Presence of clinically significant endophytic yeasts in agricultural crops: monitoring and ecological safety assessment

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Abstract. Endophytic yeasts from internal tissues of 54 names of nuts and vegetables from 36 countries were studied. In total, 74 species of yeasts were isolated from analysed agricultural crops. The study revealed high values of occurrence and abundance of clinically significant yeasts *Candida parapsilosis* and *Meyerozyma guilliermondii* in some internal tissues that may pose a potential threat to human health, primarily for immunocompromised individuals.

1. Introduction

The ubiquitous spread of yeasts in all components of natural ecosystems, i.e. soil, plant substrates, atmosphere, in various geographic zones, and the close relationship of humans with some taxa of yeasts are the most important factors of appearance of allergic diseases and mycoses. A systematic study of allergy to yeasts has begun only in the 1940s [1]. Nine genera of clinically significant yeasts regularly isolated from natural soil-plant substrates have been identified: *Candida*, *Cryptococcus* s.l., *Geotrichum*, *Meyerozyma*, *Pichia*, *Rhodotorula* s.l., *Saccharomyces* and *Trichosporon* s.l. [2-3] An exception is the lipophilic yeasts *Malassezia* which are associated exclusively with humans and animals [3]. The most common mycological diseases associated with yeasts include bronchial asthma, allergic bronchopulmonary mycosis, rhinitis, dermatitis, and alveolitis [4].

Environmental studies of the yeast complexes developing in a wide variety of natural substrates, which are transformed under the influence of anthropogenic factors and substrates with which humans in constant contact are becoming increasingly important. The health risk for people, primarily for immunocompromised individuals, upon contact or consumption of foods in which opportunistic and allergenic yeast species develop is quite high. The most important food products recommended by WHO/FAO for mandatory consumption in order to prevent various diseases as well as to prolong and improve the quality of life are fruits and vegetables [5].

The study of endophytic yeast complexes from internal tissues of fruits and vegetables are an actively developed scientific area, which important for fundamental and applied sciences. A number of studies have been devoted to this subject over the last decade [6]. The main aspects of most research concern the diversity of yeasts and their role in symbiosis, but questions of ecological safety remain
The aim of the work was to assess the level of prevalence of clinically significant yeasts in the internal tissues of agricultural crops (fresh juicy fruits, nuts and vegetables) growing in our country and imported from others.

2. Materials and methods
During the period of 2019-2020, we researched and analysed data for 54 agricultural crops from 36 countries, including those grown in Russia. The following fruit, berry, nut and vegetable cultures were analysed: almond (Azerbaijan, Georgia), apple (Argentina, Belarus, Chile, China, Moldova, Russia, Serbia, Turkey), apricot (Macedonia, Serbia, Turkey), avocado (Kenya, Mexico), banana (Ecuador), beet (India, Kyrgyzstan, Russia), black currant (China, Russia), blackthorn (Russia), damson plum (Russia), carrot (Belarus, Moldova), sour cherry (Armenia, Egypt, Russia), cucumber (Kazakhstan, Russia, Turkey), kumquat (Russia), red currant (Russia), date (Turkey), dogwood (Russia), eggplant (Iran, Israel, Turkey), gooseberry (Russia), grape (Iran, Russia, Turkey, Uzbekistan), guava (Vietnam), kiwi (Chile, Greece, Iran), lemon (Argentina, Spain), lychee (Madagascar), longan (Vietnam), tangerine (Chile, Morocco, Spain), mango (Brazil, Peru, Thailand), melon (Russia), orange (Egypt), passionfruit (Vietnam), peach (China, Turkey), peanut (Georgia), pear (Belgium, Iran, Israel, Moldova, Turkey), sweet pepper (Belarus, Iran, Israel, Turkey), persimmon (Abkhazia, Azerbaijan, Israel), pineapple (Costa Rica), pistachio (Georgia, Turkey), pitaya (Vietnam), plum (Azerbaijan, Moldova, Russia, Serbia, Turkey), pomegranate (Italy, Turkey), grapefruit (Turkey), potato (Azerbaijan, Egypt, Pakistan), pumpkin (Russia, Turkey), quince (Russia), rambutan (Vietnam), sea buckthorn (China, Russia), shadbush (Russia), vegetable marrow (Russia, Turkey), strawberry (Armenia, Russia), sweet cherry (Moldova, Russia, Serbia, Turkey), tamarillo (Colombia), tomato (China, Russia, Turkey), walnut (Russia), watermelon (Russia), zucchini (China, Turkey). Purchases of agricultural products of Russian and foreign origin were made in Moscow and Moscow region retail stores and in produce distribution centers. The sampling was carried out on the basis of visual characteristics reflecting ripeness and the absence of spoilage. A total of 1023 samples were analysed.

