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EXPERIMENTAL ARTICLES

Succession of Chitinolytic Microorganisms in Chernozem Soil

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Abstract—The chitinolytic prokaryotic and eukaryotic microbial complex of chernozem soil has been investigated in the course of a succession initiated by the introduction of chitin and humidification. The dynamics of the cell numbers of chitinolytic microorganisms and of their biomass was assessed by fluorescent microscopy and by inoculation of selective media. Emission of carbon dioxide and nitrous oxide, as well as dinitrogen fixation, was assessed by gas chromatography. It was found that, when the succession was initiated by the introduction of both chitin and humidification, it resulted in greater cell numbers and biomass of chitinolytic microorganisms and higher levels of CO_2 and N_2O emission and of nitrogen fixation than when the succession was initiated by humidification alone. As compared to the control samples, a significant (twofold) increase in the prokaryote cell number and biomass was found on the fourth day of the succession initiated by humidification and introduction of chitin. One week after the initiation of succession, the fungal biomass and length of mycelium were twice as high as those in the control samples. These results led to the conclusion that chitin utilization in chernozem soil starts during the initial stages of succession and is performed by both prokaryotic and eukaryotic microorganisms.

Key words: chitinolytic complex of soil microorganisms, chitin degradation in soils.

Chitin, a major component of invertebrate exoskeletons and of fungal hyphae, is always present in soil in concentrations of up to tenths of a percent. Although some of its physicochemical characteristics are close to those of cellulose, the presence of acetamide groups endows chitin with certain features that have important practical applications. Bacterial chitinase, for example, is known to be used to protect plants against infectious agents. The preparation based on this enzyme is a promising ecologically safe tool for controlling phytopathogenic fungi [1, 2]. Microbial attack on chitin by exoenzymes (chitinase and chitobiase) results in the formation of chitotrioses and chitobioses, which are further decomposed to monomers and *N*-acetylglucosamine [3]. Chitinase is produced by a number of bacteria [4, 5]. Actinomycetes are the first to respond to the presence of chitin by rapid growth [6, 7]. The high activity of microbial chitinases provides for the mobilization of carbon and nitrogen from not easily accessible compounds like chitin, and, thus, for the involvement of these elements in soil–atmosphere circulation. However, the issue of chitin decomposition in soils has been insufficiently studied, and the question regarding the principal groups of soil chitinolytic microorganisms most actively participating in this process remains open for discussion.

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The goal of the present work was to investigate the prokaryotic and eukaryotic microbial chitinolytic complex in chernozem soil in the course of a succession initiated by the introduction of chitin and soil humidification.

MATERIALS AND METHODS

Samples of ordinary chernozem collected in the Kamennaya Step' preserve were used in the investigation.

Microbial succession was initiated by humidification of the soil samples and the introduction of chitin. A microbial succession is termed an *initiated succession* when it starts after humidification of a dry soil sample, introduction of a certain organic compound, and incubation for prolonged time under controlled conditions of humidity and temperature [8]. The experimental procedure was as follows: a 5-g soil sample was moistened with a 0.5% chitin suspension (1 ml per 5 g soil), placed in a 15-ml vial, and incubated at 20°C for 35 days in a desiccator with water to maintain the desired humidity. The soil water pressure was maintained at a level of −0.1 MPa, corresponding to a water activity (a_w) of 0.98 [8].

Soil sampling was performed weekly at the zero moment (initiation of the succession) and on the 6th, 15th, 21st, and 34th days of the experiment. The bacterial cell numbers, length of actinomycetal and fungal

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mycelium, and the number of fungal spores were assessed by fluorescent microscopy [10], and the population dynamics of the bacteria and actinomycetes was monitored by plating serial dilutions onto agarized nutrient media [9]. A solid medium composed of 4 g of chitin, 20 g of agar, and 1 l of tap water was inoculated with diluted soil suspensions. Differential enumeration of the bacteria and actinomycetes was performed after incubation for 7 to 10 days at 28°C. The dynamics of carbon dioxide emission was determined by gas chromatography in a parallel series of experiments [10]. Humidified soil samples not supplemented with chitin were used as controls.

