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# Yeasts from Different Habitats and Their Potential as Biocontrol Agents

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**Abstract:** Ever since plant diseases began causing losses in viticulture, the control of phytopathogenic fungi has become of vital interest for winemakers. The occurrence of novel pests, fungicide resistance, and changed consumer expectations have led to an enormous demand for novel plant protection strategies. As part of integrated protection measures, antagonistic microorganisms have been investigated to a large extent. Such microorganisms can be applied not only in conventional, but also in organic farming as biological control agents (BCA). Particularly, yeasts were found to be interesting candidates for the development of BCA. Many of these eukaryotic microorganisms are found as part of the phylloplane microflora. In this study, we assessed a set of 38 yeast isolates from different habitats, including the guts of termites, for inhibitory effects against some phytopathogenic fungi that have received less attention in earlier studies. The majority of yeasts were found to interfere with fungi infecting grapevine (*Eutypa lata*, *Botrytis cinerea*, and *Roesleria subterranea*), stone fruits (*Monilinia fructicola*), or rice (*Magnaporthe oryzae*), as well in vitro and in model experiment on fruits. Although most yeast strains secreted glycoside hydrolases and proteases, attempts to demonstrate direct antagonistic activities of lytic enzymes failed. However, in culture filtrates of the termite yeast *Papiliotrema odontotermis* OO5, a low molecular thermostable antagonistic factor was detected. Iron depletion as a BCA mechanism was confirmed for strains of *Metschnikowia pulcherrima* but not for other yeasts.

**Keywords:** biological control agents; yeasts; phytopathogenic fungi; competition; lytic enzymes; pulcherrimin; termite

## 1. Introduction

Pathogenic fungi cause significant annual losses in the quality and quantity of crops, resulting in considerable economic damage. Furthermore, fungi are profound producers of secondary metabolites that are toxic to humans when consumed [1]. *Botrytis cinerea* (teleomorphic form: *Botryotinia fuckeliana*) has been identified as pathogen of more than 235 plant species, including grapes, lettuce, tomatoes, tobacco, and strawberries [2]. In viticulture, *B. cinerea* may cause serious loss (gray rot) but also improvement of wine quality (noble rot), depending on the stage of ripening in which the berries are infected and on the weather conditions [3]. A cryptic complex of species were found to cause gray mold and the core species, *B. cinerea*, has been genetically separated into distinct groups (N and S). Repeated fungicide applications have resulted in a drastic increase of multiple resistances in *Botrytis* isolates [4,5]. The filamentous ascomycete *Roesleria subterranea* causes root rot in grapevines and fruit trees. Because infected plants die suddenly after two or three years and efficient control methods are not available

at present, this pathogen leads to considerable economic losses [6]. *Eutypa lata* is the causal agent of dead arm disease on vines and is able to infect other plants, (e.g., olives and apricots) [7]. Often, years pass between infection and the first visible signs of a disease [8]. The phytopathogenic fungus *Magnaporthe oryzae* is responsible for enormous crop losses in rice plants and *Monilinia fructigena* in stone fruits [9].

It is a permanent challenge to protect crops and foodstuffs against attack by pathogens through a variety of chemical and natural methods. The demand for biological measures is constantly increasing, as chemicals are often not safe for humans and the environment [10–12]. Moreover, fungi can develop resistance to chemical antifungal agents [4,5]. In biological control, living organisms and their metabolites or botanicals are used to control pathogens on fruit and plants. This strategy appears to be a promising alternative to conventional methods alone or as part of integrated pest management [12,13]. A wide range of bacteria (e.g., *Bacillus*, *Pseudomonas*, and *Streptomyces*), filamentous fungi (e.g., *Trichoderma*, *Gliocladium*, *Chlonostachys*, and *Ulocladium*), and yeasts (e.g., *Aureobasidium* and *Candida*) have been reported to exhibit inhibitory activity against fungus-incited diseases [10–21]. Especially with regard to wine production, yeasts as natural inhabitants of grapes and vine [22] appear less problematic than bacteria and fungi. The phenomenon of antagonistic activity in yeasts was first observed in the genus *Saccharomyces cerevisiae*. Some strains are able to inhibit the growth of sensitive strains of the same species by secreting a proteinaceous “killer toxin” [23]. Ever since its discovery, this phenomenon has been studied intensively to open the way for new approaches in yeast biopesticide research. The antifungal properties are not based on a single activity pattern of the yeasts, but also involve competition for food and space and production of volatile secondary metabolites (e.g., ethanol, ethyl acetate, and CO<sub>2</sub>) or lytic enzymes [10,11,24].

Examples of currently registered biocontrol/biopreservation products consisting of yeast or yeast-like fungi as active ingredient are Aspire<sup>®</sup> (*Candida oleophila*), Candifruit<sup>®</sup> (*Candida sake*), Shemer<sup>®</sup> (*Metschnikowia fructicola*), and BoniProtect<sup>®</sup> (*Aureobasidium pullulans*). The great potential of *Pichia anomala* (syn. *Wickerhamomyces anomalus*) and other antagonistic yeasts has yet to be manifested in the form of available products for plant protection [17,18]. The successful application of a BCA depends on temperature, humidity, season, and many other environmental factors that must be considered [12,25]. Thus, the effectiveness of a product from one year or season to the next can vary to a large extent and there is a constant demand for novel products with potential biocontrol properties. Therefore, we investigated the possible inhibitory effects of yeasts originating from different environments against some phytopathogenic fungi. Particularly, we included organisms that have been paid less attention in comparable studies, such as intestinal yeasts from termites, fungi associated with serious grapevine diseases (*Eutypa lata*, *Roesleria subterranea*), and *Magnaporthe oryzae*, the most destructive agent of rice worldwide [9]. The main focus of this work was the search for possible, novel BCA candidates and their target organisms.

## 2. Materials and Methods

In this investigation, we use the terms “yeasts” and “fungi” for simplicity to differentiate between unicellular and filamentous growing fungi. All chemicals were purchased from Roth (Karlsruhe, Germany) or Sigma-Aldrich (Munich, Germany).

