ISSN 0026-2617, Microbiology, 2008, Vol. 77, No. 1, pp. 107–111. © Pleiades Publishing, Ltd., 2008.
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EXPERIMENTAL ARTICLES

Influence of *Lumbricus terrestris* Earthworms on the Structure of the Yeast Community of Forest Litter

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Received November 25, 2006; in final form, June 6, 2007

Abstract—The taxonomic structure of yeast communities was studied in forest litter and soil, as well as in substrates transformed by the activity of *Lumbricus terrestris* earthworms (leaves in heaps, the gut contents, and coproliths). The activity of *L. terrestris* has a weak effect on the total yeast abundance but results in substantial changes in the community taxonomic composition. The share of ascomycetous yeasts is significantly higher in the substrates associated with the activity of earthworms. The teleomorphic ascomycetes *Williopsis saturnus* were isolated from the gut contents. The effect of earthworms on the composition of the yeast community in the process of forest litter destruction is more pronounced than seasonal changes.

Key words: yeasts, soil invertebrates, microbial communities, forest litter, *Tilia cordata,* burrow worms.

DOI: 10.1134/S0026261708010153

The forms of interactions between invertebrate animals and yeast fungi are highly diverse [1]. Yeasts may be involved in symbiotic interactions with invertebrates and participate in their nutrition [2]. Associations of yeast with xylophages [3] or aggregates of endosymbiotic yeasts in the gut of diplopods [4] and other phyto- and saprophages [5] are some of the examples. Partial decomposition of plant material by invertebrates often results in the formation of substrates favorable for yeast development. This may be exemplified by the complex trophic interactions between yeasts and insects which utilize yeasts for feeding their larvae on decaying cactus tissue [6]. A high abundance of yeasts in the nests of leaf-cutting ants [7] and hill ants [8] was noted. The role of invertebrates, especially flying insects, in dissemination of yeasts [9–11], including the formation of successions on fermenting spring tree flux [12], is important.

Earthworms are among the animals whose vital activity plays a key role in the formation of microbial soil communities [13]. Litter and burrow worms predominantly feed on forest litter. Along with leaves, the earthworms also consume the yeasts present on their surface. Passing through the digestive tract, the yeasts may be digested, inactivated, or cast off, thus ending up in soil. Thus, the worms may take part in the formation of the soil yeast community.

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The aim of this investigation was to study the influence of *Lumbricus terrestris* activity on the development of the yeast community in litter and soil.

MATERIALS AND METHODS

The earthworm *Lumbricus terrestris* L. belonging to burrow worms was selected as the subject of the study. These worms live permanently in burrows, which attain considerable depths, and feed on the tree litter, which they arrange in piles above the burrow mouth. The studies were conducted in a tillet (*Tilia cordata* Mill.) forest with sedge on medium-soddy–medium-podzolic soil in the vicinity of the Malinki Biological Station, Severtsov Institute of Problems of Ecology and Evolution, Russian Academy of Sciences, (Moscow oblast, Narofominskii raion)

The substrates studied. To study the influence of the activity of burrow earthworms on the yeast community composition, the following substrates were analyzed: (a) those that were not subjected to the influence of worms (linden green leaves, linden waste, and the upper mineral horizon A1 down to a depth of 10 cm), and (b) the substrates transformed as a result of the activity of the earthworms (linden litter from the burrow mouths, the gut contents, and the coproliths (excrement) of the worms). These two sets of substrates may be regarded as a spatial–successional series, in which change of the initial substrate (green leaves) is affected

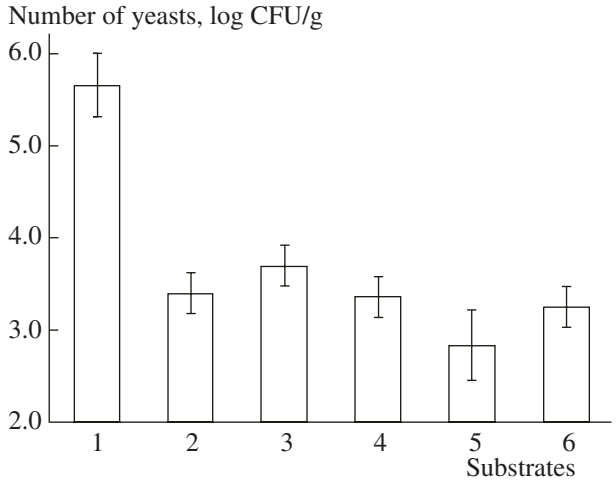

Fig. 1. The number of yeasts in different substrates: (1) leaves; (2) litter; (3) soil; (4) leaves from the burrow mouths; (5) gut contents; (*6*) coproliths.

