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EXPERIMENTAL ARTICLES

The Biometric Analysis of Bacterial Cells in Soil

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Abstract—The biometric analysis of bacterial cells in soil by light, fluorescence, and scanning electron microscopy showed that their average size is 0.8 μm in diameter, 1.4 μm in length, and 0.7 μm^3 in volume. In soil loci with enhanced microbiological activity (the rhizoplane of plants and the intestinal tract of soil invertebrates), the average size of bacterial cells was found to be 40% smaller than that of cells occurring in other parts of soil. This is the first experimental evidence showing that the metabolic activity of soil bacteria, their concentration, and allometric parameters are related.

The biometric parameters of soil microorganisms are not only of general biological interest but also of practical importance, since they are necessary for the calculation of microbial biomass in natural habitats. Research interest in this problem dates back to the works of Winogradsky [1] and Mishustin and Mirzoev [2] and is still relevant [3–6].

Most studies devoted to this problem were carried out with the aid of light microscopy. The ever-increasing practical application of scanning electron microscopy, which has a resolution capacity an order of magnitude higher than that of light microscopy, calls for the use of this advanced microscopic technique in the biometric studies of soil microorganisms.

The aim of this work was to determine the average size of active heterotrophic soil bacteria by different microscopic methods and to evaluate the statistical significance of differences in the average sizes of bacteria that inhabit contrasting soil loci.

MATERIALS AND METHODS

The size of heterotrophic bacteria in the wheat rhizoplane and in samples of humic gley and soddy podzolic soils of the Moscow region and the chernozem soil of the Kursk region was determined by the fluorescence microscopic measurement of cells vitally stained with acridine orange [7].

The size of these bacteria in the wheat rhizoplane and in samples of the soddy podzolic soil of the Moscow, Perm, and Bryansk oblast and the chernozem soil of the Kursk, Voronezh, and Samara oblast was determined with the aid of scanning electron microscopy. Each of the soils mentioned was represented by several samples taken from the upper humic horizon, 5–20 cm in depth. Along with the microorganisms of the wheat rhizoplane and soil, microorganisms inhabiting the intestinal tracts and feces of soil invertebrates, such as millipedes and pill bugs, were studied. The electron microscopic methods used for investigating soils, plants, and soil invertebrates are described in detail elsewhere [8–10].

The microscopic images of bacterial cells were photographed, and the photographs were analyzed for cell sizes with allowance made for microscopic magnification and photographic enlargement. In this case, only the microorganisms that displayed distinct features of colonial growth were accounted for, whereas single cells were considered to be allochthonous and were not taken into account. Such an approach also allowed yeasts and micromycete spores to be excluded from consideration. Yeasts that multiply by budding and exhibit pseudomycelial growth form microcolonies which clearly differ from bacterial colonies. The yeasts that multiply by fission can easily be distinguished from bacterial cells by the presence of a division septum in dividing yeast cells. As for micromycete spores, they are characterized by a specific structural organization and occur in soil singly and chaotically (unlike bacterial cells, which are arranged in microcolonies).

The biometric analysis of erythrosin-stained soil bacteria with the aid of bright-field light microscopy was carried out using the drawings of autochthonous microorganisms made by Winogradsky [1].

The results obtained were processed using the Statistica software package.

RESULTS

Size Range of Bacterial Cells

The analysis of about 1000 bacterial cells by bright-field light microscopy showed that their diameters ranged from 0.15 to 2.75 μm and their lengths (with the exception of mycelial forms) varied from 0.50 to 4.50 μm. The respective three-dimensional histogram exhibited two peaks (Fig. 1a). One of these peaks corresponded to cells with diameters <0.25 μm and lengths within 0.5–1.0 μm. The other microscopic methods employed failed to reveal this cell morphotype (Figs. 1b, 1c). The

