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Biocontrol Potential of Selected Phyllospheric Yeasts Against *Botrytis cinerea* and *Fusarium fujikuroi*

Sibusisiwe Nobuhle Nkomonde [1,2], Heinrich Wilbur du Plessis [2,*], Prashant Bhagwat [1], Ayodeji Amobonye [1,3], Zukisani Gomomo [2], Maxwell Mewa-Ngongang [2], Justin Wallace Hoff [2] and Santhosh Pillai [1]

[1] Department of Biotechnology and Food Science, Faculty of Applied Sciences, Durban University of Technology, P.O. Box 1334, Durban 4000, South Africa; 21303090@dut4life.ac.za (S.N.N.); prashantb@dut.ac.za (P.B.); santhoshk@dut.ac.za (S.P.)
[2] Post-Harvest and Agro-Processing Technologies, ARC Infruitec-Nietvoorbij (The Fruit, Vine and Wine Institute of the Agricultural Research Council), Private Bag X5026, Stellenbosch 7599, South Africa; gomomoz@cput.ac.za (Z.G.); hoffjw@arc.agric.za (J.W.H.)
[3] Department of Polymer Chemistry and Technology, Kaunas University of Technology, 50254 Kaunas, Lithuania
* Correspondence: dplessishe@arc.agric.za

Abstract

Ten phyllospheric yeast strains were studied for their potential as biocontrol agents against fruit spoilage mould. The efficacy of these yeasts against *Botrytis cinerea* and *Fusarium fujikuroi* was assessed using dual-culture, mouth-to-mouth, radial growth inhibition and post-harvest fruit assays. Additionally, their capacity for producing hydrolytic enzymes was examined. Results from the ten yeasts revealed dual culture antagonism ranging from 41% to 63% against *B. cinerea* and 23% to 48% against *F. fujikuroi*, along with radial inhibition ranging from 70% to 100% and 47% to 100%, respectively. Additionally, in vitro inhibition through the production of volatile organic compounds (VOCs) varied from 2% to 46% against *B. cinerea* and 6% to 64% against *F. fujikuroi*. Overall, *Aureobasidium melanogenum* J7, *Suhomyces pyralidae* Y1117, *Dekkera anomala* V38, and *Rhodotorula diarenensis* J43 emerged as the best-performing biocontrol yeasts. Volatile organic compounds produced by the four yeasts were also identified and included in fruit bioassays using pears and tomatoes. Various VOCs, including 1-butanol, 3-methylbutanol, and butyric acid, were linked to the antagonistic properties of the selected yeasts. Lastly, the four chosen yeast strains significantly mitigated post-harvest spoilage caused by *B. cinerea* and *F. fujikuroi* in pear and tomato fruits, with *D. anomala* V38 exhibiting the greatest inhibitory activity. These findings underscore a potential sustainable and efficient approach to reducing mould-induced post-harvest spoilage while reducing reliance on synthetic fungicides.

Keywords: biocontrol; *Botrytis cinerea*; *Fusarium fujikuroi*; fruit preservation; post-harvest; yeasts

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1. Introduction

According to the UN Sustainable Development Goal (SDG) of Responsible Consumption and Production (SDG 12), "the reduction of food waste is critical in ensuring that natural resources are used sustainably". Post-harvest loss (PHL) of fruits and other food crops contributes to the loss of valuable food as well as the wastage of the limited resources required to produce and distribute the food crops. The monetary costs of all these losses

have been noted to run into billions of dollars [1]. Post-harvest loss of food crops, during or after harvest, results in a loss of valuable food and the inputs required to produce and distribute it; therefore, reducing PHL will help create more sustainable and resilient food systems and enhance socio-economic wellbeing [2]. The factors influencing fruit PHL have been identified, which include inappropriate storage conditions, damage due to bruising, and microbial spoilage, especially during transportation [3]. Fruits and other fresh produce can be spoiled by microorganisms, especially mould, during transportation and storage; hence, controlling these spoilage moulds has become of utmost significance [4].

Fungal species within the *Aspergillus*, *Botrytis*, *Geotrichum*, *Gloeosporium*, *Monilinia*, *Mucor*, *Penicillium* and *Rhizopus* genera have been established to be responsible for a huge proportion of global fruits and vegetables' PHL [5,6]. The *Fusarium* genus is routinely associated with the pathogenesis of many fruits and other food crops; *F. fujikuroi* and other members of the *F. fujikuroi* complex, such as *F. proliferatum*, *F. acutatum*, *F. ophioides*, and *F. verticillioides*, have been identified as mycotoxigenic moulds of phytopathological significance [7,8]. Similarly, the *Botrytis* genus is made up of more than 30 phytopathogenic species, including *Botrytis cinerea*, which affects hundreds of plant species, including apples, grapes, pears, tomatoes, peppers, and berries [9], causing annual economic losses that have been estimated at USD 10 billion [10]. *Botrytis cinerea* and *F. fujikuroi* are of economic importance and have been noted to be pathogenic to hundreds of plant species, including grape, lettuce, strawberry, pear, and tomato [11,12].

Traditionally, fungal spoilage of fruits during storage is managed using chemicals such as SO_2/sulphites, dehydroacetic acid, ortho-phenylphenate, and imazalil [13]. These chemicals inhibit the growth of post-harvest microorganisms by disrupting the cell membrane or suppressing essential biochemical reactions [14]. However, many of these chemicals are toxic to humans and other non-target organisms, persist in the environment and lose efficacy over time due to the development of resistance [15]. Recently, various health risks have been reported to be linked to environmental exposure to triazole compounds, which are commonly used fungicides for fruit preservation [16]. Alternative methods, such as heat processing, cause sensory, nutritional, and textural modifications that can adversely affect the quality of fruits [17].

The biological control of spoilage mould via the antagonistic properties of some microbes, including bacteria, filamentous fungi, and yeasts, is considered a safe and effective alternative [18,19]. Yeasts can antagonistically interact with pathogenic microbes through various mechanisms, including nutrient competition, physical (contact) inhibition, the secretion of volatile organic compounds (VOCs), and the production of cell wall lytic enzymes [20]. According to Spadaro and Droby [21], there are little to no concerns about the safety of these beneficial yeasts, which are mostly isolated from the surface of healthy fruits. Various research and commercial successes have been achieved with the application of yeasts as biocontrol agents in the fruit industry [21].