by *L. terrestris* earthworms or does not involve their activity.

The experimental material was sampled during the autumn–winter period, in the middle of every month from September through December 2001, and in spring, in the middle of March 2002. The material was sampled into sterile bags, delivered to the laboratory, and analyzed immediately. In order to obtain fresh coproliths, the worms were removed from soil, washed with sterile water, and placed in sterile petri dishes for 24 h. Before removing the gut contents, the worms were washed several times with sterile water and immobilized by cooling to 0°C. The gut contents were removed in two ways. Some of the worms were dissected under sterile conditions using a dissection microscope. After dissecting the integument, the coelomic fluid was washed off with water. The gut was then dissected, and the contents of the posterior division were removed and plated on acidified wort agar as a concentrated suspension. In other cases, the gut contents were extracted by squeezing them through the anus. In the latter case, the penetration of coelomic fluid into the gut cavity through the gut wall ruptures cannot be ruled out. Coelomic fluid was reported to inhibit the growth of yeast cells [14]; however, we did not find a significant effect of the way of removing the gut contents on the abundance or the taxonomic composition of the yeasts.

Methods of yeast isolation and identification. Before inoculation, all the samples were vortex-treated. Dilutions used for different substrates varied from 1 : 50 (leaves and litter) to 1 : 5 or 1 : 10 (soil, coproliths, and gut contents).

Malt agar acidified with lactic acid (4 ml/l; pH 4–4.5) in order to inhibit bacterial growth was used for inoculations. To inhibit the growth of mycelial fungi, part of the inoculated media was incubated at about 5°C for two weeks. Using a dissection microscope, the grown yeast colonies were subdivided into different types according to their macromorphological characteristics. Several colonies of each type were isolated in pure culture and further identified on the basis of their morphological and physiological characteristics according to the keys [15–17]. Additional tests for assimilation of aromatic compounds proposed for yeast identification according to recent taxonomic studies were used [18, 19]. These characteristics allow differentiation of anamorphous dimorphic basidiomycetes at the level of orders, clades, and groups of closely related species [16, 19]. The relative abundance of the identified taxa was expressed as a share (%) of the total yeast number.

Statistical data processing. To assess the influence of earthworms on yeasts, the total abundance and the taxonomic composition of yeasts in the substrates connected with earthworm activity (leaves in the burrow mouths, coproliths) and in the intact substrates (litter, soil) were compared in sampling different periods (September, October, November, December, and March). For this purpose, the two-factor analysis of variance (ANOVA) and the two-factor multivariant analysis of variance (MANOVA) were used, with time (the time of sampling) and substrate (control substrates and substrates modified by the activity of the worms) treated as factors. For calculations, the Statistica 6.0 software package (StatSoft Inc., United States) was used.

RESULTS AND DISCUSSION

The greatest abundance (1.6×10^6 CFU/g) and diversity (10 species) of yeasts was noted on green leaves. On other substrates, the number was significantly lower ($p < 0.001$) and did not exceed 7×10^4 CFU/g (Fig. 1). The substrates related to earthworm activity did not differ from other substrates in the total abundance of yeasts. This runs counter to earlier obtained data on the increase in the number of yeasts in habitats related to the activity of soil invertebrates [5]. No significant influence of the sampling time on the total abundance of yeasts was established either.

