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EXPERIMENTAL ARTICLES

Yeasts in the Flowers of Entomophilic Plants of the Moscow Region

A. M. Glushakova, A. V. Kachalkin, and I. Yu. Chernov[1]

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

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Abstract—Dynamics of abundance and diversity of epiphytic yeasts in entomophilic flowers of 28 species of meadow, forest, and cultivated plants throughout their blooming period was determined. The number of yeasts in the flowers was shown to increase gradually during the vegetation period, and reached the maximum during summer–autumn. The total abundance and ratio of the yeast species in the flowers depended entirely on the blooming time, rather than on the taxonomic position of the plants. Three stages of development of the entomophilic yeast complexes during the vegetation period may be discerned: predominance of eurybiont nonspecific species (*Cryptococcus albidus*, *Debaryomyces hansenii*) in spring, mass development of specific nectar-associated yeasts (*Metschnikowia reukaufii*) in summer, and their substitution by widespread epiphytic species (*Rhodotorula mucilaginosa*, *Cryptococcus magnus*) in autumn.

Keywords: yeasts, entomophilic flowers, epiphytic microorganisms, entomophilic plants

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Yeasts in general are a group of microorganisms with a pronounced copiotrophic strategy. Many species, especially ascomycetous yeasts, compensate for their inability to utilize complex natural polymers (cellulose, lignin, etc.) with their capacity for rapid growth on media with high concentrations of simple sugars [1]. The nectar of entomophilic flowers is precisely the type of a natural substrate rich in simple sugars, which provides for the most active yeast growth. Nectar contains a complex of compounds, in which sugars prevail, especially sucrose, glucose, fructose, maltose, raffinose, etc. Their total concentration may reach 20%. These sugars are easily utilized by most yeasts. Apart from sugars, nectar contains amino acids, proteins, organic acids, mineral salts, phosphates, vitamins, and enzymes [2]. The number of yeast cells in nectar may exceed their average abundance on most background substrates (phyllosphere, decomposing plant debris, and soil) by several orders of magnitude, reaching 10^7–10^8 CFU/g [3]. This high abundance is caused, among other factors, by pollinating insects actively infecting the flowers during the blooming period. The taxonomic composition of yeast communities forming in such loci may be very specific, with predominance of the species not occurring or seldom occurring in other substrates. This composition may also vary significantly depending on the ontogenesis of the substrates, including the stages of budding, blossoming, and fading. In the course of several years of monitoring carried out in the Moscow region, we have previously revealed variations in the structure and abundance of the epiphytic yeast population of plant leaves associated with ontogenetic rearrangements and seasonal fluctuations of environmental factors [4, 5].

While the phyllospheric epiphytic yeast communities in Central Russia have been studied in detail, there is insufficient data on the yeast communities developing in plant nectar. *Metschnikowia reukaufii* has been long known as a common inhabitant of entomophilic flowers [6, 7]. This yeast species was originally discovered by direct microscopy of the flower nectar [8, 9]. Some rare species were found only in flowers and have never been retrieved from other habitats. For example, *Metschnikowia lunata* has been isolated only from the flowers of Central Russia [10].

The nectar yeasts of South America, Australia, India, Southeastern Asia, and Western Europe have been studied in detail. Entomophilic flowers of these regions remain a source of new yeast species [11–22]. Investigation of unique yeast communities of some entomophilic flowers of the Hawaii suggested a hypothesis of the endemic nature of certain yeast species closely associated with plants and endemic pollinator insects [23, 24].

In the present work, the results of a seasonal investigation of abundance and taxonomic structure of the yeast communities from the flowers of 28 species of meadow, forest, and cultivated entomophilic plants throughout their blooming period are presented.

MATERIALS AND METHODS

The research was carried out in 2008–2009 in Moscow (Izmailovo woodland park and Losinyi Ostrov National Park) and near the Burtsevo village, Shakhovskoi raion, Moscow oblast. The flowers were

