

For the isolation of bacteria of the *Azotobacter* genus, an elective medium was used, the Ashby culture medium, rich in organic substances, without bound nitrogen. On this solid medium, seedings from all the studied soil samples were performed using the soil grain procedure. With a sterile Pasteur pipette, soil grains 1-3 mm in diameter were introduced on the surface of the medium. In each box, 25 soil grains were seeded (Fig. 87a).

After seeding, the Petri boxes were incubated at 28º C, for 10-14 days. During the incubation period, mucilaginous colonies appeared around the soil grains, which brunified subsequently (Fig. 87b), being typical for bacteria of the *Azotobacter* genus.

These colonies were transferred to fresh media using subculture methods until pure cultures were obtained, from which nitrogen-fixing bacteria of the *Azotobacter* genus could be evidenced microscopically. These bacteria were also evidenced in the root cortical cells of orchids through transmission electron microscopy analyses (Fig. 88).

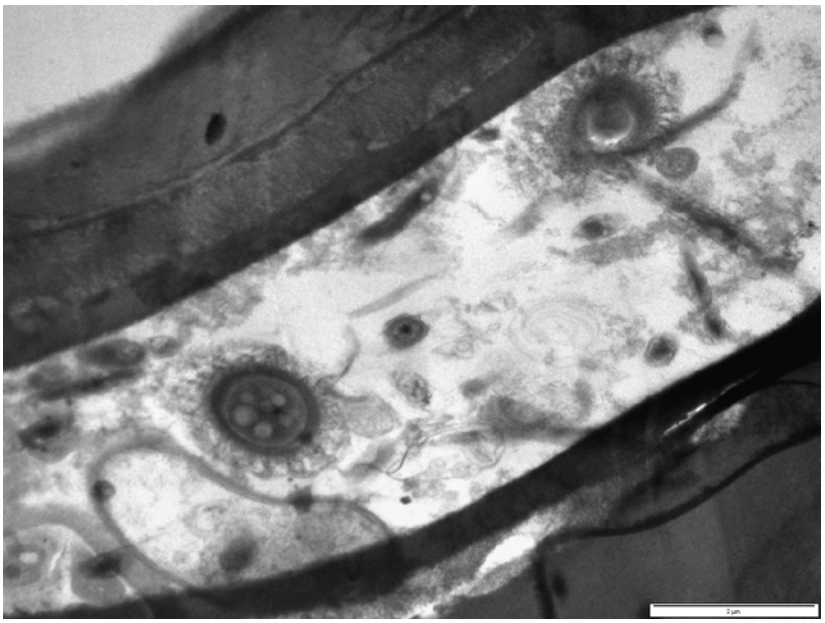

Fig. 88. Nitrogen-fixing bacteria of the *Azotobacter* genus in root cells, images obtained by transmission electron microscopy

V.5. Isolation of fungi from the soil.

In order to determine the presence of fungi (micromycetes) in the 5 analyzed soil types, two types of media were used, the Martin medium and the Csapek-Dox medium, which is used for the isolation of parasitic and saprophytic fungi [Hulea, 1969, Drăgan-Bularda, 2000].

From each analyzed type of soil (2 g), soil dilutions were performed using the dilution technique, then seedings in the culture media were carried out. The dilutions used were 10^{-4} 10^{-5},10^{-6}, for all the 5 samples. The number of hyphae was calculated by multiplying the number of the developed colonies by the inverse dilution value, in this case by 10^{-5}.

After the development of fungi in the culture medium, their identification was initiated, and macroscopic and microscopic analysis revealed the fact that in the Botanical Garden soil (Fig. 89), the *Penicillium*, but also the *Fusarium* genera were predominant, in the greenhouse soil (Fig. 90), the *Penicillium* and *Rhizopus* genera, in the Chinteni soil (Fig. 91), the *Penicillium* genus, and in the other soils (Valea Morii and orchid soils), the *Penicillium* and *Fusarium* genera were evidenced (Figs. 93, 94). The presented figures show the fact that the micromycetes in the soils belonging to the Botanical Garden area were better developed and more numerous, consequently these soils have a more complex microbiota than the natural soils (Valea Morii and Chinteni).

