Application of yeast-based biocontrol agents against fruit spoilage moulds

By

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DECLARATION

I, **Zukisani Gomomo**, know the meaning of plagiarism and declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for any degree or examination in any other university. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology, Agricultural Research Council and National Research Foundation of South Africa.

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ABSTRACT

Fruits such as apples, oranges, strawberries and grapes are of commercial importance, serving as primary sources of essential growth factors, including vitamins and minerals. However, their production, safety, and economic contributions to the agricultural sector are severely impacted by mould-induced spoilage. Pathogens such as *Botrytis cinerea* and *Penicillium* spp. cause substantial losses during pre- and post-harvest stages, with over 50% of fruit losses in developing countries attributed to these fungi. In South Africa, annual fruit losses due to mould spoilage exceed 60%.

Synthetic chemical fungicides are widely used to manage fruit spoilage fungi, but their prolonged application raises concerns regarding environmental safety, consumer health, and the development of fungicide-resistant strains. Consequently, non-*Saccharomyces* yeasts have emerged as promising, eco-friendly biocontrol agents. These yeasts utilise diverse mechanisms such as nutrient competition, parasitism, and the secretion of antimicrobial compounds to inhibit fungal growth. This study aimed to evaluate non-*Saccharomyces* yeasts for extracellular enzyme production, antifungal activity against key fruit spoilage fungi, and their viability and stability assessments on fruit surfaces under post-harvest conditions.

Among 23 yeast isolates screened for extracellular enzyme activity, five were selected for further analysis: *Aureobasidium melanogenum* (Y6), *Suhomyces pyralidae* (Y63), *Pichia kluyveri* (Y64), *Meyerozyma guilliermondii* (Y88) and *Zygoascus hellenicus* (Y89). These yeasts were tested *in vitro* using radial inhibition, dual culture, and double Petri dish assays, as well as *in vivo* post-harvest trials on apples, strawberries, and oranges. The yeasts were evaluated for antagonistic effects against three *B. cinerea* strains (B05.10, IWBT-FF1, PPRI 30807) and three *Penicillium* species (*Penicillium expansum* PPRI 5654, *P. italicum* PPRI 10380 and *P. digitatum* PPRI 30517). Compatibility and potential synergistic effects were assessed through yeast-yeast interaction assays.

Extracellular enzyme production varied among the isolates, with *Aureobasidium melanogenum* demonstrating robust activity for proteases, glucanases, chitinases, cellulases and pectinases. This yeast achieved 55%, 52% and 40% inhibition against *B. cinerea* strains B05.10, IWBT-FF1 and PPRI 30807, respectively. *Pichia kluyveri* and *M. guilliermondii* showed 100% inhibition of *B. cinerea* spore germination, while *S. pyralidae* exhibited 100% inhibition for two strains (*B. cinerea* B05.10 and IWBT-FF1), and 87% for *B. cinerea* PPRI 30807. Volatile organic compounds (VOCs) such as isobutanol, 2-phenylethanol, and isoamyl acetate, identified using solid-phase microextraction coupled with gas chromatography–mass spectrometry (SPME-GC-MS) were found to contribute to mould inhibition.

During post-harvest trials, *S. pyralidae* achieved the highest inhibition of *B. cinerea* on apples with a mean inhibition of 43%, while *M. guilliermondii* was most effective against *P. digitatum* and *P. italicum* on oranges. Commercial fungicides demonstrated higher efficacy in some instances, though yeast treatments provided viable alternative control. Stability and viability assays revealed varying levels of yeast survival on fruit surfaces, with a decrease in yeast cell concentrations observed after oven drying, while stability was maintained during the storage period. The study concludes that the selected non-*Saccharomyces* yeasts hold significant potential as biological control agents against fruit spoilage moulds. While post-harvest trials demonstrate promising results, further optimisation and field applications are recommended to enhance their efficacy and adoption in agricultural practices.

Key words: Biological control, non-*Saccharomyces* yeasts, *Aureobasidium melanogenum*, *Pichia kluyveri, Suhomyces pyralidae, Meyerozyma guilliermondii*, extracellular enzyme production, volatile organic compounds, post-harvest fruit spoilage, *Botrytis cinerea* and *Penicillium* spp.

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RESEARCH OUTPUTS

The following research outputs represent the contributions of the candidate to scientific knowledge and development during the doctoral candidacy (2022-2024):

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Conferences

- Z Gomomo, M Fanadzo, M Mewa-Ngongang, B.S Chidi, J Hoff, M van der Rijst, M.E Setati, Lucky Mokwena, H.W du Plessis. Use of non-*Saccharomyces* yeasts as biological control agents against *Botrytis cinerea.* 79th Session of the UN General Assembly (UNGA 79), Science Summit, 10^{th}-20^{th} September 2024, United States, New York, *Oral presentation*.
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DEDICATION

I sincerely dedicate this work to my Heavenly Father, my ancestors, my father Zoyisile Gomomo, my late mother Linda Gomomo, my sisters Sinovuyo and Liqhayiya Gomomo, and the entire Gomomo family under the AmaGiqwa clan name. I also extend my gratitude to close friends for their support during both challenging and joyous times, their prayers were always there motivating me and always seeing potential in me. Their encouragement throughout my studies have been helpful, and I am deeply thankful.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

GLOSSARY

Abbreviations/Symbols	Definition (units)
BCA	Biocontrol agents
GPEB	Grape pomace extract broth
MGI	Mycelial growth inhibition
MIC	Minimum inhibition concentration
MRI	Mould radial inhibition
PDA	Potato dextrose agar
RPM	Revolutions per minute (rev/min)
VOCs	Volatile organic compounds
VOCIA	Volatile organic compound inhibition activity
YMA	Yeast malt peptone agar
YMB	Yeast malt peptone broth

PREFACE OF THE THESIS

The thesis comprises six chapters, outlined as follows:

Chapter 1: Provides the background of the research topic and significance of the study. It includes the problem statement, the motivation behind conducting the research, the hypothesis, the aim, the objectives and the research questions that guided the study. Additionally, the delineation of the study is included in this section.

Chapter 2: Presents a comprehensive review of relevant literature to establish the theortical background and contextualise the study. Key research gaps within the existing body of knowledge are identified and synthesised to refine the research focus and justify the study objectives.

Chapter 3: Focuses on screening of the yeasts for their ability to produce lytic enzymes and further screen the biocontrol yeasts for inhibitory effects against three different *B. cinerea* strains on apples and strawberries.

Chapter 4: Evaluates the effectiveness of non-*Saccharomyces* yeast species as biocontrol agents against three *Penicillium* species during laboratory assays and on apples and oranges.

Chapter 5: Investigates yeast interactions, viability on fruits, and post-drying stability for biocontrol applications.

Chapter 6: Focuses on the summary and conclusions of this study, as well as future research recommendations. The achievements of the study are also covered.

CHAPTER 1

General Introduction

1.1 Background

Fruit losses represent a significant global challenge, affecting nearly every country. Post-harvest losses, predominantly due to mould spoilage caused by fungal pathogens, such as *Botrytis cinerea* and *Penicillium* species, account for 20-25% of all fruits produced globally, resulting in economic losses exceeding US$180 billion annually in the fruit and vegetable sectors (FAO, 2019; DEFF and CSIR, 2021; Post-harvest, 2022). In South Africa, approximately 66% of fruits and vegetables are lost annually, amounting to US$1.57 billion in economic losses (DEFF and CSIR, 2021).

Preservation of fruit quality is critical to the agricultural industry, as fruits such as apples, oranges, lemons, peaches, nectarines and strawberries are economically and nutritionally important (Brat *et al*., 2006; Abo-Elyousr *et al*., 2021). These fruits contribute significantly to global economic sustainability and are integral to the fresh produce economy due to their sensory and nutritional value (Holguín-Ibarra *et al.*, 2021; Sun *et al*., 2021). Ensuring high-quality fruit reaches consumers requires producers to comply with stringent quality and regulatory standards (Calvin *et al*., 2006; Jongwanich, 2009; Al-hindi *et al*., 2011).

South Africa produces approximately 4 million tonnes of apples, grapefruit, oranges, tangerines, lemons, limes, peaches, nectarines and strawberries annually (FAOSTAT, 2020). Due to their low pH, high sugar content, and nutrient density, these fruits are particularly susceptible to pre- and post-harvest mould spoilage caused by fungal genera such as *Botrytis*, *Penicillium*, *Mucor*, *Alternaria*, *Colletotrichum*, *Cladosporium*, *Rhizopus*, *Aspergillus*,

Monilinia, *Lasiodiplodia* and *Fusarium* (Sharma *et al*., 2009; Dwiastuti *et al*., 2021). These fungal pathogens lead to significant economic losses and deterioration of fruit quality and nutritional composition (Li *et al.*, 2019). Extending fruit shelf life and improving quality during pre- and post-harvest stages, as well as transportation, remain critical areas of focus in agriculture (Dwiastuti *et al*., 2021).

Currently, spoilage moulds are primarily controlled through the use of synthetic fungicides. These include triazoles, hydroanilide fenhexamid, dicarboximides, and succinate dehydrogenase inhibitors, which are incorporated into costly spraying programs (Miller and Welch, 2013; Romanazzi *et al*., 2017; Li *et al*., 2019). However, concerns over environmental safety, human health, and the emergence of fungicide-resistant mould strains have led to severe restrictions on their use (Li *et al.*, 2019; Yu *et al.*, 2020). Prolonged exposure to synthetic fungicides, even at low doses, poses risks to human health, including skin irritation, stomach pain, cardiovascular issues, and vision damage (Contarino *et al*., 2019; Dwiastuti *et al*., 2021; Zhu *et al.*, 2022). Certain countries have prohibited the use of synthetic chemicals in fruits destined for export (Benito *et al*., 2009).

In response, there is an increasing demand for safer and more sustainable alternatives to synthetic fungicides (Kumar *et al.*, 2008; Liu *et al.*, 2013; Oro *et al.*, 2014; Li *et al*., 2019; Tournas and Katsoudas, 2019). Antagonistic yeasts have emerged as promising biological control agents due to their ability to tolerate stress, minimal nutritional requirements, and capacity to colonise dry surfaces for extended periods (Liu *et al*., 2013; Spadaro and Droby, 2016). These yeasts exhibit diverse mechanisms for controlling mould growth, including nutrient competition, parasitism, secretion of antimicrobial compounds, killer toxins, and

volatile organic compounds (VOCs) (Parafati *et al*., 2017a,b; Contarino *et al*., 2019; Czarnecka *et al.,* 2019; Mewa-Ngongang *et al*., 2019b; Gomomo *et al*., 2022).

Yeasts, such as *Meyerozyma guilliermondii*, *Suhomyces pyralidae* and *Hanseniaspora* species have demonstrated significant inhibitory effects against fungal pathogens of fruits (Mewa-Ngongang *et al*., 2019b; Ruiz-Moyano *et al*., 2020; Al-Maawali *et al*., 2021; Han *et al*., 2021; Gomomo *et al*., 2022). This study investigates the potential of non-*Saccharomyces* yeasts and their extracellular metabolites as biocontrol control agents for managing fruit spoilage pathogens.

1.2 Hypotheses

- Non-*Saccharomyces* yeasts produce extracellular enzymes and VOCs that significantly inhibit *B. cinerea*.
- Non-*Saccharomyces* yeasts demonstrate comparable antifungal efficacy to chemical fungicides in inhibiting *Penicillium* spp. during *in vitro* and post-harvest trials.
- Biocontrol yeasts remain viable on fruit surfaces after harvest, maintain stability following oven drying, and effectively inhibit *Penicillium italicum* and *Botrytis cinerea* at low cell concentrations.

1.3 Research Questions

- What antifungal mechanisms (extracellular enzymes and VOCs) contribute to the antagonistic activity of non-*Saccharomyces* yeasts against *B. cinerea*?
- How effective are non-*Saccharomyces* yeasts in controlling *Penicillium* spp. under laboratory and post-harvest conditions?

- What are the effects of yeast interactions, post-harvest viability, drying stability, and the minimum concentration required for yeasts to inhibit mould growth?

1.4 Aim and Objectives

1.4.1 Aim

To evaluate the effectiveness of non-*Saccharomyces* yeasts (*Aureobasidium melanogenum*, *Suhomyces pyralidae*, *Pichia kluyveri*, *Meyerozyma guilliermondii*, and *Zygoascus hellenicus*) as biocontrol agents against *Botrytis cinerea* and *Penicillium* spp., and to assess their viability, stability, and inhibitory mechanisms.

1.4.2 Objectives

- Investigate the extracellular enzyme production, antifungal activity, and VOCs of non-*Saccharomyces* yeasts against *Botrytis cinerea*.
- Assess the efficacy of selected non-*Saccharomyces* yeasts as biocontrol agents against *Penicillium* spp. during *in vitro* assays and post-harvest fruit trials.
- Evaluate yeast interactions, viability on fruit surfaces, stability after oven drying, and the minimum concentration required for effective biocontrol applications.

1.5 Delineation of the Research

This study does not address:

- **The toxicology of the biocontrol agents:** While this research investigates the efficacy of non-*Saccharomyces* yeasts in controlling fruit spoilage moulds, it does not investigate into the potential toxicological effects of these yeasts on human health or the environment.

- **Detailed interactions between yeasts and moulds during inhibition:** Although the study evaluates extracellular enzyme activity, VOC production, and yeast viability, it does not explore the in-depth molecular pathways leading the interactions between yeasts and spoilage moulds.
- **Fruit defence mechanisms against moulds:** While this research focuses on the antagonistic properties of non-*Saccharomyces* yeasts, it does not investigate the fruit defence responses to fungal infection. Factors such as fruit-induced biochemical resistance, secondary metabolite production, and the influence of fruit microbiomes on fungal colonisation are not considered in this study.

1.6 Significance of the Research

This study offers a sustainable solution to reducing synthetic fungicide use in post-harvest fruit management by utilising environmentally friendly biocontrol agents. It aligns with global and national goals, by improving food security, reducing chemical pollution, and supporting sustainable agriculture. Scientifically, it advances knowledge on non-*Saccharomyces* yeasts antifungal mechanisms and viability post-drying, aiding commercialisation. Economically, it benefits the fruit industry by extending shelf life and enhancing export competitiveness. This research promotes safer food production, environmental sustainability, and innovation in biological control strategies.

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CHAPTER 2

Literature Review

2.1 Introduction

This chapter provides an overview of the significance of fruits in human health, emphasising their role as a sources of essential vitamins, minerals, and bioactive compounds. The discussion highlights their contributions to disease prevention and overall health promotion. Additionally, the chapter examines the economic and commercial importance of fruits in South Africa, with attention to the quality standards required for local consumption and export markets. A key focus is the impact of microbial spoilage on fruit quality and yield, and the methods employed to mitigate fungal infections.

2.2 The Importance of Fruits

Fruits, due to their perishable nature, differ from cereals, pulses, and oilseed crops in terms of storage and handling requirements. They are vital for their economic, nutritional, and medicinal contributions (WHO, 2021; Smith *et al*., 2022; FAO, 2023). Often consumed fresh, fruits are also processed into products such as juices, jellies, and preserved slices (Singh and Sharma, 2018; Kamel *et al*., 2020; Bhatta, 2021). They are rich in bioactive compounds with antioxidant properties and provide essential dietary nutrients, including vitamins (C, A, B6, thiamine, niacin, E), minerals, and dietary fibre (Kader, 2001; Yahia, *et al*., 2019; Zhu *et al*., 2019; Abo-Elyousr *et al*., 2021; Wang *et al*., 2022). These components contribute significantly to public health by reducing risks of chronic diseases such as cancer, cardiovascular diseases, and stroke (Kader, 2001; Ben-Nun, 2016; Choi *et al*., 2018; Zakrevskii, 2018; Yahia *et al*., 2019).

South Africa's fruit industry plays a pivotal role in its economy, contributing significantly to both domestic and international markets. In 2018, approximately 63% of the country's fruit production was exported, with key export markets including Europe and Russia, underscoring the sector's global competitiveness (Fruit, 2018). Around 25% of the total fruit production is processed locally, while the remaining 11% is consumed within South Africa (Fruit, 2018). This distribution highlights the strong export orientation of the industry, as well as its capacity to meet domestic consumption needs and support local processing industries. The high export rate also reflects the growing demand for South African fruit in international markets, particularly in Europe, which values the country's diverse range of high-quality fruits (FAO, 2023).

2.3 Fruit Losses due to Microbial Spoilage

Microbial spoilage poses a significant threat to fruit quality and availability. Fruits provide an ideal substrate for microbial growth due to their rich nutrient composition (Willey *et al*., 2008; Zhu *et al.*, 2019). Spoilage moulds, such as *Botrytis cinerea*, *Penicillium* spp., and others, can naturally occur or develop during post-harvest handling, causing losses of up to 25% in industrialised countries and exceeding 50% in developing regions (Droby, 2005; Nunes, 2012; Buzby *et al*., 2014; Asch *et al*., 2019; Godana *et al*., 2020; Dwiastuti *et al*., 2021; Ziv, and Fallik, 2021). Contributing factors include improper harvesting methods, rough handling, and unsuitable storage conditions, which promote microbial colonisation and degradation (Ippolito and Nigro, 2000; Sharma, *et al*., 2009; Godana *et al*., 2020). The losses affect not only economic outcomes but also consumer health, as some moulds produce mycotoxins harmful to humans (Singh and Sharma, 2018; Yu *et al*., 2020; Holguín-Ibarra *et al*., 2021). The following sections briefly discuss the major spoilage moulds (Table 2.1) and their impacts.

Table 2.1: Fungal pathogens and their effects on fruits (Li *et al*., 2019)

Fungal/Mould Species	Host Fruits	Spoilage Symptoms
Botrytis cinerea	Apples, strawberries, grapes	Grey mould, water-soaked lesions
Penicillium expansum	Apples, pears	Blue mould, mycotoxin production
Penicillium digitatum	Citrus fruits	Green mould, rapid decay
Penicillium italicum	Citrus fruits	Blue mould, rapid decay
Monilinia fructicola	Stone fruits	Soft, water-soaked spots
Monilinia fructigena	Stone and pome fruits	Small, sunken, brown lesions

2.3.1 *Botrytis cinerea*

Botrytis cinerea, a necrotrophic pathogen, is a significant cause of post-harvest grey mould in fruits such as apples and strawberries. This pathogen produces cell wall-degrading enzymes, toxins, and compounds such as oxalic acid, facilitating its pathogenicity (Williamson *et al*., 2007; Asch *et al*., 2019). It causes botrytis bunch rot, a necrotrophic disease affecting apples and strawberries (Reyes-Bravo *et al*., 2019; Shen *et al.*, 2019; Nybom *et al*., 2020; Sun *et al*., 2021). Grey mould, a significant post-harvest disease, leads to economic losses by spoiling fruits and spreading via contact, particularly in cold storage (-0.6°C to 2°C) (Xiao & Kim, 2008). Infection typically occurs via wounds or natural openings, often resulting in up to 60% losses during storage and transport (Holz *et al*., 2007; Xiao & Kim, 2008; Sardella *et al*., 2016). Symptoms include lesions and fruit rot, with losses ranging from 20% to 60% (Sholbeg *et al*., 2003; Sardella *et al*., 2016; Iqbal *et al*., 2022).

2.3.2 *Penicillium expansum*

Penicillium expansum causes blue mould in various crops, including apples and strawberries, and produces the carcinogenic mycotoxin patulin (Spadaro *et al*., 2007; Nunes, 2012; Usall *et*

al., 2016; Tournas & Katsoudas, 2019; Yu *et al*., 2020; Han *et al*., 2021). It produces patulin, a mycotoxin with significant health risks, including carcinogenic and teratogenic effects (Birgitte *et al*., 2004; Tournas and Katsoudas, 2019). It is a primary cause of post-harvest decay in pome fruits, leading to soft, musty-smelling rot (Xu & Tian, 2008). Infection typically occurs through wounds during handling or storage, with losses exceeding 50% under suboptimal conditions (Mari *et al*., 2002; Monroe, 2009; Sanzani *et al*., 2009; Sardella *et al*., 2016). Symptoms include rapidly enlarging watery spots and visible spores, especially at 20–25°C (Snowdon, 1990; Shim *et al*., 2002; Sardella *et al*., 2016).

2.3.3 *Penicillium italicum* and *Penicillium digitatum*

Penicillium italicum and *P. digitatum* are major pathogens of citrus fruits, causing green and blue moulds that lead to significant economic losses (Badawy *et al*., 2011; Florenzo *et al*., 2019; Liu *et al*., 2019). These fungi produce harmful mycotoxins and spread through wounds caused by pests or improper handling (Kellerman *et al*., 2016; Abo-Elyousr *et al*., 2021; Wang *et al*., 2022). Under optimal conditions (25°C), they generate up to two billion spores per cycle, with losses reaching 90% (Holmes & Eckert, 1999; Papoutsis *et al*., 2019; Zhu *et al*., 2019; Cheng *et al*., 2020). Infections manifest as water-soaked lesions, followed by white mycelia and distinctive spore colours (Lin *et al*., 2019; Abo-Elyousr *et al*., 2021; Bhatta, 2022; Wang *et al*., 2022).

2.3.4 *Monilinia* spp.

Monilinia species, including *M. fructicola* and *M. fructigena*, are necrotrophic pathogens causing brown rot and significant losses in stone and pome fruits (Grzegorczyk *et al*., 2017; Melgarejo *et al*., 2019; Reyes-Bravo *et al*., 2019; Nybom *et al*., 2020). Infection occurs through wounds or open floral calyxes and is exacerbated by high humidity and warm temperatures

(Bonaterra *et al*., 2003; Thomidis and Exadaktylou, 2010; Garcia-Benitez *et al*., 2016; Grzegorczyk *et al.*, 2017; Nybom *et al*., 2020). Symptoms include blossom blight, twig cankers, and fruit rot during storage (Giobbe *et al.*, 2007; Villarino *et al*., 2016). These fungi tolerate low temperatures, with conidia germinating at -4°C, contributing to their prevalence worldwide (Villarino *et al*., 2016; Tamm & Flückinger, 1993; Jemric *et al*., 2011).

2.4 Mould Spoilage Prevention Methods

2.4.1 Chemical method

Chemical fungicides are widely used to control plant diseases due to their rapid action and effectiveness in managing fruit spoilage moulds (Palou *et al*., 2008; Kamel *et al*., 2020; Zhu *et al*., 2022). However, their use poses risks, including environmental contamination, human and animal health hazards, and the development of fungicide-resistant mould strains (Ahima *et al*., 2019; Tournas and Katsoudas, 2019). Regulatory restrictions have led to a decline in approved chemicals for post-harvest applications, raising concerns about residues on fresh produce (Tahir & Nybom, 2013; Sarrocco and Vannacci, 2018; Wang *et al*., 2021). Despite these issues, various synthetic fungicides such as fludioxonil, Captan, and thiabendazole are still employed to control fruit spoilage at pre- and post-harvest stages (Zhao *et al*., 2010; Berk, 2016; Yu *et al.*, 2020).

2.4.2 Biological method

The drawbacks of synthetic fungicides have spurred interest in environmentally friendly alternatives, particularly biological control (Yu *et al*., 2020; Sun *et al*., 2021). Biological methods use living organisms, such as yeasts, bacteria, and fungi, to inhibit pathogens without leaving toxic residues (Wisniewski *et al*., 2016; Ahima *et al*., 2019; Kamel *et al*., 2020; Zhimo *et al.*, 2020; Agirman *et al.*, 2023). This approach offers eco-friendly, cost-effective solutions

with minimal risks to non-target organisms (Dukare *et al*., 2019). The advantages of yeasts as biocontrol agents include the lack of allergenic spore production, compatibility with organic farming practices and reduced environmental impact compared to chemical fungicides (Freimoser *et al*., 2019). Applying yeasts as biocontrol agents has certain limitations and challenges including inconsistent efficacy under varying environmental conditions, difficulties in large-scale production and formulation, and limited shelf-life and stability (Casas-Godoy *et al*., 2021; Zhimo *et al*., 2020).

In microbial biocontrol systems, the development of resistance by fungal pathogens poses a significant challenge. Over time, pathogens may adapt to biocontrol agents, weakening their efficacy. Understanding mould resistance mechanisms and implementing strategies to mitigate resistance development is crucial for the long-term success of biocontrol systems (Janisiewicz and Korsten, 2002; Schena *et al*., 2017; Wisniewski *et al*., 2016). Fungal pathogens can develop resistance to biocontrol agents through various mechanisms, including genetic mutations that alter target sites and modification of metabolic pathways to bypass inhibitory effects (Schena *et al*., 2017). Pathogens may adapt by forming protective biofilms or producing secondary metabolites that neutralise biocontrol agents (Parafati *et al*., 2015; Spadaro and Droby, 2016).

To mitigate the risk of resistance development in pathogens, several strategies can be employed. Rotating biocontrol agents with distinct modes of action helps prevent pathogens from adapting to a single agent, reducing selective pressure and maintaining treatment efficacy (Janisiewicz and Korsten, 2002). Similarly, combining multiple biocontrol yeasts with complementary mechanisms of action, such as *Metschnikowia pulcherrima* and *Wickerhamomyces anomalus*, has been shown to enhance efficacy and delay resistance, as demonstrated in the control of *Penicillium expansum* (Wang *et al*., 2022). Integrating

biocontrol agents with other treatments, including chemical fungicides, physical methods, and cultural practices, provides a multi-faceted approach to disease management, minimising reliance on any single method and reducing the potential for resistance (Wisniewski *et al*., 2016). Moreover, optimising application methods, such as ensuring proper coverage and timing, is crucial for maximising the effectiveness of biocontrol agents and preventing sub-lethal exposure that could encourage resistance (Schena *et al*., 2017). Collectively, these strategies emphasise the importance of diverse and well-planned disease management practices to sustain the long-term efficacy of biocontrol solutions.

2.4.2.1 Yeasts as biological control agents

Yeasts are effective biocontrol agents (BCAs) (Table 2.2) due to their resilience in adverse conditions and ability to suppress pathogens by competing for nutrients and producing antimicrobial compounds, such as volatile organic compounds (VOCs) and killer toxins (Pretscher *et al*., 2018; Kamel *et al*., 2020; Dwiastuti *et al*., 2021; Huang *et al*., 2021). They also release cell wall-degrading enzymes like chitinase and *β*-1,3-glucanase, which inhibit fungal spore germination and growth (El-Tarabily, 2006; Dukare *et al*., 2019). Effective application methods, including spraying or dipping fruits, ensure yeast colonisation and long-term pathogen suppression (Sharma *et al*., 2009; Calvo-Garrido *et al*., 2014; Lamenew *et al*., 2019). Yeast species such as *Candida guilliermondii*, *C. sake*, and *Hanseniaspora* spp. have demonstrated efficacy by producing metabolites like acetic acid and decanoic acid, which inhibit fungal growth (Lassois *et al*., 2008; Han *et al*., 2021). However, commercial adoption remains limited due to challenges in application and consistency (Köhl *et al*., 2015; Cecilia *et al*., 2020; Dwiastuti *et al*., 2021).

Mechanisms of action of biocontrol yeasts

Biocontrol yeasts inhibit moulds through several mechanisms, including nutrient competition, enzymatic activities and VOCs secretion. These modes of action often work synergistically to suppress pathogen growth and provide effective control. Nutrient competition is a key mechanism by which biocontrol yeasts suppress fungal pathogens. Biocontrol yeasts rapidly colonise surfaces, consuming available resources and creating a nutrient-depleted environment that limits mould growth (Janisiewicz and Korsten, 2002; Huang *et al*., 2021). The ability of yeasts to outcompete moulds is influenced by their rapid growth rate, high metabolic activity, and strong adhesion to the surfaces (Lassois *et al*., 2008; Spadaro and Droby, 2016).

Biocontrol yeasts produce hydrolytic enzymes that degrade fungal cell walls, directly inhibiting pathogen growth (El-Tarabily, 2006; Dukare *et al*., 2019). Key enzymes involved include glucanases, chitinases, and proteases. Glucanases target β-glucans, which are major components of fungal cell walls, while chitinases degrade chitin, another essential structural component (Arrebola *et al*., 2010; Dukare *et al*., 2019). Proteases further weaken the fungal cell wall by breaking down proteins involved in cell wall integrity (Kouassi *et al*., 2012).

