ISSN 0026-2617, Microbiology, 2015, Vol. 84, No. 5, pp. 722–725. © Pleiades Publishing, Ltd., 2015.
Original Russian Text © A.M. Glushakova, A.V. Kachalkin, T.M. Zheltikova, I.Yu. Chernov, 2015, published in Mikrobiologiya, 2015, Vol. 84, No. 5, pp. 612–615.

SHORT COMMUNICATIONS

Yeasts Associated with Wind-Pollinated Plants—Leading Pollen Allergens in Central Russia

A. M. Glushakova[a], A. V. Kachalkin[a], T. M. Zheltikova[b], and I. Yu. Chernov[a, c, 1]

[a] *Lomonosov Moscow State University, Moscow, Russia*

[b] *Research Institute of Vaccines and Sera, Russian Academy of Medical Sciences, Moscow, Russia*

[c] *Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow, Russia*

Received November 11, 2014

DOI: 10.1134/S0026261715050082

In recent years, numerous studies have dealt with the investigation of the phyllosphere and associated above-ground plant substrates. They are both of nonrecurrent and monitoring nature and relate to the taxonomic and ecological features of epiphytic and endophytic yeast communities of leaves, flowers, and fruits (Glushakova and Chernov, 2010; Glushakova et al., 2013; Isaeva et al., 2010; Herrera et al., 2009; Herzberg et al., 2002; Jindamorakot et al., 2008; Lachance et al., 1998; Lachance et al., 1999; Manson et al., 2007; Rosa et al., 1999; Rosa et al, 2007; Rosa et al., 2009; Saluja and Prasad, 2007; Sipiczki, 2010; Wang et al., 2008). However, the yeast population of some plant substrates is insufficiently studied. This is especially true for such above-ground plant substrates as tree bark, as well as pollen of wind-pollinated plants. It has been shown that typical epiphytic and euribiontic basidiomycete species of yeasts develop on the bark (Golubev and Mojilevskaya, 1982). However, these studies were carried out relatively long ago and were of nonrecurrent character. Moreover, recent changes in the approach to identification of yeast microorganisms require additional studies of this plant substrate.

The importance of analysis of microbial communities present on pollen of wind-pollinated plants stems from the fact that it contains allergens. Sensibilization to these allergens in individuals with a genetic predisposition to atopy may cause development and manifestation of various allergies (allergic rhinitis, atopic dermatitis, etc.). Association of pollen with yeasts which sometimes are allergens themselves can provoke the development of polysensibilization. This remarkably complicates the course of the disease. Moreover, fungi can act as adjuvants amplifying the effect of the pollen agents (Puc, 2003).

Thereby, it is important to know the taxonomic structure and abundance of yeast communities associated with pollen of wind-pollinated plants—the leading pollen allergens in Central Russia—as well as of those of the plant substrates associated with the pollen, especially leaves and bark, and to assess their contribution to formation of specific pollen communities.

The studies were carried out in 2012. Pollen and leaves of the following wind-pollinated plants were analyzed: *Betula pendula* Roth, *Corylus avellana* L., *Alnus glutinosa* L. (Gaertn)., *Dactylis glomerata* L., and *Phleum pratense* L., as well as the bark of *Betula pendula, Corylus avellana*, and *Alnus glutinosa*. The subjects of study were chosen due to the fact that they are considered the main pollen allergens in Central Russia.

The pollen was collected in two main types of habitats: in the city of Moscow (Yaroslavskoe highway) and in the forest zone (Dubovaya Rosha, Mytishchi district of Moscow oblast). The distance between trees in the city was about 10 m while in the forest trees formed a dense tangle. *Dactylis* and *Phleum* in the forest zone formed dense bushes, while in the city they grew at a considerable distance from each other forming small clusters.

During the period of study, 319 samples were analyzed. The pollen was collected based on method for allergen production. "Bunches" of branches with catkins (amentums)/flowers were put in water and placed in laminar flow box under sterile conditions. In the course of blooming, the pollen fell down on the sterile paper. Samples of the leaves and bark were analyzed immediately after the sampling. The plant material was homogenized, and weighed portions were transferred into test tubes and filled with sterile water to obtain 1 : 20 dilution for the bark and 1 : 50 for the leaves. Samples of the pollen in test tubes were supplemented with sterile water to obtain 1 : 10 dilution. The suspensions were treated on a Vortex («MultiReax, Heidolph», Germany) for 15 min. The samples were plated in three replicates on glucose–peptone–yeast extract medium (glucose, 20 g/L; peptone, 10 g/L; yeast extract, 5 g/L; agar 10 g/L) with addition of levomycetin (500 mg/L) to prevent bacterial growth. The

