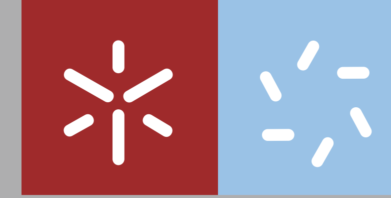
Pedro Ferraz Ramos Lopes **Biocontrol of phytopathogenic fungi relevant for cacao- and olive-derived economy**

UMinho | 2020



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Pedro Ferraz Ramos Lopes

Biocontrol of phytopathogenic fungi relevant for cacao- and olive-derived economy

janeiro de 2020

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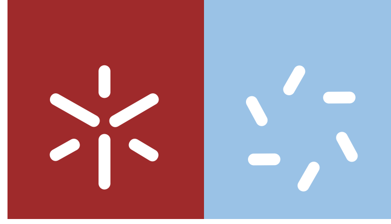

Universidade do Minho
Escola de Ciências

Pedro Ferraz Ramos Lopes

Biocontrol of phytopathogenic fungi relevant for cacao- and olive-derived economy

Tese de Doutoramento
Programa Doutoral em Biologia Molecular e Ambiental
Especialidade em Biotecnologia Molecular

Trabalho efetuado sob a orientação da
Professora Doutora Cândida Lucas
e da
Professora Doutora Fernanda Cássio

janeiro de 2020

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ACKNOWLEDGEMENTS

Em primeiro lugar, quero agradecer à Professora Cândida Lucas, por me ter acolhido no seu grupo de investigação e por me ter dado a conhecer o tema que me apaixonou desde o primeiro minuto. Agradeço todo tempo, paciência e conselhos valiosos, principalmente nos momentos difíceis, que muito me ajudaram a conseguir alcançar este marco na minha vida pessoal e profissional. À Professora Fernanda Cássio, por toda a ajuda, disponibilidade e pelas palavras de incentivo ao longo destes quatro anos.

A todos os colegas do Laboratório de Biodiversidade e do LBM II por toda a ajuda e boa disposição, em especial às minhas parceiras de bancada. À Giulia e à Joana, por me terem ajudado desde o primeiro minuto em tudo e me terem introduzido ao mundo da microbiologia, por toda a alegria e amizade. À Mariana e à Coralie, as minhas pupilas, por toda a ajuda em tantos momentos essenciais, principalmente nos mais complicados e por toda a boa disposição que tornaram o ambiente no laboratório tão bom.

A todos os colegas do Programa Doutoral, em especial à Guida, Mário, Rosana e Rui por terem partilhado comigo o estatuto de cobaias e por todo o companheirismo que sempre demonstraram.

A todas as pessoas que partilharam comigo a hora de almoço e as pausas para café nestes quatro anos, em especial à Catarina, Cláudia, Dário, Diana, Gabriel, Giulia, Joana Pereira, Joana Tulha, Maria, Mário e Rosana, por todos os momentos bons e por serem amigos que ficarão para a vida.

A todas as pessoas do Departamento de Biologia e do IB-S que, de uma forma ou de outra, me ajudaram quando necessário. Um agradecimento especial à Lídia, Manuela e Sr. Luís por toda a disponibilidade.

Aos meus pais e irmãos, por terem tornado possível este meu sonho e por me terem sempre apoiado incondicionalmente ao longo da minha vida. Muito obrigado por tudo!

À Catarina, por todo o amor, carinho e paciência, principalmente por ter aturado estes últimos tempos stressantes da escrita da tese, eu sei que não foram fáceis. Obrigado por estares sempre lá para tudo!

This work was supported by the strategic programme UID/BIA/04050/2013 (POCI-01-0145-FEDER-007569) funded by national funds through the FCT I.P. and by the ERDF through the COMPETE2020 - POCI, and the project EcoAgriFood (NORTE-01-0145-FEDER-000009), supported by the NORTE 2020 under the PORTUGAL 2020 Partnership Agreement through the European Regional Development Fund (ERDF). PF is a student of the Doctoral Programme in Applied and Environmental Microbiology (DP_AEM) and FCT grantee PD/BD/113810/2015. We thank Mariana Amorim-Rodrigues (FCT grant PD/BD/145354/2019) for the precious contributions with her research to the Chapter 4. We thank the Brazilian companies, Cerlev, Lda., Ouro Preto, MG, and Fermentec, Lda. Soluções Tecnológicas e Industriais, Piracicaba, SP, as well as Professor João Paulo Sampaio from the Portuguese Yeast Culture Collection/UCIBIO, NOVA, Portugal for kindly supplying the yeast strains used in this work. We also thank Professor Pedro Talhinhas, from LEAF - Linking Landscape, Environment, Agriculture and Food, ISA, Universidade de Lisboa, Portugal, for kindly supplying two of the fungal strains used in this work.

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

ABSTRACT

Biocontrol of phytopathogenic fungi relevant for cacao- and olive-derived economy

Plant diseases caused by fungal pathogens constitute an increasing threat to food production and security. This is the case of *Witches' Broom Disease* (WBD) of cacao, caused by the basidiomycete *Moniliophthora perniciosa*, and of Olive Anthracnose (OA), caused by a *consortium* of *Colletotrichum gloeosporioides* and *C. acutatum* species complexes, which seriously impact the economy. Both share the lack of effective containment methods. The use of chemical fungicides is increasingly more precarious and restricted. More effective eco-friendly methods are required, such as the use of biocontrol agents. This work aimed to evaluate the ability of yeasts to antagonize the fungal causal agents of both diseases. A group of yeasts originating from Brazilian sugarcane-based fermentation industries were tested against *M. perniciosa*. Two isolates from *cachaça* production fermentation and one of the most used strains in bioethanol production, efficiently kill six strains of *M. perniciosa in vitro*. Antagonistic ability is maintained in non-optimal conditions. Microscopy analysis showed that fungal cells die upon contact with these yeasts, which physically attach and fuse to the mycelium and drain the cells, in what appears like a yeast predacious behaviour. Two further observations revealed the formation of connections between yeast and fungal cells, as well as fimbriae-like connections between yeast cells. These results agree in that, at a distance, only the proliferation of mycelia is diverted through the probable secretion of a non-volatile agar diffusible compound. Several proteins were identified specifically secreted by fungi and yeasts in single culture and co-culture. The methodology now developed was further used to study the antagonism of yeasts against the OA-causing *Colletotrichum* sp.. The same group of fermentative yeasts were tested along with others originating from the olive biome in Portuguese orchards. One of these last strains showed a promising antagonistic potential, by inhibiting the growth of the three fungal strains in all the conditions tested. Moreover, the possibility of using *vinasse*, a waste product from sugarcane bioethanol production process, to control the development of *M. perniciosa* was also evaluated. Immersing or spraying the mycelium with *vinasse* either kills the fungus or impedes its proliferation. This effect is not extensive the OA-causing fungi. The results support the exploration of microbial biodiversity of the infected plant-associated biome to generate greener and more sustainable alternatives to contain phytopathogens like those causing WBD and OA, contributing to alleviate the socio-economic impact of these diseases.

Keywords: Antagonism; Biocontrol; Olive Anthracnose; *Witches' Broom Disease*; Yeasts

RESUMO

Biocontrolo de fungos fitopatogénicos importantes para a economia do cacau e da oliveira

Fitopatologias causadas por fungos são uma ameaça crescente à produção e segurança alimentares. É o caso da doença da Vassoura-de-bruxa (VB) do cacau, causada pelo fungo *Moniliophthora perniciosa*, e da Antracnose da oliveira (AO), causada pelos complexos de espécies *Colletotrichum gloeosporioides* e *C. acutatum*, que se tornaram um risco para a economia, por não haver métodos eficazes de contenção. O uso de fungicidas químicos é cada vez mais ineficaz e mais restrito, pelo que é necessário encontrar alternativas mais amigas do ambiente, como por exemplo a utilização de biocidas. Este trabalho teve como objetivo avaliar a capacidade de leveduras para antagonizar os fungos causadores destas doenças. Um grupo de leveduras proveniente da fermentação de caldo-de-cana no Brasil foi testado contra *M. perniciosa.* Dois isolados do processo de produção de cachaça e uma das leveduras mais utilizadas na produção industrial de bioetanol matam eficientemente 6 estirpes de *M. perniciosa*, *in vitro*. O antagonismo mantém-se em condições adversas. Observações de microscopia confirmaram que o fungo morre em contacto com as leveduras que aderem e se fundem com o micélio, esvaziando-o, no que parece ser um comportamento predatório. Além disso, observou-se a existência de conexões entre células de levedura e do micélio, e entre células de levedura entre si. Isto concorda com o facto de, à distância, haver apenas redireccionamento do desenvolvimento do micélio, através da difusão de um composto não-volátil, tendo sido verificado que os fungos e as leveduras segregam proteínas diferentes em cultura simples ou em co-cultura. O método desenvolvido neste trabalho foi aplicado para verificar o antagonismo de leveduras contra os agentes causadores da AO. O mesmo conjunto de leveduras fermentativas foi testado, em confronto com estirpes provenientes do bioma de olivais portugueses. Uma destas últimas leveduras mostrou grande potencial antagonista contra 3 estirpes de *Colletotrichum* sp. em todas as condições testadas. Adicionalmente, foi ensaiada com sucesso a utilização da vinhaça, o resíduo denso e líquido que resulta da destilação do etanol após a fermentação do caldo-de-cana, no controlo do desenvolvimento do *M. perniciosa*. A imersão ou a pulverização do micélio com vinhaça mata ou impede drasticamente a proliferação do fungo. Este efeito não foi extensível aos fungos causadores da AO. Em suma, a exploração da biodiversidade microbiana do bioma associado às plantas infetadas pode permitir desenvolver metodologias alternativas, mais amigas do ambiente e mais sustentáveis, para a contenção da presente progressão dos agentes fitopatogénicos, como os que provocam a VB e a AO, contribuindo para aliviar o impacto socioeconómico destas doenças.

Palavras-chave: Antagonismo; Antracnose da Oliveira; Biocontrolo; Leveduras; *Vassoura-de-bruxa*

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LIST OF COMMON ABBREVIATIONS

μg	Growth Rate (yeast)
ANOVA	Analysis of Variance
AOX	Alternative Oxidase
ATP	Adenosine Triphosphate
COD	Chemical Oxygen Demand
cyt c	Cytochrome *c*
DNA	Deoxyribonucleic Acid
dsDNA	Double-stranded Deoxyribonucleic Acid
Eno2	Enolase 2
ER	Endoplasmic Reticulum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gd	Growth diameter
Gr	Growth Rate (fungi)
GRAS	Generally Recognized As Safe
kDa	kilodalton
Kre1	Killer toxin Resistant 1
KT	Killer Toxin
MB	Methylene Blue
MDA	Malondialdehyde
ME	Malt Extract
MEA	Malt Extract-Agar
MOX	Methanol Oxidase
OA	Olive Anthracnose
OD	Optical Density
PBS	Phosphate Buffered Saline
PDA	Potato-Dextrose-Agar
PI	Propidium Iodide
PMF	Peptide Mass Fingerprinting
QSI	Quorum Sensing Inhibitor
QSM	Quorum Sensing Molecule

RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	Rotations per minute
RPS	Ribosomal Proteins of the Small subunit
Scw4	Soluble Cell Wall 4
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
SOD	Superoxide Dismutase
TCA	Tricarboxylic Acid
Tdh3	Triose-phosphate Dehydrogenase 3
TEM	Transmission Electron Microscopy
v/v	volume per volume
V-ATPase	Vacuolar-type H^+-ATPase
VOC	Volatile Compound
w/v	weight per volume
WBD	Witches' Broom Disease
Ynk1	Yeast Nucleoside diphosphate Kinase 1
YPD	Yeast Extract-Peptone-Dextrose
YPDA	Yeast Extract-Peptone-Dextrose-Agar

List of Publications

The work performed during this PhD resulted in the following peer-reviewed publications:

Ferraz, P., Cássio, F. and Lucas, C. (2019) Potential of Yeasts as Biocontrol Agents of the Phytopathogen Causing Cacao *Witches' Broom Disease*: Is Microbial Warfare a Solution? Frontiers in Microbiology 10: 1766

Ferraz, P., Amorim-Rodrigues, M., Cássio, F. and Lucas, C. *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* are able to kill *Moniliophthora perniciosa*, the causal agent of cacao *Witches' Broom Disease* (submitted)

Ferraz, P., Cássio, F. and Lucas, C. Microscopic assessment of the antagonism effect of yeasts against *Moniliophthora perniciosa*, the fungal causal agent of *Witches' Broom Disease* in cacao (manuscript in preparation)

Amorim-Rodrigues, M., Ferraz, P., Cássio, F. and Lucas, C. *Wickerhamomyces anomalus* from olive orchards microbiome efficiently antagonizes the Olive Anthracnose's causal agents, *Colletotrichum* spp. (manuscript in preparation)

Ferraz, P., Amorim-Rodrigues, M., Cássio, F. and Lucas, C. *Vinasse* waste from sugarcane-based bioethanol production plants kills *Moniliophthora perniciosa,* the causative agent of cacao *Witches' Broom Disease*. (submitted)

CHAPTER 1

General Introduction

Part of this chapter has been previously published in:

Ferraz, P., Cássio, F. and Lucas, C. (2019) Potential of Yeasts as Biocontrol Agents of the Phytopathogen Causing Cacao *Witches' Broom Disease*: Is Microbial Warfare a Solution? Frontiers in Microbiology 10: 1766

Introduction

A great number of plant diseases are responsible for major crop losses with huge socio-economic impact, causing each year a worldwide estimated losses of 40 billion dollars (Syed Ab Rahman *et al.*, 2018). Particularly, the diseases caused by fungal pathogens are increasingly recognized as a global threat to food production and security. In fact, since 2000 the number of new fungal plant pathogen alerts has increased by more than 7-fold (Fisher *et al.*, 2012). Presently, fungal-generated diseases constitute 64-67% of the total crop diseases reported globally (Fisher *et al.*, 2012; Fisher *et al.*, 2018), and account for 20% of the losses at the level of production and a further 10% at postharvest level (Fisher *et al.*, 2018). These authors estimate that fungal diseases are spreading northbound at a rate of almost 8 km/year. This could derive from increasingly common agricultural practices, such as the extensive monocultures and the use of a restricted number of plant cultivars, as well as the increased global international trade proportionating disease spreading over great distances (Fisher *et al.*, 2012). Climate change adds a burden to that equation, potentiating the development of microbes and vectors in unprecedented regions (Robert *et al.*, 2015; Fisher *et al.*, 2018).

To prevent plant diseases and protect crops from pests and pathogens, widely spread methodologies mainly correspond to applying chemical fungicides. The continued use of chemical fungicides leads to the development of fungicide resistance in the fungal pathogen (Syed Ab Rahman *et al.*, 2018) and, in the absence of other control measures, to the re-emergence of virulence (Fisher *et al.*, 2018). Therefore, in spite that the use of pesticides brought clear improvements in crop quality and quantity during more than half a century, their progressive inefficacy to treat some of the most harmful plant diseases requires the utilization of higher dosages each year (Medeiros *et al.*, 2010; Syed Ab Rahman *et al.*, 2018). The use of fungicides heavily impacts on the microflora of agrarian ecosystems, destroying beneficial microbes, such as endophytic bacteria and fungi, as well as animals important for the quality of the soils (Syed Ab Rahman *et al.*, 2018). Ultimately, the systemic use of these drugs leads to the persistence of chemical residues in the environment, proportionating low dosage toxicity and contaminating species across trophic levels (Carvalho, 2006; Dukare *et al.*, 2018).

Due to this scenario, new efficient and eco-friendly strategies to control fungal diseases are required, such as the use of biological control agents, particularly antagonistic yeasts. This possible solution offers some advantages comparing to the use of chemical fungicides, such as safer methods of application and the fact of being environmentally friendly (Dukare *et al.*, 2018). The introduction of the yeast-based biocontrol products does not harm the ecosystems due to the absence of toxic residues, and the low levels of toxicity

in combination with the high level of biodegradability make them suitable sustainable agriculture procedures and for human consumption (Ocampo-Suarez *et al.*, 2017). Besides the use in the food and agriculture, antagonistic yeast can also have an important role in medical applications, including its use as antimycotics for therapeutic treatment of human fungal infections, e.g., in the combat of pathogenic *Candida* infections (Hatoum *et al.*, 2012)

WITCHES' BROOM DISEASE OF CACAO

One of the fungal diseases with recognized high negative socio-economic impact is the pathology of cacao plant and fruit known as *Witches' Broom Disease* (WBD). This is caused by the basidiomycete fungus *Moniliophthora perniciosa* (Aime and Phillips-Mora, 2005) (formerly designated *Crinipellis perniciosa*). The severity and extent of its manifestation is endangering the rapidly expanding and very quality-demanding chocolate market. According to data from the International Cacao Organization (www.ICCO.org), more than 4 million tons of cacao beans are produced annually (Wickramasuriya and Dunwell, 2018). Cacao beans are the core raw material for the chocolate industry, although other cacao-derived products also have important world markets, such as cacao butter or liquor (Pohlan and Pérez, 2010; Wickramasuriya and Dunwell, 2018). The economic global market for chocolate reached US$ 110 billion in 2015, and the world demand is expected to grow exponentially in the next decade due to the globalization of consumption styles in expanding economies such as China and India (Squicciarini and Swinnen, 2016). Cacao is produced in countries located approximately in the same latitude interval of equatorial climate, forming the so-called Cacao Belt (Pohlan and Pérez, 2010). The biggest producers are therefore countries from Central and South America and Africa. The cacao plant is affected by several diseases, the more threatening of which is WBD (Purdy and Schmidt, 1996; Pereira, 1999; Griffith *et al.*, 2003; Aime and Phillips-Mora, 2005; Teixeira *et al.*, 2015) (Figure 1). It has severely affected South and Central America countries, where it has been responsible for major irreversible crop losses. The highest economic and social consequences of WBD are described to have occurred in Brazil. In the ten years after the onset of the disease in 1989, WBD reduced the cacao production in more than 70% (Pereira *et al.*, 1989; Santos Filho *et al.*, 1998; Trevizan and Marques, 2002; Meinhardt *et al.*, 2008; Pires *et al.*, 2009; Teixeira *et al.*, 2015), causing Brazil to shift from being the 2nd world producer to becoming a net importer of cacao beans (Bowers *et al.*, 2001; Marelli *et al.*, 2009; Teixeira *et al.*, 2015). During that

period, the most affected region of Bahia suffered losses around 90%, configuring a severe social crisis from losing more than 200,000 farm jobs (Trevizan and Marques, 2002; Teixeira *et al.*, 2015).

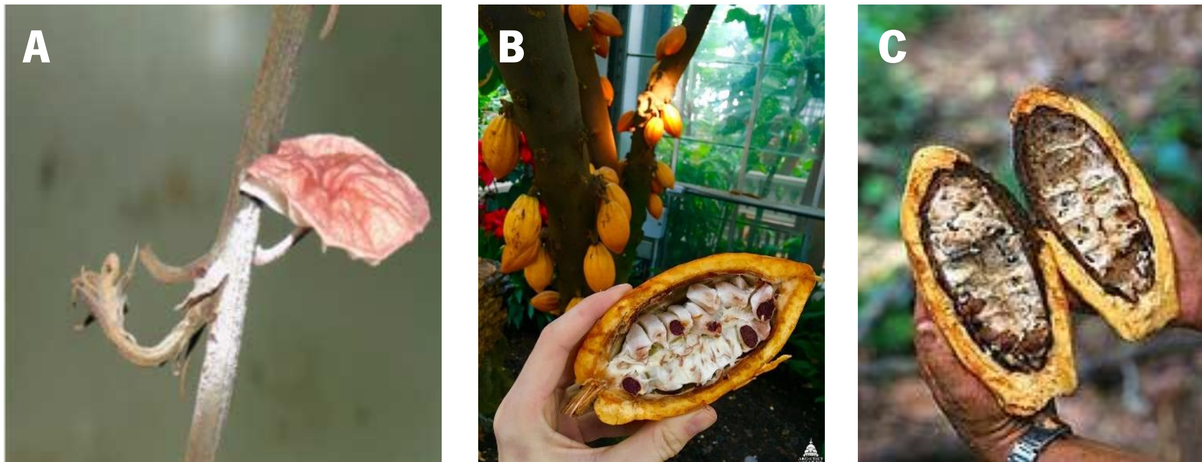

Figure 1. *M. perniciosa* basidiocarps and the released spores on a dry broom of a cacao plant (A), a healthy cacao pod (B) and the damage caused by the pathogen in the infected cacao pods (B). Adapted from Meinhardt *et al.*, 2008 (A) and Bowers *et al.*, 2001 (C).

The severity of WBD derives from several factors. *Moniliophthora perniciosa* does not form specialized infection structures such as *appressoria* like other fungal pathogens. Since it is a hemibiotrophic fungus, the full infectious cycle unfolds through two distinct phases: (i) biotrophic and (ii) saprotrophic. (i) The initial infection occurs in young meristematic tissues and susceptible actively growing tissues (e.g. buds, young leaves, flower cushions, young fruits). The fungus penetrates through the stomatal openings, the bases of damaged trichomes and the husk of young fruits (Aime and Phillips-Mora, 2005). After the initial infection, the fungus induces hypertrophy and hyperplasia, causing the loss of apical dominance. This corresponds to a disorganized proliferation of the infected vegetative meristems of axillary shoots that results in the formation of a broom-like structure of abnormal stems called a *green broom* (Aime and Phillips-Mora, 2005; Meinhardt *et al.*, 2008; Pires *et al.*, 2009). Shortly after the initial infection, the fungus starts growing intercellularly, forming a monokaryotic and parasitic mycelium without clamp connections, establishing a biotrophic relationship with the host that corresponds to its life cycle biotrophic phase. (ii) Usually 4 to 6 weeks after the development of the *green brooms*, a concerted series of infected plant cells death events occurs, and the infected tissues become necrotic forming a structure called *dry*

broom. Necrotic or dead host cells are then colonized by the fungus (Evans, 1980; Meinhardt *et al.*, 2008), which at this point, suffers major morphological changes entering its saprotrophic phase (Lawrence *et al.*, 1991; Meinhardt *et al.*, 2008). The hyphae become dikaryotic, clamp connections are formed, and the fungus begins to grow intracellularly, as well as between cells. The exact mechanisms and signaling factors that trigger the switch from the biotrophic phase to a saprotrophic phase, controlling the developmental alterations, remain unknown (Meinhardt *et al.*, 2008). After the fungus proliferation and colonization of the dead host tissues, pink-colored basidiocarps (small mushrooms) are produced on any infected necrotic tissue. Upon alternate wet and dry periods, each basidiocarp can produce 2 to 3.5 million spores (basidiospores), this way completing the fungus life cycle (Rocha and Wheeler, 1985; Almeida *et al.*, 1997). The release of the spores occurs mainly at night and is related to a high level of humidity and favorable temperature (20-30 °C). The spores are disseminated locally by water and over long distances by wind and can endure latent in the soil or inside pruned branches of the plants for long periods (Meinhardt *et al.*, 2008; Pohlan and Pérez, 2010). An overview of the disease progression in the plant and the parallel events of the fungus life cycle described above are summarized in Figure 2. The ability of *Moniliophthora perniciosa* to infect the plant in all stages of its life-cycle and the fact that virtually all the plant tissues can be infected, underlie this pest exceptional virulence. This, allied to the fungus high prevalence in the soil and plant dead material, explains why once a single plant develops symptoms the whole plantation can be compromised.

The resilience of *Moniliophthora perniciosa* relies essentially on its capacity to colonize both alive and dead plant tissue, the biotrophic and necrotrophic life cycle phases above mentioned. The shift between the two phases involves a drastic morphological and lifestyle change. Alternative Oxidase (AOXp)-respiration was associated with this transition (Thomazella *et al.*, 2012). Possibly, this type of respiration allows the fungal cell to overcome the plant host defenses generated in the first stages of the WBD, as observed with better studied model fungus *Ustilago maydis* (Cárdenas-Monroy *et al.*, 2017). The plant defenses include the production of high amounts of NO, which affect the fungal mitochondria, namely inhibiting respiration complex IV, this way inducing the production of ROS (Thomazella *et al.*, 2012). AOXp is an alternative mitochondrial oxidase that constitutes alone a bypass to respiratory chain complexes III and IV, which function prevents collapse from drugs that target these complexes like cyanide or Antimycin-A (Maxwell *et al.*, 1999; Ruy *et al.*, 2006; Vanlerberghe *et al.*, 2009). At the same time, AOXp-respiration contributes to cope with the electron flux overflow without phosphorylation, and therefore without producing ATP (Van Aken *et al.*, 2009) lowering the global energy yield of metabolism. AOX-encoding gene sequences are found in many organisms (including yeasts and fungi) (Elthon and McIntosh, 1987;

Joseph-Horne *et al.*, 2001; Stenmark and Nordlund, 2003; Chaudhuri *et al.*, 2006; McDonald *et al.*, 2009), but their physiological role in microbes is still not very well understood (Veiga *et al.*, 2003; Rogov and Zvyagilskaya, 2015). In the case of *Moniliophthora perniciosa*, AOXp activity would prevent excessive ROS accumulation inside the fungal cell induced by the host, while maintaining a low metabolism status (Thomazella *et al.*, 2012), which could explain how the fungal cell stays alive while the plant weakens.

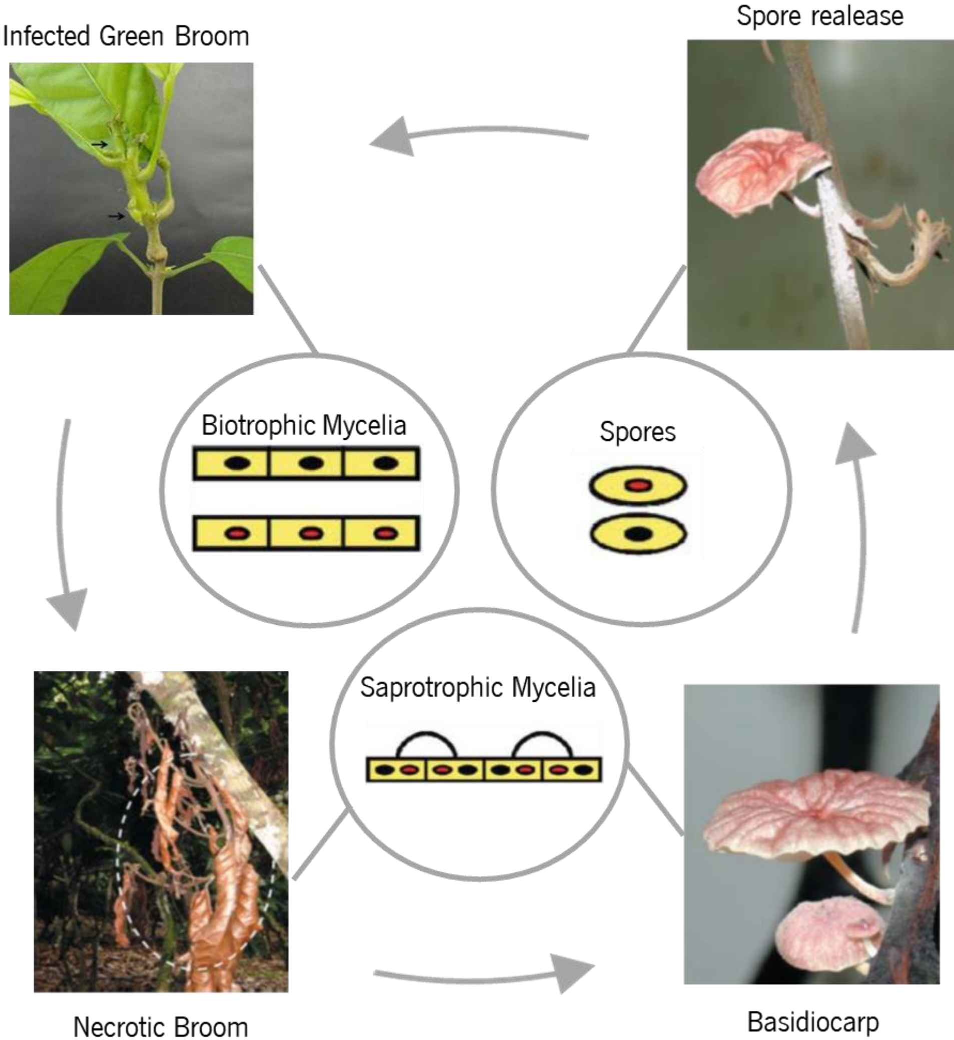

Figure 2. Schematic representation of *Witches' Broom Disease* progression in the cacao plant, and the parallel events occurring in the *M. perniciosa* life cycle. Adapted from Meinhardt *et al.*, 2008

In spite of the few *Moniliophthora perniciosa* genome surveys in the attempt to identify proteins/genes involved in this fungus infectious behavior (e.g. Mondego *et al.*, 2008; Rincones *et al.*, 2008), in fact little is known on this fungus molecular biology, mostly because as all non-model organisms it lacks the appropriate tools for genetic manipulation. Still, some physiological traits apart from AOX-respiration were shown in connection to this fungus pathosystem, like the increased secretion by the host of malondialdehyde (MDA) (usually a marker of oxidative stress (Del Rio *et al.*, 2005)) and glycerol (Scarpari *et al.*, 2005), as well as of a methanol oxidase (MOX) (de Oliveira *et al.*, 2012). Additionally, as for other fungal infections, also in this case the levels of the plant growth hormone ethylene are hypothesized to have a crucial role in the progression of the WBD, in particular in the development of the broom (Scarpari *et al.*, 2005). The biological meaning of these findings remains unknown.

OLIVE ANTHRACNOSE

Another economically important plant disease caused by fungal phytopathogens is Olive Anthracnose (OA), a phytopathology caused by *Colletotrichum gloeosporioides* and *C. acutatum* species complexes that affects the olive trees and fruits (Talhinhas *et al.*, 2011; Cacciola *et al.*, 2012). Olive tree (*Olea europaea* subsp. *europaea*) is one of the most important crops worldwide (Duarte *et al.*, 2010). It is cultivated in all the 5 continents, covering more than 10 million ha, being the Mediterranean region responsible for about 95% of the global production (Moral *et al.*, 2017). In the particular case of Portugal, the land area used for olive production is around 350,000 ha, yielding more than 100 tons of olive fruits in the last decade, most of which has been redirected to olive oil production (more than 750,000 hl in 2016) (INE 2018)[1], therein the particular impact of olive trees and fruits diseases in Portugal where high production losses can be a considerable threat to the national/regional economy. OA affects crop production, pre- and post-harvest, and oil quality (Cacciola *et al.*, 2012; Moral *et al.*, 2014). Presently, OA is spread throughout all the producing continents (Cacciola *et al.*, 2012). The disease symptoms cause serious yield losses, since it provokes premature drop and the destruction of the fruits, causing also a decrease in the vigor and productivity of the tress in the following years (Talhinhas *et al.*, 2018). Moreover, since the fruit integrity is compromised, the disease is also responsible for the deterioration of olive oil quality, affecting both its organoleptic and physicochemical properties, such as color, increased

[1] https://www.ine.pt

acidity, oxidative instability and decreased phenolic content (Carvalho *et al.*, 2008; Cacciola *et al.*, 2012; Talhinhas *et al.*, 2018). The economic consequences of OA are very severe, since in most of the affected growing areas the yield losses can be total (Gouvinhas *et al.*, 2017). Indeed, in the Mediterranean countries, epidemic outbreaks of the disease can cause yield losses varying from 80-100% of the total yearly production. As an example, the overall losses in the net income associated with olive industry due to OA is estimated to be over €80 million per year in Spain (Moral *et al.*, 2009). The disease symptoms (Figure 3) vary accordingly with the ripeness state of the olive drupes (Moral *et al.*, 2008; Cacciola *et al.*, 2012). In the case of mature drupes, symptoms appear in a first phase as circular, dark sunken lesions, with the abundant formation of an orange gelatinous mass of conidia, forming latter several concentric rings beginning at the center of the initial lesion, leading to premature fruit drop and the appearance of fruit rot and/or mummification (Talhinhas *et al.*, 2011; Cacciola *et al.*, 2012; Sharma and Kulshrestha, 2015). On the other hand, regarding ripening fruits, the brown rot of the pulp can occur before the appearance of the other symptoms in the fruit (Moral *et al.*, 2008; Talhinhas *et al.*, 2018). The disease is though not confined to the fruits, since flower blight, leaf chlorosis and necrosis, and branches and twigs defloration can occur (Cacciola *et al.*, 2012; Schena *et al.*, 2014; Talhinhas *et al.*, 2018).

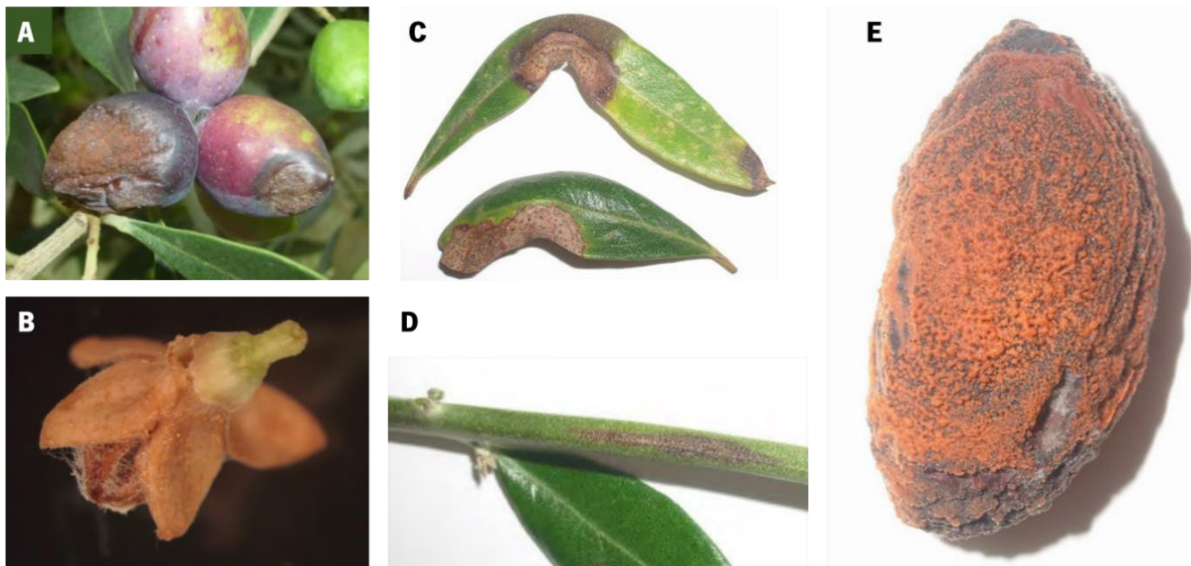

Figure 3. Symptoms of Olive Anthracnose on fruits (A), flowers (B), leaves (C) and young shoots (D), and a mummified olive fruit with a fungal spore mass (E). Adapted from Sergeeva, in press (olivedisease.com).

