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EXPERIMENTAL ARTICLES

The Composition of the Chitinolytic Microbial Complex and Its Effect on Chitin Decomposition at Various Humidity Levels

A. V. Vorob'ev, N. A. Manucharova[1], A. M. Yaroslavtsev, E. V. Belova, D. G. Zvyagintsev, and I. I. Sudnitsyn

Faculty of Soil Science, Moscow State University, Moscow, 119992 Russia

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Abstract—The dynamics of assimilation of chitin by soil microorganisms (primarily prokaryotes) as a source of carbon and nitrogen has been determined by gas chromatography and fluorescence microscopy. The highest rates of chitin decomposition in chernozem were detected at humidity levels corresponding to the pressure of soil moisture (P) of –1.4 atm. The rate of microbial consumption of chitin is three times higher than that of the carbon of soil organic matter. Fluorescence microscopy revealed that an increase in the pressure of soil moisture from P = –10 atm to P = –0.7 atm resulted in a considerable increase in the proportion of the specific surface of mycelial bacteria (actinomycetes).

Keywords: chitinolytic soil microorganisms, chitinase complex, mycelial actinobacteria, soil moisture.

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The content of chitin (a nitrogen-containing polysaccharide) in soil can be as high as several tenths of a percent of its mass [1]. Chitin is the second most widespread (after cellulose) polysaccharide in natural environments [2, 3]. It is a component of the cell walls of soil fungi and the external skeletons of invertebrates. Chitin is similar to cellulose in a number of physicochemical properties; however, the presence of acetamide groups in its molecules determines its unique and exceptionally valuable characteristics. Microorganisms act upon chitin with their exoenzymes (chitinase and chitobiase), producing chitotrioses and chitobioses; the latter then decompose to N-acetyl glucosamine [4, 5]. Many prokaryotes are able to produce chitinase [6, 7]. Mycelial prokaryotes (actinomycetes) are the first to respond to the presence of chitin by rapid reproduction [8, 9].

Due to the widespread occurrence of this biopolymer, the issue of the groups of soil microorganisms that most actively participate in chitin decomposition, as well as of the role of the various ecological factors which determine the intensity of this process (in particular, humidity levels), is of urgent interest.

The pressure of soil moisture exerts a significant effect on the chitinolytic complex of soil microorganisms and therefore on the rate and completeness of chitin decomposition in soil.

[1] Corresponding author; e-mail: manucharova@mail.ru

According to the recently published data, fungi (including primarily the members of the genera *Mortierella* and *Fusarium*), actinomycetes (*Streptomyces*, *Streptosporangium*, and *Micromonospora*), and bacteria (*Bacillus circulans*, *Arthrobacter* sp., *Cellulomonas uda*, *Clostridium* sp., and *Pseudomonas aeruginosa*) decompose chitin in dry sandy and water-logged wetland soils [10–13].

In this work, we studied the chitinolytic microbial complex of chernozem at various humidity levels.

MATERIALS AND METHODS

Microbial succession was induced in the chernozem soil samples (microcosms) by wetting and introduction of chitin. Induced microbial succession is the succession that begins after wetting a dry soil sample or supplementing it with a certain organic (inorganic) substance and proceeds in the course of incubation of this sample under specified conditions (temperature, humidity, etc.) over a prolonged period [14]. Two experimental series have been performed. Weighed portions (5 g) of soil were wetted with either 2% chitin suspension (2 g of chitin per 100 ml of water), or an ammonium sulfate solution which contained the same amount of nitrogen as the chitin suspension. We used the purified chitin preparation (poly-N-acetyl-1,4-β-*D*-glucosamine) manufactured in Germany by ICN Biomedicals, Inc. (MM 770.8, catalogue no. 101334). The

experiments were carried out at three different pressure levels of soil moisture (or humidity levels, % of the mass of absolutely dry soil, *W*): (1) $P = -10$ atm (maximum hygroscopic water capacity; $W = 20\%$), (2) $P = -1.4$ atm (field water capacity; $W = 50\%$), and (3) $P = -0.7$ atm (total water capacity; $W = 70\%$).