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

Frequency of occurrence of yeast species on leaves, bark, and pollen of the studied plants, %

Species	Pollen	Leaves	Bark
Candida albicans	20.1	0	0
Candida glabrata	16.6	0	0
Candida maltosa	19	0	0
Candida oleophila	43	27.8	6
Candida parapsilosis	14.5	0	0
Candida pimensis	12.8	0	0
Candida sake	20.7	2.3	0
Candida tropicalis	8.4	1.2	0
Candida zeylanoides	42	4.5	0
Cryptococcus magnus	83.2	66.7	18
Cryptococcus tephrensis	1.7	32.2	14
Cryptococcus victoriae	59.2	57.8	48
Cryptococcus wieringae	1.7	37.8	0
Debaryomyces hansenii	33.5	12.3	0
Metschnikowia pulcherrima	18.4	31.1	4
Meyerozyma guilliermondii	25.7	13.4	0
Ogataea cecidiorum	10.6	0	0
Rhodotorula mucilaginosa	95	95.6	34
Wickerhamomyces anomalus	21.8	5.6	0
Yamadazyma friedrichii	28.5	3.4	0

plates were incubated at room temperature for 5–7 days. The yeast colonies were grouped into morphological types with the help of binocular magnifier, and the number of colonies of each type was counted. Representatives of each colony type were isolated in pure cultures. Yeast cultures were identified according to morphological and physiological characteristics (The Yeasts..., 2011) and with the help of analysis of nucleotide sequences of D1/D2 region of 26S (LSU) rDNA.

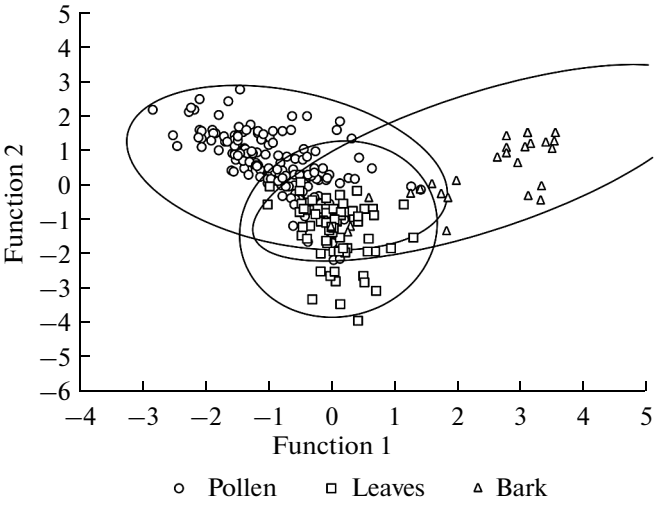
DNA isolation was carried out as follows: biomass of a 3–4-day culture (2 loopfuls) was transferred into 2-mL Eppendorf tubes and supplemented with 400 μL of glass beads (300–500 μm in diameter) and 500 μL lyzing buffer (TrisBase, 50 mM; NaCl, 250 mM; EDTA, 50 mM; SDS, 0.3%; pH 8). The mixture was shaken on a Vortex at a maximum speed for 15 min, incubated for 1 h at 65°C, repeatedly vortexed for 15 min, and centrifuged (13400 rpm) for 10 min. Then supernatant was collected.

For amplification of the rDNA region containing the D1/D2 domain of 26S rDNA region, primers ITS1f (5'-CTTGGTCATTTAGAGGAAGTA) and NL4 (5'-GGTCCGTGTTTCAAGACGG) and PCR mixtures ScreenMix (Evrogen, Moscow, Russia) were used. Amplification was carried out according to the following scheme: initial denaturation, 2 min at 96°C; then 35 cycles: denaturation, 20 s at 96°C; annealing, 50 s at 52°C; DNA synthesis, 1.5 min at 72°C; final elongation, 7 min at 72°C. Purification of the PCR product was carried out using BigDye XTerminator Purification Kit (Applied Biosystems, United States). For sequencing, the NL4 primer was used. DNA sequencing was carried out using the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, United States) with subsequent analysis of the reaction products on an Applied Biosystems 3130xl Genetic Analyzer sequencer (United States) at Syntol Co. (Moscow, Russia). Identification of obtained chromatograms was performed using the NCBI GenBank database (ncbi.nlm.nih.gov) and the CBS database (www.cbs.knaw.nl).

For each sample, the total amount of yeasts and the relative abundance of each species were determined. For yeasts species, the frequency of occurrence was calculated.

Yeast fungi were found in all studied plant samples. Their abundance varied from 10^2 to 10^4 CFU/g, with the minimal numbers (2.8×10^2 CFU/g on average) characterizing the tree bark samples. The average values of yeast abundance on leaves and pollen were about 3.7×10^3 CFU/g.

