Title: Unexplored Yeast diversity in Seed Microbiota

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Authors contribution: M. Marchi, N. Guschinskaya and M. Simonin contributed to the conception and design, analysis, interpretation of data presented in the publication, as well as to the drafting of the manuscript. S. Aligon, T. Lerenard and J. Lecorff harvested seeds from the wild Brassicaceae and isolated microorganisms from them. M. Simonin, M. Briand and C. Marais obtained and analyzed the metabarcoding data. A. Bosc-Bierne, M. Marchi and N. Guschinskaya isolated yeast from crops seed. A. Bosc-Bierne and M Marchi obtained molecular typing and M. Marchi carried out the taxonomic affiliation. A. Bosc-Bierne, N. Guschinskaya and A. Rolland contributed to the macroscopic and microscopic characterization of the yeast collection. L Gouka, V. Cordovez, T. Guillemette and P. Simoneau revised the draft manuscript.

Running Title: Unexplored Yeast diversity in Seed Microbiota

Keywords: Seed microbiota, yeast, yeast-like

Take away message:

- 219 yeasts isolated from seeds and seedlings of diverse plant species
- Most isolates are Basidiomycota yeasts, especially of the Tremellomycetes class
- Ascomycota yeasts are rarely isolated from the seed environment
- The most frequently isolated yeasts belong to *Holtermaniella*, *Vishniacozyma*, *Filobasidium* and *Sporobolomyces* genera
- The collection is representative of taxa found in seed microbiota of multiple plant species

Abstract

Yeasts are known to be fantastic biotechnological resources for medical, food, and industrial applications, but their potential remains untapped in agriculture, especially for plant biostimulation and biocontrol. In particular, yeasts have been reported as part of the core microbiome of seeds using next generation sequencing methods, but their diversity and functional roles remain largely undescribed. This study aimed to fill this knowledge gap by characterizing the diversity of seed-associated yeasts across nine

plant species (crops and non-cultivated species) using culturomics, microscopy, and metabarcoding.

Our results show that seed-associated yeasts largely belong to Basidiomycota phylum and more particularly to the Tremellomycetes class. This yeast collection covers 15 genera (2 of Ascomycota and 13 of Basidiomycota). Out of the 219 isolates described, the most frequently isolated yeasts were *Holtermaniella*, *Vishniacozyma*, *Filobasidium, Naganishia* and *Sporobolomyces.* The yeasts from these dominant genera were isolated from multiple plant species (4 to 8), except for *Naganishia* which only originated from *Solanum lycopersicum L.*. These results are also consistent with the fact that these dominant taxa were recently identified as members of the core seed microbiome, indicating their high prevalence and abundance across diverse plant hosts and environments. Compared to previous plant yeast diversity surveys, the members from Ascomycota are less frequent in seeds and only represented here by the *Aureobasidium* and *Taphrina* genera. Altogether, these results suggest that yeasts are generally well-adapted to the aboveground habitats of plants, but seeds represent a specific habitat that diverse Basidiomycota yeasts can colonize.

Introduction

There is increasing awareness of the role of plant microbiota in plant fitness and health (Compant et al., 2016; Trivedi et al., 2020). Many examples of the beneficial effects of plant-microbiome interactions originate from studies on the rhizosphere and phyllosphere, demonstrating higher nutrient acquisition, abiotic stress tolerance or disease resistance (de la Fuente Cantó et al., 2020; Vannier et al., 2019). More recently, research has been conducted on the diversity and role of seed microbiota, which has so far been neglected despite its role as a primary inoculum of plants (Abdelfattah et al., 2023; Bergmann et al., 2023; Shade et al., 2017).

Seeds are crucial for crop settlement and food production. However, only a limited number of studies have investigated the role of seed-associated microorganisms in plant germination, emergence, and tolerance to soil-borne or seed-transmitted pathogens (Figueiredo dos Santos et al., 2021; Lamichhane et al., 2018; Matsumoto et al., 2021; Rochefort et al., 2019; Simonin et al., 2023). Moreover, the available studies have focused primarily on the seed-associated bacterial community, ignoring the highly diversified fungal component. Indeed, a specificity of the seed habitat is to host similar levels of fungal and bacterial diversity, whereas other plant or soil habitats are generally dominated by bacterial taxa (Simonin et al., 2022). A recent meta-analysis of seed microbiota across 50 plant species reported the detection of more than 2000 seed fungal taxa (based on the ITS1 region), including 16 core taxa present in more than 20 plant species (Simonin et al., 2022). These core taxa were extremely abundant in most samples and were mainly affiliated with the filamentous fungi genera, *Cladosporium* and *Alternaria*. However, an interesting observation was that six seed core taxa were yeasts, affiliated with the genera *Vishniacozyma*, *Filobasidium*, *Sporobolomyces*, and *Aureobasidium*.

Yeasts are widely recognized as valuable biotechnological resources in medical, food, and industrial applications. However, their potential in agriculture,

particularly for plant biostimulation and biocontrol, remains underexplored, except for their use in post-harvest management (Ezzouggari et al., 2024; He et al., 2024). This is linked to the lack of research on plant-associated yeasts, despite their frequent detections as core or hub taxa in various plant habitats using next generation sequencing methods (Agler et al., 2016; Almario et al., 2022; Noel et al., 2022; Simonin et al., 2020). Available research on plant yeasts has been conducted in the phyllosphere, nectar, and fruit compartments (Gouka et al., 2022; Jacquemin et al., 2020) indicating adaptation to aboveground plant habitats. Still, characterization of the diversity and functions of yeast colonizing seeds has received even less attention, with few yeast isolates, genomes, or functional studies available (Bhuyan et al., 2023; Nelson et al., 2018).

This study aimed to fill this knowledge gap by characterizing the diversity of seed-associated yeasts across nine plant species (crops and non-cultivated species) using culturomics, microscopy, and metabarcoding. The following questions were addressed: What is the taxonomic diversity of the yeast and yeast-like microorganisms isolated from seeds? Which yeast taxa are shared or specific to a given plant species? How our yeast collection is representative of yeast diversity detected by metabarcoding analyses (i.e., core and flexible taxa)?

Fungal isolation was performed on seeds from three wild Brassicaceae species (*Capsella bursa-pastoris*, *Draba verna* and *Cardamine hirsuta*), and on seeds and seedlings (grown *in vitro*) from five crops (*Phaseolus vulgaris*, *Raphanus sativus*, *Brassica napus* L., *Solanum lycopersicum* L. and *Triticum aestivum*). Taxonomic and morphological identification of yeasts was conducted using ITS/D1-D2 sequencing, combined with macroscopic and microscopic observations.

We then determined if the yeast taxa isolated in our collection were commonly found in seed samples using the existing Seed Microbiota Database based on ITS1 metabarcoding. This analysis permitted to assess the retrieval of core, abundant, and rare yeast taxa using culturomics.

Material and Methods

Seed material.

Seeds from five crops and three wild *Brassicaceae* were used for yeast isolation. All details about the plant material are listed in the Table S1.

Isolation procedure.