Differently from what occurs in the case of WBD of cacao, OA is caused by a consortium of ascomycete fungi from the *Colletotrichum* genus, clustering in the *C. gloeosporioides* and *C. acutatum* species complexes (Talhinhas *et al.*, 2011; 2015). These fungi are responsible for other plant diseases, including other economic relevant crops such as papaya, mango, avocado and citrus trees (Dean *et al.*, 2012; Bautista-Rosales *et al.*, 2013; Lima *et al.*, 2013; Talhinhas *et al.*, 2015; Campos-Martínez *et al.*, 2016; Darsini *et al.*, 2017). In Portugal, the primary causal agents of OA were described as *C. nymphaeae* and *C. godetiae*, both clustering in the *C. acutatum* complex, as well as *C. gloeosporioides* sensu stricto (s.s.), which belongs to the *C. gloeosporioides* species complex (Talhinhas *et al.*, 2015). The existence of a geographic specificity for the pathogen species was also reported. Accordingly, *C. nymphaeae* prevails in most of the country; *C. godetiae* in the Northeast region of Portugal (Trás-os-Montes); and both of these species as well as *C. acutatum* s.s and *C. gloeosporioides* s.s. have an equal incidence in the southernmost regions of Portugal (Talhinhas *et al.*, 2015). The different *Colletotrichum* species are responsible for OA variable virulence: *C. acutatum* s.s. and *C. nymphaeae* are the most virulent, followed by *C. godetiae*, *C. fioriniae* and, *C. gloeosporioides* s.s. and *C. rhombiforme* are the less virulent of the complex (Schena *et al.*, 2014; Talhinhas *et al.*, 2015). The pathogens virulence also depends on the susceptibility of olive cultivars and on the producing regions (Moral *et al.*, 2008; Cacciola *et al.*, 2012). This way, the disease epidemiology depends not only in the pathogen prevalence and virulence, but also in several factors such as cultivar susceptibility, climacteric conditions and orchard design (Moral *et al.*, 2008; 2012; Talhinhas *et al.*, 2011; 2015). The life and disease cycle in the *Colletotrichum* genus are similar among species (Figure 4). The initial infection occurs with the germination of the fungal spore, which can be an ascospore, the sexual form, or a conidiospore, the asexual form. After the germination, the fungus penetrates and colonizes the plant tissues (Talhinhas *et al.*, 2018). Conidia germinate at lower rates in olive leaves and branches comparing with drupes and form long germ tubes that usually do no form appressoria. On the other hand, conidia on the fruits form a short germ tubes and appressoria with internal light spot, from which a penetration peg develops (Talhinhas *et al.*, 2018). After the ripening, a short biotrophic stage occurs, with a formation of a multi-lobed primary hypha. At this stage, intracellular hemibiotrophy occurs, and the formed hyphae develop infection vesicles inside plant epidermal and mesophyll cells, using the host metabolites as a nutrient source, without causing its death (Mendgen and Hahn, 2002). During this phase, no host defense is triggered, causing no visible symptoms. The biotrophic stage is followed by a necrotrophic phase in which some of the typical disease symptoms occur, such as tissue necrosis and the production of *acervuli.*

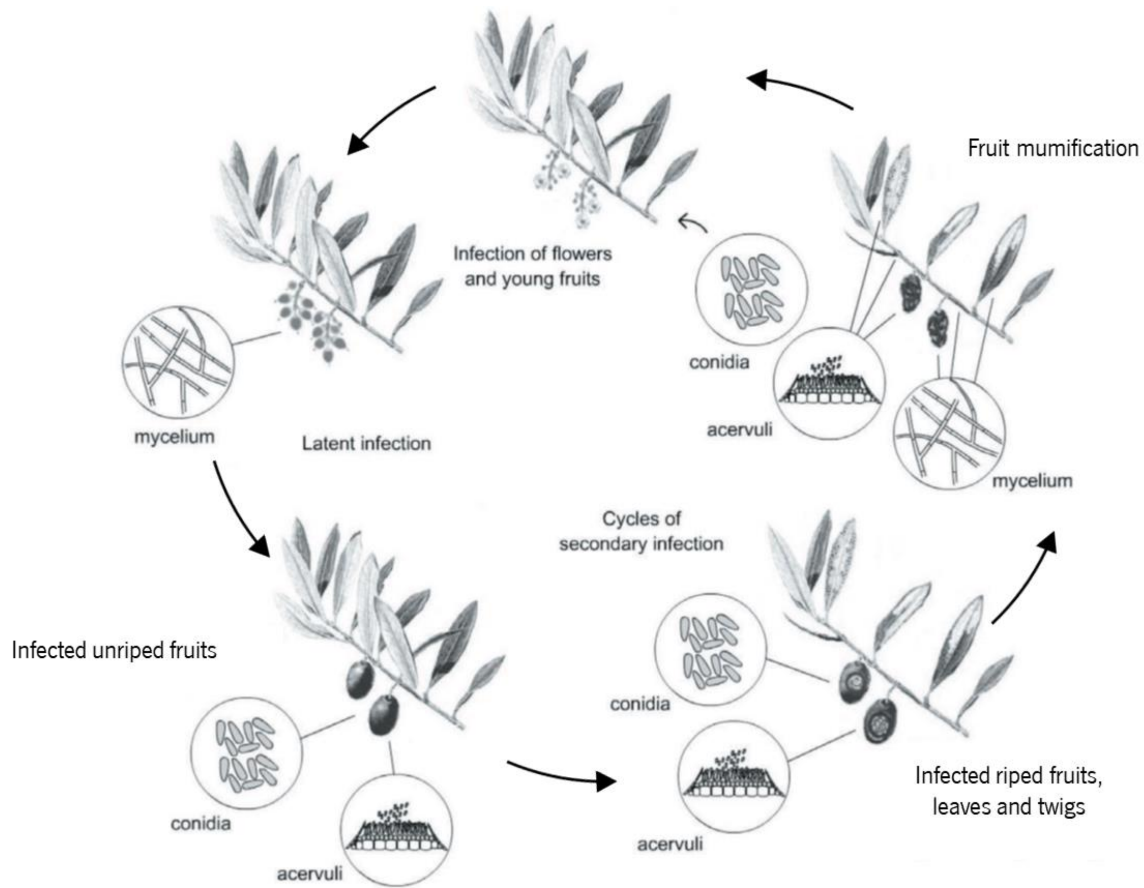

Figure 4. Schematic representation of the disease cycle of Olive Anthracnose. Adapted from Cacciola *et al.*, 2012.

During this stage subcuticular intermural necrotrophy occurs, and secondary hyphae are developed, which secrete lytic enzymes that can destroy adjacent host cells, although there is no morphological distinction between primary and secondary hyphae.

After the initial penetration, the fungus develops an intermural mycelia network within the walls of epidermal cells, and subsequently spreads inter- and intracellularly, leading to the death of the host cells. At this point, the disease symptoms, such as fruit rot or leaf necrosis, start to appear (Wharton and Diéguez-Uribeondo, 2004; Peres *et al.*, 2005). The duration of both phases and the timing of the phase transition differs according to the *Colletotrichum* species involved in the infection, the environmental conditions and also to the host maturity stage (De Silva *et al.*, 2017). An extended latency period in which the fungus remains dormant within the host without occurring fungal growth can occur after the appressoria differentiation (Cacciola *et al.*, 2012; De Silva *et al.*, 2017). This quiescent phase is not considered a significant life phase *per se*, being just a transition between the other pathogen life cycle phases (Prusky *et al.*, 2013). The transition to the necrotrophic phase after the quiescence depends on

various factors. In the first place, the accessibility of the fungus to disassembled cell wall substrates during fruit softening increases, as well as the production of ethylene. Additionally, the inducible host-defense responses decline, namely the amounts of preformed antifungal compounds such as polyphenols, phytoalexins and other fungitoxic substances (Alkan *et al.*, 2013; Prusky *et al.*, 2013). The secretion of ammonia and ammonification of host tissues could be the pathogenicity enhancement factors in mature fruit and could also be a crucial point in the shifting from the quiescent stage to a necrotrophic stage (Prusky *et al.*, 2013). Furthermore, the ammonia secretion can be triggered by changes in pH and in nitrogen content of the fruits (Prusky *et al.*, 2001). The final step of the fungal life cycle congregates the two reproductive stages: asexual/anamorph and sexual/teleomorph. Regarding the asexual stage, the conidia production occurs inside the *acervuli* in specialized hyphae known as conidiophores. These reproductive structures are embedded in an orange, gelatinous, hydrophilic matrix, and release spores at constant pulses (Talhinhas *et al.*, 2011). This matrix contains germination inhibitors and protective enzymes that defends the spores against desiccation and toxic metabolites secreted by the plant hosts, allowing conidia to remain viable for longer periods of time (Perfect *et al.*, 1999). The spores are released in conditions of high humidity and are disseminated predominantly by rain splashes or insects (Cacciola *et al.*, 2012). The majority of the *Colletotrichum* species present only the anamorph stage, but sexual reproduction has also been reported (Cacciola *et al.*, 2012; Schena *et al.*, 2014; Baroncelli *et al.*, 2017). After conjugation, the sexual spores (ascospores) are produced by meiosis inside asci, which in turn are inside dark fruiting structures named perithecia. The occurrence of sexual reproduction is usually related with unfavorable conditions, such as extreme environmental restrictions or the host senescence (De Silva *et al.*, 2017). Perithecia act as survival structures, allowing the fungi to overwinter or survive on a susceptible host. The spores are released from the perithecia under conditions of mild temperature, high humidity and on a susceptible host, and can germinate, initiating another infection cycle (De Silva *et al.*, 2017).

The disease cycle begins mainly during spring, associated with mild temperatures and high levels of humidity. Under such conditions, spores from mummified drupes or withered twigs and branches germinate and develop in the plant tissues, initially infecting developing flowers, which will later give rise to infected fruits (Moral *et al.*, 2009; Talhinhas *et al.*, 2018). Conidia are then abundantly produced on the necrotic lesions on the surface of the fruits, particularly on vulnerable olive cultivar. However, from spring to autumn the infection remains quiescent. Accordingly, during summer the temperatures are generally high, leading to less fungal activity (Talhinhas *et al.*, 2018). Long rain periods and high humidity levels, combined with high host susceptibility and ripeness state, are critical for the occurrence of an OA

epidemic (Cacciola *et al.*, 2012). Moreover, the severity of the disease varies accordingly to phytopathogens that are infecting the olive orchards (Talhinhas *et al.*, 2018). *C. acutatum* s.s. and *C. nymphaeae* are found to be more virulent, therefore more frequently related with epidemic outbreaks (Schena *et al.*, 2014; Talhinhas *et al.*, 2015). Fruit ripeness is also a crucial factor, since the ripening rates are higher and faster in trees with fewer fruits, consequently increasing the host susceptibility. Since an epidemic outbreak of OA decreases the fruit load in the subsequent year, the probability of another epidemic event increases, contributing to the severity of this disease (Moral and Trapero, 2012). The necrotrophic stage of the pathogens often causes fruit rot, which in turn results in premature fruit mummification. The majority of the mummified drupes fall to the ground and are easily decomposed, while the few that remain on the tree are capable of releasing viable conidia at a constant rate during several months, probably serving as inoculum for spring infections (Moral and Trapero, 2012; Sergeeva, 2014; Talhinhas *et al.*, 2018). These latent spring infections can, in turn, serve as the main inoculum source for autumn infections (Moral *et al.*, 2009; Talhinhas *et al.*, 2011). It has also been reported that in controlled conditions, one infected fruit per tree can affect up to 100% of the fruits in a susceptible cultivar if favorable weather conditions persevere during autumn (Moral *et al.*, 2009). All these factors combined explain why once a single plant in an olive orchard starts to develop symptoms, the whole plantation can be endangered.

FUNGAL DISEASES MANAGEMENT

The chemical fungicides generally used are either copper-based compounds, such as cuprous oxide, or azole-containing molecules, particularly tebuconazole (Oliveira and Luz, 2005; Medeiros *et al.*, 2010). These are usually used to control the spread of other fungal plant diseases, such as grapevine downy mildew and olive peacock spot, but showed very low efficiency against *M. perniciosa* (Medeiros *et al.*, 2010) .as well as the above described *C. gloeosporioides* and *C. acutatum* species complexes (Cacciola *et al.*, 2012). Copper is *per se* a non-specific anti-microbial agent able to destroy naturally occurring microorganisms, including fungi, that is for decades applied as foliar sprays (Yang *et al.*, 2011; Husak, 2015). The lethal action of copper-based fungicides derives from their ability to free copper ions that are massively internalized by the fungal cells. Intracellularly, they bind various chemical groups (imidazoles, phosphates, sulfhydryls, hydroxyls) namely in proteins, causing their denaturation and loss of function (Husak, 2015; Mirković *et al.*, 2015). Ultimately, this leads to irreversible cell damage and membrane

leakage (Husak, 2015). Yet some fungi are resistant to copper ions. The mechanisms underlying this resistance are not well understood, although several studies have suggested that they might exert a combined action: the extracellular chelation and cell wall sequester of copper ions, and their decreased intake and intracellular complexing by metallothioneins or other proteins (Cervantes and Gutierrez-Corona, 1994). This last case includes the over-expression of the superoxide dismutase (SOD) that uses copper as inorganic co-factor (Naiki, 1980). SOD has been described to display the ability to buffer copper excess independently of its superoxide scavenging function (Culotta *et al.*, 1995). Additionally, the copper-induced accumulation of glycerol was also described to be involved in its extrusion (Gadd *et al.*, 1984). Neither of these mechanisms were ever described in association with *Moniliophthora perniciosa*, although increased levels of SOD would contribute the higher resistance to the above-mentioned plant-generated ROS.

On the other hand, tebuconazole, as other azole-fungicides, acts on the synthesis of ergosterol, altering the structure and functionality of the fungal cell membrane (Price *et al.*, 2015) as well as vacuolar ion homeostasis through v-ATPase function (Zhang *et al.*, 2010b). In consequence of ergosterol synthesis disruption, mitochondrial function is also affected in its ability to form iron-sulfur clusters, which results in the deposit of insoluble iron inside mitochondria and concomitant radical formation and mitochondrial loss (Ward *et al.*, 2018). CytC harbors a *heme* group which availability for this enzyme proper assembly and function would be affected by iron homeostasis disruption, consequently affecting Complex IV function in respiration. This would justify why fungi that can respire through the AOXp could be resistant to azoles. Still, there is no reference to this possibility in the literature. Rather, in *Candida* species, the resistance to azole-fungicides implicates other types of mechanisms (Whaley *et al.*, 2017). Nevertheless, these are human commensals and pathogens, having therefore specificities that are not common with other yeasts or fungi.

Fungicides fail to control the spread of the WBD but the mechanisms underlying the resistance of *Moniliophthora perniciosa* to these drugs are not studied. The use of fungicides is therefore not a routine practice in most cacao-producing countries also due to their high cost, and the risks associated with cacao chemical contamination which hinders commercialization. The reasons underlying this include the increasingly considered negative impact of fungicides on human health and the environment. Public concerns regarding the prevalence of agronomic pesticide residues in food, and their relation with the increasing advent of pesticide resistant pathogens, not only in plants but also in humans (Droby, 2006; Pal and McSpadden Gardener, 2006; Marelli *et al.*, 2009; Verweij *et al.*, 2009; Nunes, 2012) led to restrictions in Europe. Therefore, the most commonly used methods to control the WBD are exclusively

agronomic, through phytosanitary pruning, removing as much as possible the infected material, which is though often impossible, due to hidden fungal inoculum in the soil and cut branches and leaves[2]. Therefore, more effective and eco-friendly methods and strategies are needed to satisfy the consumer demands.

In the case of OA management, it is mostly based on the use of chemical fungicides, which should be applied in a preventive manner, since the disease is nearly impossible to control once symptoms emerge (Sergeeva, 2011; Talhinhas *et al.*, 2011; Cacciola *et al.*, 2012; Moral *et al.*, 2012). Few fungicides have shown some efficacy in controlling the disease, including copper-based fungicides, dithiocarbamate, azoles (hexaconazole and tebuconazole) and strobilurins (azoxystrobin and trifloxystrobin) (Pennisi *et al.*, 1993; Sergeeva, 2011; Moral *et al.*, 2014). The effectiveness of the treatment with these drugs depends on several factors, such as the severity of the disease, the plant cultivar and environmental conditions, being therefore regionally variable. Furthermore, the timing, frequency and number of fungicide applications, as well as the preventive or curative nature of the treatment, are factors to take into account for the success of a particular chemical-based strategy (Cacciola *et al.*, 2012; Landum *et al.*, 2016). Regardless with the level of efficiency of chemical fungicides, other problems associated with their use also arise. The wash-off of the fungicides from olive crops due to rains leads to residue accumulation in soil and adjacent water areas, which could have negative and harmful effects on the surrounding ecosystem and even in human health (Komárek *et al.*, 2010; Lamichhane *et al.*, 2018). Additionally, since the active constituents of fungicides commercially exploited for disease management are chemically very similar and generally belong to the same molecular family, fungal phytopathogens easily adapt and acquire resistance (Ma and Michailides, 2005). Therefore, the overuse of these types of drugs eventually leads to their increasing inefficacy followed by loss of effect, leaving crops without possible treatment (Cacciola *et al.*, 2012). The general public awareness of food and environmental contamination caused by the misuse of chemical pesticides, as well as the risks associated to human health are pushing European policies to restrict the employment of fungicides in the control of phytopathogenic diseases. Consequently, OA management is nowadays increasingly based in agricultural strategies, which include the replacement or susceptible cultivars with resistant ones, early harvesting, strategic pruning and even orchard design (Talhinhas *et al.*, 2011; Cacciola *et al.*, 2012; Moral *et al.*, 2012; Leoni *et al.*, 2018). The efficiency of these strategies is though limited. The use of resistant cultivars should at term be the best strategy, although the complete replacement of the orchards takes a significant lag-time in production

[2] Available at: https://www.icco.org/about-cocoa/pest-a-diseases.html [Accessed February 18, 2019]

and is altogether very expensive (Landum *et al.*, 2016). Additionally, in cases where ideal conditions for fungal growth are met, apparently the infection occurs anyway (Cacciola *et al.*, 2012). Therefore, new more effective and sustainable methods to control OA are essential.

A concept that has gained considerable prominence in the agriculture sector in recent years is the use of nanoagroparticles, which are nanoparticles designed to mitigate agriculture-related problems, including plant pathologies (Parizi *et al.*, 2014; Parisi *et al.*, 2015). These nanoagroparticles include silver, copper, sulfur, zinc oxide and magnesium oxide nanoparticles (Baker *et al.*, 2017), and can act efficiently as fungicides, pesticides, herbicides and also insecticides. They can easily enter into the fungal cell wall. Once inside the cell, they act through different modes, which include (i) causing the disruption of metabolism, or of the cell membrane, with consequent loss of cellular content (Baker *et al.*, 2015; Baker *et al.*, 2017), (ii) promoting the release of toxic ions (Cd^{2+}, Zn^{2+} and Ag^{+}) that bind to sulfur-containing proteins, (iii) targeting the pathogen DNA, this way inducing cell death, (iv) interrupting electron transport, this way causing the collapse of membrane potential, (v) promoting the generation of ROS, or (vi) interfering with nutrient uptake (Alghuthaymi *et al.*, 2015). More than one of these mechanisms can occur simultaneously, conferring the ability of nanoparticles to be effective against different plant pathogens (Alghuthaymi *et al.*, 2015). Recently, nanoparticles were conjugated with some biomolecules (including biocide/killer toxins), forming bionano-hybrid agroparticles (Baker *et al.*, 2017). The free utilization of these promising phytopathology management tools still requires not only cytotoxicology studies to evaluate potential harm to human and animal health, but even more important, extensive ecotoxicology and biodegradability studies to evaluate their prevalence in the environment and food chains and their effect on the long run in the microflora of plant, soil and water. Presently few information on this regard is available (Alghuthaymi *et al.*, 2015). Nonetheless, the prospective of being able to use such a nanotool to effectively deliver a toxin and kill a phytopathogenic fungus is attractive, especially if carrying a bio-derived killing agent.

YEASTS AS BIOCONTROL AGENTS

One possible approach to fungal diseases in plants might be the use of biocides or biological control agents. In phytopathology, this term designates the use of introduced or resident living organisms to contain or suppress populations of pathogens (Pal and McSpadden Gardener, 2006). There are a few of these agents in the market, mostly used in the control of pests at small scale. They correspond to dry biomass of bacterial or filamentous fungal strains isolated from the endosphere or the rhizosphere of plants (O'Brien, 2017) that are re-hydrated and used as alive reproductive microorganisms. These include a taxonomically and biologically diverse group of endophytic fungi that are characterized by colonizing internally the plant host tissues without causing any external disease symptoms (Wilson, 1995; Rubini *et al.*, 2005). These endophytes can prevent pathogen infection and propagation directly by competition, mycoparasitism or antibiosis, or indirectly by inducing resistance responses in the plant (Bailey *et al.*, 2006). Despite this, the biocontrol agents that could be applied in phytopathology are not restricted to these two groups of organisms.

Endophytic microorganisms also include Ascomycota and Basidiomycota yeasts, found in many species of trees from very diverse climates, but also in agricultural species (reviewed by Doty, 2013). Ascomycota yeasts reproduce exclusively by budding, as the most well-known yeast *Saccharomyces cerevisiae*. Basidiomycota grow dimorphically, shifting from a monokaryotic yeast-form to a dikaryotic filamentous form (Choudhary and Johri, 2009). This is the case of *Rhodotorula* and *Cryptococcus* sp. (Table 1). Generally, endophytic yeasts apparently thrive symbiotically or mutualistically, virtually colonizing diverse plant tissues (reviewed by Doty, 2013), in which they may cause structural changes (Luna, 2017). They consume sugars and assimilate amino acids generated by the plant and contribute to the plant wellbeing and stress response in many different ways, including the production of phytopheromones, catalase or siderophores (reviewed by Joubert and Doty, 2018). Importantly, endophytic like epiphytic yeasts can antagonize phytopathogenic filamentous fungi, either by occupying their niche or by antagonizing them in more complex ways.

The antagonistic interaction of yeasts with particular phytopathogenic fungi has been described in the literature. For example, Suzzi *et al.* (1995) observed that natural wine yeast strains of *Saccharomyces* and *Zygosaccharomyces* inhibited *in vitro* the growth of 10 species of soil-borne fungal plant pathogens, namely *Cladosporium variabile*, *Rhizoctonia fragariae*, *Phomopsis longicolla*, *Colletotrichum acutatum*, *Aspergillus niger*, *Sclerotinia sclerotiorum*, *Penicillium digitatum*, *Macrophomina phaseolina*, *Trichoderma viride* and *Botrytis squamosa*. Also, Walker *et al.* (1995) reported that strains of

Saccharomyces cerevisiae and *Pichia anomala* (know designated *Wickerhamomyces anomalus*) inhibited *in vitro* the growth of several wood decay basidiomycetes including *Serpula lacrymans*, *Postia placenta*, *Lentinus lepideus* and *Ophiostoma ulmi* and phytopathogenic fungi, such as *Rhizoctonia solani*, *Fusarium equiseti*, *Botrytis fabae* and *Phytophthora infestans*. Importantly, Rosa-Magri *et al.* (2011) described the antagonism effect of the yeast *Torulaspora globosa* against the phytopathogenic mold *Colletotrichum graminicola*, the causal agent of anthracnose disease in maize. All of these cases were reported as *in vitro* studies, none were performed *in planta* or *in field*. Otherwise, yeasts have often been proposed and used for the control of microbial contaminations at the postharvest phase (Table 1).

The possibility of using yeasts as biocontrol agents of fungal or bacterial proliferation associated with food spoilage has been recognized since the early 1960s, when it was found that Saccharomyces cerevisiae strains secreted toxins that killed other yeast strains but are immune to their own toxin (Bevan and Makower, 1963). Killer toxins (KTs) can be encoded by cytoplasm-inherited double-stranded RNA viruses (Schmitt and Breinig, 2002) or linear dsDNA plasmids (Schaffrath and Meinhardt, 2005), but they can also be chromosomally encoded (Suzuki, 2005). The killer phenomenon is well characterized and studied in Saccharomyces cerevisiae. In this yeast species, KTs have been grouped into four types, K1, K2, K28, and Klus, based on their killing profiles and lack of cross-immunity (Schmitt and Breinig, 2006; Rodríguez-Cousiño *et al.*, 2011). Each strain producing one specific toxin kills strains from the other groups but has self-protective immunity (Schmitt and Breinig, 2006).

Killer toxins

The modes of action of the *Saccharomyces cerevisiae* KTs are well known, with the exception of the recently found Klus toxin (Schmitt and Breinig, 2002). K1 and K2 toxins kill sensitive yeast cells in a receptor-mediated two-step process. The first step involves a fast, energy-independent binding to a primary toxin receptor (R1), consisting of β-1,6-D-glucan (Lukša *et al.*, 2015). Though a second energy-dependent step, the toxin is translocated from the cell wall to the plasma membrane, where it interacts with a secondary membrane receptor (R2), identified in the case of K1 toxin as Kre1p, an *O*-glycosylated protein of the yeast cell surface (Breinig *et al.*, 2002; 2004). After reaching the plasma membrane, K1 and K2 toxins disrupt its function by forming cation-selective channels, and promoting the release of ATP and other metabolites, thus causing a lethal effect on the target cell (Liu *et al.*, 2015).

Table 1. Success cases of using yeasts to antagonize the spoilage of fruits by filamentous fungi.

Yeast antagonist	Host	Fungal phytopathogen(s)	References
Preharvest application[1]			
Candida (Pichia) guilliermondii	Cherry tomato	Fruit decay agents	Zhao *et al.*, 2011
Candida sake	Apple	*Penicillium expansum*	Teixidó *et al.*, 1999
Postharvest application			
Aureobasidium pullulans	Pear	*Penicillium expansum*	Robiglio *et al.*, 2011
	Apple	*Botrytis cinerea*, *Colletotrichum acutatum* and *Penicillium expansum*	Mari *et al.*, 2012
Candida (Pichia) guilliermondii	Chilli	*Colletotrichum capsici*	Chanchaichaovivat *et al.*, 2007
	Tomato	*Rhizopus nigricans*	Zhao *et al.*, 2008
		Rhizopus stolonifer	Celis *et al.*, 2014
	Kiwifruit	*Botrytis cinerea*	Sui and Liu, 2014
	Papaya	*Colletotrichum gloeosporioides*	Lima *et al.*, 2013
Candida oleophila	Banana	*Colletotrichum musae*, *Fusarium moniliforme* and *Cephalosporium* sp.	Lassois *et al.*, 2008
	Apple	*Penicillium expansum* and *Botrytis cinerea*	Liu *et al.*, 2012
Candida pelliculosa	Tomato	*Botrytis cinerea*	Dal Bello *et al.*, 2008
Candida sake	Apple	*Penicillium expansum*	Morales *et al.*, 2008
Cryptococcus infirmo-miniatus	Sweet cherry	*Monilinia fructicola*	Spotts *et al.*, 2002
Cryptococcus laurentii	Strawberry	*Botrytis cinerea*	Wei *et al.*, 2014
	Sweet cherry	Fruit decay agents	Tian *et al.*, 2004
Debaryomyces hansenii	Peach	*Rhizopus stolonifer*	Mandal *et al.*, 2007
	Mandarin, orange	*Penicillium digitatum*	Taqarort *et al.*, 2008
Metschnikowia fructicola	Apple	*Penicillium expansum*	Liu *et al.*, 2011
	Grapefruit	*Penicillium digitatum*	Hershkovitz *et al.*, 2013
Meyerozyma caribbica	Mango	*Colletotrichum gloeosporioides*	Bautista-Rosales *et al.*, 2013
Pichia membranefaciens	Apple	*Monilinia fructicola, Penicillium expansum* and *Rhizopus stolonifer*	Chan and Tian, 2005
Rhodosporidium paludigenum	Cherry tomato	*Botrytis cinerea*	Wang *et al.*, 2010
Rhodotorula mucilaginosa	Pear	*Penicillium expansum*	Hu *et al.*, 2015
Rhodotorula rubra	Tomato	*Botrytis cinerea*	Dal Bello *et al.*, 2008
Wickerhamomyces (Pichia) anomalus	Banana	*Colletotrichum musae*, *Fusarium moniliforme* and *Cephalosporium* sp.	Lassois *et al.*, 2008
	Orange	*Penicillium digitatum*	Aloui *et al.* 2015; Platania *et al.*, 2012
	Papaya	*Colletotrichum gloeosporioides*	Lima *et al.*, 2013
Commercial yeast-biocontrol products[2]			
Aureobasidium pullulans	Pome	*Penicillium, Botrytis, Monilinia*	Boni Protect®, Bio-Ferm, AT
Candida oleophila	Pome	*Penicillium, Botrytis*	Nexy®, Lesaffre, BE
Metschnikowia fructicola	Pome, table grape, stone fruits, strawberry, sweet potato	*Penicillium, Botrytis, Rhizopus, Aspergillus*	Shemer®, Bayer/Koppert, NL

[1] Preharvest application to prevent postharvest spoilage.
[2] Wisniewski *et al.*, 2016.

The K28 KT mode of action is very different, since it enters a sensitive target yeast cell by endocytosis, in a cell wall receptor-mediated manner (Schmitt and Breinig, 2006). The cell wall receptor for K28 toxin has been identified as a mannoprotein with high molecular mass (Liu *et al.*, 2015). The K28 toxin is internalized through the secretory pathway (via Golgi and ER), and after entering the cytosol the β-subunit is ubiquitinated and degraded in the proteasome. The subsequently free small α-subunit has been suggested to enter the nucleus without the help of an active nuclear import machinery, therefore by a so-called passive diffusion (Schmitt and Breinig, 2006). Once inside the nucleus, the K28 toxin kills the host cell by irreversibly blocking the DNA synthesis. The target cells arrest in early S phase of the cell cycle, forming a medium-sized bud and a single, pre-replicated nucleus in the mother cell, eventually dying (Schmitt and Breinig, 2006).

The killer phenomenon, despite being best characterized in *Saccharomyces cerevisiae*, is not confined to this yeast species, rather it is often found in other yeast species and genera (Magliani et al., 1997; Schmitt and Breinig, 2002). Some of these were described to damage the plasma membrane, very similarly to the *Saccharomyces cerevisiae* K1 toxin. This is the case of the KTs produced by *Pichia kluyveri* (Ahmed et al., 1999), *Pichia membranifaciens* (Santos and Marquina, 2004; Santos et al., 2009), *Pichia farinosa* (Suzuki et al., 2001) and *Zygosaccharomyces bailii* (Weiler and Schmitt, 2003). Other killer mechanisms include the damage of the cell wall upon the inhibition of the synthesis of β-glucans. Examples of this mode of action are the toxins produced by *Hansenula mrakii* (previously *Williopsis mrakii*) (Marquina et al., 2002), Wickerhamomyces anomalus (formerly designated *Pichia anomala* or *Hansenula anomala*) (Wang et al., 2007), *Williopsis saturnus* (Guyard et al., 2002; Peng et al., 2010) and *Kluyveromyces phaffii* (Comitini et al., 2009). Yet other yeast KTs act by blocking the cell cycle, namely the one produced by *Kluyveromyces lactis* (Klassen et al., 2004), and by triggering DNA damaging and the induction of apoptosis, which is the case of the toxins secreted by *Pichia acaciae* (Klassen and Meinhardt, 2005) and *Wingea robertsiae* (Klassen and Meinhardt, 2002). The blocking of calcium uptake was also described as killer mode of action, namely in the case of *Ustilago maydis* (Gage et al., 2001). Although there are plenty of reports in the literature regarding the interaction of non-*Saccharomyces* killer yeasts with a vast variety of sensitive targets, the actual mechanisms involved remain mostly unknown or superficially studied at the molecular level.

Lytic enzymes and mycoparasitism

Several other mechanisms of yeast antagonism have been proposed that do not involve the secretion of a peptide/protein that may be classified as a KT. Other proteins that are secreted by the yeast and antagonize filamentous fungi are lytic enzymes that destroy the fungal cell wall (Spadaro and Gullino, 2004). This kind of antagonism is considered a form of mycoparasitism. An example is the manner in which *Pichia guilliermondii* antagonizes *Botrytis cinerea* (Wisniewski *et al.*, 1991). The authors observed that the fungus cell wall glucans and a yeast-secreted β-(1–3) glucanase form a lectin-like interaction resulting in a strong attachment of the antagonist to the fungal pathogen which culminates with the lysis of fungal cells. Besides the secretion of these antifungal compounds, other modes of action can be involved in the mycoparasitism behavior. Yeasts can attack the fungi pathogens by direct physical contact, reaching the fungal cells and killing them, although not necessarily through an invasion of the target cell (Mims *et al.*, 2007). Additionally, yeasts can act as predatory mycoparasites, physically penetrating their prey cell walls, through haustoria or penetration pegs (Junker *et al.*, 2019).

