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Evaluation of Antagonistic Activity Potential in *Lachancea* Strains Against Grapevine Pathogenic Fungi

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Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

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Abbreviations

AUC Area Under the Curve

BLAST Basic Local Alignment Search Tool

DWG Days Without Growth (spore germination)

ER Extension Rate

IAC Inhibition Based on Area Under the Curve

IER Inhibition of Extension Rate

IRG Inhibition of Radial Growth

MSP-PCR Microsatellite/Minisatellite Primed-PCR

NCBI National Center for Biotechnology Information

OD Optical Density

RG Radial Growth

WL (Wallerstein Laboratory) Nutrient Agar

YPD Yeast Extract–Peptone–Dextrose Medium

Abstract

The grapevine crop *Vitis* spp. has a major worldwide economical relevance but is susceptible to several fungal phytopathogens. This threat is exacerbated by virtue of conventional chemical control measures becoming less desirable or outright banned as pathogens acquire resistances. Moreover, concerns are raised by the public and regulatory agencies regarding their negative impact in health and the environment. In consequence of that, biological control alternatives have gained attention as well as the study of their antagonist mechanisms on which the disease biocontrol potential relies. Yeasts, capable of potent antagonism, being ubiquitous in all environments, easy to cultivate and generally regarded well in terms of biosafety, are a prime target for research as biocontrol agents. The *Lachancea* genus encompasses yeasts with increasing relevance in the winemaking industry following a switch in focus for non-*Saccharomyces* yeasts as well as the oenological properties they are responsible for, through the production of compounds such as lactic acid and volatile compounds. This project highlights a high variability of antagonistic responses observed in a collection of *Lachancea* sp. strains against common grapevine pathogens *Aspergillus* sp., *Botrytis* sp., *Penicillium* sp. and *Mucor* sp. Some strains displayed specificity of action towards one target, whereas other strains boasted from a broad-spectrum antagonistic potential. Investigation of microbial volatile organic compounds and biocontrol agents in general is still in its infancy and this work aims to shed light on the potential for biocontrol present in yeasts with remarkable oenological relevance towards phytopathogens that afflict the winemaking industry.

Keywords: phytopathogens; biological control; yeasts; microbial antagonism; grapevine disease; volatile organic compounds

Resumo

As plantações da vinha *Vitis* spp. têm uma enorme importância económica a nível mundial. Não obstante, esta é uma cultura fortemente afetada por fungos fitopatogéneos. Esta tendência tem-se acentuado com o acréscimo de resistências por parte dos fungos causadores de doença, mas também pela proibição de produtos convencionalmente usados no seu controlo, face à elevação do escrutínio público e de entidades reguladoras devido aos seus impactos na saúde e no meio ambiente. Assim, elevou-se um crescente interesse em alternativas para esse controlo, com especial foco na utilização de microrganismos e no estudo dos seus mecanismos antagonistas. As leveduras exibem forte potencial antagonista, são ubíquas por todos os ecossistemas, fáceis de cultivar e geralmente consideradas seguras, tornando-se alvo de grande interesse na procura de alternativas aos métodos convencionais. O género *Lachancea* engloba leveduras com grande relevância na indústria vinícola, graças a um recente foco em leveduras não-*Saccharomyces* e ao interesse nas propriedades enológicas que conferem aos vinhos através da produção de ácido lático e compostos voláteis. Neste projeto realçam-se respostas altamente variáveis dentro de uma coleção de estirpes de *Lachancea* sp. face a fitopatogéneos comuns da vinha: *Aspergillus* sp., *Botrytis* sp., *Penicillium* sp. e *Mucor* sp. Foram descritas estirpes com uma atividade especifica para um alvo, como também estirpes com antagonismo global. A investigação de compostos voláteis orgânicos e de agentes de biocontrolo ainda está a dar os primeiros passos. Este trabalho procura conseguir um vislumbre no potencial para biocontrolo presente em leveduras pertencentes a um grupo com notável relevância enológica.

Palavras-chave: fitopatogéneos; agentes de biocontrolo; leveduras; antagonismo microbiano; doenças da videira; compostos voláteis orgânicos

1. Introduction

The potential of yeasts as antagonistic biocontrol agents, the mechanisms for doing so, as well as the various fungi and pathologies caused in grapevine (*Vitis vinifera*) organs will be the key concepts presented in this introduction. There will also be some considerations regarding current agricultural practices, related trends and whether these factors could impact an adoption of yeasts as a biocontrol agent for widespread use.

1.1. Economic relevance of grapevine and impact of fungal diseases

Yeasts are ubiquitous in all environments and play an important role in vineyards dedicated to the winemaking enterprise. This is due to yeasts making up the microbiota of grapevines as well as the soil they exist upon which will impart distinctive characteristics in the final fermented wine product (Amerine and Kunkee, 1968; Zarraonaindia, 2015). Furthermore, yeast selection is part of the modern-day process of winemaking (Suaréz-Lepe and Morata, 2012). As such, when selecting for antagonistic yeasts, competition with existing microbiota should be considered, as well as the impact such yeasts can have on the downstream processing of the grapevine berry into the final product. Not only that, but also how the application of such biocontrol could affect fermentation and the product's sensorial features. Fruits destined to be served at the table without processing, however, should not have such risks or considerations associated with biocontrol application, and thus could be an effective way to increase postharvest shelf life without disrupting the product itself. The importance of biocontrol agents for conservation at this postharvest step is supported in a report by the Food and Agriculture Organization in which the average loss of fresh fruits and vegetables during postharvest, distribution and consumption was deemed to hit a figure as high as 29% in Europe, North America, and Oceania. Such figure rose to 39% in industrialized Asia, Southeast Asia, Africa, and Latin America (FAO, 2011). Although some improvements have been made in this regard according to a more recent report from the same entity (FAO, 2019), it still indicates that the loss of fruits and vegetables made up over 20% of the food loss from post-harvest to distribution in 2016. Although there are many different underlying reasons that can contribute to this waste, such as inadequate handling, harvesting, and marketing, the main culprit is conservation, as there can be improvements made during transportation and packaging. At a retail level too, biocontrol agents can potentially extend shelf life (FAO, 2019).

An added argument in favour of adopting a more biological control-based action is the public attitude and environmental concerns towards the use of pesticides, as well as the development of powdery mildew strains resistant to different fungicides. This has led to a reduced appeal of chemical conventional methods and, consequently, to the search of alternative control methods against powdery mildews. The use of oils, salts and plant extracts capable of inducing mildews are in focus as desirable alternatives (Bélanger and Labbé, 2002, as cited by Fondevilla and Rubiales, 2012; Zhang *et al.,* 2007).

To elaborate on the previous statements, there are some shortcomings when it comes to biocontrol studies by usually being done in an isolationist manner. This limits the interaction to a species of interest against the target pathogen relevant to the study. Furthermore, such studies are carried out within the highly controlled environment of a laboratory. These factors can lead to the efficacy of an antagonist as it was expressed in the lab to translate into an only marginal efficacy in the field (Droby *et al.* 1992). It is, therefore, important to understand the mechanisms involved in the antagonistic response as well as their expression in *in vivo* field conditions, which take into consideration other factors such as competition for nutrients and space within the pre-existing microbiota. To that end, this review will also expand on some published mechanisms involved in antagonistic action by yeast, and other properties that are relevant for explaining the importance yeast can have as biocontrol agents. Such properties can be compared against other organisms like bacteria, to assess what exactly can set them apart when it comes to biocontrol potential in the field and ease of use (Droby and Chalutz 1994; Freimoser *et al.* 2019; Spadaro and Droby 2016; Wisniewski *et al.* 2007).

The rise in concern for major phytopathogens of the grapevine *Vitis vinifera* was marked by the introduction into the European crops of the powdery mildew and downy mildew back in the 19th century. The consequence of this was the following years being periods of intensive use of chemical products for protection of this valuable crop. Such practice carries on to this day in vineyards, with over 3000 publications since 1910 revolving around studies regarding this issue (Gessler, 2011).

Grapevine trunk diseases, as well, are considered destructive and very harmful for the sustainability of the winemaking heritage. These diseases cause the death of vines and prompts their replacement, affecting both mature and newly planted vines alike. Examples include Esca, Eutypa, *Botryosphaeria* dieback, Petri disease or Black-foot disease (involving species from *Campylocarpon, Cylindrocladiella, Dactylonectria, Ilyonectria* and *Neonectria* genera), all causing considerable economic loss to the industry. The ban of efficient treatments such as sodium arsenite, carbendazim and benomyl in the early 2000s escalated the impact of such diseases (OIV, 2016). Mechanisms for these are still under study. In the case of Esca disease, it is hypothesised that a microbial association between *Fomitiporia mediterranea* and *Phaeomoniella chlamydospora* is essential for the development of white-rot necrosis and with the onset of Esca. Such studies have provided insights into the switch in *P. chlamydospora* that causes it to adopt a pathogenic lifestyle in response to biotic and abiotic factors (Bruez *et al.,* 2020). These abiotic stresses too, play a major role in most of the diseases that will be explored in this review, either by directly favouring their proliferation or indirectly, by increasing crop vulnerability to disease. Another important consideration is the effect that climate change can have on arthropod migration patterns and biodiversity balance, since these organisms are important vectors for pathogens (Moyo *et al.,* 2014; Songy *et al.,* 2019).

Understanding the diseases that can affect the grapevine is important to further the advancements regarding biocontrol alternatives and what sort of diseases can be prevented with such approaches. Some are viral in nature with a genus of importance named *Vitivirus* (with over 60 grapevine-infecting viruses detected) which derives its name from the grapevine host *Vitis* spp. (Du Preez *et al*., 2011), while other diseases are fungal in origin. The latter will be the focus of this thesis, with diseases such

as powdery mildew (*Erysiphe necator*) and downy mildew (*Plasmopara viticola*) which affect the foliage and diseases such as grey mould (*Botrytis cinerea*), black rot (*Guignardia bidwellii*), black mould (*Aspergillus* section *Nigri*) and blue mould (*Penicillium* spp.) which affect mainly the grapes (Gadoury *et al.* 2011a; Gadoury *et al.* 2011b; Gessler *et al.* 2011; Pitt and Hocking, 2009; Varga *et al.* 2014; Wicht *et al.* 2012; Williamson *et al.* 2007;).

1.1.1.Powdery mildew (*Erysiphe necator*)

The etiological agent responsible for powdery mildew in grapevine, *Erysiphe necator*, is an obligate biotrophic fungus, meaning it required living plants as sources for nutrients (Gadoury *et al.,* 2011a). It has had a profound effect on the evolution of disease management due to the global importance of this crop and its susceptibility to the disease. Powdery mildew first spread throughout Europe in 1845 despite being first described in North America, leading to the introduction of copper and sulphur as ways to treat and prevent this disease in Europe (Agrios, 2005).

The disease is noticeable macroscopically as young colonies are easily visible, resembling a whitish powdery coating over the leaf surface until they coalesce, as shown in Figure 1 (DPIRD, 2020). Colonies can also appear singly if not coalescent, exhibiting a circular morphology; senescent colonies will appear more greyish. When infection reaches a severe state, the leaves can undergo necrosis, falling prematurely. Although this disease is mostly referenced as targeting leaves, young fruits can also be susceptible (Gadoury *et al.* 2011a, 2011b)

Some of the pathogen's growth characteristics could be useful when used to compare with a potential antagonistic agent being selected. To that end, the growth and sporulation interval has been reported as ranging from 23 to 30°C, with the optimum value being 26°C (Delp, 1954). However, more recent findings showed that temperatures higher than previously reported could still allow for growth, albeit at a reduced rate from 30°C to 34°C during a period of 24 hours, and with lethality effects occurring when exposure would exceed 36°C up to 44°C, at progressively lower intervals of exposure (e.g. for temperatures of 44°C, the predicted lethal exposure duration was 0.5 h, 1 h , and 1.1 h for spore germination, spore production, and colony growth, respectively) (Peduto *et al.* 2013). Additionally, exposure to radiation is also a common inhibitor of growth of *Erysiphe necator* and it is also vulnerable to this exposure as leaf surface temperature, when subjected to direct sunlight, can be 10 to 15°C higher when compared to shade (Austin, 2010; cited by Gadoury *et al.*, 2011a). The action of temperature and sunlight conditions will then result in a synergistic effect, inhibiting the growth and sporulation of this pathogen. However, shade management in a grapevine field can never be perfect and as such, leaves under shade would still be vulnerable. The proposal here is that a potential antagonistic yeast should be suited to compete and or be capable of inhibiting this fungus when applied to shade conditions.

The main concern regarding this pathogen is that, although *Vitis* as a genus is comprised of very diverse taxa, the main commercial grape production is concentrated within the species *Vitis vinifera*, a species that is native to Europe and as such, it evolved in isolation from *E. necator* which originated

from North America (Agrios, 2005). As such, *V. vinifera* proved to be highly susceptible to powdery mildew disease, even across the many different cultivars, whereas other species from the same genus in North America displayed varying degrees of resistance (Pearson and Gadoury, 1992). It is, then, important to understand what natural resistance does the host exhibit and what do we currently know regarding antagonistic agents against the *E. necator* pathogen, if any of them are yeasts and what mechanisms are decisive for their action.

We currently understand that the mechanism by which this pathogen is capable of germinating in the epidermis of photosynthesis-active tissues is by the secretion of fungal lytic enzymes, such as lipases, esterases and cutinases (Feng *et al.,* 2009). The action of these enzymes will facilitate the penetration into the cell wall of a peg that emerges from the appressorium (which is a specialized, flattened hyphal cell) and the act of invading the host cell will lead to the rise of an haustorium, a rootlike structure that absorbs water and nutrients from surrounding structures. In turn, the haustorium acts as an intracellular interface for the exchange of molecules: the parasite is able to extract hexoses, amino acids, vitamins, and other nutrients while simultaneously secreting host defence-inhibiting proteins. Afterwards, the fungus will continue to spread across the surface through the proliferation of hyphae, all the while producing more haustoria sites on the host's tissues (Delp, 1954). On the host side of things, the main strategies revolve around resisting penetration by the pathogen or programmed cell death (PCD) which can be useful on epidermis that has already been penetrated and works by blocking the nutrient supply line to the pathogen via death of invaded cells, restricting the ability of the fungus to grow (Qiu, 2015). It could be further elaborated on resistance genes present in *Vitis* species, but the relevancy of that approach would be via means of genetic engineering through the introduction of new genes into the cultivated plants or removal of susceptibility genes. Such approach would be outside the scope of biocontrol agents that are extrinsic to the cultivar itself and are meant to have a broad efficacy.

