Catching cereal killers: a multi-omics approach to disentangle yeast-Fusarium interactions in the phyllosphere

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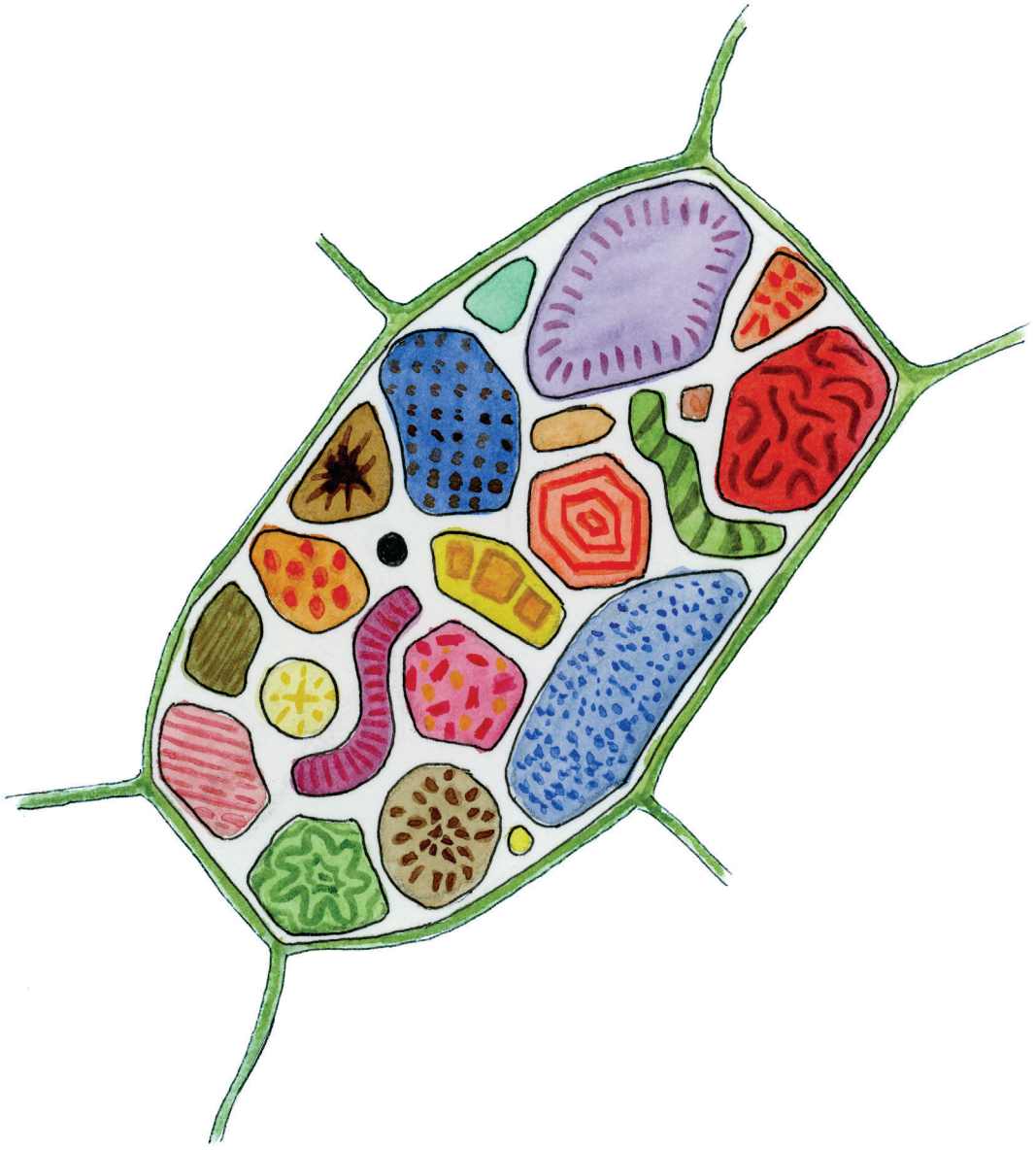

Chapter 3

Genetic, phenotypic and metabolic diversity of yeasts from wheat flag leaves

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Abstract

The phyllosphere - the aboveground part of a plant - is a harsh environment with diverse abiotic and biotic stresses, including oscillating nutrient availability and temperature as well as exposure to UV radiation. Microbial colonization of this dynamic environment requires specific adaptive traits, including tolerance to fluctuating temperatures, the production of secondary metabolites and pigments to successfully compete with other microorganisms and to withstand abiotic stresses. Here, we isolated 175 yeasts, comprising 15 different genera, from the wheat flag leaf and characterized a selection of these for various adaptive traits such as substrate utilization, tolerance to different temperatures, biofilm formation, and antagonism towards the fungal leaf pathogen *Fusarium graminearum*. Collectively our results revealed that the wheat flag leaf is a rich resource of taxonomically and phenotypically diverse yeast genera that exhibit various traits that can contribute to survival in the harsh phyllosphere environment.

Keywords

Yeast ecology, phyllosphere, (a)biotic stresses, antagonism, culturomics, functional characterization, biofilm formation, carbon utilization

Introduction

The phyllosphere is a reservoir of yet unknown microorganisms with intriguing interactions. Microorganisms colonizing the aboveground plant surfaces and tissues, including floral and vegetative parts, are referred to as the phyllosphere microbiome. The phyllosphere is considered to be a harsh environment due to the microbial exposure to limited nutrient sources, UV radiation, temperature oscillations and toxic compounds[1]. Phyllosphere microorganisms display a wide range of adaptations and antagonistic activities, which are gaining interest for sustaining plant health[2,3]. Among the phyllosphere-inhabiting microorganisms, yeasts are found to be abundant as endo- or epiphytes, reaching on average 10^3 - 10^5 colony forming units (CFU) per gram of leaf[4]. The majority of studies on the phyllosphere microbiome, so far, focuses on bacteria and filamentous fungi, especially plant pathogens. Currently, little is known about the diversity and ecological roles of phyllosphere yeasts[3].

Previous culture-dependent and independent approaches have shown a predominance of the basidiomycete yeasts *Sporobolomyces*, *Cryptococcus* (often reclassified as *Papiliotrema*) and *Pseudozyma*[5–7] in the phyllosphere of rice, corn and sugarcane. Several biotic and abiotic factors impact the abundance and diversity of yeasts in the phyllosphere. These factors include plant genetics (e.g. plant species, genotype, developmental stage) and environmental conditions such as UV radiation, moisture, geographic location and fungicides[8]. For example, the abundance of ascomycete yeasts, such as *Metschnikowia*, generally increased over time, particularly in nectar yielding flowers[9]. Additionally, an increase in nutrients found on damaged berries also increased the number of yeasts in the phyllosphere[10].

The phyllosphere is considered a resource-poor environment, thus microorganisms compete for nutrients and space. Nutrients leak from leaves or fruits into the environment, mainly consisting of carbohydrates such as fructose, glucose and sucrose, and also amino acids and methanol[11]. Yeasts are known for their versatile substrate utilization[12], they can use different carbon sources (e.g. simple sugars, methanol, methane) as well as amino acids and nitrogen sources (e.g. methylamine, ammonium salts and nitrate)[13–15], allowing them to expand their ecological niche.

A key step to elucidate the ecological roles of phyllosphere yeasts lies in determining their ability to withstand harsh environmental conditions and their interactions with other phyllosphere members. A number of mechanisms have been proposed to facilitate the adaptations of yeasts to the phyllosphere environment. For example, the majority of phyllosphere yeasts are present in highly organized multicellular communities called biofilms, which play a role in stress resilience. More specifically, biofilm formation has been implicated in resistance to fungicides and proposed as a physical barrier on plant surface injuries, preventing fungal hyphae from entering the plant tissue[16].

Currently, the majority of studies on environmental yeasts focusses on their biocontrol potential[17,18]. Several studies have shown the ability of yeasts to inhibit plant pathogens and to protect against post-harvest diseases via the production of secondary

metabolites, cell-wall-degrading enzymes, and so-called killer toxins[19]. The mechanisms underlying these antagonistic activities have been identified only for a few species. For example, the production of pulcherriminic acid by *M. pulcherrima* has been implicated in the growth inhibition of *Botrytis cinerea*[20], whereas polymers (e.g. pullulan), volatiles (e.g. ethanol, phenylethanol and ethyl acetate) and secondary metabolites (e.g. aureobasidins) produced by *A. pullulans* have been shown to inhibit the growth of *Alternaria alternata* and *B. cinerea*[21–23]. For the majority of biocontrol yeasts, however, the mechanisms underlying the antagonistic activity are still unknown[24]. Investigations of their lifestyles and adaptations to the phyllosphere environment will contribute to a better understanding of the interplay between yeasts and other phyllosphere members.

The present study aimed at investigating the taxonomic diversity of yeasts from wheat flag leaves, i.e. the last leaf before the ear emergence. The flag leaf can contribute up to 40% of the final photosynthetic capacity of wheat plants and therefore has a major impact on yield[25]. For a subset of taxonomically different flag leaf yeasts, we assessed their phenotypic and metabolic potential, including biofilm formation, substrate utilization spectrum, growth at different temperatures and antagonism towards the fungal pathogen *Fusarium graminearum*. Our results provide a first step towards characterizing the yeast diversity specifically found in wheat flag leaves and serve a foundation for further studies on the ecological roles of phyllosphere yeasts.

Material and Methods

Isolation of yeasts from the wheat flag leaf

Yeasts were isolated from the flag leaves of wheat (*Triticum aestivum*) cultivar Elixer. Samples were collected during the spring of 2020 at Taastrup, Denmark (55°38'46"N 12°17'53"E) on an untreated field plot (Gobbi et al, in prep.). Plants were sampled at the flowering stage (growth stage 61-69 according to Zadoks code). Three different methods were used to isolate both epiphytic and endophytic yeasts. For the first method a washing solution (0.5% Tween80 and 0.9% NaCl) was added to a 15 mL-tube with 33 flag leaves. Samples were vortexed for 1 min, sonicated for 2 min at 45 kHz, and vortexed again for 1 min. Leaves were transferred to a new tube and blended. The blended-washed leaves were stored in 15% (v/v) glycerol. In the second method, the washing solution was centrifuged for 5 min at 5.000 rpm, the supernatant was removed and the pellet was stored in 3 ml of 15% glycerol (v/v). The third method consisted of blending leaves directly (without the washing step) and storing in 15% glycerol (v/v). All samples were stored at -80°C.