Nitrous oxide emission was used to assess the intensity of evolution of gaseous nitrogen compounds from soil in the presence of chitin. The analysis was performed by gas chromatography [10].

Dinitrogen fixation was determined by the acetylene method with a modification developed at the Department of Soil Biology, Moscow State University. A CHROM-4-1 gas chromatograph with a flame ionization detector was used [10].

All the soil samples were analyzed in five replicates. The STATISTIKA software package was used for statistical treatment of the data.

Bergey's Manual was used for identification of the bacteria and actinomycetes [11].

The characteristics used for preliminary generic identification of the actinomycetes were both morphological (fragmentation of mycelium, location of spores on aerial and/or substrate mycelium, and occurrence of single spores and pairs or chains of spores) and chemotaxonomic (presence of L- or meso-isomers of diaminopimelic acid (DAPA) and differentiating sugars in whole cell hydrolysates). The cultures of actinomycetes exhibiting branched unfragmented mycelium, chains of nonmotile spores (3–50 spores) on aerial mycelium, no spores on substrate mycelium, and presence of L-DAPA and no differentiating sugars in the cell hydrolysates were classified as *Streptomyces.* The cultures that exhibited thin (0.5 μm) vegetative mycelium and single nonmotile spores on the substrate mycelium, had poorly developed sterile aerial mycelium or lacked it, and were characterized by the presence of meso-DAPA, xylose, and arabinose in the cell hydrolysates were classified as *Micromonospora* representatives [12].

Identification of the species of actinomycetes was performed using the manual [13].

The intensity of chitin decomposition by the cultures of actinomycetes and bacteria isolated in the course of succession was assessed from the amount of the monosaccharide *N*-acetyl-D-glucosamine [14] found in the culture liquid. The strains were grown in a liquid medium containing (g/l) chitin, 4.0; peptone, 0.4; yeast extract, 0.2; KH_2PO_4, 1.0; and $MgSO_4 \cdot 7H_2O$, 0.18 under constant shaking (180 rpm) for three days. Since published data exists concerning the positive effect of peptone on chitinase synthesis by prokaryotes, 0.4 g/l peptone was added to the nutrient medium [15, 16]. In order to determine chitinase activity, 1 ml of Tris–phosphate buffer (pH 7.2) and 6 mg of chitin were added to 1 ml of filtered culture liquid. A chitin suspension in Tris–phosphate buffer with 1 ml of water instead of the culture liquid was used as the control. The mixture was incubated for 24 h at 40°C and then filtered. Next, 3 ml of Ehrlich's reagent was added to 0.5 ml of the filtrate. The mixture was incubated at 40°C for 20 min, cooled, and the OD_{450} of the experimental and control solutions was measured using a colorimeter. To prepare Ehrlich's reagent, 10 g of 4-dimethylaminobenzaldehyde was mixed with strong acetic acid containing 12.5 vol % 10 N HCl [14]. The concentration of *N*-acetyl-D-glucosamine was determined using a calibration curve.

RESULTS AND DISCUSSION

Fluorescence microscopic examination revealed that the bacterial numbers and length of fungal and actinomycetal hyphae were higher in the samples with chitin than in the control samples, which were humidified without chitin addition. This result was found at all the stages of succession (Fig. 1). The highest values of bacterial numbers and lengths of mycelia were observed from the 7th to the 14th day of the experiment. It is worth mentioning that sharp differences between the bacterial numbers in the control and experimental samples were noticed on the 4th day (Fig. 1a). At this stage, the bacterial numbers in the samples with chitin were twice as high as those in the control samples. The same was true for mycelial prokaryotes (Fig. 1b). The differences between the lengths of fungal mycelium on the 4th day of incubation were less significant (Fig. 1c). Throughout the experiment, no reliable difference was found between the numbers of fungal spores in the control and experiment. The number of fungal spores increased at the middle and final stages of succession (14th and 30th days). By the end of the experiment (30th day), a certain decrease in bacterial numbers and in the mycelium length of the fungi and actinomycetes was observed. This circumstance may indicate a stationary state of the system.