Throughout the period of the study, 20 yeast species were isolated from phyllosphere, pollen, and bark (table). The highest number of species (20) was found on pollen, while slightly less was found on leaves (14 species). The least number of yeast species was iso-



Ordination of the samples of the studied substrates based on the taxonomic structure of the yeast population by the method of discriminant analysis.

lated from the birch, alder, and hazel bark. Only 6 species of yeasts, mostly of epiphytic and euribiotic basidiomycete and ascomycete species, were isolated from these habitats. During all of the studied period, *Rhodotorula mucilaginosa* was the dominant species on all plant substrates. This is one of the most widely spread phytobiotic yeast species, regularly isolated from different plant substrates, especially from leaves (Glushkova and Chernov, 2010). However it is regularly found in various other habitats, such as soil, food products, or dust. This species is adapted to conditions of low water activity (Glushakova et al., 2004; Glushakova et al., 2015). The group of dominant and subdominant species also included *Cryptococcus magnus, Cryptococcus victoriae*, and *Candida oleophila* (on leaves and pollen). The first two species are considered to be obligate components of most epiphytic yeast complexes. Permanently high level of occurrence of *Candida oleophila* on pollen was discovered in our previous monitor long-term study of yeast population of pollen grains (Glushakova et al., 2013). This species is known to be the dominant one in epiphytic yeast communities on above-ground plant parts. Relative abundance of this species is the highest on plant fruits and increases significantly at the end of the vegetative period (Glushakova et al., 2007).

However, together with the banal euribiont species, members of pathogenic and opportunistic ascomycete yeasts of the genus *Candida* were found on studied substrates. The latter were isolated only from the pollen collected in the anthropogenic (city) zone. Thus, the leading carriers of the pollen allergens appear to be the "carriers" of the yeast microflora potentially dangerous for humans. In general, the frequency of occurrence of ascomycete yeasts that did not belong to the group of pathogens and opportunists was higher on the pollen than on the leaves of the studied plants (the only exception was *Metschnikowia pulcherrima*).

Ordination of the samples of pollen, leaves, and bark of all studied plants according to the taxonomic structure of the yeast population by discriminant analysis revealed insignificant differences in the species structure of the yeast population of pollen and leaves. Bark samples differ from other studied substrates due to very low species diversity of the copiotrophic community that develops on the tree bark (figure). Pollen and phyllosphere form conjugate yeast communities that differ mainly in the ratio of species.

Simultaneous study of conjugate plant substrates made it possible to reveal the ecological patterns in development of the group in general and of individual species. Detection of the patterns of development of yeast species in nature and their dependence on the properties of the substrate and micro- and macroclimatic conditions makes it possible to come closer to answering the questions connected with the autecological characteristics of particular yeast species in relation to both typical and atypical habitats. Studying of yeast population closely associated with pollen is very important for medical science. Yeasts associated with pollen can increase the allergenic effects of each other, leading to a more complicated course of allergies.

This research was partially supported by the Russian Foundation for Basic Research, project no. 13-04-00822. Yeast strains identification was sponsored by the Russian Science Foundation, project no. 14-50-00029.

REFERENCES

Glushakova, A.M., Zheltikova, T.M., and Chernov, I.Yu., Groups and sources of yeasts in house dust, *Microbiology* (Moscow), 2004, vol. 73, no. 1, pp. 94–98.

Glushakova, A.M., Zheltikova, T.M., and Chernov, I.Yu., Yeasts associated with the pollen of anemophilic plants, *Mikol. Fitopatol.*, 2013, vol. 7, no. 5, pp. 294–299.

Glushakova, A.M., Kachalkin, A.V., Zheltikova, T.M., and Chernov, I.Yu., Resistance of various yeast ecological groups to prolonged storage in dry state, *Microbiology* (Moscow), 2015, vol. 84, no. 3, pp. 442–448.

Glushakova, A.M. and Chernov, I.Yu., Seasonal dynamics of the structure of epiphytic yeast communities, *Microbiology* (Moscow), 2010, vol. 79, no. 6, pp. 830–839.

Glushakova, A.M., Yurkov, A.M., and Chernov, I.Yu., Massive isolation of anamorphous ascomycete yeasts *Candida oleophila* from plant phyllosphere, *Microbiology* (Moscow), 2007, vol. 76, no. 6, pp. 799–803.

Golubev, W.I. and Mojilevskaya, L.P., Yeasts associated with bark, in *Microorganisms as Biogeocenosis Component*, Alma-Ata: KazGU, 1982, pp. 83–84.