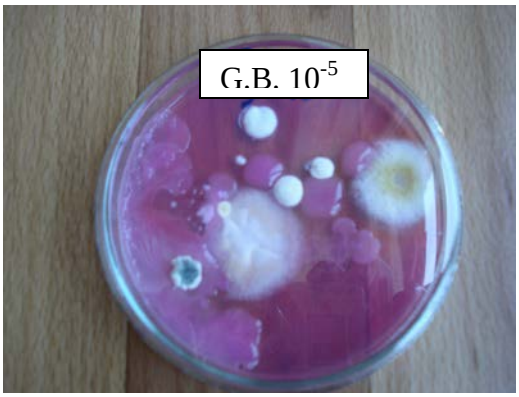

Fig. 89. Micromycetes in the Botanical Garden soil (photo C. Mocan)

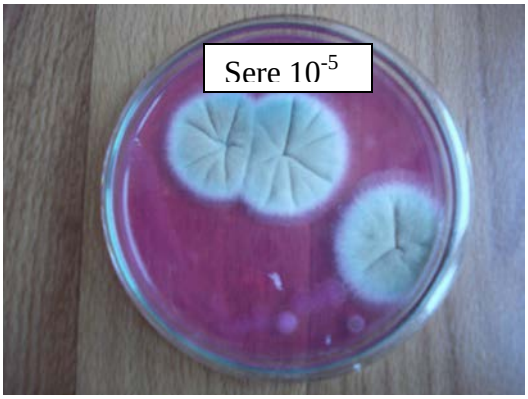

Fig. 90. Micromycetes in the greenhouse soil (photo C. Mocan)

V.7. Statistical interpretation of results.

V.7.1. The Mann-Whitney test.

This is one of the strongest non-parametric tests. It can be used both in small and large subject samples and requires only rank measurements, or when the requirements for the application of the independent t test are not met. This test operates with ordinal numbers. The test is useful for determining if the means of two groups are different.

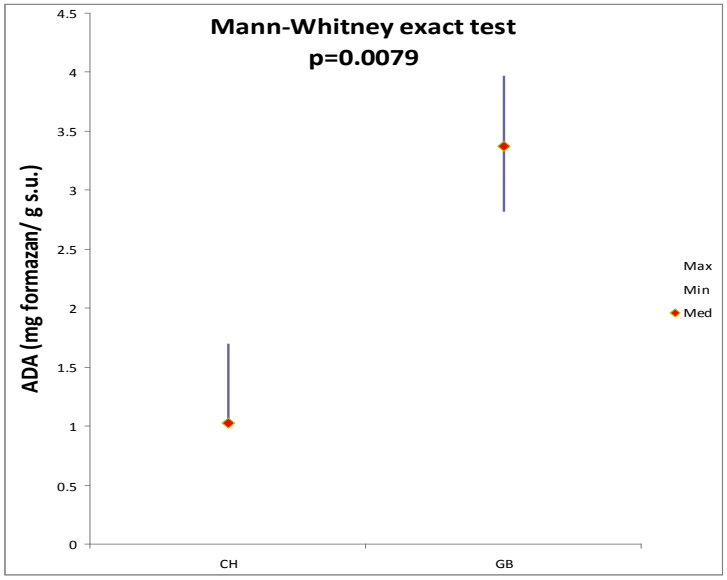

Fig. 95. Distribution of ADA expressed by maximum, minimum and median values, for the soil samples from Chinteni and the Botanical Garden

The Mann-Whitney test for actual dehydrogenase activity values recorded in the natural soils from Chinteni and Valea Morii based on the p value=0.0079 is significant (the value is <0.05), with significant location differences between the distributions of the response variable (actual dehydrogenase activity) for the two compared groups (Chinteni and Botanical Garden) (see Figs. 95 and 96).

