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Monitoring of the Chitinolytic Microbial Complex of the Phylloplane

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Abstract—A comprehensive study of chitinolytic microbial complexes of the phylloplane from cultured and forest plants has been conducted. An increase of the number and biomass of metabolically active cells of the representatives of the domain Bacteria and a decrease in fungal biomass in the experimental microcosms have been shown to occur after the introduction of chitin. The characteristic features of the taxonomic structure of metabolically active chitinolytic complexes of the phylloplane of the plants studied have been elucidated. Representatives of the phyla *Proteobacteria, Bacteroidetes,* and *Verrucomicrobia* have been shown to play important roles in the chitinolytic complexes of green leaf samples, while mycelial actinobacteria of the phylum Actinobacyteria played a similar role in needles of coniferous trees. A collection of chitinolytic microorganism cultures isolated from the phylloplane of different plant species has been created.

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Studies of the phyllosphere as a microorganism 1 habitat began in the 1950s and have constituted an important area of research both in Russia and abroad since the 1970s (Morris, 2001). Hundreds of species of 2 bacteria, mycelial fungi, yeasts, and protozoa have 1 been detected in the phyllosphere and phylloplane of plants during this time. Many of them play an important role in the life of the plant, being phytopathogens or pathogen antagonists, and producing vitamins, phytohormones, antibiotics, or toxins (Zenova, Zvyagintsev, 2002; Lindow, Brandl, 2003). Studies of the microbial community of the phylloplane of plants are of utmost importance for understanding interspecies interactions in nature; from the practical point of view, these studies provide the basis for a variety of biological pest control methods aimed at increasing the productivity of agriculture, decreasing crop loss caused by diseases and pests, etc. (Zvyagintsev et al., 1999).

Since chitin is an important structural component of the fungal cell wall, chitin-degrading microorganisms can inhibit the development of fungal mycelium. The opportunity to use chitinolytic enzymes against fungi causing plant diseases was the first to attract researchers' attention. Chitinases were considered an environmentally safer alternative to fungicides in those studies. A large number of studies conducted in recent years have demonstrated the suppression of the development of phytopathogenic fungi by chitinolytic microorganisms in vitro; as a rule, bacterial isolates from the soil and rhizosphere were used as chitinolytic microorganisms in those experiments (Gohel et al., 2006). However, chitinolytic microorganisms inhabiting the supraterrestrial parts of plants have remained virtually uncharacterized, although these parts of the plants are also vulnerable to fungal diseases.

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We focused on study of the composition and structure of the chitinolytic microbial complex from the phylloplane of both cultural and wild plants. Analysis of the composition and structure of the native complex of chitinolytic microorganisms inhabiting the plant phylloplane under normal conditions may have considerable importance for development of methods for biological control of phytopathogenic fungi.

The aim of this work was to evaluate the structure of the functional chitinolytic microbial complex inhabiting the phylloplane of various plants.

MATERIALS AND METHODS

Samples of green leaves and needles from various plants—the common spruce *Picea abies*, silver birch *Betula pendula*, sea buckthorn *Hippophae rhamnoides*, garden strawberry *Fragaria ananassa*, apple tree *Malus domestica*, stinging nettle *Urtica dioica*, common goutweed *Aegopodium podagraria*, and common raspberry *Rubus idaeus*—collected in Moscow and Moscow oblast were used as research objects. Leaf samples were collected in July and August 2011.

Microbial succession was initiated in the leaves and needles by increased humidity and chitin introduction in order for the chitinolytic complex to develop. The biomass of unicellular bacteria, filamentous bacteria and fungi in the samples was analyzed by fluorescence microscopy (Polyanskaya, 1996) after ten days; at the same time, the composition of the metabolically active prokaryotic community was analyzed by in situ hybridization of cell RNA to rRNA-specific fluorescently labeled oligonucleotide probes (FISH) (Amann, Ludwig, 2000; Manucharova et al., 2011). The most active chitinolytic microorganisms were identified by plating on culture media with chitin as the only source of carbon and nitrogen with subsequent isolation of pure cultures.