For instance, *Scheffersomyces spartinae* has been shown to be active against *Botrytis cinerea* on strawberries [22] while *Cryptococcus carnescens* has displayed remarkable inhibition against various *Fusarium* species, including *F. culmorum*, *F. graminearum*, and *F. poae* [23]. The industry has witnessed the successful commercialization of yeast-based biocontrol agents such as *Candida oleophila* (Aspire®, Ecogen, Langhorne, PA, USA and Nexy®, Agrauxine SA, Beaucouzé, France), *C. sake* (Candifruit®, IRTA, Lleida, Spain), and *Cryptococcus albidus* (YieldPlus®, Anchor yeast, Cape Town, South Africa), *Metschnikowia fructicola* (Gaia™, Lallemand, Montreal, Canada, and Noli™, NRRL Y-27328, Koppert Global, Berkel en Rodenrijs, The Netherlands) [24]. The potential of yeasts as biocontrol agents is evidenced by the numerous commercial products; however, several yeast species have not been investigated, and their antagonism against all phytopathogenic fungi is unknown. Yeast species and strains display variable levels of inhibition against different

mould species [25]; therefore, it is important to continually evaluate different yeasts. To date, biocontrol strategies against *F. fujikuroi* have not received much attention, and there are no published reports of characterising yeasts as biocontrol agents against this species. In this context, this study aimed to screen and evaluate the antagonistic activity of 10 yeast strains, primarily isolated from the phyllosphere of fruits, against *Botrytis cinerea* and *Fusarium fujikuroi*, thus providing new insights into the antagonism of yeasts against *B. cinerea* and *F. fujikuroi* through their production of hydrolytic enzymes and non-volatile and volatile compounds, as well as determining the biocontrol potential of the selected yeast strains during post-harvest trials on pears and tomatoes.

2. Materials and Methods

2.1. Microorganisms and Growth Conditions

Ten yeast strains, *namely Aureobasidium melanogenum* J7, *Debaryomyces hansenii* MY1, *Dekkera anomala* V38, *Dekkera bruxellensis* Y0106, *Dekkera bruxellensis* Y0167, *Meyerozyma guilliermondii* J26, *Pichia kluyveri* Y1125, *Rhodotorula diarenensis* J43, *Saccharomyces cerevisiae* Y0936, and *Suhomyces pyralidae* Y1117 were obtained from the culture collection and biobank of ARC Infruitec-Nietvoorbij (The Fruit, Vine and Wine Institute of the Agricultural Research Council, Stellenbosch, South Africa). These yeasts were previously isolated from wine grapes, jaboticaba fruit, mobola plums, and marula fruit and identified for their potential antagonistic activities [25]. The spoilage mould strains, namely *Botrytis cinerea* isolated from blackberries (*Rubus fruticosus*) and *Fusarium fujikuroi* isolated from garden soil, were obtained from the ARC Infruitec-Nietvoorbij and the Biotechnology and Food Science Department of the Durban University of Technology, Durban, South Africa, respectively. The potential biocontrol yeasts and the spoilage mould were cultured separately on Potato Dextrose Agar (PDA) at pH 5.6 and incubated at 28 °C for 2 and 7 days, respectively.

2.2. Preparation of Yeast Cells and Fungal Spores

Yeast cells were prepared by transferring a loopful of each culture into a test tube containing 5 mL of sterile yeast malt broth (YMB) at pH 6.2 and incubating at 28 °C for 24 h. Subsequently, 1 mL of the yeast culture broth was transferred to a sterile 2 mL microtube and centrifuged at 12,000× *g* for 6 min to recover the cells and resuspended in sterile distilled water. The yeast cells were counted using a microscope and a Neubauer haemocytometer at 400 times magnification and the concentration was adjusted to 1×10^8 cells/mL. Spores of the test moulds were prepared from PDA cultures flooded with 0.9% sterile saline; the spore suspensions were quantified by direct microscopic count as described above, and the spore concentration was adjusted 1×10^5 spores/mL [26].

2.3. Determination of Antagonistic Activity of Yeasts Against Spoilage Mould

2.3.1. Radial Growth Inhibition

The preliminary screening of the yeast cultures was conducted using the radial growth inhibition assay, as described by Tenorio-Salgado et al. [27], with some modifications. Briefly, mycelial discs (5 mm diameter) of the test mould were transferred from 7-day-old cultures using a cork borer onto the centre of a PDA plate. Afterwards, 15 µL of the yeast cell suspension (1×10^8 cells/mL) was spotted at 25 mm from the centrally positioned mycelial plug. Four different yeast cell suspensions were spotted per PDA plate and incubated at 25 °C for 7 days. The control plates were inoculated with the mould culture without the addition of any yeast suspension. The inhibition rate was calculated as fungal radial growth inhibition (FRGI) using the formula below:

$$FRGI = (D_0 - D_t/D_0) \times 100$$

D_0 = radial growth of the pathogen on the control plates (mm); D_t = radial growth of the pathogen on the test plates (mm)

2.3.2. Mouth-to-Mouth Assay

The yeast strains were also screened against *B. cinerea* and *F. fujikuroi* to produce volatile organic compounds, using the "mouth-to-mouth" method [28]. A 5 mm mycelial disc of either *B. cinerea* or *F. fujikuroi* was placed in the centre of the upper PDA plate. On the lower plate, 100 µL of yeast suspension (1×10^8 cells/mL) was spread onto PDA. The two plates were placed against each other, sealed with parafilm, and incubated at 28 °C for 7 days. The plates without yeast inoculum on the lower plate served as controls. Inhibition was determined by measuring the fungal colony diameter on day 7, and the radial inhibition (RI) was calculated as described in Section 2.3.1.

2.3.3. Dual Culture Assays

The yeast strains were further screened for inhibitory activities against *B. cinerea* and *F. fujikuroi* mycelial growth using the dual culture assay as described by Chen et al. [29]. Briefly, 5 mm mycelial discs of seven-day-old mould cultures were placed on the edge of 90 mm PDA plates. Subsequently, 20 µL of the yeast suspension (1×10^8 cells/mL) was spotted 50 mm from the pathogen and incubated at ambient temperature (±25 °C) for 7 days. The colony radii of *B. cinerea* and *F. fujikuroi* were measured, and the percentage inhibition was calculated as previously described.

2.4. Screening of Yeasts for Cell Wall Degrading Enzyme Production

The yeasts were evaluated for their ability to produce lytic enzymes, i.e., chitinase, cellulase, and proteases. A 10 µL suspension of each yeast culture ($\pm 1 \times 10^8$ cells/mL) was spotted onto agar plates containing specific substrates for each enzyme assay. Each treatment was conducted in triplicate, and agar plates without any yeast treatment served as the control for the different enzyme assays. Chitinase production was determined by replica plating the yeast on chitin agar plates containing finely ground 0.1% chitin from shrimp (Sigma-Aldrich, Kempton Park, South Africa) as the sole carbon source at 28 °C for 7 days. The plates were inspected for zones of clearance (chitinolytic zones), indicating the release of chitinase. For cellulase activity, the cultures were grown on plates containing yeast extract (10 g/L), peptone (20 g/L), carboxymethyl cellulose (CMC) (10 g/L) and agar (20 g/L) at 25 °C for 7 days. Subsequently, the plates were stained with a 0.1% Congo red solution (Sigma-Aldrich) for 30 min and then washed with a 1 M NaCl solution for 15 min. Cellulase activity was detected by the presence of degradation halos around colonies [30]. The assessment of protease production was conducted by inoculating the yeasts onto plates containing skim milk powder (28 g/L), tryptone (5 g/L), yeast extract (2.5 g/L), glucose (1 g/L), and agar (20 g/L). Growth on skim milk agar was examined for clear zones surrounding yeast colonies after incubation at 25 °C for 5–7 days [31].

