ISSN 0026-2617, Microbiology, 2009, Vol. 78, No. 3, pp. 355–359. © Pleiades Publishing, Ltd., 2009.
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EXPERIMENTAL ARTICLES

The Yeast *Candida railenensis* in the Fruits of English Oak (*Quercus robur* L.)

O. V. Isaeva, A. M. Glushakova, A. M. Yurkov, and I. Yu. Chernov[1]

Moscow State University, Soil Science Department, Leninskie Gory, Moscow, 119992 Russia

Received June 17, 2008

Abstract—The cotyledons of whole intact acorns were shown to contain yeasts; their number increased sharply before acorn germination. The yeasts in the cotyledons are mainly represented by one species, *Candida railenensis*, with the number in the germinating cotyledons reaching 10^7 CFU/g. After germination or exocarp destruction, the cotyledons were colonized by the usual epiphytic and litter yeasts *Cryptococcus albidus, Rhodotorula glutinis,* and *Cystofilobasidium capitatum.*

Key words: yeasts, acorns, oak, *Candida railenensis,* endophytic microorganisms, endophytes, seasonal dynamics.

DOI: 10.1134/S002626170903014X

Yeasts are among the most typical epiphytic microorganisms. Epiphytic yeasts exist as eccrisotrophs, consuming various easily accessible compounds (simple sugars, organic acids, sugar alcohols, and amino acids), excreted outside through stomata, hydatodes, damaged cuticles, floral and extrafloral nectaries [1–4]. An increasing body of evidence suggests that various endophytic microorganisms, especially bacteria and mycelial fungi, are constantly present in the internal organs of healthy plants [4, 5]. The data concerning endophytic yeasts are fragmentary. There are few works dedicated to the isolation of yeast cells from the internal tissues of plants. In particular, the isolation of *Rhodotorula pinicola* from the xylem of pine twigs was reported [6].

The existence of true, highly specialized endophytic species closely associated with the tissues of certain plants is unlikely among the yeasts. Most yeast fungi are typical copiotrophs, requiring sufficiently high concentrations of easily accessible compounds for growth. Therefore, yeast development is most probable in the sugar-containing, storage tissues of plants, such as the parenchymal tissue of succulent fruits. Our recent study of the yeast communities of sugary fruits indeed demonstrated the constant presence of yeasts in their tissues, and they typically belonged to the same species as on the surface. The yeasts are most abundant in the fruits which are easily penetrable due to the incompletely coalesced hypanthium, thin exocarp, damage done to them on ripening, etc. [7].

We also established that yeasts might also be present in the fruits with a thick dense covering. For example, it was found that yeasts (up to 10^7 CFU/g) developed in the cotyledons of whole intact oak acorns at certain stages of their ripening.

In this work, the results of more detailed investigations of the dynamics of the number and taxonomic composition of such endophytic yeast groupings in the fruits of English oak (*Quercus robur*), both falling off and wintering in the upper soil horizon, are represented.

MATERIALS AND METHODS

The acorns, the fruits of the oak *Quercus robur*, were collected in 2003–2005 on the territory of the Losinyi Ostrov Reserve. The acorns were sampled from the moment of their formation on fruit stalks and until their germination in soil next spring or summer. Before the germination period, whole acorns with no visible mechanical damage to the coat were sampled for analysis. In May, when mass germination of acorns began, only intact acorns were sampled. Later, in the second half of the summer, the acorns that were not germinated were analyzed, whose covering began to get ruptured. A total of 350 acorns sampled at 30 terms were analyzed, i.e., the number of repeats was 10–12 at each term.

