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Mechanisms and importance of naturally occurring antifungal resistance in the yeast-like fungus *Aureobasidium pullulans*

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Abstract

Antifungal agents inhibit the growth of or kill fungi by targeting various biochemical or biological processes. However, fungi continually develop mechanisms to evade antifungals. This doctoral thesis aimed to elucidate the antifungal resistance mechanisms of naturally occurring yeasts for the ultimate goal of discovering new plant protection strategies. The research focus was on the isolation, identification, and characterization of fungicide sensitivity of ubiquitous yeasts, molecular and functional characterization of the resistance mechanisms, and the application of these yeasts in combined disease control strategies.

Chapter 1 provides an overview of the plant protection agents, the biology of yeasts, and the importance of yeasts in ecosystems. Fungicide use and its impacts, yeast interactions, and the practical importance of these interactions are given a particular focus.

Chapter 2 documents the antifungal agents and reviews previously characterized antifungal mechanisms with a focus on four groups: demethylase inhibitors, the quinone outside inhibitors, anilinopyrimidines, and captan.

Chapter 3 demonstrates the widespread distribution of naturally occurring yeasts with tolerance to commonly used antifungal agents. Using MALDI-TOF and Sanger sequencing of the ITS region, 376 isolates from 47 taxa were identified after isolation in the presence of fungicides. Among the taxa, isolates of the yeast-like fungus *Aureobasidium pullulans* were the most abundant. The sensitivity profiles of 30 strains of this species were investigated in microbroth sensitivity assays with captan (CPN), cyprodinil (CYP), and difenoconazole (DFN). These strains' minimum inhibitory concentrations (MIC_{50}) recorded high tolerance of *A. pullulans* to CPN, CYP, and DFN.

Chapter 4 describes the antifungal tolerance mechanisms in environmental and clinical *A. pullulans*. The study in this chapter built upon the CPN, CYP, and DFN sensitivity data for 30 *A. pullulans* strains, newly generated sensitivity data for clinical *Aureobasidium* isolates, and genome sequences of 46 strains in total to characterize tolerance mechanisms. GWAS and literature-based approaches predicted possible CPN, CYP, and DFN antifungal resistance mechanisms. SNP analysis within the coding regions reported 1767 genes involved in CYP tolerance of *A. pullulans*, and only four genes for CPN were predicted. The involvement in CYP resistance of five *S. cerevisiae* gene homologs of *A. pullulans* (*STL1, LYS2, ENO1, HOL1,* and *CAC2*) using the respective gene deletion strains was confirmed experimentally by agar spot assays. Through heterologous expression of predicted *A. pullulans*' genes in *S. cerevisiae* and microbroth sensitivity assays, novel CYP tolerance mechanisms involving five newly predicted *A. pullulans* genes (*FBD, GST, HP5, HP6,* and *HP8*) were uncovered.

Chapter 5 describes a protocol of competition assays to quantify the effect of biocontrol yeasts against plant pathogenic fungi on fruits.

Chapter 6 demonstrates the synergistic interaction of CYP and a CYP-tolerant *A. pullulans* strain in controlling *Botrytis* spp. lesions on apples. The particular focus was on optimizing an artificial laboratory system for testing such synergism.

The final chapter recapitulates the study and gives future perspectives for research on antifungal resistance mechanisms and the applications of yeast-fungicide combinations.

Kurzfassung

Antimykotika hemmen das Wachstum oder töten Pilze ab, indem sie verschiedene biochemische oder biologische Prozesse hemmen. Pilze entwickeln jedoch ständig Mechanismen, um sich Antimykotika zu entziehen. Diese Doktorarbeit beabsichtigte die antimykotischen Resistenzmechanismen von natürlich vorkommender Hefe aufzuklären und letztendlich neue Pflanzenschutzstrategien zu entdecken. Der Forschungsschwerpunkt lag auf der Isolierung, Identifizierung und Charakterisierung Fungizid-tolerater Hefen, der molekularen und funktionellen Charakterisierung der Resistenzmechanismen und der Anwendung dieser Hefen in kombinierten Krankheitsbekämpfungsstrategien.

Kapitel 1 gibt einen Überblick über die Pflanzenschutzmittel, die Biologie der Hefen und die Bedeutung von Hefen in den Ökosystemen. Ein besonderer Fokus wird auf dem Einsatz von Fungiziden und seinen Auswirkungen, Hefewechselwirkungen und der praktischen Bedeutung dieser Wechselwirkungen gesetzt.

Kapitel 2 dokumentiert die Antimykotika und überprüft zuvor charakterisierte antimykotische Mechanismen mit einem Fokus auf vier Gruppen: Demethylase-Inhibitoren, externe Chinon-Inhibitoren, Anilino-Pyrimidine und Captan.

Kapitel 3 zeigt die weite Verbreitung natürlich vorkommender Hefen mit (erhöhter) Toleranz gegenüber häufig verwendeten Antimykotika. Mittels MALDI-TOF- und Sanger-Sequenzierung der ITS-Region wurden nach Isolierung in Gegenwart von Fungiziden 376 Isolate aus 47 Taxa identifiziert. Unter den Taxa waren Isolate des hefeartigen Pilzes *Aureobasidium pullulans* am häufigsten. Die Sensitivitätsprofile von 30 Stämmen dieser Spezies wurden in Microbroth-Sensitivitätstests mit Captan (CPN), Cyprodinil (CYP) und Difenoconazol (DFN) untersucht. Die minimalen Hemmkonzentrationen dieser Stämme (MHK50) wiesen eine hohe Toleranz von *A. pullulans* gegenüber CPN, CYP und DFN auf.

In Kapitel 4 werden die antimykotischen Toleranzmechanismen in *A. pullulans* Isolaten aus der Umwelt und dem klinischen Bereich chrakterisiert. Die Studie in diesem Kapitel baute auf den CPN-, CYP- und DFN-Sensitivitätsdaten für 30 *A. pullulans* Stämme, neu generierten Sensitivitätsdaten für klinische *Aureobasidium*-Isolate und Genomsequenzen von insgesamt 46 Stämmen auf, um Toleranzmechanismen zu charakterisieren. GWAS und literaturbasierte Ansätze prognostizierten mögliche CPN-, CYP- und DFN-antimykotische Resistenzmechanismen. Die SNP-Analyse ergab, dass 1767 Gene an der CYP-Toleranz von *A. pullulans* und nur 4 Gene für CPN beteiligt sind. Die Beteiligung an der CYP-Resistenz von fünf *S. cerevisiae* Gen-Homologenpaaren von *A. pullulans* (*STL1*, *LYS2*, *ENO1*, *HOL1* und *CAC2*) wurde unter Verwendung der jeweiligen Gendeletionsstämme experimentell durch Agar-Spot-Assays bestätigt. Durch heterologe Expression der vorhergesagten Gene von *A. pullulans* in *S. cerevisiae* und Microbroth-Sensitivitätstests wurden neuartige CYP-Toleranzmechanismen mit fünf neu vorausgesagten Genen von *A. pullulans* (*FBD*, *GST*, *HP5*, *HP6* und *HP8*) aufgedeckt.

Kapitel 5 beschreibt ein Protokoll von Konkurrenzassays zur Quantifizierung der Wirkung von Biokontrollhefen gegen pflanzenpathogene Pilze auf Früchten.

Kapitel 6 zeigt die synergistische Wechselwirkung von CYP und einem CYP-toleranten *A. pullulans*-Stamm bei der Kontrolle von *Botrytis* spp.-Läsionen auf Äpfeln. Der besondere Fokus lag auf der Optimierung eines künstlichen Laborsystems, um solche Synergien zu testen.

Das letzte Kapitel fasst die Studie zusammen und gibt Zukunftsperspektiven für Forschung zu antimykotischer Resistenzmechanismen und der Anwendungen von Hefe-Fungizid-Kombinationen.

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Chapter 1

Introduction

1 Agricultural pesticides with particular focus on fungicides and their impacts

1.1 Agricultural pesticides current use and trends

Pesticides are natural or synthetic compounds employed in various settings to prevent or limit harmful organisms' damage to crops, health, and property. Agriculturally used pesticides are also called plant protection products; thus, the two terms will be used interchangeably for this study. The major plant protection products are classified into the following groups: fungicides and bactericides; insecticides and acaricides; herbicides, haulm destructors, and moss killers; molluscicides; plant growth regulators; and other plant protection products [1]. Pesticide sales are used as an indicator of environmental pesticide application [2]. The current global use of agricultural pesticides is approximately 2.66 million metric tons per year (Figure 1a), with the most significant consumers being the USA, Brazil, China, Argentina, Russia, India, Thailand, Italy, France, and Canada [3]. Worldwide pesticide use increased tremendously (50%) between 1990 and 2011, after which there has been only a slight increase (Figure 1a) [3, 4] as a result of strict regulation in pesticide usage by some continents, especially in Europe. However, other countries, including those in Africa and Asia, are massively increasing their usage [5]. Similarly, although there has been a reduction in the use of some pesticide groups in various parts, other groups of pesticides are increasingly being used in some countries [4, 6, 7].

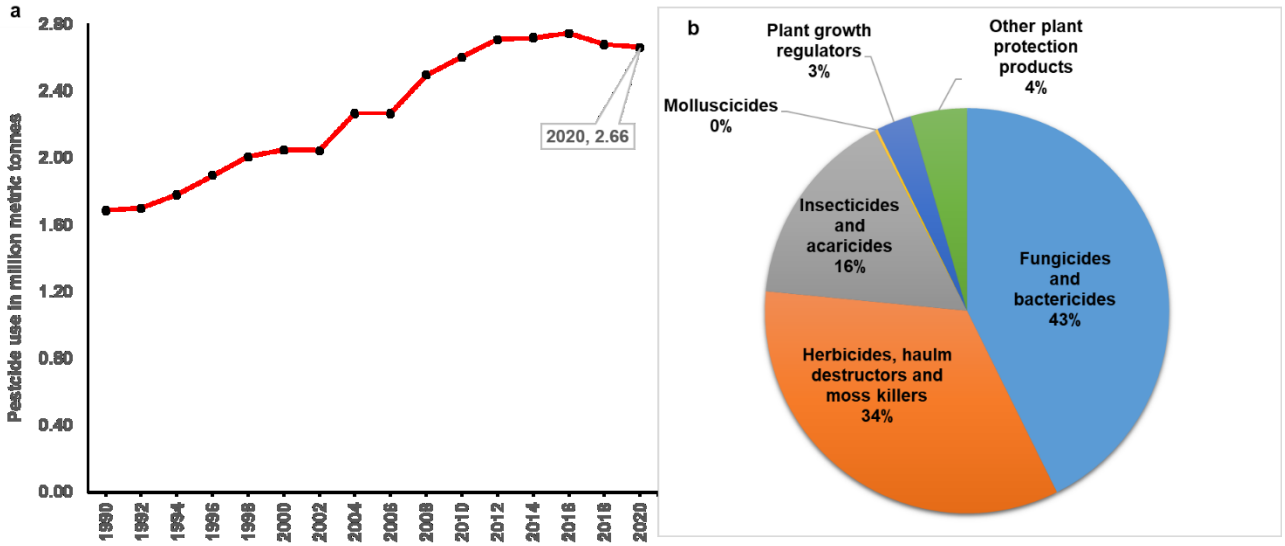

Figure 1. Pesticides use. a) The worldwide trend in total agricultural pesticide use between 1990 and 2020. b) The percentage of sales per major category of plant protection agents in Europe in 2020 (data includes EU member states with Iceland, Switzerland, and Norway). These graphs were generated based on the documented pesticide sales [3, 8].

1.2 Fungicide use and its impacts

Fungicides constitute an important group of plant protection agents. Fungicides are agents employed to prevent, control or eradicate fungi during production, storage or distribution of food crops. These agents are also refered to as antifungals and therefore, fungicides and antifungals will be used interachangebly in this study. Globally, Europe is considered the largest market for fungicide sales [9]. In the European Union (EU), as of 2020, 43% of all the agricultural pesticides sold were fungicides and bactericides, 34% were herbicides, 16% were insecticides and acaricides, while the other three groups of plant protection agents made up the

remaining 7% (Figure 1b) [8]. The high usage of fungicides in Europe is also evidenced by a recent report whereby soil sampled from 340 locations across the EU contained fungicides and herbicides at higher frequencies and concentrations than expected, but still below toxic limits [10].

Most fungicides have specific targets in the fungi, but some have multiple targets. Fungicides are classified broadly according to the mode of action and the specific biochemical functions they disrupt within the fungi [11]. Some common groups that will be encountered within this study include demethylation inhibitors (DMIs), anilinopyrimidines (APs), quinone outside inhibitors (QoIs), and phthalimides [11]. Based on the growth stage of the targeted fungus, fungicides can play a protective, eradicative, or curative role or a combination [12]. Based on the biological mode of action, protectants such as QoIs act against fungal spore germination and prevent infection and the initiation of the disease cycle. Curative fungicides such as DMIs and QoIs are effective against the early, but post penetrative stages. Eradicants such as APs act in later phases of the fungal life cycle.

Fungicides are indispensable for plant protection against fungal pathogens that threaten crop production and food security [13, 14]. They are used against economically important plant pathogens including *Botrytis cinerea, Fusarium* spp., *Puccinia* spp., *Magnaporthe oryzae, Blumeria graminis, Colletotrichum* spp., *Mycosphaerella graminicola, Ustilago maydis,* or *Melampsora lini* [15]. The most affected crops include wheat, barley, rice, cocoa, coffee, and several fruits and vegetables [12, 14]. For example, fungicide field trials to control diseases caused by *B. graminis* and *Puccinia* spp. on winter wheat increased the production from 6 to 12 Tons/ha between 1983 and 2005 in Sweden [16]. Notably, due to climate change and human activities, the number of fungal plant pathogens has increased and is predicted to continue to do so [17]. Thus, there will be more need to efficiently control fungal plant pathogens using fungicides. Better management of fungicide use is thus imperative to ensure this pesticide group's sustained effectiveness against the current and growing fungal plant pathogens.

Despite their essential role in agriculture, the extensive use of fungicides in the environment elicits serious concerns about their direct or indirect effects on humans, animals, and the environment. Using fungicides reduces the abundance of non-target fungi, such as *Bulleribasidiaceae*, or even important bacteria, such as *Hymenobacter* and *Sphingomonas*, disrupting the plants' microbiome [18]. Apart from reducing organism abundance, fungicide use also leads to the emergence of resistance in non-target, human pathogenic fungi. For example, using environmental azoles lead to the emergence of resistant *Aspergillus* spp., which causes fungal diseases that are very problematic to manage and threaten human health [19-22]. The rapid evolution of resistance in plant pathogens, which might disrupt the agricultural ecosystem, has also been observed as an adverse effect of fungicide use [23, 24]. The development of resistance also causes ineffectiveness and a shortened lifespan of fungicides [25]. Therefore, the common worldwide goal is to manage fungicide use in order to maximize effectiveness while reducing detrimental environmental effects.

2 Yeasts and their significance

Yeasts are unicellular fungal organisms defined by their budding or fission reproduction characteristics [26]. Some fungal organisms that live mostly as yeasts but develop hyphae under special conditions are still regarded as yeasts, for example, the yeast-like fungus *Aureobasiudium pullulans* [26]. Yeasts are diverse and included in two broad divisions: ascomycetes, which reproduce sexually by forming asci; and basidiomycetes which sexually produce basidiospores [26].

Yeasts make up a large group of microorganisms and due to discrepancies in methodology and species concept, the exact species number is unknown. The number has continuously grown from 164 in 1952 to 700 by 1998 and at such growth, extrapolation estimated the species number to be between 1500 and 15000 by 2016 based on different prediction models [29]. Yeasts are identified using several methods including the following: DNA sequence based methods, protein based methods, molecular genetics, chemotaxonomic, physiological and morphological methods, immunological, or chromogenic [27, 28]. The degree of differentiation among the taxonomic groups depends on the method used. For example, some methods can only differentiate yeasts at the genus level, while others can differentiate strains within a given species [27, 28].

2.1 The biology and stress tolerance of yeast

Yeasts are metabolically diverse and possess morphological and biochemical characteristics that make them robust. Thus, they occur and survive in varied environmental niches globally [31]. Yeasts possess a characteristic cell wall made of β-1,3-D-glucan (60%), mannoproteins (40%), and a tough chitin polymer (1%). The cell wall has mainly a protective role and allows interactions in different environmental conditions [32, 33]. For instance, under stressful conditions, the cell wall integrity (CWI) pathway is activated [32-34]. Activation of the CWI pathway leads to a strengthening the cell wall by increasing chitin, altering the crosslinking of cell wall components, and redistributing of chitin and glucan [32, 33]. Together these three processes help yeasts resist lytic enzymes from other organisms or cell wall-distressing agents including fungicides [32, 33, 35]. Additionally, yeasts have cell wall integrity sensors that can identify environmental stresses and activate stress signaling pathways which are relayed to the nucleus to change gene expression [36, 37]. The activation of signaling pathways allows the yeast to respond to various stress conditions [34, 37]. Metabolically, yeasts can utilize a wide range of carbon sources and survive under both aerobic and anaerobic conditions [38-40]. Yeasts grow and metabolize fast, thus giving yeast cells a colonization and nutrient competition advantage [40]. Classical reproduction in yeast occurs asexually, but some yeasts can reproduce sexually under stressful conditions [31]. The sexual lifestyle gives yeasts an adaptive advantage [41, 42]. The resourcefulness of yeasts is further depicted in their ability to switch between unicellular and filamentous morphology (e.g., *A. pullulans* and *Candida albicans*) to maximize nutrient assimilation or avoid being phagocytosed [43, 44]. Most yeast genomes are small and have ploidy plasticity, which allows the yeast to reversibly change its chromosome numbers to cope with stress. This is for example the case for *C. albicans, Cryptococcus neorfomans,* and *Saccharomyces cerevisiae* [45].

Yeasts can resist many stressors due to the several characteristics described above. As such, they are found in diverse environments and under extreme conditions. In hypersaline environments, mostly ascomycetous black yeast, e.g., *A. pullulans, Hortaea werneckii,* but also other ascomycetous and basidiomycetes yeasts have been isolated [46, 47]. Similarly, some yeast species including *Rhodotorula* spp., *Cryptococcus* spp, *A. pullulans*, and *Candida* spp. can survive in extremely cold environments such as melted ice, or subglacial and supraglacial sediments [48, 49]. Some yeasts have also been described to tolerate heavy metals (e.g., copper, zinc, and nickel), high acidity, and ethanol [50-53]. From the fungicide tolerance perspective, studies have shown how yeasts can adapt to tolerate fungicide stress [35]. Nevertheless, there is still limited knowledge on which and how many environmentally occurring yeasts tolerate fungicides and to which degree. Therefore, in this study, naturally occurring yeast will be isolated in the presence of fungicides, identified, and their individual sensitivities to fungicides characterized.

2.2 Interactions and practical significance of yeasts

Yeasts can interact with other yeasts, filamentous fungi, bacteria, plants, and animals, allowing them to thrive in different niches. These interactions might be beneficial, neutral, or detrimental to the involved organisms [54]. The various yeast interactions are significant for clinical, agricultural, and biotechnological applications. For instance, biotechnologically, the interactions between yeasts and other yeasts or bacteria are exploited in the wine and bread-making industry [30, 54, 55]. Some non-*saccharomyces* yeasts and *S. cerevisiae* synergistically interact (e.g., by modifying how nutrients are utilized), which leads to better fermentation output and wine aroma [55]. Interaction of yeasts and bacteria with humans is also of clinical importance, whereby in a healthy state, there is a balance between the microorganisms where both live commensally on the skin, in the gut, mouth, and reproductive tract [56]. However, some host factors such as antibiotic treatments, a weakened immune system, injuries to the skin, poor oral hygiene, or surgeries might cause an imbalance leading to yeast overgrowth and disease [56]. In agriculture, yeast interactions are mostly beneficial, where yeast may interact directly with plants promoting growth through the production of growth regulators and plant hormones [57-59]. Yeasts may also interact with mycorrhizae fungi stimulating hyphae growth and colonization of the later and increased nutrient uptake by the plant as a result of synergistic yeast-mycorrhizae fungi interaction [60-62]. Additionally, an agriculturally important interaction is that of yeast and filamentous fungi through antagonism, in which, the yeasts employ different strategies to suppress the growth of fungal pathogens [54, 60, 63, 64]. The practical use of yeast antagonistic characteristics will be explored during this study.

3 Combined plant protection strategies

In light of the adverse effects of pesticide use on the environment, the integrated pest management (IPM) concept was introduced [65]. IPM, as defined by Kogan, is a "decision support system for the selection and use of pest control tactics, singly or harmoniously coordinated into a management strategy, based on cost/benefit analyses that take into account the interests of and impacts on producers, society, and the environment"[66]. This definition of IPM put in plant protection context has been adapted by the European Commission to mean: carefully considering all available plant protection approaches and subsequently integrating processes that discourage the emergence of harmful organism populations, while maintaining the use of plant protection products and other intervention forms to economically and ecologically justified levels that minimize risks to human health and the environment [67]. For this study, the plant protection concept of IPM will be relevant.

3.1 Physical methods in combined disease management

Fungicides applications are combined with different approaches in order to achieve the plant protection goals. Physical methods and biological agents are employed alone or in combination with chemical fungicides to control fungal pathogens as alternative options in disease management [68-70]. Examples of physical methods include irradiation, heat (hot water dipping) treatment, low temperature, or modified atmosphere [71-74]. These strategies have minimal risk to humans or the environment since there are no residues. However, they may not be sustainable depending on how these methods are applied to control fungal pathogens. For example, the use of heat and radiation on some fruits has been associated with the induction of fungitoxic compounds (e.g., scoparone, phytoalexins, and coumarins), which enhances resistance to fungal pathogens [74-76]. Irradiation, however, might cause damage to the fruits. Storage at low temperatures slows down the metabolic processes and fungal pathogen respiration, thus fungi are only dormant and not eradicated [77].

Similarly, hot water treatment activates the biosynthesis of some antifungal compounds (e.g., phytoalexins), which only enhance the natural defence mechanism against fungi [78]. Some of these physical methods have been combined with chemical fungicides (e.g., irradiation and low concentrations of chemical fungicides) [68]. Additionally, some physical methods are also combined with biological methods for disease management [79]. Overall, the combinations of physical methods and fungicides or physical and biocontrol approaches have the disadvantages of being expensive, lack persistence and, have pathogen species-specificity, while being less preventive compared to chemical fungicides [68, 79].

3.2 Biological methods in combined disease management

Using biological agents is another viable option in IPM strategies. Biological control involves applying living organisms or their derivatives to control plant diseases by antagonistic effects and induction of resistance [80, 81]. Examples of organisms used in plant protection against fungi are *Trichoderma* species [82]. These fungi systematically induce plant resistance against other fungi and employ other antagonistic mechanisms including parasitism, competition, and hydrolytic enzymes [82]. Another example is using bacterial inducers of plant resistance such as *Bacillus mycoides* as biocontrol agents against fungal pathogens [83]. Combining *Trichoderma* spp. or *B. mycoides* with chemical fungicides led to better control of pathogens, better plant health, and reduced the number of fungicide sprays needed to manage fungal disease than either fungicides or biocontrol agents used alone [83, 84].

Other important biocontrol agents are yeasts and their derivatives. Several yeasts, both ascomycetous and basidiomycetous, have antagonistic activities against fungal pathogens [85-87]. These yeasts can control fungal pathogens through different mechanisms such as parasitism, competition for space and nutrients, biofilm formation, induction of plant host defenses, and production of metabolites against the pathogens [88-90]. Combinations of these yeasts with chemical fungicides have also been documented and showed better control of fungal pathogens than either yeast or fungicide used alone [69]. The biggest challenge in integrated disease management where biocontrol and chemical fungicide are used interchangeably or one after the other is the timing of the biocontrol application for efficient disease control [69, 91-93]. Thus, it would be interesting to know if combination in a single formulation would enable timely management of the pathogens. Additionally, some fungicides might also target yeasts; hence having a fungicide-resistant, antagonist yeast would be essential to make a compatible formulation. Therefore, it will be interesting to find a fungicide-resistant yeast with effective biocontrol activity for such combined formulations and applications.

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Chapter 2

A review of fungal resistance mechanisms to azoles, anilinopyrimidines, strobilurins, and captan

Abstract

Most antifungal agents have a specific target in the fungi. However, the fungi devise mechanisms to compromise these targets, resulting in reduced effectiveness of the antifungal agents. These mechanisms have extensively been characterized and reported in clinical and agricultural pathogenic fungi. However, there is the constant and increased use of some antifungals; thus, new resistance mechanisms are continuously evolving. Also, some fungi show resistance, yet the drug targets might be lacking, or the resistance mechanisms to those antifungal agents are yet to be understood. Here, we reviewed the resistance mechanisms to four antifungal groups: the demethylase inhibitors (DMIs), also known as azoles in the clinical setting; the strobilurins (quinone outside inhibitors (QoIs), the anilinopyrimidines (APs), and captan. Our review offers a solid basis for further mechanistic characterizations of antifungal resistances in target and non-target fungi.

1 Introduction

Antifungal agents are used in clinical and agricultural settings to manage diseases caused by pathogenic fungi that would otherwise lead to crop loss threatening food security, severe human illnesses, or even death [1-3]. These agents have particular and specific targets in the fungi, although some compounds have a multimode action [4, 5]. The various antifungals used in clinical settings are classified into eight groups based on their targets. These include ergosterol inhibitors (azoles), glucan synthesis inhibitors (echinocandins), disruptors of ergosterol function (polyenes), nucleic acid synthesis inhibitors (flucytosine), epoxidase inhibitors (squalenes), protein synthesis inhibitors (sordarins), microtubulin synthesis inhibitors (griseofulvin), and chitin synthesis inhibitors (nikkomycin and polyoxins) [5]. Of these groups, the most used antifungals against human fungal infections are the echinocandins, polyenes, flucytosine, and azoles, with azoles being the dominating group [6-8].

Agriculturally used chemical fungicides are classified broadly into ten groups based on the biochemical mode of action and further into several subgroups based on the specific target within the biosynthetic pathway of the plant pathogenic fungi [4]. Eight broad classes interfere with the following biochemical processes: nucleic acid metabolism, tubulins, actin metabolism, respiration, amino acid and protein synthesis, membrane integrity and lipid transport, signal transduction, membrane sterol biosynthesis, cell wall biosynthesis, and melanin synthesis. At the same time, two classes are made of fungicides with a multisite or unknown mode of action (i.e., phthalimides) [4]. The categorization based on specific targets is elaborate (see [4]) and only some examples will be mentioned here.

This review focuses on the clinically used azole group and four agriculturally used groups based on the specific target categorization (i.e., demethylation inhibitors (DMIs), anilinopyrimidines (APs), the quinone outside inhibitors (QoIs), and phthalimides) [4]. QoIs and DMIs have several biochemical groups and are extensively used against a broad range of fungi. For instance, the QoIs are registered in over 70 countries across the globe for use against pathogens in 80 different crops and have been regarded as the world's most sold class of fungicides [9]. Currently, DMIs and QoIs are among the commonly used fungicides in the US [10]. There has also been a remarkable rise in the usage of DMIs; for example a 434% (539-2880 metric tons) increase in triazole use was reported between 2006 and 2016 in the US [11]. With the extensive and increased use of DMIs and QoIs fungicides, the adverse effects, including resistance development, is probable. Notably, five agriculturally used triazoles are structurally similar and resistant strains show cross-resistance to clinically used azoles, (i.e., difenoconazole, tebuconazole, propiconazole, bromuconazole, epoxiconazole), which might accelerate resistance development in clinical settings [12]. APs are also a commonly used group of fungicides in agriculture since the 1990s. They are effective against pathogens resistant to QoIs, but are often used in combination with other fungicides [13, 14]. Safeguarding resistance development against APs is essential since it is one of the registered chemicals against *Botrytis* spp., which is among the most economically important fungal pathogens [15, 16]. Further, phthalimides are also widely used, for example in Japan. Captan (belonging to the phthalimides group) was reported among the majorly used chemical fungicides [17]. Due to captan's non-specific mode of action, it is effective against several pathogens, thus, monitoring for resistance development to this chemical is essential.

Fungi have several resistance mechanisms against antifungal agents, threatening the successful use of antifungals in agricultural and clinical settings [18-20]. Important to note is that some fungi show resistance

to antifungal agents, but the drug targets might be lacking, or the resistance mechanisms to those antifungal agents are yet to be understood. Therefore, we reviewed some of the characterized resistance mechanisms of QoIs, azoles (DMIs), APs, and phthalimides.

Table 1. Some groups of antifungals used in agriculture (adapted from [4]).

MOA*	Target (group name)	Chemical or biological group	Common name
Nucleic acid metabolism	Inhibition of methionine biosynthesis (proposed) anilinopyrimidines	Anilinopyrimidines (APs)	Cyprodinil (CYP), mepanipyrim, pyrimethanil
Membrane sterols synthesis	Inhibition of Sterol biosynthesis, lanosterol 14-α-demethylase (demethylation inhibitors (DMIs)	triazoles	Difenoconazole (DFN), azaconazole, bitertanol, bromuconazole, cyproconazole, diniconazole, epoxiconazole, etaconazole, fenbuconazole, triadimenol, fluquinconazole, flusilazole, flutriafol, ipconazole, hexaconazole, imibenconazole, mefentrifluconazole, metconazole, myclobutanil, triticonazole penconazole, propiconazole, simeconazole, tebuconazole, tetraconazole, triadimefon
		imidazoles	prochloraz, imazalil, oxpoconazole, pefurazoate, triflumizole
		piridines	pyrifenox, pyrisoxazole
		pyrimidines	fenarimol, nuarimol
		triazolinthiones	Prothioconazole
		piperazine	triforine
Multisite	Multi-site contact activity	phthalimides	Captan (CPN), folpet, captafol
Respiration	Bind the Qo site of the cytochrome bc1 enzyme complex inhibiting fungal respiration (quinone outside Inhibitors (QoIs))	methoxy-acrylates	azoxystrobin, coumoxystrobin, enoxastrobin, flufenoxystrobin, picoxystrobin, pyraoxystrobin
		methoxy-acetamide	mandestrobin
		methoxy-carbamates	methoxy-carbamates
		oximino-acetates	kresoxim-methyl, trifloxystrobin
		oximino-acetamides	dimoxystrobin, orysastrobin fenaminstrobin, metominostrobin
		xazolidine-diones	famoxadone
		dihydro-dioxazines	fluoxastrobin
		benzyl-carbamates	pyribencarb
		imidazolinones	fenamidone

*MOA-mode of action

2 Anilinopyrimidines

Anilinopyrimidines (APs) (e.g., cyprodinil (CYP), pyrimethanil, and mepanipyrim) are commonly used fungicides against a range of fungal pathogens, but mostly employed as botryticides. APs are described to inhibit the biosynthesis of sulphur-containing amino acids and their precursors (e.g., methionine, cysteine, cystathionine, homocysteine) (Figure 1) [14, 21-23]. In the studies mentioned above, sulphur-containing amino acids, their precursors, and other regulators thereof (e.g., alanine, leucine, isoleucine, glutamine, lysine, glycine, histidine, asparagine, arginine, threonine, α-aminobutyric, β-alanine) reversed the fungitoxicity of APs and accumulated upon APs treatment.

So far, the exact enzyme targeted and, consequently, the resistance mechanism of APs in the above-mentioned pathways is unclear. For example, cystathionine β-lyase, an enzyme involved in methionine biosynthesis and encoded by the *MetC* gene, was initially suggested as the primary target of APs in *Botrytis cinerea* based on qualitative analysis of accumulated amino acids following APs treatment [21]. However, this gene was later excluded as a target based on the lack of differences in enzyme activity and sequence polymorphism between field-sensitive and resistant strains of *B. cinerea* [24]. There were also no sequence differences in *MetC* and *MetB (*encoding the cystathionine γ synthase enzyme*)* genes between the sensitive and laboratory-induced resistant strains of *Sclerotinia sclerotiorum,* leading to the exclusion of the MetC and MetB proteins as the targets of APs [25]. Similarly, methionine biosynthesis was excluded as the primary target of APs in *Penicillium digitatum* based on the reduced toxicity of APs for this species compared to *B. cinerea* upon adding the respective amino acids in the media [26].

It is generally believed that APs target the biosynthesis of sulphur-containing amino acids, but likely also have other targets in fungi. For example, it has been suggested that APs target the secretory pathway, thereby reducing the secretion of enzymes involved in plant infection [26-28]. Nevertheless, these studies did not pin down the specific molecular targets in these pathways responsible for the effect seen with APs. Thus, the AP effect on protein secretion is still open to molecular characterization.

Apart from the described targets and resistant mechanisms above, genes that encode the various enzymes in the arginine and ornithine biosynthetic pathways have been suggested as potential targets based on transcriptomic response to treatment with sub-lethal doses of APs in *S. cerevisiae* (Figure 1) [29].

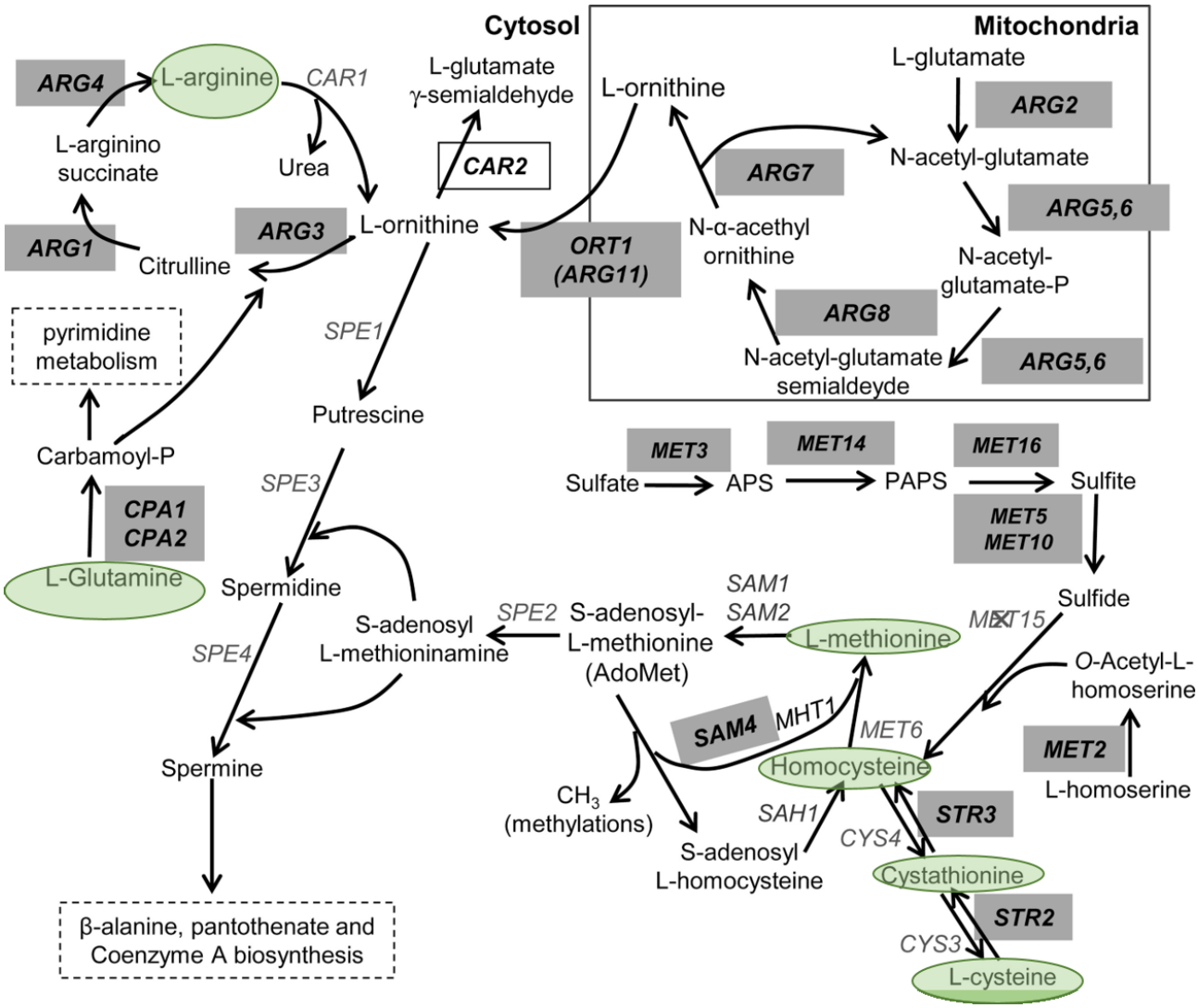

Figure 1. The sulphur-amino acids, arginine, and polyamines metabolic pathways in fungi as adapted from [29]. The green highlighted circles are some amino acids reported to reverse the fungitoxicity of APs or accumulate upon APs treatment in pathogenic fungi [14, 21-23]. *STR2* and *STR3* genes are homologs to the *MetC* and *MetB* genes in *Botrytis spp.*, which are proposed as the targets of APs [21]. APS = 5′-adenylylsulfate; PAPS = 3′-phospho-5′-adenylylsulfate. The grey boxes are the induced genes, and the white boxes are the downregulated genes upon treatment with sub-lethal APs in *S. cerevisiae* [29].

2.1 APs resistance mechanism involving mitochondrial functions

A recent study identified mitochondrial protein functions as the primary target of APs in *B. cinerea* [30]. Using laboratory-generated mutants and reverse genetics, different mutations in the nine mitochondrial genes *BcMCR1, BcMIX17, BcDNM1, BcATM1, BcPOS5, BcAFG3, BcPHB2, BcMDL1,* and *BcOLIC (ATP9)* were identified (Table 2) [30]. Mutations either involved amino acids substitutions (i.e., *Bcdnm1* ^{E450G}, *Bcafg3*^{L305P}, *Bcphb2*^{L153S}, *Bcmdl1*$^{E407K\ and\ G422R}$, *Bcmix17*$^{G83E\ and\ G79D}$, *BcoliC*^{R33C}, *Bcatm1*^{E414k}, *BcPos5* $^{L412F,L412S,\ L412V,\ G408R\ G408V\ ,\ V273I,\ P293S,\ P319A}$, *Bcmdl1*^{E407K}, and *BcMdl1* ^{S466R}) or frameshift deletions (i.e., *Bcmcr1* $^{A280\ and\ G296}$). The polymorphisms in the nine individual genes correlated with the resistance phenotype [30]. To our knowledge, this study remains the only one that molecularly characterized resistance to APs. Molecular approaches to characterize resistance mechanisms to APs based on the previously suggested target pathways thus seem necessary.

Table 2. The mutations in *B. cinerea* that correlated with APs resistance [30]

Mutation type	Mitochondrial protein function	*Gene modification*
Amino acids substitutions	Dynamin-related GTPase	*Bcdnm1* ^{E450G}
	Mitochondrial inner membrane AAA protease	*Bcafg3*^{L305P}
	Prohibitin-like protein	*Bcphb2*^{L153S}
	Mitochondrial inner membrane ABC transporter	*Bcmdl1*$^{E407K\ and\ G422R}$ and S466R
	Intermembrane space (IMS) protein	*Bcmix17*$^{G83E\ and\ G79D}$
	Subunit c of the Fo part of mitochondrial F_1Fo ATP synthase	*BcoliC*^{R33C}
	Mitochondrial ABC transporter	*Bcatm1*^{E414k}
	Mitochondrial NADH kinase	*BcPos5* L412F, L412S, L412V, G408R G408V, V273I, P293S, P319A
Frameshift deletion	Mitochondrial NADH-cytochrome b5 reductase	*Bcmcr1* A280 and G296

3 Azoles

Azoles are a preferred group of fungicides due to their low cost and broad spectrum of activity. This fungicide class is widely used in agriculture and clinics to control a wide variety of plant and human pathogenic yeasts and filamentous fungi such as *Candida* spp., *Aspergillus* spp., *Cryptococcus* spp., *Zymoseptoria tritici, Puccinia tritici*, *Fusarium* spp, *Penicillium* spp. *Mycosphaerella graminicola, B. cinerea* [31-33]. In the clinics, azoles represent an important group of antifungals that include imidazoles (i.e., ketoconazole, micanozole) and triazoles (i.e., itraconazole, voriconazole, fluconazole and posaconazole). In agriculture, azoles, due to their mode of action, are grouped as demethylation inhibitor (DMI) fungicides. Currently, azoles have six chemical groups, including triazoles, imidazoles, triaolinthiones, piridines, pyrimidines, and piperazines (Table 1) [4]. The former three are the most commonly used, with triazoles having over 20 fungicides, including tebuconazole, epoxiconazole, tetraconazole, prothioconazole, and difenoconazole [4, 11].

Clinical and agricultural azoles both target the cytochrome P450 enzyme lanosterol 14-α-demethylase (coded for by the *ERG11* or *CYP51A* gene), which catalyzes the conversion of lanosterol to ergosterol [12, 34, 35]. Ergosterol is essential for the maintenance of cell membrane permeability. Inhibition of its synthesis, in addition to the accumulation of toxic sterols, causes membrane structure disorganization and prevention of active membrane transport, which inhibits fungal growth or causes cell death [34, 36].

Resistance to azoles by both clinical and agricultural pathogens is widespread and well documented [37-40]. The mechanisms that underlie these resistances include mutations in the *ERG11* or *CYP51A* gene, increased expression of efflux pumps, overexpression of *CYP51A*, activation of stress response pathways, and aneuploidy due to genome plasticity (references in Table 3).

3.1 Mutations in the *CYP51A* gene

Mutations in the *CYP51A* gene are the commonly reported resistance mechanism in both plant and clinical pathogens. The characterization of this resistance mechanism based on transformation studies or genotyping has identified an extensive amount of amino acid substitutions in different clinical pathogens (e.g., M220R, M220I, M220K, M220V, G54W, P126L, P216L) [41-44] (and many more in references therein). Similarly, mutations in the *CYP51A* gene as a resistance mechanism to DMIs by plant pathogens have been extensively studied. These include recently characterized mutations such as Y126F and R511W, responsible for resistance

to a newer DMI (difenoconazole) in *P. expansum* and *Alternaria* spp, respectively [45, 46]. Also, the common mutation of tyrosine to phenylalanine at codon 136 (Y136F) was characterized in several species and many other mutations have been described and reviewed [47, 48].

3.2 Overexpression of the *CYP51A*

Overexpression of the *CYP51A* gene is another resistance mechanism that has been characterized for both clinical and plant-used azoles. This mechanism either solely causes resistance or is accompanied by other mechanisms such as the presence of tandem repeats at the promotor site of CYP51A [49] and mutation of *CYP51A* together with tandem repeats [50]. Aneuploidy might also lead to overexpression of *CYP51A,* as will be described later [51].

In fungal plant pathogens, for example in *P. expansum*, laboratory-resistant mutants showed a 2 to 14-fold increased expression of *CYP51A,* leading to resistance compared to the wild-type [45]. A similar resistance mechanism to DMIs has been reported in *Blumeriella jaapii, P. tritici, Z. tritici, and Venturia inaequalis,* and *P. italicum* [52-56]. Further, overexpression due to the presence of tandem repeats has been demonstrated in plant pathogens. For instance, in *P. digitatum,* a laboratory-induced resistant mutant contained five tandem repeats of a 126-base pair sequence in the promoter site of *CYP51A*, resulting in a 100-fold increased expression of *CYP51A* as compared to the sensitive strain lacking this enhancer [49]. In the same study, transforming the gene and the promotor from a *CYP51A* overexpressor into a DMI-sensitive strain led to DMI resistance.

Clinically, *CYP51A* overexpression due to *CYP51A* mutations, together with the insertion of tandem repeats, resulted in high resistance and was the leading cause of resistance in *Aspergillus* spp. [50, 57]. This resistance mechanism emerging in *Aspergillus* spp. has been linked to environmental azole use [12, 38, 57-62]. *Aspergillus* spp. strains with a four- and an eight-fold increased *CYP51A* expression, as compared to the wild-type, had tandem repeats (TR34) or tandem repeats and a point mutation (TR34/L98H) in the *CYP51A* promoter, respectively [50]. In the same study, transformants of *A. fumigatus* with TR34/L98H genotype led to an eight-fold increased *CYP51A* expression and hence azole resistance [50]. Notably, transformations to produce the TR34/L98H resistant mutant is challenging in some instances, similar to the creation of transformants of some *CYP51A* point mutation in *Aspergillus* spp. [58, 63]. Thus, most studies that report resistance due to TR34/L98H or new tandem repeats have only employed the well-established microsatellite-genotype-based method in *Aspergillus* spp. [64]. So far, several studies have reported this mutation (TR34/L98H) in *Aspergillus* spp. [41, 42, 57, 58, 62, 65]. Additionally, other tandem repeats and substitution mutations that confer azole resistance in *A. fumigatus* include TR46/Y121F/T289A, TR46/Y121F/M172I/T289A, and TR34/L98H/S297T/F495I [38, 42, 66, 67].

3.3 Aneuploidy increasing *ERG11* and efflux expression

Aneuploidy occurs in the chromosomes of some fungal species and can lead to an overexpression and thus increased number of target sites [51]. This mechanism of resistance to clinically used azoles has been reported, for example in *C. albicans* and *C. neorfomans* [68-71]. In *C. albicans,* ploidy changes involving extra copies of chromosome 5, where the *ERG11* and *TAC1* genes (encoding a transcriptional regulator) are located, led to resistance to azoles [69, 70]. Resistance to azoles in *C. neorfomans* was due to ploidy changes involving chromosome 1, and increased copy numbers of *ERG11* and *AFR1* (encoding a drug efflux transporter) [71].