To initiate succession, 2 g of chopped leaves were placed into a vial and moistened with 2 ml of water, and the experimental samples were then supplemented with 12 mg of purified chitin. Moistened samples to which chitin was not added were used as controls. The purified chitin (poly-N-acetyl-1,4- β -D-glucosamine) used in this work was from Sigma-Aldrich (United States). The amount of biomass accumulated and the composition of the prokaryotic community were determined in experimental and control samples ten days after the addition of water.

Estimation of the amount of biomass was based on the total number of cells or on the mycelium length. After incubation, samples of leaves or pine needles were suspended in water at a ratio of 1:100, and sonicated (2 min, current 0.4 A, frequency 22 kHz) to ensure cell desorption. After this, the suspension was applied to glass slides for microscopy. Samples were stained with acridine orange for assessment of the number of unicellular bacteria and actinomycete mycelium length; samples stained with calcofluor white were used to estimate the length of the fungal mycelium. The number of bacterial cells or the length of the mycelium in 1 g of the sample was calculated according to the formula

$N = S_1 a n / v S_2 c,$

where *N* is the number of cells/mycelium length in 1 g of the sample; S_1 is the preparation area, μm^2 ; *a* is the number of cells/mycelium length in one visual field (averaged for all visual fields viewed); *n* is the suspension dilution index, ml; *v* is the volume of the drop deposited on the glass, ml; S_2 is the area of the visual field of the microscope, μm^2 ; and *c* is weight of the leaf sample, g.

The dry biomass of unicellular bacteria, actinomycetes, and fungi was calculated by multiplying the total number of cells or the length of the mycelium by the average mass of a cell or a mycelium fragment. The average mass of a bacterial cell was assumed to be 2×10^{-14} g, the weight of 1 m of actinomycete mycelium with a diameter of 0.5 μ m was assumed to be 3.9 × 10⁻⁸ g, and that of 1 m of actinomycete mycelium with a diameter of 5 μ m was assumed to be 3.9 × 10⁻⁶ g (Kozhevin, 1989). The composition of the metabolically active prokaryotic community was evaluated by hybridization with specific fluorescent-labeled oligonucleotide probes (FISH, fluorescent in situ hybridization). Probes used in this work were specific for individual phylogenetic groups of the domain Bacteria or for the domain Archaea (Table 1).

After ten days of incubation, leaf samples were suspended in water at a ratio of 1:10 and sonicated (2 min, current 0.4 A, frequency 22 kHz) to ensure cell desorption. The sonicated suspension was collected in 2-ml microtubes. Fragments of leaves were removed using centrifugation at 2000 rpm for 10 min. The resulting suspension was centrifuged at 13?000 rpm for 5 min to pellet the cells. The pellet was resuspended in sterile distilled water.

Samples were fixed with formaldehyde: for this, 2 ml of the suspension were centrifuged at 10000 rpm for 5 min, the pellet was resuspended in 0.5 ml phosphate buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, and 0.2 g NaH₂PO₄ in 1 l H₂O, pH 7), and 1.5 ml of 4% formaldehyde solution in a phosphate buffer was added to the pellet and incubated at room temperature on a shaker for 1.5 h. The fixed material was collected by centrifugation (8000 rpm for 2 min), washed 2 times with phosphate buffer, resuspended in 50% ethanol in phosphate buffer, and stored at -20° C until analysis.

The fixed sample suspension $(1 \mu l)$ was applied to hybridization coverslips with windows separated by a Teflon coating and treated with a solution of lysozyme (10 mg in 1 ml 0.05 M EDTA and 0.1 M Tris-HCl, 1:1, pH 8) to increase the permeability of the bacterial cell walls. The preparations were incubated for 12–24 h at room temperature and dehydrated by incubating in a series of ethanol solutions (50, 80, and 100%). A previously developed set of rRNA-specific oligonucleotide probes for the detection of representatives of the domains Bacteria and Archaea, as well as individual phylogenetic groups within the Bacteria domain (Table 1) (Manucharova et al., 2011), was used for the hybridization. Probes labeled with the fluorescent dve Cv3 were produced by Sintol (Russia). Hybridization of the preparations with fluorescently labeled probes was performed according to the procedure of Amann (Amann et al., 1990) at 46°C (Amann, Ludwig, 2000). After hybridization, the samples were counterstained with an aqueous solution of acridine orange (diluted 1:10 000) for 2–4 minutes for the estimation of the total number of bacteria, including resting cells. The samples were analyzed using a Zeiss Mikroskop Axioskop 2 plus fluorescence microscope (Germany) with Filter set 15 for FISH probes and Filter set 09 for acridine orange staining. The abundance of the target microorganism groups in the samples was determined by counting the cells hybridized with the probes (or stained with acridine orange) in different fields of view with subsequent averaging and calculation of the numbers corresponding to 1 g of leaves.