On the fourth day after the introduction of chitin, the biomass of the bacteria and actinomycetes was almost twice that of the control samples. The changes in fungal biomass and in the length of fungal mycelium were, in that period, less significant (Fig. 2). In our earlier research with pure cultures of mycelial prokaryotes grown on medium with chitin, we observed an increase in mycelial biomass and actinomycete spore numbers by the seventh day of experiment [17].

Both in the control and the experimental samples, the fungal biomass exceeded the prokaryotic biomass throughout the experiment. On the 7th day, the eukaryotic biomass in the chitin-containing samples increased significantly compared to the control; in the middle of succession (14th day of the experiment), however,

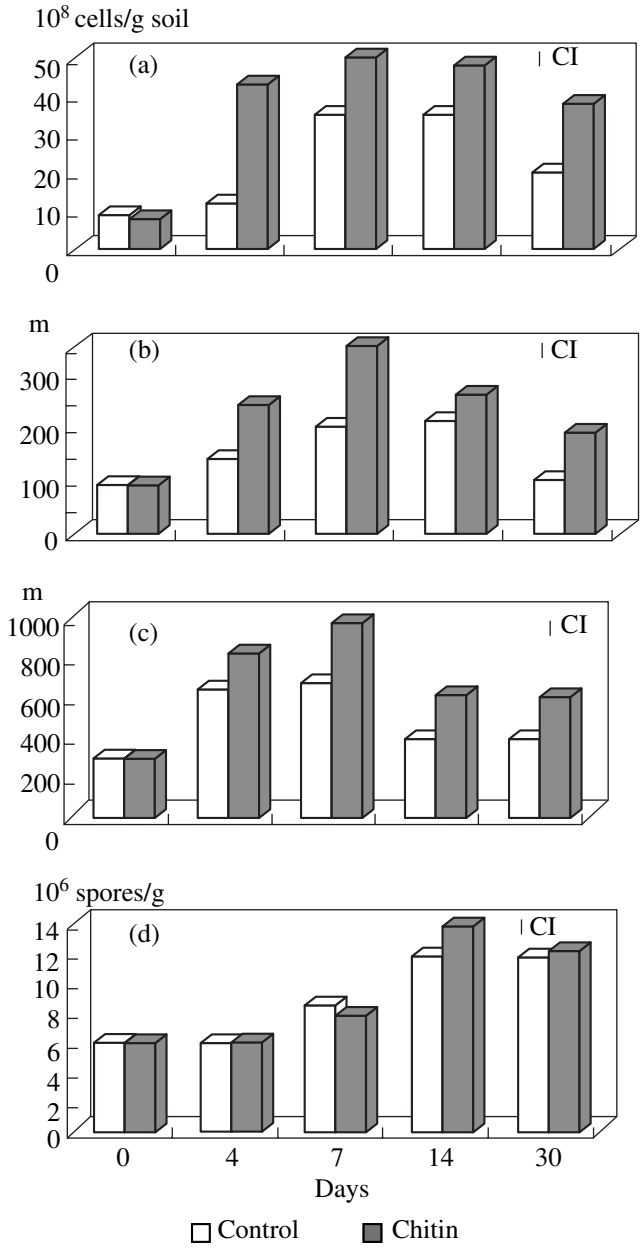

Fig. 1. (a) Bacterial numbers, length of (b) fungal and (c) actinomycetal mycelia, and (d) the number of fungal spores in ordinary chernozem with and without the addition of chitin. Here and further on, CI denotes confidence interval.