When it comes to commercial agents for the control of *E. necator*, the AQ10 Biofungicide® is composed of a proprietary strain of the fungus *Ampelomyces quisqualis*, which exhibits a modest efficacy against the disease. However, when in field conditions, *A. quisqualis* commonly parasitizes mildew colonies late in the epidemic cycle, which does not eliminate the need for earlier fungicide sprays (Falk *et al.* 1995; Gadoury *et al.,* 2011a). Several antagonistic fungi have been tested against powdery mildews as reviewed previously (Kiss, 2003) but the prospects, although promising, revealed that biocontrol of powdery mildews remained a challenge and more recent literature regarding specifically the use of biological control for *E. necator* has led to the isolation of three mycoparasites identified as *Lecanicillium antillanum, Acremonium sclerotigenum* and *Sarocladium terricola* and all three of them were positive for the production of β-1-3 glucanase, cellulase, chitinase, protease, amylase and lipase which are involved in bio-control mechanisms (Ghule *et al.,* 2019). Furthermore, the same study established that these antagonists were compatible with chitosan and sulphur. Thier joint action resulted in an increased efficacy against the *E. necator*-caused disease, when being used at later, high risk periods of the vineyard season. Beyond the secretion of enzymes, the mycoparasites exhibited a mechanism that consisted in coiling around the pathogen's hyphae that

would lead to the collapse of *E. necator* conidia and, most interesting in the presented study, was that the application of these antagonists was promising in not only lab conditions, but also in field conditions.

Figure 1. Macroscopic representation of powdery mildew resembling white powder which corresponds to coalescing fungal over the surface of the leaf. All rights reserved to DPIRD, accessed June 2021

1.1.2. Downy mildew (*Plasmopara viticola*)

Downy mildew spread throughout Europe in 1878, a few years after the appearance of *Erysiphe necator* in 1845 which also originated from North America. Both were proposed to have been introduced to Europe via American grape cuttings used to replant the French vineyards destroyed by phyloxera (Agrios, 2005; Gessler *et al.,* 2011).

The current strategy for disease control in Europe relies on the massive use of pesticides during the growing seasons, which, as previously mentioned, raises public concerns regarding health and environment safety (Bélanger and Labbé, 2002). Economically, pesticide use accounts for one of the largest shares for total cost (Koçtürk and Engindeniz 2016), in a world that is shifting to more sustainable agricultural practices as opposed to conventional practices, such as the rise of regenerative agriculture as a concept across academia (Toensmeier, 2016). Indeed, a shift in consumer trends has been hypothesized in the study carried out in Francy by Fuentes Espinoza *et al.* (2018), in which consumers were willing to trade quality for environmentally ethical winemaking at minimal economic loss for the producer. Much like is the case with *Erysiphe necator*, *V. vinifera* is susceptible to *Plasmopara viticola* and breeding programs have been conducted to introduce resistance factors to the grapevine (Figueiredo *et al.,* 2012). However, despite these efforts, the pathogen has also been able to breakthrough resistant varieties, proving that monogenic resistances are not enough to grant immunity to a crop that is meant to remain in the field for dozens of years,

even when the pathogen in Europe has lower genetic variability when compared to the population of North America (Peressotti *et al.,* 2010).

Nevertheless, volatile organic compounds (VOCs) have been proven to be effective against downy mildew symptoms with no phytotoxic effects and mechanisms ranging from direct inhibition of microbial growth, induced resistance, and associational resistance. An interesting case of associational resistance is the emission of VOCs by resistant plants promoting the resistance in neighbouring plants (Lazazzara *et al.,* 2018). Specifically, monoterpenes and sesquiterpenes demonstrated greater measures of emission in resistant grapevine genotypes when compared to susceptible cultivars such as the Pinot noir (Algarra, 2015).

An antagonistic agent has already been found for this pathogen, in the *Acremonium* genus that encompasses endophytic fungi of *Vitis vinifera* (Lo Piccolo *et al.,* 2015). Endophytic fungi can infect living plant tissues with no symptoms of disease arising, instead forming a mutualistic association. Although the presented study did not explore the mechanisms of this endophyte on the inhibition of *P. viticola*, the *in vitro* results did show strong antagonistic action by *A. persicinum* and *A. sclerotigenum*, with mentions of other *Acremonium* species also possessing similar degrees of inhibition. Naturally, *Acremonium* is not a unicellular fungus, yeast, and these findings were not reproduced *in vivo* nor was the underlying mechanism for antagonism identified. Nonetheless, it is important research in the field of biological control alternatives to conventional chemicals.

Figure 2. Visual representation of downy mildew signs in leaves, characterised by the occurrence of “oil spots”. All rights reserved to DPIRD, accessed June 2021

1.1.3. Grey mould (*Botrytis cinerea*)

The airborne plant pathogen *Botrytis cinerea* is not an exclusive pathogen to *V. vinifera* and is responsible for serious losses in over 200 crop species worldwide and, although there are fungicides for its control, the genetic plasticity of this pathogen has led to the failure of these control products. *B. cinerea* boasts from a variety of “modes of attack”, diverse inoculum sources, and survival capabilities as mycelia and/or conidia as well as sclerotia (compact masses of mycelium) in plant debris. All of this has cemented *B. cinerea* as an important necrotrophic fungi model for molecular study and a single control measure is unlikely to succeed against it. As such, it is paramount to understand the interactions between the pathogen and its host, the surrounding microenvironment, and competitors (Williamson *et al.,* 2007).

The symptoms of this disease manifest with some variance across different hosts, but in the case of grapevine, the berries become soft and watery with wet spots along the surface. As growth conditions become ideal for the fungus, its growth starts to envelop the berries. This induces a colour change in the berry’s skin as well as a mummification process giving the berries a shrivelled appearance (**Figure 3**). This fungus can also infect other organs of the grapevine, such as leaves that turn brown and necrotic.

The disease cycle of this pathogen in grapevine can be seen on Figure 4, which shows how the fungus overwinters in dead plant and fruit tissue, waiting for the ideal sporulation conditions in spring where new infection cycles occur, and the disease is then capable of developing through the whole growing season. Young shoots, leaves and flower blossoms are most susceptible to these infections, but also in older vines whose berries are wounded, which is to mean the berries were damaged and the skin broken through by abiotic factors or the action of animals like birds, insects, or even other diseases such as powdery mildew (Goldammer, 2018).

Figure 3. Visual representation of bunch rot caused by advanced fungal growth of *Botrytis cinerea* in infected grapes. The berries have become shrivelled and mummified. All rights reserved to DPIRD, accessed June 2021

Control of *B. cinerea* usually involves agronomic practices (e.g. physical protection of the vines) in combination with chemical treatments. One research (Pertot *et al.,* 2017*)* revealed a promising biocontrol efficacy delivered by different microorganisms applied to the crop at different stages: *Trichoderma* spp., a genus of fungi that is a good coloniser of dead plant tissue and, therefore, could be ideally applied at bunch closure stage, in order to colonise flower waste trapped in the bunch where *B. cinerea* is very capable of surviving, before it begins infection during ripening of the berries; *Aureobasidium pullulans,* a yeast-like fungus, which was applied when the formation of sugar increased in the berries, during early ripening stage, in order to compete with *B. cinerea* on cracks or wounds that would result from bunch compression; finally, *Bacillus* spp., gram-positive bacteria, would be applied close to harvest which is when a swift and strong action against *B. cinerea* is required the most and the antifungal metabolites and lipopeptides produced by these bacteria would be crucial for this stage.

This study was very important as it demonstrated a potential in biocontrol competence *in vivo* could be increased depending on the timing of application and with a joint action of more than one microorganism applied sequentially throughout the season. Which unfortunately, was a strategy that was receiving little attention. This would circumvent some issues that arise in mixtures of different biocontrol agents such as mutual antagonism or incompatibilities in action. Although the study alone will not prove that timing and sequential application of biocontrol agents is essential for dealing with diseases, it certainly shows great potential and perhaps a step further in the difficult process that is turning these strategies into generalised applications across large swathes of crop land, capable of replacing conventional methods for disease control.

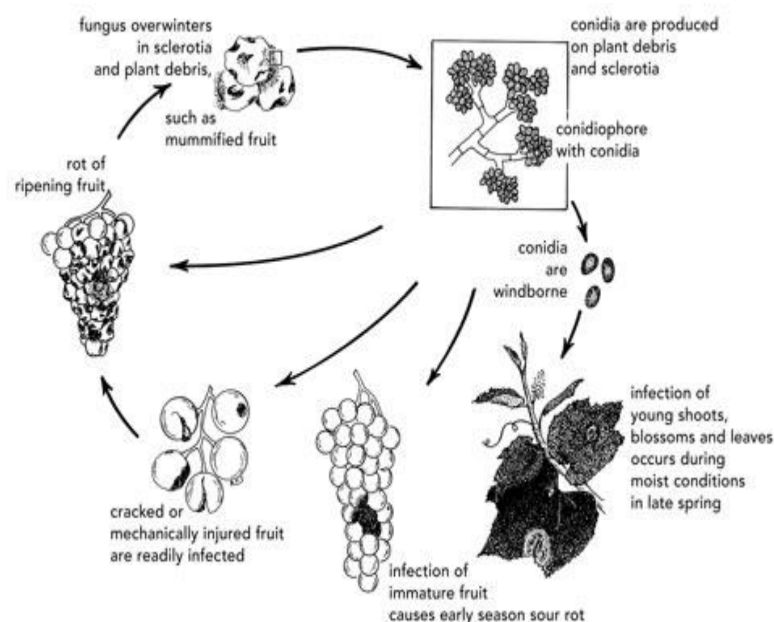

Figure 4. A representation of *Botrytis cinerea* disease cycle, where the fungus is overwintering on dead plants and fruit tissues with a production of *spores* (conidia) that are dispersed in spring via wind, allowing the fungus to infect new tissues. Young plant tissues are particularly vulnerable. All rights reserved to eVineyard, accessed June 2021

1.1.4. Black rot (*Guignardia bidwellii* and *Aspergillus* spp.)

Guignardia bidwellii, an ascomycete, is the etiological agent responsible for black rot which affects mainly grapevine (*Vitis* spp.) but also other hosts from the Vitaceae family (*Perthenocissus* spp., *Ampelopsis* spp., *Cissus* spp.). It is native to North America and was introduced to Europe through infected plant hybrids in the late 19th century (Miller 1968, cited by Wicht *et al.* 2012). The disease occurs especially in regions characterized by a humid growing season, and it spreads through a broad range of grape cultivars. Nowadays, grape black rot is considered one of the major fungal diseases affecting vineyards in several countries since it reduces plant health and can cause crop losses up to 80% (Harms *et al.* 2005, Jermini and Gessler 1996; cited by Wicht *et al.* 2012).

Black rot, caused by members of the genus *Aspergillus*, in grapes is characterized by masses of brown or black spores across the surface of the berry's skin (Figure 5). Beyond the negative impact such disease can cause in terms of crop loss, its significance increased when black *Aspergillus* were recognised as potential sources of ochratoxin A (OTA) (Abarca *et al.*, 2001; Ponsone *et al.,* 2007). A very important mycotoxin for human and animal health due to its implication in a range of toxicological effects, such as nephrotoxicity, immunotoxicity, and mutagenicity and (Pfohl-Leszkowicz and Manderville 2007, cited by Ponsone *et al.,* 2011). As such, the control strategies for *Aspergillus* section *Nigri* pathogens are of great importance as it allows for the control of the previously mentioned mycotoxin and its introduction into the food chain.

With that in mind Ponsone *et al.* (2011) aimed to select potential antagonistic agents to *Aspergillus* species from the native yeasts in Argentinian vineyards and out of a selection of 28 yeast strains, 9 showed antagonistic activity, being capable of reducing growth rate in the evaluated *Aspergillus* section *Nigri* isolates (*Aspergillus carbonarius* and *Aspergillus niger*) by about 40% *in vitro* and they were identified as belonging to *Kluyveromyces thermotolerans*, *Zygosaccharomyces fermentati, Cryptococcus flavus* and *Candida valdiviana*. It was shown that this antagonistic activity depended not only on the involved target and yeast but also in the temperature and humidity levels. Much like with Pertot *et al.,* 2017, it is clear that there are more factors to consider when using biocontrol agents than simply the formulation of the product itself.

Figure 5. A photograph in which the black spores of *Aspergillus* sp. are visible across the surface of grapes in a bunch. All rights reserved to DPIRD, accessed June 2021

1.1.5. Blue mould (*Penicillium* spp.)

Penicillium, although a well-known genus due to the capability of certain species to produce penicillin, being a very important finding that revolutionised medicine, is also one of the most common fungi associated with food spoilage that can happen at refrigeration temperatures. in contrast to other diseases mentioned, it occurs at the postharvest handling stage of the fruit's production and distribution cycle (Pitt and Hocking, 2009).

A study by Assaf *et al.* (2020) tested the use of yeasts as potential antagonistic agents for *Penicillium* species isolated from a selection of grapes showing blue mould signs, which are characterized by a discoloration on the skin of the grape, turning them brownish, making the tissue soft and eventually the coloured spores begin to spread across the surface. There was a total of 16 *Penicillium* isolates obtained, and 96 yeasts isolated from fermenting musts and the surface of healthy grapes (53 and 43, respectively). The antagonism assays were conducted *in vivo* and showed the positive activity for antagonism in 20 yeast isolates that included strains from various species: *Aureobasidium pullulans, Cryptococcus magnus, Metschnikowia pulcherrima* and *Rhodotorula glutinis*. Also, very relevant in this particular study was the temperature at which the assays were conducted to mimic postharvest conditions of the produce, something the author stated was not done in previous studies that had the assay temperatures higher than refrigeration levels.

Figure 6. A photograph depicting the effects of a *Penicillium* sp. infection in grapes, characterised by a mass of coloured spores. All rights reserved to DPIRD, accessed June 2021

1.2. Advantages of using yeast as biocontrol agents

For an organism to be considered as an active ingredient for a biocontrol product as, it must be effective against the targeted culprit of a certain disease. Although many different organisms can be considered to have such properties, other secondary characteristics can be very important for the safety and commercialization of the product itself. These properties can then be related to biosafety, the production and maintenance of cultures, formulation, and application options. It is when these characteristics are taken into account that the differences between bacteria, filamentous fungi and yeasts are highlighted, and that in itself can help to weigh the advantages and disadvantages of each of these microorganisms to better determine what makes yeasts important in biocontrol applications.