### 2.1. Yeasts, Fungi, and Culture Conditions

The yeast strains used in this investigation have been isolated from different environmental habitats over a period of several decades and are deposited at the local culture collection of the Institute of Molecular Physiology, Johannes Gutenberg-University Mainz (Table 1). Species identifications were originally performed by physiological tests and have been later confirmed for most strains by PCR amplification and sequencing of the internal transcribed spacer (ITS 1-5.8S-ITS 2) region. DNA was isolated from the pure cultured cells using an InstaGene<sup>™</sup> Matrix kit (Bio-Rad, Munich, Germany), according to the manufacturer’s protocol. Amplification of the ITS region was performed in

a mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany) with oligonucleotide primers ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS 5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'). The following PCR conditions were used: pre-denaturation for 5 min at 95 °C, followed by 35 cycles for 1 min at 94 °C, afterwards annealing for 1 min at 55 °C and extension for 1 min at 72 °C, and finally, a post-extension step for 10 min at 72 °C.

**Table 1.** Yeast strains investigated as putative antagonistic microorganisms.

Species/Strain	Habitat	References
<i>Candida sake</i> 2/42	roach	[26]
<i>Debaryomyces hansenii</i> var. <i>fabryii</i> 525	cherry	[27]
<i>Hanseniaporea uvarum</i> 469	pear	[28]
<i>Hanseniaporea uvarum</i> 470 (ATCC 64295)	raspberry	[28]
<i>Hanseniaporea uvarum</i> 471	peach	[28]
<i>Hanseniaporea uvarum</i> 473	apple	
<i>Hanseniaporea uvarum</i> 486	grape	
<i>Hanseniaporea uvarum</i> 527	grape	[29]
<i>Kluyveromyces marxianus</i> 118	grape	
<i>Kluyveromyces thermotolerans</i> 76	grape (Crete)	
<i>Metschnikowia pulcherrima</i> 152	apple	
<i>Metschnikowia pulcherrima</i> 160	grape	
<i>Metschnikowia pulcherrima</i> 192	unknown	
<i>Metschnikowia pulcherrima</i> 2305	unknown	
<i>Metschnikowia pulcherrima</i> 446	grape	[30]
<i>Metschnikowia pulcherrima</i> 523	grape	
<i>Metschnikowia pulcherrima</i> 648	grape	
<i>Pichia anomala</i> 457 (NCYC 435)	unknown	[15]
<i>Pichia kluyveri</i> 2143	apple	
<i>Pichia kluyveri</i> var. <i>kluyveri</i> 395 (CBS 7145, ATCC 66811)	grape	[29]
<i>Pichia methanolica</i> H1/3-1	cherry	
<i>Torulaspora delbrueckii</i> 3/40	cherry	
<i>Wickerhamomyces anomalus</i> 15	apple	
<i>Wickerhamomyces anomalus</i> 227	grape	[30]
<i>Wickerhamomyces anomalus</i> AS1 (DSM 28943)	grape	[31,32]
<i>Wickerhamomyces anomalus</i> H.3.2	wheat flour (Spain)	
<i>Wickerhamomyces anomalus</i> WH 1021	praline	[33]
<i>Williopsis californica</i> 3/62	cherry	
<i>Williopsis saturnus</i> var. <i>makrii</i> 458 (NCYC 500)	soil (Papua New Guinea)	[15]
<i>Zygosaccharomyces bailii</i> 412 (CBS 6708)	orange (Brasil)	
<i>Zygosaccharomyces bailii</i> 550	grape	
<i>Apiotrichum mycotoxinovorans</i> MYG	termite	[34,35]
<i>Apiotrichum mycotoxinovorans</i> MD123D	termite	[34,35]
<i>Naganishia albida</i> OO1	termite	[35]
<i>Papiliotrema odontotermitis</i> OO5 (DSM 100791 <sup>T</sup> , CBS 14181 <sup>T</sup> )	termite	[35]
<i>Saitozyma flava</i> OO2	termite	[35]
<i>Sugiyamaella mastotermitis</i> MD39V (DSM 100793 <sup>T</sup> , CBS 14182 <sup>T</sup> )	termite	[35]
<i>Sugiyamaella smithiae</i> NM1	termite	[35]

Filamentous fungi (Table 2) were obtained from CBS (Centraalbureau voor Schimmelcultuur, Westerdijk Fungal Biodiversity Centre, Utrecht, The Netherlands), ISA (Instituto Superior de Agronomia, Lisboa, Portugal), IBWF (Institut für Biotechnologie und Wirkstoff-Forschung, Kaiserslautern, Germany), or DSMZ (Leibniz-Institut DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany).

Yeasts were cultured in YPD medium (yeast extract 10 g/L, peptone 20 g/L, and glucose 20 g/L) and fungi in potato dextrose broth (PDB; Roth, Germany). Solid media were prepared with 15 g/L agar.

**Table 2.** Plant-pathogenic fungi used as target organisms.

Fungal Strain	Habitat	Source
<i>Botrytis cinerea</i> V15	<i>Vitis vinifera</i>	ISA
<i>Botrytis cinerea</i> V19	<i>Vitis vinifera</i>	ISA
<i>Botrytis cinerea</i> V27	<i>Vitis vinifera</i>	ISA
<i>Botrytis cinerea</i> B16-14	<i>Vitis vinifera</i>	ISA
<i>Botrytis cinerea</i> DSM 877	unknown	DSMZ
<i>Monilinia fructigena</i> DSM 2677	<i>Malus</i>	DSMZ
<i>Magnaporthe oryzae</i> 7015	<i>Oryza sativa</i>	IBWF
<i>Eutypa lata</i> 1190	<i>Vitis vinifera</i>	IMWF
<i>Eutypa lata</i> 16012	<i>Vitis vinifera</i>	IBWF
<i>Roesleria subterranea</i> CBS 201.25	<i>Vitis vinifera</i>	CBS
<i>Roesleria subterranea</i> CBS 271.82	<i>Populus</i>	CBS
<i>Roesleria subterranea</i> CBS 320.33	<i>Malus</i>	CBS
<i>Roesleria subterranea</i> CBS 339.96	shrub	CBS

## 2.2. Preparation of Fungal Spore Suspensions

After incubation for 14 days at 20 °C, fungal mycelia and spores were scraped from well-grown potato dextrose agar (PDA) plates and transferred to falcon® tubes containing 10 mL of sterile water. The suspensions were homogenized by vortexing to release conidia from the mycelium and filtered through two layers of Miracloth® (MilliporeSigma, Munich, Germany) in order to obtain spore suspensions ( $\approx 10^6$  spores/mL).