The secretion of VOCs by biocontrol yeasts plays a crucial role in inhibiting fungal pathogens. Volatile organic compounds are small, volatile molecules that can diffuse through the air and impact pathogens without direct contact (Arrebola *et al*., 2010; Parafati *et al*., 2017). Volatile organic compounds disrupt fungal spore germination, hyphal growth, and metabolic processes (Parafati *et al*., 2015; López-García *et al*., 2020). These compounds likely interfere with the pathogen's membrane integrity and enzyme activity, ultimately reducing its viability (Masoud and Kaltoft, 2006; Wang *et al*., 2022).

Environmental implications of using biocontrol yeasts

Yeast-based biocontrol strategies have significant environmental benefits, making them a promising tool for sustainable agriculture (Hollmann *et al*., 2006; Lobus *et al*., 2023). By reducing reliance on chemical pesticides, yeast-based biocontrol can lower the environmental impact of farming, minimising harmful residues in the ecosystem, reducing water contamination, and protecting non-target organisms, including beneficial insects and soil microorganisms (Hollmann et a*l*., 2006; Tyagi *et al*., 2024). Additionally, this approach contributes to lowering carbon footprints associated with pesticide production, transportation, and application (Lobus *et al*., 2023; Zhang *et al*., 2024). The promotion of biodiversity is another key advantage, as using biocontrol agents like yeasts enhances ecological balance and resilience, which is crucial for maintaining long-term agricultural productivity (Tyagi *et al*., 2024).

Optimisation of biocontrol applications for yeast efficacy

The optimisation of biocontrol applications for yeast efficacy involves several key methods aimed at improving the stability and effectiveness of the agents (Samsudin and Magan, 2016; Morales-Cedeño *et al*., 2021; Onwe *et al*., 2022). Yeasts tend to thrive in specific temperature ranges, with high humidity levels often promoting better survival rates during storage (Tan and van Ingen, 2004). Formulation techniques, including the use of carriers such as trehalose, have been shown to improve yeast stability during drying and storage, thereby preserving their biocontrol potential and enhancing the stability of biocontrol agents (Onwe *et al*., 2022). Additionally, the application method significantly influences yeast efficacy, as methods such as spraying, dipping, or coating fruits ensure proper yeast adherence and distribution, thereby facilitating effective pathogen control (Samsudin and Magan, 2016; Morales-Cedeño *et al*., 2021). Proper application timing and coverage are crucial to achieving optimal yeast contact

with the fruit surface and enhancing the likelihood of successful pathogen suppression (Liu *et al*., 2013). By integrating these methods environmental optimisation, formulation improvements, and effective application techniques, yeast-based biocontrol agents can be maximised for enhanced disease management.

Yeast-fruit interaction dynamics

Yeast-fruit interaction dynamics are critical to the success of biocontrol applications, as they determine the ability of yeasts to colonise fruit surfaces and effectively suppress pathogens. Yeasts colonise fruit surfaces by adhering to the wax layer, forming biofilms that enable persistence and competitive exclusion of pathogens (Sharma, 2020; Yao *et al*., 2022). Their compatibility with different fruits varies based on surface characteristics, such as pH, sugar content, and the composition of the fruit's natural microbiota; for instance, fruits with high sugar content, like grapes and strawberries, typically support effective yeast colonisation compared to more acidic surfaces, such as those of citrus fruits (Spadaro and Droby, 2016; Agirman *et al*., 2023). Fruit-specific factors, such as the antimicrobial properties of citrus essential oils, can influence yeast performance by potentially inhibiting their growth, requiring the selection of compatible yeast strains that can tolerate these compounds. (Spadaro and Droby, 2016). Understanding these interaction dynamics is essential to optimising yeast-based biocontrol strategies across various fruit types.

2.4.2.2 Microbial interactions and biocontrol development

Interactions among microbes, including antagonism and cooperation, influence the success of biocontrol strategies (Müller *et al*., 2014; Granato *et al.*, 2019; Lavrentovich and Nelson, 2019; Giometto *et al*., 2021; Agirman *et al.*, 2023). To enhance yeast-based biocontrol, integrating them with other agents or low-dose fungicides in integrated management programs has shown

promise (Droby *et al*., 2002; Lima *et al*., 2003). Effective biocontrol products require consistent performance across various conditions and target moulds (Borgeaud *et al.,* 2015; Lamenew *et al*., 2019; Zhimo *et al*., 2020).

2.4.2.3 Effects of drying on yeast viability

Preservation techniques including drying significantly impact yeast cell physiology and viability by inducing structural and oxidative stresses (Rapoport, 2017; Ippolito and Nigro, 2000; Casas-Godoy *et al.,* 2021). Yeasts can endure extended periods without water, but dehydration can affect cellular components, including membranes and organelles (Dupont *et al*., 2014; Casas-Godoy *et al.,* 2021). Incorporating carriers such as trehalose or sucrose during drying improves yeast quality and viability (Casas-Godoy *et al*., 2021). These findings are crucial for developing stable and effective yeast-based biocontrol products.

Table 2.2 Examples of biocontrol yeasts, their target moulds, host fruits, and relevant references.

Biocontrol yeast	Target mould	Fruit	Reference
Aureobasidium pullulans	*Penicillium expansum*	Pear	Spadaro and Droby (2016)
	Botrytis cinerea	Apple	Di Francesco *et al.,* (2015)
	Penicillium digitatum	Mandarin	Parafati *et al.,* (2017)
	Aspergillus tubingensis	Grape	Pantelides *et al.,* (2015)
Hanseniaspora opuntiae	*Aspergillus flavus*	Dried figs	Galván *et al.,* (2022)
Debaryomyces hansenii	*Botrytis cinerea*	Kiwi	Sui *et al.,* (2021)
	Alternaria alternata		
Pichia guilliermondii	*Rhizopus stolonife*	Peach	Li *et al.,* (2023)
Candida intermedia	*Colletotrichum gloeosporioides*	Avocado	Campos-Martínez *et al.,* (2016)
Candida membranifaciens	*Penicillium digitatum*	Orange	Terao *et al.,* (2017)
Clavispora lusitaniae	*Penicillium digitatum*	Lemon	Pereyra *et al.,* (2020)
Kluyveromyces marxianus	*Penicillium expansum*	Apple	Zheng *et al.,* (2023)
Cryptococcus laurentii	*Penicillium expansum*	Peach	Zhang *et al.,* (2017)
	Botrytis cinerea	Strawberry	Wei *et al.,* (2014)
Aureobasidium pullulans	*Erwinia amylovora*	Apple	Aktepe and Aysan (2023
Scheffersomyeces spartinae	*Botrytis cinerea*	Strawberry	Chen *et al.,* (2023)
Saccharomyces cerevisiae	*Botrytis cinerea*	Grape	Parafati *et al.,* (2015
	Penicillium digitatum	Lemon	Perez *et al.,* (2017)

2.4.3 Integrated Pest Management

Integrated Pest Management (IPM) strategy combines the advantages of biological control, such as the use of beneficial microorganisms, with agronomic practices like crop rotation, pruning, and proper irrigation, which can help manage pathogen pressure (Droby *et al*., 2002; Hjeljord and Tronsmo, 2002). When BCAs are used alongside low-dose fungicides, their effectiveness is often enhanced, as they act synergistically to prevent spoilage and reduce the need for higher chemical dosages (Papavizas, 1985; Dukare *et al*., 2019). This combined approach not only boosts the efficacy of disease control but also minimises the environmental impact associated with synthetic chemicals (Dukare *et al*., 2019; Larkin *et al*., 2019).

By reducing the overall dependence on chemical inputs, IPM strategies contribute to lower pesticide residues in the environment, which is beneficial for non-target organisms, soil health, and biodiversity (Droby *et al*., 2002; Hjeljord and Tronsmo, 2002; Sharma *et al*., 2013). Moreover, using BCAs and low-dose fungicides can help maintain long-term pest control effectiveness and reduce the likelihood of resistance development, which is a growing concern in agricultural pest management (Horsfall, 2008; Larkin *et al*., 2019). This shift towards more sustainable agricultural practices supports the growing demand for eco-friendly farming methods, which aim to balance effective pest management with environmental preservation.

2.5 Commercial Yeast-Based Biocontrol Products

Numerous unicellular fungi have been explored for their potential in biocontrol applications. Different yeast isolates undergo testing against various moulds, with the most effective microorganisms selected for further study and application as biocontrol agents (Freimoser *et al*., 2019). For yeasts to be used as active ingredients in biocontrol products, they must demonstrate strong antagonistic effects against target pathogens. However, secondary

considerations, including biosafety, regulatory requirements, production processes, formulation options, and compatibility with application equipment, are equally crucial (Freimoser *et al*., 2019).

Yeasts have been historically used in food and beverage industries, consumed directly as dietary supplements, and widely regarded as safe (Bekatorou *et al.*, 2006; Freimoser *et al*., 2019). Consequently, applying yeasts to crops and food products raises fewer safety concerns (Freimoser *et al*., 2019). Nonetheless, some yeasts, such as specific *Candida* or *Cryptococcus* species, are human pathogens and warrant caution (Miceli *et al*., 2011; Opulente *et al*., 2019). The discovery and development of effective biocontrol agents traditionally focus on isolating antagonists capable of combating multiple post-harvest pathogens across diverse crops (Zhimo *et al*., 2020). These crops exhibit significant variation in genetic makeup, physiology, susceptibility to pathogens, and pre- and post-harvest management practices (Zhimo *et al*., 2020).

Several yeast-based biocontrol products have been commercialised (Table 2.3). Their primary modes of action include competition for nutrients and space, alongside enzymatic activities like protease, chitinase, or secretion of inhibitory molecules (Bar-Shimon *et al*., 2004; Huang *et al*., 2011; Freimoser *et al*., 2019; Gore-Lloyd *et al*., 2019; Zajc *et al*., 2019).

Table 2.3 Commercial biocontrol products based on yeast species and their combinations, along with suppliers and target moulds (Agirman *et al.*, 2023).

Species names	**Product name**	**Supplier**	**Target mould**
Candida oleophila	Aspire	Ecogen Inc	*Botrytis cinerea* *Penicillium expansum* *Penicillium digitatum* *Penicillium italicum*
Candida oleophila	Nexy	Lesaffre Group	*Botrytis cinerea* *Penicillium expansum*
Aureobasidium pullulans	Boni-Protect	Bio-ferm GmbH	*Botrytis cinerea* *Penicillium expansum*
Metschnikowia fructicola	Shemer	AgroGreen	*Botrytis cinerea* *Penicillium expansum*
Cryptococcus albidus	YieldPlus	Lallemand Plant Care	*Botrytis cinerea*
Candida sake	CandiFruit	S.A. Laboratorios Syva	*Botrytis cinerea* *Penicillium expansum*
Bacillus subtilis and *Candida oleophila*	Biocontrol Blend	Novozymes BioAg Group	*Botrytis cinerea* *Penicillium expansum* *Fusarium* spp.

Bacillus amyloliquefaciens and *Aureobasidium pullulans*	EcoGuard Dual Action	Biocontrol Systems Inc	*Botrytis cinerea* *Penicillium* spp. *Alternaria alternata*
Bacillus subtilis and *Metschnikowia fructicola*	MycoGuard Plus	AgroGreen Solutions	*Botrytis cinerea* *Penicillium expansum* *Fusarium* spp.
Bacillus subtilis and *Aureobasidium pullulans*	Serenade Opti + Blossom Protect	Bayer CropScience	*Botrytis cinerea* *Monilinia* spp.
Aureobasidium pullulans strains DSM 14940 and DSM 14941	Botector	Bio-ferm GmbH	*Botrytis cinerea* *Monilinia laxa*
Metschnikowia pulcherrima and *Candida sake*	Amylo-X	Probelte Bio	*Botrytis cinerea* *Penicillium expansum* *Monilinia* spp.
Pichia guilliermondii and *Candida oleophila*	CeraMax Yeast Combo	MycoSolutions	*Botrytis cinerea* *Penicillium* spp. *Fusarium* spp.
Aureobasidium pullulans and *Metschnikowia fructicola*	FruitGard	AgraQuest Inc	*Botrytis cinerea* *Penicillium* spp. *Alternaria alternata*

2.6 Conclusions

The agricultural and food industries face significant economic losses due to mould spoilage. While chemical fungicides are the predominant control method for fruit spoilage, they pose challenges such as health concerns and resistance development among target organisms. Antagonistic yeasts, particularly naturally occurring ones, offer a promising alternative due to their antimicrobial properties. These yeasts are generally recognised as safe, being widely used in food and beverage industries as well as dietary supplements.

Non-*Saccharomyces* yeasts have demonstrated antagonistic effects against various moulds during *in vitro* and *in vivo* studies, with efficacy comparable to some chemical fungicides. However, further research is needed to develop safer, cost-effective biocontrol strategies. For instance, killer yeasts can serve as preventive and curative treatments during pre- and post-harvest stages. Their efficacy should be evaluated against multiple strains and species of spoilage moulds to assess their broad-spectrum inhibitory potential on mycelial growth and spore germination.

Research should focus on determining the minimum inhibitory concentrations (MIC) of yeasts required to suppress mould growth and identifying the specific compounds responsible for this inhibition. These compounds should then be tested individually against spoilage organisms. More extensive trials are needed to explore the effectiveness of biological agents when applied in pre- and post-harvest treatments. Additionally, studies should evaluate the stability and viability of yeasts on different fruit surfaces, especially after drying processes, to ensure they remain effective under varying conditions and post-processing scenarios.

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CHAPTER 3

The Use of Specific Non-*Saccharomyces* Yeasts as Sustainable Biocontrol Solutions Against *Botrytis cinerea* on Apples and Strawberries

Abstract

Apples and strawberries hold significant commercial and nutritional value. However, their entry into local and export markets is often hindered by pre- and post-harvest spoilage, primarily due to infections by *Botrytis cinerea.* While spoilage is conventionally managed using synthetic chemical treatments, there is a growing interest in utilising yeasts as biological control agents. The objective of this chapter was to evaluate the growth-inhibitory potential of non-*Saccharomyces* yeast species against three strains of *B. cinerea*. Five yeasts, namely *Suhomyces pyralidae, Meyerozyma guilliermondii, Pichia kluyveri, Zygoascus hellenicus* and *Aureobasidium melanogenum* were evaluated for antifungal activity against three *B. cinerea* strains B05.10, IWBT-FF1 and PPRI 30807 on agar plates and in post-harvest trials on apples and strawberries. *Aureobasidium melanogenum* exhibited a broad spectrum of extracellular enzyme production and demonstrated inhibition rates in dual culture assays, with inhibition rates of 55%, 52% and 40% against *B. cinerea* B05.10, IWBT-FF1 and PPRI 30807, respectively. Using the volatile organic compound (VOC) assays, *P. kluyveri* and *S. pyralidae* achieved 79% and 56% inhibition, respectively, against all mould strains. Seven VOCs, including isobutanol, isoamyl alcohol, 2-phenylethanol, isoamyl acetate, 2-phenethyl acetate, γ-decanolactone and methyl palmitate, were identified. In post-harvest assays, *S. pyralidae* was the most effective on apples, with inhibition rates of 64%, 40%, and 25% against *B. cinerea* B05.10, IWBT-FF1 and PPRI 30807, respectively. The commercial fungicide Captan and *S. pyralidae* achieved 100% inhibition against *B. cinerea* B05.10, while Captan and *P. kluyveri* achieved 100% inhibition against *B. cinerea* IWBT-FF1 on strawberries. These findings

highlight the potential of the selected yeast species as biological control agents against *B. cinerea*, warranting further research into their application in commercial fruit protection.

Keywords: Mould spoilage, synthetic chemicals, pre- and post-harvest control, growth inhibition, hydrolytic enzymes

3.1 Introduction

Apples (*Malus domestica*) and strawberries (*Fragaria ananassa*) are valuable for human health, serving as primary sources of essential nutrients, including vitamins and minerals, which support a healthy lifestyle (Al-Hindi *et al.,* 2011; Abo-Elyousr *et al.,* 2021; Sun *et al.,* 2021; Wang *et al.,* 2021). Their appealing sensory and nutritional characteristics make them widely consumed and processed into various products, such as cooked slices, juices, and jellies, contributing significantly to the global fresh produce market (Lutz *et al.,* 2020; Guigón-López *et al.,* 2021; Sun *et al.,* 2021). Despite their importance as major fruit crops worldwide, producers continue to encounter numerous challenges in production, storage and market distribution (Rico *et al.,* 2019; Nybom *et al.,* 2020).

Commercially grown fruits are destined for both local and export markets after harvest. However, pre- and post-harvest mould decay leads to significant economic losses (Nybom *et al.,* 2020; Sun *et al.,* 2021). Mould infections during these stages are often attributed to elevated moisture levels, excessive nutrients, low pH, and reduced fruit decay resistance as maturity progresses (Droby *et al.,* 2016). Among the most severe diseases affecting strawberries and apples is grey mould, caused by *Botrytis cinerea*, which significantly impacts yield and quality by depleting nutrients, shortening shelf life, and causing substantial financial losses (Zajc *et al.,* 2019; Sun *et al.,* 2021; Wang *et al.,* 2021; Iqbal *et al.,* 2022). Grey mould infection typically

initiates during flowering, remaining latent until fruit maturation, at which point the pathogen proliferates extensively (Kantarcioğlu and Yücel, 2002; Spadaro and Droby, 2016).

The control of *B. cinerea* presents a significant challenge due to the pathogen's high genetic plasticity, with chemical control using synthetic fungicides being the most widely used strategy (Contarino *et al.*, 2019; Guigón-López *et al.*, 2021; Huang *et al.*, 2021). While synthetic fungicides effectively reduce the pre- and post-harvest fruit losses, their use has led to increased fungicide residues on produce, the emergence of fungicide-resistant mould strains, and has raised concerns regarding human health and the environmental impact (Lutz *et al.*, 2020; Sun *et al.*, 2021; Wang *et al.*, 2021). Consequently, it is crucial to develop safe and effective alternative strategies for managing grey mould diseases in fruit crops (Sun *et al.*, 2021; Zou *et al.*, 2021).

Antagonistic fungi have proven effective against *B. cinerea*, which is susceptible to suppression by microorganisms such as non-*Saccharomyces* yeasts that produce antifungal compounds (Guigón-López *et al.*, 2021; Sun *et al.*, 2021; Gomomo *et al.*, 2022). Compared to chemical control, the use of antagonist microorganisms offers several benefits, including the absence of toxic residues, environmental safety, ease of application and cost-effectiveness (Wisniewski *et al.*, 2016; Huang *et al.*, 2021). Yeasts exhibit valuable antifungal properties, including the secretion of killer toxins such as mycocins, production of cell wall-degrading enzymes (chitinase, *β*-1,3-glucanase, protease, laminarinases, peroxidases), synthesis of volatile organic compounds (VOCs), rapid colony formation, growth within surface wounds, competition for nutrients and space, and induction of host resistance (Spadaro and Droby, 2016; Contarino *et al.*, 2019; Sun *et al.*, 2021).

Killer yeasts, including *Meyerozyma guilliermondii*, *Suhomyces pyralidae* (formerly, *Candida pyralidae*), *Pichia kluyveri*, and *Hanseniaspora* species, have shown antimicrobial activity against a range of fruit-spoilage fungi (Cordero-Bueso *et al.*, 2017; Mewa-Ngongang *et al.*, 2019b Al-Maawali *et al.*, 2021; Gomomo *et al.*, 2022). Previous research by Gomomo *et al.* (2022) evaluated non-*Saccharomyces* yeasts for their ability to inhibit mycelial growth of a strain of *B. cinerea in vitro* and on apples, with results indicating species- and strain-dependent inhibitory effects. Building on this foundation, the present study sought to screen non-*Saccharomyces* yeasts for extracellular enzyme activity and to assess the inhibition of mycelial growth and spore germination of selected yeasts against three distinct *B. cinerea* strains *in vitro* and *in vivo* on apples and strawberries.

3.2 Materials and Methods

3.2.1 Culturing conditions and inoculum preparation

Twenty-three yeast isolates (Table 3.1) were sourced from the biobank of ARC Infruitec-Nietvoorbij (Fruit, Vine and Wine Institute of the Agricultural Research Council, Stellenbosch, South Africa). The yeast selection criteria included previous research findings (Gomomo *et al.*, 2022) and results from enzyme activity screenings. The yeasts were initially cultured on yeast malt agar (YMA) media composed of 1% glucose, 0.3% malt extract, 0.3% yeast extract, 0.5% peptone, and 2% bacteriological agar and incubated at 28°C for 48 h. For inoculum preparation, using a sterile loop, a portion of each pure yeast colony was added to test tubes containing 5 mL of sterilised yeast malt broth. (YMB) (Sigma-Aldrich, South Africa) and subsequently placed in incubation at 28°C for another 48 h. Yeast cell counts were then performed using a haemocytometer under a microscope at 400x magnification to standardise the yeast inoculum concentration to 1×10^8 cells/mL.

Botrytis cinerea strains B05.10 and SAGWRI-FF1 were sourced from the South African Grape and Wine Research Institute (Stellenbosch University, South Africa), and isolate PPRI 30807 was acquired from the ARC Plant Health and Protection biobank (Pretoria, South Africa). *Botrytis cinerea* B05.10 is a wild-type strain and FF1 is a hypervirulent strain (Barkhuizen, 2019). The mould cultures were grown on potato dextrose agar (PDA, Merck, South Africa) at 25°C for 7 to 14 days. To prepare inoculum, a 5 mm mycelial disk was excised from a 5-day-old culture plate for each strain. To prepare a 50 mL spore suspension, the plate surface was gently scraped with a sterile loop and rinsed with sterile distilled water, and the suspension was transferred to a sterile 250 mL Schott bottle. The spore concentration was adjusted to 1×10^5 spores/mL using a haemocytometer and microscope at 400x magnification.

Table 3.1 Yeasts screened for the production of lytic enzymes.

Yeast code	Species name	Starch	Cellulase	Protease	Glucosidase	Chitinase	Pectinase	Lipase	*β*-1,3 Glucanase
Y6	*Aureobasidium melanogenum*	+	+	+	+	+	+	+	+
Y11	*Debaryomyces hansenii*	-	-	-	-	-	-	+	-
Y17	*Hanseniaspora occidentalis*	-	-	-	-	-	-	+	-
Y24	*Meyerozyma guilliermondii*	-	-	-	-	-	-	+	-
Y35	*Rhodotorula dairenensis*	-	-	+	-	-	-	+	+
Y39	*M. guilliermondii*	-	-	-	-	-	-	+	-
Y63	*Suhomyces pyralidae*	+	+	+	-	-	-	+	+
Y64	*Pichia kluyveri*	-	-	-	-	-	-	+	-
Y65	*Meyerozyma guilliermondii*	-	-	+	-	-	-	+	+
Y74	*Debaryomyces delbrueckii*	-	-	-	-	-	-	+	+
Y75	*Saccharomyces cerevisiae*	-	-	-	-	-	-	+	-
Y83	*Brettanomyces bruxellensis*	-	-	-	-	-	-	+	-
Y84	*Debaryomyces hansenii*	-	-	-	-	-	-	+	-
Y88	*M. guilliermondii*	+	+	+	-	-	-	+	+
Y89	*Zygoascus hellenicus*	+	+	+	-	-	-	+	+
Y91	*Zygosaccharomyces rouxii*	-	-	-	-	-	-	+	-
Y92	*Z. rouxii*	-	-	-	-	-	-	+	-
Y93	*Z. microellipsoides*	-	-	-	-	-	-	+	-
Y95	*Z. florentinus*	-	-	-	-	-	-	+	-
Y96	*Z. fermentati*	-	-	-	-	-	-	+	-
Y97	*Z. bisporus*	-	-	-	-	-	-	+	-
Y102	*Starmerella magnoliae*	-	-	-	-	-	-	+	-
Y103	*Saccharromyces cerevisiae*	-	-	-	-	-	-	+	-

*(-) no enzymes activity, (+) enzyme activity

3.2.2 Extracellular lytic enzyme activity

The yeast isolates were evaluated for their ability to produce lytic enzymes, including proteases, chitinases, glucanases, cellulase, starch-degrading amylases, pectinase and lipases. A 10 µL suspension of each yeast culture (±1×10^8 cells/mL) was spotted onto agar plates containing specific substrates for each enzyme assay (Figure 3.1). The plates were incubated at 28°C for 4-7 days, after which enzymatic activity was assessed. Each treatment was conducted in triplicate. Enzyme activity was indicated by clear halos surrounding the yeast colonies (Figure 3.1) and was recorded as either (-) for no activity or (+) for activity.

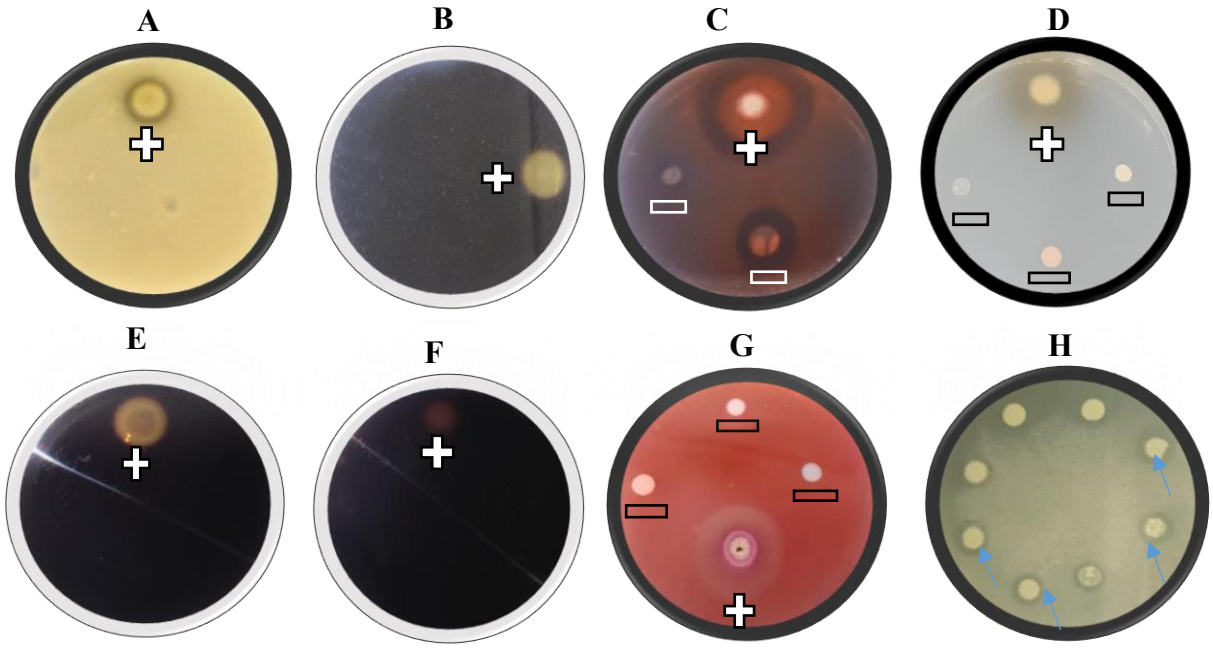

Figure 3.1: A representative example of extracellular lytic enzyme activity of selected yeast isolates, proteases (A), chitinase (B), *β*-1,3-Glucanase (C), *β*-glucosidase (D), cellulase (E), starch (F), pectinase (G) and lipase (H). Enzyme activity is denoted by a positive sign (+), whereas no enzyme activity is indicated by a negative sign (-). For lipase activity, the arrows show clear halos around the colonies. This is a representative example of three replicates.

3.2.2.1 Protease activity

Protease activity was assessed using a modified protocol based on Liu *et al.* (2019). Assays were conducted on skim milk agar plates containing 10% skim milk powder and 2% bacteriological agar. Enzymatic activity was indicated by a clear halo around the inoculated area.