For the selective isolation of yeasts, glycerol samples were 10-fold serial diluted (10^{-1} - 10^{-7}) with 0.9% NaCl and plated on different media. Suspensions were plated on Malt Extract Agar (MEA), Potato Dextrose Agar (PDA), Sabouraud Agar (SDA), 869 media supplemented with wheat flag leaf extract and Yeast Extract Peptone Dextrose (YEPD), with and without 10% lactic acid to favor yeast growth. Bacterial growth was prevented by adding 50 µl/ml chloramphenicol and 50 µl/ml tetracycline. Plates were incubated for 5 to 7 days at 25°C. At least two of each yeast colony morphologies were picked and streaked

on fresh plates (at least twice) to obtain pure cultures. Isolates were grown in YEPD broth for 2-3 days and stored in 15% glycerol (v/v). Isolates with minimal growth in liquid were streaked on plates and, after 3 to 5 days, cells were directly resuspended in glycerol. All samples were stored at -80°C for long-term storage.

Taxonomic identification of yeasts

All 175 yeast isolates were characterized by ITS rRNA gene sequencing. PCR amplifications were conducted using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3'), synthesized by Integrated DNA Technologies (IDT). Additionally, the selection of 51 isolates was also characterized by 28S rRNA gene sequencing (also known as D1/D2 sequencing) using the primers LR5 (5'-TCCTGAGGGAAACTTCG-3') and LROR (5'-ACCCGCTGAACTTAAGC-3'). DNA template for colony PCR was obtained by disrupting the cells by heating in a microwave at 600 W for 60 s, followed by vortexing and again microwaving at 600 W for 60 s in sterile demineralized-water. Samples were centrifuged at 5.000 rpm for 5 min and an aliquot of 3 µL was used as DNA template. The reaction mixture contained Go Taq G2 Hot Start Green Master Mix 2x, 0.4 µM for both primers, DNA template and nuclease-free water. The PCR conditions in the Thermal cycler were: 95°C for 2 min, followed by 30 cycles of 95°C for 1 min, 55°C for 30 s, and 72°C for 1 min 30 s, with a final extension at 72°C for 5 min and stored at 12°C. The presence of the PCR products was verified on a 1% agarose gel in TBE buffer. PCR products were purified via ethanol precipitation or using a PCR purification kit (Zymo Research fungal/bacterial DNA Miniprep Kit). Samples were sequenced at BaseClear (Leiden, The Netherlands) or Macrogen Inc. (Amsterdam, The Netherlands) using the primer ITS1 and LR5. Taxonomic characterization was based on partial ITS and 28S rRNA gene sequences using the NCBI BLAST database (https://www.ncbi.nlm.nih.gov/). For the phylogenetic analysis, sequences were aligned using the MUSCLE algorithm in the MEGA software (version 7.0) and a phylogenetic tree was constructed using Maximum Likelihood method and Tamura-Nei model with a bootstrap of 1.000 replications. The tree was visualized using iTOL (version 6). ITS and D1D2 sequences are deposited at European Nucleotide Archive (ENA) under the project number PRJEB51687.

Yeast and fungus culture conditions

For all *in vitro* assays, the fungal isolate *Fusarium graminearum* strain 8/1 was grown on Potato Dextrose Agar (PDA, pH 7) plates for 5 to7 days at 25°C. Yeast cultures were started from glycerol stocks. Isolates were plated on PDA plates and incubated at 25°C for 5 to 10 days. For all *in vitro* assays, a loop of the yeast cells was collected and inoculated in 0.9% NaCl. Cells were washed twice by centrifugation at 5.000 rpm for 5 min. An initial cell density (OD_{600nm}) of 0.1 was used for all experiments except the Biolog plate, where an OD_{600nm} of 0.01 was used. All assays were performed at 25°C, which was the temperature used for the isolation of yeasts, unless stated otherwise.

Metabolic fingerprinting

The Biolog EcoPlate™ system (Biolog Inc., Hayward, CA, USA) were used to analyze differential utilization of carbon sources and thereby providing a metabolic fingerprint for each individual strain. Every plate contains 31 different carbon sources grouped into six categories (including amino acids, amines, carbohydrates, carboxylic acids miscellaneous and polymer compounds) and a water control in three replicates. Yeast cells, described above, were adjusted to OD_{600nm} 0.01 and 100 µL per well was inoculated into the Biolog EcoPlate™. Plates were incubated at 25°C for 10 days at 180 rpm. Metabolism of specific substrates, and consequently growth of the cultures, resulted in a change in the tetrazolium dye. Cell density was measured after 2, 4, 7 and 10 days using a microtiter plate reader (OD_{590nm}), minus values were adjusted to zero. The average well color development (AWCD) was calculated by dividing the total of all values (excluding the water control) by 93. Additionally, the AWCD was calculated for each substrate group. MetaboAnalyst (Version 5.0) was used to visualize the 10 day-inoculation data. A heatmap was created following a Euclidean distance measure and a ward clustering method[26].

Growth at different temperatures

Yeast cells were collected as described above. The cell density was adjusted to OD_{600nm} 0.1 and 20 µl was added to 180 µl PDB pH 7 in a round-bottom microtiter 96-wells plate. Plates were sealed with plastic wrap and incubated at different temperatures, ranging from 4, 10, 25 to 37°C, at 200 rpm for 7 days. Cell density was measured after 2, 4 and 7 days using a microtiter plate reader (OD_{600nm}).

In vitro biofilm formation

Yeast cells were collected from plates and OD_{600nm} was adjusted to 0.1 as described above. A total of 20 µl of this suspension was added to 180 µl PDB in a flat-bottom microtiter plate. Plate was sealed and incubated statically in the dark for 3 to 4 days at 25°C. After incubation, cells were stained by adding 10 µl of 0.1% crystal violet to each well of the microtiter plate. Plate was incubated at room temperature for 15 min. Cells were washed three times with demineralized-water to remove platonic cells. After that, cells in biofilm were dissolved in 200 µl of 96% ethanol and incubated for 5 min. Cell density was measured using a microtiter plate reader at OD_{600nm}.

Antifungal activity via diffusible and volatile compounds

The effect of yeast diffusible compounds on the growth of the fungal pathogen *F. graminearum* was tested using *in vitro* dual culture assay. Yeast isolates and the fungal pathogen were grown as described above. An aliquot of 10 µL was streaked on one side of a 9-cm Petri dish (Ø 9 cm) containing PDA at pH 7 (0.5 cm from the edge of the plate). Control treatments were inoculated with 10 µL 0.9% NaCl. A total of 4 replicates was prepared. Plates were incubated for 24 h at 25°C. After that, a fungal mycelial agar plug (Ø 5 mm) was placed 24 h later at the opposite side of the Petri dish. Plates were incubated for 6 days at 25°C until the fungus on the control plates reached the edge of the plate. After 6 days, the

inhibition zone was determined by measuring the area between the fungal hyphae and the yeast colony using ImageJ (FIJI). The inhibition percentage was calculated as:

[((area of fungal growth on control) - (area of non-occupied space)) *100/
area of fungal growth on control]

The effect of volatile organic compounds (VOCs) on the growth of *F. graminearum* was investigated using two-compartment Petri dishes, which allowed the physical separation between the yeast and the fungal isolates. Yeast and fungal inocula were prepared as described above. An aliquot of 10 µl of the yeast isolates at OD_{600nm} 0.1 was inoculated on one of the compartments of the Petri dish containing PDA (pH 7) and spread evenly. Control treatments were inoculated with 10 µL 0.9% NaCl. A total of 4 replicates was prepared. Plates were incubated for 24 h at 25°C before inoculation of the fungal pathogen. A mycelium agar plug was placed (Ø 5 mm) on the other compartment of the Petri dish. Plates were sealed 3 times with plastic wrap and incubated at 25°C for 4 days. Growth inhibition was calculated by measuring the mycelial growth area after 4 days using ImageJ. The inhibition of the four replicates was calculated by [((area of fungus in control) – (area of fungus exposed to yeast VOCs)) *100/area of fungus in control]. Statistical significance was determined with one-way ANOVA, Tukey's HSD test ($P < 0.05$).

Scanning electron microscopic analysis

Scanning electron microscopy (JEOL SEM 6400 equipped with Image Convert for windows) was used to visualize the influence of yeasts on the morphological changes in the fungal hyphae of *F. graminearum*. Samples from the dual culture confrontation assay (described above) were fixed with 1.5% glutaraldehyde in PBS for 1 h while shaking. Then, samples were dehydrated in an increasing percentage of acetone for 20 min each (70%, 80%, 90%, 96% and 100% EtOH) and critical point dried (Baltec CPD-030). Afterwards, the samples were sputter coated with platina and palladium to a 20-mm-thickness and stored in a vacuum until use.

Results

Isolation and phylogenetic delineation of phyllosphere yeasts

A total of 175 yeasts were isolated from the surfaces and internal tissues of wheat flag leaves. ITS and D1/D2-amplicon sequencing revealed a total of 15 genera, representing 25 different species (Figure 1, Supplementary Table 1). Isolation on SDA and PDA media yielded the highest diversity of isolates, 10 and 9 out of 15 different genera, respectively, followed by YEPD (8 genera). The majority of the yeast isolates belonged to the phylum Basidiomycota (82.9%), including the genera *Vishniacozyma* (26,3%), *Sporobolomyces* (24,0%) and *Papiliotrema* (9,1%). Less frequent genera detected were *Pseudozyma*, *Anthracocystis*, *Dioszegia* and *Rhodotorula*. Isolates belonging to the phylum Ascomycota (17,1%) included *Aureobasidium* (16,0%) and *Metschnikowia* (1,1%). For further phenotypic and metabolic characterization, we selected 51 yeast isolates based on their phylogenetic delineation, with at least two isolates of the same genus (Figure 2).

Figure 1. Phylogeny of 175 phyllosphere yeasts isolated from the wheat flag leaf. Phylogeny is based on partial ITS sequences compared to NCBI database. Neighbor Joining tree was constructed with MEGA, MUSCLE for alignment. The iTol software was used to visualize and color-code the tree. Isolates indicated with a triangle are the 51 yeasts selected for the screening of different phenotypic and metabolic traits. *Fusarium* graminearum strain CBS 131786 was used as an outgroup. Type strains used are described in Supplementary Table S1.