Volatile compounds

Yeasts that antagonize other yeasts can also produce several volatile compounds against filamentous fungi that inhibit the target growth (Mari *et al.*, 2016). These compounds include alkenes, alcohols, ketones, benzenoids, pyrazines, sulfides and terpenes (Schulz-Bohm *et al.*, 2017). These have in common the fact of having small molecular weight and physicochemical properties which facilitate evaporation and diffusion in soil and rhizosphere environments (Mari *et al.*, 2016; Schulz-Bohm *et al.*, 2017). This is the case of the yeast *Aureobasidium pullulans* that produces 2-methyl-1-butanol, 3-methyl-1-butanol, 2-phenethyl alcohol and 2-methyl-1-propanol, with inhibitory effect against *Botrytis cinerea*, *Colletotrichum acutatum*, *Penicillium expansum*, *Penicillium digitatum* and *Penicillium italicum (Di Francesco et al., 2015)*. Interestingly, it appears that antagonizing yeasts may operate in different ways also because in some cases they strongly attach to the fungus hyphae. This was described in detail for the case of the yeasts *Pichia membranefaciens* and *Cryptococcus albidus* when challenged with three phytopathogenic fungi causing the postharvest deterioration of nectarines and apples (*Monilinia fructicola, Penicillium expansum* and *Rhizopus stolonifer*) (Chan and Tian, 2005).

Competition for nutrients and space

Another effective mechanism of antagonism and possibly the most common is competition. Microbes compete for space, for oxygen and of course for nutrients, such as carbohydrates, vitamins, minerals and amino acids (Spadaro and Droby, 2016). Yeasts grow much faster than filamentous fungi, being thus able to quickly colonize the niches that fungi can occupy, such as plant wounds or tissue lesions, forming colonies or biofilms (Andrews *et al.*, 1994). Increasingly bigger yeast populations reduce the amount of nutrients available for fungi and make them difficult to access (Zhang *et al.*, 2010a). In the case of micronutrients, iron plays a crucial role in the growth, development and virulence of the fungal pathogens (Saravanakumar *et al.*, 2008). To compete with the pathogens for iron, yeasts secrete siderophores that deplete iron from the growth medium such as pulcherrimin, produced by *Metschnikowia pulcherrima* to compete with *Botrytis cinerea*, *Alternaria alternata* and *Penicillium expansum* (Saravanakumar *et al.*, 2008).

Quorum sensing

The production and secretion of quorum sensing molecules can be also involved in yeast antagonism. The concept of quorum sensing was introduced in 1994 (Fuqua *et al.*), and consists in the cell-to-cell communication process through which each individual cells can adjust its phenotype in response to the presence of extracellular quorum sensing molecules (QSM) at a specific concentration (Wuster and Babu, 2007). When QSM reach those concentrations, they bind with a receptor, link with promotor sequences and activate the transcriptional regulators of specific genes (Mehmood *et al.*, 2019). Examples of these kind of molecules described in yeasts are 2-phenylethanol, tryptophol and tyrosol (Avbelj *et al.*, 2016). Fungi can also produce a particular type of secondary metabolites which can act as anti-microbial agents known as quorum sensing inhibitors (QSI). These microorganisms colonize several habitats interact with other organisms, such as other microbes, animals and plants. Due to the need to compete for nutrients and space, they deal with competitor organisms by producing secondary metabolites, enzymes and chemicals (Padder *et al.*, 2018). These compounds can act as QSI by degrading QSM, delaying their production or even blocking the receptors via homologs of QSM (Padder *et al.*, 2018; Mehmood *et al.*, 2019). An example of QSI is farnesol, a secondary metabolite secreted by many dimorphic yeasts (Wongsuk *et al.*, 2016; Mehmood *et al.*, 2019).

Environmental variables

Ultimately, the efficiency of the antagonism is affected by environmental constraints (Syed Ab Rahman *et al.*, 2018), and each mechanism of action depends on a specific interaction between the pathogen and the antagonizing organism but also between either and the host. It has been described that antagonizing yeasts may help the host by alleviating the production of ROS induced by the pathogen (Liu *et al.*, 2013). The production of ROS is actually not confined to plants themselves, but also happens in fruits during postharvest processes. This was observed to occur when yeasts such as *Cryptococcus laurentii* and *Rhodotorula glutinis,* which exhibit antagonistic activity against the postharvest pathogens *Botrytis cinerea* and *Penicillium expansum*, were applied to fruit surfaces and wounds (Castoria *et al.*, 2003). Concurrently, a biofilm of a *Saccharomyces cerevisiae* strain isolated from wine prevented the spoilage of apples by *Penicillium expansum* (Scherm *et al.*, 2003). The potential of killer yeasts has often been recognized, but their use seldom put to practice for economically viable processes. In biotechnology, yeast-to-yeast antagonism has been mostly successfully applied in the control of wine and brewery processes, to avoid secondary fermentations producing undesirable compounds (Hatoum *et al.*, 2012; Mehlomakulu *et al.*, 2014; Oro *et al.*, 2016). Another example of successfully commercialized application of a killer yeast is that of *Debaryomyces hansenii*, used in the prevention of spoilage of dairy products, such as milk and yogurt (Liu and Tsao, 2008), as well as in the extension of shelf-life of dry-cured meats and dry-fermented sausages (Andrade *et al.*, 2014; Núñez *et al.*, 2015). The spoilage prevention by yeasts extends to the postharvest protection of fruits from fungal spoilage, which acknowledges that yeasts can display significant antimycotic activity (Table 1). The majority of these antagonistic yeasts are naturally present on fruit and vegetable surfaces (Suzzi *et al.*, 1995) but can be also obtained from other sources such as the phyllosphere (Kalogiannis *et al.*, 2006), the rhizosphere (Long *et al.*, 2005) or the soil (Zhao *et al.*, 2012). The advantages of using naturally occurring yeasts strains are those of overcoming the negative effects of needed adaptation to the biophysical and biochemical specificities of the contaminated niches.

ADVANTAGES AND CHALLENGES OF MICROBIAL WARFARE

The use of live antagonist microorganisms or their nano-conjugated bio-derived toxins to manage phytopathogenic diseases would offer a great number of advantages over chemical fungicides or extensive pruning, including the safer field application methods and the reduced costs of production (Bonaterra *et al.*, 2012), offering an environmentally friendly and bio-sustainable alternative to manage fungal diseases, provided the right microorganism is used. Any such option has to be primarily challenged with tests *in planta* and *in field.* Moreover, the solution, as mentioned above, has to be proven unharmful to human or animal cells (Ocampo-Suarez *et al.*, 2017), as well as easily degradable so that its prevalence in the agriculture ecosystems does not generate a potential accumulation-derived harmful effect over time, and non-ecotoxic allowing the ecosystem functions to prevail (Liu *et al.*, 2013). Finally, the commercial viability of any biocontrol agent needs to be subsequently assessed in pilot, semi-commercial and large-scale commercial studies, prior to regulatory licensing from the respective regulatory agencies (Droby *et al.*, 2009). The final approval needs to be based on the actual disease control efficacy and also the evaluation of the safety of the formulated product (Dukare *et al.*, 2018). For this reason, and despite the recognition of the efficiency of microbial antagonisms and the theoretical advantages of their use, only a limited number of products based on biocontrol agents have been formulated and commercialized over the past decades (Dukare *et al.*, 2018). These products are registered for using mainly against some postharvest fungal phytopathogenic diseases of fruits and vegetables (Sharma *et al.*, 2009; Sundh and Melin, 2011; Dukare *et al.*, 2018). As an example, Nexy® (Table 1) a formulation of water dispersed granules of living biomass of the yeast *Candida oleophila*, has been approved and commercialized in the EU against postharvest spoilage of stored fruits, namely against *Penicillium expansum* and *Botrytis cinerea* in apples, *Penicillium* spp. in citrus fruits and *Colletotrichum musae* in bananas (Ballet *et al.*, 2016).

The microorganisms described to antagonize filamentous fungi include endophytic fungi, which have been suggested as biocontrol agents of several cacao plant diseases (Arnold *et al.*, 2003). These authors demonstrated that inoculating cacao plant leaf tissues with fungal endophytes isolated from naturally infected asymptomatic hosts, significantly decreased the damage provoked by the foliar pathogen *Phytophthora* sp.. Fungal endophytes isolated from healthy cacao plant tissues also displayed *in vitro* antagonism response against major pathogens of cacao, including the WBD (Mejía *et al.*, 2008). The antagonism mode of action was reported as competition for nutrients or antibiosis (Mejía *et al.*, 2008). WBD fungus was though not killed by any of the tested endophytic fungal strains. In another study (Rubini *et al.*, 2005), the endophytic fungus *Gliocladium catenulatum*, isolated from healthy cacao plants, was

able to reduce the incidence of WBD in cacao seedlings to 70% in greenhouse conditions. Again, *Moniliophthora perniciosa* was not killed by *Gliocladium catenulatum*. Instead, another endophytic isolate from *Trichoderma stromaticum* parasites internally *Moniliophthora perniciosa*, preventing its reproduction (Samuels *et al.*, 2000; Pomella *et al.*, 2007; Medeiros *et al.*, 2010). No information is presently available as to the molecular basis of this antagonism or the death process associated.

The Brazilian governmental organization CEPLAC (Executive Committee of the Cocoa Farming Plan) has produced and developed a semi-commercial product named Tricovab®, that is available to Brazilian farmers on a *by request* basis. This biocontrol agent is multiplied in silos of rice grains, in which surface the fungus multiplies. The local farmers receive the packed dry rice grains and activate the product by making a suspension in water that can be spread directly in the plantation (www.ceplac.gov.br). Despite being an alternative with relatively high efficacy compared to the other methods, it is a very expensive methodology. The high price derives from the very complex and expensive *Trichoderma stromaticum* rice-dependent production system, reason why this method is entirely subsidized by the federal government and supplied as a service from the Brazilian Ministry of Agriculture and developed at its R&D unit CEPLAC[3] (Silveira, 2013). Additionally, the waste of large amounts of rice diverted from food resource, and the unavailability of the product to local farmers located far from the CEPLAC distribution centers contribute to the impracticability of this solution, reinforcing the urgent need for strategies to control the WBD that are more sustainable. The utilization of antagonistic yeasts as biocontrol agents could be one such case. The possibility of using yeasts for this purpose is very attractive because, unlike filamentous fungi or bacteria, (i) they are ubiquitously found in the phytobiome, displaying a degree of biodiversity that allows the finding of natural and specific antagonisms, (ii) they are GRAS (Generally Recognized as Safe) for humans and animals and therefore safe to manipulate, (iii) they generally promote the wellbeing of the plants, (iv) they are environmental-friendly microorganisms, and (v) they can be cheaply multiplied to very high amounts, particularly in Brazil, in view of the long tradition of yeast-fermentation industries. Two yeast strains were previously shown to strongly antagonize *Moniliophthora perniciosa in vitro,* a *Candida* sp. strain isolated from an organic farm soil and a *Dipodascus capitatus* strain isolated from the jackfruit (Cabral *et al.*, 2009). These or other yeast strains were not further explored *in field* assays, neither was their mode of action studied. Regarding OA, the antagonistic effect of yeasts against its fungal causal agents has also been reported. A study with 580 wild yeast strains isolated from Ceará State (Brazil) were evaluated for their ability to produce killer toxins (de Lima *et al.*, 2013). From these strains, 5 provided a

[3] Available at: https://www.servicos.gov.br/servico/comprar-biofungicida-tricovab-para-a-vassoura-de-bruxa-do-cacaueiro [Accessed February 18, 2019]

significant reduction in mycelial growth and conidial germination of *C. gloeosporioides in vitro*. In the case of *C. acutatum*, Lopes *et al.* (2015) reported the efficiency of 6 isolates of *S. cerevisiae* in controlling the pathogen.

Although data in the scientific literature increasingly strongly points to the potential use of yeasts for biocontrol of fungal infections in plants as in humans (not addressed in the present review), almost no such application has been seriously explored, implemented or commercialized.

In particular, it has never been considered cost-effective, to deserve attention from the productive sector, very dependent on chemical products. Antagonist yeasts are a more eco-friendly and sustainable alternative strategy to assess fungal diseases, including WBD of cacao and the olive anthracnose, contributing to ease the associated severe social and economic implications to millions of people. The use of natural microbial biocides has in favor not only the health, environment and economic arguments above stated, but also the almost infinite microbial biodiversity available to continuously feed the search for a suitable yeast for each emerging or evolving phytopathology.

THESIS OUTLINE

Plant diseases caused by fungal pathogens are an increasing global threat to food production and security. The economic losses associated to plant diseases reach 40 billion dollars per year, two thirds of which are due to fungal diseases. The uncontrolled use of chemical fungicides to combat these diseases over the past century, led to the generalized development of fungal resistance to these drugs and, in the absence of alternative control methods, to the re-emergence of stronger fungal virulence. In the long run, the systemic use of these fungicides leads to the accumulation of chemical residues in the environment, contaminating species across trophic levels. This issue has been neglected for a long time, which has caused this problem to reach alarming proportions.

This is the case of the *Witches' Broom Disease* (WBD) of cacao, caused by the basidiomycete fungus *Moniliophthora perniciosa*, which led to severe losses in the production of cacao fruit, a major economic sustainability hurdle for tropical countries in Central and South America. Affecting a very different geographical region there is another economically important plant disease caused by fungal phytopathogens, Olive Anthracnose (OA). This phytopathology is caused by *Colletotrichum gloeosporioides* and *C. acutatum* species complexes, which affects the olive trees and fruits. This plant crop has a critical economic importance in the Mediterranean countries, particularly in Portugal, where losses have been progressively more pronounced, becoming a significant hazard to regional economy. Both diseases share the lack of effective methods for their containment and treatment, which is usually done through the use of chemical fungicides and other agronomic measures, which besides being expensive and not sustainable, are far from being appropriate to solve the problem. Therefore, more effective and eco-friendly methods and strategies are needed to face this scenario.

One possible approach to combat fungal diseases in plants might be the use of biological control agents. Several yeasts have been pointed as good candidates for the biocontrol of fungal infections. Some of these are killer yeasts, *i.e.,* they secrete a peptide toxin able to induce in the sensitive cell a death process. Other yeasts secrete enzymes that destroy the fungi cell walls, increasing their vulnerability to other biological or physicochemical agents, or toxic volatile compounds able to kill the fungi at a distance. Others yet compete for nutrients and the colonization of a given niche. Many of these yeasts are commercially used to prevent or delay post-harvest spoilage by fungi and extend shelf-life of food products. Nevertheless, so far, none has been applied to inhibit mycelium formation in the field to control or prevent fungal plagues in plants.

Considering this problem, this thesis proposed to evaluate the ability of a selected group of yeast strains to antagonize the fungal causal agents of both cacao WBD and OA, constituting the initial step towards the formulation of novel viable, eco-friendly and effective alternatives for controlling both diseases based on the application of easily cultivated yeast strains. Additionally, the present work also assessed the possible use of *vinasse*, the dark, dense liquid waste from sugarcane juice fermentations for the production of bioethanol, as an alternative to inhibit the growth of the phytopathogens that causes the WBD of cacao. Thirdly, this thesis developed an optimized and easy-to-implement protocol for the evaluation of yeast antagonism against phytopathogenic fungi, which can be used to study the potential of a wide range of yeast strains against the fungal causal agents of different phytopathogenic diseases.

The experimental work was performed in the Institute of Science and Innovation for Bio-Sustainability (IB-S) and in the Centre of Molecular and Environmental Biology (CBMA), Department of Biology, University of Minho (Portugal) under the supervision of Professor Cândida Lucas and Professor Fernanda Cássio, and in collaboration with the NUPEB research centre from the Federal University of Ouro Preto, Brazil, and CERLEV, a Brazilian company dedicated to yeast fermentations technology consultancy.

The thesis is divided in 6 chapters, according to the specific aims established and corresponding to the publications that result from this work.

Chapter 1 provides an overview on the fungal phytopathogenic diseases, particularly the *Witches' Broom Disease* of cacao and Olive Anthracnose, presenting an economic context of the impact of both diseases and the characteristics of the fungal causal agents' life cycle. The methodologies presently used for disease containment are also described, as well as the groundbreaking approaches that could be investigated. The importance and challenges that a strategy based on the use of yeasts as biocontrol agents would have is discussed and the available information on the molecular mechanisms underlying fungi antagonism by yeasts is also provided.

Chapter 2 explores the ability of a group of yeast strains originating from the industrial fermentations of *cachaça* and from the industrial production of bioethanol to antagonize *M. perniciosa*, the fungal causal agent of the *Witches' Broom Disease* of cacao. The work resulted in the choice of three industrial yeast strains as excellent and resilient antagonists that actually kill this phytopathogen, which makes them good candidates for the utilization in the biocontrol of this disease. A protocol for the evaluation of yeast antagonism against phytopathogenic fungi was developed and optimized, which can be easily implemented to assess the potential of a wide range of yeast strains against different phytopathogenic fungi.

Chapter 3 presents a microscopy analysis of the interactions between the antagonistic yeasts and the phytopathogenic fungi in the antagonism assays performed in Chapter 2. Scanning Electron Microscopy technique was used. The yeast attachment to the pathogen hyphae, the occurrence of constriction of the fungal cells and an apparent draining of the fungal cellular content was observed. Moreover, yeast fusion with the hyphae and fimbriae-like connections between the yeast and the fungal cells and also between antagonistic yeasts are described. The possibility of a predacious-like behavior exerted by the antagonistic yeasts and its role in the antagonism exerted against the WBD fungal pathogens is discussed.

Chapter 4 comprises the assessment of the capability of the group of yeast strains originating from the industrial fermentations of *cachaça* and from the industrial production of bioethanol used on Chapter 2 to antagonize *Colletotrichum gloeosporioides* and *C. acutatum*, the fungal causative agents of Olive Anthracnose. The ability of a group of yeasts from the olive biome in antagonizing the olive anthracnose fungal pathogens is also explored. A *Wickerhamomyces anomalus*, isolated from olive orchards in Portugal, is the most promising strain, being able to inhibit the growth of all *Colletotrichum* sp. tested. The validation of the application of the protocol developed and optimized in Chapter 2 for the evaluation of yeast antagonism is also presented.

Chapter 5 explores the possibility of using *vinasse*, the main waste product from sugarcane bioethanol production process, to control the development of the *Witches' Broom Disease* causal agents. The ability of *vinasse per se* to inhibit the growth and development and even kill *M. perniciosa* is reported. The potential of a solution based on the fertirrigation of cacao plantations with *vinasse*, which could contribute to contain the prevalence and spread of the disease is presented.

Chapter 6 is a conclusion chapter which summarizes all the research performed in the scope of this thesis. The results and findings of the different experimental studies are here consolidated. The future perspectives in this area related with the research work performed is also proposed and discussed.

Supplementary Material

An exploratory assessment and identification of the protein content of the yeast *vs* fungus co-culture supernatants from the antagonism assays of Chapter 2 that could be involved in the antagonism effect against *M. perniciosa* is provided.

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CHAPTER 2

Saccharomyces cerevisiae **and** ***Wickerhamomyces anomalus*** **are able to kill** ***Moniliophthora perniciosa*****, the causal agent of cacao** ***Witches' Broom Disease***

The work presented in this chapter has been submitted for publication:

Ferraz, P., Amorim-Rodrigues, M., Cássio, F. and Lucas, C. *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* are able to kill *Moniliophthora perniciosa*, the causal agent of cacao *Witches' Broom Disease*

ABSTRACT

BACKGROUND: Cacao *Witches' Broom Disease* (WBD) is caused by *Moniliophthora perniciosa,* a fungus that infects all the plant tissues, at any stage of its life cycle. Chemical fungicides are ineffective against *M. perniciosa.* Presently, WBD is tentatively controlled using a biocide, *Trichoderma stromaticum*, expensively produced by demand, and therefore economically unsustainable.

RESULTS: A group of wild yeasts from several species, isolates from Brazilian fermentation industries, were tested for their ability to antagonize *M. perniciosa* strains from South and Central America infected orchards. Tests were done *in vitro,* in solid and liquid media. Three yeasts were found that efficiently kill 6 strains of *M. perniciosa*. Death was confirmed by methylene blue and propidium iodide staining and requires the physical contact between the yeasts and the mycelium. Two yeast strains were chosen, *Wickerhamomyces anomalus* #1105 and *Saccharomyces cerevisiae* #1112 from spontaneous fermentations of the production of *cachaça,* the Brazilian spirit, and the *S. cerevisiae* PE2 from bioethanol industrial fermentations in Brazil. They are effective at three temperatures, under starvation, at different culture stages or growing old.

CONCLUSIONS: These results are the initial step towards the formulation of a new eco-friendly and effective alternative for controlling WBD, based on the application of live yeast without the need for the purification of a specific antifungal peptide/compound. Moreover, results suggest that spontaneous fermentations may provide microbial biodiversity to be locally applied in the control of the disease, improving the sustainability of small cacao producers in remote areas.

Keywords

Cacao; *Witches Broom Disease*; *Moniliophthora perniciosa*; yeast; antagonism

INTRODUCTION

The cacao plant (*Theobroma cacao* L.) is one of the most valuable crops worldwide (Pohlan and Pérez, 2010; Teixeira *et al.*, 2015), responsible for important fractions of the economic revenue of the countries from Central and South America and Africa within the Cacao Belt. *T. cacao* is affected by several diseases, the most severe being the *Witches' Broom Disease* (WBD) caused by the basidiomycete fungus *Moniliophthora perniciosa* (formerly *Crinipellis perniciosa*) (Purdy and Schmidt, 1996; Aime and Phillips-Mora, 2005; Teixeira *et al.*, 2015). This disease is responsible for major crop losses with large social-economic consequences, particularly in Brazil which cacao production decreased more than 70% in a period of 10 years after the onset of the disease (Trevizan and Marques, 2002; Meinhardt *et al.*, 2008; Teixeira *et al.*, 2015).

The severity of WBD is closely related to the virulence of *M. perniciosa*, which comes from its ability to virtually infect all cacao plant tissues at all stages of the plant life cycle (Meinhardt *et al.*, 2008; Ferraz *et al.*, 2019). As a hemibiotrophic fungus, *M. perniciosa* has two distinct phases: a biotrophic and a saprotrophic (reviewed by Ferraz *et al.*, 2019). After the initial infection, the pathogen induces hypertrophy and hyperplasia, causing a disorganized proliferation of the infected vegetative meristems of axillary shoots, which results in the formation of green brooms, a structure composed of abnormal stems. Several weeks after the development of these structures, the infected plant tissues become necrotic due to a series of cell death events, forming a structure named dry broom (Meinhardt *et al.*, 2008). *M. perniciosa* then colonizes those necrotic plant cells and generates pink-coloured basidiocarps producing 2 to 3.5 million spores each (Almeida *et al.*, 1997). The spores are mainly released at night under optimal conditions of temperature and humidity, being disseminated by water and wind, and can endure and remain latent in the soil or inside pruned plant branches for long periods of time (Meinhardt *et al.*, 2008; Pohlan and Pérez, 2010). All these factors contribute to the exceptional virulence of *M. perniciosa* and explain why a whole plantation is compromised after the initial infection of one cacao plant.

The conventional chemical fungicides used to control the spread of fungal plant diseases, such as copper or azole-based compounds, are ineffective against *M. perniciosa* (Medeiros *et al.*, 2010). In addition, the use of chemical fungicides has been restricted in most cacao producing countries due to their high cost, and to the risks associated with chemical contamination of the cacao fruits and chocolate (Marelli *et al.*, 2009; Verweij *et al.*, 2009; Nunes, 2012). Currently, WBD has only one management method implemented in Brazil, consisting in spraying the infected plants with Tricovab®, a live suspension of

Trichoderma stromaticum. This fungal competitor is apparently efficient in antagonizing *M. perniciosa*, but its production and distribution are very unpractical and expensive, having to be almost fully subsidized by the Brazilian government (reviewed by Ferraz *et al.*, 2019). It is therefore an unsustainable solution. All considering, new strategies to control the disease are essential. One such alternative could be the use of biological control agents, *i.e.,* the use of microbial antagonists to suppress the development of the phytopathogen (Pal and McSpadden Gardener, 2006).

Yeasts have been recognized as valuable tools to manage microbiological-induced decay in food and fermentation industries, namely in the post-harvest protection of fruits from decomposition (Marquina *et al.*, 2002; Liu *et al.*, 2013; Dukare *et al.*, 2018). Despite the numerous present applications, there is almost no information on how these yeasts antagonize bacteria and fungi, apart from the tacit expectation that they work as killer yeasts. These are yeasts that antagonize other yeasts through the secretion of peptidic killer toxins (Schmitt and Breinig, 2006), which are very diverse in structure, function and mode of action (reviewed by Liu *et al.*, 2015). The best characterized killer yeast is *Saccharomyces cerevisiae*, which killer strains have been clustered based on their killing profiles and lack of cross-immunity (Schmitt and Breinig, 2006; Rodríguez-Cousiño *et al.*, 2011). Nevertheless, in the case of non-*Saccharomyces* yeasts, the molecular basis of the killer phenotype is poorly studied (Liu *et al.*, 2015). Other mechanisms of yeast antagonism may occur, which involve the secretion of (i) hydrolytic enzymes like as chitinases and glucanases (Lopes *et al.*, 2015; Liu *et al.*, 2018), (ii) volatile compounds (VOCs), including alkenes, alcohols, ketones, benzenoids, pyrazines, sulphides and terpenes (Schulz-Bohm *et al.*, 2017), or (iii) quorum sensing molecules (QSM) (Wuster and Babu, 2007; Avbelj *et al.*, 2016; Mehmood *et al.*, 2019). Besides, yeasts may compete for space or for a particular nutrient. Any antagonistic activity may be the result of several of these different modes of action (Zhang *et al.*, 2011; Parafati *et al.*, 2015).

Broad killer yeasts not only kill a wide range of yeasts from genetically distant species, but may also be effective against bacteria and filamentous fungi (Magliani *et al.*, 1997; Liu *et al.*, 2015). These dominance processes must play important roles in microbial ecology, not only in natural niches but also in spontaneous fermentations (Abranches *et al.*, 1998), which prevailing strains commonly antagonize other yeasts and bacteria (Alonso-del-Real *et al.*, 2019). Many industrially strains originate from such environments (Lopes *et al.*, 2016). Exploring these niches may provide valuable biodiversity, *i.e.* strains with natural ability for many applications, including biocontrol of fungal infections in man and plants (Hatoum *et al.*, 2012; Mannazzu *et al.*, 2019). In this regard, antagonistic activity of yeasts from diverse origins against phytopathogenic fungi has been described (Suzzi *et al.*, 1995; Walker *et al.*, 1995; Rosa-

Magri *et al.*, 2011). In the particular case of WBD, *M. perniciosa* has been reported to be inhibited *in vitro* by two yeast isolates from *Candida* sp. and *Dipodascus capitatus* (Cabral *et al.*, 2009), although their ability was not explored or studied, and their mode of action remains unknown.

A group of yeasts, many of which are industrial isolates from fermentative processes, were used to test their ability to antagonize the WBD causal agent, the virulent filamentous fungus *M. perniciosa*. From this group, 3 strains were selected as efficient and resilient antagonists that kill this phytopathogen, *Wickerhamomyces anomalus* #1105 and *Saccharomyces cerevisiae* #1112 from spontaneous fermentations of the production of *cachaça,* the Brazilian spirit, and the *S. cerevisiae* PE2 from the industrial production of bioethanol in Brazil. These yeasts biocidal potential raises expectations as to their possible application in the management of this severe cacao disease, until now without effective or sustainable methods for its containment.

MATERIALS AND METHODS

Microorganisms and culture conditions

The *M. perniciosa* strains originating from KNAW (www.wi.knaw.nl) (Table 1), were cryopreserved in sterile glycerol 30% at -80 °C, maintained at 4 °C on MEA (20 g/L malt extract with 20 g/L agar), and propagated in the same media or in PDA at 30°C. Alternatively, fungi were grown in liquid ME (20 g/L malt extract), using glass tubes (13 cm x Ø 3 cm) containing 20 ml of medium at the same temperature and 200 rpm orbital shaking. Media pH was adjusted to the desired value with NaOH 2 M and HCl 37% v/v. Both solid and liquid media were inoculated using a ≈ 0.8x0.8 cm agar plug from solid media with actively growing mycelia on MEA with ≤1 week. Growth in solid media was followed measuring the micelium diameter (G_d) every 24 h under a Stereo Zoom Binocular Microscope (Leica s8 APO), and growth rate (G_r) was estimated as the ratio G_d (mm)/t (day). Latency phase was not considered for this calculation. Growth in liquid media was visually inspected, mycelia forming one or more cotton ball-like conglomerates.

Table 1. Strains of *Moniliophthora perniciosa* from CBS-KNAW (www.wi.knaw.nl), their primitive origin, and the assigned letter code used in this work.

Strain	Origin	Code
CBS 192.77	Ecuador	A
CBS 193.77	Ecuador	B
CBS 245.36	Ecuador	C
CBS 789.86	Ecuador	D
CBS 790.86	Ecuador	E
CBS 441.80	Brazil	F
CBS 442.80	Brazil	G
CBS 339.50	Venezuela	H

Yeasts strains originating from diverse sources (Table 2) were cryopreserved in sterile glycerol 30% at -80 °C, maintained at 4°C on YPDA (10 g/L yeast extract, 10 g/L bacto peptone, 20 g/L D-glucose with 20 g/L agar), and multiplied at 30 °C in the same medium for 48-72 h prior to all assays. Growth in YPD or ME was done at the same temperature with 200 rpm orbital shaking and a liquid/air ratio of 1:2.5, and followed spectrophotometrically at 600 nm, or counting the cells in suspension with a Neubauer Chamber and Light Microscope (Leica DM 300). Growth rate (μ_g (h^{-1})) was calculated from $OD_{t_x} = OD_{t_0}.e^{\mu_g . t_x}$.

Table 2. Yeast strains used in this work, their primitive origin, and their assigned code used.

Species	**Strain**	**Origin**	**Code**
Wickerhamomyces anomalus	PYCC[2] 2495	Dates	#2495
Wickerhamomyces anomalus	PYCC 2505	Grape must	#2505
Wickerhamomyces anomalus	PYCC 3294	Human secretion [1]	#3294
Wickerhamomyces anomalus	PYCC 4121	Unknown	#4121
Wickerhamomyces anomalus	PYCC 4380	Kefyr	#4380
Wickerhamomyces anomalus	PYCC 4554	Fermented figs	#4554
Wickerhamomyces anomalus	PYCC 5008	Unknown	#5008
Meyerozyma guilliermondii	PYCC 2734	Flowers	#2734
Meyerozyma guilliermondii	Cerlev 1015	Fermentations from the production of *cachaça* [3]	#1
Saccharomyces cerevisiae	Cerlev 1025		#2
Saccharomyces cerevisiae	Cerlev 1038		#3
Saccharomyces cerevisiae	Cerlev 1096		#4
Wickerhamomyces anomalus	Cerlev 1105		#5
Saccharomyces cerevisiae	Cerlev 1112		#6
Saccharomyces cerevisiae	Cerlev 1113		#7
Saccharomyces cerevisiae	CAT-1- FT280L	Industrial production of bioethanol [4]	CAT1
Saccharomyces cerevisiae	PE-2- FT134L		PE2

[1] Infected lung from a tuberculosis patient.
[2] Portuguese Yeast Culture Collection, NOVA, Lisbon, Portugal (http://pycc.bio-aware.com/).
[3] Cerlev, Lda., Ouro Preto, MG, Brasil (https://www.facebook.com/empresa.cerlev/).
[4] Fermentec, Lda. Soluções Tecnológicas e Industriais, Piracicaba, SP, Brasil (https://www.fermentec.com.br/capa.asp?pi=principal).

Evaluation of the yeast vs yeast antagonistic ability

Yeasts killer phenotype was assayed in solid media according to standard procedures (*e.g.* Aguiar and Lucas, 2000). The target strain was grown in YPD up to late exponential phase. 1 mL of the culture (≈10^6 cell/ml) was mixed with 5 mL of 0.8% melted soft agar and spread onto the surface of a plate of MEA supplemented with 0.015% methylene blue (MB) forming a thin overlay. When solid, a generous strikeout of the putative antagonist strain was applied on top. The plates were incubated at 30 °C for 10 days. Antagonism was verified through the formation of a bluish inhibition halo on yeast overlay.

Evaluation of the yeast vs fungus antagonistic ability

Solid media assays: Antagonism between yeasts and fungi was assayed in MEA or PDA, at 30°C. A fungus inoculum plug, as described above, was placed on top of a plate of medium supplemented with 0.015% methylene blue (adapted from Aguiar and Lucas, 2000). After an initial fungal growth (≈3 mm Ø), a yeast strain was inoculated on one side of the plate, corresponding to a generous strikeout of 48h YPD plate cultures. Plates were photographed and mycelial growth was registered according to an empirical classification scale with 3 levels, as described in the Results and Discussion section.

Liquid media assays: Antagonism in liquid media was evaluated inoculating an actively growing mycelium plug together with a suspension of ME-grown yeast cultures collected in exponential phase (10^8 cells/ml), using glass tubes (13 cm x Ø 3 cm) containing 20 ml of ME medium and incubating at 30°C and 200 rpm orbital shaking for 10 days. Tubes were photographed and growth of mycelia was inspected visually and registered according to the empirical classification scale described in the Results and Discussion section. The plug was washed with ultrapure water, softly shaking manually. The procedure was repeated ±10 times to obtain maximum removal of the yeasts attached to the mycelium. The plug was then placed in fresh ME and photographed.

Assessment of fungal death by staining with Methylene Blue and Propidium Iodide

At the end of the incubation period, the viability/death status of the remaining fungal cells was evaluated by staining with methylene blue (MB) and propidium iodide (PI), following well-established procedures. For MB staining, a small portion of the remaining mycelia was collected, washed with ultrapure water, added a drop of methylene blue 0.03% v/v, incubated 10 min at room temperature and observed under a light microscope (Olympus BX63F2 equipped with an Olympus DP74 camera). For PI staining, the fungal sample was washed with ultrapure water, placed in a microtube containing 500 μl PBS (Phosphate Buffered Saline) and 1 μl of PI (1 mg/ml) and incubated for 10 min in the dark at room temperature. Fluorescence was assessed with an epifluorescence microscope (Olympus BX63F2 equipped with an Olympus DP74 camera), using monochromatic light at 543 nm and an emission bandpass filter of 585–615 nm.