When it comes to culturability, both bacteria and yeasts have advantages, whereas filamentous fungi do not: bacteria due to a facilitated adhesion and biofilm formation which grants competitive leverage as well as persistence in the environment; yeasts also possess a single-celled morphology which allows for better culturability in fermentors and application options due to the lack of filamentous growth that is present on its hyphal fungal counterparts (Pandin *et al.* 2017; Rossouw *et al.* 2018). This should be enough to exclude filamentous fungi from further considerations, so only bacteria and yeasts will be contemplated in the following comparisons. Something that distinguishes yeasts from bacteria is the common presence of plasmids in the latter and general absence in the former. This provides an advantageous characteristic in favour of yeasts to be used in biocontrol products as yeasts mostly have barriers to pass on or acquire plasmid-based pathogenicity factors, toxin synthesis genes or resistances to antibiotics. Consequently, allowing for a more predictable usage

and a more stable product with minimal genetic variation. In terms of biosafety, yeasts are commonly associated with food and beverage products, being consumed either through these food items or directly as supplements, awarding yeasts with a safe connotation and lower concerns when it comes to the addition of these microorganisms in foodstuffs (EFSA, 2005). This safe reputation, however, does not invalidate the fact that some species of yeast are indeed important human pathogens, namely those in the *Candida* genus such as *Candida albicans, Candida tropicalis, Candida glabrata* and *Candida parapsilosis* (Kim and Sudbery, 2011; Wingard, 1995; Li *et al.* 2007; Trofa *et al.* 2008; Silva *et al.* 2012), as well as *Cryptococcus* (May *et al.*, 2015). There are also some valid concerns to be had over yeasts capable of displaying dimorphism (such as switching to an invasive growth form when under specific conditions) and resistance to fungicides, which are aspects that should be studied thoroughly before considering yeasts as potential new isolates for biocontrol (Freimoser *et al.* 2019; Gauthier, 2015, 2017).

1.3. Antagonistic activity mechanisms

As previously mentioned, understanding the mechanisms by which a yeast can exert a biocontrol activity upon a target pathogen is important to make an informed decision and to have a reliable biocontrol agent product. As an interest in yeasts for their antagonistic properties increases, so too should the number of studied species. Their interaction mechanisms down to the molecular level, as well as the most relevant genes which could potentially allow for a higher reliance in predictive studies into the potential of these biocontrol agents. As such, some of these mechanisms may revolve around outcompeting the targeted disease-causing fungi both in nutrients and in space (Pertot *et al.,* 2017; Ponsone *et al.,* 2011); the production of toxins, volatile organic compounds (abbreviated as VOCs) (Algarra, 2015; Lazazzara et al., 2018;), and/or enzymes (Ghule *et al.,* 2019); mycoparasitism against the target or induction of resistance in the plants afflicted by the fungi-induced disease (Daguerre *et al.* 2014; Glick, 2015; Xu *et al.* 2011).

1.3.1.Competition

Outcompeting a pathogen can be translated into a need to pre-emptively colonise crops with the chosen biocontrol agent to occupy the plant surfaces and exhaust the available nutrients, rendering the pathogen unable to grow, but competition can also occur during a cycle of infection via using an antagonistic agent that is able to rapidly outgrow the infecting pathogen and prevent the disease from increasing in severity (Glick, 2015). As it will be demonstrated in an example ahead within this introduction (Pertot *et al.,* 2017), the use of biocontrol agents that act through competition could require extra considerations than simply formulation. This would be a necessity to conciliate the pathogen’s infection cycle and the crop’s growing stages and how the pathogen could rely on or attack in a particular stage. During which the conditions could be ideal for an antagonistic agent which can rapidly grow and use sugars in the environment, as opposed to an antagonistic agent that would

be better suited for a pre-emptive colonisation of vulnerable tissue. However, not only timing plays a big role during competition strategies, but a polyphasic action using different biocontrol agents could prove to have an increased efficacy when compared to a joint action of the same antagonistic agents at the same time during the crop's growing season.

It is important to understand that such a process is difficult to study mechanistically and the results in antagonism assays can vary wildly when comparing *in vitro* trials against *in vivo* trials during which the microorganisms activate diverse survival mechanisms that can tip the scales in physical niche competition (Freimoser *et al.,* 2019). Field conditions also differ in the aspect that resources and physical space are more limited, and although yeasts grow well on agar plates, large differences in their antifungal activities can be observed (Hilber-Bodmer *et al.,* 2017; cited by Freimoser *et al.,* 2019). Competition can revolve around specific nutrients, but biofilm formation is also associated with competition strategies and this phenomenon can be observed in yeasts, since it is a strategy that revolves around microbial communities living within a surface and can be made up of a single species or several different species in a consortium. Typically, a secretion of an extracellular matrix, the formation of pseudo hyphae and certain modification to the cell wall (Costa-Orlandi *et al.*, 2017).

One case in biocontrol yeast biofilm formation is *Pichia fermentans* which has exhibited a dimorphism depending on nitrogen source, making it useful on apples to protect wounds from postharvest diseases, but the same yeast in peaches switches to a hyphal growth that causes rapid decay of inoculated fruits (Giobbe *et al.,* 2007).

1.3.2. Enzyme secretions and mycoparasitism

Enzymes capable of degrading pathogen cell wall are a common host-pathogen interaction and may be a necessary prerequisite for mycoparasitism strategies (Spadaro and Droby, 2016) and such enzymes are, therefore, of increased interest when it comes to their biocontrol potential.

Important enzymes are chitinases which have already been studied thoroughly in filamentous fungi and bacteria, which have demonstrated antagonistic activity against phytopathogenic fungi by their ability to degrade chitin. Additionally, several yeasts have expressed this activity such as those of the *Aureobasidium, Candida, Pichia* and *Saccharomyces* genera. Glucanases also share similarities to chitinases in that they degrade glucans, major cell wall components in fungi, play a role in cell adhesion and killer toxin resistance – proteins that can bind to receptors of the target microorganism, causing its death through target-specific mode of action (Mannazzu *et al.,* 2019). The lipolytic activity of lipases has also been associated with screened biocontrol yeasts and may prove to be a promising target for research. Finally, proteases have not been extensively studied in biocontrol yeasts, but some links have been established in the antagonistic activity of certain yeasts such as *Aureobasidium pullulans* and, therefore, are also important enzymes whose biocontrol potential in research is promising (Freimoser *et al.,* 2019).

1.3.3. Induction of resistance

Healthy plants usually are more resistant to diseases and pests. This is because plants possess an immune system that can be supported by adequate additives such as fertilisers and fortifiers. Nevertheless, microorganisms are also capable of inducing these resistances and have been used in combination with resistance inducers like salicylic acid. In a way, induction of resistance can be seen as a secondary or indirect resistance mechanism triggered by other factors such as secreted enzymes (Freimoser *et al.,* 2019).

1.3.4. Volatile organic compounds (VOCs)

VOCs are small molecules with high vapour pressure. These can be aldehydes, ketones, alcohols, hydrocarbons, thioalcohols, thioesters, phenols and cyclohexanes and have been produced by yeasts such as *A. pullulans*,associated with an antagonistic activity against *Botrytis cinerea* and other infections (Freimoser *et al.,* 2019).

1.3.5. Toxins

One of the reasons yeasts are not regarded poorly in terms of biosafety concerns is given their reputation for being uncommon producers of secondary metabolites. Consequently, proving to be an advantage when pursuing yeasts as an alternative to chemical control products and other biological control agents. However, this also results in a low number of known toxic molecules that may contribute to biocontrol activity. Nonetheless, some yeast killer toxins have uses in the control of spoilage yeasts, beverage, and food industries or for medical applications. *A. pullulans* has been described to produce toxins that gave it a competitive advantage in dry conditions, so it is definitely a mechanism that demonstrates potential (Freimoser *et al.,* 2019).

1.4. The genus *Lachancea*

First proposed in 2003 by Cletus P. Kurtzman, following a reclassification of several yeast genera based on phylogenetics rather than phenotypic similarities, the *Lachancea* genus is currently comprised of 11 species.

The type species for this group, *Lachancea thermotolerans* (Filippov) Kurtzman (2003) was initially classified as *Zygossacharomyces thermotolerans* Filippov (1932) and has been widely studied for its relevance towards modern winemaking ever since a shift in focus towards non-*Saccharomyces* yeast species occurred (Benito, 2018; Padilla and Manzanares, 2016; Petruzzi *et al.,* 2017; Varela, 2016).

It has been described to produce wines with increased concentrations of lactic acid, glycerol and 2-phenylethanol during mixed fermentations of grape musts and yeast-produced volatile compound profiles have been associated with oenological properties (Gobbi *et al.* 2013; Jolly *et al.,* 2014). Hranilovic *et al.* (2018) performed an extensive study on the oenological phenomes of 94 previously genotyped *L. thermotolerans* strains that revealed a remarkable difference in phenotypes related to fermentation performance, production of primary and secondary metabolites and modulations in acidity, highlighting a high intra-specific diversity for this yeast species likely associated with domestication and allopatric differentiation. This link is supported by earlier findings in which *L. thermotolerans* strains from anthropogenic and natural environments formed separate genetic clusters. Within the anthropic environment isolates, two major domestic groups mainly comprising isolates from grape and wine related environments were identified. Furthermore, strains from different regions displayed distinct oenological phenotypes, with tested traits being ethanol tolerance, SO_2 resistance, H_2S production, flocculation, fermentation rate, tritable acidity and volatile acidity (Banilas *et al.,* 2016; Hranilovic *et al.,* 2017).

In terms of lactic acid (LA) production, Gatto *et al.* (2020) analysed key genes in the LA metabolism and their promoter regions and reports a remarkable phenotypic diversity in this regard despite high similarity in terms of gene content among the strains. Thus suggesting that phenotypic differences were probably explained by single nucleotide or structural variants present in the genome, rather than presence or absence of specific genes.

Valid *Lachancea* species include *L. fermentati* Kurtzman (2003), *L. kluyveri* (Phaff, M.W. Mill. & Shifrine) Kurtzman (2003), *L. cidri* (Legakis) Kurtzman (2003), *L. waltii* (K. Kodama) Kurtzman (2003), *L. meyersii* Fell, Statzell & Kurtzman (2004), *L. dasiensis* C. F. Lee (2009), *L. nothofagi* Mestre, Ulloa, C. A. Rosa, Lachance & Fontenia (2010), *L. mirantina* L. F. Pereira, C. R. L. Costa, Brasileiro, C. A. Rosa & M. A. Morais (2011), *L. lanzarotensis* González, Alcoba-Flórez & Laich (2013), *L. quebecensis* K. C. Freel, G. Charron, J. B. Leducq, C.R. Landry & J. Schacherer (2015). **Figure 7** depicts a timeline that includes the initial characterization and the re-characterization of valid species.

Based on D1/D2 sequence analysis of the 26S rRNA, the 11 valid species of *Lachancea* are grouped into 4 phylogenetic clusters, the first being comprised of *L. thermotolerans, L. quebecensis, L. waltii, L. dasiensis, L. nothofagi, L. meyersii* and *L. lazarotensis,* while the second contains *L. kluyveri*, the third contains *L. fermentati* and *L. cidri*, and the fourth cluster contains *L. mirantina* (Porter *et al.*, 2019). When mithocondrial genes of 7 of the mentioned species were analysed, similar clustering was achieved (Friedrich *et al.,* 2012).

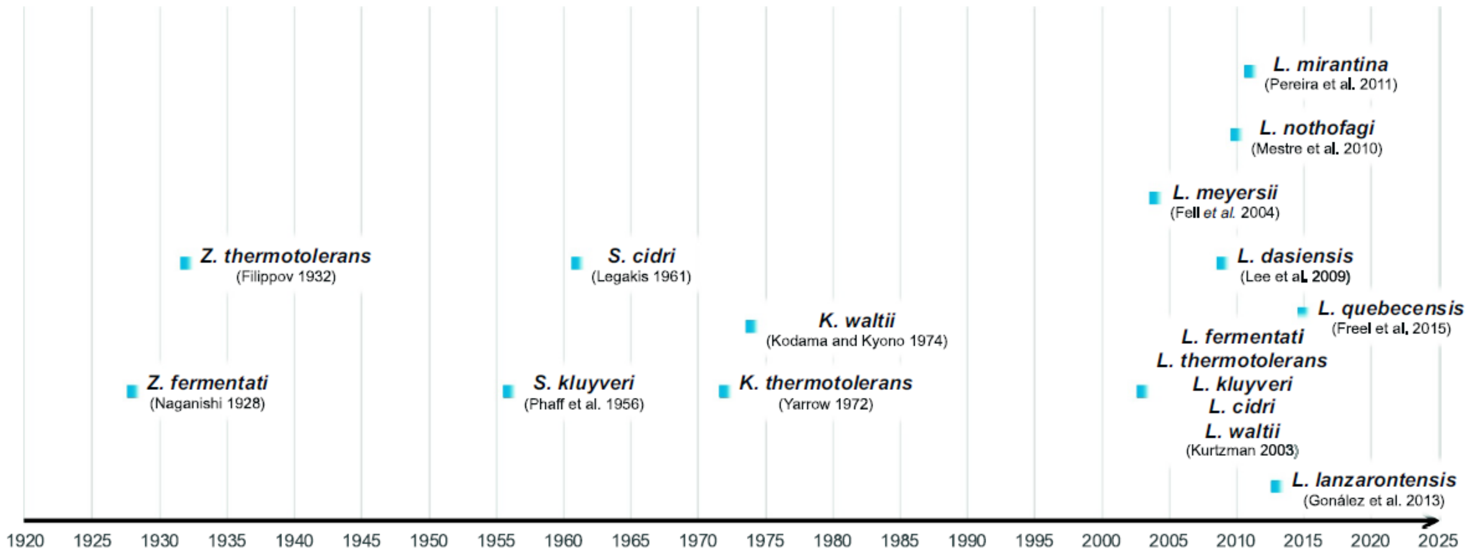

Figure 7. Timeline depicting the initial taxonomic characterization, the re-characterization of several *Lachancea* spp. and the subsequent addition of recently isolated yeast species to the *Lachancea* genus. (Porter, 2019)

1.5. Thesis outline and main objective

Presently, one of the main challenges for the wine industry is to limit the use of chemicals in its production chain to control microbiological agents responsible for vine disease and wine spoilage, all the while ensuring the quality of grapes and oenological properties of produced wine. To address this issue, a R&D project – ABCyeasts (Project n° 039/93 Norte 2020) - promoted by a consortium constituted by Proenol Indústria Biotecnológica Lda, Universidade de Trás-os-Montes e Alto Douro (UTAD), Associação Desenvolvimento da Viticultura Duriense (ADVID) and Sogrape was established, fostering the research and the development of yeast-based products to have an antagonistic action against phytopathogenic agents in the vineyard as well as against post-harvest spoilage.

In this line, this dissertation developed in the frame of this project, aims for the evaluation of the potential biocontrol activity of a collection of *Lachancea* spp. strains against *Aspergillus* sp., *Botrytis* sp., *Mucor* sp, and *Penicillium* sp., common phytopathogenic agents found in the viticultural sector. To that end, 70 wine yeast strains were evaluated in their antagonistic potential through co-culture assays. With these assays, volatile organic compound production and antagonistic effect were evaluated for each strain as well as the strength of the effect of direct competition between yeast and the target phytopathogen. With this data, a ranking of strains by antagonistic potential was built, allowing for a selection of the best candidates with special attention towards strains that prove to have a broader range of targets.