3.4 Overexpression of efflux pumps

Another azole resistance mechanism involves the overexpression of efflux pumps, which reduces the intracellular concentration of drugs and thus results in fungal resistance. The different protein pumps involved in antifungal drug resistance are of two classes: the primary (ATP-binding cassette (ABC) transporter) and the secondary transporters (major facilitator superfamily (MFS) pumps). These transporter classes, their structures, and the involvement in antifungal resistance are extensively covered [72-74]. The majority of azole resistant *Candida* spp. in the clinics exhibit this mechanism. Characterization of this mechanism has reported the involvement of different transporters in *Candida* spp. (e.g., Cdr1, Cdr2, Pdh1, Snq2, Mfs7, Mdr1, and CkAbc1p) [37, 75-84]. The same mechanism was described in other clinical pathogens. For example, upregulation of Mdr1p in *Cryptococcus neoformans* and atrF in *A. fumigatus* (both ABC transporters) was associated with azole resistance [83, 85].

This mechanism is also responsible for resistance to DMIs in plant pathogens. For example, a laboratory-developed tebuconazole-resistant strain of *Fusarium culmorum* showed a 30-fold higher expression of the *FcABC1* gene than the wild type in the presence of tebuconazole [80]. In *F. graminearum*, deletion of two ABC transporters that were upregulated upon treatment with tebuconazole led to increased sensitivity to triazoles [86, 87]. Similarly, the *M. graminicola* ABC transporters MgAtr1, MgAtr2, and MgAtr4 are involved in azole resistance. Their complementation in *S. cerevisiae* led to increased resistance to azoles compared to the control strains [88]. In *S. cerevisiae*, the ABC transporter Pdr5 has been linked azole resistance [83].

3.5 Activation of stress response pathways

The activation of stress response pathways is a general mechanism that can confer azole resistance. Under stress, for example due to fungicide exposure, the plasma membrane fluidity can be lost, which interferes with the interaction of the cell membrane with the cell wall and can cause the weakening of the cell wall [89]. This weakening causes phosphorylation and activation of protein kinases through a mitogenic-activated protein kinase (MAPKs) cascade that sends signals to the nucleus, leading to the upregulation of genes involved in cell wall biogenesis, which in turn remodels the cell wall [89-92]. This cell wall remodeling protects the fungi from fungicide stress [92]. This mechanism has been predicted through transcriptomic analysis to be involved in resistance to azoles. For example, activating Slt2-MAPK, a protein kinase in the MAPK cascade in *P. italicum*, correlated with azole resistance [56]. Similarly, characterization of the *CKA1* and *CKA2* genes involved in the response of CK2, a protein kinase from *C. albicans*, reported the involvement of this protein kinase in fluconazole resistance [93].

Table 3. Mechanisms of azole resistance characterized in different fungi.

Mechanism	**Gene**	***Species***	**Paper**
Increased expression of efflux pumps,	• ATP-binding cassette (ABC) (e.g., Cdr1, Cdr2, Pdh1, Snq2, Mfs7, Mdr1, CneMdr1p, Atrf, Pdr5 CkAbc1p, FcABC1, Atr1 MgAtr1, MgAtr2, and MgAtr4) • Major facilitator superfamily (MFS) pumps (e.g., Mfs7)	*Candida* spp. *C. neoformans* *A. fumigatus* *S. cerevisae* *F. graminearum* *F. culminareum* *M. graminicola*	[72-74] [37, 75-84] [83, 85] [80] [86, 87] [88]
Structure alterations (*CYP51A or ERG11*)	• Several reviewed elsewheresome common ones include M220R, M220I, M220K, M220V, G54W, P126L, P216L, Y126F, Y136F, and R511W	*C. albicans, A. fumigatus,* *P. expansum,* *M. graminicola,* *C. albicans, A. fumigatus,* *C. neoformans* *C. tropicalis*	[41-44] [45, 46] [47, 48]
Overexpression of *CYP51*A		*P. expansum, Z. tritici,* *P. tritici, P. italicum,* *B. jaapi, V. inaequalis*	[45], [52-56]
	• Due to tandem repeats in the promoter of the CYP51A gene, TR34/L98H TR46/Y121F/T289A, TR46/Y121F/M172I/T289A TR34/L98H/S297T/F495I	*A. fumigatus*	[50, 57] [12, 38, 57-62] [50] [41, 42, 57, 58, 62, 65] [38, 42, 66, 67]
	• 199 bp tandem repeat insertion	*P. digitatum*	[49]
Activation of stress response pathways	• Activation of Slt2-MAPK by the MAPKs cascade	*P. italicum*	[56]
	• Activation of CK2	*C. albicans*	[93].
Aneuploidy	• Increased ploidy in chromosome 5, thus, *ERG11* and *TAC1* upregulation	*C. albicans*	[68-71] [69, 70].
	• Increased ploidy in chromosome 1, thus, upregulation of *ERG11* and *AFR1*	*C. neoformans*	[71]

4 Strobilurins

The strobilurins comprise a group of agricultural fungicides (e.g., azoxystrobin, kresoxim-methyl, trifloxystrobin, fenaxamidone, pyraclostrobin) that are derived from β-methoxyacrylic acid, a natural fungicidal compound [94-96]. These fungicides control several pathogens that cause plant diseases, such as rusts, powdery mildew, downy mildew, and many other diseases [9, 97-99]. Strobilurins are also known as quinone outside inhibitors (QoIs) because of their target mechanisms. The QoIs bind the Qo site of the cytochrome bc1 enzyme complex located in the inner mitochondrial membrane of the fungi and thereby block

the electron transfer between cytochrome b and c1. These compounds thus prevent ATP synthesis by inhibiting mitochondrial respiration [94, 100-103]. The cytochrome b enzyme is encoded by the mitochondrial cytochrome b gene (*CYTB*), whose size and structure in plant pathogens was molecularly characterized [104, 105].

4.1 Mutations in the *CYTB* gene

Resistance to QoIs has been documented in several plant pathogens and attributed to point mutations in the *CYTB* gene. These mutations change the CytB peptide sequence and prevent fungicide binding. The first reported mutation in plant pathogens was a single nucleotide change from guanine to cytosine in *CYTB* [106]. This led to an amino acid substitution from glycine to alanine at position 143 (G143A) of the CytB protein in *Erysiphe graminis* sp. *tritici* [106]. This study compared the *CYTB* sequences of the resistant and sensitive isolates. Still, it did not transform a susceptible strain with the *CYTB* gene having the G143A mutation from the resistant isolate to confirm that this mutation causes resistance. However, due to the lack of any other mutation in *CYTB* of the resistant isolate, this mutation was viewed as the primary resistance mechanism [106] and has been used as a basis to detect QoIs resistance. Sequencing the *CYTB* genes consecutively identified the same G143A mutation in many plant pathogens, including *Mycosphaerella fijiensis, Colletotrichum graminicola, Plasmopara viticola, V. inaequalis, Pseudoperonospora cubensis, M. oryzae, M. graminicola, Pyricularia grisea, P. tritici-repentis* [101, 107-116]. Two other amino acid substitutions in *CYTB* that lead to QoI resistance have been described based on sequence differences in *CYTB* of resistant and sensitive isolates in plant pathogens. A substitution of phenylalanine with leucine at position 129 (F129L) has been reported to lead to QoIs resistance in *Pyricularia grisea, Pyrenophora teres, P. tritici-repentis,* and *Alternaria solani* [110, 112, 117, 118]. Additionally, a glycine to arginine change at position 137 (G137R) was reported in *P. tritici-repentis* and *Phytophthora capsici* [110, 119]. Of the three amino acid substitutions, the G143A mutation causes the highest resistance to most of the QoIs classes of fungicides [101, 112, 120]. The two other mutations are less frequent and only cause mild and, in some cases, partial resistance of the pathogen to QoIs [110]. Notably, these mechanisms have functionally been characterized in *S. cerevisiae.* For example, *CYTB* genes with the G143A and F129L mutations from *E. graminis*, *Phytophthora megasperma*, *V. inaequalis*, and *Sphaerotheca fuliginea* led to differential resistance to QoIs in *S. cerevisiae* [121]. Other amino acid substitutions that correlate with natural resistance to QoIs have been recorded in non-pathogenic fungi that naturally produce strobilurins (i.e., *Mycena galopoda* and *Strobilurus tenacellus*) [122]. Based on sequence comparison, insensitive strains of *M. galopoda* had a threonine to isoleucine change at position 127, an alanine to serine at mutation at position 153, and the well-known G143A mutation. In contrast, *S. tenacellus* only had a threonine to isoleucine switch at position 127 [122]. As extensively reviewed elsewhere, substitutions that lead to resistance have also been documented in *S. cerevisiae* [101].

4.2 Alternative oxidase (AOX) pathway

Blocking electron transfer by inhibiting the cytochrome bc1 complex activates the alternative oxidase (AOX) pathway [102, 123], which might contribute to QoIs resistance [106]. For example, *in vitro*, it has been shown that a mixture of QoIs and the fungal AOX inhibitor salicylhydroxamic acid (SHAM) leads to better control of plant pathogens [107]. *In vitro*, this mechanism has been reported to cause resistance for some species, for example *M. graminicola* [124]. However, under field conditions, the contribution of the AOX pathway to resistance is still debated or seems limited [9, 125].

4.3 AOX and other unknown mechanisms cause low *in vitro* sensitivity to trifloxystrobin in *A. pullulans*

Our analysis of a non-pathogenic yeast-like fungus, *A. pullulans*, revealed reduced sensitivity to TFS in most strains. All of the 21 *A. pullulans* strains treated with different concentrations of TFS or SHAM alone remained uncontrolled up to concentrations of 300 µg/mL and 102 µg/mL of these inhibitors, respectively (Table 4). Five of these strains (green coloured) were controlled when different concentrations of TFS were combined with 102 µg/mL of SHAM, implying that AOX might have a role in the sensitivity of these strains. Contrarily, 16 of these strains remained uncontrolled with treatments of both TFS and SHAM, indicating that their reduced sensitivity could result from other targets than cytochrome bc1. Interestingly, eight strains (red coloured) that were initially uncontrolled in the presence of TFS or low concentrations of AOX inhibitors were controlled with high SHAM concentrations (Table 4).

Table 4. Sensitivity of *A. pullulans* to trifloxystrobin

***A. pullulans* strain**	**[a]TFS + 102 µg/mL SHAM**	**[b]TFS alone or 102 µg/mL SHAM alone**	**[c]SHAM alone**
Fr1	Uncontrolled	Uncontrolled	405 µg/mL
Fr2			810 µg/mL
LC 5.2			202 µg/mL
CnL4a			405 µg/mL
LCH 2.1			405 µg/mL
LF 5.16			405 µg/mL
LF 3.10			202 µg/mL
LF 5.11			405 µg/mL
CaFr2.1			Uncontrolled
LC1.9			
LC 1.3			
LCH 10.2			
SFr4.3			
AL4e			
FFr4.3			
LF 5.10			
F2	0.0012 µg/mL		Controlled
CHF4.2	0.0045 µg/mL		
CnF4.2	0.0003 µg/mL		
FLF4.3	0.0733 µg/mL		
AF4.1	0.0045 µg/mL		

a Microbroth sensitivity assays were performed in potato dextrose broth (PDB) supplemented with different concentrations of TFS and 102 µg/mL SHAM or 2.5% dimethyl sulfoxide (DMSO) at 22°C for 36 h. The OD_{600} readings at 36 h showed that five *A. pullulans* strains (green highlighted) had more than 50% growth reduction at the indicated TFS concentrations compared to their growth in 2.5% DMSO without the inhibitors. Growth of the 16 strains highlighted in grey was not be reduced under these conditions.

b PDB was supplemented with different concentrations of TFS alone (upto 300 µg/mL) or 102 µg/mL of SHAM alone. *A. pullulans* strains cultures were incubated, and the OD_{600} readings were done similarly as in (a). All the 21 strains showed no reduction in growth in the presence of these inhibitors used separately as compared to their growth in PDB with 2.5% DMSO

c PDB was supplemented with different concentrations of SHAM alone, *A. pullulans* strains cultures were incubated, and the OD_{600} readings were done similarly as in (a). Eight strains were controlled in high concentrations of SHAM alone.

5 Captan

Captan belongs to the phthalimide group of fungicides with a multisite mode of action against fungi. Its exact mode of action is still poorly described [126]. Due to its multimode action, fungi would have to change different biological processes in order to become resistant. The risk of resistance development to captan is thus considered low [4, 127]. Nevertheless, captan-resistant *B. cinerea* strains have been reported [128, 129].

Due to the low risk of resistance development, very little work has been done to characterize resistance mechanisms to captan. Notably, the captan resistant strains exhibit multidrug resistance [128, 129], implying the involvement of general resistance mechanisms (e.g., overexpression of efflux pumps)[130]. In the non-target bacterium *Azotobacter chroococcum*, a comparison of a captan-resistant and -sensitive strain growing in captan reported the modification of glucose utilization and increased accumulation of ATP [131], which suggests the involvement glucose metabolism or transporters. Since fungal transporters are numerous, predictive tools such as genome-wide association studies (GWAS) using captan-sensitivity phenotype data could become handy in identifying the active transporters.

Additionally, an earlier study suggested increased production of thiol compounds as a captan resistance mechanism in *Botrytis* [132]. These thiol compounds are a product of captan degradation. Thus, captan resistance could also be due to increased degradation (detoxification) by the resistant fungi.

6 Conclusion

This review documents different resistance mechanisms to azoles and strobilurins for fungal pathogens and highlights a few mechanisms described for cyprodinil resistance in plant pathogens. It also emphasizes the lack of research on resistance mechanisms of non-target yeast species. Notably, the predicted resistance mechanisms to cyprodinil in a non-target yeast (*S. cerevisiae)* are yet to be characterized. Further, based on the literature, a possible multidrug resistance mechanism that involves membrane transporter pumps is suggested. A GWAS using captan-sensitivity data is proposed to decipher the exact transporters involved in this mechanism. This review provides information on the current resistance status for these four groups of antifungals.

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Chapter 3

Unconventional yeasts are tolerant to common antifungals, and *Aureobasidium pullulans* has low baseline sensitivity to captan, cyprodinil, and difenoconazole

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- Fungicide sensitivity assays and data analysis
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Abstract

Many yeasts have demonstrated intrinsic insensitivity to certain antifungal agents. Unlike the fungicide resistance of medically relevant yeasts, which is highly undesirable, intrinsic insensitivity to fungicides in antagonistic yeasts intended for use as biocontrol agents may be of great value. Understanding how frequently tolerance exists in naturally occurring yeasts and their underlying molecular mechanisms is important for exploring the potential of biocontrol yeasts and fungicide combinations for plant protection. Here, yeasts were isolated from various environmental samples in the presence of different fungicides (or without fungicide as a control) and identified by sequencing the internal transcribed spacer (ITS) region or through matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Among 376 isolates, 47 taxa were identified, and *Aureobasidium pullulans* was the most frequently isolated yeast. The baseline sensitivity of this yeast was established for 30 isolates from different environmental samples *in vitro* to captan, cyprodinil, and difenoconazole. For these isolates, the baseline minimum inhibitory concentration (MIC_{50}) values for all the fungicides were higher than the concentrations used for the control of plant pathogenic fungi. For some isolates, there was no growth inhibition at concentrations as high as 300 μg/mL for captan and 128 μg/mL for cyprodinil. This information provides insight into the presence of resistance among naturally occurring yeasts and allows the choice of strains for further mechanistic analyses and the assessment of *A. pullulans* for novel applications in combination with chemical agents and as part of integrated plant-protection strategies.

Keywords**:** fungicide; resistance; baseline sensitivity; yeasts; isolation; captan; cyprodinil; difenoconazole

1 Introduction

Fungicide resistance is an extremely important issue in medicine as well as agriculture. In both settings, the application of fungicides favours the selection of resistant strains that can consequentially become serious threats for human or crop health. Owing to these threats, fungicide resistance in human and plant pathogenic fungi is well studied at all levels, from their ecological impact and population dynamics to the molecular mechanisms involved [1–7]. By contrast, fungicide resistance and sensitivity in non-target fungal species is much less investigated. This is somewhat surprising, because these non-target fungi may reveal inherent resistance mechanisms, provide sources of resistance genes, or lead to new applications where fungicide tolerance may be a desirable trait (e.g., the decomposition of fungicides or combinations of fungicides and tolerant isolates for biocontrol applications). For example, the combinations of wild yeasts (*Rhodotorula mucilaginosa*, *R. glutinis*, and *R. graminis*) with several chemical fungicides was more effective in controlling *Botrytis cinerea* than either fungicide or biocontrol yeast alone [8]. Similar combined applications to manage head blight, powdery mildew, or different fruit-decay diseases have subsequently been reported [9–14]. It may thus be possible to develop new, commercial plant-protection strategies employing combinations of biocontrol yeasts and fungicides in order to reduce the total application rate of the latter and the development of fungicide-resistant plant pathogens.

Most fungicides used for crop protection are threatened by the development of resistance of the respective fungal pathogens [15–17]. The levels of risk vary among different fungicide chemical groups [18]. Risk management is imperative, especially in fungicide groups with a high risk of resistance development, but also needed for fungicides in medium- or low-risk groups to avoid the introduction or progression of resistance. For the study described here, we choose the three fungicides captan (CPN, phthalimide class of fungicides), cyprodinil (CYP, anilinopyrimidine class of fungicides), and difenoconazole (DFN, demethylation inhibitor (DMI) fungicide class) as representatives for commonly used fungicide classes. With respect to the risk of resistance development, these three fungicides belong to either medium- or low-risk groups [19]. CPN has multiple targets in the cell, but its exact mode of action is poorly described [18]. The multiple targets of CPN are likely the cause of the low risk of resistance development and the limited number of resistant strains that have been documented [19]. Still, there are some reports of reduced sensitivity to captan [20,21]. CPN is employed to control scab, blights, and shot hole diseases in apples, pears, cherries, and stone fruits. CYP is used to control scab and rot diseases of stone and pome fruits. Examples include apple (*V. inaequalis*) and pear scab (*V. pirina*), brown rot (*Monilinia fructicola*), and blossom blight (*Monilinia laxa*) in plums, apricots, peaches, and nectarines; and diseases caused by *B. cinerea* (e.g., *Botrytis* bunch rot and *Botrytis* fruit rot in pome fruits). Anilinopyrimidines are considered to have a medium risk for the development of resistance [18]. To date, resistance development has, for example, been reported in *Venturia* spp. and *Botrytis* spp. DFN is registered for controlling diseases such as carrot black leaf and pod spot (*Alternaria* spp.), powdery mildew (*Podosphaera* spp. and *Erysiphe* spp.), scabs (*V. inaequalis* and *V. pirina*), and rots and blights *(Monilinia* spp.) in different ,ööl,crops. DMI fungicides are a widely used class of fungicides and, despite their widespread use, still considered to have a medium risk of resistance development [18].

Few species of unconventional, non-pathogenic yeasts are currently being used in agriculture and biotechnology, but new activities and potential applications are described for a plethora of such yeasts. Many species are used for food and beverage production or as sources of enzymes and valuable chemicals. For

example, the yeast *Aureobasidium pullulans* has antifungal or antibacterial properties, is commercially used as a biocontrol agent, and produces a range of metabolites that are of biotechnological interest [22–25]. Besides *A. pullulans*, *Candida oleophila*, *Metschnikowia fructicola*, *Saccharomyces cerevisiae*, and *Cryptococcus albidus* are or have been registered in different biocontrol products [25]. In addition, some yeasts and yeast products have been explored in novel applications (e.g., in combination with antifungal/antibacterial formulations) to manage plant diseases [8–14,24,26]. However, to fully exploit the potential of unconventional yeasts for such combined applications, it is key to understand how frequent fungicide-insensitive yeasts are and what the functional or molecular mechanisms underlying this phenotype are. Although CPN, CYP, and DFN have been registered and utilised in the environment, and the mechanisms of resistance have been studied in plant pathogenic fungi, little is known about the tolerance to these fungicides for unconventional, non-pathogenic yeasts. To understand the distribution of fungicide sensitivity in natural populations and the development of fungicide resistance, it is important to establish the baseline sensitivities of natural isolates to different fungicides [27]. For many pathogenic fungi, the baseline sensitivities for CPN, CYP, and DFN have already been determined [20, 28–31], but this is not the case for non-pathogenic, naturally occurring yeasts.

The objectives of this study were, therefore, to isolate and identify naturally occurring yeasts that are tolerant to commonly used antifungals (agricultural and medical) and to establish the baseline sensitivities for CPN, CYP, and DFN for the most frequently isolated yeast, *A. pullulans*.

2 Results

2.1 Isolation and identification of naturally occurring yeasts insensitive to commonly used fungicides

2.1.1 Yeasts are tolerant to commonly used antifungal agents

Different antifungal agents, in different concentrations, were mixed with environmental samples (soil, leaves, flowers, and fruits), the suspensions were plated on potato dextrose agar (PDA) (containing antibiotics to prevent the growth of bacteria), and fungal colonies were counted and isolated. Many fungal colonies (both filamentous fungi and yeasts) were observed on the control plates, on which the samples without any antifungal agent were plated (Figure 1A). The total number of fungal colonies was reduced in the samples isolated in the presence of the antifungals amphotericin B, capsofungin, CPN, CYP, DFN, fluconazole, and tryfloxystrobin (Figure 1B). Interestingly, in the presence of all these fungicides except trifloxystrobin, the number of yeast colonies was consistently higher as compared to the colony counts for filamentous fungi (Figure 1C). For CPN, CYP, DFN, and fluconazole (for yeasts only), the total number of fungal colonies declined as the antifungal concentrations increased, but for the other compounds, this effect was not clearly observed (Figure 1C). Notably, medically used antifungals (i.e., amphotericin B, capsofungin, and fluconazole) had the least effect on overall fungal colonies, while captan had the most potent effect, resulting in almost no filamentous fungi and very few yeast colonies (Figure 1C). Overall, these results indicate that environmental yeasts can tolerate the presence of most of the fungicides tested here.

2.1.2 *Aureobasidium pullulans* is the most frequently isolated species in the presence of antifungals

Single yeast colonies were picked from plates with the highest antifungal concentrations where yeasts were still present and used for identification. A total of 359 yeast isolates belonging to 48 taxa were identified after isolation in the presence of different antifungal agents (Figure 2). *A. pullulans* was the most abundantly isolated

yeast with seven out of the eight antifungal agents (73 isolates in total), while some species were isolated only once and only with a single antifungal (Figure 2). Other commonly isolated taxa (at least 16 or more isolates, in the presence of at least five different fungicides) included *M. pulcherrima, Cryptococcus laurentii, Cyberlindnera misumaiensis, Sporidiobolus metaroseus*, and *Holtermaniella. Pichiaceae* were mostly isolated with DFN (eleven out of the total fifteen *Pichiaceae* isolates). The different yeast species were naturally occurring both in the phyllosphere (leaves, flowers, and fruits) and in the soil, but a broader diversity (38 species) was observed in the soil samples (Table 1). Interestingly, *A. pullulans, S. metaroseus,* and *Holtermaniella* were found in all the four sample types, while *M. pulcherrima* was found in all the samples except in flowers. The numbers of isolates ranged from 11 to 66 for each antifungal agent used (Table S1). Seasonal changes affected the total number but not species diversity, since most species were collected throughout the four seasons.

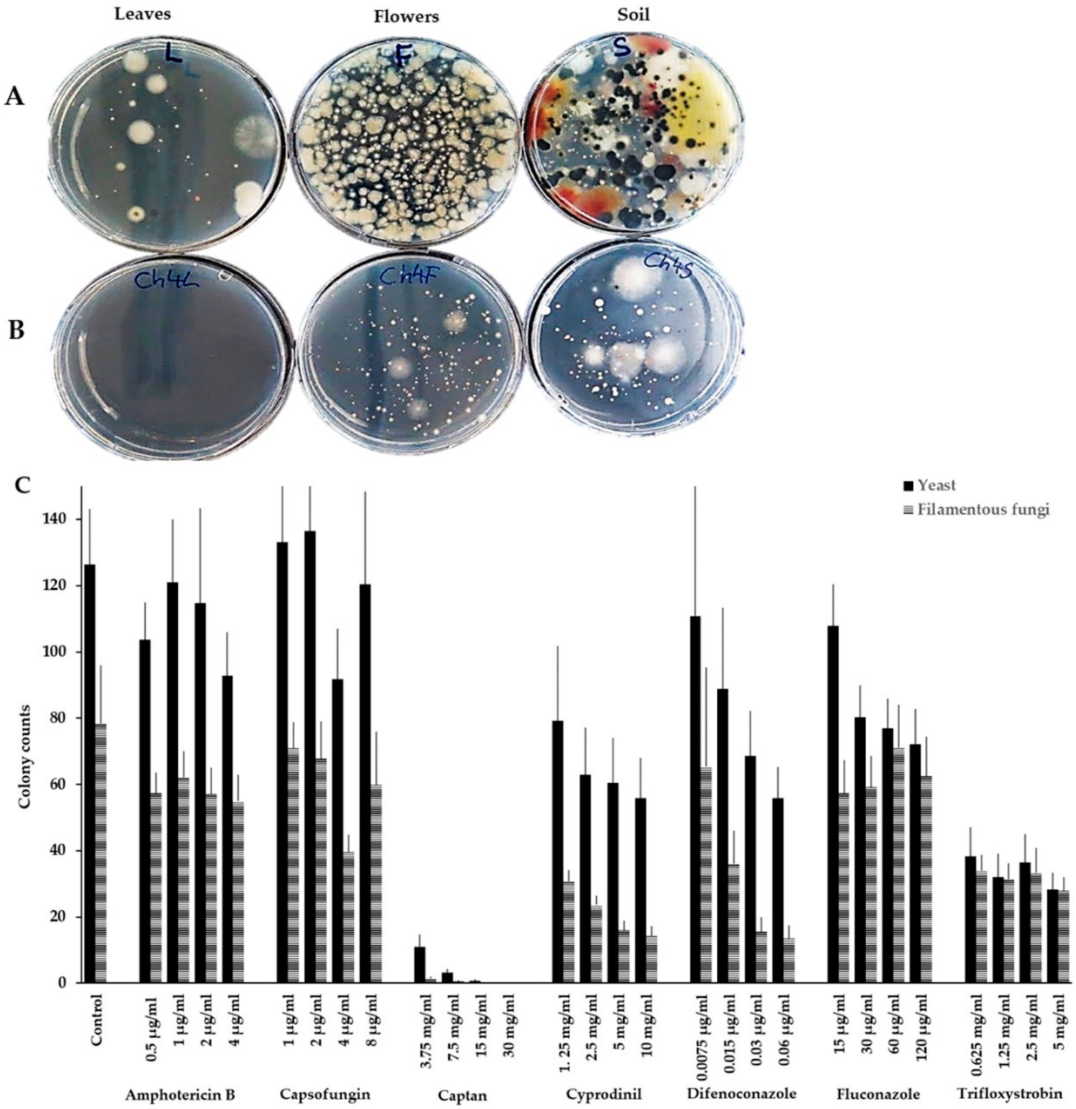

Figure 1. Yeasts tolerate commonly used antifungal agents. Yeasts from soil were isolated in 1% peptone water. These suspensions were then incubated for 1 h in 1% peptone water (A) or in peptone water containing different antifungal agents (e.g., 10 mg/mL cyprodinil (CYP)) (B). The mixtures were plated on antibiotic-supplemented potato dextrose agar (PDA) plates and incubated for 72 h. Fewer fungal colonies were isolated in the presence of antifungals as compared to the control. Colonies were counted on the control and fungicide plates. An example portraying the colony counts for filamentous fungi and yeasts in a soil sample is shown (C). The number of yeast colonies was consistently higher than that of filamentous fungi. The chart shows the average numbers and standard errors for the corresponding colony counts for three separate soil isolations, with three replicates each. All the data were pooled.

2.2 The baseline sensitivities and MIC_{50} of 30 *A. pullulans* isolates

Since *A. pullulans* was the most abundantly isolated yeast, a large number of isolates allowing the determination of baseline sensitivities to different fungicides was available. Thirty isolates of this species were thus selected for further analysis. To assess their diversity and relationship with known

Aureobasidium strains, the ITS sequences of these 30 isolates were used for a phylogenetic analysis. Based on their ITS sequences, 30 of these isolates clustered together with other, already published, *Aureobasidium* strains (Figure 3). This cluster comprised *A. pullulans*, but also species such as *A. proteae* and *A. lini*. However, the 30 isolates did not cluster with *A. namibiae*, *A. melanogenum*, and *A. sublgaciale*, which were defined as separate species [34]. Since the 30 isolates were identified as *A. pullulans* by the UNITE database (as SH1515060.08FU) and all the ITS sequences formed a cluster that also included the sequence of the neotype for *A. pullulans* var. *pullulans*, CBS 584.75, these isolates were treated as *A. pullulans* for this study.

In order to assess the fungicide sensitivity of the 30 *A. pullulans* isolates, extensive microbroth sensitivity growth assays in the presence of different concentrations of three fungicides were performed. The 30 isolates of *A. pullulans*, isolated under different conditions (sample sources, time points, and fungicides) were controlled (i.e., at least 50% growth reduction) by the experimental concentrations of CPN and DFN and the majority of the isolates (63% and 70%, respectively) had a MIC_{50} value that was below the mean MIC_{50} for the corresponding fungicide (Table 2). Additionally, for CYP, the majority of the isolates (70%) had a MIC_{50} below the mean for CYP. However, not all the isolates were controlled: isolate AL4e was insensitive to the maximal CYP concentration used here (256 μg/mL) and had a calculated MIC_{50} value of 1.45×10^{39} μg/mL. This value was thus excluded from further calculations. The resistance factors (i.e., the maximum MIC_{50} value divided by the minimum MIC_{50} value) were lower for difenoconazole (25.3) and captan (21.5) than for cyprodinil (93.0). Overall, the 30 isolates had mean MIC_{50} of 28.9 (CPN), 22.6 (CYP), and 2.2 μg/mL (DFN) (median MIC_{50} were 21.9, 8.9, and 1.4 μg/mL for CPN, CYP, and DFN, respectively). DFN had the narrowest MIC_{50} range (0.4–10.1 μg/mL), followed by CPN (5.1–109.6 μg/mL), while CYP had the widest range (2.0–186.0 μg/mL).

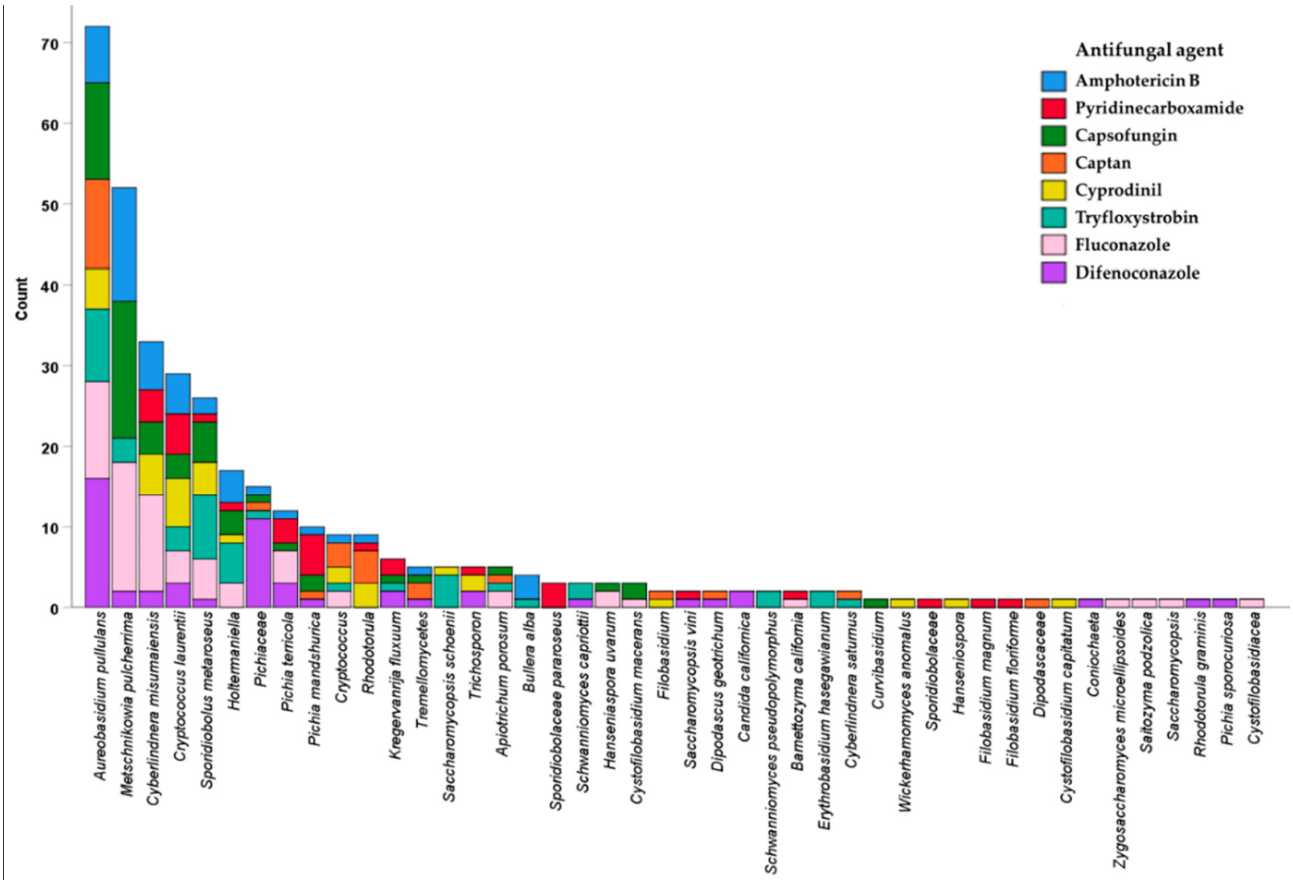

Figure 2. *Aureobasidium pullulans* is most frequently isolated species in the presence of fungicides. All the yeast taxa (species hypotheses, here referred to as species) that were identified are listed on the X-axis, while the Y-axis indicates the number of isolates obtained for each species. The color codes represent the fungicides used during the isolation, with the corresponding total numbers of isolates.

The distributions of the MIC_{50} values for all the three fungicides and the 30 isolates were skewed, since many isolates exhibited an increased sensitivity (i.e., had a lower MIC_{50} value) compared to the average for the studied population (Figure 4, Table 2). The non-transformed MIC_{50} values for all the three fungicides thus resulted in non-normal distributions (Shapiro–Wilk W = 0.82, $p = 0.0001$ (CPN); W = 0.51, $p < 0.001$ (CYP); W = 0.75, $p < 0.001$ (DFN)). Overall, the distributions of the MIC_{50} values were unimodal, potentially indicating that no disruptive resistance existed and that the *Aureobasidium* population studied here showed baseline sensitivity with significant variation.

The mean MIC_{50} values for the control of the *A. pullulans* isolates were compared with the EC_{50} values reported for applications against plant pathogenic fungi. For the control of *B. cinerea*, mean EC_{50} values of 0.9 (CPN) and 0.008 µg/mL (CYP) have been reported, while for DFN, the mean EC_{50} for controlling *Penicillium* spp. was 0.16 µg/mL [28,37,38]. The mean MIC_{50} values for the *A. pullulans* isolates were thus significantly higher (CPN: T = 7.33, df = 29, $p < 0.001$; DFN: T = 5.27, df = 29, $p < 0.001$; CYP: T = 3.41, df = 28, $p = 0.002$) than the published mean EC_{50} values for plant pathogenic fungi, suggesting that *A. pullulans* is less sensitive to these three fungicides than the plant-pathogen targets of the respective fungicides.

Table 1. Yeast species isolated from soil, flower, leaf, and fruit samples in this study. *A. pullulans*, *Holtermaniella*, and *S. metaroseus* were isolated from all sample types. The species were identified by MALDI-TOF mass spectrometry (MS) as a fast and economical alternative to DNA sequencing. Isolates that could not be identified by MALDI-TOF MS were determined based on the ITS sequence. Those identified by the ITS sequence were assigned species hypotheses (SH numbers) [32, 33].

Soil	**Flower**	**Leaf**	**Fruit**
Aureobasidium pullulans	*Aureobasidium pullulans*	*Aureobasidium pullulans*	*Aureobasidium pullulans*
Holtermaniella	*Holtermaniella*	*Holtermaniella*	*Holtermaniella*
Sporidiobulus metaroseus	*Sporidiobulus metaroseus*	*Sporodiobolus metaroseus*	*Sporidiobolus metaroseus*
Metschnikowia pulcherrima	*Metschnikowia pulcherrima*	*Metschnikowia pulcherrima*	
Cryptococcus laurentii	*Cryptococcus laurentii*		
Cyberlindnera misumaiensis	*Cyberlindneramisumaiensis*		
Hanseniaspora uvarum	*Hanseniaspora uvarum*		
Cystofilobasidium macerans	*Cystofilobasidium macerans*		
Bullera alba		*Bullera alba*	
Sporidiobolaceae pararoseus		*Sporodiobolaceae pararoseus*	
Schwanniomyces capriottii		*Schwanniomyces capriottii*	
Rhodotorula		*Rhodotorula*	
Apiotrichum porosum			
Barnettozyma california			
Candida californica			
Coniochaeta			
Cryptococcus			
Cyberlindnera saturnus			
Cystofilobasidiacea			
Cystofilobasidium capitatum			
Dipodascaceae			
Dipodascus geotrichum			
Hanseniospora			
Kregervanrija fluxuum			
Pichia mandshurica			
Pichia sporocuriosa			
Pichia terricola			
Pichiaceae			
Saccharomycopsis			
Saccharomycopsis schoenii			
Saccharomycopsis vini			
Saitozyma podzolica			
Schwanniomyces pseudopolymorphus			
Sporidiobolaceae			
Tremellomycetes			
Trichosporon			
Wickerhamomyces anomalus			
Zygosaccharomyces microellipsoides			
		Erythrobasidium hasegawianum	
		Filobasidium	
		Filobasidium floriforme	
		Filobasidium magnum	
		Rhodotorula graminis	
			Curvibasidium

2.3 The MIC_{50} values for the 30 *A. pullulans* isolates for CPN, CYP, and DFN show a significant, positive correlation

The 30 *A. pullulans* strains were initially isolated in the presence of different fungicides. However, a relationship between the initial fungicide used for isolation and the MIC_{50} values for CPN, CYP, and DFN was not apparent. For example, for CPN, the most tolerant isolate was initially isolated in fluconazole. Interestingly, three of the five strains isolated in the presence of CPN (i.e., LC 1.3, LC 1.9, and LC 5.2, all isolated from leaves) exhibited low sensitivity to all three fungicides. Isolates LCH 10.2, LCH 5.9, LCH 2.1, and ChF 4.2 were isolated with CYP but were not among the isolates most tolerant to this fungicide. By contrast, the most CYP-tolerant isolate AL 4e had initially been isolated in amphotericin B. Finally, an isolate initially isolated in DFN (e.g., SFr 4.3, LSK 2.11, FLSK 5.1, and LSK 10.4) was not more tolerant to DFN than other isolates (Table 2), likely suggesting that a pleiotropic mechanism of tolerance towards fungicides in general, as opposed to a specific resistance mechanism against a particular agent, is involved.

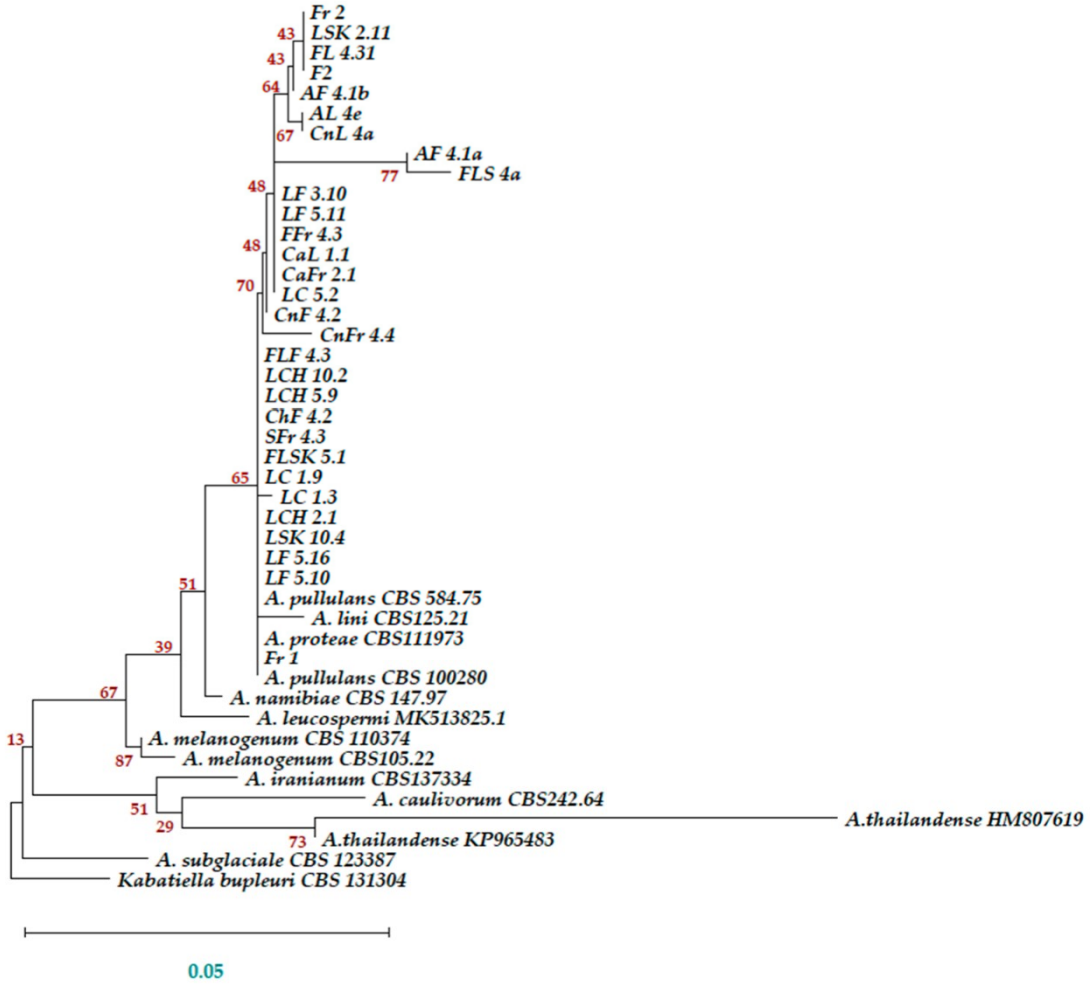

Figure 3. The ITS sequences of the 30 *A. pullulans* isolates cluster with published *A. pullulans*, *A. proteae*, and *A. lini* ITS sequences. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura–Nei model [35]. The tree with the highest log likelihood (−1641.85) is shown. The percentages of trees in which the associated taxa clustered together are shown next to the branches. The initial tree for the heuristic search was obtained automatically by applying the Maximum Parsimony method. A discrete Gamma distribution was used to model evolutionary-rate differences among sites (5 categories (+G, parameter = 0.2691)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.00% sites). The tree is drawn to scale, with branch lengths representing the numbers of substitutions per site. This analysis involved 44 nucleotide sequences. There were a total of 675 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [36].

Thus far, we have determined the MIC_{50} values for 30 *A. pullulans* strains for three fungicides and identified low sensitivity to CPN, CYP, and DFN in at least some isolates. In order to assess if tolerance was fungicide specific or if the same isolates were either sensitive or tolerant to all fungicides, the Pearson correlation coefficients (r) among the MIC_{50} values for all the isolates and the three fungicides were calculated.

In all three comparisons (i.e., CPN–CYP, CPN–DFN, and CYP–DFN,) a weak positive correlation was detected (Figure 5), suggesting that the overall tolerance for these three fungicides correlates. The correlation between the CPN and CYP MIC_{50} values was the strongest (r = 0.56) (Figure 5A), while the CPN and DFN (Figure 5B) and CYP and DFN values (Figure 5C) correlated slightly less (r = 0.43 and 0.38, respectively). All these relationships were statistically significant ($p < 0.05$). Overall, these results implied that tolerance to one fungicide goes along with lower sensitivity to the other two fungicides. Since the three fungicides used here belong to different classes and act on different targets, it is thus likely that the insensitive *A. pullulans* isolates identified here mainly exhibit pleiotropic mechanisms causing multi-drug tolerance.

Table 2. Overall MIC_{50} mean, median, and range for captan (CPN), cyprodinil (CYP), and DFN for all *A. pullulans* isolates.

