Fungistatic and fungicidal properties of *Candida pyralidae* Y1117, *Pichia kluyveri* Y1125 and *Pichia kluyveri* Y1164 on the biocontrol of *Botrytis cinerea*

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***Abstract*— The agro-processing industry is currently facing losses due to microbial spoilage of agricultural produce and associated value-added products such as beverages. To address this, synthetic chemicals that have potential human health and environmental effects have been widely used to control microbial spoilage. As a result, a bioprospecting approach that uses biological systems e.g. yeast as biocontrol agents is increasingly being considered in the food industry. The aim of the current study was to investigate the effect of varying inoculum dose (ID) of *Candida pyralidae* strain Y1117, *Pichia kluyveri* Y1125 and *Pichia kluyveri* Y1164 for the biocontrol of *Botrytis cinerea*. The headspace of the growth medium was contaminated with a fungal plug subsequent to biotreatment with different initial inoculum dose of the respective biocontrol agents. The results obtained showed that the fungistatic and fungicidal effects on the fungal pathogen was dose dependent. The fungistatic characteristics against *Botrytis cinerea* were displayed after 7 days when 10^2-10^5 cells mL^{-1} of *Candida pyralidae* Y1117, *Pichia kluyveri* Y1125 and *Pichia kluyveri Y1164* were independently used in-vitro and in-vivo. However, 10^6-10^8 cells mL^{-1} inoculum doses displayed fungicidal characteristics. Additionally, the fungicidal property of yeasts studied was also confirmed on table grape (in vivo studies) using closed jars.**

***Keywords*—** *Botrytis cinerea*, *Candida pyralidae*, Fungicidal, Fungistatic, *Pichia kluyveri*, Volatile Organic Compounds (VOCs)

I. Introduction

Fruit spoilage caused by fungal pathogens is an agricultural and post-harvest challenge [1], [2]. A significant quantity of fruit is lost annually during post-harvest processing and transportation [3]-[5]. Generally, *Botrytis cinerea* is one of the major fungal spoilers of table grapes [6], [7]. Currently, synthetic chemicals with fungicidal properties have been used in order to reduce microbial spoilage of grapes and other fruits. It has also been widely reported that the currently used synthetic chemicals pose serious health concerns that necessitates an alternative to synthetic preservatives for fruit producers and processors [8]. The use of yeasts as biological control agents is a better alternative to the use of synthetic chemicals because of the ability to compete for nutrients, space, and to grow at a faster rate than fungal pathogens [9]-[12]. The inhibitory effect of yeasts has also been attributed to the production of volatile organic compounds (VOCs) [13], [14]. Although yeasts are known to inhibit fungal growth by releasing volatile organic compounds, their fungistatic and the fungicidal effects at varying inoculum doses has never been reported for the biocontrol of *Botrytis cinerea*. The aim of the study was therefore to assess the fungistatic and fungicidal activity of the yeasts *Candida pyralidae* strain Y1117, *Pichia kluyveri* strain Y1125 and *Pichia kluyveri* strain Y1164 against *Botrytis cinerea* in-vitro and in-vivo, and to determine the minimum inoculum dose (MID) of yeast required to completely inhibit *Botrytis cinerea* in a quantified contaminated headspace, while confirming these properties on table grapes.

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II. Materials and Methods

A. Yeasts and Fungi Selection

Previously, *Candida pyralidae* strain Y1117, *Pichia kluyveri* strain Y1125 and *Pichia kluyveri* strain Y1164 were collected from the ARC Infruitec-Nietvoorbij (Post-Harvest Control Laboratory) and screened against various fruit fungal pathogens, including *Botrytis cinerea* [15]. They were then selected for the purpose of this study.

B. Microorganisms Culture Condition and Inoculum Preparation

Chenin Blanc grape pomace was collected from the ARC Infruitec-Nietvoorbij research cellar and prepared according to [15]. The yeasts were cultured in a grape pomace medium (GPM) at 150 rpm in a shaking incubator (LM-530R Orbital) for 24 hrs at 22 ^{o}C [16]. The resulting fermentation broth was centrifuged for 5 min at 5000 rpm, and the cell pellets were

collected and resuspended in sterile distilled water. The re-suspended cells were serially diluted from 10^1-10^7 cells mL^{-1}. For the grape bioassay, the cell suspensions to be sprayed were diluted in GP broth adjusted to a sugar level of 150 $g.L^{-1}$. The fungal plugs were prepared by excising a 5 mm disk from a 7 day old plate [17].

C. Volumetric Headspace Quantification

The volume of the headspace in the agar plates was calculated by considering the diameter and the height of the closed 90 mm diameter petri dish, using the approach described by [16]. The thickness (15 mm) of the empty petri dish was determined and the headspace volume was obtained by accounting for the volume of grape pomace agar (GPA) poured on the plate. The volume of the poured medium was also determined and subtracted from the total. The evaluation of the actual headspace in contact with the fungal pathogen was done to determine the fungistatic and fungicidal effect of the biological control agents when a specific inoculum dose was used in a quantified headspace.