The daily rate of CO_2 emission was determined by gas chromatography [15]. The experiments were carried out for one month. Chitin-free wet soil samples were used as controls.

Microbial cell numbers, as well as the length of actinomycete and fungal mycelium, were determined by fluorescence microscopy on days 0, 4, 7, 14, and 30.

The soil suspension (1 : 100) was treated with a UZDN-1 sonicator (Russia) at 0.40 A and 16 kHz for 3 min. The suspension (0.01 ml for bacteria and 0.02 ml for fungi) was applied with a micropipette to a thoroughly degreased slide and spread uniformly with a loop over a 4 cm^2 area (2 × 2 cm). The preparations were air-dried at room temperature, fixed by slight heating over a gas burner, and then stained with aqueous solutions of acridine orange (1 : 10000; 2–4 min) for bacterial cells or calcofluor white (1 : 10000; 15 min) for fungal cells. To remove the excessive fluorochrome, the slides were washed for 10 min in a glass or a cuvette with tap water. The stained preparations were dried at room temperature. For microscopic examination, a water drop was added and a degreased cover slip applied. The preparations were examined in an Axioskop 2 plus fluorescence microscope.

The number of microbial cells contained in one gram of soil was calculated using the following formula:

$$M = 4an \times 10^{10}/p,$$

where *M* is the cell number in one gram of soil; *a* is the average number of cells in a microscope field; *n* is the dilution index; and *p* is the microscope field area (μm^2) [15].

The biomass (mg/g soil) of bacteria, as well as of the actinomycete and fungal mycelium, was calculated using the following formulas [16]:

$Bb = 2M \times 10^{-2}$ (for bacterial biomass);

$Bam = 3.9M \times 10^{-5}$ (for the biomass of actinomycete mycelium); and

$Bfm = 2.98M \times 10^{-3}$ (for the biomass of fungal mycelium).

The specific surface of mycelial and unicellular microorganisms was also determined by calculation. The shape of the bacterial cells was arbitrarily assumed spherical and the shape of the mycelial microorganism cylindrical. We calculated the specific surface of bacterial cells using the formula $4\pi r^2 M$, where $r = 0.3$ μm and *M* is the number of bacterial cells in one gram of soil. The specific surface of fungi and actinomycetes was calculated using the formula $2\pi rl$, where $r = 0.5$ μm or 5 μm (for actinomycetes and fungi, respectively), and *l* is the length of the actinomycete or fungal mycelium in one gram of soil.

All the soil samples were analyzed in five repeats. The results were statistically examined with the STATISTICA 6.0 software package.

RESULTS AND DISCUSSION

Both in experimental soils samples supplemented with chitin and in the control samples, an increase in CO_2 emission during succession was revealed. It was shown that CO_2 emission reached its peak by the end of the first week of the experiment, indicating that microbial activity was highest within this period. This phenomenon was observed irrespective of both the level of soil humidity and the presence or absence of chitin in soil. After the first week, the process rate stabilized and subsequently decreased. At all the studied humidity levels, the rate of CO_2 emission for the samples supplemented with chitin was higher than for the control samples. However, the difference between the experimental and control variants emerged on different days of succession, depending on the humidity level. At the pressure of soil moisture (*P*) of –10 atm it was observed on day 14 of the experiment; at $P = -1.4$ atm, on day 7; and at $P = -0.7$ atm, on day 2 (Fig. 1). The differences in the duration and intensity of chitin utilization by microorganisms in the studies samples can be explained by both the amounts of water in these soil samples and the presence of available oxygen.

For a more convenient presentation of the data on the microbial transformation of chitin in soil at different humidity levels, we have calculated the coefficient of chitin transformation (CCT), which represents the ratio between the rate of CO_2 emission from chitin-containing soils and the rate of CO_2 emission from the control samples. We observed the following phenomenon: irrespective of the soil humidity, the coefficient of chitin transformation was highest by the end of the second week of the experiment. This process was most intense at the pressure of soil moisture of $P = -1.4$ atm (field water capacity). Hence, the highest intensity of chitin biotransformation in chernozem was observed on day 14 of the experiment at $P = -1.4$ atm (Fig. 2).