Isolate name	**MIC_{50} µg /ml**			**Isolating fungicide**
	Captan	Cyprodinil	Difenoconazole	
F2	5.1	2.8	0.5	None
Fr1	22.5	9	1	None
Fr2	40	7.4	0.6	None
SFr4.3	25.1	7.9	2.4	Slick (DFN)
LSK 2.11	19.7	49.3	1.8	Slick (DFN)
FLSK 5.1	18.8	7.5	1.3	Slick (DFN)
LSK 10.4	41	18.9	2.4	Slick (DFN)
LCH 10.2	20.9	34.1	7.1	Chorus (CYP)
LCH 5.9	51.5	11.9	1.3	Chorus (CYP)
ChF4.2	6.3	2.2	0.9	Chorus (CYP)
LCH 2.1	44.7	29.7	3.7	Chorus (CYP)
CaL1.1	19.1	20.6	1.1	Captan 80 WD (CPN)
CaFr2.1	19.4	3.8	1.2	Captan 80 WD (CPN)
LC 5.2	39.5	186	0.7	Captan 80 WD (CPN)
LC 1.9	38.4	59.6	5.2	Captan 80 WD (CPN)
LC 1.3	50.8	50.5	4.2	Captan 80 WD (CPN)
LF 3.10	20.6	3.4	4.8	Flint (Trifloxystrobin)
LF 5.11	23.4	14.1	2.2	Flint (Trifloxystrobin)
FFr4.3	19.9	3.2	3.1	Flint (Trifloxystrobin)
LF 5.16	28.7	42.8	1.4	Flint (Trifloxystrobin)
LF 5.10	54.4	41.9	10.1	Flint (Trifloxystrobin)
AF4.1b	17	8.9	0.4	Amphotericin B
AL4e	19.3	1.45×10^{39}	2.2	Amphotericin B
AF4.1a	5.8	3.6	0.6	Amphotericin B
CnF4.2	5.3	2	0.6	Capsofungin
CnL4a	21.4	6	1.7	Capsofungin
CnFr4.4	33.1	9	0.9	Capsofungin
FL4.31	40.2	8.3	1	Fluconazole
FLF 4.3	5.7	4.7	1.4	Fluconazole
FLS4a	109.6	6.6	1.8	Fluconazole
Mean	28.9	22.6*	2.2	
Median	21.9	8.9	1.4	
Range	5.1-109.5	2.0-186*	0.4 -10.1	

* For CYP, isolate AL4e was excluded from the mean analysis because it was not controlled at any CYP concentration used here. The fungicides and their active compounds used for isolation are indicated.

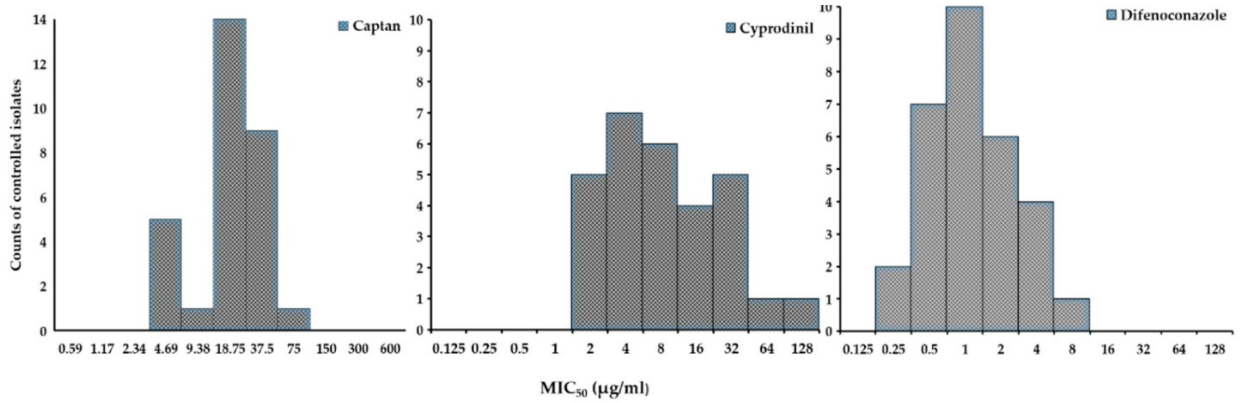

Figure 4. The frequencies of the MIC_{50} values for the 30 *A. pullulans* isolates show non-normal distributions.

The sensitivities of the 30 *A. pullulans* isolates to CPN, CYP, and DFN were determined in microbroth sensitivity assays. The minimum concentration inhibiting the growth of the yeasts by at least 50% (as determined by OD_{600} measurements; MIC_{50}) was calculated. The numbers of isolates with a particular MIC_{50} value are plotted.

In order to better visualize the different MIC_{50} values and to assess if the *A. pullulans* isolates could be grouped based on their responses to the three fungicides, a heat map was generated and a clustering analysis was performed (Figure 6). This analysis clearly identified a small cluster of highly sensitive isolates (S) that was distinguished from the intermediate and tolerant *A. pullulans* isolates (I and T, respectively). The intermediate cluster (I) had one grouping of isolates sensitive to DFN and CYP, but tolerant to CPN, and a second cluster of isolates sensitive to DFN but tolerant to CPN and CYP. Interestingly, all the isolates in cluster S (sensitive to all the three fungicides) were obtained from flowers, while all the T isolates (tolerant to all the three fungicides or tolerant to CPN and CYP) were sampled from leaves. Overall, these results document differential responses of the 30 *A. pullulans* isolates to the three fungicides and thus suggest that various, general mechanisms are likely to be involved in the insensitivity of many of the isolates studied here. However, to identify the exact mechanisms involved and compare isolates from the different clusters described here, detailed molecular analyses will be required.

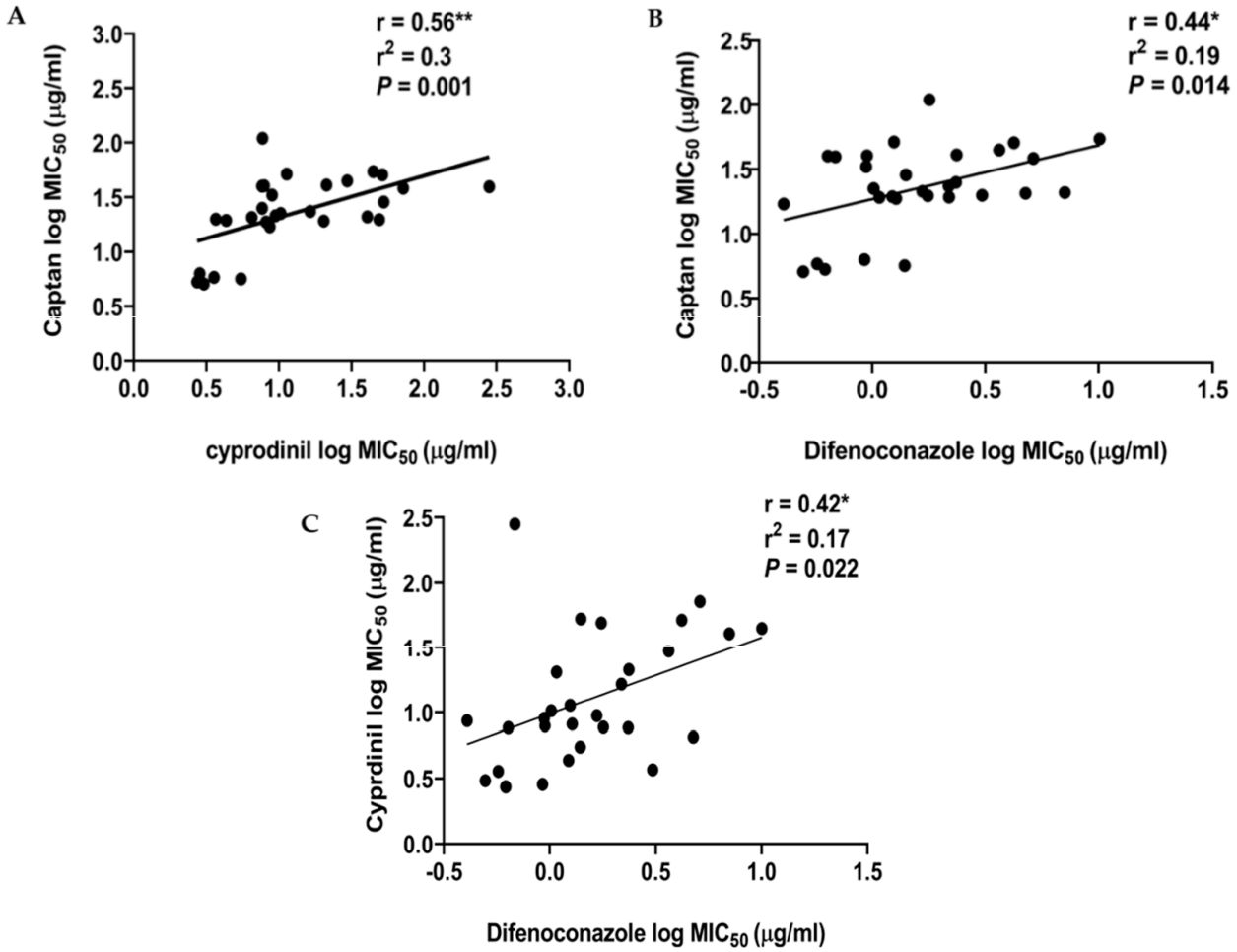

Figure 5. The MIC_{50} values of the 30 *A. pullulans* isolates for CPN, CYP, and DFN show a significant, positive correlation. Pearson's correlations portraying the relationships between the MIC_{50} values for the *A. pullulans* isolates for (**A**) CPN and CYP, (**B**) CPN and DFN, and (**C**) CYP and DFN. All the relationships were statistically significant. Correlation was determined using the log-transformed MIC_{50} values, assuming a Gaussian distribution of the data, in which the outlier (isolate AL4e with CYP) was removed. The interpolation line was fitted with linear regression (r^2) [39].

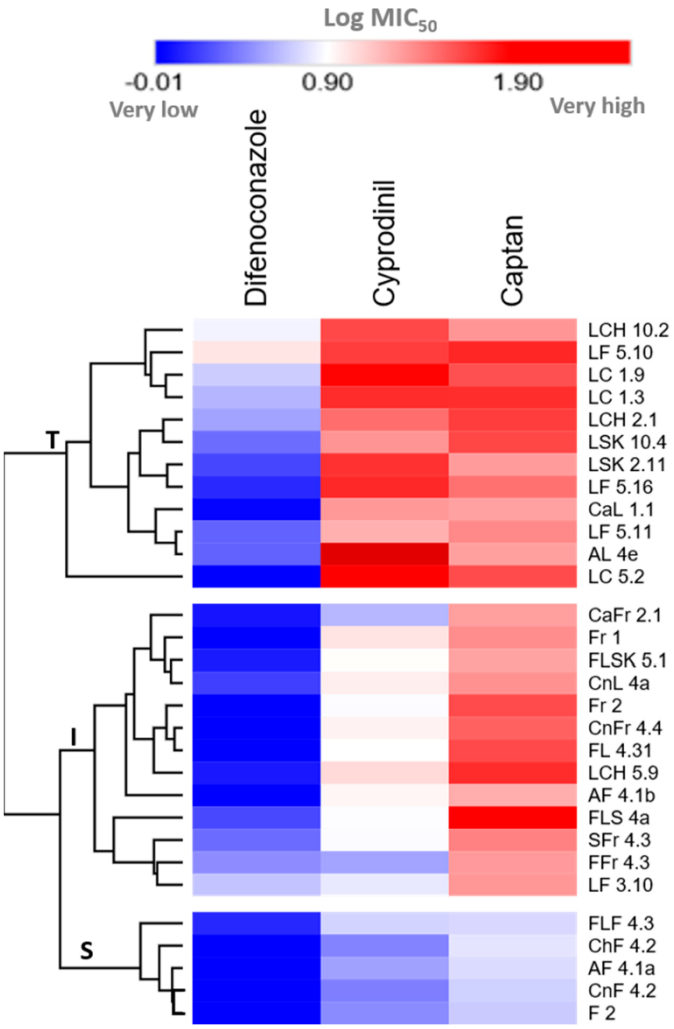

Figure 6. Clustering of the 30 *A. pullulans* isolates into tolerant (T) and sensitive (S; to one or two, or all three fungicides used here) isolates. The dendrogram was plotted using the hierarchical complete linkage clustering method (distance between clusters measured using the Euclidean distance) for the log MIC_{50} values for the three fungicides. The distributions of the sensitivities in the respective clusters based on log MIC_{50} values are highlighted in the heat map. S = sensitive (to all three fungicides), I = intermediate (sensitive to DFN and tolerant to one or two fungicides), and T = tolerant (insensitive to all the three fungicides or to CPN and CYP).

3 Discussion

Agricultural production requires the management of plant diseases, to both minimise crop losses and maintain crop quality by preventing impacts on humans and the environment as well as the development of fungicide resistance. However, consumers and regulatory agencies demand the minimal use of pesticides and crops, without residues of plant-protection agents. There is thus a strong incentive and pressure to reduce fungicide applications. This can be achieved by either reducing the dosage of fungicides or decreasing the number of applications throughout the season [40, 41]. Combining traditional fungicides with a biocontrol agent, such as an antagonistic yeast, in a disease management strategy can either reduce the number of the fungicide applications or allow the reliable application of the minimal effective dosage of the fungicide itself. Such combined treatments have been used not only to lower the number of fungicide applications, but also to reduce resistance selection [41]. Novel yeast–fungicide formulations, thus, may have the potential to reduce the amount of fungicides applied throughout the season. Such applications may also lead to a more reliable

efficacy of biocontrol organisms, save time because multiple applications are combined, and reduce chemical residues on crops. Since *A. pullulans* is already a well-established biocontrol agent and some isolates were tolerant to CPN, CYP, or DFN (or even to two or all three of these), this species could be explored for such combined applications. However, before such applications can be put into general practice, the frequency and nature of such insensitivities should be identified in order to be able to properly assess the possible risks (e.g., an increase in and spread of fungicide insensitivity and resistance). Here, we performed the first step of such an assessment by studying naturally occurring yeasts and quantifying fungicide sensitivity in the biocontrol yeast *A. pullulans*.

Wild yeasts were isolated from different agricultural samples in the presence of the fungicides CPN, CYP, and DFN. In total, 376 isolates were obtained, of which 13 different taxa were isolated from apple leaves, while eight and four taxa were obtained from flowers and fruits, respectively (Table 1). By contrast, from soil, 41 different taxa were isolated in the presence of antifungal agents (Figure 1 and Table 1). The larger number of soil yeasts isolated in the presence of fungicides may reflect the higher species diversity in soil as compared to that in the phyllosphere [42]. Soil acts as a reservoir of phyllosphere yeasts and provides a plethora of niches with different nutrients and substrates that soil yeasts can thrive in [43–46]. It is also possible that some of the many soil fungi bind or inactivate fungicides, thereby reducing their effective concentrations and thus allowing otherwise sensitive species to be isolated. In another study including herbicides, fungicides, and insecticides, only five of 11 yeast species were insensitive to fluquinconazole, while all were sensitive to prochloraz [47]. Among phyllosphere yeasts, only the four species *M. pulcherrima*, *A. pullulans*, *Pichia anomala*, and *S. cerevisiae* were identified as resistant to pesticides [48]. However, sensitivity profiles for medical antifungals have been determined for several *A. pullulans* and *Cryptococcus* isolates. Similar to that in the study presented here, the MIC_{50} for fluconazole for these environmental yeasts was higher compared to that for medically relevant yeasts [49,50].

A. pullulans was, by far, the most frequent species (76 isolates), isolated in the presence of seven out of the eight antifungal agents tested and found in all the four sample sources (soil, leaves, fruits, and flowers). This highlights the ubiquitous nature of *A. pullulans* and its ability to thrive in different habitats (e.g., soil, leaves, flowers, and fruits) and environmental conditions (e.g., hypersaline habitats, glaciers, arid conditions, and radiation sites) due to the presence of genes that confer stress tolerance [34,46,51–55]. Similarly, *M. pulcherrima, C. laurentii, C. misumaiensis,* and *S. metaroseus* are also commonly occurring and frequently isolated from the leaves of various trees, fruits, and soils of both agricultural and wild habitats, and can tolerate extreme conditions [43,46,51,56–59]. Their frequent isolation likely represents the high abundance of these species in the environment but also, likely, their tolerance to the antifungals used for isolation. Interestingly, though, the CPN, CYP, and DFN sensitivities of the 30 *A. pullulans* isolates studied here were not reflected in the initial fungicide used for isolation. For example, the isolate most tolerant to CYP was not isolated in the presence of CYP, but was in that of fluconazole. This likely implies that these yeasts rather exhibit a pleiotropic mechanism of tolerance towards fungicides as opposed to a specific resistance mechanism against a particular agent. It thus seems that high abundance, stress tolerance, and competitiveness in a broad range of environments go hand in hand with low sensitivity to antifungal agents. The unique biochemical and genetic properties rendering these yeasts particularly stress tolerant may thus also confer a general, unspecific insensitivity to antifungal compounds [60].

The mean baseline MIC_{50} values that were determined here for the 30 *A. pullulans* isolates and the three different fungicides CPN, CYP, and DFN were higher than the concentrations of the corresponding fungicides used in the field to control plant pathogens. This was particular striking for CYP, where the mean baseline MIC_{50} was 22.6 μg/mL and thus significantly higher than the concentration of 0.008 μg/mL that is used in the field against the plant pathogen *B. cinerea* [37,38] and the low EC_{50} values of some plant pathogens [61]. Similarly, the mean baseline sensitivity for DFN was 2.18 μg/mL and statistically higher than the mean EC_{50} for the control of 97 *Penicilium* spp. (0.16 μg/mL) or 44 *V. inaequalis* isolates (0.002 μg/mL) [28,31]. Although this baseline for DFN was higher for *A. pullulans* isolates, all of the isolates were controlled by DFN and only nine isolates out of the 30 showed reduced sensitivity (had MIC_{50} values above the mean). For CPN, the mean MIC_{50} value for the 30 *A. pullulans* isolates tested here was 28.9 μg/mL and thus also significantly higher than the mean EC_{50} of wildtype and resistant *B. cinerea* (0.9 and 5 μg/mL, respectively) [20,37,38]. Overall, the *A. pullulans* MIC_{50} values for CPN and DFN, CPN and CYP, and CYP and DFN correlated positively (weakly, but statistically significantly), which may also indicate a general mechanism of insensitivity of *A. pullulans* to these fungicides.

None of the three fungicides CPN, CYP, or DFN harbours a particularly high risk for the development of resistance by plant pathogenic fungi. CPN is highly effective in controlling plant pathogenic fungi, and the risk of resistance development seems low [18,19,62,63]. Nevertheless, resistance to CPN was reported after the in vitro testing of *B. cinerea* isolates from different orchards in Canada and from commercial blueberry fields in Florida [20,21]. One resistance mechanism for CPN is the increased biosynthesis of molecules containing thiol groups (i.e., glutathione), which has been described for *B. cinerea* [21] but could also be a mechanism rendering *A. pullulans* less sensitive to this fungicide. The trichloromethylthiol group of CPN non-enzymatically and irreversibly reacts with exposed thiol groups, resulting in a thiophosgene moiety and tetrahydrophthalimide (THPI) [64,65]. CPN is also sensitive to and unstable at high pH [66]. Therefore, the insensitivity of *A. pullulans* isolates to CPN might be due to the increased production of molecules with exposed thiol groups, a loss of stability in culture supernatants (e.g., due to an increase in pH), or the degradation of CPN. More detailed studies are, however, needed to understand if one or more of these mechanisms are the cause of the insensitivity of *A. pullulans* to CPN and also to identify the mechanisms conferring insensitivity to all three fungicides tested here. Resistance to CYP is rare in most orchards in the US and Europe,with the sensitivity thresholds for different pathogenic fungi in both regions set to between 0.03 and 5 mg/L [20,29,30,67,68]. Nevertheless, resistance has been noted and attributed to point mutations in the *BcmetB* gene and in nine different genes that encode mitochondrial proteins [69–71]. The wide range of MIC_{50} values for CYP that were determined for *A. pullulans* (2.82–186 μg/mL) may be explained by the complex mode of action of CYP. Resistance against DFN has been reported in laboratory-induced mutants. The mutation of tyrosine to phenylalanine at codon 126 (Y126F) in the Cyp51 protein of *Pencillium expansum* and increased expression levels of the *CYP51A1* gene were identified to correlate with DFN resistance [72,73]. Field resistance to DFN is still low but predicted to increase if proper resistance-management practices are not reinforced [74]. To extend DFN's life span, it is applied as a mixture with other compounds. The fact that the yeasts known to be particularly stress resistant seemed to be particularly insensitive to the fungicides and the positive correlation between the insensitivities to CPN, CYP, and DFN seem to suggest that *A. pullulans* is, in general, fungicide tolerant. Detailed studies at the molecular level will identify if this is indeed the case or if insensitivity correlates with specific mutations.

In summary, this study documents the widespread insensitivity of naturally occurring yeasts to different antifungals and highlights the remarkable fungicide insensitivity of at least some *A. pullulans* isolates. This property is a precondition for possible combinations and the synergistic action of a biocontrol agent and a fungicide. Since several *A. pullulans* isolates were tolerant to even the highest concentration of CYP used in the field, a combined disease-management approach (*A. pullulans* as a biocontrol agent and CYP) could be envisioned for plant protection. In general, such biocontrol–fungicide combinations may not only allow reducing the amount of fungicides applied in the field but also prevent the development of resistance against fungicides. To slow down the development of fungicide resistance and prolong the effective lifetime of a fungicide, the use of antifungal agents with different modes of action (either simultaneously, sequentially, or in a single formulation) is recommended [75–77]. For example, DFN and CYP have been combined in a single formulation, marketed as InspireSuper® (Syngenta), and used to efficiently manage disease [29]. Since we identified several *A. pullulans* isolates that exhibited low sensitivity to CPN, CYP, and DFN, we may even envision a combination of *A. pullulans* with two different fungicides. However, more studies are still necessary to understand the particular mechanisms that render *A. pullulans* tolerant to CPN, CYP, and DFN and to assess the potential applications of biocontrol–fungicide combinations in plant protection.

4 Materials and methods

4.1 Fungal isolate collection and storage

Environmental samples (cherry fruits; apple leaves and flowers; and soil from different apple and cherry orchards in Wädenswil, Switzerland) were collected from October 2018 to July 2019. Sampling was mainly performed in orchards that had never been treated with fungicides, but some samples were obtained from fields that had been treated. Amounts of 1 g of soil samples or 2 g of leaves, flowers, or fruits were mixed with 10 mL of 1% peptone water (Carl Roth GmBH, Karlsruhe, Germany) and incubated for 30 min (with vigorous shaking on an orbital shaker (Ecotron®, Infors-ht, Bottmingen, Switzerland) at 200 rpm and 22 °C). Yeasts were isolated in the presence of different, commercially available fungicides (amphotericin B, capsofungin, fluconazole, and 8-hydroxyquinoline sulfate (Fisher Scientific AG, Basel, Switzerland); Chorus® (50% cyprodinil), Slick® (250 g/L difenoconazole), and Captan 80 WDG (80% captan) (Syngenta AG, Basel, Switzerland); Flint® 500 WG (500 g/kg trifloxystrobin) (Bayer Crop Science); and boscalid (pyridine carboxamide) (BASF). The final concentrations for the seven fungicides were as follows: amphotericin B (4, 2, 1, and 0.5 μg/mL); fluconazole (120, 60, 30, and 15 μg/mL); capsofungin (8, 4, 2, and 1 μg/mL); Slick (0.06, 0.03, 0.015, and 0.0075 μg/mL); chorus (10, 5, 2.5, and 1.25 mg/mL); flint (5,2.5, 1.25, and 0.625 mg/mL); captan (30, 15, 7.5, and 3.75 mg/mL); boscalid (10, 20, 40, and 80 μg/L); 8-hydroxyquinoline sulfate (7.5, 15, 30, and 75 mg/mL). The samples were incubated for 1 h (with shaking on an orbital shaker at 22 °C and 200 rpm). An aliquot of 50 μL (25 μL for soil samples) of each dilution was plated on potato dextrose agar (PDA; Difco) dishes supplemented with chloramphenicol (0.5%) and tetracycline (0.5%) and incubated at 22 °C for 72 h. This procedure was performed in five replicates and in a manner that yielded single, well-separated fungal colonies. After incubation, yeast and filamentous colonies were counted. Yeast colonies from each replicate plate were selected (based on different morphological characteristic) and purified by sub-culturing twice on PDA to obtain pure cultures. All the isolates were stored at −80 °C in 15% (*v*/*v*) glycerol.

4.2 Fungal identification

As a faster and more economical alternative to DNA sequencing, yeast identification was first attempted using matrix-assisted laser desorption/ionization time-of-flight mass spectrophotometry (MALDI-TOF MS) as previously described [46,78], with a few modifications. Single yeast colonies were transferred onto an AXIMA-CFR MALDI-TOF target plate (Kratos, Manchester, UK) using a toothpick. The smears were left to air dry and then overlaid with 1 μL of matrix (Sinapinic acid (SA), 40 μg/mL in acetonitrile–ultra pure water (UPW)–trifluoroacetic acid (TFA) (0.6:0.4:0.003) per mL). The SA, acetonitrile, and TFA were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany; the UPW was produced by an Arium® water filter system. To create the MALDI–TOF MS reference spectra, eight replicates of the same species were spotted on the target plate and mass spectra for each spot were obtained using an AXIMA Performance MALDI-TOF MS machine (Shimadzu Schweiz GmbH, Reinach, Switzerland). All the spectra were analysed using the inbuilt AXIMA microorganism identification system (Shimadzu Schweiz GmbH). For species that could not be identified by MALDI-TOF MS, the ITS region was amplified and sequenced as previously described [79]. All the *A. pullulans* isolates studied in detail in this work were identified by sequencing the ITS region. The sequences were processed and analysed using the Genious™ software, and all the sequenced isolates were assigned a species hypothesis (SH) number according to the UNITE database [80] (see also Supplementary Table S1).

4.3 Determination of baseline MIC_{50} values

Thirty isolates of *A. pullulans*, isolated in the presence of different fungicides and from a variety of sources and locations (Table 3), were tested for sensitivity to CPN, CYP, and DFN using the microbroth sensitivity assay.

Table 3. The 30 isolates of *A. pullulans*, with sample sources and times of sampling, used for quantifying the MIC_{50} for CPN, CYP, and DFN All the isolates were identified based on the ITS sequence, which resulted in the SH number SH1515060.08FU. Isolates FLSK5.1, ChF4.2, and LF5.10 were identified by MALDI-TOF MS.

No	Isolate name	Sample	Season isolated
1	F2	Flower	Spring
2	Fr1	Fruit	Summer
3	Fr2	Fruit	Summer
4	AF4.1b	Flower	Summer
5	AL4e	Leaf	Summer
6	AF4.1a	Flower	Spring
7	LF 3.10	Leaf	Autumn
8	LF 5.11	Leaf	Autumn
9	FFr4.3	Fruit	Summer
10	CaL1.1	Leaf	Summer
11	CaFr2.1	Fruit	Summer
12	LC 5.2	Leaf	Autumn
13	CnF4.2	Flower	Spring
14	CnL4a	Leaf	Summer
15	CnFr4.4	Fruit	Summer
16	FL4.31	Leaf	Summer
17	FLF 4.3	Leaf	Spring
18	FLS4a	Leaf	Spring
19	LCH 10.2	Leaf	Autumn

No	Isolate name	Sample	Season isolated
20	LCH 5.9	Leaf	Autumn
21	ChF4.2	Flower	Spring
22	SFr4.3	Fruit	Summer
23	LSK 2.11	Leaf	Autumn
24	FLSK 5.1	Leaf	Winter
25	LC 1.9	Leaf	Autumn
26	LC 1.3	Leaf	Autumn
27	LCH 2.1	Leaf	Autumn
28	LSK 10.4	Leaf	Autumn
29	LF 5.16	Leaf	Autumn
30	LF 5.10	Leaf	Autumn

Concentrated stock solutions (5.12 mg/mL) of technical-grade DFN and CYP (Sigma-Aldrich Chemie, Schweiz, Buchs, Switzerland) were prepared in acetonitrile and methanol, respectively, and serially diluted (1:2) with the respective solvents to achieve 2× the final concentrations (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, and 256 μg/mL). Similarly, technical-grade CPN (Fisher Scientific AG, Reinach, Switzerland) was prepared in acetonitrile, adjusted to a concentrated stock solution (24 mg/mL), and diluted with acetonitrile to achieve 2× the final concentrations of 1.17, 2.34, 4.68, 9.38, 18.75, 37.5, 75, 150, 300, 600, and 1200 μg/mL. The concentration of the solvents in the controls was also kept at 2× (5%) the final concentration. The fungicide solutions or solvents were diluted with potato dextrose broth (Difco™ PDB; Becton, Dickinson and Company, Le Pont-de-Claix, France) (2× concentrated) in flat-bottomed 96-well plates (Fisher Scientific AG, Reinach, Switzerland) (total volume of 100 μL per well, with all concentrations in triplicate).

Overnight cultures of all the yeast isolates were prepared in PDB (3 mL, 22 °C, 200 rpm) using five yeast colonies maintained on PDA for 7 d after thawing from the 15% (*v*/*v*) glycerol stocks. The optical density at 600 nm (OD_{600}) was measured using a spectrophotometer (GE Healthcare NovaspecTM III, Fisher Scientific AG, Basel, Switzerland), and yeast suspensions with final densities (OD_{600}) of 1 were prepared. Of these yeast suspensions, 10 μL was added to each well. Each plate was closed with a lid and incubated in the dark for 72 h at 22 °C (shaking at 240 rpm). The OD_{600} at the 72 h time point was measured using a microplate reader (Spark®, Tecan Life Science AG, Männedorf, Switzerland) (set at 25 °C, 240 rpm (30 sec), 600 nm, and 10 flashes). These OD_{600} values were used to assess the minimum inhibitory concentrations (MIC_{50}), which were defined as the lowest concentrations of the fungicides that resulted in a 50% reduction of yeast growth (as assessed by OD_{600} measurements). Each experiment was repeated at least three times.

4.4 Statistical analyses

All the statistical analyses (unless otherwise specified) were performed in GraphPad Prism 8.4.

(GraphPad software, San Diego, CA, USA), with the level of significance set to 0.05. The means and standard deviations of three technical replicates for each isolate were determined for each experiment (these were later used as the three experimental replicates). The MIC_{50} value was calculated by non-linear regression (curve fit) of the log concentrations against the normalised mean OD_{600} responses. Descriptive statistics (mean, median, and range) were calculated for the cleaned data (one outlier was removed based on Grubb's test) [81] (pp. 26–

28) of the calculated MIC_{50} values. The frequency distribution of the sensitivity for each fungicide was determined using the log-transformed MIC_{50} values, and the presence of a Gaussian normal distribution was tested according to the Shapiro–Wilk test.

The mean MIC_{50} values for the 30 *A. pullulans* isolates were compared with the mean EC_{50} values for plant pathogenic fungi (either *B. cinerea* or *Penicillium* spp.) using *t*-tests. The mean EC_{50} values for CYP (0.008 μg/mL) and CPN (0.9 μg/mL) for the control *B. cinerea* were calculated based on published values for 6 wild strains of *B. cinerea* [37,38]. The mean EC_{50} value for *Penicillium* spp. for DFN (0.16 μg/mL) was based on published data for 97 wild *Penicillium* spp. strains [60].

Simple linear correlation coefficients (Pearson's r) [81] (p.92) were calculated to determine the relationships between the sensitivities of (a) DFN and CYP, (b) DFN and CPN, and (c) CYP and CPN. The log-transformed MIC_{50} values, which assumed a normal distribution, were used for correlation analysis. The clustering of the MIC_{50} values of all the isolates for the three fungicides was evaluated based on the log-transformed data. A hierarchical dendrogram was constructed a using complete-linkage clustering method with the Euclidean distance metric as a measure of the intervals between clusters in Morpheus (https://software.broadinstitute.org/morpheus). Evaluation for potential cross-resistance (possible similar mechanisms of resistance) was performed using the log MIC_{50} values.

4.5 Phylogenic analysis

The ITS sequences of all 30 *A. pullulans* isolates and additional, already published, strains were selected and aligned using MUSCLE, built into MEGA, version 10.1 [36]. All regions were used without gap deletion during the alignment. The phylogenetic analysis involved 44 nucleotide sequences (30 *A. pullulans* sequences, 25 published sequences of other species within the genus *Aureobasidium*, and *Kabatiella bupleuri* (CBS 131304) as the outgroup). A phylogenetic tree was constructed with the maximum-likelihood algorithm using the Tamura–Nei Model [35], and the internal branch support was assessed based on 500-bootstrapped dataset. A discrete Gamma distribution was used to model evolutionary rate differences among sites, which showed some sites to be evolutionarily invariable ([+/], 0.00% sites). All positions with missing data were eliminated, while those with gaps were included, and in the final dataset, there were a total of 675 aligned positions (including gaps).

Declarations

Author Contributions:

Fungal isolation, E.M., M.H.-B., and M.P.; ITS sequencing and identification, M.H.-B.; fungicide sensitivity assays and data analysis, E.M.; writing of the manuscript, E.M. and F.M.F.; supervision, project administration, overall conceptualization, and funding acquisition, F.M.F. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest:

The authors declare no competing interest.

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Supplementary Materials:

The following list of yeast species obtained in the course of the study (Table S1) is available online at http://www.mdpi.com/2079-6382/9/9/602/s1.

Table S1. Yeast species obtained in the course of this study

Isolate	Sample	Species	Isolating fungicide
LF 3.10	Leaf	*Aureobasidium pullulans*	Flint
LF 5.10.1	Leaf	*Aureobasidium pullulans*	Flint
LC 1.9	Leaf	*Aureobasidium pullulans*	Captan
LC 1.3	Leaf	*Aureobasidium pullulans*	Captan
LC 5.2	Leaf	*Aureobasidium pullulans*	Captan
LCH 2.1	Leaf	*Aureobasidium pullulans*	Chorus
LCH 10.2	Leaf	*Aureobasidium pullulans*	Chorus
LCH 5.9	Leaf	*Aureobasidium pullulans*	Chorus
LSK 10.4	Leaf	*Aureobasidium pullulans*	Slick
LSK 2.11	Leaf	*Aureobasidium pullulans*	Slick
SF 5.9	Soil	*Schwanniomyces pseudopolymorphus*	Flint
LF 5.11	Leaf	*Aureobasidium pullulans*	Flint
LF 5.16	Leaf	*Aureobasidium pullulans*	Flint
LF 5.3	Leaf	*Sporidiobolus metaroseus*	Flint
LF 5.8	Leaf	*Sporidiobolus metaroseus*	Flint
SF 5.9	Soil	*Schwanniomyces pseudopolymorphus*	Flint
SF 6.9	Soil	*Cryptococcus laurentii*	Flint
SF 5.19	Soil	*Cryptococcus laurentii*	Flint
SF 5.13	Soil	*Kregervanrija fluxuum*	Flint
SF 5.15	Soil	*Cryptococcus*	Flint
LF 5.10	Leaf	*Aureobasidium pullulans*	Flint
LF 5.6	Leaf	*Bullera alba*	Flint
SF 5.2	Soil	*Saccharomycopsis schoenii*	Flint
SF 5.14	Soil	*Schwanniomyces capriottii*	Flint
LF 5.14.1	Leaf	*Erythrobasidium hasegawianum*	Flint
SCH 5.6	Soil	*Wickerhamomyces anomalus*	Chorus
SF 6.18	Soil	*Saccharomycopsis schoenii*	Flint
SCH 2.1	Soil	*Cyberlindnera misumaiensis*	Chorus
LCH 10.4	Leaf	*Filobasidium*	Chorus
LCH 2.4	Leaf	*Rhodotorula*	Chorus
LCH 2.8	Leaf	*Sporidiobolus metaroseus*	Chorus
SCH 5.4	Soil	*Cryptococcus laurentii*	Chorus
SCH 10.1	Soil	*Saccharomycopsis schoenii*	Chorus
LF 5.20	Leaf	*Sporidiobolus metaroseus*	Flint
LSK 1.5	Soil	*Aureobasidium pullulans*	Slick
SF 6.6	Soil	*Cryptococcus laurentii*	Flint
LF 5.14	Leaf	*Erythrobasidium hasegawianum*	Flint
LCH10.1	Leaf	*Aureobasidium pullulans*	Chorus
LC 5.1	Leaf	*Filobasidium*	Captan
LF 9.2	Leaf	*Schwanniomyces capriottii*	Flint
SSK 2.4	Soil	*Schwanniomyces capriottii*	Slick
LCH 10.1	Leaf	*Rhodotorula*	Chorus

Isolate	Sample	Species	Isolating fungicide
LC 1.4	Leaf	*Rhodotorula*	Captan
LC 1.1	Leaf	*Rhodotorula*	Captan
LC 2.6	Leaf	*Rhodotorula*	Captan
LC 2.1	Leaf	*Rhodotorula*	Captan
LCH 5.7	Leaf	*Sporidiobolus metaroseus*	Chorus
LA 2.9	Leaf	*Bullera alba*	Amphotericin B
L 3	Leaf	*Filobasidium*	None
LA 2.1	Leaf	*Holtermanniella*	Amphotericin B
SHOL 2.6	Soil	*Cryptococcus laurentii*	8-hydroxyquinoline sulfate
SA 2.3	Soil	*Cryptococcus laurentii*	Amphotericin B
SHOL 2.3	Soil	*Rhodotorula*	8-hydroxyquinoline sulfate
SA 2.8	Soil	*Rhodotorula*	Amphotericin B
SA 2.10	Soil	*Cyberlindnera misumaiensis*	Amphotericin B
SA 2.7	Soil	*Cyberlindnera misumaiensis*	Amphotericin B
L 5	Leaf	*Bullera alba*	None
LA 2.11	Leaf	*Bullera alba*	Amphotericin B
LA 2.12	Leaf	*Bullera alba*	Amphotericin B
FLSK 1.4	Leaf	*Aureobasidium pullulans*	Slick
FLSK 5.1	Leaf	*Aureobasidium pullulans*	Slick
FLSK 5.4	Leaf	*Aureobasidium pullulans*	Slick
FLSK 1.17	Leaf	*Aureobasidium pullulans*	Slick
FSHQ 2.6	Soil	*Pichia terricola*	8-hydroxyquinoline sulfate
FSHQ 1.1	Soil	*Cyberlindnera misumaiensis*	8-hydroxyquinoline sulfate
FSHQ 1.3	Soil	*Pichia terricola*	8-hydroxyquinoline sulfate
FSHQ 1.2	Soil	*Pichia mandshurica*	8-hydroxyquinoline sulfate
FLSSK 5.2	Leaf	*Rhodotorula graminis*	Slick
FSSK 2.7	Soil	*Pichiaceae*	Slick
FSSK 5.5	Soil	*Cyberlindnera misumaiensis*	Slick
FSSK 5.6	Soil	*Candida californica*	Slick
FLHQ 1.2	Leaf	*Sporidiobolus pararoseus*	8-hydroxyquinoline sulfate
FSSK 5.1	Soil	*Dipodascus geotrichum*	Slick
FSSK 2.6	Soil	*Candida californica*	Slick
FLHQ 2.4	Leaf	*Filobasidium magnum*	8-hydroxyquinoline sulfate
FSSK 2.3	Soil	*Pichia mandshurica*	Slick
FLHQ 2.1	Leaf	*Filobasidium floriforme*	8-hydroxyquinoline sulfate
FSHQ 5.1	Soil	*Barnettozyma California*	8-hydroxyquinoline sulfate
FSCW 13	Soil	*Pichia mandshurica*	Captan
FSHQ 1.5	Soil	*Saccharomycopsis vini*	8-hydroxyquinoline sulfate
FSSK 5.4	Soil	*Kregervanrija fluxuum*	Slick
FSSK 5.2	Soil	*Saccharomycopsis vini*	Slick
FSSK 5.1	Soil	*Aureobasidium pullulans*	Slick
FSPC 2	Soil	*Sporidiobolus pararoseus*	Boscalid
FSPC 11	Soil	*Pichia terricola*	Boscalid
FSPC 19	Soil	*Sporidiobolus pararoseus*	Boscalid
FSPC 4	Soil	*Cyberlindnera misumaiensis*	Boscalid
FSPC 14	Soil	*Pichia mandshurica*	Boscalid
FSPC 15	Soil	*Pichia mandshurica*	Boscalid
FCPC 25	Soil	*Pichia mandshurica*	Boscalid
FCPC 21	Soil	*Pichia mandshurica*	Boscalid
FSPC 8	Soil	*Sporidiobolus metaroseus*	Boscalid

Isolate	Sample	Species	Isolating fungicide
FSPC 22	Soil	*Sporidiobolaceae*	Boscalid
FSPC 23	Soil	*Kregervanrija fluxuum*	Boscalid
FSPC 24	Soil	*Kregervanrija fluxuum*	Boscalid
SFC 8	Soil	*Pichia terricola*	Fluconazole
SFC 13	Soil	*Cyberlindnera misumaiensis*	Fluconazole
SFC 18	Soil	*Cyberlindnera misumaiensis*	Fluconazole
SFC 10	Soil	*Apiotrichum*	Fluconazole
SFC 11	Soil	*Pichia terricola*	Fluconazole
SFC 19	Soil	*Cyberlindnera misumaiensis*	Fluconazole
SFC 20	Soil	*Cyberlindnera misumaiensis*	Fluconazole
SFC 9	Soil	*Cyberlindnera misumaiensis*	Fluconazole
SFC 14	Soil	*Cryptococcus laurentii*	Fluconazole
CS 3	Soil	*Tremellomycetes*	Captan
CS 5	Soil	*Cryptococcus*	captan
SFC 17	Soil	*Cyberlindnera misumaiensis*	Fluconazole
SFC 27	Soil	*Cyberlindnera misumaiensis*	Fluconazole
CS 4	Soil	*Cryptococcus*	None
CS 7	Soil	*Apiotrichum porosum*	None
SFC 36	Soil	*Pichia terricola*	Fluconazole
SFC 32	Soil	*Cryptococcus laurentii*	Fluconazole
SFC 30	Soil	*Cryptococcus laurentii*	Fluconazole
SCH 2.11	Soil	*Cyberlindnera misumaiensis*	Chorus
SCH 2.20	Soil	*Cyberlindnera misumaiensis*	Chorus
SCH 2.10	Soil	*Trichosporon*	Chorus
SCH 2.12	Soil	*Trichosporon*	Chorus
SCH 2.8	Soil	*Cryptococcus laurentii*	Chorus
SCH 2.16	Soil	*Cryptococcus laurentii*	Chorus
SCH 2.19	Soil	*Hanseniospora*	Chorus
SSK 1.20	Soil	*Cyberlindnera misumaiensis*	Slick
SSK 1.12	Soil	*Pichiaceae*	Slick
SSK 1.14	Soil	*Cryptococcus laurentii*	Slick
SSK 1.9	Soil	*Cryptococcus laurentii*	Slick
SSK 1.11	Soil	*Trichosporon*	Slick
SSK 1.13	Soil	*Trichosporon*	Slick
SB 50.17	Soil	*Cyberlindnera misumaiensis*	Boscalid
SB 50.8	Soil	*Cyberlindnera misumaiensis*	Boscalid
SB 50.3	Soil	*Trichosporon*	Boscalid
SB 50.15	Soil	*Holtermanniella*	Boscalid
SB 50.1	Soil	*Cryptococcus laurentii*	Boscalid
SB 50.18	Soil	*Cryptococcus laurentii*	Boscalid
SC 15.8	Soil	*Cyberlindnera misumaiensis*	Captan
SC 15.3	Soil	*Holtermanniella*	Captan
SC 15.10	Soil	*Dipodascaceae*	Captan
SC 15.21	Soil	*Cryptococcus laurentii*	Captan
CS 6	Soil	*Chytridiales*	None
CS 1	Soil	*Cyberlindnera saturnus*	None
SCH 0.5.1	Soil	*Cyberlindnera misumaiensis*	Chorus
SCH 0.5.9	Soil	*Cryptococcus*	Chorus
SC 15.24	Soil	*Cryptococcus*	Captan
SSK 1.18	Soil	*Pichiaceae*	Slick

Isolate	Sample	Species	Isolating fungicide
SSK 1.19	Soil	*Pichiaceae*	Slick
SC 15.22	Soil	*Pichiaceae*	Captan
SSK 1.18	Soil	*Pichiaceae*	Slick
SSK 1.4	Soil	*Cryptococcus laurentii*	Slick
SB 50.12	Soil	*Cryptococcus laurentii*	Boscalid
SCH 2.1	Soil	*Cryptococcus laurentii*	Chorus
SB 50.5	Soil	*Cryptococcus laurentii*	Boscalid
SC 15.6	Soil	*Cryptococcus laurentii*	Captan
AF4.1	Flower	*Aureobasidium pullulans*	Amphotericin B
AL4.1	Leaf	*Aureobasidium pullulans*	Amphotericin B
AL4.3	Leaf	*Metschnikowia pulcherrima*	Amphotericin B
CaL1.1	Leaf	*Aureobasidium pullulans*	Capsofungin
ChF4.2	Flower	*Aureobasidium pullulans*	Chorus
ChF4.3	Flower	*Aureobasidium pullulans*	Chorus
ChS4.2	Soil	*Cryptococcus*	Chorus
ChS4.3	Soil	*Cyberlindnera misumaiensis*	Chorus
CnF4.2	Flower	*Aureobasidium pullulans*	Capsofungin
CnL4.2	Leaf	*Aureobasidium pullulans*	Capsofungin
CnL4.3	Leaf	*Metschnikowia pulcherrima*	Capsofungin
CnL4.4	Leaf	*Metschnikowia pulcherrima*	Capsofungin
CnS4.1	Soil	*Aureobasidium pullulans*	Capsofungin
CnS4.2	Soil	*Cryptococcus laurentii*	Capsofungin
CnS4.4	Soil	*Pichiaceae*	Capsofungin
F1	Flower	*Metschnikowia pulcherrima*	None
F2	Flower	*Aureobasidium pullulans*	None
FF4.1	Flower	*Aureobasidium pullulans*	Flint
FF4.2	Flower	*Aureobasidium pullulans*	Flint
FLF 4.3	Flower	*Aureobasidium pullulans*	Fluconazole
FLF4.2	Flower	*Metschnikowia pulcherrima*	Fluconazole
FLL1.3	Flower	*Aureobasidium pullulans*	Fluconazole
FLS4.1	Flower	*Cryptococcus laurentii*	Fluconazole
FLS4.10	Flower	*Cyberlindnera misumaiensis*	Fluconazole
FLS4.3	Flower	*Cyberlindnera misumaiensis*	Fluconazole
FLS4a	Flower	*Aureobasidium pullulans*	Fluconazole
FLS4.7	Flower	*Cyberlindnera misumaiensis*	Fluconazole
FLS4.9	Flower	*Aureobasidium pullulans*	Fluconazole
S2	Soil	*Pichia terricola*	None
S3	Soil	*Pichia terricola*	None
S4	Soil	*Cyberlindnera misumaiensis*	None
S5	Soil	*Bullera alba*	None
S6	Soil	*Cryptococcus laurentii*	None
L1	Leaf	*Metschnikowia pulcherrima*	None
CnS4.5	Soil	*Pichia terricola*	Capsofungin
AS4.7	Soil	*Pichia terricola*	Amphotericin B
SS4.8	Soil	*Pichia terricola*	Slick
FLS4.6	Soil	*Pichia terricola*	Fluconazole
FLS4.2	Soil	*Cystofilobasidiacea*	Fluconazole
FLL1.4	Leaf	*Metschnikowia pulcherrima*	Fluconazole
FLS4.8	Leaf	*Metschnikowia pulcherrima*	Fluconazole
AS4.9	Soil	*Aureobasidium pullulans*	Amphotericin B