The acorn's surface was sterilized with alcohol and incised with a sterile scalpel. Preliminary experiments revealed no culturable yeasts in the washout from the surface of sterilized acorns. After the fruit was opened, the storage cotyledons were separated from the seed, ground, and poured over with sterile water at a ratio of 1 : 50. The suspension was stirred with a Vortex device for 2 min. The glucose–peptone medium (glucose, 20 g/l; peptone, 10 g/l; yeast extract, 5 g/l) acidified with 40% lactic acid (4 ml/l) was inoculated with the

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

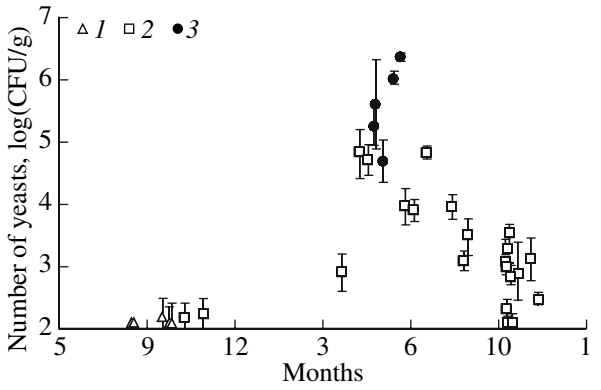

Fig. 1. Dynamics of the total number of yeasts in the acorn cotyledons; the types of acorns : in the tree (*1*); fallen off (*2*); fallen off germinating (*3*).

samples in three replicates. The cultures were incubated at room temperature for 5 to 7 days. The yeast colonies were then grouped into the morphological types under a binocular magnifier, and at least three colonies of each type were used for isolation. These cultures were identified by their morphophysiological characteristics [8] using an enlarged physiological spectrum [9, 10]. The average number of yeasts in the cotyledon expressed in CFU/g and the share of each species in the total number of yeasts were calculated for each fruit.

In order to visualize the endophytic yeasts, cotyledon preparations were made with a razor. The preparations were stained with 0.1% toluidine blue for 5 to 10 min, embedded in glycerin, and examined under the microscope.

In order to identify the yeast species dominating in the acorns, sequencing of the 26S rDNA D1D2 region was carried out. The analysis of the nucleotide sequences was carried out according to the scheme described earlier [11] using the primers ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G) and LR6 (5'-CGC CAG TTC TGC TTA CC) for initial amplification on the Uno II Thermal Cycler amplifier (Biometra, Germany). The fragment obtained was then purified and concentrated with the GFX Band Purification Kit (Amersham Biosciences). The reaction was carried out according to the standard protocols with the primers NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG) and NL4 (5'-GGT CCG TGT TTC AAG ACG G). The sequencing was carried out on a semiautomatic ALF Express II DNA analyzer sequencer (Amersham Pharmacia Biotech, Sweden) according to the standard protocol. The species was identified by comparing the nucleotide sequences with those available in the electronic databases. The sequences were aligned using the MegAlign program (DNAStar, United States), and the phylogenetic tree was constructed (the nearest mean method; the bootstrap values were obtained after 1000 iterations).

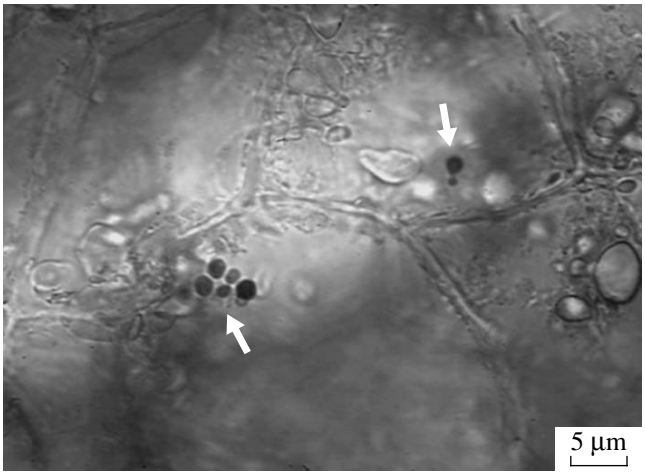

Fig. 2. Yeast cells on sections from the acorn cotyledons. Stained with toluidine blue.