Metabolic profiling of phyllosphere yeasts

To determine the metabolic diversity of the 51 selected phyllosphere yeasts, Biolog Eco-Plate was used to screen for their ability to metabolize 31 nutrient sources of which at least 20 are of plant and/or microbial origin. Growth was measured spectrophotometrically, i.e. average well color development value. The results showed that isolates classified as *Vishniacozyma* (F300, F345), *Aureobasidium* (F359, F57) or *Papiliotrema* (F301) were able to utilize various carbon sources, while none of the *Sporobolomyces* and *Dioszegia* isolates grew on any of the substrates tested (Figure 3A). More specifically, *Vishniacozyma* isolate F345 had the broadest substrate utilization spectrum, followed by *Vishniacozyma* isolates F75, F300 and F66. Intriguingly, *Fusarium graminearum* strain 8/1 was able to use 24 out of the 31 different carbon sources, indicating a broad substrate utilization spectrum for this

Isolate	ID	ITS rRNA identity	D1/D2 rRNA identity
	F52	*Holtermanniella takashimae* (99.22%)	*Holtermanniella wattica* (98.45%)
	F53	*Pseudotremella lacticolor* (87.39%)	*Pseudotremella lacticolor* (94.11%)
	F57	*Aureobasidium namibiae* (97.39%)	*Papiliotrema terrestris* (84.39%)
	F63	*Aureobasidium melanogenum* (97.57%)	*Filobasidium oeirensis* (99.79%)
	F66	*Vishniacozyma carnescens* (96.69%)	*Vishniacozyma carnescens* (96.59%)
	F73	*Holtermanniella festucosa* (99.42%)	*Holtermanniella wattica* (96.68%)
	F75	*Vishniacozyma victoriae* (98.15%)	*Vishniacozyma carnescens* (97.81%)
	F79	*Filobasidium magnum* (99.10%)	*Filobasidium magnum* (99.33%)
	F86	*Sporobolomyces roseus* (98.35%)	*Sporidiobolus metaroseus* (99.89%)
	F129	*Vishniacozyma victoriae* (97.62%)	*Vishniacozyma carnescens* (98.57%)
	F130	*Vishniacozyma victoriae* (100.0%)	*Vishniacozyma carnescens* (98.77%)
	F136	*Pseudotremella moriformis* (86.53%)	*Pseudotremella moriformis* (96.84%)
	F145	*Sporobolomyces roseus* (99.10%)	*Dioszegia rishiriensis* *(98.55%)*
	F146	*Dioszegia hungarica* (99.77%)	*Diozegia rishiriensis* (95.96%)
	F159	*Metschnikowia pulcherrima* (93.00%)	*Metschnikowia chrysoperlae* (96.96%)
	F160	*Holtermanniella festucosa* (99.74%)	*Holtermanniella wattica* (94.27%)
	F174	*Aureobasidium namibiae* (98.49%)	*Aureobasidium pullulans* (89.64%)
	F179	*Sporobolomyces roseus* (98.37%)	*Sporidiobolus metaroseus* (99.66%)
	F181	*Sporobolomyces roseus* (99.63%)	*Sporidiobolus metaroseus* (99.66%)
	F182	*Sporobolomyces roseus* (99.63%)	*Sporidiobolus metaroseus* (96.47%)
	F185	*Sporobolomyces roseus* (98.24%)	*Sporidiobolus metaroseus* (98.43%)
	F188	*Sporobolomyces roseus* (99.10%)	*Dioszegia rishiriensis* (98.47%)
	F224	*Rhodotorula babjevae* (100.0%)	*Rhodotorula glutinis* (99.77%)
	F225	*Cystobasidium slooffiae* (97.43%)	*Cystobasidium slooffiae* (99.78%)
	F253	*Filobasidium chernovii* (98.82%)	*Filobasidium globisporum* (99.45%)
	F280	*Cystobasidium psychroaquaticum* (99.77%)	*Cystobasidium psychroaquaticum* (98.30%)

Isolate	ID	ITS rRNA identity	D1/D2 rRNA identity
	F294	*Holtermanniella festucosa* (99.41%)	*Holtermanniella takashimae* (95.33%)
	F297	*Papiliotrema flavescens* (99.14%)	*Papiliotrema terrestris* (96.81%)
	F298	*Papiliotrema flavescens* (98.53%)	*Papiliotrema terrestris* (97.15%)
	F300	*Vishniacozyma tephrensis* (93.15%)	*Vishniacozyma carnescens* (99.01%)
	F301	*Papiliotrema flavescens* (99.08%)	*Vishniacozyma carnescens* (98.00%)
	F303	*Vishniacozyma victoriae* (99.54%)	*Papiliotrema terrestris* (96.60%)
	F311	*Filobasidium chernovii* (99.29%)	*Filobasidium globisporum* (99.01%)
	F312	*Pseudohyphozyma pustula* (83.16%)	*Hamamotoa lignophila* (93.74%)
	F313	*Filobasidium chernovii* (98.84%)	*Filobasidium globisporum* (98.46%)
	F314	*Vishniacozyma tephrensis* (98.41%)	*Papiliotrema terrestris* (81.32%)
	F318	*Metschnikowia pulcherrima* (94.63%)	*Metschnikowia chrysoperlae* (96.04%)
	F326	*Filobasidium chernovii* (99.17%)	*Filobasidium globisporum* (98.31%)
	F327	*Papiliotrema flavescens* (98.31%)	*Papiliotrema terrestris* (98.17%)
	F331	*Vishniacozyma carnescens* (97.93%)	*Vishniacozyma carnescens* (97.51%)
	F345	*Vishniacozyma tephrensis* (93.95%)	*Vishniacozyma victoriae* (97.31%)
	F346	*Sporobolomyces roseus* (99.45%)	*Sporidiobolus metaroseus* (97.90%)
	F347	*Papiliotrema flavescens* (98.12%)	*Papiliotrema terrestris* (97.70%)
	F355	*Papiliotrema flavescens* (99.33%)	*Papiliotrema terrestris* (96.22%)
	F359	*Aureobasidium namibiae* (99.04%)	*Aureobasidium pullulans* (99.48%)
	F376	*Holtermanniella festucosa* (99.80%)	*Holtermanniella wattica* (96.73%)
	F378	*Filobasidium wieringae* (83.18%)	*Filobasidium magnum* (95.13%)
	F382	*Filobasidium wieringae* (83.58%)	*Filobasidium magnum* (95.13%)
	F383	*Vishniacozyma victoriae* (95.50%)	*Vishniacozyma carnescens* (95.91%)
	F386	*Papiliotrema flavescens* (99.33%)	*Papiliotrema terrestris* (98.23%)
	F391	*Cystobasidium slooffiae* (97.54%)	*Cystobasidium slooffiae* (91.68%)

Figure 2. Phenotypic and taxonomic characterization of the selected yeast isolates. Pictures depict 5 to 10-day-old isolates grown on PDA at 25°C. Partial ITS and D1/D2 rRNA gene sequences were compared to the NCBI database for taxonomic identification

prevalent fungal pathogen of wheat leaves. Among the different types of carbon sources tested, carbohydrates were the preferred substrates followed by polymers and carboxylic acid; none of the 51 yeast isolates were able to use amines under the tested conditions. The most frequently metabolized carbohydrates were D-Xylose, D-Mannitol and N-Acetyl-D-Glucosamine, the polymers were Tween 40 and 80, and the carboxylic acids were D-Galacturonic acid and D-Glucosaminic acid (Figure 3B, C).

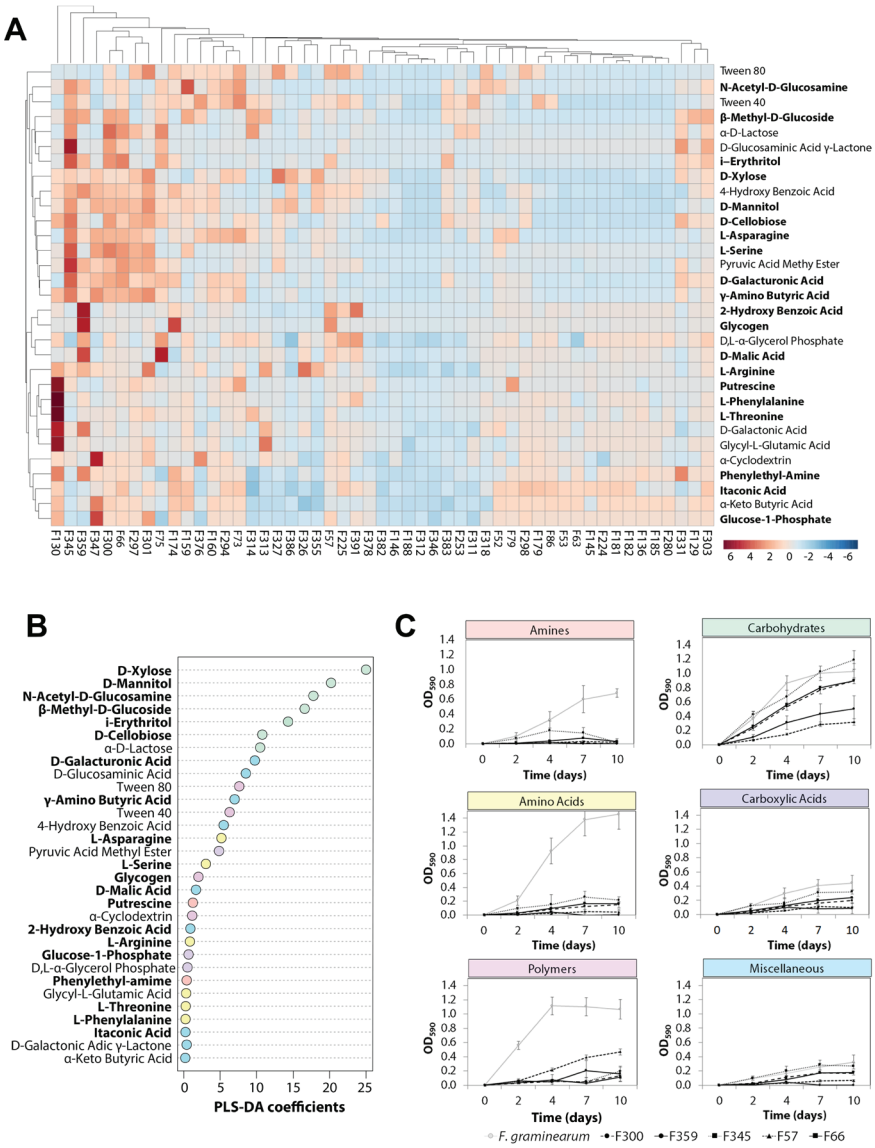

Figure 3. Metabolic profiling of the phyllosphere yeasts. **A**. Hierarchical cluster and heat-map analyses of nutrient sources utilized by the selected yeast isolates performed with MetaboAnalyst. Yeast isolates were inoculated (OD_{600} = 0.01) in the BIOLOG EcoPlates, incubated for 10 days at 25°C and 180 rpm, growth was measured with a plate reader at OD_{590nm}. Columns represent the average of three replicates of each of the 51 isolates. Rows represent the different carbon sources (blue: low abundance, red: high abundance). Compounds of plant and/or microbial origin are displayed in bold. **B**. Partial least squares-discriminant analysis (PLS-DA) indicating the importance of each carbon source. **C**. The dynamics of the top 5 isolates including *Fusarium graminearum* with the most diverse metabolic profile per carbon type.