## 2.3. Enzymatic Activities

The enzymatic activities of yeasts and fungi were visualized on agar plates containing specific enzyme substrates [36–38]. Yeasts were tested by dropping 10  $\mu$ L of an YPD culture on these plates, which were incubated at 30 °C for 3–5 days. For filamentous fungi, agar plugs of 2–4 mm<sup>2</sup> were cut out from mycelium grown on the PDA plates, placed in the center of the diagnostic enzyme plates, and incubated at 20 °C for 4–14 days.

### 2.3.1. $\beta$ -Glucosidase Activity

Agar medium (5.0 g of esculin, 20.0 g of peptone from meat, 10.0 g of yeast extract, and 12.0 g of agar) were solubilized in 990 mL of deionized water on a magnetic stirrer at 100 °C, adjusted to pH 5.0, and autoclaved (20 min at 121 °C). Before pouring into petri dishes, 10 mL of sterile-filtered ammonium iron(III) citrate solution (1.0% *w/v*) were added.

A dark brownish halo can be observed in  $\beta$ -glucosidase-positive yeasts. The enzyme  $\beta$ -glucosidase converts the esculin in the plate to esculetin and glucose. The released esculetin forms a dark complex with iron from the ammonium iron(III) citrate.

### 2.3.2. Cellulase Activity

Agar medium (5.0 g of carboxymethylcellulose sodium salt (CMC), 10.0 g of yeast extract, 20.0 g of peptone from meat, and 15.0 g of agar) were solubilized in 1000 mL of deionized water on a magnetic stirrer at 100 °C, adjusted to pH 5.0, and autoclaved (20 min at 121 °C). For evaluation, the plate was first treated with 0.2% (*w/v*) congo red solution. The dye was poured off after 10 min of exposure time, and the plate was decolorized by several treatments with 1 M NaCl. As a result, clear hydrolysis zones were visible at the sites where cellulase activity degraded the substrate, whereas the non-modified CMC background remained orange-red colored.

### 2.3.3. Amylase Activity

Agar medium (10.0 g of yeast extract, 20.0 g of peptone from meat, 20.0 g of starch, and 12.0 g of agar) were solubilized in 1000 mL of deionized water on a magnetic stirrer at 100 °C, adjusted to pH 5.0, and autoclaved (20 min at 121 °C).

The amylase plates were overlaid with Lugol's solution (iodine–potassium iodide solution) before evaluation. After 3 min of exposure, Lugol's solution was drained off, and the plates were allowed to stand for at least 30 min. Over time, a bright lysis zone was observed for amylase-positive organisms, while the rest of the plate remained violet in color as the dye solution stained the unmodified starch.

### 2.3.4. Protease Activity

Agar medium (1.0 g of yeast extract, 10.0 g of skimmed milk powder, 4.0 g of peptone from meat, and 10.0 g of agarose) were solubilized in 1000 mL of citrate–phosphate buffer (100 mM, pH 5.0: 10.1 g/L citric acid·H<sub>2</sub>O, 18.8 g/L NaHPO<sub>4</sub>·2H<sub>2</sub>O) on a magnetic stirrer at 100 °C, and autoclaved (5 min at 121 °C). For protease-positive organisms, a translucent lysis court was identified after incubation, in which the proteases degraded the milk protein.

### 2.3.5. Chitinase Activity

Agar medium (2.0 g of NaNO<sub>3</sub>, 1.0 g of K<sub>2</sub>HPO<sub>4</sub>, 0.5 g of MgSO<sub>4</sub>·7H<sub>2</sub>O; 0.5 g of KCl, 0.2 g of peptone from meat, 10.0 g of chitin, and 15.0 g of agar) were suspended in 1000 mL of deionized water on a magnetic stirrer at 100 °C, adjusted to pH 5.0, and autoclaved (20 min at 121 °C). The chitin plates were overlaid with Lugol's solution prior to evaluation. The solution was poured off after 3 min of exposure time. In the case of chitinase secretion, distinct lysis zones formed around the colonies. The remainder of the plate remained dark as the dye solution reacted with chitin, and a brownish yellow color became visible by this reaction.

## 2.4. Antagonistic Activity of Yeasts against Phytopathogenic Fungi on Agar Plates

The “killer stroke” method was performed as described elsewhere [14,39]. The antagonistic effect of the yeasts was tested on the PDA plates. In order to evaluate the effect of iron depletion on fungal growth, the PDA was prepared without and with 5.0 mg/L, 10 mg/L, and 20 mg/L FeCl<sub>3</sub> [40,41]. On dried plates, each yeast strain was applied in the form of a center streak and incubated for two days at 30 °C. The fungi were cut as 5-mm-wide and 5-mm-long mycelium plugs from well-grown PDA plates with a lancet and placed about 2–3 cm to the right and left of the yeast screed. Depending on the type of fungus, the plates were incubated for 1 to 4 weeks at 20 °C. The aim of the experiments was to find out whether fungal growth was inhibited on the PDA and whether the addition of different iron concentrations in the medium attenuated the inhibitory effect.