3.2.2.2 Chitinase activity

Chitinase activity was determined following an adapted method from Verma and Garg (2019). Chitin agar plates, prepared with 0.1% finely ground chitin derived from shrimp as the sole carbon source and 2% bacteriological agar, were used for the assay. After incubation, Gram's iodine was applied to the plates for 30 min. Chitinase activity was identified by the appearance of clear halos around the colonies.

3.2.2.3 Glucanase activity

β-1,3-Glucanase activity was evaluated using a laminarin medium consisting of 0.5% laminarin, 0.67% yeast nitrogen base and 2% bacteriological agar) (Sigma-Aldrich, South Africa) as following the method described by Strauss *et al.* (2001). After incubation, the plates were stained with 0.06% Congo red for 60 min at room temperature, and excess stain was decanted. The plates were subsequently treated with 1 mol/L NaCl for 15 min. Enzymatic hydrolysis of glucan was indicated by a yellow-orange halo surrounding the colonies.

3.2.2.4 Glucosidase activity

β-Glucosidase activity was determined on a selective medium containing 0.67% yeast nitrogen base (YNB, Difco), 0.5% arbutin and 2% bacteriological agar, as outlined by Strauss *et al.* (2001). The pH of the medium was adjusted to 5 before autoclaving. Additionally, 10 mL of a

1% ammonium ferric citrate solution (filter-sterilised) was added to the medium before plating. Colonies exhibiting β-glucosidase activity were distinguished by a brown discolouration of the medium.

3.2.2.5 Cellulase activity

Cellulase activity was evaluated on a medium containing 0.2% carboxymethyl cellulose carboxymethyl cellulose (CMC), 1% yeast nitrogen base (1%) and 2% bacteriological agar. Following incubation, the plates were flooded with Gram's iodine for 30 min. Cellulase activity was indicated by clear halos around the colonies.

3.2.2.6 Starch degrading activity

Yeasts were screened for starch-degrading activity on a medium comprising 0.67% YNB, 0.2% soluble starch and 0.2% bacteriological agar at pH 6 following the protocol by Buzzini and Martini (2002). After incubation, the plates were treated with an iodine solution, and starch hydrolysis was indicated by a pale-yellow zone surrounding the colonies.

3.2.2.7 Pectinase activity

Pectinase activity was assessed following an adapted method from McKay (1988) using a pectinase agar medium containing 1.25% pectin (Sigma), 0.68% potassium phosphate (pH 3.5), 0.67% YNB, 1% glucose and 2% bacteriological agar. Plates were stained with 0.1% ruthenium red, and colonies producing a purple halo were identified as positive for pectinase activity.

3.2.2.8 Lipase activity

Lipase activity was tested on a tributyrin agar medium containing 0.5% peptone, 0.3% yeast extract, 1% tributyrin and 2% bacteriological agar adjusted to pH 6, following the method of

Buzzini and Martini (2002). A clear halo surrounding the colony in the opaque medium signified lipase activity.

3.2.3 Dual culture assay

Dual culture assays were used to assess the inhibitory effects of yeasts on mycelial growth, following the protocol by Chen *et al.* (2018). Four yeast strains, which displayed multiple lytic enzyme activities in initial screenings, were selected for evaluation (Table 3.2). Additionally, yeast strain *P. kluyveri* (Y64), previously studied by Mewa-Ngongang *et al.* (2019a, b) and Gomomo *et al.* (2022), was included as the reference strain. A 5 mm mycelial disk was positioned at the edge of the YMA plate, and 20 µL of the yeast suspension (1×10^8 cells/mL) was spotted 40 mm away from the mycelial disk (Figure 3.2). Incubation was conducted at 25°C for 5-9 days.

Negative control plates contained only the 5 mm diameter mycelial disk of the target mould, while positive control plates included 0.5 g/L of the commercial fungicide Captan (N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide). All treatments were performed in triplicate. The percentage inhibition of mycelial growth (MGI) was calculated using the following formula:

$$MGI = [(D_0 – D_t)/D_0] \times 100$$

with D_0 representing the average horizontal growth of the mould colony in the negative control and D_t representing the average horizontal growth of the fungal colony on the yeast-treated plates (Figure 3.2), as described by Núñez *et al.* (2015).

Table 3.2. Yeasts selected for the dual assays, mould spore germination and mouth-to-mouth assays on yeast malt agar.

Yeast code	Species name	Origin
Y6	*Aureobasidium melanogenum*	Jaboticaba fruit
Y63	*Suhomyces pyralidae*	Shiraz wine fermentation
Y64	*Pichia kluyveri*	Shiraz wine fermentation
Y88	*Meyerozyma guilliermondii*	Apple
Y89	*Zygoascus hellenicus*	Apple

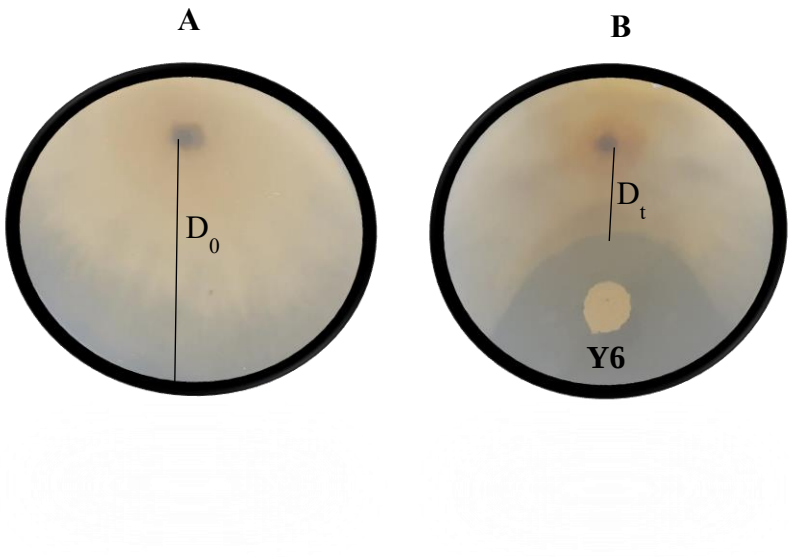

Figure 3.2: Photographic representation of *Botrytis cinerea* growth (A) and the antagonistic activity of the yeast isolate *Aureobasidium melanogenum* (Y6) against *B. cinerea* (B) on yeast malt agar. D_0 indicates the horizontal growth of the mould colony on control plates (no yeast treatment), while D_t indicates the horizontal growth on yeast-treated plates. Each plate shown is a representative example from three replicates.

3.2.4 Mould spore germination assay

The agar plate method, as described by Núñez *et al*. (2015), was used for a radial inhibition assay. Yeast cell suspensions at a concentration of 1×10^8 cells/mL were prepared from culture

broths, and 100 µL of each suspension was evenly distributed on YMA plates and allowed to dry. Subsequently, 15 µL of a *B. cinerea* spore suspension (1×10^5 spores/mL) was spotted at the centre of each plate (Figure 3.3), with each treatment conducted in triplicate. Negative control plates contained only the 15 µL spore suspension at the centre of the YMA. The plates were incubated at 25°C for 5-9 days. The mould radial inhibition (MRI) was calculated as follows:

$$MRI = [(D_0-D_t)/D_0] \times 100,$$

with D_0 representing the average diameter of the mould growth on the negative control plates and D_t representing the diameter of the mould growth on the yeast- treated plates (Núñez *et al.*, 2015).

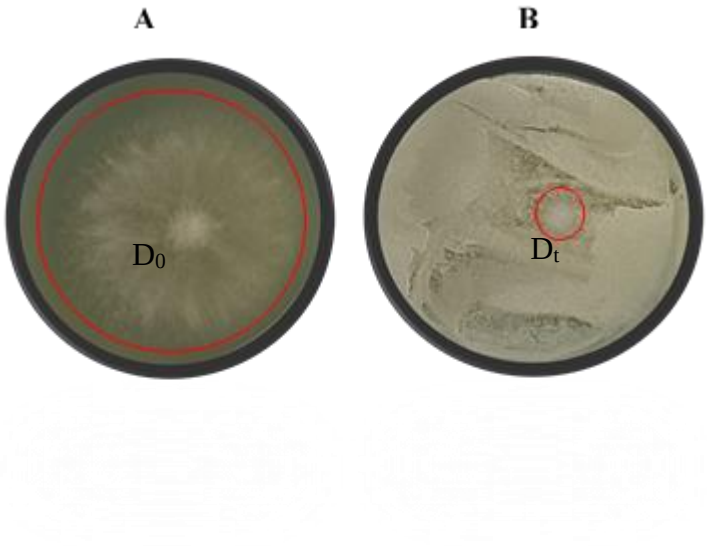

Figure 3.3: Illustration of *Botrytis cinerea* growth (A) and the inhibitory effect of the yeast isolate *Suhomyces pyralidae* (Y63) on *B. cinerea* (B) cultured on yeast malt agar. D_0 denotes the colony diameter on untreated control plates, while D_t refers to the colony diameter on plates treated with the yeast isolate. Each plate displayed is a representative sample from three replicates.

3.2.5 Volatile organic compound (VOC) production assay

The production of VOCs by selected yeast isolates was evaluated using the mouth-to-mouth assay method described by Medina-Córdova *et al*. (2016). In this method, two yeast malt agar (YMA) plates were sealed face-to-face with laboratory film. The bottom plate was inoculated with 100 µL of the yeast suspension (1×10^8 cells/mL), while a 5 mm mould mycelial disk was positioned at the centre of the top plate. For the negative control, only the mycelial disk was placed in the centre of the top plate, with no yeast applied to the bottom plate. For the positive control, 0.5 g/L of the commercial fungicide Captan was spread on one YMA plate, with the mycelial disk placed on the other. All plates were incubated at 25°C for 7 days, and each treatment was performed in triplicate. The volatile organic compound inhibition activity (VOCIA) was calculated using the following equation (Núñez *et al*., 2015):

$$VOCIA = [(D_0-D_t)/D_0] \times 100$$

with D_0 representing the average diameter of the mould colony on the negative control plates and D_t representing the diameter of the mould colony on the treated plates, as shown in Figure 3.4.

Figure 3.4: Depiction of *Botrytis cinerea* growth (A) and the inhibitory effect of the yeast isolate *Pichia kluyveri* (Y64) on *B. cinerea* (B) cultured on yeast malt agar. D_0 indicates the average colony diameter on untreated control plates, while D_t represents the colony diameter on plates treated with the yeast isolate. The plates shown are representative examples from three replicates.

3.2.6 Extraction of volatile organic compounds and gas chromatographic analyses

3.2.6.1 Sample preparation and analyses

Volatile organic compounds (VOCs) produced by *P. kluyveri* and *S. pyralidae* were analysed using headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME–GC–MS), following a modified method based on Maluleke *et al*. (2022). Two sterile YMA layers were prepared by pouring 2 mL of agar on opposite sides of each vial. A spore suspension of *B. cinerea* PPRI 30807 (1×10^5 spores/mL) was prepared, and 10 µL of the suspension was inoculated onto one side of the vial using a sterile inoculation loop (LP ITAKIAN SPA, Milan, Italy). On the opposite side, 10 µL of the yeast suspension (1×10^8 cells/mL) was inoculated. The vials were incubated at 25°C for 5 days. Control vials inoculated only with the *B. cinerea* spore suspension or the yeast cell suspension were included, and all treatments, including controls, were conducted in triplicate.

For GC–MS analysis, 50 µL of a 10 ppm solution of anisole d8 solution was added to the centre of each vial as an internal standard. The vials were subsequently incubated in an autosampler at 70°C for 10 min. A 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber (Supelco, Bellefonte, PA, USA) was then exposed to the headspace of each vial for 30 min under the same temperature conditions. Following equilibration, the fibre was inserted into the GC injector at 250°C, where compounds were thermally desorbed over 10-min period.

3.2.6.2 Chromatographic conditions

The analyses were performed using an Agilent 6890N gas chromatograph (Agilent, Palo Alto, CA, USA) coupled to an Agilent 5975B Inert XL EI/CI mass spectrometer detector (Agilent, Palo Alto, CA, USA) and equipped with a CTC Analytics PAL autosampler. Chromatographic separation was achieved using a polar ZBWax capillary column (30 m length, 0.25 mm internal diameter, 0.25 µm film thickness). The oven temperature program commenced at 40°C, held constant for 17 min, then increased at a rate of 8°C/min to 240°C, with a final hold for 5 min. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The injector operated in a splitless mode at 250°C, with a purge flow of 50 mL/min initiated after 2 min and a gas saver flow of 50 mL/min maintained for an additional 5 min. The ion source and quadrupole temperatures were set to 230°C and 150°C, respectively, while the transfer line temperature was maintained at 280°C. Compounds were identified by comparing their retention times and mass spectra to those in the NIST05 spectral library.

3.2.7 Post-harvest fruit bioassays

Post-harvest biocontrol efficacy assays were conducted on "Golden Delicious" apples and "Earliglow" strawberries across 16 treatments (Table 3.3). Each treatment included five

replicates, with each replicate comprising a rectangular fruit-packaging box containing five apples or a punnet with five strawberries. Fruit surfaces were sprayed with 70% ethanol to eliminate surface microorganisms and allowed to dry completely before wound infliction. A sterile cork borer was used to uniformly wound the fruits (approximately 5 mm diameter and 3 mm deep).

After 15 min, 15 μL of sterile purified water was applied to the wound in the control group, while the other treatments received 15 μL of the *B. cinerea* spore suspension (1×10^5 spores/mL), followed by a 30-min drying period. Then, 15 μL of a yeast inoculum (1×10^8 cells/mL) or 15 μL of the commercial fungicide Captan (0.5 g/L) was applied to the wound. The negative control was treated only with the *B. cinerea* spore suspension, without any yeast or fungicide. The treated fruits were incubated at approximately 20°C for 4–6 days at 80% relative humidity. Inhibition of mould growth was determined by the absence of visible mould development. Lesion diameters were measured, and the percentage of growth inhibition was calculated using the previously established formulas.

Table 3.3 Summary of treatments applied to apples and strawberries during post-harvest biocontrol trials.

Treatment	Description
Treatment 1	Sterile distilled water (Control)
Treatment 2	*Botrytis cinerea* B05.10
Treatment 3	*B. cinerea* IWBT-FF1
Treatment 4	*B. cinerea* PPRI 30807
Treatment 5	*B. cinerea* B05.10 and *Suhomyces pyralidae* Y63
Treatment 6	*B. cinerea* IWBT-FF1 and *S. pyralidae* Y63
Treatment 7	*B. cinerea* PPRI 30807 and *S. pyralidae* Y63
Treatment 8	*B. cinerea* B05.10 and *Pichia kluyveri* Y64
Treatment 9	*B. cinerea* IWBT-FF1 and *P. kluyveri* Y64
Treatment 10	*B. cinerea* PPRI 30807 and *P. kluyveri* Y64
Treatment 11	*B. cinerea* B05.10 and *Aureobasidium melanogenum* Y6
Treatment 12	*B. cinerea* IWBT-FF1 and *A. melanogenum* Y6
Treatment 13	*B. cinerea* PPRI 30807 and *A. melanogenum* Y6
Treatment 14	*B. cinerea* B05.10 and Captan
Treatment 15	*B. cinerea* IWBT-FF1 and Captan
Treatment 16	*B. cinerea* PPRI 30807 and Captan

3.2.8 Statistical analyses

Percentage inhibition data from each assay were analysed using a one-way analysis of variance (ANOVA) performed with the GLM procedure in SAS software (version 9.4, SAS Institute Inc, Cary, NC, USA). The normality of standardised residuals was assessed using the Shapiro-Wilk test. Fisher's least significant difference (LSD) values were calculated at a significance level of 5% ($p = 0.05$) to facilitate the comparison of treatment means. A probability level of

5% was deemed significant for all statistical tests. Statistical significance for all tests was set at $p \leq 0.05$.

3.3 Results and Discussion

3.3.1 Extracellular lytic enzymes activity

Among the 23 yeast strains examined, all displayed lipase activity, with additional enzyme activities varying across strains (Table 3.1). Notably, *A. melanogenum*, produced all the enzymes tested, while *S. pyralidae, Z. hellenicus* and *M. guilliermondii* Y88 demonstrated starch-degrading enzymes, protease, glucanase and cellulase activities. *Rhodotorula dairenensis* and *M. guilliermondii* Y65 also produced proteases and glucanases alongside lipases, whereas the remaining yeast strains exhibited activity for one additional enzyme or none.

Previous studies have documented the enzyme-producing capabilities of *Aureobasidium* species. Parafati *et al.* (2015), Zajc *et al.* (2019) and Di Francesco *et al.* (2020a) reported glucanase, pectinase, and protease activities for *A. melanogenum, A. pullulans,* and *A. subglaciale*, supporting the role of *Aureobasidium* spp. in producing lytic enzymes. Zajc *et al.* (2019) and Moura *et al.* (2021) further observed chitinase and glucanase activity in *A. melanogenum*, aligning with the current findings. The protease activity in *S. pyralidae* corroborates findings by Kantarcioğlu and Yücel (2002), Oksuz *et al.* (2007) and Mehlomakulu *et al.* (2014).

De Souza Ramos *et al.* (2015) and Yang *et al.* (2022) also reported protease and glucanase activities in *Suhomyces* spp., supporting this study's observations. Additionally, Ruas *et al.* (2019), Agirman and Erten (2020) and Lorrine *et al.* (2022) found *M. guilliermondii* capable of extracellular protease production, although this was strain dependent, consistent with the

results here. Maluleke *et al.* (2022) reported that chitinase and glucanase activities were common in yeasts with antagonistic activity against *B. cinerea*, further supporting the findings.

3.3.2 Dual culture assay

Yeasts are known to inhibit various moulds through the production of diffusible metabolites. The five yeasts tested displayed varying levels of growth inhibition against the three *B. cinerea* strains, indicating that that inhibition effectiveness varies depending on both yeast species and fungal strain (Figure 3.5). *Aureobasidium melanogenum* (Y6) was the most effective, demonstrating 55%, 52% and 40% inhibition against *B. cinerea* B05.10, IWBT-FF1 and PPRI 30807, respectively, whereas the commercial fungicide Captan achieved 57%, 41% and 34% inhibition (Figure 3.5A, B, C). This outcome aligns with Di Francesco *et al.* (2020a), although the yeast strain exhibited lower efficacy in their study compared to this study. Similarly, Gomomo *et al.,* (2022) reported that *A. melanogenum* inhibited a different strain of *B. cinerea* by 55% *in vitro*, supporting the findings of this study.

Suhomyces pyralidae (Y63) ranked second, inhibiting *B. cinerea* B05.10, IWBT-FF1, and PPRI 30807 by 56%, 38%, and 35%, respectively. Previous work by Mewa-Ngongang *et al.* (2019b) reported 100% inhibition of *S. pyralidae* on *B. cinerea* spore germination, while Gomomo *et al.* (2022) found a 62% under *in vitro* conditions, suggesting that *S. pyralidae* is more effective at preventing spore germination than controlling established mould growth.
Meyerozyma guilliermondii (Y88) inhibited *B. cinerea* strains B05.10, IWBT-FF1, and PPRI 30807 by 53%, 43% and 15%, respectively. This is consistent with findings by Wang *et al*. (2018) and Cheng *et al*. (2023), who also reported antifungal activity of *M. guilliermondii* against *B. cinerea*. The reference strain *P. kluyveri* (Y64) showed limited inhibition with an average activity of 24% against the three *B. cinerea* strains, similar to observations by Gomomo

et al. (2022), who noted its relatively weak antagonistic effect. The inhibitory effect of the highest performing yeasts, *A. melanogenum*, *S. pyralidae*, and *M. guilliermondii*, may be attributed to their production of cell wall-degrading enzymes (Table 3.1), and their ability to compete with *B. cinerea* for nutrients and space.

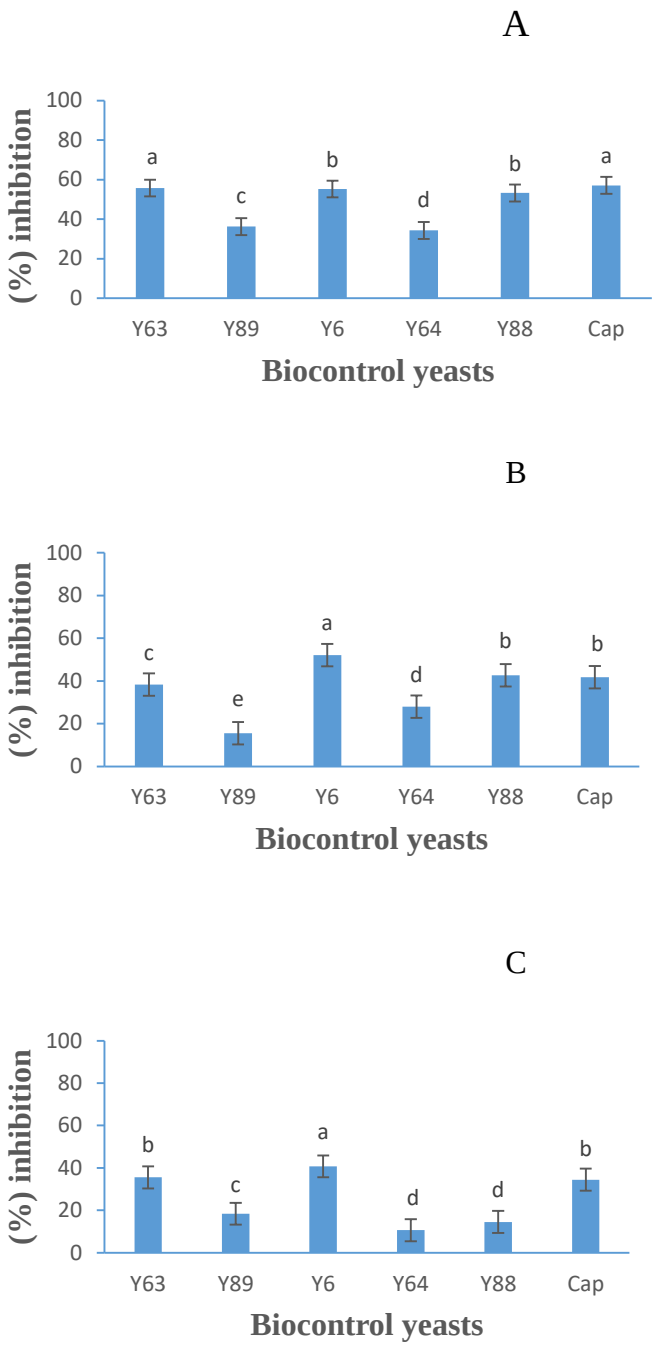

Figure 3.5: Growth inhibition activity (expressed as a percentage) of five yeasts (details listed in Table 3.2) and Captan (Cap), a commercial fungicide against *Botrytis cinerea* B05.10 (A), IWBT-FF1 (B) and PPRI 30807 (C) using the dual culture assay. The values represent the averages of three replicates, with the corresponding standard deviations also provided. Different letters denote significant differences ($p \leq 0.05$) between treatments. The plates for the negative control treatments contained only the relevant moulds and served as the baseline for assessing growth inhibition.

3.3.3 Mould spore germination assay

A radial inhibition assay was used to assess the effect of *S. pyralidae, P. kluyveri, A. melanogenum, M. guilliermondii* and *Z. hellenicus* on spore germination of three *B. cinerea* strains (Figure 3.6). Notably, the inhibitory levels observed were higher than those in the dual culture assay (Figure 3.5). Previous research by Mewa-Ngongang *et al.* (2019b) has shown that these *non-Saccharomyces* yeasts can inhibit mould growth through various mechanisms, such as rapid colonisation of surfaces and outcompeting spoilage moulds, thereby limiting mould proliferation. Both *M. guilliermondii* (Y88) and *P. kluyveri* (Y64) were highly effective, achieving 100% inhibition against all three *B. cinerea* strains (Figure 3.6A, B, C).

The increased inhibition by *P. kluyveri* compared to its mycelial growth inhibition in the dual culture assay (Figure 3.5), underscores the stronger antagonistic effect of these yeasts on spore germination, highlighting their potential as preventative treatments against moulds. This finding aligns with previous reports by Wang *et al.* (2018) and Sepúlveda *et al.* (2023), who observed a complete inhibitory effect by *M. guilliermondii* against two *B. cinerea* strains of *in vitro*. Additionally, Mewa Ngongang *et al.* (2019b; 2021) found that *P. kluyveri* effectively inhibited *B. cinerea,* corroborating findings of this study. *Suhomyces pyralidae* (Y63) demonstrated 100% inhibition against *B. cinerea* B05.10 and IWBT-FF1, and showed 87% inhibition against *B. cinerea* PPRI 30807. Similar findings were reported by Carbó *et al.* (2019), Ngongang *et al.* (2019b) and Gao *et al.* (2021), who noted various *Candida* spp. exhibited differing degrees of inhibitory activity against *B. cinerea* spoilage, consistent with results from this study.

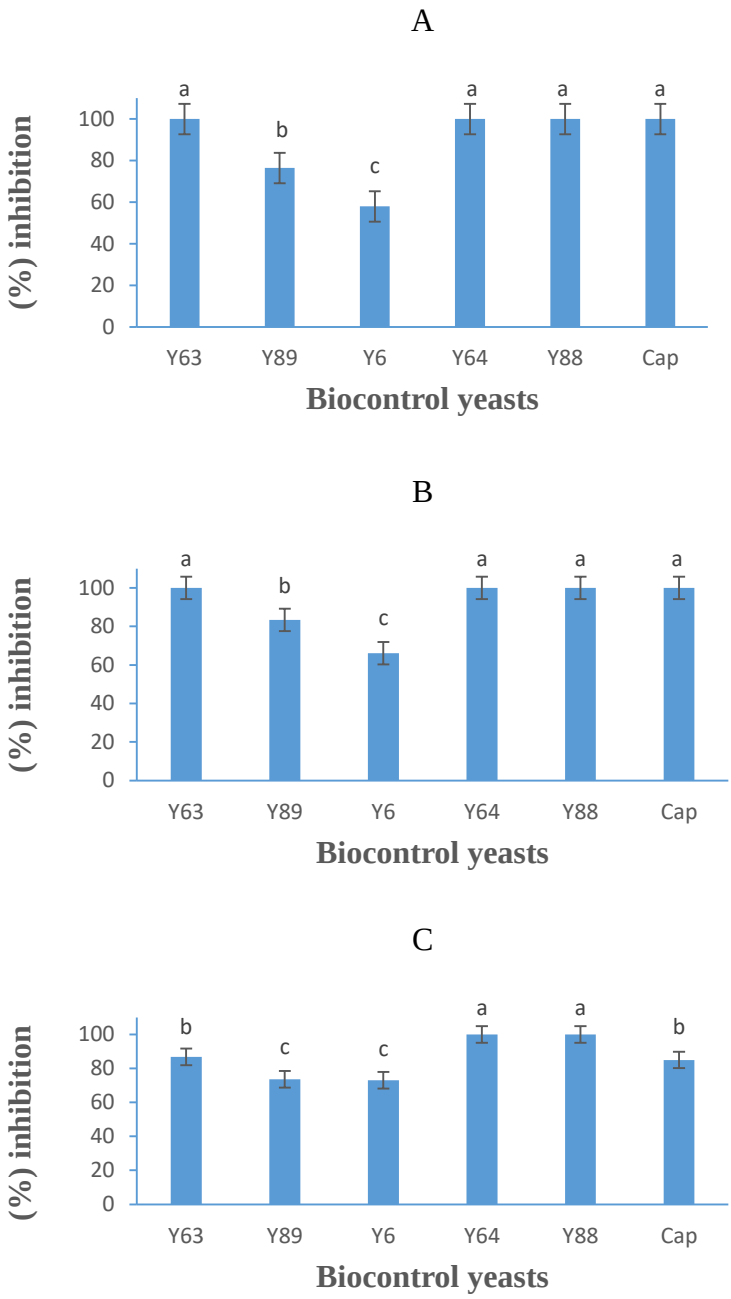

Figure 3.6: Growth inhibition activity, expressed as a percentage, of five yeast strains (details provided in Table 3.2) and Captan (Cap), a commercial fungicide against *Botrytis cinerea* B05.10 (A), IWBT-FF1 (B) and PPRI 30807 (C), assessed using a mould spore germination assay. Data represent the mean values from three independent replicates, with standard deviations shown. Treatments with distinct letters indicate statistically significant differences ($p \leq 0.05$). Negative control plates, containing only the respective *B. cinerea* strains, served as references for evaluating growth inhibition.