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

Table 1. Investigated plant species and average yeast numbers in their flowers

Plant species	Sampling period (dates)	Number of samples	Average yeast nos., log(CFU/g)
Achillea millefolium L.	18.06–18.10	130	5.0
Aegopodium podagraria L.	18.06–14.07	80	4.7
Ajuga reptans L.	11.05–12.08	60	4.4
Angelica sylvestris L.	19.07–16.08	55	4.9
Anthriscus sylvestris (L.) Hoffm.	15.06–07.07	80	4.3
Asarum europaeum L.	21.04–30.05	50	2.7
Caltha palustris L.	03.05–21.05	15	3.9
Centaurea jacea L.	19.07–25.08	50	5.8
Chrysosplenium alternifolium L.	14.04–27.05	65	3.6
Euonymus verrucosa Scop.	30.05–07.06	80	4.8
Galeobdolon luteum Huds.	11.05–07.06	70	4.3
Geum rivale L.	18.05–19.06	90	5.2
Hypericum quadrangulum L.	27.06–04.09	70	5.1
Lamium album L.	15.06–18.10	55	4.7
Lathyrus pratensis L.	18.06–22.09	150	4.4
Leontodon autumnalis L.	08.08–24.09	45	5.7
Malus domestica Borkh.	11.05–27.05	70	4.3
Narcissus pseudonarcissus L.	04.05–15.05	20	2.9
Prunus cerasus L.	11.05–27.05	55	4.0
Prunus domestica L.	05.05–18.05	20	3.1
Ranunculus cassubicus L.	04.05–29.05	25	2.5
Ranunculus repens L.	30.05–07.07	60	5.2
Ribes nigrum L.	05.05–15.05	70	3.1
Syringa vulgaris L.	12.05–27.05	60	3.2
Tanacetum vulgare L.	19.07–25.08	100	5.0
Trifolium repens L.	18.06–12.08	50	4.5
Tulipa gesneriana L	11.05–31.05	15	3.8
Tussilago farfara L.	13.04–04.05	35	2.2

examined starting at the budding period (onset of blossoming of the first entomophilic plants in the Moscow region in mid-March) to complete fading and fruit formation in the first half of October. A total of 28 plant species, both typical forest and meadow plants and cultivated plants (mostly from suburban parcels) were studied (Table 1).

The samples were collected twice a week. A total of 1745 samples were analyzed. Immediately after collection, the samples were homogenized and divided into 5–10 portions of 0.2–0.4 g. These were transferred to test tubes and sterile water was added to obtain 1 : 50 dilutions. The suspensions were vortexed (MultiReax, Heidolph, Germany) for 15 min and plated on GPY agar (glucose, 20 g/L; peptone, 10 g/L; yeast extract, 5 g/L; agar, 10 g/L) acidified with 80% lactic acid (4 mL/L) in two repeats. The plates were incubated for 5–14 days at room temperature. Morphological types of the colonies were determined and enumerated under a dissecting microscope. Members of each colony type were obtained in pure cultures. Yeast cultures were identified based on their morphological and physiological characteristics [25], as well as by using DNA barcoding by PCR amplification and sequencing of the 26S rRNA (LSU) domains D1/D2.

DNA was isolated as follows. The biomass (two loopfulls) of a 3–4-days-old culture was transferred into 2-mL Eppendorf tubes and supplemented with 400 μL glass beads (300–500 μm in diameter) and 500 μL of the lysing buffer (Tris Base, 50 mM; NaCl, 250 mM; EDTA, 50 mM; SDS, 0.3%; pH 8). The mixture was vortexed for 15 min at the maximum rate, incubated for 1 h at 65°C, vortexed for another 15 min, and centrifuged for 10 min at 13.4 rpm.

For amplification of the rDNA region containing the 26S rDNA D1/D2 domains, the primers ITS1f

(5'-CTTGGTCATTTAGAGGAAGTA-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') and the ScreenMix PCR mixture (Evrogen, Moscow, Russia) were used. The amplification protocol was as follows: initial denaturing, 2 min at 96°C; 35 cycles: denaturing, 20 s at 96°C; annealing, 50 s at 52°C; DNA synthesis, 1.5 min at 72°C; and final elongation, 7 min at 72°C. The PCR products were purified using the BigDye XTerminator Purification Kit (Applied Biosystems, United States). The NL4 primer was used for sequencing. Sequencing was carried out using the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, United States) on an Applied Biosystems 3130xl Genetic Analyzer in the facilities of Syntol (Moscow, Russia). Identification was carried out using the NCBI GenBank database (blast.ncbi.nlm.nih.gov) and the CBS database (www.cbs.knaw.nl).

Overall yeast numbers and relative abundance of each species were determined for each sample. The sequences obtained were deposited to the EMBL-EBI and NCBI databases, the accession numbers given in Table 2.

RESULTS AND DISCUSSION

Yeasts were found in 90% of the analyzed samples. Their number in entomophilic flowers averaged for the vegetation period was 1.9×10^4 CFU/g, with the maximum values of up to 10^7 CFU/g. Yeast numbers did not depend on the taxonomic position of the plants or of such properties of the flowers as size, morphology or shape lof the corolla, or their height above the ground. Dispersion analysis did not reveal reliable dependence of yeast abundance upon these parameters. Blooming time was the only factor associated with yeast abundance at a high confidence level. The lowest yeast numbers ($<10^3$ CFU/g) were revealed in the plants with the flowers blooming and actively blossoming in early spring (*Tussilago farfara, Caltha palustris, Chrysosplenium alternifolium, Narcissus pseudonarcissus, Tulipa gesneriana, Syringa vulgaris*, and *Ranunculus cassubicus*). The highest numbers were found in the flowers of the plants blooming in mid-summer and autumn (*Aegopodium podagraria, Angelica sylvestris, Centaurea jacea, Geum rivale, Hypericum quadrangulum, Lathyrus pratensis, Ranunculus repens*, and *Tanacetum vulgare*), as well as in the case of repeated blooming in late summer of the early-blooming plants (e.g., *Lamium album*). Thus, the average number of yeasts in the flowers of various plants increased gradually during the first half of summer, peaked at 10^4–10^5 CFU/g in mid-June, and remained at this level until complete cessation of the blooming in October (Fig. 1).