Yeasts were isolated from two types of biological materials (seed and seedling) using a classical isolation method (dropping seed on agar plates) or with an alternative method using seed and seedling macerates (as described below).

Classical isolation method.

- *Wild Brassicaceae*

One hundred seeds from each of the wild *Brassicaceae* plants were placed in 20 Petri dishes (90-mm diameter). Ten Petri dishes contained a Potato Dextrose Agar (PDA) (39 g/L, BIOKAR Diagnostics) including streptomycin (500 mg/L, Sigma-Aldrich) and

10 dishes contained a semi-selective medium adapted from Dhingra and Sinclair (1985) with PDA (39 g/L) including 150 mg/L of Rose Bengal and 250 mg/L of chloramphenicol (Sigma-Aldrich). Plates were incubated at 22°C in the dark and monitored daily to collect yeasts (Lerenard et al., 2024). For these Brassicaceae species only seeds, not seedlings were used for isolation.

Alternative isolation process from seeds and seedlings.

- *Crops*

Seeds from crop species and seedling (germinated in sterile conditions as described by Barret et al., 2015) were soaked in a solution of Phosphate Buffered Saline including 0.05% of Tween 20 (Fisher Scientific, 12673967) to collect both seed epiphytes and endophytes microorganisms following International Seed Testing Association (ISTA) guidelines. Three biological repetitions were used for *Solanum lycopersicum L.* (including 1g, approx. 360 seeds and 6 seedlings), *Triticum aestivum* (including 4g, approx. 100 seeds and 6 seedlings), *Raphanus sativus* (including 4 g, approx. 1000 seeds) and *Brassica napus L.* (including 2 g, approx. 400 seeds and 15 seedlings) and five biological repetitions for *Phaseolus vulgaris* (including 4 to 18 g, approx. 30 seeds and 30 seedlings).
Maceration volumes for seeds were 4, 8, 8 and 4 mL for *Solanum lycopersicum L.*, *Triticum aestivum*, *Raphanus sativus* and *Brassica napus L.* respectively. For *Phaseolus vulgaris*, given the large size variation of the seeds, the maceration volume was 2 mL/g of seeds (ranging from 7 to 36 mL). Seed maceration was conducted under agitation at 6°C for 2h30 for *Raphanus sativus* and *Brassica napus L.,* and 16h overnight for *Solanum lycopersicum L.*, *Triticum aestivum* and *Phaseolus vulgaris.* For *Solanum lycopersicum L*. and *Triticum aestivum*, after maceration, the seeds were placed in a stomacher machine for 3 minutes. For these plant species, seedlings were crushed in 2 mL sterile water to obtain a homogeneous solution.

For yeast strain isolation, the seed and seedling macerates obtained were filtered using filtration units with mesh woven filters 31 µm (SEFAR NITEX 03-31/24). The supernatant containing the microorganisms that went through the filters (mainly bacteria) was discarded to isolate only larger organisms present on the filters (yeast and filamentous fungi). The filter was washed in 5 ml of sterilized osmotic water. The suspension was diluted in liquid malt 1.5% with tetracycline 10 ml/L (Sigma-Aldrich) and streptomycin 50 mg/L (Sigma-Aldrich) to prevent bacterial growth and dilutions (1/10, 1/50, 1/100, 1/200 and 1/500) were done in 96-well plates. Plates were then incubated at 23°C for one week.
The following steps of the isolation procedure focused on microplates presenting microbial growth in half or less than half of the wells to maximize the chance to have pure culture in each well (Zhang et al., 2021). When a well presented growth, the microbial suspension was collected using a micropipette and deposited on a 1.5% malt agar plate. Plates were incubated at 23°C until the observation of fungal development. Non-filamentous isolates were selected, and yeast's shapes were checked using a

bright-field microscope. Yeast isolates were purified, and strains were stored at - 80°C in glycerol 30%.

PCR-based method (ITS and D1D2) for yeast identification.

Yeast identification was carried out based on the sequence of the D1/D2 region of the large 26S rDNA subunit using primers LROR (5'-ACCCGCTGAACTTAAGC-3') and LR5 (5'-TCCTGAGGGAAACTTCG-3') (Vilgalys & Hester, 1990) and the internal transcribed spacer (ITS) region using primers NS7 (5'-GAGGCAATAACAGGTCTGTGATGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATG-3') (White et al., 1990). Gene sequences were amplified by colony PCR using GoTaq® G2 Hot Start Polymerase from Promega Corporation in concordance with manufacturer's instructions and Sanger sequenced using Eurofins Genomics facilities.

Sequence treatment and tree inference.

All sequence alignments were produced with the server version of MAFFT (Katoh et al., 2019), with a gap open penalty of 1.0 and a gap extension penalty in the range of 0.05 to 0.1. We obtained branch support with the ultrafast bootstrap (Hoang et al., 2018) implemented in the maximum likelihood phylogenetic analysis using IQ-TREE web server (Trifinopoulos et al., 2016). Tree was obtained using Interactive Tree Of Life (iTOL)v6 (Letunic & Bork, 2021) and MEGA11: Molecular Evolutionary Genetics Analysis version 11 (Tamura et al., 2021).

Sequences accession numbers of isolates from this study and reference strains used for phylogenetic analyses are listed in Table S2 and Table S3 respectively.

Yeast Morphological Description.

Descriptions of agar-grown yeast colonies were done on Yeast Malt extract Agar (YMA) plate including texture, colour, surface, elevation and margin as described by (Kurtzman et al., 2011). Culture colour assessments were subjectively coded based on observations from 10-day-old cultures on YMA at 22°C in the dark.

Microscopic images for each yeast type strain were obtained using a transmission microscope with Differential Interference Contrast (DIC) optics (Axio Imager Z2, Carl Zeiss, Oberkochen, Germany) and ZenBlue software at x100 magnification (ROI 1024x). For sample preparation for each isolate, a single colony was picked with a sterile toothpick and transferred into a tube containing 50µL of sterile water. A 20µL drop of suspension was then placed between a microscope slide and coverslip for observation.

Prevalence and abundance of yeast isolates in seed microbiota.

To determine if the yeast taxa isolated in our collection were commonly found in seed samples, we took advantage of the existing Seed Microbiota Database (Simonin et al., 2022). This database compiles metabarcoding data from multiple seed microbiota studies, including 17 studies that characterized fungal diversity using ITS1 marker, generating data from 1037 seed samples and 34 plant species. We used the ITS1 sequences obtained from Sanger sequencing to assign taxonomy of the isolates and

trimmed them to the same ITS1 fragment compiled in the metabarcoding meta-analysis (229 bp). We obtained the final ITS1 Amplicon Sequencing Variant (ASV) corresponding to each of our isolates. Because of the short ITS1 fragment considered (genus-level resolution), several isolates possessed the same ASV and for all isolates we obtained a total of 36 unique ASVs. We then determined for each isolate ASV: the prevalence (number of samples detected), the relative abundance, and the number of plant species in which they were detected in the Seed Microbiota Database. We also compared them to the list of identified "core" fungal taxa identified in this previous study. Here, ASVs were identified as core taxa if they were detected in ≥ 20 plant species.