2.5. Production, Extraction and Analysis of VOC by Gas Chromatography

Selected yeast was grown in YPD broth prior to extraction and analyses. Each yeast sample was transferred into a 20 mL headspace vial and volatile compounds were trapped and extracted using the headspace solid-phase micro-extraction (HS-SPME) method [19]. A SPME vial was equilibrated for 10 min at 70 °C in a CTC autosampler incubator at 250 rpm. Subsequently, a 50/30 m divinylbenzene/-carboxen/-polydimethylsiloxane (DVB/CAR/PDMS) coated fibre was exposed to the sample headspace for 20 min at 50 °C. After extraction, the volatile compounds were desorbed from the fibre coating in the injection port of the gas chromatography-mass spectrometry (GC–MS) for 10 min. Separation and quantification of the volatile compounds were performed using a gas

chromatograph (Agilent 6890 N, Agilent, Palo Alto, CA, USA) coupled with an Agilent mass spectrometer detector (Agilent 5975 MS). The GC–MS system was equipped with a polar ZB-FFAP column (Model number: ZB 7KM-G009-17), with a nominal length of 30 m, an internal diameter of 250 µm, and a film thickness of 0.25 µm. Analyses were performed using helium as the carrier gas at a flow rate of 1 mL/min. The injector temperature was maintained at 250 °C. The oven program was as follows: 40 °C for 5 min, then ramped up to 240 °C at 7 °C/min and held for 5 min. The mass selective detector was operated in full scan mode and the ion source and quadrupole temperatures were maintained at 230 °C and 150 °C, respectively. The transfer line temperature was maintained at 250 °C.

2.6. Biocontrol Activities of Yeast Cultures on Infected Pears and Tomatoes Post-Harvest

The four yeasts from the previous experiments, namely *A. melanogenum* J7, *S. pyralidae* Y117, *D. anomala* V38, and *R. diarenensis* J43, were tested in vivo against *B. cinerea* and *F. fujikuroi* on pear (*Pyrus communis* L.) and tomato (*Solanum lycopersicum*). Ethanol (70% *v*/*v*) was sprayed on the fruit and allowed to dry completely prior to wound infliction. The fruits were uniformly wounded (5 mm diameter and 3 mm deep). Then, 15 µL of yeast suspension (1×10^5 cells/mL) was inoculated into the wound and allowed to dry for 15 min. The same volume of sterile distilled water served as the control. Subsequently, the wounded fruits were inoculated with the respective mould suspensions 1×10^5 spores/mL. All the fruits were incubated at ±25 °C for 7–21 days. Lesion development and percentage growth inhibition were calculated according to [32]. Positive results were characterized by the absence of fungal development on the infected fruit. This experiment was designed using a completely randomized block design with five fruit per box and five replicate boxes.

2.7. Statistical Analyses

All of the data presented are the mean ± standard deviation of at least three replicates. The variation between groups was evaluated using a one-way analysis of variance (ANOVA) using the GLM procedure of SAS software (version 9.4, SAS Institute Inc, Cary, NC, USA). The normality of standardised residuals was confirmed with the Shapiro–Wilk test. Fisher's least significant difference (LSD) values were calculated at a 5% significance level ($p = 0.05$) to allow for the comparison of treatment means. Principal Component Analysis (PCA) was performed on the enzyme production data, as well as the radial inhibition, mouth-to-mouth, the dual culture assay data for *F. fujikuroi*, using XLSTAT 2025.1.3 software (Addinsoft, New York, NY, USA).

3. Results and Discussion

3.1. Antagonistic Activity of Yeasts Against Spoilage Mould

The 10 yeast strains displayed significant inhibition activities against both *B. cinerea* and *F. fujikuroi*, with radial inhibition values ranging from 69.9 to 100% and 46.7 to 100%, respectively (Table 1). The selected yeast strains performed better against *B. cinerea* than against *F. fujikuroi*, as indicated by higher inhibition values. *Aureobasidium melanogenum* J7, *D. bruxellensis* Y0106 and *P. kluyveri* Y1125 completely prevented the growth of *B. cinerea*, with 100% inhibition for the radial assay. On the other hand, *D. anomala* V38 and *P. kluyveri* Y1125 exhibited 100% radial growth inhibition against *F. fujikuroi.* The ability of yeasts to inhibit the growth of some fungal pathogens has been established and Di Francesco et al. [33] reported on four different strains of *A. melanogenum* showing between 20 and 30% growth inhibition against *B. cinerea*. This study shows the variability in inhibition activity among yeast species against *B. cinerea* and other mould and agrees with the findings of Gomomo et al. [25]. This is the first report of yeast inhibiting the growth of *F. fujikuroi* and

inhibition is due to competition for nutrients and space. The ability of some yeast species to inhibit mould has been shown to be due to their competitive edge in utilising nutrients [34].

Table 1. In vitro antagonistic activity of *Aureobasidium melanogenum* J7, *Suhomyces pyralidae* Y1117, *Debaryomyces hansenii* MY1, *Dekkera anomala* V38, *D. bruxellensis* Y0106, *D. bruxellensis* Y0167, *Meyerozyma guilliermondii* J26, *Pichia kluyveri* Y1125, *Rhodotorula diarenensis* J43, and *Saccharomyces cerevisiae* Y0936 on mycelial growth of *Botrytis cinerea* and *Fusarium fujikuroi* determined using the different plate assays and expressed as % inhibition.