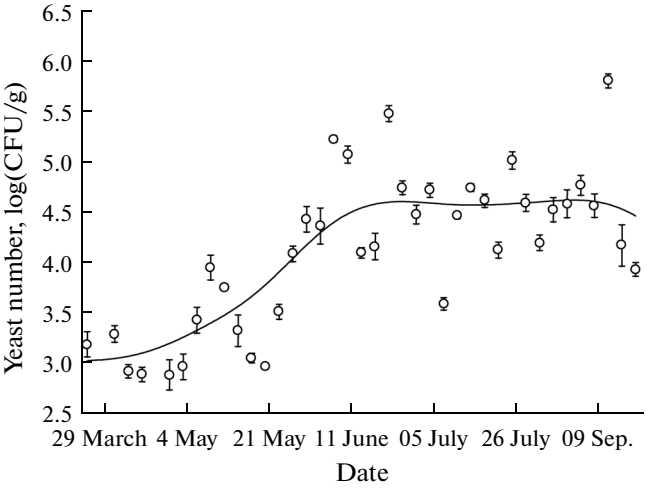

Fig. 1. Yeast abundance in flowers (averaged for all analyzed plant species) during the vegetation period.

Abundance of yeasts in the flowers is probably determined mainly by such factors as blooming duration, amount of nectar in the flowers, numbers of pollinators, and density of plant growth. The generative phase of the plants blooming in early spring is short, the number of pollinators is insufficient for massive infection of the flowers with yeast cells, and the plant density is generally thin. Moreover, most of entomophilic plants blooming in early spring are not active nectar producers. These factors are sufficient to explain the low yeast abundance in entomophilic flowers in spring. During summer and autumn, the plants with long blossoming periods (including repeatedly blooming ones) often form bushes, which favor mutual infection with yeast cells (nectar-bearing species of the families *Compositae, Umbellate, Fabaceae*, etc.). The number of pollinators, the most efficient mechanism for mutual infection of the flowers with yeasts, increases greatly during this period. Consequently, the number of yeast cells in the nectar increases significantly.

Dynamics of yeast number in the flowers of individual plant species usually exhibited a surge during the most active blossoming. In the case of plants blooming in spring and early summer, a sharp increase in yeast numbers immediately after the onset of massive blooming was typical. Shortly afterwards (in 7–14 days) the number of yeasts usually decreased drastically even before complete termination of blossoming and fading of the flowers. These dynamics were observed for most early-blooming species (Fig. 2). In late-blooming species and in those with repeated blooming, the period of elevated yeast abundance was more protracted.

A total of 33 yeast species were isolated from entomophilic flowers (Table 2), which, according to the cumulative curve of species recovery (Fig. 3), significantly coincided with their diversity in this type of habitats. Two species, *Rhodotorula mucilaginosa* and *Metschnikowia reukaufii*, were the absolute dominants occurring in about 50% of the analyzed samples. The first species is among the most typical epibionts of plant leaves [5]. As was already mentioned, the second