3.3.4 Volatile organic compound (VOC) production assay

The mode of action of VOCs in inhibiting *B. cinerea* was explored using the mouth-to-mouth assay. Results indicated that VOCs produced by the yeasts inhibited the growth of *B. cinerea*, with inhibition levels varying among yeast strains (Figure 3.7). Notably, *B. cinerea* strain PPRI 30807 exhibited higher susceptibility (mean inhibition of 73%) to yeast VOCs than strain B05.10 (mean inhibition of 38%). This pattern differed from the dual culture assay, where the inhibition of PPRI 30807 was less pronounced, suggesting that VOCs could be a primary mode of action against this particular strain.

In the VOC assay trial, *P. kluyveri* (Y64) demonstrated the highest inhibition, achieving 60%, 76%, and 100% inhibition against *B. cinerea* B05.10, IWBT-FF1, and PPRI 30807, respectively (Figure 3.7). Unlike in the diffusible metabolite assay (Figure 3.5) where *P. kluyveri* (Y64) exhibited lower inhibition, its stronger performance in the VOC assay points toward VOC production as its primary antagonistic mechanism. This observation aligns with findings by Nägeli *et al.* (2023) who reported *P. kluyveri* as an effective inhibitor of *B. cinerea* growth *in vitro*, emphasising its reliance on VOCs for mould suppression.

Other yeasts also demonstrated notable VOC-based inhibition. *Suhomyces pyralidae* (Y63) achieved inhibition rates of 32%, 55% and 82% against strains B05.10, IWBT-FF1, and PPRI 30807, respectively, while *M. guilliermondii* Y88 exhibited 29%, 47% and 79% inhibition (Figure 3.7). Previous studies by Mewa-Ngongang *et al.* (2019b) and Choińska *et al.* (2020) also reported effective VOC-mediated inhibition of *B. cinerea* by *S. pyralidae* and *M. guilliermondii*, supporting the current findings. *Zygoascus hellenicus* (Y89) showed moderate inhibition, with rates of 44%, 38% and 58% against the three *B. cinerea* strains, respectively

(Figure 3.7), further illustrating the potential of VOC production as a biological control strategy against mould proliferation.

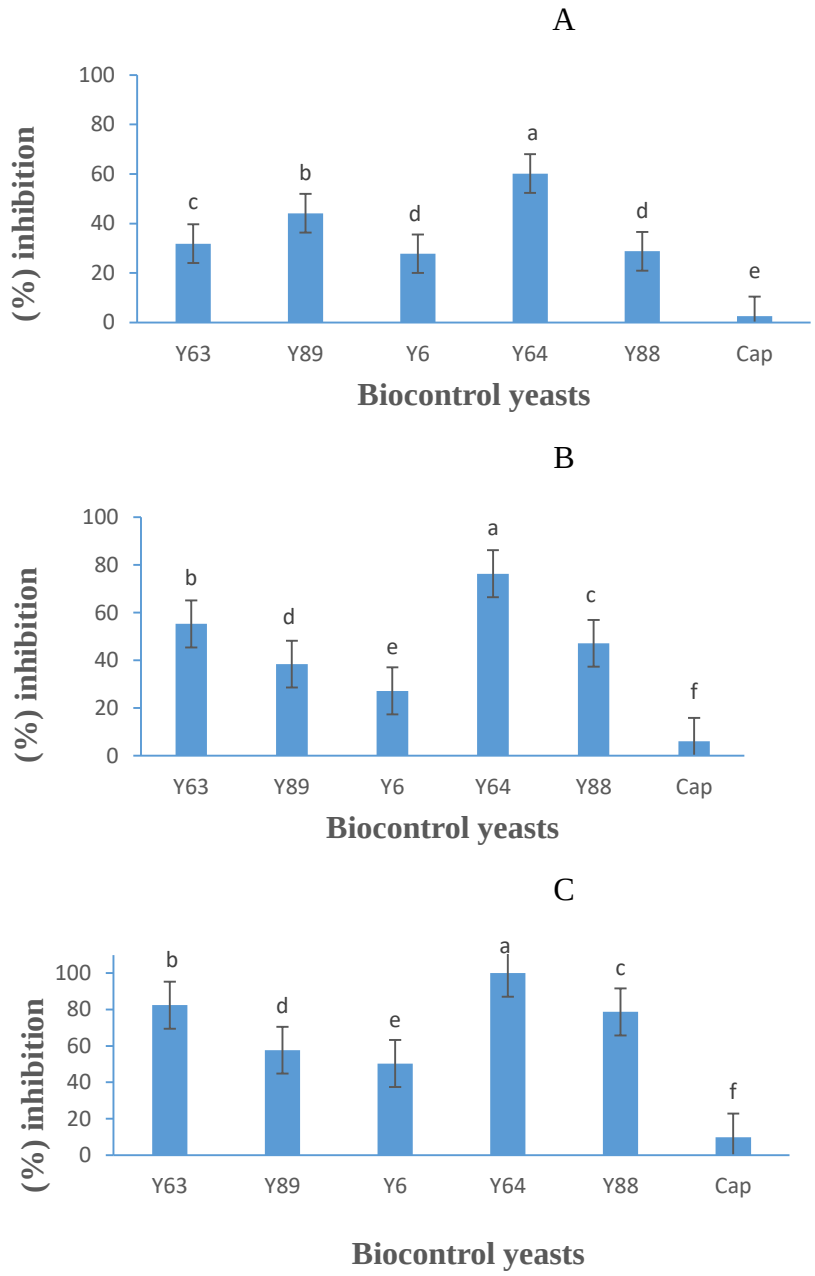

Figure 3.7: Growth inhibition activity, expressed as a percentage, of five yeast strains (details provided in Table 3.2) and Captan (Cap), a commercial fungicide, against *Botrytis cinerea* B05.10 (A), IWBT-FF1 (B) and PPRI 30807 (C), based on the production of volatile organic compounds (VOCs). Data represent mean values from three replicates, with standard deviations indicated. Different letters denote statistically significant differences ($p \leq 0.05$) between treatments. Negative control treatments, containing only the respective *B. cinerea* strains, served as references for evaluating growth inhibition.

3.3.5 Volatile organic compound (VOC) extraction and gas chromatographic analyses

The VOCs produced by *P. kluyveri* and *S. pyralidae* were shown to play a significant role in inhibiting *B. cinerea* growth during *in vitro* trials (Figure 3.7). The VOCs produced by *P. kluyveri* and *S. pyralidae* were analysed using solid-phase microextraction coupled with gas chromatography–mass spectrometry (SPME-GC–MS), identifying a total of 29 compounds, of which seven were consistently present across all replicates. The key compounds included alcohols (isobutanol, isoamyl alcohol, 2-phenylethanol), esters (isoamyl acetate, 2-phenethyl acetate), gamma butyrolactone (γ-decanolactone) and a fatty acid methyl ester (methyl palmitate) (Table 3.4). These VOCs were produced by the yeast isolates alone or in conjunction with *B. cinerea*. Notably, isoamyl alcohol, 2-phenylethanol, 2-phenethyl acetate and methyl palmitate were also detected when *B. cinerea* was cultured independently.

Suhomyces pyralidae produced all seven VOCs in monoculture, with isobutanol, isoamyl acetate and 2-phenethyl acetate concentrations slightly elevated in the presence of *B. cinerea*. These VOCs, particularly isobutanol, isoamyl acetate and 2-phenethyl acetate, are likely contributors to the inhibition of *B. cinerea*, aligning with findings of Li *et al.* (2024) who reported isoamyl acetate's antagonistic effects against grey mould on blueberries. Further, Zou *et al.* (2023) demonstrated the antifungal efficacy of isoamyl acetate against *B. cinerea* mycelial growth, and *Hanseniaspora uvarum* effectively controlled *B. cinerea* in strawberries and cherries, with 2-phenylethyl acetate identified as the predominant VOC (Ruiz-Moyano *et al.,* 2020). Phenylethyl acetate has also shown strong inhibitory activity against *Aspergillus ochraceus* and *Mucor* spp. growth (Masoud *et al.,* 2005; Choińska *et al.,* 2020).

When co-cultured with *B. cinerea*, *S. pyralidae* produced slightly lower levels of isoamyl alcohol and 2-phenylethanol, yet these VOCs continued to contribute to its antagonistic effect.

Calvo *et al.* (2020) reported complete inhibition of *B. cinerea* growth *in vivo* by isoamyl alcohol, with additional studies by Maluleke *et al.* (2022) and Zou *et al.* (2023), linking isoamyl alcohol and 2-phenylethanol to *B. cinerea* inhibition. The observed inhibition of *B. cinerea* may be due to the combined or synergistic effects of VOCs produced by *S. pyralidae*. *Pichia kluyveri*, when cultured alone, produced high concentrations of isoamyl acetate and 2-phenethyl acetate; however, these levels significantly decreased when co-cultured with *B. cinerea.* Lower concentrations of isoamyl alcohol and 2-phenylethanol were also observed in co-culture, yet these VOCs may still be central to *B. cinerea* suppression.

Previous research on biocontrol yeasts, such as *P. kudriavzevii, P. occidentalis, W. anomalus, Hanseniaspora uvarum* and *Candida intermedia*, demonstrates that VOCs effectively inhibited *B. cinerea* spore germination and mycelial growth (Contarino *et al.,* 2019; Choińska *et al.,* 2020; Maluleke *et al.,* 2022; Zhao *et al.,* 2022). Ethanol and 2-phenylethanol are specifically highlighted as potent antifungal agents against *B. cinerea* and *Alternaria alternata* (Di Francesco *et al.,* 2015; Yalage Don *et al.,* 2020). Additionally, transcinnamaldehyde has shown to inhibitory effects on *B. cinerea* mycelial growth and conidia germination, significantly reducing infections in cherry tomatoes (Guo *et al.,* 2019). VOCs such as, 3-methyl-1-butanol, 2-phenylethanol, 2-ethyl-1-hexanol, 4-methyl-ethyl ester and ethyl acetate have also been identified as effective in inhibiting *B. cinerea* spore germination and mycelial growth (Huang *et al.,* 2011; Huang *et al.,* 2012; Oro *et al.,* 2018; Di Francesco *et al.,* 2020b).

The observed decrease in VOC concentrations in the presence of *B. cinerea* may result from interspecies competition for oxygen and/or carbon dioxide within the test environment, with the biocontrol efficacy of the yeast potentially deriving from the synergistic effects of VOCs and elevated carbon dioxide levels (Altieri *et al.,* 2004; Contarino *et al.,* 2019). Additionally,

B. cinerea may produce defensive compounds that alter the metabolic pathways of biocontrol yeasts, resulting in shifts in VOC production profiles (Santos *et al.*, 2022).

Table 3.4 Major volatile compounds (VOCs) produced by *Pichia kluyveri*, *Suhomyces pyralidae* and *Botrytis cinerea* PPRI 30807.

VOCs	***B. cinerea***	***P. kluyveri***	***P. kluyveri* and *B. cinerea***	***S. pyralidae***	***S. pyralidae* and *B. cinerea***
	Average area ratio				
Isobutanol	ND	0.006	ND	0.009	0.012
Isoamyl acetate	ND	2.489	ND	0.001	0.002
Isoamyl alcohol	0.006	0.136	0.040	0.140	0.135
2-Phenethyl acetate	0.004	3.207	1.325	0.002	0.004
2-Phenylethanol	0.005	0.155	0.034	0.021	0.019
γ-Decanolactone	ND	ND	ND	0.008	0.003
Methyl palmitate	0.004	0.012	0.001	0.001	0.001

*ND-Not detected

3.3.6 Post-harvest fruit bioassays

The application of yeast-based biocontrol agents demonstrated significant efficacy in reducing the spoilage of *B. cinerea* in apples and strawberries, resulting in marked reductions in fruit decay (Figures 3.8 and 3.9). In apple trials, *S. pyralidae* (Y63) effectively inhibited the growth of *B. cinerea* strains B05.10, IWBT-FF1 and PPRI 30807 by 64%, 40%, and 25%, respectively (Figure 3.8). These findings align with prior studies by Guerrero Prieto et *al.* (2019) and Carbó

et al. (2019), which also reported the antagonistic properties of *Candida* spp., specifically *Candida oleophila* and *C. sake* against *B. cinerea* in various fruit contexts. In this study, *A. melanogenum* displayed inhibitory activity against *B. cinerea* strains B05.10, IWBT-FF1 and PPRI 30807 by 21%, 24%, and 26%, respectively, corroborating findings by Di Francesco *et al*. (2020a), who demonstrated similar antagonistic effects of *Aureobasidium* species *in vivo*. *Pichia kluyveri* inhibited *B. cinerea* B05.10, IWBT-FF1 and PPRI 30807 by 11%, 17%, and 16%, respectively. In contrast, the commercial fungicide Captan inhibited *B. cinerea* B05.10, IWBT-FF1 and PPRI 30807 by 92%, 59%, and 17%, respectively. Additionally, *P. kudriavzevii* exhibited inhibition at a minimum concentration of 1×10^2 cells/mL against *B. cinerea*, consistent with the findings of Maluleke *et al*. (2022) that *Pichia* spp. possess antimicrobial properties targeting *B. cinerea.*

In strawberry trials, yeast strains and Captan demonstrated greater inhibition, particularly against *B. cinerea* B05.10 and IWBT-FF1, compared to their performance in apples. The results demonstrate that the extent of inhibition is not solely dependent on the specific yeast or mould species but is also influenced by the type of fruit substrate. *Suhomyces pyralidae* (Y64) inhibited *B. cinerea* strains B05.10, IWBT-FF1 and PPRI 30807 by 100%, 65%, and 34%, respectively. *Aureobasidium melanogenum* (Y6) exhibited 98%, 95%, and 19% antagonistic activity against strains B05.10, IWBT-FF1 and PPRI 30807. These observations are consistent with the work of Zajc *et al*. (2020, 2022), who highlighted the inhibitory capabilities of *Aureobasidium* spp. under *in vivo* conditions. *Pichia kluyveri* exhibited 98%, 100%, and 10% inhibition of *B. cinerea* strains B05.10, IWBT-FF1 and PPRI 30807, respectively, supporting the findings of Nägeli *et al.* (2023) and Maluleke *et al.* (2022) regarding the antagonistic potential of *Pichia* spp. against *B. cinerea*. Captan, the commercial fungicide, exhibited 100%, 100%, and 23% inhibition for strains B05.10, IWBT-FF1 and PPRI 30807 respectively. This

is consistent with reports by Guerrero Prieto *et al*. (2019) on Captan's efficacy against *B. cinerea* in apples.

Overall, the study indicates that the degree of inhibition is influenced by the specific yeast or mould strain as well as the fruit type. The susceptibility of *B. cinerea* strains varied based on the host fruit, highlighting the importance of fruit type in modulating the antagonistic effects of yeast biocontrol agents. The potential application of these yeast species, either individually or in combination, offers promising alternatives to traditional chemical fungicides for managing *B. cinerea* spoilage, presenting a sustainable solution for the agricultural industry.

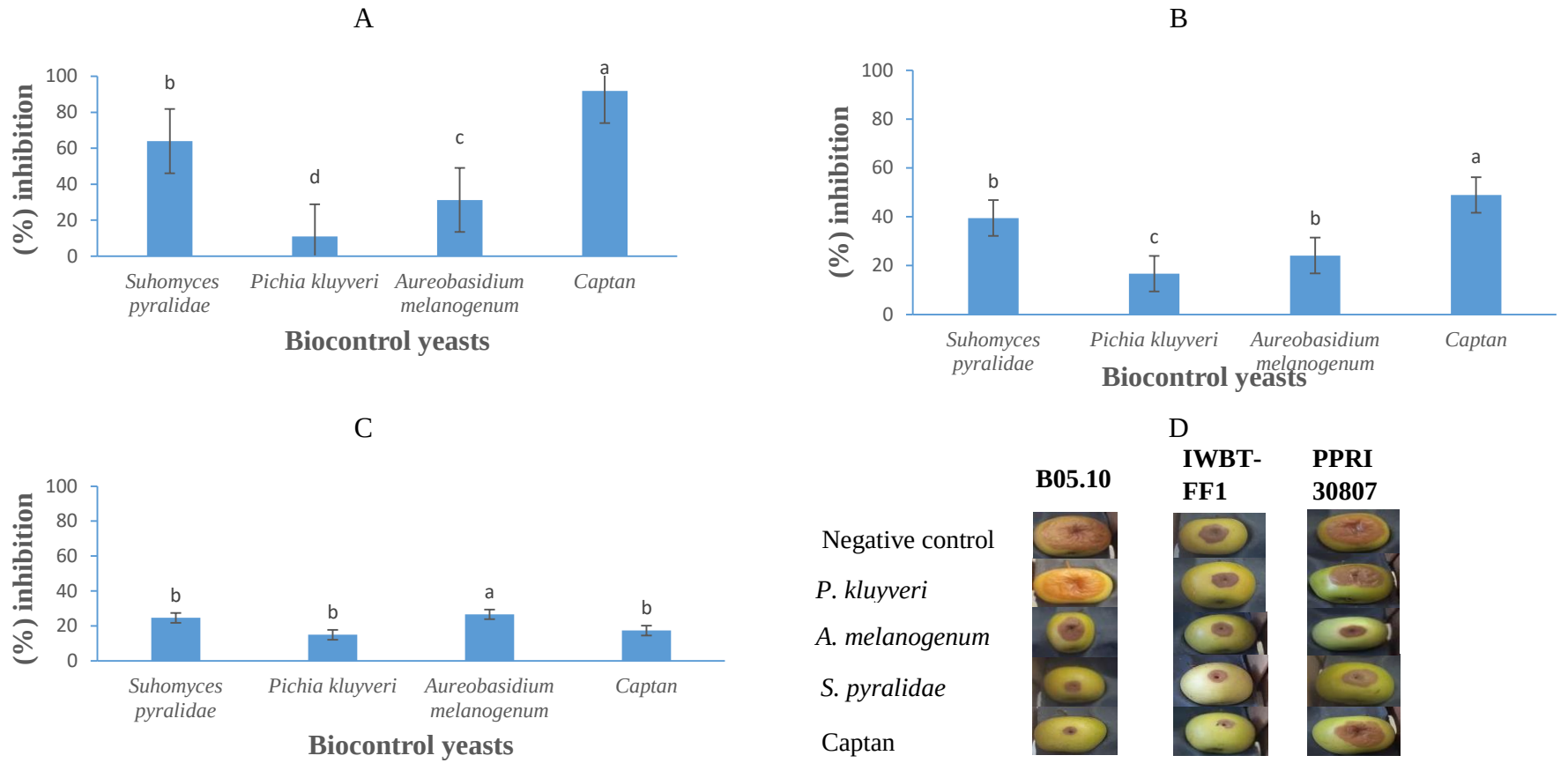

Figure 3.8: Growth inhibition activity (%) of *Suhomyces pyralidae* (Y63), *Aureobasidium melanogenum* (Y6), and *Pichia kluyveri* (Y64) against *Botrytis cinerea* strains B05.10 (A), IWBT-FF1 (B), and PPRI 30807 (C) during post-harvest trials on apples. Data represent mean values from five replicates, with standard deviations indicated. Different letters denote statistically significant differences ($p \leq 0.05$) among treatments. (D) Photographs illustrate apples with lesion diameters, with each set representing a sample of 25 apples. Negative control treatments, where apples were infected solely with the respective *B. cinerea* strains, showed no growth inhibition.

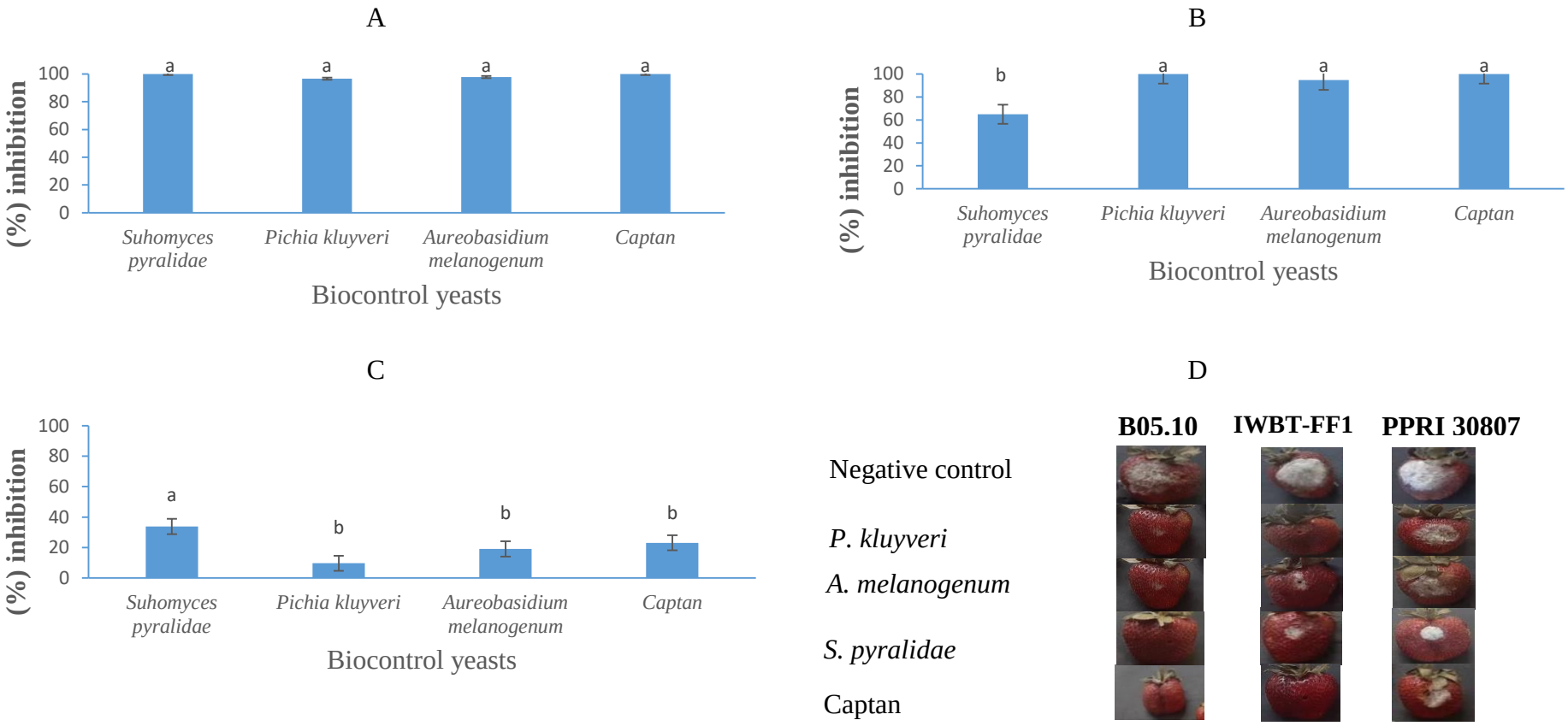

Figure 3.9: Inhibition activity (expressed as a percentage) of *Suhomyces pyralidae* (Y63), *Aureobasidium melanogenum* (Y6), and *Pichia kluyveri* (Y64) against *Botrytis cinerea* strains B05.10 (A), IWBT-FF1 (B), and PPRI 30807 (C) during post-harvest trials on strawberries. Data represent mean values from five replicates, with standard deviations included. Different letters denote statistically significant differences ($p < 0.05$) between treatments. (D) Images depict strawberries with lesion diameters, with each set representing a sample of 25 strawberries. Negative control treatments, where strawberries were inoculated only with the respective *B. cinerea* strains, showed no growth inhibition.

3.4 Conclusions

The study confirmed that *A. melanogenum* synthesises enzymes capable of hydrolysing cell walls. In the direct-contact inhibition assays, cell suspensions of *S. pyralidae* and *A. melanogenum* exhibited the strongest antagonistic effects against *B. cinerea*, with the yeasts significantly impeding spore germination. In fruit trials, *S. pyralidae*, *A. melanogenum*, and *P. kluyveri* each demonstrated distinct inhibitory capacities against *B. cinerea*, varying by fruit type, with results comparable to those of commercial fungicides. This suggests that these yeasts hold promise as biocontrol agents for reducing post-harvest spoilage in place of chemical fungicides. The antagonistic mechanism of *P. kluyveri* was linked to the production of VOCs, with isobutanol, isoamyl acetate and 2-phenethyl acetate identified as key antifungal agents. The primary VOCs contributing to this inhibition were identified as belonging to the alcohol and ester groups. This study underscores the potential of certain yeasts in controlling *Botrytis cinerea*. However, further investigations are necessary to assess their antimicrobial peptides and effectiveness against a broader range of fruit moulds. Comparative studies evaluating the efficacy of these biocontrol yeasts against commercial fungicides should also be conducted. The subsequent chapter will focus on expanding this research by examining the effectiveness of these yeasts in managing *Penicillium* species.

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CHAPTER 4

Use of *Non-Saccharomyces* Yeasts as Biocontrol Agents Against *Penicillium* Species

Abstract

Fruit producers and exporters around the world are grappling with challenges related to post-harvest fruit decay, particularly in fruits such as apples and oranges destined for export, leading to waste and financial losses. The filamentous fungi, specifically *Penicillium* species, are the primary post-harvest pathogens affecting apples and oranges. This study evaluated the use of five non-*Saccharomyces* yeast species (*Suhomyces pyralidae, Meyerozyma guilliermondii,* [1]Pichia kluyveri, *Zygoascus helenicus* and *Aureobasidium melanogenum*) as biocontrol agents against *Penicillium expansum*, *P. digitatum* and *P. italicum*. The yeasts were screened for their inhibitory effects using radial inhibition and mouth-to-mouth assays on agar plates and post-harvest trials on apples and oranges. *Aureobasidium melanogenum* exhibited the most significant growth inhibition in the radial inhibition assay, achieving 60%, 100% and 70% inhibition against *P. expansum*, *P. digitatum* and *P. italicum*, respectively. In the volatile compound assay, *Pi. kluyveri* achieved complete (100%) inhibition of *P. expansum, P. digitatum* and *P. italicum.* Seven volatile compounds were identified, isobutanol, 2-phenylethanol, isoamyl acetate, isoamyl alcohol and 2-phenethyl acetate associated with the inhibition of *P. expansum*. In post-harvest trials, the yeast treatments displayed limited inhibition activity on apples (*ca.* 15%). However, on oranges, *M. guilliermondii* demonstrated significant effectiveness, inhibiting *P. digitatum* by 72% and *P. italicum* by 77%. These findings suggest that the selected yeast species have potential as biological control agents

[1] To avoid confusion with other abbreviations, *Pichia kluyveri* will be abbreviated as '*Pi. kluyveri* ' throughout this chapter

against *P. expansum, P. digitatum* and *P. italicum*, although further research is required to fully understand and optimise their application.

Keywords: Mould spoilage, fruits, synthetic chemicals, pre- and post-harvest control, biocontrol agents (BCAs), yeasts

4.1 Introduction

The cultivation of pome fruits (apples and pears) and citrus fruits (grapefruit, oranges, tangerines, lemons and limes) represents a significant economic activity globally. These fruits are key sources of essential nutrients, including iron, zinc, vitamins C and E, as well as carotenoids (Abo-Elyousr *et al*., 2021; Wang *et al.*, 2021). Numerous studies have identified *Penicillium* spp. as the primary pathogenic agents responsible for post-harvest infections in citrus fruits and apples (Cecilia *et al.*, 2020; Youssef and Hussien, 2020; Huang *et al.*, 2021). *Penicillium digitatum* (green mould) and *P. italicum* (blue mould) are among the most economically important pathogens of citrus, causing significant post-harvest losses of up to 30% and 80%, respectively (Papoutsis *et al.*, 2019). Conversely, *P. expansum* leads to considerable financial losses in apples, with over 50% of damage occurring post-harvest (Mari *et al*., 2002; Monroe, 2009; Papoutsis *et al.*, 2019; Tournas and Katsoudas, 2019; Abo-elyousr *et al*., 2021). These moulds can cause defects in fruit appearance and deplete nutrients during both pre- and post-harvest stages (Tournas and Katsoudas, 2019; Dwiastuti *et al.*, 2021; Holguín-Ibarra *et al.*, 2021). Additionally, *Penicillium* spp. produce toxic secondary metabolites such as patulin and citrinin, which pose health risks to humans and animals (Yu *et al.*, 2020; Huang *et al.*, 2021; Zhu *et al.*, 2022).