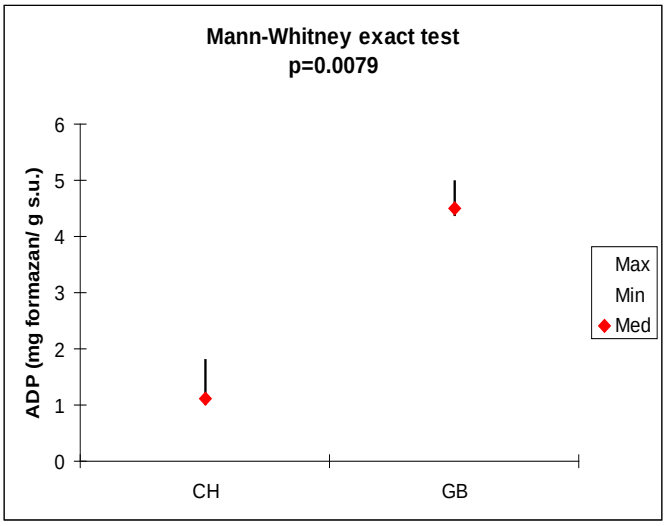

Fig. 96. Distribution of PDA expressed by maximum, minimum and median values, for the soil samples from Chinteni and the Botanical Garden

For potential dehydrogenase activity values recorded in the Chinteni and Botanical Garden soils, the Mann-Whitney test is also significant, an expected result given the actual dehydrogenase activity values.

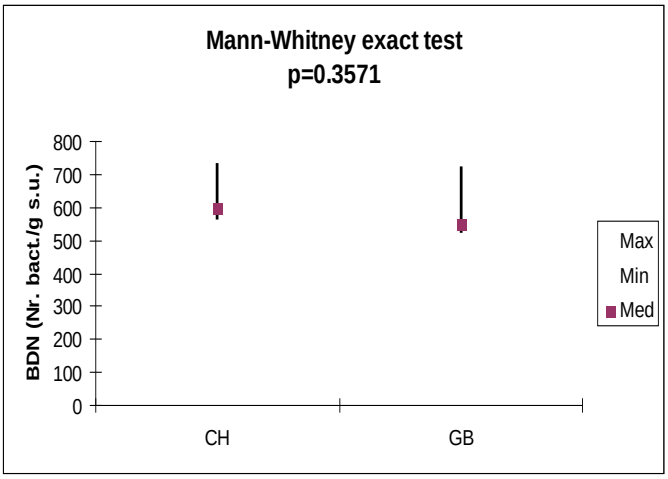

Fig. 101. Distribution of DNB expressed by maximum, minimum and median values, for the soil samples from Chinteni and the Botanical Garden

In the case of the response variable of denitrifying bacteria, for the compared groups (Chinteni and the Botanical Garden), the p value=0.3571 given by the applied test reveals the absence of significant location differences between the distributions of denitrifying bacteria values, so the test is insignificant (see Fig. 101).

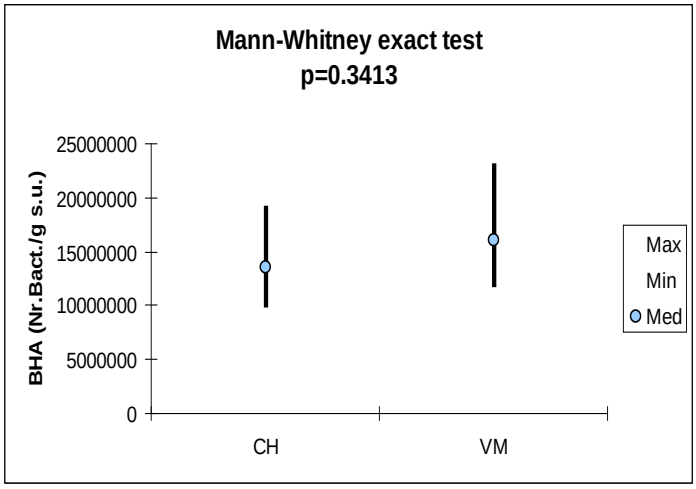

Fig. 108. Distribution of AHB expressed by maximum, minimum and median values, for the soil samples from Chinteni and Valea Morii

Regarding the distribution of aerobic heterotrophic bacteria, the Mann-Whitney test through the calculated p value=0.3413 shows no significant differences between the two compared groups, Chinteni and Valea Morii, the test being insignificant in this case (see Fig. 108).