Table 2. Isolated yeast species and their relative abundance

Species	No. NCBI	Occurrence*, %	Average rate**, %	Probability of domination***, %
*Candida boleticol*a Nakase	FR819700	0.1	0.1	0
Candida bombi Montrocher	FN667838	0.8	1.4	0.6
Candida friedrichii van Uden, Windisch	FR772348	0.7	0.9	0.9
Candida glabrata (Andersen) Meyer, Yarrow	HE572534	0.7	0.7	0.9
Candida oleophila Montrocher	FR819698	0.9	1.2	0
Candida pimensis Suh, Gibson, Blackwell	HE572533	0.9	0.8	0.9
Cryptococcas albidus (Saito) Skinner	HE572537	0.4	0.5	0.4
Cryptococcus magnus (Lodder, Kreger-van Rij) Baptist, Kurzman	FN868255	8.4	9.8	10.2
Cryptococcus tephrensis Vishniak	HE572541	0.1	0.1	0
Cryptococcus victoriae Montes et al.	FN667851	0.3	0.8	0.3
Cystofilobasidium capitatun (Fell et al.) Oberwinkler et Bandoni	FN667853	0.3	0.7	0.5
Debaryomyces hansenii (Zopf) Lodder, Kreger-van Rij	FR774535	1.9	2.6	1.8
Hanseniaspora uvarum (Niehaus) El-Tabey Shehata et al.	FR819702	2.1	2.2	2.3
Holtermannella festucosa (Golubev, Sampaio) Libkind, Wuczkowski, Turchetti, Boekhout	HE572535	0.5	0.9	0.5
Leucosporidiella yakutica (Golubev) Sampaio	HE572543	0.1	0.2	0.2
Candida parapsilosis (Ashford) Langeron, Talice	FR774537	0.2	0.3	0.2
Metschnikowia gruessii Giménes-Jurado	FN667859	2.2	3.3	3.2
Metschnikowia koreensis Hong et al.	HE572542	0.3	0.9	0.3
Metschnikowia pulcherrima Pitt, Miller	HE572532	8.1	12.5	10
Metschnikowia reukauflii Pitt, Miller	FN667860	23.2	21.6	30.4
Metschnikowia sp.	HE575194	0.4	0.5	0.6
Meyerozyma guilliermondii (Wickerham) Kurzman et M. Suzuki	FN667993	0.1	0.1	0
Ogataea cecidiorum Glushakova et al.	FJ897742	0.4	0.4	0.3
Pichia fermentans Lodder	HE572539	0.4	0.8	0.4
Pichia kluyveri Bedford var. *khiyveri*	FN667994	0.7	1.4	0.9
Pichia manshurica Saito	FN667992	0.2	0.3	0
Rhodotorula colostri (Castelli) Lodder	HE572540	0.1	0.1	0
Rhodotorula mucilaginosa (Jödrgensen) Harrison	FN667997	24.2	24	30.4
Rhodotorula pinicola Bai, Guo et Zhao	FN667998	0.4	0.5	0.6
Starmerella bombicola Rosa, Lachance	FN668001	2.1	2.8	2.8
Wickerhamomyces anomalus (Hansen) Kurtzman, Robnett, Basehoar-Powers	FR772351	4.3	5.8	5.4
Yarrowia lipolytica (Wickerham et al.) Van der Walt, von Arx	HE572538	0.4	0.6	0.3
Zygosaccharomyces rouxii (Boutroux) Yarrow	HE572536	0.4	1.2	0.2

* Share of samples in which the species was detected.

** Average share of the total yeast number.

*** Share of samples in which the species was dominant.

species was most typical of entomophilic flowers. Among other species, *Cryptococcus magnus, Metschnikowia pulcherrima, Wickerhamomyces anomalus, Metschnikowia gruessii, Starmerella bombicola, Debaryomyces hansenii*, and *Hanseniaspora uvarum* were most commonly isolated. These species formed the group of potential dominants in the flowers, so that in some samples they predominated considerably within the yeast population. The relative abundance of other species did not exceed 1%.

Most of these less common species have been repeatedly detected previously, mainly on plant leaves (*Rhodotorula pinicola, Cryptococcus tephrensis, Cryptococcus victoriae, Cystofilobasidium capitatum,* and *Candida oleophila*). Some of the rare species have not been found previously in the Moscow region. They have been retrieved from flower nectar in other geographical regions (*Metschnikowia gruessii, Starmerella bombicola, Metschnikowia koreensis, Candida bombi, Pichia manshurica*, and *Pichia kluyveri*). In spite of the long duration of the monitoring in our work, no species specific for the Moscow region were revealed in the flowers of entomophilic plants. *Metschnikowia lunata* Golubev was the only species which has been previously detected in the nectar of meadow plants in the Moscow region and has not been observed in other regions. In the present work, however, we failed to isolate this species even after analysis of over 1500 samples.

Similar to the total abundance, the species composition and the ratio of yeast species in the flowers did not depend significantly on the taxonomic and other characteristics of the plants, but were mainly determined by the time of its blooming. This may be seen from the results of ordination of the analyzed plant species according to the species structure of their yeast communities by the principal component analysis (Fig. 4). High correlation can be seen between the values of the first component, responsible for over 50% of the overall variation, and the date of the last blooming for a given plant species. In other words, the species structure of the yeast communities developing in the flowers of entomophilic plants changes gradually during the vegetation period.

Some patterns of the seasonal dynamics of the dominant species could be observed in the entomophilic yeast community (Figs. 5–7). The yeasts from early-blooming plants were mostly eurybionts *Cryptococcus albidus* and *Debaryomyces hansenii*, with its overall numbers not exceeding 10^3 CFU/g. Both species are known as eurybionts, which may be isolated from a variety of substrates with an almost equal probability.