Pure cultures of chitinolytic microorganisms were isolated from samples of green leaves using cultivation on a solid nutrient medium of the following composition: 4 g/l purified colloidal chitin, 0.3 g/l KH₂PO₄, 0.7 g/l K₂HPO₄, 0.5 g/l MgSO₄ · 7H₂O, 0.01 g/l FeSO₄ · 7H₂O, 0.01 g/l ZnSO₄ · 7H₂O, 0.01 g/l MnCl₂ · 4H₂O, and 20 g/l agar. Leaf samples were ground, suspended in water, and sonicated (2 min, current 0.4 A, frequency of 22 Hz) for cell desorption. Prokaryotic biomass was produced on WCH bacterial culture medium of the following composition: 2 g/l peptone, 2 g/l yeast extract with casein hydrolyzate, 2 g/l glucose, 5 g/l chalk, and 20 g/l agar. Eukaryotic biomass was pro-

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Table 1. rRNA-spec	ific oligonucleotide probes					
Probe	Target group of organisms	Target site of 16S rRNA	Nucleotide sequence of the probe $(5'-3')$	Formamide, %	NaCl, mM	Source
EUB338III@ EUB338III@	Bacteria	338–355	GCT GCC TCC CGT AGG AGT GC(W) CY3 GCC (W)CC CGT AGG (W)GT ^a	20	225	Amann <i>et al</i> ., 1990
ARCH915 ARC344	Archaea	915–934 344–363	GTG CTC CCC CGC CAA TTC CT TCG CGC CTG CTG CIC CCC GT	30	112	Stahl, Amann, 1991
o ALFIb ALF968	Alphaproteobacteria	19–35	CGT TCG YTC TGA GCC AG ^a	20	225	Manz <i>et al.</i> , 1992 Dedysh <i>et al.</i> , 2001
BET42a	Betaproteobacteria	1027–1043 ^b	GCC TTC CCA CTT CGT TT	35	80	Manz <i>et al.</i> , 1992
GAM42a	Gammaproteobacteria	1027-1043 ^b	GCC TTC CCA CAT CGT TT	35	80	Òî æå
SRB385Db	Deltaproteobacteria	385-402	CGG CGT TGC TGC GTC AGG	20	225	Rabus <i>et al</i> ., 1999
CF319a CFB560	Bacteroidetes (Cytophaga-Flavobacterium)	319–336 560–575	TGG TCC GTG TCT CAG TAC WCC CTT TAA ACC CAR T ^a	35 30	80 112	Manz <i>et al</i> ., 1996
HGC69a	Actinobacteria	1901–1918 ^b	TAT AGT TAC CAC CGC CGT	25	159	Roller et al., 1994
LGC354A, LGC354B, LGC354C ^d	Firmicutes	354—371	TGG GAA GAT TCC CTA CTG C, CGG GAA GAT TCC CTA CTG C, CCG GAA GAT TCC CTA CTG C	35	80	Meier <i>et al.</i> , 1999
HoAc1402	Acidobacteria	1402–1420	CTT TCG TGA TGT GAC GGG	10	450	Juretschko et al., 2002
Planctomycetes	Planctomycetes	886-906	GCC TTG CGA CCA TAC TCC C	30	112	Neef et al., 1998
Verrucomicrobia	Verrucomicrobia	138–155	CGA GCT ATT CCC CTC TTG	10	450	Dedysh et al., 2006

(a) Y = C or T, W = A or T, R = A or G; (b) 23S rRNA is the target molecule, (c) the probe was used in combination with an unlabeled oligonucleotide 5'-TATAGTGACGGCCGC-CGT-3, (d) equimolar mixture of three labeled oligonucleotides.)