Yeast strain	Radial Growth Inhibition		Mouth-to-Mouth		Dual Culture	
	B. cinerea	*F. fujikuroi*	*B. cinerea*	*F. fujikuroi*	*B. cinerea*	*F. fujikuroi*
A. melanogenum J7	100.0 ± 0.00 ^{a}	90.7 ± 2.31 ^{b}	8.0 ± 6.93 ^{c}	13.3 ± 10.84 ^{cdef}	61.11 ± 0.96 ^{a}	47.5 ± 2.50 ^{a}
S. pyralidae Y1117	86.4 ± 1.68 ^{c}	92.0 + 0.00 ^{b}	4.8 ± 1.38 ^{cde}	9.64 ± 3.61 ^{def}	62.78 ± 0.96 ^{a}	45.8 ± 1.44 ^{ab}
D. hansenii MY1	69.9 ± 1.68 ^{e}	50.7 ± 2.31 ^{e}	27.2 ± 3.67 ^{b}	19.3 ± 5.11 ^{cde}	41.67 ± 1.67 ^{c}	25.8 ± 1.44 ^{e}
D. anomala V38	89.3 ± 1.68 ^{b}	100.0 ± 0.00 ^{a}	6.4 ± 2.40 ^{cd}	27.7 ± 3.61 ^{bc}	63.33 ± 1.67 ^{a}	45.0 ± 0.00 ^{b}
D. bruxellensis Y0106	100.0 ± 0.00 ^{a}	86.7 ± 2.31 ^{c}	46.4 ± 3.67 ^{a}	63.9 ± 3.61 ^{a}	46.67 ± 1.67 ^{bc}	25.0 ± 0.00 ^{e}
D. bruxellensis Y0167	72.8 ± 1.68 ^{d}	81.3 ± 2.31 ^{d}	4.0 ± 2.40 ^{cde}	16.9 ± 7.23 ^{cde}	58.89 ± 0.96 ^{ab}	37.5 ± 0.00 ^{d}
M. guilliermondii J26	100.0 ± 0.00 ^{a}	82.7 ± 2.31 ^{d}	7.2 ± 2.77 ^{c}	20.5 ± 9.56	53.89 ± 0.96 ^{abc}	35.8 ± 1.44 ^{d}
P. kluyveri Y1125	100.0 ± 0.00 ^{a}	100.0 ± 0.00 ^{a}	4.8 ± 1.38 ^{cde}	24.1 ± 3.61 ^{cd}	41.11 ± 0.96 ^{c}	22.5 ± 2.50 ^{f}
R. diarenensis J43	98.1 ± 3.36 ^{a}	86.7 ± 2.31 ^{c}	3.2 ± 1.39 ^{de}	39.8 ± 2.09 ^{b}	57.78 ± 0.96 ^{ab}	40.0 ± 0.00 ^{c}
S. cerevisiae Y0936	90.3 ± 1.68 ^{b}	46.7 ± 2.31 ^{f}	1.6 ± 1.41 ^{e}	6.0 ± 3.61 ^{ef}	41.67 ± 0.00 ^{c}	25.0 ± 0.00 ^{e}

Each value represents the mean of three replicates (±standard deviation of the mean). Means in columns followed by different letters are significantly different ($p \leq 0.05$).

The production of VOCs has been recognised as a key yeast antagonistic mechanism against various filamentous moulds, as these compounds infiltrate cell membranes, disrupting cellular permeability, ion exchange capacity, and homeostasis [35]. The production of VOCs revealed significant variability in the biocontrol efficacy of the yeast species against *F. fujikuroi* and *B. cinerea*, as observed in the radial growth inhibition assays. In this regard, growth inhibition of 1.6 to 46.4% against *B. cinerea* and 6.0% to 63.9% against *F. fujikuroi* was recorded (Table 1). However, the efficacy of VOCs in eliciting antagonism against the two pathogens was less pronounced with *D. bruxellensis* Y0106 and *D. hansenii* MY1 showing the best inhibition against *B. cinerea*, 46.4% and 27.2%, respectively. In contrast, *D. bruxellensis* Y0106 and *R. diarenensis* J43 exhibited the best performance against *F. fujikuroi*, with growth inhibition of 63.9% and 39.8%, respectively. Compared to the inhibition values obtained for the radial inhibition assay, the lower inhibition values for the mouth-to-mouth assay indicate that the production of VOCs was most likely not the primary mode of inhibition for these yeasts against *B. cinerea* and *F. fujikuroi*. Gomomo et al. [36] reported higher levels of growth inhibition by yeasts against different *B. cinerea* strains when evaluating production of VOCs as a mode of action. Lower inhibition values can be attributed to the abilities of the different yeast species and strains that were used. The mouth-to-mouth assay is a simple and easy method to screen for the production of VOCs but is fallible if plates are not sealed properly. The observed variability in biocontrol efficacy among yeast species highlights the importance of selecting the appropriate species for effective disease management.

The dual culture plate assays also demonstrated variable degrees of antagonistic efficacy of the yeast strains against *B. cinerea* and *F. fujikuroi*, with the representative samples illustrated in Figure 1. The inhibition percentages recorded against *B. cinerea* are shown in Table 1 and ranged from 41.1% (*P. kluyveri* Y1125) to 63.3% (*D. anomala* V38). Gomomo et al. [36] reported low inhibition values (10–57%) against *B. cinerea* strains using the dual culture assays. Lower inhibition percentages were recorded against *F. fujikuroi*, with the highest inhibition of 47.5% achieved by *A. melanogenum* J7. *F. fujikuroi* was less susceptible to inhibition by the yeast strains than *B. cinerea*. *D. bruxellensis* Y0106 displayed lower growth inhibition activity against *B. cinerea* and *F. fujikuroi* during the dual culture assay than the radial inhibition assay. *D. bruxellensis* Y0167 demonstrated better growth inhibition than *D. bruxellensis* Y0106 against both mould species during the dual culture assay.

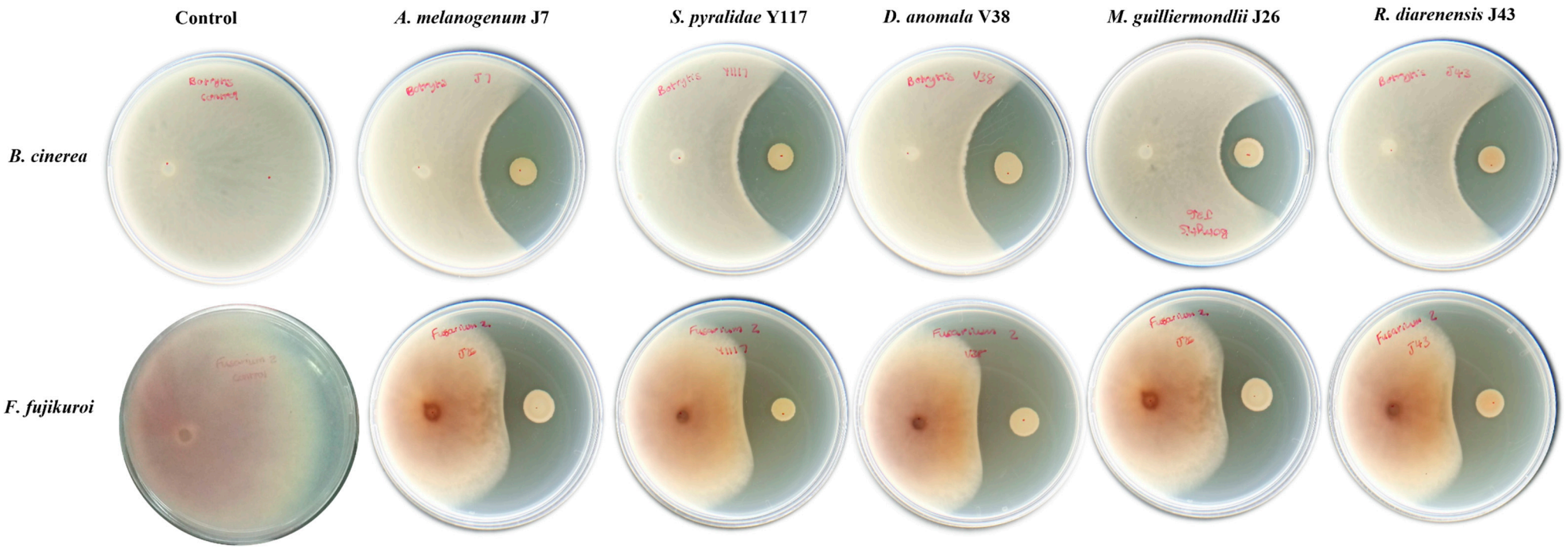

Figure 1. Dual culture test to evaluate the in vitro antagonistic activity of *Aureobasidium melanogenum* J7, *Suhomyces pyralidae* Y1117, *Dekkera anomala* V38, *Meyerozyma guilliermondii* J26, and *Rhodotorula diarenensis* J43 against *Botrytis cinerea* and *Fusarium fujikuroi.* Representative samples are shown for three repeats.