Pre-and post-harvest infections caused by *Penicillium* spp. can be managed using synthetic chemical fungicides, which are incorporated into waxes before storage (Liu *et al.*, 2019; Tournas and Katsoudas, 2019; Wang *et al.*, 2019; Yu *et al.*, 2020; Zhu *et al.*, 2022). Currently, blue and green moulds are controlled through pre- and post-harvest application of chemical fungicides such as imazalil, thiabendazole, pyrimethanil, and fludioxonil (Liu *et al.*, 2019; Papoutsis *et al.*, 2019). However, the extensive use of these fungicides has resulted in environmental pollution, increased risks to human health and the development of fungicide-resistant pathogens (Papoutsis *et al.*, 2019; Zhu *et al.*, 2019; Zhu *et al.*, 2022).

The increasing accumulation of fungicide residues on agricultural products and the associated potential health risks of fungicide build-up in the food chain have prompted researchers to seek more eco-friendly alternatives. These alternatives aim to minimise adverse effects on the environment and human health while effectively managing pre-harvest and post-harvest diseases (Liu *et al.*, 2019; Wang *et al.*, 2019; Yu *et al.*, 2020). Biological control methods utilising microbial antagonists, including yeasts, have demonstrated significant potential in mitigating fungal infections on various fruits during both pre- and post-harvest stages (Wang *et al.*, 2019; Assaf *et al*., 2020). Additionally, non-fungicide approaches such as plant-derived botanical compounds, cold plasma and pulsed light techniques have potential for controlling fruit spoilage mould (Dukare *et al*., 2022; Oztekin *et al*., 2023).

Biocontrol yeasts such as *Candida famata, C. laurentii, Debaryomyces hansenii, Meyerozyma caribbica* and *M. guilliermondii* are recognised for their lack of allergenic spore production and are generally considered safe for humans (Ocampo-Suarez *et al*., 2017; Liu *et al.*, 2019) . The mode of action of the biocontrol yeasts include competition for nutrients and space, colonising the surfaces, additionally, possess the capacity to absorb and degrade mycotoxins,

produce cell wall-hydrolysing enzymes such as chitinase, *β*-1,3-glucanase and protease and produce various defence-related enzymes that can activate host defence mechanisms (Zhu *et al.*, 2019; Godana *et al.*, 2020; Yu *et al.*, 2020; Zhu *et al.*, 2022). In our previous study, non-*Saccharomyces* yeasts were screened for their ability to inhibit the growth of a *P. expansum* strain, revealing that inhibition varied by species and strain (Gomomo *et al*., 2022). Building on that research, this study aimed to evaluate the biocontrol effectiveness of selected strains of *Suhomyces pyralidae*, *Meyerozyma guilliermondii*, *Pichia kluyveri*, *Zygoascus helenicus*, and *Aureobasidium melanogenum* against different *Penicillium species*.

4.2 Materials and Methods

4.2.1 Culturing conditions and inoculum preparation

Five yeast isolates (listed in Table 4.1) obtained from Agricultural Research Council (ARC) Infruitec-Nietvoorbij (Fruit, Vine and Wine Institute of the ARC, Stellenbosch, South Africa) were evaluated. These yeasts were selected based on findings from prior studies (Gomomo *et al*., 2022). The isolates were cultured on yeast malt agar (YMA), comprising 1% glucose, 0.3% malt extract, 0.5% peptone, and 2% bacteriological agar, at 28°C for 48 h. A loopful of each pure yeast colony was subsequently transferred into sterilised test tubes containing 5 mL of yeast malt broth (YMB) (Sigma-Aldrich, South Africa) and incubated at 28°C for an additional 48 h. The yeast cell concentration was quantified using a haemocytometer and a microscope at 400× magnification, ensuring an inoculum concentration of 1×10^8 cells/mL.

The fungal strains *Penicillium expansum* PPRI 5654, *P. italicum* PPRI 10380, and *P. digitatum* PPRI 30517 were sourced from the biobank of the ARC-Plant Health and Protection Institute (Pretoria, South Africa). These moulds were cultured on potato dextrose agar (PDA, Merck, South Africa) at 25°C for 7 to 14 days. Spore harvesting was performed by gently scraping the agar surface with a sterile loop and suspending the spores in 50 mL of sterile distilled water

within a 250 mL sterile Schott bottle. The spore suspension was adjusted to a final concentration of 1 × 10^5 spores/mL using a haemocytometer and a microscope at 400× magnification.

Table 4.1 Yeasts used for spore germination and mouth-to-mouth assays on yeast malt agar.

Yeast code	Species name	Origin	Cell free extract code
Y63	*Suhomyces pyralidae*	Shiraz Fermentation	Y63CF
Y89	*Zygoascus hellenicus*	Apple	Y89CF
Y6	*Aureobasidium melanogenum*	Jaboticaba fruit	Y6CF
Y64	*Pichia kluyveri*	Shiraz Fermentation	Y64CF
Y88	*Meyerozyma guilliermondii*	Apple	Y88CF

4.2.2 Fungal Spore Germination Assay

A radial inhibition assay was performed using the agar plate method as described by Núñez *et al*. (2015). In summary, 100 µL of yeast cell suspensions (1×10^8 cells/mL) was spread onto YMA plates and allowed to dry before the application of mould spores. Subsequently, 15 µL of the mould spore suspension (1×10^5 cells/mL) was applied to the centre of each plate, with each treatment conducted in triplicate (Figure 4.1). For the negative control plates, only 15 µL of the spore suspension was applied to the centre of the YMA plate. For the positive control plates, N-trichloromethylthio-4-cyclohexene-1,2- -dicarboximide, commercially known as Captan (800 g/kg; Universal Crop Protection (Pty) Ltd, Kempton Park, South Africa) at a concentration of 0.5 g/L, was spread onto the YMA plate. The plates were incubated at 25°C for 5-9 days.

The mould radial inhibition (MRI) was calculated using the mathematical expression:

$$MRI = (D_0\text{-}D_t/D_0) \times 100$$

with D_0 representing the average diameter of the mould colony on the negative control plates and D_t representing the diameter of the treated plates Núñez *et al*. (2015).

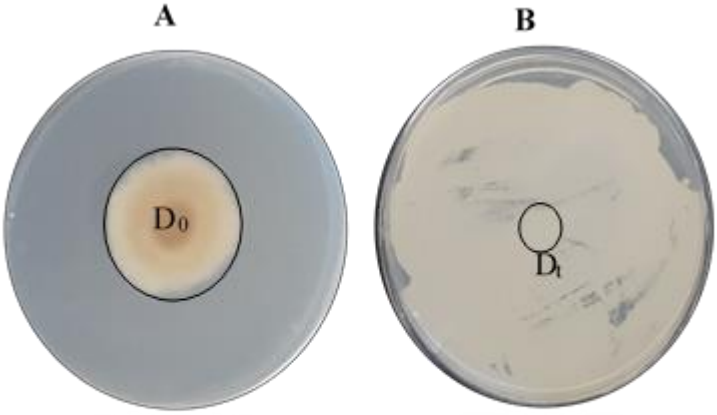

Figure 4.1: Illustration of *Penicillium expansum* growth (A) and the antagonistic activity of the yeast isolate *Meyerozyma guilliermondii* (Y88) against *P. expansum* (B) on yeast malt agar using the radial inhibition assay. D_0 indicates the colony diameter on untreated control plates, while D_t represents the colony diameter on plates treated with the yeast isolate. The plates shown are representative examples from three replicates.

4.2.3 Volatile organic compound (VOC) production

The production of volatile organic compounds (VOCs) by the selected yeasts was evaluated using the mouth-to-mouth assay described by Medina-Córdova *et al*. (2016). In this method, two YMA plates were placed face-to-face and sealed with laboratory film. The upper plate was inoculated with 100 µL of yeast suspension at a concentration of 1×10^8 cells/mL, while the lower plate was inoculated at the centre with 15 µL of mould spore suspension at a concentration of 1×10^5 spores/mL. Negative controls consisted of plates with only the mould

spore suspension (1×10^5 spores/mL) applied to the centre of the lower plate, with no yeast inoculated on the upper plate. Positive controls used the commercial fungicide Captan (0.5 g/L) applied to the YMA plate in place of yeast. All plates were incubated at 25°C for 7 days. Each treatment, including controls, was conducted in triplicate. The volatile organic compound inhibition activity (VOCIA) was calculated using the formula provided by Núñez *et al*. (2015):

$$VOCIA = (D_0\text{-}D_t/D_0) \times 100$$

with D_0 representing the average diameter of the mould colony on the negative control plates and D_t representing the diameter of the mould colony on the yeast-treated plates, as shown in Figure 4.2.

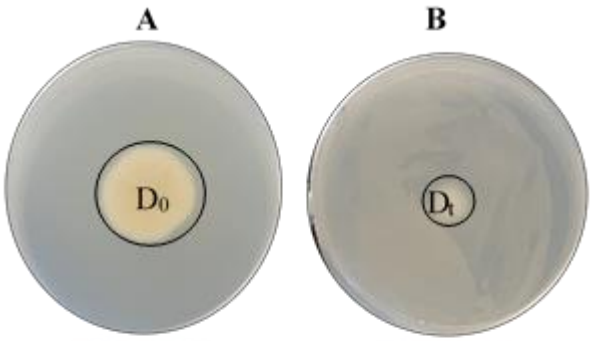

Figure 4.2: Illustration of *Penicillium italicum* spore growth (A) and the inhibitory effect of the yeast isolate *Pichia kluyveri* (Y64) against *P. italicum* (B) on yeast malt agar in the volatile organic compound (VOC) assay. D_0 represents the average colony diameter on control plates without yeast treatment, while D_t represents the colony diameter on yeast-treated plates. Each plate displayed is a representative example from three replicates.

4.2.4 Extraction of volatile organic compounds and gas chromatographic analyses

4.2.4.1 Sample preparation and analyses

Pichia kluyveri and *S. pyralidae* have been previously shown to produce VOCs that inhibit the growth of various fruit moulds, including *Penicillium expansum* (Gomomo *et al*., 2022), which

is consistent with the findings of this study. The VOCs produced by *Pi. kluyveri* and *S. pyralidae* were characterised using headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME–GC–MS). The automated sampling and analysis setup for SPME was performed according to the method described by Maluleke *et al*. (2022), with minor modifications.

Sterile YMA was prepared by pouring 2 mL of agar onto opposite sides of each vial. A spore suspension of *P. expansum* PPRI 5654, at a concentration of 1×10^5 spores/mL, was spread on one side of the vial using a 10 µL inoculation loop (LP ITAKIAN SPA, Milan, Italy). Subsequently, 10 µL of the yeast suspension (1×10^8 cells/mL) was applied to the opposite side of the vial. The vials were incubated at 25°C for 5 days. Control vials were inoculated with *P. expansum* spore suspension and yeast cell suspension separately. Three replicates were performed for each biocontrol yeast-mould combination as well as for the control treatments.

Before GC–MS analysis, 50 µL of a 10 ppm Anisole-d8 solution was added to the centre of each vial as an internal standard. The vials were then placed in the autosampler and incubated at 50°C for 5 min. Subsequently, a 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre (Supelco, Bellafonte, PA, United States) was exposed to the headspace of each vial for 30 min at the same temperature. After equilibration, the fibre was introduced into the GC injector at 250°C, where a 10-min desorption period was used to release the compounds.

4.2.4.2 Chromatographic conditions

The analysis was performed using an Agilent Gas Chromatography system, model 6890 N (Agilent, Palo Alto, CA, USA), coupled with an Agilent mass spectrometer detector (MS),

model 5975B Inert XL EI/CI (Agilent, Palo Alto, CA, USA), and equipped with a CTC Analytics PAL autosampler. Chromatographic separation of compounds was achieved using a polar J&W DB-FFAP capillary column (60 m, 0.25 mm internal diameter, 0.5 μm film thickness). The oven temperature program was as follows: an initial temperature of 40°C held for 1 min, followed by a ramp to 150°C at 25°C/min with a 3-minute hold, a further ramp to 200°C at 5°C/min with a 5-min hold, and a final ramp to 250°C at 5°C/min with a 2-min hold. The total run time was 30.54 min. Helium was used as the carrier gas, with a constant flow rate of 1.0 mL/min. The injector was operated in splitless mode and maintained at 250°C throughout the analysis. The purge flow and gas saver flow were set to 50 mL/min for 2 min and 5 min, respectively. The MS-detector was operated in single ion monitoring (SIM) mode, with the ion source and quadrupole temperatures set at 230°C and 150°C, respectively, and the transfer line at 250°C. Compounds were identified based on their GC–MS retention times and by comparing their mass spectra with the NIST05 spectral library.

4.2.5 Screening yeasts for antimicrobial peptides

Yeast preparations were conducted as described previously. Two-day-old yeast cell suspensions of each strain were transferred to Erlenmeyer flasks containing 100 mL of sterile YMB. The inocula were incubated at 28°C with agitation at 150 rpm using a rotary shaker (LM-53OR, RKC Instrument Inc., Ohta-ku Tokyo, Japan) for two days. Following incubation, samples were centrifuged at 10,000 rpm for 15 min at 4°C. The resulting cell-free supernatant was then filtered using ultrafiltration membranes with a pore size of 10 kDa (Vivaspin 4 PES, Laboratory and Scientific Equipment (Pty) Ltd, Cape Town, South Africa) by centrifugation at 4,000 rpm for 60 min at 4 °C. After ultrafiltration, the filtrate was collected and assessed for antimicrobial activity against *P. expansum, P. italicum* and *P. digitatum* using the radial inhibition assay. Negative control plates received only 15 µL of the spore suspension at the

centre of the YMA plate, while positive control plates were evenly spread with Captan at a concentration of 0.5 g/L. The plates were incubated at 28 °C for 5-7 days. Each treatment had three replicates. The codes for the cell free extracts are listed in Table 4.1.

2.4.6 Comparison of biocontrol yeasts and commercial fungicides

The comparative evaluation and analyses were conducted using the radial inhibition assay method as previously described (4.2.2 Fungal Spore Germination Assay). The yeasts *Suhomyces pyralidae* (Y63), *Meyerozyma guilliermondii* (Y88), *Pichia kluyveri* (Y64), *Zygoascus helenicus* (Y89) and *Aureobasidium melanogenum* (Y6) were prepared according to established protocols. Commercial fungicides, including N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide (trade name Captan, 800 g/kg), Pyrimethanil (anilino-pyrimidines, trade name Protector 400 SC, 400 g/L, ICA International Chemicals (Pty) Ltd, Stellenbosch, South Africa), and Pyrimethanil (aniline derivative, trade name Support 400 SC, 400 g/L, Villa Crop Protection (Pty) Ltd, Aston Manor, South Africa), were prepared in accordance with industry standards. The negative control plates were inoculated with 15 µL of the spore suspension at the centre, while the positive control plates were treated with a spread of Captan on the YMA plates. All plates were incubated at 28 °C for 5-7 days, with each treatment replicated three times.

4.2.7 Post-harvest fruit bioassays

Nineteen treatments were assessed in the post-harvest biocontrol efficacy assay conducted on "Cara Cara" navel oranges and "Golden Delicious" apples. Each experimental unit consisted of a rectangular fruit-packaging box containing five oranges and five apples, with each treatment replicated five times. The fruits were disinfected with 70% Ethanol (v/v) to remove surface microorganisms and allowed to dry completely before wounding. Uniform wounds were inflicted using a sterile cork borer: approximately 5 mm in diameter and 5 mm in depth

for oranges, and approximately 5 mm in diameter and 3 mm in depth for apples. After a 15-min period, the control treatments were inoculated with 15 µL of sterile distilled water into the wounds, while other treatments received 15 µL of the respective mould spore suspensions (1×10^5 cells/mL) and were allowed to dry for 30 min. Subsequently, 15 µL of a yeast inoculum (1×10^8 cells/mL) or 15 µL of the commercial fungicide Captan (0.5 g/L) was applied to the wounds. Negative control treatments were infected solely with the three *Penicillium* spp. without additional yeast or fungicide treatment. The treated fruits were incubated at ±20°C for 4–6 days under 80% relative humidity. Growth inhibition was assessed by the absence of mould development. Lesion diameters were recorded, and percentage growth inhibition was calculated and analysed to evaluate the effectiveness of the treatments.

4.2.8 Statistical analyses

Growth inhibition data for each assay and *Penicillium* species were analysed using a one-way analysis of variance (ANOVA) with the GLM procedure of SAS software (version 9.4, SAS Institute Inc, Cary, NC, USA). Shapiro-Wilk test was applied to the standardised residuals from the model to assess normality. Fisher's least significant difference (LSD) values were calculated at the 5% probability level (p=0.05) to compare treatment means. A significance level of 5% was used for all statistical tests.

4.3 Results and Discussion

4.3.1 Fungal spore germination assay

The yeasts *S. pyralidae, Pi. kluyveri, A. melanogenum, M. guilliermondii* and *Z. hellenicus* were evaluated for their ability to prevent the germination of three *Penicillium* spp. using radial inhibition assays. These five yeasts exhibited varying levels of effectiveness in inhibiting spore germination across the three different *Penicillium* spp. (Figure 4.3). Among the species tested,

Penicillium expansum demonstrated the highest resistance to the treatments, with a mean inhibition of 41%, while *P. digitatum* was the most sensitive with a mean inhibition of 94%. *Penicillium italicum*, with a mean inhibition of 63%, was more resistant than *P. digitatum.* These results highlight differences in sensitivity or resistance to yeast treatments and commercial fungicide among the *Penicillium* species.

Aureobasidium melanogenum (Y6) was the most effective yeast treatment, demonstrating 60%, 100% and 70% inhibition against *P. expansum*, *P. digitatum* and *P. italicum*, respectively (Figure 4.3 A, B, C). These findings align with previous studies Černoša *et al*. (2022), Gomomo *et al*. (2022) and Zajc *et al.* (2022) where the inhibitory effects of *A. pullulans, A. melanogenum,* and *A. subglaciale* were reported on the growth of *P. expansum* in both *in vitro* and *in vivo* trials. *Aureobasidium pullulans*, a well-studied species, has shown potential as a biocontrol agent for pre- and post-harvest citrus spoilage moulds, exhibiting a 23 mm zone of inhibition *in vitro* and achieving 100% inhibition in a post-harvest bioassay (Sukmawati *et al*., 2021; He *et al*. 2024). This study demonstrated that other species within the *Aureobasidium* genus possess antimicrobial properties against citrus spoilage moulds. The variation in inhibition observed may be attributed to differences in species, sources of the spoilage moulds (e.g., different citrus cultivars), and the assay methods used for screening.

Meyerozyma guilliermondii (Y88) was the second most effective yeast strain, exhibiting 56%, 100% and 61% antagonistic activity against *P. expansum*, *P. digitatum* and *P. italicum*, respectively (Figure 4.3 A, B, C). Previous studies Han *et al*. (2021) and Yang e*t al*. (2022) demonstrated the broad antagonistic potential of *M. guilliermondii* against *P. expansum* on pear fruits, thereby corroborating the results of this research. Agirman and Erten (2020) reported that *M. guilliermondii* and *A. pullulans* both exhibited antagonistic effects against *P. digitatum*

and *P. expansum,* while Wang *et al*. (2021) noted the inhibition of *P. italicum* growth by *M. guilliermondii.*

Zygoascus hellenicus (Y89) demonstrated growth inhibition of 43%, 100% and 66% against *P. expansum, P. digitatum* and *P. italicum,* respectively (Figure 4.3 A, B, C). The yeast showed better inhibition on spore germination of *P. expansum* when compared to the previous study Gomomo *et al*. (2022) when the yeast showed 35% inhibition for mycelia growth of *P. expansum*. These results illustrates that the yeast is struggling to control the *P. expansum* when it is already growing on the surface. The yeast show potential as a preventive treatment. Additionally, *Zygoascus hellenicus* has been previously shown to exert antimicrobial effect against *P. italicum* and *P. digitatum,* with a 64% mean inhibition observed on various citrus fruit cultivars (Arras *et al.,* 1998). While this data dates back 26 years ago, the current study confirmed that *Z. hellenicus* remains effective in controlling citrus spoilage moulds, highlighting the benefit of biocontrol methods where moulds do not develop resistance to biocontrol yeasts. This study further demonstrates that yeasts can inhibit mould growth by outcompeting spoilage moulds and rapidly colonising surfaces, thereby reducing fungal development.

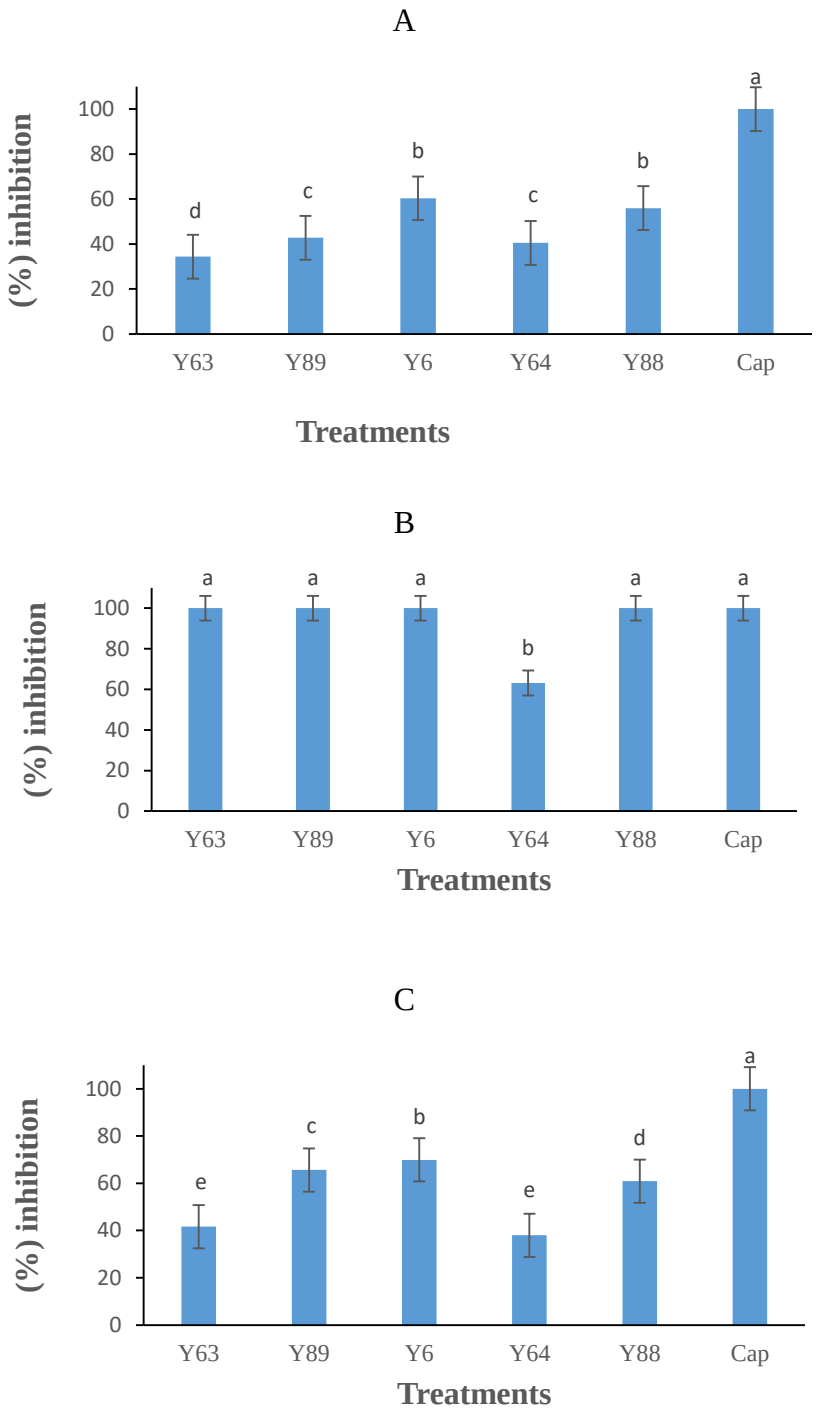

Figure 4.3: Inhibition activity (expressed as a percentage) of five yeast isolates (listed in Table 4.1) and a commercial fungicide against *Penicillium expansum* (a), *P. digitatum* (b), and *P. italicum* (c) in the radial inhibition assay. CAP refers to Captan, a commercially used chemical fungicide. Data represent the means of three replicates, with standard deviations included. Different letters denote statistically significant differences ($p \leq 0.05$) between treatments. Plates from the negative control treatments contained only the respective moulds and were used as reference treatments to calculate growth inhibition.

4.3.2 Volatile organic compound production

The production of VOCs against mould was assessed using the mouth-to-mouth assay. Variability was observed in the growth of *Penicillium* spp. and the level of inhibition due to VOC production among the yeast strains (Figure 4.4). *Penicillium digitatum* exhibited a mean inhibition of 23%, indicating lower sensitivity to the treatments compared to *P. italicum* (48% mean inhibition) and *P. expansum* (49% mean inhibition). In contrast to the results obtained from the fungal spore germination assay, the mean susceptibility of *P. digitatum* to the yeasts was lower in the mouth-to-mouth assay. This suggests that VOC production was not the primary mode of action for most of the yeast strains and that *P. digitatum* was less sensitive to VOCs than the other yeasts. A similar trend was observed in the previous study where the yeasts showed 36% mean inhibition against a different strain of *P. expansum*, confirming that *Penicillium* spp. were less sensitive to VOCs.

Pichia kluyveri (Y64) demonstrated the highest inhibitory activity, achieving 100% inhibition against *P. expansum, P. digitatum* and *P. italicum* (Figure 4.4 A, B, C). According to Gomomo *et al*. (2022), the mechanism underlying this yeast's efficacy is its capacity to produce VOCs. Additionally, Cordero-Bueso *et al*. (2017) and Choińska *et al*. (2020) reported that VOCs produced by *Pi. kluyveri* exhibited antagonistic activity against *P. expansum.* Supporting these findings, studies by Ghasemi *et al*. (2015) and Liu *et al*. (2017) demonstrated that *Pichia* spp. including *Pi. kluyveri*, possess antimicrobial properties against *P. digitatum* and *P. italicum.*

Zygoascus hellenicus (Y89) exhibited growth inhibitory activity of 57%, 7% and 45% against *P. expansum, P. digitatum* and *P. italicum,* respectively (Figure 4.4 A, B, C). Arras *et al*. (1998) also confirmed the antagonistic effects of VOCs produced by *Z. hellenicus* against these *Penicillium* species. Gomomo *et al*. (2022) also reported that *Z. hellenicus* produces VOCs that

inhibit the growth of *P. expansum.* Additionally, yeast isolate *M. guilliermondii* (Y88) demonstrated antagonistic effects of 62%, 14% and 27% against *P. expansum, P. digitatum* and *P. italicum,* respectively (Figure 4.4 A, B, C). These results are consistent with the findings of Agirman and Erten (2020) and Choińska *et al*. (2020), who reported the ability of *M. guilliermondii* to produce VOCs that inhibit the growth of *Penicillium* spp. *in vitro*. The data indicate that the level of inhibition due to VOC production varies among the yeast species.