The script for the analysis and figures generated in R is available here: https://github.com/marie-simonin/Yeast_Diversity_Seed_Microbiota.

Results.

Isolation of a large diversity of yeasts from seeds and seedling.

A total of 219 yeasts were isolated. Phylogenetic tree inference (Figure 1) includes a total of 443 strains including reference strains from Gouka et al., (2022; Li et al., (2020); Tedersoo et al., (2024) and Vu et al., (2016).

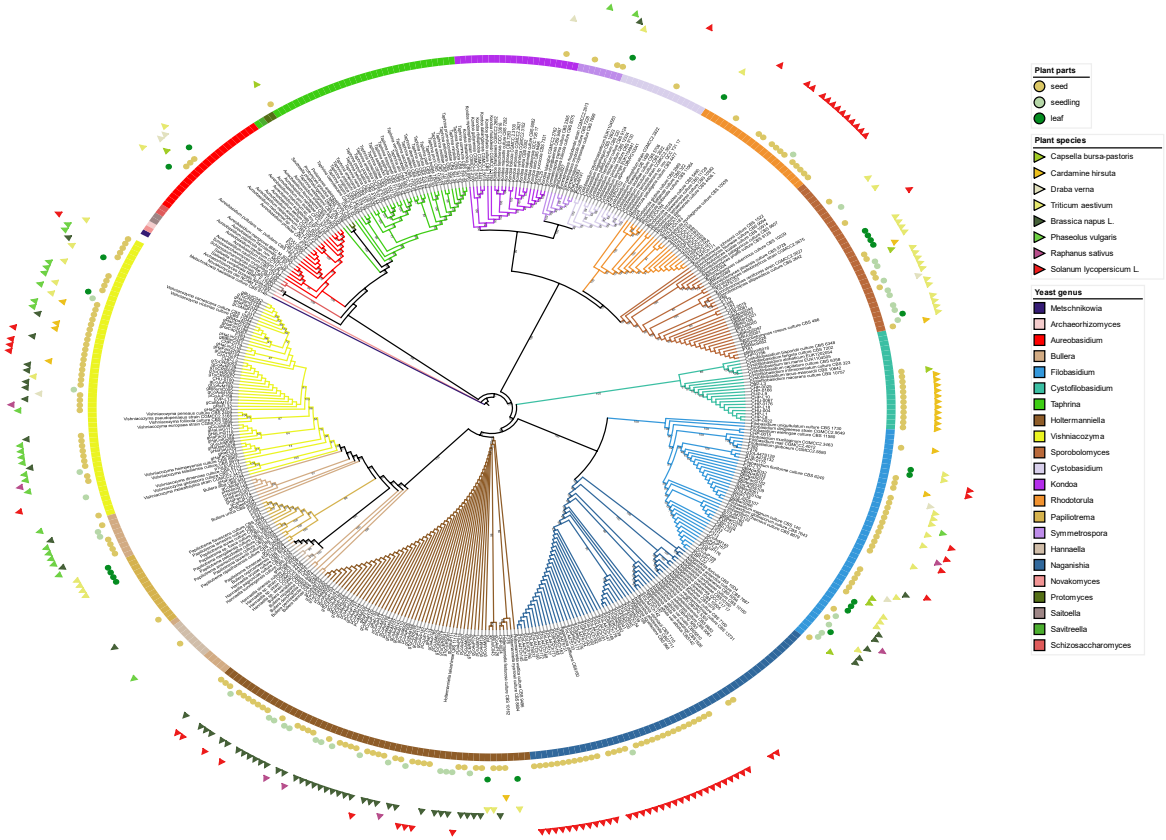

Figure 1: Circular phylogenetic tree of the 219 strains from seed and seedlings yeast collection.
Strains isolated on seed (full brown circle) of *Capsella bursa-pastoris* (light green triangle), *Draba verna* (grey triangle) and *Cardamine hirsuta* (orange triangle), and on seeds and seedlings (full light green circle) from five crops *Phaseolus vulgaris* (green triangle), *Raphanus sativus* (purple triangle), *Brassica napus L.* (black triangle), *Solanum lycopersicum L.* (red triangle), and *Triticum aestivum* (yellow triangle). *Metchnikowia* stains were used as outgroup. References strains from Li et al. (2020), Vu et al. (2016) and Tedersoo et al. (2024) were used. The tree includes also some strains from Gouka et al. (2022), isolated from *Triticum aestivum* phyllosphere (yellow triangle

for *Triticum aestivum* and dark green circle for the leaf). Phylogeny is based on the alignment and the concatenation of ITS and D1/D2 domain partial sequences. The alignments were produced with the server version of MAFFT (Katoh et al., 2019), and the maximum likelihood phylogenetic analysis using IQ-TREE web server (Trifinopoulos et al., 2016) and branch support with the ultrafast bootstrap (Hoang et al., 2018). The tree figure was obtained using Interactive Tree Of Life (iTOL)v6 (Letunic & Bork, 2021).

All the yeasts belong to the fungi kingdom to the phyla Ascomycota and Basidiomycota. Our yeast collection includes 2 genera from Ascomycota phylum (with n=4 strains) and 13 genera from Basidiomycota phylum (with n=215) (**Figure 1, Table 1 and Table S1**).

<u>Table 1:</u> List of yeast taxon and species identified in seed collection.