2. Materials and Methods

2.1. Fungal isolates

Samples obtained from shoots, buds, leaves and wine grapes collected from grapevines in several vineyards within the Douro region, were collected in 2019 by ADVID team and sent to Lab Bugworkers (M&B BioISI | FCUL) for isolation of potential phytopathogenic agents. Furthermore, convenience samples of diseased table grapes from supermarket and symptomatic vine leaves were additionally collected in Lisbon district.

2.1.1. Isolation and subculturing

Samples used in the isolations, came either from conidiophores observed through a Leica MZ9.5 stereo microscope (Leica Microsystems GmbH, Germany) or via directly plating diseased tissue on half-strength Potato Dextrose Agar (½ PDA, Type E Bacteriological Agar) (BIOKAR Diagnostics, Solabia Group, France). Cultures and subcultures for purification were incubated at 20°C under constant light source. A phenotypic analysis allowed for the subsequent identification of isolates at the genus level (Sutton, 1980; Seifert *et al.,* 2011).

2.1.2. Spore suspension and conservation

The purified *Aspergillus, Botrytis*, *Mucor* and *Penicillium* cultures were inoculated onto 250 mL conical flasks containing 30 mL of ½ PDA and subsequently incubated at 20°C under constant light for 14 days to promote sporulation. After this period, a solution of PBS buffer 1X with 0.01% Tween 20 and 50% (v/v) glycerol was added, to facilitate the dispersal of conidia, and glass beads were used to scrape the colonies. The resulting suspension was filtrated through sterile glass wool and spore counting was done using a Neubauer counting chamber as well as flow cytometry. The final volume for each spore suspension was divided into 1 mL aliquots and stored at -80°C.

2.2. Yeast strains

A collection with a total of 70 *Lachancea* spp. strains provided by UTAD-FCUL was used. Isolated from fermenting grape musts, these yeasts were isolated and collected from Portuguese wine producer regions (Alentejo, Dão, Douro, Bairrada). An additional 4 yeast strains were included in extraction, MSP-PCR and sequencing steps for dendrogram construction purposes.

These strains were recovered from cryopreserved cultures at -80°C, maintained in 20% (v/v) glycerol and from cultures preserved at 4°C in YPD slants or W.L. nutrient agar parafilm-sealed plates.

2.2.1. Isolation and subculturing

Subculturing of the *Lachancea* collection was done by streaking onto plates containing Yeast Extract Peptone Dextrose culture medium (YEPG/YPD) [1% (w/v) yeast extract (BIOKAR Diagnostics), 2% (w/v) peptone (BIOKAR Diagnostics) and 2% (w/v) glucose (MERCK), supplemented with 2% (w/v) agar (BIOKAR Diagnostics)] and subsequent incubation at 28°C for 48 hours in absence of light and subsequently kept at 4°C for follow-up experiments with periodic subculturing.

2.2.2. Collection maintenance

Collection cultures were plated from the YPD subcultures onto W.L. Nutrient Agar (CONDALAB, Spain), sealed with parafilm, incubated for 48 hours at 28°C and were then stored at 4°C. A copy of the collection was also inoculated into YPD slants in 10 mL Falcon tubes and kept at 16°C.

2.2.3. Cell suspension preparation

For the preparation of inoculum for use in co-culture assays, yeasts were transferred with a loop onto 10 mL falcon tubes with 5 mL YPD liquid medium and incubated at 28°C for 48 h after which they were stored at 16°C. Cell concentrations were determined via optical density measurement (OD) at 600 nm via a spectrophotometer. Tubes were vortexed before inoculation for co-culture assays.

2.2.4. DNA extraction and purification

Genomic DNA (gDNA) was extracted from pure cultures with 48 h growth, incubated at 28°C in the dark, following an adaptation of Löoke *et al.* (2011) (A. Amorim & L. Fernandes - Yeast Stress Bugworkers Lab Group).

A 1 mL inoculation loop was used to resuspend cells in 100 µL of 200 mM lithium acetate with 1% SDS solution within 2 mL microtubes, tubes were vortexed afterwards, followed by a 15-minute incubation at 70°C. Then, 300 µL of 100% (v/v) ethanol were added and again the microtubes were vortexed. Afterwards, the microtubes were centrifuged for 3 minutes at 14 000 rpm and room temperature, the supernatant was discarded. The pellet was washed with 50 µL of 70% (v/v) ethanol and centrifuged, for 1 minute at 14 000 rpm and room temperature, the supernatant was aspirated with a micropipette. Once the ethanol evaporated, the pellet was solubilized using 100 µL TE solution

and frozen for at least 30 minutes, after which the pellet was thawed, centrifuged for 30 seconds at 14 000 rpm and the supernatant was transferred to a new microtube.

The following DNA purification steps were based on the modified Pitcher *et al.* (1989) protocol, starting with the addition of RNAse (50 μg/mL) to the new microtubes containing the last obtained supernatant, followed by a 30-minute incubation at 37°C. Then, 100 μL solution of chloroform/isoamyl alcohol (24:1) was added, mixed by inversion, followed by a 15-minute centrifugation at 14 000 rpm, the supernatant was transferred to a new microtube (2 mL Eppendorf) and 1/10 volume (10 μL) of cold (-20°C) 3M NaAc, pH 5.2 was added and mixed by inversion, followed by a 10-minute centrifugation at 14 000 rpm, discarding the supernatant afterwards. The pellet was dried for 5 minutes at 60°C and solubilized in 50 μL TE.

An aliquot 5 μL for each sample was run via agarose gel electrophoresis to evaluate the quantity and quality of the DNA. Run conditions were 50 minutes at 90 V (5.6 V cm^{-1}), 0.8% (w/v) agarose gel (Invitrogen, United Kingdom), 0.5X TBE buffer (40 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3), 5 μL sample with 2 μL loading buffer and 5 μL molecular weight marker for use as reference, which was the 1 Kb Plus DNA Ladder, catalog no. 10787026 (Invitrogen, United Kingdom). Gel staining was done with ethidium bromide solution at a concentration of 0.5 μg ml^{-1}, with subsequent visualization with an Alliance 4.7 UV transilluminator and Alliance 15.15 software version (UVITEC, United Kingdom). DNA concentrations estimation was achieved using ImageJ 1.53i software version (Schneider *et al.*, 2012).

2.3. DNA-based studies

2.3.1. MSP-PCR fingerprinting

PCR reactions were carried out with three different primers for each run, with optimized cycling and mixture conditions on a T-Gradient Thermocycler (Biometra, Germany) with final reactions volume of 25 μL, based on Alves *et al.* (2007) and Ramírez-Castrillón *et al.* (2014). Cycling conditions were 40 cycles at 95°C for 1 minute, 50°C for 2 minutes and 72°C for 2 minutes, followed by a final extension step at 72°C for 5 minutes. The conditions were equal for all three primers used, and they were csM13 (5' – GAGGGTGGCGGTTCT – 3') (Vassart *et al.*, 1987; Ryskov *et al.*, 1988), $(GTG)_5$ (Walmsley *et al.*, 1989, Lieckfeldt *et al.*, 1993) and $[GACA]_4$ (Meyer *et al.*, 1993; Shehata *et al.*, 2008). The 25 μL PCR reactions were composed of 1x PCR buffer, 3 mM $MgCl_2$, 25 pmol of the corresponding primer, 0.2 mM of each dNTP, 1U *Taq* polymerase (Invitrogen, United Kingdom) and 2 μL of sample DNA template. Amplified products were run in an agarose gel electrophoresis after addition of loading buffer at a final volume of 5 μL, along with the 1 Kb Plus DNA Ladder, catalog no. 10787026 (Invitrogen, United Kingdom) for reference in a 1% (w/v) agarose gel in 0.5X TBE buffer (mentioned in **subsection 2.2.4**), and a constant voltage of 3.4 V cm^{-1} for 5 h, stained and visualized in the same way as described previously (**subsection 2.2.4**).

PCR-fingerprinting profiles were analysed and clustered using BioNumerics 6.6 software version (Applied Maths, Belgium), using Pearson's Correlation Coefficient and UPGMA for the generation of the similarity matrix and clustering method, respectively. Each primer profile's reproducibility was measured separately from one another based on the mean percentage of similarity between the duplicates. To that effect, duplicates for 15% of the isolates were chosen randomly using Excel 2108 software version (Microsoft Corporation, United States) and were amplified and separated in an agarose gel alongside the other isolates (Sneath and Johnson, 1972). To estimate the reproducibility cut-off level and to calculate the optimization parameters, a dendrogram was constructed with the randomly chosen isolates and their duplicates. The cut-off level was established at 90%, with optimizations defined at 2% for csM13 profiles, 2% for $[GTG]_5$ and 3% for $[GACA]_4$.

2.3.2. 26S rRNA gene amplification and sequencing

Amplification reactions were performed in a final volume of 50 µL, containing 50 to 100 ng of gDNA, 1x PCR buffer, 50 pmol of each primer, 0.2 mM of each dNTP, 2 mM MgCl2, 1 U Taq DNA polymerase and PCR-water, targeting the first two domains (D1/D2) of the large-subunit 26S rRNA gene with primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5'-GGTCCGTGTTTCAAGACCG -3´) (Kurtzman and Robnett, 1997). Amplification was performed using a PC-Personal 48 Thermocycler (Biometra, Germany) with cycling conditions being 40 cycles at 95°C for 1 minute, 52°C for 30 seconds and 72°C for 1.5 minutes, followed by a final extension step at 72°C for 10 minutes.

To assess PCR amplification and quality, along with an estimation of product concentration, amplified products were run in an agarose gel electrophoresis after addition of loading buffer at a final volume of 5 µL, along with the 1 Kb Plus DNA Ladder, catalog no. 10787026 (Invitrogen, United Kingdom) for reference in a 1% (w/v) agarose gel in 0.5X TBE buffer (mentioned in **subsection 2.2.4**), and a constant voltage of 5.6 V cm^{-1} for 1 h, stained and visualized in the same way as described previously (**subsection 2.2.4**). Gel staining was done with ethidium bromide solution at a concentration of 0.5 µg ml^{-1}, with subsequent visualization with an Alliance 4.7 UV transilluminator and Alliance 15.15 software version (UVITEC, United Kingdom). DNA concentrations estimation was achieved using ImageJ 1.53i software version (Schneider *et al.,* 2012). If concentrations were too high for sequencing, serial dilutions were performed; when concentrations were too low, another PCR reaction was done by applying different aliquots containing gDNA. Amplified PCR products were purified, and the D1/D2 region was sequenced by Eurofins (Germany), only in the forward direction using NL1 primer. Identification of possible closely related sequences from those acquired was performed by using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) provided by the National Center for Biotechnology Information's (NCBI) GenBank sequence database (Benson *et al.*, 2013).

2.4. Co-culture assays

The co-culture assays were used to evaluate the effect of direct competition (CY) and volatile organic compounds (VOCs) production on the mycelial growth and spore germination of target fungal isolates. These assays were all conducted in YPD growth media, and, to that effect, the fungal targets kept in ½ PDA were subcultured to YPD three times before being used for the assays.

The fungal targets were *Aspergillus* section *Nigri* AN1, *Botrytis* sp. BO1 and *Penicillium* sp. PE3 isolates. The *Mucor* sp. MU3 isolate was not used in the experiment described in subsection **2.4.1.** as its fast growth rate and exploratory mycelium characteristics prevented any meaningful readings.

2.4.1. Direct competition on agar plates with yeast inoculated before target (CY assay)

To evaluate the biocontrol activity of the *Lachancea* strains, co-culture assays were conducted in Ø 90 mm petri dishes, that were divided into eight radii, one of which being the control radius, with the other 7 being inoculated with 3 µL of different yeast cell suspensions. A total of 11 combinations of yeasts were randomly chosen and mirrored on all assays. Yeasts were inoculated first, then after 48 h at 28°C, the target fungi were inoculated.

In the *Aspergillus* AN1 and *Botrytis* BO1 co-culture assays, yeasts were inoculated 3 cm away from the centre of the plate where the target fungus was inoculated (**Figure 8**, **A**), whereas in the *Penicillium* PE3 co-culture assays, the yeast was inoculated 1.5 cm away from the centre of the plate (**Figure 8**, **B**). The colonies of the target fungi are well-formed and circular; therefore, measurements were taken of the colony radii to determine extension rate (Prosser and Tough, 1991). This experiment was carried out in triplicates, with daily readings at 24 h intervals, during 5 days for isolate AN1, 3 days for isolate BO1 and 7 days for isolate PE3.

2.4.2. Volatile Organic Compounds (VOCs) production

The effects of VOCs were evaluated using compartmentalized, Ø 90 mm four-quadrant petri dishes, wherein two quadrants were inoculated with yeast by heavy streaking and the remaining two were inoculated with the target fungi via inoculation needle, close to the inner vertex of the quadrant (**Figure 8**, **C**). This way, each plate acted as a duplicate of the assay, with two inoculations of the same fungi and only one yeast strain present. Subsequently to inoculation, the plates were sealed with double layer parafilm to prevent air leakage, and daily colony radii readings were taken at 24 h intervals. These measurements were taken until the mycelium growth in the control plates reached the borders of the plate. In yeast-inoculated plates where the inoculated target did not germinate during that timeframe, plates were monitored for 7 additional days to confirm inhibition of growth. After this, the parafilm layers were removed and the plates were incubated up to 5 days. This last procedure was to assess the fungistatic or fungicide mode of action of gaseous compounds. The control plates were as is displayed in **Figure 8, C**, minus the yeast-inoculated quadrants. Incubation conditions were 25°C in the dark.