To demonstrate that microorganisms utilize chitin as a carbon source, we used the following methods of succession induction in the three variants of the experiment: by wetting, by the addition of chitin, or through the addition of a nitrogen-containing mineral fertilizer (ammonium sulfate). Wet samples served as controls. The amount of nitrogen in the chitin-containing soil samples was equal to that in the soil samples with ammonium sulfate. Thus, the experimental variants differed only in their carbon content. The intensity of microbial decomposition of chitin and the mineral fertilizer was determined from the rate of CO_2 emission. During each stage of the experiment, the rate of CO_2

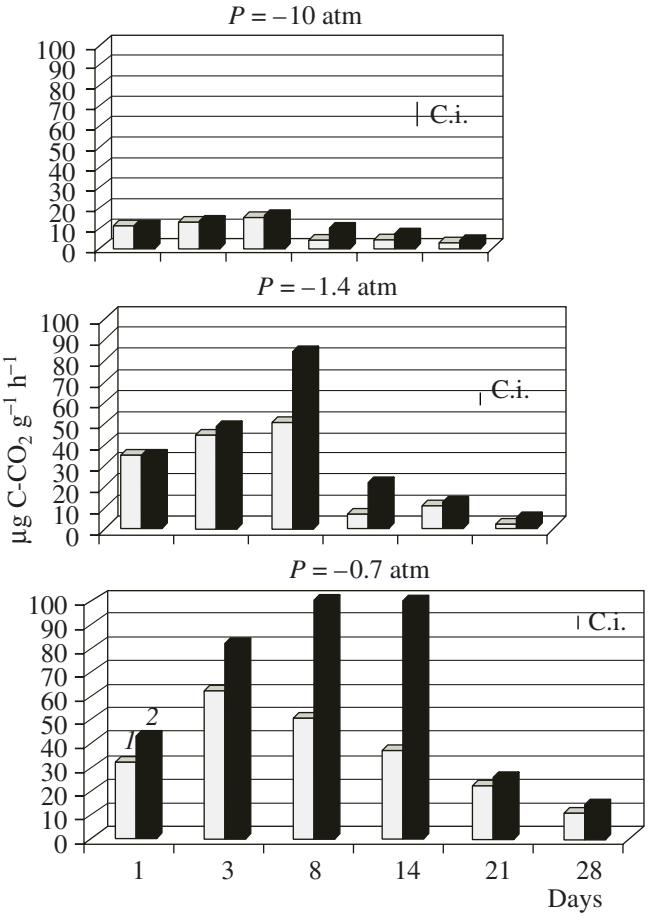

Fig. 1. Dynamics of CO_2 emission from chernozem during succession induced by the addition of chitin and various levels of humidity: (*1*) without chitin; (*2*) with chitin. Here and hereafter, C.i. stands for confidence interval.

emission from chitin-containing samples was significantly higher than that observed for the control samples and the experimental samples containing the nitrogen fertilizer. The addition of ammonium sulfate stimulated microbial respiration as compared to the controls; however, the stimulatory effect of chitin was more pronounced (Fig. 3a). The rate of CO_2 emission from chitin-containing samples was two to five times higher than from the samples containing ammonium sulfate (Fig. 3b). Hence, for microorganisms, chitin is the source of both nitrogen and, most importantly, carbon. Using the methods of mathematical statistics (based on two-factor dispersion analysis), we have demonstrated that the data on CO_2 emission resulting from all the variants (on soil supplemented with the mineral fertilizer or chitin and on wet controls), as well as the results of CCT calculation at various levels of humidity, are statistically different (Figs. 4a, 4b).

The numbers of all the studied groups of microorganisms were significantly higher in the chitin-containing samples than those in the controls and in the samples containing the mineral fertilizer. These results correlated well with the data on CO_2 emission (Fig. 5).