Phylum	Class	Order	Family	Genus	Species	Strains
Ascomycota	Dothideomycetes	Dothideales	Saccotheciaceae	*Aureobasidium*	*proteae*	CBU-L21 ; EVU0035 ; EVU-0050
	Taphrinomycetes	Taphrinales	Taphrinaceae	*Taphrina*	*sp.*	CBU-L20
Basidiomycota	Tremellomycetes	Holtermanniales		*Holtermaniella*	*takashimae*	gCoAvM132 ; gCoAvM135 ; gCoAvM143 ; gCoAvM144 ; gCoAvM188 ; gCoAvP127 ; gCoAvP163 ; gCoAvP164 ; gCoAvP165 ; gCoAvP178 ; gCoAvP179 ; gCoAvP180 ; gCoAvP187 ; gCoJuM092 ; gCoJuM093 ; gCoJuP192 ; gCoMoM107 ; gCoMoM110 ; gCoMoM120 ; gCoMoP111 ; gCoMoP112 ; gCoMoP174 ; pCoAvP007 ; pCoAvP008 ; pCoAvP009 ; pCoAvP010 ; pCoAvP014 ; pCoAvP015 ; pCoJuM020 ; pCoJuM021 ; pCoJuM022 ; pCoJuM023 ; pCoJuP157 ; pCoJuP175 ; pCoMiM004 ; pCoMiM005 ; pCoMiM006 ; gRaFL33 ; gRaFL34 ; gRaFL36 ; gToCERS029 ; gToCERS030 ; gToCERS031 ; gToCERS074 ; gToCERS075 ; gToCERS076 ; gToCERS079 ; gToCERS080 ; gToCERS081
					festucosa	CHP-0024
					sp.	gBlJulC043 ; gCoAvP126
		Filobasidiales	Filobasidiaceae	*Filobasidium*	*wieringae*	CHP-0015
				Filobasidium	*floriforme or magnum*	EVP-L12 ; gBlApaS032 ; gBlApaS033 ; gBlApaS035 ; gBlApaS037 ; gBlBarS061 ; gBlBarS066 ; gHaFacG182 ; gRaFL35 ; gToLA20S106 ; gToLA20S107 ; gToLA20S109
				Filobasidium	*sp.*	CBP-0224 ; CBP-L22 ; CBP-L23 ; EVP-0110 ; gBlBarC055 ; gCoAvM145 ; gCoMiP160 ; gRaFL31 ; gToLA47S122 ; gToLA47S129 ; gToLA47S132 ; pCoJuP159 ; pCoJuP167 ; pCoJuP176 ; pCoJuP177 ; CHP-0175
				Naganishia	*diffluens*	gToLA47Ch145 ; gToLA47Ch146 ; gToLA47Ch147 ; gToLA47Ch148 ; gToLA47Ch150 ; gToLA47Ch168 ; gToLA47Ch172 ; gToLA47Ch173 ; gToLA47S036 ; gToLA47S037 ; gToLA47S127 ; gToLA47S131 ; gToLA47S133 ; gToLA47S134 ; gToLEVCh156 ; gToLEVCh160 ; gToLEVCh181 ; gToLEVCh182 ; gToSPRCh163 ; gToSPRCh165 ; gToSPRCh175 ; gToSPRCh177 ; gToSPRCh179 ; pToP24Ch192
				Naganishia	*sp.*	gToSPRCh166; gToCRIS039; gToFERS105 ; gToFERCh154 ; gToCRIS041 ; gToSPRS002 ; gToLEVS045 ; gToCERCh139 ; gToLEVCh187 ; gToLA47Ch142
			Cystofilobasidiaceae	*Cystofilobasidium*	*sp.*	CBP-L3 ; CHP-0022 ; CHP-0025 ; CHP-0166 ; CHP-0176 ; CHP-L1 ; CHP-L10 ; CHP-L18 ; CHP-L8 ; CHP-L9 ; CHU-004 ; CHU-0067
		Tremellales	Rhynchogastremataceae	*Papiliotrema*	*sp.*	gCoJuP099
			Bulleribasidiaceae	*Vishniacozyma*	*victoriae*	CHP-0179 ; CHP-L2 ; CHU-0071 ; CHU-0100 ; EVP-L14 ; gBlApaS039 ; gBlBarC051 ; gBlSymC005 ; gCoAvM134 ; gCoAvM139 ; gCoAvM185 ; gCoAvM189 ; gCoJuP097 ; pCoJuP158 ; gCoMoM101 ; gHaCapG067 ; gHaFacG184 ; gHaFacG188 ; gHaFacG208 ; gHaLinG116 ; pHaLinG336 ; gRaFL32 ; gToCRIS052 ; gToCRIS055 ; gToCRIS057 ; gToCRIS059
				Vishniacozyma	*carnescens*	gBlJulC042 ; gCoMoP172 ; gHaFlaG334 ; gHaVanG148 ; gToCRIS058
				Vishniacozyma	*sp.*	gCoJUM081 ; gCoJuM089 ; gHaCapG073 ; gHaCotG288 ; gHaDeeG028 ; gHaFacG194 ; gHaFacG199 ; gHaFacG200 ; gHaLinG110 ; gHaLinG117 ; pHaFlaG335 ; pToLEVS111 ; gRaFL37
				Hannaella	*sp.*	gHaCaG056
			Bulleraceae	*Bullera*	*alba*	gCoAvP128 ; gCoMoM118 ; gHaDeeP004 ; gHaFacG174 ; gHaFacG201 ; pHaVanG128 ; pHaVanG149
	Agaricostilbomycetes	Agaricostilbales	Kondoaceae	*Kondoa*	*sp.*	EVI-L25
	Cystobasidiomycetes	Cystofilobasidiales	Cystobasidiaceae	*Cystobasidium*	*sp.*	gToFERCh152
				Symmetrospora	*sp.*	gCoAvM141 ; gHaCotG283 ; gHaDeeG027
	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	*Sporobolomyces*	*roseus*	CHU-0015 ; CHP-0186 ; CPB-0081 ; EVI-L24 ; gBlILLS108 ; gBlRenC021 ; pBlApaS044 ; pBlBarS052 ; pBLJulS001 ; pBlJulS005 ; pBlRecS082 ; pBlRubS097 ; pBLSymC026 ; pBlSymS016
				Sporobolomyces	*ruberrimus*	pBlRenS069
				Sporobolomyces	sp.	gBlSymC004; gToLA47S123
			Sporidiobolaceae	*Rhodotorula*	*mucilaginosa*	gToLA47Ch171 ; gToLEVCh157 ; gToLEVCh159 ; gToLEVCh161 ; gToLEVCh180 ; gToLEVCh183 ; gToLEVCh185 ; gToLEVCh188 ; gToLEVCh189 ; gToSPRCh164 ; gToSPRCh176 ; pToP24Ch193
				Rhodotorula	*sp.*	gToLEVCh184

Four genera were highly represented in our collection: *Holtermaniella* (52 isolates), *Vishniacozyma* (45 isolates), *Naganishia* (36 isolates) and *Filobasidium* (29 isolates). The other genera are less represented: *Sporobolomyces* (17 isolates), *Cystofilobasidium* (12 isolates), *Rhodotorula* (11 isolates), *Bullera* (6 isolates), *Aureobasidium* (3 isolates), *Symmetrospora* (3 isolates), *Cystobasidium* (1 isolate),

Hannaella (1 isolate), *Kondoa* (1 isolate), *Papiliotrema* (1 isolate) and *Taphrina* (1 isolate).

Details about isolate's affiliation are synthetized in the Table 1. All the strains were identified at the genus level. Most of them were affiliated at species level (n=143), for 76 isolates the identification is certain at the genus level. Two reasons could explain that result; the lack of reference species in the genus (for example CBU-L20 as member of *Taphrina* sp.) or the genetic markers genes used were not sufficiently resolutive (for example the 12 isolates affiliated to *Filobasidium floriforme* or *F. magnum*).

The phylogenetic tree includes 33 sequences from wheat phyllosphere strains from Gouka et al. (2022) study. Among them, 6 strains (F174, F57, F52, F376, F331, F181) are closed to seed and seedling isolates within *Sporobolomyces roseus*, *Vishniacozyma carnescens*, *Holtermaniella takashimae*, *Holtermaniella festucosa*, *Aureobasidium* species. These results suggest that some members of the plant yeast community could persist during plant development and colonize both leaves and seeds.

A detailed description of the phylogenetic tree is available in Supplementary Results (Taxonomic isolate description and plant origin), including information about strain clustering and ecological information of associated type strains.

Macroscopical and microscopical strains description.