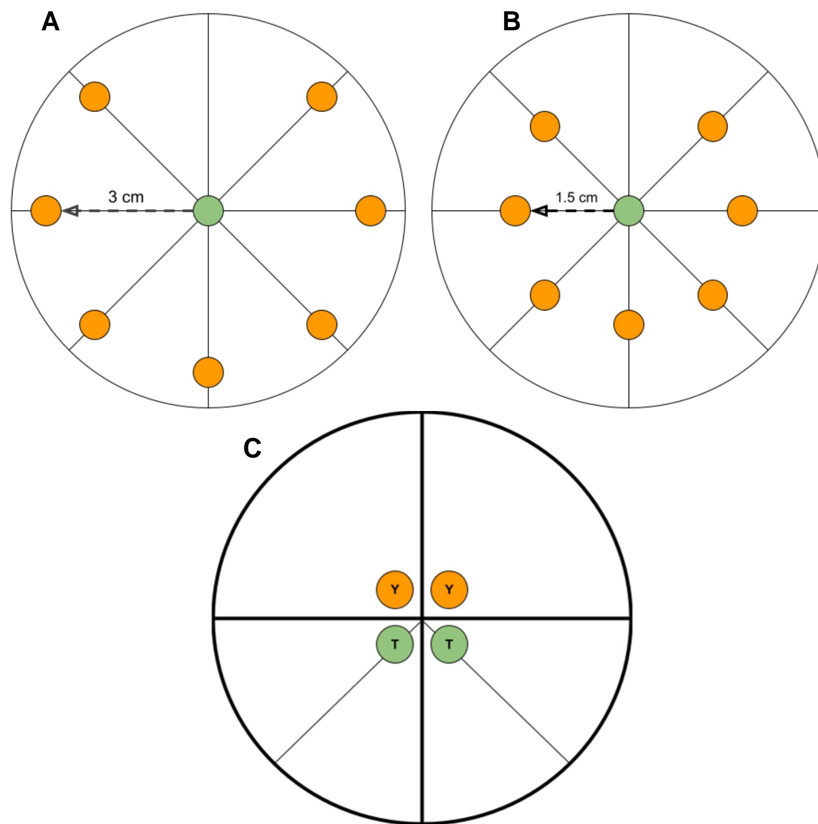

Figure 8. Schematic overview for co-culture plates used in mycelial growth inhibition assays. Direct competition assays were done in petri dishes split into 8 radii, with yeast cell suspension inoculation indicated as orange, and fungi inoculation indicated in green, distanced by 3 cm (A) or 1.5 cm (B). For the VOCs mycelial growth inhibition assay, four-quadrant compartmentalized petri dishes were used, with yeasts being inoculated via streaking on quadrants marked with an orange circle and fungal targets inoculated in the inner vertex of the respective green circle-marked quadrants (C).

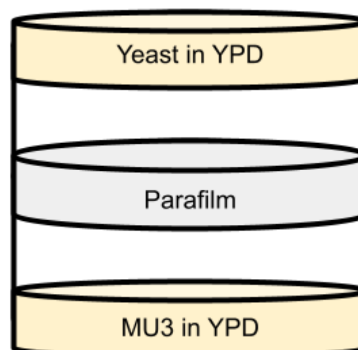

Figure 9. Schematic overview for co-culture plates used in VOCs assays for spore germination inhibition. Depicting two YPD plates, one inoculated with the target fungi by inoculation needle on the bottom part, the other with a yeast strain by streaking on the top part. Scotch tape was used to join the plates and keep them steady for subsequent parafilm sealing, indicated by a darker gray area. Figure is tri-dimensional.

Compartmentalized petri dishes were not used for *Mucor* sp. assays. Instead, a composite of two Ø 60 mm petri dishes or their respective lids was formed, with prior plating with YPD and inoculation with yeasts on one side by streaking and isolate *Mucor* sp. MU3 on the other side via inoculation needle. The composite was joined together with the aid of scotch tape and then the edges were sealed with parafilm as depicted on **Figure 9**. Incubation conditions were 25°C in the dark. Measurements were taken at 48 h. Replicates were done with the respective lids of each composite. Extension rates were determined by dividing the radius of the Petri dish by the number of hours it took in control and test groups for the fungal target to extend across the entirety of the surface.

2.4.3.Spore overlay on agar assay

Spore overlay assays were conducted for the *Mucor* sp. target (isolate MU3) using a spore suspension (5.4×10^5 spores mL^{-1}) obtained and counted as described previously (**subsection 2.1.2**), or inoculated with an inoculation needle from sporulating cultures, and the same *Lachancea* strains used in the previous assays.

Petri dishes (Ø 90 mm) were plated with 15 mL YPD agar medium, and a top agar was prepared by mixing 500 mL of YPD 0.8% agar at 50°C and 56 mL of diluted spore suspension, for a final volume of 556 mL with a spore concentration of 1×10^3 spores mL^{-1}. The top agar was poured over the existing YPD plates and, upon setting, 3 µL of yeast cell suspension were spotted on each Petri dish, 8 strains per plate and incubated at 25°C, with plates being observed for the next two days following inoculation. At the end of the assay, yeast growth was scraped off with an inoculation loop to check for growth of isolate *Mucor* sp. MU3 underneath.

2.5. Data analysis

The measurements for the previously described assays were taken based on colony diameter being an adequate form of fungal growth quantification measurement, according to Brancato and Golding (1953). For *Aspergillus, Botrytis* and *Penicillium* isolates, the colonies of the target fungi are well-formed and circular, therefore measurements were taken of the colony radii to determine extension rate (Prosser and Tough, 1991). In the particular case of *Mucor* colonies, a mean diameter of the colony was taken in light of its irregular growth in the tested conditions and only at the 48-hour mark, as growth was barely noticeable in the first 24 hours (with the exception of exploratory hyphae in some cases) and by the 64-hour mark, control groups had overtaken the entirety of the Petri dish surface. These measurements were taken on the reverse side of the Petri dishes using a ruler (±0.05 cm). The daily measurements were used to calculate the daily extension rate (ER; cm day^{-1}) by linear regression models (Zar, 1984)

The inhibition of the target fungal extension rate (IER; %) in the presence of yeast strains was calculated considering equation 1.1, indicating the reduction (%) caused by antagonistic activity.

$$IER = \left[1 - \left(\frac{ER\ Tested}{ER\ Control}\right)\right] x\ 100 \quad (1.1)$$

Similarly, the inhibition of radial growth (IRG; %) was calculated as described by Lemos Junior *et al.* (2016), comparing the target fungal colony and the control colony at the end of the incubation period (day 7 for *Penicillium*, day 5 for *Aspergillus*, day 4 for *Botrytis* and day 2 for *Mucor*), via measurement of colony diameters, using equation 1.2.

$$IRG = \left[1 - \left(\frac{RG\ Tested}{RG\ Control}\right)\right] x\ 100 \quad (1.2)$$

Purely analysing the extension rate and its inhibition, however, does not give us a complete picture, in cases where similar growth rates take place, but this growth is delayed, meaning it starts later by 1 or more days in a few cases. To contemplate such cases, a quantification of the total area under the curve (AUC), using the linear trapezoid rule which gives us the integrated fungal growth through time, was used. (Allgoewer *et al.*, 2018).

To calculate the inhibition of the fungal growth (IAC, %) in the presence of yeast strains using the AUC as a comparison basis, equation 1.3 was used.

$$IAC = \left[1 - \left(\frac{AUC\ Tested}{AUC\ Control}\right)\right] x\ 100 \quad (1.3)$$

Lastly, for the spore germination inhibition analysis, inhibition was evaluated based on the presence or absence of spore germination, in function of days till germination.

3. Results

3.1. Genomic approach

3.1.1. MSP-PCR fingerprinting of yeast collection

The obtained reproducibility value (mean similarity between duplicates) for the $(GACA)_4$ primer (75%) was considerably lower than the values obtained for the csM13 primer (96%) and the $(GTG)_5$ primer (95%). With that in mind, the chosen composite dendrogram to perform the genomic analysis of the yeast collection with was based on csM13 and $(GTG)_5$ primers, as that would yield a higher

resolution power compared to analysing both primers separately. Nevertheless, a supplementary analysis of a composite dendrogram including all three primers revealed equivalent cluster formation to that of the csM13 and $(GTG)_5$ composite dendrogram, when using a cut-off point based on the 95% calculated reproducibility value. In **Figure 10**, The reproducibility level of 95% is indicated by an arrow. Groups are distinguished by colour (in top-down order they are *Hanseniaspora uvarum*, *Candida* sp., *Lachancea* sp, and *Torulaspora delbrueckii.*.

The use of csM13, and $(GTG)_5$ primers yielded profiles rich with bands ranging from 300 to 1900 bp, whereas the $(GACA)_4$ primer yielded profiles with few bands, but a high intensity band was shared among most of the profiles with few exceptions.

3.1.2. Species-level identification of yeast isolates

To add onto the composite dendrogram crafted using csM13 and $(GTG)_5$ profiles, sequencing results of BLAST searches against the NCBI GenBank sequence database for the amplified D1/D2 region of selected yeasts were used to identify potential clusters of interest. Of the sequenced strains, 6 were from the *Lachancea* spp. collection and 4 others, identified as *Hanseniaspora uvarum*, *Candida* sp. (possible results were equally likely to be *Candida oleophila* or *Candida* zeylanoides) and *Torulaspora delbrueckii.* All 6 of the selected *Lachancea* sp. isolates (representative of the many smaller clusters of the dendrogram) for sequencing were determined to be *Lachancea thermotolerans* strains. The sequencing results for the amplified D1/D2 region compelled for a cut-off point to be defined at the 50% mark, which resulted in the formation of a main cluster in the dendrogram made up of *Lachancea* sp. isolates, with the remainder 4 sequenced yeasts functioning as outgroups, with *Torulaspora delbrueckii* appearing with the longest distance from the rest.

Figure 10. Composite dendrogram based on csM13 and (GTG)5 profiles. *Lachancea* isolates marked with an asterisk (*) were sequenced and matched with *Lachancea thermotolerans* strains.

3.2. Antagonistic activity metrics

3.2.1. Volatile organic compound production and effect

The assays for the evaluation of volatile organic compound activity yielded diverse responses within the collection of the 70 tested candidate yeasts when compared to the respective control groups (**Figure 11**). Since experimental data shows a delay of varying degrees in germination in test groups, when compared to the controls, this will be a metric henceforth labelled as DWG (days without growth). Average values of the triplicates were used to determine the extension rate (ER) in each group.

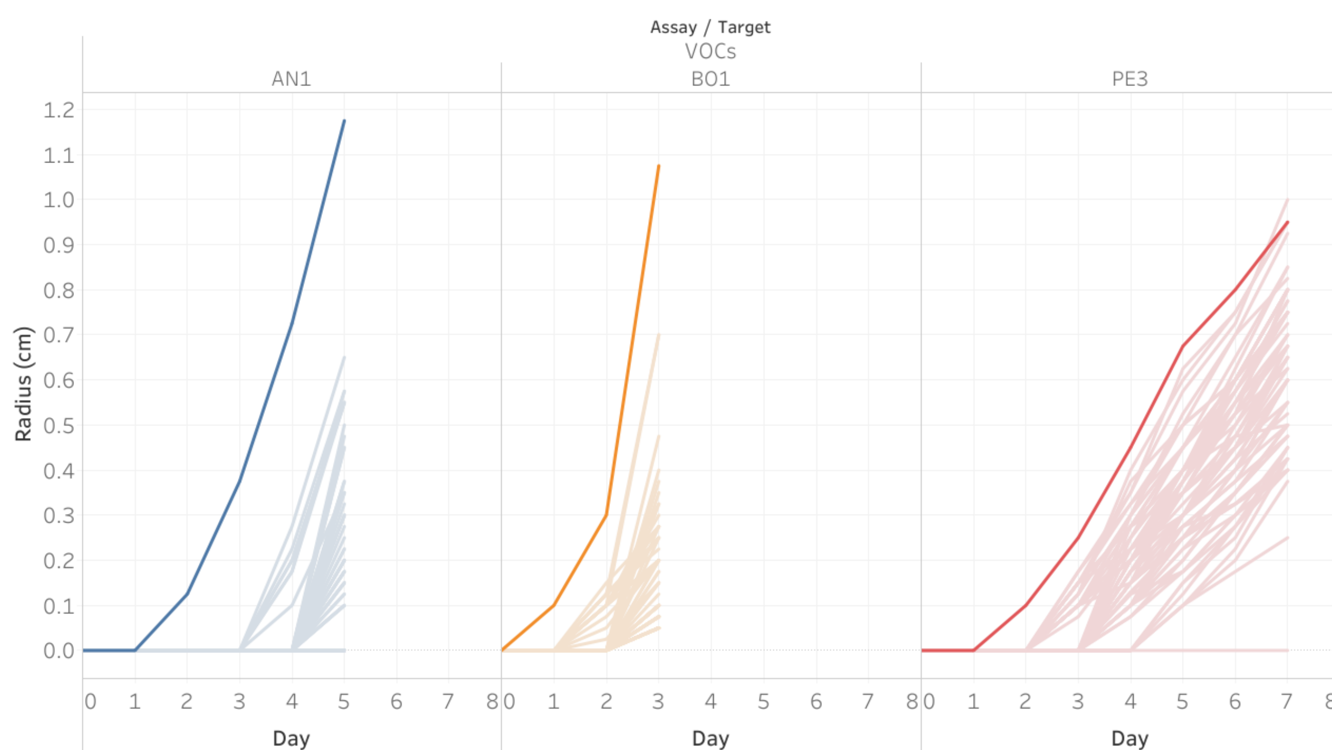

Figure 11. Fungal colony radius (cm) through time in VOCs competition assays for the different fungal targets. Target AN1 in blue, BO1 in orange and PE3 in red (*Aspergillus, Penicillium, Botrytis*, respectively). In each graphical representation, the control group is highlighted, appearing with a bolder and darker colour.

Several methods were used to ascertain antagonistic activity potential, the inhibition of extension rate **IER**, the inhibition of radial growth **IRG** and the inhibition based on the area under the curve for the extension rate **IAC** (**Figures 12 and 13**). Boxplots were used to determine the distribution of these metrics across all assays for all targets.

For the assays with *Aspergillus* sp. (AN1) as a target, 100% of the test groups had shown no signs of growth at the 3-day mark; by day 4, this figure was 89.2% of the tested groups; by the end of the assay, at the 5-day mark, 41.9% of the tested groups still showed no signs of growth. Control groups for AN1 had shown signs of growth by the 2-day mark.

No tested group displayed an extension rate higher than the control group. The median for **IER** values was around 89.8% with an interquartile range between 79.7% and 100% and a minimum antagonism value of 46.6%; 87.2% was the median value for **IRG,** having an interquartile range between 74.5% and 100% and a minimum antagonism value of 44.7%; and 95.9% was the median value for **IAC**, with an interquartile range between 91.7% and 100% and a minimum antagonism value of 66.9%.

In the case of assays with *Botrytis* sp. (BO1), although the control groups displayed signs of growth on the 1-day mark, 100% of the tested groups did not; this figure changed to 74.3% at the 2-day mark; by the end of the assay (day 3), all tested groups had shown signs of growth. No tested group displayed an extension rate higher than that of the control group. The median for **IER** values was around 89.7% with an interquartile range between 75.6% and 92.3% and a minimum antagonism value of 28.2%; 90.7% was the median value for **IRG,** having an interquartile range between 77.9% and 93.0% and a minimum antagonism value of 34.9%; and 94.4% was the median value for **IAC**, with an interquartile range between 79.6% and 95.8% and a minimum antagonism value of 46.5%.