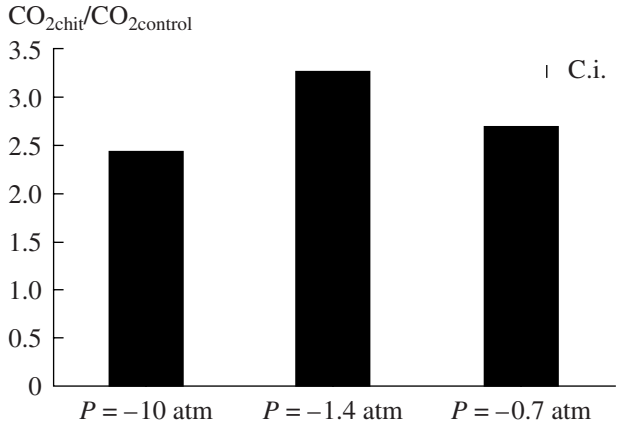

Fig. 2. Coefficient of chitin transformation in chernozem at various humidity levels (day 14 of the experiment).

Since the rate of CO_2 emission from the chitin-containing chernozem samples was higher than that from the control samples at all humidity levels, we have studied the complex of chitinolytic microorganisms at different pressures of soil humidity. As a result, it was established that the numbers of microorganisms in the chitin-containing samples were higher than those in the chitin-free control samples at all the humidity levels tested. Of particular note is the similarity between the growth dynamics of both prokaryotic and eukaryotic cells at the lowest humidity levels ($P = -10$ atm). In the chitin-containing samples, the numbers of prokaryotic cells and especially the length of the actinomycete mycelium increased with increasing humidity (Fig. 6). As to the dynamics of biomass production by microorganisms in the control and experimental samples with chitin at various humidity levels, similar processes were detected. In the chitin-containing samples, the content of prokaryotic biomass, and especially the length of actinomycete mycelium, increased with increasing humidity (Fig. 7).

To assess the structure of the microbial community involved in chitin decomposition at the studied humidity levels, the percentages of the specific surface of various microorganisms were calculated. For example, in the control samples at all the studied humidity levels, the percentage of the specific surface of all microorganisms hardly changed during succession. However, in chitin-containing samples, a threefold increase in the proportion of actinomycetes was observed as the proportion of eukaryotic cells decreased (Fig. 8).

Hence, it was found that the biomass production, as well as the mycelium length and specific surface of mycelial prokaryotes (actinomycetes), increased significantly in the course of chitin decomposition in cher-

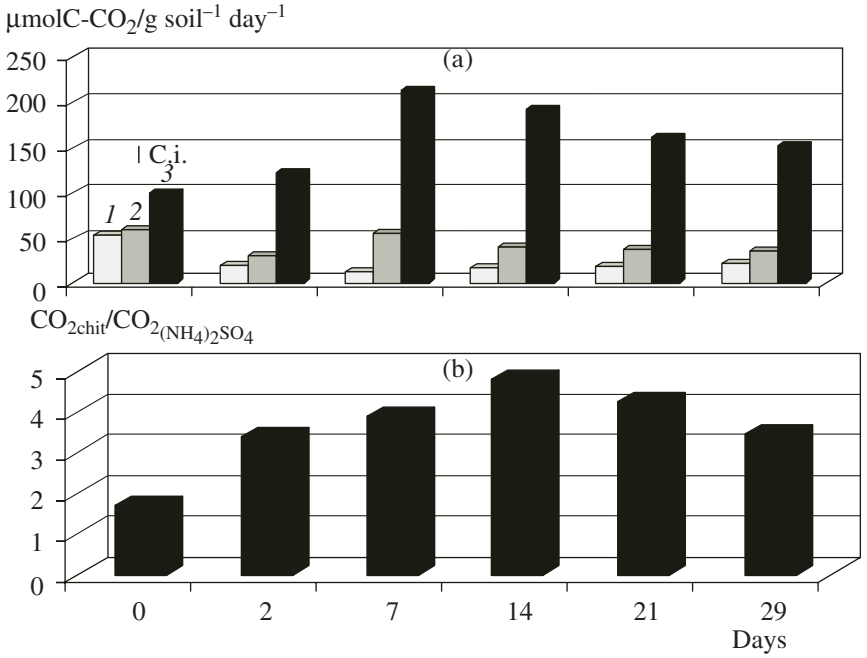

Fig. 3. (a) Dynamics of CO_2 emission (*E*) from chernozem during succession induced by wetting (*1*), addition of ammonium sulfate (*2*), or addition of chitin (*3*); (b) the ratio between CO_2 emission in the experiment with chitin and in the experiment with ammonium sulfate.