The Figure 2 presents a description of type strains (one by genus) from our yeast collection at macroscopical and microscopical scale. This description is completed by a colony morphology description detailed in the Table S1.

Most colonies cultured on YMA exhibited a butyrous texture. Notably, some colonies displayed mucoid growth (as gToFERCh152 from *Cystobasidium* genus and gToCRIS039 from *Naganishia* genus), often linked to elevated production of extracellular polysaccharides (refer to Table S1 for examples). Additionally, rare occurrences of yellow non-diffusible carotenoid pigments were observed in the isolates from genera *Holtermaniella, Cystofilobasidium* and *Hannaella* (Figure 2 and Table S1). Interestingly, 15 isolates (from *Symmetrospora*, *Sporobolomyces*, *Rhodotorula*) had red colour, the members of these three genera are known to produce red non-diffusible pigments of carotenoid composition (Chreptowicz et al., 2019; Zhao et al., 2019) (Figure 2 and Table S1). Some isolates (as gToFERCh152 close to *Cystobasidium minutum*) were observed to form creamy colonies that may become slimy with age.

Yeasts from our collection have relatively the same size, measuring between 6-10 µm. They are generally distinguished by their round, oval, or elongated shapes, and the nucleus and organelles of each cell can be easily seen through the cell wall and cytoplasmic membrane. It is possible to observe budding yeasts and the scars they leave after binary fission of the entities (Figure 2).

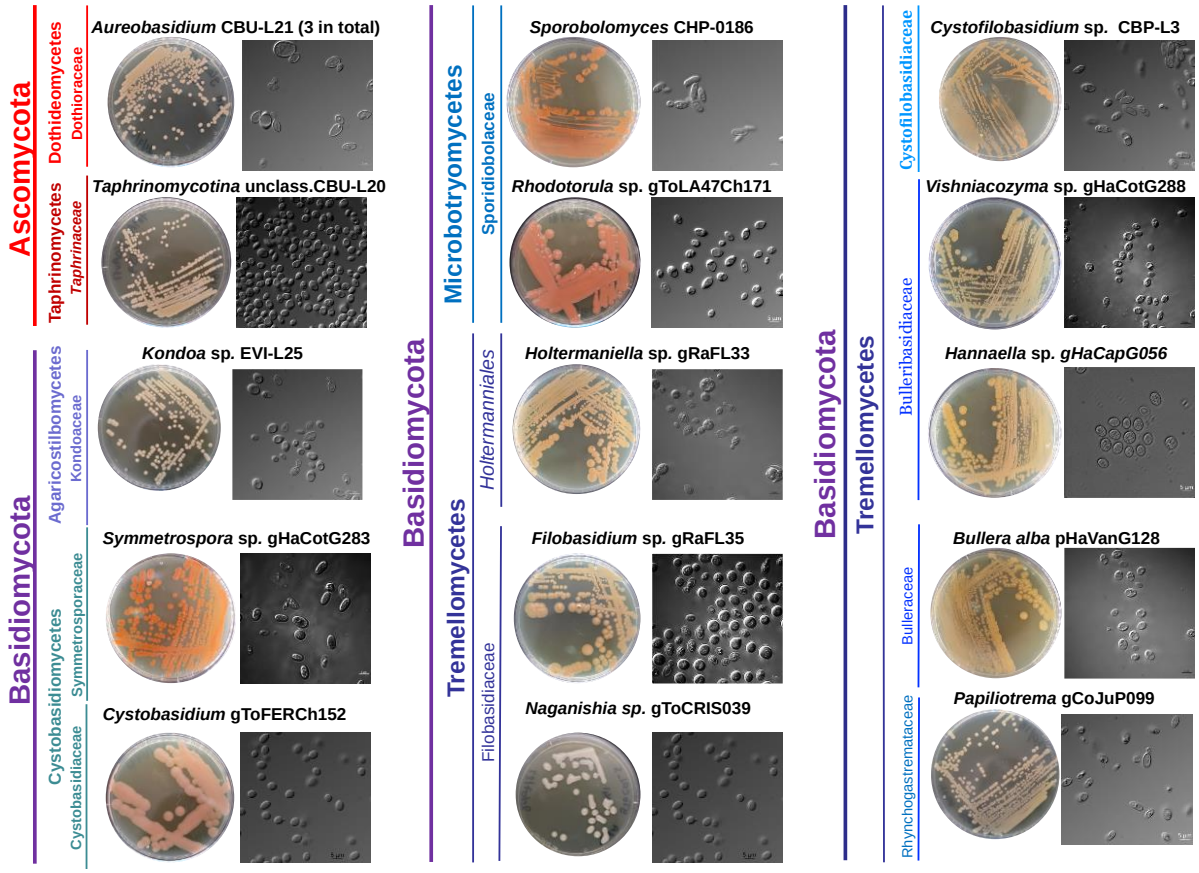

Figure 2: Phenotypic characterization of the selected yeast isolates in macroscopical and microscopical scale.

One isolate is presented for each genus. Strains are classified by phylum, class, family and genus. The pictures of agar-grown yeast colonies on Yeast Malt extract Agar (YMA) were taken after 10 days of incubation at 22°C in the dark. Microscopic images for each yeast type strain were obtained using a transmission microscope with Differential Interference Contrast (DIC) optics x100, ROI 1024, and ZenBlue software (Axio Imager Z2, Carl Zeiss, Oberkochen, Germany), scale 5 µm.

Yeast plant's specificity and ecotype.

Most yeasts from the collection were isolated from seeds (n= 183) and not from seedlings (n=36). Isolates from seed and seedling are distributed across the entire taxonomic tree (Figure 1).

Four genera were highly represented in the collection: *Holtermaniella* (52 isolates), *Vishniacozyma* (45 isolates), *Naganishia* (36 isolates) and *Filobasidium* (29 isolates). The yeasts from these dominant genera were generally isolated from multiple plant species (4 to 8), except for *Naganishia* which only originated from *Solanum lycopersicum L.* Three other genera had between 10 and 20 isolates: *Sporobolomyces* (17), *Rhodotorula* (13), *Cystofilobasidium* (12). *Sporobolomyces* isolates came from 5 plant species, while *Rhodotorula* was specific to *Solanum lycopersicum L.* and *Cystofilobasidium* to wild Brassicaceae (Figure 3). The other ten genera were represented by only a few isolates (1 to 5) generally originating from a single plant species, except for *Aureobasidium* and *Taphrina* (2 plant species).

Only 6 genera were isolated from both seeds and seedlings (grown *in vitro* for 4 crops) and they were the ones the most represented in the collection

(*Holtermaniella*, *Vishniacozyma*, *Filobasidium*, *Naganishia*, *Rhodotorula*, *Sporobolomyces*) (Figure 3).

For detailed information on the origin of each strain, its taxonomic affiliation and sequences, see Table S1 and Table S2.