Herrera, C.M., de Vega, C., Canto, A., and Pozo, M.I., Yeasts in floral nectar: a quantitative survey, *Ann. Bot.*, 2009, vol. 103, no. 9, pp. 1415–1423.

Herzberg, M., Fischer, R., and Titze, A., Conflicting results obtained by RAPD-PCR and large-subunit rDNA

sequences in determining and comparing yeast strains isolated from flowers: a comparison of two methods, *Int. J. Syst. Evol. Microbiol.*, 2002, vol. 52, pp. 1423–1433.

Isaeva, O.V., Glushakova, A.M., Garbuz, S.A., Kachalkin, A.V., and Chernov, I.Yu., Endophytic yeast fungi in plant storage tissues, *Biol. Bull.*, 2010, vol. 37, no. 1, pp. 26–34.

Jindamorakot, S., Limtong, S., Yongmanitchai, W., Tuntirungkij, M., Potacharoen, W., Kawasaki, H., Tanticharoen, M., and Nakase, T., *Candida ratchasimensis* sp. nov. and *Candida khaoyaiensis* sp. nov., two anamorphic yeast species isolated from flowers in Thailand, *FEMS Yeast Res.*, 2008, vol. 8, no. 6, pp. 955–960.

Lachance, M.A., Bowles, J.M., Starmer, W.T., and Barker, J.S., *Kodamaea kakaduensis* and *Candida tolerans*, two new ascomycetous yeast species from Australian hibiscus flowers, *Can. J. Microbiol.*, 1999, vol. 45, no. 2, pp. 172–177.

Lachance,M.A., Rosa, C.A., Starmer, W.T., Schlag-Edler, B., Barker, J.S., and Bowles, J.M., *Wickerhamiella australiensis, Wickerhamiella cacticola, Wickerhamiella occidentalis, Candida drosophilae* and *Candida lipophila*, five new related yeast species from flowers and associated insects, *Int. J. Syst.Bacteriol.*, 1998, no. 48, pp. 1431–1443.

Manson, J.S., Lachance, M.A., and Thomson, J.D., *Candida gelsemii* sp. nov., a yeast of the Metschnikowiaceae clade isolated from nectar of the poisonous Carolina jessamine, *Antonie Van Leeuwenhoek,* 2007, vol. 92, no. 1, pp. 37–42.

Puc, M., Characterization of pollen allergens, *Ann. Agr. Environ. Med.*, 2003, no. 10, pp. 143–149.

Rosa, C.A., Lachance, M.A., Starmer, W.T., Barker, J.S., Bowles, J.M., and Schlag-Edler, B., *Kodamaea nitidulidarum, Candida restingae* and *Kodamaea anthophila*, three new related yeast species from ephemeral flowers, *Int. J. Syst. Bacteriol.*, 1999, no. 49, pp. 309–318.

Rosa, C.A., Morais, P.B., Lachance, M.A., Santos, R.O., Melo, W.G., Viana, R.H., Bragança, M.A., and Pimenta, R.S., *Wickerhamomyces queroliae* sp. nov. and *Candida jalapaonensis* sp. nov., two yeast species isolated from Cerrado ecosystem in North Brazil, *Int. J. Syst. Evol. Microbiol.*, 2009, no. 59, pp. 1232–1236.

Rosa, C.A., Pagnocca, F.C., Lachance, M.A., Ruivo, C.C., Medeiros, A.O., Pimentel, M.R., Fontenelle, J.C., and Martins, R.P., *Candida flosculorum* sp. nov. and *Candida floris* sp. nov., two yeast species associated with tropical flowers, *Int. J. Syst. Evol. Microbiol.,* 2007, no. 57, pp. 2970–2974.

Saluja, P. and Prasad, G.S., *Cryptococcus rajasthanensis* sp. nov., an anamorphic yeast species related to *Cryptococcus laurentii*, isolated from Rajasthan, India, *Int. J. Syst. Evol. Microbiol.*, 2007, no. 57, pp. 414–418.

Sipiczki, M., *Candida stigmatis* sp. nov., a new anamorphic yeast species isolated from flowers, *FEMS. Yeast. Res.*, 2010, vol. 10, no. 3, pp. 362–365.

The Yeasts: A Taxonomic Study, 5th ed., Kurtzman, C.P., Fell, J.W., and Boekhout, T., Elsevier, 2011.

Wang, S.A., Jia, J.H., and Bai, F.Y., *Candida alocasiicola* sp. nov., *Candida hainanensis* sp. nov., *Candida heveicola* sp. nov. and *Candida musiphila* sp. nov., novel anamorphic, ascomycetous yeast species isolated from plants, *Antonie Van Leeuwenhoek*, 2008, vol. 94, no. 2, pp. 257–265.

Translated by E. Botchkova