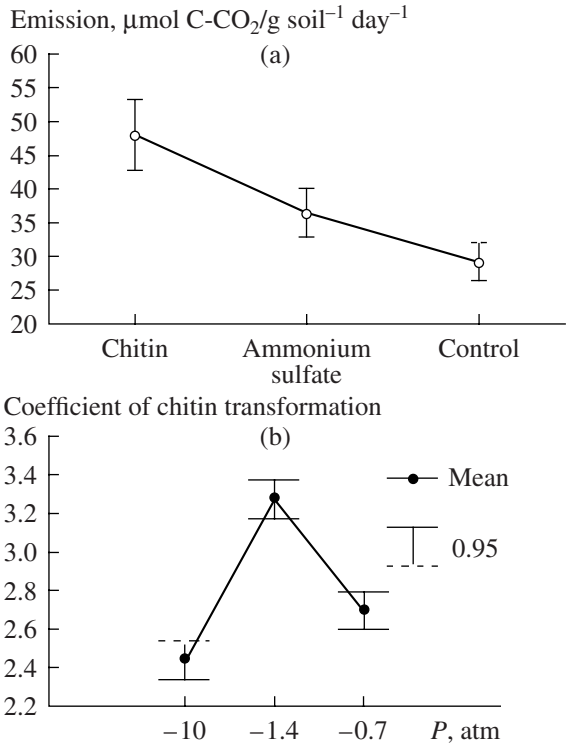

Fig. 4. (a) The rate of CO_2 emission from chernozem in the various modifications of the experiment; (b) coefficients of chitin transformation at various pressures of soil moisture; STATISTICA 6.0 software package.

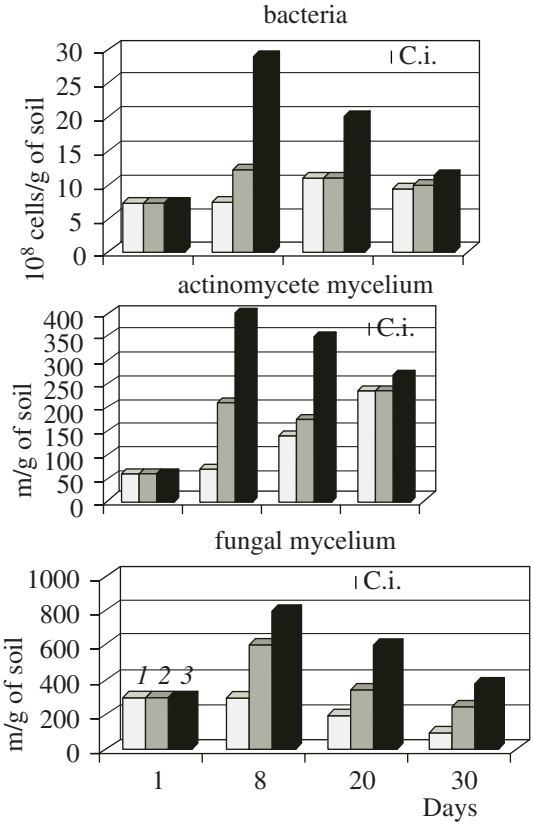

Fig. 5. Dynamics of the numbers of various groups of microorganisms determined by fluorescence microscopy in the experimental variants with chitin and ammonium sulfate (*2*), as well as in control samples (*1*), at the pressure of soil moisture of –1.4 atm.