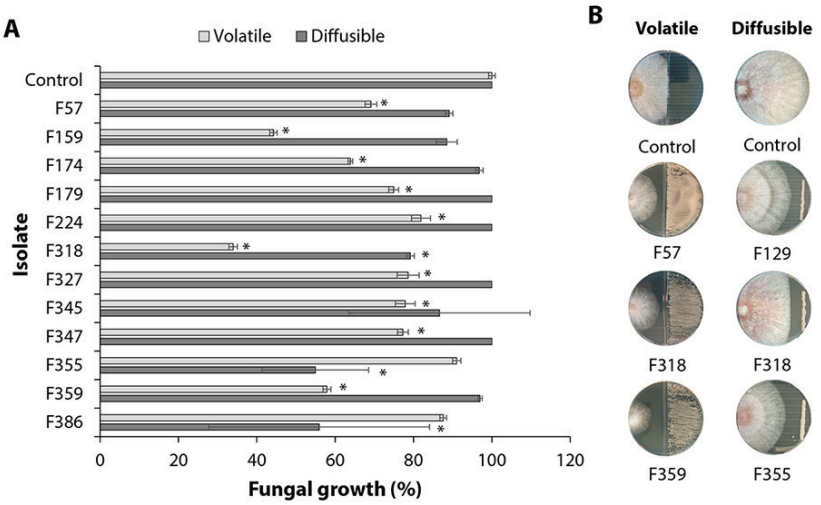

Figure 4. Antagonistic activity of phyllosphere yeasts against *Fusarium graminearum*. Each isolate was screened against *F. graminearum* for the production of volatile organic compounds (VOCs) or agar-diffusible compounds. **A.** Bars represent the standard deviation of the mean growth percentage of 4 independent replicates. Statistical differences as compared to control (exposed to medium alone) were determined using Student's t-test ($P < 0.05$) and are indicated with an asterisk (*). **B.** Pictures of the antagonistic activities by representative isolates made after 4 and 6 days of exposure for the volatile and diffusible compound, respectively.

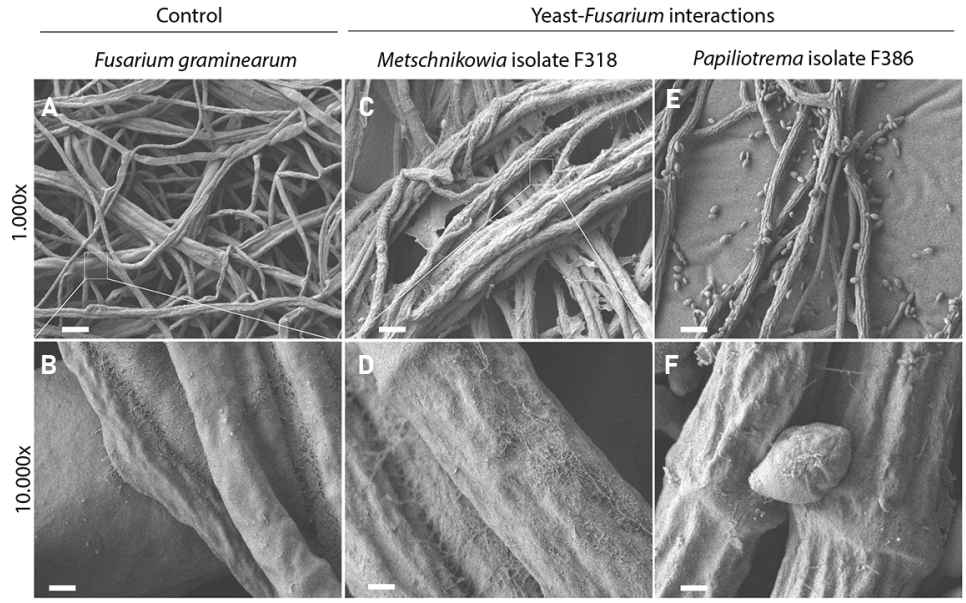

Figure 5. Scanning electron microscopic (SEM) images of the interactions between phyllosphere yeasts and the fungal pathogen *Fusarium graminearum*. Dual culture assays of *F. graminearum* growing alone (**A, B**) and exposed to *Metschnikowia* (**C, D**) and *Papiliotrema* (**E, F**) isolates. Bars represent 10 µm and 1 µm for ×1.000 (**A, C, E**) and ×10.000 (**B, D, F**) magnification images, respectively.

Temperature growth range of phyllosphere yeasts

In the phyllosphere environment, yeasts are exposed to substantial temperature oscillations. To investigate the temperature range for growth, the selected 51 isolates were tested *in vitro* at 4, 10, 25 and 37°C. All yeast isolates were able to grow at 25°C, although the growth rate ranged considerably between the different isolates (Supplementary Figure 2). Most of the isolates (94%) grew well within 2 days of incubation at 25°C, whereas *Filobasidium* isolate F253 and *Aureobasidium* isolate F63 required up to 7 days of incubation at this temperature. When the temperature was reduced to 10°C, only 27 and 45 isolates were able to grow after 48 and 96 h, respectively. At 4°C, 13 isolates were able to grow after 4 days of incubation, and an additional 31 isolates showed growth with longer incubation. Isolates F225, F280 and F391 classified as *Cystobasidium* were unable to grow at 4°C, but grew at 10°C after 7 days. None of the 51 selected yeast isolates was able to grow at 37°C.

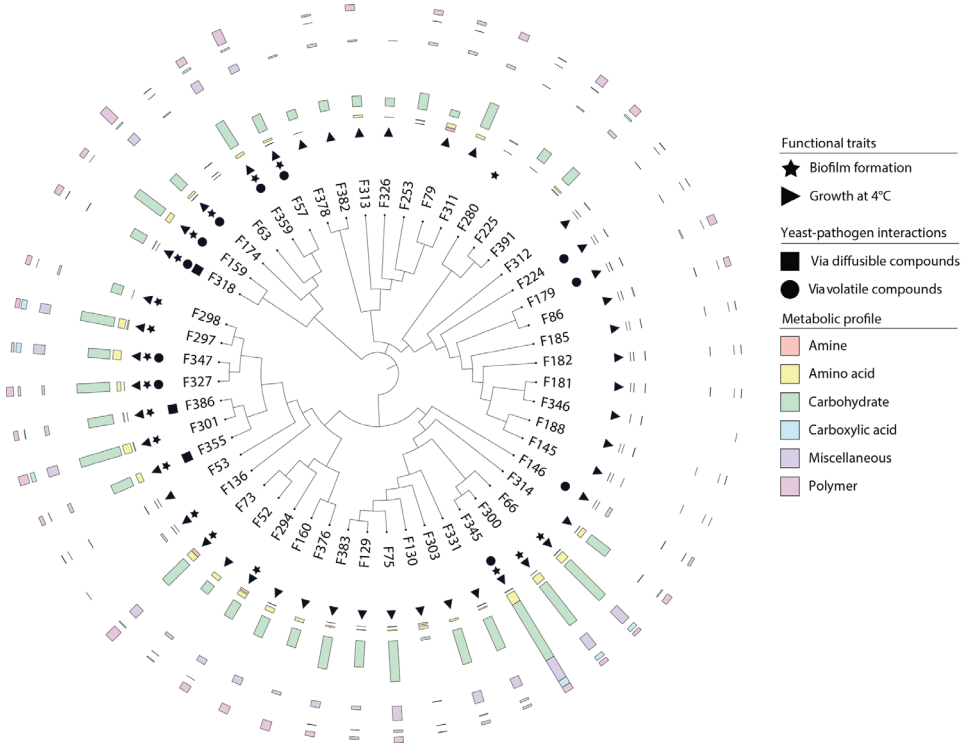

Figure 6. Overview of the adaptative traits of yeasts inhabiting the wheat flag leaf. Neighbor-joining phylogenetic tree based on partial ITS sequences of yeast isolates. Isolates were screened for different traits including biofilm formation, growth at different temperatures, competition with the fungal pathogen *Fusarium graminearum* via the production of agar-diffusible and volatile compounds; and substrate utilization.

Biofilm formation by phyllosphere yeasts

Biofilms play a vital role in stress resilience and can protect yeasts against UV radiation and fungicides. Additionally, they can form a physical barrier on leaf surfaces, preventing penetration of the leaf surface by fungal pathogens. In vitro biofilm formation was detected by crystal violet staining of liquid cultures after 7 days of growth at 25°C with a threshold of OD_{600nm} at 0.05 to prevent any false positives due to background signal. A total of 19 yeast isolates (38%) was able to form a visible biofilm in at least 2 of the 3 screenings performed (Supplementary Figure 3). Biofilm formation was detected for all *Metschnikowia* (F159, F318) and *Papiliotrema* (F297, F298, F301, F327, F347, F355 and F386) isolates, whereas no biofilm formation was detected for *Filobasidium*, *Dioszegia*, *Rhodotorula* and *Sporobolomyces* isolates under the tested conditions.