Isolate	Sample	Species	Isolating fungicide
AS4.3	Soil	*Cryptococcus laurentii*	Amphotericin B
AS4.4	Soil	*Cryptococcus laurentii*	Amphotericin B
AS4.1	Soil	*Cryptococcus*	Amphotericin B
AS4.2	Soil	*Pichiaceae*	Amphotericin B
AS1a	Soil	*Metschnikowia pulcherrima*	Amphotericin B
AL4g	Leaf	*Metschnikowia pulcherrima*	Amphotericin B
CnL3a	Leaf	*Metschnikowia pulcherrima*	Capsofungin
CnL4b	Leaf	*Metschnikowia pulcherrima*	Capsofungin
CnS3b	Soil	*Metschnikowia pulcherrima*	Capsofungin
CnS4d	Soil	*Metschnikowia pulcherrima*	Capsofungin
FIF4f	Flower	*Metschnikowia pulcherrima*	Fluconazole
FIL4a	Leaf	*Metschnikowia pulcherrima*	Fluconazole
FF4c	Flower	*Metschnikowia pulcherrima*	Flint
CnL4c	Leaf	*Aureobasidium pullulans*	Capsofungin
CnS4o	Soil	*Cyberlindnera misumaiensis*	Capsofungin
CnS4L	Soil	*Sporidiobolus metaroseus*	Capsofungin
SS2a	Soil	*Pichiaceae*	Slick
CnS4g	Soil	*Tremellomycetes*	Capsofungin
AS4d	Soil	*Cyberlindnera misumaiensis*	Amphotericin B
SS3c	Soil	*Kregervanrija fluxuum*	Slick
CnF4q	Flower	*Metschnikowia pulcherrima*	Capsofungin
FF4a	Flower	*Metschnikowia pulcherrima*	Flint
AF4d	Flower	*Metschnikowia pulcherrima*	Amphotericin B
SL1f	Leaf	*Metschnikowia pulcherrima*	Slick
SL1e	Leaf	*Metschnikowia pulcherrima*	Slick
AL4b	Leaf	*Metschnikowia pulcherrima*	Amphotericin B
CnF4v	Flower	*Metschnikowia pulcherrima*	Capsofungin
AL4c	Leaf	*Metschnikowia pulcherrima*	Amphotericin B
CnL2	Leaf	*Metschnikowia pulcherrima*	Capsofungin
CnF4d	Flower	*Metschnikowia pulcherrima*	Capsofungin
CnF4p	Flower	*Metschnikowia pulcherrima*	Capsofungin
AL4b	Leaf	*Metschnikowia pulcherrima*	Amphotericin B
AF4g	Flower	*Metschnikowia pulcherrima*	Amphotericin B
AF4b	Flower	*Metschnikowia pulcherrima*	Amphotericin B
CnF4a	Flower	*Metschnikowia pulcherrima*	Capsofungin
CnF4w	Flower	*Metschnikowia pulcherrima*	Capsofungin
CnF4h	Flower	*Metschnikowia pulcherrima*	Capsofungin
CnF4n	Flower	*Metschnikowia pulcherrima*	Capsofungin
CnF4s	Flower	*Metschnikowia pulcherrima*	Capsofungin
AF4h	Flower	*Metschnikowia pulcherrima*	Amphotericin B
AF4e	Flower	*Metschnikowia pulcherrima*	Amphotericin B
SL2s	Soil	*Aureobasidium pullulans*	Slick
SL1b	Soil	*Aureobasidium pullulans*	Slick
CnF4j	Flower	*Aureobasidium pullulans*	Capsofungin
CnF4o	Flower	*Aureobasidium pullulans*	Capsofungin
AL4e	Leaf	*Aureobasidium pullulans*	Amphotericin B
AL4a	Leaf	*Aureobasidium pullulans*	Amphotericin B
AL4f	Leaf	*Aureobasidium pullulans*	Amphotericin B
CnL4a	Leaf	*Aureobasidium pullulans*	Capsofungin
CnF4e	Flower	*Aureobasidium pullulans*	Capsofungin

Isolate	Sample	Species	Isolating fungicide
CnL3b	Leaf	*Aureobasidium pullulans*	Capsofungin
SS3a	Soil	*Pichia sporocuriosa*	Slick
SS2b	Soil	*Pichia terricola*	Slick
AS4a	Soil	*Pichia mandshurica*	Amphotericin B
CnS4f	Soil	*Pichia mandshurica*	Capsofungin
CnS4c	Soil	*Pichia mandshurica*	Capsofungin
CaS2	Soil	*Dipodascus geotrichum*	captan
CaS1b	Soil	*Tremellomycetes*	captan
CnS4a	Soil	*Sporidiobolus metaroseus*	Capsofungin
CnS4n	Soil	*Cystofilobasidium macerans*	Capsofungin
AS4e	Soil	*Cyberlindnera misumaiensis*	Amphotericin B
CnS4h	Soil	*Cyberlindnera misumaiensis*	Capsofungin
CnS4b	Soil	*Cyberlindnera misumaiensis*	Capsofungin
CnS4m	Soil	*Cyberlindnera misumaiensis*	Capsofungin
AS4c	Soil	*Cyberlindnera misumaiensis*	Amphotericin B
AS4b	Soil	*Cyberlindnera misumaiensis*	Amphotericin B
FlL3	Leaf	*Aureobasidium pullulans*	Fluconazole
Fl4c	Flower	*Aureobasidium pullulans*	Fluconazole
FlL4b	Leaf	*Aureobasidium pullulans*	Fluconazole
FF4e	Flower	*Aureobasidium pullulans*	Flint
FlF4g	Flower	*Aureobasidium pullulans*	Fluconazole
FlF4a	Flower	*Aureobasidium pullulans*	Fluconazole
FlF4c	Flower	*Aureobasidium pullulans*	Fluconazole
FF4d	Flower	*Aureobasidium pullulans*	Flint
FlL4d	Leaf	*Metschnikowia pulcherrima*	Fluconazole
FIL4c	Leaf	*Metschnikowia pulcherrima*	Fluconazole
FlF4d	Flower	*Metschnikowia pulcherrima*	Fluconazole
FF4g	Flower	*Metschnikowia pulcherrima*	Flint
FlS1	Soil	*Metschnikowia pulcherrima*	Fluconazole
FlS4a	Soil	*Cryptococcus*	Fluconazole
FlS4d	Soil	*Barnettozyma California*	Fluconazole
FlS4b	Soil	*Cyberlindnera misumaiensis*	Fluconazole
FlS4e	Soil	*Zygosaccharomyces microellipsoides*	Fluconazole
FlF4c	Flower	*Metschnikowia pulcherrima*	Fluconazole
FlF4a	Flower	*Metschnikowia pulcherrima*	Fluconazole
AF4c	Flower	*Metschnikowia pulcherrima*	Amphotericin B
AF4a	Flower	*Metschnikowia pulcherrima*	Amphotericin B
SS1.31	Soil	*Holtermanniella*	Slick
FFr4.2	Fruit	*Holtermanniella*	Flint
AS4.30	Soil	*Cryptococcus laurentii*	Amphotericin B
CnFr4.4	Fruit	*Aureobasidium pullulans*	Capsofungin
ChS4.30	Soil	*Cystofilobasidium capitatum*	Chorus
CnS3.1	Soil	*Cystofilobasidium macerans*	Capsofungin
AS4.31	Soil	*Tremellomycetes*	Amphotericin B
SS1.30	Soil	*Tremellomycetes*	Slick
CnFr4.4	Fruit	*Aureobasidium pullulans*	Capsofungin
FFr4.1	Fruit	*Sporidiobolus metaroseus*	Flint
CnL4.32	Leaf	*Sporidiobolus metaroseus*	Capsofungin
FS3b	Soil	*Sporidiobolus metaroseus*	Flint
ChF4.31	Flower	*Sporidiobolus metaroseus*	Chorus

Isolate	Sample	Species	Isolating fungicide
FS3a	Soil	*Sporidiobolus metaroseus*	Flint
SFr4.6	Fruit	*Aureobasidium pullulans*	Slick
CaFr3.3	Fruit	*Aureobasidium pullulans*	Captan
Fr2	Fruit	*Aureobasidium pullulans*	None
CaFr1.1	Fruit	*Aureobasidium pullulans*	captan
Fr1	Fruit	*Aureobasidium pullulans*	None
CaFr3.0	Fruit	*Aureobasidium pullulans*	Captan
AF4.1	Flower	*Aureobasidium pullulans*	Amphotericin B
FL4.31	Flower	*Aureobasidium pullulans*	Fluconazole
CaFr3.1	Fruit	*Aureobasidium pullulans*	Captan
SFr3.1	Fruit	*Aureobasidium pullulans*	Slick
FlS4.30	Soil	*Holtermanniella*	Fluconazole
FlS4.31	Soil	*Holtermanniella*	Fluconazole
CnF3.1	Flower	*Holtermanniella*	Capsofungin
FS4.34	Soil	*Holtermanniella*	Flint
AS4.34	Soil	*Holtermanniella*	Amphotericin B
FS4.33	Soil	*Holtermanniella*	Flint
FlFr4.1	Fruit	*Holtermanniella*	Fluconazole
SS4.30	Soil	*Pichiaceae*	Slick
SS4.32	Soil	*Pichiaceae*	Slick
SS4.33	Soil	*Pichiaceae*	Slick
FS3.2	Soil	*Pichiaceae*	Flint
SS4.34	Soil	*Pichiaceae*	Slick
SS4.31	Soil	*Pichiaceae*	Slick
FlL4.30	Leaf	*Sporidiobolus metaroseus*	Fluconazole
SFr1.1	Fruit	*Sporidiobolus metaroseus*	Slick
FlL3.2	Leaf	*Sporidiobolus metaroseus*	Fluconazole
Fl4.30	Leaf	*Sporidiobolus metaroseus*	Fluconazole
FS4.32	Soil	*Sporidiobolus metaroseus*	Flint
FlS4a	Soil	*Cryptococcus/ Naganishia*	Fluconazole
FS4.31	Soil	*Cystofilobasidium*	Flint
CnFr4.1	Fruit	*Curvibasidium*	Capsofungin
FlS4.7	Soil	*Cyberlindnera misumaiensis*	Fluconazole
FlS4.34	Soil	*Saccharomycopsis*	Fluconazole
CnS3.1	Soil	*Cryptococcus laurentii*	Capsofungin
FlS3.2	Soil	*Saitozyma podzolica*	Fluconazole
FlS4.33	Soil	*Apiotrichum porosum*	Fluconazole
FS1.3	Soil	*Apiotrichum porosum*	Flint
F1	Flower	*Metschnikowia pulcherrima*	None
SFr4.2	Fruit	*Aureobasidium pullulans*	Slick
FFr4.3	Fruit	*Aureobasidium pullulans*	Flint
SFr4.1	Fruit	*Aureobasidium pullulans*	Slick
CnL4.30	leaf	*Aureobasidium pullulans*	Capsofungin
CaFr2.2	Fruit	*Aureobasidium pullulans*	Captan
FlS4.32	Soil	*Sporidiobolus metaroseus*	Fluconazole
FlFr4.2	Fruit	*Sporidiobolus metaroseus*	Fluconazole
FFr4.4	Fruit	*Sporidiobolus metaroseus*	Flint
CnL1.1	Leaf	*Sporidiobolus metaroseus*	Capsofungin
CnFr4.6	Fruit	*Sporidiobolus metaroseus*	Capsofungin
CnF4g	Flower	*Metschnikowia pulcherrima*	Capsofungin

Isolate	Sample	Species	Isolating fungicide
ChS4.34	Soil	*Cryptococcus laurentii*	Chorus
ChS4.32	Soil	*Cryptococcus laurentii*	Chorus
AS4.32	Soil	*Cryptococcus laurentii*	Amphotericin B
CnS4.35	Soil	*Cryptococcus laurentii*	Capsofungin
ChFr4.3	Fruit	*Sporidiobolus metaroseus*	Chorus
AL4.30	Leaf	*Sporidiobolus metaroseus*	Amphotericin B
AL4.31	Leaf	*Sporidiobolus metaroseus*	Amphotericin B
CnS4.34	Soil	*Holtermanniella*	Capsofungin
CnL4.31	Leaf	*Holtermanniella*	Capsofungin
AFr4.2	Fruit	*Holtermanniella*	Amphotericin B
FS3c	Soil	*Holtermanniella*	Flint
FlF4b	Flower	*Hanseniaspora uvarum*	Fluconazole
FS4	Soil	*Saccharomycopsis schoenii*	Flint
FlF4b	Flower	*Hanseniaspora uvarum*	Fluconazole
FS4	Soil	*Saccharomycopsis schoenii*	Flint
ChS4.35	Soil	*Rhodotorula*	Chorus
CnS4.36	Soil	*Kregervanrija fluxuum*	Capsofungin
SFr4.3	Fruit	*Aureobasidium pullulans*	Slick
SFr4.4	Fruit	*Aureobasidium pullulans*	Slick
CaFr2.1	Fruit	*Aureobasidium pullulans*	Captan
CaFr3.2	Fruit	*Aureobasidium pullulans*	Captan
FF4f	Flower	*Metschnikowia pulcherrima*	Flint
AL3	Leaf	*Metschnikowia pulcherrima*	Amphotericin B
FlF4b	Flower	*Metschnikowia pulcherrima*	Fluconazole
FlF4e	Flower	*Metschnikowia pulcherrima*	Fluconazole
FlF4h	Flower	*Metschnikowia pulcherrima*	Fluconazole
FlF4k	Flower	*Metschnikowia pulcherrima*	Fluconazole
FS3c	Soil	*Holtermanniella*	Flint
ChS4.31	Soil	*Holtermanniella*	Chorus
FS1.4	Soil	*Cyberlindnera saturnus*	Flint
CnS3a	Soil	*Hanseniaspora uvarum*	Capsofungin
ChS4.33	Soil	*Apiotrichum porosum*	Capsofungin
Fl4Fb	Flower	*Cystofilobasidium macerans*	Fluconazole
SS4.8	Soil	*Pichia terricola*	Slick

Chapter 4

GWAS and functional characterizations identify new tolerance mechanisms of *Aureobasiudium pullulans* to captan and cyprodinil

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This manuscript is under preparation

Own contribution

- DNA extraction
- Genome sequence comparison
- RNA extraction and cDNA library construction
- PCR and plasmids construction
- Heterologous expression in *S. cerevisiae*
- Microbroth sensitivity assays and data analysis
- Writing of the manuscript

Abstract

Mechanisms of fungicide tolerance are rarely studied in naturally occurring, non-pathogenic, and non-target fungi. In our previous study, we have observed that environmental and ubiquitous yeasts were insensitive to commonly used antifungals and, particularly, *Aureobasidium pullulans* tolerated high concentrations of captan (CPN), cyprodinil CYP, and difenoconazole (DFN). Here, we built upon this data in order to identify the underlying mechanisms for this high tolerance. Additionally, we complemented this data with newly generated sensitivity data for 16 clinical *Aureobasidium* isolates and the fungicides CPN, CYP, and DFN. Genome data for these 16 clinical and 30 environmental *A. pullulans* isolates were genereated. SNPs-based phylogenetic analysis of the 46 genomes revealed a possible misclassification of seven *A. pullulans* isolates, which grouped with *A. melanogenum* and were thus excluded from the further analyses. This allowed us to continue with 39 environmental and clinical *A. pullulans* isolates to identify fungicide tolerance mechanisms. To accomplish this, we used GWAS and literature-based approaches to identify the potential tolerance mechanisms for the three fungicides in *A. pullulans*. We then tested these mechanisms by either heterologously expressing *A. pullulans* genes in *S. cerevisiae* or using *S. cerevisiae* gene deletion strains and determining fungicide sensitivity. A GWAS using CPN, CYP, and DFN sensitivity data of the *39 A. pullulans* genomes reported 1767 genes with significant SNPs within their coding region, which correlated to MIC_{50} of CYP and only four genes for CPN were predicted. None of the GWAS-identified significant SNPs were present in genes reported in the literature to cause CYP tolerance. CYP sensitivity assays on strains transformed with GWAS-identified genes reported complex CYP tolerance mechanisms involving five *A. pullulans* gene homologs of *S. cerevisiae* (*STL1, LYS2, ENO1, HOL1,* and *CAC2*) and five novel genes (*FBD, GST, HP5, HP6,* and *HP8).* These genes' cellular and biochemical functions ranged from substrate transporters, protein kinases, an F-box domain protein, and enzymes involved in different metabolic processes. These diverse functions point to general tolerance mechanisms in *A. pullulans* being generalized or interconnected to other already or yet-to-be-described pathways.

Keywords: antifungals, *A. pullulans*, genes, genomes, GWAS, heterologous expression, *S. cerevisiae*, SNPs

1 Introduction

Antifungal compounds are widely used in agricultural and clinical settings to control fungal diseases. Their usage in agriculture has risen in recent years [1-3]. The same has been the case in the clinical setting, where the use of some antifungal agents has also increased primarily because of the surge of immunocompromised individuals prone to opportunistic fungal infections and emerging fungal pathogens [4-7]. The extensive use of antifungals poses strong selection pressure on target and non-target fungi and can lead to the development of resistance [8-11]. A notable example is *Aspergillus* spp. resistance to azoles due to excessive use of azoles both in the environment and in clinical settings [8, 10-13]. Resistant pathogenic fungi are thus a growing problem to human, animal, and plant health worldwide that needs to be addressed [14-19]. Increased resistance to antifungals is even more concerning if non-target, ubiquitous fungi emerge as pathogenic to crops or humans [18, 20]. In contrast to fungal pathogens, naturally occurring, non-pathogenic, and non-target fungi are rarely studied in the context of fungicide resistance. Environmental yeasts belong to this group of fungi and often tolerate surprisingly high concentrations of fungicides [21-23]. However, the root cause for fungicide tolerance of these fungi has not been studied and it is thus not possible to judge the implications of this unexpected finding. Here, we studied a strain collection of the highly abundant, yeast-like fungus *Aureobasidium pullulans* to identify molecular factors implicated in the high fungicide tolerance of this species.

A. pullulans is an important yeast-like fungus that is highly abundant, readily isolated, extremotolerant, and found in diverse habitats, including hypersaline, glacial, and polluted sites [21, 24-27]. The species produces a range of antimicrobial enzymes, compounds, and metabolites, including aureobasidins, pullulan (poly-α-1,6-maltotriose biopolymer), cellulases, proteases, β-glucosidase, chitinase, glucanases, amylases, lipases, mannanases, and xylanases [28-30]. These enzymes, compounds, and metabolites have been explored biotechnologically for the production of wine, pharmaceutical drug adjuvants, and are envisioned as prospects for the production of antimicrobial agents [31-36]. In agriculture, *A. pullulans* is used as a biocontrol agent against plant pathogenic fungi and bacteria; for example, *Botrytis cinerea*, *Aspergillus*, *Penicillium* spp. *Pseudomonas syringae,* and *Phytophthora infestans* [37-41]. *A. pullulans* is also highly tolerant to commonly used antifungal agents such as captan (CPN), cyprodinil (CYP), and difenoconazole (DFN) [21], but the underlying mechanisms conferring this tolerance are unknown. It is thus not clear if the low CPN, CYP, and DFN sensitivity is a general characteristic of this species or has been selected for by repeated fungicide exposure. The latter would represent a largely neglected and unintended side-effects of fungicide use, while the former might highlight fungicide tolerant biocontrol strains for combined applications to reduce overall pesticide use [42-44]. It was therefore the goal of this study to identify and test mechanisms of CPN, CYP and DFN tolerance in *A. pullulans* in order to understand the implications of the high fungicide tolerance of this species.

DFN belongs to the demethylase inhibitors (DMIs) group of fungicides, which inhibit fungal sterol biosynthesis by targeting the lanosterol 14-α-demethylase enzyme coded for by the *CYP51A* gene [45, 46]. Characterized mechanisms of resistance to DMIs include *CYP51A* structural alterations, efflux pump overexpression, and *CYP51A* overexpression [47-51]. Additionally, the activation of the stress response pathway through the mitogenic-activated protein kinase (MAPK) cascade, (i.e., Slt2-MAPK), was predicted as a mechanism of resistance to DMIs through a transcriptomic approach [52].

CYP belongs to the anilinopyrimidines (APs) fungicide group, which is hypothesized to inhibit methionine biosynthesis, but the primary target is yet to be confirmed [53-56]. The suggested CYP target is the *METC* gene

(encoding cystathionine β-lyase). However, it is not clear how this target affects APs sensitivity in *B. cinerea*, *Penicillium digitatum*, and *Sclerotinia sclerotiorum* [53, 57-59]. APs may also target the secretory pathway, thereby reducing the production of enzymes involved in plant infection [58, 60, 61]. A recent study proposed mitochondrial protein functions as the primary target of APs in *B. cinerea* and identified mutations in nine individual genes with polymorphisms that correlated with the resistance phenotype [62]. Apart from these targets, genes that encode various enzymes of the arginine and ornithine biosynthetic pathways have been implicated in APs resistance based on the transcriptomic response to treatment with sub-lethal doses of APs in *S. cerevisiae* [63].

CPN is a multisite fungicide belonging to the phthalimides fungicide group. Due to its multisite mode of action, resistance development is regarded as unlikely [64, 65]. However, resistance to CPN has been reported in some strains that also show multidrug resistance [66, 67]. Based on transcriptome data, such a non-targeted mechanism might result from efflux pump overexpression [68]. Considering the limited understanding of resistance mechanisms against CPN, CYP, and DFN, molecular approaches that enable prediction and characterisation of the high tolerance in *A. pullulans* are necessary.

Whole-genome sequence data and downstream analysis, including genome-wide association studies (GWAS), are powerful tools for predicting and, eventually, prioritizing genes for experimental molecular characterization. The availability of phenotypic data is essential for GWAS to make such predictions [69, 70]. GWAS uses phenotypic differences (e.g., in fungicide tolerance) between strains in a population to discern genetic polymorphisms, including single nucleotide polymorphism (SNP) and small insertions and deletions (indel) related to fungicide resistance. GWAS and fungicide sensitivity data have already been used to identify azole resistance genes of the plant pathogens *Rhynchosporium commune*, *Zymoseptoria tritici*, and *Cercospora beticola* [71-73]. Apart from identifying the loci for resistance, GWAS have also been widely used to understand other mechanisms of resistance (i.e., the emergence and progression of mutations) of different plant pathogens to various fungicides, including azoles, strobilurin (QoI), benzimidazole (MBC), and succinate dehydrogenase inhibitors (SDHI) [72-74]. GWAS have also been widely used to identify resistance mechanisms of human pathogens to clinical antifungal agents [75] (and references therein). Nevertheless, to the best of our knowledge, GWAS have not been performed to understand or predict resistance mechanisms of *A. pullulans* or environmental fungi to antifungals.

In an earlier study we reported CPN, CYP, and DFN sensitivity data for 30 *A. pullulans* strains [21]. Using these data as a resource, the present study's overall goal was to identify and characterize the tolerance mechanisms of *A. pullulans* to these three fungicides. Thus, this study set out: i) to sequence whole genomes of environmental and clinical *A. pullulans* strains and analyze their genetic relatedness; ii) to use GWAS and literature search to predict possible tolerance mechanism of *A. pullulans* to CYP, DFN, and CPN; iii) to express potential *A. pullulans* resistance genes in *S. cerevisiae* and; iv) to confirm the phenotypic functions of the expressed genes in *S. cerevisiae* through CYP sensitivity assays.

2 Material and Methods

2.1 Strains and cultivation

All strains used or generated during this study are listed in Supplementary Table 1-4. 30 environmental *A. pullulans* strains have been isolated and characterized the past [21]. The reference strain *A. pullulans* NBB 7.2.1 was isolated and characterised by genome, transcriptome and secretome analyses [76]. 16 clinical *Aureobasidium* strains have been obtained from Swiss hospitals or the Westerdijk fungal culture collection (Supplementary Table 4). Unless otherwise specified, the *Saccharomyces cerevisiae* strain used for transformation was BY4741 (MATa, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*) (Euroscarf collection). Other *S. cerevisiae* strains were deletion mutants created in the BY4741 background.

A. pullulans was maintained on potato dextrose agar (PDA; Difco; Chemie Brunschwig AG, Basel, Switzerland) at 22°C, while *S. cerevisiae* strains were cultivated at 30°C on plates containing yeast nitrogen base (YNB) without amino acids (6.99 g/L; Formedium, Norfolk, UK), complete supplement mix (CSM) (790 mg/L) (Formedium, Norfolk, UK), glucose (20 g/L; Difco; Chemie Brunschwig AG, Basel, Switzerland), and agar (20 g/L; Merck, Schaffhausen, Switzerland). Cultivation of *S. cerevisiae* transformants was done on YNB agar media lacking uracil (YNB-URA) agar. The liquid media for *S. cerevisiae* cultivation contained YNB without amino acids (6.99 g/L; Formedium, Norfolk, UK), CSM (790 mg/L) (Formedium, Norfolk, UK) and glucose (20 g/L; Difco; Chemie Brunschwig AG, Basel, Switzerland) (YNB broth media). Yeasts were preserved in 15% (v/v) glycerol stocks at -80 °C.

2.2 Genome sequencing

Genomic DNA extraction was performed as previously described [76]. Whole-genome sequencing (150 bp paired end reads) for the 30 environmental and 16 clinical *A. pullulans* isolates was done at BGI Genomics (Hong Kong, China). As a reference, the high-quality, annotated genome of *A. pullulans* NBB. 7.2.1 [76] was used and the genomes of *A. pullulans* EXF-150, *A. melanogenum* CBS 110374, *A. namibiae* CBS 147.97, and *A. subglaciale* EXF-248 were used for comparison [77-79] (all available at https://mycocosm.jgi.doe.gov).

2.3 Genome analyses

De novo genome assembly was performed with SPAdes by using the default options [80]. The resulting contigs can be found on our GitLab repository (Nägeli Lukas / *Aureobasidium*_GWAS GitLab (admin.ch) (2022)). A phylogenetic and molecular evolutionary (PHaME) analysis was carried out according to the developers instructions [81]. The contig files form SPAdes were used together with the complete genome files form JGI (both available on the GitHub repository Nägeli Lukas/*Aureobasidium*_GWAS·GitLab (admin.ch) with the corresponding control file to run PHaME). The analysis was performed twice. Once for all 46 isolates and 5 references and once only with isolates belonging to the species *A. pullulans* as described in the results. The phylogenetic tree was constructed using the fasttree method with all isolates and references. For literature predicted or characterised genes, the gene identity was confirmed using protein ID from Yeastmine (yeastgenome.org) or through CD search (NCBI). Specific genes of interest were identified by blast search against the high-quality annotated genome of *A. pullulans* (NBB 7.2.1)[76] available at Mycocosm (https://mycocosm.jgi.doe.gov/AurpulNBB1/AurpulNBB1.home.html).

2.4 STagenGWAS

A GWAS was performed using the statgenGWAS [82] package in R studio (V 4.2.0) [83]. Single nucleotide polymorphisms (SNPs) were collected from pairwise comparison to the reference genome of the *A. pullulans* strain NBB. 7.2.1. To account for the haploid genome of *A. pullulans*, SNPs were coded as if present in two alleles. SNPs were filtered for duplicates in genetic patterns over all isolates, removing those SNPs, which were already present in the same isolates. This resulted in a set of marker SNPs to be analysed in the GWAS. Minimum inhibitory concentration (MIC_{50}) values for three different fungicides (as described before [21]) were used as the phenotype and the GWAS was performed separately for CPN, CYP, and DFN. Genomic control was enabled to control the inflation factor. To ensure the evaluation of every marker separately, because of the clonal reproduction of *A. pullulans,* the size inclusion region was set to 0 and the minimum linkage disequilibrium in terms of squared Pearson correlation was set to 1. Multiple testing was accounted for with the default Bonferroni correction and all other parameters were also left at the default setting. The significant markers were then used to identify all significant SNPs within exons of annotated genes in the *A. pullulans* reference strain NBB. 7.2.1. Reproducible code including prognostic figures can be found on GitHub (Nägeli Lukas/Aureobasidium_GWAS · GitLab (admin.ch) (2022)).

2.5 RNA extraction and cDNA synthesis

Total RNA was extracted from *A. pullulans* cells using the RNeasy Mini RNA isolation kit (QIAGEN, QIAGEN AG, Hombrechtikon, Switzerland) following the manufacturer's protocol and eluted in 30 µL RNase free water. RNA was quantified with a Qubit Fluorometer using 3µl of RNA and Qubit RNA HS (high sensitivity) Assay Kit (Thermo Fisher, Fisher Scientific AG, Reinach, Switzerland). DNase I treatment was performed directly on the RNeasy columns with RNase-Free DNase Set (QIAGEN, QIAGEN AG, Hombrechtikon, Switzerland). DNA-free RNA was used to synthesize cDNA libraries using SuperScript® IV (SSIV) reverse transcriptase kit (Invitrogen™, Fisher Scientific AG, Switzerland) in accordance with the manufacturer's instructions. Briefly, 10 µL (15-450 ng) of total RNA was primed with 1 µL of 50 µM Oligo dT(20) (Invitrogen™, Fisher Scientific AG, Reinach, Switzerland) in a reaction volume of 13 µL for 5 min at 65°C, then 1 min at 4°C. This was followed by synthesizing the first cDNA strand using SSIV reverse transcriptase (200 U/µL) in the presence of an RNAase inhibitor and 100 mM DTT for 10 min at 55°C, followed by inactivation of the transcriptase at 80°C for 10 min. Removal of residual RNA and subsequent second cDNA strand synthesis was achieved by adding 1 µL *E. coli* RNase H (Invitrogen™, Fisher Scientific AG, Reinach, Switzerland) and incubating at 37°C for 20 min. cDNA was stored at -20°C.

2.6 PCR amplification.

cDNA of three *A. pullulans* strains (isolates F2 (CYP sensitive), LF 5.16 (CYP tolerant) and LC5.2 (CYP tolerant), were used as templates. Primers for subsequent cloning via Golden Gate were designed by adding 5'-GCATCGTCTCATCGGTCTCATATG-3' and 5'-ATGCCGTCTCAGGTCTCAGGAT-3' at the 5' end of each forward and reverse primer, respectively. The primers for USER cloning were designed by adding an eight-base sequence containing uracil (i.e., 5'-CGTGCGAU-3' or 5'-CACGCGAU-3') at the 5' end of each forward and reverse primer, respectively (all primers and oligos used in this study are reported in supplementary Table 5. PCR was performed with 20 mM of each primer, 3 µL cDNA, and Q5U (for USER cloning) or Q5 polymerase (for Golden Gate cloning) PCR kits (New England BioLabs (NEB), Bioconept AG, Allschwil, Switzerland), using a final reaction volume of 25 µL. PCR amplification was performed with a SensoQuest

thermocycler PCR system (Witec AG, Sursee, Switzerland). PCR conditions consisted of denaturation at 98°C for 1 min, followed by 35 cycles consisting of 98°C for 30 s, annealing for 30 s, and extension at 72°C for 30 s/kb, and a final extension at 72°C for 5 min. The annealing temperatures used for amplification of different genes ranged between 62°C to 72° and were adjusted depending on the specific primers as reported in Supplementary Table 5. As positive and negative controls, PCR amplification with genomic DNA and sterile water were included for all primers. Aliquots of the PCR product (5 μL) were analyzed by electrophoresis on 1% (wt/vol) agarose gels (Agarose NEEO ultra-quality, Carl Roth GmbH, Arlesheim, Switzerland) and stained with 0.0001% (vol/vol) ethidium bromide. PCR products were cleaned using QIAquick PCR purification kit (QIAGEN, QIAGEN AG, Hombrechtikon, Switzerland), eluted in 35 μl of elution buffer, and stored at -20°C.

2.7 Cloning

All oligonucleotides and plasmids used for both USER and Golden Gate cloning are listed in Supplementary Table 6. Unless otherwise specified, all cloning (digestion, ligation, nicking linearization) enzymes and their respective buffers were from NEB (Bioconept AG, Allschwil, Switzerland). The complete cloning approach, the oligonucleotides for the USER vector, and all the constructed USER vectors for this study are described and graphically illustrated in the supplementary data (Supplementary Figure 1). All plasmids generated during this study were verified by Sanger sequencing (Microsynth AG, Balgach, Switzerland).

2.7.1 USER cloning

A USER compatible oligonucleotide cassette comprising the AsiSI and Nb.BsMI recognition sequences was cloned into the vector pYTK001 (Plasmid 1) (Supplementary Table 6). The verified USER_L1 cassette (Plasmid 6) was linearized by digesting 1-2 μg of the plasmid with 1 U AsiSI (37°C, 8 h, and reaction volume of 50 μL). This was followed by nicking with 1 U Nb.BsMI (65°C, 3 h) and heat inactivation (80°C, 15 min). The linearized vector was cleaned with the QIAquick PCR purification kit (QIAGEN, QIAGEN AG, Hombrechtikon, Switzerland) and stored at -20°C. USER II enzyme treatment was performed with 10-100 ng of PCR product, 1 μg of linearised USER_L1 vector, and 1 U USER enzyme mix (37°C, 25 min), followed by inactivation (80°C, 15 min). The entire reaction was transformed into 25 μl of chemically competent *Escherichia coli* (DH5*α*) cells by heat shocking for 90 s at 42°C, followed by 5 min on ice and directly plating on Ampicillin (100 μg/ml) amended Luria broth (LB) plates and incubation overnight at 37°C. Positive clones were identified by colony PCR using cells from eight different colonies (resuspended in 20 μL of sterile water). 4 μL of these suspensions were used as PCR templates and amplified using hot start Taq polymerase (QIAGEN, QIAGEN AG, Hombrechtikon, Switzerland) with gene-specific primers (Supplementary Table 5). The SensoQuest thermocycler PCR system (Witec Ag, Sursee, Switzerland) was used for amplification under these conditions: denaturation at 95°C for 15 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 58°C for 45 s, elongation 72°C for 30 s/kb, a final extension 72°C 10 min.

2.7.2 Golden Gate Cloning

The pYTK001 entry vector (Plasmid 1) was cloned into the intermediate vectors (Plasmid 7, 8 and 9) as follows. 1 μl PCR product (1.5-15 ng) and pYTK001 (100 ng) were simultaneous digested and ligated with 1μl T4 ligase buffer, 0.5 μl of BsmBI and 0.5 μl T4 DNA ligase in a reaction volume of 10 μl. The reaction condition consisted of 30 cycles of digestion at 42°C for 2 min and ligation at 16°C for 5 min, followed by 10 min inactivating at 60°C and 80°C, using the SensoQuest thermocycler PCR system (Witec Ag, Sursee, Switzerland). Then, 2 μl of

the ligation was transformed into 25 µl of chemically competent *E. coli* (DH5α) cells by heat shocking for 30 s at 42°C, followed by 5 min on ice, then, 1h recovery in 150 µl of LB at 37°C with moderate shaking (750 rpm). After recovery, 100 µl of the suspension was plated on chloramphenicol (25 µg/ml) amended LB plates and incubated overnight at 37°C. Positive clones were identified by picking white clones under a UV lamp (Hoefer Benchtop darkroom).

The genes in the level 0 vectors (Plasmid 7, 8 and 9) and the synthetic *GST* genes (ordered from TWIST Bioscience, San Francisco, USA) were cloned into integration vectors (Plasmid 11-15) by Bsa 1 assembly. Briefly, 1µl (approx. 100 ng) of the plasmids containing the promoter (Plasmid 2), the terminator (Plasmid 3), vector backbone with integration cassette (Plasmid 4) and gene of interest (Plasmid 7-9) were simultaneous digested and ligated using 1 µl T4 ligase buffer, 0.5 µl each of Bsa1, and T4 DNA ligase in a final volume of 10 µl. The ligation products were subsequently transformed into chemically competent *E. coli* (DH5α) and recovered as described above. 100 µl of the suspension after recovery was plated on Kanamycin (50 µg/ml) amended LB plates and incubated overnight at 37°C. Positive clones were identified by picking white clones as described above.

Overnight cultures (2-4 ml) of the positive colonies from each cloning step were pelleted and the pellets cleaned using a QIAGEN Plasmid Mini (QIAGEN AG, Hombrechtikon, Switzerland) according to the manufacturer's instructions, eluted in 35 µL of elution buffer, and the concentrations determined using a Nano drop oneC, UV spectrophotometer (Thermo Scientific, Witec Ag, Surisee, Switzerland). The plasmids with the genes of interest (plasmid 11-15) were verified by sequencing the promoter and terminator borders located 200-500 bases from the gene insertion site using primers pTHD1f and tTDH1r, respectively (Supplementary Table 5). Sequence trace files were examined for quality and manually trimmed in Benchling (Benchling Biology software [84]). The resulting sequence was assembled and compared to the corresponding *A. pullulans* genome sequence using Geneious (Vs 2021.2.2).

2.8 Transformation of *S. cerevisiae*

Integration vectors (Plasmid 11-31(Supplementary Table 6)) containing two Not I recognition sites were digested with Not I and aliquots (5 µL) were analyzed by electrophoresis on a 1% (wt/vol) agarose gel (Agarose NEEO ultra-quality, Carl Roth GmbH, Switzerland). Linearized plasmids with URA3 homologous sequences at both ends were transformed into competent cells of *S. cerevisiae* strains BY4741 or BY4741 deletion strains (i.e., Δ*car1*, Δ*car2*, Δ*arg2*, Δ*met2*) using a lithium acetate/single-stranded (LiAc/SS carrier) DNA/PEG method [85] The transformants were selected on YNB-URA plates. Gene integration was confirmed by colony PCR as earlier recommended [86] using primer pairs URA3 5'_F/URA3 5'_R and URA3 3'-F/ URA 3 3'_R (Supplementary Table 5).

2.9 Microbroth sensitivity assays for *A. pullulans* and *S. cerevisiae*

The microbroth sensitivity assays of 16 clinical isolates (Supplementary Table 4) with CYP, CPN and DFN and MIC_{50} (minimum inhibitory concentration causing reduction in growth to 50%) values were determined as previously described [21]. Similarly, microbroth sensitivity assays of *S. cerevisiae* transformants (Supplementary Table 3) to CYP and their MIC_{50} were determined as previously described protocol [21] with minor adjustments (i.e., only seven different fungicide concentrations were used (4, 8, 16, 32, 64, and 128 µg/ml)). A 20x concentrated stock (10.24 mg/mL) of technical-grade CYP (Sigma-Aldrich Chemie, Schweiz,

Buchs, Switzerland) was prepared and 2x final concentrations were obtained by serial dilution (1:2) with methanol. As a control 5% methanol (2x the final concentration) was used. A 2x YNB broth medium was used to dilute (1:2) the 2x fungicide solutions or 5% methanol. The end point OD_{600} was measured after 24 hours using a microplate reader [21].

Growth curves of *S. cerevisiae* transformants were determined with a final CYP concentration of 16 µg/mL. Assays were performed in a flat bottomed 96-well plate (total volume of 100 µL per well, with all concentrations in triplicate). The optical density of overnight cultures of yeast (grown in the 3 ml of the medium above at 30°C, 200 rpm) was determined at 600 nm using a spectrophotometer (GE Healthcare NovaspecTM III), and yeast suspensions with final OD_{600} of 0.1 were prepared. 10 µL of the yeast suspension (OD_{600} 0.1) was used to inoculate the 100 µL medium/fungicide solution. The growth kinetics were determined by measuring the OD_{600} every 3 h (for 24 h) using a microplate reader (Spark®, Tecan Life Science AG, Männedorf, Switzerland). Each experiment was repeated at least three times.

2.10 Spot assays with *S. cerevisiae* deletion mutants

Spot assays with *S. cerevisiae* deletion mutants were carried out on YNB media without CYP or amended with technical grade CYP (16 and 32 µg/mL final concentrations). Yeast suspensions were adjusted to an OD_{600} of 1 and serially diluted (1:10) with water in a 96-well plate. A 96 pin tool was used to stamp the cells on the YNB plates, which were then incubated at 30°C for 5 days. Colony growth on the different plates was evaluated.

2.11 Statistical analysis

The MIC_{50} of the *S. cerevisiae* strains were determined as before [21] using Graphpad prism (V9), with the calculation of 95% confidence interval (CI) by likelihood profiles to determine the significant differences where, an overlap of a 95% CI with the MIC_{50} of control strain indicated no statistical difference [87, 88]. The MIC_{50} of the clinical isolates were compared to that of previously determined MIC_{50} of environmental isolates using the package in R studio (V 4.2.0).

3 Results

3.1 Some clinical *A. pullulans* isolates cluster with other *Aureobasidium* species and have low tolerance to fungicides

We have previously characterised and classified 30 environmental *A. pullulans* strains into categories of sensitive (S), intermediate (I) and high tolerance (T) to CPN, CYP, and DFN [21]. Here, we complemented this strain collection with 16 clinical isolates (from Swiss hospitals and culture collections; Supplementary Table 4) and generated short-read genome data for all 46 strains. After *de novo* assembly, the PHaME pipeline [81] was used to construct a phylogenetic tree using the fasttree method in order to probe the relationship among these strains and to test if they clustered based on their origin and fungicide sensitivity.

Seven isolates from clinical samples (i.e., CBS 626.85, CBS 298.56, CBS 699.76, CBS 121327, CBS 101119, CBS 577.93, and IFIK 1931366) clustered together with the non-*pullulans* reference genomes of *A. melanogenum*, *A. subglaciale* and *A. namibiae* and exhibited a large relative distance to all other isolates (Figure 1, cluster 1). These seven isolates likely belong to the species *A. melanogenum* than to *A. pullulans*. Since large genetic distances can pose a problem for a GWAS without adjusting for population structure, these seven distantly related isolates were excluded from the further analyses. Among the *A. pullulans* isolates, five additional

clusters were drawn according to the relative genetic distance. CBS 121328 (sampled from a child's ear canal) exhibited the largest distance (Figure 1, cluster 3). Except for CBS 121328, all clinical *A. pullulans* isolates formed a single cluster together with *A. pullulans (EXF-150)* (Figure 1, cluster 4). Cluster 2 comprised environmental isolates that were very closely related to our reference isolate NBB 7.2.1. Generally, isolates of this cluster showed low resistance to all fungicides tested (Figure 1 andFigure 2, shown in blue). Interestingly, environmental *A. pullulans* strains with intermediate and low sensitivity to all the three fungicides each formed a separate cluster (cluster 6 and 5, respectively). Overall, only nine of the 16 clinical isolates likely belong to the species *A. pullulans* and the environmental isolates seemed to cluster based on their fungicide sensitivity.

The isolates grouped together in the SNP-based phylogeny showed marked differences in their sensitivity for the three fungicides CPN, CYP, and DFN (Figure 2). The clinical isolates, irrespective whether or not the isolates were *A. pullulans* or another *Aureobasidium* species, generally showed lower MIC_{50} values, in particular for CPN and DFN, as compared to environmental isolates (Figure 2). Additionally, the MIC_{50} comparison of the 16 clinical *Aureobasiudium* and 30 environmental *A. pullulans* using T-test showed a significantly lower MIC_{50} of the clinical than environmental isolates in all three fungicides (Supplementary Figure 2).