Statistical Analysis

All assays were performed at least in three independent replicates ($n \geq 3$). The data obtained was subjected to a one-way analysis of variance (one-way ANOVA) using GraphPad Prism 6 (GraphPad Software, Inc.). Statistical significance was assumed at $p \leq 0.05$.

RESULTS AND DISCUSSION

Optimization of *M. perniciosa* cultivation conditions

M. perniciosa strains used in this work (Table 1) were originally isolated from cacao plants and fruits infected with WBD in South American countries. This species of filamentous fungus is very aggressive and resilient but grows preferably within a relatively narrow range of temperatures, between 20-30 °C (ICCO.org; Purdy and Schmidt, 1996). Considering that the majority of yeasts are best cultured at 30 °C, this temperature was chosen to cultivate the fungal strains. Optimal pH, on the other hand, was determined by quantifying fungal growth rates in MEA, a well-known fungal growth media, adjusted to pH 4.0, 4.5, 5.0, 5.5 or 6.0. Results (Figure 1) showed that *M. perniciosa* strains vary considerably between each other. The fastest growing strain was F from Brazil with a specific growth rate of 2.7 mm.day^{-1} at pH 5, while the strain B from Ecuador was the slowest, growing at G_r=0.47 mm.day^{-1} at pH 4.5. No statistically significant differences were observed between the growth at pH 5 to 6. Growth rates were generally lowest at pH 4.0, with a latency phase lasting 1 day (strains C, D, F and I) or more (remaining strains). Similarly, a latency phase of 1 day was also observed for strains C, E, H and I at pH 4.5.

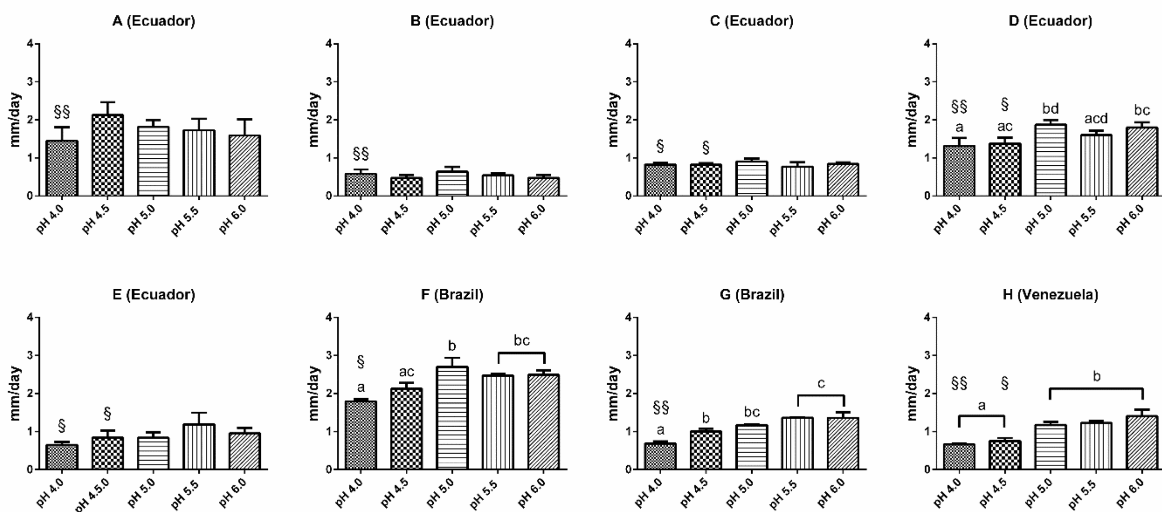


Figure 1. Variation of the growth rate of *M. perniciosa* strains in Table 1 determined in MEA at 30°C at different pH. Different letters represent significant differences across different pH values. § represents a latency phase prior to the initial mycelia growth (§ 1 day of latency; §§ >1 day of latency). Results are data from at least 3 independent replicates.

These assays were repeated in PDA and no statistically significant differences were observed between the growth rates in either media (not shown). Fungal strains were thus further cultivated on MEA or PDA at 30°C and pH 5.5 to avoid latency. From the same assays, it was also established that 10 days is enough to test fungal growth phenotypes.

Optimization of yeast vs fungi antagonism assays

The yeasts chosen for this work (Table 2) can be grouped in two sets. The first contains strains of *Wickerhamomyces anomalus* (previously *Pichia anomala* or *Hansenula anomala*) obtained from a credited culture collection (PYCC - Portuguese Yeast Culture Collection), and the second contains isolates from fermentative processes in Brazil. These yeasts were chosen based on their dominant nature in microbial mixtures, particularly the wild yeasts isolated from the spontaneous fermentations used for the production of *cachaça*, the Brazilian spirit (da Conceição *et al.*, 2015), or used in a more controlled industrial process of sugarcane juice fermentation for bioethanol production (Lopes *et al.*, 2016). Moreover, *W. anomalus* strains were chosen considering their origin from diverse natural niches, possible sources for strong antagonism (Baysal and Silme, 2018), and the fact that this is a killer species active against a large variety of other yeasts (Abranches *et al.*, 1998; Fredlund *et al.*, 2002). Broad killers are known to be also effective against bacteria and filamentous fungi, including phytopathogens (Suzzi *et al.*, 1995; Walker *et al.*, 1995; Platania *et al.*, 2012; Lima *et al.*, 2013; Parafati *et al.*, 2015).

W. anomalus strains in Table 2 have for long been kept in a culture collection, they do not originate directly from nature. Bearing in mind that they might have lost their killer ability, they were firstly assayed in that regard against a broad sensitive strain of *Meyerozyma guilliermondii* (previously *Pichia guilliermondii*) (PYCC 2734) (Aguiar and Lucas, 2000; da Silva *et al.*, 2008). *W. anomalus* strains were cultivated in the media and growth conditions established for *M. perniciosa, i.e.* on MEA with MB at pH 5.5 and 30 °C, in which conditions they displayed regular growth, and were therefore subsequently tested for their killer phenotype in these same conditions. All the strains of *W. anomalus* caused a blue inhibition halo on *M. guilliermondii* indicative of cell death (not shown), so none was excluded from the assessment of inhibitory effect over fungal growth. For this purpose, the *M. perniciosa* fastest growing strains F and G from Brazil and A from Ecuador were used. The development of mycelium was followed up to 10 days. All the *W. anomalus* strains affected the fungal growth although to a different extent. The mycelia developed freely in the opposite direction of the yeast strikeout, while the extent of its development varied

in the space between the yeast and the fungal plug. Based on this variation, three levels of response were identified, which were converted into an empirical scale of yeast/fungus interaction (Figure 2): level 0, corresponds to a high fungal resistance, in which the mycelium overgrows the yeast eventually filling the entire plate; level 1, corresponds to a weak inhibitory effect, wherein the mycelium fills the gap between the yeast and the plug and stops growing upon contact with the yeast culture; and level 2 corresponds to a clear inhibitory effect, with the formation of a blue inhibition halo facing the yeast. The results of each combination of yeast/fungal strain are presented in Table 3 according to this scale. As can be seen, *M. perniciosa* strains from Brazil were more resistant than the strain from Ecuador, which was inhibited by most of the *W. anomalus* strains. These results were obtained in MEA supplemented with MB. As controls, identical assays were made on PDA, with and without MB, and MEA without MB, plating all the strains of *W. anomalus* against the more sensitive strain of *M. perniciosa*, A from Ecuador. Results were identical to the ones in Table 3, showing that neither the medium nor the presence of MB affected the yeast-fungus antagonism (not shown). Assays were subsequently extended to the industrial yeasts in Table 2. Results (Table 3) showed a more irregular response from the fungus in the presence of these strains than with *W. anomalus*, which was weaker than predicted according to their origin (Abranches *et al.*, 1998; Alonso-del-Real *et al.*, 2019; Hatoum *et al.*, 2012; Mannazzu *et al.*, 2019). Moreover, the assays were also done using the fungal strains B and C, absent from Table 3. The growth of these fungi in the antagonism assays was extremely irregular generating unreliable results, reason why these fungi were excluded from further assays. In total, most fermentation yeasts strains were less able to antagonise *M. perniciosa* than *W. anomalus.* Noticeably, fungal strain F from Brazil stood out for displaying the highest resistance to all yeasts tested.

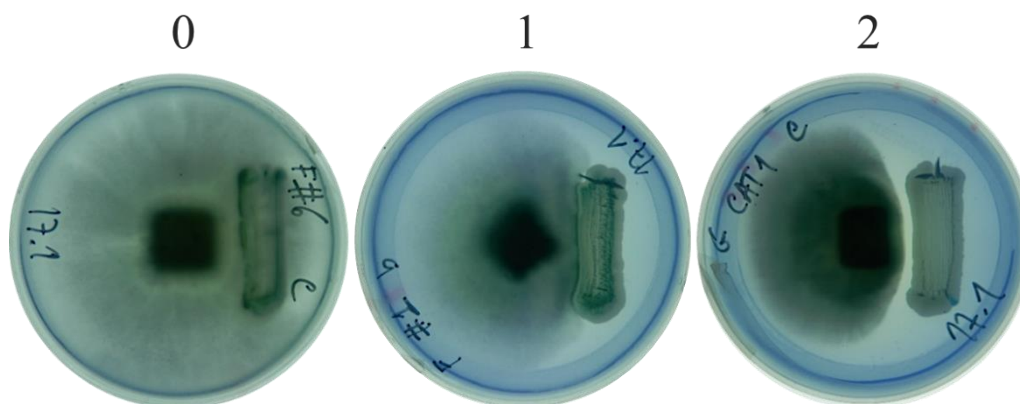


Figure 2. Empirical classification scale of antagonistic response in solid media. 0 represents the absence of inhibition of any kind, the fungus eventually growing on top of the yeast culture; 1 represents a weak inhibitory response in which case the fungus grows up to the limit of the yeast culture without overgrowing it, and 2 represents a clear antagonistic effect.

Table 3. Results of the antagonism assays between *M. perniciosa* and yeasts in solid medium. The fastest-growing fungal strains, A from Ecuador and F and G from Brazil were tested against all the yeasts in Table 2. Further assays were done using all the remaining fungal strains against the yeasts originating from fermentation processes. Results using *M. perniciosa* strains D and E from Ecuador and H from Venezuela are presented. The inhibitory effect was rated from 0 to 2, according to the empirical scale in Figure 2. Results presented were identical in three independent replicates. A table cell displaying 3 numbers (e.g. 1/1/2) represents a situation where each plate had a different result.

M. perniciosa strains	Yeasts						
	Wickerhamomyces anomalus						
	#2495	#2505	#3294	#4121	#4380	#4554	#5008
A	2	2	2	2	1	2	1
F	1	1	1	2	1	1	1
G	1	1	1	1	2	1	0

	Fermentative yeast strains								
	#1	#2	#3	#4	#5	#6	#7	CAT1	PE2
A	1	2	2	1	1	0	1	2	2
F	1	0	1	0	0	0	0	0	0
G	0	1	1/1/2	1	0	0	1	2	1
D	1	2	1	1	1	0	1	2	1
E	1	1/1/2	1	2	0	1	2	2	1/1/2
H	1	1	1	1	0	0	1	1	1

Is *M. perniciosa* growth inhibition caused by a volatile compound?

Mycelia strong growth inhibition occurred at a certain distance between the yeast and the fungal cells (*e.g.* Figure 2, antagonism level 2). This suggests that probably the yeast strains secreted some kind of compound that signals the fungal cells. Yeasts can secrete a soluble molecule which diffuses through the agar, eventually reaching the mycelium (Schmitt and Breinig, 2006; Lopes *et al.*, 2015; Liu *et al.*, 2018), or a volatile compound that may affect the fungus at some distance (Fialho *et al.*, 2016; Schulz-Bohm *et al.*, 2017). To evaluate whether the antagonistic response observed against *M. perniciosa* strains corresponded to a volatile compound secreted by the yeast, the above described antagonism assays were repeated using septate Petri dishes. These prevent the diffusion of molecules through the agar but allow the organisms in the two sides of the plate to share the atmosphere. *M. perniciosa* strain G was challenged with the presence of the yeast strains #3 and CAT1 (Table 3). The yeast strain #1 was used as negative control. Unlike before, no inhibitory response was observed for any of the combinations used (*e.g.* Figure 3), indicating that the fungal growth inhibition probably involves the diffusion of a non-volatile compound through the agar. Nevertheless, the competition for nutrients and space cannot be discarded at this point.

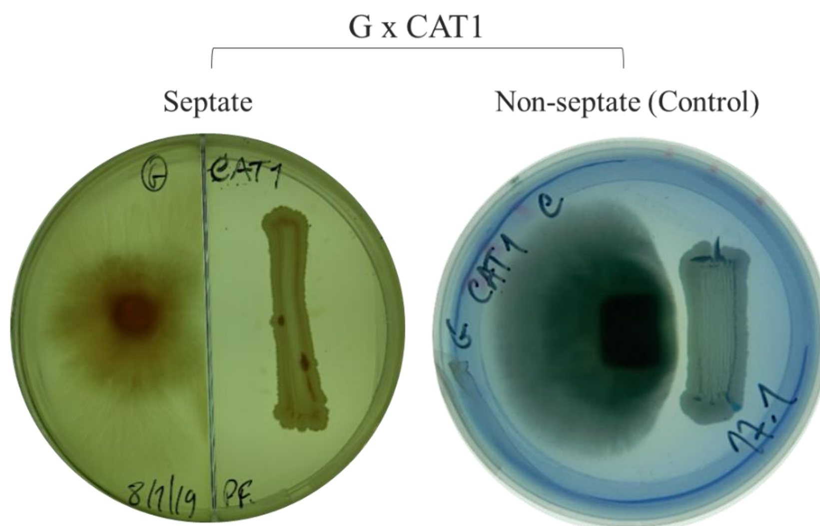


Figure 3. Example of an antagonism assay in divided Petri dishes (left picture), with the comparison with the previous tests in solid medium using the same yeast *vs* fungus combination (right picture).

Antagonism assays in liquid media

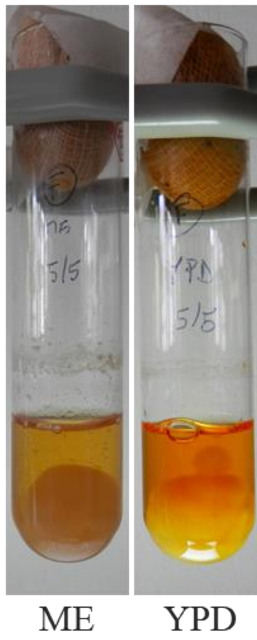
Since the fungi have a slower growth rate in comparison with yeasts, it is necessary to incubate the plates for 10 days to score the antagonism effect. This is though unfavourable to the maintenance of a fully viable yeast culture. Hence, blue halo of cell death around the yeast biomass can appear, or in some cases the whole biomass can become blue. This could be a reason underlying the diverse and irregular response depicted in Table 3, since each yeast strain will stay alive and metabolically healthy for different periods of time. For this reason, the possibility of assaying antagonism more efficiently in liquid medium, which provides a direct contact between hyphae and yeasts, was considered. Moreover, the fermentation yeasts were chosen to proceed with the antagonism in liquid media, based on that (i) the set also includes one strain of *W. anomalus* isolated from spontaneous fermentations of *cachaça* production and this way the species is represented, (ii) these strains should be more resilient bearing in mind their putative future utilization in the field, and (iii) they originate from de same geographic region of the WBD, which in case of *in field* application, could avoid the potential imbalance of the cacao plantations ecosystem caused by the introduction of an alien microbe. Importantly, since these are strains used by the industry, their legalization, commercialization and acceptance should be facilitated.

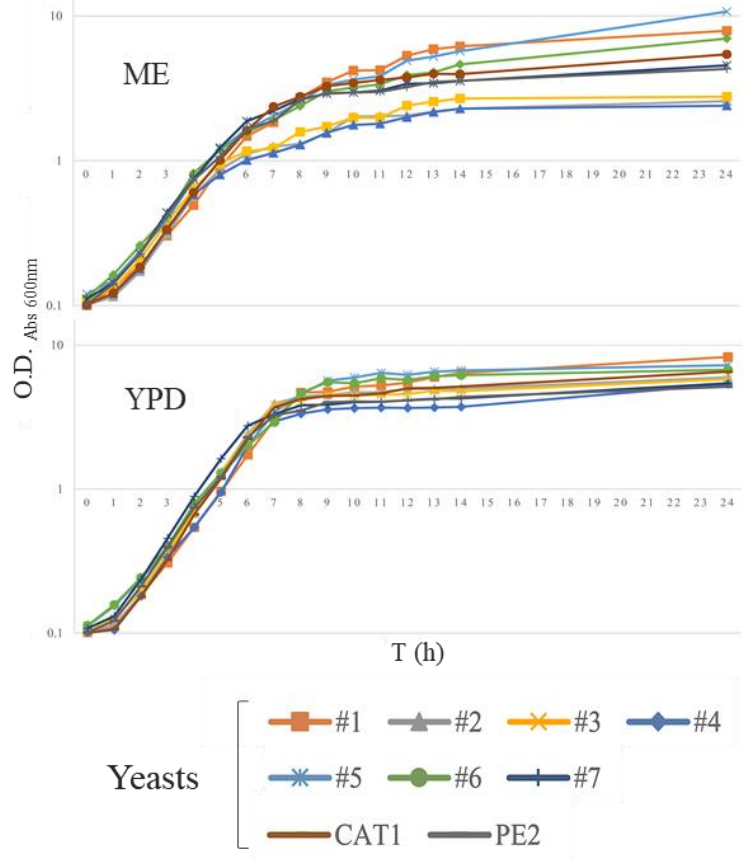
To optimize the conditions for the assays in liquid media, fungi and the industrial yeasts were firstly cultivated in ME or YPD, pH 5.5 and 30 °C. *M. perniciosa* produced abundant mycelia in the form one or several cotton ball-like large conglomerates growing in size over time (Figure 4A). Yeasts globally performed slightly better on YPD than on ME (Figure 4A), which is consistent with these yeast strains preference for glucose. Still, their growth rates allowed to consider the use of ME.

Preliminary antagonism assays were performed choosing in Table 3 the stronger antagonizing yeasts against the faster growing fungal strains F and G: strains #1 and #3 *vs* F, using CAT1 as negative control, and #3 and CAT1 *vs* G, using #1 as negative control. Separate controls of fungi and yeasts growing alone were also performed. The fungi were inoculated through a mycelium agar plug placed in a glass tube inoculated with 10^8 cells/mL from an exponentially growing yeast culture. As expected, the yeasts grew faster than the fungi, filling the growth medium. After 10 days of co-incubation the medium was decanted to check for the presence or absence of mycelium. Interestingly, unlike in solid medium, yeasts inhibited fungal growth in all the combinations tested, including the negative controls (not shown). Based on these preliminary results, antagonisms assays in liquid medium were expanded to the full set of combinations between all the fermentation yeast strains and the fungal strains F and G.

A

M. perniciosa
strain F





B

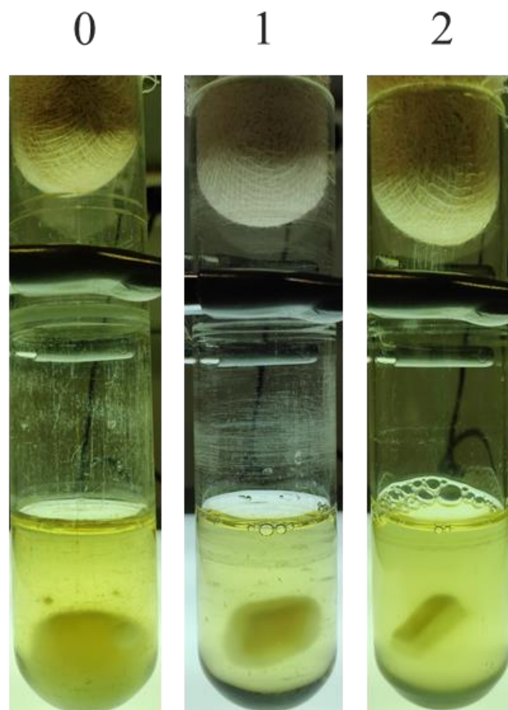


Figure 4. (A) Fungal cultivation of *M. perniciosa* strain F in ME and YPD at 30 °C, with the development of abundant mycelia in the form one or several cotton ball-like large conglomerates (left panel) and the growth curves of the industrial yeast strains in ME and YPD at 30 °C, determined by OD at 600 nm (right panel). (B) Empirical classification scale of antagonistic response in liquid media. 0 represents the absence of inhibitory effect, the fungus grows three-dimensionally producing a large conglomerate of hyphae; 1 represents a limited inhibitory response in which case some small mycelium is still formed from the agar plug, and 2 represents a strong inhibitory effect in which case no mycelium is formed. The culture media was removed and substituted to fresh ME medium to allow a better visualization of the results, since the turbidity resulting from yeast growth made it difficult to visualize the degree of inhibition at the end of the assays.

Results varied, allowing to establish a second antagonism empirical scale (Figure 4B), in which level 0 represents high fungal resistance, with the formation of the cotton ball-like conglomerates of mycelium identical to the fungal-alone control; level 1 corresponds to a weak inhibitory response, with the development of some mycelia around the fungal agar plug; and level 2 represents a strong inhibition with the total absence of fungal development. Results are scored in Table 4 according to this scale. A large increase in the inhibitory response compared to that obtained in solid medium was obtained. In fact, a strong inhibitory response was observed in 9 out of 18 combinations (50%), in opposition to the 2 out of 18 (11%) observed in solid media (Table 3). The yeast strains #1, #5, #6 and PE-2 elicited a strong inhibitory response, while CAT1 inhibitory effect was strong (level 2) or weak (level 1) in the case of strains G and F respectively.

Table 4. Antagonism assays in liquid medium using the combinations between the fermentative yeast strains and *M. perniciosa* strains F and G. The presence of the inhibitory effect was evaluated on a scale from 0 to 2 according to Figure 2. Results were identical in three independent replicates.

M. perniciosa strains	Yeasts								
	#1	#2	#3	#4	#5	#6	#7	CAT1	PE2
F	2	0	1	0	2	2	0	1	2
G	2	0	1	0	2	2	0	2	2

The difference in responses observed in solid and liquid media may derive from several factors. Liquid medium, in opposition to solid, propitiates the fast growth of the yeast population, faster than the fungal mycelium. The increasing yeast population possibly compete with the mycelium for nutrients. This was reported to occur between yeasts and phytopathogenic fungi, as one of the main modes of antagonism action (Andrews *et al.*, 1994; Saravanakumar *et al.*, 2008; Zhang *et al.*, 2010; Spadaro and Droby, 2016), showing that the inhibition of fungal growth does not always implicate death, being often reversible since the fungal cells can retain their viability (Spadaro and Droby, 2016). Additionally, liquid medium also allows the homogenous spread of the yeast cells and their contact with the hyphae. Finally, yeasts remain metabolically active for a longer period of time than in solid medium, and their permanent contact with the mycelia may trigger the production and secretion of peptides or other compounds with antifungal properties. This means that antagonism may correspond to fungal death or just an inhibition of its replication.

Evaluation of fungal death by staining with Methylene Blue and Propidium Iodide

To verify if the antagonism observed in liquid medium corresponds to actual fungal death, two well-known markers of cell death were used, Methylene Blue (MB) and Propidium Iodide (PI). MB is a cationic dye which can penetrate both live and dead cells, inside which it binds to negatively charged molecules such as nucleic acids. Living cells are able to reduce the dye and consequently remain colourless. PI can only enter the cells which membrane is disrupted; therefore, only necrotic cells are stained with red fluorescence. Cells stained with MB but not with PI lost viability but preserve the integrity of the plasma membrane, which means that they are possibly dying of apoptosis (Kwolek-Mirek and Zadrag-Tecza, 2014). Results are exemplified in Figure 5. All the yeast/fungal strain combinations evaluated that demonstrated a strong inhibitory response (level 2) presented an almost fully stained mycelium with both MB and PI, while in all the combinations with a weaker inhibitory response (level 1) the mycelium was only partially stained. These results showed that there are two mechanisms underlying *M. perniciosa* antagonism by yeasts. Yeasts kill the fungi, but in some cases, the relative amounts of yeast cells/mycelium do not appear sufficient to induce the death of the whole fungal culture in the time window of 10 days in which the assays take place. In this case, yeasts just delay fungal growth. Interestingly, it is possible to observe that isolated or clustered yeast cells appear to attach to the killed fungal hyphae,

suggesting that contact between the two organisms may be necessary to cause the effect and may even be involved in the mechanisms that cause the fungal cells death.

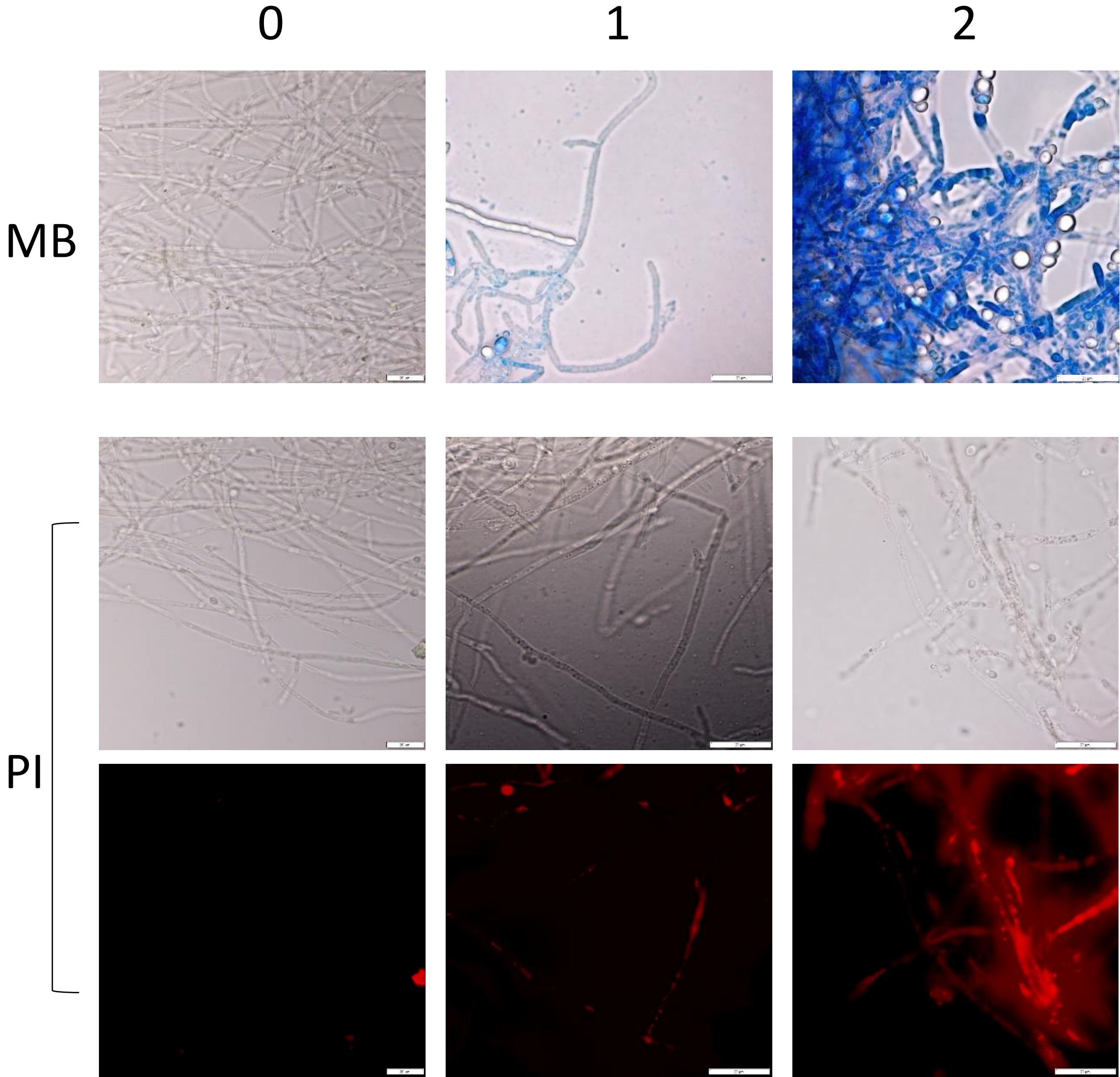


Figure 5. Example of the yeast/fungal strains cultures staining with MB (upper panel) and PI (middle and lower panels, corresponding to the observed bright field and fluorescence of the same picture, respectively). Empirical classification scale based on the intensity of the staining: 0 represents an absence of staining; 1 corresponds to a weak staining; and 2 represents a strong staining of the mycelia. Scale bar: 20 μm.

Is *M. perniciosa* killed by a yeast produced soluble compound?

The results above clearly showed that the antagonism observed in liquid medium corresponds to the actual death of the fungal cells. On the other hand, the antagonism observed in solid medium appears to correspond instead to an inhibition of fungal growth. This hypothesis is supported by the fact that the contact between yeast and fungus cells never occurs, and by the fact that the mycelium continued to grow freely in the opposite direction to the yeast inoculum. Identically to what was done for the volatile compounds, an additional assay was performed consisting in using the supernatant of the yeast culture, or of the yeast and fungal cells co-culture, to counteract the fungi. Fungal strains F and G were crossed with yeast strains #5, #6 and PE2. In order to avoid the starvation of the fungi, ME 2% (w/v) was added to the supernatants. A control consisted on using an identically ME supplemented supernatant from a fungal-alone culture. Results showed that the supernatant produced by yeasts cultured alone did not induce any fungal cell death, while the supernatant originating from a yeast/fungus co-culture varied. Fungal strain F growth was not inhibited in any of the combinations. Otherwise, the fungal strain G did not grow when incubated in the supernatant from #6/G co-incubation. Therefore, it is possible that a compound secreted to the supernatant either kills the fungus or very strongly inhibits its multiplication. This last combination was previously showed to cause cell death, inferred from MB and PI staining. Either way the #6 yeast strain appears to use a mode of action against the fungus different from the other yeasts.

Antagonism response challenged with mimicked environmental stressors

Natural field conditions for WBD development in cacao plantations may vary greatly from those used *in vitro*. The antagonistic response may therefore be different if environmental factors vary. In order to make a preliminary assessment of the resilience of the antagonistic effect, combinations between yeasts that showed a strong antagonistic ability and the two *M. perniciosa* strains used previously were challenged with various environmental stressors. The standard assay was done on ME pH 5.5 at 30 °C for 10 days and tubes were inoculated with fungi and fresh exponentially growing yeast. For each one of the stressors assays, one of the standard conditions was modified: (i) the temperature was lowered to 16 °C or 25 °C; or the fresh yeasts' inoculum was replaced by (ii) starved cells (3 days in sterile water), (iii) a 10-day old inoculum from solid medium, or (iv) a stationary phase (3-day old) liquid medium inoculum. Results summarized in Table 5 revealed that the yeast stains #5, #6 and PE2 maintained their antagonistic ability

for both fungal strains in all these non-optimal conditions, while the remaining yeasts decreased their antagonistic ability in some of them. Interestingly, the lower temperatures had no influence on the normal growth of both fungal strains which is a good prognosis for *in field* application.

Evaluation of the antagonism response against other *M. perniciosa* strains

Based on the results above, the strongest yeast antagonists against *M. perniciosa* F and G were #5, #6 and PE2. Liquid medium antagonism assays using these yeasts were therefore extended to the remaining *M. perniciosa* strains. Results (not shown) revealed strong inhibition of all the *M. perniciosa* strains tested by these yeasts, showing the broadness of their antagonistic ability, and indicating their potential for *in field* application, considering that the cacao plantations are possibly infected by several strains of *M. perniciosa*.

Yeast strain #5 is a *W. anomalus* while both #6 and PE2 are *S. cerevisiae*. These yeast species have been previously described in the literature as killers and/or antagonists (Suzzi *et al.*, 1995; Walker *et al.*, 1995; Wang *et al.*, 2007; De Ingeniis *et al.*, 2009; Platania *et al.*, 2012; Lima *et al.*, 2013). Despite this, only one registered commercial formulation exists based on *S. cerevisiae*, which is used to control plant diseases. This product, Romeo®, contains cerevisane® as the active ingredient and is recommended for the control of foliar powdery mildew, downy mildew and botrytis on grape vine and vegetables (agrauxine.com[1]). In the case of *W. anomalus*, no product based on this yeast species has yet been commercially exploited for application in the biocontrol of phytopathogens, in spite of the information on this species ability to release killer toxins (Wang *et al.*, 2007; De Ingeniis *et al.*, 2009; Platania *et al.*, 2012; Lima *et al.*, 2013) and other antimicrobial compounds, such as hydrolytic enzymes and VOCs, as well as their competition for nutrients and space and biofilm formation (Parafati *et al.*, 2015; Oro *et al.*, 2018).

[1] Available at: https://agrauxine.com/en/2018/01/19/romeo-new-biocontrol-product-registration-powdery-mildew-downy-mildew-botrytis/ (Accessed January 17, 2020)

Table 5. Antagonism assays using selected combinations of yeast and fungal strains, challenged with environmental stressors: temperatures of 16 °C or 25 °C; yeasts starved for 3 days in sterile water; 10-day old yeast inoculum from solid medium; or yeast inoculum from liquid medium from 3-day old stationary phase culture. The inhibitory effect was rated on a scale from 0 to 2 according to Figure 2. Identical results were obtained in three independent replicates.