## 2.5. Preparation of Fungal Cell Wall Components

The fungi used here were found to be susceptible in previous experiments (*B. cinerea* strains DSM 877 and B16-14). Rough cell wall preparations were obtained following the procedure described by Chan and Tian [42] with some modifications. First, 50 mL of PDA liquid medium in Erlenmeyer flasks were inoculated with 1.0 mL of a fungal spore suspension (10<sup>6</sup> spores/mL). The flasks were incubated stationary at 20 °C for 7–14 days. Thereafter, the culture suspension was rinsed through a paper filter and washed twice with sterile water. The washed biomass was placed in a 50 mL falcon<sup>®</sup> tube, filled up to the 10 mL mark with sterile water, and autoclaved (20 min at 121 °C). The inactivated fungal cells were disrupted by ultrasound treatment (Sonifier 250; Branson Ultrascall, Dietzenbach, Germany) for 10 min. The falcon<sup>®</sup> tube was frozen at –20 °C and then placed in a freeze-drying apparatus (Lyovac GT 2; Steris GmbH, Köln, Germany) for 1–2 days. After determining the mycelium dry weight, the fungal material was suspended in 10 mL of sterile water and homogenized again by

sonification. The fungal cell wall material was tested for possible induction of extracellular antagonistic factors in yeast liquid cultures.

### 2.6. Preparation of Cell-Free Culture Filtrates

In order to assess whether inhibition involves secreted antimicrobial agents or lytic enzymes, the culture filtrates of yeasts were concentrated by three different procedures: lyophilization, ultrafiltration, and extraction with ethyl acetate.

#### 2.6.1. Lyophilization

For the cultivation of yeasts, baffled flasks were used with 250 mL of YPD medium each. Putative induction of extracellular antagonistic factors was assessed in culture media prepared with 0.1% (*w/v*) fungal cell wall material. The flasks were inoculated with 1.0% (*v/v*) of a preculture and incubated at 30 °C on a shaker for five days. Subsequently, the culture suspensions were centrifuged (12 min, 8600 *g*), and the supernatants were passed through a 0.2 µm vacuum filter unit. The sterile supernatants were filled in dialysis tubing with a pore size of 3.0 kDa and dialyzed for three days at 5 °C against deionized water. Thereafter, the dialysates were poured in round-bottom flasks, frozen at −20 °C and then left for 1–2 days to freeze dry, and lyophilizates were dissolved in 10 mL of sterile water. The concentrated culture supernatants, which should contain molecules >3 kDa were tested in agar diffusion assays. Sterile YPD medium was applied as a control. The plates were incubated 4–14 days at 20 °C.

#### 2.6.2. Ultrafiltration

First, 50 mL of fresh yeast cultures were centrifuged (12 min, 8600 *g*), and the supernatants were rinsed through a paper filter. The supernatants were filtered through a 10 kDa Vivaspin® concentrator (Sartorius, Göttingen, Germany) for 3 h at 8600 *g*. The permeates of Vivaspins® were collected and freeze dried. The dry material was balanced, dissolved in 5.0 mL of water, and sterilized by filtration (pore size 0.2 µm).

The Vivaspin® retentate, the Vivaspin® permeate, and the concentrated Vivaspin® permeate were tested using the agar diffusion test. As a control, sterile YPD medium was pipetted into one of the wells. The plates were incubated for 4–14 days at 20 °C.

#### 2.6.3. Extraction with Ethyl Acetate

First, 200 mL of a three-day-old yeast culture in YPD were centrifuged (30 min at 4 °C and 17,700 *g*). The supernatant was placed in a separatory funnel, and 250 mL of ethyl acetate were added. This mixture was incubated for 30 min and shaken several times. Finally, the lower, aqueous phase was drained off. The ethyl acetate phase was transferred to a 1000 mL round-bottom flask through a paper filter to which 60 g of sodium sulfate were added to bind the water. The extract was concentrated with the aid of a rotary evaporator (Büchi, Essen, Germany) to dryness. The concentrated substances were dissolved in 3.0 mL of methanol. The solution was transferred to sample vessels and placed in a vacuum concentrator (Eppendorf, Hamburg, Germany). After evaporation of the methanol, the dry weight was determined, and the material was dissolved in 2.0 mL of sterile water and 100 µL of dimethylsulfoxide (DMSO).

The solution with potentially active substances was tested using an agar diffusion assay. As a negative control, DMSO (5.0% *v/v*) was pipetted into one of the wells. As a positive control, the herbicide BASTA® (Bayer, Leverkusen, Germany) was used in a final concentration of 2 mg/mL. The plates were incubated 4–14 days at 20 °C.

### 2.7. Agar Diffusion Assay

First, 100  $\mu\text{L}$  of a fungal spore suspension ( $10^6$  spores/mL) were spread on the PDA plates, and after incubation at room temperature for 24 h, holes were punched out and filled with 100  $\mu\text{L}$  of the test substances. The plates were incubated at 20 °C for 3–14 days, depending on the growth of the fungi. Antagonistic activity was easily recognized by a growth inhibition zone around the holes containing test substances.

### 2.8. Antagonistic Activities on Fruits

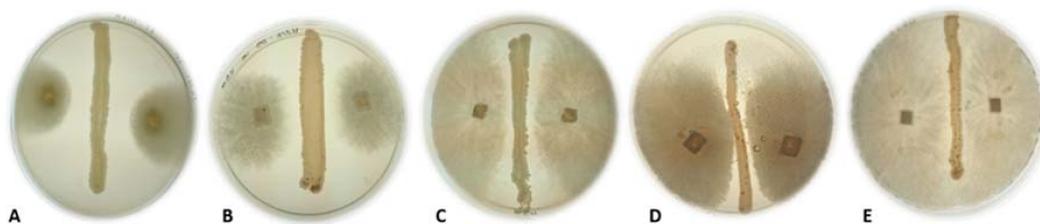
The model experiment with apples followed the procedure described by Zhang et al. [43]. Holes were punched into each of ten apples with a sterile pipette tip and filled by means of a syringe with the appropriate samples: (a) control with 200  $\mu\text{L}$  of sterile water; (b) 200  $\mu\text{L}$  of sterile filtered culture supernatant; (c) 200  $\mu\text{L}$  of fungal spore suspension; (d) 100  $\mu\text{L}$  of fungal spore suspension + 100  $\mu\text{L}$  of sterile filtered culture supernatant; and (e) 100  $\mu\text{L}$  fungal spore suspension + 100  $\mu\text{L}$  yeast suspension. The apples were incubated for seven days at room temperature. For evaluation, the fruits were cut with a knife so that the puncture holes were visible in the longitudinal section. Due to the growth of the fungus, it became brown and rotten in the apple at the puncture site. In the case of antagonistic activity of the yeast against the fungus, this rot was reduced or even prevented.