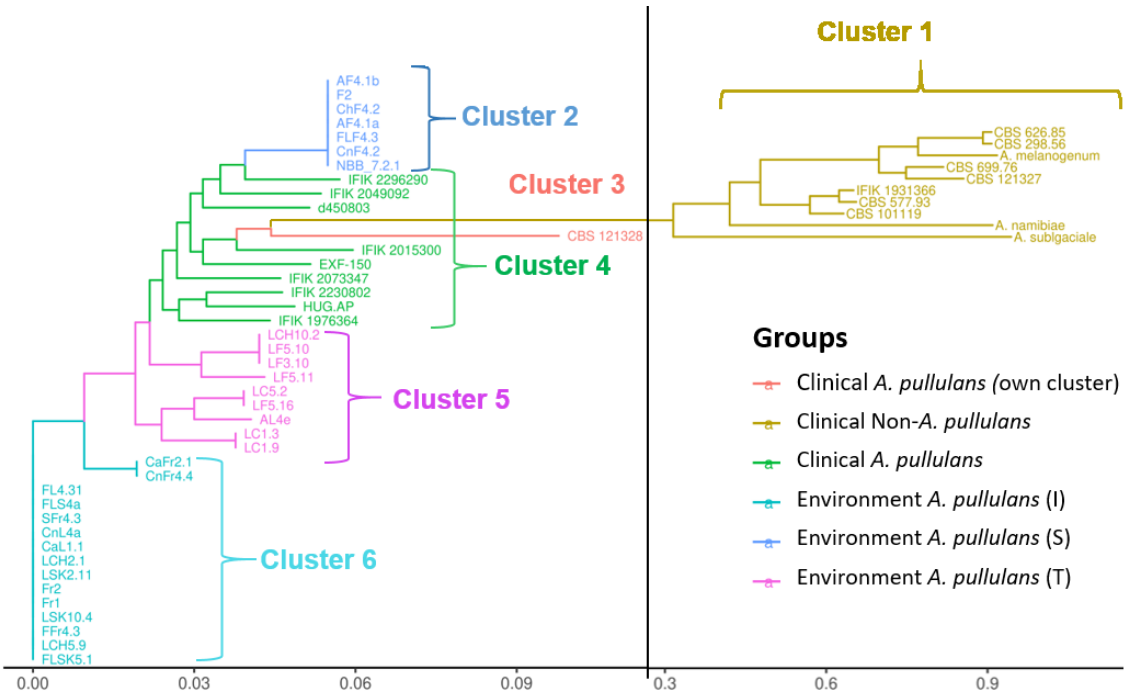

Figure 1. SNP-based phylogeny in whole genomes of 30 environmental *A. pullulans* and 16 clinical *Aureobasidium* strains. The analysis included whole genomes of 5 other *Aureobasidium* published elsewhere, *A. pullulans* (NBB 7.2.1), *A. pullulans* (EXF-150), *A. namibiae*, *A. melanogenum*, and *A. sublgaciale* [76, 89]. The X–axis represents the relative distances between one group and the next.

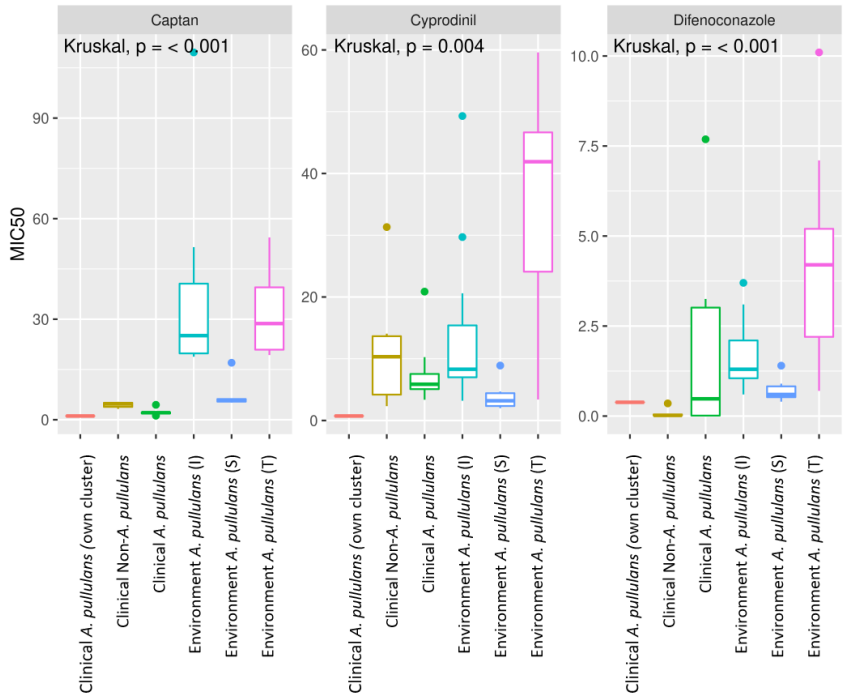

Figure 2. Boxplot of the MIC_{50} values for each cluster derived from the phylogenetic tree and all three fungicides tested. The three environmental clusters generally with the fungicide sensitivity profiles (i.e. (blue (sensitive), turquoise (intermediate) and pink (resistant). Generally, the clinical isolates show higher sensitivity especially with CPN.

In conclusion, this data show that the differences in sensitivity were also linked to genetic differences among the clusters. Overall, the clinical *Aureobasidium* strains showed higher sensitivity to CPN, CYP, and DFN, than environmental *A. pullulans* isolates. Further, knowing the genetic relationship of the clinical isolates to environmental strains allows us to select nine clinical isolates which cluster with *A. pullulans* for further analysis and experiments to understand fungicide tolerance mechanisms.

3.2 Phylogenetic and molecular evolutionary (PHaME) analysis of *A. pullulans* isolates

Based on the SNP-based phylogenetic assessment, 39 *A. pullulans* isolates were selected for further analyses in order to identify SNPs that might be responsible for tolerance to CPN, CYP, or DFN. The genome and sensitivity data for these 39 *A. pullulans* isolates were thus analysed by PHaME.

In the 39 isolates and in comparison to the reference genome of the isolate NBB 7.2.1, a total of 2'107'600 SNPs were detected, of which 882'994 were in coding DNA sequence (CDS). The core genome had a total length of 22'925'863 bp, which corresponds to 81% of the reference genome length of the isolate NBB 7.2.1. The SNPs were distributed throughout the whole genome with a tendency of increased SNP numbers at the chromosome ends and in the shorter chromosomes (Figure 3). For example, chromosomes 11 and 12 had a higher SNP density than other chromosomes. Similarly, the coverage was lower towards the ends of the chromosomes and particularly chromosome 8 had a lower coverage for most isolates (Figure 3).

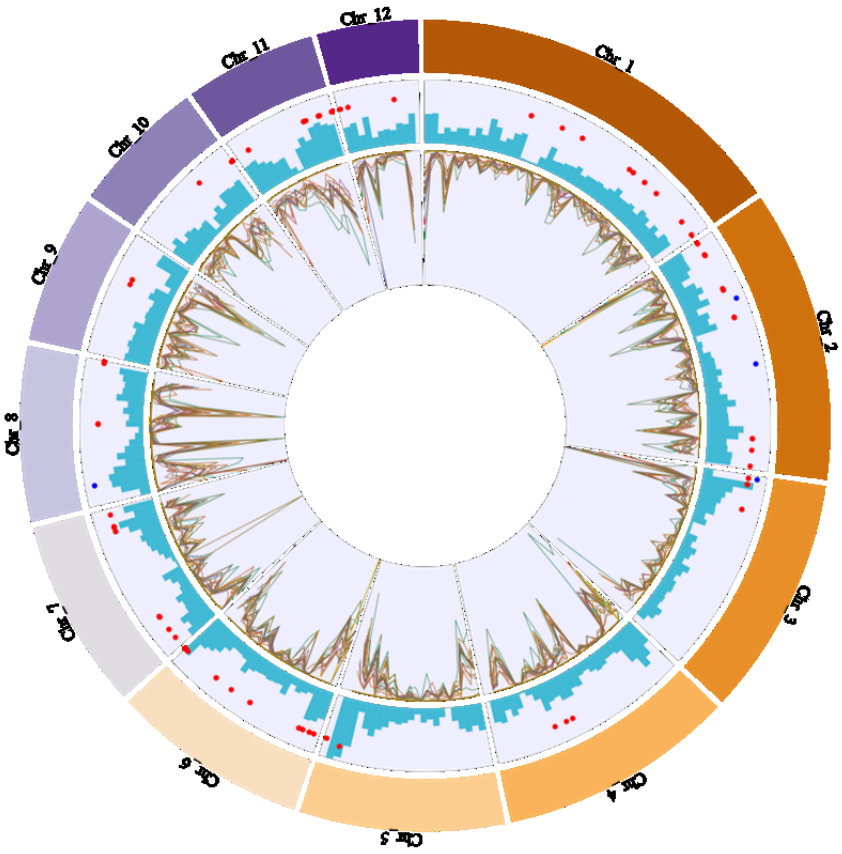

Figure 3. Circos plot showing the 12 chromosomes of *A. pullulans* from the reference strain NBB 7.2.1. The barplot shows the relative number of different SNPs within 100 kb in the specific regions. The points indicate relevant SNPs for CYP (red) and CPN (blue) tolerance according to Table 1. SNPs unique to AL4e are not indicated with points. The innermost circle shows coverage of each of the 39 isolates (each with a different colour) on the reference genome. Lines close to the center of the plot indicate low coverage.

3.3 GWAS identifies genes potentially involved in CPN and CYP sensitivity in *A. pullulans*

To identify genes potentially involvement in tolerance to CPN, CYP and DFN, a GWAS was performed with the genome and fungicide sensitivity data of the 39 *A. pullulans* isolates. All SNPs in the 39 isolates were used to identify markers; specific SNP occurrence patterns in the 39 isolates. Each marker thus had a specific pattern of presence and absence in the 39 isolates and all SNPs with the same pattern of occurrence share that specific marker.

From all SNPs, 66'087 markers with a different pattern of occurrence in the 39 isolates were selected. Among these, no significant marker was found for DFN. The GWAS with the CPN and CYP sensitivity data identified one and two significant markers, respectively (Table 1). The marker for CPN ($M1^{CPN}$) comprised 67 SNPs that were only present in the isolate FLS4a, which had a MIC_{50} value of 109.6 µg/mL (the highest value in CPN). When only considering SNPs present in exons of annotated genes (based on the NBB 7.2.1 reference genome), only 11 SNPs were unique to FLS4a and only five led to an amino acid change in the protein of four genes (Table 1). For GWAS with the CYP sensitivity data, the isolates AL4e and LC 5.2 with high MIC_{50} values (beyond the concentrations used for the experiments) were excluded. However, the precence or absence of a particular marker in these two isolates was recorded.

Two markers were identified as significant in the CYP analysis ($M1^{CYP}$and $M2^{CYP}$) (Table 1). $M1^{CYP}$ comprised SNPs only present in the isolates LSK 2.11, LF 5.16, LC 1.9 LC 1.3 and LC 5.2, while $M2^{CYP}$ was found in the same five isolates and AL4e. A total of 53 SNPs (14 of which were also present in AL4e) had identical patterns to these markers (Table 1). Only two of the 53 SNPs were located in CDS regions (one SNP for each marker) and both of these lead to an amino acid change.

Interestingly, 1'777 SNPs were unique to LC 5.2 and 272 of these SNPs led to amino acid changes in 64 proteins. Similarly, 26'112 SNPs were unique to Al4e, of which 4'658 led to an amino acid change in 1'692 proteins. Additionally, there were 67 SNPs unique to both LC 5.2 and Al4e, of which 23 led to amino acid changes in nine proteins (Table 1). These SNPs unique for LC 5.2 and AL4e were not assigned to any markers, but were still of interest because of the multiple significant SNPs within 73 genes (asterics in Table 1 (all the 73 genes found in Supplementary Table 10). The different SNPs within these genes and genes unique only to AL4e are available online (Nägeli Lukas / Aureobasidium_GWAS GitLab (admin.ch). Alltogether, our GWAS with the CYP MIC_{50} data reported 28'009 SNPs. 4'955 of these lead to amino acid changes in 1'767 proteins (Table 1).

Table 1. Significant SNPs found by the GWAS with the genome and fungicide sensitivity data for 39 *A. pullulans* isolates. Separate GWAS for the fungicides CPN, CYP, and DFN were performed

[a]Marker	Isolates with unique SNPs	Fungicide	Total Nr. of SNPs	CDS SNPs	SNPs leading to AA change	Gene(s) with AA change	[b]Example of genes (ID)
$M1^{CPN}$	FLS4a	Captan	67	11	5	4	ID 41311
$M1^{CYP}$	LSK2.11, LF5.16, LC 1.9, LC 1.3, LC 5.2	Cyprodinil	39	1	1	1	ID 86174
$M2^{CYP}$	LSK 2.11, LF 5.16, LC 1.9, LC 1.3, LC 5.2, AL4e	Cyprodinil	14	1	1	1	ID 63243
*	LC 5.2	Cyprodinil	1777	346	272	64*	41311, 73510 78168, 86861
*	LC 5.2 and AL4e	Cyprodinil	67	30	23	9*	73510
	Al4e	Cyprodinil	26112	7960	4658	1692[1]	Many[c]

a. Markers found with significant pattern: one marker for MIC_{50} CPN (^{M1}CPN); two markers for for MIC_{50} CYP ($M1^{CYP}$and $M2^{CYP}$)

b. Example of the genes with significant markers that were experimented on

c. The many genes with significant SNPs only for isolate Al4e with the complete gene list available online (Nägeli Lukas / Aureobasidium_GWAS · GitLab (admin.ch))

1. All SNPs only uinque to the strain Al4e found to associate with tolerance in CYP

*SNPs that were only unique to LC 5.2 alone or both LC 5.2 and Al4e (strains with the highest MIC_{50}) but, not present in any other isolates.

AA, amino acids; Nr, number

Some of the genes that harbored SNPs with significant patterns were selected for a more detailed analysis. A glucose substrate transporter (GST; protein ID 41311) had a unique and significant mutation in FLS4a for CPN. This gene also had two mutations unique to LC 5.2 and might be relevant in CYP tolerance. A F-box domain protein (FBD) (Protein ID 43145) had several significant SNPs in the tolerant isolates. A protein kinase (HP5) (Protein ID 73510) had five mutations that were unique to Al4e and LC 5.2 and nine mutations that were unique to LC 5.2. In both cases, the SNPs caused one amino acid change. Similarly, two hypothetical proteins HP6 (Protein ID 78168) and HP8 (Protein ID 86861), had six and ten mutations unique to LC 5.2, respectively. The proteins HP5, HP6, and HP8 contained about 10% of all the unique SNPs for LC 5.2 that led to an amino acid change. Interestingly, none of those genes have homologs in *S. cerevisiae*.

Overall, this GWAS did not uncover any SNP pattern significantly correlating with tolerance to DFN. However, one and two markers significantly correlated with sensitivity to CPN and CYP, respectively. These three markers comprised 67 and 53 SNPs and in total seven lead to an amino acid change in the corresponding protein. Additionally, genes with SNPs only present in the isolates AL4e and/or LC 5.2 might also be of interest, because these two isolates had high MIC_{50} values for CYP. Taken together this identified 73 *A. pullulans* genes that are potentially involved in mediating CPN or CYP tolerance and an addition of 1692 genes that have unique mutations for AL4e. Noteably, due to the high number of SNPs associated with the MIC_{50} for CYP as compared to the other two fungicides, further investigations including experimental work was done to understand tolerance of *A. pullulans* only to CYP.

3.4 Known CYP resistance genes did not have significant SNPs from our GWAS

In general, the mode of action of and resistance to CYP and anilinopyrimidines (APs) are not well understood. However, nine genes encoding mitochondrial protein functions were previously found to be responsible for resistance to APs in *B. cinerea.* These include *MCR1, MIX17, DNM1, ATM1, POS5, AFG3, PHB2, MDL1,* and *OLIC (ATP9)* [62]. *A. pullulans* has homologs of these nine genes, but none of the 4955 significant SNPs predicted by our GWAS analysis to be involved in CYP tolerance caused a mutation in any of these nine genes (see Supplementary Table 7). Thus, the potential CYP resistance genes identified by the GWAS analysis reported here point to a different and new mechanism of CYP tolerance.

Additionally, 23 genes had been predicted to be involved in APs resistance based on their expression patterns upon APs treatment [63]. Similarly, there were no significant SNPs found for these 23 genes. Although not containing significant SNPs based on our analysis, genes already predicted to be involved in CYP resistance may still harbor mutations that render strains more or less sensitive to CYP. We therefore extracted and compared the DNA sequences of the 23 genes from a sensitive (F2) and tolerant (LC5.2) *A. pullulans* strains (Supplementary Table 8). Our sequence comparisons of the sensitive and tolerant isolates of *A. pullulans* observed that some genes (*ARG2, CAR1, CAR2, CPA2, ARG8* and *MET2*) had SNPs that resulted in amino acid changes in the corresponding proteins (Supplementary Table 8). Thus, it was still interesting to understand the phenotypic functions of these four genes.

3.5 CYP predicted genes from literature might have an effect on CYP tolerance

Ten genes previously predicted to be involved in CYP resistance (i.e., *ARG2, ARG5,6, ARG3, ARG8, CAR1, CAR2, CPA2, MET32, MET10,* and *MET2*; Supplementary Table 8) contained different mutations in the alleles of the sensitive and tolerant strains. Of these genes, some could not be amplified because they were too long. For some genes, preliminary screening by comparing the CYP sensitivity of the deletion strain and the wild-type (WT) did not show any difference. Therefore, only four of these genes (*CAR1, CAR2, ARG2,* and *MET2*) were further investigated.

To test if the different alleles from sensitive (s) and tolerant (t) strains were responsible for CYP tolerance or sensitivity, the *CAR1, CAR2, ARG2,* and *MET2* genes were heterologously expressed in *S. cerevisiae* and the resulting strains were assessed for CYP tolerance. The gene alleles were intergrated at the *ura3* locus of the deletion strains of the respective genes. These transformations generated *S. cerevisiae* strains in which the *A. pullulans* homologs replaced the corresponding *S. cerevisiae* genes (in this study, they will be referred to as (*ApCAR2-s*, *ApCAR2-t*, *ApCAR1-s*, *ApCAR1-t*, *ApARG2-s*, *ApARG2-t*, *ApMET2-s*, *ApMET2-s*).

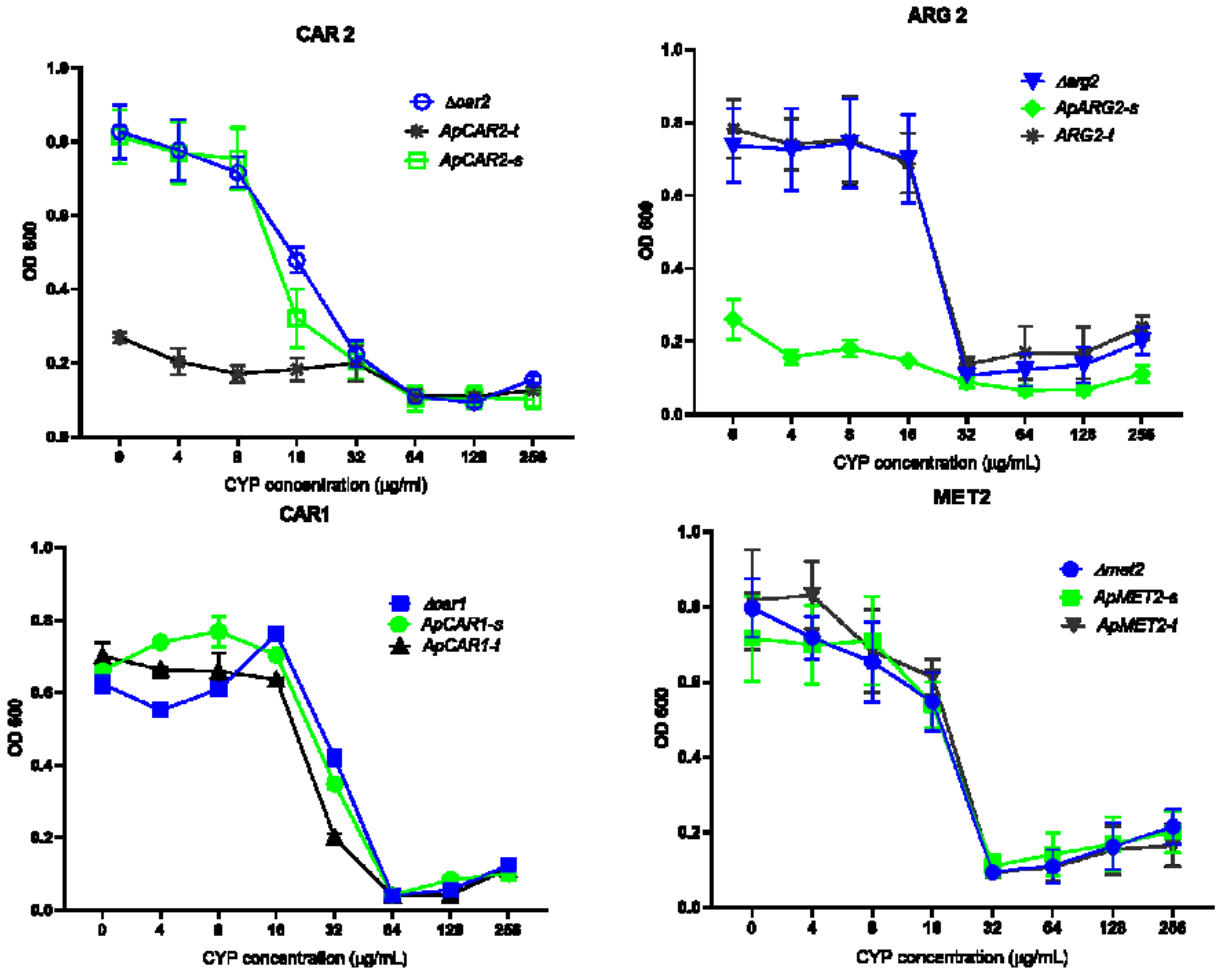

Figure 4. CYP dose response curve for *S. cerevisiae* strains transformed with *A. pullulans* gene homologs identified by literature search. *A. pullulans* homologs of *S. cerevisiae CAR2*, *CAR1*, *ARG2*, and *MET2* genes were expressed in *S. cerevisiae*. A sensitive (s) and tolerant (t) allele of each gene was included, and microbroth assays with CYP were performed. The endpoint OD_{600} for each concentration was determined after 24 h of growth. The curve was generated based on the mean OD_{600} of three independent experiments with three replicates and their 95% CI.

We observed growth differences in the presence of CYP for the *S. cerevisiae* strains containing the *A. pullulans ARG2* and *CAR2* genes and their respective control strain, while strains transformed with *MET2* and *CAR1* showed no difference (Figure 4). The *ApCAR2-s* strain grew comparably to the Δ*car2* strain in the presence of CYP and under control conditions (media without CYP). Contrarily, the *ApCAR2-t* strain containing the tolerant allele grew poorly, both with and without CYP. Interestingly, the *ApARG2-t* strain, showed higher tolerance to CYP (up to 16 µg/ml) than *ApARG-s*, but had a comparable growth as the Δ*arg2* control strain. Notably, the *ApARG-s* strain also showed generally reduced growth, even without CYP.

Overall, the *CAR2* allele from the tolerant strain and *ARG2* allele from the sensitive strain caused differences in the general growth and tolerance to CYP as compared to control. Particulary, the t allele of *ARG2* conferred a growth advantage and CYP tolerance phenotypes compared to the s allele. Thus, the mutations in the *ARG2* gene in the tolerant *A. pullulans* isolates might make these strains generally more competitive, leading to CYP tolerance in a non-targeted manner.

However, we could not explain the phenotype observed for the *CAR2* alleles. Thus, we additionally transformed a t and s allele of the *A. pullulans CAR2* gene into the WT *S. cerevisiae* strains to generate strains

where both the *S. cerevisiae CAR2* and *A. pullulans* CAR2 genes were present. The analysis of CYP sensitivity of the generated strains observed poor growth in both the transformed strains (containing either t or s alleles) under control and in the presence of CYP (Supplementary Figure 3). Still, this could not explain the differences in the t and s alleles of *A. pullulans*. Interestingly, the Δ*car2* strain was less sensitive to CYP compared to the WT, implying the involvement of this gene in CYP tolerance. Without further experiments and controls it is not possible to interpret the phenotype observed for *CAR2* gene. In particular, *CAR2* gene expression levels could be examined to explain the phenotype.

3.6 *S. cerevisiae* homologs of *A. pullulans* genes identified by GWAS affect CYP tolerance

Most of the significant SNPs identified by our GWAS affected unknown proteins without a homolog in model fungi. However, 86 of the identified genes had a predicted function. Therefore, we searched homologs of these genes in the *S. cerevisiae* genome, assessed if they are implicated in fungicide resistance, and tested deletion mutants for CYP sensitivity. Altogether, 23 *S. cerevisiae* homologs with an e-value < 10^{-10} and that corresponded to *A. pullulans* genes with significant SNPs based on our GWAS were identified (Supplementary Table 9). Of those genes, five were essential, and one represented a mitochondrial gene. Deletion mutants of the remaining 17 genes were therefore tested for CYP sensitivity in spot assays.

Under control conditions (absence of CYP), all the strains grew comparably to the WT, except for the Δ*sfp1* strain, which exhibited impaired growth. Of the 17 *S. cerevisiae* deletion strains, 13 also grew comparably to the WT in the presence of CYP (Figure 5). However, four strains (i.e., Δ*lys2*, Δ*eno1*, Δ*hol1*, Δ*cac2*) showed tolerance to the fungicide, whereas the Δ*pdb1* and Δ*stl1* strains were more sensitive to CYP than the wildtype and already inhibited at 16 μg/ml CYP.

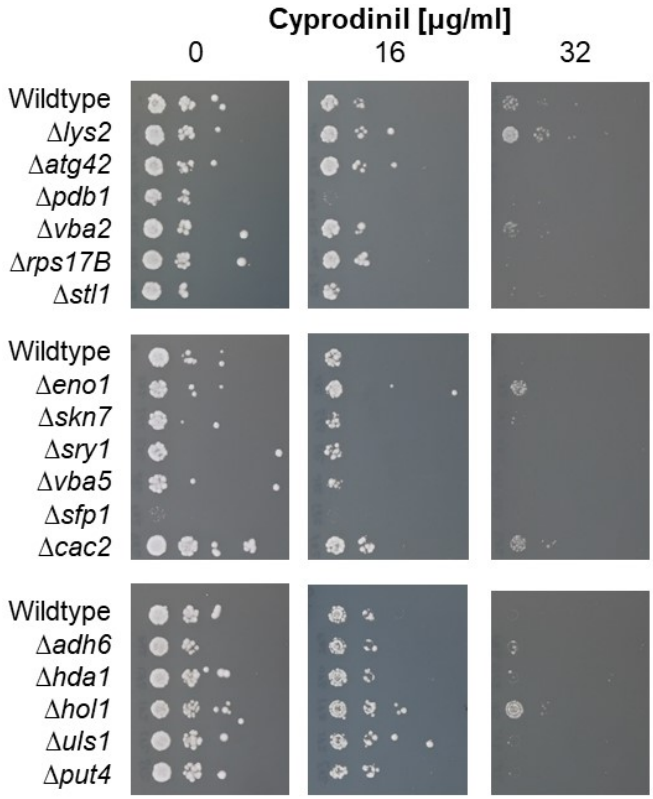

Figure 5. Agar spot assay of *S. cerevisiae* deletion strains of 17 *S. cerevisiae* genes homologous to *A. pullulans'* GWAS identified genes. Colony growth of the WT was compared to that of the deletion strains in the absence and in the presence of CYP.

The *LYS2*, *ENO1*, *HOL1*, and *CAC2* genes deleted in the strains with higher tolerance to CYP are homologs of *A. pullulans* genes with SNPs unique to the isolate LC5.2. In *A. pullulans*, *HOL1* and *ENO1* had each one significant SNP leading to an amino acid change, while *LYS2* had six such changes (Table 2). Interestingly, *ΔPDB1* had no significant marker SNPs that also change an amino acid. In contrast, *A. pullulans STL1* had two SNPs unique to the isolate LC 5.2 and one SNP unique to FLS4a and thus might be involved in both resistant mechanisms to CYP and captan. These five genes had diverse functions in *S. cerevisiae,* including glycerol proton symporter *(STL1*), putative transporter member of major facilitator superfamily (*HOL1),* Chromatin Assembly Complex (*CAC2*), biosynthesis of lysine (*LYS2*), and glycolysis and gluconeogenesis (*ENO1*).

Table 2. The five *S. cerevisiae* homologous to *A. pullulans* GWAS-identified genes with a significant effect on CYP tolerance based on spot assay

Saccharomyces gene	Name	Function in *S. cerevisiae*	Amino acid change in resistant isolate
STL1	Sugar transporter-like protein	Glycerol proton symporter of the plasma membrane.	2
HOL1	HistidinOl	Putative transporter in the major facilitator superfamily.	1
LYS2	Lysine requiring (Alpha aminoadipate reductase)	Biosynthesis of lysine.	6
CAC2	Chromatin assembly complex	Subunit of chromatin assembly factor I (CAF-1), with Rlf2p and Msi1p	5
ENO1	Enolase I, a phosphopyruvate hydratase	Involved in glycolysis and gluconeogenesis.	1

Overall, our GWAS with *A. pullulans* genome and fungicide sensitivity data identified five genes that improve or decrease CYP tolerance when deleted in *S. cerevisiae*. Thus, these genes may function to confer CYP tolerance in *A. pullulans.*

3.7 GWAS-identified *A. pullulans* genes confer CYP tolerance to *S. cerevisiae*

The majority of the *A. pullulans* genes with significant SNPs encode unknown proteins or did not have a homolog in *S. cerevisiae*. To functionally test if some of these genes are indeed involved in fungicide tolerance, five such genes (FBD (Protein ID 43145), *HP5* (Protein ID 73510), *HP6* (Protein ID 78168), *HP8* (Protein ID 86861), and *GST* (Protein ID 41311)) were heterologously expressed in *S. cerevisiae* and the fungicide sensitivity of the resulting strains was assessed.

All *S. cerevisiae* strains containing an *A. pullulans* gene had a growth advantage in the presence of 16 μg/mL CYP and showed significantly higher OD_{600} values at the 24 hour timepoint as compared to the control strain (Figure 6). This effect was not due to generally improved growth, as all the strains grew comparably in the absence of CYP (i.e., there was no significant growth difference between the control strain and the transformants) (Figure 6).

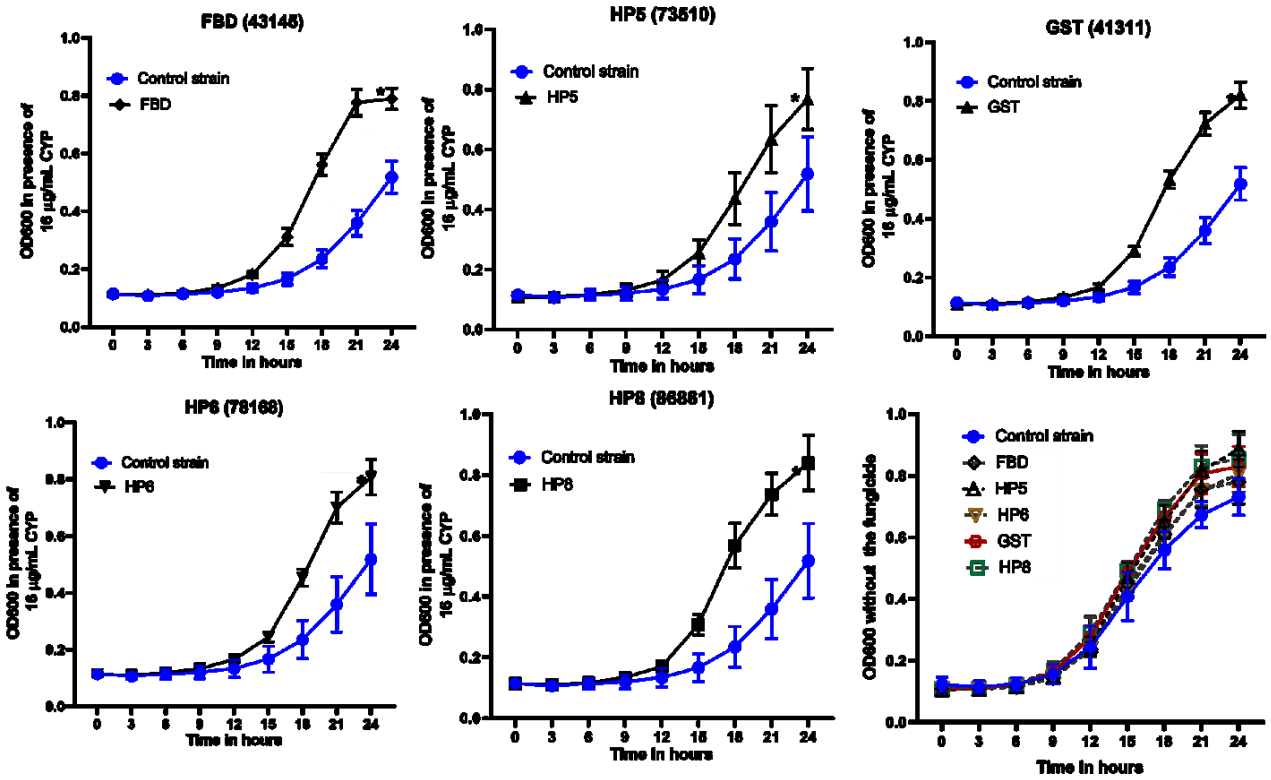

Figure 6. The growth curve in the presence of 16 µg/mL CYP of *S. cerevisiae* strains transformed with *A. pullulans* genes identified by GWAS. The curve was generated based on the mean OD_{600} of three independent experiments with three replicates and their 95% CI. *Means the curve was statistically significant from the control at 24 hours based on the 95% CI.

Microbroth sensitivity assays with different CYP concentrations allowed calculating the MIC_{50} of the *S. cerevisiae* strains expressing the different *A. pullulans* genes. The endpoint analysis after 24 hours of growth indicated lower OD_{600} values for the control strain (especially for concentration from 8 µg/ml upwards) compared to the transformants (Figure 7). Under these assay conditions, the control strain had a MIC_{50} value of 6.6 µg/mL, while this value ranged from 16 to 21 µg/mL for the *S. cerevisiae* strains expressing the five *A. pullulans* genes from isolate LC 5.2 and LF 5.16 (Table 2). Based on the 95% confidence intervals (CI), the MIC_{50} values of the strains harbouring the *A. pullulans FBD, GST, HP5, HP6*, and *HP8* genes were significantly higher than for the control (Table 3).

Based on these results, the five *A. pullulans* genes tested here have a role in CYP tolerance since their transformation into *S. cerevisiae* resulted in tolerance to CYP and significantly higher MIC_{50} values than for the control strain.

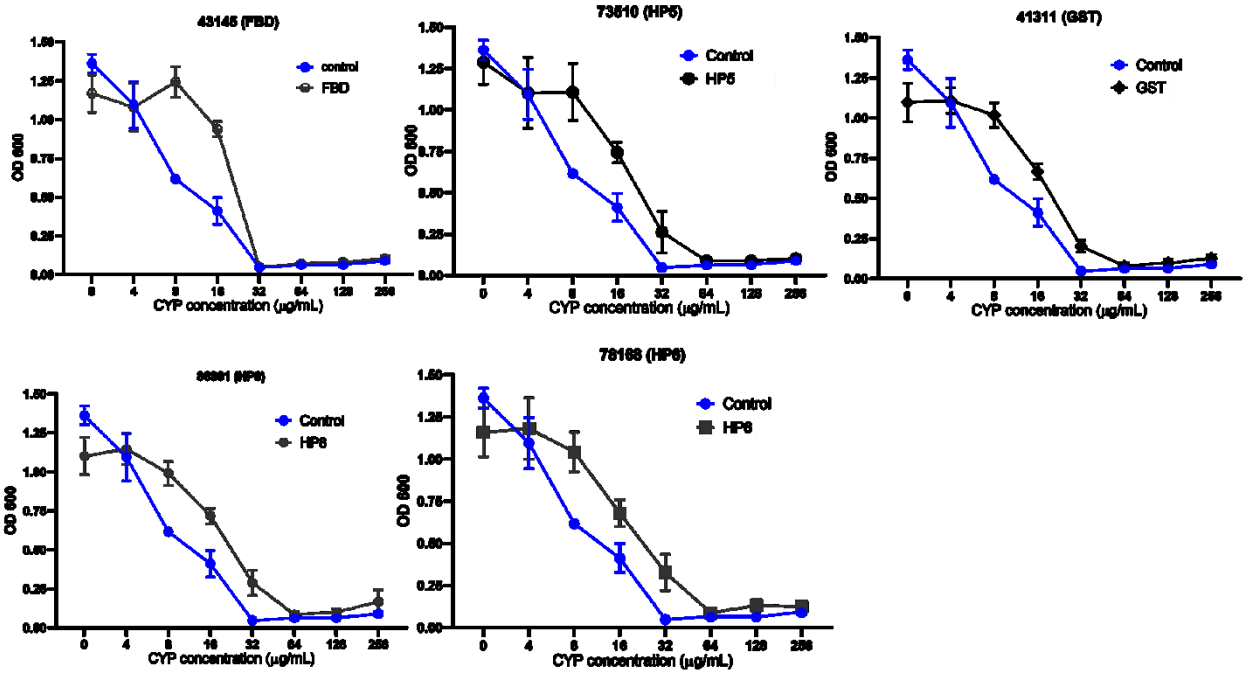

Figure 7. CYP dose response curve of *S. cerevisiae* strains containing *A. pullulans* genes identified by GWAS. The endpoint OD_{600} for each concentration was determined after 24 h of growth. The curve was generated based on the mean OD_{600} of three independent experiments with three replicates and their 95% CI determined.

3.8 Different alleles of the same *A. pullulans* genes cause CYP tolerance in *S. cerevisiae*

The *A. pullulans* genes tested for their effect on *S. cerevisiae* CYP sensitivity were identified by a GWAS analysis and with genome and fungicide sensitivity data from a collection of 46 isolates. Some isolates were highly tolerant to CYP, while others were sensitive. We, therefore, wanted to test if different alleles from tolerant and sensitive *A. pullulans* strains affected CYP sensitivity differently when expressed in *S. cerevisiae*. Therefore, we also cloned the *FBD*, *HP5*, and *GST* genes from the sensitive strain F2 and expressed these alleles in *S. cerevisiae*.

Table 3. The CYP MIC_{50} (µg/mL) values for *S. cerevisiae* strains expressing different *A. pullulans* genes.

GWAS identified protein ID	*A. pullulans* allele[2]	*S. cerevisiae* strain[1]	MIC_{50} CYP	95% CI
		Control	6.6	5.1 - 8.4
43145	F2 (S)	FBD (S)	33[a]	17- 66*
43145	LF 5.16 (T)	FBD (T)	21[a]	13 - 32*
73510	F2 (S)	HP5 (S)	8.9[b]	6.4 - 12
73510	LC 5.2 (T)	HP5 (T)	16[b]	11 - 23*
41311	F2(S)	GST (S)	23[c]	16 - 34*
41311	LC 5.2 (T)	GST (T)	18[c]	13 - 26*
78168	LC 5.2 (T)	HP6 (T)	20	14 - 28*
86861	LC 5.2 (T)	HP8 (T)	21	14 - 31*

1. *S. cerevisiae* strains expressing *A. pullulans* genes with their different alleles indicated in brackets (where; S=sensitive, T=tolerant and VT=Very tolerant).

a. Varied MIC_{50} in alleles of FBD gene but statistically insignificant.

b. The tolerant allele of the HP5 gene having double the MIC_{50} of the sensitive allele, but statistically insignificant.

c. Nearly same MIC_{50} of the S and T alleles of the GST gene

2. The original *A. pullulans* isolate from our previous study [21], from which the gene was amplified . The CYP sensitivity phenotype is indicated in brackets (where S=sensitive, T=tolerant)

* Based on the 95% confidence interval of likelihood profiles, MIC_{50} of these strains are significantly different from the control strain.

For the FBD and GST genes, the cells expressing the tolerant and sensitive alleles grew comparably and better than the control strain (as shown above). However, the dose response curves for HP5-expressing *S. cerevisiae* cells revealed marked differences between the tolerant and sensitive alleles, in particular at higher CYP concentrations (Figure 8). Despite difference in growth for HP5 expressing cells and at high CYP concentrations, the 95 % CI of the MIC_{50} values indicated no significant differences in CYP sensitivity of the strains expressing a gene from a sensitive or tolerant *A. pullulans* isolate (Table 3)

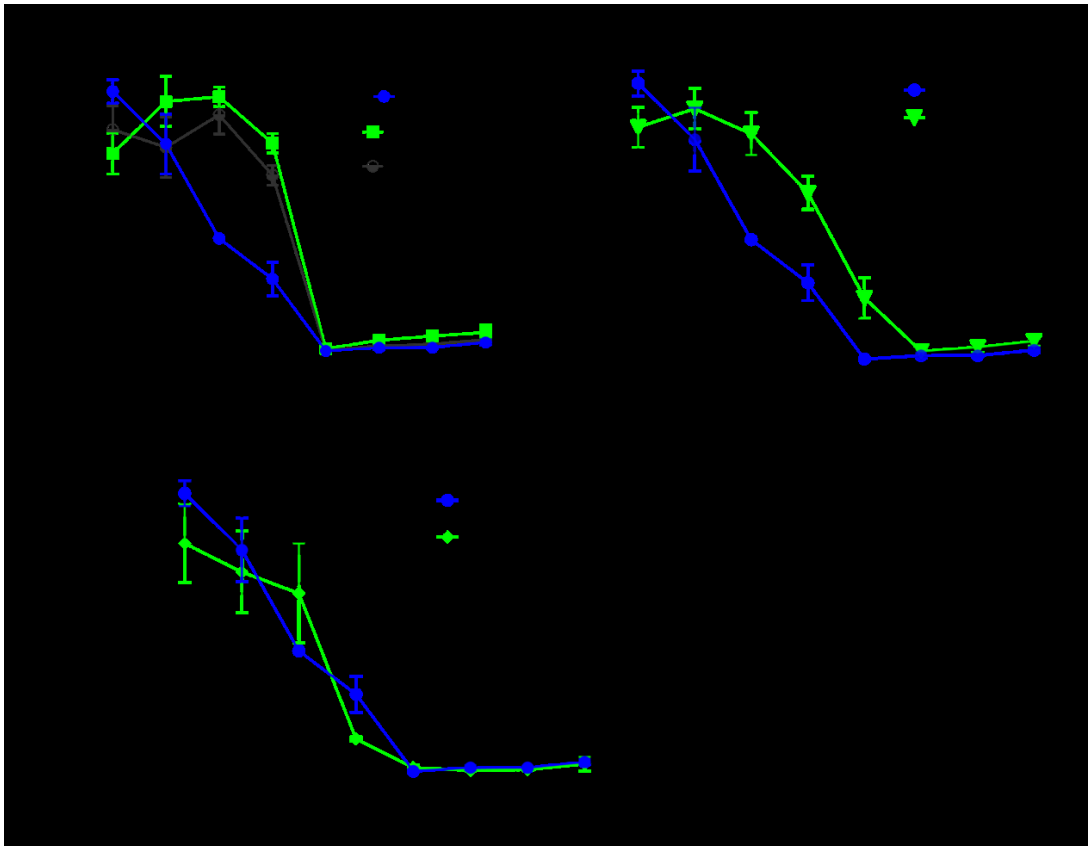

Figure 8. CYP dose response curve of *S. cerevisiae* strains containing *A. pullulans* genes identified by GWAS. Strains containing sensitive (S) and tolerant (T) alleles of each of the three genes were included in assay. The endpoint OD_{600} for each concentration was determined after 24 h of growth. The curve was generated based on the mean OD_{600} of three independent experiments with three replicates and their 95% CI determined.

Overall, there were no differences in tolerance to CYP between the S and T alleles of the three *A. pullulans* genes FBD, *GST,* and HP5 when transformed in *S. cerevisiae.* Still, both allele versions had an effect on CYP tolerance; thus, these genes might interact with other genes in *A. pullulans* to cause CYP tolerance.

4 Discussion

Antifungal compounds are essential to protect crop, human, and animal health. However, their use in the environment can cause resistance in target and non-target fungi (e.g., *A. pullulans*) and deleterious effects on the environment and humans [8-11, 17, 19]. Environmental changes could then favor some of these resistant, environmental fungi to emerge as pathogens in agriculture, animals, or immunocompromised patients [18, 20, 90]. *A. pullulans* has been considered an emerging pathogen in immunocompromised populations [90, 91]. However, in most cases, it is regarded as a contaminant from the environment rather than a pathogen due to its nature of being ubiquitous, an affinity for synthetic or surgical implants, and difficulty to disinfect from surfaces [92-94]. Recently, *A. pullulans* was reclassified into four distinct species *A. pullulans*, *A. melanogenum*, *A. subglaciale,* and *A. namibiae* [89, 95]. Most clinical *Aureobasidium* isolates are now classified as *A. melanogenum* since this species grows at 37°C and produces siderophores at this temperature, while *A. pullulans* does not exhibit properties that are important for human pathogenicity [89, 96, 97]. It has therefore been suggested that most "pathogenic" *A. pullulans* strains were misclassified [89]. Also, in the analysis presented here, seven of the 16 clinical *Aureobasidium* isolates (including six isolates obtained from culture collections) were genetically vastly different from all other *A. pullulans* isolates and clustered with reference genomes of *A. melanogenum*, *A. subglaciale*, or *A. namibiae.* Thus, this analysis confirms that clinical *Aureobasidium* isolates often do not belong to the species *A. pullulans*. At the same time, it also identifies strains from clinical samples that clearly belong to the species *A. pullulans*. Whether or not these strains were isolated as contaminants or may indeed exhibit pathogenicity to humans is currently not clear and has to be assessed in further experiments. In an earlier study we have identified environmental *A. pullulans* isolates that are tolerant to different commonly used antifungal agents and have significantly higher MIC_{50} values than plant pathogenic fungi [21]. The *A. pullulans* and non-*A. pullulans* isolates from clinical isolates included here had lower MIC_{50} values than the environmental isolates. This suggests that environmental *A. pullulans* isolates might have adapted to stresses including fungicides and implies a different environmental niche of clinical isolates. The SNP-based phylogenetic tree of the 46 *Aureobasidium* isolates showed clusters that mostly reflected the fungicide sensitivity (sensitive, tolerant, or intermediate); similar as it has been reported previously based on ITS sequences only [21]. Fungicide tolerance in *A. pullulans* is thus genetically determined. In contrast, the clinical *A. pullulans* isolates did not form a separate clade, but clustered together but within other *A. pullulans* isolates. This may imply and environmental origin of the clinical *A. pullulans* isolates and expose them rather as environmental contaminants than true human pathogens.