The isolated yeasts were assigned to the following species: the ascomycetous *Aureobasidium pullulans*, *Blastobotrys* sp.; *Lalaria* sp. (related to *Taphrina belutina*); *Pichia anomalia*; *Pichia stipitis*/*Pichia segobiensis* [20]; and *Williopsis saturnus*, as well as the basidiomycetous *Cryptococcus* aff. *magnus* [16]; *Cryptococcus* aff. *victoriae* [21]; *Cryptococcus terricola; Rhodotorula* aff. *minuta* [22]; *Rhodotorula glutinis* sensu lato [23]; *Sporobolomyces roseus*; and *Trichosporon gracile* [17]. These taxa include both the species that are reliably differentiated on the basis of their morphological and physiological characteristics at the level of homogeneous phylogenetic species [24] and the "large" species representing complex groups of closely related sister species, which cannot be reliably differen-

tiated without molecular genetic methods of identification. For the latter, references are provided to the relevant publications, in which their phylogenetic structure is considered.

By means of analysis of the taxonomic composition, the species associated with certain habitats and the groups of habitats with similar taxonomic composition of the yeasts were determined (Fig. 2). The first group is green leaves and litter. The similarity of the structure of the yeast communities in these substrates is quite understandable, because the leaves in the litter are only at the initial stage of decomposition. The second group is the gut contents and coproliths. The relationship between these substrates is determined by their common origin. The third group is soil and the litter from the mouth of the worm burrows. The similarity of these substrates is probably caused by the mixing of plant waste and mineral soil at the burrow mouth. The substrates unrelated to the activity of earthworms were colonized by yeast species which are often isolated from similar habitats [25]. Widespread eurybiont yeasts related to the species *Cryptococcus magnus* were isolated from all the substrates.

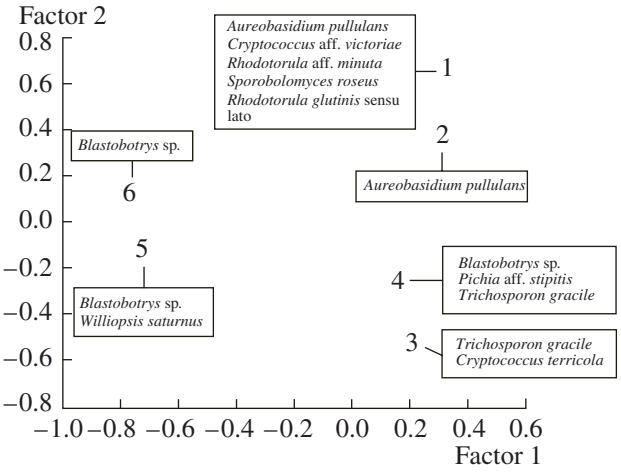

Fig. 2. Ordination using the principal components method of different substrates (see Fig. 1 for designations) by similarity of the taxonomic composition of the yeast communities (the first two factors describe 83% of the total data variance); the boxes indicate the yeast species predominating on each substrate.

Typical phytobionts dominated on the surface of green leaves: the representatives of the group of black yeasts, *Rh.* aff. *minuta*, and *Tremellales* and *Filobasidiales* anamorphous yeasts of the genus *Cryptococus*. The banal soil species *Cr. terricola* dominated in the upper organic horizons of the soil, and *Tr. gracile* was also isolated from the litter layer.

These findings agree with the results of the study of similar substrates in the same region conducted by us earlier, which revealed the dominance on the surface of freshly-fallen leaves of tremellous (*Cr. victoriae, Cr. tephrensis*) and filobasidious cryptococci (*Cr. wieringae, Cr. magnus*) and the red yeast *Rh.* aff. *minuta*, which was assigned to the closely related species *Rh. pinicola* according to the results of PCR analysis of DNA with the microsatellite primer $(GAC)_5$ [25].