MONITORING OF THE CHITINOLYTIC MICROBIAL COMPLEX



Fig. 1. Biomass of prokaryotes (a) and fungi (b) on the 10th day after the initiation of succession. 1, control; 2, samples with chitin.



Fig. 2. Biomass of the metabolically active unicellular eubacteria (1), filamentous eubacteria (2), archaea (3), and cells that did not hybridize with the probes (4) on the 10th day after the initiation of succession. I, *Picea abies*, control; II, *Picea abies*, samples with chitin; III, *Betula pendula*, control; IV, *Betula pendula*, samples with chitin.

duced on Chapek culture medium of the following composition: 0.5 g/l KCl, 0.5 g/l MgSO₄, 1 g/l KH₂PO₄, 1 g/l FeSO₄, 2 g/l NaNO₃, 20 g/l glucose, and 20 g/l agar (pH of the medium, 6.0-6.2).

The phylogenetic affiliation of the isolates was determined using methods of molecular biology— DNA isolation, target gene amplification by polymerase chain reaction (PCR), and PCR product sequencing. DNA was isolated from pure microorganism cultures using PowerSoil DNA Isolation Kit (MO-BIO, United States) according to the protocol recommended by the manufacturer. DNA solutions were stored at -18° C. Taxonomic assignment of prokaryotes was performed using the "full-length" primer pair for the 16S rRNA gene (11 forward 5'-GACGTTTGATCMTGGCTCAG-3', 1492 reverse 5'-TACGGYTACCTTGTTACGACTT-3' (Edwards et al., 1989, Burgmann et al., 2004). Taxonomic assignment of eukaryotes (filamentous fungi and yeasts) was performed using the primers for the D1 and D2 domains of large rRNA subunit (LSU; ITS1 forward 5'-GCTTCGCGTCATTTACGACGCGA-AGTA-3' and NL4 reverse 5'-GGGTCCGTGT-TTCAAGACGG-3' (Scorzetti et al., 2002)). Amplified DNA was stored at -18° C. PCR products were analyzed by electrophoresis in 2% agarose gel at a 6 V/cm intensity of electric field. Sequencing of the amplified rRNA genes was performed at the Bioengineering Center of the Russian Academy of Sciences on an automated capillary DNA sequencer (Silver Sequence d/ddNTP Mixes, Promega, United States).

RESULTS AND DISCUSSION

Analysis of the microbial biomass 10 days after the initiation of succession showed that the biomass of unicellular and mycelial prokaryotes was more abun-2 dant in chitin-supplemented samples than in control samples, while the biomass of the fungal mycelium in chitin-supplemented samples was considerably lower than in the control samples (Fig. 1). Stimulation of the chitinolytic microbial complex inhibiting growth of the fungal mycelium by chitin supplementation is the putative explanation for this observation (Feofilova, 2010). Notably, in similar experiments performed with other substrates (for example, with soil), both fungal and prokaryotic biomasses were more abundant in chitin-supplemented samples (Manucharova et al., 2005; Yaroslavtsev et al., 2009). This may be evidence of low chitinase resistance of the fungi present in the phylloplane and lower prevalence of chitinolytic fungi in this habitat.

Samples supplemented with chitin were characterized by a greater total biomass of metabolically active prokaryotes, as is evident from the results of the FISH assay, and by a higher ratio of these prokaryotes to the total prokaryotic biomass, this apparently being due to the transition of the dormant forms into a metabolically active state upon the introduction of an additional substrate. A significant difference between the phylloplanes of conifers and deciduous trees was detected: namely, the share of mycelial prokaryotes in 2 the former increased dramatically after chitin introduction, while the share of these prokaryotes in the latter was negligibly low (Fig. 2).

A set of probes allowing for estimation of the abundance of the representatives of the largest phyla of the domain Bacteria (Fig. 3) was used to assess the phylogenetic diversity of the metabolically active eubacterial complex.

Representatives of the phylum *Proteobacteria* were the most abundant in chitin-supplemented samples. Representatives of the phyla *Bacteroidetes* and *Verrucomicrobia* were also quite numerous. The proteobacteria *Verrucomicrobia* and *Planctomycetes* predominated in the control samples.