Interactions between phyllosphere yeasts and *Fusarium graminearum*

To investigate the potential of phyllosphere yeasts to inhibit the growth of the leaf pathogen *F. graminearum*, different *in vitro* assays were performed. Dual-confrontation assays showed that several yeast isolates inhibited mycelial growth of the pathogen through the production of volatile and/or diffusible metabolites (Figure 4). Ten of the 51 isolates (19,6%) exhibited significant volatile-mediated antifungal activity. Two of these antagonistic yeast isolates (F318 and F159) that were classified as *Metschnikowia* inhibited the fungal growth by 66% and 56%, respectively. Also isolates classified as *Aureobasidium*, *Papiliotrema*, *Rhodotorula*, *Sporobolomyces* or *Vishniacozyma* were able to inhibit the growth of this fungal pathogen via volatiles. Two other *Papiliotrema* isolates and one *Metschnikowia* isolate also inhibited hyphal growth via agar-diffusible metabolites, by 55%, 54% and 21%, respectively. Altogether, these results indicated that various, taxonomically diverse phyllosphere yeasts can inhibit the growth of *F. graminearum* via volatile and diffusible metabolites.

To visualize the interaction between phyllosphere yeasts and *F. graminearum*, scanning electron microscopy showed that *Metschnikowia* isolate F318 and *Papiliotrema* isolate F386 both altered hyphal morphology compared to the control conditions, where smooth fungal hyphae were observed for *F. graminearum* (Figure 5A, B). More specifically, direct confrontation between *Metschnikowia* and *F. graminearum* resulted in the production of an extracellular matrix (presumably extracellular polysaccharides (EPS)) on the hyphal surface as well as in between the hyphae (Figure 5C, D). Confrontation with *Papiliotrema* resulted in shrunken and distorted hyphae (Figure 5E, F).

Discussion

Yeasts are well-known for their biotechnological and medical importance, with *Candida* and *Saccharomyces* spp. extensively explored. Plant-associated yeasts have been de-

scribed for their biocontrol potential, especially in postharvest disease management but knowledge on yeast ecology and the mechanisms underlying their interactions with other members of the phyllosphere microbiome are still limited. In this study, we examined the diversity of culturable yeasts colonizing wheat flag leaves, their adaptive traits and ability to inhibit the growth of the fungal pathogen *F. graminearum* (Figure 6).

The wheat flag leaf harbors a taxonomically diverse reservoir of yeasts

Previous studies have shown that yeasts colonize a wide range of natural environments, including soils, oceans, insects and plants[27]. Plant-associated yeasts are receiving increased attention in the past two decades, in particular those colonizing fruits[27]. In line with previous culture-dependent and independent studies of the wheat phyllosphere[8,28,29], we found that basidiomycetes were the most frequently isolated yeasts. Yeasts belonging to the genera *Aureobasidium*, *Vishniacozyma* and *Sporobolomyces*, also found in phyllosphere of other crops, such as rice, sugarcane and corn[5,7,30], were the most abundant. Additionally, we also detected *Pseudohyphozyma* and *Pseudotremella* species, which were described previously for litter and forest soil[31], but are rarely isolated from the phyllosphere.

Here we characterized the yeast isolates based on the variable ITS region as well as the 28S domain (also known as D1/D2) for better taxonomic delineation. Yeast taxonomy has long been based on these phylogenetic markers, but the resolution is limited due to the highly conserved sequences in these regions, especially in basidiomycetous yeasts[32]. Therefore, a large number of yeast species are being reclassified[33]. For example, *Cryptococcus* species are being reclassified as *Papiliotrema* and *Filobasidium*, whereas *Candida* species can be found in multiple teleomorphic taxa with different generic names. Hence, the yeasts described here will be subjected in the near future to whole genome sequencing for improved taxonomic delineation.

Adaptations of yeasts to the harsh phyllosphere environment

The phyllosphere is a heterogeneous environment where microorganisms are exposed to different (a)biotic stresses. Here we studied a number of traits that may contribute to the yeast's adaptation to the phyllosphere, including carbon utilization, growth at different temperatures, and biofilm formation. Nutrients, such as carbon compounds, are important for growth and the synthesis of secondary metabolites[34]. A diverse metabolic profile can offer an advantage to withstand the harsh conditions in the phyllosphere, allowing microorganisms to expand their ecological niche by making better use of the available carbon sources[35]. Here we showed that the yeasts from the wheat flag leaf displayed diverse metabolic profiles with varying degradation rates. *Vishniacozyma* and *Aureobasidium* isolates, both highly abundant on the wheat flag leaf, displayed the most diverse substrate utilization profiles. Unexpectedly, *Sporobolomyces* isolates were unable to use any of the carbon sources tested under these conditions, despite their frequent and high abundances in wheat leaves found in the present and previous studies[36,37]. It is likely that *Sporobolomyces* species colonize the wheat flag leaves through the utilization of glucose, fructose or sucrose, sugars that are present in the phyllosphere and enable rapid coloni-

zation[38] and competition with leaf pathogens such as *Cochliobolus sativus*, partly by limiting glucose and amino acid availability. Also, *Metschnikowia* isolates have been shown to inhibit the post-harvest pathogen *Colletotrichum gloeosporioides* through competition for glucose and fructose[20,39]. To begin to understand ecological niche overlap or niche differentiation between the phyllosphere yeasts and the fungal pathogen *F. graminearum* strain 8/1, we also determined the metabolic profile of this pathogen. We found that *F. graminearum* is not only able to utilize most of the nutrient sources tested, but also utilizes amino acids, amines and polymers that could not be utilized by the phyllosphere yeasts tested. This versatile carbon utilization profile likely contributes to the successful infection of a wide range of cereal crops by this pathogen.

In addition to oscillating nutrient availability, phyllosphere yeasts also need to adapt to oscillating temperatures, which can differ drastically between day and night, winter and spring. Yeasts from the genera *Sporobolomyces*, *Rhodotorula* and *Vishniacozyma* have been proposed to tolerate extreme environmental conditions and to help plants adapting to cold environments[40,41]. Our results showed that all isolates from these genera grew at different temperatures, including low temperatures. Also, biofilms play an important role in stress resilience and protection against harsh environmental conditions[42], including temperature oscillations. Additionally, biofilms form a physical barrier on injuries of plant surfaces preventing the invasion by pathogenic fungi. We show that biofilm formation was wide-spread among the wheat phyllosphere yeasts, including all *Metschnikowia* and *Papiliotrema* isolates. Previous studies have proposed biofilm formation as one of the antagonistic mechanisms employed by biocontrol *Metschnikowia* isolates on grape wounds[20]. This mode of action is hypothesized to be based on growth inhibition of the pathogen by increasing colonization efficiency (e.g. competition for space). However, other biocontrol mechanisms, e.g. production of secondary metabolites, cannot be excluded to contribute to the observed antagonistic activity. Currently, data on biofilm formation by environmental yeasts is sparse[43] and our results suggest this may be a more common trait in the phyllosphere. Future investigations involving microscopy should address if these biofilms occur *in planta* and if they contribute to the phyllosphere competence of yeasts.

Interactions between yeasts and the fungal pathogen *F. graminearum* in the phyllosphere

We found that the production of secondary diffusible compounds with inhibitory activity against *F. graminearum* was not a wide-spread mechanism among the yeasts isolated from wheat flag leaves. Only two out of the 51 phyllosphere yeasts tested were able to significantly inhibit hyphal growth. However, it is still possible that these yeast isolates can inhibit the fungal pathogen by competing for nutrients and space, rather than via the production of secondary metabolites. Competition for nutrients and space have been proposed as the primary mechanism by which biocontrol yeasts inhibit pathogens[19]. Isolates F355 and F386, which showed the strongest inhibitory effects against *F. graminearum*, are closely related to *Papiliotrema flavescens*, formerly known as *Cryptococcus flavescens*/*C. nodaensis*. Earlier studies on *P. flavescens* OH182.9, isolated from wheat heads, showed no antagonism in *in vitro* experiments, but this isolate was able to reduce disease incidence

caused by *F. graminearum* by 56% in a bioassay[44]. Field experiments where different winter wheat cultivars were inoculated with this strain also demonstrated reduced disease severity of *F. graminearum* by 60%[45,46].

Scanning electron microscopy further showed hyphal morphological changes on the hyphae of *F. graminearum* during the interaction with specific yeast isolates. The interaction with *P. flavescens* (isolate F386) not only reduced hyphal growth but also changed the hyphal morphology from smooth to shrunken and distorted. This morphological change has been previously observed for *F. graminearum* confronted by different *Paenibacillus peoriae* strains, bacteria with a high potential of biocontrol and plant growth promotion[47]. Interestingly, the interaction of *F. graminearum* with *Metschnikowia* isolate F318, an isolate displaying little reduction of hyphal growth *in vitro*, led to considerable changes in hyphal morphology. These results suggest that *Metschnikowia* isolate F318 imposed stress to the fungal pathogen resulting in the production of extracellular polysaccharides (EPS). Biofilm formation by other *Fusarium* species, more specifically *F. oxysporum*, has been described to protect the fungal pathogen against several fungicides[48].

Contrary to diffusible secondary metabolites, volatile compounds with inhibitory effects on F. gramineum were more frequently produced by the phyllosphere yeasts. These low molecular weight compounds serve as a means of interspecies communication from a distance and are proposed as an effective biological control strategy against multiple pathogens[49]. The most promising volatile-producing antagonists belonged to *Metschnikowia pulcherrima* (isolates F159 and F318), *Aureobasidium pullulans* (isolates F57, F174 and F359) and *Papiliotrema flavescens* (isolates F327 and F347), inhibiting hyphal growth by up to 65%. Volatile compounds produced by *Metschnikowia* and *Aureobasidium* species have been previously described for their biocontrol potential against several fungal pathogens, including *Alternaria alternata* and *Botrytis cinerea*[50]. The volatile compounds ethanol, 2-phenylethanol and ethyl acetate have been proposed to be involved in this inhibitory activity[20–22,50] and hypothesized to disrupt the fungal membranes leading to leakage and deformed hyphae[21]. To date, however, most of the knowledge on yeast volatiles stems from studies using yeasts of biotechnological importance, e.g. the production of volatiles by yeasts and their effects on wine aroma. Further research efforts should be put on characterization of the volatile compounds produced by environmental yeasts and their ecological roles.