Seasonal changes in the taxonomic composition of the yeasts in the substrates studied were sufficiently pronounced (multivariant analysis of variance: $F = 7.5$, $p < 0.001$); however, the influence of the activity of earthworms was stronger ($F = 32.5$, $p < 0.001$). A characteristic feature of the seasonal changes in the composition of the yeast community, both in the intact litter and on leaves collected by the worms in the burrow mouths, was a decrease in species diversity in the course of destruction of plant residues. In spring, the typical phytobionts, the anamorphous fungi of the genus *Cryptococcus* related to *Cr. magnus* (more than 80% of isolates) and *Rh.* aff. *minuta*, dominated in the litter. The seasonal variation of relative abundance was determined for phytobiontic species but not for the yeasts that depend on the earthworm activity (table). In *L. terrestris* laaf heaps, the share of ascomycetous yeast fungi was higher (*Blastobotrys* sp., 20%; *P.*aff. *stipitis*, 40%). The abundance of these two species significantly depended only on the activity of the earthworms. The first species is a mycelial yeast-like fungus; the other belongs to the compact and rather specific group of yeasts capable of fermenting xylose and represented by the species *P. segobiensis*, *P. stipitis*, and *Candida shehatae* [26]. The first two species are difficult to identify exactly, since it is not possible to reliably differentiate them by RFLP analysis of the 5.8S-ITS region [27]; they differ from each other in only 1–2 nucleotide substitutions in the D1D2 region of a large ribosomal subunit [20, 28]. The most important distinction of the strains isolated in this study from *Candida shehatae* was the presence of ascospores in experimental cultures. An interesting finding is the frequent isolation of the teleomorphic species *Williopsis saturnus* from the gut of *L. terrestris*; this is a rare species of soil yeast. We did not reveal this yeast in other substrates.

The relative abundance of the ascomycetous species was significantly higher ($p < 0.001$) in all the substrates

Influence of the activity of *L. terrestris* earthworms (the substrate factor) and the sampling time (the time factor) on the relative abundance of yeasts: the results of the two-factor multivariant analysis of variance

Species	Substrate factor		Time factor	
	F	*p*	*F*	*p*
Cryptococcus aff. *magnus*	31.06	<0.001	11.09	<0.001
Blastobotrys sp.	28.94	0.023	3.16	0.035
Rhodotorula aff. *minuta*	13.21	<0.001	16.95	<0.001
Pichia aff. *stipitis*	47.55	<0.001	4.74	0.006
Trichosporon gracile	2.19	0.15	3.36	0.028

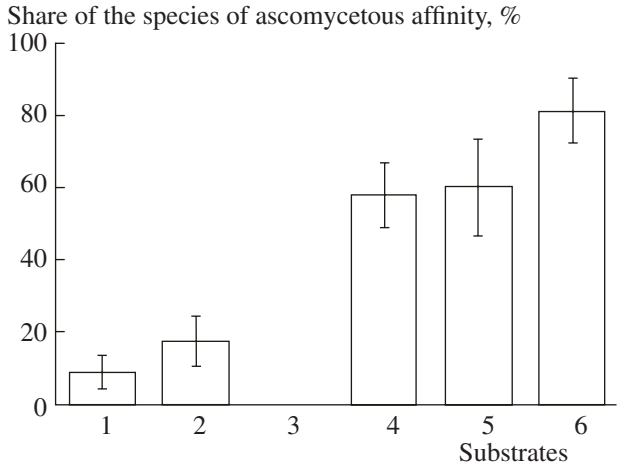

Fig. 3. The share of the ascomycete yeast species on different substrates (see Fig. 1 for designations).

connected with the earthworm activity (Fig. 3). The predominant association of ascomycetous yeasts with invertebrates has been noted earlier [1, 5], but it is the first time it has been reported for the earthworms. Most yeast species predominant in the litter were not revealed in the earthworm gut; they are probably lysed during the digestive process. However, the species *W. saturnus* seems to be resistant to the lysing action of the gut fluid and may prove to be the constant or facultative symbiont of *L. terrestris*.

We did not succeed in revealing substantial differences in the taxonomic composition of mycelial soil fungi in the intact tree litter and in *L. terrestris* laaf heaps [29]. Thus, the earthworms have an effect on different groups of soil micromycetes.

Thus, we did not find any substantial changes in the total number of yeasts in the habitats related to the activity of the *L. terrestris* earthworms. However, the activity of the worms results in significant changes in the taxonomic composition of the yeast fungi of forest litter. The species dominating in plant waste probably become lysed during the digestive process and are therefore not revealed in coproliths. It is clear that the development of some ascomycetous yeast species is stimulated by the activity of the earthworms. For these species, the relationship between abundance and the sampling time is less pronounced, which confirms their strong connection with a specific habitat created by earthworms.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research, project nos. 04-04-48713 and 05-04-48429) and the program of the Presidium of the Russian Academy of Sciences "Gene Pool Biodiversity and Dynamics."