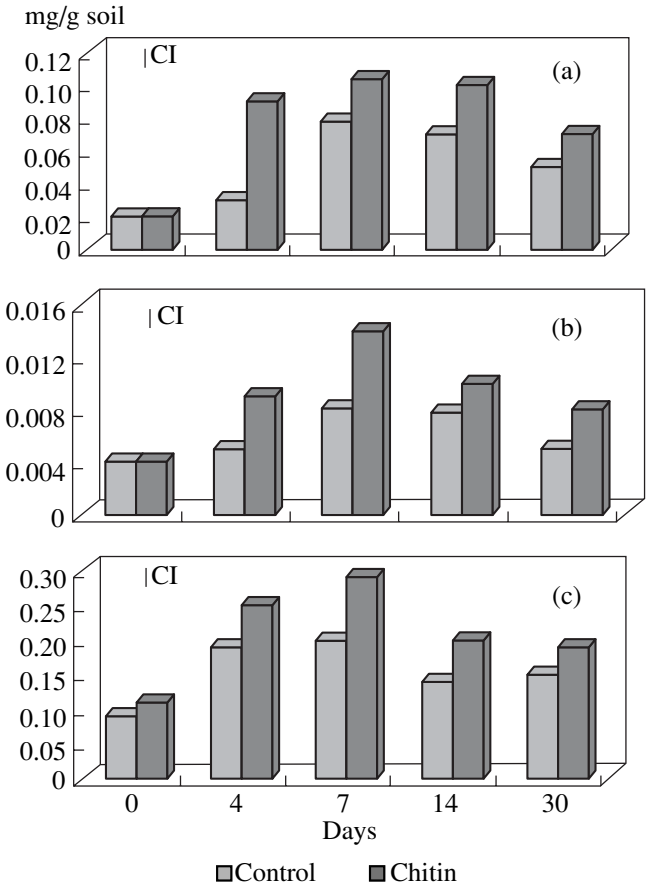

Fig. 2. Biomass of (a) bacteria, (b) actinomycetes, and (c) fungi in ordinary chernozem with and without the addition of chitin.

a decrease in fungal biomass commenced, possibly due to the processes of dying off and transition to the stage of spore formation (Fig. 2).

Investigation of the population dynamics in the course of chitin-initiated succession by plating revealed higher numbers of chitinolytic prokaryotic microorganisms at all stages of the succession in the samples with chitin as compared to the control. The increase in the CFU numbers of the actinomycetes (compared to the control) was more pronounced than that observed for nonmycelial prokaryotes (Table 1).

After succession was initiated by humidification and the introduction of chitin, the density of the nonmycelial bacterial population increased gradually over the period from the zero moment to the 15th day (Fig. 3), reaching its maximum on the 15th day of the experiment. At the beginning of the succession (zero moment), the actinomycetes were present as spores and, therefore, formed numerous colonies on agarized media. During the first six days of the succession, the CFU number of the mycelial prokaryotes somewhat decreased due to the transition of the actinomycetes from spores to vegetative mycelium. By the 7th day, most of the spores had germinated, producing mycelium, and the number of actinomycetes revealed by plating therefore decreased. Our finding that, according to data of fluorescence microscopy, the maximum length of actinomycetal mycelium was achieved on the 7th day is in accordance with the results of plating (Fig. 2). Starting from the 7th day of the experiment, the population density of the actinomycetes increased, with the maximum being reached on the 21st day. By the middle of the succession (14th–21st day), new spores had formed on the mycelium, and this caused an increase in the CFU number of the mycelial prokaryotes.

Our results indicate that, in the course of the succession, the portion of chitinolytic actinomycetes among the chitin-degrading soil bacteria had increased by the 21st day of experiment (Fig. 3).

Apart from study of the dynamics and structure of the soil microbial community, the dynamics of soil respiration activity during chitin-induced succession was determined. The samples of moistened chernozem with no chitin introduced were used as the control. In the control samples, the highest CO_2 emission was observed on the 5th day of experiment; afterwards, the rate of this process decreased. The respiration rate in this case was limited by the rate at which the soil organic matter decomposed. The rate of CO_2 emission from the soil with chitin was significantly higher than in the control samples throughout the succession. These differences were most pronounced from the 7th day onwards. The highest rate of CO_2 emission was found on the 7th day of the succession, correlating with the highest microbial numbers (Fig. 4). The differences with respect to respiration between the control and experiment can perhaps be understood as evidence of intense chitin utilization by soil microorganisms at the initial stages of the succession. The duration of the experiment (one month) was chosen because, at the end of that time, the system reached a climax, indicated by stabilization of the CO_2 emission as well as of the microbial numbers and biomass.