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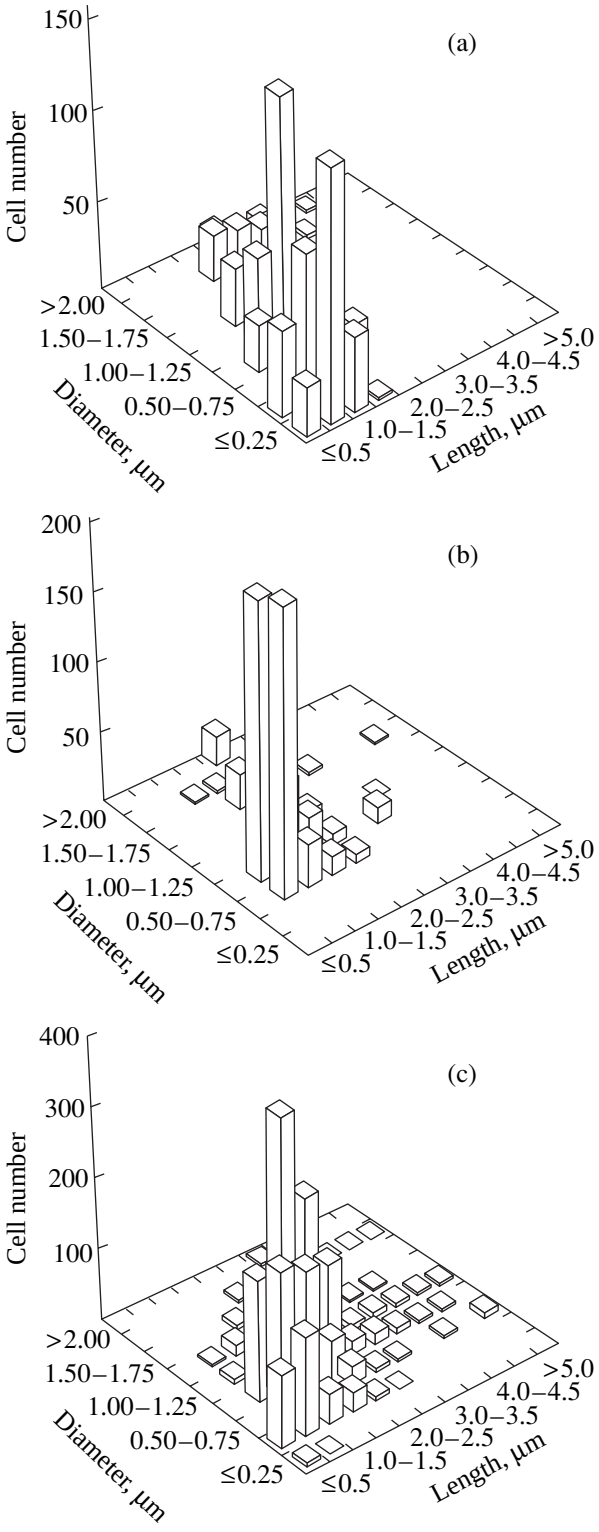

Fig. 1. Histogram showing the distribution of soil bacteria in cell sizes as derived from the data of (a) bright-field light microscopy, (b) fluorescence microscopy, and (c) scanning electron microscopy.

second peak corresponded to cells with diameters within 0.5–0.75 μm and lengths within 0.5–1.0 μm. The average diameter, length, and volume of bacterial cells calculated from bright-field light microscopy data turned out to be 0.89 ± 0.02 μm, 1.37 ± 0.02 μm, and 0.92 ± 0.05 μm^3, respectively.

The analysis of about 1200 bacterial cells by fluorescence microscopy (Fig. 1b) showed that their diameters ranged from 0.6 to 2.4 μm and their lengths (with the exception of mycelial microorganisms) varied from 0.6 to 4.8 μm. The histogram constructed with a 0.25-μm step for diameters and a 0.5-μm step for lengths had one peak, which corresponded to the most probable cell diameters and lengths within the range 0.5–1.0 μm. The average diameter, length, and volume of bacterial cells calculated from fluorescence microscopy data were 0.81 ± 0.01 μm, 1.17 ± 0.02 μm, and 0.68 ± 0.03 μm^3, respectively.

The analysis of more than 5000 bacterial cells by scanning electron microscopy (Fig. 1c) showed that their diameters ranged from 0.2 to 2.5 μm and their lengths (with the exception of mycelial microorganisms) varied from 0.3 to 7.3 μm. The cell size distribution histogram had one peak, indicating that the most frequently encountered soil bacteria have diameters within 0.75–1.0 μm and lengths within 1.0–1.5 μm. The average diameter, length, and volume of bacterial cells calculated from scanning electron microscopy data were 0.76 ± 0.01 μm, 1.46 ± 0.01 μm, and 0.77 ± 0.02 μm^3, respectively.