For the grape trials, three samples were prepared: 10 mL of yeast suspension, 10 mL of sterile filtered culture supernatant, and 10 mL of YPD medium (control). Nine grapes were prepared by washing with 70% (*v/v*) ethanol. Thereafter, they were punctured by a sterile needle eight times at regular intervals. The punctured grapes were placed in the three media for 2–3 h. The grapes were removed from the solutions under sterile conditions, left briefly to allow liquids to dry on the grape, halved, and then exposed to the PDA plates previously seeded with 100  $\mu\text{L}$  of a fungal spore suspension ( $10^6$  spores/mL). Control grapes were exposed to the PDA plates that had not previously been inoculated with the fungal spores and mycelium.

The plates were incubated at room temperature for seven days. Antagonistic activities were detected by a zone of growth inhibition around the grape.

## 3. Results and Discussion

We assessed the antagonistic activity of 38 yeasts strains from different habitats (wine, fruits, soil, and termite gut) against 12 fungal strains representing the phytopathogenic species *B. cinerea*, *M. fructigena*, *E. lata*, *R. subterranea*, and *M. oryzae*. Some typical results of the *in vitro* experiments on agar plates are shown in Figure 1 and are summarized in Table 3.



**Figure 1.** Patterns of antagonistic interaction between yeasts and phytopathogenic fungi grown on potato-dextrose agar. (A) *P. anomala* 457 vs. *E. lata* 16012 (+++); (B) *Z. bailii* 550 vs. *E. lata* 1190 (++); (C) *C. sake* H1/3-29 vs. *B. cinerea* 16-14 (+); (D) *C. sake* H1/3-29 vs. *B. cinerea* 877 ( $\pm$ ); (E) *P. methanolica* H1/3-1 vs. *R. subterranea* CBS 339.96 (–). The fungus is strongly inhibited (+++) when growing away from the yeast on both sides; the mycelium of the fungus does not reach the yeast, and the inhibition zone between fungus and yeast is about 1.0 and 2.0 cm. Medium inhibition (++) when the fungus grows away from the yeast; the inhibition zone is between 0.5 and 1.0 cm. Slight inhibition (+) when there is a distance between 0.2 and 0.5 cm between fungus and yeast. The fungus is not inhibited (–) when growing unrestrained on the agar plate in direction of yeast.

**Table 3.** Antagonistic activities of yeasts against fungi as observed on agar plates.

Yeast Strain	Fungal Strain											
	<i>B. cinerea</i> V15	<i>B. cinerea</i> V19	<i>B. cinerea</i> V27	<i>B. cinerea</i> 16-14	<i>B. cinerea</i> DSM 877	<i>M. fructigena</i> DSM 2677	<i>M. oryzae</i> 7015	<i>E. lata</i> 1190	<i>E. lata</i> 16012	<i>R. subterranea</i> CBS 271.82	<i>R. subterranea</i> CBS 320.33	<i>R. subterranea</i> CBS 339.96
<i>M. pulcherrima</i> 160	+	–	–	++	++	++	+++	+	nd	++	++	++
<i>M. pulcherrima</i> 446	++	–	–	++	+++	++	+++	+	nd	+++	++	+++
<i>M. pulcherrima</i> 648	+++	–	+	++	+++	++	+++	–	nd	+	+	+
<i>M. pulcherrima</i> 152	+++	–	+	+++	+++	++	++	–	nd	+++	+++	+++
<i>M. pulcherrima</i> 192	+++	–	+	++	+++	++	++	–	nd	++	–	–
<i>M. pulcherrima</i> 523	+	–	–	+	++	+	++	–	nd	+	–	–
<i>M. pulcherrima</i> 2305	+	–	–	++	++	+	+++	+	nd	+++	++	+
<i>C. sake</i> 2/42	–	–	+	+	+++	+	–	–	–	++	+	–
<i>P. anomala</i> 457	+++	–	+	++	–	+++	++	++	+++	nd	nd	–
<i>P. kluyveri</i> 2143	++	–	+	++	+++	–	+++	–	–	–	–	–
<i>P. kluyveri</i> 395	++	–	–	++	–	–	+++	–	–	+	–	+
<i>P. methanolica</i> H1/3-1	+	–	+++	+	++	–	+++	–	–	+	nd	–
<i>Z. bailii</i> 412	+	–	–	+	+++	+	+++	–	–	+	–	+
<i>Z. bailii</i> 550	–	–	–	–	+	–	+++	+	–	+	–	–
<i>W. anomalus</i> AS1	–	–	+++	++	+++	++	+++	–	–	++	+++	+++
<i>W. anomalus</i> H.3.2	–	–	+++	+++	+++	+	+++	–	–	+	++	+
<i>W. anomalus</i> WH 1021	–	–	+++	++	+++	+	+++	–	–	+	+	+++
<i>W. anomalus</i> 15	+	–	+++	++	+++	+	+++	–	–	++	++	+++
<i>W. anomalus</i> 227	+	+	–	++	+++	–	+++	–	–	++	+++	+++
<i>W. californica</i> 3/62	–	–	++	+	+	++	–	–	–	–	++	++
<i>W. saturnus</i> 458	–	–	–	+	++	++	–	–	–	+	+	–
<i>T. delbrueckii</i> 3/40	–	–	+++	+	++	–	+++	–	–	–	–	–
<i>K. thermotolerans</i> 76	–	–	–	–	–	–	–	–	–	–	–	–
<i>K. marxianus</i> 118	–	–	+	–	+	+	–	–	–	–	+	–
<i>H. uvarum</i> 469	–	–	++	+	+	+	++	++	+	++	–	–
<i>H. uvarum</i> 470	–	–	+	–	+	++	–	–	–	–	–	–
<i>H. uvarum</i> 471	–	–	++	–	++	++	++	+	++	–	–	–
<i>H. uvarum</i> 473	–	–	++	+	+	+	++	+	++	+	–	–
<i>H. uvarum</i> 486	–	–	–	–	++	+	–	+	+	–	–	–
<i>H. uvarum</i> 527	–	–	+	–	++	+	–	+	+	–	–	–
<i>D. hansenii</i> 525	–	–	+	–	+	–	–	–	–	–	–	–
<i>A. mycotoxinoovorans</i> MYG	–	+	+	–	+	–	–	–	–	–	–	–
<i>A. mycotoxinoovorans</i> MD123D	–	–	+	–	+	–	–	–	–	–	–	–
<i>N. albidia</i> OO1	–	–	+	+	+	+	–	–	–	–	–	+
<i>P. odontotermis</i> OO5	+	–	+	++	+	+	–	–	–	–	–	–
<i>S. flava</i> OO2	+	–	+	+	+	–	–	–	–	–	–	–
<i>S. mastotermis</i> MD39V	–	–	+	+	+	–	–	–	–	–	–	+
<i>S. smithiae</i> NM1	–	–	+	–	+	–	+	–	–	–	–	–