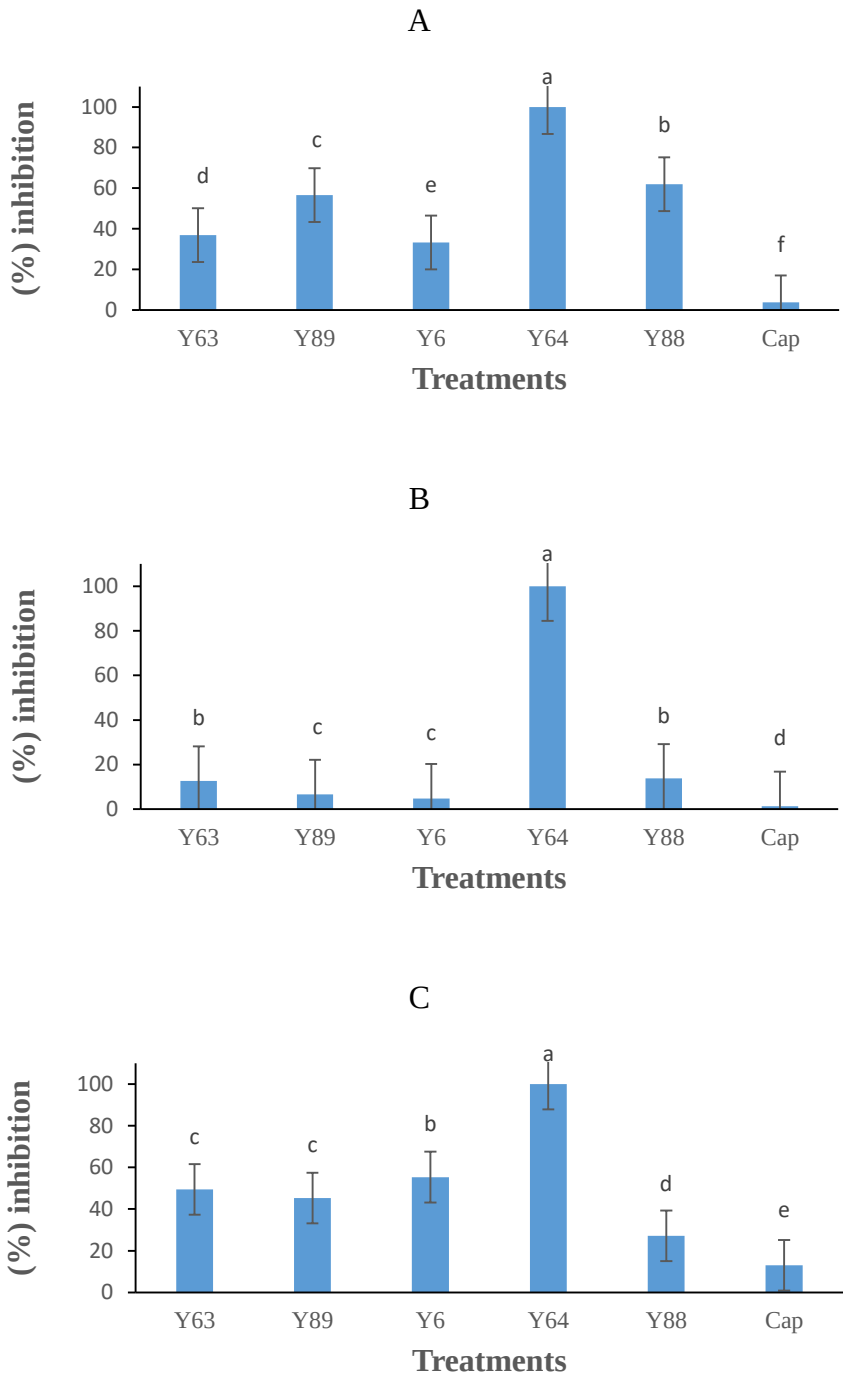

Figure 4.4: Inhibition activity (expressed as a percentage) of five yeast isolates (Table 4.1) and a commercial fungicide against *Penicillium expansum* (a), *P. digitatum* (b), and *P. italicum* (c) through the production of volatile organic compounds (VOCs). CAP represents Captan, a commercially available chemical fungicide. Results are presented as the means of three replicates, with standard deviations included. Different letters indicate statistically significant differences ($p \leq 0.05$). Negative control treatments, containing only the respective moulds, served as reference treatments for calculating growth inhibition.

4.3.3 Volatile organic compound extraction and gas chromatographic analyses

The yeasts *Pi. kluyveri* and *S. pyralidae* exhibited an antagonistic effect by producing VOCs that effectively inhibited the growth of *Penicillium* spp. (Figure 4.4). The specific VOCs responsible for inhibiting *P. expansum* were identified using SPME-GC–MS and a total of 29 compounds were detected across all the treatments. However, only seven were consistently present in all replicates. These seven organic compounds included alcohols (isobutanol, isoamyl alcohol, 2-phenylethanol), esters (isoamyl acetate, 2-phenethyl acetate), gamma butyrolactone (γ-decanolactone) and fatty acid methyl ester (methyl palmitate) (Table 4.2). The variation in VOC production among the treatments may be attributed to differences in metabolic processes, substrate utilisation, enzymatic activities, and environmental adaptation (Altieri, 2004; Contarino *et al.*, 2019).

Pichia kluyveri produced the same six VOCs in the absence and the presence of *P. expansum.* Isobutanol and 2-phenylethanol levels increased when *Pi. kluyveri* was grown together with *P. expansum.* Conversely, isoamyl alcohol, isoamyl acetate and 2-phenethyl acetate levels were higher when *Pi. kluyveri* was cultured alone, with their levels decreasing in the presence of *P. expansum* this might also help explain how *Pi. kluyveri* inhibits *P. expansum*. Isoamyl acetate and 2-phenethyl acetate levels decrease notably in the presence of *P. expansum* and are likely responsible for *P. expansum* growth inhibition.

Suhomyces pyralidae produced all seven VOCs when grown in the absence and in the presence of *P. expansum.* Although *S. pyralidae* did not demonstrate strong antagonistic activity against *P. expansum* (Figure 4.4 A), the concentrations of certain compounds, including isobutanol, isoamyl acetate, isoamyl alcohol and 2-phenethyl acetate, were elevated when the yeast was co-inoculated with *P. expansum*. Mould can potentially produce compounds that alter the

metabolic pathways of the biocontrol yeasts, leading to variations in the types and quantities of VOCs produced, additional the competition within the vial can lead the yeast to increase the production of VOCs as defence mechanism (Santos *et al*., 2022).

Isobutanol, 2-phenylethanol, isoamyl acetate, isoamyl alcohol and 2-phenethyl acetate are organic compounds produced by these two yeast isolates that are potentially linked to the inhibition of *P. expansum*. On studies conducted by Di Francesco *et al.* (2015) and Zhao *et al*. (2022) 2-phenylethanol was found to effectively inhibit the growth of *P. expansum, P. digitatum, P. italicum* and *Aspergillus carbonarius* on *in vitro* assays. These findings align with the current study, demonstrating that 2-phenylethanol produced by the biocontrol yeasts can be linked in inhibiting various fruit spoilage moulds. Additionally, Zhao *et al*. (2023) reported a 25% inhibition of blue mould on kiwifruit after 5 days of storage due to 2-phenethyl acetate.

Phenylethyl alcohol and 2-phenethyl acetate have been associated with the volatile metabolome of yeast strains exhibiting strong antifungal activity against *P. digitatum* in *in vivo* trials (de Souza *et al.,* 2018). Volatile organic compounds such as isoamyl acetate and isoamyl alcohol from *C. maltosa* were found to inhibit the spore germination of mycotoxin-producing mould *A. brasiliensis* (Ando *et al.,* 2012). These findings are consistent with the current study, which highlights the role of these VOCs in inhibiting mycotoxin-producing moulds. Additionally, isoamyl acetate was shown to completely inhibit the mycelial growth of the citrus spoilage mould *P. digitatum* under *in vitro* conditions (Pereyra *et al.,* 2022).

Table 4.2 Major volatile organic compounds (VOCs) produced by *Pichia kluyveri*, *Suhomyces pyralidae* and *P. expansum* PPRI 5654 when grown separately or together.

Volatile organic	***P. expansum***	***Pi. kluyveri***	***Pi. kluyveri* and *P. expansum***	***S. pyralidae***	***S. pyralidae* and *P. expansum***
	Average area ratio				
Isobutanol	0.005	0.006	0.013	0.009	0.014
Isoamyl acetate	ND	2.489	1.475	0.001	0.225
Isoamyl alcohol	0.057	0.136	0.102	0.140	0.224
2-Phenethyl acetate	0.016	3.207	2.665	0.002	0.024
2-Phenylethanol	0.039	0.155	0.211	0.021	0.020
γ-Decanolactone	ND	ND	ND	0.008	0.003
Methyl palmitate	0.050	0.012	0.010	0.001	0.001

*ND-Not detected

4.3.4 Evaluation of yeast antimicrobial peptides

The yeast cell-free extracts exhibited less than 10% inhibition against all three *Penicillium* spp. (Figure 4.5). These findings suggest that the modes of action are predominantly cell-associated, as evidenced by the limited inhibition of molecules smaller than 10 kDa. Macromolecules present in the supernatant above 10 kDa showed no inhibitory effects (data not shown). It is highly likely that mould inhibitors, such as proteins or peptides, are cell-associated or are not induced by the standard YM media composition. Once the cells were removed from the extract, the remaining compounds were ineffective in controlling the moulds. Although the inhibition observed with the supernatant was minimal, it indicates potential for future research to explore

the parameters influencing mould inhibitor production. Studies by Spadaro and Droby (2016) and Freimoser *et al*. (2019) demonstrated that competition is the most critical mechanism of biocontrol, and it is likely to occur when the cells are present. This aligns with the findings of this study, where higher inhibitory activity was observed in the presence of cells and not in the cell-free extracts (Figure 4.5). The antimicrobial activity of peptides is typically species- and strain-dependent, and structural differences in moulds may account for variations in sensitivity to specific peptides (Erwig and Gow, 2016; Thery *et al*., 2019).

4.3.5 Comparing the effectiveness of the yeasts and the commercial fungicides in controlling the germination of *Penicillium expansum, P. italicum* and *P. digitatum*

The yeasts and fungicides demonstrated varying degrees of inhibitory effects on the germination of *P. expansum, P. italicum* and *P. digitatum* (Figure 4.5). Captan exhibited the highest inhibitory activity, with 73% inhibition of *P. expansum* and complete (100%) inhibition of both *P. italicum* and *P. digitatum*. These findings are consistent with those of Rosenberger (2009), who also reported Captan's efficacy against *P. expansum*. Similarly, Guerrero Prieto *et al*. (2019) and Türkkan and Erper (2015) found Captan to be highly effective against various fruit spoilage moulds, outperforming yeasts and other organic compounds. The yeast *Aureobasidium melanogenum* (Y6) displayed inhibition comparable to Captan, achieving 100% inhibition of both *P. italicum* and *P. digitatum.* Additionally, the yeasts Y63 and Y88 also demonstrated significant inhibitory effects against all three moulds.

Protector 400 SC and Support 400 exhibited inhibitory effects on the germination of *Penicillium* spp. comparable to those observed with the yeasts (Figure 4.5). Similar results were reported in studies by Kanetis *et al*. (2008), Li and Xiao (2008) and Sánchez-Torres (2021), where the commercial fungicides azoxystrobin, fludioxonil and thiabendazole

effectively inhibited *P. expansum*, *P. digitatum* and *P. italicum*. These findings highlight the potential of biocontrol yeasts to be used in combination with or as alternatives to commercial fungicides. Implementing a rotation of chemical fungicides during the spraying season and/or post-harvest cleaning and disinfection is essential to mitigate the development of mould resistance to these chemicals. Additionally, alternating biocontrol yeasts with chemical treatments may help reduce the reliance on repeated applications of the same fungicide. While combining yeasts and fungicides may not always be effective due to the sensitivity of some yeasts to chemicals, the use of yeast-derived compounds in conjunction with fungicides could enhance mould control.

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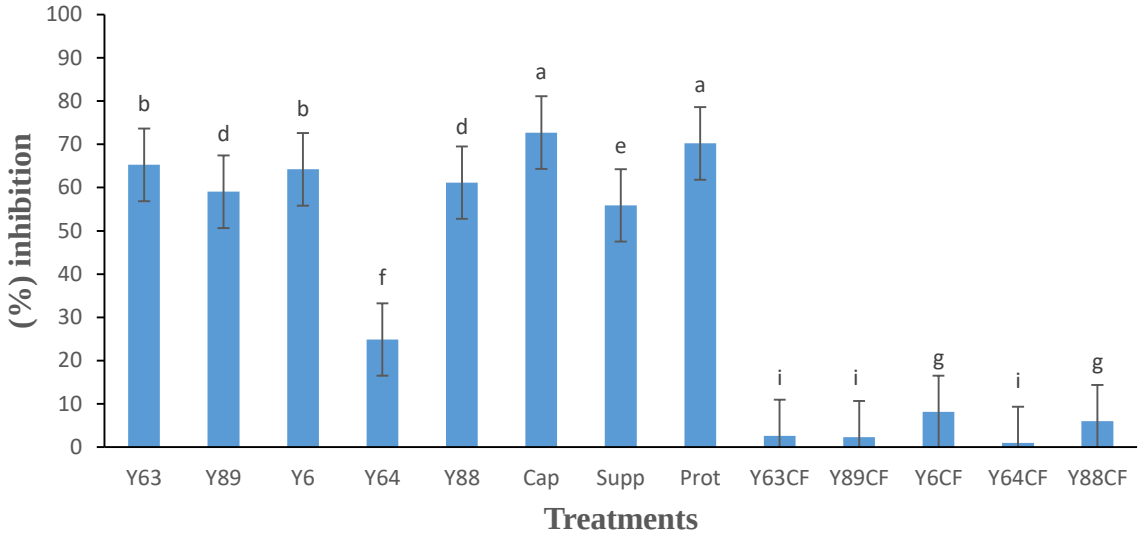
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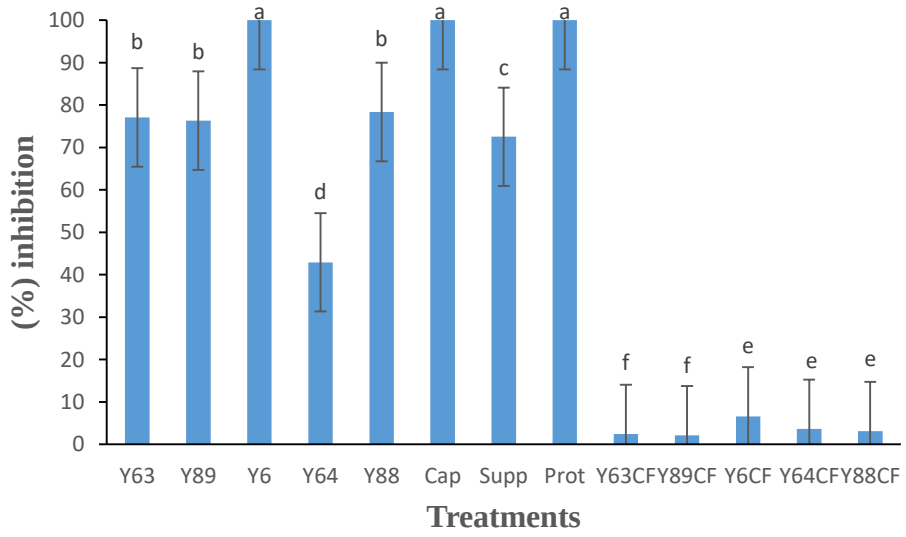
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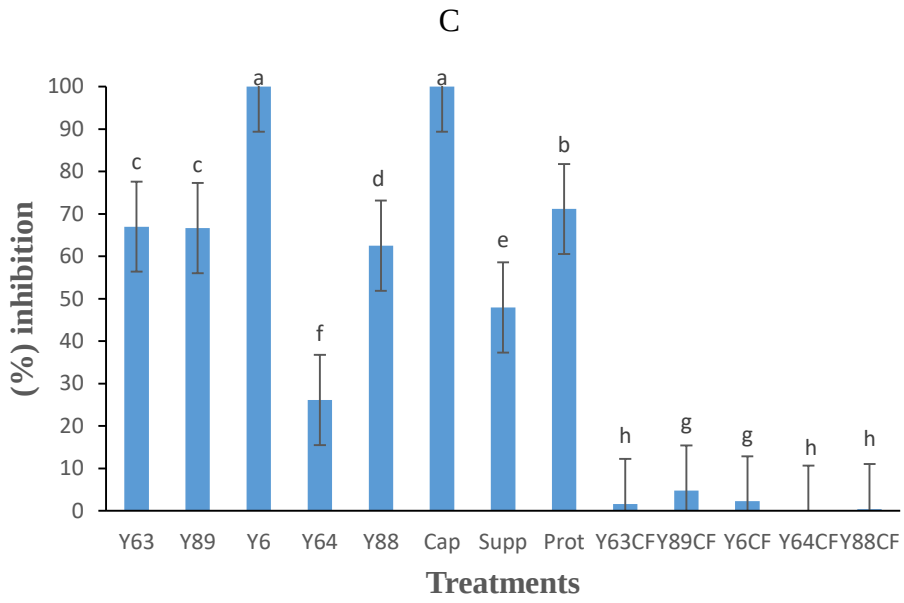

Figure 4.5: Germination inhibition activity (expressed as a percentage) of five yeasts, their cell free extracts (Table 4.1) and commercial fungicides (Captan, Support, Protection) against *Penicillium expansum* (a), *P. digitatum* (b) and *P. italicum* (c) using the radial inhibition assay. The values presented are the averages of three replicates, with standard deviations included. Different letters denote significant differences ($p \leq 0.05$) between treatments. The negative control plates contained only the corresponding moulds and acted as the baseline for evaluating growth inhibition.

4.3.6 Post-harvest fruit bioassays

The fruit bioassays revealed that *S. pyralidae* (Y63), *Pi. kluyveri* (Y64), *A. melanogenum* (Y6) and *M. guilliermondii* (Y88) were effective in controlling spoilage caused by *P. expansum*, *P. digitatum* and *P. italicum* (Figure 4.6 and 4.7). However, the inhibition of *P. expansum* observed in the *in vivo* apple bioassays (15% mean inhibition, Figure 4.6) was significantly lower compared to the *in vitro* assays (Figure 4.3 and 4.5) with Captan showing poor inhibition compared to the yeasts. This aligns with the findings by Gomomo *et al*. (2022) who also reported reduced growth inhibition activity against *P. expansum* in *in vivo* trials. Specifically, *Aureobasidium melanogenum* (Y6) demonstrated a 22% inhibition of *P. expansum* in these trials (Figure 4.6). These results are consistent with those reported by Kheireddine *et al*. (2021),

Zajc *et al.* (2022) and Cignola *et al*. (2024), who observed antagonistic effects of *Aureobasidium* spp. against *P. expansum* on apples, though with inhibition rates ranging from 40% to 90%. The variability in inhibition activity may be attributed to differences in yeast strains.

Pichia kluyveri (Y64) demonstrated a 19% inhibition of *P. expansum* on apples (Figure 4.6). Cordero-Bueso *et al*. (2017) similarly reported that *Pi. kluyveri* and other *Pichia* spp. exhibited antagonistic activity against a strain of *P. expansum* on grapes. These findings are consistent with the current study, confirming that *Pichia* spp. exert an antagonistic effect against *Penicillium* spp. under *in vivo* conditions. In contrast, the commercial fungicide showed only 7% inhibition against *P. expansum*, which was significantly lower than expected and contrary to the results observed in radial inhibition assays (Figure 4.3 and 4.5).

On oranges, *M. guilliermondii* (Y88) reduced the growth of *P. digitatum* by 72% and *P. italicum* by 77%, as shown in Figure 4.7A and 4.7B. This antagonistic effect of *M. guilliermondii* on the spore germination of *P. digitatum* and *P. italicum* on mandarin fruit has also been documented by Agirman and Erten (2020) and Wang *et al*. (2021). Similarly, *Pi. kluyveri* (Y64) inhibited *P. digitatum* and *P. italicum* by 55% and 73%, respectively. The inhibitory activity of *Pichia* species, including *Pi. kluyveri*, against these moulds on orange varieties such as Thomson navels and Newhall Sweet navels was as reported by Ghasemi *et al*. (2015) and Liu *et al*. (2017). *Suhomyces pyralidae* (Y63) exhibited 52% and 84% inhibition against *P. digitatum* and *P. italicum*, respectively, corroborating the work of Droby *et al*. (2002) and Liu *et al*. (2019), who observed similar inhibitory effects of *Suhomyces* spp. on *P. digitatum* and *P. italicum* on grapefruit and sweet oranges grown under *in vivo* conditions.

Captan proved more effective, with inhibition rates of 74% and 97%, against *P. digitatum* and *P. italicum*, respectively. In contrast, *Aureobasidium melanogenum* (Y6), which showed the highest inhibition activity in radial inhibition assays exhibited only 1% and 27% inhibition on oranges, a significantly lower efficacy compared to the fungal spore germination assay. The possible reason for *A. melanogenum* to show poor activity could be the lack of nutrients that could give the yeast an advantage to multiply and produce compounds to inhibit the mould. The antagonistic effects demonstrated by these biocontrol yeasts against *Penicillium* spp. showed their potential as alternatives to fungicides in the agricultural industry, offering a means to reduce fruit waste. Our results clearly show that some *Penicillium* species are more resistant to inhibition by yeast than others and that resistance might also differ on strain level. A biocontrol agent consisting of only one yeast species or strain might not be as effective in inhibiting different mould species or strains as a consortium.

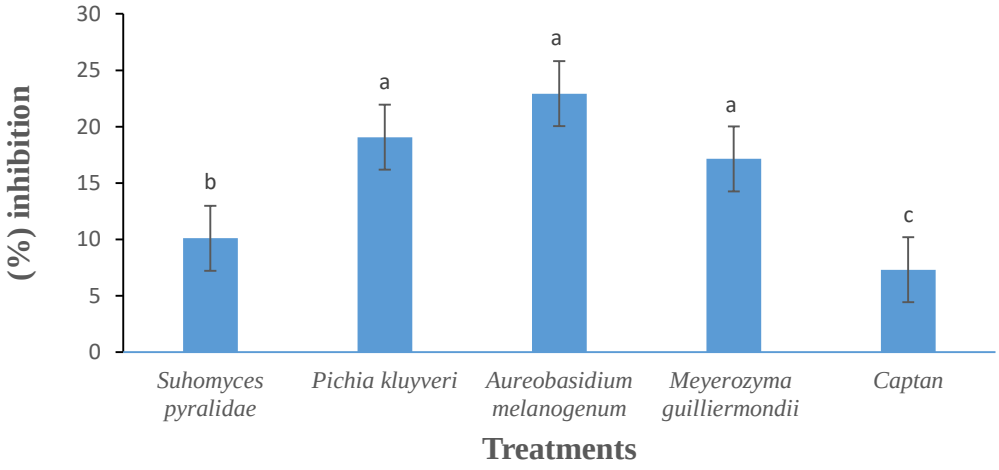

Figure 4.6: Growth inhibition activity (%) of *Meyerozyma guilliermondii* (Y88) *Suhomyces pyralidae* (Y63) *Pichia kluyveri* (Y64), and *Aureobasidium melanogenum* (Y6) against *Penicillium. expansum*, during post-harvest trials on apples. The values represent the averages from five replicates, with standard deviations included. Different letters signify significant differences ($p \leq 0.05$) between treatments. Each set corresponds to a representative sample of 25 apples. In the negative control treatments, the apples were solely infected with *P. expansum*, resulting in no observed growth inhibition. Captan is a commercially chemical fungicide.

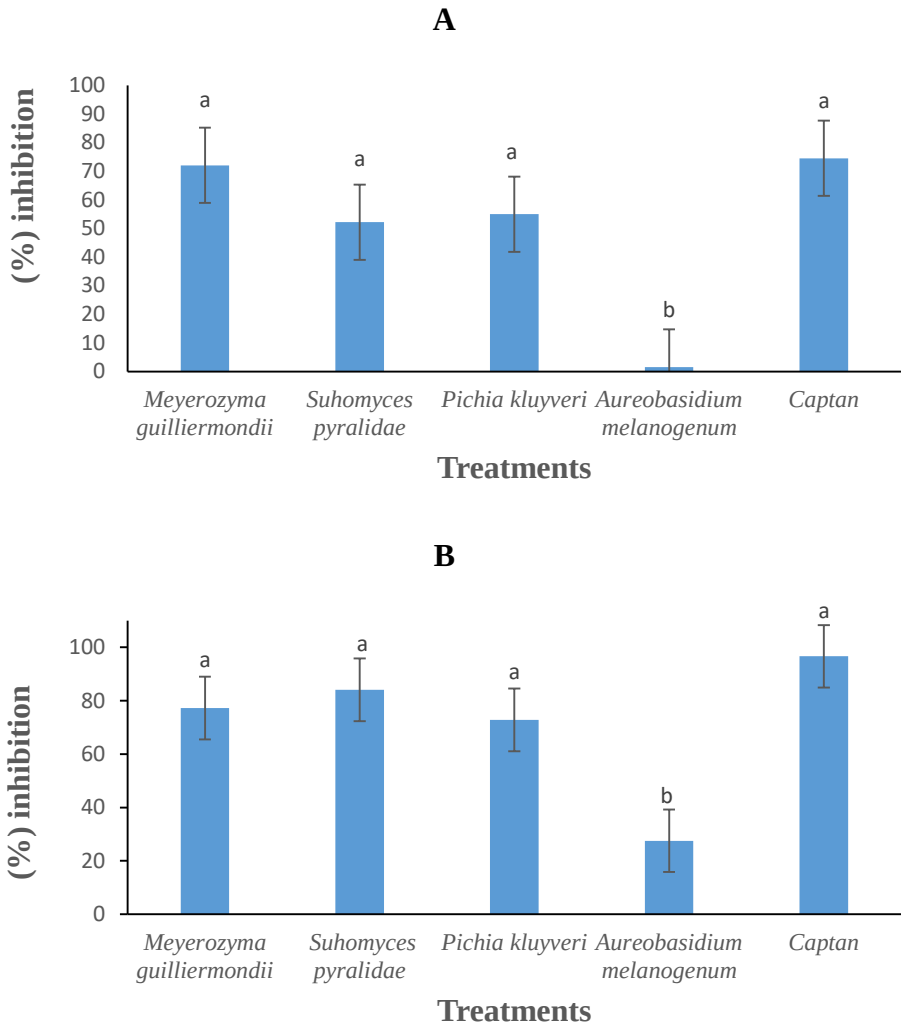

Figure 4.7: Growth inhibition activity (%) of *Meyerozyma guilliermondii* (Y88) *Suhomyces pyralidae* (Y63) *Pichia kluyveri* (Y64), and *Aureobasidium melanogenum* (Y6) against *Penicillium. digitatum* (a) and *P. italicum* (b) during post-harvest trials on oranges. The values represent the averages of five replicates, with the standard deviations provided. Different letters denote statistically significant differences ($p \leq 0.05$) between treatments. Each set represents a sample of 25 oranges. In the negative control treatments, the oranges were only inoculated with *P. digitatum* or *P. italicum*, resulting in no growth inhibition. Captan is a commercial chemical fungicide.