D. In-vitro Test: Mouth to Mouth Assay

As described by [17], the mouth to mouth method was used. In brief, two GPA plates facing each other were used in each biotreatment. The bottom plate contained the biological control yeast and the top plate contained the centred 5 mm fungal plug. The biological control plates were prepared by spreading 100 µL of each dilutions (10^1, 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 cells mL^{-1}) for different yeasts. The fungal plates were prepared in three replicates by placing the 5 mm plug at the centre of each GPA plate. All the yeast-inoculated plates were aligned (mouth to mouth) with the fungal-contaminated plates, and then sealed with laboratory film. For the negative controls, also in three replicates, the fungal plates were aligned with uninoculated GPA plates. All plates were incubated at 15 ^oC for 7 days. The fungicidal characteristics were evaluated by opening the plates for an additional 7 days under similar conditions to allow VOCs to escape from the plates.

E. In-vivo test: Jar method using table grapes

For each control and treatment, 9 jars, containing 300 g of Regal Seedless table grapes each, were wounded [14] and allowed to dry for 15 minutes. The biological control yeasts sprayed on the berries were also allowed to dry for about an hour. The treated berries previously placed on storage trays were then infected by spraying the fungal pathogen at a concentration of 10^5 spores mL^{-1} into the jars. The grapes were then placed in 500 mL volume jars and sealed with laboratory film. The negative control were free of the yeasts as biological agents. The jars were incubated at 15 ^oC for 7 days. Subsequently, after 7 days, the jars were opened and further incubated at 15 ^oC until the negative control was completely rotten. The treated jars were then compared with the negative control in order to evaluate and possibly confirm the fungicidal behaviour of the VOCs from the biocontrol yeasts. These results were assessed by monitoring the presence or absence of fungal growth after the jars had been exposed to open air.

III. Results and Discussion

A. Headspace Quantification

Biocontrol studies have been done previously but none focused on the impact of headspace on microbial inhibition. In this study, the headspace volume in which the growth inhibition occurred was quantified. The volume covered by the GPA poured onto the petri dish was found to be 12.7 mL. The actual headspace in which the efficacy of the VOCs produced by the biological control took place was 111 mL compared to 400 mL in vivo (total volume of headspace in the jar used).

B. Efficacy of the VOCs In-vitro

The evaluation of the effect of VOCs in a closed and quantifiable headspace was carried out. *Candida pyralidae* strain Y1117, *Pichia kluyveri* Y1125 and *Pichia kluyveri* Y1164 inhibited the growth of *Botrytis cinerea* at different inoculum dose. The 10^1 cell mL^{-1} treatments were mostly similar to the negative control (Fig 1 A_a, B_a and C_a) whereas the inoculum dose of 10^2- 10^5 cells mL^{-1} showed the fungistatic characteristics (Fig. 1 A, B, $C_{b,c,d,e}$). The confirmation was also made when fungal growth was observed after opening the plates. Fungal growth inhibition was also displayed in 10^6-10^8 cells mL^{-1} plates (Fig 1 A, B, $C_{f,g,h,}$). However, no fungal growth was observed after opening the plates. This was a clear indication that higher inoculum doses completely inhibit fungal growth whereas lower inoculum doses temporarily suppresses growth until VOCs escape from the container.

C. Efficacy of the VOCs In-vivo

To confirm the fungicidal effect observed in-vitro, the in-vivo test was carried-out using a grape bioassay. The fungicidal effect of *Candida pyralidae* strain Y1117, *Pichia kluyveri* Y1125 and *Pichia kluyveri* Y1164 was observed as a resultant of VOCs released (Fig. 2). Compared to the negative controls, fungal growth was still completely inhibited after leaving the jars for five weeks for all tested yeast (Fig 2). The growth inhibition of *Botrytis cinerea* by VOCs from yeasts have been reported in literature [14], [18]-[20] and comparing those findings to the results obtained in this work, it can be noted that the method used in this work and the VOCs produced by *Candida pyralidae* Y1117, *Pichia kluyveri* Y1125 and *Pichia kluyveri* Y1164 completely inhibited the growth of *Botrytis cinerea* on table grapes. Furthermore, the findings from this work clearly demonstrated the fungicidal potential of *Candida pyralidae* Y1117, *Pichia kluyveri* Y1125 and *Pichia kluyveri* Y1164 for the biocontrol of *Botrytis cinerea*. The minimum inoculum dose (MID) strategy proved beneficial in exploring the interactive relationship between yeasts and fruit fungal pathogens.