For genus abbreviations, see Tables 1 and 2. The evaluation of the results followed the scheme described in Figure 1; nd: not determined.

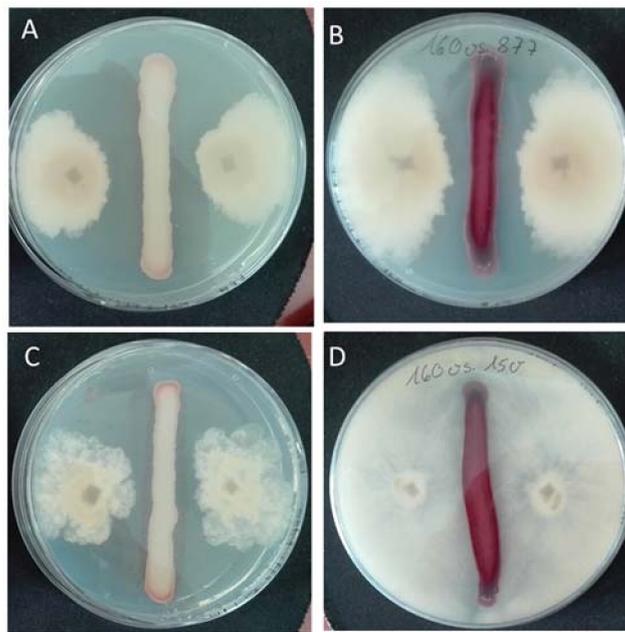
It is evident that the growth of each of the pathogenic fungi was inhibited by at least one yeast species to a different extent. Some fungal strains (*M. oryzae* 7015, *M. fructigena* DSM 2677, and *B. cinerea* strains V27, 16-14, and DSM 877) proved to be sensitive to many yeast strains, whereas others (*B. cinerea* V15 and V19; *E. lata* strains 1190 and 16012; and *R. subterranea* strains CBS 271.82, 320.33, and CBS 339.96) were inhibited by a restricted number of yeast strains. There is a remarkable difference among the sensitivity of the five *B. cinerea* strains. This divergent behavior may reflect the broad population diversity of this pathogen, which is structured not only by the host plant, but also by the cropping system, geography, and fungicide applications [44]. Strain DSM 877 may present a special case, because it is a laboratory strain used for biotechnological purposes and is probably less adapted to natural environments than the others.

Regarding the yeasts, strains of *M. pulcherrima* and *W. anomalus* showed a broad spectrum of antagonistic activities. Both species have been repeatedly isolated from grapes and other plants [22,45–48]. Strains of *M. pulcherrima* have already been suggested for biocontrol of fungal diseases on apples [49], grapes [39], sweet cherries [18], strawberries [50], mango fruits [51], and even for clinical treatment of mycoses in mammals [52].

The related species *Metschnikowia fructicola* has been reported to be an antagonistic organism against fungal diseases occurring after harvest [53,54]. Originally, it was believed that *M. pulcherrima* differs from its sister species *M. fructicola* by the production of the reddish pigment pulcherrimin [55], whereas later other authors have reported that both species are able to produce the compound [40]. Pulcherrimin is a chemical complex, formed between iron and pulcherriminic acid. In bacteria, pulcherriminic acid is synthesized via cyclodileucine by enzymatic condensation of two leucyl-tRNAs. Homologues of the *Bacillus subtilis* gene cluster could not be identified in the genome of *M. fructicola*, and so, the synthetic pathway has still to be elucidated in yeasts [56,57]. In contrast to common siderophores, which facilitate cellular iron uptake, pulcherriminic acid is water-soluble until it binds iron and then precipitates as the water-insoluble, red-brown pulcherrimin complex. Therefore, it was hypothesized that *M. pulcherrima* uses this chelating agent as a competitive factor in order to outcompete fungi and bacteria through iron depletion [40,41,58].

Our results could verify these earlier reports in part. For example, addition of iron completely reversed the growth inhibition of *B. cinerea* V15 by *M. pulcherrima* 160, while vegetative growth of *B. cinerea* DSM 877 was not affected (Figure 2). Thus, elucidation of the mechanisms of antagonism is not always straightforward [56]. Enzymatic activities may also play a role in the antagonism (e.g., the application of a chitinase from *M. fructicola* overexpressed in *Pichia pastoris* counteracts brown rot fungi in peaches) [59]. Saravanakumar et al. [60] showed that a *M. pulcherrima* strain MACH1 has the ability to secrete chitinases in different liquid media and pointed out that the enzyme could be involved in the postharvest biocontrol of *B. cinerea*. In our study, we detected chitinase activities in all *M. pulcherrima* strains and in some other yeasts (Table 4).

*Wickerhamomyces anomalus* (formerly *Pichia anomala*) is the second species in our experimental setup, for which a broad antagonistic spectrum was observed. The yeast has been extensively studied for applications against postharvest spoilage molds [15,25] and for wine biotechnology [32]. *W. anomalus* and other yeasts produce various volatile organic compounds (VOC), and the biocontrol activities of these microorganisms have been mainly ascribed to the action of ethyl acetate [11,50]. Furthermore, *W. anomalus* strains were found to inhibit the human pathogenic fungus *Aspergillus flavus* by secreting 2-phenyl ethanol, which prevents spore formation and reduces the biosynthesis of aflatoxins [24].