The rates of the processes performed by the prokaryotes were determined in order to assess the bacterial contribution to utilization of chitin as the carbon and nitrogen source in soil. The processes of dinitrogen fixation and denitrification, both in the presence and in the absence of oxygen, were used as indicators. The rate of dinitrogen fixation in the soil with chitin was higher than in the control samples (soil humidified with water) throughout the succession. Evaluation of the rates of dinitrogen fixation in the soil with and without chitin revealed that the highest rates of the process were achieved from the 7th to the 14th day of incubation (Fig. 5a). The same dynamics was revealed for the anaerobic process of nitrous oxide production in the chitin-supplemented soils. The rate reached its highest values from the 7th to the 14th day of incubation and was higher in the presence of chitin than in the control samples (Fig. 5b).

The numbers of bacteria and actinomycetes in the course of successions initiated by humidification (control) and by humidification and the introduction of chitin

Experimental conditions	Time, days			
	0	1	4	7
Bacteria, log[CFU/g soil]				
Control	5.7	5.9	6.0	6.2
Chitin	5.7	6.05	6.2	6.3
Actinomycetes, log[CFU/g soil]				
Control	5.4	5.7	5.8	6.0
Chitin	5.4	5.9	6.4	6.4

A correlation was observed between the increasing portion of prokaryotes in the microbial community and the increasing rate of the processes of respiration, denitrification, and dinitrogen fixation in soil between the 7th and 14th day of incubation with chitin.

In order to assess the intensity of chitin decomposition by pure cultures of prokaryotes, *N*-acetyl-D-glucosamine accumulation in the medium was determined. Compared to unicellular bacteria, the mycelial prokaryotes were more active in producing this monosaccharide. The highest levels of *N*-acetyl-D-glucosamine were found in the culture liquids of *Streptomyces syringini* and *Micromonospora* sp. (10 and 12 μmole/ml medium, respectively) (Fig. 6). Cytopha-

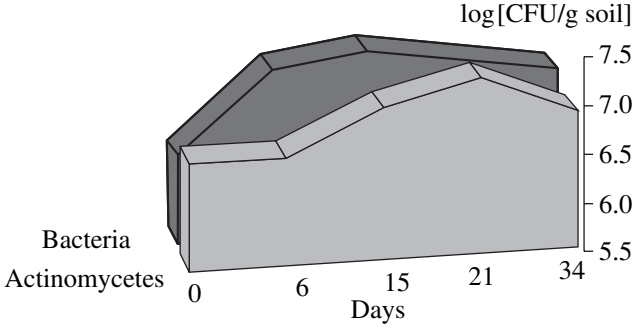

Fig. 3. Population dynamics (as revealed by plating) of bacteria and actinomycetes in the course of succession initiated by humidification and the introduction of chitin to the soil.

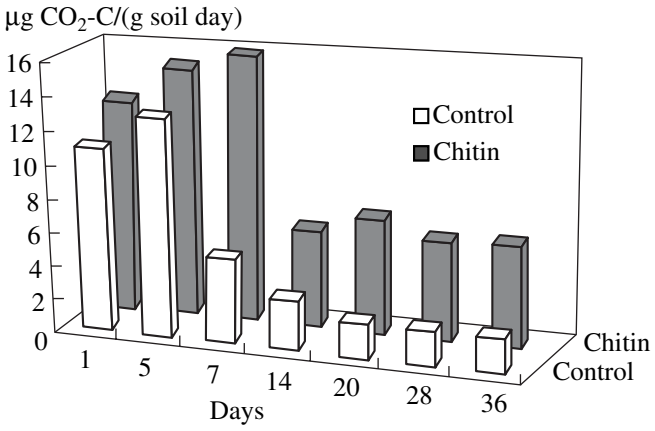

Fig. 4. Dynamics of CO_2 emission from the soil in the course of succession with and without the addition of chitin.