		Fungal / Yeast combinations									
		F *vs* #1	F *vs* #5	F *vs* #6	F *vs* CAT1	F *vs* PE2	G *vs* #1	G *vs* #5	G *vs* #6	G *vs* CAT1	G *vs* PE2
STRESSORS	Standard assay	2	2	2	1	2	2	2	2	2	2
	16 °C	2	2	2	0	2	0	2	2	0	2
	25 °C	2	2	2	0	2	0	2	2	0	2
	Starvation	2	2	2	1	2	2	2	2	2	2
	Old inoculum	2	2	2	1	2	2	2	2	1	2
	Stationary phase	2	2	2	0	2	2	2	2	1	2

CONCLUSIONS

W. anomalus #1105 (#5) and *S. cerevisiae* #1112 (#6) from spontaneous fermentations of the production of *cachaça*, as well as the PE2 strain of *S. cerevisiae* from the industrial production of bioethanol, were revealed as good candidates for the utilization in the biocontrol of WBD. This conclusion is based on that (i) they efficiently antagonize 6 strains of *M. perniciosa* originating from cacao plantations in different countries afflicted with WBD, (ii) they maintained their antagonistic ability at three different temperatures (16, 25 and 30°C), under starvation, at different culture stages, or growing old. This antagonism corresponds to actual fungal cell death. Moreover, microscopy analysis of the co-culture showed that possibly there is a need for actual contact between the yeast cells and the hyphae to achieve the antagonistic effect. Also, a clear, optimized and easy-to-implement protocol for the evaluation of yeast antagonism against phytopathogenic fungi is presented, which can be used to study the potential of a wide range of yeast strains against the fungal causal agents of different phytopathogenic diseases. All considering, this work constitutes the initial step towards the formulation of a new eco-friendly and effective alternative for controlling WBD, that can rely on the application of live yeast, without the need for the purification of a specific antifungal compound. Finally, the fact that the three best yeasts originate from fermentative processes occurring in the same region of the globe as WBD, raises an interesting possibility. Spontaneous fermentations are used to produce *cachaça* or other spirits as a generalized cultural habit throughout Central and South America countries, which might supply wild yeasts able to locally counteract WBD. Using yeasts to manage WBD would contribute to the sustainability of the small cacao producers that have less or no access to commercialized agrochemicals or other pest management strategies.

ACKNOWLEDGEMENTS

This work was supported by the strategic programme UID/BIA/04050/2013 (POCI-01-0145-FEDER-007569) funded by national funds through the FCT I.P. and by the ERDF through the COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI), and the project EcoAgriFood (NORTE-01-0145-FEDER-000009), supported by the Norte Portugal Regional Operational Programme (NORTE 2020) under the PORTUGAL 2020 Partnership Agreement through the European Regional Development Fund (ERDF). PF and MA-R are PhD students of the Doctoral Programmes in Applied and Environmental Microbiology (DP_AEM), (FCT grant PD/BD/113810/2015) and in Molecular and Environmental Biology (PDBMA), (FCT grant PD/BD/145354/2019), respectively. We thank the Brazilian companies, Cerlev, Lda., Ouro Preto, MG, and Fermentec, Lda. Soluções Tecnológicas e Industriais, Piracicaba, SP for kindly supplying the yeast strains used in this work.

SUPPLEMENTARY MATERIAL

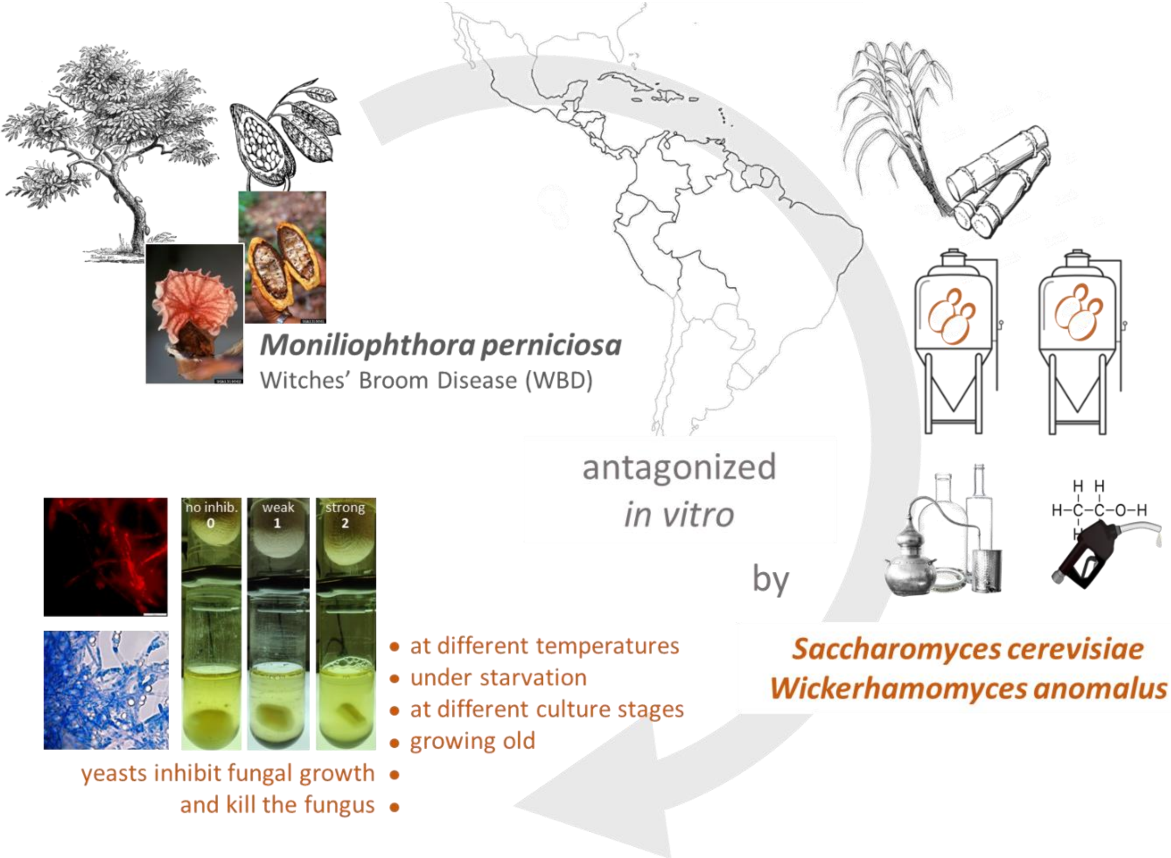


Figure S1. Graphical abstract.

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CHAPTER 3

Microscopic assessment of the antagonism effect of yeasts against *Moniliophthora perniciosa,* the fungal causal agent of *Witches' Broom Disease* in cacao

The work presented in this chapter is in preparation for publication:

Ferraz, P., Cássio, F. and Lucas, C. Microscopic assessment of the antagonism effect of yeasts against *Moniliophthora perniciosa*, the fungal causal agent of *Witches' Broom Disease* in cacao

ABSTRACT

Mycoparasitism of pathogenic fungi by yeasts could be associated with direct physical contact. Predacious yeasts can penetrate the fungal cell walls killing the fungal pathogen and/or feed on them, taking up nutrients from the fungal cells. Contact between yeasts and fungal phytopathogens was observed in antagonism yeast strains against *Moniliophthora perniciosa*, the fungal causal agent of the *Witches' Broom Disease* of cacao (Ferraz *et al.*, submitted 2). Cell analysis by Scanning Electron Microscopy (SEM) revealed yeast binding and fusing with the hyphae of the phytopathogen, causing a constriction and deformation of its cells, and even a draining of the fungal cellular content. The formation of veil-like structures possibly associated with invasion of hyphae by yeasts was also observed. Moreover, SEM micrographs showed the occurrence of fimbriae-like connections between antagonistic yeast cells and of physical tube-like connection between yeast and fungal cells, which can be related with yeast cell-cell communication and to yeast/fungus recognition or even yeast predacious behavior, respectively. These results constitute the first description of a possible predacious-like behavior in non-*Saccharomycopsis* yeasts, which can be the basis of the antagonism exerted by yeasts against the fungal pathogens that cause WBD of cacao.

Keywords

SEM, Cacao; *Witches Broom Disease*; *Moniliophthora perniciosa*; yeast; antagonism; predation

INTRODUCTION

Yeasts can antagonize other microorganisms such as other yeasts, molds, and bacteria (Hatoum *et al.*, 2012). The antagonistic effect of yeasts is based on several mechanisms of action, including the competition for nutrients and space (Parafati *et al.*, 2015) and/or the production and secretion of killer toxins (Schmitt and Breinig, 2006) and other antifungal compounds such as hydrolytic enzymes (Lopes *et al.*, 2015; Liu *et al.*, 2018), volatile compounds (Schulz-Bohm *et al.*, 2017) and quorum sensing molecules (Avbelj *et al.*, 2016). In all these mechanisms, yeasts are able to secrete compounds and exert their antagonistic effect at a distance. Despite this, some yeasts have the ability to attach to the hyphae of fungal pathogens and produce diverse extracellular cell wall lytic enzymes. This type of interaction is known as mycoparasitism (Dukare *et al.*, 2018). Some sequential events are required so that a microorganism can parasitize a fungus. First there must be close contact between the antagonist and the fungal pathogen cells followed by mutual recognition. Subsequently the antagonizing microorganism secretes lytic enzymes and ultimately, there is active growth of the antagonist into the fungal host cells through a kind of penetration peg (Spadaro and Gullino, 2004). The fungal cell wall is often targeted. The fungal cell wall is composed of polysaccharides, glucans and chitin, and glycoproteins, which together provide mechanical strength and structural integrity (Spadaro and Droby, 2016). The antagonist secretes hydrolytic enzymes, including chitinases, chitosanases, glucanases, cellulases or proteases which may act independently or in combination with each other or other enzymes (Spadaro and Droby, 2016). Ultimately, the destruction of cell wall leads to severe cytological damage, in most circumstances causing the lysis of the cell and subsequently death (Di Francesco *et al.*, 2015; Dukare *et al.*, 2018).

Some examples of mycoparasitism and its association with the biocontrol of fungal phytopathogens have been described in the literature, particularly the mycoparasitism exerted by yeasts. This is the case of the antagonism of *Botrytis cinerea* by the yeast *Meyerozyma guilliermondii* (formerly known as *Pichia guilliermondii*), in which the yeast cell attaches strongly to the host and secretes a β-(1–3) glucanase, which results in the lysis of the fungal cell (Wisniewski *et al.*, 1991). Is has also been reported that, this type of lytic enzymes also inhibits the germination of the pathogen spores and the elongation of its germ-tube (El-Tarabily and Sivasithamparam, 2006). Another example of mycoparasitism is the biocontrol of *Penicillium expansum* by the yeast *Candida oleophila* in harvested apples, associated with the secretion of an exo-β-1,3-glucanase, which results in the inhibition of conidial germination and mycelia growth (Tamayo-Urbina *et al.*, 2016).

The mycoparasites can be classified into different groups, according to their modes of action. The necrotrophic mycoparasites are very aggressive organisms with a broad range of preys, and can attack fungi at a distance by the secretion of toxins or lytic enzymes into the environment (Junker *et al.*, 2019). Contact necrotrophs attack the fungal cells by direct physical contact and can reach and kill the fungal pathogen using hyphae, although they do not necessarily invade the target cell (Mims *et al.*, 2007). On the other hand, invasive necrotrophs, also known as predatory mycoparasites, physically penetrate their prey fungal cells through haustoria or penetration pegs (Junker *et al.*, 2019). The predatory organism can kill the fungal prey, or simply take up nutrients from its cells, or both (Jeffries, 1995; Junker *et al.*, 2019).

The majority of the well-studied mycoparasites are filamentous fungi, particularly species from the *Trichoderma* genus (Schmoll *et al.*, 2016). Although many yeast species are able to secrete antagonistic peptides such as killer toxins (Schmitt and Breinig, 2006), or lytic enzymes (Spadaro and Gullino, 2004), the only yeast species described as a necrotrophic mycoparasite belong to the *Saccharomycopsis* clade (Lachance and Pang, 1997). These yeasts are able to predate a wide range of other yeasts. All share a metabolic peculiarity, the inability to use sulfate as sole sulfur source. Despite not being well studied, their mode of predatory action involves the invasion of the prey cells, mediated by small haustoria-like penetration pegs, leading the subsequent death of the prey cells (Lachance *et al.*, 2012). *S. schoenii* is considered the most aggressive predacious yeast in this clade, and it was reported to attack and kill *in vitro* several clinical isolates of pathogenic *Candida* species, including the multi-drug resistant isolates of *C. auris*, thus having a great potential in medical biocontrol applications (Junker *et al.*, 2018). Additionally, some *Saccharomycopsis* species, have been described as successful potential biocontrol agents, namely *S. schoenii*, against plant pathogens developing in the surface of oranges (Pimenta *et al.*, 2008), and *S. fibuligera* against toxic molds on pork speck (Iacumin *et al.*, 2017).

The most favorable environmental conditions that promotes predation varies significantly, depending on the predacious species. Some species are stimulated by the presence of rich nitrogenous nutrients and high concentrations of ammonium nitrogen, as well as by the presence of organic sulfur compounds, while other species may be inhibited under the same conditions (Lachance *et al.*, 2000). Moreover, the predacious yeasts are able to penetrate the cells of other described predacious species, and interestingly young cultures can penetrate older cultures of the same predacious strain (Lachance *et al.*, 2000). This is a very poorly studied phenomenon which has not been described for other yeast genera and species, and which predation modes of action have not been further studied.

In a recent study the causal agent of cacao *Witches' Broom Disease* (WBD), the filamentous fungus *Moniliophthora perniciosa*, was shown to be killed by yeast isolates from spontaneous fermentations of *cachaça* production and strains used in bioethanol industrial processes (Ferraz *et al.*, submitted 2). Death of the fungal cell occurred after the co-incubation of both organisms and detected by staining with methylene blue and propidium iodide. The work hereby presented aimed at exploring microscopically the interaction between the two types of cells, unveiling that the yeasts physically attach to the hyphae, apparently fusing their walls surface, the two cells becoming as one, and that the hyphae are drained. Moreover, the study also showed that yeasts communicate physically between each other during the process. Results were obtained observing samples stained with methylene blue, as well as using Scanning Electron Microscopy (SEM). Further assessment by staining the cytoskeleton with a specific fluorescent dye, and the use of Transmission Electron Microscopy (TEM), will allow in the future the elucidation of the cytology of the actual merge between the two cells.

MATERIALS AND METHODS

Moniliophthora perniciosa strains CBS 441.80 and 442.80 originating from infected cacao plantations in Brazil were maintained at 4 °C on MEA (20 g/L malt extract w/ 20 g/L agar), and cultivated in the same medium at 30 °C. Yeasts strains *Wickerhamomyces anomalus* #1105 and *Saccharomyces cerevisiae* PE-2-FT134L (PE2) originating from spontaneous fermentations used for the production of *cachaça* (Brazilian spirit) and the industrial production of bioethanol in Brazil, respectively (Ferraz *et al.*, submitted 1 and 2), were cryopreserved in sterile glycerol 30% at -80 °C, maintained at 4°C on YPDA (10 g/L yeast extract, 10 g/L bacto peptone, 20 g/L D-glucose with 20 g/L agar), and multiplied at 30 °C in the same medium for 48-72 h prior to the assays. Growth in ME (20 g/L malt extract) was done at the same temperature with 200 rpm orbital shaking and a liquid/air ratio of 1:2.5 and followed spectrophotometrically at 600 nm.

Antagonism assays were performed in liquid medium as previously described (Ferraz *et al.*, submitted 2), inoculating the actively growing mycelium together with a suspension of ME-grown yeast cultures collected in exponential phase (10^8 cells/ml), using glass tubes (13 cm x Ø 3 cm) containing 20 ml of ME medium and incubating at 30°C and 200 rpm orbital shaking for 10 days. The plug was washed with ultrapure water with soft manual shaking.

For Scanning Electron Microscopy (SEM) analysis, fungal samples were fixed by soaking the films in 1 mL of 2.5% v/v glutaraldehyde in PBS for 48 h at 4 °C, rinsed with 1 mL distilled water, and post-fixated with 1 mL of 1% v/v of osmium tetroxide for 1h at room temperature (adapted from Murtey and Ramasamy, 2016). Samples were dehydrated through immersion for 20 min in a series of successive ethanol-water solutions (1 mL of 20, 30, 40, 55, 70, 80, 90, 95, and 100% v/v of ethanol). Fungal samples were then dried at room temperature and coated with a thin Au/Pd layer using a sputter coater prior to scanning electron microscopic assessment with a NanoSEM (FEI Nova 200) at a 5 or 10 kV voltage and a through-lens detector (TLD). All assays were performed at least in three independent replicates (n ≥ 3).

Results and Discussion

It was previously observed that yeast strains native from industrial fermentative processes were able to cause the death of *M. perniciosa* strains, the fungal causal agent of the WBD of cacao (Ferraz *et al.*, submitted 2). In that work, the death of the fungal cells was confirmed by staining with methylene blue and propidium iodide. Additionally, it was observed that the antagonistic yeast cells appeared to attach to the hyphae, which were empty in a large fraction of the mycelia. This type of assays was repeated for further detail using the combinations between two strains of each, yeasts and fungus. The *W. anomalus* #1105 originating from spontaneous fermentation of *cachaça* (da Conceição *et al.*, 2015), and the *S. cerevisiae* strain PE2 from the industrial production of bioethanol (Lopes *et al.*, 2016) were tested against *M. perniciosa* CBS 441.80 and CBS 442.80 (Ferraz et al., submitted 2). Results showed that the yeasts in fact appear to attach to the hyphae, causing a constriction and deformation of the fungal cells (Figure 1, red arrows). It is also possible to see that in the area near the yeast-hyphae contact the hyphae appear drained of the cellular content becoming empty and flattened (Figure 1, yellow arrows).

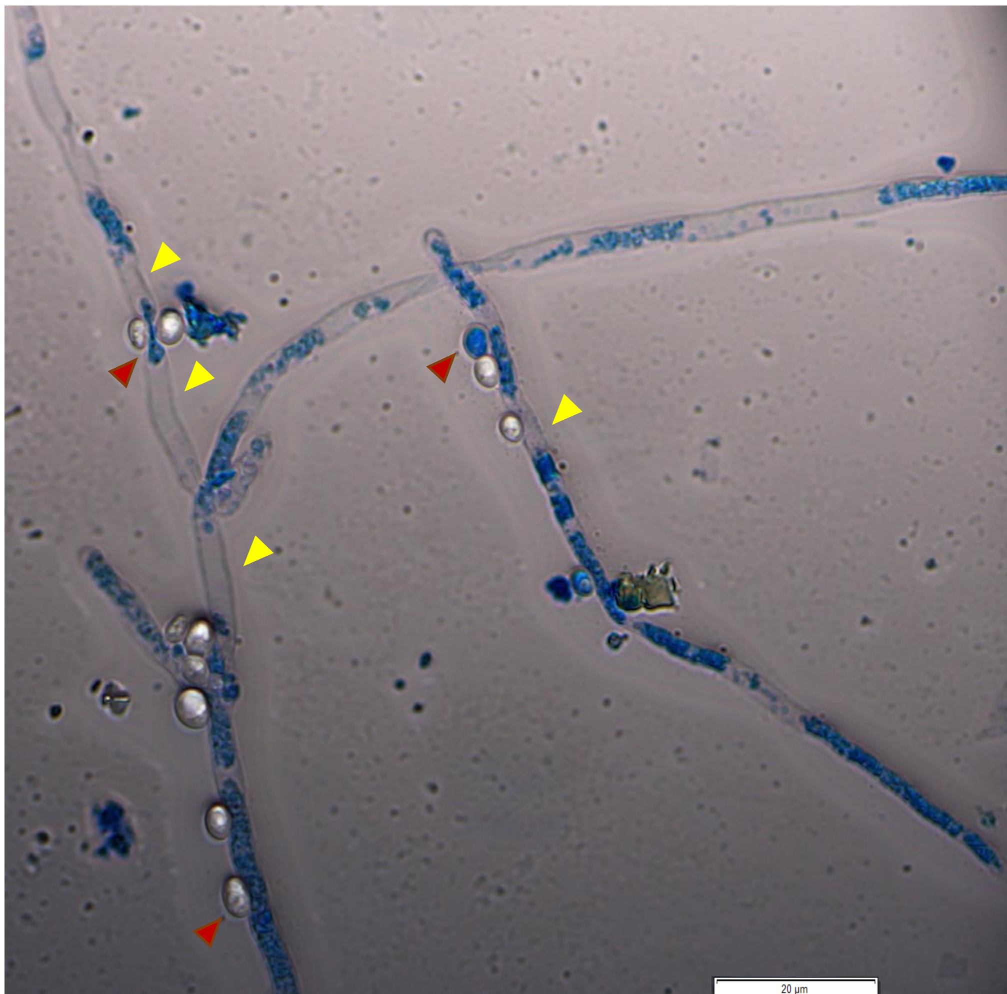


Figure 1. Example of a yeast/fungal strains co-culture showing the mycelia strongly stained with MB. Isolated or clustered yeast cells appear to attach the fungal hyphae, causing a constriction and flattening of the fungal cells by pushing the hyphae (red arrows). Hyphae are shown drained of the cellular content (yellow arrows). This particular image corresponds to the co-culture of #1105 with CBS 442.80.

To further investigate the morphological characteristics of this yeast/fungal interaction, the antagonistic assays were repeated and observed by SEM. Figure 2 shows micrographs of the control samples of either strain cultivated alone.

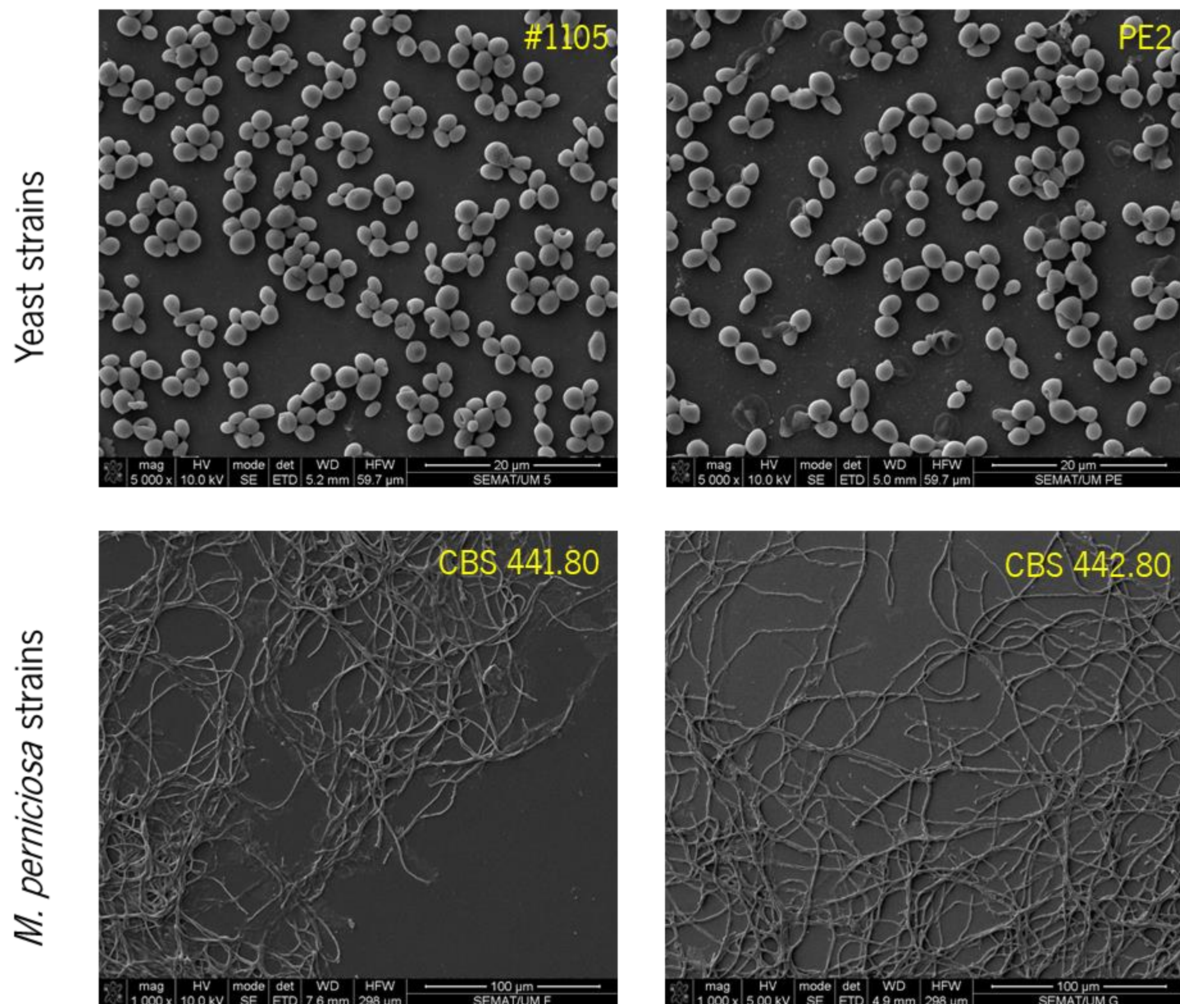


Figure 2. SEM micrographs of the control single cultures of yeasts and *M. perniciosa* strains.

The analysis of co-incubated cultures of yeasts and fungi, at increasing magnification (Figure 3 and 4), clearly shows that the yeast cells group near the hyphae, push the hyphae surface causing a compression (white arrows in Figure 4), and eventually appear to become fused with the hyphae. Moreover, empty flattened hyphae are clearly visible in Figure 4 (yellow arrows), farther from the point of yeast attachment. These results clearly show that fungal cells co-incubated with the yeasts die while yeasts attach to the hyphae, and that death is accompanied by the draining of the fungal cells, suggesting that the yeasts are predating the fungi, possibly feeding on their intracellular components. These results appeared consistent with what has been described as the behavior of predacious yeasts, associated with yeast species of the *Saccharomycopsis* clade (Lachance and Pang, 1997; Lachance *et al.*, 2012).

Three additional structures were observed in the SEM analysis, presented in detail in the micrographs of Figures 5 and 6. The first is a veil-like structure (Figure 5, red arrows), made of empty hyphae smoothly covering the yeast cell. Either the yeast cells can break the fungal cell wall and penetrate the hyphae, and the veil then corresponds to the remaining fungal cell surface involving the invasive yeast; or this was an artifact resulting from different planes of the micrograph, corresponding only to a dead and emptied hypha occasionally covering a yeast. This will need further investigation using Transmission Electron Microscopy which will enable the visualization of the internal cell structures at the point of connection, as well as fluorescence microscopy staining the yeast cytoskeleton (Higuchi-Sanabria *et al.*, 2016). Both assessments will enable to understand if the yeast cells stretch into the hyphae forming a penetration peg.

The second structure observed is a physical tube-like connection between the yeast and the fungal cells (blue arrows in Figure 5). In the literature, inter-species physical communication is to the best of our knowledge restricted to the "channel" formed by *Agrobacterium* species to promote the passage of bacterial DNA into plant cells (Fullner *et al.*, 1996; Lai and Kado, 1998). The establishment of these connections could be related to the mutual recognition by the two microorganisms or be associated with an initial stage of the possible invasion of the fungal cells by the antagonistic yeasts.

Finally, the third type of structure is shown in Figure 6. It seems a fimbriae-like connection between yeast cells, grouped in a small cell surface area (left panels, increasing magnification). Their localization apparently has no relation with a particular cell orientation or part, forming near a cell-bud ligation or in an opposite side of the cell (right panels, blue arrows). Once two cells separate (left panels) a small scar is left behind (right panels, yellow arrows). It is round, with a small rim around a shallow cavity containing a group of protrusions that might correspond to previous fimbriae.

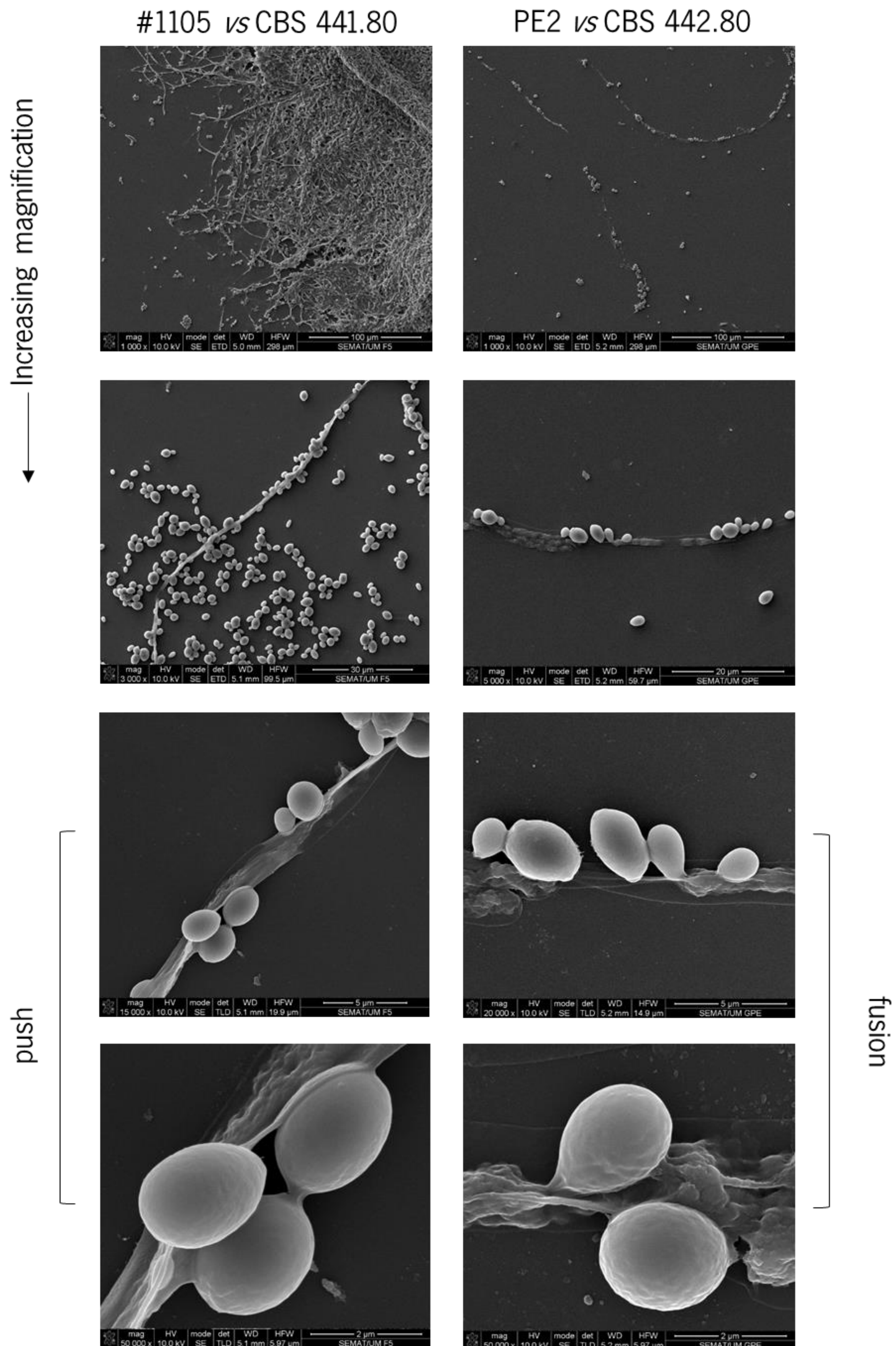
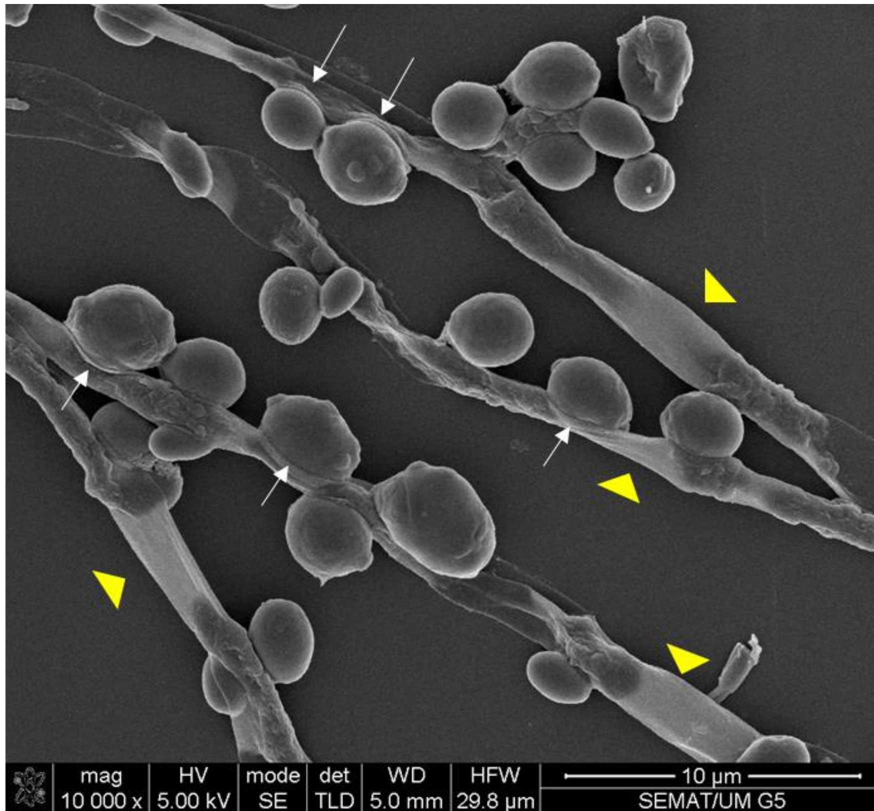


Figure 3. SEM micrographs of the yeast/fungal strains combinations #1105 *vs* CBS 441.80 and PE2 *vs* CBS 442.80 at increasing magnification, showing in detail the occurrence of yeast pushing and a constriction on the fungal cells, and fusion of the yeasts with the hyphae.