Compared to the radial inhibition assay results, the secretion of non-volatile compounds by the yeasts were not as effective in inhibiting *B. cinerea* and *F. fujikuroi*. However, generally the levels of inhibition for most of the yeasts were higher for the dual assay, than those obtained for the production of VOCs against *B. cinerea* and *F. fujikuroi*. The yeast strains showed variable levels of inhibition against the selected pathogens, and this was shown for all the different assays. These results suggest that the antagonistic activity of biocontrol yeasts and mode of action can vary depending on the target pathogen. This emphasises the importance of strain selection and tailored biocontrol strategies in agricultural applications [37].

3.2. Cell Wall-Degrading Enzyme Production

The hydrolytic activities of the cell wall degrading enzymes, cellulase, chitinase and proteases, were observed qualitatively in the various antagonistic yeasts as summarised in Table 2. While cellulolytic and proteolytic activities were observed in 80% of the yeasts, chitinolytic activity was only recorded in 20%, suggesting that both cellulases and proteases may play more prominent roles in primary metabolism, but not necessarily in the antagonistic effects of the yeasts. The presence of all three enzymes was only detected in *R. diarenensis* J43 and *S. pyralidae* Y1117. The production of cellulase, chitinase, and proteases by antagonistic yeast strains has been reported in the literature [32,38]. Gomomo et al. [37] reported more variability regarding hydrolytic enzyme activity among the antagonistic yeasts. The secretion of chitinases and proteases has been established as a defence mechanism against fungal competitors, as these enzymes degrade the chitin and protein components of the fungal cell walls, thereby inhibiting their growth [39,40]. However, the roles of cellulases are still speculative and may only be indirectly related to their growth-inhibiting potential, as highlighted for *Pichia* spp. [41] and *Sporidiobolus pararoseus* [10]. While mould generally does not contain cellulose, cellulases were hypothesised to exert antifungal activity through indirect inhibition, biofilm disruption, synergistic effects with other antifungal agents, and ecological competition [42].

Table 2. Qualitative enzyme production by yeasts used in this study.

Yeast Species	**Enzyme**		
	Cellulase	*Chitinase*	*Protease*
Aureobasidium melanogenum J7	+	-	+
Suhomyces pyralidae Y1117	+	+	+
D. hansenii MY1	+	+	-
D. anomala V38	+	-	+
D. bruxellensis Y0106	+	-	+
D. bruxellensis Y0167	-	-	+
M. guilliermondii J26	+	-	+
P. kluyveri Y1125	-	-	+
R. diarenensis J43	+	+	+
S. cerevisiae Y0936	+	-	-

(-) absence of enzyme; (+) presence of enzyme.

Subsequently, four of the ten yeast strains were selected based on the analysis of the multivariate data generated in the study. In this regard, the performance of the yeasts for radial growth inhibition, the mouth-to-mouth, and the dual culture assay against *F. fujikuroi*, as well as their enzyme production capabilities, were considered and evaluated using principal component analysis (PCA). The reason for focusing exclusively on the *F. fujikuroi* data set was to try and select the best-performing yeast strains against this mould, thus generating new information and improving the scientific value of the study. The transformed multidimensional data are shown in Figure 2. The 2-dimensional PCA plot explains ~71% of the data variability for the various assays, with the first principal

component (PC1) and second principal component (PC2) accounting for 39.17% and 32.06% of the total variables, respectively. The yeasts with the relative best performance for the various assays, grouped towards the right of the biplot. *D. bruxellensis* Y0106 showed a positive correlation for the mouth-to-mouth assay. While the yeasts *Rhodotorula diarenensis* J43 demonstrated a positive correlation for enzyme production, and *S. pyralidae* Y1117, *D. anomala* V38, and *A. melanogenum* J7 showed positive correlations for the radial inhibition and dual culture assays. These positive correlations indicate that these yeasts were the top performers overall for the four assays. *Rhodotorula diarenensis* J43, *S. pyralidae* Y1117, *D. anomala* V38, and *A. melanogenum* J7 were subsequently chosen for further investigation because of their overall performance. Only four yeasts were selected for further investigation due to financial and logistical constraints.

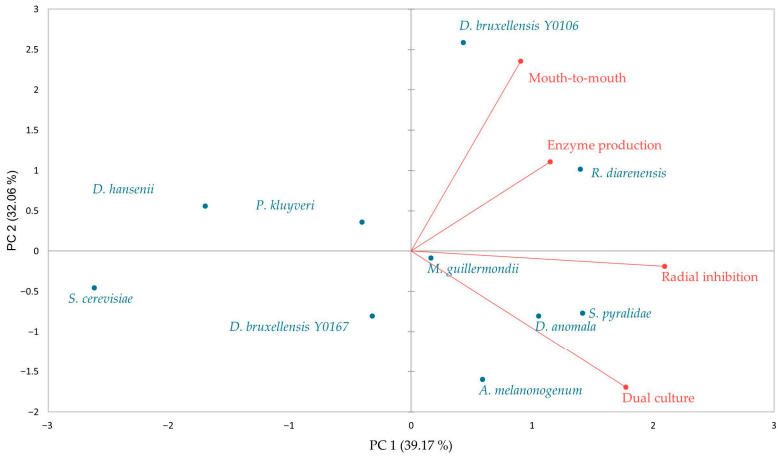

Figure 2. Principal component biplot of the biological activities (radial inhibition, mouth-to-mouth, the dual culture, and enzyme production) and yeast strains (*Aureobasidium melanogenum* J7, *Suhomyces pyralidae* Y1117, *Debaryomyces hansenii* MY1, *Dekkera anomala* V38, *D. bruxellensis* Y0106, *D. bruxellensis* Y0167, *Meyerozyma guilliermondii* J26, *Pichia kluyveri* Y1125, *Rhodotorula diarenensis* J43, and *Saccharomyces cerevisiae* Y0936).