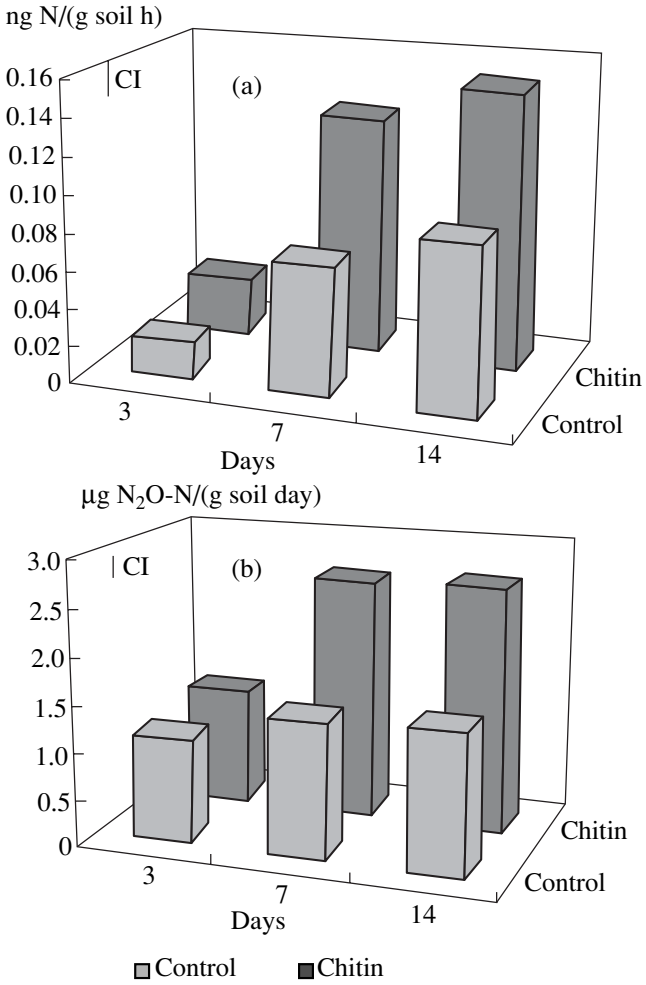

Fig. 5. Dynamics of the rates of (a) dinitrogen fixation and (b) denitrification in ordinary chernozem with and without the addition of chitin.

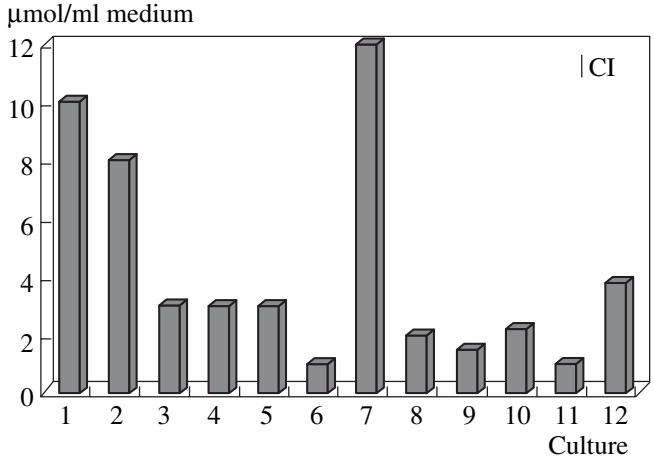

Fig. 6. Production of *N*-acetyl-D-glucosamine on a medium containing chitin by pure cultures of the prokaryotes (1) *Streptomyces syringini*, (2) *Streptomyces* strain 11, (3) *Streptomyces boarnensis*, (4) *Streptomyces nashvilensis*, (5) *Streptomyces* strain 10, (6) *Streptomyces* strain 17, (7) *Micromonospora* strain 5, (8) *Micromonospora* strain 8, (9) order *Myxobacteriales* strain 8, (10) order *Myxobacteriales* strain 3, (11) order *Myxobacteriales* strain 1, and (12) *Cytophaga*.

gas and myxobacteria were the most active among the unicellular prokaryotes.

To conclude, our investigation revealed an increase in microbial numbers and biomass during a succession initiated by humidification and the addition of chitin as compared to the same values in the course of a succession initiated by humidification alone. The prokaryotic biomass had doubled by the 4th day of the experiment, and the eukaryotic biomass had doubled by a week after the succession was initiated. The peak accumulation of microbial biomass on chitin corresponded to the highest CO_2 emission from the soil. The increase in the rates at which the processes of the nitrogen cycle occurred, which followed the peak of microbial biomass accumulation, confirms the role of prokaryotes in chitin decomposition. In the culture liquids of mycelial prokaryotes and unicellular bacteria, *N*-acetyl-D-glucosamine was determined.

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