The Biometric Characteristics of Bacteria Living in Different Soil Loci

The histograms presented in Figs. 2–7 show the biometric characteristics of bacteria living in different soil loci, which were obtained by the three microscopic methods. Experimental data are presented in these figures in a way that makes it possible to visualize the degree of difference between the average sizes of bacteria inhabiting contrasting soil loci. The standard error of the average sizes characterizes the instrumental accuracy of size measurements. Ninety-five percent confidence intervals are a measure of the statistical significance of differences between the average sizes of soil bacteria. The presentation of experimental data in the form of quartiles provide an indication of what group of bacterial cells is responsible for the differences revealed.

A set of true soil bacteria examined by bright-field light microscopy, fluorescence microscopy, and scanning electron microscopy amounted to 987, 678, and 2645 cells, respectively. The biometric parameters of soil bacteria determined by the three microscopic methods were close (Figs. 2, 3). Specifically, the average diameter, length, and volume of soil bacteria computated from bright-field light microscopy data turned out to be 0.89 μm, 1.37 μm, and 0.92 μm^3; 0.89 μm, 1.14 μm, and 0.82 μm^3 as calculated from fluorescence microscopy data; and 0.85 μm, 1.54 μm, and 0.94 μm^3 as calculated from scanning electron microscopy data.

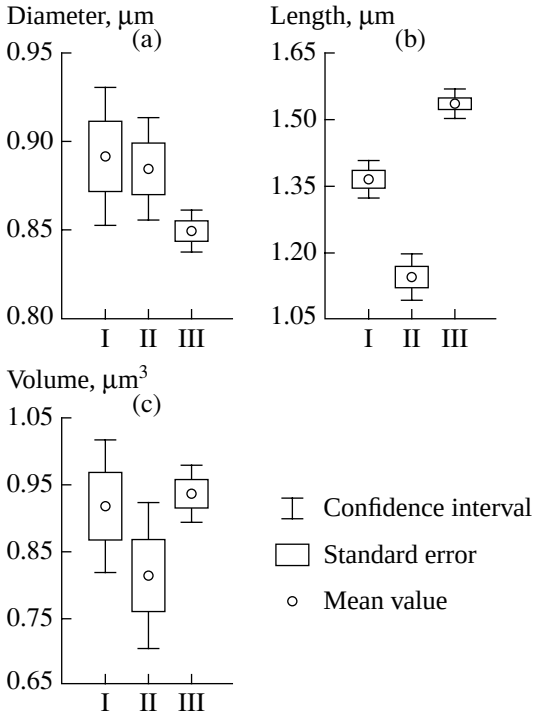

Fig. 2. The average (a) diameter, (b) length, and (c) volume of soil bacteria calculated from the data of (I) bright-field light microscopy, (II) fluorescence microscopy, and (III) scanning electron microscopy.

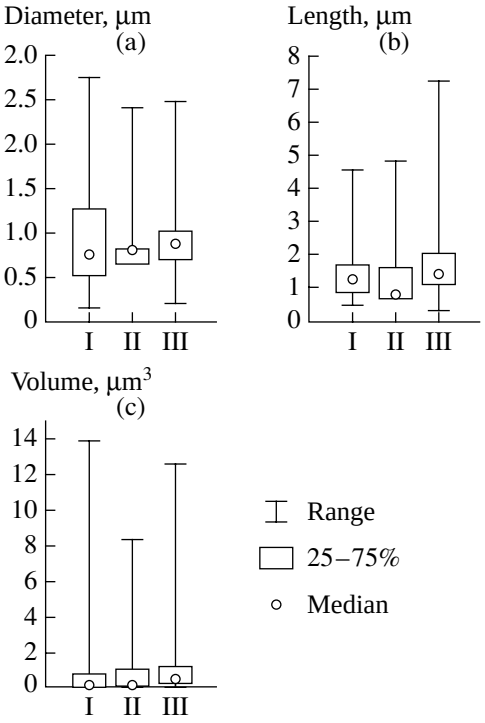

Fig. 3. The ranges of (a) the diameter, (b) the length, and (c) the volume of soil bacteria as derived from the data of (I) bright-field light microscopy, (II) fluorescence microscopy, and (III) scanning electron microscopy.

