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EXPERIMENTAL ARTICLES

Microsampling of Rhizosphere Soil and Laboratory Artifacts

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Abstract—A parallel analysis of the macrosamples of surrounding soil and microsamples of rhizosphere soil did not reveal the so-called rhizospheric effect. The data obtained showed that dilution significantly influences the results of determination of the number of soil microorganisms. The actual number of microorganisms revealed in soil samples greatly differed from the theoretically predicted values. The enumeration of microorganisms in soil microsamples by direct count and, especially, by the plating method with the use of conversion coefficients based on the degree of sample dilution gave erroneous results.

Key words: rhizosphere soil, sample, dilution, number of microorganisms.

Studies of microbial complexes in the soil–root zone and on the root surface are of primary importance for the characterization of interactions between plants and microorganisms. The rhizosphere and rhizoplane are, undoubtedly, specific microbial habitats with respect to the substrates and inhibitors present in them [1]. There is no reason to cast any doubt on the numerous data concerning the species specificity of microbial communities in the rhizosphere and rhizoplane [2–5].

On the other hand, little is known about the relationship between particular microbial populations and the total number of microorganisms in the rhizosphere soil. The widespread opinion that the microbial population density in the rhizosphere is higher than in the surrounding soil is mainly based on the analyses made by the plating method [6]; however, the number of microorganisms thus determined reflects their trophic preferences and physiological state rather than their actual population density in the rhizosphere. The results of microscopic counts reported in some publications [7–11] have called into question the aforementioned opinion. It should be noted that the number and biomass of microorganisms should not necessarily correlate with their activity: in different habitats, the biomasses of microorganisms may be the same, whereas their activities may substantially vary.

Meanwhile, many researchers consider the rhizosphere as a more favorable habitat for microorganisms than the surrounding soil (the so-called "rhizospheric effect"). However, our previous study did not reveal any noticeable rhizospheric effect [9]. This discrepancy obviously needs to be explained. A simple argument that the enumeration of soil microorganisms by the plating method is inadequate for the determination of their total population is unsatisfactory, since some investigators used direct enumeration methods as well [10, 11].

We have previously emphasized that, due to the difficulties associated with sampling rhizosphere soil in sufficient amounts, investigators use its microsamples, which may lead to laboratory artifacts as compared with the results obtained by studying the macrosamples of the surrounding soil.

The aim of this work was to study how the size of soil samples can influence the results of their microbiological analysis.

MATERIALS AND METHODS

In the present study, we attempted to test the distorting effect of the size of soil samples on the results of their microbiological analysis. Soddy podzolic soil was sampled in amounts of 1, 0.5, 0.1, 0.05, 0.02, 0.01, and 0.005 g in the field and forest. Additionally, two variants of soddy podzolic soil stored in the laboratory were sampled: air-dried soil and rhizosphere-free soil at different stages of succession. Poorly cultivated soddy podzolic soil, taken from the top horizon A, was a moderately loamy soil with a humus content of 1.9%, an absorption capacity of 17.2 mg-equivalent/100 g, and pH_{H_2O} 6.7. For comparison, samples of dry peat soil taken from a drained lowland peat bog at the Kirov meadow–bog experimental station were also studied. Such contrasting microbial habitats as soddy podzolic and peat soils were chosen because of the fact that the content of organic matter in the environment strongly influences the adsorption of microorganisms and, accordingly, the results of their enumeration.