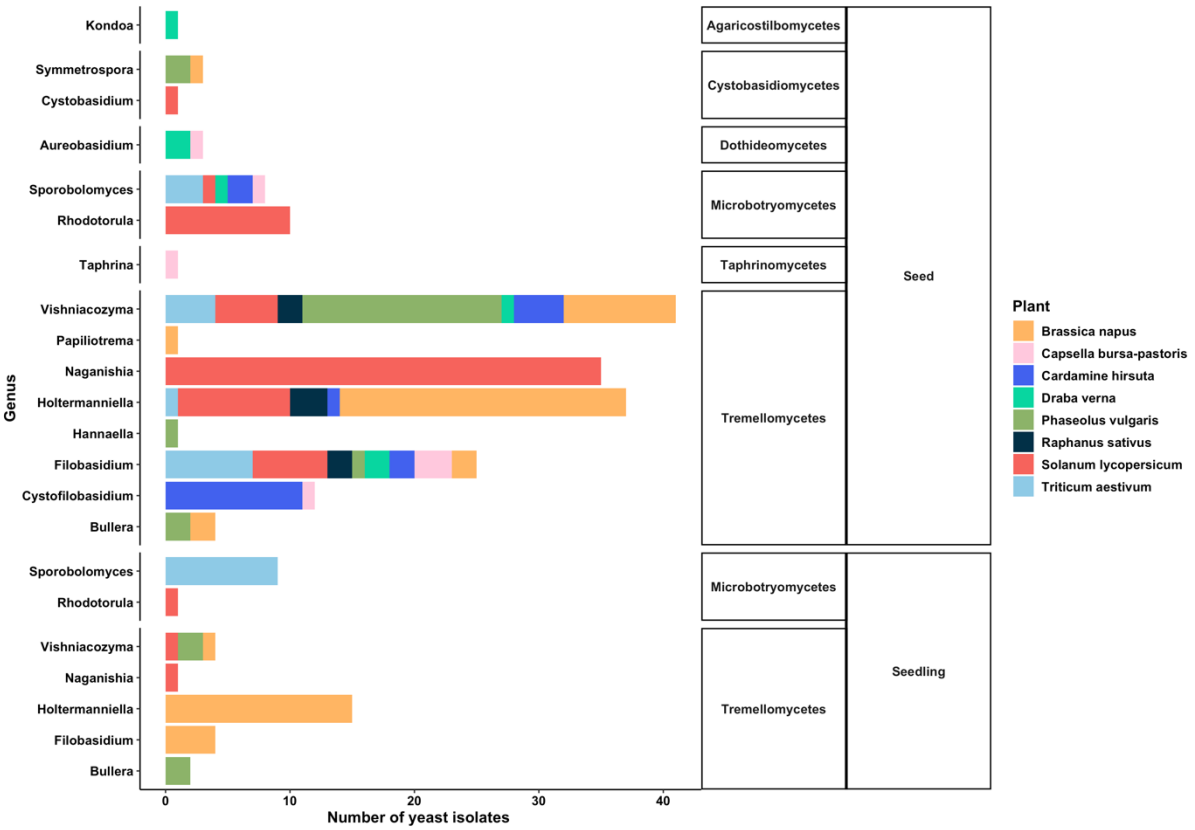

Figure 3: Number of yeast isolates for the 15 genera from our yeast collection, colored by their plant host and organized in function of the plant compartment of isolation and fungal class. Please note that yeast isolation on seedlings was only performed on four species (*Brassica napus*, *Phaseolus vulgaris*, *Solanum lycopersicum* and *Triticum aestivum*).

Yeast isolates are representative of core and flexible taxa found in seed microbiota of multiple plant species.

We took advantage of a recent meta-analysis on seed microbiota diversity to determine the prevalence and abundance of the isolated yeasts across a large diversity of plant species. After extracting the ASVs of our isolates matching the same ITS1 fragment used in the meta-analysis (Seed Microbiota Database), we determined their prevalence, relative abundance and number of plant hosts across all seed samples available (n=1037, Figure 4).

The yeast isolates represented 36 ASVs of which 25 were detected in the meta-analysis (list of detected and non-detected ASVs in Table 2). The isolate ASVs covered a wide range of abundance-occupancy patterns, ranging from ubiquitous (>

60% prevalence) to rare taxa (<1% prevalence). All isolate ASVs were detected in multiple plant species, except two, suggesting the frequent associations of these taxa with seeds originating from various hosts and environments.

In the seed microbiota meta-analysis, core ASVs were identified based on the arbitrary criterion of a detection in more than 20 plant species (Simonin et al., 2022). Sixteen fungal core ASVs were identified, including six yeast ASVs (3 *Vishniacozyma*, 1 *Filobasidium*, 1 *Sporobolomyces*, 1 *Aureobasidium*). Out of the six core ASVs, five of them were present in our yeast collection (1 Vishniacozyma ASV missing) and we often obtained multiple isolates for these taxa (3 to 20). The other 20 ASVs detected in the meta-analysis were considered as flexible taxa given their lower prevalence and generally low relative abundance (< 0.3% across all samples). Interestingly, a significant fraction of the isolate diversity (77 isolates, 11 ASVs) had not been previously detected in the Seed Microbiota Database (Table 1) suggesting their rarity or association to specific hosts or environments. Most non-detected ASVs were represented by a single isolate, except for a *Holtermaniella* ASV for which we obtained 55 isolates from different plant species.