Typical epiphytic species (*Rhodotorula mucilaginosa, Cryptococcus magnus*) were retrieved from the flowers of the plants blooming in late May–early June, while *Metschnikowia reukaufii* became the predominant species. The dynamics of abundance of this typical nectar species was characterized by two peaks, in summer and in autumn. These peaks corresponded to the periods of massive primary and secondary blooming of entomophilic grasses. The minor species *Metschnikowia gruessii* exhibited a similar dynamics, albeit with less pronounced peaks. This species has not been isolated from the substrates other than nectar-bearing plants. Another member of this genus, *Metschnikowia pulcherrima*, was also most abundant in mid-summer, although it was present in other periods as well. Unlike *Metschnikowia reukaufii* and *Metschnikowia gruessii*, this species is less closely associated with nectar and is common on plant leaves [5, 26]. These three species were the most typical components of the nectar yeast compounds; their development reflected the ontogenetic state of the flowers (active blossoming, fading, etc.) and associated variations in the abundance of the nectar and pollinators.

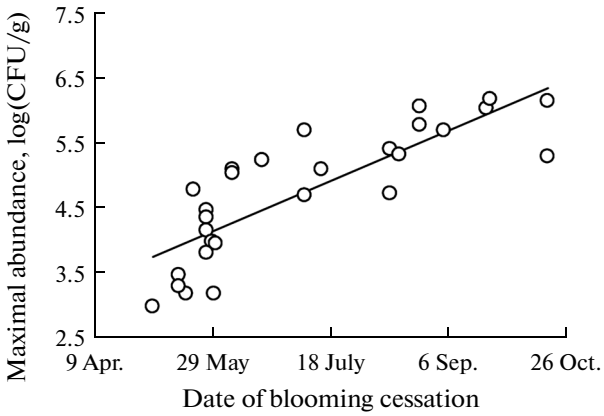

Fig. 2. Maximal recorded yeast numbers in flowers depending on the time of blooming cessation in various plant species.

The share of *Wickerhamomyces anomalus* and *Hanseniaspora uvarum* also increased in the flowers of the plants blooming in late summer and autumn. These yeasts have been repeatedly isolated from soft fruit. The number of young fruits begins to exceed the number of flowers by late summer–early autumn.

The flowers of late summer–autumn plants (*Tanacetum vulgare, Leontodon autumnalis*) exhibited significantly lower total numbers and relative abundance of *Metschnikowia reukaufii*, with the epiphytes *Rhodotorula mucilaginosa* and *Cryptococcus magnus* becoming predominant. This is certainly due to the fact that by autumn the flowers lose their specificity as sugar-rich substrates and become analogues of the phyllosphere. We have previously shown [5] a sharp increase in the numbers of these basidiomycetous yeasts on leaf surfaces in the second half of summer with a peak in autumn.

Thus, the flowers of entomophilic plants are among the major centers for formation of abundant and diverse yeast complexes. While their numbers and species structure vary significantly during the vegetation