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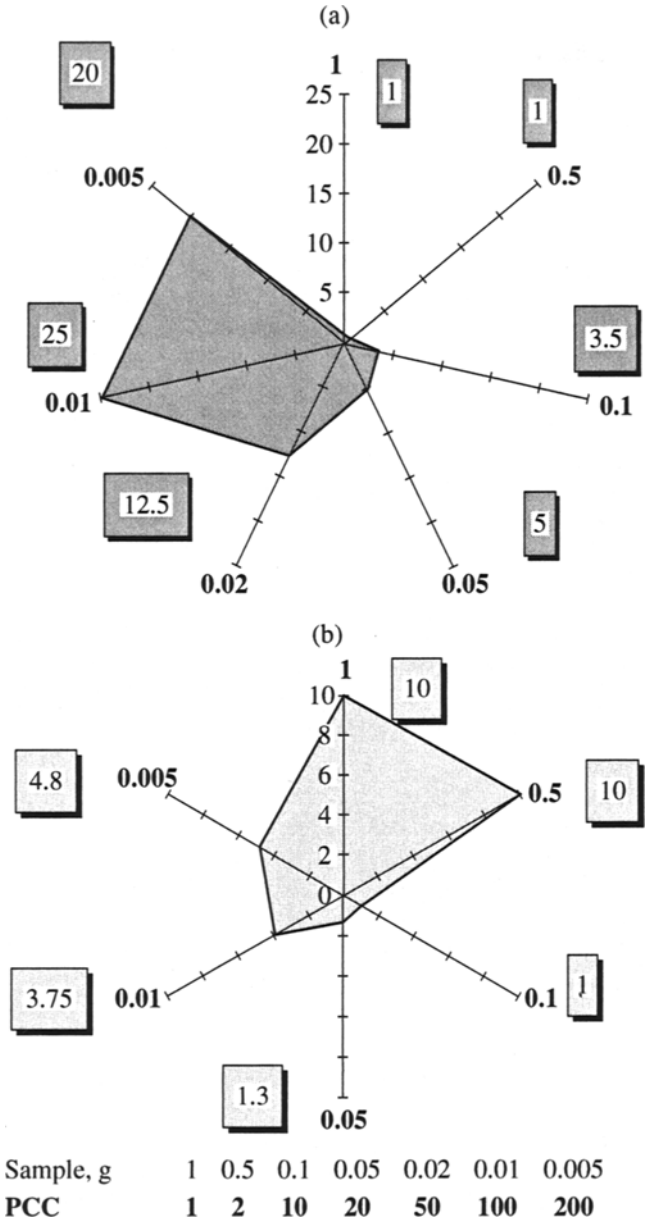

Fig. 1. Experimental conversion coefficients (ECC) for bacterial count by the direct microscopic method in (a) soddy podzolic soil and (b) peat soil. PCC are conversion coefficients predicted from the sample dilution rates.