Our GWAS found no marker that correlated to the trait of MIC_{50} for DFN in *A. pullulans* isolates. This observation was interesting considering DFN is widely used in the environment, has a similar target in clinical and plant pathogens, and is regularly detected in environmental samples, implying intense selection pressure and possible resistance development on both target and non-target organisms [98-100]. Additionally, the MIC_{50} values of DFN for *A. pullulans* used for this GWAS showed reduced sensitivity of *A. pullulans* isolates compared to pathogenic fungi [21, 101]. Therefore, it was expected that some genetic polymorphism would cause this differential sensitivity phenotype. Despite this, DFN has a very specific target (CYP51), and the range of MIC_{50} values for DFN of the isolates used in this study was small, implying that few genetic changes (if any) could explain the low sensitivity. Furthermore, since no marker nor SNP was identified to correlate with MIC_{50} values of DFN, the low sensitivity of the *A. pullulans* analyzed could be explained by other non-genetic adaptations or other gene changes that do not involve SNPs polymorphisms.

We experimented on five genes with the most significant SNPs, including an F-box domain protein, glucose transporter, a protein kinase, and 2 proteins with unknown functions from the several GWAS genes with significant markers for CYP. F-box domain proteins contain motifs that target and interact with a plethora of other proteins (e.g., cyclins, enzymes, transcription factors, structural proteins) [102]. These interactions regulate protein levels in a cell, thus affecting highly diverse cellular functions such as the cell division cycle, signal transduction, morphological mitochondrial connectivity, or tolerance to antifungal agents [102-104]. Sometimes, FBD proteins work alone as an orphan without targets [103-105]. Notably, some F-box domain proteins (e.g., Met30p) interact with transcription factors involved in methionine metabolism, regulating the methionine biosynthesis genes [106-108]. Interestingly, methionine biosynthesis is the proposed target of CYP and several genes involved in sulphur amino metabolism, including those that encode transcription functions, have been predicted to be responsible for resistance [53, 63]. The predicted FBD (protein ID 43145) was homologous to the F-box superfamily (IPR001810), but without a known biological function. Phyre2 detected an F-Box domain at the N-terminus of the FBD protein (ID 43145), but did not identify a target. Without additional experimentation, it is thus impossible to know if this protein functions alone or interacts with unidentified targets. It could be that the *A. pullulans* FBD protein interacts with METC or other proteins involved in sulphur amino metabolism [53, 63] to cause tolerance, or FBD might be acting alone as an orphan F-box protein.

The *A. pullulans* protein 73510 (HP5) had an annotated function as protein kinase (PF00069). Protein kinases are a group of enzymes that can be activated in response to several stresses, including high osmolality, heat shock, inhibition of protein glycosylation, and inhibition of protein synthesis [109]. Thereby, stress signals can be transduced to the nuclear and lead to the upregulation of genes [109-112]. Activated protein kinases phosphorylate specific proteins and can thereby prevent cell lysis or enable cell wall remodelling [109-112]. These protective roles as a result of protein kinase activation have been implicated in the enhanced tolerance of fungi to various antifungal agents [112]. Based on the proposed mode of action of CYP, which is the inhibition of methionine biosynthesis, inhibition of this pathway might cause activation of protein kinases. The mutation we observed in our resistant strain might lead to upregulation of the activated protein kinase and lead to improved protection against cell lysis, hence resistance similar to what has been observed for azoles [113]. Therefore, further experiments to quantify protein kinases in the sensitive and resistant strains under CYP pressure would further confirm this resistance mechanism

The protein 41311 (GST) is predicted to be a sugar transporter homologous to LacP (LAC12) from the yeast *Kluyveromyces lactis* and is categorized within the major facilitator superfamily (MFS) (based on transporter classification database, TCDB) [114]. The LacP protein is localized within the membrane and represents an inducible lactose permease (EC 2.A.1.1.9) with lactase and galactose as its substrates. Based on gene ontology, this group of proteins has symporter activity in yeasts and other pathogenic filamentous fungi. A blast search also recognized homologs of hexose transporters in other fungi, with the best hit being *Aspergillus clavatus NRRL 1 (*XP_001268489.1). Overexpression of MFS transporters has been implicated in resistance to different chemicals, including antifungals [115]. Notably, efflux pump overexpression has been predicted through transcriptomic data to cause multidrug resistance [68]. It is thus regarded as a generalized resistance mechanism. Further, other CPN-resistant strains with efflux pump overexpression have been shown to exhibit multidrug resistance [66, 67]. Interestingly, our GWAS identified significant SNPs in the GST protein for both CPN and CYP MIC_{50} data, also indicating a generalized resistance. Furthermore, our sequence analysis of the

resistant isolate LC 5.2, which had a high MIC_{50} for both CPN and CYP, revealed 19 amino acids within the coding region of GST, which might have led to transporter overexpression. Thus, *A. pullulans* GST can be hypothesized to non-specifically confer multidrug resistance.

S. cerevisiae deletion strains of five genes (*LYS2*, *ENO1*, *HOL1, CAC2,* and *STL1)* homologous to *A. pullulans* genes showed significant differences in sensitivity to CYP. Two of these genes *(HOL1*, a putative transporter, and *STL1*, a sugar transporter-like protein (both MSF transporters)) had transporter functions. As earlier mentioned, the involvement of transporters often results in a multi-drug resistance phenotype. The Δ*hol1* strain was demonstrated to cause resistance to chemicals and modification of arginine utilization [116, 117]. This observation agrees with our observation where *Δhol1* strain showed tolerance to CYP. *S. cerevisiae HOL1* is thus likely involved in a generalized resistance mechanism. Contrarily, based on our observation, the *Δstl1* strain was more sensitive to CYP than the WT. Interestingly, studies showed that, on minimal media, the deletion strain has a low growth competitiveness [118], which matches the phenotype we observed. Our GWAS also found significant SNPs in *STL1* that correlated with CPN tolerance. *LYS2* encodes for α-aminoadipate reductase, which is involved in lysine biosynthesis. The null mutant of this gene was also predicted to cause resistance to miconazole [119]. Notably, the accumulation of some amino acids, including lysine, was shown to reduce the toxicity of APs [53-56]. Thus, the mutations observed in the tolerant strains of *A. pullulans* might regulate lysine biosynthesis leading to CYP tolerance. *ENO1* encodes a phosphopyruvate hydratase, an enzyme involved in glycolysis as well as gluconeogenesis in the mitochondria. Null mutants of these genes have increased growth competitiveness and decreased endocytosis [118], which similarly points to a generalized resistance mechanism that this gene might impact in *A. pullulans*. *CAC2* encodes a chromatin assembly complex involved in assembling nucleosomes on newly replicated DNA. Alike the genes mentioned above, the *Δcac2* strain of *S. cerevisiae* is resistant to several chemicals such as the antifungal benomyl [120]. The mutation in the *A. pullulans CAC2* homolog might similarly confer the function of chemical resistance leading to CYP tolerance.

In summary, this study demonstrates fungicide tolerance mechanisms of *A. pullulans* with particular focus on CYP tolerance mechanisms. The results emphasize the potential of GWAS for identifying fungicide tolerance mechanisms and advance the understanding of antifungal tolerance mechanism in non-target yeast species that could be of practical importance in other species. The ten GWAS-identified genes experimented on suggest novel resistance mechanisms to CYP, some of which might function in conserved manner across different fungal species. Most of the gene functions point towards generalised fungicide resistance mechanisms that might confer multidrug/chemical tolerance. These results also highlight the complexity of CYP tolerance in *A. pullulans*, which seems to involve genes with diverse functions that might also interact with other known or undescribed pathways. Whether the interactions work in a targeted way against CYP remains to be observed and needs further analyses. Practically, these results highlight new aspects and potential mechanisms of CYP tolerance that can be further explored in other fungal species including human and plant pathogens. Further research could focus on understanding the different levels of interdependence among these genes or with other already predicted mechanisms by co-expressing two or more genes. It will also be interesting to test if the genes identified here also confer CYP tolerance to plant and human pathogenic fungi. In addition, functional characterization of more genes that were identified by GWAS herein but, not experimented in this study would be interesting area to explore

Declarations

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Author Contributions:

Genome analyses, genomic DNA extraction from clinical isolates, RNA extractions, cDNA library construction, PCR and plasmids construction, *S. cerevisiae* heterologous expression, microbroth sensitivity assays, data analysis, and writing of the manuscript (**Electine Magoye**). GWAS, genome assembly, genome analyses, and writing the manuscript for the respective parts (**Lukas Nägeli**). Spot assays of *S. cerevisiae* (**Maja Hilber-Bodmer**). Genome assembly (**Andreas Bühlmann**). Genomic DNA extractions from environmental isolates (Lena Daendiker). Isolation and collection of clinical isolates from the Swiss hospitals (P**eter Keller Konrad Mühlethaler, Arnaud Riat, Jacques Schrenzel**). Supervision, project administration, overall conceptualization, and funding acquisition, reviewing of the manuscript (**Florian Freimoser**).

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Supplementary material

Supplementary Table 1. *S. cerevisiae* deletion mutants

Strain (Orf)	Genotype	Source
Wildtype (BY4741)	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	Euroscarf collection
Δarg2 (YJL071w)	*BY4741; MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YJL071w::kanMX4*	Euroscarf collection
Δcar1 (YPL11w)	*BY4741; MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YPL11w::kanMX4*	Euroscarf collection
Δcar2 (YLR438w)	*BY4741; MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YLR438w::kanMX4*	Euroscarf collection
Δmet2 (YNL277w)	*BY4741; MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YNL277w::kanMX4*	Euroscarf collection
Δura 2 (YJL130c)	*BY4741; MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YJL130c::kanMX4*	Euroscarf collection
Δlys2 (YBR115c)	*BY4741; MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YBR115c::kanMX4*	Euroscarf collection
Δatg42 (YBR139w)	*BY4741; MATa; his3Δ1; leu2Δ0; metΔ15Δ0; ura3Δ0; YBR139w::kanMX4*	Euroscarf collection
Δpdb1 (YBR221c)	*BY4741; MATa; his3Δ1; leu2Δ0; metΔ15Δ0; ura3Δ0; YBR221c::kanMX4*	Euroscarf collection
Δvba2 (YBR293w)	*BY4741; MATa; his3Δ1; leu2Δ0; metΔ15Δ0; ura3Δ0; YBR293w::kanMX4*	Euroscarf collection
Δrps17B (YDR447c)	*BY4741; MATa; his3Δ1; leu2Δ0; metΔ15Δ0; ura3Δ0; YDR447c::kanMX4*	Euroscarf collection
Δstl1 (YDR536w)	*BY4741; MATa; his3Δ1; leu2Δ0; metΔ15Δ0; ura3Δ0; YDR536w::kanMX4*	Euroscarf collection
Δeno1 (YGR254w)	*BY4741; MATa; his3Δ1; leu2Δ0; metΔ15Δ0; ura3Δ0; YGR254w::kanMX4*	Euroscarf collection
Δskn7 (YHR206w)	*BY47Δ1; MATa; his3Δ1; leu2Δ0; metΔ15Δ0; ura3Δ0; YHR206w::kanMX4*	Euroscarf collection
Δhoc1 (YJR075w)	*BY47Δ1; MATa; his3Δ1; leu2Δ0; metΔ15Δ0; ura3Δ0; YJR075w::kanMX4*	Euroscarf collection
Δsry1 (YKL218c)	*BY47Δ1; MATa; his3Δ1; leu2Δ0; metΔ15Δ0; ura3Δ0; YKL218c::kanMX4*	Euroscarf collection
Δvba5 (YKR105c)	*BY47Δ1; MATa; his3Δ1; leu2Δ0; metΔ15Δ0; ura3Δ0; YKR105c::kanMX4*	Euroscarf collection
Δsfp1 (YLR403w)	*BY47Δ1; MATa; his3Δ1; leu2Δ0; metΔ15Δ0; ura3Δ0; YLR403w::kanMX4*	Euroscarf collection
Δcac2 (YML102w)	*BY47Δ1; MATa; his3Δ1; leu2Δ0; metΔ15Δ0; ura3Δ0; YML102w::kanMX4*	Euroscarf collection
Δadh6 (YMR318c)	*BY47Δ1; MATa; his3Δ1; leu2Δ0; metΔ15Δ0; ura3Δ0; YMR318c::kanMX4*	Euroscarf collection
Δhda1 (YNL021w)	*BY47Δ1; MATa; his3Δ1; leu2Δ0; metΔ15Δ0; ura3Δ0; YNL021w::kanMX4*	Euroscarf collection
Δhol1 (YNR055c)	*BY47Δ1; MATa; his3Δ1; leu2Δ0; metΔ15Δ0; ura3Δ0; YNR055c::kanMX4*	Euroscarf collection
Δuls1 (YOR191w)	*BY47Δ1; MATa; his3Δ1; leu2Δ0; metΔ15Δ0; ura3Δ0; YOR191w::kanMX4*	Euroscarf collection
Δput4 (YOR348c)	*BY47Δ1; MATa; his3Δ1; leu2 Δ0; metΔ15Δ0; ura3Δ0; YOR348c::kanMX4*	Euroscarf collection

Supplementary Table 2. *A. pullulans* strains used in this study

Isolate Name	MIC$_{50}$ µg/mL			Isolating Fungicide	Sample source
	Captan	Cyprodinil	Difenoconazole		
F2	5.1	2.8	0.5	None	Flower
Fr1	22.5	9	1	None	Fruit
Fr2	40	7.4	0.6	None	Fruit
SFr4.3	25.1	7.9	2.4	Slick (DFN)	Fruit
LSK 2.11	19.7	49.3	1.8	Slick (DFN)	Leaf
FLSK 5.1	18.8	7.5	1.3	Slick (DFN)	Leaf
LSK 10.4	41	18.9	2.4	Slick (DFN)	Leaf
LCH 10.2	20.9	34.1	7.1	Chorus (CYP)	Leaf
LCH 5.9	51.5	11.9	1.3	Chorus (CYP)	Leaf
ChF4.2	6.3	2.2	0.9	Chorus (CYP)	Flower
LCH 2.1	44.7	29.7	3.7	Chorus (CYP)	Leaf
CaL1.1	19.1	20.6	1.1	Captan 80 WD (CPN)	Leaf
CaFr2.1	19.4	3.8	1.2	Captan 80 WD (CPN)	Fruit
LC 5.2	39.5	186	0.7	Captan 80 WD (CPN)	Leaf
LC 1.9	38.4	59.6	5.2	Captan 80 WD (CPN)	Leaf
LC 1.3	50.8	50.5	4.2	Captan 80 WD (CPN)	Leaf
LF 3.10	20.6	3.4	4.8	Flint (Trifloxystrobin)	Leaf
LF 5.11	23.4	14.1	2.2	Flint (Trifloxystrobin)	Leaf
FFr4.3	19.9	3.2	3.1	Flint (Trifloxystrobin)	Fruit
LF 5.16	28.7	42.8	1.4	Flint (Trifloxystrobin)	Leaf
LF 5.10	54.4	41.9	10.1	Flint (Trifloxystrobin)	Leaf
AF4.1b	17	8.9	0.4	Amphotericin B	Flower
AL4e	19.3	1.45×10^{39}	2.2	Amphotericin B	Leaf
AF4.1a	5.8	3.6	0.6	Amphotericin B	Flower
CnF4.2	5.3	2	0.6	Capsofungin	Flower
CnL4a	21.4	6	1.7	Capsofungin	Leaf
CnFr4.4	33.1	9	0.9	Capsofungin	Fruit
FL4.31	40.2	8.3	1	Fluconazole	Flower
FLF 4.3	5.7	4.7	1.4	Fluconazole	Flower
FLS4a	109.6	6.6	1.8	Fluconazole	Leaf

Supplementary Table 3. *S. cerevisiae* generated during this study by heterologous expression of *A. pullulans* genes

Strain	Backgound	Assembly plasmid[1]	Promoter	Terminator	auxotrophy	*Gene-allele*	Original *A. pullulans*	Clonning method
ApCAR2-S	*Δcar2*	USER L1	pTDH3	tTDH1	ura+	*CAR2-S*	F2	USER
ApCAR2-T	*Δcar2*	USER L1	pTDH3	tTDH1	ura+	*CAR2-T*	LF 5.16	USER
ApCAR1-S	*Δcar1*	USER L1	pTDH3	tTDH1	ura+	*CAR1-S*	CnF 4.2	USER
ApCAR1-T	*Δcar1*	USER L1	pTDH3	tTDH1	ura+	*CAR1-T*	LC 5.2	USER
ApARG2-S	*Δarg2*	USER L1	pTDH3	tTDH1	ura+	*ARG2-S*	CnF 4.2	USER
ApARG2-T	*Δarg2*	USER L1	pTDH3	tTDH1	ura+	*ARG2-T*	LC 5.2	USER
ApMET2-S	*Δmet2*	USER L1	pTDH3	tTDH1	ura+	*MET2-S*	F2	USER
ApMET2-T	*Δmet2*	USER L1	pTDH3	tTDH1	ura+	*MET2-T*	LF 5.16	USER
CAR2-ApCAR2-T	BY4741	USER L1	pTDH3	tTDH1	ura+	*CAR2-T*	LF 5.16	USER
CAR2-ApCAR2-S	BY4741	USER L1	pTDH3	tTDH1	ura+	*CAR2-S*	F2	USER
FBD (S)	BY4741	pYTK096	pTDH3	tTDH1	ura+	*FBD(43145)-S*	F2	GG
FBD (T)	BY4741	pYTK096	pTDH3	tTDH1	ura+	*FBD(43145)-T*	LF 5.16	GG
HP5 (S)	BY4741	USER L1	pTDH3	tTDH1	ura+	*HP5(73510)-S*	F2	USER
HP5 (T)	BY4741	USER L1	pTDH3	tTDH1	ura+	*HP5(73510)-T*	LC 5.2	USER
HP6 (T)	BY4741	USER L1	pTDH3	tTDH1	ura+	*HP6(78168)_T*	LC 5.2	USER
HP8 (T)	BY4741	USER L1	pTDH3	tTDH1	ura+	*HP8(86861)_T*	LC 5.2	USER
GST (S)	BY4741	pYTK096	pTDH3	tTDH1	ura+	*GST(41311)_S*	F2	GG
GST (T)	BY4741	pYTK096	pTDH3	tTDH1	ura+	*GST(41311)_T*	LC 5.2	GG

Plasmids details can be found in supplementary in Supplementary Table 6

Supplementary Table 4. Clinical isolates were sampled from different body tissues and sourced from different places, the MIC_{50} for CPN, CYP, and DFN were determined, and the species assignment based on whole genomes of these isolates is also reported

Strain	**MIC_{50} (μg/ml)**			**Sample**	**Provider**	**Species assignment (SNPs based clustering)**
	Captan (CPN)	**Cyprodinil (CYP)**	**Difenoconazole (DFN)**			
IFIK 1931366	4.803	10.330	0.022	Thumb nail	a)	Non-*A. pullulans*
IFIK 1976364	1.286	5.998	3.253	Dry skin from the foot	a)	*A. pullulans*
IFIK 2015300	4.445	5.154	0.005	Nail	a)	*A. pullulans*
IFIK 2049092	1.201	10.260	0.002	Dry skin from the foot	a)	*A. pullulans*
IFIK 2073347	2.588	3.366	0.680	Dry skin from the foot	a)	*A. pullulans*
IFIK 2230802	2.124	6.630	0.016	Dry skin from the foot	a)	*A. pullulans*
IFIK 2296290	2.056	20.870	2.932	Dry skin from the foot	a)	*A. pullulans*
d450803	2.098	5.748	7.687	Thumb	b)	*A. pullulans*
CBS 101119	3.246	4.970	0.020	Man BAL fluid, patient; Netherlands	c)	Non-*A. pullulans*
CBS 121327	5.349	2.324	0.352	Ear canal of child; Greece	c)	Non-*A. pullulans*
CBS 121328	1.115	0.732	0.383	Man onychomycosis; Greece	c)	Non-*A. pullulans*
CBS 298.56	4.433	3.420	0.003	Man, lymph node; USA	c)	Non-*A. pullulans*
CBS 577.93	5.320	31.320	0.019	Man bronchial-alveolar lavage, 3-year-old boy with chronic myeloid leukemia; Sweden	c)	Non-*A. pullulans*
CBS 626.85	4.694	14.060	0.010	Man peritoneal dialysis fluid; Australia	c)	*A. pullulans (own cluster)*
CBS 699.76	3.210	13.240	0.072	Man thumb nail; Netherlands	c)	Non-*A. pullulans*
HUG.AP	2.098	4.872	0.279		d)	*A. pullulans*
Mean	**3.129**	**8.956**	**0.983**			
Range	**1.115-5.349**	**0.732-31.32**	**0.002-3.253***			
	4.153	**6.421**	**0.022**			

* Strain d450803 excluded as an outlier because it had overall reduced growth even in absence of the fungicides

a) IFIK Bern, Konrad Mühlethaler, Peter Keller; b) Klinische Bakteriologie / Mykologie, Basel

c) Westerdijk fungal biodiversity Institute Collection d) HUG, Geneva, Arnaud Riat, Jacques Schrenzel

Supplementary Table 5. Primers

Name	Conc. (uM)	Anealing temp °C	Sequence	Description	Usage
NBB_CAR 1_F	20	72	CGTGCGAUATGGACGCTCCCACTCTCC	NBB_CAR1_39236_Arginase-Fw(USER)	PCR amplification for subsequent USER cloning
NBB_CAR 1_ R	20		CACGCGAUTTAGAGCAGAGTGTCACCGAG	NBB_CAR1_39236_Arginase-rv(USER)	
NBB_Met 2_F	20	65	CGTGCGAUATGAAATACGAAAGAGTGACATCG	NBB_Met 2_78869-Homoserne-O acetyltransferase rv(USER)	
NBB_Met 2_R	20		CACGCGAUTTACCAAGCGGTAATGTCGG	NBB_Met 2_78869-Homoserne-O acetyltransferase Fw (USER)	
NBB_Arg 2_F	20	68	CGTGCGAUATGTCGACAGCATGCCTC	NBB_ARG 2_63597_N-acetylglutamatesynthase Fw (USER)	
NBB_Arg 2_R	20		CACGCGAUTCAATCAGGCTTCGCCTTG	NBB_ARG 2_63597_N-acetylglutamatesynthase rv (USER)	
NBB_CAR 2_fd	20	72	CGTGCGAUATGGCTCCCATTGCCATCCACACC	NBB_CAR2_85595_L-ornithine transaminase (OTAse)-Fw(USER)	
NBB_CAR 2_Rv	20		CACGCGAUTCAGTTGTCAACGCCGATGTGAAC	NBB_CAR2_85595_L-ornithine transaminase (OTAse) rv (USER)	
NBB_HP5_F	20	63	CGTGCGAUATGAGTTCTCGATTTTTCAAAGAC	HP5-73510_threonine protein kinase (USER)	
NBB_HP5_R	20		CACGCGAUCTAGAGCAGTTTTGCCTTTTTTAC	HP5-73510_threonine protein kinase (USER)	
NBB_HP6_F	20	72	CGTGCGAUATGGCGACGGCAGCGTCG	NBB_HP6_78168- hypothetical protein fw (USER)	
NBB_HP6_R	20		CACGCGAUCTAGTGCTTGTCGCTGAGTTTGTTGATG	NBB_HP6_78168- hypothetical protein rv (USER)	
NBB_HP8_F	20	63	CGTGCGAUATGACGACGAGAAGTACTAG	NBB_HP8-86861 - hypothetical protein fw (USER)	
NBB_HP8_R	20		CACGCGAUCTATCTCGGTGAGGAAAGAG	NBB_HP8-86861- hypothetical protein rv (USER)	
NBB_FBD_R	20	62	ATGCCGTCTCAGGTCTCAGGATCCTAAGATCCTTTTCTTTAACTCCGA	NBB Prot_ID 43145 (FBD)amplicon rv (GG)	PCR amplification for subsequent golden gate cloning
NBB_FBD_F	20		GCATCGTCTCATCGGTCTCATATGGTCGCTGGTCAAGCAACTAT	NBB Prot_ID 43145 (FBD) amplicon fwd (GG)	
URA3 3'_F	20	62.1	AGAGCACTTGAATCCACTGC	URA3' homolog fw	Primer to check integration according to [1]
URA3 3'_R	20	58.9	GATTTGGTTAGATTAGATATGGTTTC	URA3' homolog rv	
URA3 5'_F	20	61.3	GGGCGGATTACTACCGTT	URA 5' homolog fw	
URA3 5'_R	20	59.2	GTAATGTTATCCATGTGGGC	URA 5' homolog rv	
URA3 5'wt rev	20		CCTTCACCATAAATATGCCTCGC	URA3 5' WT rv	Primer to check integration
URA3 3'wt for	20		TATCCAATACCTCGCCAGAACC	URA3 3' WT rv	
pTDH3f	10	55.7	GGTGTCTGGGTGAACAGTTTATTC	pTDH3 promoter	Screening for correct plasmid assembly
tTDH1r	10	54.3	CGACGTTCTCGCCATAACT	tTDH1 terminator	
USER new 1f	100	95	GCATCGTCTCATCGGTCTCATATGCAACGGAATGCGTGCGATCGCGTGCATTCATCCTGAGACCTGAGACGGCAT	USER_F with BSA1 and BsMB1 resitriction sites	Annealing of primers to make entry vector for USER cloning
USER new 2r	100	95	ATGCCGTCTCAGGTCTCAGGATGAATGCACGCGATCGCACGCATTCCGTTGCATATGAGACCGATGAGACGATGC	USER_R with BSA1 and BsMB1 restriction sites	

1. Lee ME, DeLoache WC, Cervantes B, Dueber JE: A highly characterized yeast toolkit for modular, multipart assembly**.** *ACS synthetic biology* 2015, **4:**975-986

Supplementary Table 6. Plasmids

Plasmid Nr.	Name	Description	Type	Assembly (main parts)	*E. coli* resistance	Original user
1	pyTK001	Entry vector	Entry		Chl	(1)
2	pYTK009	Vector containing promoter sequence	Promoter		Chl	(1)
3	pyTK056	Vector containing the Terminator sequence	Terminator		Chl	(1)
4	pyTK096	Assembly vector	Assembly		Kan	(1)
5	USER_L0	Vector containing a USER fragment with AsiSI and Nb.BsMI recognition sites	Intermediate	pYTK001_primmed USER oligonucleotides	Chl	This study
6	USER_L1	Vector containing assembly parts with USER_Lo	Assembly	D56_pYTK009_USER Lo (USER primmed_pYTK001_pYTK56)	Amp	This study
7	FBD_199_l0	F-box domain _P.ID 43145_GGclonning_AP.isolat e 199	Intermediate	pYTK001_FBD199	Chl	This study
8	FBD_19_l0	F_box domain _P.ID 43145_GGclonning_AP.Isolat e 19	Intermediate	pYTK001_FBD19	Chl	This study
9	FBD_287_lo	F_box domain _P.ID 43145_GGclonning_AP.isolat e 287	Intermediate	pYTK001_FBD287	Chl	This study
10	D56	Assembly casssete	Assembly	pYTK002_pYTK047_pYTK072_pYTK 074_pYTK086_pYTK089_pyTK092	Chl	
11	FBD_199_l1	F_box domain _P.ID 43145_GGclonning_AP.isolat e 199	Integration	pYTK096_pYTK009 _L0FBD (pYTK001_FBD gene)_pYTK056	Kan	This study
12	FBD_19_l1	F_box domain _P.ID 43145_GGclonning_AP.Isolat e 19	Integration	pYTK096_pYTK009 _L0FBD (pYTK001_FBD gene)_pYTK056	Kan	This study
13	FBD_287_l1	F_box domain _P.ID 43145_GGclonning_AP.isolat e 287	Integration	pYTK096_pYTK009 _L0FBD (pYTK001_FBD gene)_pYTK056	Kan	This study
14	GST_5_l1	Glucose substrate transpoter _USER isolate 5	Integration	pYTK096_pYTK009 _GST_5_LO_pYTK056	Kan	
15	GST_211-l1	Glucose substrate transporter -USER isolate 211	Integration	pYTK096_pYTK009 _GST_211_LO_pYTK057	Kan	This study
16	CAR1_199_l2	Arginase_P.ID 39236_USERclone_AP.isolate _199	Integration	USER L1 (D56_pYTK009_USER Lo (USER primmed_pYTK001)_pYTK56)_CAR1 gene	Amp	This study
17	CAR1_287_l2	Arginase_P.ID 39236_USERclone_AP.isolate _287	Integration	USER L1 (D56_pYTK009_USER Lo (USER primmed_pYTK001)_pYTK56)_CAR1 gene	Amp	This study
18	CAR2_199_l2	L-ornithine transaminase (OTAse)_P.ID 85595_USER_AP. isolate_199	Integration	USER L1 (D56_pYTK009_USER Lo (USER primmed_pYTK001)_pYTK56)_CAR2 gene	Amp	This study
19	CAR2_19_l2	USERclone_AP. isolate_19	Integration	USER L1 (D56_pYTK009_USER Lo (USER primmed_pYTK001)_pYTK56)_CAR2 gene	Amp	This study
20	CAR2_287_l2	L-ornithine transaminase (OTAse)_P.ID- 85595_USERclone_AP. isolate_287	Integration	USER L1 (D56_pYTK009_USER Lo (USER primmed_pYTK001)_pYTK56)_CAR2 gene	Amp	This study
21	ARG2_199_L2	Acetylglutamate synthase P.ID 63597_USERclone_AP.isolate _199	Integration	USER L1 (D56_pYTK009_USER Lo (USER primmed_pYTK001)_pYTK56)_Arg 2 gene	Amp	This study
22	ARG2_5_l2	Acetylglutamate synthase P.ID	Integration	USER L1 (D56_pYTK009_USER Lo(USER	Amp	This study

Plasmid Nr.	Name	Description	Type	Assembly (main parts)	*E. coli* resistance	Original user
		63597_USERclone_AP.isolate _5		primmed_pYTK001)_pYTK56)_Arg 2 gene		
23	ARG2_287_l2	Acetylglutamate synthase P.ID 63597_USERclone_AP.isolate _287	intergartion	USER L1 (D56_pYTK009_USER Lo (USER primmed_pYTK001)_pYTK56)_Arg 2 gene	Amp	This study
24	MET 2_199_L2	*MET2* gene_uSER clone_AP.isoate 199	intergartion	USER L1 (D56_pYTK009_USER Lo (USER primmed_pYTK001)_pYTK56)_MET2 gene	Amp	This study
25	MET 2_5_L2	*MET2* gene_USERclone_AP.isolate_ 5	intergartion	USER L1 (D56_pYTK009_USER Lo(USER primmed_pYTK001)_pYTK56)_MET2 gene	Amp	This study
26	MET 2_287_L2	MET2 gene_USER_clone_AP.isolate 287	intergartion	USER L1 (D56_pYTK009_USER Lo(USER primmed_pYTK001)_pYTK56)_MET2 gene	Amp	This study
27	HP5_211_L2	Hypothethical protein_P.ID 73510_AP.isolate 211	intergartion	USER L1 (D56_pYTK009_USER Lo(USER primmed_pYTK001)_pYTK56)_HP5g ene	Amp	This study
28	HP5_5_l2_l2	Hypothethical protein_P.ID 73510_AP.isolate 5	intergartion	USER L1 (D56_pYTK009_USER Lo(USER primmed_pYTK001)_pYTK56)_HP5g ene	Amp	This study
29	HP6_5_l2_l2	Hypothethical protein_P.ID 78168_AP.isolate 5	intergartion	USER L1 (D56_pYTK009_USER Lo(USER primmed_pYTK001)_pYTK56)_HP6g ene	Amp	This study
30	HP6_287_l2	Hypothethical protein_P.ID 78168_Ap.isolate 287	intergartion	USER L1 (D56_pYTK009_USER Lo(USER primmed_pYTK001)_pYTK56)_HP6g ene	Amp	This study
31	HP8_5	Hypothethical protein_P.ID 78168_AP.isolate 5	intergartion	USER L1 (D56_pYTK009_USER Lo(USER primmed_pYTK001)_pYTK56)_HP8g ene	Amp	This study
Synthethic gene						
	GST_211_L0	Codon optimised for golden gate at Twist	Entry			This study
	GST_5_L0	Codon optimised for golden gate at Twist	Entry			This study
Oligonucleotides						
	USER 1f	USER_F with BSA1 and BsMB1 resitriction sites and AsiSI and Nb.BsMI recognition sites	Oligo-nucleotides			This study

Supplementary Table 7. Confirmed resistance mechanisms but no SNPs found in GWAS

GENE	Function in B. cinerea	Location in NBB	NBB protein ID
DNM1	Dynamin-related GTPase	Chr_5:700429-703002	71988
AFG3	Mitochondrial inner membrane AAA protease	Chr_6:841417-844144	48143
PHB2	Prohibitin-like protein	Chr_12:850305-851365	57573
MDL1	Mitochondrial inner membrane ABC transporter	Chr_1:1598958-1601400	36016
MIX17	Intermembrane space (IMS) protein	Chr_2:955604-956154	78901
OLI	Subunit c of the FO part of mitrochondrial F1Fo ATP synthase	Not found	Not found
ATM	Mitochondrial ABC transporter	Chr_2:2009963-2012247	59984
POS5	Mitochondrial NADH kinase		
MCR1	Mitochondrial NADH-cytochrome b5 reductase	Chr_8:550984-552137	51229

Supplementary Table 8. Literature-identified genes and sequence comparison between resistant and sensitive *A. pullulans*

Gene	**Functions**	**Protein ID**	**Sequence difference between the sensitive and resistant *A. pullulans***
CPA2	Large subunit of carbamoyl phosphate synthetase	48243	D495N, K602R,D613E, S624T, E629D, D1057E
URA2	Bifunctional carbamoylphosphate synthetase/aspartate transcarbamylase	56266	No difference
ARG2	Acetylglutamate synthase	63597	I8T, T29A, S38N, S39N, 3 Amino acid insertion (D, P, N) at codon 543, R640K.
MET16	3'-phosphoadenylsulfate reductase	69324	No difference
MET28	bZIP transcriptional activator in the Cbf1p-Met4p-Met28p complex	70424	No difference
SAM4	S-adenosylmethionine-homocysteine methyltransferase	65333	No difference
ARG3 (ARG F)	Ornithine carbamoyltransferase;	60964	T240A, 18 amino acid insertaion at codon 352
ARG8	Acetylornithine aminotransferase	85549	G35A, T280K, D322N, P344S
ARG1 (ARG10)	Arginosuccinate synthetase	61298	No different
ARG4	Argininosuccinate lyase	80658	No difference
STR2	cystathione y syntahse (same as *METC* in filamentous fungi)	52770	Not found
MET32	Zinc-finger DNA-binding transcription facto	80402	L661P
MET5	Sulfite reductase beta subunit	40385	Not found
ADI1	Acireductone dioxygenease	47814	No difference
ARG7	Mitochondrial ornithine acetyltransferase	38841	No difference
STR3	cystathione B Lyase (same as METB)	39912	No difference
CPA1	Small subunit of carbamoyl phosphate synthetase	38809	no difference
MET2	L-homoserine-O-acetyltransferase	78869	T7S, I470V
CAR2	L-ornithine transaminase (OTAse)	85595	K254R
MET10	Subunit alpha of assimilatory sulfite reductase	39370	A580S, E612O, T1020M
CAR1	Arginase	39236	H202R
ARG5,6	Acetylglutamate kinase and N-acetyl-gamma-glutamyl-phosphate reductase	67317	V165, S166L, F204S, F238S, V250A, P237L, L237L, L296S, P307L, T322I, Q349P, L353P, F420C, R422L, L426P, P440H, T451M, P486L, S491F, N526S, S540L, W644L, Y717C, V875A
ORT1 (ARG11)	Ornithine transporter of the mitochondrial inner membrane	45076	Not found

Supplementary Table 9. GWAS-identified genes with homolog in *S. cerevisiae*

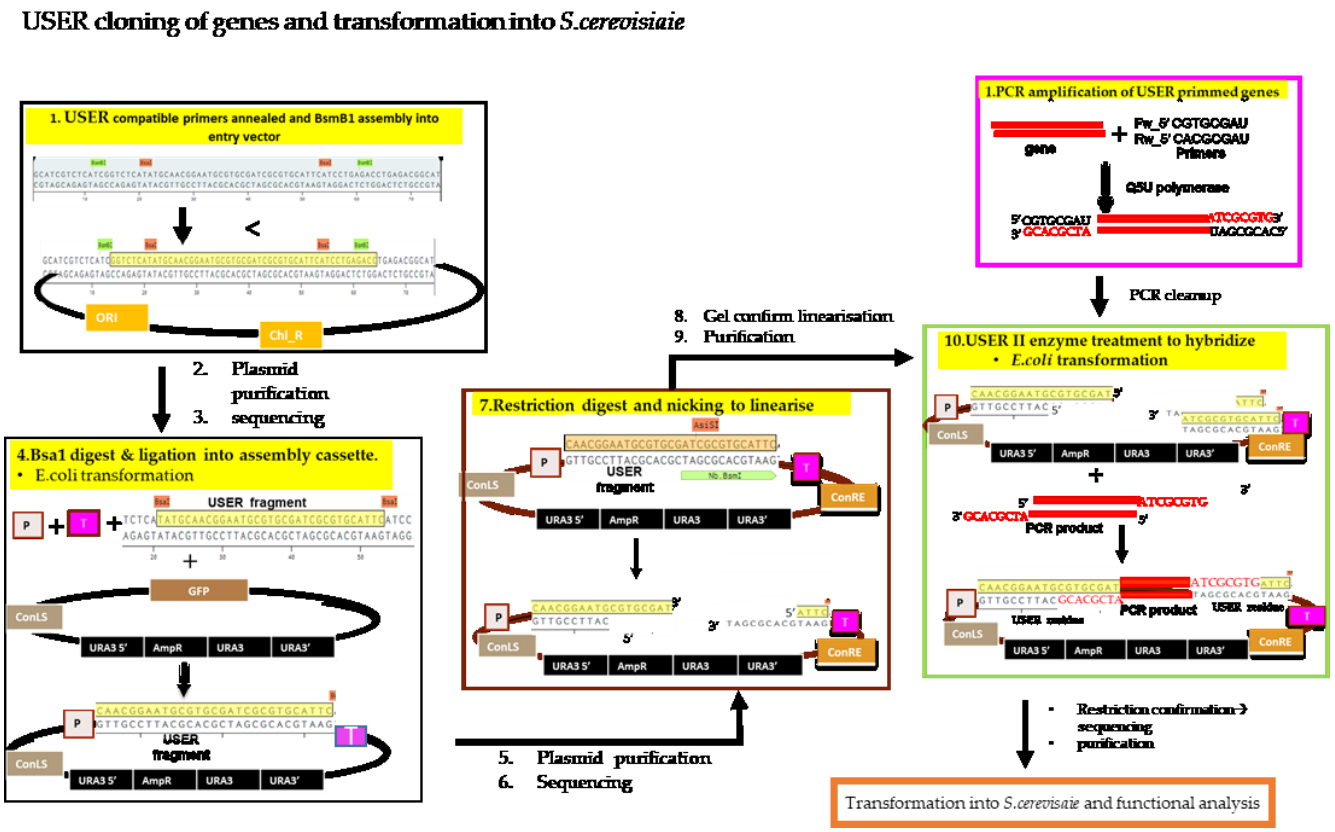
Gene	ID in NBB	Function	Nr. of significant SNPs with a.as changes
LYS2	65659	Lysine requiring (Alpha aminoadipate reductase)	6
ATG42	46183	Alpha/Beta hydrolase fold	8
PDB1	50975	Thiamin diphosphate-binding fold	0
VBA2	56221	Major facilitator superfamily domain-containing protein	2
RPS17B	79859	Ribosomal protein S17e	0
STL1	80840	Sugar transporter-like protein	3
ENO1	53332	enolase/allergen Asp F 22	1
SKN7	37964	hypothetical protein	11
HOC1	63440	Nucleotide-diphospho-sugar transferase	9
SRY1	34703	putative threonine dehydratase	2
VBA5	68119	Major facilitator superfamily domain-containing protein	4
SFP1	35695	hypothetical protein	0
CAC2	11929	chromatin assembly factor 1 protein	0
ADH6	55687	NADPH-dependent medium chain alcohol dehydrogenase	0
HDA1	41910	histone deacetylase	1
HOL1	55918	Major facilitator superfamily domain-containing protein	1
ULS1	47349	SNF2 family N-terminal domain-domain containing protein	6
PUT4	83824	Amino acid permease-domain containing protein	10

Supplementary Table 10. Genes with significant SNPs

Protein ID	Prot_name in NBB 7.2.1	Trait (MIC_{50})	Chromosome	Chromosome position
78908	Aspartyl-tRNA synthetase-like protein	CPN	Chr_2	966311
59949	Hypothetical protein	CPN	Chr_2	1925624
41311	General substrate transporter	CPN	Chr_3	43684
31132	Putative glucan 1%2C3-beta-glucosidase precursor	CPN	Chr_8	274754
32926	Hypothetical protein	CYP	Chr_2	3146883
34703	Putative threonine dehydratase	CYP	Chr_1	4460341
37964	Hypothetical protein	CYP	Chr_2	215770
39317	Hypothetical protein	CYP	Chr_2	763909
41910	Histone deacetylase	CYP	Chr_4	1683739
44970	P-loop containing nucleoside triphosphate hydrolase protein	CYP	Chr_5	2361793
46183	Alpha/Beta hydrolase fold	CYP	Chr_6	2298204
46274	Hypothetical protein	CYP	Chr_6	298760
47349	SNF2 family N-terminal domain-domain containing protein	CYP	Chr_6	1098348
48442	1-aminocyclopropane-1-carboxylate deaminase	CYP	Chr_7	1886046
49547	Hypothetical protein	CYP	Chr_7	512069
53332	Enolase/allergen Asp F 22	CYP	Chr_9	1076311
54470	Fungal specific transcription factor domain-domain containing protein	CYP	Chr_10	1569263
55687	NADPH-dependent medium chain alcohol dehydrogenase	CYP	Chr_11	1513854
55918	Major facilitator superfamily domain-containing protein	CYP	Chr_11	1508249
58460	Hypothetical protein	CYP	Chr_1	2354196
58779	Hypothetical protein	CYP	Chr_1	3234901

Protein ID	Prot_name in NBB 7.2.1	Trait (MIC_{50})	Chromosome	Chromosome position
58942	Hypothetical protein	CYP	Chr_1	3682170
59293	Hypothetical protein	CYP	Chr_2	171063
59313	Hypothetical protein	CYP	Chr_2	226073
59517	Tannase/feruloyl esterase	CYP	Chr_2	788776
59684	Hypothetical protein	CYP	Chr_2	1212564
60611	Hypothetical protein	CYP	Chr_3	343914
62041	Hypothetical protein	CYP	Chr_4	1583752
62148	Hypothetical protein	CYP	Chr_4	1863745
63243	Hypothetical protein	CYP	Chr_5	2157118
63379	Hypothetical protein	CYP	Chr_6	144812
63417	WD40-repeat-containing domain protein	CYP	Chr_6	244817
63440	Nucleotide-diphospho-sugar transferase	CYP	Chr_6	301326
63834	Hypothetical protein	CYP	Chr_6	1423948
63937	Hypothetical protein	CYP	Chr_6	1699926
64151	Hypothetical protein	CYP	Chr_6	2268928
64265	Hypothetical protein	CYP	Chr_7	278466
64868	AAA family ATPase	CYP	Chr_7	1942170
65659	Putative nonribosomal peptide synthase	CYP	Chr_8	2023002
66077	Hypothetical protein	CYP	Chr_9	1150283
66915	Hypothetical protein	CYP	Chr_11	190698
67220	Hypothetical protein	CYP	Chr_11	1082595
67306	Hypothetical protein	CYP	Chr_11	1327600
67388	FAD binding domain protein	CYP	Chr_12	25138
67431	Hypothetical protein	CYP	Chr_12	135431
68119	Major facilitator superfamily domain-containing protein	CYP	Chr_1	1551005
68832	Hypothetical protein	CYP	Chr_2	26532
69911	Hypothetical protein	CYP	Chr_2	2980140
72616	Acyltransferase 3	CYP	Chr_6	303789
73510	Hypothetical protein	CYP	Chr_7	505528
74620	Cysteine desulfurase	CYP	Chr_8	1696155
74751	Leptomycin B resistance protein pmd1	CYP	Chr_8	2018875
76323	Hypothetical protein	CYP	Chr_11	1118335
76752	Hypothetical protein	CYP	Chr_12	807906
76959	Glycoside hydrolase superfamily	CYP	Chr_1	256905
76959	Glycoside hydrolase superfamily	CYP	Chr_1	256905
77635	Hypothetical protein	CYP	Chr_1	2029675
78060	Hypothetical protein	CYP	Chr_1	3163831
78168	Hypothetical protein	CYP	Chr_1	3450985
79002	Hypothetical protein	CYP	Chr_2	1207425
79810	Hypothetical protein	CYP	Chr_2	3375296
79872	Hypothetical protein	CYP	Chr_3	133328
79872	Hypothetical protein	CYP	Chr_3	133360
80019	Hypothetical protein	CYP	Chr_3	499704
82792	Hypothetical protein	CYP	Chr_6	75395
83408	Hypothetical protein	CYP	Chr_6	1696173
83683	Hypothetical protein	CYP	Chr_7	130695
83824	Amino acid permease-domain containing protein	CYP	Chr_7	499190

Protein ID	Prot_name in NBB 7.2.1	Trait (MIC_{50})	Chromosome	Chromosome position
84383	Hypothetical protein	CYP	Chr_7	1958997
84444	Hypothetical protein	CYP	Chr_7	2134543
84856	Hypothetical protein	CYP	Chr_8	1145441
86174	Hypothetical protein	CYP	Chr_10	1021059
86394	Cytochrome P450	CYP	Chr_10	1570882
86779	Hypothetical protein	CYP	Chr_11	1089966
86861	Hypothetical protein	CYP	Chr_11	1310436

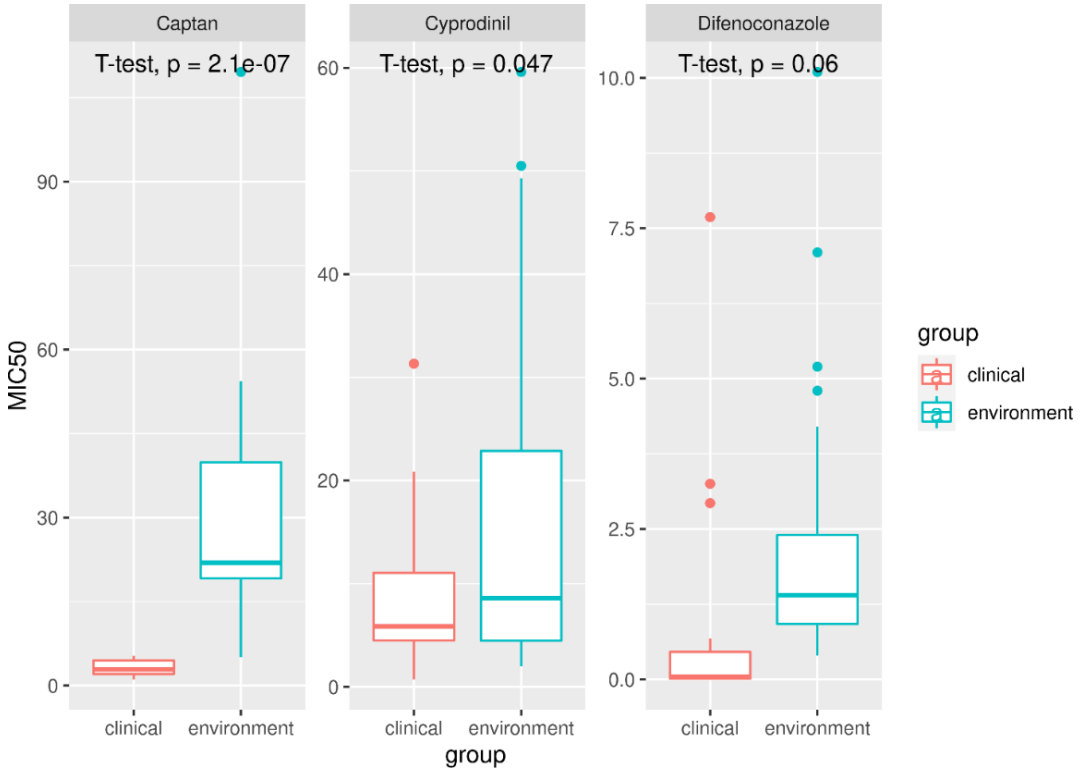


Supplementary Figure 1. User cloning

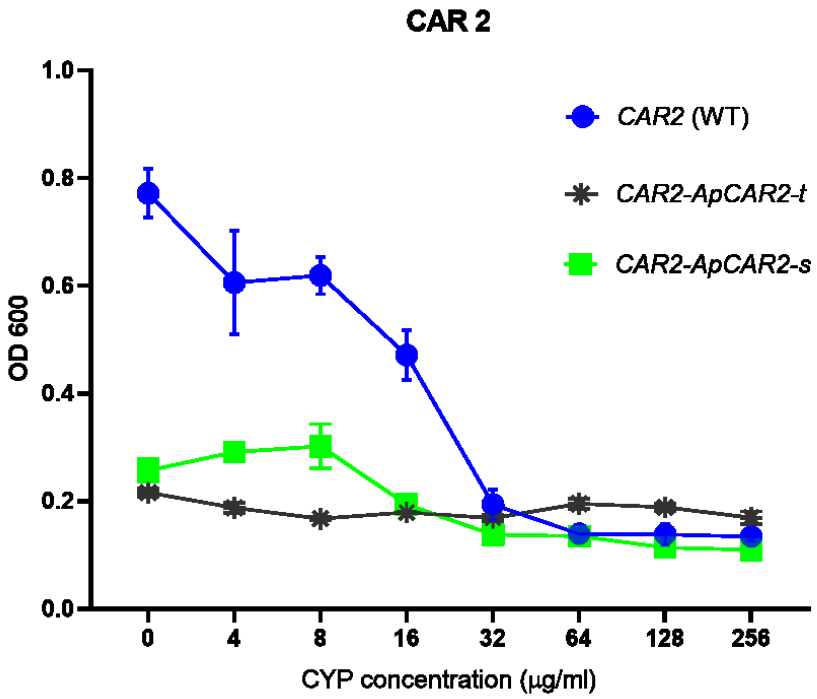
Note: unless otherwise specified all restriction and ligation enzymes and the compatible buffers were from NEB. All the plasmid description and number are found in Supplementary Table 6 and all primers used in Supplementary Table 5.