Confidence intervals for the average cell diameters and volumes calculated from the data of the three microscopic methods overlapped (Figs. 2a, 2c), indicating that these average sizes differ insignificantly. At the same time, the differences in the average lengths of bacterial cells calculated from the data of the three methods were statistically significant. The smallest mean length was obtained from fluorescence microscopy data, while the greatest mean length was obtained from scanning electron microscopy data (Fig. 2b). A comparison between the medians and the distribution of cells over quartiles confirmed the closeness of the mean sizes of bacterial cells obtained by the three methods (Fig. 3). The overestimation of the average length of bacterial cells, as calculated from the data of scanning electron microscopy, may be explained by the fact that this kind of microscopy preferentially detects long cells (Fig. 3b). Conversely, fluorescence microscopy likely overestimates the occurrence frequency of short cells. Bright-field light microscopy gives intermediate results.

A set of bacterial cells analyzed by fluorescence microscopy in the wheat rhizoplane consisted of 486 cells. The average diameter, length, and volume of these cells calculated from fluorescence microscopy data turned out to be 0.70 μm, 1.21 μm, and 0.48 μm^3, respectively (Fig. 4). A comparison between these values and the respective parameters of true soil bacteria presented above showed that these bacteria are larger than the rhizoplane-associated bacteria by 27% in average diameter and by 69% in average volume (Figs. 4a, 4c). The analysis of cell distribution over quartiles showed that the larger values of the average diameter and volume of soil bacteria are primarily due to the large number of cells in the third quartile (Fig. 5). As is evident from the overlapping of the respective confidence intervals, the difference between the average lengths of soil and rhizoplane-associated bacteria is statistically insignificant (Fig. 4b).

The scanning electron microscopic analysis of 397 bacterial cells in the wheat rhizoplane and 1953 bacterial cells associated with the intestinal tract or feces of soil invertebrates showed that the rhizoplane-associated bacteria had an average diameter of 0.64 μm, an average length of 1.45 μm, and an average volume of 0.50 μm^3 (Fig. 5), whereas the invertebrate-associated bacteria had an average diameter of 0.66 μm, an average length of 1.35 μm, and an average volume of 0.60 μm^3.

A comparison between these values and the respective parameters of true soil bacteria showed that the latter are larger than the rhizoplane- and invertebrate-associated bacteria in all three parameters—the average diameter (Fig. 6a), length (Fig. 6b), and volume (Fig. 6c). The mean diameter of soil bacteria was larger than the mean diameters of rhizoplane- and invertebrate-associated bacteria by 32 and 29%, respectively. The mean length of soil bacteria was larger by 6 and 14%, while their mean volume by 87 and 57%, than the respective

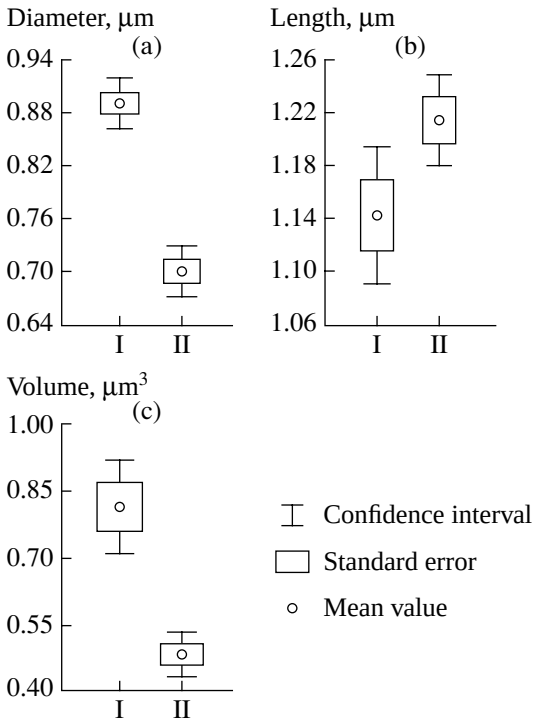

Fig. 4. The average (a) diameter, (b) length, and (c) volume of bacterial cells living in (I) soil and (II) the plant rhizosphere as calculated from fluorescence microscopy data.