These data reveal obvious differences between the chitinolytic complexes from phylloplane and the soil: *Actinobacteria* and *Firmicutes* were the most abundant

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Fig. 3. The number of representatives of the phylogenetic groups of the domain Bacteria in control samples (1) and samples with chitin (P). 1, *Alphaproteobacteria*; 2 *Betaproteobacteria*; 3, *Gammaproteobacteria*; 4, *Deltaproteobacteria*; 5, *Actinobacteria*; 6, *Bacteroidetes*; 7, *Acidobacteria*; 8, *Firmicutes*; 9, *Verrucomicrobia*; 10, Planctomycetes.

in the latter, many of them being active soil hydrolytics (Manucharova et al., 2011), while proteobacteria, the dominant group in the native microbial complex of leaves, played a key role in the phylloplane. The same bacteria, together with *Bacteroidetes* and *Verrucomicrobia*, were the most numerous in the succession initiated by chitin introduction.

Chitinolytic microorganism isolates were obtained by cultivation on a solid nutrient medium with chitin as the sole source of carbon and nitrogen. Green leaves of six plant species—silver birch, apple-tree, sea buckthorn, common raspberry, stinging nettle, and garden strawberry—were sampled. Most of the prokaryotic strains isolated belonged to the class *Gammaproteobacteria*; in addition, representatives of the class *Alphaproteobacteria* and phyla *Actinobacteria* and *Firmicutes* were detected (Table 2). The absence of actinomycetes among the isolated strains is noteworthy, because these organisms are active destructors of chitin in the soil and rhizosphere and are often isolated from these substrates using the above-named method. Filamentous fungi of the genera *Mucor*, *Penicillium*, and *Cladosporium*, as well as askomycete yeast, were among the eukaryotes isolated using chitin-containing media (Table 2).

CONCLUSION

This study using microbial cultivation and methods of molecular biology allowed for the elucidation of some of the characteristic features of the composition and structure of the chitinolytic microbial complex from the phylloplane of various plants, as well as for the assessment of the contribution of different groups of microorganisms to this complex, and for the identification of a number of distinctions between the chitinolytic microbial complexes from the phylloplane of deciduous and coniferous plants, and from that of different deciduous plants. Gram-negative prokaryotes belonging to the group of proteobacteria were found to play a significant role in the chitinolytic microbial complex of the phylloplane. Gammaproteobacterial species

 Table 2. Chitinolytic microorganisms predominating in the phylloplane of the plants examined

Plant	Bacteria	Fungi and yeast
³ Apple tree <i>Malus domestica</i>	Clavibacter michiganensis (редко),@ Acinetobacter iohnsonii.	Cladosporium sp., Mucor circinelloides, Aureobasidium pullulans
4	Pseudomonas rhizosphaerae (обильно), @ P. graminis, Micrococcus luteus, Brevundimonas sp.,	ſ
Silver birch Betula pendula	Pseudomonas sp., Bacillus subtilis.	_
Garden strawberry Fragaria ananassa	Stenotrophomonas sp.	_
Common raspberry Rubus idaeus	P. rhizosphaerae, Rhizobium huautlense	Cladosporium sp., Sporobolomyces roseus
Sea buckthorn Hippophae rhamnoides	Pseudomonas sp.	Cladosporium sp., M. circinelloides, S. ruberrimus, Metschnikowia fructicola
Stinging nettle Urtica dioica	Pantoea sp.	Penicillium sp.

Note: "-" means strains were not isolated.

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were shown to predominate among the cultures of chitinolytic prokaryotes of the phylloplane. Actinobacteria and firmicutes, which actively degrade chitin in other habitats, were less numerous among the phylloplane chitinilytics. Chitinolytic eukaryotes were represented by askomycete yeast along with filamentous fungi.

A significant decrease in fungal biomass concomitantly with an increase in prokaryote biomass was detected in model experiments involving chitin supplementation, this probably being evidence of mycolytic activity of the phylloplane chitinolytics. Thus, the microbial population of the phylloplane forms a welldeveloped chitinolytic complex, which is different from the chitinolytic complexes of the soil and rhizosphere with regard to composition and is of a certain interest for biotechnology research as a tool against phytopathogenic fungi.

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SPELL: 1. phyllosphere, 2. mycelial, 3. редко, 4. обильно