RESULTS AND DISCUSSION

Almost no yeasts were revealed in the fresh acorn cotyledons during the whole period from their formation until their falling off in autumn. Very rarely, single yeast colonies were present in the platings. In the wintered whole acorns, yeasts occurred more often and in greater numbers, sometimes as high as 10^3 CFU/g. However, in May, immediately before acorn germination, yeasts were detected in almost all the cotyledons analyzed; their average number was 10^5 CFU/g, in some cotyledons it reached 10^7 CFU/g (Fig. 1).

When the sections of cotyledon collected in this period were stained with toluidine blue and examined under the microscope, round and short oval yeast cells 2–3 μm in diameter were shown to occur both in the intercellular space and inside the plant cells (Fig. 2). In the period before germination and in the germinating cotyledons, most of the cells budded; aggregates of cells were often seen, giving evidence of their active multiplication.

Beginning with June, the number of yeasts in acorns that were not germinated gradually decreased. In the period of late summer–early autumn, the integrity of the covering of most of the acorns that were not germinated was disrupted; however, the number of yeasts in the cotyledons continued to decrease. The minimal number was observed in November in decomposing fruits and in those that had not entered the germination phase. Thus, the increase in the number of endophytic yeasts in the acorns was noted in the period preceding their germination. Active hydrolysis of starch with the release of simple sugars required for the seed embryo to develop is known to occur in the parenchymal storage tissues before germination. Apparently, an increase in the amount of available nutrients stimulated intense yeast development.

A total of seven yeast species were isolated from the acorns (table). Of the young acorns in the tree, we suc-

Average relative abundance (%) of the yeast species isolated from the acorn cotyledons

Species	In the tree	Fallen off before germination	Germinating	Not germinated, decomposing
Cryptococcus albidus	0	0	13.6	62.8
Rhodotorula glutinis	0	2	5.2	12.1
Candida railenensis	100	98	74.1	1.2
Rhodotorula pilati	0	0	0	0.1
Rhodotorula mucilaginosa	0	0	0	1.6
Cystofilobasidium capitatum	0	0	7.1	20.8
Metschnikowia pulcherrima	0	0	0	1.4

ceeded in isolating the yeast of only one anamorphous ascomycetous species. The same species was also revealed as an absolute dominant in the fallen off wintered acorns before the period of their germination. All the isolated strains of this species had almost identical morphological and physiological characteristics. In contrast to the other species revealed, the phenotypic identification of these strains caused certain difficulties. According to the standard combination of the morphological and physiological traits, they appeared to be close to the species *Candida oleophila* and *C. railenensis*; however, they exhibited certain differences from the standard descriptions of these species.

Identification of one of the isolated strains (EF608212) based on the nucleotide sequence of the rDNA D1D2 region showed that it differed from the type strain of *C. railenensis* (CBS 8164, NRRL Y-17762; U45800) only in one nucleotide substitution, which is consistent with the criterion of nonspecificity adopted in genosystematics [12]. Complete homology of the nucleotide sequences of the D1D2 region was revealed between the strain EF608212 isolated by us and the strain *C. railenensis* KCTC 7835 (AF257274) isolated from plum fruits in Korea [13]. All known isolations of this species are of a fortuitous nature and do not allow a conclusion as to the peculiarities of its prevalence to be made. By the D1D2 sequence, this species is the closest to *C. oleophila*, from which it differs phenotypically only in its ability to ferment trehalose.