In the *Penicillium* sp. (PE3) assays, the control groups displayed signs of growth at the 2-day mark, but 100% of the tested groups did not; this figure changed to 59.5% at day 3; at the 4-day mark, only 9.5% of the tested groups showed no signs of growth; a single test group (yeast 731) went on without displaying any signs of growth by the end of the assay (7-day mark). A total of 3 tested groups (yeasts 594, 653 and 713A) displayed higher extension rates when compared to the control group, with one of the 3 (yeast 713A), surpassing the radial growth observed in the control groups. The median for **IER** values was around 33.2% with an interquartile range between 19% and 48.7% and maximum antagonism values of 100% and 74.3%; 34.2% was the median value for **IRG,** having an interquartile range between 21.1% and 50% and maximum antagonism values of 100% and 73.7%; and 50% was the median value for **IAC**, with an interquartile range between 39,1% and 64,5% and a minimum antagonism value of 13.6% and maximum values of 100% and 85.5%.

As the *Mucor* sp. (MU3) assays were undertaken differently, with measurements taken at the 48-hour mark only, a graph is not presented here. For the assay with MU3, averages values of the duplicates were used, and experimental data shows that by the 48-hour mark, control groups had grown 4 cm, whereas test groups in that same time frame had only grown between 0.3 and 1.83 cm. Control groups extended across the entirety of the Petri dish surface area by the 64-hour mark and test groups did so at the 72-hour mark. The median for **IRG** values was around 78% with an interquartile range between 71,75% and 81,75% and a minimum antagonism value of 54.3% and a maximum antagonism of 92.5%**.** Based on time till coverage of Petri dish, **IER** was calculated to be 11.1%, representative of all tested groups.

Test groups that displayed no signs of growth by end of the respective assays were monitored for another 7 days, after which point the sealing parafilm was removed from each closed system. After the removal of the parafilm, all tested groups displayed signs of growth.

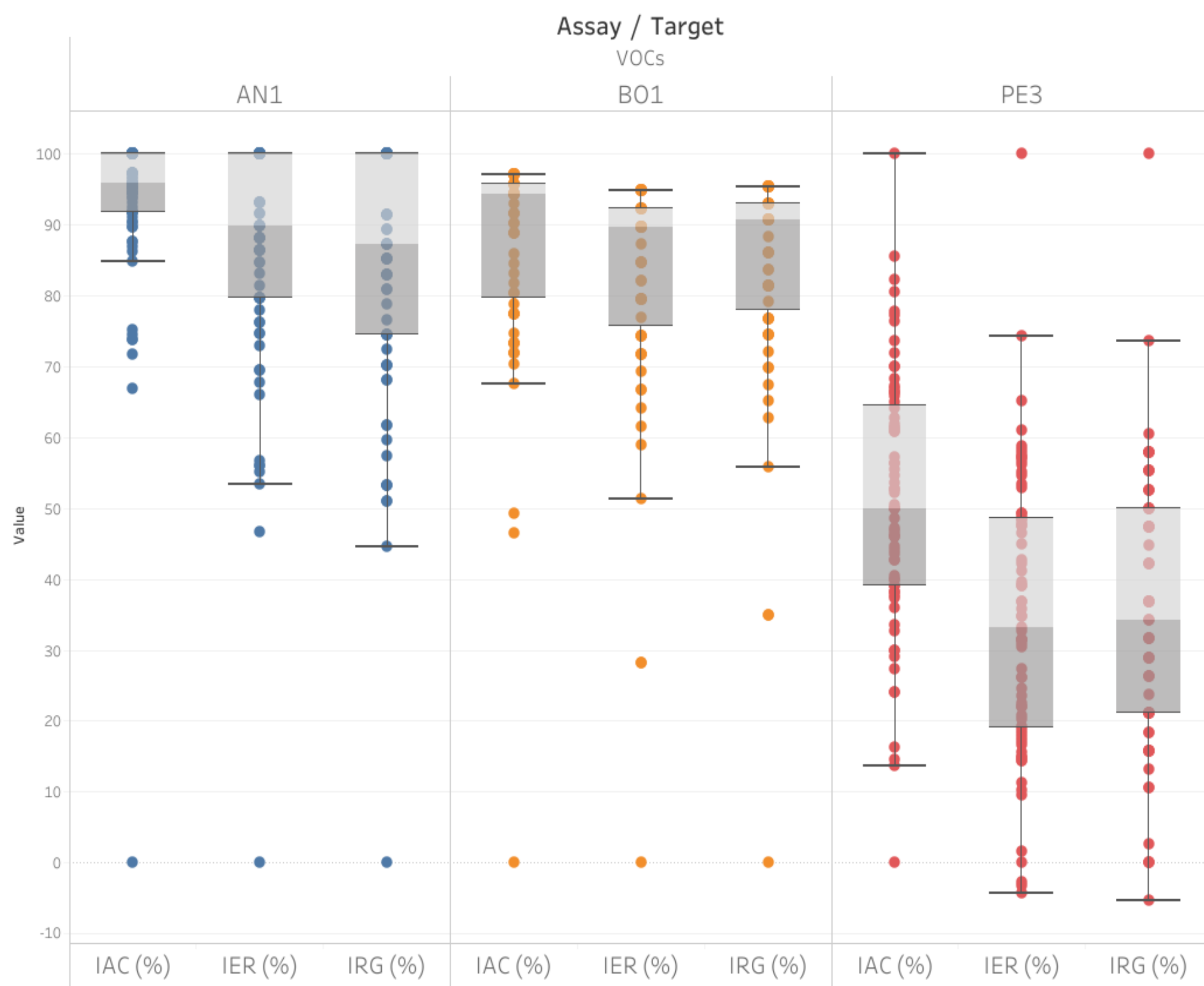

Figure 12. Data dispersion for the different metrics used to evaluate antagonistic activity on VOCs assays. Fungal targets AN1 in blue, BO1 in orange and PE3 in red (*Aspergillus, Penicillium, Botrytis*, respectively).

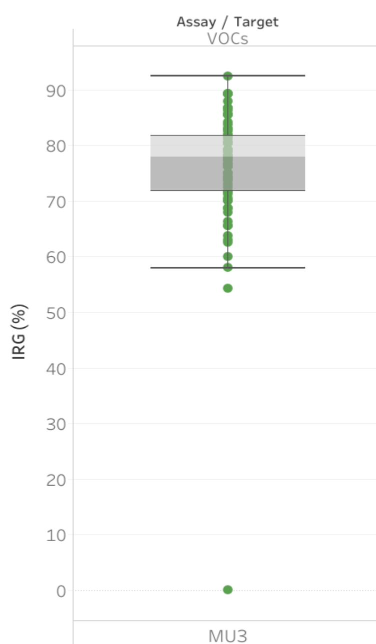

Figure 13. Data dispersion of IRG for *Mucor* sp. assays. The median for **IRG** values was around 78% with an interquartile range between 71,75% and 82,75%

3.2.1. Target inoculated after yeast (CY) assays

The assays for the evaluation of the antagonistic effect exhibited by the collection of 70 yeasts yielded both negative and positive results as, in some cases, the presence of specific yeasts promoted growth of the phytopathogen (*Mucor* was not included in this assay) when compared to the control groups. (**Figure 14**).

Several methods were used to ascertain antagonistic activity potential, the inhibition of extension rate **IER**, the inhibition of radial growth **IRG** and the inhibition based on the area under the curve for the extension rate **IAC** (**Figure 15**). Boxplots were used to determine the distribution of these metrics across all assays for all targets.

For the assays with *Aspergillus* sp. (AN1) as a target, the median for **IER** values was around 15.3% with an interquartile range between 6,5% and 18,4%, and a maximum value of 35.7%; 13.3% was the median value for **IRG,** having an interquartile range between 4.5% and 18.1% and a maximum antagonism value of 30%; and 9% was the median value for **IAC**, with an interquartile range between 2.1% and 13.6% and a maximum antagonism value of 22.5%.

In the case of assays with *Botrytis* sp. (BO1), the median for **IER** values was 4.8% with an interquartile range between -4,2% and 17.2% and a maximum antagonism value of 34.8%; 9.9% was the median value for **IRG,** having an interquartile range between -1.2% and 22.9% and a maximum antagonism value of 38.5%; and 14.9% was the median value for **IAC**, with an interquartile range between 0.5% and 29.3% and a maximum antagonism value of 43.7%.

In the *Penicillium* sp. (PE3) assays, the median for **IER** values was around 10.1% with an interquartile range between 5.2% and 17.4% and maximum antagonism value of 34.4%; 12.2% was the median value for **IRG,** having an interquartile range between 7.8% and 18.8% and maximum antagonism value of 34.2%; and 14.7% was the median value for **IAC**, with an interquartile range between 7.1% and 24.4% and a maximum antagonism value of 40.9%.

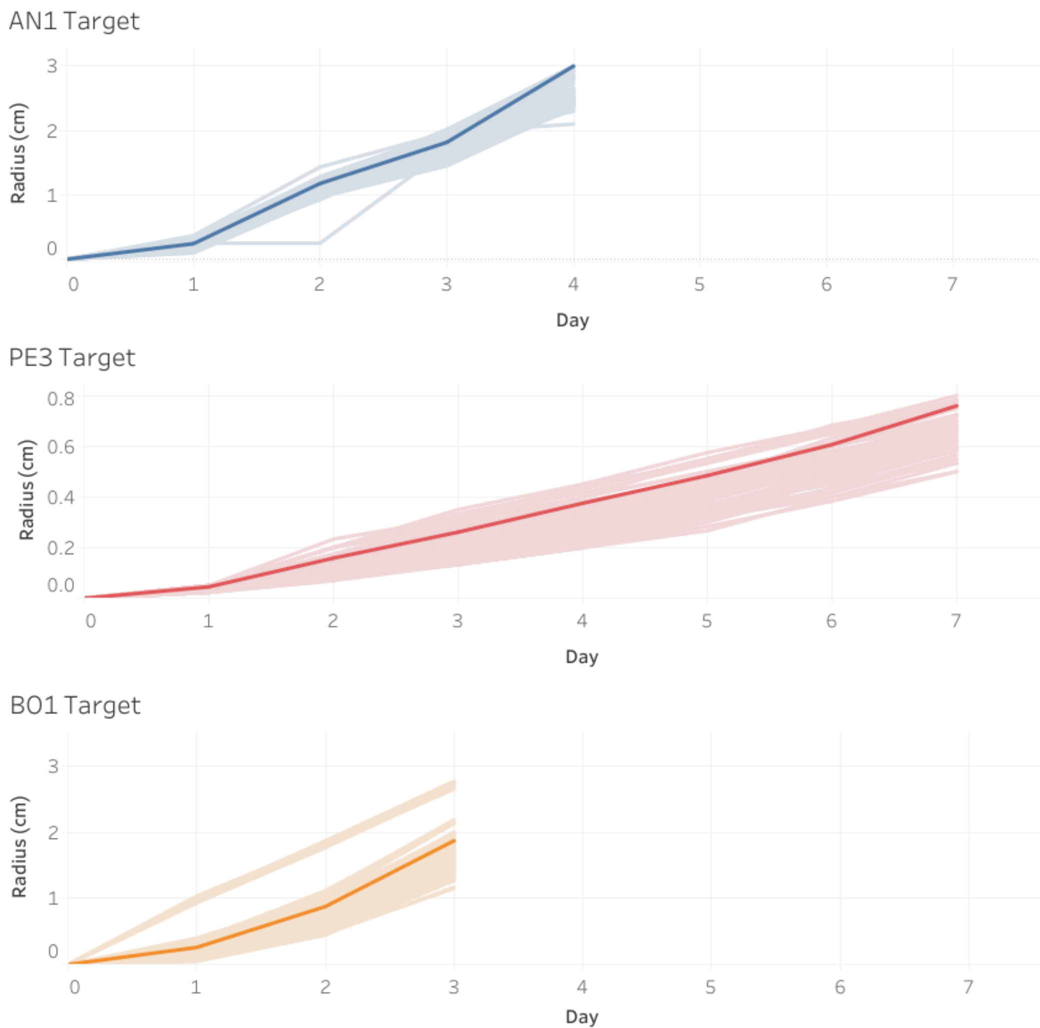

Figure 14. Fungal colony radius (cm) through time in CY competition assays for the different fungal targets. Target AN1 in blue, PE3 in red and BO1 in orange (*Aspergillus, Penicillium, Botrytis*, respectively). In each graphical representation, the control group is highlighted, appearing with a bolder and darker colour.

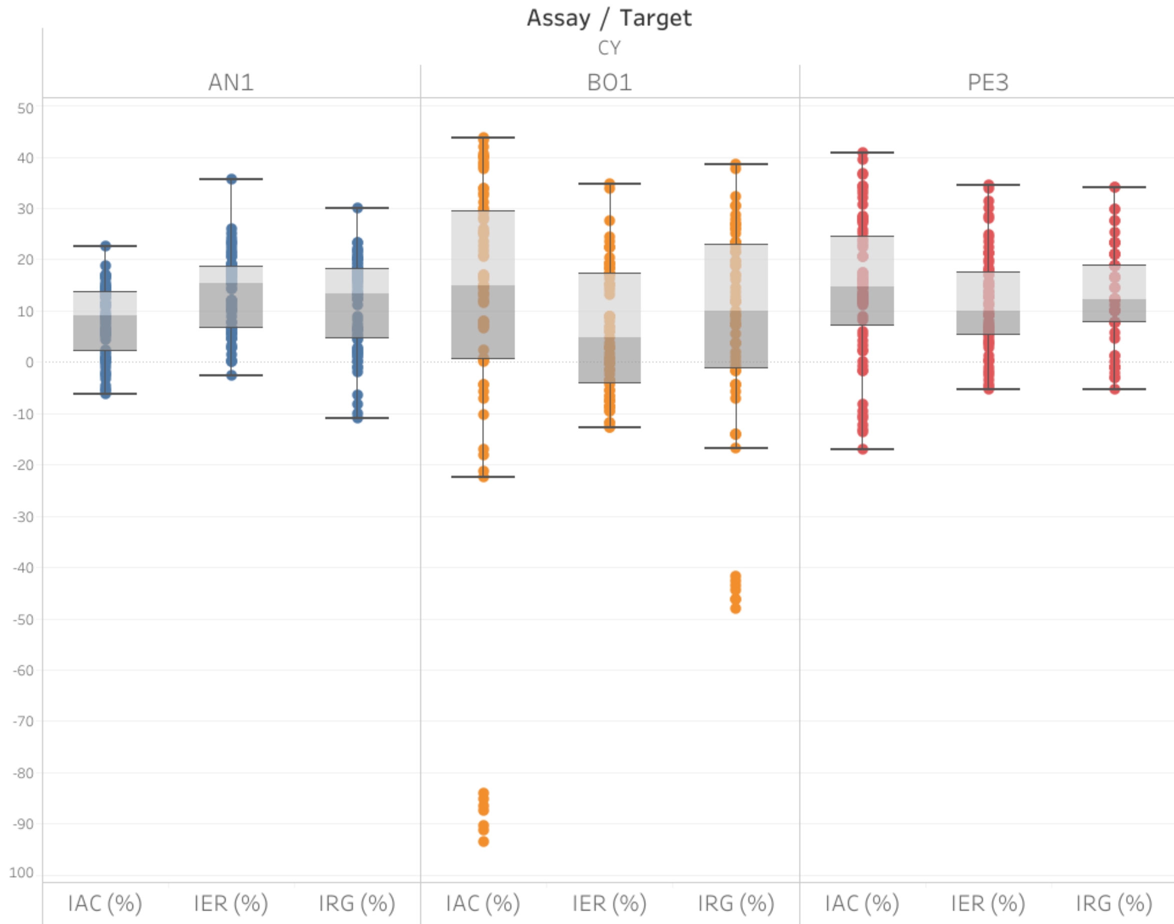

Figure 15. Data dispersion for the different metrics used to evaluate antagonistic activity on CY assays. Fungal targets AN1 in blue, BO1 in orange and PE3 in red (*Aspergillus, Penicillium, Botrytis*, respectively).