For all yeasts, the MID of 10^6 cells $mL^{-1}$was sufficient to display fungicidal activity against *Botrytis cinerea*. Since a more preventative approach against *Botrytis cinerea* was adopted in this study, the inoculum dose and headspace findings could be used to model and optimise biofungicide activities of yeasts under commercial transportation and storage condition.

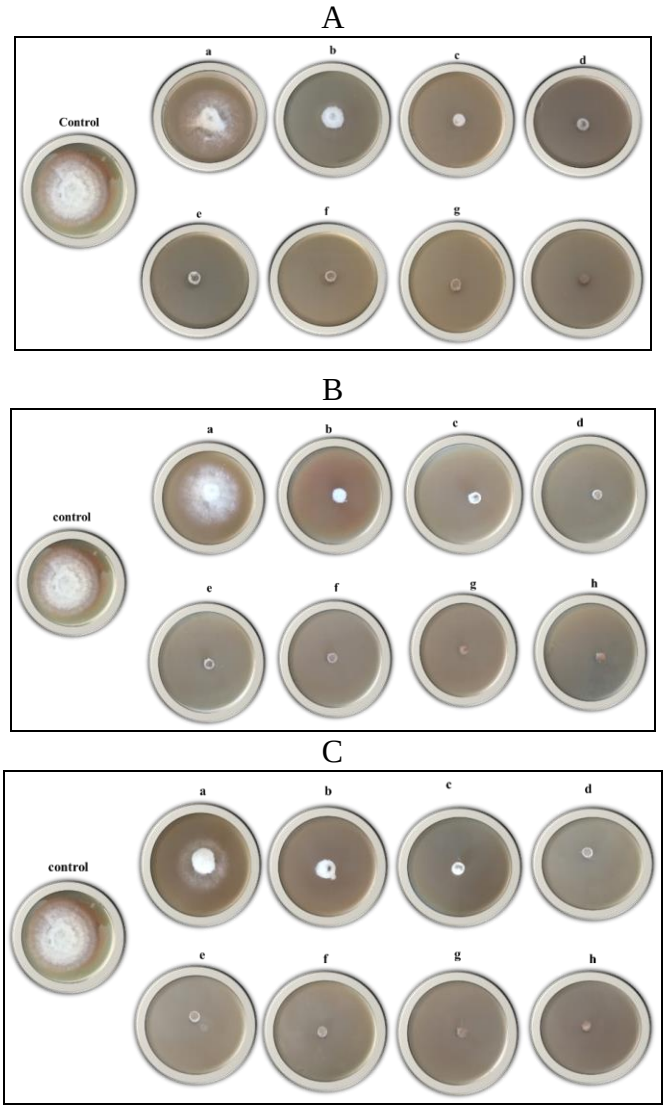

Fig. 1: Representative agar plates showing the in-vitro fungistatic and fungicidal effect of *Candida pyralidae* Y1117 (A), *Pichia kluyveri* Y1125 (B) and *P. kluyveri* Y1164 (C) on the biocontrol of *Botrytis cinerea*. (a) represents the plates spread with initial inoculum of 10^1 cells mL^{-1}; b, c d and e depict the initial inoculum of 10^2, 10^3, 10^4 and 10^5 cells mL^{-1} respectively. f, g, h correspond to the initial inoculum of 10^6, 10^7 and 10^8 cells mL^{-1}. Assays were conducted in triplicates.

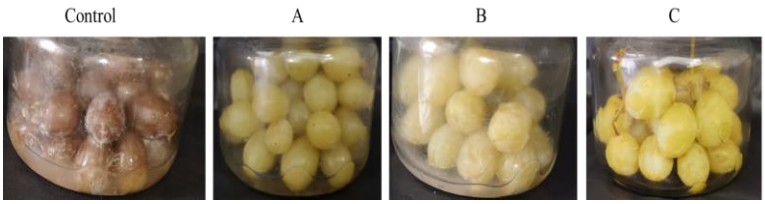

Fig. 2: Representative jars showing the fungicidal effects of *Candida pyralidae* Y1117 (A), *Pichia kluyveri* Y1125 (B) and *P. kluyveri* Y1164 (C) on the biocontrol of *Botrytis cinerea*. Only one jar per treatment was selected as a representative.

IV. Conclusion

This study demonstrated the fungistatic and fungicidal potential of the biocontrol yeasts. The VOCs produced by *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 when trapped in a closed environment were found to be responsible for the fungicidal effect on the growth of *B. cinerea* both in-vitro and in-vivo. The use of yeast with biocontrol activity presents a potential alternative to synthetic chemicals currently used as fungicides on fruit and other fruit derived beverages.

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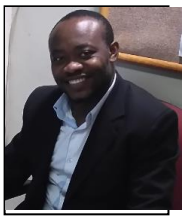

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