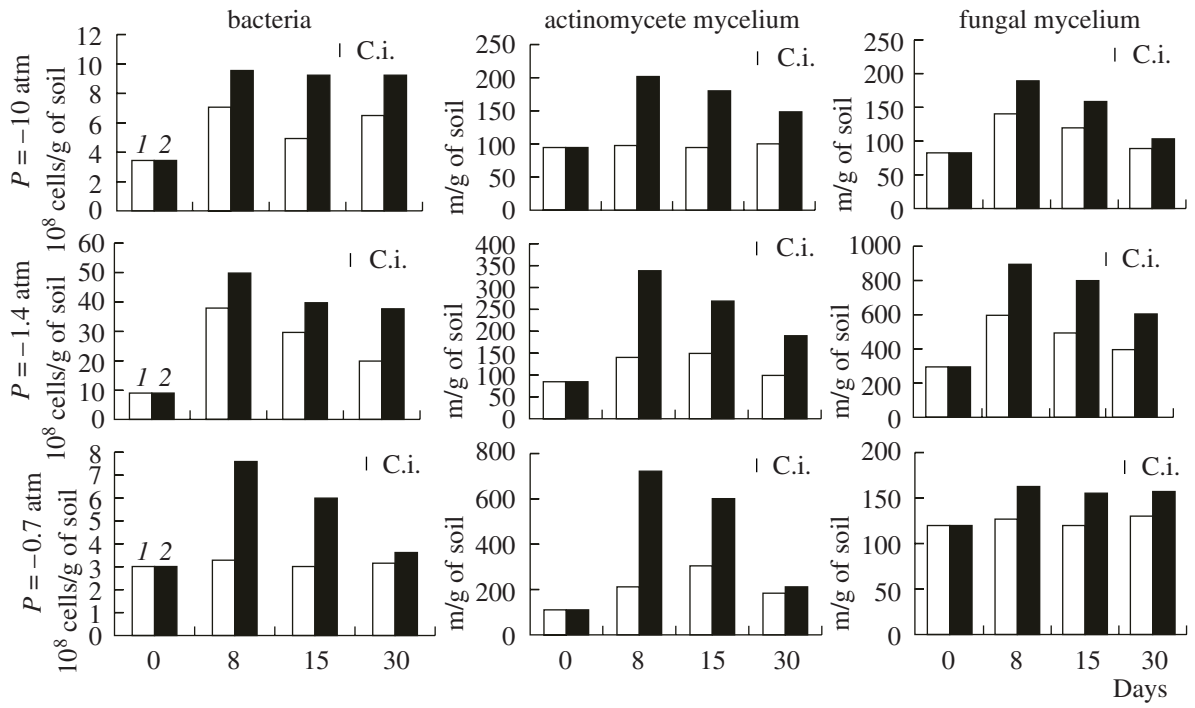

Fig. 6. Dynamics of the numbers of various groups of microorganisms from chernozem determined by fluorescence microscopy at various levels of soil humidity: (*1*) control; (*2*) chitin.