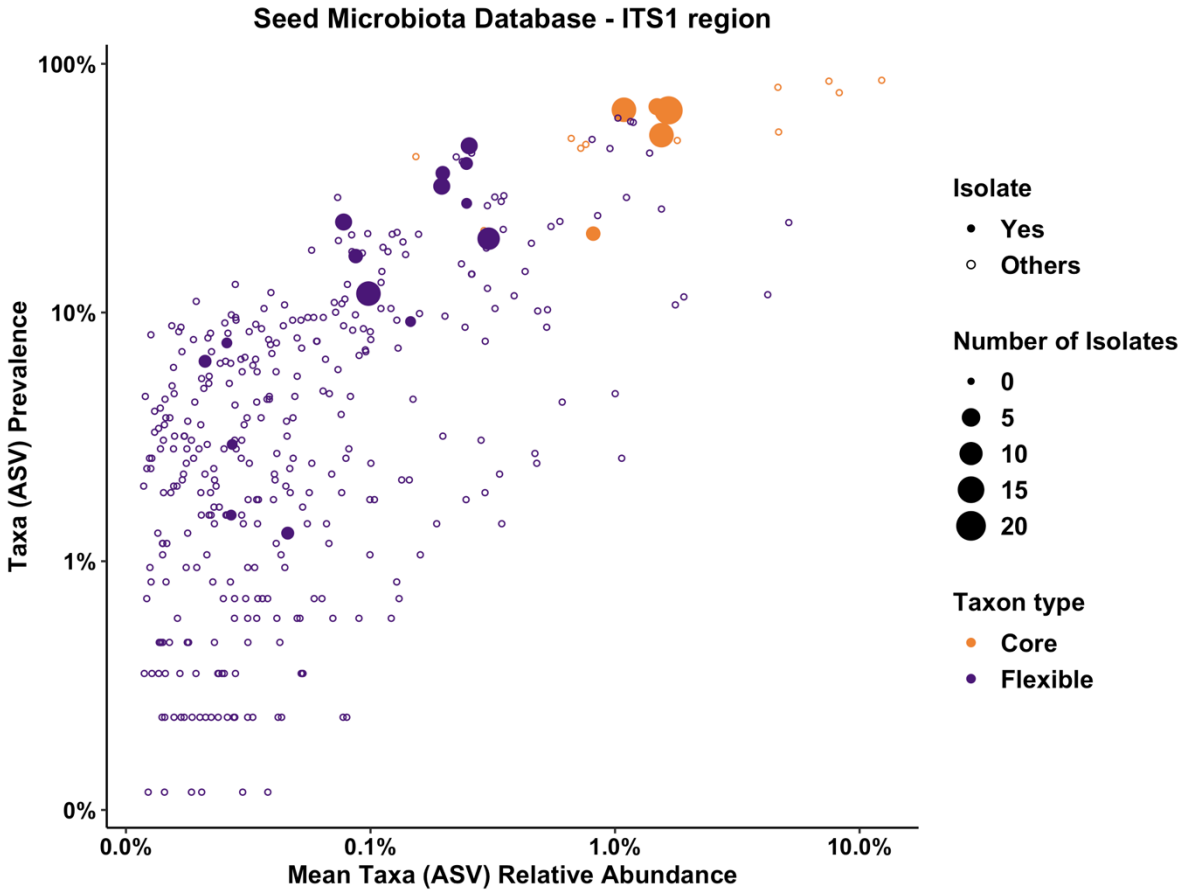

Figure 4: Abundance–occupancy curves of all fungal amplicon sequence variants (ASVs, including filamentous fungi and yeasts) detected in the Seed Microbiota Database (Simonin et al., 2022) using ITS1 region metabarcoding (ASVs < 100 reads were excluded). Each point represents an ASV, and the shapes indicate ASVs for which we have yeast isolates, the points are coloured based on their characterization as a core (detected in > 20 plant species) or flexible taxon. The size of the point is proportional to the number of yeast isolates in the collection.

<u>Table 2:</u> List of yeast ASVs detected or not in Seed Microbiota Database.

The 25 detected ASVs in the database collectively represent 8.4% of the relative abundance of the meta-analysis dataset and include 5 core ASVs (out of the six yeast core ASVs) detected in 24 to 27 plant species.

ASV ID ITS1	Taxon Type	Number of Plant Species	Relative abundance across all samples	Prevalence across all samples	Number of isolates	Phylum	Class	Family	Genus
b8fe600476c530d38dc5d7f3a450cfae	Core	27	1.0609 %	65.33 %	14	Basidiomycota	Tremellomycetes	Filobasidiaceae	Filobasidium
a59945f9d0e60ecbeb7f551b02f6e185	Core	26	1.5915 %	64.98 %	20	Basidiomycota	Tremellomycetes	Bulleribasidiaceae	Vishniacozyma
f42fce2c183220e1926f415acc2b08a4	Core	26	1.5280 %	51.65 %	14	Basidiomycota	Microbotryomycetes	Sporidiobolaceae	Sporobolomyces
39e2a4c2eaa5a0acecf79fe777dd26ec	Core	24	1.4525 %	67.22 %	5	Basidiomycota	Tremellomycetes	Bulleribasidiaceae	Vishniacozyma
a49eabdb23d4ba49e6801e9edfc3754a	Core	24	0.7859 %	20.75 %	3	Ascomycota	Dothideomycetes	Aureobasidiaceae	Aureobasidium
77eaf84cba19f572269297e708e34b12	Flexible	19	0.1338 %	9.20 %	1	Basidiomycota	Tremellomycetes	Filobasidiaceae	Filobasidium
95c3b8960cd248aad6592f917a2fd33b	Flexible	19	0.2927 %	19.81 %	11	Basidiomycota	Tremellomycetes	Cystofilobasidiaceae	Cystofilobasidium
fa001c50dbea5e2a88f0aae657a3135d	Flexible	17	0.0924 %	11.91 %	14	Basidiomycota	Tremellomycetes	Filobasidiaceae	Filobasidium
461613cbf8808c9b082843b8d6e46fb8	Flexible	15	0.1908 %	36.32 %	3	Basidiomycota	Tremellomycetes	Filobasidiaceae	Filobasidium
87a064b3f8daff8a08a02c2845da4279	Flexible	12	0.2456 %	46.82 %	5	Basidiomycota	Tremellomycetes	Bulleribasidiaceae	Vishniacozyma
07226812dae41889a9855c0ae4b37719	Flexible	10	0.2433 %	39.74 %	2	Basidiomycota	Cystobasidiomycetes	Erythrobasidiaceae	Symmetrospora
8940797ab300032c5a89e232a13913be	Flexible	10	0.0249 %	7.55 %	1	Basidiomycota	Tremellomycetes	Cystofilobasidiaceae	Cystofilobasidium
08585ef1ed23d6010596ff6ddb224240	Flexible	9	0.2446 %	27.48 %	1	Basidiomycota	Agaricostilbomycetes	Kondoaceae	Kondoa
3ebd9324cf5a646de856f615123123c5	Flexible	9	0.0838 %	16.86 %	3	Basidiomycota	Tremellomycetes	Bulleribasidiaceae	Vishniacozyma
3efbb18c351289b9a7380690fb045510	Flexible	9	0.0204 %	6.37 %	2	Basidiomycota	Microbotryomycetes	Sporidiobolaceae	Sporobolomyces
4b972a0a28bc7bc835597f5627f8cbb0	Flexible	9	0.1872 %	32.19 %	5	Basidiomycota	Tremellomycetes	Bulleribasidiaceae	Vishniacozyma
292db1aa132490bdfbdfe41c9e7e55fc	Flexible	7	0.0747 %	23.11 %	5	Basidiomycota	Tremellomycetes	Bulleribasidiaceae	Vishniacozyma
8179e3920b27f827595d8561fad7bff3	Flexible	7	0.0249 %	1.53 %	1	Basidiomycota	Tremellomycetes	Bulleribasidiaceae	Vishniacozyma
41c2ee554aae1369a29d2f7e18fe8a83	Flexible	6	0.0258 %	2.95 %	1	Basidiomycota	Microbotryomycetes	Sporidiobolaceae	Sporobolomyces
34c6473f0ace2216c640ba0d29bde8df	Flexible	5	0.0073 %	2.95 %	2	Basidiomycota	Microbotryomycetes	Sporidiobolaceae	Sporobolomyces
9c3e743be137cb5d41cc97a4680c0292	Flexible	4	0.0421 %	1.30 %	2	Basidiomycota	Tremellomycetes	Filobasidiaceae	Naganishia
fbc0ba9e116183387544a48e9968b212	Flexible	3	0.0020 %	0.59 %	13	Basidiomycota	Microbotryomycetes	Sporidiobolaceae	Rhodotorula
bd42f5161cb6389345eba8dea09f0774	Flexible	2	0.0002 %	0.24 %	1	Basidiomycota	Microbotryomycetes	Sporidiobolaceae	Rhodotorula
a22c92bab7b246bfbfcc88e8e1e789e0	Flexible	1	0.0003 %	0.24 %	24	Basidiomycota	Tremellomycetes	Filobasidiaceae	Naganishia
f62540968019a7a555665636d3e440be	Flexible	1	0.0002 %	0.12 %	1	Basidiomycota	Tremellomycetes	Bulleribasidiaceae	Vishniacozyma
ASVs not detected in Meta-analysis									
1e0aafc1e1c22e392a1e7c3d9c0af6a0	Rare	Not detected in Seed Microbiota Database			1	Basidiomycota	Cystobasidiomycetes	Erythrobasidiaceae	Bannoa
329686a976b16aa40101eb8f2821cfab	Rare				1	Ascomycota	Taphrinomycotina	Taphrinaceae	Taphrina
5f5ab80b6d93797813e640823cb6b3b7	Rare				1	Basidiomycota	Tremellomycetes	Bulleribasidiaceae	Vishniacozyma