Concluding remarks

The wheat flag leaf is a diverse reservoir of yeasts which can withstand the harsh conditions found in the phyllosphere through different mechanisms. Here we provide a first insight into the genetic and phenotypic diversity of yeasts living on and in the wheat flag leaf. Further research should focus on the molecular mechanisms underlying the interactions between yeasts, including the identification of genes and metabolites involved in antifungal activity. To this end, current approaches and tools already in use for model yeasts can be employed for comparative genomics and functional analysis of the phyllosphere yeasts. Unraveling the mechanisms underlying the antagonistic activities would not only improve our understanding of the ecology of phyllosphere yeasts, but also contribute to the discovery of novel yeast-based biocontrol products.

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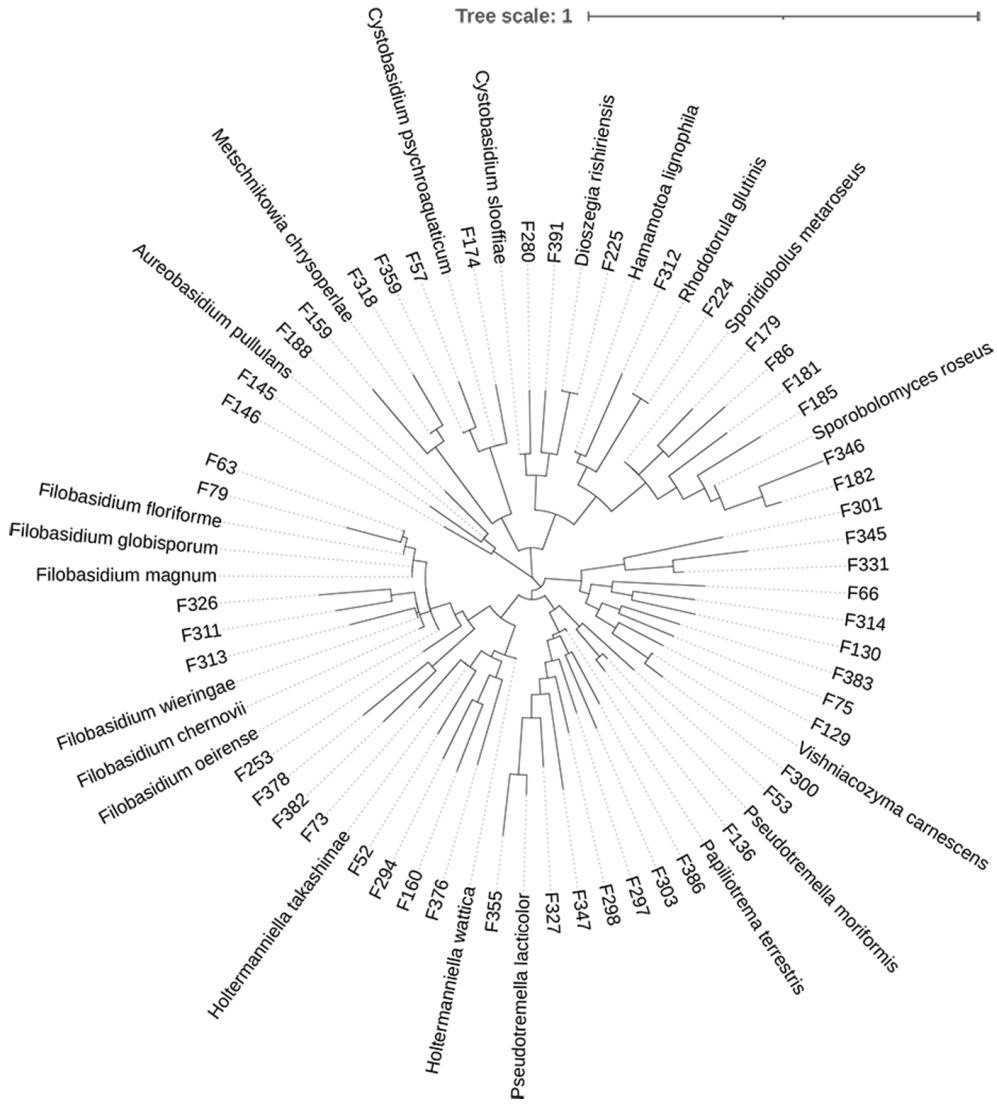
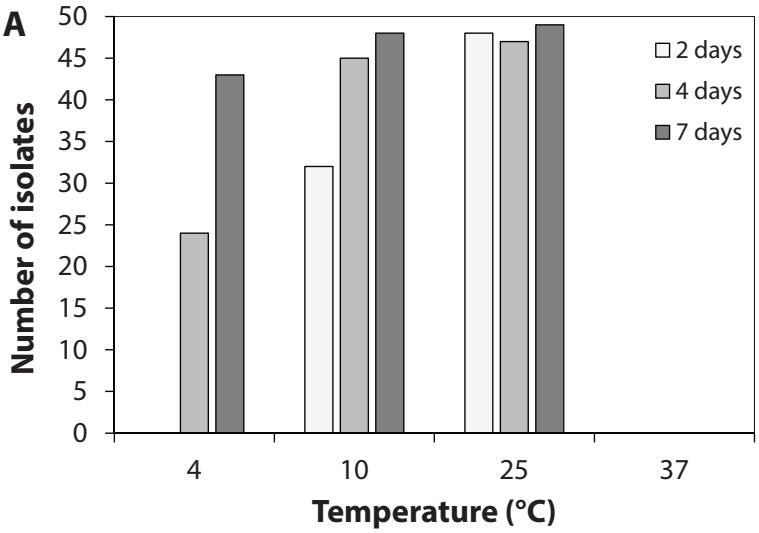
Author contributions

LG and VC designed the study. LG, CV, and VC performed the experiments. LG and VC processed and interpreted the data. LG and VC drafted the manuscript. All authors discussed the results, provided feedback and contributed to the final manuscript.

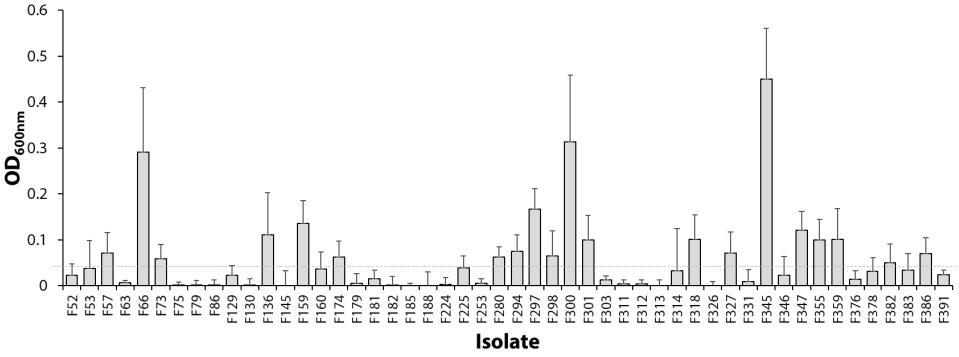
Supplementary Figures & Tables



Supplementary Figure 1. Phylogeny of 51 phyllosphere yeast isolates selected for the screening of different phenotypic and metabolic traits. Phylogeny is based on D1/D2 domains of the large subunit of the 26S rRNA gene sequences compared to NCBI database. Neighbor Joining tree was constructed with MEGA, MUSCLE for alignment.



Supplementary Figure 2. Growth of phyllosphere yeasts at different temperatures. Bars represent the number of phyllosphere yeasts able to grow in the different temperature range. Additional information can be found in Supplementary Table S3.



Supplementary Figure 3. Biofilm formation by phyllosphere yeasts. Optical density (OD_{600nm}) of cells in biofilm was determined after 7 days. Bars present the standard deviation of the means of 4 replicates and at least 2 independent experiments. Isolates displaying an optical density higher than 0.05 were considered to form biofilm.

Supplementary Table S1. Taxonomy of the 175 yeasts isolated from wheat flag leaves.

Isolate			Closest BLAST hit (NCBI)			
ID	Target	Primer	Species	Length (bp)	Identity (%)	Accession
F52	ITS	ITS1	*Holtermanniella takashimae*	1048	99.22	NR_137721.1
	LSU	LRO5	*Holtermanniella wattica*	903	98.45	NG_058307.1
F53	ITS	ITS1	*Pseudotremella lacticolor*	852	87.39	NR_158875.1
	LSU	LRO5	*Pseudotremella lacticolor*	850	94.11	NG_058379.1
F54	ITS	ITS1	*Vishniacozyma tephrensis*	468	96.59	NR_144812.1
F55	ITS	ITS1	*Vishniacozyma tephrensis*	1041	96.72	NR_144812.1
F56	ITS	ITS1	*Vishniacozyma tephrensis*	458	97.1	NR_144812.1
F57	ITS	ITS1	*Aureobasidium namibiae*	1109	97.39	NR_147362.1
	LSU	LRO5	*Aureobasidium leucospermi*	870	97.92	NG_058566.1
F58	ITS	ITS1	*Aureobasidium namibiae*	990	99.04	NR_147362.1
F59	ITS	ITS1	*Aureobasidium namibiae*	1045	98.12	NR_147362.1
F61	ITS	ITS1	*Vishniacozyma carnescens*	978	97.66	NR_130695.1
F63	ITS	ITS1	*Aureobasidium melanogenum*	1099	97.57	NR_159598.1
	LSU	LRO5	*Filobasidium oeirense*	488	99.79	NG_070508.1
F66	ITS	ITS1	*Vishniacozyma carnescens*	455	96.69	NR_130695.1
	LSU	LRO5	*Vishniacozyma carnescens*	1432	96.59	NG_058430.1
F67	ITS	ITS1	*Aureobasidium namibiae*	1020	98.68	NR_147362.1
F68	ITS	ITS1	*Vishniacozyma tephrensis*	470	98.3	NR_144812.1
F73	ITS	ITS1	*Holtermanniella festucosa*	515	99.42	NR_155179.1
	LSU	LRO5	*Holtermanniella wattica*	1219	96.68	NG_058307.1
F74	ITS	ITS1	*Aureobasidium namibiae*	1071	98.32	NR_147362.1
F75	ITS	ITS1	*Vishniacozyma victoriae*	999	98.15	NR_073260.1
	LSU	LRO5	*Vishniacozyma carnescens*	925	97.81	NG_058430.1
F76	ITS	ITS1	*Holtermanniella festucosa*	1014	95.98	NR_155179.1
F78	ITS	ITS1	*Holtermanniella festucosa*	1050	99.05	NR_155179.1
F79	ITS	ITS1	*Filobasidium magnum*	565	99.1	NR_130655.1
	LR05	LR05	*Filobasidium magnum*	900	99.33	NG_069409.1
F80	ITS	ITS1	*Papiliotrema flavescens*	472	99.33	NR_130696.1
F81	ITS	ITS1	*Aureobasidium namibiae*	1005	98.5	NR_147362.1
F82	ITS	ITS1	*Vishniacozyma victoriae*	471	99.54	NR_073260.1
F83	ITS	ITS1	*Aureobasidium namibiae*	1110	98.47	NR_147362.1
F84	ITS	ITS1	*Aureobasidium namibiae*	1082	98.49	NR_147362.1
F85	ITS	ITS1	*Sporobolomyces roseus*	755	98.92	NR_155845.1
F86	ITS	ITS1	*Sporobolomyces roseus*	547	98.35	NR_155845.1
	LR05	LR05	*Sporidiobolus metaroseus*	878	99.89	NG_069415.1
F87	ITS	ITS1	*Sporidiobolus metaroseus*	520	98.84	NR_137686.1
F88	ITS	ITS1	*Sporobolomyces roseus*	547	99.45	NR_155845.1