The study of endophytic yeast communities was carried out according to the standard technique [7-9]. The fruit surface was sterilized according to the previously described and tested procedure [7]. After removing the integumentary tissues with a sterile scalpel, we cut out and crushed sections of the internal tissues, then poured in sterile water at a ratio of 1:5-1:10. The resulting suspensions were vortexed on a MultiReax vortex (Heidolph, Germany) for 15 min and plated on glucose-peptone-yeast extract-agar medium (GPYA) in triplicate. Plates were incubated at 20-22 °C for 5-7 days. Colonies were grouped according to their morphology into morphological types using dissection microscopy, counted and two or three representatives of each colony type were transferred into pure culture.

The identification of yeasts was performed on the basis of the analysis of the nucleotide sequence of the ITS region of rDNA. DNA isolation and PCR were performed according to the previously described procedure [9]. DNA sequencing was performed on a 3130xl Genetic Analyzer with the Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, USA) at the Evrogen company (Moscow, Russia). For sequencing, the ITS5 primer (5’ – GGA AGT AAA AGT CGT AAC AAG G) was used. For species identification, nucleotide sequences were compared with those in public databases using the BLAST NCBI (www.ncbi.nlm.nih.gov) and the MycoID (www.mycobank.org) tools.

The statistical processing of the results was performed using the STATISTICA 8 software (StatSoft, USA).

3. Results and discussion
Despite the absence of microbiological indicators of yeast numbers in the "Hygienic Requirements for the Safety and Nutritional Value of Food Products" (Sanitary Rules and Norms 2.3.2.1078-01) for fresh fruits and vegetables, but safety values 100-500 CFU/g are existing for frozen, vacuum packed and processed fruits and vegetables. Our research demonstrated that numbers more than 500 CFU/g in 84% studied samples of fruit and vegetable products from retails. The average number of yeasts in the
studied fruit and vegetable crops was $2.20 \times 10^3$ CFU/g. Out of the 54 agricultural crops under study, 74 yeast species were isolated: 32 ascomycetes and 42 basidiomycetes (table 1).

Table 1. The frequency of occurrence (FO, %) of endophytic yeasts in the studied crops.

| Ascomycetes | FO, % | Basidiomycetes | FO, % |
|-------------|-------|----------------|-------|
| Ambrosiozyma sp. KBP YE-0733 | 0.20 | Buckleyzyma aurantiaca | 0.20 |
| Aureobasidium pullulans | 23.17 | Bullera alba | 3.52 |
| Candida friedrichii | 0.10 | Colacogloae sp. KBP YE-0268 | 0.29 |
| Candida oleophila | 2.74 | Curvibasidium cygneicollum | 0.10 |
| Candida parapsilosis | 6.06 | Curvibasidium pallidicorallinum | 1.86 |
| Candida pellucida | 0.49 | Cystobasidium laryngis | 0.10 |
| Candida railenensis | 2.93 | Cystobasidium lysinophilum | 0.10 |
| Candida saitoana | 1.56 | Cystobasidium sp. KBP YE-0710 | 3.52 |
| Candida sake | 0.98 | Cystofilobasidium capitatum | 4.40 |
| Candida sp. KBP YE-0711 | 0.88 | Cyst. infirmominiatum | 0.49 |
| Candida zeylanoides | 26.20 | Cystofilobasidium macerans | 1.47 |
| Cyberlindnera misumaiensis | 3.03 | Filobasidium magnum | 7.72 |
| Cyberlindnera dauci | 0.68 | Filobasidium stepposus | 0.78 |
| Debaryomyces fabryi | 19.75 | Filobasidium unigutulatum | 0.29 |
| Debaryomyces hansenii | 11.05 | Filobasidium wieringae | 4.59 |
| Debaryomyces subglobosus | 2.54 | Graphiola sp. | 0.29 |
| Dothiora prunorum | 2.15 | Krasilnikovozyma huempii | 1.08 |
| Dothiora sp. KBP YE-0249 | 1.17 | Kwonienia pini | 2.93 |
| Groenewaldozyma tartarivorans | 0.20 | Leucosporidium scottii | 2.83 |
| Hanseniaspora thailandica | 3.42 | Moeziomyces aphidis | 2.05 |
| Hanseniaspora uvarum | 14.96 | Moeziomyces rugulosus | 1.47 |
| Metschnikowia chrysoperlae | 0.29 | Naganishia albida | 2.05 |
| Metschnikowia pulcherrima | 22.87 | Naganishia globosa | 0.10 |
| Metschnikowia reukaufii | 0.10 | Papiliotrema flavescentes | 5.38 |
| Meyerozyma carribica | 19.94 | Rhodosporidiobolus colostri | 5.67 |
| Meyerozyma guillermondii | 10.95 | Rhodorula babjevae | 7.82 |
| Nadsonia starkeyi-henricii | 0.78 | Rhodorula graminis | 2.54 |
| Taphrina populina | 0.10 | Rhodorula mucilaginosa | 16.52 |
| Taphrina sp. | 0.10 | Rhodorula pacifica | 0.68 |
| Wickerhamomyces onychis | 0.68 | Rhodorula taiwanesis | 0.49 |
| Yarrowia deformans | 0.29 | Sporidiobolus metaroseus | 3.71 |
| Yarrowia galli | 4.11 | Sporidiobolus pararoseus | 0.29 |
| Sporobolomyces roseus | 0.78 | Symmetrospora coprosmae | 0.10 |
| Sympodiomycopsis kandeliae | 5.96 | Tremella sp. KBP YE-0255 | 0.10 |
| Vishniacozyma carncens | 5.28 | Vishniacozyma phoenicis | 0.59 |
| Vishniacozyma sp. KBP YE-0008 | 0.88 | Vishniacozyma sp. KBP YE-0724 | 1.47 |
| Vishniacozyma tephrensis | 0.20 | Vishniacozyma victoriae | 5.47 |