**Figure 2.** Antagonistic interactions on the PDA plates. *M. pulcherrima* 160 and *B. cinerea* DSM 877 in the presence of (A) 5 mg/L FeCl<sub>3</sub> and (B) 20 mg/L FeCl<sub>3</sub>; *M. pulcherrima* 160 and *B. cinerea* V15 in the presence of (C) 5 mg/L FeCl<sub>3</sub> and (D) 20 mg/L FeCl<sub>3</sub>.

**Table 4.** Extracellular enzymatic activities of the strains investigated.

Yeast	Secreted Enzymatic Activity				
	Amylase	Cellulase	β-Glucosidase	Chitinase	Protease
<i>M. pulcherrima</i> 160	++	—	+++	+	++
<i>M. pulcherrima</i> 446	+	—	+++	+	++
<i>M. pulcherrima</i> 648	+++	—	+++	+	+++
<i>M. pulcherrima</i> 152	+	—	+++	+	+
<i>M. pulcherrima</i> 192	+	—	+++	+	++
<i>M. pulcherrima</i> 523	+	—	+++	+	+++
<i>M. pulcherrima</i> 2305	++	—	+++	+	++
<i>C. sake</i> 2/42	+++	—	++	—	+
<i>P. anomala</i> 457	+++	—	++	+	—
<i>P. kluyveri</i> 2143	++	—	++	—	+
<i>P. kluyveri</i> 395	++	+	+	—	++
<i>P. methanolica</i> H1/3-1	+++	—	+++	+++	+++
<i>Z. bailii</i> 412	++	+	++	—	++
<i>Z. bailii</i> 550	+	—	+	—	—
<i>W. anomalus</i> AS1	+	+	+	+	+++
<i>W. anomalus</i> H.3.2	+	+	+	+	++
<i>W. anomalus</i> WH 1021	+	+	+	—	++
<i>W. anomalus</i> 15	+	+	+	+++	++
<i>W. anomalus</i> 227	+	+	+	++	+++
<i>W. californica</i> 3/62	+	+	+	—	+
<i>W. saturnus</i> 458	+	+	+	++	—
<i>T. delbrueckii</i> 3/40	+	+	+	+++	+
<i>K. thermotolerans</i> 76	+	+	+	+	+
<i>K. marxianus</i> 118	+	—	+	+	—
<i>H. uvarum</i> 469	—	+	+	+	+
<i>H. uvarum</i> 470	—	+	+	—	+
<i>H. uvarum</i> 471	—	+	+	+	—
<i>H. uvarum</i> 473	—	+	+	+	+
<i>H. uvarum</i> 486	—	—	+	—	+
<i>H. uvarum</i> 527	—	+	+	+	+

Table 4. Cont.

Yeast	Secreted Enzymatic Activity				
	Amylase	Cellulase	$\beta$ -Glucosidase	Chitinase	Protease
<i>D. hansenii</i> 525	++	+	+	–	+
<i>A. mycotoxinovorans</i> MYG	+++	–	++	–	++
<i>A. mycotoxinovorans</i> MD123D	+++	–	+++	–	+
<i>N. albida</i> OO1	+++	+	++	–	+
<i>P. odontotermitis</i> OO5	+++	+	++	+	++
<i>S. flava</i> OO2	–	–	+	+	–
<i>S. mastotermitis</i> MD39V	+++	+	++	–	+
<i>S. smithiae</i> NM1	+	+	+	+	+
<b>Fungus</b>					
<i>B. cinerea</i> V15	+	–	+	+	–
<i>B. cinerea</i> V19	+	–	+	+	–
<i>B. cinerea</i> V27	+	–	–	+	+
<i>B. cinerea</i> 16-14	+	–	+	+	+
<i>B. cinerea</i> 877	+	–	+	+	+
<i>M. fructigena</i> DSM 2677	+	–	+	+	+
<i>M. oryzae</i> 7015	+	–	+	+	+
<i>E. lata</i> 1190	+	–	+	+	+
<i>E. lata</i> 16012	+	–	+	+	+
<i>R. subterranea</i> CBS 271.82	+	–	+	+	+
<i>R. subterranea</i> CBS 320.33	+	–	+	+	–
<i>R. subterranea</i> CBS 339.96	+	–	+	+	–

For genus abbreviations, see Tables 1 and 2. Evaluation: (+), (++) and (+++) mean slight, medium, and strong positive reaction zones (>1 cm) around the yeast colonies, respectively; (–) means no detectable activity.

Antagonistic activities were also detected in the genera *Candida*, *Debaryomyces*, *Pichia*, *Hanseniaspora*, *Kluyveromyces*, *Williopsis*, and *Zygosaccharomyces* (Table 3), which is in line with earlier observations [10,11,14,28,29].

Yeasts that exhibited antimycotic activities on the agar plates were tested under more environmentally related conditions. As shown in Figures 3 and 4, fruits treated with yeast suspensions efficiently prevented growth of the co-inoculated phytopathogenic fungus.



**Figure 3.** Antagonistic activity on fruits. The grape was soaked with a suspension of *P. methanolica* H1/3-1 and placed on PDA inoculated with spores of *B. cinerea* 887. Around the treated grape, a clear zone of fungal growth inhibition is evident.



**Figure 4.** Antagonistic activity on fruits. Apple inoculated with *P. odontotermitis* OO5 (A), *B. cinerea* B16-14 (B), and *P. odontotermitis* OO5 + *B. cinerea* B16-14 (C). Inoculation with the fungal strain caused a brown lesion zone, which was significantly reduced by co-inoculation with the yeast.