Isolate			Closest BLAST hit (NCBI)			
ID	Target	Primer	Species	Length (bp)	Identity (%)	Accession
F89	ITS	ITS1	*Sporobolomyces roseus*	548	99.45	NR_155845.1
F90	ITS	ITS1	*Sporobolomyces roseus*	549	99.45	NR_155845.1
F125	ITS	ITS1	*Vishniacozyma victoriae*	798	96.91	NR_073260.1
F126	ITS	ITS1	*Holtermanniella festucosa*	514	100	NR_155179.1
F129	ITS	ITS1	*Vishniacozyma victoriae*	464	97.62	NR_073260.1
	LSU	LRO5	*Vishniacozyma carnescens*	933	98.57	NG_058430.1
F130	ITS	ITS1	*Vishniacozyma victoriae*	467	100	NR_073260.1
	LSU	LRO5	*Vishniacozyma carnescens*	896	98.77	NG_058430.1
F136	ITS	ITS1	*Pseudotremella moriformis*	1049	86.53	NR_155685.1
	LSU	LRO5	*Pseudotremella moriformis*	942	96.84	NG_058379.1
F137	ITS	ITS1	*Papiliotrema flavescens*	953	98.33	NR_130696.1
F140	ITS	ITS1	*Papiliotrema flavescens*	465	99.32	NR_130696.1
F145	ITS	ITS1	*Sporobolomyces roseus*	1004	99.1	NR_155845.1
	LR05	LR05	*Diozegia rishiriensis*	895	98.55	NG_059156.1
F146	ITS	ITS1	*Dioszegia hungarica*	427	99.77	NR_073227.1
	LSU	LRO5	*Diozegia rishiriensis*	1096	95.96	NG_059156.1
F147	ITS	ITS1	*Vishniacozyma tephrensis*	458	98.25	NR_144812.1
F148	ITS	ITS1	*Vishniacozyma tephrensis*	459	98.26	NR_144812.1
F149	ITS	ITS1	*Vishniacozyma tephrensis*	460	98.26	NR_144812.1
F150	ITS	ITS1	*Vishniacozyma victoriae*	443	100	NR_073260.1
F151	ITS	ITS1	*Filobasidium oeirense*	579	100	NR_077106.1
F152	ITS	ITS1	*Vishniacozyma tephrensis*	469	98.29	NR_144812.1
F153	ITS	ITS1	*Vishniacozyma tephrensis*	955	94.85	NR_144812.1
F154	ITS	ITS1	*Vishniacozyma tephrensis*	944	94.44	NR_144812.1
F155	ITS	ITS1	*Vishniacozyma victoriae*	466	99.36	NR_073260.1
F156	ITS	ITS1	*Vishniacozyma tephrensis*	464	98.06	NR_144812.1
F157	ITS	ITS1	*Sporobolomyces roseus*	550	99.45	NR_155845.1
F158	ITS	ITS1	*Sporobolomyces roseus*	552	99.27	NR_155845.1
F159	ITS	ITS1	*Metschnikowia pulcherrima*	350	93	NR_164379.1
	LSU	LRO5	*Metschnikowia chrysoperlae*	831	96.96	NG_058336.1
F160	ITS	ITS1	*Holtermanniella festucosa*	387	99.74	NR_155179.1
	LSU	LRO5	*Holtermanniella wattica*	1315	94.27	NG_058307.1
F162	ITS	ITS1	*Aureobasidium melanogenum*	982	98.33	NR_159598.1
F163	ITS	ITS1	*Vishniacozyma tephrensis*	469	96.6	NR_144812.1
F164	ITS	ITS1	*Vishniacozyma tephrensis*	951	95.33	NR_144812.1
F165	ITS	ITS1	*Vishniacozyma victoriae*	1000	96.2	NR_073260.1
F166	ITS	ITS1	*Vishniacozyma tephrensis*	466	96.79	NR_144812.1
F167	ITS	ITS1	*Papiliotrema flavescens*	465	99.32	NR_130696.1

Isolate			Closest BLAST hit (NCBI)			
ID	Target	Primer	Species	Length (bp)	Identity (%)	Accession
F169	ITS	ITS1	*Holtermanniella festucosa*	512	99.41	NR_155179.1
F171	ITS	ITS1	*Vishniacozyma tephrensis*	1054	95.4	NR_144812.1
F172	ITS	ITS1	*Aureobasidium namibiae*	1094	98.32	NR_159598.1
F173	ITS	ITS1	*Aureobasidium namibiae*	1105	98.49	NR_147362.1
F174	ITS	ITS1	*Aureobasidium namibiae*	1081	98.49	NR_147362.1
	LSU	LRO5	*Aureobasidium leucospermi*	909	97.95	NG_058566.1
F175	ITS	ITS1	*Aureobasidium namibiae*	1111	99.04	NR_147362.1
F179	ITS	ITS1	*Sporobolomyces roseus*	555	98.37	NR_155845.1
	LSU	LRO5	*Sporidiobolus metaroseus*	899	99.66	NG_069415.1
F180	ITS	ITS1	*Sporobolomyces roseus*	1081	98.92	NR_155845.1
F181	ITS	ITS1	*Sporobolomyces roseus*	551	99.63	NR_155845.1
	LSU	LRO5	*Sporidiobolus metaroseus*	939	99.66	NG_069415.1
F182	ITS	ITS1	*Sporobolomyces roseus*	539	99.63	NR_155845.1
	LSU	LRO5	*Sporidiobolus metaroseus*	562	96.47	NG_069415.1
F183	ITS	ITS1	*Sporobolomyces roseus*	542	98.52	NR_155845.1
F184	ITS	ITS1	*Sporobolomyces roseus*	556	98.55	NR_155845.1
F185	ITS	ITS1	*Sporobolomyces roseus*	994	98.24	NR_155845.1
	LSU	LRO5	*Sporidiobolus metaroseus*	1023	98.43	NG_069415.1
F186	ITS	ITS1	*Sporobolomyces roseus*	549	99.63	NR_155845.1
F187	ITS	ITS1	*Sporobolomyces roseus*	539	99.63	NR_155845.1
F188	ITS	ITS1	*Sporobolomyces roseus*	952	99.1	NR_155845.1
	LSU	LRO5	*Dioszegia rishiriensis*	1201	98.47	NG_059156.1
F189	ITS	ITS1	*Sporobolomyces roseus*	822	98.57	NR_155845.1
F190	ITS	ITS1	*Sporobolomyces roseus*	1165	98.19	NR_155845.1
F224	ITS	ITS1	*Rhodotorula babjevae*	557	100	NR_077096.1
	LSU	LRO5	*Rhodotorula glutinis*	869	99.77	NG_055728.1
F225	ITS	ITS1	*Cystobasidium slooffiae*	518	97.43	NR_103568.1
	LSU	LRO5	*Cystobasidium slooffiae*	901	99.78	NG_059008.1
F226	ITS	ITS1	*Sporobolomyces roseus*	551	98.36	NR_155845.1
F228	ITS	ITS1	*Sporobolomyces roseus*	546	99.45	NR_155845.1
F229	ITS	ITS1	*Sporobolomyces roseus*	546	99.45	NR_155845.1
F230	ITS	ITS1	*Sporobolomyces roseus*	540	99.63	NR_155845.1
F231	ITS	ITS1	*Sporobolomyces roseus*	555	98.37	NR_155845.1
F232	ITS	ITS1	*Sporobolomyces roseus*	547	99.45	NR_155845.1
F233	ITS	ITS1	*Sporobolomyces roseus*	531	99.62	NR_155845.1
F234	ITS	ITS1	*Sporobolomyces roseus*	679	97.75	NR_155845.1
F235	ITS	ITS1	*Sporobolomyces roseus*	552	99.64	NR_155845.1
F236	ITS	ITS1	*Vishniacozyma victoriae*	456	99.34	NR_073260.1