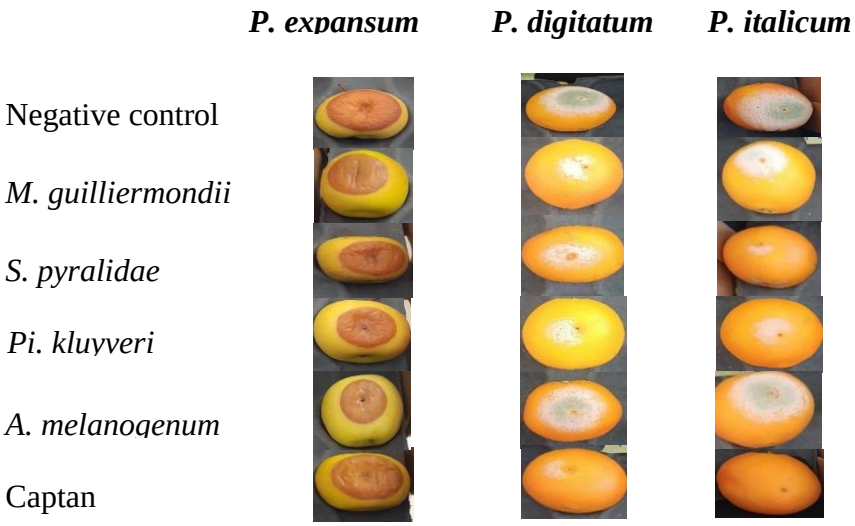

Figure 4.8: Representative samples of apples and oranges showing lesion diameters used to determine growth inhibition activity (%) of *Meyerozyma guilliermondii* (Y88) *Suhomyces pyralidae* (Y63) *Pichia kluyveri* (Y64), and *Aureobasidium melanogenum* (Y6) against *Penicillium expansum*, *P. digitatum* (B) and *P. italicum* (C) during post-harvest trials on oranges.

4.4 Conclusions

The cell suspensions of yeast strains *A. melanogenum* and *M. guilliermondii* exhibited a direct inhibitory effect on the spore germination of *P. expansum*, *P. digitatum* and *P. italicum.* Their efficacy was comparable to that of the commercial fungicides. *Pichia kluyveri* demonstrated an antagonistic effect against *Penicillium* spp. through the emission of VOCs. The primary VOCs responsible for this inhibitory activity were 2-phenylethanol, isoamyl acetate, isoamyl alcohol and 2-phenethyl acetate. During *in vivo* studies conducted on oranges, *M. guilliermondii*, *Pi. kluyveri* and *S. pyralidae* demonstrated the most effective antimicrobial properties against *P. digitatum* and *P. italicum.* The results also showed that *P. expansum* strain used in this study was less sensitive to yeast inhibition than the *P. digitatum* and *P. italicum* strains. Confirming how important it is to screen potential biocontrol agents against different

mould species and strains. The findings suggest that the application of these yeasts for biological control represents a viable alternative to chemical fungicides or can be considered for use in combination with chemical fungicides to mitigate post-harvest mould spoilage of fruits. This chapter, along with previous chapter highlights the potential of these yeasts as effective biocontrol agents against various fruit mould strains and species. However, additional research is required to better understand their interactions and long-term viability on fruit surfaces. The next chapter will delve into these areas, examining yeast interactions, post-drying viability and stability, as well as evaluating their Minimum Inhibitory Concentrations (MIC).

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CHAPTER 5

Evaluating Non-*Saccharomyces* Yeasts for Post-Harvest Biocontrol: Viability, Interactions, and Stability on Apples and Oranges

Abstract

Non-*Saccharomyces* yeasts exhibit potential as biocontrol agents for managing post-harvest diseases in apples and oranges. Despite extensive research on biocontrol methods, achieving sustainable agricultural practices require effective implementation and a comprehensive understanding of the interactions among biocontrol yeasts, host fruits and environmental conditions. The aim of this study was to investigate the interactions and efficacy of selected non-*Saccharomyces* yeasts on fruit surfaces, evaluate their stability following oven drying, and determine the minimum inhibitory concentration (MIC) required to inhibit mould growth. Three yeast strains, *Suhomyces pyralidae* (Y63), *Pichia kluyveri* (Y64), and *Meyerozyma guilliermondii* (Y88) were screened for compatibility using the growth inhibition seeding assay and co-inoculated in liquid medium. The yeasts were applied to fruit and their viability evaluated. Their viability on fruit surfaces and stability post-drying were also assessed. The MIC values were evaluated against *Penicillium italicum* and *Botrytis cinerea* using a radial inhibition assay. Results showed no antagonistic interactions between yeast strains; however, competition was observed when co-inoculated in liquid medium. The yeast isolates exhibited varying levels of survival on both apples and oranges. Viability on apples was highest for *S. pyralidae* (91%), followed by *M. guilliermondii* (38%) and *P kluyveri* (26%). On oranges, all yeast exhibited reduced viability, averaging 6%. The drying process led to a reduction in yeast viability, with *P. kluyveri* and *M. guilliermondii* maintaining 45% and 19% viability, respectively, while *S. pyralidae* showed a 99% decrease. In terms of inhibitory activity, *S. pyralidae* and *P. kluyveri* achieved complete (100%) inhibition of *B. cinerea* spore germination

at a concentration of 10^5 cells/mL, while *M. guilliermondii* demonstrated 96% inhibition at the same concentration. These findings indicate that these yeasts show potential as effective biocontrol agents for post-harvest disease management.

Keywords: Biocontrol yeasts, yeast interactions, post-harvest, oven drying, viability and minimum inhibition concentration

5.1 Introduction

Effective crop protection is vital for sustaining high productivity and crop quality. Fungal pathogens pose a major pre- and post-harvest threat, leading to significant economic losses and potential health risks. (Lamenew *et al.,* 2019; Sun *et al.,* 2021; Iqbal *et al.,* 2022). Controlling plant pathogens is therefore crucial, and microorganisms and their metabolites offer promising biotechnological solutions for sustainable crop protection across the food chain (Lamenew *et al.,* 2019; Pereyra *et al.,* 2020; Comitini *et al.,* 2023). Biological control has emerged as an environmentally and economically viable alternative to chemical fungicides (Wisniewski *et al.,* 2016; Lamenew *et al.,* 2019; Pereyra *et al.,* 2020; Huang *et al.,* 2021). Yeasts, which naturally inhabit diverse environments including fruit and vegetable surfaces, have been widely explored for biocontrol applications against various plant pathogens (Freimoser *et al*., 2019; Lamenew *et al*., 2019; Zhimo *et al*., 2020 Gao *et al*., 2021).

Interest in non-*Saccharomyces* yeasts has grown, with numerous studies highlighting their potential as biocontrol agents against specific fungal pathogens (Freimoser *et al*., 2019; Lamenew *et al*., 2019; Casas-Godoy *et al.,* 2021). These yeasts combine strong antifungal activity with advantageous properties such as antagonistic efficacy, culturability, formulatability, ease of application and stress resistance, enhancing their suitability for

biocontrol (Wisniewski and Droby 2012; Freimoser *et al*., 2019; Hernandez-Montiel *et al*., 2021). Given that major post-harvest diseases are typically caused by moulds, many biocontrol agents target these pathogens effectively by competing for essential resources such as nutrients, oxygen and space (Zhang *et al*., 2010; Spadaro and Droby, 2016; Lamenew *et al*., 2019).

Biocontrol yeasts interact with other microorganisms within their own or different species, and with host fruits during post-harvest stages, providing essential ecological functions (Topalović and Heuer, 2019; Agirman *et al*., 2023). Interactions between these antagonist yeasts and the fruit hosts have been extensively studied, revealing their critical role in various biocontrol systems (Sui *et al.,* 2015; Spadaro and Droby, 2016). The potential of biocontrol yeasts in reducing fruit decay depends on their ability to colonise fruit surfaces and adapt to diverse environmental conditions in the field and during storage (Tian *et al.,* 2004; Sharma *et al.,* 2014; Pereyra *et al.,* 2020).

Rapid colonisation is influenced by yeast concentration and host fruit species, as specific antagonists have nutrient preferences (Lamenew *et al.,* 2019). Species such as *Candida oleophila, Clavispora lusitaniae* and *Pichia fermentans* serve as biocontrol models, with several commercial products available to control post-harvest fruit diseases (Jijakli *et al.,* 1993; Droby *et al.,* 1998; Lahlali *et al.,* 2004; Pe´rez *et al.,* 2016, 2017, 2019). The response varies by fruit and cultivar, and depends on physiological maturity (Spadaro and Droby, 2016).

A primary challenge in using biocontrol yeasts is their limited tolerance to fluctuating environmental conditions and the difficulties in developing a stable formulation; in addition, proprietary restrictions limit accessible information on microorganism formulation (Carbó *et*

al., 2018; Palazzini *et al.*, 2020). During the production process, biocontrol agents encounter various severe abiotic and biotic stresses that affect their viability (Sui *et al.*, 2015; Lorenz *et al.*, 2020). Preservation techniques, such as drying, have been used to enhance biocontrol yeast stability (Casas-Godoy *et al.*, 2021). However, further research is required to understand effects of environmental factors on biocontrol systems, especially regarding yeast viability and efficacy (Lahlali and Jijakli, 2009; Liu *et al.*, 2013; Sui *et al.*, 2015). This study aimed to investigate the interactions between *S. pyralidae, P. kluyveri* and *M. guilliermondii*, evaluate their viability on apple and orange surfaces, assess their stability post-drying, and determine the minimum inhibitory concentration against *P. italicum* and *B. cinerea.*

5.2 Materials and Methods

5.2.1. Selection and screening of yeast strains

Three yeast strains, *Suhomyces pyralidae* (Y63), *Pichia kluyveri* (Y64) and *Meyerozyma guilliermondii* (Y88) were obtained from the culture collection of the Agricultural Research Council (ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa). These yeasts were selected based on prior research by Gomomo *et al*. (2022). A cross-screening procedure was performed whereby the selected yeasts were screened against each other. To prepare the yeast cultures, a loopful of each pure yeast colony from the yeast malt agar (YMA) plates was transferred to test tubes containing 5 mL of sterilised yeast malt broth (YMB) and incubated at 28°C for 2 days, as described by Gomomo *et al.* (2022). A modified seeding growth inhibition assay was applied, following the methodology of Mewa-Ngongang *et al*. (2019b).

In this assay, YMA plates were seeded with each yeast strain (*S. pyralidae*, *P. kluyveri*, or *M. guilliermondii*) at a concentration of 1×10^6 cells/mL, used as the sensitive yeast. The same yeasts were then tested as antagonistic ("killer") yeasts against each other, with the cell

concentrations adjusted to 1×10^8 cells/mL. Ten microlitre of each culture were spotted onto the seeded YMA plates. As a positive control, the commercial fungicide, N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide, commonly known as Captan (800 g/kg; Universal Crop Protection (Pty) Ltd, Kempton Park, South Africa) was applied at a concentration of 0.5 g/L. The plates were then incubated at 28°C for four days, with each treatment replicated three times. Inhibition zones, indicating antimicrobial activity, were identified by clear areas surrounding the yeast colonies or the Captan control (Figure 5.1).

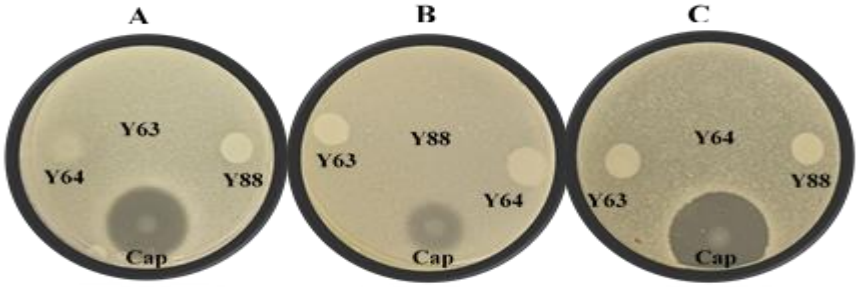

Figure 5.1: (A) Antagonistic activity of *Pichia kluyveri* (Y64) and *Meyerozyma guilliermondii* (Y88) against *Suhomyces pyralidae* (Y63). (B) Antagonistic activity of *S. pyralidae* and *P. kluyveri* (Y64) against *M. guilliermondii* (Y88). (C) Antagonistic activity *S. pyralidae* and *M. guilliermondii* against *P. kluyveri.* Each plate is a representative example of three YMA plates replicates. Cap – the commercial fungicide (N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide), common name Captan at a concentration of 0.5 g/L.

5.2.2 Biocontrol yeasts interactions

The yeast strains *S. pyralidae* Y63, *P. kluyveri* Y64 and *M. guilliermondii* Y88 were cultured by transferring pure yeast colonies into test tubes containing 5 mL of sterilised yeast malt broth (YMB) and incubating them at 28°C for 2 days. Cells were counted as described by Gomomo *et al*. (2022). To prepare individual and combined inocula, 1×10^6 cells/mL of each yeast strain was transferred into fresh YMB. For the co-culture experiments, each yeast strain was co-inoculated at 1×10^6 cells/mL into the same YMB. The yeast treatment conditions are detailed in Table 5.1, with each treatment replicated three times. The test tubes were incubated at 28°C

for 6 days, and samples were plated on YMA after 1 day and after 6 days of incubation. The YMA plates were further incubated at 28°C for 2-3 days to observe yeast growth and interactions.

Table 5.1 Treatments used in test tubes that contain yeast malt broth during yeast interaction assay.

Treatments	Treatment description
1	*Suhomyces pyralidae*
2	*Pichia kluyveri*
3	*Meyerozyma guilliermondii*
4	*S. pyralidae + P. kluyveri*
5	*S. pyralidae + M. guilliermondii*
6	*P. kluyveri+ M. guilliermondii*
7	*S. pyralidae + P. kluyveri + M. guilliermondii*

5.2.3 Yeast viability on fruits

Two-day-old yeast cell suspensions (5 mL) of *S. pyralidae*, *P. kluyveri* and *M. guilliermondii* were transferred to Erlenmeyer flasks containing 100 mL of sterile YMB and incubated at 28°C with constant agitation at 150 rpm using a rotary shaker (LM-53OR, RKC Instrument Inc., Ohta-ku Tokyo, Japan) for 2 days. After incubation, the cultures were centrifuged at 10,000 rpm for 15 min at 4°C. The cell-free supernatant was discarded, and the yeast cell pellets were stored in a -20°C freezer for 1 day. The yeast pellets were then resuspended in 500 mL of sterile distilled water. The mixed treatment containing all three yeasts was prepared in a 1:1:1 ratio.

Five treatments were evaluated in a post-harvest biocontrol viability assay on "Cara Cara" navel oranges and "Golden Delicious" apples. Each experimental unit consisted of a rectangular fruit-packaging box holding five oranges or five apples, with each treatment replicated six times. The fruits were washed, surface-sterilised by spraying with 70% ethanol (v/v) and allowed to dry. After drying, the fruits were submerged in the yeast cell formulation for 2 min to ensure even coating, following the method by Lahlali *et al*. (2009) (see Figure 5.2).

The control treatments were submerged in sterile purified water. The treated fruits were incubated at ±20°C for 7 days. After incubation, the fruits were washed with 30 mL saline solution, agitated at 150 rpm using a rotary shaker (LM-53OR, RKC Instrument Inc., Ohta-ku Tokyo, Japan) for 20 min, with rotations at 5 min intervals. Samples were then plated out on YMA, and 1 mL aliquot of each washing solution was collected for serial dilutions. Each treatment was plated on YMA after 1 day and again after 7 days, with plates incubated at 25°C for 2-3 days to assess yeast viability on the fruit surfaces.

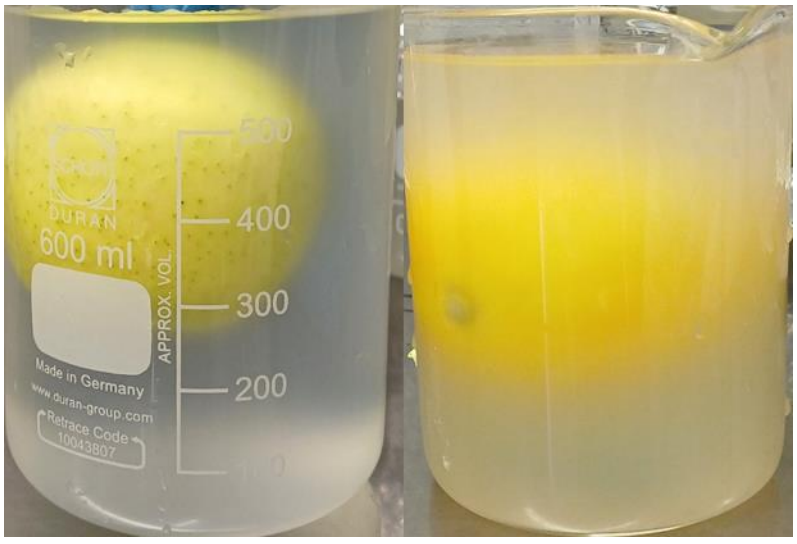

Figure 5.2: Representative samples showing a submerged apple and an orange in yeast cell formulation.

5.2.4 Drying and stability of yeasts

Yeasts cultures were prepared by transferring 100 µL of the 2-day-old culture into fresh 10 mL of YMB and incubating for 3 days. One millilitre of each culture was then transferred to a sterile 2 mL microtube and centrifuged at 13,400 rpm for 5 min. The supernatant was discarded, and the resulting yeast pellets were dried in a laboratory oven at 33°C for 3 days, with three replicates for each treatment. Yeast viability was assessed by plating the cultures before and after drying. Dried yeast pellets were stored at room temperature (22°C) and plated to assess viability at 3, 7 and 21 days post-drying. The YMA plates were incubated at 28°C for 2-3 days to determine yeast survival.

5.2.5 Evaluation of Minimum Inhibiting Concentration

The minimum yeast cell concentration of *S. pyralidae, P. kluyveri* and *M. guilliermondii* required to inhibit the growth of *Penicillium italicum* PPRI 10380 and *Botrytis cinerea* PPRI 30807 was determined using radial inhibition assay, described by Núñez *et al*. (2015). Fresh yeast cell suspensions (5 mL) were prepared as previously detailed, with serial dilutions ranging from 10^2 to 10^7 cells/mL for each yeast strain. The diluted yeast suspensions were plated on YMA, and once the plates were dry, a 20 µL spore suspension (1×10^5 spores/mL) of *P. italicum* and *B. cinerea* was spotted at the centre of each YMA plate (Figure 5.3). Negative control plates received only the spore solution, while positive controls contained the fungicide Captan at a concentration of 0.5 g/L. The YMA plates were incubated at 25°C for 4 days to observe mould inhibition.

The mould radial inhibition (MRI) was calculated using the mathematical expression:

$$MRI = [(D_0-D_t)/D_0] \times 100$$

with D_0 representing the average diameter of the mould growth on the negative control plates and D_t representing the diameter of the mould growth on the yeast-treated plates (Núñez *et al.*, 2015).

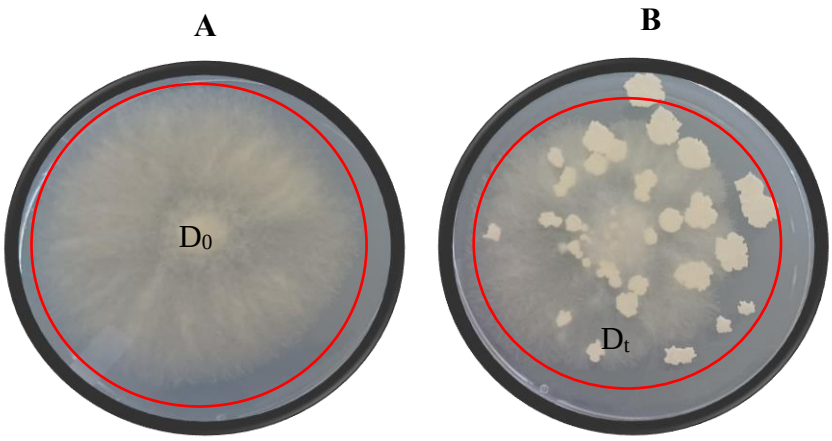

Figure 5.3: Illustration of *Botrytis cinerea* growth (A) and the inhibitory effect of the yeast isolate on *B. cinerea* (B) on yeast malt agar. D_0 denotes the colony diameter on the untreated control plates, while D_t indicates the colony diameter on plates treated with the yeast isolate. Each plate shown is a representative example from three replicates.

5.3 Results and Discussion

5.3.1 Cross-screening of biocontrol yeasts

Cross-screening assays were conducted to assess potential antagonistic activity among *S. pyralidae* Y63, *P. kluyveri* Y64, and *M. guilliermondii* Y88 on YMA plates. Results indicated that no inhibition zones were observed between any of the yeast strains (Figure 5.1), suggesting no antagonistic effects among them. This compatibility supports the potential use of these strains as a combined biocontrol consortium to combat fruit spoilage moulds, as also noted by Sipiczki (2016), who highlighted that yeast interactions often yield inhibition "halos" or zones due to nutrient competition or inhibitory compound release.

The potential of the individual yeast strains for pathogen inhibition, previously documented by Gomomo *et al.* (2022) and current studies, underscores the possible enhanced biocontrol effectiveness when applied as a combination. Interestingly, *S. pyralidae* did not exhibit antagonistic effects against *M. guilliermondii* Y88, contrasting prior findings by Mewa-Ngongang *et al*. (2019b), where *S. pyralidae* inhibited *M. guilliermondii* Y0848 *in vitro* assays. This suggests that antagonistic activity may be strain dependent. Mewa-Ngongang *et al*. (2019b) demonstrated that *S. pyralidae* and *P. kluyveri* exhibited no antimicrobial activity against each other, a finding corroborated in the current study. The commercial fungicide Captan, used as the positive control, produced inhibition zones for all three yeast strains, with *M. guilliermondii* Y88 showing comparatively lower sensitivity. This observation implies that further studies should investigate fungicide tolerance in these biocontrol yeasts to assess their compatibility with chemical fungicides.

5.3.2 Biocontrol yeast interactions

The interactions of the biocontrol yeasts when co-cultured in a liquid medium were further examined, revealing both direct and indirect dynamics shaped by mutualism and competition (Topalović & Heuer, 2019; Zhang *et al.*, 2020). The interactions showed that after inoculation, *S. pyralidae* showed notable growth when grown alone, with cell counts rising from 1.9×10^7 to 5.7×10^7 cells/mL. However, co-inoculation with *P. kluyveri* and *M. guilliermondii* led to reduced cell counts, likely due to nutrient competition (Figure 5.4A).

Pichia kluyveri, exhibiting high individual growth (7.8×10^7 cells/mL), showed a notable decrease when co-inoculated with *S. pyralidae*, with cell counts declining from 3.9×10^7 to 1.0×10^7 cells/mL (Figure 5.4B). Conversely, co-inoculation with *M. guilliermondii* stimulated

growth of *P. kluyveri* from 2.2×10^7 to 3.9×10^7 cells/mL, although subsequent co-culturing with both *M. guilliermondii* and *S. pyralidae* resulted in declines over time (Figure 5.4B).

For *M. guilliermondii*, cell counts initially dropped from 2.3×10^7 to 1.8×10^7 cells/mL when cultured alone. However, co-inoculation with *S. pyralidae* resulted in a modest increase from 9×10^6 to 1.7×10^7 cells/mL (Figure 5.4C). While *S. pyralidae* and *P. kluyveri* thrived when inoculated individually, both showed diminished growth when cultured with other strains, possibly due to resource competition (Figure 5.4). Although no inhibition zones were observed on solid media (Figure 5.1), these results from liquid medium reveal that resource competition can still lead to growth disadvantages in mixed cultures.

Schmitt and Breinig (2002) and Sipiczki (2016) found that the inhibition among yeast strains was due to the antagonistic isolate secreting a toxic agent into the medium, which killed the sensitive yeast. Additionally, Giometto *et al*. (2021) showed that competitive antagonism in a liquid medium allows stronger antagonists to outcompete weaker ones if initial population thresholds are met. Some yeasts may also promote the growth of others by breaking down complex substrates, such sugars and proteins, into simpler molecules that can be accessible to other yeast species (Sipiczki, 2016). This may explain the modest growth observed when *M. guilliermondii* was co-cultured with *S. pyralidae* (Figure 5.4A, C). Factors such as nutrient availability and spatial constraints influence yeast interactions, with varying inhibitory compound production in each strain (Celik Ozgen *et al.*, 2018; Granato *et al.*, 2019; Giometto *et al*., 2021).

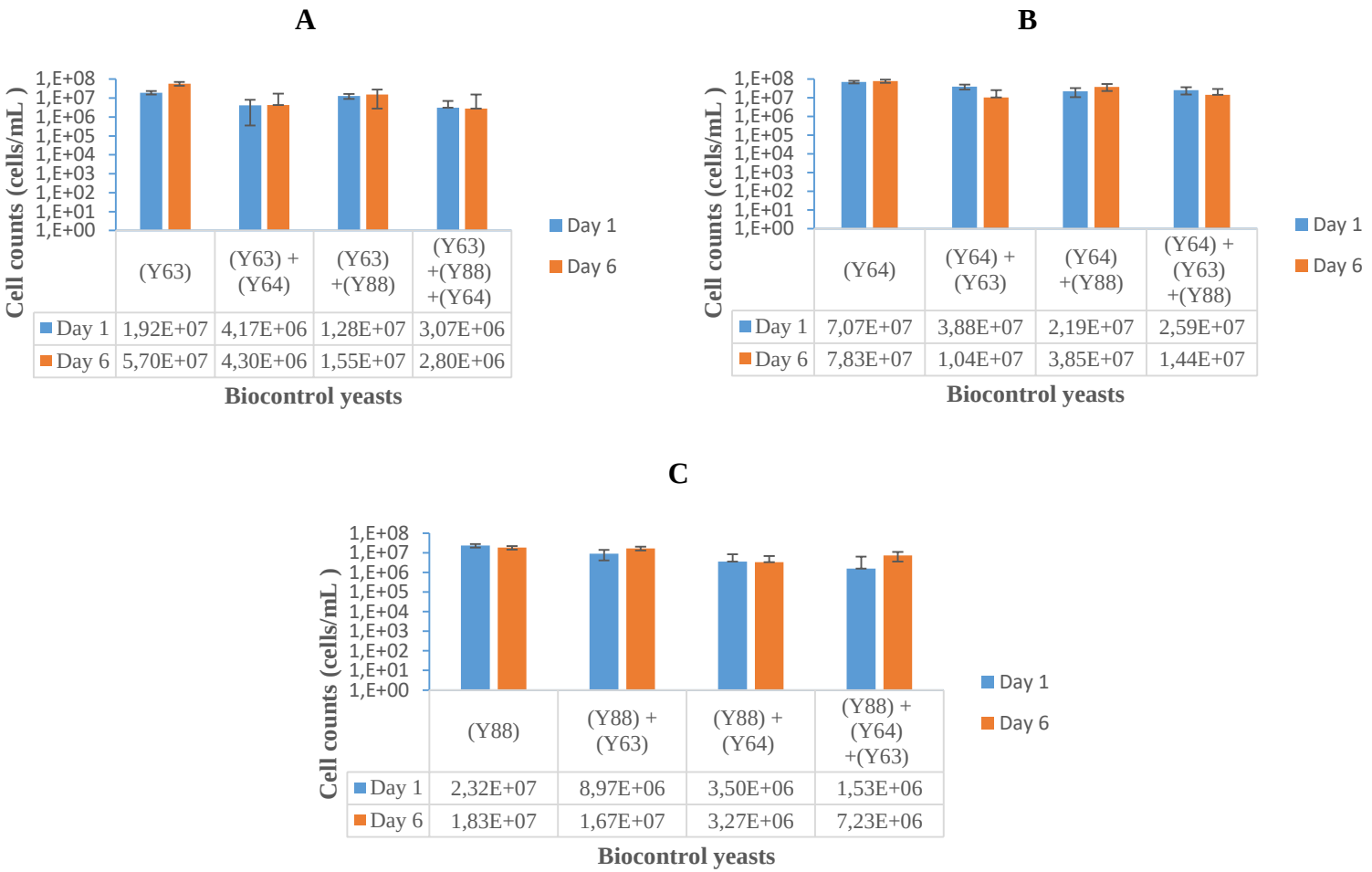

Figure 5.4: Yeast cell counts after (A) *Suhomyces pyralidae* (Y63), (B) *Pichia kluyveri* (Y64) and (C) *Meyerozyma guilliermondii* (Y88) were inoculated individually and with the other two yeast and grown for 6 days.