1. The oligonucleotide cassettes were annealed to each other using 1 uL each of 100 µM of USER IF and USER 1R, 1 µL T4 DNA ligase buffer and water to a final volume of 9 µL. Annealing was done at 95°C for 5 minutes, then the temperature ramped at 5°C/min to 25 °C. This was followed by simultaneous digestion of the cassette and ligation into entry vector 1 using 1 µL BSmBI and T4 DNA ligase, 1 ul of 100 ng/mL entry vector 1 (plamid 1). This ligation product was transformed into *E. coli* and plated on chloramphenicol ammended LB plates to create the USER L0 entry vector.
2. The USER Lo plasmid was purified after overnight culturing of positive clone in chloramphenicol at 37°C.
3. The plasmid was prepared for sequencing in a final volume of 15 µL (1 µL of 10 µM primer78 ,1 ul of 50- 100 µg /mL plasmid and 13 µL water) sent for sequencing at Microsynth.
4. BSA1 digestion and ligation was performed using USER Lo, promoter P (plasmid 3), terminator (plasmid 4) and assembly cassette D56 (plasmid 5). This ligation products were transformed into *E. coli* and plated on ampicillin amended LB plates to create USER L1 assembly cassette.

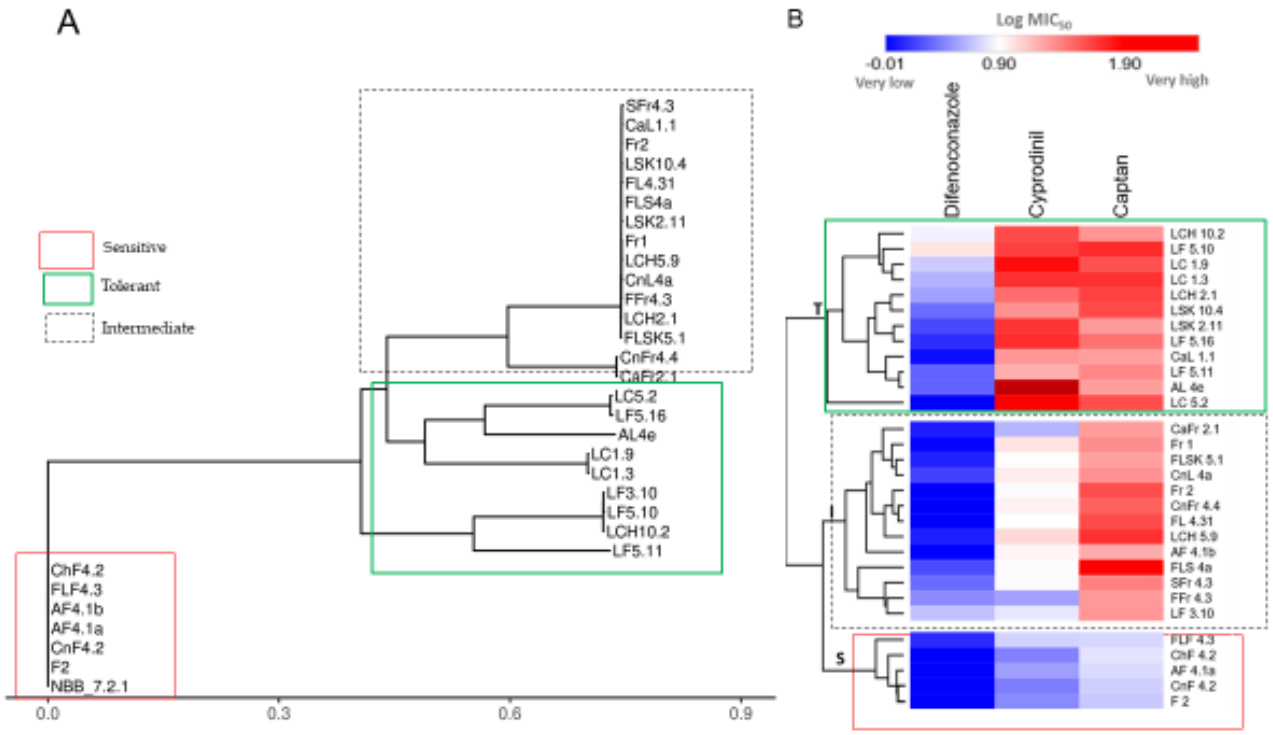
5. The plasmid was purified as elaborated in step 2
6. The purified USER L1 (plasmid 6) was prepared and sent for sequencing as elaborated in step 3
7. The verified USER_L1 cassette (Plasmid 6) was linearized by digesting 1-2 µg of the plasmid with 1 U AsiSI (37°C, 8 h, and reaction volume of 50 µL). This was followed by nicking with 1 U Nb.BsMI (65°C, 3 h) and heat inactivation (80°C, 15 min).
8. An agarose gel was run for 1h, 65V to check for complete linearization
9. The linearized vector was cleaned with the QIAquick PCR purification kit (QIAGEN, QIAGEN AG, Hombrechtikon, Switzerland) and aliquots stored at -20°C.
10. USER II enzyme treatment was performed with 10-100 ng of PCR product (amplified using USER compatible primers as shown in the right step1), 1 µg of linearised USER_L1 vector, and 1 U USER enzyme mix(37°C, 25 min), followed by inactivation (80°C, 15 min). The entire reaction was transformed into 25 µl of chemically competent *Escherichia coli* (DH5*α*) cells by heat shocking for 90 s at 42°C followed by 5 min on ice and directly plating on Ampicillin (100 µg/ml) amended LB plates and incubation overnight at 37°C. Positive clones were identified by colony PCR using cells from eight different colonies (resuspended in 20 µL of sterile water).



Supplementary Figure 2. MIC_{50} comparison of the clinical *Aureobasiudium* and environmental *A. pullulans* using T-test. The clinical isolates had a significantly lower MIC_{50} than of environmental isolates for all the three fungicides



Supplementary Figure 3. CYP dose response curve for *S. cerevisiae* strains transformed with *A. pullulans* gene homologs identified by literature search. *A. pullulans* homologs of *S. cerevisiae* CAR2 genes were expressed in *S. cerevisiae* with a sensitive(s) and tolerant (t) allele of each gene was included, and microbroth assays in CYP were performed. The endpoint OD_{600} for each concentration was determined after 24 h of growth. The curve was generated based on the mean OD_{600} of three independent experiments with three replicates and their 95% CI.



Supplementary Figure 4. A SNP-based tree of 30 *A. pullulans* genomes (A) shows groups that are comparable to the MIC_{50}-based groups (B)

Chapter 5

Competition assays to quantify the effect of biocontrol yeasts against plant pathogenic fungi on fruits

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Own contribution

- Writing the protocol

- Experiments

- Manuscript preparation

Abstract

Yeasts such as *Aureobasidium pullulans* are unicellular fungi that occur in all environments and play important roles in biotechnology, medicine, food and beverage production, research, and agriculture. In the latter, yeasts are explored as biocontrol agents for the control of plant pathogenic fungi (e.g., *Botry-tis cinerea*, *Fusarium sp.*); mainly on flowers and fruits. Eventually, such yeasts must be evaluated under field conditions, but such trials require a lot of time and resources and are often difficult to con-trol. Experimental systems of intermediate complexity, between in vitro Petri dish assays and field tri-als, are thus required. For pre- and post-harvest applications, competition assays on fruits are repro-ducible, economical and thus widely used. Here, we present a general protocol for competition assays with fruits that can be adapted depending on the biocontrol yeast, plant pathogen, type of assay or fruit to be studied.

Keywords: Microbiology, Fungi, Yeast, *Aureobasidium pullulans*, Competition assay, Antagonism, Biocontrol, Plant pathogen, Fruit.

1 Background

Biocontrol, the use of organisms to control plant pathogens, pests, and weeds, is an attractive alternative to conventional plant protection methods and can help reduce the amount of chemical pesticides employed in agriculture. Most biocontrol organisms are first evaluated *in vitro*, under laboratory conditions, but eventually will have to prove their efficacy in extensive field trials over many years. Experimental systems which more closely resemble the conditions of an eventual application than a Petri dish and are at the same time, more defined and less time- and resource-demanding than a field trial, are therefore required for both research and the development of novel biocontrol applications. The bio-protocol presented here represents a combination and adaptation of earlier methods and is specifically aimed at testing the antagonistic activity of yeasts against postharvest diseases of different fruits. Such assays have for example been used to test for antagonistic activity of various yeasts in oranges [1], cherries [2, 3], peach [4], nectarines [5], plums [6], pears [7], grapes [8] and apples [9]. Apart from antagonism testing, a similar method has also been used to assess postharvest disease resistance of apples [10]. The protocol can also be adapted for testing the combined application of biocontrol yeasts with antifungal agents, formulation compounds, plant resistance inducers, or other additives that are envisioned to improve biocontrol efficacy [11-13]

The method presented here is thus easily reproducible, efficient and extensively adaptable for testing and evaluating disease control in a variety of fruits.

2 Materials and Reagents

1. 1.5 ml microcentrifuge tubes (Eppendorf™ 3810X microcentrifuge tubes, Fisher Scientific, catalog number: 10451043)
2. 15 ml centrifuge tubes (Fisherbrand™ PP Centrifuge Tube, Fisher Scientific, catalog number: 11889640)
3. Hemocytometer cover glasses (Huberlab, 24 x 24 x 0.4 mm, catalog number: 10.0440.24)
4. Trays (Thermo Scientific™ Nalgene™ Autoclavable Polypropylene Pans, 1260 x 159 x 64 mm, catalog number: 6902-1000)
5. Sterile miracloth (autoclaved), pore size 22-25 μm (VWR, Millipore sigma, catalog number: EM475855-1R)
6. Inoculating loops, inoculating sterile disposable loops (VWR, catalog number: 612-9358P)
7. Disposable cuvettes (semi-micro cuvette 10 x 10 x 45 mm, Fisher Scientific, Greiner Bio One, catalog number: 613101)
8. Tissue papers
9. Fruit pack trays (for apple assays)
10. 30 cm ruler
11. Custom-made consisting of a wooden handle, a metal cover and a metal nail protruding 3 mm from the tip of the tool; resulting in 3 mm deep and 2.5 mm wide lesions
12. Biocontrol yeast (*e.g.*, *A. pullulans;* available from the Culture Collection of Switzerland under CCOS1008) (maintained at 22 °C on Potato Dextrose Agar plate (PDA) for 72 h)

13. Fungal plant pathogen (*e.g.*, *B. cinerea* (available from the German Collection of Microorganisms and Cell Cultures, DSMZ, under DSM 877), *Fusarium* sp. (CCOS1020)) (maintained at 22 °C on PDA for 5-7 days)
14. Whole fruits (*e.g.*, apples, cherries, plums)
15. Potato dextrose agar (CM0139B Potato Dextrose Agar [EP/USP/JP/BP] [Dehydrated], Thermo Scientific™, catalog number: 10197602)
16. Glycerol
17. 70% ethanol
18. Distilled water

2.1 Equipment

1. Light microscope (Leitz-Wetzlar; Ortholux)
2. Hemocytometer (Neubauer improved, 0.1 mm depth, Huberlab, catalog number: 10.0442.04)
3. Spectrophotometer (GE Healthcare Novaspec™ III visible spectrophotometer, Fisher Scientific, catalog number: 10773457)
4. Vortex (Ika® vortex, Genius 3, Fisher Scientific, catalog number: 10132562)
5. Micropipette P10, P1000 (Socorex: Acura® manual 825 autoclavable adjustable micropipette)
6. 3 mm cork drill (cork-drilling kits, Huberlab, catalog number: 13.1118.06)
7. Forceps
8. Wide bottomed measuring cylinder (3 L)
9. -80 °C freezer

2.2 Procedure

A. Surface sterilization and preparation of fruits

1. Wipe off any dirt from the fruits using wet tissue paper with 70% ethanol. As a standard, we use 6 fruits (replicates) for each treatment but, depending on the variability of the assay, more or less replicates may be required.
2. Line the propylene trays with tissue paper and arrange them on a clean surface.
3. Spray 70% ethanol onto the tissue paper laid out in the trays until the entire tissue surface is covered.
4. Add 1 L of 70% ethanol into the 3 L measuring cylinder.
5. Dip the fruits in 70% ethanol for 1 min (do not wipe after dipping) by hand (using surface sterilized gloves), gently turn the fruits so that the entire fruit surface is covered, carefully transfer fruits to the sterilized tray by-hand (using surface sterilized gloves).
6. Let them dry for 1 h in the sterilized trays. Label each tray with the different treatments. When using larger fruits (*i.e.*, apples), apply the label using a permanent marker pen to the fruit itself.

B. Wounding of fruits

1. Sterilize the custom-made tool using 70% ethanol and use it to create a 2.5 mm wide and 3 mm deep wound in each fruit (Figure 1A - Figure 1B).
2. Let the inflicted wound dry for 1 h.

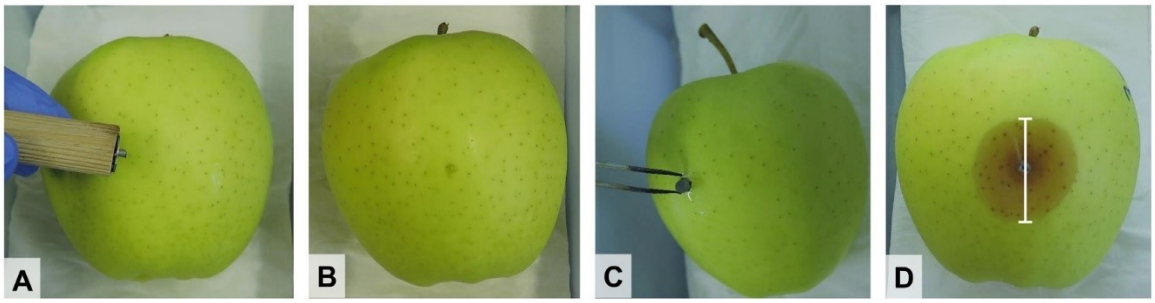

Figure 1. Wounding and infection of an apple fruit using mycelium. A. Wound infliction on an apple using a custom-made tool. B. Wound created before infection. C. Application of mycelium into the wound to start the infection. D. Measuring of the lesion at day 5 post-infection.

C. Preparation of yeast suspension

1. Defreeze the biocontrol yeast initially preserved in 30% glycerol at -80 °C. Transfer 10 µl to a PDA plate and spread using an inoculation loop. Incubate at 22 °C for 72 h.
2. Using an inoculation loop, pick 5 colonies of the biocontrol yeast cells that have been grown on PDA for 72 h.
3. Transfer the colonies into 1 ml of distilled water.
4. Vortex the suspension for 1 min or until the yeast cells solution is homogeneous.
5. Dilute the yeast suspension 10 times inside the disposable cuvettes (*i.e.*, mix 100 µl yeast solution with 900 µl distilled water).
6. Using the spectrophotometer, measure the absorbance at 600 nm (OD_{600}) and record this OD_{600} reading.
7. Adjust the cell suspension to a final OD_{600} of 2 in 1,000 µl of distilled water as follows:

$$Volume_{yeast} = \frac{Volume_{final} \times Final\ OD_{600}}{Measured\ OD_{600}}$$

Example: If the OD_{600} reading is 5.6, then 357 µl ((1,000 µl x 2)/5.6) of the yeast cell suspension and 643 µl (1,000 µl - 357 µl) of distilled water are mixed in a clean 1.5 ml microcentrifuge tube.

Note: The final OD_{600} can vary depending on the yeast, fruit, and pathogen.

D. Yeast application to wounds

1. Add 10 µl of the yeast suspension to the wound (ensure the solution is pipetted inside the wound and not flowing out).

2. Let the wounds dry for 3 h (do not move the trays to avoid yeast solution flowing out of the wound). Depending on the goal of the assay, inoculate yeasts and pathogens simultaneously or give the yeast more time to establish in the wound (*i.e.*, inoculate the pathogen after 3 h, 12 h or 1 day).

Note: Some yeasts may benefit from a longer time to establish before the pathogen is added.

E. Preparation of the fungal plant pathogen

Defreeze the fungal pathogen mycelium plugs that were initially preserved in 30% glycerol at -80 °C. Take out the mycelium plugs using sterile loops and transfer them on a PDA plate. Place 1 mycelium plug per plate (in the center of the plate) and incubate at 22 °C.

Depending on the pathogen of choice, use either conidia or mycelium for infection.

1. Preparation of conidia (e.g., *Fusarium sp.*)
   a. Let the fungus grow on 5 cm PDA plates (or the medium most suitable to the particular fungal pathogen in question) for 7-14 days depending on the fungal pathogen.
   b. Add 10 ml of distilled water onto the 5 cm PDA plate containing the pathogen.
   c. Using the inoculation loop, gently scrape the surface of the plate to suspend the conidia in the water.
   d. Filter the conidia using sterile miracloth and collect the filtrate in a sterile 15 ml centrifuge tube.

   Note: The conidia are in the filtrate, while hyphae remain in the miracloth.

   e. Transfer 1 ml of the filtrate into a 1.5 ml microcentrifuge tube.
   f. Clean the glass cover slips and hemocytometer using 70% ethanol.
   g. Place the glass cover slips over the counting chambers of the hemocytometer (apply sufficient force, so that the slide is intact and forms 'Newton colour rings').
   h. Pipette 10 µl of the conidia suspension between the fixed cover slip and the hemocytometer (pipetting slowly at the edge will allow the solution to seep through).
   i. View the hemocytometer under the microscope at magnification 125x (objective 10x, ocular 12.5x).
   j. Count the number of conidia in the large corner squares and calculate their density based on the specifications of the specific hemocytometer used.
   k. Adjust the concentration of conidia to 5 x 10^4 conidia per ml in a defined volume (1,000 µl is usually sufficient) as follows:

$$Volume_{Conidia} = \frac{Volume_{final} \times Conidia\ conentration_{final}}{Conidia\ concentration_{Calculated}}$$

   l. Example: If the calculated conidia concentration is 1 x 10^5 conidia/ml, then 500 µl (1,000 µl x 5 x 10^4/1 x 10^5) of the conidia suspension and 500 µl (1,000 µl-500 µl) of distilled water are mixed in a 1.5 ml microcentrifuge tube.

Note: The conidia can be stored in 30% glycerol at -80 °C for subsequent use. However, if stored for a longer period they should be tested for viability (plate on PDA medium and after 24 h, microscopically check for germination and growth).

2. Preparation of mycelium (e.g., *B. cinerea*)
    a. Use a 7 days old fungal culture maintained on PDA.
    b. Using a 3 mm cork drill, careful cut out mycelium plugs from the edge of the colony, where the mycelium is young (Figure 2).

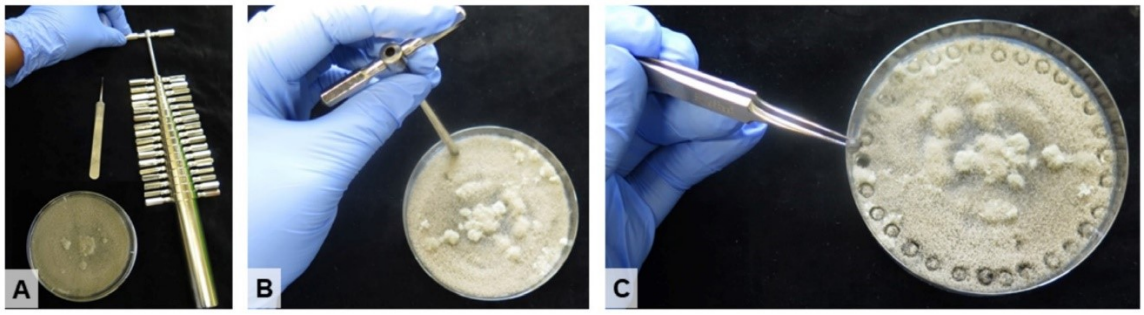

Figure 2. Preparation of mycelium plugs. A. Tools and material needed for mycelium preparation (forceps, cork drill and a pathogen growing on PDA plate). B. Using the 3mm cork drill to cut out a mycelium plug. C. A mycelium plug is being picked from the plate using forceps and will be used for wound infection.

F. Wound infection
    1. With conidia
        a. Use the conidia suspension with the adjusted concentrations for infection.
        b. Carefully pipette 10 µl of the conidia solutions into the wound and let it dry for 1 h (do not move the trays to avoid the pathogen flowing out of the wound).
    2. With mycelium

    Transfer the mycelium plug using forceps and place it upside down onto the wound (this ensures the mycelium grows into the wound (Figure 1C).

G. Incubation
    1. Maintain bigger fruits (*e.g.*, apples, oranges, nectarines) on fruit pack trays. Evenly distribute the different treatments across all boxes and cover the boxes with an autoclave bag. Keep the smaller fruits (*e.g.*, cherries, plums) in plastic incubation boxes that are tightly covered with a lid (in all cases ensure the infected side is facing upwards).
    2. Incubate at room temperature for 3-7 days.

    Note: Depending on the type and ripeness of the fruit and the pathogen, rotting and lesions will develop at different time points.

H. Evaluation and measurement
    1. Measure the diameter of the lesion/rot using a ruler perpendicularly to the stem (Figure 1D)

2. Record the measurement.

2.3 Data analysis

1. Subtract the diameter of the wound without infection or yeast from all the other recorded values.
2. Calculate the means and the standard deviation for the lesion/rot diameters of each treatment (Table 1).
3. Normalize the means of all the treatments with the 'pathogen only (PO)' treatment by dividing each average value by the average of PO treatment.

   Note: The normalized value for the PO treatment will always be 1, while the value for treatment with biocontrol yeast is ideally less than 1.
4. Statistically analyze the data (ideally, there should be a statistically significant difference between the pathogen only (PO) treatment and 'biocontrol yeast + pathogen' treatment).

Table 1. The diameter of *B. cinerea* lesions (in mm) with various treatments on apple fruits

Lesion/rot diameter (mm)									Normalized
	i	ii	iii	iv	v	vi	Mean	SD	Mean SD
Botrytis	17	17	22	20	17	16	18.2	2.3	1
Botrytis+ Yeast	2	3	2	3	2	1	2.2*	0.8	0.1
Yeast	0	0	0	0	0	0	0.0*	0	0
No infection/yeast	0	0	0	0	0	0	0.0*	0	0

*Statistically significant mean difference from the pathogen only (PO) treatment.

5. Present the data as histograms, plot the percentage relative size of the lesion Y-axis) and the different treatments (X-axis) (Figure 3).

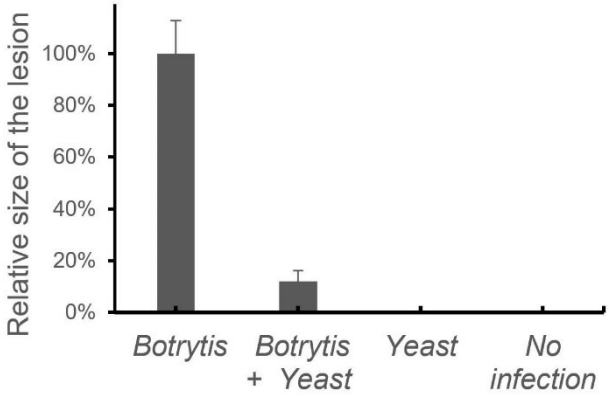

Figure 3. The inhibition of *Botrytis* lesions in apple fruits in the presence of a biocontrol yeast (*A. pullulans*). The histogram shows the relative size of *Botrytis* lesions (normalised means of six independent replicates). Addition of *A. pullulans* to the apple wound suppressed the development of lesions/rot by *B. cinerea* to 10%. Wounding alone or addition of the yeast alone did not cause the development of a lesion.

Notes

Although the normalized value for treatment with biocontrol yeast is ideally less than 1 (meaning reduction in pathogen lesion formation upon treatment with a biocontrol yeast), in some cases a value of 1 or more than 1 can be obtained. This is for the case of yeasts that have a stimulatory effect (value larger than 1) or no effect (value of 1) on the pathogen.

Declarations

Acknowledgments

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Competing interests

The authors declare no competing interests.

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Chapter 6

Synergestic effect of a fungicide tolerant *Aureobasidium pullulans* strain and low concentration of cyprodinil against *Botrytis caroliniana* on apples

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This is experimental work is part of the already published protocol annexed in Chapter 5.

Own contribution

- Binary competition assays

- Apple assay

- Writing the manuscript

Abstract

The successful commercial application of biocontrol agents depends on characteristics such as the persistence in the field, antagonistic activity, or resistance to chemicals used in the environment. The antifungal tolerance trait of yeasts could be exploited in combined yeast-fungicide applications and formulations to control diseases. Here, we evaluated the antagonistic effect of 48 yeast strains isolated in precence of antifungals and belonging to 16 different species against *Botrytis caroliniana* and *Fusarium*. This evaluation aimed to identify suitable strains for use in combined yeast-fungicide disease control experiments. We observed that all *A. pullulans* strains had an inhibitory effect against both filamentous fungi. Consequently, we selected two *A. pullulans* (a cyprodinil (CYP)-sensitive (F2) and a CYP-tolerant (LC5.2)) strains for use in combined yeast-fungicide apple assays. These experiments were specifically designed to achieve two goals. Firstly, we evaluated the potential synergy of a yeast and fungicide application (as compared to the yeast or fungicide used alone) to control laboratory-induced *B. caroliniana* lesions. Secondly, we compared the the fungicide-tolerant and -sensitive *A. pullulans* strains with respect to this synergistic effect. Our results showed that the co-inoculation of *B. caroliniana* with low concentrations of the tolerant or sensitive *A. pullulans* isolates (using 1.06×10^5 cells/ml, ≈ OD 0.067) did not affect lesion size. Similarly, treatment with 0.167 μg/mL CYP alone did not cause a reduction of the lesion. However, combined treatment with the sensitive *A. pullulans* strain F2 and CYP slightly reduced the growth of the lesion, but this reduction was not significant. In contrast, the combination of the tolerant *A. pullulans* isolate LC 5.2 with CYP significantly reduced the lesion to indistinguishable levels from the water control. This study further advances the possibility of using fungicide-tolerant *A. pullulans* in integrated CYP-*A. pullulans* biocontrol products for disease management.

1 Introduction

Plant pathogens cause massive crop losses and are thus a significant threat to food security [1]. *Botrytis* spp. are important plant pathogens that infect over 200 plant species and cause massive crop damage [2, 3]. This genus comprises several species, including the most destructive *B. cinerea* and a recently isolated new species *B. caroliniana* [3-5]. These ubiquitously occurring necrotrophic fungi cause diseases (such as blue and grey mould) on several plant parts, including stems, leaves, blossoms, flowers, and fruits, leading to substantial pre- and post-harvest crop losses [3, 6]. Several processes lead to the infection and invasion of *Botrytis* spp. The members of *Botrytis* spp. produce asexual spores, which germinate upon landing on the plant surface and form an appressorium [3,8,9]. Penetration of these fungi into the plant is preceded by the pressure exerted by the appressorium, for example due to increased generation of fungal superoxoide dismutases or melanin build-up and forming a penetration peg [7-12]. Complete penetration is then achieved with the help of degradative enzymes (e.g., cutinases, cellulases, pectinases, and proteases), which break down the plant's cell wall and cuticle [7]. Apart from this process, *Botrytis* may also infect the plant through cuts, cracks, or wounds (caused mechanically or by insects) [3]. Post-penetrative processes include the production of toxic metabolites (e.g., botrydial, oxalic acid), oxidative damage, and suppression of the plant's immune defence, which eventually lead to plant cell death [13, 14].

Chemical fungicides are widely used to manage *Botrytis* infections and, so far, are the most successful means of controlling diseases caused by this pathogen [15]. The different groups of fungicides used against *Botrytis* spp. target and control various stages of the *Botrytis* spp. disease cycle [15]. For example, anilinopyrimidines (APs) (registered botryticides), target the post-penetrative stages of the *Botrytis* spp. disease cycle [16, 17]. The application rates of different fungicide groups vary, with some chemical groups being applied at higher rates (i.e., between one to twenty sprays per season) to ensure sufficient disease control [15]. This intensive use of chemical fungicides to control *Botrytis* spp. has led to detrimental effects, such as resistance development, which reduces the fungicides' effectiveness [18-23]. Therefore, fungicide resistance stewardship of chemicals used in the control of *Botrytis* spp. is necessitated to ensure continued effectiveness [24]. These recommendations include reducing the number of fungicide applications per season. In addition to maintaining the fungicide effectiveness, strategies that minimize other detrimental human and environmental effects are equally important [25-26]. All these strategies are within the EU definition of integrated pest management, which directs consideration of all available options in controlling the disease in a cost-effective way with minimalized risk to the environment, health, and resistance development of both target and non-target organisms [27].

One possible option is using biocontrol agents alone or in combination with chemical agents. Biocontrol yeasts have received considerable attention for this application [28, 29]. So far, several yeast species have been described to have biocontrol activity against pathogenic fungi, including *Aureobasidium pullulans*, *Metschnikowia pulcherrima*, *Metschnikowia fructicola*, *Rhodotorula* spp., *Candida* spp., *Hanseniaspora* sp., *Cyberlindnera sargentensis*, *Cryptococcus* sp. and *Pichia* sp. [30-35]. The advancement of biocontrol has been experienced through the formulation of biocontrol yeasts into commercial products such as BlossomProtect™ containing *A. pullulans*, and Aspire™ comprising *Candida oleophila* as the active ingredients [29,36]. Although several yeast species have already been identified as potential biocontrol organisms, ubiquitously occurring yeasts offer a vast reservoir for potential biocontrol agents that are yet to be explored [37]. Notably, some

unique characteristics are recommended for the successful use of an agent in commercial biocontrol programs. Such traits include resistance to chemicals that are commonly used in the environment [28]. Interestingly, our previous study established that many *A. pullulans* isolates are tolerant to the fungicides captan (CPN), cyprodinil (CYP) and difenoconazole (DFN) [38]. Thus, we wanted to test if some of these *A. pullulans* strains can be explored for biocontrol applications in combination with fungicides.

Combining biocontrol agents (such as antagonistic yeasts) and chemical agents is an important approach to managing diseases [36, 39, 40]. This approach aims at reducing overall pesticide application while maintaining effective disease control [28, 39-42]. The use of such combinations has been demonstrated with different yeasts and fungicides. For example, the combination of the biocontrol yeast *Cryptococcus laurentii* with thiabendazole (264 µg/mL) led to better control of *Penicillium expansum* than either thiabendazole (528 µg/mL) or yeast used alone [43]. Similarly, combined treatment of *A. pullulans* and different chemical fungicides led to an almost three times better control of *Monilinia laxa* than chemical fungicides alone [44]. Another notable example is the combination of *Rhodotorula glutinis* and vinclozolin to control *B. cinerea* [45]. Other successful combinations of biocontrol yeast and fungicides to control plant diseases are documented or extensively reviewed elsewhere [39, 40, 46, 47]. Nevertheless, limited studies show that using a chemical-resistant strain of antagonistic yeast gives better disease control than a chemical-insensitive strain. This is an untapped research area, considering that chemical resistance and compatibility are essential in considering agents for commercial biocontrol and combined fungicide-biocontrol formulations [28, 46]. Thus, this study specifically designed experiments to understand synergistic effects of combined fungicide and yeast applications in controlling laboratory-induced *B. caroliniana* lesions.

2 Material and Methods

2.1 Strains and cultivation

The 50 yeast strains used in this study are listed in Supplementary Supplementary Table S1. The two plant pathogenic, filamentous fungi *Botrytis caroliniana* (isolate EC 1.05) and *Gibberella fujikuroi* (*Fusarium*; isolate BC 8.14) [30, 48] were used. All the yeasts and filamentous fungi were maintained on potato dextrose agar (PDA) (Difco; Chemie Brunschwig AG, Basel, Switzerland) medium at 22°C. Cultures were preserved in 15% (v/v) glycerol at -80°C. *B. caroliniana* was kept in the dark to encourage sporulation.

2.2 Competition assays on agar plates

In vitro competition assays between the 50 yeast strains (Supplementary Table S1) and the filamentous fungi (*B. caroliniana* (isolate EC 1.05) and *Fusarium)* were performed as previously described [30]. The growth area of the filamentous fungi was measured four days post-inoculation using a planimeter (Planix 5, Tamaya Technics Inc., Tokyo, Japan). Three experiments were performed for all the yeasts, with three replicates for each experiment.

2.3 Fruit assays

Golden Delicious apples were used. These apples were grown under normal orchard conditions and were, after harvesting, stored at 4°C. All the apples used in the experiments were harvested from a the same orchard. Healthy apples (free of visible disease or damage) and yellow (ripe) in colour were chosen for the experiments.

Fruit assays were performed with *A. pullulans* (strains LC 5.2 and F2), CYP, and *Botrytis* (isolate EC 1.05) on apples by adapting our published protocol (Chapter 5)) [49]. Briefly, a concentrated stock solution (0.5 mg/mL) of technical-grade CYP (Sigma-Aldrich Chemie, Schweiz, Buchs, Switzerland) was prepared in methanol and diluted (1000x) in the same solvent to achieve the 3× stock concentration (0.5 µg/mL). *A. pullulans (*LC 5.2 and F2) were grown for 72 h on PDA and five colonies were picked and suspended in water. The cell density was determined using a spectrophotometer (Fisher Scientific, GE healthcare Novaspec™ III visible spectrophotometer) and adjusted to create a stock solution of $OD_{600} \approx 0.2$. The cell numbers were confirmed by hemocytometer counting and adjusted to 3.18 $\times 10^5$ cells/mL. Similarly, the *Botrytis* conidia (EC 1.05) were prepared as previously described and the final cell number was adjusted to 1×10^5 conidia/mL.

The surfaces of Golden Delicious apples (nine individual apples were used for each treatment) were sterilized as described [49]. The wounding was then done using a 3 mm diameter custom-made tool as described [49]. Immediately, inoculation and treatment with the respective solutions were carried out using the scheme outlined below (Table 1). The inoculated apples were incubated in the dark for seven days and the lesion was determined as outlined before [49].

Table 1. Concentrations and volumes of inoculation for each treatment on apples.

Treatment	**Suspensions and concentration (3X final)**			
	B. caroliniana (EC 1.05), 1×10^5 conidia/mL	*A. pullulans* (LC 5.2/ F2), 3.18 $\times 10^5$ cells/mL	Cyprodinil (CYP), 0.5 µg/mL	sterile water
B. caroliniana (EC 1.05)	10 µL			20 µL
B. caroliniana + *A. pullulans* (LC 5.2 / F2)	10 µL	10 µL		10 µL
B. caroliniana + cyprodinil (CYP)	10 µL		10 µL	10 µL
B. caroliniana + LC 5.2 + CYP	10 µL	10 µL	10 µL	
Water				30 µL

2.4 Statistical analysis

All statistical analyses were performed using Graphpad Prism (V9). The means, SD, and SEM of the growth area of the filamentous fungi were calculated for each experiment. The mean growth area of the filamentous fungi in the presence of each yeast strain was normalized to that of the filamentous fungi alone (control treatment without yeast and fungicide) and presented as a percentage of relative growth. Where 100% growth meant the growth area was identical to the filamentous fungi without inhibition by yeast and 0% meant complete inhibition by the yeast; thus, no growth of filamentous fungi. ANOVA was used to test for significant differences in the growth of filamentous fungi in the presence of different yeast strains compared to growth in the absence of a yeast. The median and the 25-75% interquartile ranges (IQR) for the nine replicates of the apple assay from one representative experiment were determined and reported according to the recommendation for reporting non-normally distributed data [50].

3 Results

3.1 All *A. pullulans* strains had strong antagonism against both *Fusarium* and *B. caroliniana* on agar plates.

To select a suitable fungicide tolerant strain for combination assays, binary competition assays of 50 yeasts against two filamentous fungal pathogens were performed. These yeast strains had previously been isolated in the presence of different fungicides (captan (CPN), chorus (CYP), slick (DFN), flint (trifloxystrobin), and amphotericin B) from either flowers, leaves or soil samples and based on species hypothesis numbers

identified as 16 individual species (Supplementary Table S2) [33]) The *A. pullulans* strain F2 was isolated without fungicides. The *A. pullulans* strain NBB 7.2.1 has already been established to have potent antagonistic activity in the competition assay experiments against *B. caroliniana* [25] and was used as a reference. The strains belonged to the following 16 species: *A. pullulans* (16 strains), *Bullera alba* (2 strains), *Candida californica*, *Coniochaeta*, *Cryptococcus*, *C. laurentii* (4 strains), *Cyberlindnera misumaiensis, Filobasidium* (2 strains), *Kregervanrija fluxuum* (2 strains), *Pichia, Rhodotorula* (6 strains), *Saccharomycopsis shoenii* (3 strains), *Schwanniomyces capriottii* (3 strains), *Schwanniomyces pseudopolymorphus, Sporidiobolus metaroseus* (5 strains) and *Wickerhamomyces anomalus* (Figure 1a).

The two species *S. metaroseus* and *S. capriottii* had strains that showed generally weak inhibition of *Botrytis* and *Fusarium* (Figure 1b). Notably, strains of *S. capriottii* showed huge variation in controlling both pathogens. For example, *S. capriottii* strain SF 5.13 strongly inhibited (3% growth) *B. caroliniana*, while it only weakly inhibited (88% growth) *Fusarium.* Thus, these strains might not be suitable for combination with fungicide for synergistic experiments.

W. anomalus, *C. californica, K. fluxuum* and two strains of *S. schoenii* strongly inhibited (less than 10% growth) both *B. caroliniana* and *Fusarium*. Interestingly, all the 16 strains of *A. pullulans* used in this experiment strongly inhibited *B. caroliniana (*to 3-5%), while *Fusarium* growth was less affected (7-26% growth area). Still, all the *A. pullulans* strains tested here showed a strong inhibition effect against both *Fusarium* and *B. caroliniana.* Generally, the inhibition of both *B. caroliniana* and *Fusarium* by all strains of the 14 species was relatively consistent, ranging from very strong to moderate inhibition. Thus, the strains from all the 14 species could potentially be used in the combined fungicide-yeast assays to control the pathogens.

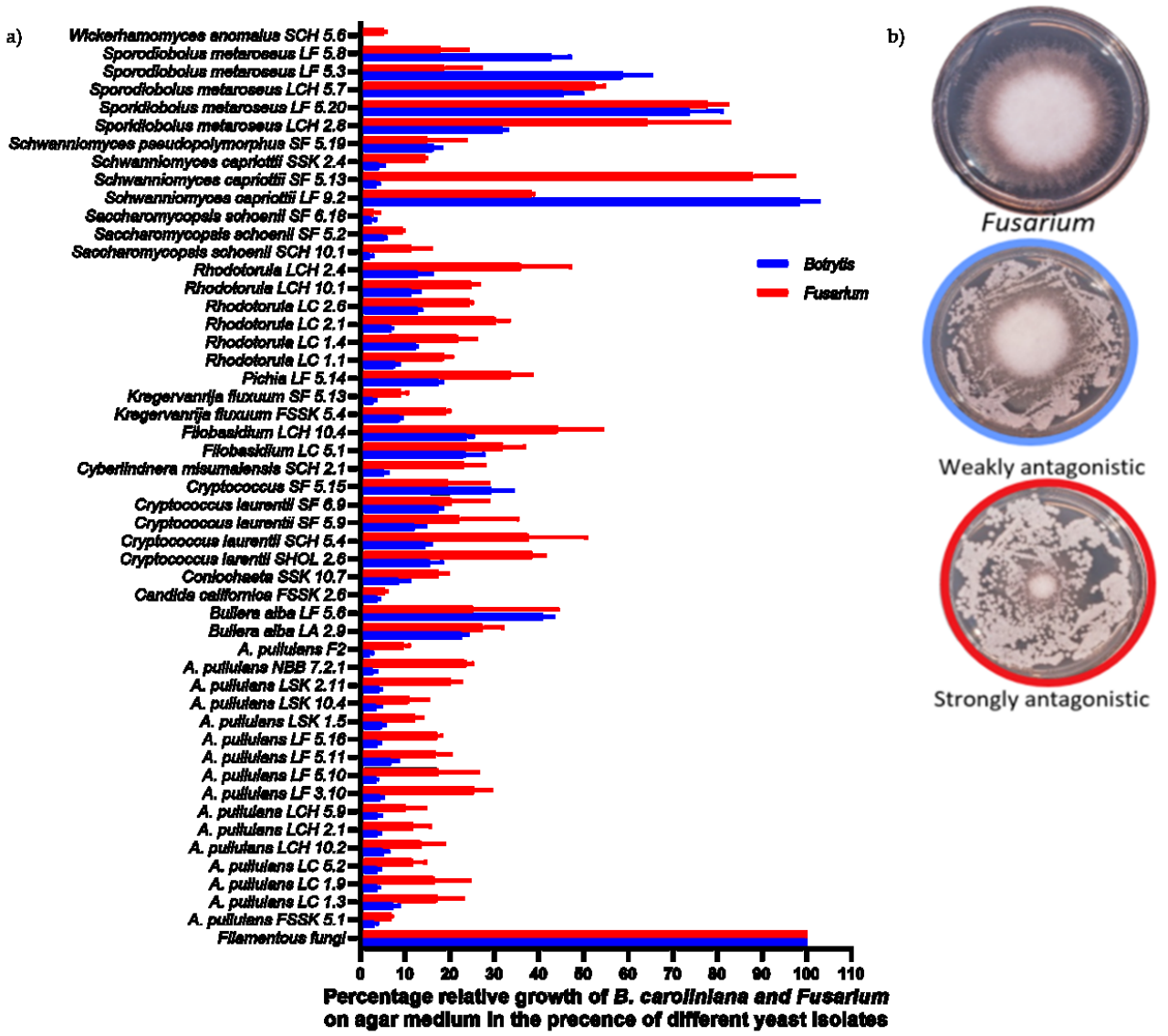

Figure 1: a) Relative growth of *Botrytis caroliniana* and *Fusarium* in the presence of 50 yeast strains (2 were isolated without and 48 with fungicides). b) Example of competition assay with *Fusarium* showing weak and strong inhibitory effect of the yeast

3.2 Low cyprodinil (CYP) concentration and a tolerant *A. pullulans* strain act synergistically against *Botrytis*

In order to test if combining a biocontrol yeast and a fungicide causes a synergistic effect, assays were performed on apples and with CYP as the fungicide. Two *A. pullulans* strains were used for these assays; a tolerant strain (*A. pullulans* LC 5.2), which had an MIC_{50} for CYP of 186 µg/mL, and a sensitive strain (*A. pullulans* F2) with an MIC_{50} for CYP of 2.8 µg/mL CYP [33]. The fungicide concentration, cell densities and inoculation times were adjusted in order to reveal a possible synergistic effect.