61ac26b896c5bb05411c0ce699315ff3	Rare	1	Basidiomycota	Microbotryomycetes	Sporidiobolaceae	Rhodotorula
95e79b5243a161c9ae5e081f0e6899e3	Rare	1	Basidiomycota	Tremellomycetes	Filobasidiaceae	Naganishia
a6d51bad65d066b6a8bad4f083ab9197	Rare	7	Basidiomycota	Tremellomycetes	Bulleraceae	Bullera
ac478cb9c9356c6e7b3ec38b78dc94e4	Rare	1	Basidiomycota	Tremellomycetes	Holtermanniales	Holtermanniella
dac0603c0d797778f2ffed431e70acbc	Rare	1	Basidiomycota	Tremellomycetes	Filobasidiaceae	Naganishia
ebe1a96fdcd18f714253e1946200f181	Rare	1	Basidiomycota	Tremellomycetes	Holtermanniales	Holtermanniella
f3a2f71a1bc012d10601fdc9ebb32369	Rare	55	Basidiomycota	Tremellomycetes	Holtermanniales	Holtermanniella
f8d698bb5e8840844966207fdaf7f9d5	Rare	7	Basidiomycota	Tremellomycetes	Filobasidiaceae	Naganishia

Discussion

Based on our culturomic survey and metabarcoding analyses, seed-associated yeasts largely belong to Basidiomycota and more particularly to the Tremellomycetes class. The most frequently isolated yeasts belong to *Holtermaniella*, *Vishniacozyma*, *Filobasidium* and *Sporobolomyces*, which is consistent with previous seed metabarcoding studies (Morales Moreira et al., 2021). This observation is also consistent with the fact that these taxa were recently identified as members of the core seed microbiome (Simonin et al., 2022). Our strain collection contains multiple isolates sharing the same ITS1 sequences with these core taxa, indicating their high prevalence and abundance across diverse plant hosts and environments. It should be noted that these same genera are also frequently detected in the phyllosphere of diverse plants (Gouka et al., 2022) which encourage further investigation in the potential vertical transmission of yeasts from other aboveground plant compartments to seeds.

However, compared to previous phyllosphere, fruit or flower yeast diversity surveys (Gouka et al., 2022), the yeast from Ascomycota phylum are less frequent in seeds and only represented here by the *Aureobasidium* genus (which is also a seed core taxon) (Simonin et al., 2022) and *Taphrina* sp. 98% isolates from the collection described here belong to Basidiomycota phylum, including red-pigmented isolates. Their coloration is attributed to the production of red, non-diffusible carotenoid pigments, which are abundantly synthesized in strains from *Rhodotorula, Sporobolomyces and Symmetrospora* genera (Frengova & Beshkova, 2009). Carotenoids are thought to protect yeast against oxidative stress, particularly under high UV exposure that they can experience on plant surfaces. This is in line with previous work highlighting that oxidative and hydric stress resistance are crucial for seed microbial colonization (Iacomi-Vasilescu et al., 2008; Pochon et al., 2013). However, determinants conferring yeast seed colonization success remain largely unknown and further work is needed to identify their specific molecular mechanisms and the potential role of morphogenetic adaptations (switch from hyphae to yeast).

In addition to ubiquitous yeast genera, our collection also contains isolates which were specific to one particular plant species. The clearest examples are the

case of isolates belonging to *Naganishia* and *Rhodotorula* genera which are specific to *Solanum lycopersicum L.* seeds. This specificity might be due to the wet fruit and seed type of *Solanum lycopersicum L.* that represent a very distinct habitat compared to the other dry seeds included in the survey. The members of *Rhodotorula* and *Naganishia* genera are commonly isolated from different type of environments: water, air, milk etc (Mussagy et al., 2022; Turchetti et al., 2008; Zhao et al., 2019) and not specifically of plant habitats.

Altogether, these results suggest that yeasts are generally well-adapted to the aboveground habitats of plants, but that seeds represent a specific habitat that diverse Basidiomycota yeasts can colonize.

Perspectives for using yeasts in agriculture

This study presents a previously unexplored yeast diversity on seeds with many genera that had never been observed or isolated in this habitat before. These findings encourage future studies to understand the ecological roles of these yeasts for plant health, especially of the taxa identified as part of the core seed microbiome. The fact that these core taxa are present on numerous plant hosts and environments, suggest a potential co-evolution between the plant and these microbiota members (Risely, 2020). Moreover, some core members and two other genera (*Holtermaniella*, *Vishniacozyma*, *Filobasidium*, *Naganishia*, *Rhodotorula* and *Sporobolomyces*) were isolated from seedlings germinated *in vitro*, which indicates that they could be transmitted between generations from seeds to seedlings. Limited knowledge is available on the persistence and role of yeasts during plant microbiota assembly from seed to seed during two plant generations, but these first results encourage further investigations.

From a more applied point of view, these yeast taxa and especially the ones transmitted to the seedlings are of interest for performing plant microbiota engineering through seed inoculations of single strains or synthetic communities (Arnault et al., 2024; Joubert et al., 2024). Seed yeasts represent an untapped biotechnological resource for seed agriculture that currently mainly rely on bacteria and some filamentous fungi (e.g. *Trichoderma*, *Beauvaria*) for biostimulation and biocontrol (Kepler et al., 2017). The relative ease of cultivating and manipulating these yeasts, compared to other fungi, opens up numerous promising opportunities for large-scale production of valuable metabolites or field inoculations. Leveraging the deep expertise of the food and beverage industry, yeast-based innovations hold great potential for advancing seed technology, particularly in the areas of crop protection and biofertilizer development.

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Supplementary data.

Table S1: Supplementary table including strain information (host species, plant's varieties, culture conditions and geographic localization), macroscopic colony description and taxonomic assignation from the phylogenomic tree and from DADA2 (based on ITS1 metabarcoding sequence).

Table S2: Yeast collection accession number for partial SSU + ITS and D1/D2 domain deposited on NCBI

Table S3: List of strains used in the taxonomic tree and the sequences accession number.

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