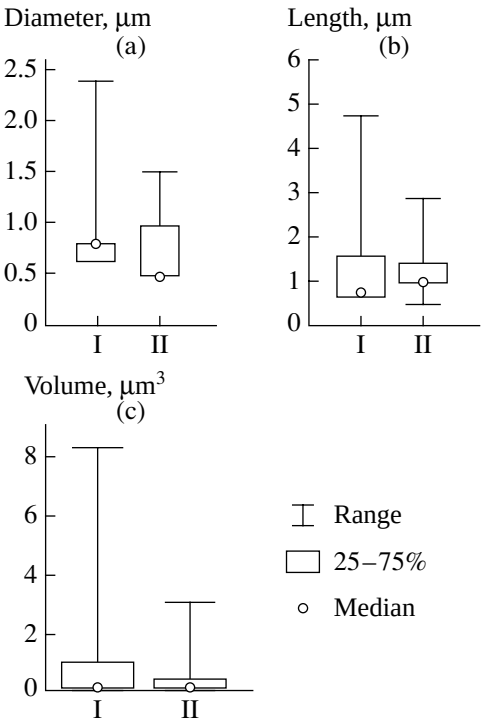

Fig. 5. The ranges of (a) the diameter, (b) the length, and (c) the volume of bacterial cells living in (I) soil and (II) the plant rhizosphere as derived from fluorescence microscopy data.

parameters of the rhizoplane-associated and the invertebrate-associated bacteria. The analysis of the distribution of these kinds of bacterial cells over quartiles (Fig. 7) showed that the larger average sizes of soil bacteria as compared with those of the rhizoplane- and invertebrate-associated bacteria are due to the prevalence of large cells in the second and third quartiles.

DISCUSSION

Analysis of the data obtained by the three microscopic methods makes it possible to assess the instrumental limitations of each of these methods. A comparison between the results obtained in this study (Fig. 1) and those obtained by Winogradsky by the bright-field light microscopic examination of soil suspensions stained with erythrosin shows that this kind of light microscopy does not allow the detection of large bacterial cells with lengths exceeding 3 μm, but, in contrast, detects a great number of small rodlike cells with diameters of less than 0.25 μm and lengths of about 1 μm. This finding agrees well with the differences in the experimental conditions. Indeed, Winogradsky did not take into account the so-called zymogenous soil microorganisms, whereas we examined all microorganisms occurring in soil. As for the group of small erythrosin-stained rodlike cells, we assume that they actually represent small colloid particles, which were mistakenly considered to be bacterial cells because of the low resolution capacity of light microscopes. Confirmation of this assumption comes from the fact that such small saprotrophic microorganisms are not described in comprehensive Bergey's manual [11].

The set of bacterial cells detected by fluorescence microscopy is characterized by the absence of small (smaller than 0.5 μm in both diameter and length) cells (Fig. 1) and by the underestimation of the average length of soil bacteria (Fig. 2). The inability of fluorescence microscopy to detect small cells can be explained by the overestimation of the actual size of microscopic objects by this kind of microscopy because of the bright halo around the objects [12]. This effect is especially profound when objects' sizes are close to the resolution threshold of the microscope. As a result, small cells mistakenly fall into a group of larger cells. The underestimation of the mean size of bacterial cells is caused by another factor. It is known that the fluorescent acridine orange stain used in fluorescence microscopy preferably binds to nucleic acids, which are concentrated in the nucleoid region [12]. Due to this effect, the size of a bacterial cell estimated with the aid of this fluorescent stain reflects the size of the nucleoid region rather than the actual size of this cell.

The major advantage of electron microscopy is its high resolution capacity, due to which the standard error of electron microscopic measurements is approximately three times smaller than that of light micro-

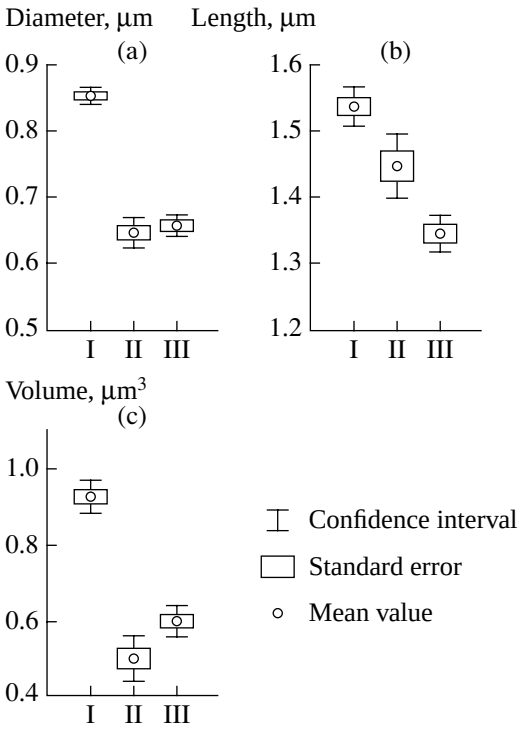

Fig. 6. The average (a) diameter, (b) length, and (c) volume of bacterial cells living in (I) soil, (II) the plant rhizosphere, and (III) the intestinal tract of soil invertebrates, as calculated from scanning electron microscopy data.