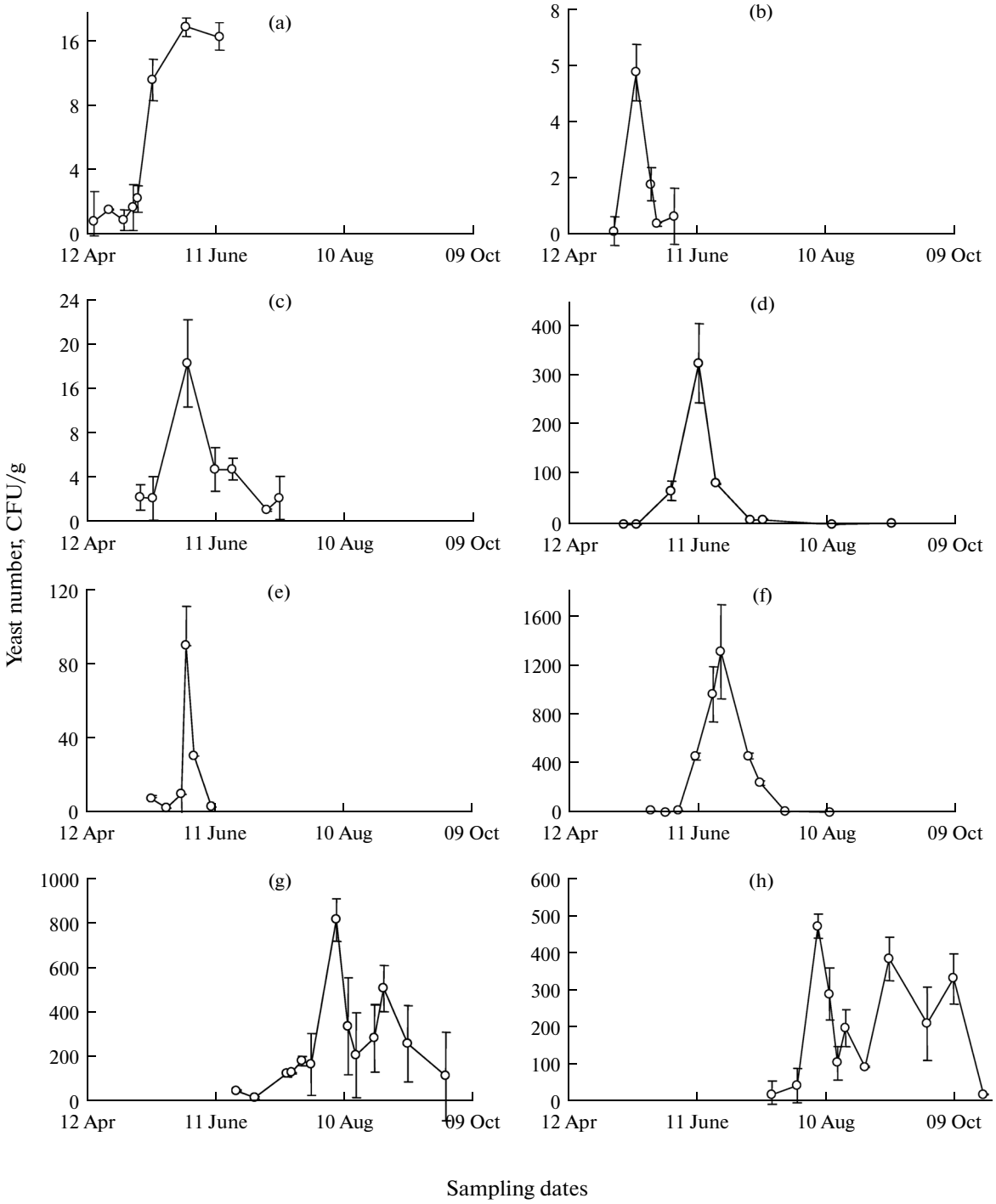

Fig. 3. Dynamics of yeast abundance in the flowers of different plant species (a–h).

period, different plant species blooming at the same time have, on average, a similar yeast composition in nectar-bearing flowers.

Investigation in tropical and subtropical regions revealed highly specific mutualistic complexes of entomophilic yeasts and entomophilic plants, which were supported by the closely associated insect species. The insects act as vectors for yeast transfer, providing for the primary contamination by certain specific species [23, 24]. In the moderate zone, epiphytic yeast species closely associated with specific plant species are probably not present. Abundance of the different epiphytic yeast species depends mostly on the hydrothermal conditions and on the ontogenetic stage

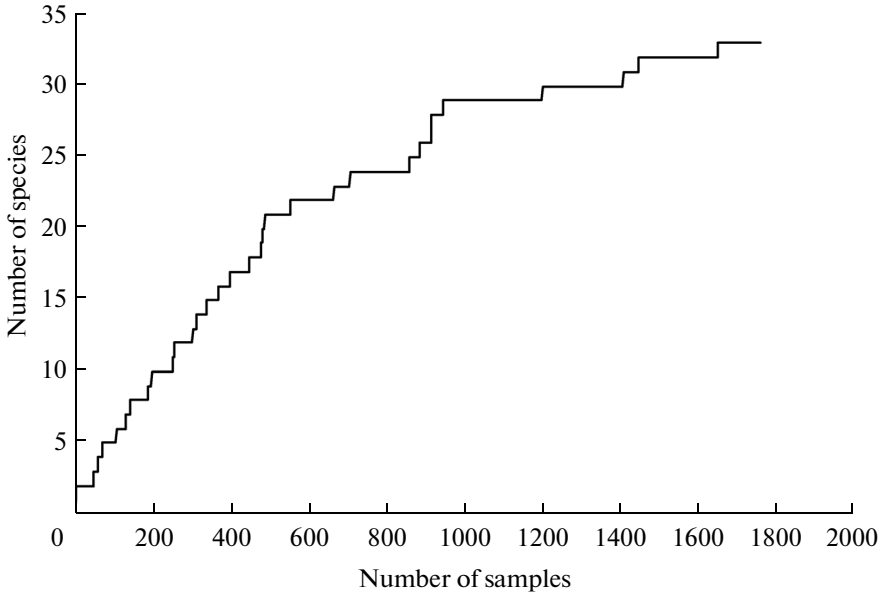

Fig. 4. Number of detected yeast species depending on the number of analyzed flower samples.