V.7.2. Logistic regression.

In experimental sciences, therefore in biology, the variations of two parameters, i.e. two quantities are studied within the same statistical population. We used this test in order to find which of the enzymological or bacterial parameters has an influence on the presence of orchids in the soil.

It was found that at actual dehydrogenase activity values higher than 2, the chances of appearance of orchids increase significantly. Regarding phosphatase activity, at an increase in the values of this activity over 7.5, the chances of appearance of orchids decrease progressively.

Ammonifying bacteria values lower than 5000, the number recorded in all analyzed samples, decrease the chances of appearance of orchids.

Denitrifying bacteria values higher than 200 bact./2.5 g d.s. decrease the chances of appearance of orchids.

When the number of aerobic heterotrophic bacteria increases, the chances of appearance of orchids also increase. At nitrite bacteria values higher than 125 bact./2.5 g d.s., the chances of appearance of orchids increase.

Regarding the other parameters: PDA, CA, NaB, it can be said that there is no relationship between the values obtained following analysis and the chances of appearance of orchids, the test being insignificant.

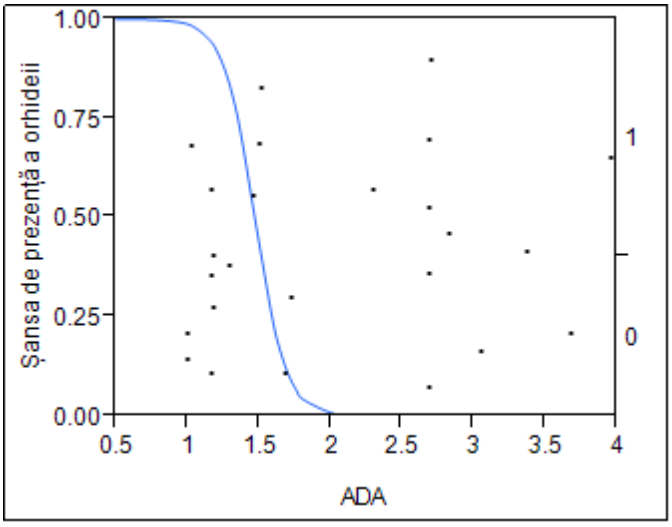

Fig. 122. Estimation of the effect of the ADA variable on the probability of appearance of orchids and adjustment quality indicators

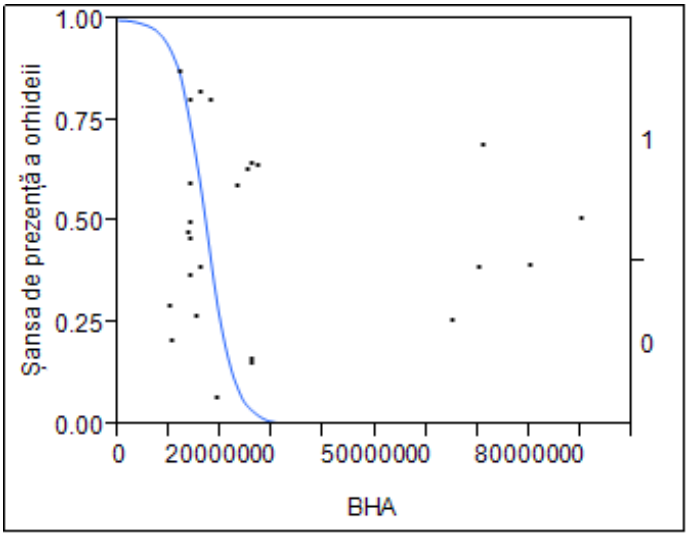

Fig. 126. Estimation of the effect of the AHB variable on the probability of appearance of orchids and adjustment quality indicators

The diagram presented above (Fig. 126) shows the fact that when the number of aerobic heterotrophic bacteria increases, the chances of appearance of orchids also increase.