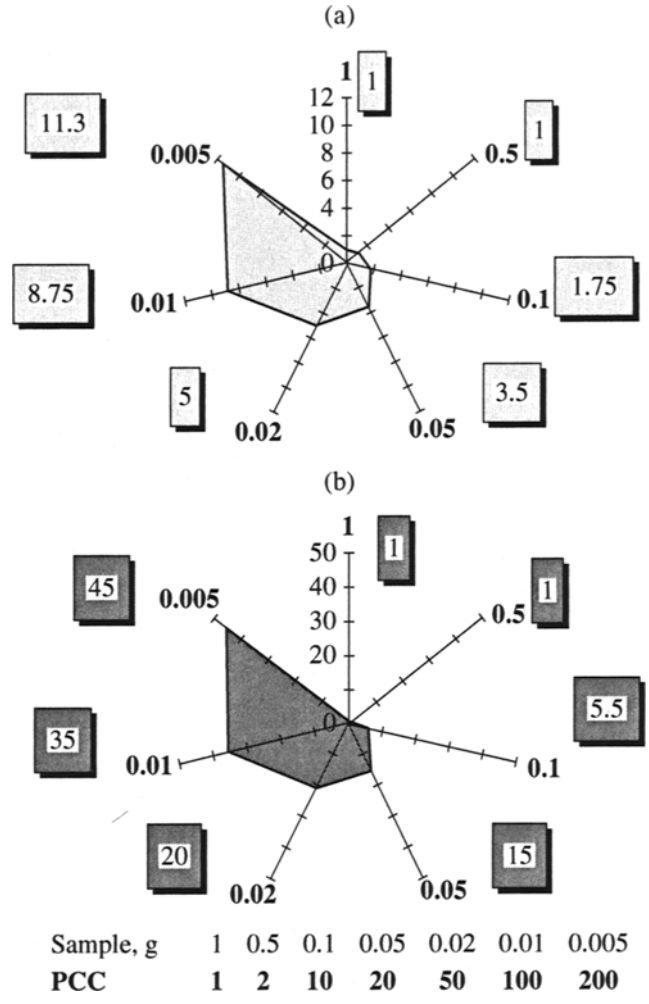

Fig. 2. Experimental conversion coefficients (ECC) for (a) mycelium and (b) spores of fungi in soddy podzolic soil and peat soil as estimated by the direct microscopic examination of soil samples. PCC are conversion coefficients predicted from the sample dilution rates.

The preliminary treatment of samples for microbiological analysis involved their ultrasonic dispersion on a UZDN-1 low-frequency disintegrator (22 kHz; 0.44 A; 2 min) [12]. Total numbers of microorganisms were determined by luminescence microscopy using specimens prepared by the routine procedure [13]. The specimens were stained with an aqueous solution of acridine orange for the enumeration of soil bacteria and actinomycetes or with calcofluor white for the enumeration of fungi [14]. The standard error did not exceed 5–10% for the count of bacteria and 15% for the count of fungi and actinomycetes.

In addition to the direct microscopic enumeration of soil microorganisms, they were also counted by the plating technique. Bacteria and actinomycetes were counted on the medium described in the handbook [13]. The medium used for bacterial count was supplemented with 0.1 mg/ml nystatin to inhibit the growth of fungi. Colonies were enumerated after two weeks of cultivation at room temperature. The total numbers of microorganisms were expressed as CFU/g dry soil (CFU, colony-forming unit). Micromycetes were counted on Czapek medium supplemented with 100 U/ml streptomycin sulfate [13]. Micromycete colonies were counted on day 8 of cultivation at room temperature.

RESULTS AND DISCUSSION

Our experiments showed that the so-called dilution effect is extremely important for the correct enumeration of soil bacteria. The actual number of bacteria in

Table 1. The number of bacteria (million CFU/g) and fungi (thousand CFU/g) in soddy podzolic soil samples as a function of their dilution

Microorganisms	Sample weight, g	Dilution		
Bacteria		1 : 100	1 : 1000	1 : 10000
Fungi		1 : 100	1 : 200	1 : 500
Bacteria	1	7.5	32	79
Fungi		5	6	5.5
Bacteria	0.5	12	36	–
Fungi		5	7.5	–
Bacteria	0.1	41	84	–
Fungi		8	10	–
Bacteria	0.01	160	165	–
Fungi		15	16	–

soil samples significantly differed from the predicted values; for instance, the estimated numbers of bacteria in 1- and 0.5-g soil samples were almost the same, since, presumably, a more complete desorption of bacterial cells was achieved in the second sample. When the weight of the soil samples was reduced from 1 g to 0.1 g, 0.05 g, and 0.005 g, the estimated number of bacteria decreased by 3.5, 5, and 20 times instead of the expected 10, 20, and 200 times (Fig. 1a). The results of the examination of all five variants of soddy podzolic soil under study were almost the same. The examination of dry peat soil samples showed that the ratio of soil particles to bacterial cells is of great importance in order to achieve a correct bacterial count. In the case of 1- and 0.5-g peat soil samples, each microscopic field contained a large number of soil particles and more than 40 bacterial cells; this made a correct enumeration of bacterial cells impossible. Conversely, in 0.1-g peat soil samples, the ratio of soil particles to bacterial cells was optimal for a correct bacterial count. This size of the sample can be recommended for the microscopic estimation of bacteria in dry peat soil (Fig. 1b).

In general, the regularities established for soddy podzolic soil were also valid for peat soil samples: with microsamples, the expected conversion coefficients due to sample reduction did not correspond to the actual situation, and the distorting effect of the sample size increased as the weight of samples decreased.