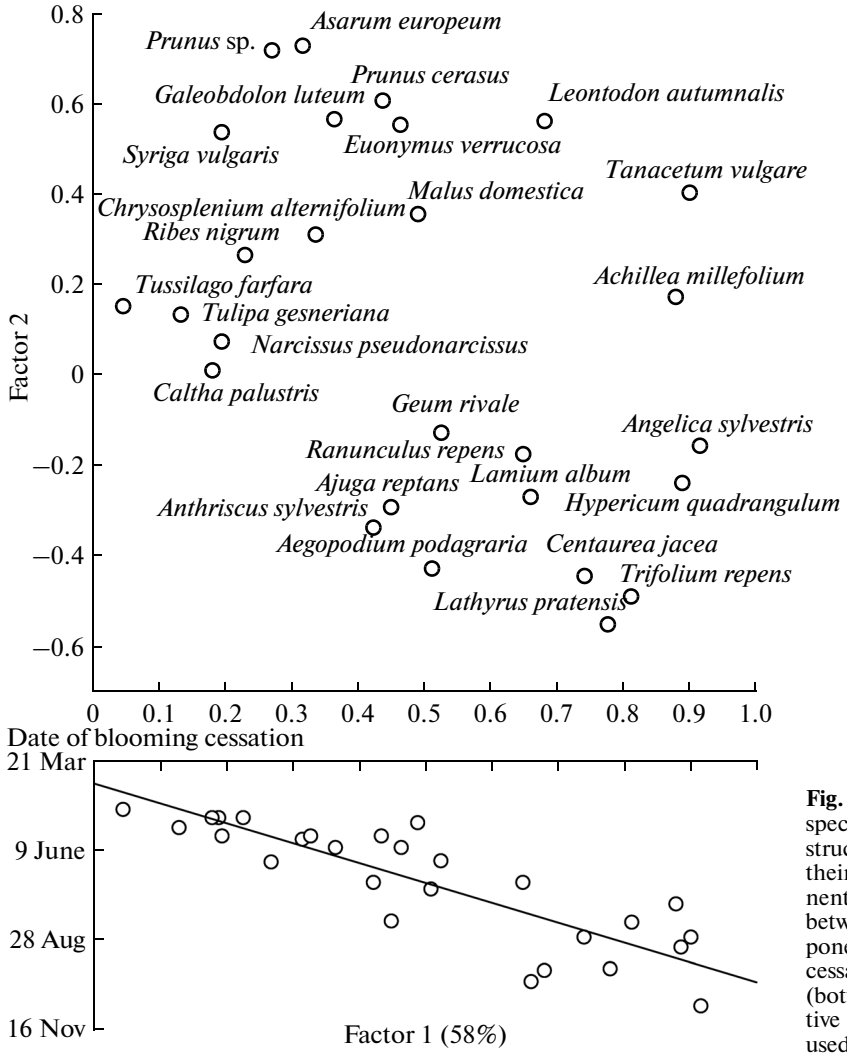

Fig. 5. Ordination of various plant species according to the species structure of the yeast population of their flowers by the principal component analysis (top) and correlation between the values of the first component and the dates of blooming cessation for different plant species (bottom). The values of average relative abundance yeast species were used as variables.

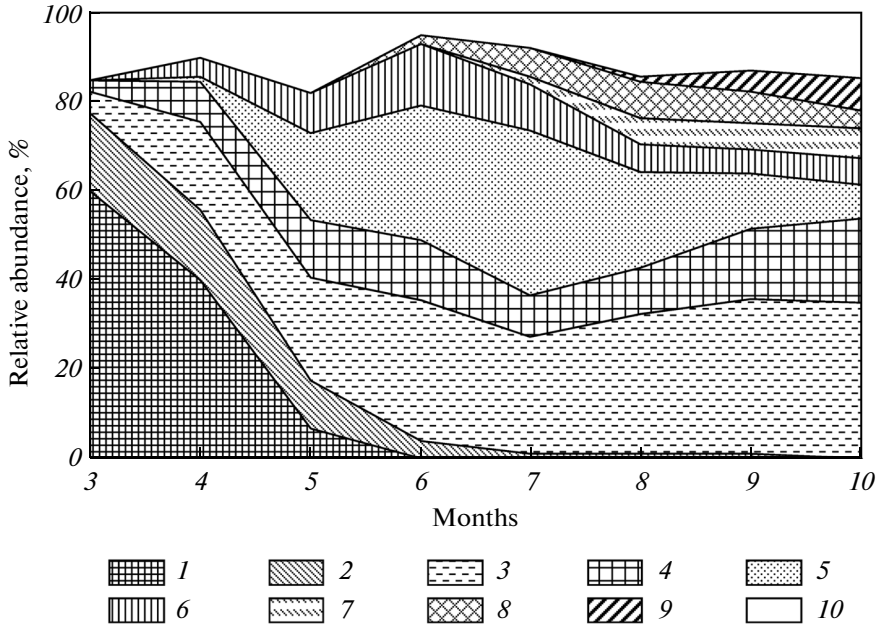

Fig. 6. Species structure of the yeast communities of the flowers during the vegetation period (averaged for all plant species): *Cryptococcus albidus* (*1*), *Debaryomyces hansenii* (*2*), *Rhodotorula mucilaginosa* (*3*), *Cryptococcus magnus* (*4*), *Metschnikowia reukaufii* (*5*), *Metschnikowia pulcherrima* (*6*), *Hanseniaspora uvarum* (*7*), *Wickerhamomyces anomalus* (*8*), *Stramerella bombicola* (*9*), and others (*10*).