Our results showed that under these conditions, inoculating the apples with 1×10^5 conidia/mL of *B. caroliniana* caused brown lesions that covered approximately half of the apple surface, seven days post-infection (Figure

2a). Treatment with sterile water caused no lesion seven days post-infection and resulted in the 3mm wound that was inflicted at the beginning of experiments

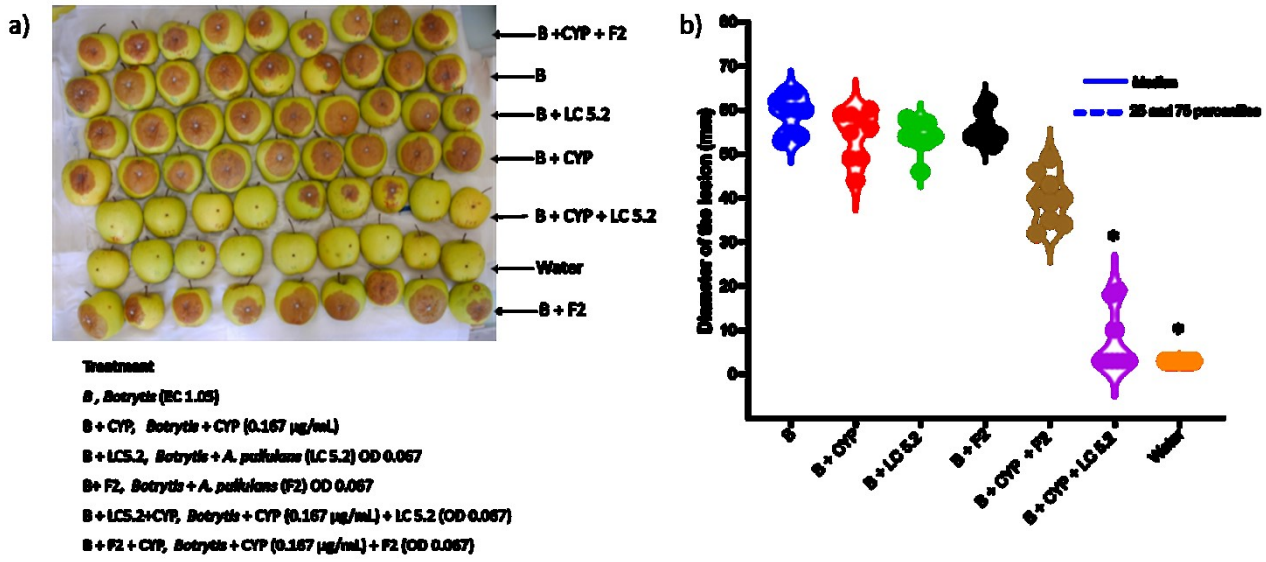

Figure 2. The combination of a low CYP concentration and *A. pullulans* LC 5.2 reduced *B. caroliniana* lesions on apples, while the use of either CYP or LC 5.2 alone or the combination of a low CYP concentration with *A. pullulans* F2 did not. a) The apples from different treatments show *Botrytis* lesions seven days post-infection. Contrarily, the apples from sterile water treatment had no lesions and only the 3 mm wound initially inflicted at day zero was visible. Six apples from the treatment with the combination of a low CYP concentration and *A. pullulans* LC 5.2 had no lesions, and three had significantly reduced lesions. b) The lesion median (full line) and 25-75% interquartile range (IQR) (broken line) for the nine replicates from each treatment were recorded. *The values for the treatment with water and a combination of a low CYP concentration and LC 5.2 were significantly different from those of *Botrytis*-only treatment.

The median diameter of the *B. caroliniana* lesion (without additional yeast or fungicide treatment) was 60 mm (IQR 55-63 mm) (Figure 2b). Co-inoculation with tolerant and sensitive *A. pullulans* isolates (LC 5.2 or F2, respectively; 1.06×10^5 cells/ml, ≈ OD_{600} 0.067) did not affect the lesion size (54 mm (IQR 53-57 mm) and 55 mm (IQR 54-58 mm), respectively). Similarly, co-treatment with 0.167 µg/mL CYP alone did not cause a reduction in the lesion (56 mm (IQR 49-59 mm). Combined treatment with the sensitive *A. pullulans* strain F2 and CYP reduced the growth of the lesion slightly (40 mm (IQR 35-45 mm), but this reduction was not significant. In contrast, the combination of the tolerant *A. pullulans* isolate LC 5.2 with CYP significantly reduced the lesion diameter to 3 mm (IQR 3-14 mm), which was statistically indistinguishable from the water control (median diameter of 3 mm; the size of the wound initially inflicted on the apples). Overall, using a fungicide-sensitive strain (F2) in combination with CYP (even at a lower concentration than what this yeast is sensitive to) did not significantly affect the diameter of the lesion. In contrast, using low concentrations of the tolerant strain LC 5.2 and CYP to treat laboratory-induced apple lesions synergistically inhibited *B. caroliniana.* Interesting to note is that the yeast cell densities were critical for being able to observe a synergistic effect. When a slightly higher cell density of LC 5.2 (OD_{600} 0.13) was used, the effect of the yeast masked the effect of CYP (0.125 µg/mL) (Figure 3). Notably, under laboratory conditions, the fungicide concentration that was required to control the lesion was high (2 mg/mL).

a) b)

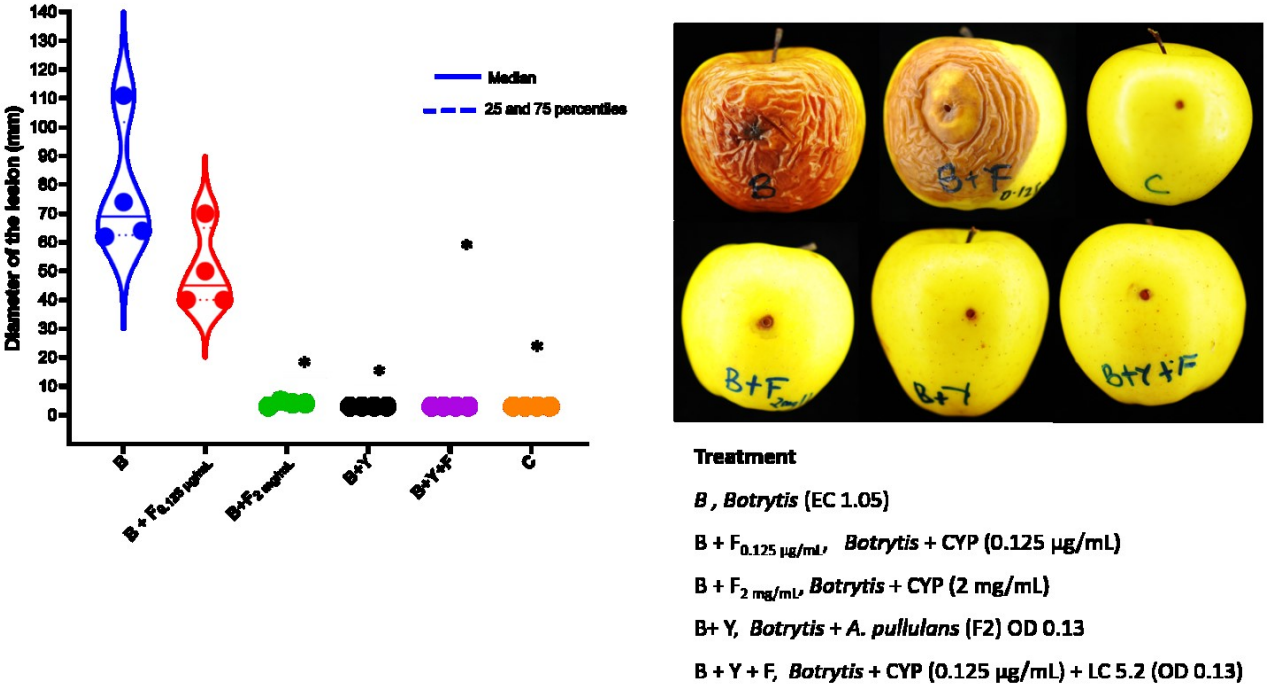

Figure 3. Application of a slightly higher concentration of yeast cells led to complete control of *B. caroliniana* lesion and masked the effect of the fungicide combination. a) The median lesion size (full line) and 25-75% interquartile range (IQR) (broken line) for the nine replicates from each treatment were recorded. *The values for the treatment with water and a combination of a low CYP concentration and LC 5.2 were significantly different from those of *Botrytis*-only treatment. b) The apples from different treatments show *Botrytis* lesions seven days post-infection.

Additionally, the time of inoculation of the pathogen was important. For example, when the yeast and fungicide were applied onto the wound 1h or 5h before *B. caroliniana* infection, the effect of the yeast was strong and the effect of CYP could not be observed (data not shown). Overall, to observe this synergetic effect under laboratory conditions, it was critical to adjust the cell and fungicide concentrations as well as the inoculation timepoints.

4 Discussion

Biocontrol agents are important for protecting plants against plant pathogens [28, 35, 39, 51]. Most of the ubiquitous yeasts species we experimented on have been documented to have antagonistic activity against plant pathogens [30, 32-35, 42, 52, 53]. However, to our knowledge, no study documents *S. capriottii* as an antagonist. Still, evaluating it for the potential antagonistic effect was interesting since many ubiquitous yeasts are yet to be identified as biocontrol agents [37]. From our experiments, S. *capriottii* only weakly inhibited both *Botrytis* and *Fusarium*, which implied that this species might not have antagonistic activity. This is likely the reason why S. *capriottii* has not been reported before. Notably, unlike previous studies, most strains used here except two (F2 and NBB 7.2.1) were previously isolated in the presence of different antifungal products [38]. Surprisingly, *S. metaroseus* showed a generally weak and inconsistent antagonistic effect against *B. caroliniana* and *Fusarium*, despite having some desirable biocontrol characteristics (e.g., stress tolerance and high activity of hydrolytic enzymes) [54, 55]. However, some studies have reported *S. metaroseus* as an antagonist. For example, *Sporobolomyces roseus*, an anamorph of *S. metaroseus*, had an antagonistic effect against *P. expansum* in apples [51]. The weak and inconsistent antagonism in different strains of *S. metaroseus* concluded that strains from this species and *S. capriottii* might not be reliable for combination with fungicide for synergistic pathogen

control. Interestingly, all isolates from the 14 other species had very strong to moderate antagonistic effects against both *B. caroliniana* and *Fusarium*, making them good candidates for use in the combined fungicide-yeast assays against pathogens.

In this study, we used CYP, which belongs to the Anilinopyrimidines (APs) group of fungicides and is registered as a botryticide [16]. A potential target of APs is the biosynthesis of sulphur amino acids or their precursor [56-59]. The addition or accumulation of some sulphur amino acids and their precursor reversed the fungitoxicity of the APs, thus inhibiting their synthesis would improve the toxicity of these fungicides [56-59]. However, the fungitoxicity of APs to *B. cinerea* by this mechanism was only achieved partially or when a high concentration (1 μg/mL) was used. Therefore, this mechanism might have little contribution to controlling *B. cinerea* [59, 60]. In addition, APs are proposed to inhibit the secretion of degrading enzymes in *Botrytis* [61, 62]. Such enzymes are involved in the later stages of *Botrytis* infection. Inhibiting the secretion of degrading enzymes reduces host cell lysis and stops the expansion of infection, thereby eradicating *Botrytis* [17]. The complex (and still insufficiently understood) CYP mode of action is probably why this compound is more effective in controlling *Botrytis* spp. in combination with other fungicides such as fludioxonil [63]. Thus, CYP appealed to us as a good fungicide candidate for combination with compatible biocontrol agents for integrated disease management. Interestingly, the application of 0.167 μg/mL CYP alone did not likely affect the *B. caroliniana* lesion because the concentration was too low. We experimentally confirmed this hypothesis by using a higher CYP concentration (2 μg/mL) which controlled the *B. caroliniana* lesion completely.

In this study we used *A. pullulans* for the apple assays. In an earlier study, we quantified the sensitivity of 30 different *A. pullulans* strains to CYP [38], which was the basis for selecting a CYP tolerant (LC 5.2) and sensitive strain (F2) (MIC_{50} 186 μg/mL and 2 μg/mL, respectively) for the experiments reported here. Further, *A. pullulans* has traits that make it successful in controlling *B. caroliniana.* For instance, *A. pullulans* is poly-extremotolerant, adheres well to surfaces (e.g., fruits) due to extracellular polysaccharides, successfully colonizes fruit surfaces, and adapts to low temperature and water potential [64-66]. For example, *A. pullulans* survived, gradually decreased in colony number, and controlled lesions on apples for up to 6 months in cold storage [64]. We also demonstrated the ability of *A. pullulans* to adapt and control *B. caroliniana* lesions of apples alone or in combination with fungicides for up to 5 months at 4°C storage (Figure S1). Although, we could not quantify the number of yeasts at the final stage. All these factors attracted our interest in this species for use in our assays and made *A. pullulans* a good candidate for future potential combined formulations to control diseases under different environments.

In our experiments, the application of *A. pullulans* (F2 or LC 5.2) alone had no effect, likely because of the low cell densities (1.06×10^5 cells/ml (≈ OD 0.067) used. Notably, when a slightly higher *A. pullulans* cell density was used (OD_{600} ≈ 0.13, for example in Figure 3), *B. caroliniana* lesions were controlled. *A. pullulans* might have utilized several reported mechanisms against *Botrytis* spp., for example, competition for space and nutrients, which is considered the primary mode of most biocontrol yeasts [42, 68, 69]. *A. pullulans* also controls *Botrytis* by secretion of cellular degrading enzymes such proteases. For example, the *A. pullulans'* alkaline serine protease inhibited spore germination of *B. cinerea in vitro* and reduced the lesions in apples [69, 70]. Additionally, *A. pullulans* produces volatile organic compounds, which inhibit *B. cinerea* conidia germination *in vitro* and reduce the growth of *B. cinerea* lesions in apples [71].

No synergistic effect of *A. pullulans* F2 and low CYP combinations was detectable, despite the use of a CYP concentration (0.167 μg/mL) below the MIC_{50} value for F2 (2.8 μg/mL). Nevertheless, the lesion spread, possibly because the low concentration of CYP was already toxic to the very low yeast cell numbers of this highly sensitivity strain. In contrast, we observed significant control of the *B. caroliniana* lesion when the CYP-tolerant isolate (LC5.2) was used even in low concentration, probably due to the yeast and fungicide synergistic mechanism. As mentioned above, the described *A. pullulans* mechanisms against *Botrytis* spp. seems to strongly target the early stages of infection (conidia germination) [69-71]. In contrast, APs are described to cause minor early control and significant control of *Botrytis* spp. at the post-penetrative stages [17, 61, 62]. Thus, CYP might employ its minor-early mechanisms against the infection, stressing the pathogen, which allows the yeast to successfully colonize and strongly employ its antagonistic mechanisms even at a lower concentration; consequently, the two work synergistically to control the pathogen.

In summary, our experiments explored the antagonistic activity of yeasts isolated in the presence of fungicides and draw attention to the strains of *A. pullulans* which all had strong antagonistic effect against *Fusarium* and *B. caroliniana*. Further, they imply that information about fungicide sensitivity of a particular yeast and screening strains for antagonism is critical for selecting and developing combined fungicide-yeast applications for plant protection. Importantly, the results here optimize the artificial laboratory system of experimentally testing synergy of combined yeast and fungicide applications in controlling apple disease. However, such systems would need to be specifically adapted for each yeast, fungicide and pathosystem. Further, it advances the possibility of using tolerant *A. pullulans* in integrated CYP-*A. pullulans* biocontrol products for disease management.

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Supplementary material

S1. Strains used

Supplementary Table S1. All the yeast strains used in this experiment, their species hypothesis number (SH) and isolating conditions

Strain	SH identification number	Species	Isolating fungicide	Sample
LF 3.10	SH1515060.08FU	*A. pullulans*	Flint (trifloxystrobin)	Leaf
LF 5.10	SH1515060.08FU	*A. pullulans*	Flint (trifloxystrobin)	Leaf
LC 1.9	SH1515060.08FU	*A. pullulans*	Captan 80 WD	Leaf
LC 1.3	SH1515060.08FU	*A. pullulans*	Captan 80 WD	Leaf
LC 5.2	SH1515060.08FU	*A. pullulans*	Captan 80 WD	Leaf
LCH 2.1	SH1515060.08FU	*A. pullulans*	Chorus (cyprodinil)	Leaf
LCH 10.2	SH1515060.08FU	*A. pullulans*	Chorus (cyprodinil)	Leaf
LCH 5.9	SH1515060.08FU	*A. pullulans*	Chorus (cyprodinil)	Leaf
LSK 10.4	SH1515060.08FU	*A. pullulans*	Slick (difenoconazole)	Leaf
LSK 2.11	SH1515060.08FU	*A. pullulans*	Slick (difenoconazole)	Leaf
LF 5.11	SH1515060.08FU	*A. pullulans*	Flint (trifloxystrobin)	Leaf
LF 5.16	SH1515060.08FU	*A. pullulans*	Flint (trifloxystrobin)	Leaf
LSK 1.5	SH1515060.08FU	*A. pullulans*	Slick (difenoconazole)	Leaf
FSSK 5.1	SH1515060.08FU	*A. pullulans*	Slick (difenoconazole)	Flower
NBB7.21	SH195774.07FU	*A. pullulans*	None	Soil
F2	Maldi-TOF	*A. pullulans*	None	Flower
LF 5.6	SH1574527.08FU	*Bullera alba*	Flint (trifloxystrobin)	Leaf
LA 2.9	SH1574527.08FU	*Bullera alba*	Amphotericin B	Leaf
FSSK 2.6	SH1569227.08FU	*Candida californica*	Slick (difenoconazole)	Flower
SSK 10.7	SH1645100.08FU	*Coniochaeta*	Slick (difenoconazole)	Soil
SHOL 2.6	SH1576892.08FU	*C. larentii*		Soil
SF 5.9	SH205045.07FU	*C. larentii*	Flint (trifloxystrobin)	Soil
SCH 5.4	SH1576892.08FU	*C. larentii*	Chorus (cyprodinil)	Soil
SF 6.9	SH1576892.08FU	*C. larentii*	Flint (trifloxystrobin)	Soil
SF5.15	SH1557457.08FU	*Cryptococcus sp.*	Flint (trifloxystrobin)	Soil
SCH 2.1	SH208527.07FU	*Cyberlindnera misumaiensis*	Chorus (cyprodinil)	Leaf
LCH 10.4	SH1631612.08FU	*Filobasidium sp.*	Chorus (fiprodinil)	Leaf
LC 5.1	SH1631612.08FU	*Filobasidium sp.*	Captan 80 WD	Leaf
SF 5.13	SH180904.07FU	*Kregervanrija fluxuum*	Flint (trifloxystrobin)	Soil
FSSK 5.4	SH1505790.08FU	*Kregervanrija fluxuum*	Slick (difenoconazole)	Flower
LF 5.14	SH487917.07FU	*Pichia sp.*	Flint (trifloxystrobin)	Leaf
LCH 2.4	SH1558727.08FU	*Rhodotorula sp.*	Chorus (cypronil)	Leaf
LCH 10.1	SH1558727.08FU	*Rhodotorula sp.*	Chorus (cypronil)	Leaf
LC 1.4	SH1558727.08FU	*Rhodotorula sp.*	Captan 80 WD	Leaf
LC 1.1	SH1558727.08FU	*Rhodotorula sp.*	Captan 80 WD	Leaf
LC 2.6	SH1558727.08FU	*Rhodotorula sp.*	Captan 80 WD	Leaf
LC 2.1	SH1558727.08FU	*Rhodotorula sp.*	Captan 80 WD	Leaf
SF 5.2	SH179232.07FU	*Saccharomycopsis schoenii*	Flint (trifloxystrobin)	Soil
SF 6.18	SH1513832.08FU	*Saccharomycopsis schoenii*	Flint (trifloxystrobin)	Soil
SCH 10.1	SH1513832.08FU	*Saccharomycopsis schoenii*	Chorus (cyprodinil)	Soil
SF 5.13	SH190095.07FU	*Schwanniomyces capriottii*	Flint (trifloxystrobin)	Soil
LF 9.2	SH1516577.08FU	*Schwanniomyces capriottii*	Flint (trifloxystrobin)	Leaf
SSK 2.4	SH1516577.08FU	*Schwanniomyces capriottii*	Chorus (cyprodinil)	Soil

Strain	SH identification number	Species	Isolating fungicide	Sample
SF 5.19	SH194739.07FU	*Schwanniomyces pseudopolymorphus*	Flint (trifloxystrobin)	Soil
LCH 2.8	SH1575137.08FU	*Sporidiobolus metaroseus*	Chorus (cyprodinil)	Leaf
LF 5.20	SH194991.07FU	*Sporidiobolus metaroseus*	Flint (trifloxystrobin)	Leaf
LF 5.3	SH1575137.08FU	*Sporodiobolus metaroseus*	Flint (trifloxystrobin)	Leaf
LF 5.8	SH1575137.08FU	*Sporodiobolus metaroseus*	Flint (trifloxystrobin)	Leaf
LCH 5.7	SH1575137.08FU	*Sporodiobolus metaroseus*	Chorus (cyprodinil)	Leaf
SCH 5.6	SH1514738.08FU	*Wickerhamomyces anomalus*	Chorus (cyprodinil)	Soil

S2. *A. pullulans* alone or in combination with trifloxystrobin (TFS) was able to control *B. caroliniana* lesions for up to 5 months in at 4°C storage conditions

We had established that 21 *A. pullulans* strains are insensitive to trifloxystrobin (Chapter 2). Thus, TFS was used alone or in combination with the fungicide insensitive *A. pullulans* strain LF5.11 to treat apples wounds infected with *B. caroliniana.* After the initial evaluation, the inoculated apples were stored at 4°C for five months to understand how long the lesion could be controlled with *A. pullulans* and in the combination treatment.

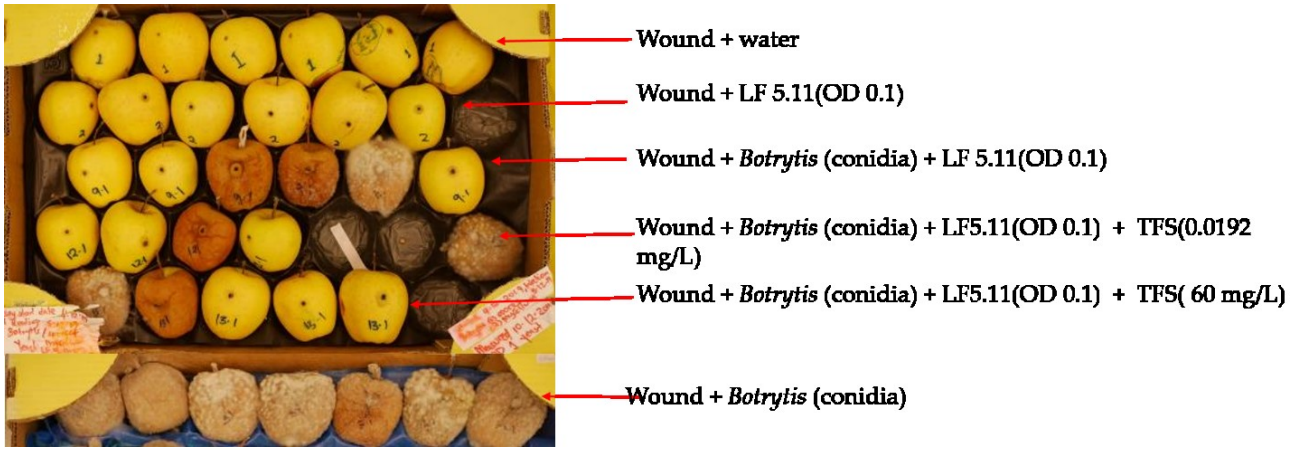

Figure S1. After the initial evaluation, the inoculated apples were stored at 4°C for five months. *A. pullulans* (LF 5.11) at OD 0.1 alone or in combination with trifloxystrobin (TFS) (0.0192 or 60 mg/L) controlled *Botrytis* lesion growth.

Chapter 7

Conclusion and future perspectives

This PhD aimed at the characterization of fungicide resistance mechanisms of naturally occurring yeasts and the utilization of their fungicide resistance characteristics for combined disease management.

There has been an increased use of fungicides, especially for two decades starting from the 1990s (Chapter 1 - Figure 1). With this increase, adverse effects, including reduced fungicide effectiveness due to resistance, have been observed. Subsequently, measures have been implemented to maintain the effectiveness of fungicides and sustain food production with minimal risk to health or the environment (as elaborated in Chapter 1). As part of these strategies, using biological control agents (such as yeasts) combined with chemical fungicides for integrated disease management has become a field of interest [1-3]. Therefore, we highlighted some unique morphological and biochemical characteristics that enable naturally occurring yeasts to interact in different ecosystems and be used as biocontrol agents (Chapter 1). It is well established that naturally occurring yeasts can tolerate different stresses due to these characteristics (Chapter 1). However, studies on yeasts' ability to tolerate fungicides were still limited. Also unknown were the mechanisms that govern these tolerances in natural yeasts. The third aspect was the challenge to combine yeasts and-fungicides for plant disease management. Thus, the key results on these aspects will be presented in three parts and future research potential areas will be highlighted.

Naturally occurring antagonistic yeast have high tolerance to commonly used antifungals

The results in chapter 3 answered the questions of how many and which natural occurring yeast could be isolated in the presence of antifungals and how tolerant *A. pullulans* isolates are to some of these antifungals. MALDI-TOF and fungal ITS region sequencing were used to identify fungal taxa in this study. Our results established that many naturally occurring yeasts can be isolated in the presence of antifungal agents: 376 yeasts belonging to 44 different taxa were identified (Chapter 3). Strikingly, most of the yeasts that were readily isolated and in high frequency in the presence of antifungals (such as *Aureobasidium pullulans, Metschnikowia pulcherrima, Cryptococcus laurentii, Cyberlindnera misumaiensis,* and *Sporidiobolus metaroseus)* are also commonly isolated and tolerant to different stresses [4-7]. In view of that, we argued that stress tolerance and abundance go hand in hand. One of the polyextremotolerant yeasts that was highly abundant in our study was *A. pullulans* (76 strains). Due to its high abudance and isolation in all the antifungal agents, the degree of sensitivity of *A. pullulans* to different antifungal agents of this species was interrogated. We established that many *A. pullulans* strains could tolerate very high concentrations of captan (CPN), cyprodinil (CYP), and difenoconazole (DFN).

These results move toward reassuring that the use of recommended dosages of fungicides in the environment might not kill or eradicate some beneficial yeast such as *A. pullulans.* Therefore, fungicides might not affect the plants' microbiome involving yeasts. Still, it would be noteworthy to investigate how fungicides affect yeasts and other plant microbiome players. Further, our study highlights that the *A. pullulans* strains tolerate much higher concentrations than the established baselines in pathogenic fungi (Chapter 3). Since the reduction in recommended dosages might select for resistance in plant pathogens [8], we infer that *A. pullulans* could even be combined with chemical fungicides at the recommended dosages for controlling plant pathogens. However, this would increase costs and it is not clear if better disease control could be achieved. Further experiments could dissect the degree of antifungal sensitivity in other commonly occurring yeasts. Additionally, our study only covered three groups of antifungal agents to understand the degree of sensitivity; further studies could include more antifungal agents.

Further, chapter 3 of this study interrogated if isolation in a specific fungicide meant low insensitivity to that particular isolating antifungal. We highlighted the tolerance of individual *A. pullulans* strains to multiple fungicides and documented that the highest tolerance was not for the isolating fungicide (Chapter 3). From this observation, we construed that *A. pullulans'* mechanism of tolerance to fungicides could be related to overall stress tolerance. We confronted this question in chapter 4. Noteworthy is that not all *A. pullulans* were tolerant, which implied that fungicide tolerance differences in naturally occurring yeasts, even within species, do occur. This observation opens the question of whether antifungal resistance in natural yeasts results from fungicide selection or generally increased competitiveness due to the innate stress tolerance ability of some strains within a species. These hypotheses highlighted the importance of characterizing the tolerance mechanisms in naturally occurring yeasts and call for more research into this area.

GWAS, literature and functional characterisation uncover the tolerance mechanism in *A. pullulans*

As a first step to understanding the antifungal resistance mechanisms in *A. pullulans*, we reviewed the documented and characterized or predicted mechanisms with a focus on Anilinopyrimidines (APs), demethylase inhibitors (DMIs), the quinone outside inhibitors (QoIs), and captan (Chapter 2). In this review, it became evident that fungi continually develop mechanisms to evade the antifungals, such as altering the target site, promoter changes and genome plasticity resulting in upregulation of the target site, overexpression of efflux pumps, which decreases intracellular drug accumulation, and differential expression of stress response pathways. Additionally, we highlighted that these resistance mechanisms did not develop exclusively in target pathogenic fungi, but were also present or predicted in non-target fungi such as *S. cerevisiae* [9-11]. Moreso, we evidenced that unique antifungal resistance mechanisms could emerge in non-target fungi as in the case of DMIs use and the emergence of resistance *Aspergillus* spp. in the clinical setting [12-15]. In light of this information from chapter 2, our study questioned the presence of antifungal tolerance mechanisms that are yet to be described in the non-target species *A. pullulans* (Chapter 4). Additionally, the questions of whether other non-pathogenic fungi develop resistance that is unique to them and whether this resistance has a functional role in plant or human disease control also became clear.

To characterize resistance mechanisms in *A. pullulans*, we used sensitivity and genome data from 46 *A. pullulans* isolates (Chapter 4). Our study highlights the genetic involvement in fungicide tolerance of *A. pullulans* by SNP-based phylogeny using 46 *Aureobasidium* genomes, which clustered groups that mostly reflected the fungicide sensitivity phenotypes (sensitive, tolerant, or intermediate) determined in chapter 3. Similarly, the comparable clusters based on ITS sequences had already been reported (Chapter 3), reemphasizing the genetic determination of the fungicide sensitivity phenotype in *A. pullulans*. Therefore, this study employed a genome-wide association study (GWAS) to predict genes involved in the tolerance of *A. pullulans* to CPN, CYP, and DFN using these fungicides' phenotype sensitivity data. First, we questioned the presence of the already reported mechanism (chapter 2) in *A. pullulans.* Our GWAS, however, did not find any significant single nucleotide polymorphism (SNPs) in the previously reported genes. This lack of SNPs implicated other new or different mechanisms employed by *A. pullulans* in fungicide tolerance.

Noteworthy, our GWAS predicted 4955 significant SNPs within the coding region of several genes, which correlated to *A. pullulans* tolerance to CYP. However, only five SNPs in four genes were predicted for CPN tolerance. No SNPs were predicted to associate with tolerance to DFN despite our observation in chapter 3 of reduced sensitivity in *A. pullulans*. This implied that the low *A. pullulans* sensitivity could be explained by

other non-genetic adaptations or other gene changes that do not involve aneuploidy, SNPs or insertion and deletion (indel) polymorphisms (which are captured in GWAS). Thus, further studies could try to look at other such mechanisms (e.g., epigenetic changes) to dissect the underlying tolerance mechanisms of *A. pullulans* to DFN. Overall, these results highlight the effectiveness of such predictive tools to link MIC_{50} phenotype data to genetic changes.

Further, our study experimentally tested the GWAS- and literature-predicted genes by either heterologously expressing *A. pullulans* genes in *S. cerevisiae* or using *S. cerevisiae* gene deletion strains. The study then characterized the generated strains' sensitivity to CYP using microbroth sensitivity and agar spot assays (Chapter 4). Overall, our results provide experimental evidence for a function in CYP tolerance mechanisms for 10 new genes. Five of these genes (*LYS2, ENO1, HOL1, CAC2* and *STL1*) had homologs in *S. cerevisiae* and were tested with deletion strains. The five genes without homologs (*FBD* (Protein ID 43145), *HP5* (Protein ID 73510), *HP6* (Protein ID 78168), *HP8* (Protein ID 86861), and *GST* (Protein ID 41311)) were heterologously expressed in *S. cerevisiae* and also affected CYP sensitivity in this model organism. The complex CYP tolerance mechanisms in *A. pullulans* are implicated by these genes' diverse functions in fungi, including; substrate transporters (*GST, STL1, HOL1*), F-box domain protein (*FBD*), protein kinases (*HP5*), metabolic functions (*ENO1, LYS2*), and chromatin assembly (*CAC2*). At the same time, two genes were hypothetical with unknown functions (*HP8* and *HP6*).

Our observation argued that the mechanisms involving the genes *GST, HP5 STL1, HOL1,* and*CAC2* are generalized fungicide resistance mechanisms that might confer multi-drug/chemical tolerance. Thus, it implicates a non-targeted CYP resistance development in *A. pullulans* isolates. Moreso, from our studies, we can hypothesize that the proteins encoded by *FBD, ENO1,* and *LYS2* might interact in other known or undescribed pathways. However, whether these interactions work in a targeted way against CYP remains to be observed and needs further analysis. Therefore, future research in understanding CYP tolerance could focus on understanding the different levels of interdependence among these genes or other already predicted mechanisms by co-expressing two or more genes. Further, an interesting area to pursue would be the practical implication of our characterized tolerance mechanisms in plant and human pathogenic fungi. For instance, if such mechanisms are also present in pathogenic fungi and if they cause antifungal resistance to APs or other antifungal agents.

Combination of *A. pullulans* and chemical fungicide is a viable option for disease management

This study's ultimate goal was to exploit the tolerance characteristics of yeasts for combined disease management strategies, as was demonstrated in chapter 5. Therefore, possible challenges and success for future use of the biocontrol yeast in potential combined formulations were considered during this study. Our study results highlighted most of these recommended characteristics, such as effectiveness at low concentration, rapid colonization of the fruit wounds, broad antagonistic activity, tolerance to commonly used environmental chemicals, and non-pathogenicity [16] as will be presented below.

The tolerance of environmental *A. pullulans* isolates to commonly used fungicides was demonstrated in chapter 3, thus implicating the attractiveness of *A. pullulans* as a biocontrol agent. Notably, some studies have considered *A. pullulans* as an emerging pathogen in immunocompromised populations, although in most cases, it is a contaminant [17-20]. In such cases, the biosafety concerns for successful use of *A. pullulans* in

formulations that end up in the environment and food system would be raised. Thanks to the clinical isolates we received from Swiss hospitals and culture collections, this study clarified the misclassification of some clinical *Aureobasidium* isolates by clustering seven of the 16 clinical isolates with other species of *Aureobasidium* (Chapter 4). We argued that most of the clinical strains do not belong to *A. pullulans,* but to one of the other three species that were recently reclassified (e.g., *A. melanogenum*, *A. subglaciale*, or *A. namibiae)* [21, 22]. However, whether these clinical *Aureobasidium* isolates are contaminants or pathogens remains unclear and needs further investigation. Future work could investigate these strains by molecularly assessing virulence genes [23] and, infecting sterile cells with them to understand their pathogenic capacity. Additionally, our results recorded lower fungicide tolerance of all the clinical *Aureobasidium* as compared to the environmental isolates (Chapter 4). These results led us to question the origin of clinical isolates and we argue that they might be from a fungicide-naïve environment. Thus, more population studies are needed to understand the ecological background of these clinical strains.

Further, the results herein (chapter 4) highlighted the multi-drug resistance phenotype of *A. pullulans*, making this species a good candidate for combined formulation in disease control. For instance, the tolerance mechanisms we elucidated in chapter 4 identified genes that might confer multi-drug/chemical tolerance in *A. pullulans i*solates. Furthermore, the multi-drug resistance was also evidenced by the GWAS identification of genes with significant SNPs associated with tolerance to both CPN and CYP (Chapter 4). These results emphasize the multi-chemical tolerance ability of *A. pullulans,* which implies that *A. pullulans* could be used in combination with several fungicide formulations. However, more studies are needed to understand the possible compatibilities. The high competitiveness and broad antagonistic activity of *A. pullulans* were demonstrated in binary competition assays with two pathogenic fungi (Chapter 5). Markedly, our study not only reaffirmed the antagonism of *A. pullulans,* but also established the use of fungicide-tolerant *A. pullulans* as an antagonist.

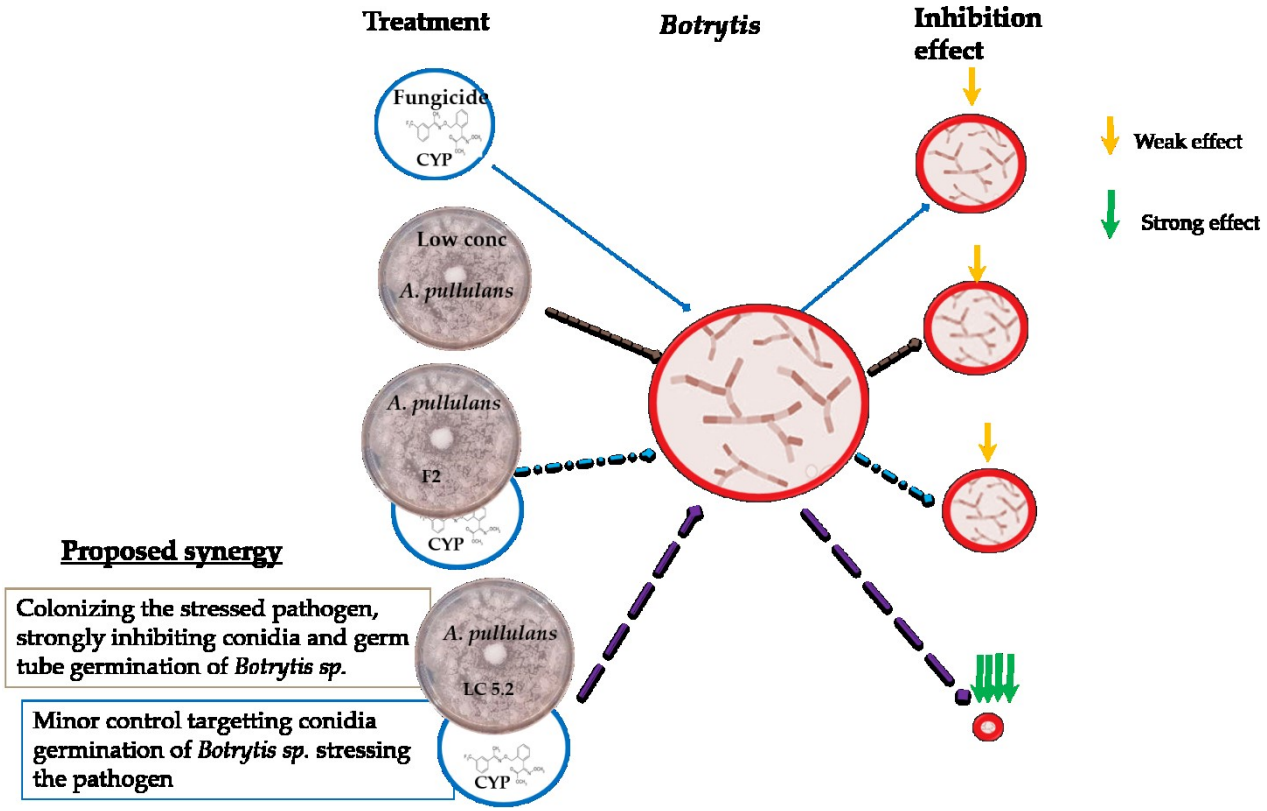

Figure 1. Proposed synergy between fungicide-tolerant *A. pullulans* and an anilinopyrimidines (CYP) in controlling *Botrytis spp.*

Strikingly, we demonstrated a synergistic effect when combining a fungicide-tolerant *A. pullulans* isolate and CYP in bioassays with apples (Chapter 5). We optimized an artificial laboratory system where fungicide-tolerant *A. pullulans* and a low CYP concentrationcould synergistically control *B. caroliniana* (Figure 1). Contrarily, a fungicide-sensitive *A. pullulans* could not achieve the same synergistic effect. Based on these results, we argued that information about the fungicide sensitivity of a particular yeast and screening strains for antagonism is critical for selecting and developing combined fungicide-yeast applications for plant protection. The underlying mechanisms for the synergistic effect we observed is not entirely clear. So far, studies have shown that *A. pullulans* targets the early stages of *Botrytis* spp. infections (i.e., conidia germination) [24-26]. At the same time, APs cause minor control in the early infection stages of *Botrytis* spp., while significant control by APs for this genus works at the post-penetrative stages [27-29]. Therefore, our study infers that the control of *B. caroliniana* might be due to minor-early control mechanisms by the fungicide against the infection, stressing the pathogen. Thus, the yeast can aggressively colonize and employ its antagonistic mechanisms on weakened conidia even at a lower concentration (Figure 1).

Nevertheless, our research leaves some open questions:

1. We hypothesized the possible synergistic mechanism that leads to disease control; hence, further research needs to characterize the synergism between the fungicide and yeast in controlling *Botrytis* spp. lesions of apples.

2. The system we used here is purely artificial and specific to *Botrytis* spp. Therefore, such systems would need to be specifically adapted for each yeast, fungicide, and pathosystem. Also, once this is achieved, scaling up this system to a practical application example, greenhouse testing, would be a logical step.

3. Our study demonstrated the possibility of using fungicide-tolerant *A. pullulans* in integrated CYP-*A. pullulans* biocontrol products for disease management. However, our results are only at the preliminary stage of any biocontrol product development based on the generalized pipeline of developing a biocontrol product [30]. For instance, our study achieved the following steps in product development; the isolation, identification and screening of the antagonist *(A. pullulans)*, the molecular characterization of the chemical resistance for compatibility with fungicide and *in vitro* and fruit bioassays at a laboratory scale. Still, more experiments and work are needed until the practical application of the system we optimized in this study reaches the stages of technology development and transfer and, commercialization.

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Curriculum Vitae

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Education

- 2018 – 2022 PhD in Microbiology, Agroscope & ETH Zürich, Switzerland
  Mechanisms and importance of naturally occurring antifungal resistance in yeasts
- 2017 – 2018 MSc in Molecular Life Sciences, Institute of Plant Science, University of Bern, Switzerland
  Principal subjects: Plant physiology; Genome size and cytological study of *Eragrosits spp*
- 2013 – 2015 MSc in Crop Biotechnology and Entrepreneurship, University of Nottigham, Kuala Lua-pur, Malaysia
  Principal subject: Crop biotechnology (biotechnology technology transfer project)
- 2007 – 2011 BSc in Biochemistry, Egerton University, Nairobi, Kenya
  Principal subjects: Biochemistry and molecular biology (major) and botany (minor)

Employment history

- 2015 – 2017 Curriculum / content development consultant at 3viewGroup, Singapore & Kenya
  Manage and supervise a team of instructional designers; Oversee the creation and development of educational and training content; Research & development reporting; Proposal preparation for public and private corporations.
- 2015 Research assistant, Advanced agriecological research sdn bhd, Malaysia
  Evaluate the protocol for detection of coconut cadang-cadang viroid (CCCVd) in oil palm
- 2011 – 2012 Laboratory analyst, Spectre International, Kenya
  Microbial fermentation of alcohol; microbial and chemical analyses in raw water; Monitoring vital parameters in bioreactors during fermentation and analysis of waste water; Reagent preparation and calibration of equipment.
- 2010 Internship, Kenya Agricultural Research Institute, Kenya

Awards

- 2021 Best poster award for the best poster at the Agroscope PhD/Post-DOC Symposium 2021
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Publications

- Magoye, E., Hilber-Bodmer, M., Pfister, M., & Freimoser, F. M. (2020). Unconventional yeasts are tolerant to common antifungals, and *Aureobasidium pullulans* has low baseline sensitivity to captan, cyprodinil, and difenoconazole. Antibiotics, 9(9), 602.
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