The phylogenetic relationship between *C. railenensis* and *C. oleophila* is illustrated by the tree (Fig. 3), including the *C. oleophila* strain (EF452234), which was isolated by us from the plant phyllosphere in the same region as the *C. railenensis* strain (EF608212). As it was shown by us earlier, *C. oleophila* is constantly present on the leaves of various plant species [14]. Apparently, the closely related *C. railenensis* and *C. oleophila* should be regarded as varieties of one

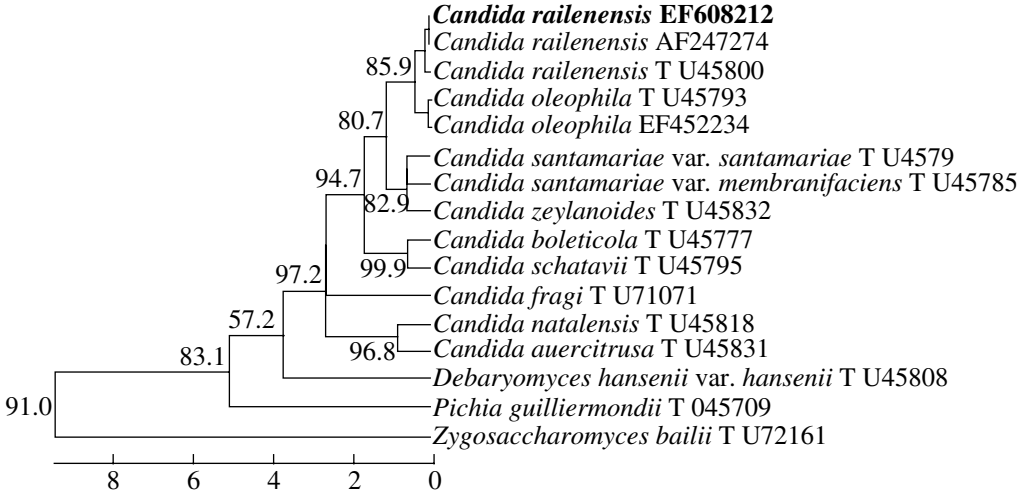

Fig. 3. Phylogenetic position of the yeast species isolated from the acorns (strain EF608212). The results of sequencing of the rDNA D1D2 region; the scale corresponds to the number of nucleotide replacements per 100 bp, the bootstrap values less than 50 are not shown.