The same was true for the direct microscopic enumeration of soil fungi. With the exception of the extremely small 0.005-g samples, the conversion coefficients for fungal mycelium and spores were almost the same in the cases of soddy podzolic and peat soils (Fig. 2).

With the plating method of bacterial count, the dilution effect manifests itself already at the stage of preparation of soil suspensions [15]. According to our data, the estimated number of bacterial and fungal colonies decreased by 2–4 and 1.5–2 times, respectively, after each subsequent tenfold dilution of soil suspensions (Table 1).

It should be noted that dilution may also affect the results of the direct luminescent microscopic analysis of pure cultures of *Arthrobacter* sp. and *Aquaspirillum* sp. (Table 2).

In the subsequent experiments, all the microsamples of soddy podzolic soil were diluted prior to inoculation by 1000 times for the enumeration of bacteria and by 200 times for the enumeration of fungi. With the plating method, the difference between the expected and actual conversion coefficients was greater than in the case of direct microscopic count (Fig. 3).

Therefore, in order to obtain a correct count for different groups of microorganisms in various habitats, samples should be of equal or at least comparable size. Otherwise, various laboratory artifacts, such as the "rhizospheric effect," may arise. Taking account of the "effect of microsampling" is of extreme methodological importance for the correct evaluation of microorganisms in the soil and other environments.

Thus, parallel analysis of the macrosamples of the surrounding soil and microsamples of the rhizosphere soil did not reveal the so-called rhizospheric effect. The data obtained suggest that dilution can substantially influence the results of determination of the number of soil microorganisms. The actual number of microor-

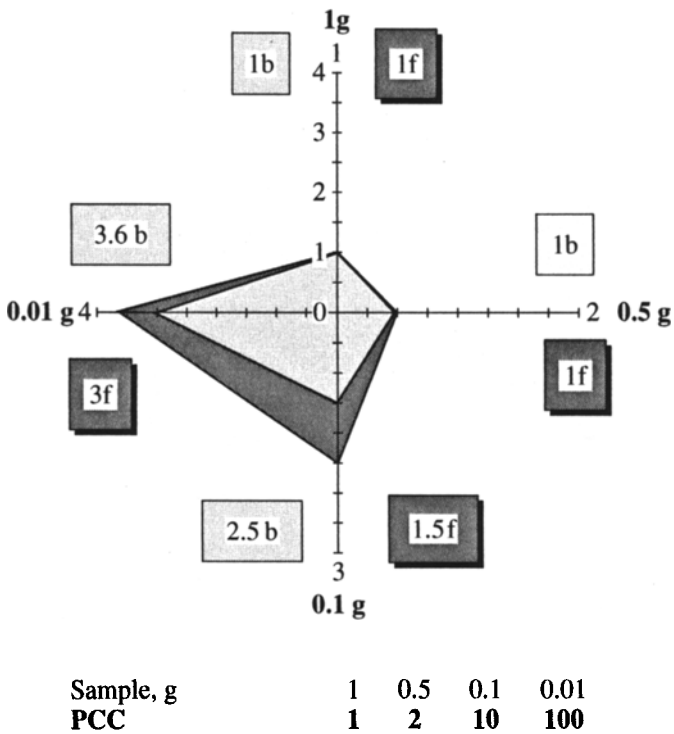

Fig. 3. Experimental conversion coefficients (ECC) for the enumeration of bacteria (coefficients are marked by the letter b) and fungi (coefficients are marked by the letter f) in soddy podzolic soil as estimated by the plating method. PCC are conversion coefficients predicted from the sample dilution rates.