#1105 *vs* CBS 442.80



PE 2 *vs* CBS 441.80

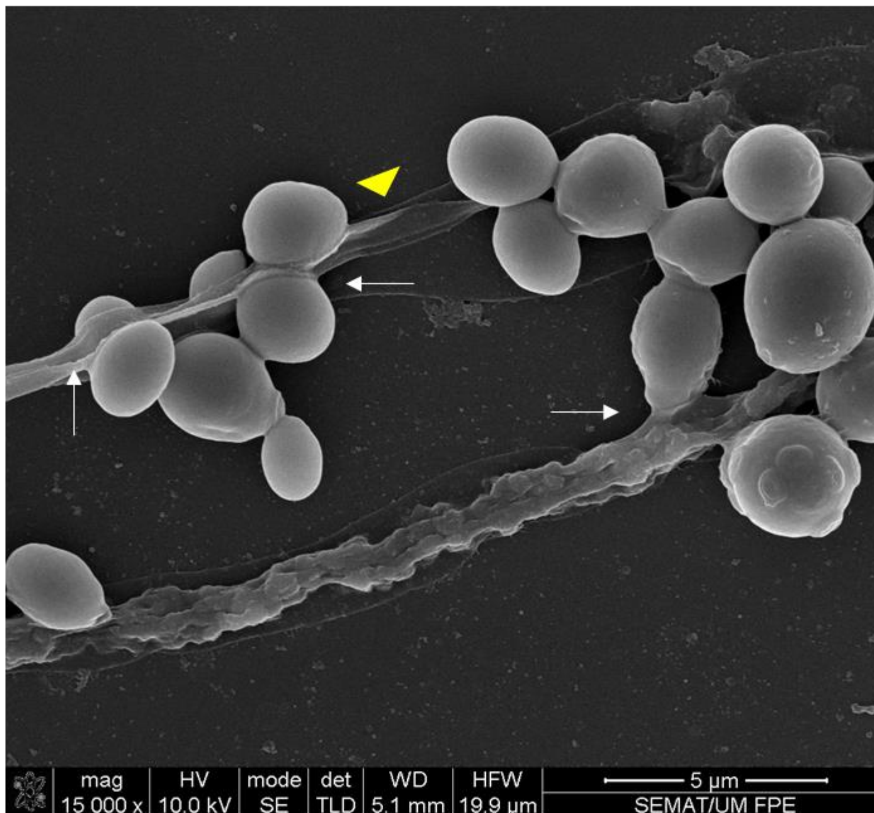
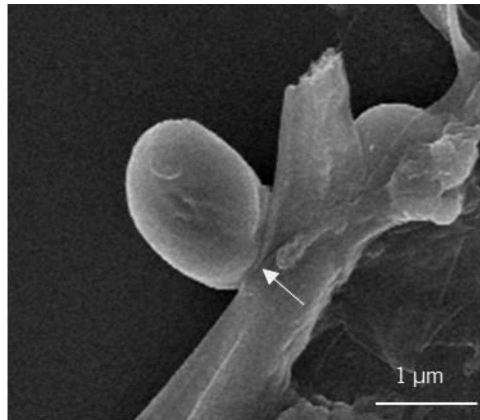
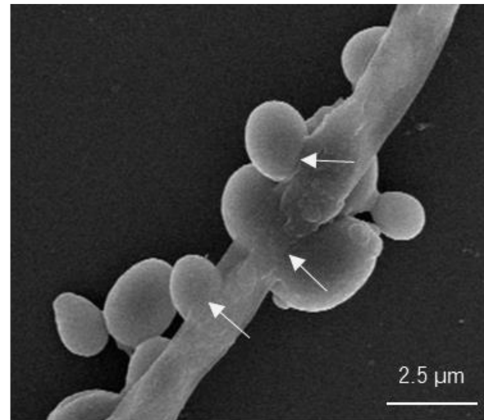
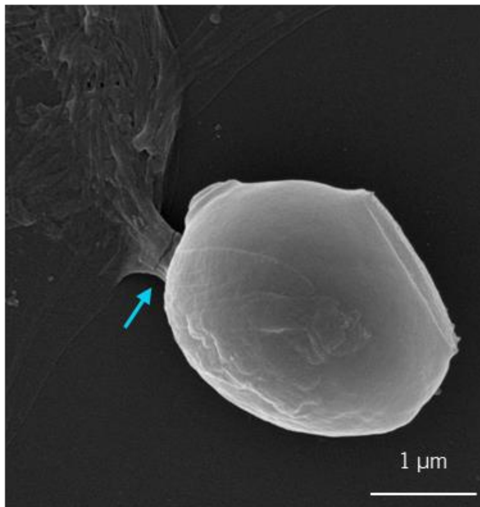
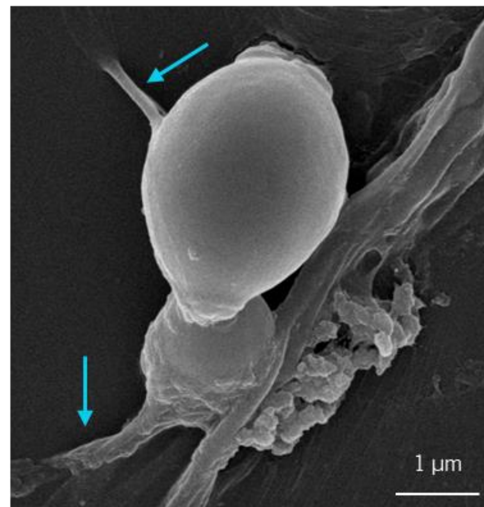


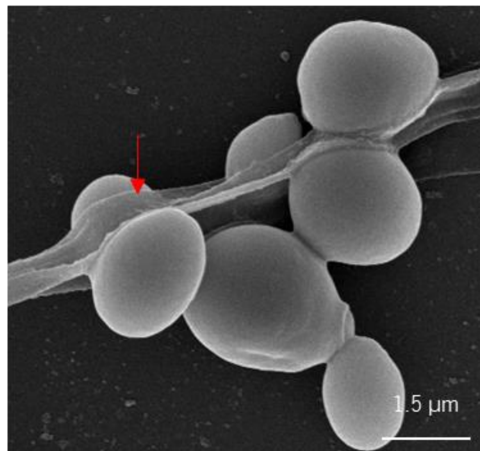
Figure 4. SEM micrographs of the yeast/fungal strains combinations #1105 *vs* CBS 442.80 and PE2 *vs* CBS 441.80, showing in detail the occurrence of yeast pushing and fusion with the hyphae (white arrows) and the disappearance of the fungal intracellular content in the areas adjacent to the presence of the antagonistic yeasts (yellow arrows).











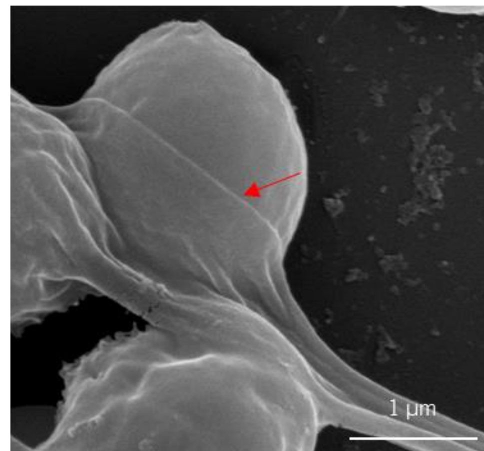


Figure 5. SEM micrographs of the yeast/fungal strains combinations #1105 *vs* CBS 441.80 and PE2 *vs* CBS 441.80, showing in close detail different morphological structures of the interaction: yeast pushing the hyphae and fusion between the cells of both organisms (white arrows); connections between the yeast and the fungal cells (blue arrows); and formation of a veil-like structure (red arrows).

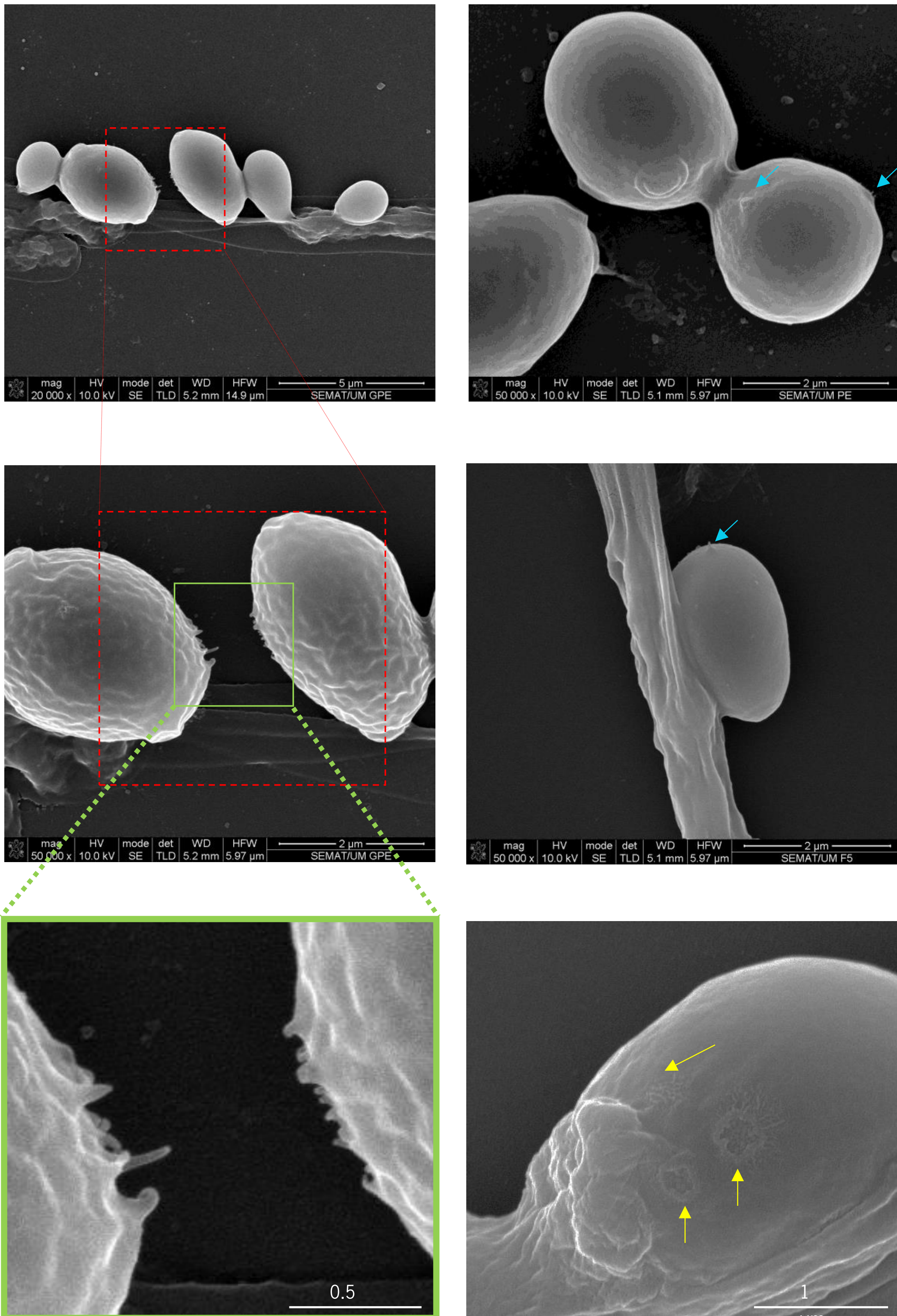


Figure 6. SEM micrographs of the connection between yeast cells. Left panels - Details of two yeast cells that apparently were connected and their small fimbriae-like structures on the cell surface. Right panels - Fimbriae-like structures in the surface of the yeast cells (blue arrows) and locations of previous connection structures (yellow arrows).

Fimbriae and pili have abundantly been described in bacteria (Carter *et al.*, 2016) as hair-like structures protruding from bacteria cell wall. They are involved in adherence to inert surfaces or living tissues, in cell-cell communication, as well as in the conjugational transfer of DNA between two cells (reviewed by Berne et al., 2018). In the case of yeasts, connection fibrils between two cells from the same species and strain have been reported in biofilms (Mamvura *et al.*, 2017) or in starved cell (Varon and Choder, 2000). To these fibrils have been attributed a role in the colony formation of some *Candida* species (Vargas *et al.*, 2004; Furlaneto *et al.*, 2012). Information is though very scarce, and there are no detailed studies on which yeasts are able or not to form them, on how or when they are formed, or on their structure and composition.

CONCLUSIONS

The present study allowed the visualization of (i) the antagonizing yeast cell binding and fusing to the dead hyphae of the phytopathogen, (ii) the constriction and deformation of the fungal cells caused by yeast attachment, (iii) the draining of the fungal cellular content in areas adjacent to the yeast attachment. Moreover, the analysis by SEM revealed (i) the formation of a veil-like structure that could be related with a possible invasion of hyphae by yeasts, (ii) the occurrence of a physical tube-like connection between yeast and fungal cells which can be related to yeast/fungus recognition or even associated with yeast predacious behavior, and (iii) the existence of fimbriae-like connections between antagonistic yeast cells, which can be associated with a mechanism of yeast cell-cell communication. Although neither of the antagonistic yeasts tested belong to the *Saccharomycopsis* clade, these characteristics resemble those of the predacious yeasts.

This work presents the first description of a possible predacious-like behavior in non-*Saccharomycopsis* yeasts and might provide a basis to understand why the industrial yeasts are so resilient to harsh environments and prevail over other strains in their natural/industrial habitat. Nevertheless, a predacious behavior may not be independent of the secretion of some antifungal compounds by these yeasts, which may act synergistically, causing the antagonism effect and leading to the pathogen's death. Future analysis by TEM and fluorescence microscopy can improve the understanding of this antagonism and clarify these yeasts mode of action.

ACKNOWLEDGEMENTS

This work was supported by the strategic programme UID/BIA/04050/2013 (POCI-01-0145-FEDER-007569) funded by national funds through the FCT I.P. and by the ERDF through the COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI), and the project EcoAgriFood (NORTE-01-0145-FEDER-000009) from Norte Portugal Regional Operational Programme (NORTE 2020) under the PORTUGAL 2020 Partnership Agreement through the European Regional Development Fund (ERDF). PF is a PhD student of the Doctoral Programme in Applied and Environmental Microbiology (DP-AEM), (FCT grant PD/BD/113810/2015). We thank the Brazilian companies, Cerlev, Lda., Ouro Preto, MG, and Fermentec, Lda. Soluções Tecnológicas e Industriais, Piracicaba, SP for kindly supplying the yeast strains used in this work.

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CHAPTER 4

Wickerhamomyces anomalus **from olive orchards microbiome efficiently antagonizes the Olive Anthracnose's causal agents, *Colletotrichum spp.***

The work presented in this chapter is in preparation for publication:

Amorim-Rodrigues, M., Ferraz, P., Cássio, F. and Lucas, C. *Wickerhamomyces anomalus* from olive orchards microbiome efficiently antagonizes the Olive Anthracnose's causal agents, *Colletotrichum* spp.

ABSTRACT

Olive Anthracnose (OA) is a phytopathology severely affecting olive orchards particularly in the Mediterranean region. OA is caused by fungi from the *Colletotrichum gloeosporioides* and *C. acutatum* species complexes. Application of chemical fungicides is the main current management strategy, although these are ineffective and harmful for the environment and human health. Yeasts are known to antagonize other microorganisms including fungi. The present work evaluates the potential of yeast strains, originating from Portuguese olive trees microbiome, as well as well-known fermentation industrial processes, against *C. gloeosporioides* sensu stricto, *C. godetiae* and *C. nymphaeae* originating from infected orchards in Portugal and Italy. Antagonism was assessed in co-cultures in solid and liquid media. The strongest inhibition was observed using an isolate of *Wickerhamomyces anomalus* from a Portuguese olive orchard. Further work is under way to assess the mode of action of this yeast, aiming at the development of an eco-friendly method based on this yeast as a living biocidal agent in the management of Olive Anthracnose in Portugal.

Keywords: Antagonism; Biocontrol; Olive Anthracnose; *W. anomalus*; *Colletotrichum* spp.

INTRODUCTION

Olive tree (*Olea europaea* subsp. *europaea*) products, olives and olive oil, are of major economic relevance mostly in the Mediterranean region, though it is spreading to other regions, meeting an increasing market demand (Moral *et al.*, 2017). Olive trees are susceptible to a number of pests and diseases, some of which extremely virulent, eventually causing the death of the tree, as is the case of the leprosy caused by the bacteria *Xylella fastidiosa* (Sicard *et al.*, 2018), or the destruction of a year's crop by a plague of the olive fruit fly *Bactrocera oleae* (Marchini et al., 2017), or the perennial persistence and spread of a debilitating fungal infection like anthracnose (Talhinhas *et al.*, 2018). Protecting the olive trees from increasingly more serious threats is a challenge.

Olive anthracnose (OA) is a disease that affects trees and fruits, causing great losses in the production of olives and olive oil (Talhinhas *et al.*, 2018). It was first described to occur in Portugal in the 19th century (Almeida, 1899), and it affects crop production, pre- and post-harvest, and oil quality (Cacciola *et al.*, 2012; Talhinhas *et al.*, 2018). Total crop losses are common during severe outbreaks, translating in important economical shortfalls (Cacciola *et al.*, 2012; Talhinhas *et al.*, 2018). For example, in Spain the estimated overall losses per year due to OA have reached over €80 million (Moral *et al.*, 2009).

The causal agents of OA are a *consortium* of *Colletotrichum* spp. fungi, namely *C. gloeosporioides* sensu stricto, *C. nymphaeae* and *C. godetiae*, varying in prevalence and also virulence (Talhinhas *et al.*, 2011). These fungi are hemibiotrophic, presenting a biotrophic and a necrotrophic phase. During the biotrophic stage, occurring through spring and summer, the fungus develops asymptomatically inside the infected flowers and developing fruits. In autumn, the mild and humid weather, coupled with the ripening of the fruits, triggers the necrotrophic stage, leading to the appearance of the symptoms (Moral *et al.*, 2008; Cacciola *et al.*, 2012). Symptoms are present in all organs of the plant, mostly in the mature drupes, where they appear as small, dark sunken lesions, profusely producing an orange mass of conidia in concentric rings (Moral *et al.*, 2008; Cacciola *et al.*, 2012). At this point, if the weather conditions are conducive and the inoculum pressure is high, the conidia can germinate and cause secondary infections, greatly increasing yield losses (Moral *et al.*, 2008; Cacciola *et al.*, 2012). These symptoms lead to premature drop of the drupes or their mummification. Mummified drupes that remain on the tree will act as inoculum reservoir during the winter, causing the flower infections in the spring, beginning a new cycle of infection (Moral and Trapero, 2012; Sergeeva, 2014; Talhinhas *et al.*, 2018).The early fruit drop, alongside with the modifications that occur on the olive fruits, can result in low quality of the olives and

the olive oil, causing colour variation, increased acidity and oxidative instability (Talhinhas *et al.*, 2005; Hernández *et al.*, 2008; Cacciola *et al.*, 2012).

To combat and overcome the harmful effects caused by OA, several methods of disease management are applied. Commonly, these strategies include (i) early harvesting and use of late-ripening cultivars, (ii) pruning of trees and wide orchard design, (iii) replacing existent trees with resistant cultivars, and most frequently (iv) the application of chemical fungicides (Rosa *et al.*, 2010; Cacciola *et al.*, 2012; de Lima *et al.*, 2013). Several classes of fungicides can be applied, including the copper-based fungicides, dithiocarbamate (ziram), azoles (hexaconazole, tebuconazole) and strobilurins (azoxystrobin, trifloxystrobin) (Pennisi *et al.*, 1993; Sergeeva, 2011; Moral *et al.*, 2014). Nevertheless, few of these have proven to be efficient in controlling the disease, and their efficacy substantially varies regionally, since it depends on the disease severity, the olive cultivar and particular environmental conditions. Moreover, success of using a particular agrochemical also depends on timing, frequency and number of applications as well as the strategy, *i.e.* whether it is meant to be protective/preventive or curative (Cacciola *et al.*, 2012; Landum *et al.*, 2016). Protective fungicides, both organic and inorganic must be applied in a preventive way, frequently multiple times in order to maximise their effect (Landum *et al.*, 2016). The rainy weather conditions that prevail during the most common treatment season aggravate host susceptibility and the run-off of the fungicides, enhancing fungal development (Cacciola *et al.*, 2012). Further recognised limitations of the chemical fungicides are the acquired fungal resistance and incomplete protection of most of the susceptible cultivars (Cacciola *et al.*, 2012).

Increasingly public awareness over pesticide-promoted environmental contamination and health-problems is pushing public opinion and UE directives, namely the Directive 2009/128/EC (EUR- Lex, 2018), towards an increasing discouragement of applying fungicides in agroecosystems. This generates a new market demand for alternative, greener, more sustainable and effective ways to control phytopathogens. New alternatives include the use of natural antagonists such as microorganisms like bacteria, viruses, fungi or yeasts. Alternatively, products resulting from their biological processes, like antibiotics or other toxins, can also be used once purified (Pal and McSpadden Gardener, 2006; Hagens and Loessner, 2010; Liu *et al.*, 2015).

Yeasts can be applied directly, alive, or can be used to produce lethal toxins (Hatoum *et al.*, 2012). Several mechanisms underlie the yeast antagonistic ability, including the competition for nutrients and space, and the secretion of antifungal compounds such as killer toxins, hydrolytic enzymes like and toxic volatile compounds (reviewed by Ferraz *et al.*, 2019). Due to their lack of production of allergenic spores or mycotoxins, and the absence of associated bacteria-like health hazards, yeasts are more often the

choice to control undesirable microorganisms, such as other yeasts, bacteria and filamentous fungi (moulds and pathogens). The employment of yeasts as biocontrol agents is widespread in the food and beverage industry, as well as in agriculture, for post-harvest spoilage prevention or retarding (Hatoum *et al.*, 2012; Syed Ab Rahman *et al.*, 2018). Some products directed to this type of application are commercialised (Lima *et al.*, 2013; Nigro *et al.*, 2018). For example, Nexy® and Candifruit® are yeast-based formulations used to control postharvest spoilage of pome and citrus fruits (Sundh and Melin, 2011; Lima *et al.*, 2013). These last authors showed that yeast strains from *Wickerhamomyces anomalus* and *Meyerozyma guilliermondii* are able to inhibit the germination of spores from *C. gloeosporioides*, causal agent of papaya anthracnose, raising the expectation that yeasts could act as efficient antagonists of the OA causative agents. Nevertheless, regarding the pre-harvest application of yeasts as biocontrol agents, it has never been consistently and successfully used, although it is a very promising alternative (Oro *et al.*, 2014; Leoni *et al.*, 2018).

In this work, several yeasts, originating from olive biome and biotechnology fermentation-based industry environments, were used to test their capacity to antagonize *in vitro* the OA causal agents: *C. gloeosporioides* s.s., *C. nymphaeae* and *C. godetiae*. A total of 63 yeast/fungal combinations were tested in solid and liquid media. From these, one *W. anomalus* strain from the Portuguese olive biome showed an excellent antagonistic ability against the OA causal agents, being the only yeast able to inhibit the growth of the three fungal strains in all the conditions tested. The antagonism effect of this yeast strain raises expectations as to its possible utilization in the management of OA, thus allowing to address the lack of effective and sustainable methods for its control and contributing to ease the social-economic impact of the disease in the Mediterranean area.

MATERIALS AND METHODS

Microorganisms culture conditions

Yeast and filamentous fungal strains (Table 1) were cultivated and maintained as described by Ferraz *et al.* (submitted). Growth of yeasts in liquid media was followed spectrophotometrically at 600 nm. Fungal growth in solid media was followed until mycelia covered the whole plate or developed *acervuli*. In liquid medium it was visually inspected for the formation of a dense fungal mass, eventually replacing most of the culture media, and forming a ring of mycelium on the glass tube walls. The yeast and fungal growth rates were determined as described before (Ferraz *et al.*, submitted).

Evaluation of the antagonistic ability

Each combination of yeast /fungal strain was assayed in solid and liquid media as previously described (Ferraz et al., submitted). Antagonism in solid media was evaluated by an empirical scale of classification defined in the same study, as well as by determining the percentage of inhibition. For this purpose, the mycelium growth radius was measured on opposite sides of the inoculum plug, away from (R1) and facing (R2) a yeast strike. Inhibition was quantified according to Royse and Ries (1978), as Inhibition (%) = ((R1-R2)/R1) x 100. Antagonism in liquid media was assessed by co-culturing a plug of actively growing mycelium with a suspension of grown yeast cultures collected in the exponential phase ($1x10^8$ cells). The tubes were incubated at 25 °C and 200 rpm orbital shaking for 4 days. Antagonism was evaluated using a second similar empirical classification scale also previously defined (Ferraz et al., submitted).

Statistical Analysis

All the results correspond to at least three independent replicates. To test the effect of culture media and pH on the fungal growth rates, an analysis of variance (two-way ANOVA) was performed. Since no interaction was found between two factors, multiple comparisons were performed by Tukey's test at 5 % probability, using R packages multcomp v1.4-8 (Hothorn et al., 2008) in R v3.3.3 (R Core Team, 2018) environment, using RStudio v1.1.383 (RStudio Team, 2015). Graphical display was performed with Microsoft® Office Excel and R Studio (R Core Team, 2018), using the packages, ggplot2 v2.2.1 (Wickham, 2016), cowplot v0.0.1 (Wilke, 2017).

Table 1. Phytopathogenic filamentous fungi and yeast strains and their origin.

	Species	Strain	Origin		Present Designation
Filamentous Fungi	*Colletotrichum gloeosporioides* s.s.	CBS 100471	CBS-KNAW [1]		C1
	Colletotrichum godetiae	–	ISA [2]		C2
	Colletotrichum nymphaeae	–			C3
Fermentative yeasts	*Pichia guilliermondii*	Cerlev 1015	*Cachaça* wort [3]		#1
	Saccharomyces cerevisiae	Cerlev 1025			#2
	Saccharomyces cerevisiae	Cerlev 1038			#3
	Saccharomyces cerevisiae	Cerlev 1096			#4
	Wickerhamomyces anomalus	Cerlev 1105			#5
	Saccharomyces cerevisiae	Cerlev 1112			#6
	Saccharomyces cerevisiae	Cerlev 1113			#7
	Saccharomyces cerevisiae	CAT1-FT280L	Sugarcane juice fermentation [4]		CAT1
	Saccharomyces cerevisiae	PE2-FT134L			PE2
Yeasts from olive trees biome	*Solicoccozyma phenolicans*	PYCC 7188	Olives on tree	PYCC [5]	#11
	Zygosaccharomyces bailii	PYCC 7190	Fallen leaves		#12
	Kodamaea ohmeri	PYCC 7192	Soil under tree		#13
	Torulaspora delbrueckii	PYCC 7193	Fallen leaves		#14
	Zygosaccharomyces bailii	PYCC 7197	Fallen ripe olives		#15
	Zygosaccharomyces rouxii	PYCC 7198	Soil under tree		#16
	Kluyveromyces lactis	PYCC 7201	Leaves on tree		#17
	Wickerhamomyces anomalus	PYCC 7203	Fallen ripe olives		#18
	Lachancea thermotolerans	PYCC 7205	Olives on tree		#19
	Saccharomyces cerevisiae	PYCC 8114	Olives on tree		#20
	Saccharomyces cerevisiae	PYCC 8114	Olives on tree		#21
	Saccharomyces cerevisiae	PYCC 8114	Olives on tree		#22

[1] CBS-KNAW Collections, The Netherlands (http://www.westerdijkinstitute.nl/Collections/DefaultInfo.aspx?Page=Home)

[2] ISA -Instituto Superior de Agronomia, Univ. de Lisboa, Portugal (see Acknowledgements)

[3] Cerlev, Lda., Ouro Preto, MG, Brasil (https://www.facebook.com/empresa.cerlev/)

[4] Fermentec, Lda. Soluções Tecnológicas e Industriais, Piracicaba, SP, Brasil (https://www.fermentec.com.br/capa.asp?pi=principal)

[5] Portuguese Yeast Culture Collection, NOVA, Lisbon, Portugal (http://pycc.bio-aware.com/)

RESULTS AND DISCUSSION

Optimization of fungal cultivation conditions

Yeast strains used in the present work (Table 1) can be divided in two groups. The first contains strains which were isolated from the olive biome of Portuguese orchards. The second group includes strains isolated from sugarcane-based spontaneous and industrial fermentation processes. The fungal strains that were used have different origins. The strain of *C. gloeosporioides* s.s. was originally isolated from infected orchards in Italy. The strains of *C. godetiae* and *C. nymphaeae* originate from infected olive trees in Portugal. Prior to the antagonism assays, the *in vitro* optimal culture conditions of fungal and yeasts strains were determined. Firstly, the fungal growth rates were assessed in MEA and PDA, at different pH (4.0, 4.5, 5.0, 5.5 and 6.0) and 30 °C. Only *C. gloeosporioides* s.s. was able to grow at this temperature. Therefore, assays were repeated at 25°C. In agreement with the literature (Wharton and Diéguez-Uribeondo, 2004; Talhinhas *et al.*, 2005), *C. godetiae* and *C. nymphaeae* (from the *C. acutatum* species complex) grew much slower than *C. gloeosporioides* s.s. (Table S1). Although this last actually grew better at 30 °C than at 25 °C, the difference was not significant (not shown). The selected temperature for further assaying all the fungi was therefore defined as 25 °C. Furthermore, differences in growth rates between assays in MEA or PDA (not shown) and at different pH (Table S1) were not significant. Based on these results, the optimal conditions were established for all the fungal strains as MEA or PDA, and pH 5.5 at 25 °C. From the same assays, it was also shown that 4 to 8 days is enough to assess fungal growth.

Antagonism in solid media

Antagonism was tested using the yeasts and the phytopathogenic fungal strains in Table 1. Results were captured after 8 days of incubation (Table 2) and expressed according to the empirical scale of levels 0, 1 and 2 described in Ferraz *et al.* (submitted). A more detailed assessment of antagonism followed, determining the percentage of fungal growth inhibition (Table 3). As can be seen in these Tables, all the yeasts were able to inhibit the growth of at least one of the fungal strains, in most cases causing a low inhibitory response (level 1). Accordingly, some yeasts behaved generally as weak antagonists, like the strains #1 and PE2. In opposition, the yeast strain causing the strongest inhibitory response (level 2) was #18, which inhibited the growth of the three fungal strains in more than 70 % (Table 3).

Table 2. Results of antagonism assays in solid medium, using the yeast strains from Table 1 against *Colletotrichum gloeosporioides* s.s. (C1), *C. godetiae* (C2) and *C. nymphaeae* (C3). Assays were performed in MEA at 25 °C. The numbers displayed translate results according to the empirical scale described in Ferraz *et al.* (submitted). Single numbers represent the inhibitory response of 3 replicates. A table cell displaying 3 numbers (e.g. 1/0/0) represents a situation where each plate had a different result.

Phytopathogens	**Yeasts**										
	#1	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**CAT1**	**PE2**	**#11**	**#12**
C1	0	1/0/0	1/0/0	0	2/1/1	1	0	1/1/0	0	0	1/1/0
C2	1	1/1/0	1/1/0	1/1/0	1	2/1/0	1	1	1/0/0	1	1
C3	1	1/0/0	1/0/0	1/1/0	1/1/0	1/1/0	1	1/0/0	0	1/1/1	1/1/0
	#13	**#14**	**#15**	**#16**	**#17**	**#18**	**#19**	**#20**	**#21**	**#22**	
C1	1/0/0	1/1/0	1/0/0	1/0/0	1/1/0	2/2/1	1/1/0	1	1/1/0	1	
C2	1	2/1/0	1	1/1/0	1	2	1	1	1	1	
C3	1	1/0/0	1/1/0	1/1/0	1	2	1	1/1/0	1	1/1/0	

The inhibition of the fungal growth was observed to occur at a certain distance as exemplified in Figure 1A, suggesting that yeasts possibly secrete a compound that affects the fungal multiplication. There are two types of possible compounds: (i) soluble, diffusing through the agar, such as killer toxins and lytic enzymes (Parafati *et al.*, 2015; Oro *et al.*, 2018); and (ii) volatile (Toffano *et al.*, 2017). To assess which might be the case, the antagonism on solid media was repeated in MEA using septate Petri dishes, equidistantly inoculated with the mycelial plug on one side of the septum, and the yeast streak on the other. This only allows the fungus and the yeast to share the atmosphere inside the dish. No inhibitory response was observed up to 2 weeks of incubation, as exemplified in Figure 1B. Moreover, the fungal strains, except *C. godetiae*, went over the *septum*, eventually growing on top of the yeast and covering the whole dish. This result strongly suggested that the inhibition of fungal growth by this yeast requires the diffusion of a molecule through the agar. Accordingly, Lima and collaborators (2013) found that when *W. anomalus* and *M. guilliermondii* cultures were killed by autoclaving and filtered, they lost the ability to inhibit the spore germination of *C. gloeosporioides* s.s., which concurs with the need for yeast to be metabolically active abovementioned. After 2 weeks of incubation, most of the yeast population is most probably dying or dead, consistently with the MB coloration of the yeast streak (not shown).

Table 3. Antagonism between the OA causative agents *Colletotrichum gloeosporioides* s.s. (C1), *C. godetiae* (C2) and *C. nymphaeae* (C3) and the yeast strains in Table 1. Results are percentage of inhibition in solid media, according to Royse & Ries (1978). Values are average across replicates N≥3 and standard deviation. Shaded cells show highest inhibition of all fungi.