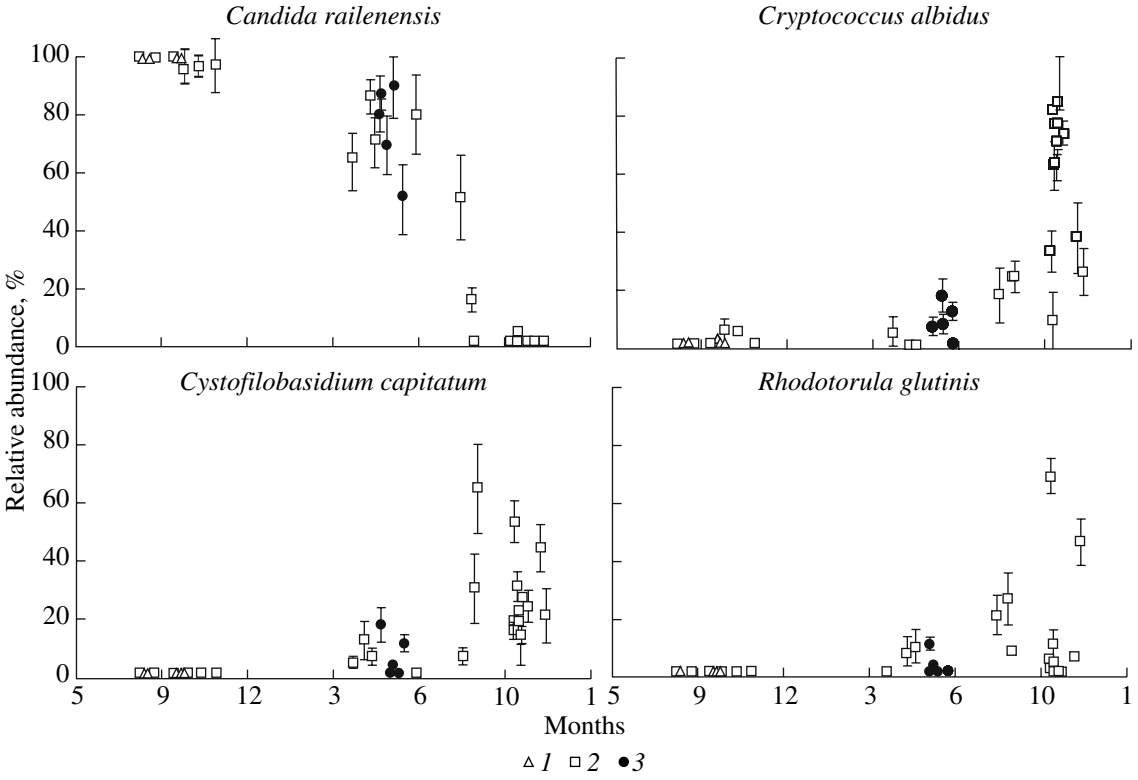

Fig. 4. Dynamics of the relative abundance of the dominating yeast species in the acorn cotyledons. See Fig. 1 for the designations.

large anamorphous species whose main habitat is the plant surface. It cannot be ruled out that in the process of development, one of these varieties became adjusted to endophytic existence, while the other remained a nonspecialized inhabitant of the plant phyllosphere. In this case, we see an example of how separation of the ecological niches of anamorphous yeasts may be a factor of sympatric speciation.

Gradually, as the acorns germinated or decomposed, the relative abundance of *C. railenensis* in them decreased, and after the exocarp disruption, the acorns were colonized by the typical epiphytic and litter species (Fig. 4). The yeast population of the decomposing cotyledons in the late autumn–early winter period did not differ in the species composition of the dominants from that of the litter: basidiomycetous yeasts *Cryptococcus albidus, Rhodotorula glutinis*, and *Cystofilobasidium capitatum* predominated, i.e., the species prevalent on the oak leaves and in its litter [15].

It was shown earlier that yeasts are regularly found in the internal tissues of many succulent fruits, their number sharply increasing after fruit ripening [7, 16]. In the process, the yeasts growing in the fruit flesh are represented by the same species as those on the surface. One of the variants of such a nonspecific endophytic yeast community was studied by us by the example of the fruits of the wild rose [7]. As a rule, succulent fruits do not possess dense covering. Therefore, it is possible for the epiphytic yeast cells to penetrate regularly into the fruits as a result of microscopic disruption of the integrity of their covering tissues. A similar taxonomic composition and the specifics of the seasonal dynamics of the yeast groupings on the fruit surface and inside them are the consequence of this [7].

The dense leathery covering of acorns is evidently a more serious hindrance to yeast cell penetration. Therefore, along with the fortuitous penetration of yeast cells through microinjuries of the covering, it may be assumed that yeasts penetrate an acorn from an oak flower in the process of its formation, are retained in the cotyledons, and begin to grow when the process of embryo development (which is known to be accompanied by starch hydrolysis) is activated. This agrees with the notion of seasonal endophytic development of saprophytic microorganisms as the realization of the strategy of avoiding unfavorable factors [4]. It is not excluded that yeasts also play a certain part in the germination of acorns. For example, it was shown that certain yeasts capable of auxin synthesis accelerate the development of the embryo root. Such a phenomenon was noted for the yeast *Williopsis saturnus* artificially inoculated into the maize tissues [17].

The results obtained correct the existing notions of the peculiarities of the distribution of yeasts in natural habitats and allow us to regard the internal storage tissues of plants as a promising source for the search for

new taxa and as a new interesting model for investigating the coevolving microbial–plant associations.

ACKNOWLEDGMENTS

We are grateful to I. Spencer-Martins, A. Fonseka, and J.P. Sampaio, researchers from the Lisbon University, for technical support in molecular identification.

This work was supported by the Russian Foundation for Basic Research, project no. 07-04-00481, and the Russian Federation President's Program to support the Russian Federation leading scientific schools, project no. NSh-2227.2008.4.

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