An analysis of the frequency of occurrence (proportion of samples containing yeast species)
showed the predominance of ascomycetes yeasts in the endophytic community of all studied crops: *A. pullulans*, *C. zeylanoides*, *D. fabryi*, *D. hansenii*, *H. uvarum*, *M. pulcherrima*, *Mey. carribica*, *Mey. guilliermondii*, and *Rh. mucilaginosa*. Among these predominance yeasts with the frequency of occurrence more than 10% only *Rh. mucilaginosa* belongs to basidiomycetes.

A comparison of the dominant yeasts in crops of domestic and foreign origin according to their frequency of occurrence showed in table 2. For *A. pullulans*, *F. magnum*, *H. uvarum*, *R. colostri*, *Rh. babjevae*, *Sym. kandeliae* and *V. victoriae*, the occurrence in imported and domestic cultures had similar values. *B. alba*, *Cystobasidium sp.*, *K. pini*, *N. albida*, and *P. flavescens* were found predominantly in domestic fruits and vegetables, while *C. parapsilosis*, *C. zeylanoides*, *D. fabryi*, *D. hansenii*, *F. wieringae*, *Mey. carribica*, *Mey. guilliermondii*, *Rh. mucilaginosa*, and *V. carnescens* – predominantly in the imported crops. This separation does not show all the features of yeast communities, and differentiation of endophytic yeast communities in crops from different regions does take place, especially for rare yeast species. This is especially evident for fruits from tropical regions. For example, the species *H. thailandica* was found only in fruits from Vietnam; the yeast-like fungi of the genus *Graphiola* were isolated only in dates.

**Table 2. The frequency of occurrence species (%) in crops of various origin.**

| Species                 | Import | Russia |
|-------------------------|--------|--------|
| *Aureobasidium pullulans* | 19.04  | 35.86  |
| *Bullera alba*          | 1.42   | 9.96   |
| *Candida parapsilosis*  | 6.99   | 3.19   |
| *Candida zeylanoides*   | 31.61  | 9.56   |
| *Cystobasidium sp. KBP YE-0710* | 1.94 | 8.37 |
| *Debaryomyces fabryi*   | 23.96  | 6.77   |
| *Debaryomyces hansenii* | 12.95  | 5.18   |
| *Filobasidium magnum*   | 8.03   | 6.77   |
| *Filobasidium wieringae*| 5.18   | 2.79   |
| *Hanseniaspora uvarum*  | 16.06  | 11.55  |
| *Kwoniella pini*        | 0.39   | 10.76  |
| *Metschnikowia pulcherrima* | 25.13 | 15.94 |
| *Meyerozyma carribica*  | 23.19  | 9.96   |
| *Meyerozyma guilliermondii* | 14.12 | 1.20 |
| *Naganishia albida*     | 0.39   | 7.17   |
| *Papiliotrema flavescens* | 4.27 | 8.76 |
| *Rhodosporidiobolus colostri* | 5.44 | 6.37 |
| *Rhodotorula babjevae*  | 7.90   | 7.57   |
| *Rhodotorula mucilaginosa* | 18.78 | 9.56 |
| *Symposiomyces kandeliae* | 6.48 | 4.38 |
| *Vishniacozyma carnescens* | 6.87 | 0.40 |
| *Vishniacozyma victoriae* | 5.96 | 3.98 |

Obviously, not all of the yeast species we have isolated are true endophytes. We should consider some of them as contaminating species. Among the contaminants, clinically significant yeasts deserve special attention.