3.3. Identification of Volatile Organic Compounds (VOCs) Produced by Yeasts

Volatile organic compounds (VOCs) play a significant role in the biological activities of yeasts against other microorganisms, including bacteria and moulds [43]. The role of VOCs in the antagonism of the yeasts was demonstrated by the mouth-to-mouth assay. GC-MS analysis of the VOCs produced by the four selected yeast strains identified twelve different metabolites in varying proportions (Table 3). These VOCs include alcohols, carboxylic acids, and ester groups. As observed in the area ratio, butyric acid was the most abundant VOC produced by *A. melanogenum* J7 (0.16), while 3-methylbutanol was the highest compound produced by *D. anomala* V38 (0.19), *R. diarenensis* J43 (0.46), and *S. pyralidae* Y1117 (0.15). The variation in the proportion of metabolite production among the four yeasts could be attributed to differences in their metabolism, substrate utilization, enzymatic activities, as well as environmental adaptation [44]. Interestingly, 3-methylbutanol was also observed at a high concentration in *A. melanogenum* J7 with an area ratio of 0.14, thus highlighting the importance of this metabolite across all the biocontrol agents. Recent studies by Paszkot et al. [45] and Ramírez-Ordorica et al. [46] have reported higher production of 3-methylbutanol from different biocontrol yeasts compared to other compounds. The compound, 3-methylbutanol, showed significant inhibitory activities against the fungal plant pathogen, *Phytophthora capsica*, and prevented spore germination of the food contaminant, *Aspergillus brasiliensis* [47]. Furthermore, the antifungal activities of 1-butanol, 1-propanol, 2-phenylethanol and 2,3-butanediol were reported by [36,48]. Similarly, the carboxylic acids, including those recorded in this study, such as acetic acid, butyric acid,

isobutyric acid, isovaleric acid, and propionic acid, are notable antifungal agents with immense industrial potential [49].

Table 3. Major volatile organic compounds (VOCs) produced by selected yeast strains used in this study.

			Aureobasidium melanogenum J7	*Suhomyces pyralidae* Y1117	*Dekkera anomala* V38	*Rhodotorula diarenensis* J43
VOC	**Chemical Formula**	**Molar Mass (g/mol)**	**Area Ratio**			
1-Butanol	C_4H_9OH	74.12	0.011	0.009	0.042	0.017
1-Propanol	C_3H_7OH	60.1	0.039	0.072	0.081	0.051
2-Phenylethanol	C_8H_9OH	122.16	0.133	0.013	0.022	0.029
2,3-Butandiol	$(CH_3CHOH)_2$	90.121	0.054	0.059	0.082	0.143
3-Methylbutanol	$C_5H_{11}OH$	88.15	0.138	0.149	0.189	0.459
Acetic acid	CH_3COOH	60.05	0.154	0.080	0.156	0.164
Butyric acid	C_3H_7COOH	88.11	0.160	0.089	0.117	0.069
Ethyl lactate	$C_5H_{10}O_3$	118.13	0.000	0.000	0.014	0.000
Isobutanol	C_4H_9OH	74.12	0.007	0.007	0.007	0.007
Isobutyric acid	$(CH_3)_2CHCOOH$	88.11	0.028	0.020	0.132	0.057
Isovaleric acid	$(CH_3)_2CHCH_2COOH$	102.13	0.144	0.053	0.171	0.168
Propionic acid	$CH_3CH_2CO_2H$	74.08	0.014	0.008	0.014	0.013

3.4. Application of Biocontrol Yeasts in Preventing Fruit Spoilage

The potential of the yeasts, *A. melanogenum* J7, *S. pyralidae* Y1117, *D. anomala* V38, and *R. diarenensis* J43 to control *B. cinerea* and *F. fujikuroi* on pear and tomato fruits was demonstrated. The pear bioassay showed that *S. pyralidae* Y1117 (68.4%) and *D. anomala* V38 (67.6%) were the most effective yeasts in mitigating spoilage by *B. cinerea*, and there was no significant difference between the performance of both strains (Figure 3). *R. diarenensis* J43 and *A. melanogenum* J7 displayed 60% and 39% inhibition against *B. cinerea.* While the performance of these two yeasts was acceptable, it was significantly less effective than *S. pyralidae* Y1117 and *D. anomala* V38. These results support the observations of the dual culture assay, where *D. anomala* V38 and *S. pyralidae* Y1117 demonstrated slightly better inhibition than *A. melanogenum* J7 and *R. diarenensis* J43 (Table 1). The results for the pear bioassay are contradictory to those of the radial inhibition assay because *A. melanogenum* J7 and *R. diarenensis* J43 performed significantly better than *D. anomala* V38 and *S. pyralidae* Y1117.

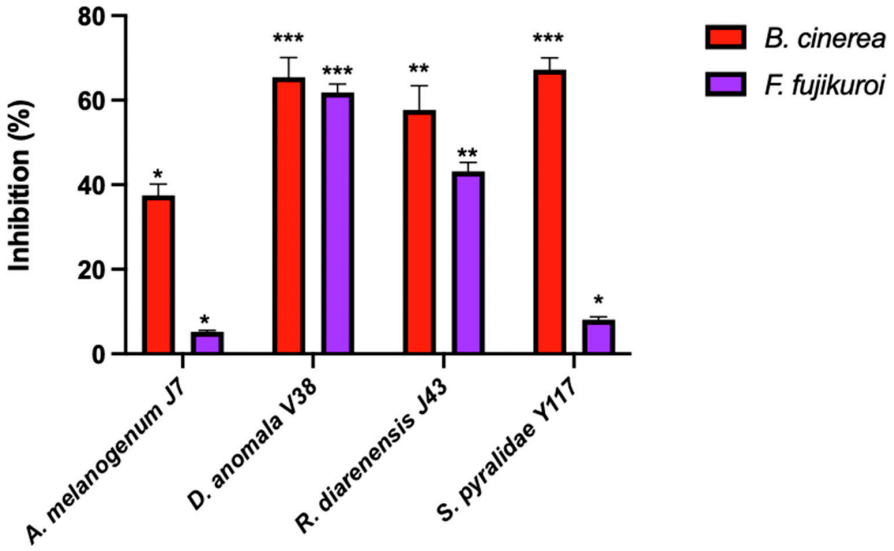

Figure 3. Efficacy (% inhibition) of *Aureobasidium melanogenum* J7, *Dekkera anomala* V38, *Rhodotorula diarenensis* J43, and *Suhomyces pyralidae* Y1117 against *Botrytis cinerea* and *Fusarium fujikuroi* on infected pears. Asterisks (*, ** and ***) indicate significant differences ($p \leq 0.05$) among the yeast treatments against *B. cinerea* (red columns) or *F. fujikuroi* (purple columns).