REFERENCES

1. Blackwell, M. and Jones, K., Taxonomic Diversity and Interactions of Insect-Associated Ascomycetes, *Biodivers. Conservat.*, 1997, vol. 6, no. 5, pp. 689–699.
2. Suh, S.O., Noda, H., and Blackwell, M., Insect Symbiosis: Derivation of Yeast-Like Endosymbionts within an Entomopathogenic Filamentous Lineage, *Mol. Biol. Evol.*, 2001, vol. 18, no. (6), pp. 995–1000.
3. Phaff, H.J and Starmer, W.T, Yeasts Associated with Plants, Insects, and Soil, in *The Yeasts*, Rose, A.H. and Harrison, J.S., Eds., London: Academic, 1987.
4. Byzov, B.A., Vu Nguen Thanh, Bab'Eva I.P. Interrelationships between Yeasts and Soil Diplopods, *Soil Biol. Biochem.*, 1993, vol. 25, no. 8, pp. 1119–1126.
5. Terenina, E.E. and Chernov, I.Yu., Taxonomic Structure of the Yeast Communities Associated with Invertebrates, *Mikol. Fitopatol.*, 2001, no. 4, pp. 65–73.
6. Starmer, W.T., Lachance, M.A., Phaff, H.J., and Heed, W.B., The Biogeography of Yeasts Associated with Decaying Cactus Tissue in North America, the Caribbean and Northern Venezuela, *Evol. Biol.*, 1990, vol. 24, pp. 253–296.
7. Carreiro, S.C., Pagnocca, F.C., Bueno, O.C., Bacci, M.J., Aparecida Hebling, M.J., and Silva, O.A., Yeasts Associated with Nests of the Leaf-Cutting Ant *Atta sexdens rubropilosa* Forel, 1908, *Antonie van Leeuwenhoek*, 1997, vol. 71, pp. 243–248.
8. Golubev, V.I. and Bab'eva, I.P., The Yeasts *Debaryomyces* Klock. in the Nests of *Formica rufa* L. Ants, *Ekologiya*, 1972, no. 1, pp. 78–82.
9. Lachance, M.A., Starmer, W.T., Rosa, C.A., Bowles, J.M., Barker, J.S.F., and Janzen, D.H., Biogeography of the Yeasts of Ephemeral Flowers and Their Insects, *FEMS Yeast Res.*, 2001, vol. 1, no. 1, pp. 1–8.
10. Rosa, C.A., Lachance, M.A., Silva, J.O.C., Teixeira, A.C.P., Marini, M.M., Antonini, Y., Rogerio, P., and Martins, R.P., Yeast Communities Associated with Stingless Bees, *FEMS Yeast Res.*, 2003, vol. 4, pp. 271–275.
11. Brysch-Herzberg, M., Ecology of Yeasts in Plant-Bumblebee Mutualism in Central Europe, *FEMS Microbiol. Ecol.*, 2004, vol. 50, pp. 87–100.
12. Golubev, V.I., Bab'eva, I.P., and Novik, S.N., Yeast Succession in Fluxes of Birch Sap, *Ekologiya*, 1977, no. 5, pp. 23–28.
13. Edwards, C.A. and Fletcher, K.E., Interactions between Earthworms and Microorganisms in Organic Matter Breakdown, *Agricult. Ecosyst. Environm.*, 1988, vol. 24, pp. 235–247.
14. Vu Nguyen Thanh and Byzov, B.A., The Effect of Coelomic Fluid of the Earthworm *Eisenia fetida* on Yeast Growth, *Soil Organisms and Soil Health. Abstr. XI Intern. Coll. on Soil Zoology. Juvaskyla*, 1992, p. 91.
15. *The Yeasts, a Taxonomic Study. Fourth Revised and Enlarged Edition*, Kurtzman, C.P. and Fell, J.W. Eds, Amsterdam: Elsevier Science B.V, 1998.
16. Fonseca, A., Scorzetti, G., and Fell, J.W., Diversity in the Yeast *Cryptococcus albidus* and Related Species as Revealed by Ribosomal DNA Sequence Analysis, *Can. J. Microbiol.,* 2000, vol. 46, pp. 7–27.
17. Middelhoven, W.J., *Trichosporon wieringae* sp. nov., an Anamorphic Basidiomycetous Yeast from Soil, and