Clinically significant yeasts of the BSL-2 level were isolated from fruits and vegetables of foreign and domestic origin. Among such yeasts, *C. parapsilosis* was the most common species (tables 1 and 2). This is a species from the group of opportunistic and allergenic yeasts; it is a regular part of the normal microbiota of human skin. *C. parapsilosis* is also isolated from the skin affected by mycoses;
in case of immune system disorders, it causes damage to the skin and mucous membranes [10]. *C. parapsilosis* has previously been found in the yeast complex of ripe apple and pear fruits growing in city parks and along highways, as well as in some contaminated soils in the city of Moscow [9, 11]. In the current research *C. parapsilosis* often was found in crops imported to Russia rather than in domestically grown (table 2). The high abundance of this species may indicate the presence of unfavorable conditions for the growth of agricultural crops associated with a high anthropogenic load [12].

The analysis of *C. parapsilosis* abundance (proportion of yeast species based on colony counts) in the yeast communities of the studied crops demonstrates its significant representation among vegetables, i.e. root vegetables, tubers and melons (table 3). Perhaps, such a high abundance in vegetables is associated with violations in the cultivation of crops and the use of unprepared fertilizers from sewage sludge or manure.

**Table 3.** Abundance (A > 1%) of *C. parapsilosis* and *Mey. guilliermondii* in studied crops.

| Country | Crop          | Candida parapsilosis A, % | Country | Crop          | Meyerozyma guilliermondii A, % |
|---------|---------------|---------------------------|---------|---------------|-------------------------------|
| India   | beet          | 47.77                     | Vietnam | longan        | 99.80                         |
| Russia  | beet          | 33.65                     | Egypt   | orange        | 33.63                         |
| Kenya   | avocado       | 23.38                     | Russia  | beet          | 19.63                         |
| Egypt   | potato        | 12.97                     | Chile   | mandarin      | 18.92                         |
| Azerbaijan | potato      | 11.54                     | Turkey  | peach         | 18.31                         |
| Pakistan| potato        | 5.00                      | Kazakhstan | cucumber    | 17.32                         |
| Russia  | vegetable marrow | 3.44                   | Spain   | mandarin      | 15.29                         |
| Kyrgyzstan | beet         | 3.22                      | India   | beet          | 15.22                         |
| Moldova | carrot        | 2.25                      | Spain   | lemon         | 11.84                         |
| China   | vegetable marrow | 2.14                 | Belarus | apple         | 10.99                         |
| Spain   | lemon         | 1.81                      | Uzbekistan | grape       | 4.50                          |
| Turkey  | eggplant      | 1.70                      | China   | apple         | 4.49                          |
| Iran    | eggplant      | 1.58                      | Turkey  | cucumber      | 3.42                          |
| Turkey  | vegetable marrow | 1.40                 | Iran    | pear          | 3.24                          |
| Spain   | tangerine     | 1.39                      | Israel  | pepper        | 3.22                          |
|         |               |                           | Argentina | apple        | 2.73                          |
|         |               |                           | Chile   | apple         | 2.47                          |
|         |               |                           | Turkey  | pear          | 1.94                          |
|         |               |                           | Iran    | eggplant      | 1.20                          |
|         |               |                           | China   | zucchini      | 1.04                          |
|         |               |                           | Turkey  | pumpkin       | 1.03                          |

Among the endophytic yeasts isolated from agricultural crops, in addition to *C. parapsilosis*, we should mention the clinically significant species *Mey. guilliermondii* [13]. Likewise, in case of immune system disorders, it can also be a causative agent of candidal infection. This species we previously found in the urban soils in some southern regions of Russia with a high anthropogenic load [14]. *Mey. guilliermondii*, as well as *C. parapsilosis*, was often isolated from imported fruits and vegetables to Russia rather than domestically grown ones.

5. Conclusion
As noted earlier, the presence of opportunistic and clinically significant yeasts in natural samples may indicate a high anthropogenic load and pollution of the natural environment [12]. We consider that *C. parapsilosis* and *Mey. guilliermondii* belong to such contaminant species. An additional concern is the fact that many natural strains of opportunistic *Candida* and *Meyerozyma* yeasts, including those that are isolated from the internal tissues of fruits and vegetables, are increasingly showing resistance
to widely used antibiotics, that became a substantial clinical problem [15]. In addition, the questions remain, where and how the contamination occurred, and whether these cultures are adapted to the endophytic lifestyle. These questions are requiring further research. However, our results demonstrated the need for additional microbiological control of agricultural crops and the development of new standards that assess the presence of clinically significant yeasts.

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