Isolate			Closest BLAST hit (NCBI)			
ID	Target	Primer	Species	Length (bp)	Identity (%)	Accession
F237	ITS	ITS1	*Vishniacozyma victoriae*	1004	99.38	NR_073260.1
F239	ITS	ITS1	*Vishniacozyma victoriae*	465	99.54	NR_073260.1
F241	ITS	ITS1	*Vishniacozyma tephrensis*	465	96.57	NR_144812.1
F245	ITS	ITS1	*Filobasidium stepposum*	484	99.79	NR_111207.1
F246	ITS	ITS1	*Aureobasidium melanogenum*	528	98.29	NR_159598.1
F249	ITS	ITS1	*Holtermanniella takashimae*	502	98.99	NR_137721.1
F250	ITS	ITS1	*Vishniacozyma victoriae*	459	99.54	NR_073260.1
F253	ITS	ITS1	*Filobasidium chernovii*	1276	98.82	NR_073223.1
	LSU	LRO5	*Filobasidium globisporum*	1235	99.45	NG_070553.1
F254	ITS	ITS1	*Filobasidium chernovii*	571	99.48	NR_073223.1
F268	ITS	ITS1	*Pseudozyma flocculosa*	624	91.45	NR_154663.1
F272	ITS	ITS1	*Sporobolomyces roseus*	965	98.38	NR_155845.1
F273	ITS	ITS1	*Sporobolomyces roseus*	1065	98.92	NR_155845.1
F274	ITS	ITS1	*Sporobolomyces roseus*	547	99.45	NR_155845.1
F275	ITS	ITS1	*Sporobolomyces roseus*	929	98.74	NR_155845.1
F276	ITS	ITS1	*Sporobolomyces roseus*	594	97.56	NR_155845.1
F277	ITS	ITS1	*Sporobolomyces roseus*	526	99.62	NR_155845.1
F278	ITS	ITS1	*Sporobolomyces roseus*	550	99.63	NR_155845.1
F279	ITS	ITS1	*Sporobolomyces roseus*	547	99.63	NR_155845.1
F280	ITS	ITS1	*Cystobasidium psychroaquaticum*	516	99.77	NR_171727.1
	LSU	LRO5	*Cystobasidium psychroaquaticum*	1177	98.3	NG_059007.1
F282	ITS	ITS1	*Aureobasidium melanogenum*	896	98.5	NR_159598.1
F283	ITS	ITS1	*Aureobasidium namibiae*	1105	98.49	NR_147362.1
F284	ITS	ITS1	*Aureobasidium namibiae*	1126	98.66	NR_147362.1
F286	ITS	ITS1	*Aureobasidium melanogenum*	1074	98.5	NR_159598.1
F287	ITS	ITS1	*Aureobasidium melanogenum*	855	98.5	NR_159598.1
F288	ITS	ITS1	*Aureobasidium namibiae*	1105	99.04	NR_147362.1
F291	ITS	ITS1	*Aureobasidium melanogenum*	789	98.32	NR_159598.1
F293	ITS	ITS1	*Aureobasidium namibiae*	889	98.85	NR_147362.1
F294	ITS	ITS1	*Holtermanniella festucosa*	514	99.41	NR_155179.1
	LSU	LRO5	*Holtermanniella takashimae*	677	95.33	NG_060626.1
F295	ITS	ITS1	*Vishniacozyma tephrensis*	469	96.6	NR_144812.1
F296	ITS	ITS1	*Vishniacozyma victoriae*	485	97.66	NR_073260.1
F297	ITS	ITS1	*Papiliotrema flavescens*	972	99.14	NR_130696.1
	LSU	LRO5	*Papiliotrema terrestris*	1247	96.81	NG_058367.1
F298	ITS	ITS1	*Papiliotrema flavescens*	975	98.53	NR_130696.1

Isolate			Closest BLAST hit (NCBI)			
ID	Target	Primer	Species	Length (bp)	Identity (%)	Accession
F298	LSU	LRO5	*Papiliotrema terrestris*	1242	97.15	NG_058367.1
F299	ITS	ITS1	*Filobasidium chernovii*	951	98.21	NR_073223.1
F300	ITS	ITS1	*Vishniacozyma tephrensis*	1007	93.15	NR_144812.1
	LSU	LRO5	*Vishniacozyma carnescens*	934	99.01	NG_058430.1
F301	ITS	ITS1	*Papiliotrema flavescens*	457	99.08	NR_130696.1
	LSU	LRO5	*Vishniacozyma carnescens*	1238	98	NG_058430.1
F302	ITS	ITS1	*Vishniacozyma victoriae*	442	100	NR_073260.1
F303	ITS	ITS1	*Vishniacozyma victoriae*	467	99.54	NR_073260.1
	LSU	LRO5	*Papiliotrema terrestris*	1500	96.6	NG_058367.1
F304	ITS	ITS1	*Papiliotrema flavescens*	437	99.29	NR_130696.1
F305	ITS	ITS1	*Papiliotrema flavescens*	471	99.33	NR_130696.1
F306	ITS	ITS1	*Papiliotrema flavescens*	467	99.1	NR_130696.1
F307	ITS	ITS1	*Vishniacozyma tephrensis*	467	96.58	NR_144812.1
F308	ITS	ITS1	*Papiliotrema flavescens*	456	99.31	NR_130696.1
F309	ITS	ITS1	*Vishniacozyma victoriae*	459	97.33	NR_144812.1
F310	ITS	ITS1	*Vishniacozyma victoriae*	456	99.34	NR_073260.1
F311	ITS	ITS1	*Filobasidium chernovii*	563	99.29	NR_073223.1
	LSU	LRO5	*Filobasidium globisporum*	1207	99.01	NG_070553.1
F312	ITS	ITS1	*Pseudohyphozyma pustula*	1352	83.16	NR_073288.1
	LSU	LRO5	*Hamamotoa lignophila*	1235	93.74	NG_070402.1
F313	ITS	ITS1	*Filobasidium chernovii*	981	98.84	NR_073223.1
	LSU	LRO5	*Filobasidium globisporum*	1227	98.46	NG_070553.1
F314	ITS	ITS1	*Vishniacozyma tephrensis*	441	98.41	NR_144812.1
	LSU	LRO5	*Vishniacozyma carnescens*	895	97.99	NG_058430.1
F315	ITS	ITS1	*Filobasidium chernovii*	571	99.48	NR_073223.1
F316	ITS	ITS1	*Holtermanniella takashimae*	492	100	NR_137721.1
F317	ITS	ITS1	*Papiliotrema flavescens*	965	98.13	NR_130696.1
F318	ITS	ITS1	*Metschnikowia pulcherrima*	678	94.63	NR_164379.1
	LSU	LRO5	*Metschnikowia chrysoperlae*	829	96.04	NG_058336.1
F325	ITS	ITS1	*Filobasidium oeirense*	1086	98.67	NR_077106.1
F326	ITS	ITS1	*Filobasidium chernovii*	1117	99.17	NR_073223.1
	LSU	LRO5	*Filobasidium globisporum*	1286	98.31	NG_070553.1
F327	ITS	ITS1	*Papiliotrema flavescens*	996	98.31	NR_130696.1
	LSU	LRO5	*Papiliotrema terrestris*	1116	98.17	NG_058367.1
F328	ITS	ITS1	*Filobasidium chernovii*	1267	99.15	NR_073223.1
F329	ITS	ITS1	*Holtermanniella festucosa*	329	99.81	NR_155179.1
F330	ITS	ITS1	*Vishniacozyma tephrensis*	456	98.25	NR_144812.1
F331	ITS	ITS1	*Vishniacozyma carnescens*	910	97.93	NR_130695.1
	LSU	LRO5	*Vishniacozyma carnescens*	563	97.51	NG_058430.1

Isolate			Closest BLAST hit (NCBI)			
ID	Target	Primer	Species	Length (bp)	Identity (%)	Accession
F332	ITS	ITS1	*Vishniacozyma tephrensis*	468	96.59	NR_144812.1
F334	ITS	ITS1	*Anthracocystis grodzinskae*	1022	91.01	NR_154694.1
F342	ITS	ITS1	*Aureobasidium namibiae*	910	97.28	NR_159598.1
F343	ITS	ITS1	*Aureobasidium namibiae*	821	99.04	NR_147362.1
F344	ITS	ITS1	*Aureobasidium melanogenum*	506	98.41	NR_159598.1
F345	ITS	ITS1	*Vishniacozyma tephrensis*	940	93.95	NR_144812.1
F345	LSU	LRO5	*Vishniacozyma victoriae*	924	97.31	NG_057678.1
F346	ITS	ITS1	*Sporobolomyces roseus*	550	99.45	NR_155845.1
	LSU	LRO5	*Sporidiobolus metaroseus*	872	97.9	NG_069415.1
F347	ITS	ITS1	*Papiliotrema flavescens*	959	98.12	NR_130696.1
	LSU	LRO5	*Papiliotrema terrestris*	1159	97.7	NG_058367.1
F353	ITS	ITS1	*Sporobolomyces roseus*	930	98.39	NR_155845.1
F354	ITS	ITS1	*Filobasidium chernovii*	602	99.32	NR_073223.1
F355	ITS	ITS1	*Papiliotrema flavescens*	472	99.33	NR_130696.1
	LSU	LRO5	*Papiliotrema terrestris*	1037	96.22	NG_058367.1
F357	ITS	ITS1	*Sporobolomyces roseus*	1043	98.4	NR_155845.1
F359	ITS	ITS1	*Aureobasidium namibiae*	895	99.04	NR_147362.1
	LSU	LRO5	*Aureobasidium pullulans*	389	99.48	NG_055734.1
F360	ITS	ITS1	*Sporobolomyces roseus*	540	99.63	NR_155845.1
F362	ITS	ITS1	*Aureobasidium melanogenum*	790	98.5	NR_159598.1
F363	ITS	ITS1	*Holtermanniella festucosa*	513	99.8	NR_155179.1
F364	ITS	ITS1	*Sporobolomyces roseus*	540	98.51	NR_155845.1
F366	ITS	ITS1	*Vishniacozyma tephrensis*	459	97.33	NR_144812.1
F375	ITS	ITS1	*Vishniacozyma tephrensis*	462	97.11	NR_144812.1
F376	ITS	ITS1	*Holtermanniella festucosa*	513	99.8	NR_155179.1
	LSU	LRO5	*Holtermanniella wattica*	842	96.73	NG_058307.1
F378	ITS	ITS1	*Filobasidium wieringae*	1245	83.18	NR_077105.1
	LSU	LRO5	*Filobasidium magnum*	968	95.87	NG_069409.1
F379	ITS	ITS1	*Holtermanniella festucosa*	513	99.41	NR_155179.1
F381	ITS	ITS1	*Holtermanniella festucosa*	1027	99.24	NR_155179.1
F382	ITS	ITS1	*Filobasidium wieringae*	1091	83.58	NR_077105.1
	LSU	LRO5	*Filobasidium magnum*	1206	95.13	NG_069409.1
F383	ITS	ITS1	*Vishniacozyma victoriae*	875	95.5	NR_073260.1
	LSU	LRO5	*Vishniacozyma carnescens*	1222	95.91	NG_058430.1
F386	ITS	ITS1	*Papiliotrema flavescens*	471	99.33	NR_130696.1
	LSU	LRO5	*Papiliotrema terrestris*	919	98.23	NG_058367.1
F391	ITS	ITS1	*Cystobasidium slooffiae*	528	97.54	NR_103568.1
	LSU	LRO5	*Cystobasidium slooffiae*	910	99.56	NG_059008.1

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