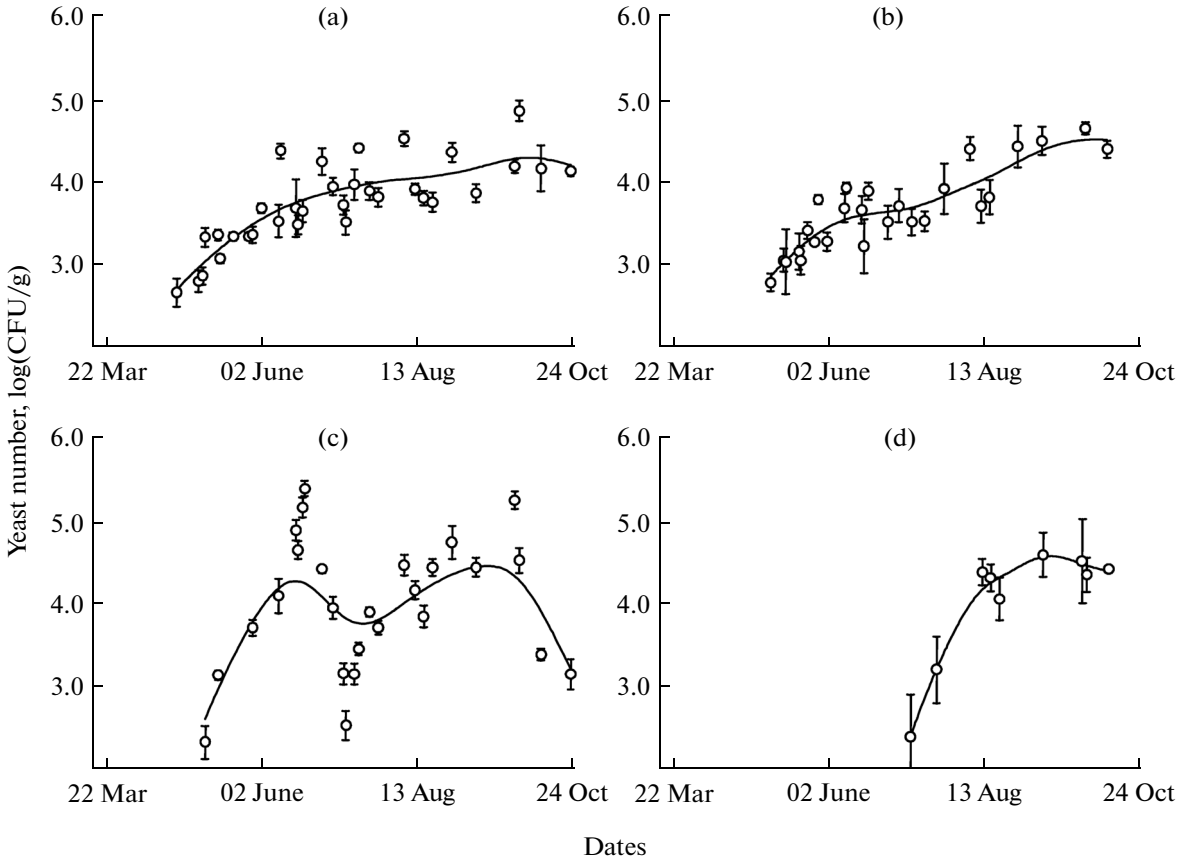

Fig. 7. Dynamics of abundance of some dominant yeast species in the flowers during the vegetation period (averaged for all plant species): *Rhodotorula mucilaginosa* (a), *Cryptococcus magnus* (b), *Metschnikowia reukaufii* (c), and *Wickerhamomyces anomalus* (d).

of development of a given plant, while it is but weakly associated with the taxonomic position of the plant. We have previously shown that the averaged annual composition of the dominant yeast species on the leaves of different plant species was approximately the same [5]. This phenomenon was observed for entomophilic flowers, although the taxonomic composition of the yeasts was somewhat different. Moreover, abundance and composition of the dominant yeast species in the flowers showed considerable seasonal variations. This was probably due to the climate of the temperate zone, with pronounced seasonal variations and considerable differences in temperature and humidity between the seasons, which had more effect than the differences in the nectar composition. Under such conditions, yeast species with pronounced adaptive capacities and relatively weak specialization gain an advantage. Moreover, specialized pollinators are less numerous in the temperate zone than in tropical forests with high diversity of plants. This results in mutual contamination of different plant species by the same yeast species.

Thus, the species composition of the yeasts inhabiting the flowers of entomophilic plants in the moderate zone is determined primarily by the seasonal factors. Development of the entomophilic yeast complex throughout the vegetation period may be subdivided into three stages: predominance of nonspecific eurybionts in spring; mass development of specific nectar yeasts in summer, with predominance of *Metschnikowia reukaufii*; and their replacement by typical epiphytic species an autumn.

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