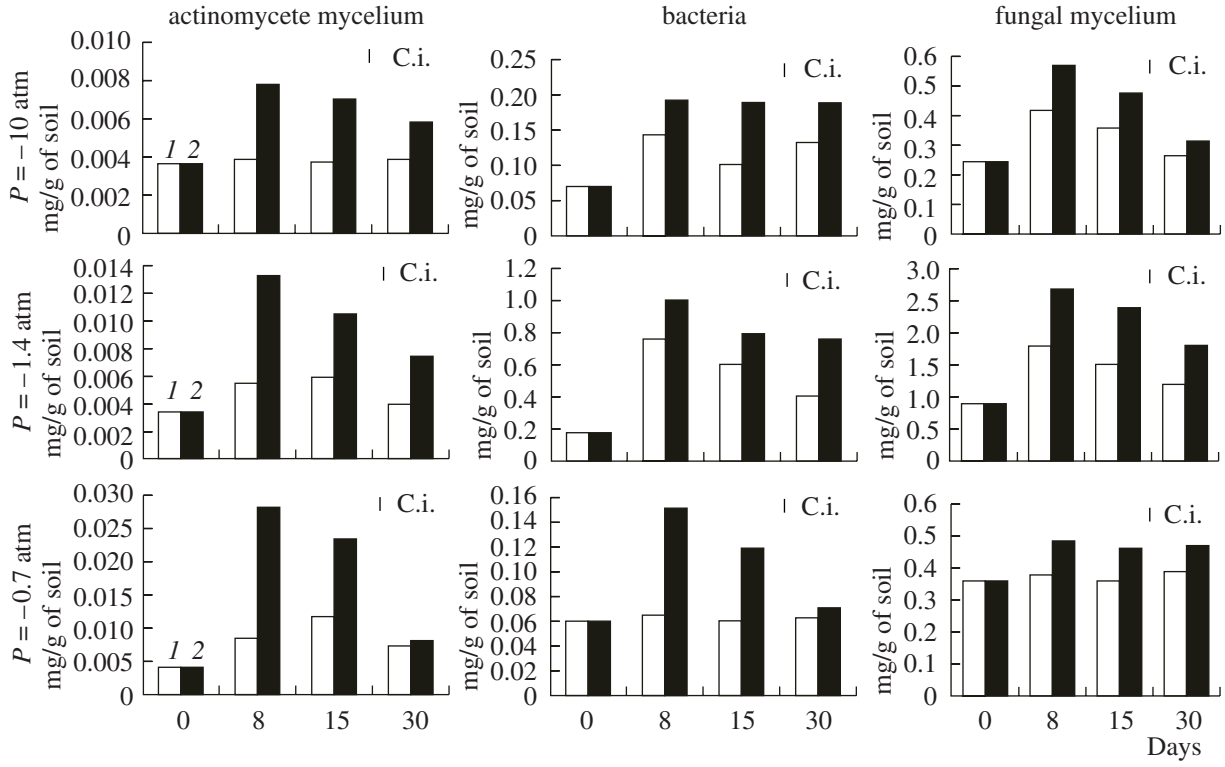

Fig. 7. Dynamics of biomass production by various groups of microorganisms from chernozem determined by fluorescence microscopy at various levels of soil humidity: (*1*) control; (*2*) chitin.

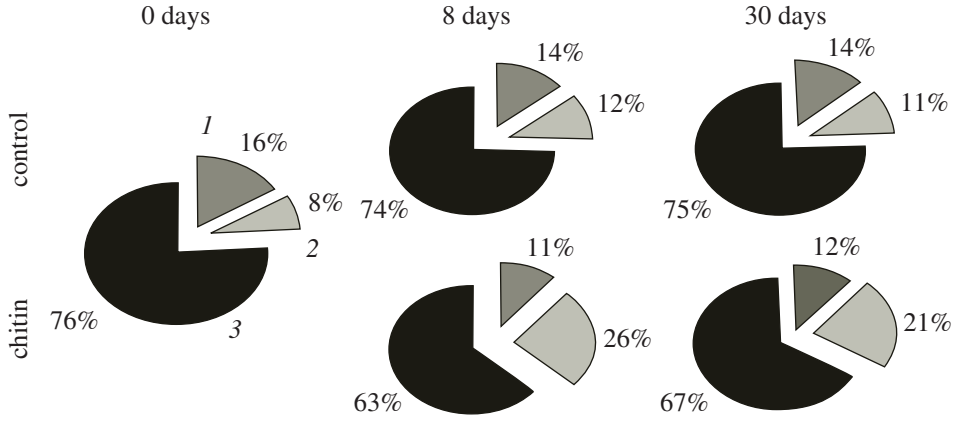

Fig. 8. Dynamics of the percent ratio between the specific surface of prokaryotes and that of eukaryotes in chernozem during succession induced by wetting and addition of chitin (P = –1.4 atm): (*1*) bacteria; (*2*) actinomycetes; (*3*) fungi.

nozem as the pressure of soil moisture increased from –10 to –0.7 atm.