Phytopathogens	Yeasts										
	#1	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**CAT1**	**PE2**	**#11**	**#12**
C1	3.2 ± 5.5	17.5 ± 7.3	19.1 ± 4.8	25.4 ± 15.3	70.4 ± 1.4	46.0 ± 22.5	23.8 ± 4.8	57.1 ± 24.7	19.1 ± 4.8	0.0 ± 0.0	28.6 ± 31.2
C2	40.9 ± 7.9	22.7 ± 25.3	33.3 ± 26.6	25.8 ± 9.5	28.8 ± 9.5	21.2 ± 11.4	59.1 ± 4.6	10.6 ± 14.6	9.1 ± 4.6	43.2 ± 18.4	46.1 ± 17.9
C3	25.9 ± 3.6	33.3 ± 24.7	47.2 ± 17.5	54.7 ± 2.1	62.9 ± 7.6	51.2 ± 22.8	25.3 ± 17.1	37.9 ± 20.3	14.6 ± 12.4	30.2 ± 2.8	55.7 ± 3.1
	#13	**#14**	**#15**	**#16**	**#17**	**#18**	**#19**	**#20**	**#21**	**#22**	
C1	22.2 ± 22.5	31.8 ± 28.7	11.1 ± 7.3	14.3 ± 12.6	33.3 ± 35.9	70.8 ± 7.2	22.2 ± 5.5	28.6 ± 0.0	25.4 ± 24.0	28.6 ± 4.8	
C2	38.1 ± 5.7	32.1 ± 27.8	33.3 ± 11.6	48.2 ± 5.6	55.2 ± 18.9	70.5 ± 0.8	41.9 ± 7.3	44.1 ± 10.6	52.2 ± 10.6	55.9 ± 16.0	
C3	56.3 ± 5.5	65.9 ± 3.0	54.0 ± 9.7	43.6 ± 19.4	52.8 ± 14.1	74.5 ± 4.3	49.1 ± 4.3	61.3 ± 5.2	62.2 ± 7.9	65.4 ± 3.8	

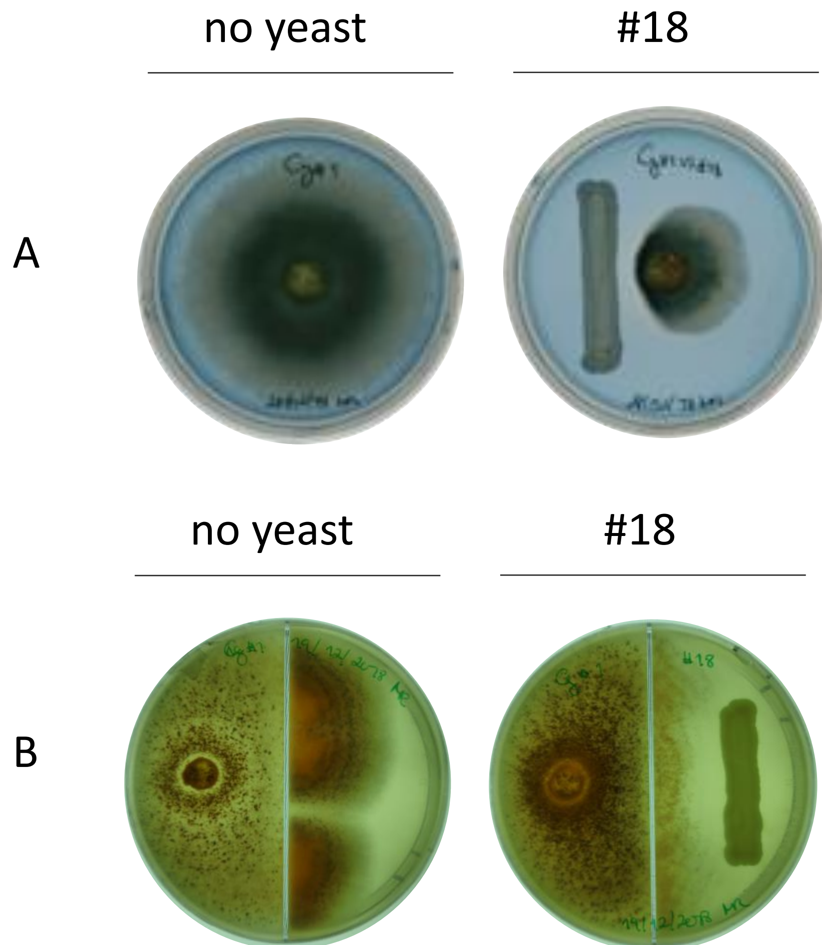

Figure 1. Yeast strain #18 against *Colletotrichum gloeosporioides* s.s. strain. Inhibitory response at a distance (A) and none in a septate petri dish (B).

Antagonism in liquid media

The antagonism assays were subsequently repeated in liquid medium. If a metabolite of some kind is produced and secreted by the yeasts that inhibits the fungal growth, its diffusion should be much more efficient in liquid medium and therefore might cause stronger inhibition. Moreover, the production of some antifungal metabolites is greater at the exponential growth (Lopes *et al.*, 2015), which is only possible in liquid cultures. Moreover, these also allow a greater nutrient availability promoted by the constant agitation, according to which yeasts should stay metabolically active for longer periods of time. Finally, the efficient antagonism in some cases can rely on physical contact between the inhibitor and the inhibited strains, as previously demonstrated with another phytopathogen (Ferraz *et al.*, submitted).

The three OA fungal strains were challenged with a metabolically active yeast inoculum and incubated for 4 days. Results of the antagonistic response using empirical scale with 0, 1, 2 levels of inhibition previously described (Ferraz *et al.*, submitted) are shown in Table 4, and represent the types of inhibition exemplified in Figure 2.

Table 4. Antagonism between the phytopathogenic fungal strains *Colletotrichum gloeosporioides* s.s. (C1), *C. godetiae* (C2) and *C. nymphaeae* (C3) and the yeast strains in Table 1 in liquid media. The numbers displayed translate results according to a previously established empirical scale (Ferraz *et al.*, submitted; 0 = no inhibition; 1 = weak inhibition;2 = full inhibition) and represent at least three independent replicates with identical result.

Phytopathogens	**Yeasts**										
	#1	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**CAT1**	**PE2**	**#11**	**#12**
C1	2	0	0	0	2	2	2	2	2	0	1
C2	2	1	0	0	2	2	2	2	2	0	1
C3	2	0	0	0	2	2	2	2	2	0	1
	#13	**#14**	**#15**	**#16**	**#17**	**#18**	**#19**	**#20**	**#21**	**#22**	
C1	2	1	2	0	1	2	2	2	2	2	
C2	2	0	2	1	2	2	2	2	2	2	
C3	2	1	2	0	1	2	2	2	2	2	

In general, as expected, the inhibitory response increased comparing to that obtained in solid media. In solid media, a strong inhibitory response (level 2 in Table 2; >70% in Table 3) was only observed in 6 out of 63 combinations, while in liquid medium, the fungi were strongly inhibited (level 2) in 39 out of 63 combinations. Results in Table 4 also show that 3 yeasts did not inhibit the growth of any of the fungal strains: #3, #4, and #11, and the #2 and #16 only weakly inhibited one of them. Based on these results, these yeasts were not further considered.

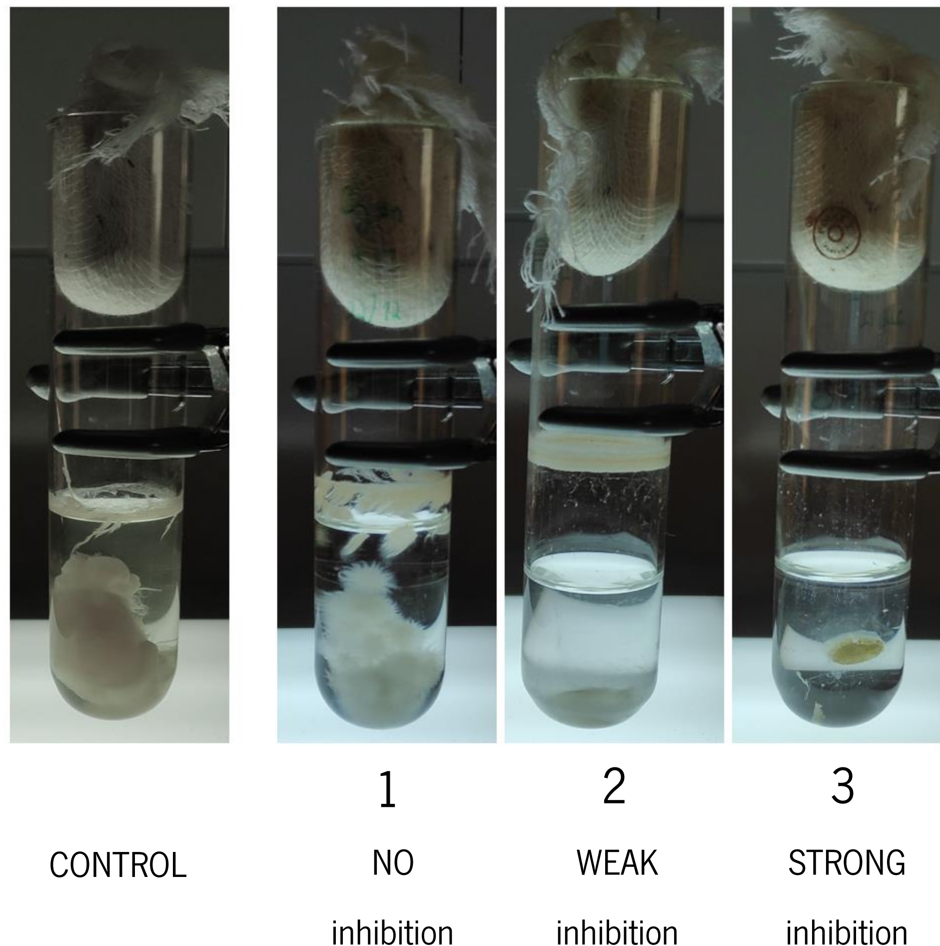

Figure 2. Liquid media antagonism assay against *Colletotrichum gloeosporioides* s.s. strain. Examples of the type of inhibitory response.

Exploratory assays of confirmation of the death of the fungal cells were conducted. The fungal inoculum plug, after the co-incubation in liquid medium with the strongest antagonizing yeast strains, was stained with Methylene Blue (MB) and Propidium Iodide (PI) (not shown). The first dye binds to negatively charged molecules when it is not reduced, thus staining dead cells, and the second only enters the cell when the plasma membrane is disrupted (Kwolek-Mirek and Zadrag-Tecza, 2014). In some combinations both mycelium and spores were well stained, while other combinations showed no or weak staining. These preliminary results suggest that some yeast strains could be killing the fungi, but in the combinations that were not stained, yeast strains are probably just inhibiting fungal proliferation, allowing these to retain viability. This exploratory microscopic inspection of all combinations did not reveal yeasts attaching to the hyphae as previously observed with another phytopathogen (Ferraz *et al.,* submitted).

CONCLUSIONS

All taken, results suggested that fungal growth inhibition might occur through the secretion of a diffusible compound by antagonizing yeasts. For this to happen, yeasts have to be metabolically active. Whether this compound causes the death of the fungi, or simply inhibits their replication, remains to be confirmed. Moreover, results also showed that yeast strain #18, a *W. anomalus* isolated from olive orchards in Portugal, has a strong ability to antagonize the three *Colletotrichum* sp. strains used in this work. These results agree with other studies proposing the potential of *W. anomalus* (formerly known as *Pichia anomala* or *Hansenula anomala*) as biocontrol agent (Friel *et al.*, 2007; Oro *et al.*, 2014; Parafati *et al.*, 2015). This species possesses other desirable characteristics, like the ability to grow under high osmotic pressure, at low pH, and at a broad range of temperatures (Fredlund *et al.*, 2002).

The fact that the best yeast candidate originates from the olive trees biome suggests that, better than using well-known yeasts for long captive of laboratory or industrial processes and accordingly domesticated, the exploitation of an appropriate niche biodiversity yields better strains, able to efficiently colonise the olive tree orchards. The niches where the natural wild yeast and the phytopathogens co-exist are most probably ideal. The results in this work suggested as much and can be considered a steppingstone for the formulation of a new eco-friendly and effective alternative for controlling OA. Further studies are required in order to fully understand the mechanisms by which these yeasts are able to antagonize the phytopathogenic fungi.

ACKNOWLEDGEMENTS

This work was supported by the strategic programme UID/BIA/04050/2013 (POCI-01-0145-FEDER-007569) funded by national funds through the FCT I.P. and by the ERDF through the COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI), and the project EcoAgriFood (NORTE-01-0145-FEDER-000009), supported by the Norte Portugal Regional Operational Programme (NORTE 2020) under the PORTUGAL 2020 Partnership Agreement through the European Regional Development Fund (ERDF). PF and MA-R are PhD students of the Doctoral Programmes in Applied and Environmental Microbiology (DP_AEM), (FCT grant PD/BD/113810/2015) and in Molecular and Environmental Biology (PDBMA), (FCT grant PD/BD/145354/2019), respectively. We thank the Brazilian companies, Cerlev, Lda., Ouro Preto, MG, and Fermentec, Lda. Soluções Tecnológicas e Industriais, Piracicaba, SP, as well as Professor João Paulo Sampaio from the Portuguese Yeast Culture Collection/UCIBIO, NOVA, Portugal for kindly supplying the yeast strains used in this work. We also thank Professor Pedro Talhinhas, from LEAF - Linking Landscape, Environment, Agriculture and Food, ISA, Universidade de Lisboa, Portugal, for kindly supplying two of the fungal strains used in this work.

SUPPLEMENTARY MATERIAL

Table S1. Growth rates of the phytopathogenic fungal strains *Colletotrichum gloeosporioides* s.s., *C. godetiae* and *C. nymphaeae* at 25 and 30 °C, in two culture media and at different pH values.

			Growth rate (mm.day $^{-1}$)				
			pH 4.0	**pH 4.5**	**pH 5.0**	**pH 5.5**	**pH 6.0**
30 ºC	***C. gloeosporioides***	PDA	3.90	4.43	5.31	5.13	5.86
		MEA	4.10	5.89	6.14	5.76	6.14
25 ºC	***C. gloeosporioides***	PDA	3.17	4.84	5.00	5.38	5.08
		MEA	4.06	4.80	5.56	5.89	5.76
	C. godetiae	PDA	1.88	2.77	1.77	2.68	2.56
		MEA	1.77	2.21	2.76	2.25	2.46
	C. nymphaeae	PDA	2.17	2.47	2.71	3.15	2.54
		MEA	2.41	2.39	3.18	2.90	2.64

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CHAPTER 5

Vinasse waste from sugarcane-based bioethanol production plants kills *Moniliophthora perniciosa*, the causative agent of cacao *Witches' Broom Disease*

The work presented in this chapter has been submitted for publication:

Ferraz, P., Amorim-Rodrigues, M., Cássio, F. and Lucas, C. *Vinasse* waste from sugarcane-based bioethanol production plants kills *Moniliophthora perniciosa,* the causative agent of cacao *Witches' Broom Disease*.

ABSTRACT

Bioethanol production based on sugarcane juice fermentation yields *vinasse*, a dark, dense liquid waste high in potassium, used for fertirrigation. *Vinasse* production largely exceeds this application, constituting a high environmental hazard, particularly in Brazil, where this long-used practice increasingly damages soils and superficial and ground waters. Alternatives are urgently needed. *Vinasses* from bioethanol plants from Brazil were tested for their ability to contain the proliferation of *Moniliophthora perniciosa*, responsible for cacao *Witches' Broom Disease*. Immersing or spraying the mycelium with *vinasse* either kills the fungus at varying time/dosage or impedes its proliferation. Two fungal strains presented identical results. Identically testing another phytopathogen showed this effect is not that of a generalized fungicide. The well-ordered utilization of *vinasse* for fertirrigation of cacao plantations could help containing/reverting the prevalence of *Witches' Broom Disease*, thus contributing to the sustainability of the bioethanol industry.

Keywords

Vinasse; bioethanol; cacao; *Witches Broom Disease*; *Moniliophthora perniciosa*

INTRODUCTION

Moniliophthora perniciosa is a filamentous fungus that causes a devastating disease in the cacao plant (*Theobroma cacao*) and fruit known as *Witches' Broom Disease* (Aime and Phillips-Mora, 2005). This was responsible for the steep production fall-out in South and Central America countries, mainly in Brazil, where it poses a serious social-economic crisis in the cacao producing region. The Brazilian production fell 70% in a period of 10 years after the onset of the disease in 1989 (Meinhardt *et al.,* 2008; Teixeira *et al.,* 2015). This scenario had a huge impact on the Brazilian economy, leading Brazil to shift from being the 2nd world producer to become a net importer of cacao beans (Marelli *et al.,* 2009; Teixeira *et al.,* 2015).

M. perniciosa is a very aggressive fungus, virtually impossible to eradicate. The exceptional virulence of the fungus is intrinsically related to its ability to infect all the pant tissues in all the stages of the cacao plant life-cycle (Meinhardt *et al.,* 2008; Pohlan and Pérez, 2010; Ferraz *et al.,* 2019). *M. perniciosa* is a hemibiotrophic fungus, which infectious cycle has two distinct phases: a biotrophic and a saprotrophic. After the initial infection, a series of cell death events occur in the infected tissues causing these to become necrotic and to form a particular structure called dry broom (Meinhardt *et al.,* 2008). Those necrotic or dead plant cells are then colonized by the fungus and pink-coloured basidiocarps are produced. In alternating periods of drought and humidity, each basidiocarp can produce 2 to 3.5 million spores (Almeida *et al.,* 1997). The spores are released mainly at night and are locally dispersed by water and over long distances by wind and can remain latent in the soil or inside pruned plant branches for long periods of time (Meinhardt *et al.,* 2008; Pohlan and Pérez, 2010).

Classical chemical fungicides, usually azole or copper-based compounds, are not effective against *M. perniciosa* (Medeiros *et al.,* 2010). Presently, cacao *Witches' Broom Disease* has one single functional management technique, which consists in spraying the affected plantations with Tricovab®, a *Trichoderma stromaticum* live suspension (reviewed by Ferraz *et al.,* 2019). This other fungus is very efficient in antagonizing *M. perniciosa*, but it has to be multiplied in rice grains surface, being supplied lyophilized, as a service on demand, by an agro-support governmental organization (www.ceplac.gov.br). This is therefore a very expensive and unsustainable solution, only possible since it is almost fully subsidised by the Brazilian government. Alternatives could consist in using other microbes, easier and cheaper to cultivate, as biofungicides (reviewed by Ferraz *et al.,* 2019), or possibly implementing a sustainability-prone solution controlling the fungus with an inexpensive agro-friendly industrial waste that is produced in very high amounts.

Brazil produces bioethanol mostly from yeast fermentation of sugarcane juice. The process involves the physical extraction of the juice from the cane which is then fermented by yeasts. After each fermentation cycle, the must is centrifuged, and the yeast biomass separated and recycled. The subsequent distillation process yields a dark liquid waste called *vinasse* (Carrilho et al., 2016). Brazilian industry generates an average 12 L of *vinasse* per litre of ethanol produced (Lopes *et al.,* 2016), meaning that each year the country generates around 300-400 billion litres of *vinasse* (Carrilho *et al.,* 2016). This residue has been used for more than 50 years mostly for fertirrigation (fertilization + irrigation) of the sugarcane fields (Carrilho *et al.,* 2016). This appears to be a virtuous cycle, since *vinasse* is rich in potassium (Naspolini *et al.*, 2017), avoiding the need for chemical fertilization (Prado *et al.,* 2016), which in the long run is beneficial for soil quality and sugarcane production yield (Naspolini *et al.*, 2017). But in reality, it configures a serious environmental problem since the amounts of *vinasse* produced are far bigger than the fields can absorb, leading to their saturation. Additionally, the *vinasse's* low pH (3.5 – 5.0) and the corrosive nature (Fuess *et al.*, 2017) are promoting undesirable changes of soil composition and physicochemical properties (Christofoletti *et al.,* 2013), and serious contamination of ground water (Botelho *et al.,* 2012). These problems are exacerbated by illicit discharges and inappropriate storage.

The most studied possibilities of *vinasse* utilization relate with its reuse in the energy equation of the ethanol plant (Naspolini *et al.*, 2017; Rodrigues Reis and Hu, 2017). Nevertheless, *vinasse* is rich in minerals and organic matter (Goldemberg *et al.,* 2008; Nitayarvardhana *et al.,* 2013; Carrilho *et al.,* 2016), around 100 – 130 g/L (COD) (Goldemberg *et al.,* 2008), and includes sugars, organic acids, ethanol and glycerol (Nitayarvardhana *et al.,* 2013; Carrilho *et al.,* 2016), which suggest *vinasse* should be fit for microbial growth. Studies proposing this possibility include the cultivation of economically promising filamentous fungi such as the edible *Rhizopus oligosporus* (Nitayarvardhana *et al.,* 2013), or the biotechnology relevant *Aspergilus oryzae* (Money, 2016; Gmoser *et al.,* 2018) and *Neurospora intermedia* (Nair and Taherzadeh, 2016). In the present work, we explored the possibility of using *vinasse* for controlling the proliferation of *M. perniciosa*.

Three different *vinasse*s were tested against two strains of the fungus originating from Brazil *via* a credited international culture collection. The fungi did not grow on *vinasse*. Instead, both died upon immersing or spraying with two of them, although at varying time/dosage, and were inhibited from proliferating by the third. One of the three tested *vinasses* inhibited the growth of the fungi. This killing effect of *vinasse* was not shared by another genetically distant phytopathogenic fungus suggesting specificity. Results suggest that using *vinasse* for fertirrigation of cacao plantations could, in time, clean the resident fungus inoculum. If not eradicating it, it could at least reduce the disease to manageable levels.

MATERIALS AND METHODS

Moniliophthora perniciosa strains CBS 441.80 and 442.80, and *Colletotrichum gloeosporioides* CBS 100471 were purchased from CBS-KNAW Collections, The Netherlands. They were maintained at 4 °C on MEA (20 g/L malt extract w/ 20 g/L agar), and cultivated in the same medium at 30 °C. *Vinasse* was obtained from Fermentec Lda (https://www.fermentec.com.br/), and originated from three different bioethanol producing plants in the State of São Paulo, Brazil: Usina Alta Mogiana (http://www.altamogiana.com.br/), Usina Batatais (http://www.usinabatatais.com.br/) and Usina da Pedra (https://www.pedraagroindustrial.com.br/). *Vinasse* was stored at 4 °C. Prior to utilization it was centrifuged for 30 min at 13.000 x g and 4°C to eliminate particles in suspension, and autoclaved (121 ºC, 1 atm, 20 min). Growth assays were performed inoculating 20 mL of *vinasse* in a glass tube (Ø 3 cm; 13 cm height) with a one-week ME-grown fungus agar plug of approximately 0.8x0.8 cm. Tubes were incubated at 30 ºC and 200 rpm orbital shaking. Cultures identically inoculated and incubated in liquid ME medium were used as control. Growth was assessed after 10 days, decanting the culture supernatant and checking for mycelium development. The putative viability of any remaining fungal cells was assessed incubating this agar plug in MEA at 30°C for 1 week. These assays were repeated in the same manner, using *vinasse* supplemented with 2% of malt extract as a supplementary nutrient source. Death along incubation time in *vinasse* was assessed using a fully-grown fungal culture previously grown in liquid ME medium in a glass tube (until the formation of a globular mycelia), which was then transferred to a glass tube containing 20 mL of *vinasse*, under the same conditions described above. Each sample/incubation time corresponding to an independent tube identically inoculated from fully-grown fungal cultures. The incubation period varied between 1 and 10 days (T1 to T10), and at increasing time points, the mycelia was taken from the *vinasse*, gently washed with sterile water and its viability assayed in MEA at 30°C for 1 week. The death-inducing effect without immersing the cells in *vinasse* was performed by spraying *vinasse* on a MEA plate containing fully grown mycelia. The assay was performed during 10 days, applying a single spray at T0, or repeating it once each 24h. The plates were then incubated at 30°C for 3 days. All assays were performed at least in three independent replicates ($n \geq 3$).

RESULTS AND DISCUSSION

The *vinasse* used in this work originated from three bioethanol plants from the State of São Paulo in Brazil, all of which identically producing bioethanol from a sugarcane juice fermentation process. According to the literature, the composition and physical properties of *vinasses* do not differ significantly (e.g. Goldemberg *et al.,* 2008; Nitayarvardhana *et al.,* 2013). The three *vinasses* used in this work have ±6 % solids in suspension (mostly ashes), pH ≈ 4.5, 0.115 ± 0.060 % (w/v) sugars and 0.9 % (w/v) organic acids (information kindly supplied by Fermentec, Lda (https://www.fermentec.com.br/), values that fall within published data.

The pH of *vinasse* is adequate to support the growth of *M. perniciosa* which optimal growth occurs at pH between 4.5 and 5.5 (our unpublished results), and the presence of carbon source (totalling ±0.5% (w/v)) should allow some fungal multiplication. Therefore, *vinasse* was centrifuged, to remove the solids in suspension, and the remaining liquid fraction was sterilized and used to inoculate the two *Moniliophthora perniciosa* strains. After 10 days (Figure 1A) there was no visible growth of either fungus.

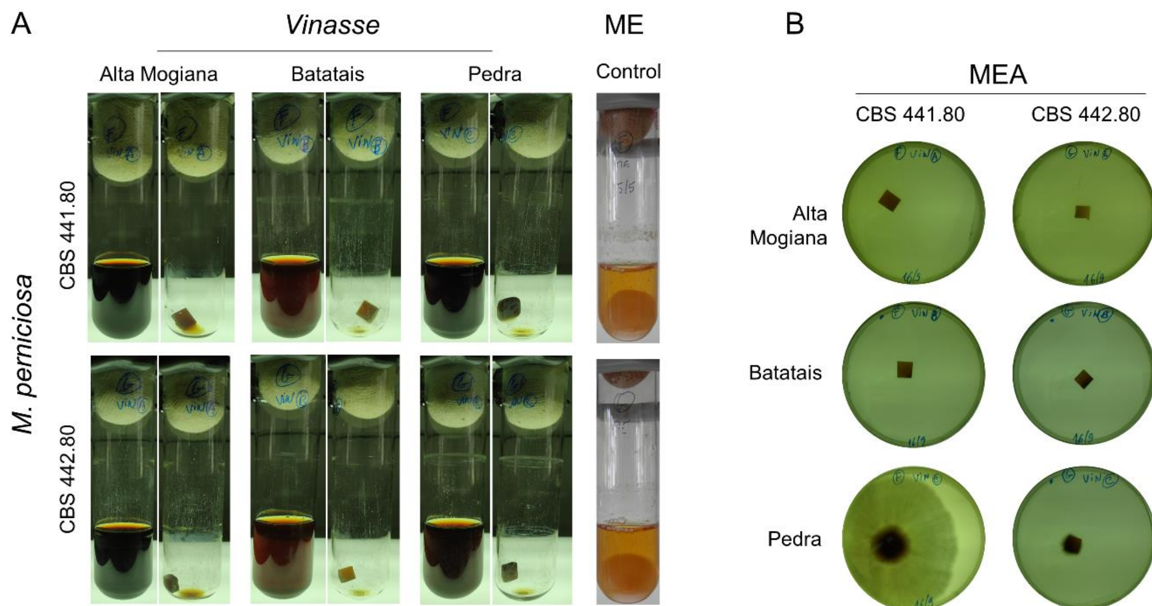
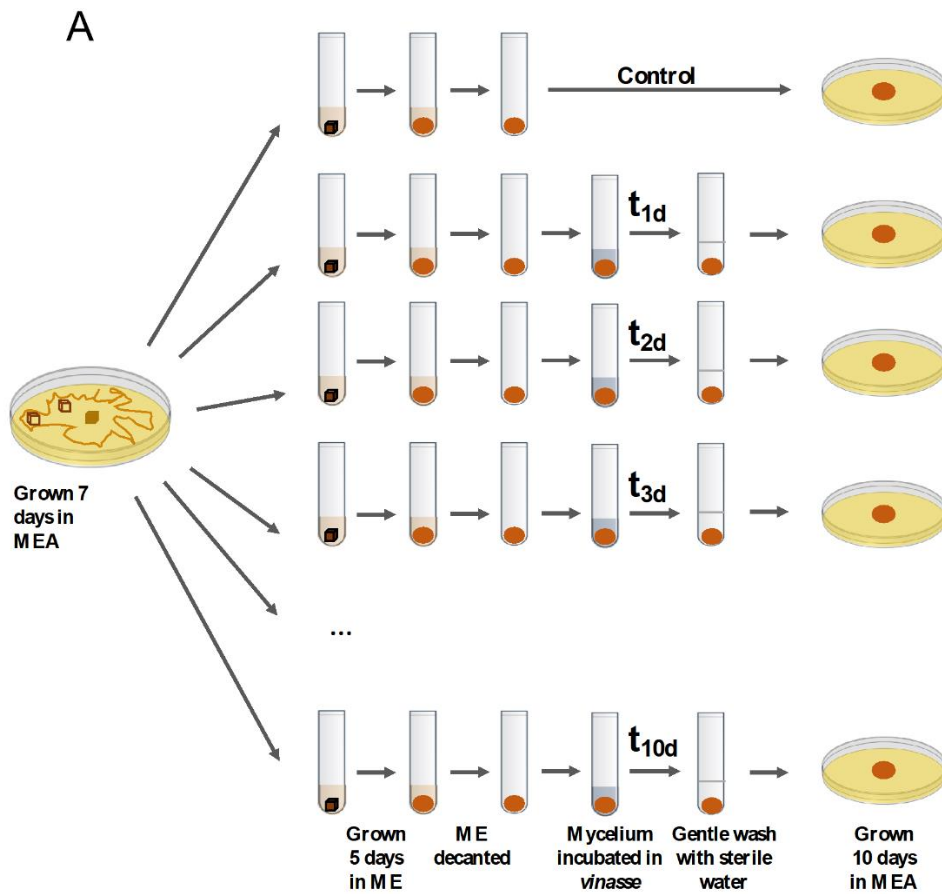


Figure 1. Two *M. perniciosa* strains originating from contaminated cacao fruits and trees in Brazil were grown in unsupplemented *vinasse* originating from three Brazilian bioethanol plants. (A) Results were scored after a 10 days incubation at 30°C, before and after decanting the *vinasse* showing the inoculum agar plug. Control growth in identical period and temperature in MEA showing the fungal biomass ball. (B) The plugs from (A) were inoculated in MEA solid medium. Only the plugs originating from the incubation in *vinasse* from Pedra showed mycelia growth.

Microscopic inspection confirmed the absence of hyphae associated with the agar plugs used as inocula. To check whether there were still residual viable mycelia, the fungal plugs were taken from the *vinasse* (Figure 1A), re-inoculated in solid MEA medium and incubated for a further 10 days at 30°C. As can be seen in Figure 1B, the plugs from the *vinasses* of Alta Mogiana and Batatais did not develop any growth, suggesting the fungus was killed. The fungal plug taken from the *vinasse* from Pedra developed mycelia, showing that it did not exert a killing effect, but only a strong inhibition of fungal growth.

Considering that the carbon source present in *vinasse* might not be sufficient to allow the fungal to develop generously, and/or that the death of mycelia could occur at such different speed that the 10 days contact with the *vinasse* would not be enough to detect death of the mycelia, two different assays were performed. The first consisted in increasing the amount of carbon source to support survival and growth in *vinasse* by adding malt extract 2% (w/v) and repeating the incubation in the same conditions used in the previous assay. Results were identical to the previous ones (not shown), indicating that the growth inhibition observed when inoculating the fungal plugs in *vinasse* is not related with nutrient deprivation. The second approach aimed to establish how fast *M. perniciosa* was dying on *vinasse*. The assay consisted in having ME-grown mycelia and replacing ME with vinasse (Figure 2A). At increasing time points, the mycelia were taken from the *vinasse* and their viability was assayed in solid MEA medium as before. This was done using the *vinasse* originating from Alta Mogiana plant, for having proved able to kill the two fungal strains. Results showed that the complete death of the mycelia from the two fungal strains indeed occurred when the fungi were immersed in *vinasse*, but at different times. The strain CBS 441.80 lost full viability between day 6 and day 7 (Figure 2B), while the strain CBS 442.80 lost full viability faster, between day 2 and day 3 (Figure 2B). *Vinasse* has thus a different time/dosage effect on the two strains of *M. perniciosa*, which must be considered for the putative application in the field.

In order to check whether the death-inducing effect could be obtained without the need to immerse the mycelium in *vinasse*, this was used to spray MEA fully grown mycelia. The spray was applied once every 24h for 10 days and the evolution of the mycelium was photographed. (Figure 3). This time the three *vinasses* were used. Mycelium progressively shrunk, becoming darker coloured. *Vinasses* from the different plants had different time/dosage effect. The *vinasse* from Batatais killed the two strains faster than the one from Alta Mogiana, and Pedra's presented the weaker inhibitory effect. Moreover, it was also possible to verify that each *vinasse* had a different time-effect on each of the *M. perniciosa* strains, being CBS 442.80 more sensitive since the mycelium disappeared between days 5 and 10 (except when using the Pedra plant *vinasse*).



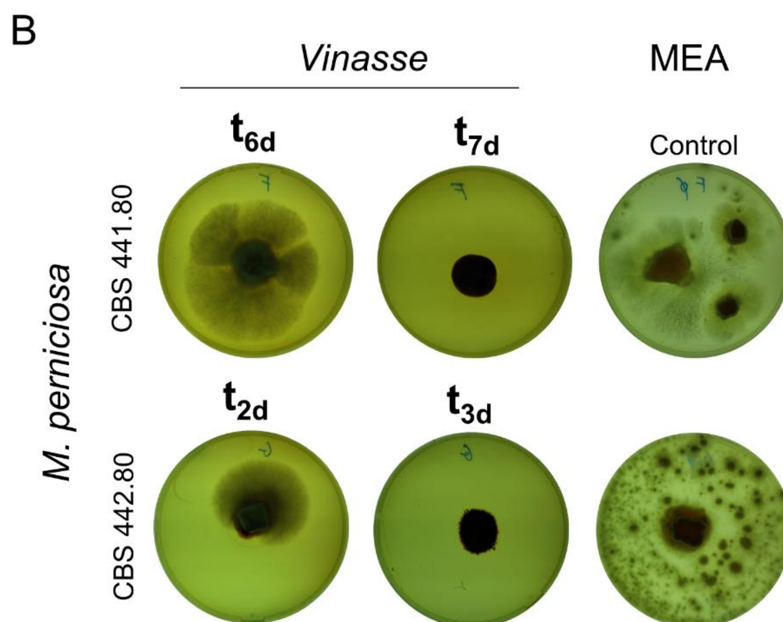


Figure 2. (A) Scheme of the procedure used to assay the effect of *vinasse* on the viability of the *M. perniciosa* along time. (B) Representative results of the turning point in the loss of viability which occurs at different time points for each fungal strain. The controls show the growth of the mycelium ball fragmented, which occurs whenever the inoculum is large.