GENERAL CONCLUSIONS

Through this thesis, the study on mycorrhizal symbiosis in several orchid species (three species from the spontaneous Romanian flora and three species cultivated in the greenhouses of the ”Alexandru Borza” Botanical Garden of Cluj-Napoca), as well as the study of the microbiology of soils from various areas populated with orchids was initiated in Romania. The thesis analyzes for the first time the results of a complex approach to the microbial ecology of orchid soils, in which the bacteria involved in the nitrogen cycle were studied.

Thus, based on the data collected so far, the following conclusions can be drawn:

Of the studied orchids, five species presented mycorrhizae (*Dactylorhiza fucsii*, *Epipactis palustris*, *Cypripedium calceolus*, *Anoectochilus regalis*, *Paphiopedilum insigne*) throughout the biological cycle, the only exception being the epiphytic species – *Epidendrum radicans*, which had no mycorrhizae in any of the biological cycle stages.

The route of entry into the cortical cells was demonstrated to be in the highest proportion the absorbing hairs, when these were present.

Mycorrhizal fungi proved to be present in high numbers before and during the flowering of orchids.

Our results demonstrated that the fungi present inside the root cells underwent all the stages of intracellular evolution, developing pelotons in cortical cells and manifesting the talyphophagy phenomenon.

Mycorrhizal fungi were demonstrated never to affect the endodermis and the central cylinder of the orchid root.

In native species, the presence of numerous mycorrhizal fungi is in accordance with the presence of many lateral ramified roots.

Mycorrhizal fungi in all studied cases correspond to the description of those belonging to the *Rhizoctonia* group.

The soils cultivated with orchids had an alkaline pH and a high conductivity, while the Chinteni soil without orchids had the lowest conductivity (45.3 μS/cm) and the lowest pH (5.95), being the most acid soil of the five studied soils.

The amount of nitrogen in the orchid soils did not vary compared to that of soils without orchids, which indicates the fact that mycorrhizae improve the nitrogen nutrition of orchids.

The amount of phosphorus determined by us was the highest in the soils of the Botanical Garden, while in the other soils the phosphorus level was much lower.

The enzymatic activities determined by us were present in all studied soil samples, with differentiations depending on the type of soil.

Actual dehydrogenase activity, potential dehydrogenase activity and catalase activity were higher in soils with orchids.

Interestingly, phosphatase activity was higher in the soils without orchids studied by us (Chinteni, greenhouse soils).

The enzymatic indicator of soil quality through the values recorded in our research shows that orchid soils are qualitatively better regarding their microbial potential.

The five qualitative enzymatic activities were present in all soil samples, their intensity being different depending on the soil type, demonstrating the presence of a complex enzymatic potential.

Aerobic heterotrophic bacteria, nitrate bacteria and nitrite bacteria were present in the highest numbers in orchid soils, while denitrifying bacteria proved to be in smaller numbers.

According to results, the bacterial indicator of soil quality proved to be higher in orchid soils, which demonstrates a higher bacterial load of these soils.

Bacteria of the *Azotobacter* genus were evidenced both in orchid soils and in the two other soils, but the highest percentage of these bacteria in the soil was found in the Botanical Garden soil, followed by the Valea Morii soil and the orchid soil.

Our electron microscopy data demonstrated that bacteria of the *Azotobacter* genus were present not only in the rhizosphere soil, but also inside root cells in orchids.

The analyses for the determination of micromycetes in the studied soils revealed their presence in high numbers in all the five studied soils, with higher values in orchid soils, i.e. the Botanical Garden soil and the orchid greenhouse soil. Of the evidenced micromycetes genera, the most frequently found in the analyzed soils were the *Penicillium* and *Fusarium* genera.

The statistical analyses performed show the fact that the best predictor for the appearance of orchids in a soil is the actual dehydrogenase activity (ADA) variable, proving that this enzymatic activity of the soil through its recorded values is of the greatest importance in the prediction of the appearance and presence of orchids in a certain soil and can be considered the reference in this situation.

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