REFERENCES

1. Terekhov, A.S., Zenova, G.M., Kozhevin, P.A., and Mikhailova, N.V., Multisubstrate Testing of Soil Actinomycetes, *Vestnik Moskovskogo Universiteta*, Ser. 19, "Soil Science", 2001, no. 2, pp. 10–13.
2. De Boer, W., Klein Gunnewiek, P.J.A., Lafeber, P., Janse, J.D., Spit, B.E., and Woldendorp, J.W., Antifungal Properties of Chitinolytic Dune Soil Bacteria, *Soil Biol. Biochem.*, 1998, vol. 30, pp. 193–203.
3. Chen, K.S., Lee, K.K., and Chen, H.C., A Rapid Method for Detection of N-acetglucosaminidase-Type Chitinase Activity in Crossed Immunoelectrophoresis and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Gels Using 4-methylumbelliferyl-N-acetyl-B-glucosaminide as Substrate, *Electrophoresis*, 1994, vol. 15, pp. 622–665.
4. Eltarabily, K.A., Soliman, M.H., Nassar, A.H., and Alhassani, H.A., Biologycal Control of *Sclerotinia minor* Using a Chitinolytic Bacterium and Actinomycetes, *Plant Pathol.*, 2000, vol. 49, iss. 5, pp. 573–583.
5. *Sovremennaya mikrobiologiya (prokarioty)* (Modern Microbiology (Prokaryotes)), Lengeler, I. et al., Moscow: Mir, 2005.
6. Aktuganov, G.E., Melent'ev, A.I., Kuz'mina, L.Yu., Galimzyanova, N.F., and Shirokov, A.V., The Chitinolytic Activity of *Bacillus* Cohn Bacteria Antagonistic to Phytopathogenic Fungi, *Mikrobiologiya*, 2003, vol. 72, no. 3, pp. 356–360 [*Microbiology* (Engl. Transl.), vol. 72, no 3, pp. 313–317].
7. Gomez Ramirez, M., Rojas Avelizapa, L.I., Rojas Avelizapa, N.G., and Cruz Camarillo, R., Colloidal Chitin Stained with Remazol Brilliant Blue R, a Useful Substrate to Select Chitinolytic Microorganisms and to Evaluate Chitinases, *J. Microbiol. Meth.*, 2004, vol. 56, iss. 2, pp. 213–219.
8. Kalakutskii, L.V. and Agre, N.S., *Razvitie aktinomitsetov* (Development of Actinomycetes), Moscow: Nauka, 1977.
9. Hildgund Schrempf. Recognition and Degradation of Chitin by Streptomycetes, *A. van Leeuwenhoek*, 2001, vol. 79 (3–4), pp. 285–289.
10. Doroshenko, E.A., Zenova, G.M., Zvyagintsev, D.G., and Sudnitsyn, I.I., Spore Germination and Mycelial Growth of Streptomycetes at Different Humidity Levels, *Mikrobiologiya*, 2005, vol. 74, no 6, pp. 795–799 [*Microbiology* (Engl. Transl.), vol. 74, no 6, pp. 690–694].
11. Folders, J., Algra, J., Roelofs, M.S., van Loon, L.C., Tommassen, J., and Bitter, W., Characterization of *Pseudomonas aeruginosa* Chitinase, a Gradually Secreted Protein, *J. Bacteriol.*, 2001, vol. 183, no. 24, pp. 7044–7052.
12. Patidar, P., Agrawal, D., Banerjee, T., and Patil, S., Optimization of Process Parameters for Chitinase Production by Soil Isolates of *Penicillium chrysogenum* under Solid Substrate Fermentation, *Process Biochem.*, 2005, vol. 40, iss. 9, pp. 2962–2967.
13. Dobrovol'skaya, T.G., *Struktura bakterial'nykh soobshchestv pochv* (Structure of Soil Bacterial Communities), Moscow: Akademkniga, 2002.
14. Zvyagintsev, D.G., *Pochvy i mikroorganizmy* (Soils and Microorganisms), Moscow: Mosk. Gos. Univ., 1987.
15. *Metody pochvennoi mikrobiologii i biokhimii* (Methods of Soil Microbiology and Biochemistry), Moscow: Mosk. Gos. Univ., 1991, p. 303.
16. Kozhevin, P.A., *Mikrobnye populyatsii v prirode* (Microbial Populations in Nature), Moscow: Mosk. Gos. Univ., 1989.