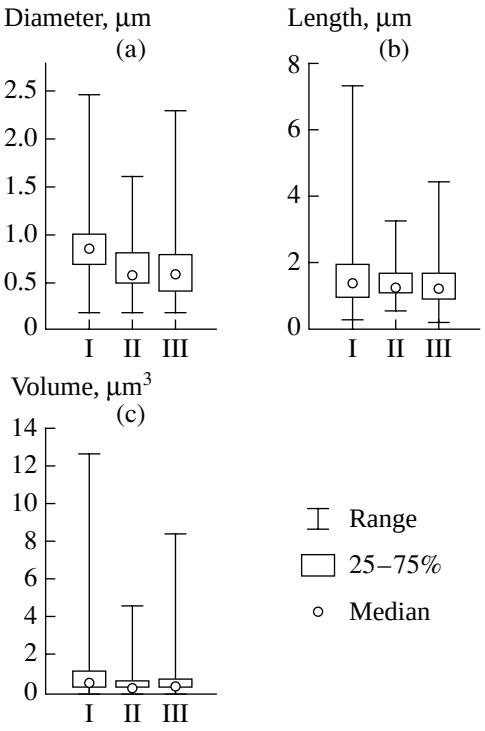

Fig. 7. The ranges of (a) the diameter, (b) the length, and (c) the volume of bacterial cells living in (I) soil, (II) the plant rhizosphere, and (III) the intestinal tract of soil invertebrates, as derived from scanning electron microscopy data.

scopic methods (Figs. 2a, 2c). The major source of error in scanning electron microscopy is the vacuum in electron microscopes, due to which bacterial cells may somewhat shrink [13]. This leads to an underestimation of the actual size of bacterial cells, their diameter in particular, by scanning electron microscopy (Fig. 2a). It should be noted, however, that the difference in the mean diameters of soil bacteria obtained by the three microscopic methods is not statistically significant. This can be explained by the fact that the shrinkage of soil bacteria in vacuum is presumably low because of the high rigidity of the cell wall of these bacteria.

In spite of some differences, the average sizes of soil heterotrophic bacteria obtained by the three microscopic methods are close, averaging 0.8 μm in diameter, 1.4 μm in length, and 0.7 μm^3 in volume. These values are in agreement with the data obtained by other researchers [4, 6]. In general, the mean volume of bacterial cells is considered to be 0.1 μm^3 [3, 5], confirming the view that soil bacteria are smaller than the bacteria grown in laboratories in specially designed nutrient media [14]. Some discrepancy between the reported mean sizes of bacterial cells can be explained by two factors. First, the microscopic examination of bacterial cells in soil suspensions, in which colonial growth of bacteria is impossible, will lead to a situation where some colloidal particles present in soil will be mistaken for bacterial cells. Second, soil always contains a considerable number of allochthonous microorganisms, including the so-called ultramicrobacteria, nannobacteria, L-forms, viable but nonculturable bacteria, and so on [15, 16], which are not functional and are not directly related to soil and the processes occurring there.

Soil is a heterogeneous system that contains loci with enhanced microbiological activity, such as the plant rhizosphere and zoosphere. The rhizosphere of higher plants is characterized by a high metabolic activity of microorganisms due to the nutritionally rich exudates of plant roots [17]. The intestinal tract of soil invertebrates is also a conducive medium for the growth of symbiotic microorganisms [18]. Some researchers relate the high metabolic activity of bacteria in these loci with their specific systematic position and increased population density [19, 20].

It is an accepted fact that the unique metabolic properties of microbes are due to their small sizes, since the increased proportions between their surface area and volume are favorable for diffusion processes and, hence, are conducive to the rapid exchange of matter between the cell interior and the environment [21]. The estimation of the average sizes of heterotrophic soil bacteria living in contrasting soil loci (soil itself, the plant rhizosphere, and the intestinal tract of soil invertebrates) showed that these differ. Specifically, soil loci with high microbiological activity are characterized by a smaller average size of inhabiting microorganisms. This finding is in agreement with the view in general

ecology that a habitat is either beneficial or not with respect to the size of inhabiting organisms [22]. A habitat with intense competition of organisms favors the growth of larger organisms, whereas *r*-strategy populations are characterized by lower sizes of their members.

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