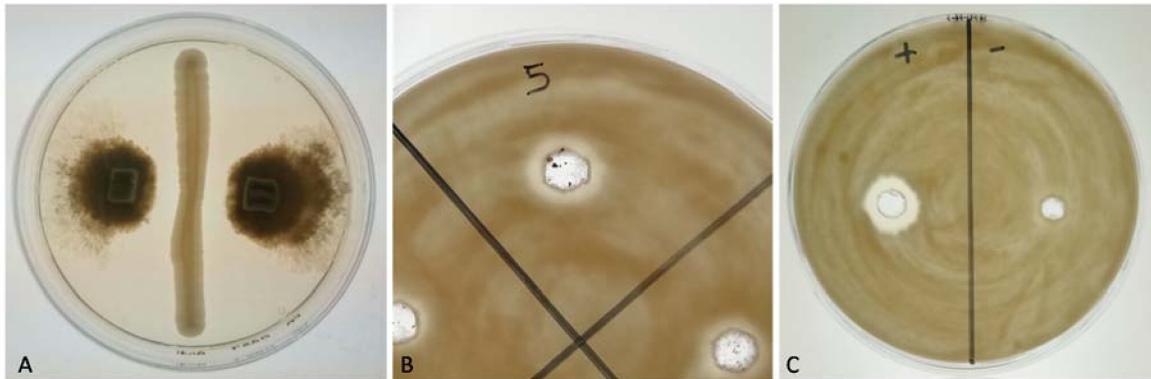
Apart from secondary metabolites, exo- $\beta$ -1,3 glucanases seem to be involved in the antagonistic action of *W. anomalus* (strain K) against *B. cinerea* and *Penicillium expansum* on apples, *B. cinerea* on grapes, *Penicillium digitatum* on oranges, and *Colletotrichum gloeosporioides* on papayas [11]. Nevertheless, the role of lytic enzymes as antagonistic “toxins” against fungi is a matter of debate. The results of Grevesse et al. [61] ruled out any involvement of exo- $\beta$ -1,3 glucanases, whereas Friel et al. [62] demonstrated, by gene knock-out experiments, that exoglucanase PaExg2 of strain K is essential for the inhibition of *B. cinerea*. Zhang et al. [63,64] reported antifungal activities of an exo-1,3- $\beta$ -glucanase secreted by the antagonistic yeast *Pichia guilliermondii* and of an alkaline serine protease released by the biocontrol agent *Aureobasidium pullulans* PL5. It should be considered that enzyme systems for fungal cell lysis are usually a mixture of several different enzymes, including one or more  $\beta$ -1,3- and  $\beta$ -1,6-glucanases, proteases, mannanases, or chitinases, acting synergistically to lyse the cell wall [65]. In this context, we screened for lytic exoenzymes in the organisms investigated and found significant secreted activities of different glycoside hydrolases and proteases (Table 4). The  $\beta$ -glucosidase activities may simultaneously indicate the presence of  $\beta$ -glucanases, as found for *W. anomalus* AS1 in our previous study [32].

Surprisingly, all attempts to demonstrate direct antagonistic activities of lytic enzymes in the yeast culture filtrates by the agar diffusion assay failed, regardless of culture conditions (non-induced or induced with fungal cell walls), and the concentration method used (lyophilization or ultrafiltration). Possible explanations may be low activities, low stabilities, diffusion barriers or enzymatic (proteolytic) inactivation by the fungi.

In contrast, we detected non-enzymatic antagonistic activities in the cultures of yeasts isolated from the guts of termites. To our knowledge, this is one of the first reports on the biocontrol potential of intestinal yeasts. Termites fed on wood, plant decay products, grass, soil, and dung. For the digestion of these materials, these insects require hydrolytic enzymes, such as cellulases and xylanases, which are predominantly supplied by microorganisms living in their digestive systems. In addition, microbes in the intestines of organisms often protect termites and other insects from attack by harmful fungi [66]. This implies potential antagonistic effects of termite yeasts against competitors. Therefore, intestinal yeasts from termites were tested in this work for antifungal activity. Termite yeasts were found to inhibit growth of *B. cinerea* strains on agar plates in an effective manner (Table 3, Figure 4A). In ethyl

acetate extracts and in the concentrated ultrafiltrate fraction (<10 kDa), we detected antagonistic activity in *P. odontotermidis* OO5 (Figure 4B), which is presumably due to a low molecular thermostable factor.

The termite yeasts included in this study are not obligate gut symbionts and can be efficiently cultivated under aerobic laboratory conditions. In a model experiment with apples, *P. odontotermidis* OO5 successfully counteracted infection by *B. cinerea* (Figure 5).



**Figure 5.** Antagonistic activity of *P. odontotermidis* OO5 against *B. cinerea* B16-14. (A) Growth inhibition on PDA; (B) agar diffusion assay with culture filtrate extracted with ethyl acetate; and (C) positive control (BASTA<sup>®</sup>) and negative control (5% v/v DMSO).

Thus, yeasts from termite gut may present a promising group of biocontrol organisms. A practical example is *A. mycotoxinivorans*, which was found to degrade mycotoxins, such as ochratoxin A and zearalenone. This termite yeast has been assessed as a safe to be used as a probiotic food additive for toxin decontamination in animal feed [34,67].

#### 4. Conclusions

The inhibitory effects of yeasts vary according to antagonist and environmental conditions and are based on different principles [11]. Verified inhibitory mechanisms of some yeast genera, such as the iron complexation by *M. pulcherrima*, are not valid for other yeasts studied here. Although exoenzyme production of the investigated yeasts is versatile, the involvement of lytic enzymes in antagonistic reactions remains essentially unclear and may depend on experimental and environmental conditions. The inhibitory activities of the yeasts were confirmed in natural environments, such as table grapes and apples, even after the freeze-drying of the yeast cultures [25]. Furthermore, mycotoxin production is generally of little concern in yeasts compared with filamentous fungi [17], and most genera have GRAS (Generally Recognized as Safe) status and are useful for food fermentations [68]. Yeasts from the original environment of the pathogens may be more active in terms of antagonistic activity than commercial killer yeasts, such as *Candida oleophila*. Nevertheless in the experiments presented here, termite yeasts were antagonistic to selected phytopathogenic fungi both in vitro and in model plant/fruit experiments. In particular, the observed inhibitory effect of *P. odontotermidis* OO5 against *B. cinerea* appears promising. Therefore, it is mandatory to investigate the identity and molecular basis of yeast antagonistic factors in more detail.

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**Conflicts of Interest:** The authors declare no conflict of interests.

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