3.2.2. Spore agar overlay assay

In this assay, growth of the *Mucor* spores incorporated in the agar overlay was observed over a period or 72 hours. In the first 24 hours, growth was observable with a naked eye as hyphae began to extend through the growth media. At the 48-hour mark, the entire surface area of the growth media had been covered by *Mucor* mycelium. No inhibition halos were observable around any of the 70 yeasts and growth was identical to that of the control groups. Yeast colonies were carefully scraped off to reveal the presence or absence of *Mucor* growth underneath them, as seen on **Figure 16**.

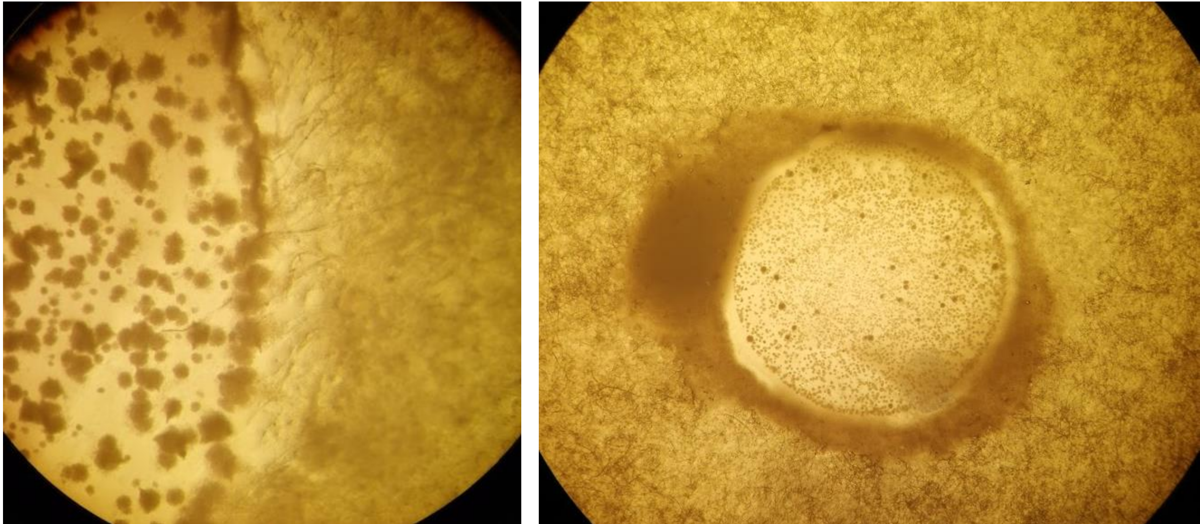

Figure 16. Observation of *Mucor* hyphae growth through a stereo microscope, on a growth media overlay. The growing yeast colony was scraped off to ascertain *Mucor* growth underneath it.

3.2.3. Dendrogram-based heatmap for antagonism metrics

To conciliate genomic closeness and antagonistic potential, a heatmap was produced based on selected methods for antagonism measurement (**DWG** and **IAC** for *Aspergillus, Penicillium* and *Botrytis* VOCs assays) and subsequently was linked with the dendrogram obtained in subsection **3.1** (**Figure 17**).

An overlap can be observed overall when comparing **DWG** and the corresponding **IAC** for the same target, but **IAC** appears as a more discriminatory measurement, particularly in the assays with *Botrytis* as a target.

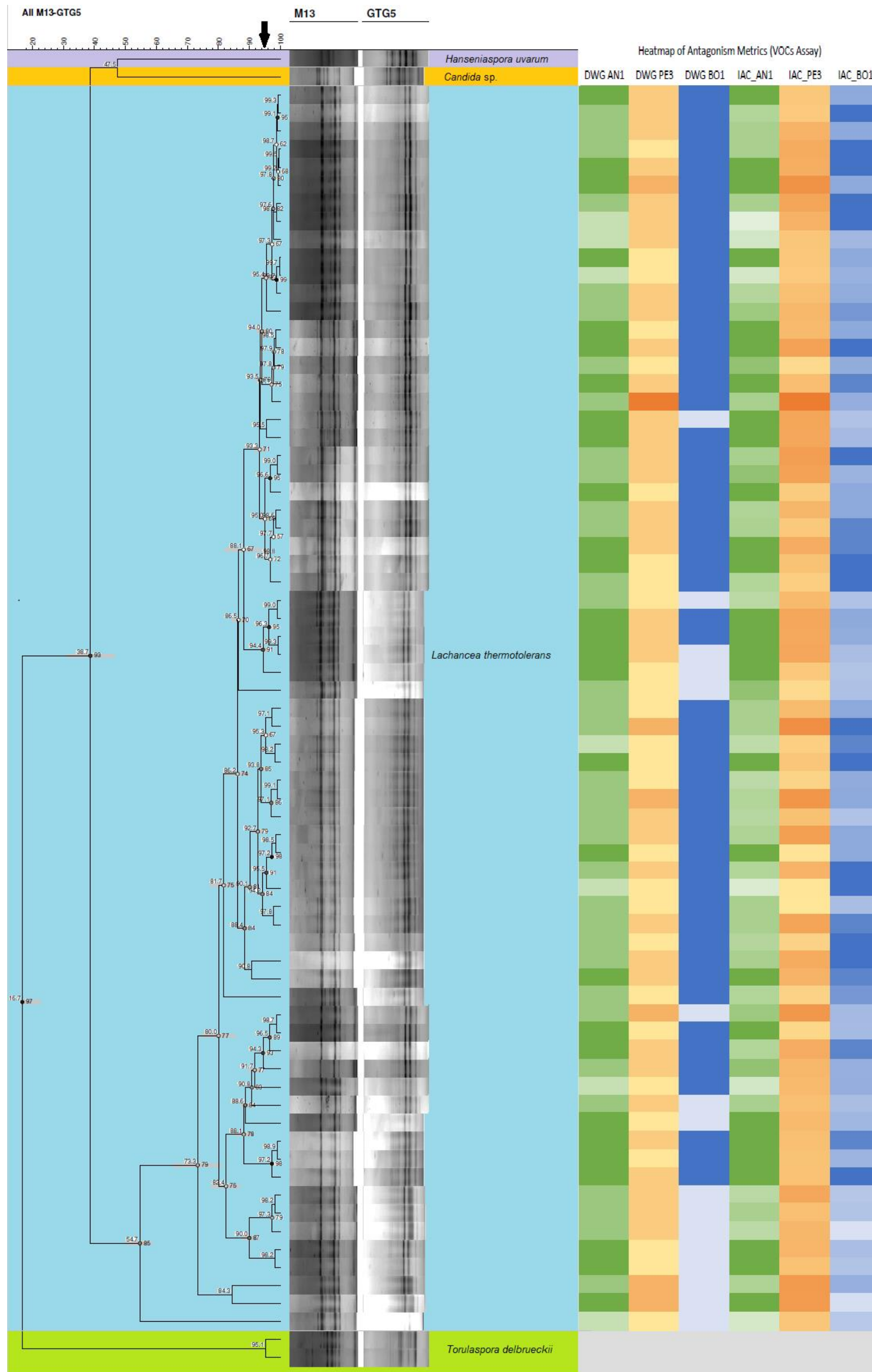

Figure 17. Side-by-side comparison corresponding each tested strain's antagonistic performance (VOCs assay) in a selection of metrics, days without growth (**DWG**) and inhibition based on the area under the curve for extension rate (**IAC**). These were associated with each target: *Aspergillus* AN1 (associated with green tones), *Penicillium* PE3 (associated with yellow and orange tones) and *Botrytis* BO1 (associated with blue tones). Darker tones signify a higher antagonism score for that specific metric.

4. Discussion

In the following section, a detailed discussion will take place regarding the obtained results. A genomic analysis will be conciliated with antagonistic findings, and criteria will be set for the selection of the most competent tested strains for antagonistic properties.

4.1. Genomic analysis

The fingerprinting profiles obtained using the $(GACA)_4$ primer yielded, in general, few bands when compared with the other primers. The lower reproducibility (75%) attached to that profile, as well as its much lower discriminatory power led to the exclusion of the $(GACA)_4$ profile from a composite dendrogram including all 3 profiles. Thus, csM13 and $(GTG)_5$ primer generated the most discriminatory, informative, and reproducible profiles with a combined analysis boasting a higher discriminatory power when compared to each individual profile's analysis.

In the dendrogram shown at **Figure 10**, the *Torulaspora delbrueckii* cluster appears further away from the remaining groups, which was an unexpected result. *T. delbrueckii* shares the *Saccharomycetaceae* family alongside the genus *Lachancea*, unlike *Candida oleophila* or *Candida. zeylanoides* and *H. uvarum* which belong to different families altogether in *Debaryomycetaceae* and *Saccharomycodaceae,* respectively (Schoch *et al.,* 2020). Therefore, the dendrogram does not represent current phylogenetic closeness by not showing a higher closeness between the *Lachancea* sp. and *Torulaspora* sp. clusters. The *Candida* sp. and *Hanseniaspora* sp. clusters should be the furthest from *Lachancea* sp. This is supported by phylogenies exhibited in works by Kurtzman (2011) and Mühlhausen and Kollmar (2014). Subclusters can be formed based on the sequenced strains.

Furthermore, within the *Lachancea* sp. cluster, all 6 of the sequenced isolates matched *Lachancea thermotolerans* strains, despite these selected strains appearing at different subclusters within the dendrogram. As such, due to the location within the dendrogram of the sequenced *L. thermotolerans* strains as well as the homogeneity of fingerprinting profiles within clusters, the *Lachancea* spp. collection can be proposed to be regarded as a collection of *L. thermotolerans* strains instead. The tested collection represents a close-knit group of strains, that nonetheless is capable of remarkable intra-specific diversity, which has been shown to translate into phenotype diversity in different parameters such as the production capabilities of lactic acid (Gatto *et al.,* 2020), fermentation performance, primary and secondary metabolite production, H_2S production, flocculation phenotype and volatile acidity as well as resistance traits to ethanol and SO_2 (Banilas *et al.,* 2016; Hranilovic *et al.,* 2017). Hranilovic *et al.* (2018) also showed that *L. thermotolerans* metabolome studies indicate that this diversity is present not only when comparing 'wild' isolated with 'domestic' isolates, but also between 'domestic' isolates themselves in relation to the isolate region of origin. Such intra-specificity between 'domestic' strains was observable with one group of strains exhibiting superior lactate production which translates into a pronounced acidification of the environment, whereas another group of 'domestic' strains had the lowest lactate yield of the study and thus was also responsible for the lowest pH values observed in the study. However, contrary to findings in *S. cerevisiae* that show

varying degrees of domestication, with increased specialisation in beer strains (i.e, decreased ability to grow in nature-like environments) (Gallone *et al.,* 2016), all *L. thermotolerans* strains were capable of proliferating in an oenological environment and 'domestic' strains were capable of proliferating in the presence of different carbon sources and physiochemical conditions which, altogether, suggests an absence of niche specialisation (Hranilovic *et al.,* 2017).

It is expected, then, for a group of 70 *Lachancea* isolates to exhibit a diverse antagonistic activity phenotype linked to a diversity potential regarding metabolite production and sugar consumption rates (Hranilovic *et al.,* 2018).

4.2. Antagonistic activity analysis

In the present work, inhibitory phenotypes were characterized either by a reduction of growth rate (extension of the mycelium) when compared to the control groups, comparing the final radius of colonies in test groups when compared to the control groups, and by the total of number of days where fungi growth was unable to occur. Inhibitory values were, as such, calculated as the relative inhibition effect and within each group of tests, a dispersion of these inhibitory values was analysed to facilitate comparison of any single yeast's antagonistic potential across the plethora of tests carried out.

4.2.1. Volatile organic compound production

The effects studied in the VOCs production assays were those of extension rate inhibition and days without growth (**DWG**). At a first glance, **DWG** appears as the most impactful effect as it translates into a complete suppression of a target fungus' ability to proliferate, albeit temporarily. The value of such an effect would guarantee that, in a hypothetical *in vivo* situation, crops could be protected during the timeframes more crucial to target pathogen proliferation and resist yield loss for the season. In harvested produce as well, this effect could be valuable during transportation and storage, potentially extending the lifespan of such items. The inhibition of the extension rate could potentially facilitate the surrounding microbiota to outcompete the invading pathogen or for another subsequent measure for control of the pathogen to be more effective, in a hypothetical *in vivo* situation. Depending on concentration of the active metabolite or metabolites responsible for such inhibitory activity, the effect can be stronger in a practical application.

For this assay, different antagonism values obtained in the various methods were compared, namely, **IER, IRG** and **IAC** with particular interest in **IAC** as it provides a more accurate representation for the fungal extension rates by using the area under the curve rather than the slope of the linear regression. Area under the curve is made more relevant in this assay by virtue of there being a delay of fungal target growth in a substantial amount of test groups (**DWG**). When compared to **IER** values, **IAC** methodology also, therefore, yielded higher antagonism results.

The tested strains had an overall strong antagonistic action against *Aspergillus* and *Botrytis* but results against *Penicillium* were not as impactful. Indeed, the range of antagonism against *Penicillium* was great and variable with some suppression of spore germination being observed. However, some of the test groups in which spore germination was suppressed resulted in the growth of the target fungus quickly achieving colony radii like the control groups. In contrast, in *Aspergillus* and *Botrytis* assays, a wide gap in colony radius between control and test groups is maintained throughout the duration of the assays. Strains that displayed strong antagonism against *Aspergillus* and *Botrytis* but not *Penicillium* could indicate a lack of affinity or specificity towards the latter.