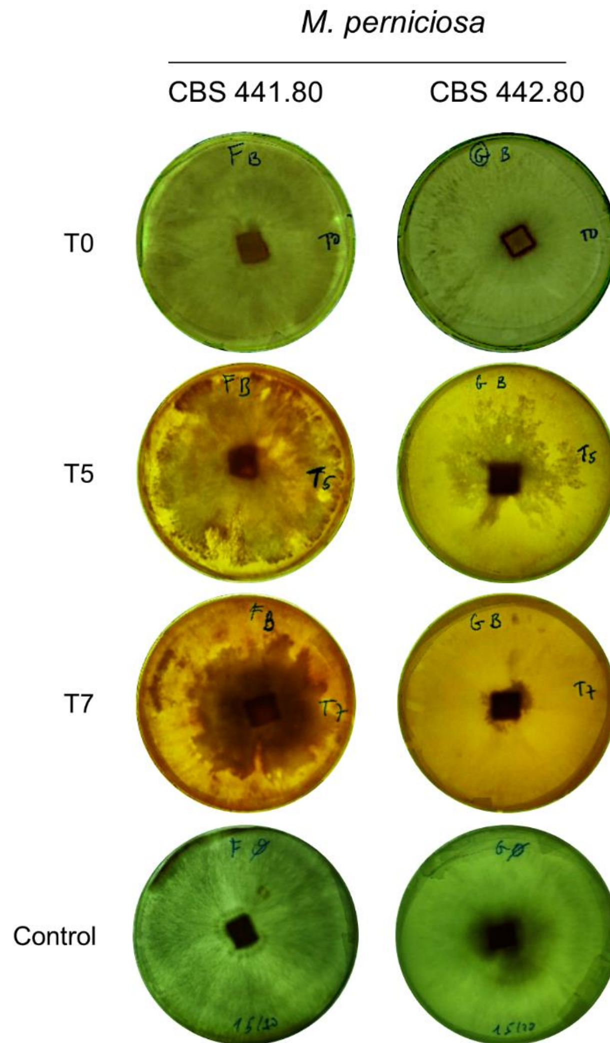


Figure 3. Chosen examples of the effect of spraying *vinasse* on the top of fully-grown mycelia on MEA once every 24h. Arrows indicate shrunk residual mycelium after 7 applications of *vinasse*.

These results are consistent with the ones obtained by immersing mycelium in *vinasse*. Results further indicate that the spraying has to be repeated over time in order to observe the desired effect, corroborating the hypothesis that the continuity of the treatment could contribute to clean the field of hidden mycelium over time.

Finally, as an attempt to understand whether the ability of *vinasse* to kill filamentous fungi was specific for *M. perniciosa* and/or the correspondent taxa, the assays of fungal growth in *vinasse* for 10 days were repeated using another phytopathogenic fungus, *Colletotrichum gloeosporioides* (Weir et al., 2012). This is one of the causative agents of olive anthracnose, a disease causing serious economic losses in the Mediterranean region (Talhinhas et al., 2018) and it was chosen because it is a very resilient fungal

species. Results (Figure 4) showed that *C. gloeosporioides* was able to grow without the need for nutrient supplementation, fully occupying the volume of *vinasse* with mycelium which thus became altogether a hard-solid mass. These results indicate that the ability of *vinasse* to kill *M. perniciosa* does not correspond to a generalized antifungal activity, and therefore depends on some specific trait of *M. perniciosa* biology.

Vinasse chemical composition is very complex (Rodrigues Reis and Hu, 2017). Future approaches might allow the identification of the compounds or chemical groups in *vinasse* that are active against *M. perniciosa*, eventually enabling the creation of an engineered simpler and more efficient solution which considers the environmental effects in the long run. Moreover, the evaluation of the potential spectrum of *vinasse* application as a specific fungicide will have to be addressed experimentally. In particular, there is one possibility that stands out, which is that *vinasse* might affect the proliferation of *M. roreri* (Barbosa *et al.,* 2018), another phytopathogenic filamentous fungus genetically very close to *M. perniciosa,* also causing a severe and economically threatening disease in cacao fruits known as *moniliasis* or frosty pod rot (Bailey *et al.,* 2018).

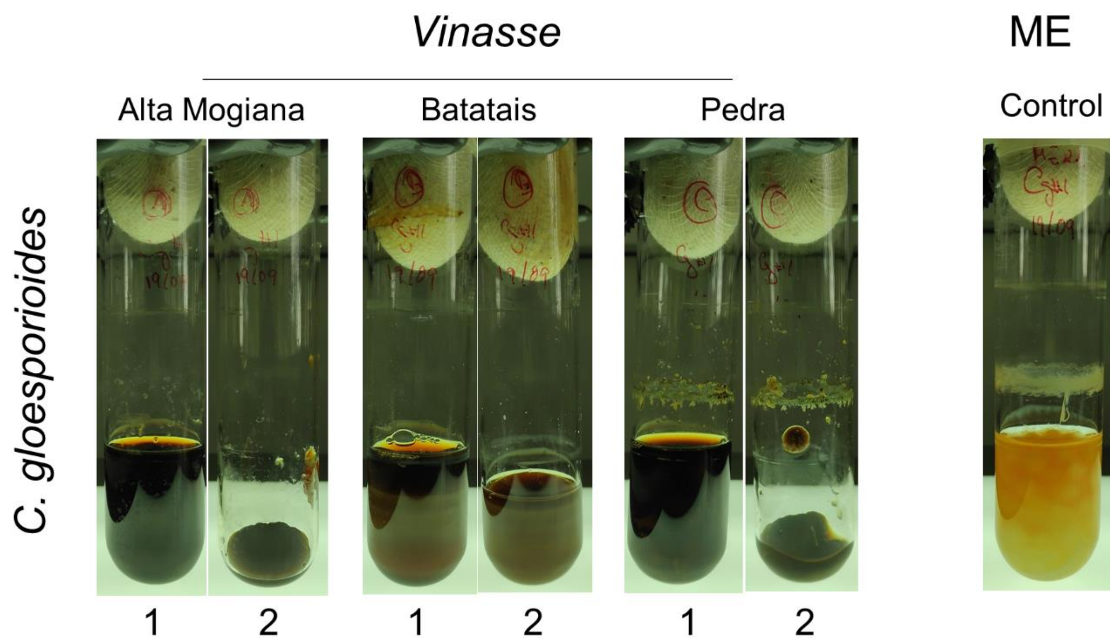


Figure 4. *C. gloeosporioides* grown in unsupplemented *vinasse* originating from three different bioethanol plants from Brazil. Inoculum was done using a fully-grown mycelia agar plug as used for *M. perniciosa*. Results were scored after a 10 days incubation at 30°C (1), and after decanting the remaining *vinasse* showing the fungal mycelium that was formed (2). Control growth was performed in MEA in identical period and temperature. *C. gloeosporioides* did not form a mycelium ball, instead it spread eventually filling the full liquid volume available.

CONCLUSIONS

Vinasse is the main waste product from sugarcane bioethanol production process. It has a very high fertilization ability, but if applied in excess it becomes a critical pollution problem for soils and groundwater. Many solutions for *vinasse* disposal have been suggested in literature, mainly biotechnological applications, including the growth of some economically interesting microorganisms or aerobic/anaerobic digestion. Additionally, many suggestions in the literature regard solutions that promote the reduction of the amount of *vinasse* produced per litre of ethanol, its concentration, clearing or neutralization. For now, the most cost-effective application for *vinasse* keeps being fertirrigation, in time improving soil quality and crop productivity, provided it is done carefully and responsibly, guaranteeing the control over all the environmental implications.

Results clearly show the potential of *vinasse* to control the filamentous fungus *M. perniciosa*, cacao *Witches' Broom Disease* causative agent. Considering the huge amounts of *vinasse* produced in Brazil every year, and the fact that this country was the most affected by the disease, the utilization of *vinasse* for its containment is an expressive possibility. There may be logistic and cost problems hindering the utilization of *vinasse* in the short term deriving from the distances separating the bioethanol plants from the cacao fields, associated with this waste physicochemical properties, in particular its pH and corrosive nature. Nevertheless, these problems should in time be technically overcome in view of the advantage of contributing to solve two serious and urgent problems with a single procedure: introducing a new alternative for the disposal of high amounts of *vinasse*, reducing the serious danger to the environment and groundwater of unlawful discharges and precarious storage, and the containment of cacao *Witches' Broom Disease*. Together, the procedure should impact positively in the overall economical equation and contribute significantly to the socio-economic recovery of the regions of Brazil that most suffered with the crisis caused by cacao production fall-out in the last decades.

ACKNOWLEDGEMENTS

Funding: This work was supported by the strategic programme UID/BIA/04050/2013 (POCI-01-0145-FEDER-007569) funded by national funds through the FCT I.P. and by the ERDF through the COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI), and the project EcoAgriFood (NORTE-01-0145-FEDER-000009) from Norte Portugal Regional Operational Programme (NORTE 2020) under the PORTUGAL 2020 Partnership Agreement through the European Regional Development Fund (ERDF). PF and MA-R are PhD students of the Doctoral Programmes in Applied and Environmental Microbiology (DP_AEM), (FCT grant PD/BD/113810/2015) and in Molecular and Environmental Biology (PDBMA), (FCT grant PD/BD/145354/2019), respectively.

The images of *Witches' Broom Disease* and cacao tree and fruit used in the Graphical Abstract were obtained at https://www.forestryimages.org/: credit by Scott Bauer, USDA Agricultural Research Service, Bugwood.org, licensed under a Creative Commons Attribution 3.0 License.

SUPPLEMENTARY MATERIAL

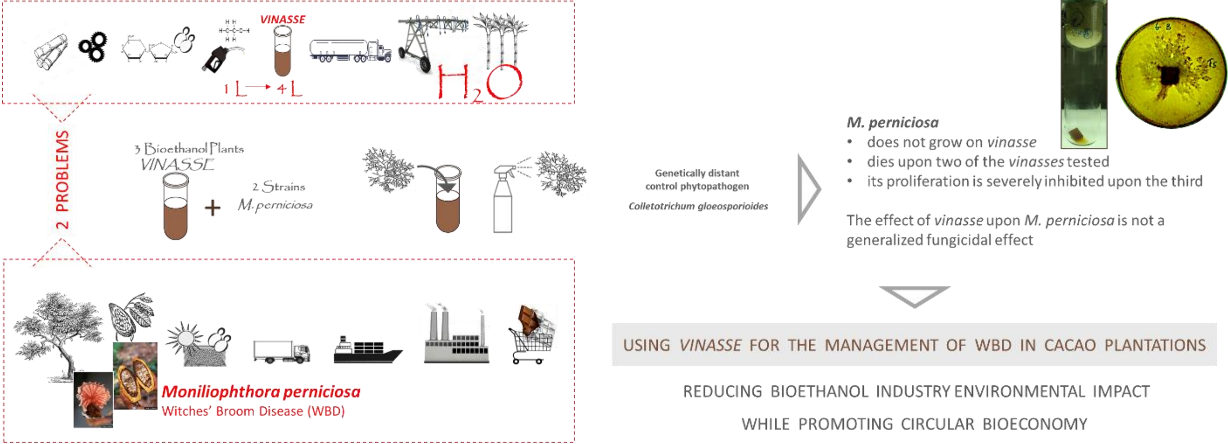


Figure S1. Graphical abstract.

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CHAPTER 6

Conclusions and Future Perspectives

CONCLUSIONS AND FUTURE PERSPECTIVES

The socio-economic impact associated with plant diseases is undeniable. In fact, 10% of the global food production is lost to plant diseases, with annual losses of up to 40 billion dollars been estimated to occur worldwide (Strange and Scott, 2005; Syed Ab Rahman *et al.*, 2018). These diseases are caused by several groups of pathogens, including other parasitic plants, viruses, bacteria, nematodes, and most importantly fungi. Fungal pathogens represent a huge threat to agricultural production, since they account for about two-thirds of annual losses (Fisher *et al.*, 2018). To overcome the harmful effects of fungal phytopathogenic agents, several methods of disease management and treatment have been implemented, and the use of chemical fungicides has prevailed during the last century. Despite having helped to solve this problem relatively effectively, and having brought clear improvements in crop quality and quantity, the rampant application of agrochemicals has led to the appearance of new and equally serious problems, namely the development of resistance to fungicides (Syed Ab Rahman *et al.*, 2018) and, in the absence of other control methods, to the re-emergence of virulence (Fisher *et al.*, 2018). Moreover, the uncontrolled use of fungicides has led to the accumulation of chemical residues in the environment and in the food products, proportionating low dosage toxicity and contaminating species across trophic levels (Dukare *et al.*, 2018). Although being neglected for a long time, the problem of fungicide overuse has been gaining more attention in recent years. This is intimately related with increasing awareness of the general public regarding the prevalence of the aforementioned problems, which has led the European authorities to increasingly impose restrictions on the employment of chemical fungicides in agroecosystems (Droby, 2006; Verweij *et al.*, 2009; Nunes, 2012). This situation generated a new market niche for alternative eco-friendly, more sustainable and effective methods to control phytopathologies.

An alternative could be the use of natural antagonists such as microorganisms. Examples of antagonistic organisms with potential to be used against many diseases are yeasts, which can antagonize a fungal phytopathogen through a variety of mechanisms of action, including the competition for nutrients and space and the secretion of antifungal compounds such as killer toxins, hydrolytic enzymes and volatile compounds (reviewed by Ferraz et al., 2019). Although some examples of antagonistic yeasts have been reported in the literature, none of the yeasts identified so far as inhibitors of mycelium formation have been applied in the field to control or prevent fungal plagues.

Taking into account all the potential that this type of alternative may have, this thesis aimed to evaluate the capacity of a selected group of yeast strains to antagonize the fungal causal agents of cacao *Witches' Broom Disease* (WBD) and Olive Anthracnose (OA). The first is caused by the basidiomycete fungus *Moniliophthora perniciosa* and severely affects the cacao plant and fruit production, leading to a major socio-economical problem to the tropical countries in Central and South America (Aime and Phillips-Mora, 2005). OA is caused by *Colletotrichum gloeosporioides* and *C. acutatum* species complexes, affecting olive production in the Mediterranean countries, progressively threatening the regional economy (Talhinhas *et al.*, 2011; Cacciola *et al.*, 2012). Despite being caused by pathogens from different classes, both diseases share a lack of effective methods for their treatments, mostly carried out using agrochemicals and other agronomic methods that besides being economically not viable and not sustainable, are ineffective in solving the problem (Droby, 2006; Marelli *et al.*, 2009; Cacciola *et al.*, 2012).

A group of yeasts, isolated from the spontaneous fermentations used for the production of *cachaça* and from the industrial process of sugarcane juice fermentation for bioethanol production, were used to test their ability to antagonize the WBD causal agent. From these, *Wickerhamomyces anomalus* #1105 and *Saccharomyces cerevisiae* #1112 from spontaneous fermentations of *cachaça* as well as the *S. cerevisiae* PE2 strain of from the industrial production of bioethanol were revealed as the best candidates to be used in the biocontrol of WBD. These 3 strains efficiently antagonize 6 strains of *M. perniciosa* originating from cacao plantations in different countries, and maintained their antagonistic ability when challenged at three different temperatures (16, 25 and 30 °C), under starvation, at different culture stages, or growing old. The results obtained clearly showed that the antagonism effect corresponds to actual fungal cell death, since in all the strong antagonism combinations observed, fungal mycelia were fully stained with methylene blue and propidium iodide, two well-known markers of cell death. Interestingly, microscopy analysis of the co-culture showed that isolated or clustered yeast cells appear to attach the killed fungal hyphae, suggesting that the contact between antagonistic yeasts and the fungal pathogen is needed to trigger the antagonistic effect.

The findings of this work might be the initial step towards the formulation of a new greener, effective and sustainable alternative for controlling WBD, which may be based on the application of live yeast, not requiring the purification of a specific antifungal compound. The industrial fermentative nature of these yeast isolates reinforces the potential of their application. The production of *cachaça* and other spirit drinks is a generalized cultural habit throughout Central and South America countries. The traditionally

craft fermentations performed by the small cacao producers might supply wild yeasts with a potential ability to locally counteract WBD at some extent. This could ultimately contribute to the sustainability of the process and have an important socio-economic impact in the small cacao producers in economically fragile and remote regions, who have less or no access to commercialized chemical fungicides or other pest management compounds.

In addition, to elucidate the capacity of some industrial yeasts used to antagonize the WBD causal agent, this part of the work raised some intriguing questions related to the observation of contact and attachment of yeasts with the fungal cells. Although there is a wide range of antagonism mechanisms, only some forms of mycoparasitism require the physical contact between antagonist and pathogen. This is the case of two groups of mycoparasites, the contact necrotrophs (Mims *et al.*, 2007) and the invasive necrotrophs (Junker *et al.*, 2019). To further investigate the morphological characteristics of this yeast/fungal interaction observed, the above-mentioned antagonistic assays were repeated and analyzed by Scanning Electron Microscopy (SEM). The results allowed the visualization of some unexpected morphological structures. Firstly, the antagonist yeast cell binding to the dead fungal cells was confirmed. Surprisingly, a phenomenon of yeast cell fusing to the dead hyphae of the phytopathogen was revealed. By attaching to the hyphae, yeasts cause a constriction and deformation of the fungal cells, ultimately leading to the draining of mycelia cellular content in areas nearby the yeast attachment local. Moreover, SEM analysis showed the formation of a veil-like structure that could be related with a possible invasion of the fungal cells. Remarkably, the occurrence of a physical tube-like connection between yeast and fungal cells, an inter-species connection which has not been previously described involving yeasts. This structure could be related to a process of yeast/fungus mutual recognition, but can also be associated with yeast predacious behavior, being the initial stage of the possible invasion of the pathogen by a predacious yeast. Finally, SEM observations revealed the existence of fimbriae-like connections between yeast cells, which can be related to a yeast cell-cell communication mechanism. Although this type of yeast-to-yeast connections has been reported in the case of biofilm formation (Mamvura *et al.*, 2017) or in starved cells (Varon and Choder, 2000), there are no detailed descriptions on their mechanisms of formation or on their structure and composition. The only yeast species described as a necrotrophic mycoparasite belong to the *Saccharomycopsis* clade (Lachance and Pang, 1997). This work proposes for the first time a possible predacious-like behavior in non-*Saccharomycopsis* yeasts. Moreover, the possible predacious nature of the fermentative yeasts could explain their remarkable resilience to harsh fermentation environments and their prevalence over other species in their natural or industrial habitats. However, the predacious behavior of these strains may not be independent of the production and secretion of antifungal

compounds, which can act in a synergistic manner and be the basis of the antagonistic effect observed. Although this SEM assessment has been essential to reveal the occurrence of this type of structures, future analysis by Transmission Electron Microscopy (TEM) and by fluorescence microscopy, staining the cytoskeleton with a specific fluorescent dye, will allow the elucidation of the cytology features underling the antagonism observed and contribute to clarify the yeasts mode of action.

The antagonism assays not only revealed that physical contact between yeasts and fungal cells was involved in mycelium death, but also showed that the inhibition of fungal proliferation at a distance occurred most likely through a non-volatile solid medium diffusible compound. This prompted the exploratory assessment of the peptides putatively secreted by the antagonizing yeast when in contact with *M. perniciosa*. To assess these, the media resulting from the co-culture of yeasts and fungal strains were analyzed by SDS-PAGE followed by mass spectrometry identification. The results revealed that yeasts in contact and in the absence of the fungi secrete different proteins in apparently different concentrations. Moreover, this preliminary assessment indicates that fungal strains in co-culture secrete different proteins compared to those secreted in single cultures and that different fungal strains secrete different peptides, suggesting physiologic/genetic variability. Although these results need to be confirmed, they indicate that in addition to the contact between yeasts and fungal pathogens, which can be involved in a potential predatory behavior, yeast antagonism may also engage the secretion of protein compounds which may be or not directly involved in the mycelium death.

As a supplementary output of the above work, a simple, optimized and easy-to-implement protocol for the evaluation of yeast antagonism against phytopathogenic fungi was developed. The characteristics of the protocol make it suitable to be applied in the assessment of the potential of a wide range of yeast strains against the fungal causal agents of different phytopathogenic diseases. This was used to assay the antagonism of yeasts against the fungi causing OA. The same set of industrial yeast strains from the spontaneous fermentations for production of *cachaça* and from the industrial production of bioethanol production previously used was tested against strains of *C. gloeosporioides* s.s., *C. nymphaeae* and *C. godetiae* originating from Portugal and Italy. Interestingly, neither of these fermentative yeasts strongly inhibited the fungal growth in solid media although the inhibitory effect increased considerably in liquid media. The antagonism assays were expanded using yeast strains originating from the olive biome of Portuguese orchards. Results revealed that *Wickerhamomyces anomalus* PYCC 7203 strain isolated from olive orchards in Portugal, has the strongest ability to antagonize the three *Colletotrichum* sp. strains used in this work. Exploratory assessments of the fungal cell death through microscopic analysis of the

yeast/fungi co-culture indicated that this yeast strain causes fungal cell death and that yeast cells do not appear to attach to the fungal mycelium. Accordingly, fungal growth inhibition was shown to occur via non-volatile medium-diffused compound(s). The fact that this yeast strain originates from the olive biome and the above strongest WBD antagonists originate from regional industrial ecosystems call the attention to the value of explore the microbial biodiversity to find effective antagonists. Moreover, this raises expectations as to their regional application not facing too severe regulation constraints.

Finally, in the present work, an alternative was tested for the control of WBD, which consisted in the use of *vinasse*, the dark, dense liquid waste resulting from the production of bioethanol based on the fermentation of sugarcane juice (Carrilho *et al.*, 2016) as an inhibitor of the growth of the phytopathogens. This strategy was based on that *vinasse* is presently produced in far excess in relation to its utilization in sugarcane fields fertirrigation, configurating a serious environmental hazard for soil and for ground and superficial water in Brazil (Carrilho *et al.*, 2016), and considering that WBD is also a serious problem in adjacent regional areas. Three different lots of *vinasse* were assessed against two strains of *M. perniciosa* originating from Brazil. Remarkably, both fungal strains did not grow on *vinasse*. Additionally, both died after being immersed or sprayed with two of the lots used, and were inhibited from developing by the third. The killing effect of *vinasse* on *M. perniciosa* was not observed using *C. gloeosporioides* s.s., a fungal causal agent of OA, a *M. perniciosa* genetically distant phytopathogenic fungus, which suggests the effect of *vinasse* is specific. *Vinasse* could thus be used for a controlled fertirrigation of the cacao plantations, contributing in the long run to clean the resident inoculum of *M. perniciosa*., helping to reduce WBD to manageable levels. Despite the probable logistic and economic problems that the utilization of *vinasse* may rise, technical investment in such a solution could contribute to solve two urgent problems with a single approach, channeling at least a part of the annual surplus of *vinasse* by contributing to cope with the cacao production problem.

As a whole, the results present in this thesis support the possibility of exploring new more eco-friendly and sustainable alternative strategies to assess fungal diseases, namely WBD of cacao and OA, this way contributing to ease the associated socio-economic implications that these phytopathogenic diseases cause. These new methods could be based on the use of potential fungicidal effect of industrial wastes like *vinasse*, or better, on the use natural microbial biocides, such as the antagonistic yeasts described in these studies. This last option also has in its favor the almost infinite microbial biodiversity available to constantly supply the search for a suitable yeast for different and emerging phytopathogenic diseases. The protocol developed within this thesis is readily applicable in the survey of the antagonism ability of

such microbes. All considering, it will be important in the future to assess how broad is these yeasts spectrum of action, to elucidate their mode of action, and to evaluate the impact of their introduction in the agroecosystems and associated ground and superficial waters. The immediate next steps needed to support the use of the most promising industrial strains reported in this work for WBD biocontrol should be the *in vivo* and *in field* assessment of the antagonistic ability. Additionally, the evaluation of the ability of the promising yeast originating from the olive biome to actual kill the fungal causative agents of OA should validate these findings and constitute an alternative to manage this plant disease.

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SUPPLEMENTARY MATERIAL

IDENTIFICATION OF THE PROTEINS SECRETED BY YEASTS AND PHYTOPATHOGENIC FUNGI IN CO-CULTURE CONDITIONS

The results described in Chapter 2 showed that some yeasts are able to kill *Moniliophthora perniciosa*, the filamentous fungus causing the cacao *Witches' Broom Disease*. Those assays showed that (i) the death of the fungus occurs when it is co-cultured with these yeasts in liquid medium; (ii) in solid medium there is rather the inhibition of growth at a distance, which requires the secretion of a medium-diffused compound; and (iii) the yeasts that kill the fungus have also been described as killer strains therefore secreting a killer toxin. It is thus probable that these yeasts secrete a peptide in response to the presence of the fungal pathogen, and that this might be responsible for killing the fungus, either alone or in combination with other mechanisms of antagonism.

To perform an exploratory assessment of the putative peptides that the antagonistic yeasts secrete when in contact with the *M. perniciosa*, the media resulting from the co-culture of yeasts and fungal strains were analyzed by SDS-PAGE. Two assays were made, using the supernatants of co-cultures from the combinations of the *M. perniciosa* CBS 441.80 with the *Saccharomyces cerevisiae* #1038 and CAT-1-FT280L, and of the *M. perniciosa* CBS 442.80 with the *Meyerozyma guilliermondii* #1015 and *Wickerhamomyces anomalus* #1105. As controls, fungal and yeasts strains were cultivated alone. Yeasts for this purpose were cultivated for 24h.

All cultures and co-cultures were performed as described in Chapter 2. These were centrifuged at 6500 x g for 10 min at 4 °C, and the resulting supernatant was freeze-dried. These samples were subsequently rehydrated in 1 mL of ultrapure water, and proteins within were precipitated with 10% v/v TCA, incubated at 4 °C for 10 min and then centrifuged at 18000 x g for 10 min at 4 °C. Pellets were resuspended in 50 µL of loading buffer (10% w/v SDS, 10 mM β-mercapto-ethanol, 20% v/v glycerol, 0.2 M Tris-HCl pH 6.8, 0.05% w/v bromophenol blue) and stored at -20 °C. Prior to the running of the SDS-PAGE, samples were boiled for 10 min at 95 °C. 10% and 8-16% gradient polyacrylamide gels were run at a constant current flow of 15 mA for approximately 2 h, and stained with colloidal Coomassie Blue or silver as previously described (Yan *et al.*, 2000; Dyballa and Metzger, 2009). The resulting gels (not shown) revealed that yeasts in the presence and in the absence of the fungal strains apparently secrete different proteins in what seems different concentration. In a similar way, some of the proteins secreted by the *M. perniciosa* strains when cultured alone are apparently inhibited by the presence of yeast antagonist. Importantly, also the *M. perniciosa* strains did not yield the same SDS-PAGE bands suggesting that they differ physiologically and possibly also genetically. Selected bands were excised from the gel and sent for

identification by Peptide Mass Fingerprinting (PMF) and fragmentation at the Unit of Proteomics from the Complutense University of Madrid. The excised bands positively identified are shown in Table 1.

Table 1. Proteins from yeasts/fungal strains co-cultures and control fungal single culture. SDS-PAGE bands were analyzed by PMF and fragmentation. The respective score, coverage, protein mass and NCBI accession number are presented.

		Protein	Protein Mass (KDa)	Score	Coverage (%)	Accession number
M. perniciosa CBS 441.80	Without yeast	Hypothetical protein MPER_14396	16	126	26	gi|238571636
	S. cerevisiae #1038	Rps0b	28	129	21	gi|6323077
		Rps8a	22	84	23	gi|6319399
		Rps19b	16	93	42	gi|6324027
	S. cerevisiae CAT1- FT280L	Scw4	40	130	17	gi|323304771
		Eno2	47	230	32	gi|6321968
		Tdh3	35	218	61	gi|6321631
M. perniciosa CBS 442.80	Without yeast	Hypothetical protein MPER_05184	24	238	46	gi|238599370
		Hypothetical protein MPER_05065	9	153	19	gi|238599752
	M. guilliermondii #1015	Nucleoside diphosphate kinase	17	143	31	gi|146423220
	W. anomalus #1112	Hypothetical protein MPER_13064	41	213	43	gi|238576275

An interesting result is that of the protein MPER_14396 that is secreted by *M. perniciosa* CBS 441.80 when cultured alone and which secretion is apparently inhibited by the presence of the yeasts. The designation MPER corresponds to *M. perniciosa*. The *M. perniciosa* genome has been sequenced (Mondego *et al.*, 2008; Barbosa *et al.*, 2018) but is not yet publicly available. Therefore, the only possibility

to identify a peptide from this fungus is by searching the annotated genome of the closest relative species, *M. roreri.* MPER_14396 is a protein with 98% identity to the central part of choline dehydrogenase from *M. roreri*. This enzyme, although not very well characterized in fungi, is known to be involved in the biosynthesis of the osmoprotectant betaine, produced by plants, animals and many bacteria in conditions of drought, high salinity or high temperature (Waditee *et al.*, 2003).

Noticeably, all the proteins identified in relation to *S. cerevisiae* #1038 are ribosomal proteins. These are not reported in the literature as regularly secreted proteins, although they were at least once previously identified in *S. cerevisiae* secreted proteome (Faria-Oliveira *et al.*, 2014). There is thus no information relating to any putative non-ribosomal activity of these proteins.

In the case of the yeast CAT1- FT280L, the bands identified correspond to Scw4, Eno2, and Tdh3 proteins, all of which well-known *S. cerevisiae* secreted proteins. Scw4 is a glucan-binding cell wall glucanase involved in survival during stationary phase and cell size regulation (Grbavac *et al.,* 2017). The full extent of this protein function is though not available. It has a paralog SCW10 that is putatively implicated in the regulation of a transcription factor activated by signaling pathways involved in pseudohyphal/invasive growth and mating (Zeitlinger *et al.*, 2003). Eno2 and Tdh3 are glycolytic/gluconeogenic enzymes, enolase II and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) isoform 3, respectively. These are well-recognized moonlighters, *i.e.,* they belong to a special class of multifunctional proteins that can perform different functions in different locations, including outside of the cell (reviewed by Gancedo *et al.*, 2016). Although there is no information about Eno2 alternative functions in yeasts, in mammalian cells α-enolase binds plasminogen, promoting its cleavage to plasmin, which activates metalloproteases that degrade the extracellular matrix being consequently involved in tissue invasion, inflammation and cancer progression (Díaz-Ramos *et al.*, 2012). On the other hand, in what concerns Tdh3, *S. cerevisiae* has been described to secrete a natural biocide (saccharomycin) which is composed of two main anionic peptides derived from the cleavage of GAPDH, AMP1 and AMP2/3 (Branco *et al.*, 2014). These cause the death of several non-*Saccharomyces* yeasts by perturbing plasma membrane permeability, by increasing the influx of protons and inhibiting their efflux, thus leading to a drop of the intracellular pH (Branco *et al.*, 2018). In a recent study, the same authors observed that the GAPDH-derived AMPs secreted by *S. cerevisiae* accumulates on the cell surface, inducing death of a competitor yeast species by direct cell-to-cell contact (Branco *et al.*, 2017). Apart from these works, nothing is described in the literature regarding the involvement of yeast Tdh3 or any of the other yeast proteins in Table 1 in the antagonism of filamentous fungi, although the filamentous form of the yeast human commensal and pathogen *Candida albicans*, was reported to be killed by the GAPDH N2-32 amino-acids peptide (Wagener *et al.*, 2013).

A second assessment was performed using *M. perniciosa* CBS 442.80 combined with the yeasts *Meyerozyma guilliermondii* #1015, *Wickerhamomyces anomalus* #1105, and *Saccharomyces cerevisiae* #1112 and PE-2- FT134L. These were the combinations in which the yeast strains exerted the strongest antagonistic effect (Chapter 2). The supernatants of the fungus and the yeasts growing alone were used as controls. In this case, the bands of interest from combinations with yeast strains *W. anomalus* #1105 and *S. cerevisiae* PE-2- FT134L, were not positively identified, possibly due to the low concentration of the proteins in the samples and/or the small size of the proteins, even though a more concentrated gel was used for better separation of low molecular weight proteins. The successfully identified proteins for the *M. perniciosa* CBS 442.80 combinations are also shown in Table 1.

The above-mentioned *M. perniciosa* CBS 441.80 fungal band that apparently disappears in the presence of the yeasts does not coincide with the ones identified for *M. perniciosa* CBS 442.80. This fungal strain secretes two distinct MPER proteins that are also absent from the co-culture supernatants, but lack recognizable conserved domains, turning their identification impossible. On the other hand, another MPER protein was found in the yeast /fungus combination using *W. anomalus* #1105. In opposition to the above MPER proteins, this was found in the co-culture but not in the culture of the fungus alone. This MPER_13064 has 88% identity to a glucose oxidase (GOD) of *M. roreri*. GOD is a glycoprotein which catalyzes the oxidation of β-D-glucose to D-glucono-1,5-lactone and hydrogen peroxide. This enzyme has been purified from a range of different fungal sources, mainly from the genus *Aspergillus* and *Penicillium* and used in large scale for technological applications since the early 1950s (Fiedurek and Gromada, 1997; Bhat *et al.*, 2013). Nevertheless, no information is available in the literature related to it having other biological functions, namely in the protection of fungal cells from competing antagonistic microorganisms.

Finally, the protein identified in the co-culture with *M. guilliermondii* #1015 is a nucleoside diphosphate kinase, a protein with broad substrate specificity that is involved in both DNA and RNA metabolism. Not much information is available related to the secretion or alternative functions of this kinase in *M. guilliermondii*, although the nucleoside diphosphate kinase (YNK1) of *S. cerevisiae* was previously identified in the secreted proteome (Faria-Oliveira *et al.*, 2014). Nucleoside diphosphate kinases are considered ubiquitous and highly-conserved enzymes, which abundance increases in response to DNA replication stress in *S. cerevisiae* (Tkach *et al.*, 2012).

Taken together, the results from this exploratory assessment revealed that the antagonistic yeasts strains produce and secrete proteins in response to the presence of the fungal pathogen. Moreover, the fungal

strains in co-culture secrete different proteins compared to those secreted when they are alone in culture, and different strains secrete different proteins. Much more research, probably involving a full secreted proteome characterization, will be needed to understand the putative involvement of the secreted proteome in the yeast /fungal antagonism.

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