Dekkera anomala V38 also showed the highest activity on the *F. fujikuroi*-infected pears with inhibition of 61.7%, followed by *R. diarenensis* J43 with 43.6% (Figure 3). This result supports those of the radial inhibition assay, where *D. anomala* V38 was also one of the best-performing yeasts. For the dual culture assay, *A. melanogenum* J7 displayed the highest inhibition activity against *F. fujikuroi* (Table 1), while the opposite was observed for the pear assay (Figure 3). *Dekkera anomala* V38 showed the most potential against both mould strains. In comparison to the control, the progression of spoilage of the pear fruits inoculated separately with both *B. cinerea* and *F. fujikuroi* was remarkably suppressed by the yeast treatment (Figure 4). The spoilage mould was also observed to cause extensive necrosis in the areas surrounding the inoculation site in the control pears, with lesion diameters that were twice the size of the least performing treated group. Conversely, the progression of necrosis in the yeast-treated pears was less relative to the control group, thus providing evidence for yeast-induced suppression of necrotic development on pears (Figure 4). In general, a higher reduction in disease incidence was observed for the four yeasts against *B. cinerea* compared to *F. fujikuroi*, as demonstrated by their respective inhibitory activities (Figure 3).

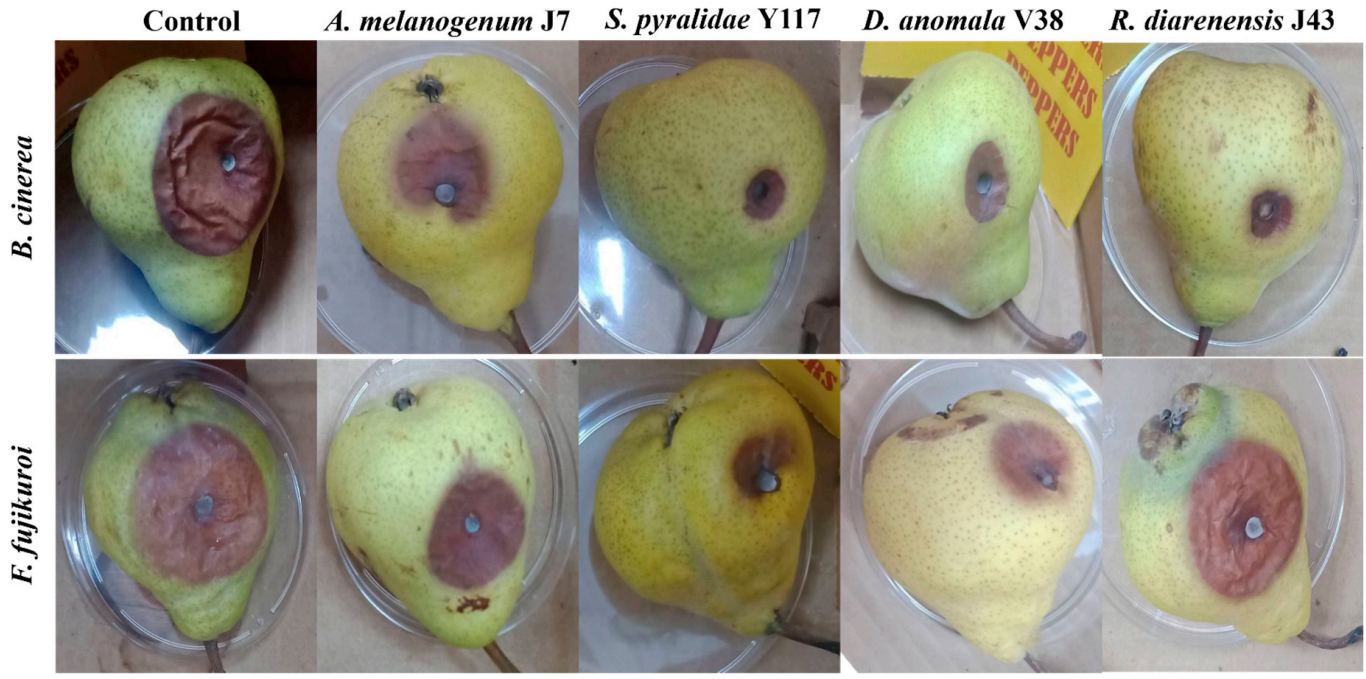

Figure 4. Reduction in lesion diameter on pears infected with *Botrytis cinerea* or *Fusarium fujikuroi* and treated with *Aureobasidium melanogenum* J7, *Suhomyces pyralidae* Y1117, *Dekkera anomala* V38, or *Rhodotorula diarenensis* J43. Representative samples of 25 fruits for each treatment.

The efficacy of yeasts in preventing fungal spoilage of tomatoes is illustrated in Figures 5 and 6. Similarly, to the pear assay, *D. anomala* V38 showed the highest efficacy against *B. cinerea* tomato spoilage with 96% inhibition, followed by *R. diarenensis* J43 with 88.4% (Figure 5). These results support the observation of the dual culture and pear assay, highlighting the potential of *D. anomala* V38 as a biocontrol agent against *B. cinerea.*

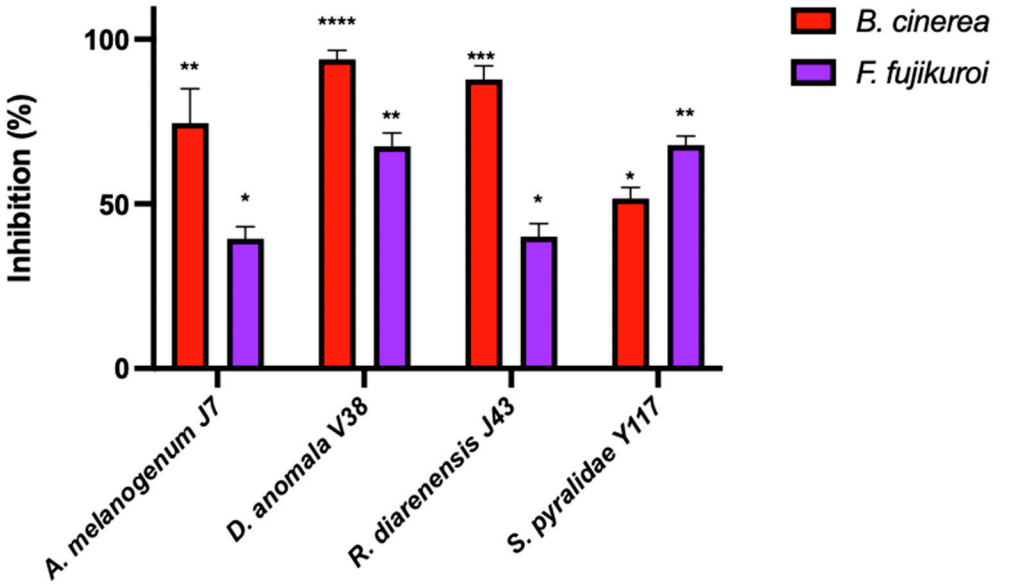

Figure 5. Efficacy (% inhibition) of *Aureobasidium melanogenum* J7, *Dekkera anomala* V38, *Rhodotorula diarenensis* J43 and *Suhomyces pyralidae* Y1117 against *Botrytis cinerea* and *Fusarium fujikuroi* on infected tomatoes. Asterisks (*, **, *** and ****) indicate significant differences ($p \leq 0.05$) among the yeast treatments against *B. cinerea* (red columns) or *F. fujikuroi* (purple columns).