4.2.2. Target inoculated after yeast (CY) assays

Beyond a weaker overall antagonism when compared to the VOC assays, in CY the opposite effect of antagonism was observed in some test groups, with fungal extension rates exceeding those observed in control groups. Indeed, such events have been observed previously in other yeast-fungal interactions, highlighting the complexity underlying antagonism assays. The presence of a yeast can increase the availability of a particular carbon source for the target fungi in the growth medium, facilitating uptake and subsequent growth in a phenomenon known as crossfeeding, i.e. breaking down a disaccharide into monosaccharides usable by the other organism (Horváth *et al.,* 2020; Sipiczki, 2016).

Although synergistic effects are undesirable, at least initially, studying and understanding them can provide insight into possible synergistic effects that, in nature, facilitate proliferation of pathogens and, as such, can also be targeted for control in a two-way action that seeks to both antagonise the target pathogen and the surrounding organism interactions that synergise with it.

Regarding antagonism in this assay, a high potential (>30% inhibition) for such was observed in *Botrytis* and *Penicillium* targets, which can complement the results obtained in VOCs assays for the selection of high interest strains.

4.2.3. Spore agar overlay assay

The assay had the goal of evaluating antagonistic effect by yeast strains upon a media incorporated with spores from *Mucor* sp. target fungi. The antagonism, if any, would be mostly through diffusion of metabolites through the shared growth media, or via direct competition between the two organisms. Expected inhibitory effects would be the formation of halos around the inoculated yeasts where *Mucor* growth would not occur.

From the 70 tested *Lachancea* strains, none lead to the creation of such halos when observed through a stereo microscope, instead the target *Mucor* isolate proliferated throughout the entirety of the growth media, covering its surface. Underneath the yeast colonies, however, there was no filamentous fungal growth. This could be explained by nutrient depletion, or possibly some metabolite

diffusion did occur. By the 48-hour mark a lawn of *Mucor* had been formed across the growth media, which coincides with the incubation period for the yeasts in the CY assays before inoculating the target fungi. It could be that possible metabolites with antagonistic activity, if any, would not have had time to be produced and diffused through the growth media or perhaps the concentration of *Mucor* spores was too great and inadequate for such assay.

An approach that could overcome difficulties in this assay would be to use a filtrate obtained from each strain after 48 h of incubation as described in Spadaro *et al.* (2002). This approach proved promising in a more recent study by Mekbib *et al.* (2011) in which *Penicillium digitatum* spore germination was inhibited in such an assay.

4.2.4. Is there predictability of antagonistic potential based on genomic closeness?

Through the analysis of the heat map on **Figure 17**, most strains, when displaying higher values for antagonism, tend to do so only against one or in some cases two targets. However, some exceptions did occur, with strains exhibiting a strong antagonistic activity against all three targets, raising particular interest. Furthermore, certain groups of genomically close strains exhibited similarly strong antagonism, but with a cut-off point defined at 95% (obtained from reproducibility MSP-PCR fingerprinting calculations), these clusters cannot be determined to be integrated by different strains, instead no such conclusions can be taken through this methodology for genomic relatedness. The MSP-PCR fingerprinting functioned to raise insights on the variability of the tested collection, but greater knowledge regarding this group and its variability can be obtained through whole-genome sequencing and functional annotation can be performed using BlastKOALA which is a tool that assigns sequence data against non-redundant KEGG genes, exemplified in previous studies (Gatto *et al.,* 2020).

4.2.5. Global analysis

Overall, volatile compounds assays led to the highest levels for inhibition of fungal target growth with magnitudes 2 to 4 times greater when compared to the same test groups in CY assays, with the added effect of suppression of spore germination. Knowing that a big challenge in biocontrol assays is that *in vitro* studies do notnecessarily translate into field applications, it should still nonetheless be worthwhile to primarily focus on the organisms and metabolites that exhibit the most impactful results *in vitro* (Feimoser *at al.,* 2019). As such, and since the VOCs assays displayed the highest antagonistic potential in this study, when assessing the overall antagonistic potential of a strain in this study, a strong inhibitory effect obtained through VOCs should weigh more in that assessment than a strong inhibitory effect obtained from a CY assay.

With that in mind, many studies have been conducted with the goal of evaluating microbial volatilomes and their applications and effectiveness. Indeed, important findings reside in the fact that volatile blends have proved capable of high effectiveness even at low concentrations, all the while

posing a negligible hazardous effect on both animals and the environment. A great benefit as well is the volatility property which enables a wide and homogeneous diffusion when applied either below- or above-ground. This property, however, also poses a major challenge to their application but also to the evaluation of the specific effect in massive open-field agricultural and horticultural practices when compared to greenhouse or *in vitro* conditions. Nonetheless, open field application of the 3-pentanol and 2-butanone in a cucumber field has demonstrated a significant effectiveness against the bacterial angular leaf spot pathogen *Pseudomonas syringae* pv. *lachrymans* by inducing plant systemic acquired resistance mechanisms. In turn, the activation of the defense-related gene CsLOX stimulated the oxylipin pathway, which plays a role in recruiting *Coccinella septempunctata*, a natural enemy of the sucking insect aphid, *Myzus persicae*. Other studies yielded similar results in drastically different environments and crops, such as a treatment of red pepper leaves with *Bacillus amyloliquefaciens* strain IN937a which resulted in an antagonistic effect against *Xanthomonas axonopodis* pv. *vesicatoria*. The application of VOCs is facilitated under air-tight conditions for the control and prevention of storage pathogens, the atmosphere is quickly saturated and allows for the maintenance of inhibitory concentration levels of volatile blends (Mansurova *et al.,* 2018; Tilocca *et al.,* 2020).

This is to say that investigation of microbial VOCs and their applications and effects is still in its infancy, volatiles are linked to a complex network of interactions between and within species and without understanding the biotic and abiotic factors that could play a role in this regard, *in vitro* results might not translate into field results for now, but research into these compounds can certainly facilitate such applications and help to achieve results in field that are not dissimilar to those obtained *in vitro*.

4.3. Selection of best biocontrol candidate yeast strains

An important part of this project would be the selection of the strains with the most relevant antagonistic action towards the various targets studied. In that regard, no target was more relevant or important for this assessment. Instead, a combination of strong results across assay types and targets will be considered as ideal characteristics for selection.

As a first step for selection, strains were filtered based on the antagonistic scores displayed in their respective VOCs and CY assays, using **IAC**, and **IER** methods for all targets. To that end, the dispersions obtained with boxplots were paramount, as the filtering was done based on the 75th percentile, strains above the upper quartile for all assays were selected. The second criteria would be selecting strains that fulfilled the previous criterion for more than one target. Due to the large collection of tested strains, many candidates were found to fulfil those criteria and, as such, it was decided that the strains that had antagonistic scores above the 75th percentile on 4 or more assays would be selected, this ensured that such strains had a strong antagonistic score for each target on at least one assay.

Based on these criteria, a total of 11 strains were selected and a final score was attributed based on the fulfilment of selection criteria, illustrated in **Table 1.** They were as follows: Y351, Y390, Y452, Y540, Y572, Y632, Y682, Y1477 and finally, Y1479 which was the only tested yeast to exhibit antagonistic activity in the upper quartile on all assays across all targets.

Table 1. Selected strains and matching fulfilled criteria of having an antagonistic activity above the 75th percentile for each assay and respective target. Positives are filled with a green colour, Blank means that the strain did not fulfil the criterion for that assay.

	Aspergillus CY	*Aspergillus* VOCs	*Penicillium* CY	*Penicillium* VOCs	*Botrytis* CY	*Botrytis* VOCs
Y351	✓	✓	✓	✓		✓
Y390			✓	✓	✓	✓
Y452	✓	✓		✓		✓
Y533	✓	✓		✓	✓	✓
Y540			✓	✓	✓	✓
Y572		✓	✓		✓	✓
Y632			✓	✓	✓	✓
Y682	✓		✓	✓	✓	
Y1468			✓	✓	✓	✓
Y1477	✓	✓	✓	✓		✓
Y1479	✓	✓	✓	✓	✓	✓

5. Concluding remarks

A major focus in the food industry has been the minimisation of food loss, which, to this day and with the great advancement of conservation and transportation technology, is still an important issue to address (FAO, 2016, cited by FAO, 2019). Throughout the years, the use of chemical control agents in the form of pesticides and fungicides in agriculture has been raising concerns with the general public as environmental and health awareness becomes the trend. This is supported by research establishing a correlation between negative health and environmental impacts to the heavy use of chemical control agents in modern day practices. On top of that, the rise of resistant strains to these chemicals drives and the need for alternatives that would be more difficult for a pathogen to tolerate or become resistant to over time (Bélanger and Labbé, 2002; Fondevilla and Rubiales, 2012; Zhang *et al.,* 2007).

To that end, biological forms of disease and pest control have been gaining attention in the industry and the scientific community. These involve bacteria, filamentous fungi and yeasts that exhibited an

antagonistic activity both *in vitro* and in field conditions. In this work we have explored what distinguishes these organisms from each other in their potential for biocontrol properties and yeasts are arguably the most promising of the three by virtue of being easy to cultivate and maintain in culture like bacteria are (Pandin *et al.,* 2017; Rossouw *et al.,* 2018), but also by not being regarded as prolific producers of secondary metabolites. In addition, yeasts have been used since ancient times and are to this day very important in food and beverage industries, but also in medicine. These factors contribute to yeasts being regarded as generally safe (despite the existence of pathogenic strains) (EFSA, 2005) albeit with some concerns that need to be considered when selecting a yeast for biocontrol agents, such as the propensity for displaying dimorphism and to acquire resistance to fungicides (Freimoser *et al.,* 2019; Gauthier, 2015, 2017).

Vitis vinifera is the most common grapevine crop and has a great economic value and importance in the world. It's a crop that can be very negatively impacted by attacks of phytopathogenic fungi that cause diseases commonly known as powdery mildews, downy mildews, grey moulds, black mould, and blue moulds, among a whole plethora of rots and diseases that can afflict the various organs of the crop. For winemaking, this crop heavily relies on yeasts with the manifestation of "good" rots such as the noble rot, that contrast with the diseases mentioned throughout this work. Yeasts isolated from grapevine often express antagonistic properties (Zarraonaindia, 2015). This work was focused on diseases in *Vitis* spp., and it was possible to see the results that some of the biocontrol assays had on this crop. Such results were also observed in other research by using biocontrol agents that acted with varied mechanisms ranging from mycoparasitism, release of volatile compounds or just competition against the target fungus (Algara, 2015; Ghule *et al.,* 2019; Lazazzara *et al.,* 2018; Pertot *et al.,* 2017; Ponsone *et al.,* 2011).

A major challenge in biocontrol assays remains and it is the need for field condition studies that often exhibit results that contrast a lot from *in vitro* studies in which certain mechanisms may not be activated at all (Freimoser *et al.,* 2019). Furthermore, the technique behind the application of the biocontrol agents themselves could be decisive for the efficacy of the strategy, as was exemplified by Petot *et al.* (2017), demonstrating a polyphasic strategy involving three different microorganisms (*Trichoderma* spp., *A. pullulans*, and *Bacillus* spp.). In this study, the strategy was focused on conciliating the disease cycle of *Botrytis cinerea* with the conditions present in each of the growth stages of the crop that are critical for the success of *Botrytis cinerea*'s pathogenicity. In short, a simple formulation that includes a single or even a mixture of biocontrol agents could be lacking in field conditions where the competition for space and nutrients varies drastically. Instead, a more complete relationship between biocontrol strategy and abiotic factors, in relation to the disease cycle, must be established.

With the various examples seen in this work highlighting the use of biocontrol agents, and what different mechanisms and application strategies can be decisive in boosting efficacy, there is certainly potential to be discovered. Nonetheless, more research into the underlying mechanisms for disease and disease control is in demand. Furthermore, an important takeaway is that, although antagonism *in vitro* results can prove insufficient at times, it might not be entirely enough to discard a yeast's

potential in a real field condition. As such, future assays could stand to gain much by experimenting with join action of biocontrol agents (bearing in mind that some antagonistic yeasts can be incompatible with each other) and mimicking different tissues and nutrient abundance or scarcity conditions *in vitro*. Identification of bioactive molecules along with the dynamic cross-talk that volatile compounds are involved in would greatly facilitate the development of suitable chemical forms (i.e., immobilized molecules, pro-bioactive compounds) that should allow better handling, storage, and safe delivery to open-fields (Tilocca *et al.,* 2020, Mansurova *et al.,* 2018).

The collection of *Lachancea thermotolerans* strains studied here provided much insight into the potential for antagonism and how it can vary depending on which mechanisms are isolated. Some strains had a very specific affinity toward one target, others had a broad action. Others even exhibited synergistic action toward a target. All within the same species *L. thermotolerans*. Great expectations are set for other species of *Lachancea* as this group's potential for oenological properties and biocontrol competence are further explored.

6. References

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7. Image references

(Figure 1). Macroscopic representation of powdery mildew resembling white powder which corresponds to the fungi colonies. All rights reserved to Department of Primary Industries and Regional Development, <https://www.agric.wa.gov.au/table-grapes/powdery-mildew-grapevines-western-australia> accessed June 2021.

(Figure 2). Visual representation of downy mildew signs in leave, characterised by the occurrence of “oil spots” All rights reserved to Department of Primary Industries and Regional Development, <https://www.agric.wa.gov.au/table-grapes/ downy-mildew-grapevines> accessed June 2021.

(Figure 3). Visual representation of fungal growth caused by advanced *Botrytis cinerea* bunch rot in infected grapes. The berries have become shrivelled and mummified. All rights reserved to Department of Primary Industries and Regional Development, <https://www.agric.wa.gov.au/summer/know-your-grapevine-bunch-rots> accessed June 2021.

(Figure 4). A representation of *Botrytis cinerea* disease cycle, where we can observe the fungus overwintering on dead plant and fruit tissues with a production of spores (conidia) come spring that are dispersed by wind and allows the fungus to infect new tissue. Young plant tissue is particularly

vulnerable. All rights reserved to eVineyard, <https://www.evineyardapp.com/blog/2015/11/27/gray-mold-of-grape-botrytis-cinerea> accessed June 2021.

(Figure 5). A photograph in which the black spores of Aspergillus spp. Are visible across the surface of grapes in a bunch. All rights reserved to Department of Primary Industries and Regional Development, <https://www.agric.wa.gov.au/summer/know-your-grapevine-bunch-rots> accessed June 2021

(Figure 6). A photograph depicting the effects of an Aspergillus infection in grapes, characterised by a mass of coloured spores and a strong odour. All rights reserved to Department of Primary Industries and Regional Development, <https://www.agric.wa.gov.au/summer/know-your-grapevine-bunch-rots> accessed June 2021