Assimilation of Some Phenolic Compounds, Polysaccharides and Other Non-Conventional Carbon Sources by Saprophytic *Trichosporon* Species, *Antonie van Leeuwenhoek*, 2004, vol. 86, pp. 329–337.

18. Fonseca, A., Utilization of Tartaric Acid and Related Compounds by Yeasts: Taxonomic Implications, *Can. J. Microbiol.*, 1992, vol. 38, pp. 1242–1251.
19. Sampaio, J.P., Utilization of Low Molecular Weight Aromatic Compounds by Heterobasidiomycetous Yeasts: Taxonomic Implications, *Can. J. Microbiol.*, 1999, vol. 45, pp. 491–512.
20. Kurtzman, C.P., *Candida shehatae*–Genetic Diversity and Phylogenetic Relationships with Other Xylose-Fermenting Yeasts, *Antonie van Leeuwenhoek*, 1990, vol. 57, pp. 215–222.
21. Takashima, M., Sugita, T., Shinoda, T., and Nakase, T., Three New Combinations from the *Cryptococcus laurentii* Complex: *Cryptococcus aureus, Cryptococcus carnescens* and *Cryptococcus peneaus, Int. J. Sys. Evol. Microbiol.*, 2003, vol. 53, pp. 1187–1194.
22. Sampaio, J.P., Diversity, Phylogeny and Classification of Basidiomycetous Yeasts, *Frontiers* in *Basidiomycote Mycology Eds. Agerer A., Blanz M., Piepenbring M. IHW-Verlag*, 2004, pp. 49–80.
23. Gadanho, M. and Sampaio, J.P., Polyphasic Taxonomy of the Basidiomycetous Yeast Genus *Rhodotorula: Rh. glutinis* sensu stricto and *Rh. dairenensis* comb. nov, *FEMS Yeast Res*, 2002, vol. 2, pp. 47–58.
24. Kurtzman, C.P. and Robnett, C.J., Identification and Phylogeny of Ascomycetous Yeasts from Analysis of Nuclear Large Subunit (26S) Ribosomal DNA Partial Sequences, *Antonie van Leeuwenhoek*, 1998, vol. 73, pp. 331–371.
25. Yurkov, A.M., Comparative Analysis of Yeast Communities in Birch Forests of the European Part of Russia and Western Siberia, *Cand. Sci. (Biol.) Dissertation*, Moscow: Mosk. Gos. Univ., 2006.
26. Suh, S.O., Marshall, C.J., McHugh, J.V., and Blackwell, M., Wood Ingestion by Passalid Beetles in the Presence of Xylose-Fermenting Gut Yeasts, *Mol. Ecol.*, 2003, vol. 12, pp. 3137–3145.
27. Esteve-Zarzoso, B., Belloch, C., Uruburu, F., and Querol, A., Identification of Yeasts by RFLP Analysis of the 5.8S RRNA Gene and the Two Ribosomal Internal Transcribed Spacers, *Int. J. Sys. Bacteriol.*, 1999, vol. 49, pp. 329–337.
28. Kurtzman, C.P. and Robnett, C.J., Identification of Clinically Important Ascomycetous Yeasts Based on Nucleotide Divergence in the 5' End of the Large-Subunit (26S) Ribosomal DNA Gene, *J. Clin. Microbiol.*, 1997, vol. 35, no. 5, pp. 1216–1223.
29. Orazova, M.K., Semenova, T.A., and Tiunov, A.V., The Microfungal Community of *Lumbricus terrestris* Middens in a Linden (*Tilia cordata*) Forest, *Pedobiologia*, 2003, vol. 47, pp. 27–32.