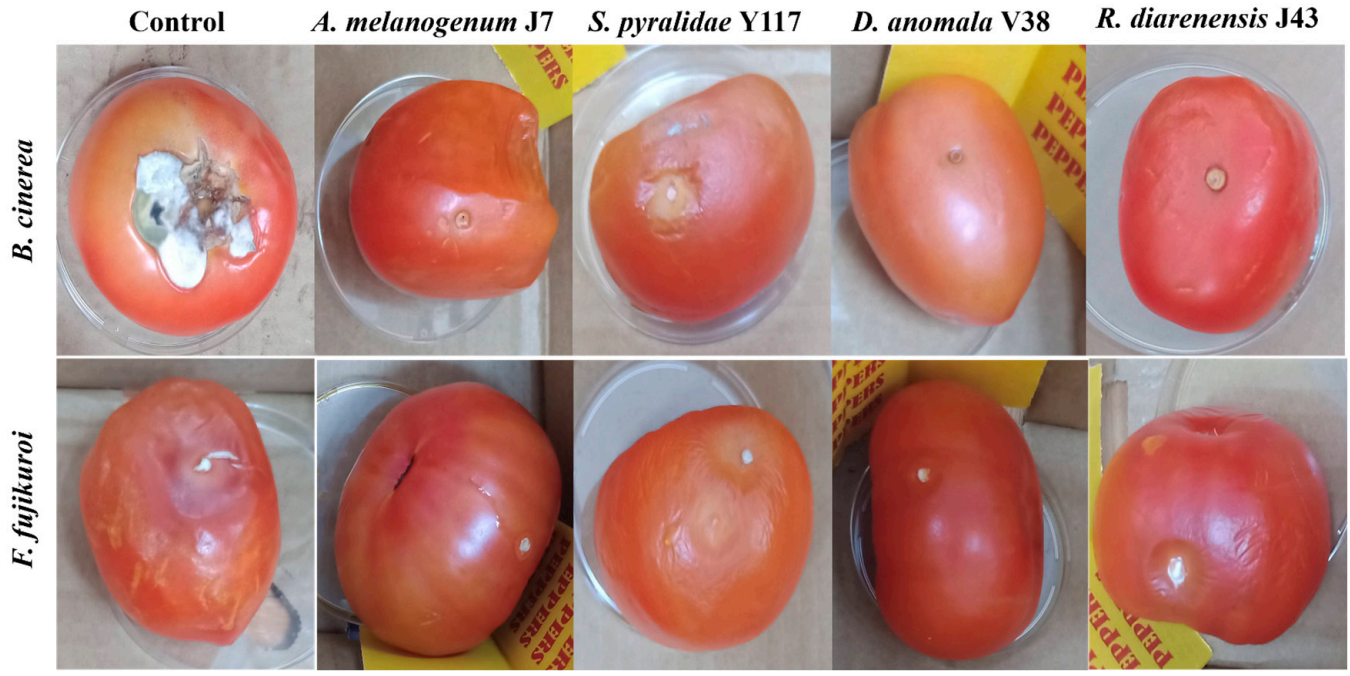

Figure 6. Reduction in lesion diameter on tomatoes inoculated into wounds with *Aureobasidium melanogenum* J7, *Suhomyces pyralidae* Y1117, *Dekkera anomala* V38, and *Rhodotorula diarenensis* J43 against *Botrytis cinerea* or *Fusarium fujikuroi*. Representative samples of 25 fruits for each treatment.

Conversely, *S. pyralidae* (70.4%) displayed the highest inhibition against *F. fujikuroi* followed by *D. anomala* V38 with 69.1%, (Figure 5). The effectiveness of these yeast strains against *F. fujikuroi* are confirmed by the results of radial inhibition and dual culture assay (Table 1). At the end of the 21-day storage period, little to no signs of mould spoilage and mycelial proliferation were observed on the tomato fruits treated with the yeasts compared to the untreated control (Figure 6). During the same period, significant changes and spoilage were observed in the non-yeast-treated tomatoes (negative control), including tis-

sue softening around the wound, skin rupture with liquid exudation, and the proliferation of fungal mycelium.

The results further confirm that the efficacy of phyllospheric yeasts as biocontrol agents varies depending on the target pathogen and the host involved. Overall, *D. anomala* V38 significantly suppressed the growth of *B. cinerea* and *F. fujikuroi* on both fruits, suggesting its potential as a broad-spectrum biocontrol agent. Interestingly, *R. diarenensis* J43 displayed high inhibition against *B. cinerea* but lower inhibition against *F. fujikuroi*, suggesting a pathogen-specific biocontrol mechanism. *Suhomyces pyralidae* Y1117 showed lower inhibition against *B. cinerea* but performed better against *F. fujikuroi*, indicating its potential as a biocontrol agent with broad efficacy.

In general, the four yeasts (*D. anomala* V38, *R. diarenensis* J43, *Suhomyces pyralidae* Y1117, and *A. melanogenum* J7) demonstrated higher inhibition activity against *B. cinerea* compared to *F. fujikuroi* and this trend was observed for the in vivo and in vitro experiments. The data also clearly show that there were significant differences in the abilities of these yeasts to control the growth of *B. cinerea* and *F. fujikuroi* during in vivo and in vitro trials. *D. anomala* V38 and *R. diarenensis* J43 showed good inhibition activity and should be investigated further for application against *B. cinerea*, while more yeasts should be screened against *F. fujikuroi* to achieve better growth inhibition.

4. Conclusions

This study highlights the potential of phyllospheric yeasts in combating spoilage mould, offering practical applications in fruit preservation and post-harvest disease control. Yeasts isolated from various fruits demonstrated inhibition activity against *B. cinerea* and *F. fujikuroi* in multiple assays. Moreover, the presence of cell wall-degrading enzymes essentially enhanced biocontrol activities. *D. anomala* V38 was the most bioactive yeast against *B. cinerea* and *F. fujikuroi*. The susceptibility of *B. cinerea* to the biocontrol yeasts across all assays contributes key information to the development of yeast-based biocontrol agents against this notorious mould. This is the first report on using yeasts to control the growth of *F. fujikuroi.* Future research should investigate the efficacy of these yeasts, either individually or as a consortium, against other spoilage organisms. Moreover, thorough safety assessments are necessary before deploying these yeast strains in agricultural practices. Understanding the mechanistic aspects of yeast–fungus interactions will further enhance their practical applications in post-harvest management of fruits.

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Conflicts of Interest: The authors hereby declare that they have no conflicts of interest.

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