5.3.3 Viability of biocontrol of yeasts on fruits

To determine the survival duration of biocontrol yeasts on fruit surfaces, the yeasts were applied to apples and oranges, and monitored over 7 days. The initial cell concentration of the yeasts ranged from 10^5 to 10^6 cells/mL (Figure 5.5). The yeasts were applied individually and as a mixture of the three yeasts (*S. pyralidae*, *P. kluyveri* and *M. guilliermondii*) at a ratio of 1:1:1. *Suhomyces pyralidae* started at a concentration of 3.3×10^5 cells/mL on both apples and oranges (Figure 5.5A, B). On apples, it adapted well, increasing to 1.7×10^6 cells/mL after 1 day (Figure 5.5A). However, after 7 days, its cell count dropped to 3×10^5 cells/mL, maintaining 91% viability. On oranges, *S. pyralidae* showed a rapid decline of cell numbers to 1×10^4 cells/mL after day 1, resulting in 97% viability loss and struggled to adapt, showing only 10% viability with 3.2×10^4 cells/mL by day 7 (Figure 5.5B).

Meyerozyma guilliermondii, with an initial concentration of 1.8×10^6 cells/mL, decreased to 5.4×10^5 cells/mL after day 1, reflecting 30% viability on apples (Figure 5.5A). By day 7, cell concentration reached 7×10^5 cells/mL, an 8% increase in viability compared to day 1. On oranges, its concentration decreased to 1.2×10^5 cells/mL (7% viability) after day 1, and it maintained a similar trend through day 7 with a concentration of 1.3×10^5 cells/mL (Figure 5.5B).

The initial cell concentration of *P. kluyveri* was 3.7×10^5 cells/mL, which was lower than *M. guilliermondii* but higher than *S. pyralidae*. On apples, *P. kluyveri* displayed moderate adaptation, increasing to 3.8×10^5 cells/mL by day 1, but then decreased to 9.7×10^4 cells/mL by day 7, displaying 26% viability (Figure 5.5A). On oranges, *P. kluyveri* showed a poor response, decreasing to 3.6×10^3 cells/mL by day 1 (99% viability loss), with cell counts of 6.4×10^3 cells/mL by day 7, and only 2% viability. Across both fruit types, *M. guilliermondii* was the

most stable yeast over time, while *P. kluyveri* was the least viable, with *S. pyralidae* performing moderately, but thriving more on apples (Figure 5.5A, B).

The initial cell concentration for the yeast mixture was 2.2×10^6 cells/mL. On apples, the population decreased to 1.2×10^6 cells/mL by day 1 (55% viability), potentially indicating early competitive or environmental stress (Figure 5.5A). By day 7, the mixture's cell count was 3.6×10^5 cells/mL, maintaining a higher population than *S. pyralidae* and *P. kluyveri* individually, though its viability was only 17%, likely due to resource-sharing dynamics as suggested by Sui *et al*. (2015) and Spadaro and Droby (2016).

On oranges, the mixture declined to 3.1×10^5 cells/mL after day 1, representing 14% viability (Figure 5.5B), suggesting that oranges may be less favourable due to their high acidity (Haïssam, 2011; Liu *et al.*, 2012; Sui *et al.*, 2015) and natural antimicrobial compounds, such as flavonoids and essential oils (Jing *et al*., 2014. Calo *et al*., 2015; Spadaro and Droby, 2016). By day 7, the cell count was 1.8×10^5 cells/mL, outperforming *S. pyralidae* (3×10^4 cells/mL), *P. kluyveri* (6×10^3 cells/mL) and *M. guilliermondii* (3×10^4 cells/mL) applied individually (Figure 5.5B), although its viability (8%) remained low compared *S. pyralidae* individually.

Qian *et al*. (2020) reported that the population of the yeast *Rhodotorula mucilaginosa* on apple surfaces incubated at 20°C exhibited a gradual increase from 0 to 48 h. This observation supports their findings that certain yeast species experience a slight initial population growth within the first 24 h of incubation, although this can vary depending on the yeast species and environmental conditions. Similar studies Lahlali *et al.* (2009) and Li *et al.*, (2011) observed population growth of the yeasts *Pichia anomala* and *Rhodotorula mucilaginosa* on apples. However, the current study reveals a different trend. Lahlali *et al*. (2011) and Aloui *et al.* (2015)

found that the yeasts *Pichia guilliermondii* and *Wickerhamomyces anomalus* remained stable for the first 24 h of incubation on oranges, followed by an increase reaching a maximum concentration of 3.4×10^7 cells/mL, which contrasts with findings of this study These discrepancies may stem from differences in yeast species, environmental factors, and the physiological characteristics of the fruit, all of which are crucial for yeast viability (Spadaro and Droby, 2016).

Studies on *Candida sake* show that populations increased significantly on grape berries within 24 h of incubation under both controlled and outdoor conditions (Calvo-Garrido *et al.*, 2014). Quantitative analyses of cultivable populations showed notable increases during grape ripening (Martins *et al.*, 2015). However, it should be noted that grape berries differ from other fruits like apples and oranges in terms of surface characteristics and environmental interactions, which can affect yeast population dynamics and viability. Understanding the factors influencing yeast survival and stability on fruit surfaces is essential, as viability is crucial for sustaining biocontrol efficacy. Yeasts need to remain viable on fruit surfaces to effectively inhibit the growth of spoilage microorganisms and pathogens (Hershkovitz *et al.*, 2013; Massart *et al.*, 2015; Spadaro and Droby, 2016). Any decrease in cell numbers could reduce the antagonistic effect of the biocontrol yeasts.

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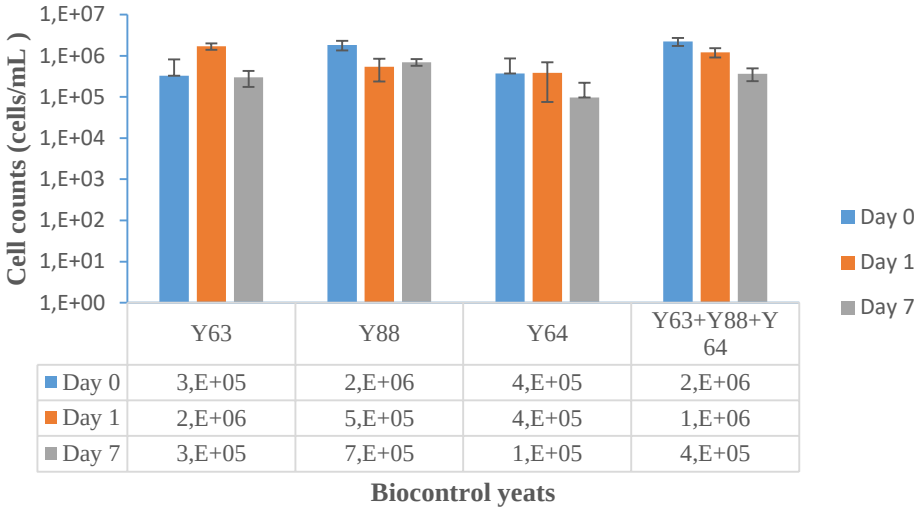
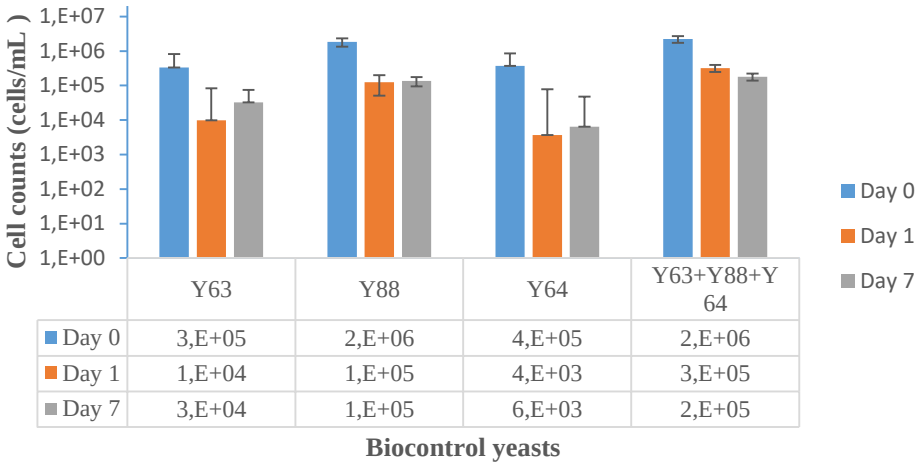


	Y63	Y88	Y64	Y63+Y88+Y64
Day 0	3,E+05	2,E+06	4,E+05	2,E+06
Day 1	2,E+06	5,E+05	4,E+05	1,E+06
Day 7	3,E+05	7,E+05	1,E+05	4,E+05

Biocontrol yeats

B



	Y63	Y88	Y64	Y63+Y88+Y64
Day 0	3,E+05	2,E+06	4,E+05	2,E+06
Day 1	1,E+04	1,E+05	4,E+03	3,E+05
Day 7	3,E+04	1,E+05	6,E+03	2,E+05

Biocontrol yeasts

Figure 5.5: *Suhomyces pyralidae* (Y63), *Pichia kluyveri* (Y64), *Meyerozyma guilliermondii* (Y88) viability in colony forming units/mL on apples (A) and oranges (B) tested individually and in combination with each other.

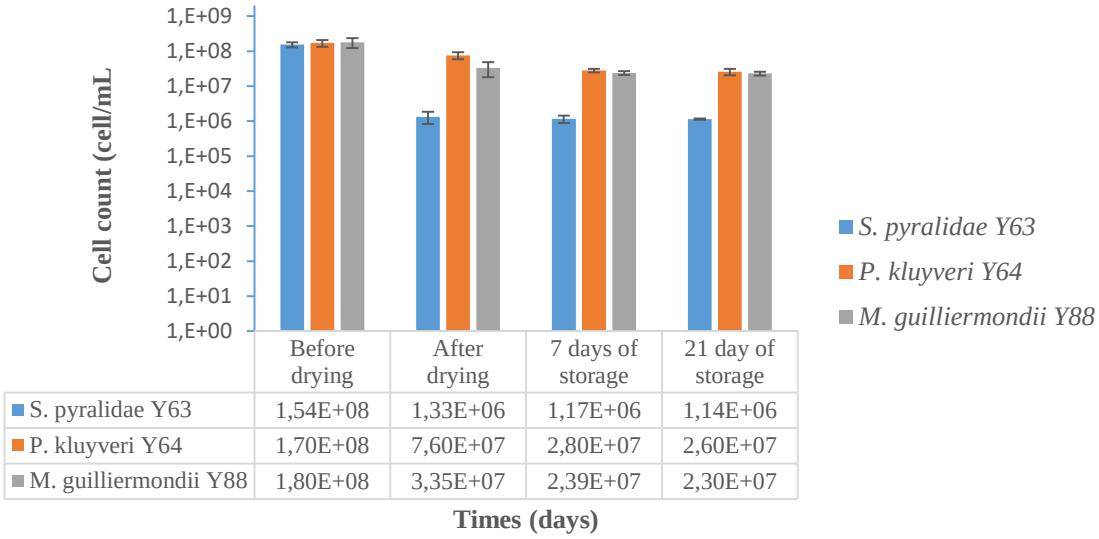
5.3.4 Post-drying stability of biocontrol yeasts

The stability of *S. pyralidae*, *P. kluyveri* and *M. guilliermondii* was assessed following drying at 33°C. The yeast *S. pyralidae* began with an initial cell concentration of 1.5×10^8 cells/mL, which decreased to 1.3×10^6 cells/mL after 3 days of drying and further to 1.2×10^6 cells/mL after 7 days. By day 21, viable cells decreased to 1.1×10^6 cells/mL (Figure 5.6), indicating a 99% average viability loss from the drying process through storage.

Pichia kluyveri, initially at 1.7×10^8 cells/mL, showed a decline in cell concentration to its lowest count of 2.6×10^7 cells/mL after 21 days (Figure 5.6). After drying, *P. kluyveri* showed 45% viability, which decreased to 16% over the storage period. *Meyerozyma guilliermondii*, with an initial concentration of 1.8×10^8 cells/mL, dropped to 3.3×10^7 cells/mL after drying, reflecting 19% viability. Viability declined to 2.4×10^7 cells/mL after 7 days, and further to 2.3×10^7 cells/mL after 21 days of storage (Figure 5.6), maintaining an average viability of 13% viability over this period. All three yeasts showed a rapid initial viability loss after drying but reached stability, with minimal decline observed during storage.

According to Alp and Bulantekin (2021), microbial cell viability is more stable in a dry state. During the drying process, yeasts experience mechanical, structural, and oxidative constraints that impact cell components including the cell wall, plasma membrane, mitochondria, vacuoles, peroxisomes, lipid droplets, and nucleus (Abee and Wouters, 1999; Van De Guchte *et al.*, 2002; Rapoport *et al.*, 2019; Casas-Godoy *et al.*, 2021). This stress likely contributed to the initial rapid viability decrease observed in this study. Júnior *et al*. (2018) and Casas-Godoy *et al*. (2021) reported that the yeasts can survive prolonged dry periods by entering an anhydrobiotic state, reducing metabolic activity, which may explain the limited decline in cell numbers during storage.

Previous studies on *Naumovia castellii* and *Lachancea kluyveri* showed an average of 50% viability after drying, with cells dried in the presence of trehalose 16% viability increase, suggesting the supplement's role in preserving yeast viability and efficacy when applied to fruit surfaces (Rodríguez-Porrata *et al*., 2010). Notably, the drying impact on viability varies by yeast species, reflecting species-specific metabolic and life cycle characteristics (Casas-Godoy *et al.*, 2021). Factors such as drying conditions and physical properties such as pellet size (Van Engeland *et al.*, 2019), can affect post-drying viability. Additionally, incorporating solid carriers before drying can improve yeast quality (Casas-Godoy et *al.*, 2021). The ability of these yeasts to endure drying and remain stable during storage highlights their potential as viable biological control agents in agricultural applications.



	Before drying	After drying	7 days of storage	21 day of storage
S. pyralidae Y63	1,54E+08	1,33E+06	1,17E+06	1,14E+06
P. kluyveri Y64	1,70E+08	7,60E+07	2,80E+07	2,60E+07
M. guilliermondii Y88	1,80E+08	3,35E+07	2,39E+07	2,30E+07

Figure 5.6: *Suhomyces pyralidae* (Y63), *Pichia kluyveri* (Y64), *Meyerozyma guilliermondii* (Y88) viability in colony forming units/mL before and after drying.

5.3.5 Evaluation of the Minimum Inhibitory Concentrations

The minimum inhibitory concentrations (MICs) were evaluated for *S. pyralidae* Y63, *P. kluyveri* Y64 and *M. guilliermondii* Y88. Recent studies confirm that these yeast strains effectively inhibit *P. italicum* and *B. cinerea* growth and maintain viability on fruit surfaces (Figure 5.5), with continued survival following drying and storage (Figure 5.6). *Suhomyces*

pyralidae achieved complete (100%) inhibition of *B. cinerea* PPRI 30807 spore germination at a concentration of 10^5 and 53% inhibition at 10^4 cells/mL. At concentrations between 10^2 and 10^3 cells/mL, the yeast demonstrated an average inhibition of 19% (Figure 5.7A).

Pichia kluyveri completely inhibited *B. cinerea* PPRI 30807 growth at a minimum concentration of 10^5 cells/mL, while achieving 37% inhibition at 10^4 cells/mL. The yeast displayed an average inhibition of 9% at cell concentrations ranging from 10^2 to 10^3 cells/mL (Figure 5.7A). *Meyerozyma guilliermondii* showed 96%, 35% and 19% inhibition against *B. cinerea* PPRI 30807 at cell concentrations 10^5, 10^4 and 10^3 cells/mL, respectively (Figure 5.7A). All three yeasts displayed 100% inhibition at concentrations 10^6 and 10^7 cells/ mL against *B. cinerea*.

In contrast, inhibition levels were reduced against *P. italicum* PPRI 10380, and none of the yeasts showed complete inhibition (Figure 5.7B). *Suhomyces pyralidae* demonstrated 86%, 45% and 32% inhibition of *P. italicum* PPRI 10380 spores at cell concentrations 10^7, 10^6 and 10^5 cells/mL, respectively, with an average inhibition of 14% at concentrations ranging from 10^2 to 10^4 cells/mL (Figure 5.7B). *Pichia kluyveri* exhibited 42%, 34% and 24% inhibition against *P. italicum* PPRI 10380 at cell concentrations 10^7, 10^6 and 10^5 cells/mL, respectively, and achieved an average inhibition of 15% at lower concentrations (10^2, 10^3 and 10^4 cells/mL) (Figure 5.7B). The yeast *M. guilliermondii* achieved 27% average inhibition against *P. italicum* PPRI 10380 at concentrations ranging from 10^2 to 10^7 cells/mL.

The results suggest that a concentration of 10^5 cells/mL is sufficient for the three yeast isolates to inhibit *B. cinerea* spore germination. However, even at a concentration of 10^7 cells/mL, they could not fully prevent *P. italicum* spore germination. These findings are consistent with

Cordero-Bueso *et al*. (2017), who reported that, *P. kluyveri* and *M. guilliermondii* inhibit the growth of *B. cinerea* within a similar concentration range of 10^3 to 10^6 cells/mL. Additionally, Cordero-Bueso *et al*. (2017) observed that, *P. kluyveri* and *M. guilliermondii* effectively inhibit the growth of *P. expansum* within the same concentration range, supporting the findings of this study on the antagonistic activity of these yeasts against *Penicillium* species. Maluleke *et al.* (2022) further reported that *Pichia kudriavzevii* inhibits *B. cinerea* at concentrations as low as 10^2 cells/mL.

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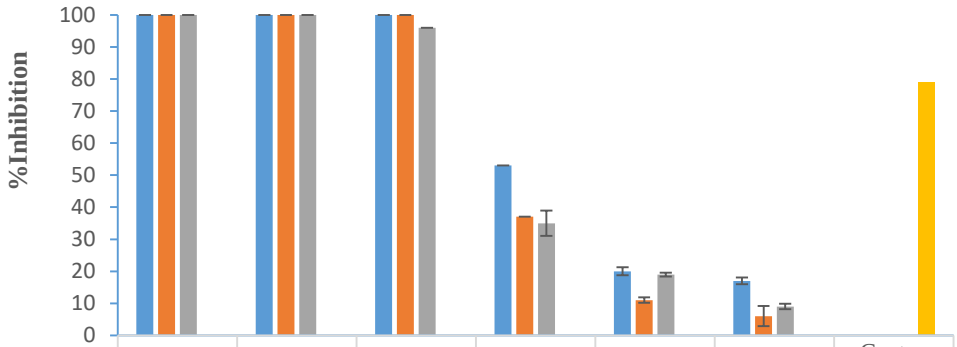
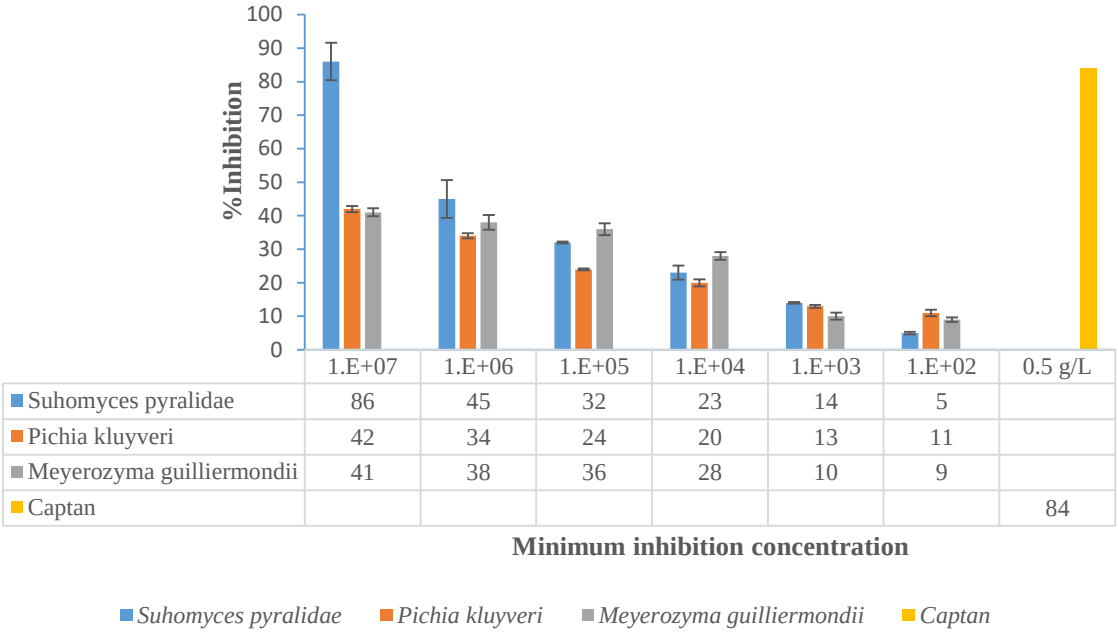


	1.E+07	1.E+06	1.E+05	1.E+04	1.E+03	1.E+02	Captan (0,5 g/L)
Suhomyces pyralidae	100	100	100	53	20	17	
Pichia kluyveri	100	100	100	37	11	6	
Meyerozyma guilliermondii	100	100	96	35	19	9	
Captan							79

B



	1.E+07	1.E+06	1.E+05	1.E+04	1.E+03	1.E+02	0.5 g/L
Suhomyces pyralidae	86	45	32	23	14	5	
Pichia kluyveri	42	34	24	20	13	11	
Meyerozyma guilliermondii	41	38	36	28	10	9	
Captan							84

Figure 5.7: Minimum inhibitory concentration (MIC) of three yeast isolates (*Suhomyces pyralidae* Y63, *Pichia kluyveri* Y64, *Meyerozyma guilliermondii* Y88) in colony-forming units/mL, and Captan (Cap), a commercial fungicide, against *Botrytis cinerea* PPRI 30807 (A) and *P. italicum* PPRI 10380 (B) determined through the radial inhibition assay. Data represent the means of three replicates, with standard deviations included.

5.4 Conclusion

The study demonstrated that the three yeast strains (*S. pyralidae*, *P. kluyveri*, and *M. guilliermondii*) did not exhibit antagonistic effects against each other, suggesting their potential for co-application without adverse interactions that could compromise their efficacy. However, it was noted that the commercial fungicide inhibited all three yeasts, indicating they cannot be co-applied with the commercial fungicide at the industrial concentrations (0.5 g/L). A synergistic relationship was observed between *S. pyralidae* and *M. guilliermondii* as co-inoculation resulted in an increase in cell numbers for both species. Furthermore, *M. guilliermondii* demonstrated a gradual recovery in cell population over time when inoculated together with the other yeasts. *Suhomyces pyralidae* adapted well to the apple surface, exhibiting an increase in cell numbers and maintaining the highest viability among the three

yeasts. In contrast, *P. kluyveri* showed the highest viability after the drying process, and all three yeasts showed stability throughout the storage period. These findings suggest that further investigation into the interactions among these yeast strains could provide valuable insights into the underlying mechanisms contributing to their observed behaviour.

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CHAPTER 6

General Conclusions and Recommendations

6.1 Summary and Conclusions

The agricultural industry plays a critical role in global food security, with fruits serving as a vital component of a balanced diet and a significant contributor to the global economy. Ensuring the quality of fruits throughout production, harvest, transportation, and storage is essential for meeting the nutritional and economic demands of a growing global population. To ensure fruit quality, it is essential to protect them from mould-induced spoilage at all stages. Traditional chemical fungicides are effective but present challenges such as fungicide resistance, health risks, and environmental concerns.

This study investigates the potential of biocontrol yeasts as sustainable and environmentally friendly alternatives to chemical fungicides. It evaluates the antifungal activity of *Suhomyces pyralidae, Aureobasidium melanogenum, Meyerozyma guilliermondii*, and *Zygoascus hellenicus* against multiple strains of *Botrytis cinerea* and species of *Penicillium*. The findings offer a viable solution to integrated pest management (IPM) by presenting yeasts as effective, natural biocontrol agents.

This study confirmed that the selected yeasts inhibit mould growth through various mechanisms, including competition for nutrients and space, rapid colonisation, secretion of extracellular enzymes (proteases, chitinases, glucanases, pectinases), and the release of volatile organic compounds (VOCs), as evidenced by dual culture, spore germination, and VOC assays. Dual culture, spore germination, and VOC assays revealed that these mechanisms effectively inhibit the growth and spore germination of *B. cinerea* and *Penicillium* species.

Novel findings included the discovery of *Pichia kluyveri* (Y64) achieving complete inhibition of various *Penicillium* species. The VOCs identified, including alcohols (isobutanol, isoamyl alcohol, 2-phenylethanol) and esters (isoamyl acetate, 2-phenethyl acetate), displayed inhibitory effects that varied with the level of competition between yeast and mould. Synergistic effects were observed with less-studied VOCs, such as isobutanol, which contributed to enhanced mould. Specific VOCs identified in this study and their unique inhibitory effects against these pathogens had not been reported.

Yeasts perform comparably to commercial fungicides, with added advantages such as multiple antifungal mechanisms and the potential for rotation with chemical fungicides to mitigate resistance. The study highlighted the yeasts' effectiveness in controlling spoilage on apples, strawberries, and oranges, emphasising the role of fruit characteristics on the efficacy of biocontrol.

Preventive and curative applications of yeasts were both effective. Preventive treatments inhibited spore germination, while curative treatments controlled mould growth even after infection had begun. Furthermore, yeast-to-yeast interactions showed no antagonism. The findings here contribute new insights into the potential for combining yeasts in biocontrol formulations, particularly for enhancing efficacy. This research also identified the importance of post-harvest viability and stability of yeasts. Although the yeasts survived post-harvest drying processes, further optimisation is required to enhance their viability for commercial applications. Protective agents and improved drying methods may strengthen yeast formulations, ensuring long-term efficacy during storage and transportation. The study underscores the potential of biocontrol yeasts to reduce reliance on chemical fungicides, improve food safety, and support sustainable agricultural practices.

This research demonstrates that yeasts such as *A. melanogenum*, *S. pyralidae*, *Z. hellenicus*, and *M. guilliermondii* employ diverse antifungal mechanisms, including enzymatic activity, space and nutrient competition, and VOC production, to inhibit mould spoilage.

- *A. melanogenum* produced antimicrobial compounds effective against mycelial growth, while *S. pyralidae*, *M. guilliermondii*, and *P. kluyveri* inhibited spore germination of *B. cinerea* and *Penicillium* species.
- *P. kluyveri* exhibited unique VOC-mediated antagonism, achieving complete inhibition of certain *Penicillium* species.
- *S. pyralidae*, *A. melanogenum*, and *P. kluyveri* inhibited *B. cinerea* on apples and strawberries, while *M. guilliermondii* was most effective against *P. digitatum* and *P. italicum* on oranges.

These yeasts maintained viability after drying and demonstrated strong antifungal activity even at lower concentrations, comparable to commercial fungicides. The findings highlight their potential as sustainable alternatives for post-harvest mould control in the agricultural industry.

6.2 Recommendations

To advance the practical application of biocontrol yeasts, future research should focus on several key areas. Efficacy assessment should involve investigating the individual and combined efficacy of biocontrol yeasts across a broader range of fruits and under various pre- and post-harvest conditions. Additionally, the synergistic effects of volatile organic compounds (VOCs) and their specific roles in mould inhibition should be evaluated. Integrated approaches should explore the combination of biocontrol yeasts with other microorganisms, such as antagonistic bacteria, to enhance antifungal efficacy. It is also important to assess the feasibility of integrating biocontrol yeasts with reduced concentrations of commercial fungicides to minimise resistance development.

Formulation development should focus on optimising the drying processes of biocontrol yeasts, particularly those explored in the current study, and investigating protective agents to improve yeast viability and stability during storage and transport. Furthermore, the development of powdered formulations capable of maintaining antifungal activity under diverse environmental conditions is essential. Large-scale field trials should be conducted to validate laboratory findings on the effectiveness of yeast applications in preventing fungal diseases during pre-harvest. These trials should also assess the long-term stability and efficacy of biocontrol yeasts in enhancing crop protection before harvest. By addressing these areas, biocontrol yeasts can be effectively integrated into agricultural practices, promoting environmentally sustainable and economically viable mould management solutions.