Table 2. The number of bacteria in the original cell suspensions and in their dilutions as estimated by luminescence microscopy

	Dilution					
	Original suspension	1 : 10	1 : 100	1 : 1000	1 : 10000	1 : 100000
	Arthrobacter sp.					
Number of cells/field of vision	224	51	20	6.5	3.5	3.0
Number of cells/ml	9×10^{10}	20×10^{10}	80×10^{10}	26×10^{11}	14×10^{12}	12×10^{13}
	Aquaspirillum sp.					
Number of cells/field of vision	120	50	6.5	5.0	3.0	3.0
Number of cells/ml	5×10^{10}	20×10^{10}	26×10^{10}	20×10^{11}	12×10^{12}	12×10^{13}

ganisms revealed in the soil samples significantly differed from the theoretically predicted values. The enumeration of microorganisms in soil microsamples by direct count and, especially, by the plating method with allowance for the degree of sample dilution gave erroneous results.

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REFERENCES

1. Lynch, J.M. and Whipps, J.M., Substrate Flow in the Rhizosphere, *Plant Soil*, 1990, vol. 129, pp. 1–10.
2. Krasil'nikov, N.A., *Mikroorganizmy pochvy i vysshie rasteniya* (Soil Microorganisms and High Plants), Moscow: Acad. Nauk SSSR, 1958.
3. Litvinov, M.A., About the Problem of Specificity of the Rhizosphere and Rhizoplane Soil Microscopic Fungi, *Mikol. Fitopatol.*, 1967, vol. 1, pp. 201–214.
4. Katznelson, H., Nature and Importance of the Rhizosphere, *Ecology of Soil-Borne Plant Pathogens*, Baker, K.F. and Snyder, W.C., Eds., Berkeley: Univ. Calif., 1965, pp. 187–209.
5. Foster, R.C., The Ultrastructure of the Rhizoplane and Rhizosphere, *Ann. Rev. Phytopathol.*, 1986, vol. 24, pp. 211–234.
6. Rovira, A.D. and Davey, C.B., Biology of the Rhizosphere, *The Plant Root and Its Environment*, Carson, E.W., Ed., Charlottesville: Univ. of Virginia, 1974.
7. Polyanskaya, L.M., Orazova, M.Kh., Mirchink, T.G., and Zvyagintsev, D.G., Population Dynamics and Composition of Microbial Complexes in the Pea Rhizosphere, *Mikrobiologiya*, 1994, vol. 63, no. 2, pp. 314–325.
8. Orazova, M.Kh., Polyanskaya, L.M., and Zvyagintsev, D.G., Heterogeneity of Roots as Microbial Habitats, *Mikrobiologiya*, 1994, vol. 63, no. 4, pp. 706–714.
9. Orazova, M.Kh., Polyanskaya, L.M., and Zvyagintsev, D.G., Microbial Complexes of the Barley Rhizosphere, *Mikrobiologiya*, 1999, vol. 68, no. 1, pp. 127–133.
10. Van Vuurde, J.W.L. and de Lange, A., The Rhizosphere Microflora of Wheat Grown under Controlled Conditions. II. Influence of the Stage of Growth of the Plant, Soil Fertility and Leaf Treatment with Urea on the Rhizosphere Soil Microflora, *Plant Soil*, 1978, vol. 50, pp. 461–472.
11. Curl, E.A. and Truelove, B., *The Rhizosphere*, Berlin: Springer, 1986.
12. Zvyagintsev, D.G., Dmitriev, E.A., and Kozhevin, P.A., About the Study of Soil Microorganisms by Luminescence Microscopy, *Mikrobiologiya*, 1978, vol. 47, pp. 1091–1096.
13. *Metody pochvennoi mikrobiologii i biokhimii* (Methods of Soil Microbiology and Biochemistry), Moscow: Mosk. Gos. Univ., 1991.
14. Polyanskaya, L.M., Microbial Succession in Soil, *Doctoral (Biol.) Dissertation*, Moscow, 1996.
15. Zvyagintsev, D.G., *Vzaimodeistvie mikroorganizmov s tverdymi poverkhnostyami* (Interaction of Microorganisms with Solid Surfaces), Moscow: Mosk. Gos. Univ., 1973.